DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION OF SOME NOVEL ANTI-CANCER AND ANTI-HIV CHEMOTHERAPEUTIC AGENTS.

A thesis presented by

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in partial fulfilment of the requirements of the

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Department of Chemistry,
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Finally, I am especially grateful to my brother and sisters; David, Dawn, Jean and Julie for all their help and encouragement.

Dedication

Dedicated to my late parents :

John Morgan Jones

and

Sarah Margaret Jones

Rwy'n dy caru ti Bernadette! For holding my hand when I needed a hand to hold and for giving me a mountain of happiness. ✿

DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION OF SOME NOVEL ANTI-CANCER AND ANTI-HIV CHEMOTHERAPEUTIC AGENTS.

by

Bryan Colin Nicholas Morgan Jones

Abstract

The work presented in this thesis has been directed towards the synthesis of uncharged, masked phosphate derivatives of the anti-cancer nucleoside analogue araC, the anti-HIV nucleoside analogue AZT and some other modified nucleosides which are of biological interest, that is, 3'-fluoro-3'-deoxythymidine, 3'-iodo-3'-deoxythymidine and 3'-acetylthymidine. It was hoped that this approach would achieve cellular penetration while overcoming some of the mechanisms of resistance to treatment which are observed for the parent nucleosides.

The first chapter, the review, presents the background data and knowledge pertaining to cancer (especially leukaemia) and to the aquired immunodeficiency syndrome. The chemistry and biology of the compounds employed, along with related compounds are described.

In chapter two, the synthesis of symmetric 5'-dialkyl phosphate triester derivatives of araC is described, along with a lipophilicity study and biological testing in mammalian epithelial cells. The compounds showed interesting activity which correlated with increasing lipophilicity. An attempted X-ray crystallographic study of 1-β-D-arabinofuranosylcytosine-5'-diethyl phosphate is also presented.

In the third chapter, the synthesis of assymetric 5'-dialkyl phosphate triester, phosphoramidate and phosphorodiamidate derivatives of araC are described. The results of biological testing in mammalian epithelial cells are discussed. The attempted combination of the active moiety of the alkylating agent cyclophosphamide with araC is described. The reason why protection of araC may be necesary is outlined and a novel base protected derivative of araC is presented. A Nuclear Overhauser Experiment is corelated with molecular graphics results in order to assign stereochemistry to the separated diasteroisomers of 1-β-D-arabinofuranosylcytosine-5'-ethyl-2,2,2-trichloroethyl phosphate.

The fourth chapter describes the syntheses of AZT, 3'-iodo-3'-deoxythymidine and 3'-acetylthymidine and the subsequent use of a modified Yoshikawa reaction to the production of phosphorodiamidate derivatives of the aforementioned nucleoside analogues and of 3'-fluoro-3'-deoxythymidine. In-vitro anti-HIV biological testing was carried out on the AZT phosphorodiamidates. As the method by which the active AZT phosphorodiamidates act as inhibitors of HIV probably involves the hydrolyses of the phosphate moiety to either the 5'-monophosphate or the parent nucleoside, some studies on the hydrolysis of four of the analogues in the AZT series were carried out in human plasma.

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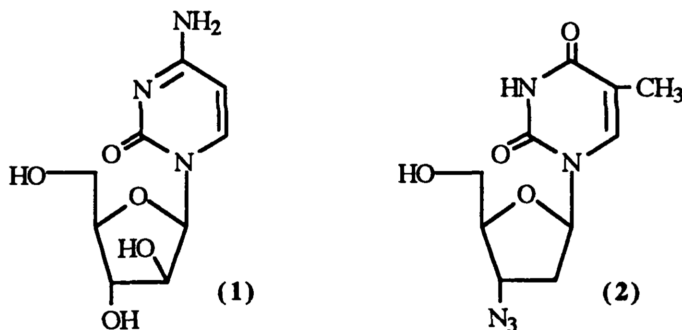
1.0 Review

1.1 Introduction

The work described in this thesis is concerned with the design, synthesis and biological evaluation of phosphoramidates, phosphorodiamidates and symmetric and asymmetric 5'-dialkyl phosphate derivatives of the anti-cancer drug 1-β-D-arabinofuranosylcytosine (araC, cytarabine, cytosine arabinoside, cytosar) **(1)** and phosphorodiamidate derivatives of the anti-HIV drug AZT **(2)** along with the investigation into other novel chemotherapeutic compounds. The object of the review is to highlight the background knowledge on which the work presented in this thesis is based.



This review includes published literature data which deals with the structure-activity correlation studies of nucleoside analogues with various cancer cell lines and various HIV strains, which are relevant to the work presented in later chapters.

1.2 Cancer

The disease of cancer goes back millions of years, fossil evidence has established that cancer was present in the pre-historic dinosaur.[1] The Egyptians (5,000 BC) understood the medical use of many drugs, including opium and hemlock, they founded the first National Health Service. Cancer has been found

in mummies and in Egyptian papyrus it stated 'If thou examinest a man having bulging tumors on his breast and thou findest that swellings have spread over the breast.....there is no treatment'. To treat a disease without first understanding its nature almost always produces poor results. It was not until the Greek doctor Galen (c130 to 200 AD) introduced the idea of humours that the treatment of cancer was based on a theory of cancer. However, the theory was incorrect. Galen's idea prevailed into the nineteenth century.[2]

The twentieth century has taken great strides towards understanding and treating cancers. Cancer or neoplasm (from the Greek, υεοζ, new, and πλασμα, formation) refers not to a single disease but to over 100 related but unique forms of a disease of cells.[3] Cancerous cells are characterized by their growth advantage over normal cells, this allows them to proliferate continuously not only in their site of origin but also in other environments. Damage to the host occurs at a variety of levels by pressure effects, destruction of involved tissues both physically and in terms of normal function and by systemic effects secondary to the localized growths.[4]

Cancer may be caused by several agents; certain chemical compounds, radiant energy, certain viruses (retroviruses, Section 1.6), pollution agents (from water, air and food), alimentary deficiency, hereditary factors and cellular mutation of unknown origin but involving oncogenes. There are two principle groups of cancer, solid tumors and malignant haematological diseases. Solid tumors are localized initially in concrete tissues and organs. 'Benign' tumors are those that stay in the same place and are easier to deal with. As time goes on a tumor may become 'malignant', cancer cells separate themselves from the primary tumor and are carried by the lymphatic system or blood stream to distant sites in the organism, where they divide and form secondary tumors. This

characteristic dissemination phase of the disease is called metastasis. Malignant haematological diseases affect blood and lymphatic systems and for this reason, often are disseminated from the beginning.[5]

Cancer is also classified by the type of tissue in which it originates:

1. Sarcoma: Connective tissue and muscles, such as bone or cartilage.
2. Carcinoma: Lining tissue, such as tissue that makes up the skin or lines the intestine, kidney, mouth, uterus, lung and other organs.
3. Lymphoma: Lymphatic tissue.
4. Leukaemia: Blood forming tissue, in the bone marrow and lymph nodes.

The major types of cancer listed above are further divided into sub-types[4,5], which are not included here.

The behavioral characteristics of cancer cells which are unlike normal cells are summarized as follows :

1. Uncontrolled cell proliferation
2. Undifferentiated cells
3. Able to invade surrounding tissue
4. Capable of metastases

Mechanisms underlying these abnormal characteristics must be understood for the development of a therapy to eradicate cancers.[6]

Figure 1 Schematic representation of a normal cell cycle (Adapted from the literature.[6,7,8])

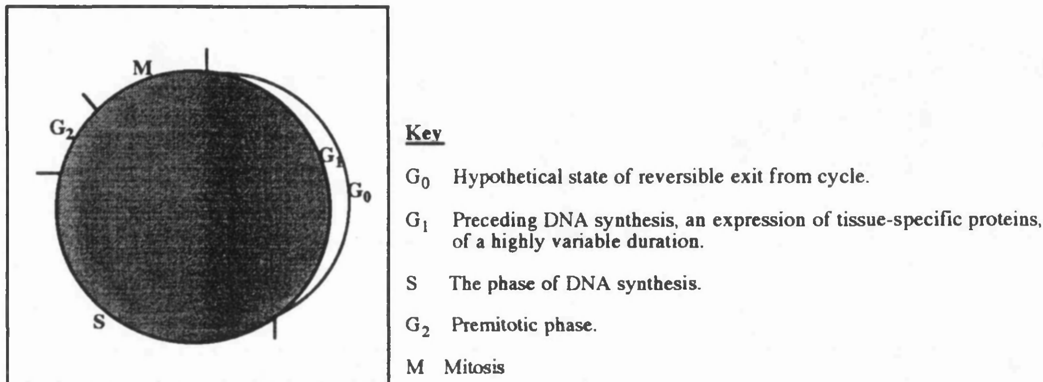


Figure 1 is a view of the division of a mammalian cell derived from the study of cell growth in tissue culture conditions in which the influence of variables can be minimised. Complications following cellular differentiation and proliferation (found in proliferating cells *in vivo*) have been avoided by the use of cell lines adapted to tissue culture in which every cell divides with a similar intermitotic time.[6]

The length of the cell cycle varies considerably from tissue to tissue in proliferating cells. Under optimum culture conditions, certain mammalian cell lines can be made to divide in 10 to 12 hours. The majority of cell lines require approximately 1 to 2 days, although under certain conditions cell cycle times can be as long as months.[7]

Animal cells have only two phases of cell division; a short period of actual cell division (mitosis) between an apparent resting phase (interphase).

In mitosis, there are four separate phases:

1. Prophase: The nuclear membrane appears to disintigrate and the chromosomes begin to 'clump'.

2. Metaphase: The chromosomes align in the middle of the cell.

3. Anaphase: The chromosomes appear to separate and move to the centrioles.

4. Telophase: Actual cell division results in the production of two cells, one new identical 'daughter' cell.

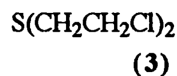
After mitosis, daughter cells predominantly differentiate into functional cells, forming a particular type of tissue making up a particular organ. They rarely retain the proliferative capacity of their parent cells. Thus the normally small stem cell population is maintained.[8]

During interphase (the so called 'resting' phase), scientists have found that DNA synthesis (S) occurs in a portion of interphase with two gaps on either side of this period, termed G_1 (gap1) and G_2 (gap2).

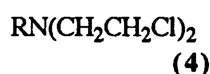
The times for the S (10 to 20 hours), G_2 (2 to 10 hours) and M (0.5 to 1 hour) phases are constant regardless of cell-cycle times, however G_1 phase can vary from being undetectable in rapid proliferating systems to a number of months. G_1 phase variation is believed to be due to a hypothetical state of reversible exit from the cycle; G_0 where the cell enters a resting state and is not actively committed to cell division.[6,7,8]

The initial use of cytotoxic agents in the treatment of cancer stems from the chemical warfare of World War I. During the 1st World War, medical attention was focused on the action of certain chemicals, such as sulphur mustard (**3**), on

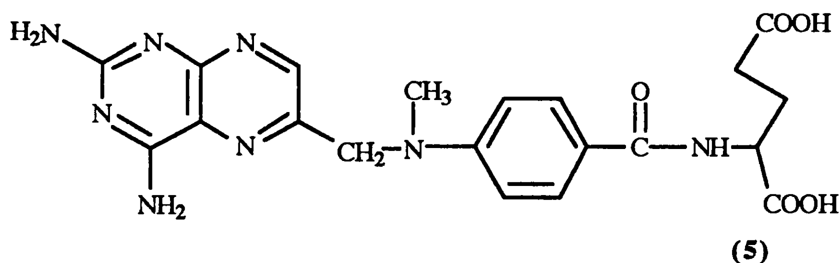
the skin, eyes and respiratory tract. It was found that poisoning caused by **(3)** is characterized by leukopenia and in autopsy cases by aplasia of the bone marrow.[9]



The marked cytotoxic action on lymphoid tissue prompted studies into the effect of nitrogen mustards **(4)** on transplanted lymphosarcoma in mice.[10]



The use of chemotherapeutic agents in cancer therapy has made enormous progress since the initial application of chemicals in the late 1940's when Farber[11] prescribed methotrexate **(5)** to treat childhood leukaemia. Today in the 1990's there is a vast array of chemotherapeutic agents with varying modes of action against various forms of cancer.

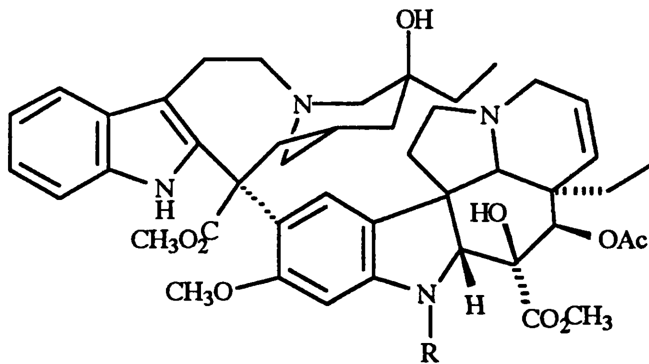


Chemotherapeutic agents can be categorized into functional sub-groups: There are cell-cycle (phase)-specific drugs (CCS) (Table 1) and cell-cycle (phase)-nonspecific drugs (CCNS) (Table 2).[12]

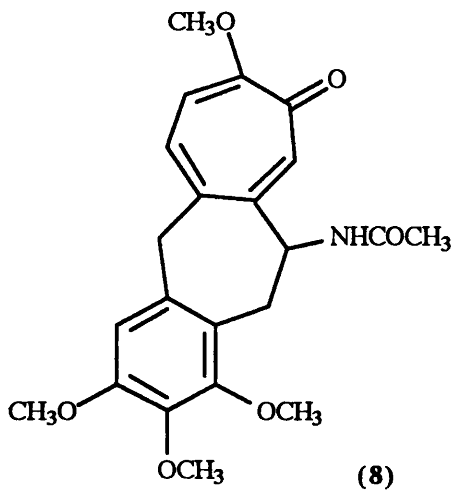
Table 1 Common cell-cycle (phase)-specific drugs

Dependent phase:

M	G1	G2	S
Vincristine (6) Vinblastine (7) Colchicine (8) Etoposide (VP-16) (9) Teniposide (VM-26) (10)	Asparaginase (11)	Bleomycin (12)	AraC(1) Fluorouracil (13) Mercaptopurine (14) Methotrexate (5) Thioguanine (15) Hydroxyurea(16) Prednisone (17) Procarbazine (18)



R = CH_3 (6)
R = CHO (7)



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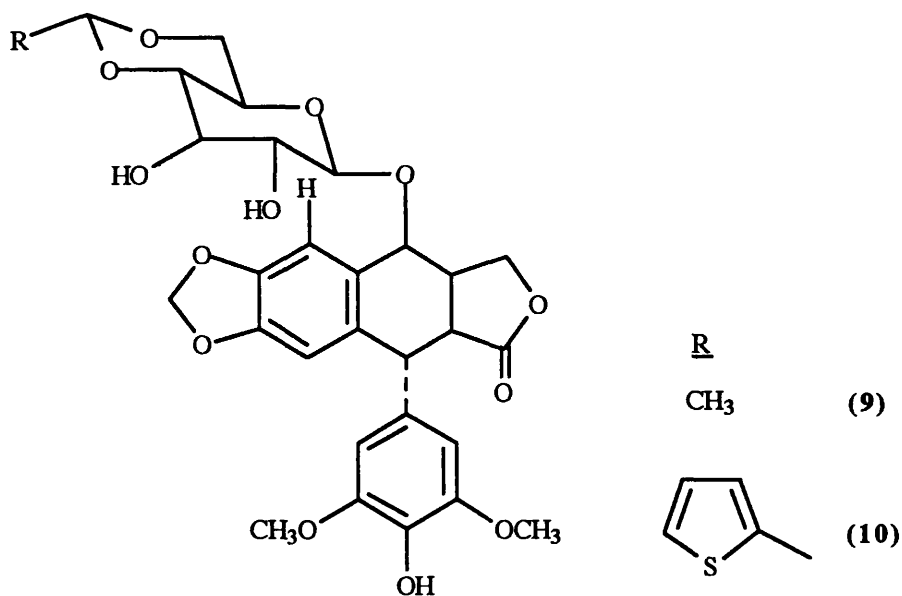
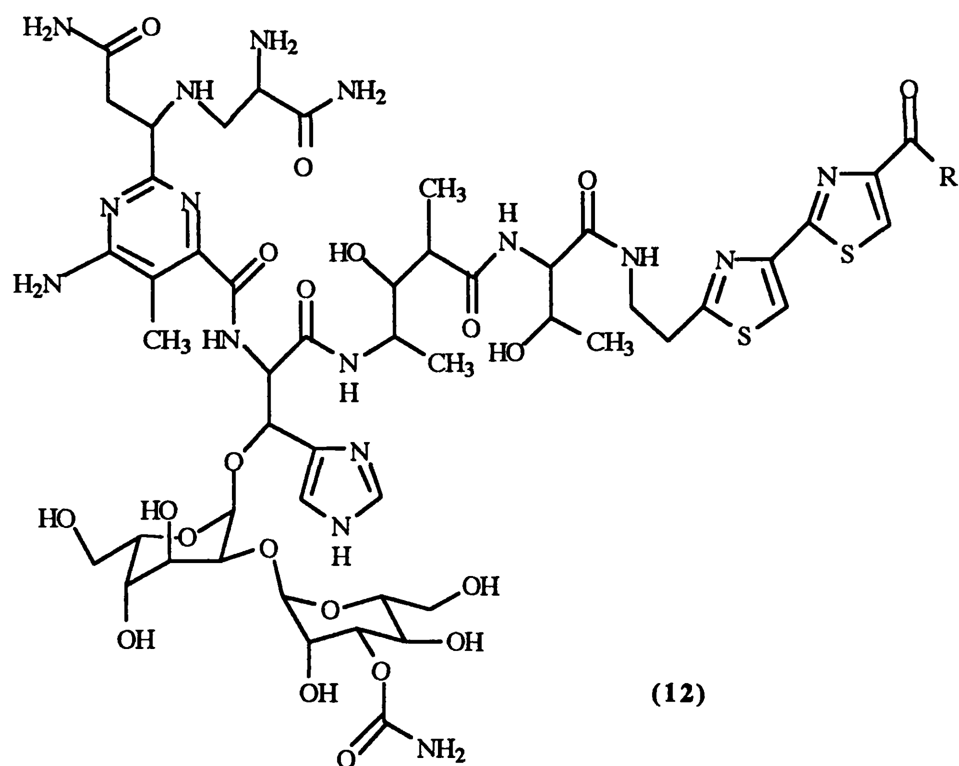
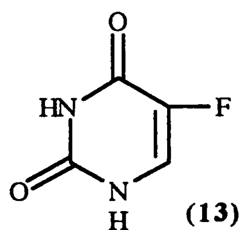


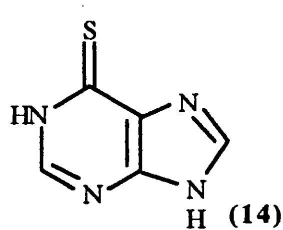


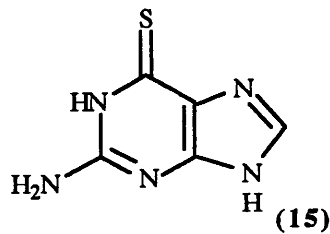
Bleomycinic acid: R = OH

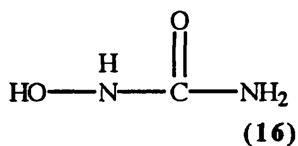
Bleomycin A_2: R = $NHCH_2CH_2CH_2S$+$(CH_3)_2$

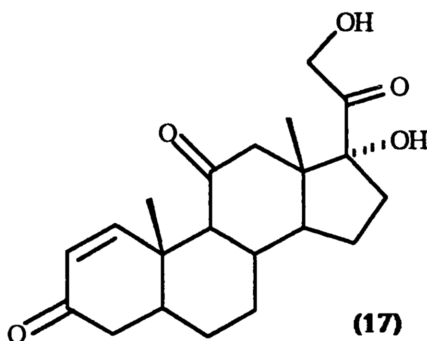
Bleomycin B_2: R = $NHCH_2CH_2CH_2CH_2NHC(NH)(NH_2)$

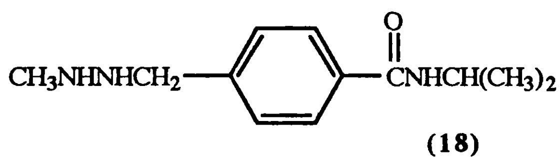








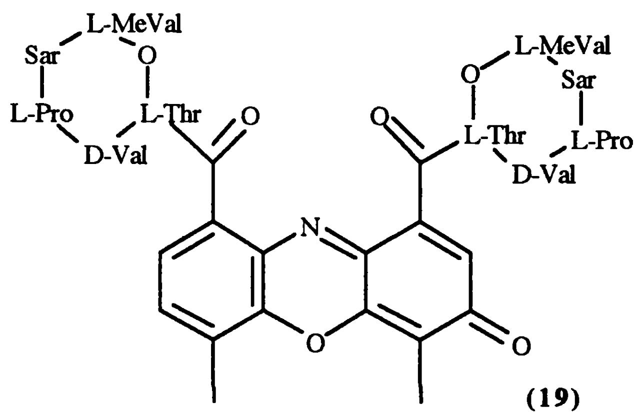


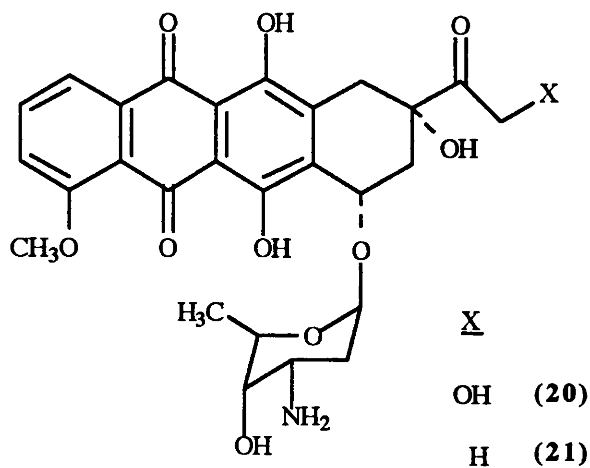


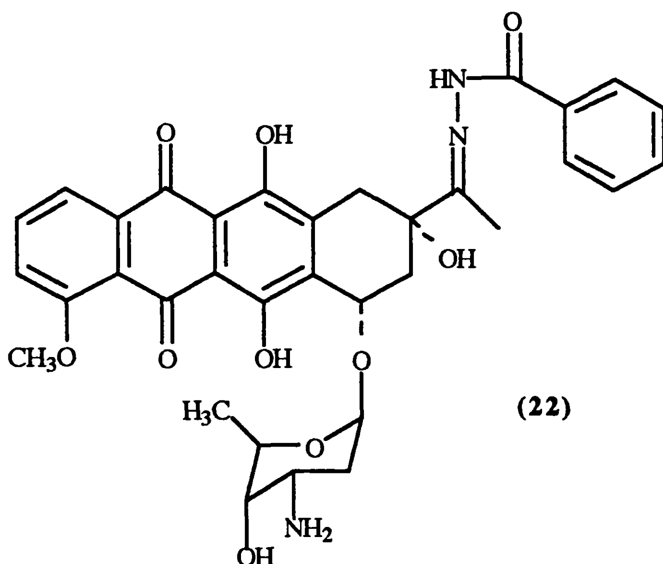
In the resting phase, the cell is desensitised towards cytotoxic drugs. Drugs blocking mitosis act only on cells in the M state, while chemotherapeutic attacks on DNA synthesis are made during S phase. Thus, knowledge about factors controlling changes from one state to another in the cell cycle are very important. A critical problem with tumor annihilation is that all the cells in the tumor are not at the same phase of the cycle.[13]

Table 2 Common cell-cycle (phase)-non specific drugs

Antitumor antibiotics	Alkylating agents	Nitrosoureas	Miscellaneous
Dactinomycin (19) Doxorubicin (20) Daunorubicin (21) Rubidazone (22)	Busulfan (23) Chlorombucil (24) Cyclophosphamide (25) Melphalan (26) Mechlorethamine (27)	Semustine (28) Cormustine (29) Lomustine (30)	Dacarbazine (31) Cisplatin (32)







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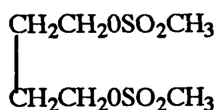

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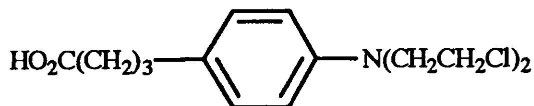

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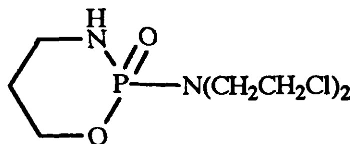

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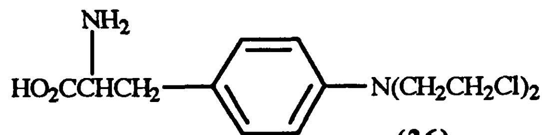

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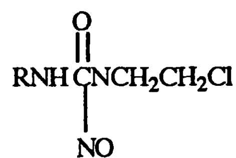

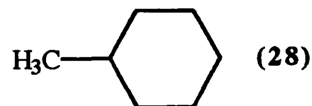

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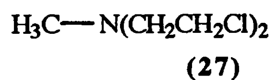

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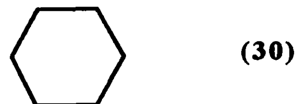

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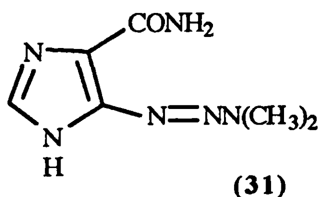

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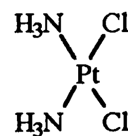

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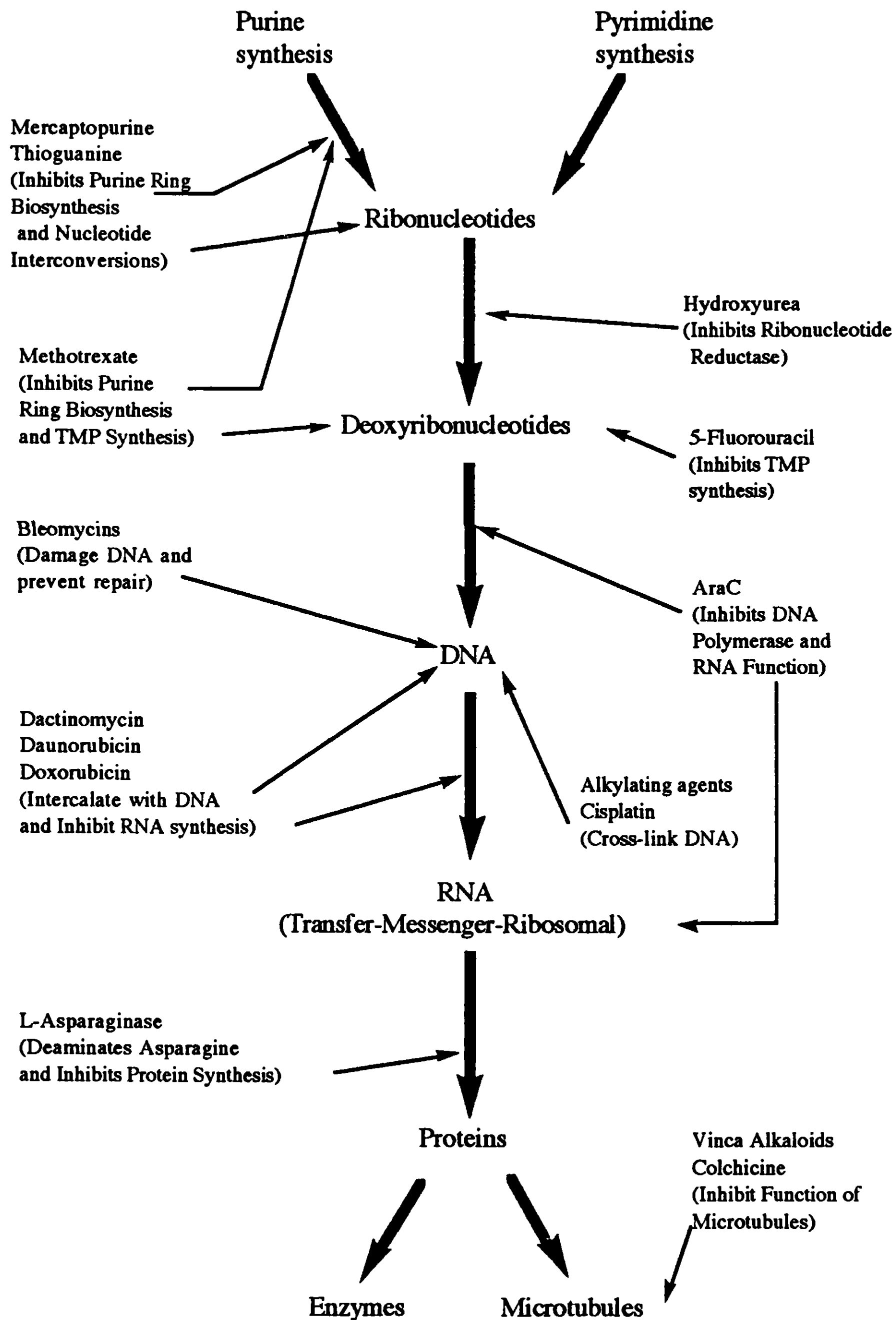
Figure 2 Schematic representation of the mechanisms and sites of action of some anti-neoplastic chemotherapeutic agents (Adapted from the literature.[6,12])



At the present time at least 10 neoplasms can be treated with good expectation of a cure: acute leukaemia in children, Burkitt's lymphoma, choriocarcinoma, Ewing's sarcoma, Hodgkin's disease, lymphosarcoma, mycosis fungoides, rhabdomyosarcoma, retinablastoma in children and testicular carcinoma. However the future does not appear promising for patients with such common tumors as those of the liver, pancreas, colon and lung. Numerous other tumors show a partial response to chemotherapy but still remain a serious cause of death, such as breast cancer.[14]

Surgery is the prime mode of treatment for solid tumors and radiation is used for skin cancer and localized deeper tumors. However, chemotherapy is usually used as an adjuvant with these other forms of treatment especially when metastasis is suspected.[15,16]

The most common rationale for the use of chemotherapy is control of tumor growth by a cell-kill mechanism[17] which is limited by the toxicity shown by these cytotoxic agents on normal cells. A major challenge in this field is to make tumor-specific therapy.

One approach to the problem of lack of selectivity is combination chemotherapy. The combination of chemotherapeutic agents that might act synergistically on cancer cells but not on normal tissues, has been attempted by combining agents with different mechanisms of action. An excellent example is the MOPP regimen ((**27**), (**6**), (**18**) and (**17**)) used succesfully in the treatment of advanced Hodgkin's disease.[18] Monotherapy in cancer treatment is employed very exceptionally. Indeed, only two types of cancer, choriocarcinoma and Burkitt's lymphoma have been cured with a single agent. As a rule, combinations of different cytostatics are employed, they offer better

results than single drug therapy, with reduction of toxic effects, enhancement of anti-tumor action and delay of the onset of drug resistance.[19]

More recently, a new form of treatment employing differentiating agents has been reported. The process by which a cancer cell returns to normality is called differentiation and dimethyl sulfoxide, DMSO (**33**), was one of the earliest differentiating agents. One of the possible advantages of differentiating agents is that they may work with far fewer side-effects than conventional chemotherapy. Because they act to correct a defect in a cell rather than destroy the whole cell, they have a gentler effect on the body as a whole. From the initial lead compound, a better differentiating agent was produced, hexamethylene bisacetamide, HMBA (**34**), which was effective against a range of human cancers *in vitro*.[20]

1.3 Leukaemia

Leukaemias are cancers that originate in the blood forming tissue of the bone marrow or the lymph nodes. The white blood cell forming tissues produce immature white blood cells in endless numbers, which replace the normal white blood cells, so that the body may become overwhelmed by infectious disease. The leukaemic cells proliferate without the control exhibited by normal cells, crowding out platelets and red blood cells so that the body bleeds easily and receives insufficient supplies of oxygen. Thus patients with leukaemia often die from secondary causes such as rampant infection or massive internal bleeding.[5]

Leukaemias which involve the lymphocyte precursors are called lymphocytic or lymphoid leukaemias; those involving the granulocytes (or myeloid cell) precursors are termed myelocytic, myeloid or granulocytic leukaemia. Both types can be acute or chronic, the former being rapidly fatal

disorders, characterized by the presence in the blood of poorly differentiated cells called blasts. In such cases, the average course from onset to death is reached within months. Chronic leukaemias are of a longer duration, usually lasting 2 to 6 years and are characterized by the presence of more differentiated leukocytes and few or no blasts.[21,22]

Thus there are four major forms of leukaemia :

Lymphocytic Leukaemias

1. **Acute lymphocytic leukaemia (ALL)**

i) Null cell type

ii) T-cell type

iii) B-cell type

2. **Chronic lymphocytic leukaemia (CLL)**

i) B-cell type

ii) T-cell type

iii) PCL

iv) IC

v) HCL

Myelocytic Leukaemias

1. **Acute non-lymphocytic leukaemia (ANLL)**

i) Acute myelocytic leukaemia (AML) (with maturation) M1, (without maturation) M2

ii) Acute promyelocytic leukaemia (APL) M3

iii) Acute myelomonocytic leukaemia (AMML) M4

iv) Acute monocytic leukaemia (AMoL) M5

v) Rare type - erythroleukaemia M6

2. Chronic myelocytic leukaemia (CML)

i) Ph' positive

ii) Ph' negative

Acute lymphocytic leukaemia (ALL).[23] This cancer generally occurs in children. This leukaemia is characterized by excessive accumulation of lymphoblasts and their progenitors in the bone marrow. The disease is heterogenous, the malignant cells expressing diverse phenotypes and respond variably to chemotherapy. ALL classification is based on immunologic criteria, the leukaemic lymphoblasts express antigens corresponding to different stages of B- or T-cell development. Approximately 80% of cases of ALL arise from the B-cell lineage. 15% of ALL cases arise from the T-cell lineage and ALL without B- or T-cell features are termed null-cell ALL. Children have a higher proportion of B-cell, whereas adults are more likely to have T- or null cell ALL.[24]

Identification of prognostic factors has allowed classification of the disease into low-, average and high-risk forms that require different therapeutic approaches. Hence, substantial advances have been made in treatment, where previously ALL in children was a 100% fatal disease, to one that is curable in almost 70% of patients and up to one third in adults. Combinations of drugs are administered to leukaemic children in large doses over long periods. Prophylactic antibiotics are prescribed along with the chemotherapeutic agents and blood transfusions or platlet transfusions are given to control haemorrhaging. Some leukaemic cells may spread to the brain, where treatment by drugs becomes less feasible. Thus the brain is irradiated to combat the

leukaemic cells. Occasionally acute lymphocytic leukaemia may be treated by bone marrow transplantation, where the patients diseased marrow is destroyed with large doses of radiation and replaced with healthy tissue that is compatable with the patient.

Chronic lymphocytic leukaemia(lymphoma), (CLL).[25] This cancer represents 25% of all leukaemias in the west and is probably the most frequent of all adult leukaemias. It is a heterogeneous group, there are B-type CLL (B-CLL), T-cell CLL (T-CLL), prolymphocytic leukaemia (PLL), immunocytoma (IC) and hairy cell leukaemia (HCL). CLL and PLL usually affect patients over the age of fifty years, whilst the HCL peak incidence is between forty and fifty years. This group of leukaemias are diagnosed as diffuse well differentiated lymphoma. The major limitation in the treatment of CLL is the inability to critically determine when remission is achieved, that is, when all the malignant cells have been eradicated.

Acute non-lymphocytic leukaemia, (ANLL).[26] Of this group the most comonly occuring leukaemia is AML. AML (M1 and M2) is a heterogenous disease differing considerably among patients in respect to cytogenetic abnormalities, cellular phenotype and response to therapy. In 20% of patients the leukaemic cells have characteristics of both myeloid and lymphoid cells. These cases have been termed hybrid or mixed lineage leukaemias. AML results from malignant transformation of a haematopoietic progenitor cell followed by clonal proliferation and accumulation of the transformed cells. AML is found to occur twice as often as ALL in adults, that is approximately 40% of all acute adult leukaemias.

Chronic myeloid leukaemia, (CML).[21,22] Like acute myeloid leukaemia, this cancer occurs mainly in adults. CML is a neoplastic disease of the

haematopoietic system that results from the mutation in a single pluripotential stem cell. The abnormal stem cell receives a growth advantage, while for unknown reasons the proliferation of regular haematopoiesis is inhibited. Excessive and unrestrained growth of blood progenitor cells leads to an accumulation of mature and immature cells in the peripheral blood and organ infiltration.

Compared to other neoplasias, CML has some unique features which are of importance in selecting treatment. In over 90% of patients with a clinical diagnosis of CML, these cells have a specific chromosomal abnormality, the Philadelphia chromosome (Ph').[21]

The high frequency of the Ph' chromosome in clinical CML and the specificity of this abnormality for CML suggests that Ph' chromosome must be in some way related to the pathogenesis of the disease. CML is found to proceed in two completely different phases, a chronic phase and an acute phase. During the chronic phase, the disease follows a relatively benign course and is oligo- or asymptomatic lasting some years. The proliferation of haematopoietic cells can be suppressed by cytotoxic drugs in most cases. Nevertheless, the disease progresses to an accelerated phase, where by, an almost abrupt transformation has occurred, characterized by a rise in the number of blasts and promyelocytes, that is cells which have lost their capacity to mature normally. In most cases the blast cell crisis (CML-BC) prognosis is extremely poor and treatment largely ineffective.

The objectives of leukaemia treatment are related to the type of leukaemia. For the acute leukaemias, the therapeutic goal is to completely eradicate the leukaemia cells and restore normal haematopoiesis. These objectives are best accomplished by the administration of relatively high-doses of antileukaemic

drugs followed by the cyclic re-administration of chemotherapy with or without treatment of the central nervous system to prevent leukaemia relapse. Bone marrow transplantation is an alternative approach.

The principles of chronic leukaemia therapy, that is CLL, CML and HCL are somewhat different. In most instances, the intent is not to cure but rather to control the disease and restore normal haematopoiesis. Thus the treatment of CML and CLL generally involves relatively low-dose chemotherapy, and HCL usually by splenectomy alone.

The clinical application of the treatment of leukaemia has followed a strategy which has been applied over the past twenty years. This strategy comprises three phases:

The initial phase is termed remission induction and is designed to reduce the number of leukaemic cells below the level of detecton in the blood, bone marrow and extramedullary sites, by conventional techniques and normalize haemopoiesis. Without the induction of complete remission (CR), anything beyond a few months survival is very rarely possible. By definition, no leukaemic cells are detected in CR, however, substantial numbers of malignant cells probably persist in most patients and lead to leukaemia relapse if effective post remission therapy is not administered.[27]

The second phase of treatment is called post-remission therapy and is designed to eradicate clinically undetectable leukaemic cells and prevent relapse. It comes in two forms; consolidation and intensification. Consolidation refers to one or more courses of therapy administered with the same drugs as were used to induce remission but usually in lower doses. Intensification is early or late, the former involves high dose chemotherapy with drugs that the patient has not

previously received and late intensification involves one or more courses of similarly intensive chemotherapy with new drugs but given after a delay of six to twelve months.

The third phase is maintenance chemotherapy, which is generally conceived of as low dose frequent courses with the same or different drugs, either cyclically or continuously given over several months or years.

There are numerous chemotherapeutic agents and strategies employed for the treatment of the various leukaemias, some of the most commonly employed are as follows. Compound **(5)** is a major drug in the treatment of acute leukaemia, primarily ALL, where it is used for maintenance chemotherapy and CNS prophylaxis. High-dose **(5)** with **(11)** rescue has activity in resistant ALL and AML. Compound **(5)** is competitive for the folate binding site of dihydrofolate reductase, so that the true substrate, folic acid, cannot replace it to any extent. It inhibits the synthesis of tetrahydrofolate and depletes reduced folate cofactors resulting in decreased synthesis of thymidine **(42)** and purine nucleotide and ultimately in decreasing DNA synthesis and cell death. Adverse reactions include myelosupression, gastrointestinal tract symptoms, heptic and renal toxicity associated with high-dose therapy.[28,29]

There are two purine analogues useful in clinical treatment of leukaemia, **(14)** and **(15)**.[30] Although they are structurally similar and cross-resistance between the two is common, they differ in their pharmacology and clinical toxicities. Compound **(14)** requires activation to the ribonucleotide form, *via* intensive intracellular metabolism to mercaptopurine ribose phosphate (6-MPRP) due to the action of the enzyme xanthine oxidase, for its anti-leukaemic effect. 6-MPRP is able to inhibit glutamine-5-phosphoribosylpyro phosphate (PRPP) amidotransferase, the enzyme which initiates *de novo* purine synthesis, by

feedback inhibition. It also interferes with the conversion of inosinic acid into adenylic and guanylic acid. The adverse effects of **(14)** include myelosuppresion, gastrointestinal and hepatic toxicity. It is primarily used for maintenance chemotherapy in ALL while in AML treatment has been replaced by **(15)**, it also requires activation to its ribonucleotide form and acts in much the same way as **(14)**, however **(15)** is a weaker inhibitor of PRPP amidotransferase. Compound **(15)** is commonly used in combination chemotherapy regimens for remission induction in AML where it is known to act synergistically with **(1)**. Compound **(15)** has adverse effects of myelosuppression, gastrointestinal toxicity and hepatotoxicity.[31]

A natural product, **(21)** is used to treat AML as an intravenous high dose pulse schedule.[32] Compound **(21)** acts by inhibiting DNA replication by intercalation of base pairs and it is also known to be converted to free radicals which create oxygen radicals and alkylating drug species, which bind to cell membranes and directly affect membrane structure and function and avidly chelate transition metal ions which damage cell membranes and DNA. Adverse reactions include myelosuppression, cardiac toxicity, alopecia and gastrointestinal dysfunction.[33]

Compound **(9)** has been used extensively as a single agent and in combination chemotherapy to treat many different malignancies, amongst which are the acute leukaemias, in combination with **(1)**.[34] Compound **(9)** is a premitotic inhibitor that has been demonstrated to slow the progression of cells through late S phase and early G_2 phase, with cellular arrest in G_2, thus it is cell-cycle specific.[35]

In leukaemia treatment, **(6)** and **(7)** are the two most commonly used vinca alkaloids, which are plant alkaloids of the periwinkle plant.[36] Although these

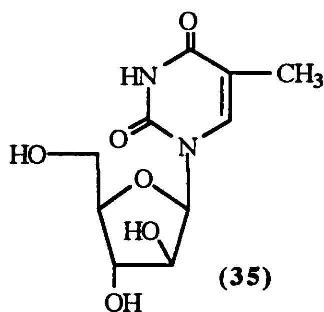
drugs differ only in one side chain, this leads to markedly different biological activities, that is, in dose limiting toxicities and in some instances, to a lack of clinical cross-resistance among the drugs. Compound **(6)** is the principle vinca alkaloid used in antileukaemic therapy because of its virtual lack of myelosuppresive toxicity, whereas **(7)** is a potent marow toxin.[37,38] Compound **(6)** is given intravenously and is commonly used to treat ALL and CLL.[39]

Other agents which act on acute leukaemias are the family of adrenocorticosteroids such as **(17)** which act by an initial interaction of the steroid with a specific cytoplasmic receptor protein, however the full mechanism of action is unclear.[40] Undesirable side effects with short term treatment include sodium and water retention, potassium loss, psychosis and exacerbation of diabetes mellitus.[41,42]

Agents which act on chronic leukaemias include, **(23)**, an alkyl sulphonate with primary use in the treatment of CML.[43] Compound **(23)** is generally given as a single agent and is highly effective in controlling white blood cell count, but does not usually produce remissions, it also has myelosuppression as its major toxic reaction.[12] The main alkylating agents used to treat CLL are **(24)** and **(25)**. Compound **(24)** is well tolerated but its adverse effects include bone marrow and immune suppression, gastrointestinal symptoms, hepatotoxicity and cardiac damage in very high doses. Compound **(25)** is inactive *per se* and requires bio-transformation in the liver to 4-hydroxycyclophosphamide **(60)**.[44,45] Toxicity occurs primarily in rapidly dividing tissues like the bone marrow and nausea, vomiting and alopecia are the main side effects.[12]

1.4 1-β-D-arabinofuranosylcytosine (1)

The discovery of spongothymidine, (araT) (**35**) and spongouridine, (araU) (**36**) in the sponge *Cryptotethya crypta* yielded the first aranucleosides.[46] These naturally occurring D-aranucleosides[47] stimulated interest to produce synthetic aranucleosides such as (**1**)[48], 9-β-D-arabinofuranosyladenine, (araA) (**37**)[49] and 9-β-D-arabinofuranosylguanine, (araG) (**38**). The chemical properties of the D-arabinosyl nucleosides and early studies of their biological properties have been reviewed in some detail.[50,51,52]



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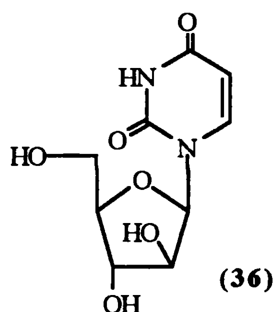

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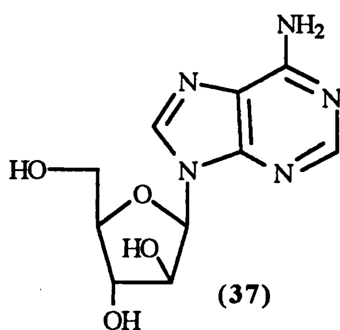

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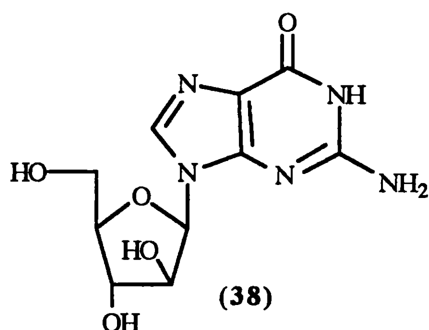
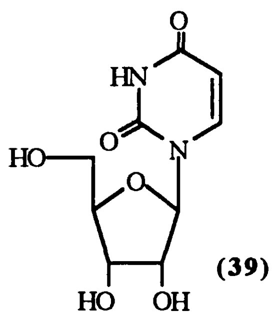
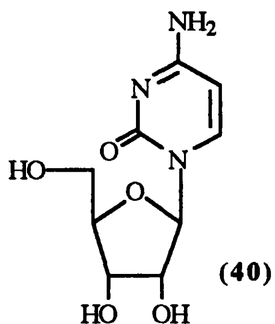


(38)

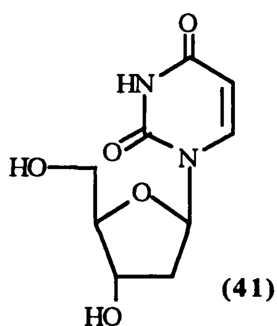
Structure of the common natural nucleosides[53]

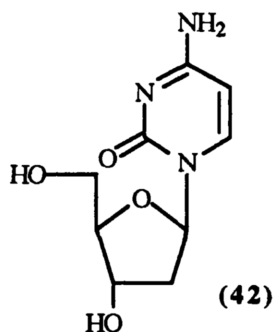


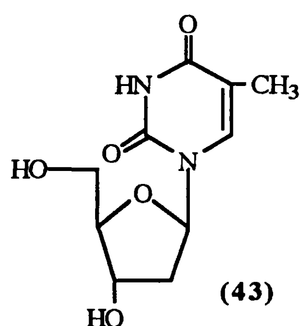
Uridine



Cytidine



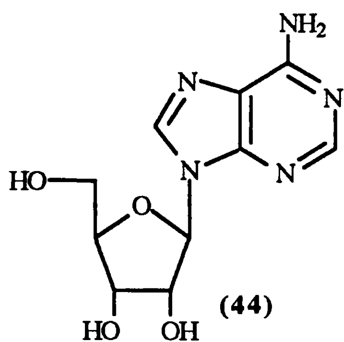


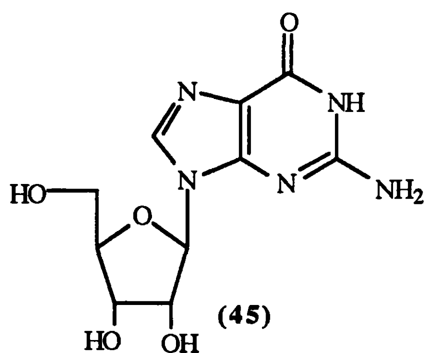


2'-Deoxyuridine

2'-Deoxycytidine

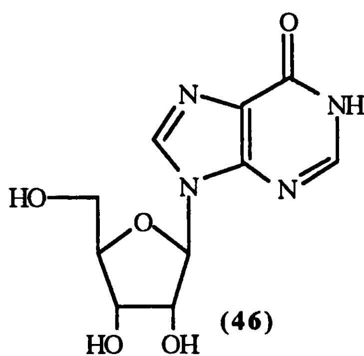
Thymidine



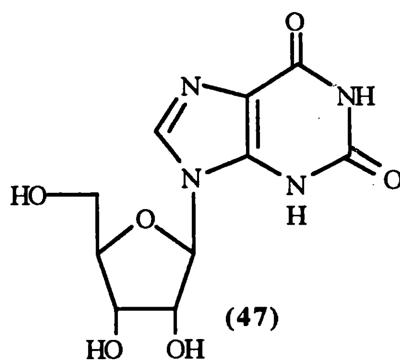


Adenosine

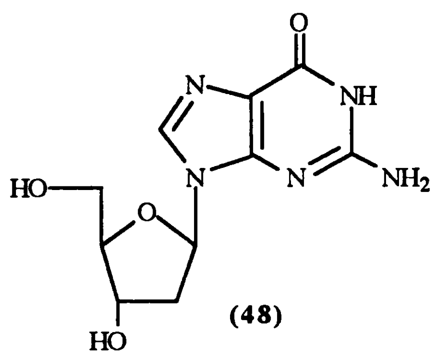
Guanosine



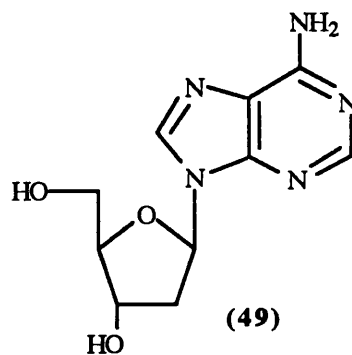
Inosine



Xanthosine



2'-Deoxyguanosine



2'-Deoxyadenosine

Many investigators have found interesting *in vivo* and *in vitro* antitumor activities in experimental neoplasms with **(1)**.[54-56] The structure of the common natural nucleosides are shown above. Compound **(1)** is a congener of cytidine **(40)** and 2'-deoxycytidine **(42)** with the 2'-hydroxyl in a position *trans* to the 3'-hydroxyl of the sugar. The 2'-hydroxyl causes steric hindrance to the rotation of the pyrimidine base around the glycosidic bond. The bases of polyarabinonucleotides cannot stack normally as do the bases of polydeoxynucleotides. This might lead to nucleic acid dysfunction if **(1)** residues replace **(42)** in nucleic acids.[57,58] As with most purine and pyrimidine antimetabolites, **(1)** must be activated to the nucleoside 5'-triphosphate (Figure 3).[59,60]

Figure 3 Activation of (1) *via* phosphorylation to araCTP (52)[59,60]

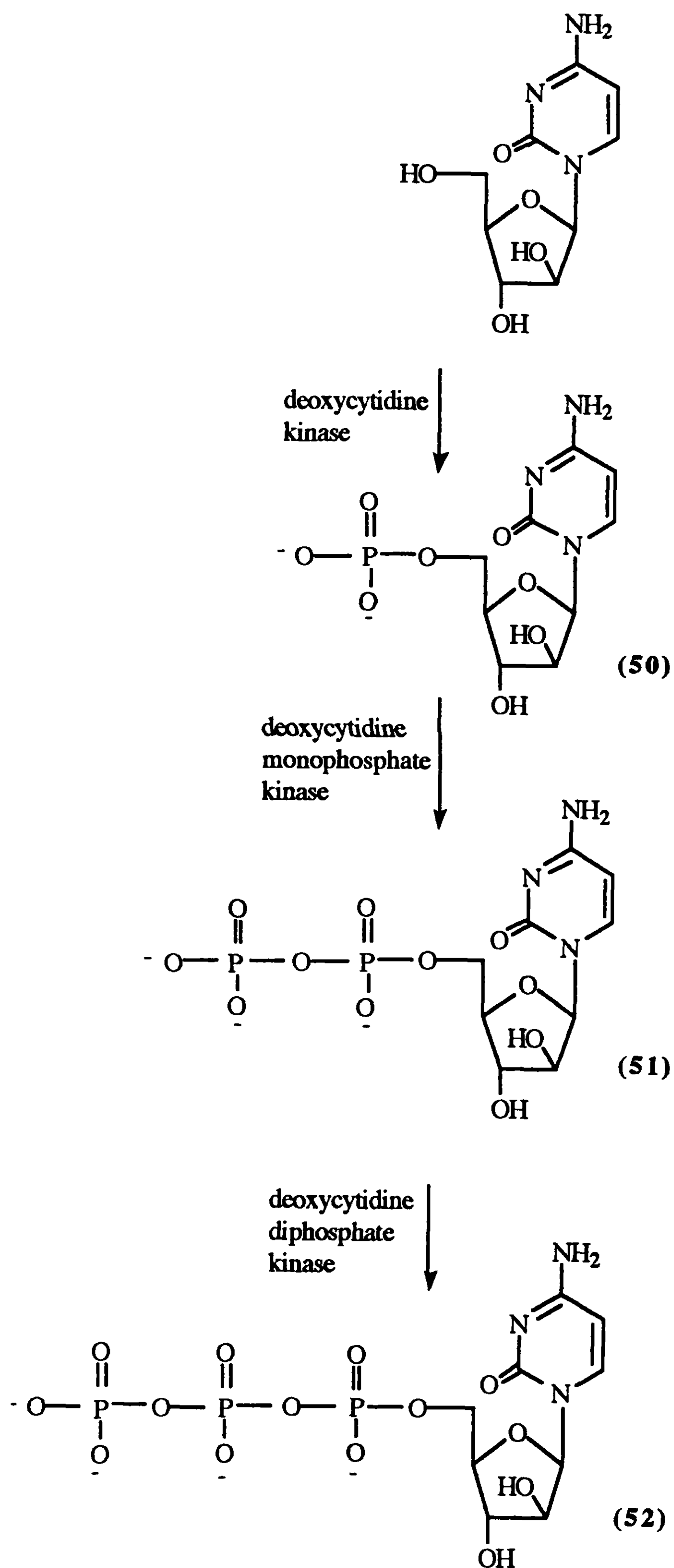
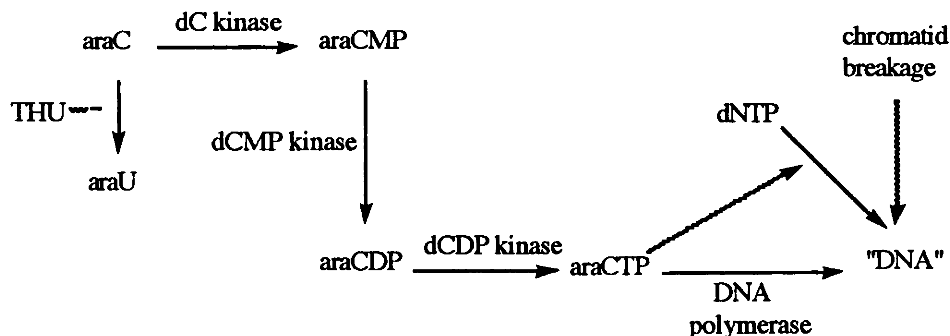


Figure 4 Schematic representation of the metabolism of (1)[62]



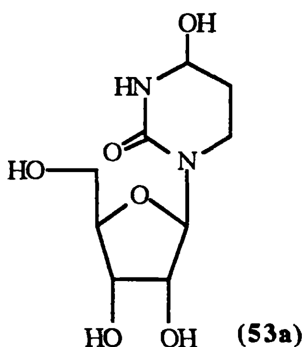
The cytotoxicity of **(1)** has been given several explanations. Early investigations suggested a block of ribonucleoside diphosphate reductase by a nucleotide of **(1)** as a crucial effect.[61] *In vivo* **(1)** is metabolized by kinases to ultimately form the triphosphorylated compound **(52)**. This metabolite acts as a competitive inhibitor of DNA polymerase.[62] Other cytotoxic mechanisms of action may involve the inhibition of certain acid kinases and the incorporation of small amounts of drug into nucleic acids and this lesion impedes DNA synthesis by, for example, providing a poor primer for further chain elongation.[57,58]

However compound **(1)** has its limitations:

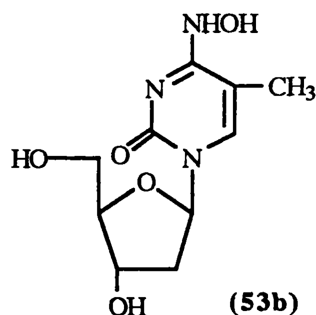
1. Deoxycytidine kinase enzymes are needed to phosphorylate **(1)** to **(52)**. Cells with a low level of these enzymes show a poor response to treatment.[63]

2. Compound **(1)** is rapidly deaminated to an inactive form, **(36)**, by cytidine deaminase.[64,65] In an effort to overcome this major problem, potent inhibitors of cytidine deaminase have been sought and found, such as tetrahydrouridine **(53a)**[66a] which requires preincubation with the enzyme for maximum effectiveness and produces noncompetitive inhibition of cytidine deaminase. A hydroxylamino analog, 4-hydroxylamino-5-methylpyrimidin-2-one-2'-deoxy-

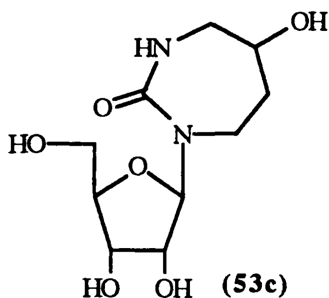
ribonucleoside **(53b)**[66b], acts as a competitive inhibitor and undergoes direct dehydroxylamination. More recently a diazepinone nucleoside **(53c)**[66c] and an aromatic 2-oxopyrimidine riboside **(53d)**[66d] have been shown to be potent inhibitors of cytidine deaminase. Compound **(53d)** is the only cytidine deaminase inhibitor that is endowed with antitumor activity. Co-administration of **(1)** and a cytidine deaminase inhibitor greatly increases the plasma half-life of the drug, and may increase its therapeutic effect. However, the long term inhibition of crucial metabolic enzymes may have toxic side-effects.[67]



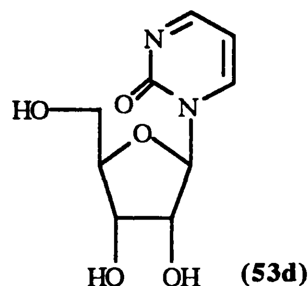
(53a)



(53b)



(53c)



(53d)

3. Compound **(1)** is S-phase cell cycle specific, thus it has to be given continuously due to its short half-life which is very inconvenient and sometimes very painful for the patient. It has low selectivity to cancer cells and hence has toxic side-effects. The destruction of normal white blood cells also leads to a weak immune system, leading to infections.[68]

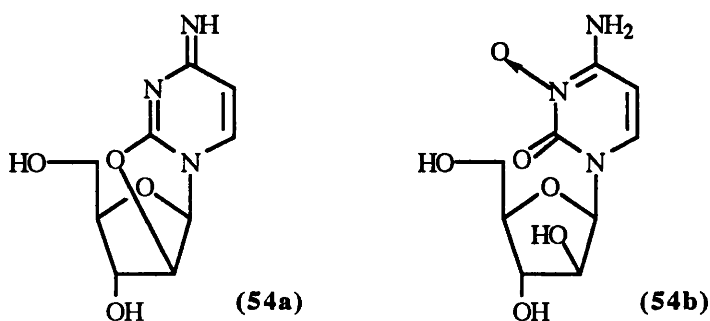
Compound **(1)** has found widespread use in the treatment of a number of cancers, in particular various leukaemias, including acute lymphoblastic, acute myeloid and chronic myeloid (blast phase)[69]. As with many anti-cancer drugs,

(1) is especially useful in combination with other agents, in particular compound (**25**), for example in the treatment of acute lymphoblastic leukaemia of childhood.[70] A number of recent studies have demonstrated the value of i.p chemotherapy with (**1**) for patients with ovarian cancer who have low or minimal bulk disease confined to peritoneal structures.[71] In the treatment of a number of viral infections, (**1**) inhibits the replication of most DNA viruses.[72] All the herpes viruses examined are sensitive to (**1**). These include the human herpes virus, herpes zoster and cytomegalo viruses, as well as HSV. Vaccinia virus[74] and SV40[75] are also inhibited by (**1**), while adeno viruses[72] have been the only DNA viruses found to be either insensitive, or only slightly sensitive to (**1**). Most RNA viruses are insensitive to (**1**), the exceptions to this are the oncorna viruses whose replication requires DNA synthesis *via* reverse transcriptase, and the rhabdo viruses.[72]

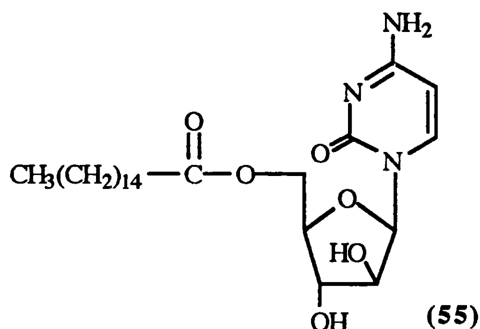
If administered by mouth (**1**) is not effective, because less than 20% of a dose is absorbed from the gastrointestinal tract.[76] After intravenous injection it disappears rapidly from the plasma, 80% being excreted in the urine within 24 hours, 90% as the inactive uracil arabinoside and less than 10% as the unchanged compound. Compound (**1**) diffuses across the blood-brain barrier only moderately. After deamination, mainly in the liver, the drug disappears from the blood in two phases, the first with a half-life of 12 minutes and the second of about 2 hours.[65]

Several prodrugs of (**1**) have been prepared by a number of researchers, in an attempt to combine deaminase resistance with prolonged tissue retention. An important prodrug of (**1**) is the 2,2'-anhydro derivative, cycloC (**54a**), which has been shown to have positive therapeutic value both in experimental animal models and in man.[77a] Sustained levels of free (**1**) are observed in plasma

following treatment with **(54a)** because the 4-imino group in this molecule is deaminase stable and because cleavage of the 2,2'-anhydro bond to form **(1)** occurs relatively slowly, by chemical as opposed to enzymatic cleavage. The N-oxide **(54b)** also resists cytidine deaminase and is converted slowly to **(1)** by reduction.[77b]



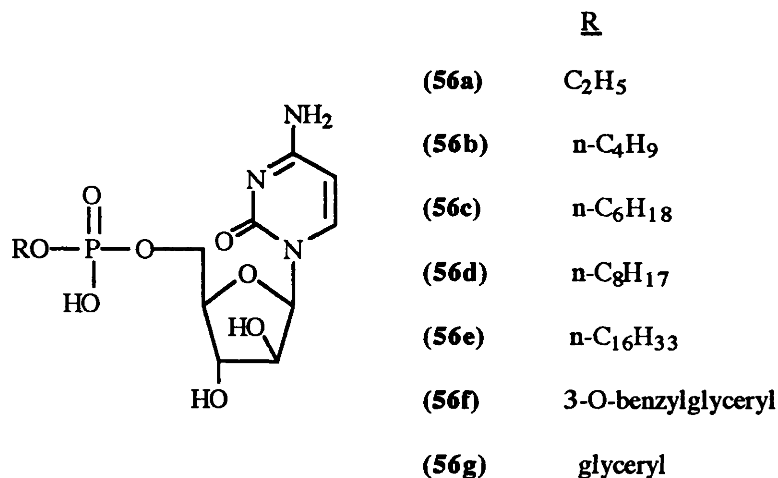
More extensive latentiation has also been achieved with long chain 5'-O-acyl derivatives and more recently with 'multiply latentiated' derivatives of **(1)** *via* acylation of the 2' and 3' or N-acylation, which were prepared with a view to increasing water solubility and prolonged life of the active moiety.[78-82] A number of prodrug derivatives possess greater antitumor activity against L1210 leukaemia than either **(1)** or **(54a-b)** and are essentially schedule independent. The most noteworthy 5'-ester derivative of **(1)** seems to be 5'-O-palmitoyl-araC **(55)**.[78]



In vivo studies showed that the optimum dose and schedule for **(1)** gave an ILS% of 147, whilst the optimum dose and schedule for **(55)** gave an ILS% of 139. It has been proven that such derivatives may protect the parent drug from

catabolic degradation; however, this *in vivo* study indicated no therapeutic advantage of **(55)**, perhaps due to poor drug absorption

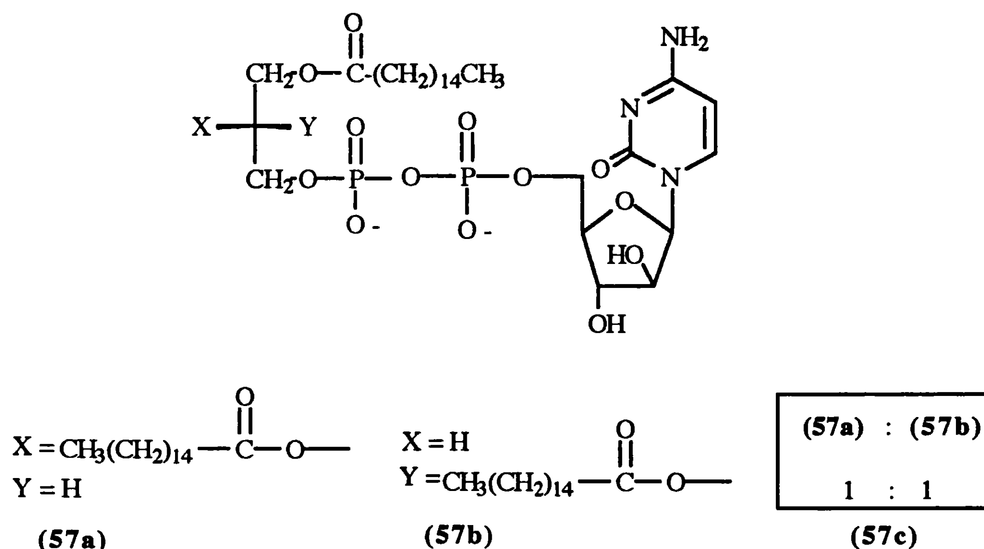
Another attempted approach was the preparation of 5'-phosphodiester derivatives of araC which was investigated in order to overcome cytidine deaminase deactivation and to by-pass kinase based resistance. Compounds **(56a-g)** were prepared and tested for growth inhibition of cultured L1210 leukaemia, and their activity was compared to that of **(50)**.[79] The cytotoxicity of the simple 5'-(alkyl phosphate) esters **(56a-e)** toward L1210 cells appeared to obey an inverse structure activity relationship with respect to alkyl chain length, with the n-butyl **(56b)** and the n-hexyl **(56c)** derivatives being about half as active on a molar basis as the ethyl ester **(56a)**. The severalfold higher activity of the glyceryl **(56g)** and the O-benzylglyceryl **(56f)** may be due to the β-hydroxy group facilitating hydrolysis of the phosphate bond *via* intramolecular nucleophilic catalysis, thus allowing the activation of the prodrug.



