UNIVERSITY OF EDINBURGH

College of Science and Engineering
School of Chemistry

A supported phosphorylation reagent for the synthesis of triphosphates and prodrugs

By

Carole Brückler

Doctor of Philosophy

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ABSTRACT

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A solid-supported phosphorylation reagent was developed, based on Ludwig-Eckstein chemistry. Different resin supports were evaluated and microporous polystyrene with 1 % crosslinking was found to give the best results. The supported reagent was characterised by ^{31}P Gel-phase NMR and phosphorus content and applied to the synthesis of DNA triphosphates. The triphosphates were isolated in 28-50 % yields, often without the need for ion exchange chromatography, using preparative HPLC for purification. Propargylamino linked triphosphates were also prepared using this approach.

The reagent was successfully applied to the synthesis of a fluorescein labelled thymidine triphosphate. Using the supported reagent, complete phosphitylation of the alcohol could be demonstrated. The product was isolated by preparative HPLC and its identity proven by ^{31}P NMR and mass spectrometry.

Additionally, the reagent's potential as a handle to access different phosphorylated compounds, such as mono-*H*-phosphonate esters, phosphonate diesters, phosphate triesters and diphosphoramidates was explored and its application to the synthesis of nucleoside prodrugs is discussed.

Declaration

The research described in this thesis was carried out by the author under the supervision of Prof. Mark Bradley at the University of Southampton between October 2002 and December 2004 and at the University of Edinburgh between January 2005 and August 2006. No part of this thesis has been previously submitted at this or any other university for any other degree or a professional qualification. Part of this work has been protected under UK patent 0606038.8.

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And finally thanks to my family for the support and to Richard for making this years so infinitely more pleasant.

Abbreviations

Å	Angstrom
A	adenine, adenosine
Ac	acetyl
Ahx	6-amino hexanoic acid
AIDS	Acquired immunodeficiency syndrome
ATP	adenosine triphosphate
AZT	3'-azido-3'-deoxy-thymidine
AZTMP	3'-azido-3'-deoxy-thymidine monophosphate
aq	aqueous
Bn	benzyl
bp	boiling point
br	broad
Bu	butyl
^{i}Bu	iso-butyryl
^{t}Bu	tert-butyl
Bz	benzoyl
°C	degrees Celsius
C	cytosine, cytidine
calcd	calculated
CPG	Controlled Pore Glass
Cy	cyclohexyl
δ	chemical shift in ppm downfield from tetramethylsilane
d	doublet, days
d4T	2',3'-dideoxy-2',3'-didehydrothymidine
dATP	deoxyadenosine triphosphate
DBU	1,8-diazabicyclo(5.4.0)undec-7-ene
dCTP	deoxycytidine triphosphate
DCC	dicyclohexylcarbodiimide
DEAE	diethylaminoethyl
ddATP	dideoxyadenosine triphosphate
ddNTP	dideoxynucleoside triphosphate
dGTP	deoxyguanosine triphosphate
DIAD	diisopropyl azodicarboxylate
DIPEA	N,N-diisopropyl-N-ethylamine
DIC	diisopropylcarbodiimide
DMAP	4-(dimethylamino)-pyridine
DMF	dimethylformamide
Dmt	4,4'-dimethoxytrityl
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate

dsDNA	double-stranded DNA
dTTP	deoxythymidine triphosphate
ELSD	evaporative light scattering detector
eq	equivalent
ES	electrospray mass spectrometry
Et	ethyl
fDNA	functionalised DNA
FISH	Fluorescene *in situ* hybridisation
Fmoc	9-fluorenylmethoxycarbonyl
g	grams
G	guanine, guanosine
h	hours
HIV	Human Immunodeficiency Virus
HOBt	1-hydroxybenzotriazole
HRMS	high resolution mass spectrometry
Hz	Hertz
iC	5-methylisocytidine
ICP	Inductively coupled plasma
iG	isoguanosine
^{i}Pr	isopropyl
IPA	isopropyl alcohol
IR	infrared
J	coupling constant
L	litres
λ	wavelength
m	multiplet, metres
M	moles *per* litre
MALDI-MS	Matrix-assisted laser desorption ionisation mass spectrometry
MAS	magic angle spinning
Me	methyl
min	minutes
mol	moles
mp	melting point
mRNA	messenger ribonucleic acid
MS	mass spectrometry
mw	molecular weight
m/z	mass to charge ratio
n.d.	not determined
NDP	nucleoside diphosphate
NHS	N-hydroxy succinimide
NNRTI	non-nucleoside reverse transcriptase inhibitor
NMP	nucleoside monophosphate
NMR	nuclear magnetic resonance
NRTI	nucleoside reverse transcriptase inhibitor

NTP	nucleoside triphosphate
NOESY	nuclear Overhauser effect spectroscopy
Nuc	nucleoside
PCR	Polymerase Chain Reaction
PEG	polyethyleneglycol
Ph	phenyl
Piv	pivaloyl
ppm	parts *per* million
Pr	propyl
Proton sponge	1,8-bis(dimethylamino)-naphthalene
PS	polystyrene
PS-PEG	polystyrene-polyethyleneglycol copolymer
q	quartet
R_f	retention factor (movement of compound/solvent front)
RNA	ribonucleic acid
RP-HPLC	reverse-phase high performance liquid chromatography
rRNA	ribosomal ribonucleic acid
rt	room temperature
RT	reverse transcriptase
s	singlet, seconds
SBS	sequencing by synthesis
SELEX	systematic evolution of ligands by an exponential enrichment
SM	starting material
SNP	single nucleotide polymorphism
ssDNA	single-stranded DNA
t	triplet
T	thymine, thymidine
TBA	tetrabutyl ammonium
TCA	trichloroacetic acid
TEA	triethylamine
TEAB	triethylammonium bicarbonate
Tfa	trifluoroacetate
TFA	trifluoroacetic acid
THF	tetrahydrofuran
tlc	thin layer chromatography
t_R	elution time of compound on HPLC
tRNA	transfer ribonucleic acid
U	uracil, uridine
UV	ultraviolet

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1 Introduction

1.1 Nucleic acids

1.1.1 Introduction

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) belong to the group of biopolymers referred to as nucleic acids. DNA, located in the cell nucleus, is the permanent storage medium for genetic information, while various types of RNA are involved in the production of proteins. The DNA code is transcribed to messenger RNA (mRNA). Protein assembly is carried out by transfer RNAs (tRNA) and ribosomal RNA (rRNA) using mRNA as template.

1.1.2 General structure of a nucleotide

A nucleoside consists of a ribose sugar and a base. When phosphorylated, it is referred to as a nucleotide, the general structure of which is shown below (Figure 1.1). The accepted nomenclature for the ribose positions and the phosphate groups are illustrated below; the nomenclature for the bases is shown later (Figure 1.2).

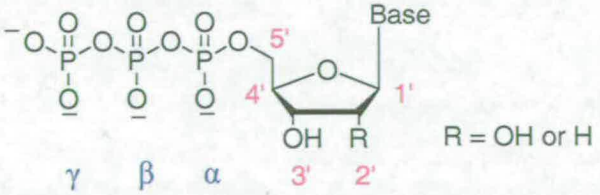


Figure 1.1 Structure of an RNA nucleotide

The major structural difference between RNA and DNA is located on the ribose sugar. RNA nucleosides have hydroxyl groups on the 2' and 3' positions, whereas DNA bases are 2' deoxygenated. The lack of the second hydroxyl group renders DNA more stable to backbone cleavage under basic conditions. If both 2' and 3' are deoxygenated, they are referred to as dideoxynucleotides, which have found applications in DNA sequencing.[1]

Bases are classed into two groups of heterocycles: the pyrimidines, cytosine (**C**), uracil (**U**) and thymine (**T**), and the purines, which are pyrimidines fused to an imidazole, adenine (**A**) and guanine (**G**) (Figure 1.2). Here another difference is

observed between RNA and DNA: RNA incorporates uracil, whereas DNA uses thymine, which differs from it by a methyl substitution at C-5.

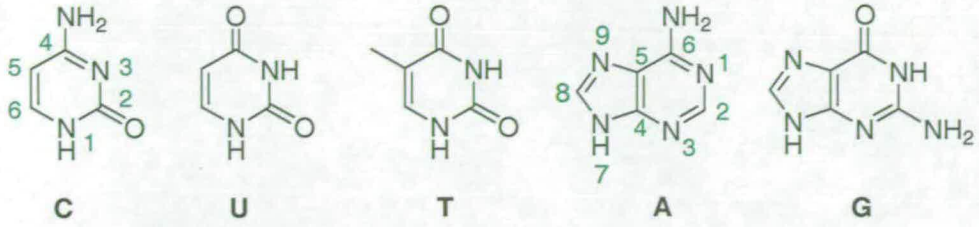


Figure 1.2 Structures of bases

The 5' and 3' positions of the nucleotide can carry phosphate groups. The free hydroxyl group is joined to phosphate groups through successive kinase catalysed condensations. The first phosphate to be condensed is the α phosphate, subsequent ones are referred to as β and γ respectively. Mono-, di- and triphosphates play roles in various metabolic pathways. It is however the triphosphates which are of particular interest in this thesis, as the triphosphates are required for DNA polymerases in the synthesis of DNA.

1.1.3 Watson-Crick base pairing

The 3D structure of DNA remained elusive until 1953, when Watson and Crick solved its structure based on X-ray pictures of hydrated DNA. They correctly proposed that it consists of two intertwining strands which form a double helix.[2] The Watson-Crick model states that a purine base of one strand pairs with a pyrimidine base of the other strand. The complementary base pairs are GC and AT. Thymine forms two hydrogen bonds to adenine and cytosine forms three hydrogen bonds to guanine (Figure 1.3).

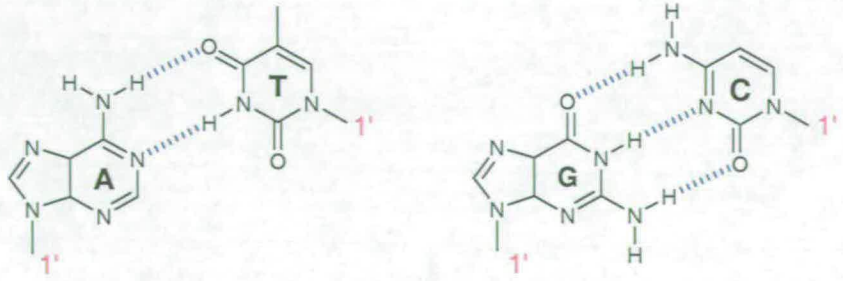


Figure 1.3 Hydrogen-bonding in the DNA base pairs

1.2 Triphosphates

Synthetic triphosphates have been coveted molecules ever since their potential for therapeutic and diagnostic applications was recognized and of particular importance are labelled dideoxynucleotide triphosphates (ddNTPs) as chain terminators for DNA sequencing.[1,3] Triphosphates of deoxynucleotides (dNTPs) are required for enzymatic DNA synthesis,[4,5] as they are essential building blocks for different biochemical applications, such as the Polymerase Chain Reaction (PCR)[6] or fluorescence *in situ* hybridisation (FISH).[7] This section describes the different applications and the varied modifications that are used on the triphosphates.

1.2.1 Enzymatic DNA synthesis

1.2.1.1 *Introduction*

DNA is synthesised enzymatically by providing a DNA polymerase with a template and all four dNTPs in a suitable buffer and at a suitable temperature. The template for DNA synthesis is annealed to a short, specific primer of usually 15-30 nucleotides, complementary to the sequence on the template (Figure 1.4).

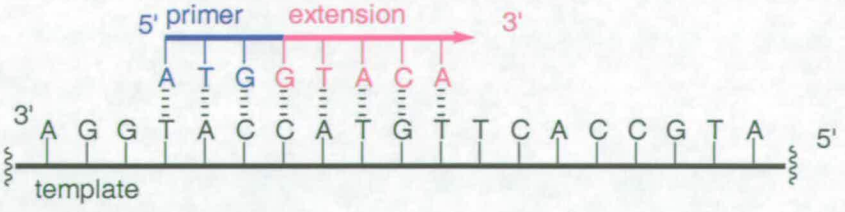


Figure 1.4 Template-dependent DNA synthesis

If the template provided is DNA based, the enzyme is referred to as a DNA polymerase, if it is RNA based, as a reverse transcriptase. All polymerases synthesise DNA in the 5'→3' direction, which means that incoming nucleotides are added to the 3' end of the previous nucleotide (Figure 1.4).

In living cells, DNA polymerases also fulfil other functions, thus they proof-read and repair cellular DNA and correct errors during the replication process. Enzymes for use in the synthesis of modified DNA often have such exonuclease activity suppressed. Additionally, they should be able to accept synthetic triphosphates, not

dissociate from the strand at an appreciable rate and ideally be active at raised temperatures which disrupt secondary structures in the DNA template. Naturally occurring DNA polymerases have been modified accordingly through chemical modifications or genetic engineering.[8]

Either full or partial substitution of a dNTP is possible during enzymatic synthesis. Substituted dNTPs can have modifications on the base, the sugar ring or the triphosphate group. Replacement of more than one dNTP with a synthetic analogue during the enzymatic synthesis is also feasible. A few pertinent examples with applications are discussed below.

1.2.1.2 *Fluorescent labelling of DNA*

Labelling of DNA has been achieved by treating DNA with a nuclease which creates random nicks (cuts) in both DNA stands. DNA strands can be labelled by partially substituting dTTP, with a labelled analogue[4,5] in an extension reaction, resulting in a tagged sequence (Figure 1.5). This is referred to as nick translation.

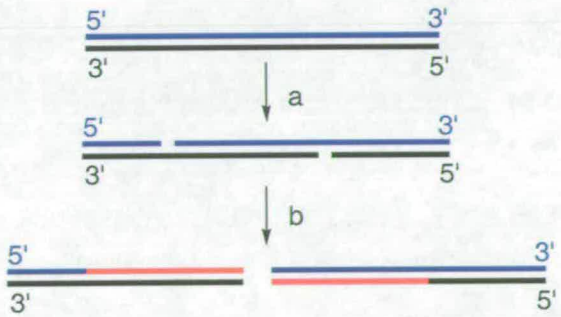


Figure 1.5 Nick translation: (a) Creation of nicks, (b) extension in the presence of a labelled dNTP

The resulting DNA segments can be used in fluorescent *in situ* hybridisation (FISH).[7] Prior to the development of fluorescent labels, radio-isotopes were used, but due to safety concerns and it not being possible to perform dual labelling, these have fallen out of favour.

An alternative labelling approach involves indirect probes based on hapten molecules, which are detected in a separate step through a fluorogenically labelled secondary molecule, such as an antibody. Another example is the incorporation of biotin into DNA, which is visualised using labelled streptavidin.

FISH is a highly sensitive technique used to detect and localise DNA sequences on chromosomes. The applications include mapping of sequences to specific positions on a chromosome, identification of chromosomal abnormalities and chromosome painting. Chromosome painting is achieved by using a multitude of small probes and with one colour specific to each chromosome, this technique gives rise to an easily assigned spectral karyotype (Figure 1.6).

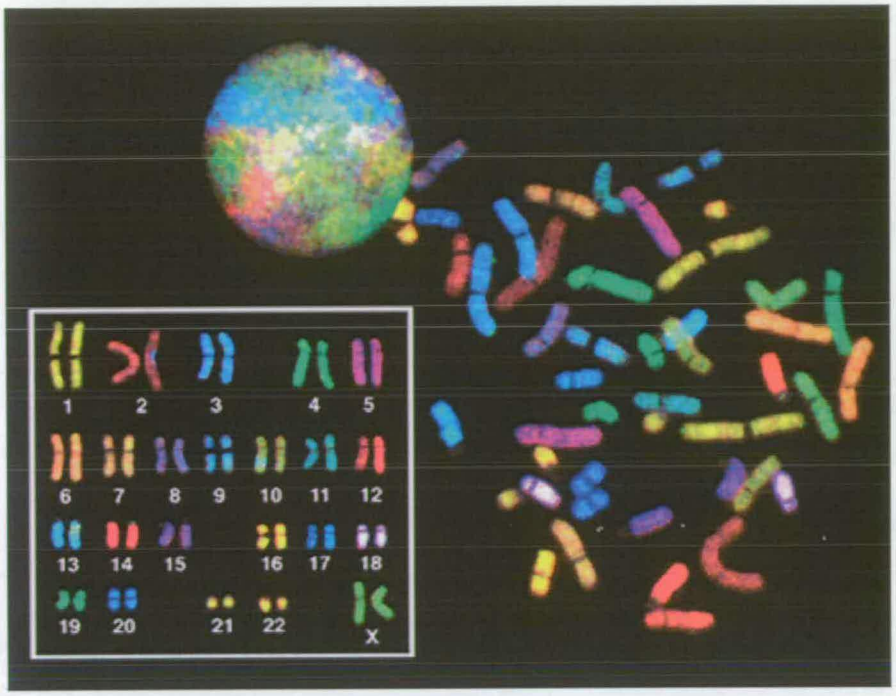


Figure 1.6 Spectral Karyotyping, reproduced with permission[9]

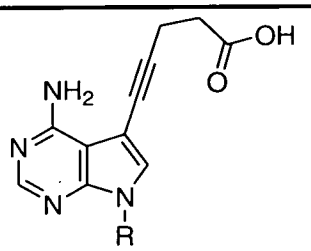
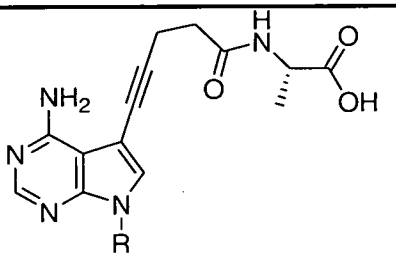
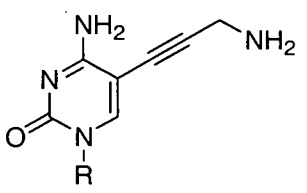
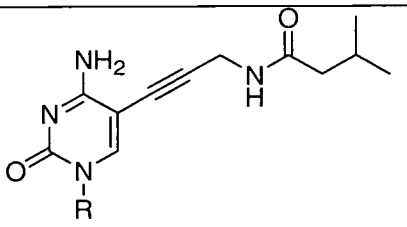
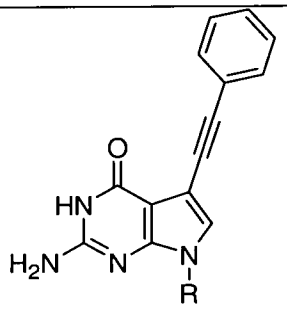
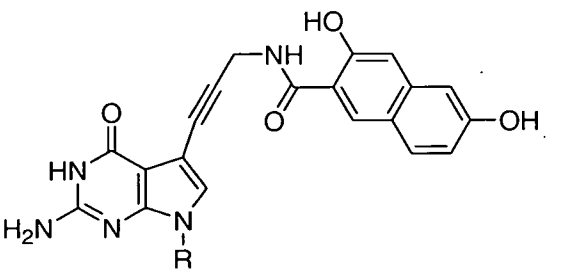
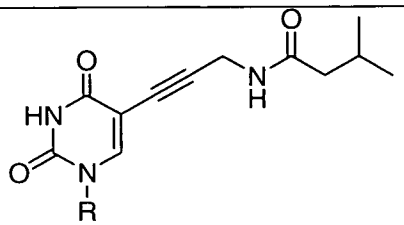
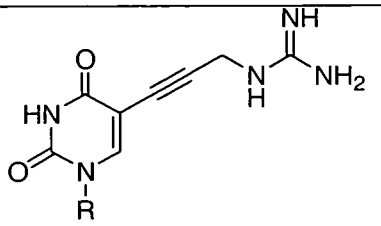
1.2.1.3 *Functionalization of DNA*

DNA is being studied for a range of applications, such as nanotechnology, material science, computing, and biotechnology. Widening the diversity of DNA by incorporating analogues for one, two or all four nucleotides is crucial to accessing wider chemical space. The incorporation of analogues with base modifications has been studied to see how tolerant polymerases are of these modifications and under which conditions the desired full-length products can be produced. Other studies have replaced the natural bases outright with unnatural analogues, such as benzimidazoles.[10]

A recent study by *Jager and Famulok* demonstrated that enzymatic synthesis of high-density functionalised DNA (fDNA) was possible. This is important because

long DNA strands (>100 base pairs) are produced chemically only with great difficulty. They showed that fDNA strands could be obtained from a natural DNA strand by substituting all natural dNTPs with triphosphates **A1-T1** (Table 1.1).[11] This fDNA strand could then be used as a template in the presence of a second set of triphosphates **A2-T2** to give highly-functionalised double-stranded DNA (dsDNA)[11] or could be copied using natural dNTPs to give the original strand in high fidelity, as asserted by sequencing.[12]

Table 1.1 Structures of functionalised nucleotides, R = 2'-deoxy ribose 5' triphosphate

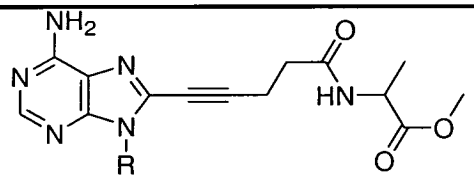
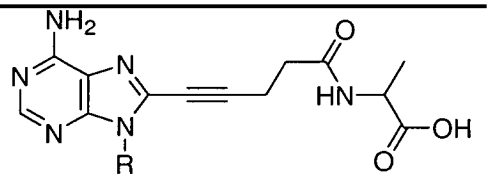
	1	2
A		
C		
G		
T		

In general, for enzymatic DNA synthesis, purines modified either at the 7 or 8 positions appear to be accepted by polymerases. While purines modified at the 8

position destabilise dsDNA secondary structure to cause a slight decrease in melting temperature, 7-modified-7-deazapurines are well accommodated in the major groove.[13] This has led to most research groups adopting the synthetically much more challenging 7-modified-7-deazapurines rather than commercial 8-modified purines.

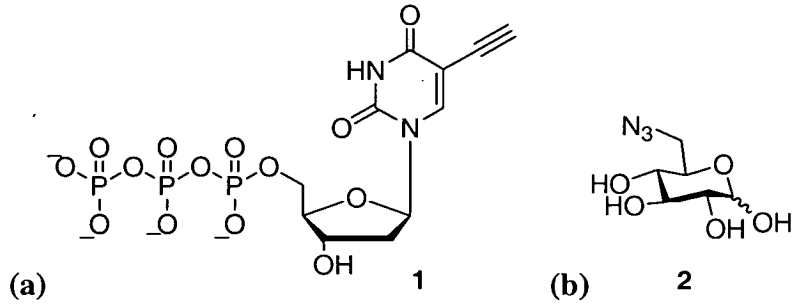
Applied to fDNA synthesis, nucleotide triphosphates **C1**, **G1**, **T2** in the presence of either **A3** or **A4** did not yield a full length product, due to lack of incorporation of the 8-modified purine analogues (Table 1.2).[13] The 7-analogue **A2** however was incorporated successfully under the same conditions.[12]

Table 1.2 Structures of 8-modified functionalised adenosines, R = 2'-deoxy ribose 5' triphosphate

	3	**4**
A		

1.2.1.4 *DNA metallization*

DNA is being studied in nanoelectronics and metal ions need to be added to increase its conductivity for it to act as a molecular wire. To improve on current uniform metallization procedures, Burley *et al.* developed a procedure to introduce selectivity though aldehyde labelling of DNA (Scheme 1.1).[14]



(a) **1** (b) **2**

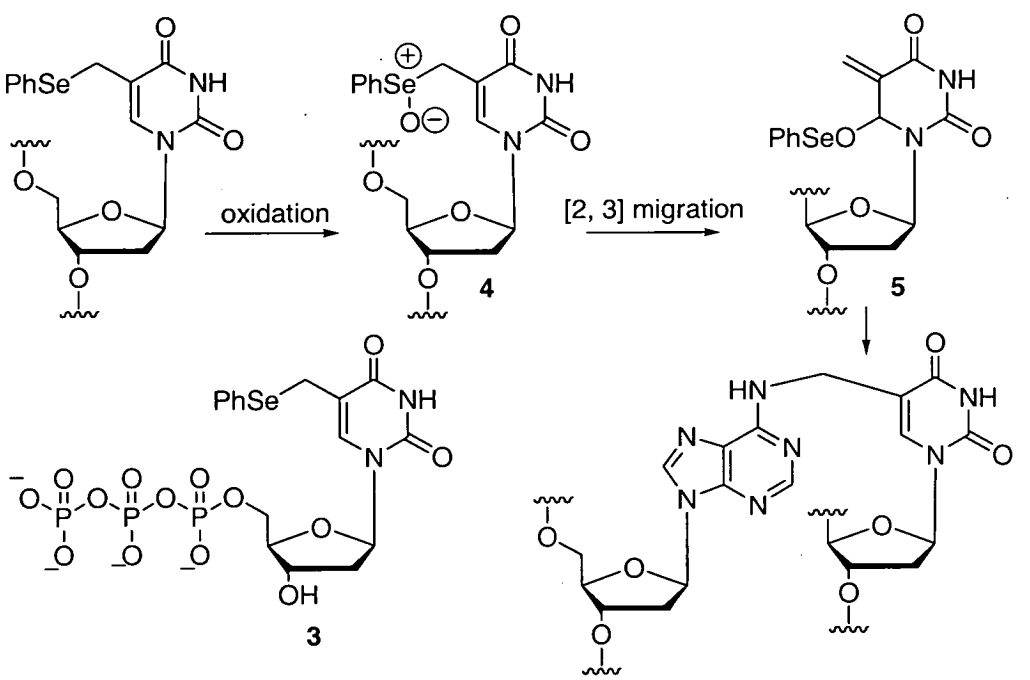
Scheme 1.1 Monomers for the synthesis of DNA susceptible to silver deposition: (a) alkynyl triphosphate, (b) galactose azide

Alkynyl modified triphosphate **1** was incorporated by a DNA polymerase into DNA opposite adenine. The alkyne was reacted with galactose azide **2**, a hemi-

acetal protected aldehyde, through Huisgen click chemistry and silver (0) was deposited on the DNA using Tollen reaction (a redox reaction between an aqueous diamine silver (I) complex and an aldehyde).[15]

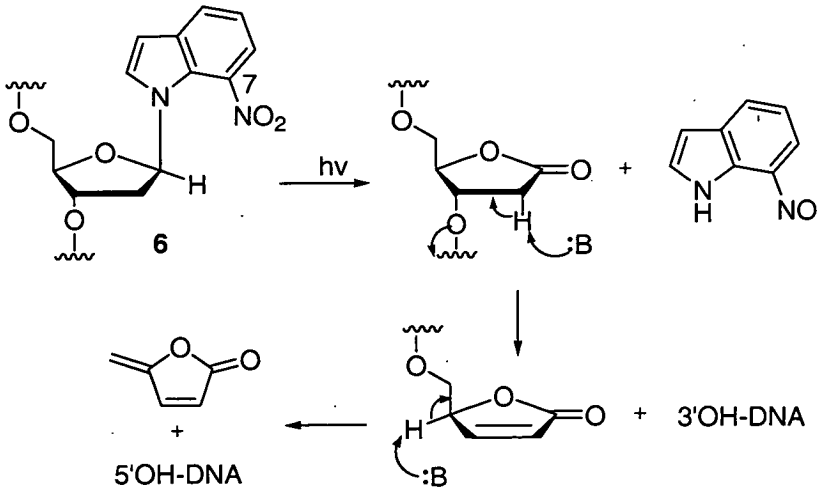
1.2.1.5 *DNA damage*

Death of cancer cells can be induced by provoking DNA damage through inter-strand cross-links, which can subsequently lead to backbone cleavage. Examples of such agents which have been incorporated into DNA are seleno-modified thymidine triphosphate **3**, which was found to sensitise DNA towards γ-irradiation at low radiation doses of 2-10 Gy by producing inter-strand cross-links.[16] Greenberg *et al.* postulated that the mechanism by which the γ-rays provoke the cross-links proceeded *via* oxidation to the selenoxide **4**, followed by a [2,3] migration to give the electrophile **5** (Scheme 1.2).



Scheme 1.2 Interstrand cross-link formation

Another set of DNA fragmentation tools are photocleavable nucleosides, which, when inserted into the DNA, trigger DNA backbone cleavage upon irradiation which can lead to sequence-independent backbone cleavage (Scheme 1.3). The triphosphate of 7-nitroindole **6** is one such nucleotide which can be inserted opposite all natural bases, albeit at a reduced rate.[17]



Scheme 1.3 Backbone cleavage of DNA following irradiation under alkaline conditions

1.2.1.6 *X-ray structure analysis of nucleic acids*

X-Ray crystallography is dogged by the difficulty of growing crystals of molecules of interest and of phasing problems. As the issue of phasing was addressed for proteins in 1990 by incorporating seleno-methionine into proteins,[18] an analogous approach for nucleic acids was developed.

PSe/PO oligonucleotide chimeras showed that selenium atoms only needed to be included every 30 base pairs to improve crystallography.[19] Selenium could be incorporated instead of oxygen in most positions by appropriate synthesis of the nucleosides. One of the easiest sites for incorporation was on the α position of the triphosphate (Figure 1.7). The Klenow fragment of DNA polymerase I could incorporate both diastereoisomers at phosphorus;[20] this is unlike for sulfur, where only S_P-diastereoisomers are recognised substrates.

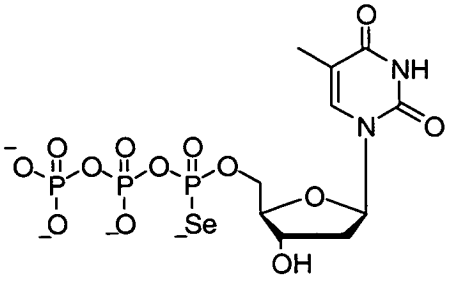


Figure 1.7 Structure of an α–selenium triphosphate

1.2.2 Polymerase Chain Reaction

1.2.2.1 *Introduction*

The polymerase chain reaction (PCR) is a technique to replicate DNA enzymatically *in vitro*. The quantity of DNA is amplified exponentially and as a result, PCR is commonplace in medical and biological research labs.

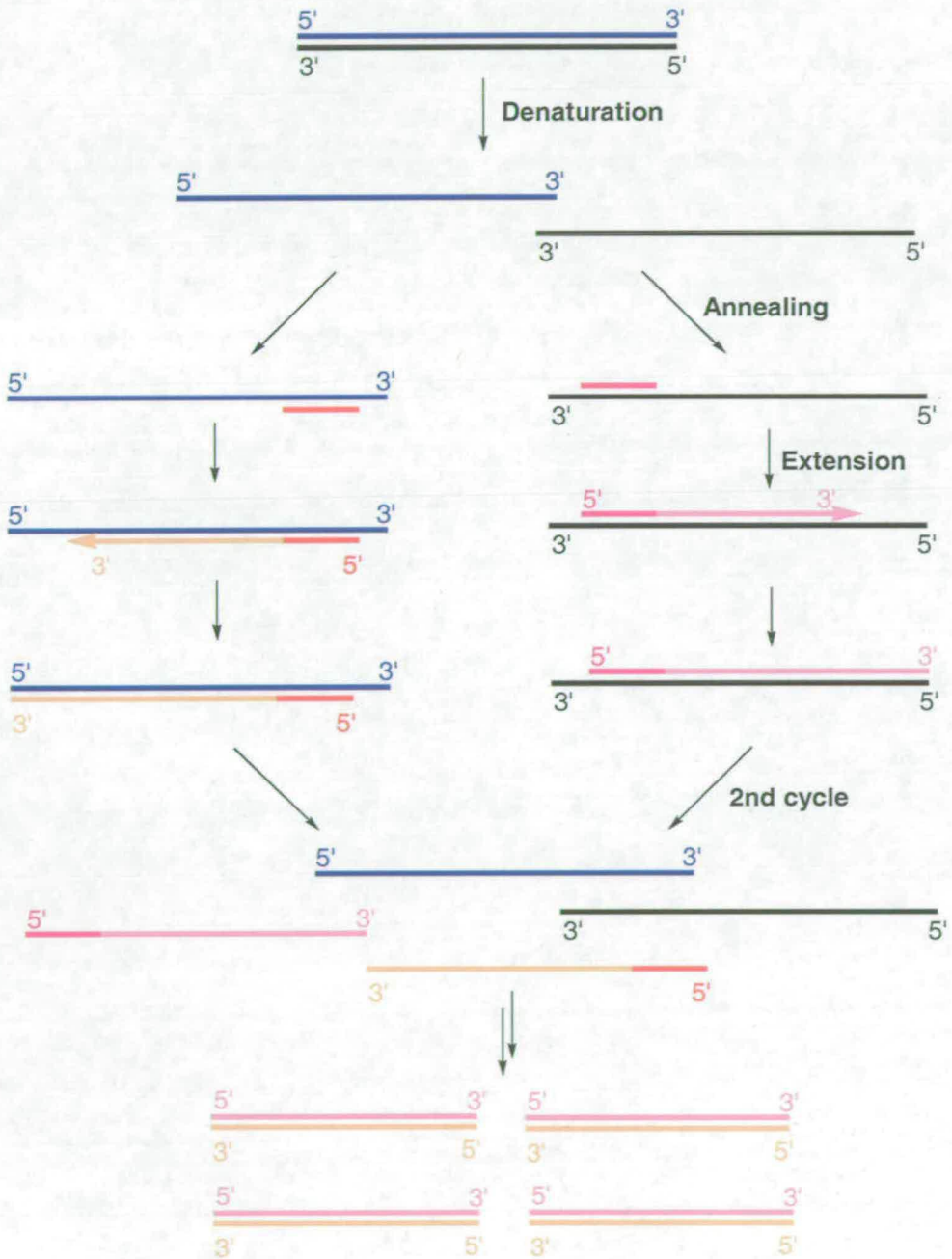


Figure 1.8 Schematic drawing of a PCR cycle

The PCR procedure starts from a small amount, potentially even a single molecule of dsDNA (Figure 1.8). To initiate replication, it is separated to give two lengths of single-stranded DNA (ssDNA) by heating (Step 1). The temperature is then lowered so the primers can anneal to the ssDNA (Step 2). The third step in the PCR process is the elongation of the DNA strands. The polymerase adds dNTPs to the 3' end of the primer and replicates along the template strand. When the elongation process is finished, the strands are again denatured, annealed and elongated. Typically between 20 and 35 cycles are performed to obtain the required amount of DNA.[6]

1.2.2.2 *Real-time PCR* via *selective labelling of DNA amplicons*

PCR is very successful at exponential amplification of DNA, however no quantification is possible, as the amount of DNA produced *per* cycle plateaus off, as the primers are used up. Real-time PCR has been developed to address this and thus enables genotyping, mutation detection and chimerism analysis.[21] Detection of PCR products is achieved by measuring fluorescence changes in the PCR mixture as the reaction progresses. Fluorescence is generated either by adding a dsDNA-specific dye or a ssDNA sequence specific fluorescent probe, such as TaqMan,[22] Molecular Beacons[23] and hybridization probes.[24] As they rely on hybridisation with the PCR product, the probes and primers need to be designed to avoid problems with intramolecular structures and the sequence to be amplified needs to be known.

A novel real-time PCR monitoring method has been described recently, based on selective incorporation of a third base pair, isoguanosine (iG):5-methylisocytidine (iC).[25] Specific incorporation of a quencher linked to iG opposite iC, which was linked to a fluorophore, lead to a decrease of fluorescence thus quantitation of the PCR product in a sealed-tube format.[26]

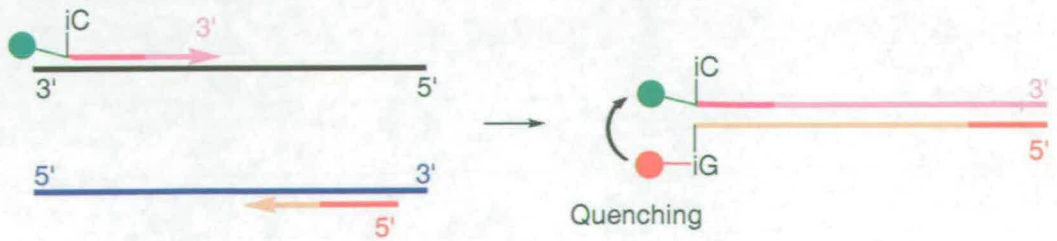


Figure 1.9 Real-time PCR using site-specific quenching during amplification

The target to be amplified was annealed to a primer labelled at the 5' end of iC with a fluorophore (Figure 1.9). Amplification was performed in the presence of dabcyl-diGTP (Figure 1.10) and site-specific incorporation of the quencher occurred opposite iC. The close proximity of fluorophore and quencher reduced the fluorescence intensity over 35 cycles. The percentage of quenching varied from 71 to 35 % depending on the fluorophore used.[26]

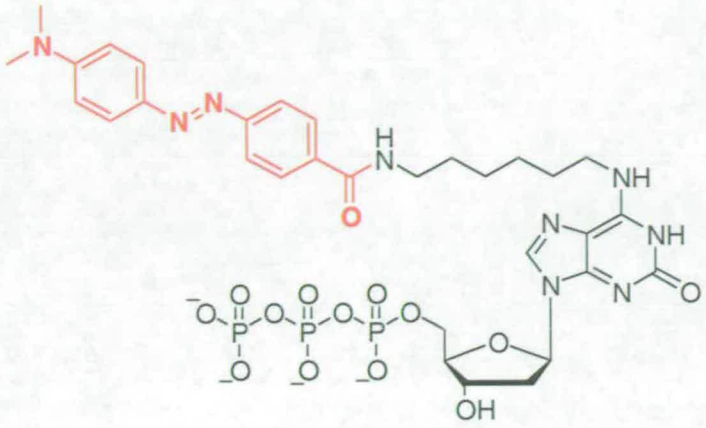


Figure 1.10 Structure of quencher dabcyl-diGTP

1.2.2.3 *Aptamers*

Aptamers are RNA or DNA sequences that bind tightly to a specific entity for which they have been selected by adopting a complex three-dimensional shape through intramolecular interactions that allows binding to the target. High-affinity aptamers have been identified for enzymes, growth factors, antibodies, amino acids, drugs, organic dyes, antibiotics, nucleotides and peptides.[27]

Aptamers are developed using a systematic evolution of ligands by an exponential enrichment (SELEX) process. A random-sequence combinatorial library of 10^{14} to 10^{15} individual sequences, comprising a 40 nucleotide random sequence, is incubated with the target, immobilised on a solid support. The sequences that bind are retained, while the other sequences are removed by washing. The hits are amplified by 'error-prone' PCR, which introduces a small degree of mutations to enrich the pool. After 8 to 15 cycles of selection and amplification, the sequences are cloned and sequenced.

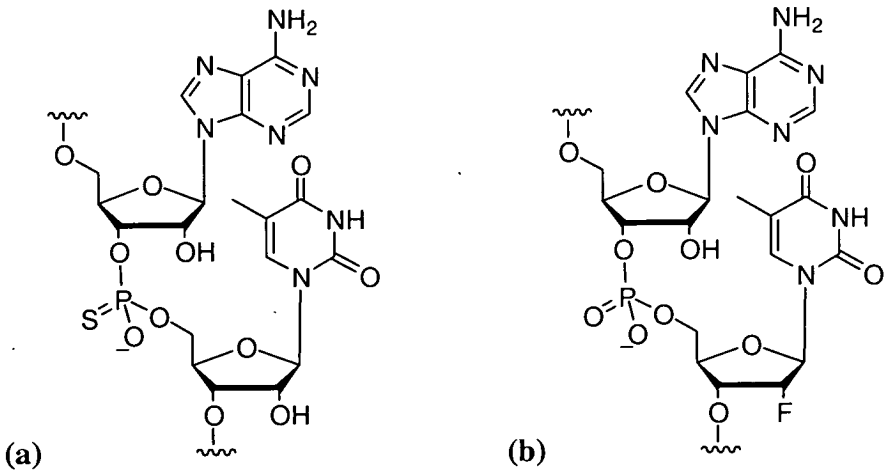


Figure 1.11 Endonuclease resistant RNA aptamers

For use of aptamers in biological fluids, i.e. for medicinal applications, they need resistance towards nucleases which degrade RNA readily, especially next to pyrimidine bases. This can be achieved by introducing phosphorothioate linkages[28] (Figure 1.11a), substitution of 2' position on pyrimidine nucleotides with amino[29] and fluoro[30] groups (Figure 1.11b) or 4'-thio substitution on the sugar (Figure 1.12).

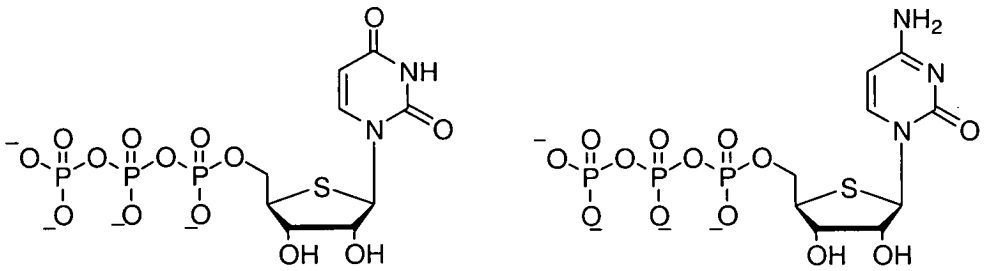


Figure 1.12 Structures 4'-thiopyrmidine triphosphates

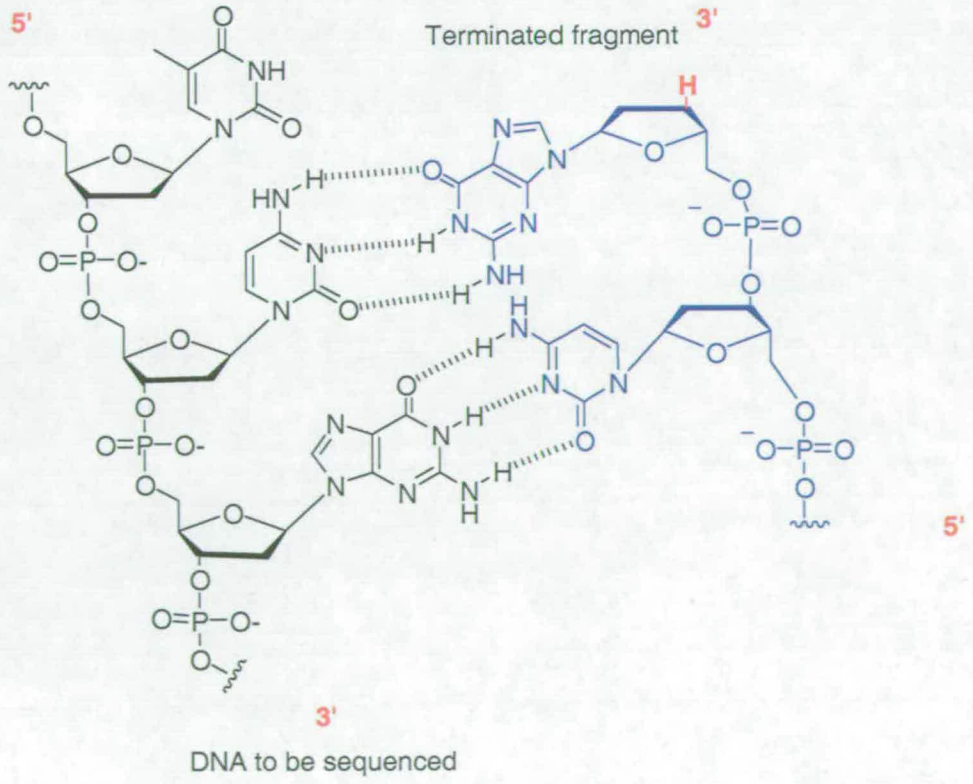
Added diversity over the natural bases can be achieved by incorporating unnatural base pairs.[31] Examples include alternative *H*-bonding pairs such as iC/iG[25] and 2,6-diaminopyrimidine/xanthine.[32] Base pair analogues which are shape mimics and do not rely on hydrogen-bonding were reported by Kool *et al.* to be replicated successfully.[33]

1.2.3 DNA sequencing

The advent of DNA sequencing has revolutionised biology and enabled high-profile research projects, such as the Human Genome project.[34] In 1977 two separate methods enabling analysis of genes at the nucleotide level were reported by Maxam & Gilbert[35] and Sanger.[1] Other methods developed since then, are pyrosequencing,[36,37,38] single molecule sequencing with an exonuclease[39] and DNA sequencing by MALDI-MS.[40] For high-throughput DNA sequencing, a level of automation is required, which so far had only been achieved for the Sanger method.[3] Recently, an automated instrument has also become available based on Pyrosequencing, enabling increased throughput.[41]

1.2.3.1 *Sanger's dideoxynucleotide chain termination method*

The Sanger chain termination method is the most influential and widely used method to date and led to the award of a Nobel Prize to F. Sanger in 1980. It can be summarized as an enzyme catalysed reaction that synthesises DNA fragments complementary to a DNA template of unknown sequence. A brief overview of the technique is given below.



Scheme 1.4 Chain terminated sequence via *incorporation of a dideoxynucleotide*

Originally a labelled primer was annealed to a specific region on the DNA sequence of interest and subjected to DNA polymerisation in the presence of all four dNTPs and a small percentage of a ddNTP. Dideoxy NTPs (ddNTPs) differ from dNTPs by having a hydrogen atom attached to the 3' carbon instead of a hydroxyl group. They are substrates for polymerases, but terminate elongation as they cannot form the subsequent phosphodiester bond (Scheme 1.4).

The reaction was carried out four times in parallel to yield four pools of DNA fragments of different lengths, each terminating with a different ddNTP. All DNA fragments obtained have the same 5' end, as they were started on the same primer (Figure 1.13a). As illustrated for A, the DNA strand to be sequenced is treated with all four dNTPs, as well as ddATP to give controlled chain terminated fragments. As all fragments were started from the same primer, their lengths can be compared by four-lane electrophoresis. In this technique, the smallest fragment moves farthest from the loading point on the gel and the 3' end nucleotides can be read off with increasing size (Figure 1.13b).

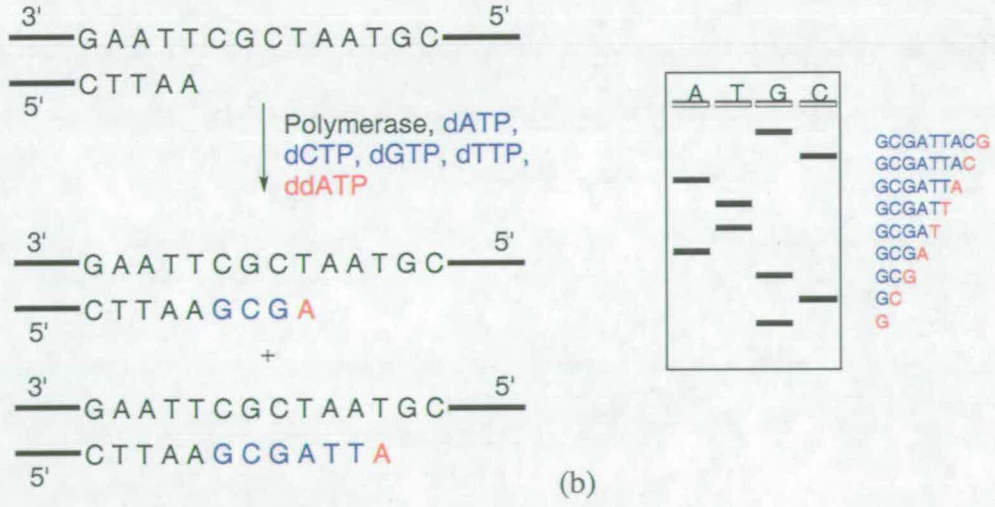


Figure 1.13 (a) Synthesis of chain terminated fragments for A; (b) Four lane electrophoresis to read the DNA sequence

1.2.3.2 *Fluorescently labelled chain terminators*

In Sanger's original dideoxy chain termination method, DNA fragments were visualised by incorporating ^{32}P or ^{35}S radioisotopes into the primer, but radio-labels have now been superseded by fluorescent labels. Two methods to do so have evolved: dye-labelled primer sequencing where the fluorescent dye is attached to

the 5' end of the primer[42] and more recently dye-labelled terminator sequencing where the terminating ddNTPs carry the fluorescent dye. [42,43] A typical ddNTP chain terminator, as used by Prober *et al.* is shown below (Figure 1.14). In some studies different speeds for incorporating labelled ddNTPs and un-labelled dNTPs were observed, giving rise to peaks of different intensities which could result in errors during automated base calling.[42]

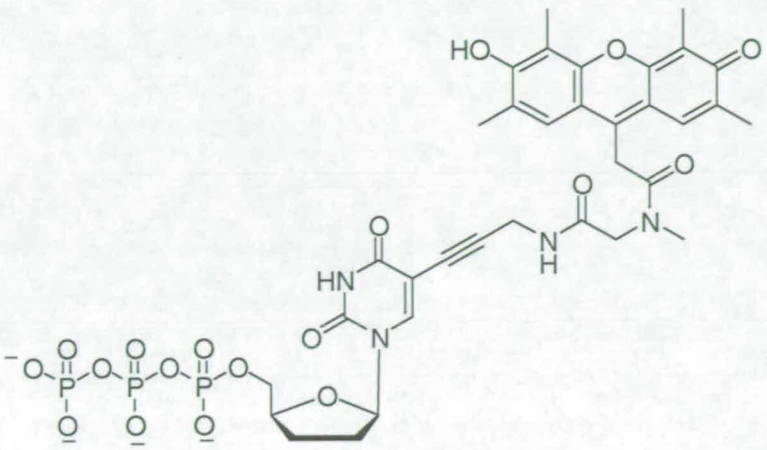


Figure 1.14 Structure of fluorescently labelled chain terminator

1.2.3.3 *Automation*

The advantage of fluorescence labelling over radio-labels is the ability to automate DNA sequencing, as each nucleotide is identified through a different fluorophore and all four families of terminated DNA fragments are synthesised in one pot and analysed by array capillary electrophoresis with laser-induced fluorescence detection. This is the state of the art technology that was used for the Human Genome Project. This technique is referred to as 'Four Colour DNA sequencing.' Modern automated DNA sequencers can analyse as many as 384 fluorescently labelled samples in a run and perform as many as 24 runs a day.

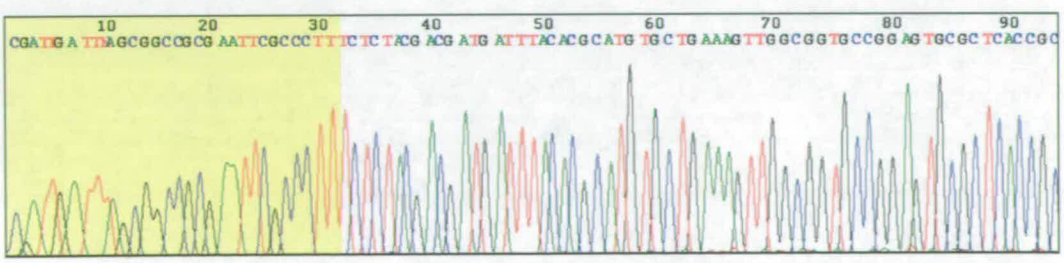


Figure 1.15 Four colour DNA sequencing electropherogram, reproduced with permission[44]

The data from the sequencing is obtained as an electropherogram. An example of the start of a Sanger sequencing read is given (Figure 1.15). The four bases are detected using different fluorescent labels, which are represented as peaks of different colours, which can then be interpreted to determine the base sequence.

The limitations of the Four Colour DNA sequencing are a relatively high cost for on-demand sequencing which makes the potential associated with personalised medicine prohibitively expensive.

Electrophoresis based separations are also limited by band compression in GC rich regions and the technology is ambiguous in assigning heterozogytes (single nucleotide polymorphisms). Several approaches are being pursued to circumvent electrophoresis and reduce cost,[45] three of which will be discussed below.

1.2.4 DNA sequencing by MALDI-MS

This technique makes use of the strongest non-covalent protein/substrate interactions known in nature: the binding between biotin and streptavidin. Biotin is used as a purification handle to obtain Sanger DNA fragments in a purity sufficient for MS. The fragments are generated using biotin labelled ddNTPs in the polymerase reaction. (Figure 1.16)

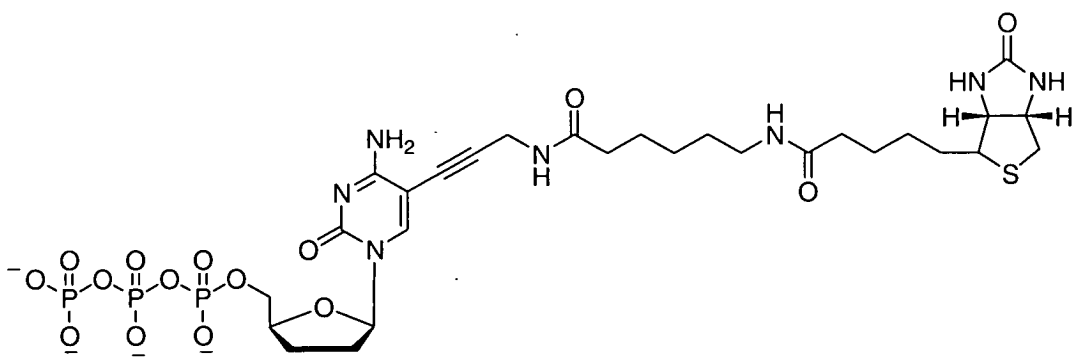


Figure 1.16 Example of a biotin labelled chain terminator

The polymerase reaction is carried out in the presence of all four dNTPs and all four ddNTPs in one pot, then all the terminated DNA fragments with 3'-biotin incorporation are isolated on streptavidin covered magnetic beads. Excess primer, polymerase and falsely terminated DNA fragments are washed off the support, thus removing all factors which in previous methods contributed to difficulties in the interpretation of mass spectra.[46] The purified DNA fragments are released from the

beads and analysed by MALDI-TOF-MS. The mass difference between fragment peaks determines the identity of individual nucleosides, thus the sequence. Mass spectrometry is a fast method that provides a high-throughput method for DNA sequencing for up to 100 base pairs with current instrumentation.[47]

1.2.5 DNA sequencing by synthesis

DNA sequencing by synthesis (SBS) on a chip is an interesting alternative to Four Colour sequencing. The concept of SBS was first discussed in 1988 by Hyman,[48] and recent developments in microarray technology mean the potential for this high-throughput method have come within reach.

SBS involves detecting the identity of each nucleotide as it is incorporated into the nascent DNA chain during the polymerase reaction. The principle can be broken down into three steps: probing, read-out and label removal (Figure 1.17).

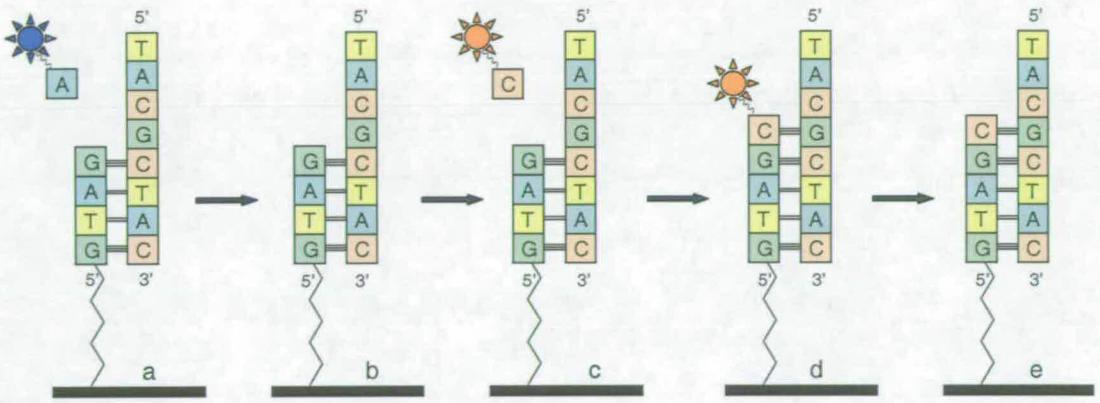


Figure 1.17 Principle of DNA SBS. (a) Probing, (b) negative read-out, (c) probing, (d) positive read-out, (e) label removal

A primer is immobilised on a surface and hybridised with an oligonucleotide with a sequence of interest. This sequence is probed with a labelled dNTP in the presence of a DNA polymerase (a). If the dNTP is not complementary, the read-out is negative and no signal is detected (b). Probing continues with the other labelled dNTPs until fluorescence is observed (d). This allows assigning one nucleotide and after removal of the label (e), the cycle can be repeated.

Fluorescence detection offers great sensitivity, provided the label can be destroyed to avoid interference with the subsequent nucleoside read-out. To date, removal of fluorescence from the label has been achieved by photochemical bleaching[49] and

by use of a photo-cleavable linker.[50,51] Four-colour DNA sequencing by synthesis was recently published using four photocleavable fluorescent nucleotides.[50] The nucleotide analogues were labelled with a different fluorophore, *via* the 7 position for purines and the 5 position for pyrimidines.

The method enabled identification of 12 contiguous bases. The template used did however not contain homopolymeric runs, thus problems such as double incorporation of a dNTP would not have been identified. It is likely that the nucleotide analogues described do not act as chain terminators, despite their steric bulk, as the same group more recently described the synthesis and application of a 3' allyl protected analogue to DNA SBS (Figure 1.18).[52]

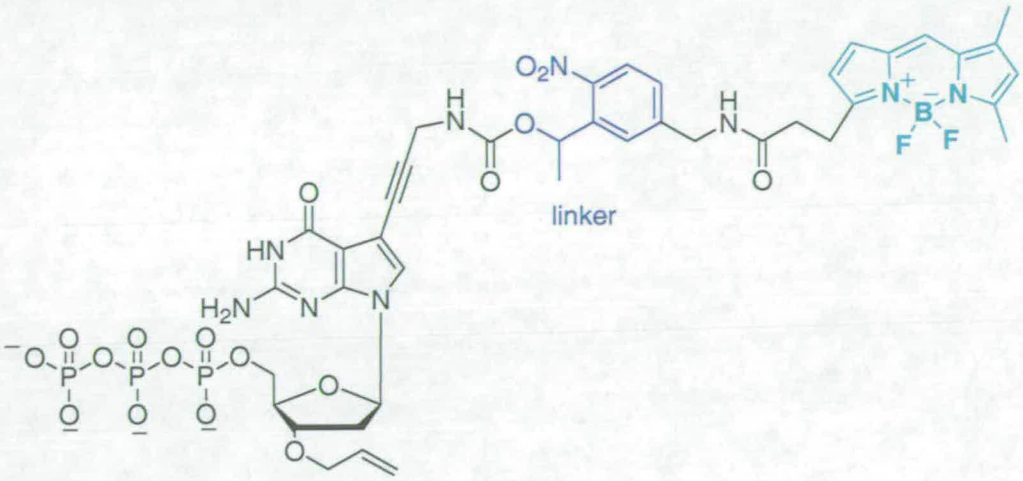


Figure 1.18 Structure of 3'-allyl-dGTP-PC-Bodipy

1.2.6 Polony sequencing

In this technique, amplification of the DNA is localized on a glass slide, by performing PCR in a polyacrylamide gel. Due to restricted diffusion, the amplicons remain close to the template and lead to 'PCR colonies' or polonies.[53] Polonies can be sequenced by performing cycles of primer extension with reversibly labelled fluorescent dNTPs in a highly parallel fashion on the slide. DNA strands are immobilised onto the gel, denatured and hybridised to a universal primer. The primers are extended by sequential treatment with one of four fluorescently labelled dNTPs (Figure 1.19). The fluorescent label is removed by cleaving the disulfide linker under reducing conditions.[54] Current limitations are detachment of the gel from the slide after 10-14 cycles and multiple incorporations for homologous runs, i.e. the dNTPs are not acting as chain terminators.

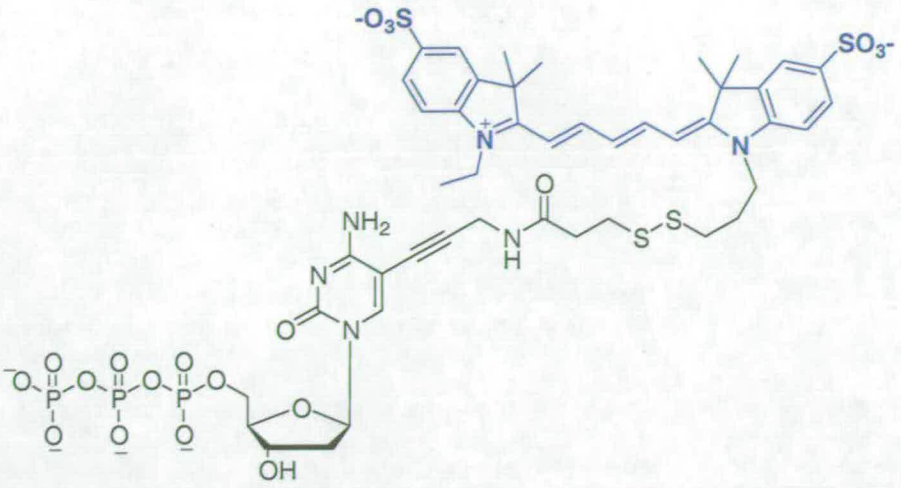


Figure 1.19 Structure of Cy5-SS-dCTP

1.2.7 High-throughput SNP analysis

Single nucleotide polymorphisms (SNPs) account for 90 % of all human genetic variations and occur on average once every 1000 to 2000 nucleotides. The fully annotated sequence resulting form the Human Genome Project contained some 1.4 million sites of variation along the 3.2 billion base pairs.[55] SNPs are of great value to biomedical research as they can affect how humans develop diseases and respond to pathogens and drugs. SNPs can be detected using a range of techniques.[56] A few of the techniques have been transposed onto a multiplexing platform to meet the high-throughput requirements of large-scale genetic studies.[57]

1.2.7.1 *Allele specific primer elongation on microarrays*

This SNP detection protocol relies on the specificity of Taq DNA polymerase to discriminate against single base mismatches. ssDNA targets hybridised to a primer, immobilised on a DNA chip, serve as templates in elongation reactions. The match and mismatch sequences differ in their 3' base. Elongation and thus incorporation of a labelled dNTP takes place only if the variable nucleotide is complementary to the primer (Figure 1.20).[58] Specific elongation of the primer took place in the presence of three natural dNTPs and a labelled dTTP analogue and sites of polymerase extension, thus SNPs, were detected by confocal laser scanning.

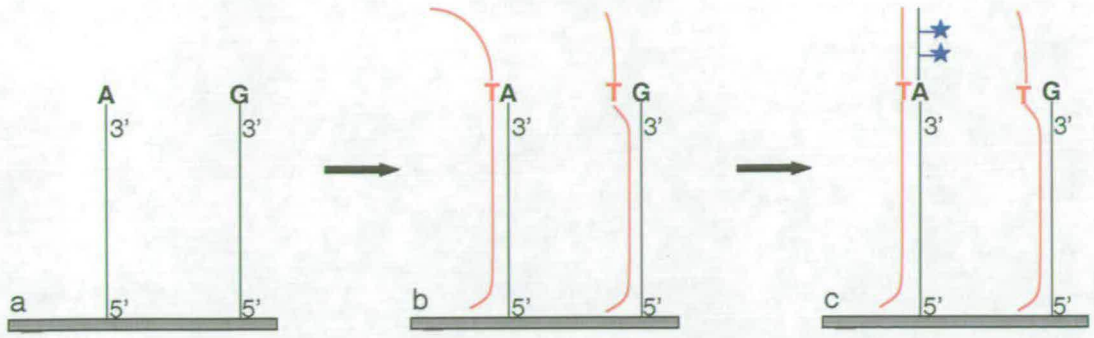


Figure 1.20 Allele-specific primer elongation: (a) immobilisation, (b) hybridisation, (c) allele specific elongation with labelled dNTP

1.2.7.2 *Mini-sequencing*

An alternative technique for SNP detection is mini-sequencing. It is analogous to SBS, with the difference that terminators such as ddNTPs are used. The primers are annealed immediately next to a SNP of interest, followed by a single labelled ddNTP base extension.

On the microarray, the technique was first demonstrated for ^{33}P-ddNTP terminators for the detection of 9 SNPs.[59] An advantage over allele-specific extension is the fact that only one primer is required *per* SNP.

Another application of mini-sequencing is the use of tag-arrays. The primer is attached to a 5' tag, which enables the extension of the primer hybridised to the target ssDNA, to be performed in solution. To decode the extension products they are then hybridised onto DNA chips, which have been covalently modified with anti-tags, complementary oligonucleotides to the tag. Using this tag technology, mini-sequencing was performed using four differently labelled ddNTPs, thus giving Four Colour Mini-sequencing.[60]

Mini-sequencing has also been applied to flow-cytometry based separation of microspheres. Fluorescein labelled microspheres were coupled to anti-tags, while primers were extended in solution with fluorogenically labelled ddNTPs and captured onto the beads.[61,62]

1.3 Syntheses of nucleotide triphosphates

The first synthesis and isolation of the triphosphate ATP, was a formidable challenge in 1949.[63] In practical terms, the synthesis and isolation of triphosphates has moved on a great deal in this time from successive barium salt precipitations, but the area of synthesis is still dogged with the inherent problems of the chemistry. It requires reaction between lipophilic protected nucleosides and multiply charged pyrophosphate ions under rigorously dry conditions, followed by ion-exchange chromatography and then HPLC purification. Added to the synthesis issues, is the fact that triphosphates are not stable and some are known to decompose at room temperature.[64]

1.3.1 Retrosynthesis of a triphosphate

A range of procedures have been reported for the conversion of a nucleoside to its triphosphate. A comprehensive review of synthetically useful methods for triphosphate preparation was published in 2000 by Burgess and Cook.[64] Based on this review, the different approaches can be summarized in a few general routes, exemplified by the disconnection of the triphosphate moiety. (Figure 1.21)

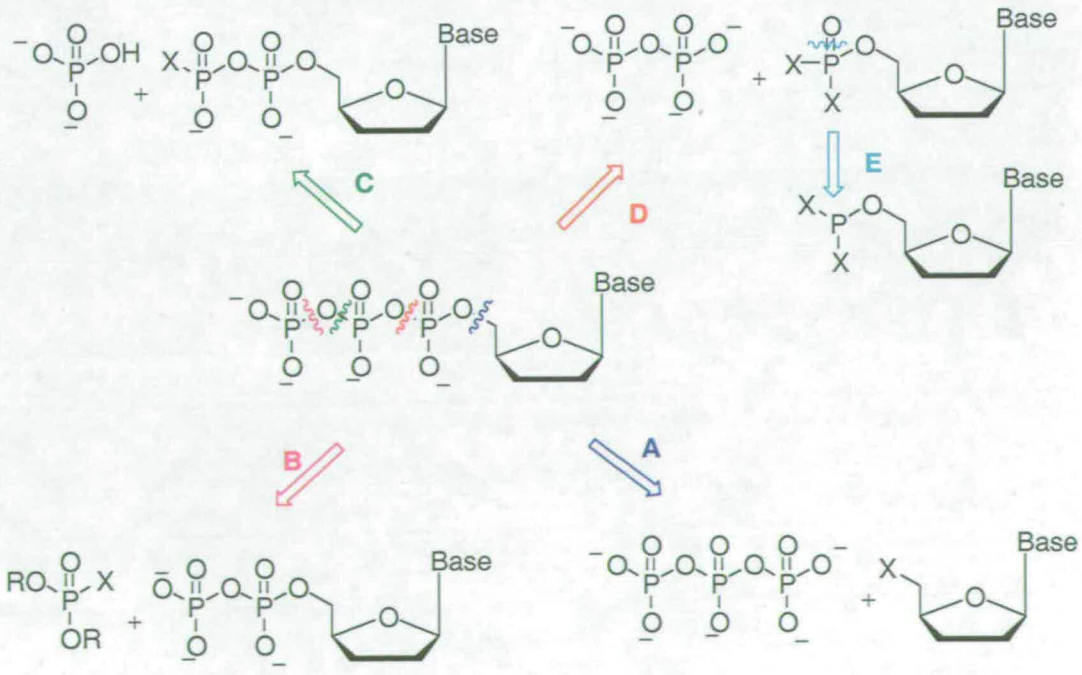
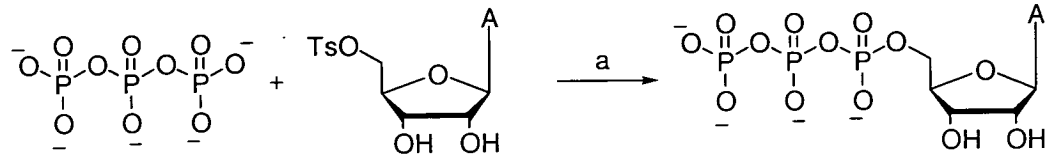


Figure 1.21 Retrosynthesis of a triphosphate

1.3.2 A: Nucleophilic attack of triphosphate on an activated nucleoside

This is the route that conceptually looks most efficient, however only few examples have been shown to be synthetically useful. This is in part due to the instability of triphosphate ions and has only been shown to be practical for the synthesis of ATP.[65,66]



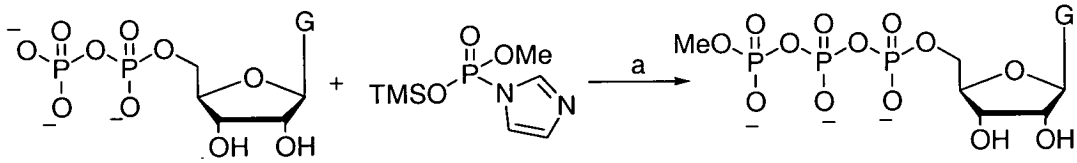
(a) MeCN, rt, 48 h, 72 %

Scheme 1.5 Synthesis of a triphosphate via *triphosphate attack on an activated nucleoside*

Interesting examples exist for triphosphate analogues though, where the oxygens between the phosphorus atoms have been replaced with methylene groups[67] or nitrogens.

1.3.3 B: Nucleophilic attack of a nucleoside diphosphate on an activated phosphate

The unprotected nucleoside diphosphate is added to an activated phosphate, here a phosphoroimidazolide (Scheme 1.6) to give the methyl triphosphate of guanosine in good yield.



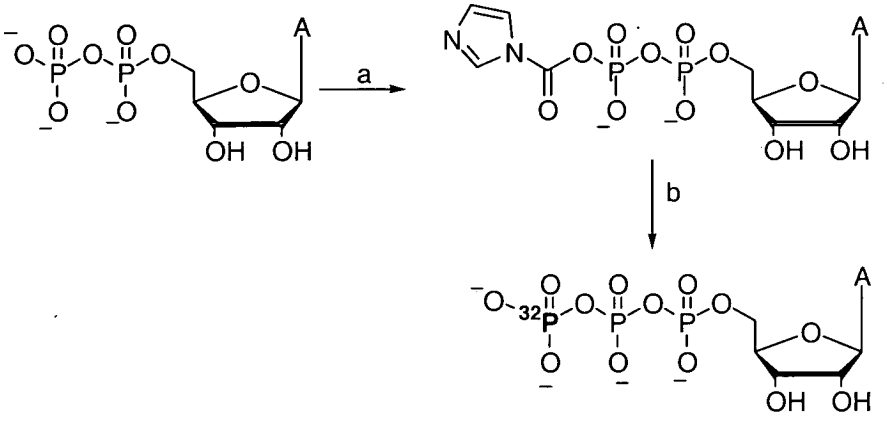
(a) $ZnCl_2$, DMF, 4 h, rt, 86 % yield

Scheme 1.6 Synthesis of a triphosphate via *a diphosphate attack on an activated phosphate*

It was found that for the reaction to progress at a reasonable rate, Lewis acidic metal ions had to be added.[68] No further examples using this disconnection have been reported.

1.3.4 C: Nucleophilic attack of a phosphate on an activated nucleoside diphosphate

This route also involves a nucleoside diphosphate and a monophosphate; however in this case, the roles of nucleophile and electrophile are reversed. Syntheses using this disconnection have chiefly been employed for the introduction of a radio-labelled ^{32}P isotope at the γ phosphorus. Thus a nucleoside diphosphate was treated with 1,1'-carbonyldiimidazole to activate specifically the β position, which was then treated with a phosphate.[69] Selective activation of the β rather than α position was verified incidentally when radio-labelled phosphorus was found only in the γ position (Scheme 1.7).



(a) (i) $(Im)_2CO$, DMF, rt, 12 h, (ii) MeOH; (b) $HNBu_3^+H_2{}^{32}PO_4^-$, DMF, rt, 36 h

Scheme 1.7 Synthesis of a triphosphate via *phosphate attack on an activated diphosphate*

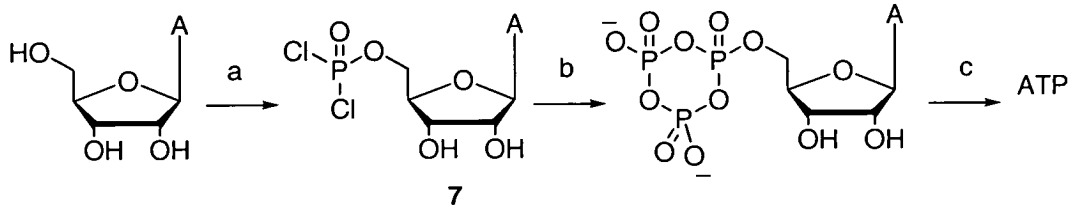
This method has found no general applications, probably because, as a group of compounds, nucleoside diphosphates are even harder to synthesise.

1.3.5 D: Nucleophilic attack of pyrophosphate on an activated nucleoside monophosphate

The greatest number of procedures can be grouped under route D. The monophosphate nucleotide is activated to pyrophosphate attack by introducing a leaving group on the monophosphate. Examples of the most commonly used leaving groups are given below.

1.3.5.1 *Dichlorophosphates*

Early work by Yoshikawa using $POCl_3$/trialkylphosphate mixtures to selectively monophosphorylate at the 5'-OH position,[70] were exploited by others by treating the activated dichloromonophosphate **7** with a pyrophosphate solution to give a cyclic triphosphate, which was quenched to yield the triphosphate (Scheme 1.8).[71]



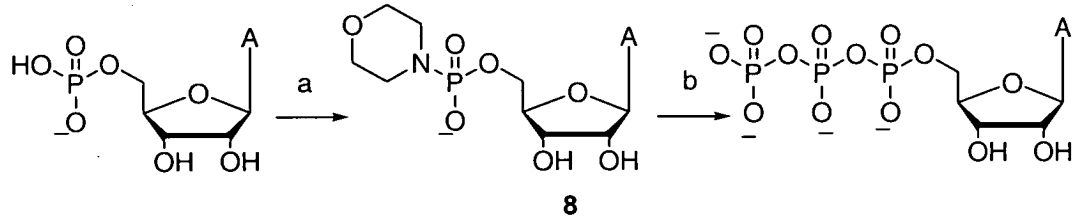
(a) $POCl_3$, $(MeO)_3PO$, 0 °C, 1.5 h; (b) $(HNBu_3)_2H_2P_2O_7$, Bu_3N, rt, 1 min; (c) TEAB buffer

Scheme 1.8 Synthesis of a triphosphate via *a dichlorophosphate*

By replacing $POCl_3$ with $PSCl_3$, α-thio triphosphates can also be accessed.[72] While this chemistry has an appealing 'one pot-three step' efficiency, it does have drawbacks in that steps are very substrate-dependent and the monophosporylation step is often not as selective as in the first reported cases.

1.3.5.2 *Phosphoramidates*

An alternative leaving group that can be introduced is an amine, which is typically morpholine, to give the nucleoside phosphoramidate. The appeal of the methodology lies in the fact that it starts from the monophosphate. Mofatt *et al.*[73] used DCC and morpholine to produce the phosphoroamidate **8**, which reacts with bis-(tributylammonium) pyrophosphate to give the triphosphate, but di and tetraphosphates are formed through disproportionation reactions. This is reduced in the presence of DMSO,[74] but reaction times of a few days are necessary, to reach yields of 70 to 80 % (Scheme 1.9).

