Synthetic Routes to the Tumour Proliferation Biomarker FLT and ProTide Analogues for PET Imaging

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Acknowledgements

Abstract

Being one of the most rapidly advancing cancer imaging techniques in recent years, Positron Emission Tomography (PET) represents a standard of excellence with respect to sensitivity and resolution for the non-invasive *in vivo* molecular imaging of solid tumours via the detection of radiotracer molecules. Amongst these radiotracers, radiolabelled fluorinated nucleosides such as 3'-Deoxy-3'-[18F]-fluorothymidine (18F-FLT) has been widely recognized as a key, specific biomarker for tumour cellular proliferation. Current methods for the commercial production of [18F]FLT are characterized by low overall yields and time-consuming high-performance liquid chromatography (HPLC) purification. These disadvantages could be rectified by the development of a fast, efficient synthetic route to FLT that could enhance the productivity and reduce the reaction time of the fluoridation step necessary for the synthesis of short-lived radioisotope, ^{18}F ($t_{1/2}$=110 min) installed nucleoside.

This project focussed mainly on the development of a new efficient chemical synthetic route to FLT. The synthesis and *in vitro* evaluation of a series of pro-nucleotide (ProTide) analogues of FLT as new potential therapeutic agents was also carried out.

Various studies regarding FLT synthesis have been carried out on the cold (non-radioactive) ^{19}F and radioactive ^{18}F isomer by varying and optimizing conditions for the incorporation of different protecting groups and also different fluoridation reactions by nucleophilic displacement.

Further optimization studies were made for the fluoridation step and the synthesis of [18F]FLT-ProTide analogues as new diagnostic PET imaging agents by variation of different chemical parameters on the phosphoramidate group was attempted.

The synthesis of ^{19}F- FLT based ProTides as new therapeutic agents were initiated by the introduction of the phosphoramidate group at the 5'-position of the furanosyl group of thymidine under basic conditions followed by fluoridation to generate the desired analogues.

In addition to that, biological evaluation of the newly developed ^{19}F-FLT ProTide analogues for anti-HIV 1 and anti-HIV 2 activities was undertaken on CEM cells. The results in those models indicated that the synthesized compounds were less potent than the parent nucleoside FLT. However in TK$^-$ (HIV-2) cells, the analogues retained biological activity in contrast to FLT. This suggested that the FLT ProTides bypassed the first phosphorylation step.

However, the therapeutic *in vitro* evaluation for anti-tumour activity on L1210, CEM and HeLa cells showed no significant activity.

Contents

Abbreviations

^{o}C:	degree Celsius
%:	percentage
$t_{1/2}$:	half-life
µM:	micromolar
AgCl:	silver chloride
aq:	aqueous
Ar:	aryl
ALL:	acute lymphoblastic leukemia
ATP:	adenosine triphosphate
AZT:	azidothymidine
Bq:	becquerel
bs:	broad singlet
^{t}BuMgCl	tertiary butyl magnesium chloride
CEM:	human T-lymphocyte
CH_3Cl:	chloroform
CH_3CN:	acetonitrile
CsF:	cesium fluoride
CT:	computed tomography

cycloSal:	cyclosaligenyl
CYP:	cytochrome P450
d:	doublet
d4A:	2',3'-didehydro-2',3'-dideoxyadenosine
d4T:	2',3'-didehydro-2',3'-dideoxythymidine
DAST:	diethylaminosulphur trifluoride
DBU:	1,8-Diazabicycloundec-7-ene
dCK:	deoxycytidine kinase
dCMPK:	deoxycytidine monophosphate kinase
DCM:	dichloromethane
dd:	doublet of doublets
ddA:	dideoxyadenosine
ddU:	dideoxyuridine
dFdC:	2',2'-difluorodeoxycytidine
DIAD:	diisopropyl azodicarboxylate
DIPEA:	N,N-diisopropylethylamine
DMF:	dimethylformamide
DMSO:	dimethylsulfoxide
DMTr:	4,4'-dimethoxytriphenylmethyl
DNA:	deoxyribonucleic acid
dTMP:	2'-deoxythymidine-5'-monophosphate

dUMP:	2'-deoxyuridine-5'-monophosphate
EC_{50}:	half maximal effective concentration
ED_{50}:	median effective dose
eq:	equivalent
EtOAc:	ethyl acetate
EWG:	electron-withdrawing group
FDG:	fluorodeoxyglucose
3'-FddC:	3'-fluoro-2',3'-dideoxycytidine
3'-FddG:	3'-fluoro-2',3'-dideoxyguanosine
3'-FddU:	3'-fluoro-2',3'-dideoxyuridine
FdUR:	5-fluoro-2'-deoxyuridine
FLT:	3'-fluoro-3'-deoxythymidine
L-FMAU:	1-(2-fluoro-5-methyl-beta-L-arabino-furanosyl)uracil
5-FU:	5-fluorouracil
GBq:	gigabecquerel
GI tract:	gastrointestinal tract
GMP:	good manufacturing practice
HBV:	hepatitis B virus
HCl:	hydrochloric acid
HeLa:	cervical cancer cell line
HF:	hydrogen fluoride

HIV:	Human immunodeficiency virus
HPLC:	high performance liquid chromatography
HSV:	herpes simplex virus
K_{222}:	kryptofix-222
keV:	kilo electronvolt
KF:	potassium fluoride
$KHCO_3$:	potassium bicarbonate
MBq:	megabecquerel
MeOH:	methanol
mesyl:	methanesulfonyl
$MgSO_4$:	magnesium sulfate
MRI:	Magnetic resonance imaging
MW:	molecular weight
NaOH:	sodium hydroxide
Na_2SO_4:	sodium sulfate
NDPK:	nucleotide diphosphate kinase
NMI:	1-methylimidazole
NMR:	Nuclear magnetic resonance
nosyl:	4-nitrobenzenesulfonyl
NRTI:	nucleoside reverse transcriptase inhibitor
NT:	nucleoside transporter

PET:	Positron emission tomography
PETIC:	Wales research and diagnostic positron emission tomography imaging centre
PMBz:	paramethoxybenzoyl
POM:	pivaloyloxymethyl
ProTide:	phosphoramidate pronucleotide
RCY:	radiochemical yield
RNA:	ribonucleic acid
RNR:	ribonucleotide reductase
RT:	reverse transcriptase
SPECT:	Single photon emission computed tomography
TBAF:	tetra-N-butylammonium fluoride
TD_{50}:	median toxic dose
THF:	tetrahydrofuran
TK1:	thymidine kinase 1
Tr:	triphenylmethyl
TS:	thymidylate synthase
UV:	ultraviolet

Chapter 1

1. Introduction

1.1. Biological aspects of fluorinated nucleosides

For decades, modified nucleosides especially fluorinated ones, have been the subject of many medicinal studies due to their predicted potential activity as enzyme inhibitors resulting in mainly anti-tumour[1] and anti-viral[2] activities. Apart from the predominance of fluorinated nucleosides as therapeutics, the role of some fluorinated nucleosides such as [^{18}F]-fluorodeoxyglucose (FDG) and [^{18}F]-3'-deoxy-3'-fluorothymidine (FLT, ^{18}Fluorine-labeled thymidine) as clinical diagnostic biomarkers in oncology has been widely recognized. These fluorinated nucleoside biomarkers find their primary cancer biomarker application in Positron Emission Tomography (PET) imaging, a nuclear medicine imaging technique that offers the ability to monitor tumour proliferation and other cancer hallmarks non-invasively in cancer patients.[3]

1.1.1. Therapeutic aspects of fluorinated nucleosides

Fluorinated nucleosides and their derivatives constitute a fundamentally important class of non-natural compounds that have been used in a wide variety of biological applications such as anti-viral and anti-tumour therapy, and as diagnostics. Natural nucleosides, which are the building blocks of both nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), are of fundamental importance in all living

systems.[4,5] These nucleosides are composed of a nitrogen heterocycle base (**Figure 1.1**) linked to a sugar moiety (deoxyribose or ribose) by a β-N-glycosidic bond. The nitrogen base is either a purine or pyrimidine.
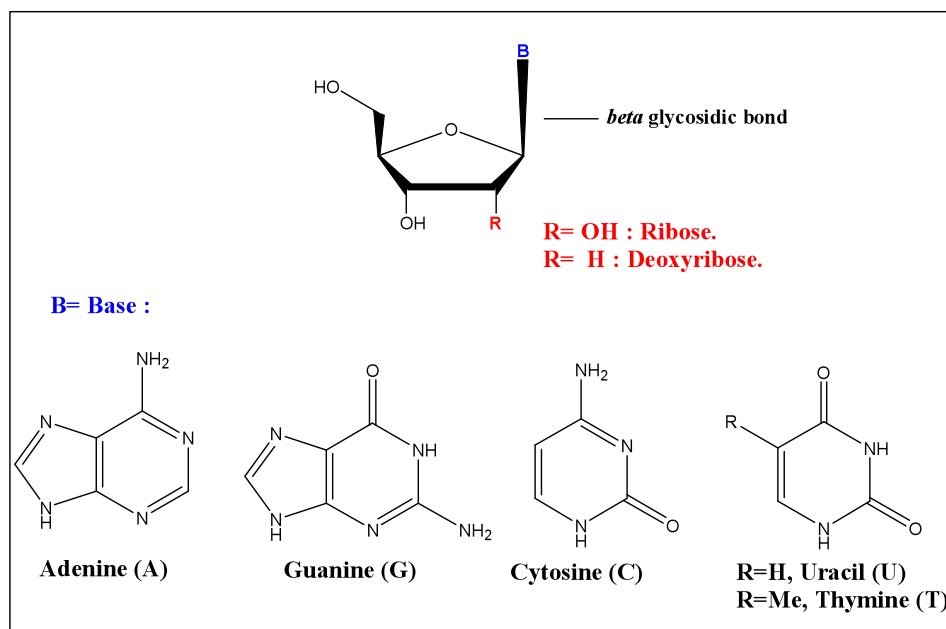
Figure 1.1: Structures of ribose, 2'-deoxyribose nucleosides and nitrogen bases.

Adenine and guanine are purine bases, which are present in both DNA and RNA, as is the pyrimidine base cytosine. However, the pyrimidine base uracil occurs only in RNA whereas the pyrimidine base thymine is present only in DNA. Regarding the sugar moiety, it varies from DNA to RNA; i.e. the sugar unit present in DNA is a 2-deoxyribose while in RNA it is a ribose sugar molecule (**Figure 1.1**).[6] Many biological applications such as anticancer and antiviral activities have been implemented by inducing small modifications to the structures of the naturally occurring nucleosides. The structural modifications could take place at either the heterocyclic base ring and/or at the sugar. These nucleoside analogues are phosphorylated at the 5'-position to form

active nucleotides that can function as inhibitors of viral and cellular proliferation processes.[7] The initial, rate-limiting phosphorylation step is carried out by cytosolic kinase enzymes such as thymidine kinase 1 (TK1) and deoxycytidine kinase (dCK), to form the nucleoside monophosphate. The biochemical substrate specificity of nucleosides for kinase enzymes varies. For instance, thymidine, **1** and its analogues such as azidothymidine (AZT), **2** are substrates for TK1, while cytidine, **3** and its analogues such as gemcitabine, **4** are substrates for dCK.
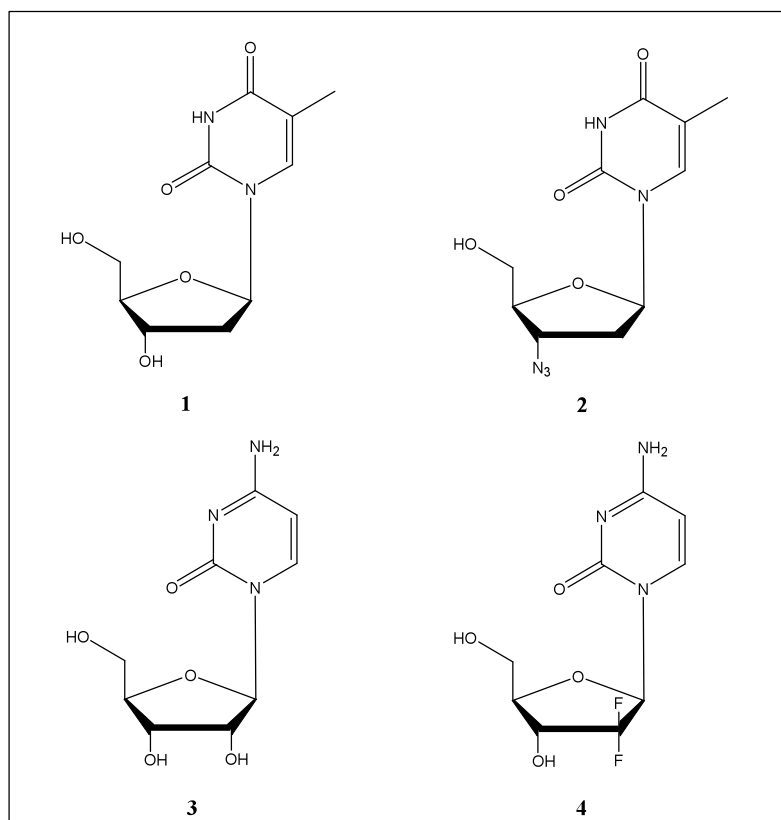
Figure 1.2: The nucleosides thymidine (**1**) and cytidine (**3**) with their respective analogues, AZT (**2**) and gemcitabine (**4**).

The monophosphorylation of nucleosides leads to the trapping of charged nucleotides inside the cellular cytoplasm. Since nucleosides are hydrophilic and diffuse slowly across cell membranes, cells have evolved a complex transport system consisting of multiple carrier proteins called nucleoside transporters (NTs) to facilitate the cellular uptake of these nucleoside analogues for nucleotide biosynthesis. The physiological roles of these transporters include the salvaging of DNA and RNA synthetic precursors.[8] Consecutively, the nucleoside monophosphates are further phosphorylated by kinases to form their respective diphosphates and finally the triphosphates which participate in the DNA and RNA biosynthesis.

1.1.1.1. Impact of Fluorine Substitution

Although fluorine is the most abundant halogen found on earth, it occurs extremely rarely in biologically active natural products and is completely absent in biomolecules such as nucleotides or nucleosides.[9] Nature must have found it difficult to incorporate fluorine into naturally occurring molecules, perhaps because of the very strong energy of solvation of fluoride ions in water.[10] However, many non-natural organo-fluorine compounds with indispensable biological properties have been synthesized.[11] The idea of introducing fluorine into nucleosides at a specific position has gained much appreciation in the pharmaceutical pipeline over the past few decades. This is mainly due to the positive influence of fluorine on the physicochemical, pharmacokinetic and pharmacological properties of the resulting compounds.[12]

The selective introduction of fluorine into a bioactive nucleoside as an isosteric replacement for hydrogen or as an isopolar mimic of a hydroxyl group can change the

inherent biological, physical and chemical properties of the molecule dramatically. It is generally thought that when hydrogen is substituted by fluorine, it exerts minimal steric constraints at the receptor sites due to the slight difference in their sizes.[13,14,15]

Being one of the strongest bonds in organic chemistry, carbon-fluorine bond formation would result in the increase in metabolic and thermal stability. The C-F bond is less susceptible to oxidative metabolism caused by liver enzymes such as cytochrome P450 (CYP) and hence contributes to metabolic stability.[16] Fluorine substitution has often resulted in the enhanced biological activity of drugs by improving binding affinity with the target enzyme or receptor.[17, 18] Another major impact is the increased hydrophobicity or the lipophilicity of the resulting compounds leading to greater passive diffusion of drugs across the membranes.[19] As a matter of fact, these important properties are still being exploited in cutting edge research for the design and discovery of novel potential fluorinated nucleoside drug candidates.

When nucleophilic fluorine substitution occurs at the 2'- or 3'-position of the bioactive nucleoside furanosyl (sugar) unit replacing hydrogen or the hydroxyl group, the chemical and biological stability of the nucleoside analogues are known to increase, since the strength of the resultant C-F bond is much greater than the C-H and C-O bonds. In particular, target binding activity can be exceeded in an acidic environment since the C-F bond is a hydrogen bond acceptor.[20] In addition to this factor; metabolic stability is another favourable effect that results from this phenomenon.[21]

1.1.1.2. Antitumour activity of fluorinated nucleosides

Fluorinated nucleosides are well documented and explored as potential anti-cancer agents.[22] Studies suggest that the induction of apoptosis is the final pathway through which these drugs exert their anti-tumour activity.[23] These nucleoside analogues mimic natural nucleosides in terms of uptake and metabolism. Further, these molecules can be incorporated into newly synthesized DNA resulting in chain termination. Some of these drugs also inhibit key enzymes involved in the biosynthesis of DNA and RNA synthesis, such as polymerases, leading to the activation of apoptosis by the anti-cancer agent. Some of the most relevant anticancer fluorinated nucleosides are shown in **Figure 1.3**.[24] Recent studies indicated the importance of one of the fluorinated nucleosides, clofarabine (2-chloro-2'-deoxy-fluoro-β-D-arabinofuranosyladenine), **5** as a new-generation nucleoside analogue developed for acute lymphoblastic leukemia (ALL).[25] It exerts its anti-cancer effect by inducing apoptosis following blockade of DNA synthesis as well as through inhibition of DNA repair.
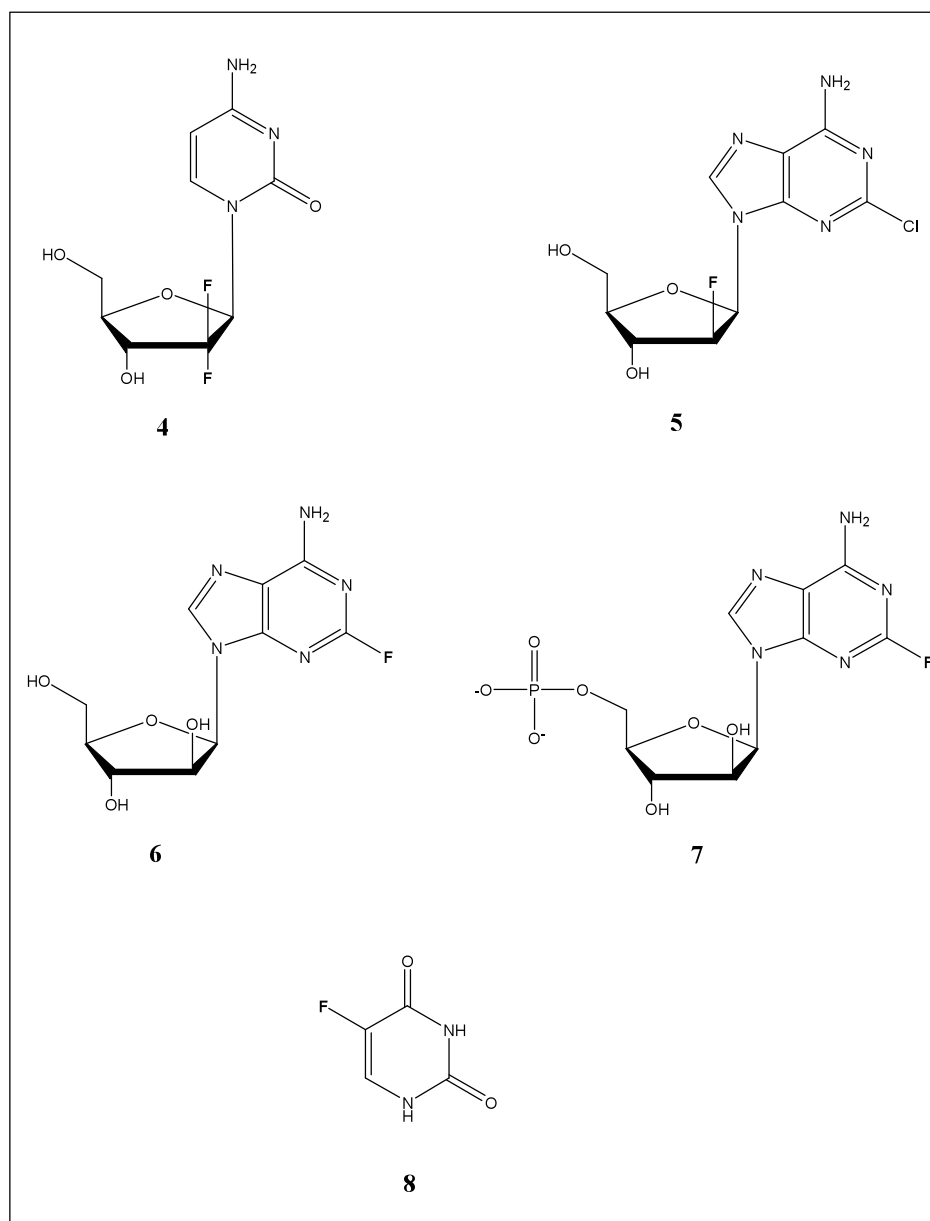
Figure 1.3: Some important anti-cancer fluorinated nucleoside analogues such as gemcitabine (**4**), clofarabine (**5**), fludarabine (**6**), fludarabine monophosphate (**7**) and 5-fluorouracil (5-FU, **8**).[24]

Clofarabine is a new generation nucleoside anticancer agent structurally related to the nucleoside, fludarabine, **6**.[26] The nucleotide monophosphate derivative of **6**, fludarabine phosphate, **7** is a water-soluble prodrug that is rapidly dephosphorylated to 2-fluoro-vidarabine. Subsequently, it is transported actively into cells and rephosphorylated via deoxycytidine kinase to the active triphosphate derivative 2F-ara-adenosine triphosphate (2F-ara-ATP). This derivative competitively inhibits DNA synthesis via inhibition of DNA polymerase, ribonucleotide reductase, DNA primase and DNA ligase; hence preventing elongation of DNA strands through its direct incorporation into DNA as a fraudulent nucleotide.[27,28] However, the degree of inhibition of these enzymes is stronger in the case of clofarabine. An illustration of the clofarabine mechanism of action that is reproduced from Bonate et al. *Nat Rev Drug Discovery* is shown in **Figure 1.4**.[26] The rate-limiting mono phosphorylation step of clofarabine is substantially more efficient than that of fludarabine.[29] Additionally, the intracellular retention of clofarabine triphosphate in acute myeloid leukemia is longer than that of fludarabine, which contributes to the higher potency of clofarabine as an anti-cancer agent.[30] In recent years, the combination of radiotherapy and chemotherapy has emerged as a potential tool in cancer therapy. The current experimental evidence showed that a moderate dose of clofarabine along with radiotherapy led to a significant inhibition of tumour growth.[31]
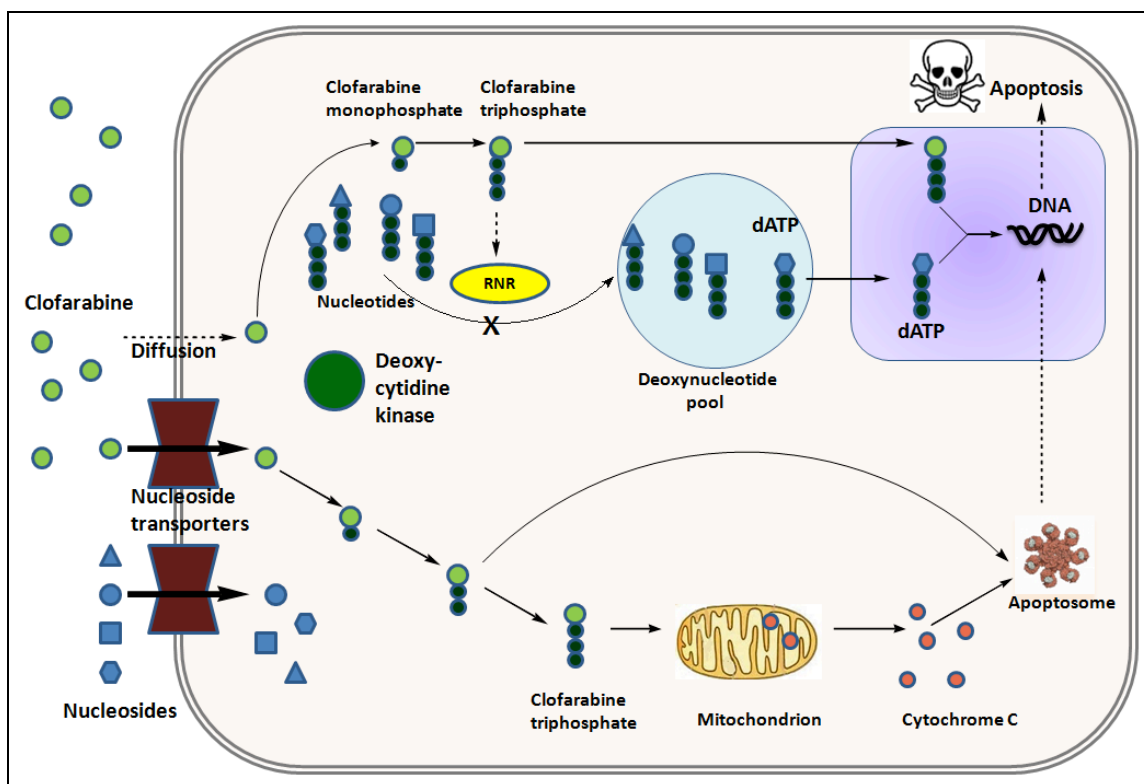
Figure 1.4: Clofarabine, which crosses the cell membrane by both diffusion and NT facilitated active transport, is monophosphorylated by dCK and by further phosphorylations, the active clofarabine triphosphate is formed. The triphosphate terminates DNA chain elongation and constricts repair through its incorporation into the DNA chain by competitive inhibition of DNA polymerases. It also inhibits ribonucleotide reductase (RNR) hindering the formation of deoxynucleotide triphosphates which are DNA precursors, and induces apoptosis through direct and indirect action on mitochondria by releasing cytochrome *c* and other apoptotic signals triggering cell death.[26]

The well known 5-fluorouracil (5-FU), **8** which has been used in cancer treatment for more than 60 years, was first synthesized in 1957 by Heidelberger et al. as an antimetabolite.[32] The active form is a uridine 5'-phosphate analogue, which is generated

in vivo. This prodrug is an active inhibitor of the enzyme, thymidylate synthase (TS) that converts 2'-deoxyuridine-5'-monophosphate (dUMP) to 2'-deoxythymidine-5'-monophosphate (dTMP) via C5-methylation and thus blocks the formation of dTMP, which is a building block for DNA-synthesis and repair.[33] 5-FU has been extensively used for skin cancer treatments and a variety of solid tumours such as breast, colorectal and gastric tumours. Unfortunately, it has been found to cause neurotoxic and cardiotoxic side effects deriving from the lack of selectivity of 5-FU towards tumours and can lead to resistance by the excess production of dUMP in the cell to compete with the drug for the active site.

Another milestone in anti-cancer fluorinated nucleoside therapeutics is the development of gemcitabine (2',2'-difluorodeoxycytidine, dFdC), **4** by Eli Lilly in 1996 with a broad spectrum of activities against solid tumours such as pancreatic and breast cancers. Once transported into the cell, the pro-drug gemcitabine must be phosphorylated by the enzyme dCK to form the active species. The incorporation of the phosphates of **4** into DNA is considered to be the major mechanism by which cell death is caused and hence obstructing DNA synthesis by inhibiting DNA polymerases. [34]

1.1.1.3. Antiviral activity of fluorinated nucleosides

The first anti-HIV (human immunodeficiency virus) nucleoside drug that was clinically approved for the treatment of AIDS (acquired immunodeficiency syndrome) was AZT (Azidothymidine/ Zidovudine), which is a nucleoside analogue that inhibits the functioning of HIV reverse transcriptase (RT).[35] Since the development of AZT, there

has been extensive research into identifying nucleoside based compounds with good inhibitory activities of HIV reverse transcriptase and such compounds are classified as nucleoside reverse transcriptase inhibitors (NRTIs). The NRTIs include those compounds which can mimic endogenous pyrimidine or purine nucleosides, and all of them require intracellular phosphorylations to their respective triphosphates for therapeutic activity.[36]
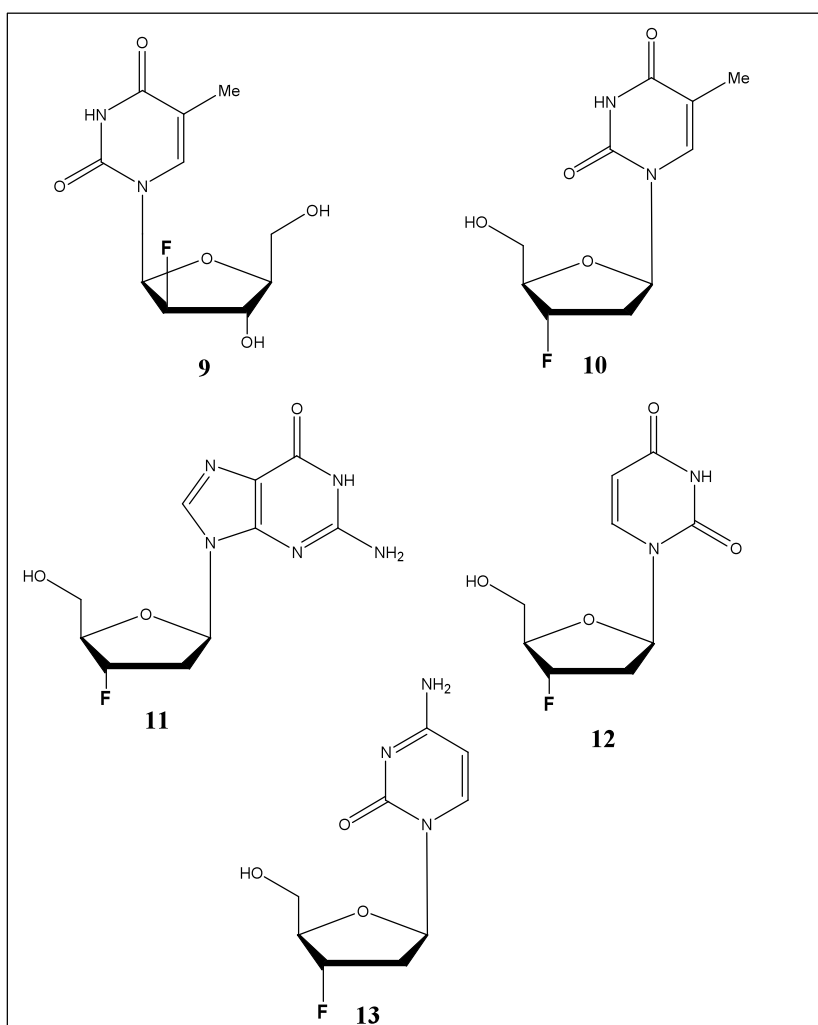
Figure 1.5: Some of the most potent anti-viral fluorinated nucleosides such as L-FMAU (1-[2-fluoro-5-methyl-beta-L-arabino-furanosyl]uracil, **9**), FLT (3'-fluoro-3'-

deoxythymidine, **10**), 3'-FddG (3'-fluoro-2',3'-dideoxyguanosine, **11**), 3'-FddU (3'-fluoro-2',3'-dideoxyuridine, **12**) and 3'-FddC (3'-fluoro-2',3'-dideoxycytidine, **13**).[37, 38]

The presence of a fluoro group in NRTIs makes them unique in terms of their chemical, biological and structural properties.

The active fluorinated nucleotide triphosphate moiety is incorporated into the growing DNA by cellular polymerases. Mostly, when fluorine is substituted at the 3'-position of the nucleosides, it often leads to compounds imparting good antiviral activities as a result of the DNA chain termination effect that the absence of the 3'-hydroxyl group causes. As most of the NRTIs follow the same mechanism of inhibition of the HIV reverse transcriptase, a representative schematic diagram (**Figure 1.6**) illustrating the mechanism of action of 3'-FddC, **13** that is reproduced from *J Clin Vir* by De Clercq is given below.[37]
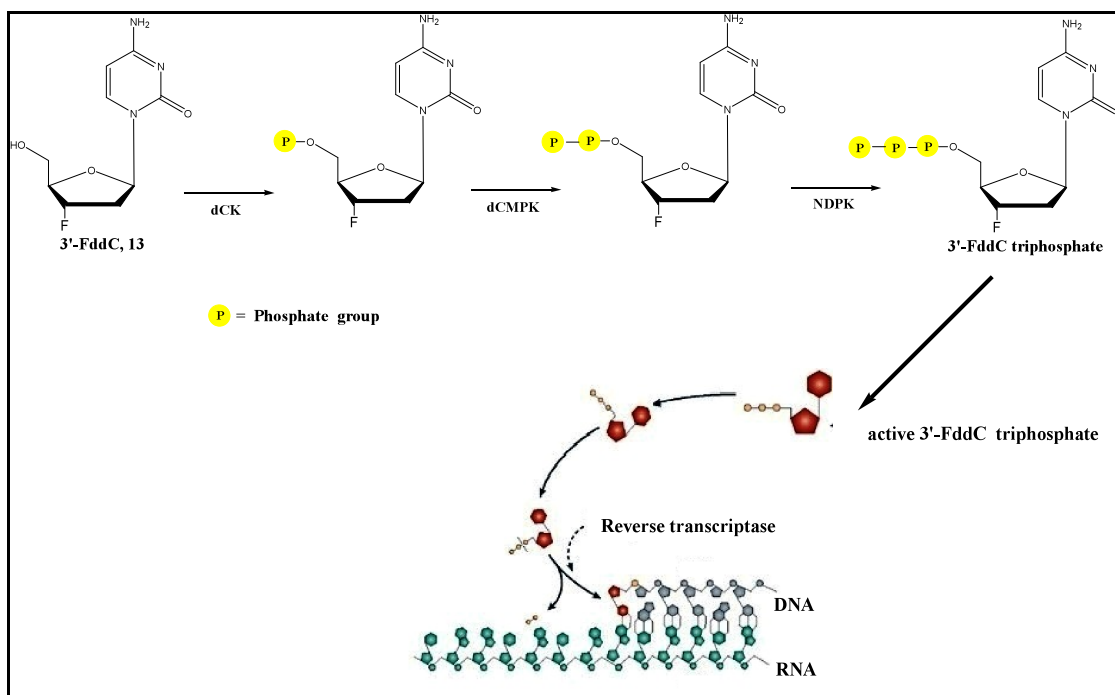
Figure 1.6: 3'FddC is phosphorylated by deoxycytidine kinase (dCK), deoxycytidine monophosphate kinase (dCMPK) and nucleotide diphosphate kinase (NDPK) to form FddC-monophosphate, FddC-diphosphate and the active FddC-triphosphate respectively. The viral enzyme reverse transcriptase catalyses the transcription of the retroviral RNA into DNA. By implanting the NRTI, FddC into DNA, the elongation of the growing viral DNA double strand is terminated via RT inhibition.[37]

It is worth noting that since the first phosphorylation step (monophosphorylation) of the nucleoside analogue is rate-limiting, certain therapeutic nucleosides exhibit poor antiviral activity due to the deprived monophosphorylation resulting from the hydrophilic nature of nucleosides. Consequently, their membrane permeability potential and bioavailability are lowered. Therefore, employing a prodrug strategy that could successfully deliver the nucleoside monophosphate analogues into cells by bypassing the rate-limiting monophosphorylation could boost the antiviral activity. Nowadays,

several prodrug strategies exist, that deliver nucleoside monophosphate analogues into cells and these are discussed in *Chapter 4.*

L-FMAU, **9** (clevudine) is a 2'-fluorinated L-nucleoside derivative of thymidine with potent anti-HBV (hepatitis B virus) activity. It is an oral antiviral agent for the treatment of chronic HBV.[38] Another clinically used antiviral drug is 3'-FddG, **11** the 3'-fluoro analogue of dideoxyguanosine that showed potent anti-HIV and anti-HBV activities *in vitro.*[39]

3'-fluoro-3'-deoxythymidine, **10** (FLT/ alovudine) has more potent antiretroviral activity *in vitro* than AZT (zidovudine). The substitution of fluorine for the azido group at the 3'-position of the deoxyribose ring generated FLT that was up to ten times more potent than AZT in preclinical studies.[40] FLT inhibits replication of highly NRTI-resistant HIV strains *in vitro* initially tested in the early 1990s.[41] However, dose-dependent drug safety concerns resulted in the initial development of alovudine to be halted due to toxicity.[42] One of the main aims of this research project was to apply the pro-nucleotide strategy (ProTide technology) on to FLT as elaborated in *Chapter 4*, in order to deliver the FLT monophosphate into target cells at high concentration with minimal toxicity.

1.1.2. Diagnostic aspects of fluorinated nucleosides

The application of fluorinated nucleosides as non-invasive imaging biomarkers of tumour cellular proliferation has been widely acknowledged. Biomarkers are playing an increasing role in the evaluation and validation of drug actions.[43] A biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention.[44] Non-invasive imaging biomarkers are the subset of biomarkers that are detectable and measurable through a variety of imaging technologies developed by health professionals and researchers to image *in vivo* body structures and functions for the purpose of disease diagnosis, staging, and drug discovery. The imaging techniques include x-ray diffraction, optical, ultrasound, radiographs, CT (Computed Tomography), PET (Positron Emission Tomography), SPECT (Single Photon Emission Computed Tomography) and MRI (Magnetic Resonance Imaging).[45]

Each of these modalities has its own advantages and disadvantages, as well as unique applications. Although a greater spatial resolution is obtained with optical, X-ray and MRI studies, these techniques are inherently insensitive for tracer studies, requiring tracer concentrations of $>10^{-3}$ molar. Whereas, for nuclear based imaging techniques, SPECT and PET require the use of radiopharmaceutical molecules that are labeled with radioactive nuclides and are injected at tracer concentrations of 10^{-11} molar for SPECT and 10^{-12} molar for PET respectively which would essentially have no pharmacologic effect. Therefore, PET emerges as a highly sensitive imaging technique. In combination with X-ray CT, PET is becoming an imaging modality of increasing demand.[46]

1.1.2.1. Positron Emission Tomography (PET)

PET is currently an invaluable tool used frequently in oncology, providing diagnosis, monitoring of treatment response, and imaging of tumour metabolic pathways.[47] The ability to image and measure biological and/or biochemical processes in a non-invasive manner could be of great value; especially at the stage of diagnosis and treatment of malignancies in the case of cancer and PET is rapidly becoming the imaging technique of choice.[48,49] Further evidence of the emerging importance of PET is provided by the continuing development of new PET centres at major hospital sites in UK, such as the Wolfson Brain Imaging Centre in Cambridge, Wolfson Molecular Imaging Centre in Manchester and PETIC at the University Hospital of Wales (established in 2009). The first medical application of positron emitters was reported more than 50 years ago in 1951 by Sweet at Massachusetts General Hospital, in which a simple probe was used to localize brain tumours with the use of coincidence detectors.[50]

1.1.2.1.1. Basic Principles of PET

This technology relies mainly on positron emitting short-lived radionuclides, which are radioactive atoms/isotopes that consist of an unstable nucleus undergoing spontaneous radioactive decay by the emission of γ rays and/or subatomic particles; hence achieving stability.

Positron emission is a type of beta decay characteristic of low or medium mass neutron-deficient nuclei. So in this radioactive decay, a proton (p) is converted to a neutron (n), a positron (e^+) and a neutrino (v) as shown in the formula below:

Positron emission $\quad p \longrightarrow n + e^+ + \nu$
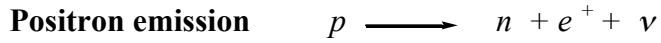
Positron emission occurs only if an energy of 1022 keV or greater is available; otherwise electron capture (EC) would take place.

Electron capture $\quad p + e^- \longrightarrow n + \nu$
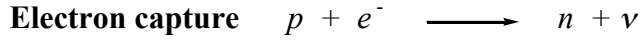
During positron emission, the emitted positron collides with the surrounding electron and as they are antiparticles, they annihilate each other. In the annihilation process, two gamma quanta of 511 keV are generated that travel in opposite directions at a relative angle of 180 ^{0}C and measured coincidently by a pair of detectors. These detector cameras, which are combined with powerful computing systems, convert the gamma-rays into electronic signals for data acquisition and image reconstruction and these consequently provide information about the distribution of the radiotracer in tissue.[51] In **Figure 1.7** that is reproduced from Diploma work by Heins, the basic principle of PET imaging is demonstrated.[52]
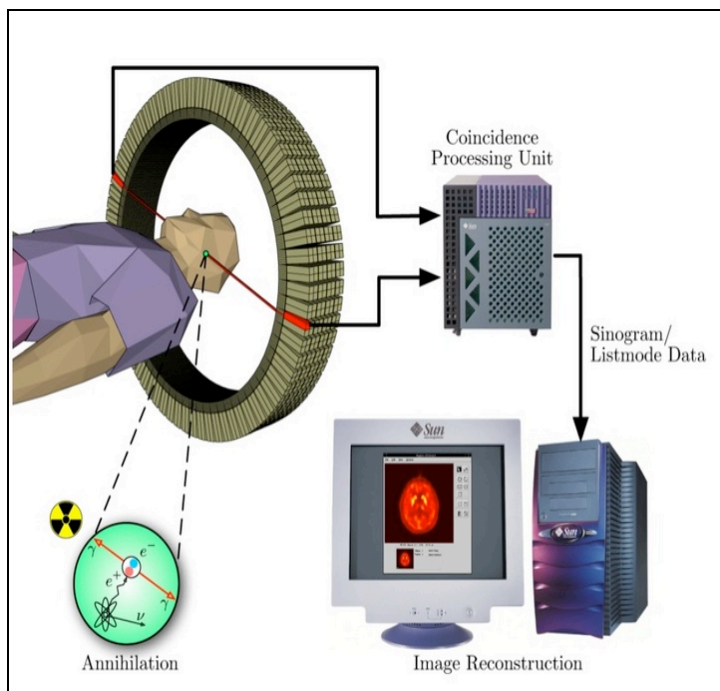
Figure 1.7: The basic principle of PET imaging.[52]

1.1.2.1.2. PET radiotracers

Conventionally, PET employs radiotracer molecules that are biomolecules such as glucose analogues, proteins or DNA-components (eg. thymidine) labeled with positron emitting radionuclides of fairly short half-lives. The most commonly used radionuclides are: ^{11}C ($t_{1/2}$ = 20 min), ^{13}N ($t_{1/2}$ = 10 min), ^{15}O ($t_{1/2}$ = 2 min), and ^{18}F ($t_{1/2}$ = 110 min). All these radioisotopes are produced by a cyclotron, which is ideally located as close as possible to the PET scanner because of the short half-lives of the positron-emitting radionuclides. Hence, amongst commonly used (non-metal) PET radioisotopes only ^{18}F can be transported from its production site as a result of its longer half-life compared to others.

The most common way of producing $^{18}F^-$ ions inside the cyclotron is the ^{18}O (p,n) ^{18}F reaction, in which $H_2{}^{18}O$ (oxygen-18-enriched water (>90%)) acts as the target material to be bombarded with proton beams to generate the $^{18}F^-$ ion in water and a neutron as shown in **Figure 1.8**. The required radioactive fluoride ion is available for further reactions to produce ^{18}F-based radiotracer molecules after separation from water. Since ^{18}F is generated as an anion, it is normally introduced into molecules via nucleophilic reactions.
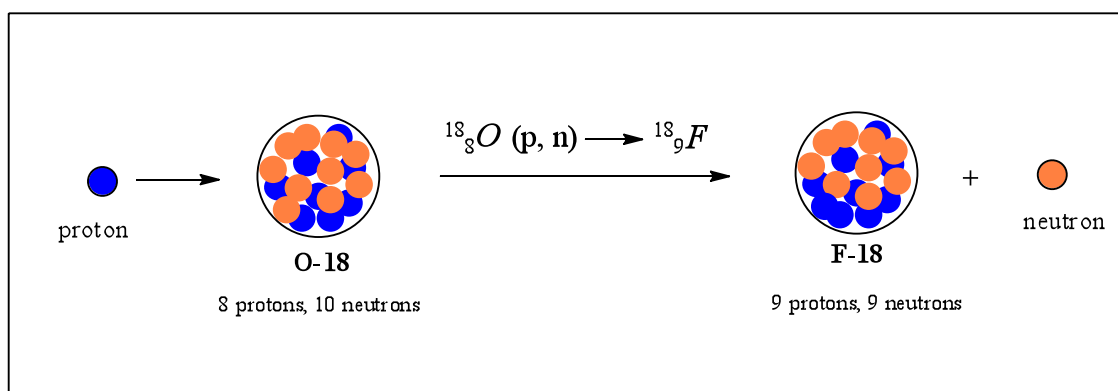
Figure 1.8: Production of an ^{18}F nucleus inside the cyclotron by the bombardment of a proton with oxygen-18-enriched water to form the radioactive ^{18}F nucleus consisting of nine protons and nine neutrons with the release of a neutron.

According to nature, carbon, oxygen, nitrogen, and hydrogen are the main elements of life and building blocks of nearly every molecule of biological importance. But since hydrogen has no radioactive isotope undergoing positron emission, the fluorine isotope is often used to replace the hydrogen atom for many positive reasons. The ^{18}F isotope is mostly considered as an ideal positron emitter as its cyclotron production is efficient and high activity is generated. Its longer optimum half-life allows multi-step synthesis of their respective tracers, purification and shipment. High specific activity, which is the

amount of radioactivity per gram or mole; high positron yield (e^+ 96.7%), low positron energy (635 keV), hence enabling image reconstruction at high resolution, are its additional advantages.[51] Another important fact is that the presence of the small and highly electronegative fluorine isotope, can lead to a more metabolically stable radiotracer molecule. For these purposes, investigators in the field of nuclear medicine have been striving to develop effective ^{18}F radiotracers for more than 30 years.[49, 53]

When designing a novel radiotracer molecule, factors such as an appropriate target, high *in vitro* affinity and selectivity for target, reliable radiolabeling, target accessibility, and low non-specific binding have to be considered. Hence the ideal radiotracer should result in good signal to noise ratio *in vivo*, appropriate *in vivo* pharmacokinetics, distribution and pharmacology, use of low quantities of labeled metabolites and finally should exhibit sensitivity and selectivity towards the target.[54]

Due to the relatively longer half-life of the positron emitting isotope fluorine-18 ($t_{1/2}=$ 110 min), fluorinated PET probes have emerged to be more prevalent.[55] The most widely used clinical PET tracer molecule to date is the ^{18}F-labeled fluorodeoxyglucose ([^{18}F] FDG, **14**) as seen in **Figure 1.9**. However, ^{18}F-FDG has not satisfied the need for a specific indicator of tumour cellular proliferation although it is very effective in locating many types of human solid tumours.[56] Once injected intravenously, this tracer molecule is transported into tumour cells and once internalised, **14** is rapidly and irreversibly phosphorylated by hexokinase. It cannot be further phosphorylated because of the presence of the fluorine atom and as a result ^{18}F-FDG-5'-monophosphate is trapped intracellularly. Thus, an [^{18}F] FDG PET image reveals those locations where glucose metabolic activity is highest.[57]
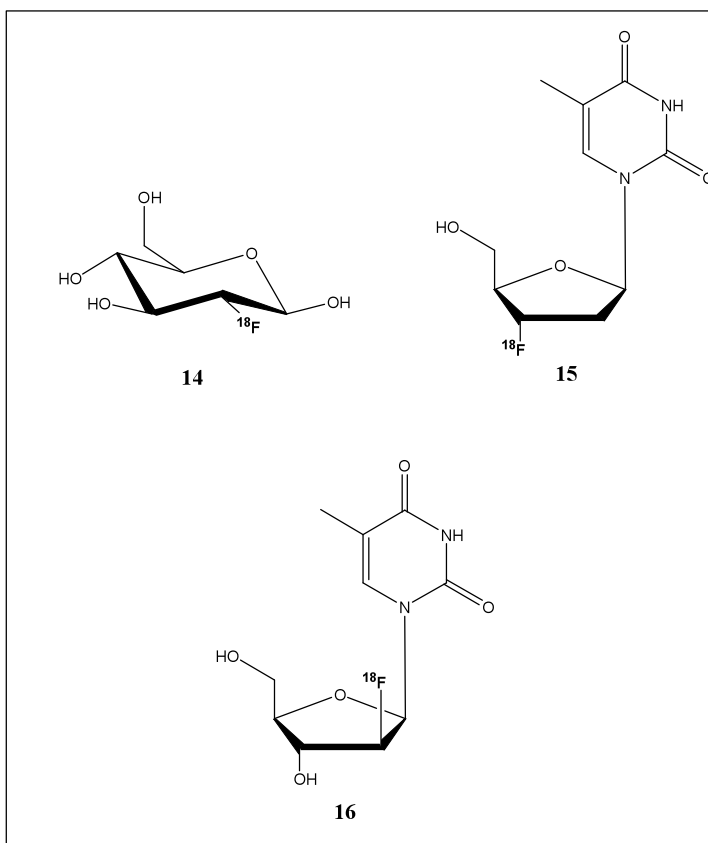
Figure 1.9: Some currently used clinical fluorinated PET biomarkers such as ^{18}F-FDG ([^{18}F]-2'-fluorodeoxyglucose, 1**4**), ^{18}F-FLT ([^{18}F]-3'-fluoro-3'-deoxythymidine, 1**5**) and ^{18}F-FMAU ([^{18}F]-1-[2'-fluoro-5-methyl-β-L-arabinofuranosyl]uracil, **16**).

Since 1998, the introduction of 3'-deoxy-3'-[^{18}F]fluorothymidine ([^{18}F]FLT, **15**) has drawn much attention as a cellular proliferation imaging biomarker in oncology for the purpose of PET imaging. [^{18}F]FLT, **15** is also one of the most commonly used fluorinated nucleoside PET tracer molecule and was developed as an alternative to the derivative [^{11}C]thymidine in order to overcome the *in vivo* catabolism of thymidine.[58] The PET tracer molecule, **15** is subject to intracellular phosphorylation by the enzyme

thymidine kinase-1 (TK1), which is expressed during the DNA synthesis phase (S-phase) of the cell cycle. Consequently, the resulting ^{18}F-labeled thymidine monophosphate gets trapped within the proliferating cells. Thus it has been shown that the selective uptake of **15** by tumour cells reflects the cell proliferation rate better than the widely used tumour-imaging agent, [^{18}F]FDG (**14**).[59] Their selective uptake in proliferating tumour cells is thought to be based on two mechanisms which are either the over-expression of human nucleoside transporters (hNT 1) that aids in the transport of FLT across the tumour cell membranes or the increased activity of proliferation-dependent thymidine kinase 1 enzyme that results in the FLT phosphorylation and accumulation of FLT monophosphates in tumour cells.[60]

[^{18}F]FLT has been reported to be inert to metabolic degradation unlike ^{18}F FDG.[61, 62] It has also been used to investigate various types of cancers for imaging tumour proliferation with PET and is currently in clinical use.[63] Besides tumour imaging, this radiotracer has also proved to be very useful to measure early response to therapy, which has been performed in both animal models and humans.[64,65] For the quantitative measurement of tumour cellular proliferation, [^{18}F]FLT PET was found to be superior to that of [^{18}F]FDG PET.[66]

1.2. Synthesis of ^{18}F-labeled Nucleosides for PET Imaging

1.2.1. Radioactive labeling with Fluorine-18

Amongst the radioactive nuclides present in existing PET biomarkers, fluorine-18 (^{18}F) has the most favourable nuclear properties and therefore it is the most widely used radioisotope for PET imaging. It has a relatively long half-life of 110 minutes compared to other positron-emitting radionuclides (e.g. 20 minutes for ^{11}C) which allows fluorine to be produced in a regional centre and to be distributed to local hospitals, provided that the radiochemical synthesis of the tracer molecule is rapid and efficient. For example, transporting a 1 GBq dose of ^{18}F activity gives a 250 MBq dose at a site four hours away. The half-life of ^{18}F also provides sufficient decay time for imaging and pharmacokinetic studies to be conducted. The ^{18}F radioisotope also gives the highest resolution PET images out of all the available positron emitters due to its relatively low positron energy (635 keV). In addition to that, both the high specific activity (radioactivity per gram of a radioactive material; units are Becquerel/gram or Bq/g) and high positron yield (β^+ 96.7%) of the ^{18}F radioisotope, make it an ideal positron emitter. Another advantage is that, the small and highly electronegative fluorine atom imparts some improved molecular properties to small molecules such as resistance to CYP-induced metabolism. A list of available positron emitting isotopes for PET imaging has been provided in **Table 1.1**.[67]

Table 1.1. Positron-emitting isotopes for PET imaging[67]

Isotope	$t_{1/2}$	Positron energy/ yield
^{86}Y	14.74 h	1254 keV (12.4 %)
^{64}Cu	12.7 h	653 keV (17.9 %)
^{66}Ga	9.4 h	4153 keV (49.3 %)
^{18}F	109.8 min	633 keV (96.7 %)
^{11}C	20.39 min	960 keV (99.8 %)
^{13}N	10 min	1199 keV (99.8 %)
^{15}O	2 min	1732 keV (99.8 %)

It can be noted from **Table 1.1** that transition metal isotopes such as ^{86}Y and ^{64}Cu have half-lives lasting for several hours, which may not be optimal as patients are exposed to a higher risk due to the prolonged exposure to radioactivity. Hence, these isotopes have not emerged as relevant in imaging biomolecules.

However, there are certain practical limitations involved in the chemical synthesis of ^{18}F-labeled molecules. Most syntheses of non-radiolabeled fluorinated drug molecules (containing the natural ^{19}F isomer) have the fluorine atom present at an early stage of synthesis for a multi-step process. Considering the longer exposure to radioactivity and the decay time of the fluorine isotope, ^{18}F incorporation into molecules at an early stage

is not feasible for the multi-step synthesis of ^{18}F-labeled compounds. The half-life of the positron-emitting ^{18}F atom (110 minutes) means that its introduction has to be near the end of the synthetic sequence, while the speed of reaction and purification have to be efficient and GMP (good manufacturing practice) compliant if high quality patient PET data are to be obtained. In general, the final radiolabeled tracer molecule should be synthesized, purified and formulated to pharmaceutical (injectable) quality within the half-life of the PET isotope.