A further investigation by another team of researchers was to prepare phospholipid derivatives of **(1)** as prodrugs with enhanced catabolic stability and as with the series **(56a-g)**, in an attempt to overcome kinase based resistance. AraCDP-L-dipalmitin **(57a)**, araCDP-D-dipalmitin **(57b)** and the

racemic mixture araCDP-D,L-dipalmitin (**57c**) were tested *in vivo* with mice, the results were expressed in terms of %ILS.[80]

Some favourable properties found for **(57a)** are; superior antitumor activity, independent of the treatment schedule, sustained release form, resistance to hydrolysis by cytidine deaminase, rapid interaction with serum lipoprotein, which may have a role in the transport of the conjugate and solution by sonication.



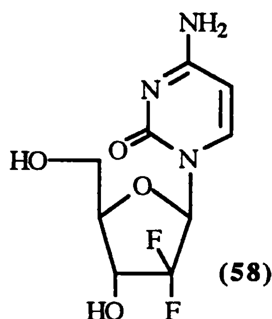
X = $CH_3(CH_2)_{14}$—C—O—
Y = H
(57a)

X = H
Y = $CH_3(CH_2)_{14}$—C—O—
(57b)

(57a) : **(57b)**
1 : 1

(57c)

Recently, another novel analogue of **(42)** has been reported, 2',2'-difluorodeoxycytidine, dFdC **(58)** with good activity against human leukaemic cell lines and murine solid tumors.[84]



(58)

Compounds **(1)** and **(58)** each differ from the parent nucleoside deoxycytidine by a modification at the 2' position of the sugar moiety. Both

nucleoside analogues inhibit cellular proliferation in S phase which causes cells to accumulate at the G_1-S phase boundary. The finding that the cytotoxicity of both drugs may be reversed by **(42)** suggests that **(1)** and **(58)** may be activated by a common metabolic pathway.

The fact that **(1)** must be present in the envirionment of the cancer cells for a sufficient period of time so that all or most of the cancer cells would have attempted to divide at least once for optimal cell kill has already been emphazised due to the prodrug's short half-life of about 2 hours. An interesting tool for slow-release intrathecal therapy has been the employment of multivesicular liposomes containing **(1)**. The intrathecal half life of the liposome-encapsulated drug was found to be 148 hours in a Sprague-Dawley rat model. The prolonged maintenance of a therapeutic drug level may increase efficiacy and the elimination of the very high peak level may decrease toxicity.[85,86]

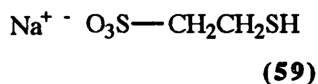
1.5 Cyclophosphamide (25)

(IUPAC systematic name: 2-[bis-(2-chloroethyl) amino] tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide monohydrate).

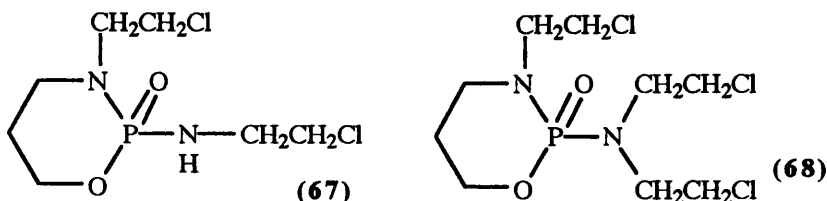
The first reported synthesis of **(25)**[87] was *via* treatment of N,N-bis-(β-chloroethyl) phosphamide dichloride with propanolamine in the presence of triethylamine and dioxane. Compound **(25)** has a wide spectrum of documented clinical use. As a single agent it is potentially curative in Burkitt's lymphoma.[88] In combination with other anti-neoplastic agents, as in the combination regimen for the induction therapy of non-Hodgkin's lymphomas, for example, BACOP[89] or COP[90] and adult acute leukaemia, for example, COAP[91] and sometimes replaces **(27)** in the MOPP therapy of Hodgkin's disease.[92]

Compound **(25)** is frequently used with **(20)** in the treatment of breast cancer.[93]

Compound **(25)** is a prodrug, thus by definition it has no action of its own. In the liver it is converted to 4-hydroxycyclophosphamide **(60)**, this metabolite, in its open chain form **(62)**, decomposes non-enzymatically to phosphoramide mustard **(63)** and acrolein **(66)**.[94,95] The urotoxicity of **(25)** is prevented by ingestion of mesna **(59)** which de-toxifies **(66)**.[96]

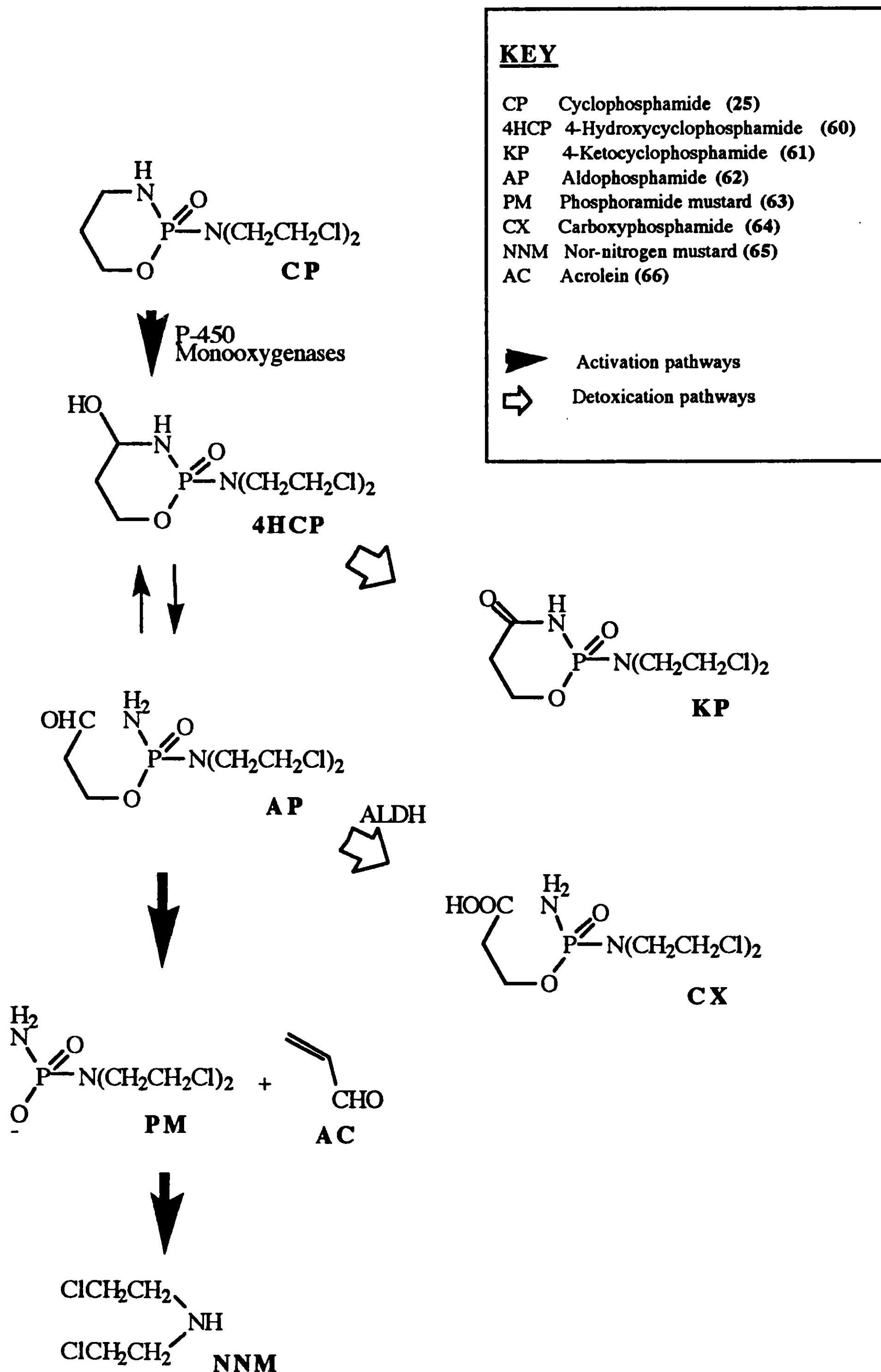


Excretion of **(25)** (50 to 70% of a dose) occurs in the urine within 48 hours, one third as parent drug and two thirds as metabolites. Its plasma half life is 4 to 6 hours. Peak concentrations are reached within 1 hour after an oral dose.[97] Although a great number of analogues of **(25)** have been synthesized and tested, only two have shown sufficient promise to receive significant clinical testing, isophosphamide **(67)** and trilophosphamide **(68)**.[98]

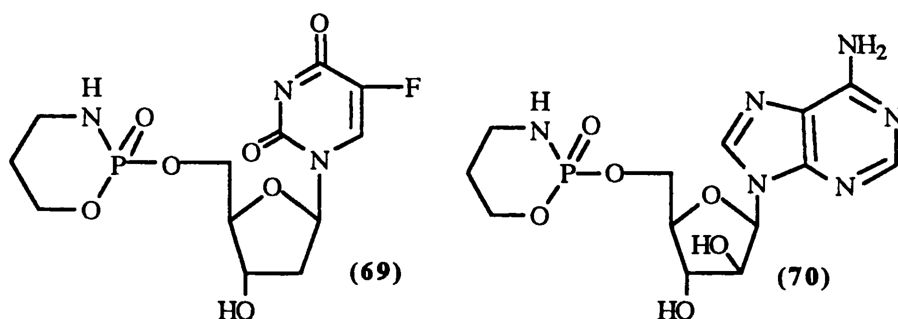


Compound **(68)** appears to be similar to **(25)** in its effects but has been suggested to be better tolerated orally. Compound **(67)** has been the more extensively used clinically of the two and has been claimed to be more effective against solid tumors. However, the drug has not been compared directly with **(25)** at equally toxic doses. The metabolism of **(67)** and **(68)** are analogous to that of **(25)**.

Figure 5 Schematic representation of the metabolism of (25)[99]



The mechanism of action of **(25)**, is somewhat different than other alkylating agents due to its enzymatic activation. Thus researchers have attempted to use the structural part of **(25)** to allow the enzymatic activation of other chemotherapeutic agents. 5-Fluoro-5'-(2-oxo-1,3,2-oxazaphosphorinan-2-yl)-2'-deoxyuridine **(69)** and 9-[5'-(2-oxoazaphosphorinan-2-yl)-β-D-arabinosyl]adenine **(70)** were prepared in the hope that they would undergo activation *in vivo*, by hepatic P-450 dependent mixed function oxidases in an analogous manner to **(25)**.[100a-b]



However, both **(69)** and **(70)** were only minimally biotransformed when incubated in-vitro with mouse hepatic microsomal preparations under conditions where **(25)** was extensively degraded. The poor substrate properties of **(69)** and **(70)** for the mixed function oxidases are most likely due to their low lipid solubility. It has been reported that lipophilic compounds are usually more readily biotransformed than their hydrophilic analogues, presumably because the cytochrome P-450 complex is intimately associated with lipoidal membranes of the endoplasmic reticulum.[101]

1.6 Retroviruses

The study of retroviruses has made an important contribution in the understanding of the molecular basis of cancers in recent years. The main feature

of a retrovirus is that its genetic material is not DNA , but RNA. The virus inserts its strand of RNA into host cells and then a viral enzyme called reverse transcriptase makes a DNA copy of the chain of RNA. The viral DNA then becomes part of the cell's DNA.[102]

In several animal species, including cats, chickens and mice, retrovirus-induced lymphomas and leukaemias are amongst the commonest naturally occuring neoplasms. The search for a related virus in man led eventually to the isolation of human retroviruses. All human retroviruses isolated and characterized to date are T-lymphotropic. The family of human T-lymphotropic retroviruses is HTLV and three distinct sub-types have been identified. HTLV-I is the etiological agent of adult T-cell leukaemia, HTLV-II was first isolated from a patient with hairy cell leukaemia but has not been linked to any human disease. More recently, a third member of this family, HTLV-III, has been shown to be the etiological agent of the aquired immunodeficiency syndrome, AIDS[103,104] (Section 1.7).

The most important contribution to cancer research to emerge from the study of retroviruses was the discovery that certain viruses contained transforming genes (v-oncs) that were ultimately derived from cellular genes. The cellular equivalent of the oncogenes (c-oncs) have been implicated in a wide range of viral and non-viral cancers.[102-104]

The idea that virally induced leukaemias do occur in the absence of extensive viral replication unexpectedly emerged from the bovine leukaemia system. There the etiological agent BLV was detected only in cells *in vitro.* Further studies showed that the BLV provirus is completely "silent" *in vivo.* A point of great interest is the fact that BLV causes B-cell lymphomas in cows, but when inoculated into sheep it often produces T-cell cancers. Recently it was reported

that rabbits treated with BLV can develop an AIDS-like immunosuppression. Thus , the same virus can cause three types of disorders of lymphocytes in three different systems: hyperproliferation of B-cells, hyperproliferation of T-cells and a suppression of T-cells. A cat virus FeLV also offers interesting lessons and questions. FeLV was shown to cause cat leukaemia and recently FeLV has been shown to cause an AIDS-like disease more frequently than it causes leukaemia. Thus, within the same species (cat) the different effects are achieved. The exact mechanism of action and the reason for the particular disease caused by the retrovirus in different systems and in the same system are yet to be determined.[103]

1.7 Acquired immunodeficiency syndrome (AIDS)

It was not until 1981 that acquired immunodeficiency syndrome (AIDS) was first recognised as a distinct clinical entity, alerting the world to a new and fatal pandemic immunosuppressive disease.[105] The clinical signs indicative of AIDS include weight loss, generalised lymphadenopathy and the patient develops a number of life threatening opportunistic infections and malignancies,[106] primarily *Pneumocystis carinii* pneumonia (PCP) and Kaposi's sarcoma.[107] A number of other bacterial (e.g. *Mycobacterium avium-intracellulare*), viral (e.g. cytomegalovirus, herpes simplex virus, Epstein-Barr virus) and fungal (e.g. *Candida* species) infections take advantage of a weak immune system. Some AIDS patients also suffer from neurological diseases which include peripheral neuropathy and fulminant dementia.[108]

Two related retroviruses can cause AIDS, called HIV-1 and HIV-2, both believed to originate from Africa, there also appear to be several different strains of HIV-1 and HIV-2. HIV-1 is found predominantly in Central Africa and HIV-2 appears more frequently in West Africa. The genomes of HIV-1 and HIV-2

are only about 50% homologous at the nucleotide level. However, the two viruses contain the same complement of genes and appear to attack and kill the same human cells by much the same mechanisms.[109]

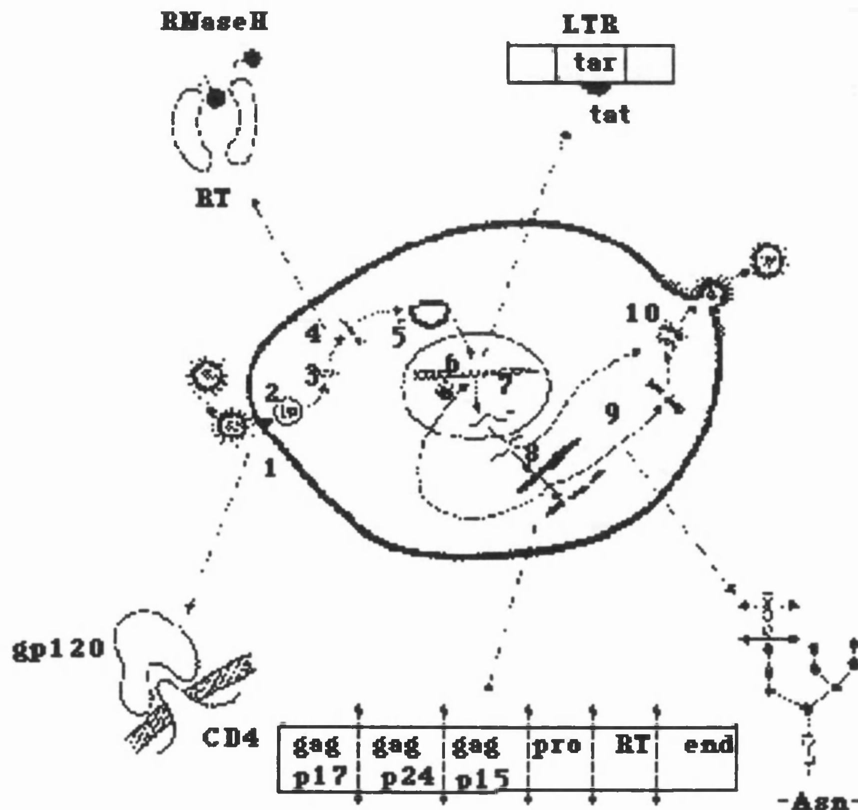
HIV-1 is the virus that was isolated in 1983 and has gone by several names, including human T-cell lymphotropic virus type III (HTLV-III), lymphadenopathy-associated virus (LAV) or AIDS-associated retrovirus (ARV). virtually all AIDS cases in the U.S. are associated with HIV-1 infection. It is the virus upon which most research has been done and hence the most characterized virus.[110,111,112] HIV-2 was isolated in 1986 from West African AIDS patients.[113,114] The two viruses share similar biological properties, most of the knowledge gained on HIV-1 research seems to apply to HIV-2. HIV-1 and HIV-2 are spread by either intimate sexual contact, or the administration of infected blood products or by the maternal-foetal route. For an unknown time-span after initial infection the carrier may remain healthy and will not have produced antibodies to the virus and the patient is termed antibody-negative. Some patients sero-convert and become antibody positive. Patients may develop subclinical illnesses known as AIDS related complex (ARC) which includes lethargy, skin ailments and general reduction of immune function. Other patients will go on to develop 'full blown' AIDS. AIDS in almost all cases eventually leads to death of the patient.[115,116] In this thesis, HIV will be used to refer to HIV-1 since most of the research discussed applies specifically to HIV-1, HIV-2 will be distinguished only when necessary.

A similar disease to AIDS is found in African green monkeys and the causative agent was designated simian immunodeficiency virus, SIV also called simian T-cell lymphotropic virus type III, STLV-III. Research has established that SIV has genes that encode structural and regulatory proteins analogous to

those found in the human viruses. The research also indicated that SIV and HIV-2 are more closely related evolutionary to each other than either is to HIV-1. An hypothesis of HIV origin is that SIV crossed species and adapted to humans, and are now spreading throughout their new environmental niche.[117]

HIV destroys the body's immune system. HIV has a long latency period , that is, people can be infected for several years, spreading it to others, before they exhibit the symptoms that typify AIDS. This delay between infection and the appearance of the disease in the patient is characteristic of a group of viruses known as lentiviruses. "Lenti" means "slow", it refers to the time it takes these viruses to cause disease to develop.[118] Several strategies are employed to combat HIV and are based on the different targets in the virus replicative cycle.

Figure 6 Schematic representation of HIV replicative cycle[119]



Key

gp120, viral envelope glycoprotein; **CD4**, T4 cell surface receptor; **RT**, reverse trancriptase; **tat**, trans acting transcriptional activator; **LTR**, long terminal repeat; **tar**, transactivator responsive region; **gag**, group-specific antigens; **pro**, protease; **end**, endonuclease.

Stage (Possible treatment)

1. Binding to target cell[120] (Antibodies to virus or cellular receptors: genetically engineered soluble CD4 proteins **(71)**.[121,122] AL-721 **(72)**,[123,124] may hinder binding by fluidising the host cell membrane, dextran sulphate **(73)**,[125] blocks HIV association to host cells).

   Fusion of virus with target cell (Antibodies or drugs that block the fusogenic domain of the virus, silicotungstate **(74)** may modify cell membrane).[126]

2. + 3. Entry into target cell and uncoating of RNA (Uncoating inhibitors, e.g. amantadine **(75)**).[127]

4. Transcription of RNA to DNA by reverse transcriptase[128] (reverse transcriptase inhibitors, e.g. **(2)** and other dideoxy or didehydro-dideoxynucleoside congeners (Section 1.8)).

   Degradation of RNA by RNase activity (encoded by viral pol gene) (RNase H inhibitors).

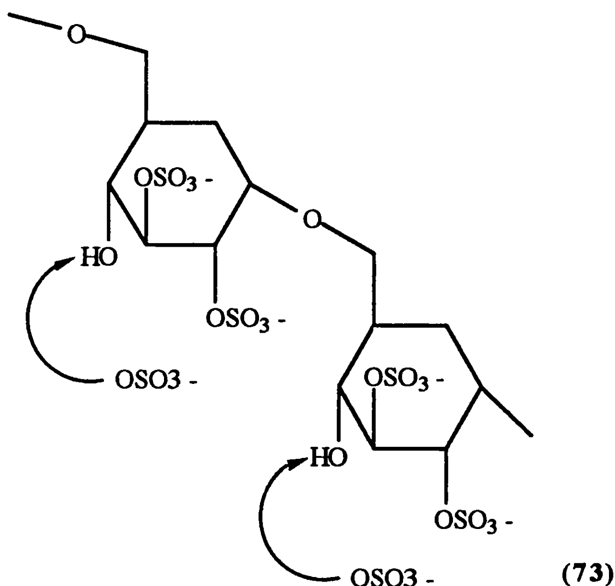
5. Circularization and integration of pro viral DNA into cellular genome (Agents that inhibit pol-encoded integrase function; as yet unidentified).[129]

6. Replication of proviral DNA (if integrated, concomitantly with the replication of cellular genome).

7 + 8. Transcription of proviral DNA/Translation of viral RNA (Inhibitors of tat protein; mutant tat protein molecules; TAR inhibitors; "Pseudo" TAR molecules; rev inhibitors; anti-sense oligonucleotides against tat or rev; stimulator of the nef gene).[130-134]

9. Viral component production (Myristylation, glycosylation inhibitors (e.g. castanospermine (**76**) and inhibitors of trimming glucosidase); protease inhibitors (e.g. synthetic peptide analogues; aspartyl protease-specific inhibitors)).[135,136]

10. Packaging (Antisense construct against the gene). Viral budding ((**75**) and interferons (**77**) or interferon inducers; antibodies to viral antigens which may be associated with viral release).[137]



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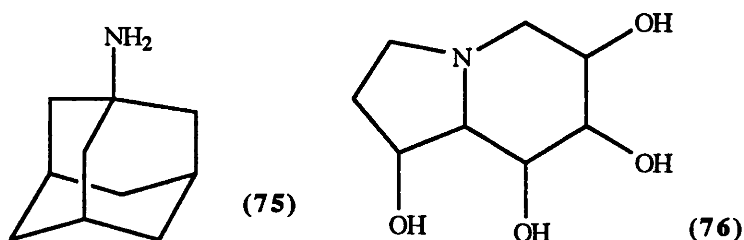
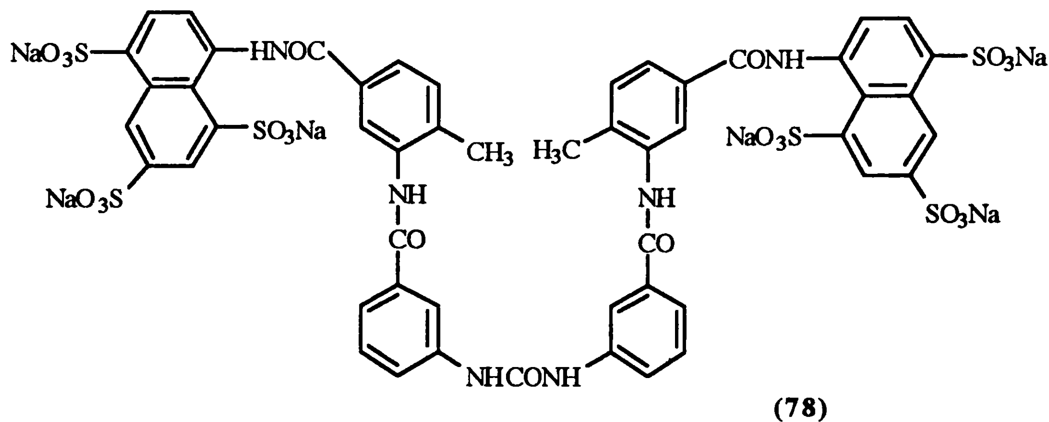


Compounds acting at different targets may show synergistic activity when used in combination. Even compounds acting on the same target but which differ in pharmacological or toxicological behaviour may prove benificial in combined or alternate use. Such procedures may maintain efficiacy and reduce drug dosage and thus diminish toxicity.

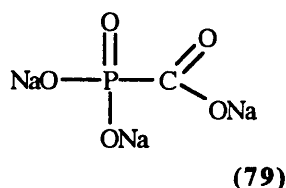
1.8 Reverse transciptase (RT) inhibitors

Suramin **(78)** was known as a potent inhibitor of retrovirus-associated reverse transcriptase before the discovery of HIV and was the first anti-HIV chemotherapeutic agent to be used in the treatment of AIDS.[138]



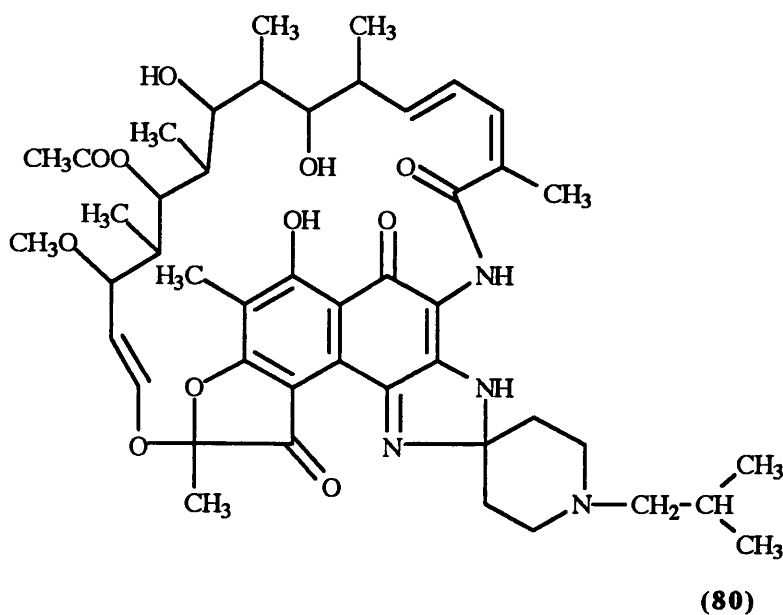
Compound **(78)** was found to inhibit HIV *in vitro* and in AIDS patients. However a treatment course with **(78)** did not improve the immune system of patients and prolonged treatment proved to be extreamly toxic so its use in the treatment of AIDS was terminated.[124]

Phosphonoformate (or foscarnet) **(79)** is known to inhibit RT *in vitro* by interacting with a site where pyrophosphate is split off during the polymerisation process.[139,140] Clinical trials with **(79)** are currently in progress.[141]



(79)

Rifabutin (or ansamycin) **(80)** has been shown to inhibit RT and is also able to inhibit HIV *in vitro*.[142] However no anti-viral response was noted in a clinical trial with **(80)**.[124] Antimoniotungstate (HPA-23) **(81)** has also been found to inhibit RT *in vitro*. Clinical trials have shown a slight improvement in symptoms.[143]



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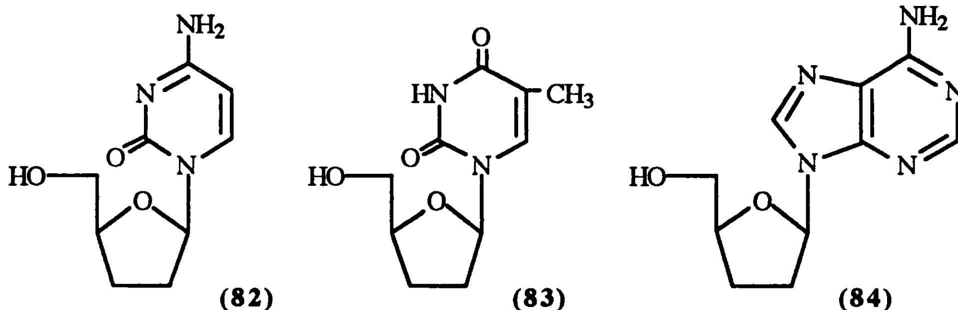
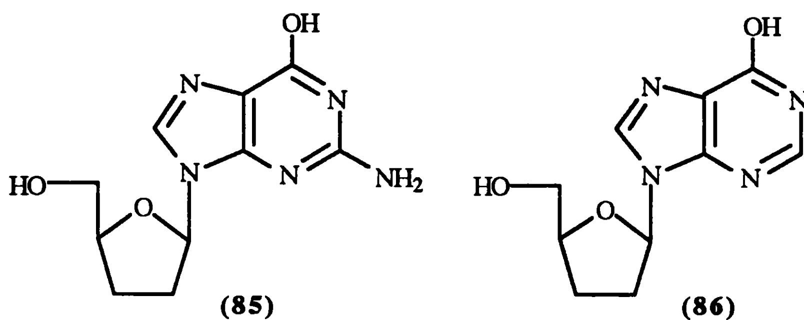
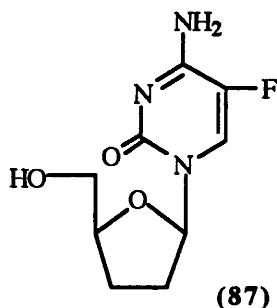
The search for effective inhibitors of RT and thus of HIV replication has led to the discovery of a wide variety of nucleoside analogues with activity.

2',3'-dideoxynucleosides:

2',3'-dideoxycytidine (**82**), 3'-deoxythymidine (**83**), 2',3'-dideoxyadenosine (**84**), 2',3'-dideoxyguanosine (**85**), 2',3'-dideoxyinosine (**86**), 5-fluoro-2',3'-dideoxycytidine (**87**).

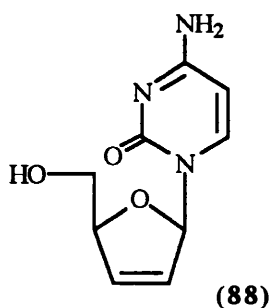






2',3'-didehydro-2',3'-dideoxynucleosides:

2',3'-didehydro-2',3'-dideoxycytidine (**88**), 2',3'-didehydro-3'-deoxy-thymidine (**89**), 2',3'-didehydro-2',3'-dideoxyadenosine (**90**), 2',3'-didehydro-2',3'-dideoxyguanosine (**91**), 2',3'-didehydro-2',3'-dideoxyuridine (**92**), 2',3'-didehydro-2',3'-dideoxyinosine (**93**).



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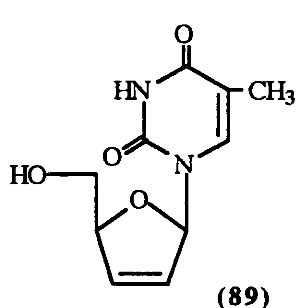

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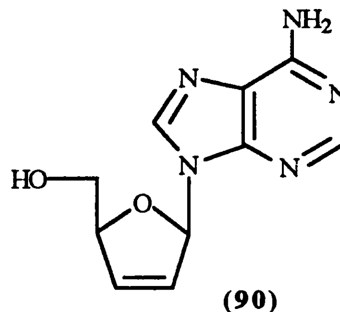

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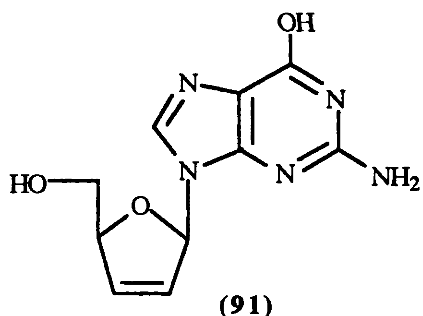

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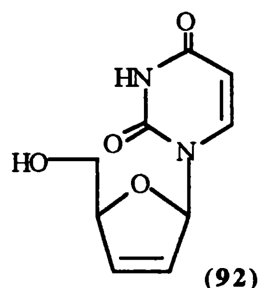

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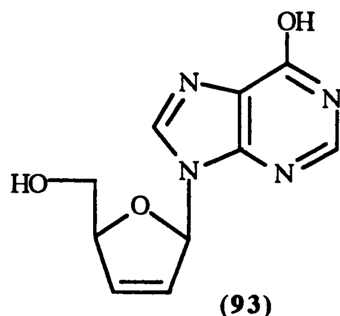

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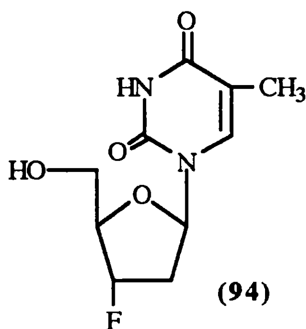
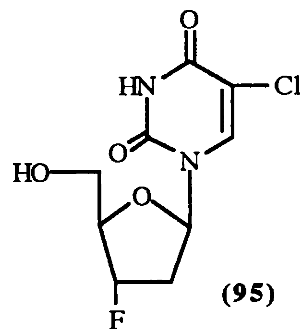
3'-fluoro-2',3'-dideoxynucleosides:

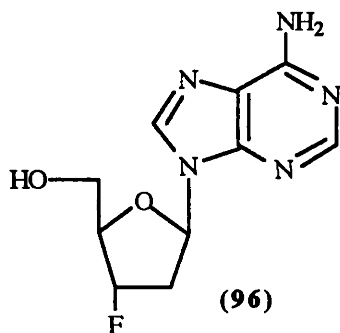
3'-fluoro-3'-deoxythymidine (**94**), 3'-fluoro-2',3'-dideoxy-5-chlorouridine (**95**), 3'-fluoro-2',3'-dideoxyadenosine (**96**), 3'-fluoro-2',3'-dideoxyuridine (**97**).



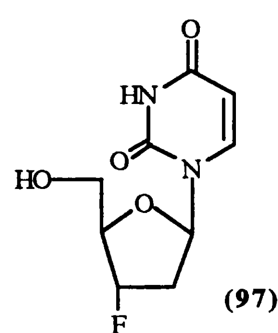
(**94**)



(**95**)



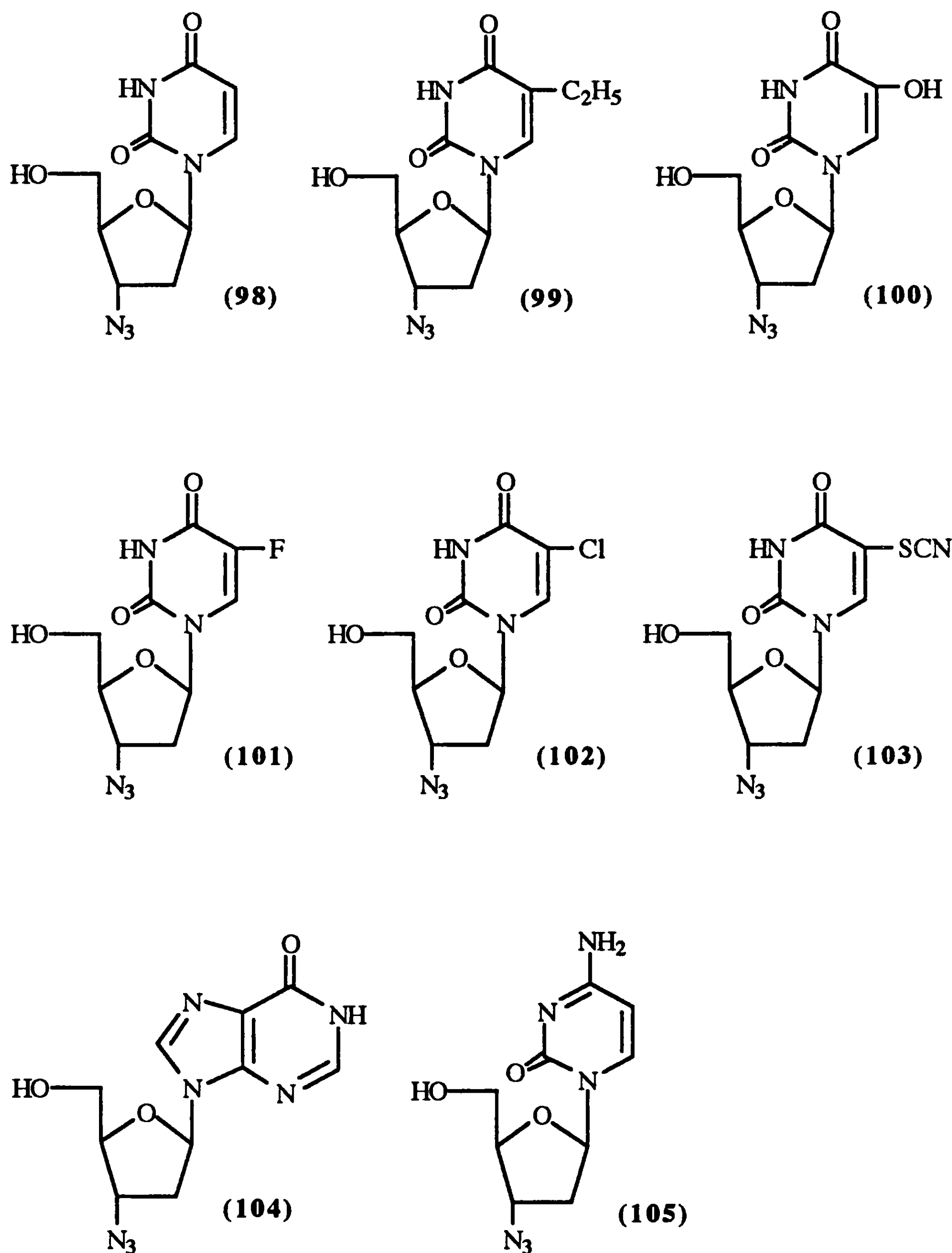
(**96**)



(**97**)

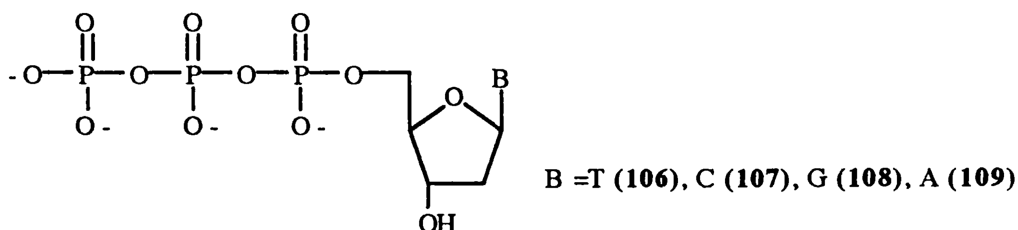
3'-azido-2',3'-dideoxynucleosides:

3'-azido-3'-deoxythymidine (**2**), 3'-azido-2',3'-dideoxyuridine (**98**), 3'-azido-2',3'-dideoxy-5-ethyluridine (**99**), 3'-azido-2',3'-dideoxy-5-hydroxyuridine (**100**), 3'-azido-2',3'-dideoxy-5-fluorouridine (**101**), 3'-azido-2',3'-dideoxy-5-chlorouridine (**102**), 3'-azido-2',3'-dideoxy-5-thiocyanouridine (**103**), 3'-azido-2',3'-dideoxy-2,6-diaminopurineriboside (**104**), 3'-azido-2',3'-dideoxy cytidine (**105**).



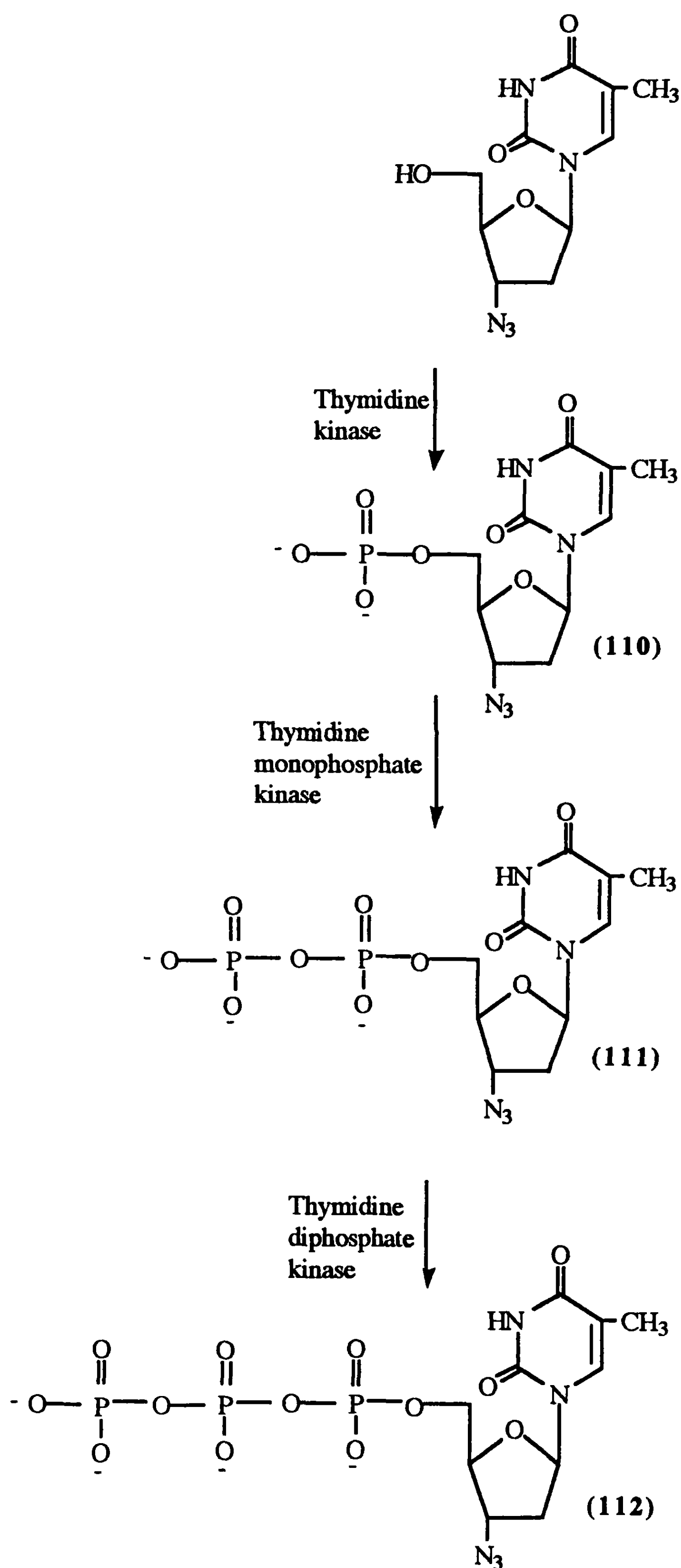
Compound (2) was prepared first in 1964[144] and was shown to inhibit the retrovirus Friend lymphatic leukaemia helper virus.[145] At present (2) is the only anti-HIV drug receiving wide clinical usage. Despite (2) being discovered early amongst nucleoside inhibitors of HIV, it remains one of the most potent and is the only one approved for general use in the treatment of HIV.

Compound **(2)** and other 2',3'-dideoxynucleoside analogues are believed to require activation to their triphosphate[146-148] (Figure 7) and then act at the reverse transcriptase step, where they can act in a dual fashion, either as competitive inhibitors with respect to the natural substrates (TTP **(106)**, dCTP **(107)**, dGTP **(108)**, dATP **(109)**) or alternative substrates leading to chain termination of DNA synthesis. Although both processes stop the production of viral DNA, it is not clear which is most important.



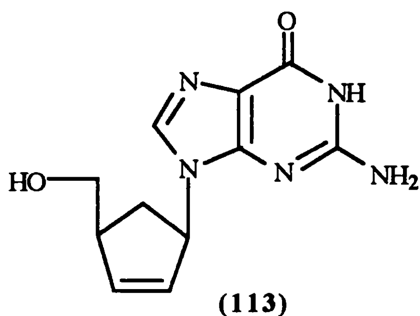
The 2',3'-dideoxynucleoside 5'-triphosphates exhibit a much greater affinity for the viral reverse transcriptase and cellular DNA polymerase γ, than for the cellular DNA polymerase α and β.[149] Only a few 2',3'-dideoxynucleoside analogues equal or approach **(2)** in potency against HIV in vitro. Some of these compounds, **(82)**, **(86)** and **(89)** have already been studied in clinical trials. However the clinical usefullness of **(82)** is limited by peripheral neuropathy.[150] The use of **(86)** is sometimes complicated by acute pancreatitis. There are some 2',3'-dideoxynucleosides, such as **(89)** and **(95)**, which are less potent than **(2)** but also less toxic to the bone marrow than **(2)** and thus have advantages over **(2)** in certain circumstances. Side effects of **(2)** include headaches, lowered white blood cell counts and suppression of bone marrow cell formation. Additionally, its short half life in the body necessitates frequent administration to maintain therapeutically effective levels.[151]

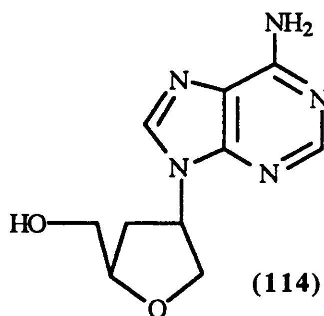
Figure 7 Activation of (2) *via* phosphorylation to AZTTP (112)[152]

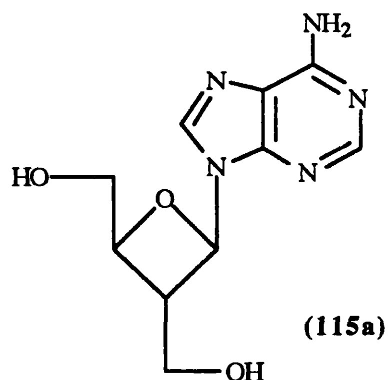


One theory to the myelotoxicity of **(2)** is that it leads to an accumulation of **(110)** in cells that have been exposed to the drug.[153] Compound **(110)** accumulates because it is a potent inhibitor of the enzyme TMP kinase that is needed for its further phosphorylation to **(111)**. The inhibition of TMP kinase results in the reduction in the supply of **(106)** and thus an inhibition of host cell DNA synthesis.

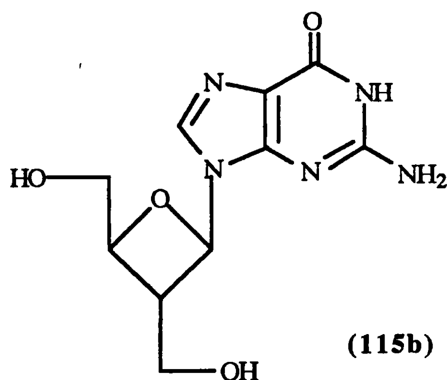
The news of resistant HIV strains[154] to **(2)** promoted the search for more unusual anti-HIV nucleoside analogues. Some acyclic and carbocyclic nucleoside analogues were shown to be promising for the treatment of retrovirus infections. Carbocyclic 2',3'-didehydro-2',3'-dideoxyguanosine **(113)**, IsoddA **(114)** and the carbocyclic oxetanocin analogues cyclobut-A **(115a)** and cyclobut-G **(115b)** are active against HIV.[155] The in vitro anti-HIV activity of 1-(2'-hydroxymethyl-5'-(1,3-oxathiolanyl))cytosine **(116a)** has been reported with potency equivalent to the most active dideoxynucleosides.[156] Replacing the sulphur by oxygen to give compound **(116b)** decreased the activity, while the sulphoxide gave inactive compounds.



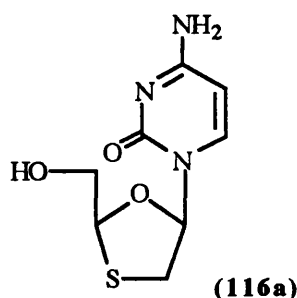




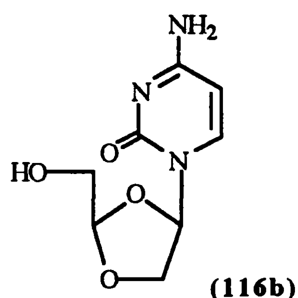
(115a)



(115b)

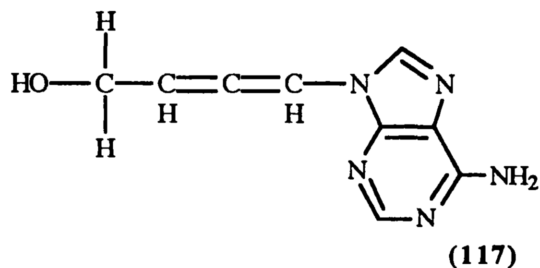


(116a)

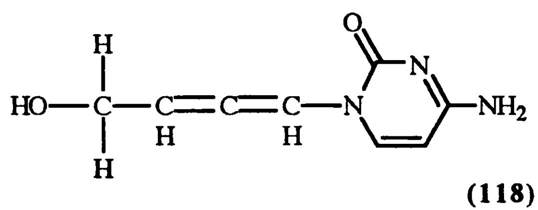


(116b)

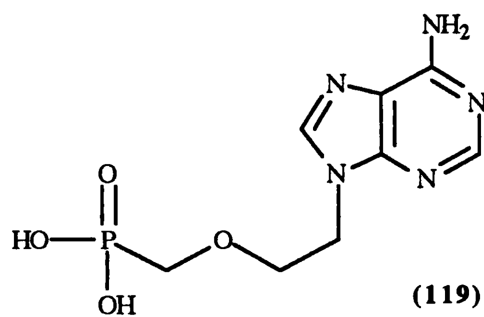
Acyclic nucleoside analogues adenallene **(117)**, cytallene **(118)**, 1-[9-(2-phosphonylmethoxyethyl)-adenine] (PMEA) **(119)** and [9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine] (PMEDAP) **(120)** have antiretroviral potential.[157,158] Compounds **(119)** and **(120)** are targeted at reverse transcriptase, presumably they are phosphorylated to their triphosphate in a similar manner to **(2)**, the thymidine kinase stage of this phosphorylation sequence being by-passed.



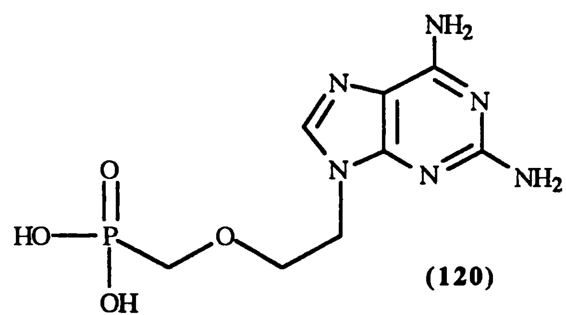
(117)



(118)



(119)



(120)

There is a large difference in the activities of the closely related nucleoside analogues to kill HIV (Table 3), which is of great interest.

Table 3 Inhibition of HIV by some RT inhibitors[151]

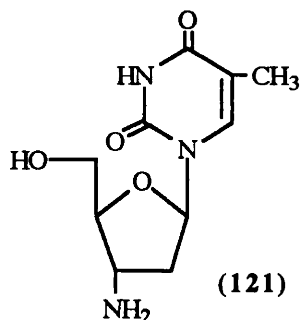
No.	ED_{50}/(μM)/MT-4 cells
(2)	0.003
(82)	0.06
(83)	0.2
(84)	6.3
(88)	0.13
(89)	0.05
(94)	0.001
(95)	0.38
(96)	50
(97)	0.04
(98)	0.36
(102)	0.72
(104)	0.3
(105)	3.1

Three possible explanations have been explored to account for the differences in activity:

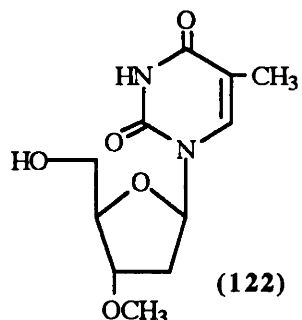
1. The nucleoside analogues may be able to affect the pools of physiological 2'-deoxynucleosides present in the cell to differing extents.
2. The nucleoside analogues may differ in their abilty to generate their corresponding 5'-triphosphates to inhibit RT.
3. There may be a difference in the abilty of these 5'-triphosphates to inhibit RT.

Some 2',3'-dideoxynucleosides and related compounds have been investigated in order to correlate activity with one of the above explanations.[159] The compounds studied were **(2)**, **(82)**, **(83)**, **(84)** and **(85)**. No correlation was found betwen anti-viral activity and the ability of the test compounds to modify the physiological 2'-deoxynucleoside pool sizes. Nor was there a correlation between anti-viral activity and the ability of the 5'-triphosphates of these compounds to inhibit purified RT, the 5'-triphosphates tested were practically equal in their ability to inhibit RT. However there was a correlation between the anti-viral activity of the compounds tested and their ability to generate their 5'-triphosphates.[160] Compound **(2)** generated a relatively high level of its triphosphate intracellularly, which correlates with its activity. The ability of **(83)** to generate its 5'-triphosphate was relatively poor and this correlates with the relatively low anti-HIV activity of **(83)**.

Other studies have shown that some compounds such as 3'-amino-3'-deoxythymidine **(121)**[161] and 3'-O-methylthymidine **(122)**[162] in their 5'-triphosphate form inhibit purified HIV-RT *in vitro*. Whilst the unphosphorylated form of **(121)** and **(122)** did not show any activity against HIV *in vitro*. A possible explanation for this result is that **(121)** and **(122)** are not phosphorylated efficiently (if at all) to their respective 5'-triphosphates by host kinases.



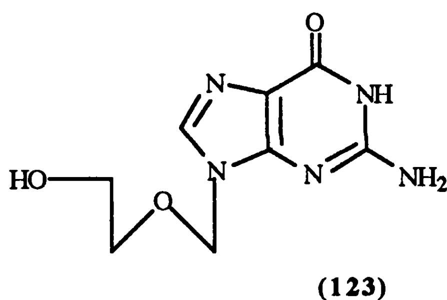
(121)



(122)

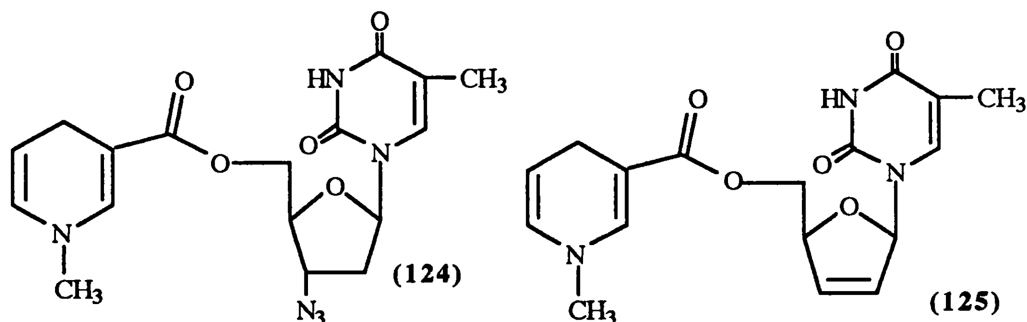
It thus seems conclusive that the activity of the nucleoside analogues against HIV depends on phosphorylation *in vivo* to their 5'-triphosphates by host kinase. The variation as substrates for host kinase seems to result in the variation in anti-HIV activity.

An anti-herpes nucleoside analogue acyclovir **(123)** is a poor substrate for cellular kinase, however its active form is the triphosphate, it must be activated to its triphosphate and this is achieved by viral kinases making **(123)** a very selective anti-viral agent. However, not used as an anti-HIV agent because **(123)** would not be activated to its corresponding triphosphate due to the lack of an appropriate kinase system.[163]

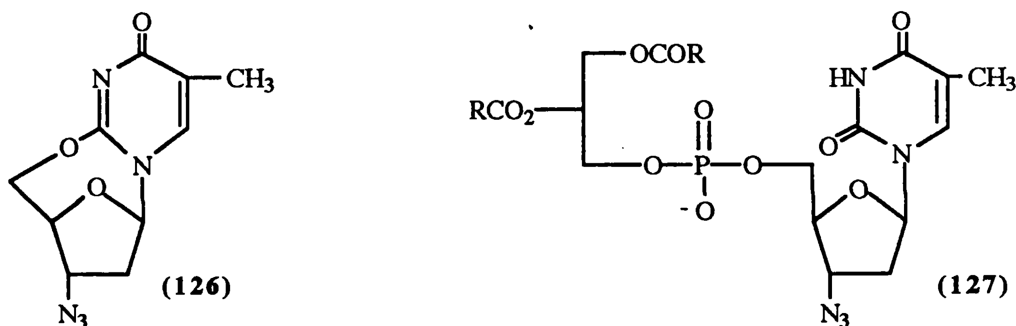


(123)

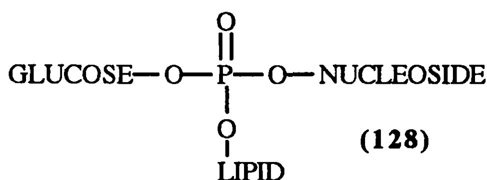
Interesting prodrugs of **(2)** have been reported in the literature. 5'-(1,4-Dihydro-1-methyl-3-pyridinylcarbonyl)-3'-azido-3'-deoxy-thymidine **(124)**.[164] This dihydropyridine prodrug can cross the blood brain barrier and this is of interest since HIV is known to infect the brain causing neurological disorders. Compound **(124)** has been shown to be more active against HIV and less toxic to bone marrow cells than **(2)**. 5'-(1,4-Dihydro-1-methyl-3-pyridinylcarbonyl)-2',3'-didehydro-3'-deoxythymidine **(125)** has also been prepared in an attempt to increase the efficiacy of **(89)**.[165]

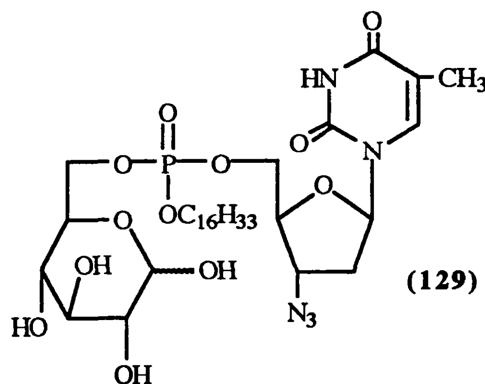


Another analogue of **(2)** is 2,5'-anhydro-3'-azido-3'-deoxythymidine **(126)** has been found to be an inhibitor of HIV *in vitro.*[166] **(126)** was slightly less active than **(2)** and it may be that **(126)** acts as a prodrug of **(2)** by undergoing intracellular cleavage of the 2,5'-anhydro linkage.

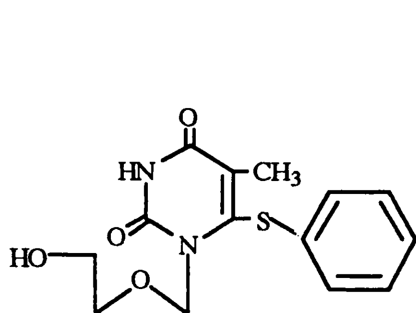


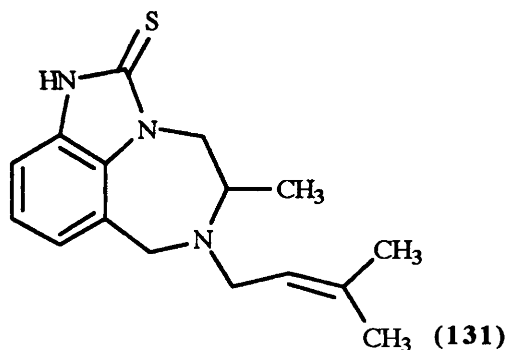
As mentioned in section 1.4, since chemotherapeutic nucleosides exert their biological effect as their corresponding nucleotides, prodrugs of these nucleotides may offer greater potential as chemotherapeutic agents. Phosphate diesters **(127)**, phospholipid derivatives of **(2)** have been shown to inhibit HIV *in vitro.*[167] Phosphate diester derivatives suffer from one disadvantage, they are charged and therefore may not be able to penetrate the cell membrane easily. Phosphate triesters such as **(128)** may have potential as drug transport and drug targeting prodrugs.[168] An analogue of this glucose-phospholipid drug delivery system **(129)** has been reported to inhibit HIV *in vitro,* further investigations into this derivative are currently in progress.[169]



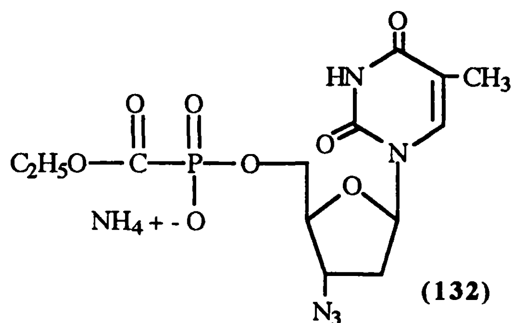


An interesting effect of the new derivatives HEPT **(130)**[170,171] and TIBO **(131)**[172] is that they have differing inhibitory effects on HIV-1 and HIV-2. Compounds **(130)** and **(131)** show a marked preference for HIV-1, the reasons for this are unclear. Both **(130)** and **(131)** act on RT but it is thought that they act in a different manner to most anti-HIV nucleoside analogues.





Another area of research is to combine RT inhibitors in the same structure such as the water soluble ammonium salt of 3'-azido-5'-(O-ethoxycarbonylphosphinyl)-3'-deoxythymidine **(132)**.[173]



The rationale for the synthesis of **(132)** was that it may be cleaved intracellularly to **(2)** and **(79)** *via* hydrolysis of the phosphate ester bond or to **(110)** by oxidative cleavage of the carbon-phosphorus bond. Compound **(132)** may be viewed as the prototype of a new class of 5'-blocked antiviral nucleoside analogues in which the blocking group itself has antiviral activity. Compound **(132)** is less active than **(2)** against HIV but is also less cytotoxic than **(2)**.

Even though HIV is a relatively new target for medicinal chemists, seemingly a never-ending list of novel nucleoside analogues are being reported to be active anti-HIV agents. Basically for the most part these novel nucleoside analogues work in much the same way as **(2)**. Recently published reviews may be consulted for a fuller account of all the nucleoside analogues active and inactive against HIV *in vitro*, produced to date.[151,155,174-176]

Most nucleosides with anti-HIV activity are 2',3'-dideoxy pyrimidines and purines and to a lesser extent their acyclic analogues. Many substitutions on the 2'- and/or 3'- of various ddN's have been done, 2'- or 3'- azido or fluoro substitutions, 3'-amino and 3'-O-methyl nucleosides. 3'- Azido or fluoro enhanced the activity of the ddpyrimidine nucleosides, but decreased the activity of ddpurine nucleosides. 3'-substitution with amino, alkyl, cyano, alkoxy, thioalkyl, thiocyanayo and non-fluorine halogens gave inactive compounds.

A 2'-ara fluoro substituent increased the activity of purine ddN's to greater extent than the pyrimidine ddN's. A 2'-fluoro in the erythro configuration in ddN's gave inactive compounds. A 3'-fluoro in the threo configuration resulted in inactive compounds while a 3'-fluoro in the erythro configuration gave active

compounds. A 3'-difluoro substitution gave an inactive compound, while 2',3'-difluoro substitution gave less activity than the 3'-fluoro derivative.

Introduction of a 2'-OH in the active 3'-azidopyrimidine ddN's abolished activity. A 2',3'-double bond inactivated purine but not pyrimidine ddN's. Fluorine substitution on the uridine ddN's base gave inactive compounds. The monomethylation of the 6-amino group in ddA or the 4-imino group in ddC enhances the activity of these compounds while introduction of ethyl, two methyl groups or benzyl abolishes activity. An unsubstituted sugar in purine ddN's is optimal for activity, while a halogen on the purine base results in less active and more toxic analogues.

Carbocyclic purine ddN's but not pyrimidine ddN's have anti-HIV activity. Acyclonucleosides of purine and pyrimidine have shown anti-HIV activity. Replacement of the 3'-carbon with S or O, but not N resulted in active purine and pyrimidine ddN's. Purine but not pyrimidine ddN's in which 3'-carbon and ring oxygen are transposed gave active compounds. A four membered sugar ring and its carbocyclic ddN analogues have shown anti-HIV activity.

Other compounds against targets other than RT must be found if the plague of HIV is to be comfortably controlled and much work is being conducted with this aim. Research is also being conducted for a vaccine against HIV, which is going to be essential, in the long term, to stop the spread of HIV.

1.9 Aims and objectives

The aims and objectives of this thesis is the synthesis of 5'-phosphate triester derivatives of **(1)** and **(2)** with a view to these compounds being inhibitors of cancer cells and HIV respectively. It was thought that groups could be found which would be hydrolysed intracellularly generating the free 5'-phosphate thereby in some instances by-passing kinase based resistance. Moreover, the reason for the lack of anti-HIV activity of many nucleoside analogues is that they are incapable of being phosphorylated by cellular kinases. Thus, preparation of some phosphate triester derivatives of some inactive nucleoside analogues are of great interest, in order to overcome their inactivity against HIV.

5'-Phosphate derivatives of **(1)** may also be resistant to cytidine deaminase and thus degradation to **(36)** may be avoided thereby increasing the efficacy of **(1)** as an anti-cancer agent. The probability that phosphate triesters of **(1)** and **(2)** may be more lipophilic than the nucleosides themselves suggests that the lipophilic derivatives would pass the blood brain barrier with greater ease.