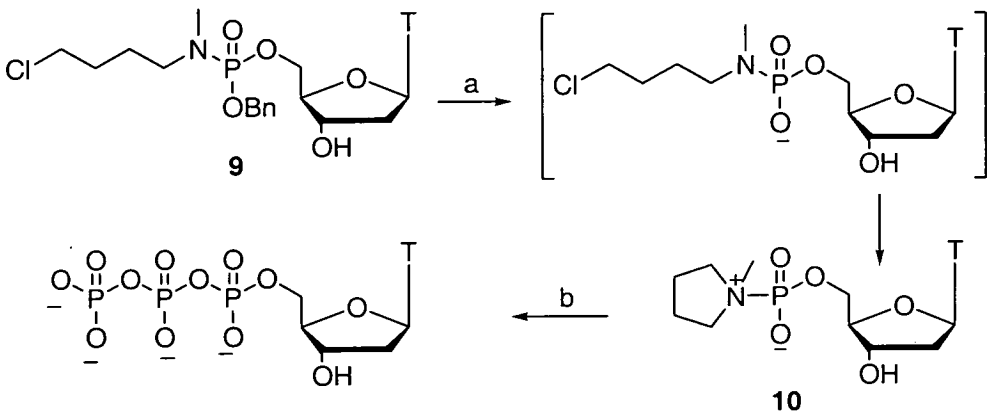


(a) DCC, morpholine, $^tBuOH/H_2O$, reflux, 6 h; (b) $(HNBu_3)_2H_2P_2O_7$, DMSO, rt, 2-4 d

Scheme 1.9 Synthesis of a triphosphate via *a phosphoramidate*

1.3.5.3 *Charged phosphoramidates*

Borch *et al.*[75] recently applied a more reactive phosphoramidate to triphosphate synthesis, which progresses *via* a highly reactive zwitterionic intermediate. Following a nine-step solution phase procedure[76] to obtain phosphoramidate **9**, the phosphate ester is deprotected catalytically, which activates the alkyl chloride side chain to nucleophilic attack from the nitrogen. Zwitterionic pyrrolidinium **10** is displaced to form the triphosphate. (Scheme 1.10)



(a) H_2, Pd/C, THF, rt; (b) $(NBu_4)_3HP_2O_7$, THF; 77 % yield

Scheme 1.10 Synthesis of a triphosphate via *a charged phosphoroamidate*

This method was applied to the synthesis of dTTP, dCTP, UTP and two nucleoside drugs in 55-77 % yields. Apart for the lengthy preparation of the starting material, no reference is made to the synthesis of purine triphosphates and to the base protection requirements of nucleophilic amino groups.

1.3.5.4 *Other activation methods*

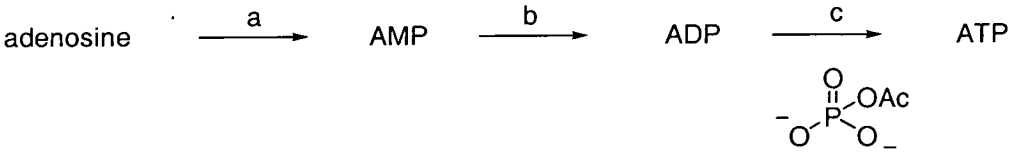
Dervan *et al.*[25] activated the monophosphate with 1,1'-carbonyldiimidazole and subjected it to tributylammonium pyrophosphate to yield the triphosphate. Other activation methods for monophosphates are triazoles,[77] mixed phosphate anhydrides with $(^tBu)_2PSCl$,[78] $(PhO)_2POCl$[79] or trifluoroacetic anhydride.[80]

3'-azido-thymidine was triphosphorylated with tris-imidazolylphosphate, followed by MeOH addition to give 5'-imidazolylmethylphosphate and pyrophosphate attack gave the triphosphate in a 77 % yield. The high yield was attributed to minimisation of side-reaction by MeOH addition.[81]

1.3.6 . Enzymatic reactions

Reactions which fall in the realm of biocatalysis are mentioned at this point, as the principle can be applied to any of the previous routes, availability of an enzyme performing the required reaction and recognition of the desired substrate permitting. An enzymatic approach is almost perfect in some cases, while it is utterly unsuited in others.

The approach for the synthesis of ATP is exemplified (Scheme 1.11), where the total conversion was 98 %.[82] However, the overall efficiency still depends on the isolation procedure and enzyme access.



(a) adenosine kinase; (b) adenylate kinase, cat. ATP; (c) acetate kinase

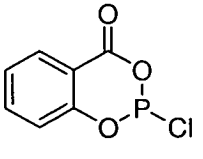
Scheme 1.11 Synthesis of a triphosphate via *biocatalysis*

The use of enzymatic reactions in triphosphorylation requires suitable enzymes to accept synthetic substrates and is also not always successful. This initial investment is most suitable for bulk synthesis of triphosphates.

1.3.7 E: Nucleophilic attack of pyrophosphate on an activated nucleoside phosphite

1.3.7.1 *The Ludwig-Eckstein reagent*

An attractive alternative to the previously mentioned methods is the one-pot Ludwig-Eckstein method,[83] where the nucleoside is reacted *in situ* with salicyl phosphorochloridite **11** (Figure 1.22).

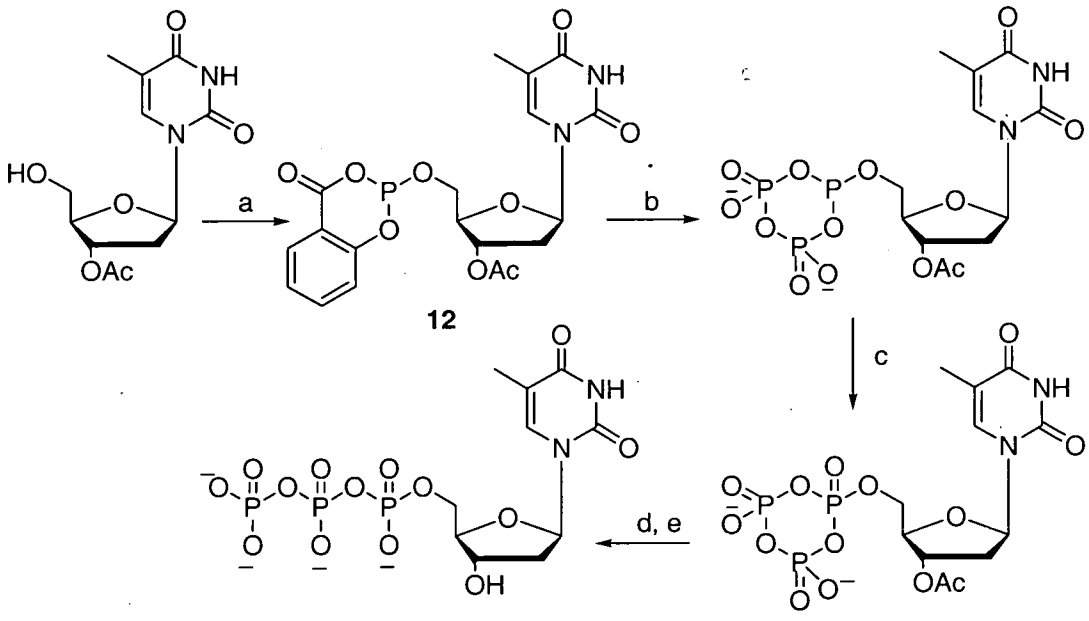


11

Figure 1.22 Structure of 2-chloro-benzo[1,3,2]dioxaphosphinin-4-one 11

The phosphitylated nucleoside intermediate **12** undergoes two subsequent nucleophilic substitutions with pyrophosphate to give a cyclic triphosphite, which

is oxidised with iodine/pyridine and hydrolysed with aqueous ammonia (Scheme 1.12). Base protection has not been found to be necessary, while 2' and 3' OH groups need protection, as even at -78 °C little selectivity for primary alcohols over secondary alcohols was observed.[83] (Counterions omitted for clarity)



(a) **11** , py/THF; (b) pyrophosphate; (c) I_2 in pyridine/H_2O; (d) H_2O; (e) NH_3 in H_2O

Scheme 1.12 Thymidine triphosphate synthesis by Ludwig and Eckstein

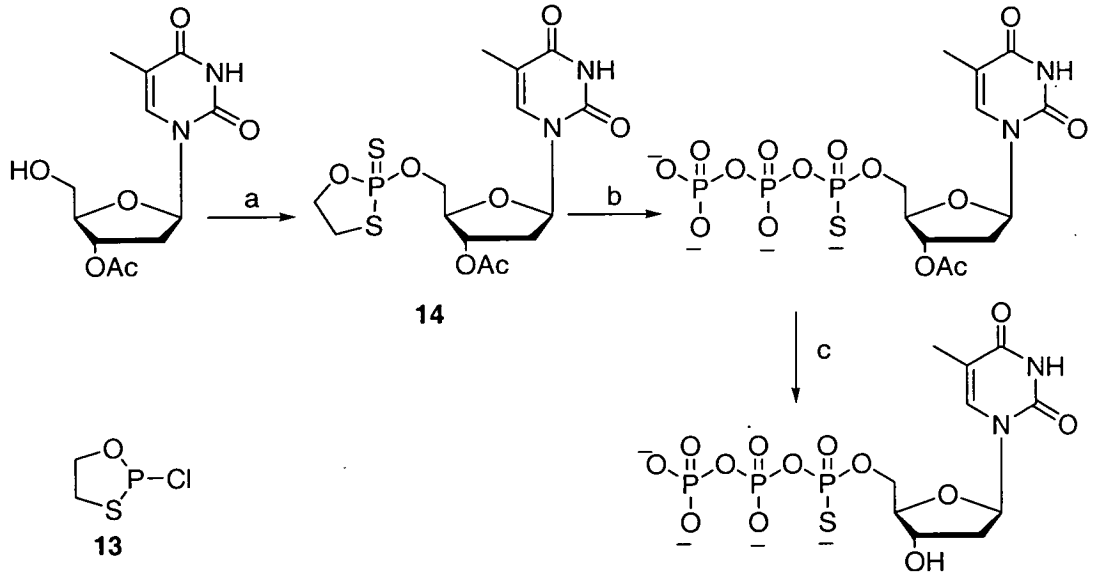
This method is widely used for synthesis of nucleoside triphosphates. It has also been applied to the synthesis of modified triphosphate analogues such as α-thio,[83] α-borano,[84] α-thio-α-borano,[85] α,α-dithio and α,γ-dithio triphosphates.[86] It has also been used to access α-thio, α-borano and α-seleno diphosphates[87] and dinucleoside tetra- and pentaphosphates.[88] A recent literature survey showed that over 600 examples of its use in synthesis had been reported.[89]

Even though it was a significant improvement on other methods, the Ludwig-Eckstein method still requires the resultant triphosphate to be purified by ion-exchange chromatography, followed by preparative HPLC. Isolated yields for simple triphosphates can be as high as 50-70 %, but for modified nucleoside or fluorophore labelled nucleosides yields can be very low, if the reaction works at all. This has been attributed to a sluggish phosphitylation of the primary alcohol.

1.3.7.2 *Oxathiaphospholane methodology*

This method also relies on the activation of a nucleoside monophosphite towards nucleophilic attack. First reports of oxathiophospholane **13** centered on using intermediates such as **14** for the generation of PO/PS chimeric oligonucleotides.

Its application to triphosphate synthesis has recently been reported.[90] Protection of exocylic amines was not necessary.[91] The nucleoside alcohol is phosphitylated and *in situ* oxidised to the thio analogue or the seleno analogue (Scheme 1.13). This is followed by nucleophilic displacement with pyrophosphate and deprotection to give 48-78 % conversion by ^{31}P NMR, but only 10-30 % isolated products for the DNA and RNA α-thiotriphosphates.



(a) **13** , S_8, py/THF; (b) pyrophosphate, DBU; (c) pyridine/H_2O; (d) H_2O; (e) NH_3 in H_2O; 18 % yield

Scheme 1.13 Thymidine α-thiotriphosphate synthesis using oxathiaphospholane 13

Analogously, the α-selenotriphosphate of adenosine could be accessed in 31 % yield and the unmodified triphosphate of adenosine could be formed by oxidising its thio-analogue with H_2O_2 in 65 % yield. No discussion was however made with regards to applying this reagent directly to synthesis of unmodified triphosphates.

1.4 Solid-supported reagents

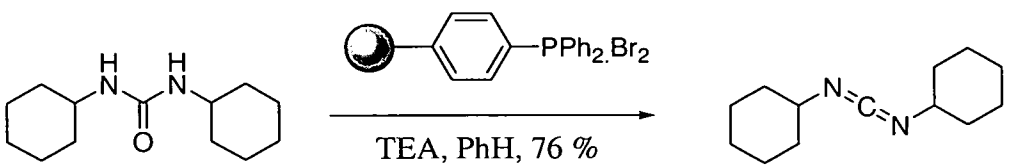
High-throughput synthesis is of huge importance in the generation of small molecules. These are required to interact with potential therapeutic targets, which have been uncovered by recent progresses in genomic and proteomic sciences.[92] Not only do the molecules need to be synthesised in a pure and timely fashion, there is also pressure to do this in an environmentally friendly manner and with minimum manipulation. One technology able to provide the tools to satisfy these goals is the use of solid-supported reagents.

1.4.1 Immobilised reagents in solution phase synthesis

Solid-supported reagents were developed to combine the advantages of combinatorial chemistry and solution phase chemistry. Immobilised reagents can be added to the substrate of interest without compromising the purity of the product. Additionally, all the real-time monitoring procedures like tlc, HPLC and MS of solution phase chemistry can be applied. As supported reagents have gained in popularity, so have their applications. The general topics will be briefly discussed below and illustrated with an example. The field of polymer supported reagents and its aspects have been extensively reviewed. [92,93,94,95]

1.4.1.1 *As a reagent*

The supported reagent is used in an excess relative to the substrate of interest, thus, the reaction is pushed to completion. Excess reagent is simply removed by filtration and does not complicate isolation. With appropriate protocols, the reaction product can then reacted subsequently without further purification. Examples in this area cover oxidations and reductions, halogenations, C-C bond forming reactions, nucleophilic substitution reactions and deprotection and dehydrating reagents.[95]



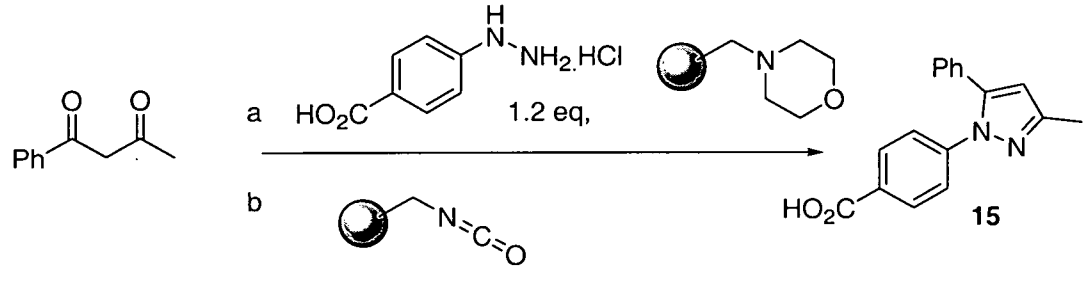
Scheme 1.14 Synthesis of carbodiimides using a supported triphenylphosphine complex

Triphenylphosphine has many applications in organic synthesis, but this is marred by purification problems caused by its by-product triphenylphosphine oxide as it is an especially difficult purification and the use of polymer-supported triphenylphosphine has been able to remedy this. A PS-PPh_3-Br_2 complex has been employed for the formation of carbodiimides from ureas (Scheme 1.14).[96]

1.4.1.2 *As a scavenger*

There is also scope to use supports to clean up reaction mixtures in a rapid and efficient manner. By adding a supported reagent complementary to an undesirable component in the reaction mixture, it is bound to the support either through ionic interactions or covalent bonds and removed through filtration. Scavenging reagents can be grouped into two groups: scavengers for electrophiles or nucleophiles *via* covalent bonds and acidic or basic scavengers which form ionic interactions.[97,98]

In the synthesis of pyrazole **15**, a polymer-bound amine was used to neutralise the salt and then excess hydrazine was scavenged by a polymer-bound isocyanate to give a supported urea-type adduct, which was readily removed to give the pyrazole in excellent purity (Scheme 1.15).[99]



(a) MeOH, rt, 2.5 h; (b) MeOH, rt, 16 h; 48 % yield, 97 % HPLC purity

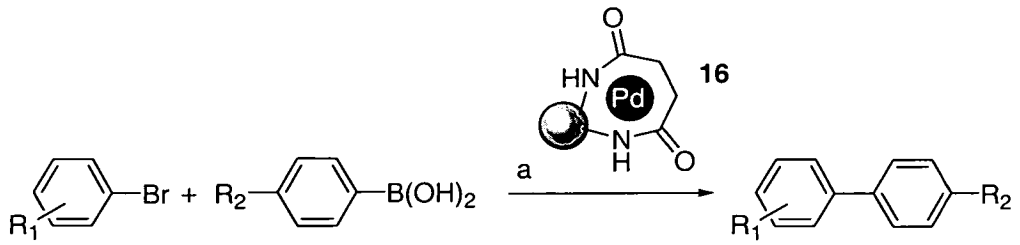
Scheme 1.15 Synthesis of pyrazole using an electrophilic scavenger

1.4.1.3 *As a catalyst*

The same principles as for reagents can also be applied to catalytic systems. The active catalyst is encapsulated or bound to the support and removed by filtration after the reaction and re-used.

In a recent example described by Bradley *et al.*, palladium (0) was encapsulated inside a Tentagel resin. Palladium leaching out of solid-supports was addressed by entrapping palladium as nanoparticles. Aqueous Suzuki couplings to aryl bromides

could be performed at 10 mol % of catalyst in good to excellent yields (Scheme 1.16). The catalyst was recycled up to six times without loss of activity.[100]

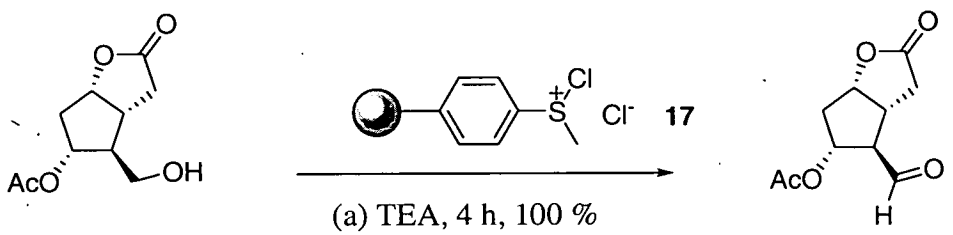


(a) 3 eq K_2CO_3, 10 mol % **16** , H_2O, 80 °C, 4-16 h, 10 examples, 62-99 % yield

Scheme 1.16 Aqueous Suzuki couplings using supported Palladium nanoparticles

1.4.1.4 *To reduce hazards*

A range of chemical transformations involve reagents or by-products that are either toxic, smelly or hazardous. By immobilising them, their hazardous nature is obviously greatly reduced or removed and thus making them much easier and safer to handle. This was demonstrated by the elimination of the volatile sulfur compounds of a Swern oxidation. Phenylmethlychlorosulfonium salt **17** was immobilised and used successfully to obtain the desired aldehyde (Scheme 1.17).[101]

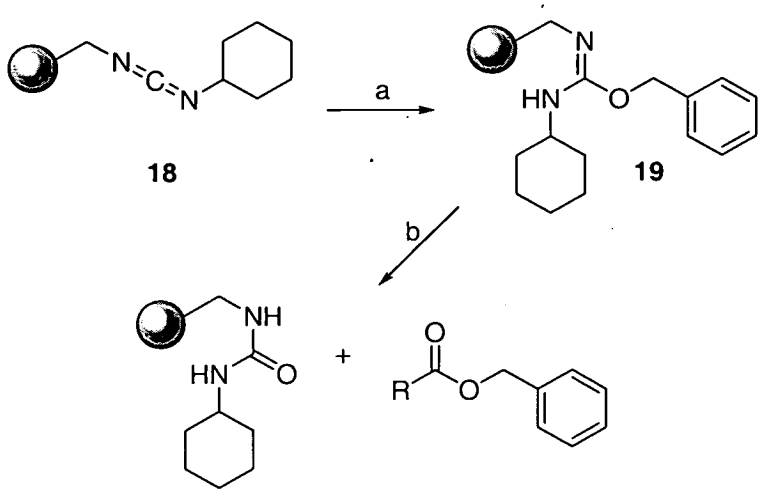


Scheme 1.17 Oxidation of a primary alcohol using a supported Swern-type oxidation

1.4.1.5 *Catch-and-release resins*

This methodology combines polymer supported synthesis and scavenging protocols. Functionalised polymers have been developed which trap the substrate as a supported reactive intermediate. After washing the support to remove soluble by-products, the intermediate is subjected to a second reaction which provokes a chemical transformation and concomitant release from the resin. Acyl and sulfonyl transfers, alkylations, ring closing cleavage reactions and selenium based reactions have been reviewed.[102] A few pertinent examples are discussed below.

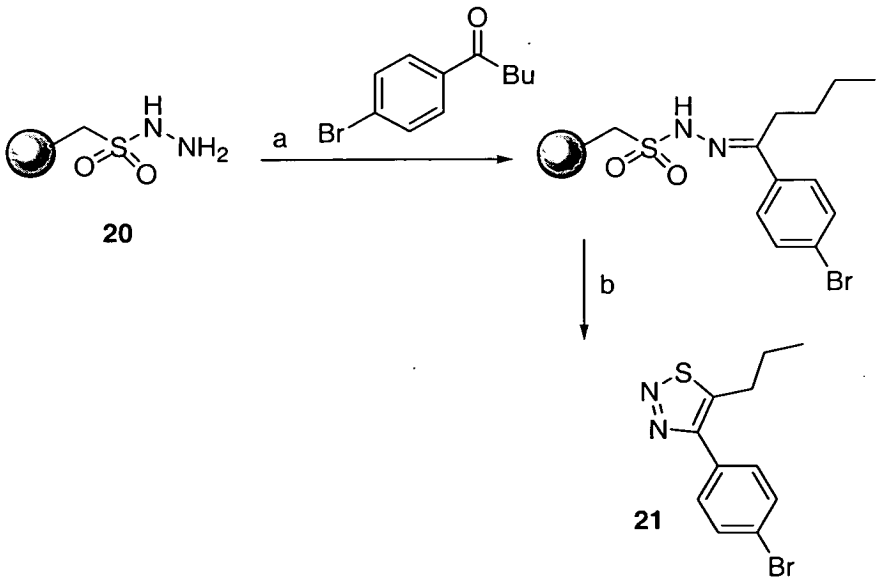
Polymer-supported carbodiimide **18** was reacted with various alcohols under Cu (II) catalysis to yield the corresponding *O*-alkyl isourea **19**, which was treated with carboxylic acids to give esters in excellent yield (>90 %) and purity (>95 %) under heating or microwave irradiation (Scheme 1.18).[103]



(a) (i) BnOH, $Cu(OTf)_2$, THF, 16 h, (ii) TMEDA, CH_2Cl_2; (b) RCO_2H, 125 °C, μw, 3 min

Scheme 1.18 Synthesis of esters using polymer supported O*-alkylisoureas*

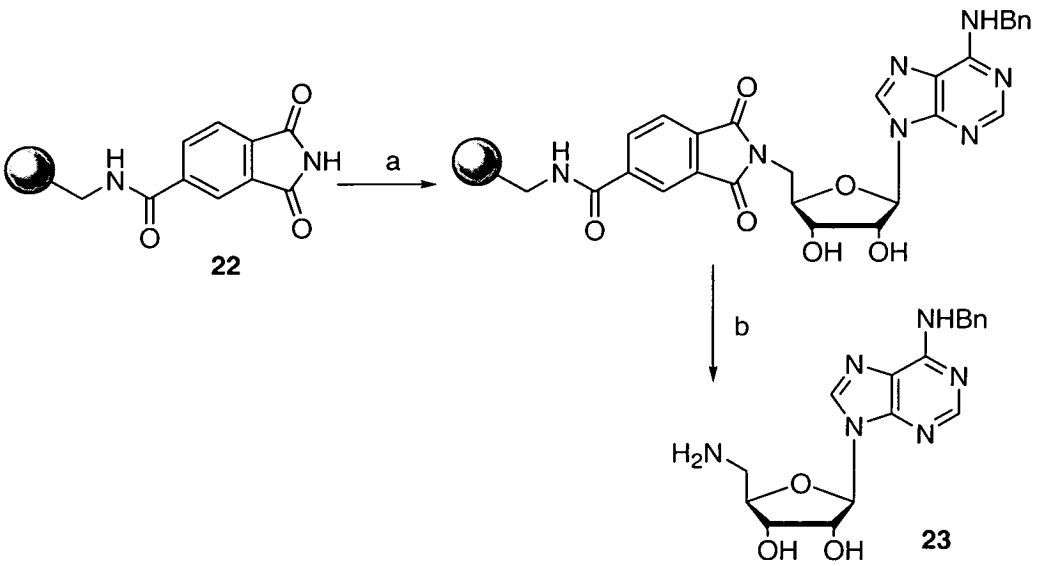
Various ketones were trapped as sulfonyl hydrazones on PS-sulfonyl-hydrazide resin **20**. Hurd-Mori reaction[104] and concomitant cleavage was initiated by treatment with $SOCl_2$ to yield the desired 1,2,3-thiadiazole **21** in good yield and purity (Scheme 1.19).[105]



(a) AcOH/THF, 50 °C; (b) $SOCl_2$, $(CH_2Cl)_2$, 60 °C; 82 % yield, 94 % GC purity

Scheme 1.19 Polymer-assisted synthesis of 1,2,3-thiadiazoles

Of particular relevance to nucleoside chemistry is a Mitsunobu protocol applied recently to the conversion of a primary alcohol to an amine. Polymer-supported phthamlimide **22** was coupled to the 5' hydroxyl group of adenosine. The resultant 5'-amino nucleoside **23** was released by hyrdazinolysis in excellent yield (Scheme 1.20).[106]

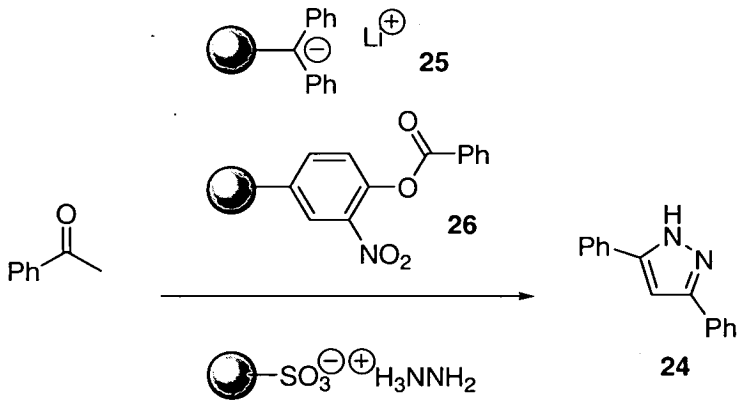


(a) A^{Bn}, PPh_3, DEAD, THF; (b) N_2H_4, EtOH, CH_2Cl_2; >96 % yield

Scheme 1.20 A polymer supported Mitsunobu reaction

1.4.1.6 *Multi-step reactions*

The potential to exploit reactions in a one-pot-multi-step reaction sequence, where the individual reagents are mutually incompatible, but while bound on a support do not interact has also been realised.



THF; 91 % yield

*Scheme 1.21 Simultaneous use of mutually incompatible reagents to prepare pyrazole **24***

One of the first examples was reported by Patchornik *et al.*[107] Two insoluble polymers containing functionalities which react when mixed in solution, could be employed in a one-pot reaction sequence. The strong base **25** and the acylation reagent **26** were reacted together in the presence of acetophenone to yield a diketone anion, which was treated immediately with a supported hydrazine to yield the substituted pyrazole **24** in excellent yield (Scheme 1.21).

1.4.2 Synthesis and properties of resins

Work has been concentrated mainly on polystyrene-divinylbenzene co-polymers as a support, however graft polymers of other monomers, as well as glass, paper and zeolites have also found their uses. The inherent properties of the resins vary significantly; therefore careful selection of the polymer is often necessary.

1.4.2.1 *Polystyrene-based resins*

The first resins to come into synthetic use were polystyrene (PS) based. Cross-linking was found to be necessary to impart mechanical stability. Currently used resins contain 1-2 % cross-linking introduced using divinylbenzene (DVB) **28** added during the polymerisation with vinylbenzene **27** and 0.5 to 3.5 mmolg^{-1} loading introduced either *via* the addition of a suitable monomer, like chloromethylvinylbenzene **29**, in the desired portion or subsequent chemical modification of the benzene ring through Friedel-Crafts chemistry (Figure 1.23).

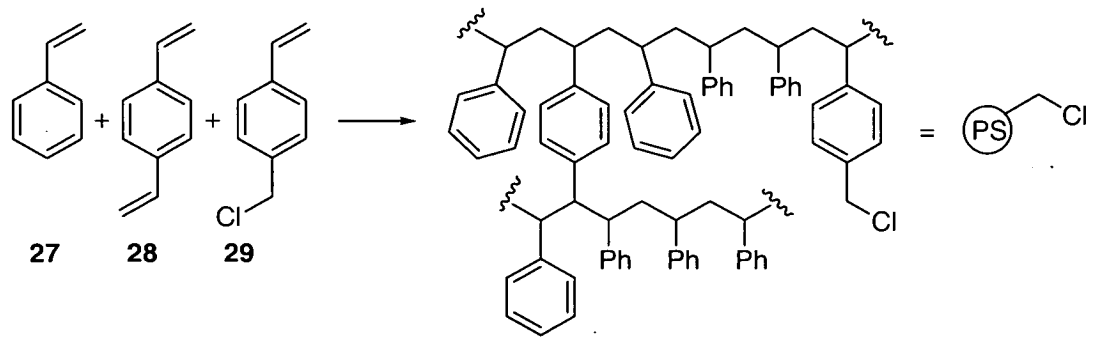


Figure 1.23 Structure of monomers and of chloromethyl polystyrene resin

The hydrophobic properties of polystyrene make the resin swell in non-polar organic solvents up to five times its dry volume.[108] The resin, however, suffers from poor swelling in water and some polar solvents, thus reactions cannot

proceed. To date polystyrene is arguably the most widely used type of resin, however due to its lack of swelling in aqueous and polar solvents, alternatives have been developed.

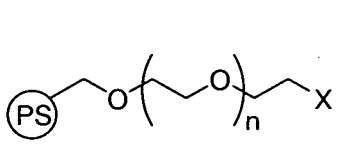
A low cross-linked resin which, when swollen, forms a gel and thus makes all functionalisation sites throughout the resin bead accessible. This type of resin is referred to as microporous resin.

1.4.2.2 *Highly cross-linked PS resins*

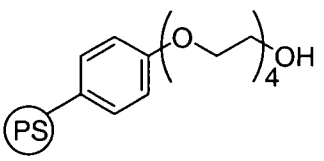
By increasing the proportion of the cross-linking monomer to 20 or 30 % w/v, the resin appreciably changes its swelling properties and becomes rigid. When a porogen is added during the polymerisation, it is referred to as a macroporous resin. The accessible resin functionalities are reached through the rigid pores. Highly cross-linked PS resins are used mostly as support for scavengers and for synthesis of oligonucleotides.

1.4.2.3 *Grafted PS resins*

Graft resins combining the properties of hydrophobic microporous PS and water soluble polyethylene-glycol (PEG) chains were developed to access more hydrophilic resins. They contain a PS core which is modified by linking a PEG chain through an ether bond. PS-PEG is commercialised as Tentagel **30** and contains around 70-80 % by weight of PEG (Figure 1.24). Disadvantages are loading levels, the potential of the PEG chains to complex Lewis acids and the potential instability of PEG and the tendency to become sticky and difficult to handle as the synthesis progresses.



30



31

Figure 1.24 Structures of PS-PEG resins: Tentagel 30 and Quadragel **31**

The properties of PS-PEG resins such as Tentagel have been modulated with a view to lessening these issues, by incorporating a defined tetraethylene glycol unit. This resin is being marketed as Quadragel **31** for a microporous PS core and

Quadrapore for the macroporous analogue. Quadragel could be employed favourably over Argogel, a PS-PEG resin analogous to Tentagel with ca. 60 ethylene glycol units, when a Lewis acid ($SnCl_4$) mediated reaction needed to be performed.[109] This was attributed to reduced amount of Lewis basic ethylene glycol chains.

1.4.2.4 *Controlled Pore Glasss*

Controlled Pore Glass (CPG) is a rigid glass-derived material compatible with any type of solvent and extremes of pressure and temperature that are occasionally used in synthesis. Loading levels are low as all functionalities are located on the surfaces. CPG is formed using a Si-O network and is functionalised with short carbon chains and an amine, such as propylamine.

In CPG beads a considerable amount of the surface silanols are linked to vincinal silanols. Only free silylic acids can be functionalised with silanes. Loadings are typically between 0.1 and 0.2 $mmolg^{-1}$, which is suitable for oligonucleotide synthesis.

Higher loading levels can be achieved by functionalising silica, which differs from CPG by having all surface silanols free and thus available for functionalisation. The difference in surface properties between CPG and silica arises from the difference in temperature used in their respective production processes. Loading of commercial silica range between 0.5 and 1.5 $mmolg^{-1}$ and they can be used as scavengers, catalysts and reagents.

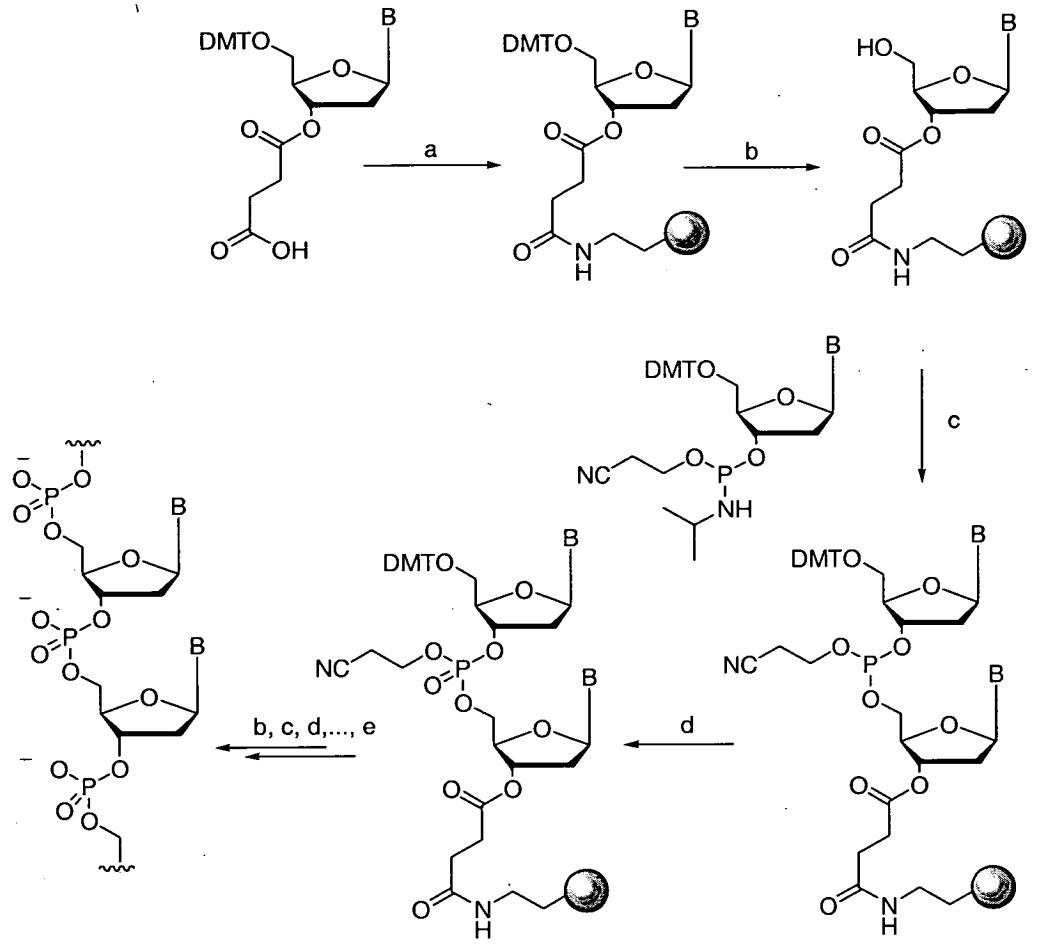
1.5 Solid phase approaches to phosphorylated nucleosides

The vast majority of nucleic acid chemistry on solid support reported is in the area of oligonucleotide synthesis, but there is also research into facilitating synthesis of phosphorylated nucleosides using solid phase approaches.

1.5.1 Synthesis of oligonucleotides

The first reported synthesis of an oligonucleotide reaches back to Todd and Michelson synthesis of a dinucleotide in 1955.[110] Despite some heroic efforts in developing new protecting groups, coupling agents and synthesis strategies, the single most important aspect of nucleic acid chemistry was the use of solid

supports for oligonucleotide synthesis.[111] The first report in 1965 used the phosphotriester approach,[112] but this chemistry has now been superseded by phosphoramidite chemistry (Scheme 1.22).



(a) aminopropyl-CPG; (b) 3 % trichloroacetic acid; (c) condensation; (d) I_2 in pyridine/H_2O; (e) NH_3 in H_2O

Scheme 1.22 Synthesis of oligonucleotides using phosphoramidite chemistry

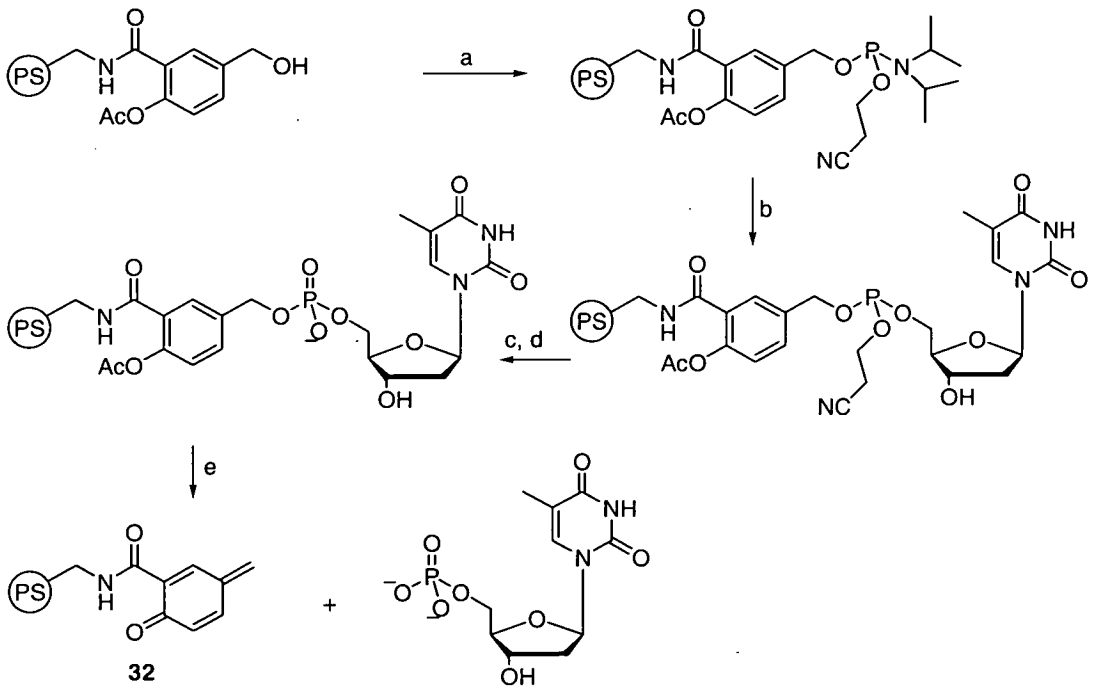
The synthesis is most commonly performed on CPG[113] or macroporous polystyrene.[114] The beads are coupled to the linker on the starting nucleoside, then after 5'-OH deprotection, the resin is treated with a phosphoramidite monomer. Typically, the phosphate and the bases are protected with base labile protecting groups. After successful coupling, the phosphite triester is oxidized to the phosphate triester and the next coupling cycle can start. After the required amount of cycles, the oligonucleotide is cleaved and deprotected (Scheme 1.22).

This chemistry is used most widely for oligonucleotide synthesis and 50mers are no longer a synthetic challenge. This was made possible with the development of automated synthesizers and readily available and cheap starting materials.

1.5.2 Solid phase approaches to phosphorylated nucleosides

1.5.2.1 *Monophosphates*

Access to nucleoside and carbohydrate monophosphates is a requirement for studying fundamental biological pathways. A supported monophosphitylating procedure for synthesising monophosphates has been developed by Parang *et al.*[115] The method is based on phosphoramidite chemistry and involves construction of an activated monophosphite on the resin. The resin was loaded with the alcohol, then oxidised, deprotected and cleaved to yield the monophosphate in 41-67 % yield (Scheme 1.23). The trapped methine quinone **32** can be recycled and reused.



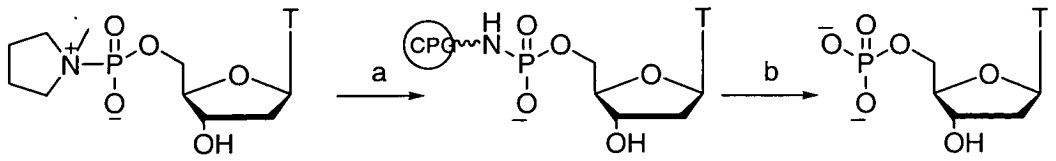
(a) $(^{i}Pr)_2NP(Cl)OCH_2CH_2CN$, DIPEA, THF; (b) dT, THF, DMSO, 1*H*-tetrazole, 24 h; (c) $^{t}BuOOH$, THF, 1 h; (d) DBU, THF, 48 h; (e) 50:50 TFA:CH_2Cl_2, 30 min; 55 % yield

Scheme 1.23 Synthesis of nucleoside monophosphate on solid support

Another solid-supported mono-phosphitylation reagent was published by the same group, relying on a similar principle.[116]

1.5.2.2 *Immobilisation of nucleotide monophosphates*

Borch *et al.* have applied their method for triphosphate synthesis (discussed in 1.3.5.3) also to nucleotide monophosphates.[117] The charged pyrrolidinium **10** was intercepted with nucleophilic amine immobilised on CPG to yield the immobilised nucleotide **33** (Scheme 1.24). It could be released in acidic hydrolysis conditions.



10 33

(a) CPG-NH_2, TEA, THF, 15 h; (b) 50:50 HCO_2H:H_2O, 8 h; 55 % yield based on loading

Scheme 1.24 Immobilisation and release of a nucleotide monophosphate

1.5.3 Solid phase approaches to nucleoside triphosphates

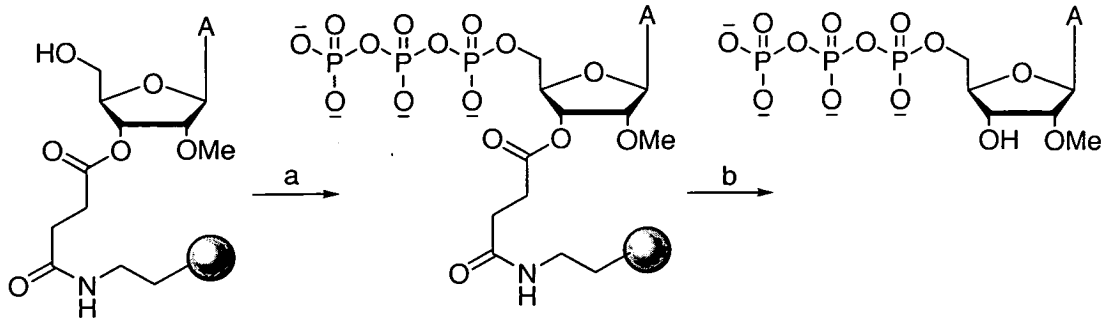
The shortcomings of the triphosphate syntheses described in Section 1.3, have been recognised by many researchers. An efficient and general synthesis of triphosphates would indeed be a very desirable procedure. Several methods have been reported previously to utilize resins for more efficient triphosphate synthesis.

There are two approaches to immobilise the nucleoside onto the resin: (i) attachment *via* a linker to the sugar or base or (ii) attachment *via* the alcohol to release the triphosphate from the resin after formation. Both approaches will be discussed in turn and their relative merits highlighted.

1.5.3.1 *Attachment* via *a linker*

The use of a linker implies that there is a functional group on the nucleoside that can be involved in this bond. This condition has resulted previously in utilising the 2' or 3' positions on the sugar for linker attachment. This unfortunately excludes all dideoxy and dideoxy-didehydro nucleoside analogues, thus such a method can never be universal. Nonetheless it has been shown to be valuable for synthesis of specific groups.

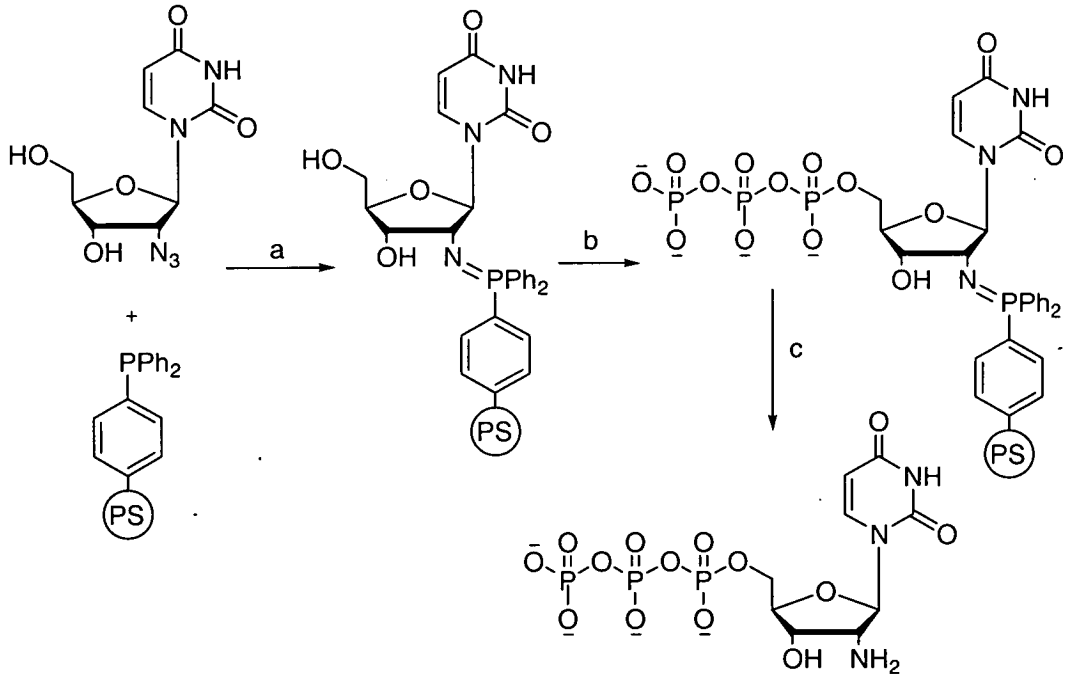
2'O-methyl RNA triphosphates were accessed by Gaur *et al.* by applying the linker and resins commonly using in oligonucleotide synthesis.[118] The 3' hydroxyl group was functionalised with a succinimidyl linker which was then attached to amino-propyl functionalised CPG, then the resin was deprotected and yielded the supported nucleoside triphosphate under Ludwig-Eckstein conditions. The triphosphate was released from the resin using ammonolysis at elevated temperature in good yield (Scheme 1.25).



(a) (i) **11**, py/THF, (ii) pyrophosphate, (iii) I_2 in pyridine/H_2O; (b) NH_3/H_2O, 50 °C, 2 h

Scheme 1.25 Gaur's method of supported synthesis of triphosphates

2'-amino triphosphates were synthesised by Schoetzau *et al.* from their 2'-azido analogues in a one pot triphosphorylation/reduction sequence.[119] The method was based on Staudinger chemistry, where the azide was immobilised on polystyrene-triphenylphosphine resin, phosphorylated according to the Ludwig-Eckstein method and cleaved to yield the triphosphate (Scheme 1.26).



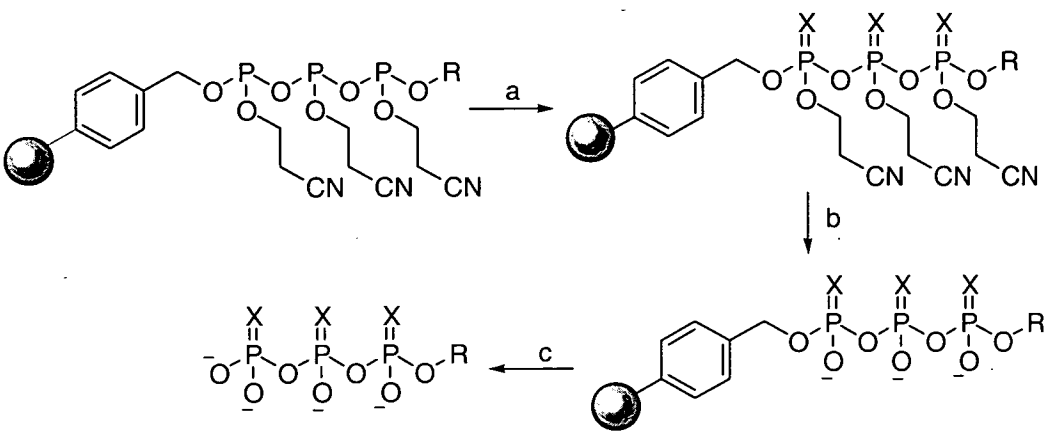
(a) dioxane; (b) (i) **11**, py/THF, (ii) pyrophosphate, (iii) I_2 in pyridine/H_2O, (iv) H_2O; (c) NH_3 in H_2O

Scheme 1.26 Schoetzau's method for synthesis of amino-nucleoside triphosphates

1.5.3.2 *Attachment* via *the alcohol*

This approach has the advantage that it could be applicable to all molecules of interest as it does not rely on functionality to attach the linker. Parang *et al.*[120],

published an example of such a method recently. It is analogous to the method discussed in 1.5.2.1 and utilises phosphoramidite chemistry, where a cyanoethyl protected triphosphite phosphoramidite is constructed on the resin, which is then treated with an alcohol (R-OH), oxidised either to the triphosphate or the tri-thiophosphate, deprotected and cleaved to yield carbohydrate or nucleoside triphosphates in typically 45 – 80 % yields. While this method has been shown to work for simple molecules such as unmodified nucleosides and carbohydrates, this method has not been demonstrated to be applicable to molecules with more elaborate functionalities.



(a) ^{t}BuOOH, THF (X=O) or Beaucage's reagent, MeCN (X=S); (b) DBU, THF; (c) TFA, DCM, H_2O

Scheme 1.27 Parang's method for supported synthesis of carbohydrate and nucleoside triphosphates

1.6 Aims of the project

As shown in the above section (1.3), a range of triphosphate syntheses have been developed which indicates, quite rightly, that none of them is wholly satisfactory. There is a strong need for a general triphosphorylation reagent which enables a straightforward conversion of any alcohol, especially nucleosides to their triphosphate. Using an excess of phosphorylating reagent, then even more sluggish substrates will be able to be pushed to completion.

The aim of the project was to develop such a phosphorylating reagent and we sought to develop it as a supported reagent to take advantage of the ease of purification and the chance to drive reactions to completion. The chemistry will be based on the Ludwig-Eckstein method (section 1.3.7.1), as it is the most well established technique in triphosphate synthesis.

To transpose the chemistry from solution to solid phase, a suitable resin has to be designed and synthesised. The target will be a resin coupled to 2,4-dihydroxy benzoic acid which can then be converted to the supported Ludwig-Eckstein reagent (Figure 1.25). An ether bond was chosen to link the resin to the salicylic acid linker, as it was judged to be most suitable to resist Lewis acids such as PCl_3.

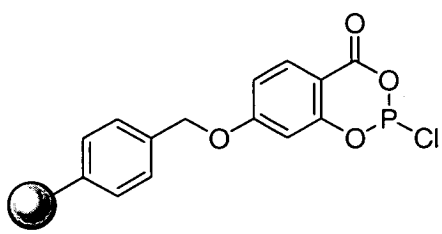


Figure 1.25 Supported Ludwig-Eckstein based resin

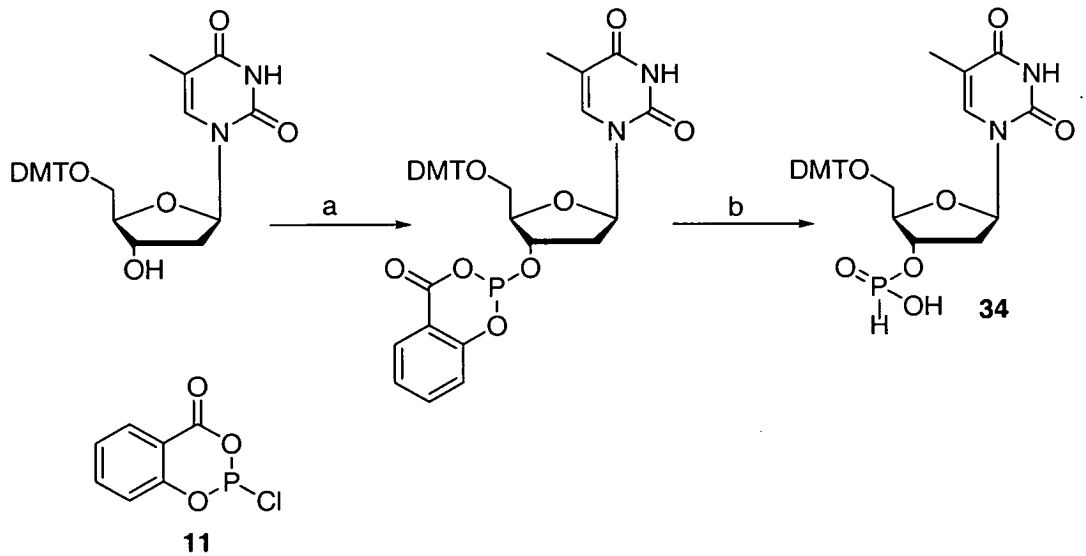
Different resins are available for developing a supported reagent, so different supports will be investigated with the hope to identify the properties required from the resin to give an optimal yield.

2 Development of a supported Ludwig-Eckstein reagent

2.1 The Ludwig-Eckstein reagent

2.1.1 Introduction

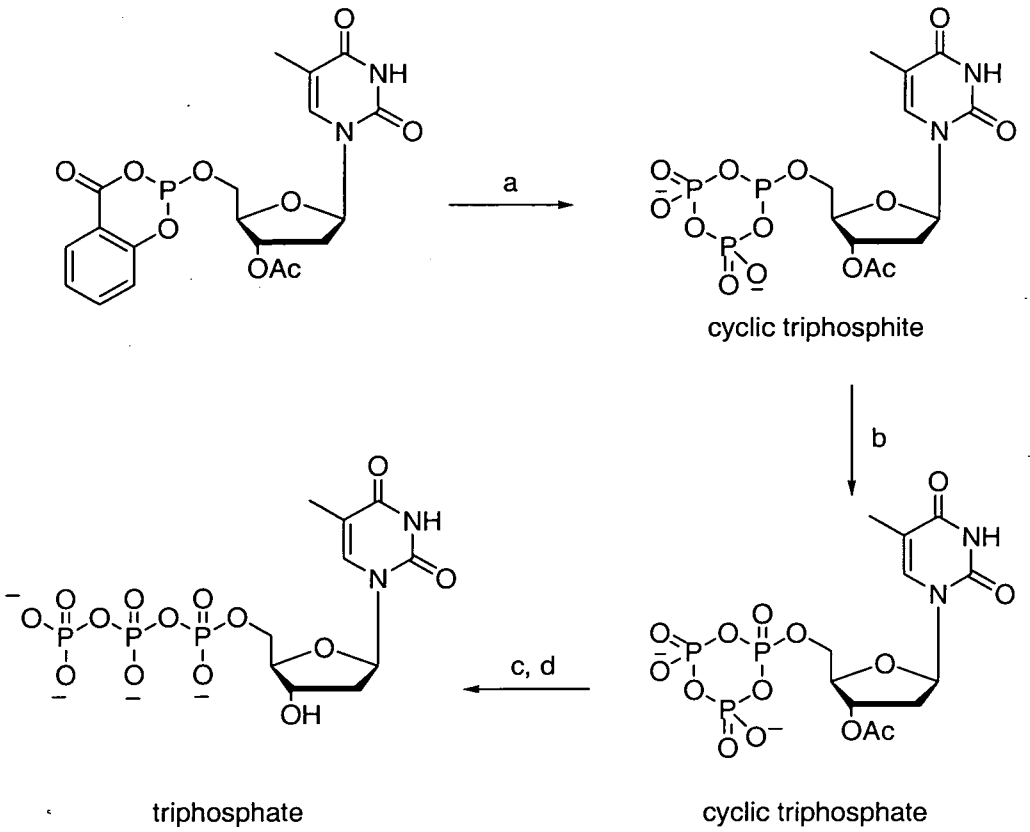
The Ludwig-Eckstein reagent **11** was first synthesised in 1887 by Anschütz *et al.*[121] but its structure and fundamental chemistry were only established in 1952 by Young.[122] Its first application to nucleoside chemistry was reported by Marugg *et al.*[123] for the synthesis of 3'O-mono-*H*-phosphonate **34**. (Scheme 2.1)



(a) 1.25 eq **11**, 5 min; (b) H_2O

*Scheme 2.1 Synthesis of 3'O-H-phosphonates using **11***

The full scope of the reagent was realised and exploited by the seminal work of Ludwig and Eckstein who recognised that the phosphitylated alcohol could undergo two further substitutions.[83] Pyrophosphate could attack twice on the P(III) centre, yielding a cyclic triphosphite which could then be oxidised to a cyclic triphosphate. Hydrolysis, followed by ammonolysis gave access to thymidine triphosphate in a 72 % overall yield after Sephadex DEAE purification and lyophilisation. (Scheme 2.2)

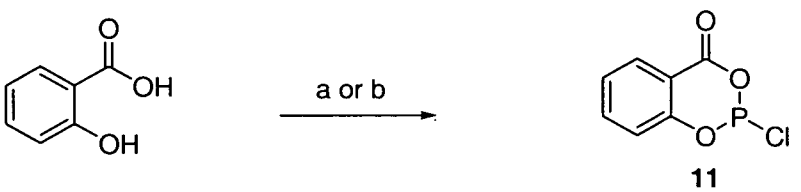


(a) pyrophosphate; (b) I_2 in pyridine/H_2O; (c) H_2O; (d) NH_3 in H_2O

Scheme 2.2 Triphosphate synthesis according to Ludwig and Eckstein

2.1.2 Synthesis

Two reaction conditions have been reported for the formation of the Ludwig-Eckstein reagent.[124,125] The first method refluxes phosphorus trichloride with salicylic acid in the presence of a dry solvent. In the second method, salicylic acid is dissolved in a dry solvent with pyridine which is then added to a cooled solution of PCl_3. During the course of my PhD, both methods were reproduced successfully to prepare reagent **11** (Scheme 2.3). The reagent is also available commercially, but it was found to be of variable quality and direct synthesis was preferred.



(a) 2 eq PCl_3, THF, 60 °C, 2 h, 80%; (b) 1 eq PCl_3, 2 eq pyridine, THF, -15 °C to rt, 15 h, 45 %

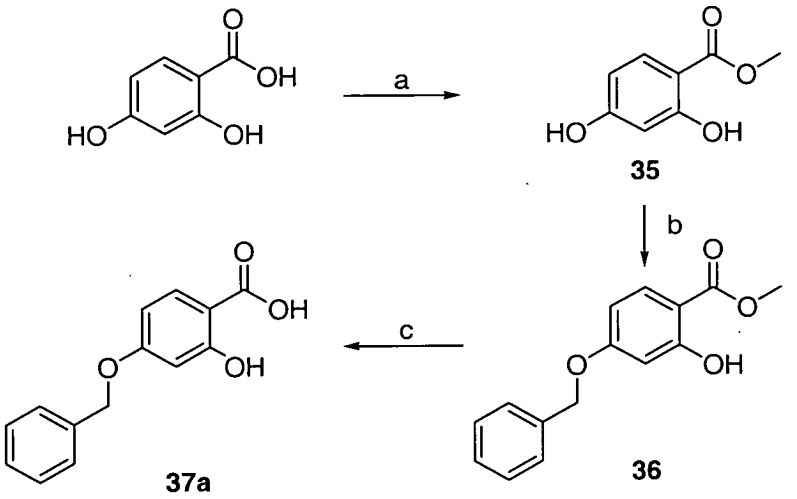
Scheme 2.3 Synthesis of the solution phase reagent

Using method (a), **11** was isolated using vacuum distillation to remove THF, then excess phosphorus trichloride to furnish the desired compound. Using method (b), distillation was preceded by a filtration to remove precipitated salts. In both cases, care had to be exercised to keep the isolated product air and moisture-free, as **11** could be readily oxidised and hydrolysed.

2.2 Solution phase model

2.2.1 Benzylation of 4-hydroxy salicylic acid

In a solution model, the selectivity of the benzylation of 2,4-dihydroxy benzoic acid was assessed *via* methyl ester protection of the acid as *per* Astles *et al.*[126] followed by benzylation under Finkelstein conditions.



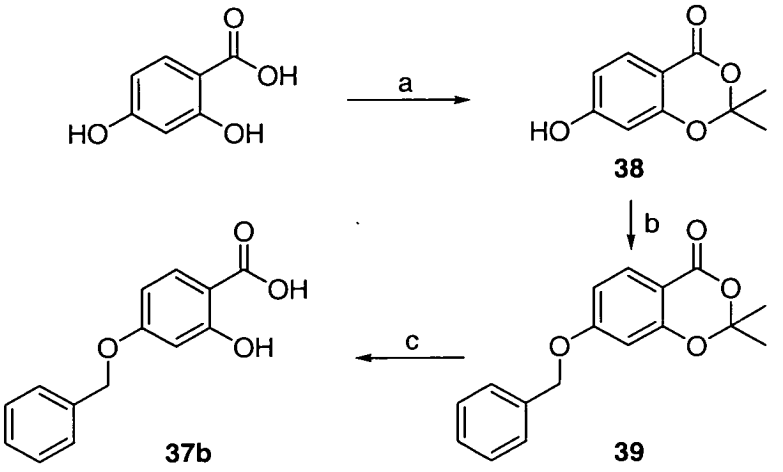
(a) MeOH, conc. H_2SO_4, reflux, 66 h, 79 %; (b) BnCl, K_2CO_3, KI, TBA-Cl, acetone, reflux, 10 h, 72 %; (c) 2 M NaOH, dioxane, reflux, 5 h, 91 %

Scheme 2.4 Benzylation via *a methyl ester derivative*

NOESY experiments on the methyl ester **36** to determine which regio-isomer was formed; therefore a second route was explored *via* an acetonide protection of the carboxylic acid and the 2-hydroxy group. (Scheme 2.5)

Acetonide **38** was accessed using dehydrating conditions[127] and the benzylation of the *para*-OH group was readily performed *via* a Mitsunobu reaction.[128] The deprotection of the acetonide was not straightforward as classical methods for acetonide deprotection, such as refluxing 2 M HCl, or refluxing 2 M NaOH gave low yields of the desired product (typically <20 %) with the remainder of the

material producing an unidentified side-product, but presumably a poly-ester. Deprotection was finally achieved in good yield using a method of Danishefsky *et al.*[129] The compounds synthesised by both routes were analytically and spectroscopically identical.



(a) Acetone, Piv_2O, cat. H_2SO_4, 10 h, 47 %; (b) BnOH, DIAD, PPh_3, THF, 0°C to rt, 15 h, 96 %; (c) 48 % KOH, DMSO, 60 °C, 30 min, 85 %

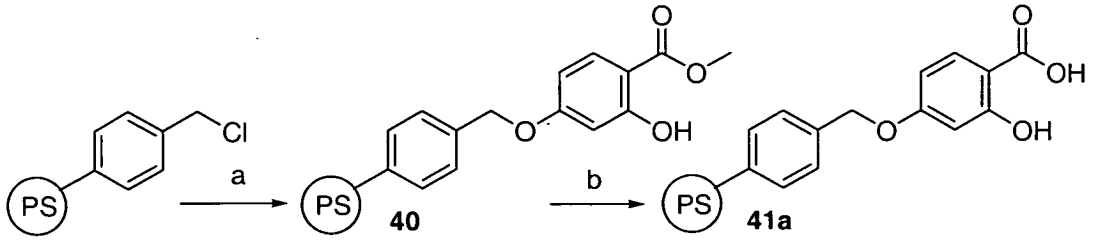
Scheme 2.5 Benzylation via *an acetonide derivative*

2.3 Synthesis of supported reagent on microporous PS

Different resins were evaluated as supports for the linker as their different properties were expected to fundamentally influence the chemistry.

2.3.1 Attachment of the linker to microporous PS resin

Microporous polystyrene (1 % DVB cross-linking, 75-150 μm, 1.75 mmolg^{-1}) was chosen as the first resin type to explore the chemistry developed above to allow immobilisation of the desired linker **35**. (Scheme 2.6)



(a) 3 eq **35**, acetone, K_2CO_3, KI, 60 °C, 2 d; (b) $Bu_4N^+OH^-$, THF, 60°C, 2 d, then 2 M HCl

Scheme 2.6 Synthesis of supported 2-hydroxy benzoic acid using Finkelstein conditions

The reaction was monitored by ^{13}C Gel-Phase NMR and after 2 days, the Finkelstein coupling of the phenol had gone to completion. (^{1}H MAS NMR was also attempted, but the observed signals obtained were too broad to be useful.) The deprotection conditions were modified and tetrabutylammonium hydroxide was chosen over NaOH as a deprotection reagent, as it is more suitable for resin chemistry. Acidification of the carboxylate was found to be essential to enable the resin construct to be visible by Gel-Phase NMR.

Comparison of the Gel-Phase NMR of PS-supported 2-hydroxy benzoic acid **41** and 4-benzyloxy-2-hydroxy benzoic acid **37** (Figure 2.1) showed all the expected signals. Additional signals were assigned to the polystyrene resin backbone (40-50, 125-130 ppm) and residual Et_2O (15, 66 ppm).

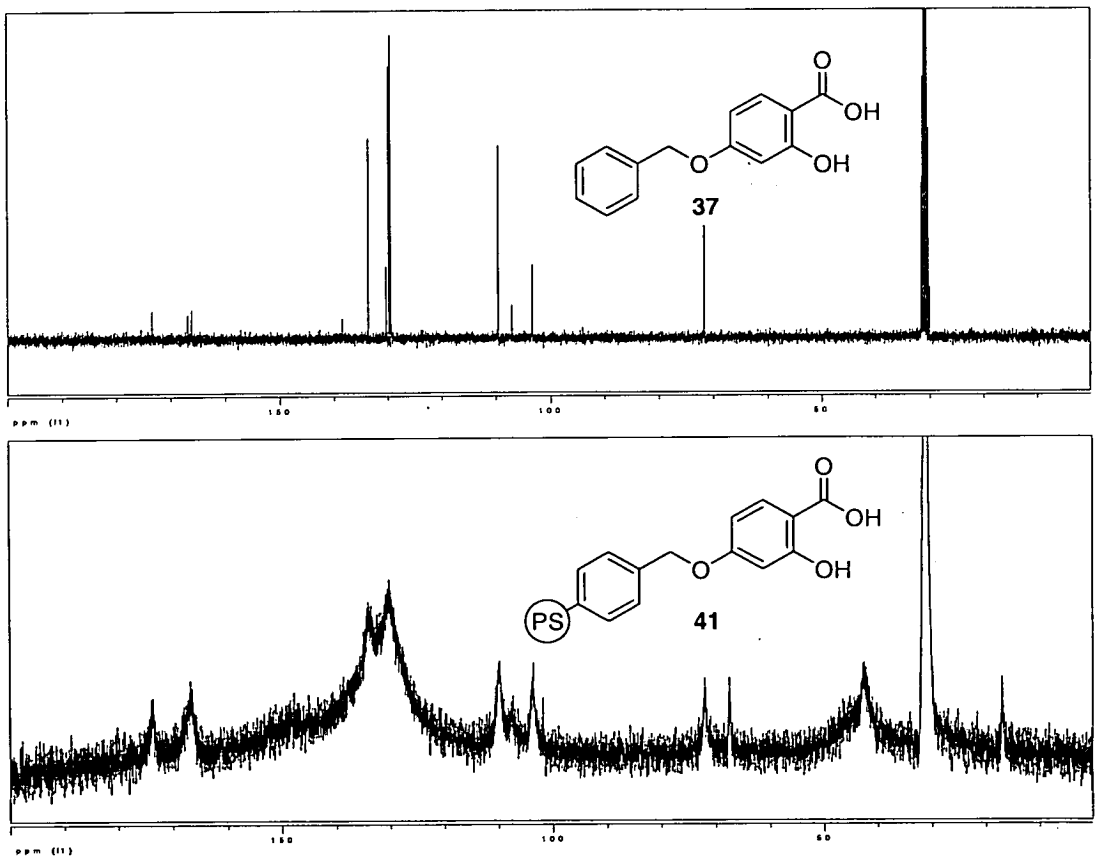
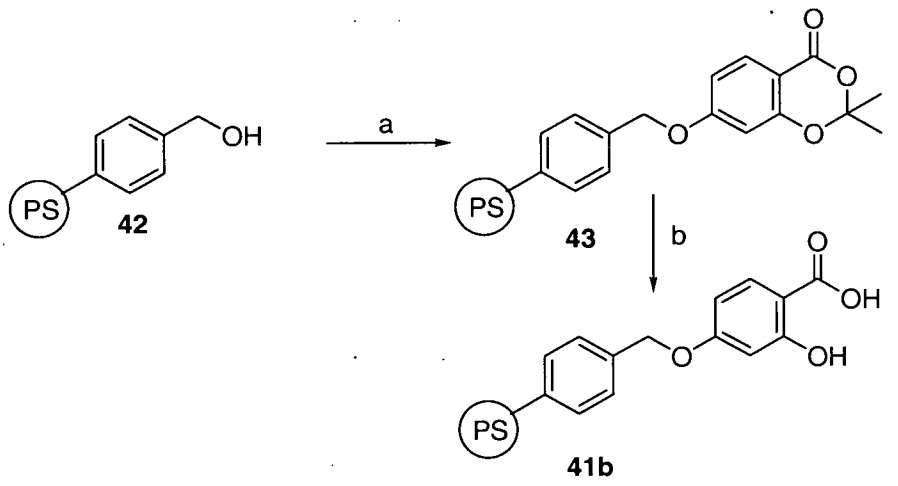


*Figure 2.1 ^{13}C Gel Phase NMR spectra of **37** and **41** in d_6-acetone*

Different solvents were used for the gel-phase NMR studies. The ideal solvent requires swelling of the resin to enable recording of the spectra, however if swelling is too large, lower concentrations will be exposed to the magnetic field, thus 'diluting' the signal. $CDCl_3$ usually fulfils these requirements adequately,

however one drawback of its use with PS, is the fact that the resin floats, thus excess solvent needs to be removed from the bottom of the NMR tube, which can be difficult without introducing air bubbles into the resin bed. d_6-acetone was found to be a suitable substitute, as the resin sinks in it, while still giving adequate signals.

The alternative route using an acetonide was also transposed to the solid phase. Hydroxymethyl PS resin **42** was treated with acetonide **38** under Mitsunobu conditions. After 15 h, the coupling was not complete, as judged by Gel-Phase NMR, but was pushed to completion after 2 days. The deprotection was incomplete after 3 h, but after a total of 7 h, the resin was fully deprotected. (Scheme 2.7)



(a) 5 eq **38**, PPh_3, DIAD, THF, 2 d; (b) 48 % KOH, THF, 60 °C, 7 h, then 2 M HCl

Scheme 2.7 Synthesis of supported 2-hydroxy benzoic acid using Mitsunobu conditions

Both routes were shown to be viable, however the route *via* the methyl ester protection was preferred as the deprotection step was less likely to cause problems and this route was used in all further resin functionalisations, where a methyl ester protection of the carboxylic acid was appropriate.

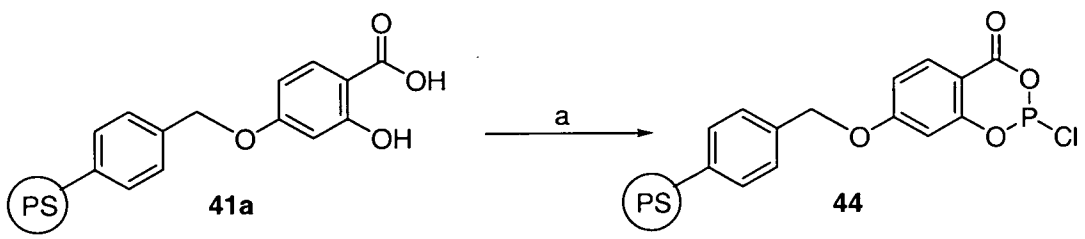
2.3.2 Synthesis of a solid-supported Ludwig-Eckstein reagent

The literature presents two methods for preparing the reagent, thus it was important to investigate both methods for transposition to the solid phase. The adaptation to the support requires taking into account reactions that have longer reaction times on the resin, while on the other hand purification can be greatly simplified as excess reagents can simply be washed off, provided that the resin is adequately

swollen, the washing steps are thorough and the by-products are soluble in the solvent used for rinsing the resin.

2.3.2.1 *Method A: refluxing PCl_3*

Method A: Refluxing PCl_3 with salicylic acid in a dry solvent for 15 h (Scheme 2.3a). To adapt it to the resin, it was found that the easiest approach experimentally was to heat the resin in a syringe immersed in an oil bath. After formation of the reagent, the resin was rinsed with dry solvents using positive nitrogen pressure.



(a) 4 eq PCl_3, THF, 2 h, 60 °C

Scheme 2.8 Formation of Ludwig-Eckstein reagent on the resin using method A

The supported Ludwig-Eckstein reagent was formed successfully after reaction for 2 h in dry THF with an excess of PCl_3 at 60 °C (Scheme 2.8). Resin **44** was characterised by ICP (phosphorus content) and combustion analysis (chlorine content). The reaction was performed at 2 different concentrations of phosphorus trichloride; at both levels, the conversion based on phosphorus was excellent (90 % from the starting Merrifield resin). The accuracy of the chlorine analysis was however less convincing, especially as a higher loading was obtained for a reduced concentration. (Table 2.1)

Table 2.1 Combustion results for formation of reagent on resin

Entry	Time (h)	Conc. (M)	P (%)	P ($mmolg^{-1}$)	yield (%)[a]	Cl (%)	Cl ($mmolg^{-1}$)	yield (%)[a]
1	2	0.8	3.45	1.11	90	1.72	0.485	40
2	2	0.27	3.45	1.11	90	2.29	0.646	53

[a] *theoretical yield 1.23 $mmolg^{-1}$ based on manfacturer's loading of Merrifield resin*

To verify the microanalysis data, resin **44** was also characterised by ^{31}P Gel Phase NMR. $CDCl_3$ was used as a solvent to reduce hydrolysis expected from the use of d_6-acetone. Gratifyingly, it showed the expected signal centered at 149 ppm (see Figure 2.2). A second signal at -2.5 ppm can be attributed to a hydrolysis product. Integration shows 82 % of the correct ^{31}P species on the resin. The lower than

expected chlorine result was thus not reflected in the NMR data, suggesting hydrolysis regarding the Cl microanalysis of the reactive material.

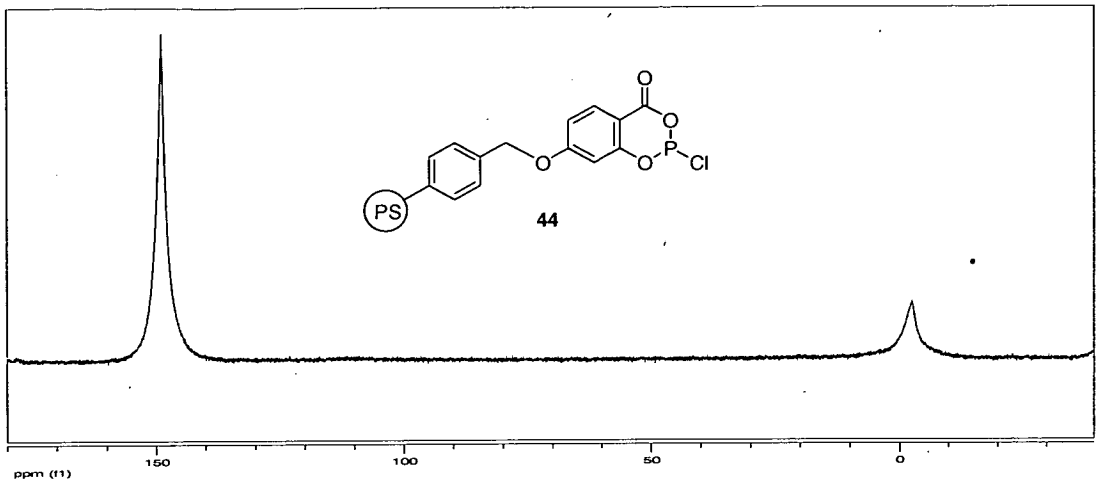


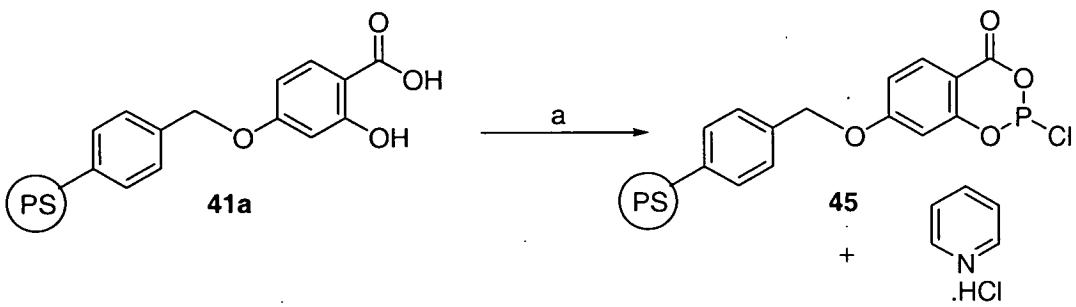
*Figure 2.2 ^{31}P Gel Phase NMR of resin **44** in $CDCl_3$ at 145 MHz*

Thus it could be concluded that, using method A, the solid-supported Ludwig-Eckstein reagent could be successfully synthesised from Merrifield resin in three steps in an overall 90 % yield, based on P loading.