1.2.1.1. Strategies for ^{18}F Radiolabeling

The variety of chemical methods by which the ^{18}F radioactive nuclide can be introduced into target molecules is rather limited. The two distinct areas, into which the main synthetic strategies behind ^{18}F labeling are crudely divided, are given below:

i.   direct fluorination, where the ^{18}F isotope is incorporated "directly" into the target molecule in one step; and

ii.   indirect fluorination which utilizes ^{18}F prosthetic groups and requires a multistep synthetic approach.

These prosthetic groups are typically small ^{18}F labeled alkyl or aryl groups that have reactive functional groups. They are exploited to react with more complex biological molecules, which are very difficult or unstable to undergo direct fluorination methods.[67-70] An example of indirect fluorination is the click reaction firstly adopted by Marik, Sutclife et al.[71] for the preparation of ^{18}F-labeled peptide fragments as shown in **Scheme 1.1**. In this method, an ^{18}F-labeled alkyne **18** was prepared by the ^{18}F$^-$ nucleophilic substitution reaction of an alkyne tosylate **17**. As conventional copper (I)-catalysed 1,3-

dipolar cycloadditions of azides and alkynes require reaction times of several hours to obtain high yields, rapid syntheses of PET biomarkers may not be feasible. However, the vast stoichiometric excess of the Cu^I catalyst and azide compared to the [^{18}F] alkyne **18,** resulted in good radiochemical yield (RCY: 54-99%) of the ^{18}F-labeled peptide **19** within 10 minutes at room temperature.[71]
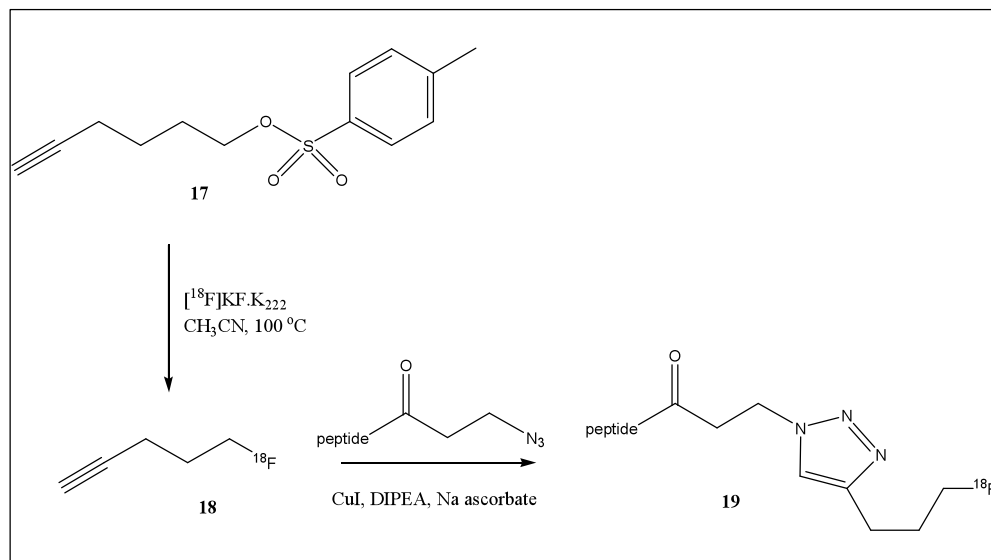
Scheme 1.1: Formation of ^{18}F-labeled peptide, **19** through the indirect ^{18}F labeling of the prosthetic alkyne group, **18**.

The direct ^{18}F labeling strategies can be subdivided into two main areas of fluorination, namely nucleophilic and electrophilic. Nevertheless, among these two methods, nucleophilic ^{18}F-fluorination reactions have emerged to be more dominant and important due to their greater selectivity and capability to give radioactive compounds.

1.2.1.2. Electrophilic Fluorine-18 Labeling Reactions

Nowadays, electrophilic ^{18}F-fluorinations are less favoured for the synthesis of PET tracer molecules mainly for two reasons. First of all, the disadvantages associated with the use of electrophilic reagents such as ^{18}F-F for labeling are considerable, as handling of fluorine poses significant safety issues resulting from the use of this highly toxic gas. Secondly, the high reactivity of ^{18}F-F in electrophilic substitution reactions can result in unpredictable and unspecific mixtures (e.g. through low regioselectivities) of ^{18}F-labeled products and hence the necessity of further product purification steps. It is also to be noted that the use of ^{18}F-F leads to mixtures of [19]F- and ^{18}F-containing product resulting in variable radiochemical yields and low specific activity.[72]

1.2.1.3. Nucleophilic ^{18}F-Substitution Reactions

Some of the most important ^{18}F nucleoside PET radiotracers such as [^{18}F]FLT ([^{18}F]-3'-fluoro-3'-deoxythymidine, **15**)[73], [^{18}F]FMAU ([^{18}F]-2'-fluoro-5-methyl-1-beta-D-arabinofuranosyl uracil, **20**)[74] and [^{18}F]FUdR ([^{18}F]-2'-fluoro-2'-deoxyuridine, **22**)[75] are routinely synthesized using nucleophilic ^{18}F-fluorination reactions. A selection of the most well-studied and utilized ^{18}F-labeled nucleoside tracers is shown in **Figure 1.10**.
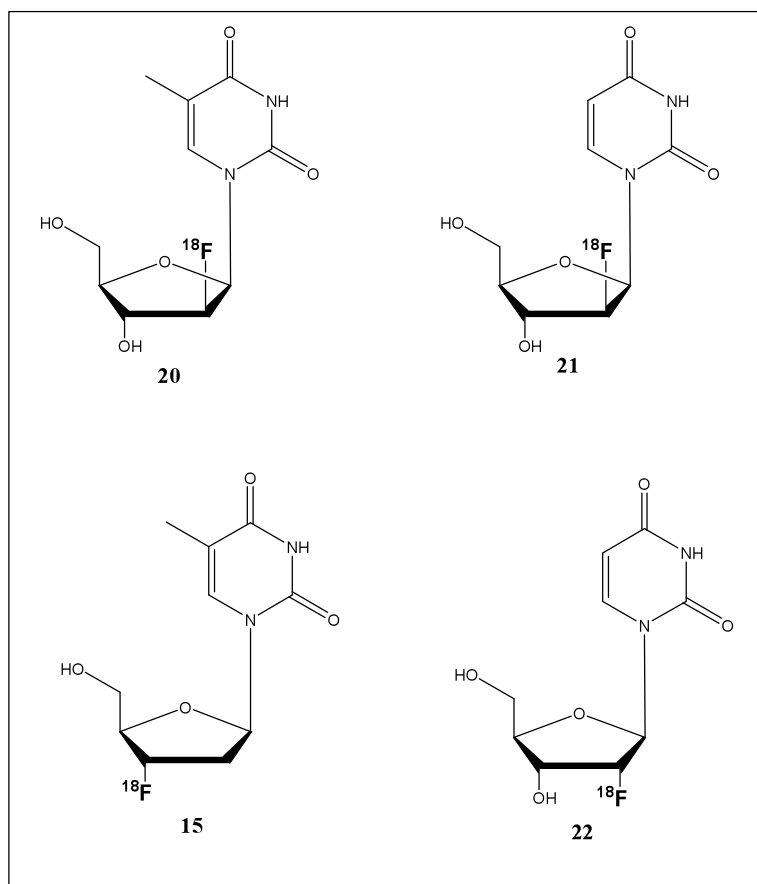
Figure 1.10: A selection of important ^{18}F-labeled nucleoside PET radiotracers including [^{18}F]FLT ([^{18}F]-3'-fluoro-3'-deoxythymidine, **15**), [^{18}F]FMAU ([^{18}F]-2'-fluoro-5-methyl-1-beta-D-arabinofuranosyl uracil, **20**), [^{18}F]FAU ([^{18}F]-2'-fluoro-1-beta-D-arabinofuranosyl uracil, **21**) and [^{18}F]FUdR ([^{18}F]-2'-fluoro-2'-deoxyuridine, **22**).

The nucleophilic [18]F⁻ is commonly produced by the nuclear reaction [18]O(p,n)[18]F from enriched [^{18}O]H$_2$O as mentioned in section *1.1.2.1.2*. The radioactive fluoride, [18]F⁻ from the target is then trapped on an ion exchange column which allows the recovery of [^{18}O]H$_2$O. The trapped [18]F⁻ is then eluted from the ion-exchange resin using potassium carbonate in a water/acetonitrile solution. However, the aqueous fluoride obtained is a

poor nucleophile because of its high degree of solvation. The subsequent addition of the phase-transfer reagent kryptofix-222 (K_{222}), followed by the removal of water through azeotropic evaporation with anhydrous acetonitrile has proven to be a crucial step in improving the nucleophilic reactivity of the [^{18}F]fluoride ion for nucleophilic substitution reactions. The azacryptand K_{222} forms a strong complex with the potassium cation as depicted in **Figure 1.11** and leaves the fluoride ion exposed and more nucleophilic when dissolved in a polar non-protic solvent such as DMF, DMSO, or acetonitrile.
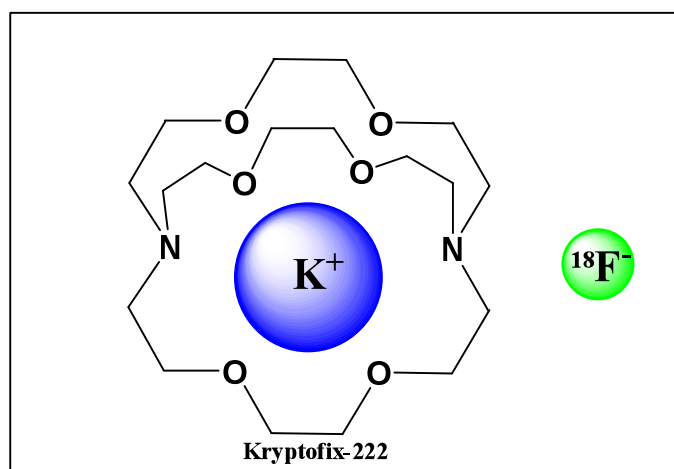
Figure 1.11: Complexation of the potassium ion (K^+) by the azacryptand kryptofix-222 isolating the radiolabeled fluoride ion ($^{18}F^-$).

In addition to [^{18}F]fluoride activation, the reacting precursor molecule is required to have a suitable leaving group. Direct ^{18}F nucleophilic labeling can be subdivided into two categories: aliphatic ^{18}F labeling and aromatic ^{18}F labeling strategies.[67]

1.2.1.3.1. Aromatic ^{18}F Nucleophilic Reactions

In comparison to the widely encountered electrophilic aromatic substitutions, direct nucleophilic aromatic substitution (S_NAr) reactions that are mainly used to generate ^{18}F-labeled aromatic compounds are inherently difficult reactions. This effect is due to the electrostatic repulsion between the electron-rich domain of most aromatic rings and an electron-rich/negatively-charged nucleophile (e.g. fluoride). Thus the aromatic rings are suitably activated by strong electron-withdrawing groups (e.g. nitro, cyano or acyl) on the *ortho* and/or *para* positions relative to the leaving group **(Scheme 1.2)**. Common leaving groups used in nucleophilic aromatic ^{18}F-fluorination reactions include nitro, trialkylamine, halogen, mesylate, tosylate, triflate or iodinium salts. Harsh reaction conditions such as temperature ranging from 120 to 160 ^oC and reaction solvents, DMSO or DMF are employed for this type of reaction.[76]

EWG (Electron withdrawing group): NO_2, CN, RC=O etc.

LG (Leaving group): NO_2, N$\overset{+}{\text{M}}$e$_3$, I+Ph etc.
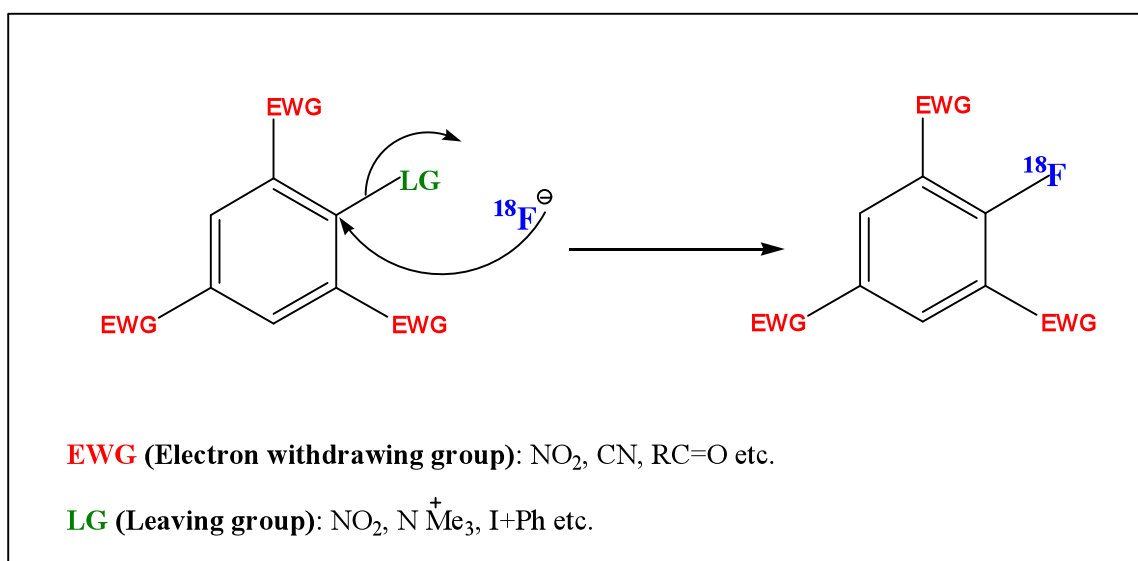
Scheme 1.2: Aromatic nucleophilic substitution of a leaving group facilitated by [^{18}F]Fluoride in the presence of EWGs on the aromatic ring.[76]

In recent years, the use of diaryl iodinium salts as precursors in nucleophilic aromatic ^{18}F-substitution reactions has emerged extremely useful for the synthesis of a range of simple [^{18}F]fluoroaromatic compounds in good radiochemical yields (RCY) and in short reaction times. An example is given in **Scheme 1.3**, which depicts the synthesis of 1-bromo-4-[^{18}F]fluorobenzene, **23**.[77]
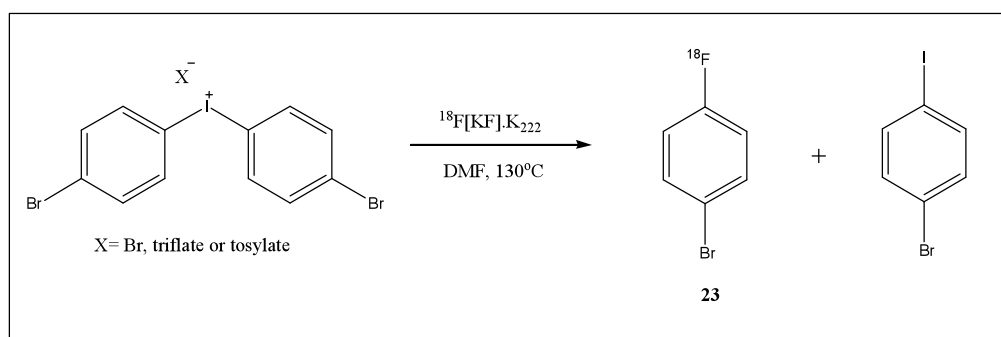
Scheme 1.3: Synthesis of **23** from bis(4-bromophenyl)iodonium salts.

1.2.1.3.2. Aliphatic ^{18}F Nucleophilic Substitution Reactions

For the introduction of ^{18}F into a range of molecules, especially nucleosides, direct aliphatic nucleophilic substitution reactions are generally used. The only requirement for aliphatic ^{18}F nucleophilic substitutions is a good leaving group, such as a triflate, tosylate, mesylate or nosylate. However, there is a need to protect any potentially competing sites of nucleophilic attack in the molecule (principally alcohol or amine groups). Hence, additional synthesis and purification steps are required resulting in longer synthesis times. The substrates react according to a pattern of typical S_N2 substitution reactions. Alternatively, certain constrained cyclic systems can be opened efficiently with [^{18}F]fluoride ion, for example, in the synthesis of the dimethoxytrityl

(DMTr) protected ^{18}F-labeled nucleoside, [^{18}F]5'-*O*-DMTrFLT, **25** from 5'-*O*-DMTr-2,3'-anhydrothymidine, **24** as shown in **Scheme 1.4**.
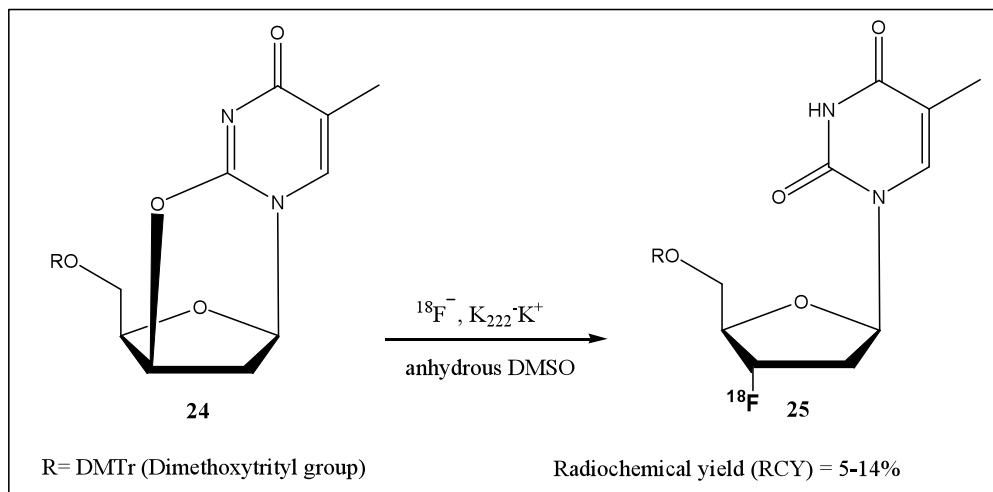
Scheme 1.4: [^{18}F]FLT synthesis from the ring opening of 5'-*O*-DMTr-2,3'-anhydrothymidine, **24** by [^{18}F]fluoride and deprotection of 5'-*O*-DMTr protected FLT, **25**.[79]

Aliphatic nucleophilic substitution reactions with [^{18}F]-fluoride ion are usually performed in a polar aprotic solvent such as acetonitrile, dimethylformamide (DMF), tetrahydrofuran (THF), dimethylsulfoxide (DMSO) etc. Amongst these solvents, acetonitrile is highly preferred as it can be easily removed *in vacuo*. Generally, protic solvents such as alcohols are not used for nucleophilic substitution reactions because of their ability to solvate the nucleophile, [^{18}F]fluoride and hence retarding its reactivity.[78,79]

1.2.2. Some new methods for Chemistry with [^{18}F]Fluoride ion

1.2.2.1. Enzyme-Catalyzed [^{18}F]Fluorination

The fact that enzyme-catalyzed chemical transformations produce organic or bioorganic molecules in a stereo-, regio-, and chemo-selective manner and that enzymes operate at physiological temperature in aqueous solution have been beneficial for application to the synthesis of ^{18}F-labeled tracers.[80] The first example of the formation of a C-F bond by an enzyme called fluorinase, which was isolated from the bacterium, *Streptomyces cattleya*, was reported by O'Hagan and co-workers.[81] Initial reports using the fluorinase enzyme for radiofluorinations gave low RCYs of about 1%. Subsequently, recent studies of ^{18}F-labeling using the over-expressed fluorinase enzyme for the synthesis of [^{18}F]-5'-fluoro-5'-deoxyadenine ([^{18}F]5'-FDA, **26**) brought about major improvements with 95% of radiochemical yield (RCY) in two hours (**Scheme 1.5**). However, the radiofluorination reaction was found to be in equilibrium. In order to give a good RCY, this equilibrium was to be shifted towards the synthesis of **26** by introducing a second enzyme, L-amino acid oxidase, which removes the L-methionine from the reaction. Hence, the equilibrium is shifted towards the right and thereby improving both the rate of reaction and overall enzyme expression. Such enzymatic methods for preparing radiolabeled compounds are particularly enticing, because of the high chemospecificity of enzymes and the simple purification methods utilized to remove the side products.[82]
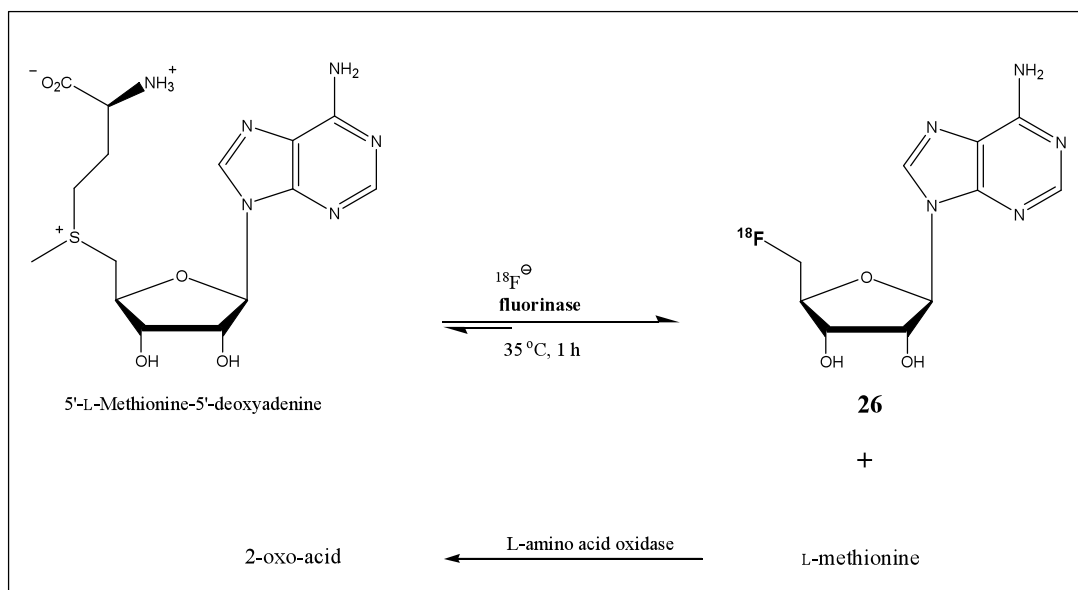
Scheme 1.5: The coupled fluorinase-oxidase enzymatic reaction for the synthesis of [^{18}F]-5'-fluoro-5'-deoxyadenine, ([^{18}F]5'-FDA, **26**).[82]

1.2.2.2. Microwave Enhancement of ^{18}F Incorporation

The use of microwaves to enhance radiochemistry has been gaining popularity as it offers speed, selectivity and efficiency of reactions. Upon the availability of controlled and user-friendly commercial microwave systems, earlier concerns on the safety regarding pressure and temperature controls have been addressed. Currently, some instruments also provide visual monitoring of the reaction. In comparison to conventional heating modes, the reaction times can be remarkably reduced from hours to minutes and seconds by adopting the microwave technology, which is ideally suited to the chemistry of ^{18}F incorporation, given the time constraints imposed by the half-life of ^{18}F isotope (110 min). Decreases in reaction time and in the amount of required precursors, and improved radiochemical yields (RCY) have proved to be of greater

advantage in the multi-step syntheses of radiotracer molecules. The decreased amount of precursor not only saves precious starting materials, but also reduces the product purification steps.[83-86] Examples of [^{18}F]- radiolabeling reactions under microwave conditions are shown in **Scheme 1.6**.[87-88]
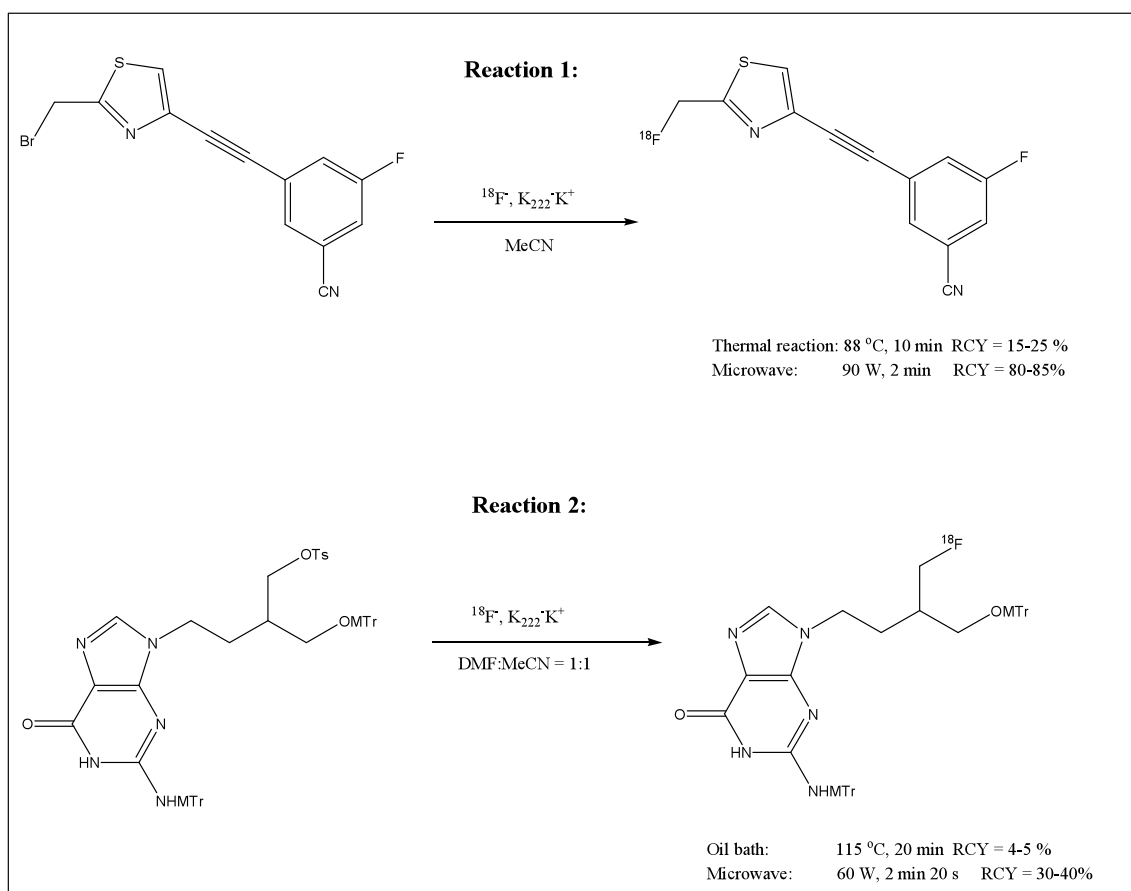
Scheme 1.6: Examples of microwave-enhanced nucleophilic substitutions used in the syntheses of ^{18}F-labeled tracers. In reaction 1, the microwave-assisted [^{18}F]-radiolabeling by bromine substitution resulted in better yields of 80-85% compared to the conventional thermal reaction (15-25%). In reaction 2, the microwave-assisted tosyl substitution by the [^{18}F]fluoro group, which is the first step for the radiosynthesis of the

radiotracer, 9-(4-[^{18}F]fluoro-3-hydroxymethylbutyl)guanine ([^{18}F]FHBG) gave better yields (30-40%) than the corresponding procedure with conventional heating (4-5%).

1.3. Main Aims and Objectives

The ability to monitor tumour progression and treatment response in cancer patients using the currently most frequently used PET imaging technique, has become a significant objective of modern cancer diagnosis and treatment. Although 3'-[^{18}F]fluoro-3'-deoxythymidine ([^{18}F]FLT) is one of the most important clinical PET biomarkers, the current methods for the radiosynthesis of [^{18}F]FLT have been facing obstacles such as low overall yields and time-consuming synthesis.

Thus, one of the main aims of this project was the development of a new efficient radiochemical synthetic route to the tracer molecule, [^{18}F]FLT. Initial studies for FLT synthesis were focused on the non-radioactive ^{19}F isomer by varying and optimizing conditions for the incorporation of different protecting groups and also fluoridation reactions. The introduction of fluorine into the synthesized precursor molecules by nucleophilic displacement reaction was to be carried out using both conventional thermal methods and microwave-assisted techniques. Afterwards, the feasible reaction conditions were to be applied for the radiosynthesis of [^{18}F]fluorine labeled FLT.

Another objective was to implement optimization studies on the fluoridation step and the synthesis of [18F]FLT Pronucleotide phosphoramidates ([18F]FLT ProTides) as a new class of diagnostic PET imaging agents using conventional thermal methods, which can be applied later on for the radioactive [18F] labeling studies of [18F]FLT ProTide molecules.

The synthesis of a series of 19F-FLT based ProTide analogues and their biological *in vitro* evaluation in various human cell lines as potential new therapeutic agents that can efficiently cross cell membranes and exhibit minimal toxicity than the parent nucleoside FLT, was also one of the major goals of this project.

References:

1. Manta, S.; Agelis, G.; Botic, T.; Cencic, A.; Komiotis, D. Unsaturated fluoro-ketopyranosyl nucleosides: synthesis and biological evaluation of 3-fluoro-4-keto-beta-d-glucopyranosyl derivatives of N(4)-benzoyl cytosine and N(6)-benzoyl adenine. *Eur J Med Chem.* **2008**, *43* (2), 420-428.

2. Li, W.; Yin, X.; Schneller, S. W. 5'-Fluoro-5'-deoxyaristeromycin. *Bioorg Med Chem Lett.* **2008**, *18* (1), 220-222.

3. Alauddin, M. M.; Gelovani, J. G. Pyrimidine Nucleosides in Molecular PET Imaging of Tumour Proliferation. *Curr Med Chem.* **2010**, *17*, 1010-1029.

4. Chu, C.K.; Baker, D.C. *Nucleosides, Nucleotides as Antitumour and Antiviral Agents.*, Plenum Press: New York, **1993**.

5. Van Rompay, A. R.; Johansson, M.; Karlsson, A. Substrate specificity and phosphorylation of antiviral and anticancer nucleoside analogues by human deoxyribonucleoside kinases and ribonucleoside kinases. *Pharmacology and Therapeutics.* **2003**, *100*, 119-139.

6. Berg, J. M.; Tymoczko, J. L.; Stryer, L. *Biochemistry*, 5th ed.; W.H. Freeman and Company: New York, **2003**, 118-142.

7. Balzarini, J.; Egberink, H.; Hartmann, K.; Cahard, D.; Vahlenkamp, T.; Thormar, H.; De Clercq, E.; McGuigan, C. Antiretrovirus specificity and intracellular metabolism of 2′,3′-didehydro-2′, 3′-dideoxythymidine (d4T, stavudine) and its d4T 5′-monophosphate triester prodrug. *Mol Pharmacol.* **1996**, *50*, 1207−1213.

8. Al-Madhoun, A.S.; Tjarks, W.; Erikson, S. The role of thymidine kinases in the activation of pyrimidine nucleoside analogues. *Mini-Rev Med Chem.* **2004**, *4*, 341-50.

9. Deng, H.; O'Hagan, D.; Schaffrath, C. Fluorometabolite biosynthesis and the fluorinase from Streptomyces cattleya, *Nat Prod Rep.* **2004**, *21*, 773-784.

10. Dolbier, W.R. Fluorine chemistry at the millennium. *J Fluor Chem.* **2005**, *126*, 157-163.

11. Hunter, L. The C–F bond as a conformational tool in organic and biological chemistry. *Beilstein J Org Chem.* **2010**, *6* (38), 1-14.

12. Liu, P.; Sharon, A.; Chu, C.K. Fluorinated nucleosides: Synthesis and biological implications. *J Fluor Chem.* **2008**, *129*, 743-766.

13. Soloshonok, V.A. *Fluorine-containing Synthons,* ACS Symposium Series 911, Ed. Oxford University Press: Washington DC, **2005**.

14. O'Neill, P. M.; Kitteringham, N. R.; Park, K.B. Metabolism of Fluorine-containing drugs. *Annu Rev Pharm. Tox.* **2001**, *41,* 443-470.

15. O'Hagan, D.; Harper, D.B. Fluorine containing natural products, *J Fluor Chem.,* **1999**, *100,* 127-133.

16. Berkowitz, D.B.; Bose, M. (Alpha-monofluoroalkyl) phosphonates: a class of isoacidic and tunable mimics of biological phosphonates, *J Fluor Chem.*, **2001**, *112,* 13-33.

17. Fischman, A.J.; Babich, J.W.; Bonab, A.A. et al. Pharmacokinetics of [^{18}F] trovafloxacin in healthy human subjects studied with positron emission tomography. *Antimicrob Agents Chemother.,* **1998**, *42*, 2048-2054.

18. Ojima, I.; McCarthy, J.R.; Welch, J.T. *Biomedical Frontiers of Fluorine Chemistry,* **1996***, 639*, ACS Symposium Series.

19. Lee, K.; Choi, Y.; Gumina, G.; Zhou, W.; Schinazi, R.F.; Chu, C.K. Structure-activity relationships of 2'-fluoro-2',3'-unsaturated D-nucleosides as anti-HIV-1 agents. *J Med Chem.* **2002***, 45,* 1313-1320.

20. Zhou, W.; Gumina, G.; Choi, Y.; Wang, J.; Schinazi, R.F.; Chu, C.K. Synthesis, structure-activity relationships, and drug resistance of beta-D-3-fluoro-2,3-unsaturated nucleosides as anti-HIV Agents. *J Med Chem.* **2004**, *47*, 3399-3408.

21. Heidelberger, C.; Danenberg, P. V.; Moran, R. G. Fluorinated pyrimidines and their nucleosides. *Adv Enzymol Relat Areas Mol Biol.*, **1983**, *54*, 58–119.

22. Huang, P.; Robertson, L. E.; Wright, S.; Plunkett, W. High molecular weight DNA fragmentation: a critical event in nucleoside analogue-induced apoptosis in leukemia cells. *Clin Cancer Res,* **1995**, *1* (9)*,* 1005–1013.

23. Liu, P.; Sharon, A.; Chu, C. K. Fluorinated Nucleosides: Synthesis and Biological implications. *J Fluor Chem.* **2008**, *129* (9), 743-766.

24. Montgomery, J. A.; Shortnacy-Fowler, A. T.; Clayton, S. D., Riordan, J. M.; Secrist, J. A. 3rd. Synthesis and biological activity of 2'-fluoro-2-halo derivatives of 9-beta-D-arabinofuranosyladenine. *J Med Chem.* **1992**, *35*, 397–401.

25. Faderl, S.; Gandhi, V.; Kantarjian, H. M. Potential role of novel nucleoside analogs in the treatment of acute myeloid leukemia. *Curr Opin Hematol.* **2008**, *15*, 101–107.

26. Bonate, P. L.; Arthaud, L.; Cantrell, W. R. Jr.; Stephenson, K.; Secrist, J. A. III.; Weitman, S. Discovery and development of clofarabine: a nucleoside analogue for treating cancer. *Nat Rev Drug Discov.,* **2006**, *5*, 855-863.

27. Duschinsky, R.; Plevan, E.; Heidelberger, C. The synthesis of 5-fluoro pyrimidines. *J Am Chem Soc.* **1957**, *79*, 4559–4560.

28. Isanbor, C.; O'Hagan, D. Fluorine in medicinal chemistry: a review of anti-cancer agents. *J Fluor Chem.* **2006**, *127*, 303–319.

29. Xie, C.; Plunkett, W. Metabolism and actions of 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl) adenine in human lymphoblastoid cells. *Cancer Res.* **1995**, *55*, 2847–2852.

30. Cariveau, M. J.; Stackhouse, M.; Cui, X. L.; Tiwari, K.; Waud, W.; Secrist, J. A. 3rd.; Xu, B. Clofarabine acts as radiosensitizer in vitro and in vivo by interfering with DNA damage response. *Int J Rad Onco Bio Phys.* **2008**, *70*, 213–220.

31. Heidelberger, C.; Chaudhuari, N.K.; Danenberg, P. et al. Fluorinated pyrimidines: a new class of tumour inhibitory compounds. *Nature,* **1957**, *179,* 663–666.

32. Peters, G.J.; Backus, H.H.J.; Freemanthle, S.; Van der Triest, B. *et al.* Induction of thymididylate synthase as a 5-fluorouracil resistance mechanism. *Biochem Biophys Acta*. **2002,** *1587,* 194–205.

33. Mader, R. M.; Mueller, M. S.; Steger, G.G. Resistance to 5-Fluorouracil. *Gen Pharm,* **1998**, *31*, 661–666.

34. Plunkett, W.; Huang, P.; Xu, Y. Z.; Heinemann, V.; Grunewald, R.; Gandhi, V. Gemcitabine: metabolism, mechanisms of action, and self-potentiation. *Semin Oncol*, **1995**, *22*, 3–10.

35. De Clercq, E. New approaches towards anti-HIV chemotherapy. *J Med Chem,* **2005**, *48*, 1297-1313.

36. Hao, Z.; Cooney, D. A.; Farquhar, D.; Perno, C. F.; Zhang, K.; Masood, R.; Wilson, Y.; Hartman, N. R.; Balzarini, J.; Johns, D. G. Potent DNA chain termination activity and selective inhibition of human immunodeficiency virus reverse transcriptase by 2', 3'-dideoxyuridine-5'-triphosphate. *Mol Pharm.* **1989**, *37,* 157-163.

37. De Clercq, E. Antiviral drugs in current clinical use. *J Clin Virol.* **2004**, *30,* 115-133.

38. Marcellin, P.; Mommeja-Marin, H.; Sacks, S. L.; Lau, G. K.; Sereni, D.; Bronowicki, J. P.; Conway, B.; Trepo, C.; Blum, M. R.; Yoo, B. C.; Mondou, E.; Sorbel, J.; Snow, A.; Rousseau, F.; Lee, H. S. A phase II dose-escalating trial of clevudine in patients with chronic hepatitis B. *Hepatology*, **2004**, *40*, 140–148.

39. Herdewijn, P.; Balzarini, J.; Baba, M.; Pauwels, R.; Van Aerschot, A.; Janssen, G.; De Clercq, E. Synthesis and anti-HIV activity of different sugar-modified pyrimidine and purine nucleosides. *J Med Chem*, **1988**, *31,* 2040-2048.

40. Kong, X. B.; Zhu, Q. Y.; Vidal, P. M. ; Watanabe , K.A. ; Polsky, B. ; Armstrong, D ; Ostrander, M. ; Lang Jr, S.A. ; Muchmore, E. ; Chou, T.C. Comparisons of anti-human immunodeficiency virus activities, cellular

transport, plasma and intracellular pharmacokinetics of 3'-fluoro-3'-deoxythymidine and 3'azido-3'-deoxythymidine. *Antimicrob Agents Chemother,* **1992**, *36,* 808-18.

41. Ghosn, J.; Quinson, A. M.; Sabo, N.D. et al. Antiviral activity of low-dose alovudine in antiretroviral- experienced patients: results from a 4-week randomized, double-blind, placebo-controlled dose-ranging trial. *HIV Medicine,* **2007**, *8, 142-147*.

42. Flexner, C.; Van der Horst, C.; Jacobson, M. A. et al. Relationship between plasma concentrations of 3'-deoxy-3'-fluorothymidine (Alovudine) and antiretroviral activity in two concentration controlled trials. *J Infect Dis,* **1994**, *170,* 1394–1403.

43. Neves, A. A.; Brindle, K. M. Assessing responses to cancer therapy using molecular imaging. *Biochim Biophys Acta*, **2006**, *1766*, 242–61.

44. Strimbu, K.; Tavel, J.A. What are biomarkers. *Curr Opin HIV AIDS*, **2010**, *5*, 463-466.

45. Waerzeggers, Y. Specific biomarkers of receptors, pathways of inhibition and targeted therapies: clinical applications. *Brit J Radiol.* **2011**, *84*, 179-195.

46. Alford, R.; Ogawa, M.; Choykea, P. L.; Kobayashi, H. Molecular probes for the *in vivo* imaging of cancer. *Mol BioSyst,* **2009**, *5*, 1279-91.

47. Strauss, L.G.; Conti, P. S. The Applications of PET in clinical oncology. *J Nucl Med.* **1991**, *32*, 623-48.

48. Christman, D.; Crawford, E.J.; Friedkin, M.; Wolf, A.P. Detection of DNA synthesis in intact organisms with positron-emitting [*Methyl*-11C]thymidine. *Proc Natl Acad Sci.* **1972**, *69*, 988-92.

49. Sun, H.; Sloan, A.; Mangner, T.J.; Vaishampayan, U.; Muzik, O.; Collins, J.M.; Douglas, K.; Shields, A.F. Imaging DNA synthesis with [18F]FMAU and positron emission tomography in patients with cancer. *Eur J Nucl Med Mol Imaging,* **2005**, *32*, 15-22.

50. Sweet, W.H. The use of nuclear disintegration in diagnosis and treatment of brain tumours. *N Engl J Med.,* **1951**, *245,* 875-878.

51. Vaalburg, W.; Paans, A.M.J.; vanWaarde, A.; Elsinga, P.H.; Willemsen, A.T.M. Positron emission tomography: the conceptual idea using a multidisciplinary approach. *Methods,* **2002**, *27*, 195-207.

52. Heins, P. Sparse Model-Based Reconstruction in dynamic Positron Emission Tomography, Diploma work, Wilheims University Muenster, **2011**, 2.

53. Crawford, E. J.; Christman, D.; Atkins, H.; Friedkin, M.; Wolf, A. P. Scintigraphy with positron-emitting compounds. *Int J Nucl Med Biol.,* **1978**, *5*, 61-69.

54. Gee, A.D. Principles of Radiotracer design for Positron Emission Tomography (PET). *Am Pharm Rev.,* 1-3.

55. Adam, M.J.; Wilbur, D.S. Radiohalogens for imaging and therapy. *Chem Soc Rev.,* **2005**, *34*, 153-163.

56. Moon, B.S.; Lee, K.C.; An, C. I. et al. Preparation of 3'-deoxy-3'-[18F]fluorothymidine in ionic liquid, [bmim][OTf], *J Label Compd Radiopharm.,* **2006**, *49*, 287-293.

57. Daniels, S.; MdTohid, S. F.; Velanguparackel, W; Westwell, A.D. The role and future potential of fluorinated biomarkers in positron emission tomography. *Expert Opin Drug Discov.,* **2010**, *5*, 291-304.

58. Grierson, J.R.; Shields, A.F., Radiosynthesis of 3'-deoxy-3'-[^{18}F]fluorothymidine: [^{18}F]FLT for imaging of cellular proliferation *in vivo*. *Nucl Med Biol.,* **2000,** *27*, 143-156.

59. Windhorst, A. D.; Klein, P.J.; Eisenbarth, J.; Oeser, T.; Kruijer, P.S.; Eisenhut, M. 3' Sulfonylesters of

2,5' anhydro 1 (2 deoxy beta d threo pentofuranosyl)thymine as

precursors for the synthesis of [18F]FLT:syntheses and radiofluorination trials. *Nucl Med Biol.,* **2008**, *35*, 413-423.

60. Cass, C.E.; Paproski, R.J.; Ng, A.M.L.; Yao, S.Y.M.; Graham, K.; Young, J.D. The role of Human Nucleoside Transporters in Uptake of 3'-Deoxy-3'-fluorothymidine. *Mol Pharm.,* **2008**, *74*, 1372-1380.

61. Shields, A.F.; Grierson, J. R.; Dohmen, B.M. et al. Imaging proliferation *in vivo* with [^{18}F]FLT and positron emission tomography. *Nat Med.,* **1998**, *4*, 1334-1336.

62. Salskov, A.; Tammisetti, V.S.; Grierson, J.; Vesselle, H. FLT: Measuring Tumour Cell Proliferation In Vivo with Positron Emission Tomography and 3'-Deoxy-3'-[^{18}F]Fluorothymidine. *Seminars in Nuclear Medicine,* **2007**, 429-439.

63. Moon, D.H.; Ryu, J.S.; Yeo, J.S. et al, 3'-deoxy-3'-[^{18}F]fluorothymidine PET for the diagnosis and grading of brain tumours. *Eur J Nucl Med Mol Imaging,* **2005**, *32*, 653-659.

64. Kenny, L.; Coombes, R.C.; Vigushin, D.M.; Al-Nahhas, A.; Shousha, S.; Aboagye, E.O., Imaging early changes in proliferation at 1 week post chemotherapy: a pilot study in breast cancer patients with 3'-deoxy-3'-[18F]fluorothymidine positron emission tomography. *Eur J Nucl Med Mol Imaging.,* **2007,** *34*, 1334-1338.

65. Pio, B. S.; Park, C.K.; Pietras, R.; Hsueh, W.; Satyamurthy, N.; Silverman, D.H.S., Usefulness of 3'-deoxy-3'-[18F]fluorothymidine with positron emission tomography in predicting breast cancer response to therapy. *Mol Imag Biol.,* **2006**, *8,* 36-42.

66. Collindridge, D.R. 3'-deoxy-3'-[18F]fluorothymidine as a new marker for monitoring tumour response to antiproliferative therapy *in vivo* with positron emission tomography. *Cancer Res.,* **2003**, *63,* 3791-3798.

67. Miller, P. W. ; Long, N. J. ; Vilar, R. ; Gee, A. D. Synthesis of ^{11}C, ^{18}F, ^{15}O, and ^{13}N Radiolabels for Positron Emission Tomography. *Angew. Chem. Int. Ed.* **2008**, *47,* 8998-9033.

68. Vaalburg, W.; Paans, A.M.J.; vanWaarde, A.; Elsinga, P.H.; Willemsen, A.T.M. Positron emission tomography: the conceptual idea using a multidisciplinary approach. *Methods,* **2002**, *27*, 195-207.

69. Shah, P.; Westwell, A. D. The role of fluorine in medicinal chemistry. *J Enz Inhib Med Chem.* **2007**, *22*, 527-540.

70. Pike, V. W.; Lu, S.; Cai, L. Chemistry with [^{18}F]Fluoride Ion. *Eur J Org Chem.* **2008**, 2853-2873.

71. Marik, J.; Hausner, S.H.; Fix, L.A.; Gagnon, M.K.J.; Sutcliffe, J.L. Solid phase synthesis of 2-[18F]fluoropropionyl peptides. *Bioconjugate Chem.* **2006**, *17*, 1017.

72. Yun, M. High radiochemical yield synthesis of 3'-deoxy-3'-[^{18}F]fluorothymidine using(5'-O-dimethoxytrityl-2'-deoxy-3'-O-nosyl-ß-D-threopentofuranosyl)thymine and its 3-N-BOC-protected analogue as a labeling precursor. *Nucl Med Biol.* **2003**, *30*, 151-7.

73. Price, P. M.; Aboagye, E.O.; Kenny, L. M. Positron Emission Tomography Imaging of Cell Proliferation in Oncology. *Clin Oncol.* **2004**, *16,* 176-185.

74. Shields, A.F.; Anderson, L.; Klecker, R.W.; Mangner, T.J. Synthesis of 2'-deoxy-2'-[18F]fluoro-beta-D-arabinofuranosyl nucleosides, [18F]FAU, [18F]FMAU, [18F]FBAU and [18F]FIAU as potential PET agents for imaging cellular proliferation. Synthesis of [18F]labeled FAU, FMAU, FBAU, FIAU. *Nucl Med Biol.* **2003**, *30*, 215-224.

75. Deng, W. P. Molecular imaging with 123I-FIAU, 18F-FUdR, 18F-FET and 18F-FDG for monitoring herpex simplex virus type 1 thymidine kinase and ganciclovir prodrug activation gene therapy of cancer. *J Nucl Med.* **2006**, *47*, 1161-71.

76. Daniels, S.; Md Tohid, S. F.; Velanguparackel, W.; Westwell, A. D. The role and future potential of fluorinated biomarkers in positron emission tomography. *Expert Opin Drug Discov.* **2010**, *5*, 291-304.

77. Ermert, J.; Hocke, C.; Ludwig, T.; Gail, R.; Coenen, H.H. Comparison of pathways to the versatile synthon of no-carrier-added 1-bromo-4-[^{18}F]fluorobenzene. *J Lab Compd Radiopharm.* **2004**, *47*, 429-441.

78. Pike, V. W.; Lu, S.; Cai, L. Chemistry with [^{18}F]Fluoride Ion. *Eur J Org Chem.* **2008**, 2853-2873.

79. Machulla, H. J.; Blocher, A.; Kuntzsch, M.; Piert, M.; Wei, R.; Grierson, J. R. Simplified labeling approach for synthesizing 3'-deoxy-3'-[^{18}F]fluorothymidine ([^{18}F]FLT). *J Radioanal Nucl Chem.* **2000**, *243*, 843–846.

80. Koeller, K. M.; Wong, C. H. Enzymes for chemical synthesis. *Nature,* **2001**, *409*, 232–240.

81. Martarello, L.; Schaffrath, C.; Deng, H.; Gee, A. D.; Lockhart, A.; O'Hagan D. The first enzymatic method for C-^{18}F bond formation: the synthesis of 5'-[^{18}F]-fluoro-5'-deoxyadenosine for imaging with PET. *J Lab Compd Radiopharm.,* **2003**, *46*, 1181-9.

82. Deng, H.; Cobb, S. L.; Gee, A. D.; Lockhart, A.; Martarello, L.; McGlinchey, R. P.; O'Hagan, D.; Onega, M. Fluorinase mediated C-18F bond formation, an enzymatic tool for PET labeling. *Chem Comm.* **2006**, 652–654.

83. Elander, N.; Jones, J. R; Lu, S. Y.; Stone-Elander, S. Microwave-enhanced radiochemistry. *Chem Soc Rev.* **2000**, *29*, 239–249.

84. Stone-Elander, S.; Elander, N. Microwave applications in radiolabeling with short-lived positron emitting radionuclides. *J Lab Compd Radiopharm.* **2002**, *45*, 715–746.

85. Jones, J. R.; Lu, S. Y. Microwaves in Organic Chemistry, 2nd edition (Ed.: A. Loupy), Wiley-VCH, Weinheim, **2006**, 820–859.

86. Kappe, C. O.; Stadler, A. Microwaves in Organic and Medicinal Chemistry, Wiley-VCH, Weinheim, **2005**.

87. Lu, S. Y.; Hong, J.; Musachio, J. L.; Chin, F. T.; Vermeulen, E. S.; Wikström, H. V.; Pike V. W. Alternative methods for labeling the 5-HT$_{1A}$ receptor agonist, 1-[2-(4 fluorobenzoylamino)ethyl]-4-(7-methoxynaphthyl)piperazine (S14506), with carbon-11 or fluorine-18, *J Lab Compd Radiopharm.* **2005**, *48*, 971–981.

88. Lemaire, J. C.; Cantineau, R.; Guillaume, M.; Plenevaux, A.; Christiaens, L. Fluorine-18-Altanserin: A radioligand for the study of serotonin receptors with PET: Radiolabeling and *in vivo* biological behavior in rats. *J Nucl Med.* **1991**, *32*, 2266–2272.

Chapter 2

2. Synthetic routes to ^{19}F-FLT

2.1. Brief rationale

One of the main purposes of this project included the development and optimization of a fast and efficient chemical synthetic route to ^{19}F- FLT, which can be adapted to the radioactive production of the clinically used tumour proliferation biomarker 3'-[^{18}F]-fluoro-3'deoxythymidine, [^{18}F]-FLT. Fortunately, the synthetic chemistry of the positron emitting isomer, ^{18}F mirrors that of the non-radioactive natural isomer, ^{19}F. Therefore, the establishment and optimization of reaction conditions can be carried out under conventional thermal conditions without the need for radiochemical facilities, with final transfer of the optimal synthetic route and purification conditions to the radiochemical facility of Cardiff University, Wales Research and Diagnostic Positron Emission Tomography Imaging Centre (PETIC) at the University hospital.

This chapter has mainly two different approaches for synthesizing ^{19}F-FLT:

1. Use of different nucleophilic fluorides using various protecting group strategies and reaction conditions, in which work was designed to lay the framework for translation of [^{18}F]fluoride radiochemistry to [^{18}F]FLT.
2. Use of electrophilic fluorinating agents such as DAST (diethylaminosulphur trifluoride) or XTalFluor reagents to generate FLT in sufficient quantities for ProTide studies.

2.2. ^{19}F- FLT synthesis for [^{18}F]fluoride radiochemistry studies

2.2.1. FLT synthesis via Mitsunobu reaction with 5'-O-protection

Since the furanosyl moiety of the thymidine nucleoside consists of both primary and secondary hydroxyl groups at its 5' and 3' positions respectively, the primary hydroxyl functionality had to be protected with a suitable protecting group that allows the regioselective halogenation at the 3' position. In order to carry out a successful reaction, it was essential that the appropriate protecting group met certain conditions such as

- i. simple and straightforward process of protection
- ii. stability to reaction conditions
- iii. easy and fast removal ensuring a good yield of the expected product.