Another aim of this thesis was to study the structure activity relationship of the 5'-phosphate triester derivatives of **(1)** and **(2)** which were prepared and to study the mechanism of action of these novel prodrugs. Thus, if the efficacy of **(1)** and **(2)** could be increased as chemotherapeutic agents, this approach and methodology would be applicable to a number of other nucleoside analogues.

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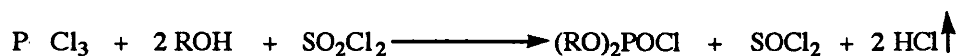
2.0 Design and synthesis of novel symmetric 5'-dialkyl phosphate triester derivatives of (1)

2.1 Introduction

A number of novel n-alkyl phosphate triester derivatives of the anti-cancer nucleoside analogue (1) were prepared by a rapid two step procedure, not necessitating prior sugar protection.[1]

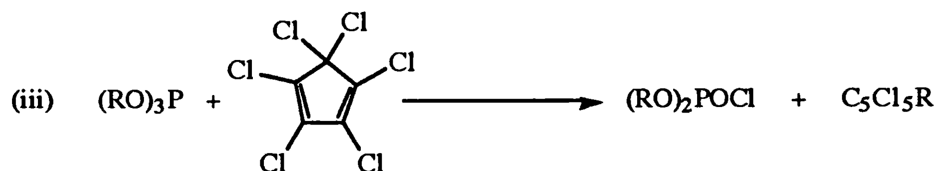
The first step involved the preparation of a series of dialkyl phosphorochloridates, which can be prepared by several alternative methods. Some of the methods for making dialkyl phosphorochloridates include:

(1) The reaction of two molar equivalents of alcohol with phosphorus trichloride in the presence of sulphuryl chloride using benzene as a solvent.[2]

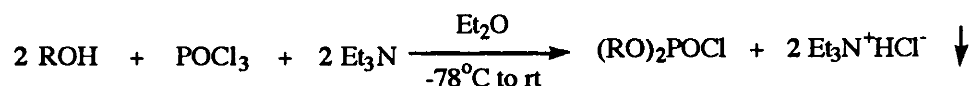


(2) Direct chlorination of dialkyl phosphites by N,N-dichloro-benzenesulphonamide.[3]

(3) The reaction of trialkyl phosphites with (i) sulphuryl chloride[4], (ii) thionyl chloride[5] or (iii) hexachlorocyclopentadiene[6].

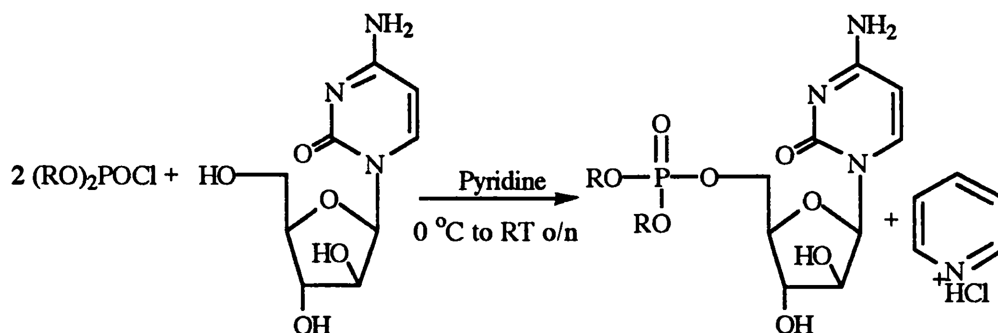


There are other methods for the preparation of dialkyl phosphorodichloridates.[7] However, the method that was chosen for the preparation was the reaction of an ethereal solution of two molar equivalents of the normal alcohol, in the presence of the base triethylamine, with phosphoryl chloride.[8]

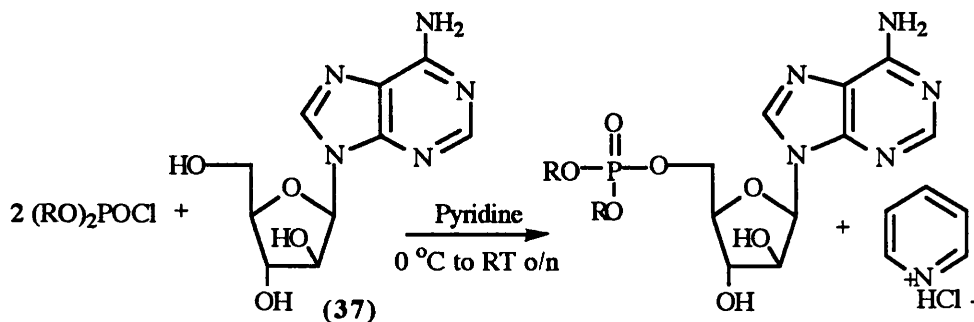


Pyridine has been reported as being the base employed in this type of reaction, but the phosphorochloridates can react with pyridine or pyridine hydrochloride on heating to yield alkyl halides and this causes problems if the products are to be purified by distillation.[9] This procedure was chosen from the array of alternative methods, because it was relatively easy to control, also the starting materials were easy to handle and very accessible.

The second step involved the reaction of the phosphorochloridates with **(1)** in pyridine to give a series of 5'-dialkyl esters of araCMP. Pyridine is often used as the solvent and base in similar reactions due to the lack of solubility of most nucleosides in other common solvents.[10] Compound **(1)** is insoluble in most solvents and has to be heated to reflux in pyridine and then cooled in ice to give a super-saturated solution.



The main disadvantage of this route is that the phosphorochloridate can react not only at the 5'-OH but also at the 2'-OH, 3'-OH and/or the NH_2 of the base. Selective protection and deprotection of these reactive groups would have left the 5'-OH as the only free reactive centre. The primary 5'-OH is more reactive than the secondary 2'-OH and 3'-OH, whilst the base NH_2 is the least reactive, thus protection was not thought to be necessary. Previously, within this Department, a similar reaction had been shown to proceed well, with unprotected **(37)**.[11]

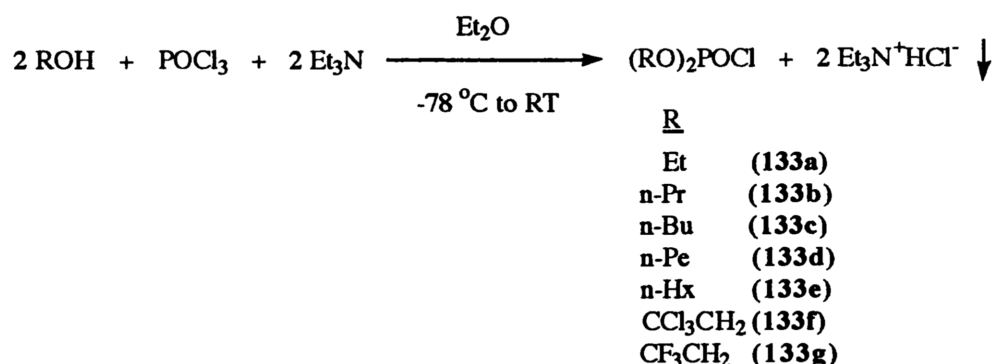


The series of 5'-dialkyl araCMP analogues produced were tested biologically with mammalian epithelial cells *in vitro* and the activties were correlated with lipophilicity.

2.2 Results and discussion

A series of n-alkyl phosphate triesters of the anti-cancer nucleoside analogue **(1)** was prepared *via* the simple two step procedure outlined in the introduction to this chapter.

The dialkyl phosphorochloridates (**133a-g**) were prepared by the reaction of two equivalents of alcohol with phosphoryl chloride in the presence of two molar equivalents of triethylamine with diethyl ether as the solvent. The impurities which are possibly produced in this reaction are the monoalkyl phosphorodichloridate and the trialkyl phosphate, also some hydrolysed products (if there is some water in the system). To minimise the formation of these unwanted products, all reagents were scrupulously dried, the reaction was carried out at -78 °C and the alcohol and base, in ether, were added slowly to the phosphoryl chloride, which was also in ether.



The reaction mixture was allowed to warm to ambient temperature and stirred overnight, then the precipitated triethylamine hydrochloride was removed by filtration under nitrogen. Compounds (**133a-d**) were all distilled under vacuum to give yields between 55% and 75%. It was necessary to distill (**133d**) very carefully as it was noted to decompose if overheated.

Even greater care was taken to prepare (**133e**) because distillation was expected to be very difficult. The preparation of (**133e**) was performed under dry nitrogen by extremly slow addition of hexanol to phosphoryl chloride at -78 °C in ether, this allowed isolation of pure (**133e**). Compound (**133f**) proved to be very difficult to produce in a pure form. 2,2,2-Trichloroethanol seemed to be a very reactive species, even at -78 °C and after several attempts there was

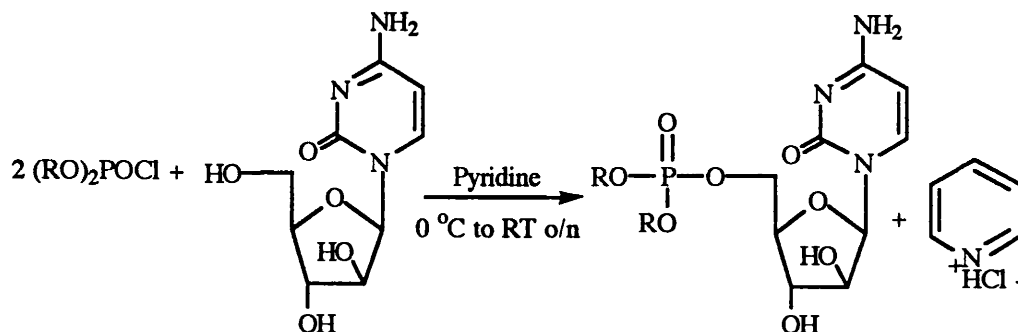
always a large amount of dichloridate and some trialkyl phosphate. Removal of the dichloridate under high vacuum was accomplished and the isolation of **(133f)** by Kugelruhr distillation was achieved to give the product as a white solid in a 20% yield. Compound **(133g)** was isolated after careful addition of 2,2,2-trifluoroethanol to phosphoryl chloride at -78 ^{o}C in diethyl ether followed by simple vacuum distillation, giving a yield of 66%.

Confirming their identity and purity, all the phosphorochloridates gave one peak ^{31}P NMR spectra with chemical shifts close to literature values.[12,13,14] The ^{13}C NMR and ^{1}H NMR spectra of **(133a-g)** confirmed their formation and purity. Phosphorus coupling was observed in the ^{13}C NMR spectra of all the compounds, but only to the two carbon atoms nearest to the phosphorus atom. An interesting facet of the ^{13}C NMR of **(133b-d)** series was that the nearer the carbon was to the phosphorus, the chemical shift would be more downfield. However with **(133e)**, the 2nd carbon from the phosphorus was more upfield than the 3rd carbon. In all the compounds **(133a-d)** and **(133f-g)**, the three bond coupling was slightly larger than the two bond coupling, probably due to the angular dependence of coupling constants[15], however with compound **(133e)**, the two bond coupling was slightly larger than the three bond coupling.

The 2,2,2-trihaloethoxy compounds **(133f-g)** also showed some interesting NMR spectra. The ^{13}C NMR of **(133f)** showed that the 2nd carbon from the phosphorus was heavily shifted downfield due to the deshielding effect of the chlorines, the peak being of low intensity due to the lack of protons to aid full relaxation between pulses during NMR acquisition. The ^{13}C NMR of **(133g)** showed that the 2nd carbon from the phosphorus was shifted far downfield as compared to the appropriate carbon in **(133a)** and **(133f)**. Both the CF_3 and the CH_2 carbons were not only split by phosphorus but also split

by fluorine. The ^{1}H NMR spectra of the series (**133a-g**), showed that the protons within three bonds to the phosphorus were found to be more complicated than would be expected, due to the fact that the protons were coupled to the phosphorus giving multiplets, rather than triplets. The mass spectra of the series (**133a-e**) provided interesting data, all showed a parent ion and fragments due to sequential loss of carbon fragments from the alkyl chains. The expected chlorine isotope pattern was present in all peaks assigned to chlorine containing fragments and confirmed that the molecule contained only one chlorine atom. Satisfactory microanalytical data were also obtained for each of the phosphorochloridates.

The 1-β-D-arabinofuranosylcytosine-5'-dialkyl phosphate series (**134a-g**) were prepared by treating a solution of (**1**) in pyridine with two molar equivalents of the appropriate phosphorochloridate (**133a-g**). Two molar equivalents were employed because this had proven in the past to be required with analogous reactions in this Department.[11]



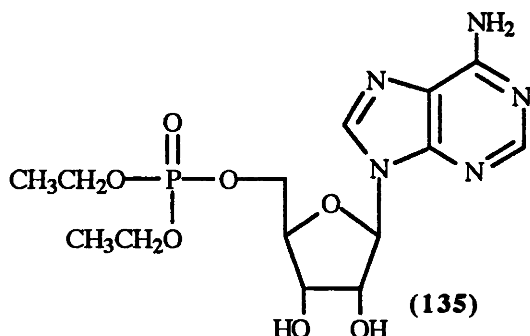
R	
Et	(**133a**)
n-Pr	(**133b**)
n-Bu	(**133c**)
n-Pe	(**133d**)
n-Hx	(**133e**)
TCE	(**133f**)
TFE	(**133g**)

R	
Et	(**134a**)
n-Pr	(**134b**)
n-Bu	(**134c**)
n-Pe	(**134d**)
n-Hx	(**134e**)
TCE	(**134f**)
TFE	(**134g**)

Two equivalents of **(133a)** were added to **(1)** in pyridine. In order to allow some temperature control over the reaction, it was carried out at about 4 ^{o}C. After addition, the reaction was stirred at ambient temperature until all of **(1)** had reacted. The reaction mixture was quenched with water after 5 hours, in order to react with any excess **(133a)**, before the pyridine was removed under reduced pressure. Purification of the resulting residue by column chromatography led to the isolation of **(134a)** in 49% yield. A sample of the product was crystallised slowly by dissolving in the minimum of warm ethanol followed by filtration. The solution was left to stand in a desiccator over a bath of ethyl acetate. Fine white crystals were obtained and a single crystal was selected and considered to be of sufficient quality for investigation by X-ray diffraction.

In order to elucidate the electronic and conformational perturbations arising from phosphate esterification, which may be the basis of the biochemical and biological effects of **(134a)**, the X-ray structure of the triesterified nucleotide was undertaken.

The crystal structure and conformation of the phosphotriester adenosine-5'-diethyl phosphate **(135)** has been reported.[16]



(135)

Comparison of the methylene groups of **(134a)** and **(135)** showed that the bond angles C1P1 and C3P1 in **(135)** were around 124° whereas in **(134a)** were between 95° and 149°. The bond angles O1C2 and O3C4 in **(135)** were between 111° and 113° whereas in **(134a)** were between 120° and 126°. The bond lengths O1C1 and O3C3 in **(135)** were 1.4 Å, whilst in **(134a)** they were between 1.1 to 1.3 Å. However, other bond angles and bond lengths compared well with the literature. Thus, analysis of the data set revealed extensive disorder of the ethoxy substituent on the phosphorus atom even in the solid state. Attempts to refine chemically reasonable models by least squares analysis led to the observation of anomolous bond lengths and bond angles at the methylene carbon atoms of the ethyl groups. Another system of crystallisation may form crystals with a more fixed conformation and thus allow a better X-ray structure to be obtained in the future.

Figure 8 Computer generated X-ray structure of (134a)

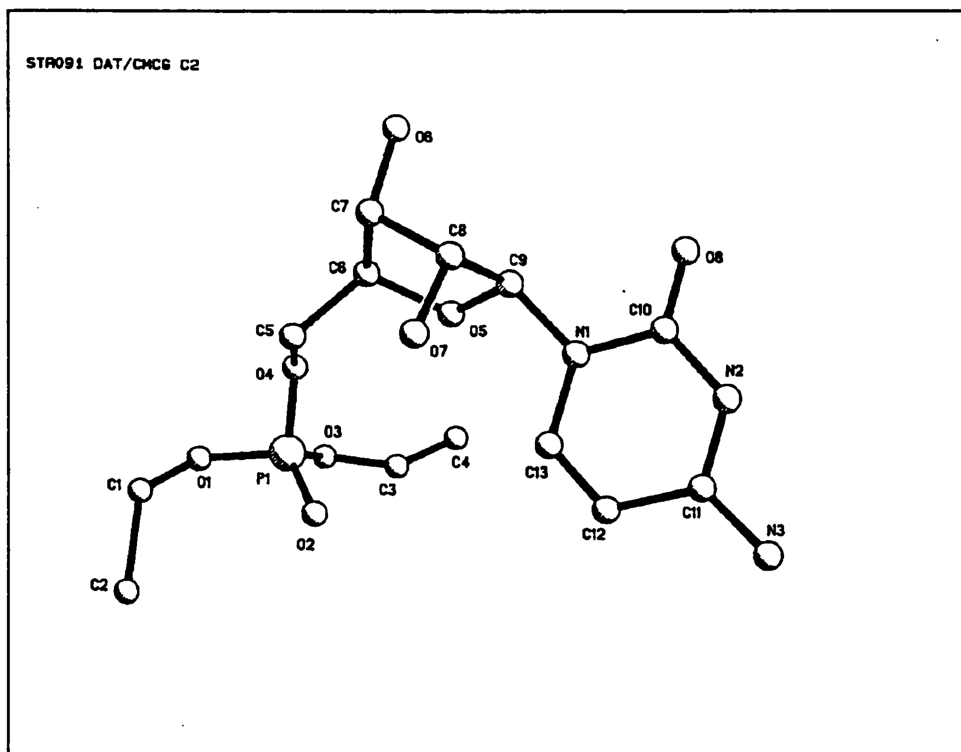


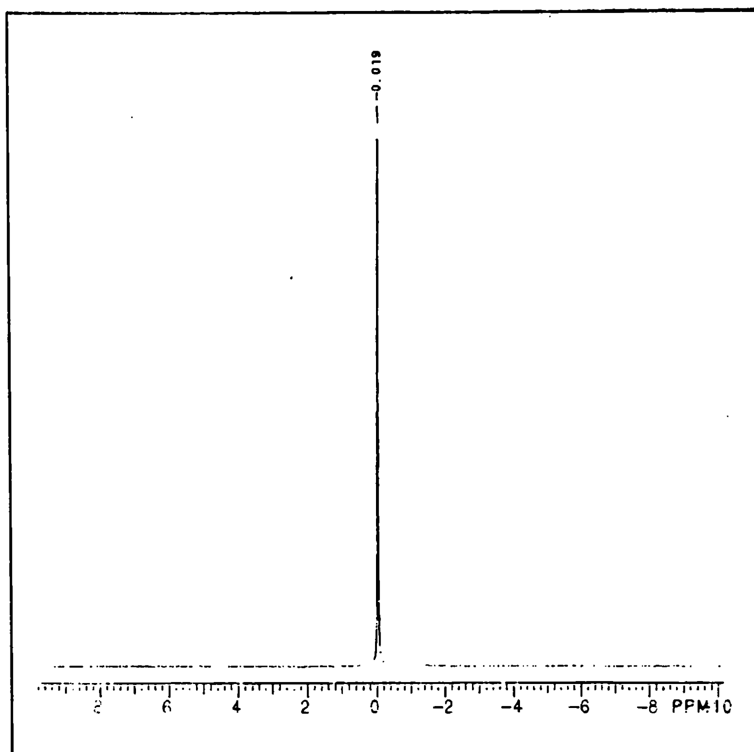
Table 4 Bond lengths and bond angles derived by least squares analysis on the X-ray crystallography of a single crystal of (134a)

Bond length	Angstroms	Bond angle	Degrees
P1 O1	1.548	O1 O2	115.1
P1 O2	1.446	O1 O3	98.5
P1 O3	1.561	O1 O4	103.7
P1 O4	1.506	O2 O3	117.2
O1 C1	1.131	O2 O4	111.4
O3 C3	1.314	O3 O4	109.5
O4 C5	1.483	C1 P1	149.2
O5 C6	1.458	C3 P1	95.2
O5 C9	1.432	C5 P1	127.4
O6 C7	1.396	C6 C9	108.0
O7 C8	1.393	C9 C10	117.4
O8 C10	1.203	C9 C13	120.4
N1 C9	1.472	C10 C13	121.8
N1 C10	1.394	C10 C11	121.8
N1 C13	1.410	O1 C2	126.3
N2 C10	1.360	O3 C4	119.8
N2 C11	1.343	O4 C6	102.5
N3 C11	1.340	C5 O5	113.2
C1 C2	1.698	O5 C7	106.4
C3 C4	1.417	C5 C7	111.7
C5 C6	1.522	C6 O6	110.7
C6 C7	1.550	O6 C8	108.1
C7 C8	1.535	C6 C8	102.4
C8 C9	1.516	C7 O7	107.9
C11 C12	1.476	O7 C9	111.7
C12 C13	1.300	C7 C9	100.3
		N1 O5	108.5
		O5 C8	106.5
		N1 C8	113.3
		N1 O8	120.1
		N2 O8	122.2
		N1 N2	117.6
		N2 N3	119.8
		N2 C12	119.9
		N3 C12	120.3
		C11 C13	118.6
		N1 C12	120.2

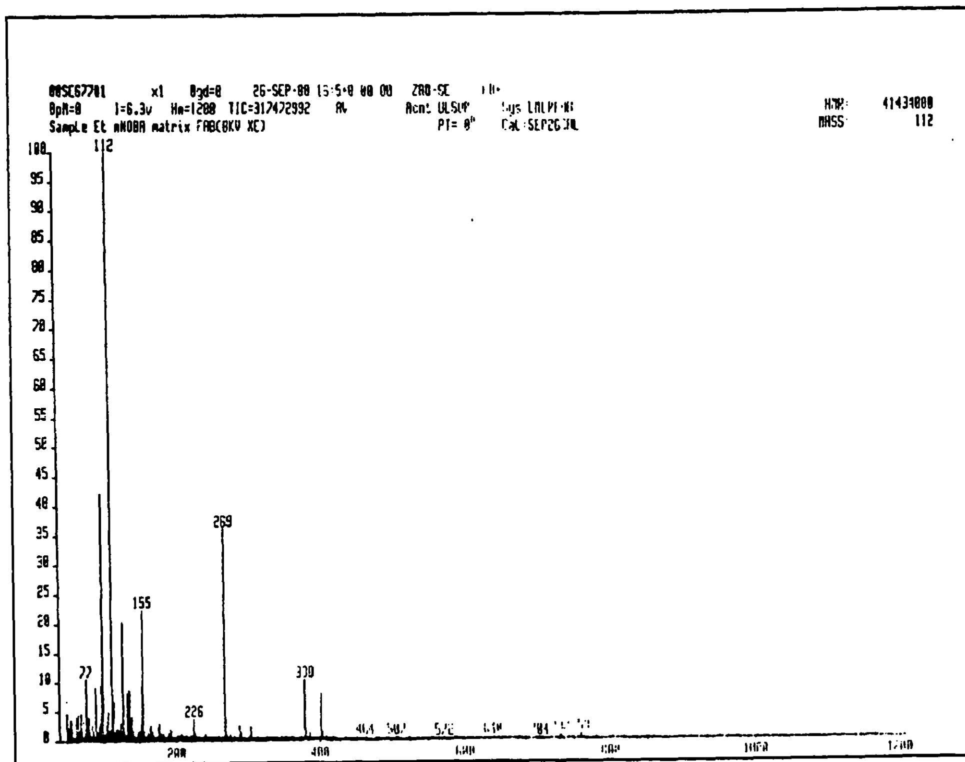
Further data on **(134a)** included a ^{31}P NMR spectrum with one signal at essentialy zero which is in the region that trialkyl phosphates are known to resonate.[17] The ^{1}H NMR spectrum of **(134a)** was assigned by comparison to a spectrum of **(1)**. The base protons H6 and H5 are coupled to each other giving doublets at δ7.91 and δ5.99 both doublets split by 8.1 Hz. H1' is a doublet, coupled to H2' giving a coupling constant of 3.7 Hz, H5', OH, H4', H3', H2' and CH_2OP signals are a mass of peaks around δ4.15, the methyl signal being

at δ1.35. A ^{13}C NMR was fully consistent with the formation of **(134a)**, the spectrum was assigned by comparison with the known spectrum of **(1)**. Evidence that **(134a)** was the product of 5'-phosphorylation of **(1)** and not 3'-phosphorylation was obtained from the ^{13}C NMR spectrum. Phosphorus-carbon coupling was seen for C5' and C4' with coupling constants of 5.6 Hz and 6.7 Hz respectively. These values are within the expected range of these types of phosphorus-carbon coupling[14] and it is consistent that the three bond coupling constant is of greater magnitude than the two-bond coupling constant. As noted above for **(133a)** phosphorus-carbon coupling is observed for the methyl and methylene carbons of the ethoxy chain. A FAB mass spectrum of **(134a)** showed a peak for a protonated dimer of the parent molecule, a protonated parent ion was observed and the base peak was due to the base cytosine. Microanalysis was consistent with a hydrated form of **(134a)**.

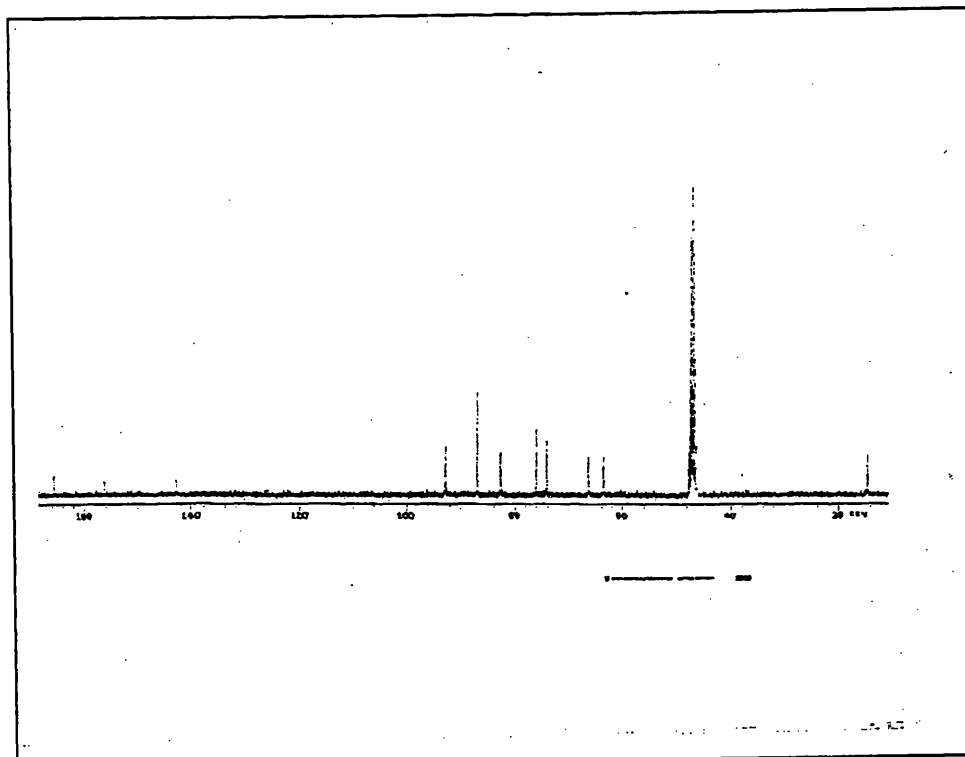
^{31}P NMR spectrum of (134a)



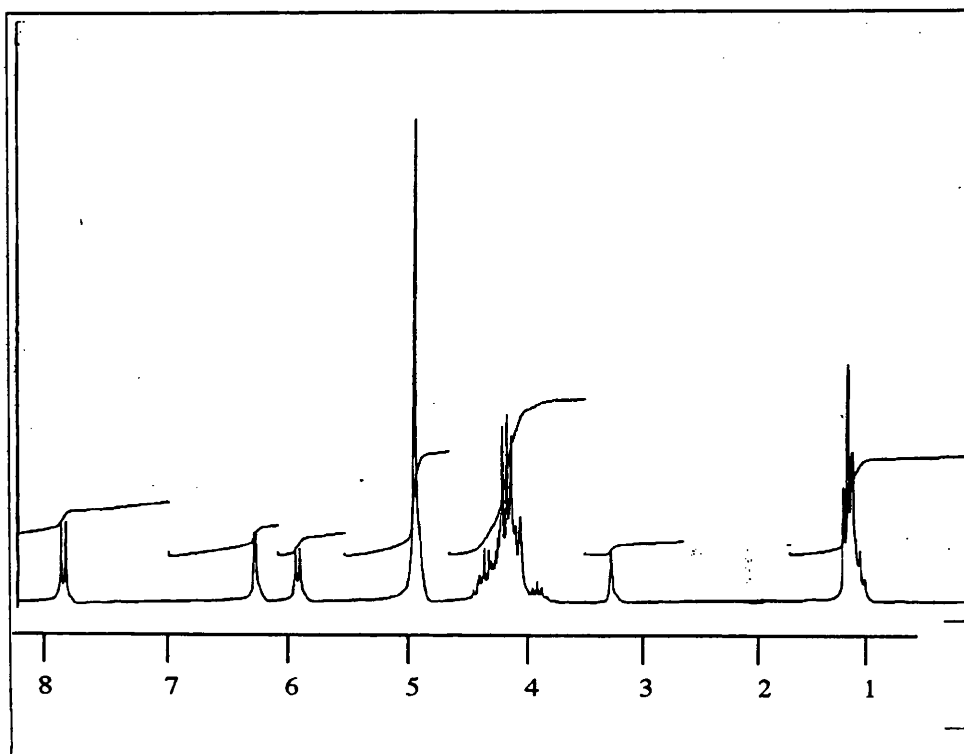
Mass spectrum of (134a)



^{13}C NMR spectrum of (134a)



^{1}H NMR spectrum of (134a)



Compound **(134b)** was prepared in an entirely analogous way to **(134a)** with the exception that the reaction was stirred for 17 hours with a yield of 58%. ^{31}P NMR revealed **(134b)** to have a chemical shift of δ0.179, slightly different to the value for **(134a)**. A ^{1}H NMR of **(134b)** was as expected very similar to the spectrum obtained for **(134a)**. However the C$\underline{H_3}$CH$_2$CH$_2$OP signal was resolved as a multiplet or two closely spaced triplets, probably due to the non-equivalence of the alkyl chains. This non-equivalence was not evident for other protons of the alkyl chain. A ^{13}C NMR spectrum of **(134b)** was fully assigned and was very similar to the spectrum obtained for **(134a)**. The signals of C4' and C5' were observed as doublets, again as a result of phosphorus-carbon coupling. However this time the CH_3 was a singlet and only the two methylene carbons of the propyl chain were split due to phosphorus coupling. A FAB mass spectrum of **(134b)** showed a peak for a protonated dimer of the parent molecule, a protonated parent ion was observed and the base peak was due to

the base cytosine as for **(134a)**. Microanalysis was consistent with a hydrated form of **(134b)**.

The triester **(134c)** was prepared by an identical method to **(134a-b)** except that the reaction was stirred for 6 hours and **(134c)** was isolated in 78% yield after purification by column chromatography. The ^{31}P NMR spectrum of **(134c)** was similar to that for **(134b)**, with one signal at δ0.185. In the ^{1}H NMR spectrum of **(134c)**, apart from the characteristic signals due to the nucleoside, a similar double triplet was observed for the methyl of the butyl chain, again due to the non-equivalence of the alkyl chains. A ^{13}C NMR spectrum of **(134c)** was fully assigned and was very similar to the spectrum obtained for **(134a-b)**. The signals of C4' and C5' were observed as doublets, again as a result of phosphorus-carbon coupling and the two methylene carbons of the butyl chain nearest to the phosphorus were split due to phosphorus coupling. A FAB mass spectrum of **(134c)** showed a peak for a protonated dimer of the parent molecule, a protonated parent ion was observed and the base peak was due to the base cytosine as for **(134a-b)**. Microanalysis was consistent with a hydrated form of **(134c)**.

Compound **(134d)** was prepared by an analogous method to **(134b)** except that a different method of crystallisation was attempted but failed, **(134d)** was isolated in 6% yield after purification by column chromatography. The low yield was due to the modified workup, a better yield, more consistent with **(134a-c)** would be expected with the same workup as for **(134a-c)**. The ^{31}P NMR spectrum of **(134d)** was similar to the ^{31}P NMR spectrum for **(134b-c)**, with one signal at δ0.186. In the ^{1}H NMR spectrum of **(134d)**, apart from the characteristic signals due to the nucleoside, a similar double triplet was observed for the methyl of the pentyl chain, again due to the non-

equivalence of the alkyl chains. The ^{13}C NMR spectrum for **(134d)** was fully assigned and it was evident that the further the carbon was from the phosphorus, the more upfield the chemical shift, this was also the case with **(134a-c)**. A FAB mass spectrum of **(134d)** showed a peak for a protonated dimer of the parent molecule, a protonated parent ion was observed and the base peak was due to a fragment ion at 99 m/e, the peak due to cytosine was 90% intensity relative to the base peak. Microanalysis was consistent with a hydrated form of **(134d)**.

The triester **(134e)** was prepared by an identical method to **(134b)** except that an attempted recrystallisation failed and **(134e)** was isolated in a 31% yield after purification by column chromatography. The ^{31}P NMR spectrum of **(134e)** was similar to that for **(134b-d)**, with one signal at δ0.193. The ^{1}H NMR of **(134e)** was similar to others in this series **(134a-d)** and was consistent with the structure of **(134e)**, a similar double triplet was observed for the methyl of the hexyl chain, again due to the non-equivalence of the alkyl chains. The ^{13}C NMR spectrum for **(134e)** was fully assigned and it was observed that the 2nd methylene carbon from the phosphorus was more upfield than the 3rd carbon. In all the compounds **(134a-d and 134f-g)**, the three bond coupling was slightly larger than the two bond coupling, probably due to the angular dependence of coupling constants[15], this was with the exception of **(133e)** where the two bond coupling was slightly larger than the three bond coupling, the reason for this is not fully determined. A FAB mass spectrum of **(134e)** showed a peak for a protonated dimer of the parent molecule, a protonated parent ion was observed and the base peak was due to the base cytosine and a fragment ion at 99 m/e was seen with an 83% intensity relative to the base peak. Microanalysis was consistent with a hydrated form of **(134e)**.

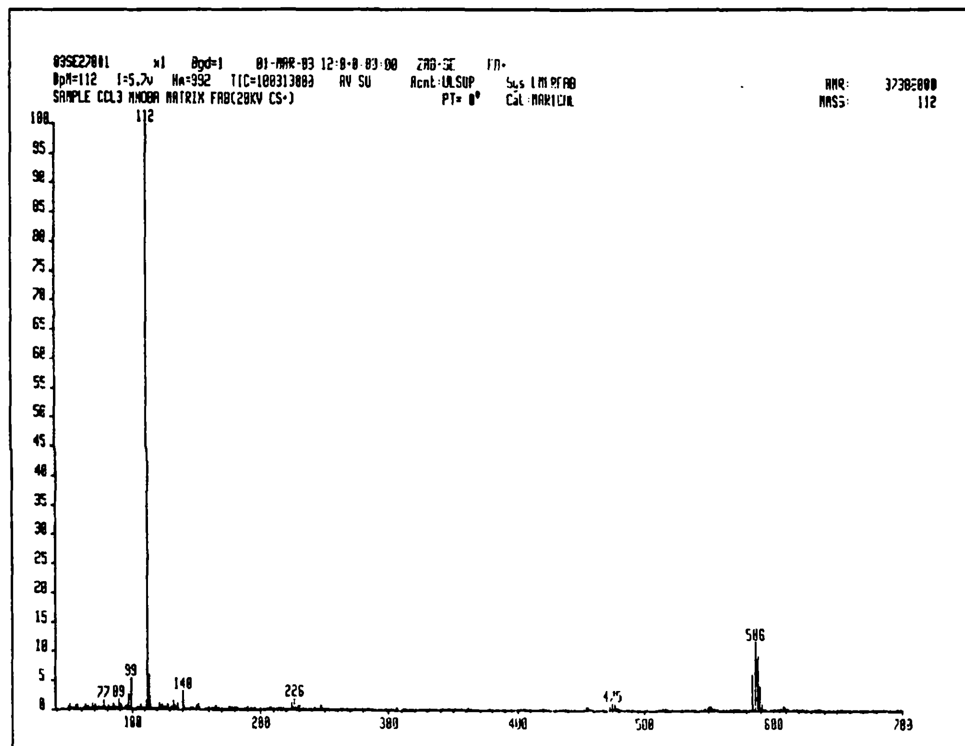
It was decided that the preparation of triesters with alkyl groups with greater lability may afford compounds with substantially different biological activities compared to the unsubstituted alkyl chains. Substitution of the α-carbon with halogen atoms would afford the greatest increase in leaving group ability, but this would require the use of the unstable alcohols CX_3OH, in the first step of the simple two step procedure. Thus it was decided to prepare β-halo derivatives because of the greater ease of synthesis, although the leaving group propensity of the esterifying group is not as greatly enhanced.

The novel triesters **(134f)** and **(134g)** possess electron withdrawing halogen atoms at the β-carbon of the phosphate-esterifying group. Compound **(134f)** was prepared by an analogous reaction to **(134a)** except that **(134f)** was stirred for 17 hours with two equivalents of **(133f)**, a further one molar equivalent of **(133f)** was added prior to stirring for another 2 days. The product **(134f)** was isolated in a 63% yield, no attempt was made to crystallise this product.

The ^{31}P NMR signal of **(134f)** was at δ-2.34 which is more upfield than the ^{31}P NMR signal of its counterpart **(134a)**, presumably the difference is brought about by the influence of the chlorine containing moiety on the phosphorus atom. The ^{1}H NMR of **(134f)** was very similar to the spectrum of **(134a)**, however the CCl_3CH_2OP signal was seen as two doublets almost coincident with each other., which may be explained by this signal being generated by two non-equivalent methylene groups, whose protons resonate at slightly different chemical shifts. In addition, both methylene groups experience proton-phosphorus coupling. The ^{13}C NMR spectrum for **(134f)** was fully assigned and compared well with the ^{13}C NMR spectrum of **(134a)**, however the $\underline{C}Cl_3CH_2OP$ resonance, downfield at δ96.02, was observed as a doublet.

This was due to phosphorus-carbon coupling, but the magnitude of the coupling constant displayed by this signal (11.3 Hz) was much greater than the coupling constant displayed by the CH_3CH_2OP resonance in the ^{13}C NMR spectrum of **(134a)**.

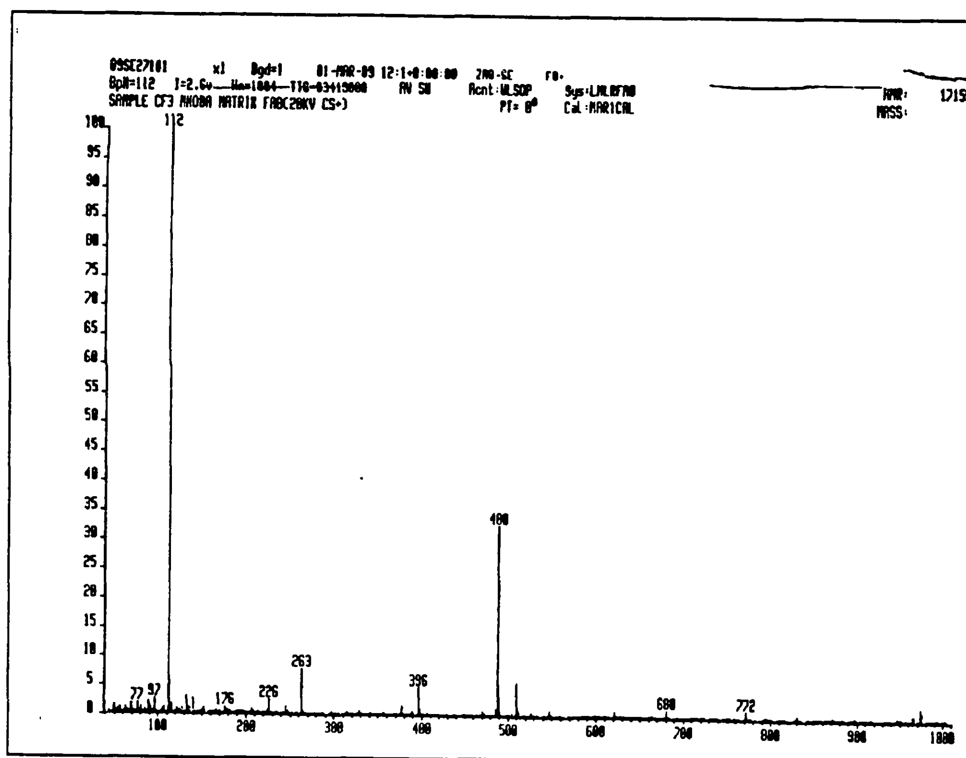
Mass spectrum of (134f)



A FAB mass spectrum of **(134f)** showed many interesting features. The spectrum was obtained by dissolving a sample in nitrobenzyl alcohol and this gave rise to protonated molecular ion species rather than the molecular ion itself. In addition a number of signals were seen in the molecular ion region of **(134f)**. The theoretical pattern of these signals may be readily calculated from the theoretical abundance of isotopes and this may be compared to the pattern observed in the spectrum itself. An isotope cluster abundance calculation was carried out on this sample and was found to be consistent with **(134f)** having six chlorine atoms. It should be noted that "satellite peaks" were observed one

mass unit higher than the main peaks. This may be due to the fact that the isotope ^{13}C, can give rise to peaks at one mass unit higher than the main peaks in a mass spectrum. Alternatively, these "satellite peaks" may be due to doubly protonated molecular ion species. In this spectrum of **(134f)** the base peak was due to the base cytosine. Microanalysis was consistent with the structure of **(134f)** monohydrate.

Mass spectrum of (134g)



Compound **(134g)** was prepared by an analogous reaction to **(134a)** except that in the preparation of **(134g)** only 1.6 molar equivalents of **(133g)** were used. The product **(134g)** was isolated in a 22% yield, no attempt was made to crystallise this product.

The ^{31}P NMR signal of **(134g)** was at δ-2.05 which is more upfield than the ^{31}P NMR signal of its counterpart **(134a)**, presumably the difference is brought about by the influence of the fluorine containing moiety on the

phosphorus atom. The ^{1}H NMR of **(134g)** was very similar to the spectrum of **(134a)**, however the CF_3CH_2OP signal was seen as a multiplet, which may be explained by this signal being generated by two non-equivalent methylene groups, whose protons resonate at slightly different chemical shifts. In addition, both methylene groups experience proton-phosphorus coupling and proton-fluorine coupling. The ^{13}C NMR spectrum for **(134g)** was fully assigned and compared well with the ^{13}C NMR spectrum of **(134a)**, however it revealed that the $\underline{C}F_3CH_2OP$ resonance, downfield at δ125.80, was observed as a split quartet, resulting from coupling to three equivalent fluorine atoms and to the phosphorus atom with coupling constants of 278.2 Hz and 8.5 Hz respectively. The $CF_3\underline{C}H_2OP$ resonated at a chemical shift of δ64.29 again a split quartet from coupling to three equivalent fluorine atoms and to the phosphorus atom with coupling constants of 38.3 Hz and 3.7 Hz respectively. A FAB mass spectrum of **(134g)** showed a protonated parent ion, the base peak was attributed to the base cytosine. The microanalysis was consistent with the composition of **(134g)**.

Biological evaluation of the compounds **(134a-g)** was performed, employing an assay developed by Riley and co-workers.[18] The procedure depends on the fact that extracellular thymidine diffuses across the cell membrane and is then phosphorylated by cell kinases to give thymidine-5'-triphosphate which is incorporated into DNA during the S-phase of the cell cycle.[19] The use of tritiated thymidine allows the possibilty of measuring the amount of thymidine incorporated into DNA over a period of time and this acts as a measure of DNA synthesis. Measurement of thymidine uptake relies on extracting the DNA from the cells, then the radioactivity caused by β-emission from tritium in the sample is measured by scintillation counting.

Mammalian epithelial cells from the cell line CNCM-1.221 were employed in the experiment and were routinely sub-cultured at weekly intervals. Only cells of passage number 21 to 30 were used for experiments, because cells can undergo transformation over long periods involving many sub-cultures; changes can be minimised by keeping a small range of passage numbers.

Mycoplasma is a genus of the family *Mycoplasmataceae* that contains minute polymorphic gram-negative nonmotile microorganisms without cell walls that are intermediate in some respects between viruses and bacteria and are usually parasitic in mammals.[20] Cells taken from liquid nitrogen storage were tested for the presence of *Mycoplasma* because contamination can affect thymidine uptake. The test for *Mycoplasma* contamination involved growing cells on a microscope slide in growth medium, labelling them with tritiated thymidine for a period of time and then carefully washing the slide to remove any unincorporated label. After drying, the slide was coated with a thin layer of photographic emulsion, left for 7 days, then developed and fixed. The slide was stained with Giemsa which enables the radiolabel to be seen as dark spots under a microscope. Large clumps of extracellular thymidine suggest mycoplasma infection whereas normal incorporation into cellular DNA is seen as nuclear clusters of label. All the cells tested were found to be free from mycoplasma contamination.

Cells were grown to sub-confluence in multiwell trays over a period of 48 hours. Solutions of **(1)** and **(134a-g)** were freshly prepared prior to use and diluted to the required concentrations, that is to give a final concentration of 30 μM in the cells. Aliquots of 10% DMSO and the test compounds were added to four replicate wells and the cultures were incubated at 37 °C in 2% CO_2 atmosphere for 30 minutes. An aliquot of a sterile solution of tritiated thymidine

in PBS was then added to each well and the cultures were incubated for a further 30 minutes. The cultures were carefully washed with PBS several times to remove any unincorporated label which would affect the scintillation counts, and then fixed with dilute trichloroacetic acid, washed again and carefully dried. The cells were digested with sodium hydroxide solution to release the contents and then an aliquot from each was mixed with scintillator solution and the radioactivity measured. The scintillation counts are proportional to the amount of tritiated thymidine incorporated into DNA.

The remainder of the digests of each set of four wells was pooled and a measurement of the absorbance at 280 nm taken, as an estimate of the total cellular protein. This gives some indication of the regularity of cell seeding and also ensures that cells have not been lost during washing. If the absorbance values are very variable, a correction can be made by dividing the mean counts per minute by the absorbance for that set of wells, no correction was necessary in the experiments described.

Each experiment was performed three times on cells of different passage number and the mean and SEM of each set of inhibition values calculated to give some indication of the reproducibility of the experiment. For each experiment, the results were normalised by expressing the inhibition as a percentage of the inhibition produced by 10% DMSO. Column graphs were plotted of lipophilicity against compound number and % inhibition (relative to solvent) against compound number. Graphs of lipophilicity and log (lipophilicity) against % inhibition (relative to solvent with deviation bars) were also plotted.

The column graph figure 9 illustrates that the triesters (**134a-e**) are all more lipophilic than (**1**) and as would be expected lipophilicity increases with increasing chain length. Figure 10 shows that increasing chain length correlates

with increasing inhibition of DNA synthesis from ethyl to hexyl. However as can be clearly seen on this column graph **(1)** is much more active than any of the triesters **(134a-e)**. Figures 11 and 12 show graphs which correlate the inhibition of DNA and lipophilicity in the series **(134a-e)**. It is apparent that inhibition of DNA increases with increasing lipophilicity, that is, inhibition is directly proportional to increasing lipophilicity in the series **(134a-e)**.

The lipophilicity of the halogenated dialkyl derivatives of **(1)**, that is, **(134f-g)** were not determined thus the only graph which may be plotted for these compounds is shown in figure 13, which is a column graph of inhibition against compound number. However, figure 13 illustrates rather well, the fact that **(134f)** and **(134g)** are much more active than their unhalogenated counterpart **(134a)**. Indeed **(134f)** is even more active than **(1)** in this experiment.

Figure 9 Lipophilicity (P) against compound number

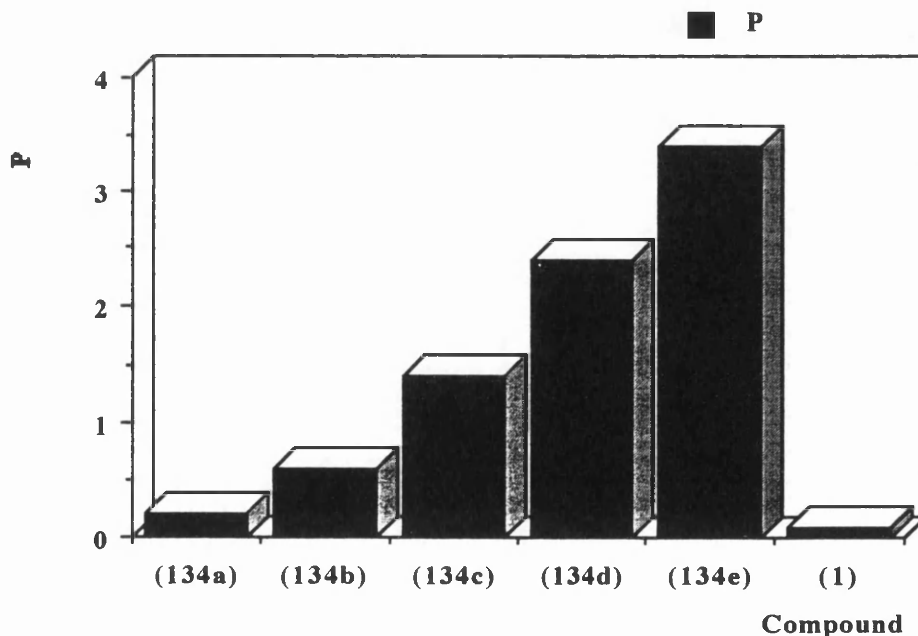


Figure 10 %Inhibition (relative to solvent) against compound number

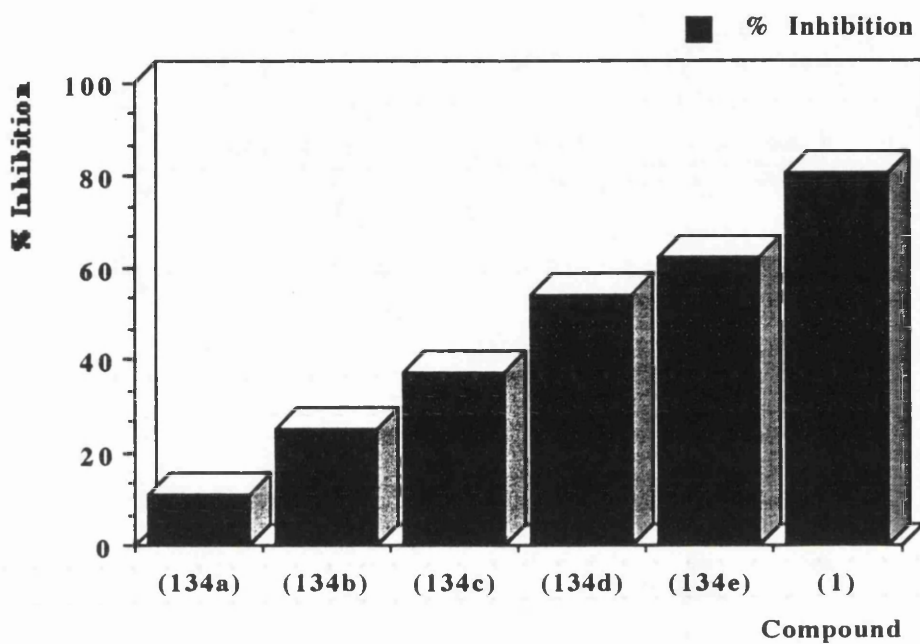


Figure 11 %Inhibition (relative to solvent) against log of lipophilicity (P)

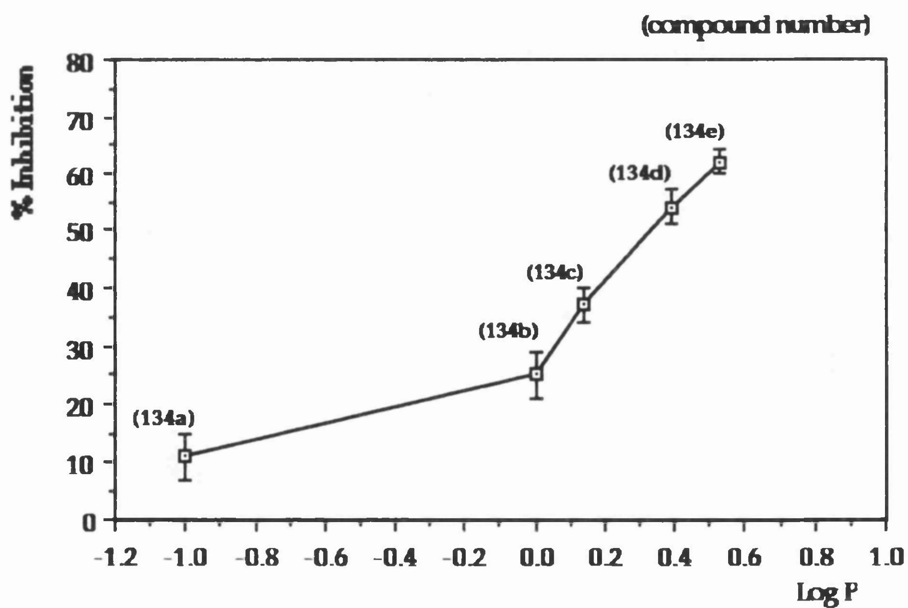


Figure 12 %Inhibition (relative to solvent) against lipophilicity (P)

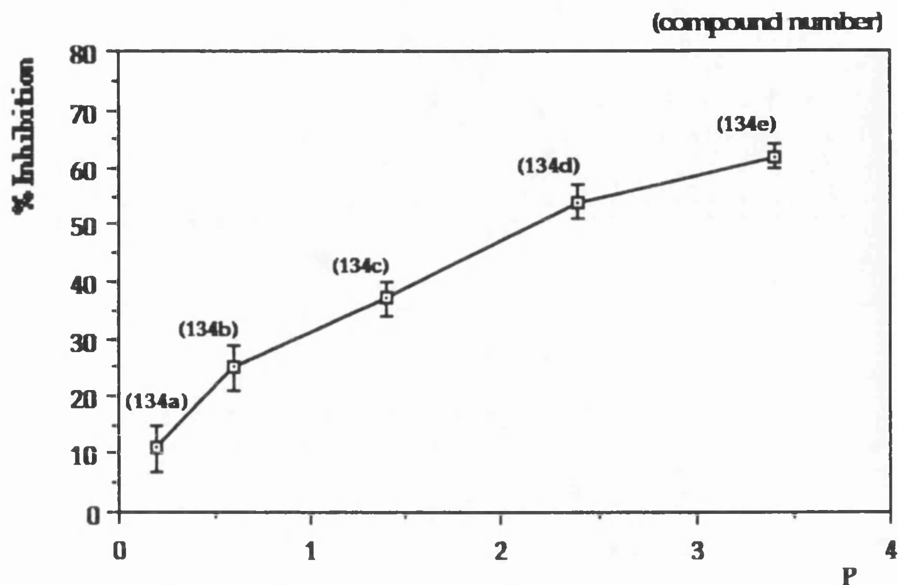
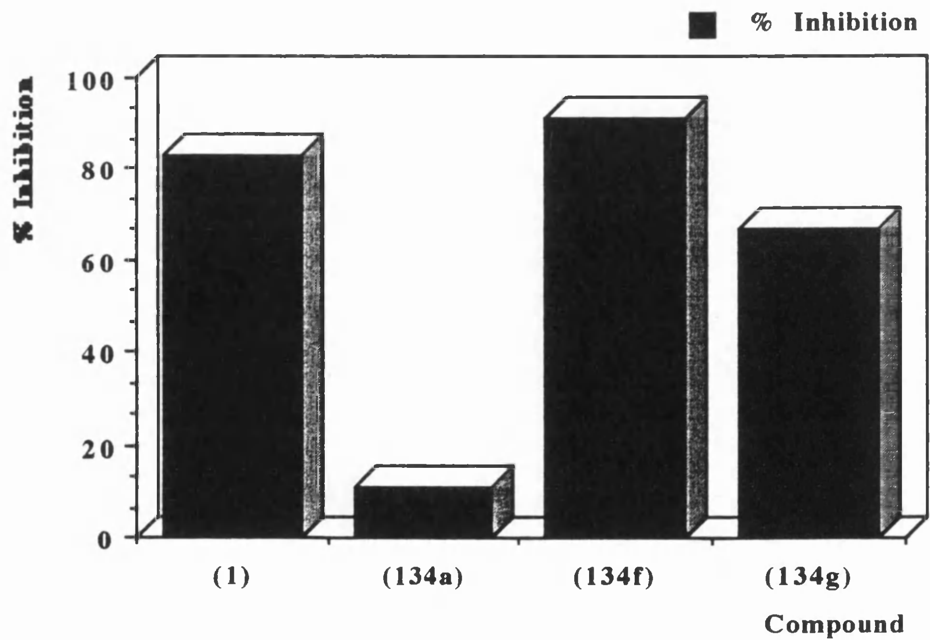


Figure 13 %Inhibition (relative to solvent) against compound number



Thus it would seem that there is a correlation with lipophilicity (P) for the simple dialkyl esters. Compounds **(134f)** and **(134g)** show interesting activity, it may be that the halogenated compounds have a greater susceptibility to hydrolysis than the simple dialkyl esters. The mechanism of action of **(134a-g)** is not determined, however it is believed that the triesters may be chemically hydrolysed inside the cell and that the resulting diester is enzymatically hydrolysed by cellular phosphodiesterase to give the free nucleotide or nucleoside. However it is not ruled out that these triesters may show activity without hydrolysis. The review discussed how simple 5'-phosphate diester derivatives of **(1)** were found to be inactive.[21] Although this was a different biological system than the one employed to evaluate the 5'-triester derivatives of **(1)**, it may be possible to compare the results obtained and infer that triesters being uncharged, have the advantage over diester derivatives in that their passive diffusion through the cell membrane is much faster. However, one possibilty is that the triesters could be interfering with the transport of the exogenously added thymidine across the cell membrane, rather than actually exerting an effect on DNA synthesis. This could be investigated by growing cultures of cells in the absence and presence of the triesters and counting actual cell numbers over a period of several cell cycles. If the triesters were interfering with the replication process of the cell this would be observed in the relative growth rates.

In order to determine whether the phosphorus attached to **(1)** in the triesters actually becomes incorporated into DNA, one of the triesters using phosphoryl chloride with radiolabelled ^{32}P would have to be prepared and assayed in the cell culture experiment (in the absence of tritiated thymidine). Scintillation counting of the cell digests would determine whether the labelled phosphorus had crossed the cell membrane and if this was the case, it would

imply that the labelled compound was being transported intact, since it is unlikely that the charged fragments, such as the free nucleotide or the phosphate diester moiety would enter the cells by passive diffusion. If the compound could cross the cell membrane intact, it would then presumably be metabolised either to **(1)** or **(50)**, but in either case would have evaded extracellular deactivation by cytidine deaminase. Further work is being carried out, by other members in this Department, to investigate the mechanism of action of similar compounds to the series **(134a-g)**.

Experimental

2.3.1 Dialkyl phosphorylating agents

2.3.1.1

Diethyl phosphorochloridate (133a)

Phosphoryl chloride (10 ml, 16.45 g, 0.1073 mol) was dissolved in diethyl ether (100 ml). A solution of triethylamine (30 ml, 21.78 g, 0.2152 mol) and ethanol (12.59 ml, 9.89 g, 0.2146 mol) in diethyl ether (100 ml) was added dropwise at -78 ^oC. The reaction mixture was stirred at room temperature for 17 hours. The mixture was filtered and the filtrate was rotary evaporated to yield a clear yellow oil (12.34 g) which was distilled under vacuum to give a clear colourless oil (bpt: 41 ^oC at 0.05 mm Hg)(10.18 g, 55%).

^1H NMR δ($CDCl_3$, 200 MHz, TMS) 4.32(2H, m, CH_2OP), 1.42(3H, t, CH_3)

^{31}P NMR δ($CDCl_3$, 80 MHz, H_3PO_4) 2.48

^{13}C NMR δ($CDCl_3$, 200 MHz, TMS) 65.86(d, CH_2, J=6.8 Hz), 15.95(d, CH_3, J=7.4 Hz)

EI MS m/e 175(MH^+,^{37}Cl, 0.42%), 174(M^+,^{37}Cl, 0.10), 173(MH^+, ^{35}Cl, 1.31), 172(M^+, ^{35}Cl, 0.35), 147(MH^+ - C_2H_4, ^{37}Cl, 21.58), 145(MH^+ - C_2H_4,^{35}Cl, 65.42), 119(MH^+ - (2 x C_2H_4),^{37}Cl, 32.16), 117(MH^+ - (2 x C_2H_4),^{35}Cl, bp), 81($OP(OH)_2^+$, 32.01)

Found C 27.42%, H 5.72, Cl 20.41, P 18.31;

$C_4H_{10}ClO_3P$ requires C 27.84, H 5.84, Cl 20.55, P 17.95.

2.3.1.2

Di-n-propyl phosphorochloridate (133b)

Phosphoryl chloride (11 ml, 18.1 g, 0.118 mol) was dissolved in diethyl ether (100 ml). A solution of triethylamine (33 ml, 23.96 g, 0.237 mol) and n-propanol (18 ml, 14.5 g, 0.240 mol) in diethyl ether (100 ml) was added dropwise at -78 °C. The reaction mixture was stirred at room temperature for 3 days. The mixture was filtered and the filtrate was rotary evaporated to yield a clear yellow oil (20.44 g) which was distilled under vacuum to give a clear colourless oil (bpt: 59 °C at 0.25 mm Hg)(15.61 g, 66%).

^{1}H NMR δ($CDCl_3$, 200 MHz, TMS) 4.14(2H, m, CH_2OP), 1.76(2H, sextet, $CH_3\underline{CH_2}$), 0.99(3H, t, CH_3)

^{31}P NMR δ($CDCl_3$, 80 MHz, H_3PO_4) 1.63

^{13}C NMR δ($CDCl_3$, 200 MHz, TMS) 70.81(d, CH_2OP, J=7.1 Hz), 23.22(d, $CH_3\underline{C}H_2$, J=7.6 Hz), 9.84(CH_3)

EI MS m/e 225([$(C_3H_7O)_3PO$]H^+, 2.56%), 203(MH^+, ^{37}Cl, 9.49), 201(MH^+, ^{35}Cl, 31.58), 161(MH^+ - C_3H_6, ^{37}Cl, 6.98), 159(MH^+ - C_3H_6, ^{35}Cl, 22.09), 145(MH^+ - C_3H_7 - CH_3, ^{37}Cl, 0.72), 143(MH^+ - C_3H_7 - CH_3, ^{35}Cl, 3.00), 131(MH^+ - C_3H_7 - C_2H_5, ^{37}Cl, 9.40), 129(MH^+ - C_3H_7 - C_2H_5, ^{35}Cl, 24.33), 119(MH^+ - 2 x C_3H_6, ^{37}Cl, 62.28), 117(MH^+ - 2 x C_3H_6, ^{35}Cl, bp), 101(MH^+ - (2 x C_3H_6) - H_2O, ^{37}Cl, 3.43), 99(MH^+ - (2 x C_3H_6) - H_2O, ^{35}Cl, 17.59)

Found C 35.53, H 6.77, Cl 17.88, P 15.81;
$C_6H_{14}ClO_3P$ requires C 35.92%, H 7.03, Cl 17.67, P 15.44.

2.3.1.3

Di-n-butyl phosphorochloridate (133c)

Phosphoryl chloride (10 ml, 16.45 g, 0.1073 mol) was dissolved in diethyl ether (100 ml). A solution of triethylamine (30 ml, 21.78 g, 0.2152 mol) and n-butanol (20 ml, 16.18 g, 0.2183 mol) in diethyl ether (100 ml) was added dropwise at -70 °C. The reaction mixture was stirred at room temperature for 17 hours. The mixture was filtered and the filtrate was rotary evaporated to yield a clear yellow oil (21.94 g) which was distilled under vacuum to give a clear colourless oil (bpt: 79 °C at 0.25 mm Hg)(18.38 g, 75%).

^1H NMR δ($CDCl_3$, 200 MHz, TMS) 4.12(2H, m, CH_2OP), 1.56(4H, m, $CH_3C\underline{H}_2C\underline{H}_2$), 0.92(3H, t, CH_3)

^{31}P NMR δ($CDCl_3$, 80 MHz, H_3PO_4) 1.84

^{13}C NMR δ($CDCl_3$, 200 MHz, TMS) 70.19(d, <u>C</u>H_2OP, J=7.1 Hz), 31.50(d, <u>C</u>H_2CH_2OP, J=7.5 Hz), 18.46(CH_3<u>C</u>H_2), 13.42 (CH_3)

EI MS m/e 231(MH^+, ^{37}Cl, 0.04%), 229(MH^+, ^{35}Cl, 0.30), 175(MH^+ - Bu + H^+, ^{37}Cl, 1.31), 174(MH^+ - Bu, ^{37}Cl, 0.83), 173(MH^+ - Bu + H^+, ^{35}Cl, 4.67), 172(MH^+ - Bu, ^{35}Cl, 2.31), 157(M^+ - BuO, ^{37}Cl, 1.31), 155(M^+ - BuO, ^{35}Cl, 2.62), 145(MH^+ - Bu - Et, ^{37}Cl, 3.36), 143(MH^+ - Bu - Et, ^{35}Cl, 8.56), 137(MH^+ - Bu - ^{35}Cl, 6.59), 132(MH^+ - Bu - Pr + H^+, ^{37}Cl, 2.98), 130(MH^+ - Bu - Pr + H^+, ^{35}Cl, 8.85)

Found C 42.12%, H 8.29, Cl 15.88, P 13.51;
$C_8H_{18}ClO_3P$ requires C 42.02, H 7.93, Cl 15.50, P 13.55.

2.3.1.4

Di-n-pentyl phosphorochloridate (133d)

Phosphoryl chloride (10 ml, 16.45 g, 0.1073 mol) was dissolved in diethyl ether (100 ml). A solution of triethylamine (30 ml, 21.78 g, 0.2152 mol) and n-pentanol (23 ml, 18.65 g, 0.2116 mol) in diethyl ether (100 ml) was added dropwise at -66 °C. The reaction mixture was stirred at room temperature for 17 hours. The mixture was filtered and the filtrate was rotary evaporated to yield a clear yellow oil (22.16 g) which was distilled under vacuum to give a clear colourless oil (bpt: 102 °C at 0.07 mm Hg)(15.24 g, 55%).