2.3.2.2 *Method B: PCl_3 in pyridine at room temperature*

The results using method A looked very promising, however refluxing with PCl_3 may not be suitable for other resins; therefore an alternative method was also investigated.

Method B in solution is performed by treating salicylic acid with pyridine at -10 °C, followed by the addition of phosphorus trichloride. As cooling resins requires greater manipulation and, more crucially, makes dry conditions difficult to maintain, formation of the resin using this method was performed at rt.



(a) 4 eq PCl_3, 16 eq py, THF, rt, 0.8 M

Scheme 2.9 Formation of Ludwig-Eckstein reagent on the resin using method B

This method produces pyridinium hydrochloride as a by-product. (Scheme 2.9) It was found that it cannot be removed from the resin, as all solvents which would dissolve it (H_2O, MeOH, EtOH) would hydrolyse the acid chloride on the resin.

While this causes problems with characterisation, the presence of pyridinium hydrochloride salt was hoped to be of no significance in the loading and cleavage reactions. The resin produced using method B was thus not characterised at this stage.

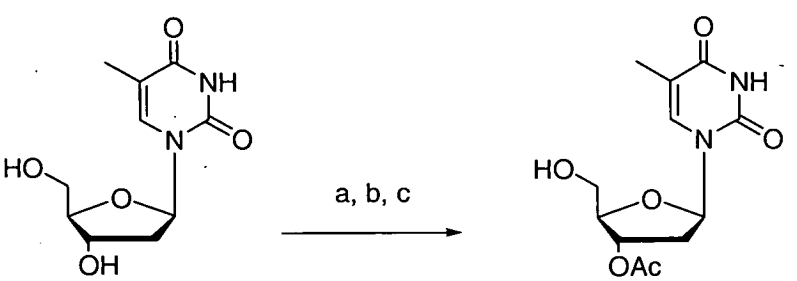
The conditions required for complete substitution of the chlorine were evaluated using the attachment of a nucleoside, followed by nucleophilic attack of water for resins prepared *via* both methods (see sections 2.3.4 and 2.3.5).

2.3.3 Synthesis and characterisation of nucleoside loaded resin

2.3.3.1 *Synthesis of model nucleoside to assess the supported reagent*

Ludwig-Eckstein derived reagents were tested using a simple nucleoside. Thymidine **46** was chosen, since 2'-deoxy riboses are less susceptible to hydrolysis than riboses.

The Ludwig-Eckstein reagent has been shown not to be selective for the primary 5'-OH over the secondary 3'-OH,[83] thus thymidine **46** was protected by selective reaction on the primary 5'-OH group with DMT-Cl, followed by acetylation of the 3'-OH group, with 3'-OAc-T **49** was obtained by acidic hydrolysis of the DMT group (Scheme 2.10).



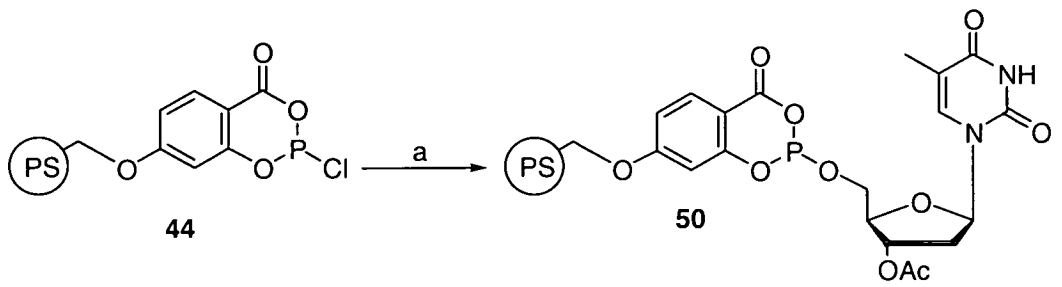
46 **49**

(a) DMT-Cl, pyridine, 2 h, 93 %; (b) Ac_2O, DMAP, TEA, pyridine, 45 min, 99 %; (c) MeOH:TFA 5:1, 1 h, 54 %

*Scheme 2.10 Synthesis of 3'-OAc-T **49***

2.3.3.2 *Loading of the solid-supported Ludwig-Eckstein reagent*

The synthesis and characterisation of the resin was based on the supported reagent derived *via* Method A. To enable full loading of the nucleoside on the resin, supported reagent **44** was derivatised by displacement with 2 eq of 3'-OAc-T **49** at 0.27 M in THF (Scheme 2.11) to yield **50** (Table 2.2).



(a) 2 eq 3'-OAc-T **49**, THF, 0.27 M, see table

Scheme 2.11 Displacement of Ludwig-Eckstein reagent on the resin with 3'OAc-T

Usually, the nucleoside would have been dissolved in pyridine:THF (1:3), so that HCl generated in the attachment is neutralised to form pyridinium hydrochloride. This was not suitable in this case, as its presence would have distorted the analysis.

Table 2.2 Combustion results for displacement reaction

Entry	Time (min)	Conc. (M)	P (%)	P ($mmolg^{-1}$)	yield (%)[a]
1	60	0.27	2.67	0.861	91

[a] *theoretical yield for coupling 0.945 mmolg*$^{-1}$

The nucleoside loaded resin was rinsed with THF and dried. ICP analysis of the phosphorus content indicated a loading 0.861 $mmolg^{-1}$ (*cf.* starting loading on Merrifield resin 1.75 $mmolg^{-1}$). Nucleoside derived resin **50** was also characterised by ^{31}P Gel-Phase NMR and showed the expected signal centered at 127 ppm and all reactive sites functionalised as judged by the absence of a signal at 148 ppm (see Figure 2.3).

The observed signal was broad, as resin **50** is a diasteromeric mixture at phosphorus; the ^{31}P NMR signals have been reported at 127.6 ppm and 126.3 ppm respectively.[83] Integration of all ^{31}P NMR signals gives 82 % of nucleoside loading on the phosphorinone species. Peaks in the 7 to 11 ppm range can be attributed to hydrolysis products, affected by residual water present in $CDCl_3$.

It can therefore be concluded that the supported Ludwig-Eckstein reagent was successfully loaded with a deoxy-nucleoside.

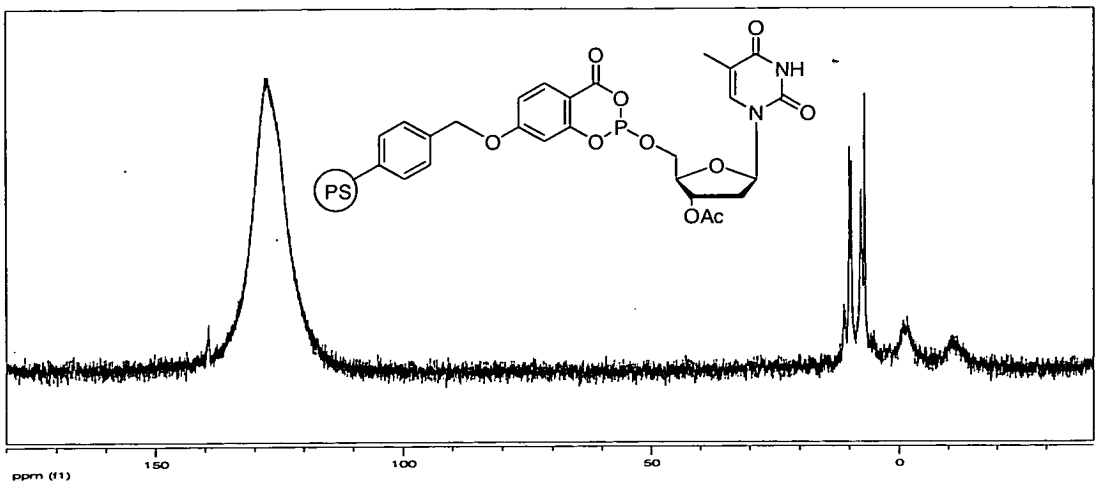


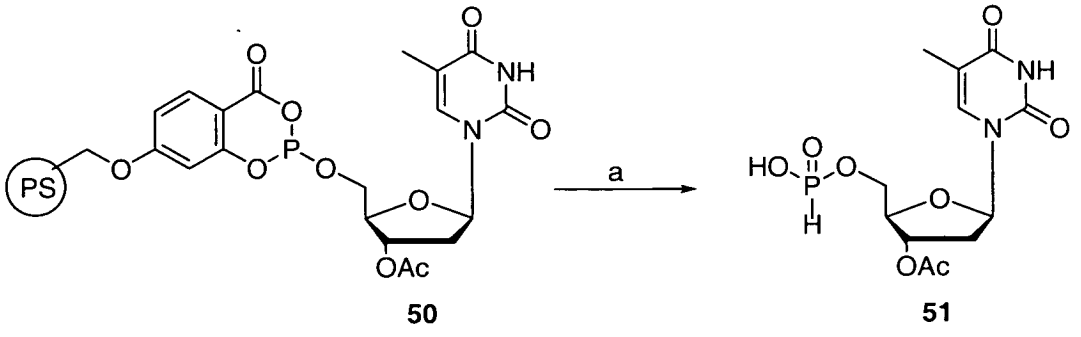
*Figure 2.3 ^{31}P Gel Phase NMR of resin **50** in $CDCl_3$ at 145 MHz*

2.3.4 Cleavage of nucleoside loaded resin

Several ways were considered for following progress of the reactions on the resins and subsequent product analyses. ^{31}P NMR of cleaved product mixtures looked promising, however, while the signals are indicative of the phosphorus group, they have little dependence on an alkyl group attached to them, thus for example the difference in the ^{31}P NMR signal between a phosphonate salt and mono-*H*-phosphonate **51** is about 1 ppm, which leads to inconclusive results. Therefore HPLC was found to be the best option, by comparing crude reaction mixtures to known standards.

2.3.4.1 *Cleavage using water*

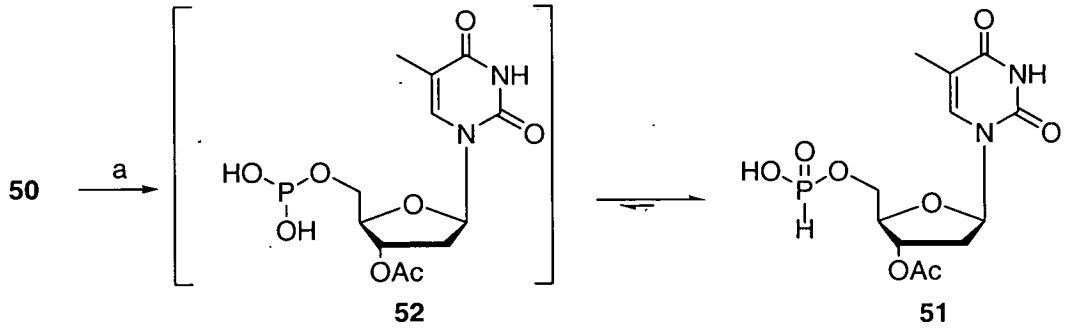
Nucleophilic attack of water on the nucleoside loaded resin **50** should yield mono-*H*-phosphonate **51** (Scheme 2.12). This was exploited to indirectly optimise formation of the Ludwig-Eckstein reagent on the resin and attachment of the nucleoside to it. Formation of mono-*H*-phosphonate **51** was monitored by RP-HPLC on a C18 column eluting with a 0.1 M TEAB/MeCN gradient (see experimental section for details).



(a) double H_2O attack

*Scheme 2.12 Hydrolysis of nucleoside loaded resin **50** to yield mono-H-phosphonate*

The immediate product of the cleavage reaction is thought to be the triphosphite monoester **52**. As the P=O double bond is thermodynamically more stable than the P-O single bond, the triphosphite tautomerises to the favoured tetrahedral *H*-phosphonate form, which is the one that was isolated (Scheme 2.13).

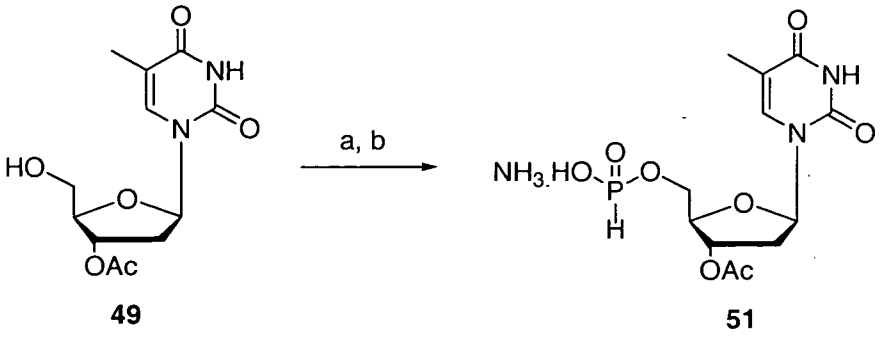


(a) H_2O

Scheme 2.13 Tautomerism of triphosphite monoester and mono-H-phosphonate ester

2.3.4.2 *Synthesis of mono-H-phosphonate **51** in solution phase*

To enable monitoring of the formation of mono-*H*-phosphonate **51**, it was synthesised in solution using the method reported by Marugg *et al.* (Scheme 2.14).[123] Several methods were attempted for the purification of **51**. Silica chromatography eluting with a MeOH/CH_2Cl_2 gradient has been reported for a protected guanosine analogue[130] and a DMT protected thymidine analogue[131], however in this instance the increased hydrophilicity of **51**, rendered elution impossible even with gradients up to 30:70 MeOH:CH_2Cl_2. Sephadex DEAE ion exchange chromatography was investigated as an alternative; at ionic strengths as low as 0.05 M TEAB neither salicylic acid, nor mono-*H*-phosphonate **51** could be retained on the column.



49 **51**

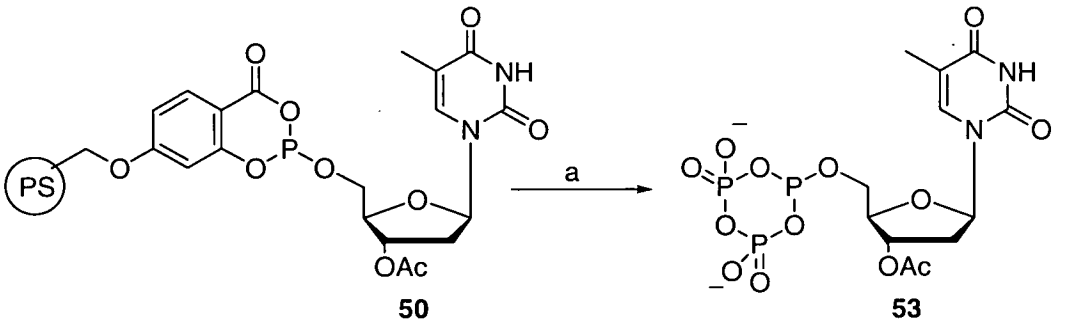
(a) 1.5 eq **11**, py/THF, 10 min; (b) H_2O, 5 min; 23 %

Scheme 2.14 Solution phase preparation of the mono-H-phosphonate 51

The mono-*H*-phosphonate **51** was purified eventually using Prep HPLC on an Xterra C18 column. Purification was found to be easier when changing the buffer from TEAB to NH_4OAc, as separation from residual starting material 3'-OAc-T **49** was greater. The product was isolated as its ammonium salt in good yield after excess buffer had been removed by lyophilisation.

2.3.4.3 *Cleavage using pyrophosphate*

Nucleoside resin **50** can be used in a more synthetically useful way and yield cyclic triphosphite after nucleophilic attack by pyrophosphate (Scheme 2.15).



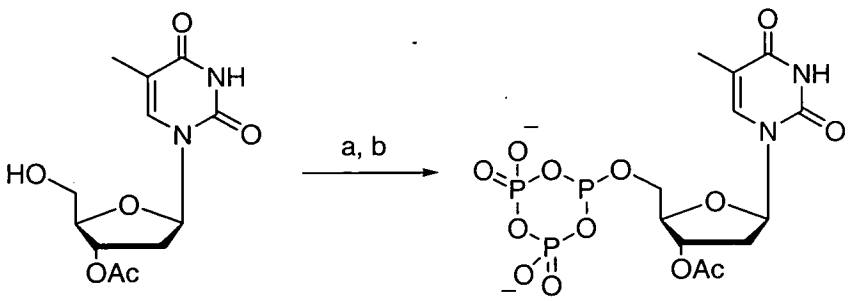
50 **53**

(a) pyrophosphate ion

Scheme 2.15 Cleavage with pyrophosphate of nucleoside resin 50

2.3.4.4 *Synthesis of cyclic triphosphite 53 in solution phase*

The compound was first synthesised in solution to obtain an HPLC standard to monitor cleavage of the compound from the resin and optimise that step. Cyclic triphosphite **53** was synthesised as shown below (Scheme 2.16).

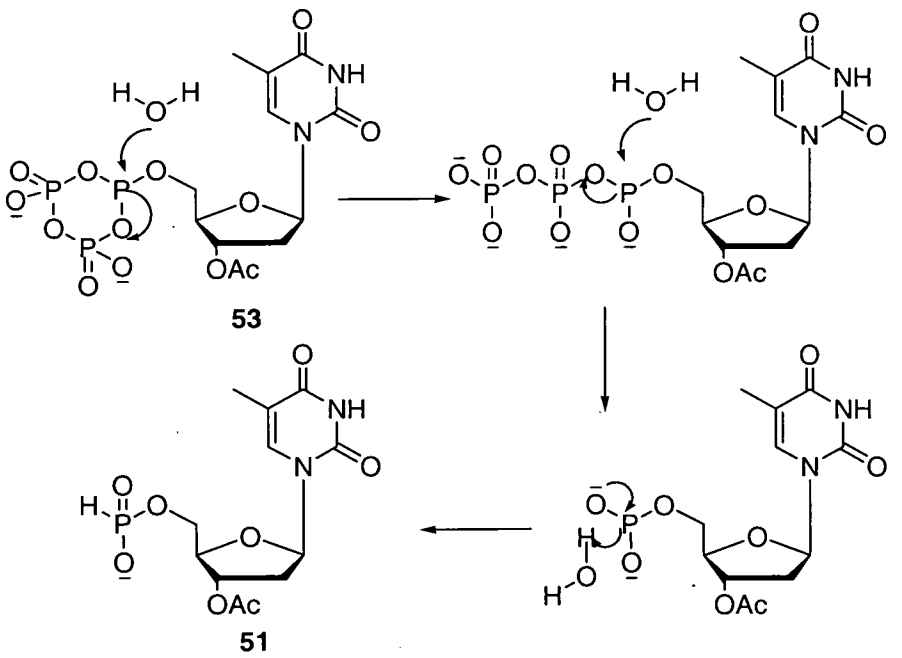


49 **53**

(a) 1.5 eq **11**, py/THF, 10 min; (b) 1.5 eq 0.5 M $(Bu_3N)_2$ pyrophosphate in DMF, 4 eq Bu_3N, 30 min; not isolated

Scheme 2.16 Solution phase preparation of cyclic triphosphite 53

^{31}P NMR of the crude reaction mixture showed the characteristic signals ^{31}P NMR signals of the triphosphite ring as reported by Ludwig and Eckstein[83] (105.7 (t), -19.1 (q), -19.3 (q)). However, subjecting the crude mixture to a CH_2Cl_2/0.1M TEAB extraction gave the mono-*H*-phosphonate **51**.



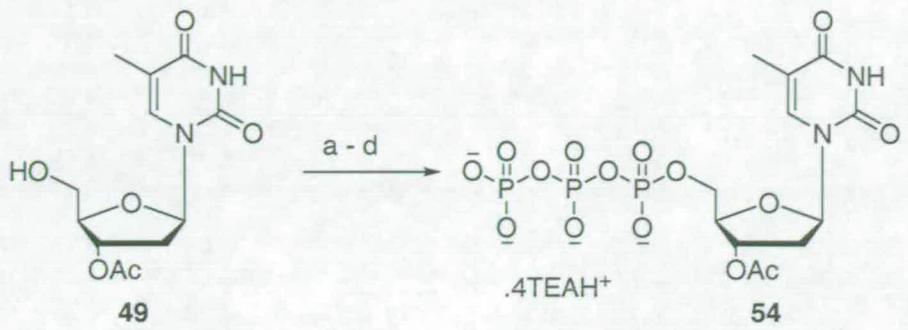
53 **51**

Scheme 2.17 Decomposition of cyclic triphosphite 53 to mono-H-phosphonate 51

It was thus postulated that the cyclic triphosphite **53** is susceptible to nucleophilic attack by water which leads to disproportionation to the mono-*H*-phosphonate **51** and pyrophosphate (Scheme 2.17). This can be rationalised as pyrophosphate is a good leaving group. This observation was corrobated by HPLC, as the chromatogram of the crude reaction mixture only showed mono-*H*-phosphonate and no additional peaks.

2.3.4.5 *Synthesis of triphosphate **54** in solution phase*

As it was not possible to monitor the direct cleavage product by HPLC, the next step was added before HPLC analysis. Thus, the cyclic triphosphite was oxidised with iodine in aqueous pyridine, followed by ring opening under aqueous conditions to yield 3'-OAc-dTTP **54** (Scheme 2.18). 3'-OAc-dTTP **54** was purified by Prep HPLC and isolated as its tetrakis [triethylammonium] salt after lyophilisation in 24 % overall yield.



(a) 1.5 eq **11**, py/THF, 10 min; (b) 1.5 eq 0.5 M $(Bu_3N)_2$ pyrophosphate in DMF, 4 eq Bu_3N, 30 min; (c) 1.5 eq 0.5 M I_2 in 98:2 pyridine:H_2O; (d) H_2O; 24 % yield

*Scheme 2.18 Solution phase preparation of 3'OAc triphosphate **54***

2.3.4.6 *HPLC traces of standards*

The HPLC traces of nucleoside derived compounds were monitored and recorded at 282 nm run using a gradient using 0 to 100 % MeCN in 0.1 M TEAB buffer over 25 min. HPLC traces for all standards run: 3'-OAc-T **49** (blue), mono-*H*-phosphonate **51** (red) and 3'-OAc-dTTP **54** (green) (Figure 2.4). It is important to note, that retention times shift, since the buffer is volatile which leads to pH changes.

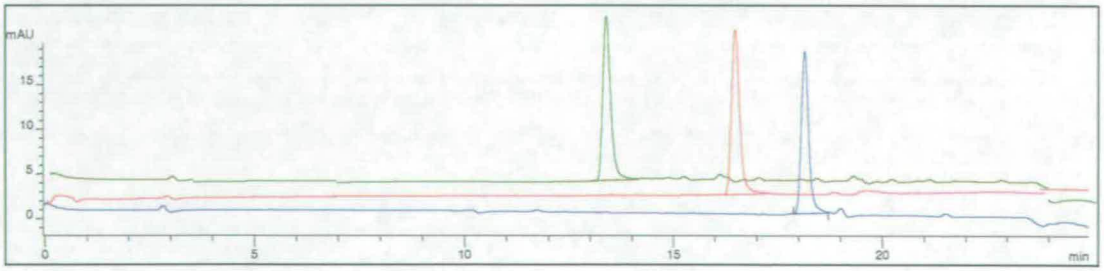


Figure 2.4 HPLC chromatograms of standards

2.3.5 Optimisations for cleavage of nucleoside resin with water

2.3.5.1 *General*

Optimisation of a reaction on the resin requires a choice of washing routine which removes excess reagents and by-products, while leaving the resin functionalisation intact. It was therefore important to use dry solvents throughout and filter the resin with positive nitrogen pressure to minimise premature hydrolysis from the salicylate linker. Optimisation reactions were carried out on 100 mg of resin and 3 eq of resin were used relative to the nucleoside, with all reagents stored in a desicator over P_2O_5 prior to use. The resin was washed with dry solvents prior to starting the reaction sequence.

2.3.5.2 *Swelling and rinsing time of resin*

Initial studies on the rinsing of resin **44,** after formation of the supported reagent, showed that too short a rinsing time caused side-products: ^{1}H NMR of the nucleoside after attachement and hydrolysis indicated racemisation at the H-1' position. Acetyl deprotected nucleoside was also isolated from some reactions. Both problems were eliminated when supported reagent **44** was swollen twice for 5 min in THF before loading the nucleoside.

2.3.5.3 *Attachment of nucleoside to supported reagent from method A*

For the preparation of the Gel Phase NMR samples of nucleoside resin **50** (2.3.3.2), the nucleoside was used in excess. This will however not be desirable in future, as the resin is more expendable. Furthermore the phosphorinone species formed on the resin after chloride displacement is a powerful electrophile able to react with an excess of alcohol. In the initial tests, the solvent volume used to swell the resin was maintained and nucleoside was added in the desired amount. However it soon became apparent that with a lowered concentration, reactions were not going to completion. It was necessary to dry the resin after formation of the supported reagent by flushing with nitrogen prior to adding the nucleoside solution, to reach a high enough concentration. The nucleoside was fully loaded when used at 0.1 M for 1 h, as judged by absence of starting material in the HPLC chromatogram (Table 2.3).

Table 2.3 Reaction conditions used for method A

Reaction	eq	Conc. (M)	Time (h)	Temp. (°C)	Outcome
Formation of reagent	4	0.27	2	60	-
Loading of 3'-OAc-T	0.33	0.10	1	rt	complete

2.3.5.4 *Attachment of nucleoside to supported reagent from method B*

The same reaction conditions as for the loading step were applied. Completion of the reaction was again judged by absence of starting material 3'-OAc-T **49** in the drained solution. The loading of the nucleoside did not now go to completion (Table 2.4). The reactions were more sluggish, which was probably due to pyridinium hydrochloride impeding the diffusion into and out of the polymer gel. It was decided at this point to pursue method A to form the supported reagent on the resin.

Table 2.4 Reaction conditions used for method B

Entry	Reaction	eq	Conc. (M)	Time (h)	Temp. (°C)	Outcome
1	Formation of reagent	4	0.27	0.25	rt	-
	Loading of 3'-OAc-T	0.33	0.10	1	rt	incomplete
2	Formation of reagent	4	0.27	2	rt	-
	Loading of 3'-OAc-T	0.33	0.10	1	rt	incomplete

2.3.5.5 *Isolation of products*

As the supported reagent could be loaded successfully, the reaction conditions were scaled up and performed on enough resin to load 0.1 mmol of nucleoside. The purity of the cleaved mixture was excellent and the only nucleoside based impurity present was some remaining starting material (Figure 2.5).

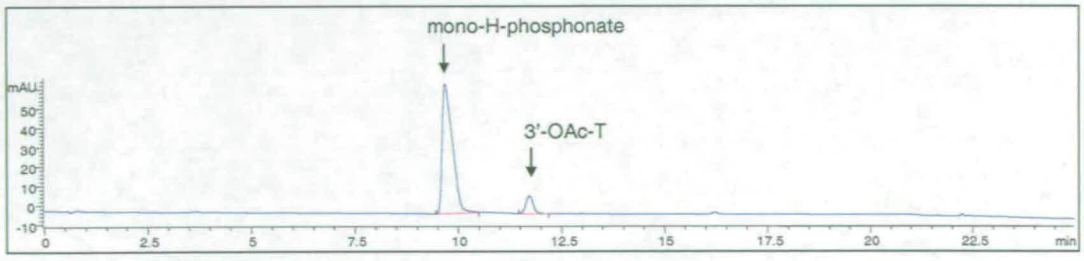
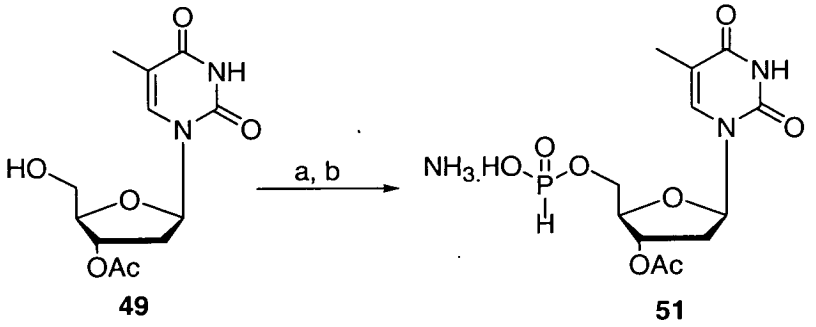


Figure 2.5 HPLC chromatogram of crude mono-H-phosphonate cleavage

Mono-*H*-phosphonate **51** was isolated by Prep HPLC (eluting with 0.1 M NH_4OAc/MeCN gradient) in a 58 % yield. (Scheme 2.19)



49 **51**

(a) 3 eq PS-P-Cl **44**, py/THF 1/3, rt, 1 h, 0.10 M; (b) THF:H_2O 1:1, 2 x 5 min; 58 % yield

*Scheme 2.19 Synthesis of mono-*H*-phosphonate **51** using supported reagent*

2.3.5.6 *Summary*

At this point, it can be concluded that the supported reagent was prepared and used successfully to load a nucleoside with subsequent cleavage as its mono-*H*-phosphonate **51**. The reaction conditions developed for loading of the nucleoside are summarized below (Table 2.5).

Table 2.5 Reaction conditions developed

Reaction	**eq**	**Conc. M**	**Time h**	**Temp. °C**
Formation of reagent (A)	4	0.27	2	60
Loading of 3'-OAc-T	0.33	0.10	1	rt
Hydrolysis	-	-	0.17	rt

2.3.6 Optimisations for cleavage of nucleoside resin with pyrophosphate

2.3.6.1 *General*

Using the reaction conditions established to synthesise and load the supported reagent, it was next attempted to perform the cleavage with pyrophosphate to obtain the protected triphosphate after oxidation.

It became very soon all too apparent that the success of this chemistry hinges on the quality and dryness of all reagents utilised. The phosphorinone on resin **50** is in fact a very powerful electrophile which will readily react with any nucleophile

present. To minimise side-reactions, the reagents used had to be purified painstakingly as discussed below.

2.3.6.2 *Tributylamine*

Tributylamine is used as a base during the pyrophosphorylation to dissolve salts formed during the two previous steps. Samples of even the purest grade available were positive by Ninhydrin test, indicating presence of primary amines. These could be removed by storing over ninhydrin, followed by vacuum distillation. The purified amine was stored in the dark over 4 Å MS at rt and was still dry and free of primary amines after 6 months.

2.3.6.3 *Preparation of pyrophosphate salt*

A pyrophosphate salt with 1.6 mol of tributylamine *per* mol of pyrophosphate is available commercially (Sigma). Its ^{31}P NMR indicated however that it is contains only about 75 % pyrophosphate, the remainder being a phosphate, which yielded close-running impurities (Figure 2.6). The mono-*H*-phosphonate peak results from water present in the reaction mixture.

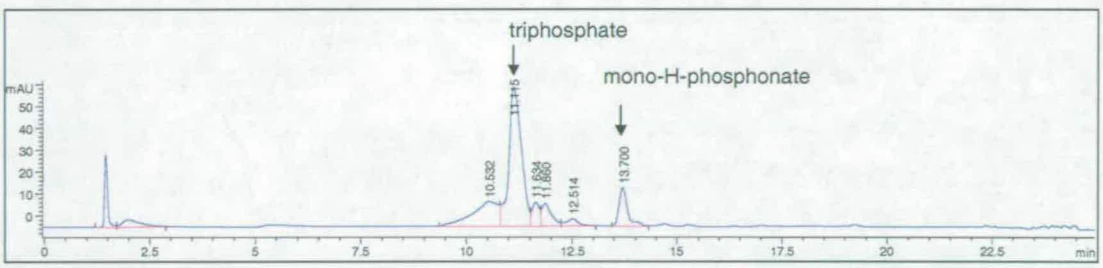


Figure 2.6 HPLC of crude reaction mixture when using commercial pyrophosphate salt

Bis (tributylammonium) pyrophosphate was therefore prepared by adapting the method by Ludwig and Eckstein.[83] The pyrophosphate salt was eluted off an ion exchange column to give pyrophosphoric acid, which was treated with tributylamine and lyophilised until the oily residue turned into a white powder. It was then dissolved in dry DMF and transferred by canula onto cooled molecular sieves which had been activated by microwave irradiation. This preparation contained typically >95 % of pyrophosphate, as judged by ^{31}P NMR. This compares more than favourably with the commercial batch. (Figure 2.7)

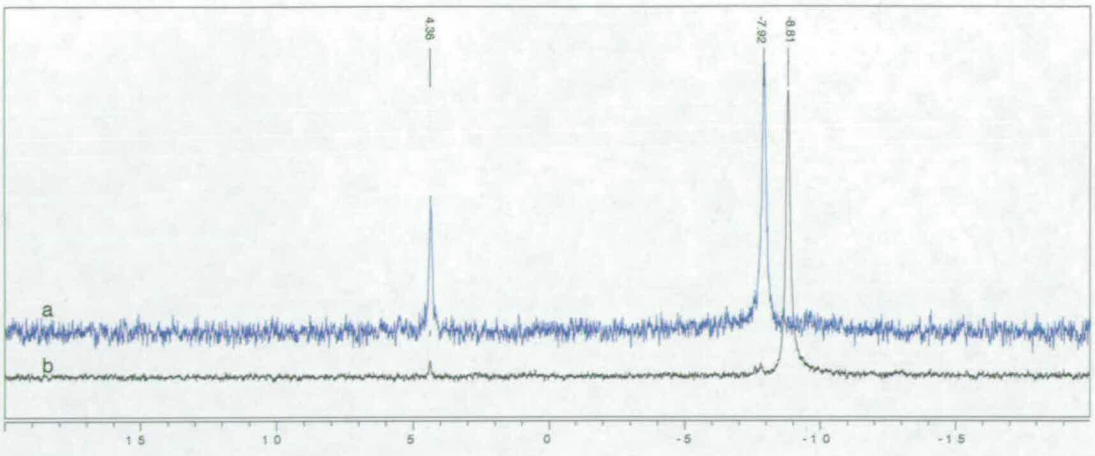


Figure 2.7 ^{31}P NMRs of (a) commercial and (b) in-house prepared bis (tributylammonium) pyrophosphate

2.3.6.4 *Reaction conditions for pyrophosphate attack*

Reaction conditions were used as described earlier (Table 2.5). For the pyrophosphate cleavage, the reagents were prepared as for the solution phase method and added onto the nitrogen flushed resin, giving a concentration of 0.37 M for the pyrophosphate ion.

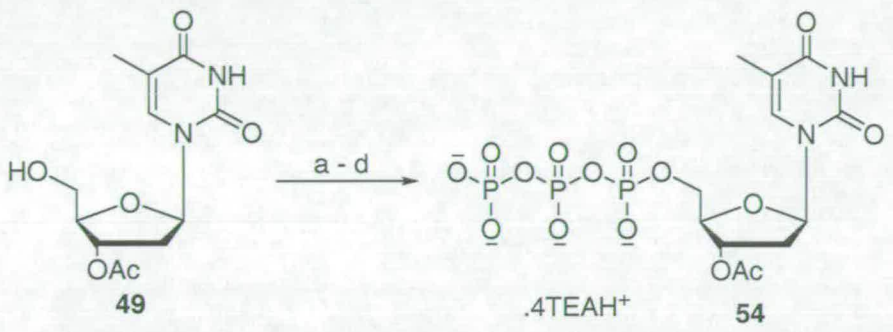
Table 2.6 Reaction conditions used for pyrophosphate attack

Reaction name	**eq**	**Conc. (M)**	**Time (h)**	**Temp. (°C)**
Formation of reagent	4	0.27	2	60
Loading of 3'-OAc-T	0.33	0.10	1	rt
Pyrophosphate cleavage	1.5	0.37	see text	rt
Oxidation	1.5	0.37	0.50	rt
Hydrolysis	-	-	0.25	rt

Cleavage of the resin with pyrophosphate was performed for 30 min, 1 h and 2 h. The ratio of triphosphate to mono-*H*-phosphonate looked most favourable for triphosphate formation after 30 min cleavage time. This judgement was made due to the presence of mono-*H*-phosphonate **51** when the resin was treated with water after the pyrophosphate cleavage had finished. The necessity for short cleavage times can be rationalised by the fact that the labile cyclic triphosphite decomposes to the mono-*H*-phosphonate during prolonged reaction times. It was therefore desirable to minimise cleavage times to maximise the yield.

2.3.6.5 *Isolation of product*

The synthesis was scaled up to 0.10 mmol nucleoside. It was realised when performing the cleavage reaction that it was more efficient to subject the resin to three x 10 minute cleavages rather than one 30 minute cleavage. The triphosphate was synthesised (Scheme 2.20) and isolated after CH_2Cl_2/0.1 M TEAB work-up and Prep HPLC. The work-up was found to be essential; otherwise the sample needed to be dissolved in too much MeCN, which resulted in column-breakthrough. Ac-dTTP **54** was obtained as its tetrakis [triethylammonium] salt after lyophilisation in a 14 % yield. This yield was acceptable, though lower than the conversions by HPLC would have suggested (Figure 2.8).



(a) 3 eq PS-P-Cl **44**, py/THF 1/3, rt, 1 h, 0.10 M; (b) 1.5 eq 0.5 M $(Bu_3N)_2$ pyrophosphate in DMF, 4 eq Bu_3N, rt, 3 x 10 min; (c) 1.5 eq 0.5 M I_2/98:2 pyridine:H_2O, 15 min; (d) H_2O, 30 min; 14 % yield

*Scheme 2.20 Synthesis of 3'-OAc-dTTP **54** using supported reagent*

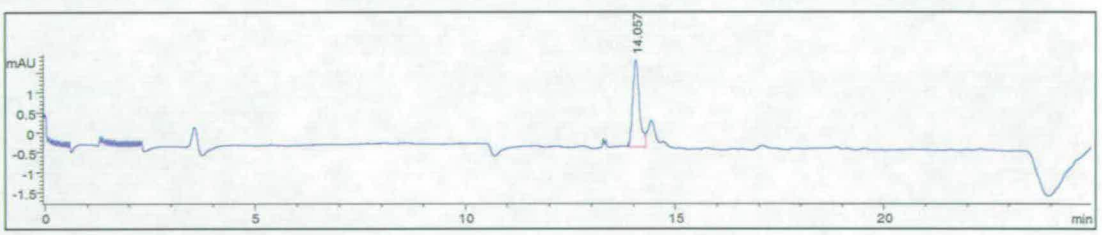
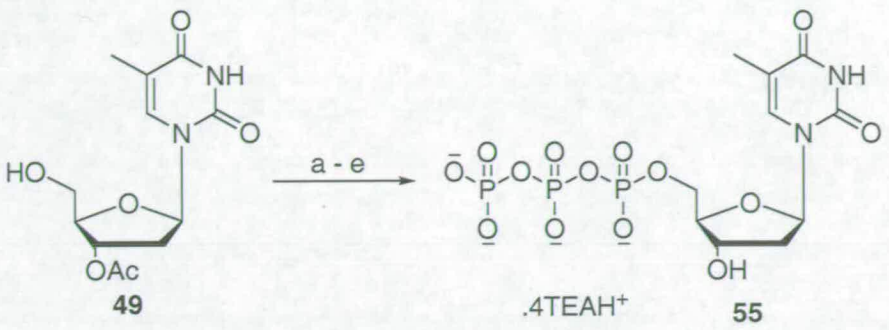


*Figure 2.8 HPLC chromatogram of crude 3'OAc-dTTP **54***

2.3.6.6 *Synthesis of dTTP **55** after deprotection*

The final step of the synthesis, the acetyl deprotection, was then added onto the synthetic sequence (Scheme 2.21). The crude HPLC indicated dTTP **55** to be of acceptable purity (Figure 2.9). Close-running impurities were readily separated by Prep HPLC (Figure 2.10) and dTTP **55** was isolated in a 50 % overall yield from 3'OAc-T **49**.

The difference in yields sems to suggest that the triphosphate is easier isolated in its unprotected form, maybe due to solubility difficulties. It could also be that the acetate protecting group on the 3' hydroxyl group is not stable to the synthesis or the HPLC purification conditions, which is performed at pH 8-9 buffer and thus faciliates partial cleavage of the acetate group.



(a) 3 eq PS-P-Cl **44**, py/THF 1/3, rt, 1 h, 0.10 M; (b) 1.5 eq 0.5 M $(Bu_3N)_2$ pyrophosphate in DMF, 4 eq Bu_3N, rt, 3 x 10 min; (c) 1.5 eq 0.5 M I_2/98:2 pyridine:H_2O, 15 min; (d) H_2O, 30 min; (e) NH_3/H_2O, 1 h; 50 % yield

*Scheme 2.21 Synthesis of dTTP **55** using the supported reagent on Gel PS*

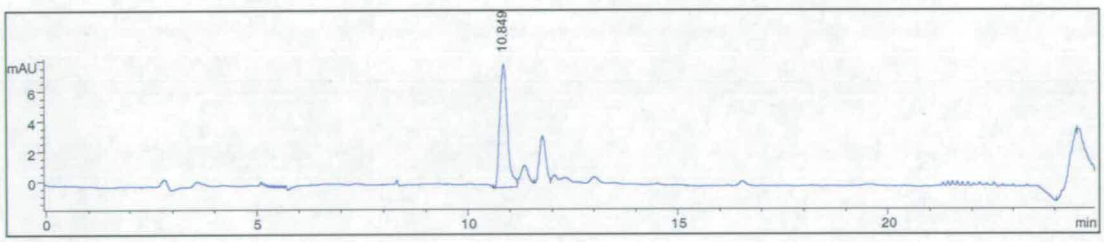


*Figure 2.9 Analytical HPLC chromatogram of crude dTTP **55***

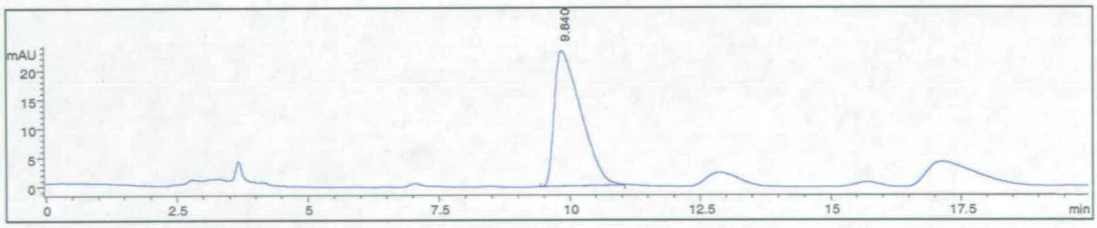


*Figure 2.10 Prep HPLC chromatogram of dTTP **55***

2.3.6.7 *Comparison to solution phase synthesis*

It was of course important to evaluate how this new method compared to the established procedure. The compounds were again isolated using a work-up, followed by Prep HPLC. The synthesis for 3'-OAc-dTTP **54** gave a 24 % yield

(Scheme 2.18), whereas for dTTP **55**, a yield of 36 % was obtained after ammonolysis of the acetate group. These yields show that isolation of the fully deprotected dTTP **55** compares favourably to the solution phase procedure.

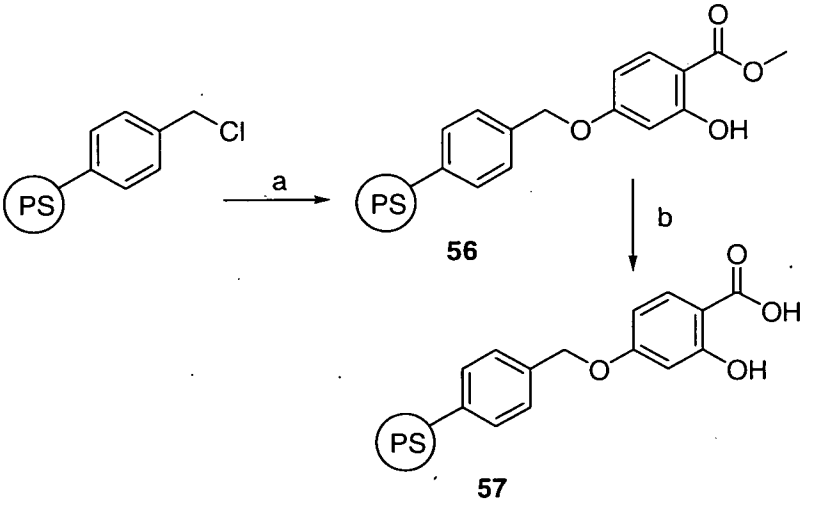
2.3.7 Conclusion

Microporous polystyrene was used successfully as a support for the Ludwig-Eckstein reagent. It was used to synthesise thymidine triphosphate **55** as its tetrakis [triethylammonium] salt in a 50 % yield from its 3'-O-acetyl protected analogue. This yield compares favourably to the solution phase methodology, where in our hands a yield of 36 % could be obtained using Prep HPLC as isolation method.

To understand the factors involved in determining the yield obtained with PS resin, other resins were studied. In the next few sections, the synthesis and application of such resins to the supported reagent will be discussed. The same reaction conditions used for all resin types to allow direct comparison. Results and yields obtained for all resins are summarised and rationalised in Section 2.7.

2.4 Attachment of the linker to macroporous PS

Macroporous polystyrene typically has a DVB crosslinking of 20-30 % resulting in a non-swellable resin, thus making the chemistry more accessible in hydrophilic solvents as the sites remain available. In the context of the pyrophosphate cleavage, this should ensure rapid and complete cleavage of the cyclic triphosphite.



(a) 3 eq **35**, acetone, K_2CO_3, KI, 60 °C, 90 h; (b) TBA-OH, THF, 60°C, 2 d, then 2 M HCl

Scheme 2.22 Synthesis of supported 2-hydroxy benzoic acid on Macroporous PS

The resin (20 % DVB cross-linking, 150-350 μm, 2.0 mmolg^{-1}) was functionalised with the same method as for the microporous resin (Scheme 2.22). Monitoring by Gel-Phase NMR was not possible here as the resin does not solvate and therefore it is not possible to record any spectra. Instead, couplings were monitored by elemental analysis of remaining halogen content.

As expected, couplings to macroporous resin were sluggish compared to microporous resin. After 24 h, a 43 % conversion was obtained which could pushed up to 76 % by reacting for a total of 90 h. (Table 2.7)

The methyl ester was deprotected using the method established previously, giving a macroporous resin **57** with a loading of 0.93 mmolg^{-1}.

Table 2.7 Monitoring of Finkelstein coupling on macroporous PS

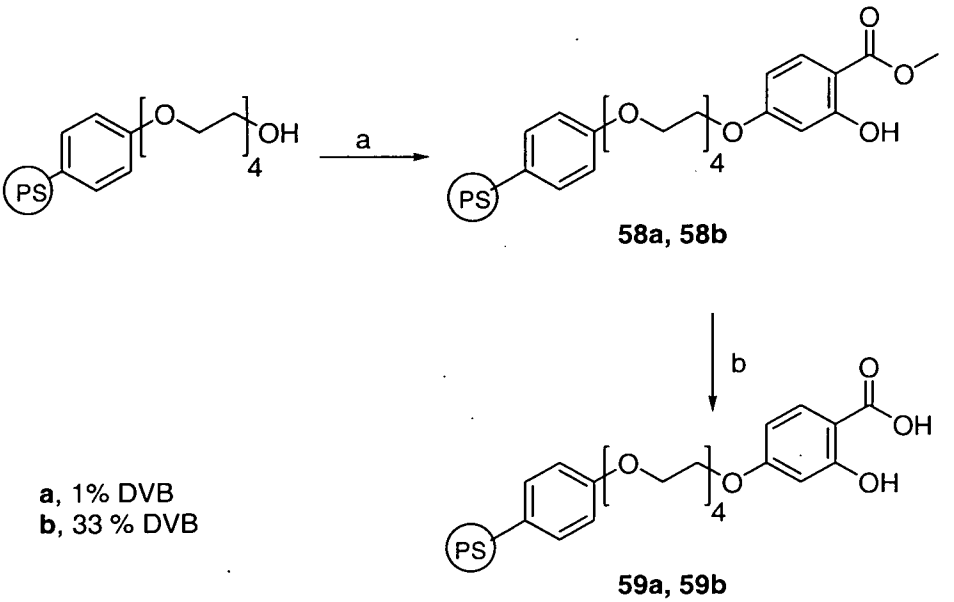
t (h)	Cl %[b]	Cl mmolg^{-1}[c]	Cl %[d]	Cl mmolg^{-1}	I %[b]	I mmolg^{-1}	loading mmolg^{-1}[f]	loading %[f]
0	8.04	2.27	7.09	2.0[a]				
24	3.61	1.02	2.66	0.75[e]	1.88	0.15	0.68	43
90	1.99	0.56	1.04	0.29[e]	1.15	0.09	1.20	76

[a]available loading (value supplied by manufacturer), [b]measured by elemental analysis, [c]total Cl loading, [d]loading available for substitution (obtained by subtracting residual Cl (0.27 mmolg^{-1} or 0.95 %) from experimental value), [e]Cl loading available for substitution, [f]theoretical yield 1.58 mmolg^{-1} based on loading available for substitution

2.5 Attachment of the linker to Quadragel and Quadrapore

Quadragel and Quadrapore resins have a polystyrene core which has been functionalized with a tetraethyleneglycol unit. This is claimed to impart advantages to the resin over polystyrene, such as compatibility with polar and non-polar solvents, better accessibility of sites leading to purer peptides and oligonucleotides.[132] Quadragel is a gel polymer with a low-crosslinking of 1 % DVB, whereas Quadrapore has a cross-linking of 33 %.

The hydroxyl functionalised resins were coupled to the methyl ester protected salicylate linker **35** under Mitsunobu conditions, as previously used for microporous PS (Scheme 2.23). The resins were checked for completion using DMT tests,[133] which gave 98 % and 95 % loadings after 12 h.



(a) 4 eq **35**, PPh_3, DIAD, THF, 12 h; (b) TBA-OH, THF, 65 °C, 2 d

Scheme 2.23 Attachment of linker onto Quadragel & Quadrapore resins

The identity of the resin was checked by Gel-Phase ^{13}C NMR. High-quality spectra could be obtained for Quadragel. It compared favourably to polystyrene, as the added tetraethylene glycol linker makes the functionality much more flexible, thus easier to visualise by NMR. (Figure 2.11)

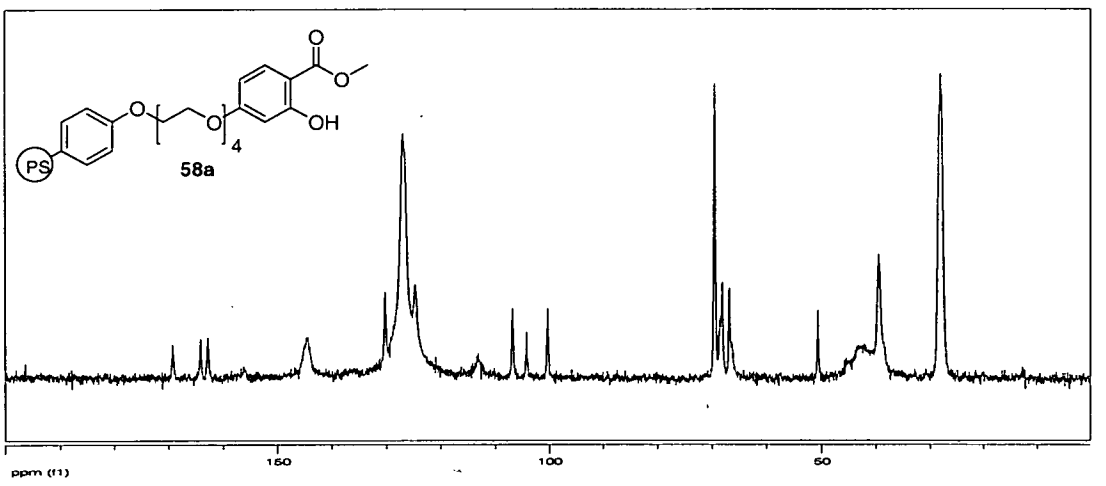


Figure 2.11 ^{13}C Gel Phase NMR of Quadragel resin 58a

The deprotection conditions were used as previously described for the PS resins. The completion of the reaction was verified by ^{13}C Gel-Phase NMR. The methyl ester signal which is visible at 50 ppm (Figure 2.11), was not present (Figure 2.12).

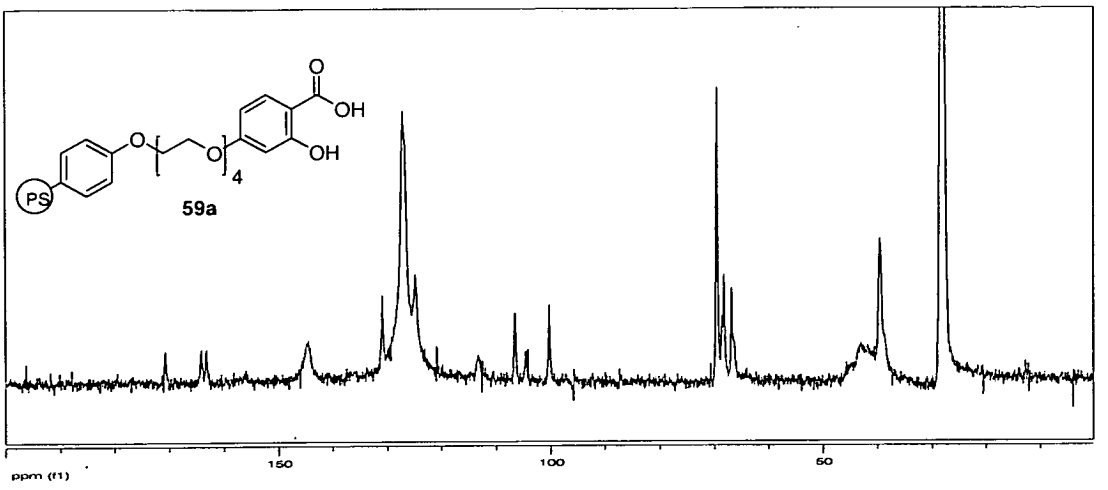


*Figure 2.12 ^{13}C Gel Phase NMR of Quadragel resin **59a***

For the macroporous analogue, Quadrapore, it was not possible to perform Gel-Phase NMR, despite the tetrathylene glycol linker. The Mitsunobu coupling was monitored by the DMT test and the deprotection was performed as for Quadragel.

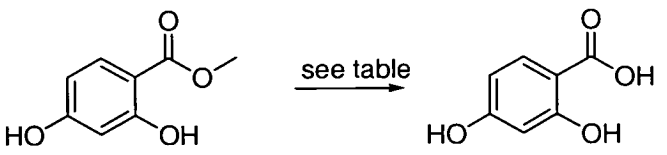
2.6 Attachment of the linker to silica based resin

2.6.1 Development of salicylate linker for silica

CPG is formed of a Si-O network and is typically surface-functionalised with short carbon chains and an amine, such as propylamine. The protection group strategy for the carboxylic acid on the salicylic acid had to be reconsidered, as aqueous base is not suitable; under these conditions all functionalisation will be cleaved off the resin and the resin eventually dissolved.

2.6.1.1 *Screening for non-basic deprotection conditions of methyl 2,4-dihydroxy benzoate*

In the first instance, an attempt was made to find alternative deprotection conditions for methyl ester **35** (Scheme 2.24). A well published alternative to basic conditions is nucleophilic attack of on iodide ion on the methyl group to give MeI and the carboxylate.[134]



Scheme 2.24 Deprotection of methyl ester

Various conditions were attempted, but none of them were satisfactory for on-resin deprotection (Table 2.8). The most promising set of conditions involved *in situ* generation of TMS-I *via* formation of the TMS ester, which is readily hydrolysed to the carboxylic acid. The model reaction is not identical, but the adjacent phenol was an identical mimic to that observed in the real compound. The methyl ester was abandoned as a protecting group for CPG, as the cleavage was deemed to be poor on CPG.

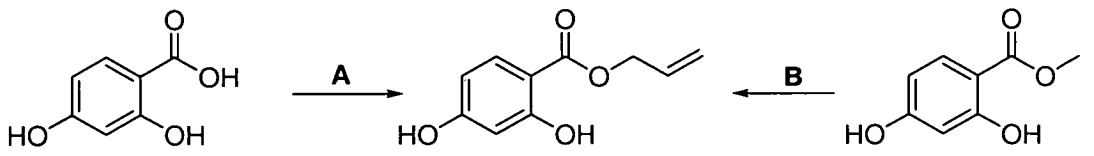
Table 2.8 Attempted conditions for deprotection

Conditions	temp °C	time	product %[a]	impurity total %[a]	SM %[a]	Ref
3 eq LiI, EtOAc, 0.2 M	65	15 h	29	-	71	135
3 eq LiI, EtOAc, 0.2 M	65	3 d	30	19	51	135
3 eq CaI_2, Toluene, 0.3 M	95	18 h	-	-	100	136
TMS-Cl, NaI, MeCN	65	15 h	55	15	30	137
TMS-Cl, LiI, MeCN	65	24 h	4	1	95	
3 eq KO^{t}Bu, DMSO, 0.3 M	rt	18 h	6	46	48	138
LiOH, THF:MeOH, 20:1, 0.2 M	rt	15 h	-	-	100	
Pig liver esterase, PBS buffer	35	3 h	-	-	100	139

[a] *Conversions by HPLC peak area in UV at 254 nm*

2.6.1.2 *Synthesis of allyl 2,4-dihydroxy benzoic acid*

It was then decided to synthesise the allyl ester, which is readily cleaved under neutral conditions with Pd (0) activating it to nucleophilic attack. Typical species used for intercepting the Pd-allyl complex are carbon nucleophiles, amines, thiols, carboxylates and hydride donors.



60

Scheme 2.25 Protection as allyl ester

Allyl esters can be accessed *via* esterification of the corresponding carboxylic acid (route A) or *via* trans-esterification of an existing ester (route B) (Scheme 2.25). Both approaches were considered. Synthesis of allyl ester was attempted *via* acid-catalysed esterification under Dean-Stark conditions (Table 2.9). Using toluene as the solvent resulted in a charred mixture, due to the temperature required for reflux

(Entry 1). Using benzene, that problem was solved, but isolated yields were low and the product obtained was of less than optimal purity (Entry 2). Longer reaction times (6 days), while increasing the overall yield, also increased the amount of co-purifying impurities.

Table 2.9 Conditions for allyl ester synthesis

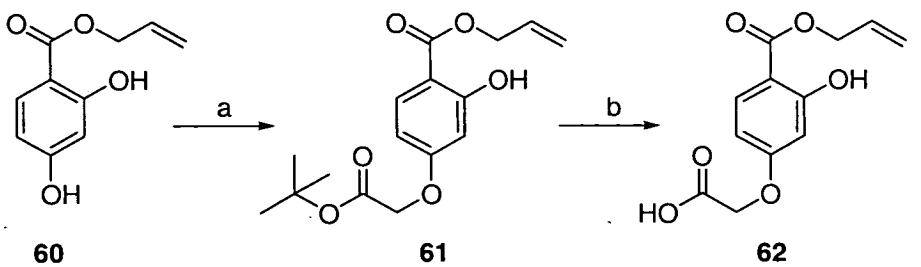
Entry		Conditions	temp. °C	time	product %	SM %	Ref
1	A	2 eq allyl alcohol, 10 % *p*-TsOH, Dean-Stark, PhMe	130	3 d	charred		
2	A	3 eq allyl alcohol, 10 % *p*-TsOH, Dean-Stark, PhH, 0.6 M	115	3 d	13[a]		140
3	B	10 % $(Bu_2Sn)O$, 100 eq AllOH	95	24 h	10[b]	90[b]	141
4	A	0.95 eq Proton sponge, 1.5 eq allyl bromide, DMF, 0.45 M	70	1 h	78[a]		

[a]*Isolated yield,* [b]*Conversions by HPLC peak area in UV at 254 nm*

A dibutyltinoxide mediated transesterification gave only a low conversion over 24 h, even though for salicylic acid quantitative conversion had been reported within 5 h (Entry 3). The most suitable method developed used proton sponge which deprotonated the carboxylic acid and resulted in alkylation in good yield and product isolation in excellent purity after work-up (Entry 4).

2.6.2 Synthesis of an allyl ester based linker and coupling to aminoproppyl-CPG

Allyl ester **60** was alkylated on the *para*-phenol position after deprotonation with sodium hydride with ^{t}butyl bromoacetate to give the alkylated product **61** in good yield. Selective deprotection was performed with TFA in CH_2Cl_2 to yield the linker **62** needed to couple to aminopropyl-CPG. (Scheme 2.26)

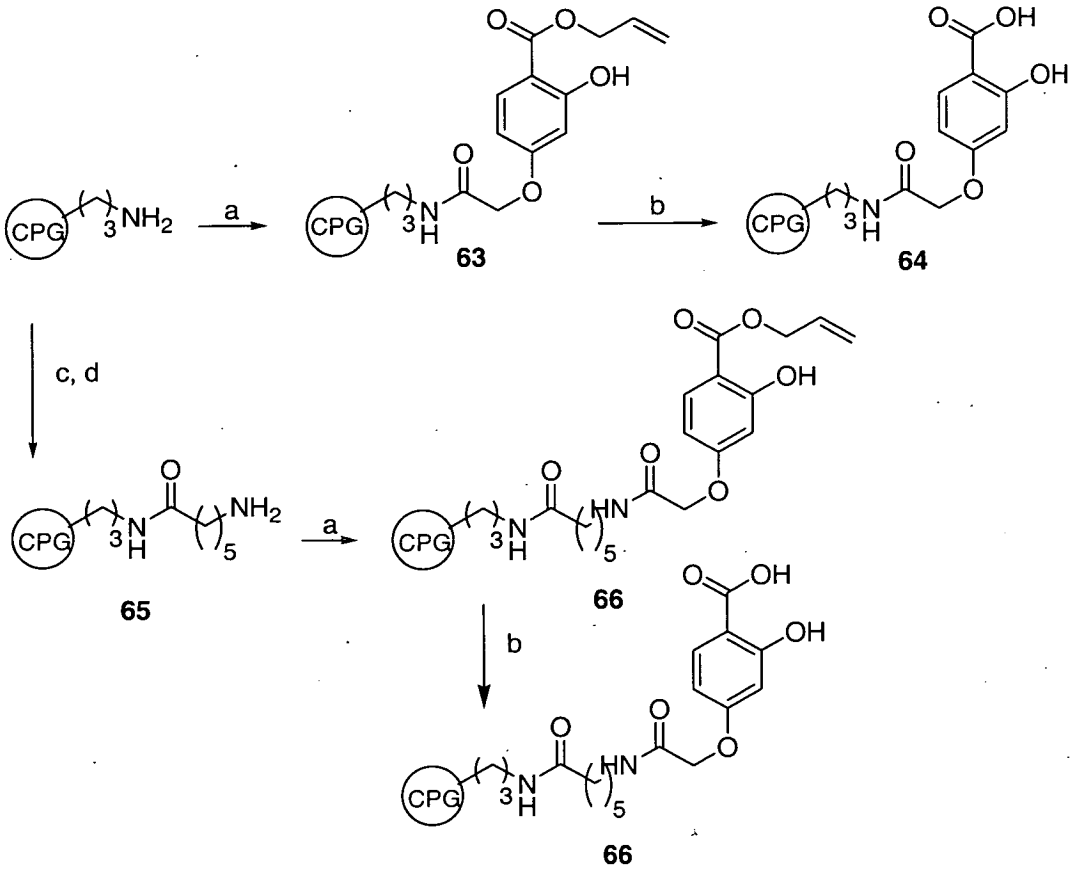


60 **61** **62**

(a) ^{t}butyl bromoacetate, 1 eq NaH, DMF, 1 h, 64 %; (b) 30 % TFA in CH_2Cl_2, 2.5 eq Et_3SiH, 45 min, 68 %

Scheme 2.26 Synthesis of carboxylic acid functionalised salicylic acid derivative

CPG used was of 50-100 μm diameter with 46 nm pore size, as supplied by Trisoperl. Functionalisation of the aminopropyl CPG is known to be sluggish, therefore couplings were performed at raised temperature (Scheme 2.27).



(a) 10 eq linker **62**, DIC/HOBt, DCM/DMF 4/1, 60 °C, 3.5 h; (b) 1 % $[Pd(PPh_3)_4]$, 1.5 eq morpholine, THF, 1 h; (c) 10 eq Fmoc-Ahx-OH, DIC/HOBt, DCM/DMF 4/1, 60 °C, 24 h; (d) 20 % piperidine/DMF

Scheme 2.27 Synthesis of CPG based 2-hydroxy benzoic acids

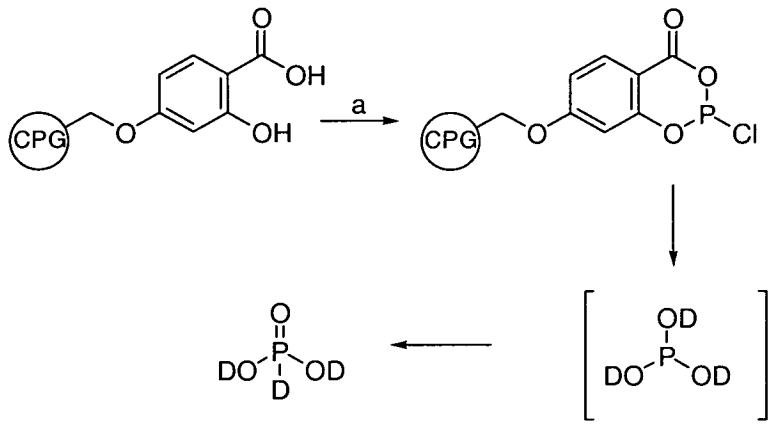
Progression of reaction was monitored by a qualitative ninhydrin test. Moreover, to reduce surface interactions between the resin and the functionality, an additional linker, 6-amino-hexanoic acid, was coupled to the glass surface prior to adding the salicylate linker. Using this strategy, two supports **64** and **67** were obtained after allyl ester deprotection for testing.

2.6.3 Synthesis of the solid-supported reagent on CPG

As monitoring of the allyl ester deprotection was not possible, the two supports **64** and **67** were immediately tested to see whether they could form the supported reagent. Attempts were made to form the Ludwig-Eckstein reagent using method B

(PCl_3/pyridine in THF at rt), as refluxing PCl_3 was not deemed suitable for the CPG construct which contained amide bonds.

The reagent was formed on the resin using method B and quenched with D_2O. This would enable assement of whether the reagent had been formed on the resin, as a P-D coupling would be observed for the phosphonate salt formed (Scheme 2.28). For resin **64** no P-D phosphonate salt was formed. To verify whether this observation was due to absence of the reagent on the resin or steric surface interactions, it was repeated for resin **67**. Again no P-D couplings were observed.



(a) 16 eq pyridine, 4 eq PCl_3, THF, 10 min, 0.27 M; (b) D_2O

Scheme 2.28 Displacement of Ludwig-Eckstein reagent on the resin with D_2O

The microporous PS based reagent **44** was readily quenched and drained to yield a phosphonate salt with P-D coupling as judged by ^{31}P NMR. As this however was not possible for either of the CPG supports, it was decided at this point not to pursue CPG further.

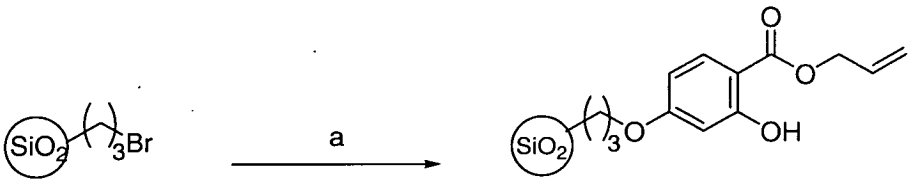
A few reasons for the lack of success in the use of CPG can be cited:

- The method for preparing the reagent involves production of pyridinium hydrochloride, which might have lead to blocking of the pores.
- The success of the allyl ester deprotection reaction could not be verified, i.e. the nucleophile might not have been suitable, as it is a base.
- The amide bonds in the resin construct could have undergone Vilsmeier-Haack type reactions with the PCl_3.

2.6.4 Attachment of the linker to high-loading silica

As attempts on commercial aminopropyl-functionalised CPG were unsuccessful, attention was focused onto silica supports. Functionalised silica was obtained with a diameter of 40-63 μm with typical loadings of 1.4 mmolg^{-1} from Silicycle.

Two silica supports were selected for coupling to the salicylate linker by forming phenol ether bonds. The methods used previously for PS resin (K_2CO_3/acetone) were not suitable for silica, as the base was expected to cause undesired side reactions by dissolving the silica. The first resin was functionalised with propyl bromide and was loaded with allyl ester protected linker **60** in the presence of DIPEA (Scheme 2.29). The yield, determined by residual bromine elemental analysis, was 0.29 mmolg^{-1}, which equates to a 25 % loading. (Table 2.10)



68

(a) 5 eq **60**, DIPEA, 40 °C, 34 h, THF, 0.9 M

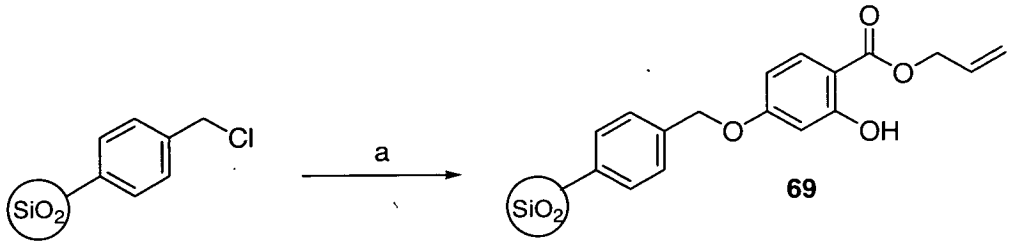
Scheme 2.29 Functionalisation of bromo-propyl-silica with allyl ester protected linker

Table 2.10 Monitoring of silica functionalisation by elemental analysis

Time	Br (%)	Br (mmolg^{-1})	loading (%)^c	loading (mmolg^{-1})
SM		1.43^a		
SM	8.61	1.08^b		
10 h	8.04	1.01	18	0.23
34 h	7.03	0.88	29	0.35

^{a} *Value supplied by manufacturer,* ^{b}*derived by elemental analysis,* ^{c}*theoretical yield for coupling 1.23 mmolg^{-1}*

The starting material was also tested for bromine content and there was in fact a considerable underloading on the stated value, thus the yields and loadings are based on that determined experimental value. The second resin chosen contained a benzyl chloride group and was functionalised with the preformed sodium phenolate of **60** to give a loading of 0.46 mmolg^{-1} (45 % loading).



69

(a) 5 eq **60**, 5 eq NaH, 50 °C, 48 h, THF, 0.9 M

Scheme 2.30 Functionalisation of silica with allyl ester protected linker

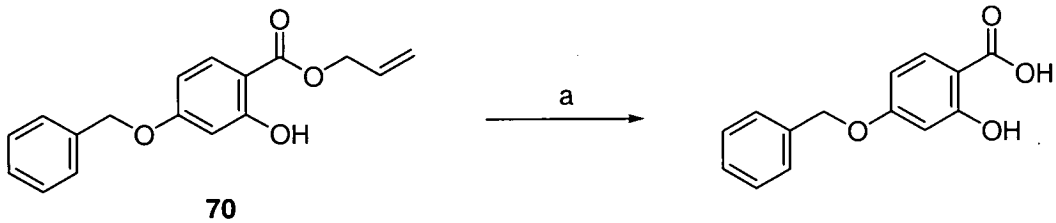
Table 2.11 Monitoring of silica functionalisation by elemental analysis

Time	Cl (%)	Cl (mmolg^{-1})	loading (%)[c]	loading (mmolg^{-1})
SM		1.34[a]		
SM	3.64	1.03[b]		
24 h	2.48	0.70	37	0.41
48 h	1.98	0.56	50	0.55

[a] *Value supplied by manufacturer,* [b] *derived by elemental analysis,* [c] *theoretical yield for coupling 1.11 mmolg^{-1}*

2.6.4.1 *Screening for deprotection conditions of allyl ester in solution*

The deprotection conditions were modelled in solution by deprotection of a *p*-benzylated analogue of the allyl ester, analogous to resin **69**. The resin mimic **70** was obtained from **60** under Finkelstein conditions in a 69 % yield. The palladium source chosen was palladium triphenylphosphine tetrakis complex, as this is soluble in organic solvents, while other sources such as $Pd(OAc)_2$ are not.



70

(a) 5 % $[Pd(PPh_3)_4]$, 0.14 M, 1.5 eq nucleophile

Scheme 2.31 Deprotection of solution model of the allyl ester

The reactions were run in parallel with different nucleophiles and monitored by HPLC after 15 and 45 minutes. The products were isolated through a work-up for the reactions that had gone to completion. The purity of the crude product was assessed by ^{1}H NMR. (Table 2.12)

Table 2.12 Deprotection conditions for allyl ester

Nucleophile	solvent	Conversion %[a]	Yield %[b]	Purity (NMR)	Ref
Pyrrolidine	THF	91	-	-	142
Morpholine	THF	100	51	no product	143
Dimedone	THF	100	6	moderate	
Barbituric acid	THF	100	52	good	
Phenyl silane	THF	100	96	good	144
p-toluene sulfinic acid, sodium salt	THF/ MeOH	100	73	good	145

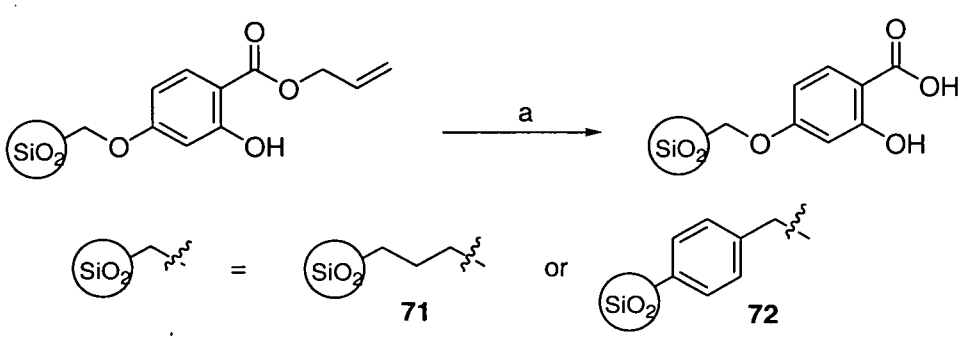
[a]*HPLC conversions by peak area in ELSD after 45 min,* [b]*Isolated yield after work-up*

Good yields and purity of the isolated product were obtained for phenylsilane and the sodium salt of *p*-toluenesulfinic acid.

In light of unsuccessful attempts to use an aminopropyl-CPG as a support (Section 2.6.3), it is interesting to note that when morpholine was used as the nucleophile, the correct product was not isolated.

2.6.4.2 *Deprotection of resins*

Out of the two possible scavengers, it was decided to use phenylsilane for the on-resin deprotection of the supported allyl esters, as this reagent generates the silyl ester *in situ*, which is then readily cleaved with water. The resins were obtained with theoretical yields of 0.350 molg^{-1} (**71**) and 0.560 mmolg^{-1} (**72**) (Scheme 2.32).



(a) 5 % $Pd(PPh_3)_4$, 5 eq $PhSiH_3$, THF; H_2O

Scheme 2.32 Allyl ester deprotection of silica resins

2.7 Comparison of resins

2.7.1 Yields using solid-supported Ludwig-Eckstein reagent

The new supports were tested using the reaction conditions in Section 2.3.6.5, as developed for microporous polystyrene. The test compound remained dTTP **55**. The results obtained for all the isolated dTTP products from the syntheses are summarized below (Table 2.13).

Table 2.13 Summary of syntheses of dTTP using different resins

	Resin	Type	loading mmolg^{-1}	Yield %[a]	Bead size μm
41a	Microporous PS	gel	1.34	50	75-150
57	Macroporous PS	rigid	0.93	15	150-350
59a	Quadragel	gel	0.75	19	150-300
59b	Quadrapore	rigid	0.65	26	150-300
71	Silica	rigid	0.35	8	40-63
72	Silica	rigid	0.56	4	40-63

[a]*Isolated yield after Prep HPLC purification*

Macroporous PS based reagent **57** was readily amenable to the synthetic sequence. No problems were observed during the synthesis and a yield of 15 % could be obtained after purification.

