

Welsh School of Pharmacy – Cardiff University



Design and Synthesis of Potential Inhibitors Against dUTPase, a Novel Drug Target for the Control of Protozoal and Bacterial Infections

Thesis submitted to Cardiff University for the degree of

Doctor of Philosophy

By

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Abstract

The enzyme deoxyuridine triphosphate nucleotidohydrolase (dUTPase) is a ubiquitous enzyme that can be considered as the first line of defence against misincorporation of uracil into DNA. Inhibition of dUTPase is lethal in *E. coli*, *S. cerevisiae*[38,39] and is also likely to be lethal in many other organisms including *Plasmodium* spp. and *Leishmania* spp., which are the causative agents of malaria and leishmaniasis respectively.

By taking advantage of the differences in the structures of the enzymes in human and in the protozoa, dUTPase was chosen as a chemotherapeutic target. The main aim of this research project was to develop selective inhibitors against leishmanial and plasmodium dUTPases.

The research was focused on building a structure-activity relationship study making analogues of previously identified inhibitors within our research group. The work was also supported by molecular modelling studies on the *P. falciparum* enzyme. The synthesised analogues were nucleosides derivatives of dUMP, which is a proven inhibitor of the leishmanial dUTPase. The different series of compounds included 5'-tritylated nucleosides and acyclic derivatives of dUMP.

Three acyclic derivatives showed particular promise against the enzyme with sub-μM *Ki* values against *P. falciparum* dUTPase. Some compounds also inhibited *in vitro* the *P. falciparum* growth with IC_{50} values in the μM range. Promising results were obtained also against *L. donovani* parasite with IC_{50} values in the 10-50 μM range.

An antimicrobial screening of compounds previously made in the group was performed by the author against five bacteria strains (*E. coli*, *Ps. aeruginosa*, *E. faecalis*, *P. vulgaris* and *S. aureus*) to evaluate the potential application as an antibacterial agent.

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This work is dedicated to Sabrina who supported me for all this time, also to my Mother, my brother and sisters, and to the memory of my beloved Father.

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Abbreviation & Acronyms

ADME	Administration Distribution Metabolism Elimination
AP	Apyrimidinic
BER	Base Excision Repair
BMD	Broth Microdilution Method
Bn	Benzyl
BomCl	Benzyl chloromethyl ether
BSAC	British Society for Antimicrobial Chemotherapy
Bz	Benzoyl
CbzCl	Benzyl chloroformate
Cfu	Colony forming units
COSY	Correlation Spectroscopy
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
DHP	3,4-dihydro-α-pyran
DIAD	Diisopropylazodicarboxylate
DIBAL-H	Diisobutyl Aluminum Hydride
DIPEA	Diisopropylethylamine
DMAP	*N,N*-Dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
EIAV	Equine Infectus Anaemia Virus
FIV	Feline Immunodeficiency Virus
HLM	Human Liver Microsome
HSV	Herpes Simplex Virus
IC_{50}	Inhibitory Concentration
LAH	Lithium Aluminum Hydride
Lg	Leaving Group
MIC	Minimum Inhibitory Concentration
MMTr	4-monomethoxytrityl

NB	Nutrient Broth
NCCLS	National Committee for Clinical Laboratory Standards
NOESY	Nuclear Overhauser Effect Spectroscopy
OD_{500}	Optical Density at 500 nm wavelength
PPi	Inorganic Pyroshosphate
PPTS	Pyridinium *para*-toluenesulfonate
Py	Pyridine
PyTr	PyridylTrityl
r.t.	Room temperature
SAR	Structure Activity Relationship
TBAF	Tetrabutylammonium Fluoride
TBAI	Tetrabutylammonium Iodide
TBDMS	*tert*-butyldimethylsilyl
TBDPS	*tert*-butyldiphenylsilyl
TDR	Tropical Drug Research
TEA	Triethylamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
THP	Tetrahydropyranyl
TMEDA	*N,N,N',N'*-tetramethylethylenediamine
Tr	Triphenylmethyl (trityl)
TSA	Triptone Soya Agar
TSB	Triptone Soya Broth
UNG	Uracil DNA Glycosylase
WHO	World Health Organization
WSP	Welsh School of Pharmacy

Chapter 1 Background

1.1 Tropical Diseases

Countries of the tropical regions of the world, which are generally developing or undeveloped countries, are often affected by a range of diseases commonly referred to as "Tropical Diseases". Examples of the most important, according to the World Health Organization are given in Table 1-1. Climatic conditions and lack of resources (e.g. economic, health infrastructures) contribute to aggravating the burden of these diseases, that are for the most part endemic, in those countries[1].

Disease	Infective Organism	Disease Burden (DALYs*)	Cases (worldwide)	Deaths/Year (worldwide)
African sleeping sickness	*Trypanosome brucei*	1.8 million	300,000-500,000	41,000
Chaga's disease	*Trypanosome cruzi*	680,000	16-18 million	21,000
Dengue fever	Flaviviridae viruses	433,000	50 million	12,000
Leishmaniasis	*Leishmania donovani*	1.7 million	500,000	60,000
Leprosy	*Mycobacterium leprae*	141,000	740,000	2,000
Lymphatic filariasis	Wuchereria bancrofiti, Brugia timori and others	5.5 million	120 million	0
Malaria	Plasmodium spp	40.2 million	300 million	1,080,000
Onchocerciasis	*Onchocerca volvulus*	951,000	20 million	0
Schistosomiasis	Schistosoma mansoni and additional S. spp	1.6 million	200 million	50,000
Tuberculosis	*Mycobacterium tuberculosis*	35.8 million	62 million	1,660,000

Table 1-1 WHO Tropical Diseases Research (TDR) list of Diseases[2], *DALYs - Disability Adjusted Life Years (the number of healthy years of life lost due to premature death and disability). The figures reported in the last column (Deaths/Year) are estimations, and it is acknowledged that the real values are likely higher to be than those indicated in the table.

From Table 1-1 we can see that Malaria and Leishmaniasis are two of the most widespread parasitic infections in terms of number of cases. They are second in number of deaths/year only to Tuberculosis.

Companies are unwilling to target these diseases for drug development because of the lack of economic incentive. Of the 1393 new drugs introduced between 1975 and 1999, only 16 were indicated for treatment of tropical diseases that the World Health Organization, Tropical Drug Research (TDR) division, has placed on its list of targeted diseases[1].

Therefore there is a continuous need to develop new drugs against these kinds of diseases; this is exacerbated by the raising of new drug resistant strains of parasites and bacteria, the increasing number of deaths per year and the inefficacy of the old treatments, some of which were introduced more than fifty years ago.

1.2 Malaria

Malaria remains among the most challenging disease that afflicts humankind, both in its prevalence and in the morbidity and mortality it causes[3].

Human malaria is caused by infection with intracellular parasites of the genus Plasmodium that are transmitted by Anopheles mosquitoes. Of the four species of *Plasmodium* (*P. falciparum, P. vivax, P. malaria* and *P. ovale*) which infect humans, *Plasmodium falciparum* is the most lethal. The parasite *P. falciparum* is responsible for hundreds of millions of cases of malaria, and kills more than one million African children annually[2].

Despite more than a century of efforts to eradicate or control malaria, the disease remains a major and growing threat to the public health and economic development of countries in the tropical and subtropical regions of the world. Approximately 40% of the world population lives in areas where malaria is transmitted and there are an estimated 300-500 million cases with up to 1 million deaths from malaria each year. The mortality levels are greatest in sub-Saharan Africa, where children under 5 years of age account for 90% of all deaths due to malaria[2].

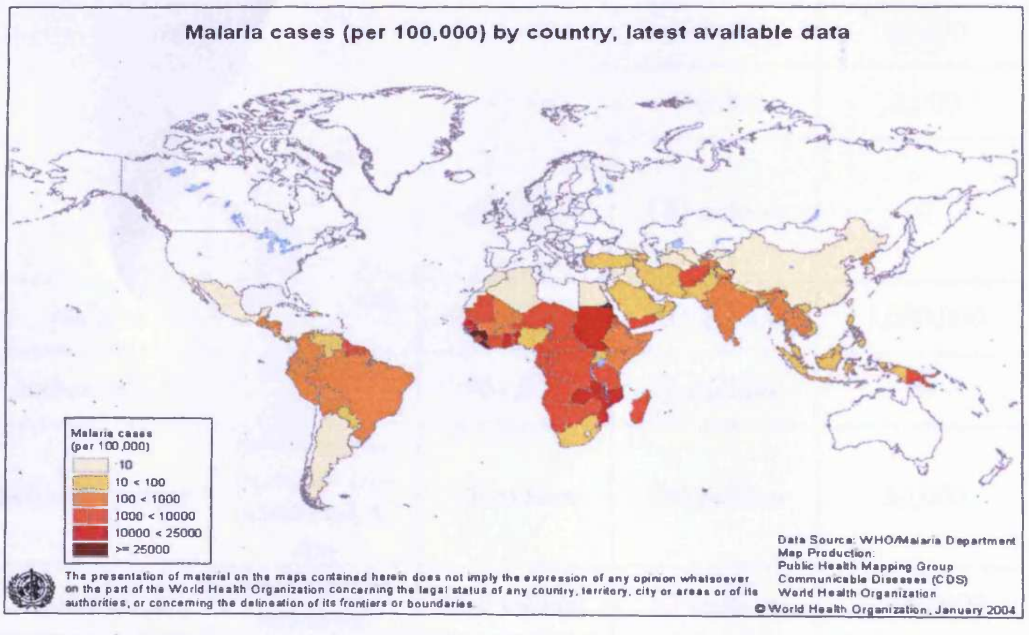


Figure 1-1 Malaria distribution in the world[4]

1.2.1 The disease & Plasmodium parasite

The disease is characterized by shaking chills, relapsing fever, mental or physical exhaustion, splenomegaly and anaemia. The infective stage of the parasite, the sporozoite, is injected into the bloodstream by a mosquito from its salivary gland while it is feeding on mammalian blood (see Figure 1-2).

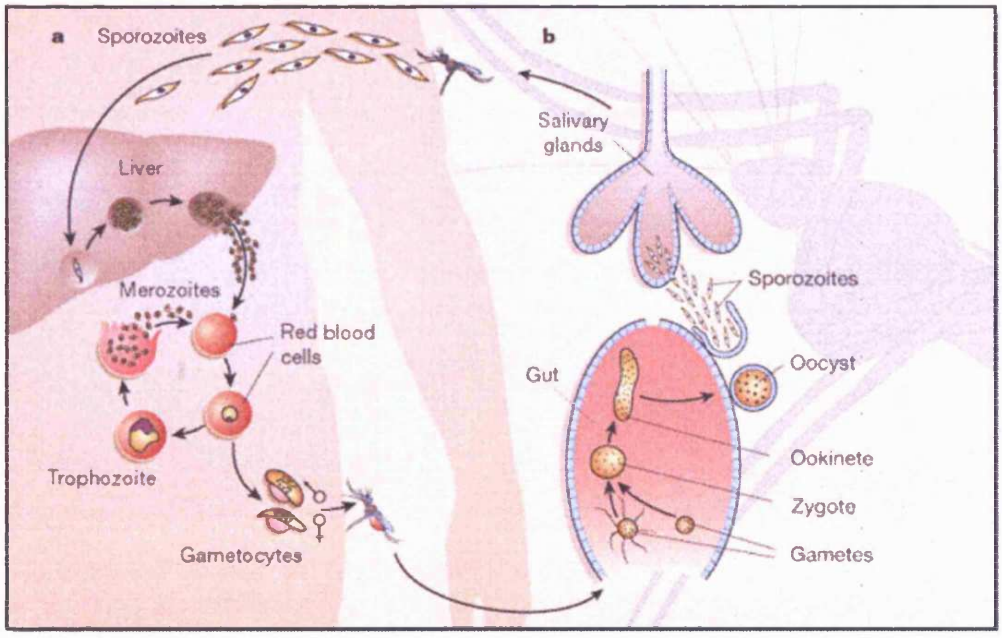


Figure 1-2 Life Cycle of Plasmodium[5]; (a) in the human host; (b) in the insect vector

After about 1 hour of circulating in the mammalian bloodstream the sporozoites enter the hepatic microcirculation and then move into hepatocytes. Development of the parasites in the hepatocytes lasts for different durations (5-13 days) depending on the type of *Plasmodium* species. This stage is termed exoerythrocytic schizogony when the parasites multiply rapidly, eventually forming thousands of merozoites. These are released into the bloodstream and enter the erythrocytes through specific receptors, thus establishing the erythrocytic cycle.

There are important differences between parasite species as to how the hepatic stage ends. In *P. falciparum* and *P. malariae* the liver stage terminates at the beginning of the erythrocytic stage. In *P. vivax* and *P. ovale* the liver form persists, differentiating into stages termed hypnozoites. The dormant hypnozoites remain in the hepatocytes for some time. They undergo schizogony and cause the relapse of the disease when they invade the red cells again. The merozoite stage in the

erythrocyte grows and develops into a ring-shaped trophozoite and the multinucleated erythrocytic schizonts. These latter multiply (8-12 per schizont) and develop in the infected erythrocytes until they destroy the erythrocytes and are released into the bloodstream to continue the infectious cycle by invading more erythrocytes. After several erythrocytic cycles some merozoites develop into female and male gametocytes, which are transmitted to the stomach of the mosquito as it sucks blood from the mammalian host. In the mosquito's stomach fertilization occurs to form zygotes, which develop into ookinetes, which penetrate the mosquito stomach wall and multiply, forming a growing oocyst. This stage eventually will rupture, releasing sporozoites that migrate to and accumulate in the salivary gland of the mosquito ready for infecting the next victim.

Effective chemotherapy against malaria must be targeted not only to the species of the parasite but to specific stages of parasite development. Therefore an ideal drug against malaria should be active against all stages of the parasite.

1.2.2 Current treatments

Resistance to anti-malaria drugs and insecticides, the decay of public health infrastructure, population movements, political unrest, and environmental changes are contributing to the spread of malaria. In countries with endemic malaria, the annual economic growth rates over a 25-year period were 1.3% lower than in other countries. Malaria cases may double in the next 20 years if new methods of control are not devised and implemented[6].

In the following tables are reported the main current treatment against malaria, according to the "British National Formulary 2004", and the structures of the main antimalarial compounds.

In order to combat the increasing problems of drug resistance, the latest treatments introduced against malaria are combinations. The two components are chosen to act synergistically, targeting different metabolic pathway of the parasite. The combinations approved by the BNF are reported in Table 1-4. The structures for the components are also included in Figure 1-3.

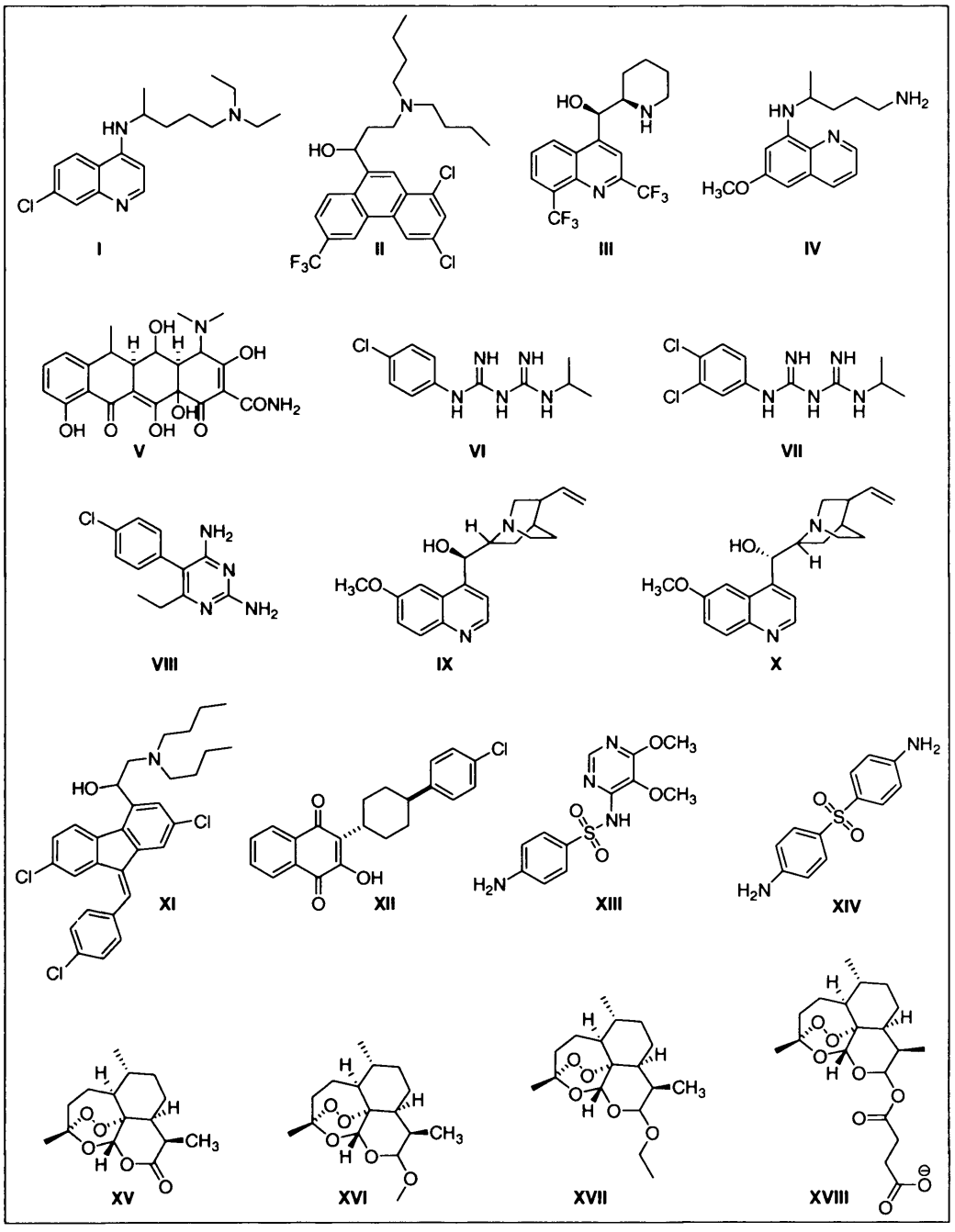


Figure 1-3 Structures of the main antimalarial drugs: (I) Chloroquine, (II) Halofantrine, (III) Mefloquine, (IV) Primaquine, (V) Doxycycline, (VI) Proguanil, (VII) Chloroproguanil, (VIII) Pyrimethamine, (IX) Quinine, (X) Quinidine, (XI) Lumefantrine, (XII) Atovaquone, (XIII) Sufadoxine, (XIV) Dapsone, (XV) Artemisinin, (XVI) Artemether, (XVII) Arteether, (XVIII) Artesunate.

Name	Structure	Chemical class	Indication	Mechanism of Action	Disadvantages	Biological Activity	References
Artemether	16	Sesquiterpene acetal with endoperoxide	Treatment of severe chloroquine resistant malaria	Oxidative stress and alkylation of heme	Probable neurotoxicity	Blood Schizontocide	7,8,9
Artesunate	18	Sesquiterpene ester endoperoxide	Treatment of severe chloroquine resistant malaria	Oxidative stress and alkylation of heme	Low stability at pH 7, intravenous administration	Blood Schizontocide	7,8,9
Chloroquine	1	4-aminoquinoline	Chemoprophylaxis and treatment of malaria	Inhibits Hemozoin formation	Widespread resistance	Blood Schizontocide	7,10,11,12, 13,14,15,16
Mefloquine	3	4-quinoline methanol	Chemoprophylaxis and treatment of uncomplicated falciparum malaria and vivax malaria	Inhibits Hemozoin formation	Neurotoxicity, increase the probability of stillbirth in pregnant women, high costs.	Blood Schizontocide	7,16
Primaquine	4	8-aminoquinoline	Adjunct in the treatment of *P. ovale* and *P. vivax* malaria	Probable interference with the parasite's energy metabolism in the mitochondria	Widespread resistance. Can induce intravascular haemolysis not suitable for pregnant women and G6DP deficient patients	Tissue schizontocide and hypnozoiticide	7,16,17

Table 1-2 Principal antimalarial drugs suggested for treatment and the prophylaxis of malaria according to the BNF vol 46

Name	Structure	Chemical class	Indication	Mechanism of Action	Disadvantages	Biological Activity	References
Proguanil	6	Bis-guanidine	Chemoprophylaxis of malaria	Inhibition of dihydrofolate reductase	Resistance, nephrotoxicity	Blood and Tissue Schizontocide	7
Quinine	9	Quinoline	Treatment of falciparum malaria	Inhibits Hemozoin formation	Resistance, not suitable for children under 2 years, cardiotoxicity, very toxic in overdosage.	Blood Schizontocide	7,16
Pyrimethamine	8	2,4-diaminopyrimidine	Treatment of falciparum malaria	Inhibition of dihydrofolate reductase	Easy development of resistance when used alone.	Blood Schizontocide	7,16
Doxycycline	5	Tetracycline	Prophylaxis of malaria, adjunct to quinine in treatment of falciparum malaria after quinine	Inhibition of the parasite's mitochondrial protein synthesis	Slow-acting drug, adverse effects on bones and teeth, not administrable during pregnancy and to children under 8 years of age.	Blood and Tissue Schizontocide	7

Table 1-3 Principal antimalarial drugs suggested for the treatment and the prophylaxis of malaria according to the BNF N° 46

Names	Structures	Commercial Name	Indication	Mechanism of Action	Disadvantages	Biological Activity	References
Pyrimethamine with Sulfadoxine	8 & 13	Fansidar®	Adjunct to quinine in treatment of falciparum malaria	Inhibition of DHFR and DHPS (dihydropteroate synthase) Antifolate	Same as pyrimethamine, severe side-effects on long term use.	Blood and Tissue Schizontocide	7,18
Proguanil with Atovaquone	6 & 12	Malarone®	Prophylaxis and treatment of acute uncomplicated falciparum malaria	Inhibition of DHFR and inhibition of dihydroorate dehydrogenase, possible interference with the energy metabolism in the mitochondria	Nephrotoxicity, not administrable during pregnancy and during breast feeding	Blood and Tissue Schizontocide	7
Arthemeter with Lumefantrine	16 & 11	Riamet®	Treatment of acute uncomplicated malaria	Oxidative stress, alkylation of heme	Not suitable in case of cardiac problems (arrythmias, bradycardias, etc.) and during breast feeding.	Blood Schizontocide	7
Chloroproguanil with Dapsone	7 & 14	LapDap®	Treatment of uncomplicated falciparum malaria	Inhibition of DHFR and DHPS (dihydropteroate synthase) Antifolate	Not suitable during breast feeding	Blood and Tissue Schizontocide	7,18

Table 1-4 Main combinations used for the treatment of malaria according to the BNF N° 46

1.3 Leishmaniasis

Leishmaniasis is a parasitic infection, transmitted by the bite of an infected female sandfly, whose host are animals such as dogs, rodents, or human beings. The parasite may survive for decades in asymptomatic infected people, who are of great importance for the transmission since they can spread visceral *leishmaniasis* indirectly through the sandfly. The parasites can also be transmitted directly from person to person through the sharing of infected needles which is often the case with *leishmania*/HIV co-infection[19].

1.3.1 The disease

Leishmaniasis is caused by different species belonging to the genus *Leishmania*, a protozoa of the *Trypanosomatidae* family; the name is derived from W. B. Leishman, who in 1901 gave one of the first accounts of the parasite's association with the initial symptoms of the disease.[20]

The disease has four main forms, depending on the parasitic species and the cellular immune system of the patient:

- *Cutaneous Leishmaniasis.* Cutaneous leishmaniasis produces skin lesions mainly on the face, arms and legs. Although this form is often self-healing, it can create serious disabilities and permanent scars. After recovery or successful treatment, cutaneous Leishmaniasis can induce immunity to re-infection by the species of leishmania that caused the disease. This form of the disease is mainly caused by *L.tropica* or *L. mexicana*, and it represents 50-75% of all new cases.
- *Diffuse Cutaneous Leishmaniasis.* Diffuse cutaneous leishmaniasis is difficult to treat due to disseminated lesions that resemble leprosy and do not heal spontaneously. This form is especially related to a defective immune system and it is often characterized by relapses after treatment.
- *Mucocutaneous Leishmaniasis.* Mucocutaneous leishmaniasis, also called "espundia" in South America, causes disfiguring lesions to the face; it destroys the mucous membranes of the nose, mouth and throat.

Reconstructive surgery of deformities is an important part of the therapy. This form is caused by L. *braziliensis.*

- *Visceral Leishmaniasis.* Visceral leishmaniasis, also know as "kala azar", is characterized by irregular fever, weight loss, swelling of the liver and spleen anaemia. It is the most severe form of leishmaniasis, and is usually fatal if left untreated. The incubation period can be months or years and, unlike the cutaneous forms of leishmaniasis, it involves the internal organs. After treatment and recovery, patients may develop chronic cutaneous leishmaniasis that requires long and expensive treatment. This form is caused by the *L. donovani, L. infantum or L. chagasi.*

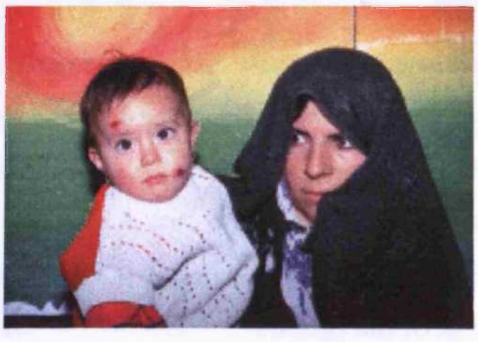


Figure 1-4 example of cutaneous leishmaniasis[21]

Today, an estimated 12 million cases of leishmaniasis exist worldwide with an estimated number of 1.5-2 million new cases occurring annually; 1-1.5 million cases of cutaneous leishmaniasis and 0.5 million cases of visceral leishmaniasis[19].

The geographical distribution of leishmaniasis is restricted to tropical and temperate regions, the habitat of the sandfly. Leishmaniasis is endemic in 88 countries (16 developed countries and 72 developing countries) and on four continents (see Figure 1-5, Figure 1-6).

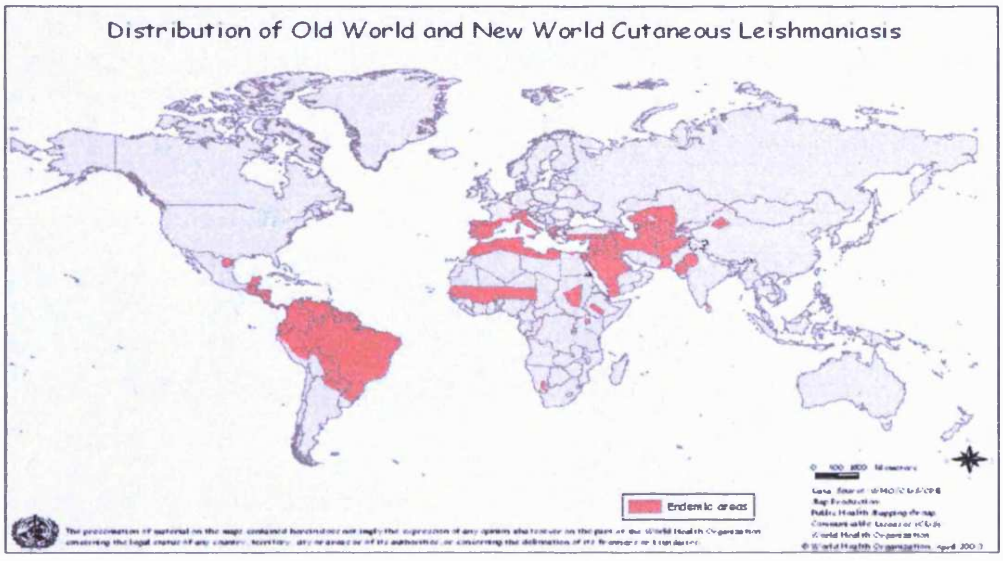


Figure 1-5 World distribution of cutaneous Leishmaniasis[4]

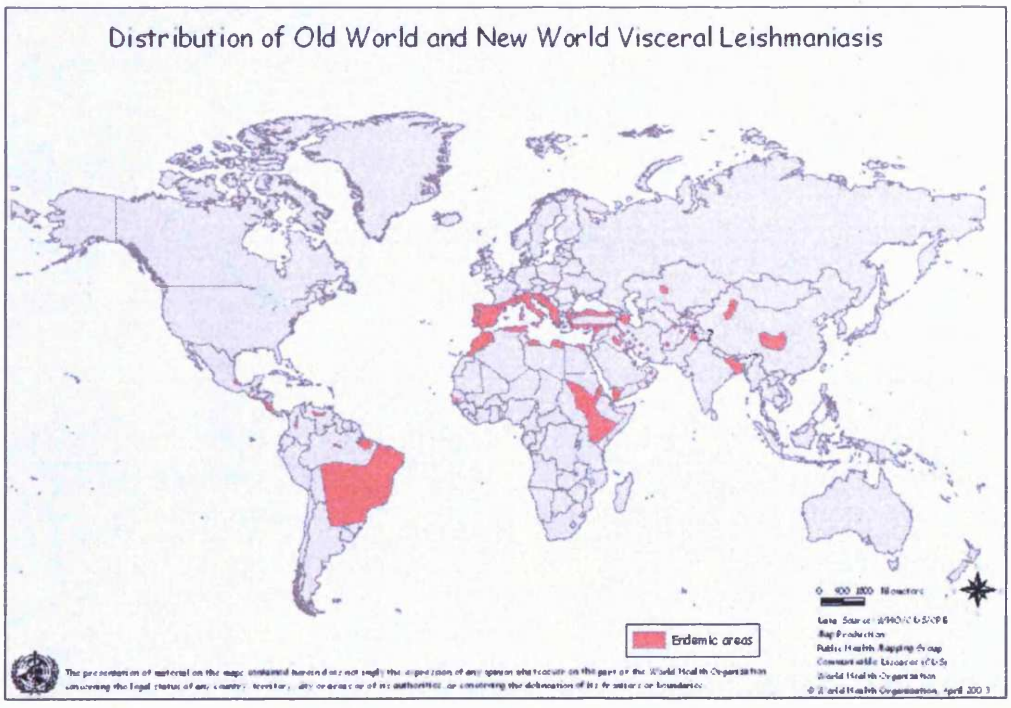


Figure 1-6 Overlapping areas of visceral leishmaniasis/HIV co-infections[4]

It is believed that the number of cases of *Leishmaniasis* is increasing. This is partly due to the man-made environmental changes such as extracting timber, mining, building dams, widening areas under cultivation, new irrigation schemes, road construction in primary forests such as the Amazon, widespread migration

from rural to urban areas and fast urbanization worldwide which are among the main causes for an increased exposure to the sandfly vector[19].

1.3.2 The Leishmania parasite

Leishmania parasites exist in one of two distinct morphological forms: amastigote in the vertebrate host; promastigote in the vector.

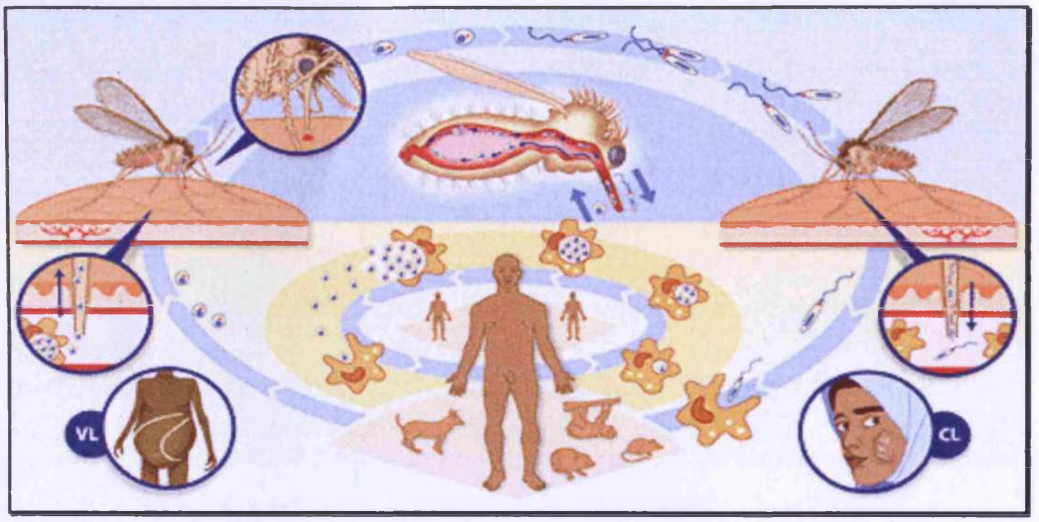


Figure 1-7 Leishmania life cycle[22]. The top of the figure illustrates the parasite life cycle in the insect vector. The lower side describes the parasite life cycle in the human host and in particular the cycle inside the macrophages.

The female sandfly ingests infected macrophages or released amastigotes along with a blood meal taken from an infected vertebrate host. These transform in the insect's midgut to flagellated promastigotes which divide by repeated binary longitudinal fission before migrating to the vector's proboscis ready for inoculating a fresh vertebrate host. Promastigotes rapidly parasitize the reticuloendothelial system of an inoculated vertebrate host; they are rapidly engulfed by macrophages in which they transform to amastigotes to divide repeatedly, eventually causing the macrophage to rupture. Some of the released amastigotes infect other macrophages and division and rupture is repeated while some remain available for vector uptake and transmission[19].

1.3.3 Current treatments

The leishmaniasis are complex diseases caused by different species of protozoan parasite *Leishmania.*

Chemotherapy of these parasites remains unsatisfactory and most of the drugs in use were derived empirically many years ago. New treatments are becoming available however they have some disadvantages such as adverse side effects or high costs.

Unfortunately, it may be that the previous ambitions to develop a single drug effective against all forms of leishmaniasis may have been too optimistic. This is due to the differences in drug sensitivity for different *Leishmania* species and the different sites of infection in cutaneous and visceral leishmaniasis, which requires different pharmacokinetic properties for the drug[23].

Pentavalent antimonials

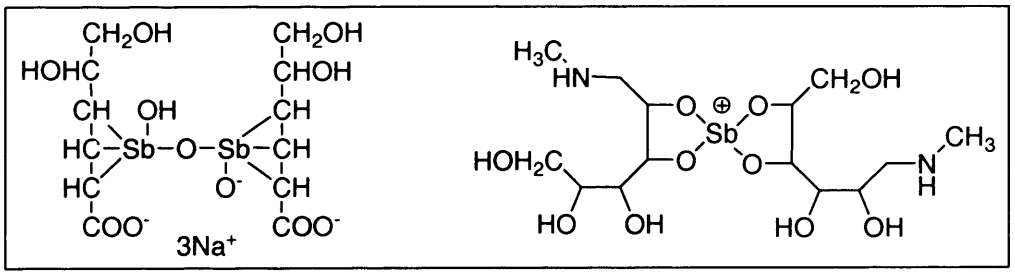


Figure 1-8 Sodium Stibogluconate (left), Meglumine Antimonate (right)

These two derivatives of antimony have been the first choice treatment against Leishmanias for the past 50 years. The drugs are prepared by reacting gluconic acid (sodium stibogluconate) or meglumine (Meglumine antimonite) with pentavalent antimony. It is important to note that the compounds shown in Figure 1-8, are the main compounds, but not the only ones, present in the polymeric mixture.

Sodium stibogluconate and meglumine antimonite are prodrugs, requiring the reduction of the Sb^{V} to Sb^{III} [24,25]. The activation occurs selectively in the amastigote stage of the parasite life cycle, so they are active only against this form of the *Leishmania* parasite[26].

The mechanism of action of these compounds is still unknown and although there are some theories about the mechanism, none of them has been experimentally validated. A recent study suggests that Sb^{III} might compromise the thiol redox potential of the cell by inducing the efflux of intracellular thiols and by inhibiting the enzyme trypanothione reductase[26]. Severe side effects are often associated with therapy using these antimonial derivatives and they also require long treatment (at least 28 days) with parenteral administration[23].

Amphotericin B

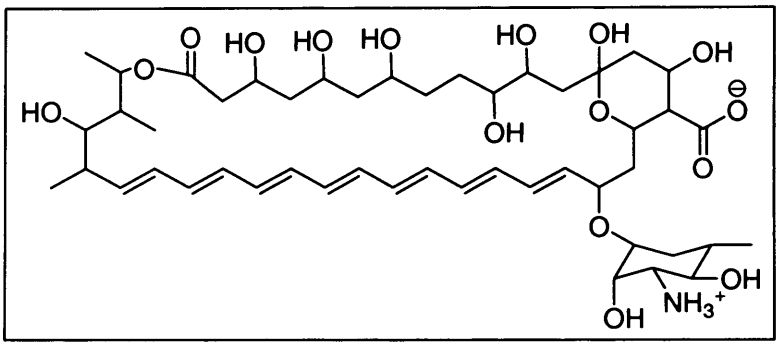


Figure 1-9 Amphotericin B

Amphotericin B is a polyene antibiotic which has been used mainly in antifungal treatments. It is active against antimony resistant strains of *Leishmania*[27]. Amphotericin is effective against all type of leishmaniasis, but the problems concerned with this drug are the high costs, the only parenteral administration in hospital settings, the long period of treatment required and the severe side effects. Recently a new lipid formulation has been introduced in the market with lower toxic effect and retained activity. However the formulation is more expensive than the simple amphotericin B.

The drug acts by interacting with the ergosterol in the cell membrane of the parasite. These interactions create pores in the membrane, allowing the lacking of ions in and out of the parasite, leading to cell death[28]. When resistance to Amphotericin B arises, it is due to a decreased concentration of ergosterol in the parasite's membrane, or to a substitution of ergosterol with another steroid structurally different to which the drug does not bind. Amphotericin B is generally a very toxic drug and one of the main side effects is nephrotoxicity.

Pentamidine

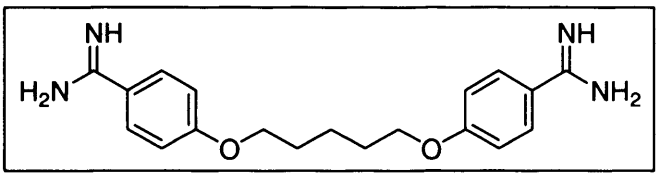


Figure 1-10 Pentamidine

Isethionate or methansulphonate salts of pentamidine are considered as the second line drug treatment against leishmaniasis after Sb^V treatment[26]. The cellular target of pentamidine is still unknown, although recent studies have shown that the drug accumulates in the parasite's mitochondria[29]. Because of the increasing resistance and the severe side effects (hypotension, hypoglycemia, diabetes, nephrotoxicity) the use of pentamidine is being abandoned.

Miltefosine

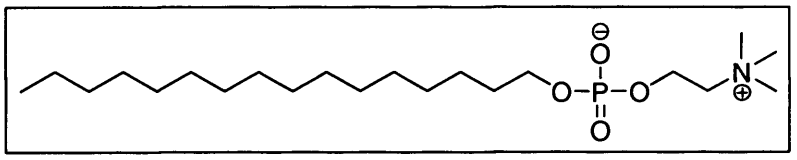


Figure 1-11 Miltefosine

Miltefosine is a simple isosteric ether for ester of phosphatidylcholine, initially developed as antitumor agent[26]. Miltefosine is the most promising drug for the treatment of leishmaniasis. After good results in phase I and phase II[30] clinical trials against Indian leishmaniasis, the drug was licensed in India in 2002.

One of the main advantages of Miltefosine is that it is orally administrable, unlike all the other anti-leishmania drugs. It is active against both the visceral and the cutaneous form of leishmaniasis. The mode of action of this drug is not known precisely, but it seems associated with changes in the alkyl-lipid metabolism and phospholipid biosynthesis[31].

The major limitation of milfetosine is teratogenicity which excludes its use in pregnant women. The other side effects are significant but generally tolerable. Acute effects are gastrointestinal distress, vomiting and diarrhea[1].

Chapter 2 The Enzyme dUTPase

The dUTP nucleotidohydrolase (dUTPase) is a ubiquitous enzyme present in almost all organisms, including eukaryotes, prokaryotes and some viruses. dUTPase catalyses the reaction of hydrolysis of dUTP into dUMP and PPi (Figure 2-1), and plays an important role for the viability in the cell[32,33].

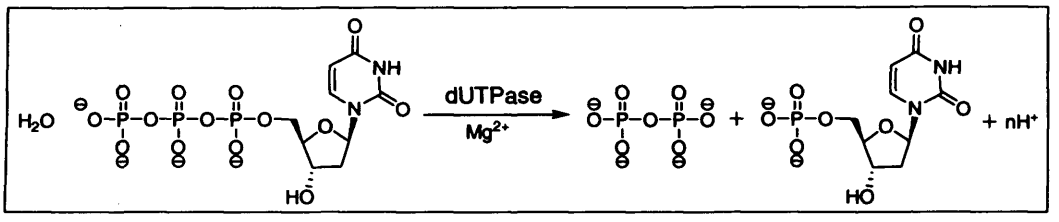


Figure 2-1 Hydrolysis reaction of dUTP into dUMP and PPi

2.1 Catalytic reaction

The dUTPase-catalyzed reaction is part of the nucleotides metabolism. The concentration of dUTP in the cells is set through a balance between the reactions by which dUTP is produced and those by which it is consumed (Figure 2-2)[34]. The enzyme provides two main roles in the cell viability:

1. dUTPase produces dUMP for the enzyme thymidylate synthase, necessary for the synthesis of dTMP, and consequently of DNA.
2. Maintains a low dUTP/dTTP ratio to avoid the erroneous incorporation of the uracil base into DNA.

The enzyme requires the presence of divalent ion (Mg^{2+}) as a cofactor in the catalysis. Without the magnesium the dUTPase seems to be inactive. The ion is mainly involved in the co-ordination of the triphosphate group of the substrate, thus assisting in the right orientation and recognition of the substrate at the active site[35,36].

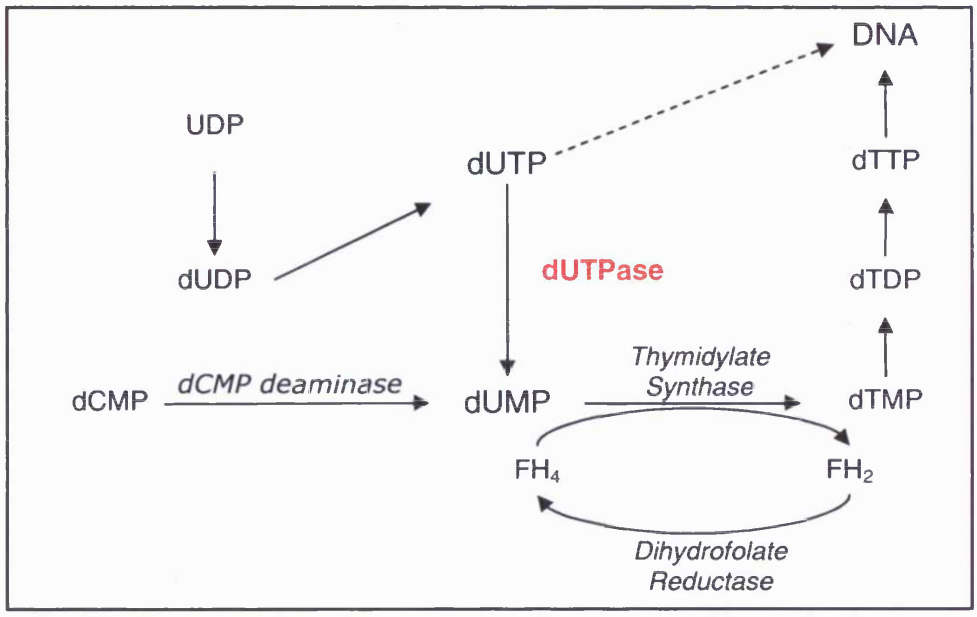


Figure 2-2 Metabolic pathways of nucleosides[34]

2.2 Physiological function

In most, if not all cellular systems, uracil is not a normal component of DNA. Although the uracil has the same base-paring and coding properties as thymine, its extensive incorporation into DNA is lethal in bacteria[37], yeast and likely all cellular systems[38].

Uracil in DNA results from either (i) deamination of cytosine (Figure 2-3), creating a premutagenic U:G mispair, or (ii) from misincorporation of deoxyuridine instead of deoxythymidine by the enzyme DNA polymerase using dUTP instead of dTTP, hence creating a U:A pair[39].

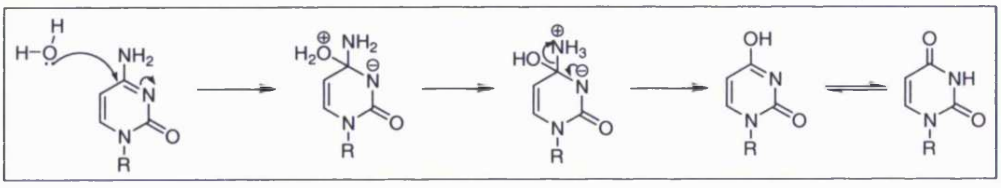


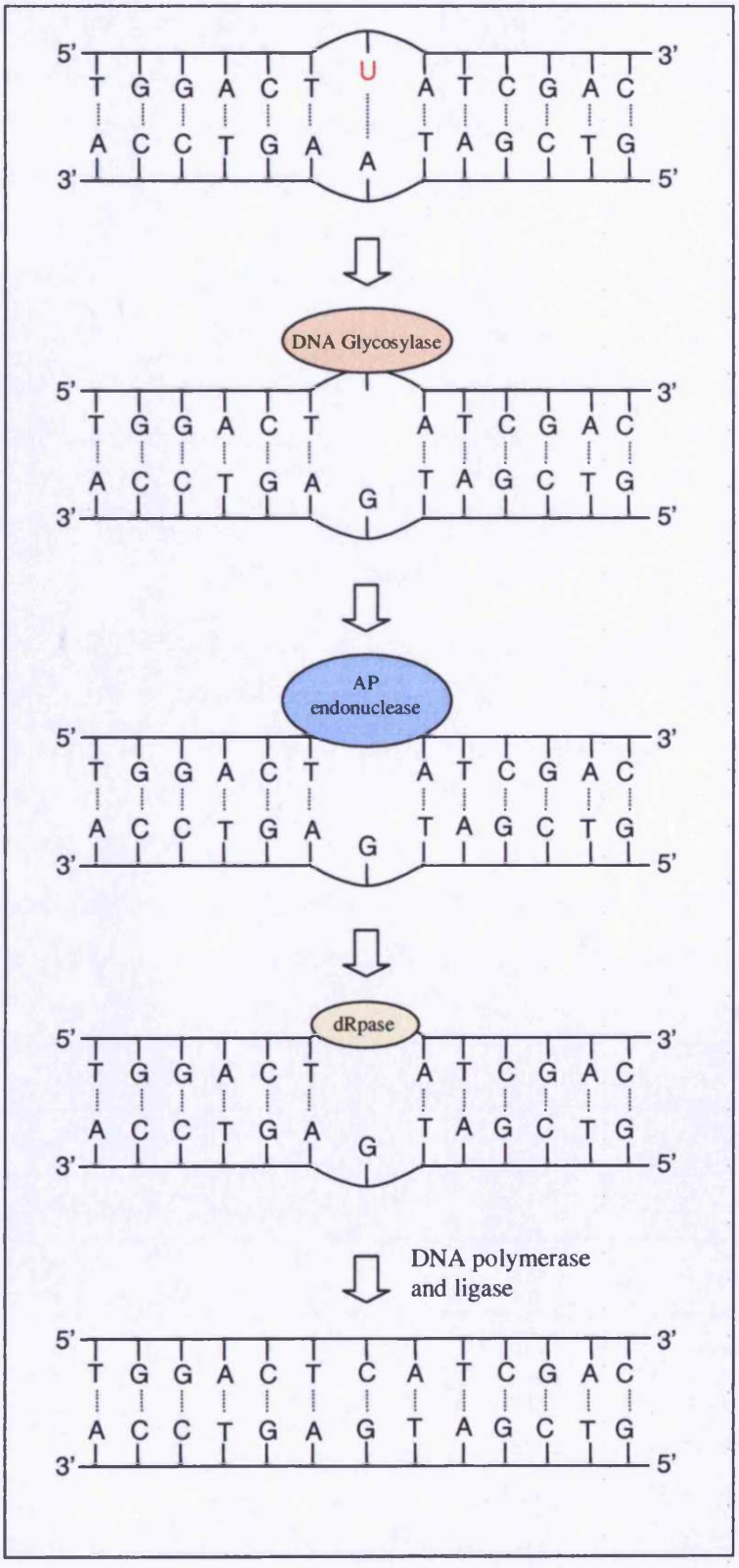
Figure 2-3 Mechanism for the deamination of cytosine to uracil, this process is catalysed by the enzyme cytosine deaminase.

If not replaced, the U:G mispair can, after replication, lead to an A:T transition thus undermining the integrity of the cell genome. Therefore the "wrong" base uracil has to be replaced by the base excision repair (BER) system[40] with the correct base thymidine or cytosine. The BER system is extremely complex and involves several enzymes and several steps. It is simplified in Scheme 2-1.

In the first step of the process the enzyme uracil-DNA glycosylase (UNG) is involved. This enzyme can selectively break the glycosidic bond between the uracil base and the 2'-deoxyribose of the backbone, generating an apyrimidinic site (AP) in the DNA. In the second phase an AP endonuclease then hydrolyses the phosphodiester bond immediately 5' or 3' to each AP site and the deoxyribose-phosphate backbone is removed from DNA by the action of deoxyribophosphodiesterase (dRpase) enzyme (third step). In the final step the enzyme DNA polymerase fills the gap with the missing nucleotide or nucleotides and then the ligase seals the repaired strain[41]. By means of this repair system the cells are able to preserve the integrity of the genome avoiding mutations.

The inhibition of dUTPase greatly influences the uracil metabolism promoting a pronounced increase in intracellular dUTP concentration and the incorporation of uridylate into DNA. In fact the DNA polymerases are not able to discriminate between dUTP and dTTP[42], and they use the nucleotide which is present in higher quantities in the dNTP pool. This has been documented in prokaryotes and eukaryotes, where deletion of the dUTPase gene in *E. coli*[37] and *S. cerivisiae*[43] leads to an increased incorporation of dUMP into DNA. Consequently Uracil-DNA glycosylase starts the base excision pathway at the sites where uracil has been incorporated. However this mechanism becomes self-perpetuating due to the elevated concentration of dUTP in the dNTP pool when, in the last step of the repair process, the enzyme DNA polymerase re-inserts dUTP instead of dTTP into DNA. As final result this cyclic, self-perpetuating process will generate numerous single/double-strand breaks[44], will delay the complete replication of DNA[45,46] and will cause cell death.

So far, the enzyme dUTPase can be considered as the first line of defence against the misincorporation of uracil into DNA, making dUTP unavailable to DNA polymerase[46].



Scheme 2-1 Schematic representation of the Base Excision Repair system[41]

The consequences produced by the inhibition of the enzyme dUTPase are similar to those produced by inhibitors of thymidine synthesis (e.g. methotrexate, 5-fluorouracil). These compounds block the biosynthesis of the thymidine triphosphate nucleotide and increase the ratio dUTP:dTTP in the nucleotide pool augmenting the incorporation of uracil into DNA.

Some studies have shown some correlation between the resistance to thymidylate synthase inhibitors and dUTPase activity in human lymphoblastoid cell lines[47] and colorectal tumour cells[48]. In these works it has been found that high levels of dUTPase expression result in tumour resistance to chemotherapy.