^1H NMR δ($CDCl_3$, 200 MHz, TMS) 4.1(2H, m, CH_2OP), 1.5(6H, m, $CH_3C\underline{H}_2C\underline{H}_2C\underline{H}_2$), 0.9(3H, t, CH_3)

^{31}P NMR δ($CDCl_3$, 80 MHz, H_3PO_4) 2.67

^{13}C NMR δ($CDCl_3$, 200 MHz, TMS) 69.85(d, $\underline{C}H_2OP$, J=7.2 Hz), 29.40(d, $\underline{C}H_2CH_2OP$, J=7.6 Hz), 27.33($CH_3CH_2\underline{C}H_2$), 22.17($CH_3\underline{C}H_2$), 13.72(CH_3)

EI MS m/e 257(MH^+, ^{35}Cl, 0.03%), 189 (MH^+ - C_5H_{10},^{37}Cl, 1.19), 187 (MH^+ - C_5H_{10}, ^{35}Cl, 4.70), 119 (MH^+ - (2 x C_5H_{10}), ^{37}Cl, 57.31), 117 (MH^+ - (2 x C_5H_{10}), ^{35}Cl, bp), 81($OP(OH)_2^+$, 1.66)

Found C 46.57%, H 8.66, Cl 13.95;

$C_{10}H_{22}ClO_3P$ requires C 46.79, H 8.64, Cl 13.81.

2.3.1.5

Di-n-hexyl phosphorochloridate (133e)

Phosphoryl chloride (10 ml, 16.45 g, 0.1073 mol) was dissolved in diethyl ether (150 ml). A solution of triethylamine (30 ml, 21.78 g, 0.2152 mol) and n-hexanol (27 ml, 21.98 g, 0.2151 mol) in diethyl ether (150 ml) was added dropwise at -78 °C under an atmosphere of dry nitrogen. The reaction mixture was stirred at room temperature for 17 hours under an atmosphere of dry nitrogen. The mixture was filtered and the filtrate was rotary evaporated to yield a clear yellow oil (26.41 g, 86%).

^1H NMR δ($CDCl_3$, 200 MHz, TMS) 4.20(2H, m, CH_2OP), 1.72(2H, m, $C\underline{H}_2CH_2OP$), 1.32(6H, m, $CH_3C\underline{H}_2C\underline{H}_2C\underline{H}_2$), 0.88(3H, t, CH_3)

^{31}P NMR δ($CDCl_3$, 80 MHz, H_3PO_4) 4.41

^{13}C NMR δ($CDCl_3$, 200 MHz, TMS) 69.82(d, $\underline{C}H_2OP$, J=6.9 Hz), 31.35($\underline{C}H_2CH_2CH_2OP$), 30.14(d, $CH_2\underline{C}H_2CH_2OP$, J=5.4 Hz), 26.18($CH_3CH_2\underline{C}H_2$), 23.51($CH_3\underline{C}H_2$), 13.27($CH_3$)

EI MS m/e 287(MH^+,^{37}Cl, 0.52%), 285(MH^+, ^{35}Cl, 1.81), 119 (MH^+ - (2 x C_6H_{12}), ^{37}Cl, 28), 117 (MH^+ - (2 x C_6H_{12}), ^{35}Cl, bp), 81($OP(OH)_2^+$, 12)

Found C 50.20%, H 9.34;

$C_{12}H_{26}ClO_3P$ requires C 50.61, H 9.20.

2.3.1.6

Bis-(2,2,2-trichloroethyl) phosphorochloridate (133f)

Phosphoryl chloride (10 ml, 16.45 g, 0.1073 mol) was dissolved in diethyl ether (100 ml). A solution of triethylamine (30 ml, 21.78 g, 0.2152 mol) and 2,2,2-trichloroethanol (20.6 ml, 32.06 g, 0.2146 mol) in diethyl ether (100 ml) was added dropwise at -78 °C. The reaction mixture was stirred at room temperature for 17 hours. The mixture was filtered and the filtrate was rotary evaporated to yield a clear yellow oil (35.73 g). The yellow oil was stirred vigorously on a high vacuum pump at room temperature for 4 hours. The residue was distilled by short path distillation to give a white solid (8.14 g, 20%).

^1H NMR δ($CDCl_3$, 200 MHz, TMS) 4.41 (4H, m, CH_2)

^{31}P NMR δ($CDCl_3$, 80 MHz, H_3PO_4) 2.52

^{13}C NMR δ($CDCl_3$, 200 MHz, TMS) 93.57 (d, $\underline{C}Cl_3CH_2$, J=11.3 Hz), 70.55 (d, $CCl_3\underline{C}H_2$, J=4.2 Hz)

Found C 13.09%, H 1.38;

$C_4H_4Cl_7O_3P$ requires C 12.67, H 1.06.

2.3.1.7

Bis-(2,2,2-trifluroethyl) phosphorochloridate (133g)

Phosphoryl chloride (5 ml, 8.23 g, 0.0536 mol) was dissolved in diethyl ether (100 ml). A solution of triethylamine (15 ml, 10.89 g, 0.1076 mol) and 2,2,2-trifluroethanol (7.8 ml, 10.73 g, 0.1072 mol) in diethyl ether (100 ml) was added dropwise at -78 ^{o}C. The reaction mixture was stirred at room temperature for 17 hours. The mixture was filtered and the filtrate was rotary evaporated to yield a clear yellow oil which was purified by vacuum distillation to give the product as a colourless liquid (bpt: 38 ^{o}C at 0.8 mm Hg)(9.94 g, 66%).

^{1}H NMR δ($CDCl_3$, 200 MHz, TMS) 4.53(4H, m, CH_2)

^{31}P NMR δ($CDCl_3$, 80 MHz, H_3PO_4) 5.96

^{13}C NMR δ($CDCl_3$, 200 MHz, TMS) 123.71(qd, CF_3, J_{CCOP}=10.1 Hz; J_{CF}=277.5 Hz)), 64.52(qd, CH_2, J_{COP}=4.5 Hz; J_{CCF}=38.5 Hz)

Found C 17.59%, H 1.72;

$C_4H_4F_6ClO_3P$ requires C 17.13, H 1.44.

2.3.2 Phosphorylation of 1-β-D-Arabinofuranosylcytosine

2.3.2.1

1-β-D-Arabinofuranosylcytosine-5'-diethyl phosphate (134a)

Compound (1) (0.50 g, 2.06 mmol) was dissolved in pyridine (100 ml), and diethyl phosphorochloridate (0.60 ml, 0.72 g, 4.15 mmol) added dropwise with vigorous stirring at *ca.* 4 °C. After stirring for 5 hours at ambient temperature, the reaction was quenched with water (75 µl, 4.14 mmol), and the solvent was removed under reduced pressure. The resulting oil was dried in vacuum, and purified by flash column chromatography on silica. Elution with 15% methanol in chloroform, followed by pooling and evaporation of appropriate fractions gave an impure product (0.62 g). This was re-chromatographed, using first chloroform (150 ml), and then 20% methanol in chloroform as eluant. Pooling and evaporation of appropriate fractions of the latter eluant gave the product as a white solid (0.38 g, 49%). A sample of the product (100 mg) was successfully recrystallised by dissolving in the minimum of warm ethanol followed by filtration. The solution was then left in a desiccator over a bath of ethyl acetate for 2 weeks. Fine white crystals were collected (73 mg).

^{1}H NMR δ(CD_3OD, 200 MHz, TMS) 7.91(1H, d, H6, J=8.1 Hz), 6.26(1H, d, H1', J=3.7 Hz), 5.99(1H, d, H5, J=8.1 Hz), 4.15 to 3.81(11H, m, H5', CH_2OP, 2 x OH, H4', H2', H3'), 1.35(6H, m, CH_3)

^{31}P NMR δ(CD_3OD, 160 MHz, H_3PO_4) -0.019

^{13}C NMR δ(CD_3OD, 100 MHz, TMS) 166.38(C4), 156.81(C2), 145.19(C6), 94.91(C5), 88.92(C1'), 84.94(d, C4', J=6.7 Hz), 77.85(C2'),

76.16(C3'), 68.26(d, C5', J=5.6 Hz), 65.85(d, CH_2OP, J=5.5 Hz), 16.50(d, CH_3, J=6.4 Hz)

FAB MS m/e 759(M_2H^+, 2%), 380(MH^+, 12), 112(cytosineH^+, bp)

Found C 39.33%, H 5.83, N 10.32, P 7.79;
$C_{13}H_{22}N_3O_8P.H_2O$ requires C 39.30, H 6.09, N10.58, P 7.80.

2.3.2.2

1-β-D-Arabinofuranosylcytosine-5'-di-n-propyl phosphate (134b)

Compound **(1)** (0.50 g, 2.06 mmol) was dissolved in pyridine (100 ml), and di-n-propyl phosphorochloridate (0.74 ml, 0.83 g, 4.14 mmol) added dropwise with vigorous stirring at *ca.* 4 °C. After stirring for 17 hours at ambient temperature, the reaction was quenched with water (75 μl, 4.14 mmol), and the solvent was removed under reduced pressure. The resulting oil was dried in vacuum, and purified by flash column chromatography on silica. Elution with 15% methanol in chloroform, followed by pooling and evaporation of appropriate fractions gave the product as a white solid (0.49 g, 58%). A sample of the product (100 mg) was successfully recrystallised by dissolving in the minimum of warm ethanol followed by filtration. The solution was then left in a desiccator over a bath of ethyl acetate for 2 weeks. Fine white crystals were collected (68 mg).

^1H NMR δ(CD_3OD, 200 MHz, TMS) 7.85(1H, d, H6, J=8.1 Hz), 6.26(1H, d, H1', J=3.7 Hz), 5.91(1H, d, H5, J=8.1 Hz), 4.07 to 3.31(11H, m, H5', CH_2OP, 2 x OH, H4', H2', H3'), 1.74(4H, m, $CH_3\underline{CH_2}$), 0.98(6H, m, CH_3)

^{31}P NMR δ(CD_3OD, 160 MHz, H_3PO_4) 0.179

^{13}C NMR δ(CD_3OD, 100 MHz, TMS) 167.45(C4), 158.06(C2), 144.67(C6), 94.81(C5), 88.85(C1'), 84.71(d, C4', J=6.8 Hz), 77.86(C2'), 76.16(C3'), 70.99(d, CH_2OP, J=4.4 Hz), 68.34(d, C5', J=5.7 Hz), 24.68(d, $CH_3\underline{C}H_2$, J=5.2 Hz), 10.36(CH_3)

FAB MS m/e 815(M_2H^+, 5.5%), 408(MH^+, 27.9), 112(cytosineH^+, bp)

Found C 42.48%, H 6.54, N 9.62, P 7.93;
$C_{15}H_{26}N_3O_8P.H_2O$ requires C 42.35, H 6.64, N 9.88, P 7.28.

2.3.2.3

1-β-D-Arabinofuranosylcytosine-5'-di-n-butyl phosphate (134c)

Compound (**1**) (0.50 g, 2.06 mmol) was dissolved in pyridine (100 ml), and di-n-butyl phosphorochloridate (0.87 ml, 0.94 g, 4.11 mmol) added dropwise with vigorous stirring at *ca.* 4 °C. After stirring for 6 hours at ambient temperature, the reaction was quenched with water (75 μl, 4.14 mmol), and the solvent was removed under reduced pressure. The resulting oil was dried in vacuum, and purified by flash column chromatography on silica. Elution with 20% methanol in chloroform, followed by pooling and evaporation of appropriate fractions gave an impure product (0.47 g). This was re-chromatographed, using first chloroform (150 ml), and then 20% methanol in chloroform as eluant. Pooling and evaporation of appropriate fractions of the latter eluant gave the product as a white solid (0.32 g, 36%). A sample of the product (100 mg) was successfully recrystallised by dissolving in the minimum of warm ethanol followed by filtration. The solution was then left in a desiccator

over a bath of ethyl acetate for 2 weeks. Fine white crystals were collected (61 mg).

^1H NMR δ(CD_3OD, 200 MHz, TMS) 7.84(1H, d, H6, J=8.1 Hz), 6.26(1H, d, H1', J=3.7 Hz), 5.90(1H, d, H5, J=8.1 Hz), 4.11 to 3.31(11H, m, H5', CH_2OP, 2 x OH, H4', H2', H3'), 1.68(4H, m, $C\underline{H}_2CH_2OP$),1.43(4H, m, $CH_3C\underline{H}_2$), 0.96(6H, m, CH_3)

^{31}P NMR δ(CD_3OD, 160 MHz, H_3PO_4) 0.185

^{13}C NMR δ(CD_3OD, 100 MHz, TMS) 167.21(C4), 158.12(C2), 144.34(C6), 94.34(C5), 87.77(C1'), 83.67(d, C4', J=6.7 Hz), 76.73(C2'), 75.67(C3'), 68.45(d, CH_2OP, J=5.3 Hz), 67.33(d, C5', J=5.6 Hz), 32.50(d, $\underline{C}H_2CH_2OP$, J=6.8 Hz), 18.87($CH_3\underline{C}H_2$), 13.61(CH_3)

FAB MS m/e 871(M_2H^+, 1%), 436(MH^+, 9), 112(cytosineH^+, bp)

Found C 45.75%, H 7.09, N 8.83, P 6.51;
$C_{17}H_{30}N_3O_8P.[H_2O]_{0.8}$ requires C 45.39, H 7.08, N9.34, P 6.89.

2.3.2.4

1-β-D-Arabinofuranosylcytosine-5'-di-n-pentyl phosphate (134d)

Compound (1) (0.50 g, 2.06 mmol) was dissolved in pyridine (100 ml), and di-n-pentyl phosphorochloridate (1 ml, 1.03 g, 4.10 mmol) added dropwise with vigorous stirring at *ca.* 4 °C. After stirring for 17 hours at ambient temperature, the reaction was quenched with water (75 µl, 4.14 mmol), and the solvent was removed under reduced pressure. The resulting oil was dried in vacuum, and purified by flash column chromatography on silica. Elution with

20% methanol in chloroform, followed by pooling and evaporation of appropriate fractions gave an impure product (0.63 g). An attempt was made to recrystallise the crude product by dissolving it in the minimum of methanol (20 ml), and a trace amount of undissolved solid was filtered. Decolourising charcoal was added to the filtrate and the mixture was boiled. The charcoal was filtered and distilled water (10 ml) was added to the filtrate until it went slightly cloudy. Hot methanol (3 ml) was added to give a colourless solution. The solution was filtered and the filtrate was cooled at *ca.* 4 ^{o}C for 48 hours. No crystals formed so it was decided to rotary evaporate some of the methanol until the solution became cloudy. The product was left at *ca.* 4 ^{o}C for 48 hours and fine white crystals (0.23 g, 24%) fell out of solution. The recrystallised product still contained some non-nucleoside impurity. The crude product was re-chromatographed, using first chloroform (150 ml), and then 20% methanol in chloroform as eluant. Pooling and evaporation of appropriate fractions gave the product as a white solid (0.06 g, 6%).

^{1}H NMR δ(CD_3OD, 200 MHz, TMS) 7.83(1H, d, H6, J=8.1 Hz), 6.26(1H, d, H1', J=3.7 Hz), 5.90(1H, d, H5, J=8.1 Hz), 4.10 to 3.31(11H, m, H5', CH_2OP, 2 x OH, H4', H2', H3'), 1.69(4H, m, $C\underline{H}_2CH_2OP$), 1.36(8H, m, $CH_3C\underline{H}_2C\underline{H}_2$), 0.92(6H, m, CH_3)

^{31}P NMR δ(CD_3OD, 160 MHz, H_3PO_4) 0.186

^{13}C NMR δ(CD_3OD, 100 MHz, TMS) 167.43(C4), 157.97(C2), 144.77(C6), 94.74(C5), 88.88(C1'), 84.73(d, C4', J=6.7 Hz), 77.99(C2'), 76.15(C3'), 69.47(d, CH_2OP, J=6.5 Hz), 68.48(d, C5', J=5.6 Hz), 31.05(d, $\underline{C}H_2CH_2OP$, J=2.2 Hz), 28.74($\underline{C}H_2CH_2CH_2OP$), 23.25($CH_3\underline{C}H_2$), 14.34($CH_3$)

FAB MS m/e 928($M_2H_2^+$, 3%), 464(MH^+, 10), 99($C_5H_7O_2^+$, bp)

Found C 47.70%, H 7.46, N 8.84, P 6.43;

$C_{13}H_{22}N_3O_8P.H_2O$ requires C 47.40, H 7.54, N 8.73, P 6.43.

2.3.2.5

1-β-D-Arabinofuranosylcytosine-5'-di-n-hexyl phosphate (134e)

Compound (**1**) (0.38 g, 1.56 mmol) was dissolved in pyridine (80 ml), and di-n-hexyl phosphorochloridate (0.8 ml, 0.87 g, 3.06 mmol) added dropwise with vigorous stirring at *ca.* 4 °C. After stirring for 17 hours at ambient temperature, the reaction was quenched with water (55 μl, 3.06 mmol), and the solvent was removed under reduced pressure. The resulting oil was dried in vacuum, and purified by flash column chromatography on silica. Elution with 20% methanol in chloroform, followed by pooling and evaporation of appropriate fractions gave an impure product (0.39 g). An attempt was made to recrystallise the crude product by dissolving it in the minimum of ethanol (5 ml). The ethanolic solution was placed in a desiccator which contained diethyl ether (30 ml) and n-hexane (30 ml). The desiccator was closed to the air and the solvents were allowed to condense into the flask containing the ethanolic solution of the product over a period of 8 days, this did not produce any crystals. The solution was rotary evaporated and re-chromatographed, using first chloroform (150 ml), and then 20% methanol in chloroform as eluant. Pooling and evaporation of appropriate fractions of the latter eluant gave the product as a white solid (0.24 g, 31%).

^{1}H NMR δ(CD_3OD, 200 MHz, TMS) 7.87(1H, d, H6, J=8.1 Hz), 6.27(1H, d, H1', J=3.7 Hz), 5.92(1H, d, H5, J=8.1 Hz), 4.10 to 3.31((11H, m, H5', CH_2OP, 2 x OH, H4', H2', H3'), 1.70(4H, m, $C\underline{H}_2CH_2OP$), 1.34(12H, m, $CH_3C\underline{H}_2C\underline{H}_2C\underline{H}_2$), 0.91(6H, m, CH_3)

^{31}P NMR δ(CD_3OD, 160 MHz, H_3PO_4) 0.193

^{13}C NMR δ(CD_3OD, 100 MHz, TMS) 167.26(C4), 157.76(C2), 144.88(C6), 94.79(C5), 88.92(C1'), 84.82(d, C4', J=6.5 Hz), 78.01(C2'), 76.14(C3'), 69.51(d, CH_2OP, J=6.6 Hz), 68.45(d, C5', J=5.5 Hz), 32.46($\underline{C}H_2CH_2CH_2OP$), 31.25(d, $CH_2\underline{C}H_2CH_2OP$, J=2.3 Hz), 26.27($CH_3CH_2\underline{C}H_2$), 23.63($CH_3\underline{C}H_2$), 14.39($CH_3$)

FAB MS m/e 982(M_2^+, 0.5%), 491(M^+, 3), 112(cytosineH^+, bp)

Found C 50.28%, H 7.83, N 8.26, P 6.38;
$C_{21}H_{38}N_3O_8P.[H_2O]_{0.5}$ requires C 50.39, H 7.85, N 8.40, P 6.19.

2.3.2.6

1-β-D-Arabinofuranosylcytosine-5'-bis-(2,2,2-trichloroethyl) phosphate (134f)

Compound **(1)** (1.00 g, 4.12 mmol) was dissolved in pyridine (150 ml), and bis-(2,2,2-trichloroethyl) phosphorochloridate (1.56 g, 4.14 mmol) added dropwise with vigorous stirring at *ca.* 4 °C. After stirring for 17 hours at ambient temperature, more bis-(2,2,2-trichloroethyl) phosphorochloridate (0.78 g, 2.07 mmol) was added. The reaction was stirred for a further 2 days at *ca.* 4 °C, then the reaction was quenched with water (150 μl, 8.24 mmol), and the solvent was removed under reduced pressure. The resulting oil was dried in

vacuum, and purified by flash column chromatography on silica. Elution with 15% methanol in chloroform, followed by pooling and evaporation of appropriate fractions gave an impure product (1.79 g). This was re-chromatographed, using first chloroform (500 ml), and then 10% methanol in chloroform as eluant. Pooling and evaporation of appropriate fractions of the latter eluant gave the product as a white solid (1.48 g, 63%).

^{1}H NMR δ(CD_3OD, 200 MHz, TMS) 7.75(1H, d, H6, J=7.5 Hz), 6.16(1H, d, H1', J=3.5 Hz), 5.79(1H, d, H5, J=7.5 Hz), 4.66(4H, 2xd, CH_2OP, J=4.1 Hz, J=4.1 Hz), 4.48 to 3.98(7H, m, H5', 2 x OH, H4', H2', H3')

^{31}P NMR δ(CD_3OD, 160 MHz, H_3PO_4) -2.34

^{13}C NMR δ(CD_3OD, 100 MHz, TMS) 167.60(C4), 158.15(C2), 144.69(C6), 96.02(d, CCl_3, J=11.3Hz), 94.90(C5), 88.81(C1'), 84.47(d, C4', J=6.6 Hz), 78.48(d, CH_2OP, J=4.4 Hz), 78.06(C2'), 76.12(C3'), 70.04(d, C5', J=6.0 Hz)

FAB MS m/e 594(MH^+, (^{37}Cl x 5) + (^{35}Cl x 1), 0.2%), 593(M^+, (^{37}Cl x 5) + (^{35}Cl x 1), 0.2), 592(MH^+, (^{37}Cl x 4) + (^{35}Cl x 2), 1), 591(M^+, (^{37}Cl x 4) + (^{35}Cl x 2), 0.5), 590(MH^+, (^{37}Cl x 3) + (^{35}Cl x 3), 4), 589(M^+, (^{37}Cl x 3) + (^{35}Cl x 3), 1), 588(MH^+, (^{37}Cl x 2) + (^{35}Cl x 4), 10), 587(M^+, (^{37}Cl x 2) + (^{35}Cl x 4), 2), 586(MH^+, (^{37}Cl x 1) + (^{35}Cl x 5), 12), 585(M^+, (^{37}Cl x 1) + (^{35}Cl x 5), 1), 584(MH^+, (^{35}Cl x 6), 6), 582(M-H^+, (^{35}Cl x 6), 1), 112(cytosineH^+, bp)

Found C 25.91%, H 2.94, N 6.57;
$C_{13}H_{16}N_3Cl_6O_8P.H_2O$ requires C 25.85, H 3.01, N 6.96.

2.3.2.7

1-β-D-Arabinofuranosylcytosine-5'-bis-(2,2,2-trifluoroethyl) phosphate (134g)

Compound (**1**) (0.5 g, 2.056 mmol) was dissolved in pyridine (60 ml), and bis-(2,2,2-trifluroethyl) phosphorochloridate (0.91 g, 3.25 mmol) added dropwise with vigorous stirring at *ca.* 4 °C. After stirring for 17 hours at ambient temperature, the reaction was quenched with water (75 μl, 4.12 mmol), and the solvent was removed under reduced pressure. The resulting oil was dried in vacuum, and purified by flash column chromatography on silica. Elution with chloroform (500 ml) and then 20% methanol in chloroform, followed by pooling and evaporation of appropriate fractions gave the product as a white solid (0.22 g, 22%).

^{1}H NMR δ(CD_3OD, 200 MHz, TMS) 7.73(1H, d, H6, J=7.5 Hz), 6.14(1H, d, H1', J=3.9 Hz), 5.79(1H, d, H5, J=7.5 Hz), 4.52(4H, m, CH_2OP), 4.45 to 3.96(7H, m, H5', 2 x OH, H4', H2', H3')

^{31}P NMR δ(CD_3OD, 160 MHz, H_3PO_4) -2.05

^{13}C NMR δ(CD_3OD, 100 MHz, TMS) 166.56(C4), 157.01(C2), 143.03(C6), 125.80(qd, CF_3, J_{CCOP}=8.5 Hz; J_{CF}=278.2 Hz), 93.98(C5), 88.21(C1'), 83.87(d, C4', J=6.5 Hz), 77.26(C2'), 75.21(C3'), 69.27(d, C5', J=5.8 Hz), 64.14(qd, CH_2OP, J_{COP}=3.7 Hz; J_{CCF}=38.3 Hz)

FAB MS m/e 975(M_2H^+, 3%), 488(MH^+, 32), 112(cytosineH^+, bp)

Found C 31.64%, H 3.67, N 7.43;
$C_{13}H_{16}N_3F_6O_8P$ requires C 32.05 , H 3.31 , N 8.62.

2.4 Biological Testing

2.4.1 Materials and Procedures

(a) Cell Culture

The cell line used was the mammalian epithelial cell line CNCM-1.221 (Collection Nationale de Cultures de Microorganisms Institute Pasteur, Paris), which was established on the 22nd of March 1972. Cells taken from liquid nitrogen storage were reconstituted by rapid thawing in warm water and then placed in growth medium. The following day the medium was replaced and after the first sub-culture, some cells were sub-cultured further for experiments and some were screened for contamination by autoradiography. A microscope slide in a close-fitting plastic case inside a square petri dish was seeded at a density of 5×10^4 cells/ml by placing growth medium (5 ml) on the slide and then carefully running a suspension (0.25 ml) containing 10^6 cells/ml down the centre of the slide. The slide was incubated at 37 ^oC in a 2% CO_2 atmosphere for 48 hours and then labelled with (methyl ^3H)thymidine (50 μl of a 100 $\mu Ci/ml$ solution, giving a final concentration of 1.0 $\mu Ci/ml$). After 5 hours the slide was washed five times with phosphate buffered saline (composition: NaCl, 8.0 g; KCl, 0.2 g; $CaCl_2.2H_2O$, 0.132 g; $MgCl_2.H_2O$, 0.1 g; $Na_2HPO_4.2H_2O$, 1.15 g; KH_2PO_4, 0.2 g; made up to 990 ml with distilled water). The pH was adjusted to 7.40 and the volume made up to 1.0 litre. The solution was filter sterilised before use and fixed with 4% glutaraldehyde for 10 minutes. Glutaraldehyde was prepared by diluting 4 ml of a 25% stock solution (TAAB Laboratory Equipment Ltd.) with 25 ml Sorensen's buffer, pH 7.4 (19.6 ml of 6.7×10^{-2} M KH_2PO_4 and 80.4 ml of 6.7 x 10-4 M Na_2HPO_4). The slide was then

washed sequentially with distilled water, 70% alcohol, 95% alcohol and absolute alcohol.

The slide was coated and developed by Len Bowler of Middlesex Hospital Pathology Unit. The slide was coated in a dark room. A heaped spatula of emulsion (Emulsion K2 in gel form, Ilford Ltd.), was mixed with an equal volume of distilled water (previously heated to 37 °C in a water bath) to form a thick slurry and kept warm. The emulsion was poured down the vertically held slide, to form an even coat. The slide was then placed in a rack in a drying cabinet for 10 minutes. The slide was then transferred to a light-proof box, sealed and left at 4 °C for 7 days. The developing of the slide was also performed in a dark room. Developer (Contrast FF developer, Ilford Ltd.) (15 ml) was added to distilled water (100 ml) at room temperature and then poured into the slide box and mixed with the lid on. The box was left for 15 minutes with mixing every 5 minutes, and then washed with distilled water. Hypam fixative (Ilford Ltd.) was added and the slide left for 5 minutes with mixing. The slide was removed from the dark room and then thoroughly washed with water and rinsed with distilled water. The dry slide was stained with freshly filtered giemsa R66 (BDH) for 20 minutes and then sequentially washed and dehydrated with distilled water, 70% alcohol, 95% alcohol, absolute alcohol (for 1 minute), absolute alcohol (for 2 minute) and xylene (2 x 1 minute) to clear. The slide was air dried and mounted in Deepex. The cells were found to be mycoplasma free as assessed by microscopic examination.

Some cells were re-frozen for storage. On freezing, cells were protected from low temperature damage by the addition of DMSO to the medium. The cells were spun down and the pellet resuspended in 7.5% DMSO to give a cell concentration of about 2×10^6 cells/ml. The suspensions were transferred in 1-2

ml aliquotes to sterile 2 ml polypropylene tubes and placed in a polystyrene container. Stepwise freezing was carried out for 3 hours or overnight at 4 °C, then 30 minutes at -18 °C, then overnight at -70 °C, and then the ampoules were transferred to liquid nitrogen for long term storage.

The cells were routinely cultivated in polystyrene flasks (Falcon Scientific Supplies Ltd.) in growth medium consisting of minimum essential medium (Eagle with Earle's salts, Flow Labs Ltd.), supplemented with foetal bovine serum (10%, Flow Labs Ltd.), L-glutamine (2 mM), sodium bicarbonate (0.03%), penicillin (Glaxo, 100 units/ml) and streptomycin (Glaxo, 100 mg/ml) and buffered to pH 7.4, with HEPES (20 mM). Cells were grown in a 37 °C incubator, with a 2% CO_2 in air atmosphere, and routinely sub-cultured at weekly intervals after detachment using 5% trypsin (Trypsin powder, Hopkin and Williams 883600) from a 10% stock solution (in 6 mM glucose and 1 mM phosphate buffer) which was diluted with serum free medium.

For one large falcon (30 ml) of confluent cells, the spent medium was poured away. Trypsin (pH adjusted, 1.0 ml) and SFM (1.0 ml) were added at room temperature until the cells started to detach when agitated. The cells were then transfered to a centrifuge tube and washed in Growth medium (2 ml). The tube was spun for about 4 minutes maximum at 800 rpm. The supernatant was poured off and the cells were resuspended in Growth medium (1 ml) and counted in a Coulter counter. The cells were then seeded into clean falcons with Growth medium (30 ml), the normal seeding density for a large falcon was 2 x 10^3 cells/ml.

(b) (Methyl-^{3}H)thymidine Incorporation Well Experiment

Day 1: A large stock falcon (30 ml) of cells was sub cultured, as described in (a) for the following week at a seeding density of 2 x 10^3 cells/ml. Cell counts were made using a coulter counter, model ZBI, calibrated with ragweed pollen, using the mean of three counts to calculate cell numbers. Cell suspension in Growth medium (4 ml, 10^6 cells/ml) was added to Growth medium (80 ml) to give a cell suspension in Growth medium of 5 x 10^4 cells/ml. Experiments were performed in multiwell trays, with a growth area of 2.1 cm^2/well, SFM (1 ml) was added to the outer 16 wells/culture tray whilst the inner 8 wells/culture tray were seeded at 5 x 10^4 cells/ml in Growth medium (1 ml/well in a 'snake fashion'). Then the trays were placed in a CO_2 incubator for 48 hours unstacked.

Day 2: The test compounds **(134a-e)** and the control **(1)** were made up in sterile 10% DMSO to a concentration of 300 μM and the reagents and equipment for the following day were prepared.

Day 3: All the solutions to be added to the 8 centre wells were at 37 °C. Each compound (100 μl) was added to one set of four wells and mixed by gentle rocking. The cells were incubated in the carbon dioxide incubator for 30 minutes. Then (methyl-^{3}H)thymidine (specific activity 925 GBq/mmol, Amersham International PLC) solution (50 μl, 20 μCi/ml) was added to each well (final concentration per well 1 μCi/ml) and mixed by gentle rocking. The trays were left in a CO_2 incubator for 30 minutes. At the end of this incubation the cells were washed gently five times with phosphate buffered saline and fixed by leaving in a fridge (4-7 °C, 30 minutes) with TCA solution (1 ml/well, 5%). The trays were then washed twice with PBS, drained well and dried under a

warm air dryer. Then NaOH (1 M, 250 μl/well) was added and allowed to digest overnight in a sealed plastic box (to minimise the evaporation) in a 37 °C incubator.

Day 4: The trays were removed from the plastic box, some digest (100 μl) from each well were added separately to HCl (1 M, 100 μl) and EcosintA (4 ml) in plastic tubes, mixed well, for taking the radioactive counts per minute. The left over digest for each set of four wells were pooled (4 x 150 μl = 600 μl) and added seperately to a cuvette containing NaOH (1 M, 2400 μl) the absorbance at λ = 280 nm taken on a UV/VIS Spectrophotometer (Unicam SP500 series 2) which had been zeroed with a cuvette containing NaOH (1 M, 3000 μl). The absorbance was taken to check the amount of protein that was present to ensure that the wells had been seeded uniformly. The scintillation counts were measured in an Intertechnique ABAC SL40 scintillation counter. The results of the assay are expressed as mean counts per minute for each set of 4 replicate wells. From the data, the mean % inhibition of thymidine incorporation into DNA was calculated, with respect to 10% DMSO control.

(c) General cleaning and sterilisation procedures

All glassware and plastic containers used for tissue culture were immersed in cleaning liquid (Decon) overnight, rinsed with water, left overnight in 2% HCl, washed with water, rinsed three times with distilled water and dried. Graduated pipettes were left in 2% bleach solution (Chloros) overnight, rinsed with water then distilled water and dried. Caps were boiled in distilled water containing detergent for 5 minutes, rinsed with distilled water and dried. The work area was washed with Precept (containing 1000 ppm chlorine) and 1% Hycolin was used for the general cleaning of the laboratory. Plastics were sterilised by autoclaving for 15 minutes. Non-plastic or non-rubber materials

were sterilised by hot air oven at 160 °C for 2 hours. Falcons were sterilised by gamma irradiation. Pipettes were plugged with non-adsorbant cotton wool, placed in canisters and sterilised by hot air oven.

2.4.2 Biological results

Table 5 The inhibition of the incorporation of tritiated thymidine into DNA in the presence of the compounds listed at a final concentration of 30 μM, relative to 10% DMSO control

Compound	%I (SEM)
(1)	81 (3)
(134a)	11 (4)
(134b)	25 (2)
(134c)	37 (2)
(134d)	54 (3)
(134e)	62 (4)

The biological evaluation of **(134f-g)** was carried out by an identical method employed for **(134a-e)**.

Compound	%I (SEM)
(1)	83 (5)
(134f)	91 (3)
(134g)	67 (6)

2.4.3 Lipophilicity studies

Partition coefficients of (1) and the series (134a-e)

Samples (*ca.* 50 μM) of compounds **(1)** and **(134a-e)** were partitioned between distilled water (25 ml) and n-octanol (25 ml) at ambient temperature. In some cases emulsion formation was noted; being overcome by the addition of solid sodium chloride. Two samples of each separate layer were taken in each case, and their UV spectra recorded. Each experiment was carried out three times. Absorbances were averaged and the mean lipophilicity, P values were calculated.

Table 6 Lipophilicity values, P and logP for (1) and (134a-e)

Compound	P	logP
(1)	0.1	-1.00
(134a)	0.2	-0.68
(134b)	0.6	-0.19
(134c)	1.4	0.14
(134d)	2.4	0.39
(134e)	3.4	0.53

2.5 References

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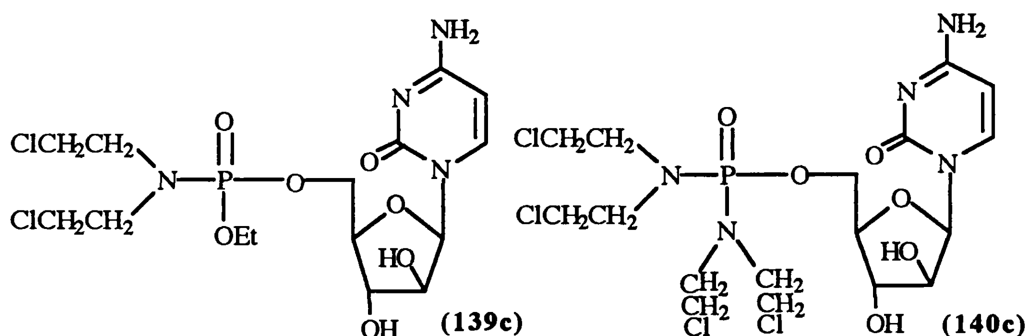
3.0 Design and synthesis of novel phosphoramidate and phosphorodiamidate and asymmetric 5'-dialkyl phosphate triester derivatives of (1)

3.1 Introduction

The main aim of this part of the project was to produce a compound containing the alkylating properties of **(25)** and the antimetabolic character of (1). The target compounds being **(139c)** and **(140c)**.



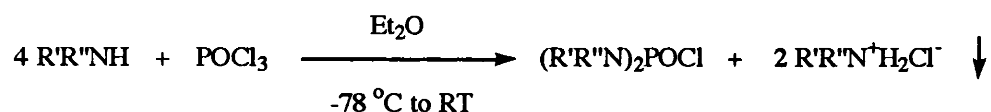
Many model reactions were carried out with primary and secondary amines in order to ultimately find a route to these interesting novel 'hybrid' anti-cancer agents. The phosphoramidate and phosphorodiamidate model compounds were of interest in themselves as lipophilic forms of (1). Nucleoside phosphorodiamidates are relatively unknown phosphate derivatives.[1] By contrast, phosphoramidates have been widely used in the synthesis of oligophosphates,[2,3] and their kinetics of hydrolysis have been thoroughly studied.[4] In water, phosphorodiamidates behave in a manner similar to that of the phosphoramidates; that is, they are relatively labile and are hydrolyzed to the parent phosphates at rates that are influenced by both the nature of the amino group and the pH of the medium.[1]

An attempt was made to prepare phosphorodiamidate derivatives of **(1)** *via* several methods.

The initial approach was to attempt a similar reaction used for the preparation of symmetric 5'-dialkyl phosphate triester derivatives of (1) (Chapter 2). The first step of this approach involved the preparation of a bis-dialkylamino phosphorochloridate, for which there are a few methods.[5] One method is the reaction of phosphoryl chloride with amine hydrochlorides.[5]

These reactions are carried out under mild reflux, for several hours, until the amine salt suspension becomes a clear solution.The slowness of the attack permits a clean preparation of the target compound. However undue heating may cause decomposition to the imido derivative [$(RNH)_2PO:NR$].[5]

The method employed to make bis-dialkylamino phosphorochloridate was the reaction in an ethereal solution of 4 molar equivalents of an amine with phosphoryl chloride.



Primary and secondary amines react smoothly with phosphorus oxychloride to form the corresponding amides. Somewhat elevated temperatures are required to produce bis-dialkylamino phosphorochloridates relative to the production of the analogous dialkyl phosphorochloridates.[5]

The isolated bis-dialkylamino phosphorochloridate was then put into a reaction with **(1)** in pyridine as both solvent and base, as for the reaction of the dialkyl phosphorochloridates with **(1)** (Chapter 2). However the phosphorus

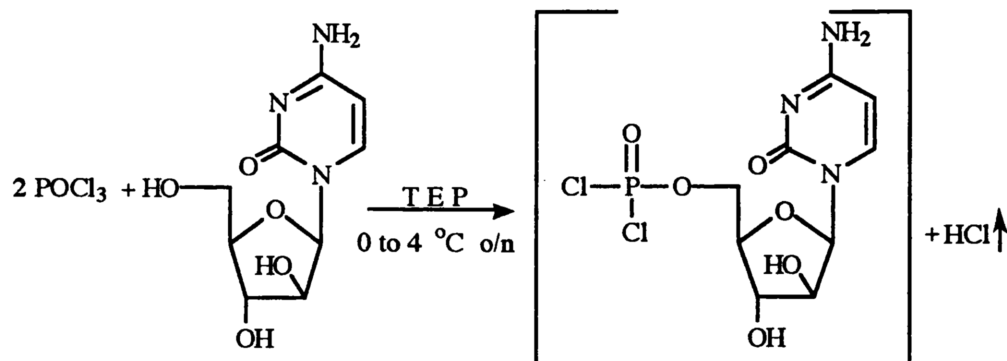
was probably deactivated by the lone pairs on the nitrogens of the amino groups in the phosphorylating agent and the reaction did not proceed to give the target compound, unlike the similar reaction with dialkyl phosphorochloridate.[5]

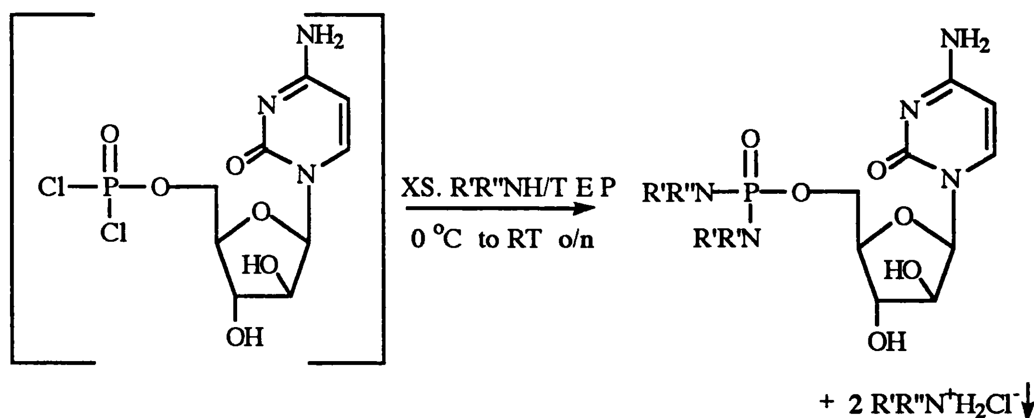
The second approach to make phosphorodiamidate derivatives of **(1)** was to react phosphoryl chloride with **(1)** in triethyl phosphate and then to react the appropriate amine with the unisolated intermediate.

Triethyl phosphate (TEP) has been widely used in this type of chemistry ever since Yoshikawa et al noted the 5'-specific reaction of phosphoryl chloride with nucleosides in TEP.[6,7]

Similar reactions have been reported in the production of phosphorodiamidate derivatives of 2'-deoxyuridine and thymidine. Although in the literature, the nucleosides were protected at the 3'-OH by an acyl group and then deprotected after phosphorylation and amination.[8-10]

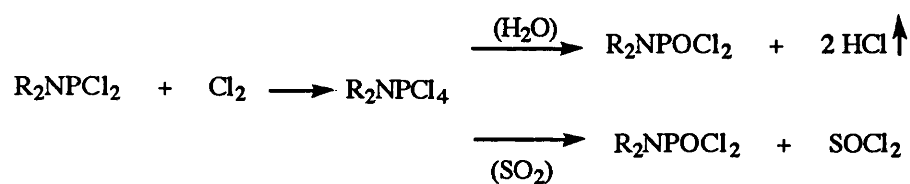
A high yield of reaction was not a primary aim, as the main interest were the biological properties, thus the reaction was carried out on unprotected **(1)**.





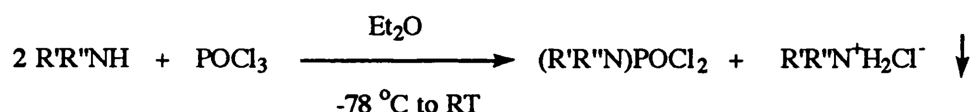
The preparation of (alkyl)(amino), asymmetric, derivatives of araCMP were attempted by several methods. A series of asymmetric phosphorylating agents, that is, (alkyl)(amino)phosphorochloridates were prepared in order to react them with (**1**). The preparation of the asymmetric phosphorylating agents involved either, the preparation of an alkyl phosphorodichloridate and its subsequent reaction with the required amine, or the preparation of an amino phosphorodichloridate and its subsequent reaction with the required alcohol, both routes were attempted. As with most phosphorylating agents there are numerous methods for the preparation of these phosphorodichloridates.[5]

In common with other trivalent phosphorus derivatives, the amidohalophosphites, their neutral esters, and the amides of phosphonous acids readily add chlorine. Usually only the derivatives of secondary amines can be used because of the side reactions that take place at the NH of the primary amine analogues. The products can be easily converted to the target amino phosphorodichloridates by mild hydrolysis, usually exposure to moist air. However a cleaner product is given when SO_2 is used rather than water.[5]

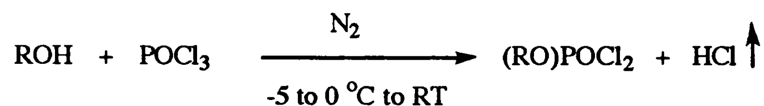


The reaction of phosphoryl chloride with amine hydrochlorides as for the preparation of bis-dialkylamino phosphorochloridates can be achieved by the use of one molar equivalent of the amine hydrochloride.

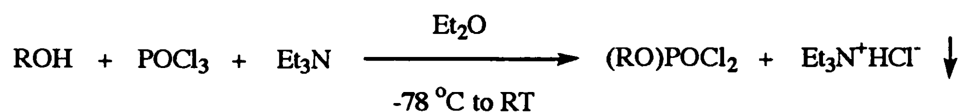
The method of choice was the reaction of two molar equivalents of the amine with phosphoryl chloride in an etheral solution in an analogous method to the preparation of the bis-dialkylamino phosphorylating agents. However, the reaction was not heated, in order to avoid the reaction of the amine salt with the target compound.



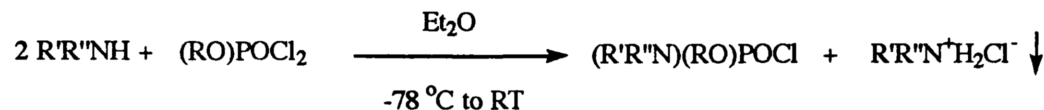
One of the simplest and most commonly used method to prepare alkyl phosphorochloridates is to react the appropriate alcohol with phosphoryl chloride, with removal of the HCl by-product *via* a rapid flow of dry nitrogen through the system.[11]

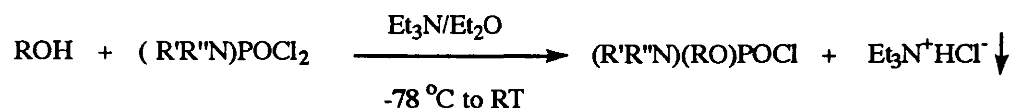


The reaction of one molar equivalent of an alcohol with phosphoryl chloride in an inert solvent with a tertiary organic base to take up the hydrogen chloride, usually below room temperature to control the reaction is often used, the products are isolated after filtration.[5]



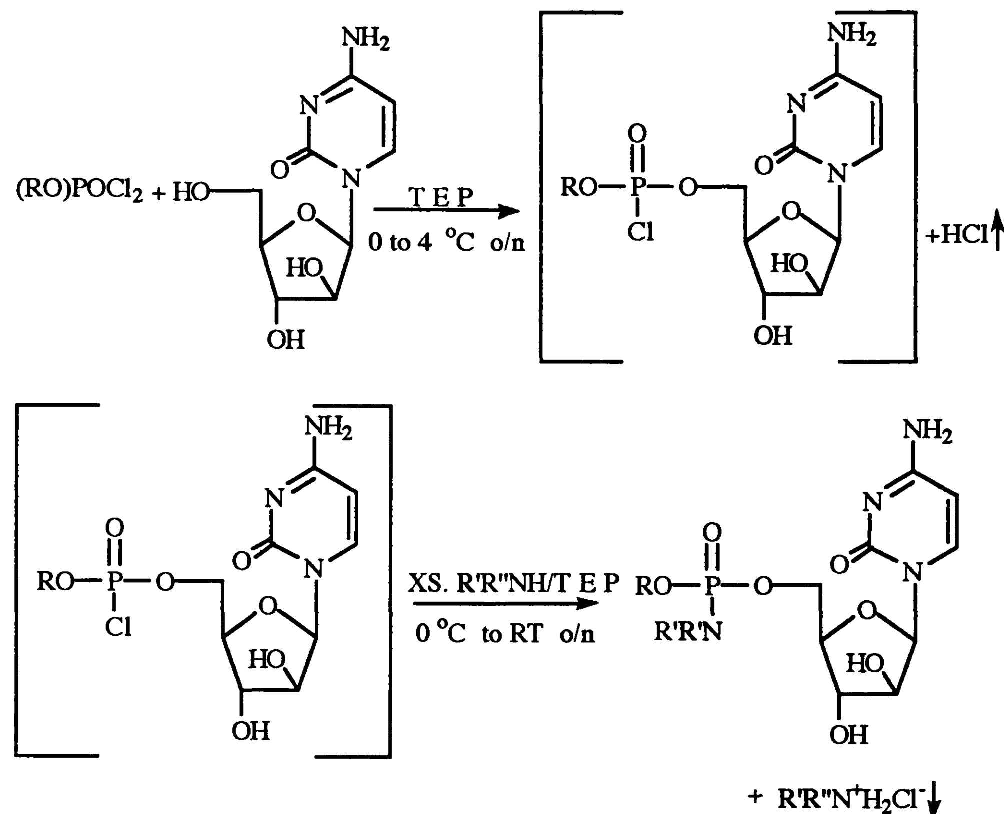
The reaction of an amino phosphorodichloridate with an alcohol or the reaction of an alkyl phosphorodichloridate with an amine allowed the isolation of the target asymmetric phosphorylating agents.





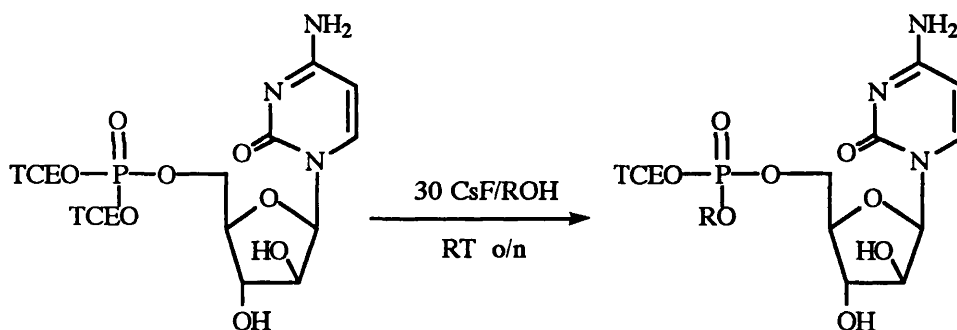
The asymmetric phosphorylating agents were put into an analogous reaction for dialkyl phosphorochloridates with (1) (Chapter 2).

Another modification to the Yoshikawa reaction was the attempted reaction of alkyl phosphorodichloridate with (1) in TEP and the subsequent reaction of the required amine with the unisolated intermediate.

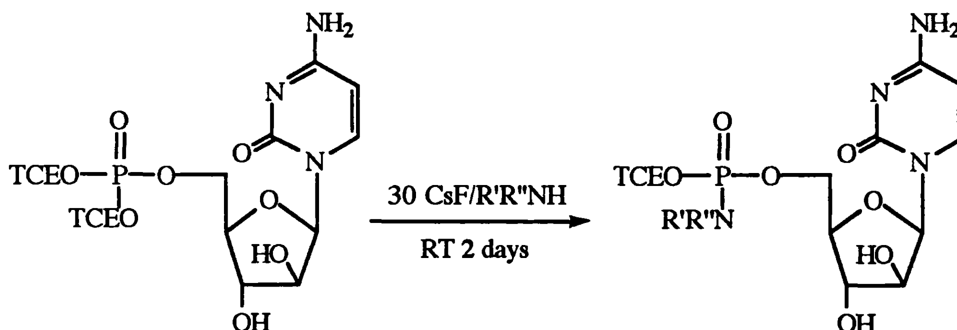


Yet another procedure employed to the synthesis of asymmetric derivatives of (**1**) was a transesterification reaction based on the work of Ogilvie et al[21] for the synthesis of mixed trialkyl phosphates. Ogilvie et al reported numerous compounds of the type [(TCE)$_2$PO(OR)] which were reacted with an alcohol [R'OH] and an excess of cesium fluoride. The alcohol acted as both reactant and solvent, to give products of the type [(TCE)PO(OR)(OR')]. Ogilvie suggested an unisolated intermediate of the type [(TCE)PO(OR)(F)] was formed during these reactions.[13]

1-β-D-Arabinofuranosylcytosine-5'-bis-(2,2,2-trichloroethyl) phosphate (Chapter 2) was employed in these transesterification reactions



An adaption to this reaction was discovered, in that primary amines were found to undertake a similar reaction to the normal alcohols.



Molecular graphics and NOE experiments were carried out on the HPLC separated diastereoisomers of 1-β-D-arabinofuranosyl cytosine-5'-2,2,2-

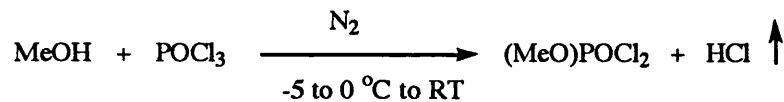
trichloroethylethyl phosphate and the results are duly presented.The compounds isolated were tested for their biological activity with mammalian epithelial cells *in vitro*.

A novel base protected derivative of **(1)** was produced, which may be the starting point for future research in this area. An analogous protection of cytidine has been reported.[14]

3.2 Results and discussion

A number of reactions were carried out with the aim of producing asymmetric derivatives of the anti-cancer nucleoside analogue **(1)** as outlined in the introduction to this chapter.

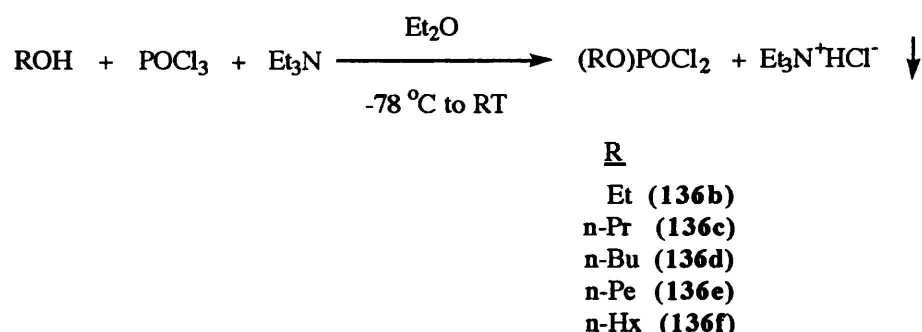
A series of n-alkyl phosphorodichloridates **(136a-f)** was prepared in order that asymmetric phosphorylating agents could be subsequently formed. Compound **(136a)** was produced by the reaction of one equivalent of methanol with phosphoryl chloride, the HCl by-product was removed and the product was isolated by vacuum distillation in a 46% yield.



(136a)

The ^{31}P NMR showed one peak at δ5.26 which agreed with the literature value.[15] ^{13}C and ^{1}H NMR were also fully consistent with the structure of **(136a)**. ^{13}C NMR was a doublet at δ57.63 with a coupling constant of 8.6 Hz. ^{1}H NMR gave a doublet at δ3.89 coupling to phosphorus with a coupling constant of 17 Hz.

Compounds **(136b-f)** were prepared by the addition of one molar equivalent of the appropriate alcohol to phosphoryl chloride in the presence of one molar equivalent of triethylamine with diethyl ether as the solvent. The impurities which are possibly produced in this reaction are the dialkyl phosphorochloridates along with unreacted phosphoryl chloride, also some hydrolysed products (if there is some water in the system). To minimise the formation of these unwanted products, all reagents were scrupulously dried and the reaction was carried out at -78 °C and the alcohol and base, in ether, were added slowly to the phosphoryl chloride, which was also in ether.

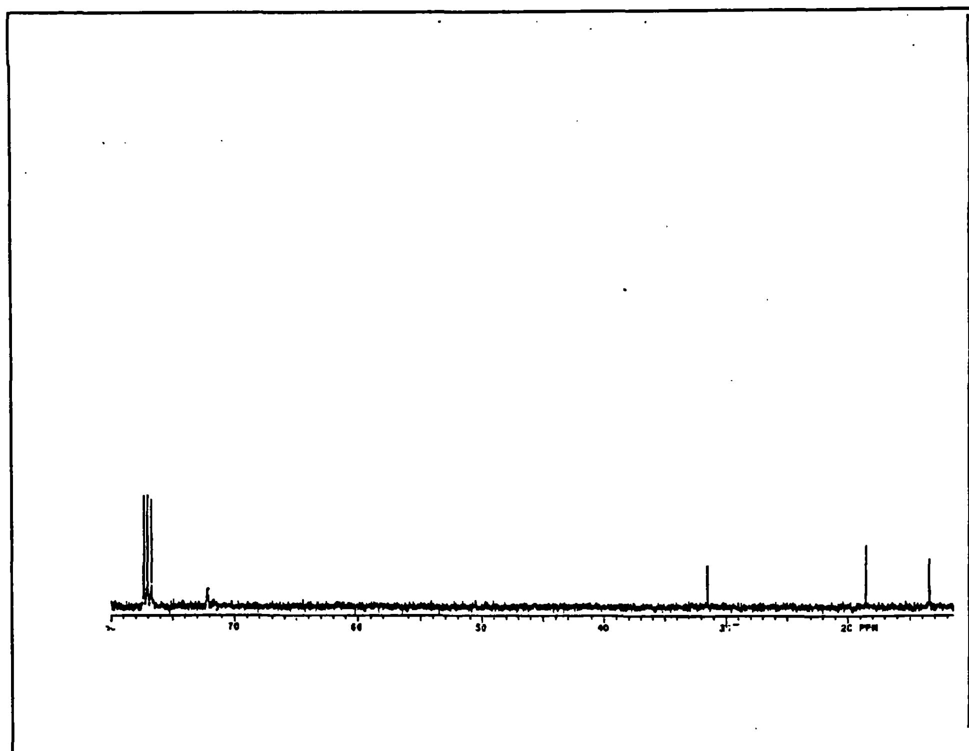


The reaction mixture was allowed to warm to ambient temperature and stirred overnight, then the precipitated triethylamine hydrochloride was removed by filtration under nitrogen. Compounds **(136b-f)** were all isolated pure crude with yields of *ca.* 80%.

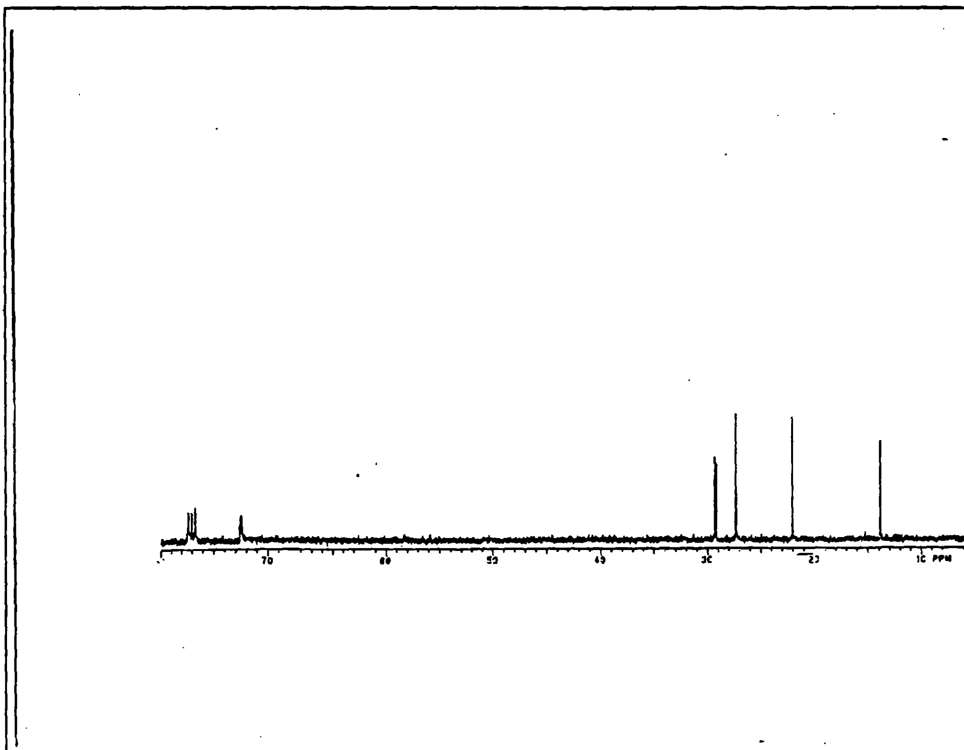
Confirming their identity and purity, all the alkyl phosphorodichloridates gave one peak ^{31}P NMR spectra with chemical shifts close to literature values.[15] The ^{13}C NMR and ^{1}H NMR spectra of **(136b-f)** confirmed their formation and purity. Phosphorus coupling was observed in the ^{13}C NMR spectra of all the compounds, but only the two nearest carbon atoms to the phosphorus atom. An interesting facet of the ^{13}C NMR of **(136b-e)** series was that the nearer the carbon was to the phosphorus, the chemical shift would be

more downfield. However with **(136f)**, the 2nd carbon from the phosphorus was more upfield than the 3rd carbon. The three bond coupling was slightly larger than the two bond coupling, probably due to the angular dependence of coupling constants[16], this was with the exception of **(136f)** where the two bond coupling was slightly larger than the three bond coupling. These phenomenon were also observed for the similar dialkyl phosphorochloridates **(133a-e)**.

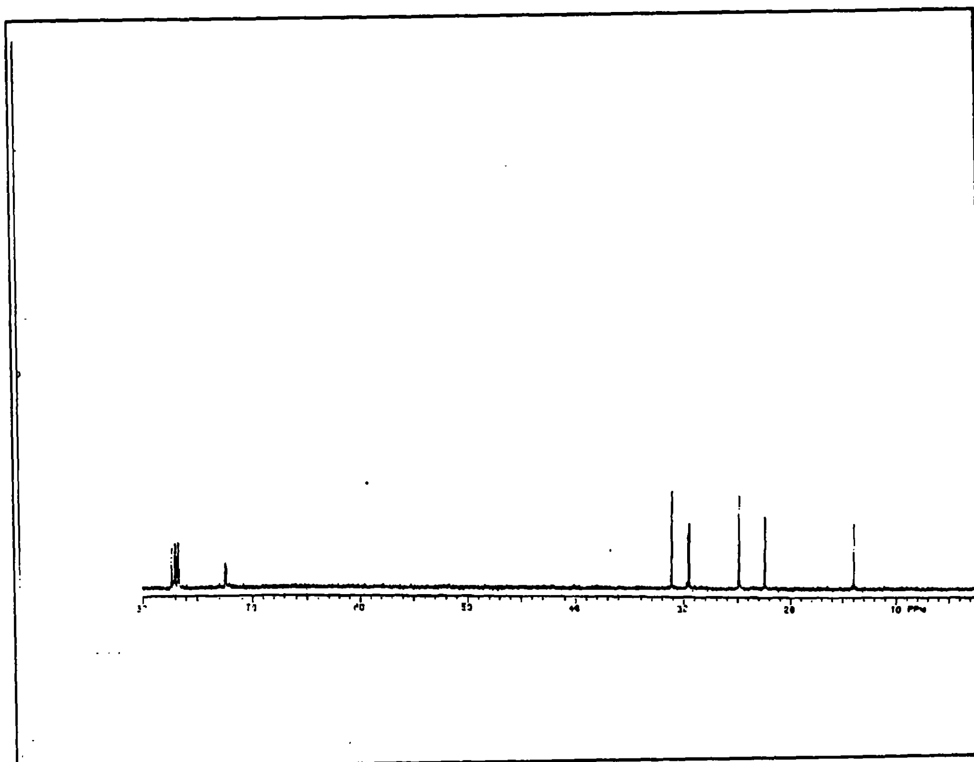
^{13}C NMR spectrum of (136d)



^{13}C NMR spectrum of (136e)

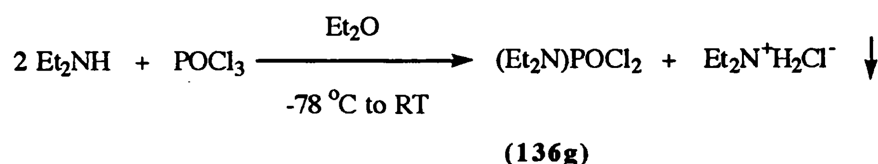


^{13}C NMR spectrum of (136f)



^{1}H NMR spectra of the series **(136b-f)**, showed that the protons within three bonds to the phosphorus were complicated by coupling to the phosphorus.

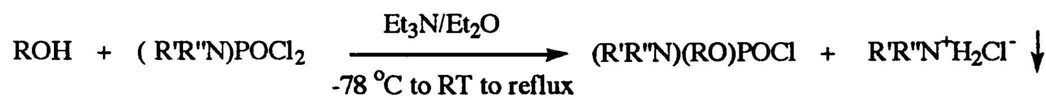
One dialkylamino phosphorodichloridate was prepared, diethylamino phosphorodichloridate, **(136g)**. Compound **(136g)** was prepared by a similar method to the alkyl phosphorodichloridate series **(136b-f)** except that two equivalents of diethylamine were employed, rather than one equivalent of diethylamine and one equivalent of triethylamine.[5]



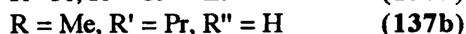
After filtration and evaporation of the filtrate the target phosphorodichloridate **(136g)** was obtained without further purification in a 74% yield. The ^{31}P NMR of **(136g)** was consistent with the literature and was noted to be dramatically downfield compared to the alkyl phosphorodichloridates **(136a-f)** with a chemical shift of δ13.79 which was a quintet on hydrogen coupling, as would be expected.[15]

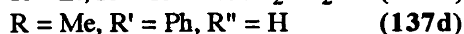
The ^{13}C NMR and the ^{1}H NMR of **(136g)** were fully consistent with its structure, with two and three bond coupling to phosphorus. The methyl and methylene shifts in the diethylamino group of **(136g)** were slightly upfield relative to the ones in the ethoxy group of **(136b)**.

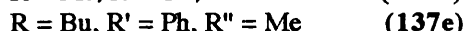
The preparation of the asymmetric phosphorylating agents involved either, the preparation of an amino phosphorodichloridate and its subsequent reaction with the required alcohol,



or the preparation of an alkyl phosphorodichloridate and its subsequent reaction with the required amine.





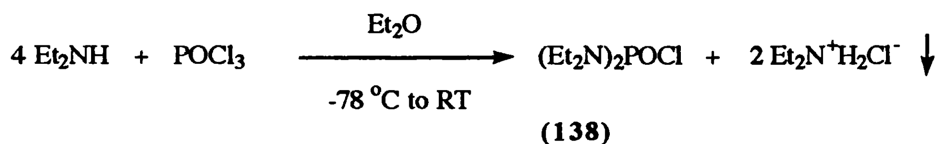


Both routes were attempted for **(137a)**, however subsequent analogous reactions involved the latter reaction because this proceeded without heating at a quicker rate than the former, however both routes gave similar yields. The yields for the preparation of **(137a-f)** were within 59% to 98%.