The Quadragel based reagent **59a** exihibited different swelling properties than microporous PS; an increased swelling of the resin was observed in THF, which would be expected to facilitate reactions. For Quadrapore **59b** no swelling was observed, as is usual for a macroporous resin. The yields were 19 % and 26 % respectively, which might indicate that the macroporous nature of it could impart advantages over the gel type resin for this transformation.

The silica based materials **71** and **72** on the hand gave even lower yields. It is likely that this is due to steric interactions with the resin surface. This means that either more forcing condtions are necessary for the loading and cleavage reactions to proceed or that the reagent needs to be distanced further from the support to enable a more solution phase like synthesis. This could be achieved by coupling a polyethylene glycol linker to the resin prior to coupling the salicylate linker.

The results show that microporous polystyrene as a support for the Ludwig-Eckstein reagent produces the highest yield, by a significant margin. It is in a way

almost surprising that the synthesis works this well, considering that the key step, the pyrophosphate cleavage, relies on a charged species interacting favourably with the highly hydrophobic polystyrene support. Intuitively this combination would be expected to give less favourable results than macroporous resins or polyethylene glygol grafted resins.

One clue towards explaining the observations comes from considering the bead size for the polystyrene based resins. The batch of microporous polystyrene was 100-200 mesh, i.e. a bead diameter of 75-150 μm, whereas the other resins had much higher size specifications, more poignantly illustrated below (Figure 2.13).

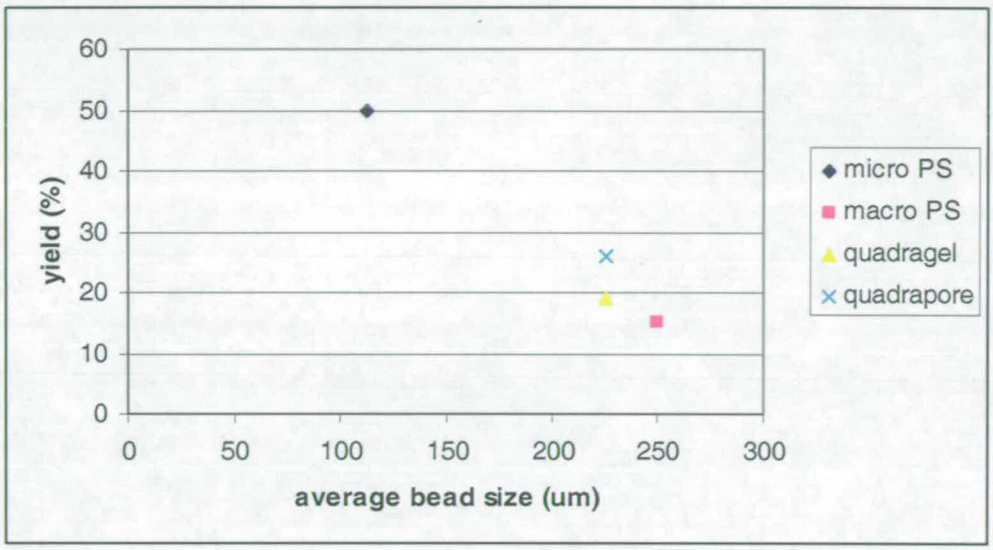


Figure 2.13 Corrolation between yield and average bead size

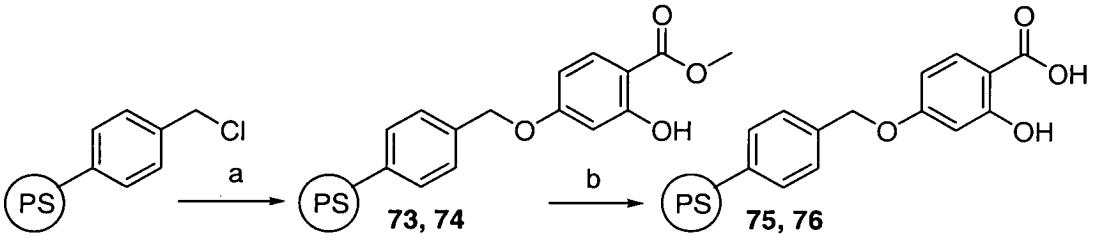
For the polystyrene based resins, leaving aside all other factors momentarily, a correlation seems to exist between bead size and yield. Of course, this statement must be made only very catiously, as there are indeed many other factors differentiating the resins, such as nature of support, level of crosslinking, loading and presence or absence of a PEG linker.

Thus it can be summarized at this point that no firm conclusions can be drawn across the different types of resins. If a direct comparison between resins is sought, further resins need to be made and tested to allow a more valid comparison between them.

2.7.2 Synthesis and evaluation of further microporous polystyrene resins

As the bead size seemed to be one factor impacting on the triphosphate yield obtained, a more complete series of supported reagents was synthesised for microporous polystyrene resin. These resins were chosen, as additional particle sizes were available commercially and, while the yield was already good, there was scope for further improvement with a smaller bead size.

The synthetic scheme was followed as developed earlier (Scheme 2.33) and the resins were used as supported reagents for dTTP synthesis as described in Section 2.3.6.5. The yields obtained are tabulated below (Table 2.14).



(a) 3 eq **35**, acetone, K_2CO_3, KI, 60 °C, 2 d; (b) TBAOH, THF, 60°C, 2 d, then 2 M HCl

Scheme 2.33 Synthesis of microporous resins

When the reactions using the resin **75** with 38-75 μm bead size were carried out, it was noticed that this resin was blocking the frist and more diffcult to handle than the previously used 75-150 μm bead size, as the pressure required to drain the vessel increased considerably. This is not unexpected and has been reported.[108] The yield obtained was also considerably lower than for the 75-150 μm beads, due to difficult washing and the prolonged handling required.

Table 2.14 Summary of syntheses of dTTP using different microporous PS resins

	Bead size (μm)	Resin	Type	loading (mmol g^{-1})	Average Yield (%)
75	38-75	Microporous PS	gel	1.05	13
41a	75-150	Microporous PS	gel	1.34	50
76	150-300	Microporous PS	gel	1.42	20

For the bigger bead size resin **76**, no handling issues were observed and the reactions proceeded smoothly. The yield obtained for dTTP **55** synthesis was 20 % after lyophilisation, which is a reduction on the yield observed previously. Thus it is clear that a lowering of the bead size for microporous PS does not give the resin

an improved yield, on the contrary, it leads to mechanical damage and much impaired yield.

2.7.3 Comparison between similar bead sizes

For all four resins in the ~150-300 μm region, it is apparent that the yields obtained were very similar (Figure 2.14).

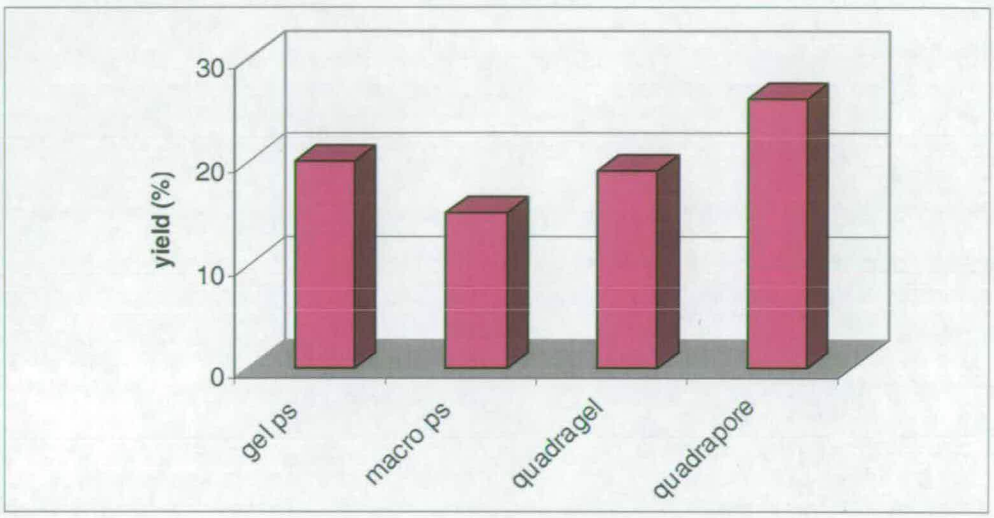


Figure 2.14 Yield for resins in the 150-300 μm bead size range

2.8 Conclusion

In summary, a supported phosphorylation reagent has been synthesised and its application to triphosphate synthesis has been developed and optimized using thymidine triphosphate. A range of different resins were functionalized and employed as a basis for the support.

The bead size was found to an important factor that governed the yields obtained, presumably due to the fact that cleavage times had to be short and the intermediate formed is labile. Microporous polystyrene of 75-150 μm gave a 50 % yield after HPLC purification for the trial compound and desalting and ion exchange chromatography was not necessary.

The reaction conditions developed are tabulated below (Table 2.15). The scope of the reagent will be described in the next chapters with a view to accessing other

triphosphates, labeled triphosphate nucleosides and other phosphorylated nucleosides.

Table 2.15 Reaction conditions

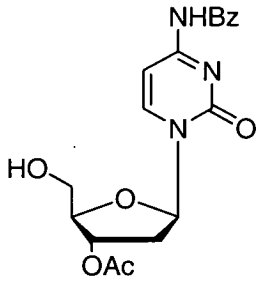
Reaction name	**eq**	**Conc. (M)**	**Time (min)**	**Temp. (°C)**
Loading of nucleoside	0.33	0.10	60	rt
Pyrophosphate cleavage	1.5	0.37	3 x 10	rt
Oxidation	1.5	0.37	15	rt
Hydrolysis	-	-	30	rt
Deprotection	-	-	60	rt

3 Application to triphosphate synthesis

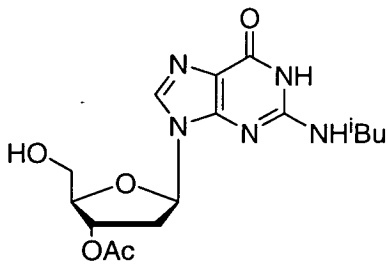
3.1 Synthesis of DNA triphosphates

3.1.1 Nucleosides

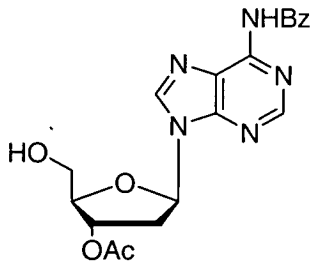
The use of the supported phosphorylation reagent was extended to the synthesis of the other three DNA nucleosides. The Ludwig-Eckstein procedure established the need for protection of the 3' hydroxyl group, whereas protection of the exocyclic amino groups was reported not to be necessary.[83] Since removal of the base protection groups (Bz and ^{i}Bu) can be performed in the same step as the acetate removal, it was opted to use the commercially available nucleosides shown below (Figure 3.1).



77



78



79

Figure 3.1 Protected DNA nucleosides

3.1.2 Phosphorylation reactions

The phosphorylation conditions developed in Chapter 2 were used to phosphorylate the other DNA nucleosides (Table 2.15).

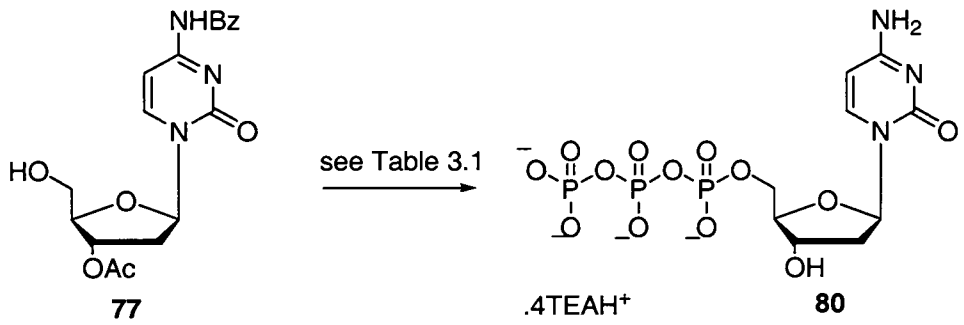
Table 3.1 Reaction conditions used for triphosphate synthesis

Reaction name	eq	Conc. (M)	Time (min)	Temp. (°C)
Loading of nucleoside	0.33	0.10	60	rt
Pyrophosphate cleavage	1.5	0.37	3 x 10	rt
Oxidation	1.5	0.37	15	rt
Hydrolysis	-	-	30	rt
Deprotection	-	-	60	rt

3.1.3 dCTP

Cytdine nucleoside **77** was stored over P_2O_5 in a desicator for a week prior to use. The phosphorylation conditions were used as detailed in Table 2.15 and the intermediates were not characterised in this instance.

It was found that nucleoside **77** was not fully soluble in 1/3 pyridine/THF at the desired 0.1 M concentration, thus the solvent for the loading step was modified to include dry DMF in a ratio of 1/1/2 DMF/pyrdine/THF. This is in line with Ludwig and Eckstein, who also added DMF to solubilise cytidine.[83] The pyrophosphate cleavage, oxidation and hydrolysis steps proceeded smoothly (Scheme 3.1).



*Scheme 3.1 Synthesis of cytidine triphosphate using PS-P-Cl **44***

The final deprotection step required to deprotect the 3' acetate as well as a benzoyl group on the base. It was found that a one hour of treatment with concentrated aqueous ammonia was sufficient to effect full deprotection to yield the free cytidine triphosphate. After an aqueous wash, the crude triphosphate was purified by Prep HPLC. Inspection of the HPLC chromatogram of the resultant compound (Figure 3.2) shows that it had excellent purity. The yield obtained after several lyophilisation steps was however too elevated and ^{1}H NMR confirmed presence of further triethylammonium salts.

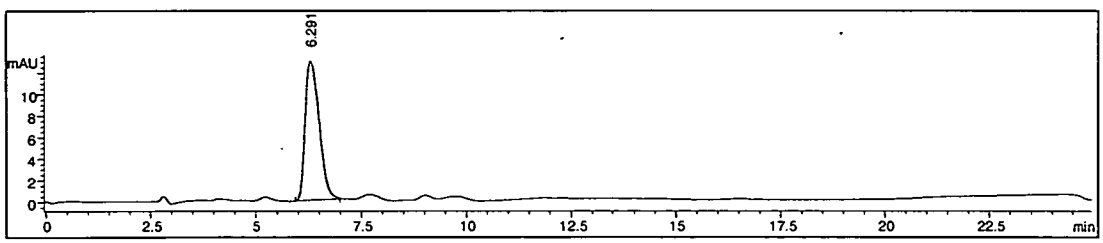


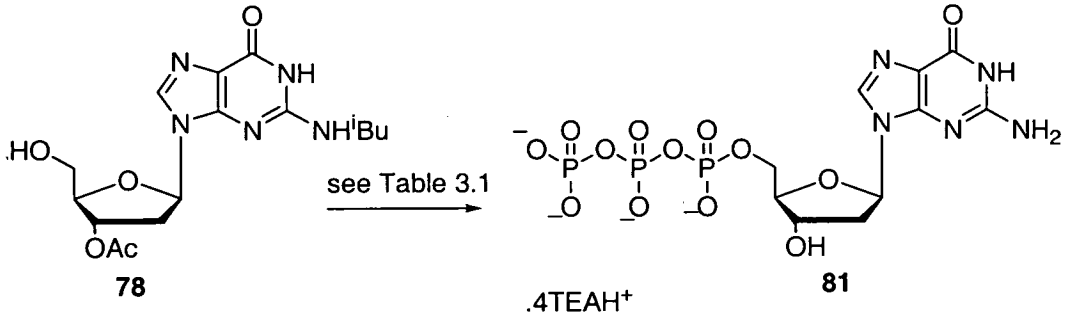
*Figure 3.2 HPLC chromatogram of dCTP **80** after Prep HPLC purification*

The ^{1}H NMR showed that the ratio of counterion to nucleoside peaks was too high, thus it seemed reasonable to conclude that salts present in the crude mixture were not completely removed by Prep HPLC. This is possible as, under the HPLC purification conditions used the crude cytidine triphosphate eluted quickly at *ca.* 3 min, thus separation from salts must have been insufficient. A second Prep HPLC purification was thus performed and since the bulk of the impurities had already been removed, the retention time now slowed to 6.3 min under the same elution conditions and cytidine triphosphate **80** could be isolated in a 12 % yield as its tetrakis triethylammonium salt after two Prep HPLC purifications.

This yield is lower than would have been hoped for considering the thymidine triphosphate yield. The lowered yield can be attributed to be fact that two Prep HPLC purifications had to be performed, and as mass recovery on a Prep HPLC system is not perfect, this contributed to lowering the yield.

3.1.4 dGTP

Guanidine nucleoside **78** was readily soluble in py/THF and after drying over P_2O_5 in a desicator was phosphorylated as detailed in Table 3.1. The following steps also proceeded as expected, but the final deprotection step was found to require longer time. The reaction was stirred for 15 h before evaporating and was then purified using Prep HPLC.



78 **81**

Scheme 3.2 Synthesis of guanosine triphosphate using PS-P-Cl ***44***

The crude reaction mixture showed good purity in the HPLC (Figure 3.3) and was isolated and dried as previously. Guanosine triphosphate **81** was isolated as its tetrakis triethylammonium salt in a good 28 % yield after lyophilisation.

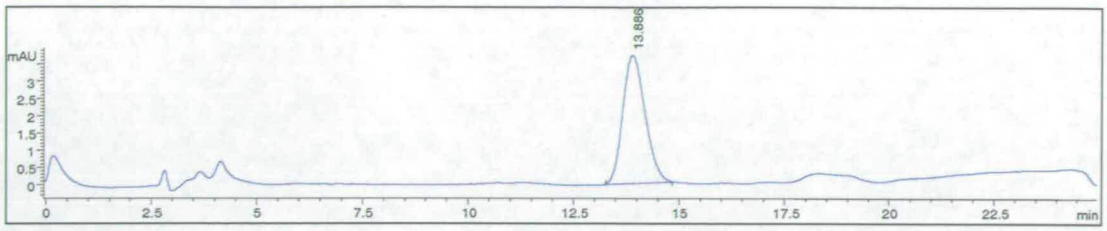
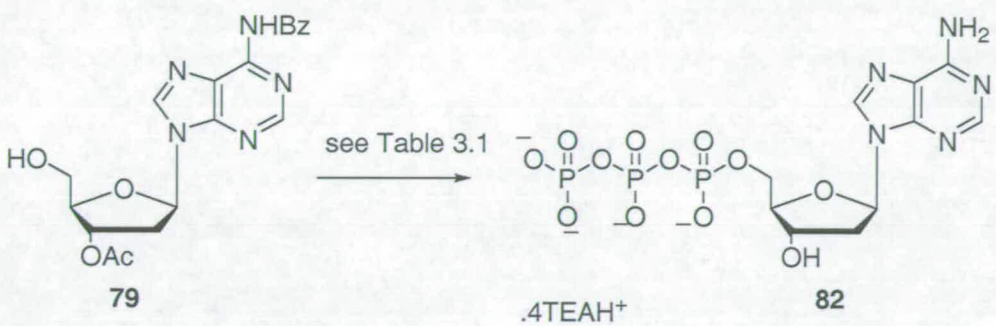


Figure 3.3 Crude HPLC chromatogram of dGTP ***81***

3.1.5 dATP

The synthesis and purification for adenosine triphosphate **82** was carried out analogously to the other triphosphates (Scheme 3.3). Again, the deprotection step of the exocyclic amine required prolonged reaction time, shown by MS analysis after 2.5 h, where the molecular ion corresponding to the benzoyl protected analogue was present.



Scheme 3.3 Synthesis of adenosine triphosphate using PS-P-Cl ***44***

The detrotection reaction was thus left to stir for a total of 15 h, before isolation of the product. Again, an HPLC of excellent purity was measured (Figure 3.4) and adenosine triphosphate was isolated in an excellent 45 % yield after lyophilisations.

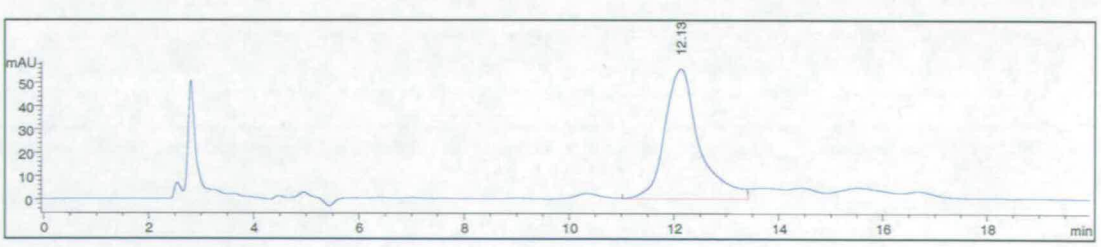
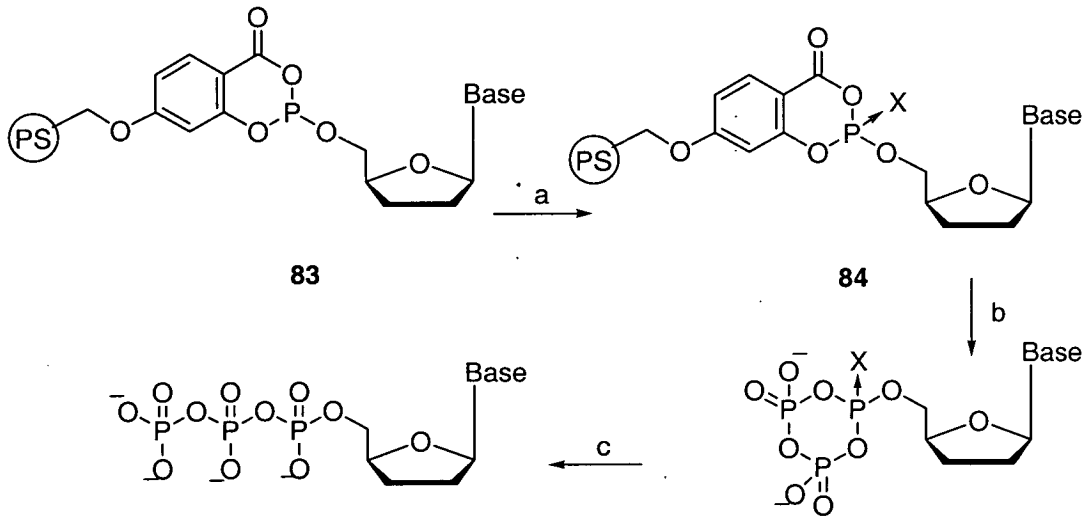


Figure 3.4 Crude HPLC chromatogram of dATP ***82***

3.2 On-resin oxidation

3.2.1 Introduction

During the course of the development of this approach, a different reaction sequence was also investigated. Rather than performing a solution phase oxidation on the cyclic triphosphite, it was decided to establish whether it was possible to convert the immobilised phosphitylated nucleoside **83** to a phosphorylated nucleoside **84** and whether this analogue was still electrophilic enough to be cleaved by pyrophosphate ions (Scheme 3.4).

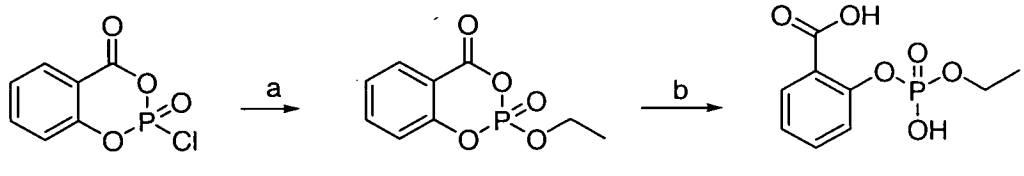


83 **84**

(a) Oxidation; (b) nucleophilic cleavage; (c) hydrolysis

Scheme 3.4 Projected reaction sequence using on-resin oxidation

A reaction relevant to this study was reported by Cremlyn *et al.*[146] who found that salicyl phosphorochloridate **85** could be treated with a range of alcohols (EtOH, PrOH, ^{i}PrOH, BnOH) to yield the alkoxy derivative **86** in excellent yield, which in turn hydrolysed to the 2-carboxyphenyl phosphate **87** (Scheme 3.5).



85 **86** **87**

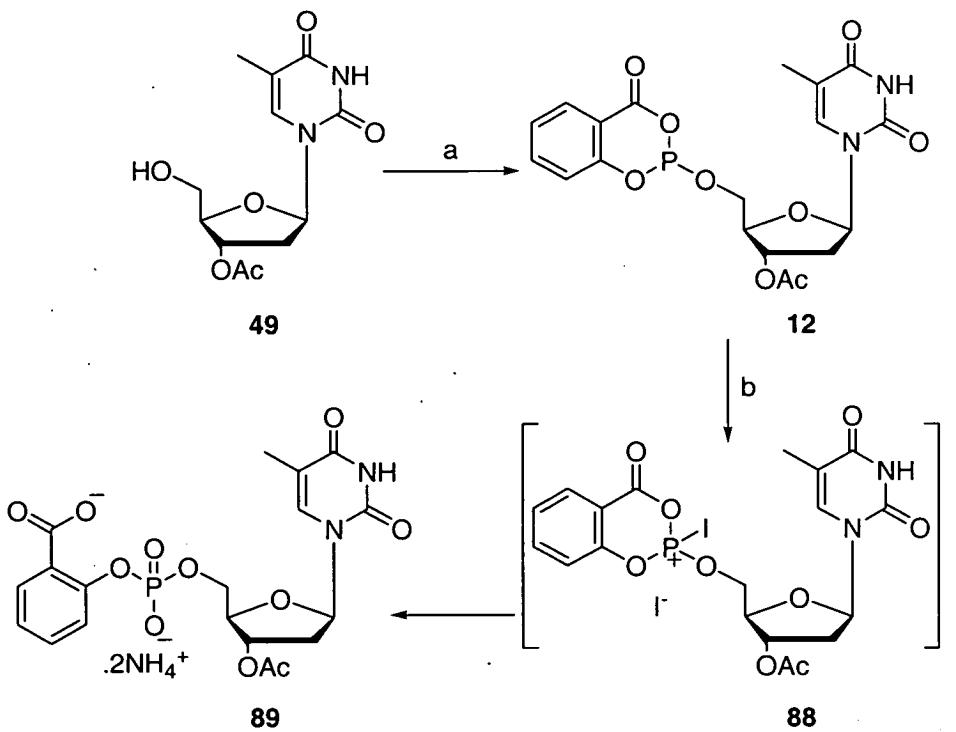
(a) 1 eq EtOH, 1 eq py, PhH, 2 h, 100 %; (b) 30 % aq dioxane, rt, 7 d, 58 %

Scheme 3.5 Nucleophilic reactions on salicyl phosphorochloridate 85

Therefore, the on-resin oxidation sequence could have a significant advantage over the original method. Premature hydrolysis of the nucleoside loaded resin **83**, which is a problem controlled by using dry solvents, would be reduced. As the phosphorus centre is less electrophilic in the oxidised state, premature hydrolysis would not only be dimished, but additionally, when it does occur, the product would not be released into solution remaining immobilised on the support, which should lead to a cleaner triphosphate product.

3.2.2 In solution

The model nucleoside 3'OAc-T **49** was treated in solution with the phosphitylation reagent and oxidised to prove that the salicyl phosphate ester could be obtained. 3'OAc-T **49** was readily phosphitylated as previously to yield **12**, which was oxidised *in situ* with 0.5 M I_2 in 98:2 py:H_2O. The intermediate formed is the phosphonium iodide salt **88**. Addition of water displaces the iodine from phosphorus and hydrolyses the P-O bond to give the desired 2-carboxyphenyl phosphate **89** in a 27 % yield after Prep HPLC purification (Scheme 3.6).



(a) 1.5 eq **11**, py/THF, 0.19 M, 10 min; (b) 1.5 eq 0.5 M I_2 in py:H_2O (98:2), 15 min; (c) H_2O, 5 min; 27 %

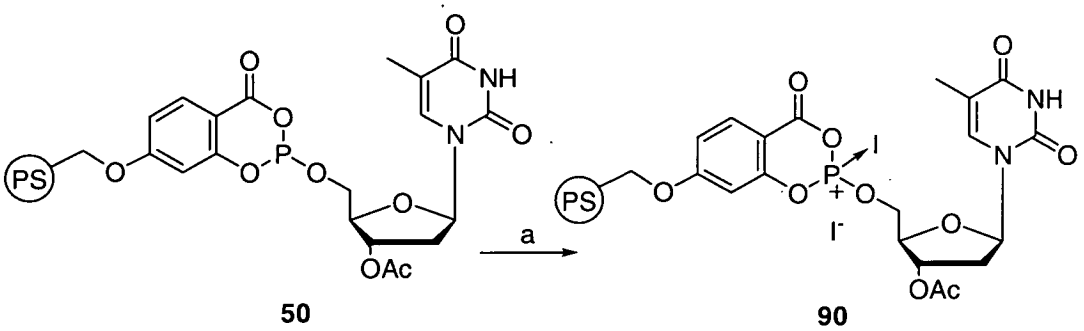
Scheme 3.6 Solution phase study of on-resin oxidation

3.2.3 On the resin

The oxidation step was then transferred onto the resin and studied on immobilised phosphitylated nucleoside **50**. In order to ensure complete conversion, different oxidation agents were considered and the intermediates verified by ^{31}P gel phase NMR.

3.2.3.1 *Investigation of an oxidising agent*

The nucleoside loaded resin **50** was treated with 0.5 M I_2 in pyridine (Scheme 3.7). Excess iodine was washing off the resin with DCM and THF. A minium of iodine remained though, which left the resin a brown colour. Phosphonium iodide salt **90** was characterised by ^{31}P Gel-Phase NMR.



50 **90**

(a) 5 eq 0.5 M I_2/pyridine, THF, 0.33 M, 1 h

Scheme 3.7 On-resin oxidation using iodine

The ^{31}P Gel-Phase NMR obtained was unsatisfactory; not only were two species present, but the signals observed were also very broad (Figure 3.5).

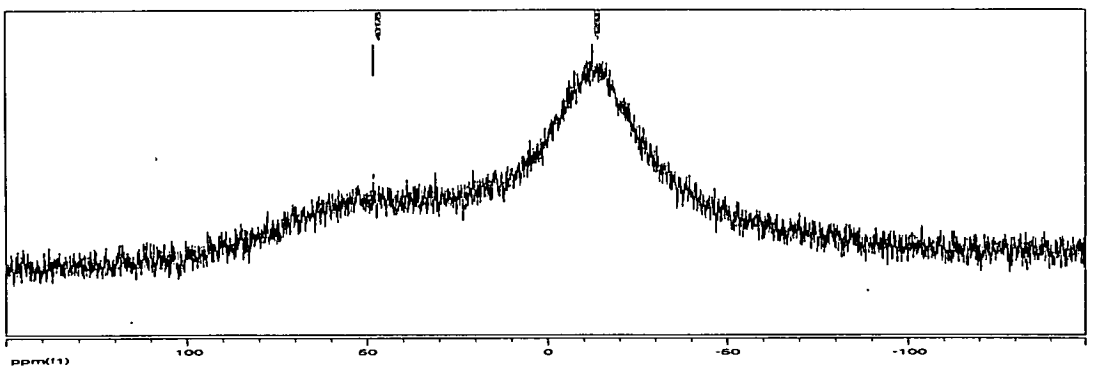
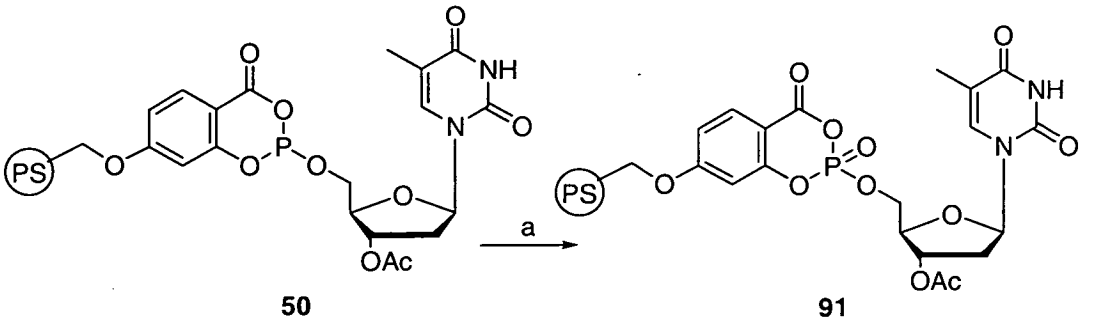


Figure 3.5 ^{31}P Gel-Phase NMR using iodine oxidation

Alternative oxidiation reagents for P (III) exist, one of which, ^{t}butylhydroperoxide, has previously been applied to solid phase chemistry.[120] This was used at the same

concentration as in the previous reaction for iodine (Scheme 3.8). The resulting phosphate triester **91** was characterised by ^{31}P Gel-Phase NMR.



50 **91**

(a) 5 eq 5-6 M ^{t}BuOOH in nonane, THF, 0.33 M, 1 h

Scheme 3.8 On-resin oxidation using ^{t}butylhydroperoxide

Using ^{t}butylhydroperoxide as the oxidant, a ^{31}P gel-phase NMR of excellent purity was obtained (Figure 3.6). It also shows that the previous oxidiation method probably resulted in incomplete oxidation.

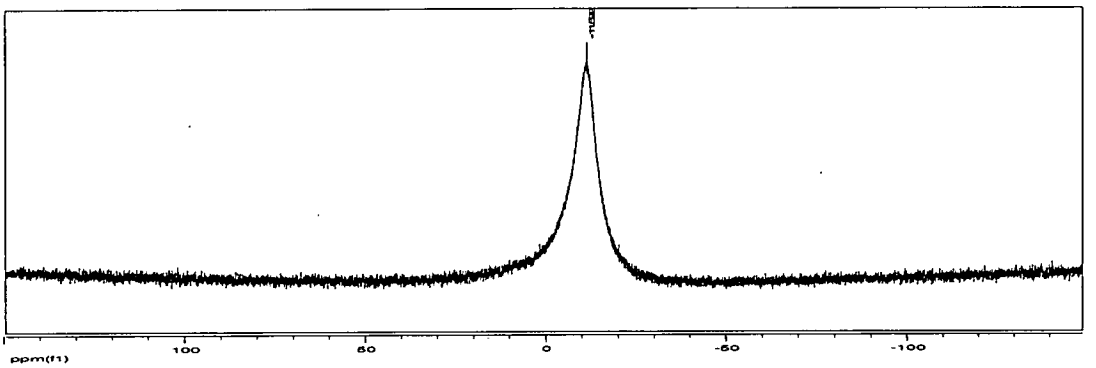


Figure 3.6 ^{31}P Gel-Phase NMR using ^{t}butylhydroperoxide oxidation

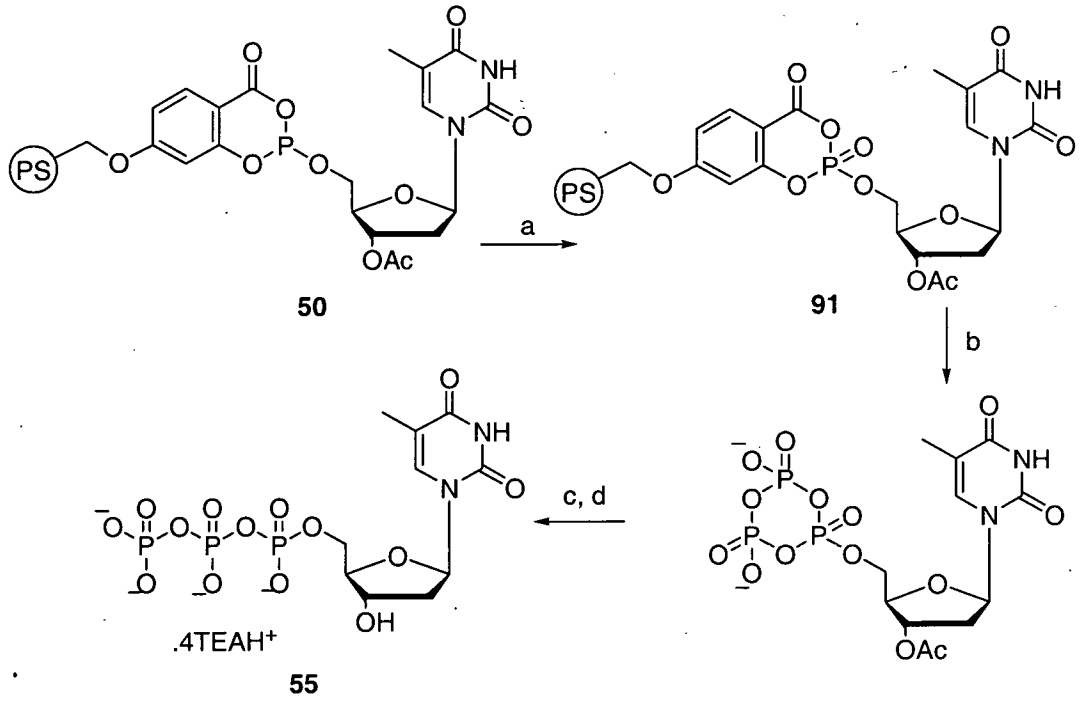
3.2.3.2 *Application to triphosphate synthesis*

On the basis of the ^{31}P NMRs of the resins, ^{t}butylhydroperoxide looked as the more promising oxidant for application to triphosphate syntyhesis. It was thus applied in the synthesis of dTTP using microprous PS, with the details given below (Table 3.2). The nucleoside was loaded onto the resin as previously and after rinsing, was treated with ^{t}butylhydroperoxide. The phosphate resin **91** was then subjected to pyrophosphate cleavage (Scheme 3.9). In light of the reduced electrophilicity of the phosphorus, it was expected that the conditions developed earlier would not be sufficient to cleave the nucleoside fully.

Table 3.2 Reaction conditions for triphosphate synthesis using on-resin oxidation

Scheme 3.9	eq	Conc. (M)	Time (min)	Temp. (°C)
Loading of nucleoside	0.33	0.10	60	rt
Oxidation (^{t}BuOOH)	1.5	0.33	60	rt
Pyrophosphate cleavage	1.5	0.37	3 x 10	rt
Hydrolysis	-	-	30	rt
Deprotection	-	-	60	rt

The pyrophosphate cleavage reaction was carried out as previously developed using three 10 minute cleavage steps and the resulting drained solution was subjected to ammonolysis to yield triphosphate **55**.



(a) 5 eq 5-6 M ^{t}BuOOH in nonane, THF, 0.33 M, 1 h, rt, 1 h; (b) 1.5 eq 0.5 M $(Bu_3N)_2$ pyrophosphate in DMF, 4 eq Bu_3N, rt, 3 x 10 min; (c) H_2O, 30 min; (d) NH_3/H_2O, 1 h; 3 % yield

Scheme 3.9 Synthesis of dTTP 55 using on-resin oxidation

An HPLC of the crude product after work-up and lyophilisation (Figure 3.7) compared favourably with the HPLC chromatogram of crude dTTP **55** obtained from the solution phase oxidation with iodine (Figure 3.8), although the dTTP was isolated by Prep HPLC in only a 3 % yield. This indicates that due to the lowered electrophilicity of the phosphorus centre, the cleavage conditions will need to be re-evaluated to push the reaction to completion with material remaining attached to the resin. However the purity was still excellent.

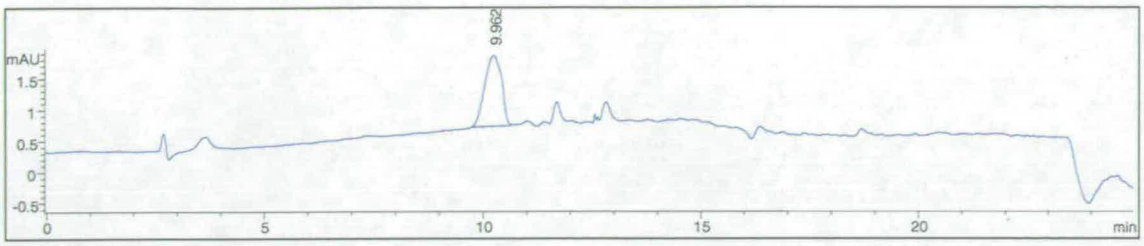


Figure 3.7 Analytical HPLC of crude dTTP ***55*** *using on-resin oxidation*

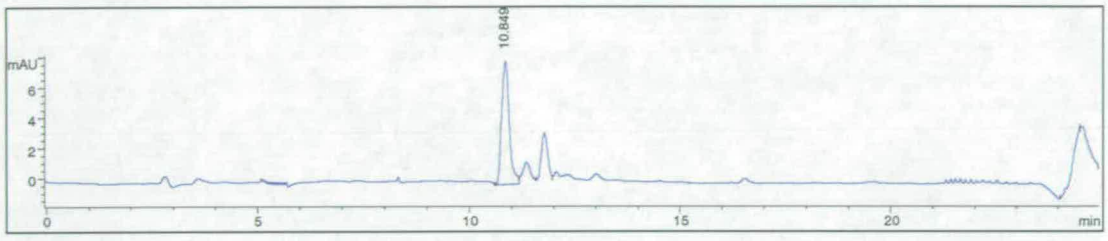


Figure 3.8 Analytical HPLC chromatogram of crude dTTP ***55*** *using solution phase oxidation*

3.2.3.3 *Investigation of pyrophosphate counterion*

An alternative to optimising the cleavage time, temperature or equivalents of the pyrophosphate ion used is to re-consider the counter-ion used with the pyrophosphate. Up until now, a bis (tri-*n*-butylammonium) salt of pyrophosphate was used.

Tris (tetra-n-butylammonium) hydrogen pyrophosphate has been proposed for synthesis of isoprenoid diphosphates[147] and triphosphate synthesis using the oxathiaphopholane approach[90] or using activated nucleoside phosphoramidates.[75] In those cases tri-*n*-butylammonium had been investigated as a counterion, but found not to be sufficiently nucleophilic. It was also reported that a displacement which was complete in 4 hours using the bis (tri-*n*-butylammonium) salt, took less than 10 minutes using the tris (tetra-n-butylammonium) salt.

With this in mind, tris (tetra-n-butylammonium) hydrogen pyrophosphate was investigated to cleave the nucleoside as a triphosphate from the resin. As the reagent is commercially available, this was considered first, however analysis by ^{31}P NMR precluded its use, due to its abysmal 'purity' (Figure 3.9a). Tris (tetra-n-butylammonium) hydrogen pyrophosphate was thus synthesised according to the procedure published by Poulter *et al.*[147] which involved ion exchange

chromatography to give pyrophosphoric acid, which was neutralised with aqueous tetrabutylammonium hydroxide. ^{31}P NMR of thus prepared pyrophosphate gave a much purer profile (Figure 3.9b).

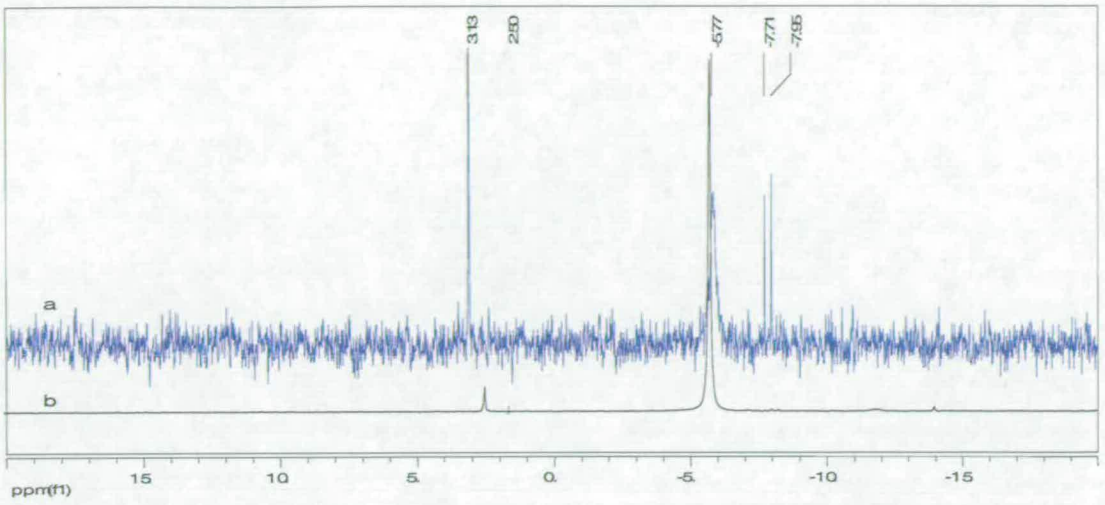


Figure 3.9 ^{31}P NMR of (a) commercial and (b) in-house prepared tris (tetrabutylammonium) pyrophosphate in $CDCl_3$

The only concern remained the water of hydration; it has been reported that tris (tetra-n-butylammonium) hydrogen pyrophosphate contains at least 3 molecules of water *per* pyrophosphate if re-crystallised from EtOAc.[147] Aezotropic removal of water by repeated evaporation from dry MeCN is possible,[147] but was found to leave still too much water for attack on oxathiaphopholane nucleosides;[90] drying by molecular sieves of a MeCN solution was thus advocated by these authors.

With this previous work in mind, the tris (tetra-n-butylammonium) hydrogen pyrophosphate salt was dissolved in $CHCl_3$ at a 0.5 M concentration. Chloroform was chosen over DMF as it enables better swelling of the resin and with the added hydrophobic counter-ions the salt was fully soluble in chloroform. This compares more than favourably to the tributylammonium salt, which requires sonication to dissolve even in DMF. The chloroform solution was then dried over 4 Å molecular sieves prior to use.

The triphosphorylation conditions were repeated as *per* Scheme 3.9 and Table 3.2, apart for the cleavage step; three ten minute cleavage steps were used without added base. Then, the collected solution was treated with water to hydrolyse the cyclic triphosphate and the acetate group was removed under ammonolysis. An HPLC chromatogram of the cleaved and deprotected tymdine triphosphate showed

only a very small amount of triphosphate present (data not shown). A purification by Sephadex DEAE chromatography was preformed; however no compound could be recovered.

This would leave the conclusion that most likely the pyrophosphate preparation with the tetrabutylammonium counter-ion was not sufficiently dry and hydrolysis of the supported phosphorylated nucleoside **91** would have led to an immoblised salicylate phosphate ester, analogous to **89**. Other solvents, which favour substitution chemistry (as before) should also be explored.

3.2.4 Conclusion

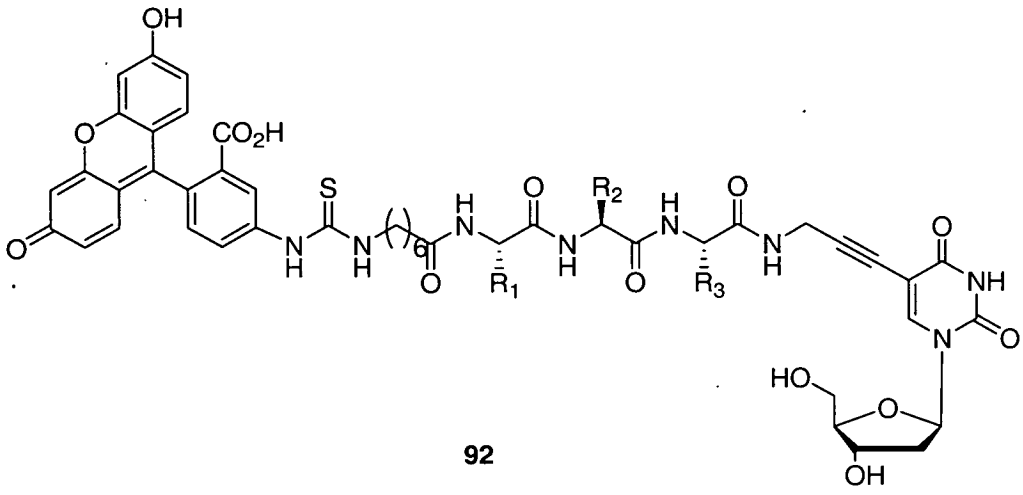
The possibility of performing the oxidation of the phosphitylated nucleoside was explored in this section. *Tert*-butyl hydroperoxide was found to be able to affect the oxidation step efficiently and cleanly as asserted be ^{31}P Gel Phase NMR of the intermediate.

The cyclic triphosphate could be cleaved off the resin in low yield, but excellent purity using the same cleavage conditions as for the P (III) analogue. Due to the reduced electrophilicity of P (III), a reduced yield can be rationalised. It is expected that the yield could be significantly improved upon if longer reaction times or higher temperatures or even more equivalents were to be used. These investigations were not performed; instead the method developed in Chapter 2 was used for the further work.

3.3 Synthesis of propargylamino linked triphosphates

3.3.1 Protease cleavable nucleosides

There is a long-standing interest in the group to synthesise triphosphates of nucleosides with a dye-peptide modification for DNA sequencing by synthesis applications. However, direct triphosphorylation of compounds such as **92** was found to be impossible.[148] This part of the work will discuss synthesis and triphosphorylation of linker modified pyrimidine bases, which can be used subsequently to react with an activated ester of the dye-peptide, such as an *N*-hydroxy succinimide ester (NHS).



92

Figure 3.10 Peptide modified nucleoside

3.3.2 Linkers for attaching reporter groups

Modification of the base is required for attachment of a fluorophore or other reporter molecule. Alkynyl based linkers are easily accessed using Sonogashira chemistry and were used in the first studies into fluorophore labelled dideoxynucleotides.[43] Additionally, alkenyl- and alkyl linkers have been synthesised.[149] With regards to spacer length, apart from the usual three carbon spacer, 5 and 12 carbons were investigated.[150]

A vital property of the spacer is that their presence does not affect DNA polymerases. Incorporation of 5-modified purine nucleosides is dependent on the flexibility, stereochemistry and the length of the linker group. Considering all

factors, propargylamine introduced through Pd cross-coupling is the favoured linker for general applications, due to its ease of synthesis and acceptance by polymerases.

3.3.3 Aims of the work

In the previous chapter a solid-supported triphosphorylation reagent was developed which incorporates the advantages of working on solid support, such a being able to push reactions to completion by using an excess of reagent without it interfering with the reaction. The plan was to apply this method to triphosphorylation of propargylamino modified nucleosides, illustrated below (Figure 3.11).

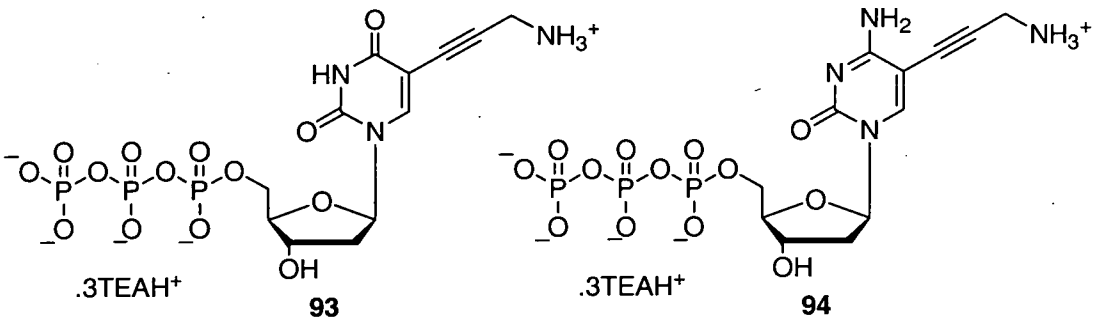
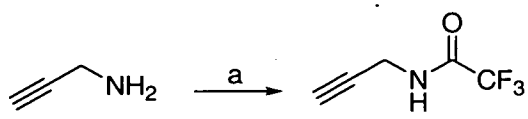


Figure 3.11 Structure of targeted triphosphates

3.3.4 Synthesis of propargylaminouridine

Propargylamine was protected as its trifluoroacetate with trifluoroacetic anhydride (Scheme 3.10). The product was readily isolated by column chromatography in good yield.



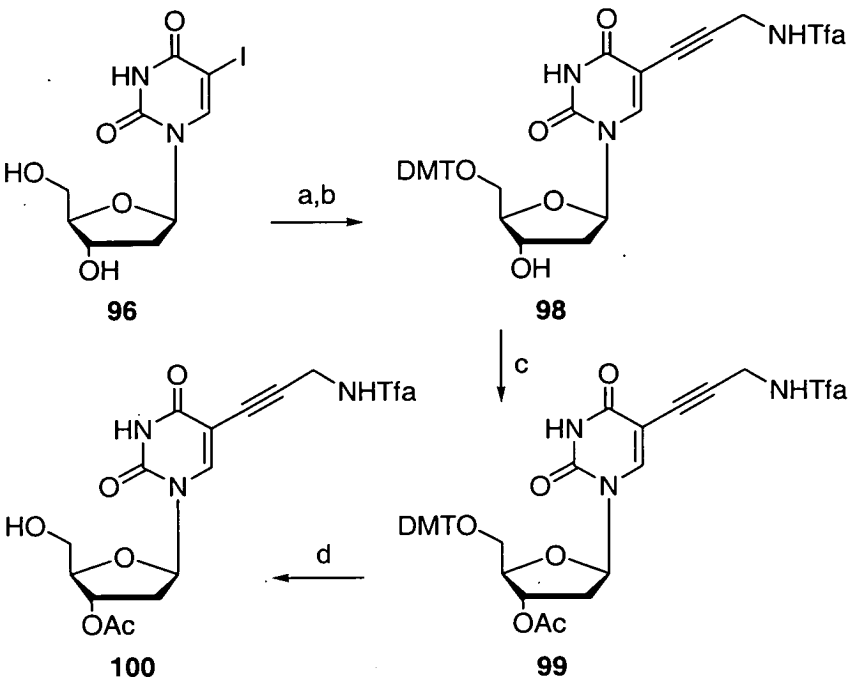
95

(a) 1.12 eq TFAA, 2 eq TEA, CH_2Cl_2, 0 °C to rt, 1.1 M, 14 h, 74 %.

*Scheme 3.10 Synthesis of protected propargylamino linker **95***

The thymidine analogue was accessed from commercial 5-iodo-uridine **96** *via* selective protection of the primary 5' alcohol with DMT-Cl to give **97** in good yield. Subsequent coupling of the 5-iodo to trifluoroacetate protected propargylamine **95** was executed under Sonogashira conditions to give **98**. A small

amount of a close-running non-identified side-product could be separated by column chromatography; in light of a similar observation for cytidine (Scheme 3.14), it is expected that it is a reduction product of the iodo group. Capping of the secondary 3' alcohol with acetic anhydride accessed fully protected thymidine analogue **99** with a three carbon linker (Scheme 3.11). Selective deprotection of DMT group was readily performed using 3 % trichloroacetic acid (TCA) to yield the desired product initially as an oil. As having a dry nucleoside is vital for the phosphorylation, **100** was subjected to lyophilisation to result in an off-white powder.



(a) DMT-Cl, 0.2 eq DMAP, pyridine, 0.28 M, 3 h, 76 %; (b) 2.5 eq **95**, 0.1 eq $[Pd(PPh_3)_4]$, 0.2 eq CuI, 5 eq TEA, DMF, 2.5 h, 0.5 M, 69 %; (c) 1.0 eq Ac_2O, 0.25 eq DMAP, 2.5 eq TEA, pyridine, 0.15 M, 2 h, 93 %; (d) 3 % TCA/CH_2Cl_2, MeOH, 98 %.

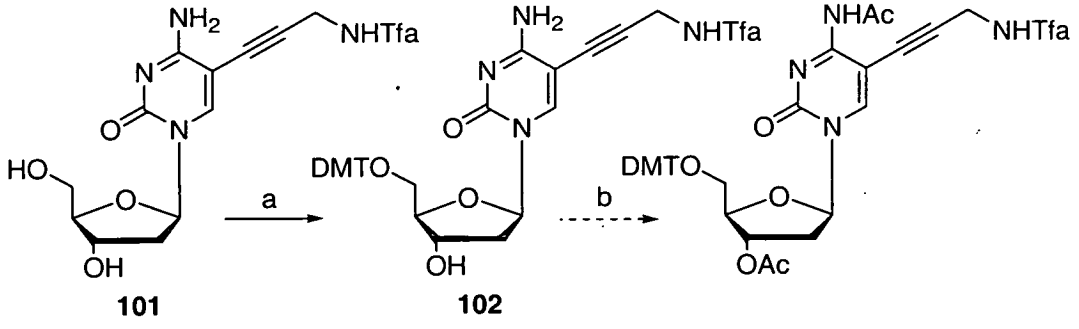
*Scheme 3.11 Synthesis of protected uridine analogue **100***

3.3.5 Synthesis of propargylaminocytidine

The synthesis of a propargylamino modified cytidine analogue required consideration of the exocyclic amine of the cytosine base. Base protection is not required for the phosphorylation step, however it may be easier to protect it in order to achieve protection of the 3' hydroxyl group.

The first attempts towards the desired nucleoside were made from spacer modified cytosine **101**. Again selective protection with DMT-Cl was carried out, but the

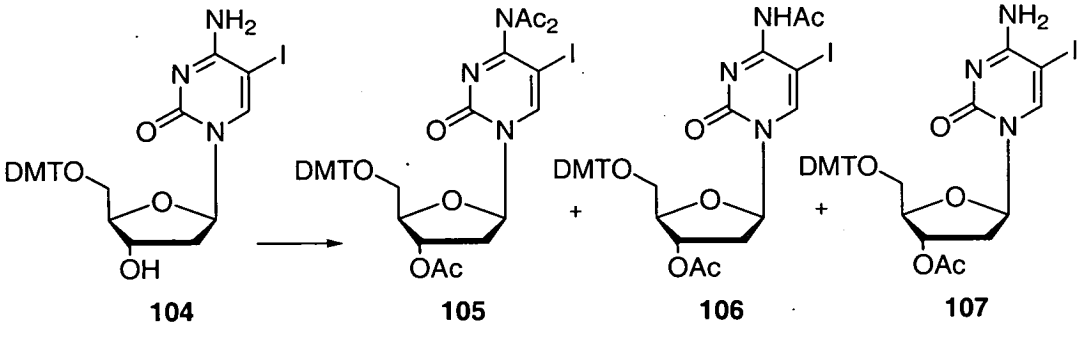
reaction was rather sluggish and did not go to completion in 24 h; nonetheless **102** was isolated in acceptable yield (Scheme 3.12). The obtained cytidine analogue **102** was then treated with an excess of acetic anhydride under DMAP catalysis to acetylate both the 3' hydroxyl and the exocyclic amine. This however yielded complex mixtures and multiple acetylations, as asserted by LC-MS analysis. More vigorous conditions using acetyl chloride acetylated additionally the NH of propargylamine and gave rise to a series of compounds where the Tfa had been exchanged for an acetyl.



(a) DMT-Cl, 0.2 eq DMAP, pyridine, 0.25 M, 24 h, 55 %; (b) 5 eq Ac_2O, 0.25 eq DMAP DCM, not isolated.

Scheme 3.12 Attempted synthesis of protected cytidine analogue with linker

A second approach was taken to circumvent acetylisation once the linker had been introduced. Commercial 5-iodo-cytidine **103** was protected on the 5' hydroxy postion with DMT-Cl to yield **104** in a 61 % yield. Suitable acetylation conditions could now be developed on **104** (Scheme 3.13, Table 3.3).



Scheme 3.13 Acetylation of cytidine analogue

Treatement of **104** with excess acetic anhydride under DMAP catalysis gave tri-acetylated cytidine **105** in 31 % and di-acetylated cytidine **106** in 20 % yield (Entry 1). This showed that both the 3'OH and the amine were readily acetylated. A 0.05 M nucleoside solution was then treated with 1.0 eq of acetic anhydride. Analysis by

tlc showed spot-to-spot conversion, which was isolated to give **107** with 3' acetylation in very good yield (Entry 2). Neat addition of a 2nd eq of Ac_2O yielded a mixture of **105** and **106**. However, dropwise addition of a 2 eq Ac_2O solution performed at higher dilution produced **106** selectively (data not shown).

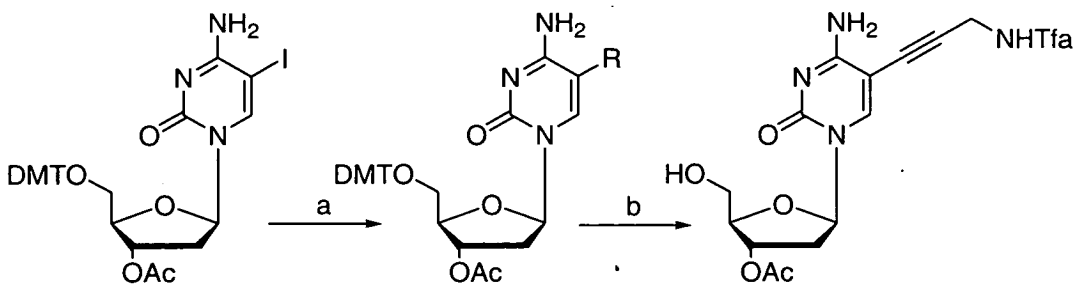
Table 3.3 Acetylation conditions

Entry	Conditions	Conc. (M)	Time (min)	105 %[a]	106 %[a]	107 %[a]
1	5 eq Ac_2O, 0.25 eq DMAP, CH_2Cl_2	0.2		31	20	/
2	1 eq Ac_2O, 0.25 eq DMAP, CH_2Cl_2	0.05	30	/	3	89

[a]*Isolated yield*

With the essential 3' protection in place, compounds, either with or without base protection, were available to take forward to couple the linker. Monoacetyl-base protected cytidine **106** was subjected to Sonogashira conditions (2.5 eq **95**, 0.1 eq $[Pd(PPh_3)_4]$, 0.2 eq CuI, 5 eq TEA, DMF, 0.5 M). The reaction, monitored by tlc, gave however rise to a complex mixture of products, even before the starting material had been used up.

A more satisfying result could be obtained with base unprotected **107**, which was readily converted to the linker modified analogue **108** in good yield (Scheme 3.14). A minor side-product was the reduced cytidine **109**, which could be separated from the desired compound by careful column chromatography. It is expected that its presence could be reduced or eliminated altogether by lowering the catalyst loading.



107 R = $CCCH_2NHTfa$, **108**, 79 %; H, **109**, 7 % **110**

(a) 2.5 eq **95**, 0.1 eq $[Pd(PPh_3)_4]$, 0.5 eq CuI, 5 eq TEA, DMF, 2.5 h, 0.5 M; (b) 3 % TCA/CH_2Cl_2, 95 %

*Scheme 3.14 Attachement of linker to cytidine analogue **110***

As the final step, the DMT group was removed by the standard conditions (3 % TCA in CH_2Cl_2) in excellent yield to give the desired nucleoside **110** as an oil which was isolated as a yellow powder after lyophilisation.

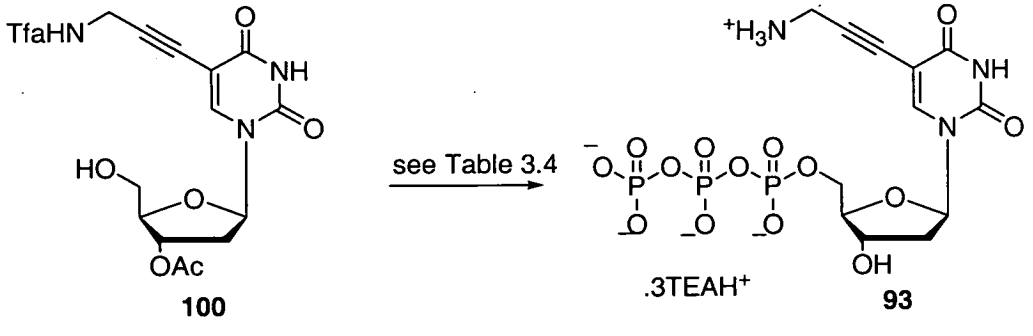
3.3.6 Phosphorylation of propargylaminouridine

The triphosphate synthesis conditions developed previously are summarised below (Table 3.4) and were used as such for the phosphorylation of the pyrimidine nucleosides.

Table 3.4 General reaction conditions for triphosphate synthesis

Reaction name	eq	Conc. (M)	Time (min)	Temp. (°C)
Loading of nucleoside	0.33	0.10	60	rt
Pyrophosphate cleavage	1.5	0.37	3 x 10	rt
Oxidation	1.5	0.37	15	rt
Hydrolysis	-	-	30	rt
Deprotection	-	-	60	rt

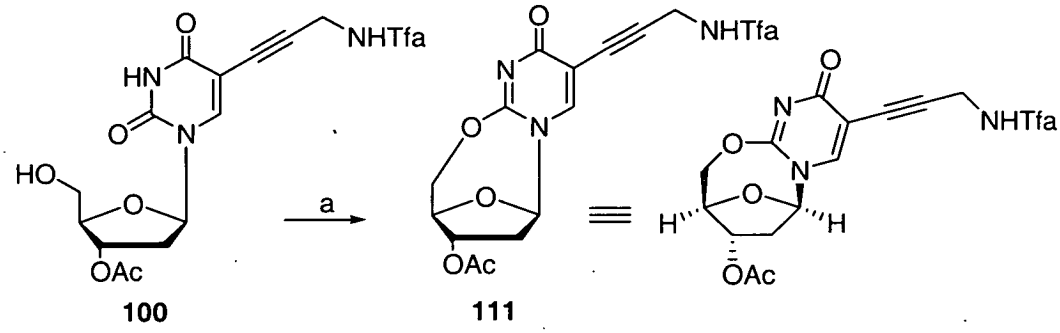
Propargyl amino linked uridine nucleoside **100** was subjected to the phosphorylation conditions (Scheme 3.15); however this was met with no success in detecting any triphosphate in the cleaved solution by Prep HPLC or Sephadex ion exchange chromatography. Initially this was attributed to the nucleoside not being sufficiently dried. This was eliminated as a possible explanation after experiments with prolonged drying of the nucleoside were equally unsuccessful. Increased steric bulk was considered as the cause, but after experiments on cytidine were more successful, this also looked unlikely.



Scheme 3.15 Synthesis of propagylamino linked uridine triphosphate

Finally, the drained solution from the nucleoside loading onto the resin was examined (Scheme 3.16). Apart from the starting nucleoside, a lower running spot

was detected, which exhibited chemical shift changes of H-6 and H-5' by ^{1}H NMR. It was tenatitively assigned to be 2,5'-anhydronucleoside **111**. By HPLC, the anhydronucleoside **111** could be seen to be decomposing to starting nucleoside **100** when TEA had been added. However, further characterisation of this anhydronucleoside **111** was not possible as it could only be isolated in small quantities and decomposed quickly.

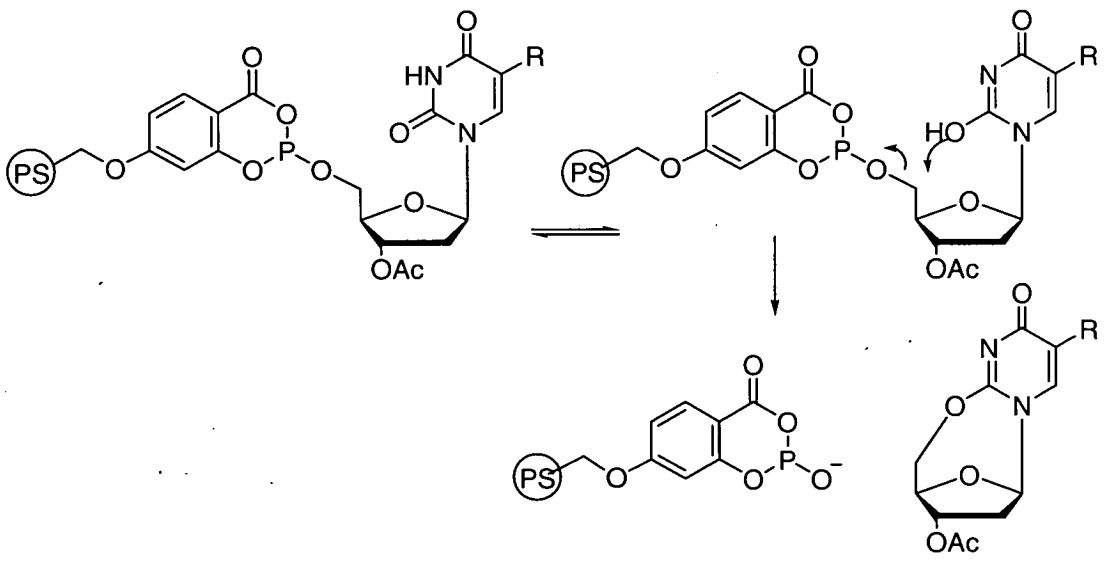


100 **111**

(a) 3 eq PS-P-Cl **44**, 0.1 M py/THF 1/3, 1h, rt.

Scheme 3.16 Synthesis of as 2,5'-anhydrouridine analogue

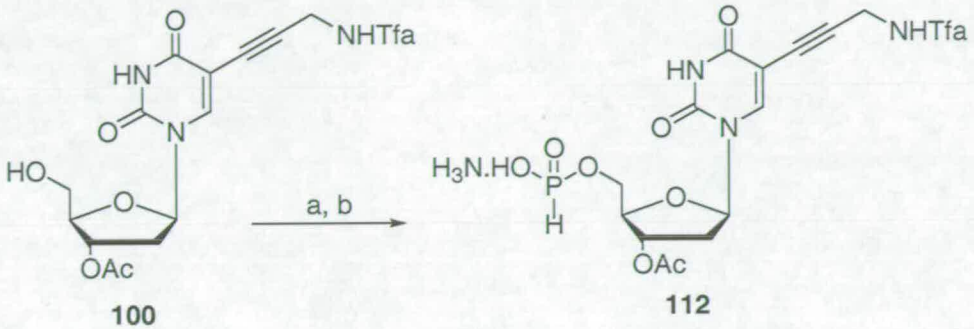
Previous syntheses of 2,5'-anhydrouridines activated the 5' hydroxyl group either as the tosylate, which yielded the corresponding 2,5'-anhydrouridine nucleoside upon treatment with DBU in 40 % yield over two steps,[151] or as the 5' naphalenesulfonyl derivative with subsequent NaO^{t}Bu treatment.[152]



Scheme 3.17 Proposed mechanism for formation of 2,5'-anhydrouridine nucleoside

In this instance, it is surmised that the enol tautomer of uridine attacks the phosphitylated 5' methylene. The oxygen, activated as a leaving group, remains on the resin, while the anhydro nucleoside is cleaved off the resin (Scheme 3.17).

To assess the quantity of urdine nucleoside **100** that remained on the resin after 'self-cleavage' under the phosphitylation conditions, the nucleoside was loaded onto 3 eq of the resin **44** for 1 h, as previously. The resin was drained, rinsed and cleaved with aq THF (Scheme 3.18) and the residue analysed by HPLC.



(a) 3 eq PS-P-Cl **44**, 0.1 M py/THF 1/3, 1h, rt; (b) THF/H_2O 1/1, 10 min, 57 % yield.

Scheme 3.18 Synthesis of propagylamino linked uridine-H-phosphonate

Inspection of the crude chromatogram indicated a major product at 5.97 min (Figure 3.12), which was shown to be the desired product by MS. Comparison of retention times with the starting material **100** showed that it was barely present (retention time of 4.5 min). The product was isolated by Prep HPLC eluted in a 0.1 M ammonium acetate/MeCN gradient. The other two products remain un-identified.

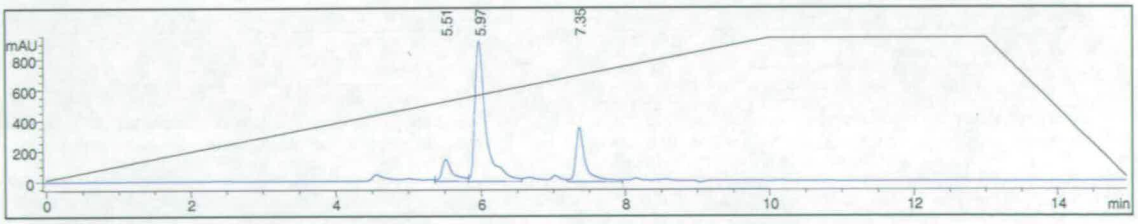


*Figure 3.12 Crude HPLC chromatogram of uridine phosphonate **112***

After repeated lypohilisation, the *H*-phosphonate **112** was isolated in a 57 % yield as its ammonium salt. Comparison with the formation of the *H*-phosphonate of 3'OAc-T **49** under the same conditions is favourable as the yield obtained for this simpler substrate was 58 % yield. Thus, it can be concluded that the

propargylamino linker does not interfere with the phosphitylation reaction of the primary alcohol. Any issues arising with this substrate thus are most likely to be due to solubility issues.

It was next attempted to isolate the triphosphate. The resin was loaded and cleaved as previously and the collected solution oxidised and the remaining iodine quenched. The resulting cyclic triphosphate was hydrolysed, which resulted in a clear yellowish solution. As no solubility problems were observed at this stage, it was decided to evaporate the solution and proceed to the ammonolysis step. The resulting crude reaction mixture was washed with CH_2Cl_2 and analysed by HPLC (Figure 3.13).

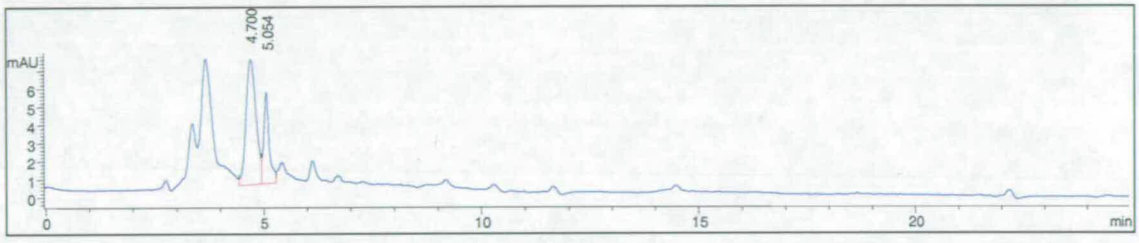


Figure 3.13 Crude HPLC chromatogram of uridine propgargylamino triphosphate ***93***

The peak at 4.70 min was found to be the desired compound and the crude product was purified by Prep HPLC to yield the desired product in a 23 % yield as a triethylammonium salt.

Analysis of the ^{1}H NMR of the resulting product however indicated that some triethylammonium salts were not purified out of the crude, as the integration of the triethylammonium relative to the nucleoside peaks was higher than expected. It would thus be necessary to perform a desalting step prior to Prep HPLC purification.

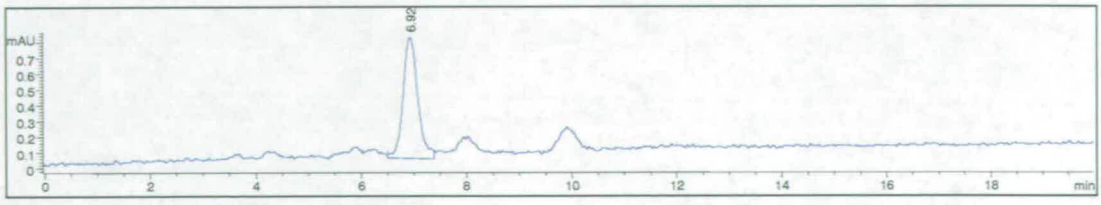


Figure 3.14 Crude HPLC chromatogram of uridine triphosphate ***93*** *after Prep HPLC*

Instead, it was opted to re-purify the resulting compound by a second Prep HPLC. Inspection of the subsequent HPLC trace shows that with the majority of the impurities removed in the first Prep, the retention time of the compound slowed down to 6.22 min (Figure 3.14). The remaining impurities were thus readily separated and **93** could be isolated readily as a white powder.

The yield was estimated by UV-Vis absorbance to deduce the concentration, using the method by Battersby *et al.* who used an extinction coefficient of $\varepsilon_{290} = 9000\ M^{-1}\ cm^{-1}$ to determine the yield for their synthesis of **93**.[153] The amount obtained was calculated to be 680 nmol, which gives approximately a 1 % yield from **100**. A comparison with the synthesis from Battersby *et al.* is not possible as the authors did not provide a yield.