2.3 Structure and classification

Forty years have elapsed since the discovery of dUTPase. During this time many articles have been published about this enzyme. The first dUTPases reported were found to be highly specific towards dUTP but with more enzymes isolated and characterized, dUTPases nowadays form a group that is heterogeneous and extended to comprise also less specific enzymes.

All known dUTPases fall into one of three distinct families which each show a characteristic form: monomeric, homodimeric and homotrimeric (see Table 2-1).

Subunit composition	Sources
Monomers	Herpes virus
Dimers	*Leishmania, Trypanosomes, Campylobacter*
Trimers	Human, Bacteria, Fungi, Equine Infectious, Anaemia virus, *Plasmodium*

Table 2-1 Structures of dUTPases form several sources

To date seven dUTPases have been purified and characterized from different sources: *E. coli*[36,49]; *Bacillus subtilis*[50]; *Human*[51]; *Herpes viruses*[52]; *Leishmania major*[53]; *Trypanosome cruzi*[54]. For some of those the crystal structure has been solved: *E. coli*[55]; *Bacillus subtilis*[50]; *Human*[35]; Feline Immunodeficiency Virus (FIV)[56]; Equine Infectious Anaemia Virus (EIAV)[57]; *Trypanosome cruzi*[54,58], *Mycobacterium tubercolosis*[59], *Plasmodium falciparum*[60], *Campylobacter jejuni*[70].

2.3.1 Trimeric dUTPase

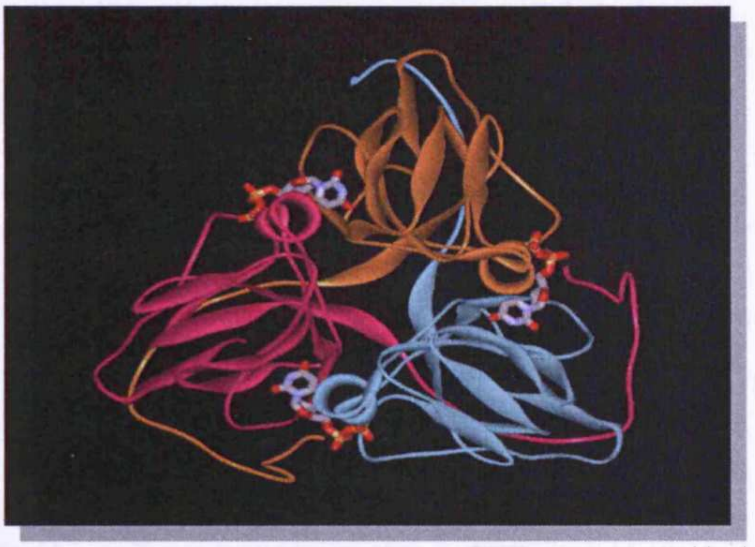


Figure 2-4 Crystal structure of homotrimeric Human dUTPase complex with the substrate analogue (dUDP)[61]. The three chains are highlighted in different colours; the active sites are located at the interfaces of the adjacent subunits.

The homotrimeric form is by far the most common, and is found in most prokaryotes, eukaryotes and some viruses. Six X-ray structures of this form have been published so far: from *E. Coli*[36,61], *Human*[35], FIV[56] (Feline Immunodeficiency Virus), EIAV[57] (Equine Infectus Anemia Virus), *M. tuberculosis*[59] and *P. falciparum*[60]. Trimeric dUTPases are built from three identical subunit polypeptides, each of approximately 150 amino acid residues. The active sites lie at the interface of the adjacent units and are made up from highly conserved motifs (Figure 2-6), which can be considered as signature sequences typical of trimeric and monomeric dUTPases.

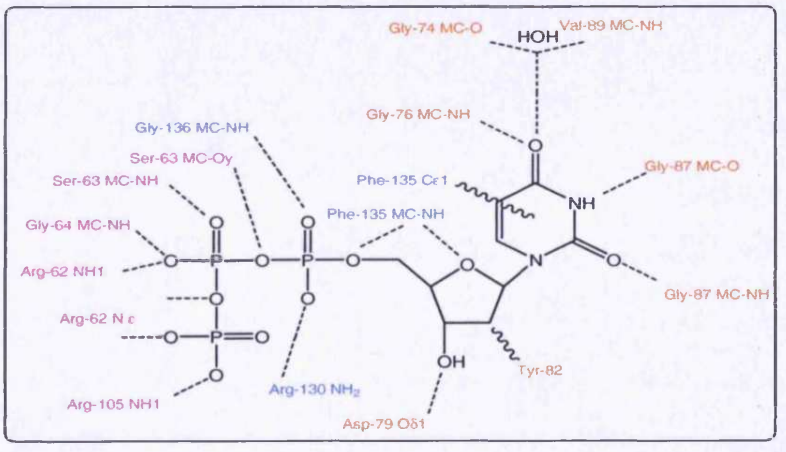


Figure 2-5 Schematic representation of the main network of interactions between enzyme and substrate in *E. coli* dUTPase. Different colours indicate residues from different monomeric unit.

Amongst the homotrimeric enzymes, the five conserved motifs contain residues that line the active site and contribute to substrate binding and catalysis[38]. Each active site is made of the five conserved motifs belonging to each of the monomers of the protein. For example, in Figure 2-7 analysing a generic active site in the clefts between the monomers A (yellow) and B (blue) there is: motif 3 from monomer A which is involved in uracil and sugar recognition and interactions. The conserved motifs 1, 2 and 4 from the monomer B which are responsible for the interactions with the phosphate groups, and the fifth motif from the monomer C (red) which is also involved in the phosphate recognition. Therefore the correct architecture of the active site and the overall activity of the enzyme depend on the correct oligomerization of the homotrimer[36].

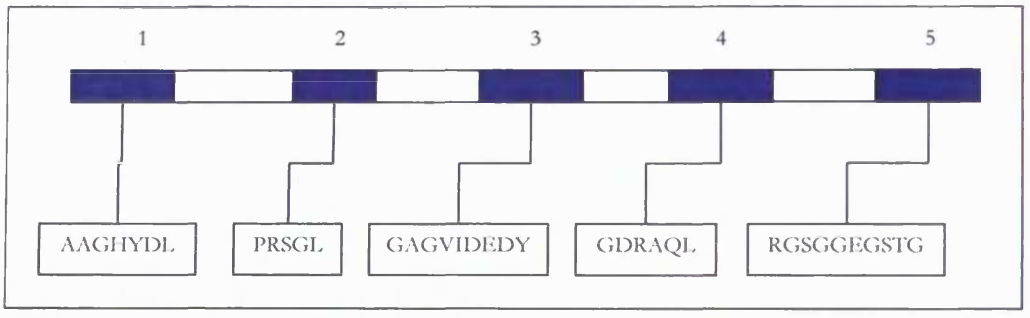


Figure 2-6 Schematic representation of five conserved motifs in trimeric dUTPases[38]

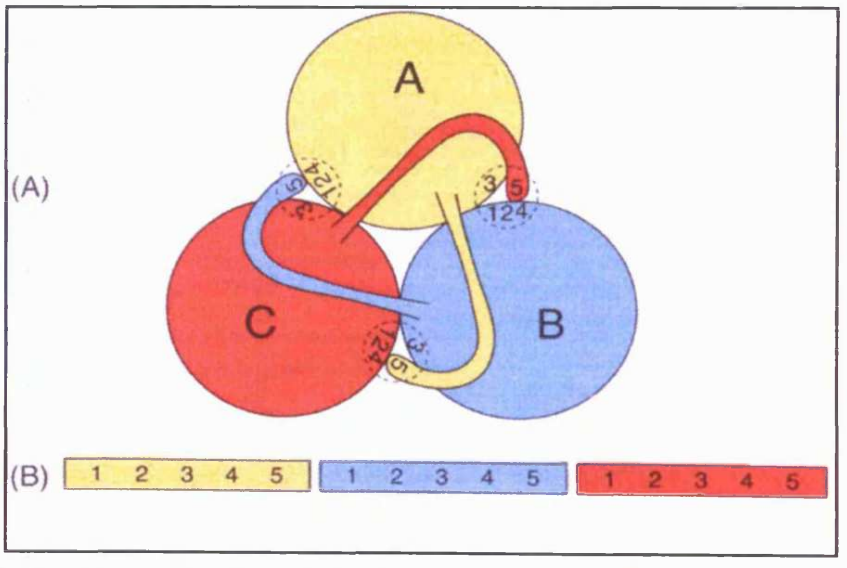


Figure 2-7 Model of active sites organization in homotrimeric dUTPase. (A) represents the oligomer with the three active sites at the interfaces of two monomers. The numbers indicate the five conserved motifs and how they contribute to the active site structure. (B) is a linear schematic representation of the monomers with the five motifs[62].

In human cells there are two different isoforms of dUTPase: nuclear and mitochondrial[63,64]. The two forms are largely identical, differing only in a short region in the amino terminal sequence. The nuclear isoform has a higher molecular weight and is approximately 30-fold more abundant than the mitochondrial isoform. Both are encoded by the same nuclear gene[65] and arise by the use of alternative 5' exons[64]. The nuclear form of dUTPase is regulated in a growth-dependent manner and is detected in replicating cell-types; whereas the mitochondrial form is expressed constitutively and is present in higher levels in metabolically active tissues.

2.3.2 Mechanism of catalysis for *E. coli* dUTPase

Using water labelled with ^{18}O it was proved that the nucleophilic attack is on the α-phosphorous, in fact the labelled oxygen was detected bonded to the dUMP and not to the pyrophosphate[66]. It was also demonstrated that a strictly conserved residue (Asp 90 in *E. coli* dUTPase) is involved in the mechanism of hydrolysis, activating the water molecule for the nucleophilic attack[36]. This study was carried out by Barabas and co-workers using an isostere of the substrate dUTP which is the α-β-imino-dUTP[36]. In this compound the bridge oxygen between the α and β phosphates is replaced with a NH group. Such substitution has nearly the same bond length (1.63 and 1.68 Å, for P-O and P-N respectively) and bond angles (128.7° and 127.2°, for P-O-P and P-N-P respectively) of the normal substrate dUTP. The lower electronegativity of the nitrogen atom results in lower reactivity of the phosphate ester imino analogues. Nevertheless dUTPase is able to hydrolyse, even if very slowly, the imino linkage of α-β-imino-dUTP, whilst no detectable hydrolysis of β-γ bond was found, thus confirming the exclusive specificity of dUTPase for α-β bond cleavage, as shown in Table 2-2.

In the work they also investigated the role of the Asp 90 residue, which in the crystal structure coordinates the water molecule responsible for the nucleophilic attack. Both carboxyl oxygens of aspartate may participate in hydrogen bonding to the water molecule, as shown in Figure 2-8. This residue was replaced with a asparagine in a mutant version of *E. coli* dUTPase and subsequent experiments on enzyme activity showed that the mutation had largely compromised the catalytic

efficiency of dUTPase, supporting the hypothesis of the role played by the Asp 90 residue, as reported in Table 2-2 .

Nucleotide	Wild Type			Asp90→Asn mutant		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
dUTP	11	0.5	1.4×10^{7}	2.55×10^{-4}	0.45	850
α-β-imino dUTP	2×10^{-5}	1.0	20	ND	ND	ND

Table 2-2 Kinetic and ligand binding data for wild type and Asp^{90} → Asn mutant *E.coli* dUTPase[36], ND= not determined

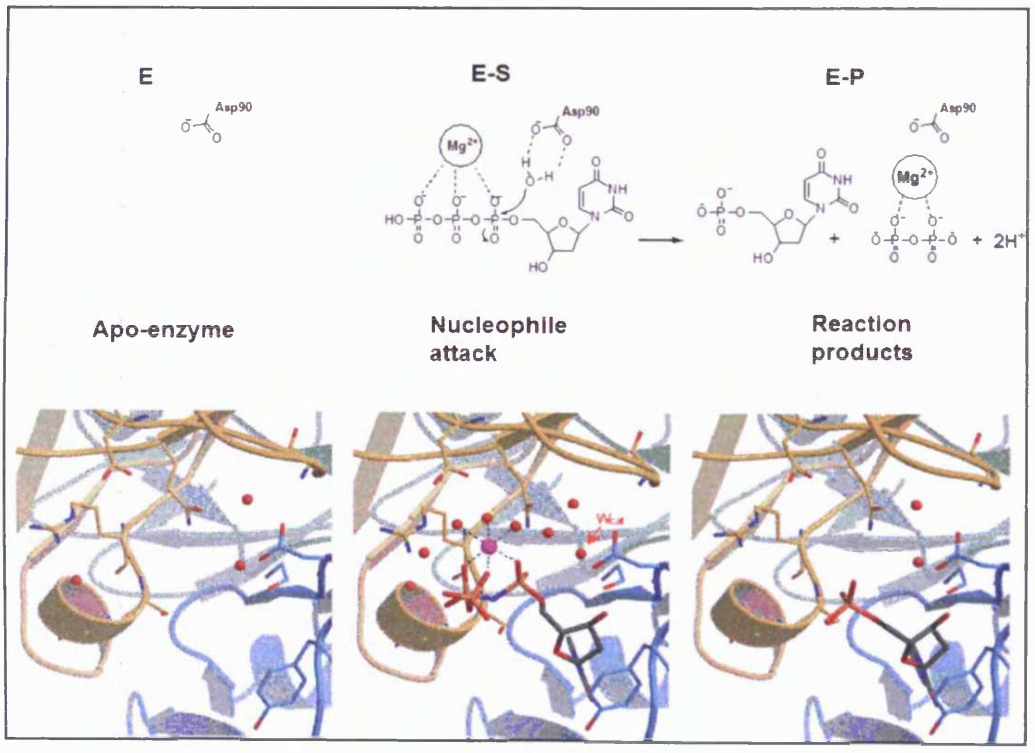


Figure 2-8 Theoretical reaction scheme for *E.coli* dUTPase with crystal structure of the active site (apo-enzyme on the left) the complex enzyme-substrate-cofactor (centre) and the enzyme-products of reaction (right). "E" represents the free active site of the enzyme. "E-S" is the enzyme-substrate complex with the water molecule coordinated by the Asp90 residue, which starts the nucleophilic attack on the α phosphate. "E-P" represents the final product of the reaction dUMP and the free enzyme active site[36].

In the same work they elucidated the role of the co-factor Mg^{2+}. The metal ion coordinates the three phosphate groups and three molecules of water allowing the right orientation for the nucleophilic attack, as shown in Figure 2-8. This was also

proved by Larsson *et al.*[66] by studies of hydrolysis of several substrate analogues. One of the tested dUTP analogues was dUTPαS; in this compound one of the nonbridging oxygens on the α-phosphorous was replaced at random with a sulphur atom, generating a mixture of two enantiomers. Only one enantiomer was found to be a substrate for the enzyme. This was mostly understood in view of a model where the Mg^{2+} has to bind to the α-phosphate in order to achieve the productive enzyme-substrate complex. Because magnesium does not bind easily to the sulphur, only one of the enantiomers of dUTPαS can bind the metal in the correct position.

Another controversial study on the role of the magnesium ion in *E.coli* dUTPase[67], appeared in the literature. This study proved that the enzyme can catalyze the reaction in the absence of divalent metal ions. The magnesium only enhances the catalytic action and, therefore, does not act as obligatory cofactor; as shown in Table 2-3.

Metal ion	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1} \times 10^6$)
No metal ion	4.5 ± 0.1	0.49 ± 0.1
Mg^{2+}	14.7 ± 2.2	25.1 ± 7.4
Mn^{2+}	14.5 ± 2.2	Not determined
VO^{2+}	27.3 ± 4.1	2.8 ± 0.9
$VO(acac)_2$	24.2 ± 3.6	2.4 ± 0.7

Table 2-3 Comparison of the influence of divalent metal ions on steady-state kinetic parameters governing hydrolysis of dUTP catalyzed by recombinant *E. coli* dUTPase[67]

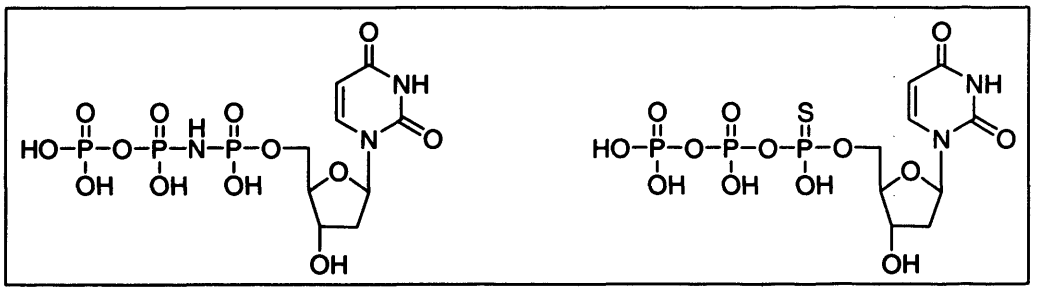


Figure 2-9 Structure of α-β-imino dUTP (left) and dUTPαS (right)

2.3.3 Dimeric dUTPases

The other group of dUTPases consists of the enzymes from trypanosomatid protozoa, for example *Leishmania and Trypanosome spp.*[53,68]. Another recently discovered enzyme of this class is from *Campylobacter jejuni*[69,70]. They occur as homodimeric aggregates and have a broader enzymatic specificity. The *Leishmania major* and *Trypanosoma cruzi* enzymes are capable of hydrolyzing not only dUTP but also dUDP[68]. To date the only dimeric dUTPase crystal structures solved are from the parasite *Trypanosoma cruzi*, in both native form and complexed with the ligand dUDP[58] and from *C. jejuni* complexed with the non-hydrolysable substrate α-β-imino-dUDP[70].

Each monomer of this completely different class of dUTPases is made of about 260-270 amino acid residues and do not contain any of the five conserved motifs present in the active site of the trimeric enzymes. Also the secondary structure of dimeric dUTPase is mainly made of α helices in comparison to the trimeric forms, which are predominantly β-pleated sheets. No significant structural similarity could be established with the two families of dUTPases[68,71].

As with the trimeric forms of dUTPase, the dimeric requires a metal ion as cofactor. In absence of Mg^{2+} ions the *L. major* UTPase is completely inactive. No enzymatic activity was also observed when the Mg^{2+} was substituted with Ca^{2+} or Cu^{2+}; however in the presence of Co^{2+} and Mn^{2+} the dimeric dUTPase retains or increases its activity[68].

It has been proved that dUTPase is essential for the viability in *L. major* and *T. brucei*. In the former antisense RNA oligonucleotide[68] and in the latter RNAi[72], caused downregulation of the protein expression and affected the normal growth of the parasites in culture cell. With these experiments it was demonstrated that the enzyme is indispensable for the viability of *T. brucei* in both stages of the parasites (the blood form in the mammalian host and the procyclic form in the insect)[72].

Some studies on the localization of dUTPase in the protozoal cell were carried out by Gonzalez-Pacanowska et al[72]. In this work the dimeric enzyme was found to localize only in the nucleus of the *T. brucei* cell, with no presence in the mitochondria. These findings suggest further differences with the corresponding human enzyme which has two isoforms (mitochondrial and nuclear).

2.3.4 *Trypanosome cruzi* dUTPase

The *T. cruzi* crystal structure has been solved by Wilson and co-workers, in both native form and as a complex with the substrate dUDP[58]. The main features of this enzyme are the differences between the two forms. In fact the enzyme almost changes totally shape when the substrate is bound in the active site.

The amino acid sequences of *T. cruzi* and *L. major* dUTPases share 50% of identity and 75% of similarity[73] which suggest that the two enzymes may have similar structures. The structure is predominantly α helical; each monomer is arranged in two distinct domains termed mobile and rigid, with an angle of *circa* 60° to each other. The rigid domain of one monomer interacts with the corresponding domain of the other monomer, forming the rigid core of the enzyme (see Figure 2-10).

The active sites lie in the groove formed at the interface of the mobile and rigid domains within individual subunits. In the native form the overall shape is an open structure where the active sites are exposed to the environment. When the nucleotides bind to the enzyme the mobile domains undergo structural changes closing on themselves engulfing the nucleotides in the active sites, as shown in Figure 2-11.

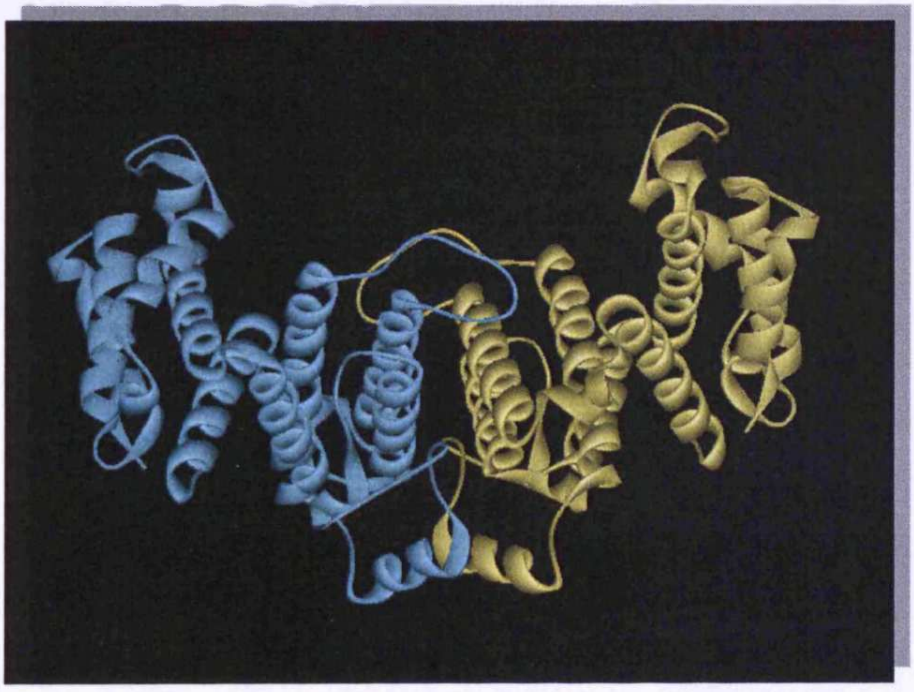


Figure 2-10 Crystal structure of *T. cruzi* dUTPase native form. The two monomers are illustrated in two different colors[58]

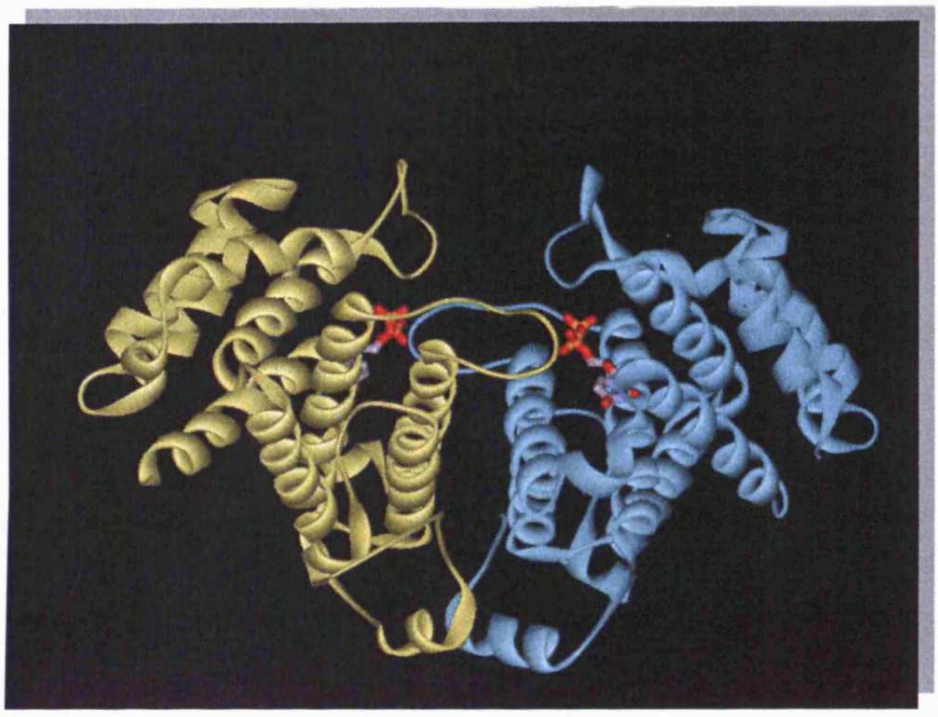
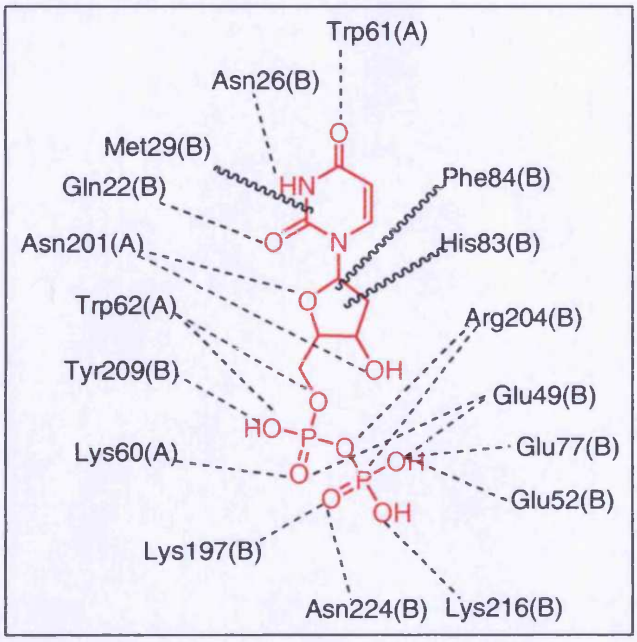


Figure 2-11 Crystal structure of homodimeric *T. cruzi* dUTPase complex with the substrate (dUDP)[58]

The interactions of the substrate in the active sites are illustrated in Scheme 2-2, where the uracil base is involved in a network of hydrogen bonding with the side chain atoms of the polypeptide (Asn26, Gln22 and Trp61). The uracil is kept in the site by interaction with the side chain of Met29. The sugar moiety is recognized by two hydrogen bonds with the Asn201 and two interactions with the aromatic rings of Phe84 and His83. The phosphate groups are surrounded with several polar residues, mainly glutamates, which probably also bind the metal ion(s).



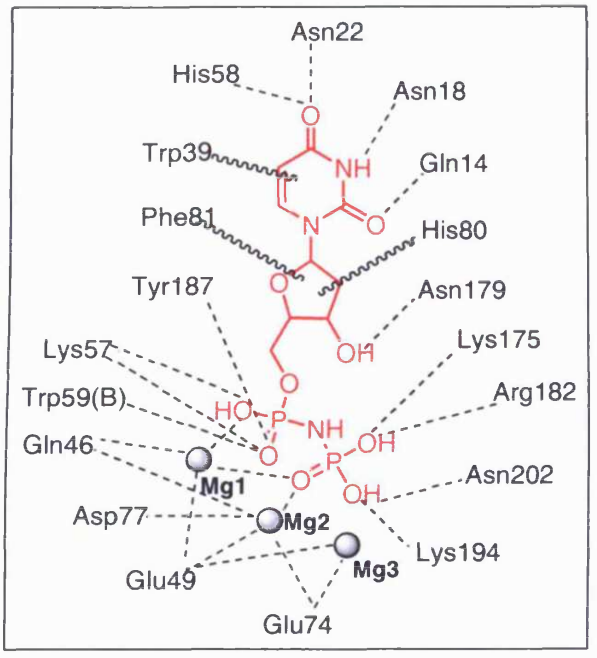
Scheme 2-2 Two dimensional schematic rappresentation of the main interactions of the substrate dUDP in the active site of *T. cruzi* dUTPase

Further information can be acquired, and more hypotheses can be made by comparison of the *T. cruzi* crystal structure with the dimeric enzyme found in *C. jejuni*.

2.3.5 *Campylobacter jejuni* dUTPase

The *C. jejuni* dUTPase is the second dimeric dUTPase which has been crystallised and diffracted. The crystal structure available for this enzyme is complexed with the non-hydrolysable substrate α-β-imino dUDP in presence of magnesium ions[70], and therefore corresponds to the closed conformation as previously described for *T. cruzi* dUTPase.

Noteworthy in this crystal structure, it is possible to see the three magnesium ions present in the active site. The three divalent metals are coordinated between the phosphate groups, the conserved glutamate residues and one aspartate, as shown in Scheme 2-3.



Scheme 2-3 Schematic representation of the network of interactions between the substrate dUDP in the *C. jejuni* active site in the presence of Magnesium[70].

The overall network of interactions is very similar to the one present in the *T. cruzi* dUTPase. One of the few differences present is the Asn22 residue, which is involved in two hydrogen bonds with the oxygen of the uracil, and the π interaction between Trp39 and the uracil ring. The sugar moiety is locked in the active site by the same two interactions with Phe81 and His80 seen in *T. cruzi* dUTPase. The remaining phosphate groups are surrounded by a series of polar residues aspartates, glutamates and lysines which also coordinate the three magnesium ions. There is also present a water molecule (not illustrated in the scheme), coordinated by two magnesium ions, which could be considered as an ideal candidate for the nucleophilic attack during the hydrolysis reaction.

2.3.6 *Plasmodium falciparum* dUTPase

Although *Plasmodium falciparum* belongs to the protozoa family, it has been found that the *P. falciparum* dUTPase is not dimeric like *L. major* or *T. cruzi*, but trimeric like the human enzyme. This finding would suggest a different phylogenetic evolution for this parasite compared to the trypanosomatid protozoa. Despite the fact that plasmodial and human dUTPases fall in the same

structural class there is some diversity that could be exploited for selective inhibition of the *Plasmodium* enzyme.

The crystal structure of *P. falciparum* dUTPase was recently obtained by Wilson and co-workers[60] using one of the inhibitors developed in our group[74]. This allowed crystallisation of the enzyme with a substrate analogue bound in the active site.

The amino acid sequences of human and plasmodial dUTPases have only 28.4% identity. In particular there is an insertion of 25 amino acids adjacent to the active site. This kind of insertion, also called Low Complexity Region (LCR)[75], seems to be a characteristic of *P. falciparum* proteins and could affect the shape/charge structure or could change the mechanism of the substrate recognition compared to other trimeric forms. Many plasmodium proteins contain insertions which often contain distinctive homopeptide repeats of hydrophilic amino acid residues[60]. The following picture (Figure 2-12 illustrates the two trimeric structures of *P. falciparum* and *human* dUTPase.

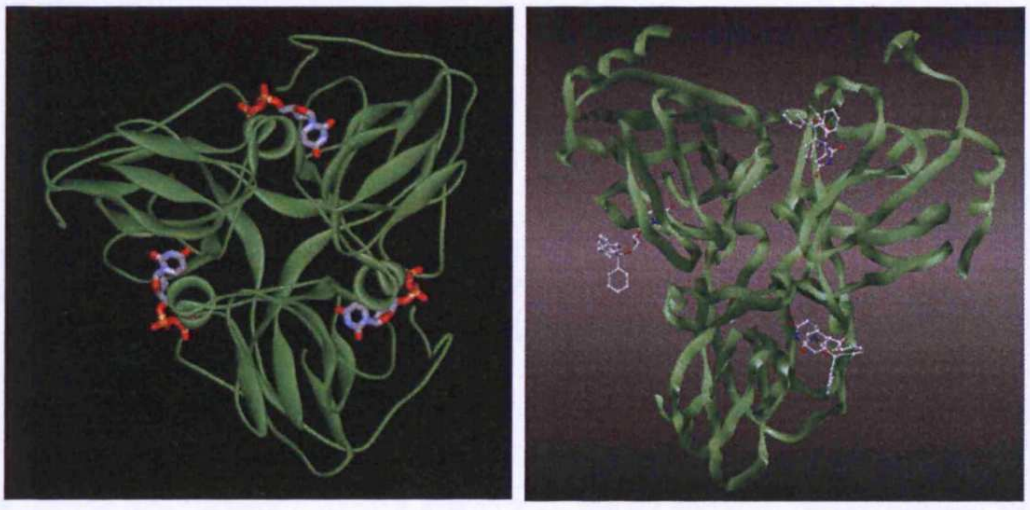


Figure 2-12 Three dimensional structure of *P. falciparum* dUTPase (left) and *Human* dUTPase (right)[60,35]

Studies with fluorescent antibodies allowed investigation of the cellular location within the parasite cell. It was found that contrary to the human enzyme, *P. falciparum* dUTPase is not associated with organelles like the nucleus or mitochondria, but is mainly located in the parasite food vacuole[60]. This organelle is a subcellular compartment where the parasite processes substances acquired

from the host, such as haemoglobin. In recent years many observation have indicated a number of significant biological processes within the parasite food vacuole that are important for the parasites survival. Therefore it could be possible that dUTPase is secreted by Plasmodium in order to hydrolyse host/parasite dUTP to the dUMP necessary for thymidylate biosynthesis[41].

2.3.7 Monomeric dUTPase

The third group contains monomeric dUTPases which are encoded by mammalian herpes virus. The enzymes in this class have the same conserved motifs as the trimeric dUTPases but in a different order, and structurally correspond to one of the monomeric units forming the homotrimer. For this reason it is likely that the active site of this monomeric form has structural features similar to those of the trimeric enzyme[76]. So far no crystal structures are available for this class of dUTPases.

2.3.8 Selectivity and inhibition

dUTPase is a very selective enzyme which can discriminate between the four different nucleobases and the sugar moiety. In fact none of the other five nucleotides present in the nucleotide pool are substrates for the enzyme. This should not be surprising considering that hydrolysis of nucleotide triphosphates would be an energy waste for the cell metabolism.

The only dUTPases having broader substrate specificity are the trypanosomal dUTPases, which are able to hydrolyse dUTP and dUDP. Deoxyuridine diphosphate is instead a competitive inhibitor for all the other species of dUTPases.

Nucleotide	*T. cruzi*	*L. major*	*E. coli*	EIAV	HSV-1*	MMTV**
dUTP K_m (k_{cat}/K_m)	0.53 (5.2×10^6)	2.11 (2.3×10^7)	0.2 (4.0×10^3)	1.1 (2.0×10^7)	0.3 (2.0×10^7)	0.8 (2.0×10^6)
dTTP K_m (k_{cat}/K_m)	>800 (2.5×10^3)	1514 (5.5×10^3)	>2000 (34)	260 (<2000)	400 (1000)	ND
dCTP K_m (k_{cat}/K_m)	ND	>2500 (5.0×10^3)	4000 (<100)	3000 (1000)	1000 (2000)	ND
UTP K_m (k_{cat}/K_m)	ND	>2500 (2.0×10^3)	2500 (<100)	ND	1000 (200)	ND
dUDP K_m (k_{cat}/K_m)	6.27 (1.7×10^6)	62.7 (1.0×10^7)	K_i= 15	K_i= 3.6	K_i= 17	ND
dUMP K_{in}	18.4	13.05	1500	130	170	ND

Table 2-4 Michealis-Menten, Specificity and Inhibition Constants of Viral, Bacterial and Trypanosomatid dUTPases for different Nucleotides. Units for K_m and K_i are μM and for k_{cat}/K_m $M^{-1}s^{-1}$ [68], * HSV-1: Herpes Simplex Virus Type 1; ** MMTV: Mouse Mammary Tumor Virus. ND- not determined

In Table 2-4 are reported kinetic parameters for different nucleotides and enzymes from different sources, K_m is the Michaelis constant and is an indicator of the affinity that an enzyme has for a given substrate, and hence the stability of the enzyme-substrate complex. The ratio (k_{cat}/K_m) is the specificity constant or

catalytic efficiency and indicates the efficiency with which the enzyme converts the substrate into product.

According to the last row of the table the substrate dUMP is a moderate competitive inhibitor of trypanosomatid dUTPases, with K_i of 18.4 μM for *T. cruzi* and 13.05 μM for *L. major* whilst the K_i values for the trimeric enzymes are at least 10 times higher. On the other hand the nucleoside dUDP can be hydrolysed by the dimeric dUTPases, but the same compound is reported to be an inhibitor for the other trimeric dUTPases with K_is of 15 μM, 3.6 μM and 17 μM respectively for *E. coli*, EIAV and HSV-1. This suggests a strong product inhibition of the dimeric enzyme and also proves that selective inhibition of the dimeric dUTPase versus the trimeric form is possible.

Compound	*L. major*	*T. cruzi*	*E. coli*	EIAV	HSV-1	HSV-2
5-mercury dUTP	-	-	-	-	Ki=27 [77]	Ki=21 [77]
M-dUTP	-	-	Ki=2.5 [78]	-	-	-
BM-dUTP	-	-	Ki=0.3 [78]	-	-	-
α-β-imido dUTP	Ki=0.89[68]	-	Ki=5 [79]	-	-	-
α-β-imido dUDP	-	Ki=0.24[71]	-	-	-	-

Table 2-5 Inhibition constants (*Ki*) for some known inhibitors of dUTPases (all values are μM)

In Table 2-5 are reported some inhibition constants of known inhibitors of dUTPase enzymes. Those inhibitors are nucleotide triphosphate analogues in which the bond between the α and β phosphate is nonhydrolyzable (imino or methylene) or they contain a heavy metal like mercury in position 5 of the uracil ring. These compounds are not suitable drug candidates as they have multiple charges which would prevent the penetration across membranes; they are enzymatically and chemically unstable and toxic. Particularly the nucleotide analogue α-β-imido dUTP can inhibit the *L. major* dUTPase with a K_i of 0.89 μM, indicating that the enzyme can bind nucleotides analogues which lack a

hydrolysable bond between the α and β phosphate groups. The same α-β-imido dUTP is also a good inhibitor of the trimeric dUTPase from *E. coli*[79]. *T. cruzi* dUTPase has been tested with the diphosphate analogue α-β-imido dUPD, and also this compound has shown the capacity to inhibit the trypanosamal enzyme with a K_i of 0.24 μM.

Confirming the differences among the dimeric form compared to the trimeric dUTPase, the DMT-dU (5'-O-(4,4'-dimethoxytrity)-2'-deoxyuridine (Figure 2-13) which is a strong inhibitors of *E. coli* dUTPase, does not show any activity against *L. major* and *T. cruzi* dUTPases[68,71].

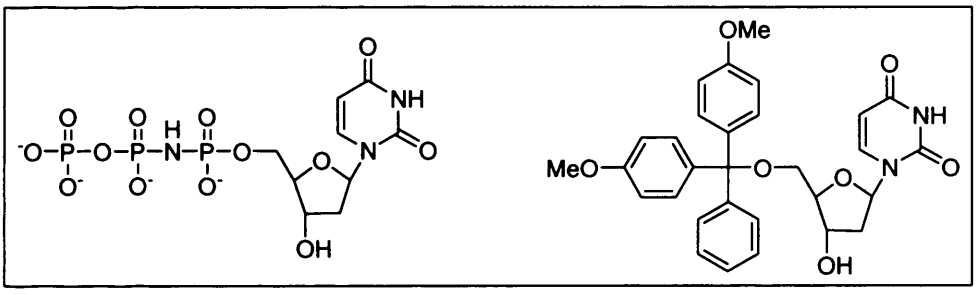


Figure 2-13 The two structures for the inhibitors α-β-imido dUTP (left) and the DMT-dU (right)

Other inhibitors of *Leishmania* dUTPase and *Plasmodium* dUTPase not known in the literature are illustrated in Table 2-6. They were identified by screening of a library of nucleotide analogues synthesised in a pharmaceutical company (Medivir AB Sweden)[72] collaborating with this project, and another library coming from the Welsh School of Pharmacy.

The three inhibitors of *L. major* enzyme, MV002291, MV002293, MV002295, are thymidylate derivatives, with an isosteric group for the triphosphate moiety. The other *P. falciparum* inhibitors are analogues of uridilate, with a bulky lipophilic group instead of the phosphate moiety in 5' position. WSP869 was used to obtain the crystal structure for *P. falciparum* dUTPase[60].

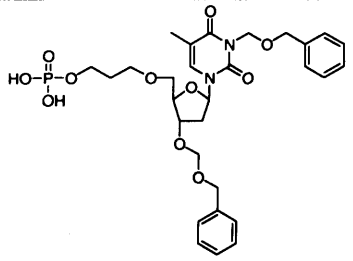
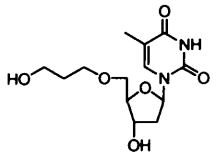
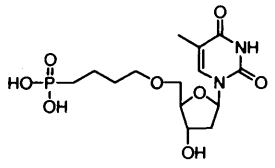
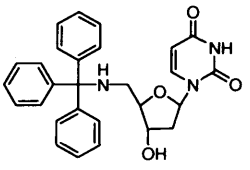
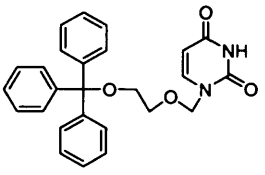
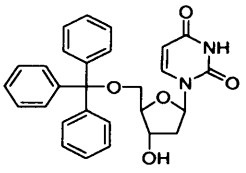
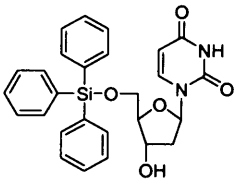
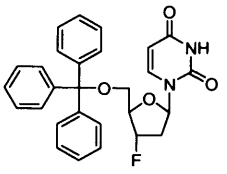
Structure	Name	*P. falciparum*	*L. major*
	MV002291	-	Ki=37.5 μM
	MV002293	-	Ki=63 μM
	MV002295	-	Ki=134 μM
	MV025212 WSP871	Ki=0.21 μM	Ki=300 μM
	WSP870	Ki=0.67 μM	Ki=304 μM
	WSP483	Ki=1.8 μM	Ki=285 μM
	WSP949	Ki=2.8 μM	-
	WSP869	Ki=4.9 μM	Ki=177 μM

Table 2-6 dUTPase inhibitors unknown in literature[72]

2.4 dUTPase as a chemotherapeutic target

Much attention has been focused on the biosynthesis of thymidilate triphosphate nucleotide and many inhibitors of this metabolic pathway (Thymidylate Synthase or Dihydrofolate Reductase inhibitors, see Figure 2-2) have been discovered and used for chemotherapy (i.e. 5-fluorouracil, methotrexate, and pyrimethamine). The mechanism of action of these compounds results in a depletion of intracellular levels of dTTP followed by cell death. This process is therefore called "Thymidineless death", but the real action of such a process is very similar to the effects caused by the inhibition of the enzyme dUTPase. In particular the low cellular concentration of dTTP in the cell will increase the incorporation of dUTP in the DNA leading to the same consequences described previously in paragraph 2.2.

Despite many publications presented in literature regarding the enzyme dUTPase from different organisms, regarding dUTPase as potential drug target for cancer or viral therapy and also the crystal structures available in the protein database; not much attention has been focused on the synthesis of potential drugs against dUTPase, which could be used in chemotherapy. Therefore this known biological target, which has also been validated as a drug target[37,43,68,72], represents a relative new research field for medicinal chemists who wants to develop selective inhibitors for this enzyme.

It should be possible to exploit the structural differences between the parasite (*leishmanial, trypanosome*, and *plasmodium*) enzymes and the host (*human*) enzyme to develop selective inhibitors. These compounds could eventually be used for medical treatment against some of the most devastating tropical diseases. In addition, it has been suggested that human dUTPase could be a drug target for the treatment of cancer[42].

In summary the enzyme dUTPase, due to its essential role in cell viability, can be considered as a good target in chemotherapeutic drug development against cancer, viruses or the tropical diseases.

Chapter 3 Aims and Objectives

Based on the available knowledge on the enzyme dUTPase and on the experience acquired in the group the main aims of this project was to develop selective inhibitors against the dUTPase enzymes. The research was focused initially on the *Leishmania* and *Plasmodium* dUTPases.

It has been shown that inhibition of dUTPase can be lethal for bacteria[37], yeast[43] and some protozoa[68,72]. Some substrate analogue inhibitors are known in literature, and several crystal structures are also available. Hence different approaches can be exploited in order to achieve the proposed objectives.

First the *substrate-based approach*, which consists of modifications of the structure of the natural substrate of the enzyme to get some insight into important structural requirements of the protein.

Second, the *inhibitor-based approach*, where some known inhibitors of the enzyme are chosen as lead compounds to develop structure-activity relationships, in order to increase inhibition, selectivity and also pharmacokinetic properties of drug-like structures.

Third, the *structure-based approach* which consists of molecular modelling studies. That is the use of software tools and crystal structures of proteins to give some ideas and suggestions about the structural features of compounds which could have good affinity and inhibition of the enzyme.

The substrate-based approach had already been followed previously in the group, when some dUTP analogues were synthesised and tested against different dUTPase enzymes[74]. This led to the identification of various inhibitors of the *P. falciparum* dUTPase; some of them are illustrated in Figure 3-1. The compounds discovered in the group are drug-like, contrary to the dUTP analogues used for crystallising the enzyme, which are isosteres of the triphosphate nucleotide with chemical and enzymatic instability, poor bioavailability and difficulties to penetrate cellular membranes.

Therefore the last two approaches will be exploted in this project.

3.1 P. falciparum dUTPase targets

In Figure 3-1 are the structures for three of the inhibitors previously discovered in the group. These compounds showed inhibition constants (*Ki*) in the μM range and IC_{50} values between 1 and 5 μM. Whereas the *Ki* gives a quantitative estimation of the structural affinity of a substrate for the enzyme, the IC_{50} is by definition the concentration inhibiting 50% of the cellular growth (in this case parasite growth). Considering those two parameters the three compounds illustrated in Figure 3-4 could be considered as good "starting points" for an inhibitor-based approach against *P. falciparum* dUTPase.

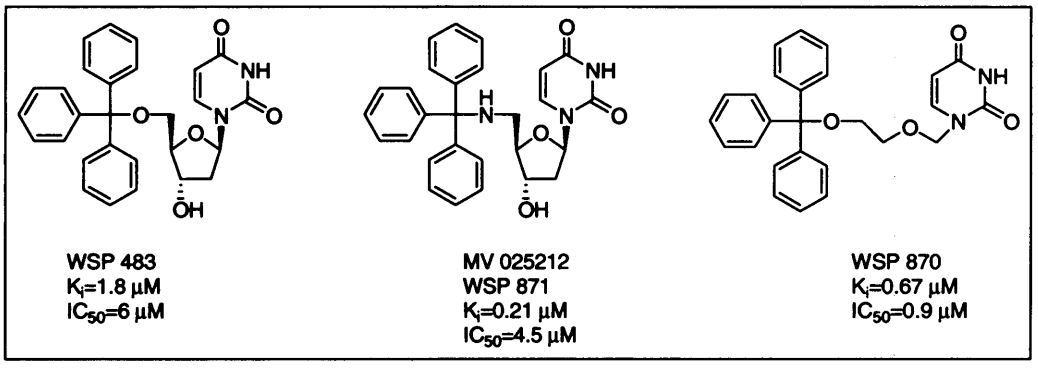


Figure 3-1 *P. falciparum* dUTPase inhibitors discovered in a previous work carried out in the group

3.1.1 Acyclic uridine derivatives

Initially it was decided to investigate further the acyclic uracil derivative WSP870. Despite the good activity against the enzyme and the growth inhibition of the parasite, this compound did not have very good results in ADME (Administration, Distribution, Metabolism and Elimination) studies. In particular, the Human Liver Microsome test (HLM), which gives an idea of the metabolic stability, suggested that this compound may have certain metabolic instability[80]. To overcome this problem, it was decided to introduce a simple aliphatic chain without oxygen (structure **A** in Figure 3-2). This type of structure should have better pharmacokinetic properties with increased metabolic stability.

It was also proposed to explore the effect of double bonds in the aliphatic chain spacer between the uracil base and the terminator group (**B** in Figure 3-2);

this work could benefit molecular modelling studies, by which it could be possible to pre-investigate what should be the best position and conformation (*cis* or *trans*) of the double bond within the aliphatic chain.

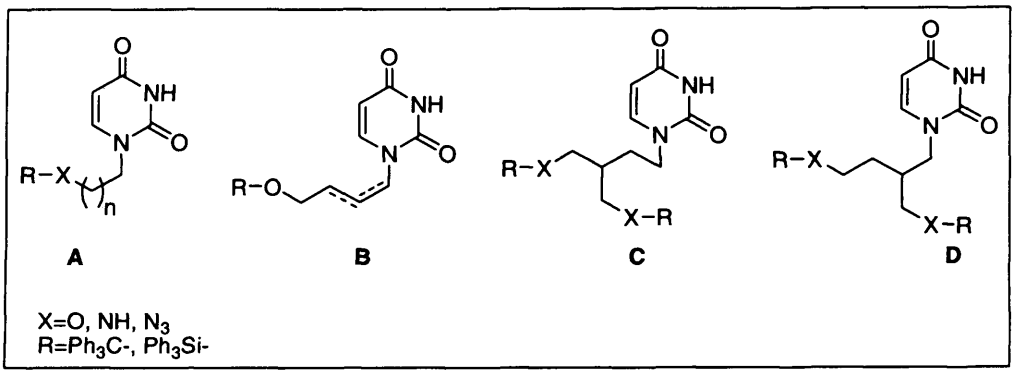


Figure 3-2 Proposed modifications for the WSP870 analogues

It was also proposed to prepare analogues with added side-chains, in order to introduce new functionality and exploit more interactions with the enzyme active site (structures **C** & **D** in Figure 3-2).

In all the proposed targets, the modifications were limitated to the linker between the uracil base and the terminator group (trityl or triphenylsilyl); no modifications were proposed on these latter two components since, from our previous experience, they seemed to be essential for a good inhibitory effect and selectivity against the *P. falciparum* dUTPase.

3.1.2 3'-Modified-2'-deoxyuridine derivatives

To get more information regarding the 3' position of the sugar moiety, it was decided to replace the OH group with an amino function and synthesise some analogues of WSP483, (structure **E** in Figure 3-3). The NH_2 group in the 2' position should retain some properties of the OH such as being a hydrogen bond donor and having similar steric properties, but at the same time introduces a new positive charge in that position because, at physiological pH, it is likely that the nitrogen is protonated. From the chemistry point of view the amino function is also more suitable for further introduction of substituents such as amides or sulfonamides (**F** in Figure 3-3).

Another interesting modification is the introduction of a methylene group between the sugar and the hydroxyl group in 3' position, (structure **G** in Figure 3-3).

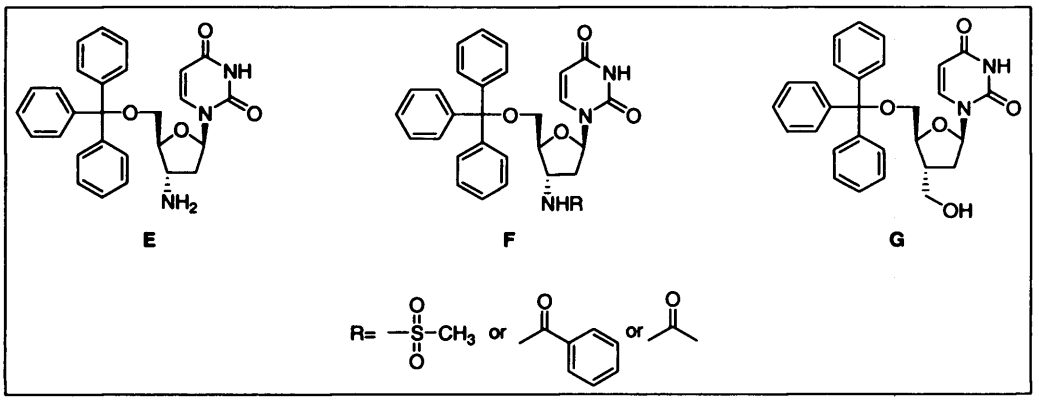


Figure 3-3 Proposed modifications in 3' position of the sugar

3.2 L. major dUTPase targets

No very active compounds were previously found in our group against *Leishmania* dUTPase. However some potential lead compounds were discovered by screening a library of compounds from one of our collaborators on this project, the pharmaceutical company Medivir AB[80]. These three compounds are illustrated in Figure 3-4. They all are thymidine derivatives, with a phosphate/phosphonate group in the position 5'.