Confirming their identity and purity, all the asymmetric phosphorochloridates gave one peak ^{31}P NMR spectra with chemical shifts close to literature values.[15] An interesting comparison of the ^{31}P NMR signal of the compounds with a phenyl amino group and compounds with an n-alkyl amino group showed that the signal of the former phosphorylating agents were more upfield by approximately 3 ppm. Satisfactory microanalytical data were obtained for each of the asymmetric phosphorochloridates. The ^{13}C NMR and ^{1}H NMR spectra of **(137a-f)** confirmed their formation and purity. Phosphorus coupling was observed in the ^{13}C NMR spectra of all the compounds, but only the two nearest carbon atoms to the phosphorus atom. The mass spectrum of compound **(137a)** showed a parent ion at 213 m/e and 215 m/e in a 3:1 ratio consistent with its structure. Similarly, the mass spectrum of compound **(137d)** showed a protonated parent ion at 206 m/e and 208 m/e in a

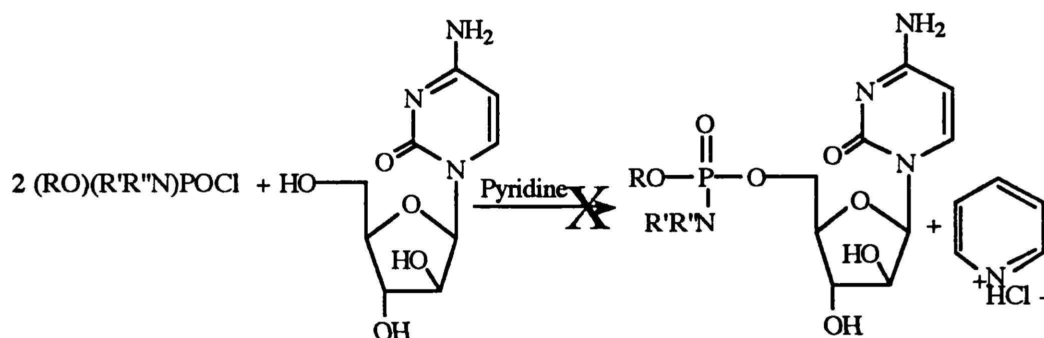
3:1 ratio consistent with its structure, also the base peak was due to the parent ion at 205 m/e which was in a 3:1 ratio with the expected peak at 207 m/e. Interestingly, the mass spectrum of compound **(137e)** showed a weak signal for the parent ion and the base peak was due to $C_6H_5NCH_3$, other fragment ions confirmed the presence of one chlorine in the structure of **(137e)**.

Bis-diethylamino phosphorochloridate **(138)** was the only bis-dialkylamino phosphorylating agent produced. Compound **(138)** was prepared by a similar method to the production of the dialkyl phosphorochloridate series **(133a-g)**. However, diethylamine was employed as reactant and base rather than using triethylamine as the base. The reaction to prepare **(138)** was refluxed for 4 days compared to the overnight reactions at room temperature for the dialkyl phosphorochloridate series. Compound **(138)** was isolated after vacuum distillation to yield a pure sample in a 28% yield.



The ^{31}P NMR showed one peak at δ24.52 which was consistent with similar compounds in the literature,[15] it is interesting that the presence of one diethylamino group in an asymmetric phosphorylating agent moves the ^{31}P NMR signal about 10 ppm downfield compared to a dialkyl phosphorochloridate, whilst the ^{31}P NMR signal for **(138)** was found to be *ca.* 20 ppm downfield compared to a dialkyl phosphorochloridate. ^{13}C and ^{1}H NMR were also fully consistent with the structure of **(138)**. The mass spectrum of compound **(138)** showed a parent ion at 226 m/e and 228 m/e in a 3:1 ratio consistent with its structure. Satisfactory microanalytical data was obtained for **(138)**.

The asymmetric phosphorylating agents **(137a-f)** and compound **(138)** were put into an analogous reaction for dialkyl phosphorochloridates **(133a-g)** with **(1)** in pyridine as both solvent and base. However the phosphorus was probably deactivated by the lone pairs on the nitrogens of the amino groups in the phosphorylating agent and the reactions did not proceed to give the target compounds, unlike the similar reactions with a dialkyl phosphorochloridate.



R =Pr, R' = R" = Et	**(137a)**	R =Pr, R' = R" = Et	**(139a)**
R = Me, R' = Pr, R" = H	**(137b)**	R = Me, R' = Pr, R" = H	**(139b)**
R = Et, R' = R" = $ClCH_2CH_2$	**(137c)**	R = Et, R' = R" = $ClCH_2CH_2$	**(139c)**
R = Me, R' = Ph, R" = H	**(137d)**	R = Me, R' = Ph, R" = H	**(139d)**
R = Bu, R' = Ph, R" = Me	**(137e)**	R = Bu, R' = Ph, R" = Me	**(139e)**

An attempt to prepare **(139a)** was made by reacting two equivalents of **(137a)** with **(1)** in pyridine. The reaction was carried out at about 4 ^{o}C, after addition, the reaction was stirred at ambient temperature and then heated at 50 ^{o}C for 3 days. The reaction mixture was quenched with water. The residue was found to be extremely messy on TLC. ^{31}P NMR spectrum of the crude material showed several signals ranging from δ11.28 to δ-12.11. The reaction was not worked up further.

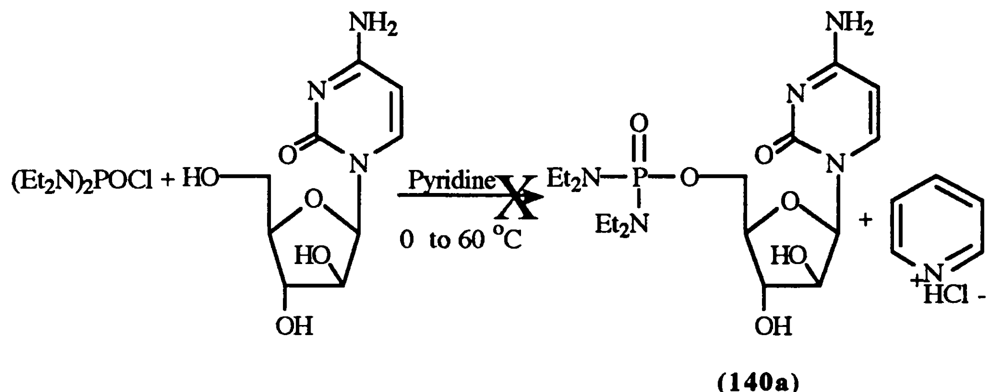
From the attempted preparation of **(137a)** it was decided that a less bulky phosphorylating agent should be attempted. An attempt to prepare **(139b)** was made by reacting two equivalents of **(137b)** with **(1)** in pyridine. The reaction was carried out at about 4 ^{o}C, after addition, the reaction was stirred at ambient temperature for 5 days. The reaction mixture was quenched with water. The residue was found to be extremely messy on TLC and the reaction was not worked up further.

Even though the model reactions to prepare **(139a-b)** were unsuccessful, an attempt was made to prepare the target 'hybrid' anti-cancer compound **(139c)**. However, the preparation of **(139c)** was found to be even more messy than the model reactions, another method of preparation had to be investigated.

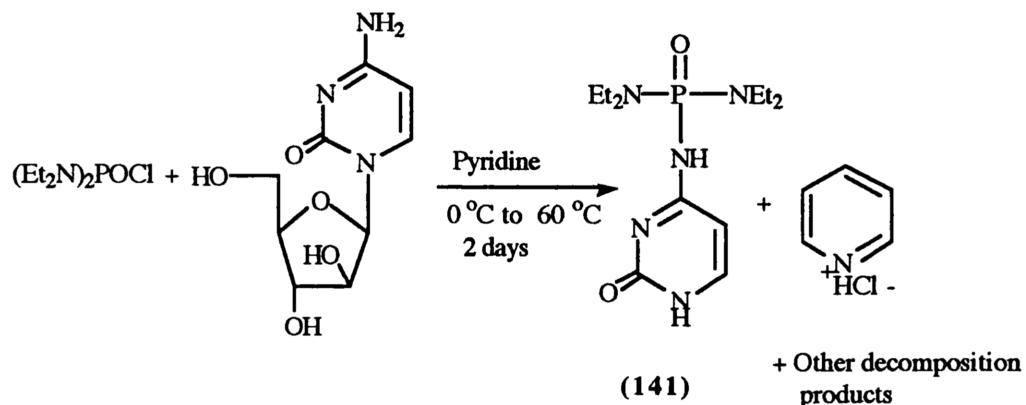
Thus from the attempted preparation of **(137a-c)** it was decided that the amino group was deactivating the phosphorylating agent compared to the alkoxy groups in the series **(133a-g)**. A small study of whether the inactivity of **(137a-c)** were due to the donation of the lone pair of the nitrogen was undertaken. The phosphorylating agents **(137d-e)** were prepared in the belief that the lone pair on the nitrogen would be delocalised into the phenyl ring and thus the activity of the phosphorylating agent would be enhanced. The attempted preparation of **(139a-d)** employing a similar reaction to the preparation of **(139b)** was found to be extremley messy on TLC; reactants, solvent and products all having an UV chromophore, the target product was again not isolated.

In order to investigate whether a bis-dialkylamino phosphorochloridate was more unreactive than the asymmetric phosphorylating agents **(137a-e)**, two

molar equivalents of compound **(138)** was reacted with **(1)** in pyridine. This reaction was attempted twice, the first attempt was very messy and the reaction was not worked up.



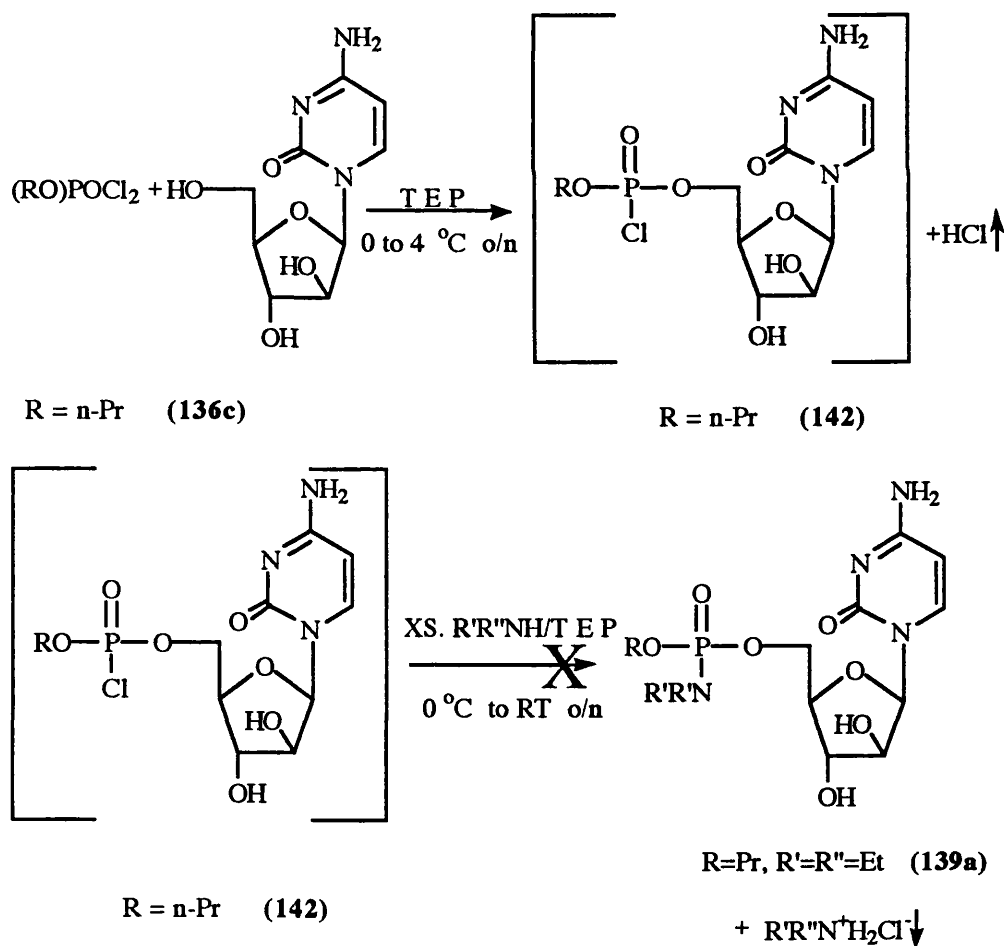
The second attempt to prepare **(140a)** gave numerous products with one major product faster than compound **(1)** on TLC. The major product was isolated by flash column chromatography and was found to be a decomposition product, cytosine-N-bis-diethylamino phosphate **(141)** in a 13% yield.



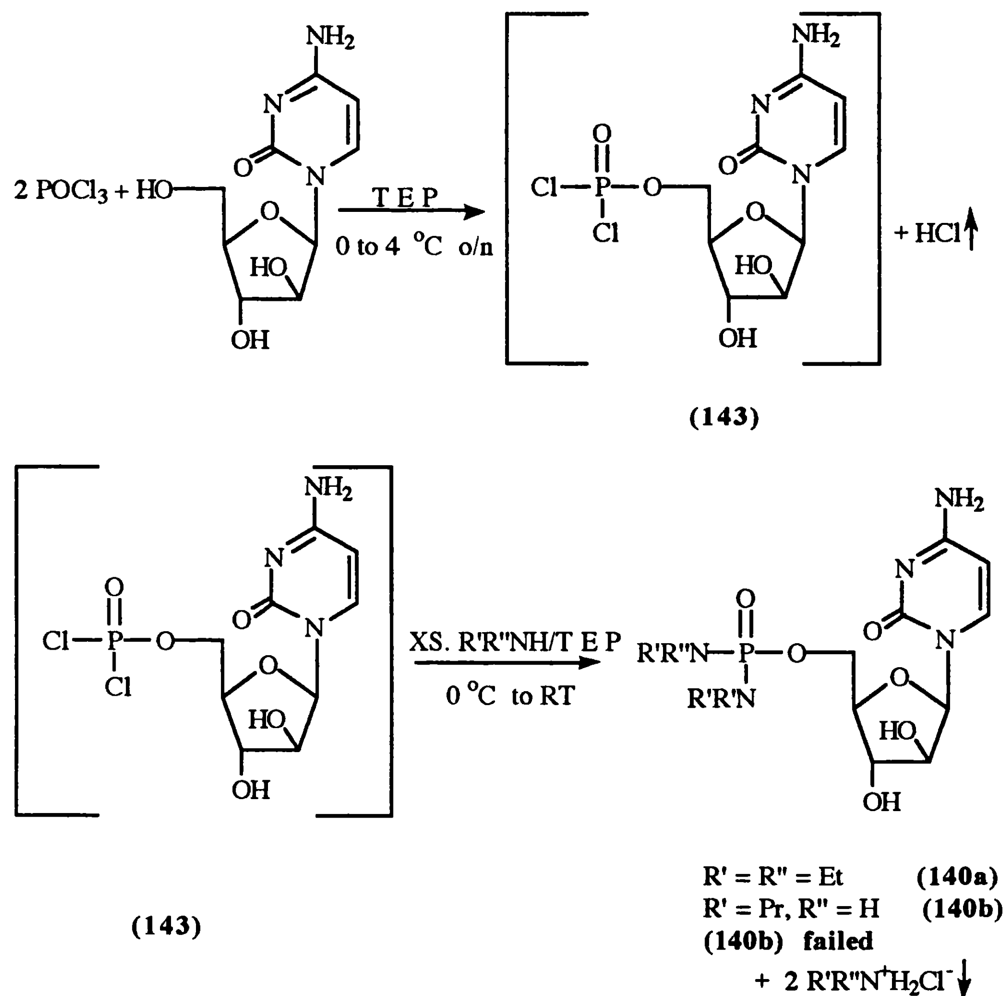
The ^{31}P NMR signal of **(141)** was interesting, even though there are three P-N bonds, the shift is analogous to if there was only one alkylamino group attached to the phosphorus, this may be due to the fact that substituents on phosphorus containing a carbonyl group seem to cause a shift upfield.[15] The ^{1}H NMR and ^{13}C NMR were fully consistent with the proposed decomposition product. It was apparent from the ^{13}C NMR that the NH_2 had been

phosphorylated and not any other position in cytosine because, C4 and C5 were split due to phosphorus coupling. The mass spectrum of **(141)** showed a parent ion at 301 m/e with an exact mass relating to that calculated for the proposed structure. Microanalysis was consistent with the structure of **(141)**.

The preparation of a phosphoramidate derivative of **(1)** was attempted *via* the initial reaction of the nucleoside with **(136c)** in triethyl phosphate and then to react the proposed intermediate **(142)** with an excess of diethylamine, with the aim of producing **(139a)**. However the reaction mixture was found to be very messy on inspection with TLC, the reaction was not worked up further.

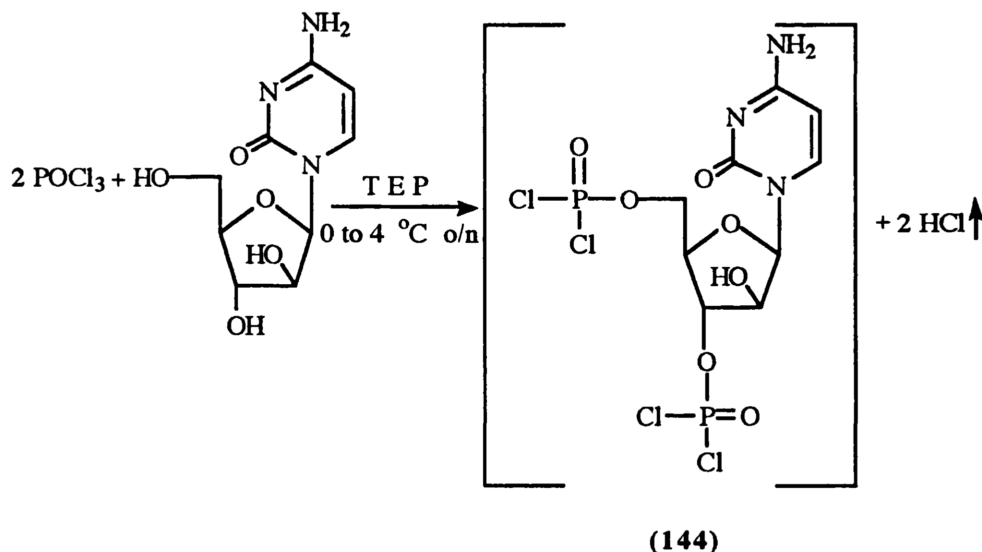


The preparation of phosphorodiamidates was attempted *via* the initial reaction of phosphoryl chloride with **(1)** to give the unisolated, proposed intermediate **(143)**, followed by the subsequent reaction of the intermediate with an excess of amine. Thus, **(140a)** was prepared by the reaction of diethylamine with **(143)**. The reaction was extre mly messy and compound **(140a)** was isolated in a very low yield.

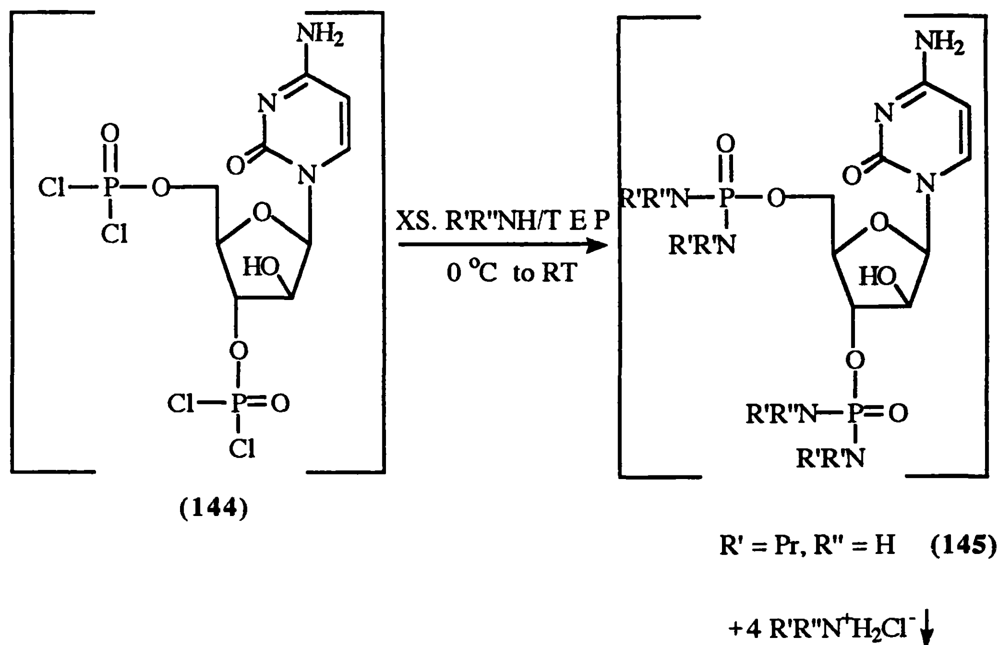


The ^{31}P NMR of compound **(140a)** showed one peak at δ18.61 which was consistent with other similar compounds in the literature.[15] A ^{1}H NMR of **(140a)** was as expected very similar to the spectrum obtained for **(134a)**. However the methyl and methylene protons were more upfield in **(140a)**

compared to (**134a**). A ^{13}C NMR spectrum of (**140a**) was fully assigned and was very similar to the spectrum obtained for (**134a**). The signals of C4' and C5' were observed as doublets, again as a result of phosphorus-carbon coupling. The methyl and methylene carbons were split but were more upfield than observed for compound (**134a**). A FAB mass spectrum of (**140a**) showed a peak for protonated parent ion and the base peak was due to the diethylamino group. Microanalysis was consistent with a hydrated form of (**140a**). An attempt was made to prepare (**140b**) in an entirely analogous method to produce (**140a**). However the target compound was not isolated. It is presumed that the major intermediate product was (**144**). Thus on reacting an excess of n-propylamine with the intermediate reaction mixture, the major product formed was (**145**), which was isolated by column chromatography in the belief that it was compound (**140b**). Thus (**145**) was isolated in a 10% yield.

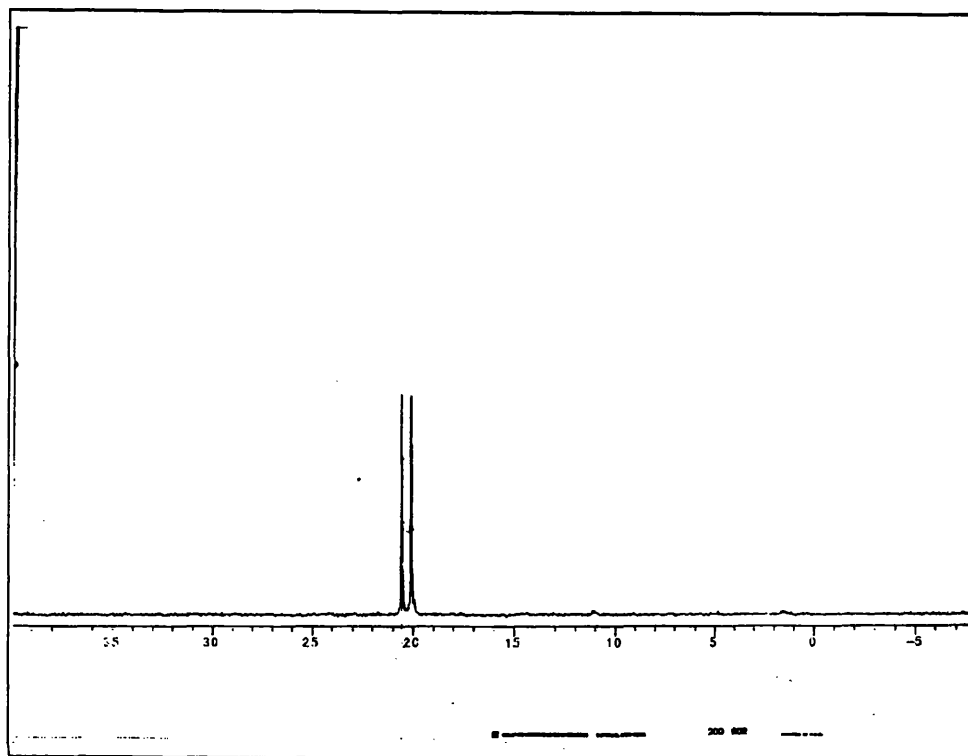


(**144**)

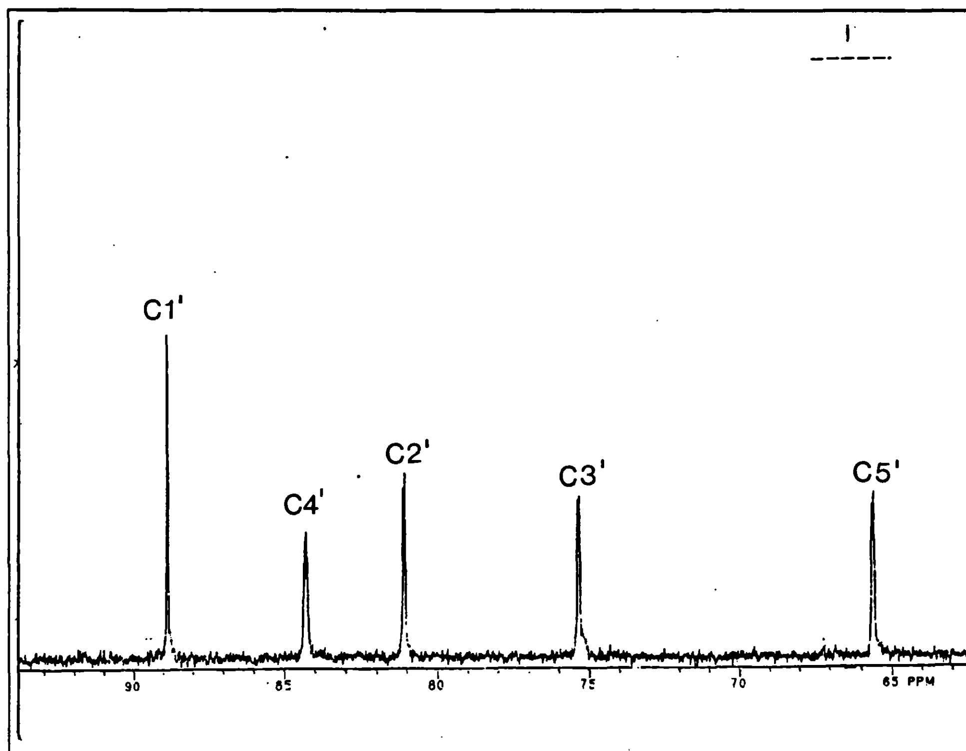


The ^{31}P NMR of compound **(145)** was two signals 0.5 ppm apart and in a 1:1 ratio, which is consistent with the proposed structure. The ^{1}H NMR of **(145)** showed an interesting characteristic, the sugar protons were all slightly downfield compared to the same protons in the series of compounds **(134a-e)**. The ^{13}C NMR was highly informative and confirmed the proposed structure of **(145)**, the C5' was a doublet as expected, however the C4' was found to be a triplet due to the 5' and 3' phosphorus coupling. The C2' was split into a doublet and shifted far downfield compared to the 5'-phosphorylated compounds **(134a-e)**, providing further evidence of a change at the 2' position in the sugar. The C3' was split into a doublet due to three bond coupling to the 2' phosphorus, however the three bond coupling was almost equal to the two bond coupling constant of the C2' doublet, unlike the much greater three bond coupling constants observed for the series **(134a-d)** and the larger three bond coupling constant of C4' compared to C5' in all the 5'-phosphorylated compounds.

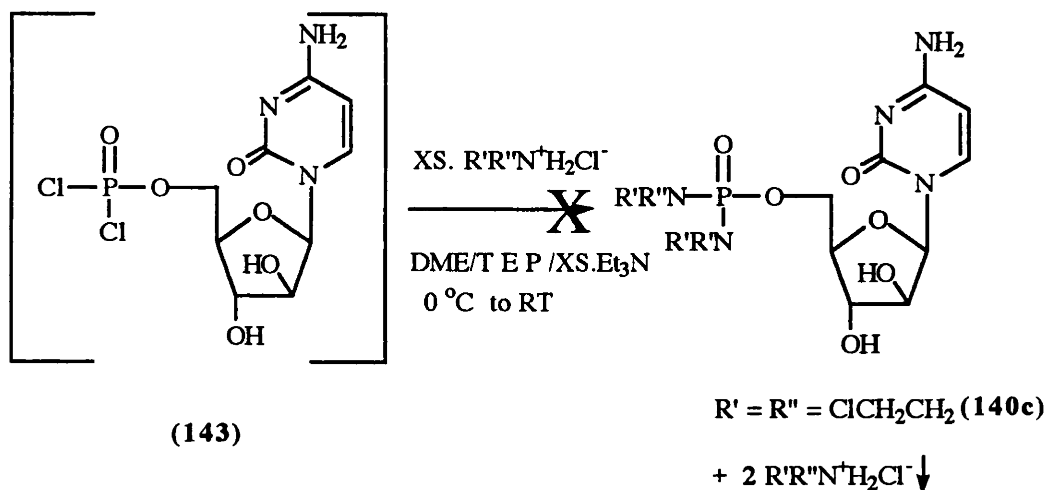
^{31}P NMR spectrum of (145)

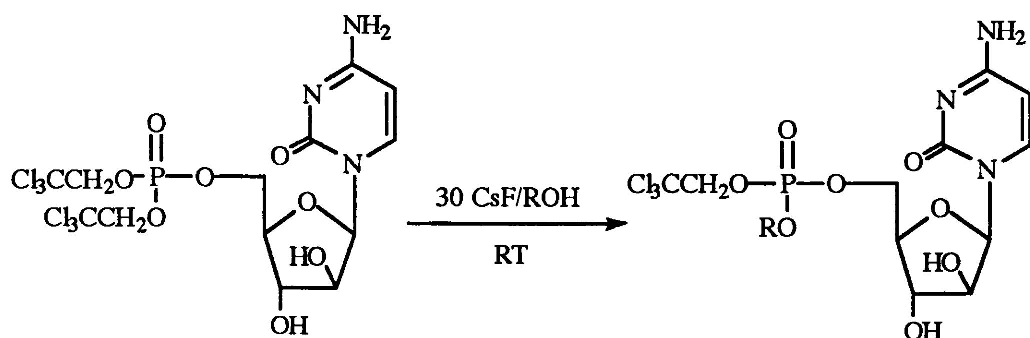


^{13}C NMR spectrum of (145) (SUGAR CARBONS)



An attempt was made to produce the 'hybrid' anti-cancer compound (**140c**), *via* the same methodology used to make (**140a**), except that (2-chloroethyl)amine hydrochloride was added to a solution of (**143**) in triethyl phosphate and triethylamine in dimethoxyethane was added. Petrol rather than ether was employed to precipitate the mixture of products, leaving TEP in the petrol. The resulting yellow solid contained numerous products on TLC and the target compound (**140c**) was not isolated.





R	
Me	(**146a**)
Et	(**146b**)
n-Pr	(**146c**)
n-Bu	(**146d**)

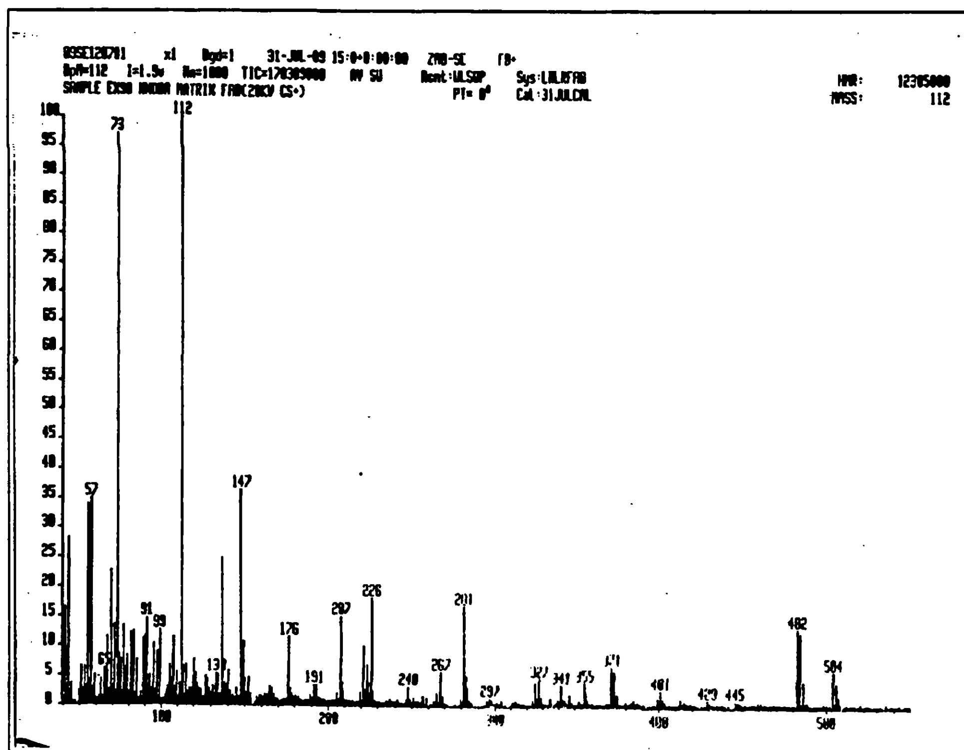
Further attempts to produce asymmetric 5'-phosphorylated derivatives of **(1)** involved a series of transesterification reactions employing compound **(134f)** as a reactant with an excess of CsF along with an alcohol or amine as solvent and reactant as described in the introduction to this chapter.

Thus compound **(146a)** was prepared by adding CsF to a methanolic solution of **(134f)** and the mixture was stirred for two days at ambient temperature. The workup was rotary evaporation and column chromatography to give a 41% yield of the target compound. The ^{31}P NMR of **(146a)** was one signal at δ1.67 which was slightly more downfield than the starting material. The ^{1}H NMR spectrum for **(146a)** was analogous to compound **(134f)**, except that a doublet was seen at δ3.72 with a coupling constant of 10.91 Hz due to coupling of the methyl with phosphorus. A ^{13}C NMR spectrum of **(146a)** was fully assigned and was similar to the ^{13}C NMR of **(134f)** except that there was a doublet at δ56.9 due to the methyl. It was interesting to note that compound **(146a)** was not a diastereomeric mixture, unless all signals were coincident in the ^{31}P and ^{13}C NMR. The mass spectrum for **(146a)** showed a cluster of signals for the parent ion plus sodium from the matrix at 490 m/e also observed was a cluster of signals for the parent ion at 467 m/e, the base peak was due to protonated cytosine. Microanalysis was consistent with a hydrated form of **(146a)**.

The preparation of **(146b)** was performed in an entirely analogous manner to compound **(146a)**, except that an ethanolic solution of **(134f)** was employed. The mass spectrum of **(146b)** showed a cluster of signals for the parent ion plus sodium from the matrix at 504 m/e also observed was a cluster of signals for the protonated parent ion at 482 m/e, the base peak was at 112 m/e due to protonated cytosine. Microanalysis was consistent with a hydrated form

of **(146b)**. The ^{31}P NMR of **(146b)** was two signals at δ-2.66 and δ-2.62. The ^{1}H NMR and the ^{13}C NMR were fully assigned and some signals were found to be duplicated. Duplication of peaks was especially clear with some signals in the ^{13}C NMR such as the C6, C5, C1', C2' and C3'. Thus, unlike compound **(146a)**, compound **(146b)** was found to be a diastereoisomeric mixture which was separated into two isomerically pure samples *via* preparative HPLC. The slow compound in the HPLC system employed was found to give the most upfield signal on investigation by ^{31}P NMR. Experimental and theoretical studies were carried out on the separated isomers in order to determine their conformation about the phosphorus.

Mass spectrum of (146b)



Essentialy, three experimental methods are available to determine the conformation of relatively large molecules in the solid, gaseous (isolated state) and liquid state.[17] X-ray diffraction on single crystals for the solid state, quantum chemical and empirical calculations for the isolated state and nuclear

magnetic resonance (NMR) analysis for the liquid or dissolved state. X-ray diffraction on single crystals is the method of choice for the precise determination of the 3-D structure of a molecule. More specifically, it yields the co-ordinates of each individual atom within the space of the crystalographic unit cell. From this the bond lengths, bond angles and torsion angles are easily calculated. NMR is a powerful method for determining the shape of molecules although not as precise as X-ray diffraction. There are two main theoretical approaches to studying the three-dimensional aspects of drug molecules. One is molecular mechanics and the other is quantum mechanics. Molecular mechanics considers a molecule to be an assembly of atoms held together by elastic forces. These forces can be described by classical potential energy functions involving stretching and bending of bond lengths and bond angles, twisting about bonds, non-bonded and electrostatic interactions. Molecular mechanics is a highly popular method in those instances where only the preferred conformations are required. Quantum mechanical calculations, which on average are about two to three orders of magnitude slower than molecular mechanics calculations, yield not only the preferred conformations but also charge distributions and many other quantities which can be derived from the wave functions. These two theoretical methods supplement each other in present day theoretical conformation analysis.

Thus, NMR in the form of a Nuclear Overhauser Experiment (NOE)[18] and molecular mechanics calculations[19,20] were performed to determine the relative conformations of the separated diastereoisomers of **(146b)**, that is **(146bI)** (R-conformation about the phosphorus) and **(146bII)** (S-conformation about the phosphorus).

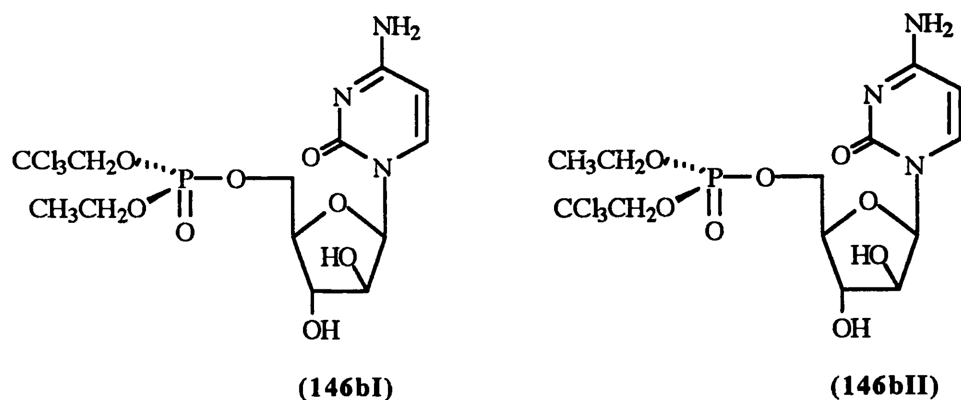
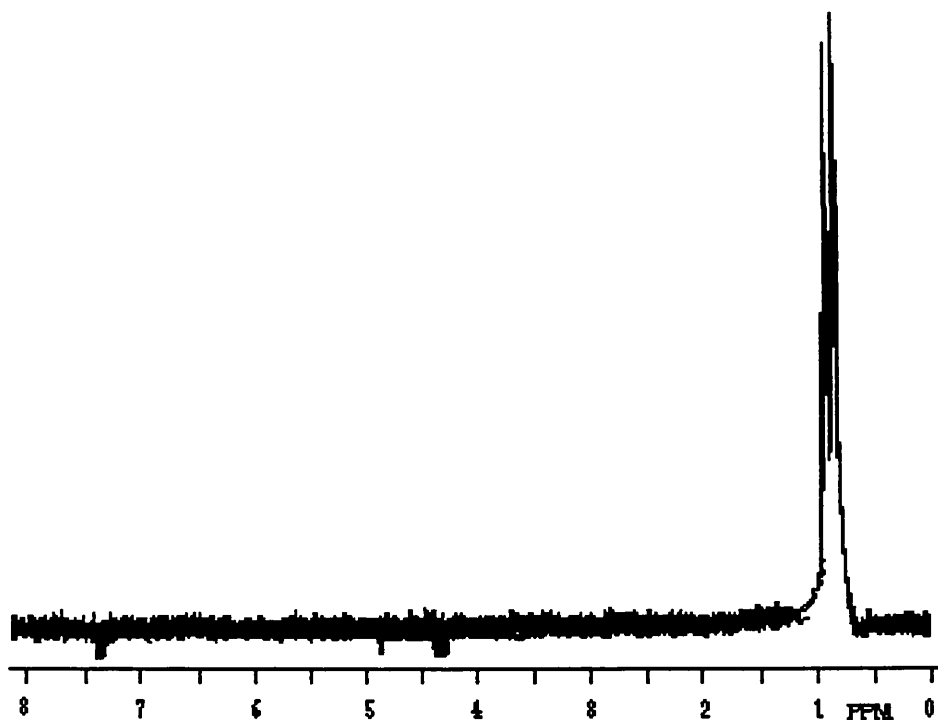


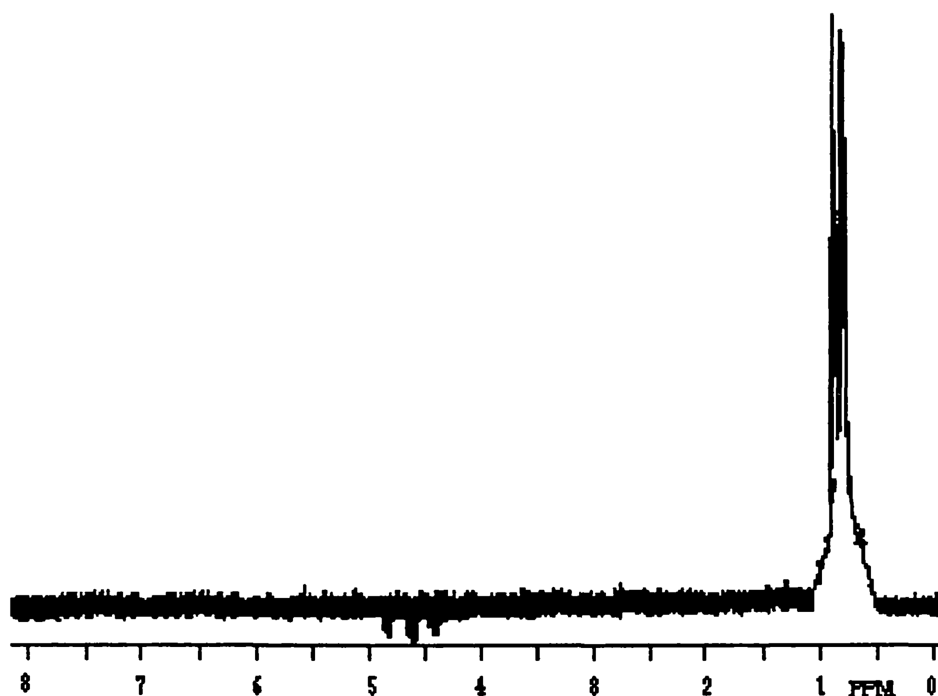
Figure 14: NOE difference spectrum obtained by the irradiation of the CH_3 protons in the (Slow) isomer of (146b)



The NOE difference spectrum of the slow isomer shown above revealed a distinct NOE effect in the region of H6 when the region of CH_3 was irradiated and also in the region of the CH_2 of the ethoxy chain and the CH_2 of the trichloroethoxy chain and in the region of H5' and H4'. The nuclei of the irradiated proton and the affected proton must be as close as *ca.* 3 Å in order that

an NOE effect is observed. Thus, the conclusion seems to be that the slow isomer may spend time in two or more conformations, however it may be inferred that the CH_3 in one of its preferred conformations is very close to H6.

Figure 15: NOE difference spectrum obtained by the irradiation of the CH_3 protons in the (Fast) isomer of (146b)



The NOE difference spectrum of the fast isomer shown above revealed no effect in the region of H6 when the region of CH_3 was irradiated. There was seen an NOE effect in the region of the methylene moieties of the ethoxy, trichloroethoxy chains and in the region of H5' and H4' when the region of CH_3 was irradiated. It is probable that the slow isomer spends time in two or more conformations in solution, however the CH_3 of the fast isomer is never close enough to H6 to show an effect.

The fact that the slow isomer had an NOE effect between CH_3 and H6, whilst the fast isomer had no NOE effect between CH_3 and H6 was investigated by molecular mechanics.

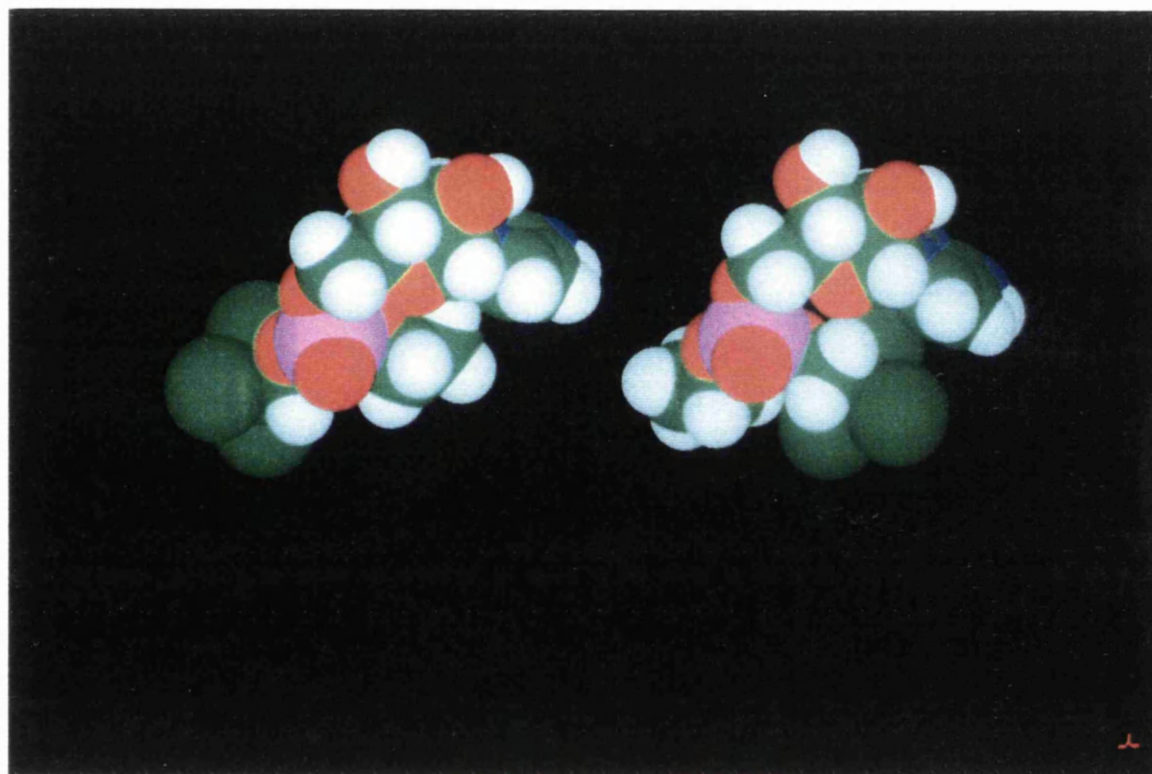
Figure16: Photograph (taken from a Silicon Graphics 4D25G workstation) of the Cylindrical bond models of the R (left) and S (right) isomeric conformation about the phosphorus, (146bI) and (146bII) respectively. The structures have been treated with molecular mechanics calculations and a preferred conformation is shown for each isomer. The distances between the carbon of the methyl of the ethoxy chain and the H6 of the base are clearly shown for both isomers in their preferred conformation. The protons of the methyl moiety of the R-isomer (left) being within the distance required for an NOE effect. However, the protons of the methyl moiety of the S-isomer (right) being well outside the required distance for an NOE effect.

Figure17: Photograph (taken from a Silicon Graphics 4D25G workstation) of the Space filling models of the R (left) and S (right) isomeric conformation about the phosphorus, (146bI) and (146bII) respectively. The structures have been treated with molecular mechanics calculations and a preferred conformation is shown for each isomer.

Key to spheres

Large green	Cl
Small green	C
Red	O
Blue	N
White	H
Purple	P

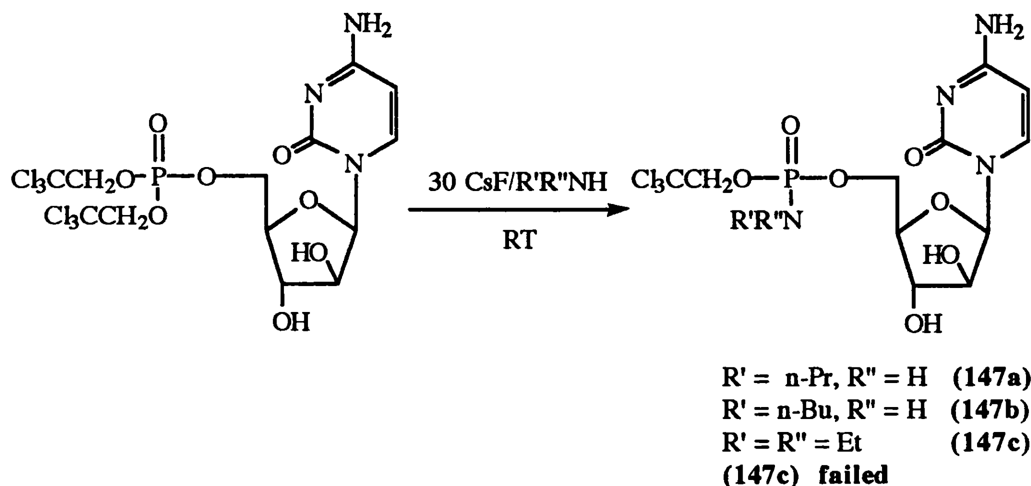
The Space filling models show clearly the dramatic difference between the R(left) and S(right) with regards to the distance between the methyl moiety and the H6 in these particular preferred conformations.



In summary it may be assumed that there are several preferred conformations for **(146bI)** and **(146bII)** from the NOE experiments. However it seems that the slow isomer of **(146b)** has a preferred conformation within which an NOE effect between the methyl moiety of the ethoxy chain and H6 of the base is possible, whilst the fast isomer of **(146b)** does not have a preferred conformation which allows such an effect. It is taken into account that there may be several conformations preferred by the isomers of **(146b)** and that under different experimental conditions or techniques, a different result may be seen. However the conclusion inferred from the results obtained may be that the slow isomer has an R-conformation about the phosphorus and the fast isomer has an S-conformation about the phosphorus.

Compounds **(146c)** and **(146d)** were prepared in an entirely analogous manner to the preparation of compound **(146a)**, duplication of peaks was not observed by NMR for **(146c)** and **(146d)**. The ^{31}P NMR of **(146c)** was one broad signal at δ-1.47 and for **(146d)** was one signal at δ-1.92 which were analogous to other similar compounds.[15] The ^{1}H and ^{13}C NMR of **(146c)** and **(146d)** were fully assigned and were consistent with the target compounds. The mass spectra of **(146c)** and **(146d)** showed a cluster of signals for the parent ions at 495 m/e and 509 m/e respectively and both had protonated cytosine as the base peak. Microanalysis were consistent with the hydrated form of **(146c)** and **(146d)**.

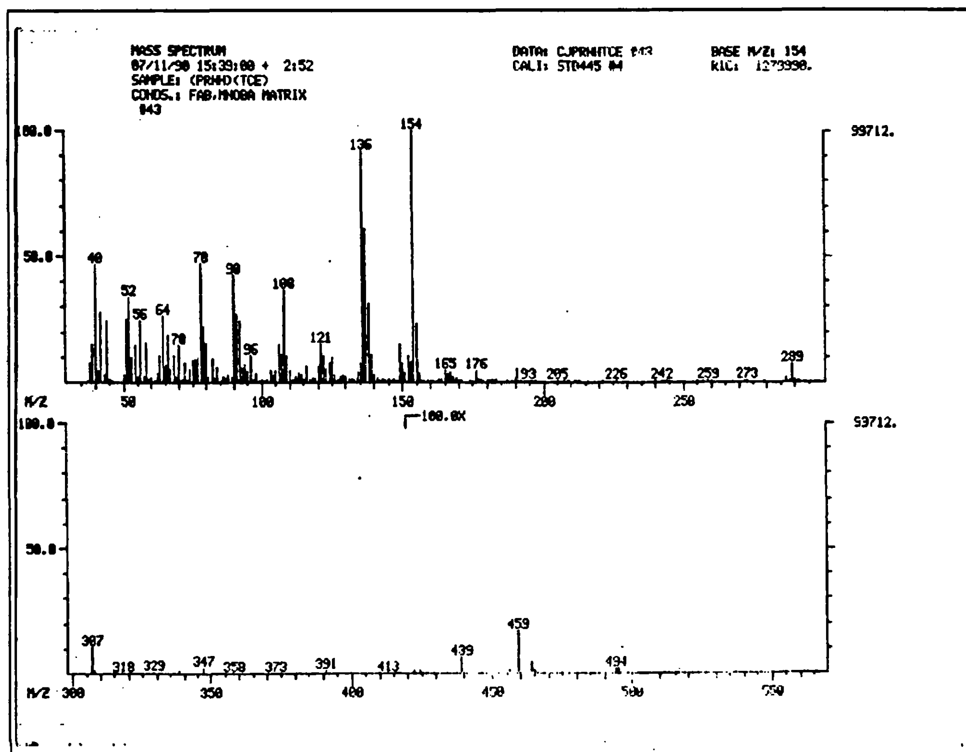
In order to produce phosphoramidate derivatives of **(1)**, the application of the transesterification reaction employed to produce **(146a-d)** was modified. A variety of amines were used as both reactant and solvent with CsF and compound **(134f)**.



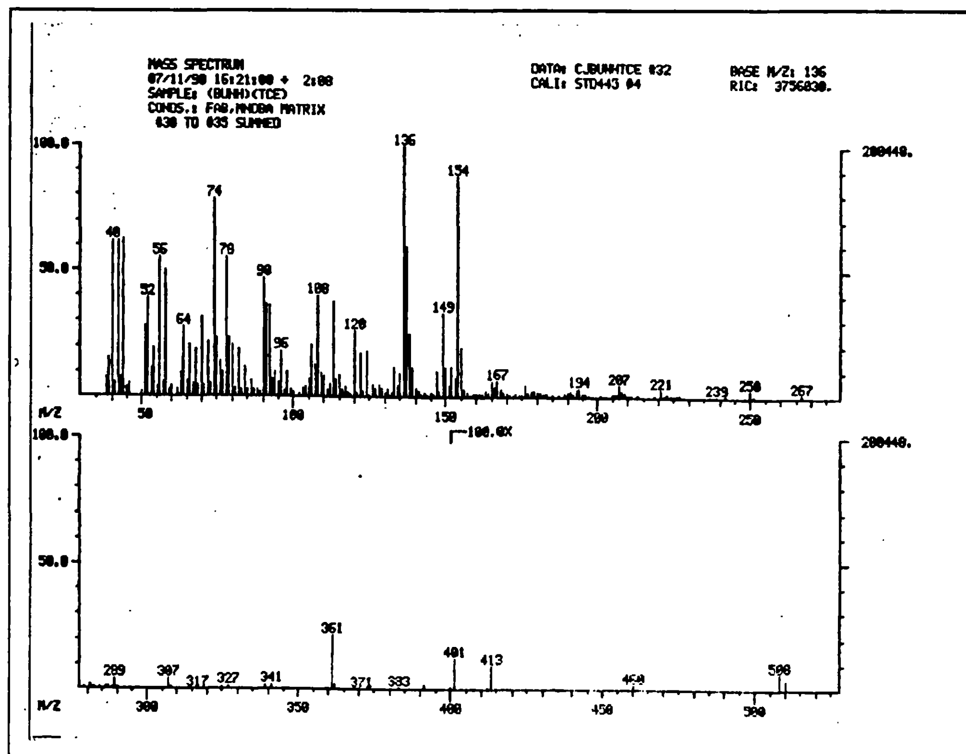
R' = n-Pr, R" = H **(147a)**
R' = n-Bu, R" = H **(147b)**
R' = R" = Et **(147c)**
(147c) failed

Compound **(147a)** was prepared by the novel removal of a trichloroethoxy group from **(134f)** and the incorporation of an n-propylamino group presumably *via* a P-F intermediate.[12,13] The reaction was stirred for double the time allowed for the series of compounds **(136a-d)**. The ^{31}P NMR of **(147a)** was analogous to other similar compounds in the literature[15] and it is interesting to note that a nitrogen directly attached to the phosphorus causes a dramatic shift downfield compared to phosphorus compounds with only P-O bonds. The ^{1}H and ^{13}C NMR of **(147a)** were fully assigned and duplication of signals was not observed, suggesting that the sample was diastereomerically pure. In the ^{13}C NMR two and three bond phosphorus coupling was observed and it was interesting to note that the methylene carbons were more upfield than the methylene carbons of **(134b)**. The mass spectrum showed two of the three signals expected for the parent ion at 494 m/e and 496 m/e, the base peak was at 136 m/e. Microanalysis was consistent with the hydrated form of **(147a)**.

Mass spectrum of (147a)



Mass spectrum of (147b)

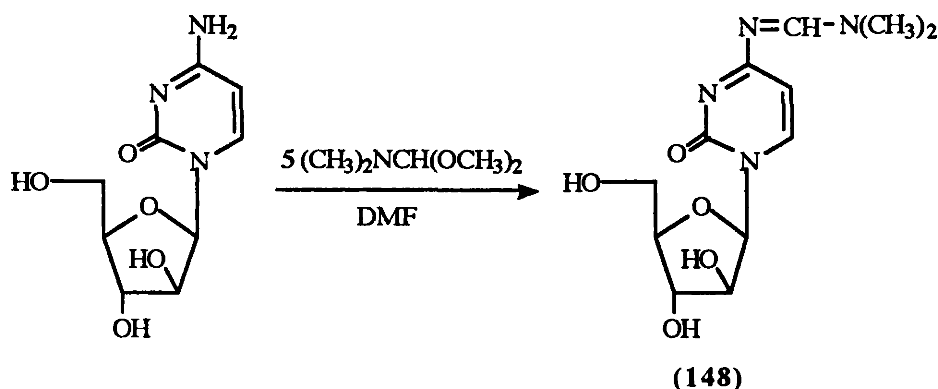


Compound **(147b)** was prepared in a method analogous to the preparation of **(147a)**, except that n-butylamine was used as a solvent and reactant. The ^{31}P NMR of **(147b)** was one signal similar to compound **(147a)**. The ^{1}H NMR and the ^{13}C NMR confirmed the production of **(147b)**. The methylene carbons of the butyl chain being upfield compared to the methylene carbons in the ^{13}C NMR of **(134c)**. The mass spectrum showed two of the three signals expected for the parent ion at 508 m/e and 510 m/e, the base peak was at 136 m/e. Microanalysis was consistent with the formation of (147b).

An attempt was made to produce compound **(147c)** by an entirely analogous method to compounds **(147a-b)**, except that diethylamine was used as a solvent and reactant and the reaction mixture was heated to reflux for 16 hours because it did not proceed at ambient temperature. Compound **(147c)** was not produced, maybe diethylamine is too bulky to allow transesterification, which is the reported case for t-butyl alcohol.[12]

The interesting feature and an enigma of the series of compounds **(146a-d)** and the compounds **(147a-b)** was that the only compound isolated as a diastereomeric mixture was compound **(146b)**. The yield of compound **(146b)** was almost double the yield of compounds **(146a)**, **(146c-d)** and **(147a)** and three times the yield of compound **(147b)**. Thus from the yields there may be a suggestion that all the transesterification reactions may produce diastereoisomeric mixtures but only one isomer is isolated in the workup for the majority of products. Different workup procedures may be applied in the future in order to determine whether the yields can be improved along with the isolation of the proposed and expected diastereoisomers.

Some of the above reactions have proven to produce numerous products, in many instances isolation of the target compound was made very difficult and thus some reactions were not worked up. In order that 'cleaner' reactions may be carried out in the future, the protection of some of the reactive sites in **(1)** may be necessary. 1-β-D-arabinofuranosyl-N-dimethylaminomethylenecytosine **(148)**, a novel base protected derivative of **(1)** was prepared. A suspension of **(1)** in dimethylformamide was shaken with an excess of dimethylformamide dimethylacetal at room temperature overnight and the resulting clear solution was evaporated under vacuum, the target product was isolated after purification by precipitation from ethanol by the addition of ether to give an almost quantitative yield.



(148)

The ^{1}H NMR was fully assigned with the N=CH signal being a singlet at δ8.31 and the methyl protons being two singlets between δ2 and δ3. The ^{13}C NMR was further evidence that **(148)** had been produced, with the base carbons; C4, C6 and C5 slightly further downfield than for derivatives of (1) unchanged at the base. A UV spectrum of the product also gave an indication that there was a change in the base of this derivative of **(1)**. The FAB mass spectrum of **(148)** gave a signal for the protonated parent ion at 299 m/e and the base peak at 167 m/e due to the protonated N-dimethylamino methylenecytosine fragment. Microanalysis was consistent with the form of **(148)**.

Biological testing was performed on compounds **(140a)**, **(146a)**, **(146bI)**, **(146bII)**, **(146c)**, **(147a)** and **(147b)** in an entirely analogous manner to the procedure employed for the testing of compounds **(134a-f)** (Chapter 2). Each experiment was performed three times on cells of different passage number and the mean and SEM of each set of three inhibition values calculated to give some indication of the reproducibility of the experiment. For each experiment, the results were normalised by expressing the inhibition as a percentage of the inhibition produced by 10% DMSO. A column graph of inhibition against compound number for all the compounds tested against mammalian epithelial cells in vitro (from chapter 2 and chapter 3, figure 18) shows clearly the relationship between the compounds and their biological activity.

Figure 18 Column graph of %Inhibition (relative to solvent) against compound number for all compounds tested against mammalian epithelial cells in Ch 2 and Ch 3

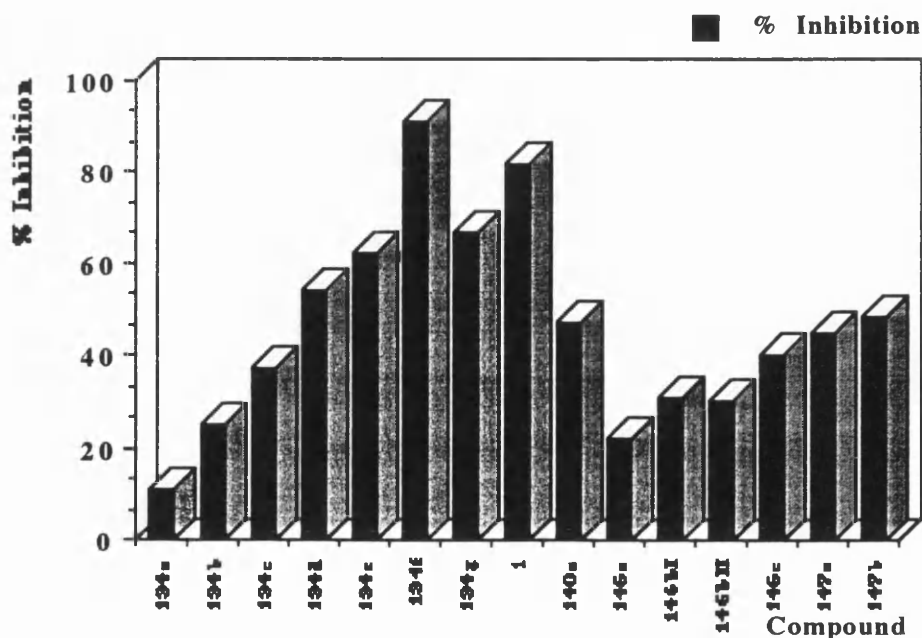


Figure 18 illustrates the increasing biological activity of the 1-β-D-arabinofuranosylcytosine-5'-dialkyl phosphate series **(134a-e)** with increasing chain length and the exceptional activity of the 1-β-D-arabinofuranosylcytosine-5'-bis-(2,2,2-trihaloalkyl) phosphate series **(134f-g)**. 1-β-D-Arabinofuranosylcytosine-5'-bis-(diethylamino) phosphate **(140a)** showed activity between the activities of **(134b-c)** but was not as active in this system as might be expected in a tumor where a more acidic environment would be available to allow P-N cleavage and thence the release of **(1)** and/or **(50)** allowing the compound to be activated to **(52)**.[1]

The series of asymmetric compounds **(146a-c)** and **(147a-b)** exhibited interesting activities. The separated diastereoisomers **(146bI-II)** did not show any significant difference in activities, which may indicate that hydrolysis of the alkyl or trichloroalkyl moieties are unaltered by their configuration at the phosphorus, at least in compound **(146b)** where the chains are not very bulky. The activities of **(146a-c)** again showed increasing activity with increasing chain length, however if compounds **(134a)** and **(146b)** are compared along with **(134b)** and **(146c)**, it is apparent that replacing an alkyl chain with a trichloroethyl group enhances activity. Comparing compounds **(134b)**, **(146c)** and **(147a)** demonstrates that replacing the n-propyl groups of **(134b)** with a trichloroethyl group and an n-propylamino increases the activity of the resulting compound. Another interesting result was that there was no significant difference in going from **(147a)** to **(147b)** within the standard error of the mean, unlike the difference obtained between **(134b)** to **(136c)**.

Thus, there are interesting structure activity relationships between the compounds synthesised and tested, providing possible leads for further research

and development. Especially interesting is the trichloroethoxy moiety, which for compound (**134f**) seems to improve the activity of the parent drug.

3.3 Experimental

3.3.1 Direct phosphorylation of araC

3.3.1.1 Phosphorodichloridates

3.3.1.1.1

Methyl phosphorodichloridate (136a)

Phosphoryl chloride (25 ml, 41.13 g) was placed in a three necked flask. Methanol (10.5 ml, 8.31 g) was added dropwise at 0 to -5 °C under nitrogen, over a period of 25 minutes. The bulk of the HCl by-product was removed at room temperature by rapid stirring with a vigorous nitrogen bubble. Further removal was achieved by application of a vacuum(10 mmHg) at room temperature. The product was distilled to yield a colourless oil (bpt 47 °C @ 10 mmHg)(15.93 g, 46%).

^{1}H NMR δ($CDCl_3$, 200 MHz, TMS) 3.89 (3H, d, CH_3, J=17.4 Hz)

^{31}P NMR δ($CDCl_3$, 80 MHz, H_3PO_4) 5.26

^{13}C NMR δ($CDCl_3$, 100 MHz, TMS) 57.63 (d, CH_3, J=8.6 Hz)

3.3.1.1.2

Ethyl phosphorodichloridate (136b)

Phosphoryl chloride (20 ml, 32.9 g) was dissolved in diethyl ether (400 ml). A solution of triethylamine (30 ml, 21.78 g) and ethanol (12.5 ml, 9.81 g)

in diethyl ether (400 ml) was added dropwise at -78 °C under an atmosphere of nitrogen. The reaction mixture was allowed to warm to room temperature and stirred overnight. The mixture was filtered and the filtrate was rotary evaporated to yield a yellowish oil (28.84 g, 83%).