A generic representation of the synthetic route to FLT involving protection and deprotection of the hydroxyl group at the 5'- position of the thymidine nucleoside, **1** is shown in **Scheme 2.1**. The route consists of four major steps:

- (a) protection of the 5'-hydroxyl group of thymidine
- (b) creation of a more electrophilic centre at the 3'-nucleoside position by the formation of a six-membered 2,3'-anhydro ring through Mitsunobu reaction
- (c$_1$) nucleophilic fluoridation reaction at the 3' position after formation of the anhydro ring or
- (c$_2$) direct nucleophilic substitution of the 3' hydroxyl group and
- (d) deprotection of 5'-hydroxyl group to generate FLT, **10**.
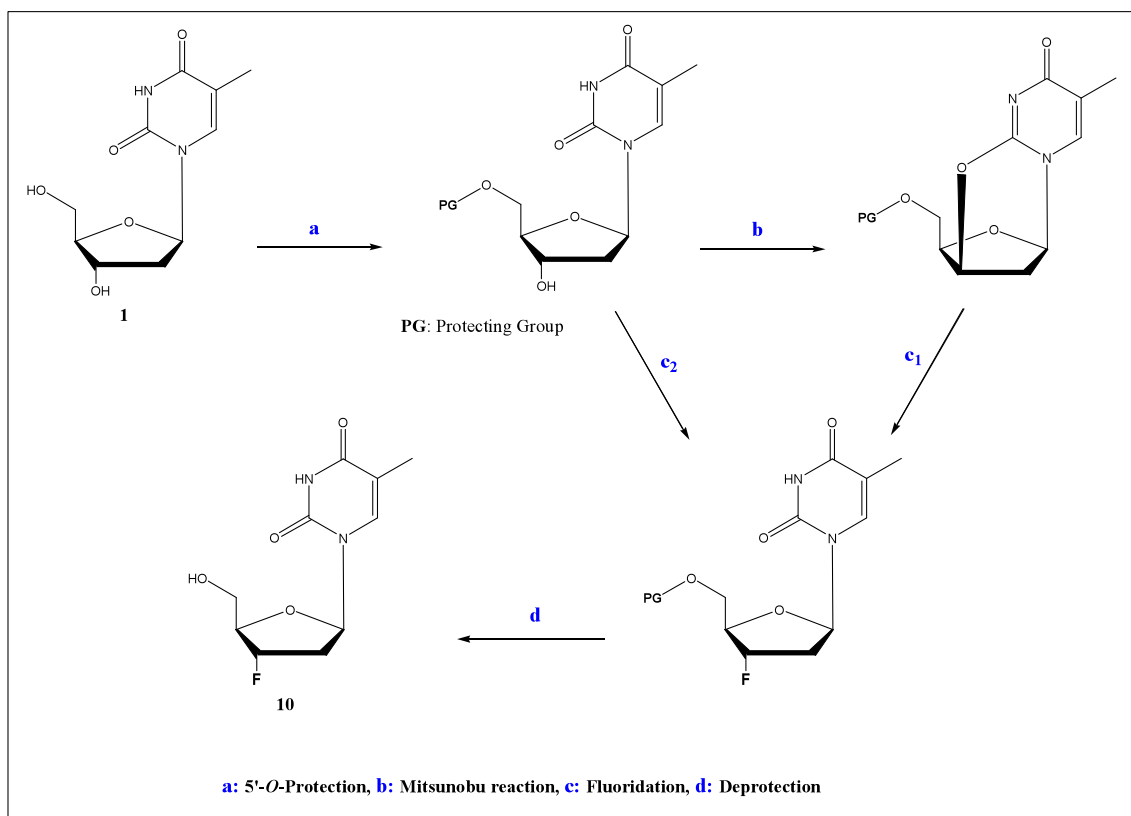
Scheme 2.1: Generic route for the synthesis of FLT, **10** involving 5'-*O*-protection of the thymidine nucleoside **1**, nucleophilic displacement by fluorine and deprotection of the protecting group (PG).

Various types of protecting groups for the protection of the 5'-hydroxyl group were studied, namely the *p*-methoxybenzoyl (PMBz) group, the dimethoxytriphenylmethyl (DMTr) group and the triphenylmethyl (Tr) group, which are common protecting groups for 5'-hydroxyl groups of nucleosides and their analogues.

The mechanism of the Mitsunobu reaction, which allows the intramolecular conversion of the secondary alcohol group present at the 3'-position of thymidine into a six-membered 2,3'-anhydro cycle, is shown in **Scheme 2.2**. The triphenyl phosphine, **27** combines with diisopropyl azodicarboxylate (DIAD), **28** to generate a zwitterionic phosphonium intermediate, **29**. Subsequently, the intermediate deprotonates the 3'-secondary alcohol group on thymidine to form the anionic nucleophile, **30**. This nucleophile binds to the phosphonium ion and the resulting nucleophile performs an S_N2 attack to yield the final substitution product 5'-*O*-protected-2,3'-anhydrothymidine, **31** with an inverted stereochemistry at C-3'. The formation of a strong P=O bond in the by-product triphenylphosphine oxide, **32** helps to drive the reaction forward.[1, 2]
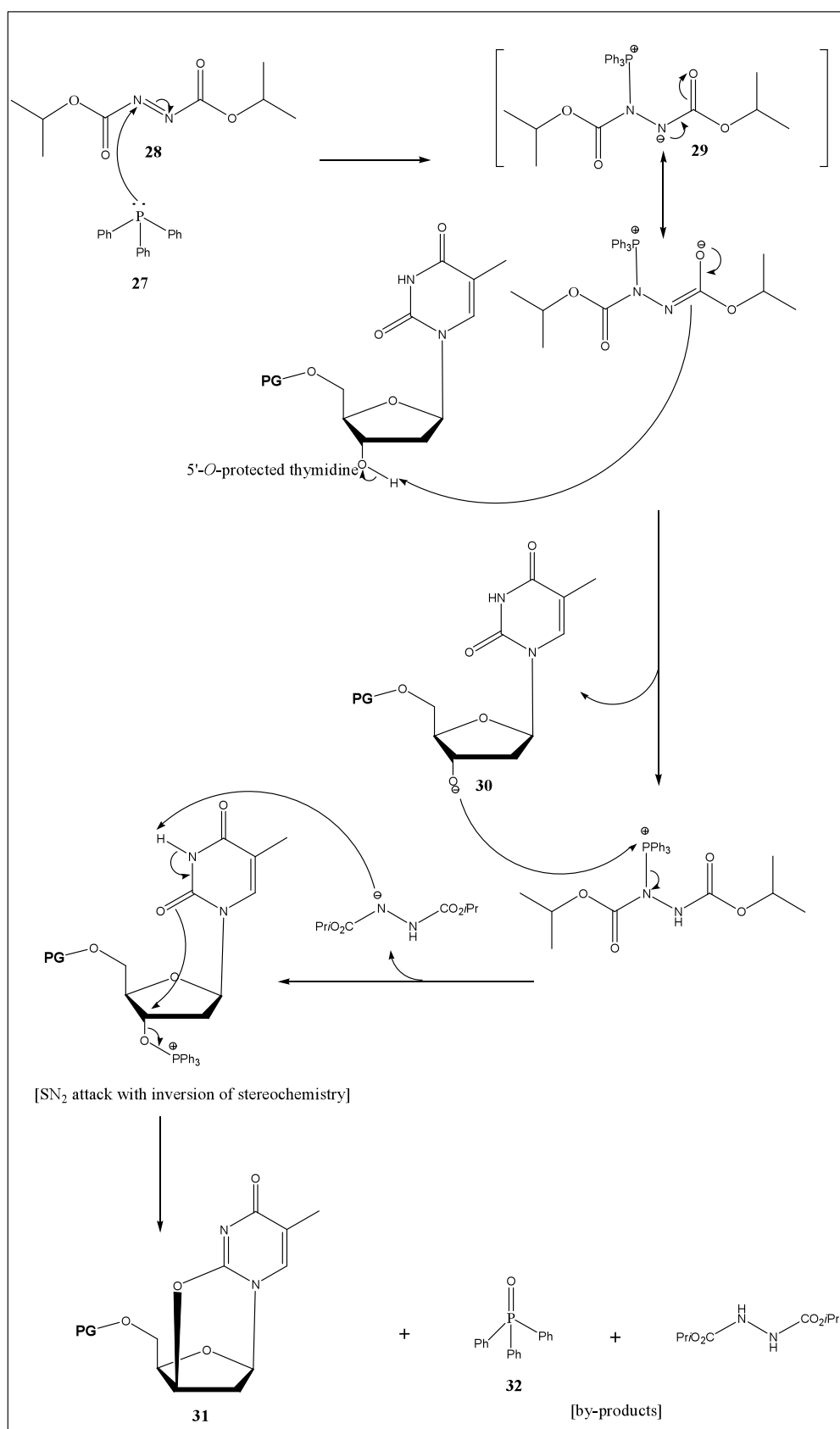
Scheme 2.2: Mitsunobu reaction mechanism of a 5'-*O*-protected thymidine to form **31**.

2.2.1.1. Protecting group: *p*-methoxybenzoyl (PMBz)

With reference to previous studies carried out for uridine analogues, the PMBz group was employed for the protection of the primary hydroxyl group.[3] An electrophilic, 2,3'-anhydro cyclic intermediate was generated under Mitsunobu conditions (**Scheme 2.3**), which later on served as an effective precursor for the fluoridation reaction.
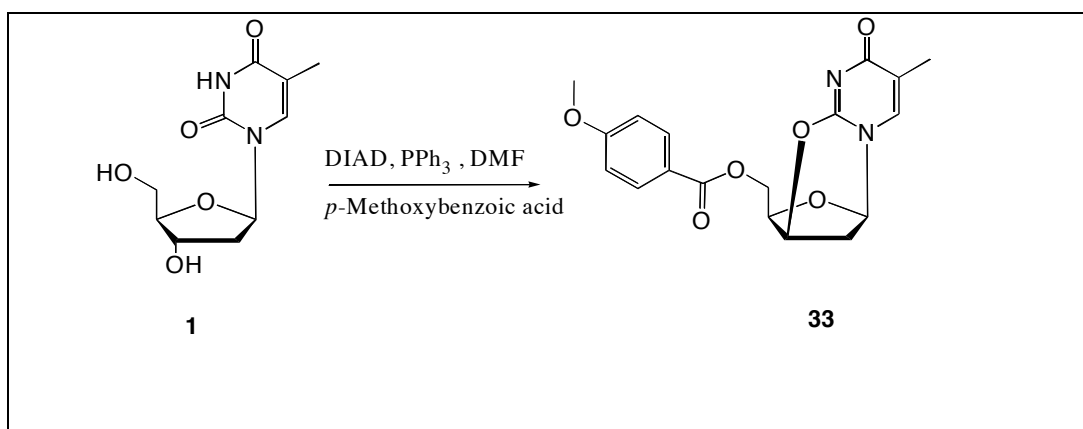
Scheme 2.3: Mitsunobu reaction performing both 5'-*O*-PMBz protection and intramolecular cyclization on thymidine **1**.

The synthesis of 5'-*O*-*p*-methoxybenzoyl-2,3'-anhydrothymidine, **33** from thymidine, **1** comprised of a one-pot reaction, which involved *p*-methoxybenzoic acid, triphenyl-phosphine, DIAD and DMF solvent. Hence both the PMBz protection reaction of the 5'-OH group and the formation of the 2,3'-anhydro ring were performed together. The reagent DIAD was added in two portions; first at the start of the reaction and the second after 1.5 hours maintaining the temperature at 0^0 C. Upon the second addition, the desired compound started to form and was filtered out to obtain white powder of the product in 93% yield after washing with brine and ether. It is noteworthy that the reaction proceeded with a stereogenic inversion at the 3' position forming an anhydro

bond between the 3'-position of furanosyl unit and 2-position of the thymine as illustrated in **Scheme 2.3**. The formation of the protected 2,3'-anhydro thymidine was confirmed by analyzing its exact mass using NMR spectroscopy and mass spectrometry.

The next step was to fluoridate the 3'-position of the 2,3'-anhydrothymidine formed after PMBz protection, which was successful using cesium fluoride (CsF) in DMF solvent followed by stereogenic inversion at the 3'-position. It is observed that the rate of the nucleophilic fluoridation reaction is higher and faster through the formation of the 2,3'- anhydro ring since the resulting 6-membered arrangement is in a thermodynamically strained state and hence more susceptible than the non-cyclic counterpart towards nucleophilic attack as shown in **Scheme 2.4**.
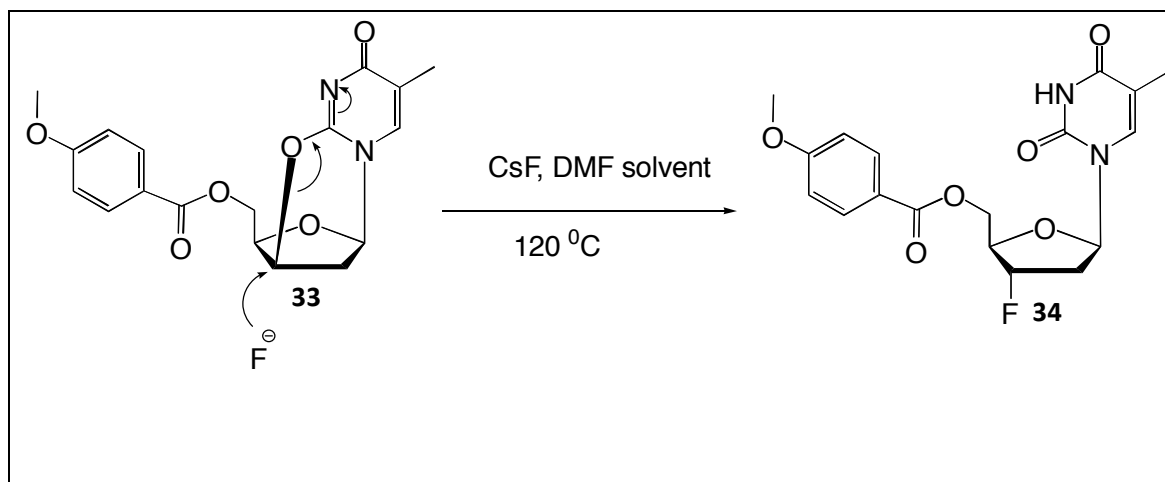
Scheme 2.4: S_N2 mechanism of 3'-fluoridation reaction.

Although the fluoridation reaction was carried out for 24 hours at 120 $^{\circ}$C, the reaction yielded 91% of the pure compound merely by filtering the reaction precipitate and washing it with ether.

Other fluoridating reagents and conditions were also tested as shown in **Table 2.1**.

Table 2.1: Fluoridation reactions of 5'-*O*-PMBz-2,3'-anhydrothymidine, **33**.

F⁻ source	solvent	reaction time (h)	yield
CsF	DMF	24	**91%**
KF	DMF	24	/
TBAF	DMF	12-24	/

In consideration of the half-life of ^{18}F ($t_{1/2}$ = 110 min), a reduced fluoridation reaction time was essential. The use of potassium fluoride (KF) and (tetra-*N*-butylammonium fluoride) TBAF as fluoridating agents was not successful as the starting material was retained completely with no progress in reaction. Hence microwave promoted fluoridation studies of 5'-*O*-PMBz-protected thymidine, **33** were carried out as shown in **Table 2.2**: Microwave promoted reactions.

Reaction mixture	result	time	yield (%)
33 (1 mmol), CsF (3.138 mmol), DMF (3.5 mL)	decomposed product	1 hour	/
	pure product after filtration	30 min	60
	minor impurities	15 min	80

Microwave experiments were performed in the Department of Chemistry, Cardiff University in the laboratory of Dr. Mark Bagley. For all the three microwave experiments, the compound **33** (0.36g, 1 mmol) and CsF (0.48g, 3.138 mmol) were dissolved in anhydrous DMF (3.5 mL), stirred and sonicated to form a homogeneous solution mixture. The sealed vessel containing the reaction mixture was then placed in the microwave and irradiated at 120 $^{\circ}$C with a power of 150 W and pressure of 100 psi at varying reaction times as shown in **Table 2.2**. However, the microwave reaction which lasted 1 hour led to the decomposition, as the resulting solid compound was black. The reactions which lasted 30 and 15 minutes were able to generate the desired compound, **34** with yields of 60% and 80% respectively.

The final step was to remove the PMBz protecting group from the 5'-*O*-position of 3'-fluorothymidine using sodium methoxide in methanol. However, the deprotection reaction was not successful as peaks of the aromatic protons of PMBz-group were still detected on the ^{1}H-NMR spectrum even after purification by preparative column chromatography.

Hence the DMTr protecting group was used afterwards.

2.2.1.2. Protecting group: 4,4'-dimethoxytriphenylmethyl (DMTr)

As suggested from the literature, the dimethoxytrityl group was used to protect the 5'-hydroxyl group of thymidine, since it was more reliable in terms of simple and fast deprotection conditions.[4,5] This protecting group can also impart crystallinity to many low-melting solids.[6] However, the conditions used for the protection and purification of the resulting 5'-*O*-DMTr-thymidine were more time-consuming compared to the PMBz-group.

The dimethoxytrityl group was preferred over the trityl moiety since it is more acid sensitive and removed more rapidly at lower temperature.[7] The protection was carried out using dimethoxytrityl chloride and anhydrous pyridine at room temperature as depicted in **Scheme 2.5**. Although the reaction time was only three hours, the purification of the desired compound was time consuming. The purified DMTr-protected thymidine, **35** was isolated by preparative column chromatography with 59 % yield. The structure of the compound was confirmed by NMR spectroscopic and mass spectrometric analysis.

2.2.1.2.1. Fluoridation without a precursor

Initially, it was thought to attempt the fluoridation reaction by the direct replacement of the hydroxyl group on the 5'-*O*-DMTr protected thymidine, **35** by treating it with a suitable fluoride such as CsF as represented in **Scheme 2.5**. Direct replacement of the OH-group at the 3'-position of **35** by a fluoride emerged to be a highly challenging task as the nucleophilic substitution of the hydroxyl group upon fluoride attack is less

favourable compared to leaving groups such as mesyl or tosyl. This is mainly because the hydroxyl group is a poor leaving group. Also the reaction was expected to be very slow and low-yielding. An authentic sample of FLT was purchased from the supplier Carbosynth as reference and an NMR spectrum of the reference FLT in d_4-MeOD was obtained to assess the viability of fluoridation studies. It is thought that if the 5'-O-DMTr protected thymidine is converted into a 2,3'-anhydro complex, the rate of the fluoridation reaction could be enhanced. Several solvents such as DMF and DMSO were tested to identify the most efficient reaction medium. It was found that when acetonitrile and methanol are combined in a 1:1 ratio, fluoridation emerged to be more favourable. The formation of 5'-*O*-DMTr-3'-fluorothymidine, **36** was confirmed by NMR spectroscopy followed by stereogenic inversion at the 3' position and the attack of the fluoride nucleophile (F$^-$) occurred from the β-face of the nucleoside. At this point, it was confirmed that the fluoridation reaction would result in the stereogenic inversion of the configuration of the stereocenter as it is a typical nucleophilic S_N2 reaction (substitution reaction, 2nd order). But for the stereoselective synthesis of FLT, it was necessary for the fluoride nucleophile to attack from the α-face of the nucleoside. Therefore, a stronger β-configured electrophile had to be introduced at the 3'-position of the 5'-*O*-protected thymidine for the fluoride to attack from the α-face in an S_N2 nucleophilic manner.

The removal of this particular protecting group was found to be more advantageous than the previous group used for the protection of 5'- hydroxyl functionality as this involved simple drop-wise addition of aqueous 1N HCl over 5 minutes and raising the temperature to 50 ^{0}C. The dimethoxy trityl group was released as a cation with a characteristic bright red-orange colour. The white precipitate that resulted from the

reaction showed no peaks for the DMTr protons in its ^{1}H-NMR spectrum, confirming the complete deprotection of the DMTr group from the 5'-*O*-position of the nucleoside. But unfortunately, the peaks did not correspond to the original structure of the expected product, FLT due to decomposition.
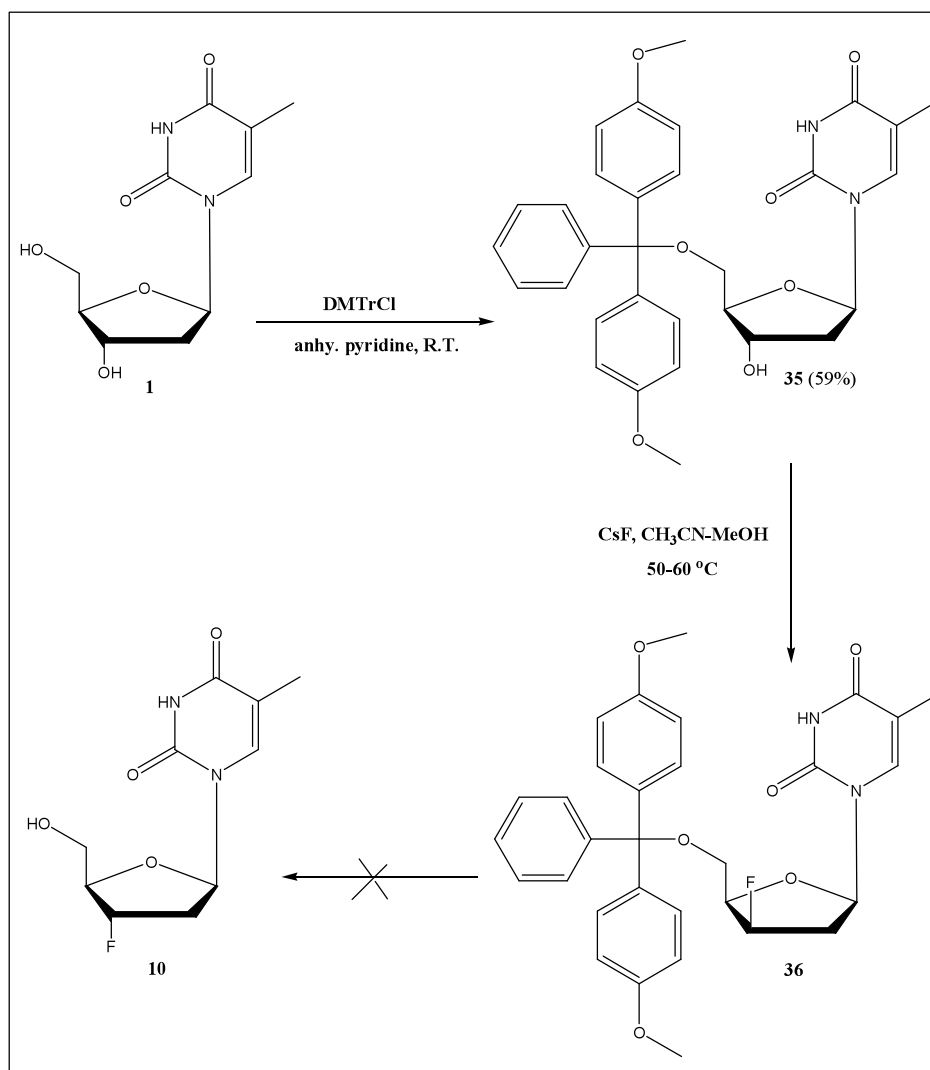
Scheme 2.5: Fluoridation of 5'-*O*-DMTr-thymidine, **35**.

2.2.1.2.2. Fluoridation following precursor formation

In order to improve the rate of the fluoridation reaction and conduct stereoselective FLT synthesis, the analogy of using the precursor 2,3'-anhydro derivative of the 5'-*O*-DMTr protected thymidine was applied based on the same principle carried out previously for the PMBz-protected thymidine.

Again with the main intention of generating an electrophilic center at the 3'- nucleoside position, the 5'-*O*-(4,4'-dimethoxytrityl)-2,3'-anhydrothymidine, **37** was prepared using Mitsunobu reaction conditions.[8] The solvent used for this particular reaction was ethyl acetate. The dropwise addition of DIAD (1.5eq) into the reaction mixture was carried out at $0\ ^0$C, and a better yield of the expected product was obtained when the temperature at which the addition took place was kept between 0-2 ^{0}C. Once all the DIAD addition was completed, the reaction was continued for three hours at room temperature.

From the TLC analysis, it was evident that the product appeared at a different R_f value, 0.1 (EtOAc-MeOH, 9:1 v/v) compared with the starting material 5'-*O*-DMTr-thymidine appearing at 0.7. Thus preparative column purification enabled a good isolation of the white amorphous product with 70% yield.

Subsequently, it was decided to test this precursor anhydro molecule with various fluorides in different polar aprotic solvents as shown in **Scheme 2.6**. As mentioned previously, these types of solvents were preferred to render the nucleophilic characteristics of the respective fluoride used.
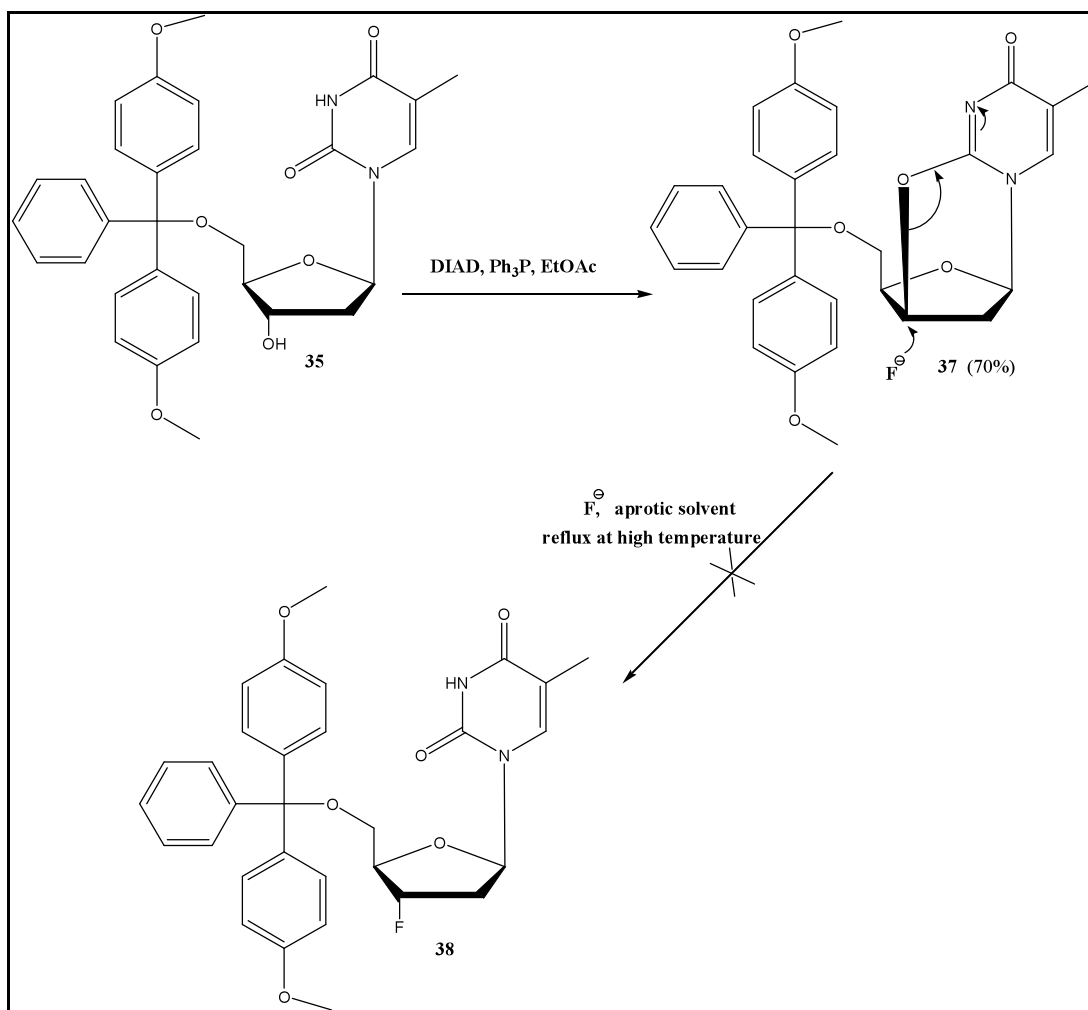
Scheme 2.6: Formation and nucleophilic fluoridation of 5'-*O*-DMTr-2,3'-

anhydrothymidine, **37**.

In the following **Table 2.3**, a summary of the different reaction conditions that were examined is given below using the fluorides, CsF and TBAF.3H$_2$O (Tetra-N-butylammonium fluoride).

Table 2.3: Fluoridation reactions of 5'-*O*-DMTr-2,3'-anhydrothymidine, **37**.

Reaction	Starting material (300mg)	Fluoride source (3eq)	Solvent (anhydrous) (4ml)	Progress	Time (hr)	Temp. (^{0}C)
1.	Compound **37**	CsF	DMSO	**X**	12	160
2.		TBAF.3H$_2$O		**X**	1/2	170
3.			DMF	Formed a different analogue (dehydro)	5	150

X= expected product not formed, starting material remaining.

The first reaction was an overnight experiment, in which CsF was the nucleophilic fluoride and DMSO was the solvent, since only starting material could be detected by TLC analysis (DCM:MeOH, 9.6:0.4 v/v) carried out at intervals of 2 hours. After collecting a cream precipitate by pouring into excess water, ^{1}H and ^{19}F- NMR studies were done and revealed that the expected product, **38** was not formed since the peaks in the spectra corresponded to that of the starting material. The DMSO used here was purchased as anhydrous (was a fresh bottle used). However given the notorious hygroscopic nature of DMSO, it is possible that small traces of water might have compromised the nucleophilicity of fluoride anion in this reaction, leading to return of unreacted starting material.

While conducting the second reaction, where tetra-*n*-butylammonium fluoride trihydrate (TBAF.3H$_2$O) and the same solvent were utilized, TLC analysis after 30 minutes showed the appearance of a new spot (R_f=0.43, DCM-MeOH 9.6:0.4 v/v) different to that of starting material (R_f = 0.1). Hence the reaction was stopped and preparative column purification was done to isolate the newly formed molecule. Although the same reaction conditions were employed in a recently published journal by S.K. Nandy and M.G.R Rajan[9] for the introduction of the radioactive fluoride to synthesize [^{18}F]-FLT, these conditions proved to be unsuccessful for the thermal fluoridation reaction as no peak was detected in the ^{19}F- NMR and hence indicating the absence of fluorine at the expected 3' nucleoside position. In the ^{1}H-NMR, the H$_{2'}$ peaks were absent and hence it is suspected that another unknown analogue might have formed.

For the final reaction, where the 5'-*O*-DMTr-protected anhydro precursor, **37** was heated to 150 ^{0}C under reflux with the same fluoride, TBAF.3H$_2$O and a different anhydrous solvent, DMF, TLC analysis after one hour showed a new spot at R_f = 0.25 (DCM:MeOH, 9.6:0.4 v/v). The reaction was quenched after 5 hours by pouring the reaction mixture into excess water and thereby forming a white precipitate, which was collected by filtration. The white amorphous crude product (61%) was purified by column chromatography to isolate the unknown product as a white amorphous powder (40%) and was subjected to NMR analysis. But in ^{1}H-NMR, both the H$_{2'}$ and H$_{3'}$ were found to be absent. It is suspected that the TBAF trihydrate, being a strong base, is attacking at the H$_{2'}$ as shown in **Scheme 2.7** to form a double bond between C$_{2'}$ and C$_{3'}$ and thereby opening the six-membered anhydro ring. This same mechanism was seen in

the Tetrahedron paper by P. Langen *et al.*[10] where they used the same reaction conditions to obtain 3'-deoxy-2',3'-didehydrothymidine. Here in this case, NMR results confirmed that it had gone through the same mechanistic pathway and formed the didehydro analogue, **39** as TBAF, a trihydrate, contains water which will reduce the nucleophilicity of fluoride anion, leading to either no reaction or elimination reaction.
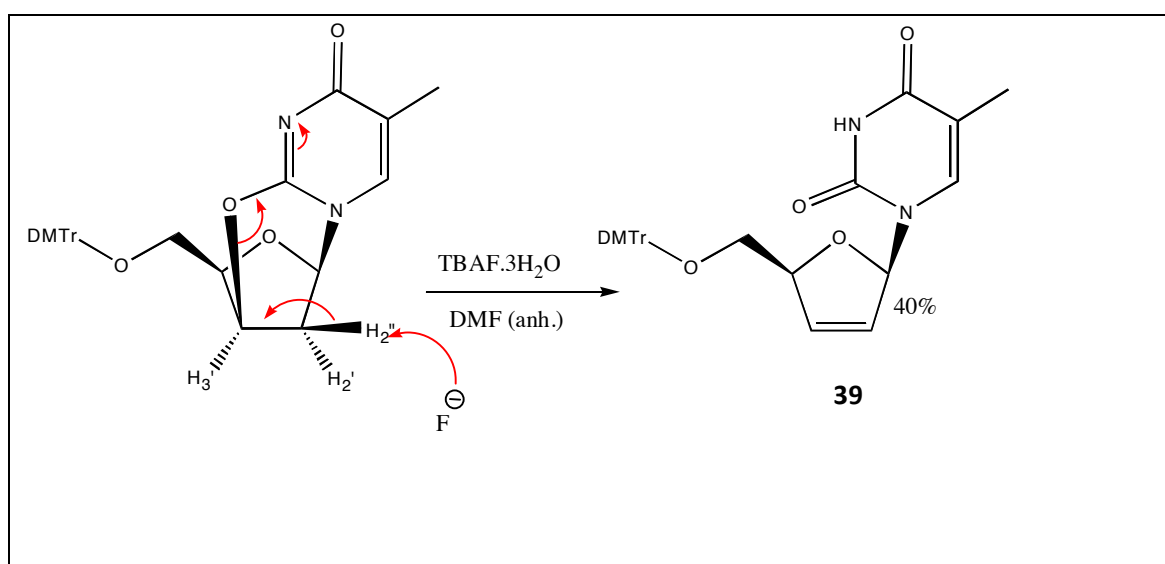
Scheme 2.7: Elimination reaction by the fluoride ion to form 5'-*O*-DMTr-3'-deoxy-2',3'-didehydrothymidine, **39**.

As the above reactions failed to obtain the desired product, 5'-*O*-DMTr-3'-fluoro-3'-deoxy-thymidine **38**, other reaction conditions were tested with the same fluorides in acetonitrile and pyridine as solvents. But unfortunately, the use of anhydro precursor molecule was rather unsuccessful as these all resulted in the unwanted elimination product, **39**. Hence more favourable reaction conditions were examined for the incorporation of fluoride at the 3' nucleoside position.

2.2.1.2.3. Deprotection of DMTr- group

The final step consists of the acid- catalysed removal of the dimethoxytriphenylmethyl group at the 5'-*O*-position of **39** by treating it with a suitable acid such as HCl in solvents such as methanol or acetonitrile.

Although the same deprotecting conditions that were used previously for the DMTr removal (**Scheme 2.5**), were employed this time as well for deprotection of **39**, there were no identifiable products from proton NMR.

2.2.1.3. Protecting group: Trityl (Tr)

While carrying out column chromatography purifications of DMTr protected compounds, the DMTr group dissociated quite easily despite adding small amounts of triethylamine base to the eluent to neutralize the slight acidity of silica. As the dimethoxytrityl (DMTr) group is known to be very acid labile, the more stable and commercially available protecting group, Trityl was trialled instead. As the use of the anhydro precursor was unsuccessful, a different strategy was undertaken where the trityl ether of the thymidine nucleoside was first converted into its mesylate analogue, **40** of 3'-OH and thereby converting it into a good leaving group to enable the formation of the lyxo configured 5'-*O*-trityl thymidine, **41** through a base mediated reflux reaction, followed by a stereogenic inversion of configuration as illustrated in **Scheme 2.8**.

The transformation of the sterically least hindered hydroxyl group (5'-OH) of thymidine, **1** into its trityl ether was achieved selectively under the presence of trityl chloride and pyridine, which acts as both base and solvent, along with a catalytic amount of 4-DMAP. The reaction was carried out for 8 hours under anhydrous conditions and heated at 80 ^{0}C. Both the tritylation at 5'-OH and mesylation at 3'-OH groups of thymidine were performed as a one-pot reaction; maintaining the same solvent pyridine, the reaction mixture after tritylation reaction was cooled down to 0 ^{0}C and mesyl chloride was added drop wise and stirred for one hour. [11] Aqueous work-up was required after the reaction was complete and the product, **40** was obtained in 73 % yield after column purification. The trityl protected mesylate analogue of thymidine was then subjected to a hydroxylation reaction with sodium hydroxide (10 eq) as the hydroxylating agent and ethanol as the solvent. The nucleophilic substitution of the hydroxide resulted in the stereogenic inversion of configuration at the 3' position of the nucleoside generating the product, **41** with an 85 % yield after silica gel column chromatography purification.

This particular synthetic approach was aimed to be implemented for ^{18}F-FLT synthesis as a rapid fluoridation reaction was required considering the half-life of [18]F.

Since the conversion of 5'-*O*-protected and unprotected 2,3'-anhydrothymidine into the 3'-fluoro-3'deoxythymidine analogue was unfavourable as mentioned previously, a different precursor molecule with a better leaving group was sought for the attack of nucleophilic fluoride.

The use of nosyl group as a good leaving group is well established in literature. Hence the idea was to convert **41** into a 4-nitrobenzene sulfonyl substituted nosyl analogue **42** that would spontaneously react with the nucleophilic fluoride as presented in the **Scheme 2.8**.

Nosylation was carried out in the presence of a halide-abstracting reagent, silver triflate (AgOTf) that could drive the reaction faster and in the presence of the base, pyridine.[12] The nosyl precursor **42** was obtained in 60% as a yellow solid after column purification. This was then subjected to various fluoridation reactions with different nucleophilic fluoride sources. The treatment of **42** with three equivalents of tetrabutyl ammonium fluoride trihydrate (TBAF.3H$_2$O) gave the desired fluorinated intermediate **43** (25%) after 30 minutes of heating under reflux with DMF as the solvent along with Kryptofix [222] to enhance the nucleophilicity of fluoride and drive the reaction forward.
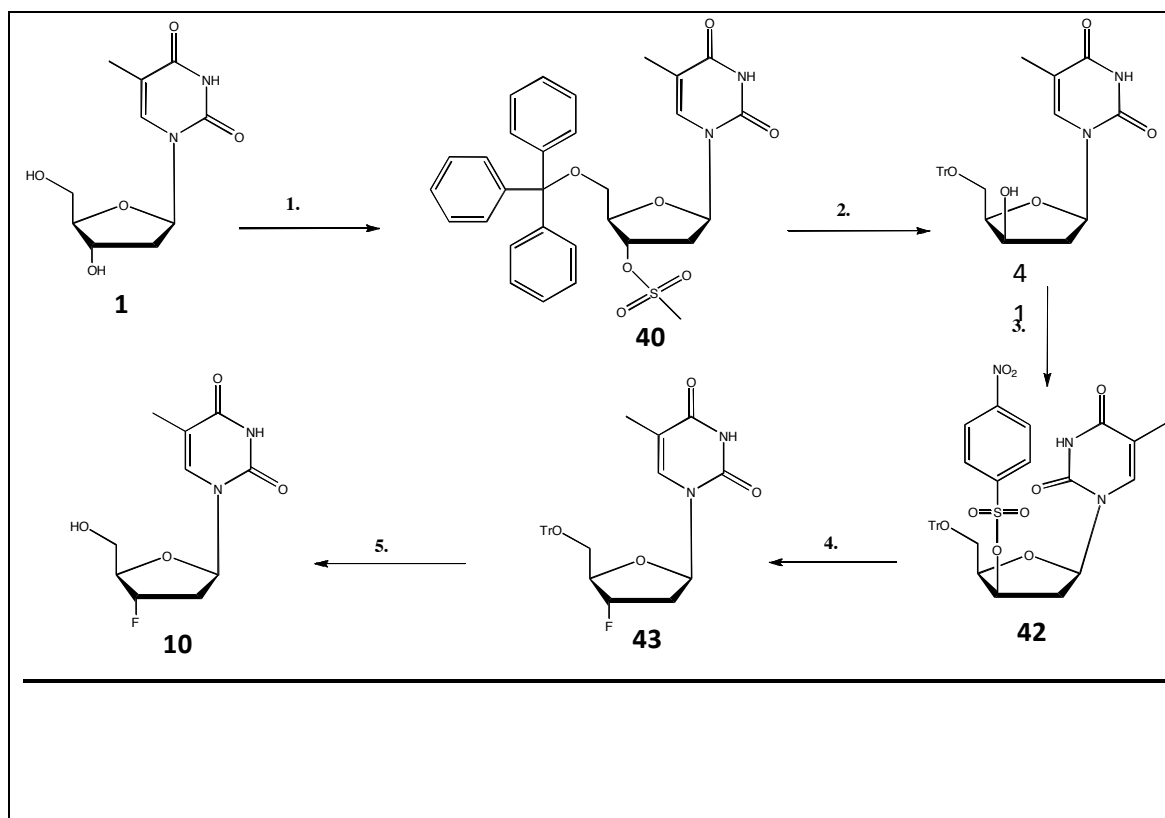
Reagents and conditions: 1. (i) TrCl (1.6 eq), 4-DMAP (0.06 eq), anhydrous pyridine, 80 °C, 8h
(ii) Mesyl chloride (2 eq), 0 °C, overnight

2. NaOH, 90% aq. EtOH, reflux, 2hr

3. NsCl (2 eq), AgOTf (2 eq), anhydrous pyridine, 0 °C, 2 hrs

4. TBAF.3H₂O (3 eq), Kryptofix (1.8 eq), DMF, reflux

5. 80% aq. AcOH, 90 °C, 0.5 hr

**Scheme 2.8:** Synthesis of FLT, **10** via the nosyl precursor **42**.

The acid sensitive trityl group was removed by heating compound **43** with 80% acetic acid under reflux condition for half an hour. FLT, **10** was generated after column purification with a yield of 50%.

2.2.2. FLT synthesis without 5'-O- protection

With the main intention of reducing the number of synthetic steps by excluding the protecting group on the 5'-position of thymidine and hence eliminating the protection and deprotection steps, the idea of generating FLT was attempted from just two steps, i.e.

1. intramolecular cyclization
2. nucleophilic fluoridation reaction.

As shown in **Scheme 2.9**, the unprotected thymidine was converted into 2,3'-anhydrothymidine, **44** using Mitsunobu reaction conditions to accomplish intramolecular cyclization for creating an electrophilic center at the 3'-position. The reaction was carried out using different solvents such as DMF and acetonitrile. It was found that upon using acetonitrile as the solvent, a better yield of 18% was obtained than using DMF which resulted in only 10% yield of **44**. Also when drop-wise addition of DIAD was performed at a temperature range of -15 to -5 ^{0}C and the reaction was allowed to run at 0 ^{0}C, a higher yield of the desired product (30%) was observed than when running the reaction at room temperature (18%) with acetonitrile as the solvent.[13]
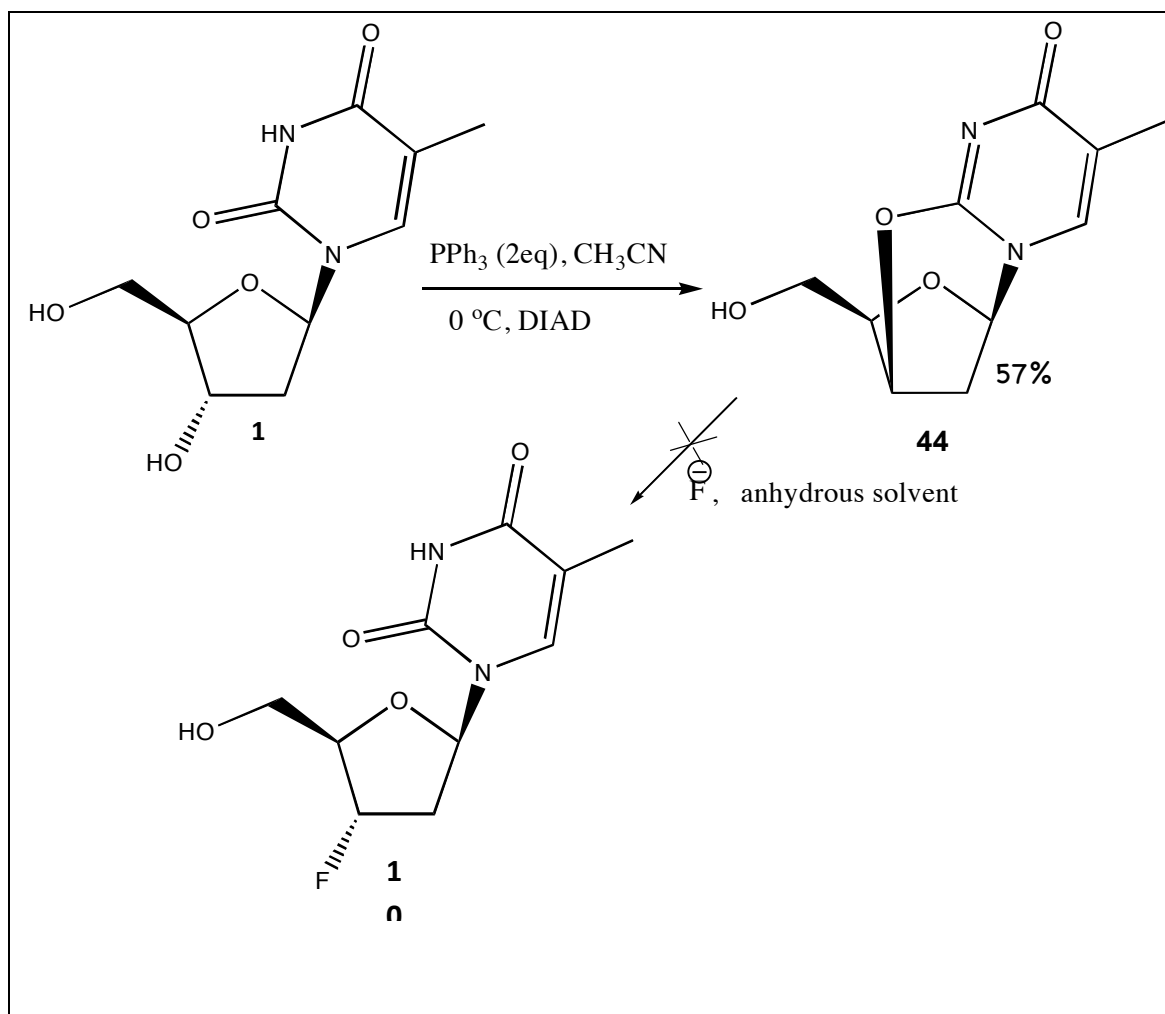
Scheme 2.9: Formation and fluoridation of the unprotected 2,3'-anhydrothymidine, **44**.

The purification of the compound **44** consisted of its precipitation in ethyl acetate and collection by filtration. Further purification was done by column chromatography using DCM-MeOH as the eluent in 8:2 v/v ratio to isolate the desired precursor molecule as a white amorphous powder in 57% yield with R_f=0.3.

The next and final step was to treat the 2,3'-anhydrothymidine precursor, **44** with a suitable nucleophilic fluorinating reagent to allow fluoridation. Hence, it was tested against different nucleophilic fluorides such as CsF, TBAF.3H$_2$O and KF. A summary of the various reaction conditions used for different fluorides is shown in the **Table 2.4**.

Table 2.4: A summary of fluoridation reactions with 2,3'-Anhydrothymidine, **44.**

Expt.	Starting material (200mg)	Fluoride source (3eq)	Solvent (anhydrous) (2ml)	Time (hr)	Temp. (^{0}C)
1.			DMSO	1	120
2.		CsF	DMF	1	100
3.			PYRIDINE	2	100
4.	Compound **44**		DMSO	2	120
5.		TBAF.3H$_2$O	DMF	1.5	150
6.		KF	DMF	6	100
7.		KF + Kryptofix [222]	DMF	0.5	80

At first, compound **44** was heated with CsF and pyridine to about 120 ^{0}C under N$_2$ atmosphere and reflux for two hours. After TLC analysis, it was observed that a new spot appeared at R$_f$ = 0.5 which was different to that of **44** (R$_f$=0.1) in DCM-MeOH (9.5:0.5 v/v). Thus column chromatographic purification enabled the isolation of the crude compound as a white amorphous powder in 58% yield. A clear NMR spectrum was generated representing successful purification with no other impurities present. It

was concluded that the same unwanted elimination reaction had occurred as with previously mentioned 5'-*O*-DMTr-2,3'-anhydrothymidine, **37** to form 3'-Deoxy-2',3'-didehydrothymidine, **45** as shown in **Scheme 2.10**.

The treatment of **44** with TBAF.3H$_2$O and DMF solvent provided the same disappointing results as different side reactions occurred, which were obvious from TLC analysis after 90 minutes. Considering the importance of the azacryptand Kryptofix [222] in radiochemistry to improve the nucleophilicity of the [^{18}F]fluoride ion as mentioned in Chapter 1, K$_{222}$ was used along with KF in one of the reactions to increase the nucleophilicity of the fluoride ion. But a progress in reaction could not be observed. The spots proved very difficult to separate even after column chromatographic purification. As all the resulting products from the different fluoridation reactions were analyzed, it was confirmed that the desired product was not formed.
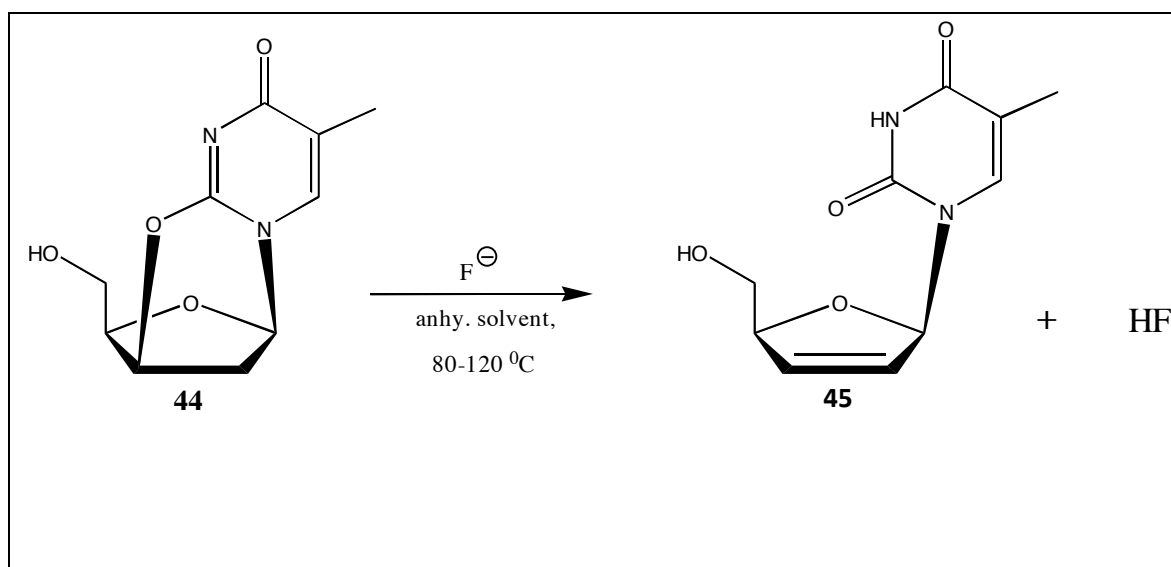
Scheme 2.10: Schematic representation of the didehydro thymidine analogue formation.

2.3. ^{19}F- FLT synthesis for ProTide studies

For the purpose of ProTide studies as detailed in Chapter 4, it was necessary to generate FLT in larger quantities of minimum 5 g. Even though FLT was commercially available, it was a very expensive nucleoside to be purchased on a large scale.

The direct replacement of oxygen by fluoride in organic molecules is commonly referred to as deoxofluorination and was implemented in this case, using electrophilic fluorinating reagents. DAST and Deoxo-Fluoro (**Figure 2.1)** are the most widely used deoxofluorinating reagents. But these reagents were extremely thermally unstable and highly explosive in nature. DAST degraded more rapidly with somewhat larger heat evolution. These are also liquids that discolour with aging and require redistillation, hence the decomposition can easily be observed.[14] However, DAST was used in this case for the selective deoxofluorination of compound **41** at 3' position. But when 0.1 g of compound **41** was used for the reaction, only 50% of the required product **43** was obtained and when used on a large scale, the selectivity was driven towards side product formation resulting in no product.
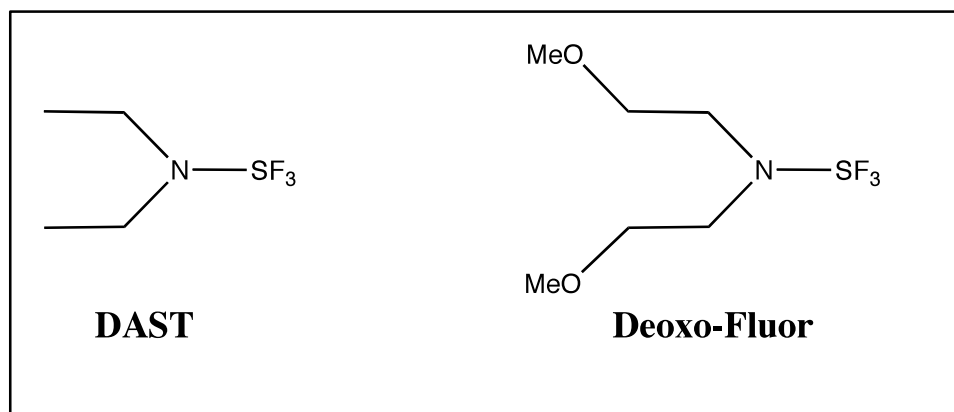

Figure 2.1: Structures of popular deoxo-fluorinating reagents

In the quest for safe and stable deoxofluorinating reagents capable of incorporating fluorine in a controlled fashion, dialkylamino difluorosulfinium (XTal Fluor-E) and morpholinodifluorosulfinium tetrafluorobarate (XTal Fluor-M) salts were found to act as surrogates for aminosulfur trifluorides and they emerged to be safer and more efficient alternative reagents. When used in conjunction with exogenous fluoride source or promoters such as Et$_3$N.3HF, Et$_3$N.2HF, or DBU, these reagents were able to effectively convert alcohols to alkyl fluorides; thereby leading to fewer elimination byproducts as compared to DAST.[15]

The synthesis of Xtal Fluor reagents (**Figure 2.2**), were first reported over three decades ago by Markovskii et al.[16] In recent years, the use of Xtal Fluor-M as a deoxofluorinating reagent was reported in literature by Pashinnik et al. for the deoxofluorination of an allylic alcohol in acetonitrile to yield the corresponding fluoride in 85% as a mixture of epimers.[17] Since then various types of alcohols have been converted to their respective fluorides with the use of Xtal Fluor deoxofluorinating reagents. Xtal Fluor reagents have demonstrated not only enhanced thermal stability over DAST, but also improved preparatory methods excluding hazardous distillations which otherwise were required for synthesizing DAST. On the contrary to DAST, these reagents were more selective in providing less elimination side products and can be easily handled as free-flowing solid which do not generate highly toxic and corrosive HF.[18]
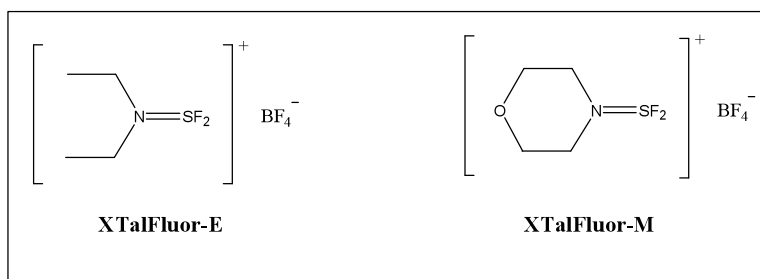
Figure 2.2: Structures of XTal Fluor reagents.