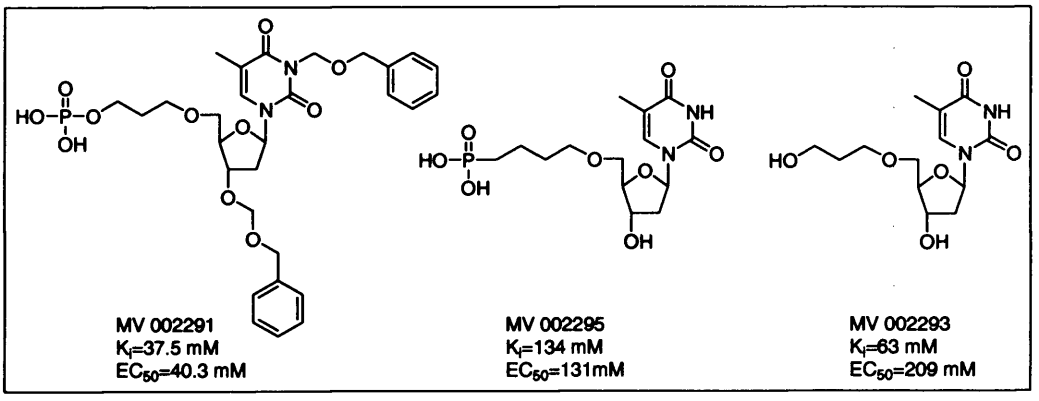


Figure 3-4 Three inhibitors of *L. major* enzyme found by screening a Medivir library[80]

Although their Ki values and the effective dose (EC_{50}), against the amastigote form of the parasite, are not exceptional (in the range of 30-100 μM); they were, so far, the best available results in our hands.

3.2.1 Medivir analogues or dUMP analogues

Having chosen the three Medivir compounds as leads, the most obvious suggestion, coming from the illustrated structures, was to synthesise the corresponding uracil derivatives.

In previous chapters it has been shown that the enzyme dUTPase is extremely selective toward all the nucleotides and is able to hydrolise only dUTP, therefore uridine analogues of the three MV compounds shoud have a better biological activity.

It was also attempted to investigate the effect of different chains in the 5' position with phosphate or phosphonate groups as terminators, as shown in Figure 3-5.

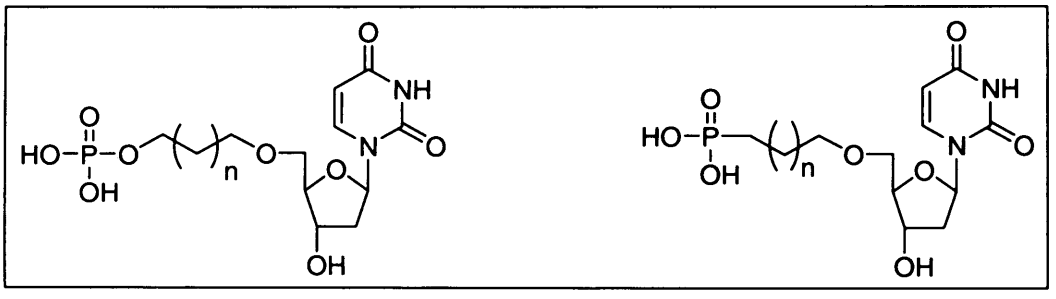


Figure 3-5 Proposed analogues of the three Medivir compounds

3.2.2 Acyclic phosphates and phosphonates

As a second objective it was decided to study the effect of structures with the deoxyribose sugar replaced with simple aliphatic chain of different lengths and phosphates or phosphonates groups as terminator as illustrated in the scheme below.

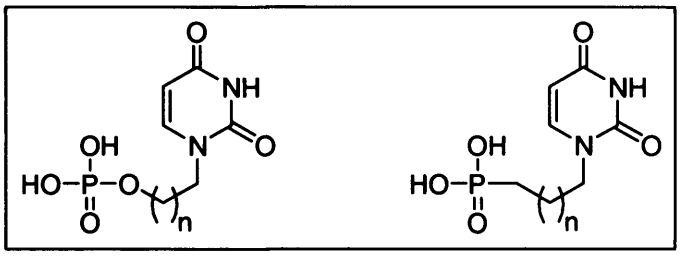


Figure 3-6 Proposed structures of phosphate and phosphonates acyclic derivatives

In this class of structures, the polar terminator at the end of the spacer chain should interact with the polar area of the active site, the one responsible for phosphate recognition.

3.3 Microbiology

As reported in the earlier chapters the enzyme dUTPase is ubiquitous and therefore is found in bacteria. One of the ideas at the beginning of this project was to investigate the enzyme dUTPase as a target within the bacteria. Therefore a small complementary project was developed in order to evaluate the antimicrobial activity of compound libraries readily available in our group. This part of the research was carried out by the author at the University of Brighton under the supervision of Dr. Jean-Yves Maillard. The main aim of this research was to test chemical compounds from the Welsh School of Pharmacy (WSP) against some standard bacterial strains in order to determinate their Minimum Inhibitory Concentration (MIC) values. This piece of work will be described in detail in Chapter 9.

Chapter 4 Synthesis of 2'-deoxyuridine Analogues

The three Medivir compounds were chosen as lead compounds against *L. major* dUTPase, their structures are shown in Figure 4-1.

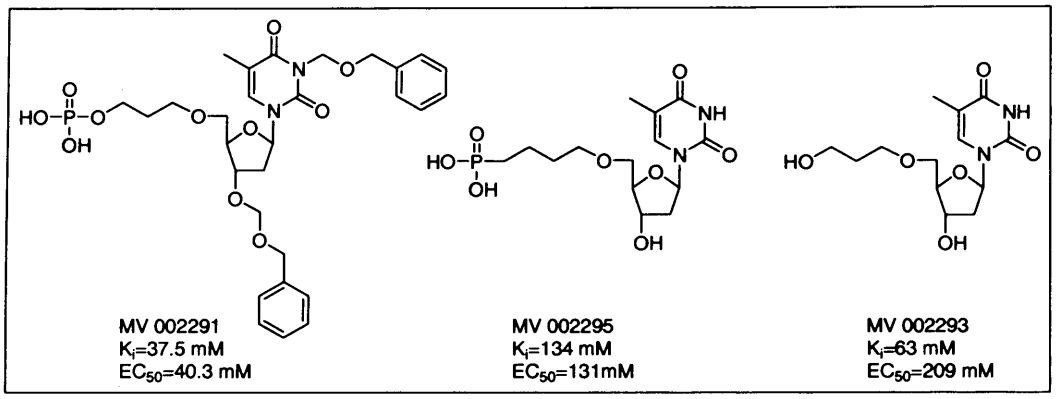
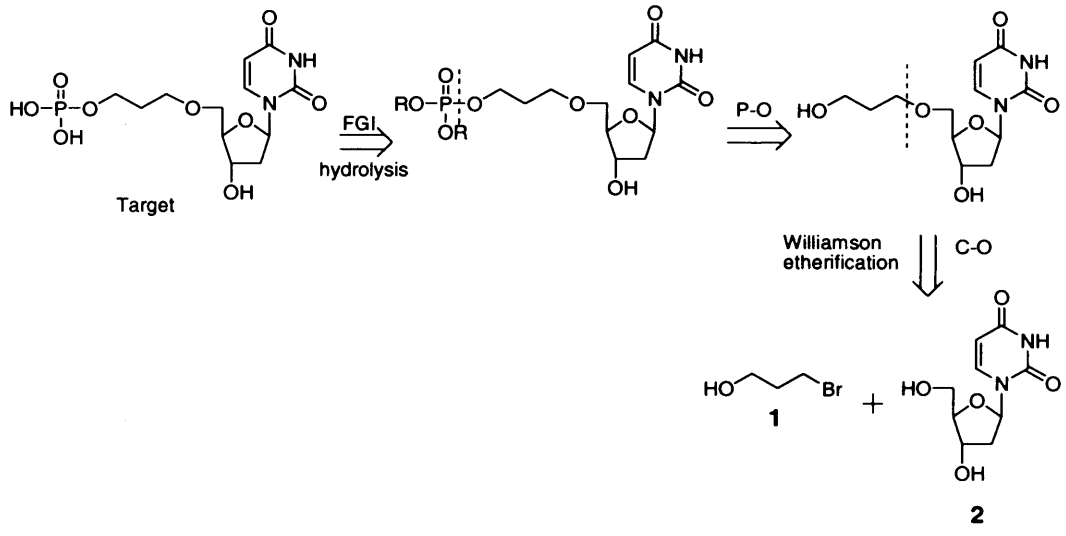


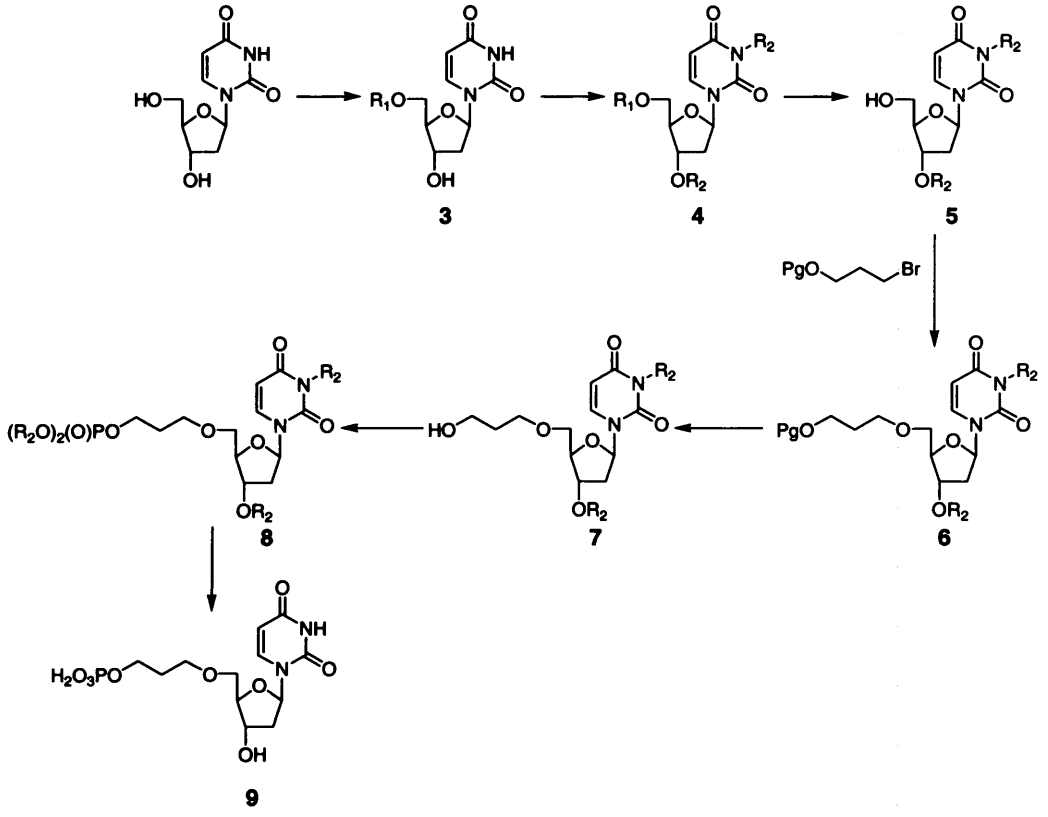
Figure 4-1 Chosen lead compounds against *L. major* dUTPase

Since they are thymidine derivatives the simplest modification was the synthesis of the corresponding uracil derivatives. The retrosynthetic analysis is shown in Scheme 4-1.



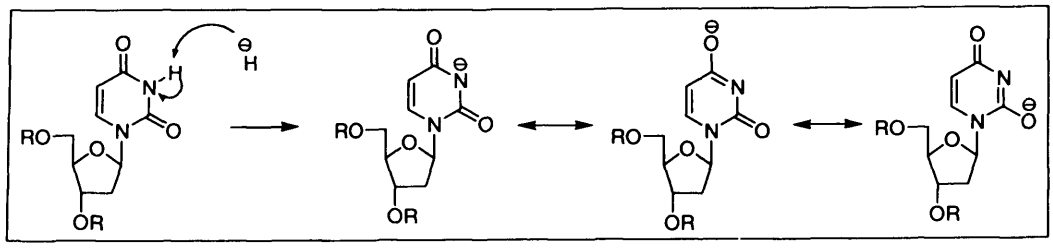
Scheme 4-1 Retrosynthetic analysis for the target molecule

The target compound could be synthesised starting from 2'-deoxyuridine **2** and 3-bromo-1-propanol **1**. The other two Medivir structures can be isolated as intermediates during the synthesis of the target compound. This retrosynthesis corresponds to a coupling reaction of the alkoxide of the primary alcohol of the 2'-deoxyribose with the protected bromo alcohol **1**, by a Williamson's etherification, followed by introduction of the phosphate moiety as a tri-ester and as final step, deprotection of the phosphate tri-ester. The synthetic route is depicted in Scheme 4-2.



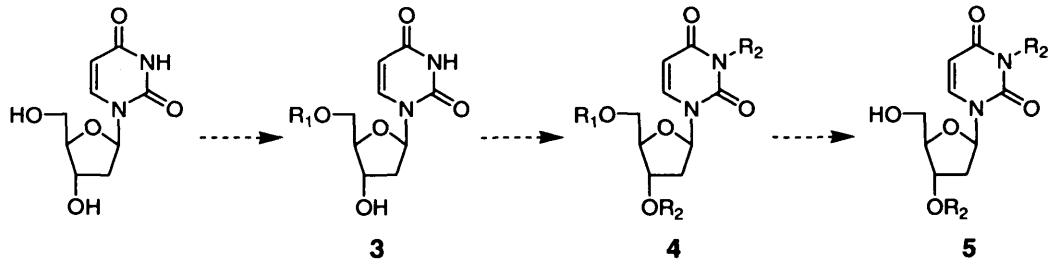
Scheme 4-2 Proposed synthesis of the target compound **9**

Unfortunately, from previous experience in the group[74], the synthesis of 5'-functionalised nucleoside by Williamson's etherification gave some problems when unprotected 2'-deoxyuridine on the N-3 position was used. The nitrogen proton is quite acidic and can be removed in the presence of a strong base like sodium hydride (Scheme 4-3).



Scheme 4-3 Deprotonation of the N-3 in the uracil base

Therefore the synthesis required the protection of both the secondary alcohol in 3' position and the amide function in position 3 of the uracil ring, as illustrated in Scheme 4-4. Once obtained, the intermediates **5** can be used for further modification on the 5' position of the sugar, such as a Williamson etherification or other reactions.

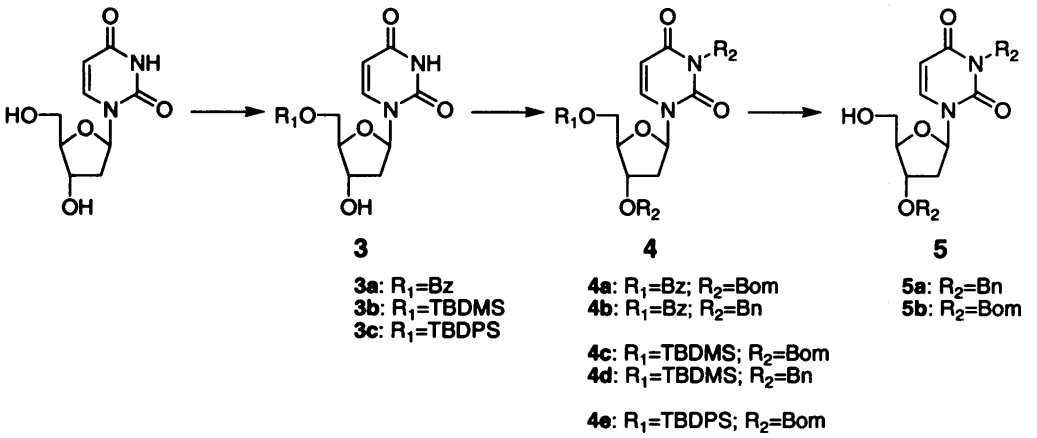


Scheme 4-4 Proposed synthesis of diprotected 2'-deoxyuridine

Such synthesis required the use of orthogonal protective groups. An orthogonal system is defined as a set of completely independent classes of protecting groups, which can be removed in any order and in the presence of the all the other classes[81]. The protection of the primary 5'-hydroxyl, followed by two steps of protection/deprotection to obtain the intermediates **5** shown in Scheme 4-4, in this case is required.

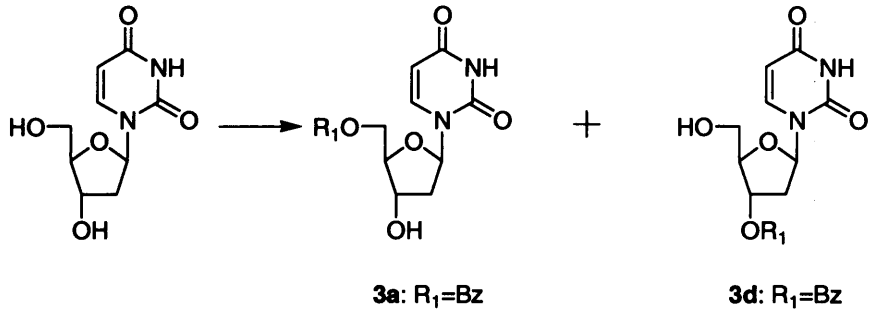
For the selective protection of the primary alcohol in 5' position it was decided to try the benzoyl group first. The reaction was carried out with benzoyl chloride using standard conditions[82], and cooling the system to 0°C in order to increase the selectivity for the primary alcohol rather than the secondary. After the work-up the ^{1}H-NMR of the crude residue showed the presence of some di-protected 2'-deoxyuridine, with integration in the aromatic region higher than expected. This was also confirmed with two signals, around 130 ppm in the ^{13}C-NMR, due to the

presence of two quaternary carbons in the aromatic region. However after chromatographic purification, compound **3a** was obtained in 50% yield.



Scheme 4-5 **3a**: BzCl, DCM/Py, 0°C, 1h. **3b**: TBMSCl, Imidazole, DMF, 0°C, 3h. **3c**: TBDPSCl, Imidazole, DMF, 0°C, 3h. **4b**, **4d**: BnBr, NaH, DMF, 25°C **4a**, **4c**, **4e**: BomCl, DIPEA, 1,2-dichloroethane, 56 °C, o.n..

It is noteworthy that in the scale-up of this protection a small amount of 3'-protected nucleoside was recovered, (Scheme 4-6). A small spot with lower Rf than the main product was visible on the TLC plate. The NMR of the corresponding fractions had almost the same shifts as compound **3a**, with the exception of the chemical shift of the proton in 3'-position, now moved to lower field. The low resolution mass spectrum also, confirmed the same molecular weight of the corresponding isomers **3a**, and therefore it was concluded that the side product of the reaction was the corresponding 3'-benzoyl-2'-deoxyuridine **3d**.



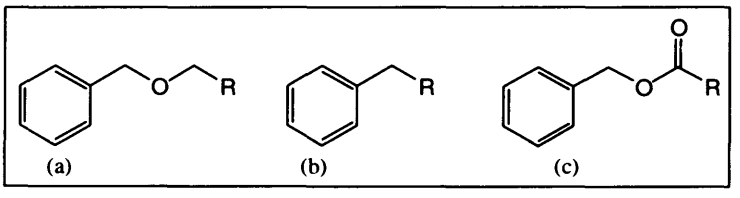
Scheme 4-6 Products **3a** and **3d** of the first protections of the 2-deoxyuridine with benzoyl chloride

As a second attempt the *tert*-butyldimethylsilyl (TBDMS) group was chosen to protect the primary alcohol. In this case, TBDMS-chloride was used as silylating

reagent in the presence of imidazole as base and DMF as solvent. This gave a higher yield of compound **3b** (78%) and a smaller quantity of diprotected compound which was more easily removed by chromatography.

The third protective group investigated for the protection of the 5'-position was the *tert*-butyldiphenylsilyl ether (TBDPS). It was introduced by the same procedure as the TBDMS group. The advantage of this silyl ether is that due to its hindrance it is more selective for the primary alcohols. In fact it provided a more effective result, when compound **3c** could be isolated in 90% yield, after chromatographic purification to remove the traces of diprotected 2'-deoxyuridine.

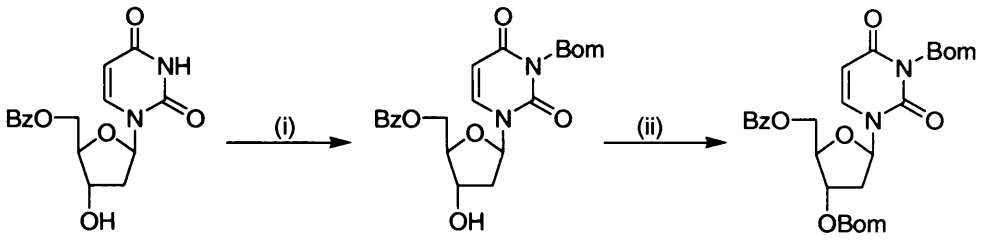
To continue the synthesis, it was then necessary to protect both the 3'-OH of the sugar and the 3-NH of the nucleobase with an orthogonal protective group. As the first protective groups were base (benzoyl) and acid labile (silyl ethers), the choice of the second was restricted to those protecting groups which are stable in these conditions. It was decided to use the benzyloxymethyl ether (Bom), benzyl (Bn) and carbobenzyloxy (Cbz) groups. All of them are cleavable by catalytic hydrogenation and stable to the conditions used to cleave the Benzoyl, TBDMS and TBDPS groups.



Scheme 4-7 (a) Bom; (b) Bn; (c) Cbz

This choice was made to introduce the phosphate moiety as a benzyl ester, which can be cleaved by catalytic hydrogenation to liberate the phosphate group. Therefore this step could be combined together with the cleavage of the protective group used on the secondary alcohol and on NH of the uracil (Bom, Benzyl or Cbz) saving one step in the total synthesis of the target compound.

In the first attempt to introduce the Bom group a two step procedure[83] was first followed (Scheme 4-8). That was starting from the 5'-protected deoxyuridine, using dry acetone, potassium carbonate and Bom-chloride.



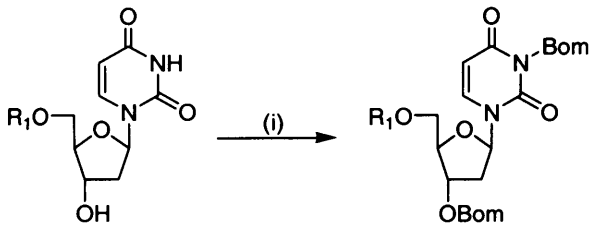
3a **3h** **4a**

Scheme 4-8 (i): BomCl, K_2CO_3, Acetone, 56 °C, 12 h; (ii): DIPEA, BomCl, Toluene, 50 °C, 1h.

Heating the reaction for 12 hours at 56 °C the 3-N protected compound **3h** was obtained after purification by flash chromatography in 57% yield. The proton NMR confirmed the outcome with the disappearance of the NH signal at δ 11.30, and the still visible broad singlet of the OH at δ 5.58 using deuterated DMSO as solvent. The aromatic region at δ 7.45-7.58 integrated to 10 protons, consistent with the required product.

The next step was performed with DIPEA as base, Bom-chloride and toluene as solvent. After warming for 1 hour at 50 °C the reaction seemed to be complete, there were no traces of starting material **3h** in the TLC. Unfortunately, after cooling the reaction and following the suggested work-up[83], the final product **4a** was recovered in very low yield (<20% final yield) after purification. The reason for this failure could be due to the reagent Bom-Cl, which only comes in 67% purity.

According to a second method[84] (Scheme 4-9) it is possible to introduce the Bom group in one step. This procedure required the use of Bom chloride, DIPEA as base and 1,2-dichloroethane as solvent, warming to 56° C overnight. It was first tested on the 5'-benzoyl deoxyuridine **3a**, and after chromatographic purification, the desired product was obtained in 58% yield. The same procedure was then applied to the 5'-silylated nucleosides **3b** and **3c**. The result for the TBDMS intermediate was superior to the benzoyl with 72% yield; this could be attributed to the better solubility of the TBDMS derivatives in dichloroethane. Unfortunately, the TBDPS was not as good as the previous, with only 48% as final yield, although no particular reasons can be found to explain the result.



3a: R_1=Bz
3b: R_1=TBDMS
3c: R_1=TBDPS

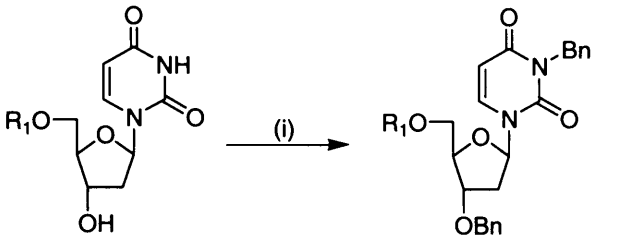
4a: R_1=Bz
4c: R_1=TBDMS
4e: R_1=TBDPS

Scheme 4-9 (i): BomCl, DIPEA, 1,2-dichloroethane, 56 °C, 24 h

All the NMR analyses confirmed the presence of compounds with the disappearance of the NH signal at δ 11, a higher integration in the aromatic region and the four singlets between δ 5.50-4.50 for the four CH_2 of the Bom groups.

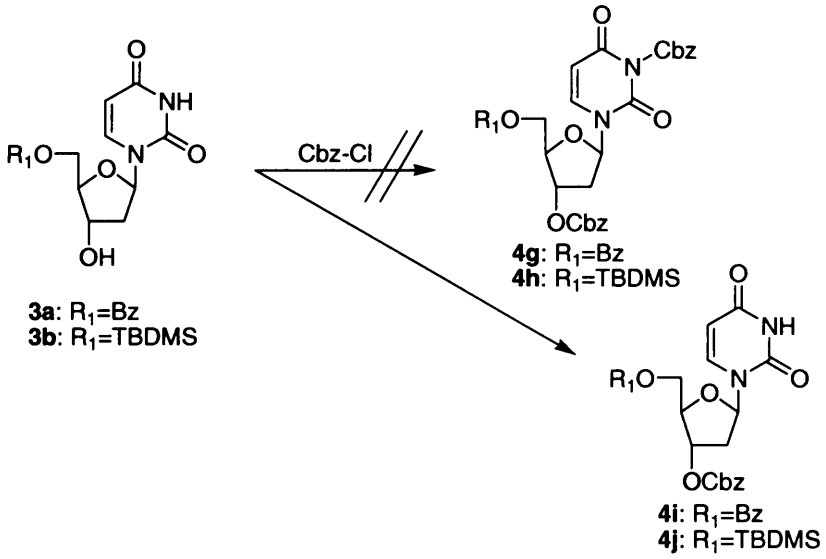
Since compound MV002291 has two Bom groups (Figure 4-1) and is one of the lead compounds with the lowest *Ki* value (37.5 μM) against L. *major* dUTPase; it was decided to explore the effect of other protective groups, in order to understand the biological effects correlated with the presence these moieties in the molecule. The benzyl group was the second protective group studied. This is also cleavable by hydrogenolysis, but should be more stable in the cellular environment.

The standard procedure[82] used to introduce the benzyl group onto an amino function; requires the use of benzyl bromide (BnBr), a strong base such as sodium hydride (NaH) in a polar aprotic solvent like dimethylformamide (DMF). These conditions are harsher than those required to introduce the same protective group on alcohols and therefore with two equivalents of BnBr and sodium hydride it was possible to achieve the double protection in a one step reaction. After stirring the reaction for 2-12 hours (depending on the substrate) at room temperature, the desired compounds were isolated in 43%, 52% and 30% yields, respectively, for **4b**, **4d** and **4f**, (Scheme 4-10).



Scheme 4-10 (i): BnBr, DMF, 25°C, 2-12 h.

A third protective group cleavable by reduction with hydrogen is the benzyloxycarbonyl (Cbz) group. This group would introduce further structural diversity in the molecule since it would create a carbamate function with the amino group and a carbonate function with the alcohol. Several attempts were made to introduce the Cbz on substrates **3a** and **3b**; in all cases benzyl chlorofomate was used as the reagent, with different bases and solvents as shown in Table 4-1.



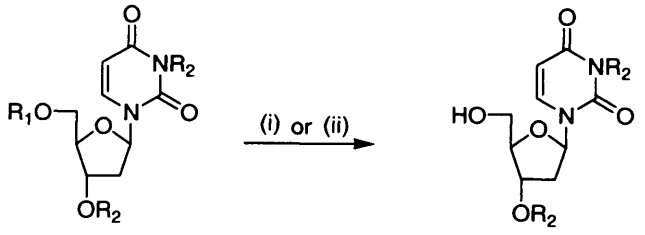
Scheme 4-11 Attempted protection with benzyl chloroformate (Cbz-Cl) led only to compounds **4i** and **4j**

Substrate	Base	Solvent	Product/Yield
3a	DMAP	DCM/THF	**4i**
3a	DIPEA	THF	**4i**
3a	NaH	DMF	N. R.
3b	DMAP	DCM	**4j**
3b	DIPEA	1,2-dichloroethane	**4j**

Table 4-1 Conditions tested to introduce the Cbz group; N.R=no reaction

None of them was successful and only the protection of the OH in 3' was obtained. This was proved by NMR where it was possible to see the broad singlet due to the NH proton around δ 10-9. Also the integration of the aromatic region gave only 10 protons for compound **4i** and 5 for compound **4j**, instead of the expected 15 and 10, respectively. The low resolution mass spectrum indicated only the molecular weight of the monoprotected compounds. Hence after these failures the Cbz protective group strategy was abandoned.

The next step in the synthetic route was the deprotection of the 5'-OH. The cleavage of the benzoyl group in compound **4a** was achieved according to Krecmerova *et al.*[83] with sodium methoxide in methanol, and neutralization of the excess base with Dowex ion-exchange resin. The intermediate **5b** was obtained in 72% yield, after purification to remove the methyl benzoate by-product of the reaction. Both ^{1}H and ^{13}C NMR were according to the required structure. In the proton NMR the integration for the aromatic region was 20 protons, and in the carbon the disappearance of the carbonyl at δ 166 was observed, consistent with loss of the benzoyl group.



4a: R_1= Bz; R_2=Bom
4c: R_1=TBDMS; R_2=Bom
4d: R_1=TBDMS; R_2=Bn
4e: R_1=TBDPS; R_2=Bom

5a: R_2=Bn
5b: R_2=Bom

Scheme 4-12 (i): 1) MeONa/MeOH 1M, r.t.; 2) Dowex ion exchange. (ii): TBAF 0.1 M in THF, r.t.

To cleave the TBDMS group two different conditions were tested, one using a solution of tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF) with intermediate **4d**, the second using solid TBAF added to a solution of compound **4c** in THF. Although both procedures gave the desired compounds, when the already prepared solution was used it was more effective, and with the same reaction times, the yields were higher. The reactions were monitored by TLC, stirred at room temperature until the disappearance of the starting material was observed on TLC and the new spot for the product at lower R_f was present. The NMR analysis confirmed the purity of the product, as no signs of the characteristic singlets at δ 1.00 and δ 0.00 for the *tert*-butyldimethyl silyl group were present.

Also for the deprotection of TBDPS the standard procedure with TBAF was used. The cleavage was achieved in 76% yield for compound **4e** (Scheme 4-12) with a solution of TBAF in THF. The only problem encountered with this reaction was the difficulty in monitoring the reaction by TLC, since the by-product of the reaction *tert*-butyldiphenylsilyl fluoride had almost the same Rf as the starting material, therefore it was not completely clear when the reaction had reached completion. However the result was supported by ^{1}H-NMR where the singlet for the 9 protons of the *tert*-butyl group was absent, and the integration for the aromatic multiplet was for 20 protons.

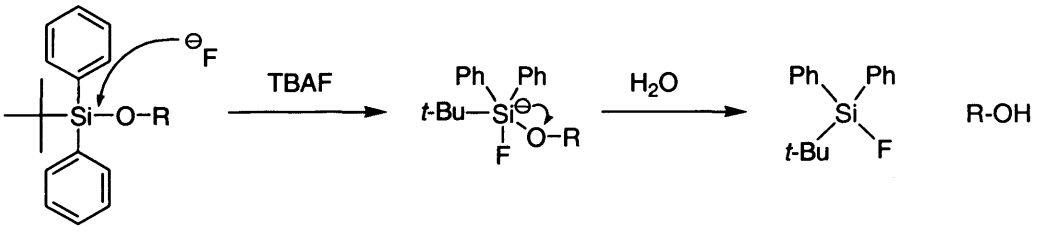
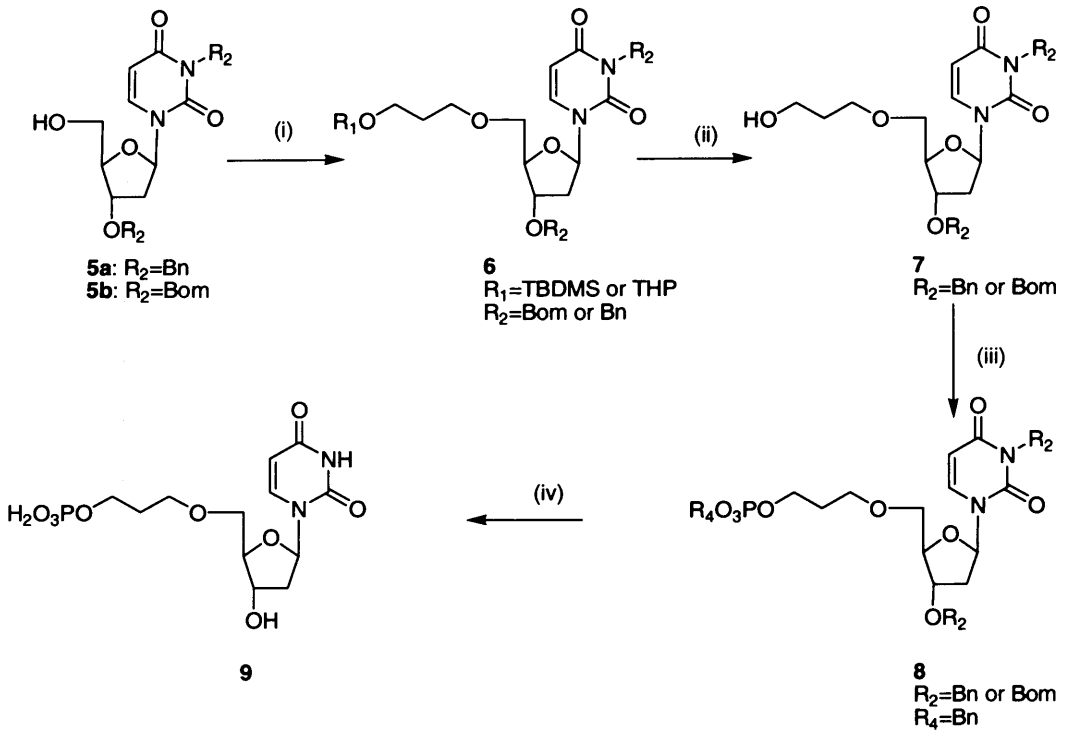


Figure 4-2 Proposed mechanism for the deprotection of silyl ethers with TBAF

At this stage the intermediates **5a** and **5b** could be used to achieve the functionalization of the 5' position of the sugar; in particular it would be possible to carry on with the planned synthesis as shown in Scheme 4-2.

4.1.1 Attempted phosphate synthesis



Scheme 4-13 Proposed linear synthesis of the phosphate derivatives. (i) $R_1O(CH_2)_3Br$, NaH, THF. (ii) TBAF in THF or HCl in MeOH. (iii) $(BnO)_2P(O)Cl$, Pyridine. (iv) H_2, Pd/C, MeOH.

According to the linear strategy (Scheme 4-13) the next step was the etherification *via* Williamson's reaction, Figure 4-3. In this kind of reaction the alkoxide ion is generated by reaction of the alcohol with a strong base like sodium hydride, and after it displaces the halogen (or other leaving group) from the alkyl halide, generates a new C-O bond[85]. The reaction is a nucleophilic substitution where the nucleophile is the alkoxide ion, and in our case the alkoxide is generated from compounds **5a** and **5b**.

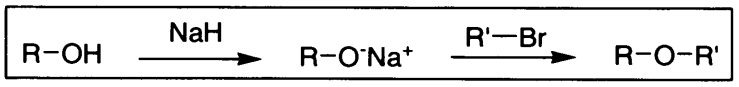
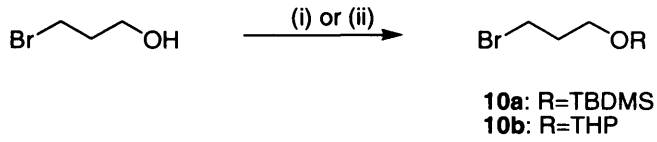


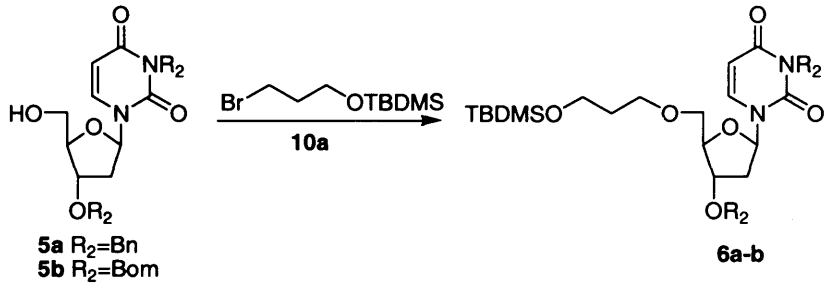
Figure 4-3 General Williamson etherification

The commercially available 3-bromo-1-propanol was chosen as alkyl halide, since this substrate has a hydroxyl function it was required to protect it to avoid any self condensation side reactions.



Scheme 4-14 (i): TBDMSCl; Imidazole, DMF, 25 °C, 12 h; (ii): DHP, PPTs, DCM, 25°C.

The TBDMS protected bromo-alcohol **10a** was obtained in 90% yield without necessity of chromatographic purification. The work-up with basic and acidic washing was enough to remove the by-products of the reaction, affording the product in a reasonable purity. The following step was the etherification with compounds **5a-b**, (Scheme 4-15). Despite the many different conditions attempted (Table 4-2), none of them gave the desired compound in good yield. The few times that it was possible to recover some of intermediate **6a** the amount was very low (< 15%) and it was not possible to carry on with successive steps.



Scheme 4-15 Attempted etherification with the TBDMS bromo-alcohol and the intermediates **5a** and **5b**

Substrate 1	Substrate 2	Base	Solvent	Result
5a	**10a**	NaH	DMF	Low yield
5a	**10a**	NaH	THF	N. R.
5a	**10a**	K_2CO_3	THF	N. R.
5b	**10a**	NaH	DMF	Low yield
5b	**10a**	NaH/Imidazole	DMF	Low yield
5b	**10a**	NaH	THF	N. R.
5b	**10a**	DBU	THF	N. R.
5b	**10a**	K_2CO_3	CH_3CN	N. R.

Table 4-2 Conditions tested in the reaction of etherification. N.R.: no reaction

The problems encountered in this stage were different: no reaction was observed on TLC, when any solvent other than DMF was used. The only effective base was found to be sodium hydride. When the reaction was stirred for longer than 18 hours with more than two equivalents of base, decomposition of the starting materials was observed on TLC, which showed several spots between the Rfs of the starting materials. Perhaps the reason for the failures could be attributed to the lability of the TBDMS group under the reaction conditions, and to the sensitivity of the di-protected uridine with the excess of strong base.

To avoid one of the aforementioned problems it was decide to change the protecting group of the hydroxyl function to the tetrahydropyranyl group (THP), which is more stable in basic conditions and can be cleaved by acidic treatment. It was introduced using the standard procedure[82] with DHP, dichloromethane and pyridinium *para*toluensulfonate as acid catalyst (Scheme 4-14). The THP protected bromo-alcohol **10b**, was obtained in 73% yield as a mixture of enantiomers after purification by chromatography. In fact, the introduction of the THP function in the molecule is accomplished with the introduction of a new chiral centre (Figure 4-4).

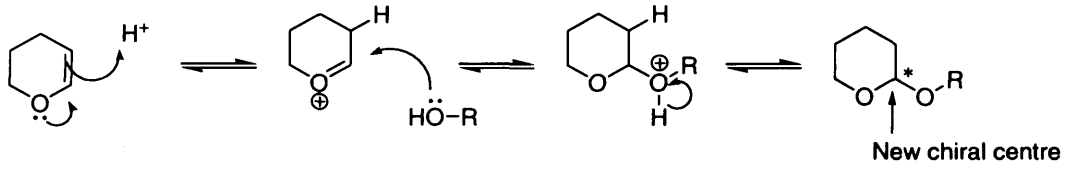


Figure 4-4 Reaction mechanism of THP protection

For the etherification with substrates **5a-b** different conditions were tested. Following the observations of Aspinall *et al.*[86] some crown ether was added to the reaction with sodium hydride in THF. The effect of this compound is to coordinate the Na^+cation, within itself, making the oxygen of the alkoxide more available for the nucleophilic attack, as shown in Figure 4-5.

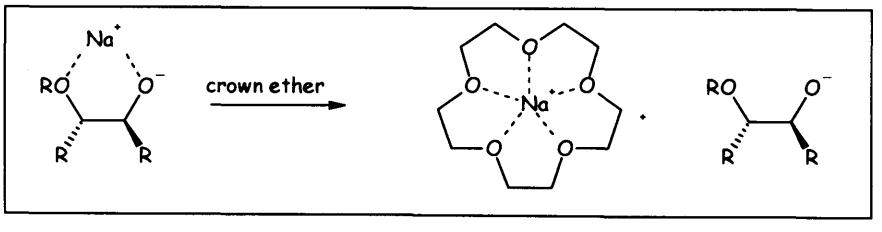
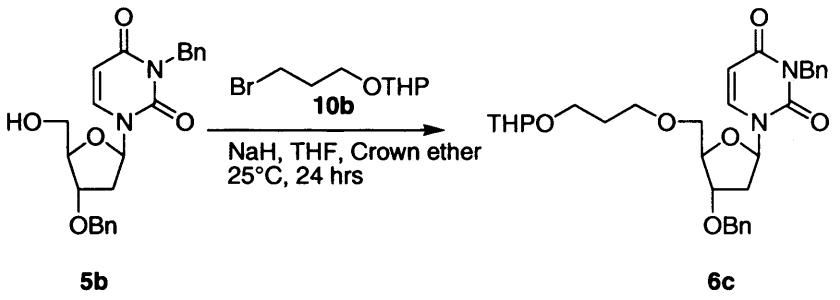


Figure 4-5 Chelating action of the 15-crown-5-ether on the sodium cation[86]



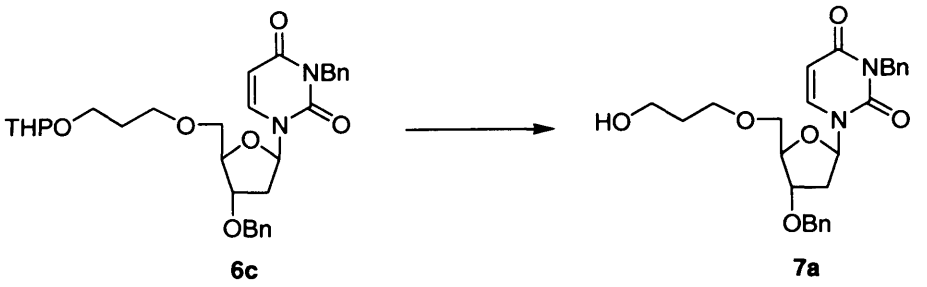
5b **6c**

Scheme 4-16 Etherification with THP bromo-alcohol **10b**; sodium hydride; THF and 15 –crown-5-ether

Under these conditions the compound **6c** (Scheme 4-16) was obtained in 43% yield. The result was supported by NMR analysis. Although the proton NMR was quite complicated, it was possible to identify the new signals due to the presence of the three carbon aliphatic chain and the THP group through the higher integration in the regions between δ 4.5-3.5 and δ 2.0-1.0. The low resolution mass analysis also confirmed the result of the reaction with one peak at 573.1 m/z for the ion $[M+Na]^+$.

Once the intermediate **6c** was obtained the synthesis was continued with the introduction of the phosphate moiety at the end of the aliphatic chain in 5' position, after cleavage of the THP group.

For deprotection, **6c** was treated with dilute HCl in methanol and after a few hours, at room temperature, the reaction was complete, (Scheme 4-17). The total disappearance of the starting material was observed together with the appearance of a new spot at a lower Rf. The solvent and the by-products were removed under reduced pressure and the desired compound was obtained in quantitative yield without further purification. The mass analysis showed only one peak at m/z 489.1 corresponding to the $[M+Na]^+$ ion. The ^{1}H-NMR was in agreement with the desired compound showing a quintet for the CH_2 in the middle of the aliphatic chain at δ 1.7-1.5. The ^{13}C-NMR also confirmed the outcome of the reaction with all the signals for the three carbons of the chain at 69.7, 60.7 and 32.7 ppm respectively.



6c **7a**

Scheme 4-17 THP deprotection with 3M HCl in MeOH at room temperature

A common way to convert alcohols into phosphate esters is to prepare a chloro derivative of a di-substituted phosphate and react it with an alcohol in the presence of a base to neutralise the HCl released[87]. In our case we wanted to introduce the phosphate protected as benzyl ester to save one step with the final hydrogenolysis which would cleave all the protecting groups at once. Another advantage of this procedure would be to avoid a tedious chromatographic purification, because the catalytic hydrogenation is a clean reaction where the catalyst can be removed by simple filtration and the solvent and by-product can be removed *in vacuo*. Therefore we required the chloro derivative of the dibenzylphosphite, which can be prepared by reaction of sulfuryl chloride and the commercially available dibenzylphosphite, as depicted in Figure 4-6.

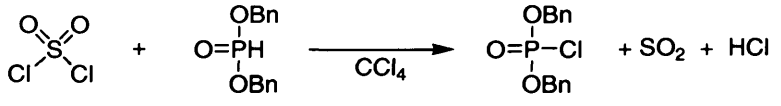
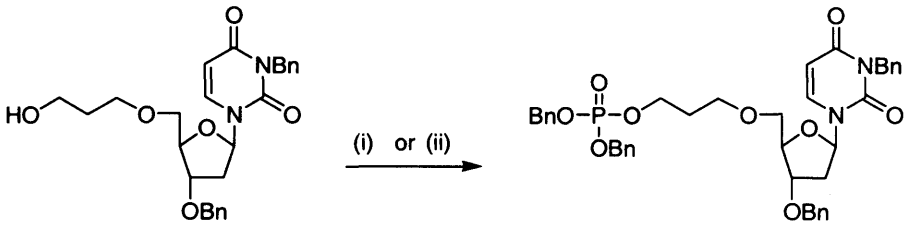


Figure 4-6 *in situ* preparation of dibenzylchlorophosphite

The reaction was monitored by TLC and, when complete, the *in situ* prepared dialkyl phosphochlorohydrates was used directly after evaporation of the by-products. It was added by syringe to an ice-bath cooled solution of **7a** in dry pyridine. The system was stirred under nitrogen and warmed to room temperature. The TLC showed the complete disappearance of the starting alcohol after two hours, and several new spots with higher R_f's. Since the scale of the reaction was small no work-up was attempted and the concentrated residue was purified by chromatography affording a main fraction, not completely pure, in approximately 38% yield.



Scheme 4-18 (i): Dibenzylchlorophosphite, pyridine, r.t. (ii): Tribenzyl phosphite, Iodine, Pyridine, DCM, r.t.

Spectroscopic analysis of the product was in accordance with the desired structure: one singlet in the decoupled ^{31}P-NMR at δ 0.56, corresponding to the phosphate group; a multiplet integrating to 20 protons in the ^{1}H-NMR experiment and a multiplet with complex structure present around δ 5.05, for the new two methylenes of the benzyl groups. The ^{13}C-NMR was very complex, due to the coupling between the C and P, especially in the aromatic region between 128-140 ppm. Nevertheless it was possible to identify the main signals for the uracil moiety and the sugar ring.

In order to achieve better and more replicable results a second procedure was attempted. Gefflaut *et al.*[88] used a combination of tribenzyl phosphite and iodine to convert hydroxyl groups into phosphate dibenzyl esters. According to this oxidating/phosphorylating procedure the species P(III) reacts first with iodine to afford a phosphoryl iodide and then the alcohol displaces the halogen with a nucleophilic substitution on the phosphorus[89], Figure 4-7.

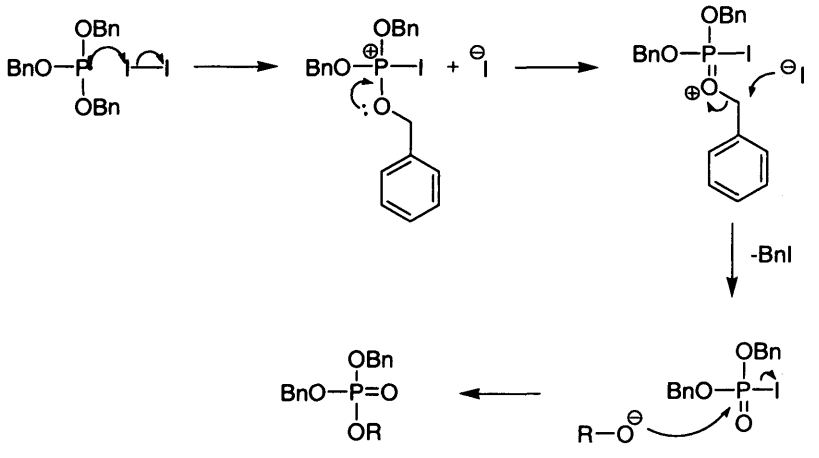
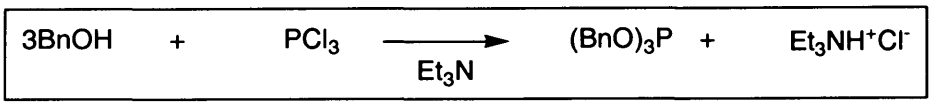


Figure 4-7 Proposed mechanism for the phosphorylation with tribenzyl phosphite and iodine

The tribenzyl phosphite was prepared from benzyl alcohol and phosphorous trichloride in the presence of triethylamine. Although the reaction is simple, it requires attention because of the moisture sensitivity and the high susceptibility to oxidation of the product. After purification by chromatography, the TLC showed two spots, but in the ^{31}P-NMR there was only one peak at 140 ppm, corresponding to the phosphorous (III) derivative, and also in the proton NMR there were no traces of impurity, so it was assumed that the second spot with a R_f lower than the main compound was due to oxidation of the product on the TLC plate. The obtained tribenzyl phosphite was stored under nitrogen at 4 °C.