^{1}H NMR δ($CDCl_3$, 200 MHz, TMS) 4.29 (2H, m, CH_2), 1.38 (3H, t, CH_3)

^{31}P NMR δ($CDCl_3$, 80 MHz, H_3PO_4) 4.53

^{13}C NMR δ($CDCl_3$, 100 MHz, TMS) 68.84 (broad s, CH_2), 15.58 (d, CH_3, J=8.7 Hz)

3.3.1.1.3

n-Propyl phosphorodichloridate (136c)

Phosphoryl chloride (20 ml, 32.9 g) was dissolved in diethyl ether (400 ml). A solution of triethylamine (30 ml, 21.78 g) and n-propanol (16 ml, 12.86 g) in diethyl ether (400 ml) was added dropwise at -78 °C under an atmosphere of nitrogen. The reaction mixture was allowed to warm to room temperature and stirred overnight. The mixture was filtered and the filtrate was rotary evaporated to yield a clear colourless oil (32.27 g, 85%).

^{1}H NMR δ($CDCl_3$, 200 MHz, TMS) 4.29 (2H, m, CH_2), 1.79 (2H, sextet, $CH_3C\underline{H_2}$), 0.99 (3H, t, CH_3)

^{31}P NMR δ($CDCl_3$, 80 MHz, H_3PO_4) 4.60

^{13}C NMR δ($CDCl_3$, 100 MHz, TMS) 73.89 (broad s, CH_2), 23.04 (d, $CH_3\underline{C}H_2$, J=8.9 Hz), 9.89 (CH_3)

3.3.1.1.4

n-Butyl phosphorodichloridate (136d)

Phosphoryl chloride (20 ml, 32.9 g) was dissolved in diethyl ether (400 ml). A solution of triethylamine (30 ml, 21.78 g) and n-butanol (19.5 ml, 15.79 g) in diethyl ether (400 ml) was added dropwise at -78 ^oC under an atmosphere of nitrogen. The reaction mixture was allowed to warm to room temperature and stirred overnight. The mixture was filtered and the filtrate was rotary evaporated to yield a yellowish oil (33.19 g, 81%).

^1H NMR δ($CDCl_3$, 200 MHz, TMS) 4.35 (2H, m, $CH_3CH_2CH_2CH_2OP$), 1.81 (2H, m, $CH_3CH_2C\underline{H_2}$), 1.49 (2H, m, $CH_3C\underline{H_2}$), 0.99 (3H, t, CH_3)

^{31}P NMR δ($CDCl_3$, 80 MHz, H_3PO_4) 4.91

^{13}C NMR δ($CDCl_3$, 100 MHz, TMS) 72.11 (d, $CH_2CH_2\underline{C}H_2$, J=7.5 Hz), 31.58 (d, $CH_3CH_2\underline{C}H_2$, J=8.7 Hz), 18.54 ($CH_3\underline{C}H_2$), 13.44 (CH_3)

3.3.1.1.5

n-Pentyl phosphorodichloridate (136e)

Phosphoryl chloride (20 ml, 32.9 g) was dissolved in diethyl ether (400 ml). A solution of triethylamine (30 ml, 21.78 g) and n-pentanol (23 ml, 18.65 g) in diethyl ether (400 ml) was added dropwise at -78 ^oC under an atmosphere of nitrogen. The reaction mixture was allowed to warm to room temperature and stirred overnight. The mixture was filtered and the filtrate was rotary evaporated to yield a yellowish oil (32.27 g, 85%).

^1H NMR δ($CDCl_3$, 200 MHz, TMS) 4.37(2H, m, $CH_3CH_2CH_2CH_2C\underline{H}_2$), 1.82(2H, m, $CH_3CH_2CH_2C\underline{H}_2$), 1.41(4H, m, $CH_3C\underline{H}_2C\underline{H}_2CH_2$), 0.95(3H, t, CH_3)

^{31}P NMR δ($CDCl_3$, 80 MHz, H_3PO_4) 4.83

^{13}C NMR δ($CDCl_3$, 100 MHz, TMS) 73.57(d, $CH_3CH_2CH_2CH_2\underline{C}H_2$, J=7.0 Hz), 29.33(d, $CH_3CH_2CH_2\underline{C}H_2$, J=8.8 Hz), 27.36($CH_3CH_2\underline{C}H_2$), 22.09($CH_3\underline{C}H_2$), 13.89($CH_3$)

3.3.1.1.6

n-Hexyl phosphorodichloridate (136f)

Phosphoryl chloride (20 ml, 32.9 g) was dissolved in diethyl ether (400 ml). A solution of triethylamine (30 ml, 21.78 g) and n-hexanol (27 ml, 21.98 g) in diethyl ether (400 ml) was added dropwise at -78 °C under an atmosphere of nitrogen. The reaction mixture was allowed to warm to room temperature and stirred overnight. The mixture was filtered and the filtrate was rotary evaporated to yield a yellowish oil (39.48 g, 84%).

^1H NMR δ($CDCl_3$, 200 MHz, TMS) 4.33(2H, m, $CH_3CH_2CH_2CH_2CH_2C\underline{H}_2$), 1.81(2H, m, $CH_3CH_2CH_2CH_2C\underline{H}_2$), 1.74(6H, m, $CH_3C\underline{H}_2C\underline{H}_2C\underline{H}_2$), 0.91(3H, t, CH_3)

^{31}P NMR δ($CDCl_3$, 80 MHz, H_3PO_4) 4.84

^{13}C NMR δ($CDCl_3$, 100 MHz, TMS) 72.56(d, $\underline{C}H_2OP$, J=9.5 Hz), 31.13($CH_3CH_2CH_2\underline{C}H_2$), 29.60(d, $CH_3CH_2CH_2CH_2\underline{C}H_2$, J=8.9 Hz), 24.93($CH_3CH_2\underline{C}H_2$), 22.47($CH_3\underline{C}H_2$), 13.98($CH_3$)

3.3.1.1.7

Diethylamino phosphorodichloridate (136g)

Phosphoryl chloride (10 ml, 16.45 g) was dissolved in diethyl ether (250 ml). A solution of diethylamine (25 ml, 17.68 g) in diethyl ether (400 ml) was added dropwise at -78 ^oC under an atmosphere of nitrogen. The reaction mixture was allowed to warm to room temperature and stirred overnight. The mixture was filtered and the filtrate was rotary evaporated to yield a yellowish oil (15.07 g, 74%).

^1H NMR δ($CDCl_3$, 200 MHz, TMS) 3.36(2H, m, $CH_3\underline{C}\underline{H}_2N$), 1.28(3H, t, CH_3)

^{31}P NMR δ($CDCl_3$, 80 MHz, H_3PO_4) 13.79 (quintet on H coupling)

^{13}C NMR δ($CDCl_3$, 100 MHz, TMS) 40.46(d, $CH_3\underline{C}H_2N$, J=5.7 Hz), 13.78(d, CH_3, J=7.6 Hz)

3.3.1.2 (Alkyl)(alkylamino) phosphorochloridates

3.3.1.2.1

Diethylamino n-propyl phosphorochloridate (Method 1) (137a)

Diethylamino phosphorodichloridate (1.6 ml, 2 g) was dissolved in diethyl ether (50 ml). A solution of triethylamine (1.3 ml, 1.06 g) and n-propanol (0.78 ml, 0.63 g) in diethyl ether (50 ml) was added dropwise at -70 ^oC. The mixture was stirred at room temperature for 17 hours. Then the mixture was refluxed for

2 days, filtered and the filtrate was rotary evaporated to yield a clear yellowish oil (1.52 g, 68%).

^{1}H NMR δ($CDCl_3$, 200 MHz, TMS) 4.27(2H, m, $CH_3CH_2C\underline{H}_2$), 3.24(4H, m, CH_2NH), 1.82(2H, m, $CH_3C\underline{H}_2CH_2$), 1.17(6H, t, CH_3), 0.99(3H, t, $C\underline{H}_3CH_2CH_2$)

^{31}P NMR δ($CDCl_3$, 80 MHz, H_3PO_4) 13.48

3.3.1.2.2

Diethylamino n-propyl phosphorochloridate (Method 2) (137a)

n-Propyl phosphorodichloridate (2 ml, 3 g) was dissolved in diethyl ether (50 ml). A solution of diethylamine (4 ml, 2.83 g) in diethyl ether (50 ml) was added dropwise at -70 °C. The mixture was stirred at room temperature for 17 hours. Then the mixture was filtered and the filtrate was rotary evaporated to yield a clear yellowish oil (2.63 g, 73%).

^{1}H NMR δ($CDCl_3$, 200 MHz, TMS) 4.11(2H, m, $CH_3CH_2C\underline{H}_2$), 3.18(4H, m, CH_2NH), 1.75(2H, m, $CH_3C\underline{H}_2CH_2$), 1.18(6H, t, CH_3), 0.99(3H, t, $C\underline{H}_3CH_2CH_2$)

^{31}P NMR δ($CDCl_3$, 80 MHz, H_3PO_4) 13.40

^{13}C NMR δ($CDCl_3$, 100 MHz, TMS) 74.66 (broad s, $CH_3CH_2C\underline{H}_2$), 40.33(d, $CH_3\underline{C}H_2N$, J = 5.5 Hz), 24.19 (d, $CH_3\underline{C}H_2CH_2$, J=8.7 Hz), 13.56(d, $C\underline{H}_3CH_2N$, J =7.3 Hz), 8.97 ($C\underline{H}_3CH_2CH_2$)

EI MS m/e 215(M^+, ^{37}Cl, 1.57%), 213(M^+, ^{35}Cl, 6.88), 200(M^+- Me, ^{37}Cl, 7.23), 198(M^+- Me, ^{35}Cl, 23.83), 178(M^+- Cl, 1.57), 172(M^+- Pr, ^{37}Cl,

1.26), 170(M^+- Pr, ^{35}Cl, 2.17), 158(M^+- PrO, ^{37}Cl, 38.17), 156(M^+- PrO, ^{35}Cl, bp)

Found C 39.57%, H 8.37, N 6.51, Cl 16.55, P 14.26;

$C_7H_{17}ClNO_2P$ requires C 39.35, H 8.02, N 6.56, Cl 16.59, P 14.50.

3.3.1.2.3

Methyl n-propylamino phosphorochloridate (137b)

Methyl phosphorodichloridate (5 ml, 7.44 g, 0.049 mol) was dissolved in diethyl ether (80 ml). A solution of n-propylamine (8 ml, 5.75 g, 0.097mol) in diethyl ether (80 ml) was added dropwise at -78 °C. The mixture was stirred at room temperature for 17 hours. A precipitate formed rapidly, the mixture was filtered and the filtrate was rotary evaporated to yield a clear yellowish oil (8.26 g, 98%).

^1H NMR δ($CDCl_3$, 200 MHz, TMS) 4.67(1H, broad s, NH), 3.72(3H, d, CH_3O, J=17.2 Hz), 2.82(2H, m, CH_2NH), 1.45(2H, m, $C\underline{H}_2CH_2NH$), 0.92(3H, t, $C\underline{H}_3CH_2$)

^{31}P NMR δ($CDCl_3$, 80 MHz, H_3PO_4) 15.83

^{13}C NMR δ($CDCl_3$, 100 MHz, TMS) 53.47(d, CH_3O, J=8.1 Hz), 43.42(d, CH_2NH, J=5.6 Hz), 23.68(d, $\underline{C}H_2CH_2NH$, J=7.6 Hz), 11.05($\underline{C}H_3CH_2$)

Found C 28.69%, H 6.57, N 9.91;

$C_4H_{11}ClNO_2P$ requires C 28.00, H 6.46, N 8.16.

3.3.1.2.4

Bis-(2-chloroethyl)amino ethyl phosphorochloridate (137c)

Ethyl phosphorodichloridate (6.13g) and bis-(2-chloroethyl)-amine hydrochloride (6.7 g) were stirred in a reaction vessel with diethyl ether (150 ml). A solution of triethylamine (10.5 ml, 7.6 g) in diethyl ether (150 ml) was added dropwise at -78 °C. The mixture was stirred at room temperature for 2 days. Then the mixture was filtered and the filtrate was rotary evaporated to yield a clear yellowish oil (8.97 g, 89%).

^{1}H NMR δ($CDCl_3$, 200 MHz, TMS) 4.32(2H, m, CH_2O), 3.68(4H, m, $ClCH_2$ x 2), 3.54(4H, m, CH_2N x 2), 1.41(3H, t, CH_3)

^{31}P NMR δ($CDCl_3$, 80 MHz, H_3PO_4) 13.49

^{13}C NMR δ($CDCl_3$, 100 MHz, TMS) 65.04(d, CH_2O, J=6.2 Hz), 49.83 (d, CH_2N, J=4.2 Hz), 41.52(d, $ClCH_2O$, J=8.4 Hz), 15.75(d, CH_3, J=8.3 Hz)

Found C 26.29%, H 4.96 , N 5.08;
$C_6H_{13}Cl_3NO_2P$ requires C 26.84, H 4.88, N 5.22.

3.3.1.2.5

Methyl N-phenylamino phosphorochloridate (137d)

Methyl phosphorodichloridate (3 ml, 4.46 g, 0.03 mol) was dissolved in diethyl ether (100 ml). A solution of aniline (5.5 ml, 5.62 g, 0.06 mol) in diethyl ether (100 ml) was added dropwise at -60 °C. The reaction mixture was

covered with aluminium foil and stirred overnight. A precipitate formed gradually, the mixture was filtered and the filtrate was rotary evaporated to yield a reddish solid (5.83 g, 94%).

^{1}H NMR δ($CDCl_3$, 200 MHz, TMS) 7.54(1H, d, NH), 6.92(5H, m, Ph), 3.70(3H, d, CH_3, J=14.1 Hz)

^{31}P NMR δ($CDCl_3$, 80 MHz, H_3PO_4) 9.03

EI MS m/e 208(MH^+, ^{37}Cl, 1.59%), 207(M^+, ^{37}Cl, 32.35), 206(MH^+, ^{35}Cl, 7.70), 205(M^+, ^{35}Cl, bp), 189(M^+ - Me - H, ^{35}Cl, <1)169(38), 91(49)

Found C 40.54%, H 4.92, N 6.25;
$C_7H_9ClNO_2P$ requires C 40.90, H 4.41, N 6.81.

3.3.1.2.6

n-Butyl N-methyl-N-phenylamino phosphorochloridate (137e)

n-Butyl phosphorodichloridate (5 ml, 6.51 g, 0.034 mol) was dissolved in diethyl ether (100 ml). N-methylaniline (5.5 ml, 5.62 g, 0.06 mol) in diethyl ether (100 ml) was added dropwise at -60 °C. The reaction mixture was covered with aluminium foil and stirred overnight. A precipitate formed gradually, the mixture was filtered and the filtrate was rotary evaporated to yield a brown solid (8.37 g, 95%).

^{1}H NMR δ($CDCl_3$, 200 MHz, TMS) 7.21(5H, m, Ph), 4.29(2H, m, $CH_3CH_2CH_2C\underline{H}_2$), 2.84(3H, d, CH_3N, J=12.4 Hz), 1.79(2H, m, $CH_3CH_2C\underline{H}_2$), 1.32(2H, m, $CH_3C\underline{H}_2$), 0.94(3H, m, $C\underline{H}_3CH_2$)

^{31}P NMR δ($CDCl_3$, 80 MHz, H_3PO_4) 10.79

EI MS m/e 261(M^+, ^{35}Cl, 1.59%), 207(M^+ - C_4H_9, ^{37}Cl, 6), 205(M^+ - C_4H_9, ^{35}Cl, 20), 120(M^+ - $C_6H_5NCH_3$ - Cl, 16), 106($C_6H_5NCH_3^+$, bp), 77($C_6H_5^+$, 30)

Found C 49.97%, H 6.94, N 4.88;
$C_{11}H_{17}ClNO_2P$ requires C 50.49, H 6.55, N 5.35.

3.3.1.2.7

Methyl diethylamino phosphorochloridate (137f)

Methyl phosphorodichloridate (10 ml, 14.88 g) was dissolved in diethyl ether (150 ml). A solution of diethylamine (21 ml, 14.9 g) in diethyl ether (150 ml) was added dropwise at -78 °C. The mixture was stirred at room temperature for 17 hours. A precipitate formed rapidly, the mixture was filtered and the filtrate was rotary evaporated to yield a clear yellowish oil (10.98 g, 59%).

^1H NMR δ($CDCl_3$, 200 MHz, TMS) 3.76(3H, d, CH_3O, J=17.5 Hz), 3.18(4H, m, CH_2NH), 1.17(6H, t, $C\underline{H}_3CH_2$)

^{31}P NMR δ($CDCl_3$, 80 MHz, H_3PO_4) 15.90

^{13}C NMR δ($CDCl_3$, 100 MHz, TMS) 53.87(d, CH_3O, J=8.3 Hz), 40.35(d, CH_2NH, J=5.7 Hz), 13.56(d, $C\underline{H}_3CH_2$, J=7.3 Hz)

Found C 31.59%, H 7.72, N 6.92;
$C_5H_{13}ClNO_2P$ requires C 32.36, H 7.06, N 7.55.

3.3.1.3 Bis-dialkylamino phosphorylating agents

3.3.1.3.1

Bis-diethylamino phosphorochloridate (138)

A solution of diethylamine (50 ml, 35,35 g, 0.4853 moles) in diethyl ether was added dropwise at -70 °C to a stirred solution of phosphoryl chloride (10 ml, 16.45 g, 0.1072 moles) in diethyl ether (150 ml). After 5 hours the reaction was allowed to warm to room temperature and left to stir for 4 days. The reaction was monitored at intervals by ^{31}P NMR. The reaction was refluxed for 4 days, filtered and the filtrate was rotary evaporated to yield a clear yellow oil (14.33 g, 59%). The crude product was distilled to yield a pure sample of bis-diethylamino phosphorochloridate (87-89 °C @ 0.15 mmHg)(6.71 g, 28%).

^{1}H NMR δ($CDCl_3$, 60 MHz, TMS) 3.23(2H, split quartet, $CH_3C\underline{H}_2N$), 1.27(3H, t, CH_3)

^{31}P NMR δ($CDCl_3$, 160 MHz, H_3PO_4) 24.52

^{13}C NMR δ($CDCl_3$, 100 MHz, TMS) 40.39(d, $CH_3\underline{C}H_2N$, J=5.6 Hz), 13.62(d, CH_3, J=7.3 Hz)

FAB MS m/e 228(M^+, ^{37}Cl, 1.38%), 226(M^+, ^{35}Cl, 4.93), 213(M^+ - Me, ^{37}Cl, 24.72), 211(M^+ - Me, ^{35}Cl, 76.25), 197(M^+ - Et, ^{35}Cl, 0.17), 191(M^+ - Cl, 22.27), 185(M^+ - Me - C_2H_4, ^{37}Cl, 2.41), 183(M^+ - Me - C_2H_4, ^{35}Cl, 9.92), 72(bp)

Found C 41.07%, H 8.75, N 11.98, Cl 15.18, P 13.89;
$C_8H_{20}ClN_2OP$ requires C 42.39, H 8.89, N12.36, Cl 15.64, P 13.66.

3.3.1.4 Phosphorylation of araC

3.3.1.4.1

Attempt to prepare 1-β-D-Arabinofuranosylcytosine-5'-diethyl-amino n-propyl phosphate (139a)

Compound (**1**) (0.50g, 2.06 mmol) was dissolved in pyridine (100 ml), and diethylamino-n-propyl phosphorochloridate (0.75 ml, 0.81g, 4.11 mmol) was added dropwise with vigorous stirring at *ca.* 4 °C. After stirring for 17 hours at ambient temperature, the reaction was heated for 3 days at 50 °C. The mixture was allowed to return to ambient temperature and a small amount of solid precipitated from solution. The reaction was quenched with water (75 μl, 4.14 mmol), filtered and the filtrate was evaporated under reduced pressure. A trace of pyridine was removed by co-evaporation with toluene. The reaction was not worked up further.

^{31}P NMR δ(CD_3OD, 80 MHz, H_3PO_4) 11.28(9%), 11.10(10), 8.62(7), 1.49(1), 1.28(3), 0.35(11), 0.12(15), -11.82(23), -12.11(21),

3.3.1.4.2

Attempt to prepare 1-β-D-Arabinofuranosylcytosine-5'-methyl n-propylamino phosphate (139b)

Compound (**1**) (0.20g, 0.822 mmol) was dissolved in pyridine (50 ml), and methyl-n-propylamino phosphorochloridate (0.18 ml, 0.21 g, 1.22 mmol) was added dropwise with vigorous stirring at *ca.* 4 °C. After stirring for 17 hours at ambient temperature, more methyl-n-propylamino phosphorochloridate (0.06 ml, 0.07 g, 0.41 mmol) was added and the reaction was stirred for a

further 4 days at ambient temperature. The reaction was quenched with distilled water (75 μl, 4.14 mmol), and the solvent was removed under reduced pressure. TLC (20% methanol in chlorofrom) showed that the residue contained numerous products, the reaction was not worked up further.

3.3.1.4.3

Attempt to prepare 1-β-D-Arabinofuranosylcytosine-5'-bis-(2-chloroethyl)amino ethyl phosphate (139c)

Compound (1) (0.096g, 0.4 mmol) was dissolved in pyridine (20 ml), and bis-(2-chloroethyl)amino ethyl phosphorochloridate (0.23 g, 0.87 mmol) was added dropwise with vigorous stirring at *ca.* 4 °C. After stirring for 17 hours at ambient temperature, more bis-(2-chloroethyl)amino ethyl phosphorochloridate (0.23 g, 0.87 mmol) was added and the reaction was stirred for a further 3 days at ambient temperature. The reaction was quenched with distilled water (29 μl, 1.6 mmol), and the solvent was removed under reduced pressure. TLC (50% methanol in chlorofrom) showed that the residue contained only a baseline product which was not isolated.

3.3.1.4.4

Attempt to prepare 1-β-D-Arabinofuranosylcytosine-5'-methyl N-phenylamino phosphate (139d)

Compound (1) (0.50g, 2.06 mmol) was dissolved in pyridine (80 ml), and methyl N-phenylamino phosphorochloridate (0.86g, 4.18 mmol) in pyridine (10 ml) was added dropwise with vigorous stirring at *ca.* 4 °C. The reaction was

stirred for 3 days at room temperature and then quenched with water (75 μl, 4.14 mmol), and the solvent was removed under reduced pressure. TLC (20% methanol in chlorofrom) showed that the residue contained numerous products, the target compound was not isolated.

3.3.1.4.5

Attempt to prepare 1-β-D-Arabinofuranosylcytosine-5'-n-butyl N-methyl-N-phenylamino phosphate (139e)

Compound **(1)** (0.50g, 2.06 mmol) was dissolved in pyridine (80 ml), and n-butyl N-methyl-N-phenylamino phosphorochloridate (1.09 g, 4.18 mmol) in pyridine (10 ml) was added dropwise with vigorous stirring at *ca.* 4 °C. The reaction was stirred for 4 days at ambient temperature and then quenched with water (75 μl, 4.14 mmol), and the solvent was removed under reduced pressure. TLC (20% methanol in chlorofrom) showed that the residue contained numerous products, the target compound was not isolated.

3.3.1.4.6

Attempt to prepare 1-β-D-Arabinofuranosylcytosine-5'-bis-diethylamino phosphate (140a)

Compound **(1)** (0.50g, 2.06 mmol) was dissolved in pyridine (100 ml), and bis-diethylamino phosphorochloridate (0.87 ml, 0.93g, 4.11 mmol) was added dropwise with vigorous stirring at *ca.* 4 °C. After stirring for 17 hours at ambient temperature, the reaction was heated for 3 days at 60 °C and then quenched with water (75 μl, 4.14 mmol), and the solvent was removed under

reduced pressure. TLC (20% methanol in chlorofrom) showed that the residue contained numerous products, the reaction was not worked up further.

3.3.1.4.7

Attempt to prepare 1-β-D-Arabinofuranosylcytosine-5'-bis-diethylamino phosphate (140a) and isolation of cytosine-N^4-bis-diethylamino phosphate (141)

Compound **(1)** (0.50g, 2.06 mmol) was dissolved in pyridine (100 ml), and bis-diethylamino phosphorochloridate (0.87 ml, 0.93g, 4.11 mmol) was added dropwise with vigorous stirring at *ca.* 4 °C. After stirring for 5 hours at ambient temperature, the reaction was heated for 2 days at 60 °C and then quenched with water (75 µl, 4.14 mmol), and the solvent was removed under reduced pressure. The resulting oil was dried in vacuum, and purified by flash column chromatography. Elution with 20% methanol in chloroform, followed by pooling and evaporation of appropriate fractions gave an off-white solid (0.08 g, 13%).

^{1}H NMR δ(CD_3OD, 400 MHz, TMS) 7.88(1H, d, H6, J=7.7 Hz), 6.13(1H, d, H5, J=8.2 Hz), 3.18(8H, m, $CH_3C\underline{H}_2$), 1.07(12H, t, CH_3)

^{31}P NMR δ(CD_3OD, 80 MHz, H_3PO_4) 14.18

^{13}C NMR δ(CD_3OD, 80 MHz, TMS) 160.52(d, C4, J=5.6 Hz), 154.81(C2), 144.19(C6), 94.09(d, C5, J=8.3 Hz), 42.15(d, $CH_3\underline{C}H_2$, J=4.8 Hz), 13.04(d, CH_3, J=7.0 Hz)

FAB MS m/e 302(MH^+, 2.61%), 301(M^+, 1.34), 273(MH^+ - Et, 3.27), 230(MH^+ - NEt_2, 68.31), 216(MH^+ - NEt_2 - Me - H, 1.46), 159(MH^+ - NE_2^+ x 2 + H^+, 51.08), 112(MH^+ - O=P$(NEt)_2$ + H^+, 35.46), 72($(NEt_2)^+$, bp)

Found C 47.50%, H 8.12, N 22.36, P 10.42;

$C_{12}H_{24}N_5O_2P$ requires C 47.82, H 8.03, N 23.24, P 10.28.

3.3.2 Modified Yoshikawa reactions

3.3.2.1 Phosphoramidates

3.3.2.1.1

Attempt to prepare 1-β-D-arabinofuranosylcytosine-5'-diethyl amino n-propyl phosphate (139a)

n-Propyl phosphorodichloridate (470 µl, 0.71 g, 3.99 mmol) was dissolved in triethyl phosphate (5 ml) and cooled to 0 to 4 °C. Compound **(1)** (0.5 g, 2.056 mmol) was added to the stirred solution under an atmosphere of nitrogen. The reaction was stirred at 4 to 7 °C for 16 hours and then at room temperature for 2 days. Excess n-propyl phosphorodichloridate and HCl were removed by heating and vigorous stirring under reduced pressure (38 °C at 0.1 mm Hg). Diethylamine (2.5 ml, 1.77 g, 0.0242 mol) was added dropwise at -17 °C to the solution of the first stage product (2.056 mmol) in triethyl phosphate (6 ml) and stirred for 5 days at room temperature, a salt formed. The salt was filtered and the filtrate was evaporated to give the triethyl phosphate (5 ml) residue. TLC (20% methanol in chloroform) showed that there were numerous products, the target compound was not isolated.

3.3.2.2 Phosphorodiamidates

3.3.2.2.1

1-β-D-arabinofuranosylcytosine-5'-bis-(diethylamino) phosphate (140a)

Phosphoryl chloride (370 μl, 1.97 mol equ.) was dissolved in triethyl phosphate (5 ml) and cooled to 0 to 4 °C. Compound **(1)** (0.5 g, 2.056 mmol) was added to the solution under an atmosphere of nitrogen. The reaction was stirred at 4 to 7 °C for 16 hours. Excess phosphoryl chloride and the HCl by-product were removed under high vacuum at room temperature for 4 hours. Diethylamine (2.5 ml, 11.75 mol equ.) was added dropwise at room temperature to the solution of the first stage product (2.056 mmol) in triethyl phosphate (5 ml). After the addition was completed, the reaction was stirred at room temperature for 6 days.

The salt was filtered and the filtrate was evaporated to give the triethyl phosphate (5 ml) residue containing the product. This residue was poured into diethyl ether (60 ml), a cream coloured tar fell out of solution. The ether was decanted and the tar was dissolved in methanol and rotary evaporated to give a yellow oil. The oil was purified by flash column chromatography (Silica 65 g), eluted with chloroform (500 ml). The column was then eluted with 20% methanol in chloroform to give a white solid (0.08 g, 9%).

^{1}H NMR δ(CD_3OD, 200 MHz, TMS) 7.89(1H, d, H6, J=8.1 Hz), 6.26(1H, d, H1', J=3.8 Hz), 5.98(1H, d, H5, J=8.1 Hz), 4.19 to 3.28(7H, m, H5', 2 x OH, H4', H2', H3'), 3.15(8H, m, $CH_3C\underline{H_2}$), 1.04(12H, t, CH_3)

^{31}P NMR δ(CD_3OD, 160 MHz, H_3PO_4) 18.61

^{13}C NMR δ(CD_3OD, 100 MHz, TMS) 166.20(C4), 156.35(C2), 144.21(C6), 94.68(C5), 88.71(C1'), 84.57(d, C4', J=6.4 Hz), 77.34(C2'), 75.79(C3'), 67.89(d, C5', J=5.3 Hz), 42.81(d, $CH_3\underline{C}H_2$, J=4.6 Hz), 12.94(d, CH_3, J=6.9 Hz)

FAB MS m/e 434(MH^+, 7.39%), 405(MH^+ - Et, 2.74), 362(MH^+ - NEt_2, 52.84), 316(M^+, 25.82), 289(MH^+, 41.61), 72($(NEt2)^+$, bp)

Found C 45.87%, H 7.13, N 15.37;

$C_{17}H_{32}N_5O_6P.[H_2O]_{0.5}$ requires C 46.15, H 7.52, N 15.83.

3.3.2.2.2

Attempt to prepare 1-β-D-arabinofuranosylcytosine-5'-bis-(n-propylamino) phosphate (140b) and isolation of 1-β-D-arabinofuranosylcytosine-3'-bis-(n-propylamino) phosphate 5'-bis-(n-propylamino) phosphate (145)

Phosphoryl chloride (370 μl, 1.97 mol equ.) was dissolved in triethyl phosphate (2 ml) and cooled to 0 to 4 °C. Compound (**1**) (0.5 g, 2.056 mmol) was added to the solution under an atmosphere of nitrogen. The reaction was stirred at 4 to 7 °C for 16 hours. Excess phosphoryl chloride and the HCl by-product were removed under high vacuum at room temperature for 4 hours. The solution of the first stage product (2.056 mmol) in triethyl phosphate (2 ml) was added dropwise to n-propylamine (1 ml, 5.92 mol equ.) and dimethoxyethane (8 ml). After the addition was completed, the reaction was stirred at 4 to 7 °C for 2 days.

The salt was filtered and the filtrate was evaporated to give the triethyl phosphate (2 ml) residue containing the product. This residue was purified by flash column chromatography (Silica 65 g), eluted with chloroform (500 ml). The column was then eluted with 20% methanol in chloroform. A pure sample of the main product was not obtained. Another silica column was performed with 10% methanol in chloroform as eluant. The main product was isolated as a white solid (0.12 g, 10%).

^{1}H NMR δ(CD_3OD, 200 MHz, TMS) 7.85(1H, d, H6, J=7.6 Hz), 6.21(1H, d, H1', J=3.5 Hz), 5.92(1H, d, H5, J=7.5 Hz), 4.61 to 4.30(7H, m, H5', 2 x OH, H4', H2', H3'), 2.84(8H, m, CH_2NH), 1.51(8H, m, $C\underline{H}_2CH_2NH$), 0.93(12H, m, CH_3)

^{31}P NMR δ(CD_3OD, 160 MHz, H_3PO_4) 20.57, 20.07 (1:1)

^{13}C NMR δ(CD_3OD, 100 MHz, TMS) 167.74(C4), 158.21(C2), 144.95(C6), 95.23(C5), 88.86(C1'), 84.29(t, C4'), 81.12(d, C2', J=4.8 Hz), 75.38(d, C3', J=4.6 Hz), 65.08(d, C5', J=5.2 Hz), 44.31(t, CH_2NH), 26.48(t, $\underline{C}H_2CH_2NH$), 12.05 (CH_3)

3.3.2.2.4

Attempt to prepare 1-β-D-arabinofuranosylcytosine-5'-bis-(bis-(2-chloroethyl)amino) phosphate (140c)

Phosphoryl chloride (370 μl, 1.97 mol equ.) was dissolved in triethyl phosphate (3 ml) and cooled to 0 to 4 °C. Compound **(1)** (0.5 g, 2.056 mmol) was added to the solution under an atmosphere of nitrogen. The reaction was stirred at 4 to 7 °C for 16 hours. Excess phosphoryl chloride and the HCl by-

product were removed under high vacuum at room temperature for 4 hours. Bis-(2-chloroethyl)amine hydrochloride (1.15 g, 3.13 mol equ.) was added to the solution of the first stage product (2.056 mmol) in triethyl phosphate (3 ml) and triethylamine (1.60 ml, 5.58 mol equ.) in DME (10 ml) was added dropwise at 0 to 4 °C. After the addition was completed, the reaction was stirred at 4 to 7 °C for 6 days. The reaction was worked up, the precipitated solids were collected by filtration and washed with DME (4 ml) and then with ether (4 ml). The filtrate was concentrated to 3 ml at room temperature under reduced pressure.

The residue was dissolved in chloroform (10 ml) and added slowly to a flask containing petrol (250 ml, bpt 30 to 40 °C) which was simultaneously stirred vigorously. A yellow solid product fell out of solution and the flask was left in a cold store (0 to -5 °C) overnight. The petrol was decanted off and rotary evaporated, TLC (8% methanol in chloroform) of the petrol residue showed that there was no UV emitting product present. The yellow solid precipitate contained several product spots on investigation with the same TLC system. The reaction was not worked up further.

3.3.3 'Ogilvie' transesterification reactions

3.3.3.1 Asymmetric phosphate triesters

3.3.3.1.1

1-β-D-Arabinofuranosylcytosine-5'-(2,2,2-trichloroethyl) methyl phosphate (146a)

Compound **(134f)** (120 mg, 0.21 mmol) was dissolved in methanol (12 ml). Cesium fluoride (0.91 g, 5.99 mmol, 28.5 mol equ.) was added and the reaction was stirred at ambient temperature for 2 days. The reaction mixture was

rotary evaporated at room temperature @ 20 mmHg and the white solid residue was purified by flash column chromatography. Elution with 10% methanol in chloroform, followed by pooling and evaporation of appropriate fractions gave a white solid (0.04 g, 41%), .

^1H NMR δ(CD_3OD, 200 MHz, TMS) 7.75(1H, d, H6, J=7.5 Hz), 6.15(1H, d, H1', J=3.6 Hz), 5.78(1H, d, H5, J=7.5 Hz), 4.68(2H, t, CH_2OP), 4.34 to 3.95(7H, m, H5', 2 x OH, H4', H2', H3'), 3.72(3H, d, CH_3, J=10.91 Hz)

^{31}P NMR δ(CH_3OD, 160 MHz, H_3PO_4) 1.67

^{13}C NMR δ(CD_3OD, 100 MHz, TMS) 167.63(C4), 158.15(C2), 144.63(C6), 95.89(d, CCl_3, J=11.3 Hz), 94.85(C5), 88.81(C1'), 84.57(d, C4', J=6.8 Hz), 78.51(d, CH_2OP, J=4.4 Hz), 77.91(C2'), 76.19(C3'), 70.08(d, C5', J=5.8 Hz), 56.9 (d, CH_3, J=8.6 Hz)

FAB MS m/e 494(MNa^+, (^{37}Cl x 2) + (^{35}Cl x 1), <1%), 492(MNa^+, (^{37}Cl x 1) + (^{35}Cl x 2), 3), 490(MNa^+, 5), 471(M^+, (^{37}Cl x 2) + (^{35}Cl x 1), 3), 469(M^+, (^{37}Cl x 1) + (^{35}Cl x 2), 6), 467(M^+, 8), 112(cytosineH^+, bp)

Found C 29.14%, H 4.39, N 8.43;
$C_{12}H_{17}N_3Cl_3O_8P.H_2O$ requires C 29.62, H 3.94, N 8.63.

3.3.3.1.2

1-β-D-Arabinofuranosylcytosine-5'-(2,2,2-trichloroethyl) ethyl phosphate (146b)

Compound **(134f)** (150 mg, 0.26 mmol) was dissolved in ethanol (12 ml). Cesium fluoride (0.91 g, 5.99 mmol, 23.1 mol equ.) was added and the reaction

was stirred at ambient temperature for 2 days. The reaction mixture was rotary evaporated at room temperature @ 20 mmHg and the white solid residue was purified by flash column chromatography. Elution with 10% methanol in chloroform, followed by pooling and evaporation of appropriate fractions gave a white solid (0.11 g, 87%), The product was found to be a diastereoisomeric mixture which was separated into two isomerically pure samples *via* preparative HPLC, using a Spherisorb CN 5 μm column and an eluant mixture of water (A)/acetonitrile (B), with a gradient run (Time = 0 mins; 85% (A), 15% (B). Time = 12 mins; 85% (A), 15% (B). Time = 30 mins; 20% (A), 80% (B).). Nuclear Overhauser experiments and molecular mechanics calculations were carried out on the separated isomers in order to determine their conformation about the phosphorus.

^{1}H NMR δ(CD_3OD, 200 MHz, TMS) 7.74[7.73](1H, d, H6, J=7.5 Hz), 6.16[6.15](1H, d, H1', J=3.7 Hz), 5.75(1H, d, H5, J=7.5 Hz), 4.66(2H, m, CH_2OP), 4.42 to 4.04(9H, m, H5', 2 x OH, H4', H2', H3', $CH_3C\underline{H}_2OP$), 1.39(3H, m, CH_3)

^{31}P NMR δ(CH_3OD, 160 MHz, H_3PO_4) -2.66, -2.62 (1:1)

^{13}C NMR δ(CD_3OD, 100 MHz, TMS) 167.65(C4), 158.17(C2), 144.56[144.53](C6), 95.92(d, CCl_3, J=11.3 Hz), 94.92[94.88](C5), 88.84[88.72](C1'), 84.53(d, C4', J=6.6 Hz), 78.50(d, CH_2OP, J=4.4 Hz), 77.89[77.38](C2'), 76.16[76.08](C3'), 70.60(d, C5', J=6.0 Hz), 64.83(d, CH_2OP, J=5.1 Hz), 15.98(d, CH_3,.J=6.2 Hz)

(Peaks in [ ] are due to the diastereoisomeric mixture)

FAB MS m/e 506(MNa^+, (^{37}Cl x 1) + (^{35}Cl x 2), 4%), 504(MNa^+, 6), 486(MH^+, (^{37}Cl x 2) + (^{35}Cl x 1), 4), 484(MH^+, (^{37}Cl x 1) + (^{35}Cl x 2), 10), 482(MH^+, 11), 112(cytosineH^+, bp)

Found C 30.73%, H 4.66, N 8.10;

$C_{13}H_{19}N_3Cl_3O_8P.H_2O$ requires C 31.19, H 4.23, N 8.39.

Separated isomers:

HPLC RT [Spherisorb CN 5 μm column and an eluant mixture of water (A)/acetonitrile (B), with a gradient run (Time = 0 mins; 85% (A), 15% (B). Time = 12 mins; 85% (A), 15% (B). Time = 30 mins; 20% (A), 80% (B).)]

11.56 (Fast), 11.84 (Slow)

^{31}P NMR δ(CH_3OD, 160 MHz, H_3PO_4) -2.62(Fast), -2.66(Slow)

Isomer	**Observed NOE (Irradiation of CH_3)**
Slow	H6, H4', H5', $CH_3C\underline{H_2}$, $CCl_3C\underline{H_2}$
Fast	H4', H5', $CH_3C\underline{H_2}$, $CCl_3C\underline{H_2}$

3.3.3.1.3

1-β-D-Arabinofuranosylcytosine-5'-(2,2,2-trichloroethyl) n-propyl phosphate (146c)

Compound **(134f)** (120 mg, 0.21 mmol) was dissolved in propanol (12 ml). Cesium fluoride (0.91 g, 5.99 mmol, 28.5 mol equ.) was added and the reaction was stirred at ambient temperature for 2 days. The reaction mixture was

rotary evaporated at room temperature @ 20 mmHg and the white solid residue was purified by flash column chromatography. Elution with 10% methanol in chloroform, followed by pooling and evaporation of appropriate fractions gave a white solid (0.04 g, 38%).

^{1}H NMR δ(CD_3OD, 200 MHz, TMS) 7.69(1H, d, H6, J=7.5 Hz), 6.13(1H, d, H1', J=3.7 Hz), 5.77(1H, d, H5, J=7.5 Hz), 4.66(2H, m, CH_2OP), 4.44 to 4.04(9H, m, H5', 2 x OH, H4', H2', H3', $CH_3C\underline{H}_2OP$), 1.84(2H, m, $CH_3C\underline{H}_2$), 0.99(3H, m, CH_3)

^{31}P NMR δ(CH_3OD, 160 MHz, H_3PO_4) -1.47

^{13}C NMR δ(CD_3OD, 100 MHz, TMS) 167.65(C4), 158.17(C2), 144.69(C6), 95.87(d, CCl_3, J=11.3 Hz), 94.91(C5), 88.75(C1'), 84.43(d, C4', J=6.6 Hz), 78.43(d, CH_2OP, J=4.4 Hz), 78.06(C2'), 76.10(C3'), 71.99(d, $CH_2\underline{C}H_2OP$, J=4.1 Hz), 70.04(d, C5', J=6.0 Hz), 25.69($CH_3\underline{C}H_2$), 11.35(CH_3)

FAB MS m/e 499(M^+, (^{37}Cl x 2) + (^{35}Cl x 1), 3%), 497(M^+, (^{37}Cl x 1) + (^{35}Cl x 2), 7), 495(M^+, 10), 112(cytosineH^+, bp)

Found C 32.48%, H 4.62, N 7.52;
$C_{14}H_{21}N_3Cl_3O_8P.H_2O$ requires C 32.67, H 4.51, N 8.16.

3.3.3.1.4

1-β-D-Arabinofuranosylcytosine-5'-(2,2,2-trichloroethyl) n-butyl phosphate (146d)

Compound **(134f)** (120 mg, 0.21 mmol) was dissolved in n-butanol (12 ml). Cesium fluoride (0.91 g, 5.99 mmol, 28.5 mol equ.) was added and the

reaction was stirred at ambient temperature for 2 days. The reaction mixture was rotary evaporated at room temperature @ 20 mmHg and the white solid residue was purified by flash column chromatography. Elution with 10% methanol in chloroform, followed by pooling and evaporation of appropriate fractions gave a white solid (0.05g, 47%).

^{1}H NMR δ(CD_3OD, 200 MHz, TMS) 7.71(1H, d, H6, J=7.5 Hz), 6.14(1H, d, H1', J=3.6 Hz), 5.74(1H, d, H5, J=7.5 Hz), 4.65(2H, m, CH_2OP), 4.40 to 4.04(9H, m, H5', 2 x OH, H4', H2', H3', $CH_3C\underline{H}_2OP$), 1.72(2H, m, $C\underline{H}_2CH_2OP$), 1.34(2H, m, $CH_3C\underline{H}_2$), 0.97(3H, m, CH_3)

^{31}P NMR δ(CH_3OD, 160 MHz, H_3PO_4) -1.92

^{13}C NMR δ(CD_3OD, 100 MHz, TMS) 167.60(C4), 158.13(C2), 144.68(C6), 95.89(d, CCl_3, J=11.3 Hz), 94.92(C5), 88.76(C1'), 84.51(d, C4', J=6.6 Hz), 78.43(d, CH_2OP, J=4.4 Hz), 78.06(C2'), 76.11(C3'), 70.09(d, C5', J=6.0 Hz), 68.48(d, $CH_2\underline{C}H_2OP$, J=5.5 Hz), 31.40(d, $\underline{C}H_2CH_2OP$, J=6.4 Hz), 18.67($CH_3\underline{C}H_2$), 13.91(CH_3)

FAB MS m/e 513(M^+, (^{37}Cl x 2) + (^{35}Cl x 1), 1%), 511(M^+, (^{37}Cl x 1) + (^{35}Cl x 2), 5), 509(M^+, 10), 112(cytosineH^+, bp)

Found C 34.87%, H 4.82, N 7.79;
$C_{15}H_{23}N_3Cl_3O_8P$ requires C 35.28, H 4.54, N 8.23.

3.3.3.2 Phosphoramidates

3.3.3.2.1

1-β-D-Arabinofuranosylcytosine-5'-(2,2,2-trichloroethyl) n-propylamino phosphate (147a)

Compound (**134f**) (120 mg, 0.21 mmol) was dissolved in n-propylamine (12 ml). Cesium fluoride (0.91 g, 5.99 mmol, 28.5 mol equ.) was added and the reaction was stirred at ambient temperature for 4 days. The reaction mixture was rotary evaporated at room temperature @ 20 mmHg and the white solid residue was purified by flash column chromatography. Elution with 10% methanol in chloroform, followed by pooling and evaporation of appropriate fractions gave a white solid. The crude product was re-columned on flash silica and eluted with 5% methanol in chloroform to yield a pure white solid (0.05 g, 48%).

^1H NMR δ(CD_3OD, 200 MHz, TMS) 7.85(1H, d, H6, J=7.5 Hz), 5.91(1H, d, H1', J=3.6 Hz), 5.81(1H, d, H5, J=7.5 Hz), 4.70(2H, m, CH_2OP), 4.34 to 3.85(7H, m, H5', 2 x OH, H4', H2', H3'), 2.49(2H, m, $CH_2\underline{CH_2}N$), 2.07(2H, m, $\underline{CH_2}CH_2N$), 0.69(3H, t, CH_3)

^{31}P NMR δ(CH_3OD, 160 MHz, H_3PO_4) 12.72

^{13}C NMR δ(CD_3OD, 100 MHz, TMS) 167.62(C4), 158.11(C2), 144.66(C6), 95.97(d, CCl_3, J=11.3 Hz), 94.86(C5), 88.78(C1'), 84.34(d, C4', J=6.6 Hz), 78.47(d, CH_2OP, J=4.4 Hz), 78.09(C2'), 76.12(C3'), 70.16(d, C5', J=6.0 Hz), 48.94(d, CH_2NH, J=4.7 Hz), 27.35(d, $\underline{C}H_2CH_2NH$, J=6.2 Hz), 12.75(CH_3)

FAB MS m/e 496(M^+, (^{37}Cl x 1) + (^{35}Cl x 2), <1%), 494(M^+, <1), 154(bp), 136(92)

Found C 32.18%, H 5.02, N 10.47;

$C_{14}H_{22}N_4Cl_3O_7P.H_2O$ requires C 32.73, H 4.71, N 10.91.

3.3.3.2.2

1-β-D-Arabinofuranosylcytosine-5'-(2,2,2-trichloroethyl) n-butylamino phosphate (147b)

Compound **(134f)** (120 mg, 0.21 mmol) was dissolved in n-butylamine (12 ml). Cesium fluoride (0.91 g, 5.99 mmol, 28.5 mol equ.) was added and the reaction was stirred at ambient temperature for 4 days. The reaction mixture was rotary evaporated at room temperature @ 20 mmHg and the white solid residue was purified by flash column chromatography. Elution with 5% methanol in chloroform, followed by pooling and evaporation of appropriate fractions gave a white solid (0.03 g, 28%).

^1H NMR δ(CD_3OD, 200 MHz, TMS) 7.82(1H, d, H6, J=7.5 Hz), 6.01(1H, d, H1', J=3.6 Hz), 5.80(1H, d, H5, J=7.5 Hz), 4.68(2H, m, CH_2O), 4.35 to 3.76(7H, m, H5', 2 x OH, H4', H2', H3'), 2.56(2H, m, $CH_2\underline{CH_2}N$), 2.09(4H, m, $\underline{CH_2}\underline{CH_2}N$), 0.67(3H, t, CH_3)

^{31}P NMR δ(CH_3OD, 160 MHz, H_3PO_4) 11.70

^{13}C NMR δ(CD_3OD, 100 MHz, TMS) 167.61(C4), 158.12(C2), 144.63(C6), 95.93(d, CCl_3, J=11.3 Hz), 94.85(C5), 88.79(C1'), 84.37(d, C4', J=6.6 Hz), 78.48(d, CH_2OP, J=4.4 Hz), 78.06(C2'), 76.14(C3'), 70.17(d, C5', J=6.0 Hz), 44.71(d, CH_2NH, J=4.5 Hz), 36.67(d, $\underline{C}H_2CH_2NH$, J=6.8 Hz), 24.54($CH_3\underline{C}H_2$), 15.82(CH_3)

FAB MS m/e 510(M^+, (^{37}Cl x 1) + (^{35}Cl x 2), <1%), 508(M^+, <1), 154(90) 136(bp)

Found C 34.98%, H 5.17, N 10.13;

$C_{15}H_{24}N_4Cl_3O_7P$ requires C 35.35, H 4.75, N 10.99.

3.3.3.2.3

Attempt to prepare 1-β-D-Arabinofuranosylcytosine-5'-(2,2,2-trichloroethyl) diethylamino phosphate (147c)

Compound **(134f)** (120 mg, 0.21 mmol) was dissolved in diethylamine (12 ml). Cesium fluoride (0.91 g, 5.99 mmol, 28.5 mol equ.) was added and the reaction was stirred at ambient temperature for 6 days. There was only baseline material observed by TLC (20% methanol in chloroform), there was no sign of any target compound being produced. The mixture was then heated to reflux for 16 hours but no further reaction was observed.

3.3.4

N^4-Dimethylaminomethylene-1-β-D-arabinofuranosylcytosine (148)

A suspension of **(1)** (300 mg, 0.0012 mol) in dimethylformamide (5 ml) was shaken with a fivefold excess of dimethylformamide dimethylacetal at room temperature overnight and the resulting clear solution was evaporated under vacuum of an oil pump. The residual sirup was diluted with ethanol (6 ml) and the solution was precipitated by the addition of ether (90 ml). The precipitate

was collected with suction and dried at 0.2 mmHg (room temperature) to yield a white solid (0.35 g, 90%).

^{1}H NMR δ(CD_3OD, 200 MHz, TMS) 8.31(1H, s, N=CH), 7.65(1H, d, H6, J=7.3 Hz), 5.84(1H, d, H1', J=3.4 Hz), 5.78(1H, d, H5, J=7.3 Hz), 3.88 to 3.49(5H, m, H4', H2', H3', H5'), 2.85(3H, s, CH_3), 2.76(3H, s, CH_3)

^{13}C NMR δ(CD_3OD, 100 MHz, TMS) 171.16(C4), 158.20(C2), 156.12(N=CH), 143.54(C6), 100.90(C5), 87.16(C1'), 84.87(C4'), 76.36(C2'), 74.63(C3'), 60.98(C5'), 40.71(CH_3), 34.31(CH_3)

FAB MS m/e 299(MH^+, 0.01%), 167($C_7H_{11}N_4O$, bp)

UV λ max (nm) Product (306), araC (269)

Found C 47.14%, H 6.33, N 18.24;
$C_{12}H_{18}N_4O_5$ requires C 48.32, H 6.08, N 18.78.

3.4 Biological results (Method: cf. Chapter 2)

Table 7 The inhibition of the incorporation of tritiated thymidine into DNA in the presence of the compounds listed at a final concentration of 30 μM, relative to 10% DMSO control

Compound	%I (SEM)
(1)	83 (5)
(140a)	47 (6)
(146a)	22 (6)
(146bI)	31 (2)
(146bII)	30 (4)
(146c)	40 (4)
(147a)	45 (3)
(147b)	48 (7)

3.5 References

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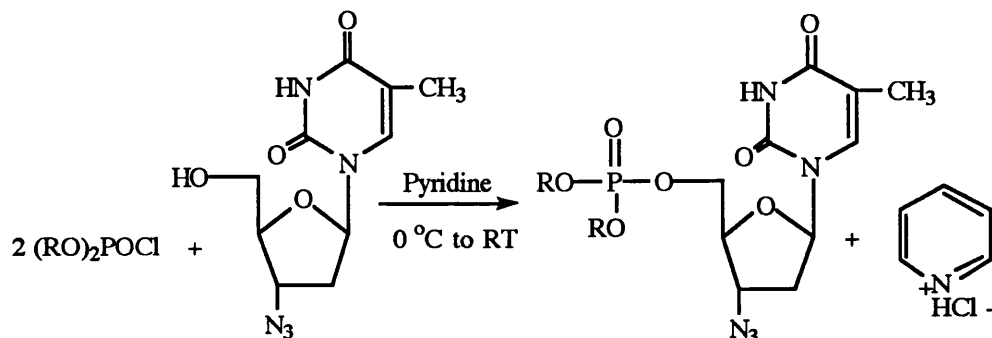
4.0 Design and synthesis of novel anti-HIV chemotherapeutic agents

4.1 Introduction

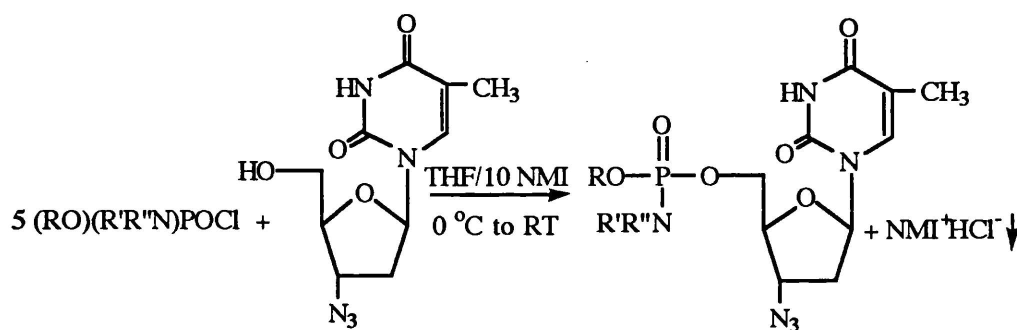
As with other nucleoside analogues, including other anti-HIV agents, **(2)** acts only after intracellular conversion to its 5'-triphosphate.[1] The consequential dependence on host kinases is problematic, since low kinase activity will result in a poor clinical response.[2] The straightforward use of simple nucleotides to by-pass the kinase dependence is precluded on account of their poor membrane penetration.[3,4] However there has been much interest in the use of masked phosphate esters as membrane soluble depot forms of the bio-active nucleotides of several chemotherapeutic nucleoside analogues.[5-8] Recently these studies have been extended to derivatives of **(2)**.[9,10] It has been noted that simple dialkyl phosphate derivatives of **(2)** are inactive as anti-HIV agents,[11] whereas certain phosphoramidate analogues are potent inhibitors of viral proliferation.[12] The initial rational behind the synthesis of phosphoramidates was the idea that HIV aspartate proteinase might specifically hydrolyse these membrane soluble pro-drugs.[13] The resulting phosphate diesters would be trapped inside the (infected) cell, where phosphodiesterase action may yield either **(2)** or **(110)** or both. The activity of the phosphoramidates suggested that further novel species of similar structure may be of interest. Thus the synthesis and potent anti-HIV activity of analogous phosphorodiamidate derivatives was achieved; where both free phosphate valencies of **(110)** were linked to the amino groups of amines in particular amino acids.

The synthetic route employed to make 5'-phosphate derivatives of **(2)** involves the reaction of the free nucleoside with the appropriate

phosphorochloridate. For dialkyl phosphorochloridates, this reaction proceeded well in pyridine,[11]



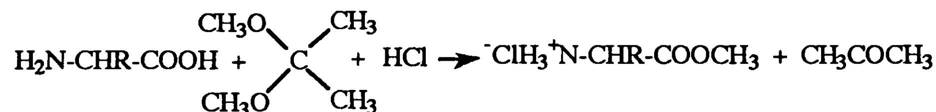
whereas for alkyl alkylamino phosphorochloridates these conditions gave a poor reaction and N-methylimidazole in tetrahydrofuran was necessary.[12]



Initial studies with bis-(alkylamino) phosphorochloridates revealed that even the latter conditions were poor. Thus an alternative synthetic strategy was required. In a similar situation, with the anti cancer agent (**1**) (chapter 3), a method based on the initial reaction of the nucleoside with phosphoryl chloride was adopted.

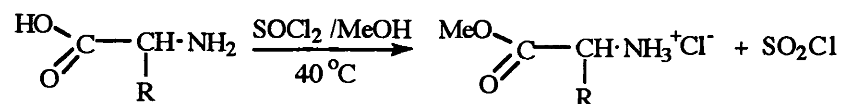
The reaction of **(2)** with phosphoryl chloride followed by amino acid methyl esters gave novel diamidate derivatives of **(110)**. It was hoped that the 5'-phosphorodiamidates might act as membrane soluble pro-drugs of the bio-active free nucleotides of **(2)**. Different amino acids were employed, covering a range of structures and polarities. The reaction was also conducted with propylamine and with diethylamine. This procedure was also extended to other nucleoside analogues, 3'-O-acylthymidine **(149)**, 3'-deoxy-3'-iodothymidine **(150)** and 3'-deoxy-3'-fluorothymidine **(94)**

The first requirement was to prepare amino acid alkyl ester hydrochlorides; which may be prepared *via* numerous methods. One of the methods include, the reaction of the amino acid with 2,2-dimethoxypropane and hydrogen chloride.[14]



Another involves the introduction of dry HCl gas to a suspension of the amino acid in ethanol without cooling until a clear solution forms.[15]

However the method chosen was the reaction of amino acid with methanol and thionyl chloride to give the methyl ester hydrochloride of a series of amino acids.[16]



This was a simple method that could be employed for the range of amino acids used. Starting materials were easily accesible and the product was isolated in approximately 75% yield.

The second requirement was to prepare the nucleoside analogues, **(149)**, **(150)** and **(2)**. The routes to the production of these nucleoside analogues of thymidine were chosen on the known reliability of the reactions employed .

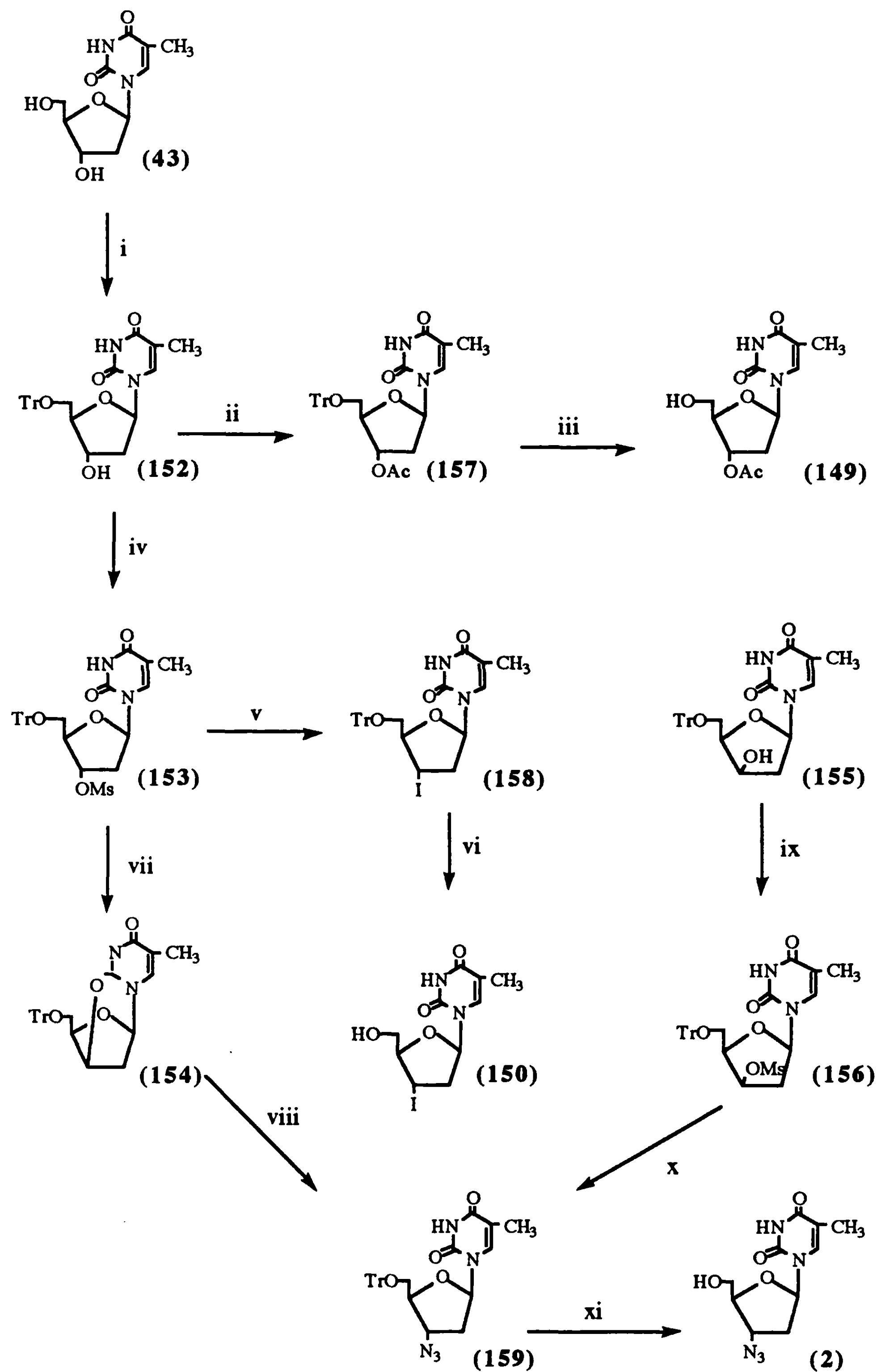
Compound **(149)** was prepared (Figure 19, steps i,ii and iii),[17] by the adition of trityl chloride to thymidine in pyridine produced **(152)**. This was followed by the addition of acetic anhydride at room temperature to the isolated **(152)**. 80% Acetic acid was used for detritylation to give **(149)**.

The method chosen for the production of **(150)** was the reaction of **(153)** with sodium iodide in acetone (Figure 19, steps i,iv,v and vi).[18]

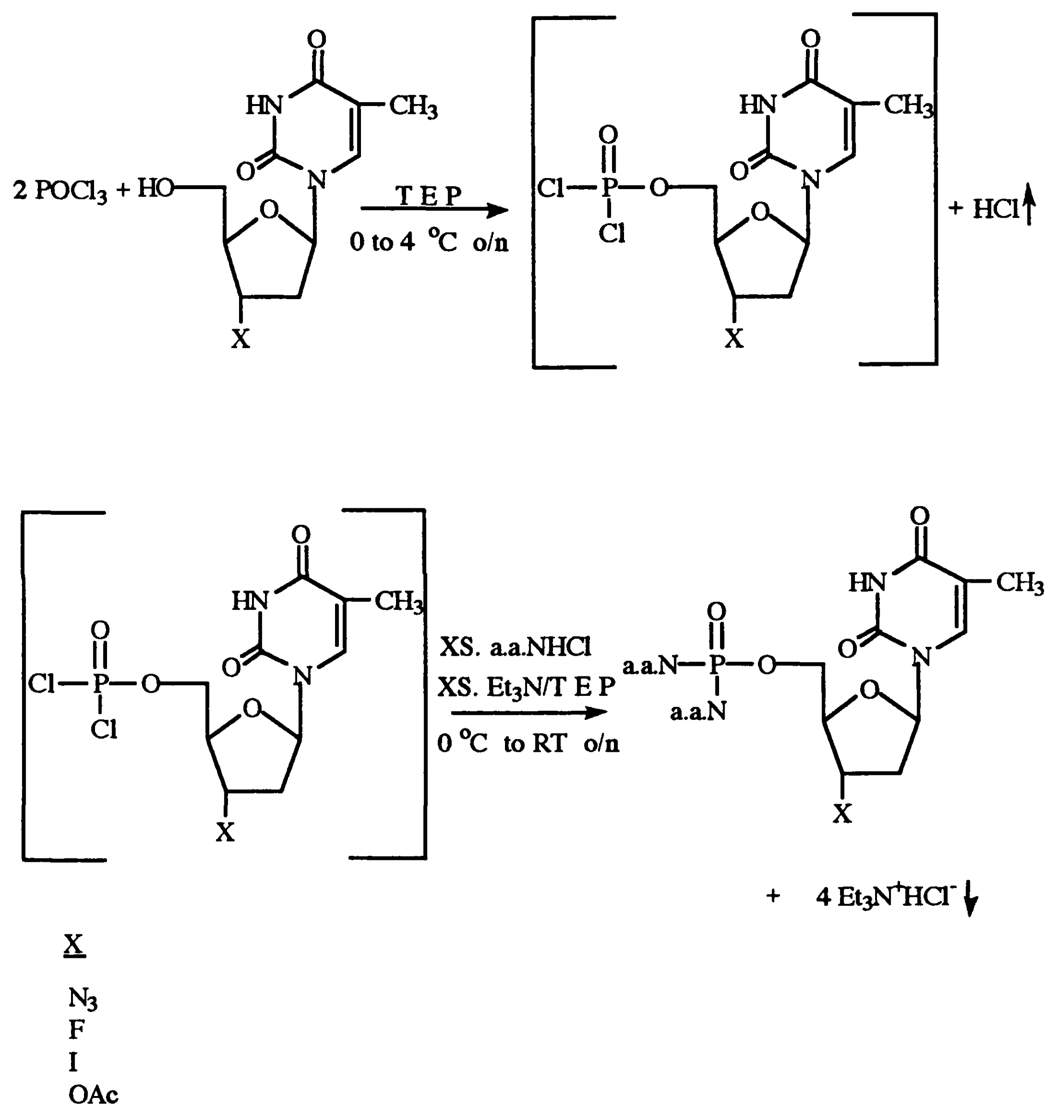
Compound **(2)** was prepared *via* two separate routes. The first route (Figure 19, steps i,iv,vii,viii and xi)[19,20] entailed the protection of the 5'-OH of thymidine with trityl chloride in pyridine. The 3'-position was activated by the use of mesyl chloride giving **(153)**. Then **(154)** was prepared by reaction with 1,5-diazobicyclo[5.4.0]undec-7-ene (DBU). Compound **(154)** was reacted without purification with sodium azide in DMF, followed by detritylation with 80% acetic acid to give **(2)**.