In this case, the morpholinodifluorosulfinium tetrafluoroborate (XtalFluor-M), which was commercially available as a white crystalline salt, was used to perform deoxofluorination on compound **41** and yielded **43** with a comparatively better yield of 75% when used on a large scale starting with 5g of **41** as described in **Scheme 2.11**. Procedures 1 (tritylation and mesylation), 2 (hydroxylation) and 4 (deprotection of Tr group) were followed in the same manner as detailed in section 2.2.1.3. A good yield (93%) of the desired final product FLT, **10** was obtained after column purification with dichloromethane: methanol eluent. The nucleoside product was further used for the synthesis of various FLT phosphoramidates.
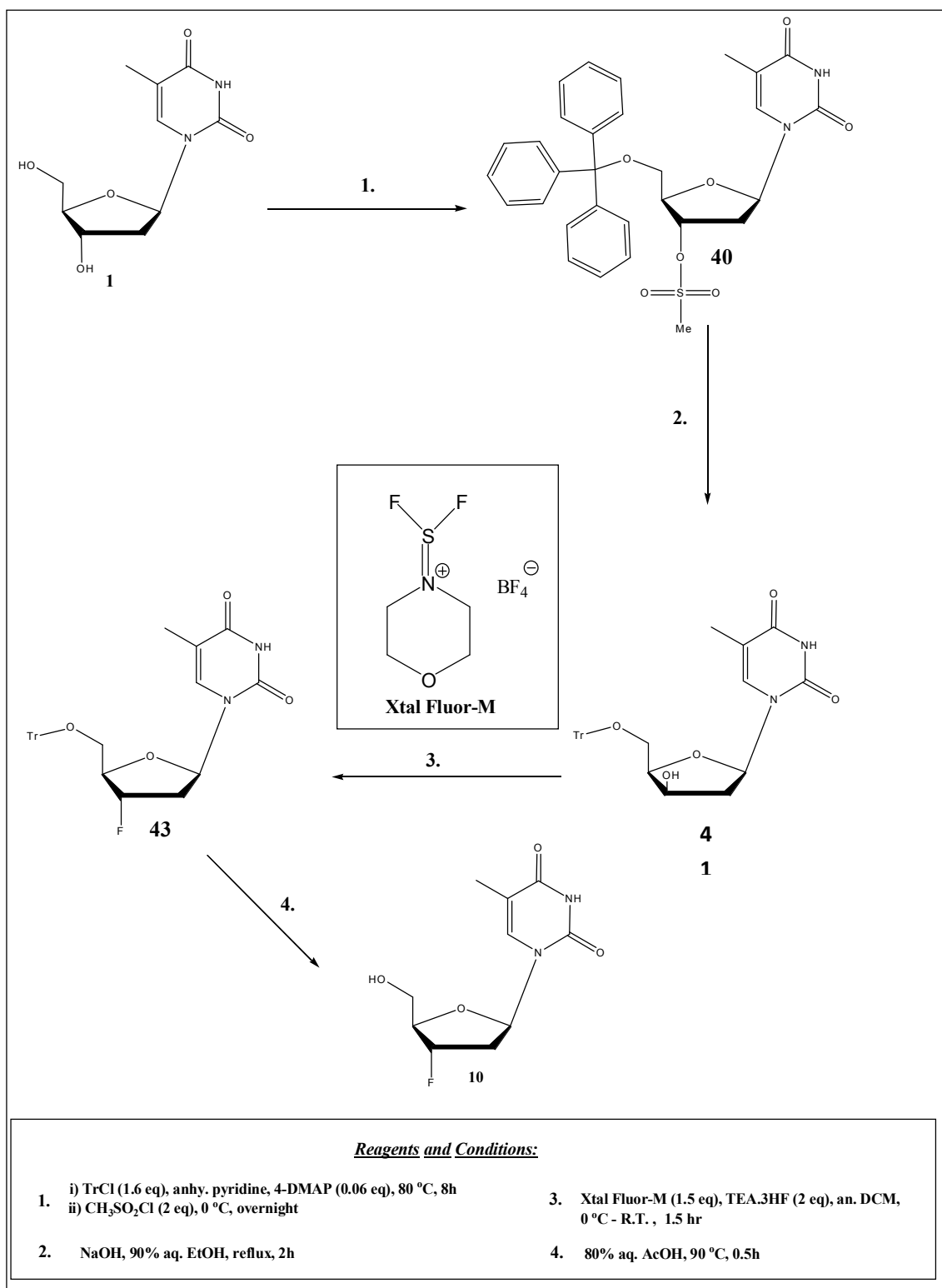
Scheme 2.11: Synthesis of FLT, **10** via deoxofluorination of **41**.

2.4. Summary

The synthesis of ^{19}F-FLT, **10** for the [^{18}F]fluoride radiochemistry studies was achieved using 5'-*O*-trityl protected nosyl precursor molecule using TBAF.3H$_2$O as the fluoride source. Although, ^{19}F-FLT synthesis was unsuccessful with 5'-*O*-PMBz and DMTr protected precursor molecules consisting of the anhydro ring at the 3'-position as the leaving group for the nucleophilic substitution by the fluoride ion, these methods were repeated under radiochemical conditions as described in Chapter 3.

For the purpose of ProTide studies (Chapter 4), ^{19}F-FLT was synthesized in larger quantities using the electrophilic reagent, XTalFluor-M.

References:

1. Mitsunobu, O.; Yamada, M.; Mukaiyama, T. Preparation of esters of carboxylic and phosphoric acid by the reaction of trivalent phosphorus compounds with diethyl azodicarboxylate in the presence of alcohols. *Bull Chem Soc Jpn.,* **1967**, *40*, 935-939.

2. Mitsunobu, O.; Yamada, M. Preparation of esters of carboxylic and phosphoric acid via quaternary phosphonium salts. *Bull Chem Soc Jpn.,* **1967**, *40*, 2380-2382.

3. Mehellou, Y. The design, synthesis and biological evaluation of novel antiviral nucleosides and nucleotides. Ph.D. thesis, **2008**, Cardiff University.

4. Martin, S.J.; Eisenbarth, J.A.; Wagner-Utermann, U. et al. A new precursor for the radiosynthesis of [F-18] FLT. *Nucl Med Biol.,* **2002,** *29, 263-273.*

5. Blocher, A. et al. Synthesis and labeling of 5'-O-(4,4'-dimethoxytrityl)-2,3'-anhydrothymidine for ^{18}F-FLT preparation. *J Radioanal Nucl Chem.;* **2002**, *251 (1),* 55-58.

6. McGuigan, C.; Wang, Y. K. A new protocol for the removal of dimethoxytrityl ether groups derived from primary alcohols. *Synth Commun.* **1997**, 27, 3829-3833.

7. Yang, G.S. Facile selective detritylation of 5'-primary alcohols of pyrimidine nucleosides using tetra-n-butylammonium peroxydisulfate. *Heteroatom Chemistry,* **1997**, *8 (5)*, 435-438.

8. Gaynor, J.W.; Bentley, J.; Cosstick, R. Synthesis of the 3'-thio-nucleosides and subsequent automated synthesis of oligodeoxynucleotides containing a 3'-S-phosphorothiolate linkage *Nature Protocols,* **2007**, *2 (12)*, 3122-3135.

9. Nandy, S.K.; Rajan, M.G.R. Fully automated and simplified radiosynthesis of 3'-deoxy-3'-[18F]fluorothymidine using anhydro precursor and single neutral alumina column purification. *J Radioanal Nucl Chem.,* **2010**, *283*, 741-748.

10. Langen, P. Antimetabolites of Nucleic Acid Metabolism*,* **1975**, New York, Gordon & Breach.

11. Kumar, R.; Tyrell, D.L.; Agrawal, B.; Mak, M.; Shakya, N.; Srivastav, N.C. Antiviral activity of various 1-(2'-Deoxy-β-D-lyxofuranosyl), 1-(2'-Fluoro-β-D-lyxofuranosyl), 1-(3'-Fluoro-β-D-arabinofuranosyl), and 2'-Fluoro-2',3'-didehydro-2',3'-dideoxyribose Pyrimidine Nucleoside Analogues against Duck Hepatitis B Virus (DHBV) and Human Hepatitis B Virus (HBV) Replication. *J Med Chem.,* **2010**, *53,* 7156-7166.

12. Grierson, J.R.; Shields, A.F. Radiosynthesis of 3'-deoxy-3'-[18F]-fluorothymidine: [18F]FLT for imaging of cellular proliferation in vivo, *Nucl Med Biol.,* **2000**, *27,* 143-156.

13. Balagopala, M.I.; Ollapally, A.P.; Lee, H.J. An improved synthesis of azidothymidine. *Nucleosides and Nucleotides,* **1996**, *15(4)*, 899-906.

14. Fauq, A.H; Singh, R.P.; and Meshri, D.T. In *Handbook of Reagents for Organic Synthesis-Fluorine Containing Reagents;* Paquette, L.A., Ed.; John Wiley & Sons Ltd.: England, **2007**, 180-194.

15. L' Heureux, A.; Beaulieu, F.; Beauregard, L.P.; Courchesne, G.; LaFlamme, F.; Couturier, M. Aminodifluorosulfinium Tetrafluoroborate Salts as stable and crystalline deoxofluorinating reagents. *Organic Letters,* **2009***, 11 (21),* 5050-5053.

16. Markovskii, L.N.; Pashinnik, V.E.; Saenko, E.P. *Zh. Org. Khim.,* **1977**, *13*, 1116.

17. Pashinnik, V.E.; Martynyuk, E.G.; Shermolovich, Y.G. *Ukr. Khim. Zh.(rss. Ed.),* **2002**, *68*, 83.

18. L' Heureux, A.; Beaulieu, F.; Bennett, C.; Bill, D.R.; Clayton, S.; LaFlamme, F.; Mirmehrabi, M.; Tadayon, S.; Tovell, D.; Couturier, M. Aminodifluorosulfinium Salts: Selective Fluorination Reagents with Enhanced Thermal Stability and Ease of Handling. *J Org Chem.,* **2010***, 75,* 3401-3411.

Chapter 3

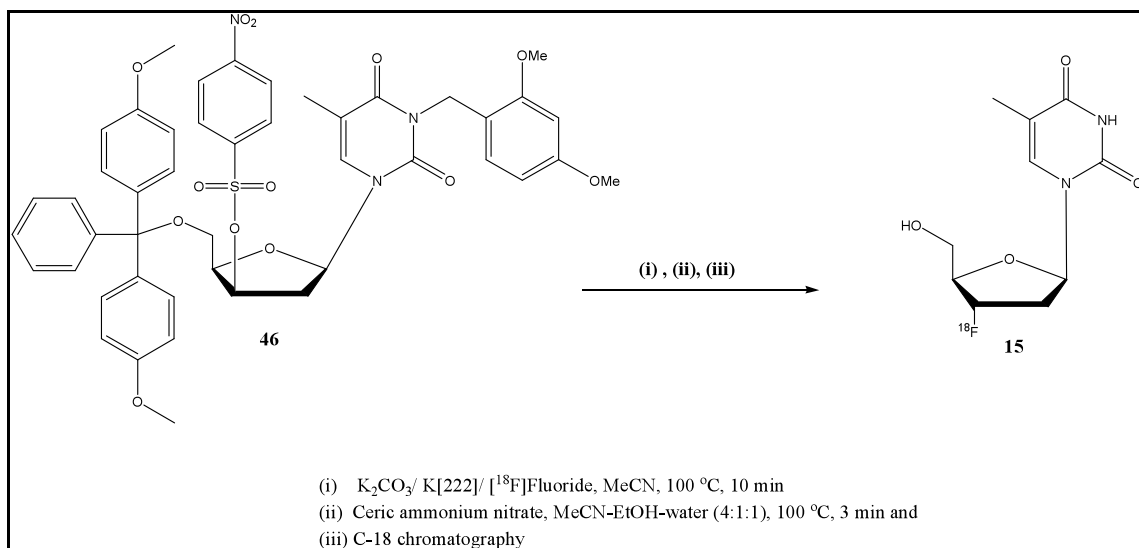# 3. Radiosynthetic routes to [^{18}F]-FLT and its ProTides

3.1. Radiosynthesis of [^{18}F]-FLT

In spite of currently being the most promising and widely used PET tracer molecule for monitoring cellular proliferation in tumours, the clinical applicability of [^{18}F]-3'-fluoro-3'-deoxythymidine ([^{18}F]FLT) has been limited by a number of radiosynthetic drawbacks. These include the lengthy synthesis of the precursor molecule, time-consuming purification techniques such as preparative high performance liquid chromatography (HPLC) and low radiochemical yields of the tracer molecule ranging from 5-20%.[1] Therefore, it was felt that there is an urgent need to overcome these limitations. Hence, the first and foremost objective was the development of a fast and efficient synthetic route for the radiosynthesis of [^{18}F]FLT at the new Wales PET Imaging Centre (PETIC) located at the University Hospital of Wales.

3.1.1. Previous studies

One of the earliest examples of [^{18}F]FLT radiosynthesis was reported by Grierson *et al.*, using a complex precursor molecule, 1-(2-deoxy-3-*O*-(4-nitrobenzenesulfonyl)-5-*O*-(4,4'-dimethoxytrityl)-β-D-lyxofuranosyl)-3-*N*-(2,4-dimethoxybenzyl) thymine. In this method, a solution of [^{18}F]fluoride, potassium carbonate and Kryptofix [2.2.2] was dried by azeotropic distillation with acetonitrile under argon at 100 $^{\circ}$C as the complete absence of atmospheric moisture was required to enhance the nucleophilicity of

[^{18}F]fluoride. The solution mixture was then solubilized in anhydrous acetonitrile and treated with the nosylate labeling precursor, **46** as shown in the **Scheme 3.1** for 10 minutes. To the resulting residue, ceric ammonium nitrate (CAN) in 4:1:1 MeCN/EtOH/water was added at 100 ^{o}C to remove the 4,4'-dimethoxytrityl (DMTr) and 2,4-dimethoxybenzyl protecting groups. Subsequently, the crude mixture was filtered and then purified in a semi-preparative C-18 HPLC eluted with 10% EtOH in sterile water to yield [^{18}F]FLT, **15** with a radiochemical yield (RCY) of 13% by the nucleophilic displacement of 3'-*O*-nosyl group on the precursor molecule with [^{18}F]fluoride. However, many disadvantages have been associated with this particular synthetic methodology such as time consuming deprotection steps, excessive [^{18}F]FLT degradation to [^{18}F]fluoride by the use of CAN and the formation of precipitating ceric salts, which would represent a difficult procedure for an automated synthesis.[2]

(i)   K$_2$CO$_3$/ K[222]/ [^{18}F]Fluoride, MeCN, 100 ^{o}C, 10 min
(ii) Ceric ammonium nitrate, MeCN-EtOH-water (4:1:1), 100 ^{o}C, 3 min and
(iii) C-18 chromatography

Scheme 3.1: Synthesis and purification of [^{18}F]FLT described by Grierson and Shields.[2]

Thereafter, many alternative synthetic methodologies have been brought forward using different precursor molecules in the nucleophilic [18F]fluoridation reactions to generate [18F]FLT, **15**. One such notable study was the use of 5'-*O*-(4,4'-dimethoxytrityl)-2,3'-anhydrothymidine, **37** by Machulla and coworkers as a precursor for the radiosynthesis of [18F]FLT, which is illustrated in **Scheme 3.2** to generate 5'-*O*-DMTr-3'-[18F]fluorothymidine, **47**. The anhydro structure acts as both an electrophilic center at the 3'- position and as a pyrimidine protecting group. This led to simplifications regarding the precursor synthesis, labeling and purification of [18F]FLT. However the synthesis times ranged between 90 min and 140 min.[3] It is worth mentioning that the introduction of other leaving groups such as methylsulfonyl (mesyl=Ms), *p*-toluenesulfonyl (tosyl=Ts) and trifluoromethanesulfonyloxy (triflate=Tf) to create an electrophilic center at the 3'-position, have also been recorded in literature. Conventionally, the OH-group at the 5'-position of the precursor molecule is protected with either of the easily removable triphenylmethyl (Tr) or 4,4'-dimethoxytriphenylmethyl (DMTr) protecting groups, since the hydroxylic groups are thought to decrease the nucleophilicity of [18F]fluoride.[4]
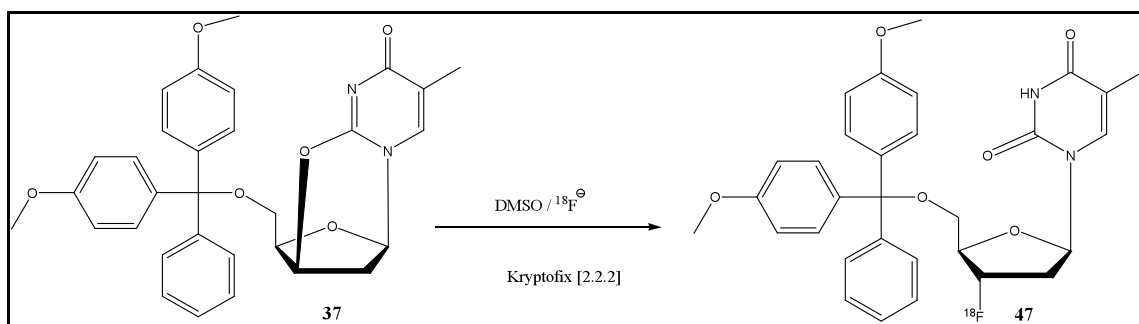
Scheme 3.2 : Synthetic method for 18F-FLT developed by Machulla and coworkers.[3]

Organic solvents such as anhydrous DMSO, acetonitrile or DMF are often used as the reaction medium. For the nucleophilic fluoridation reaction, phase transfer reagents such as kryptofix 222, K_2CO_3, TBA-HCO$_3$, and $CsCO_3$ are used along with the radioactive fluoride ion, $^{18}F^-$ that is produced from the cyclotron, in order to increase the nucleophilicity of the fluoride ion and accelerate the rate of $[^{18}F]$-radiolabeling reactions.[5]

3.1.2. Radiosynthetic routes for [^{18}F]FLT using different precursors

In this study, preliminary work was focussed on optimising existing reaction conditions for $[^{18}F]$FLT synthesis from previously prepared precursor molecules as described in *chapter 2*. The idea was to incorporate the radioactive fluoride that is generated from the cyclotron into the previously prepared precursor molecules in an automated process. This was carried out in an Eckert and Ziegler Nuclear Interface Module (Modular Lab) as shown in **Figure 3.1** which was configured for general-purpose fluoridation, deprotection and HPLC purification; finally generating the pure PET tracer molecule $[^{18}F]$ FLT. All these steps were automated in the modules using developed software programs. It was necessary to use an automated radiosynthesis system performed by the Modular Lab application software and place these inside a hot cell for radiation safety purposes due to the production of radiolabeled compounds with high activities. The hot cell is self-shielded and large enough to accommodate the modular lab synthesizer providing sufficient shielding against radiation in all directions.
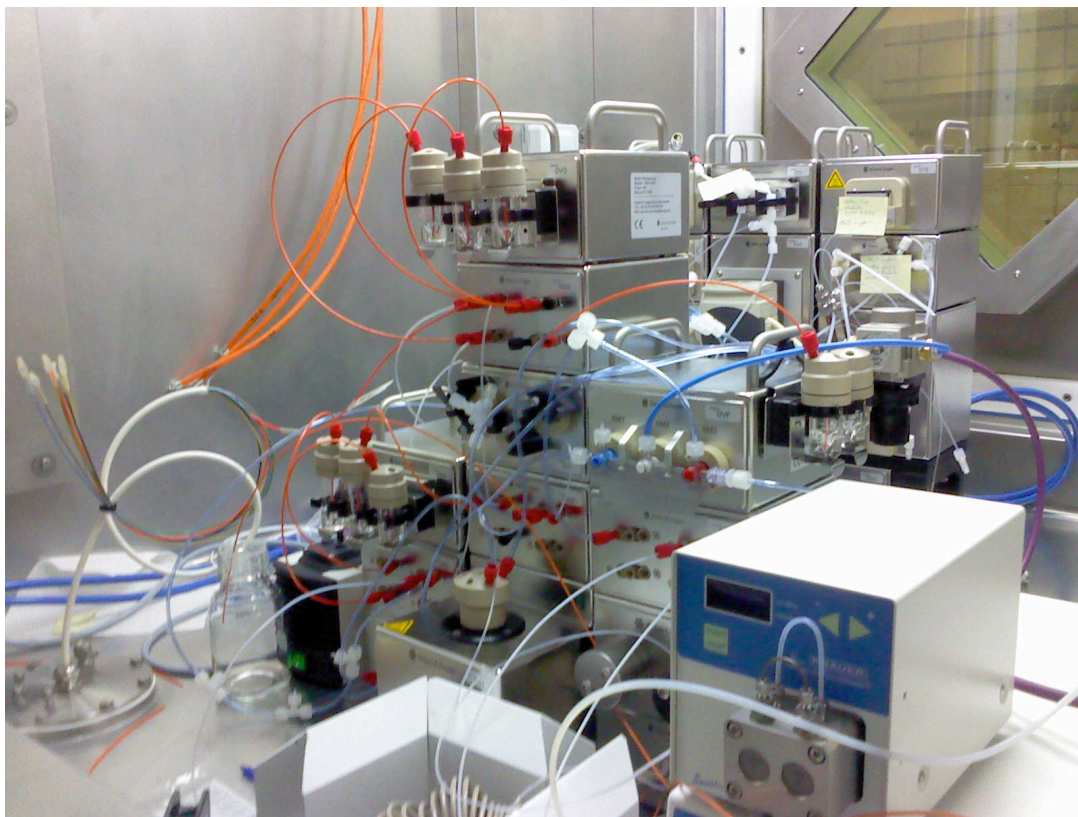
Figure 3.1: Modular Lab Synthesizer placed inside a hot cell at PETIC.

Each of the previously prepared precursor molecules were subjected to different reaction conditions such as varying temperature, reactant concentration, solvents and reaction time in order to achieve the best optimised [^{18}F]FLT synthetic route. A general representation of the radiochemical preparation of [^{18}F]FLT, **15** is given in **Scheme 3.3** below.
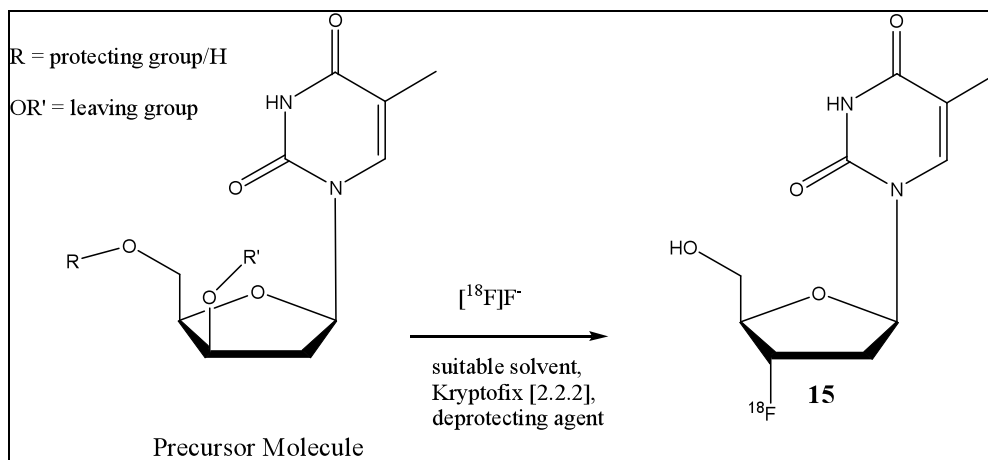
Scheme 3.3: General schematic representation of [^{18}F]FLT, **15** synthesis

As the project required further technical knowledge of radiochemical synthetic processes using Modular Lab software and module equipments, special training was given by Dr. Stephen Paisey prior to carrying out work at PETIC.

Repeated automated processes were trialled for optimizing reaction conditions such as the maximum temperature for solvent evaporation, reaction solvents and also to ensure module equipments were performing according to required specifications.

3.1.2.1. Module for remote [^{18}F]FLT synthesis

The [^{18}F]FLT module consisted of nine main components as marked on **Figure 3.2**:

(A) reaction vial

(B) QMA (quaternary methyl ammonium) anion exchanger cartridge, which was preconditioned using 5 mL of 8.4% aqueous NaHCO$_3$ solution followed by 10 mL of water, in order to trap ^{18}F$^-$ from the cyclotron

(C) Kryptofix [2.2.2] (K$_{222}$) vial

(D) anhydrous acetonitrile (CH$_3$CN) vial for the purpose of azeotropic evaporation

(E) precursor vial, which was charged with the precursor molecule dissolved in the reaction solvent

(F) acid vial containing 0.05M HCl for deprotection

(G) base vial filled with 0.25M NaOH for neutralization

(H) vacuum pump for solvent removal and

(I) final product vessel for product isolation.

The same modular set-up was utilised for each set of experiments using different precursor molecules.
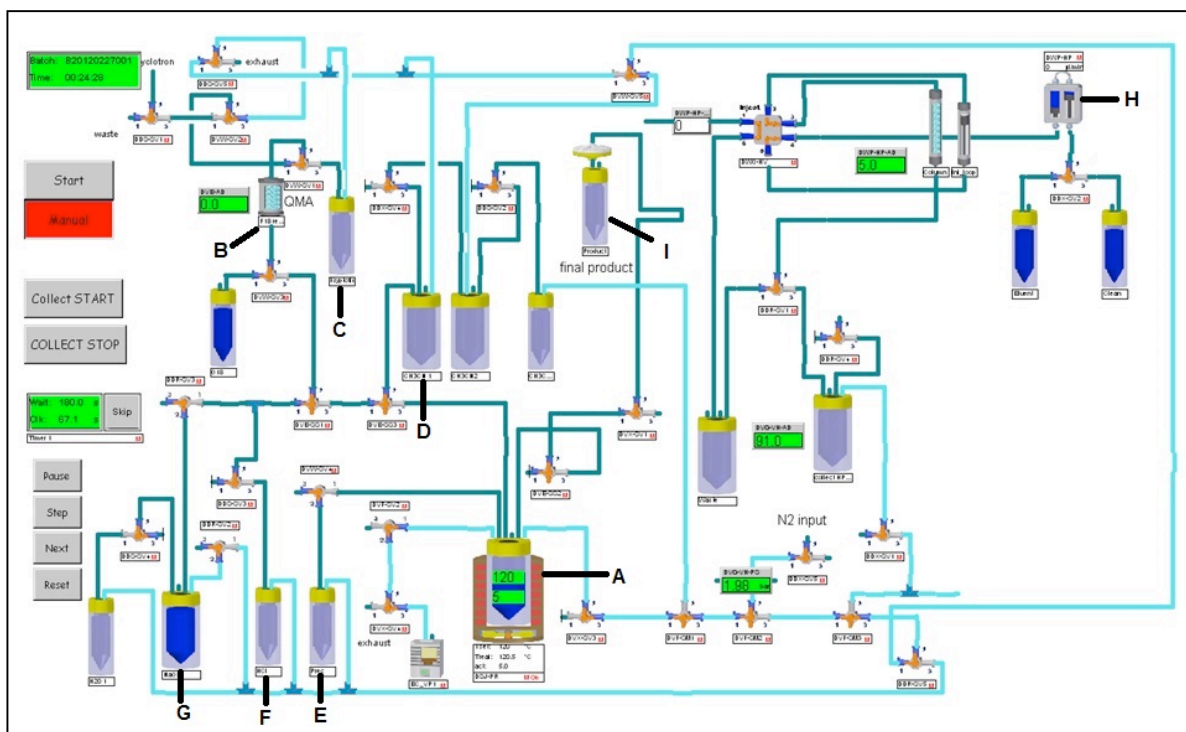
Figure 3.2: Schematic sketch of the Modular Lab synthetic set-up on the computer with **A**= reaction vial, **B**= QMA cartridge, **C**= K$_{222}$ vial, **D**= CH$_3$CN vial, **E**= precursor vial, **F**= acid vial, **G**= base vial, **H**= vacuum pump, and **I**= product vial.

A flow chart of the automated process performed by the software program is depicted in **Figure 3.3** below. The automatically driven process consisted of several steps that were followed in a consecutive manner. The initial step was the trapping of the fluoride ion by the QMA cartridge (**B**), which was mixed with K$_{222}$ in vial **C** and transferred into the reaction vial **A**. Anhydrous CH$_3$CN was injected from vial **D** into vial **A** and the mixture was azeotropically dried. It is noteworthy that the liquids were transferred automatically inside the modular lab through connecting tubes using pressure differences enabled by nitrogen gas input. In the next step, the precursor molecule which was dissolved in 1 mL of the reaction solvent, was added into vial **A** from vial **E**. After the reaction was carried

out, the vacuum pump (**H**) was initialized for 5 minutes to remove the reaction solvent at 140 ^{o}C. The deprotecting agent was then transferred from vial **F** into vial **A** to remove the protecting group and neutralized to obtain the crude product. The final step involved the preparative HPLC purification of the crude mixture to separate the final product, which was to be transferred into vial **I**.
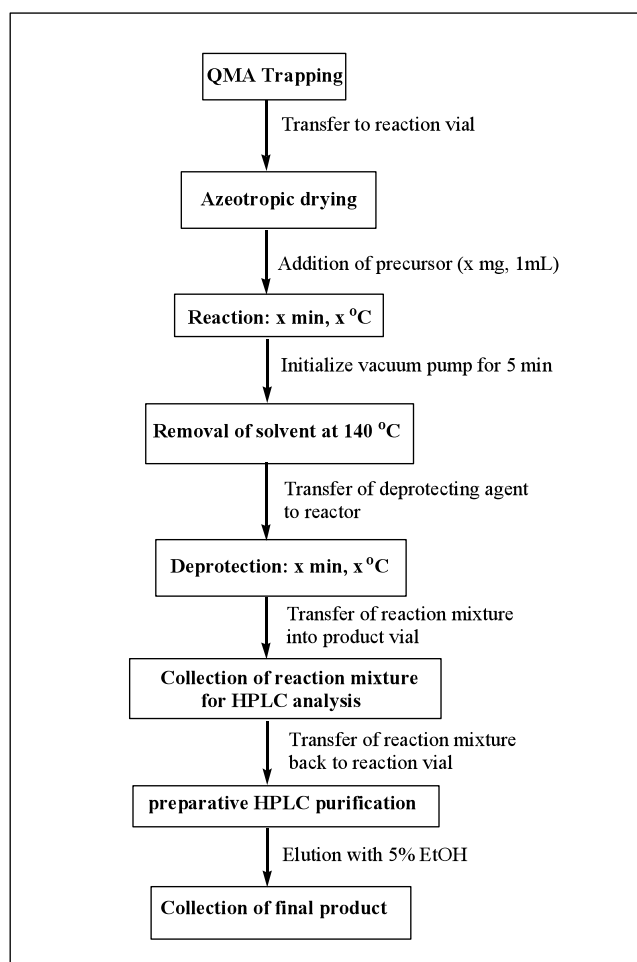
Figure 3.3: Flow chart describing the general synthetic steps in modular lab.

3.1.2.2. [^{18}F]FLT synthesis using 5'-*O*-Bz-2,3'-anhydrothymidine

Although a viable method was developed by the Wolfson Molecular Imaging Centre group[6] using the precursor molecule 5'-*O*-benzoyl-2,3'-anhydrothymidine, **48** as depicted in **Scheme 3.4,** the experiment was repeated under the same reaction conditions to assess the feasibility of the reaction using the modular set-up shown in **Figure 3.2.**
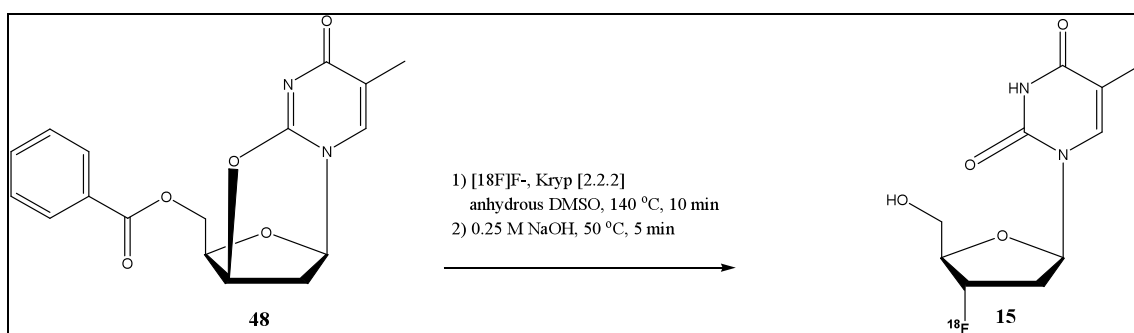
Scheme 3.4: ^{18}F-FLT synthesis using the precursor 5'-*O*-Bz-2,3'-anhydrothymidine, **48**.

For this procedure, the precursor molecule 5'-*O*-benzoyl-2,3'-anhydrothymidine, **48** was synthesized under Mitsunobu reaction conditions in a one-pot reaction as outlined in **Scheme 3.5** performing both benzoyl protection at the 5'-*O*-position and 2,3'anhydro ring formation at the 3'-position in one pot.
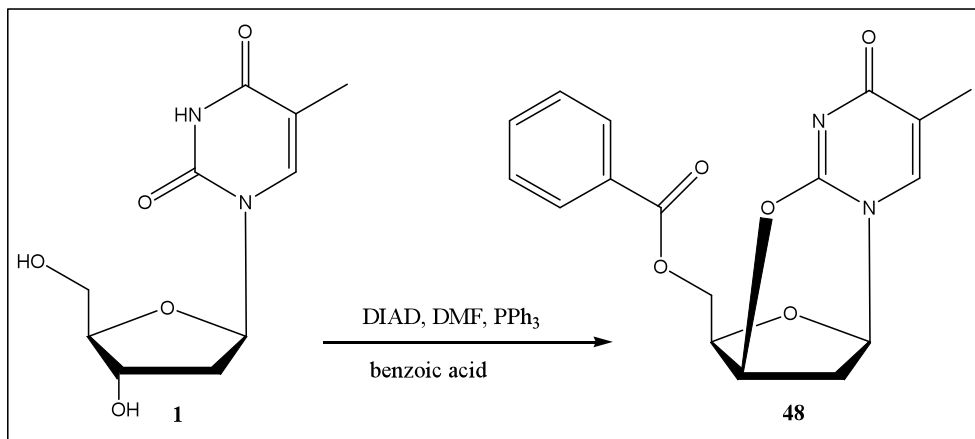
Scheme 3.5: Formation of precursor **48** via Mitsunobu reaction.

The Modular Lab software program was set up for a reaction time of 10 minutes. All the vials in the Modular Lab were filled with 1 mL each of the solutions or solvents as mentioned in section 3.1.2.1. The reaction vial (**A**) was filled with 30 mg of the synthesized precursor **48** dissolved and sonicated in 1 mL of anhydrous DMSO. Thereafter, the automated radiosynthesis was allowed to proceed by operating the Modular Lab software. For the purpose of radiolabeling, 2 GBq of $[^{18}F]F^-$ in $H_2^{18}O$ was delivered from the cyclotron into the hot cell containing the Modular synthetic kit and passed through the activated QMA ion exchange cartridge (**B**) to isolate the $[^{18}F]F^-$ anion. The ^{18}F-fluoride was then passed into vial (**C**) automatically to complex with the Kryptofix [2.2.2] solution and dried azeotropically with dry acetonitrile, after which it was transferred into the reaction vial (**A**). The reaction progress was monitored using analytical radio-HPLC with 10% EtOH as the eluent as described in the experimental section *6.1.4*. In **Figure 3.4**, the radio-HPLC radioactivity and UV traces of the reaction mixture with an activity of 0.89 GBq containing $[^{18}F]FLT$ are given along with the

96

HPLC data of the reference non-radioactive ^{19}F-FLT for comparative study. As seen in **Figure 3.4**, the peak for [^{18}F]FLT was detected at 7.5 minutes and was corresponding to the retention time of ^{19}F-FLT. The other peaks detected at UV 254 nm were assigned to the following compounds: Kryptofix [2.2.2]/ potassium salts (4.5 to 6.5 minutes), elimination product (8.5 to 10 minutes) and thymidine (10 to 10.5 minutes). The retention times of thymidine and K_{222} were analyzed previously with 10% EtOH on analytical HPLC. Unfortunately, [^{18}F]FLT could not be purified from the reaction mixture by preparative HPLC due to technical issues with the HPLC instrument but the formation of [^{18}F]FLT was confirmed by analytical radio-HPLC with a radiochemical yield (RCY) of 44.5%. The radioactivity of the crude product was calculated using the following equation:

Bq = [moles of ^{18}F$^-$ x N_a x ln (2)] / $t_{1/2}$; given that 1GBq of Activity = 10^9 Bq, $t_{1/2}$ of ^{18}F= 109.8 minutes, N_a = 6.022x10^{23} mol^{-1} and ln(2)=0.693.

RCY (%) = [radioactivity of the product / starting radioactivity of ^{18}F$^-$] x 100
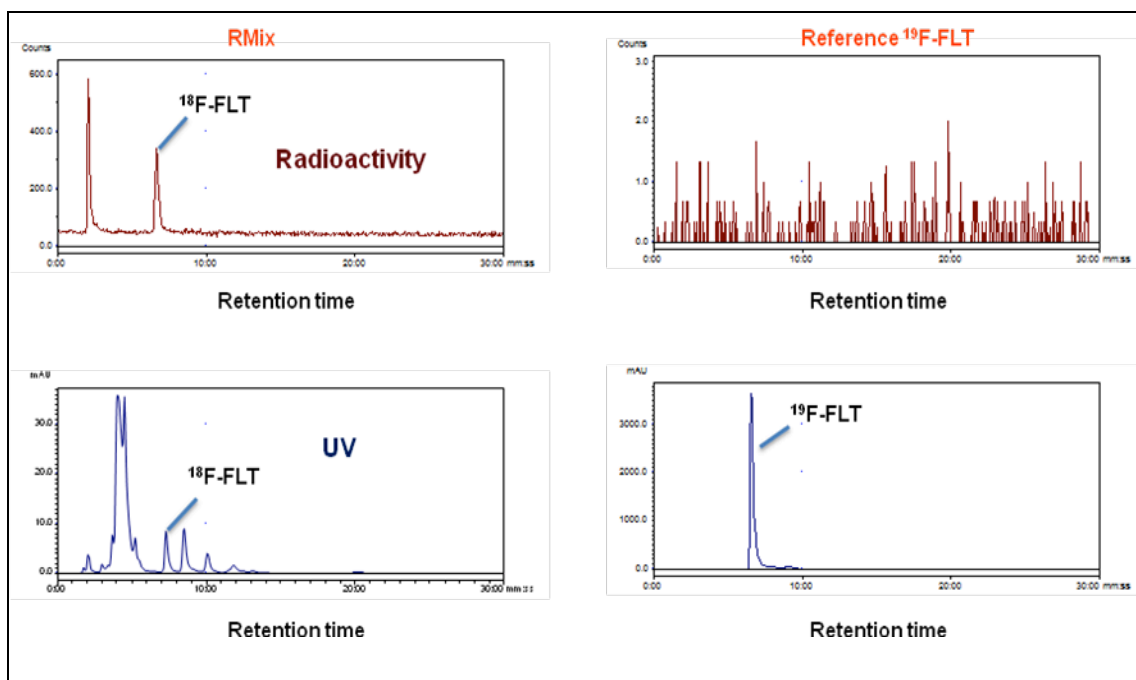
Figure 3.4: Analytical radioactive and UV (254 nm) HPLC traces of [^{18}F]FLT, **15** synthesized from precursor **48** compared with UV HPLC trace for reference sample of ^{19}F-FLT, **10**; column : ZORBAX Eclipse Plus C18 (4.6 x 250 mm) 5µm;

eluent: 10% ethanol in 90% water; flow rate: 1 mL min^{-1}. The main product **15** has been labeled in the figure above and is detected at a retention time of 7.5 minutes.

3.1.2.3. [^{18}F]FLT synthesis using 5'-*O-p*MeOBz-2,3'-anhydrothymidine

In continuation of the efforts to develop efficient synthetic methods for [^{18}F]FLT, a further improved and similar synthetic approach was sought for the minimization of reaction time. Hence the 5'-*O*-para-methoxybenzoyl-2,3'-anhydrothymidine precursor, **33** was studied for [^{18}F]FLT radiosynthesis due to the easier removal of the protecting group and the novelty of the use of this precursor. Although, the study of fluoridation

reactions of this precursor molecule under thermal conditions have not been successful as discussed in chapter 2, a positive outcome was still anticipated under radiosynthetic conditions to synthesize [^{18}F]FLT, **15**.

The radiosynthesis was performed under the conditions given in **Scheme 3.6** using the same Modular Lab set up described in the section above. However, the reaction time was varied from 5 minutes to 10 minutes and the reaction temperature ranging from 140°C to 160°C. Therefore, a number of experiments had to be carried out, during which technical issues with the Modular Lab kit were encountered. For instance, failure of the modular software, electrical faults in the generator connections and formation of precipitate causing blockage in modular lab tubes, prompted the experiments to be repeated.
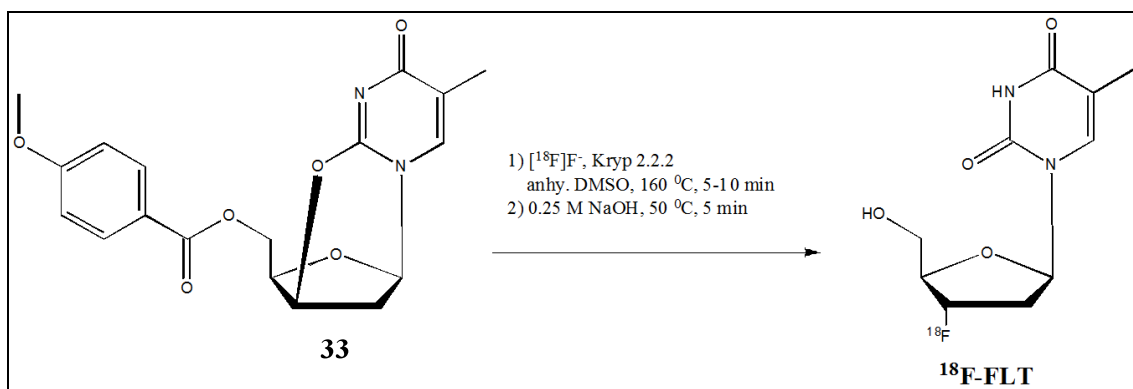
Scheme 3.6: ^{18}F-FLT synthesis using 5'-*O*-pMeOBz-2,3'-anhydrothymidine, **33**.

A table of comparison of synthesis yields achieved using different concentrations of the precursor molecule **33**, in 1 mL of anhydrous DMSO, varying reaction times and

temperature is shown below. From **Table 3.1**, it can be noted that for the reaction to become feasible, at the least 30 mg of the precursor **33** had to be used. Another observation was that, while undertaking the experiments at a reaction time of 160 °C, decomposition of the precursor occurred, which was apparent from the dark brown discolouration of the reaction vial (A). Surprisingly, the reaction progressed successfully at a reaction temperature of 140 °C within both 5 minutes and 10 minutes of reaction time. For the 10 minutes experiment, 2 GBq of activity was used at the start leaving 0.5 GBq of [^{18}F]-activity at the end and hence RCY of 25%. However, the 5 minutes experiment, which started off with 2 GBq of activity as well produced a lower RCY of 7.1%, presumably due to non-completion of the radio-fluorination reaction.

Table 3.1: Comparison of synthesis yields from different procedures using precursor **33**.

Precursor Amount (mg)	Reaction time (min)	Temperature (°C)	No. of trials	Reaction progress	RCY (%)
10	5	160	1	X	/
		140	2	X	/
	10	160	1	X	/
		140	1	X	/
20	5	160	2	X	/
		140	3	X	/
	10	160	2	X	/
		140	2	X	/
30	5	160	1	X	/
		140	3	☑	7.1
	10	160	2	X	/
		140	3	☑	25

The reaction mixture was analysed with radio-HPLC using 10% of EtOH as the eluent and the formation of trace amounts of [^{18}F]FLT was verified by comparing the data with the HPLC data of the reaction mixture sample spiked with reference material, ^{19}F-FLT as shown in **Figure 3.5**.
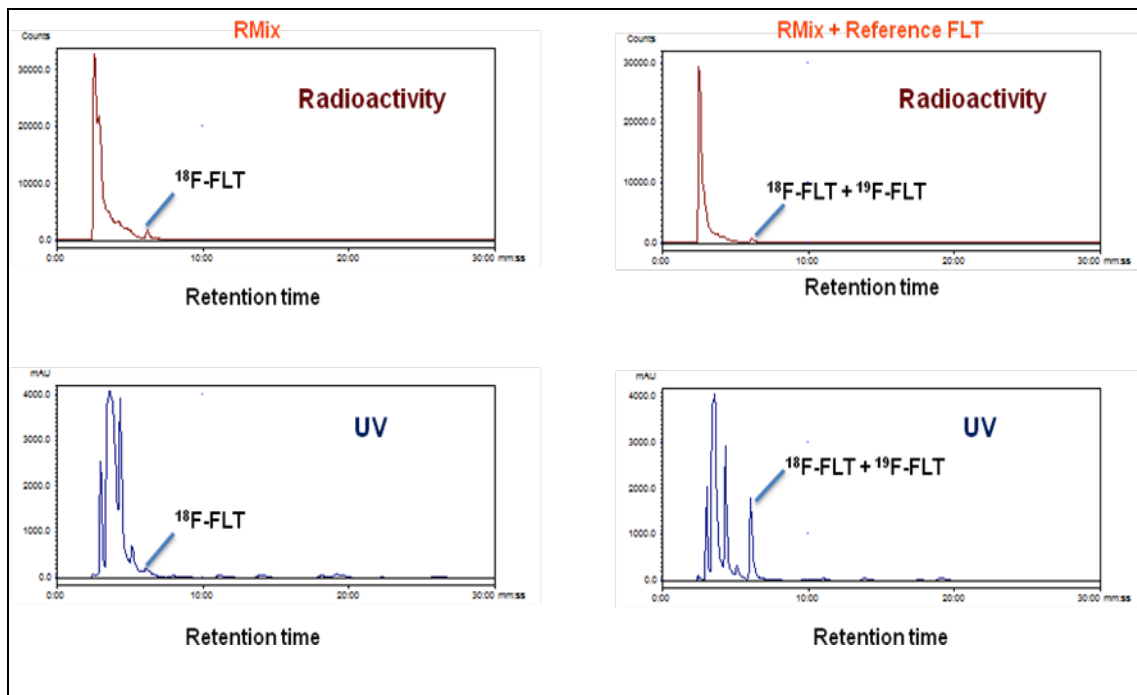

Figure 3.5: Analytical radioactive and UV (254 nm) HPLC traces of [^{18}F]FLT, **15** synthesized from precursor **33** and compared with HPLC traces for [^{18}F]FLT spiked with reference ^{19}F-FLT, **10**. column : ZORBAX Eclipse Plus C18 (4.6 x 250 mm) 5μm; eluent: 10% ethanol in 90% water; flow rate: 1 mL min^{-1}.

3.1.2.4. [^{18}F]FLT synthesis using 5'-*O*-DMTr-2,3'-anhydrothymidine

Further to the investigation of a suitable method, the next step was to conduct experiments using the precursor molecule, 5'-*O*-Dimethoxytrityl-2,3'-anhydrothymidine, **37** under the reaction conditions given in **Scheme 3.7**. However, this precursor has been used previously by Machulla and coworkers[3] for [^{18}F]FLT synthesis. But, due to comparatively longer synthesis times (90-140 min), the study was again initiated by variations in some reaction conditions. In the previously studied method, anhydrous DMSO was used as the solvent and since the removal of DMSO can take longer, it was decided to use anhydrous DMF in order to reduce the overall reaction time. As a consequence of the previous experiments conducted at PETIC, led to decomposition when the precursor molecule was subjected to the reaction temperature of 160 ^{o}C, the radiosyntheses were carried out at a maximum reaction temperature of 140 ^{o}C. Also a minimum of 30 mg of the precursor compound, **37** was used in the ensuing experiments, which was dissolved and sonicated with 1 mL of anhydrous DMF in the reaction vial **A**. Subsequently, the radiosynthesis experiments were allowed to proceed using the Modular Lab synthetic kit with varying reaction times of 5 minutes and 10 minutes. Unfortunately, none of the experiments showed satisfactory results as the expected product, [^{18}F]FLT peak was not detected using analytical HPLC. This could be due to the use of anhydrous DMF as the reaction solvent, which might have been less effective than anhydrous DMSO in solvating the precursor molecule **37**.
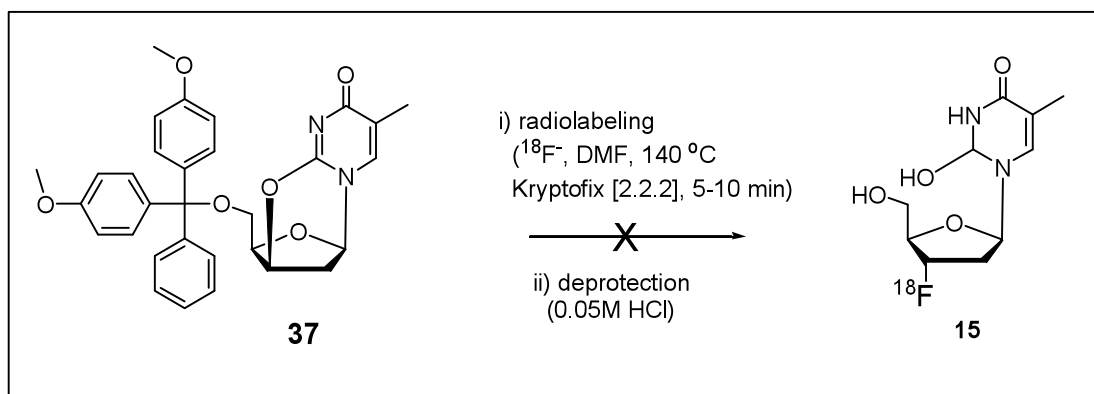
Scheme 3.7: Radiosynthetic conditions for [^{18}F]FLT synthesis using precursor **37**.

3.1.2.5. [^{18}F]FLT synthesis using 5'-*O*-Tr-3'-nosylthymidine

At this stage, it was decided to investigate further with the radiosynthesis studies using the previously synthesized precursor molecule, 5'-*O*-trityl-3'-nosyl thymidine, **42**. It was hoped that the successful synthesis of ^{19}F-FLT from the precursor **42** under conventional thermal conditions as discussed in chapter 2, could also have a positive impact under radiosynthesis conditions as outlined in **Scheme 3.8**. Although the synthesis of **15** using the precursor **42** has been studied previously and recorded in literature (Grierson and Shields method[2]), the reaction solvent, temperature and protecting groups have been varied to conduct a comparative study. The radiosynthesis experiments were carried out using the Modular Lab set-up consisting of the reaction vial **A** filled with 30 mg of precursor **42** dissolved in 1 mL of anhydrous DMF. The reaction times were varied from 5 minutes to 10 minutes, but the reaction temperature of 140 ^{o}C was kept constant. A number of experiments that were carried out failed due to technical issues with the Modular lab kit, such as formation of precipitates in the

modular tubes causing blockage and obstructing the automated process. Therefore, the radiosynthesis experiments had to be repeated. However, the outcome of these experiments were all negative as [^{18}F]FLT peak was not detected after HPLC analysis. As it can be noted from the literature, the NH group on thymine base of the precursor was masked with a protecting group as the nosyl moiety is a very good leaving group and can be easily attacked by the base instead of being substituted by the nucleophile ^{18}F$^-$. Hence the failure of this experiment can be rationalized by the absence of a suitable protecting group on NH.
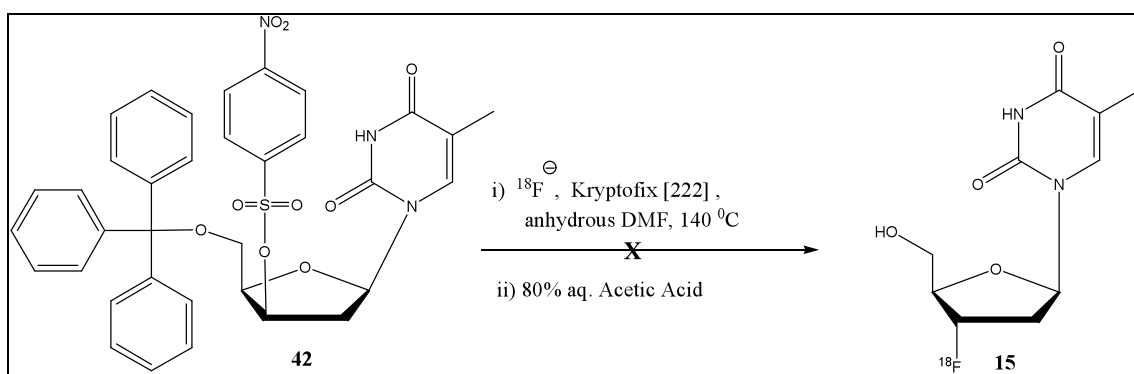

Scheme 3.8: Radiosynthesis conditions for [^{18}F]FLT synthesis using precursor **42**.