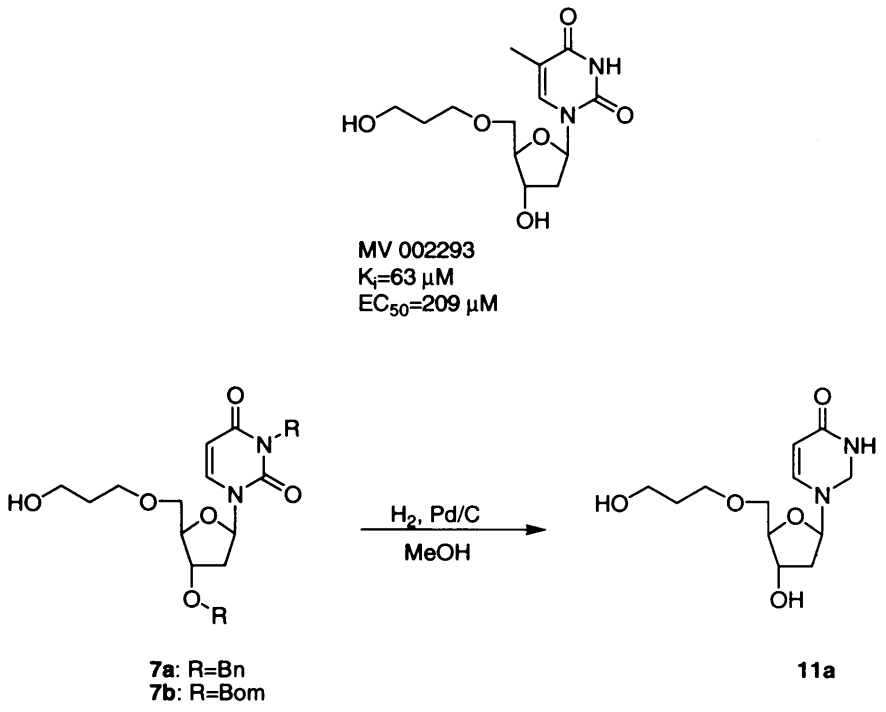


Scheme 4-19 Synthesis of tribenzyl phosphite: Benzyl alcohol, PCl_3, triethylamine, diethyl ether, 24 h

The oxidation/phosphorylation was then tested on a small scale with intermediate **7a**. The phosphoryl iodide was prepared by reaction of tribenzyl phosphite and iodine in a separate flask and then it was added to a solution of **7a** in dry pyridine. The reaction was monitored by TLC, the complete disappearance of the starting material was observed after two hours at room temperature. The mixture was purified by chromatography, effording a pure product compared with the previous synthesis, but the final yield was disappointingly low, (25%). The NMR analysis had the same characteristic signal found also for the previous reaction. However further attempts in the scale up of this procedure did not succeed, and the desired compound could not be isolated in a reasonable quantity and purity.

After the last two consecutive partial failures with the planned synthesis the attempts were stopped, in order to search for an alternative synthetic pathway with better yields in order to obtain more intermediate **8** to complete the synthesis.

At this stage the intermediates **7** could be used to synthesise the first analogue of MV002293. This was achieved by simple reduction with palladium on carbon as the catalyst and hydrogen gas.

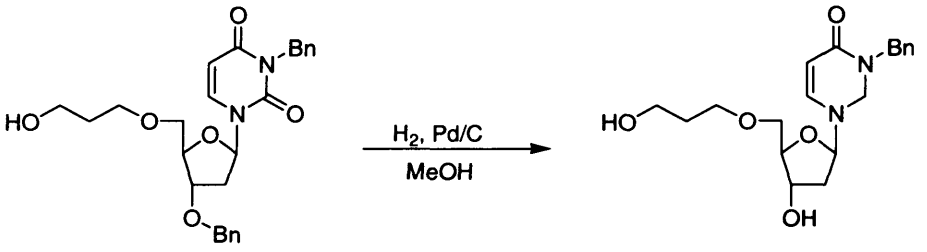


Scheme 4-20 Cleavage of the Bom and Bn group to obtain the MV002293 analogue **11a**

The two intermediates were dissolved in methanol and the catalyst (palladium 10% on charcoal) was added to the solution. The hydrogen was introduced with a balloon and the reactions were stirred at room temperature until all the starting materials were completely reacted.

The intermediate **11a** was successfully obtained after overnight stirring. The TLC of the reaction mixture clearly showed the presence of two new spots with lower Rfs and no spot for the starting Bom derivative was visible on the plate. After filtration the residue was purified by chromatography, to remove the small traces of by-products, affording the desired compound **11a** in 80%. The mass analysis confirmed the result of the reaction showing only one peak at 309 m/z for the ion $[M+Na]^+$ using low resolution electrospray analysis. This was also confirmed by NMR spectroscopy where in the proton NMR there was a quintuplet at δ 1.82 integrating 2H for the CH_2 group in the middle of the aliphatic chain, no sign of aromatic protons, and two multiplets in the region between 3.5-4.0 ppm corresponding to the two methylene groups bound to oxygens. In the carbon spectra all the signals for the aromatic carbons around 127-128 ppm disappeared and three new peaks at δ 72.1, 69.7 and 34.0 indicated the presence of the three carbon aliphatic chain.

The deprotection of dibenzylated intermediate **7a** did not have the same outcome as the previous reaction. After stirring overnight in the presence of the catalyst and a slight pressure of hydrogen only one spot with R_f 0.20 was present on TLC. Since the reaction seemed clear the catalyst was removed by filtration and the reaction mixture concentrated under vacuum. Unfortunately the NMR of the crude residue still showed the presence of a benzyl group with a multiplet around δ 7.30, thus proving that only a partial deprotection was achieved (Scheme 4-21). Searching in the literature it was found that this problem can be encountered in the cleavage of *N*-benzyl pyrimidine nucleosides[90,91].



7a **11b**

Scheme 4-21 Partial de-benzylation of the intermediate **7a**

Alternative methods for the cleavage of benzyl group from amide are either with a mixture of sodium in ammonia or with boron tribromide in benzene[92]. However such procedures are not easy to apply since they require the use of ammonia gas or long heating with benzene. Therefore, due to the small amount of monobenzylated intermediate **7a**, no further attempts could be made to cleave the benzyl group from the uracil ring.

4.1.2 Attempted phosphonate synthesis

A second planned modification of the Medivir structures was the substitution of the phosphate moiety with a phosphonate group. Phosphonates have similar physical properties to phosphates. The bond distance and the angle distance differ only slightly. The main difference between the two groups is the stability of the C-P bond compared to the P-O bond. In fact phosphate groups are susceptible to enzymatic hydrolysis in the cellular environment, whilst the phosphonate cannot be hydrolysed by esterase enzymes present in the cellular environment. Therefore phosphonates are considered as the most direct isostere of a phosphate group.

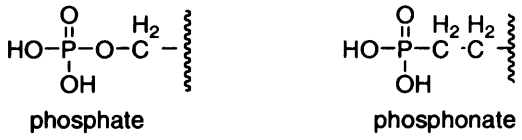


Figure 4-8 Phosphate and phosphonate structures

Consequently, simultaneously to the phosphate synthesis, it was decided to attempt the synthesis of some phosphonates. The proposed synthetic route to these compounds is depicted in Figure 4-9.

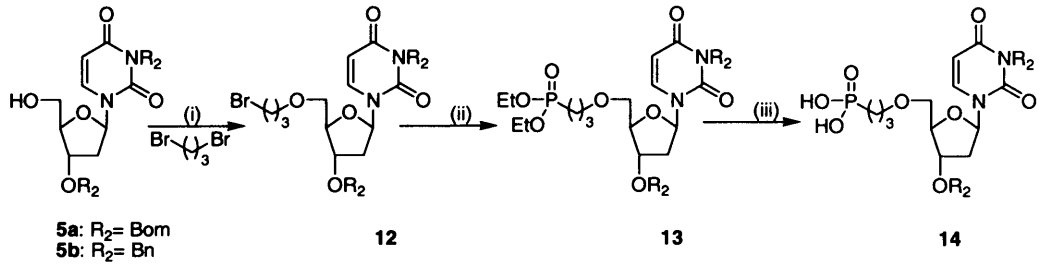


Figure 4-9 Proposed route to the synthesis of phosphonate **14**; (i) NaH, 1,3-dibromopropane, DMF; (ii) $P(OEt)_3$; (iii) Me_3SiBr.

It consists of four steps: coupling of the intermediates **5a-b** with a 1,3-dibromopropane, followed by a Michaelis-Arbuzov reaction and as the final step, the hydrolysis of the ethyl groups with trimethylsilyl bromide.

The Michaelis-Arbuzov reaction[87] is a common procedure to convert alkyl halides into phosphonates. It consists of nucleophilic attack of the phosphorus of

a trialkyl phosphite on the alkyl halide. This leads to a positively charged intermediate which undergoes formation of P-O double bond and elimination of the more volatile alkyl halide, see Figure 4-10.

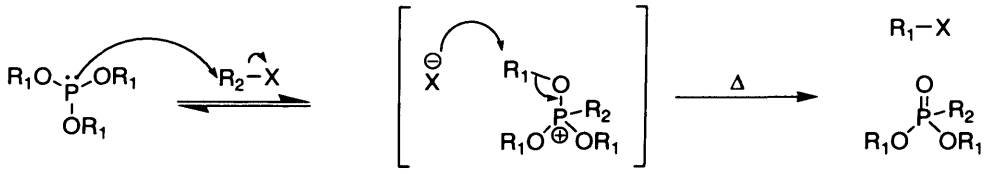
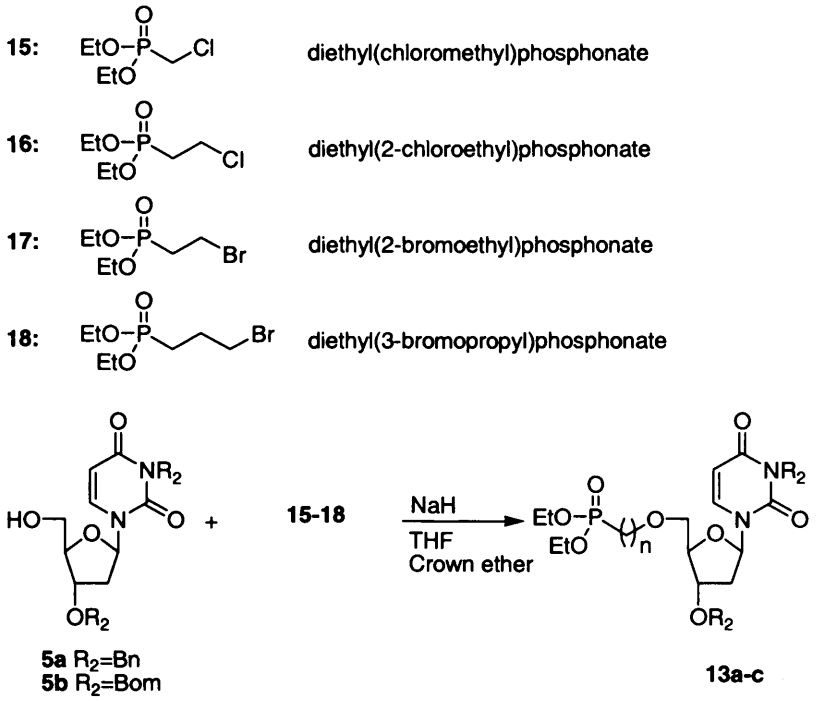


Figure 4-10 Mechanism of the Michaelis-Arbuzov reaction

We attempted the synthesis of the alkyl bromide nucleoside **12** using the same conditions explored for the Williamson etherification. The *bis*-bom protected nucleoside **5a** was treated with sodium hydride in DMF. After generation of the alkoxide, the dibromopropane was added in a slight excess. Unfortunately it was not possible to monitor clearly the reaction by TLC, since the halide was not UV active and no staining agent was found able to visualise the compound on the TLC plate. Consequently, the reaction was stopped after 3 days with traces of starting material **5a**. Further problems were encountered in the purification of the residue, when it was difficult to remove the DMF left from the reaction. After chromatography the collected fraction showed the probable presence of the desired compound **12**, although it was not completely pure, with a yield around 15%.

To avoid two synthetic steps it was decide to use some commercially available diethyl-halophosphonates **15-18** as shown in the next scheme.



Scheme 4-22 Alternative strategy using the four commercially available phosphonates **15-18**

Several reaction conditions were tested in a similar manner as described for the Williamson etherification, (see Table 4-3).

Substrate 1	Substrate 2	Reaction conditions	Result
5a	**15**	NaH, DMF, 48 h, r.t	N. R.
5b	**15**	NaH, DMF, 48 h, r.t	N. R.
5a	**16**	K_2CO_3, DMF	N. R.
5a	**16**	NaH, THF, crown ether, 48 h, r.t.	51 %
5a	**17**	NaH, THF, crown ether, 48 h, r.t	mixture
5b	**17**	NaH, THF, crown ether, 48 h, r.t	mixture
5a	**18**	NaH, THF, crown ether, 24 h, r.t	mixture
5b	**18**	NaH, THF, crown ether, 24 h, r.t	mixture

Table 4-3 Conditions tested for the synthesis of the phosphonate analogues. N.R. no reaction

With the diethyl(chloromethyl)phosphonate **15** and the two substrates **5a-b**, no reaction was observed by TLC after 48 hours at room temperature, and no higher temperatures were tested due to safety reasons.

The only positive result was obtained with diethyl(2-chloroethyl)phosphonate **16** affording the desired compound **13d** in 51% yield. All the NMR, ^{1}H, ^{13}C and ^{31}P data were consistent with the required structure. In the proton spectrum there was a clear triplet integrating to six protons for the CH_3 of the ethyl ester at δ 1.35. The carbon spectrum showed splitting pattern for the ethyl carbons due to the coupling with the phosphorous nucleus. In the ^{31}P NMR spectrum there was only one singlet at δ 29.06 proving the success of the reaction.

Decomposition was observed, and mixtures of different compounds were isolated from the reactions between the substrates **5a-b** and the diethyl(3-bromoethyl)phosphonate **17**. The reason for these failures could be attributed to the starting material which was too sensitive to the strong basic conditions used for the reactions. In all cases the reaction was monitored by ^{31}P-NMR and two new signals appeared at δ 28 and δ 19, the first perhaps corresponding to the desired compound and the second due to a by-product. The main problem of this coupling reaction is competition between substitution of the bromine and elimination. In fact the bromophosphonate **17** and **18** (and also the chloride derivatives) can undergo elimination in basic conditions in the presence of hindered bases.

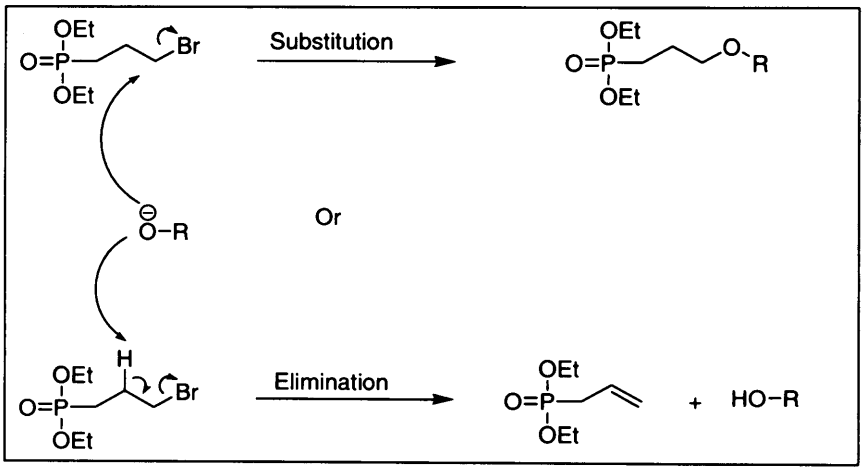


Figure 4-11 Competition between substitution and elimination

The elimination reaction is favoured in the presence of strong hindered bases; in this case perhaps the generated alkoxide ion was acting as a base instead of a nucleophile. This conversion of 2-bromoethane phosphonate into vinyl

phosphonate has been reported in literature by Kosolapoff[93] and can be achieved by simple treatment of the phosphonate with alcoholic potassium hydroxide.

The same problems were encountered with compound **18**, which should be less prone to elimination compared to **17**, which has an allylic structure. In the reaction of the 3-bromopropylphosphonate **18** a mixture of compounds was recovered after 24 hours. Although in the TLC only one spot was present (under UV or PMA stain), the ^{1}H-NMR showed the presence of the typical complex structure for the alkene proton in the region around δ 6. The integration for the CH_3 and CH_2 group of the ethyl ester were higher than the expected values, therefore confirming that it was a mixture.

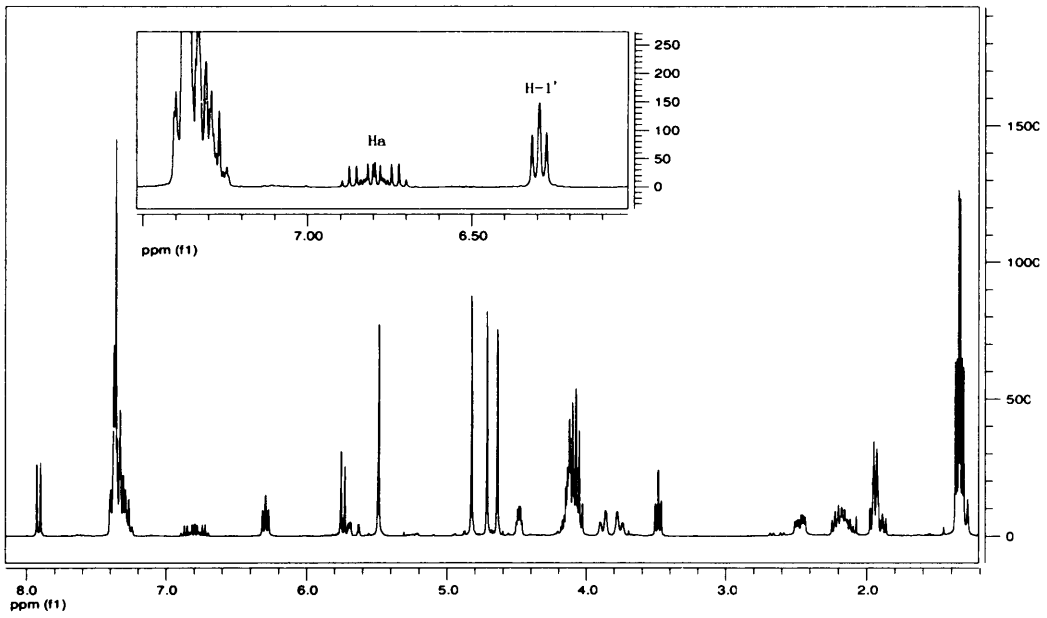


Figure 4-12 ^{1}H-NMR of the isolated mixture

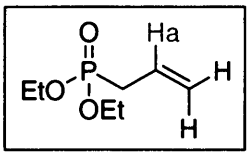
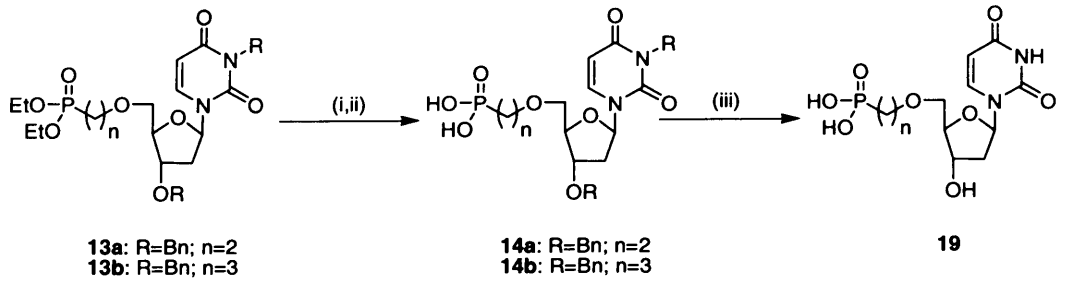


Figure 4-13 Probable diethyl phosphonate formed as byproduct of the reaction with substrate **18**



13a: R=Bn; n=2
13b: R=Bn; n=3

14a: R=Bn; n=2
14b: R=Bn; n=3

19

Scheme 4-23 (i): Me_3SiBr; DCM; (ii): EtOH/Water × 3; (iii): H_2, 10% Pd/C, MeOH

The next step in the synthesis was the hydrolysis of the ester functions, followed by deprotection of the benzyl groups. To cleave the ethyl groups from compound **13a** it was decided to use the procedure suggested by Mikolajczyk and co-workers[94]. The compound was treated with trimethylsilyl bromide in dichloromethane. After 24 hours the TLC showed the disappearance of the starting material and only one spot present at the baseline of the plate. The work-up was a simple co-evaporation of the by-products with a mixture of 1:1 of EtOH/Water. In order to verify the result of the reaction, the residue was analysed by reverse-phase thin layer chromatography, using a mixture of acetonitrile/water. Disappointingly it was discovered that the compound was not pure, due to the presence of other spots in the TLC. Consequent attempts at purification via reverse phase chromatography failed twice, and the compound was not recovered from the column due to the small scale of the reaction.

Concluding in this first part of the project presented in this chapter, some synthesis of derivatives of 2'-deoxyuridine-5'-alkylphosphate and phosphonate were attempted, using a linear multi-step synthetic pathway. This led to the synthesis of different protected 2-deoxyuridine derivatives (5'-protected and 3-N-3'-O-diprotected) in moderate and good yields. Functionalization of the 5' position of the diprotected derivatives was partially achieved in very low yield. and all the attempted methods to introduce phosphate or phosphonate group in the molecules did not succeed. Unfortunately only one of the final targets was achieved (compound **11a**).

To increase the data for the structure-activity relationship study, against the enzymes dUTPase, some of the protected intermediates synthesised in this section were sent for biological evaluation together with the only final target **11**. All of them are illustrated in the following Figure 4-14.

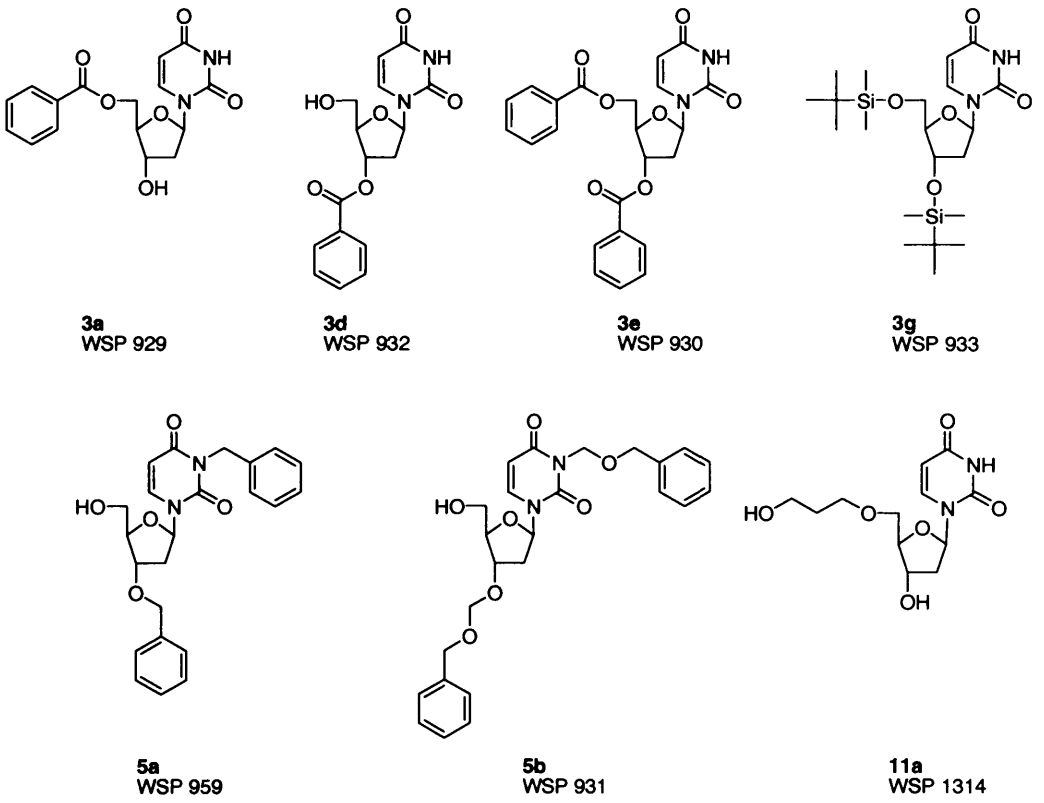
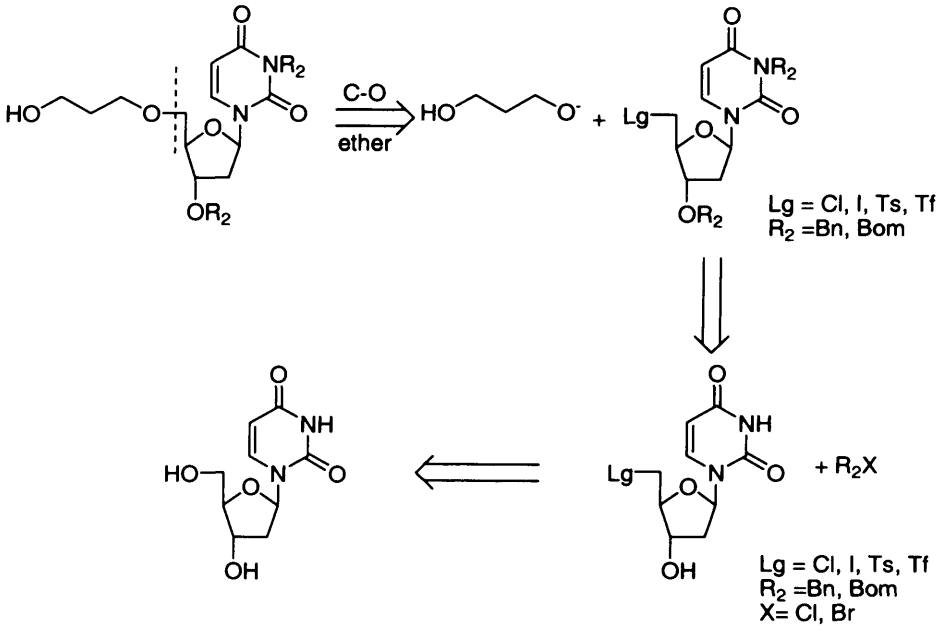


Figure 4-14 Compounds submitted for biological evaluation

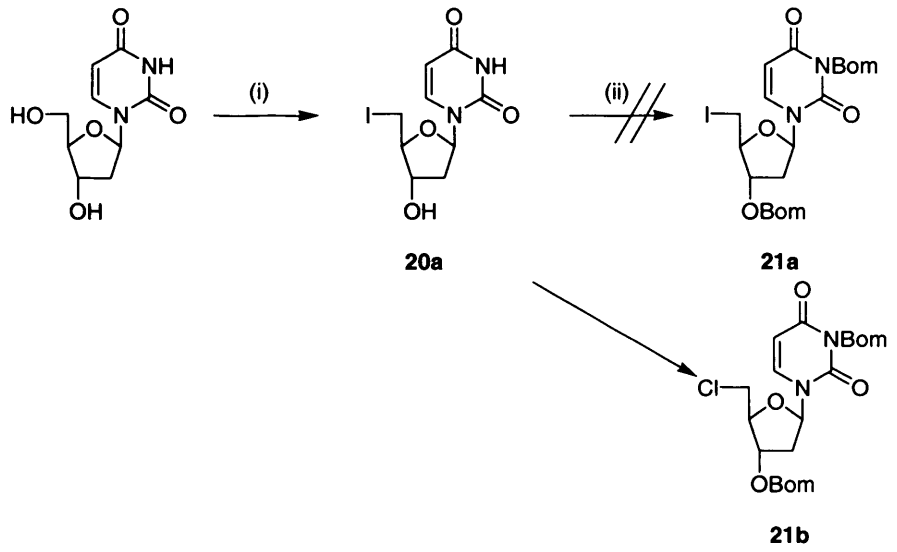
4.1.3 Alternative strategies

The partial failures in the previously described synthesis of phosphate and phosphonates made us plan another synthetic route starting from a different retrosynthetic approach. This second retrosynthetic analysis is shown in Scheme 4-24. It consists of the inversion of the two synthons for the synthesis of the ether bond. In this case the 5'-modified nucleoside would have a leaving group in that position (i. e. iodide, tosylate or triflate), whilst the alkoxide would be generated from a simple monoprotected diol. The synthesis would therefore require the introduction of good leaving group in the 5' position of the 2'-deoxyuridine, followed by protection of the secondary alcohol and the amide nitrogen of the uracil. This would be followed by coupling with the monoprotected diol in a Williamson etherification reaction.



Scheme 4-24 Alternative strategy for the phosphate synthesis. Lg: leaving group

The 2'-deoxyuridine was first converted into 5'-iodo-2'-deoxyuridine using the standard procedure[95] with iodine and triphenylphosphine (Scheme 4-25).



Scheme 4-25 (i): PPh_3, I_2, dioxane, r.t.; (ii): Bom-Cl, DIPEA, 1,2-dichloroethane, 56 °C.

Compound **20a** was obtained after recrystallization from diethyl-ether in 49%. The mass spectrum showed the presence of the desired compound with a peak at 336.9 m/z for the ion $[M+Na]^+$, also the proton NMR confirmed the results showing the disappearance of the OH signal in deuterated DMSO.

In the next step, the previously described procedure (Chapter 4) was used to introduce the Bom in 3-N and 3'-OH position. After purification by flash chromatography two spots which ran closely were recovered. Although the NMR analysis seemed to be in agreement with the structure of compound **21a**, the mass spectrum of the major spot showed a peak at 509.1 m/z with 100% intensity and a second peak at m/z 510.9 with 30% intensity, which indicates the substitution of the iodine with the chlorine, in fact the intensities of the two peaks correspond to the isotopic abundance ratio of the chlorine (Figure 4-15). Therefore the real product of the reaction was the chlorinated compound **21b**. Due to this negative result, this strategy was abandoned.

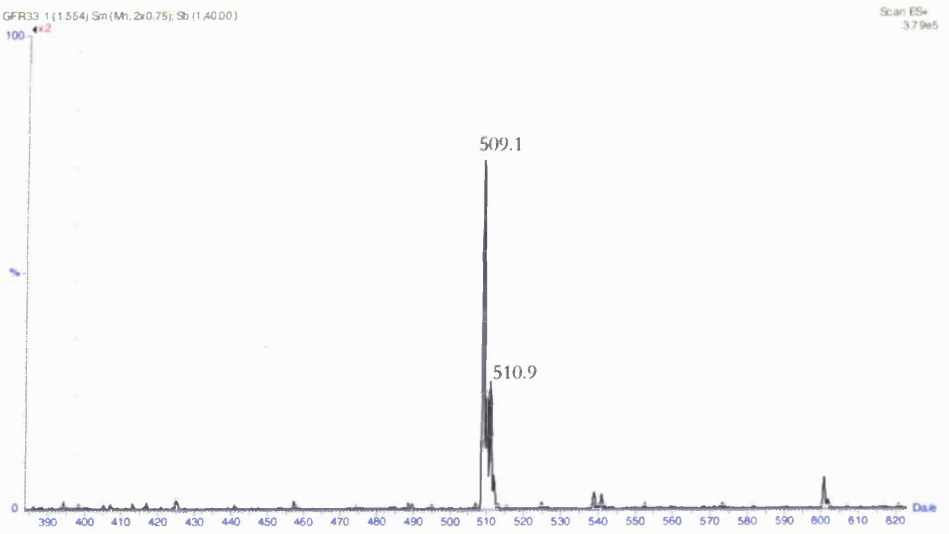
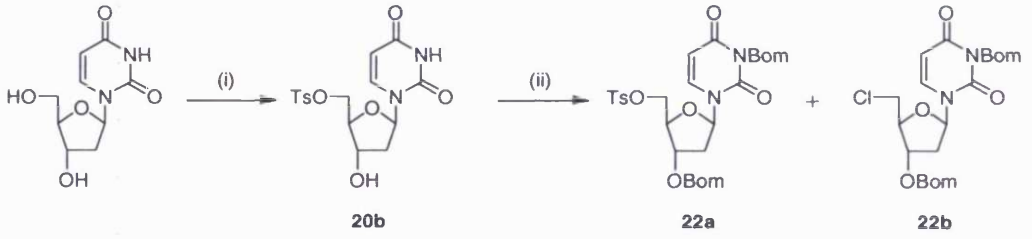


Figure 4-15 Low Resolution Mass Spectrum for compound **21**

The second leaving group investigated was the tosylate. The group was introduced following the standard procedure with *para*-toluenesulfonyl chloride and pyridine. The reaction worked well and the 5'-tosyl-2'-deoxyuridine **20b** was obtained pure, in quite good yield (40%) after recrystallization from petroleum ether.



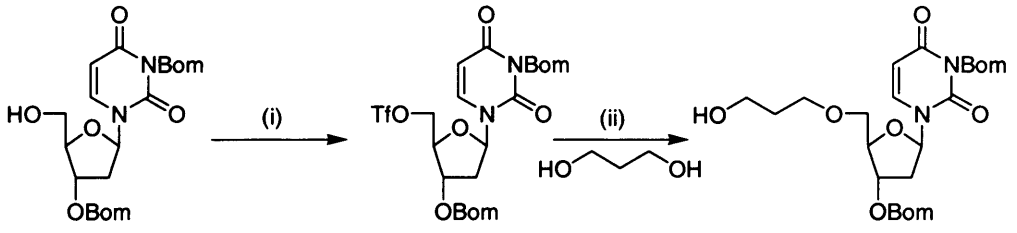
20b **22a** **22b**

Scheme 4-26 (i): *p*ara-toluenesufonyl chloride, pyridine, DCM, 0°C. (ii): Bom-Cl, DIPEA, 1,2-dichloro ethene, 56 °C.

In the subsequent step, the protection with the Bom chloride, the chlorine released during the reaction had partially displaced the tosylate. This was observed by TLC with two new spots at higher R_fs, and also by low resolution mass spectrometry. The desired compound **22a** was obtained only in 25% yield, the 5'-chlorinated **22b** by-product amounted to 32%.

In order to avoid the displacement of the leaving group during the protection of the 3' hydroxyl and the 3-NH of the uracil ring it was decided to introduce the leaving group on the previously protected intermediates **5b**, Scheme 4-27. This

time it was decided to use a triflate. The reaction was tested on a small scale. After treating the bis-Bom derivative **5b** with trifluoromethanesulfonic anhydride and diisopropylethylenediamine (DIPEA) for 1 hour the complete conversion into compound **23** was observed by TLC. The reaction was quenched with water, the phases were separated and the organic layer was dried and concentrated under reduced pressure without warming. Due to its high reactivity the triflate **23** was used directly for the next step without further purification. To reduce the chances of dialkylation on both sides of the diol, a large excess of 1,3-propane diol was used and was then treated with sodium hydride in DMF in order to generate the monoalkoxide ion. When the release of hydrogen was finished the reaction was cooled to -50 °C and the triflate dissolved in THF was added by syringe. In two hours the complete disappearance of the starting triflate **23** was observed by TLC. The reaction was stopped with water and extracted with ethyl acetate. After concentration the residue was purified by chromatography affording 540 mg (48%) of compound **24**. The isolated compound had similar spectral properties found for the previously obtained compound **7a**.



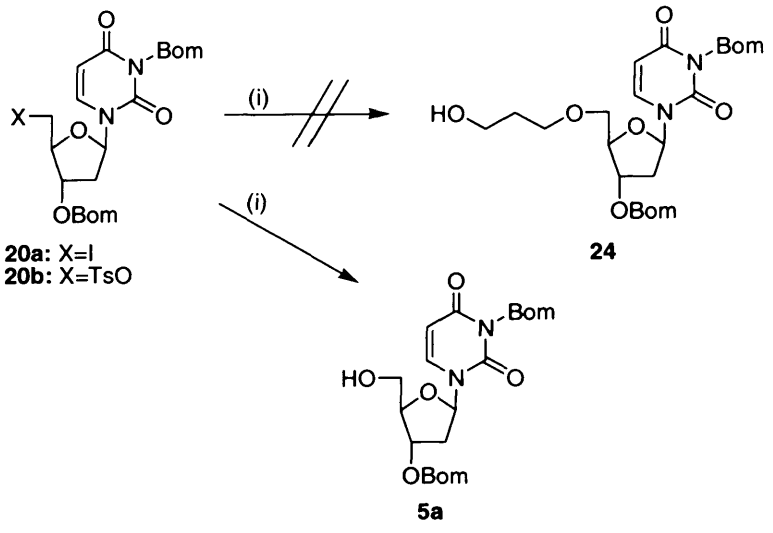
5b **23** **24**

Scheme 4-27 (i): Trifluoromethanesulfonic anhydride, DIPEA, DCM, -78 °C. (ii): 1,3-propane diol, NaH, THF.

In the ^{1}H-NMR a new multiplet at δ 1.82 was present due to the methylene group in the middle of the aliphatic chain; the other two CH_2 group were overlapped as a multiplet in the region between 3.65 and 3.45 ppm. The carbon NMR experiment showed the presence of three new carbons at 31.8, 67.6 and 69.4 ppm. Despite the good result obtained in the small scale reaction, the 540 milligrams was not enough to carry on with following steps, and therefore some scale up of the reaction was attempted in order to get some more of intermediate **24**. Unfortunately those attempts were not as successful as the first one. The

desired compound was recovered only in very low yield (<20%) and the increased quantity of by-product made the purification more complex.

The same procedure with 1,3-propane diol and NaH was applied to the two intermediates **20a** and **20b**. This time the reactions were attempted on small scale, due to the low quantity of available intermediates, (Scheme 4-28).



Scheme 4-28 1,3-propane diol, NaH, DMF or THF, 25 °C

Unfortunately these attempts did not succeed either, and after chromatographic purification the only product recovered was the starting material 3'-O-3-N-*bis*bom-2'-deoxyuridine **5a**. This failure could be attributed to the conditions not being totally anhydrous. The starting diol is quite moisture sensitive and the water present in it reacted first with the sodium hydride generating OH^- anions. These should react as nucleophiles after the intermediates **20 a-b** were added to the mixtures and displace the leaving groups (tosylate or iodide) forming the starting material **5a**.

It is known in organic chemistry that a convergent multistep organic synthesis has a better total yield compared to the same linear synthesis[96].

A → B → C → D → E → F → G → H → I → J → TM **(a)**

A → B → C → D → E
F → G → H → I → J
} → K → TM **(b)**

Figure 4-16 Comparison, between, linear and convergent synthesis. In linear synthesis **(a)** with ten steps, each having 90% yield, the overall yield will be 34%. In a convergent synthesis **(b)** the overall yield will be 53%[96].

To overcome the problem of the low yields faced so far, it was thought to investigate the use of a convergent synthesis, (Figure 4-17). The coupling of the intermediates **5a-b** directly with a previously synthesised phosphate ester was explored.

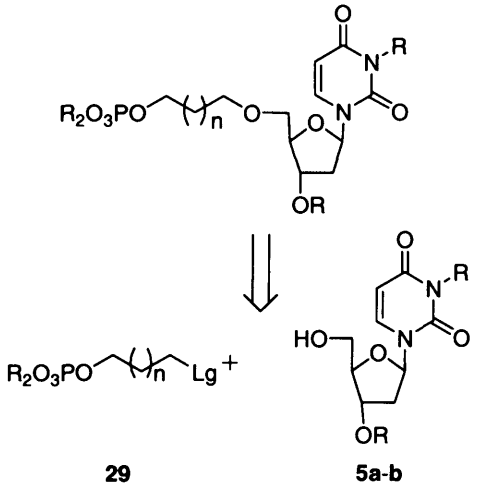
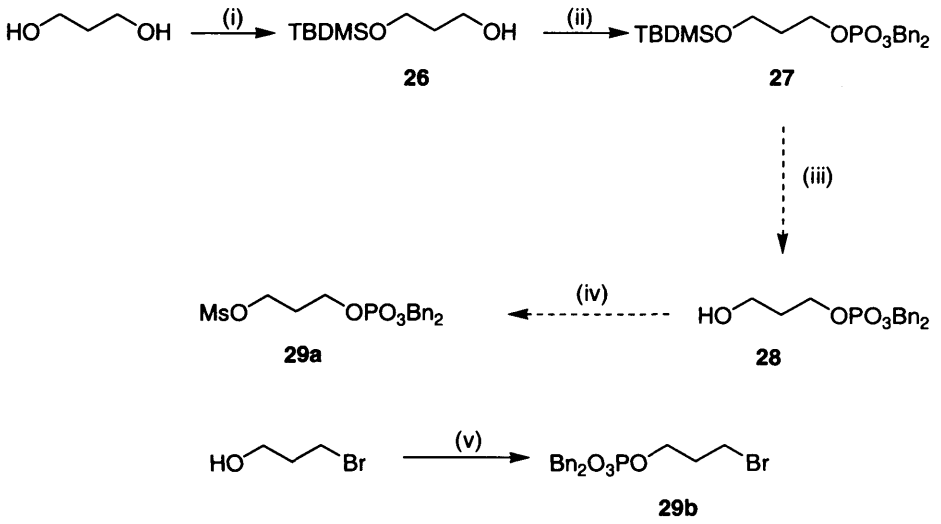


Figure 4-17 Alternative coupling with a pre-synthesised phosphate **29**

The phosphate ester **29** could be synthesised either from the 1,3-propane diol or directly from the 3-bromo-1-propane with a shorter route.



Scheme 4-29 (i): TBDMSCl 0.5 eq., Imidazole, DMF. (ii): $(BnO)_2P(O)Cl$, Pyridine, DCM; (iii): TBAF, THF; (iv): MsCl, Pyridine; (v): $(BnO)_3P$, I_2, Pyridine.

The monoprotection of the diol was obtained using an excess of starting material using the classical conditions for the introduction of the TBDMS group. After chromatographic purification, the intermediate **26** was obtained in 42%

yield. In the successive step the dibenzylchlorophosphonate was used as a phosphorylating agent, but the compound **27** was obtained pure in only 9% yield purity confirmed by ^{31}P-NMR showing only one singlet at δ 0.5. Due to this result this synthetic way was not explored further.

With the 3-bromo-1-propanol, introduction of the dibenzyl phosphate group was attempted with the tribenzyl phosphite and iodine, because this procedure[88] is also suitable for bromo-alcohol substrates.

The reaction was tested in a small scale affording the dibenzyl(3-bromo-propyl) phosphate **29b** in 26% after purification. The phosphorous NMR showed only one signal at δ 0.36, corresponding to the phosphate. The presence of the bromine was confirmed by mass analysis which showed the typical peaks for the isotopic concentration of the halide at m/z 420.9 and 422.9 $[M+Na]^+$, with almost the same intensity, as shown in the spectrum below.

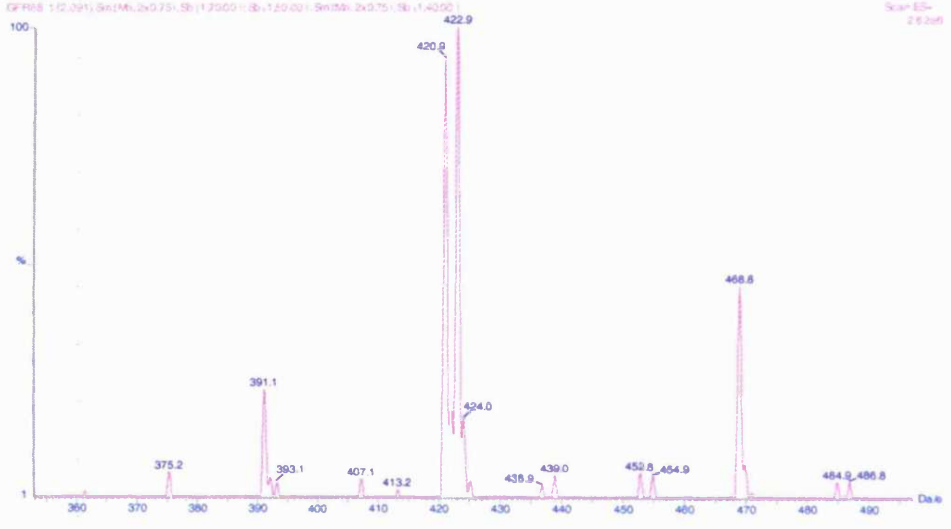
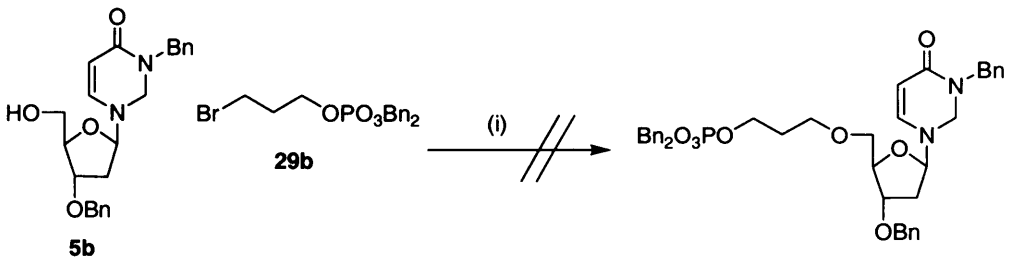


Figure 4-18 Low Resolution Mass Spectrum for compound **29b**

The intermediate **29b** was then used for the coupling with the diprotected 2'-deoxyuridine **5b** using sodium hydride and 15-crown-5-ether, (Scheme 4-30). Although in the TLC the disappearance of the starting material **29b** was observed, after the work up with water, only the starting nucleoside was recovered. The ^{31}P NMR of the crude residue did not show any presence of phosphorous. Probably the phosphate ester intermediate was not stable in the harsh basic conditions used for the reaction.



Scheme 4-30 (i) NaH, 15-crown-5-ether, THF, 25 °C

Summarizing, the synthesis of the two main building blocks **5b** and **29b** was achieved, in two and three steps respectively. Unfortunately the key reaction did not work as expected and the final targets could not be reached. Although other routes could have been investigated, no other attempts of synthesis of the target phosphates and phosphonates were made. This was due to the lack of results obtained and the need of compounds for biological evaluation and SAR studies. Consequently the chemistry was then focused on the synthesis of the other proposed structures presented earlier in Chapter 3.

Chapter 5 Synthesis of Acyclouridine Derivatives

It has previously been shown that compound WSP870 (Figure 5-1) had good inhibitory effect against both *P. falciparum* dUTPase and good IC_{50} values against parasitic growth[74]. Some preliminary studies on the pharmacokinetic properties of this compound revealed that in a Human Liver Microsome (HLM) test it has a quite fast time of intrinsic clearance which means lower stability in the liver. One of the reasons of this partial metabolic instability could be attributed to the oxygen present in the aliphatic chain. This oxygen, in fact, is quite susceptible to protonation in physiological conditions and therefore the bond can be hydrolysed in the cellular environment.

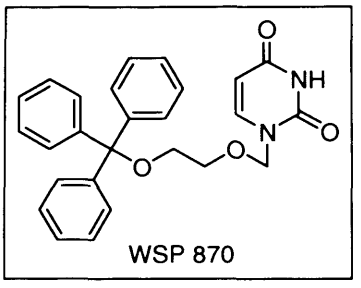


Figure 5-1 WSP870 lead compound against *P. falciparum* dUTPase

In this chapter the synthesis of some analogues of the lead compound WSP870, will be discussed. The aim was to prepare analogues with an increased biological activity and increased metabolic stability. The simplest modification to introduce is the substitution of the heteroatom with a carbon atom; therefore the first syntheses will be focused on analogues having an aliphatic chain bonded to the uracil ring. As terminators, at the end of the aliphatic chain, different groups could be investigated either bulky and lipophilic, like the trityl present in WSP870, or polar, hydrophilic substituents such as phosphates and phosphonates. A further variable to play with was the length of the chain.

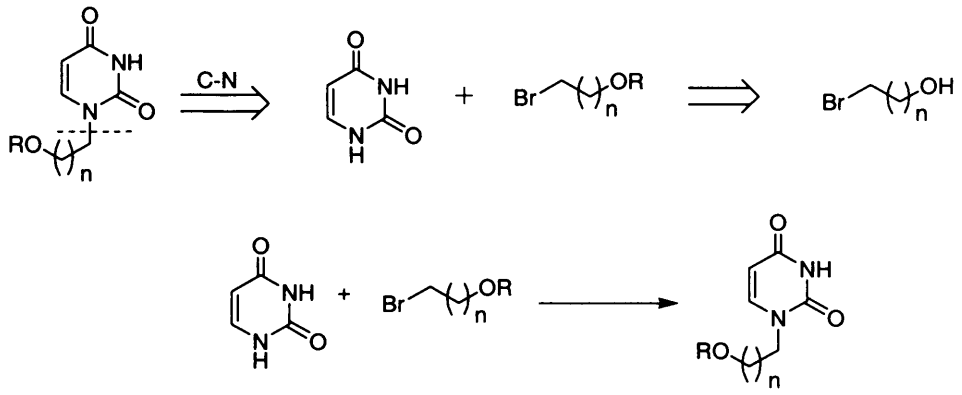
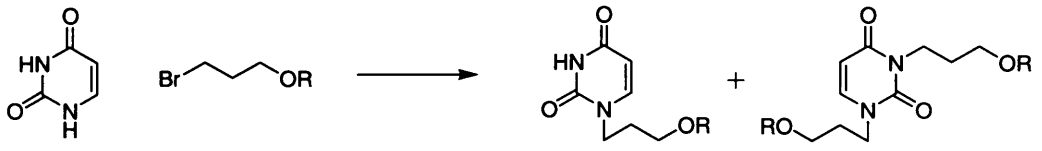


Figure 5-2 Retrosynthetic analysis and proposed synthesis of acyclic uracil analogues

The retrosynthesis and the corresponding synthesis for the preparation of acyclic uracil derivatives is reported in Figure 5-2. The key step is the coupling of the uracil ring with the aliphatic chain. In the literature five main methods for the coupling of nucleobases with sugars or other alkyl halides[97] are described. These procedures generally require the use of polar aprotic solvents (DMSO, DMF) and strong inorganic bases (NaH, Na_2CO_3 or Cs_2CO_3), and protected nucleobases.

In our effort to synthesise the acyclo derivatives of uridine, it was decided to find a simple and versatile method to couple the uracil with different substrates, in order to be able to synthesise a variety of structures using the same kind of chemistry. It was planned to use the protected 3-bromo-1-propanol as a test reaction to find the optimal conditions for the coupling with the unprotected heterocyclic base, Scheme 5-1.



10
a: R=TBDMS
b: R=THP
c: R=Trt

30
a: R=TBDMS
b: R=THP
c: R=Trt

31
a: R=TBDMS
b: R=THP
c: R=Trt

Scheme 5-1 Coupling between uracil base and protected bromo-alcohols

Several conditions were experimented with, and the most effective was found to be the use of caesium carbonate in DMF, moderate heating and a slight excess of uracil. The main side reaction was the formation of dialkylated uracil on

the N1 and N3 position, this could be minimized by using a slight excess of uracil base (1.5 equivalents).

Alcohol	Base	Solvent	T	Monoalkylated **30**	Dialkylated **31**
10b	Na_2CO_3 (1.2 eq.)	THF	50°	n.r	
10b	Na_2CO_3 (1.2 eq.)	DMF	90°	n.r.	
10b	Cs_2CO_3 (1.2 eq.)	DMF	50°	20%	22%
10b	Cs_2CO_3 (1.2 eq.)	THF	50°	n.r.	
10a	Cs_2CO_3 (1 eq.)	DMF	50°	33%	>10%
10a	Cs_2CO_3 (1 eq.)	THF	50°	n.r.	
10c	Cs_2CO_3 (1eq.)	DMF	40°	31%	15%

Table 5-1 Conditions tested for the coupling reactions, n.r.: no reaction

The protected bromo alcohols **10a-c** were added to a suspension of uracil and caesium carbonate in DMF. The reaction was warmed to 40 °C first, and checked by TLC. After a three hours two new spots were present on the TLC plate, with R_f values higher than the starting materials, if no new spots were visible the temperature was increased to 50 °C and then 90 °C. When the reactions were complete, no traces of bromo alcohol were present on TLC. The reaction mixture was diluted with water and extracted with ethyl acetate. Even though it was not possible to completely avoid the formation of dialkylated uracil, it was possible to separate this by-product from the desired compound by simple chromatography. The reactions times were within 4 and 12 hours, depending on the aliphatic chain used. The procedure was found to be compatible with all the protecting groups tested (THP, TBDMS and Trityl).