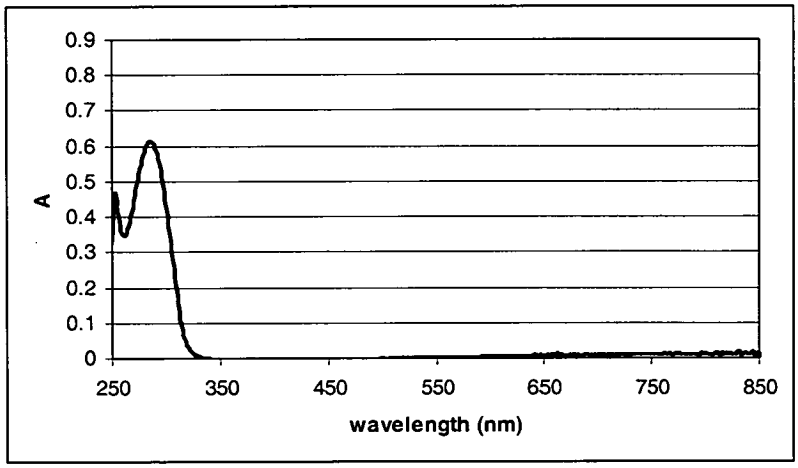
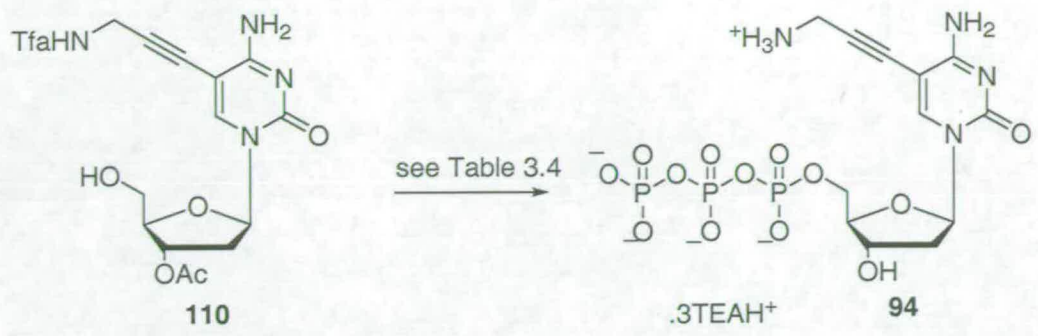


*Figure 3.15 UV-Vis spectrum of **93** in H_2O*

3.3.7 Phosphorylation of propargylaminocytidine

The synthesis of propagylamino linked cytidine triphosphate **94** involved triphosphosphorylation of the corresponding 3' acetate protected nucleoside **110**, which had been dried carefully before attempting the triphosphorylation (Scheme 3.19). The general reaction conditions as developed in the Chapter 2 and illustrated in Table 3.4 were applied to the synthesis.



Scheme 3.19 Synthesis of propagylamino linked cytidine triphosphate

The crude HPLC chromatogram showed four main peaks (Figure 3.16). They were separated by Prep HPLC, but could not be assigned to a particular compound using ES-MS. The remainder of the crude product was resuspended in NH_3/H_2O. It was noted at that point that a considerable amount of the product was not soluble. After 1 h treatment with NH_3/H_2O, an aliquot was analysed by HPLC, however the peaks remained essentially unchanged.

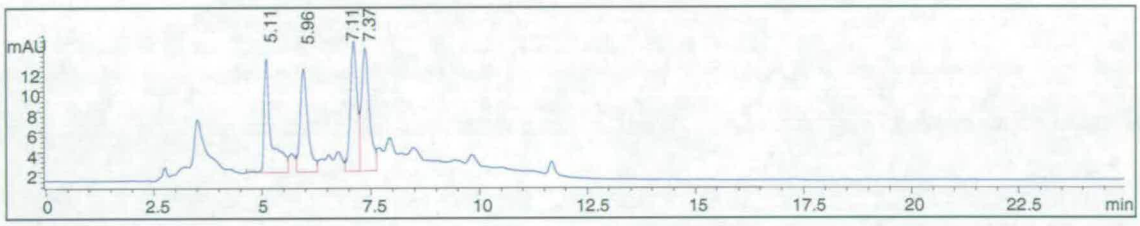


Figure 3.16 Crude HPLC of propargylamino linked cytidine triphosphate ***94***

The synthesis was thus repeated, while taking care at each step that all the compound was fully dissolved. Thus, the loading of the cytidine nucleoside was easily achieved, as judged by the absence of nucleoside in the drained solution. The pyrophosphate cleavage step yielded a yellow solution which was treated with iodine solution to affect oxidation. After quenching of excess iodine with $NaHSO_3$, the solution was dissolved in water to hydrolyse to the cyclic triphosphate to the desired linear compound. After 30 minutes, the solution was reduced *in vacuo* to a oil with white solids. The final step was the deprotection step. Thus adding concentrated aqueous ammonia was required to deprotect the acetate and trifluoroacetate groups. However, it was noted that upon addition of the required amount of ammonia, a considerable amount of solids remained. Addition of 5 mL of water and brief sonication however managed to fully dissolve these solids and the milky-yellow solution was stirred for 90 minutes.

The crude reaction mixture was subjected to the usual work-up procedure, and then purification by Prep HPLC was attempted. The crude HPLC shows a single main product, which was very satisfying, especially as it corresponded to one of the peaks from the previous attempt (Figure 3.17). Frustatingly enough, the HPLC peak could not be isolated without co-running with the impurities. Re-purification after the first Prep did in this case not improve the purity, thus in the future ion exchange chromatography on Sephadex will not be avoidable.

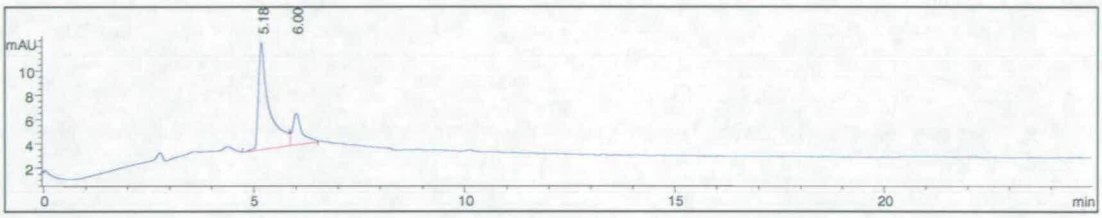


Figure 3.17 Crude HPLC chromatogram of cytidine propgargylamino triphosphate ***94***

3.4 Synthesis of a fluorophore labelled triphosphate

3.4.1 Triphosphate synthesis

Triphosphates of dideoxy and deoxy nucleosides are compounds with many important applications. Their straightforward synthesis is frustrated by the inability to form the triphosphate moiety if a large lipophilic group such as a fluorophore is present. In a recent study by Burgess *et al.*[154] this property of triphosphate synthesis was observed when their synthetic efforts to convert the rigid fluorescein labelled nucleosides **113** and **114** to their triphosphates were unsuccessful. They attributed their failure at performing these synthetic transformations to several issues. Most important were solubility problems which impacted on purification using ion exchange chromatography and HPLC purifications.

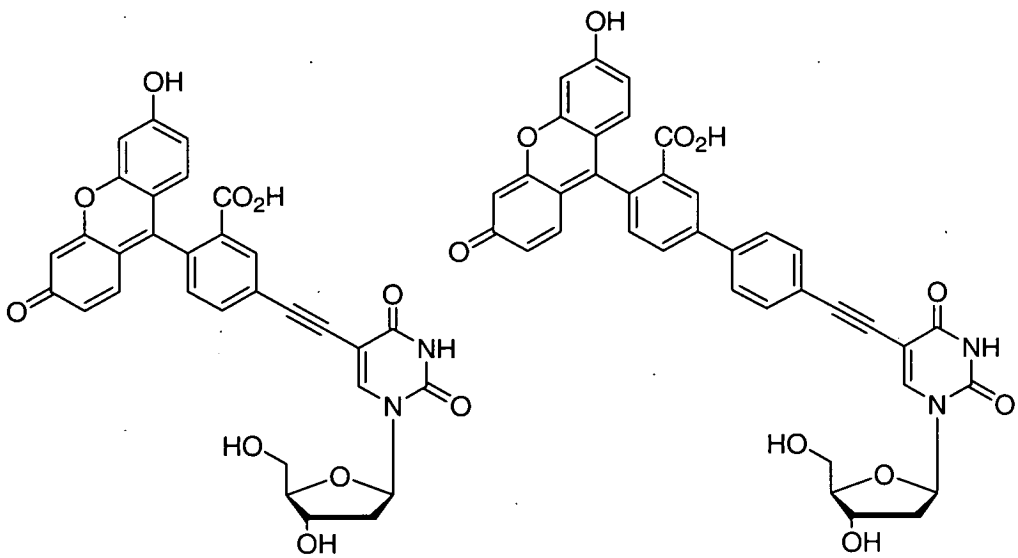


Figure 3.18 Rigid fluoroscein nucleosides

3.4.2 Aims of the work

In the previous chapter a solid-supported triphosphorylation reagent was developed which incorporates the advantages of working on solid support, such a being able to push reactions to completion by using an excess of reagent without it interfering with the reaction. The plan is to develop a method to perform a direct triphosphorylation on a labelled nucleoside in a synthetically useful yield,

something which so far has not been possible. The deoxy and dideoxy nucleoside triphosphates which are envisaged in this study are illustrated below (Figure 3.19). The flurorophore envisaged was carboxy-fluorescein, which is commercially available as a mixture of regioisomers.

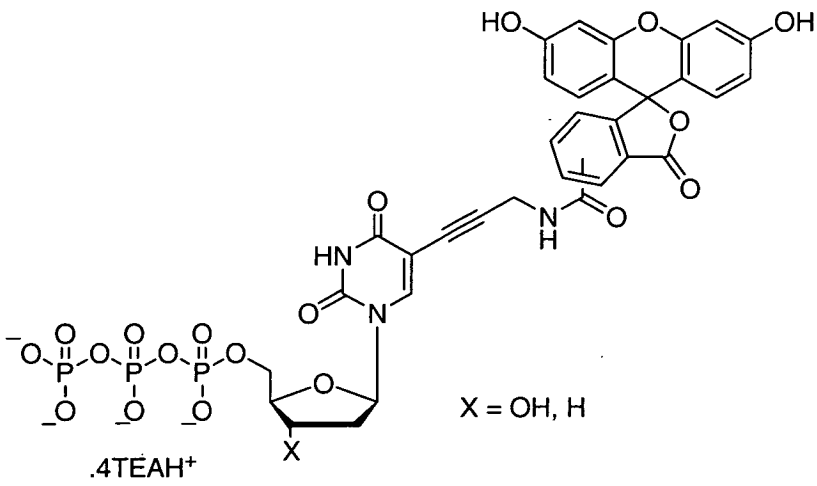


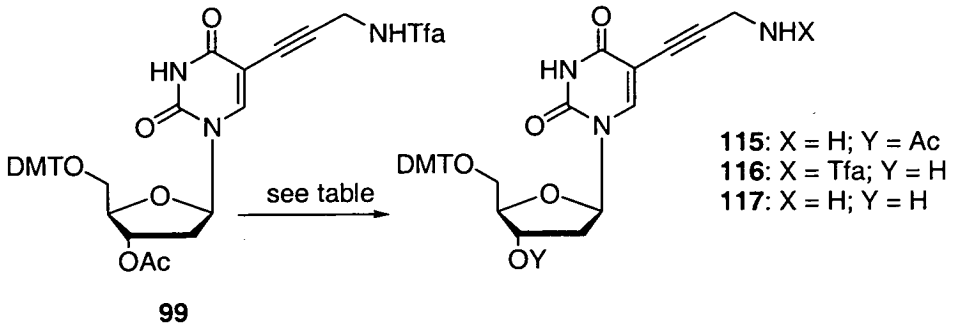
Figure 3.19 Structure of targeted triphosphates

3.4.3 Synthesis of a labelled 2'-deoxy nucleoside

In order to attempt a triphosphorylation of a fluorogenically labelled nucleoside, such a compound was synthesised. The chosen fluorophore, carboxyfluorescein, needed to be coupled to an amino modified linker on the nucleoside, so the initial target was an amino-modified thymidine analogue.

3.4.3.1 *Synthesis of propargylamino-uridine*

Fully protected uridine **99** synthesised in the previous section (3.3.4) was used as a starting point for a thymidine analogue with a free amine group. Conditions for deprotecting the Tfa group in the presence of the acetate group were investigated and monitored by HPLC (Scheme 3.20). Apart from the desired product **115**, two side products were observed: **116**, where both protection groups had been removed and **117**, where the acetate group had been deprotected in favour of the Tfa group. The literature conditions known for selective deprotection were not succesful for this substrate (Table 2.9).



99

Scheme 3.20 Deprotection of the Tfa group in the presence of the Ac group

In fact, a reversal of the selectivity was observed (Entry 1). Deprotection conditions under basic pH were also unsuccessful, as acetate deprotection was favoured (Entry 2). Attention was then turned to reducing conditions (Entry 3). Using $NaBH_4$ the desired compound was observed with a good conversion, when fused calcium chloride was added to the reaction mixture to keep the reaction dry (Entry 4). (Presence of water hydrolyses $NaBH_4$ to yield a basic solution.)

Table 3.5 Conditions for trifluoroacetamide deprotection

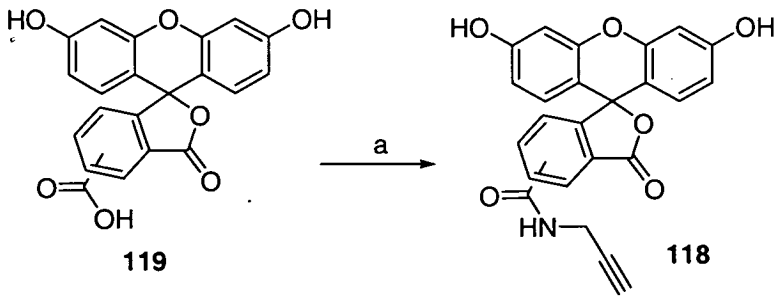
	Conditions	time	99 % [a] SM	115 % [a] -Tfa	116 % [a] -Ac	117 % [a] -both	Ref
1	10 % K_2CO_3, 5:2 MeOH:H_2O	25 min	6	-	66	24	155
2	phosphate pH 8:MeOH 1:1	17 h	80	-	18	-	
3	13 eq $NaBH_4$, EtOH	30 min	17	37	20	18	156
4	5 eq $NaBH_4$, 5 eq $CaCl_2$, EtOH	3 h	43	45	8	5	157
5	5 eq $NaBH_4$, 5 eq $CaCl_2$, EtOH	3 h	7	64	8	21	

[a] *Conversions obtained by peak area in UV at 282 nm*

Nonetheless, the reaction was not very reproducible (entries 4 and 5) and the isolated yield was low (23 % for Entry 5). It was therefore decided to look at performing the Sonogshira coupling directly on carboxyfluorescein derivatised propargylamine **118**.

3.4.3.2 *Sonogashira coupling to alkynyl fluorescein*

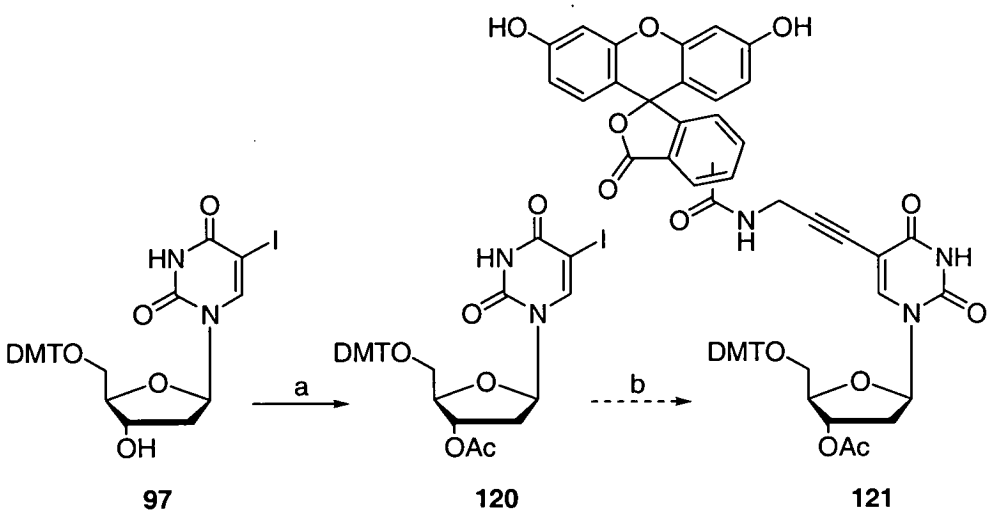
To pursue this second strategy, 5(6)-carboxyfluorescein **119** was activated with PyBOP and treated with the amine under basic conditions (Scheme 3.21). The conversion was quantitative and after several precipitations **118** was isolated with only a minimum of phosphoramidate, a side-product of PyBOP, present by NMR.



(a) 0.98 eq PyBOP, 1 eq propargylamine, 2 eq DIPEA, DMF, 0.125 M, 77 %

Scheme 3.21 Derviatisation of carboxy-fluorescein to its alkynyl derivative

In order to have the required acetate group on the 3' OH, 5'-ODmt protected 5-iodo uridine **97** was acetylated to give **120** in a quantitative yield. The Sonogashira reaction was monitored by tlc and HPLC, but very little formation of coupling product **121** could be observed in the mixture (<5 %, HPLC) (Scheme 3.22).



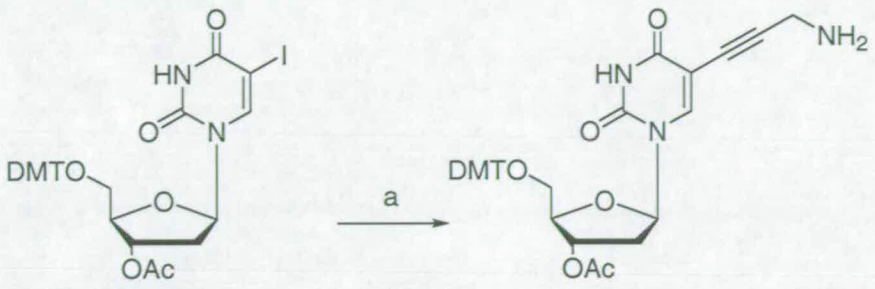
(a) Ac_2O, 0.25 eq DMAP, 2.5 eq TEA, pyridine, 0.15 M, 2 h, 100 %; (b) 2.5 eq **95**, 0.1 eq $[Pd(PPh_3)_4]$, 0.2 eq CuI, 5 eq TEA, DMF, 0.5 M, 48 h, not reacted

*Scheme 3.22 Sonogashira coupling of uridine derivative **120** to alkynyl fluorescein **118***

3.4.3.3 *Coupling of unprotected alkynyl linker*

With both initial strategies unsuccessful, attention was then turned to introducing the alkynyl linker without a protecting group. Sonogashira couplings of 5-iodo purines to propargylamine are known if the ratio of copper co-catalyst to Pd catalyst is adjusted to 5:1 from the customary 2:1 ratio.[150] This method has not found any general applicability as the ammonium salts generated are hard to remove from the polar products. However, the use of a supported amine in the Sonogashira reaction can readily circumvent purification problems.[158]

Propargylamino modified uridine **122**, with 3' and 5' protection, was accessed from **120** using the modified ratio of Pd/Cu catalysts to give the desired product in excellent yield (Scheme 3.23). Presence of the DMT group on the 5' postion is believed to have aided isolation of the product greatly, as it rendered it much more hydrophobic, as it was possible to perform a column chromatography without adding an amine into the eluent.



120 **122**

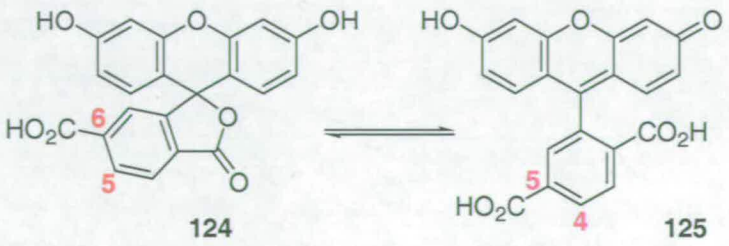
(a) 10 % $[Pd(PPh_3)_4]$, 50 % CuI, 3 eq propargylamine, 2 eq Amberlite IRA-67, 0.2 DMF, rt, 2 h, 87 % yield

*Scheme 3.23 Synthesis of propargylamino linked thymidine **122***

3.4.3.4 *Synthesis of Ahx-fluorescein **123***

Before attempting coupling of 5(6)-carboxyfluorescein **119** to amino-modified uridine **122**, it was modified by derivatising it with an amino-hexanoic acid linker. This is known to improve its reactivity, as the carboxylic acid on the alkyl chain is more reactive than the benzoic acid of fluorescein.

Carboxyfluorescein exists in two tautomeric forms, lactone **124** and ring-opened form **125** (Figure 3.20). While it is the ring-opened form that exerts fluorescence through its conjugated π-system, the exact position of the equilibrium depends on the pH.

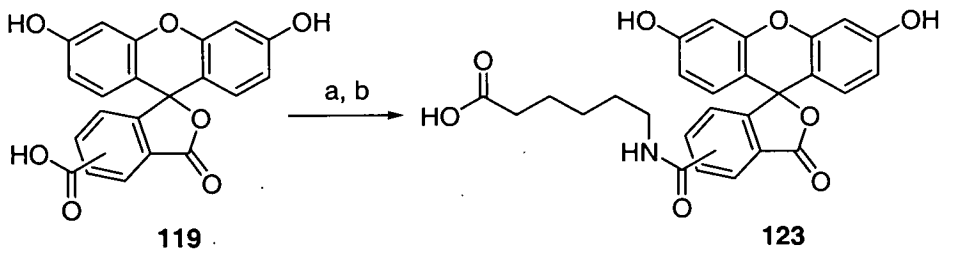


124 **125**

Figure 3.20 Tautomerism of 6-carboxyfluorescein

The numbering of the benzofuran ring of lactone **124** starts of at the *spiro* carbon, thus giving positions 5 and 6 for the carboxy modified variant, whereas for the ring-opened variant **125**, it starts at the *ipso* carbon of the benzene ring, giving postions 4 and 5 respectively. Carboxyfluorescein will be represented in its lactone form and the positions on the benzofuran numbered accordingly. Carboxyfluorescein is commercially available as a 40/60 mixture of 5 and 6-carboxyfluorescein, which was used as such unless specified otherwise.

Thus, 5(6)-carboxyfluorescein **119** was activated with PyBOP and treated with the methyl ester of the linker. The intermediate methyl ester was separated by acid precipitation from the by-product and readily deprotected under basic hydrolysis to yield the desired product **123** in good yield over two steps (Scheme 3.24). The hydrolysis step also served to remove phenolic esters side-products formed in the ester coupling step resulting from cross-coupling.



119 **123**

(a) 0.98 eq PyBOP, 1 eq H-Ahx-OMe.HCl, 3 eq DIPEA, DMF, 0.13 M; (b) 2 M NaOH, DMF, 2 h, rt, 70 % yield

Scheme 3.24 Coupling of carboxy-fluorescein to an amino-hexanoic acid linker

3.4.3.5 *Coupling of amino-modified uridine **122** to Ahx-fluorescein **123***

The coupling reaction between amino-modified uridine **122** and Ahx-fluorescein **123** was investigated using a few different reaction conditions. Initial attempts to form the *N*-hydroxysuccinimide ester of Ahx-fluorescein were only moderately successful.

Attention was then turned to carbodiimide mediated amide bond couplings (Figure 3.21). Treatment of a carboxylic acid with a carbodiimide can yield an *O*-acyl isourea **126**, an active ester, which reacts with an amine to yield amide **127** (route A). If however the coupling reaction is sluggish, *O*-acyl isoureas **126** rearrange to give the *N*-acylurea **129** (route C). This removes the carboxylic acid from the productive pathway and lowers the overall yield and purity of the product. This can

be prevented by using an additive such as *N*-hydroxybenzotriazole (HOBt), which intercepts the *O*-acyl isourea **126** to form the HOBt-ester **128**, which is subsequently transformed into the desired amide (route B). The addition of HOBt also suppresses enantiomerisation during peptide couplings.

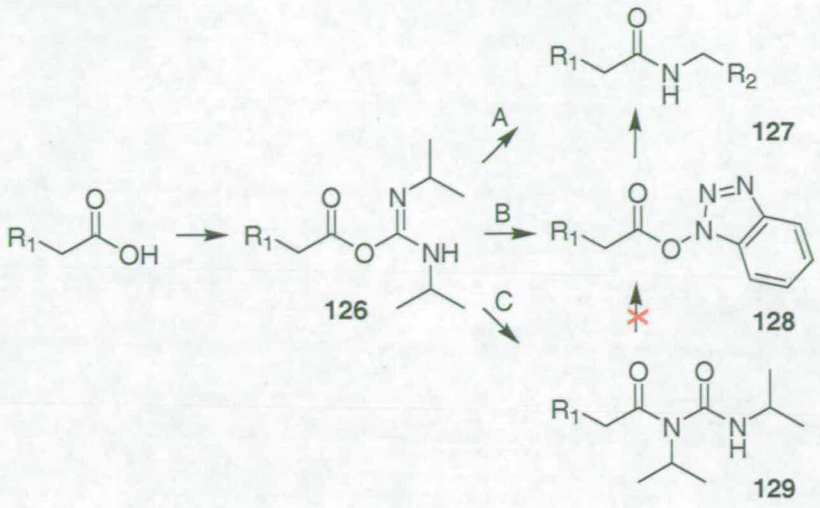
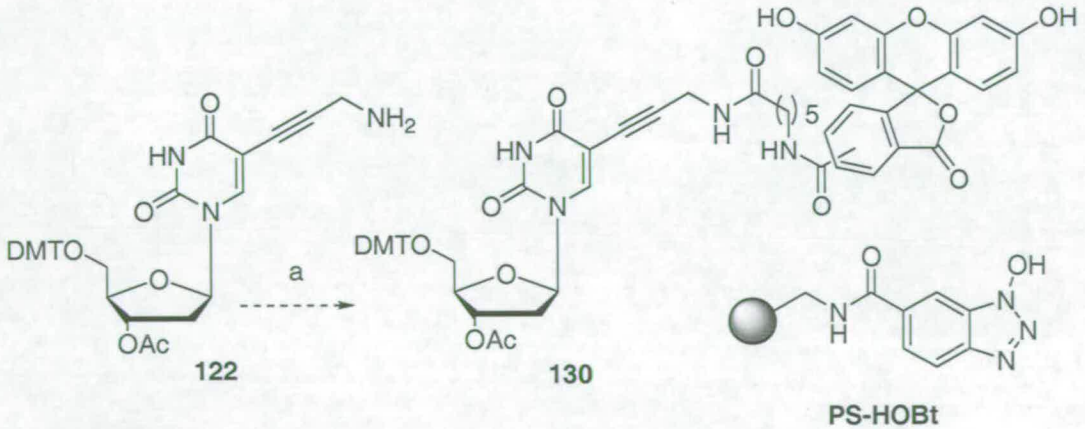


Figure 3.21 Carbodiimide mediated amide bond formation

As couplings with Ahx-fluorescein **123** with a carbodiimide were rather sluggish, addition of HOBt was necessary. A solid-supported analogue of HOBt was used to avoid it being extracted into the organic phase with 2-propanol/CH_2Cl_2, the preferred solvent for fluorescein derivatives.



(a) 1 eq **123**, 1.5 eq **PS-HOBt**, 4 eq DIC, DMF, 0.1 M, not isolated

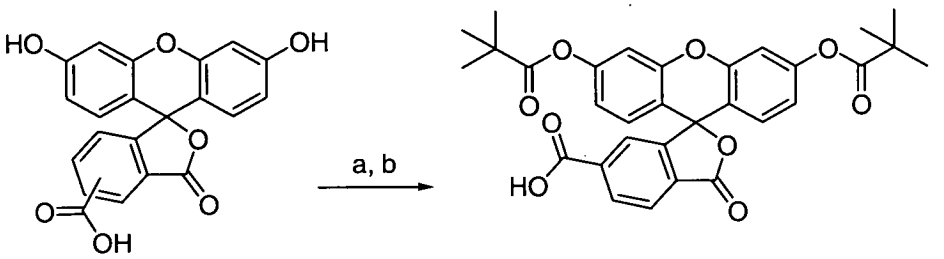
*Scheme 3.25 Coupling of Ahx-fluorescein **123** to aminopropargyl-uridine **122***

PS-HOBt, a commercially available resin, was investigated. The supplier advocates a catch-and-release strategy, which pre-activates Ahx-fluorescein **123** as its PS-HOBt ester, which is cleaved off the resin with a sub-stoichimetric amount of

amine. This procedure was found to yield a considerable amount of hydrolysis product when washing the loaded resin, it was thus also attempted to add PS-HOBt as additive into the reaction mixture (Scheme 3.25). While the reaction was believed to successful to some extent, the resulting mixture was rather complex and could not be purified.

3.4.3.6 *Isomer purification of 5(6)-carboxy fluorescein*

As the purification of fluorgenically labelled nucleoside was unduly complicated by the isomers of carboxyfluorescein, 5(6)-carboxyfluorescein **119** was separated. Most methods achieve separation by column chromatography or Prep HPLC of phenolic fluorescein esters. A facile and simple method involving selective precipitation of the 6-isomer as its dipivalate diisopropylammonium salt[159] was successfully applied to yield 6-carboxyfluorescein dipivalate **131** after subsequent acidification of the diisopropylammonium salt (Scheme 3.26).



119 **131**

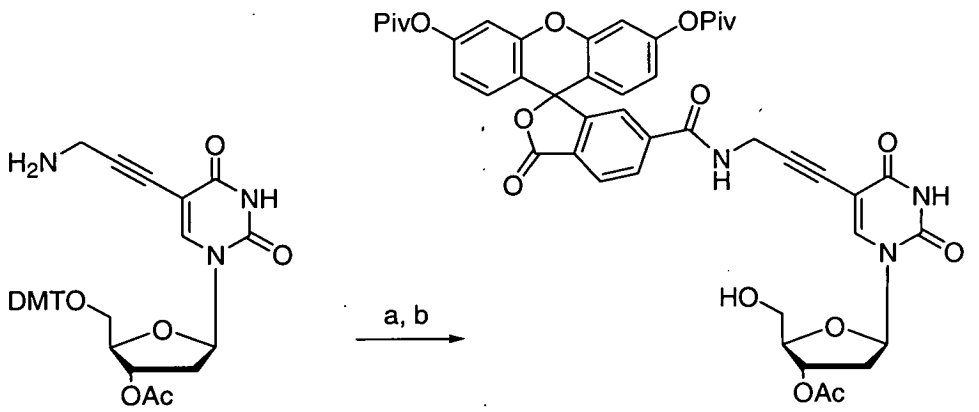
(a) Piv_2O, 130 °C, 2 h; EtOH, $^iPr_2NH$, -20 °C; (b) 1 M HCl; 16 % yield

Scheme 3.26 Isomer separation of 5(6)-carboxyfluorescein

3.4.3.7 *Synthesis of fluorescein labelled nucleoside **132***

Carbodiimide mediated amide bond formation was then applied to couple the fluorophore to nucleoside **122**. As the phenol groups had been protected for the isomer separation, this was taken advantage of for the coupling reaction. The reaction was performed in CH_2Cl_2 and while several components of the reaction mixture were thought to be due to decomposition of the protecting groups, the desired product was isolated by column chromatography (Scheme 3.27).

The urea of the carbodiimide co-purified, however, and could only be removed through column chromatography after the DMT deprotection step. The desired nucleoside **132** was thus isolated in a 37 % yield over two steps.



122 **132**

1.1 eq **131**, 1.1 eq DIC, 0.1 M CH_2Cl_2; (b) 3 % TCA/CH_2Cl_2, 37 % yield over 2 steps

Scheme 3.27 Synthesis of fluorescein labelled uridine analogue 132

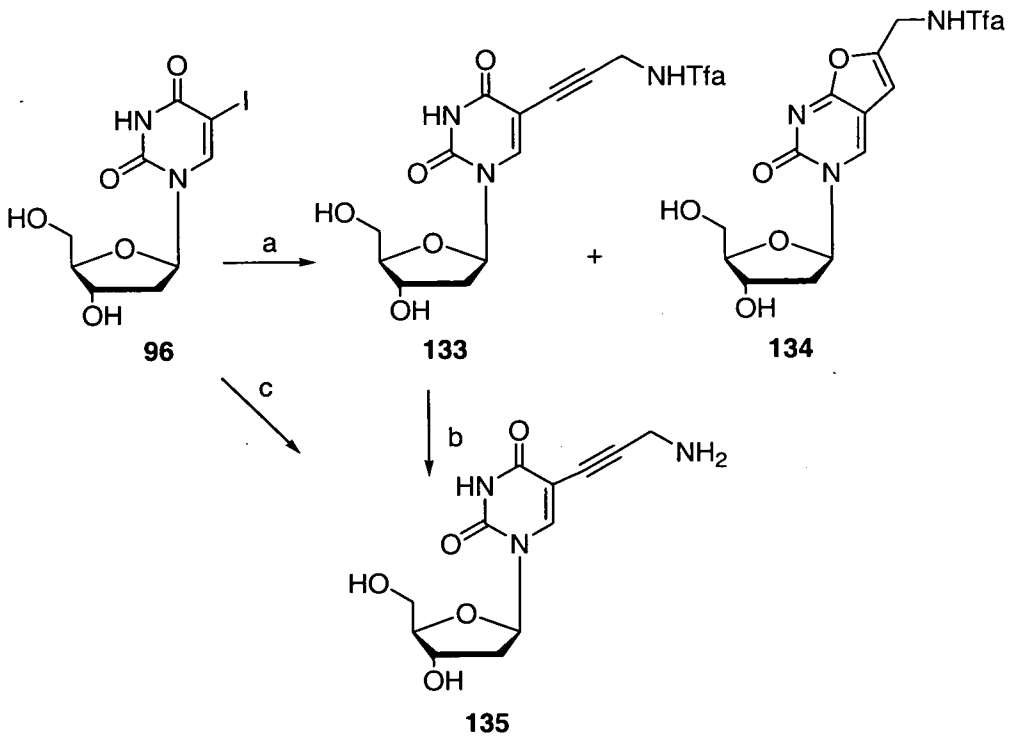
3.4.4 Synthesis of a labelled 2',3'-dideoxy nucleoside

Additionally to the 2'deoxy nucleoside, synthesis of a 2',3'-dideoxy nucleoside was also envisaged. The synthetic scheme could be shortened, as there was no requirement to protect the primary alcohol to protect the secondary 3' position. Therefore, the Sonogshira coupling to the propargylamino linker was the first synthetic step.

As there is a much higher cost associated with dideoxy nucleosides, the synthesis was optimised on the 2'-deoxy analogue first. The couplings were performed on both the protected and the unprotected propargylamino group for comparison (Scheme 3.28).

5-Iodo-2'-deoxy-uridine **96** could be coupled efficiently to the trifluoroacetamide protected linker **95** to yield **133** in good yield. A small amount of the fluorescent furano-pyrimidine side-product **134** was detected by tlc; its appearance under Sonogshira conditions has been associated with prolonged heating and/or reaction times of the reaction mixture.[160] The trifluoroacetamide group could be readily removed with aqueous ammonia to yield **135**.

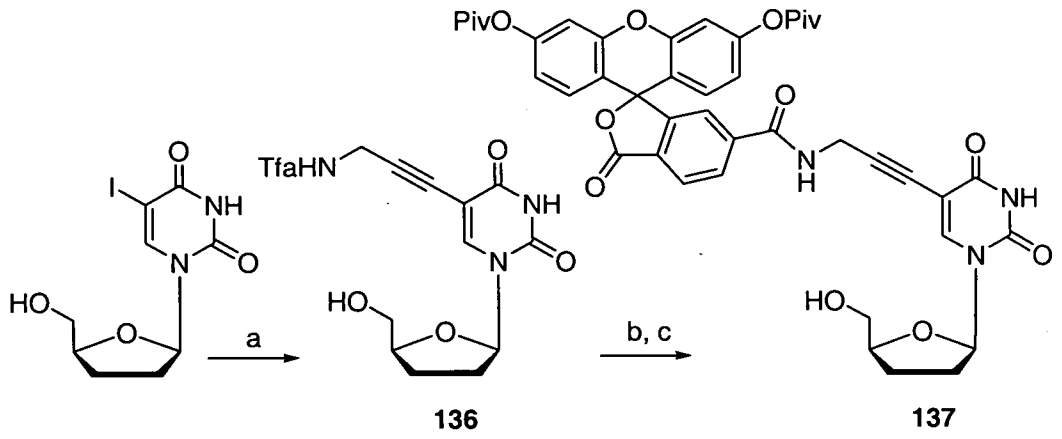
The Sonogashira product with the unprotected linker **135** could be accessed directly only in moderate yield (43 %) and was isolated by column chromatography eluted with 20 % ammonia saturated MeOH as eluant. Due to the eluant used in the isolation, **135** did contain traces of silica.



(a) 5 % $[Pd(PPh_3)_4]$, 0.2 eq CuI, 2 eq **95**, 5 eq Amberlite IRA-67, 0.1 DMF, 60 °C, 14 h, 80 %; (b) NH_3/H_2O, 80 min, 68 %; (c) 10 % $[Pd(PPh_3)_4]$, 50 % CuI, 3 eq propargylamine, 2 eq Amberlite IRA-67, 0.2 DMF, rt, 2 h, 43 %

Scheme 3.28 Optimisation on 2'-deoxy-5-iodouridine

From the optimisation on the 2-deoxy model it was clear that it was easier to isolate the linker modified nucleoside in its protected form. Thus commercial dideoxy-5-iodouridine was coupled under Sonogashira conditions to the Tfa protected propargylamino linker **136** (Scheme 3.29).



(a) propargylamine-trifluoroacetamide, $[Pd(PPh_3)_4]$, PS-TEA, DMF, 57 %; (b) NH_3 in H_2O; (c) 1.1 eq **131**, 1.1 eq DIC, CH_2Cl_2, ~ 30 %.

Scheme 3.29 Application to 2',3'-dideoxy-5-iodouridine

The Sonogashira reaction was carried out under the same conditions as for the 2'-deoxy analogue, however did not go to completion within 22 h at rt. The product was isolated at this stage, as it was found to readily cyclise to the furanopyrimidine if heated. The Tfa-propargylamino linked dideoxyuridine **136** was thus isolated in a 57 % yield, based on recovered starting material.

The Tfa group was then cleaved using aqueous ammonia and the resulting product was used immediately in carbodiimide mediated amide bond formation to yield the desired fluorescent dideoxy nucleoside **137**. The yield obtained was rather low, estimated at around 30 % yield. As there was only a small amount of nucleoside obtained, it was decided to only pursue the deoxynucleoside for phopshorylation.

3.4.5 Phosphorylation

The triphosphorylation conditions developed in the earlier chapter formed again the basis for the triphosphorylation of fluorescein modified nucleoside **132**. As mentioned previously, phosphorylations of fluorophore labelled nucleosides are notoriously difficult, which has been attributed by to a lack of phosphitylation of the primary alcohol. As this first step was thought to be the most difficult step, it was thus decided to concentrate first on finding suitable phosphitylation conditions to synthesise mono-*H*-phosphonate **138**.

Thus, as the reaction was thought to be difficult, more equivalents of resin were used to push the reaction to completion. In this instance, 6 eq of phosphitylation resin **44** were used with all the other parameters, such as concentration, solvent and reaction time remaining the same.

After 1 h, the solution was drained and the resin washed with dry THF. The eluate will be referred to as the drained solution. Then, the resin was treated with a H_2O/THF (1/1) mixture to cleave the phosphitylated nucleoside off as its mono-*H*-phosphonate **138**. This will be referred to as the cleaved solution. Both samples were analysed by LC-MS and ^{1}H and ^{31}P NMR to assess the extent of phosphitylation.

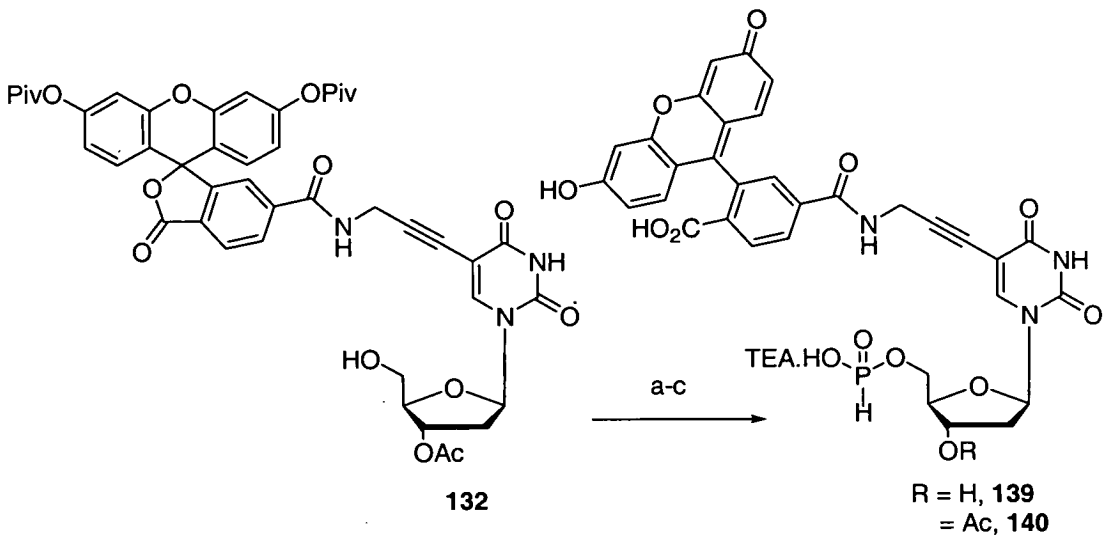
The drained solution contained no ^{31}P NMR peak corresponding to phosphonate (*ca.* 7 ppm), thus it can be concluded that the loading of the nucleoside **132** was carried out under dry conditions, as there was no premature hydrolysis. LC-MS of

this solution indicated the presence of the starting nucleoside **132**, thus the loading on the resin was not complete.

Analysis of the cleaved solution by ^{31}P NMR revealed a phosphonate peak at 7 ppm; however this is entirely expected as, there were 5 eq in excess of the nucleoside and the chemical shift does not indicate whether the phosphonate is attached to the nucleoside. LC-MS analysis of the sample showed no peak corresponding to the nucleoside phosphonate **138** or its pivaloyl deprotected analogues.

The drained solution was purified by column chromatography to recover the staring nucleoside **132** and to assess the level of unattached nucleoside. Careful purification of the solution yielded a 50 % recovery of starting material, as well as substantial amount of pivaloyl deprotected nucleosides and other unidentified products, which leaves the conclusion that no or only very little of the compound was phosphitylated.

The cleaved solution was thus examined with a view to isolate the mono-*H*-phophonate, if any, that was present. However, as the mixture proved to be insoluble in aqueous buffer, it was decided to deprotect the pivaloyl esters.



132

R = H, **139**
= Ac, **140**

(a) 6 eq PS-P-Cl **44**, py/THF 1/3, 0.1 M, 1 h, rt; (b) H_2O/THF 1/1, 2 x 10 min; (c) NH_3/H_2O, 30 sec.

*Scheme 3.30 Synthesis of mono-*H*-phosphonate of fluorescein labelled uridine*

The mixture was treated with ammonium hydroxide for 30 sec and evaporated. Gratifyingly, the solution turned yellow-greenish from deprotection of the phenol

groups. Analysis of the resulting mixture revealed two peaks at 15.1 min and 18.3 min (Figure 3.22). Using MS, they were assigned to the fully deprotected mono-*H*-phosphonate **139** and 3'OAc-protected mono-*H*-phosphonate respectively **140**. No further characterisation was performed. It shows that the brief ammnonia treatment had also produced the unplanned, but not unexpected, acetyl deprotection.

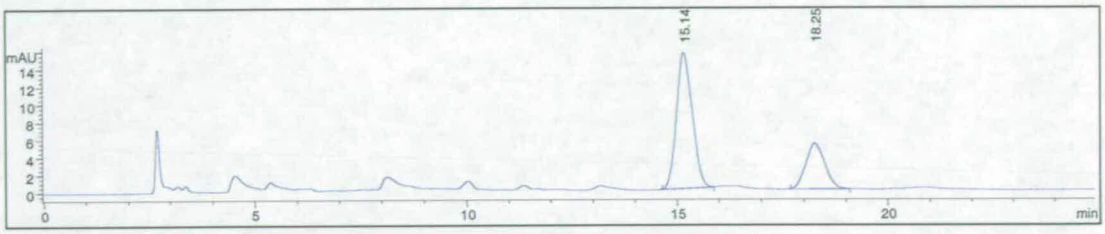
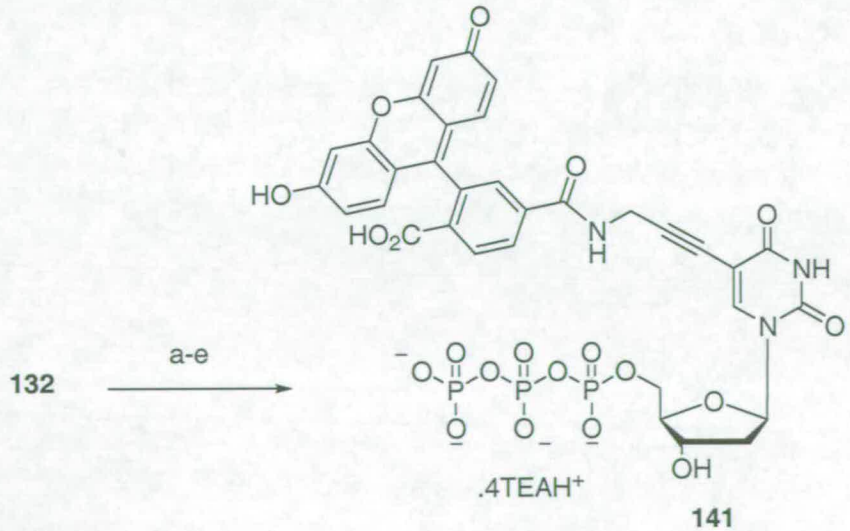


Figure 3.22 Crude HPLC chromatogram from resin hydrolysis

This showed that the phosphitylation step, while difficult to achieve, was possible to some extent and thus efforts concentrated to optimise this step, before proceeding to attempting the triphosphorylation step. To push the phosphitylation step to completion, nucleoside **132** was dissolved in 1/3 pyridine/THF, but now to a more concentrated 0.2 M concentration. Additionally, the reaction was left for 2 h, as opposed to the usual 1 h.



141

(a) 6 eq PS-P-Cl **44**, py/THF 1/3, 0.2 M, 2 h, rt; (b) 1.5 eq 0.5 M $(Bu_3N)_2$ pyrophosphate in DMF, 4 eq Bu_3N, rt, 3 x 10 min; (c) 1.5 eq 0.5 M I_2/98:2 pyridine:H_2O, 15 min; (d) H_2O, 30 min; (e) NH_3/H_2O, 1 h.

Scheme 3.31 Synthesis of fluorescein labelled uridine triphosphate ***141***

The drained solution after 2 h, was analysed for presence of the starting material and gratifyingly, no starting nucleoside **132** could be detected. It was thus decided to treat the resin with the customary cleavage, oxidation and hydrolysis conditions. When the resulting crude was treated with ammonia solution, the reaction colour immediately turned the characteristic fluorescein colour. The reaction was evaporated after 1 h, then analysed by HPLC (Figure 3.23).

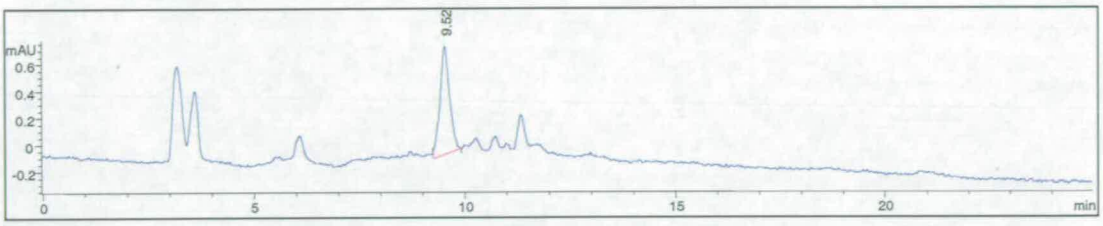


*Figure 3.23 Crude HPLC chromatogram for dUTP-6-FAM **141***

Inspection of the HPLC chromatogram revealed a major peak a 9.52 min. As the HPLC was run at identical conditions as for the phosphonates, it can be asserted with confidence then neither phosphonates are present in the reaction mixture, which indicates that all precautions to prevent premature hydrolysis of the resin were successful.

The peak was isolated by Prep HPLC and lyophilised to yield an orange residue. A ^{31}P NMR spectrum provided the confirmation that the triphosphate group was present by exhibiting the characteristic signals at -6, -11 and -22 ppm (Figure 3.24), while the ^{1}H NMR spectrum proved too complicated to interpret.

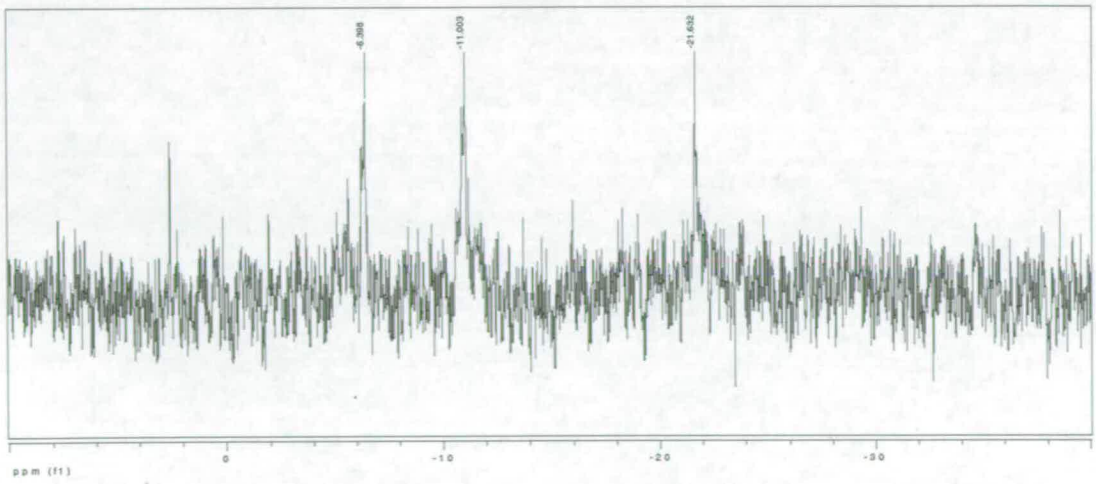


*Figure 3.24 ^{31}P NMR spectrum of dUTP-6-FAM **141***

The yield of **141** obtained could not be measured directly, as the mass obtained could not be reliably measured. An estimation of the yield was thus performed by measuring its UV-Vis absorbance and from that deducing the concentration, using a literature value for the extinction coefficient of ε_{495} = 80000 M^{-1} cm^{-1}.[161] The amount of **141** obtained was dissolved in 0.1 M TEAB buffer (Figure 3.25). The number of moles was calculated to be ~50 nmol in the entire sample, sufficient for many biological assays. By comparing with starting amount of 44 μmol, an estimated yield of *ca.* 1 % could be deduced.

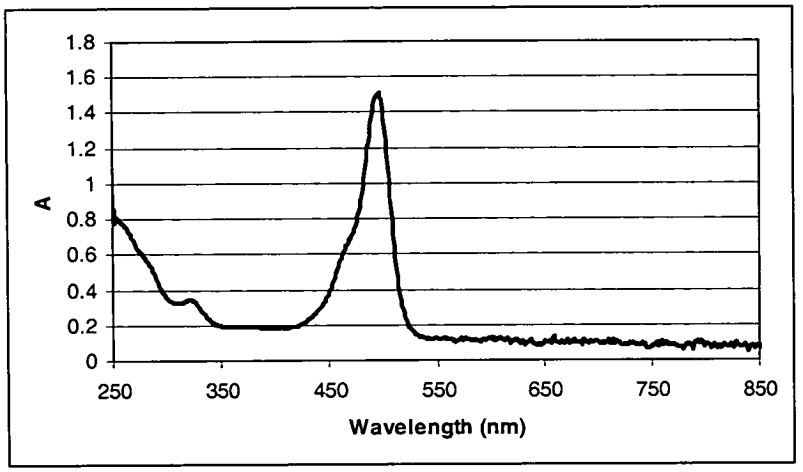


*Figure 3.25 UV-Vis spectrum of **141** in 0.1 M TEAB buffer*

4 Application to synthesis of nucleoside prodrugs

4.1 Introduction

4.1.1 HIV reverse transcriptase inhibitors

The human immunodeficiency virus (HIV) virus has claimed millions of victims since the 1980's. Currently neither vaccine nor cure exists, but once infection takes place its spread can be contolled inside the host to delay the on-set of AIDS (aquired immuno deficiency syndrome). Knowledge of virus replication has enabled inhibition at various stages. HIV is a retro-virus, meaning that it carries its genetic information as RNA. When a host cell is hijacked, a reverse transcriptase (RT) of HIV translates it into DNA, which is in turn integrated into the host's DNA by a integrase. Transcription/translation of this DNA generates a peptide which is cleaved by a protease, which enables production of more virions. RT inhibition is especially interesting as host cells do not have a RT, therefore this is a virus-specific pathway. Nucleoside RT inhibitors (NRTIs) constituted the first approved HIV drugs (Figure 4.1).[162]

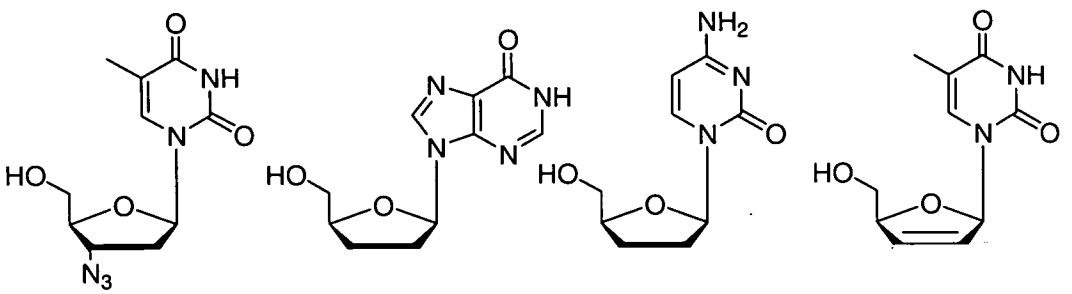
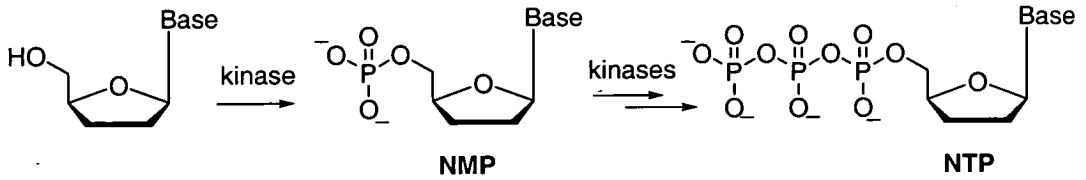


Figure 4.1 Selected NRTIs with year in which they were first FDA approved

The biosynthetic synthesis of dNTPs progresses *via* three kinases. Nucleosides are converted to the NMP (nucleoside monophosphate) with a specific nucleoside kinase and then the NMP is tranformed to the NDP by a specific NMP kinase. The NTP is obtained by an NDP kinase catalysed phosphoryl transfer from ATP (Scheme 4.1). Unlike for the previous two enzymes, the NDP kinase has little base specificity, but a strong binding to the sugar.[163] Mechanistic studies have shown

that this phosphoryl transfer is stabilised by the interaction of the 3'OH with the β-phosphate *via* an *H*-bonding interaction.[164]



Scheme 4.1 Biosynthetic activation of a nucleoside

NRTIs need to go through the same enzymatic activation to be converted into the triphosphate, which means that drugs for therapeutic applications need to be good substrates for the kinases as well as for the RT. Low conversion to TPs has meant that a lot of drug candidates have fallen at this hurdle. Even the ones that have succeeded need to be administered in high doses that lead to toxicity problems.

The phosphorylation pattern of nucleosides strongly depends on their structure. The conversion of AZT to AZTMP by thymidine kinase occurs with similar efficiency to natural thymidine, whereas for d4T, the efficiency is at least 200 times lower.[165] To circumvent the problems associated with NRTIs, introduction of nucleosides as masked monophosphate inhibitors has been investigated.

4.1.2 Prodrugs

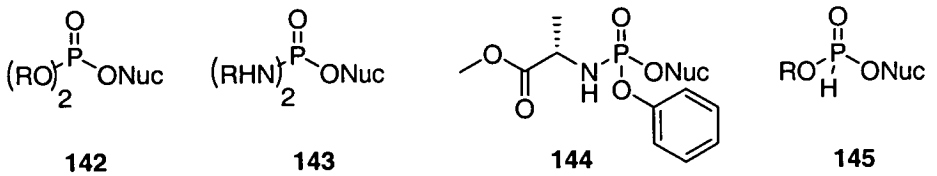
The reliance on host kinases is an inherent drawback of administering NRTIs. Direct circumvention of phosphorylations is not possible as the nucleoside phosphates are susceptible to non-specific dephosphorylations in extra-cellular fluids and due to their charge can not penetrate the cell. Several approaches have been proposed to introduce prodrugs of the nucleoside monophosphate.

The concept of prodrugs was introduced to medicinal chemistry in 1957 and is defined as molecules without intrinisic biological activity but which are activated to give a biologically active drug.[162] The transformations necessary for activation involve quite often enzymes such as hydrolases which release the active component *in situ*.

4.1.3 Masked phosphate prodrugs

Two types of prodrug approaches can be useful in nucleoside drug administration: 5' carboxylic esters which transiently increase lipophilicity, cellular uptake and general bioavailabity and release the nucleoside through esterase activity.[166] The other approach is referred to as kinase by-pass prodrugs, which release the nucleoside monophosphate intra-cellularly. The masked pronucleotides can readily cross cell membranes and are converted to the active nucleotides by enzymatic or chemical activation. A few examples of 5' phosphate prodrugs are described below; for a full discussion, the reader is referred to reviews on the topic.[162,165,167]

Dialkyl phosphate esters **142** were proposed as prodrugs by McGuigan *et al.*, however their antiviral activity was generally lower than that of the parent nucleoside due to the metabolic stability of the alkyl phosphate.[168] Diaryl phosphate esters showed more promise, but anti-viral activity was strongly dependent on para-substitution and was not maintained in kinase deficient cell lines.[169] Diphosphoramidates **143** of AZT displayed some anti-viral activity,[170] but due to low synthetic yields were not pursued further.[171] Aryloxy phosphoramidate esters **144** were more successful; they retained activity in kinase deficient cells, where the nucleoside itself was inactive.[172]



142 **143** **144** **145**

Figure 4.2 Proposed nucleoside prodrugs

The intra-cellular release for aryloxy phosphoramidate esters **144** was described as proceeding *via* an esterase mediated ester hydrolysis. The loss of the phenol group was induced by intramolecular carboxylate attack on phosphorus. The resulting cyclic intermediate spontaneously ring opened to the amino acid phosphoramidate, which was indentified in vitro.[173] For the hydrolysis of the P-N bond, a putative phosphoramidase was invoked, even though no pure enzyme could be isolated.[171]

Hydrogen phosphonate derivatives of nucleosides **145** have also been explored. In particular, Phosphazid (R = H, Nuc = AZT) is in clinical trials as anti-HIV drug. The presence of the phosphonate, while lowering the anti-viral activity, increases

its selectivity relative to AZT. For the mechanism of action an intra-cellular oxidation has been proposed,[174] but is more likely that these compounds simply deliver the nucleoside.[175] *H*-phosphonate diesters (R = ^{i}Pr, Cy) of various drugs (AZT, d4T, ddA) have also been proposed, with some have them having higher selectivity indexes than the parent nucleoside.[176]

Another potential group of prodrugs are boranophosphates. The rationale behind this is the significant inhibition of excision from terminated DNA chains observed with borano substitution. Thus borano substitution could offer a handle to treat variant RT in drug-resistant HIV strains.[177] Amino acid boranophosphoramidates **146** were proposed by Shaw *et al.*[178] as a novel prodrugs class, as they not only exhibit nuclease inhibition, they also have greater lipophilicity.

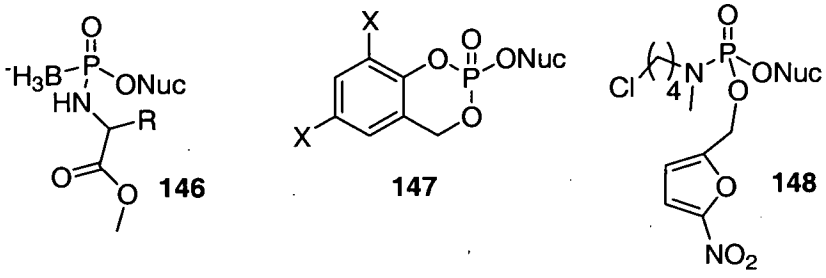


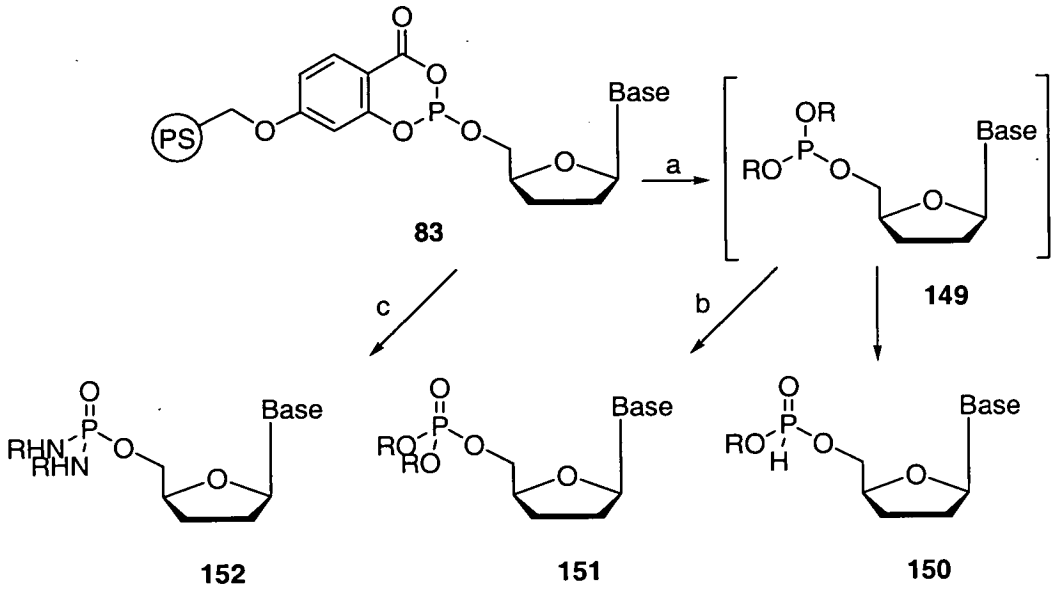
Figure 4.3 Proposed nucleoside prodrugs

Other prodrugs concepts involve pH dependant intra-cellular release of masked phosphates, such as the cycloSal derivatives **147**, which release the phosphate under mild basic conditions. The half-life of the prodrugs could be modulated by appropriate substitutions on the benzene ring and anti-viral activity could be observed in kinase-deficient cells.[179]

As a final example, Borch *et al.* developed cellular delivery of anti-cancer drugs based on enzymatic reduction and spontaneous explusion of one substituent (the nitrofuryl group), which then enables nucleophilic cyclisation of the nitrogen with concomitant halide expulsion.[180] The resulting charged phosphoramidate acts as a leaving group to deliver the nucleotide. Analogues of **148** with 2'-deoxy-5-fluorouridine as nucleoside retained their cell proliferation activity when tested on kinase deficient cells.[180]

4.2 Aims of the work

The aims of this part of the work were to establish whether the nucleoside resin **83** could serve as starting point for the synthesis of nucleoside prodrugs. Such a property would be particularly advantageous if different types of prodrugs could be accessed from the same intermediate. It is envisaged that the phosphorus on **83** is sufficiently electrophilic to be displaced by nucleophiles other than pyrophosphate and water.



(a) ROH; (b) oxidation; (c) (i) RNH_2; (ii) oxidation

Scheme 4.2 Nucleoside prodrug synthesis based on nucleoside resin

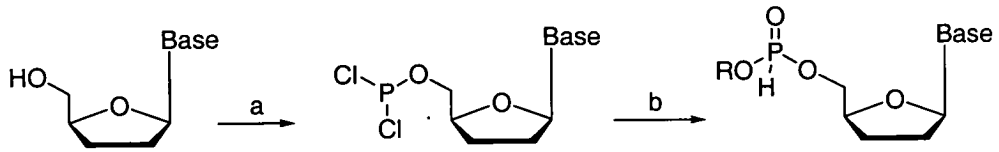
Using an alcohol as a nucleophile, the initial product, the phosphite triester **149**, reacts further through Arbuzov chemistry to give the phosphonate diester **150**. If the reaction is intercepted prior to the dealkylation, the phosphorus can be oxidised to yield phosphate triester **151**. Additionally, more nucleophilic amines should give access to diphosphoramidate phosphate esters **152** after oxidation (Scheme 3.4). These ideas were investigated using the nucleoside 3'-OAc-T **49** (for preparation, see Scheme 2.10).

4.3 Nucleoside *H*-phosphonate diesters

4.3.1 Previous syntheses

Nucleoside *H*-phosphonate diesters have been accessed by various methods. The mono-*H*-phosphonate ester, treated with a coupling agent, such as oxalyl chloride[181] or pivalyl chloride,[182] gave a mixed anhydride, which yielded the mixed diester after addition of the alcohol. Transesterification of diphenylphosphite has also been proposed to give the double- or mono-exchange *H*-phosphonates.[183] Selectivity issues arise for asymmetric targets, but they can be controlled by using diphenylphosphite in excess or by its slow addition.[184]

A one-pot synthesis of nucleoside *H*-phosphonate diesters was recently proposed. The nucleoside was treated with an excess of phosphorus trichloride, followed by alcoholysis to give the mixed phosphite triester (Scheme 4.3). Dealkylation through Arbuzov chemistry proceded spontaneously by involvement of the HCl generated previously to generate the alkylchloride and the phosphonate ester which was isolated by careful column chromatography.[185]



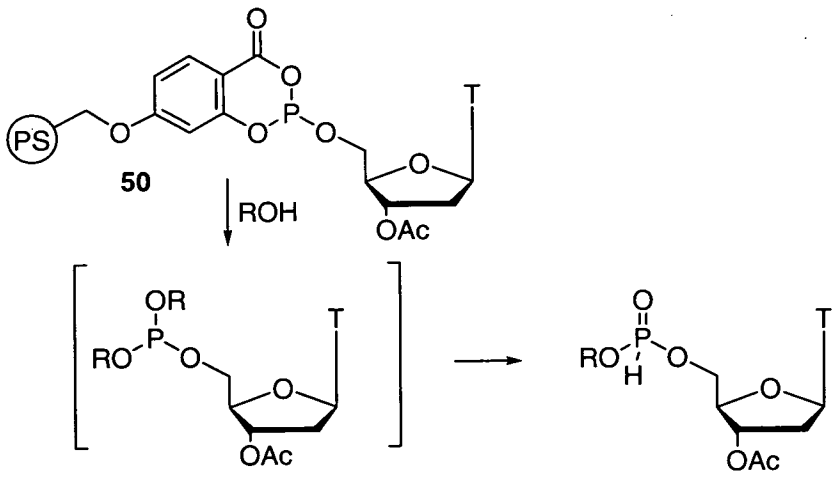
(a) 10 eq PCl_3, CH_2Cl_2, -30 °C, 1 h, then 6 h, rt; (b) 2.5 eq ROH, CH_2Cl_2, 0 °C, 30 min, then 2 eq TEA; 18 – 88 % yield.

Scheme 4.3 Synthesis of nucleoside H*-phosphonate diesters*

4.3.2 Synthesis using a supported Ludwig-Eckstein reagent

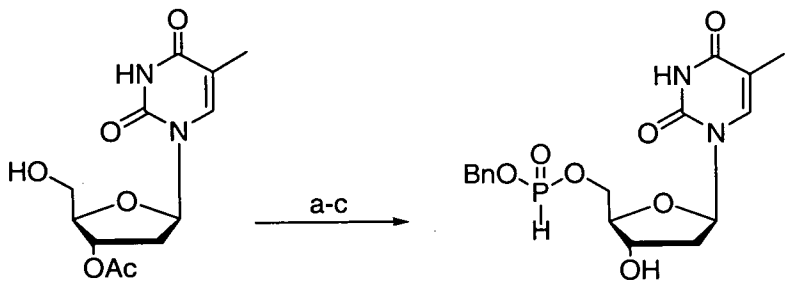
The potential for application to nucleoside prodrugs synthesis was explored by starting from nucleoside resin **50** synthesised and characterised in Chapter 2. The resin was subjected to alcohol cleavage as illustrated below (Scheme 4.4).

One example of cleavage with a hydroxyl group, namely with water, was already discussed in Chapter 2, where the mono-*H*-phosphonate ammonium salt of 3'-OAc-T **51** was isolated in a 58 % yield (Entry 1, Table 4.1). Thus extending the cleavage to alcohols appeared straightforward.



Scheme 4.4 Nucleoside H*-phosphonate diester synthesis based on nucleoside resin*

The supported phosphitylation reagent **44** was loaded with nucleoside **49** to give **50**, as described in Chapter 2, then was treated with benzyl alcohol. Analogously to the water cleavage, the reaction time was 10 min, which was enough to cleave the nucleoside off, as evidenced by absence of nucleoside on the resin. Alcohol cleavage gave the mixed phosphite triester **153**, which was not characterised, but it could be detected in the crude ^{31}P NMR (145 ppm). The eluate was stirred at rt for 30 min for the dealkylation reaction to proceed (Scheme 4.5).



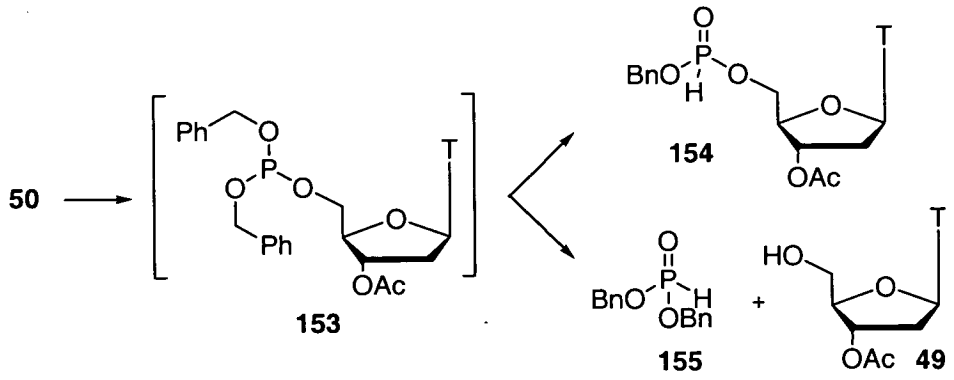
(a) 3 eq PS-P-Cl **44**, 0.1 M py/THF 1/3, 1h, rt; (b) BnOH, 10 min; (c) 30 min; see Table 4.1.

Scheme 4.5 Synthesis of H*-phosphonate ester 154*

As the phosphite carries three ester groups, there are three possible CH_2 groups to dealkylate. This will generate a mixture of the desired mixed *H*-phosphonate diester **154** to starting material **49** and symmetrical *H*-phosphonate dibenzyl ester **155** (Scheme 4.6).

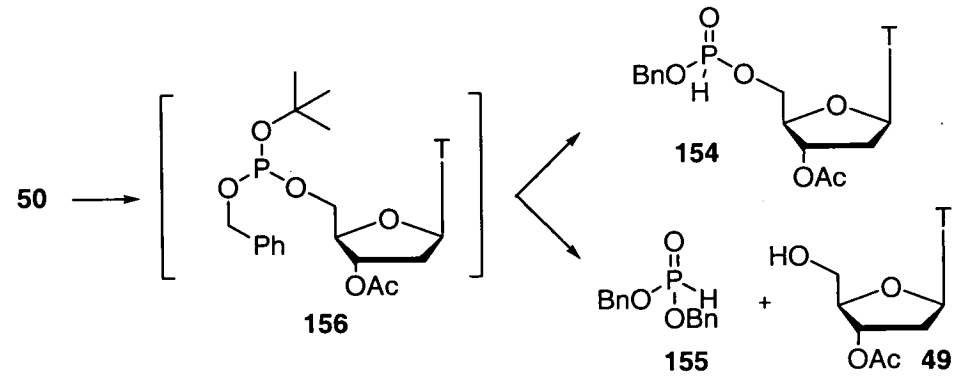
The reaction was carried out at a 0.1 mmol scale with commercial anhydrous benzyl alcohol. The reaction mixture was purified by column chromatography to

yield the desired compound **154** in an 8 % yield and recovered starting material **49** in a 24 % yield (Entry 2). Water present in the benzyl alcohol resulted in a significant amount of mono-*H*-phosphonate **51**, as evidenced by a baseline spot on the tlc plate. The benzyl *H*-phosphonate diester **154** was obtained as a diastereomeric mixture at phosphorus, as shown by having two signals in the ^{31}P NMR spectrum and two peaks for several protons in the ^{1}H proton spectrum.



Scheme 4.6 Nucleoside H*-phosphonate diester synthesis based on nucleoside resin*

A method published recently for *H*-phosphonate diester synthesis relied also on dealkylation of mixed phosphite triesters.[185] To circumvent undesired dealkylation, the authors proposed using a 1:1 mixture of the desired alcohol and ^{t}butanol and thus improved the yield for the ethyl *H*-phosphonate of AZT from 22 % for ethanol alone to 61 % for this mixture.[185] They explained this observation by preferential dealkylation of the ^{t}butyl cation over either of the secondary groups.



Scheme 4.7 Nucleoside H*-phosphonate diester synthesis using ^{t}butanol as additive*

It was thus decided to attempt to reproduce this work for this method. The cleavage solution constituted of a 1:1 mixture of anhydrous benzyl alcohol and tert-butyl alcohol. Purification of the resulting reaction mixture resulted however in an unchanged recovery of the desired product **154** as well as an improved recovery of

starting material **49**. Additionally, the by-product dibenzyl phosphite **155** was isolated and was recovered in a 30 % yield based on the nucleoside loading. It is equally possible for **155** to result from benzyl alcohol being phosphitylated by the remaining functionality on the resin, followed by subsequent cleavage.

The results for the *H*-phosphonate esters are summarised below (Table 4.1). It can be concluded that it is possible to use an immobilised phosphitylated nucleoside such as **50** as a handle for the synthesis of *H*-phosphonate esters. It was shown that mono-esters such as **51** as well as the more challenging diesters, exemplified by **154**, can be accessed. In this instance, the yield was frustrated by the level of water contained in the alcohol used. Thus to fully exploit this approach, it will be necessary to source dry alcohol.

Table 4.1 Summary of H-*phosphonate ester synthesis*

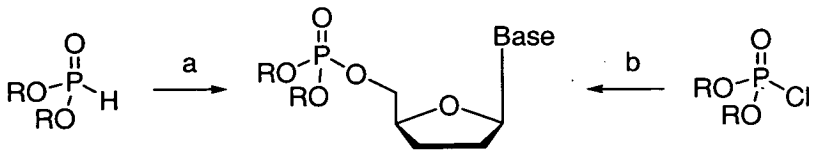
Entry	Cleavage solvent	Amount	Cleavage Time (min)	Yield %[a]	Yield %[a]	SM 49 %[a]
1	water	1 mL	10	58 (**51**)	-	-
2	benzyl alcohol	1 mL	10	8 (**154**)	n.d.	24
3	benzyl alcohol: ^{t}butanol (1:1)	1 mL	10	7 (**154**)	30[b] (**155**)	48

[a] *Isolated yield,* [b] *yield based on nucleoside loading*

4.4 Nucleoside phosphate triesters

4.4.1 Previous syntheses

Phosphate triesters have been accessed essentially by two methods: Mitsunobu coupling of the *H*-phosphonate diester to an alcohol[186] or reaction with the corresponding phosphorochloridate,[168] which in turn can be prepared from the *H*-phosphonate diester and *N*-chlorosuccinimide.[181]

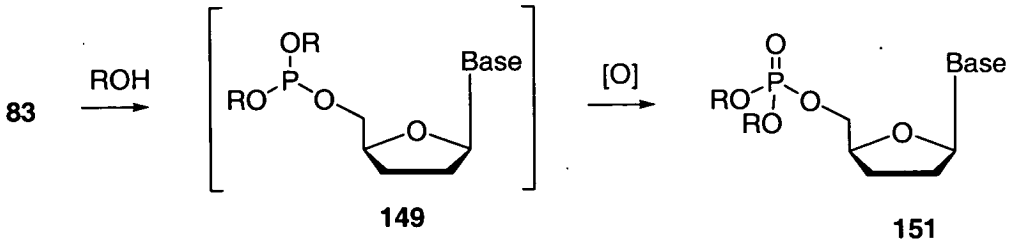


(a) DIAD, PPh_3, THF, alcohol (R = Bn); (b) pyridine, alcohol (R = Et, Pr, Bu, Bn).