3.1.2.6. [^{18}F]FLT synthesis using 2,3'-anhydrothymidine

The final set of radiosynthesis experiments were studied using the previously synthesized precursor molecule, 2,3'-anhydrothymidine, **44**. The particular aspect that proved to be advantageous by the use of this precursor **44** is the omission of the deprotecting step as the 5'-*O*-position of the precursor molecule is not protected. Hence, the overall reaction time can be reduced. Although the fluoridation reactions performed

on the compound **44** under thermal conditions as mentioned in chapter 2, did not result in the formation of FLT, it was still agreed to conduct radiosynthesis experiments as the reactions are performed in the airtight Eckert-Ziegler modular lab kit enclosed by lead-shielded hot cell, which would increase the nucleophilicity/ reactivity of the [^{18}F] fluoride ion and hence leading to better results. The reaction conditions for the synthesis of [^{18}F]FLT using the 2,3'-anhydro precursor are described in **Scheme 3.9**.
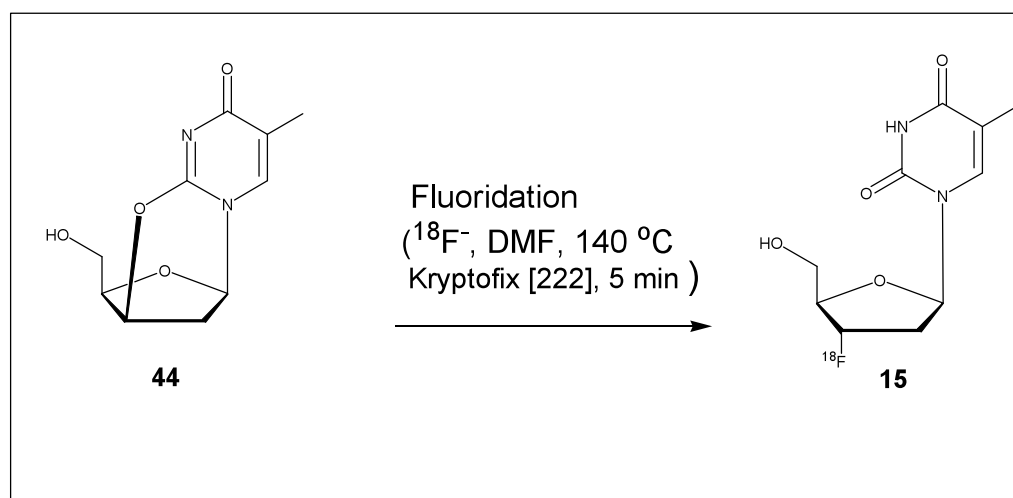
Scheme 3.9: Radiosynthesis conditions for [^{18}F]FLT, **15** synthesis using 2,3'-anhydro precursor, **44**.

The Modular lab was set up for a reaction time of 5 minutes at the start and all the vials were filled accordingly including the reaction vial **A** with 30 mg of the precursor molecule, **44**. For the radiolabeling, an activity of 1.2 GBq was available from the cyclotron. With the use of the Modular lab software the reaction was allowed to proceed under the reaction temperature of 140 ^{o}C. At the end of the reaction, the mixture was analyzed with radio-HPLC and interestingly the radioactive and UV traces of [^{18}F]FLT,

15 were detected as depicted in **Figure 3.6**. For further verification, the reaction mixture was analyzed with reference ^{19}F-FLT sample.
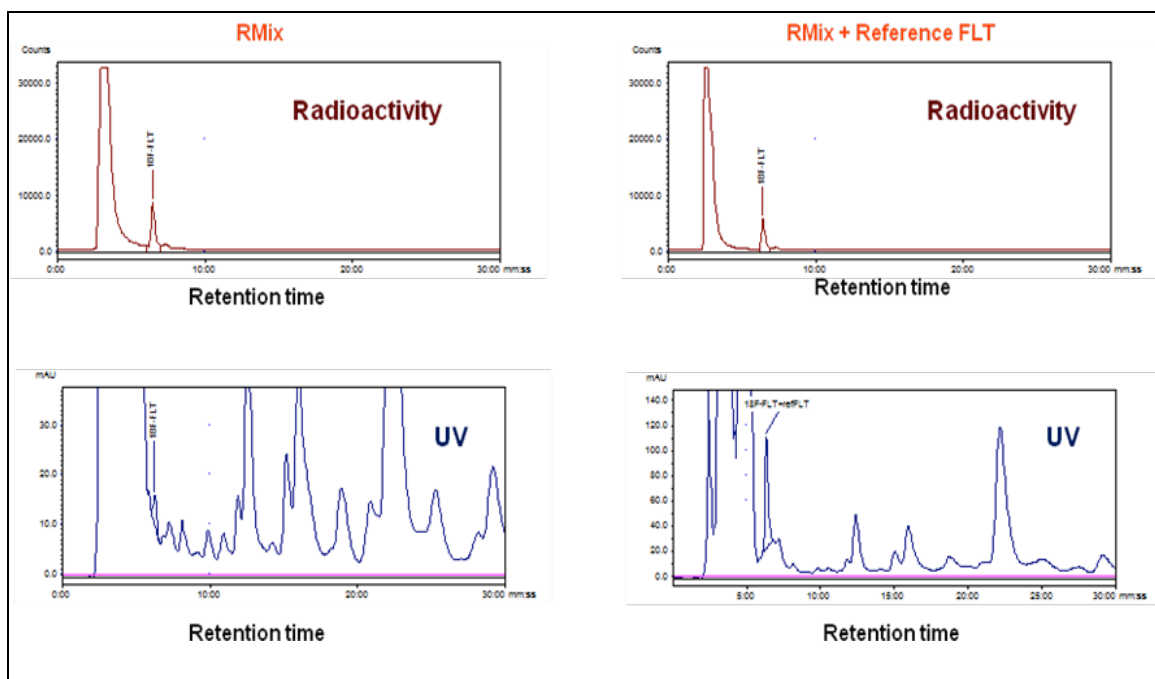
Figure 3.6: Analytical radioactive and UV HPLC traces of [^{18}F]FLT compared with HPLC traces for [^{18}F]FLT, **15** spiked with reference ^{19}F-FLT, **10**. column : ZORBAX Eclipse Plus C18 (4.6 x 250 mm) 5μm; eluent: 10% ethanol in 90% water; flow rate: 1 mL min^{-1}.

However, the radiosynthetic yield (RCY) of 1.5 % was very low. Although [^{18}F]FLT was detected at a retention time of 7.3 minutes on the analytical HPLC data along with a number of unexpected side products, the desired product [^{18}F]FLT could be purified using preparative HPLC. Unfortunately, the preparative HPLC could not be operated due to faults in the HPLC apparatus and hence purification of the product was not pursued.

3.1.2.7. Summary of results

Among the numerous experiments conducted using the previously synthesized precursor molecules, only 5'-*O*-Bz-2,3'-anhydrothymidine, **48**, its derivative 5'-*O*-pMeBz-2,3'-anhydrothymidine, **33** and 2,3'-anhydrothymidine, **44** gave positive results. Precursor molecule **48** gave the highest RCY of 44.5%, while precursor **29** is to be noted for its novel use as a precursor for $[^{18}F]FLT$ radiosynthesis. Also the omission of the deprotecting step with the use of precursor **44** for $[^{18}F]FLT$ radiosynthesis proved to be advantageous due to the reduction in the reaction time. DMF was found to be the optimum solvent for the radiolabeling reaction with a reaction temperature of 140 ^{o}C. The minimum reaction time that could be reduced to, was 5 minutes as further reduction in time could lead to incomplete reaction.

3.2. Concept of [^{18}F]-FLT ProTides

In this section, focus is given to the synthetic attempt of novel ^{18}F-incorporated FLT phosphoramidates pronucleotides (ProTides) as potential diagnostic PET imaging agents that could effectively cross cell membranes and represent a model system to visualize the pharmacological and biological effect of these compounds *in vivo*. The ProTide technology is a successful pro-drug strategy developed by Prof. Chris McGuigan and his group in 1992 to improve the cellular permeability of biologically active nucleosides and thereby improve the bioavailability of nucleoside drugs. The study of FLT ProTides as therapeutic agents has been elaborated in chapter 4. The initial stages of the synthesis were focussed on converting the 3'-position of the starting material, thymidine, **1** into a suitable leaving group (e.g. mesyl) which is labile for nucleophilic attack by the fluoride, ^{18}F$^-$; along with the SN$_2$ inversion of stereochemical configuration. The expertise of McGuigan group helped to analyze the fact that the phosphoramidate group was most likely to be prone to decomposition over multiple synthetic steps involving preparative column chromatographic purification. Therefore, the masking of the 5'-OH group of thymidine with a phosphoramidate group was carried out during the intermediate stages of the synthetic route to generate the ProTide precursor (**Scheme 3.10)**. Considering the half-life of the radionuclide ^{18}F, the aim was to introduce the fluoride ^{18}F$^-$ into the 3'-position of the ProTide precursor molecule during the final stages of the synthesis. A general description of how ProTide precursor molecules were attempted to be synthesized is depicted in **Scheme 3.10**. All the fluoridation reactions were planned to be performed under thermal conditions using different nucleophilic fluorides and reaction conditions such as varying temperature and reaction solvents, as

the optimization study could lay the framework for translation to ^{18}F-Fluoride radiochemistry and eventually the ^{18}F-FLT ProTide radioactive synthesis.
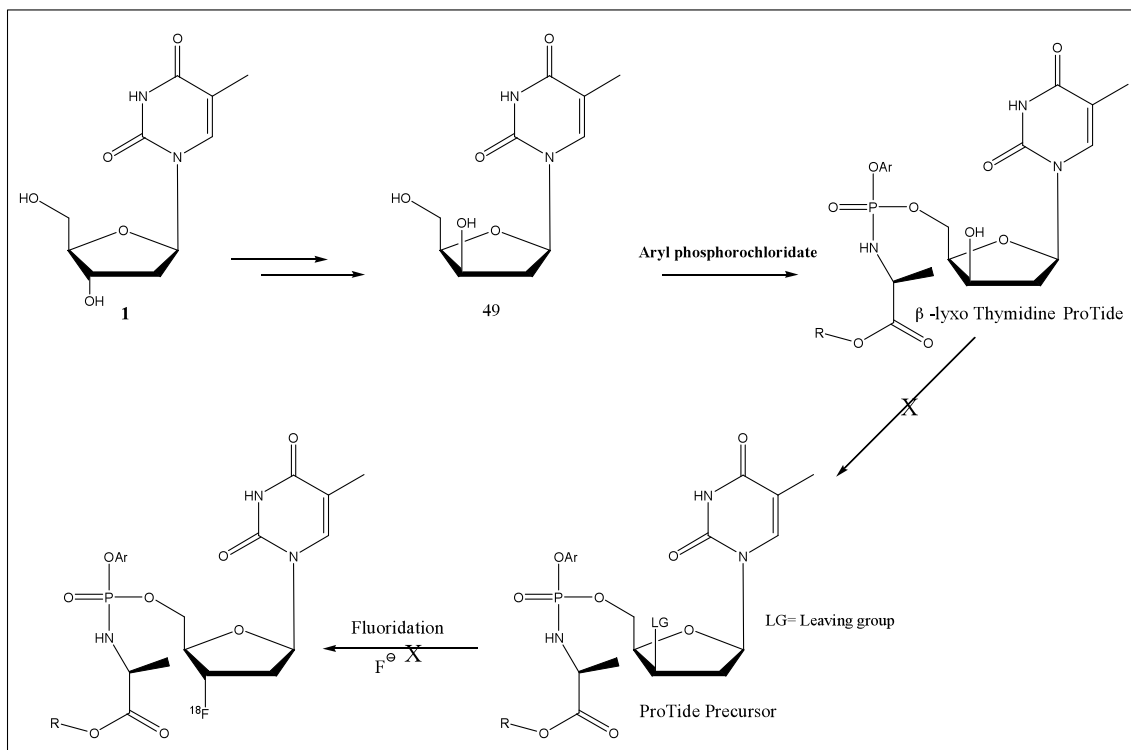
Scheme 3.10: General schematic plan for ^{18}F-FLT ProTide synthesis starting from thymidine, **1** converted to β-lyxo thymidine, **49** using methods explained in the next section. The thymidine analogue **49** is then protected with phosphoramidate group at 5'-*O*-position using standard procedure *E* (section **6.2**), in order to synthesize the ProTide precursor. Various reaction conditions have been applied for fluoridation reactions of the precursor molecule, which have been detailed in the following sections.

3.2.1. Fluoridation attempt via ProTide mesyl precursor

Initially, **1** was converted to **44** using Mitsunobu reaction conditions as described in **Scheme 3.11**. In order to introduce a hydroxyl group at the 3'-position of the nucleoside **44**, to form the ProTide analogue **50**, hydroxylation reaction using NaOH [25M] was carried out on **44** to yield the nucleoside **49**, which is the epimer of **1**. Consecutively, **50** was synthesized from **49** using the standard procedure E (chapter 6). Unfortunately, the incorporation of mesyl group at the 3'-position of **50** to form the ProTide precursor **51** using mesyl chloride was not successful. It was suspected that mesylation occurred on the phosphoramidate group by analyzing with NMR spectrum. Hence, fluoridation studies could not be performed on **51** to form the desired compound **52.**
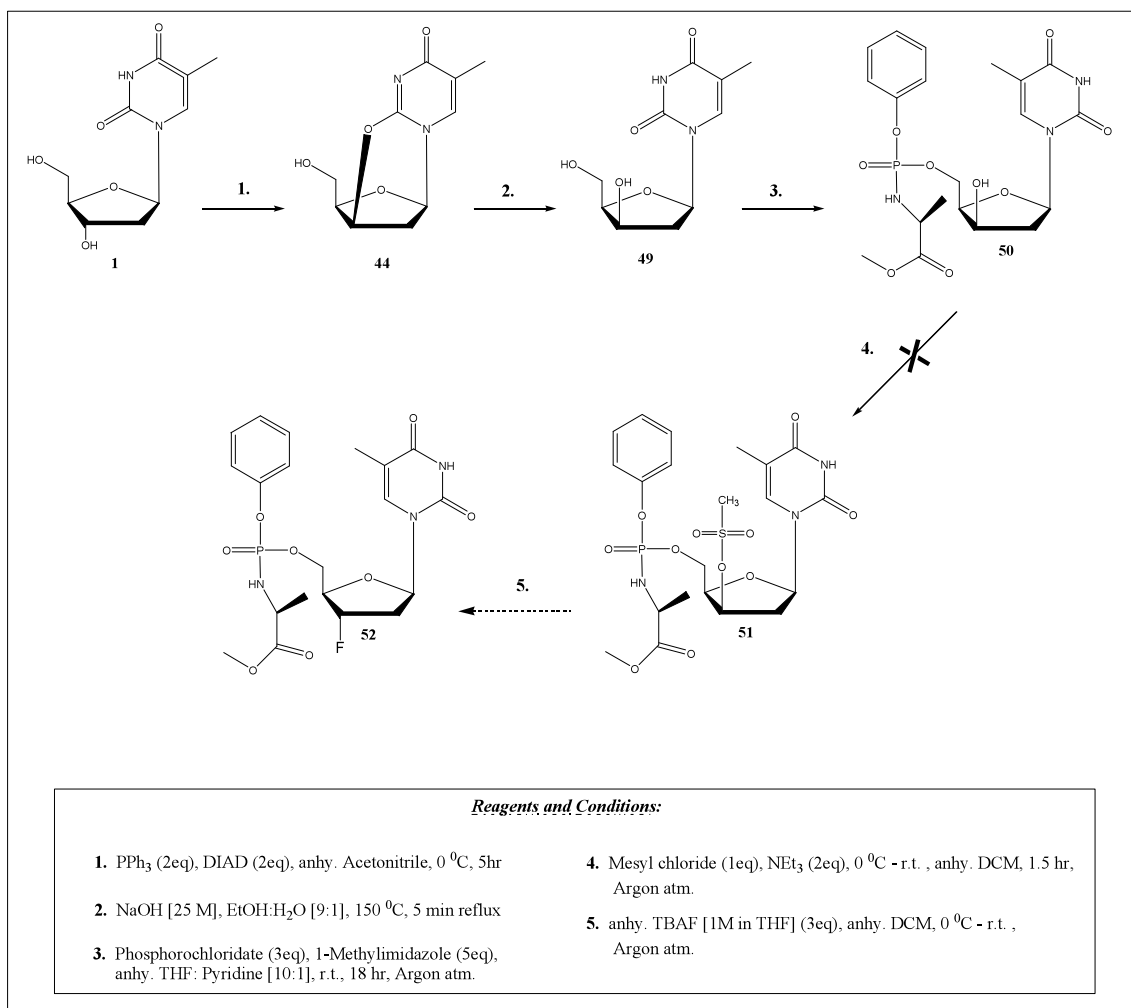
Scheme 3.11: Synthetic procedure for [¹⁸F]FLT ProTide via the mesyl precursor, **51**.

3.2.2. Fluoridation attempt via ProTide anhydro precursor

The next stage was preparing [^{18}F]FLT ProTide **52**, using 2,3'-anhydro precursor. As the anhydro group has proven to be a good leaving group by the nucleophilic ^{18}F$^-$ attack in [^{18}F]FLT synthesis, it was anticipated that by generating ProTide anhydro precursor, the synthesis of [^{18}F]FLT ProTide could also be achieved by fluoridation. As shown in

Scheme 3.12, compound **53** failed to be synthesized from **44** as no reaction occurred retaining 100% of the starting material. Although tetrahydrofuran (THF) is the ideal solvent for synthesizing ProTides, the reaction was repeated by dissolving compound **44** in a 1:1 mixture of THF and pyridine as **44** was not dissolving in THF. But a progress in reaction could not be observed leading again to the failed attempt of synthesizing [^{18}F]FLT ProTides.
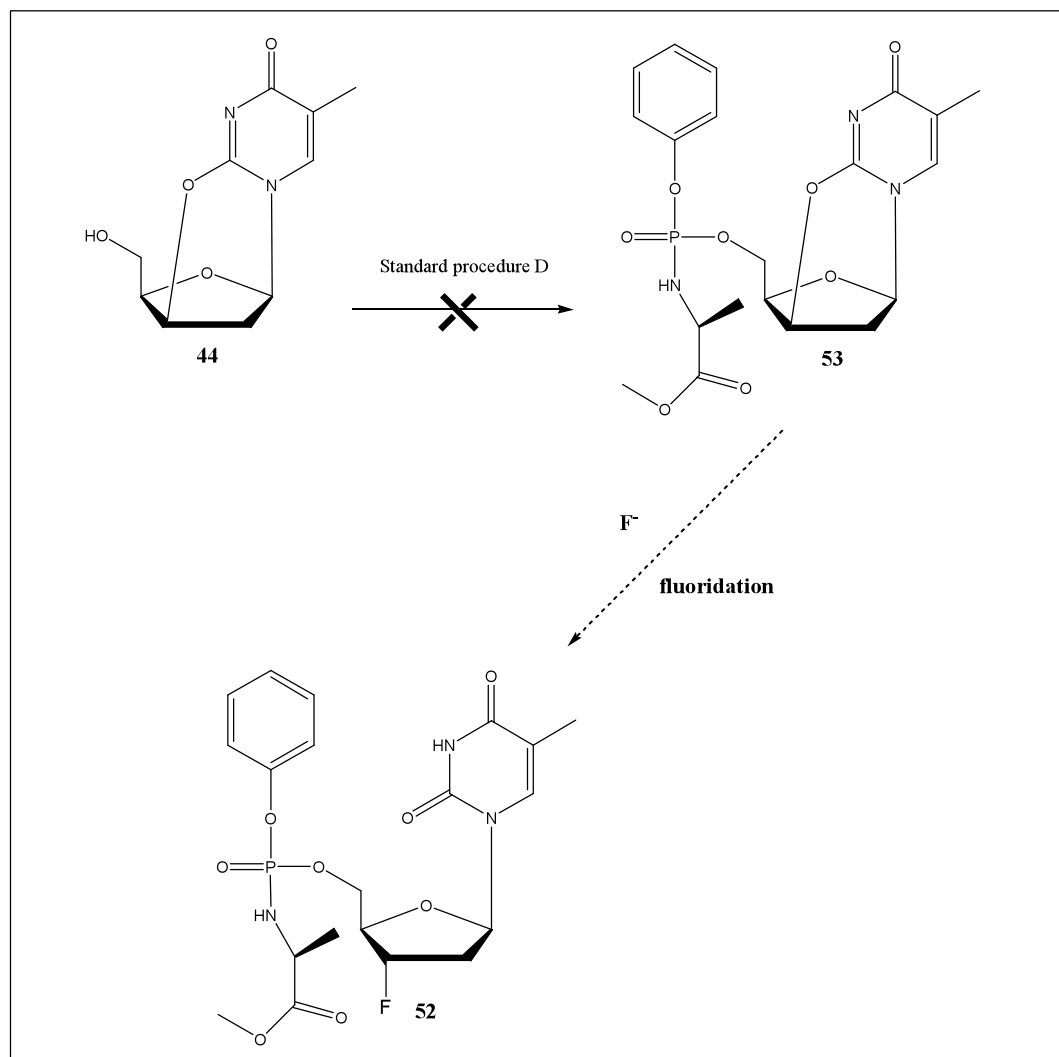
Scheme 3.12: Synthetic attempt of [^{18}F]FLT ProTide, **52** via the anhydro precursor, **53**.

References:

1. Ballon, D. J.; Goldsmith, S. J.; Vallabhajosula, S.; Suehiro, M. Investigation of the role of the base in the synthesis of [^{18}F]FLT. *Appl Radiat Isot.,* **2007**, *65*, 1350-1358.

2. Grierson, J. R.; Shields, A. F. Radiosynthesis of 3'-deoxy-3'-[^{18}F]fluorothymidine: [^{18}F]FLT for imaging of cellular proliferation in vivo. *Nucl Med Biol.,* **2000**, *27*, 143-156.

3. Machulla, H. J.; Blocher, A.; Kuntzsch, M.; Piert, M.; Wei, R.; Grierson, J. R. Simplified labeling approach for synthesizing 3'-deoxy-3'-[^{18}F]fluorothymidine ([^{18}F]FLT), *J Radioanal Nucl Chem.,* **2000**, *243*, 843-846.

4. Martin, S. J.; Eisenbarth, J. A.; Wagner-Utermann, U.; Mier, W.; Henze, M.; Pritzkow, H.; Haberkorn, U.; Eisenhut, M. A new precursor for the radiosynthesis of [^{18}F]FLT, *Nucl Med Biol.,* **2002**, *29,* 263-273.

5. Kilbourn, M.R. *Nucl. Sci. Series, NAS-NS3203,* **1990**, National Academy Press*,* Washington DC.

6. Radigois, M. 3'-deoxy-3'-[^{18}F]fluorothymidine (FLT) production-standard operating procedure. Personal communication. Wolfson Molecular Imaging Centre group protocol. The University of Manchester.

Chapter 4

4. ^{19}F-FLT Phosphoramidates

4.1. Nucleotide Prodrugs

Since the discovery of many fluorinated nucleosides and nucleotides with potent anti-viral and anti-cancer activities, increased attention has been given to their intracellular delivery.[1] Despite difficult delivery and formulation issues, nucleosides account for a large proportion of registered anticancer and antiviral drugs. In general, nucleosides become biologically active once they are converted into their respective 5'-*O*-triphosphates through three separate nucleoside kinases. However, the initial phosphorylation (monophosphorylation) step is usually rate limiting, due to the absolute need for nucleoside kinase-mediated activation, and often results in poor pharmacokinetic properties for the nucleoside drug candidate that need to be addressed during drug development.[2] Also, as nucleotides are charged molecules, their membrane permeability is very poor and they exhibit low bioavailability. In order to circumvent the latter issue and also bypass the conversion of nucleosides into their monophosphates, numerous nucleotide prodrug (pro-nucleotide or "ProTide") strategies have been designed, which were found to be effective in successfully delivering nucleotides to their intracellular target sites. These approaches involve prodrugs that are designed to mask the negatively charged oxygens of the nucleotide analogue 5'-*O*-monophosphate and stabilize it. The diffusion of lipophilic pro-nucleoside analogues across the cell membrane occurs through passive diffusion or is facilitated by nucleoside transporters which are complex transport systems consisting of multiple carrier proteins.[3] Nucleoside

transporters play a crucial role for the cellular uptake of nucleoside drugs, which may be expressed at various levels for different cell types. Upon entering the cell, the masking groups are cleaved to release the monophosphate followed by further intracellular phosphorylations as illustrated below to generate the corresponding nucleotide analogue triphosphate (**Figure 4.1**).[4,5] Thus the aim of this concept was to synthesize lipophilic prodrugs of the nucleoside analogues, which after active transport and enzymatic cleavage, release the free monophosphate at high intracellular concentrations.
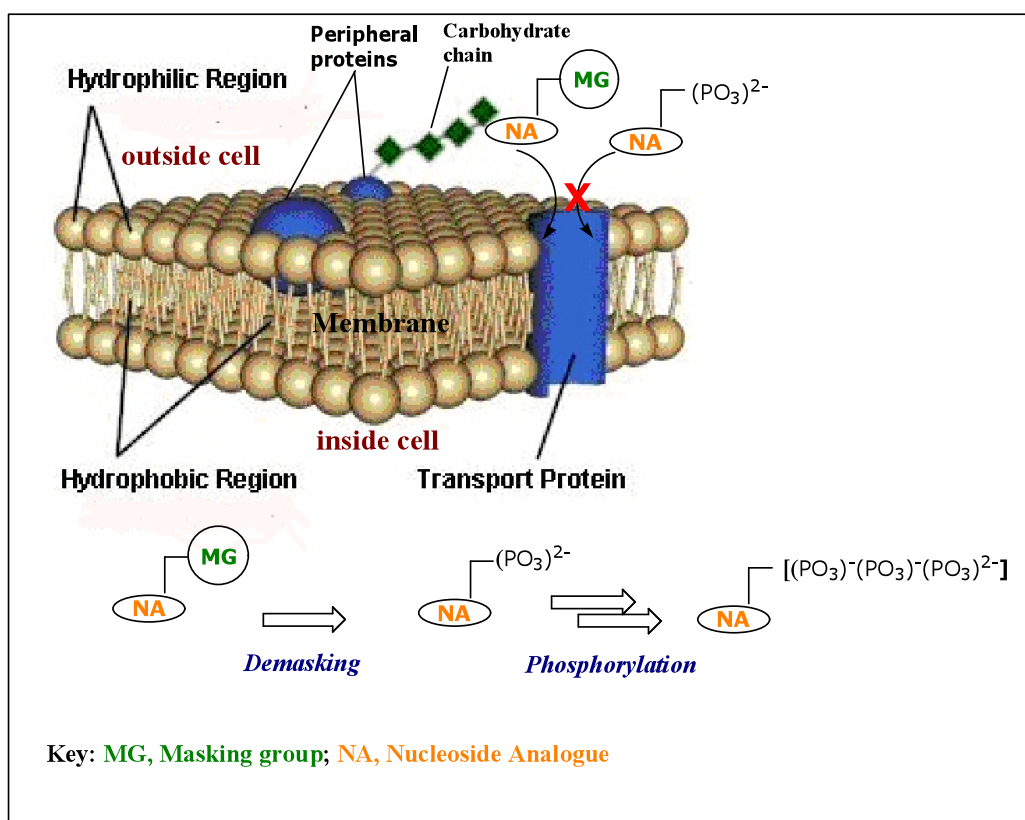

Figure 4.1: General scheme for the activation of nucleoside prodrugs.[4,5]

In this chapter, some of the currently available nucleotide prodrug strategies with a particular focus on the aryloxyphosphoramidates of FLT will be discussed.

4.2 Nucleotides as Drug Candidates

In contrast to nucleosides, nucleotides are already phosphorylated species which do not require the first enzyme (nucleoside kinase) mediated phosphorylation step for their metabolic activation. Therefore, nucleotides possess certain advantages over nucleosides considering their amenability as drug candidates. Firstly, nucleotides are expected to show activity in cases where the parent nucleoside is inactive due to the poor first intracellular phosphorylation as a result of reduced levels of activating enzyme (kinase) or reduced transporter-mediated entry of the nucleoside analogue into cells.[6] Secondly, nucleotides retain activity against certain nucleoside resistant mutants (e.g. HSV TK). Another advantage of using nucleotides rather than nucleosides as drugs is in cases where nucleosides are metabolically deactivated *in vivo* due to the actions of certain enzymes. For example, nucleosides are often substrates for phosphorylase-induced glycosidic bond cleavage, whereas nucleotides are not.[7]

After Cohen and Plunkett showed that ara-A monophosphate could enter cells directly (albeit poorly)[8], the field of nucleotides started to flourish with the discovery by Holy and De Clercq of phosphonomethylester nucleosides, a class of nucleotide analogues with interesting biological activities.[9]

Nucleotides also have some disadvantages over nucleosides as drug candidates. As nucleotides are charged species, they possess poor membrane permeability and hence, show very poor or no activity *in vitro*.[8] The negative charges of nucleotides also contribute to their poor bioavailability[10] and instability in biological media as they are rapidly dephosphorylated by phosphatases.[8] Thus utilizing successful nucleotide

prodrug strategies that can potentially overcome these shortages would lead to a better generation of antiviral and anticancer drugs.

4.3. Current Pro-nucleotide Strategies

4.3.1. The Bis (POM) Approach

The bis (pivaloyloxymethyl) [Bis(POM)] phosphotriester approach, firstly described by Farquhar *et.al,* is one of the possible ways of delivering the nucleotide analogue monophosphate derivatives into cells.[11-13] The mechanism of release of the nucleotide analogue monophosphate is thought to consist of two steps (**Figure 4.2**). The initial step involves an esterase enzyme that cleaves one of the two POM groups to give the highly reactive hydroxymethyl phosphotriester, **54** which spontaneously dissociates with the elimination of formaldehyde, **55** to give the phosphodiester, **56**. The cleavage of the second POM group is performed by cellular carboxyesterases resulting in the generation of the parent nucleoside monophosphate, **57**.[14]

But there are numerous issues related to the use of this prodrug strategy. Since the esterase enzymes involved in the activation of this class of drugs are present at high levels in many tissues including the small intestine, prodrug cleavage prior to absorption could limit the oral bioavailability of this class of prodrugs. Also the by-products released from the metabolism of bis (POM), *i.e.* formaldehyde and pivalic acid, are relatively toxic.
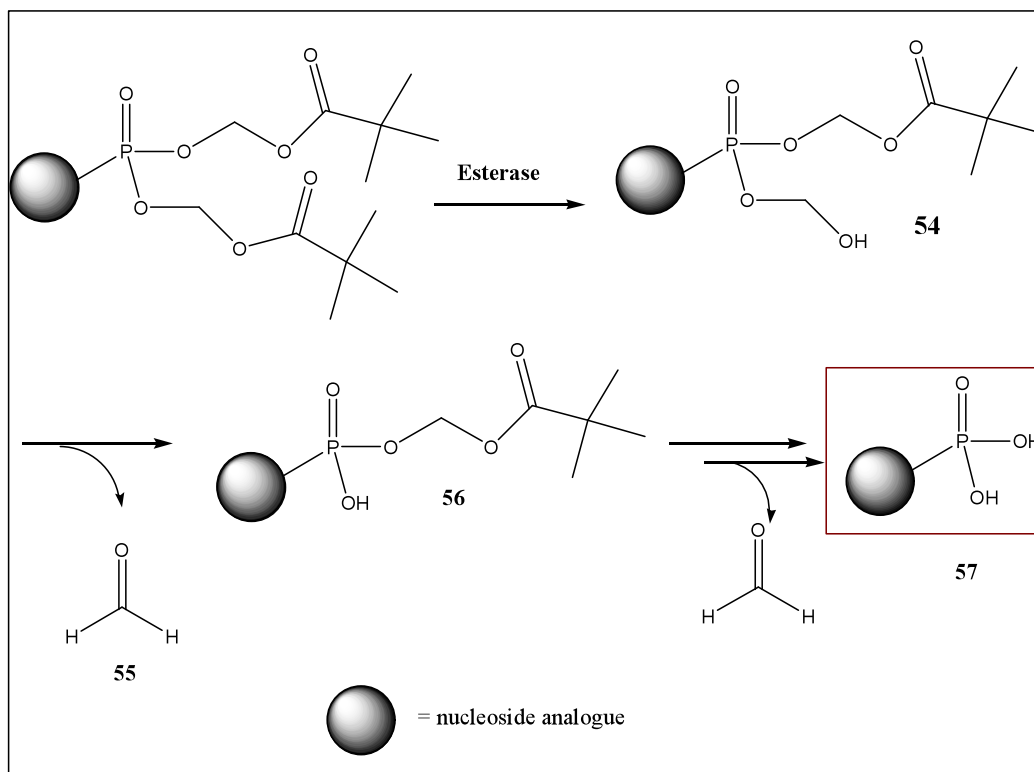
Figure 4.2: The activation process of the bis(POM).

However, this pronucleotide approach has been applied to some antiviral and anticancer drugs with variable success. An example is Bis(POM) PMEA (9-(2-phosphomethoxypropyl)adenine), which is clinically approved for the treatment of HBV (Viread[TM]).[15] In addition to that, the application of this strategy to other nucleosides including thymidine[16] and d4T[17] has been reported.

In terms of anticancer activity, the application of the bis (POM) approach to the antitumor active compound 2'-deoxy-5-fluorouridine-5'-monophosphate (FdUMP) was found to be useful in inhibiting the growth of cell lines resistant towards the originally used nucleoside drug, 5-fluorouracil.[18, 19]

4.3.2. The CycloSal Approach

While most of the nucleotide prodrugs present to date are activated by enzymes to liberate the corresponding nucleotide, some pronucleotide strategies are designed to involve chemically driven hydrolysis in their activation process. One such approach is the *cyclo*Sal method of Meier.[20] In this strategy, the cyclosaligenyl (*cyclo*Sal) moiety is chemically cleaved during the activation, although the second generation of this prodrug approach has been designed to also involve esterase cleavage. The metabolism of the first generation *cyclo*Sal prodrugs to release the attached nucleoside proceeds in two consecutive steps, firstly by chemical cleavage of the phenyl phosphotriester, **58** and then the benzyl phosphodiester, **59** (**Figure 4.3).** This concept is based on the different stability of the phenyl and benzyl phosphate esters.[21] The phenyl ester bond is the most labile one among the three P-O bonds, as the negative charge of the departing phenoxide ion can be delocalized in the benzene ring. This cleavage leads to the formation of the 2-hydroxybenzyl phosphodiester, **59**. The next step proceeds faster than the prior one because the ortho-hydroxy group mesomerically stabilizes the benzocarbocation. This would accelerate the formation of the monophosphate, **57** and salicyl alcohol, **60**.

This strategy has been applied to several nucleosides, *e.g.* d4T[22], FdU[23], AZT[24] and PMEA[25]. *In vitro* antiviral activity has been shown to correlate with the hydrolytic stability of the pronucleotide; 3- and 5-methyl substituents on the benzene ring enhance the anti-viral activity against HIV-1 and HIV-2 infected cells. These compounds are hydrolytically more stable than their unsubstituted counterparts, which suggest that reasonably slow hydrolysis is a prerequisite for markedly increased cellular uptake. The methyl substituted compounds also retain their activity in thymidine kinase-deficient

cells compared to the unsubstituted *cyclo*Sal derivatives. A clear advantage of the first generation *cyclo*Sal prodrug strategy over esterase-dependant approaches is that no potentially toxic side products are liberated upon cleavage.
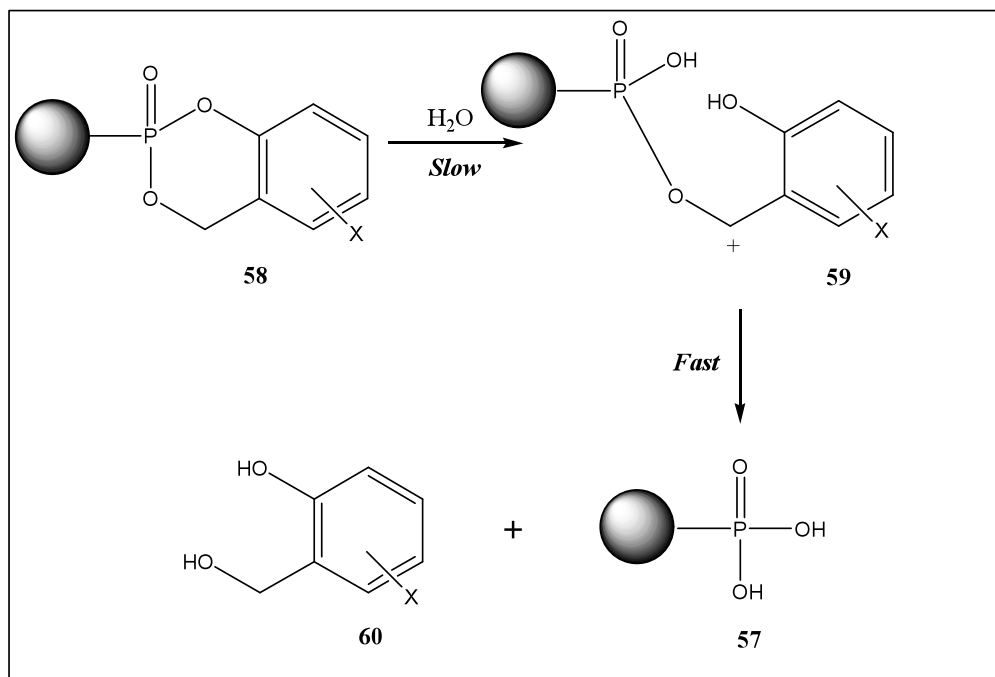
Figure 4.3: Activation of *cyclo*Sal prodrugs to release the nucleoside monophosphate, **57**.

4.3.3 The Phosphoramidate Diester Approach

This particular approach, developed by Wagner *et.al.,* is a prodrug strategy for nucleosides where only one of the phosphate charges is masked by an amino acid ester.[26] This strategy is derived from the pronucleotide method developed by McGuigan *et.al.*, in which both phosphate charges are masked to improve cellular uptake.[27] The unmasked negative charge in the phosphoramidate diester approach contributes to the water-solubility and stability of the prodrugs in human blood. The activation proceeds

directly to the monophosphate via the action of an enzyme, phosphoramidase, identified as Hint-1, without the intermediate action of carboxyesterases (**Figure 4.4**).[28]

This approach has been applied on various nucleosides including d4T[29], AZT[30] and FdU[31]. Although an improvement in *in vitro* activity has been observed, in some cases, some monoester phosphoramidates had poor bioavailability and underwent a substantial degradation to the parent nucleoside in the GI tract.[32]
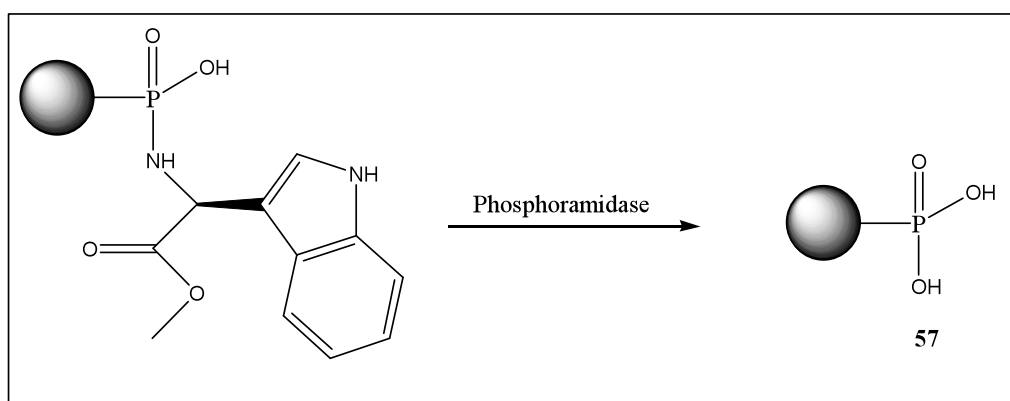
Figure 4.4: Proposed activation of monoester phosphoramidates to release **57.**

4.3.4. The Aryl Phosphoramidate Approach

This methodology was firstly applied in 1992, when McGuigan *et.al* used this to improve the anti-HIV activity of the nucleoside analogue AZT. Indeed, this approach improved significantly the activity of AZT against HIV-1 and HIV-2 in AZT-resistant cells. In addition to that, AZT-phosphoramidates were shown to retain full anti-HIV activity in cell lines deficient of thymidine kinase whereas AZT failed to do so.[33] This observation served as a proof of the phosphoramidate ability to overcome the first

phosphorylation step, which is the most rate-limiting step for many nucleoside analogues with interesting biological profiles. Since then, this strategy has been employed to improve the biological activities of nucleoside analogues that are unable to efficiently undergo the first phosphorylation step.

One of the early successes of this pronucleotide phosphoramidate (ProTide) approach was observed in the mid 1990s, when a completely inactive nucleoside, 2'3'-dideoxyuridine (ddU) was turned into an active one, proving that monophosphorylation was the rate-limiting step and its triphosphate was active. Thus ddU phosphoramidates, **61 (Figure 4.5)** were found to exert significant anti-HIV activity[34], while there was no activity seen with the free nucleoside analogue, ddU.[35] It is worth noting that the antiviral activity of ddU aryloxy phosphoramidates is attributed to the TK1 kinase by-pass.
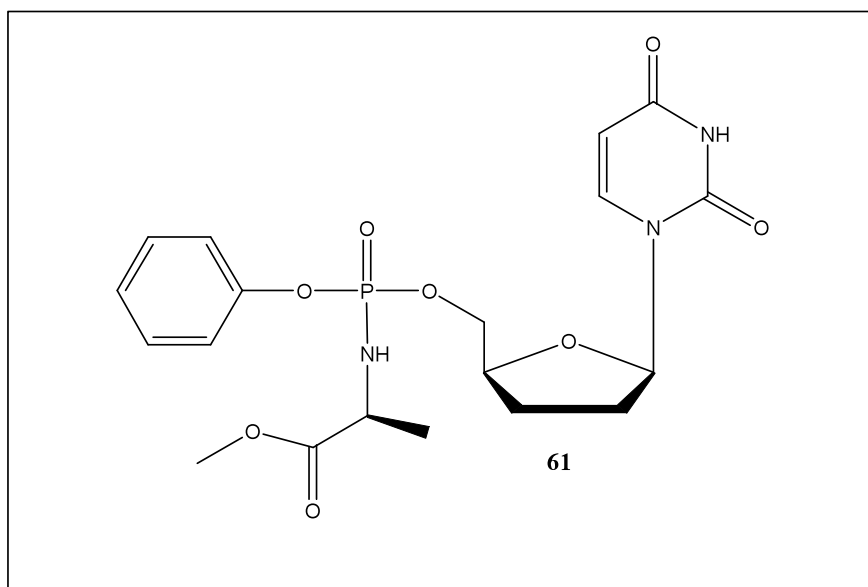
Figure 4.5: The structure of a potent ddU phosphoramidate.

These successes led to the application of ProTide technology on a wide range of both therapeutic anti-viral and anti-cancer nucleosides with the aim of improving their biological activities. Such nucleosides include d4T[36] (**62**), d4A[37] (**63**), ddA[37] (**64**), 2'-deoxy-2',2'-difluoro-5-halouridine[38] (**66**) and FUDR[39] (**65**) shown in **Figure 4.6**.
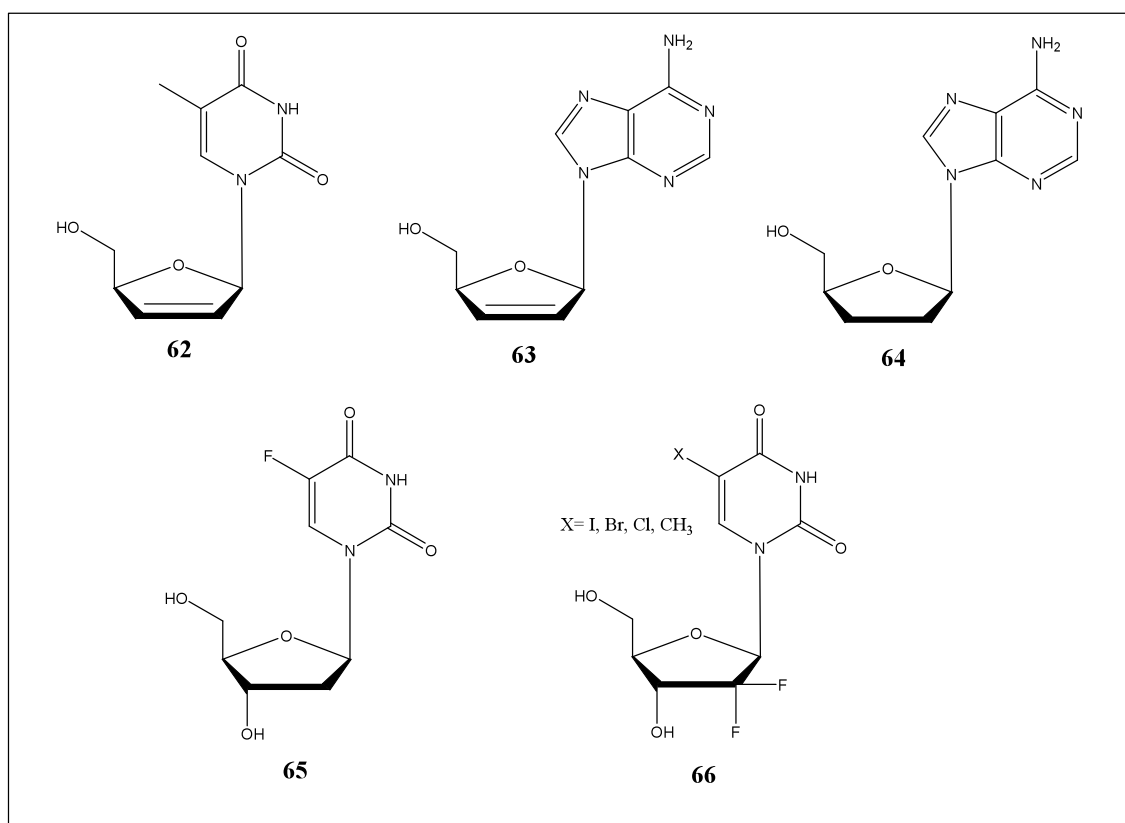
Figure 4.6.: Structures of some nucleoside analogues on which ProTide strategy has been applied.

Structurally, phosphoramidates consist of an aryloxy group and an amino acid carboxylic ester moiety, which are both attached to the phosphorus atom, which in turn is attached to the 5'-*O*-position of the nucleoside, as depicted in **Figure 4.7**. The aryl

functionalities in the nucleoside phosphoramidates are either substituted or unsubstituted phenyls and naphthyls. Regarding the substitutions in some cases, studies show that strongly electron-withdrawing substitutions, e.g. *p*-CN and *p*-NO$_2$, lead to slight reductions in potency whilst several substitutions, such as *p*-COOMe, and particularly *p*-Cl groups showed significant potency boosts for some nucleoside analogues depending on the SAR (structure-activity relationship) of the nucleoside phosphoramidates.[40]

Figure 4.7: SAR of Aryloxy nucleoside phosphoramidates.

As for the amino acid unit variations and their stereochemistry, great differences in activity have been observed in the literature. The amino acid motif is essential for sustaining the activity of phosphoramidates as early studies indicated that its

replacement with simple n-alkylamines resulted in complete loss of activity. In terms of the amino acid stereochemistry, generally L-amino acids seem to give better biological results compared to their D-counterparts from conclusion of previous studies.[41]

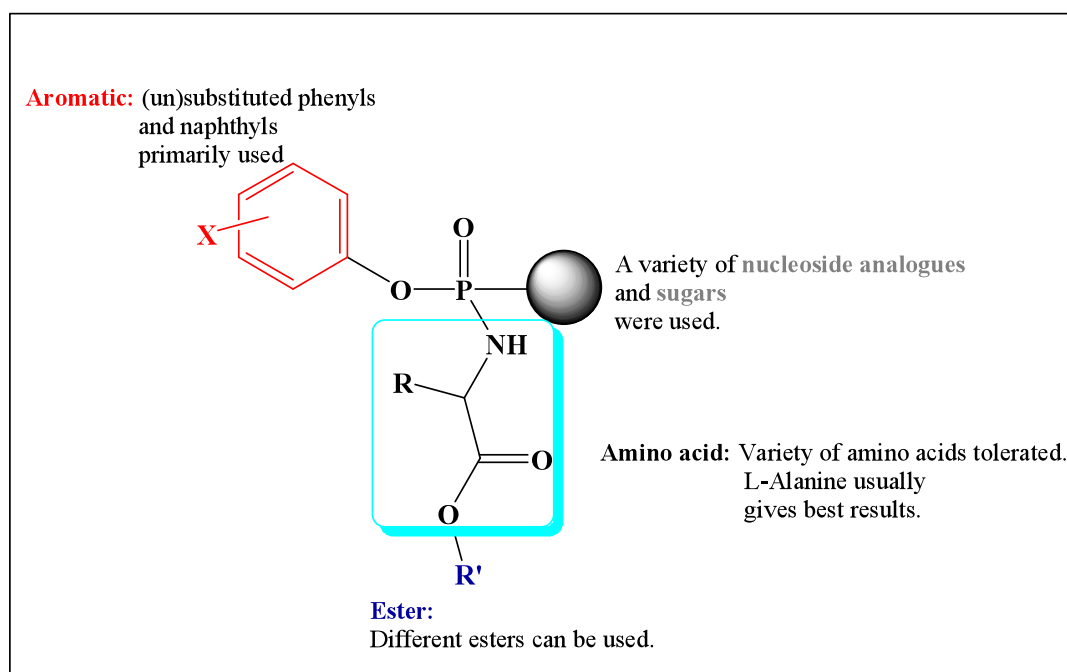Concerning the ester part, different types of esters have been explored and amongst them benzyl ester phosphoramidates were found to be more effective than the other ester types from literature studies. Also methyl and ethyl esters gave good results in some cases. However, depending on the nucleoside and amino acid ester variables, the biological activities also vary.

The activation of aryloxy phosphoramidates to release the monophosphate species is thought to consist of mainly four steps; two of which are enzyme-mediated and the other two being spontaneous reactions (**Figure 4.8**). The activation process is initiated by the hydrolytic cleavage of the carboxylic ester moiety hypothesized to be mediated by a carboxyesterase – type enzyme. Scientists at Gilead identified cathepsin A as the major enzyme responsible for ester cleavage.[42] Interestingly, phosphoramidates consisting of alanine were found to be bioprocessed quicker than those with branched side chains as cathepsin A was found to be less selective towards branched side chain amino acids and the reason behind the hydrolyzing selectivity remains unclear. A depiction of the mechanistic action involved during these processes for the ProTide of the nucleoside, FUDR (5-fluoro-2'-deoxyuridine), **67** is shown in **Figure 4.8**.[39] Upon esterase hydrolysis in the second step (b), an intracellular nucleophilic displacement of the aryloxy group by the carboxylate anion **68** on the phosphorus centre**,** takes place resulting from the spontaneous formation of a five-membered anhydride ring, **69**. In the third step (c), the unstable cyclic intermediate opens spontaneously via water mediated

hydrolysis to generate the intermediate **70**. At this stage, hydrolysis can proceed either by attacking the phosphorus atom (Attack 2)[44] or the carbonyl function (Attack 1)[43]. However, the hydrolysis often proceeds by the attack of the carbonyl function to release the carboxylate. The final activation step (d) is the P-N bond cleavage to generate FUDR monophosphate, **71** and this step is believed to result from an intracellular activity of the enzyme phosphoramidase.[45]

In order for aryloxyphosphoramidates to progress as drug candidates, issues such as by-product toxicity and stereochemistry should be addressed. The metabolism of aryloxyphosphoramidates generates phenol/naphthol aryl derivatives as by-products, which are considered to be highly toxic. But it was found that they are not mutagenic.