The chemoselectivity between the 1-N and the 3-N positions in the uracil for the alkylation was verified by 2D ^{1}H-NMR spectroscopy, see Figure 5-3. With the NOESY experiment (2D Nuclear Overhauser Effect Spectroscopy) it is possible to understand which protons are close in space, thus providing information about geometry of molecules[98], contrary to the COSY experiment (Correlation Spectroscopy) which gives information about the spin-spin coupled protons and therefore information about through-bond interactions. In the NOESY

spectrum the off-diagonal peaks result from protons close in space which exchange resonance energy. Because the energy exchange can happen only if the nuclei are close enough, only protons that are separated by no more than 5 Å will show an off-diagonal signal. This experiment was used to prove that the H-6 of the uracil is near to the second CH_2 of the aliphatic chain, named H-7 (Figure 5-4), thus confirming that the structure of the obtained compound was **30b** instead of **32**. It is noteworthy, that if the highlighted signal was not present in the spectra illustrated, no conclusion could have been made, since the conformation assumed by the compound could have had a distance between the H-6 and the H-7 greater than 5 Å, and therefore no interaction would have been detected.

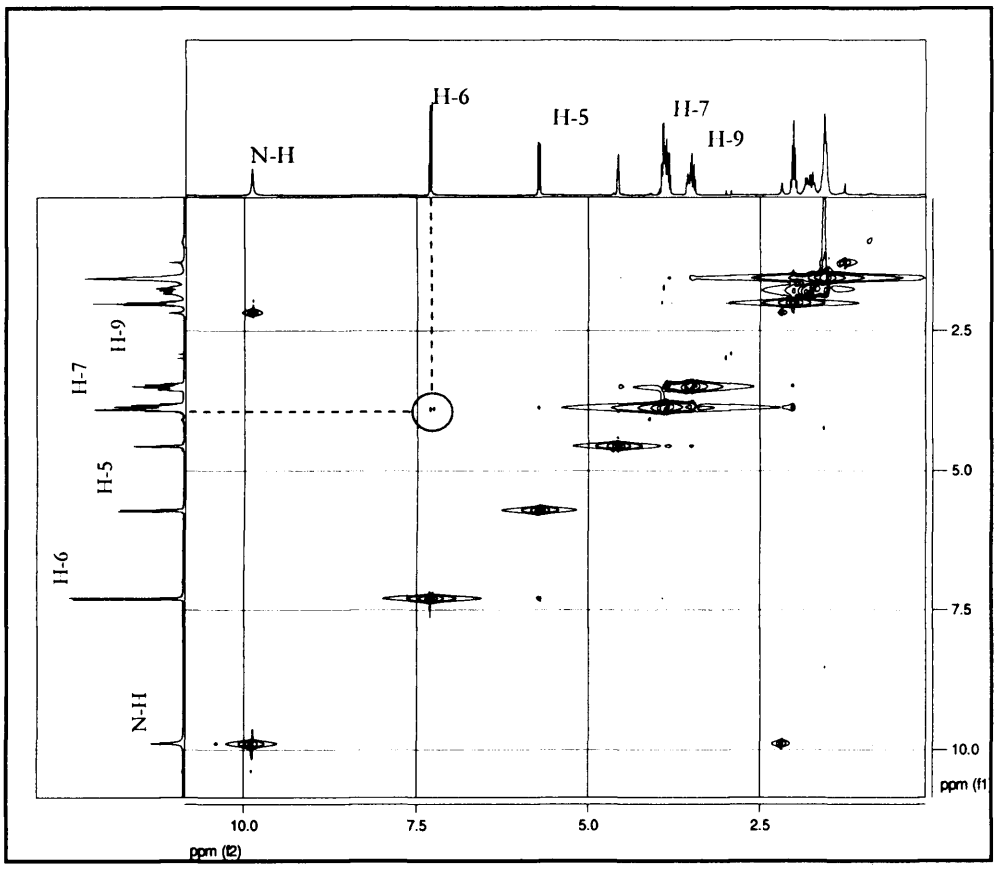


Figure 5-3 NOESY spectrum of the compound **30b**. Highlighted the "coupling" between the H-6 of the heterocyclic base and the CH_2 of the aliphatic chain

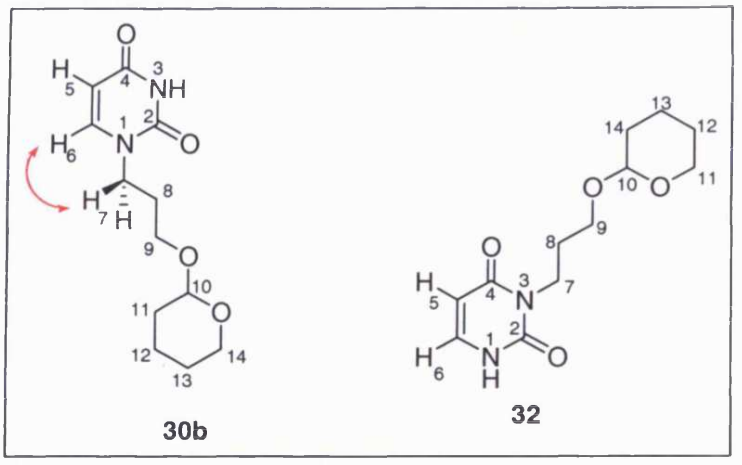


Figure 5-4 Comparison of the two possible structures obtained by alkylation of unprotected uracil

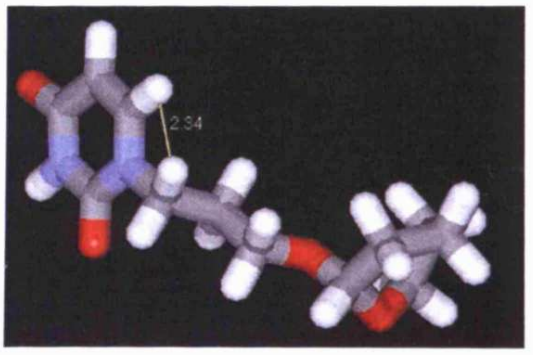
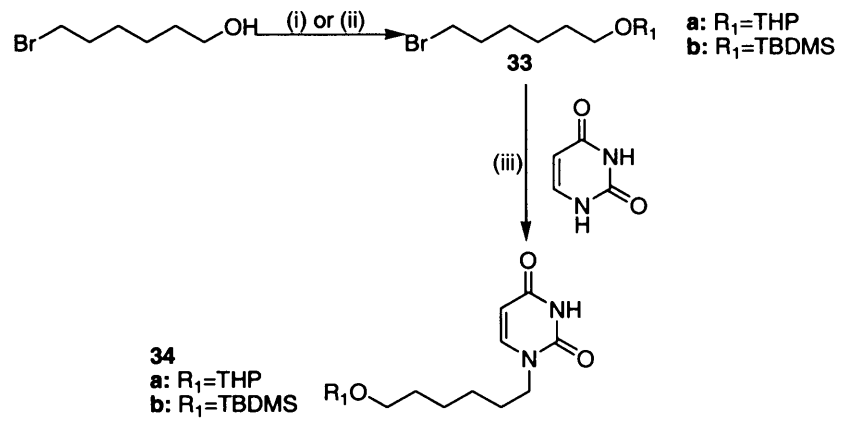


Figure 5-5 Energy minimized 3D structures of compound **30b**

The coupling method of uracil with alkyl halide was extended to other commercially available substrates, Scheme 5-2. With 6-bromo-hexanol the results were similar to those obtained with 3-bromo-propanol. 6-Bromo-1-hexanol was first protected as THP ether **33a** and silyl ether **33b** using the standard procedures. The coupling with uracil did not work as well as before and two products **34a-b** were isolated with lower yield (18% and 17%) The reason for the partial failure was not well understood, since not much dialkylated by-product was obtained.



Scheme 5-2 (i): DHP, PPTS, DCM, r.t; (ii): TBDMSCl, imidazole, DMF, r.t.; (iii): Uracil, Cs_2CO_3, DMF, 40-50 °C. 4-12 h.

The drawback of this method is the lack of commercially available bromo alcohols with different chain lengths. In particular alcohol halides with chain of 4 and 5 carbons, are not available probably due to their tendency to undergo intramolecular cyclization producing five and six-member rings.

An alternative strategy to overcome this problem is to use diols instead of bromo alcohol derivatives. Diols are cheap and readily available starting materials, with a range of chain lengths from 2 up to 6 carbon atoms. Coupling a monoprotected diol to uracil ring would produce the same compound obtained with the previously described method. A synthetic route which allows the alkylation of an amide function with an alcohol is the Mitsunobu reaction.

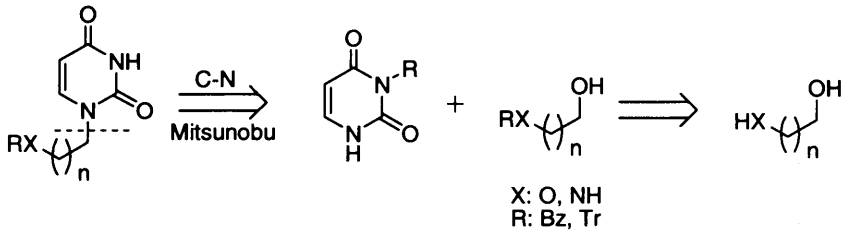
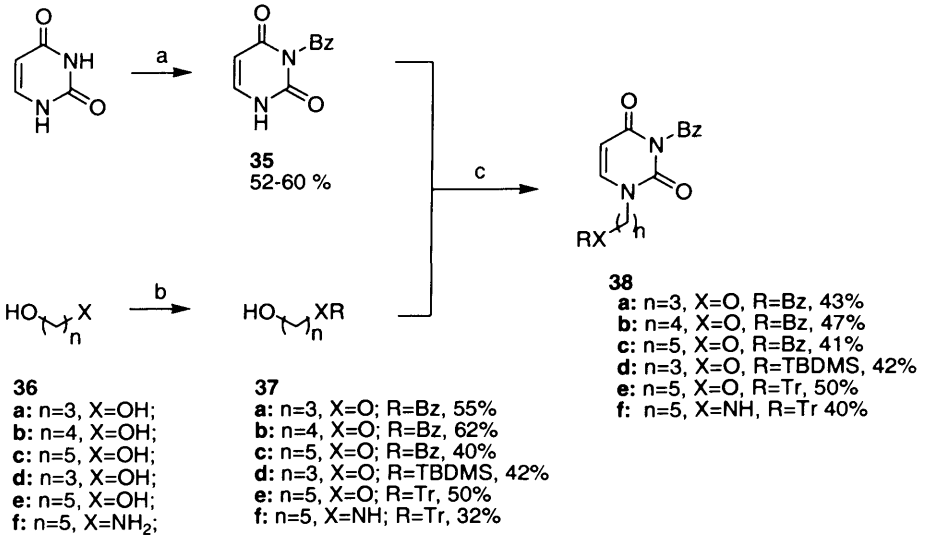


Figure 5-6 Retrosynthetic approach to Mitsunobu coupling of uracil with alcohols

Hernandez and co-workers[99] developed a procedure to couple a pyrimidine base with an alcohol using polymer supported triphenylphosphine (PS-PPh_3) and diisopropylazadicarboxylate (DIAD) in THF.

With this procedure several monoprotected diols and amino alcohols **37** could be used for the coupling with the protected uracil base **35**, leading to the synthesis of several different structures **38**, as shown in Scheme 5-3. After deprotection of

the second functional group at the end of the chain, the obtained products **39** could further be functionalised.



Scheme 5-3 Route to the synthesis of a small library of acyclic tritylated acyclic derivatives. (a) 1. BzCl, Pyridine, acetonitrile 2. K_2CO_3, Dioxane. (b) TBDMSCl, imidazole, DMF or BzCl, Dimethyltin dichloride, K_2CO_3, THF or TrCl, TEA, DCM. (c) PS-PPh_3, DIAD, THF.

The method required the selective protection of the N-3 in the uracil ring to avoid the double alkylation. This was achieved with a two step procedure: protection and selective cleavage as reported in the literature[100] (Scheme 5-3, a). The uracil was treated with 2 equivalents of benzyl chloride and potassium carbonate in acetonitrile. After 24 hours the TLC showed the complete reaction with no uracil left and two new spots at higher R_f, showing that the dibenzylated uracil was formed, with probably some traces of monoprotected. After extraction with dichloromethane the residue was concentrated, dissolved in dioxane and treated with a diluted solution of potassium carbonate to cleave selectively the benzyl group from the N-1 position. After 30 minutes the higher spot in the TLC disappeared. The desired compound **35** was then crystallised from aqueous acetone as reported by Frieden and co-workers. Although the melting point measured for our compound (214-217 °C) was very different from the one reported in literature the spectroscopic analysis were exactly the same as those reported in literature[100,101]. The ^{1}H-NMR showed a broad singlet at δ 11.61 for the amide proton on the N-1 of the uracil, using deuterated DMSO as solvent. The characteristic doublets for the two CH of the uracil at δ 7.63 and δ 5.69 were present. The ^{13}C experiment showed a chemical shift at δ 100.4 for the C-5,

consistent with the structure of the 3-N-benzoylated uracil; since in the literature is reported a chemical shift at δ 103.6 for the same carbon in the 1-N benzoylated isomer[100,101]. As further confirmation of the result, it was proven the neighbouring of the H-6 with the 1-NH protons by a NOESY experiment.

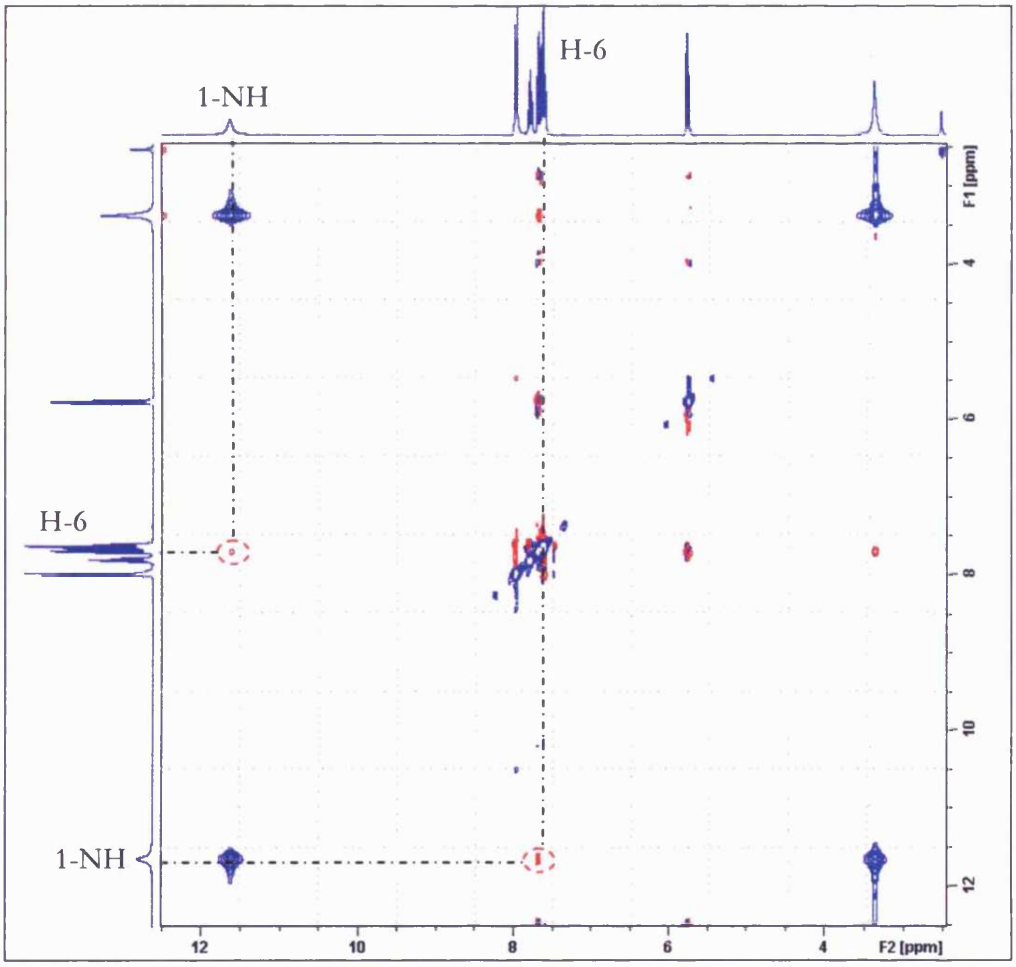
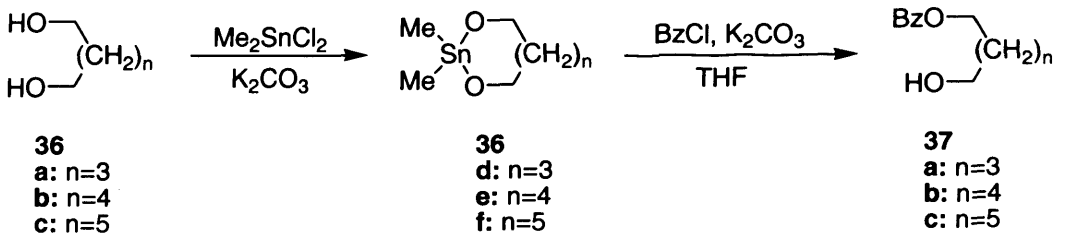


Figure 5-7 NOESY spectrum of the 3-N-benzoyl uracil **35**

The three diols **36a-c** were monobenzylated using benzoyl chloride in the presence of dimethyltin dichloride as the catalyst to favour the formation of the monoprotected compounds **37a-c**, as suggested by Maky and co-workers[102].



36
a: n=3
b: n=4
c: n=5

36
d: n=3
e: n=4
f: n=5

37
a: n=3
b: n=4
c: n=5

Figure 5-8 Monoprotection of diols using Me_2SnCl_2

The effect of the tin is to form acetal-like complexes (**36g-e**) which react with benzoyl chloride regioselectively forming mainly the monobenzoylated products **37a-b**. The precise origin of the regioselectivity is not known. However the hypothesis is that the stannylene complexes associate with themselves creating dimeric structure like the one illustrated in Figure 5-9[103]. In this type of structure one of the two oxygens is more nucleophilic than the other, and therefore is favourable to react with the benzoyl chloride.

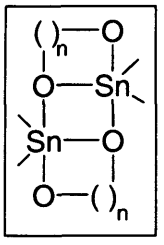


Figure 5-9 Dimeric structure of stannylene complexes[103]

The procedure worked quite well and the three compounds **37a-c** were isolated in yields varying from 40% to 62%. All of them were characterized by ^{1}H-NMR and low resolution mass spectroscopy, which agreed with the desired structures.

In the next step DIAD and polymer supported triphenylphosphine were used for the coupling between the intermediate N^3-benzoyl uracil **35** and the intermediates and **37a-f** (Scheme 5-3). The advantage of using a polymer supported reagent like the triphenylphosphine resin is to simplify the purification step. In the particular case of the Mitsunobu reaction, one of the by-products is the triphenylphosphine oxide which can, sometimes, be difficult to separate from the main product of the reaction. With the PS-triphenylphosphine, that by-product will be linked to the insoluble resin; consequently at the end of the reaction it can be removed by simple filtration of the polymeric suspension. On

the other hand, the main disadvantage with the use of supported reagents is the reaction conditions. A suspension of insoluble polymer is a heterogeneous system where the collisions of the particles are not as efficient as in a homogeneous phase, leading to an increased reaction time; an increased ratio of equivalents for the supported reagent and sometimes difficulties for the reaction to reach completion. The mechanism for this reaction is described in Figure 5-10.

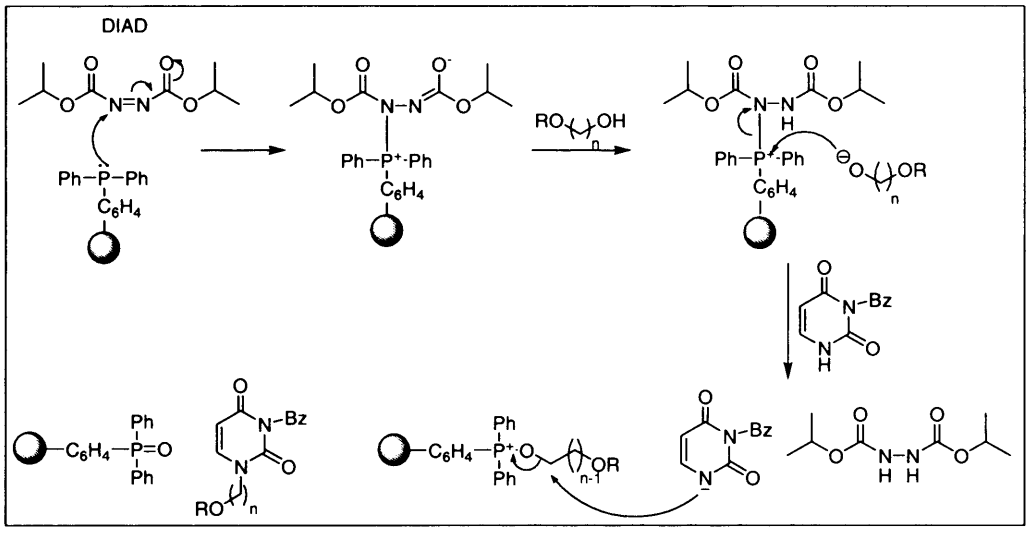
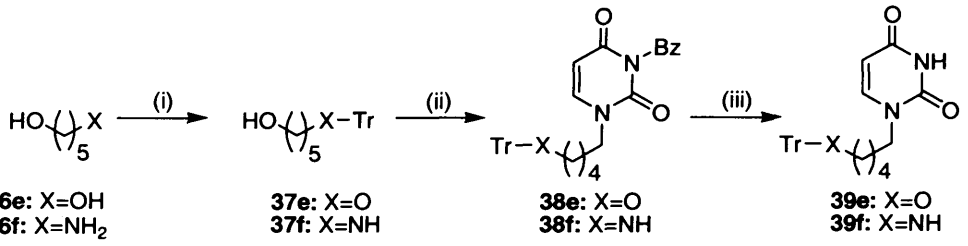


Figure 5-10 Proposed mechanism for the Mitsunobu reaction with PS-PPh_3

Fortunately no problems were encountered in the coupling of the substrates **37a-f** and **35**. The reactions were shaken instead of the conventional magnetic stirring so as to not compromise the integrity of the resin beds. DIAD was added to the suspension of the resin containing the protected uracil and the alcohols. TLC showed several spots at higher R_f, probably due to side reactions or by products, whilst only one spot with R_f, between the uracil and the alcohol, probably the desired product. The reactions were stopped when the complete disappearance of the alcohol was observed by TLC. After filtration of the resin, the residue was concentrated and purified by chromatography. All the attempts were successful and the products were obtained in yields between 41-50% after chromatography. Although the reaction times were probably longer than the classical Mitsunobu, (24-36 hours), no problem of contamination by triphenylphosphine oxide was observed. In the proton NMRs there was a multiplet integrating to 10 hydrogens in the aromatic region around δ 7-8. The

low resolution mass spectrum also confirmed the results showing the m/z for the molecular ion plus sodium $[M+Na]^+$ for all three products.

As mentioned earlier one of the aims for this part of the project was to synthesise analogues of WSP870 with aliphatic chains of different lengths with different atoms in the end of the chain and a trityl group as terminator. Therefore to complete a series synthesised by another member of our group, the Mitsunobu procedure was applied with the substrates **36e** and **36f** 1,5-pentane diol and 5-amino-1-pentanol respectively, as shown in Scheme 5-4.

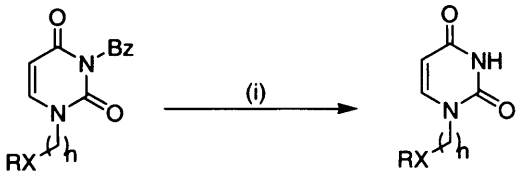


Scheme 5-4 (i) TrCl- TEA, DCM; (ii) N^3-Bz Uracil, DIAD, THF; (iii) NaOH, dioxane or MeONa, MeOH

For this case the two substrates **36e-f** were directly protected with trityl group to save one step in the total synthesis. The monoprotection was achieved using the standard procedure with trityl chloride, triethylamine, and an excess of diol **36e** or amino alcohol **36f** to limit the formation of diprotected compounds. However the formation of some di-tritylated compound was not completely avoided since two upper spots were visible on the TLC plates. This was also proved by NMR analysis of the two fractions collected after chromatography. The fractions with the higher R_f, showed an integration of 30 protons at δ 7 according to the presence of two trityl moiety; whilst the second two fractions had only 15 protons as required for the desired products **37e-f**. In deuterated DMSO a triplet at δ 4.33 was clearly visible integrating for 1 proton, for the unprotected OH for compound **37f**.

The next step, the cleavage of the two benzoyl groups, was performed under two different conditions, the first with sodium methoxide in methanol and the second with a mixture of Dioxane/NaOH 1:1, (Scheme 5-5). Although both the procedures worked fine, the first gave the better yields and easier purification. All the intermediates **39a-c** and **39e-f** were synthesised with yields between 52-80%.

The only failure was encountered in the deprotection of the orthogonal protective group TBDMS in the intermediate **37d**, (Scheme 5-5 and Scheme 5-3). The reason of this unsuccessful attempt is probably due to the small scale of the reaction (40 milligrams). Despite the TLC showing the complete disappearance of the starting material, all attempts to purify the N-3 benzoylated acyclic nucleoside failed.



38
a: n=3, X=O, R=Bz
b: n=4, X=O, R=Bz
c: n=5, X=O, R=Bz
d: n=3, X=O, R=TBDMS
e: n=5, X=O, R=Tr
f: n=5, X=NH, R=Tr

39
a: n=3, X=O.R=H, 74%
b: n=4, X=O. R=H, 53%
c: n=5, X=O. R=H, 80%
d: n=3,X=O. R=TBDMS, 0%
e: n=5, X=O. R=Tr, 60%
f: n=5, X=NH; R=Tr, 52%

Scheme 5-5 (i) MeONa in MeOH, r.t. or NaOH, Dioxane r.t.

The structures of all the intermediates were verified by NMR spectroscopy. In the ^1H experiments there were no traces of the aromatic signals of the benzoyl group. Also the mass spectroscopy confirmed the success of the reactions, with the electrospray positive ionization mode, the compounds showed a nice peak for the molecular ion + sodium.

The intermediates **39a-c** could have been used to carry out a SAR studies on substituents of the OH group. The synthesis of phosphates and phosphonates with this type of structure is discussed in the next chapter.

The two compounds **39e** and **39f** were sent for biological evaluation. They completed a series of acyclic derivatives having the general structure reported below.

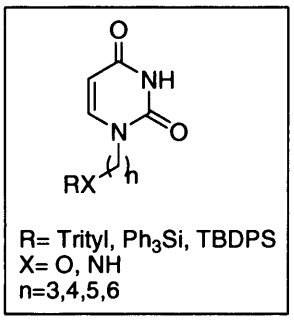
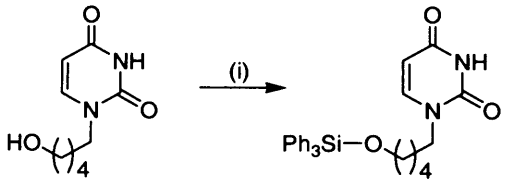


Figure 5-11 General structures of the acyclic series of uracil derivatives synthesised in the group

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The series was also expanded with a triphenylsilyl derivative synthesised from intermediate **39c** by reaction with triphenylsilyl chloride, (Scheme 5-6). Although the reaction appeared to be straightforward, some problems were encountered when the uracil alcohol **39c** was treated with the silylating agent in pyridine. After the reaction was stirred at room temperature for 24 hours significant amounts of starting alcohol were still present, even though a new spot with R_f between the silyl chloride and the alcohol was present. On warming to 90 °C decomposition of the starting materials was observed on the TLC, which showed several spots. A second attempt made using imidazole as a base in DMF, according to the standard procedure used to introduce silyl group in alcohol, did not work at all and unreacted starting material was recovered after 24 hours. Consequently it was decided to revert to the first procedure and the reaction was stopped after stirring overnight at 25 °C, although not all the starting material had reacted. Chromatographic purification of the crude residue afforded 30 mg (26%) of the desired compound **40**.



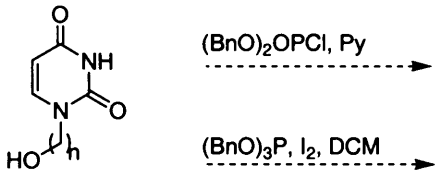
39c **40**

Scheme 5-6 (i) Triphenylsilyl chloride, Pyridine, 25 °C

The low resolution mass spectrum presented a peak at m/z 479 for the $[M+Na]^+$, proton and carbon NMR also agreed with the structure.

5.1.1 Acyclic phosphates

We wanted to investigate the biological effects of polar groups at the end of the aliphatic chain, as these should bind in the phosphate binding site. Thus we attempted the synthesis of some phosphates starting from the previously described uracil-alcohols **39a-c** and **39g**. In the first case it was decided to use the same procedure followed for the synthesis of the 2'-deoxyuridine phosphate analogues, as earlier described. The alcohols **39** were treated either with *in situ* prepared dibenzylchloro phosphite or tribenzyl phosphite and iodine, Scheme 5-7. Unfortunately these procedures did not succeed, since several spots were present on the TLC, purification of the mixtures was almost impossible, and also the ^{31}P-NMR had several signals showing many by-products. Therefore, after two unsuccessful attempts with the dibenzyl chlorophosphonate and the tribenzyl phosphite[88], an alternative route was investigated.



39
a: n=3,
b: n=4,
c: n=5,
g: n=6

Scheme 5-7 Attempted synthesis of phosphonates using dibenzylchloro phosphite or tribenzylphosphite

One of the procedures used in carbohydrate chemistry to introduce a dibenzylphosphate ester is using a phosphorous (III) reagent: dibenzyl N,N-diisopropylphosphoramidite, in the presence of 1-H-tetrazole[104]. The reaction of the alcohol with the phosphoramidite creates a new P-O bond, generating an intermediate phosphite ester. The phosphorous (III) can then be oxidised to phosphorous (V) with *meta*-chloro peroxybenzoic acid (MCPBA) or *tert*-butylhydroperoxide, to afford the desired dibenzylphosphate, as shown in Figure 5-12[105].

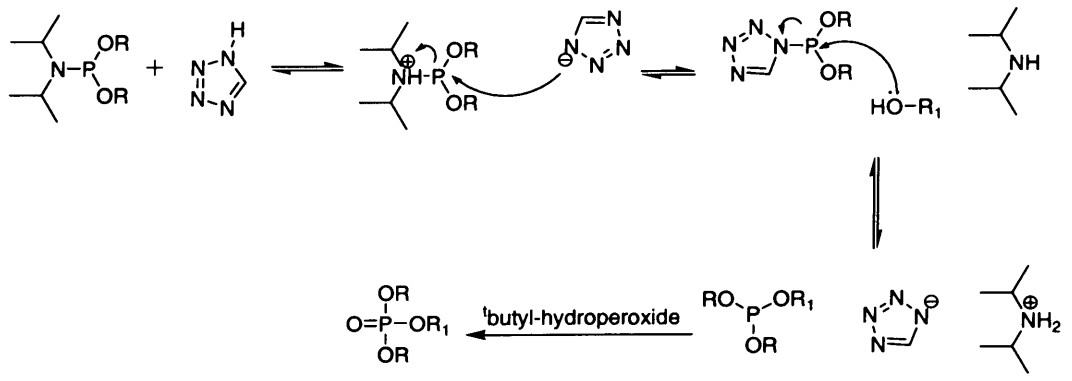
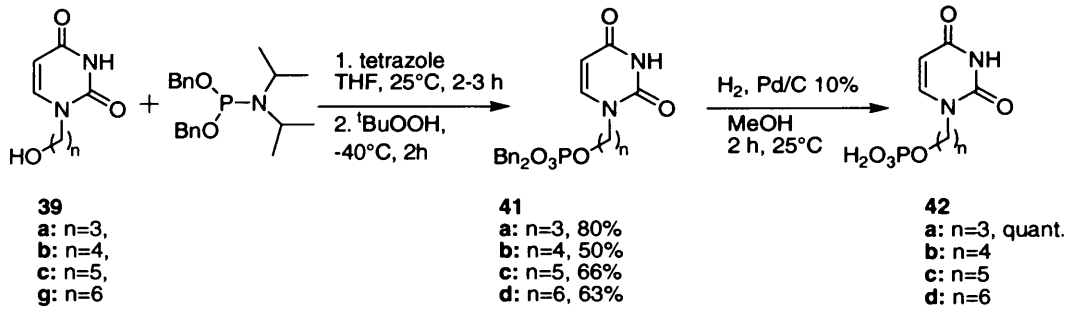


Figure 5-12 Reaction mechanism for the phosphitylation/oxidation step

This method allowed the conversion of the four intermediates **39a-g** to the four corresponding dibenzyl phosphates, with quite good yields (50-80%) after purification by flash chromatography.



39
a: n=3,
b: n=4,
c: n=5,
g: n=6

41
a: n=3, 80%
b: n=4, 50%
c: n=5, 66%
d: n=6, 63%

42
a: n=3, quant.
b: n=4
c: n=5
d: n=6

Scheme 5-8 Introduction of the phosphate moiety with the phosphoramidite chemistry

The phosphoramidite was added to solutions of the starting alcohols in THF in the presence of tetrazole. After few hours the starting alcohol was completely converted into the corresponding phosphite, according by TLC. Then the solutions were cooled to -40 °C, and the oxidising agent *tert*-butyl hydroperoxide was added to the mixture. All the structures were confirmed by ^{1}H, ^{13}C, ^{31}P NMRs and mass spectrometry. The chemical shift for the phosphorus ester was around δ 0.5 in the ^{31}P-NMR. A C-P coupling could be observed in all the carbon spectra, for the two benzylic CH_2, the last methylene of the aliphatic chain, and for the quaternary carbons of the phenyl groups. In the low resolution mass spectra all the compound showed a strong peak for the ion $[M+Na]^+$ with the positive electrospray ionization mode.

Finally, to cleave the benzyl ester moiety a classical hydrogenation was used, with 10% palladium on charcoal as catalyst, (Scheme 5-8). The reactions were complete after 2 hours; only one spot on the baseline of the TLC was visible and the starting material had completely disappeared. The product was then analysed with reverse phase TLC, which also presented a single spot. The catalyst was filtered off and the final compounds were isolated without further purification after evaporation of the solvent and the by-product toluene.

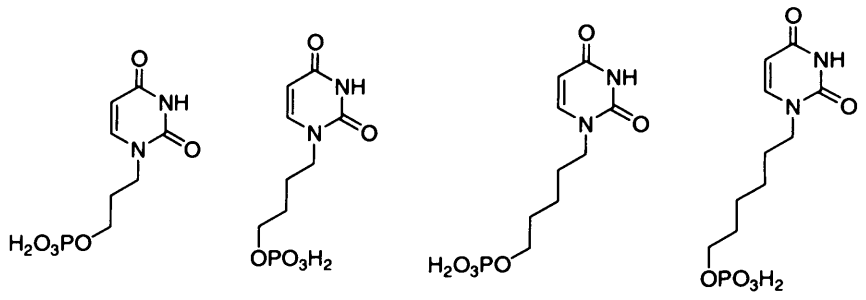
Unfortunately NMR spectroscopy showed the presence of some impurities in the two products **42b** and **42c**. In the proton NMR there was a singlet at 10 ppm; which was initially thought to be due to the NH of the uracil. However the ^{13}C-NMR showed a peak at 140 ppm, which was not consistent with the right structure. Therefore analysing the NMRs of the starting materials, it was discovered that the two compounds **42b-c** were contaminated by some tetrazole left from the phosphitylation. In fact it was not possible to detect the heterocycle by TLC because it is not UV active and also oxidizing staining agents were not able to reveal it.

Since the tetrazole is sold as solution in acetonitrile, the solubility of compounds **42b** and **42c** was tested in this. After discovering that they were not very soluble in acetonitrile, the traces of tetrazole could have been removed by washing the solids with acetonitrile.

All the phosphates were subjected to mass spectral analysis with electrospray negative ionization mode; for all of them a clear peak for the molecular ion $[M]^-$ was recorded, plus a series of dimeric aggregates corresponding to the ions $[2M]^-$.

In the ^{31}P-NMR spectra the chemical shift for the free phosphate had shift of *circa* 1 ppm, and all the compounds showed a resonance singlet at δ 1.5. The proton showed the complete disappearance of the benzyl groups, with no signal in the aromatic region around δ 7.5 and the disappearance of the singlet for the CH_2 groups at δ 5 which were due to the benzylic methylene groups.

Summarizing, from the intermediates **39a-g**, synthesised with the Mitsunobu based synthetic route, four acyclic nucleosides were synthesised, with different chain lengths and different terminator groups. These were sent for biological evaluation.

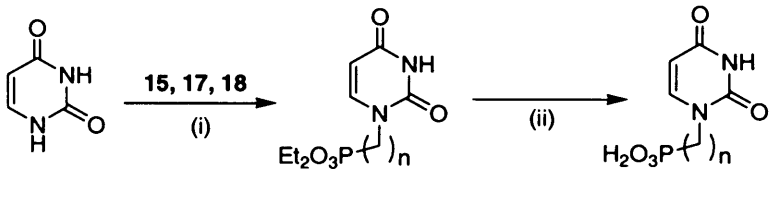


42a WSP1146 **42b** WSP1143 **42c** WSP1144 **42d** WSP1225

Figure 5-13 Summary of the phosphates synthesised

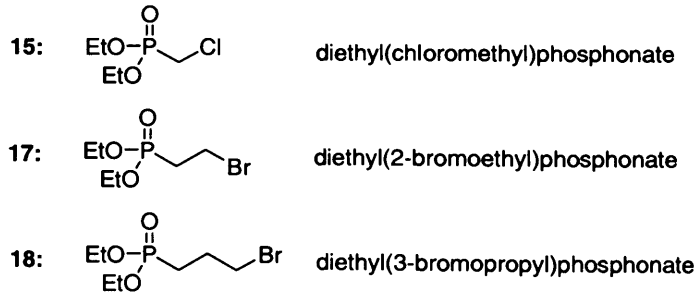
5.1.2 Acyclic phosphonates

The second polar group we wanted to examine as terminator was the phosphonate. As mentioned earlier phosphate and phosphonate are structurally similar, but the second group has the advantage to be metabolically more stable. This can be achieved by simple coupling of uracil base with the commercially available halophosphonesters **15**, **17** and **18**, as shown in Scheme 5-9. Using the same conditions developed for the coupling with halo-alcohols and described in Chapter 5, we managed to synthesise a small library of compounds, where the main difference was the chain length between the uracil moiety and the phosphonate group.



43a: n=2
43b: n=3

44a: n=2
44b: n=3



15: diethyl(chloromethyl)phosphonate

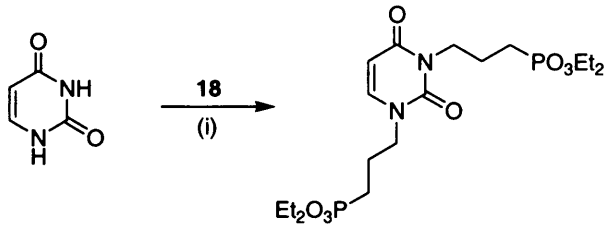
17: diethyl(2-bromoethyl)phosphonate

18: diethyl(3-bromopropyl)phosphonate

Scheme 5-9 (i): Cs_2CO_3, DMF, 12-72 h; (ii): a) Me_3SiBr, DCM; b) $EtOH/H_2O$.

The first reaction with the diethylchloromethyl phosphonate **15** did not work at all. After 12 hours at 50 °C, the TLC showed many spots very close to each other. Reducing the reaction time did not affect the result, whilst at room temperature no reaction was observed by TLC. All the attempts to separate the compounds by chromatography were unsuccessful and, since the mass spectrum of the crude residue did not show the presence of the desired compound, no more effort was put in the synthesis of this compound.

The second phosphonate **17** gave the best results. Although this reaction was slower than the others, heating at 50 °C for five days gave the desired compound in 38% yield after purification. In the ^{1}H-NMR together with the two doublets for the two protons of the uracil, the triplet for the ethyl ester was clearly visible at δ 1.3, all the other signals were overlapped in multiplets due to coupling with the phosphorous. In the carbon spectrum it was also possible to identify the coupling between the P and the two CH_2's of the aliphatic chain. The ^{31}P-NMR confirmed the purity, with only one peak at δ 28.64.



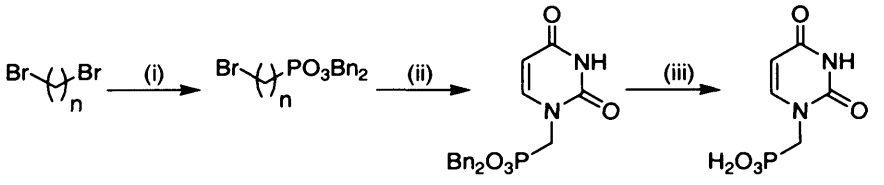
43c

Scheme 5-10 (i) Cs_2CO_3, DMF, 50 °C

The third phosphonate **18** showed completely different behaviour compared to compound **17** (Scheme 5-10). In the first attempt, the reaction was warmed as usual to 50°C and after 4 hours the complete disappearance of the starting phosphonate was observed. The reaction was stopped and the same work up was followed, but after purification by chromatography, the dialkylated product was recovered as the main product. This was confirmed by the proton NMR, which showed double integration for the ethyl groups and by the ^{31}P-NMR experiment with two signals having a chemical shift at δ 30. Also the low resolution mass presented two signals at m/z 469.2 for the ion $[M+H]^+$ and at m/z 491.2 corresponding to the $[M+Na]^+$. In the second attempt the addition of the starting diethyl(3-bromopropyl)phosphonate **18** was done at 0 °C, in an ice bath, and then

the reaction was left to warm to 25 °C and stirred for 12 hours. After that time in the TLC there was no presence of starting material, but two different spots very close to each other were clearly visible. Again the same work up was applied and after a long and slow chromatographic column the compound **43b** was isolated in 32% yield. All the analyses confirmed the result of the reaction.

The bromophosphonates with a chain length of 4 or 5 carbons were not commercially available. They were prepared starting from 1,4-dibromo butane **45** and 1,5-dibromopentane **46**, which are cheap and easily available starting materials. The planned synthetic route is depicted in Scheme 5-11. After replacing one bromine atom with a phosphonate moiety following the Arbuzov reaction, the bromo-intermediates **47a-b** can be used directly for the coupling with the uracil.



45: n=4 **46**: n=5 | **47a**: n=4 **47b**: n=5 | **48a**: n=4 **48b**: n=5 | **49a**: n=4 **49b**: n=5

Scheme 5-11 (i): Dibenzylphosphite, Cs_2CO_3, DMF; (ii): Uracil, Cs_2CO_3, DMF, 0°C → 25°C; (iii):H_2, Pd/C 10% MeOH.

One of the drawbacks of Michelis-Arbuzov reaction is the extensive heating required to eliminate the alkyl halide generated by the reaction, and is limited to the use of aliphatic trialkyl phosphites in order to form more volatile alkyl halides.

In the search for a better procedure a method has been developed by Cohen *et al.*[106]. According to this new milder synthesis, the phosphonates can be obtained by reacting first a dialkyl phosphite with a strong base such as caesium carbonate and subsequently with the desired alkyl halide, in the presence of a polar aprotic solvent (DMF) at room temperature. In this case the base removes the proton from the dialkylphosphite generating thus a phosphite anion that undergoes nucleophilic attack on the alkyl halide, as shown in Figure 5-14.

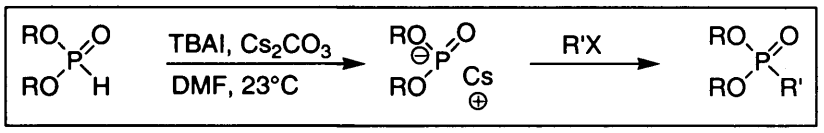


Figure 5-14 Milder phosphonate synthesis with caesium carbonate and TBAI in DMF. The reaction does not required heating, because the preformed phosphite anion can directly displace the halide

The procedure does not require heating since the released halide reacts with the cation Cs^+ to form a salt. This by-product can then be easily removed in the work-up by aqueous wash.

In the first step the dibromoalkanes **45** and **46** were treated with dibenzyl phosphite, caesium carbonate and tetra butyl ammonium iodide (TBAI) in DMF. This new synthesis of phosphonates allowed the intermediates **47a-b** to be obtained in 32% and 52% yields respectively.

No problems were encountered in the next coupling with the uracil base, the two intermediates **48a-b** were obtained pure after chromatographic purification in 65 and 41% yields, no detectable dialkylated product was observed and both the desired compounds showed a clean NMR spectra. In the ^{13}C-NMR experiment most of the signals were split into doublets due to the coupling with the ^{31}P nucleus; however the measured *J* values were in accordance with those reported in literature. The highest coupling constant was registered for the α carbon to the phosphorus (*J*=140 Hz, *circa*).

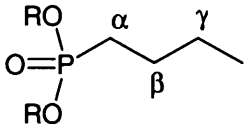
	|J(P-Cα)|: ≈140 Hz
	|J(P-Cβ)|: ≈5 Hz
	|J(P-Cγ)|: ≈16 Hz

Table 5-2 Typical C-P coupling constant (*J*) for alkyl phosphonates[107]

To complete the planned synthesis it was necessary to hydrolyse the ester functions. The ethyl esters of the intermediates **43a-b** were removed using the procedure with trimethylsilyl bromide followed by co-evaporation with ethanol and water, Scheme 5-9. No problems were encountered with this reaction, after stirring overnight at room temperature and after the suggested work up the two

phosphonates **44a-b** (Scheme 5-9) were obtained in quantitative yield, without further purification.

The two benzyl esters in the compounds **48a-b** were cleaved by hydrogenolysis, using Pd/C 10% as the catalyst, methanol and hydrogen (Scheme 5-11). Also in these reactions no problems were encountered and the phosphonic acids **49a-b** were recovered pure after filtration of the catalyst and evaporation of solvent and by-products in quantitative yield. All the synthesised phosphonates were analysed by negative electrospray (ES-) low resolution mass spectrometry which always showed the presence of the desired compounds with the molecular ions $[M]^-$, $[2M]^-$ and $[3M]^-$, due to the dimerisation and trimerisation of the sample in the ionization chamber of the spectrometer. The ^{31}P-NMRs had only one signal, proving the absence of other phosphorus impurities, and the chemical shifts were within the range of 20-30 δ.

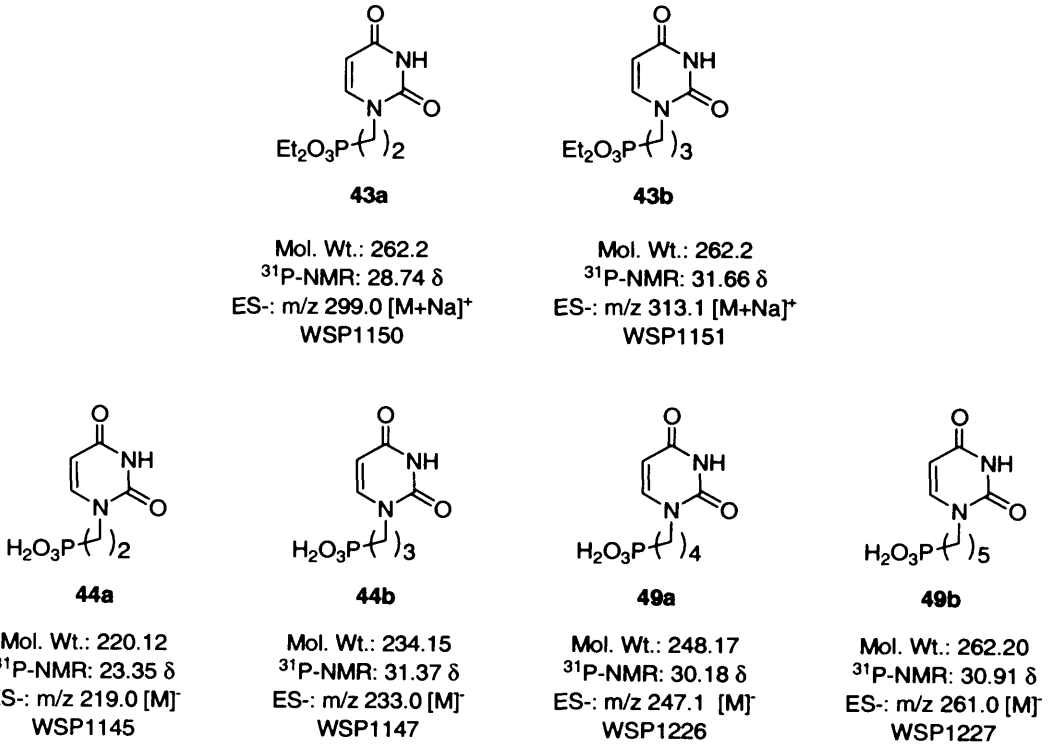
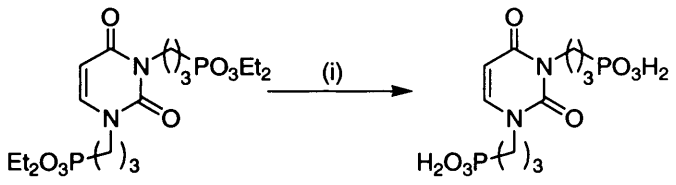


Figure 5-15 The six acyclic phosphonates synthesised

The small library was then sent for biological tests and the results will be discussed in the biological chapter.

In addition to the six compounds illustrated in Figure 5-15, another phosphonate was sent for testing.



43c **51**

WSP1148

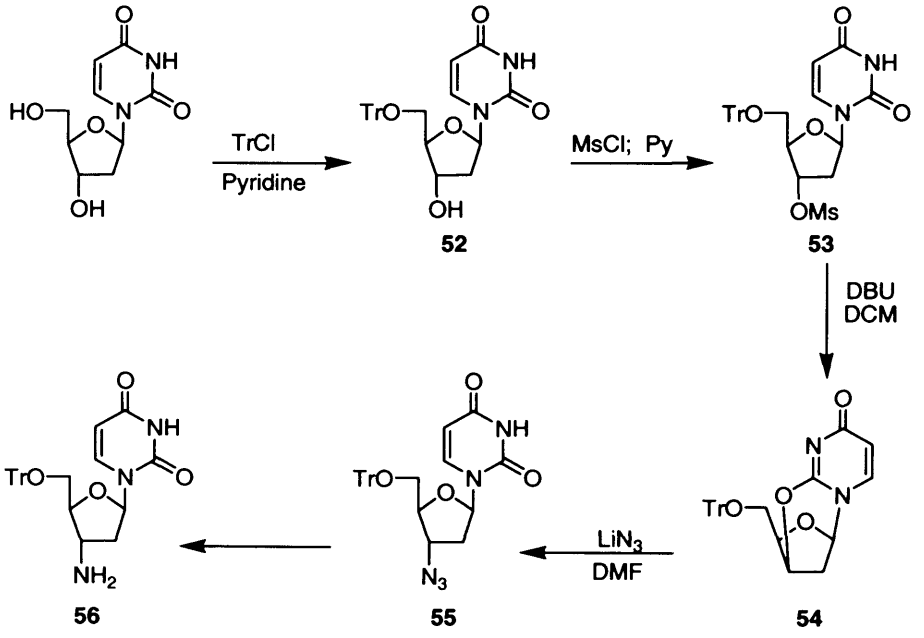
Scheme 5-12 The by-product **43c** isolated from the synthesis of compound **43b**, deprotected to get compound **51** and sent for biological evaluation.