Scheme 4.8 Synthesis of nucleoside phosphate triesters

4.4.2 Synthesis using a supported Ludwig-Eckstein reagent

Phosphate triester synthesis using alcohol cleavage, followed by solution-phase oxidation, as proposed below requires that the oxidation step needs to be more rapid than the competing dealkylation reaction, which was exploited in the previous section (Scheme 4.9).



149 **151**

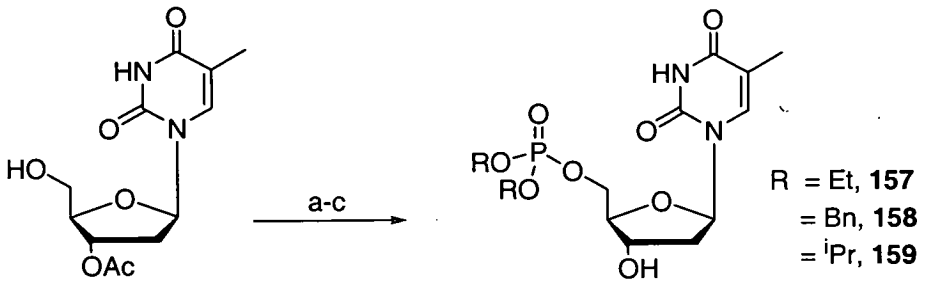
Scheme 4.9 Nucleoside phosphate triester synthesis based on nucleoside resin

Nonetheless, the cleavage reaction requires a certain amount of time to complete, thus a compromise needs to be struck to cleave all the nucleoside off as the phosphite triester, but without it decomposing before the oxidation step. It is expected that different alcohols will have different relative reaction rates for the cleavage and dealkylation steps, so a selection will be attempted to compare them.

Before performing the reactions on the resin, it was attempted to determine the relative reaction times required for the dealkylation and oxidation steps. This was

attempted using the solution phase phosphitylation reagent **11**. However, this approach was limited as the reaction yielded, as expected, the desired phosphate triester as well as starting material 3'-OAc-T **49**. In the solution phase though, it was not possible to determine whether **49** resulted from incomplete phosphitylation or dealkylation.

Thus, the reactions were performed directly on the resin instead; using this approach complete phosphitylation could be confidently achieved. As alcohol, initially ethanol was used with a cleavage time of 10 min as in the previous section.



49

(a) 3 eq PS-P-Cl **44**, 0.1 M py/THF 1/3, 1h, rt; (b) ROH, 10 min; (c) 0.5 M I_2 in pyridine:H_2O, 98:2, 15 min, then 5 % $NaHSO_3$; see Table 4.2.

Scheme 4.10 Synthesis of phosphate triesters

The crude reaction mixture was purified by column chromatography eluted with with a $MeOH/CH_2Cl_2$ gradient, however it was not possible to separate product from starting material. The ratio of both compounds was thus determined by measuring the integration of the H-1' signal in the ^1H proton spectrum of the mixture. It was thus determined that the ratio of starting material **49** to product **157** was 1.00 to 0.13. This shows that for ethanol the dealkylation reaction is already mostly complete by the time the reaction mixture is exposed to the oxidant.

Thus, it seemed valuable to explore a shorter cleavage time for ethanol. The loaded resin was therefore cleaved with three 30 sec cleavage steps and after oxidation and work-up showed a much more favourable ratio of starting material to product, giving an isolated yield 15 % for the product **157**.

Other than the primary alcohol ethanol, other alcohols were explored. Thus, benzyl alcohol, as well as a secondary alcohol, iso-propanol, and a tertiary alcohol, tert-butyl alcohol, were used to synthesise their respective phosphate triesters.

Benzyl alcohol and 2-propanol both resulted in giving the desired phosphate triesters **158** in 27 % and **159** in 18 % yields respectively. In both instances, no starting material was visible by tlc, thus the respective phosphite triesters seemed to be stable during the 10 minute cleavage step. When using the tertiary tert-butyl alcohol however, neither product, nor starting material could be detected, indicating that sterics interfered with cleaving the phosphite triester **50**.

The results for the phosphate triesters are summarised below (Table 2.15). As can be seen, a range of phosphate esters can be accessed using this methodology. The alcohols used in this study were not dry enough to attain the maxium yields possible from the chemistry and were thus limited by producing the mono-*H*-phosphonate esters as side-products.

Table 4.2 Summary of phosphate triester synthesis

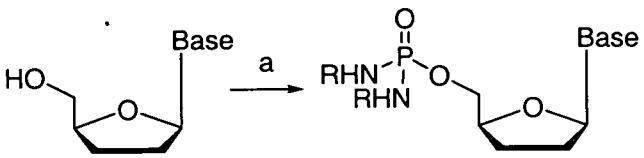
Alcohol	**Volume**	**Cleavage Time (min)**	**Yield %[a]**	**SM 49 %[a]**
Ethanol	3 x 1 mL	3 x 0.5	15 (**157**)	10
benzyl alcohol	1 mL	10	27 (**158**)	/
2-propanol	1 mL	10	18 (**159**)	/
^{t}butanol	1 mL	10	/	/

[a] *Isolated yields*

4.5 Nucleoside diphosphoramidates

4.5.1 Previous syntheses

Synthesis of nucleoside diphosphoramidates is generally not very straightforward. Due to the deactivating effect of the nitrogen, treatment of a phosphorochloridate is not successful.[187] Thus McGuigan *et al.* proposed treating the nucleoside with $POCl_3$ to give the dichlorophosphate intermediate with subsequent treatment with an excess of amine, such as amino acids or alkyl amines.[187] The same strategy was applied to morpholino and methoxyamino-nucleoside derivatives.[188]

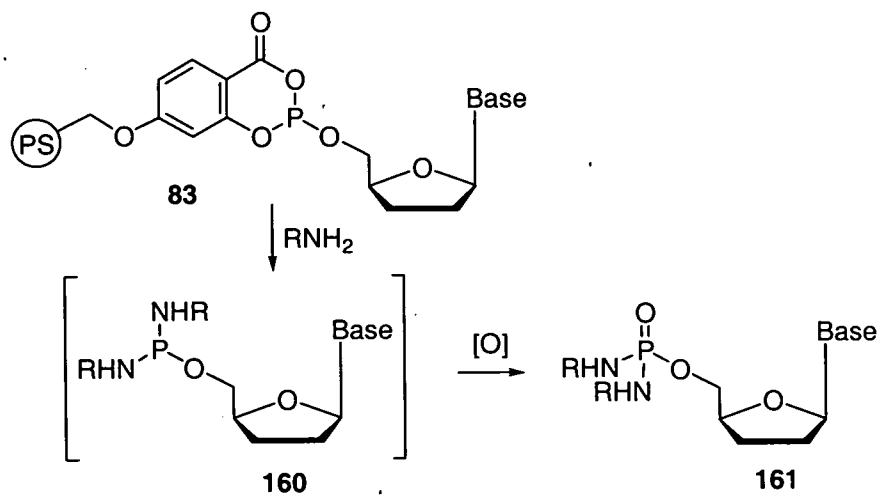


(a) 2 eq $POCl_3$, $PO(OEt)_3$, 0 °C, then 5 eq RNH_2 in $(CH_3OCH_2)_2$, 10 eq TEA, 4 °C, 5 d; 21 - 44 % yield.

Scheme 4.11 Synthesis of nucleoside diphosphoramidates

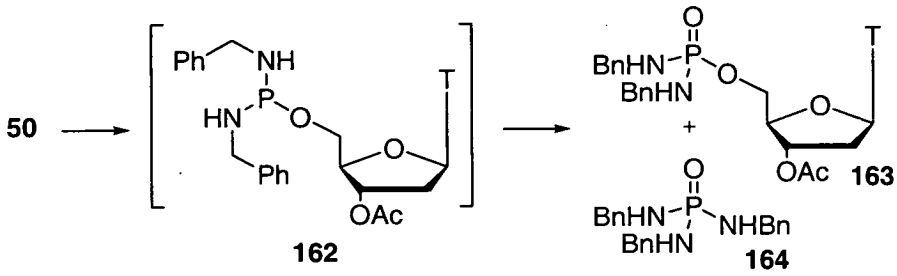
4.5.2 Synthesis using a supported Ludwig-Eckstein reagent

The putative scheme is outlined below. As amines are rather more powerful nucleophiles than alcohols, they should be able to readily achieve cleavage of the phosphitylated nucleoside **83** to give phosphoramidite **160**. It is then expected that the oxidation can be performed under analogous conditions than previously.



Scheme 4.12 Nucleoside diphosphoramidate synthesis based on nucleoside resin

The methodology was explored using benzylamine as a nucleophile. The nucleoside 3'OAc-T **49** was loaded onto the supported reagent **44** to give nucleoside resin **50**. The resin was then subjected to 20 eq of benzylamine dissolved in THF at a 1.75 M concentration for 10 min. No attempts were made to identify **162**, instead the solution was oxidised with 0.5 M iodine in aqueous pyridine and quenched after 15 min. ^{31}P NMR of the crude mixture revealed two major peaks at 15 and 17 ppm, tentatively assigned to **163** and **164**. Their presence was further corrobated by LC-MS analysis. Purification by column chromatography was attempted, but due to the volume of eluent required, the products **163** and **164** could not be detected in the fractions.



Scheme 4.13 Nucleoside diphosphoramidate synthesis based on nucleoside resin

This seems to indicate that the cleavage can be successfully performed with benzylamine. The resulting phosphoramidite is also readily oxidised under the same conditions as for the phosphate triesters. Nonetheless, the purification was not met with any success and Prep HPLC purification would be required to advance this work.

5 Conclusions

The aims of this thesis were to establish whether the method for synthesising nucleoside triphosphates of Ludwig and Eckstein, could be improved by transposing it to the solid phase. By developing the Ludwig-Eckstein reagent as a solid-supported reagent, the benefits associated with supported reagents could be incorporated and hopefully extend its applications.

The envisaged solid-supported reagent was synthesised by attaching a salicylate linker through an ether bond to Merrifield resin. The benzyl ether bond was robust enough to survive the conditions required to convert the linker into the reagent; this was most efficiently achieved by refluxing the resin with phosphorus trichloride in dry conditions.

Different solid-supports have established themselves for various applications in solid-phase chemistry, thus some were examined in turn. Microporous polystyrene was found to give the most satifactory results, as measured by the yield obtained for the synthesis of the model substrate 2'deoxy-thymidine triphosphate. Other supports investigated were macroporous polystyrene, polystyrene-poly ethyleneglycol graft resins, as well as silica based resins. The silica based resins were thought to give lower yields due to surface interactions with rigid silica surface. For the other resins, a factor that was noted to influence the yield obtained was the bead size. As the cleavage step generated a rather unstable species, cleavage times were kept short and thus it is expected that for bigger bead size this contributing towards lower yields.

Additionally to an easier and higher yielding synthesis, it was also expected that the purification of triphosphates could be simplified. Most synthesis protocols require a desalting step using ion exchange chromatography prior to preparative HPLC purification. Using the supported reagent, it was found that the Prep HPLC step could be preceeded with an aqueous work-up and thymidine triphosphate was isolated as its tetrakis alkylammonium salt in an excellent 50 % yield.

An alternative reaction sequence was also explored. As opposed to oxidising the cyclic triphosphite ring in solution, on-resin oxidation was explored. As the P (III) is kept in a trigonal configuration, it was readily oxidised to P (V) using anhydrous

tert-butyl hydroperoxide. The pyrophosphate cleavage was not sufficient to drive the reaction to completion, resulting in a low isolation yield. However the crude triphosphate obtained by on-resin oxidiation was purer, as asserted by HPLC.

The method developed was then applied to the synthesis of the other three DNA triphosphates. Simple DNA triphosphates could be isolated after Prep HPLC purification in good to excellent yield. Some limiations were observed for fast running nucleoside triphosphates such as cytidine triphosphate. This could be circumvented by duplicating the Prep HPLC purification, although this lead to loss in yield. The same observation could be made for proparglyamino modified pyrimidine nucleoside triphosphates. Uridine triphosphate could be obtained in pure form after two Prep HPLCs, whereas the even more fast-running cytidine analogue could not be isolated using this method and requires ion exchange chromatography prior to HPLC purification.

The method was also tested on the most challenging targets, the fluorescently labelled nucleoside triphosphates. A fluorescein labelled uridine analogue was synthesised and phosphitylation of the primary alcohol was found to be possible to yield the mono-*H*-phosphonate. Full loading of the nucleoside onto the resin could be achieved by increasing the amount of resin, the concentration and the reaction time. Treatment of the resin yielded a compound, which could be assigned to the expected triphosphate on the basis of its ^{31}P NMR.

The applications of the supported reagent could also be extended to synthesis of nucleoside prodrugs. The immbolised phosphitylated nucleoside was shown to be used as a handle to access mixed phosphonate diesters and phosphate triesters using alcohols as cleavage reagents. The dryness of the alcohol was found to be very important and presence of water contributed to a lowering of the yield. Initial attempts were also made towards synthesising nucleoside phosphoramidates. Their presence could be verified spectroscopically, but the purification remained elusive.

In conclusion, a solid-supported phosphorylation reagent was developed which enabled to the synthesis of the natural DNA triphosphates in high yields and which could also be applied successfully to the very challenging fluorescein labelled nucleoside triphosphates.

6 Experimental

6.1 General Information

NMR spectra were recorded on a Bruker ARX250, AV300, DPX360 and DPX 400 spectrometers operating at 250 MHz, 300 MHz, 360 MHz and 400 MHz for ^{1}H and 62.5 MHz, 75 MHz, 90 MHz and 100 MHz for ^{13}C spectra respectively. ^{31}P NMR spectra were recorded on Bruker ARX250 at 101 MHz, referenced to 85 % H_3PO_4 in D_2O. ^{1}H MAS NMR spectra were recorded on a Bruker DPX400. Gel phase ^{13}C NMR spectra were recorded on a Bruker DPX 360 or AV300 and and ^{31}P NMR Gel phase spectra on a Bruker DPX 360. Chemical shifts (δ), referenced to the residual protonated solvent as an internal standard, are quoted in ppm, coupling constants (*J*) are reported in Hz. Triphosphate containing compounds were run in D_2O for ^{1}H and ^{13}C spectra; for ^{31}P spectra 50 μL of 2 M TEAB buffer was added. The resulting triethylammonium peaks are omitted from the NMR data. The ^{13}C NMR data of Dmt-containing compounds have been assigned as far as possible. However, due to the chiral environment, the Dmt group displays more signals depending on the structure

LRES mass spectra were recorded on a Fisons VG platform quadropole or a Waters ZMD single quadropole, equipped with an electronspray ionisation source, measuring monoisotopic masses. Major peaks are reported with percentage intensities of the base peak. HRMS were run on a Bruker APEX3.

The Smith Synthesiser from Personal Chemsitry was used to perform microwave irradiations. Infra-red spectra were recorded on a BIORAD FTS 135 with a Golden Gate ATR access. All samples were run neat. Melting points were determined using a Gallenkamp melting point apparatus and are uncorrected. UV-Vis spectra were recorded using a 8452 A diode array detector. Microanalysis were performed by MEDAC Ltd., Surrey.

Dry solvents were obtained by passing through an alumina column. Reagents were used as purchased from commercial sources unless otherwise specified. TLC analysis was carried out using foil-backed sheets coated with silica gel (0.25 mm) and containing the fluorescent indicator UV_{254}. Flash column chromatography was performed, on Sorbsil C60, 40-60 mesh silica.

RP-HPLC was performed on a Hewlett Packard HP1100 Chemstation eluting with (A) 0.1 % TFA/H_2O, (B) 0.04 % TFA/MeCN, (C) 0.1 % formic acid/H_2O and (D) 0.1 % formic acid /MeOH using the methods below.

Method 1: Discovery (Supelco) C18 ODS analytical column, 4.6 mm × 30 mm i.d. 5 μm, flow rate 1.0 mL min^{-1}, gradient: 90 % (A) to 10 % (A) in (B) over 3 min, detection by UV at 220, 254, 260, 282 and 440 nm.

Method 2: Phenomonex (Prodigy) C18 ODS analytical column, 4.6 mm × 150 mm i.d. 5 μm, flow rate 0.5 mL min^{-1}, gradient: 90 % (A) to 10 % (A) in (B) over 10 min, detection by UV at 220, 254, 260, 282 and 440 nm.

Method 3: Phenomonex (Gemini) C18 ODS analytical column, 4.6 mm × 100 mm i.d. 5 μm, flow rate 1.0 mL min^{-1}, gradient: 90 % (C) to 10 % (C) in (D) over 6 min, detection by UV at 214, 220, 254, 280 and 650 nm and ELSD (evaporative light scattering detection).

Preparative RP-HPLC was performed on a Hewlett Packard HP1100 Chemstation eluting with (A) 0.1 M TEAB in H_2O, (B) MeCN and (C) 0.1 M NH_4OAc in H_2O on a Waters (Xterra) RP18 OBD preparative column, 19 mm × 150 mm i.d. 5 μm at a flow rate of 10 mL min^{-1}, using the methods below. The fractions were collected using a Gilson G2250A robot.

Method 4: gradient: 100 % (A) for 5 min, 100 % (A) to 80 % (A) over 15 min, 80 % (A) to 0 % (A) in (B) over 4 min, detection by UV at 230, 254, 260 and 282 nm.

Method 5: 100 % (A) for 20 min, detection by UV at 254, 260 and 282 nm.

Method 6: gradient: 100 % (A) to 97 % (A) over 8 min, then isocratic for 14 min in (B), detection by UV at 230, 254, 260 and 282 nm.

Method 7: gradient: 100 % (C) for 5 min, 100 % (C) to 80 % (C) over 15 min, 80 % (C) to 0 % (C) in (B) over 4 min, detection by UV at 230, 254, 260 and 282 nm.

Method 8: gradient: 100 % (C) to 95 % (C) over 8 min, then isocratic for 14 min in (B), detection by UV at 254, 260, 282 and 440 nm.

Method 9: gradient: 100 % (C) to 0 % (C) over 10 min in (B), detection by UV at 214, 220, 254, 280 and 400 nm.

6.2 General solid-phase chemistry procedures

6.2.1 Calculation of theoretical loading

The theoretical loading of a resin after a reaction was calculated using the equation:

New loading (mmol/g) = [Old loading /(1+(Old loading x Mass added x 10^{-3})]

where 'Old loading' is the loading of the starting resin (mmol/g) and 'Mass added' is the mass of compound added onto the resin.

6.2.2 Colorimetric tests

6.2.2.1 *Quantitative Ninhydrin test*

A known mass of resin (ca. 5 mg) in a small test tube was treated with 3 drops of reagent A and 1 drop of reagent B and heated at 100 °C for 10 min. The test tube was cooled and 60 % aq EtOH (2 mL) added. The resin was removed by filtration through cotton wool and the deep blue filtrate collected in a 25 mL volumetric flask. The resin was washed with a solution of Et_4NCl (0.5 M in CH_2Cl_2, 2 x 0.5 mL) and the sample made up to 25 mL with 60 % aq EtOH. The absorbance at 570 nm was measured against a reagent blank. The level of amine present was calculated using the equation:

Amount of amine present (mmol/g) = $[(A_{570} \times V)/(\varepsilon_{570} \times m)] \times 10^3$

where A_{570} is the recorded absorbance at 570 nm, V is the final volume (mL), ε_{570} is an extinction coefficient suitable for most peptides (1.5×10^4 M^{-1} cm^{-1}) and m is the mass of the resin sample (mg).

Reagent A

- Solution 1: Phenol (40.0 g) was dissolved in EtOH (10 mL) with warming and then stirred over Amberlite mixed-bed resin MB-3 (4.0 g) for 45 min. The mixture was filtered.
- Solution 2: Potassium cyanide (65 mg) was dissolved in H_2O (100 mL). A 2 mL aliquot of this solution was diluted with pyridine (freshly distilled from ninhydrin) and stirred over Amberlite mixed-bed resin MB-3 (4.0 g). The solution was filtered and mixed with 'Solution 1' to give reagent A.

Reagent B

Ninhydrin (2.5 g) was dissolved in absolute EtOH (50 mL).

6.2.2.2 *Quantitative Fmoc test*

A known mass of resin (ca. 5 mg) was weighed into a 1 mL SPE syringe and treated with 20 % piperidine in DMF by percolating through the resin bed into a 25 mL volumetric flask. The filtrate was made up to volume with 20 % piperidine in DMF. The absorbance at 302 nm was measured against 20 % piperidine in DMF. The loading was calculated using the equation:

$$\text{Loading (mmol/g)} = [(A_{302} \times V)/(\varepsilon_{302} \times m)] \times 10^3$$

where A_{302} is the recorded absorbance at 302 nm, V is the final volume (mL), ε_{302} is the extinction coefficient of the piperidyl-fulvene adduct (7.8×10^3 M^{-1} cm^{-1}) and m is the mass of the resin sample (mg).

6.2.2.3 *Qualitative chloranil test*

To a small amount of resin (ca. 1-3 mg), one drop of 2 % acetaldehyde in DMF was added followed by one drop of 2 % *p*-chloranil in DMF and was allowed to stand for 5 min. Blue stained beads indicated the presence of secondary amines.

6.2.2.4 *Quantitative DMT test*

A small amout of resin (ca. 5 - 10 mg) in 1 mL SPE syringe was treated with 0.5 M DMT-Cl in CH_2Cl_2 (0.5 mL), 0.5 M $TBANO_3$ in CH_2Cl_2 (0.5 mL) and dry pyridine (2 drops) for 15 minutes. The resin was drained, rinsed with dry CH_2Cl_2 (3 x 2 mL) and vacuum dried. The resin was weighed into a syringe and treated with 3 % trichloroacetic acid in CH_2Cl_2 by percolating through the resin bed into a 25 mL volumetric flask and made up to volume.

The absorbance at 502 nm was measured against 3 % trichloroacetic acid in CH_2Cl_2. The level of hydroxyl groups present was calculated using the equation:

$$\text{Loading (mmol/g)} = [(A_{502} \times V)/(\varepsilon_{502} \times m)] \times 10^3$$

where A_{502} is the recorded absorbance at 502 nm, V is the final volume (mL), ε_{502} is an extinction coefficient of the DMT cation (7.6×10^4 M^{-1} cm^{-1}) and m is the mass of the resin sample (mg).

6.3 Experimental for Chapter 2

6.3.1 General methods

6.3.1.1 *Preparation of buffers*

TEAB buffer

Deionised water (650 mL) and TEA (278 mL) were mixed and stirred over an ice-bath. Dry ice was placed in a concial flask and with tubing fitted to the top was left to bubble through the TEA/water solution until pH reached pH 7-8. The solution was diluted to 1 L to give a 2 M TEAB buffer and was stored at 4 °C.

Ammonium acetate buffer

Ammonium acetate powder (77 g, high purity grade) was dissolved in deionised water (500 mL) to a 2 M Ammonium acetate buffer.

Both buffers were diluted to the required concentration immediately prior to use.

6.3.1.2 *Preparation and purification of reagents for phosphorylation reactions*

Resins

After attachment of the linker, resins were dried in a vacuum oven at 40 °C for 1 day, then stored over P_2O_5 in a desicator prior to use.

Nucleosides

Nucleosides were synthesised using methods described below or purchased from Chemgenes Corp (3'-OAc-A^{Bz}, 3'-OAc-C^{Bz} and 3'-OAc-G^{iBu}). Nucleosides were lyophilised from MeCN/H_2O if obtained as an oil and then stored over P_2O_5 in a desicator prior to use.

Tributylamine

Tributylamine of >99.5 % purity was suspended in ninhydrin powder and stored for 1 week with intermittent shaking. The yellow liquid was filtered off and distilled from a Kugelrohr @ 0.1 mbar and 40 °C. The clear liquid obtained was negative by ninhydrin test and stored over 4Å MS.

Bis-tributylammonium pyrophosphate

Dowex 50 resin (50 g, 71 % moisture content) was soaked repeatedly with MeOH, NH_3 sat. MeOH, H_2O, 2 M HCl and H_2O until all solutions were running clear. The

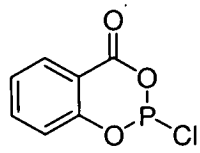
dried resin was filled into a 150 mL SPE reservoir. Tetra sodium pyrophosphate decahydrate (2.23 g, 5.0 mmol) dissolved in H_2O (50 mL) was added onto the column and was eluted with H_2O (ca. 80 mL) until the pH rose to pH 5. The elutate was dropped directly into a stirred solution of Bu_3N (2.38 mL, 10.0 mmol) in EtOH (20 mL) stirred over an ice-bath. The eluate was freeze-dried until a white solid appeared (*ca.* 1 week) and dissolved in anhydrous DMF (10 mL) with activated 4 Å MS to yield a 0.5 M bis(tributylammonium) pyrophosphate solution. **^{31}P NMR** (121.5 MHz, $CDCl_3$) δ_P –11.24.

Tris-tetrabutylammonium pyrophosphate

Disodium dihydrogen pyrophosphate (2.22 g, 10.0 mmol) dissolved in H_2O (50 mL) was applied to a Dowex 50 column and was eluted with H_2O (ca. 80 mL) until the pH rose to pH 5. The elutate was neutralised to pH 7 with addition of 40 % w/v aqueous tetrabutylammonium hydroxide solution (18 mL) and then freeze-dried until a white solid appeared (*ca.* 1 week) and dissolved in anhydrous $CHCl_3$ (10 mL) with activated 4 Å MS to yield a 0.5 M tris(tetrabutylammonium) pyrophoshate solution. **^{31}P NMR** (121.5 MHz, $CDCl_3$) δ_P –5.72.

6.3.2 Synthesis of compounds and resins

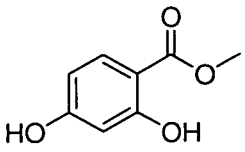
2-Chloro-5,6-benzo[1,3,2]dioxaphosphorinan-4-one (11)



Method 1: PCl_3 (900 μL, 10.3 mmol) was added to dry THF (10 mL) at -15 °C, then a salicylic acid (1.38 g, 10.0 mmol) solution in dry pyridine (1.6 mL) and dry THF (5 mL) was added dropwise over 5 min. The reaction mixture was warmed to rt and stirred for 15 h. The solid precipitate was filtered off through oven dried glassware and the filtrate concentrated *in vacuo* to yield a white solid (916 mg, 4.52 mmol, 45 % yield). **Method 2:** Salicylic acid (6.98 g, 50.5 mmol) was dissolved in dry THF (10 mL) under a N_2 atmosphere at 70 °C. PCl_3 (9.0 mL, 103 mmol) was added down the condenser. HCl gas was trapped by bubbling through 6 M NaOH solution (100 mL). After 2 h, the reaction was cooled down to rt and THF and PCl_3 were evaporated *in vacuo*. A clear oil (9.46 g, 46.7 mmol, 92 % yield)

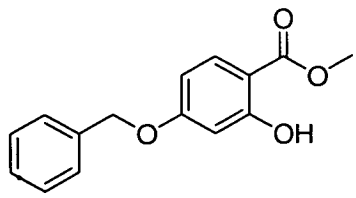
was distilled off at 110 °C, 0.57 mbar in a Kugelrohr. **bp** 110 °C at 0.57 mbar, *lit.*[125]– 75 °C at 0.1 mm Hg, **^{1}H NMR** (300 MHz, C_6D_6) δ_H 7.93 (1H, d, J = 7.5 Hz, CH), 7.07 (1H, t, J = 7.5 Hz, CH), 6.78 (1H, t, J = 7.5 Hz, CH), 6.68 (1H, d, J = 8.1 Hz, CH), **^{13}C NMR** (75 MHz, C_6D_6) δ_C 153.6 (d, J_{PC} = 6.9 Hz, CO_2), 153.2 (d, J_{PC} = 6.4 Hz, CO), 137.1 (CH), 131.2 (d, J_{PC} = 2.0 Hz, CH), 125.6 (CH), 120.0 (CH), 117.0 (d, J_{PC} = 13.4 Hz, CCO_2), **^{31}P NMR** (121.5 MHz, C_6D_6) δ_P 149.03.

Methyl 2,4-dihydroxybenzoate (35)



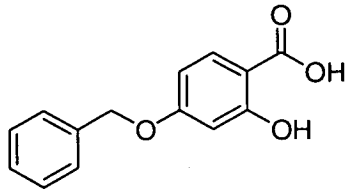
Method 1: 2,4-dihydroxybenzoic acid (20.0 g, 0.130 mol) in MeOH (150 mL) was treated with conc. H_2SO_4 (5 mL) and refluxed at 70 °C for 66 h. The reaction was cooled to rt, neutralised by adding $NaHCO_3$ powder and evaporated *in vacuo*. H_2O (500 mL) was added and extracted into CH_2Cl_2 (600 mL). The CH_2Cl_2 layer was washed with brine (500 mL), dried (Mg_2SO_4) and evaporated *in vacuo* to yield an off-white powder (17.3 g, 0.103 mol, 79 % yield). **Method 2:** To a cooled solution of 2,4-dihydroxybenzoic acid (4.66 g, 30.3 mmol) in MeOH (40 mL), $SOCl_2$ (10 mL, 137 mmol, 4.5 eq.) was added dropwise while stirring at 0 °C. The solution was refluxed at 50 °C for 2 d and evaporated *in vacuo*. The residue was dissolved in CH_2Cl_2 (50 mL), washed with 5 % $NaHCO_3$ (100 mL) solution, dried (Na_2SO_4) and concentrated *in vacuo* to give a salmon powder which was recrystallised from MeOH/H_2O to yield an off-white powder (4.18 g, 24.9 mmol, 82 % yield). **mp** 113-114 °C (aq MeOH), *lit.*[189] – 116-117 °C (benzene), **IR** (neat) 3331 (m), 3176 (m), 1741 (w), 1637 (s), 1615 (s), 1434 (s), 1145 (s), 1095 (s) cm^{-1}, **^{1}H NMR** (300 MHz, CD_3CN) δ_H 10.80 (1H, br signal, *o*-OH), 7.68 (1H, d, *J* = 8.7 Hz, *o*-CH), 6.40 (1H, dd, *J* = 8.7, 2.2 Hz, *m*-CH), 6.34 (1H, d, *J* = 2.2 Hz, *m*-CH), 3.87 (3H, s, CH_3), **^{13}C NMR** (75 MHz, CD_3CN) δ_C 171.4 (C=O), 164.8 (COH), 164.6 (COH), 132.8 (*o*-CH), 109.1 (*m*-CH), 106.0 (C), 103.6 (*m*-CH), 52.8 (CH_3), **ES$^-$** m/z (%) 281.1 (M+TFA-H$^-$, 100), **HPLC** (λ_{220}) t_R 3.24 min (Method 1), **R_f** 0.44 (7:3 Hexane:EtOAc, UV).

Methyl 2-hydroxy-4-benzyloxybenzoate (36)



Using a procedure by Astles *et al.*[126], phenol **35** (5.02 g, 29.9 mmol) dissolved in acetone (35 mL), treated with KI (2.55 g, 15.4 mmol), Bu_4NCl (51 mg, 0.19 mmol), K_2CO_3 (4.74 g, 34.3 mmol) and BnCl (34 mL, 29.5 mmol), was refluxed at 60 °C for 10 h. The mixture was filtered and the filtrate evaporated *in vacuo*. The pale orange residue was suspended in MeOH (10 mL), sonicated and filtered off to yield a white powder (5.53 g, 21.4 mmol, 72 % yield). **mp** 101-102 °, lit.[126] – 101-102 °C, **IR** (neat) 3332 (m), 3180 (m), 1738 (w), 1665 (s), 1621 (s), 1437 (s), 1344 (s), 1247 (s), 1094 (s) cm^{-1}, **^{1}H NMR** (300 MHz, $CDCl_3$) δ_H 11.00 (1H, s, *o*-OH), 7.76 (1H, d, J = 8.8 Hz, *o*-CH), 7.21 – 7.43 (5H, m, Ph), 6.56 - 6.50 (2H, m, *m*-CH), 5.09 (2H, s, CH_2), 3.93 (3H, s, CH_3), **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C 170.6 (C=O), 164.8 (C-O), 163.9 (C-O), 136.2 (*o*-CH), 131.4 (C), 128.9 (CH), 128.8 (CH), 127.7 (CH), 108.3 (*m*-CH), 105.8 (C), 101.8 (*m*-CH), 70.5 (CH_2), 52.2 (CH_3), **ES$^-$** m/z (%) 257.1 ($M\text{-}H^-$, 100), **HPLC** (λ_{220}) t_R 4.51 min (Method 1), **R_f** 0.65 (7:3 Hexane:EtOAc, UV).

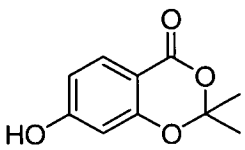
2-Hydroxy-4-benzyloxybenzoic acid (37)



Method 1: Using a procedure by Astles *et al.*[126], ester **36** (5.48 g, 21.3 mmol) dissolved in 1,4-dioxane (50 mL) and 2 M NaOH solution (25 mL) was refluxed for 5 h. The mixture was concentrated *in vacuo* to give a white residue, which was dissolved in water (100 mL) and washed with EtOAc (50 mL). The pH of the aqueous layer was adjusted to pH 1 with conc. HCl (*ca.* 10 mL), after which a precipitate formed which was isolated by filtration as a white powder (4.74 g, 19.4 mmol, 91 % yield). **Method 2:** Using a procedure as by Dushin & Danishefsky[129], acetonide **39** (25 mg, 89 μmol) in DMSO (0.3 mL) was treated with 48 % aqueous

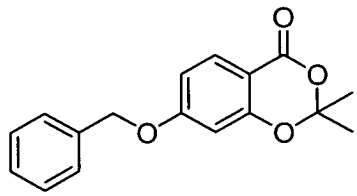
KOH (50 μL) and the mixture was heated at 60 °C for 30 min. Upon cooling, the solution was acidified with 1 M HCl and extracted with EtOAc (3 x 5 mL). The extracts were washed with water (5 mL) and brine (5 mL), dried (Na_2SO_4) and evaporated *in vacuo* to yield a white powder (18 mg, 75 μmol, 85 % yield). **mp** 184-185.5 °C, lit.[126]– 182 °C, **IR** (neat) 3012 (w), 2549 (w), 1738 (m), 1655 (s), 1618 (s), 1453 (s), 1432 (s), 1378 (s), 1237 (s), 1149 (s), 1024 (s) cm^{-1}, **^{1}H NMR** (300 MHz, $(CD_3)_2CO$) δ_H 11.33 (1H, br signal, *o*-OH), 7.77 (1H, d, *o*-CH, J = 8.8 Hz), 7.28 – 7.47 (5H, m, Ph), 6.53 – 6.58 (2H, m, 2 *m*-CH), 5.16 (2H, s, CH_2), **^{13}C NMR** (75 MHz, $(CD_3)_2CO$) δ_C 172.3 (C=O), 165.7 (COH), 165.0 (COH), 137.4 (*o*-CH), 132.4 (C), 129.4 (CH), 128.7 (CH), 128.3 (CH), 108.4 (*m*-CH), 105.9 (C), 102.2 (*m*-CH), 70.5 (CH_2), **ES$^-$** m/z (%) 243.1 ($M\text{-}H^-$, 100), **HPLC** (λ_{220}) t_R 3.94 min (Method 1), **R_f** 0.24 (1:1:0.01 Hexane:EtOAc:AcOH, UV).

7-Hydroxy-2,2-dimethyl-benzo[1,3]dioxin-4-one (38)



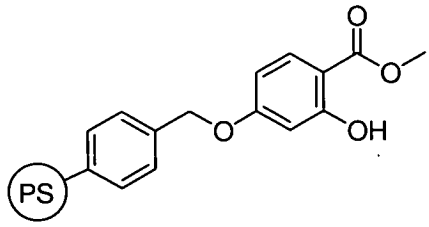
Using a procedure as *per* Japan Patent 09194474[127], 2,4-dihydroxybenzoic acid (10.0 g, 64.9 mmol) dissolved in dry acetone (20 mL), Piv_2O (14.3 mL, 70.5 mmol, 1.1 eq) and conc. H_2SO_4 (0.2 mL) was stirred under N_2 for 18 h. The mixture was evaporated *in vacuo* and adjusted to pH 6 with 5 % aq K_2CO_3 (*ca.* 50 mL). The organic layer was extracted with EtOAc (100 mL), washed with H_2O (100 mL) and brine (100 mL), dried (Na_2SO_4) and evaporated *in vacuo*. The resulting slurry was dissolved in CH_2Cl_2:hexane:EtOAc (33 mL, 5:5:1), resulting in a white precipitate and a yellow solution, which was stored at 5 °C for 15 h. The precipitate was filtered off and rinsed with hexane:CH_2Cl_2 (4:1) to give an off-white powder (5.97 g, 30.73 mmol, 47 % yield). **mp** 203-204 °C - lit[190] 203-204 °C (EtOAc), **IR** (neat) 3126 (w), 1690 (s), 1590 (s), 1481 (m), 1310 (s), 1255 (s), 1188 (s) cm^{-1}, **^{1}H NMR** (400 MHz, $(CD_3)_2CO$) δ_H 7.69 (1H, d, J = 8.4 Hz, 5-CH), 6.61 (1H, dd, J = 8.4, 2.2 Hz, 6-CH), 6.39 (1H, d, J = 2.2 Hz, 8-CH), 1.63 (6H, s, CH_3), **^{13}C NMR** (100 MHz, $(CD_3)_2CO$) δ_C 166.5 (C=O), 161.7 (CO), 159.7 (CO), 132.9 (*o*-CH), 112.9 (*m*-CH), 107.6 (C), 107.3 (C), 104.5 (*m*-CH), 26.7 (CH_3), **ES$^-$** m/z (%) 193.0 ($M\text{-}H^-$, 100), **HPLC** (λ_{220}) t_R 3.12 min (Method 1), **R_f** 0.47 (1:1 EtOAc:hexane, UV).

7-Benzyloxy-2,2-dimethyl-benzo[1,3]dioxin-4-one (39)



Using a procedure by Dushin & Danishefsky[129], acetonide **38** (1.01 g, 5.20 mmol), PPh_3 (1.46 g, 5.57 mmol, 1.1 eq) and anhydrous BnOH (0.6 mL, 5.80 mmol, 1.1 eq) were dissolved in dry THF (20 mL) at 0 °C. DIAD (1.5 mL, 90 % tech grade, 6.99 mmol, 1.3 eq) was added in 7 aliquots over 1.5 h. The mixture was warmed to rt and stirred for 15 h. The evaporated residue was purified by column chromatography eluted with 9:1 hexane:EtOAc to give a white powder (1.41 g, 4.98 mmol, 96 % yield). **mp** 72-73 °C, **IR** (neat) 3070 (w), 1720 (s), 1613 (s), 1586 (s), 1279 (s) cm^{-1}, **^{1}H NMR** (300 MHz, $CDCl_3$) δ_H 7.87 (1H, d, J = 8.8 Hz, 5-CH), 7.3 - 7.4 (5H, m, Ph), 6.72 (1H, dd, J = 8.8, 2.6 Hz, 6-CH), 6.50 (1H, d, J = 2.6 Hz, 8-CH), 5.09 (2H, s, CH_2), 1.72 (6H, s, CH_3), **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C 165.6 (C=O), 161.1 (CO), 158.0 (CO), 135.8 (C), 131.4 (*o*-CH), 128.9 (CH), 128.6 (CH), 127.7 (CH), 111.0 (*m*-CH), 106.6 (C), 106.5 (C), 102.1 (*m*-CH), 70.6 (CH_2), 26.0 (CH_3), **EI** m/z 284 (M^+, 4), **HRMS** (EI) for $C_{17}H_{16}O_4$ M^+: calcd 284.10486, found 284.10475, **Anal.** Calcd. for $C_{17}H_{16}O_4$: C 71.82, H 5.67, found: C 71.60, H 5.65, **HPLC** (λ_{220}) t_R 4.32 min (Method 1), **R_f** 0.58 (2:1 hexane:EtOAc, UV).

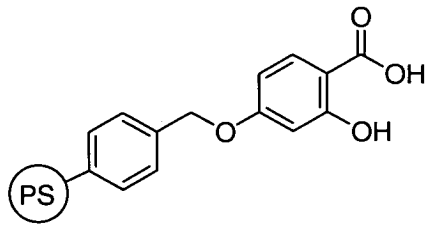
Methyl 2-hydroxy-benzoate 4-oxymethyl microporous polystyrene resin, 75-150 μm (40)



Merrifield resin (20.4 g, 1.75 $mmolg^{-1}$, 35.7 mmol) suspended in acetone (250 mL) with KI (6.4 g, 38.6 mmol, 1 eq), K_2CO_3 (14.8 g, 0.11 mol, 3 eq) and **35** (18.0 g, 0.11 mol, 3 eq) was refluxed at 60 °C for 2 days. The resin was drained, rinsed with H_2O, DMF/ H_2O, MeOH/H_2O, DMF, MeOH, CH_2Cl_2 and Et_2O (3 x each), vacuum oven dried and stored over P_2O_5. Excess linker was recovered from the drained

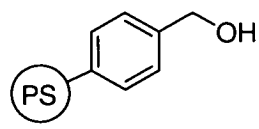
reaction mixture, by evaporation *in vacuo*, neutralisation with 2 M HCl to pH 7, extraction into CH_2Cl_2, washing with sat. $NaHCO_3$ and brine and evaporation *in vacuo*. **IR** (neat) 3026 (w), 2921 (w), 1668 (s), 1620 (m), 1439 (m), 1253 (s) cm^{-1}, **Gel-Phase ^{13}C NMR** (75 MHz, $(CD_3)_2CO$) δ_C 170.8 (C=O), 164.3 (2 C-O), 125 – 135 (resin), 108.6 (*m*-CH), 106.0 (C), 102.4 (*m*-CH), CH_2 signal not observed, 52.1 (CH_3).

2-Hydroxy-benzoic acid 4-oxymethyl microporous polystyrene resin, 75-150 μm (41a)



Resin **40** (ca. 20 g, 35.7 mmol) suspended in THF (100 mL) with tetrabutylammonium hydroxide solution (50 mL, 40 % w/v in H_2O) was refluxed at 70 °C for 2 d. The light orange resin was cooled to r.t., drained, rinsed with H_2O, THF, 2 M HCl/THF (1/1), H_2O, THF/H_2O, THF, MeOH, DMF, CH_2Cl_2 and Et_2O, vacuum oven dried and stored over P_2O_5. **IR** (neat) 3025 (w), 2923 (w), 1666 (m), 1620 (m), 1493 (m), 1240 (s) cm^{-1}, **Gel-Phase ^{13}C NMR** (75 MHz, $(CD_3)_2CO$) δ_C 172.4 (C=O), 165.1 (2 C-O), 132.4 (*o*-CH), 128 – 132 (4 CH + resin), 108.5 (*m*-CH), 106.0 (C), 102.2 (*m*-CH), 70.6 (CH_2), 41.1 (resin).

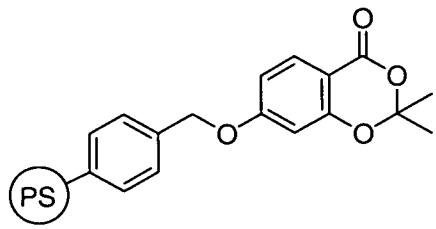
Hydroxymethyl microporous polystyrene resin, 75-150 μm (42)



Using a procedure by Basso[191], Merrifield resin (3.02 g, 1.60 $mmolg^{-1}$, 4.83 mmol) suspended in DMF (20 mL) was treated with anhydrous cesium acetate (3.16 g, 16.5 mmol, 3.4 eq) and KI (70 mg, 0.42 mmol) and the resin was stirred at 50 °C for 2 days. The resin was drained, washed with H_2O, MeOH, DMF, CH_2Cl_2 and Et_2O and oven dried. Analysis by IR showed C=O stretch at 1735 cm^{-1}. Resin suspended in THF (40 mL) and aq. Bu_4NOH solution (40 % w/v in H_2O, 15 mL) was shaken for 2 days, filtered, washed with H_2O, MeOH, DMF, CH_2Cl_2 and Et_2O

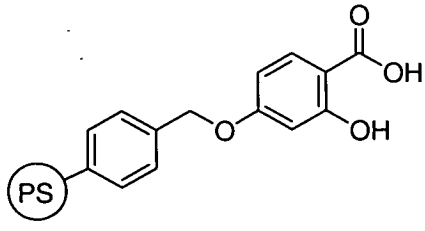
and oven dried. **IR** (neat) 3024 (w), 2921 (m), 2853 (w), 1601 (w), 1493 (s), 1452 (s), 1028 (m) cm^{-1}.

2,2-dimethyl-benzo[1,3]dioxin-4-one 4-oxymethyl microporous polystyrene resin, 75-150 μm (43)



Using a procedure by Basso,[191] hydroxymethyl polystyrene resin **42** (theoretical loading 1.64 $mmolg^{-1}$, 0.98 g, 1.61 mmol) was washed in dry THF (3 x 15 mL). A solution of acetonide **38** (1.12 g, 5.78 mmol) and PPh_3 (1.62 g, 6.18 mmol) in dry THF (12 mL) was added and the suspension was treated with DIAD (1.11 mL, 5.75 mmol) added in 5 aliquots over 30 min and shaken at r.t. for 2 d. The resin was filtered, washed with THF, MeOH, DMF, CH_2Cl_2 and Et_2O and oven dried. **IR** (neat) 3026 (m), 2921 (m), 1738 (s), 1617 (s), 1493 (s) cm^{-1}, **MAS ^{1}H NMR** (400 MHz, $CDCl_3$) δ_H 7.9 (1H, s, 5-CH), 6.3 - 7.2 (resin background), 5.0 (2H, s, CH_2), 1.3 – 2.0 (resin), 1.7 (6H, s, 2 CH_3), **Gel-Phase ^{13}C NMR** (75 MHz, C_6D_6) δ_C 165.7 (C=O), 160.3 (C-O), 158.3 (C-O), 131.6 (*o*-CH), 128 – 132 (4 CH + resin), 110.8 (*m*-CH), 107.2 (C), 106.1 (C), 102.2 (*m*-CH), 70.4 (CH_2), 40.9 (resin), 25.6 (CH_3), 21.9 (resin).

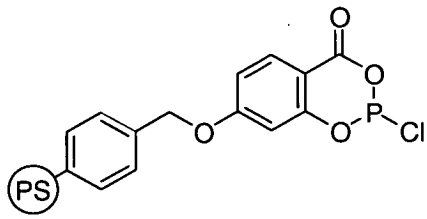
2-hydroxy-benzoic acid 4-oxymethyl microporous polystyrene resin, 75-150 μm (41b)



Resin **43** (theoretical loading 1.27 $mmolg^{-1}$, 1.26 g, 1.60 mmol) was swollen in THF (15 mL) for 10 min. 48 % w/v KOH solution (5 mL) was added and stirred at 60 °C for 7 h. The resin was filtered, washed with H_2O and CH_2Cl_2, swollen in CH_2Cl_2, acidified with 3 M HCl/CH_2Cl_2 (1/1) (2x) and rinsed with H_2O, MeOH,

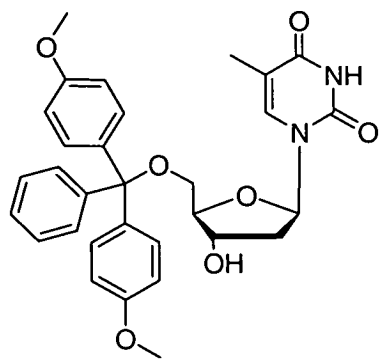
DMF, CH_2Cl_2 and Et_2O. **IR** (neat) 3025 (w), 2922 (w), 1620 (m), 1589 (m), 1493 (m), 1370 (s) cm^{-1}, **Gel-Phase ^{13}C NMR** (75 MHz, $(CD_3)_2CO$) δ_C 172.4 (C=O), 165.7 (C-O), 164.8 (C-O), 132.4 (*o*-CH), 128 – 132 (4 CH + resin), 108.4 (*m*-CH), 106.0 (C), 102.2 (*m*-CH), 70.7 (CH_2), 41.1 (resin), 22.2 (resin).

2-Chloro-4-oxo-benzo[1,3,2]dioxaphosphinine 7-oxymethyl microporous polystyrene resin, 75-150 μm (44)



Resin **41a** (300 mg, 0.402.mmol, theoretical loading 1.34 $mmolg^{-1}$) was swollen in dry CH_2Cl_2 and dry THF (2 x 2 mL for 5 min each) at 60 °C. PCl_3 (95 μL, 4 eq) was added to the resin in dry THF (4 mL) and the syringe was heated at 60 °C for 2 h. The resin was drained and rinsed with dry CH_2Cl_2 and dry THF (2 x 2 mL for 5 min each) and dried over P_2O_5 in a desicator. **Gel Phase ^{31}P NMR** (162 MHz, $CDCl_3$) δ_P 148.8 ppm (82 % by integration), **ICP analysis:** P (%) 3.45, **Loading obtained:** 1.11 $mmolg^{-1}$, 90 % yield.

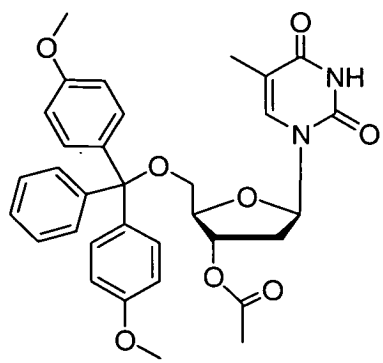
5'-*O*-(4,4'-Dimethoxytrityl)-thymidine (47)



Thymidine (2.52 g, 10.4 mmol) was suspended in dry pyridine (5 mL) and DMT-Cl (3.52 g, 10.4 mmol, 1.0 eq) in dry pyridine (10 mL) was added *via* canula over 1 min. The reaction was stirred under N_2 for 2 h, evaporated *in vacuo* and dissolved in $CHCl_3$ (20 mL) and H_2O (10 mL). The organic layer was separated, washed with H_2O (10 mL) and brine (10 mL), dried (Na_2SO_4), evaporated *in vacuo* and purified by column chromatography eluted with 0 - 2 % MeOH/CH_2Cl_2 to yield a white

foam (5.24 g, 9.63 mmol, 93 % yield). **IR** (neat) 2929 (w), 1678 (s), 1607 (m), 1508 (m), 1464 (s), 1249 (s), 1032 (s) cm^{-1}, **^{1}H NMR** (400 MHz, $CDCl_3$) δ_H 8.72 (1H, s, H-3), 7.60 (1H, d, *J* = 1.0 Hz, H-6), 7.18 -7.43 (9H, m, 9 Ar), 6.86 (4H, d, *J* = 8.8 Hz, 4 Ph), 6.43 (1H, dd, *J* = 7.7, 6.1 Hz, H-1'), 4.59 (1H, m, H-4'), 4.08 (1H, q, J = 3.0 Hz, H-3'), 3.82 (6H, s, CH_3O), 3.50 (1H, dd, H-5', *J* = 10.3, 3.3 Hz), 3.42 (1H, dd, H-5', *J* = 10.6, 3.0 Hz), 2.84 (1H, s, OH), 2.30 - 2.46 (2H, m, H-2'), 1.51 (3H, s, CH_3), **^{13}C NMR** (100 MHz, $CDCl_3$) δ_C 163.3 (C-4), 158.5 (COMe), 158.3 (COMe), 150.0 (C-2), 144.0 (C), 135.3 (C-6), 135.1 (C), 135.1 (C), 129.8 (CH), 128.8 (CH), 127.8 (CH), 127.7 (CH), 127.5 (CH), 127.5 (CH), 126.9 (CH), 126.8 (CH), 113.4 (CH), 112.9 (CH), 110.9 (C-5), 86.7 (C), 85.8 (C-4'), 84.4 (C-1'), 72.2 (C-3'), 63.3 (C-5'), 55.0 (CH_3O), 40.6 (C-2'), 11.5 (CH_3), **HPLC** (λ_{282}) t_R 3.89 min (Method 1), **R_f** 0.19 (4 % MeOH/CH_2Cl_2, ammonium molybdate).

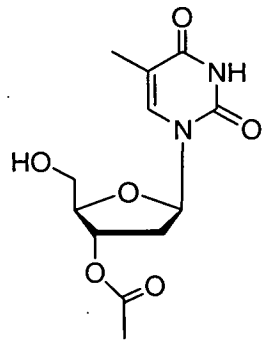
3'-*O*-Acetyl-5'-*O*-(4,4'-dimethoxytrityl)-thymidine (48)



Nucleoside **47** (5.24 g, 9.63 mmol) dissolved in dry pyridine (50 mL) was treated with DMAP (283 mg, 2.31 mmol, 0.24 eq), TEA (2.70 mL, 19.2 mmol, 2.0 eq) and Ac_2O (909 µL, 9.62 mmol, 1.0 eq). The mixture was stirred for 45 min, evaporated *in vacuo*, dissolved in EtOAc (100 mL), washed with 5 % $NaHCO_3$ solution (100 mL), H_2O (50 mL) and brine (50 mL), dried (Na_2SO_4), evaporated *in vacuo* and purified by column chromatography eluted with 2 % MeOH/CH_2Cl_2 to yield a white foam (5.32 g, 9.60 mmol, 99 % yield). **IR** (neat) 3330 (w), 3198 (m), 1687 (s), 1599 (m), 1480 (m), 1372 (m), 1096 (m) cm^{-1}, **^{1}H NMR** (300 MHz, $CDCl_3$) δ_H 9.50 (1H, s, H-3), 7.70 (1H, s, H-6), 7.28 -7.45 (9H, m, 9 Ar), 6.88 (4H, d, *J* = 8.8 Hz, 4 Ph), 6.50 (1H, dd, *J* = 8.4, 6.2 Hz, H-1'), 5.49 (1H, m, H-4'), 4.18 (1H, s, H-3'), 3.83 (6H, s, CH_3O), 3.51 (2H, m, H-5'), 2.50 (2H, m, H-2'), 2.13 (3H, s, $COCH_3$), 1.43 (3H, s, CH_3), **^{13}C NMR** (75 MHz, $CDCl_3$) δ_C 170.7 (*C*=OCH_3), 164.1 (C-4), 158.9 (COMe), 150.8 (C-2), 144.4 (C), 135.5 (C-6), 135.4 (C), 135.3

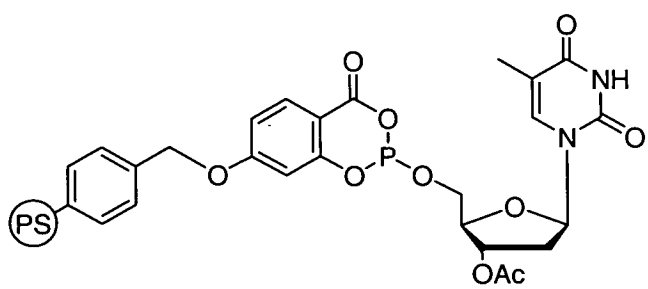
(C), 130.2 (CH), 128.3 (CH), 128.2 (CH), 127.4 (CH), 113.5 (CH), 111.9 (C-5), 87.3 (C), 84.5 (C-4'), 84.2 (C-1'), 75.6 (C-3'), 63.9 (C-5'), 55.4 (CH_3O), 38.1 (C-2'), 21.2 (CH_3O), 12.0 (CH_3), **HPLC** (λ_{282}) t_R 4.23 min (Method 1), $\mathbf{R_f}$ 0.63 (10 % MeOH/CH_2Cl_2, ammonium molybdate).

3'-*O*-Acetyl-thymidine (49)



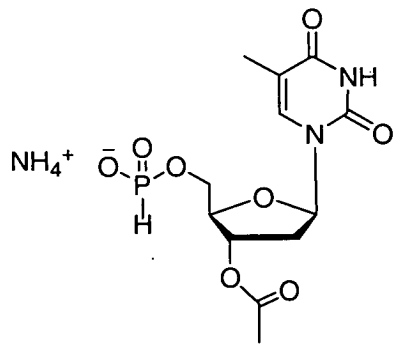
Nucleoside **48** (5.32 g, 9.60 mmol), dissolved in MeOH (50 mL), treated with TFA (10 mL), stirred for 1 h, evaporated *in vacuo* and purified by column chromatography eluted with 0-5 % MeOH in 1% AcOH/CH_2Cl_2 to yield an off-white powder (1.48 g, 5.22 mmol, 54 % yield). **mp** 175-176 °C (MeOH), lit.[192] – 174 °C, **IR** (neat) 3326 (w), 3187 (m), 1705 (s), 1686 (m), 1660 (s), 1480 (m), 1232 (s), 1096 (m) cm^{-1}, **^{1}H NMR** (300 MHz, CD_3OD) δ_H 7.84 (1H, d, J = 1.3 Hz, H-6), 6.28 (1H, dd, J = 8.2, 6.2 Hz, H-1'), 5.31 (1H, dt, J = 2.9, 2.2 Hz, H-4'), 4.10 (1H, dt, J = 2.9, 2.2 Hz, H-3'), 3.80 (2H, d, J = 2.9 Hz, H-5'), 2.33 (2H, m, H-2'), 2.09 (3H, s, $COCH_3$), 1.88 (3H, d, J = 1.3 Hz, CH_3), **^{13}C NMR** (75 MHz, CD_3OD) δ_C 172.2 (C=OCH_3), 166.3 (C-4), 152.4 (C-2), 137.9 (C-6), 111.9 (C-5), 86.7 (C-4'), 86.2 (C-1'), 75.4 (C-3'), 63.0 (C-5'), 38.4 (C-2'), 20.9 (CH_3O), 12.4 (CH_3), **HPLC** (λ_{282}) t_R 2.13 min (Method 1), $\mathbf{R_f}$ 0.26 (10 % MeOH/CH_2Cl_2, ammonium molybdate).

2-[3'-O-Acetylthymidine]-4-oxo-benzo[1,3,2]dioxaphosphinine 7-oxymethyl resin, 75-150 μm (50)



Resin **41a** (301 mg, 0.361 mmol, theoretical loading 1.34 $mmolg^{-1}$) was swollen in dry CH_2Cl_2 and dry THF (2 x 2 mL for 5 min each) at 60 °C. PCl_3 (95 μL, 4 eq) was added to the resin in dry THF (4 mL) and the syringe was heated at 60 °C for 2 h. The resin was drained and rinsed with dry CH_2Cl_2 and dry THF (2 x 2 mL for 5 min each). 3'OAc-T **49** (235 mg, 0.827 mmol, 2 eq) was evaporated *in vacuo* from dry THF (2 mL), dried over P_2O_5 in a desiccator for 1 h *in vacuo* and dissolved in dry THF (3 mL). The N_2 flushed resin was treated with the nucleoside solution for 1 h at rt, drained and rinsed with dry THF (2 x 2 mL for 5 min), then dried over P_2O_5 in a desicator. **Gel Phase ^{31}P NMR** (162 MHz, $CDCl_3$) δ_P 127.7 (82 %), 6.7 (hydrolysed P-Cl reagent), **ICP analysis:** P (%) 2.67, **Loading obtained:** 0.861 $mmolg^{-1}$, 91 % yield.

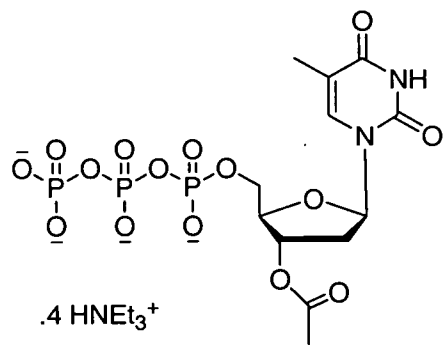
3'-*O*-Acetyl-thymidine 5'-*H*-phosphonate ammonium salt (51)



Solution phase procedure: 3'OAc-T **49** (50 mg, 180 μmol) was dissolved in dry pyridine (2 mL), evaporated *in vacuo* and stored under vacuum over P_2O_5 for 1 h. The residue, dissolved in dry pyridine (0.3 mL) and diluted with dry THF (0.9 mL), was treated with freshly prepared 1.0 M 2-chloro-benzo[1,3,2]dioxaphosphinin-4-one **11** solution in dry THF (355 μL, 0.355 mmol), stirred for 10 min, treated with H_2O (1 mL) and evaporated *in vacuo*. The residue was separated between CH_2Cl_2 (3 mL) and 0.1 M NH_4OAc (3 mL). The aqueous phase was purified by Prep HPLC eluted with a 0.1 M NH_4OAc/MeCN gradient to yield a white hygroscopic solid (15 mg, 41 μmol, 23 % yield). **Procedure using supported reagent:** Resin **41a** (207 mg, 0.277 mmol, 1 eq) was swollen in dry CH_2Cl_2 and dry THF (2 x 2 mL for 5 min each) at 60 °C. PCl_3 (95 μL, 4 eq) was added to the resin in dry THF (4 mL) and the syringe was heated at 60 °C for 2 h. 3'OAc-T **49** (27 mg, 95 μmol, 0.33 eq) was evaporated *in vacuo* from dry pyridine (1 mL), dried over P_2O_5 in a desiccator for 1 h *in vacuo* and dissolved in dry pyridine (0.25 mL) and dry THF

(0.75 mL). The resin was drained and rinsed with dry CH_2Cl_2 and dry THF (2 x 2 mL for 5 min each). The N_2 flushed resin was treated with the nucleoside solution for 1 h at rt, drained, rinsed with dry THF (2 x 2 mL for 5 min) and treated with THF/H_2O (1/1, 2 mL) for 10 min. The cleaved product was collected in an RB, evaporated *in vacuo* and separated between CH_2Cl_2 (3 mL) and 0.1 M NH_4OAc (3 mL). The aqueous layer was washed with CH_2Cl_2 (2 x 3 mL) and purified by Prep HPLC eluted with 0.1 M NH_4OAc/MeCN gradient to yield a white hygroscopic foam (20 mg, 55 μmol, 58 % yield). **mp** 72-74 °C, **^{1}H NMR** (250 MHz, D_2O) δ_H 7.72 (1H, d, J = 1.2 Hz, H-6), 6.35 (1H, dd, J = 8.3, 6.4 Hz, H-1'), 6.76 (1H, d, J = 637.7 Hz, PH), 5.35 (1H, dt, J = 4.8, 2.3 Hz, H-4'), 4.32 – 4.36 (1H, m, H-3'), 4.08 (2H, dd, J = 6.1, 2.9 Hz, H-5'), 2.34 – 2.52 (2H, m, H-2'), 2.11 (3H, s, $COCH_3$), 1.70 (3H, d, J = 1.2 Hz, CH_3), **^{13}C NMR** (62.5 MHz, D_2O) δ_C 173.9 (C=OCH_3), 166.9 (C-4), 152.1 (C-2), 137.4 (C-6), 112.2 (C-5), 85.4 (C-1'), 83.7 (d, J = 8.4 Hz, C-4'), 75.7 (C-3'), 63.8 (d, J_{PC} = 3.9 Hz, C-5'), 36.7 (C-2'), 20.8 (CH_3O), 12.0 (CH_3), **^{31}P NMR** (101 MHz, D_2O) δ_P 7.60, **ES$^-$** m/z (%) 347.2 ($M-H^-$, 100), **HRMS** (FAB) for $C_{12}H_{18}N_2O_8P_1$ $(M+H)^+$: calcd 349.08008, found 349.08073, **HPLC** (λ_{282}) t_R 8.1 min (Method 7).

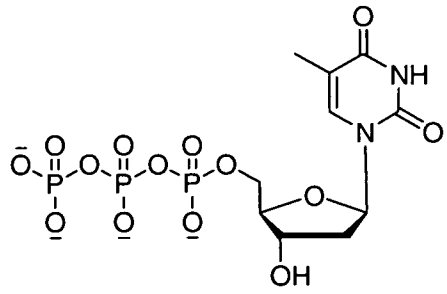
3'-*O*-Acetyl-thymidine 5'-triphosphate tetraethylammonium salt (54)



Solution phase procedure: 3'OAc-T **49** (25 mg, 88 μmol) was dissolved in dry pyridine (1 mL), evaporated *in vacuo* and stored under vacuum over P_2O_5 for 1 h. The residue, dissolved in dry pyridine (0.3 mL) and diluted with dry THF (0.9 mL), was treated with freshly prepared 1.0 M 2-chloro-benzo[1,3,2]dioxaphosphinin-4-one **11** solution in dry THF (132 μL, 0.132 mmol, 1.5 eq), stirred for 10 min, treated with 0.5 M bis (tributyl ammonium) pyrophosphate in DMF (265 μL, 1.5 eq) and Bu_3N (85 μL, 4 eq) at rt for 10 min, then 0.5 M I_2/pyridine:H_2O 98:2 (265 μL,1.5 eq) was added. After 15 min, excess iodine was destroyed by adding a few

drops of a 5 % aqueous solution of $NaHSO_3$ and the reaction solution was evaporated *in vacuo*. The residue was dissolved in H_2O (10 mL), stirred for 30 min, washed with CH_2Cl_2 (3 x 5 mL) and purified by Prep HPLC eluted with 0.5 % MeCN in 0.1 M TEAB yield a white solid (20 mg, 21.5 μmol, 24 % yield). **Procedure using supported reagent:** Resin **41a** (205 mg, 0.275 mmol, 1 eq) was swollen in dry THF (2 x 2 mL for 5 min) at 60 °C. PCl_3 (95 μL, 4 eq) was added to the resin in dry THF (4 mL) and the syringe was heated at 60 °C for 2 h. 3'OAc-T **49** (26 mg, 91 μmol, 0.33 eq) was evaporated *in vacuo* from dry pyridine (1 mL), dried over P_2O_5 in a desiccator for 1 h *in vacuo* and dissolved in dry pyridine (0.25 mL) and dry THF (0.75 mL). The resin was drained and rinsed with dry THF (2 x 2 mL for 5 min). The N_2 flushed resin was treated with the nucleoside solution for 1 h at rt, drained and rinsed with dry THF (2 x 2 mL for 5 min). The N_2 flushed resin was treated three times with 0.5 M bis (tributyl ammonium) pyrophosphate in DMF (800 μL, 1.5 eq) and Bu_3N (255 μL, 4 eq) at rt for 10 min each. The cleaved product was collected in an RB with 0.5 M I_2/pyridine:H_2O 98:2 (800 μL, 1.5 eq). 15 min after the last cleavage, excess iodine was destroyed by adding a few drops of a 5 % aqueous solution of $NaHSO_3$ and the reaction solution was evaporated *in vacuo*. The residue was dissolved in 0.1 M TEAB (3 mL), stirred for 30 min, washed with CH_2Cl_2 (3 x 3 mL) and purified by Prep HPLC eluted with 0.5 % MeCN in 0.1 M TEAB yield a white solid (12 mg, 13 μmol, 13 % yield). **^{1}H NMR** (250 MHz, D_2O) δ_H 7.78 (1H, d, J = 1.1 Hz, H-6), 6.34 (1H, t, J = 7.3 Hz, H-1'), 5.40 – 5.44 (1H, m, H-4'), 4.37 (1H, dd, J = 3.4, 1.7 Hz, H-3'), 4.21 (2H, dd, J = 7.8, 4.6 Hz, H-5'), 2.42 (2H, dd, J = 7.3, 4.0 Hz, H-2'), 2.10 (3H, s, $COCH_3$), 1.90 (3H, d, J = 1.1 Hz, CH_3), **^{13}C NMR** (91 MHz, D_2O) δ_C 174.4 (C=OCH_3), 167.6 (C-4), 152.8 (C-2), 138.2 (C-6), 113.0 (C-5), 85.9 (C-1'), 84.1 (d, J_{PC} = 9.2 Hz, C-4'), 76.8 (C-3'), 66.9 (d, J_{PC} = 5.3 Hz, C-5'), 37.4 (C-2'), 21.5 (CH_3O), 12.6 (CH_3), **^{31}P NMR** (101 MHz, D_2O/2 M TEAB) δ_P -5.40 (d, J = 21.1 Hz, P-γ), -10.52 (d, J = 19.4 Hz, P-α), -21.52 (t, J = 19.8 Hz, P-β), **ES$^-$** m/z (%) 523.2 ($M\text{-}H^-$, 100), **HPLC** (λ_{282}) t_R 17.5 min (Method 6).

Thymidine 5'-triphosphate tetraethylammonium salt (55)

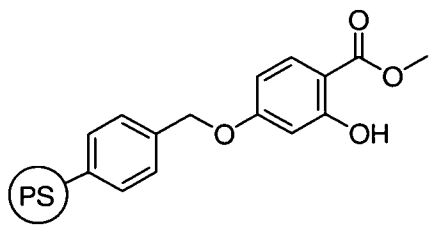


.4 $HNEt_3^+$

Solution phase procedure: 3'OAc-T **49** (26 mg, 92 μmol) was dissolved in dry pyridine (1 mL), evaporated *in vacuo* and stored under vacuum over P_2O_5 for 1 h. The residue, dissolved in dry pyridine (0.3 mL) and diluted with dry THF (0.9 mL), was treated with freshly prepared 1.0 M 2-chloro-benzo[1,3,2]dioxaphosphinin-4-one **11** solution in dry THF (135 μL, 0.135 mmol, 1.5 eq), stirred for 10 min, treated with 0.5 M bis (tributyl ammonium) pyrophosphate in DMF (265 μL, 1.5 eq) and Bu_3N (85 μL, 4 eq) at rt for 10 min, then 0.5 M I_2/pyridine:H_2O 98:2 (265 μL,1.5 eq) was added. After 15 min, excess iodine was destroyed by adding a few drops of a 5 % aqueous solution of $NaHSO_3$ and the reaction solution was evaporated *in vacuo*. The residue was dissolved in H_2O (10 mL), stirred for 30 min, then concentrated ammonia (20 mL) was added. After 1 h the solution was evaporated *in vacuo*, the residue dissolved in 0.1 M TEAB (3 mL), extracted with CH_2Cl_2 (3 x 3 mL) and purified by Prep HPLC eluted with 0.5 % MeCN in 0.1 M TEAB yield a white solid (29 mg, 32.7 μmol, 36 % yield). **Procedure using supported reagent:** Resin **41a** (202 mg, 0.271 mmol, 1 eq) was swollen in dry THF (2 x 2 mL for 5 min) at 60 °C. PCl_3 (95 μL, 4 eq) was added to the resin in dry THF (4 mL) and the syringe was heated at 60 °C for 2 h. 3'OAc-T **49** (25 mg, 88 μmol, 0.33 eq) was evaporated *in vacuo* from dry pyridine (1 mL), dried over P_2O_5 in a desiccator for 1 h *in vacuo* and dissolved in dry pyridine (0.25 mL) and dry THF (0.75 mL). The resin was drained and rinsed with dry THF (2 x 2 mL for 5 min). The N_2 flushed resin was treated with the nucleoside solution for 1 h at rt, drained and rinsed with dry THF (2 x 2 mL for 5 min). The N_2 flushed resin was treated three times with 0.5 M bis (tributyl ammonium) pyrophosphate in DMF (800 μL, 1.5 eq) and Bu_3N (255 μL, 4 eq) at rt for 10 min each. The cleaved product was collected in an RB with 0.5 M I_2/pyridine:H_2O 98:2 (800 μL, 1.5 eq). 15 min after the last cleavage, excess iodine was destroyed by adding a few drops

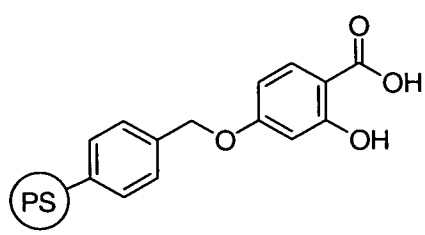
of a 5 % aqueous solution of $NaHSO_3$. The reaction mixture was diluted with H_2O (20 mL), stirred for 30 min and evaporated *in vacuo*. The residue was dissolved in concentrated ammonia (20 mL). After 1 h the solution was evaporated *in vacuo*, the residue dissolved in 0.1 M TEAB (5 mL), washed with CH_2Cl_2 (3 x 5 mL), lyophilised and purified by Prep HPLC eluted with 0.1 M TEAB. The fractions containing triphosphate were pooled and lyophilised to remove the buffer to yield a hygroscopic solid (42 mg, 47 μmol, 50 % yield). **^{1}H NMR** (250 MHz, D_2O) δ_H 7.68 (1H, d, J = 1.1 Hz, H-6), 6.31 (1H, t, J = 6.9 Hz, H-1'), 4.65 (1H, dd, J = 8.4, 5.0 Hz, H-4'), 4.09 – 4.25 (3H, m, H-3' + H-5'), 2.27 - 2.34 (2H, m, H-2'), 1.86 (3H, d, J = 1.1 Hz, CH_3), **^{13}C NMR** (62.5 MHz, D_2O), δ_C 167.0 (C-4), 152.1 (C-2), 137.8 (C-6), 112.1 (C-5), 85.8 (d, J = 9.4 Hz, C-4'), 85.2 (C-1'), 71.1 (C-3'), 65.8 (d, J_{PC} = 5.6 Hz, C-5'), 38.9 (C-2'), 12.0 (CH_3), **^{31}P NMR** (101 MHz, D_2O/2 M TEAB) δ_P -5.23 (d, J = 21.1 Hz, P-γ), -10.32 (d, J = 20.1 Hz, P-α), -21.45 (t, J = 20.8 Hz, P-β), **ES$^-$** m/z (%) 480.1 ($M-H^-$, 80), **HPLC** (λ_{282}) t_R 9.4 min (Method 5).

Methyl 2-hydroxy-4-benzyloxybenzoate macroporous polystyrene resin, 150-350 μm (56)



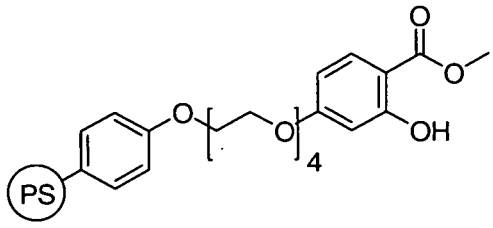
Agropore-Cl resin (5.01 g, available loading 2.0 $mmolg^{-1}$, 10.02 mmol) suspended in acetone (100 mL) with KI (1.63g, 9.82 mmol, 1.0 eq), K_2CO_3 (4.16 g, 30.10 mmol, 3.0 eq) and **35** (5.04 g, 29.8 mmol, 3.0 eq) was refluxed at 70 °C for 90 h. The resin was drained, rinsed with DMF/H_2O, MeOH/H_2O, DMF, MeOH, DMF, CH_2Cl_2 and Et_2O (3 x 50 mL each). **Anal.** Found: Cl 1.99, I 1.15, **Loading obtained:** 1.20 $mmolg^{-1}$.

2-Hydroxy-4-benzyloxybenzoic acid polystyrene resin (57)



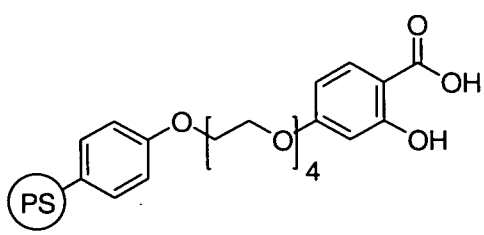
Resin **56** (4.00 g, 1.20 mmolg^{-1}, 4.80 mmol) suspended in THF (20 mL) was treated with tetrabutylammonium hydroxide (20 mL, 40 % w/v in H_2O) and heated at 75 °C for 24 h. The resin was drained, rinsed with THF, 2 M HCl/THF (1/1), THF, DMF/H_2O, MeOH/H_2O, DMF, MeOH, DMF, CH_2Cl_2 and Et_2O (3 x 50 mL each) and vacuum dried. **IR** (neat) 2925 (w), 1738 (s), 1669 (w), 1603 (w), 1366 (m), 1217 (s) cm^{-1}, **Theorectical loading obtained:** 1.22 mmolg^{-1}.

Methyl 2-hydroxy-benzoate 4-tetraethyleneglycol-oxymethyl microporous polystyrene resin, 150-300 μm (58a)



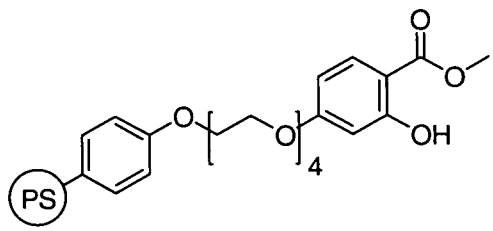
Quadragel resin (11.1 g, 0.894 mmolg^{-1}, 9.92 mmol), swollen twice in dry THF (2 x 150 mL), was treated with PPh_3 (11.3 g, 43.1 mmol, 4.3 eq) and **35** (6.97 g, 41.5 mmol, 4.2 eq). DIAD (7.8 mL, 39.7 mmol, 4.0 eq) was added in 3 aliquots over 5 min and was shaken for 12 h. A quantitative DMT test showed the coupling to be 98 % complete. The resin was drained, rinsed with THF, DMF, MeOH, DMF, CH_2Cl_2 and Et_2O (3 x 50 mL each) and vacuum dried. **IR** (neat) 3025 (w), 2970 (w), 1739 (s), 1667 (m), 1217 (s) cm^{-1}, **Gel-Phase ^{13}C NMR** (91 MHz, $(CD_3)_2CO$) δ_C 172.0 (C=O), 165.6 (C-O), 165.5 (C-O), 148.1 – 147.2 (*C*-O-CH_2), 133.0 (*o*-CH), 127.3 – 129.8 (resin), 109.5 (*m*-CH), 106.9 (C), 103.0 (*m*-CH), 72.0 (CH_2), 70.8 (CH_2), 69.5 (CH_2), remainder of CH_2 signals obscured, 53.4 (CH_3), 42.0 - 40.0 (resin), **Loading obtained:** 0.740 mmolg^{-1}.