The second route to produce **(2)** (Figure 19, steps ix,x and xi)[21] was the mesylation of **(155)** which afforded **(156)** in high yield. Treatment of the sulfonate with sodium azide in DMF followed by detritylation in 80% acetic acid led to the isolation of **(2)**.

Figure 19: Preparation of nucleoside analogues of thymidine



Once the starting materials had been produced, that is, the amino acid methyl ester hydrochlorides and the nucleoside analogues, the production of phosphorodiamidate derivatives of **(2)**, **(149)**, **(150)** and **(94)** were prepared *via* the methodology of Yoshikawa et al.[22-26]



The isolated phosphorodiamidate derivatives of **(2)** were tested for their inhibitory effect on HIV-1 proliferation in a human lymphoblastoid cell line. The amino acid derivatives of **(2)** were potent inhibitors of viral proliferation; small changes in strucure leading to marked changes in activity.[27]

The mechanism of action of these phosphorodiamidates of **(2)** has been studied by human plasma kinetic studies, employing the use of HPLC and ^{31}P NMR.

4.2 Results and Discussion

A number of amino acid methyl ester hydrochloride salts were prepared by the initial dropwise addition of thionyl chloride to methanol at -10 °C, followed by a slow addition of an amino acid. Then the mixture was allowed to warm to room temperature and heated at 40 °C overnight. The resulting yellowish oil was evaporated under reduced pressure to yield a white solid. The white solid was dissolved in the minimum of warm methanol and then ten times the volume of diethyl ether was added to give the product as white crystals which were filtered and air-dried. Thus **(151a-e)** were prepared and isolated in a 75% to 81% yield. The ^1H NMR for the series **(151a-e)** were fully assigned and confirmed the esterification of the relevant amino acid.

$$HO(O=)C-CH(R)-NH_2 \xrightarrow[40\,^{\circ}C\ o/n]{2\ SOCl_2/8\ MeOH} MeO(O=)C-CH(R)-NH_3^+Cl^- + SO_2Cl$$

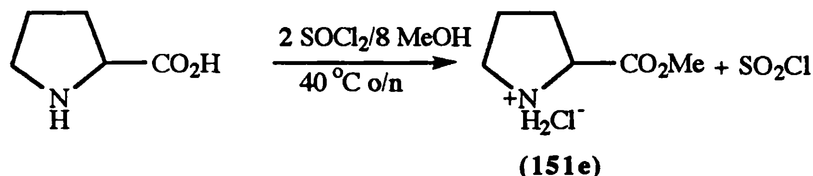
R	
$CH(CH_3)_2$	**(151a)**
CH_3	**(151b)**
CH_2Ph	**(151c)**

The ^1H NMR of compounds **(151a-c)** showed the methyl ester singlet at δ3.6 to 3.7 along with the expected signals for the relevant amino acid.

$$HO(O=)C-CH(CH_2R)-NH_2 \xrightarrow[40\,^{\circ}C\ o/n]{4\ SOCl_2/16\ MeOH} MeO(O=)C-CH(CH_2R')-NH_3^+Cl^- + 2\ SO_2Cl + HCl$$

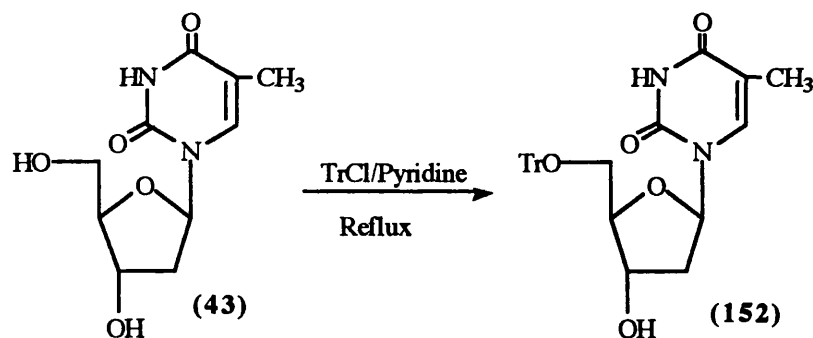
R = CO_2H R' = CO_2CH_3 **(151d)**

Compound (**151d**) was esterified at both carboxylic acid moieties ^{1}H NMR showed two singlets at around δ3.7 to 3.9 along with the expected signals for aspartic acid.



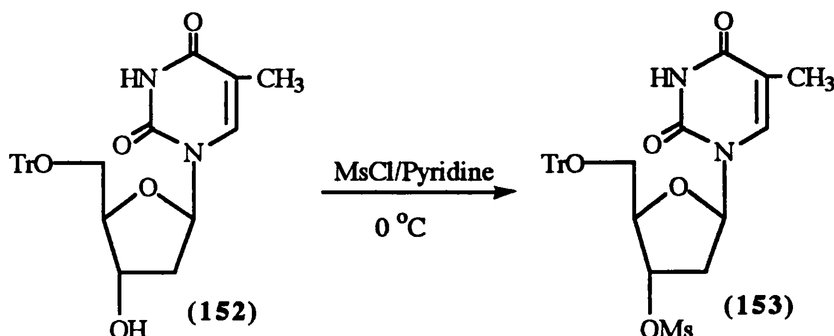
There was some difficulty encountered in producing (**151e**) in the form of a solid. Eventually, the isolated white gum was triturated in ether and allowed to stand at 0 to 4 °C for 3 days to give the product as a white solid which on investigation by ^{1}H NMR displayed all the signals expected for L-proline along with a singlet at around δ3.5.

Compound (**152**) was prepared, with an 83% yield, by the reaction of trityl chloride with (**43**) with pyridine as both solvent and base.



The ^{1}H and ^{13}C NMR spectra were fully consistent with the structure of (**152**). In the ^{1}H NMR, the signals expected for (**43**) were observed along with the signals for the phenyl protons at δ7.19 integrating for 15 protons. The ^{13}C NMR showed signals expected for thymidine along with signals for the trityl protecting group between δ144 to 127, for the phenyl carbons and a signal at δ86.82, for the methyl carbon.

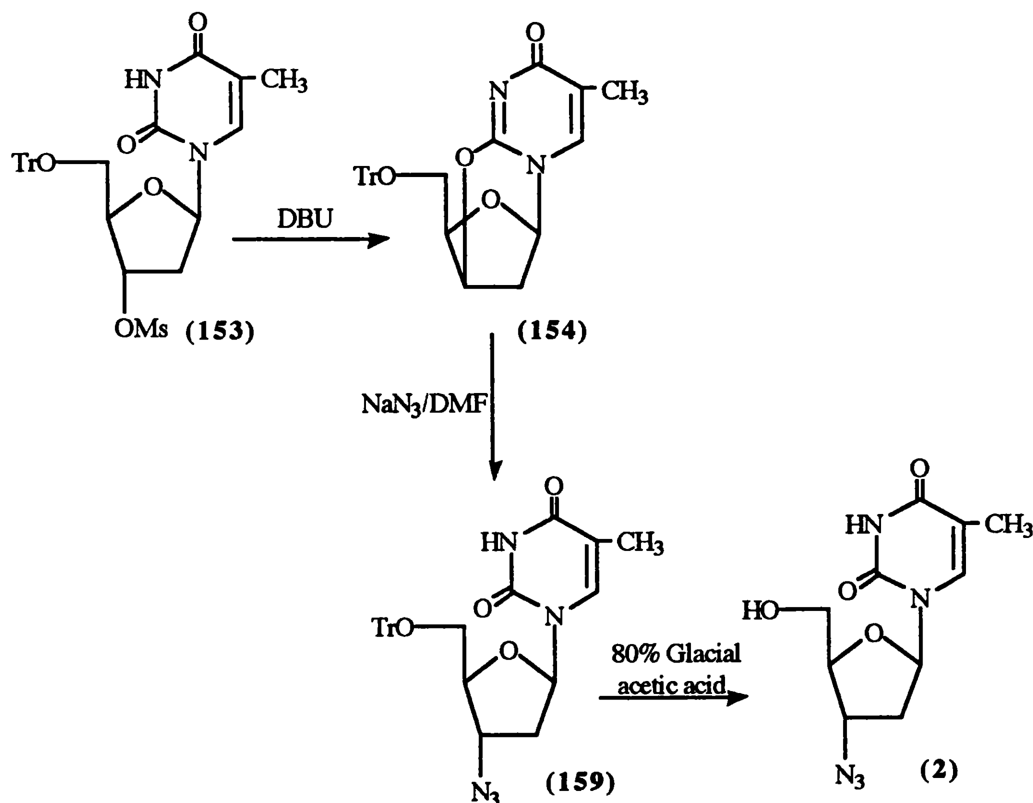
Compound **(153)** was prepared, with an 89% yield, by the reaction of mesyl chloride with **(152)** with pyridine as both solvent and base.



The ^{1}H NMR and ^{13}C NMR spectra of compound **(153)** were similar to compound **(152)** with the addition of a singlet due to the methyl of the mesyl group at δ3.12, for the ^{1}H NMR and a singlet at δ37.46, for the ^{13}C NMR.

Compound **(2)** was prepared by two separate routes, the first route involved the formation of the anhydro compound **(154)** in a 89% yield by the treatment of compound **(153)** with 1,5-diazabicyclo [5.4.0] undec-7-ene. The formation of **(154)** was presumed and the product was used without characterisation in a reaction to produce **(159)**.

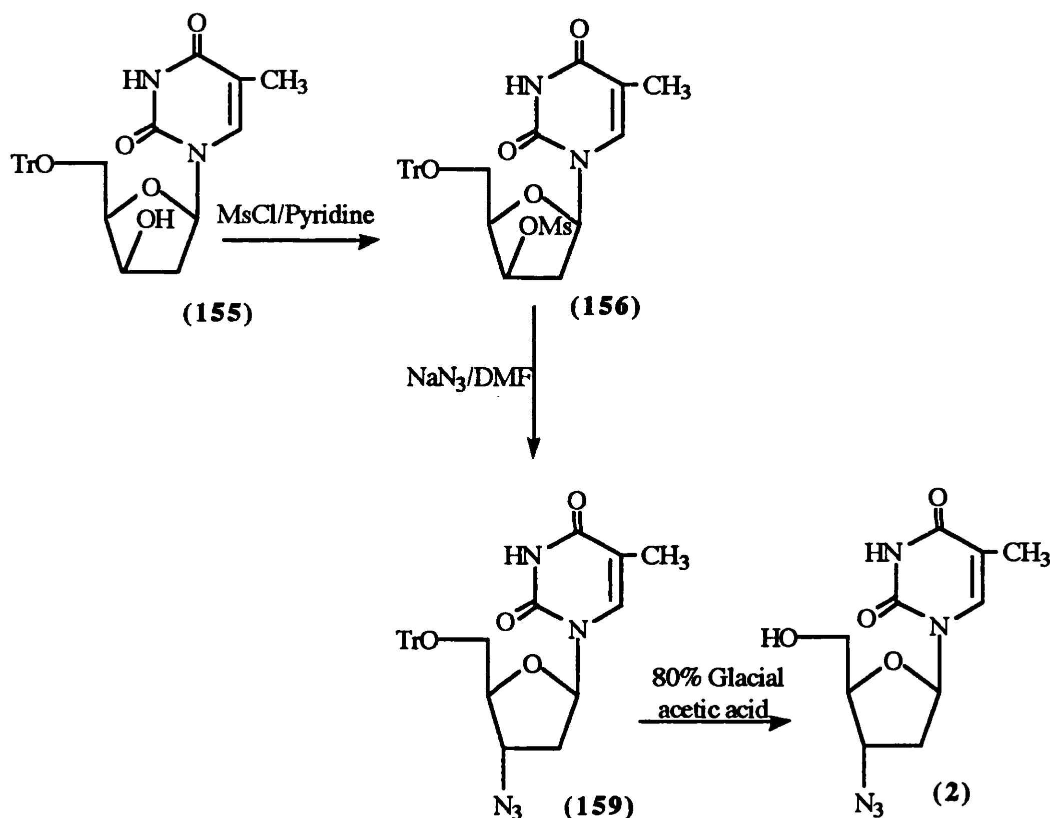
Compound **(154)** was reacted with sodium azide in dimethylformamide, a solid was isolated after workup and presumed to be **(159)**. The solid was suspended in 80% glacial acetic acid and refluxed for 40 minutes, it was important to stop the reaction as soon as the deprotection of **(159)** was complete, otherwise thymine would appear in the reaction mixture. The solvent was removed under reduced pressure and the residue was shaken several times with ether. The ether insoluble material was chromatographed on flash silica gel and **(2)** was isolated as an off-white solid by pooling and evaporation of appropriate fractions in a 63% yield from **(154)**.



The ^{1}H and the ^{13}C NMR were fully assigned and consistent with the structure of **(2)**, with all the peaks expected for **(43)** with the exception that a 3'OH was not observed in the ^{1}H NMR and the C3' was more upfield in the ^{13}C spectrum of **(2)** compared to the C3' of **(43)**.

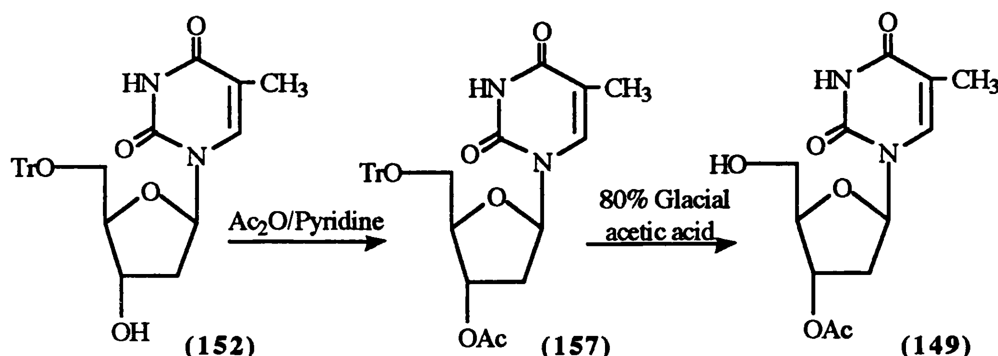
The second route employed to produce **(2)** was the mesylation of **(155)** to give the assumed product **(156)** in an 88% yield. Then the reaction of **(156)** with sodium azide in DMF under an atmosphere of nitrogen and at 100 °C. This displacement of a mesyl group by azide operates principally by an SN2 mechanism and thereby leads to inversion of configuration at the C3' atom.[17,18] The isolated solid product was suspended in 80% glacial acetic acid and heated at 80 °C for 40 mins in order to deprotect the 5'OH. The product was evaporated under reduced pressure and the residue was shaken several times with diethyl ether. The solvent was removed under reduced pressure and the

residue was shaken several times with ether. The ether insoluble material was chromatographed on flash silica gel and **(2)** was isolated as a white solid by pooling and evaporation of appropriate fractions in a 76% yield from **(156)**. The product compared well with an authentic sample, on investigation by analytical thin layer chromatography.

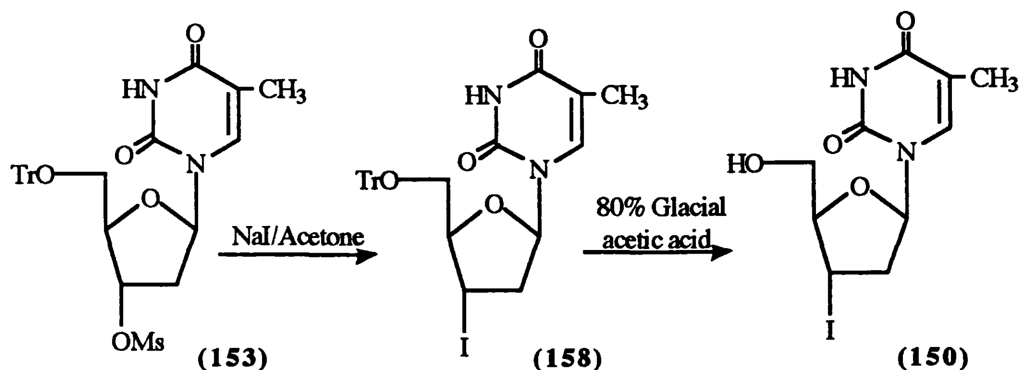


Compound **(149)** was prepared by the addition of acetic anhydride to a solution of **(152)** in pyridine. The isolated precipitate was suspended in 80% acetic acid and heated to 80 °C for 40 minutes, to remove the trityl protecting group. The solvent was then removed under reduced pressure and the residue was purified by chromatography on silica, with elution by 5% methanol in chloroform. Pooling and evaporation of appropriate fractions gave the product as a yellowish solid, which was recrystallised from acetone-petroleum ether to give a white crystalline solid in an 81% yield. The ^{1}H NMR showed the acetyl protons at δ2.09 with the H3' being more downfield than would be seen in

(43), all other peaks expected for **(43)** were seen except for the 3'OH. The ^{13}C NMR showed the signals expected for **(43)**, however the C3' was more downfield than would be observed for **(43)**. The ^{13}C NMR also showed the $CH_3\underline{C}O$ signal at δ170.67 with the $\underline{C}H_3CO$ signal at δ21.07.

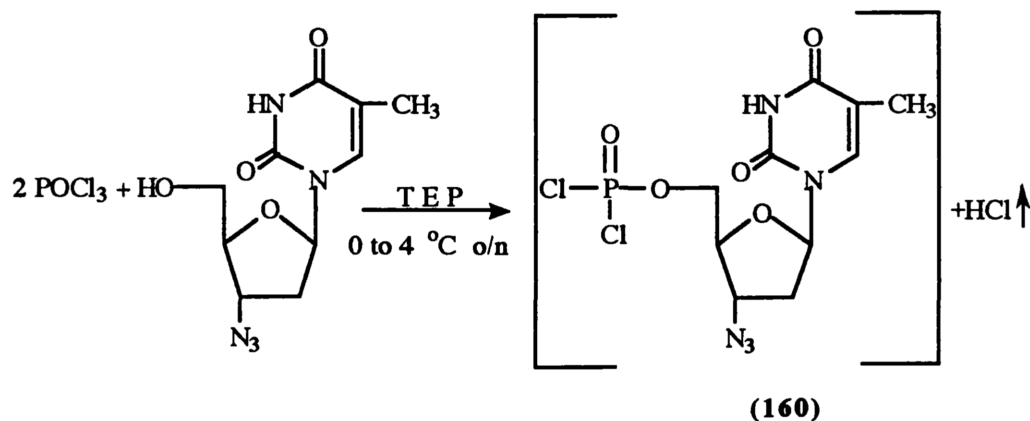


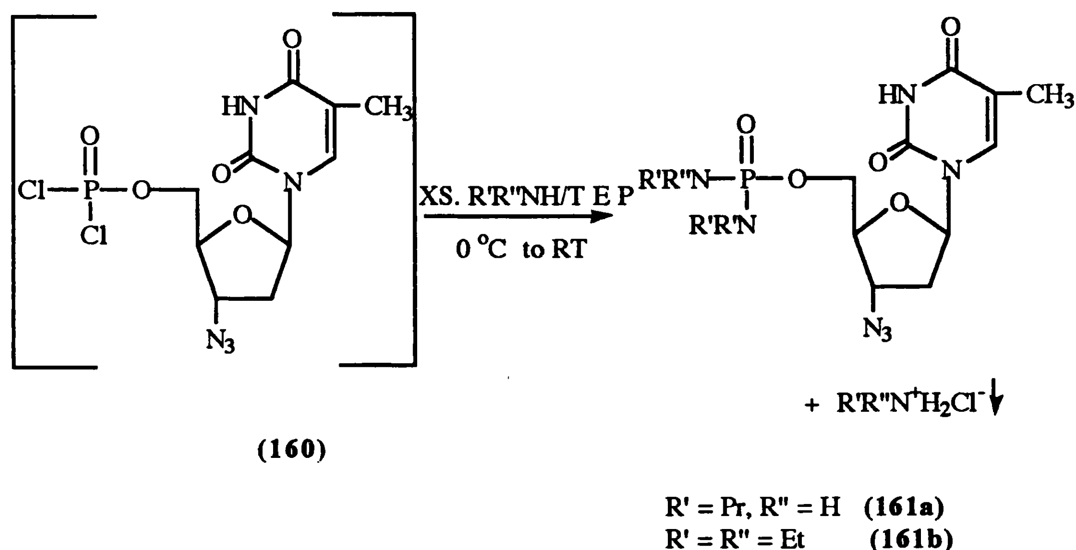
Compound **(150)** was produced *via* the initial reaction of **(153)** with sodium iodide in acetone at 100 °C *via* the methodology of Michelson and Todd,[18] the configuration of the 3'-halogen was assumed to be of the ribo form. Michelson and Todd postulated that "A change in configuration at position 3' appears to be involved...". It is almost certain from their experiments that in the formation of their halogeno derivatives, a double Walden inversion had occurred *via* an anhydronucleoside intermediate such as **(154)**. The yellow amorphous solid was collected, well washed with water and suspended in 80% glacial acetic acid and refluxed for 40 minutes. The product was evaporated under reduced pressure and the residue was shaken several times with ether to remove triphenyl methanol. The ether insoluble residue was chromatographed on flash silica gel to give a 39% yield of **(150)** as a white crystalline solid. The ^{1}H NMR and the ^{13}C NMR were fully assigned and the major difference between **(150)** and **(43)** was that **(150)** showed H3' and C3' dramatically shifted upfield. A mass spectrum of a sample of **(150)** was also obtained which gave a signal for the parent ion at 352 m/e along with fragment ions consistent with the target compound.



Having isolated the required starting materials, reactions were attempted to prepare novel phosphorodiamidate derivatives of the nucleoside analogues **(2)**, **(94)**, **(149)** and **(150)**.

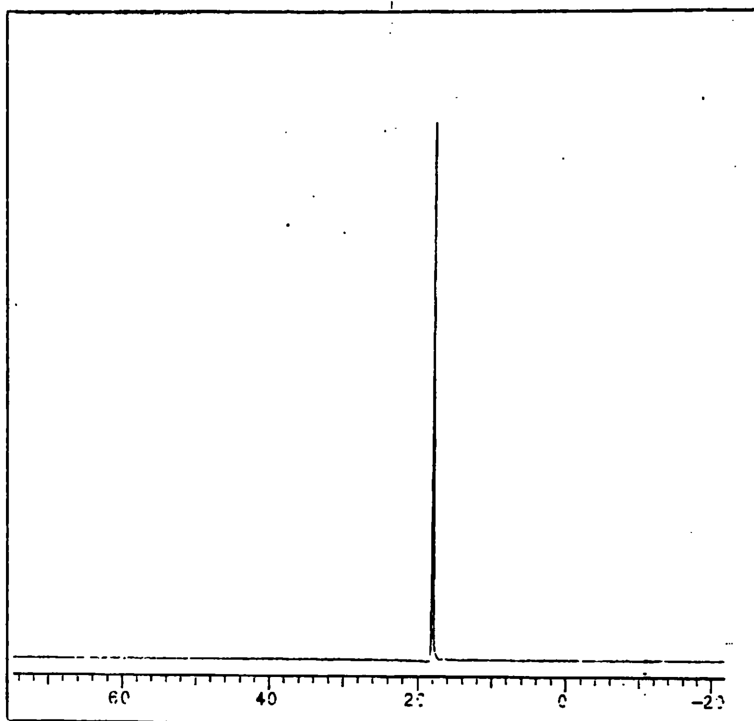
The preparation of **(161a)** and **(161b)** involved the initial treatment of **(2)** with phosphoryl chloride in triethyl phosphate. After the removal of unreacted phosphoryl chloride, the intermediate 3'-azido-3'-deoxythymidine 5'-phosphorodichloridate **(160)** was not isolated but was treated with an excess of the appropriate amine in DME to give the phosphorodiamidate derivatives of **(2)**.[27]



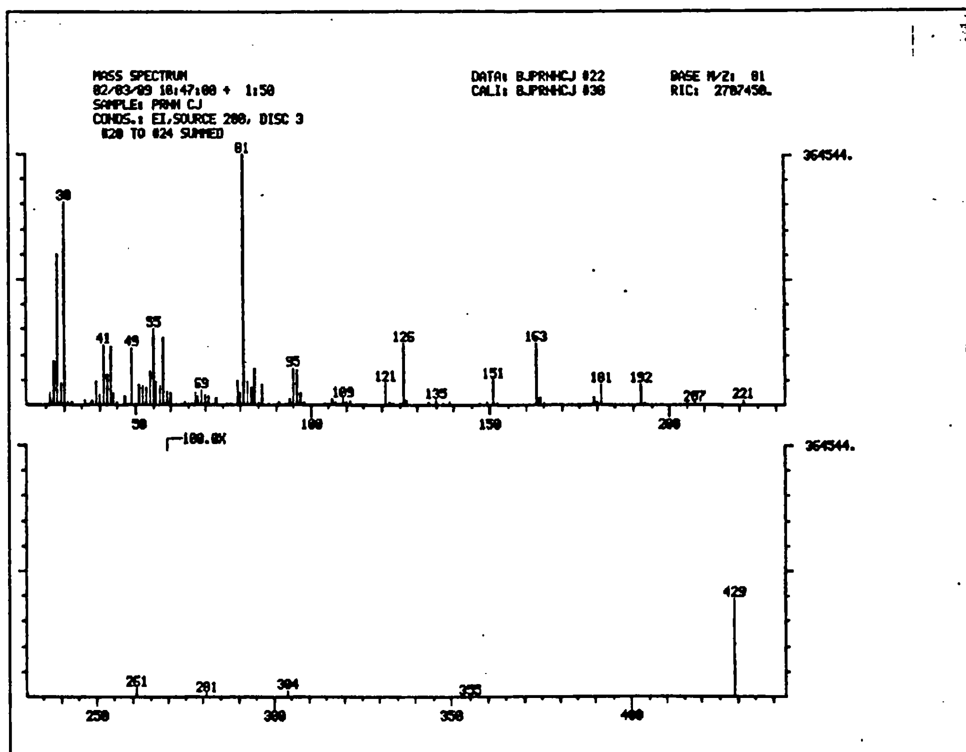


Compounds **(161a)** and **(161b)** were isolated in a 44% and 23% yield respectively by flash column chromatography. Analytical HPLC revealed a trace of compound **(2)** to be present, and the products were therefore subjected to preparative HPLC to obtain extremely pure samples for biological evaluation. The ^{31}P NMR of compounds **(161a)** and **(161b)** displayed a single resonance at approximately δ19 consistent with the proposed structures.[28] The ^{13}C NMR spectra were particularly informative with 2- and 3-bond phosphorus coupling being noted where appropriate. The ^{1}H NMR spectra and mass spectra for compounds **(161a)** and **(161b)** were also fully consistent with the proposed structures, the purity of the samples were confirmed by microanalysis.

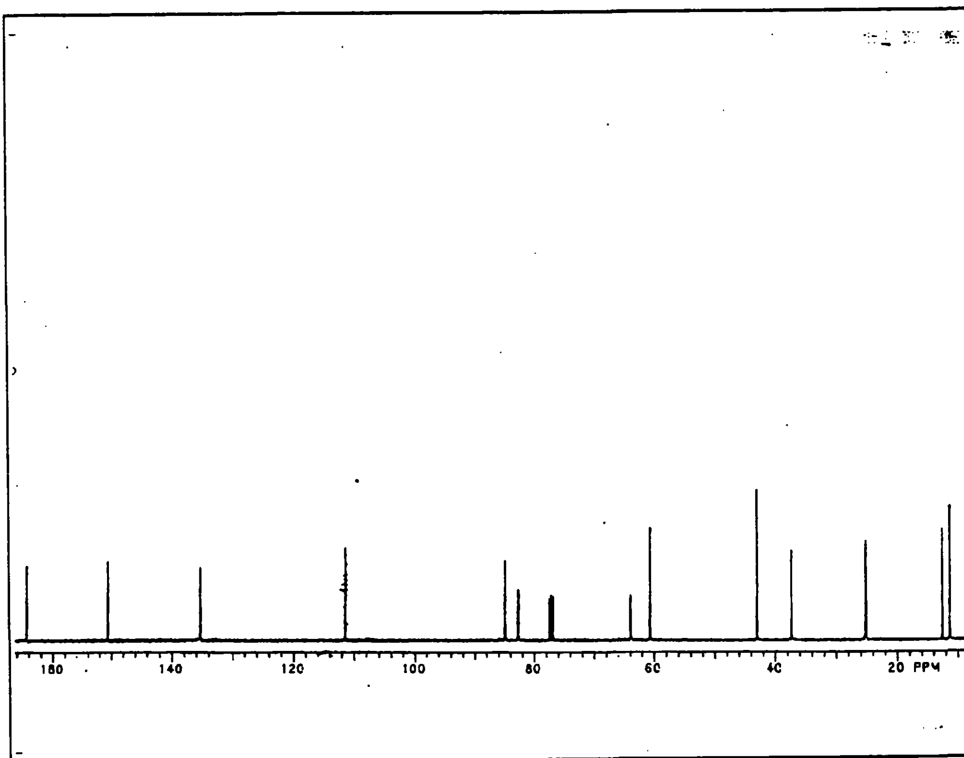
^{31}P NMR spectrum of (161a)



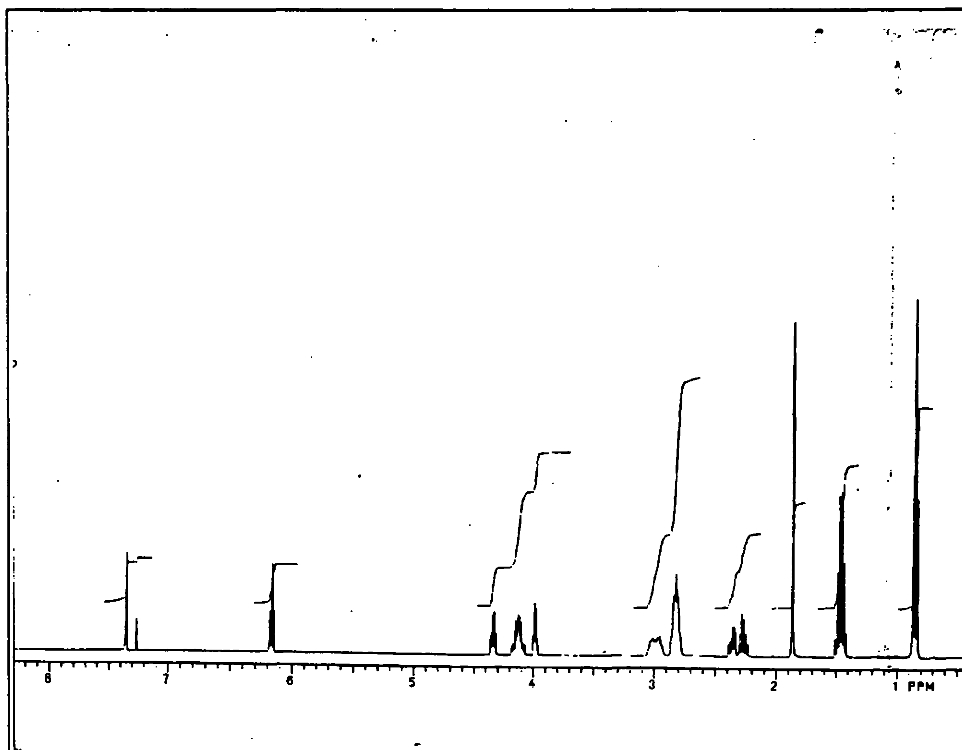
Mass spectrum of (161a)



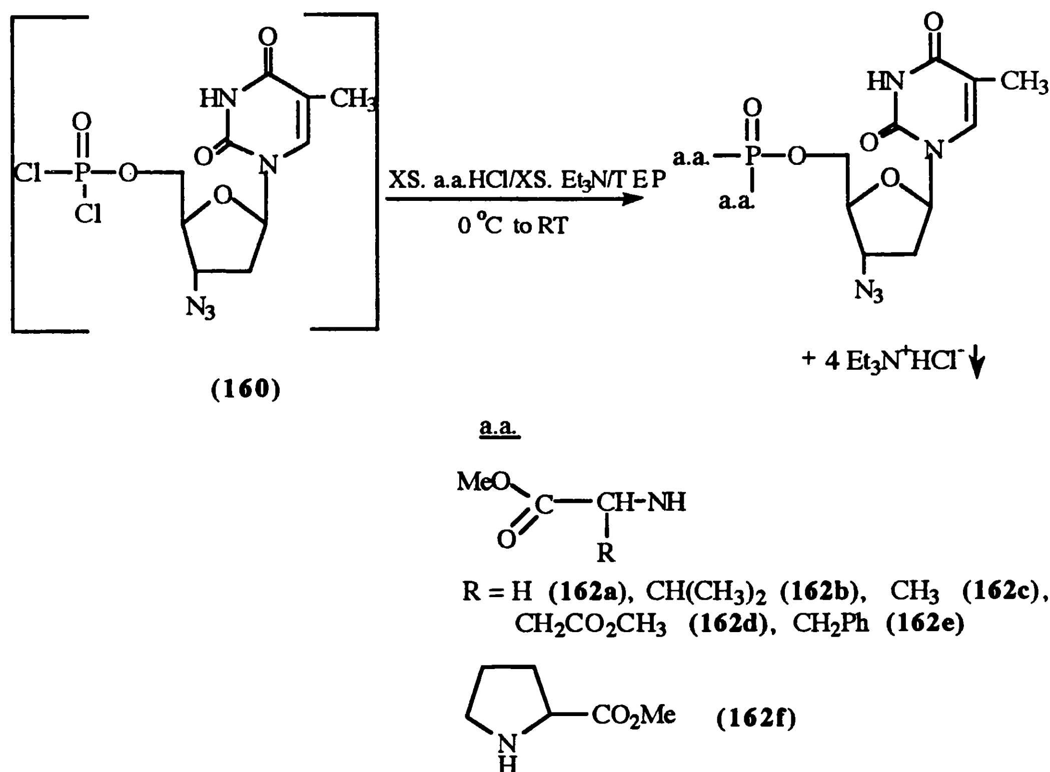
^{13}C **NMR spectrum of (161a)**



^{1}H **NMR spectrum of (161a)**



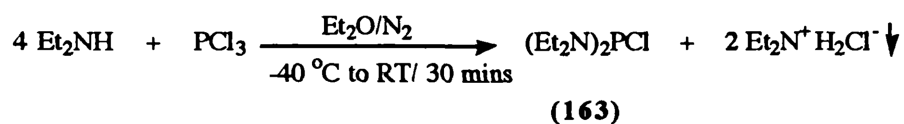
Compounds **(162a-f)** were also synthesized by the initial preparation of **(160)**, which was not isolated but was treated with an excess of the appropriate amino acid methyl ester hydrochloride and an excess of triethylamine in DME to give the phosphorodiamidate derivatives of **(2)**.



Compounds **(162a-f)** were isolated within a yield of 21% to 43% by flash column chromatography. Analytical HPLC revealed a trace of compound **(2)** to be present in all the products, and they were therefore subjected to preparative HPLC to obtain extremely pure samples. The ^{31}P NMR of compounds **(162a-f)** displayed a single resonance at approximately δ11 consistent with the proposed structures.[28] The ^{13}C NMR spectra were again particularly informative with 2- and 3-bond phosphorus coupling being noted where

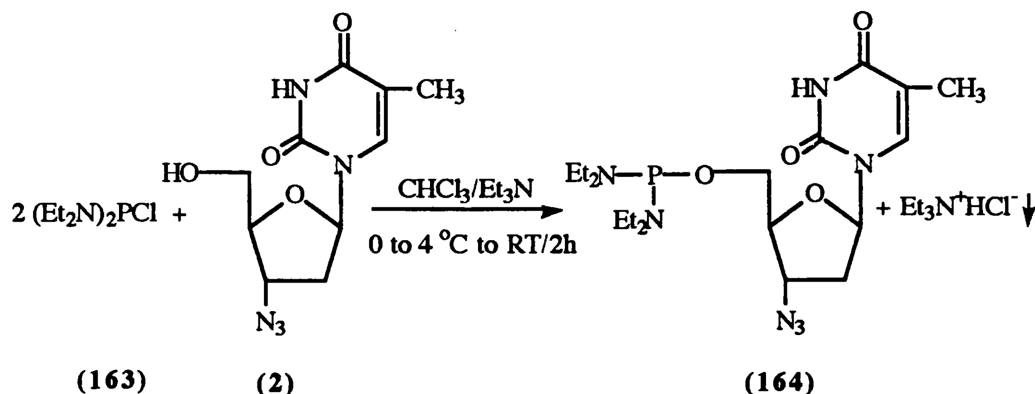
appropriate. The ^1H NMR spectra and mass spectra for compounds **(162a-f)** were also fully consistent with the proposed structures, the purity of the samples were confirmed by microanalysis.

In order to increase the yield of phosphorodiamidate derivatives of **(2)** and to try to reduce the time required to produce these derivatives, P(III) chemistry was investigated.[29,30] Bis-(diethylamino) phosphorochloridite, **(163)** was prepared by the reaction of phosphorus trichloride with four molar equivalents of diethylamine in diethyl ether. After 30 mins the mixture was filtered and the filtrate was evaporated under reduced pressure to give a clear yellowish oil in a 61% yield.



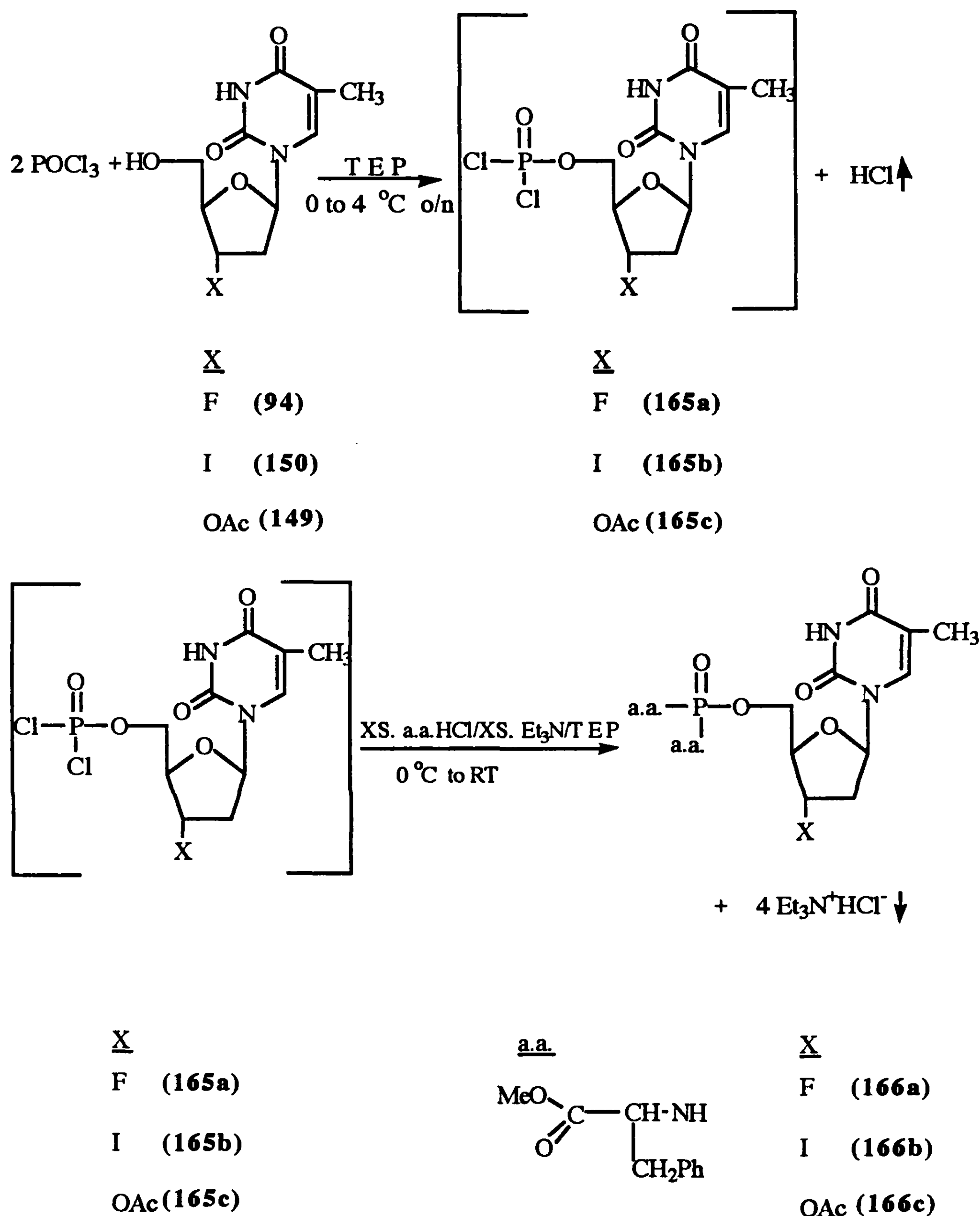
The ^{31}P NMR was one signal at δ156.01 which was consistent with the structure of **(163)**.[28] It is interesting to note the difference between the ^{31}P chemical shifts of P(III) and P(V) in that the equivalent P(V) compound, **(138)** has a chemical shift of δ24.52. The ^1H NMR and the ^{13}C NMR displayed signals for methyl and methylene moieties, which were fully consistent with the proposed structure.

The attempt to prepare 3'-azidothymidine-5'-bis-(diethylamino) phosphite, **(164)** involved the reaction of **(163)** with **(2)** in dry chloroform and triethylamine. The reaction was stirred at room temperature for 2 hours, then the reaction was left at 0 to 4 °C under nitrogen overnight.



The ^{31}P NMR indicated that probably only P(V) and pyrophosphate compounds were present in the crude product which was discarded. If this reaction is performed in the future, the phosphorylation and workup should all be done within a working day, otherwise from TLC observation the target P(III) compound probably decomposes. It is probable that this reaction would work with further careful attempts, allowing P=O, P=S and P=Se compounds to be prepared.

The preparation of phosphorodiamidate derivatives of **(94)**, **(149)** and **(150)** were prepared by an entirely analogous manner to the production of phosphorodiamidate derivatives of **(2)**. Thus the preparation of **(166a-c)** involved the initial treatment of the appropriate 3'-modified thymidine analogue with phosphoryl chloride in triethyl phosphate. After the removal of unreacted phosphoryl chloride and the by-product hydrogen chloride, the intermediate 3'-modified-thymidine phosphorodichloridates **(165a-c)** were not isolated but were treated with an excess of **(151c)** and an excess of triethylamine in DME to give the target compounds.



Compounds **(166a-c)** were isolated within 26% to 42% yield by flash column chromatography. The ^{31}P NMR of compounds **(162a-f)** displayed a single resonance at within δ10 to 12 which was consistent with the proposed structures.[28] The ^{13}C NMR spectra were again particularly informative with 2- and 3-bond phosphorus coupling being noted where appropriate. The ^{1}H NMR

spectra for compounds (**166a-c**) were also fully consistent with the proposed structures. Thus the above route to phosphorodiamidates seems to be applicable to a number of 3'-modified thymidine derivatives. In the future it may be possible to prepare a number of phosphorodiamidate derivatives of a variety of chemotherapeutic or non-chemotherapeutic nucleoside analogues, in order to enhance or introduce activity.

All of the compounds tested were active in the antiviral assay; the amino acid derivatives were particularly active. For the non amino acid compounds, the primary and secondary amines appeared to be equally efficacious. However, the latter compound (**161b**) showed toxicity towards uninfected cells; indeed, there appeared to be no sepqration between the effective and toxic doses (10 μM) for this compound. None of the other compounds in this series (**161a**) or (**162a-f**) displayed any toxicity towards uninfected cells, up to the maximum concentration tested (100 μM). The origins of the toxicity associated with the introduction of the secondary amine moiety remains unclear.

For the amino acid compounds there were major changes in activity with relatively minor changes in structure. In particular, non-polar amino acid side chains appeared to correlate, in most cases with the greatest antiviral activity. This is in agreement with earlier observations on phosphoramidate derivatives by other members in this department.[12] In conclusion, phosphate triester derivatives of (**2**) bearing two amino acid moieties possess marked anti-HIV activity in this assay system. Different amino acid groups confer changes in activity. A simple primary amine may substitute for an amino acid, with reduced efficiacy, and a secondary amine may substitute also, but here with marked toxicity.

Figure 20 Column graph of anti-HIV ED_{50} versus compound number

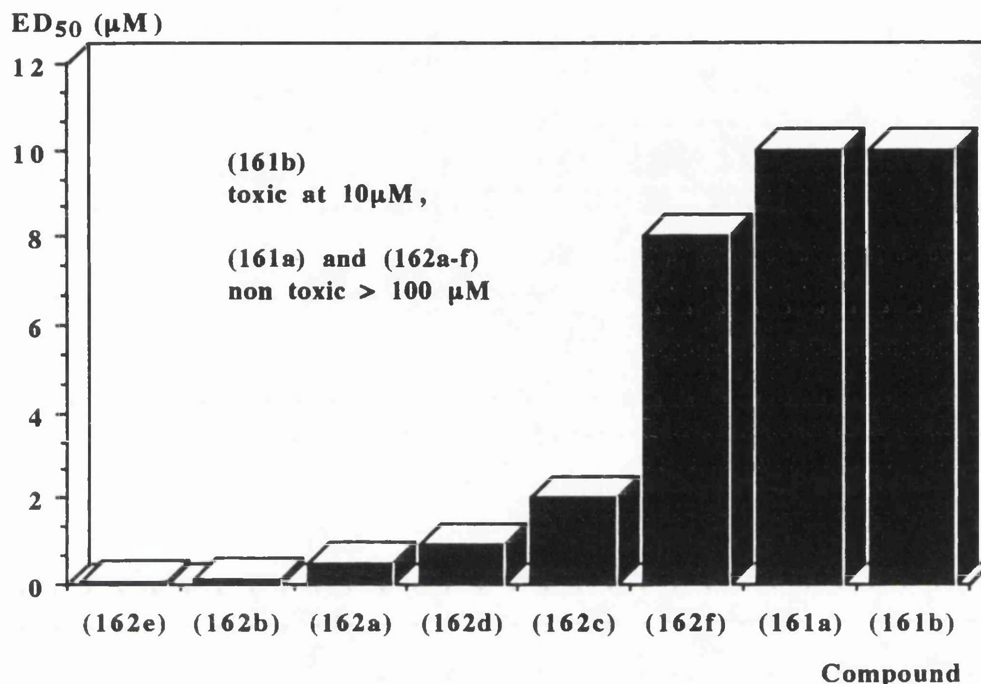


Figure 20 above illustrates the variation in the ED_{50} for the compounds tested against HIV. It is interesting to note that **(162f)**, a secondary amino acid being much less active than the primary amino acids but slightly more active than its corresponding secondary amine and also non-toxic at a 100 µM compared to the toxicity of **(161b)** at 10 µM. In an attempt to investigate the mechanism by which these derivatives exert their anti-HIV activity, a human plasma kinetic study was performed on compounds **(161a)**, **(162a)**, **(162d)** and **(162e)**.

The activity of the compounds which were in the human plasma kinetic study and the release of **(2)** in plasma at 360 hours did not correlate. This lack of direct correlation may have been expected, in that so many factors are involved. The timing and conditions of the biological system were very different from the human plasma system, the lipophilicty, structure and the rate of decay and decomposition products of the appropriate starting material seemed to vary greatly. There may be an inference that other structures, such as **(110)**, are

present in the decomposition mixture of the phosphorodiamidates, thus resulting in activity depending on the removal of the amidate moiety. Thus, this would explain the relative low activity of compound **(161a)** which can be clearly seen from figure 22 to be extremely stable in the human plasma system, even after 720 hours. However, compound **(162a)** decomposes quite rapidly in the human plasma system producing numerous decomposition products and is found to be the second most active phosphorodiamidate derivative of **(2)**. Compound **(162d)** and **(162e)** seem to decompose at a similar rate, however with some difference in the decomposition products formed. The difference in anti-HIV activity between **(162d)** and **(162e)** is sharply evident, this may be due to the difference in decomposition products or due to the difference in the expected lipophilicity of these compounds.

Figure 21 Human plasma kinetic study, HPLC measurement of the amount of AZT in the human plasma/phosphorodiamidate mixture at 360 hours. A plot of %AZT (9.7 retention time) versus compound number.

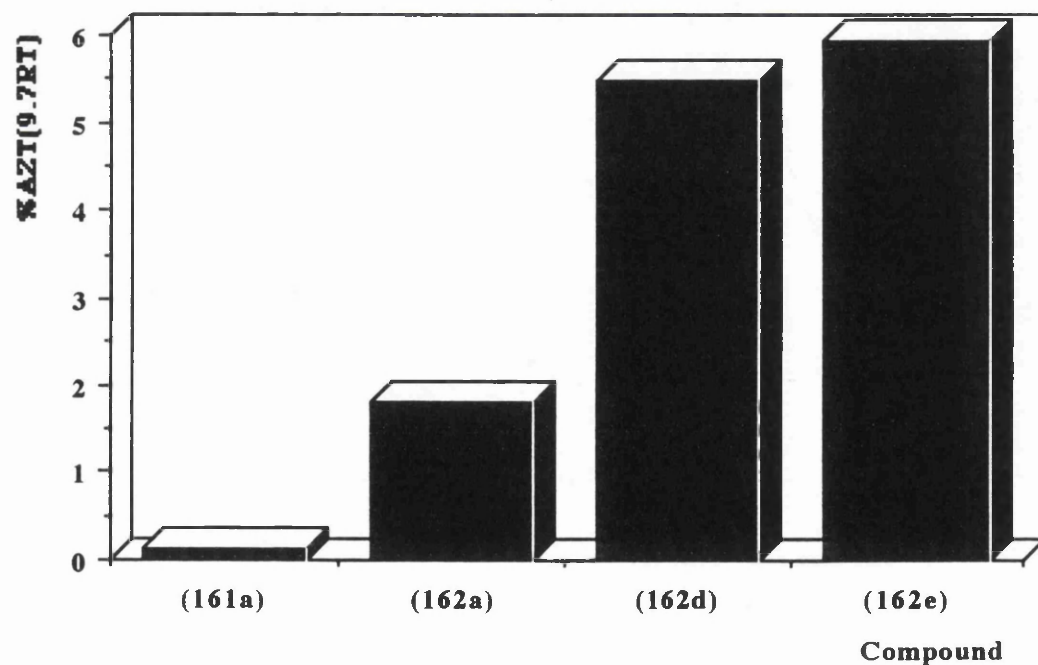


Figure 22 The % SM (Starting material, (161a)) in the human plasma, measured by ^{31}P NMR versus Time in hours

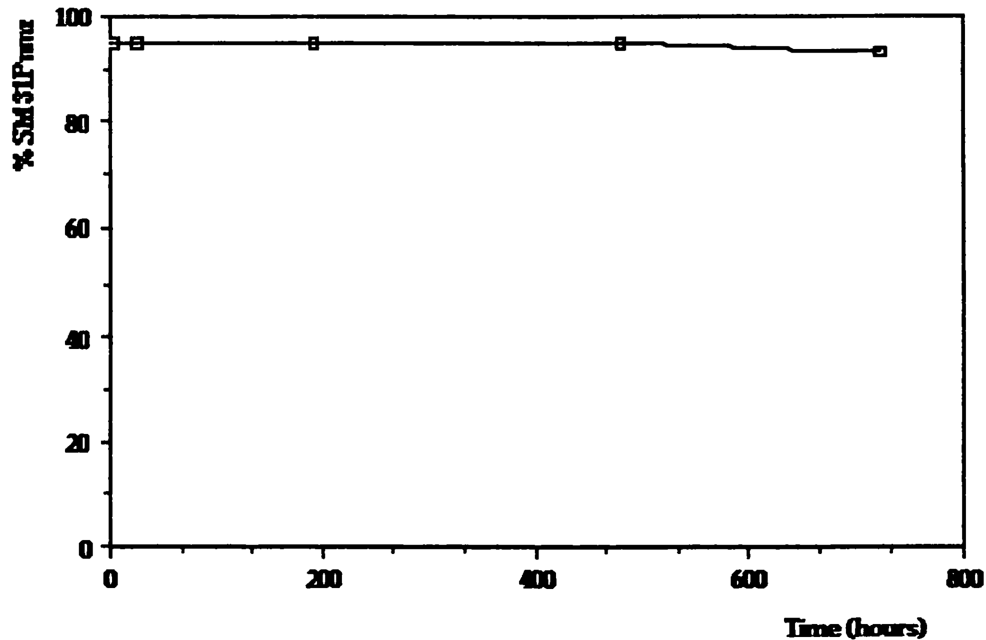


Figure 23 The % SM (Starting material, (162a)) in the human plasma, measured by ^{31}P NMR versus Time in hours

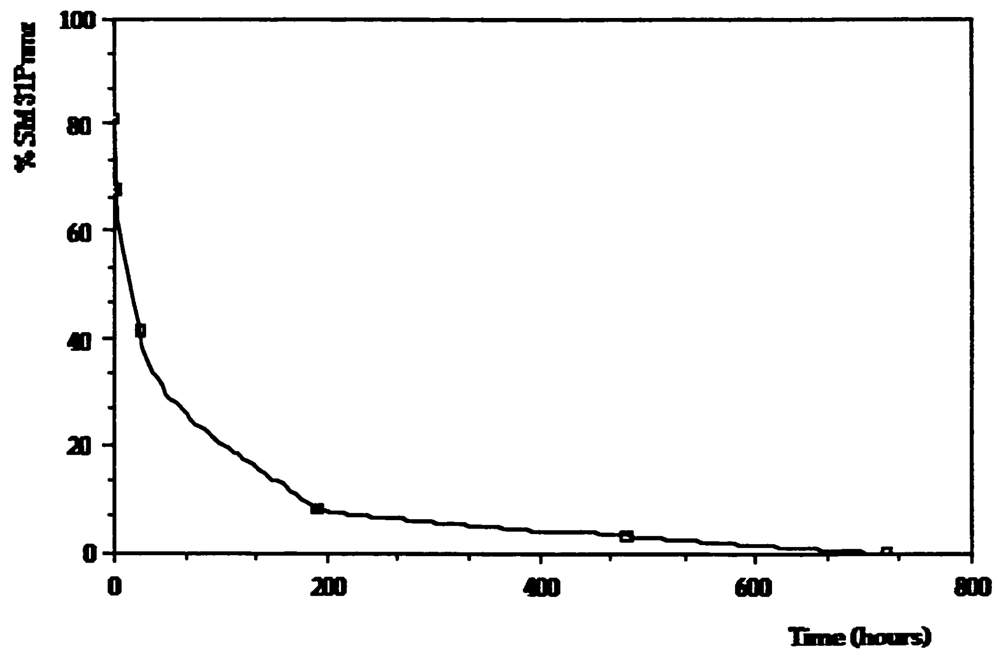


Figure 24 The % SM (Starting material, (162d)) in the human plasma, measured by ^{31}P NMR versus Time in hours

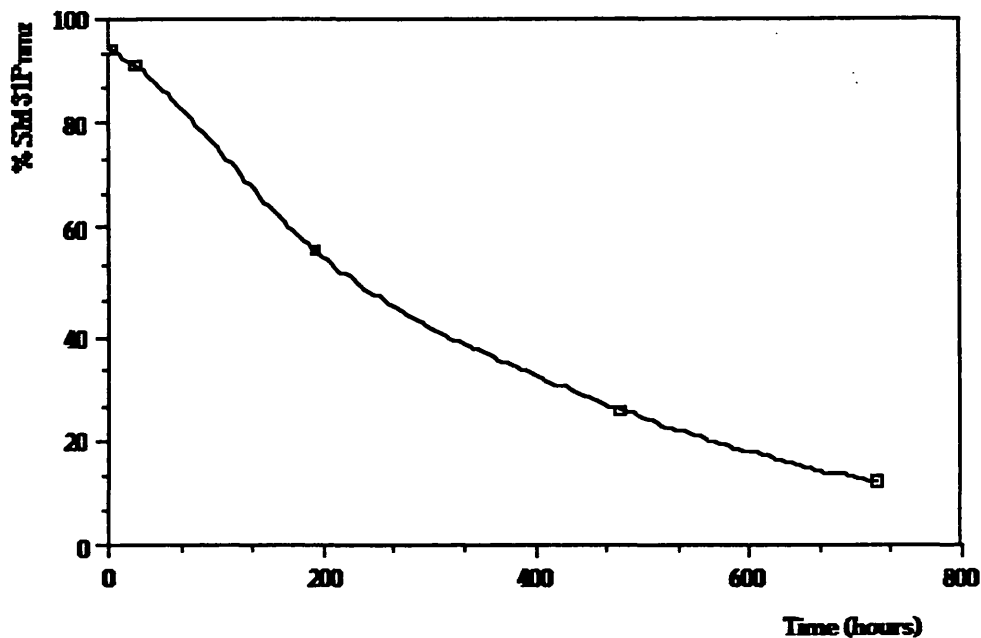
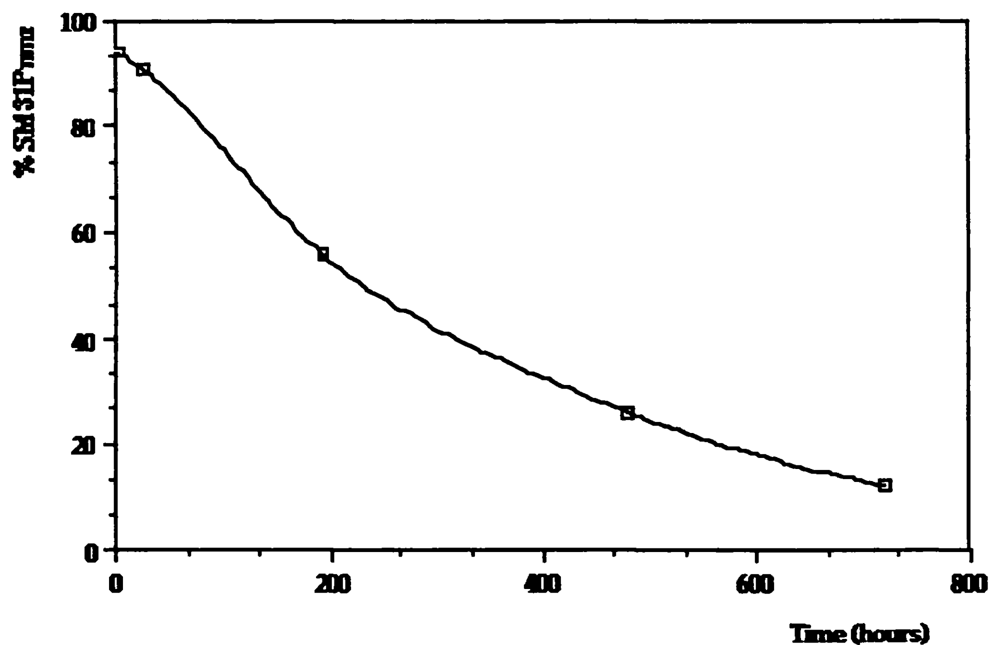


Figure 25 The % SM (Starting material, (162e)) in the human plasma, measured by ^{31}P NMR versus Time in hours



Thus the human plasma kinetic study seems to raise many questions and some results which provide an initial step to the understanding of the mechanism

of action of the phosphorodiamidate derivatives of **(2)**. It is probable that removal of the amidate moiety may be required for activation of the analogues. However the derivatives acting in themselves without decomposition may still remain a possibility.

4.3 Experimental

4.3.1 Preparation of L-amino acid methyl ester hydrochlorides

4.3.1.1

L-Valine methyl ester hydrochloride (151a)

Thionyl chloride (23.21 ml, 0.32 mol, 2 mol equ.) was added dropwise to methanol (51.8 ml, 8 mol equ.) at -10 °C over 45 mins. L-Valine (18.74 g, 0.16 mol) was added slowly and then the mixture was allowed to warm to room temperature and then heated at 40 °C overnight. The yellowish oil was evaporated under reduced pressure to yield a white solid. The white solid was dissolved in the minimum of warm methanol and then ten times the volume of diethyl ether was added to give the product as white crystals which were filtered and air-dried (21.14 g, 78%).

^{1}H NMR δ($CDCl_3$, 60 MHz, TMS) 8.65(3H, broad s, NH_3^+), 4.22(1H, broad s, *CH), 3.91(1H, m, *CHC$\underline{H}$), 3.72(3H, s, CO_2CH_3), 1.12(6H, d, CH_3, J=8.1 Hz)

4.3.1.2

L-Alanine methyl ester hydrochloride (151b)

Thionyl chloride (23.21 ml, 0.32 mol, 2 mol equ.) was added dropwise to methanol (51.8 ml, 8 mol equ.) at -10 °C over 45 mins. L-Alanine (14.25 g, 0.16 mol) was added slowly and then the mixture was allowed to warm to room temperature and then heated at 40 °C overnight. The yellowish oil was evaporated under reduced pressure to yield a cream coloured solid. The solid was dissolved in the minimum of warm methanol and then ten times the volume of diethyl ether was added to give the product as white crystals which were filtered and air-dried (17.76 g, 76%).

^1H NMR δ($CDCl_3$, 60 MHz, TMS) 8.51(3H, broad s, NH_3^+), 4.15(1H, broad s, *CH), 3.64(3H, s, CO_2CH_3), 1.61(3H, d, *CHC<u>H</u>$_3$, J= 7.2 Hz)

4.3.1.3

L-Phenylalanine methyl ester hydrochloride (151c)

Thionyl chloride (11.61 ml, 0.16 mol, 2 mol equ.) was added dropwise to methanol (25.9 ml, 8 mol equ.) at -10 °C over 45 mins. L-Phenylalanine (13.22 g, 0.08 mol) was added slowly and then the mixture was allowed to warm to room temperature and then heated at 40 °C overnight. The yellowish oil was evaporated under reduced pressure to yield a white solid. The white solid was dissolved in the minimum of warm methanol and then ten times the volume of diethyl ether was added to give the product as white crystals which were filtered and air-dried (13.97 g, 81%).

^{1}H NMR δ($CDCl_3$, 60 MHz, TMS) 8.12(3H, broad s, NH_3^+), 7.31(5H, m, phenyl protons), 4.36(1H, m, *CH), 3.74(1H, s, OCH_3), 3.39(2H, m, CH_2)

4.3.1.4

L-Aspartic acid methyl diester hydrochloride (151d)

Thionyl chloride (23.21 ml, 0.32 mol, 4 mol equ.) was added dropwise to methanol (51.8 ml, 16 mol equ.) at -10 °C over 45 mins. L-Aspartic acid (10.65 g, 0.08 mol) was added slowly and then the mixture was allowed to warm to room temperature and then heated at 40 °C overnight. The yellowish oil was evaporated under reduced pressure to yield a white solid. The white solid was dissolved in the minimum of warm methanol and then ten times the volume of diethyl ether was added to give the product as white crystals which were filtered and air-dried (11.86 g, 75%).

^{1}H NMR δ($CDCl_3$, 60 MHz, TMS) 8.79(3H, broad s, NH_3^+), 4.61(1H, m, *CH), 3.85(3H, s, CO_2CH_3), 3.74(3H, s, CO_2CH_3), 3.31(2H, m, CH_2)

4.3.1.5

L-Proline methyl ester hydrochloride (151e)

Thionyl chloride (23.21 ml, 0.32 mol, 4 mol equ.) was added dropwise to methanol (51.8 ml, 16 mol equ.) at -10 °C over 45 mins. L-Proline (18.42 g, 0.16 mol) was added slowly and then the mixture was allowed to warm to room temperature and then heated at 40 °C overnight. The yellowish oil was

evaporated under reduced pressure to yield a white gum. The white gum was triturated in ether and allowed to stand at 0 to 4 ^oC for 3 days to give the product as a white solid (20.68 g, 78%).

^1H NMR δ($CDCl_3$, 60 MHz, TMS) 8.31(2H, broad s, NH_2^+), 4.26(1H, bs, *CH), 3.53(3H, s, CO_2CH_3), 3.12(2H, bs, CH_2NH), 2.37(4H, bs, CH_2CH_2)

4.3.2 Preparation of nucleoside analogues

4.3.2.1

5'-O-Tritylthymidine (152)

Trityl chloride (12.70 g, 0.0454 mol) was added to a solution of thymidine (10.0 g, 0.0413 mol) in pyridine (100 ml) and the mixture was refluxed for 6 hours. The solution was then cooled to ambient temperature and poured into well stirred ice-water (1000 ml). The water washed gummy material was dissolved in ethyl acetate and dried over magnesium sulphate. After filtration, the solution was concentrated to a glass-like solid. The solid was recrystallised from benzene to give a white crystalline solid (16.64 g, 83%).

^1H NMR δ($CDCl_3$, 200 MHz, $CDCl_3$) 9.86(1H, bs, NH), 7.47(1H, s, H6), 7.19(15H, m, Ph), 6.28(1H, t, H1'), 4.55(1H, m, 3'OH), 4.26(1H, m, H4'), 3.72(2H, m, H5'), 3.47(1H, m, H3'), 2.40 to 2.10(2H, m, H2'), 1.65(3H, s, $5CH_3$)

^{13}C NMR δ($CDCl_3$, 100 MHz, $CDCl_3$) 164.53(C2), 150.72(C4), 143.69 (Ph), 135.58(C6), 128.32(Ph), 128.14(Ph), 127.37(Ph), 111.48(C5),

86.82($Ph_3\underline{C}$), 85.09(C1'), 83.46(C4'), 64.36(C5'), 60.27(C3'), 37.41(C2'), 12.42($5CH_3$)

4.3.2.2

3'-O-Methanesulphonyl-5'-O-tritylthymidine (153)

Methanesulphonyl chloride (5.58 ml, 0.0721 mol) was added to a solution of **(152)** (10g, 0.0206 mol) in pyridine (100 ml), and the mixture kept at 0 °C overnight with exclusion of moisture. Ice-water (1 ml) was then added and after 1 hour at 0 °C the mixture was poured into ice-water (1000 ml) with vigorous stirring. The precipitate of **(153)** was filtered off and well washed with water. The solid was dissolved in chloroform and dried over magnesium sulphate. Filtration and evaporation of the filtrate under reduced pressure yielded a yellowish oil. Diethyl ether was added to the oil to yield an off-white solid (10.32 g, 89%).