This compound shown in Scheme 5-12, was recovered as a by-product in the synthesis of **43b**, and it was decided to evaluate its biological activity in order to have more data about the enzyme specificity.

Chapter 6 Synthesis of 3' Modified Deoxyuridine Analogues

To investigate the role of the 3' position of the sugar in substrate binding/recognition, it was decided to explore some substitution of the secondary hydroxyl group in the 2'-deoxyuridine with an amino function. Such substitution could eventually be further functionalized as amide or sulphonamide.

This part of the project was started with the help of an undergraduate project student (Yvette Verbeeck). The planned synthetic route is reported in Scheme 6-1. It is based on the route for the synthesis of the anti-HIV drug AZT developed by Horwitz and co-workers[108].



Scheme 6-1 Planned synthetic route to the 3'-amino-2',3'-dideoxyuridine

Starting from the 2'-deoxyuridine with protection of the 5' primary alcohol as trityl ether **52**, using the standard procedure[82], the next step was the transformation of the 3' OH into a good leaving group such as mesylate **53**.

Trityl ethers have been extensively used in nucleoside chemistry, due to their selectivity in the protection of primary alcohols compared to secondary. They can be introduced by reacting the nucleoside with trityl chloride in the presence of

pyridine as base and solvent under moderate heating. The reaction did not give particular problems and the intermediate **52** was obtained in 93% yield. The ^{1}H-NMR of the compound recovered after the work-up showed a good purity, although some diprotected nucleoside or triphenylmethyl alcohol was present, since the integration in the aromatic region was slightly higher than (20H) the expected value. However the tritylated intermediate was used for the next step without further purification, since the eventual impurities should not interfere with the following reaction, and they could be removed later by chromatography.

The mesylate was chosen to convert the 3'-secondary alcohol of the nucleoside into a good leaving group. Again the reaction worked well, and after treating the 5'-triphenylmethoxy-2'-deoxyuridine **52** with a little excess of mesyl chloride in pyridine the 3'-mesylate **53** was obtained in very good yield (83%). The proton NMR confirmed the success of the reaction and showed no particular impurities; therefore also this intermediate was used for the subsequent reaction without undergoing chromatography.

The formation of the anhydronucleoside **54** intermediate was required in order to retain the conformation of the carbon in 3' position; in fact the direct substitution of the mesylate by the azide group would change the configuration of the carbon in 3'-position from the α (down) to the β (up) position. The anhydronucleoside controls the attack of the nucleophile azide, which can occur only from the bottom face of the sugar giving as only the product the 3'-azido derivative **55** with the desired α configuration. The synthesis of the intermediate **54** was achieved by treating the 3'-mesyl derivatives **53** with DBU [1,8-diazobicyclo[5.4.0]undec-7-ene] in DCM at room temperature. After 24 hours the TLC showed the complete disappearance of the starting material, and the anhydronucleoside **54** was recovered in 82% yield after purification. The success of the reaction was proved by mass spectrometry and NMR. In the proton NMR the expected triplet for the H-1' became a doublet. It was reasoned that this was probably due to the rigid conformation of the molecule, leading to a dihedral angle with one of the hydrogens in the 2' position of about 90°. This would lead to a *J* value close to zero, as it can be estimated looking at the Karplus diagram and Karplus equation[109], which correlates the coupling constant values with the dihedral angle between atoms[98].

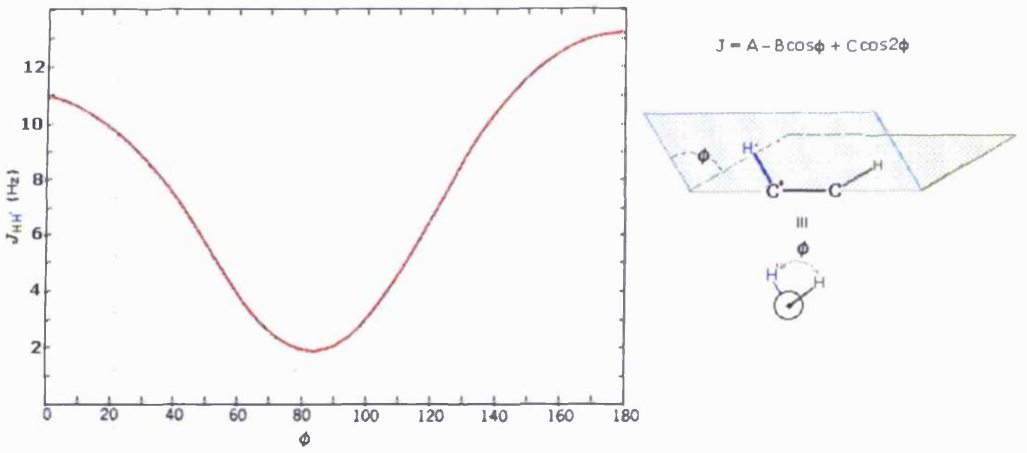


Figure 6-1 Karplus diagram and dihedral angle

This hypothesis was partially confirmed by a modelling study. Thus using the computer program Chem3D (from the ChemOffice© package), the dihedral angle for the minimized structure of the anhydronucleoside **54** is about 76° and not 90° as required from the Karplus diagram, as shown in the Figure 6-2. However it was close enough to 80°, where the coupling constant is near the minimum value, therefore in the recorded spectrum the expected triplet appeared as a doublet.

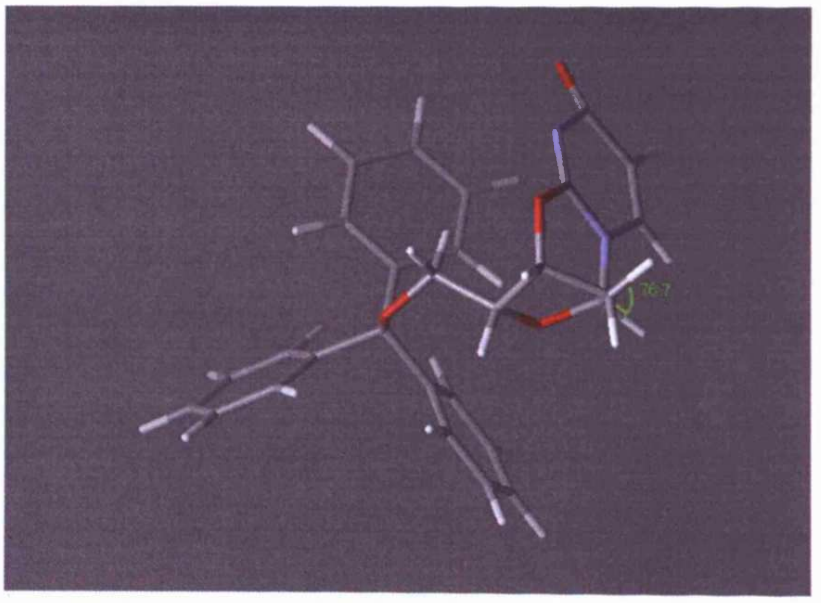


Figure 6-2 3D structure of the anhydronucleoside **54** with highlighted the dihedral angle between H1' and H2'.

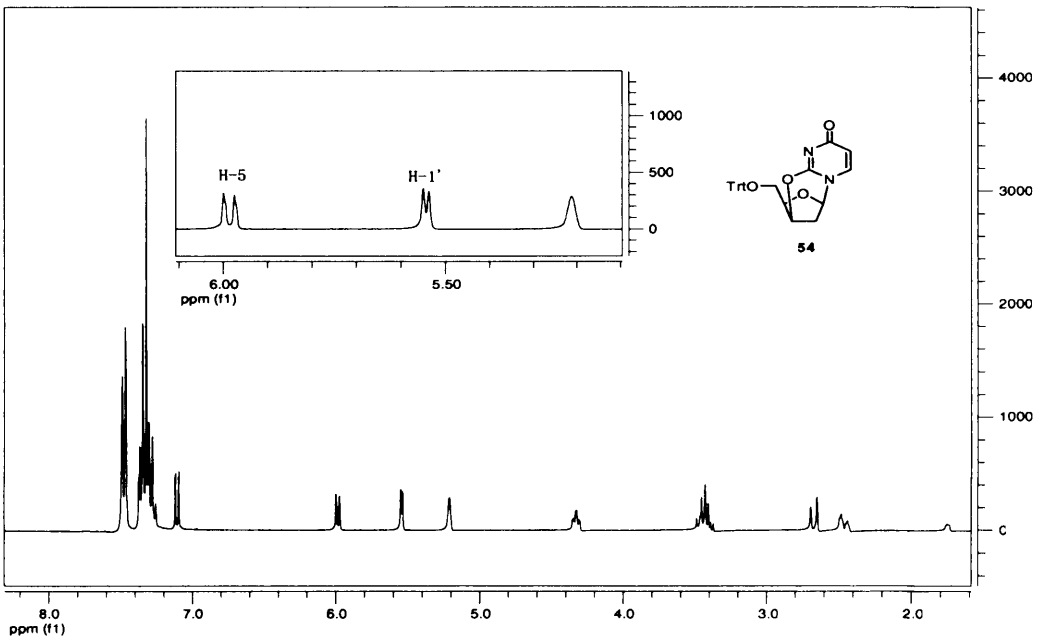


Figure 6-3 ^{1}H-NMR for compound **54**, in the expansion at δ 5.55 is highlighted the unexpected doublet for the H-1' of the sugar

The opening of the anhydride was achieved using lithium azide, generated *in situ*, to afford the 3'-azide nucleoside **55**. The advantage in the use of lithium azide instead of the cheaper sodium azide is in the higher solubility of the former in DMF compared to the latter, thus influencing the rate of the reaction, as reported by Finnegan et al[110].

The lithium azide was generated *in situ* from lithium fluoride and azidotrimethylsilane in the presence of tetramethylethylenediamine (TMEDA), as suggested by Kirschenheuter and co-workers[111]. This technique, should help to have a cleaner reaction, since when commercially available lithium azide was used for the synthesis of azidonucleosides, several by-products were reported[111].

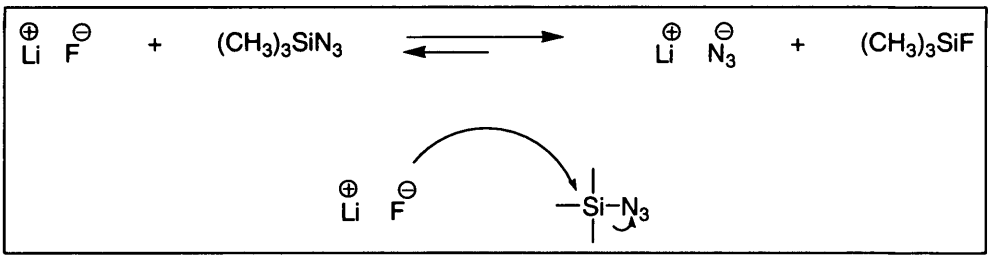


Figure 6-4 *In situ* formation of lithium azide

The role of the TMEDA is to form a complex with the lithium cations, making the azide anion more available for the nucleophilic substitution, as shown in Figure 6-5.

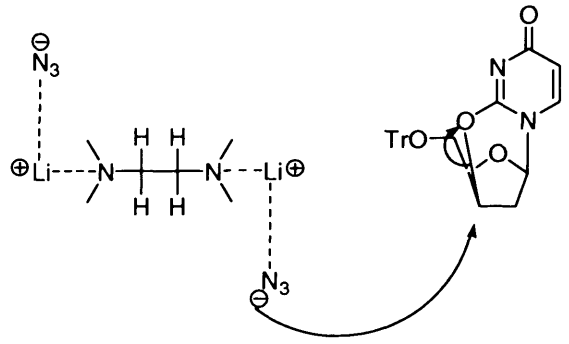
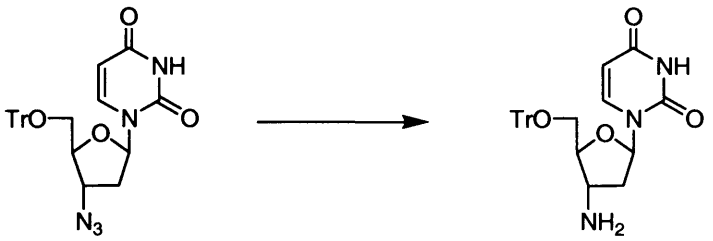


Figure 6-5 Complex between the TMEDA and lithium azide

After heating at 110 °C for 20 hours, a new spot was visible on TLC with R_f higher than the starting material. Following the suggested work up the 3'-azido nucleoside was obtained in 56% yield. When in the work up the extraction was made using ethyl acetate, instead of the suggested chloroform, the yield increased to 65%. Compound **55** was characterized either by NMR, mass spectrometry and IR where it was possible to identify the typical band for the azido group at 2000 cm^{-1}. In the proton NMR the signal for the H-1' was a triplet, as it is for normal 2'-deoxy nucleoside structures. The only problem was in the microanalysis of the sample. The expected percentage for the nitrogen in the compound was 14.13%, but only 12.53% was found with the analysis.

6.1 Conversion of the 3'-azido-2',3'-dideoxyuridine (55) into 3'-amino-2',3'-dideoxyuridine (56) and further derivatizations

The next stage was the conversion of the azide into an amine. In literature the most commonly used ways to obtain this transformation are hydrogenolysis or reaction with triphenylphosphine[112].



55 **56**

Scheme 6-2 Conversion of the azido group into amino function

Substrate	Conditions	Solvent	Results
55	10% Pd/C, H_2	MeOH	Decomposition
55	5% Pd/C, H_2	MeOH	56%
55	Lindlar's, H_2	EtOH	70%
55	PS-PPh_3, NH_4OH	Pyridine	Decomposition
55	PPh_3, H_2O	THF	84%

Table 6-1 Reaction conditions tested for the reduction of the azide **55** to the 3'-amino deoxyuridine **56**

The hydrogenation should be a clean reaction, where the work-up usually consists of filtration of the catalyst and evaporation of the solvent, to afford a clean product without chromatographic purification.

For the first attempt, 10% Pd/C was used as catalyst in MeOH with a hydrogen balloon, according to the general procedure for hydrogenation[113]. After the first hour a new spot with lower R_f was observed in the TLC, but significant levels of starting material were also present; therefore the reaction was left overnight at room temperature. Unfortunately TLC indicated a mixture of spots probably due to the cleavage of the trityl group from the 5' position; in fact trityl ethers are not very stable for prolonged periods under hydrogenation conditions with 10% Pd/C.

In order to overcome this problem the use of a milder hydrogenation catalyst, 5% Pd/C, with hydrogen was investigated. Again the appearance of a new spot at lower R_f was observed after 1 hour; however after 6 hours, TLC showed decomposition of the starting material.

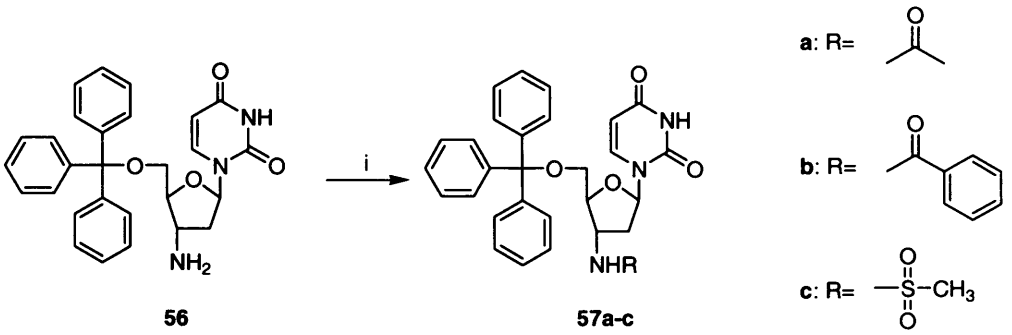
Lindlar catalyst is Pd on calcium carbonate poisoned with lead, which should allow a milder hydrogenation of the azide function. Five hours after the addition

of hydrogen the new spot was observed, but still starting material was present, consequently the catalyst was filtered off, replaced with new and the reaction was left stirring for a further 3 hours. This time no decomposition was observed and the desired compound **56** could be isolated after chromatographic purification in 70% yield.

The second procedure to convert azides into amines is the reaction with triphenylphosphine, the Staudinger reaction[112]. In this approach a polymer bound triphenylphosphine in pyridine as solvent was used, following the procedure of Holletz *et al.*[114], a procedure specific for nucleosides. Unfortunately problems in monitoring the reaction were encountered, due to the inapplicability of TLC technique and it was not possible to understand how the reaction was proceeding. Therefore the same timing described in the reference was followed and after 12 hours ammonia was added. After the work up and chromatographic purification none of the collected fraction appeared to be the desired compound

As a final attempt to improve the reaction for the conversion of the azide to the amine, the reaction with triphenylphosphine in homogeneous phase was tried. In this case it was possible to monitor the reaction by TLC. When the disappearance of the starting material was observed (after 12 hours) water was added and the reaction was warmed to 60 °C. Also in this case the 3'-amino-2',3'-dideoxyuridine **56** was obtained in a good yield (84%) after purification by chromatography. The success of the reaction was proved by mass spectrosmetry where the main peak was at m/z 518.0 for the ion $[M+Na]^+$. Further confirmation came from the ^{13}C-NMR with a new chemical shift for the C-3', now moved to high field at δ 50.7 (δ 59.8 for the starting azide).

A final goal of this part of work was the functionalization of the amino function to probe further the active site. Compound **56** was used to make different derivatives such as acetylamino **57a**, benzoylamino **57b** and the sulphonamide **57c** as shown in Scheme 6-3.



56 57a-c

Scheme 6-3 i-a: AcO_2, Et_3N, DCM; i-b: BzCl, Et_3N; DCM; i-c: MsCl; Et_3N; DCM.

Further derivatives could be made depending on the assay results for these first compounds.

The starting material **56** was split into different tubes, and the reactions were carried out in parallel, using a Radleys 12 tube carousel.

No problems were encountered in the synthesis of the acetylamino derivatives **57a**. Treating the 3'-amino deoxyuridine **56** with acetic anhydride in the presence of triethylamine as base and DCM as solvent, the reaction reached completion in 3 hours stirring at room temperature. The desired amide was obtained in 95% after purification by chromatography. NMRs were in agreement with the structure: a broad singlet at δ 9.82 confirmed the presence of the hydrogen on the nitrogen in the uracil base. Another singlet at δ 2.04 integrating for three protons, due to the methyl group, proved that only one equivalent of acetic anhydride reacted with the amino function. The low resolution mass spectrum also confirmed the result, with a peak at m/z 533.8 for the ion $[M+Na]^+$, with 20% of intensity due to the lost of the trityl group, which could be revealed by another peak at m/z 243. Finally from the microanalysis the compound appeared to be a hydrochloride salt.

Different results were obtained for the benzoylation, using the same conditions as for the acetylation. Benzoyl chloride, triethylamine and DCM were used for the reaction, but after 4 hours at room temperature, many new spots with higher R_f than the starting material were present on the TLC. After the work up and the chromatographic purification it was possible only to recover the most intense spot in the TLC. Spectroscopic analysis of the collected fraction did not confirm the presence of the desired compound **57b**. In the NMR the integration in the aromatic region was higher than the expected (30H instead of 20H), indicating

that perhaps two equivalents of benzoyl chloride had reacted with the amine. In the mass spectrum there was no peak corresponding to $[M+H]^+$ or $[M+Na]^+$ ions using the positive electrospray ionization mode; only a small peak at m/z 699 was visible with a low intensity (25% ca) due to the *bis*-benzoylated derivative.

The last derivative was the sulfonamide **57c**. Sulfonamides are interesting group in medicinal chemistry because of their ability to be donor and/or acceptor for hydrogen bonds. For this reason their presence in a potential inhibitor can help to achieve more interactions within the active site of the enzyme, hence increasing the affinity and consequently the inhibition of the biological target.

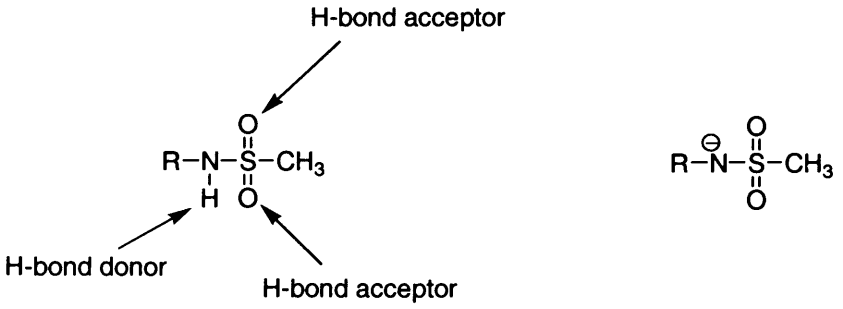
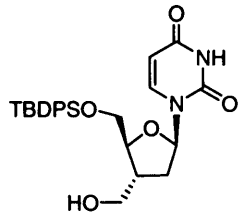


Figure 6-6 Structure of sulfonamide and its ionised form at physiological pH

With the same conditions adopted for the acetylation, the sulphonamide derivative was obtained in reasonable yield (51%). The reaction was left only one hour to avoid the formation of side products as for the benzoylation. NMR analysis confirmed the presence of the sulphonamide moiety with a singlet at δ 2.90 integrating for 3 protons as was expected for the methyl group of the sulfonyl group. The low resolution mass spectrum showed a peak for m/z 569 for the ion $[M+Na]^+$, detected also with the high resolution mass spectrometry.

6.2 Further modification of the 3'-position

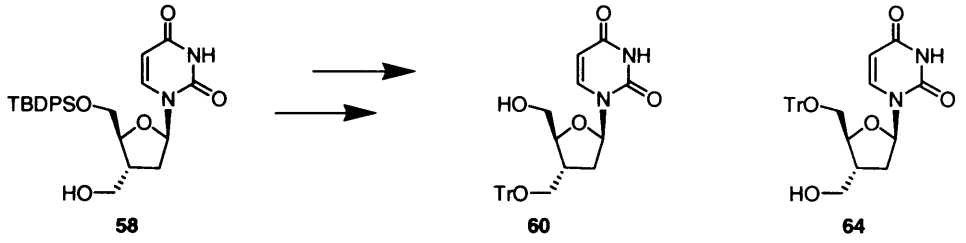
A further modification of the 3' position of the 2'-deoxyuridine was achieved starting from the intermediate **58** provided to us by Medivir, one of our partners collaborating on this project, Figure 6-7.



58

Figure 6-7 3'-(α)-methylenehydroxy-2',3'-dideoxyuridine derivate, provided by Medivir

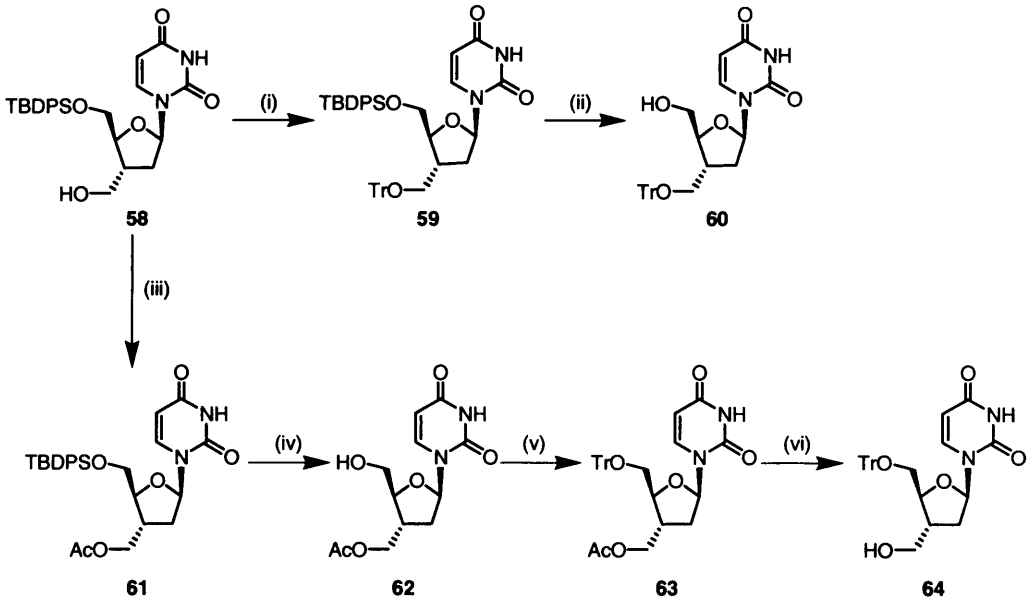
Such a compound could have been transformed either into 5'-trityl or 3'-trityl derivatives; compounds **60** and **64** respectively. Since both possess a primary alcohol function, and therefore their three dimensional structures share important similarities, the two isomers could give important information by probing the enzyme active site.



58 60 64

Figure 6-8 Possible conversion of compound **58** into tritylated derivatives **60** and **64**

The synthesis of these compounds again required the use of orthogonal protecting groups as shown in Scheme 6-4.



61 62 63 64

Scheme 6-4 (i): TrCl, Pyridine, 80 °C; (ii): TBAF, THF, r.t.; (iii): Ac_2O, Pyridine, r.t.; (iv): TBAF, THF, r.t.; (v): TrCl, Pyridine, 80 °C; (vi): MeONa, MeOH, r.t..

Since the trityl ether is compatible with the conditions used for the cleavage of the *tert*-butyldiphenylsilyl ether, the synthesis of compound **60** was straightforward. The triphenylmethyl moiety was introduced on the primary alcohol in the 3' position using the standard procedure with trityl chloride in pyridine. The reaction needed to be warmed to 80 °C overnight, since in the first attempt at room temperature the TLC did not show any new spot after several hours. Whilst warming the mixture the formation of the product **59** was already visible on TLC after one hour, with a new spot with Rf between the two starting materials (trityl chloride and nucleoside **58**). The intermediate **59** was recovered in 77% yield after purification. The proton NMR confirmed the results showing a multiplet around δ 7.5 integrating 25, for the five phenyl rings. As in the case of the anhydronucleoside **54**, the expected triplet for the H-1' appeared as a double doublet using a 500 MHz spectrometer. With a bigger magnetic field it was possible to solve the structure for this signal and measure both the coupling constants (J_1=6.8 Hz and J_2=3.3 Hz). Also the doublet for the H-5 of the uracil ring was a double doublet, due to the coupling with adjacent H-6 and with the amide hydrogen in position 3.

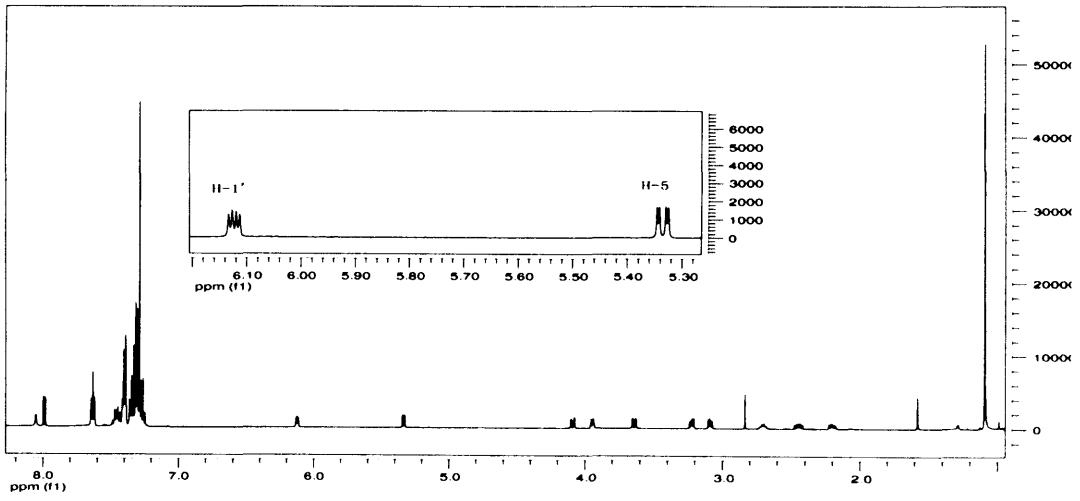


Figure 6-9 ^{1}H-NMR for compound **59**, with expansion of the region for the H-1' and the H-5

The low resolution mass spectrum showed only one peak with m/z 745.4 for the ion $[M+Na]^+$. The following step was the deprotection of the hydroxyl group in the 5' position. This was achieved, according to the standard procedure, with TBAF in THF, at room temperature, affording the desired compound **60** with a 60% yield. NMR spectroscopy confirmed the loss of the TBDPS group with the

lack of the typical singlet at δ 1 for *tert*-butyl group and with a lower integration (15 protons) in the aromatic region of the spectrum. The only peak visible in the low resolution mass spectrum was at m/z 243, which corresponded to the triphenylmethyl cation which was cleaved in the ionization chamber, and its intensity covered other peaks. Fortunately with high resolution analysis the ion $[M+NH_4]^+$ was detected, using a positive electrospray ionization mode.

The synthesis of compound **64** was a little more complicated and required a four step synthesis. As orthogonal protective group the acetyl ester was chosen for the pseudo 3' hydroxyl group. It was introduced using acetic anhydride, in pyridine. The reaction worked well at room temperature, and in 3 hours the desired intermediate **61** was obtained in 61% yield. According to the NMR the intermediate was enough pure for the following steps. The cleavage of the silyl ether was performed as previously described with TBAF in THF. The acetyl ester was stable in the conditions used and compound **62** was obtained in 56% yield after chromatography. No aromatic rings were detectable in the proton NMR, and also the signal for the *tert*-butyl group was missing.

Continuing with the synthesis the intermediate **62** was treated with trityl chloride in pyridine and heated to 80 °C for 24 hours. The procedure worked well and after 24 hours the starting material had completely reacted, and only two spots were present on TLC. One with lower R_f due to the desired product, the second with the higher R_f due to the excess of trityl chloride used. The reaction mixture was concentrated under reduced pressure, and he intermediate **63** was then isolated after chromatographic purification in 95% yield.

For the final step, the cleavage of the acetyl group, it was decided to use a solution of sodium methoxide in methanol. The advantages of this procedure are that the by-product of the reaction is the volatile methyl acetate that can be removed *in vacuo*. The acetylated intermediate **63** was stirred in a solution 0.2 M sodium methoxide at room temperature. Compound **64** was obtained after evaporation of the solvent and the by-product. The ^{1}H-NMR appeared similar to the one recorded for compound **60**, a multiplet in the aromatic region integrating to 15 protons, a double doublet for the H-1'of the sugar with a chemical shift moved to down field at δ 6.11 (δ 6.06 for compound **60**), also the multiplet for the H-3' was shifted to the left of the spectrum δ 2.65 compared to the 3'-

tritylated analogue **60** (δ 2.56); while the CH_2 group in 5' position gave two double doublets at higher field δ 3.59 and 3.47 compared to compound **60** where it was possible to distinguish two multiplet at δ 4.00 and 3.80. The two melting points were not very different with 106-108 °C for the **64** and 107-110 °C for **60**. The microanalysis showed that the 5'-tritylated derivative **64** was a hydrochloride salt, probably due to the reaction with the trityl chloride which released HCl during the reaction.

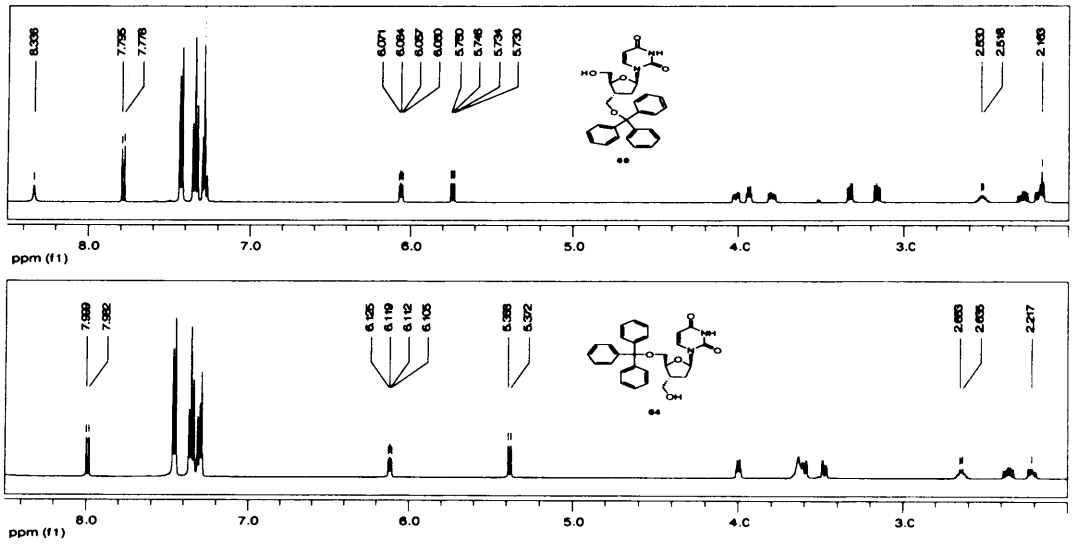
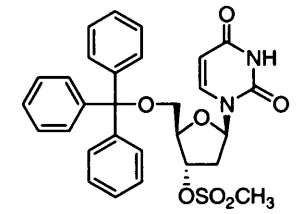
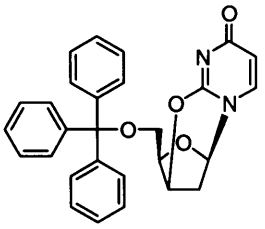


Figure 6-10 ^{1}H-NMR spectra of the two isomers **60** and **64**

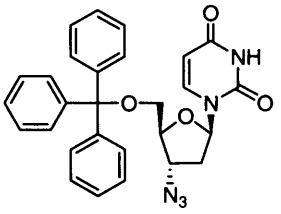
Concluding this part of the project a small series of 3'-modified 2'-deoxyuridine derivatives (Figure 6-11) have been synthesised and sent for biological evaluation.



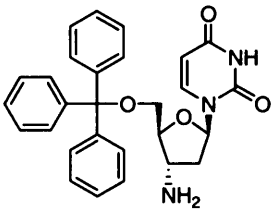
53
WSP 1203



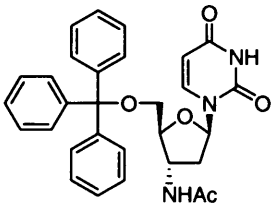
54
WSP 1204



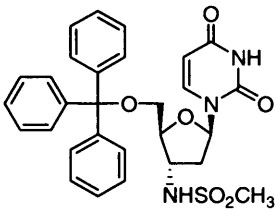
55
WSP 1205



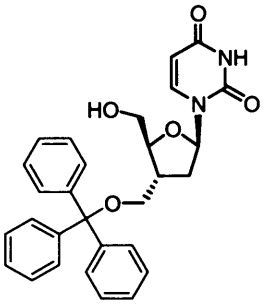
56
WSP 1206



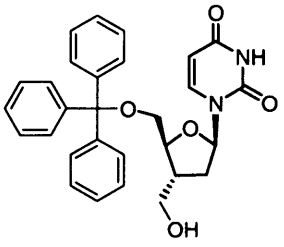
57a
WSP 1207



57c
WSP 1229



60
WSP1323



64
WSP1324

Figure 6-11 3'-modified nucleosides sent for biological tests

Chapter 7 Unsaturated and Branched Acyclic Uracil Derivatives

7.1 Unsaturated acyclic nucleosides - Modelling study

From the study of the *P. falciparum* dUTPase crystal structure it was found that one of the key amino acid residue for the interaction with the substrate dUTP is the tyrosine 112 (Figure 7-1). The phenyl ring of this amino acid interacts with the oxygen of the sugar ring. In acyclic uracil analogues with a simple aliphatic chain as spacer, this interaction is lost.

The introduction of a double bond in the spacer between the uracil moiety and the trityl group, (Figure 7-2) would lead to unsaturated compounds that should be able, in theory, to restore the lost interaction with the tyrosine by a π-stacking interaction. Such kinds of structures should therefore have a stronger binding mode with the enzyme active site.

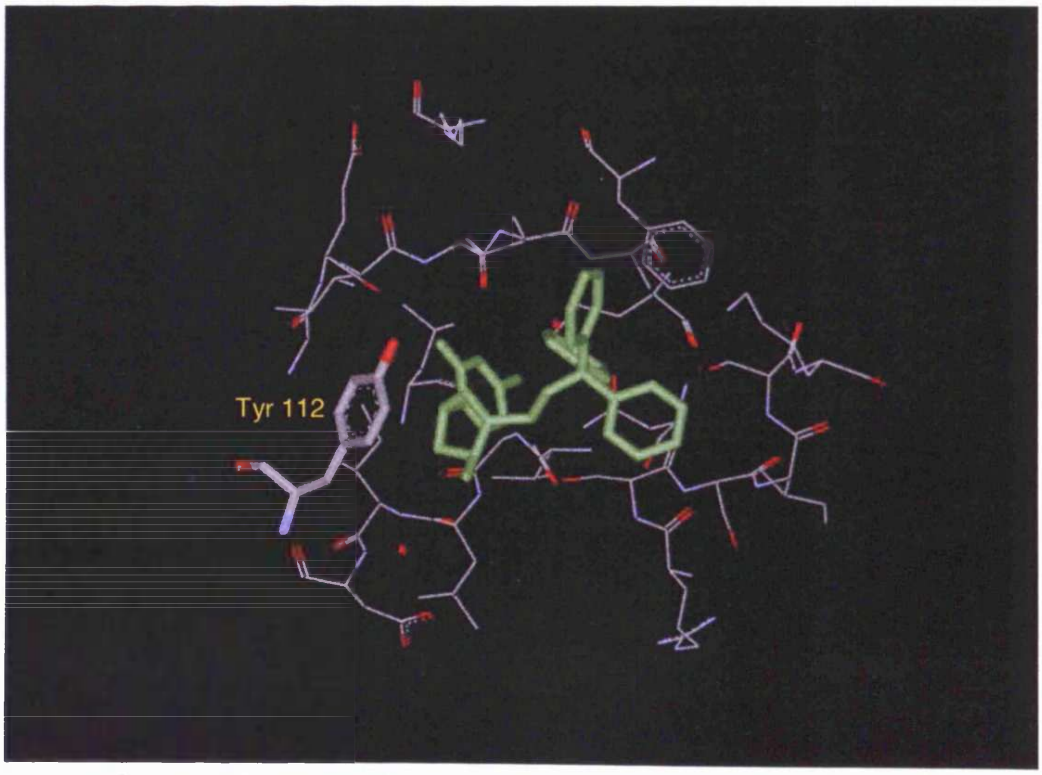


Figure 7-1 Active site of *P. falciparum* dUTPase with the inhibitor WSP 869 co-crystallised and the tyrosine 112 residue highlighted on the left of the picture

By the help of molecular modelling it was possible to have an idea of the structural features that might increase the binding strength of a ligand into the active site. This could be achieved by performing a so called "virtual screening", that involves docking and scoring several molecules within a relatively short time frame[115]. Technically speaking the positioning of a molecule into an active site is referred as "docking", whereas the prediction of the affinity is defined as "scoring". The computer programs would therefore be able to predict the binding mode of a structure in the active site, and the binding affinity of compounds[115].

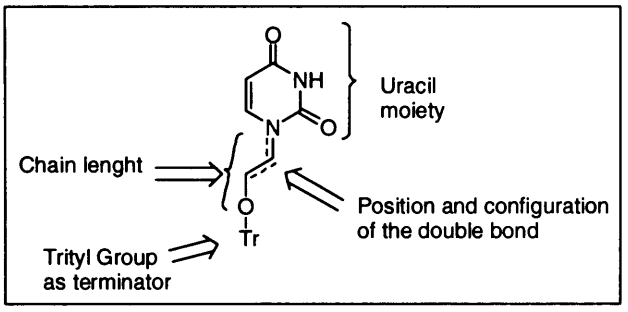


Figure 7-2 Common features and investigated details of the compounds virtually screened.

It was decided to perform a virtual screening of a small virtual library of structures. These compounds shared three common features: the uracil base, the trityl group as terminator and an unsaturated aliphatic chain as spacer. The screening was focused on *P. falciparum* dUTPase, using only a portion of *circa* 10 Å around the active site, to evaluate the effect of the chain length, the position and the conformation (*Z* or *E*) of the double bond, as shown in Figure 7-2. A total of 20 different structures were docked into *P. falciparum* active site.

Since the crystal structure of the enzyme was available with an inhibitor already in the active site containing a uracil ring and trityl group (Figure 7-1), this could be used to predict the binding mode of the molecules. Conformations of compounds which docked in a very different mode to WSP 869 were not considered.

The program chosen for the calculation was FlexX, which is based on an algorithm developed by Rarey and co-workers[116]. The choice was guided by the availability of the program, the short time required for the calculation and the experience developed in our group in the use of this software.

The FlexX algorithm considers the protein as a rigid structure which cannot move, whilst the ligand is considered as a flexible entity which can move to fit and to achieve the best interaction within the active site. However the conformational flexibility is modelled discretely. Bond lengths and angles are used as given in the input structure. Therefore, reasonably minimized structures should be used[116].

The protocol followed to perform the docking is illustrated in following scheme:

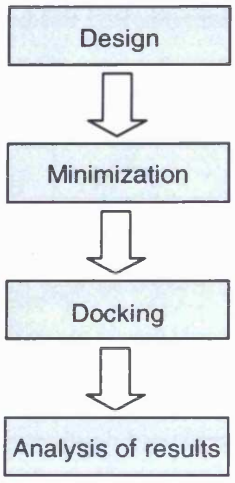


Figure 7-3 Flow chart of the procedure followed for the molecular modelling studies

The structures of the virtual library were drawn in a 2-dimensional format using the software ChemDraw. The two-dimensional structures were then imported in Chem3D, which can generate a three-dimensional structure from a conventional two-dimensional sketch. With this program the structures were minimized using a molecular mechanics method (MM2).

The minimized database of compounds was then loaded in the program FlexX. The program performed the docking using a section of the active site of *P. falciparum* dUTPase. The results of the screening were a series of complex enzyme/substrate interactions with the corresponding scores as energy.

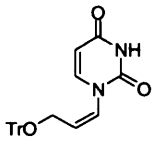
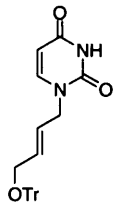
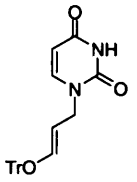
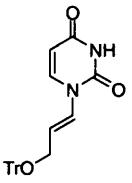
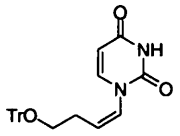
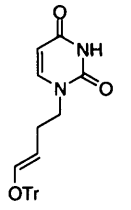
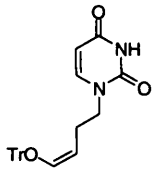
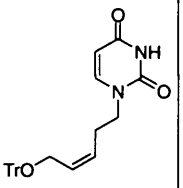
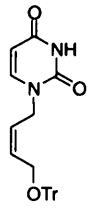
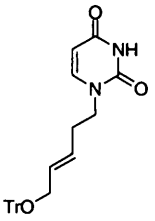
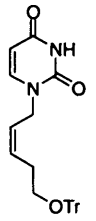
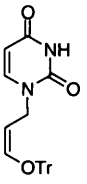
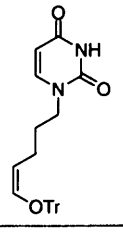
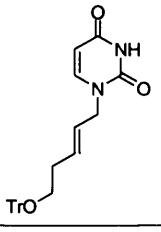
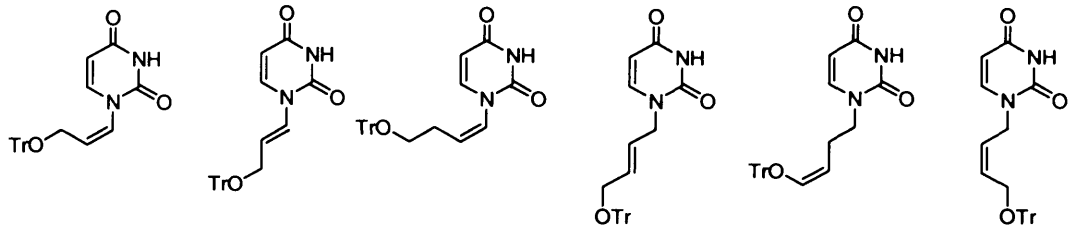
	Structure	Score		Structure	Score
65		-19.9	**72**		-17.1
66		-19.2	**73**		-16.1
67		-19.2	**74**		-14.7
68		-18.9	**75**		-14.5
69		-18.1	**76**		-14
70		-17.4	**77**		-13.6
71		-17.1	**78**		-13.6

Table 7-1 Scores for the docking of the library of unsaturated acyclic nucleosides. The scores are estimations of the energies for the complexes enzyme-substrate and are reported as kJ/mol, even although they are not absolute values[116].

Some results (scores) are reported in Table 7-1 with the corresponding structures. The table shows the best 14 structures. As mentioned earlier the score is not the only result of a virtual screening procedure, since the positioning within the active site has to be taken in to account.

Although the table shows the structures in order of the best scores obtained, some of the compounds illustrated in the table did not have a similar binding mode to the X-ray structure; in fact their docking resulted in positions outside of the enzyme active site.

Analysing the results from the energy point of view the structures in the table had better scores when the double bond is in a *Z* configuration instead of the *E* isomer; 6 out of the first 7 compound have a *Z* double bond in their structures. The variation of the chain length seems to favour structure with 3 or 4 carbon atoms, while structures with chains of 5 and 6 carbons were less favourable according to values of the scores. Not much information could be obtained regarding the position of the double bond, since it can be either between the α and the β carbon or the β and the γ and the differences in the score values are not significant. For example compounds **65**, **66**, **67** and **68** in Table 7-1 have double bonds in three different positions, but all the scores were comparable.



65 **73** **67** **72** **68** **69**

Figure 7-4 Compounds with the six best scores obtained from the docking

From the docking point of view the structures in general superimposed well with the reference inhibitor WSP 869. The analysis of the dockings suggests a good binding mode for almost all the designed structures, with only some exceptions. This would indicate that the presence of the double bond can be compatible with binding in the enzyme active site. Pictures for the best docking are shown in the following figures. These are for the compounds with chains of 3 and 4 carbons, see Table 7-1.

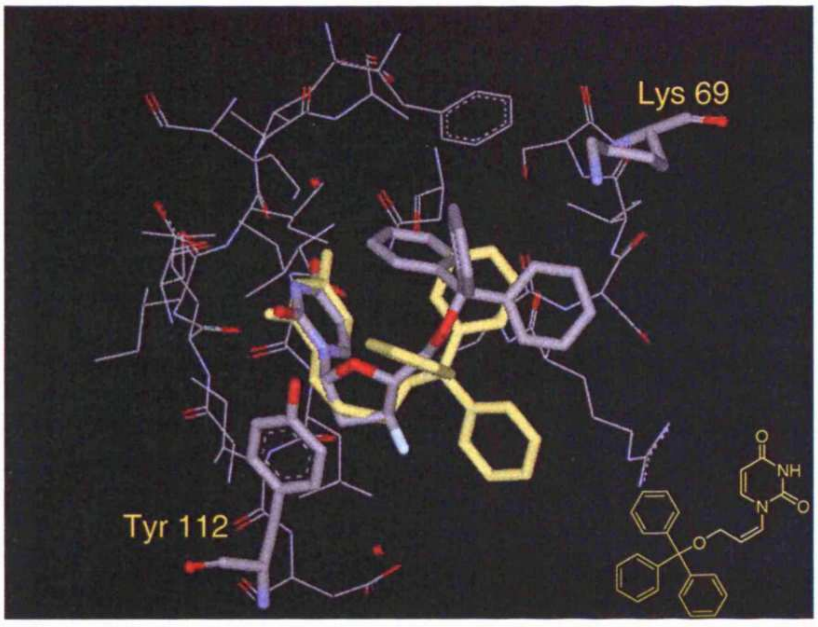


Figure 7-5 Docking of **65** (yellow) in the *P. falciparum* active site, superimposed with the inhibitor WSP 869 (grey) co-crystallised in the enzyme active site

Figure 7-5 shows the docking for compound **65** (score -19.9 in Table 7-1) superimposed with the inhibitor WSP 869. The two uracil rings superimpose well overlapped in the bottom of the active site. The main difference is in the position of the trityl groups. Due to the short 3-carbon chain the triphenylmethyl group of compound **65** (yellow) is closer to the tyrosine residue and further away from the lysine 69 which interacts with the trityl group of the ligand in the original crystal structure.

The docking for **73** is illustrated in Figure 7-6, this structure is an isomer of **65** with 3 carbon chain and the double bond in *trans* configuration. Despite the lower affinity prediction, (score -16.1) **73** superimposed better with the reference inhibitor. In particular the double bond is positioned very close to the position occupied by the oxygen of the ribose, facing the tyrosine 112 residue with which it could have an interaction. Also the trityl group has a position similar to the one adopted by the trityl group of the inhibitor in the crystal structure, closer to the lysine residue on the top-right of the picture.

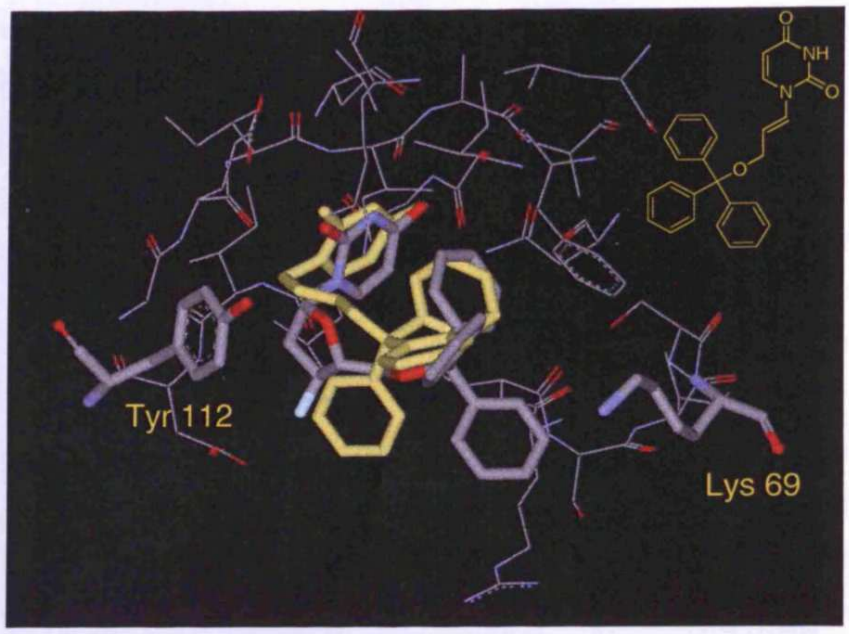


Figure 7-6 Docking of compound **73** (yellow) in the *P. falciparum* active site, superimposed with the inhibitor WSP 869 (grey) co-crystallised in the enzyme active site

Contrary to the score value obtained for compound **69** (score -18.1), the overlapping with the reference in the crystal structure was not as good as expected, Figure 7-7. The aliphatic chain assumes a very different position compared to the ribose in WSP 869. The trityl group is pointing in an opposite direction, and also the superimposition between the two uracil bases is poor.