2-Hydroxy-benzoic acid 4-tetraethyleneglycol-oxymethyl microporous polystyrene resin, 150-300 μm (59a)



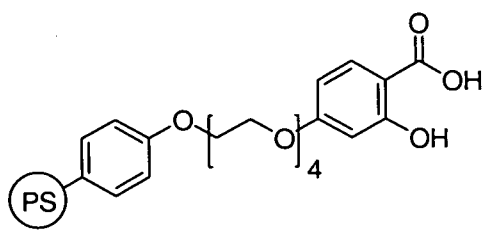
Resin **58a** (9.45 g, 0.740 mmolg^{-1}, 6.99 mmol), swollen THF (150 mL) was treated with tetrabutylammonium hydroxide (30 mL, 40 % w/v in H_2O) and was heated at 65 °C for 2 d. The resin was drained, rinsed with THF, 2 M HCl/THF (1/1), THF, DMF/H_2O, MeOH/H_2O, DMF, MeOH, DMF, CH_2Cl_2 and Et_2O (3 x 100 mL each) and vacuum dried. **IR** (neat) 3025 (w), 2918 (w), 1738 (s), 1666 (m), 1238 (s) cm^{-1}, **Gel-Phase ^{13}C NMR** (91 MHz, $(CD_3)_2CO$) δ_C 173.9 (C=O), 166.9 (C-O), 166.0 (C-O), 147.3 (*C*-O-CH_2), 133.7 (*o*-CH), 127.6 – 130.7 (resin), 109.3 (*m*-CH), 107.0 (C), 103.1 (*m*-CH), 72.3 (CH_2), 71.5 (CH_2), 71.3 (CH_2), 71.0 (CH_2), 69.7 (CH_2), 69.4 (CH_2), 69.3 (CH_2), 69.1 (CH_2), 42.2 – 42.3 (resin), **Theoretical loading obtained:** 0.748 mmolg^{-1}.

Methyl 2-hydroxy-benzoate 4-tetraethyleneglycol-oxymethyl macroporous polystyrene resin, 150-300 μm (58b)



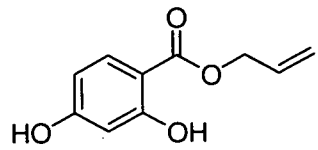
Quadrapore resin (2.80 g, 0.756 mmolg^{-1}, 2.12 mmol), swollen twice in dry THF (2 x 40 mL), was treated with PPh_3 (2.24 g, 8.53 mmol, 4.0 eq) and **35** (1.46 g, 41.5 mmol, 4.1 eq). DIAD (1.67 mL, 8.48 mmol, 4.0 eq) was added in 3 aliquots over 5 min and was shaken for 12 h. A quantitative DMT test showed the coupling to be 95 % complete. The resin was drained, rinsed with THF, DMF, MeOH, DMF, CH_2Cl_2 and Et_2O (3 x 50 mL each) and vacuum dried. **IR** (neat) 3024 (w), 2921 (w), 1738 (s), 1669 (m), 1228 (s) cm^{-1}, **Loading obtained:** 0.644 mmolg^{-1}.

2-Hydroxy-benzoic acid 4-tetraethyleneglycol-oxymethyl macroporous polystyrene resin, 150-300 μm (59b)



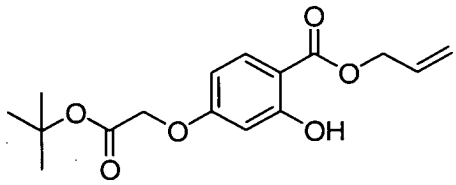
Resin **58b** (1.50 g, 0.64 mmolg^{-1}, 0.97 mmol), swollen THF (40 mL) was treated with tetrabutylammonium hydroxide (8 mL, 40 % w/v in H_2O) and heated at 65 °C for 2 d. The resin was drained, rinsed with THF, 2 M HCl/THF (1/1), THF, DMF/H_2O, MeOH/H_2O, DMF, MeOH, DMF, CH_2Cl_2 and Et_2O (3 x 20 mL each) and vacuum dried. **IR** (neat) 3024 (w), 2920 (w), 1737 (m), 1669 (m), 1240 (s) cm^{-1}, **Theoretical loading obtained:** 0.650 mmolg^{-1}.

Allyl 2,4-dihydroxy benzoate (60)



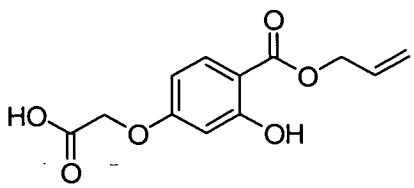
2,4-Dihydroxy benzoic acid (21.1 g, 137 mmol) was dissolved in DMF (200 mL) at 70 °C. Proton sponge (27.9 g, 130 mmol) dissolved in DMF (100 mL) was added, followed by allylbromide (1.5 eq). The mixture was heated at 70 °C for 1 h, cooled to rt and diluted with EtOAc (300 mL) and sat. $NaHCO_3$ solution (500 mL). The aqueous phase was extracted with EtOAc (2 x 100 mL) and the combined organic phases were washed with 2 M HCl (2 x 100 mL) and brine (100 mL), dried ($MgSO_4$) and evaporated *in vacuo* to give a purple oil, which solidified upon standing. The residue was triturated with hexane to yield an off-white powder (19.8 g, 102 mmol, 78 % yield). **mp** 41 - 42 °C, **IR** (neat) 3335 (w), 1666 (s), 1623 (s), 1597 (s), 1136 (s) cm^{-1}, **^{1}H NMR** (250 MHz, $CDCl_3$) δ_H 10.97 (1H, s, *o*-OH), 7.78 (1H, dd, J = 8.4, 0.7 Hz, *o*-CH), 6.36 – 6.41 (2H, m, 2 *m*-CH), 6.02 (1H, ddt, J = 17.2, 10.4, 5.6 Hz, C*H*=CH_2), 5.61 (1H, s, *p*-OH), 5.41 (1H, dq, J = 17.2, 1.4 Hz, CH=CH_2 trans), 5.31 (1H, dq, J = 10.4, 1.4 Hz, CH=CH_2 cis), 4.82 (2H, dt, J = 5.7, 1.4 Hz, CH_2-CH=CH_2), **^{13}C NMR** (62.5 MHz, $CDCl_3$) δ_C 169.7 (C=O), 163.4 (C-O), 162.2 (C-O), 132.0 (CH), 131.6 (CH), 118.7 (CH_2), 108.0 (CH), 105.7 (C), 103.1 (CH), 65.6 (CH_2), **ES$^-$** m/z (%) 193.1 (M-H$^-$, 100), **HRMS** (FAB) for $C_{10}H_{11}O_4$ $(M+H)^+$: calcd 195.0652, found 195.0660, **HPLC** (λ_{254}) t_R 7.41 min (Method 3), **R_f** 0.68 (Et_2O, $KMnO_4$).

Allyl 4-(2-*tert*-butoxy-2-oxoethoxy)-2-hydroxybenzoate (61)



Phenol **60** (1.02 g, 5.23 mmol) dissolved in DMF (20 mL) was treated with NaH dispersion (60 % in mineral oil, 204 mg, 5.10 mmol) for 5 min, then ^{t}butyl bromoacetate (770 μL, 5.21 mmol) was added and stirred at rt for 1 h. The reaction was diluted with 10 % w/v LiCl solution (50 mL), extracted with EtOAc (2 x 50 mL), dried ($MgSO_4$) and evaporated *in vacuo* to yield a yellow oil which was purified by column chromatography eluted with CH_2Cl_2 to yield a clear oil (1.03 g, 3.33 mmol, 64 % yield). **IR** (neat) 2979 (w), 1752 (s), 1732 (w), 1666 (s), 1621 (s), 1135 (s) cm^{-1}, **^{1}H NMR** (400 MHz, $CDCl_3$) δ_H 10.92 (1H, s, *o*-OH), 7.80 (1H, d, J = 8.9 Hz, *o*-CH), 6.47 (1H, dd, J = 8.8, 2.5 Hz, *m*-CH), 6.40 (1H, d, J = 2.5 Hz, *m*-CH), 5.97 - 6.07 (1H, m, C*H*=CH_2), 5.41 (1H, ddt, J = 17.2, 1.5, 1.5 Hz, CH=C*H*$_2$ trans), 5.31 (1H, ddt, J = 10.4, 1.5, 1.5 Hz, CH=C*H*$_2$ cis), 4.82 (2H, dt, J = 4.5, 1.4 Hz, C*H*$_2$-CH=CH_2), 4.53 (2H, s, CH_2O), 1.49 (9H, s, $3CH_3$), **^{13}C NMR** (100 MHz, $CDCl_3$) δ_C 169.5 (C=O), 167.1 (C=O), 163.8 (C-O), 163.8 (C-O), 132.0 (CH), 131.8 (CH), 131.4 (CH), 118.7 (CH_2), 107.7 (CH), 106.2 (C), 101.6 (CH), 82.8 (C), 65.5 (CH_2), 65.5 (CH_2), 28.0 (CH_3), **APCI$^-$** m/z (%) 307.2 (M-H$^-$, 100), **HPLC** (λ_{254}) t_R 8.33 min (Method 3), **R_f** 0.74 (EtOAc, $KMnO_4$).

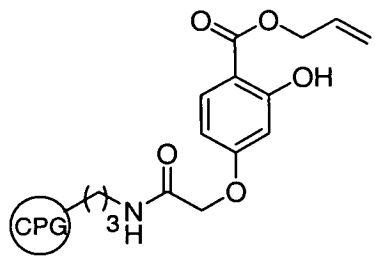
Allyl 4-carboxymethoxy-2-hydroxy-benzoate (62)



Ester **61** (650 mg, 2.11 mmol) dissolved in CH_2Cl_2 (4 mL) was treated with Et_3SiH (800 μL, 5.01 mmol, 2.4 eq) and TFA (2 mL, 26.0 mmol, 12.3 eq) and stirred for 2.75 h. The reaction mixture was evaporated *in vacuo* to to yield an off-white powder which was purified by column chromatography eluted with hexane:EtOAc:AcOH (2:1:0.02, 1:1:0.02) to yield a white solid (360 mg, 1.43 mmol, 68 % yield). **^{1}H NMR** (400 MHz, CD_3OD) δ_H 7.81 (1H, d, J = 8.9 Hz, *o*-

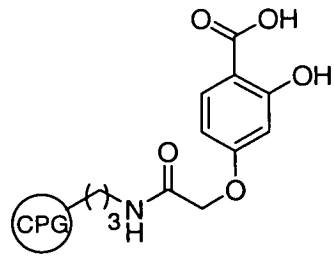
CH), 6.53 (1H, dd, J = 8.9, 2.5 Hz, *m*-CH), 6.45 (1H, d, J = 2.5 Hz, *m*-CH), 6.02 - 6.11 (1H, m, C*H*=CH_2), 5.41 (1H, d, J = 17.2 Hz, CH=CH_2 trans), 5.29 (1H, d, J = 10.5, Hz, CH=CH_2 cis), 4.83 (2H, d, J = 5.5 Hz, CH_2-CH=CH_2), 4.81 (2H, s, CH_2O), **^{13}C NMR** (100 MHz, $CDCl_3$) δ_C 171.9 (C=O), 170.8 (C=O), 165.5 (C-O), 164.9 (C-O), 133.4 (CH), 132.6 (CH), 118.9 (CH_2), 108.6 (CH), 107.3 (C), 102.8 (CH), 66.6 (CH_2), 65.9 (CH_2), **ES$^-$** m/z (%) 251.1 (M-H$^-$, 100), **HRMS** (FAB) for $C_{12}H_{13}O_6$ $(M+H)^+$: calcd 253.07121, found 253.07119, **R_f** 0.51 (98:2 EtOAc:AcOH, bromophenol blue).

Allyl 2-hydroxy-4-(propylcarbamoylmethoxy)-benzoate CPG (63)



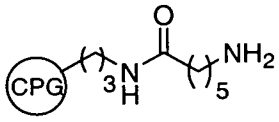
Trisoperl CPG (340 mg, 0.225 mmolg^{-1}, 68 μmol) in CH_2Cl_2/DMF (4/1, 5 mL) was treated with linker **62** (169 mg, 670 μmol), DIC (93 μL, 633 μmol) and HOBt (106 mg, 693 μmol) and was heated at 60 °C for 3.5 h. The resin was drained, rinsed with CH_2Cl_2, DMF, MeOH and CH_2Cl_2 (3 x 10 mL each) and vacuum dried. **Ninhydrin test:** negative, **Theoretical loading:** 0.214 mmolg^{-1}.

2-Hydroxy-4-(propylcarbamoylmethoxy)-benzoic acid CPG (64)



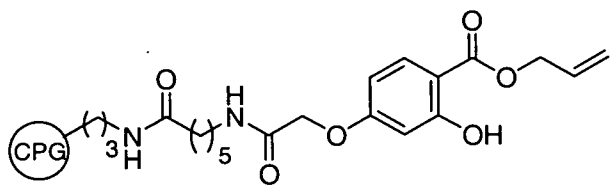
Resin **63** (300 mg, 0.214 mmolg^{-1}, 64 μmol) in THF (5 mL) was treated with morpholine (20 μL, 0.229 mmol, 3.6 eq) and $[Pd(PPh_3)_4]$ (2.7 mg, 2.3 μmol, 3.7 mol %) for 1 h at rt. The resin was drained, rinsed with CH_2Cl_2, DMF, MeOH and CH_2Cl_2, then suspended in CH_2Cl_2, treated with CH_2Cl_2/AcOH 4/1, then rinsed with CH_2Cl_2, DMF, MeOH and CH_2Cl_2 (3 x 10 mL each) and vacuum dried. **Theoretical loading:** 0.227 mmolg^{-1}.

5-Amino-hexanoic acid propylamide CPG (65)



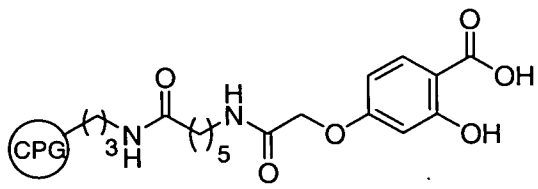
Trisoperl CPG (515 mg, 0.225 mmolg^{-1}, 0.116 mmol) in CH_2Cl_2/DMF (4/1, 10 mL) was treated with Fmoc-Ahx-OH (408 mg, 1.15 mmol), DIC (162 μL, 1.16 mmol) and HOBt (202 mg, 1.16 mmol) and was heated at 60 °C. After 7 h, a further 10 eq of DIC were added. The resin was drained after 24 h, rinsed with DMF, MeOH, CH_2Cl_2 and Et_2O (3 x 10 mL each). The resin was capped by shaking with Ac_2O/pyridine (2 mL, 1/1) in CH_2Cl_2 (3 mL) for 2 h. The resin was drained, rinsed with DMF/H_2O, MeOH/H_2O, DMF, MeOH, CH_2Cl_2 and Et_2O (3 x 10 mL each) and vacuum dried. The resin was deprotected with 20 % piperidine in DMF (3 x 10 min), drained and rinsed with DMF, MeOH, CH_2Cl_2 and Et_2O (3 x 10 mL each) and vacuum dried. **Loading obtained:** 0.162 mmolg^{-1} (72 % yield) (Quantitative Fmoc test).

Allyl 2-hydroxy-4-[(5-propylcarbamoyl-pentylcarbamoyl)-methoxy]-benzoate CPG (66)



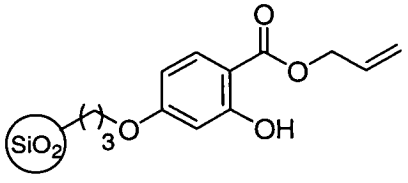
Resin **65** (510 mg, 0.162 mmolg^{-1}, 83 μmol) in CH_2Cl_2/DMF (4/1, 5 mL) was treated with linker **62** (206 mg, 0.817 mmol), DIC (116 μL, 0.833 mmol) and HOBt (137 mg, 0.895 mmol) and was heated at 60 °C for 4 h. The resin was drained, rinsed with CH_2Cl_2, DMF, MeOH and CH_2Cl_2 (3 x 10 mL each) and vacuum dried. **Ninhydrin test:** negative, **Theoretical loading:** 0.156 mmolg^{-1}.

2-Hydroxy-4-[(5-propylcarbamoyl-pentylcarbamoyl)-methoxy]-benzoic acid CPG (67)



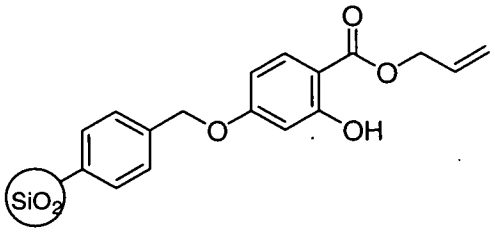
Resin **66** (532 mg, 0.156 mmolg^{-1}, 83 μmol) was treated with [$Pd(PPh_3)_4$] (2.9 mg, 2.5 μmol, 3.0 mol %), morpholine (22 μL, 252 μmol, 3.0 eq) in THF (5 mL) and shaken at rt for 90 min. The resin was drained, rinsed with CH_2Cl_2, DMF, MeOH and CH_2Cl_2, then suspended in CH_2Cl_2, treated with CH_2Cl_2/AcOH 4/1, then rinsed with CH_2Cl_2, DMF, MeOH and CH_2Cl_2 (3 x 10 mL each) and vacuum dried. **Theoretical loading:** 0.157 mmolg^{-1}.

Allyl 2-hydroxy-4-(propyloxy)-benzoate silica (68)



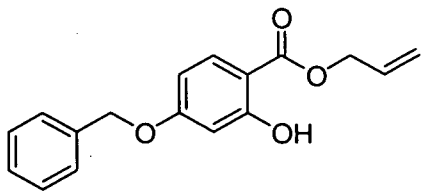
Bromopropylsilica (3.27 g, 1.43 mmolg^{-1}, 4.68 mmol) was suspended with linker **60** (4.54 g, 23.4 mmol, 5 eq) and DIPEA (4 mL, 23.4 mmol, 5 eq) in THF (25 mL) and was heated at 40 °C for 34 h. The resin was drained, rinsed with THF, CH_2Cl_2, MeOH, DMF, CH_2Cl_2 and Et_2O and vacuum dried. **Anal.** Found: Br 7.03, **Loading obtained:** 0.350 mmolg^{-1}.

Allyl 2-hydroxy-4-(benzyloxy)-benzoate silica (69)



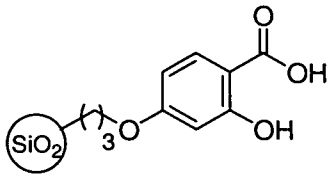
Allyl 2,4-dihydroxybenzoate **60** (4.52 g, 23.3 mmol, 5 eq) in THF (25 mL) was treated with NaH (60 % in mineral oil, 946 mg, 23.7 mmol, 5.2 eq) for 5 min to give a green solution. Chlorobenzylsilica (3.37 g, 1.34 mmolg^{-1}, 4.52 mmol) was added and heated at 50 °C for 48 h. The resin was quenched with MeOH, drained, rinsed with DMF/H_2O, MeOH/H_2O, DMF, MeOH, CH_2Cl_2, and Et_2O and vacuum dried. **Anal.** Found: Cl 1.98, **Loading obtained:** 0.550 mmolg^{-1}.

Allyl 4-benzyloxy-2-hydroxy benzoate (70)



Phenol **60** (1.04 g, 5.33 mmol) in acetone (20 mL), treated with K_2CO_3 (3.28 g, 23.8 mmol) and BnBr (650 μL, 5.47 mmol, 1.03 eq), was refluxed at 75 °C for 3 h until complete (HPLC). The reaction mixture was evaporated *in vacuo*, quenched with 2 M HCl (75 mL) and extracted into Et_2O (75 mL). The organic layer was washed with sat. $NaHCO_3$ solution (50 mL) and brine (50 mL), dried ($MgSO_4$) and evaporated *in vacuo*. The oily residue was sonicated in MeOH to yield a white suspension which was filtered off and dried to yield a fine white powder (670 mg). The filtrate was evaporated and purified by colun chromatography eluted with a 0 – 10 % Et_2O:hexane to yield white crystals (3ℰ᜵ mg). (Combined yield: 1.05 g, 3.69 mmol, 69 % yield). **mp** 60-61 °C, **IR** (neat) 3069 (w), 1652 (s), 1613 (s), 1369 (s), 1247 (s) cm^{-1}, **^{1}H NMR** (250 MHz, $CDCl_3$) δ_H 10.97 (1H, s, *o*-OH), 7.80 (1H, dt, J = 8.9, 1.0 Hz, *o*-CH), 7.22 – 7.37 (5H, m, Ar), 6.42 – 6.46 (2H, m, 2 *m*-CH), 6.03 (1H, ddt, J = 17.2, 10.4, 5.6 Hz, C*H*=CH_2), 5.42 (1H, ddt, J = 17.2, 1.5, 1.5 Hz, CH=CH_2 trans), 5.31 (1H, ddt, J = 10.4, 1.5, 1.5 Hz, CH=CH_2 cis), 5.08 (2H, s, CH_2O), 4.82 (2H, dt, J = 5.6, 1.4 Hz, CH_2-CH=CH_2), **^{13}C NMR** (62.5 MHz, $CDCl_3$) δ_C 169.6 (C=O), 164.7 (C-O), 163.8 (C-O), 136.0 (C), 131.8 (CH), 131.3 (CH), 128.6 (CH), 128.2 (CH), 127.5 (CH), 118.6 (CH_2), 108.1 (CH), 105.6 (C), 101.6 (CH), 70.1 (CH_2), 65.5 (CH_2), **APCI$^-$** m/z (%) 283.2 (M-H$^-$, 100), **HRMS** (FAB) for $C_{17}H_{17}O_4$ $(M+H)^+$: calcd 285.1127, found 285.1138, **HPLC** (λ_{254}) t_R 4.57 min (Method 1), 8.39 min (Method 3), **R_f** 0.28 (5:95 Et_2O:hexane, PMA).

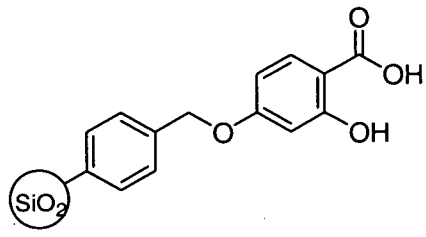
2-Hydroxy-4-(propyloxy)-benzoic acid silica (71)



Silica **68** (*ca.* 3.0 g, 0.350 $mmolg^{-1}$, *ca.* 1.0 mmol) in THF (10 mL) was treated with phenylsilane (620 μL, 5.02 mmol, 5 eq) and [$Pd(PPh_3)_4$] (58 mg, 50 μmol, 5

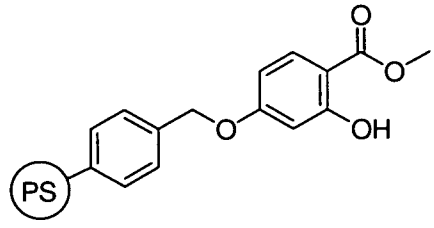
mol %) in THF (1 mL) for 1 h at rt. The resin was drained, rinsed with THF, THF/H_2O, CH_2Cl_2 and vacuum dried. **Theoretical loading:** 0.355 $mmolg^{-1}$.

2-Hydroxy-4-(benzyloxy)-benzoic acid silica (72)



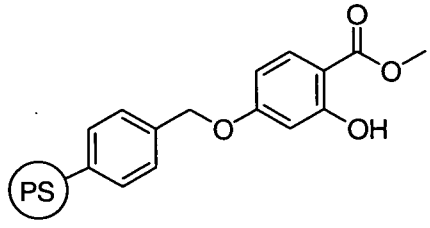
Silica **69** (*ca.* 3.0 g, 0.550 $mmolg^{-1}$, *ca.* 1.7 mmol) in THF (10 mL) was treated with phenylsilane (940 μL, 7.62 mmol, 5 eq) and $[Pd(PPh_3)_4]$ (92 mg, 80 μmol, 5 mol %) in THF (1 mL) for 1 h at rt. The resin was drained, rinsed with THF, THF/H_2O, CH_2Cl_2 and vacuum dried. **Theoretical loading:** 0.560 $mmolg^{-1}$.

Methyl 2-hydroxy-4-benzyloxybenzoate microporous polystyrene resin, 38-75 μm (73)



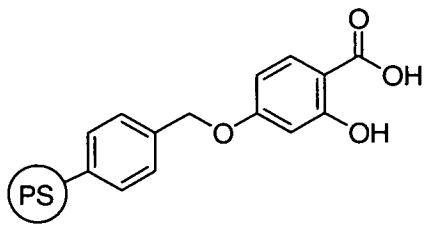
Merrifield resin (24.0 g, 1.2 $mmolg^{-1}$, 28.8 mmol) was suspended in acetone (250 mL) with KI (5.5 g, 33.1 mmol, 1.1 eq), K_2CO_3 (13.4 g, 97.0 mmol, 3.4 eq) and **35** (14.8 g, 88.0 mmol, 3.0 eq) and refluxed at 70 °C for 2 days. The resin was drained, rinsed with acetone/H_2O, H_2O, MeOH/H_2O, DMF, MeOH, DMF, CH_2Cl_2 and Et_2O (3 x each). **IR** (neat) 3024 (w), 2918 (w), 1719 (s), 1667 (s), 1252 (s) cm^{-1}, **Anal.** Found: Cl 0.98, I <0.10, **Loading obtained:** 1.01 $mmolg^{-1}$.

Methyl 2-hydroxy-4-benzyloxybenzoate microporous polystyrene resin, 150-300 μm (74)



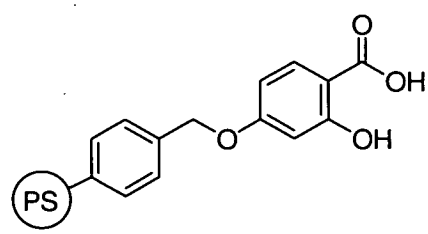
Merrifield resin (5.1 g, 1.7 $mmolg^{-1}$, 8.67 mmol) was suspended in acetone (50 mL) with KI (1.4 g, 8.4 mmol), K_2CO_3 (3.6 g, 26.0 mmol, 3.0 eq) and **35** (14.4 g, 85.6 mmol, 3.0 eq) and refluxed at 70 °C for 2 days. The resin was drained, rinsed with acetone/H_2O, H_2O, MeOH/H_2O, DMF, MeOH, DMF, CH_2Cl_2 and Et_2O (3 x each). **IR** (neat) 3026 (w), 2920 (w), 1669 (s), 1662 (m), 1348 (m), 1254 (s) cm^{-1}, **Anal.** Found: Cl 1.60, I <0.10, **Loading obtained:** 1.39 $mmolg^{-1}$.

2-Hydroxy-benzoic acid 4-oxymethyl microporous polystyrene resin, 38-75 μm (75)



Resin **73** (*ca.* 24 g, 28.8 mmol) suspended in THF (250 mL) with tetrabutylammonium hydroxide solution (50 mL, 40 % w/v in H_2O) was refluxed at 70 °C for 2 d. The light orange resin was cooled to rt, drained, rinsed with H_2O, THF, 2 M HCl/THF (1/1), H_2O, THF/H_2O, THF, MeOH, DMF, CH_2Cl_2 and Et_2O. **IR** (neat) 3024 (w), 2919 (w), 1666 (s), 1600 (m), 1492 (m) cm^{-1}, **Theorectical loading:** 1.05 $mmolg^{-1}$.

2-Hydroxy-4-benzyloxybenzoic acid microporous polystyrene resin, 150-300 μm (76)



Resin **74** (*ca.* 5 g, 8.67 mmol) suspended in THF (50 mL) with tetrabutylammonium hydroxide solution (10 mL, 40 % w/v in H_2O) was refluxed at 70 °C for 2 d. The light orange resin was cooled to rt, drained, rinsed with H_2O, THF, 2 M HCl/THF (1/1), H_2O, THF/H_2O, THF, MeOH, DMF, CH_2Cl_2 and Et_2O. **IR** (neat) 3026 (w), 2924 (w), 1740 (m), 1666 (s), 1604 (m), 1367 (m), 1219 (s) cm^{-1}, **Theorectical loading:** 1.42 $mmolg^{-1}$.

6.3.3 Phosphorylations using different resin supports

General method: synthesis of dTTP

Resin (0.27 mmol, 1 eq) was swollen in dry THF (2 x 2 mL for 5 min) at 60 °C. PCl_3 (95 μL, 4 eq) was added to the resin in dry THF (4 mL) and the syringe was heated at 60 °C for 2 h. 3'OAc-T **49** (90 μmol, 0.33 eq) was evaporated *in vacuo* from dry pyridine (1 mL), dried over P_2O_5 in a desiccator for 1 h *in vacuo* and dissolved in dry pyridine (0.25 mL) and dry THF (0.75 mL). The resin was drained and rinsed with dry THF (2 x 2 mL for 5 min). The N_2 flushed resin was treated with the nucleoside solution for 1 h at rt, drained and rinsed with dry THF (2 x 2 mL for 5 min). The N_2 flushed resin was treated three times with 0.5 M bis (tributyl ammonium) pyrophosphate in DMF (800 μL, 1.5 eq) and Bu_3N (255 μL, 4 eq) at rt for 10 min each. The cleaved product was collected in an RB with 0.5 M I_2/pyridine:H_2O 98:2 (800 μL, 1.5 eq). 15 min after the last cleavage, excess iodine was destroyed by adding a few drops of a 5 % aqueous solution of $NaHSO_3$. The reaction mixture was diluted with H_2O (20 mL), stirred for 30 min and evaporated *in vacuo*. The residue was dissolved in concentrated ammonia (20 mL). After 1 h the solution was evaporated *in vacuo*, the residue dissolved in 0.1 M TEAB (5 mL), washed with CH_2Cl_2 (3 x 5 mL), lyophilised and purified by Prep HPLC eluted with 0.1 M TEAB on a C18 column. The fractions containing triphosphate were pooled and lyophilised to remove the buffer to yield a hygroscopic solid.

Table 6.1 Summary of syntheses for dTTP using different resins

	Resin	Type	Bead size (μm)	Loading (mmol g^{-1})	Resin (mg)	Yield (%)
41a	Microporous PS	gel	75-150	1.34	201	50
57	Macroporous PS	rigid	150-350	0.93	289	15
59a	Quadragel	gel	150-300	0.75	372	19
59b	Quadrapore	rigid	150-300	0.65	413	26
71	Silica	rigid	40-63	0.35	774	8
72	Silica	rigid	40-63	0.56	435	4
75	Microporous PS	gel	38-75	1.05	290	13
76	Microporous PS	gel	150-300	1.42	192	20

6.4 Experimental for Chapter 3

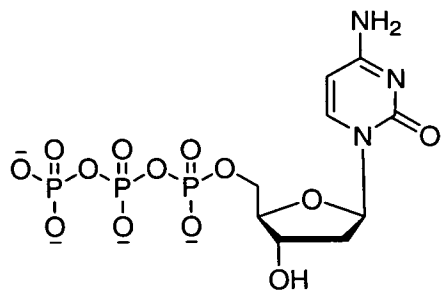
6.4.1 General methods

Triphosphate synthesis: 0.1 mmol scale

Resin **41a** (0.27 mmol, 1 eq) was swollen in dry THF (2 x 2 mL for 5 min) at 60 °C. PCl_3 (95 μL, 4 eq) was added to the resin in dry THF (4 mL) and the syringe was heated at 60 °C for 2 h. Nucleoside (90 μmol, 0.33 eq) was evaporated *in vacuo* from dry pyridine (1 mL), dried over P_2O_5 in a desiccator for 1 h *in vacuo* and dissolved in dry pyridine (0.25 mL) and dry THF (0.75 mL). The resin was drained and rinsed with dry THF (2 x 2 mL for 5 min). The N_2 flushed resin was treated with the nucleoside solution for 1 h at rt, drained and rinsed with dry THF (2 x 2 mL for 5 min). The N_2 flushed resin was treated three times with 0.5 M bis (tributyl ammonium) pyrophosphate in DMF (800 μL, 1.5 eq) and Bu_3N (255 μL, 4 eq) at rt for 10 min each. The cleaved product was collected in an RB with 0.5 M I_2/pyridine:H_2O 98:2 (800 μL, 1.5 eq). 15 min after the last cleavage, excess iodine was destroyed by adding a few drops of a 5 % aqueous solution of $NaHSO_3$. The reaction mixture was diluted with H_2O (20 mL), stirred for 30 min and evaporated *in vacuo*. The residue was dissolved in concentrated ammonia (20 mL). After 1 h the solution was evaporated *in vacuo*, the residue dissolved in 0.1 M TEAB (5 mL), washed with CH_2Cl_2 (3 x 5 mL), lyophilised and purified by Prep HPLC eluted with 0.1 M TEAB on a C18 column. The fractions containing triphosphate were pooled and lyophilised to remove the buffer to yield a hygroscopic solid.

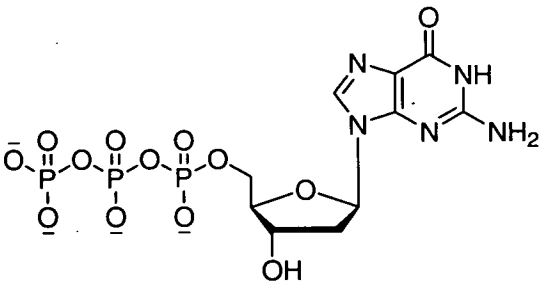
6.4.2 Synthesis of compounds and resins

Cytidine 5'-triphosphate tetraethylammonium salt (80)



Using general procedure: 3'OAc-C^{Bz} **77** (33 mg, 89 μmol) and resin **41a** (199 mg, 0.270 mmol, 1 eq) were used. The nucleoside was dissolved in dry pyridine (0.25 mL), dry DMF (0.25 mL) and dry THF (0.50 mL) to load onto the resin. The final deprotection step was left for 1 h and the residue was purified twice by Prep HPLC eluted with 0.1 M TEAB to yield a white hygroscopic solid (9 mg, 10 μmol, 12 % yield). **^{1}H NMR** (250 MHz, D_2O) δ_H 8.14 (1H, d, J = 7.7 Hz, H-5), 6.43 (1H, t, J = 6.7 Hz, H-1'), 6.29 (1H, d, J = 7.5 Hz, H-4), 4.73 (1H, t, J = 5.2 Hz, H-4'), 4.31 – 4.33 (3H, m, H-3' + H-5'), 2.54 (1H, ddd, J = 14.1, 6.3, 3.8 Hz, H-2'), 2.37 - 2.48 (1H, m, H-2'),**^{13}C NMR** (62.5 MHz, D_2O) δ_C 164.9 (C-4), 153.6 (C-2), 142.6 (C-6), 96.7 (C-5), 86.0 (d, J_{PC} = 9.2 Hz, C-4'), 85.9 (C-1'), 71.0 (C-3'), 65.6 (d, J_{PC} = 5.9 Hz, C-5'), 39.8 (C-2'), **^{31}P NMR** (101 MHz, D_2O) δ_P -9.62 (d, J = 18.8 Hz, P-γ), -10.24 (d, J = 20.2 Hz, P-α), -22.08 (t, J = 18.1 Hz, P-β), **ES$^-$** m/z (%) 466.2 ($M-H^-$, 100), **HPLC** (λ_{280}) t_R 6.29 min (Method 5).

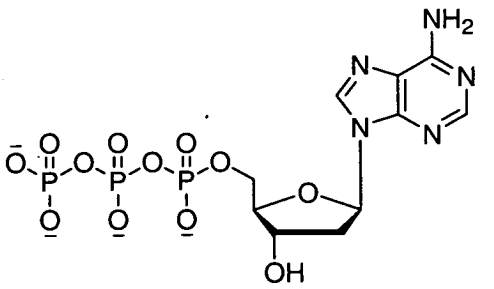
Guanidine 5'-triphosphate tetraethylammonium salt (81)



.4 $HNEt_3^+$

Using general procedure: 3'OAc-G^{iBu} **78** (33 mg, 87 μmol) and resin **41a** (205 mg, 0.272 mmol, 1 eq) were used. The final deprotection step was left for 15 h and the residue was purified by Prep HPLC eluted with 0.1 M TEAB to yield a white hygroscopic solid (22 mg, 24 μmol, 28 % yield). **^{1}H NMR** (250 MHz, D_2O) δ_H 7.94 (1H, s, H-8), 6.21 (1H, dd, J = 8.1, 6.0 Hz, H-1'), 4.63 - 4.67 (1H, m, H-4'), 4.14 (1H, t, J = 3.7 Hz, H-3'), 4.06 (2H, dd, J = 9.7, 5.2 Hz, H-5'), 2.68 (1H, ddd, J = 14.3, 8.1, 6.2 H, H-2'), 2.39 (1H, ddd, J = 14.1, 6.3, 3.1 Hz, H-2'), **^{31}P NMR** (101 MHz, D_2O) δ_P 0.01 (d, J = 19.3 Hz, P-γ), -10.25 (d, J = 20.3 Hz, P-α), -21.66 (t, J = 19.6 Hz, P-β), **ES$^-$** m/z (%) 506.1 ($M-H^-$, 100), **HPLC** (λ_{280}) t_R 13.89 min (Method 5).

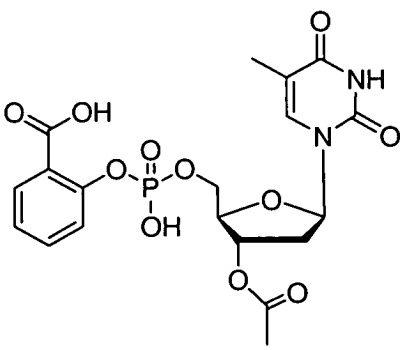
Adenosine 5'-triphosphate tetraethylammonium salt (82)



.4 $HNEt_3^+$

Using general procedure: 3'OAc-A^{Bz} **79** (38 mg, 95 μmol) and resin **41a** (205 mg, 0.272 mmol, 1 eq) were used. The final deprotection step was left for 15 h and the residue was purified by Prep HPLC eluted with 0.1 M TEAB to yield a white hygroscopic solid (38 mg, 42 μmol, 45 % yield). **^{1}H NMR** (250 MHz, D_2O) δ_H 8.31 (1H, s, H-2), 8.06 (1H, s, H-8), 6.32 (1H, t, J = 6.5 Hz, H-1'), H-4' obscured by H_2O peak, 3.94 – 4.12 (3H, m, H-3' + H-5'), 2.61 – 2.72 (1H, m, H-2'), 2.37 - 2.47 (1H, m, H-2'), **^{31}P NMR** (101 MHz, D_2O) δ_P -5.60 (d, J = 20.7 Hz, P-γ), -9.99 (d, J = 19.7 Hz, P-α), -21.15 (t, J = 20.5 Hz, P-β), **ES$^-$** m/z (%) 490.1 ($M\text{-}H^-$, 40), **HPLC** (λ_{280}) t_R 6.29 min (Method 5).

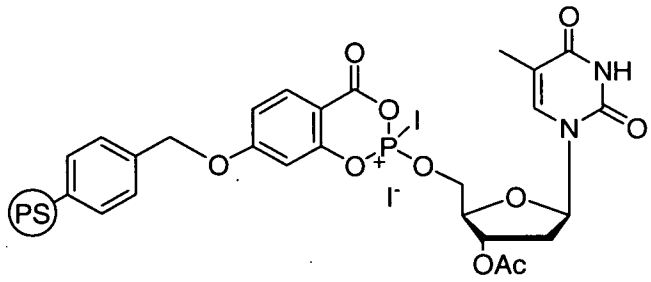
3'-*O*-Acetyl-thymidine 5'-(salicyl)-phosphate ammonium salt (89)



3'-OAc-T **49** (55 mg, 0.19 mmol) was dissolved in dry pyridine (2 mL), evaporated *in vacuo* and stored under vacuum over P_2O_5 for 1 h. The residue, dissolved in dry pyridine (0.25 mL) and diluted with dry THF (0.75 mL), was treated with freshly prepared 1.0 M 2-chloro-benzo[1,3,2]dioxaphosphinin-4-one **11** solution in dry THF (290 μL, 290 μmol, 1.5 eq). After 10 min, 0.5 M I_2 in 98:2 pyridine:H_2O (580 μL, 0.290 mmol, 1.5 eq) was added. The reaction was quenched with H_2O (1 mL) after 5 min and evaporated *in vacuo*. The residue was dissolved in 0.1 M TEAB buffer (10 mL), washed with CH_2Cl_2 (3 x 5 mL) and lyophilised. The residue was

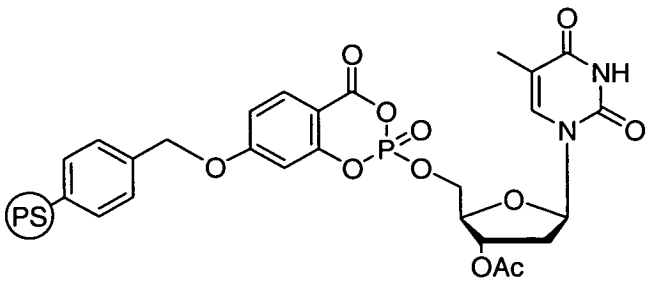
purified by Prep HPLC eluted with 5 % MeCN in 0.1 M ammonium acetate to yield a white hygroscopic solid (25 mg, 50 μmol, 27 % yield). **^{1}H NMR** (250 MHz, D_2O) δ_H 7.33 (1H, d, J = 1.1 Hz, H-6), 7.25 (1H, d, J = 7.7 Hz, CH), 6.99 – 7.11 (2H, m, CH), 6.85 (1H, td, J = 7.2, 1.2 Hz, CH), 6.01 (1H, dd, J = 8.1, 6.4 Hz, H-1'), 4.99 (1H, dt, J = 4.5, 2.2 Hz, H-4'), 4.04 (1H, dt, J = 5.0, 2.6 Hz, H-3'), 3.91 (1H, d, J = 3.8 Hz, H-5'), 3.88 (1H, d, J = 4.3 Hz, H-5'), 1.99 – 2.14 (2H, m, H-2'), 1.79 (3H, s, $COCH_3$), 1.42 (3H, d, J = 1.1 Hz, CH_3), **^{13}C NMR** (62.5 MHz, D_2O) δ_C 173.9 (C=O), 173.9 (C=O), 166.8 (C-4), 152.1 (C-2), 149.5 (d, J_{PC} = 6.5 Hz, C-O), 137.6 (C-6), 132.1 (CH), 130.1 (CH), 124.3 (CH), 120.1 (d, J_{PC} = 1.9 Hz, CH), 112.1 (C-5), 85.5 (C-1'), 83.5 (d, J_{PC} = 9.2 Hz, C-4'), 75.8 (C-3'), 66.2 (d, J_{PC} = 4.9 Hz, C-5'), 36.6 (C-2'), 20.8 (CH_3O), 11.9 (CH_3), **^{31}P NMR** (101 MHz, D_2O) δ_P -3.91, **ES$^-$** m/z (%) 483.1 (M-H$^-$, 100), **HPLC** (λ_{282}) t_R 13.6 min (Method 8 (tp5g25)).

2-[3'-O-Acetylthymidine]-2-iodo-4-oxo-benzo[1,3,2]dioxaphosphonium iodide 7-oxymethyl resin (90)



Resin **41a** (310 mg, 0.420 mmol, theoretical loading 1.34 mmolg^{-1}) was swollen in dry THF (2 x 2 mL for 5 min) at 60 °C. PCl_3 (142 μL, 4 eq) was added to the resin in dry THF (4 mL) and the syringe was heated at 60 °C for 2 h. The resin was drained and rinsed with dry THF (2 x 2 mL for 5 min each). 3'OAc-T **49** (225 mg, 0.792 mmol, 2.0 eq) was evaporated *in vacuo* from dry THF (5 mL), dried over P_2O_5 in a desiccator for 1 h *in vacuo* and dissolved in dry THF (2.8 mL). The N_2 flushed resin was treated with the nucleoside solution for 1 h at rt, drained and rinsed with dry THF (2 x 2 mL for 5 min). The resin was swollen in dry THF (2 mL), treated with 0.5 M I_2 in pyridine (4 mL, 5 eq) for 1 h, drained, rinsed with CH_2Cl_2 (4 x 2 mL for 5 min) and dry THF (4 x 2 mL for 5 min) and dried over P_2O_5 in a desicator. **IR** (neat) 3024 (w), 1738 (s), 1685 (s), 1608 (s), 1230 (s) cm^{-1}, **Gel Phase ^{31}P NMR** (162 MHz, $CDCl_3$) δ_P 46, -12.

2-[3'-O-Acetylthymidine]-2-oxo-4-oxo-benzo[1,3,2]dioxaphosphinone 7-oxymethyl resin (91)



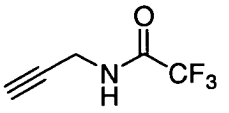
Resin **41a** (315 mg, 0.422 mmol, theoretical loading 1.34 $mmolg^{-1}$) was swollen in dry THF (2 x 2 mL for 5 min) at 60 °C. PCl_3 (142 μL, 4 eq) was added to the resin in dry THF (4 mL) and the syringe was heated at 60 °C for 2 h. The resin was drained and rinsed with dry THF (2 x 2 mL for 5 min). 3'OAc-T **49** (227 mg, 0.800 mmol, 2.0 eq) was evaporated *in vacuo* from dry THF (5 mL), dried over P_2O_5 in a desiccator for 1 h *in vacuo* and dissolved in dry THF (2.8 mL). The N_2 flushed resin was treated with the nucleoside solution for 1 h at rt, drained and rinsed with dry THF (2 x 2 mL for 5 min). The resin was swollen in dry THF (6 mL), treated with 5.5 M ^tBuOOH in nonane (363 μL, 2 mmol, 5 eq) for 1 h, drained, rinsed with CH_2Cl_2 (4 x 2 mL for 5 min) and dry THF (4 x 2 mL for 5 min) and dried over P_2O_5 in a desicator. **IR** (neat) 3026 (w), 1774 (m), 1688 (s), 1615 (s), 1232 (s) cm^{-1}, **Gel Phase ^{31}P NMR** (162 MHz, $CDCl_3$) δ_P -11.5, **ICP analysis:** P (%) 2.57, 0.829 $mmolg^{-1}$, 89 % yield.

Thymidine triphosphate synthesis using on-resin oxidation

Resin **41a** (201 mg, 0.27 mmol, 1 eq) was swollen in dry THF (2 x 2 mL for 5 min) at 60 °C. PCl_3 (95 μL, 4 eq) was added to the resin in dry THF (4 mL) and the syringe was heated at 60 °C for 2 h. 3'-OAc-T **49** (26 mg, 92 μmol, 0.33 eq) was evaporated *in vacuo* from dry pyridine (1 mL), dried over P_2O_5 in a desiccator for 1 h *in vacuo* and dissolved in dry pyridine (0.25 mL) and dry THF (0.75 mL). The resin was drained and rinsed with dry THF (2 x 2 mL for 5 min). The N_2 flushed resin was treated with the nucleoside solution for 1 h at rt, drained and rinsed with dry THF (2 x 2 mL for 5 min). The resin was swollen in dry THF (4 mL), treated with 5.5 M ^tBuOOH in nonane (245 μL, 2 mmol, 5 eq) for 1 h, drained, rinsed with dry THF (2 x 2 mL for 5 min). The N_2 flushed resin was treated three times with 0.5 M bis (tributyl ammonium) pyrophosphate in DMF (800 μL, 1.5 eq) and Bu_3N

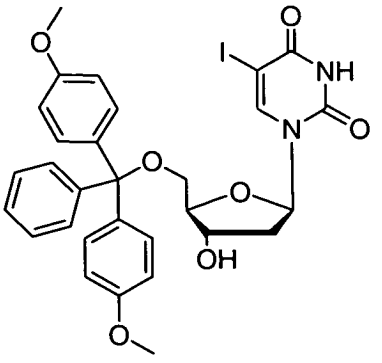
(255 μL, 4 eq) at rt for 10 min each. The filtrate was stirred with H_2O (20 mL), then treated with concentrated ammonia (20 mL) for 1 h and evaporated *in vacuo*. The residue was dissolved in 0.1 M TEAB (5 mL), washed with CH_2Cl_2 (3 x 5 mL), lyophilised and purified by Prep HPLC eluted with 0.1 M TEAB. The fractions containing triphosphate were pooled and lyophilised to remove the buffer to yield a hygroscopic solid (2.0 mg, 2.3 μmol, 3 % yield).

2,2,2-Trifluoro-*N*-prop-2-ynyl-acetamide (95)



Propargylamine (8.84 g, 160 mmol) and TEA (45 mL, 320 mmol, 2 eq) were dissolved in CH_2Cl_2 (50 mL) at 0 °C. Trifluoroacetic anhydride (25 mL, 180 mmol, 1.13 eq) was added dropwise over 30 min. The reaction mixture was warmed to r.t., stirred for 14 h and evaporated *in vacuo* to a brown oil. Et_2O (200 mL) and 1 M NH_4OAc (300 mL) were added. The organic layer was separated and washed with H_2O (100 mL), dried (Na_2SO_4) and purified by column chromatography eluted with CH_2Cl_2 to yield a yellow oil (17.9 g, 118 mmol, 74 % yield). **IR** (neat) 3301 (m), 3093 (w), 1705 (s), 1549 (m), 1153 (s) cm^{-1}, **^{1}H NMR** (250 MHz, $CDCl_3$) δ_H 7.37 (1H, br signal, NH), 4.11 (2H, ddd, J = 5.5, 2.6, 0.4 Hz, CH_2), 2.30 (1H, t, J = 2.6 Hz, CH), **^{13}C NMR** (62.5 MHz, $CDCl_3$) δ_C 157.2 (q, J = 37.9 Hz, $COCF_3$), 115.5 (q, J = 287.2 Hz, CF_3), 77.0 (C), 72.8 (CH), 29.5 (CH_2), **ES$^-$** m/z (%) 150.2 ($M-H^-$, 100), **HPLC** (λ_{220}) t_R 4.63 min (Method 3), **R_f** 0.44 (CH_2Cl_2, $KMnO_4$).

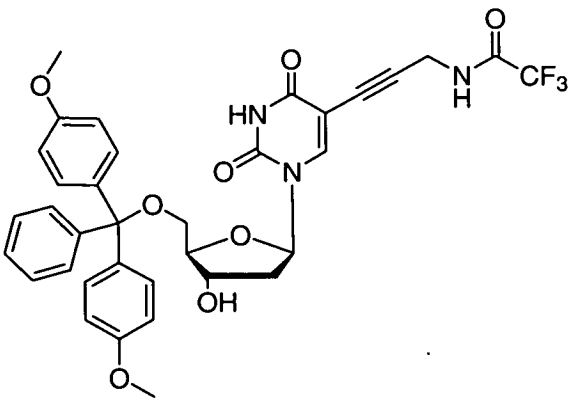
5'-*O*-(4,4'-Dimethoxytrityl)-5-iodo-2'-deoxyuridine (97)



5-Iodo-2'-deoxyuridine **96** (2.98 g, 8.40 mmol), evaporated *in vacuo* from dry pyridine (10 mL), was treated with DMAP (210 mg, 1.72 mmol, 0.2 eq) and sealed

under a N_2 atmosphere. The solids were dissolved in dry pyridine (10 mL) and treated dropwise with a solution of DMT-Cl (2.86 g, 8.43 mmol, 1.0 eq) in dry pyridine (20 mL) over 30 min *via* canula transfer. The reaction mixture was stirred for a total of 3 h until complete (tlc) and evaporated *in vacuo*. The residue was dissolved in CH_2Cl_2 (100 mL), washed with H_2O (2 x 50 mL) and brine (100 mL), dried (Na_2SO_4), evaporated *in vacuo* and purified by column chromatography eluted with 0-3 % MeOH/CH_2Cl_2 to yield an off-white foam (4.17 g, 6.35 mmol, 76 % yield). **IR** (neat) 1685 (s), 1676 (s), 1507 (m), 1210 (s) cm^{-1}, **^{1}H NMR** (250 MHz, $CDCl_3$) δ_H 9.24 (1H, br signal, H-3), 8.15 (1H, s, H-6), 7.22 - 7.43 (9H, m, Ph + *p*-CH of DMT), 6.85 (4H, d, J = 9 Hz, 4 *o*-CH of DMT), 6.32 (1H, dd, J = 8, 6 Hz, H-1'), 4.55 (1H, dt, J = 5, 3 Hz, H-3'), 4.11 (1H, dt, J = 3, 3 Hz, H-4'), 3.78 (6H, s, CH_3O), 3.41 (1H, dd, J = 11, 3 Hz, H-5'), 3.35 (1H, dd, J = 11, 3 Hz, H-5'), 2.51 (1H, ddd, J = 14, 6, 3 Hz, H-2'), 2.29 (1H, ddd, J = 14, 8, 6 Hz, H-2'), 1.78 (1H, br signal, OH), **^{13}C NMR** (62.5 MHz, $CDCl_3$) δ_C 160.0 (C-4), 158.6 (COMe), 150.0 (C-2), 144.3 (C-6, C), 135.4 (C), 135.3 (C), 130.1 (CH), 128.1 (CH), 128.0 (CH), 127.1 (CH), 113.4 (CH), 87.0 (C-Ar_3), 86.5 (C-4'), 85.6 (C-1'), 72.5 (C-3'), 68.6 (C-5), 63.5 (C-5'), 55.3 (CH_3O), 41.4 (C-2'), **APCI$^-$** m/z (%) 655.1 (M-H$^-$, 100), **HPLC** (λ_{282}) t_R 2.55 min (Method 1), 6.59 min (Method 2), **R_f** 0.35 (10 % MeOH/CH_2Cl_2, ammonium molybdate).

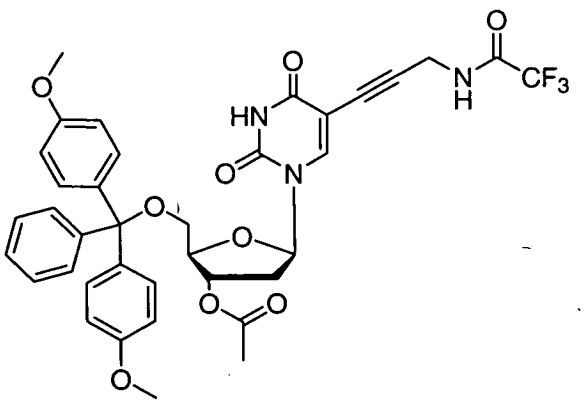
5-(3''-Trifluoroacetamidopropynyl)-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxyuridine (98)



Nucleoside **97** (3.88 g, 5.91 mmol), [Pd (PPh_3)$_4$] (682 mg, 0.590 mmol, 0.1 eq) and copper (I) iodide (226 mg, 1.187 mmol, 0.2 eq) were sealed under a N_2 atmosphere. Trifluoroacetylpropargylamine **95** (2.24 g, 14.85 mmol, 2.5 eq) in DMF (11.8 mL) and TEA (4.2 mL, 5 eq) were injected through the septum. The reaction mixture

was stirred for 2.5 h until complete (tlc) and evaporated *in vacuo*. The residue was dissolved in CH_2Cl_2 (100 mL), washed with 5 % w/v EDTA (3 x 100 mL), 5 % w/v $NaHSO_3$ (100 mL) and brine (100 mL), dried (Na_2SO_4), evaporated *in vacuo* and purified by column chromatography eluted with 0-3 % MeOH/CH_2Cl_2 to yield a yellow powder (2.77 g, 4.08 mmol, 69 % yield). **mp** 98-102 °C, **IR** (neat) 1708 (s), 1674 (s), 1508 (m), 1462 (s), 1282 (s) cm^{-1}, **^{1}H NMR** (250 MHz, $CDCl_3$) δ_H 9.43 (1H, br signal, H-3), 8.24 (1H, s, H-6), 7.19 – 7.43 (10H, m, Ph + *p*-CH of DMT + H-9), 6.84 (4H, d, J = 9 Hz, 4 *o*-CH of DMT), 6.35 (1H, dd, J = 7, 6 Hz, H-1'), 4.60 (1H, m, H-3'), 4.13 (1H, dt, J = 3, 3 Hz, H-4'), 3.96 (1H, dd, J = 18, 5 Hz, H-9), 3.87 (1H, dd, J = 18, 5 Hz, H-9), 3.77 (6H, s, CH_3O), 3.40 (1H, dd, J = 11, 3 Hz, H-5'), 3.34 (1H, dd, J = 11, 3 Hz, H-5'), 2.98 (1H, d, J = 4.4Hz, OH), 2.56 (1H, ddd, J = 14, 6, 3 Hz, H-2'), 2.33 (1H, m, H-2'), **^{13}C NMR** (62.5 MHz, $CDCl_3$) δ_C 162.7 (C-4), 158.5 (*C*OMe), 158.5 (*C*OMe), 156.6 (q, J = 37.6 Hz, *C*OCF_3), 149.4 (C-2), 144.4 (C), 143.5 (C-6), 135.4 (C), 135.3 (C), 129.9 (CH), 129.8 (CH), 127.9 (CH), 127.8 (CH), 126.9 (CH), 115.5 (q, J = 289.7 Hz, CF_3), 113.2 (CH); 98.8 (C-5), 87.4 (C-Ar_3), 86.9 (C-7, C-4'), 85.9 (C-1'), 75.1 (C-8), 72.1 (C-3'), 63.5 (C-5'), 55.1 (CH_3O), 41.5 (C-2'), 30.3 (C-9), **APCI$^-$** m/z (%) 678.2 (M-H$^-$, 100), **HPLC** (λ_{282}) t_R 2.51 min (Method 1), 6.47 min (Method 2), **R_f** 0.33 (10 % MeOH/CH_2Cl_2, Ninhydrin).

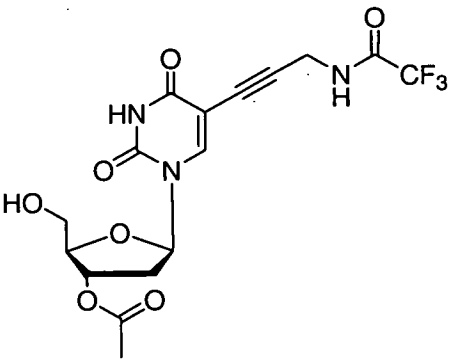
3'-*O*-Acetyl-5-(3''-trifluoroacetamidopropynyl)-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxyuridine (99)



Nucleoside **98** (2.56 g, 3.78 mmol) was treated with DMAP (115 mg, 0.94 mmol, 0.25 eq) and dissolved in pyridine (25 mL). TEA (1.33 mL, 9.46 mmol, 2.5 eq) and Ac_2O (385 μL, 3.78 mmol, 1.0 eq) were added and the reaction mixture was stirred for 2 h until complete (tlc) and evaporated *in vacuo*. The residue was dissolved in

EtOAc (100 mL), washed with H_2O (3 x 100 mL), dried (Na_2SO_4) and evaporated *in vacuo* to yield a brown foam (2.53 g, 3.51 mmol, 93 % yield). **mp** 108-110 °C, **IR** (neat) 3065 (w), 1692 (s), 1608 (m), 1508 (w), 1246 (s), 1174 cm^{-1}, **^{1}H NMR** (250 MHz, $CDCl_3$) δ_H 9.78 (1H, br signal, H-3), 8.25 (1H, s, H-6), 7.22 - 7.44 (9H, m, Ph + *p*-CH of DMT), 7.21 (1H, br signal, H-9), 6.85 (4H, d, J = 9 Hz, 4 *o*-CH of DMT), 6.34 (1H, dd, J = 8, 6 Hz, H-1'), 5.46 (1H, d, J = 6 Hz, H-3'), 4.18 (1H, m, H-4'), 3.99 (1H, dd, J = 18, 5 Hz, H-9), 3.88 (1H, dd, J = 18, 5 Hz, H-9), 3.78 (6H, s, CH_3O), 3.45 (1H, dd, J = 11, 3 Hz, H-5'), 3.38 (1H, dd, J = 11, 3 Hz, H-5'), 2.59 (1H, m, H-2'), 2.42 (1H, ddd, J = 14, 8, 6 Hz, H-2'), 2.09 (3H, s, CH_3), **^{13}C NMR** (90 MHz, $CDCl_3$) δ_C 170.4 (*C*=OCH_3), 162.2 (C-4), 158.6 (COMe), 156.6 (q, J = 37.7 Hz, *C*OCF_3), 149.2 (C-2), 144.3 (C), 143.4 (C-6), 135.3 (C), 135.1 (C), 129.9 (CH), 129.8 (CH), 128.1 (CH), 127.7 (CH), 127.0 (CH), 115.5 (q, J = 287.7 Hz, CF_3), 113.3 (CH), 99.4 (C-5), 87.3 (C-Ar_3), 87.3 (C-7), 85.4 (C-4'), 84.6 (C-1'), 75.0 (C-8), 74.9 (C-3'), 63.5 (C-5'), 55.2 (CH_3O), 38.8 (C-2'), 30.3 (C-9), 20.9 (CH_3), **APCI$^-$** m/z (%) 720.2 (M-H$^-$, 100), **HPLC** (λ_{282}) t_R 2.71 min (Method 1), 7.19 min (Method 2), **R_f** 0.56 (10 % MeOH/CH_2Cl_2, ammonium molybdate).

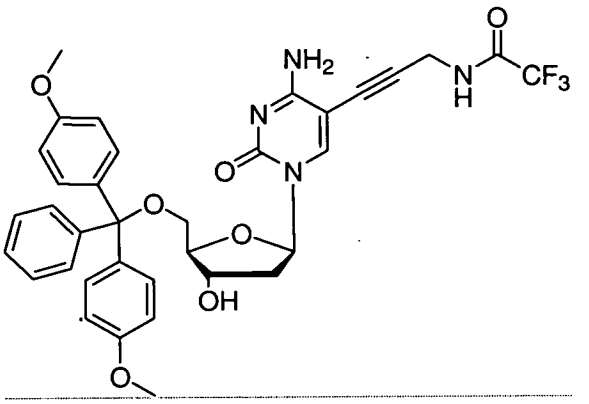
3'-*O*-Acetyl-5-(3''-trifluoroacetamidopropynyl)-2'-deoxyuridine (100)



Nucleoside **99** (1.62 g, 2.24 mmol) was dissolved in 3 % TCA/CH_2Cl_2 (50 mL) to give a dark--red solution. MeOH (5 mL) was added after 1 min to yield a yellow-orange solution, evaporated *in vacuo* and purified by column chromatography eluted with 0 – 3 % MeOH in CH_2Cl_2 to yield a yellow powder (920 mg, 2.19 mmol, 98 % yield). **mp** 145-148 °C, **IR** (neat) 3050 (w), 1690 (s), 1555 (m), 1288 (s), 1239 (s), 1208 (s), 1186 (s) cm^{-1}, **^{1}H NMR** (250 MHz, $CDCl_3$/CD_3OD) δ_H 9.44 (1H, br signal, NH), 8.32 (1H, s, H-6), 6.24 (1H, dd, J = 8.3, 5.7 Hz, H-1'), 5.28 (1H, dt, J = 6.0, 1.5 Hz, H-3'), 4.20 (2H, s, H-9), 4.08 (1H, q, J = 2.2 Hz, H-4'), 3.84 (1H, dd, J = 11.5, 1.7 Hz, H-5'), 3.79 (1H, dd, J = 11.5, 1.9 Hz, H-5'), 2.41

(1H, ddd, J = 14.0, 5.7, 1.7 Hz, H-2'), 2.24 (1H, m, H-2'), 2.06 (3H, s, CH_3), **^{13}C NMR** (90 MHz, $CDCl_3/CD_3OD$) δ_C 170.7 (C=OCH_3), 162.9 (C-4), 157.2 (q, J = 37 Hz, $COCF_3$), 149.3 (C-2), 144.0 (C-6), 115.6 (q, J = 287 Hz, CF_3), 98.7 (C-5), 87.1 (C-7), 85.5 (C-4'), 85.4 (C-1'), 75.0 (C-3'), 74.8 (C-6), 61.5 (C-5'), 38.1 (C-2'), 29.9 (C-9), 20.5 (CH_3), **ES$^-$** m/z (%) 418.2 (M-H$^-$, 100), **HPLC** (λ_{280}) t_R 5.96 min (Method 3), **R_f** 0.33 (10 % $MeOH/CH_2Cl_2$, $KMnO_4$).

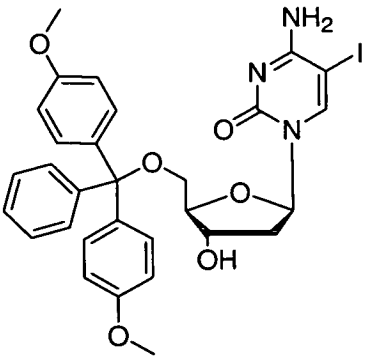
5-(3''-Trifluoroacetamidopropynyl)-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxycytidine (102)



5-(3''-Trifluoroacetamidopropynyl)-2'-deoxycytidine **101** (295 mg, 0.784 mmol), evaporated *in vacuo* from dry pyridine (5 mL), was treated with DMAP (26 mg, 0.213 mmol, 0.27 eq) and sealed under a N_2 atmosphere. The solids were dissolved in dry pyridine (3 mL) and treated with DMT-Cl (282 mg, 0.832 mmol, 1.06 eq). After 24 h, the reaction mixture was evaporated *in vacuo*. The residue was dissolved in EtOAc (15 mL), washed with H_2O (2 x 10 mL) and brine (10 mL), dried (Na_2SO_4), evaporated *in vacuo* and purified by column chromatography eluted with 0-5 % $MeOH/CH_2Cl_2$ to yield an off-white powder (292 mg, 0.430 mmol, 55 % yield). **mp** 117-120 °C, **IR** (neat) 3064 (w), 1716 (m), 1642 (s), 1605 (m), 1505 (s), 1299 (m), 1174 (s) cm^{-1}, **^{1}H NMR** (250 MHz, $CDCl_3$) δ_H 8.22 (1H, s, H-6), 7.49 (1H, t, J = 5.0 Hz, NHTfa), 7.21 -7.44 (9H, m, Ph + p-CH of DMT), 7.00 (1H, br signal, OH), 6.84 (4H, d, J = 8.9 Hz, 4 o-CH of DMT), 6.26 (1H, t, J = 6.1 Hz, H-1'), 5.99 (2H, br signal, NH_2), 4.51 (1H, dt, J = 6.9, 3.5 Hz, H-3'), 4.12 (1H, q, J = 3.0 Hz, H-4'), 4.08 (1H, m, H-9), 3.96 (1H, dd, J = 17.9, 4.9 Hz, H-9), 3.78 (6H, s, CH_3O), 3.37 (1H, dd, J = 10.8, 2.9 Hz, H-5'), 3.30 (1H, dd, J = 10.7, 4.0 Hz, H-5'), 2.67 (1H, ddd, J = 13.8, 6.1, 3.7 Hz, H-2'), 2.19-2.27 (1H, m, H-2'), one H-9 signal partially obscured by H-4' signal, **^{13}C NMR** (62.5 MHz, $CDCl_3$) δ_C

164.7 (C-4), 158.4 (COMe), 157.6 (q, J = 37.2 Hz, $COCF_3$), 154.7 (C-2), 144.4 (C-6), 135.8 (C), 135.7 (C), 130.1 (CH), 129.9 (CH), 128.0 (CH), 126.9 (CH), 116.3 (q, J = 287.4 Hz, CF_3), 113.3 (CH), 90.4 (C-5/C-7), 89.4 (C-5/C-7), 86.9 (C-4'), 86.8 (C-Ar_3), 86.5 (C-1'), 75.1 (C-8), 71.8 (C-3'), 63.5 (C-5'), 55.3 (CH_3O), 42.1 (C-2'), 30.3 (C-9), **APCI**$^-$ m/z (%) 677.5 (M-H^-, 100), **HRMS** (FAB) for $C_{35}H_{34}N_4O_7F_3$ $(M+H)^+$: calcd 679.23796, found 679.23751, **HPLC** (λ_{280}) t_R 7.90 min (Method 3), $\mathbf{R_f}$ 0.22 (10 % MeOH/CH_2Cl_2, ammonium molybdate).

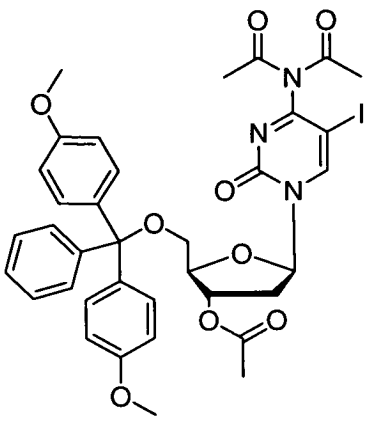
5'-*O*-(4,4'-Dimethoxytrityl)-5-iodo-2'-deoxycytidine (104)

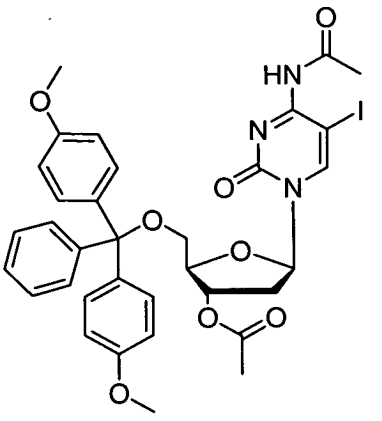


5-Iodo-2'-deoxycytidine **103** (639 mg, 1.81 mmol), evaporated *in vacuo* from dry pyridine (4 mL), was treated with DMAP (50 mg, 0.409 mmol, 0.23 eq) and sealed under a N_2 atmosphere. The solids were dissolved in dry pyridine (2 mL) and treated dropwise with a solution of DMT-Cl (630 mg, 1.86 mmol, 1.03 eq) in dry pyridine (5.4 mL) over 45 min *via* canula transfer. The reaction mixture was stirred for a total of 3 h and evaporated *in vacuo*. The residue was dissolved in CH_2Cl_2/EtOAc (1/1, 150 mL), washed with H_2O (50 mL) and brine (50 mL), dried (Na_2SO_4), evaporated *in vacuo* and purified by column chromatography eluted with 0-5 % MeOH/CH_2Cl_2 to yield a white flaky powder (722 mg, 1.10 mmol, 61 % yield). **mp** 176-178 °C, **IR** (neat) 3054 (w), 1658 (m), 1631 (s), 1605 (m), 1507 (m), 1489 (m), 1249 (m) cm^{-1}, **^{1}H NMR** (250 MHz, $CDCl_3$) δ_H 8.18 (1H, s, H-6), 7.18 - 7.39 (9H, m, Ph + *p*-CH of DMT), 6.80 (4H, d, J = 9.0 Hz, 4 *o*-CH of DMT), 6.18 (1H, dd, J = 7.4, 5.7 Hz, H-1'), 4.40 (1H, dt, J = 5.8, 2.9 Hz, H-3'), 4.08 (1H, q, J = 3.1 Hz, H-4'), 3.75 (6H, s, CH_3O), 3.37 (1H, dd, J = 10.6, 3.0 Hz, H-5'), 3.26 (1H, dd, J = 10.7, 3.4 Hz, H-5'), 2.54 (1H, ddd, J = 13.7, 5.7, 2.9 Hz, H-2'), 2.14 (1H, ddd, J = 13.5, 7.3, 6.0 Hz, H-2'), **^{13}C NMR** (62.5 MHz, $CDCl_3$) δ_C 163.6 (C-4), 158.5 (COMe), 158.4 (COMe), 155.2 (C-2), 146.6 (C-6), 144.3 (C), 135.6 (C), 135.5 (C), 130.0 (CH), 128.0 (CH), 127.9 (CH), 126.9 (CH), 113.2 (CH), 86.8

(C-Ar_3), 86.7 (C-4'), 86.6 (C-1'), 71.7 (C-3'), 63.4 (C-5'), 55.2 (CH_3O), 42.0 (C-2'), C-5 signal not observed, **APCI**$^-$ m/z (%) 654.4 (M-H$^-$, 100), **HPLC** (λ_{280}) t_R 8.00 min (Method 3), $\mathbf{R_f}$ 0.15 (10 % MeOH/CH_2Cl_2).

5'-*O*-(4,4'-Dimethoxytrityl)-3-*O*-acetyl-4-*N*,*N*-diacetyl-5-iodo-2'-deoxycytidine (105) and 5'-*O*-(4,4'-dimethoxytrityl)-3-*O*-acetyl-4-*N*-acetyl-5-iodo-2'-deoxycytidine (106)

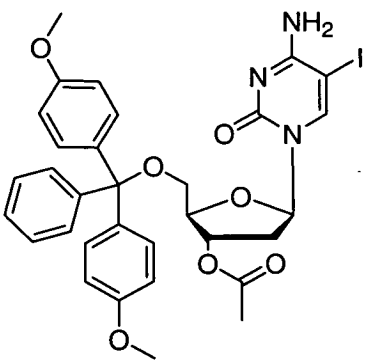




Nucleoside **104** (50 mg, 77 μmol) and DMAP (13 mg, 0.11 mmol, 1.4 eq) dissolved in dry CH_2Cl_2 (1.2 mL) were treated with TEA (110 μL, 0.782 mmol, 10 eq) and Ac_2O (40 μL, 0.392 mmol, 5 eq) and stirred at r.t. for 14 h. The reaction mixture was purified by column chromatography eluted with 0-2 % MeOH/CH_2Cl_2 to yield a yellow amorphous powder **105** (19 mg, 24 μmol, 31 % yield) and a clear glass **106** (12 mg, 16 μmol, 20 % yield). **Data for 105: mp:** 75-78 °C, **IR** (neat) 2931 (w), 1729 (s), 1669 (s), 1606 (m), 1222 (s) cm^{-1}, **^{1}H NMR** (250 MHz, $CDCl_3$) δ_H 8.69 (1H, s, H-6), 7.22 - 7.41 (9H, m, Ph + *p*-CH of DMT), 6.84 (4H, dd, J = 9.0, 1.2 Hz, 4 *o*-CH of DMT), 6.26 (1H, dd, J = 8.0, 5.5 Hz, H-1'), 5.39 (1H, dt, J = 6.1, 1.7 Hz, H-3'), 4.29 (1H, q, J = 2.7 Hz, H-4'), 3.79 (6H, s, CH_3O), 3.47 (1H, dd, J = 10.9, 2.8 Hz, H-5'), 3.41 (1H, dd, J = 10.9, 3.0 Hz, H-5'), 2.92 (1H, ddd, J = 14.2, 5.5, 1.6 Hz, H-2'), 2.40 – 2.45 (1H, m, H-2'), 2.35 (6H, s, Ac_2), 2.09 (3H, s, Ac), **^{13}C NMR** (62.5 MHz, $CDCl_3$) δ_C 170.4 (C=O), 170.2 (C), 158.7 (COMe), 153.9 (C-2), 151.0 (C-6), 144.0 (C), 135.3 (C), 135.1 (C), 130.0 (CH), 129.9 (CH), 128.2 (CH), 128.0 (CH), 128.0 (CH), 113.5 (CH), 88.3 (C-Ar_3), 87.3 (C-4'), 85.5 (C-1'), 74.9 (C-3'), 66.7 (C-5), 63.2 (C-5'), 55.3 (CH_3O), 39.6 (C-2'), 26.6 (CH_3), 20.9 (CH_3), C-4 signal not observed, **APCI**$^-$ m/z (%) 780.4 (M-H$^-$, 40), **HRMS** (FAB) for $C_{36}H_{37}N_3O_9I_1$ $(M+H)^+$: calcd 782.15746, found 782.15807, **HPLC** (λ_{280}) t_R 8.25 min (Method 3), $\mathbf{R_f}$ 0.68 (10 % MeOH/CH_2Cl_2); **Data for 106: mp:** 72-75

°C, **IR** (neat) 2933 (w), 1707 (m), 1660 (s), 1607 (s), 1506 (s), 1233 (s) cm^{-1}, **^{1}H NMR** (250 MHz, $CDCl_3$) δ_H 8.40 (1H, s, H-6), 7.75 (1H, s, NH), 7.20 - 7.40 (9H, m, Ph + *p*-CH of DMT), 6.84 (4H, dd, *J* = 9.0, 0.6 Hz, 4 *o*-CH of DMT), 6.30 (1H, dd, *J* = 8.3, 5.5 Hz, H-1'), 5.39 (1H, dt, *J* = 6.1, 1.8 Hz, H-3'), 4.22 (1H, m, H-4'), 3.79 (6H, s, CH_3O), 3.42 (2H, d, *J* = 2.7 Hz, H-5'), 2.80 (1H, dd, *J* = 13.7, 4.9 Hz, H-2'), 2.68 (3H, s, Ac), 2.31 – 2.40 (1H, m, H-2'), 2.09 (3H, s, Ac), **^{13}C NMR** (62.5 MHz, $CDCl_3$) δ_C 171.1 (C=O), 169.4 (C), 157.7 (COMe), 152.4 (C-2), 147.6 (C-6), 143.2 (C), 134.3 (C), 134.2 (C), 129.0 (CH), 127.1 (CH), 127.0 CH), 126.2 (CH), 112.4 (CH), 86.2 (C-Ar_3, C-4'), 84.0 (C-1'), 74.1 (C-3'), 66.7 (C-5), 62.4 (C-5'), 54.3 (CH_3O), 38.6 (C-2'), 25.1 (CH_3), 20.0 (CH_3), C-4, C-5 signals not observed, **APCI$^-$** m/z (%) 738.3.4 (M-H$^-$, 40), **HPLC** (λ_{280}) t_R 8.28 min (Method 3), **R_f** 0.55 (10 % MeOH/CH_2Cl_2).