Since the phosphorus centre is chiral, often phosphoramidates are synthesized as a mixture of diastereoisomers. Although, from a drug development point of view, it is very much desired for drugs with chiral centres to be developed as a single stereoisomer than a mixture as diastereoisomers often show different biological properties. In this case, the phosphorus centre stereogenicity overcomes these issues as both the diastereoisomers of the ProTide break down to a single product, which is the nucleotide monophosphate.
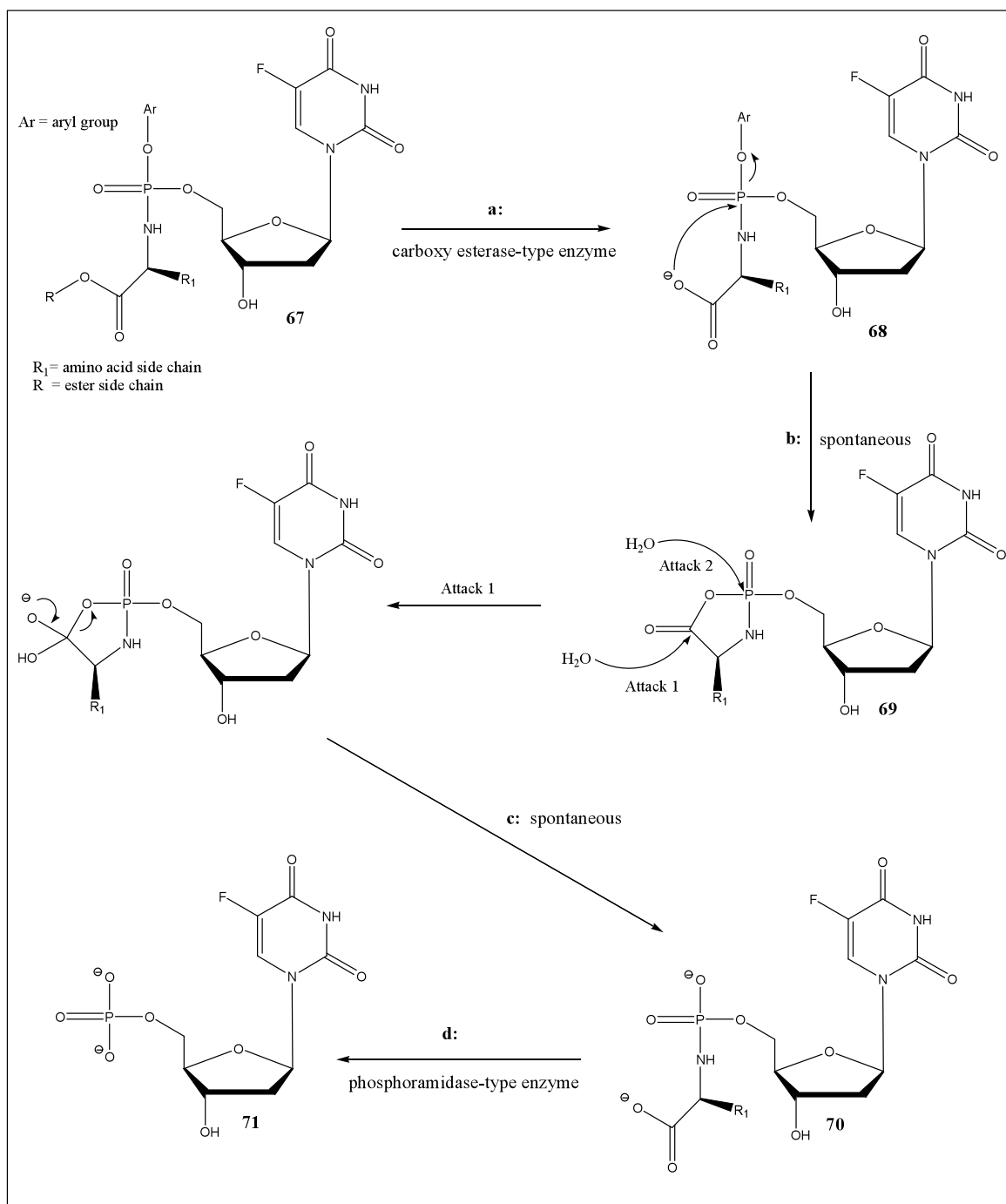
Figure 4.8: Proposed activation pathway of FUDR Protides.

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4.4 ^{19}F-FLT Phosphoramidates/ ProTides

In this thesis, mainly the synthesis of unsubstituted phenyloxy/naphthyloxyalanine based phosphoramidates of the nucleoside FLT, **10** is focussed on, in order to conduct a comparative study of any improvements in the biological properties of FLT. In order to obtain a better insight into the mechanism by which FLT acts as a proliferation biomarker and to identify novel therapeutic analogues of FLT, the synthesis of lipophilic FLT phosphoramidate pronucleotides (ProTides) was carried out that are able to cross the cell membranes by passive diffusion and rapidly break down to deliver the respective nucleotide monophosphates; hence bypassing the rate-limiting monophosphorylation step. A general structure of FLT phosphoramidate is shown in **Figure 4.9**.

Ar= aromatic substituent
R= sidechain of the amino acid
R'= sidechain of the carboxylic acid ester
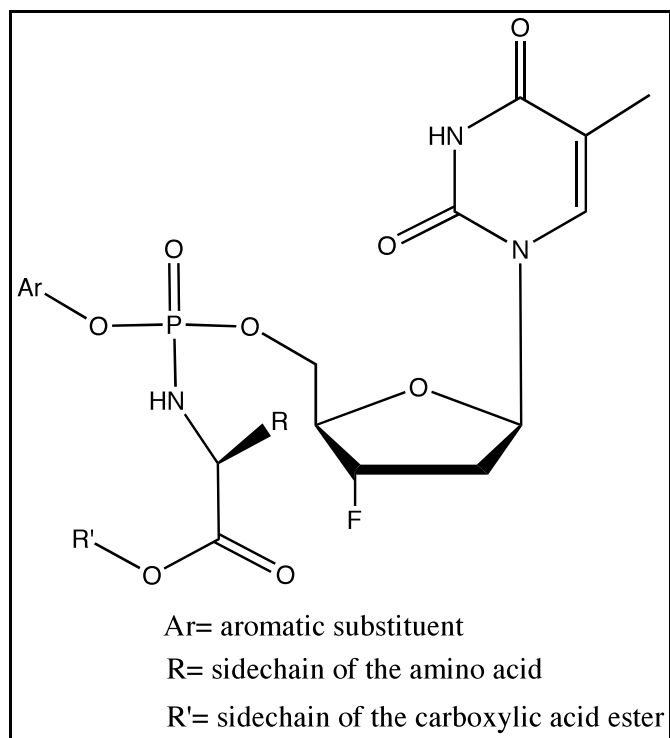
Figure 4.9: General structure of FLT phosphoramidates

FLT, also known as alovudine in medical terms is a very potent inhibitor of HIV[46], even more potent than the anti-retroviral agent, AZT. **10** inhibits replication of highly NRTI-resistant HIV strains *in vitro*. However due to safety concerns, the use of **10** as an anti-viral agent has been restricted as it exerts toxicity.[47] With the insight of improving the biological properties of FLT, Prof. McGuigan and group synthesized L-phenylalaninyl Protides and diamidates prodrugs of FLT as shown in **Scheme 4.1** and these were tested for anti-viral activity in human T lymphocytes. Unfortunately, the prodrugs are less potent than their parent nucleoside, **10** (**Table 4.1**)[48].

Table 4.1: Anti-HIV-1 activity of the protides synthesized in McGuigan laboratory as detailed in **Scheme 4.1**.

Compound	ED_{50} (μM)	TD_{50} (μM)
10	0.005	100
72	0.01	100
73	0.3	>100
74	0.3	>100
75	5	>100

Therefore, the goal of this project was to synthesize new FLT protides with L-Alanine esters and unsubstituted aryloxy group (Phenoxy and Naphthoxy) on the phosphorous atom as most of the ProTides generated by McGuigan group containing these groups exerted improved biological activities over the parent nucleosides.
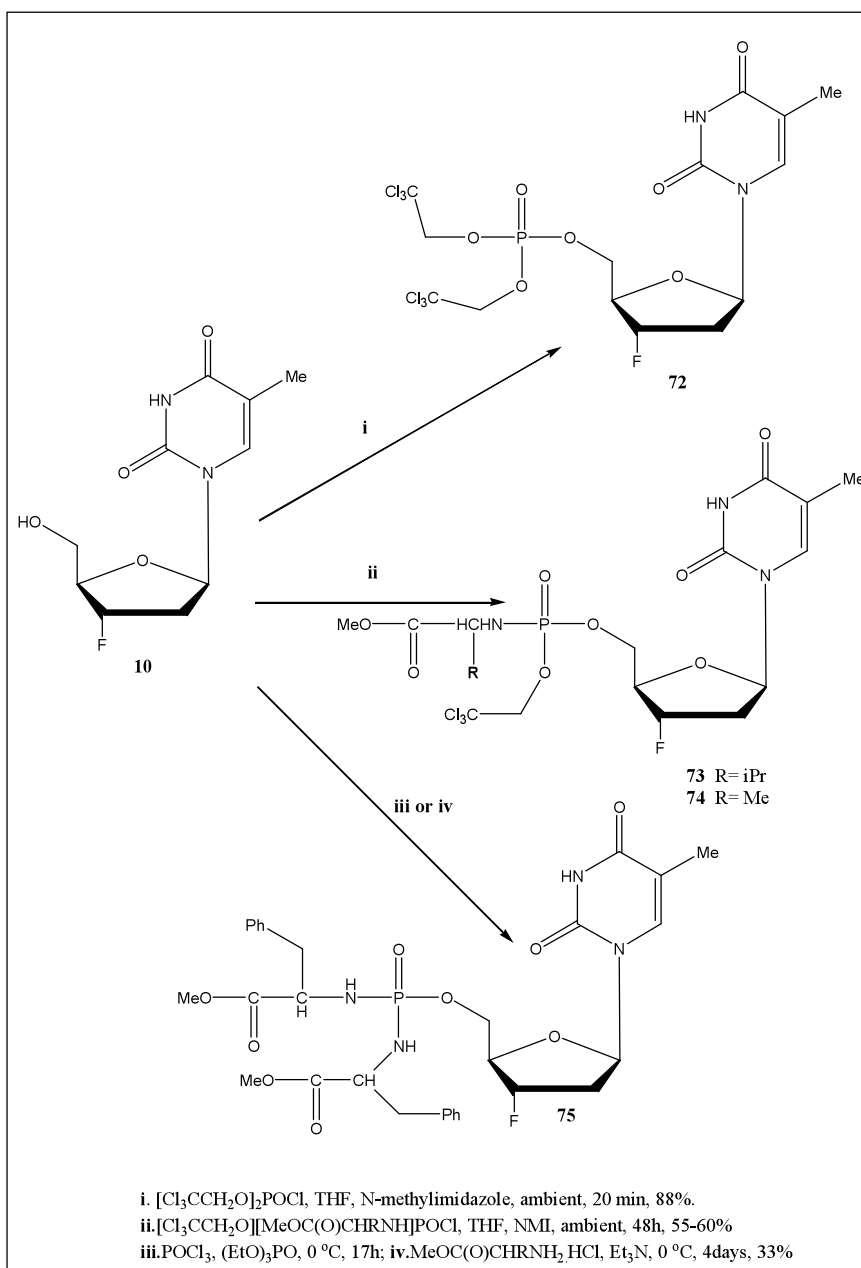
Scheme 4.1: Protides and diamidates prodrugs of FLT, **10**, which have been already synthesized in the laboratory of Prof. McGuigan.

Anti-cancer activity of alovudine **10** has not been reported in literature. Therefore the anti-viral and anti-cancer activity of FLT and the corresponding ProTides which were

going to be synthesized were agreed to be assessed biologically by Prof. Jan Balzarini group.

4.4.1. Synthesis of Phosphorylating Agents

It is clear from the structure activity relationship studies of phophoramidates, that there are mainly three variables that could be changed; the aryl moiety, the amino acid and the ester as can be noted from **Figure 4.7**.

Literature suggested that most of the aryloxy phosphoramidates consisting of phenyls and naphthyls as the aryl moieties showed good pharmacological activities and from comparison with all the natural (L) and unnatural (D) amino acids that have been tested so far, L-alanine appeared to be the most active in most of the cases.

Regarding the ester motif variability, benzyl esters seem to be always among the most active phosphoramidates, possibly due to the high lipophilicity generated by the presence of aromatic groups. Overall several FLT phosphoramidates were synthesized with mainly L-alanine as the amino acid, and the aryl moiety being either phenyl or naphthyl group with different carboxylic acid ester sidechains in the amino acid.

For the synthesis of FLT phosphoramidates, it was essential to prepare the phosphorylating agents, aryl phosphorochloridates, which were then reacted with FLT.

4.4.1.1. Synthesis of Phenyl Phosphorochloridates

The phenyl phosphorochloridates were synthesized according to the procedures reported by *McGuigan et al.*[49] The amino acid ester was used as a hydrochloride salt, of L-alanine, which was dissolved in anhydrous DCM under an argon atmosphere and cooled down to -78 ^{0}C after the addition of the commercially available phenyl dichlorophosphate in equimolar quantity to the amino acid ester salt. Triethyl-amine (two equivalents) was used as the base to trigger the attack of the nucleophilic amine at the electrophilic phosphorus (V) center to generate the desired phenyl phosphochloridates as represented in **Scheme 4.2** below. The amino acid ester salts were commercially available with the exception of L-Ala isopropyl ester hydrochloride, **76** and hence it was synthesized.
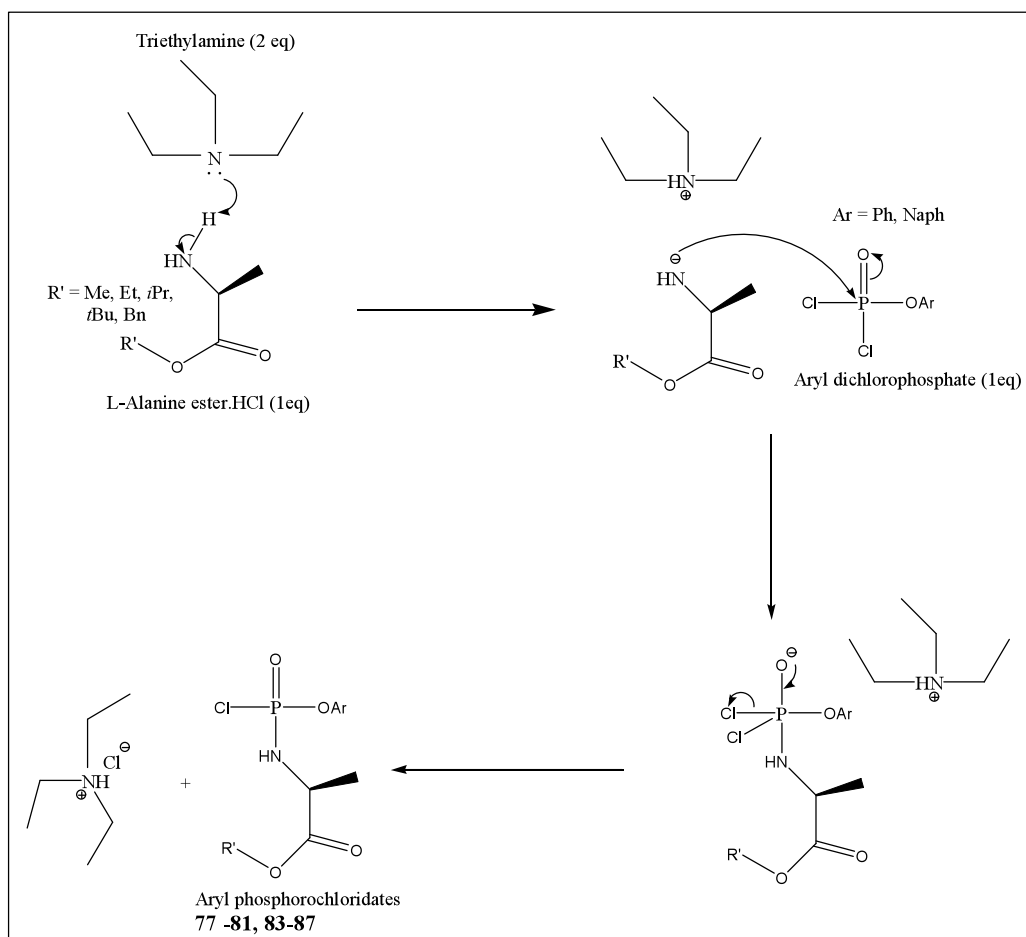
Scheme 4.2: General mechanism for Aryl L-alanine ester phosphorochloridate

synthesis.

After 30 minutes of stirring, the temperature was raised to room temperature and stirred for 2-3 hours. The white precipitate side product, triethylammonium chloride salt was filtered off after the reaction. The same reaction conditions were adapted for the preparation of all the aryl phosphorochloridates. In this case, five different phenyl phosphorochloridates were synthesized as listed in **Table 4.2**.

Table 4.2: List of synthesized phenyl L-alanine ester phosphorochloridates.

Compound	Ester (R')	Yield (%)	^{31}P-NMR (ppm) in CDCl$_3$
77	Methyl	50	7.96, 8.15
78	Ethyl	45	7.84, 8.13
79	*i*Propyl	76	8.06, 8.25
80	*t*Butyl	64	7.96, 8.33
81	Benzyl	40	7.85, 8.09

Upon the synthesis of these phosphorylating agents, always a 1:1 mixture of diastereoisomers was generated due to the non-specificity at the chiral phosphorus centre as can be seen from the ^{31}P-NMR data listed in **Table 4.2**.

4.4.1.2. Synthesis of 1-Naphthyl Phosphorochloridates

The preparation of 1-naphthyl phosphorochloridates involves the synthesis of 1-naphthyl dichlorophosphate (**82**), which is not commercially available unlike the phenyl phosphorodichloridate and it is obtained by first dissolving 1-naphthol (1 eq) and phosphorus(V)oxychloride in dry ether under argon atmosphere with the successive dropwise addition of triethylamine (1 eq) at -78 ^{0}C, which again acts as the base to promote the nucleophilic attack of the naphthoxide anion at the electrophilic phosphorus

(V) center to generate the required intermediate product. The synthesized crude product **82** was used for the preparation of 1-naphthyl phosphorochloridates without any further purification. A similar procedure and mechanism to that used for the preparation of phenyl phosphorochloridates, was followed up for synthesizing the five different 1-naphthyl L-alanine ester phosphorochloridates.

Table 4.3.: List of synthesized 1-Naphthyl L-alanine ester phosphorochloridates

Compound	Ester (R')	Yield (%)	^{31}P-NMR (ppm) in CDCl$_3$
83	Methyl	89	8.06, 8.21
84	Ethyl	82	8.15, 8.43
85	*i*Propyl	75	8.16, 8.35
86	*t*Butyl	60	8.29, 8.56
87	Benzyl	55	8.37, 8.65

4.4.2. Synthesis of ^{19}F-FLT Phosphoramidates

The preparation of FLT ProTides was carried out according to the procedure developed by *McGuigan et al.*[49] The prepared nucleoside, FLT (**10**) (one molar equivalent) was first dissolved in anhydrous tetrahydrofuran (THF) under argon atmosphere.

Subsequently, the Grignard reagent, *t*BuMgCl (1.5 - 2 equivalents) was added dropwise at room temperature, which acts as the base to abstract the proton from the 5'-*OH* group of the nucleoside. This initiates the nucleophilic attack of the nucleoside at the electrophilic phosphorus center of the appropriate aryloxy phosphorochloridate to form the respective phosphoramidates as shown in **Scheme 4.3**. The aim was to prepare FLT phosphoramidates from all the prepared phenyl and naphthyl phosphorochloridates, and hence generate the respective FLT ProTides. The FLT phosphoramidates were prepared with percentage yields ranging from 5-20 % after column purification of the crude product as listed in **Table 4.4**. The final compounds were obtained as a mixture of R and S diastereomers at the phosphorus center, which were not separated for final biological assays. Once all the phosphoramidates were synthesized and purified, they were subjected to biological evaluation for the identification of any potential therapeutic activity.
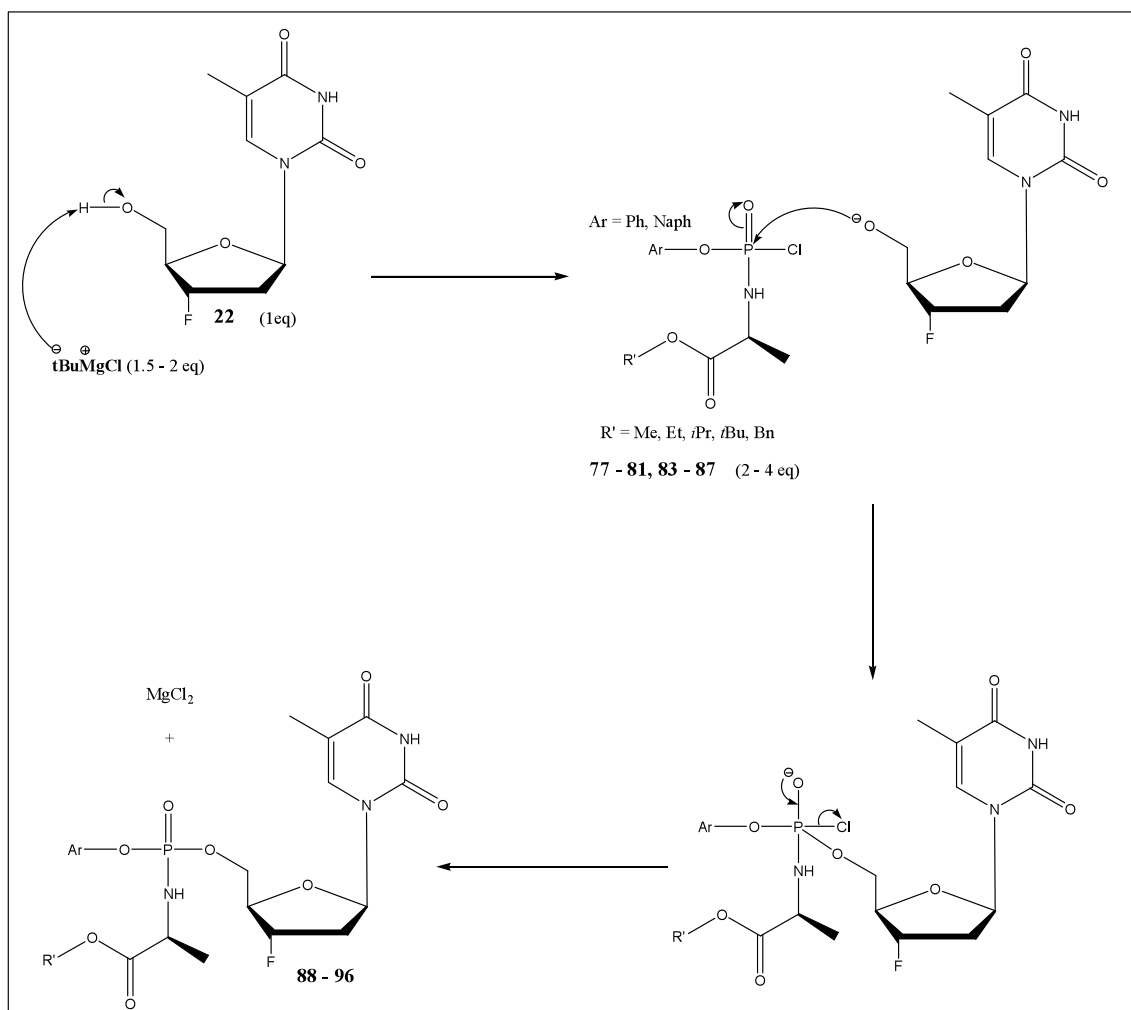
Scheme 4.3. General mechanistic pathway for FLT phosphoramidates.

Table 4.4 .: Summary of the synthesized FLT phosphoramidates.

Compound	Ar	Ester (R')	Yield (%)	^{31}P-NMR (ppm) in CDCl$_3$	^{19}F-NMR (ppm) in CDCl$_3$
88	Phenyl	Methyl	20	3.71, 3.99	-174.94, -176.37
89	Phenyl	Ethyl	10	2.54, 2.79	-174.88, -175.19
90	Phenyl	*i*Propyl	13	2.76, 3.55	-174.83, -175.18
91	Phenyl	*t*Butyl	5	2.93, 3.82	-175.06, -175.15
92	Phenyl	Benzyl	7	2.70, 3.52	-174.86, -175.01
93	Naphthyl	Methyl	47	2.95, 3.10	-174.92, -175.22
94	Naphthyl	Ethyl	50	3.05, 3.17	-174.93, -175.23
95	Naphthyl	*i*Propyl	22	2.98, 3.14	-174.90, -175.19
96	Naphthyl	*t*Butyl	36	3.12, 3.26	-174.85, -175.13

4.5. Biological Data

4.5.1. Anti-HIV data of FLT-ProTides

The synthesized FLT phosphoramidates were tested biologically by Balzarini *et al* in human T-lymphocyte (CEM) cells for anti-HIV 1 and anti-HIV 2 activities. The results indicated that the compounds exhibited an EC_{50} ranging from 0.1 to 146 µM which were less potent than the parent nucleoside FLT as shown in **Table 4.5**. However, when biological testing was performed in the presence of the thymidine kinase (TK minus) enzyme, most of the synthesized compounds retained biological activity, while the parent nucleoside showed an EC_{50} (µM) $\geq$50. This confirmed the hypothesis that the conversion of FLT into its monophosphate derivative is the rate- limiting step in the activation of this nucleoside analogue. In addition, the results proved the success of FLT phosphoramidates to be useful in bypassing the first phosphorylation step. Interestingly, the data revealed that the phenyl phosphoramidates of FLT had a relatively better activity than their naphthyl counterparts. Among them, the ethyl carboxyester ProTide, **89** showed best activity along with benzyl carboxyester ProTide, **92**.

The compounds were also tested against a broad series of DNA and RNA viruses such as Herpes simplex viruses 1 and 2, Vaccinia virus and Vesicular stomatitis virus as shown in **Table 4.6**. Consequently, none of them showed any significant anti-viral activity or cytotoxicity. For a reasonable comparison of anti-viral activity, the compounds were tested along with some of the predominant anti-viral drugs such as Brivudin, Cidofovir, Acyclovir and Ganciclovir.

Table 4.5 : Anti-HIV-1 and -HIV-2 data of FLT phosphoramidates

in human T-lymphocyte (CEM) cells.

| Compound | EC$_{50}$ (µM) | | CEM/TK$^-$ |
| | CEM/0 | | |
	HIV-1	HIV-2	HIV-2
88	0.55 ± 0.21	1.3 ± 0.14	4.7 ± 4.8
89	0.10 ± 0.068	0.24 ± 0.23	1.3 ± 0.71
90	0.75 ± 0.0	1.1 ± 0.23	28 ± 2.1
91	0.29 ± 0.16	0.90 ± 0.71	>50
92	0.13 ± 0.11	0.18 ± 0.049	2.0 ± 0.0
93	0.43 ± 0.35	0.65 ± 0.35	3.0 ± 1.3
94	0.16 ± 0.028	0.18 ± 0.071	5.2 ± 0.92
95	0.56 ± 0.23	1.0 ± 0.27	≥50
96	2.3 ± 1.4	4.7 ± 2.4	>50
10	0.0062 ± 0.0021	0.018 ± 0.014	≥50

EC$_{50}$ = The effective concentration or concentration required to protect CEM cells

against the cytopathogenicity of HIV by 50 %

Table 4.6: Cytotoxicity and antiviral activity of compounds in HEL cells

(Human erythroleukemia cell lines).

Compound	Minimum cytotoxic concentration[a] (μM)	EC_{50}[b] (μM)				
		Herpes simplex virus-1 (KOS)	Herpes simplex virus-2 (G)	Vaccinia virus	Vesicular stomatitis virus	Herpes simplex virus-1 TK[-] KOS ACV[r]
88	>100	>100	>100	>100	>100	>100
89	>100	>100	>100	>100	>100	>100
90	>100	>100	>100	>100	>100	>100
91	>100	>100	>100	>100	>100	>100
92	>100	>100	>100	>100	>100	>100
93	>100	>100	>100	>100	>100	>100
94	>100	>100	>100	>100	>100	>100
95	>100	>100	>100	>100	>100	>100
96	>100	>100	>100	>100	>100	>100
10	>100	>100	>100	>100	>100	>100
Brivudin	>250	0.02	112	10	>250	50
Cidofovir	>250	2	1	50	>250	2
Acyclovir	>250	0.4	0.1	>250	>250	10
Ganciclovir	>100	0.03	0.03	>100	>100	4

[a]Required to cause a microscopically detectable alteration of normal

cell morphology.

[b]Required to reduce virus-induced

cytopathogenicity by 50 %.

4.5.2. Anti-tumour data of FLT-ProTides

The inhibitory effects of the synthesized FLT ProTide compounds on the proliferation

of murine leukemia cells (L1210), human T-lymphocyte cells (CEM) and human cervix

carcinoma cells (HeLa) were assessed as presented in **Table 4.7**. However, the

phosphoramidates exerted poor activity as they were 50 to 100 fold less effective than the parent nucleoside and they lose activity in TK minus tumour cells. These results indicated that the ProTide compounds, which should bypass TK mediated activation, appear to have the requirement for TK in order to be active in tumour cells. As TK mediated monophosphorylation is the most rate-limiting step, the ProTide analogues also exhibited poor anti-tumour activity.

Table 4.7: Inhibitory effects of compounds on the proliferation of murine leukemia cells (L1210), human T-lymphocyte cells (CEM).

Compound	$IC_{50}{}^{*}$ (μM)			
	L1210/0	L1210/TK⁻	CEM/0	CEM/TK⁻
88	8.2 ± 3.7	> 250	≥ 250	≥ 250
89	4.4 ± 1.4	> 250	≥ 250	> 250
90	23 ± 1	220 ± 42	145 ± 38	> 250
91	6.3 ± 0.6	184 ± 20	124 ± 58	> 250
92	5.8 ± 0.6	223 ± 39	117 ± 88	172 ± 59
93	3.0 ± 1.2	110 ± 30	66 ± 10	112 ± 52
94	6.0 ± 0.9	108 ± 32	81 ± 1	138 ± 5
95	24 ± 2	176 ± 35	206 ± 62	> 250
96	36 ± 3	170 ± 30	148 ± 17	> 250
10	0.081 ± 0.037	176 ± 4	39 ± 26	> 250

*50% inhibitory concentration.

References:

1. Holy, A. Isopolar phosphorus-modified nucleotide analogues. In: De Clercq (Ed.). *Adv Antivir Drug Des.,* **1993**, Vol. I, JAI Press, 179-231.

2. Al-Madhoun, A.S.; Tjarks, W.; Erikson, S. The role of thymidine kinases in the activation of pyrimidine nucleoside analogues. *Mini-Rev Med Chem.,* **2004**, *4*, 341-350.

3. Kong, W.; Enge, K.; Wang, J. Mammalian nucleoside transporters. *Curr Drug Metabol.*, **2004**, *5*, 63-84.

4. Balzarini, J.; Kruining, J.; Wedgwood, O.; Pannecouque, C.; Aquaro, S.; Perno, C.F.; Naesens, L.; Witvrouw, M.; Heijtink, R.; De Clercq, E.; McGuigan, C. Conversion of 2',3'-dideoxyadenosine (ddA) and 2',3'-didehydro-2',3'-dideoxyadenosine (d4A) to their corresponding aryloxyphosphoramidate derivatives markedly potentiates their activity against human immunodeficiency virus and hepatitis B virus. *FEBS Lett.* **1997**, *410*, 324-328.

5. McGuigan, C.; Cahard, D.; Sheeka, H.M.; De Clercq, E.; Balzarini, J. Aryl phosphoramidate derivatives of d4T have improved anti-HIV efficacy in tissue culture and may act by the generation of a novel intracellular metabolite. *J Med Chem.,* **1996**, *39*, 1748-1753.

6. (a) Holland, J. F.; Frei, E.; Pizzorno, G.;Diasio, R. B.; Cheng, Y.C. *Cancer Medicine*, 7th ed.; BC Decker: Hamilton, Ontario, Canada, **2006**. (b) Parker, W. B. Enzymology of purine and pyrimidine antimetabolites used in the treatment

of cancer. *Chem. Rev.* **2009**, *109*, 2880–2893. (c) Ensminger, W. D.; Gyves, J. W. Clinical pharmacology of hepatic arterial chemotherapy. *Semin Oncol.* **1983**, *10*, 176–182.

7. Saffhil, R; Hume, W. J. The degradation of 5-iododeoxyuridine and 5-bromodeoxyuridine by serum from different sources and its consequences for the use of these compounds for incorporation into DNA. *Chem Biol Interactions.* **1986**, *57,* 347-355.

8. Cohen, S. S.; Plunkett, W. The utilisation of nucleotides by animal cells. Annals of the New York Academy of Sciences. **1975**, 255, 269-286.

9. Holy, A. Isopolar phosphorus-modified nucleotide analogues. *Adv Antivir Drug Des.,* **1993**, *1*, 179-231.

10. Bischofberger, N; Prisbe, E.J.; Arimilli, M.N.; Cundy, K.C.; Lee, W.A. Evaluation of nucleoside prodrugs as inhibitors of viral replication. *Antiviral Research.* **1993**, *20*, 142.

11. Sastry, J. K.; Nehet, P. N.; Khan, S.; Nowak, B. J.; Plunkett, W.; Arlinghaus, R. B.; Farquhar, D. Membrane-permeable dideoxyuridine 5'-monophosphate analogue inhibits human immunodeficiency virus infection. *Molecular Pharmacology.* **1992**, *41*, 441-445.

12. Farquhar, D.; Khan, S.; Srivastva, D. N.; Saunders, P. P. Synthesis and antitumour evaluation of Bis[(pivaloyloxy)methyl] 2'-deoxy-5-fluorouridine 5'-monophosphate (FdUMP): a strategy to introduce nucleotides into cells. *J Med Chem.,* **1994**, *37*, 3902-3909.

13. Farquhar, D.; Chen, R.; Khan, S. 5'-[4-(Pivaloyloxy)-1,3,2-dioxaphosphorinan-2-yl]-2'-deoxy-5-fluorouridine: a membrane permeable prodrug of 5-fluoro-2'-uridylic acid (FdUMP). *J Med Chem.,* **1995**, *38*, 488-495.

14. Liederer, B. M.; Borchardt, R. T. Enzymes involved in the bioconversion of ester-based prodrugs. *J Pharm Sciences.* **2006**, *95*, 1177-1195.

15. Starrett, J. E. Jr.; Tortolani, D. R.; Russell, J.; Hitchcock, M. J.; Whiterock, V.; Martin, J. C.; Mansuri, M. M. Synthesis, oral bioavailability determination, and in vitro evaluation of prodrugs of the antiviral agent 9-[2-(phosphonomethoxy)ethyl] adenine (PMEA). *J Med Chem.,* **1994**, *37,* 1857-1864.

16. Khan, S. R.; Nowak, B.; Plunkett, W.; Farquhar, D. Bis (pivaloyloxymethyl) thymidine 5'-phosphate is a cell membrane-permeable precursor of thymidine 5'-phosphate in thymidine kinase deficient CCRF CEM cells. *J Med Chem.,* **2005**, *69*, 1307-1313.

17. Pompon, A.; Lefebvre, I.; Imbach, J. L.; Khan, S.; Farquhar, D. Decomposition pathways of the mono- and bis (pivaloyloxymethyl) esters of azidothymidine 5'-monophosphate in cell extract and in tissue culture medium: an application of the on-line ISRP-cleaning' HPLC technique. *Antivir Chem Chemother.,* **1994**, *5*, 91-98.

18. Farquhar, D.; Srivastva, D.N.; Kattesch, N. J.; Saunders, P. P. Biologically reversible phosphate-protective groups. *J Pharm Sciences.* **1983**, *72,* 324-325.

19. Freed, J. J.; Farquhar, D.; Hampton, A. Evidence for acyloxymethyl esters of pyrimidine 5'-deoxyribonucleotides as extracellular sources of active 5'-

deoxyribonucleotides in cultured cells. *Biochemical Pharmacology.* **1989**, *38,* 3193-3198.

20. Meier, C. 2-Nucleos-5′-*O*-yl-4*H*-1,2,3-benzodioxaphosphinin-2-oxides: a new concept for lipophilic, potential prodrugs of biologically active nucleoside monophosphates. *Angew Chem Int Ed Engl.,* **1996**, *35,* 70−72.

21. Meier, C. Pro-Nucleotides- Recent Advances in the Design of Efficient Tools for the delivery of biologically active nucleoside monophosphates. *Synlett*. **1998**, *3,* 233-242.

22. Balzarini, J.; Aquaro, S.; Knispel, T.; Rampazzo, C.; Bianchi, V.; Perno, C. F.; De Clercq, E.; Meier, C. Cyclosaligenyl-2',3'-didehydro-2',3'-dideoxythymidine monophosphate: efficient intracellular delivery of d4TMP. *Mol Pharmacology.* **2000**, *58,* 928-935.

23. Lorey, M.; Meier, C.; De Clercq, E.; Balzarini, J. New synthesis and antitumour activity of cycloSal-Derivatives of 5-Fluoro-2'-deoxyuridinemonophosphate. *Nucleosides Nucleotides.* **1997**, *16,* 789-792.

24. Meier, C.; De Clercq, E.; Balzarini, J. Nucleotide delivery from cycloSaligenyl-3'-azido-3'-deoxythymidine monophosphates (cycloSal-AZTMP). *Eur J Org Chem.,* **1998**, *5,* 837-846.

25. Meier, C.; Goerbig, U.; Mueller, C.; Balzarini, J. CycloSal-PMEA and cycloAmb-PMEA: potentially new phosphonate prodrugs based on the cycloSal-pronucleotide approach. *J Med Chem.,* **2005***, 48,* 8079-8086.

26. Wagner, C. R.; Iyer, V. V.; McIntee, E. J. Pronucleotides: toward the in vivo delivery of antiviral and anticancer nucleosides. *Medical Research Reviews.* **2000**, *20,* 417-451.

27. Cahard, D.; McGuigan, C.; Balzarini, J. Aryl phosphoramidate triesters as ProTides. *Mini-Rev Med Chem.,* **2004**, *4,* 371-482.

28. Chou, T. F.; Baraniak, J.; Kaczmarek, R.; Zhou, X.; Cheng, J.; Ghosh, B.; Wagner, C. R. Phosphoramidate pronucleotides: a comparison of the phosphoramidase substrate specificity of human and Escherichia coli histidine triad nucleotide binding proteins. *Molecular Pharmaceutics.* **2007**, *4*, 208-217.

29. Abraham, T. W.; Wagner, C.R. A phosphoramidate- based synthesis of phosphoramidate amino acid diesters of antiviral nucleosides. *Nucelosides and Nucleotides.* **1994**, *13,* 1891-1903.

30. Wagner, C. R.; McIntee, E. J.; Schinazi, R.F.; Abraham, T. W. Aromatic amino acid phosphoramidate di- and triesters of 3'-azido-3'-deoxythymidine (AZT) are non-toxic inhibitors of HIV-1 replication. *Bioorg Med Chem letters.* **1995**, *5*, 1819-1824.

31. Abraham, T. W.; Kalman, T. I.; McIntee, E. J.; Wagner, C. R. Synthesis and biological activity of aromatic amino acid phosphoramidates of 5-fluoro-2'-deoxyuridine and 1-beta-arabinofuranosylcytosine: evidence of phosphoramidase activity. *J Med Chem.,* **1996**, *39*, 4569-4575.

32. Song, H.; Griesgraber, G. W.; Wagner, C. R.; Zimmerman, C. L. Pharmacokinetics of amino acid phosphoramidate monoesters of zidovudine in rats. *Antimicr Agents Chemotherapy.* **2002**, *46,* 1357-1363.

33. McGuigan, C.; Pathirana, R.; Mahmood, N.; Devine, K. G.; Hay, A.J. Aryl phosphate derivatives of AZT retain activity against HIV-1 in cell lines which are resistant to the action of AZT. *Antiviral Research* **1992**, *17,* 311-321.

34. McGuigan, C.; Bellevergue, P.; Sheeka, H.; Mahmood, N.; Hay, A.J. Certain phosphoramidate derivatives of deoxyuridine (ddU) are active against HIV and successfully bypass thymidine kinase. *FEBS Letters.* **1994**, *351*, 11-14.

35. Johns, D.G.; Broder, S.; Hao, Z. et al. Factors determining the activity of 2',3'-dideoxynucleosides in suppressing human immunodeficiency virus in vitro. *Molecular Pharmacology*, **1988**, *34*, 431-435.

36. Siddiqui, A. Q.; McGuigan, C.; Ballatore, C.; Zuccotto, F.; Gilbert, I. H.; De Clercq, E.; Balzarini, J. Design and synthesis of lipophilic phosphoramidate d4T-MP prodrugs expressing high potency against HIV in cell culture: structural determinants for in vitro activity and QSAR. *J Med Chem.* **1999**, *42,* 4122-4128.

37. Balzarini, J.; Kruining, J.; Wedgwood, O.; Pannecouque, C.; Aquaro, S.; Perno, C. F.; Naesens, L.; Witvrouw, M.; Heijtink, R.; De Clercq, E.; McGuigan, C. Conversion of 2',3'-dideoxyadenosine (ddA) and 2',3'-didehydro-2',3'-dideoxyadenosine (d4A) to their corresponding aryloxyphosphoramidate derivatives markedly potentiates their activity against human immunodeficiency virus and hepatitis B virus. *FEBS Letters.* **1997**, *30*, 324-328.

38. Quintiliani, M.; Persoons, L.; Solaroli, N.; Karlsson, A.; Andrei, G.; Snoeck, R.; Balzarini, J.; McGuigan, C. Design, synthesis and biological evaluation of 2'-deoxy-2',2'-difluoro-5-halouridine phosphoramidate ProTides. *Bioorg Med Chem.,* **2011**, *19*, 4338-4345.

39. McGuigan, C.; Murziani, P.; Slusarczyk, M.; Gonczy, B.; Voorde, J. V.; Liekens, S.; Balzarini, J. Phosphoramidate ProTides of the Anticancer Agent FUDR successfully deliver the preformed bioactive monophosphate in cells and confer advantage over the parent nucleoside. *J Med Chem.,* **2011**, *54, 7247-7258.*

40. McGuigan, C.; Cahard, D.; Salgado, A.; De Clercq, E.; and Balzarini, J. Phosphoramidates as potent prodrugs for anti-HIV nucleotides: Studies in the amino region. *Antivir Chem Chemotherapy.* **1996**, *7,* 31-36.

41. Curley, D.; McGuigan, C.; Devine, K.G.; O'Connor, T.J.; Jeffries, D.J.; and Kinchington, D. Synthesis and anti-HIV evaluation of some phosphoramidate derivatives of AZT: studies on the effect of chain elongation on biological activity. *Antiviral Research,* **1990**, *14,* 345-356.

42. Birkus, G.; Wang, R.; McDermott, M. et al. Cathepsin A is the major hydrolase catalyzing the intracellular hydrolysis of the antiretroviral nucleotide phosphonoamidate prodrugs GS-7340 and GS-9131. *Antimicr Agents Chemother.,* **2007**, *51,* 543-550.

43. DiSabato, G.; Jencks, W.P. Mechanism and Catalysis of reactions of Acyl Phosphates. II. Hydrolysis. *J Am Chem Soc,* **1961**, *83,* 4400-4405.

44. Clark, V.M.; Kirky, A.J. Neighbouring group participation in phosphate ester hydrolysis. *J Am Chem Soc,* **1963**, *85,* 3705-3706.

45. Balzarini, J.; De Clercq, E.; McGuigan, C. et al. Characterization of the activation pathway of phosphoramidate triester prodrugs of stavudine and zidovudine. *Molecular Pharmacology,* **1999**, *56,* 693-704.

46. Herdewijn, P.; Balzarini, J.; De Clercq, E.; Pauwels, R.; Baba, M.; Broder, S.; Vanderhaeghe, H. 3'-substituted 2',3'-dideoxynucleoside analogues as potential anti-HIV (HTLV-III/LAV) agents. *J Med Chem.,* **1987**, *30*, 1270-1278.

47. Katlama, C.; Valdez, H.; Mardh, G. et al. Antiviral activity of low-dose alovudine in antiretroviral-experienced patients: results from a 4-week randomized, double-blind, placebo-controlled dose-ranging trial. *HIV Medicine*. **2007**, *8*, 142-147.

48. McGuigan, C.; Jones, B.C.N.M.; Devine, K.G.; Nicholls, S.R.; O'Connor, T.J.; Kinchington, D. Attempts to introduce chemotherapeutic nucleotides into cells: Studies on the anti-HIV agent FdT. *Bioorg Med Chem Lett*., **1991,** *1*, 12, 729–732.

49. McGuigan, C.; Madela, K.; Aljarah, M.; Bourdin, C.; Arrica, M.; Barrett, E.; Jones, S.; Kolykhalov, A. Phosphorodiamidates as a promising new phosphate prodrug motif for antiviral drug discovery: Application to anti-HCV agents. *J Med Chem.,* **2011**, *54,* 8632-8645.

Chapter 5

5. Conclusions and Future Perspectives

5.1. Conclusions

In recent years, 3'-Deoxy-3-[^{18}F]-fluorothymidine (^{18}F-FLT) has been widely recognized as a key, specific diagnostic biomarker for tumour cellular proliferation. However, many disadvantages have been associated with the current methods for the commercial production of [^{18}F]FLT and these include low overall yields and time-consuming high-performance liquid chromatography (HPLC) purification. These disadvantages could be rectified by the development of a fast, efficient synthetic route to FLT.

Initial studies were mainly focussed on the development of a new and efficient chemical synthetic route to FLT, **10** under thermal conditions featuring microwave-promoted chemistry to accelerate the fluoridation reaction, which then later on can be applied for the radiolabeled synthesis of the PET imaging agent, [^{18}F] FLT, **15**. It was found that the microwave reactions led to decomposition of the precursor molecule under high temperature and pressure. Since a high temperature of 140 ^{o}C was required for fluoridation reactions, further investigations were carried out devoid of microwave technology. Hence, precursor molecules with various protecting groups were subjected to different fluorides under varying temperatures, reaction solvents and reaction times. However, the synthesis of ^{19}F-FLT under thermal conditions using the fluorinating agents, TBAF.3H$_2$O, DAST and XtalFluor-M was achieved.

Although the thermal fluoridation reactions of the synthesized precursor molecules were not all successful, the same synthetic methodologies were extended to the radiosynthesis of $[^{18}F]$FLT using the synthesized precursor molecules.

Amongst these radiosynthetic experiments, only the precursor molecules with a 2,3'-anhydro group at 3'-position gave positive results, with precursor molecule **48** giving the highest RCY, while precursor **33** is to be noted for its novel use as a precursor for $[^{18}F]$FLT radiosynthesis. Also the omission of the deprotecting step with the use precursor **44** for $[^{18}F]$FLT radiosynthesis proved to be advantageous. DMF was found to be the optimum solvent for the radiolabeling reaction with a reaction temperature of 140 ^{o}C. The minimum reaction time that could be reduced to, was 5 minutes, which turned out to be expedient in terms of the short-lived radioisotope, ^{18}F ($t_{1/2}$=110 minutes).

ProTide analogues of ^{19}F-FLT for therapeutic purpose were prepared by reacting FLT with the synthesized phenyl and naphthyl L-alanine phosphorochloridates containing different esters using the Grignard reagent, tBuMgCl. Subsequently, biological evaluation of the newly developed ^{19}F-FLT ProTide analogues for anti-HIV 1 and anti-HIV 2 activities were assessed on human T-lymphocyte (CEM) cells. Although the results indicated that the synthesized compounds were less potent than the parent nucleoside FLT, the analogues retained biological activity in contrast to FLT. This proved that the FLT ProTides bypassed the first phosphorylation step. However, the therapeutic evaluation for anti-tumour activity on L1210, CEM and HeLa cells showed no significant activity.

5.2. Recommendations for future work

Although the biological evaluation results for anti-tumour activity of this particular class of FLT ProTides synthesized for this project were not promising, future work could be focussed on generating other classes of FLT ProTides by reacting FLT with phosphorochloridates containing different ester, amino acid or aryl group.

It is also worth considering that the study of ^{18}F incorporated FLT ProTides as new diagnostic PET imaging agents could be extended by the incorporation of more labile leaving groups such as tosyl and nosyl into the 3'-*O*-position of the phosphoramidate protected nucleosides. After a suitable ProTide precursor has been generated, various ^{18}F-fluoridation studies could be done using different reaction conditions such temperature, solvents and precursor concentrations. Once an optimized radioactive reaction condition for the most therapeutically interesting FLT ProTide has been established, the radiosynthesis of the [^{18}F]FLT ProTide can be carried out. If the radiosynthetic studies unfold to be successful, a new class of theragnostic FLT ProTides could be generated for the purpose of both therapeutic and diagnostic activity.

Chapter 6

6. Experimental

6.1. General Experimental Conditions

6.1.1. Solvents and reagents

All reagents and solvents were used as supplied from Sigma-Aldrich except thymidine and amino acid esters were purchased from Carbosynth. All glassware was oven dried at 100 ^{0}C for several hours or overnight and allowed to cool under a steam of dry nitrogen.

6.1.2. Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) was performed on commercially available Merck silica gel 60-F254 pre-coated aluminium plates and separated components were visualised using ultra violet light (245 and 366 nm).

6.1.3. Column Chromatography (CC)

Column chromatography was performed using Fluka silica (35-70 μM) as stationary phase. Glass columns were slurry packed in the appropriate eluant under gravity. Samples were applied as a concentrated solution in the same eluant, or pre-absorbed onto silica gel. Fractions containing the product were identified by TLC, pooled and the solvent removed *in vacuo*.

6.1.4. High Performance Liquid Chromatography (HPLC)

Analytical procedures were run on a high-flow system with radio-, UV (254 nm) and refractive index (RI) detectors using a ZORBAX Eclipse Plus C18 (4.6 x 250 mm), 5

µm column; elution was performed using a mobile phase consisting of water/ethanol (90:10) in gradient.

6.1.5. Nuclear Magnetic Resonance (NMR)

^{1}H, ^{13}C, ^{31}P and ^{19}F NMR spectra were recorded on a Bruker Advance 500 MHz spectrometer with operating frequencies of 500, 125, 202 and 470 MHz respectively. Chemical shifts (δ) were recorded in parts per million (ppm) with tetramethylsilane (TMS) as the internal standard for ^{1}H and ^{13}C spectra. ^{31}P-NMR are reported in units of d relative to 85% phosphoric acid as external standard, positive shifts are downfield. The following abbreviations were used for NMR peak designation; s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet).

6.1.6. Mass Spectroscopy (MS)

High and low resolution mass spectroscopy using electroionisation (EI) were run on a Waters GCT Premier, and using electrospray (ES) on a Waters LCT Premier XE. Mass spectroscopy was performed as a service provided by Cardiff University, School of Chemistry.

6.1.7. Microwave Chemistry

Microwave synthesis was performed using a CEM Discover Labmate. Temperature was measured *in situ.*

6.1.8. Melting Points

Melting points were measured on a Griffen melting point apparatus.

6.2. Standard Procedures

Standard Procedure A: Radiosynthesis of [^{18}F]FLT

[^{18}F]Fluoride activity (1.5-2 GBq) was transferred from the cyclotron to a 5 ml vial fitted with a septum and passed through the QMA cartridge B. Kryptofix [2.2.2] (15 mg, 40 μmol) and KHCO$_3$ (4% aqueous solution, 75 μL, 30 μmol) were placed in vial C containing the [^{18}F]Fluoride solution in anhydrous acetonitrile (1 mL) which was transferred automatically from B. The solvents were evaporated under a slight flow of nitrogen at 100 $^{\circ}$C. A second azeotropic distillation with anhydrous CH$_3$CN (1 mL) was performed to remove traces of water. There-after, the appropriate labeling precursor (30 mg) dissolved and sonicated in the reaction solvent, anhydrous DMSO or DMF (1 mL) was added to the reaction vial A and heated at 140 $^{\circ}$C for 5-10 minutes. The reaction mixture was neutralized by the addition of aq. sodium hydroxide (0.25 M, 0.7 mL) solution.

Standard Procedure B: Synthesis of Phosphorodichloridates

To a stirring solution of phosphorous oxychloride (1 eq) and the appropriate phenol/ naphthol (1 eq) under argon atmosphere in anhydrous diethyl ether, was added dropwise at -78 ^{0}C, anhydrous triethylamine (1 eq). Following the addition, the reaction mixture was stirred at -78 ^{0}C for 30 min, then allowed to warm up to room temperature. The reaction was left for stirring overnight and the formation of the product was monitored by ^{31}P-NMR. Once the reaction was complete, the mixture was filtered under nitrogen

and the corresponding filterate was concentrated under reduced pressure to give the crude product as oil, which was then used in making phosphorochloridates without any further purifications.

Standard Procedure C: Synthesis of Aryloxy Phosphorochloridates

Anhydrous triethylamine (2 eq) was added dropwise after 15 min to a stirring solution mixture of the appropriate aryl dichlorophosphate (1 eq) and the appropriate amino acid ester salt (1 eq) in anhydrous dichloromethane at -78 ^{0}C under argon atmosphere. Following the addition, the reaction mixture was stirred at -78 ^{0}C for 30 min, then at room temperature for 2.5 h. The formation of the product was monitored by ^{31}P-NMR. Once the reaction was complete, the solvent was removed under reduced pressure and the residue was triturated with dry dietyl ether. The precipitate was filtered under nitrogen and the corresponding filterate was concentrated under reduced pressure to give the crude product as oil. Most of the synthesized phosphorochloridates were purified by flash column chromatography (eluting with ethyl acetate/ petroleum ether, 6:4 v/v).