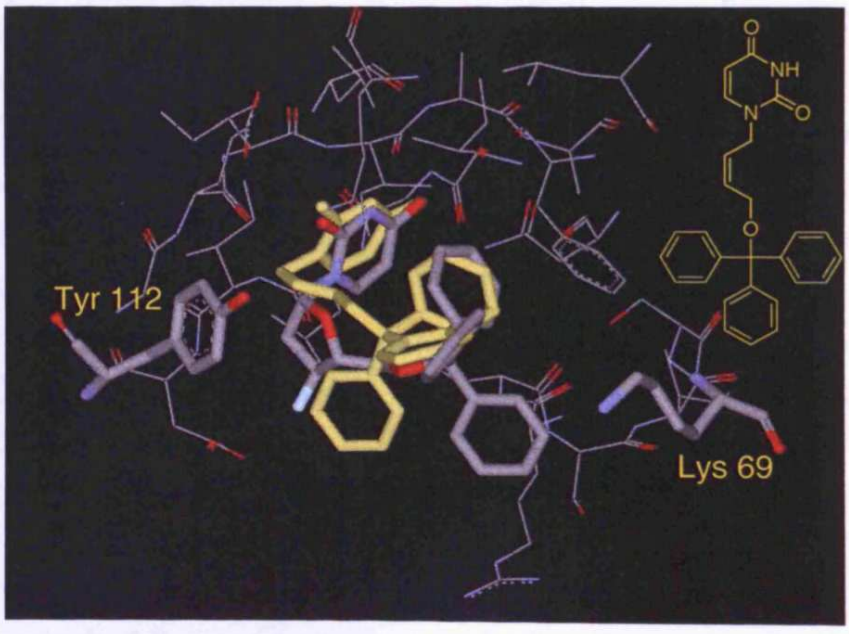


Figure 7-7 Docking of compound **69** (yellow) in the *P. falciparum* active site, superimposed with the inhibitor WSP 869 (grey) co-crystallised in the enzyme active site

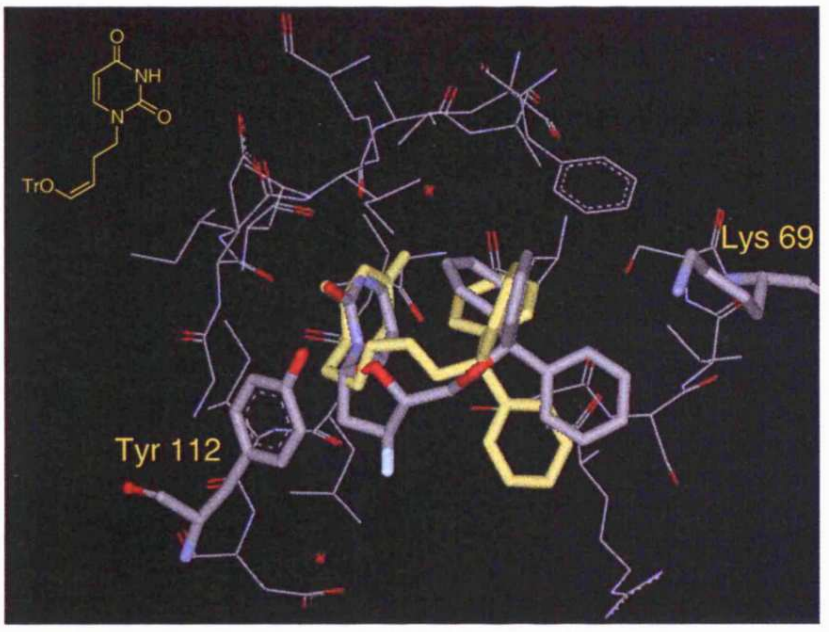
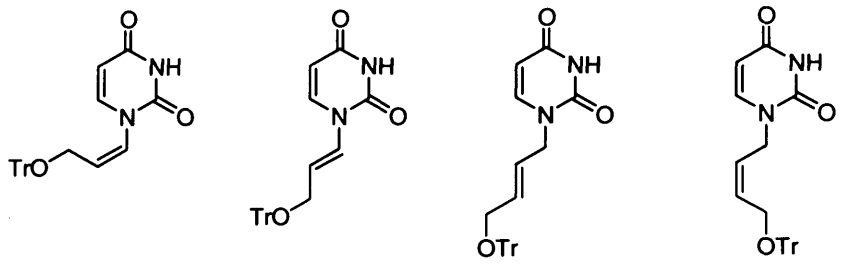


Figure 7-8 Docking of compound **68** (yellow) in the *P. falciparum* active site, superimposed with the inhibitor WSP 869 (grey) co-crystallised in the enzyme active site

Probably the best result for both score (-18.9) and binding interactions (Figure 7-8) was compound **68**. For this compound the trityl group is almost in the same position as the inhibitor WSP 869, the uracil ring is in the bottom of the active site pocket very well overlapped with the original ligand. In addition the carbon chain is mimicking the sugar backbone, in a flat position parallel to the phenyl ring of the tyrosine 112 residue (see Figure 7-8), and predicted to have a good interaction with it.

The other structures presented in Table 7-1 presented poor binding modes compared to the reference WSP869 co-crystallised with the protein. Some of them docked completely outside of the active site or the superimposition with the structure of WSP869 was not very good, due to a partial fitting in the active site. Therefore those results were not considered.

From the virtual screening study it was then decided to attempt the chemical synthesis of several targets; which are a compromise between the results from the modelling study and achievable chemical synthesis.

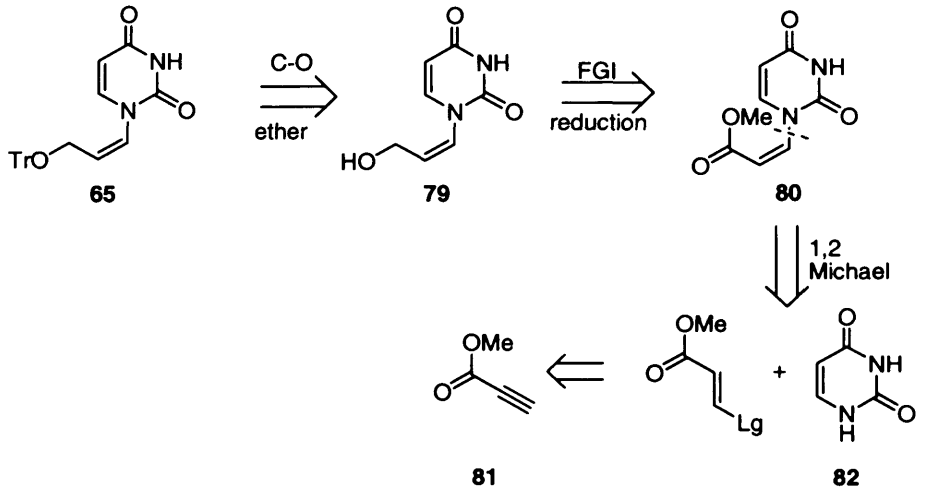


65 73 72 69

Figure 7-9 Chosen structures for chemical synthesis

7.2 Synthesis of unsaturated acyclic nucleosides

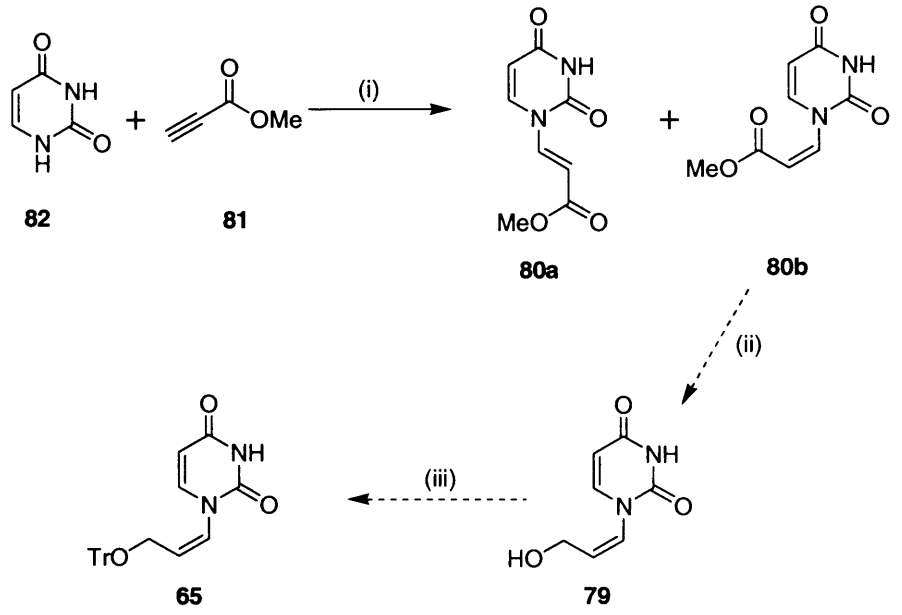
A first retrosynthetic approach to the synthesis of compound **65** is depicted in Scheme 7-1.



65 79 80 81 82

Scheme 7-1 Retrosynthetic analysis of *cis*-unsaturated nucleoside **65**

The retrosynthesis starts with a conversion of the trityl ether into the original alcohol **79**, followed by a functional group interconversion (FGI) to go back to the ester function in compound **80**. This interconversion allows the next disconnection between the uracil base and the first carbon of the chain, giving the commercially available starting materials uracil **82** and methyl propiolate **81**.



Scheme 7-2 (i) Caesium carbonate; DMF; r.t.; (ii) $LiAlH_4$, THF, -78 °C; (iii) TrCl, Imidazole, DMF

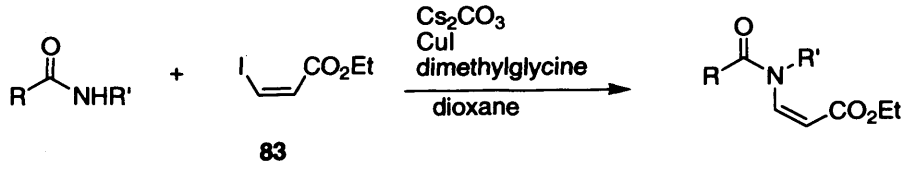
Redwane *et al.*[117] reported a Michael addition of nucleobases to an α-β unsaturated carbonyl group like the one present in the methyl propiolate **81**. This reaction was not regiospecific and the product was a mixture of *E* and *Z* alkenes which could be purified by chromatography. Although in literature this reaction required the use of K_2CO_3 as base and a mixture of 1,4-dioxane/DMF as solvent, in our case it was decided to use the same conditions developed in our group (Chapter 5) to couple the uracil with the aliphatic chain: caesium carbonate as base and DMF as solvent.

Methyl propiolate **81** was added to a suspension of uracil and Cs_2CO_3 in DMF at room temperature. Although two new spots at higher R_f were present on the TLC plate; some problems were encountered in the work-up of the reaction, when after washing the organic layer (EtOAc) the products were mainly found in the aqueous phase. Evaporation of the aqueous layer gave a residue which was purified by flash chromatography affording two fractions. The fraction with the higher R_f was the *bis*-alkylated product at the N1 and N3 positions in the uracil ring.

The fraction with the lower R_f was, instead, the *E* monoalkylated uracil **80a**. The configuration of the double bond was proved by measuring the coupling constant of the two doublets at δ 8.20 and δ 6.20 for the two ethylenic protons.

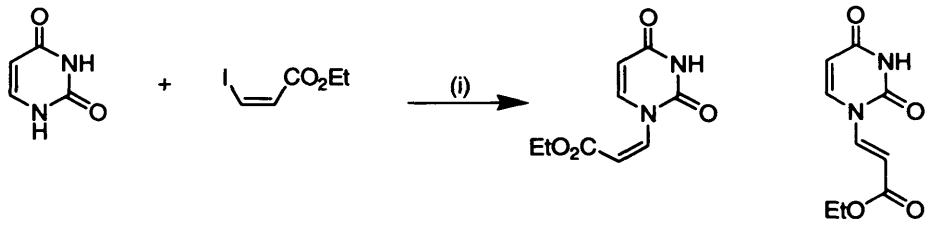
They were 14 Hz suggesting a *trans* configuration as reported in the literature[117]. No *Z* isomer **80b** was isolated.

As alternative strategy for the synthesis of the *Z* isomer was attempted following the procedure developed by Pan *et al.*[118] for the coupling of amides with vinyl halides **83**. This should retain the configuration of the double bond, (Scheme 7-3).



Scheme 7-3 Coupling of amides with vinyl halides as reported by Pan et al[118].

This is a Ulmmann type reaction catalysed by copper iodide and *N,N*-dimethylglycine. The idea was to use the amide function present in the uracil ring to exploit this reaction and to obtain the *Z* intermediate **84**.

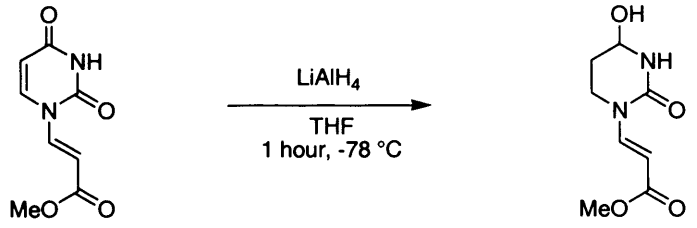


Scheme 7-4 (i): Caesium carbonate, dimethylglycine, CuI

Unfortunately the method did not work in the desired way and the only isolated compound, was again the *E* isomer **85**, in very low yield (13%) after chromatographic purification. This compound had the same NMR properties as **80a** synthesised from methyl propiolate **81**, except for the fact that **85** is an ethyl ester.

The synthesis was then carried on, to investigate the reactivity of the unsaturated ester with a reducing agent. The common procedures used to reduce an ester to the corresponding alcohol are with lithium aluminium hydride or with DIBAL-H (diisobutyl aluminium hydride). While the first reagent is a quite strong, the second one is slightly milder due to its more hindering substituents, and often is used to reduce an ester function to the corresponding aldehyde.

The following step would be the selective reduction of the ester. In theory, the ester function at the end of the chain should be more reactive and susceptible to reduction than the amide functions present in the uracil moiety. $LiAlH_4$ was investigated as reductive agent in the first attempt. The temperature was kept at -78 °C and only two equivalents of reducing agent were used. Nevertheless after 1 hour at -78°C the only product recovered was the reduced uracil ring with the ester and double bond functions un-touched **86**, (Scheme 7-5).



80a **86**

Scheme 7-5 Failed attempt of reduction of the ester function in compound **80a**

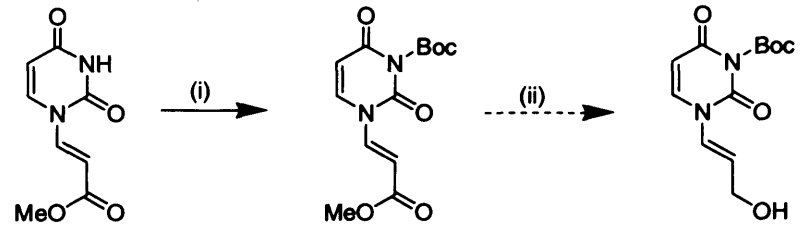
This was proven by NMR spectroscopy which showed the complete disappearance of the two doublets at δ 7.97 and δ 5.96 typical of the H-5 and H-6 of the uracil heterocycle. A new triplet was also present around δ 5, integrating for 1 proton and corresponding to the "new" hydrogen in position 4 of the uracil ring. The negative result of the reaction was also confirmed by ^{13}C-NMR and mass specrometry where there was only one peak at m/z 223 corresponding to $[M+Na]^+$ for compound **86**.

This partial failure could be attributed to the reducing reagent, which is perhaps too strong and un-hindered to give selectivity. Therefore in the second attempt the milder DIBAL-H was investigated. Compound **85** was dissolved in THF and cooled to -78 °C. DIBAL-H was added by a syringe.

TLC analysis after a few hours showed the complete disappearance of the starting material, and the presence of several spots, at higher and lower R_f compared to the starting ester, proving that the reaction was not clean. It was decided to carry on and try to isolate the compounds corresponding to the main spots. The reaction was quenched with water and filtered through a Celite pad. Unfortunately all the attempts to purify the crude residue failed, due to the complexity of the mixture and the low scale of the reaction. One of the hypotheses advanced was that the amide function of the uracil was interfering,

giving side reaction and creating a complex mixture which was impossible to purify.

In order to avoid these problems, it was decided to protect the amide function in position 3 of the uracil base. As a protecting group the classical *tert*-butylcarbamate (Boc) was chosen. It was introduced following the standard procedure reported in the literature[82], (Scheme 7-6).



80a **87** **88**

Scheme 7-6 (i): Boc anhydride, TEA, DMAP, THF, r.t.; (ii): DIBAL-H, THF, -78 °C→ r.t..

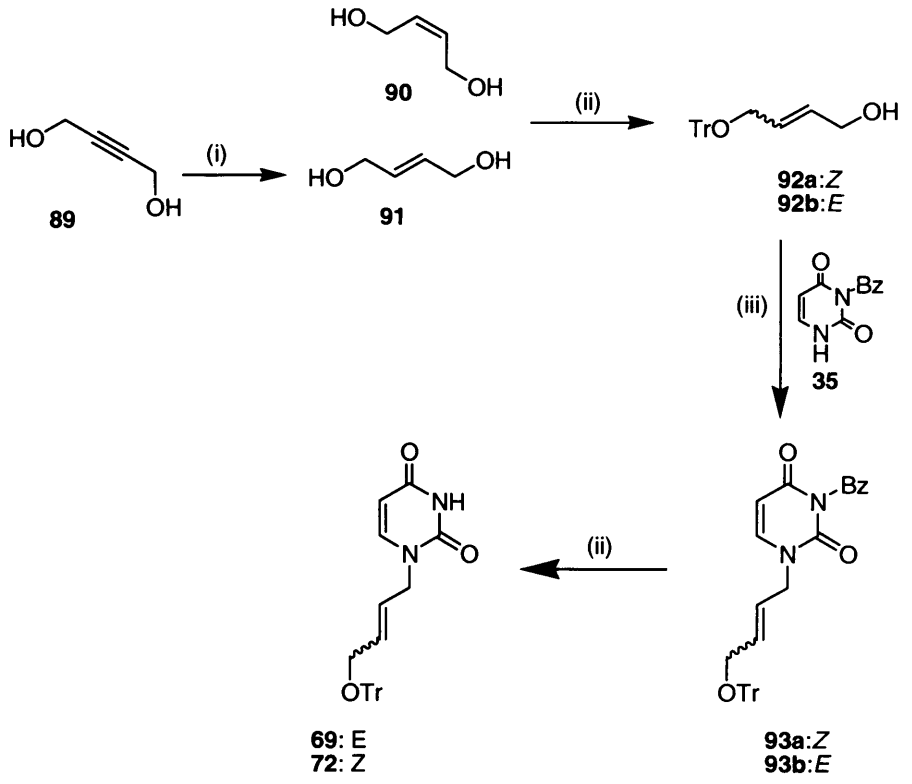
Compound **80a** was treated with Boc anhydride in THF, in the presence of TEA and catalytic amount of 4-(*N*,*N*-dimethylamino)pyridine (DMAP). After the work-up and the chromatographic purification compound **87** was obtained in 86% yield. The presence of the Boc group was confirmed by ^{1}H-NMR with a singlet at δ 1.67 for the *tert*-butyl group, and in the carbon with three more signals at δ 159, 88 and 27 corresponding to carbonyl, the quaternary carbon and the three methyl groups respectively.

The protected intermediate **87** was then treated following the same procedure described earlier for the reduction with DIBAL-H. TLC of the mixture after 24 hours still showed the presence of starting material and another equivalent of DIBAL-H was added. The mixture was stirred for a further 12 hours; again several new spots were present on the TLC plate. Due to the small amount of crude residue (20 mg) obtained from the work-up, it was decided to purify it by preparative TLC. Unfortunately the purification did not succeed and the spots ran very close to each other creating a long tail on the TLC plate. The attempt to scratch the main spot, afford a tiny quantity of a residue (5 mg) which was not possible to identify by qualitative analysis (NMR and mass spectrometry).

Since the failures in the synthesis of the targets **65** and **66**, the chemistry was then focused on the synthesis of the other two targets with longer carbon chains

69 and **72**. The synthesis of these structures seemed to be more achievable, since they could be obtained using the Mitsunobu coupling developed by Hernandez and co-workers[99], and already described in the earlier chapters.

The chosen starting materials were the N3-benzoyl uracil **35**, and the two diols but-2-yne-1,4-diol **89** and *(Z)*-but-2-ene-1,4-diol **90**, (Scheme 7-7).



Scheme 7-7 (i): LAH, THF, -78 °C. (ii): TrCl, TEA, DMAP, DCM. (iii) DEAD, PS-PPh_3, THF. (iv): MeONa, MeOH.

As the *(E)*-but-2-ene-1,4-diol **91** is not commercially available, it was prepared from the corresponding alkyne **89**. This was obtained according to the classical reduction of triple bonds of propargylic alcohol with lithium aluminium hydride (LAH), which gives alkenes in *E* configuration[119]. LAH was added to a cooled solution of the alkyne and stirred for three hours. After chromatography the desired alkene was obtained in 79% yield. Although the NMR spectrum was not very resolved, it was consistent with the required structure with a broad singlet at δ 5.87 (2H) and a doublet at δ 4.07 (4H) in deuterated MeOH. Low resolution mass analysis confirmed the addition of two hydrogens with a peak at m/z 111.0 corresponding to the ion $[M+Na]^+$.

The next protective step was conducted in a parallel way on both diols **90** and **91**. This was achieved according to the general procedure[82] for the tritylation of primary alcohols. To minimize the formation of diprotected derivatives, the two diols were treated with a half equivalent of trityl chloride in the presence of TEA and DMAP as bases. The *E* substrate **91** required a shorter reaction time and after 3 hours the reaction went to completion, according to the TLC; while the *Z* substrate **90** was left stirring overnight. The same work-up was applied for both reactions and the two desired compounds **92a-b** were obtained in 71% and 81% yields, respectively. The two tritylated isomers had different R_f, and different spectral properties. The proton NMR of the *E* isomer **92a** showed, even if not completely solved, two double triplet at δ 6.07 (J_1=15.5 Hz, J_2=5.4Hz) and 5.87 (J_1=15.5 Hz, J_2=4.9 Hz) for the two hydrogens of the double bond, whilst the second regioisomer **92b** had the two protons of the double bond collapsed in a multiplet around δ 5.80. In the carbon spectra no significant difference could be spotted; the chemical shift for the sp^2 carbons were at δ 130.5 in the *E* isomer **92a** and at δ 131.5 in the *Z* **92b,** however the two sp^3 carbons of the chain were shifted to higher field in the *Z* isomer (δ 60.6 and 59.3) compared to the *E* (δ 64.5 and 63.8).

The following step was the coupling of the tritylated substrates **92a-b** with the benzoyluracil **35**. The same procedure described in Chapter 5 was applied, with DIAD and polymer supported triphenylphosphine. Both the reactions were shaken overnight at room temperature and the two desired compounds were obtained with not very high yields (48% for the *Z* **93a** and 29% for the *E* **93b)**. The ^{1}H-NMR of **93b** was very clear (top spectrum in Figure 7-11) showing two new doublets at δ 7.9 and δ 5.7 corresponding to the H-5 and H-6 of the uracil base, and the other two ethylenic protons split into two double triplets (dt) at δ 6.09 (dt, J_1=11.0 Hz, J_2=6.5 Hz), and δ 5.68 (dt, J_1=10.9 Hz, J_2=5.7 Hz) due to the coupling with the neighbouring hydrogens as shown in the coupling pattern in Figure 7-10.

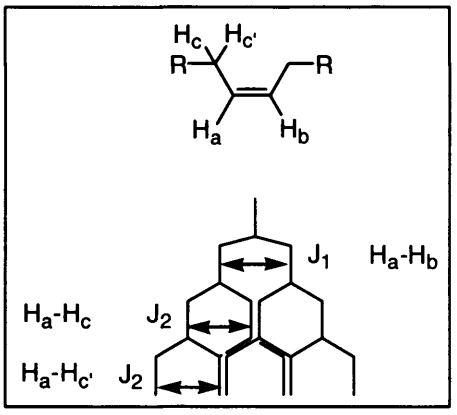


Figure 7-10 Coupling pattern for the ethylenic proton with adjacent methylenic group

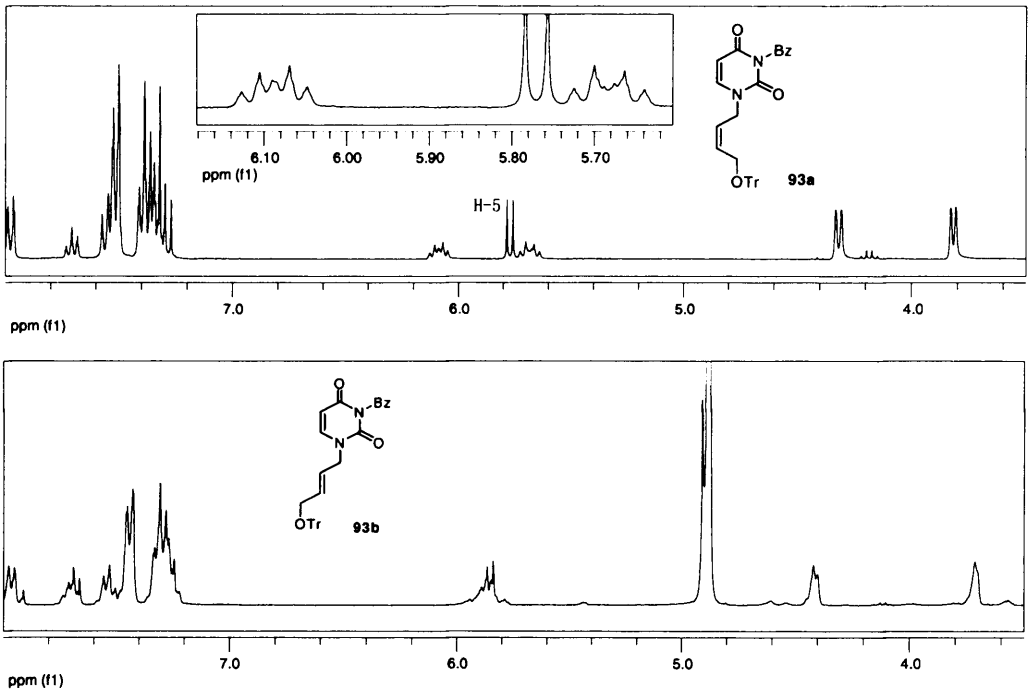
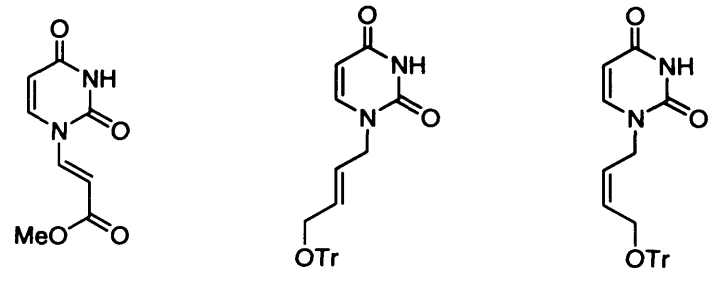


Figure 7-11 ^{1}H-NMR for compound **93a** (bottom spectrum) in $CDCl_3$ and compound **93b** (top spectrum) in deuterated methanol

The *Z* regioisomer presented, instead, a complex multiplet around δ 5.85 corresponding to the H-5 of the uracil ring together with the two ethylenic protons, as shown in the bottom spectra of Figure 7-11.

In the final step, the cleavage of the benzoyl group from the uracil ring, was achieved using sodium methoxide in methanol. Despite the long time for the reactions (24 hours) at room temperature they worked reasonably well and compounds **68** and **70** were obtained in very good yield (90%). The ^{1}H-NMR spectra presented a lower integration (15H) in the aromatic region, corresponding

to the three phenyl rings of the trityl group, confirming thus the cleavage of the benzoylamide for both compounds. The two ethylenic protons again formed a multiplet around δ 5.88 for compound **72**, next to the doublet at δ 5.77 for the H-5 of the uracil base. The *E* isomer **69**, instead, showed a multiplet with a structure similar to a double triplet at δ 6.05, but not sufficiently resolved to allow the measuring of the *J* values. This multiplet integrated for 1 proton, corresponding to one of the two CH of the double bond (presumably the one closer to the oxygen); another multiplet was present around 5.25 ppm, integrating for 2 protons, corresponding to the second CH of the ethylenic group plus the H-5 of uracil base (Figure 7-12).



80a **72** **69**

Figure 7-12 Three unsaturated acyclic uracil derivatives synthesised and sent for testing

The two compounds also had different melting points with 65-68 °C for compound **72** and 70-72 °C for compound **69**. The mass spectrum showed the presence of a peak at m/z 447.2 for ion $[M+Na]^+$ in both cases.

Concluding, only the synthesis of two targets (**69** and **72**) was achieved, problems were encountered in the synthesis of the shorter analogues **65** and **66**. The three compounds illustrated in Figure 7-12 were sent for biological evaluation, even though compound **80a** was an intermediate, and due to the absence of the trityl moiety as terminator poor biological activity was expected.

7.3 Branched acyclic nucleosides

To increase the chemical diversity in the modification of the acyclic WSP870 lead compound, it was decided to explore the effects of extension in the aliphatic chain of the spacer (Figure 7-13). In particular making branched chain analogues as found in penciclovir and famciclovir (Figure 7-14). All these branched chain analogues or derivatives of them might have additional interactions in the enzyme active site.

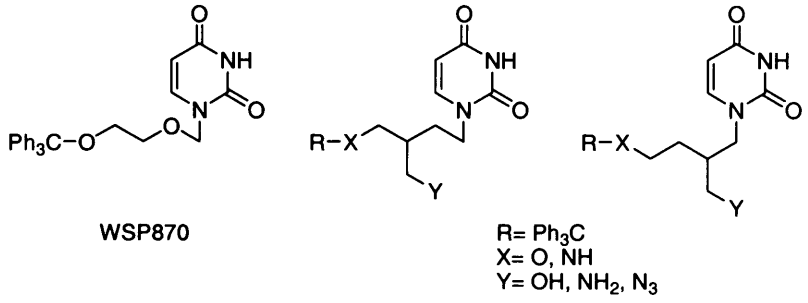


Figure 7-13 Lead compound WSP870 and proposed branched structures.

Such branched structures should partially mimic the backbone of the 2'-deoxyribose in the natural substrate dUTP and also allows different combinations of functional groups which can, hopefully, form additional interactions within the enzyme active site.

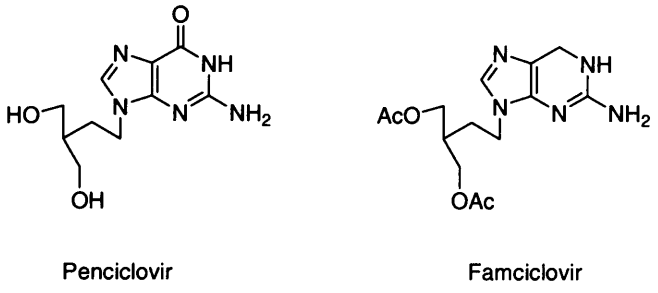


Figure 7-14 Examples of branched acyclic nucleosides pencivlovir and famciclovir

This part of the project started from some intermediates kindly provided by our partner Medivir. The structures are illustrated in Figure 7-15.

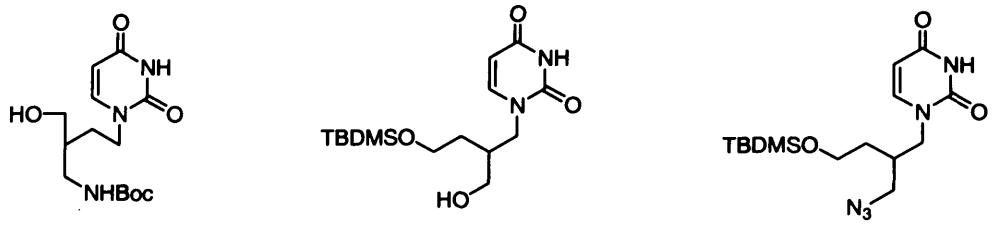


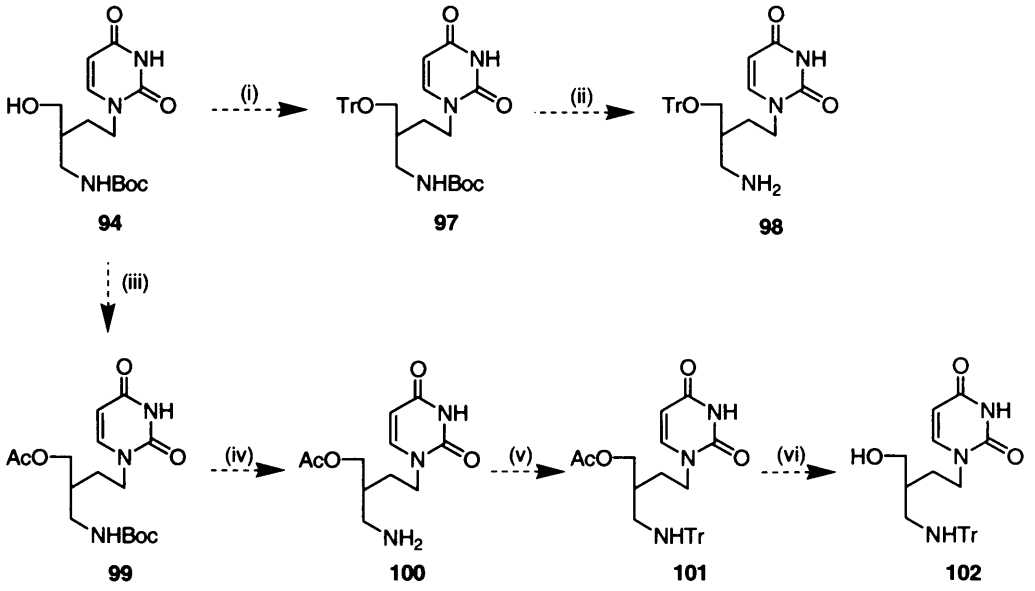
Figure 7-15 The three branched acyclic intermediates provided by Medivir.

As the three reported structures have one chiral centre each, it was decided, at this early stage of this part of the project, to ignore the problem of the stereochemistry. Thus the provided compounds were used as mixtures of the two enantiomers and no attempt was made to separate them. Should the compounds have potent activity then attempts will be made to test the individual enantiomers.

The work on this family of compounds will be divided in two series according to the two main structures of the branched aliphatic chain. However the synthesis and the attempted synthesis were carried out simultaneously since the same procedures were applied to different substrates (i.e. tritylation of alcohol, deprotection and so on) using a Radley 12 tube carousel apparatus.

7.3.1 First series of acyclic derivatives

The intermediate **94** could be converted into either the trityloxy derivative **98** or the corresponding tritylamino compound **102**, (Scheme 7-8).



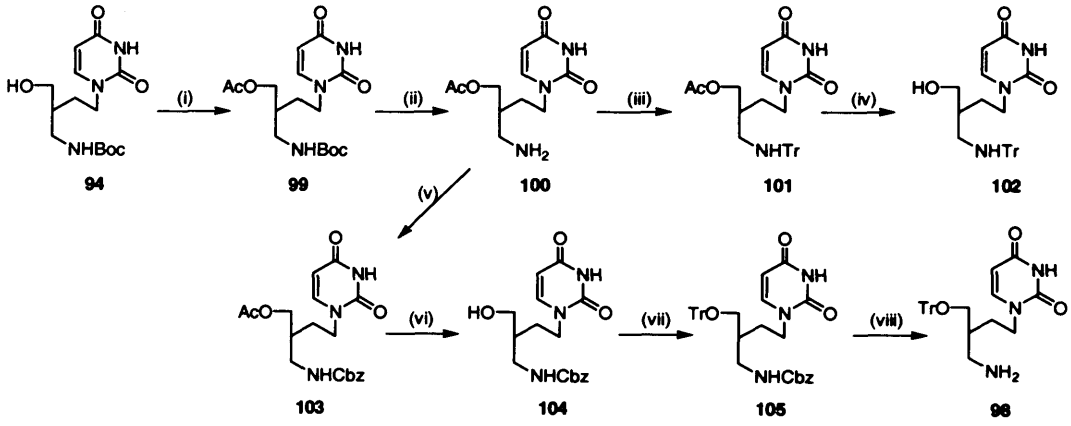
Scheme 7-8 Proposed modification for the intermediate **94** (i): TrCl, base; (ii): TFA, DCM; (iii): Ac_2O, Py; (iv): TFA, DCM; (v): TrCl, base; (vi): MeONa, MeOH.

The two targets could be obtained using a multistep synthesis made of a series of protections and deprotections of orthogonal protecting groups. The introduction of the trityl moiety to obtain compound **97** was not as simple as expected. In the first attempt using the standard procedure with trityl chloride and TEA in DCM, the TLC still showed presence of the starting material after stirring the reaction 24 hours at room temperature. Some more trityl chloride was added and the reaction was stirred for further 48 hours. Unfortunately after chromatography only unreacted starting material was recovered, as the proton NMR did not show any presence of aromatic protons around δ 7.5. Changing solvent and base, and heating the reaction gave a slightly better result. The trityl chloride was added to a solution of the starting intermediate **94** in pyridine, and the mixture was warmed to 100 °C for 48 hours. TLC then showed the almost complete disappearance of the starting alcohol and a new spot at higher Rf for the forming product. Nevertheless the desired compound **97** was isolated only with a 40% yield, proving the reaction was still far from completion. However the protected alcohol **97** was obtained quite pure as the NMR demonstrated with a clear doublet at δ 7.48 integrating 6 protons, corresponding with the *para*-hydrogens of the three phenyl rings; another multiplet around δ 7.35, integrating to 10 protons, corresponding to the remaining aromatic protons plus the H-6 of

the uracil base and finally the singlet at 1.47 ppm for the *tert*-butyl group of the Boc, integrating for 9 protons.

To improve this synthetic step, it was decided to exploit the advantages of using microwaves in organic chemistry. It is well known[120] that microwave irradiation can help to reduce dramatically the reaction time, and can also increase the reactivity of poorly reactive substrates. This is due to the more efficient heating of the system by the microwaves which are absorbed by solvent and reagent having a dipole moment[120].

The same tritylation of the intermediate **94** was repeated in pyridine using 3 cycles of microwave irradiation at 140 °C for 5 minutes. After such short periods of time the spots on the TLC where much more intense for the formed product and fainter for the starting material, compared to the spots on the TLC for the classical reaction. In fact, following the same chromatographic purification the desired product was obtained in 79%.



Scheme 7-9 (i): Ac_2O, Py, r.t. 3h; (ii): TFA, DCM, r.t; (iii): TrCl, TEA, DCM, r.t.; (iv): MeONa, MeOH, r.t. 2-3 h; (v): Cbz-Cl, TEA, DCM; (vi): MeONa, MeOH, r.t.; (vii): TrCl, Py, 80 °C, 24 h; (viii): H_2, Pd/C 5%, EtOH/MeOH, 8h.

The selective cleavage the Boc group was attempted with trifluoacetic acid (TFA). It was expected that the triyl group might have been unstable to the usual conditions required for the deprotection of a Boc carbamate (TFA 10% in DCM), since there has been reported a deprotection of trityl ethers by trifluoroacetic acid in the presence of silica[121].

Therefore, it was decided to use a diluted solution of TFA 5% in DCM. Although the TLC showed only one spot with lower R_f when the tritylated

intermediate **97** was stirred at room temperature for 30 minutes; the isolation of the compound by column chromatography showed that it was not the desired product. NMR analysis proved that during the reaction the trityl group was cleaved, while the Boc was still present. This was clearly visible due to the total absence of any aromatic protons around δ 7.5 and with the typical singlet at δ 1.5 for the *tert*-butoxycarbamate.

Since the selective cleavage did not seem achievable, a new approach to compound **98** was envisaged. As reported in Scheme 7-9, the desired target could be synthesised by replacing the Boc protecting group with a Cbz protecting group. This procedure shares the first two steps with the synthetic route to the second target, the tritylamino derivative **102**. Consequently the synthesis of both targets was carried out in parallel.

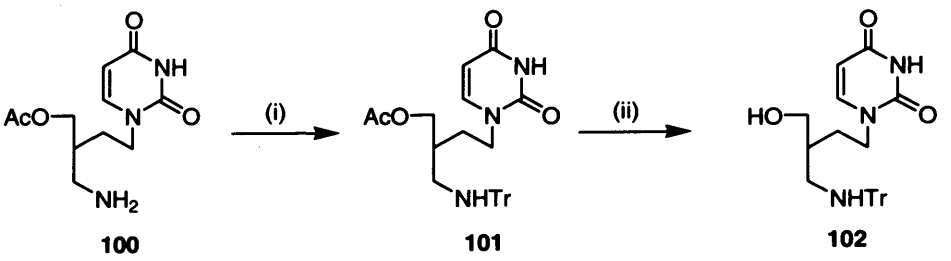
The acetyl ester is widely used as protecting group of alcohols in many field of organic chemistry from sugars to nucleoside chemistry. It can be introduced with several different methods from acidic to basic conditions, using the acetic anhydride or the corresponding chloride. Acetyl esters are stable in the conditions used to remove the Boc group, and they can be cleaved by simple basic hydrolysis. The cleavage conditions would thus be compatible with the presence of a trityl group in the molecule.

In the first step the acetylation of the hydroxyl group was easily obtained with acetic anhydride in pyridine[82], to give the acetyloxy derivative **99** in 88% after chromatography. The structure was verified by ^{1}H-NMR which showed the singlet, integrating for 3 protons, at δ 2.11 corresponding to the methyl group next to the carbonyl of the acetate.

The next step was cleavage of the Boc carbamate. This time the solution of TFA 10% in DCM worked without problems, in 3 hours the complete disappearance of the starting material was observed on TLC and only one new spot with lower R_f was present. No work-up was carried out and the crude compound **100**, obtained by evaporation of the solvents, was directly used for the next steps; there was no sign of the singlet corresponding to the *tert*-butyl group in the ^{1}H-NMR. The intermediate **100** was then used for both synthetic routes.

Triphenylmethylamino alcohol (102)

The tritylamino compound **102** required two further steps, one to introduce the triphenylmethyl moiety and the second to cleave the acetyl ester, releasing the free hydroxyl group in the pseudo 5' position, (Scheme 7-10).



100 **101** **102**

Scheme 7-10 (i): TrCl, TEA, DCM, r.t. 6h (ii): MeONa/MeOH, r.t. 2h

Amines are more nucleophilic than alcohols, hence the conditions applied for the tritylation of the intermediate **100** were slightly milder than those used for the synthesis of the trityloxy derivative **97**. Triethylamine was chosen as base and dichloromethane as solvent, which is the general method to tritylate amine[82]. Further addition of trityl chloride was required. After 24 hours at room temperature the product was obtained in 70% yield. The NMR was very clear and showed all the couplings between the methylenic protons in the structure of compound **101**. Low resolution mass spectrometry by chemical ionization (CI) showed the required peak at m/z 498 for the molecular ion $[M+H]^+$.

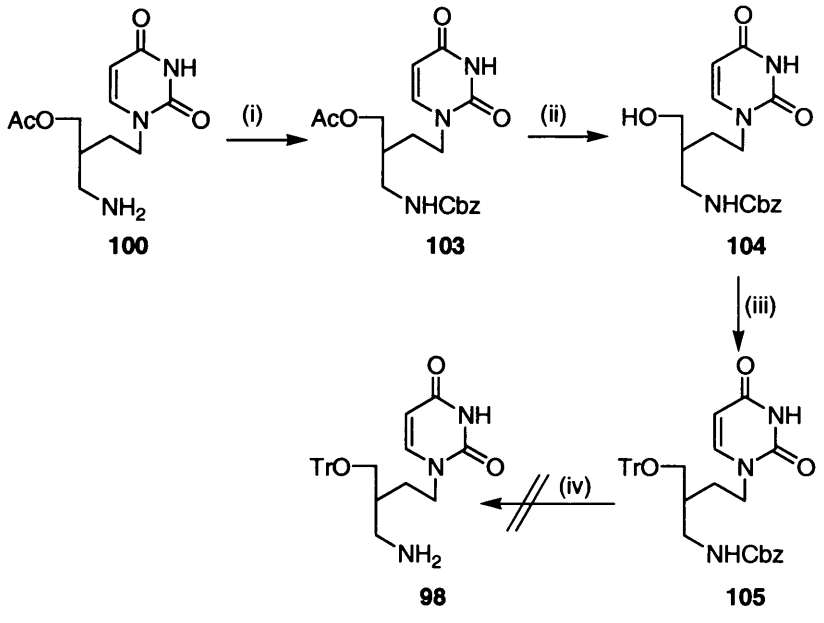
The last step of this synthesis was the hydrolysis of the acetyl group on the OH. Although the most common way used to cleave the acetyl group is perhaps with aqueous bases[82] (i.e. K_2CO_3 in $MeOH/H_2O$), it was decided to use the same procedure applied for the cleavage of benzoyl esters, with sodium methoxide in methanol. This is because the alkoxide base would form the corresponding methyl acetate which is a quite volatile compound (bp 57° C) and could therefore be eliminated by reduced pressure, simplifying the final purification. The second by-product of the alcohol would be the slight excess of sodium methoxide that can be neutralised by Dowex ion exchange resins (H^+ form), and the remaining traces of sodium salts could be removed by a short flash chromatography.

The acetylated intermediate **101** was dissolved and stirred at room temperature in a 2M solution of sodium methoxide in methanol. Monitoring the reaction by TLC showed after a few hours, no starting material was left, and only one spot

with lower R_f was present on the silica plate. The solution was neutralised with ion exchange resin, by simple addition of pre-washed resin into the flask and stirring for few minutes. The pH of the solution was checked with pH paper and when it was 7, the reaction was filtered, concentrated and purified by chromatography to produce the target compound **102** in 99% yield. The product was characterised by ^1H, ^{13}C-NMR, low and high resolution mass spectrometry which were all consistent with the structure required.

Unfortunately the microanalysis was quite different than the theoretic values, suggesting that some inorganic impurity was present. The probable contaminant was sodium chloride; the chloride could have arisen from the trityl chloride used for the tritylation and the sodium from the NaOMe. Unfortunately insufficient sample was left for a further microanalysis which could confirm the conclusion. On the other hand NaCl should not affect the eventual biological test due to its low toxicity and the compound **102** was biologically tested anyway.

Triphenylmethoxy amino derivative (98)



Scheme 7-11 (i): CbzCl, TEA, DCM, 0 °C→r.t.; (ii): MeONa, MeOH, r.t.; (iii): TrCl, Py, 80 °C, 24h; (iv): H_2, 5% Pd/C, EtOH, MeOH.

In the synthesis of the trityloxy target **98** it was decided to use the benzyl carbamate (Cbz or Z group in honour of L. Zervas its developer). Carbamates

have found wide application in peptide chemistry; several different kinds have been developed[82]. The benzylcarbamate is cleaved by hydrogenolysis and is stable in acidic and basic conditions.

The protection of the amino group was followed by hydrolysis of the acetate ester **104**, introduction of the triphenylmethyl group **105** and final hydrogenation to get the free amine **98**, Scheme 7-11.

The Cbz-protected amine **103** was obtained starting from benzylchloroformate and triethylamine in DCM. The reaction worked well and the desired compound was obtained in 53% yield after chromatography. The proton NMR of the product showed a multiplet in the aromatic region, and the classical singlet at δ 5.06 corresponding to the benzylic CH_2 of the carbamate moiety. In the mass spectrum there was only one peak at m/z 389.3 corresponding to the molecular ion $[M]^+$.

The cleavage of the acetyl ester was achieved following the same procedure as described earlier in the synthesis of compound **102**. With similar success the intermediate **104** was isolated in 94% yield. NMR and mass spectra were clear and in accordance with the desired structure.

On the basis of previous experience, the tritylation of the pseudo 5' position was performed using trityl chloride in pyridine and heating the reaction at 80 °C overnight. The attempt was successful and the trityloxymethyl derivative **105** was obtained in good yield (69%).

Finally the last step of the synthetic pathway required the cleavage of the Cbz group from the amine function in the pseudo 3' position of the branched chain. As mentioned before this can be done by hydrogenation with a metal catalyst (e.g. Pd-C, $CaNi_5$) in the presence either of hydrogen gas or a hydrogen donor such ammonium formate, cyclohexane, formic acid.

Since the trityl group can be sensitive to hydrogenation, in our case it was decide to use Pd/C 5% with hydrogen gas. Many solvents are compatible with hydrogenation reactions. Generally the most used is methanol, but again due to the problems experienced during the synthesis of the 5'-trityl-3'-amino-2'-deoxyuridine **56** (Chapter 6) suggested use of a mixture of ethanol and methanol, since with these solvents the trityl group seemed to more stable. The reaction was carried out applying a slight pressure of hydrogen provided by a balloon.

After 12 hours the spot corresponding to starting carbamate had much lower intensity, therefore the reaction was stopped in order to avoid unwanted side reactions like the cleavage of the trityl group. The catalyst was separated by filtration and the crude residue was purified by chromatography.

Unfortunately NMR analysis of the fraction corresponding to the spot close to the baseline of the TLC was a mixture. Almost all the expected signals for compound **98** were duplicated, as shown in Figure 7-16.

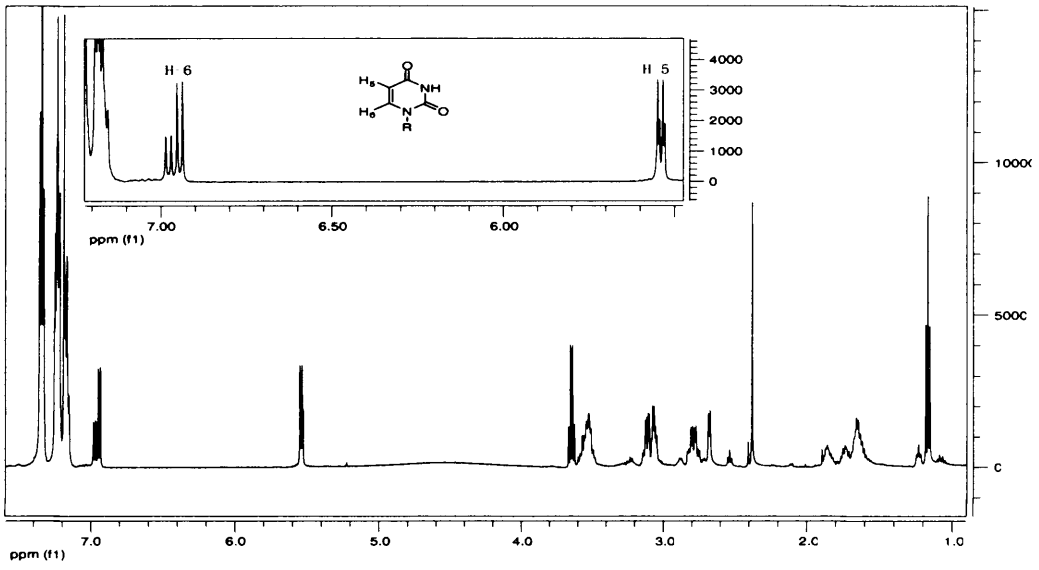


Figure 7-16 ^{1}H-NMR of compound **98**. In the expansion the H-5 & H-6 of the uracil ring are highlighted.

The ^{13}C experiment also presented double signals. A deeper analysis by TLC of the isolated compound revealed that the spot observed close to the baseline (using only chloroform/MeOH 90:10) consisted of two spots very close each other (using chloroform/MeOH/TEA 90:9.9:0.1). Unfortunately all the attempts to separate the two spot failed due to the small amount of sample available and the little difference in the R_f of the compound ($\Delta R_f < 0.1$). However the low resolution mass spectrum presented only one peak for the ion $[M+Na]^+$, therefore the hypothesis was that the product was a mixture of trityiloxy and tritylamino derivatives, probably due to a migration of the group from the hydroxyl to the amino group.