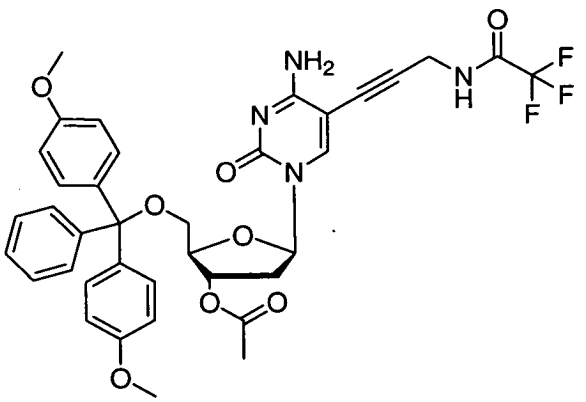
5'-*O*-(4,4'-Dimethoxytrityl)-3-*O*-acetyl-5-iodo-2'-deoxycytidine (107)

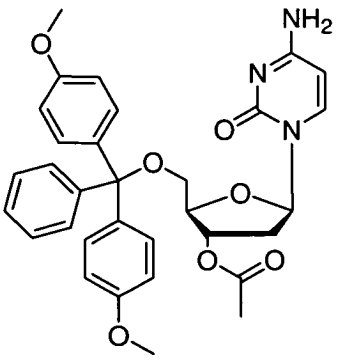


Nucleoside **104** (200 mg, 0.306 mmol) and DMAP (8.4 mg, 69 μmol, 0.22 eq) suspended in dry CH_2Cl_2 (6.1 mL) and TEA (430 μL, 3.06 mmol, 10 eq). Ac_2O (31 μL, 0.304 mmol, 1 eq) was added and stirred at r.t. for 15 min. The reaction mixture was evaporated *in vacuo* and purified by column chromatography eluted with 0-2.5 % MeOH/CH_2Cl_2 to yield a clear glass **106** (7 mg, 9.5 μmol, 3 % yield) and a white foam **107** (190 mg, 0.273 mmol, 89 % yield). **mp** 115-118 °C, **IR** (neat) 2928 (w), 1739 (m), 1626 (s), 1508 (s), 1488 (m), 1235 (s) cm^{-1}, **^{1}H NMR** (250 MHz, $CDCl_3$) δ_H 8.58 (1H, s, NH), 8.12 (1H, s, H-6), 7.15 - 7.38 (9H, m, Ph + *p*-CH of DMT), 6.80 (4H, d, *J* = 8.8 Hz, 4 *o*-CH of DMT), 6.28 (1H, dd, *J* = 8.4, 5.3 Hz, H-1'), 5.60 (1H, s, NH), 5.32 (1H, d, *J* = 5.8 Hz, H-3'), 4.13 (1H, m, H-4'), 3.74 (6H, s, CH_3O), 3.38 (1H, dd, *J* = 10.1, 2.8 Hz, H-5'), 3.34 (1H, dd, *J* = 10.0, 3.0 Hz, H-5'), 2.60 (1H, dd, *J* = 13.7, 5.2 Hz, H-2'), 2.23 (1H, ddd, *J* = 14.4, 8.5, 6.3, H-2'), 2.02 (3H, s, Ac), **^{13}C NMR** (62.5 MHz, $CDCl_3$) δ_C 170.3 (C=O), 163.7

(C-4), 158.6 (COMe), 154.6 (C-2), 146.2 (C-6), 144.2 (C), 135.4 (C), 135.3 (C), 130.0 (CH), 128.0 (CH), 127.0 (CH), 113.3 (CH), 87.0 (C-Ar_3), 86.3 (C-4'), 84.3 (C-1'), 75.2 (C-3'), 63.4 (C-5'), 56.7 (C-5), 55.2 (CH_3O), 39.3 (C-2'), 20.9 (CH_3), **APCI**$^-$ m/z (%) 696.4 (M-H$^-$, 100), **HRMS** (FAB) for $C_{32}H_{33}N_3O_7I_1$ $(M+H)^+$: calcd 698.1358, found 698.1355, **HPLC** (λ_{280}) t_R 8.25 min (Method 3), $\mathbf{R_f}$ 0.39 (10 % MeOH/CH_2Cl_2).

5'-*O*-(4,4'-Dimethoxytrityl)-3-*O*-acetyl-5-(3''-trifluoroacetamidopropynyl)-2'-deoxycytidine (108) and 5'-*O*-(4,4'-dimethoxytrityl)-3-*O*-acetyl-2'-deoxycytidine (109)

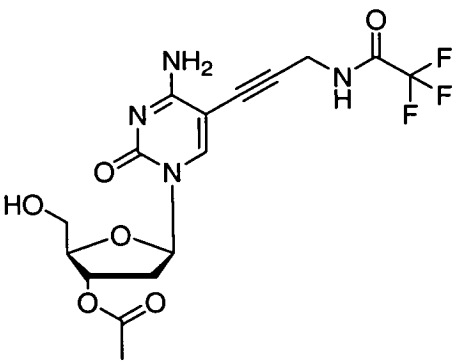




Nucleoside **107** (323 mg, 0.463 mmol), [Pd (PPh_3)$_4$] (56 mg, 48.5 μmol, 0.1 eq) and copper (I) iodide (50 mg, 0.263 mmol, 0.5 eq) were sealed under a N_2 atmosphere. Trifluoroacetylpropargylamine **95** (200 mg, 1.20 mmol, 2.5 eq), DMF (925 μL) and TEA (325 μL, 2.33 mmol, 5 eq) were added and the reaction mixture was stirred for 2 h until complete (tlc) and evaporated *in vacuo*. The reaction mixture was purified by column chromatography eluted with 0-5 % MeOH/CH_2Cl_2 to yield a brown foam **108** (263 mg, 0.364 mmol, 79 % yield) and a brown oil **109** (18 mg, 32 μmol, 7 % yield). **Data for 108: mp** 98-101 °C, **IR** (neat) 1719 (m), 1643 (s), 1504 (s), 1174 (s) cm^{-1}, **^{1}H NMR** (250 MHz, $CDCl_3$) δ_H 8.13 (1H, s, H-6), 7.84 (1H, t, J = 5.0 Hz, NH), 7.18 - 7.42 (9H, m, Ph + *p*-CH of DMT), 6.84 (4H, d, J = 8.9 Hz, 4 *o*-CH of DMT), 6.26 (1H, dd, J = 7.6, 5.8 Hz, H-1'), 6.16 (1H, s, NH), 5.36 (1H, d, J = 6.1 Hz, H-3'), 4.17 (1H, q, J = 2.2 Hz, H-4'), 4.12 (1H, dd, J = 18.1, 5.2 Hz, H-9), 4.01 (1H, dd, J = 17.7, 4.6 Hz, H-9), 3.78 (6H, s, CH_3O), 3.39 (1H, dd, J = 10.5, 3.3 Hz, H-5'), 3.32 (1H, dd, J = 9.3, 2.8 Hz, H-5'), 2.67 (1H, ddd, J = 14.0, 5.7, 1.3 Hz, H-2'), 2.23 - 2.35 (1H, m, H-2'), 2.06 (3H, s, Ac), **^{13}C NMR** (62.5 MHz, $CDCl_3$) δ_C 170.4 (C=O), 164.4 (C-4), 158.5 (COMe), 157.0 (q, J

= 37.7 Hz, *C*OCF$_3$), 154.3 (C-2), 144.4 (C-6), 144.2 (C), 135.6 (C), 135.5 (C), 129.9 (CH), 129.9 (CH), 128.0 (CH), 127.9 (CH), 127.0 (CH), 116.1 (q, J = 287.3 Hz, CF$_3$), 113.3 (CH), 90.5 (C-5/C-7), 89.5 (C-5/C-7), 87.1 (C-Ar$_3$), 86.6 (C-4'), 84.4 (C-1'), 74.9 (C-8), 74.8 (C-3'), 63.5 (C-5'), 55.3 (CH$_3$O), 39.5 (C-2'), 30.3 (C-9), 20.9 (CH$_3$), **APCI**$^-$ m/z (%) 719.5 (M-H$^-$, 100), **HRMS** (FAB) for $C_{37}H_{36}N_4O_8F_3$ $(M+H)^+$: calcd 721.2480, found 721.2470, **HPLC** (λ_{280}) t_R 8.16 min (Method 3), **R$_f$** 0.34 (10 % MeOH/CH$_2$Cl$_2$); **Data for 109: mp** 95-98 °C, **IR** (neat) 2931 (w), 1737 (s), 1645 (s), 1508 (m), 1245 (s), 1032 (m) cm^{-1}, **^{1}H NMR** (250 MHz, CDCl$_3$) δ_H 7.80 (1H, d, J = 7.5 Hz, H-6), 7.22 - 7.39 (9H, m, Ph + *p*-CH of DMT), 6.83 (4H, d, J = 8.8 Hz, 4 *o*-CH of DMT), 6.32 (1H, dd, J = 7.4, 5.8 Hz, H-1'), 5.55 (1H, d, J = 7.5 Hz, H-5), 5.36 (1H, d, J = 6.2 Hz, H-3'), 4.16 (1H, q, J = 2.6 Hz, H-4'), 3.79 (6H, s, CH$_3$O), 3.43 (2H, d, J = 2.9 Hz, H-5'), 2.59 (1H, ddd, J = 14.1, 5.5, 2.1 Hz, H-2'), 2.20-2.31 (1H, m, H-2'), 2.05 (3H, s, Ac), **^{13}C NMR** (62.5 MHz, CDCl$_3$) δ_C 170.8 (C=O), 165.4 (C-4), 159.1 (COMe), 156.1 (C-2), 144.7 (C-6), 141.6 (C), 135.7 (C), 135.6 (C), 130.5 (CH), 128.5 (CH), 128.4 (CH), 127.5 (CH), 113.8 (CH), 113.7 (CH), 95.1 (C-5), 87.4 (C-4'), 86.4 (C-1'), 84.4 (C-Ar$_3$), 75.0 (C-3'), 63.7 (C-5'), 55.7 (CH$_3$O), 39.5 (C-2'), 21.4 (CH$_3$), **APCI**$^-$ m/z (%) 570.4 (M-H$^-$, 100), **HRMS** (FAB) for $C_{32}H_{34}N_3O_7$ $(M+H)^+$: calcd 572.23968, found 572.23907, **HPLC** (λ_{280}) t_R 7.37 min (Method 3), **R$_f$** 0.33 (10 % MeOH/CH$_2$Cl$_2$).

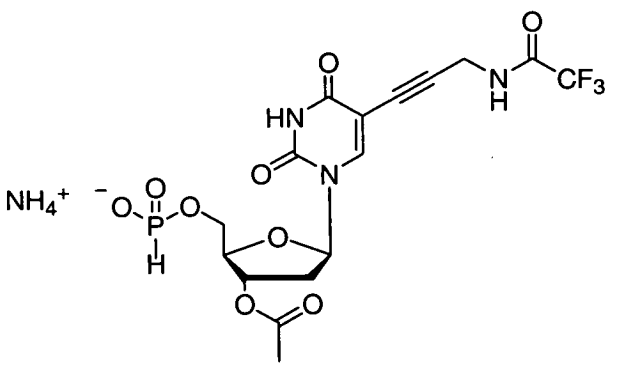
3'-*O*-Acetyl-5-(3''-trifluoroacetamidopropynyl)-2'-deoxycytidine (110)



Nucleoside **108** (262 mg, 0.364 mmol) was dissolved in 3 % TCA in CH$_2$Cl$_2$ (10 mL). After 5 min, MeOH (5 mL) was added, evaporated *in vacuo* and purified by column chromatography eluted with 0-7.5 % MeOH/CH$_2$Cl$_2$ to yield a yellow oil, which was lyophilized to yield a yellow powder (145 mg, 0.347 mmol, 95 % yield). **mp** 105-108 °C, **IR** (neat) 3205 (w), 1717 (s), 1647 (s), 1603 (m), 1506 (s),

1186 (s), 1157 (s) cm^{-1}, **^{1}H NMR** (250 MHz, CD_3OD) δ_H 8.37 (1H, s, H-6), 6.22 (1H, dd, J = 7.9, 5.7 Hz, H-1'), 5.28 (1H, dt, J = 6.2, 1.9 Hz, H-3'), 4.33 (2H, s, H-9), 4.15 (1H, q, J = 2.8 Hz, H-4'), 3.85 (1H, dd, J = 11.9, 2.7 Hz, H-5'), 3.79 (1H, dd, J = 11.9, 3.0 Hz, H-5'), 2.52 (1H, ddd, J = 14.0, 5.7, 1.9 Hz, H-2'), 2.26 (1H, ddd, J = 14.2, 7.8, 6.4 Hz, H-2'), 2.09 (3H, s, CH_3), **^{13}C NMR** (62.5 MHz, CD_3OD) δ_C 172.2 (C=OCH_3), 165.7 (C-4), 158.8 (q, J = 37.4 Hz, $COCF_3$), 155.7 (C-2), 146.5 (C-6), 117.3 (q, J = 286.3 Hz, CF_3), 92.4 (C-5), 91.4 (C-7), 88.1 (C-4'), 87.3 (C-1'), 76.3 (C-3'), 75.0 (C-6), 62.7 (C-5'), 39.7 (C-2'), 31.0 (C-9), 20.9 (CH_3), **APCI$^-$** m/z (%) 417.2 ($M\text{-}H^-$, 100), **HRMS** (FAB) for $C_{16}H_{18}N_4O_6F_3$ $(M+H)^+$: calcd 419.1173, found 419.1166, **HPLC** (λ_{280}) t_R 5.72 min (Method 3), **R_f** 0.10 (10 % $MeOH/CH_2Cl_2$).

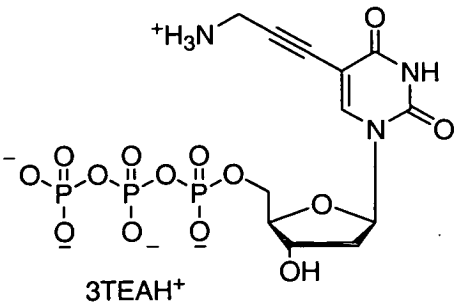
3'-*O*-Acetyl-5-(3''-trifluoroacetamidopropynyl)-2'-deoxyuridine 5'-*H*-phosphonate ammonium salt (112)



Nucleoside **100** (40 mg, 95 μmol) was evaporated *in vacuo* from dry pyridine (1 mL), dried over P_2O_5 in a desiccator for 1 h *in vacuo* and dissolved in dry pyridine (0.25 mL) and dry THF (0.75 mL). Resin **41a** (0.27 mmol, 3 eq) was swollen in dry THF (2 x 2 mL for 5 min) at 60 °C. PCl_3 (95 μL, 12 eq) was added to the resin in dry THF (4 mL) and the syringe was heated at 60 °C for 2 h. The resin was drained and rinsed with dry THF (2 x 2 mL for 5 min). The N_2 flushed resin was treated with the nucleoside solution for 1 h at rt, drained and rinsed with dry THF (2 x 2 mL for 5 min). The resin was suspended with THF/H_2O (1/1, 4 mL) for 10 min, then drained and evaporated *in vacuo*. The residue was purified by Prep HPLC eluted with an MeCN/0.1 M ammonium acetate gradient to yield a white hygroscopic powder (27 mg, 54 μmol, 57 % yield). **^{1}H NMR** (250 MHz, D_2O) δ_H 8.33 (1H, s, H-6), 6.91 (1H, d, J = 638.9 Hz, PH), 6.42 (1H, dd, J = 8.0, 6.0 Hz, H-1'), 5.49 (1H, dt, J = 6.0, 2.0 Hz, H-3'), 4.53 – 4.58 (1H, m, H-4'), 4.46 (2H, s, H-

9), 4.23 (1H, dd, J = 5.9, 3.1 Hz, H-5'), 2.72 (1H, ddd, J = 14.7, 6.0, 2.0 Hz, H-2'), 2.56 (1H, ddd, J = 14.8, 8.2, 6.4 Hz, H-2'), 2.26 (3H, s, CH_3), **^{13}C NMR** (90 MHz, D_2O) δ_C 173.9 (C=OCH_3), 164.9 (C-4), 150.8 (C-2), 145.3 (C-6), 99.2 (C-5), 88.4 (C-7), 86.6 (C-1'), 84.2 (d, J_{PC} = 8.4 Hz, C-4'), 75.6 (C-3'), 74.7 (C-6), 63.7 (d, J_{PC} = 4.1 Hz, C-5'), 37.5 (C-2'), 30.3 (C-9), 20.8 (CH_3), $COCF_3$ signals not visible due to low concentration, **^{31}P NMR** (101 MHz, D_2O) δ_P 7.44, **ES$^-$** m/z (%) 482.1 ($M\text{-}H^-$, 100), **HPLC** (λ_{280}) t_R 5.97 min (Method 9).

5-propargylammonium 5'-triphosphate tetraethylammonium salt (93)



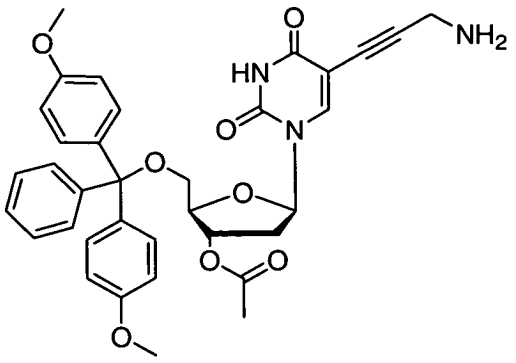
Using general procedure: Nucleoside **100** (37 mg, 88 μmol) and resin **41a** (209 mg, 0.280 mmol, 1 eq) were used. The final deprotection step was left for 1 h and the residue was purified twice by Prep HPLC eluted with 0.1 M TEAB to yield a white solid. **^{1}H NMR** (250 MHz, D_2O) δ_H 8.49 (1H, s, H-6), 6.30 – 6.35 (1H, m, H-1'), 4.69 – 4.70 (1H, m, H-3'), 4.31 – 4.32 (1H, m, H-4'), 4.24 – 4.26 (2H, m, H-9), 4.05 (2H, s, H-5'), 2.45 – 2.47 (2H, m, H-2'), **^{31}P NMR** (101 MHz, D_2O) δ_P -5.1 (m, P-γ), -10.2 (m, P-α), -21.6 (m, P-β), **ES$^-$** m/z (%) 520.1 ($M\text{-}H^-$, 100), **HPLC** (λ_{280}) t_R 6.92 min (Method 5). The yield was estimated using UV/Vis spectrometry. **93** was dissolved in 0.1 M TEAB buffer to 10 mL and the absorbance value measured (Table 6.3). The number of moles was calculated to give an estimated yield of 1 %.

Table 6.2 Determination of the yield using UV-Vis

Dilution mL	A	conc M	n μmol	yield % [b]
10	0.6134	$6.82\ 10^{-5}$	0.68	0.8

[a] ε_{495} = 9000 M^{-1} cm^{-1}, [b] based on starting material 88 μmol

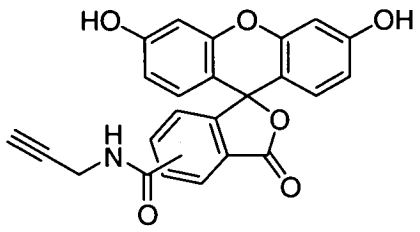
3'-*O*-Acetyl-5-(3''-aminopropynyl)-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxyuridine (115)



Method 1: $NaBH_4$ (61 mg, 1.61 mmol, 5.7 eq) and anhydrous $CaCl_2$ (161 mg, 1.45 mmol, 5.2 eq) were flushed with N_2. Nucleoside **99** (203 mg, 0.281 mmol, 1.0 eq) was evaporated from dry EtOH (5 mL), flushed with N_2, dissolved in dry EtOH (5.6 mL) and added dropwise onto the powders stirred over an ice-bath. The reaction was kept at 0 °C for 10 min until the effervescence subsided and was then stirred at rt for a further 20 min. The reaction was quenched with the addition of 0.1 M NH_4OAc (20 mL), partially evaporated *in vacuo* and extracted with CH_2Cl_2 (3 x 10 mL). The organic layer was dried (Na_2SO_4), evaporated *in vacuo* and purified by column chromatography eluted with 0-3 % MeOH in 1%TEA/CH_2Cl_2 to yield a yellow oil (40 mg, 0.064 mmol, 23 % yield). **Method 2:** A vessel was charged with nucleoside **120** (1.00 g, 1.43 mmol), [Pd (PPh_3)$_4$] (167 mg, 0.145 mmol, 0.1 eq), copper (I) iodide (137 mg, 0.719 mmol, 0.5 eq), propargylamine (295 μL, 4.30 mmol, 3.0 eq), Amberlite IRA-67 resin (526 mg, 5.6 meq/g, 2.96 mmol, 2.1 eq) and DMF (7.2 mL). The reaction mixture was purged with N_2 and stirred for 2 h. The solution was diluted with MeOH (5 mL) and TEA (1 mL), filtered through a silica plug with MeOH (20 mL) and evaporated *in vacuo*. The residue was purified by column chromatography eluted with 0-6 % MeOH/CH_2Cl_2 to yield a brown powder (778 mg, 1.24 mmol, 87 % yield). **mp** 132-135 °C, **IR** (neat) 2930 (w), 1682 (s), 1606 (m), 1506 (m), 1280 (s) cm^{-1}, **^{1}H NMR** (250 MHz, $CD_3OD/CDCl_3$) δ_H 8.22 (1H, s, H-6), 7.19 - 7.45 (9H, m, Ph + *p*-CH of DMT), 6.86 (4H, d, J = 8.7 Hz, 4 *o*-CH of DMT), 6.26 (1H, dd, J = 7.8, 5.9 Hz, H-1'), 5.42 (1H, dt, J = 5.9, 2.0 Hz, H-3'), 4.17 (1H, q, J = 2.1 Hz, H-4'), 3.77 (6H, s, CH_3O), 3.67 (2H, s, H-9), 3.44 (1H, dd, J = 10.8, 3.0 Hz, H-5'), 3.38 (1H, dd, J = 11.0, 2.8 Hz, H-5'), 2.59 (1H, ddd, J = 14.5, 5.5, 1.5 Hz, H-2'), 2.40 - 2.51 (1H, m, H-2'),

2.06 (3H, s, CH_3), **^{13}C NMR** (62.5 MHz, $CD_3OD/CDCl_3$) δ_C 171.8 (C=OCH_3), 164.2 (C-4), 159.8 (COMe), 150.7 (C-2), 145.6 (C), 144.4 (C-6), 136.5 (C), 136.4 (C), 130.9 (CH), 128.8 (CH), 127.8 (CH), 114.2 (CH), 99.6 (C-5), 88.1 (C-Ar_3), 87.8 (C-7), 86.7 (C-4'), 85.6 (C-1'), 77.5 (C-8), 75.9 (C-3'), 64.4 (C-5'), 55.8 (CH_3O), 39.4 (C-2'), 31.1 (C-9), 21.0 (CH_3), **ES$^+$** m/z (%) 626.2 ($M+H^+$, 100), **HRMS** (FAB) for $C_{35}H_{36}N_3O_8$ $(M+H)^+$: calcd 626.2502, found 626.2505, **HPLC** (λ_{282}) t_R 2.13 min (Method 1), 5.35 min (Method 2), 6.16 min (Method 3), **R_f** 0.32 (10 % MeOH/CH_2Cl_2, Ninhydrin), 0.47 (10 % MeOH in 1%TEA/CH_2Cl_2, Ninhydrin).

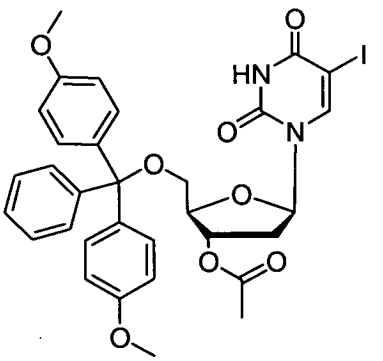
3-[5(6)-Carboxy-fluorescein]-amido-propyne (118)



5(6)-Carboxyfluorescein **119** (2.00 g, 5.32 mmol) in DMF (40 mL) was treated with PyBOP (2.72 g, 5.22 mmol, 0.98 eq) for 1 min, then propargylamine (365 µL, 5.32 mmol, 1.0 eq) and DIPEA (1.85 mL, 10.62 mmol, 2.0 eq) were added and the mixture was stirred for 2 h, then 2 M NaOH (10 mL) was added and stirred for 90 min. The reaction mixture was evaporated *in vacuo*, dissolved in H_2O (100 mL) and MeOH (200 mL). The greenish-yellow solution was acidified with 2 M HCl (*ca.* 50 mL) and partially evaporated until the solution turned cloudy. The flask was cooled to 4 °C for 15 h and the yellow powder was filtered off (1.69 g, 4.09 mmol, 77 % yield). **IR** (neat) 3042 (w), 1716 (w), 1637 (s), 1586 (s), 1490 (s) cm^{-1}, **^{1}H NMR** (250 MHz, $(CD_3)_2SO$) δ_H 9.36 (1H, t, J = 5.5 Hz, NH, isomer A), 9.20 (1H, t, J = 5.5 Hz, NH, isomer B), 8.48 (1H, d, J = 0.7 Hz, CH, C_4 isomer 5), 8.27 (1H, dd, J = 8.0, 1.5 Hz, CH, C_6 isomer 5), 8.19 (1H, dd, J = 8.0, 1.2 Hz, CH, C_5 isomer 6), 8.09 (1H, d, J = 8.0 Hz, CH, C_4 isomer 6), 7.71 (1H, s, C_7 isomer 6), 7.39 (1H, d, J = 8.0 Hz, CH, C_7 isomer 5), 6.73 - 6.74 (2H, m, *m*-CH, isomers 5 & 6), 6.53 – 6.64 (4H, m, *o*-CH, isomers 5 & 6), 4.11 (2H, dd, J = 5.4, 2.4 Hz, CH_2, isomer A), 3.99 (2H, dd, J = 5.2, 2.6 Hz, CH_2, isomer B), 3.17 (1H, t, J = 2.4 Hz, H-C≡C, isomer A), 3.10 (1H, t, J = 2.4 Hz, H-C≡C, isomer B), **^{13}C NMR** (62 MHz, $(CD_3)_2SO$) δ_C 168.5 (C=O), 168.4 (C=O), 164.8 (C=O), 164.6 (C=O), 160.3 (C),

160.1 (C), 155.1 (C), 152.4 (C), 152.3 (C), 152.2 (C), 140.2 (C), 135.9 (C), 135.0 (CH, C_6 isomer 5), 129.9 (CH, C_5 isomer 6), 129.6 (*o*-CH), 129.5 (*o*-CH), 128.9 (C), 127.0 (C), 125.4 (CH, C_4 isomer 6), 124.8 (CH, C_7 isomer 5), 123.9 (CH, C_4 isomer 5), 122.8 (CH, C_7 isomer 6), 113.2 (*o*-CH), 113.1 (*o*-CH), 109.5 (C), 109.4 (C), 102.7 (*m*-CH), 81.3 (C), 81.1 (C), 73.5 (*C*≡CH), 73.5 (*C*≡CH), 29.1 (CH_2), 29.0 (CH_2), C≡*C*H signal obscured by DMSO peak, **ES**$^-$ m/z (%) 412.1 ($M\text{-}H^-$, 95), **HRMS** (FAB) for $C_{24}H_{15}N_1O_6$ $(M+H)^+$: calcd 413.08994, found 413.09032, **HPLC** (λ_{254}) t_R 6.44, 6.56 min (Method 3), $\mathbf{R_f}$ 0.25 (10 % MeOH:CH_2Cl_2).

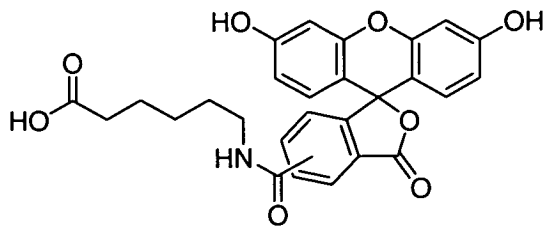
3'-*O*-Acetyl-5'-*O*-(4,4'-dimethoxytrityl)-5-iodo-2'-deoxyuridine (120)



Nucleoside **97** (2.00 g, 3.05 mmol) was dissolved in pyridine (20 mL) and treated with DMAP (95 mg, 0.78 mmol, 0.25 eq), TEA (1.1 mL, 7.83 mmol, 2.5 eq) and Ac_2O (310 μL, 3.04 mmol, 1.0 eq) and stirred for 2 h until complete (tlc). The reaction mixture was evaporated *in vacuo,* dissolved in EtOAc (100 mL), washed with H_2O (3 x 100 mL), dried (Na_2SO_4), evaporated *in vacuo* and purified by column chromatography eluted with 0-3 % MeOH/CH_2Cl_2 to yield a white foam (2.20 g, 3.10 mmol, 100 % yield). **IR** (neat) 3058 (w), 2834 (w), 1682 (s), 1606 (m), 1507 (w), 1300 (s) cm^{-1}, **^{1}H NMR** (250 MHz, $CDCl_3$) δ_H 9.50 (1H, br signal, H-3), 8.17 (1H, s, H-6), 7.23 – 7.44 (9H, m, Ph + *p*-CH of DMT), 6.86 (4H, d, *J* = 8.5 Hz, 4 *o*-CH of DMT), 6.35 (1H, dd, *J* = 9, 5 Hz, H-1'), 5.44 (1H, d, *J* = 6 Hz, H-3'), 4.15 (1H, m, H-4'), 3.79 (6H, s, CH_3O), 3.47 (1H, dd, *J* = 11, 3 Hz, H-5'), 3.40 (1H, dd, *J* = 11, 3 Hz, H-5'), 2.56 (1H, dd, *J* = 14, 5 Hz, H-2'), 2.39 (1H, m, H-2'), 2.09 (3H, s, CH_3), **^{13}C NMR** (90 MHz, $CDCl_3$) δ_C 170.3 (*C*=OCH_3), 159.9 (C-4), 158.6 (COMe), 150.1 (C-2), 144.1 (C-6), 143.9 (C), 135.3 (C), 135.1 (C), 130.0 (CH), 128.1 (CH), 128.0 (CH), 127.1 (CH), 113.3 (CH), 87.2 (C-Ar_3), 85.1 (C-4'), 84.4 (C-1'), 75.1 (C-3'), 69.1 (C-5), 63.4 (C-5'), 55.2 (CH_3O), 38.6 (C-2'),

20.9 (CH_3), **APCI**$^-$ m/z (%) 697.1 (M-H$^-$, 100), **HPLC** (λ_{282}) t_R 4.26 min (Method 1), 16.98 min (Method 2), $\mathbf{R_f}$ 0.62 (10 % MeOH/CH_2Cl_2, ammonium molybdate).

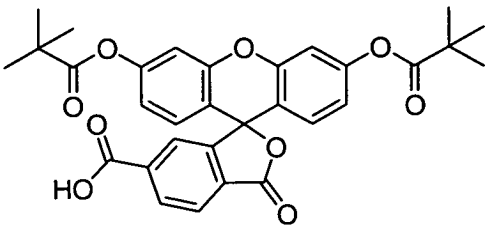
3-[5(6)-Carboxy-fluorescein]-amido-hexanoic acid (123)



5(6)-Carboxyfluorescein **119** (201 mg, 0.535 mmol) in DMF (4 mL) was treated with PyBOP (277 mg, 0.532 mmol) for 1 min, then 6-aminohexanoic acid methyl ester hydrochloride (98 mg, 0.534 mmol) and DIPEA (300 μL, 1.72 mmol, 3.2 eq) were added and the mixture was stirred for 2 h until complete (HPLC). The reaction mixture was evaporated *in vacuo*, dissolved in CH_2Cl_2 (5 mL) and 2 M HCl (5 mL) was added. The brown precipitate was filtered off, washed with H_2O (5 mL) and dried to give a brown powder. The residue was dissolved in DMF (5 mL) and was stirred with 2 M NaOH (1 mL) for 2 h. The reaction mixture was evaporated *in vacuo*, the residue dissolved in H_2O (5 mL), 2 M HCl (2 mL) was added, the precipitate filtered off and rinsed with H_2O to yield an orange-brown solid (184 mg, 0.376 mmol, 70 % yield). **IR** (neat) 2931 (w), 1707 (m), 1587 (s), 1538 (s), 1453 (s), 1205 (s), 1115 (s) cm^{-1}, **^{1}H NMR** (400 MHz, $(CD_3)_2SO$) δ_H 9.49 (1H, br signal, COOH, isomers 5 & 6), 8.81 (1H, t, J = 5.4 Hz, NH, isomer A), 8.67 (1H, t, J = 5.4 Hz, NH, isomer B), 8.45 (1H, d, J = 1.3 Hz, CH, C_4 isomer 5), 8.23 (1H, dd, J = 8.0, 1.3 Hz, CH, C_6 isomer 5), 8.17 (1H, dd, J = 8.0, 1.2 Hz, CH, C_5 isomer 6), 8.06 (1H, d, J = 8.0 Hz, CH, C_4 isomer 6), 7.67 (1H, d, J = 1.2 Hz, CH, C_7 isomer 6), 7.35 (1H, d, J = 8.0 Hz, CH, C_7 isomer 5), 6.71 - 6.72 (2H, m, *m*-CH, isomers 5 & 6), 6.56 – 6.57 (4H, m, *o*-CH, isomers 5 & 6), 3.28 – 3.33 (2H, m, CH_2, C_A isomer A), 3.16 – 3.21 (2H, m, CH_2, C_A isomer B), 2.22 (2H, t, J = 7.3 Hz, CH_2, C_E isomer A), 2.16 (2H, t, J = 7.4 Hz, CH_2, C_E isomer B), 1.51 – 1.58 (4H, m, CH_2, C_B + C_D isomer A), 1.42 – 1.49 (4H, m, CH_2, C_B + C_D, isomer B), 1.30 – 1.38 (2H, m, CH_2, C_C isomer A), 1.23 – 1.28 (2H, m, CH_2, C_C isomer B), **^{13}C NMR** (100 MHz, $(CD_3)_2SO$) δ_C 174.5 (CO_2H), 174.4 (CO_2H), 168.2 (C=O), 168.1 (C=O), 164.5 (C=O), 164.3 (C=O), 159.7 (C), 154.6 (C), 152.7 (C), 151.9 (C), 140.8 (C), 136.4 (C), 134.6 (CH, C_6 isomer 5), 129.4 (CH, C_5 isomer 6), 129.2

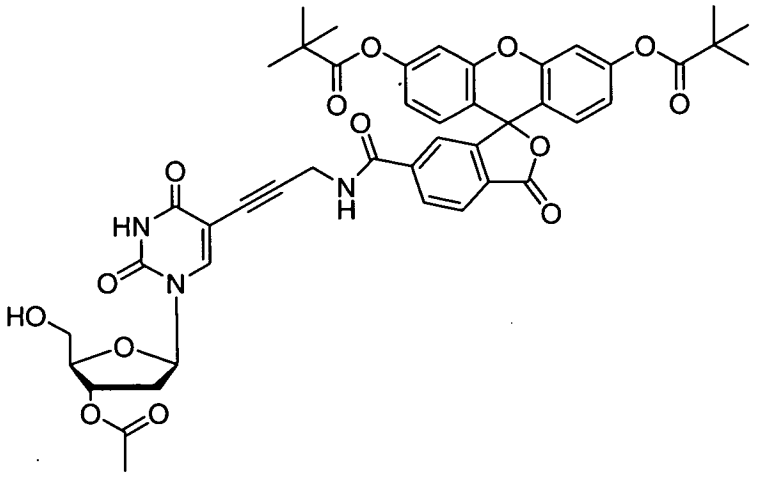
(*o*-CH), 129.1 (*o*-CH), 128.2 (C), 126.5 (C), 124.8 (CH, C_4 isomer 6), 124.2 (CH, C_7 isomer 5), 123.2 (CH, C_4 isomer 5), 122.2 (CH, C_7 isomer 6), 112.8 (*o*-CH), 112.7 (*o*-CH), 109.2 (C), 109.1 (C), 102.3 (*m*-CH), 83.4 (C), 39.3 (CH_2), 39.3 (CH_2), 33.7 (CH_2), 33.6 (CH_2), 28.7 (CH_2), 28.6 (CH_2), 26.0 (2 CH_2), 24.3 (CH_2), 24.2 (CH_2), **ES**$^+$ m/z (%) 490.2 ($M+H^+$, 100), **HPLC** (λ_{440}) t_R 6.89 min (Method 2), $\mathbf{R_f}$ 0.10 (5:95:2 MeOH:CH_2Cl_2:AcOH).

6-Carboxyfluorescein dipivalate (131)



5(6)-Carboxyfluorescein **119** (5.15 g, 13.7 mmol) was refluxed in Piv_2O (10 mL) for 2 h at 130 °C, cooled to rt, diluted with THF (10 mL) and H_2O (10 mL) and stirred at rt for 1 h. Et_2O (50 mL) was added and the lower phase separated. The organic layer was washed with 1 M pH 7 sodium phosphate buffer (20 mL), brine (2 x 25 mL), 1 M HCl (25 mL), brine (25 mL), dried ($MgSO_4$) and evaporated *in vacuo*. The yellow oil was dissolved in EtOH (50 mL), diisopropylamine (5 mL) was added and the solution cooled to -20 °C for 2 h. An off-white precipitate was filtered off, washed with ice-cold EtOH and acetone and vacuum-oven dried (1.89 g). The powder was dissolved in CH_2Cl_2 (50 mL), washed with 1 M HCl (2 x 25 mL), dried ($MgSO_4$) and evaporated *in vacuo* to yield a pale yellow foam (1.17 g, 2.16 mmol, 16 % yield). **IR** (neat) 2972 (w), 1756 (m), 1733 (s), 1480 (s), 1158 (s) cm^{-1}, **^{1}H NMR** (250 MHz, $CDCl_3$) δ_H 8.34 (1H, dd, J = 8.0, 1.3 Hz, CH, C_5), 8.12 (1H, dd, J = 8.0, 0.7 Hz, CH, C_4), 7.86 (1H, dd, J = 1.3, 0.8 Hz, CH, C_7), 7.08 (2H, *t*, J = 1.3 Hz, CH), 6.79 (4H, d, J = 1.3 Hz, CH), 1.35 (18H, s, $C(CH_3)_3$), **^{13}C NMR** (62 MHz, $CDCl_3$) δ_C 176.5 (C=O), 169.3 (C=O), 168.1 (C=O), 153.1 (C), 152.8 (C), 151.5 (C), 135.7 (C), 131.8 (CH, C_5), 130.2 (C), 128.7 (CH), 125.9 (CH, C_4), 125.5 (CH, C_7), 117.9 (CH), 115.3 (C), 110.5 (CH), 82.2 (*spiro*C), 39.2 ($C(CH_3)_3$), 27.0 (CH_3), **APCI**$^-$ m/z (%) 543.3 ($M-H^-$, 100), **HPLC** (λ_{254}) t_R 9.45 min (Method 3), $\mathbf{R_f}$ 0.35 (10 % MeOH:CH_2Cl_2).

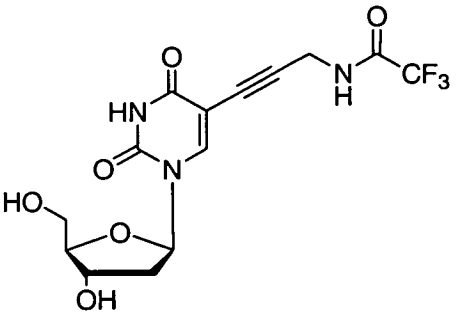
3'-*O*-Acetyl-5-(3''-(dipivaloyl-6-carboxamidofluorescein)-propynyl)-2'-deoxyuridine (132)



Nucleoside **122** (50 mg, 81 μmol) and 6-carboxyfluorescein dipivalate **131** (45 mg, 83 μmol) were dissolved in dry CH_2Cl_2 (0.4 mL) and treated with DIC (25 μL). The mixture was stirred for 2 h, evaporated *in vacuo*, purified by column chromatography eluted with 0-2 % MeOH in CH_2Cl_2 to yield a white powder (43 mg, ~46 % yield, $\mathbf{R_f}$ 0.68 (10 % MeOH/CH_2Cl_2)). The powder was dissolved in 3 % TCA in CH_2Cl_2 (3 mL), MeOH (1mL) was added and after 5 min, was evaporated *in vacuo*. The residue was purified by column chromatography eluted with 0-2 % MeOH in CH_2Cl_2 to yield a white powder (30 mg, 31 μmol, 37 % over two steps). **mp** 150 °C (decomp.), **IR** (neat) 2977 (w), 1753 (s), 1661 (s), 1616 (m), 1242 (m), 1113 (s) cm^{-1}, **^{1}H NMR** (250 MHz, CD_3OD) δ_H 8.13 (1H, s, H-6), 8.07 (1H, dd, J = 8.0, 1.3 Hz, CH, C_5), 8.01 (1H, dd, J = 8.0, 0.7 Hz, CH, C_4), 7.65 (1H, dd, J = 1.1, 0.8 Hz, CH, C_7), 7.02 - 7.03 (2H, m, CH), 6.80 (2H, dd, J = 8.7, 0.6 Hz, CH), 6.74 (2H, ddd, J = 8.7, 2.1, 0.9 Hz, CH), 6.07 (1H, dd, J = 8.2, 5.8 Hz, H-1'), 5.15 (1H, dt, J = 6.0, 1.9 Hz, H-3'), 4.07 (2H, s, H-9), 4.17 (1H, q, J = 2.5 Hz, H-4'), 3.65 (2H, d, J = 2.8 Hz, H-5'), 2.28 (1H, ddd, J = 14.0, 5.8, 1.9 Hz, H-2'), 2.15 (1H, ddd, J = 14.3, 8.3, 6.2, H-2'), 1.97 (3H, s, CH_3), 1.24 (18H, s, $C(CH_3)_3$), **^{13}C NMR** (62 MHz, CD_3OD) δ_C 178.4 (C=O), 172.5 (C=OCH_3), 170.3 (C=O), 167.8 (C=O), 164.9 (C-4), 155.0 (C), 154.8 (C), 153.3 (C), 151.4 (C-2), 145.7 (C-6), 142.5 (C), 131.5 (CH, C_5), 130.8 (CH), 130.1 (C), 126.9 (CH, C_4), 124.5 (CH, C_7), 119.8 (CH), 117.5 (C), 112.0 (C), 110.5 (CH), 100.5 (C-5), 90.2 (C-7), 87.5 (C-4'), 87.3 (C-1'), 83.8 (*spiro*C), 76.8 (C-3'), 76.0 (C-8), 63.2 (C-5'), 40.6 ($C(CH_3)_3$), 39.5 (C-2'), 31.5 (C-9), 27.8 (CH_3), 21.3 (CH_3), **APCI$^-$** m/z (%) 848.6 (M-H$^-$, 100),

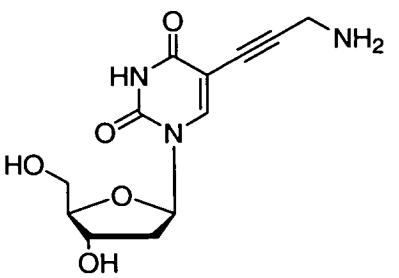
HRMS (FAB) for $C_{45}H_{44}N_3O_{14}$ $(M+H)^+$: calcd 850.2818, found 850.2844, **HPLC** (λ_{280}) t_R 8.41 min (Method 3), $\mathbf{R_f}$ 0.55 (10 % MeOH/CH_2Cl_2).

5-(3''-trifluoroacetamidopropynyl)-2'-deoxyuridine (133)



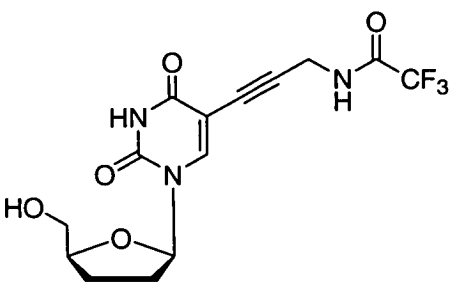
A vessel was charged with 5-iodo-2'-deoxyuridine **96** (90 mg, 0.253 mmol), [Pd $(PPh_3)_4$] (14 mg, 0.012 mmol, 0.047 eq), copper (I) iodide (13 mg, 0.068 mmol, 0.26 eq), trifluoroacetamidopropargylamine **95** (76 g, 0.50 mmol, 2.0 eq), Amberlite IRA-67 resin (230 mg, 1.29 mmol, 5.1 eq) and DMF (2.5 mL). The reaction mixture was purged with N_2 and stirred for 14 h at 60 °C. The solution was cooled to rt, filtered through a silica plug with MeOH/CH_2Cl_2 (1/2, 20 mL) and evaporated *in vacuo*. The residue was purified by column chromatography eluted with 0-15 % MeOH in CH_2Cl_2 to yield a yellow oil (73 mg, 0.20 mmol, 80 % yield). **^{1}H NMR** (250 MHz, CD_3OD) δ_H 8.32 (1H, s, H-6), 6.22 (1H, t, J = 6.5 Hz, H-1'), 4.39 (1H, dt, J = 6.8, 3.6 Hz, H-3'), 4.27 (2H, s, H-9), 3.94 (1H, q, J = 3.3 Hz, H-4'), 3.81 (1H, dd, J = 12.1, 2.9 Hz, H-5'), 3.73 (1H, dd, J = 12.0, 3.5 Hz, H-5'), 2.32 (1H, ddd, J = 14.5, 6.6, 4.2 Hz, H-2'), 2.21 (1H, dd, J = 13.6, 6.5 Hz, H-2'), **^{13}C NMR** (90 MHz, $CDCl_3$) δ_C 164.6 (C-4), 158.5 (q, J = 37.5 Hz, $COCF_3$), 151.1 (C-2), 145.8 (C-6), 117.4 (q, J = 286.6 Hz, CF_3), 99.5 (C-5), 89.2 (C-4'), 88.4 (C-7), 87.2 (C-1'), 76.1 (C-8), 72.0 (C-3'), 62.6 (C-5'), 41.7 (C-2'), 30.8 (C-9), **HPLC** (λ_{282}) t_R 2.38 min (Method 1), 9.50 min (Method 2), $\mathbf{R_f}$ 0.19 (10 % MeOH/CH_2Cl_2, Ninhydrin).

5-(3''-aminopropynyl)-2'-deoxyuridine (135)



Method 1: A vessel was charged with 5-iodo-2'-deoxyuridine **96** (449 mg, 1.27 mmol), [Pd $(PPh_3)_4$] (146 mg, 0.126 mmol, 0.10 eq), copper (I) iodide (120 mg, 0.630 mmol, 0.50 eq), propargylamine (255 μL, 3.72 mmol, 3.0 eq), Amberlite IRA-67 resin (446 mg, 2.50 mmol, 2 eq) and DMF (6.25 mL). The reaction mixture was purged with N_2 and stirred for 2 h. The solution was diluted with NH_3 sat. MeOH (5 mL) filtered twice through a silica plug with NH_3 sat. MeOH (20 mL) and evaporated *in vacuo*. The residue was purified by column chromatography eluted with 20 - 30 % NH_3 sat. MeOH in CH_2Cl_2 to yield a white powder (152 mg, 0.541 mmol, 43 % yield). **Method 2:** Nucleoside **133** (61 mg, 0.220 mmol) was dissolved in NH_4OH (10 mL) and stirred for 80 min until complete (tlc). The reaction was evaporated *in vacuo* and purified by column chromatography eluted with 20 - 30 % NH_3 sat. MeOH in CH_2Cl_2 to yield a white powder (42 mg, 0.149 mmol, 68 % yield). **IR** (neat) 3023 (w), 2840 (w), 1709 (s), 1684 (s) cm^{-1}, **^{1}H NMR** (250 MHz, $(CD_3)_2SO$) δ_H 8.39 (1H, s, H-3), 8.22 (1H, s, H-6), 6.10 (1H, t, J = 6.6 Hz, H-1'), 4.21- 4.26 (1H, m, H-3'), 3.80 (1H, q, J = 3.1 Hz, H-4'), 3.64 (2H, s, H-9), 3.56 (1H, dd, J = 12.0, 3.5 Hz, H-5'), 2.08 -2.13 (2H, m, H-2'), one H-5' signal obscured by H-9 signal, **^{13}C NMR** (90 MHz, $(CD_3)_2SO$) δ_C 161.6 (C-4), 149.4 (C-2), 143.7 (C-6), 98.0 (C-5), 90.8 (C-7), 87.6 (C-4'), 84.7 (C-1'), 75.8 (C-8), 70.2 (C-3'), 60.9 (C-5'), 41.2 (C-2'), 30.4 (C-9), **ES$^+$** m/z (%) 563.4 ($2M+H^+$, 100), **HRMS** (FAB) for $C_{12}H_{15}N_3O_5$ $(M)^+$: calcd 281.10117, found 281.10198, **HPLC** (λ_{282}) t_R 0.75 min (Method 1), 3.80 min (Method 2), 1.34 min (Method 3), **R_f** 0.09 (20 % NH_3 sat. MeOH/CH_2Cl_2, Ninhydrin).

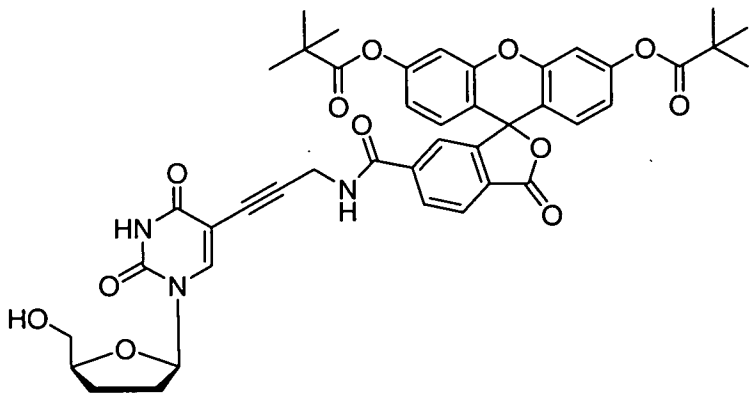
5-(3''-trifluoroacetamidopropynyl)-2',3'-dideoxyuridine (136)



A vessel was charged with 5-iodo-2',3'-dideoxyuridine (30 mg, 87.5 μmol), [Pd $(PPh_3)_4$] (4.5 mg, 3.9 μmol, 0.045 eq), copper (I) iodide (3.6 mg, 18.9 μmol, 0.22 eq), trifluoroacetamidopropargylamine **95** (27 mg, 0.178 mmol, 2.0 eq), Amberlite IRA-67 resin (77 mg, 0.431 mmol, 4.9 eq) and DMF (875 μL). The reaction

mixture was purged with N_2 and stirred for 22 h at rt. The solution was filtered through a silica plug with MeOH/CH_2Cl_2 (1/2, 20 mL) and evaporated *in vacuo*. The residue was purified by column chromatography eluted with 0-2 % MeOH in CH_2Cl_2 to yield starting material (15 mg, 44 μmol, 51 % yield) and **136** as a clear oil (9 mg, 25 μmol, 28 % yield). **^{1}H NMR** (250 MHz, CD_3OD) δ_H 8.47 (1H, s, H-6), 6.00 (1H, dd, *J* = 6.6, 3.0 Hz, H-1'), 4.27 (2H, s, H-9), 4.12 - 4.20 (1H, m, H-4'), 3.91 (1H, dd, *J* = 12.2, 2.8 Hz, H-5'), 3.68 (1H, dd, *J* = 12.3, 3.6 Hz, H-5'), 2.33 - 2.49 (1H, m, H-2'), 2.06 – 2.17 (1H, m, H-2'), 1.96 (1H, dd, *J* = 12.1, 4.5 Hz, H-3'), 1.93 (1H, dd, *J* = 14.1, 1.5 Hz, H-3'), **^{13}C NMR** (90 MHz, $CDCl_3/CD_3OD$) δ_C 163.8 (C-4), 149.9 (C-2), 144.9 (C-6), 98.0 (C-5), 87.4 (C-4'/C-1'), 87.3 (C-7), 82.9 (C-1'/C-4'), 75.7 (C-6), 62.4 (C-5'), 33.6 (C-2'/C-3'), 30.5 (C-9), 24.4 (C-2'/C-3'), $COCF_3$ signals not visible due to low concentration, **APCI$^-$** m/z (%) 360.2 (M-H$^-$, 100), **HPLC** (λ_{280}) t_R 5.54 min (Method 3), **R_f** 0.18 (10 % MeOH/CH_2Cl_2, $KMnO_4$).

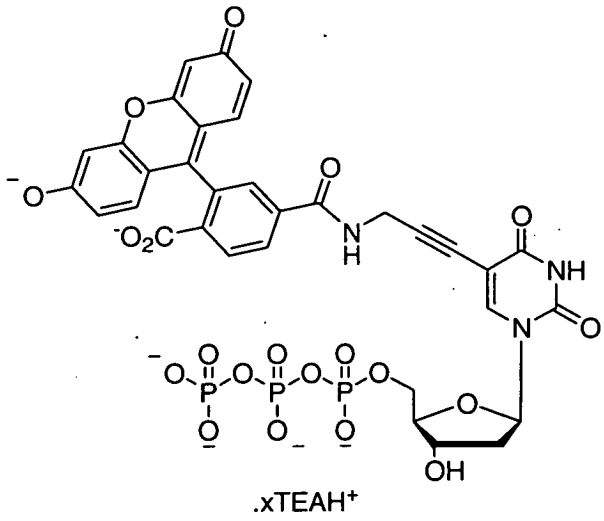
5-(3''-(dipivaloyl-6-carboxamidofluorescein)-propynyl)-2'-deoxyuridine (137)



Nucleoside **136** (9 mg, 25 μmol) was dissolved in NH_4OH (10 mL) and stirred for 1h 45 min and evaporated in vacuo. 6-Carboxyfluorescein dipivalate **131** (15 mg, 27 μmol) was dissolved in dry DMF/CH_2Cl_2 (1/2, 0.4 mL) and treated with DIC (4 μL) for 5 min, added to the dried nucleoside and stirred for 3 h. The mixture was evaporated *in vacuo*, purified by column chromatography eluted with 0-2 % MeOH in CH_2Cl_2 to yield a white powder (6 mg, ~30 % yield). Compound contained diisopropylurea, which co-purified. **^{1}H NMR** (250 MHz, CD_3OD) δ_H 7.94 (1H, s, H-6), 7.73 (1H, dd, *J* = 8.0, 1.4 Hz, CH, C_5), 7.67 (1H, dd, *J* = 8.1, 0.8 Hz, CH, C_4), 7.25 (1H, dd, *J* = 1.3, 0.8 Hz, CH, C_7), 6.69 (2H, d, *J* = 2.1 Hz, CH), 6.45 (2H, d, *J* = 8.6 Hz, CH), 6.40 (2H, dd, *J* = 8.7, 2.1 Hz, CH), 5.50 (1H, dd, *J* = 6.7, 3.0

Hz, H-1'), 3.78 (2H, s, H-9), 3.61 – 3.70 (1H, m, H-4'), 3.16 (1H, dd, J = 12.2, 3.5 Hz, H-5'), 1.83 - 1.98 (1H, m, H-2'), 1.54 – 1.65 (1H, m, H-2'), 1.39 -1.48 (2H, m, H-3'), 0.98 (18H, s, $C(CH_3)_3$), one H-5' signal obscured by CD_3OD signal.

5-propargylamido-(6-carboxyfluorescein) 5'-triphosphate ethylammonium salt (141)



Using general procedure: Nucleoside **132** (38 mg, 44 μmol) and resin **41a** (199 mg, 0.270 mmol, 1 eq) were used. The nucleoside was dissolved in dry pyridine/ dry THF (1/3, 0.25 mL) to load onto the resin. The loading was performed for 2 h. The final deprotection step was left for 1 h and the residue was purified by Prep HPLC eluted with 0.1 M TEAB to yield an orange solid. **^{31}P NMR** (101 MHz, D_2O/2 M TEAB) δ_P -6.40 (m, P-γ), -11.00 (m, P-α), -21.63 (m, P-β), **MALDI**$^-$ m/z (%) 878.31 ($M\text{-}H^-$, 80), **HPLC** (λ_{280}) t_R 9.52 min (Method 5). The yield was estimated using UV/Vis spectrometry. **141** was dissolved in 0.1 M TEAB buffer to 25 mL. Two further dilutions were performed and the absorbance values tabulated below (Table 6.3). The number of moles was calculated to give an estimated yield of 1 %.

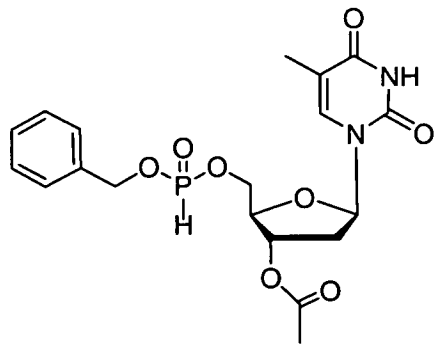
Table 6.3 Determination of the yield using UV-Vis

Dilution mL	A	conc M	n μmol	yield % [b]
25	1.5156	$1.89\ 10^{-5}$	0.47	1.1
62.5	0.6134	$7.67\ 10^{-6}$	0.48	1.1
125	0.3409	$4.26\ 10^{-6}$	0.53	1.2

[a] ε_{495} = 80000 M^{-1} cm^{-1}, [b] *based on starting material 44 μmol*

6.5 Experimental for Chapter 4

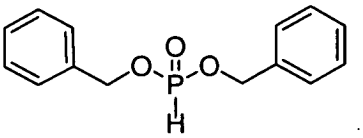
3'-*O*-Acetyl-5'-*O*-benzyloxy-thymidine 5'-*H*-phosphonate (154)



Resin **41a** (207 mg, 0.277 mmol, 1 eq) was swollen in dry THF (2 x 2 mL for 5 min each) at 60 °C. PCl_3 (95 μL, 4 eq) was added to the resin in dry THF (4 mL) and the syringe was heated at 60 °C for 2 h. 3'OAc-T **49** (25 mg, 88 μmol, 0.33 eq) was evaporated *in vacuo* from dry pyridine (1 mL), dried over P_2O_5 in a desiccator for 1 h *in vacuo* and dissolved in dry pyridine (0.25 mL) and dry THF (0.75 mL). The resin was drained and rinsed with dry THF (2 x 2 mL for 5 min each). The N_2 flushed resin was treated with the nucleoside solution for 1 h at rt, drained, rinsed with dry THF (2 x 2 mL for 5 min) and treated with anhydrous BnOH (1 mL) for 10 min. The cleaved product was collected in an RB, stirred at rt for 30 min, evaporated *in vacuo* and purified by column chromatography eluted with 0 – 4 % MeOH in CH_2Cl_2 to yield a clear oil **154**(3 mg, 7 μmol, 8 % yield) and recovered starting material **49** (6 mg, 21 μmol, 24 % yield). **IR** (neat) 2927 (m), 1739 (s), 1686 (s), 1469 (s), 1235 (s) cm^{-1}, **^{1}H NMR** (250 MHz, $CDCl_3$) δ_H 8.51 (1H, br signal, H-3), 7.44 (1H, d, J = 1.1 Hz, H-6), 7.39 (5H, m, Ph), 6.96 (1H, d, J = 708.5Hz, PH), 6.34 (1H, dd, J = 9.1, 5.3 Hz, H-1'), 5.18 (2H, s, CH_2), 5.14 (1H, s, H-3'), 4.21 – 4.37 (2H, m, H-5'), 4.14 (1H, dd, J = 4.3, 2.1, H-4'), 2.37 (1H, dtd, J = 10.4, 5.4, 1.5 Hz, H-2'), 2.10 (3H, s, $COCH_3$), 2.01 – 2.14 (1H, m, H-2'), 1.91 (3H, d, J = 1.2 Hz, CH_3), **^{13}C NMR** (62.5 MHz, D_2O) δ_C 170.5 (C=OCH_3), 164.4 (C-4), 152.7 (C-2), 134.9 (d, J = 6.8 Hz, *i*-C), 135.0 (C-6), 129.0 (CH), 128.8 (CH), 128.2 (CH), 111.8 (C-5), 84.5 (C-1'), 82.6 (d, J = 7.0 Hz, C-4'), 74.1 (C-3'), 69.8 (d, J = 11.7 Hz, Ph-CH_2), 65.1 (d, J_{PC} = 4.7 Hz, C-5'), 37.0 (C-2'), 20.8 (CH_3O), 12.3 (CH_3), **^{31}P NMR** (101 MHz, $CDCl_3$) δ_P 10.25, 9.05, **ES$^+$** m/z (%) 439.2 ($M+H^+$, 100), **HRMS** (FAB) for $C_{32}H_{33}N_3O_7I_1$ $(M+H)^+$: calcd 698.1358, found

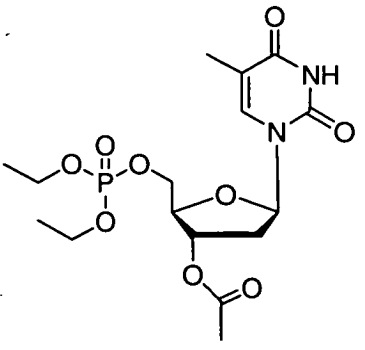
698.1355, **HPLC** (λ_{280}) t_R 6.28 min (Method 3), $\mathbf{R_f}$ 0.26 (10 % MeOH/CH_2Cl_2, ammonium molybdate).

Dibenzyl phosphite (155)



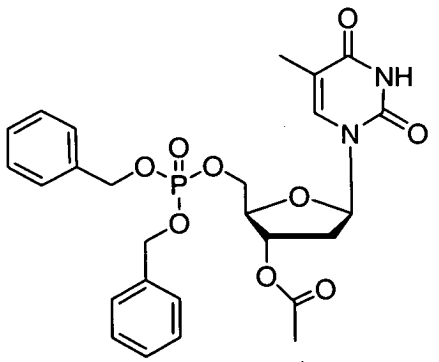
Resin **41a** (205 mg, 0.275 mmol, 1 eq) was swollen in dry THF (2 x 2 mL for 5 min) at 60 °C. PCl_3 (95 μL, 4 eq) was added to the resin in dry THF (4 mL) and the syringe was heated at 60 °C for 2 h. 3'OAc-T **49** (25 mg, 88 μmol, 0.33 eq) was evaporated *in vacuo* from dry pyridine (1 mL), dried over P_2O_5 in a desiccator for 1 h *in vacuo* and dissolved in dry pyridine (0.25 mL) and dry THF (0.75 mL). The resin was drained and rinsed with dry THF (2 x 2 mL for 5 min). The N_2 flushed resin was treated with the nucleoside solution for 1 h at rt, drained, rinsed with dry THF (2 x 2 mL for 5 min) and treated with anhydrous BnOH:^{t}BuOH (1:1, 1 mL) for 10 min. The cleaved product was collected in an RB, stirred at rt for 30 min, evaporated *in vacuo* and purified by column chromatography eluted with 0 – 4 % MeOH in CH_2Cl_2 to yield dibenzylphosphite **155** as a clear oil (7 mg, 27 μmol, 30 % yield), a clear oil **154** (3 mg, 7 μmol, 7 % yield) and recovered starting material **49** (12 mg, 42 μmol, 48 % yield). **^{1}H NMR** (250 MHz, $CDCl_3$) δ_H 7.52 (10H, m, Ph), 6.94 (1H, d, J = 706.7 Hz, PH), 5.10 (2H, dd, J = 11.9 , 9.8 Hz, CH_2), 5.03 (2H, dd, J = 11.8, 9.4 Hz, CH_2), **^{13}C NMR** (62.5 MHz, $CDCl_3$) δ_C 146.3 (CH), 128.6 (CH), 128.0 (CH), 67.3 (d, J = 5.7 Hz, Ph-CH_2), *ipso*-C not visible, **^{31}P NMR** (101 MHz, $CDCl_3$) δ_P 8.94, $\mathbf{R_f}$ 0.39 (10 % MeOH/CH_2Cl_2, $KMnO_4$).

3'-*O*-Acetyl-5'-*O*-diethoxyphosphoryl-thymidine (157)



Resin **41a** (204 mg, 0.272 mmol, 1 eq) was swollen in dry THF (2 x 2 mL for 5 min each) at 60 °C. PCl_3 (95 μL, 4 eq) was added to the resin in dry THF (4 mL) and the syringe was heated at 60 °C for 2 h. 3'OAc-T **49** (25 mg, 89 μmol, 0.33 eq) was evaporated *in vacuo* from dry pyridine (1 mL), dried over P_2O_5 in a desiccator for 1 h *in vacuo* and dissolved in dry pyridine (0.25 mL) and dry THF (0.75 mL). The resin was drained and rinsed with dry THF (2 x 2 mL for 5 min each). The N_2 flushed resin was treated with the nucleoside solution for 1 h at rt, drained, rinsed with dry THF (2 x 2 mL for 5 min) and treated with dry EtOH (1 mL) for 10 min. The cleaved product was collected in an RB with 0.5 M I_2 in pyridine (800 μL). After 15 min, excess iodine was quenched with a few drops of 5 % $NaHSO_3$ solution. The reaction mixture was extracted with CH_2Cl_2 (3 x 3 mL), dried (Na_2SO_4) and purified by column chromatography eluted with 0-20 % acetone in Et_2O to yield recovered starting material (3 mg, 9 μmol, 10 % yield) and **157** as a clear oil (6 mg, 14 μmol, 15 % yield). **^{1}H NMR** (250 MHz, $CDCl_3$) δ_H 8.92 (1H, s, H-3), 7.51 (1H, d, J = 1.3 Hz, H-6), 6.40 (1H, dd, J = 9.2, 5.5 Hz, H-1'), 5.28 (1H, dt, J = 6.4, 1.3 Hz, H-4'), 4.32 (1H, ddd, J = 11.1, 5.1, 2.6 Hz, H-5'), 4.25 (1H, ddd, J = 11.0, 5.1, 2.3 Hz, H-5'), 4.10 (4H, dqd, J = 8.1, 7.1, 1.0 Hz, CH_2CH_3), 4.05 – 4.15 (1H, m, H-3'), 2.40 (1H, ddd, J = 14.0, 5.5, 1.3 Hz, H-2'), 2.16 (1H, ddd, J = 11.7, 7.9, 5.1, H-2'), 2.10 (3H, s, $COCH_3$), 1.94 (3H, d, J = 1.2 Hz, CH_3), 1.35 (3H, td, J = 7.1, 2.1 Hz, CH_2CH_3), 1.34 (3H, td, J = 7.1, 2.1 Hz, CH_2CH_3), **^{31}P NMR** (101 MHz, $CDCl_3$) δ_P 0.20, **ES$^-$** m/z (%) 421.2 ($M+H^+$, 100), **HPLC** (λ_{280}) t_R 6.38 min (Method 3), **R_f** 0.36 (20 % acetone/Et_2O, PMA).

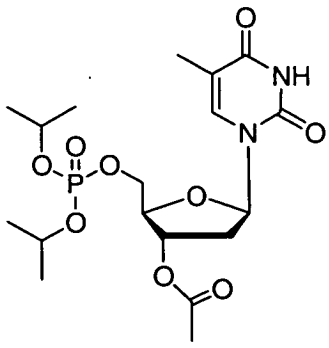
3'-*O*-Acetyl-5'-*O*-dibenzyloxyphosphoryl-thymidine (158)



Resin **41a** (205 mg, 0.275 mmol, 1 eq) was swollen in dry THF (2 x 2 mL for 5 min) at 60 °C. PCl_3 (95 μL, 4 eq) was added to the resin in dry THF (4 mL) and the syringe was heated at 60 °C for 2 h. 3'OAc-T **49** (25 mg, 88 μmol, 0.33 eq) was

evaporated *in vacuo* from dry pyridine (1 mL), dried over P_2O_5 in a desiccator for 1 h *in vacuo* and dissolved in dry pyridine (0.25 mL) and dry THF (0.75 mL). The resin was drained and rinsed with dry THF (2 x 2 mL for 5 min). The N_2 flushed resin was treated with the nucleoside solution for 1 h at rt, drained, rinsed with dry THF (2 x 2 mL for 5 min) and treated with dry BnOH (1 mL) for 10 min. The cleaved product was collected in an RB with 0.5 M I_2 in 98:2 pyridine:H_2O (800 μL, 1.5 eq). After 15 min, excess iodine was quenched with a few drops of 5 % $NaHSO_3$ solution. The reaction mixture was evaporated *in vacuo*, dissolved in CH_2Cl_2, washed with H_2O (3 x 3 mL), dried (Na_2SO_4) and purified by column chromatography eluted with 0 - 30 % acetone in Et_2O to yield a clear film (13 mg, 24 μmol, 27 % yield). **IR** (neat) 2957 (w), 1738 (m), 1686 (s), 1497 (w), 1236 (s) cm^{-1}, **^{1}H NMR** (250 MHz, $CDCl_3$) δ_H 8.44 (1H, s, H-3), 7.50 (1H, d, J = 1.1 Hz, H-6), 7.34 (10H, d, J = 1.2 Hz, Ph), 6.33 (1H, dd, J = 9.3, 5.4 Hz, H-1'), 4.99 – 5.14 (5H, m, CH_2 + H-4'), 4.23 (1H, ddd, J = 11.1, 5.0, 2.5 Hz, H-5'), 4.16 (1H, ddd, J = 11.1, 5.2, 2.3 Hz, H-5'), 4.08 (1H, td, J = 4.1, 2.3, H-3'), 2.27 (1H, ddd, J = 14.0, 5.4, 1.0 Hz, H-2'), 2.09 (3H, s, $COCH_3$), 1.88-1.93 (1H, m, H-2'), 1.84 (3H, d, J = 1.2 Hz, CH_3), **^{13}C NMR** (62.5 MHz, D_2O) δ_C 170.4 (*C*=OCH_3), 163.3 (C-4), 150.2 (C-2), 135.3 (d, J = 5.9 Hz, *i*-C), 135.0 (C-6), 128.9 (CH), 128.7 (CH), 128.1 (CH), 111.7 (C-5), 84.4 (C-1'), 83.7 (d, J = 8.4 Hz, C-4'), 74.6 (C-3'), 69.8 (d, J = 4.9 Hz, Ph-*CH*$_2$), 67.1 (d, J_{PC} = 5.8 Hz, C-5'), 37.1 (C-2'), 20.9 (CH_3O), 12.3 (CH_3), **^{31}P NMR** (101 MHz, D_2O) δ_P 0.59, **ES$^+$** m/z (%) 545.3 ($M+H^+$, 100), **HPLC** (λ_{280}) t_R 7.20 min (Method 3), **R_f** 0.39 (20 % acetone/Et_2O, PMA).

3'-*O*-Acetyl-5'-*O*-diisopropyloxyphosphoryl-thymidine (159)



Resin **41a** (201 mg, 0.269 mmol, 1 eq) was swollen in dry THF (2 x 2 mL for 5 min) at 60 °C. PCl_3 (95 μL, 4 eq) was added to the resin in dry THF (4 mL) and the syringe was heated at 60 °C for 2 h. 3'OAc-T **49** (25 mg, 88 μmol, 0.33 eq) was

evaporated *in vacuo* from dry pyridine (1 mL), dried over P_2O_5 in a desiccator for 1 h *in vacuo* and dissolved in dry pyridine (0.25 mL) and dry THF (0.75 mL). The resin was drained and rinsed with dry THF (2 x 2 mL for 5 min). The N_2 flushed resin was treated with the nucleoside solution for 1 h at rt, drained, rinsed with dry THF (2 x 2 mL for 5 min) and treated with dry *iso*-propanol (1 mL) for 10 min. The cleaved product was collected in an RB with 0.5 M I_2 in 98:2 pyridine:H_2O (800 μL, 1.5 eq). After 15 min, excess iodine was quenched with a few drops of 5 % $NaHSO_3$ solution. The reaction mixture was evaporated *in vacuo*, dissolved in CH_2Cl_2, washed with H_2O (3 x 3 mL), dried (Na_2SO_4) and purified by column chromatography eluted with 0 - 20 % acetone in Et_2O to yield a clear film (7 mg, 16 μmol, 18 % yield). **IR** (neat) 2925 (w), 1740 (m), 1692 (s), 1467 (s), 1239 (s) cm^{-1}, **^{1}H NMR** (360 MHz, $CDCl_3$) δ_H 8.21 (1H, s, H-3), 7.55 (1H, d, J = 1.3 Hz, H-6), 6.42 (1H, dd, J = 9.3, 5.4 Hz, H-1'), 5.31 (1H, dt, J = 6.2, 2.3 Hz, H-3'), 4.64 – 4.74 (2H, m, CH), 4.30 (1H, ddd, J = 11.1, 4.9, 2.4 Hz, H-5'), 4.21 (1H, ddd, J = 11.1, 4.9, 2.4 Hz, H-5'), 4.17 (1H, td, J = 4.1, 2.2, H-4'), 2.40 (1H, ddd, J = 14.0, 5.4, 1.2 Hz, H-2'), 2.18 (1H, ddd, J = 13.5, 9.3, 6.3 Hz, H-2'), 2.11 (3H, s, $COCH_3$), 1.97 (3H, d, J = 1.2 Hz, CH_3), 1.33 – 1.36 (12H, m, 4 CH_3), **^{13}C NMR** (90 MHz, $CDCl_3$) δ_C 170.4 (C=OCH_3), 163.2 (C-4), 150.1 (C-2), 135.0 (C-6), 111.7 (C-5), 84.4 (C-1'), 83.0 (d, J_{PC} = 8.4 Hz, C-4'), 74.8 (C-3'), 73.1 (d, J_{PC} = 5.4 Hz, CH), 66.7 (d, J_{PC} = 5.9 Hz, C-5'), 37.2 (C-2'), 29.6 (CH_3), 20.8 (CH_3O), 12.3 (CH_3), **^{31}P NMR** (101 MHz, D_2O) δ_P -1.67, **ES$^+$** m/z (%) 449.2 ($M+H^+$, 100), **HPLC** (λ_{280}) t_R 6.52 min (Method 3), **R_f** 0.38 (20 % acetone/Et_2O, PMA).

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Appendix

Publications

- UK patent 0606038.8 filed March 2006.
- Oral Presentation: Supported phosphorylation reagents - synthesis of nucleoside triphosphates, ACS National Meeting, Atlanta, USA (Mar 2006).
- Poster Presentation: Thiohydantoins – A technique for labeling peptides and proteins, Eurocombi-3, Winchester, UK (Jul 2005).
- Poster Presentation: Thiohydantoins – A technique for labeling peptides and proteins, 19th American Peptide Symposium, San Diego, USA (Jun 2005).
- Oral Presentation: A universal reagent for synthesis of triphosphates, Group Presentation, Edinburgh (May 2005).
- Oral Presentation: Thiohydantoins – A useful approach to protein labeling, 3rd year talk, Firbush (Apr 2005).
- Poster Presentation: Thiohydantoins – A useful way of labelling peptides and proteins, South West RSC Regional Meeting, Bristol, UK (Dec 2004).
- Poster Presentation: Thiohydantoins – A useful way of labelling peptides and proteins, 2nd Year Poster Presentation, Southampton, UK (Oct 2004).

Abstract ACS Conference

Supported phosphorylation reagents - synthesis of nucleoside triphosphates

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The importance of triphosphates and their analogues in DNA sequencing and enzyme and inhibition studies has led to various approaches for their synthesis, none of them wholly satisfactory for a wide range of alcohols and none of them suitable for use in a library synthesis. To address this issue, a supported phosphorylation reagent has been developed. The use of polystyrene based supports allows direct access to phosphonate mono and diesters, which could be oxidized to their phosphate esters. The effect of the nature of the solid support on the chemistry was studied and is discussed with respect to the synthesis of nucleoside triphosphates.