^{1}H NMR δ($CDCl_3$, 200 MHz, $CDCl_3$) 8.91(1H, bs, NH), 7.31(1H, s, H6), 7.19(15H, m, Ph), 6.28(1H, t, H1'), 5.38(1H, m, H3'), 4.30 to 3.42 (3H, m, H4', H5'), 3.12(3H, s, CH_3SO_2), 2.40 to 2.10(2H, m, H2'), 1.89(3H, s, $5CH_3$)

^{13}C NMR δ($CDCl_3$, 100 MHz, $CDCl_3$) 164.48(C2), 150.76(C4), 143.72 (Ph), 135.41(C6), 128.33(Ph), 128.18(Ph), 127.42(Ph), 111.39(C5), 87.04($Ph_3\underline{C}$), 84.89(C1'), 83.63(C4'), 72.28(C3'), 64.42(C5'), 37.46(CH_3SO_2), 37.12(C2'), 12.39($5CH_3$)

4.3.2.3

3'-Azido-3'-deoxythymidine (2)

Method (1)

Compound **(153)** (5.00 g, 8.87 mmol) in 1,5-diazabicyclo [5.4.0] undec-7-ene (8 ml) was left standing overnight at 37 °C. The solution was evaporated to dryness under reduced pressure and washed with cold water. The solid product was recrystallised from methanol to give a white crystalline solid of **(154)** (3.71 g 92%). This product was used without further purification in succeeding transformations.

Sodium azide (2.83 g, 0.0435 mol) in dimethylformamide was added to a vigorously stirred solution of **(154)** (3.50 g, 7.25 mmol) in dimethylformamide (50 ml) at room temperature. The mixture was refluxed for 6 hours, allowed to cool to ambient temperature and then poured into ice-water (800 ml) with vigorous stirring. The solid was collected and well washed with water. The solid was then suspended in 80% glacial acetic acid (50 ml) and refluxed for 40 minutes. The solvent was removed under reduced pressure and the residue was shaken several times with ether. The ether insoluble material was chromatographed with 5% methanol in chloroform as eluant. **(2)** (1.22 g, 63%; from **(154)**) was isolated as an off-white solid by pooling and evaporation of appropriate fractions. mpt 118 to 120 °C

^{1}H NMR δ($CDCl_3$, 200 MHz, $CDCl_3$) 8.43(1H, bs, NH), 7.35(1H, s, H6), 6.07(1H, t, H1'), 4.26(1H, m, H3'), 4.12 to 3.65(3H, m, H4', H5'), 2.69(1H, bs, 5'OH), 2.53 to 2.31 (3H, m, H2', 5'OH), 1.90(3H, s, $5CH_3$)

^{13}C NMR δ($CDCl_3$, 100 MHz, $CDCl_3$) 164.53(C2), 150.61(C4), 136.45(C6), 111.06(C5), 85.03(C4'), 83.14(C1'), 64.89(C5'), 60.27(C3'), 37.41(C2'), 12.33($5CH_3$)

Method (2)

To a cold solution of 1-(2'-deoxy-5'-O-trityl-β-D-lyxosyl) thymine **(155)** (5 g, 0.0103 mol) in pyridine (28 ml) was added methanesulfonyl chloride (2.3 ml). The mixture was stirred at 0 to 4 °C for 16 hours. The amber solution was allowed to reach room temperature and stirred for 3 hours. The reaction mixture was again cooled to 0 °C, treated with ice-water (*ca.* 1 ml), refrigerated for an additional hour, and finally poured slowly, with stirring into ice-water (800 ml). After 1 hour of stirring the product 1-(2'-deoxy-3')-mesyl-5'-O-trityl-β-D-lyxosyl) thymine **(156)** (5.11 g, 88%) was collected, air-dried and used without further purification for succeeding transformations.

A solution of **(156)** (5.00 g, 8.88 mmol) in DMF (30 ml) which contained sodium azide (2 g) was stirred for 34 hours at 100 °C under an atmosphere of nitrogen. The reaction was allowed to cool to ambient temperature and poured into ice-water (900 ml) and the product was suspended in 80% glacial acetic acid and heated at 80 °C for 40 mins. The product was evaporated under reduced pressure and the residue was shaken several times with diethyl ether. The ether insoluble material was purified by chromatography with 5% methanol in chloroform as eluant. Compound **(2)** (1.8 g, 76%; from **(156)**) was isolated as a white solid by pooling and evaporation of appropriate fractions. The product compared well with an authentic sample, on investigation by analytical thin layer chromatography.

4.3.2.4

3'-O-Acetylthymidine (149)

Acetic anhydride (6.32 g, 0.0619 mol) was added at ambient temperature to a solution of (**152**) (6.00 g, 0.0124 mol) in pyridine (100 ml) and the mixture was stirred overnight and then poured into ice-water (1000 ml) with vigorous stirring. The precipitate was filtered, suspended in 80% acetic acid (50 ml) and heated to 80 °C for 40 minutes. The solvent was then removed under reduced pressure and the residue was purified by chromatography with 5% methanol in chloroform as eluant. Pooling and evaporation of appropriate fractions gave the product as a yellowish solid, which was recrystallised from acetone-petroleum ether (bpt 40 to 60 °C), to give a white crystalline solid (2.86 g, 81%).

^{1}H NMR δ($CDCl_3$, 200 MHz, $CDCl_3$) 8.91(1H, bs, NH), 7.42(1H, s, H6), 6.33(1H, t, H1'), 5.30(1H, m, H3'), 4.29 to 3.58(3H, m, H4', H5'), 2.69(1H, bs, 5'OH), 2.40 to 2.10 (2H, m, H2'), 2.09(3H, s, CH_3CO), 1.88(3H, s, $5CH_3$)

^{13}C NMR δ($CDCl_3$, 100 MHz, $CDCl_3$) 170.67($CH_3\underline{C}O$), 163.74(C2), 150.31(C4), 135.28(C6), 111.42(C5), 85.98(C4'), 85.82(C1'), 73.56(C3'), 64.58(C5'), 37.36(C2'), 21.07($\underline{C}H_3CO$), 12.45($5CH_3$)

4.3.2.5

3'-O-Deoxy-3'-O-iodothymidine (150)

A solution of (**153**) (5 g, 8.87 mmol) and sodium iodide (2.66 g) in anhydrous acetone (50 ml) was heated at 100 °C for 2 hours. The mixture was

filtered and the filtrate taken to dryness under reduced pressure, water (100 ml) was added to the residue, and the mixture shaken vigorously. The yellow amorphous solid was collected, well washed with water and suspended in 80% glacial acetic acid (50 ml) and refluxed for 40 minutes. The product was evaporated under reduced pressure and the residue was shaken several times with ether to remove triphenyl methanol. The ether insoluble residue was chromatographed with 5% methanol in chloroform as eluant, pooling and evaporation of appropriate fractions yielded **(150)** as a white crystalline solid (1.28 g, 39%).

^{1}H NMR δ(CD_3OD, 200 MHz, CD_3OD) 7.65(1H, s, H6), 5.89(1H, t, H1'), 4.32(1H, m, H4'), 4.11(2H, m, H5'), 2.93 to 2.24(3H, m, H2', H3'), 1.73(3H, s, $5CH_3$)

^{13}C NMR δ(CD_3OD, 100 MHz, CD_3OD) 164.82(C2), 150.62(C4), 136.45(C6), 110.18(C5), 89.76(C1'), 85.15(C4'), 58.62(C5'), 44.49(C2'), 12.41($5CH_3$), 11.74(C3')

EI MS m/e 352(M^+,3%), 227(M^+ - $C_5H_6N_2O_2$(base), 67), 126 (thymine, 83), 99(bp)

4.3.3 Phosphorodiamidate derivatives of (2)

4.3.3.1

3'-Azido-3'-deoxythymidine-5'-bis-(n-propylamino) phosphate (161a)

Compound **(2)** (0.30 g, 1.14 mmol) was added to a solution of phosphoryl chloride (0.21 ml, 2.25 mmol) in anhydrous triethyl phosphate (1 ml), with

stirring at 0 °C. After 17 hours, the solution was subjected to high vacuum, with stirring, at ambient temperature. Anhydrous dimethoxyethane (4 ml) was added, the mixture was cooled to 0 °C, followed by the addition of n-propylamine (530 ml 0.38 g, 6.45 mmol) with vigorous stirring. After stirring in the cold for 3 days, the mixture was filtered and the precipitate washed with dimethoxyethane (30 ml) and diethyl ether (2 ml). The combined filtrate and washings were evaporated to *ca.* 1 ml under reduced pressure. This solution was dissolved in chloroform (10 ml) and added dropwise with vigorous stirring to petroleum ether (300 ml, bpt 30 to 40 °C). After cooling to 0 °C overnight, the solvent was decanted off, and the residue purified by flash column chromatography on silica, using 5% methanol in chloroform as eluant. Pooling and rotary evaporation of appropriate fractions gave the crude product (0.21 g, 44%). Analytical HPLC revealed a trace of compound **(2)** to be present, and the product was therefore subjected to preparative HPLC using a Spherisorb CN 5 μm column and an eluant mixture of water (A)/5% water in acetonitrile (B), with a linear gradient from 5% (B) to 90% (B), the product was isolated as a white gum.

^{1}H NMR δ($CDCl_3$, 200 MHz, $CDCl_3$) 10.37(1H, bs, NH), 7.35(1H, s, H6), 6.16(1H, t, H1'), 4.33(1H, m, H4'), 4.13(2H, m, H5'), 3.99(1H, m, H3'), 2.96(2H, bs, NH), 2.82(4H, m, CH_2N), 2.32(2H, m, H2'), 1.87(3H, s, $5CH_3$), 1.45(4H, m, $CH_3\underline{CH_2}$), 0.85(6H, m, CH_3)

^{31}P NMR δ($CDCl_3$, 160 MHz, H_3PO_4) 18.01

^{13}C NMR δ($CDCl_3$, 100 MHz, $CDCl_3$) 164.29(C2), 150.65(C4), 135.30(C6), 111.33(C5), 84.84(C1'), 82.70(d, C4', J=7.7 Hz), 63.93(d, C5', J=3.9 Hz), 60.64(C3'), 43.01(d, CH_2NH, J=4.6 Hz), 37.13(C2'), 25.07(d, $CH_3\underline{C}H_2$, J=6.2 Hz), 12.56($5CH_3$), 11.24(CH_3)

EI MS m/e 429(M^+, 0.4%), 355(<0.1), 304(<0.1), 261(4), 163(24), 126(24), 81(bp), 42(13), 30(81)

Found C 43.93%, H 6.43, N (16.27);
$C_{16}H_{28}N_7O_5P.[H_2O]_{0.5}$ requires C 43.83, H 6.67, N 22.36.

4.3.3.2

3'-Azido-3'-deoxythymidine-5'-bis-(diethylamino) phosphate (161b)

Compound **(2)** (0.30 g, 1.14 mmol) was added to a solution of phosphoryl chloride (0.21 ml, 2.25 mmol) in anhydrous triethyl phosphate (1 ml), with stirring at 0 °C. After 17 hours, the solution was subjected to high vacuum, with stirring, at ambient temperature. Anhydrous dimethoxyethane (4 ml) was added, the mixture was cooled to 0 °C, followed by the addition of diethylamine (660 ml, 5.6 mol equ.) with vigorous stirring. After stirring in the cold for 2 days, the mixture was filtered and the precipitate washed with dimethoxyethane (30 ml) and diethyl ether (2 ml). The combined filtrate and washings were evaporated to *ca.* 1 ml under reduced pressure. This solution was dissolved in chloroform (10 ml) and added dropwise with vigorous stirring to petroleum ether (300 ml, bpt 30 to 40 °C). After cooling to 0 °C overnight, the solvent was decanted off, and the residue purified by flash column chromatography on silica, using 5% methanol in chloroform as eluant. Pooling and rotary evaporation of appropriate fractions gave the crude product (0.12 g, 23%). Analytical HPLC revealed a trace of compound **(2)** to be present, and the product was therefore subjected to preparative HPLC using a Spherisorb CN 5 μm column and an

eluant mixture of water (A)/5% water in acetonitrile (B), with a linear gradient from 5% (B) to 90% (B), the product was isolated as a white gum.

^{1}H NMR δ($CDCl_3$, 200 MHz, $CDCl_3$) 10.37(1H, bs, NH), 7.30(1H, s, H6), 6.18(1H, t, H1'), 4.32(1H, m, H4'), 4.03(3H, m, H3', H5'), 3.00(8H, m, CH_2N), 2.35(2H, m, H2'), 1.84(3H, s, $5CH_3$), 1.04(12H, m, CH_3)

^{31}P NMR δ($CDCl_3$, 160 MHz, H_3PO_4) 19.54

^{13}C NMR δ($CDCl_3$, 100 MHz, $CDCl_3$) 164.31(C2), 150.67(C4), 135.17(C6), 111.41(C5), 84.80(C1'), 82.63(d, C4', J=7.8 Hz), 63.73(d, C5', J=3.4 Hz), 60.79(C3'), 41.86(d, CH_2, J=4.4 Hz), 37.32(C2'), 14.43(d, CH_3, J=5.7 Hz), 12.71($5CH_3$)

FAB MS m/e 457(M^+, 4%), 429(1), 428(<0.1), 332(0.1), 356(0.2), 251(0.6), 125(0.6), 124(0.4), 72(bp)

Found C 45.06%, H 7.11 N (14.08);
$C_{18}H_{32}N_7O_5P.H_2O$ requires C 45.47 , H 7.21, N 20.62.

4.3.3.3

3'-Azido-3'-deoxythymidine-5'-bis-(methoxyglycinyl) phosphate (162a)

Compound **(2)** (0.30 g, 1.14 mmol) was added to a solution of phosphoryl chloride (0.21 ml, 2.25 mmol) in anhydrous triethyl phosphate (1 ml), with stirring at 0 °C. After 17 hours, the solution was subjected to high vacuum, with stirring, at ambient temperature. Anhydrous dimethoxyethane (4 ml) was added, followed by L-glycine methyl ester hydrochloride (0.81 g, 6.44 mmol). The mixture was cooled to 0 °C and triethylamine (1.60 ml, 11.5 mmol) added dropwise with vigorous stirring. After stirring in the cold for 5 days, the mixture

was filtered and the precipitate washed with dimethoxyethane (30 ml) and diethyl ether (2ml). The combined filtrate and washings were evaporated to *ca.* 1ml under reduced pressure. This solution was dissolved in chloroform (10 ml) and added dropwise with vigorous stirring to petroleum ether (500 ml, bpt 30 to 40 °C). After cooling to 0 °C overnight, the solvent was decanted off, and the residue purified by flash column chromatography on silica, using 5% methanol in chloroform as eluant. Pooling and rotary evaporation of appropriate fractions gave the crude product (0.18 g, 32%). Analytical HPLC revealed a trace of compound (**2**) to be present, and the product was therefore subjected to preparative HPLC using a Spherisorb CN 5 µm column and a eluant mixture of water (A)/5% water in acetonitrile (B), with a linear gradient from 5% (B) to 90% (B), the product was isolated as a colourless gum.

^{1}H NMR δ($CDCl_3$, 200 MHz, $CDCl_3$) 9.61(1H, bs, NH), 7.35(1H, s, H6), 6.14(1H, t, H1'), 4.39(1H, m, H4'), 4.24(2H, m, H5'), 3.99(1H, m, H3'), 3.80 to 3.60(12H, m, OMe, CH_2N, NH), 2.39(2H, m, H2'), 1.91(3H, s, $5CH_3$)

^{31}P NMR δ($CDCl_3$, 160 MHz, H_3PO_4) 12.53

^{13}C NMR δ($CDCl_3$, 100 MHz, $CDCl_3$) 172.91(d, C=O, J=4.6 Hz), 163.91(C2), 150.42(C4), 135.64(C6), 111.56(C5), 85.28(C1'), 82.51(d, C4', J=7.4 Hz), 64.50(d, C5', J=4.5 Hz), 60.24(C3'), 52.43(OCH_3), 42.55(d, CH_2, J=6.1 Hz), 37.12(C2'), 12.40($5CH_3$)

EI MS m/e 490(MH^+, 1.2%), 489(M^+, 0.4), 429(2), 355(5), 312(1), 223(42), 166(86), 42(17), 30(bp)

Found C 37.98%, H 4.65, N (12.39);
$C_{16}H_{24}N_7O_9P.H_2O$ requires C 37.88 , H 5.16, N 19.32.

4.3.3.4

3'-Azido-3'-deoxythymidine-5'-bis-(methoxyvalinyl) phosphate (162b)

Compound (**2**) (0.30 g, 1.14 mmol) was added to a solution of phosphoryl chloride (0.21 ml, 2.25 mmol) in anhydrous triethyl phosphate (1 ml), with stirring at 0 °C. After 17 hours, the solution was subjected to high vacuum, with stirring, at ambient temperature. Anhydrous dimethoxyethane (4 ml) was added, followed by L-valine methyl ester hydrochloride (1.08 g, 6.44 mmol). The mixture was cooled to 0 °C and triethylamine (1.60 ml, 11.5 mmol) added dropwise with vigorous stirring. After stirring in the cold for 4 days and at room temperature for 3 days, the mixture was filtered and the precipitate washed with dimethoxyethane (30 ml) and diethyl ether (2 ml). The combined filtrate and washings were evaporated to *ca.* 1 ml under reduced pressure. This solution was dissolved in chloroform (10 ml) and added dropwise with vigorous stirring to petroleun ether (300 ml, bpt 30 to 40 °C). After cooling to 0 °C overnight, the solvent was decanted off, and the residue purified by flash column chromatography on silica, using 5% methanol in chloroform as eluant. Pooling and rotary evaporation of appropriate fractions gave the crude product (0.18 g, 28%). Analytical HPLC revealed a trace of compound (**2**) to be present, and the product was therefore subjected to preparative HPLC using a Spherisorb CN 5 μm column and an eluant mixture of water (A)/5% water in acetonitrile (B), with a linear gradient from 5% (B) to 90% (B), the product was isolated as a white gum.

^{1}H NMR δ($CDCl_3$, 200 MHz, $CDCl_3$) 9.86(1H, bs, NH), 7.36(1H, s, H6), 6.16(1H, t, H1'), 4.32(1H, m, H4'), 4.19(2H, m, H5'), 3.97(1H, m, H3'), 3.73(8H, s, OMe, *CH), 3.46(2H, m, NH), 2.4 to 2.1(4H, m, H2', iPrCH), 1.91(3H, s, $5CH_3$), 0.90 (12H, m, iPrCH_3)

^{31}P NMR δ($CDCl_3$, 160 MHz, H_3PO_4) 12.48

^{13}C NMR δ($CDCl_3$, 100 MHz, $CDCl_3$) 173.91(d, C=O, J=8.5 Hz), 163.70(C2), 150.27(C4), 135.55(C6), 111.69(C5), 85.15(C1'), 82.44(d, C4', J=7.4 Hz), 64.56(d, C5', J=4.5 Hz), 60.20(C3'), 59.52(*CH), 52.24(OCH_3), 37.09(C2'), 31.92(CH), 19.07(CH_3), 17.37(CH_3), 12.46($5CH_3$)

FAB MS m/e 574(MH^+, 46%), 559(12), 544(37), 542(4), 531(20), 126(6), 81(bp)

Found C 44.98%, H 6.65, N (14.56);
$C_{22}H_{36}N_7O_9P.H_2O$ requires C 44.67 , H 6.47, N 16.57.

4.3.3.5

3'-Azido-3'-deoxythymidine-5'-bis-(methoxyalaninyl) phosphate (162c)

Compound **(2)** (0.30 g, 1.14 mmol) was added to a solution of phosphoryl chloride (0.21 ml, 2.25 mmol) in anhydrous triethyl phosphate (1 ml), with stirring at 0 °C. After 17 hours, the solution was subjected to high vacuum, with stirring, at ambient temperature. Anhydrous dimethoxyethane (4 ml) was added, followed by L-alanine methyl ester hydrochloride (0.9 g, 6.44 mmol). The mixture was cooled to 0 °C and triethylamine (1.60 ml, 11.5 mmol) added

dropwise with vigorous stirring. After stirring in the cold for 4 days and at room temperature for 3 days, the mixture was filtered and the precipitate washed with dimethoxyethane (30 ml) and diethyl ether (2 ml). The combined filtrate and washings were evaporated to *ca.* 1 ml under reduced pressure. This solution was dissolved in chloroform (10 ml) and added dropwise with vigorous stirring to petroleun ether (300 ml, bpt 30 to 40 °C). After cooling to 0 °C overnight, the solvent was decanted off, and the residue purified by flash column chromatography on silica, using 5% methanol in chloroform as eluant. Pooling and rotary evaporation of appropriate fractions gave the crude product (0.12 g, 21%). Analytical HPLC revealed a trace of compound **(2)** to be present, and the product was therefore subjected to preparative HPLC using a Spherisorb CN 5 µm column and an eluant mixture of water (A)/5% water in acetonitrile (B), with a linear gradient from 5% (B) to 90% (B), the product was isolated as a white gum.

^{1}H NMR δ($CDCl_3$, 200 MHz, $CDCl_3$) 8.87(1H, bs, NH), 7.34(1H, s, H6), 6.14(1H, t, H1'), 4.39(1H, m, H4'), 4.21(2H, m, H5'), 3.99(3H, m, H3', *CH), 3.74(6H, s, OMe), 3.47(2H, m, NH), 2.42(2H, m, H2'), 1.95(3H, s, $5CH_3$), 1.40 (6H, m, ala CH_3)

^{31}P NMR δ($CDCl_3$, 160 MHz, H_3PO_4) 10.45

^{13}C NMR δ($CDCl_3$, 100 MHz, $CDCl_3$) 174.78(d, C=O, J=6.6 Hz), 163.38(C2), 150.06(C4), 135.83(C6), 111.63(C5), 85.70(C1'), 82.52(d, C4', J=7.2 Hz), 64.25(d, C5', J=4.6 Hz), 60.07(C3'), 52.59(*CH), 50.01(OCH_3), 37.17(C2'), 21.11(CH_3), 12.45($5CH_3$)

EI MS m/e 517(M^+, 1%), 458(2), 250(2), 218(4), 181(6), 166(7), 126(24), 96(12), 81(bp)

Found C 42.07%, H 5.73, N (16.47);

$C_{18}H_{28}N_7O_9P$ requires C 41.78 , H 5.45, N 18.95.

4.3.3.6

3'-Azido-3'-deoxythymidine-5'-bis-(dimethoxyaspartyl) phosphate (162d)

Compound (**2**) (0.30 g, 1.14 mmol) was added to a solution of phosphoryl chloride (0.21 ml, 2.25 mmol) in anhydrous triethyl phosphate (1 ml), with stirring at 0 °C. After 17 hours, the solution was subjected to high vacuum, with stirring, at ambient temperature. Anhydrous dimethoxyethane (4 ml) was added, followed by L-aspartic acid dimethyl ester hydrochloride (1.28 g, 6.44 mmol). The mixture was cooled to 0 °C and triethylamine (1.60 ml, 11.5 mmol) added dropwise with vigorous stirring. After stirring in the cold for 3.5 days, the mixture was filtered and the precipitate washed with dimethoxyethane (30 ml) and diethyl ether (2 ml). The combined filtrate and washings were evaporated to *ca.* 1 ml under reduced pressure. This solution was dissolved in chloroform (10 ml) and added dropwise with vigorous stirring to petroleum ether (300 ml, bpt 30 to 40 °C). After cooling to 0 °C overnight, the solvent was decanted off, and the residue purified by flash column chromatography on silica, using 5% methanol in chloroform as eluant. Pooling and rotary evaporation of appropriate fractions gave the crude product (0.26 g, 37%). Analytical HPLC revealed a trace of compound (**2**) to be present, and the product was therefore subjected to preparative HPLC using a Spherisorb CN 5 μm column and an eluant mixture of water (A)/5% water in acetonitrile (B), with a linear gradient from 5% (B) to 90% (B), the product was isolated as a white gum.

^{1}H NMR δ($CDCl_3$, 200 MHz, $CDCl_3$) 8.58(1H, bs, NH), 7.27(1H, s, H6), 6.07(1H, t, H1'), 4.37(1H, m, H4'), 4.22(2H, m, H5'), 4.05(5H, m, H3', NH, *CH), 3.72(6H, s, OMe), 3.67(6H, s, OMe), 2.79(4H, m, CH_2), 2.35(2H, m, H2'), 1.86(3H, s, $5CH_3$)

^{31}P NMR δ($CDCl_3$, 160 MHz, H_3PO_4) 10.83

^{13}C NMR δ($CDCl_3$, 100 MHz, $CDCl_3$) 172.40(d, C=O, J=9.9 Hz), 171.31(C=O), 163.66(C2), 150.23(C4), 135.83(C6), 111.46(C5), 85.51(C1'), 82.44(d, C4', J=6.8 Hz), 64.51(d, C5', J=4.9 Hz), 60.09(C3'), 52.84(*CH), 52.09(OCH_3), 50.47(OCH_3), 38.35(d, CH_2, 7.2 Hz) 37.05(C2'), 12.34($5CH_3$)

EI MS m/e 634(MH^+, 54%), 603(22), 572(41), 128(11), 81(bp)

Found C 41.11%, H 5.33 N (12.77);

$C_{22}H_{32}N_7O_{13}P$ requires C 41.71 , H 5.09, N 15.48.

4.3.3.7

3'-Azido-3'-deoxythymidine-5'-bis-(methoxyphenylalaninyl) phosphate (162e)

Compound **(2)** (0.30 g, 1.14 mmol) was added to a solution of phosphoryl chloride (0.21 ml, 2.25 mmol) in anhydrous triethyl phosphate (1 ml), with stirring at 0 °C. After 17 hours, the solution was subjected to high vacuum, with stirring, at ambient temperature. Anhydrous dimethoxyethane (4 ml) was added, followed by L-phenylalanine methyl ester hydrochloride (1.39 g, 6.44 mmol). The mixture was cooled to 0 °C and triethylamine (1.60 ml, 11.5 mmol) added

dropwise with vigorous stirring. After stirring in the cold for 4 days, the mixture was filtered and the precipitate washed with dimethoxyethane (30 ml) and diethyl ether (2 ml). The combined filtrate and washings were evaporated to *ca.* 1 ml under reduced pressure. This solution was dissolved in chloroform (10 ml) and added dropwise with vigorous stirring to petroleum ether (300 ml, bpt 30 to 40 ^{o}C). After cooling to 0 ^{o}C overnight, the solvent was decanted off, and the residue purified by flash column chromatography on silica, using 5% methanol in chloroform as eluant. Pooling and rotary evaporation of appropriate fractions gave the crude product (0.31 g, 43%). Analytical HPLC revealed a trace of compound (**2**) to be present, and the product was therefore subjected to preparative HPLC using a Spherisorb CN 5 µm column and an eluant mixture of water (A)/5% water in acetonitrile (B), with a linear gradient from 5% (B) to 90% (B), the product was isolated as a white gum.

^{1}H NMR δ($CDCl_3$, 200 MHz, $CDCl_3$) 9.95(1H, bs, NH), 7.0 to 7.2(11H, m, Ph, H6), 6.06(1H, t, H1'), 4.07(1H, m, H4'), 3.97(2H, m, H5'), 3.81(3H, m, H3', *CH), 3.6 to 3.75(8H, s, OMe, NH), 2.83(4H, m, $PhCH_2$), 2.30(2H, m, H2'), 1.87(3H, s, $5CH_3$)

^{31}P NMR δ($CDCl_3$, 160 MHz, H_3PO_4) 10.48

^{13}C NMR δ($CDCl_3$, 100 MHz, $CDCl_3$) 173.91(d, C=O, J=5.2 Hz), 163.88(C2), 150.29(C4), 136.18(Ph C), 135.87(C6), 129.33 to 126.86(Ph C), 111.27(C5), 84.77(C1'), 82.08(d, C4', J=7.6 Hz), 64.36(d, C5', J=4.7 Hz), 60.30(C3'), 59.71(*CH), 52.14(OCH_3), 39.99 (d, CH_2, J=7.5 Hz), 37.16(C2'), 12.20($5CH_3$)

EI MS m/e 670(MH^+, 64%), 638(2), 610(3), 491(4), 180(57), 126(8), 81(bp)

Found C 51.74%, H 6.07; N (12.72);

$C_{30}H_{36}N_7O_9P.[H_2O]_{1.5}$ requires C 51.72 , H 5.64, N 14.07.

4.3.3.8

3'-Azido-3'-deoxythymidine-5'-bis-(methoxyprolinyl) phosphate (162f)

Compound (**2**) (0.30 g, 1.14 mmol) was added to a solution of phosphoryl chloride (0.21 ml, 2.25 mmol) in anhydrous triethyl phosphate (1 ml), with stirring at 0 °C. After 17 hours, the solution was subjected to high vacuum, with stirring, at ambient temperature. Anhydrous dimethoxyethane (4 ml) was added, followed by L-proline methyl ester hydrochloride (1.07 g, 6.44 mmol). The mixture was cooled to 0 °C and triethylamine (1.60 ml, 11.5 mmol) added dropwise with vigorous stirring. After stirring in the cold for 4 days, the mixture was filtered and the precipitate washed with dimethoxyethane (30 ml) and diethyl ether (2 ml). The combined filtrate and washings were evaporated to *ca.* 1 ml under reduced pressure. This solution was dissolved in chloroform (10 ml) and added dropwise with vigorous stirring to petroleum ether (300 ml, bpt 30 to 40 °C). After cooling to 0 °C overnight, the solvent was decanted off, and the residue purified by flash column chromatography on silica, using 5% methanol in chloroform as eluant. Pooling and rotary evaporation of appropriate fractions gave the crude product (0.22 g, 34%). Analytical HPLC revealed a trace of compound (**2**) to be present, and the product was therefore subjected to preparative HPLC using a Spherisorb CN 5 μm column and an eluant mixture of water (A)/5% water in acetonitrile (B), with a linear gradient from 5% (B) to 90% (B), the product was isolated as a white gum.

^{1}H NMR δ($CDCl_3$, 200 MHz, $CDCl_3$) 10.01(1H, bs, NH), 7.37(1H, s, H6), 6.12(1H, t, H1'), 4.38(1H, m, H4'), 4.20 to 3.81(5H, m, H5', H3', *CH), 3.48(6H, s, OMe), 3.09(4H, m, C$\underline{H}_2$NH), 2.42 to 2.29(10H, m, C$\underline{H}_2$C$\underline{H}_2$, H2'), 1.89(3H, s, 5CH_3)

^{31}P NMR δ($CDCl_3$, 160 MHz, H_3PO_4) 10.39

^{13}C NMR δ($CDCl_3$, 100 MHz, $CDCl_3$) 170.15(d, C=O, J=4.9 Hz), 163.84(C2), 150.46(C4), 136.08(C6), 111.28(C5), 84.61(C1'), 82.31(d, C4', J=7.2 Hz), 64.16(d, C5', J=4.9 Hz), 60.30(C3'), 59.92(*CH), 52.90(OCH_3), 46.22(d, CH_2, J=6.2 Hz), 37.35(C2'), 28.25(d, CH_2, 7.2Hz), 23.59(d, CH_2, 7.3Hz) 12.40(5CH_3)

4.3.4 Phosphite reaction with (2)

4.3.4.1

Bis-(diethylamino) phosphorochloridite (163)

Phosphorus trichloride (6.6 ml, 10.3 g, 0.075 mol) was dissolved in diethyl ether (40 ml). A solution of diethylamine (31 ml, 21.94 g, 0.3 mol) in diethyl ether (60 ml) was added dropwise at -40 °C under an atmosphere of dry nitrogen. The reaction mixture was allowed to warm to room temperature and stirred for 30 mins. The mixture was filtered and the filtrate was evaporated under reduced pressure to yield a clear yellowish oil (10.43 g, 61%).

^{1}H NMR δ($CDCl_3$, 200 MHz, $CDCl_3$) 3.4(2H, m, CH_2), 1.3(3H, t, CH_3)

^{31}P NMR δ($CDCl_3$, 160 MHz, H_3PO_4) 156.01

^{13}C NMR δ($CDCl_3$, 100 MHz, $CDCl_3$) 48.93(d, CH_2, J=5.7 Hz), 16.41 (d, CH_3, J=4.1 Hz)

4.3.4.2

Attempt to prepare 3'-azidothymidine-5'-bis-(diethylamino) phosphite (164)

Compound (**2**) (300 mg, 1.12 mmol) was dissolved in dry chloroform (40 ml) and triethylamine (390 µl, 0.28 g, 2.8 mmol) and cooled to 0 to 4 °C. Compound (**163**) (620 mg, 2.92 mmol) was added dropwise with vigorous stirring. The reaction was stirred at room temperature for 2 hours. The main product appeared above (**2**) in a co-spot on TLC (solvent: 8% methanol in chloroform). Reaction spot showed UV at 0.36 rf, 0.63 rf (main product), 0.69 rf, none of the spots corresponded to (**2**) in a co-spot. However the main product tailed into the region of (**2**), to ensure that all the (**2**) had reacted, the reaction was left at 0 to 4 °C under nitrogen overnight. On the following day, the TLC (solvent: 8% methanol in chloroform) indicated that the main product 0.55 rf was below (**2**) 0.59 rf and there was an intense spot on the baseline. Water (30 ml) was added to the reaction mixture, the chloroform layer was separated and dried over anhydrous magnesium sulphate and then filtered and the filtrate was evaporated under reduced pressure to give a tary product. The crude product was discarded.

^{31}P NMR δ($CDCl_3$, 160 MHz, H_3PO_4) 39.18(10%), 36.97(4), 26.29(15), 22.60(27), 18.60(13), 4.31(7), 0.45(4), -5.23(6), -11.67(14)

4.3.5 Phosphorodiamidate derivatives of other potential anti-HIV nucleoside analogues

4.3.5.1

3'-Fluoro-3'-deoxythymidine-5'-bis-(methoxy phenylalaninyl) phosphate (166a)

Compound **(94)** (160 mg, 0.66 mmol) was added to a solution of phosphoryl chloride (0.12 ml, 1.31 mmol) in anhydrous triethyl phosphate (1 ml), with stirring at 0 °C. After 17 hours, the solution was subjected to high vacuum, with stirring, at ambient temperature. Anhydrous dimethoxyethane (4 ml) was added, followed by L-phenylalanine methyl ester hydrochloride (0.79 g, 3.7 mmol). The mixture was cooled to 0 °C and triethylamine (0.92 ml, 6.61 mmol) added dropwise with vigorous stirring. After stirring in the cold for 4 days, the mixture was filtered and the precipitate washed with dimethoxyethane (30 ml) and diethyl ether (2 ml). The combined filtrate and washings were evaporated to *ca.* 1 ml under reduced pressure. This solution was dissolved in chloroform (10 ml) and added dropwise with vigorous stirring to petroleum ether (300 ml, bpt 30 to 40 °C). After cooling to 0 °C overnight, the solvent was decanted off, and the residue purified by flash column chromatography on silica, using 5% methanol in chloroform as eluant. Pooling and rotary evaporation of appropriate fractions gave a white gum (0.14 g, 33%).

^{1}H NMR δ($CDCl_3$, 200 MHz, $CDCl_3$) 9.08(1H, bs, NH), 7.0 to 7.2(11H, m, Ph, H6), 6.26(1H, t, H1'), 5.31(1H, m, H3'), 4.36(2H, m, H5'), 4.08(1H, m, H4'), 3.79(2H, m, *CH), 3.60 to 3.77(8H, s, OMe, NH), 2.85(4H, m, $PhCH_2$), 2.51 to 2.19(2H, m, H2'), 1.88(3H, s, $5CH_3$)

^{31}P NMR δ($CDCl_3$, 160 MHz, H_3PO_4) 11.03

^{13}C NMR δ($CDCl_3$, 100 MHz, $CDCl_3$) 174.16(d, C=O, J=5.2 Hz), 163.61(C2), 150.27(C4), 136.16(Ph C), 135.83(C6), 129.30 to 126.85(Ph C), 111.87(C5), 93.24(d, C3', J_{CF}=185.0 Hz), 85.29(C1'), 82.43(dd, C4', J_{CCF}=29.7 Hz, J=7.7 Hz), 66.83(dd, C5', J_{CCCF}=11.3 Hz, J=5.7 Hz), 59.64(*CH), 51.08(OCH_3), 39.98 (d, CH_2, J=7.5 Hz), 37.62(d, C2', J_{CCF}=21.46 Hz), 12.40(5CH_3)

4.3.5.2

3'-Iodo-3'-deoxythymidine-5'-bis-(methoxy phenylalaninyl) phosphate (166b)

Compound **(150)** (300 mg, 0.85 mmol) was added to a solution of phosphoryl chloride (0.16 ml, 1.73 mmol) in anhydrous triethyl phosphate (1 ml), with stirring at 0 °C. After 17 hours, the solution was subjected to high vacuum, with stirring, at ambient temperature. Anhydrous dimethoxyethane (4 ml) was added, followed by L-phenylalanine methyl ester hydrochloride (1.05 g, 4.90 mmol). The mixture was cooled to 0 °C and triethylamine (1.23 ml, 8.83 mmol) added dropwise with vigorous stirring. After stirring in the cold for 4 days, the mixture was filtered and the precipitate washed with dimethoxyethane (30 ml) and diethyl ether (2 ml). The combined filtrate and washings were evaporated to *ca.* 1 ml under reduced pressure. This solution was dissolved in chloroform (10 ml) and added dropwise with vigorous stirring to petroleum ether (300 ml, bpt 30 to 40 °C). After cooling to 0 °C overnight, the solvent was decanted off, and the residue purified by flash column chromatography on silica, using 8% methanol in chloroform as eluant. Pooling and rotary evaporation of appropriate fractions gave a white gum (0.27 g, 42%).

^{1}H NMR δ($CDCl_3$, 200 MHz, $CDCl_3$) 9.64(1H, bs, NH), 7.0 to 7.2(11H, m, Ph, H6), 5.97(1H, t, H1'), 4.29 to 4.00(3H, m, H4', H5'), 3.79(2H, m, *CH), 3.6 to 3.75(8H, s, OMe, NH), 2.81(5H, m, $PhCH_2$, H3'), 2.27(2H, m, H2'), 1.79(3H, s, $5CH_3$)

^{31}P NMR δ($CDCl_3$, 160 MHz, H_3PO_4) 10.92

^{13}C NMR δ($CDCl_3$, 100 MHz, $CDCl_3$) 173.91(d, C=O, J=5.2 Hz), 163.85(C2), 150.26(C4), 136.18(Ph C), 136.27(C6), 129.33 to 126.86(Ph C), 111.03(C5), 88.62(C1'), 84.75(d, C4', J=7.4 Hz), 62.30(d, C5', J=4.7 Hz), 59.69(*CH), 52.14(OCH_3), 39.96 (d, CH_2, J=7.5 Hz), 42.39(C2'), 12.30($5CH_3$), 11.72 (C3')

4.3.5.3

3'-Acetylthymidine-5'-bis-(methoxy phenylalaninyl) phosphate (166c)

Compound (**149**) (300 mg, 1.06 mmol) was added to a solution of phosphoryl chloride (0.20 ml, 2.14 mmol) in anhydrous triethyl phosphate (1 ml), with stirring at 0 °C. After 17 hours, the solution was subjected to high vacuum, with stirring, at ambient temperature. Anhydrous dimethoxyethane (4 ml) was added, followed by L-phenylalanine methyl ester hydrochloride (1.30 g, 6.1 mmol). The mixture was cooled to 0 °C and triethylamine (1.52 ml, 0.0109 mol) added dropwise with vigorous stirring. After stirring in the cold for 4 days, the mixture was filtered and the precipitate washed with dimethoxyethane (30 ml) and diethyl ether (2 ml). The combined filtrate and washings were evaporated to *ca.* 1 ml under reduced pressure. This solution

was dissolved in chloroform (10 ml) and added dropwise with vigorous stirring to petroleum ether (300 ml, bpt 30 to 40 °C). After cooling to 0 °C overnight, the solvent was decanted off, and the residue purified by flash column chromatography on silica, using 5% methanol in chloroform as eluant. Pooling and rotary evaporation of appropriate fractions gave an off-white gum (0.19 g, 26%).

^{1}H NMR δ($CDCl_3$, 200 MHz, $CDCl_3$) 9.05(1H, bs, NH), 7.41 to 7.03(11H, m, Ph, H6), 6.24(1H, t, H1'), 5.37(1H, m, H3'), 4.29 to 3.55(3H, m, H4', H5'), 3.77(2H, m, *CH), 3.6 to 3.75(8H, s, OMe, NH), 2.78(4H, m, $PhCH_2$), 2.40 to 2.10(2H, m, H2'), 1.84(3H, s, $5CH_3$)

^{31}P NMR δ($CDCl_3$, 160 MHz, H_3PO_4) 12.38

^{13}C NMR δ($CDCl_3$, 100 MHz, $CDCl_3$) 173.94(d, C=O, J=5.1 Hz), 170.67($CH_3\underline{C}O$), 163.82(C2), 150.29(C4), 136.16(Ph C), 135.62(C6), 129.33 to 126.86(Ph C), 111.29(C5), 85.63(d, C4', J=7.2 Hz), 84.75(C1'), 72.91(C3'), 64.49(d, C5', J=4.7 Hz), 59.70(*CH), 52.08(OCH_3), 39.57 (d, CH_2, J=7.5 Hz), 37.24(C2'), 20.38($\underline{C}H_3CO$)12.31($5CH_3$)

4.4 Biological evaluation of anti-HIV compounds

4.4.1 Materials and methods

High titre virus stocks of the human immunodeficiency virus HIV-1 (RF strain of HTLV III) were grown in H9 cells with RPMI 1640 (Flow laboratories) supplemented with 10% foetal calf serum, penicillin (100 iu/l) and streptomycin (100 μg/ml). Cell debris was removed by low speed centrifugation, and the supernatant stored at -70 °C until required. The target cell

used in these assays was the C8166 $CD4^+$ lymphoblastoid cell line. In a typical asay C8166 cells were incubated with 10 $TCID_{50}$ HIV-1 at 37 °C for 90 minutes and then washed three times with phosphate buffered saline (PBSA, Dulbecco A). Cell aliquotes (2×10^5) were resuspended in 1.5 ml growth medium in 6 ml tubes, and compounds in half log dilutions (100 μM to 0.1 μM) were added immediately. The nucleoside phosphate triesters were sparingly soluble in aqueous solution, and 10 mM stock solutions of each compound were made up in DMSO. The final DMSO concentration in the tissue culture medium was 1%. The cells were then incubated at 37 °C in a 95% air/5% CO_2 incubator.

At 72 hours post-infection 200 μl of supernatent was taken from each culture and assayed for HIV using an antigen capture ELISA (Coulter, Luton, UK). The following controls were used: supernatents taken from uninfected, and infected cells, infected cells treated with AZT (Roche Products UK Ltd.), and ddCyd (Roche Products UK Ltd.). The activities of AZT and ddCyd on infected cells consistently gave an ED_{50} of *ca.* 0.03 and 0.3 μM respectively. The ELISA plates were read with a Biorad spectrophotometer. Compounds were tested in duplicate at each concentration, and each compound was tested on at least two different occasions. To test for compound toxicity, 2×10^5 aliquotes of uninfected cells were cultured with the compounds in the same half log dilutions for 72 hours. The cells were then washed with PBSA and resuspended in 200 μl of growth medium containing ^{14}C protein hydrolysate. After 12 hours the cells were harvested and the ^{14}C incorporation measured. Uninfected, untreated cells were used as controls.

4.4.2 Biological results

Table 8 ED_{50} values of the phosphate triesters (161a-b) and (162af), evaluated as inhibitors of HIV-1: this is the minimum concentration of drug in μM which will reduce HIV antigen production by 50%

Compound	ED_{50} (μM)
(2)	0.004
(161a)	10
(161b)	10^{T}
(162a)	0.5
(162b)	0.1
(162c)	2
(162d)	0.9
(162e)	0.05
(162f)	8

T: This compound inhibits ^{14}C protein hydrolysate uptake at 10 μM; its apparent antiviral activity is therefore attributed to toxic effects.

4.4.3 Stability study: Human plasma kinetic study

Human plasma (No. P-9523 Sigma Chemical Co.), Lot 17F-9412. To lyophilized solids from human plasma (10 ml), containing 1/10 volume of 3.8% trisodium citrate, were added D_2O (2 ml) and distilled water (4 ml), the volume was made up in a volumetric flask to 10 ml with distilled water. The plasma was stored at 0 to 4 ^{o}C prior to use. The appropriate compound (4 x 10^{-5} mol) was added to an aliquot of human plasma (2.5 ml) in an NMR tube (10 mm BB). The mixture was agitated and the 0 hours ^{31}P NMR spectrum was recorded at 37 ^{o}C. The compound plus human plasma kinetic studies were observed by ^{31}P NMR at 2, 24, 192, 480 and 720 hours at 37 ^{o}C. This procedure was performed on compounds **(161a)**, **(162a)**, **(162d)** and **(162e)**, all samples were also stored at 37 ^{o}C. A control of each compound was checked by ^{31}P NMR in $CDCl_3$ to ensure that the compound produced one signal and a blank plasma, without any compound was also checked to ensure that any peaks due to the plasma alone would be recognized.

Table 9 HPLC of the human plasma kinetic study after 360 hours.

Compound	**% AZT (9.7 RT)**
(161a)	0.13
(162a)	1.80
(162d)	5.48
(162e)	5.94

Conditions used in HPLC:
Spherisorb ODS2 5 µm (50 + 250 x 4.6 mm).254 nm
A = Water/Acetonitrile 82/18
B = Acetonitrile/Water 95/5

Gradient:

Time/mins	%B	Flow
0	0	1
10	0	1
20	80	1.5
30	80	1.5

Table 10 Human plasma ^{31}P NMR study

	^{31}P nmr δ(Human plasma, D_2O, 160 MHz, H_3PO_4) [ppm(%)]			
[Time]	[Compound (**161a**)]	[Compound (**162a**)]	[Compound (**162d**)]	[Compound (**162e**)]
0	19.78(95), 3.69(3), 1.98(1), -0.35(1).	18.05(d,2), 17.77(81), 17.27(1), 12.81(2), 7.91(5), 3.69(6), 1.98(2), -0.34(1).	15.34(92), 3.64(5), 1.62(2), -0.21(1).	15.25(94), 3.64(4), 1.62(1), -0.19(1).
2 hours	19.78(95), 3.69(3), 1.98(1), -0.35(1).	18.04(d,4), 17.76(68), 17.27(2), 12.11(d,5), 8.51(5), 7.91(6), 3.63(7), 1.99(2), -0.34(1).	15.34(92), 3.64(5), 1.62(2), -0.21(1).	15.25(94), 3.64(4), 1.62(1), -0.19(1).
24 hours	19.78(95), 3.69(3), 1.98(1), -0.35(1).	18.04(m,6), 17.82(41), 17.65(2), 12.74(d,4), 12.12(d,4), 8.56(22) 7.95(14), 3.34(5), 1.98(1), -0.34(1).	15.34(89), 8.13(2), 7.59(1), 3.64(5), 1.62(2), -0.21(1).	15.25(91), 8.57(2) 6.27(1), 3.64(4), 1.62(1), -0.19(1).
192 hours	19.78(95), 3.69(2), 1.98(1), -0.35(1).	17.84(8), 12.69(d,9), 12.27(d,9), 8.49(43), 8.25(4), 7.91(22), 3.43(3), 1.96(1), -0.33(1).	15.93(1), 15.85(2), 15.34(49), 8.13(29), 7.59(11), 3.64(6), 1.62(1), -0.21(1).	15.39(m,4), 15.25(56), 9.25(1), 8.34(32), 3.64(4), 1.62(1) -0.19(1).
480 hours	19.78(95), 3.69(3), 1.98(1), -0.35(1).	17.84(3), 12.69(d,7), 12.27(d,9), 8.49(45), 8.25(4), 7.92(23), 3.43(3), 2.51(m,4), 1.96(1), -0.33(1).	15.93(1), 15.85(1), 15.34(20), 8.13(48), 7.59(m,13), 3.64(6), 2.16(m,9), 1.62(1), -0.21(1).	15.39(m,7), 15.25(26), 9.25(1), 8.34(58), 7.96(m,4), 3.64(4), 1.62(1), -0.19(1).
720 hours	19.78(93), 8.35(2) 3.69(3), 1.98(1), -0.35(1).	12.70(d,5), 12.26(d,10), 8.57(46), 8.32(2), 7.96(d,25), 3.43(3), 2.51(m,7), 1.96(1), -0.33(1).	15.93(2), 15.85(3), 15.34(2), 8.13(58), 7.59(m,15), 3.64(6), 2.16(m,12), 1.62(1), -0.21(1).	15.39(m,11), 15.25 (12),9.25(2), 8.34(62), 7.96(m,4), 3.64(4), 3.27(m,4), 1.62(1), -0.19(1).

4.5 References

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Appendix I

Abbreviations

araC	1-β-D-Arabinofuranosylcytosine
AIDS	Aquired Immunodeficiency Syndrome
ARC	AIDS Related Complex
AZT	3'-Azido-3'-deoxythymidine
bp	base peak
bpt	boiling point
CDP	Cytidine-5'-diphosphate
CMP	Cytidine-5'-monophosphate
CTP	Cytidine-5'-triphosphate
DMF	N,N-Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EI	Electron Impact
FAB	Fast Atom Bombardment
ip	Intraperitoneal
HMBA	Hexamethylene-bis-acetamide
HEPT	1-(2-Hydroxyethoxymethyl)-6-phenylthio thymine
HPLC	High Performance Liquid Chromatography
HIV	Human Immunodeficiency Virus
MS	Mass Spectrometry
mpt	melting point
mRNA	messenger RNA
min(s)	minute(s)

NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Experiment
ppm	parts per million
RT	Reverse Transcriptase
RNA	Ribonucleic Acid
TMS	Tetramethylsilane
TIBO	Tetrahydro-imidazo[4,5,1-jk][1,4]-benzodiazepin-2(1H)-one
TLC	Thin Layer Chromatography
tRNA	transfer RNA
UV	Ultra Violet

Appendix II

Apparatus and reagents

Microanalysis were carried out by the microanalytical section of the Chemistry Department, University College London; the nucleoside derivatives were noted to be hygroscopic and analytical data are presented appropriately. Electron Impact Mass spectra were recorded on a VG707OH mass spectrometer with Finnigan Incos II data system. Fast atom bombardment mass spectra were recorded on a VG Zab1F spectrometer by the University of London Mass Spectrometry Service.

Biological testing on the anti-cancer compounds was carried out on the apparatus of the cell culture unit of the Unit of Chemical Pathology at the Middlesex Hospital, Euston, London; coulter counter, spectrophotometer and

^{3}HT counter. Biological testing on the anti-HIV compounds was carried out by the Virology Unit of St. Mary's Hospital, Paddington, London.

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian XL-200 (200 MHz) or a Varian VXR-400 (400 MHz) spectrometer and are reported in δ values relative to tetramethylsilane as an internal standard or relative to the solvent employed. Carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Varian XL-200 (50 MHz) or a Varian VXR-400 (100 MHz) spectrometer and are reported in δ values relative to tetramethylsilane as an internal standard or relative to the solvent employed. Phosphorus nuclear magnetic resonance (^{31}P NMR) spectra were recorded on a Varian XL-200 (80 MHz) or a Varian VXR-400 (160 MHz) spectrometer and are reported in δ values relative to phosphoric acid (H_3PO_4) as an external standard. All peaks reported are singlets unless otherwise stated. Carbon and phosphorus NMR spectra were heteronuclear decoupled unless otherwise stated. The NMR spectra were recorded in either deuterochloroform ($CDCl_3$) or deuteromethanol (CH_3OD or CD_3OD). The following abbreviations are used in signal assignments: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), b (broad).

X ray crystallography was performed on a Nicolet R3m/v diffractometer operating at room temperature with graphite-monochromated Mo-K_{α} radiation.

Molecular graphics were performed with the programmes COSMIC and ASTRAL, originally written at SK & F (UK) but are now housed and administered through the University of Nottingham, Department of Pharmaceutical Sciences. The code used was developed by Dr. A Vinter on an IBM 386 PC system under FTN77 (DBOS) and INTERACTER (Graphics

interface). The high resolution version (including ASTRAL) was implemented on a Silicon Graphics 4D25G workstation.

Electronic ultra violet spectra were recorded on a Perkin-Elmer 554 spectrophotometer, fitted with a Perkin-Elmer temperature control. Commercially available Merck Kieselgel 60 F_{254} plates were used for analytical thin layer chromatography (TLC). They were visualised with ultra violet light or iodine. Column chromatography was performed using Woelm silica (32-63 μM) as the stationary phase. The ratio of silica:compound was between 40:1 and 100:1 (w/w).

All experiments involving water sensitive compounds were done under scrupulously dry conditions. Reagents were dried as follows prior to use; pyridine was distilled from calcium hydride and stored over activated 4 Å molecular sieves. Triethylamine was heated to reflux over calcium hydride for several hours and then freshly distilled before use. Diethyl ether was heated to reflux over calcium hydride for several hours, then distilled onto 3 Å molecular sieves. Phosphoryl chloride, thionyl chloride, phosphorus trichloride, acetic anhydride and mesyl chloride were freshly distilled prior to use. Triethyl phosphate was distilled under reduced pressure and stored over 4 Å molecular sieves. Nucleosides were dried at *ca.* 50 °C / 0.1 mm Hg and amino acids were dried at *ca.* 50-100 °C / 0.1 mm Hg for several hours. Methanol was dried by heating to reflux with Mg/I_2 and then distilled onto 3 Å molecular sieves. Ethanol, 2,2,2-trifluoroethanol and 2,2,2-trichloroethanol were distilled and stored over activated 3 Å molecular sieves. All other alcohols were dried over 4 Å molecular sieves for at least 24 hours prior to use. Molecular sieves were activated by heating to 160 °C at 0.1 mm Hg for at least 5 hours.

Appendix III

Compounds (Listed by Number)

Number	Name
1	1-β-D-Arabinofuranosylcytosine
2	3'-Azido-3'-deoxythymidine
3	Sulphur Mustard
4	Nitrogen Mustard
5	Methotrexate
6	Vincristine
7	Vinblastine
8	Colchicine
9	Etoposide
10	Teniposide
11	Asparaginase
12	Bleomycin
13	Fluorouracil
14	Mercaptopurine
15	Thioguanine
16	Hydroxyurea
17	Predinisone
18	Procarbazine
19	Dactinomycin
20	Doxorubicin
21	Daunorubicin
22	Rubidazone
23	Busulfan
24	Chlorambucil

25	Cyclophosphamide
26	Melphalan
27	Mechlorethamine
28	Semustine
29	Cormustine
30	Lomustine
31	Dacarbazine
32	Cisplatin
33	DMSO
34	HMBA
35	1-β-D-Arabinofuranosylthymine
36	1-β-D-Arabinofuranosyluracil
37	9-β-D-Arabinofuranosyladenine
38	9-β-D-Arabinofuranosylguanine
39	Uridine
40	Cytidine
41	2'-Deoxyuridine
42	2'-Deoxycytidine
43	2'-Deoxythymidine
44	Adenosine
45	Guanosine
46	Inosine
47	Xanthosine
48	2'-Deoxyguanosine
49	2'-Deoxyadenosine
50	1-β-D-Arabinofuranosylcytosine monophosphate
51	1-β-D-Arabinofuranosylcytosine diphosphate

52	1-β-D-Arabinofuranosylcytosine triphosphate
53a	Tetrahydrouridine
53b	4-Hydroxylamino-5-methylpyrimidin-2-one-2'-deoxy-ribonucleoside
53c	Diazepinone nucleoside
53d	Aromatic 2-oxopyrimidine riboside
54a	2,2'-O-Cyclocytidine
54b	1-β-D-Arabinofuranosylcytosine-N-oxide
55	5'-O-Palmitoyl-1-β-D-arabinofuranosylcytosine
56a-e	5'-(Alkyl phosphate) esters of 1-β-D-Arabinofuranosyl-cytosine
56f	5'-(3-O-Benzylglyceryl) ester of 1-β-D-Arabinofuranosyl-cytosine
56g	5'-(Glyceryl) ester of 1-β-D-Arabinofuranosyl-cytosine
57a-c	1-β-D-Arabinofuranosylcytosine diphosphate dipalmitin; L,D and Racemic Mixture
58	2',2'-Difluorodeoxycytidine
59	Mesna
60	4-Hydroxycyclophosphamide
61	Ketophosphamide
62	Aldophosphamide
63	Phosphoramide mustard
64	Carboxyphosphamide
65	Nor-nitrogen mustard
66	Acrolein
67	Isophosphamide
68	Trilophosphamide
69	5-Fluoro-5'-(2-oxo-1,3,2-oxazaphosphorinan-2-yl)-2'-deoxyuridine

70	9-[5'-(2-oxoazaphosphorinan-2-yl)-β-D-arabinosyl]adenine
71	CD4 proteins
72	AL-721
73	Dextran sulphate
74	Silicotungstate
75	Amantadine
76	Castanospermine
77	Interferons
78	Suramin
79	Foscarnet
80	Ribabutin
81	Antimoniotungstate (HPA-23)
82	2',3'-Dideoxycytidine
83	2',3'-Dideoxythymidine
84	2',3'-Dideoxyadenosine
85	2',3'-Dideoxyguanosine
86	2',3'-Dideoxyinosine
87	5-fluoro-2',3'-dideoxycytidine
88	2',3'-Didehydro-2',3'-dideoxycytidine
89	2',3'-Didehydro-2',3'-dideoxythymidine
90	2',3'-Didehydro-2',3'-dideoxyadenosine
91	2',3'-Didehydro-2',3'-dideoxyguanosine
92	2',3'-Didehydro-2',3'-dideoxyuridine
93	2',3'-Didehydro-2',3'-dideoxyinosine
94	3'-Fluoro-3'-deoxythymidine
95	3'-Fluoro-2',3'-dideoxy-5-chlorouridine
96	3'-Fluoro-2',3'-dideoxyadenosine

97	3'-Fluoro-2',3'-dideoxyuridine
98	3'-Azido-3'-deoxyuridine
99	3'-Azido-2',3'-dideoxy-5-ethyluridine
100	3'-Azido-2',3'-dideoxy-5-hydroxyuridine
101	3'-Azido-2',3'-dideoxy-5-fluorouridine
102	3'-Azido-2',3'-dideoxy-5-chlorouridine
103	3'-Azido-2',3'-dideoxy-5-thiocyanouridine
104	3'-azido-2',3'-dideoxy-2,6-diaminopurine-riboside
105	3'-Azido-3'-deoxycytidine
106	Thymidine triphosphate
107	2'-Deoxycytidine triphosphate
108	2'-Deoxyguanosine triphosphate
109	2'-Deoxyadenosine triphosphate
110	3'-Azido-3'-deoxythymidine monophosphate
111	3'-Azido-3'-deoxythymidine diphosphate
112	3'-Azido-3'-deoxythymidine triphosphate
113	Carbocyclic 2',3'-didehydro-2',3'-dideoxyguanosine
114	Iso 2',3'-dideoxyadenosine
115	Cyclobutadenosine
116	Cyclobutguanosine
117	Adenallene
118	Cytallene
119	1-[9-(2-Phosphonylmethoxyethyl)-adenine]
120	[9-(2-Phosphonylmethoxyethyl)-2,6-diaminopurine]
121	3'-Amino-3'-deoxythymidine
122	3'-O-methylthymidine
123	Acyclovir

124	5'-(1,4-Dihydro-1-methyl-3-pyridinylcarbonyl)-3'-azido-3'-deoxy-thymidine
125	5'-(1,4-Dihydro-1-methyl-3-pyridinylcarbonyl)-2',3'-didehydro-3'-deoxythymidine
126	2,5'-Anhydro-3'-azido-3'-deoxythymidine
127	Phospholipid derivative of 3'-azido-3'-deoxythymidine
128	Phosphate triester (General)
129	Glucose-phospholipid drug delivery system
130	HEPT
131	TIBO
132	3'-Azido-5'-(O-ethoxycarbonylphosphinyl)-3'-deoxythymidine
133a	Diethyl phosphorochloridate
133b	Di-n-propyl phosphorochloridate
133c	Di-n-butyl phosphorochloridate
133d	Di-n-pentyl phosphorochloridate
133e	Di-n-hexyl phosphorochloridate
133f	Bis-(2,2,2-trichloroethyl) phosphorochloridate
133g	Bis-(2,2,2-trifluroethyl) phosphorochloridate
134a	1-β-D-Arabinofuranosylcytosine-5'-diethyl phosphate
134b	1-β-D-Arabinofuranosylcytosine-5'-di-n-propyl phosphate
134c	1-β-D-Arabinofuranosylcytosine-5'-di-n-butyl phosphate
134d	1-β-D-Arabinofuranosylcytosine-5'-di-n-pentyl phosphate
134e	1-β-D-Arabinofuranosylcytosine-5'-di-n-hexyl phosphate
134f	1-β-D-Arabinofuranosylcytosine-5'-bis-(2,2,2-trichloro-ethyl) phosphate
134g	1-β-D-Arabinofuranosylcytosine-5'-bis-(2,2,2-trifluoro-ethyl) phosphate
135	Adenosine-5'-diethyl phosphate
136a	Methyl phosphorodichloridate

136b	Ethyl phosphorodichloridate
136c	n-Propyl phosphorodichloridate
136d	n-Butyl phosphorodichloridate
136e	n-Pentyl phosphorodichloridate
136f	n-Hexyl phosphorodichloridate
136g	Diethylamino phosphorodichloridate
137a	Diethylamino-n-propyl phosphorochloridate
137b	Methyl-n-propylamino phosphorochloridate
137c	Bis-(2-chloroethyl)amino ethyl phosphorochloridate
137d	Methyl N-phenylamino phosphorochloridate
137e	n-Butyl N-methyl-N-phenylamino phosphorochloridate
137f	Methyl diethylamino phosphorochloridate
138	Bis-diethylamino phosphorochloridate
139a	1-β-D-Arabinofuranosylcytosine-5'-diethylamino n-propyl phosphate
139b	1-β-D-Arabinofuranosylcytosine-5'-methyl n-propylamino phosphate
139c	1-β-D-Arabinofuranosylcytosine-5'-bis-(2-chloroethyl)-amino ethyl phosphate
139d	1-β-D-Arabinofuranosylcytosine-5'-methyl N-phenylamino phosphate
139e	1-β-D-Arabinofuranosylcytosine-5'-n-butyl N-methyl-N-phenylamino phosphate
140a	1-β-D-Arabinofuranosylcytosine-5'-bis-diethylamino phosphate
140b	1-β-D-arabinofuranosylcytosine-5'-bis-(n-propylamino) phosphate
140c	1-β-D-arabinofuranosylcytosine-5'-bis-(bis-(2-chloroethyl)-amino) phosphate
141	Cytosine-N^4-bis-diethylamino phosphate
142	1-β-D-Arabinofuranosylcytosine-5'-n-propyl phosphorochloridate

143	1-β-D-Arabinofuranosylcytosine-5'-phosphorodichloridate
144	1-β-D-arabinofuranosylcytosine-3'-phosphorodichloridate 5'-phosphorodichloridate
145	1-β-D-arabinofuranosylcytosine-3'-bis-(n-propylamino) phosphate 5'-bis-(n-propylamino) phosphate
146a	1-β-D-Arabinofuranosylcytosine-5'-(2,2,2-trichloroethyl) methyl phosphate
146b	1-β-D-Arabinofuranosylcytosine-5'-(2,2,2-trichloroethyl) ethyl phosphate
146c	1-β-D-Arabinofuranosylcytosine-5'-(2,2,2-trichloroethyl) n-propyl phosphate
146d	1-β-D-Arabinofuranosylcytosine-5'-(2,2,2-trichloroethyl) n-butyl phosphate
147a	1-β-D-Arabinofuranosylcytosine-5'-(2,2,2-trichloroethyl) n-propylamino phosphate
147b	1-β-D-Arabinofuranosylcytosine-5'-(2,2,2-trichloroethyl) n-butylamino phosphate
147c	1-β-D-Arabinofuranosylcytosine-5'-(2,2,2-trichloroethyl) diethylamino phosphate
148	N^4-Dimethylaminomethylene-1-β-D-arabinofuranosyl cytosine
149	3'-O-Acetylthymidine
150	3'-O-Deoxy-3'-O-iodothymidine
151a	L-Valine methyl ester hydrochloride
151b	L-Alanine methyl ester hydrochloride
151c	L-Phenylalanine methyl ester hydrochloride
151d	L-Aspartic acid methyl diester hydrochloride
151e	L-Proline methyl ester hydrochloride
152	5'-O-Tritylthymidine
153	3'-O-Methanesulphonyl-5'-O-tritylthymidine
154	5'-O-Trityl-2,3'-anhydrothymidine
155	1-(2'-Deoxy-5'-O-trityl-β-D-lyxosyl)thymine

156	1-(2'-Deoxy-5'-O-trityl-3'-O-mesyl-β-D-lyxosyl)thymine
157	5'-O-Trityl-3'-O-acetylthymidine
158	5'-O-Trityl-3'-O-iodothymidine
159	5'-O-Trityl-3'-azido-3'-deoxythymidine
160	3'-Azido-3'-deoxythymidine-5'-phosphorodichloridate
161a	3'-Azido-3'-deoxythymidine-5'-bis-(n-propylamino) phosphate
161b	3'-Azido-3'-deoxythymidine-5'-bis-(diethylamino) phosphate
162a	3'-Azido-3'-deoxythymidine-5'-bis-(methoxyglycinyl) phosphate
162b	3'-Azido-3'-deoxythymidine-5'-bis-(methoxyvalinyl) phosphate
162c	3'-Azido-3'-deoxythymidine-5'-bis-(methoxyalaninyl) phosphate
162d	3'-Azido-3'-deoxythymidine-5'-bis-(dimethoxyaspartyl) phosphate
162e	3'-Azido-3'-deoxythymidine-5'-bis-(methoxyphenylalaninyl) phosphate
162f	3'-Azido-3'-deoxythymidine-5'-bis-(methoxyprolinyl) phosphate
163	Bis-(diethylamino) phosphorochloridite
164	3'-Azidothymidine-5'-bis-(diethylamino) phosphite
165a	3'-Fluoro-3'-deoxythymidine-5'-phosphorodichloridate
165b	3'-Iodo-3'-deoxythymidine-5'-phosphorodichloridate
165c	3'-Acetylthymidine-5'-phosphorodichloridate
166a	3'-Fluoro-3'-deoxythymidine-5'-bis-(methoxy phenyl alaninyl) phosphate
166b	3'-Iodo-3'-deoxythymidine-5'-bis-(methoxy phenylalaninyl) phosphate
166c	3'-Acetylthymidine-5'-bis-(methoxy phenylalaninyl) phosphate