Standard Procedure D: Synthesis of FLT Phosphoramidates (ᵗBuMgCl method)

To a stirring solution of FLT (1 eq) in anhydrous THF was added dropwise under argon atmosphere, ᵗBuMgCl in 1.0 M THF solution (1.5 eq) and the reaction mixture was stirred at room temperature for 30 min. Then a solution of the appropriate phosphorochloridate (2 eq) in anhydrous THF was added dropwise to the reaction mixture and was left for stirring overnight. The solvent was removed *in vacuo* and the

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residue was purified by column chromatography eluting with DCM/MeOH in different proportions. The compounds synthesized were generated as a mixture of diastereoisomers (P_S and P_R) but were unable to be separated.

Standard Procedure E: Synthesis of Phosphoramidates (NMI method)

To a stirring solution of nucleoside (1 eq) in anhydrous THF, was added dropwise under argon atmosphere NMI (1-methylimidazole, 5 eq). After 15 minutes of stirring, the appropriate phosphorochloridate (3 eq) was added dropwise at room temperature and the reaction mixture was stirred overnight at room temperature. After monitoring the reaction progress by TLC with DCM/ MeOH (9.8:0.2) as the eluent, the solvent was removed under reduced pressure. The residue was dissolved in DCM, washed with water (twice) and 0.5 N HCl (twice). The organic phase was dried over $MgSO_4$, filtered, reduced to dryness and the crude product was purified by column chromatography eluting with DCM/ MeOH in different proportions.

6.3. Synthesized compounds

5'-*O*-(4-methoxybenzoyl)-2,3'-anhydrothymidine (33)
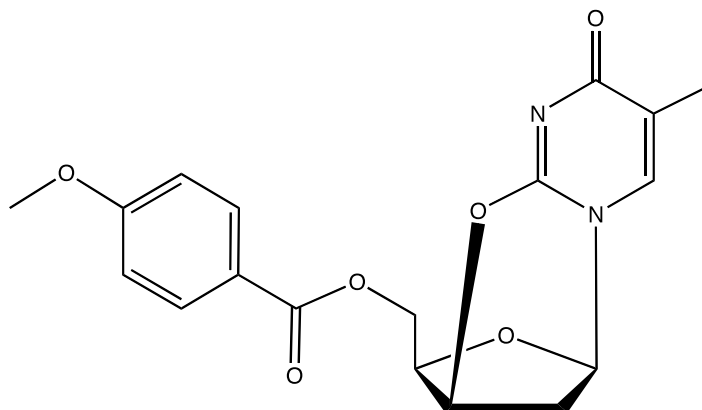
$C_{18}H_{18}N_2O_6$, MW: 358.35 gmol^{-1}

A mixture containing thymidine, **1** (5.0 g, 20.64 mmol) and triphenylphosphine (12.61 g, 48.08 mmol, 2.2eq.) was dissolved in DMF (60 mL) and stirred to cool down at 0° C. Meanwhile, a solution comprising of DIAD (5.8 g, 28.68 mmol, 5.2 mL, 1.2eq.), 4-methoxybenzoic acid (3.7 g, 24.32 mmol, 1.1eq.) and DMF (20 mL) was prepared to get a yellow solution. This solution was then added drop-wise into the reaction mixture maintaining the temperature of 0 °C. Afterwards it was allowed to stir for 1.5 hours at room temperature. After that again an aliquot of DIAD (5.5 g, 27.2 mmol, 1.2eq.) and 4-methoxybenzoic acid (0.5 g, 3.28 mmol) in DMF (10 mL) was added to the reaction mixture at 0° C and was left for stirring at room temperature for another 2 hours. Upon the second addition of DIAD, a precipitate started to form. After this, the mixture was

poured into flask containing ether (400 mL) and stirred for 10 minutes at room temperature. The resulting suspension was stored in the fridge at a temperature less than 4° C for 48 hours. The white amorphous precipitate was collected by filteration and washed with ether and brine to give the title compound **33** (6.84 g, 93%) as a fine white powder: mp 238-240 °C.

^{1}H-NMR (d$_6$-DMSO): δ 7.87 (2H, *d, J* = 9Hz, O-Ph), 7.59 (1H, *s*, H-6), 7.02 (2H, *d, J* = 8.5Hz, O-Ph), 5.92 (1H, *d, J* = 3.5Hz, H-1'), 5.42 (1H, *s,* H-3'), 4.58 (1H, *m*, H-4'), 4.50 (1H, *dd, J* =5Hz, *J* =10Hz, H-5'), 4.33 (1H, *dd, J* = 6.5Hz, *J* = 11.5Hz, H-5"), 3.84 (3H, *s*, OCH$_3$), 2.62 (1H, *m,* H-2'), 2.54 (1H, *m,* H-2"), 1.74 (3H, *s*, CH$_3$);

^{13}C-NMR (d$_6$-DMSO): δ 170.9 (C=O), 164.9 (C=O Bz), 163.2 (C-4"), 153.3 (C2), 136.6 (C6), 131.3 (CH$_3$), 121.2 (C2"), 116.1 (C3"), 113.9 (C1"), 86.8 (C5), 81.9 (C4'), 77.2 (C3'), 62.3 (C5'), 55.5 (CH$_3$O), 32.7 (C2') .

ESI MS m/z (+): Calculated for C$_{18}$H$_{18}$N$_2$O$_6$: 358.1165 found: 381.11 [M+Na].

5'-*O*-*p*-methoxybenzoyl-3'-fluoro-3'-deoxythymidine (34)
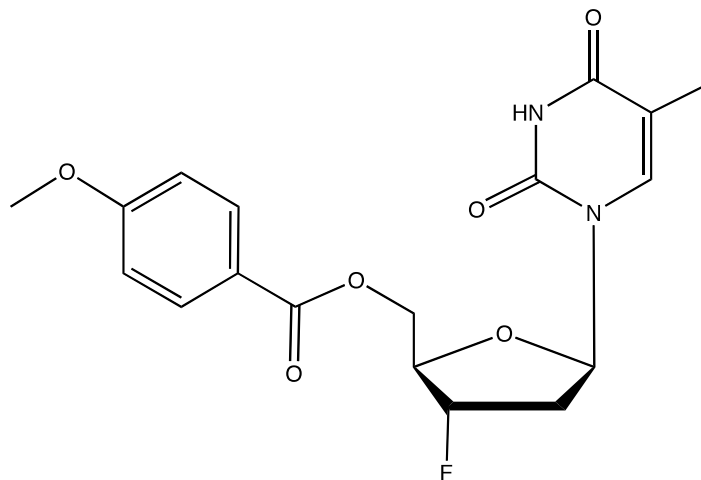
$C_{18}H_{19}FN_2O_6$, MW: 378.35 gmol^{-1}

The compound **33** (3.6 g, 10.05 mmol) was dissolved in DMF (30 mL) and stirred to get a cloudy solution. Subsequently, cesium fluoride (4.76 g, 31.38 mmol, 3eq.) was added to the reaction mixture and was heated to 120^0 C under reflux for 24 hours. The resulting mixture containing a white precipitate was allowed to cool down to room temperature. The precipitate was collected by Buchner filteration, washed with ether and dried to give the desired compound, **34** (4.56 g, 91%) as a fine cream powder: mp 208-210 ^{0}C;

^{1}H-NMR (d$_6$-DMSO): δ 7.87 (2H, *d, J* = 9Hz, o-Ph), 7.59 (1H, *s*, H-6), 7.02 (2H, *d, J* = 8.5Hz, m-Ph), 5.92 (1H, *d, J* = 3.5Hz, H-1'), 5.42 (1H, *s,* H-3'), 4.58 (1H, *m*, H-4'), 4.50 (1H, *dd, J* =5Hz, *J* =10Hz, H-5'), 4.33 (1H, *dd, J* = 6.5Hz, *J* = 11.5Hz, H-5"), 3.84 (3H, *s*, OCH$_3$), 2.62 (1H, *m,* H-2'), 2.54 (1H, *m*, H-2"), 1.74 (3H, *s*, CH$_3$).

^{19}F-NMR (d$_6$-DMSO): δ -141.63.

5'-*O*-(4,4'-dimethoxytrityl)-thymidine (35)

$C_{31}H_{32}N_2O_7$, MW: 544.59 gmol^{-1}
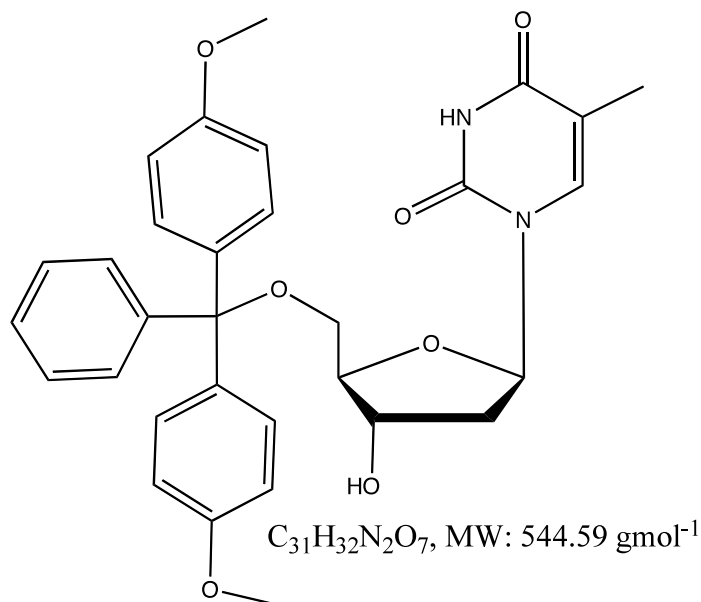
A mixture of thymidine, **1** (0.515 g, 2.126 mmol) and dimethoxytrityl chloride (DMTrCl, 0.792 g, 2.339 mmol, 1.1 eq.) was dissolved in anhydrous pyridine (10 mL) under inert gas atmosphere and stirred at room temperaure for three hours. The reaction mixture was then diluted with ethyl acetate (100 mL) and extracted with copper sulphate solution (Cu(II)SO$_4$, 5x25 mL) and brine (50 mL). The yellow-brown solution was dried over anhydrous MgSO$_4$ powder and the solvent was evapourated in vacuo. The crude product was subjected to preparative column purification using EtOAc:Hexane (7:3, v/v) as the eluent to afford the pure title compound, **35** (0.67g, 59%) as a white amorphous powder: mp 119^{0}C (lit. mp 119-122 ^{0}C)[1].

TLC (ethyl acetate : hexane, 7:3 v/v): R$_f$ = 0.25.

¹H-NMR (CDCl₃): δ 9.61 (1H, *s*, NH), 7.63 (1H, *s*, H-6), 7.41 (6H, *m*, Ar-H), 6.46 (2H, *t*, Ar-H), 5.35 (1H, *s*, H-1'), 4.59 (1H, *dd, J* =3Hz, *J* = 6Hz, H-3'), 4.10 (1H, *d*, H-4'), 3.49 (6H, *s*, OCH₃), 3.39 (1H, *dd, J* =2.5Hz, *J* = 10.5Hz, H-5'), 2.47 (1H, *m*, H$_e$-2'), 2.43 (1H, *m*, H$_x$-2'), 1.48 (3H, *s*, CH₃).

ESI MS m/z (+): Calculated for C₃₁H₃₂N₂O₇: 544.59 gmol⁻¹, found: 567.55 [M+Na].

5'-*O*-(4,4'-dimethoxytrityl)-2,3'-anhydrothymidine (37)

C₃₁H₃₀N₂O₆, MW: 526.58 gmol⁻¹
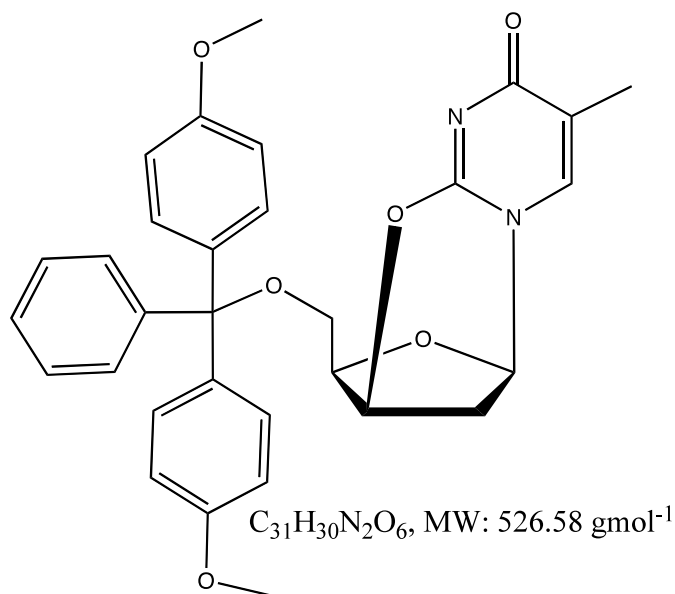
The synthesized compound **35** (0.93g, 1.71 mmol) was weighed into a 50 mL round-bottom flask and EtOAc (10 mL) was added to effect dissolution. To this mixture, Ph₃P (0.69g, 2.63 mmol, 1.5 eq) was added and stirred vigorously. Meanwhile, the solution was allowed to cool down to 0 ^{0}C and a solution of DIAD (0.5 mL, 2.63 mmol, 1.5 eq) in EtOAc (3.3 mL) was prepared and added dropwise to the reaction mixture maintaining the temperature between 0-2 ^{0}C. Once all the DIAD solution was added, the mixture was left for stirring at room temperature for 3 h. Afterwards, the solvent was removed using rotary evaporator. Column purification was carried out using EtOAc / MeOH mixture (4:1 v/v) containing 0.5% Et₃N as the eluent to isolate the product, **37** (0.63g, 70%) as a white amorphous foam[2].

TLC (EtOAc:MeOH 9:1 v/v): R_f=0.1.

¹H-NMR (CDCl₃): δ 1.91 (3H, *s*, CH₃), 2.35 (1H, *ddd*, *J* = 12.92 Hz, *J* = 3.8 Hz, *J* = 3.04 Hz, H-2'), 2.64 (1H, *dd*, *J* = 12.78 Hz, *J* = 0.86 Hz, H-2"), 3.31 (2H, *m*, H-5', H-5"), 3.77 (6H, *s*, OMe), 4.32 (1H, *m*, H-4'), 5.15 (1H, *s*, H-3'), 5.49 (1H, *d*, *J* = 3.8 Hz, H-1'), 6.79 (4H, *m*, ArH), 6.91 (1H, *s*, H-5), 7.17-7.40 (9H, *m*, ArH).

¹³C-NMR (CDCl₃): δ 13.8 (CH₃), 34.1 (C-2'), 55.6 (OMe), 62.6 (C-5'), 77.2 (C-3'), 85.0 (C-4'), 87.2 (C-7), 87.9 (C-1'), 113.6 (ArC), 118.8 (C-5), 127.3-130.4 (ArC), 135.5 (C-6), 135.8-144.9 (ArC), 153.7 (C-2), 159.0 (ArC), 172.0 (C-4).

MS (*m/z*): 549 (M+Na)⁺, 100%; 1075 (2M+Na)⁺, 33%.

5'-*O*-(4,4'-dimethoxytrityl)-3'-fluoro-3'-deoxythymidine (36)

$C_{31}H_{31}FN_2O_6$, MW: 546.59 gmol^{-1}
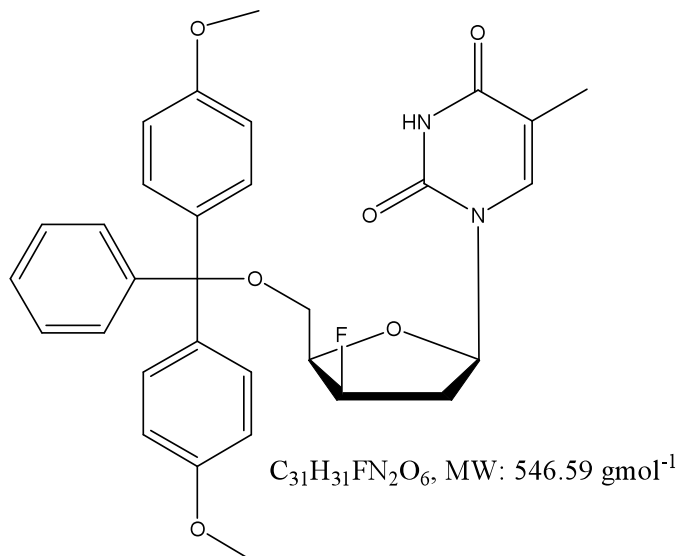
The compound **35** (74 mg, 0.136 mmol) was diluted in a solvent system comprising of acetonitrile and methanol in a 1:1 ratio (1.0mL) and CsF (98 mg, 0.64 mmol) was added to it. The mixture was heated to 60 ^{0}C under reflux and left for stirring overnight. The resulting violet slurry was diluted with MeOH and dried under vacuum.[3] The obtained crude product was subjected to column purification using DCM: MeOH (9.5:0.5) as the eluent to form 50 mg of the desired compound, **36** as light violet powder with R$_f$ value of 0.38.

^{1}H-NMR (d$_4$-MeOD): δ 7.71 (1H, *s*, H-6), 7.41 (6H, *m*, Ar-H), 6.46 (2H, *t*, Ar-H), 6.35 (1H, *t*, H-1'), 4.56 (1H, *dd, J* =3Hz, *J* = 6Hz, H-3'), 4.10 (1H, *d*, H-4'), 3.75 (6H, *s*, OCH$_3$), 3.39 (2H, *m*, H-5', H-5"), 2.47 (1H, *m*, H$_e$-2'), 2.43 (1H, *m*, H$_x$-2'), 1.48 (3H, *s*, CH$_3$).

^{19}F-NMR (d$_4$-MeOD): δ -150.65.

5'-*O*-(4,4'-dimethoxytrityl)-3'deoxy-2',3'-didehydrothymidine (39)

C$_{31}$H$_{30}$N$_2$O$_6$, MW: 526.58 gmol^{-1}
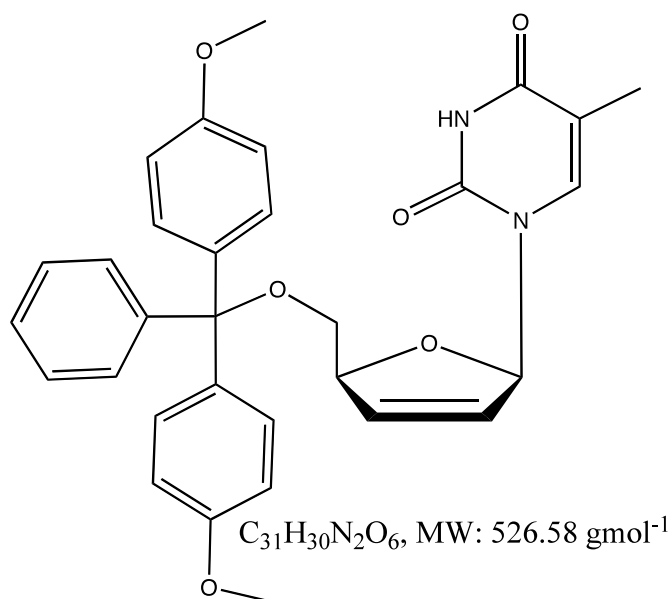
The compound **37** (0.52 g, 0.98 mmol) was dissolved in anhydrous DMF (4 mL) in a 25 mL round-bottom flask. To this solution, TBAF.3H$_2$O (0.94 g, 2.96 mmol, 3 eq) was added and the reaction mixture was heated to 150 ^{0}C under reflux conditions. The reaction was left to run for 5 hours. After this, the mixture was poured into excess water and a white crude precipitate was collected by filteration.[3] It was purified by column chromatography and a white amorphous powder (50 mg, 10%) was isolated:

mp 123-125 ^{0}C.

TLC (DCM:MeOH 9.6:0.4 v/v): R_f=0.27

^{1}**H-NMR** (CDCl$_3$): δ 1.21 (3H, s, CH$_3$), 3.32 (2H, *m,* H-5', H-5"), 3.71 (6H, *s*, 2x OMe), 4.95 (1H, *br s*, H-4'), 6.01 (1H, *d*, *J* =3.8 Hz, H-1'), 6.50 (1H, *s*, H-5), 6.85 (4H, *m*, ArH), 7.21-7.45 (9H, *m*, ArH).

2,3'-anhydrothymidine (44)
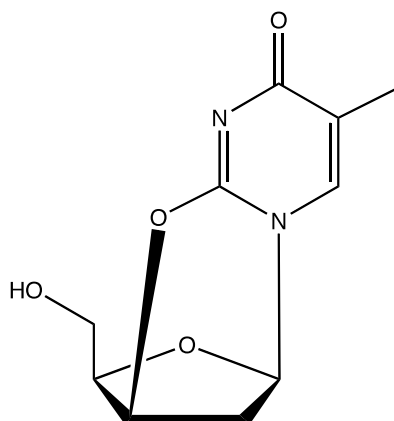
C$_{10}$H$_{12}$N$_2$O$_4$, MW: 224.21 gmol^{-1}

A mixture of **1** (2 g, 8.26 mmol) and PPh$_3$ (4.33 g, 16.51 mmol, 2 eq) was suspended in anhydrous acetonitrile (60 mL) and cooled down to -15 ^{0}C in ice-MeOH/Acetone bath. To this mixture, DIAD (3.25 mL, 16.51 mmol, 2 eq), which was dissolved in acetonitrile (5 mL), was added dropwise maintaining the temperature below -5 ^{0}C with vigorous stirring. After the addition, the reaction was left to stir for another 5 hours at 0 ^{0}C. Following this, the mixture was again cooled down to -20 ^{0}C and was poured into a conical flask containing ethyl acetate (100 mL) at the same temperature and stirred for 15 min. The white precipitate that was formed was collected by Buechner filteration and washed with cold ethyl acetate. The obtained compound was again purified by column chromatography using DCM-MeOH (8:2 v/v) as the eluent to separate the desired product, **44** (0.55 g, 30%): mp 229-230 ^{0}C, lit. mp 230 ^{0}C.[4]

TLC (DCM:MeOH 8:2 v/v): R$_f$ = 0.30.

^{1}H-NMR (d$_6$-DMSO): δ 1.76 (3H, *d, J* = 1.1 Hz, CH$_3$), 2.45 (1H, *ddd, J$_{1,8}$* = 19 Hz, *J$_{1,4}$* =6.7 Hz, *J$_{1,2}$*= 3 Hz, H-2"), 2.57 (1H, *d, J* = 1.2, H-2'), 3.50 (2H, *m*, H-5', H-5"), 4.20 (1H, *m*, H-4'), 5.03 (1H, *t*, C-5'OH), 5.25 (1H, *br s*, H-3'), 5.82 (1H, *d, J* = 3.9 Hz, H-1'), 7.56 (1H, *d, J* = 1.2 Hz, ArH).

3'-*O*-(Methylsulfonyl)-5'-*O*-trityl-2'-deoxythymidine (40)
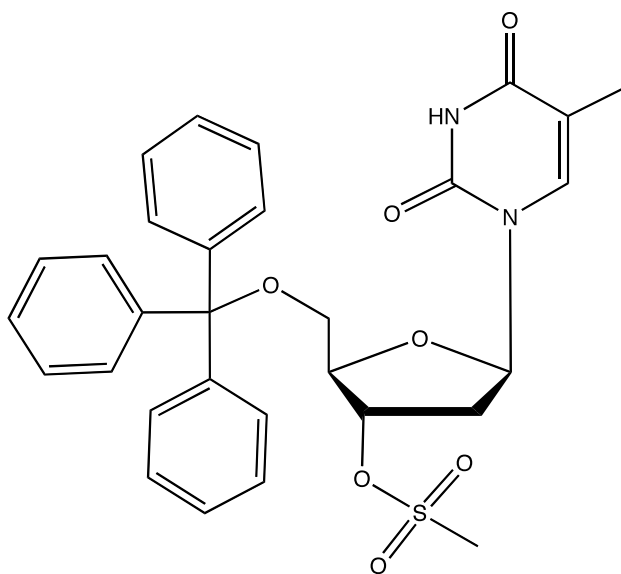
C$_{30}$H$_{30}$N$_2$O$_7$S, MW: 562.63 gmol^{-1}

A dried mixture of thymidine, **1** (4.3 g, 17.75 mmol), trityl chloride (7.4 g, 26.54 mmol), and 4-(dimethylamino)pyridine (0.14 g, 1.10 mmol) was dissolved in anhydrous pyridine (80 mL) and heated at 80 ^{0}C for 8 h. The reaction mixture was then cooled to 0 ^{0}C in an ice bath, and methanesulphonyl chloride (2.75 mL, 35.50 mmol) was added dropwise to the reaction flask with constant stirring. The mixture was left for stirring at 0 ^{0}C for 1 h and then kept in the refrigerator overnight. The solvent was removed in vacuo, and then 100 mL of ice-water mixture was added to the residue. The resulting

aqueous solution was extracted with ethyl acetate (3 x 100 mL). The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated to give the crude product, which was purified on a silica gel column using EtOAc/Hexane (1:1, v/v) as eluent to give **40** (7.25 g, 73 %) as a white solid, mp: 121-123 ^{0}C, lit. mp: 120-122 ^{0}C. [5]

TLC (EtOAc:Hexane, 7:3 v/v): R_f = 0.45.

^{1}H-NMR (CDCl$_3$): δ 1.48 (s, 3H, CH$_3$), 2.43-2.51 (m, 1H, H-2'), 2.65-2.74 (m, 1H, H-2"), 3.03 (s, 3H, CH$_3$SO$_2$), 3.45-3.58 (m, 2H, 2 x H-5'), 4.34 (s, 1H, H-4'), 5.42 (m, 1H, H-3'), 6.45 (m, 1H, H-1'), 7.29-7.41 (m, 15H, 5'-*O*-trityl), 7.55 (s, 1H, H-6), 8.48 (s, 1H, NH).

1-(5'-*O*-Trityl-2'-deoxy-*β*-D-lyxofuranosyl)thymine (41)
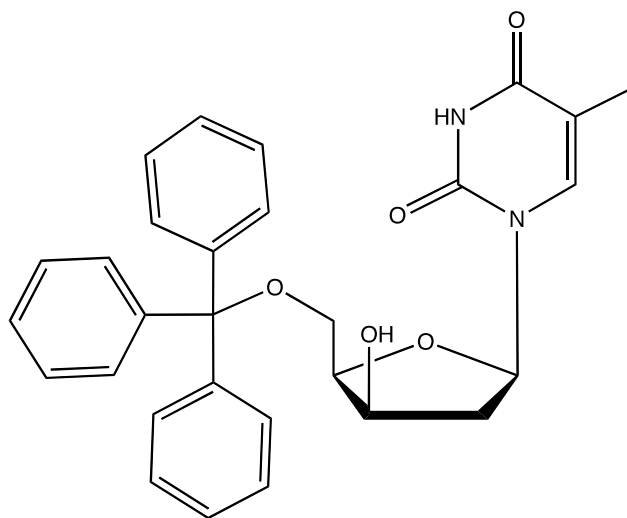
$C_{29}H_{28}N_2O_5$, MW: 484.54 gmol^{-1}

NaOH (5.49 g, 137.13 mmol, 10 eq) was added to a solution of compound **40** (7.25g, 12.89 mmol) in 90% aqueous ethanol (200 mL), and the reaction mixture was refluxed for 2 h at 90 ^{0}C. Afterwards, the reaction flask containing the mixture was cooled in an ice-bath for 15 min and neutralized with 80% acetic acid. The solvent was removed under reduced pressure in rotary evaporator. Methanol (100 mL) was added to the resulting residue and the pure product **41** (10.55 g, 85%) precipitated out as a white powder, which was filtered under vacuum and nitrogen, mp: 241-243 ^{0}C, lit. mp: 240-244 ^{0}C. [6]

¹H-NMR (DMSO-d_6**):** δ 1.63 (s, 3H, CH₃), 1.84 (d, J = 14.65 Hz, 1H, H-2'), 2.50-2.58 (m, 1H, H-2"), 3.16-3.42 (m, 2H, H-5'), 4.09 (m, 1H, H-4'), 4.18 (m, 1H, H-3'), 5.22 (br s, 1H, 3'-OH), 6.12 (d, J = 6.91 Hz, 1H, H-1'), 7.21-7.48 (m, 15H, 5'-*O*-Trityl), 7.60 (s, 1H, H-6), 11.28 (br s, 1H, NH).

¹³C-NMR (DMSO- d_6): δ 12.43 (CH₃), 40.76 (C-2'), 63.03 (C-5'), 68.92 (C-3'), 83.21 (C-1'), 84.11 (C-4'), 86.10 (C-7), 108.30 (C-5), 126.98-128.29 (ArC), 136.75 (C-6), 150.53 (C-2), 163.84 (C-4).

5'-*O*-Trityl-3'-deoxy-3'-fluoro-thymidine (43)
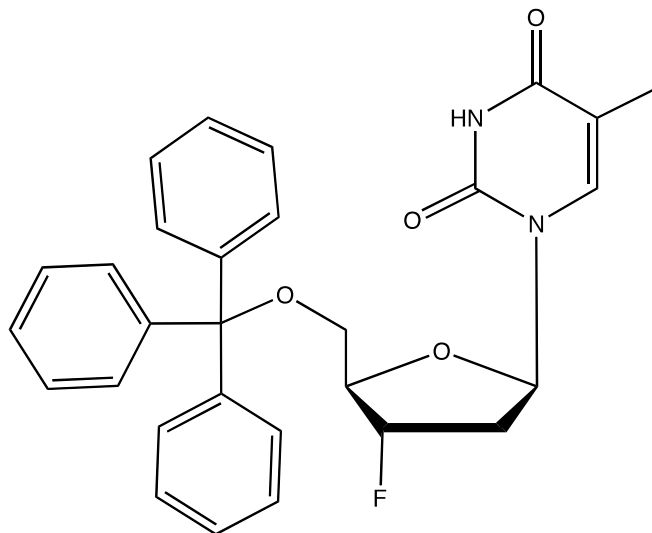
$C_{29}H_{27}FN_2O_4$, MW: 486.53 gmol⁻¹

Method 1: Fluoridation with direct displacement of 3'-OH group

To a solution of triethylamine trihydrofluoride (TEA.3HF; 1.1 mL, 6.19 mmol, 2eq) in anhydrous dichloromethane (20 mL) at room temperature were successively added Xtal Fluor-M (1.13g, 4.64 mmol, 1.5 eq) and compound **41** (1.50g, 3.11 mmol, 1eq). The reaction mixture was left to stir for 1.5 h. After that, it was quenched with 5% aqueous sodium bicarbonate solution (20 mL) and stirred for another 15 min. The resulting mixture was extracted twice with DCM. The organic phases were combined and dried over anhydrous $MgSO_4$ and filtered. The filterate was concentrated *in vacuo* and the residue was purified on a silica gel column using EtOAc/Hexane (1:1, v/v) as the eluent to yield **43** (0.75 g, 50 %) as white foam. [7]

TLC (EtOAc:Hexane, 1:1 v/v): R_f = 0.5.

Method 2: Fluoridation via Nosyl precursor

The prepared nosyl precursor **42** (0.15g, 0.22 mmol) was dissolved in anhydrous DMF (10 mL) and subsequently a mixture of Tetrabutylammonium fluoride trihydrate (TBAF.3H$_2$O; 0.21 g, 0.67 mmol, 3 eq) and Kryptofix [222] (0.12 g, 0.34 mmol, 1.5 eq) was added to the reaction flask. The reaction mixture was stirred and heated at 90 ^{0}C for 30 min. After that, the reaction was stopped and the solvent was removed under vacuum. Column purification was performed with EtOAc/Hexane (1:1, v/v) as the eluent to obtain **43** (0.03 g, 25 %) as white foam.

TLC (EtOAc:Hexane, 1:1 v/v): R_f = 0.5.

¹H-NMR (CDCl₃): δ 1.49 (s, 3H, CH₃), 2.28-2.45 (m, 1H, H-2'), 2.64-2.75 (m, 1H, H-2"), 3.41-3.55 (m, 2H, H-5', H-5"), 4.34 (d, J = 30 Hz, 1H, H-4'), 5.33 (dd, $J_{1,2}$ = 10 Hz, $J_{1,3}$ = 52 Hz, 1H, H-3'), 6.51 (m, 1H, H-1'), 7.22-7.45 (m, 15H, Tr-H), 7.61 (s, 1H, H-6), 9.72 (s, 1H, NH).

¹⁹F-NMR (CDCl₃): δ -172.95.

1-[5'-*O*-Trityl-2'-deoxy-3'-*O*-(4-nitrobenzenesulfonyl)-*β*-D-*threo* pentofuranosyl]thymine (42)
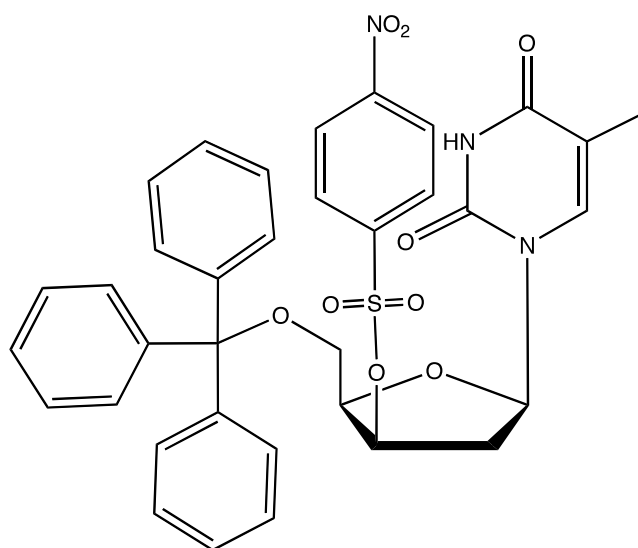
C₃₅H₃₁N₃O₉S, MW: 669.70 gmol⁻¹

The solid **41** (1.16 g, 2.34 mmol) was dissolved in anhydrous pyridine (20 mL) at 0 ^{0}C and a mixture of 4-nitrobenzenesulfonyl chloride (Nosyl chloride; 1.06 g, 4.79 mmol, 2 eq) and silver trifluoromethanesulfonate (1.23 g, 4.79 mmol, 2 eq) was added to the solution. The reaction mixture was left for stirring at 0 ^{0}C for 50 min. Then the temperature was adjusted to room temperature and the reaction was quenched after 40 min. The mixture was diluted with EtOAc (50 mL) and the precipitate (AgCl) that was formed, was filtered. The filterate was extracted with brine (70 mL) and water (70mL). The organic layer was dried with anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel with ethyl acetate: hexane (4:1, v/v) to give **42** as a yellow solid (1.88 g, 60%), mp: 120-122 ^{0}C. [8]

TLC (EtOAc:Hexane, 4:1 v/v): R_f = 0.8.

^{1}H-NMR (CDCl$_3$): δ 1.75 (s, 3H, CH$_3$), 2.48 (m, 1H, H-2'), 2.77 (m, 1H, H-2"), 3.21 (m, 1H, H-5'), 3.59 (m, 1H, H-5"), 4.22 (m, 1H, H-4'), 5.20 (m, 1H, H-3'), 6.23 (m, 1H, H-1'), 7.21 (s, 1H, H-6), 7.25 (m, 15H, TrH), 7.89 (d, J = 6.32 Hz, 2H, H$_a$-Nos), 8.24 (d, J = 6.32 Hz, 2H, H$_b$-Nos), 8.71 (s, 1H, NH).

^{13}C-NMR (CDCl$_3$): δ 12.46 (CH$_3$), 39.46 (C-2'), 61.46 (C-5'), 80.28 (C-3'), 81.50 (C-1'), 84.05 (C-4'), 111.02 (C-5), 124.60 (Nos-C), 127.46-128.86 (Tr-C), 134.82 (C-6), 150.42 (C-2), 163.35 (C-4).

3'-deoxy-3'-fluorothymidine (10)
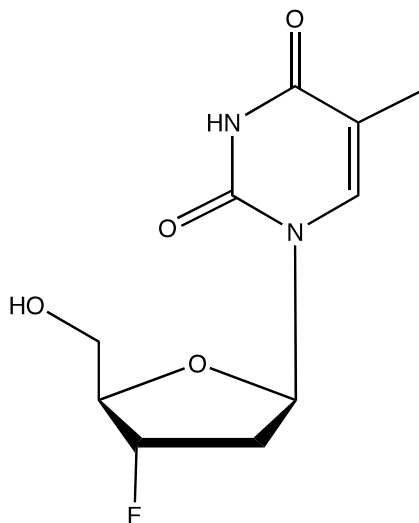
$C_{10}H_{13}FN_2O_4$, MW: 244.22 gmol^{-1}

The compound **43** (0.30g, 0.62 mmol) was dissolved in 80% aqueous acetic acid (10 mL) and heated at 90 ^{0}C for 30 min under reflux. The solvent was removed under reduced pressure and the crude product was purified on silica gel column using DCM/ MeOH (9.5:0.5 v/v) as eluent to give the desired product, **10** (0.14g, 93%). mp: 176-177^{0}C, lit. mp: 176 ^{0}C. [3]
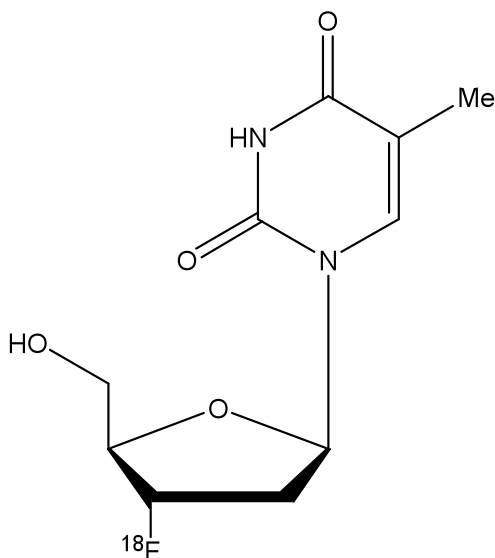
TLC (DCM: MeOH 9.5:0.5 v/v): R_f = 0.4.

^{1}H-NMR (d$_4$-MeOD): δ 1.91 (*s*, 3H, CH$_3$), 2.23-2.62 (*m,* 2H, H-2', H-2"), 3.76 (*m*, 2H H-5', H-5"), 4.27 (d*t*, *J* = 35 Hz, 1H, H-4'), 5.01 (*bs*, 1H, OH), 5.37 (*dd*, $J_{1,2}$ = 4.95 Hz, $J_{1,3}$= 50 Hz, 1H, H-3'), 6.32 (m, 1H, H-1'), 7.81 (s, 1H, H-6).

^{13}C-NMR (d$_4$-MeOD): δ 12.28 (CH$_3$), 39.11 (C-2'), 62.74 (C-5'), 86.82 ($J_{3',F}$= 172.8 Hz, C-3'), 95.24 (C-1'), 96.64 (C-4'), 111.90 (C-5), 137.89 (C-6), 152.42 (C-2), 166.33 (C-4).

¹⁹F-NMR (d₄-MeOD): δ -176.48.
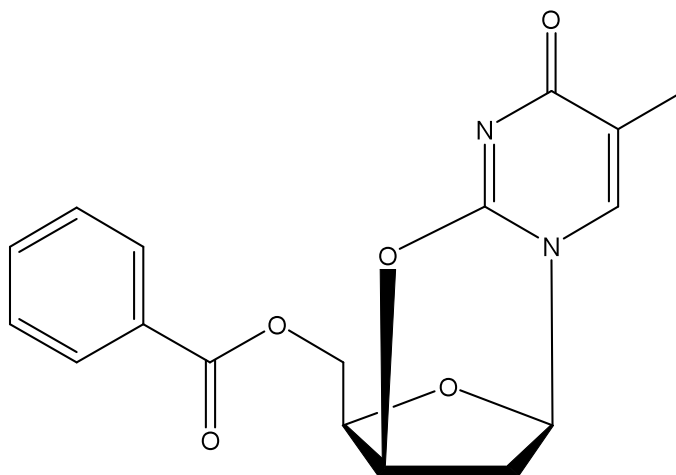
[¹⁸F]- 3'-deoxy-3'-fluorothymidine (15)

$C_{10}H_{13}{}^{18}FN_2O_4$, MW: 243.22 gmol^{-1}

The compound **15** was radiolabeled according to *Procedure A* from precursor molecules **48** [9], **33** and **44** with RCY of 44.5%, 25% and 1.5 % respectively.

Radio-HPLC analysis:

Retention time ([¹⁸F]-FLT): 7.5 minutes

5'-*O*-benzoyl-2,3'-anhydrothymidine (48)

$C_{17}H_{16}N_2O_5$, MW: 328.32 gmol^{-1}

A mixture containing thymidine, **1** (5.0 g, 20.64 mmol) and triphenylphosphine (12.61 g, 48.08 mmol, 2.2eq.) was dried under Argon, dissolved in DMF (60 mL) and stirred to cool down at 0° C. Meanwhile, a solution comprising of DIAD (5.8 g, 28.68 mmol, 5.2 mL, 1.2eq.), benzoic acid (4.54g, 1.5eq.) and DMF (10 mL) was prepared to get a yellow solution. This solution was then added drop-wise into the reaction mixture maintaining the temperature of 0 °C. Afterwards it was allowed to stir for 4 hours at room temperature. After this, the mixture was poured into flask containing ether (400 mL) and stirred for 10 minutes at room temperature. The resulting suspension was stored in the fridge at a temperature less than 4 °C for 48 hours. The white amorphous precipitate was collected by filteration and washed with ether and brine to give the compound **48** (5.85 g, 79 %) as a fine white powder. mp: 241-242 °C, lit. mp: 242 °C. [10]

¹H-NMR (d₆-DMSO): δ 7.87 (2H, *d, J* = 9Hz, O-Ph), 7.66 (1H, *m*, O-Ph), 7.59 (1H, *s*, H-6), 7.02 (2H, *d, J* = 8.5Hz, O-Ph), 5.92 (1H, *d, J* = 3.5Hz, H-1'), 5.42 (1H, *s*, H-3'), 4.58 (1H, *m*, H-4'), 4.50 (1H, *dd, J* =5Hz, *J* =10Hz, H-5'), 4.33 (1H, *dd, J* = 6.5Hz, *J* = 11.5Hz, H-5"), 2.62 (1H, *m,* H-2'), 2.54 (1H, *m,* H-2"), 1.74 (3H, *s*, CH₃);

1-(2-deoxy-*β*-D-lyxofuranosyl)thymine (49)
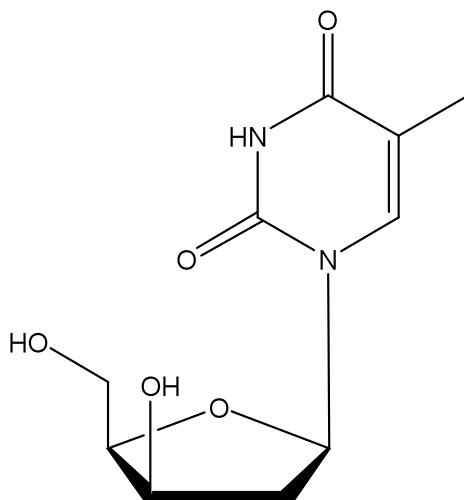

C₁₀H₁₄N₂O₅, MW: 242.23 gmol⁻¹

A mixture of **44** (0.6 g, 2.67 mmol) and aq. NaOH (1M, 10 mL) was stirred in steambath under reflux for three hours. The clear solution changed to gold brown upon

heating and the progress of the reaction was monitored by TLC analysis with DCM:MeOH (9.5 : 0.5, v/v) as the eluent. The solvent was removed by evaporation under reduced pressure.[4] The crude compound was purified by column chromatography using DCM:MeOH (9.5 : 0.5, v/v) as the eluent to obtain **49** in 57% yield.

mp: 172-174 °C

¹H-NMR (d$_6$-DMSO): δ 11.25 (1H, *s*, NH), 7.79 (1H, *s*, H-6), 6.06 (1H, *dd*, *J* = 8.5Hz, *J*= 2.44 Hz, H-1'), 5.25 (1H, *d*, *J* = 3.35Hz, 3'-OH), 4.69 (1H, *t*, *J*= 5.49 Hz, 5'-OH), 4.22 (1H, *m*, H-3'), 3.58-3.82 (3H, *m*, H-4', H-5' and H-5''), 2.55-2.59 (1H, *m*, H-2''), 1.84 (1H, *dd*, *J*= 14.95 Hz, *J*= 2.14 Hz, H-2'), 1.76 (3H, *s*, CH$_3$);

1-(5'-*O*-[benzyl-(methoxy-alaninyl)-phosphate]-β-D-lyxofuranosyl)thymine (50)
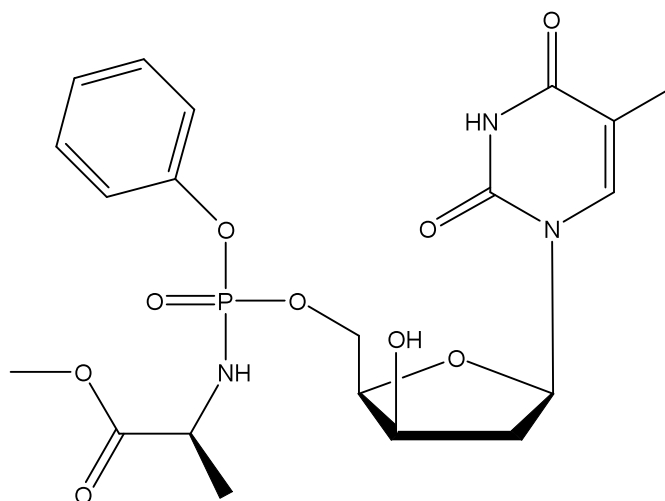

C$_{20}$H$_{26}$N$_3$O$_9$P, MW: 483.41 gmol^{-1}

Prepared according to *Procedure E* from **49** (2.5g, 10.32 mmol, 1eq), NMI (4.12 mL, 51.60 mmol, 5 eq) and **77** (8.5 g, 30.62 mmol, 3 eq) in anhydrous THF and pyridine (1:1 v/v, 50 mL). After addition of the phosphorochloridate **77**, the colour of the reaction mixture changed from cloudy white to light yellow. Column chromatography purification using CHCl$_3$-MeOH (9.8:0.2) as the eluent afforded the product **50** (0.36 g, 7 %) as oil.

^{1}H-NMR (CDCl$_3$): δ 7.58 (d, J = 7 Hz, 1H, H-6), 7.35-7.43 (m, 2H, Ar-H), 7.20-7.29 (m, 3H, Ar-H), 6.25-6.32 (m, 1H, H-1'), 5.22 (m, 1H, 3'-OH), 4.94 (m, 1H, H-3'), 4.31-4.52 (m, 2H, NH, H-4'), 3.95-4.03 (m, 1H, CH), 3.68 (s, 3H, OCH$_3$), 3.35 (d, J = 16 Hz, 2H, H-5', H-5"), 2.48-2.51 (m, 1H, H-2"), 2.09-2.25 (m, 1H, H-2'), 1.85 (d, J = 10 Hz, 3H, CH$_3$ -Thy), 1.31-1.42 (m, 3H, CH$_3$ -Ala).

^{31}P-NMR (CDCl$_3$): δ 3.58, 3.71.

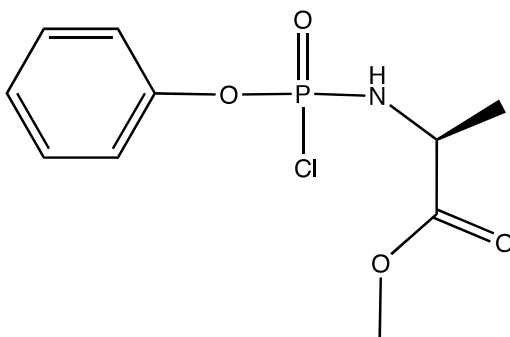**L-Alanine isopropyl ester hydrochloride (76)**

$C_6H_{14}ClNO_2$, MW: 167.63 gmol^{-1}
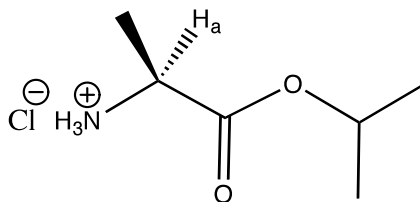
Thionyl chloride (SOCl$_2$; 16.38 mL, 0.23 mol, 2 eq) was added dropwise at 0 ^{0}C to isopropyl alcohol (128.9 mL, 1.68 mol, 15 eq) and left to stir. After 30 min under stirring, the mixture was allowed to warm to room temperature and L-Alanine (10 g, 0.12 mol) was added. The reaction mixture was heated at 70 ^{0}C under reflux overnight. The volatiles were removed under reduced pressure to yield after precipitation with diethyl ether, the desired ester hydrochloride salt **76** (25 g, 70%) as a white amorphous powder.

^{1}H-NMR (CDCl$_3$): δ 1.32 (d, J = 6.21 Hz, 6H, 2xCH$_3$ iPr), 1.54 (d, J = 7.19 Hz, 3H, CH$_3$ Ala), 4.18 (t, 1H, Hα), 5.12 (m, 1H, CH ester), 8.73 (s, 3H, NH$_3$).

^{13}C-NMR (CDCl$_3$): δ 15.95 (CH$_3$-iPr), 21.59 (CH$_3$-iPr), 49.34 (CH$_3$-Ala), 70.33 (CH-ester), 169.51 (C=O).

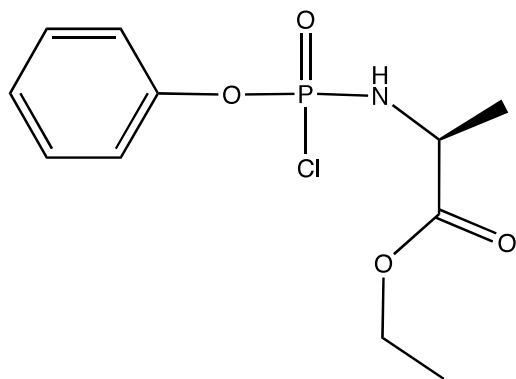## (2*S*)-methyl-2-(chloro(phenoxy)phosphorylamino)propanoate (77)

$C_{10}H_{13}ClNO_4P$, MW: 277.64 gmol^{-1}

The compound **77** was prepared according to *Procedure C*, from commercially available phenyl dichlorophosphate (2.82g, 13.39 mmol, 1.6 mL, 1 eq), L-alanine methyl ester hydrochloride salt (1.87 g, 13.30 mmol, 1 eq), and anhydrous triethylamine (2.71 g, 26.77 mmol, 3.73 mL, 2 eq) in dry DCM (20 mL). The product was obtained as clear oil (3.55 g, 50 %).

^{31}P-NMR (CDCl$_3$): δ 7.96, 8.15.

^{1}H-NMR (CDCl$_3$): δ 1.52 (m, 3H, CH$_3$-Ala), 3.75 (d, *J* = 20Hz, 3H, OCH$_3$), 4.21 (m, 1H, CH), 4.65 (m, 1H, NH), 7.18-7.27 (m, 3H, Ar-H), 7.32-7.39 (m, 2H, Ar-H).

**(2*S*)-ethyl-2-(chloro(phenoxy)phosphorylamino)propanoate (78)**

$C_{11}H_{15}ClNO_4P$, MW: 291.67 gmol^{-1}

The compound **78** was prepared according to *Procedure C*, from phenyl dichlorophosphate (3.17 g, 15.00 mmol, 2.24 mL, 1 eq), L-alanine ethyl ester hydrochloride salt (2.30 g, 15.00 mmol, 1 eq), and anhydrous triethylamine (4.20 mL, 30.00 mmol, 2 eq) in dry DCM (50 mL). The product was obtained as clear oil (3.94 g, 45 %).

^{31}P-NMR (CDCl$_3$): δ 7.84, 8.13.

^{1}H-NMR (CDCl$_3$): δ 1.25 (m, 3H, CH$_3$), 1.51 (m, 3H, CH$_3$), 3.95 (m, 2H, CH$_2$), 4.21 (m, 1H, CH), 4.53 (m, 1H, NH), 7.21-7.30 (m, 3H, Ar-H), 7.35-7.41 (m, 2H, Ar-H).

(2*S*)-isopropyl-2-(chloro(phenoxy)phosphorylamino)propanoate (79)
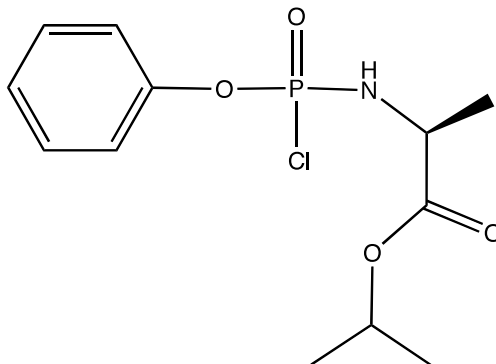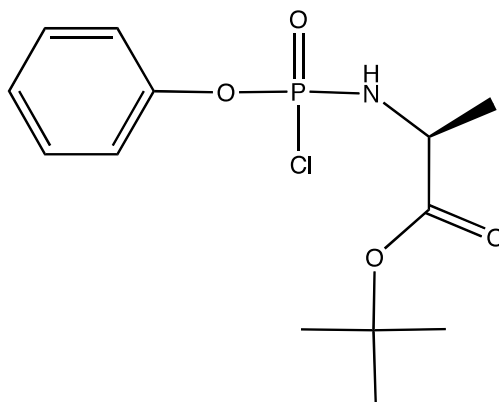
$C_{12}H_{17}ClNO_4P$, MW: 305.69 gmol^{-1}

The compound **79** was prepared according to *Procedure C*, from phenyl dichlorophosphate (2.92 g, 13.85 mmol, 2.00 mL, 1 eq), L-alanine isopropyl ester hydrochloride salt (**76**, 2.24 g, 13.85 mmol, 1 eq), and anhydrous triethylamine (3.73 mL, 26.77 mmol, 2 eq) in dry DCM (50 mL). The product was obtained as clear oil (6.21 g, 76 %).

^{31}P-NMR (CDCl$_3$): δ 8.06, 8.25.

^{1}H-NMR (CDCl$_3$): δ 1.15-1.25 (m, 6H, 2xCH$_3$-*i*Pr), 1.51 (m, 3H, CH$_3$-Ala), 4.13 (m, 1H, CH), 4.53 (m, 1H, NH), 5.05-5.12 (m, 1H, CH-*i*Pr), 7.23-7.29 (m, 3H, Ar-H), 7.39-7.45 (m, 2H, Ar-H).

## (2S)-tert-butyl-2-(chloro(phenoxy)phosphorylamino)propanoate (80)

$C_{13}H_{19}ClNO_4P$, MW: 319.72 gmol^{-1}

The compound **80** was prepared according to *Procedure C*, from phenyl dichlorophosphate (2.92 g, 13.39 mmol, 2.00 mL, 1 eq), L-alanine *tert*-butyl ester hydrochloride salt (2.43 g, 13.39 mmol, 1 eq), and anhydrous triethylamine (3.73 mL, 26.77 mmol, 2 eq) in dry DCM (50 mL). The product was obtained as clear oil (5.47 g, 64 %).

^{31}P-NMR (CDCl$_3$): δ 7.96, 8.33.

^{1}H-NMR (CDCl$_3$): δ 1.52 (m, 12H, 4xCH$_3$), 4.15 (m, 1H, CH), 4.35 (m, 1H, NH), 7.23-7.29 (m, 3H, Ar-H), 7.39-7.45 (m, 2H, Ar-H).

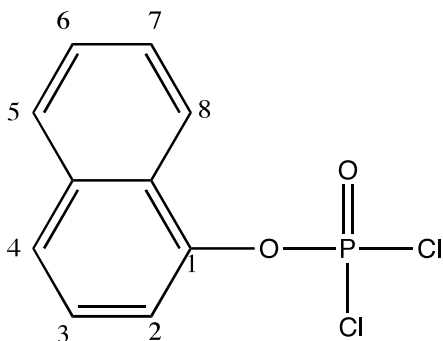**(2*S*)-benzyl-2-(chloro(phenoxy)phosphorylamino)propanoate (81)**
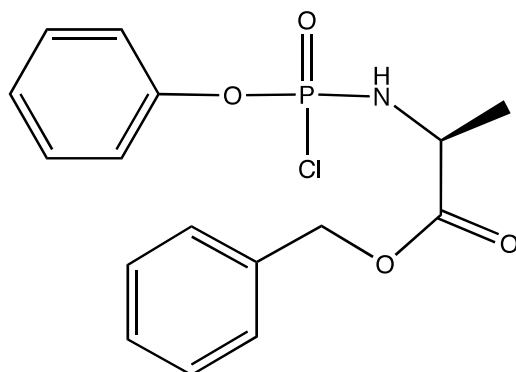
$C_{16}H_{17}ClNO_4P$, MW: 353.74 gmol^{-1}

The compound **81** was prepared according to *Procedure C*, from phenyl dichlorophosphate (2.92 g, 13.39 mmol, 2.00 mL, 1 eq), L-alanine benzyl ester hydrochloride salt (2.89 g, 13.39 mmol, 1 eq), and anhydrous triethylamine (3.73 mL, 26.77 mmol, 2 eq) in dry DCM (60 mL). The product was obtained as clear oil (3.79 g, 40 %).

^{31}P-NMR (CDCl$_3$): δ 7.85, 8.09.

^{1}H-NMR (CDCl$_3$): δ 1.55 (m, 3H, CH$_3$), 4.23 (m, 1H, CH), 4.52 (m, 1H, NH), 5.22 (d, *J* = 15 Hz, 2H, BnCH$_2$), 7.23-7.29 (m, 3H, Ar-H), 7.39-7.49 (m, 7H, Ar-H).

1-Naphthyl dichlorophosphate (82)

$C_{10}H_7Cl_2O_2P$, MW: 261.04 gmol^{-1}

Compound **82** was prepared according to *Procedure B* from 1-naphthol (8.00 g, 55.49 mmol, 1 eq), POCl$_3$ (8.51 g, 55.49 mmol, 5.18 mL, 1 eq) and anhydrous triethylamine (7.73 mL, 55.49 mmol, 1 eq) in dry diethyl ether (120 mL). The product was isolated as light yellow oil (15.25 g, 52 %).

^{31}P-NMR (CDCl$_3$): δ 3.89.

^{1}H-NMR (CDCl$_3$): δ 7.45-7.59 (m, 4H, H-2, H-3, H-6, H-7), 7.73-7.76 (m, 1H, H-4), 7.91-7.95 (m, 1H, H-5), 8.12-8.15 (m, 1H, H-8).

(2*S*)-methyl-2-(chloro(naphthalen-1-yloxy)phosphorylamino)propanoate (83)

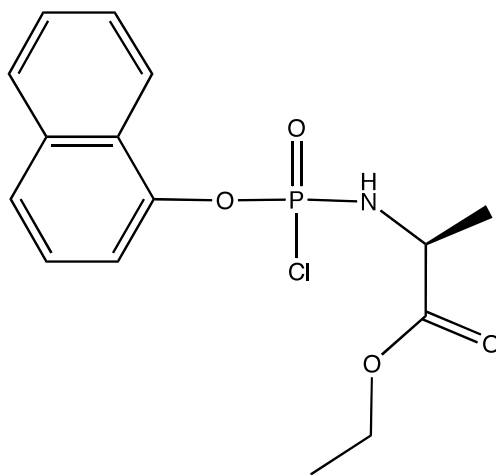
C$_{14}$H$_{15}$ClNO$_4$P, MW: 327.70 gmol^{-1}
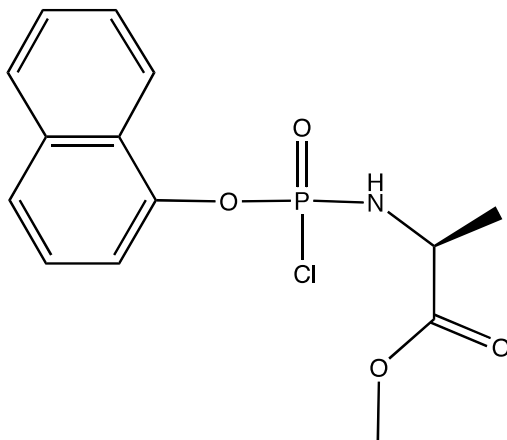
The compound **83** was prepared according to *Procedure C*, from 1-naphthyl dichlorophosphate (**82**, 1.87 g, 7.16 mmol, 1 eq), L-alanine methyl ester hydrochloride salt (1.35 g, 7.16 mmol, 1 eq), and anhydrous triethylamine (2.00 mL, 14.33 mmol, 2 eq) in dry DCM (40 mL). The product was obtained as clear oil (4.18 g, 89 %).

^{31}P-NMR (CDCl$_3$): δ 8.06, 8.21.

^{1}H-NMR (CDCl$_3$): δ 1.22 (m, 3H, CH$_3$-Ala), 3.75 (d, *J* = 45 Hz, 3H, OCH$_3$), 4.21 (m, 1H, CH), 4.65 (m, 1H, NH), 7.45 (m, 1H, Ar-H), 7.52-7.61 (m, 3H, Ar-H), 7.69-7.72 (d, *J* = 8.5 Hz, 1H, Ar-H), 7.88-7.92 (d, *J* = 8Hz, 1H, Ar-H), 8.08-8.12 (t, 1H, Ar-H).

**(2*S*)-ethyl-2-(chloro(naphthalen-1-yloxy)phosphorylamino)propanoate (84)**

C$_{15}$H$_{17}$ClNO$_4$P, MW: 341.73 gmol^{-1}

The compound **84** was prepared according to *Procedure C*, from 1-naphthyl dichlorophosphate (**82**, 1.70 g, 6.51 mmol, 1 eq), L-alanine ethyl ester hydrochloride salt (1.00 g, 6.51 mmol, 1 eq), and anhydrous triethylamine (1.32g, 13.03 mmol, 1.82 mL, 2 eq) in dry DCM (40 mL). The product was obtained as light yellow oil (3.65 g, 82 %).

^{31}P-NMR (CDCl$_3$): δ 8.15, 8.43.

^{1}H-NMR (CDCl$_3$): δ 1.22 (m, 3H, CH$_3$), 1.62 (m, 3H, CH$_3$), 4.12 (m, 1H, CH), 4.20-4.39 (m, 2H, CH$_2$), 4.55-4.61 (m, 1H, NH), 7.45 (m, 1H, Ar-H), 7.52-7.61 (m, 3H, Ar-H), 7.69-7.72 (d, *J* = 8.5 Hz, 1H, Ar-H), 7.88-7.92 (d, *J* = 8Hz, 1H, Ar-H), 8.08-8.12 (t, 1H, Ar-H).

**(2*S*)-isopropyl-2-(chloro(naphthalen-1-yloxy)phosphorylamino)propanoate (85)**
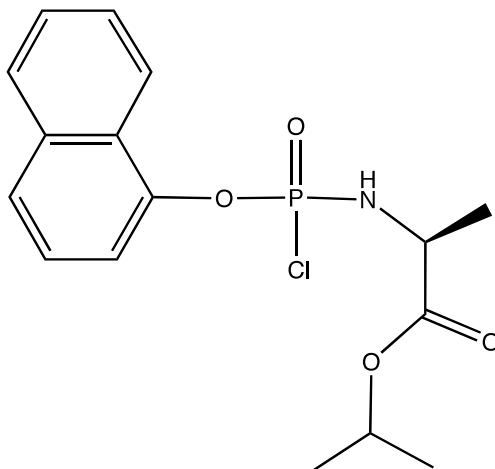
$C_{16}H_{19}ClNO_4P$, MW: 355.75 gmol^{-1}

The compound **85** was prepared according to *Procedure C*, from 1-naphthyl dichlorophosphate (**82**, 1.56 g, 5.97 mmol, 1 eq), L-alanine isopropyl ester hydrochloride salt (1.00 g, 5.97 mmol, 1 eq), and anhydrous triethylamine (1.21 g, 11.93 mmol, 1.67 mL, 2 eq) in dry DCM (40 mL). The product was obtained as light yellow oil (3.18 g, 75 %).

^{31}**P-NMR** (CDCl$_3$): δ 8.16, 8.35.

^{1}**H-NMR (**CDCl$_3$): δ 1.12-1.39 (m, 6H, 2 x CH$_3$), 1.58 (m, 3H, CH$_3$), 4.22 (m, 1H, CH), 4.55-4.61 (m, 1H, NH), 4.93-5.03 (m, 1H, CH-*i*Pr), 7.45 (m, 1H, Ar-H), 7.52-7.61 (m, 3H, Ar-H), 7.69-7.72 (d, *J* = 8.5 Hz, 1H, Ar-H), 7.88-7.92 (d, *J* = 8Hz, 1H, Ar-H), 8.08-8.12 (t, 1H, Ar-H).

(2*S*)-*tert*-butyl-2-(chloro(naphthalen-1-yloxy)phosphorylamino)propanoate (86)
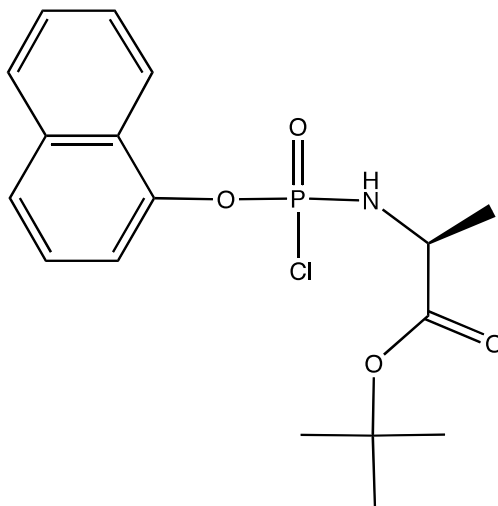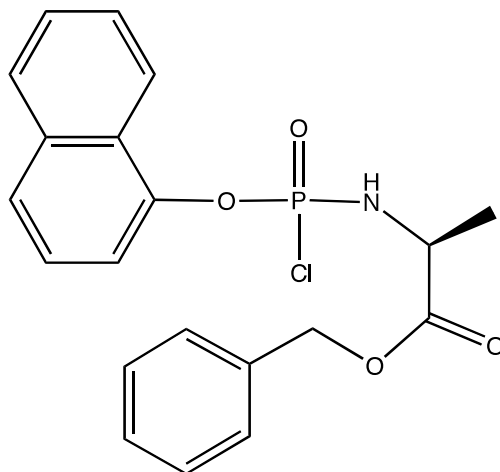

$C_{17}H_{21}ClNO_4P$, MW: 369.78 gmol^{-1}

The compound **86** was prepared according to *Procedure C*, from 1-naphthyl dichlorophosphate (**82**, 2.16 g, 8.26 mmol, 1 eq), L-alanine *tert*-butyl ester hydrochloride salt (1.50 g, 8.26 mmol, 1 eq), and anhydrous triethylamine (1.67 g, 16.51 mmol, 2.30 mL, 2 eq) in dry DCM (50 mL). The product was obtained as clear yellow oil (3.66 g, 60 %).

^{31}P-NMR (CDCl$_3$): δ 8.29, 8.56.

^{1}H-NMR (CDCl$_3$): δ 1.37 (m, 3H, CH$_3$), 1.45 (m, 9H, 3 x CH$_3$), 4.11 (m, 1H, CH), 4.55-4.61 (m, 1H, NH), 7.45 (m, 1H, Ar-H), 7.52-7.61 (m, 3H, Ar-H), 7.69-7.72 (d, *J* = 8.5 Hz, 1H, Ar-H), 7.88-7.92 (d, *J* = 8Hz, 1H, Ar-H), 8.08-8.12 (t, 1H, Ar-H).

(2*S*)-benzyl-2-(chloro(naphthalen-1-yloxy)phosphorylamino)propanoate (87)

$C_{20}H_{19}ClNO_4P$, MW: 403.80 gmol^{-1}

The compound **87** was prepared according to *Procedure C*, from 1-naphthyl dichlorophosphate (**82**, 2.42 g, 9.27 mmol, 1 eq), L-alanine benzyl ester hydrochloride salt (2.00 g, 9.27 mmol, 1 eq), and anhydrous triethylamine (1.88 g, 18.55 mmol, 2.60 mL, 2 eq) in dry DCM (50 mL). The product was obtained as clear yellow oil (4.12 g, 55 %).

^{31}P-NMR (CDCl$_3$): δ 8.37, 8.65.

^{1}H-NMR (CDCl$_3$): δ 1.56-1.60 (m, 3H, CH$_3$), 4.11-4.15 (m, 1H, CH), 4.65-4.72 (m, 1H, NH), 5.17-5.26 (m, 2H, Bn-CH$_2$), 7.45-8.15 (m, 12H, Ar-H).

3'-Fluoro-3'-deoxy-5'-*O*-[benzyl-(methoxy-alaninyl)-phosphate]thymidine (88)
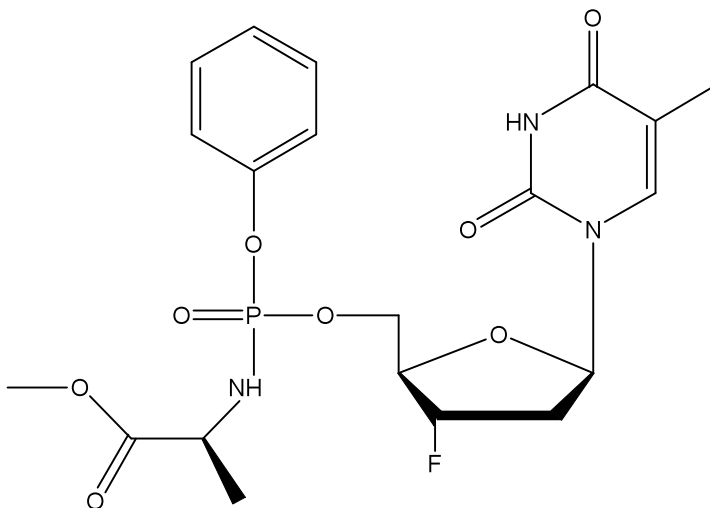
$C_{20}H_{25}FN_3O_8P$, MW: 485.40 gmol^{-1}

Prepared according to *Procedure D* from **10** (0.15g, 0.61 mmol, 1eq), ^{t}BuMgCl (1.0M, 0.11 g, 0.92 mmol, 0.12 mL, 1.5 eq) and **77** (0.34 g, 1.23 mmol, 2 eq) in anhydrous THF (15 mL). Column chromatography purification using DCM-MeOH (9.7:0.3) as the eluent afforded the title compound **88** (0.12 g, 20 %) as oil.

^{1}H-NMR (CDCl$_3$): δ 7.58 (d, *J* = 7 Hz, 1H, H-6), 7.35-7.43 (m, 2H, Ar-H), 7.20-7.29 (m, 3H, Ar-H), 6.25-6.32 (m, 1H, H-1'), 5.33 (m, 1H, H-3'), 4.31-4.52 (m, 2H, NH, H-4'), 3.95-4.03 (m, 1H, CH), 3.68 (s, 3H, OCH$_3$), 3.35 (d, *J* = 16 Hz, 2H, H-5', H-5"), 2.48-2.51 (m, 1H, H-2"), 2.09-2.25 (m, 1H, H-2'), 1.85 (d, *J* = 10 Hz, 3H, CH$_3$ -Thy), 1.31-1.42 (m, 3H, CH$_3$ -Ala).

¹³C-NMR (CDCl₃): δ 175.60 (C=O, Ala), 166.26 (C-4), 152.03-152.28 (C-2), 137.26, 137.27 (C-6, diastereoisomers), 121.38-130.90 (Ar-C), 112.05, 112.10 (C-5), 94.41-96.07 (C-4'), 86.55, 86.67 (C-1'), 67.22-67.54 (C-5'), 54.83 (OCH₃), 52.83 (CH), 51.66 (C-3'), 38.68-38.90 (C-2'), 20.25-20.50 (CH₃-Ala), 12.54, 12.58 (CH₃-Thy, diastereoisomers).
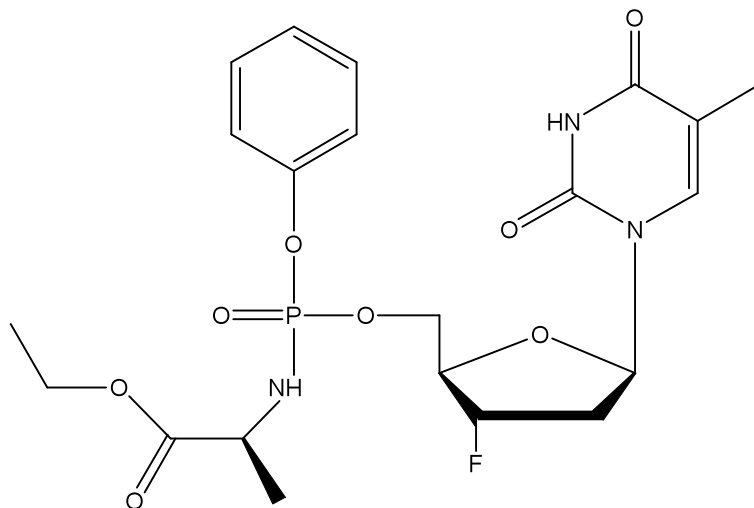
¹⁹F-NMR(CDCl₃): δ -174.94, -176.37.

³¹P-NMR (CDCl₃): δ 3.71, 3.99.

MS (ES-) *m/z:* 485.05

Reverse HPLC eluting with H₂O/MeOH from 90/10 to 0/100 in 25 min: t_R= 17.28 and 17.96 min (98%).

3'-Fluoro-3'-deoxy-5'-*O*-[benzyl-(ethyloxy-alaninyl)-phosphate]thymidine (89)

$C_{21}H_{27}FN_3O_8P$, MW: 499.43 gmol^{-1}

Prepared according to *Procedure D* from **10** (0.05g, 0.21 mmol, 1eq), ^{t}BuMgCl (1.0M, 0.41 mmol, 0.05 mL, 2 eq) and **78** (0.24 g, 0.82 mmol, 4 eq) in anhydrous THF (10 mL). Column chromatography purification using DCM-MeOH (9.7:0.3) as the eluent generated the final compound **89** (0.02 g, 10 %) as oil.

^{1}H-NMR (CDCl$_3$): δ 8.61 (br s, 1H, NH), 7.58 (s, 1H, H-6), 7.35-7.43 (m, 2H, Ar-H), 7.20-7.29 (m, 3H, Ar-H), 6.25-6.32 (m, 1H, H-1'), 5.33 (m, 1H, H-3'), 4.43-4.53 (m, 1H, NH, H-4'), 4.21 (m, 1H, CH), 3.95 (m, 2H, CH$_2$), 3.35-3.51 (m, 2H, H-5', H-5"), 2.48-2.51 (m, 1H, H-2"), 2.09-2.25 (m, 1H, H-2'), 1.51 (m, 3H, CH$_3$), 1.25 (m, 3H, CH$_3$).

¹³C-NMR (CDCl₃): δ 172.50 (C=O, Ala), 163.41 (C-4), 150.17 (C-2), 135.11, 135.07 (C-6, diastereoisomers), 130.90 – 121.38 (Ar-C), 111.49, 111.53 (C-5, diastereoisomers), 94.29-92.65 (C-3'), 85.11, 84.84 (C-1', diastereoisomers), 83.11, 82.93 (dd, J = 7.8 Hz, C-4', diastereoisomers), 77.27, 77.02 (C-5', diastereoisomers), 61.85, 61.82 (OEt, diastereoisomers), 50.46, 50.29 (CH Ala, diastereoisomers), 38.18, 37.86 (C-2', diastereoisomers), 21.10, 20.97 (CH₃-Ala), 14.10 (OEt), 12.48, 12.36(CH₃-Thy, diastereoisomers).
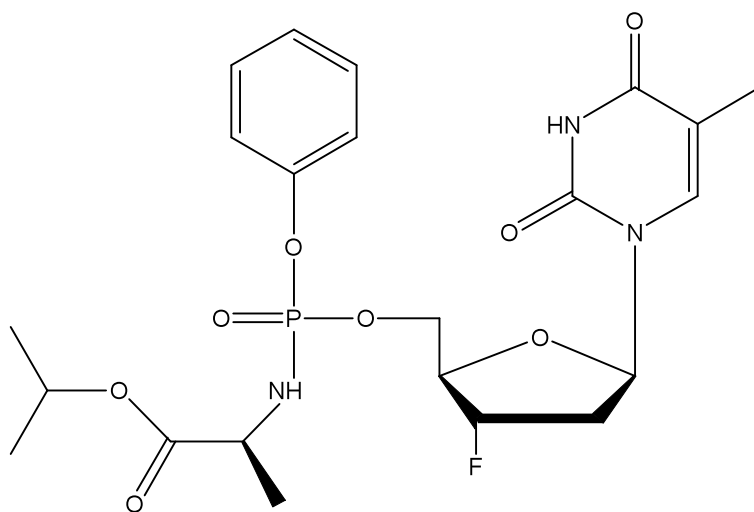
¹⁹F-NMR(CDCl₃): δ -174.88, -175.19.

³¹P-NMR (CDCl₃): δ 2.54, 2.79.

MS (ES-) *m/z:* 499.09

Reverse HPLC eluting with H₂O/MeOH from 90/10 to 0/100 in 25 min: t_R= 17.85 and 18.17 min (97%).

3'-Fluoro-3'-deoxy-5'-*O*-[benzyl-(isopropyloxy-alaninyl)-phosphate]thymidine (90)

$C_{22}H_{29}FN_3O_8P$, MW: 513.45 gmol^{-1}

Prepared according to *Procedure D* from **10** (0.07g, 0.29 mmol, 1eq), ^{t}BuMgCl (1.0M, 0.07g, 0.57 mmol, 0.08 mL, 2 eq) and **79** (0.35 g, 1.15 mmol, 4 eq) in anhydrous THF (10 mL). Column chromatography purification using DCM-MeOH (9.7:0.3) as the eluent generated the desired compound **90** (0.04 g, 13 %) as oil.

^{1}H-NMR (CDCl$_3$): δ 8.15 (br s, 1H, NH), 7.45 (s, 1H, H-6), 7.35-7.28 (m, 2H, Ar-H), 7.25-7.15 (m, 3H, Ar-H), 6.51-6.38 (m, 1H, H-1'), 5.35-5.17 (m, 1H, H-3'), 4.51 (m, 1H, CH *iPr),* 4.45-4.22 (m, 1H, NH, H-4'), 4.15 (m, 1H, CH Ala), 3.55-3.51 (m, 2H, H-

5', H-5"), 2.48-2.51 (m, 1H, H-2"), 2.65-2.49 (m, 1H, H-2'), 1.92 (s, 3H, CH$_3$ Thy), 1.51 (s, 3H, CH$_3$ Ala), 1.26 (s, 6H, 2xCH$_3$ *iPr*).

^{13}**C-NMR** (CDCl$_3$): δ 173.95 (C=O, Ala), 163.78 (C-4), 150.86 (C-2), 136.11, 136.12 (C-6, diastereoisomers), 131.90 –122.81 (Ar-C), 110.49, 110.23 (C-5, diastereoisomers), 94.29, 93.65 (d, *J*= 7.1 Hz, C-3', diastereoisomers), 85.12, 84.74 (C-1', diastereoisomers), 83.11, 82.93 (d, *J*= 7.5 Hz, C-4', diastereoisomers), 77.27, 77.02 (C-5', diastereoisomers), 69.85, 69.82 (CH *iPr*, diastereoisomers), 50.46, 49.29 (CH Ala, diastereoisomers), 38.18, 37.86 (C-2', diastereoisomers), 21.65, 21.91 (2x CH$_3$-*iPr*, diastereoisomers), 19.95, 20.13 (CH$_3$-Ala), 12.45, 12.32(CH$_3$-Thy, diastereoisomers).
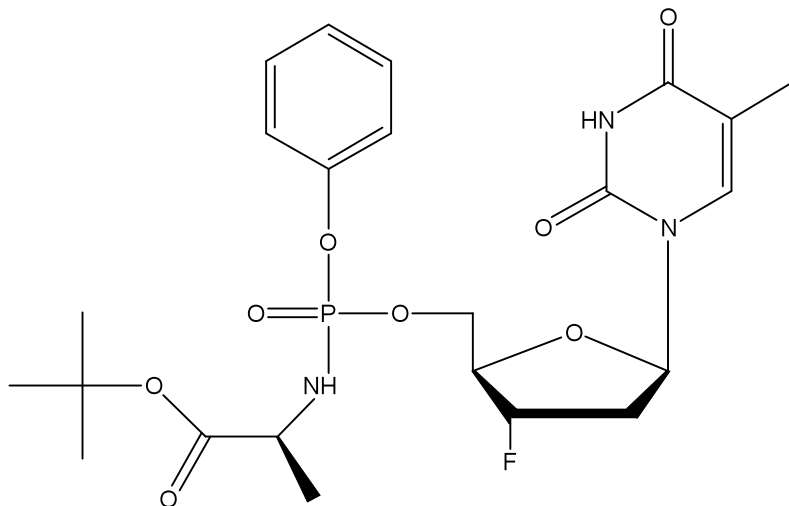
^{19}**F-NMR**(CDCl$_3$): δ -174.83, -175.18.

^{31}**P-NMR** (CDCl$_3$): δ 2.76, 3.55.

MS (ES-) *m/z:* 513.12

Reverse HPLC eluting with H$_2$O/MeOH from 90/10 to 0/100 in 25 min: t_R= 18.85 and 19.17 min (95%).

3'-Fluoro-3'-deoxy-5'-*O*-[benzyl-(tert-butyloxy-alaninyl)-phosphate]thymidine (91)

$C_{23}H_{31}FN_3O_8P$, MW: 527.48 gmol^{-1}

Prepared according to *Procedure D* from **10** (0.08g, 0.33 mmol, 1eq), ^tBuMgCl (1.0M, 0.08g, 0.66 mmol, 0.09 mL, 2 eq) and **80** (0.42 g, 1.31 mmol, 4 eq) in anhydrous THF (10 mL). Column chromatography purification using DCM-MeOH (9.7:0.3) as the eluent generated the title compound **91** (0.02 g, 5 %) as oil.

^{1}H-NMR (CDCl$_3$): δ 8.68 (br s, 1H, NH), 7.48 (s, 1H, H-6), 7.35-7.28 (m, 2H, Ar-H), 7.25-7.15 (m, 3H, Ar-H), 6.51-6.38 (m, 1H, H-1'), 5.35-5.17 (m, 1H, H-3'), 4.45-4.22 (m, 1H, NH, H-4'), 4.15 (m, 1H, CH Ala), 3.55-3.51 (m, 2H, H-5', H-5"), 2.48-2.51 (m, 1H, H-2"), 2.65-2.49 (m, 1H, H-2'), 1.92 (s, 3H, CH$_3$ Thy), 1.51 (d, *J*= 8.5Hz, 3H, CH$_3$ Ala), 1.46 (s, 9H, 3x CH$_3$ *tBu*).

¹³C-NMR (CDCl₃): δ 174.69 (C=O, Ala), 163.78 (C-4), 150.86 (C-2), 135.09, 135.02 (C-6, diastereoisomers), 131.90 –122.81 (Ar-C), 110.49, 110.23 (C-5, diastereoisomers), 94.29, 93.65 (d, *J=* 7.1 Hz, C-3', diastereoisomers), 85.12, 84.74 (C-1', diastereoisomers), 83.11, 82.93 (d, *J=* 7.5 Hz, C-4', diastereoisomers), 77.27, 77.02 (C-5', diastereoisomers), 73.86, 73.02 (C-*tBu*, diastereoisomers), 50.46, 49.29 (CH Ala, diastereoisomers), 38.18, 37.86 (C-2', diastereoisomers), 27.91, 27.05 (3 x CH₃-*tBu*, diastereoisomers*)* 21.22, 21.08 (CH₃-Ala), 12.51, 12.36(CH₃-Thy, diastereoisomers).

¹⁹F-NMR(CDCl₃): δ -175.06, -175.15.

³¹P-NMR (CDCl₃): δ 3.82, 2.93.

MS (ES-) *m/z:* 527.15

Reverse HPLC eluting with H₂O/MeOH from 90/10 to 0/100 in 25 min: t_R= 19.55 and 19.97 min (97%).

3'-Fluoro-3'-deoxy-5'-*O*-[benzyl-(benzoxy-alaninyl)-phosphate]thymidine (92)
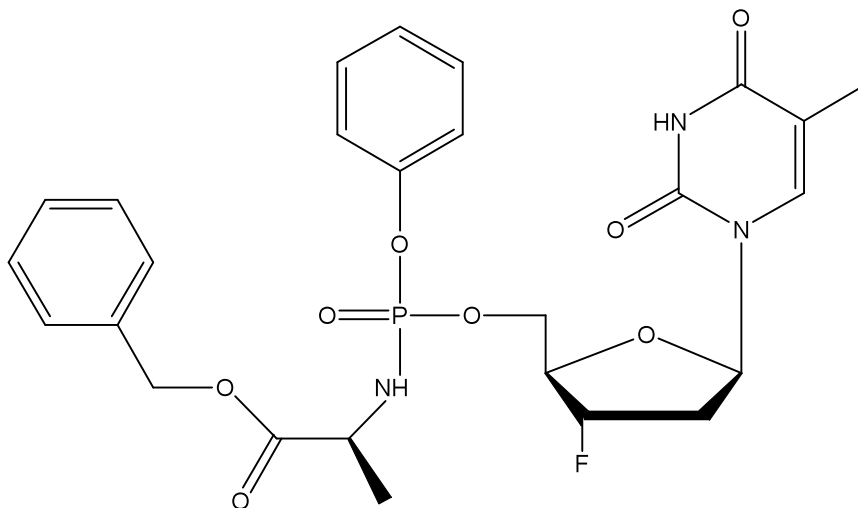
$C_{26}H_{29}FN_3O_8P$, MW: 561.50 gmol^{-1}

Prepared according to *Procedure D* from **10** (0.07g, 0.33 mmol, 1eq), ^{t}BuMgCl (1.0M, 0.49 mmol, 0.06 mL, 1.5 eq) and **81** (0.24 g, 0.66 mmol, 2 eq) in anhydrous THF (10 mL). Column chromatography purification using DCM-MeOH (9.7:0.3) as the eluent generated the title compound **92** (0.03 g, 7 %) as oil.

^{1}**H-NMR** (CDCl$_3$): δ 8.62 (br s, 1H, NH), 7.49-7.38 (m, 6H, Ar-H + H-6), 7.35-7.28 (m, 2H, Ar-H), 7.25-7.15 (m, 3H, Ar-H), 6.38-6.25 (m, 1H, H-1'), 5.35-5.17 (m, 3H,CH$_2$Ph + H-3'), 4.45-4.22 (m, 2H, NH + H-4'), 4.15 (m, 1H, CH Ala), 3.55-3.51 (m, 2H, H-5', H-5"), 2.48-2.51 (m, 1H, H-2"), 2.65-2.49 (m, 1H, H-2'), 1.92 (s, 3H, CH$_3$ Thy), 1.51 (d, *J*= 8.5Hz, 3H, CH$_3$ Ala).

13C-NMR (CDCl₃): δ 173.77 (C=O, Ala), 163.78, 163.56 (C-4,diastereoisomers), 150.86 (C-2), 135.09, 135.02 (C-6, diastereoisomers), 131.90 −122.81 (Ar-C), 110.49, 110.23 (C-5, diastereoisomers), 94.29, 93.65 (d, J= 7.1 Hz, C-3', diastereoisomers), 85.12, 84.74 (C-1', diastereoisomers), 83.11, 82.93 (d, J= 7.5 Hz, C-4', diastereoisomers), 77.27, 77.02 (C-5', diastereoisomers), 72.2 (CH₂Ph), 50.46, 49.29 (CH Ala, diastereoisomers), 38.18, 37.86 (C-2', diastereoisomers), 21.22, 21.08 (CH₃-Ala, diastereoisomers), 12.51, 12.36(CH₃-Thy, diastereoisomers).
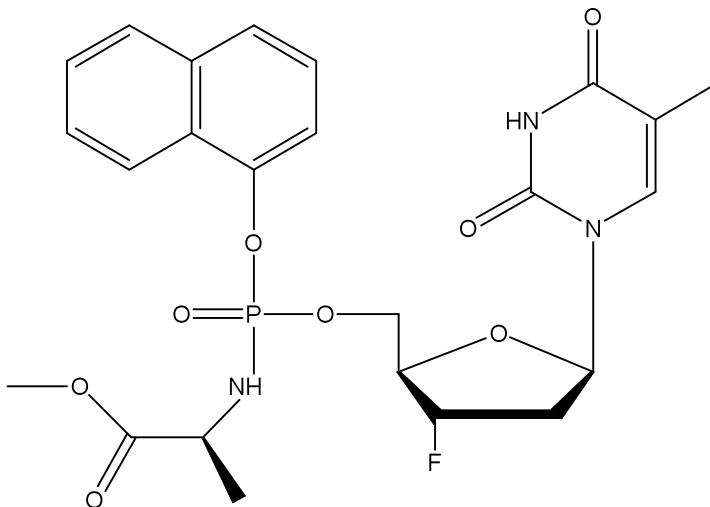
19F-NMR(CDCl₃): δ -174.86, -175.01.

31P-NMR (CDCl₃): δ 3.52, 2.70.

MS (ES-) *m/z:* 561.16

Reverse HPLC eluting with H₂O/MeOH from 90/10 to 0/100 in 25 min: t$_R$= 20.64 and 21.42 min (96%).

3'-Fluoro-3'-deoxy-5'-*O*-[naphthyl-(methoxy-alaninyl)-phosphate]thymidine (93)

$C_{24}H_{27}FN_3O_8P$, MW: 535.46 gmol^{-1}

Prepared according to *Procedure D* from **10** (0.15g, 0.61 mmol, 1eq), ^{t}BuMgCl (1.0M, 0.11 g, 0.92 mmol, 0.12 mL, 1.5 eq) and **83** (0.34 g, 1.02 mmol, 2.5 eq) in anhydrous THF (15 mL). Column chromatography purification using DCM-MeOH (9.7:0.3) as the eluent afforded the title compound **93** (0.18 g, 47 %) as white foam.

^{1}H-NMR (CDCl$_3$): δ 8.68 (br s, 1H, NH), 8.17-8.14 (m, 1H, Ar-H), 7.89-7.85 (m, 1H, Ar-H), 7.71-7.67 (t, *J*= 8.5 Hz, 1H, Ar-H), 7.58-7.51 (m, 3H, Ar-H + H-6), 7.39-7.25 (m, 2H, Ar-H), 6.25-6.32 (tt, 1H, H-1'), 5.33-5.05 (m, 1H, H-3'), 4.31-4.52 (m, 2H, NH, H-4'), 3.95-4.03 (m, 1H, CH), 3.68 (s, 3H, OCH$_3$), 3.35 (d, *J* = 16 Hz, 2H, H-5', H-5''),

2.48-2.51 (m, 1H, H-2"), 2.09-2.25 (m, 1H, H-2'), 1.85 (d, J = 10 Hz, 3H, CH$_3$ -Thy), 1.45-1.32 (m, 3H, CH$_3$ -Ala).

^{13}**C-NMR** (CDCl$_3$): δ 175.60 (C=O, Ala), 166.26 (C-4), 152.03-152.28 (C-2), 137.26, 137.27 (C-6, diastereoisomers), 130.90-126.54 (Ar-C), 125.95-115.67 (Ar-C), 112.05, 112.10 (C-5), 94.41-96.07 (C-4'), 86.55, 86.67 (C-1'), 67.22-67.54 (C-5'), 51.33, 50.99 (OCH$_3$, diastereoisomers), 52.83 (CH), 51.66 (C-3'), 38.68-38.90 (C-2'), 20.25-20.50 (CH$_3$-Ala), 12.54, 12.58 (CH$_3$-Thy, diastereoisomers).
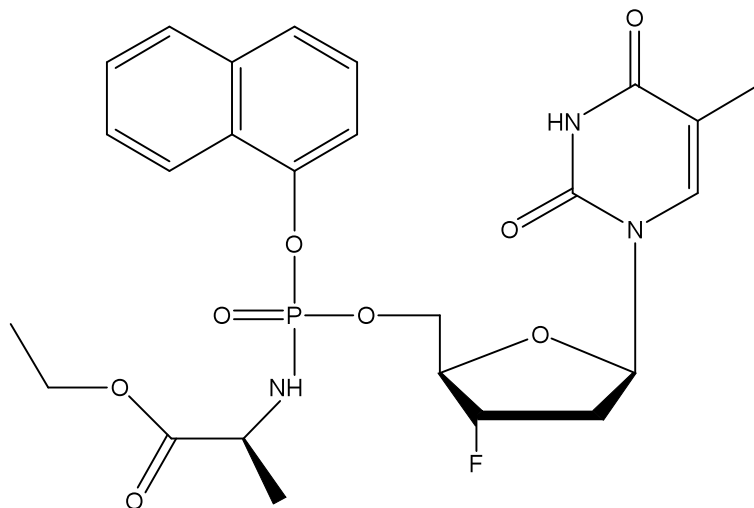
^{19}**F-NMR**(CDCl$_3$): δ -174.92, -175.22.

^{31}**P-NMR** (CDCl$_3$): δ 2.95, 3.10.

MS (ES-) *m/z:* 535.12

Reverse HPLC eluting with H$_2$O/MeOH from 90/10 to 0/100 in 25 min: t_R= 22.14 and 22.42 min (98%).

3'-Fluoro-3'-deoxy-5'-*O*-[naphthyl-(ethoxy-alaninyl)-phosphate]thymidine (94)

$C_{25}H_{29}FN_3O_8P$, MW: 549.49 gmol^{-1}

Prepared according to *Procedure D* from **10** (0.10g, 0.42 mmol, 1eq), ^{t}BuMgCl (1.0M, 0.62 mmol, 0.08 mL, 1.5 eq) and **84** (0.35 g, 1.02 mmol, 2.5 eq) in anhydrous THF (10 mL). Column chromatography purification using DCM-MeOH (9.7:0.3) as the eluent generated the final compound **94** (0.15 g, 50 %) as white foam.

^{1}H-NMR (CDCl$_3$): δ 8.68 (br s, 1H, NH), 8.17-8.14 (m, 1H, Ar-H), 7.89-7.85 (m, 1H, Ar-H), 7.71-7.67 (t, *J*= 8.5 Hz, 1H, Ar-H), 7.58-7.51 (m, 3H, Ar-H + H-6), 7.39-7.25 (m, 2H, Ar-H), 6.25-6.32 (tt, 1H, H-1'), 5.33 (m, 1H, H-3'), 4.43-4.53 (m, 1H, NH, H-4'), 4.21 (m, 1H, CH), 3.95 (m, 2H, CH$_2$), 3.35-3.51 (m, 2H, H-5', H-5"), 2.48-2.51 (m, 1H, H-2"), 2.09-2.25 (m, 1H, H-2'), 1.51 (m, 3H, CH$_3$), 1.25 (m, 3H, CH$_3$).

¹³C-NMR (CDCl$_3$): δ 175.60 (C=O, Ala), 166.26 (C-4), 152.03-152.28 (C-2), 137.26, 137.27 (C-6, diastereoisomers), 130.90-126.54 (Ar-C), 125.95-115.67 (Ar-C), 111.49, 111.53 (C-5, diastereoisomers), 94.29-92.65 (C-3'), 85.11, 84.84 (C-1', diastereoisomers), 83.11, 82.93 (dd, *J*= 7.8 Hz, C-4', diastereoisomers), 77.27, 77.02 (C-5', diastereoisomers), 61.85, 61.82 (OEt, diastereoisomers), 50.46, 50.29 (CH Ala, diastereoisomers), 38.18, 37.86 (C-2', diastereoisomers), 21.10, 20.97 (CH$_3$-Ala), 14.10 (OEt), 12.48, 12.36(CH$_3$-Thy, diastereoisomers).

¹⁹F-NMR(CDCl$_3$): δ -174.93, -175.23.
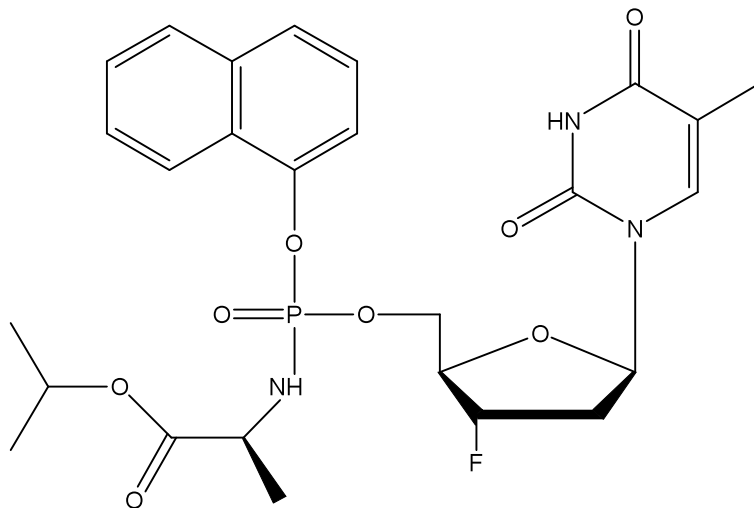
³¹P-NMR (CDCl$_3$): δ 3.05, 3.17.

MS (ES-) *m/z:* 549.16

Reverse HPLC eluting with H$_2$O/MeOH from 90/10 to 0/100 in 25 min: t$_R$= 22.24 and 22.65 min (98%).

3'-Fluoro-3'-deoxy-5'-*O*-[naphthyl-(isopropoxy-alaninyl)-phosphate]thymidine

(95)

$C_{26}H_{31}FN_3O_8P$, MW: 563.51 gmol^{-1}

Prepared according to *Procedure D* from **10** (0.10g, 0.42 mmol, 1eq), ^{t}BuMgCl (1.0M, 0.61 mmol, 0.08 mL, 1.5 eq) and **85** (0.35 g, 1.15 mmol, 2.3 eq) in anhydrous THF (10 mL). Column chromatography purification using DCM-MeOH (9.7:0.3) as the eluent generated the desired compound **95** (0.04 g, 22 %) as oil.

^{1}H-NMR (CDCl$_3$): δ 8.68 (br s, 1H, NH), 8.17-8.14 (m, 1H, Ar-H), 7.89-7.85 (m, 1H, Ar-H), 7.71-7.67 (t, *J*= 8.5 Hz, 1H, Ar-H), 7.58-7.51 (m, 3H, Ar-H + H-6), 7.39-7.25 (m, 2H, Ar-H), 6.25-6.32 (tt, 1H, H-1'), 5.35-5.17 (m, 1H, H-3'), 4.51 (m, 1H, CH *iPr),* 4.45-4.22 (m, 1H, NH, H-4'), 4.15 (m, 1H, CH Ala), 3.55-3.51 (m, 2H, H-5', H-5"),

2.48-2.51 (m, 1H, H-2"), 2.65-2.49 (m, 1H, H-2'), 1.92 (s, 3H, CH$_3$ Thy), 1.51 (s, 3H, CH$_3$ Ala), 1.26 (s, 6H, 2xCH$_3$ *iPr*).

^{13}C-NMR (CDCl$_3$): δ 175.60 (C=O, Ala), 166.26 (C-4), 152.03-152.28 (C-2), 137.26, 137.27 (C-6, diastereoisomers), 130.90-126.54 (Ar-C), 125.95-115.67 (Ar-C), 110.49, 110.23 (C-5, diastereoisomers), 94.29, 93.65 (d, *J*= 7.1 Hz, C-3', diastereoisomers), 85.12, 84.74 (C-1', diastereoisomers), 83.11, 82.93 (d, *J*= 7.5 Hz, C-4', diastereoisomers), 77.27, 77.02 (C-5', diastereoisomers), 69.85, 69.82 (CH *iPr*, diastereoisomers), 50.46, 49.29 (CH Ala, diastereoisomers), 38.18, 37.86 (C-2', diastereoisomers), 21.65, 21.91 (2x CH$_3$-*iPr*, diastereoisomers), 19.95, 20.13 (CH$_3$-Ala), 12.45, 12.32(CH$_3$-Thy, diastereoisomers).

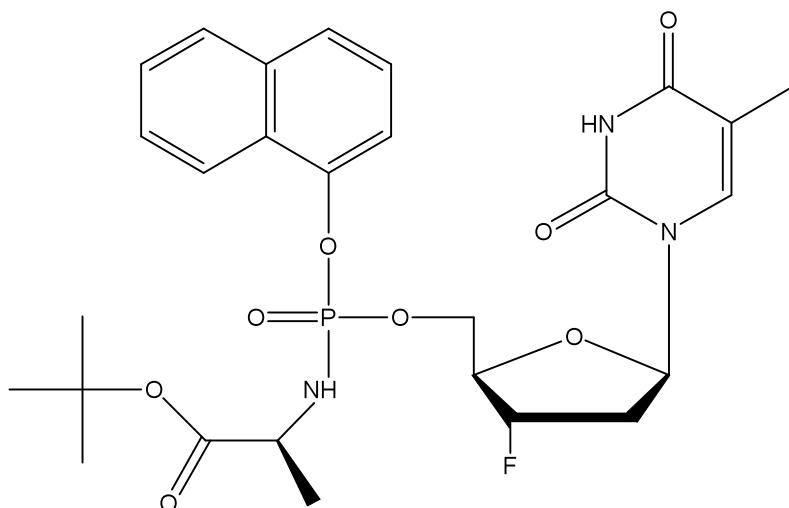**^{19}F-NMR**(CDCl$_3$): δ -174.90, -175.19.

^{31}P-NMR (CDCl$_3$): δ 2.98, 3.14.

MS (ES-) *m/z:* 563.65

Reverse HPLC eluting with H$_2$O/MeOH from 90/10 to 0/100 in 25 min: t$_R$= 22.29 and 22.78 min (97%).

3'-Fluoro-3'-deoxy-5'-*O*-[naphthyl-(tert-butoxy-alaninyl)-phosphate]thymidine

(96)

$C_{27}H_{33}FN_3O_8P$, MW: 577.54 gmol^{-1}

Prepared according to *Procedure D* from **10** (0.10g, 0.42 mmol, 1eq), ^{t}BuMgCl (1.0M, 0.62 mmol, 0.08 mL, 1.5 eq) and **86** (0.30 g, 0.82 mmol, 2 eq) in anhydrous THF (10 mL). Column chromatography purification using DCM-MeOH (9.7:0.3) as the eluent generated the title compound **96** (0.08 g, 36 %) as oil.

^{1}H-NMR (CDCl$_3$): δ 8.68 (br s, 1H, NH), 8.17-8.14 (m, 1H, Ar-H), 7.89-7.85 (m, 1H, Ar-H), 7.71-7.67 (t, *J*= 8.5 Hz, 1H, Ar-H), 7.58-7.51 (m, 3H, Ar-H + H-6), 7.39-7.25 (m, 2H, Ar-H), 6.25-6.32 (tt, 1H, H-1'), 5.35-5.17 (m, 1H, H-3'), 4.45-4.22 (m, 1H, NH, H-4'), 4.15 (m, 1H, CH Ala), 3.55-3.51 (m, 2H, H-5', H-5"), 2.48-2.51 (m, 1H, H-2"),

2.65-2.49 (m, 1H, H-2'), 1.92 (s, 3H, CH$_3$ Thy), 1.51 (d, $J=$ 8.5Hz, 3H, CH$_3$ Ala), 1.46 (s, 9H, 3x CH$_3$ *tBu*).

^{13}C-NMR (CDCl$_3$): δ 174.70 (C=O, Ala), 166.26 (C-4), 152.03-152.28 (C-2), 137.26, 137.27 (C-6, diastereoisomers), 130.90-126.54 (Ar-C), 125.95-115.67 (Ar-C), 110.49, 110.23 (C-5, diastereoisomers), 94.29, 93.65 (d, $J=$ 7.1 Hz, C-3', diastereoisomers), 85.12, 84.74 (C-1', diastereoisomers), 83.11, 82.93 (d, $J=$ 7.5 Hz, C-4', diastereoisomers), 77.27, 77.02 (C-5', diastereoisomers), 73.86, 73.02 (C-*tBu*, diastereoisomers), 50.46, 49.29 (CH Ala, diastereoisomers), 38.18, 37.86 (C-2', diastereoisomers), 27.91, 27.05 (3 x CH$_3$-*tBu*, diastereoisomers*)* 21.22, 21.08 (CH$_3$-Ala), 12.51, 12.36(CH$_3$-Thy, diastereoisomers).

^{19}F-NMR(CDCl$_3$): δ -174.85, -175.13.

^{31}P-NMR (CDCl$_3$): δ 3.12, 3.26.

MS (ES-) *m/z:* 577.22

Reverse HPLC eluting with H$_2$O/MeOH from 90/10 to 0/100 in 25 min: t$_R=$ 23.29 and 23.78 min (99%).

References:

1. Hansen, A.S.; Thalhammer, A.; El-Sagheer, A.H.; Brown, T.; Schofield, C.J. Improved synthesis of 5-hydroxymethyl-2'-deoxycytidine phosphoramidite using a 2'-deoxyuridine to 2'-deoxycytidine conversion without temporary protecting groups. *Bioorg Med Chem Lett.* **2011**, *21*, 1181-1184.

2. Gaynor, J.W.; Bentley, J.; Cosstick, R. Synthesis of the 3'-thio-nucleosides and subsequent automated synthesis of oligodeoxynucleotides containing a 3-S-phosphorothiolate linkage. *Nature Protocols.* **2007**, *2*, 3122-3135.

3. Yun, M.; Oh, S.J.; Ha, H.; Ryu, J.; Moon, D. High radiochemical yield synthesis of 3'-deoxy-3'-[18F]fluorothymidine using (5'-*O*-dimethoxytrityl-2'-deoxy-3'-*O*-nosyl-β-D-threo-pentofuranosyl)thymine and its 3-N-BOC-protected analog as a labeling precursor. *Nucl Med Biol.,* **2003**, *30*, 151-157.

4. Krizmanic, I.; Visnjevac, A.; Luic, M.; Glavas-Obrovac, L.; Zinic, M.; Zinic, B. Synthesis, structure and biological evaluation of C-2 sulfonamido pyrimidine nucleosides. *Tetrahedron*, **2003**, *59*, 4047-4057.

5. Shakya, N.; Srivastav, N.C.; Desroches, N.; Agrawal, B.; Kunimoto, D.Y.; Kumar, R. 3'-Bromo analogues of pyrimidine nucleosides as a new class of potent inhibitors of mycobacterium tuberculosis. *J Med Chem.,* **2010**, *53*, 4130-4140.

6. Cosford, N.D.P.; Schinazi, R.F. Selenium nucleophiles for the preparation of antiviral nucleosides. *J Org Chem.,* **1991**, *56,* 2161-2165.

7. Agarwal, H. K.; Parang, K. Application of solid-phase chemistry for the synthesis of 3'-fluoro-3'-deoxythymidine. *Nucleosides, Nucleotides and Nucleic Acids,* **2007**, *26*, 317-322.

8. Eisenhut, M. *et al.* A new precursor for the radio-synthesis of [18F]FLT. *Nucl Med Biol.,* **2002**, *29*, 263-273.

9. Radigois, M. 3'-deoxy-3'-[¹⁸F]fluorothymidine (FLT) production-standard operating procedure. Personal communication. Wolfson Molecular Imaging Centre group protocol. The University of Manchester.

10. Czernecki, S.; Valery, J.M. One-step conversion of thymidine into 2,3'-anhydro derivatives. *J Chem Soc.,* **1990**, *11*, 801-802.