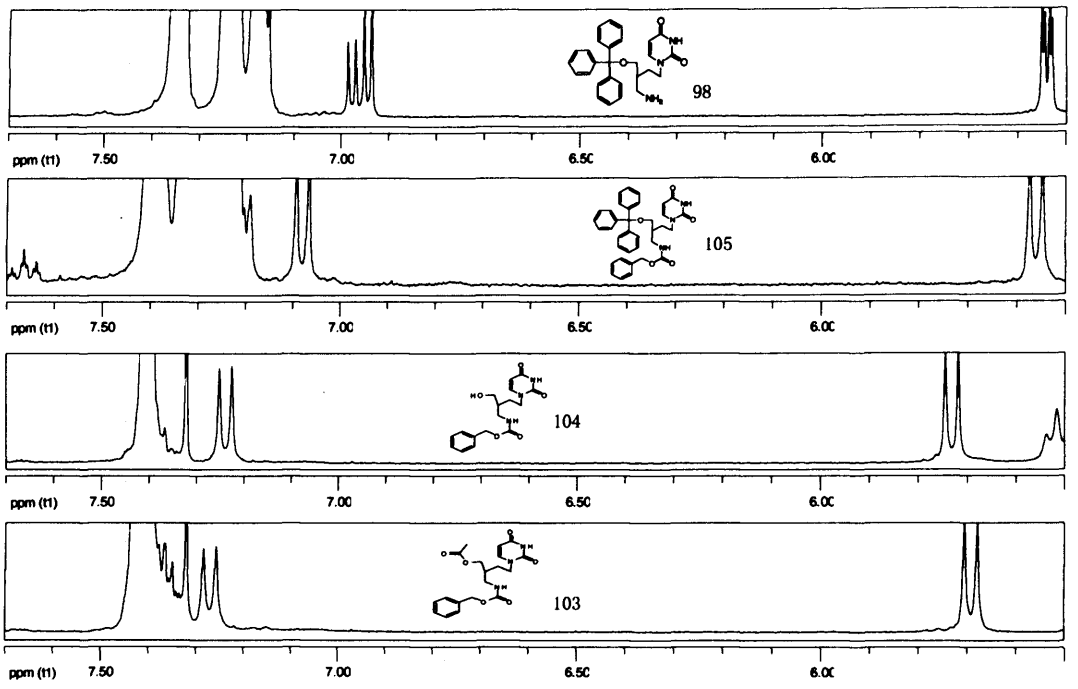


Figure 7-17 Comparison of the ^{1}H-NMR spectra for compounds **103, 104, 105** and the mixture **98**

7.3.2 Second series of acyclic uracil derivatives

This section will discuss the modifications made on the remaining two acyclic intermediates **95** and **96**, (Figure 7-18).

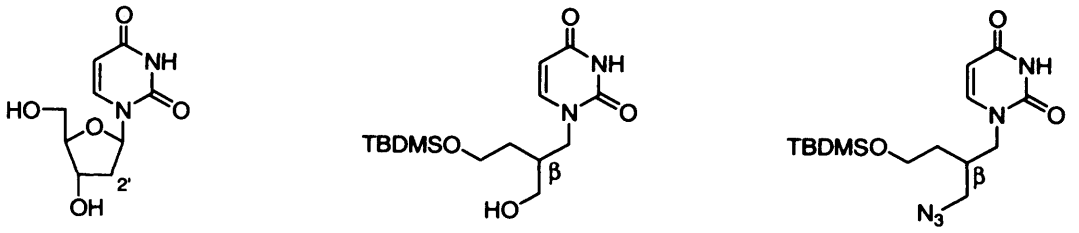
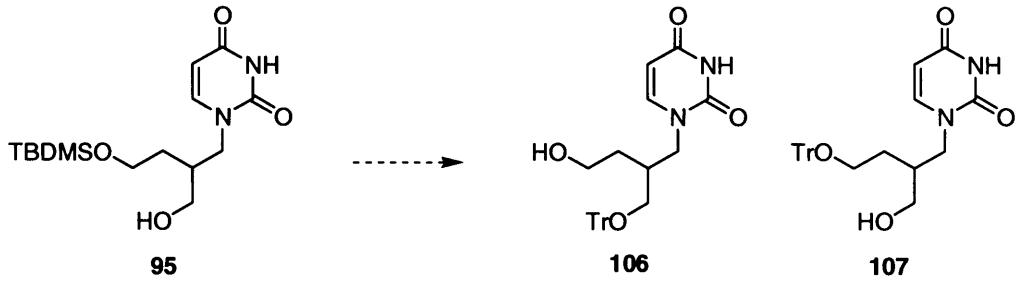


Figure 7-18 2'-deoxyuridine (left) and the two acyclic derivatives **95** and **96**

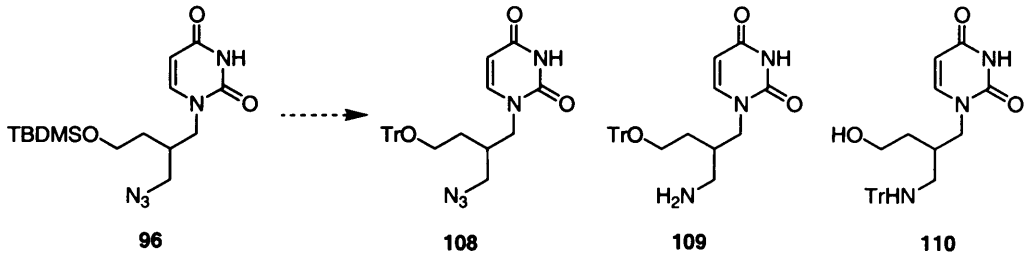
As shown in Figure 7-18 these compounds have an extension on the β carbon which could mimic the 2'-position of the corresponding 2'-deoxyuridine.

As described earlier the idea was to introduce a triphenylmethyl moiety into these compounds in order to complete the series of branched acyclic derivatives, thus intermediate **95** could be used to synthesise the two targets **106** and **107**, (Scheme 7-12).



Scheme 7-12 Possible targets that can be obtained from the intermediate **95**

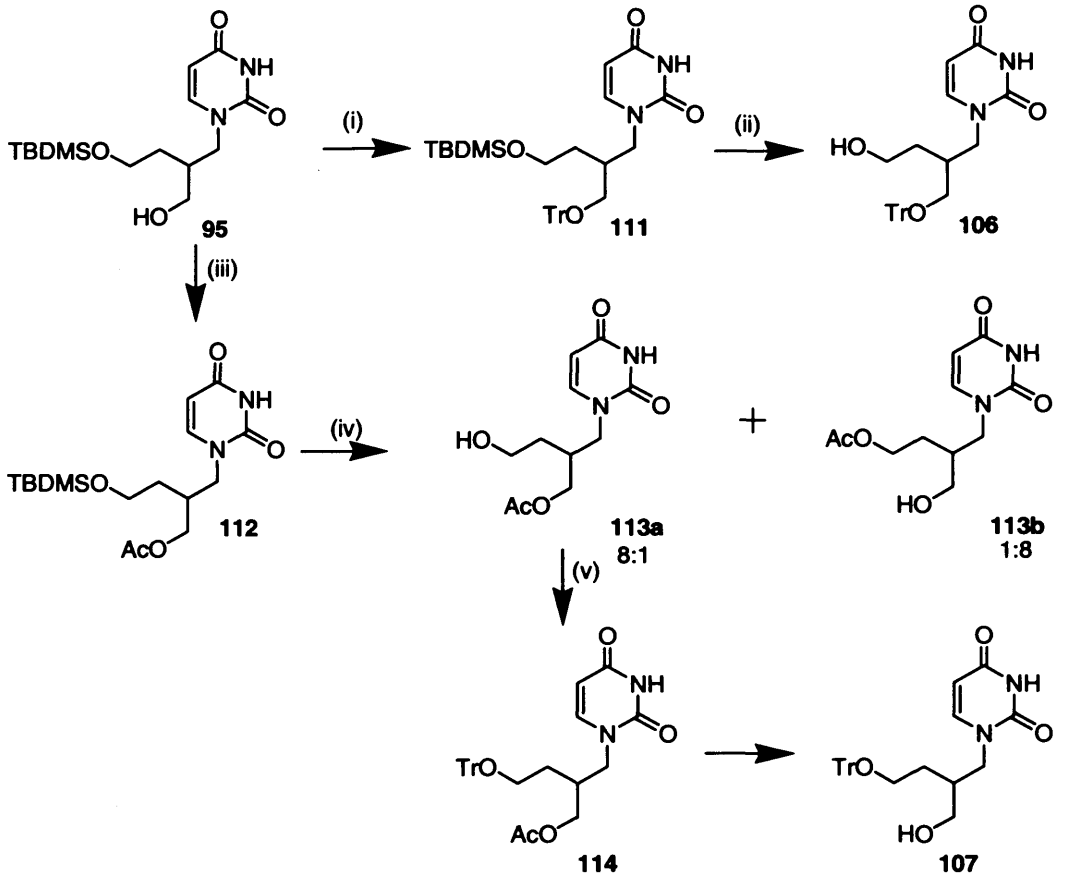
In the same way the intermediate **96** could be converted in the trityloxy-azido derivative **108**, the trityloxy-amino **109** and the tritylamino-hydroxyl **110** (Scheme 7-13).



Scheme 7-13 Proposed targets that can be obtained starting from the intermediate **96**

Triphenylmethyl derivatives (106) and (107)

Compound **95** was split in two tubes and left to react with either trityl chloride in pyridine or acetic anhydride in pyridine as solvent (Scheme 7-14).



Scheme 7-14 (i): TrCl, Py, 80 °C, 12h; (ii): TBAF, THF, r.t.; (iii): Ac_2O, Py, r.t.; (iv): TBAF, THF, r.t.; (v): MeONa/MeOH, r.t.

The tritylation required stirring and heating overnight at 80 °C and gave the desired product **111** in 79% yield after chromatographic purification. The acetylation did not require heating and the reaction time (3 hours) was shorter than the corresponding tritylation. The intermediate **112** was obtained in 67% yield, without further purification.

Both compounds **111** and **112** were then treated with TBAF in THF at room temperature. After stirring the reactions overnight the starting materials had completely reacted, as shown by TLC. Evaporation of the solvent and chromatographic purification afforded the target trityloxy/hydroxyl derivative **106** in only 47% yield.

The substrate **112**, instead, gave a mixture of products which gave two separate spots with lower R_f compared to the starting material. Fortunately it was possible to isolate the two compounds by chromatography. Spectrometric analysis of the two fractions gave the spectra illustrated in Figure 7-19.

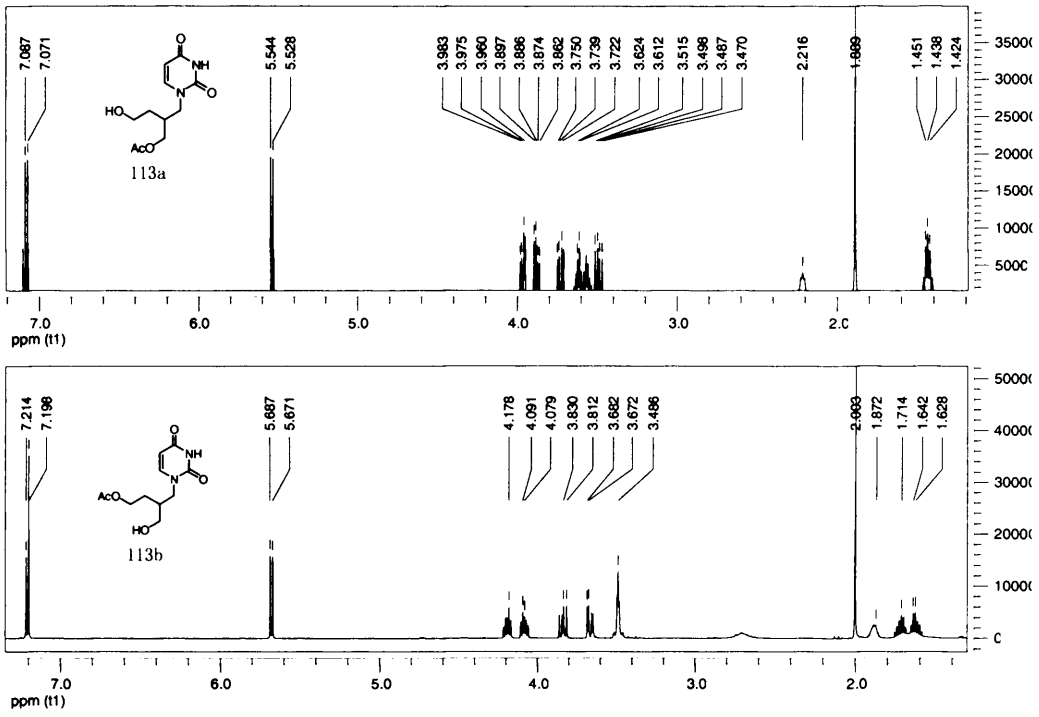


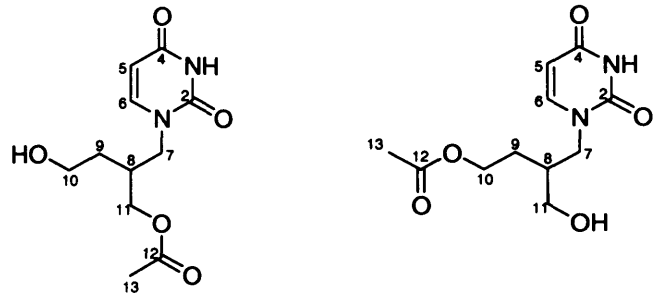
Figure 7-19 Proton NMR spectra for compounds **113a** (top) and **113b** (bottom)

Interpretation of the spectra led to the conclusion that the two components of the mixture were the regioisomers **113a** and **113b**; due to the probable migration of the acetyl group in the reaction conditions.

One of the key signals for the interpretation of the spectra was the multiplet corresponding to the chiral centre of the molecules (H number 8 in Figure 7-20). In the top spectrum (Figure 7-19), this multiplet is around δ 2.2, while in the bottom spectrum it is shifted to a higher field (δ 1.8). Since in compound **113a** the chiral centre is closer to the acetyl group than in compound **113b**, it is more likely that its chemical shift is the one at lower field (δ 2.2) and therefore the top spectrum should correspond to the structure **113a**. This was also confirmed by the fact that the same chemical shift (δ 2.25) for the H-8 was observed in the starting material **112**, thus confirming the assignment. On the other hand, in the structure **113b** the acetyl group in the pseudo 5'-position (now called 10 in Figure 7-20)

should exploit its de-shielding effect on the CH_2 (named 9 in Figure 7-20) adjacent to the chiral centre. This was clearly visible in the bottom spectrum where the two methylenic protons (H-9) were split in two multiplets around δ 1.7 and 1.6. In the top spectrum, instead, the same methylene group gave a multiplet at higher field (δ 1.4), according to the structure **113a**. Consequently the structure **113b** was attributed to the bottom NMR in Figure 7-19.

Moreover the main product, with 80% yield, was the compound **113a**, whilst the structure **113b** gave a total yield of 10%.



113a **113b**

Figure 7-20 The structures of the two regioisomers **113a** and **113b**

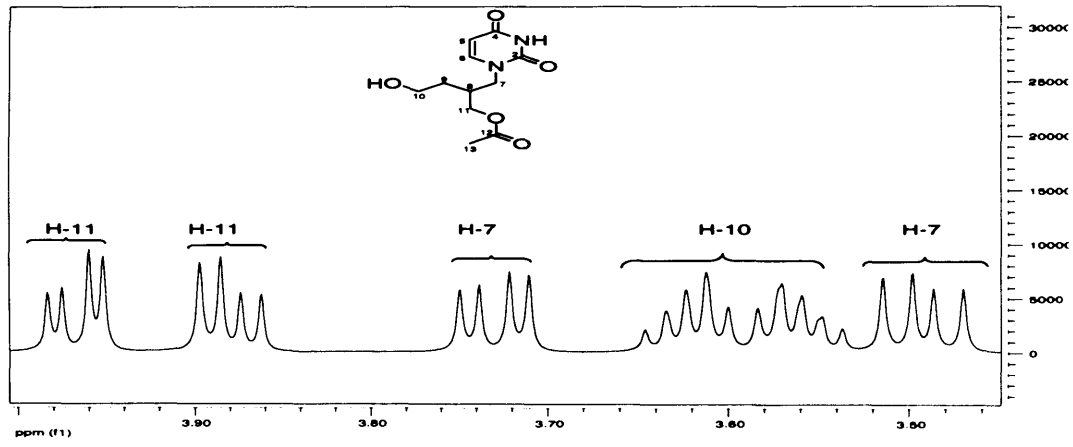


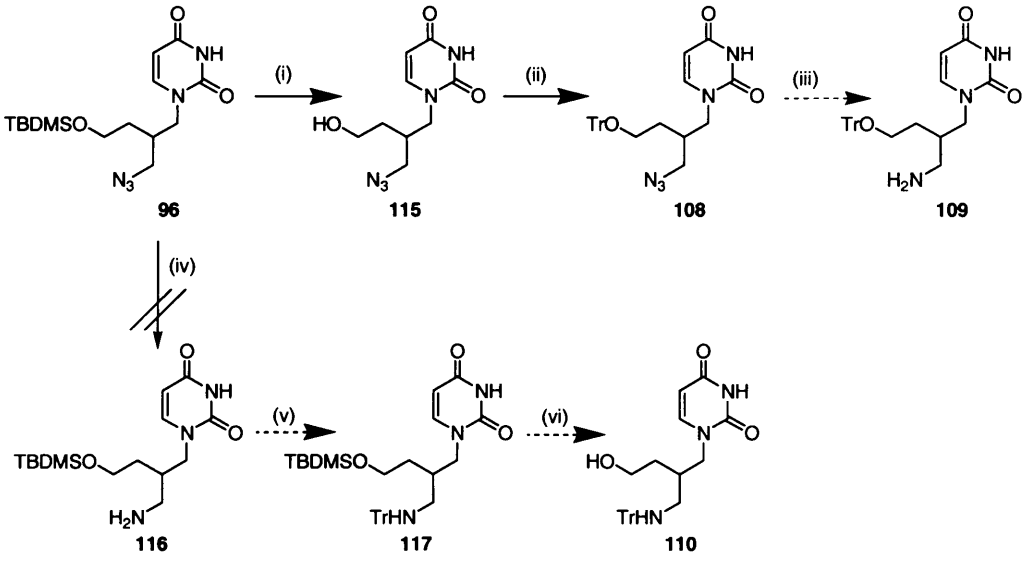
Figure 7-21 Expansion of the spectrum for compound **113a**, with further assignments of the signals to the structure. For the full interpretation with *J*s values see experimental section

Continuing with the synthesis the intermediate **113a** was tritylated with the usual conditions. The reaction worked well and the triphenylmethoxy derivative **114** was obtained with a yield of 90%.

Finally the last step was the cleavage of the acetyl ester. Within 4 hours at room temperature the starting ester was completely reacted. Only one spot for a more polar compound, compared to the starting material, was present on the TLC

plate. Neutralization of the pH with dowex resin, filtration and chromatographic purification afforded 73 mg (57%) of a white solid. NMR analysis of the isolated product confirmed the structure for compound **107**; the proton spectrum gave three multiplets in the aromatic region (δ 7.3) for the trityl group with an integration of 15H, according with the 3 phenyl rings. The chemical shift for the proton of the chiral centre moved to upfield (δ 2.05), due to the absence of the de-shielding effects produced by the acetyl group. In ^{13}C-NMR the signal at 171 ppm, corresponding to the carbonyl carbon of the acetate, disappeared together with the methyl group at δ 20.8. Low resolution mass spectrum also proved the success of the reaction with a peak at m/z 479.2 for the ion $[M+Na]^+$.

1-(2-(azidomethyl)-4-(trityloxy)-butyl) uracil (108), 1-(2-(methylamino)-4-(trityloxy)-butyl) uracil (109) and 1-(2-(triphenylmethylamino)-4-(hydroxy)-butyl) (110)



Scheme 7-15 (i): TBAF, THF, r.t.; (ii): TrCl, Py, 80 °C, o.n.; (iii) & (iv): H_2, Pd/C, MeOH/EtOH; (v): TrCl, TEA, DCM, r.t.; (vi): TBAF, THF, r.t..

These three targets could have been prepared according to the synthesis depicted in Scheme 7-15. The triphenylmethoxy azide uracil **108** can be readily prepared in two steps from the starting material **96**. Once the compound **115** is obtained, the azide could be converted into amine to get the triphenylmethyloxy amine **109**. The starting azido intermediate **96** could also be directly converted

into the amine **116**, which could be used to obtain the triphenylmethylamino uracil alcohol **110** *via* tritylation and deprotection.

The removal of the *tert*-butyldimethylsilyl group **96** was performed as before with TBAF in THF. The reaction was very clean and after 4 hours, only one spot was present on TLC. The NMR analysis confirmed that the structure of the obtained product was the desired intermediate **115**. The final yield was only 58%, and the reaction had to be repeated another time, in order to get a reasonable amount of intermediate for the following reactions.

The intermediate **115** was directly taken for the next step with trityl chloride in pyridine. The reaction was carried out in a parallel apparatus with all the other tritylations as described earlier. The desired product **108** was isolated as a white solid in 82% yield. The product was analysed by ^{1}H-NMR and ^{13}C-NMR; both spectra supported the structure of the tritylated intermediate. To confirm the presence of the azide infrared spectroscopy was used. It is well known that organic azides show a typical absorption around 2100 cm^{-1} of the infrared region, which corresponds to the stretching frequency of the bond[98] ($-N=N^{+}=N^{-}$). Indeed the recorded spectrum for compound **108** presented a very intense band at 2102 cm^{-1}. Finally the low resolution mass spectrometry had a clear peak at m/z 504.3 for the ion $[M+Na]^{+}$.

Due to the small amount available of the azide intermediate **108**, (163 mg) it was decided to first attempt the conversion of the azide into amine using the starting material **96**. In the earlier chapters the chemical transformations of the azide group into the amino group was described. This can be achieved either with a catalytic hydrogenation[112,113] or by reaction with triphenylphosphine to form the corresponding iminophosphorane and subsequent hydrolysis to release the amine, a procedure known as Staudinger reaction (Figure 7-22). Other methods are not very efficient (i.e. with sodium borohydride[112]) and/or require the use of strong or very toxic reagents like lithium aluminium hydride and hydrogen sulphide[112].

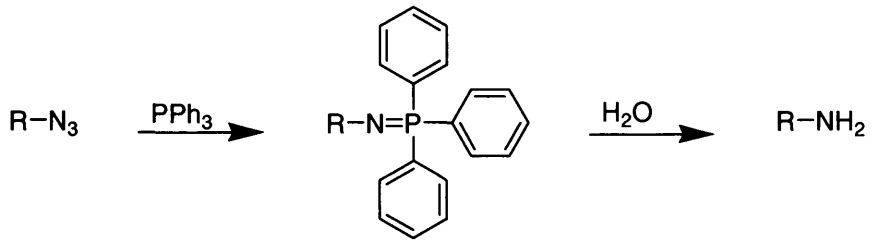
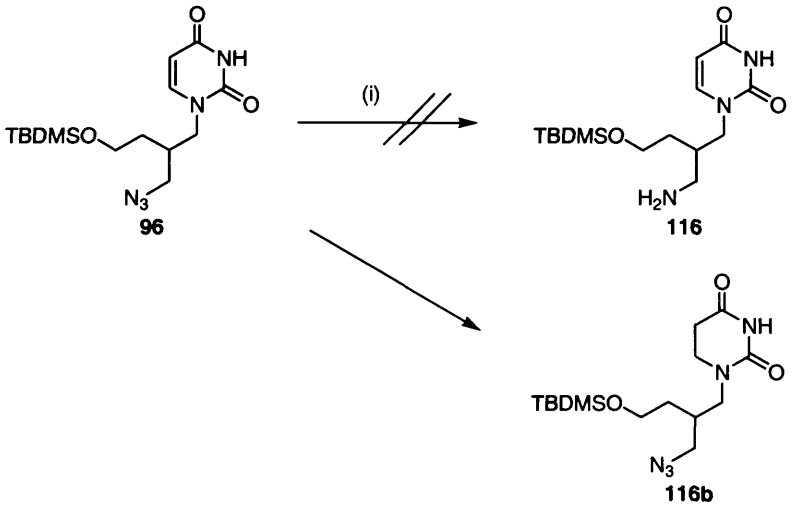


Figure 7-22 The Staudinger reaction for the conversion of azides into amines with triphenylphosphine

In the first attempt the catalytic hydrogenation was investigated on a small scale reaction. Pd 5% on charcoal was the chosen catalyst with methanol as solvent. The starting azide **96** was stirred at room temperature in a flask with a slight pressure of hydrogen gas, applied by a balloon. The reaction was closely monitored by TLC and after six hours at room temperature the starting material was completely reacted. A new spot at lower R_f, detectable only with ninhydrin as staining agent, was visible on the TLC plate. Since ninhydrin dye is sensitive to amines, it was thought that the procedure had been successful. However ^{1}H-NMR analysis of the crude residue obtained after filtration and evaporation, showed all the expected signals with the exception of the two characteristic doublets for the uracil ring (H-5 and H-6). Probably the reduction had occurred on the double bond of the uracil ring, leading to a 5,6-dihydrouracil derivative **116b**, although this was not completely proved by purification. Further analysis of the residue was not possible due to the small amount of compound available.



Scheme 7-16 (i) 5% Pd/C, MeOH

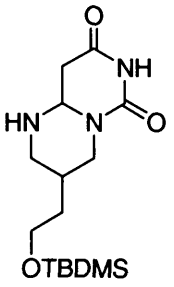
As a second attempt the Staudinger reaction[112] was undertaken with triphenyl phosphine. This procedure was used to convert the 3'-azido-2',3'-dideoxyuridine **55** into 3'-amino-2',3'-dideoxyuridine **56** as reported earlier in Chapter 6.

After stirring overnight compound **96** in the presence of triphenylphosphine, the formation of the imidophosphorane was observed by TLC. Consequently water was added and the mixture was warmed to 50°C for 6 hours, as reported in the procedure[122]. Chromatographic purification of the crude mixture led to three main fractions.

The NMR of the less polar fraction, suggested that the compound was a by-product, perhaps some unreacted imidophosphorane contaminated with triphenylphosphine, due to the high integration (> 30H) of the multiplet present in the aromatic region.

Also the fraction with R_f at 0.38 was a minor by-product of the reaction. In this case the proton NMR presented a triplet around δ 7.3 where the doublet for the H-6 of the uracil ring was expected. The two singlets for the TBDMS group at δ 0.0 and 0.8 gave integrations higher than the values they should have, and also in the region between 1.5 ppm and 3.5 ppm lots of complex signals were present, with none of them recognizable for the expected structure.

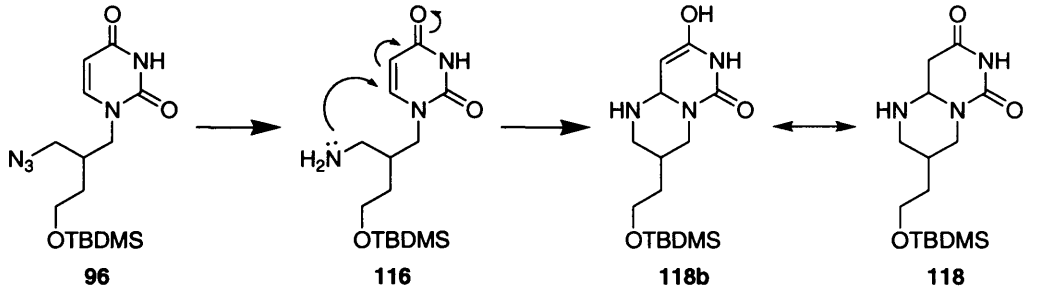
The third fraction, with R_f 0.46, was the main product of the reaction. Since this fraction corresponded to the spot on TLC visible only using ninhydrin, it was thought it could have been the desired product. Unfortunately the spectroscopic analysis did not confirm success of the reaction. In the ^{1}H-NMR the two signals for the H-5 and H-6 of the uracil were missing. The TBDMS group was still present with the two singlets at δ 0.0 and 0.8. Further analysis by COSY and heterocorrelated (H-C) spectroscopy suggested that the isolated compound could have the structure illustrated in Figure 7-23.



118

Figure 7-23 Probable structure for the isolated product from the reduction of the azide **96** to amine

Such a structure could be obtained by nucleophilic attack of the generated amine on the α-β unsaturated carbonyl group of the uracil ring, as depicted in Scheme 7-17, affording a condensed 6 member ring. This could explain the loss of UV activity, as the loss of conjugated double bound would shift the electronic transition associated with the absorption of electromagnetic radiation to higher energy, consequently to higher frequencies, and therefore the standard wavelength (254 nm) of the UV lamp used to observe the TLC plates would not be able to promote the electronic transition.



Scheme 7-17 Proposed mechanism for the formation of the structure **112** during the reduction of the azide **96** to amine **118**

Unfortunately low resolution mass spectrum of the sample did not give further clues since the two isomers **116** and **118** have the same molecular weight (mw 327.5), and only one peak at m/z 328 for the ion $[M+H]^+$ was present.

After this second failure no further attempts to convert the azide into amine were made. Due to the lack of time available for the laboratory work the synthesis of branched uracil derivatives was stopped here and the following compounds were submitted for biological evaluation.

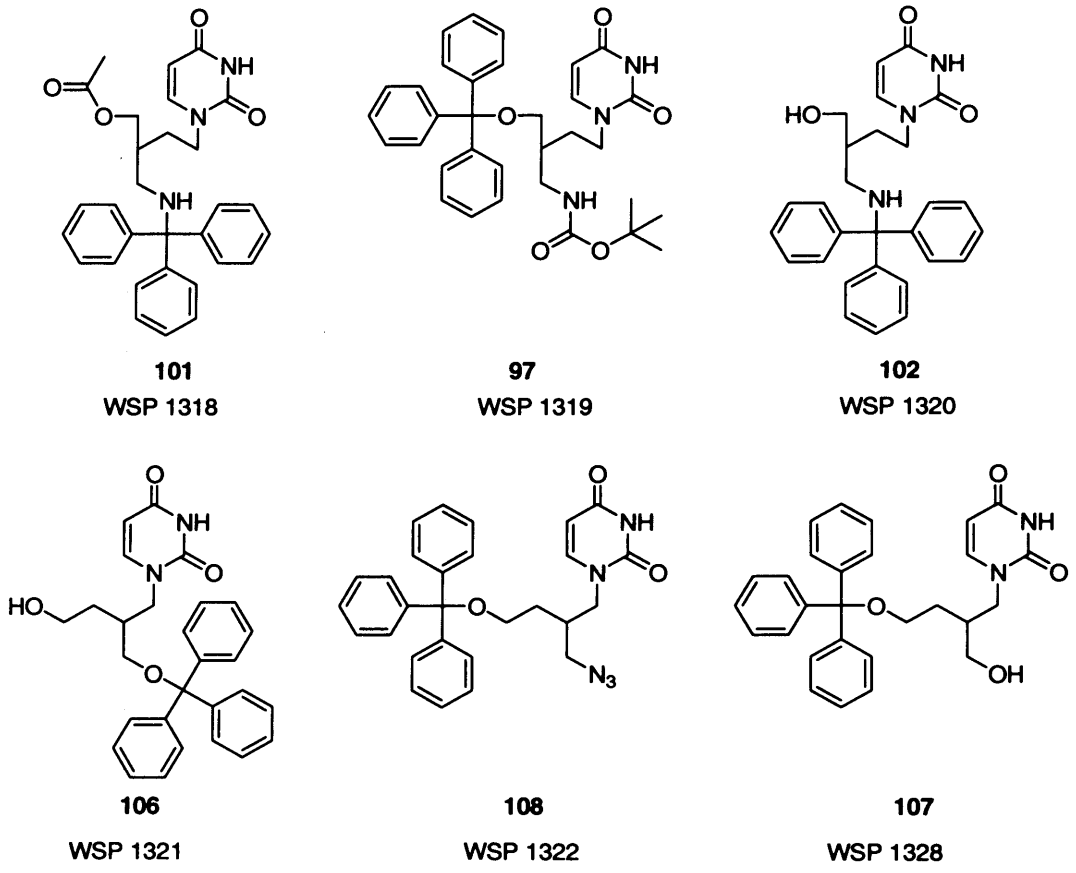


Figure 7-24 The six branched uracil derivatives sent for biological evaluation

Chapter 8 Biological Results

The synthesised compounds were tested against recombinant dUTPases from various sources, (*P. falciparum*, *L. major* and *Human*) and *in vitro* against culture of parasites (*P. falciparum* K1 strain and *L. donovani* amastigote MHOM-ET-67/L82 strain).

The enzyme assays were carried out at the "Instituto de Parasitologia y Biomedicina Consejo Superior de Investigaviones Cientificas" Granada (Spain). The *in vitro* parasite growth inhibition tests were performed at the "Swiss Tropical Institute" of Basel (Switzerland). The procedures followed for the biological tests are reported in the appendix to this thesis.

So far 42 compounds were synthesised, purified and sent for biological evaluation by the author. However, as mentioned in earlier chapter, other people and other groups collaborated to this project; consequently more than 200 compounds have been synthesised and tested to date.

This discussion of biological results will be focused mainly on the compounds prepared and submitted by the author, and sometimes it will be expanded to other compounds tested; with the purpose of giving a better and more complete structure-activity relationship analysis.

The list of the 42 structures evaluated is illustrated in the tables below, divided in four main classes:

- 2'-deoxyuridine analogues (Figure 8-1 & Table 8-1)
- Unsaturated and branched acyclic uracil derivatives (Figure 8-2 & Table 8-2)
- Acyclic phosphates and phosphonate uracil derivatives (Figure 8-3 & Table 8-3)
- Aliphatic acyclic and dialkylated uracil derivatives (Figure 8-4 & Table 8-4)

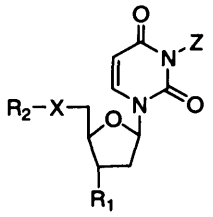


Figure 8-1 General reference structure for compounds illustrated in Table 8-1

Compound	WSP	X	Z	R_1	R_2	MW
3a	**929**	O	-	-OH	Bz	332.3
3e	**930**	O	-	-OBz	Bz	436.4
5b	**931**	O	Bom	-OBom	H	468.5
3d	**932**	O	-	-OBz	H	332.3
3g	**933**	O	-	-OTBDMS	TBDMS	456.7
5a	**959**	O	Bn	-OBn	H	408.4
53	**1203**	O	-	$-OSO_2CH_3$	Trityl	548.6
54	**1204**	O	-	-O-Uracil	Trityl	452.5
55	**1205**	O	-	$-N_3$	Trityl	495.5
56	**1206**	O	-	-NH	Trityl	469.5
57a	**1207**	O	-	-NHAc	Trityl	511.6
57c	**1229**	O	-	$-NHSO_2CH_3$	Trityl	547.6
11	**1314**	O	-	-OH	$-(CH_2)_3OPO_3H_2$	286.28
60	**1323**	O	-	$-CH_2OH$	Trityl	484.5
64	**1324**	O	-	$-CH_2O$Tritly	H	484.5

Table 8-1 List of 2'-deoxyuridine derivatives sent for biological evaluation. For structures see Figure 8-1

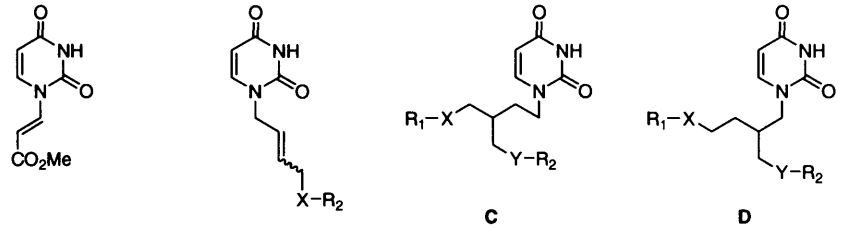


Figure 8-2 Structures of unsaturated and branched acyclic uracil derivatives

Compound	Structure	WSP	X	Y	N	R_1	R_2	MW
69	**B**	**1315**	O	-	4 (*Z*)	-	Trityl	424.5
80a	**A**	**1316**	O	-	3 (*E*)	-	$MeCO_2$-	196.2
72	**B**	**1317**	O	-	4 (*E*)	-	Trityl	424.6
101	**C**	**1318**	O	NH		Ac	Trityl	497.6
97	**C**	**1319**	O	NH		Trityl	Boc	555.7
102	**C**	**1320**	O	NH		H	Trityl	455.6
106	**D**	**1321**	O	O		H	Trityl	456.5
108	**D**	**1322**	O	N_3		Trityl		481.6
107	**D**	**1328**	O	O		Trityl	H	456.5

Table 8-2 List of unsaturated and branched acyclic uracil derivatives sent for biological evaluation. For full structures see Figure 8-2

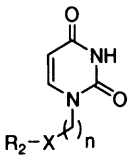


Figure 8-3 General structure for acyclic phosphate/phosphonate uracil derivatives

Compound	WSP	X	n	R_1	R_2	MW
42b	**1143**	O	4	-	$-OPO_3H_2$	264.2
42c	**1144**	O	5	-	$-OPO_3H_2$	278.2
44a	**1145**	-	2	-	$-PO_3H_2$	220.1
42a	**1146**	O	3	-	$-OPO_3H_2$	250.1
44b	**1147**	-	3	-	$-PO_3H_2$	234.1
43a	**1150**	-	2	-	$-PO_3Et_2$	276.23
43b	**1151**	-	3	-	$-PO_3Et_2$	290.2
42d	**1225**	O	6	-	$-OPO_3H_2$	292.2
49a	**1226**	-	4	-	$-PO_3H_2$	248.2
49b	**1227**	-	5	-	$-PO_3H_2$	262.2

Table 8-3 List of phosphate/phosphonate uracil derivatives submitted for biological evaluation. For structures see Figure 8-3

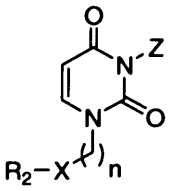


Figure 8-4 General structure of acyclic uracil derivatives and acyclic dialkylated uracil derivative

Compound	WSP	X	Z	n	R_2	MW
31c	**960**	O	$-(CH_2)_3OTr$	3	Trityl	712.9
30c	**961**	O	H	3	Trityl	412.5
30a	**962**	O	H	3	TBDMS	284.4
39e	**1142**	O	H	5	Trityl	440.5
51	**1148**	-	$-(CH_2)_3PO_3H_2$	3	$-PO_3H_2$	356.2
39c	**1208**	O	H	5	H	198.2
39f	**1224**	NH	H	5	Trityl	439.6
40	**1228**	O	H	5	Ph_3Si	456.6

Table 8-4 List of acyclic uracil derivatives and dialkylated acyclic uracil derivatives submitted for biological evaluation. For a complete structure see Figure 8-4

8.1 P. falciparum results

As reported in the aims and objectives (Chapter 3), the lead compounds against *P. falciparum* dUTPase were the acyclic WSP 870 and the two 2'-deoxyuridine analogues WSP 871 and WSP 483.

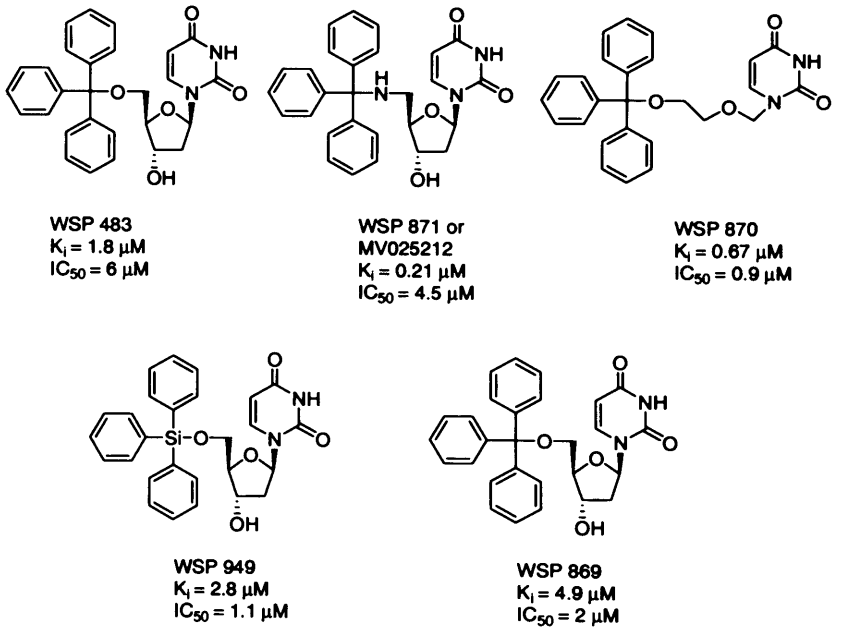


Figure 8-5 Lead compounds and known inhibitors of *P. falciparum* dUTPase

Two other inhibitors are illustrated in Figure 8-5, these are: WSP 949 and WSP 869. In particular the latter was co-crystallized with the *P. falciparum* dUTPase.

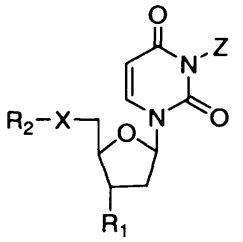
The inhibition constant is a measure of the affinity of a substrate for the enzyme active site. The lower the value of the constant and stronger is the binding of the compound to the enzyme. The compounds in Figure 8-5 have *Ki* values between 0.2 and 4.9 μM. They can also inhibit the parasite's growth in culture with IC_{50} values between 0.9 and 6 μM.

In the next tables are reported the *Ki*, the IC_{50} and also the selectivity index and cytotoxicity for all the compound sent for biological evaluation.

The selectivity (S) is a measure of the selectivity of a compound between the *Human* dUTPase and the parasite *P. falciparum* dUTPase and is obtained from the ratio of the two inhibition constants *Ki*s ($Ki_{human}/Ki_{P.\ falciparum}$).

The cytoxicity was measured against L6 cell line, and gives indication about the toxicity of a compound for human cells. It is therefore correlated with the cellular selectivity, since a compound with a very low IC_{50} value and a high cytotoxicity value would kill the parasites rather than the human cells.

8.1.1 2'-deoxyuridine analogues



	Cmpd	Enzyme K_i	*In vitro* IC_{50}	Selectivity	Toxicity	X	Z	R_1	R_2
i	56	1.27	0.99	>787	3.02	O	H	$-NH_2$	Trityl
ii	57	1.43	2.15	>699	44.17	O	H	-NHAc	Trityl
iii	64	2.11	3.05	55.9	28.6	O	H	CH_2OH	Trityl
iv	57c	4.22	1.31	>236	18.4	O	H	$-NHSO_2CH_3$	Trityl
v	53	25.5	0.73	>39	37.7	O	H	OSO_2CH_3	Trityl
vi	55	48.1	1.34	>20	25.8	O	H	$-N_3$	Trityl
vii	3a	206.3	9.8	>4.8	-	O	H	OH	Bz
viii	3g	268.5	3	>3.7	11	O	H	OTBDMS	TBDMS
ix	119	515.2	25	>1.9	>154	O	H	OTBDMS	Trityl
x	3e	>1mM	>11.5	>0.9	95.7	O	H	OBz	Bz
xi	5b	>1mM	-	>1	-	O	Bom	OBom	H
xii	3d	>1mM	>15	>1	34	O	H	OBz	H
xiii	5a	>1mM	>12	>1	124	O	Bn	OBn	H
xiv	54	>1mM	0.56	>1	54.6	O	H	-*-	Trityl
xv	11	>1mM	>17.5	>1	>314	O	H	OH	$H_2O_3PO(CH_2)_3$-
xvi	60	>1mM	0.48	>1	20.8	O	H	CH_2OTr	H

Table 8-5 Biological results for the 2'-deoxyuridine analogues against *P. falciparum* dUTPase and *P. falciparum in vitro* cultures. All the values are in μM. -*- anhydronucleoside **54**

In the nucleoside analogues the best inhibition constants are those obtained for the 3'-amino derivatives **56** and the corresponding 3-acetamide **57** (entries **i** & **ii**), which had *Ki* of 1.27 and 1.43 μM, respectively. Unfortunately such values are still higher than the corresponding lead compound 5'-tritylamino-2'-deoxyuridine WSP 871 (*Ki* = 0.21 μM). Besides, both compounds retained a good selectivity index, both above 600. They also showed a good inhibition of the parasitic growth with IC_{50} of 0.99 and 2.15 μM. It is notable that the corresponding acetylated compound has a much lower cellular toxicity respect to the free 3'-amino **56**.

Substitution of the 3' OH with a methylenehydroxyl group (compound **64**, entry **iii** in Table 8-5) produced an inhibition of the enzyme (*Ki* 2.11 μM), comparable to the lead WSP 483 with OH in 3' position (*Ki* 1.8 μM), thus indicating that the elongation of the carbon chain in such position is not relevant for the enzyme inhibition. However, compound **64** had marginally better activity in the *in vitro* inhibition (IC_{50} 3.05 μM) compared to the WSP 483 (6 μM).

The general trend on the enzyme inhibition produced by different substituents in 3'-position seems to favour small polar groups (OH or NHR) instead of bulky groups (polar or apolar), since the latter had the worst results in the enzyme inhibition as shown by the entries **viii-xvi** in Table 8-5; this is clearly proved by compound **119** (entry **ix**) the 3'-TBDMS protected analogue of the lead WSP 483. This silylated compound had a *Ki* value (515 μM) nearly 250-fold higher than the parent compound.

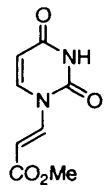
Although the first three strctures in the table present a correlation for both *Ki* and IC_{50} values, this trend is not generally true for the remaining compounds. For example the 3'-mesylate **53** (entry **v**) and the anhydronucleoside **54** (entry **xiv**) are poor inhibitors of the enzyme (*Ki* 25.5 μM and >1000 μM respectively), but can still inhibit the parasite growth with IC_{50} values in the range of 0.5 μM; thus indicating that perhaps other factors such as membrane permeation and metabolic instability are influencing the effect on parasites or the compounds are acting on a different metabolic pathway.

A similar situation arises for compound **60** (entry **xvi**) which has a free OH in 5'-position and another primary alcohol in 3'-position protected as trityl ether. The enzyme inhibition was very weak (*Ki*>1 mM), but growth inhibition of the parasite was potent (IC_{50}=0.48 μM,) so far the best for this series. On the other hand compound **64**, which has the trityl group swapped compared to compound **60** (entry **xvi**) had a much better result against the enzyme (*Ki* 2.11 μM), but IC_{50} (3.05 μM) not as good as the regio-isomer **60**.

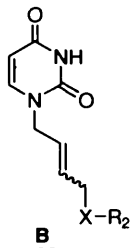
It is clear that the trityl group is essential for the compounds, in order to have an appreciable activity against the enzyme dUTPase. The first six entries reported in the table have a trityl group in 5'-position. If this group is replaced with a lipophilic one, but without aromaticity such as TBDMS (compound **3g**, entry **viii**) or with a simple aromatic benzoyl ester (compounds **3a** & **3e**, entries **vii** & **x**) the

enzyme inhibition is significantly compromised and *Ki* values are above 200 μM or more.

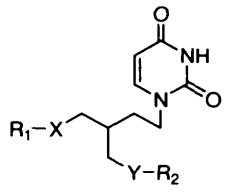
8.1.2 Branched and unsaturated acyclic uracil derivatives



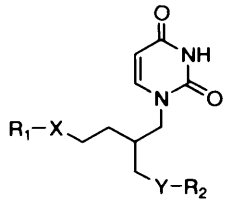
A
80a



B
69
72



C
101
97
102



D
106
108
107

	Structure	Cmpd	Enzyme K_i	In vitro IC_{50}	Selectivity	Toxicity	X	Y	n	R_1	R_2
i	C	102	0.24	3.17	23.9	55.4	O	NH		H	Trityl
ii	B	72	0.61	3.65	>1000	33.9	O	-	4 (*E*)	-	Trityl
iii	D	106	0.9	1.85	23.7	57.1	O	O		H	Trityl
iv	B	69	1.2	3.46	4.3	38.7	O	-	4 (*Z*)	-	Trityl
v	C	101	1.33	2.84	55.5	37.3	O	NH		Ac	Trityl
vi	D	107	2.15	1.15	26	32.6	O	O		Trityl	H
vii	C	97	2.48	0.89	>403	14.7	O	NH		Trityl	Boc
viii	D	108	23.1	0.64	>43.2	18.4	O	N_3		Trityl	
xi	A	80a	>1000	23.14	>1000	206.7	O	-	3 (*E*)	-	$MeCO_2$-

Table 8-6 Biological results for the unsaturated and branched acyclic uracil derivatives against *P. falciparum*. All the values are in μM

To date this series of compounds has given the best results in terms of enzyme inhibition.

The γ-branched tritylamino hydroxyl derivatives **102** (entry **i** in Table 8-6) had a *Ki* value (0.24 μM) comparable to the one reported for the lead WSP 871 (0.20 μM). However this good result is partially contrasted by the loss of selectivity, with a value (23.9) approximately 10-fold lower than the corresponding lead WSP 871 (S=231).

The other two γ-branched derivatives **101** and **97** (entries **v** & **vii**), the former with an acetyl ester on the OH and the latter with a triphenylmethyl ether on the OH and a Boc carbamate on the amino, showed higher values of *Ki* for the

enzyme inhibition (*Ki* 1.33 and 2.48 μM respectively), indicating a slightly lower affinity for the enzyme active site compared to the amino derivative **102** (entry **i**). On the other hand, these two compounds are better inhibitors of the parasite's growth with IC_{50} values of 2.84 and 0.89 μM, respectively for **101** and **97**, than compound **102** (IC_{50} 3.17 μM). Such results could be explained by considering the two structures as a pro-drug.

A pro-drug is a "masked-drug" compound, which is able to release the corresponding drug once inside in the cellular environment. The release can be achieved either enzymatically or chemically, depending on the masking groups present on the molecules.

In the case of **97** and **101** the ester function in the first compound and the carbamate on the second might be cleaved once they are inside the cell, releasing the parental compounds with the OH and NH_2 groups free. The presence of the ester and the carbamate decreases the total polarity of the compound and might therefore help to cross the cellular membrane, increasing the chances of reaching the target.

When the 2'-deoxyribose moiety of the lead WSP 871 was replaced with a γ-branched aliphatic chain as found in compounds **97**, **101** and **102** (WSP 1318, 1319 and 1320, Figure 8-6), these retained both activity *in vitro* and against the enzyme, but a considerable loss of selectivity was obtained compared to the lead 871, with the exception of **97**.

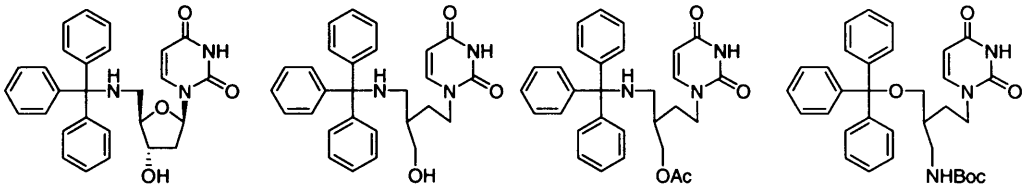


Figure 8-6 γ-branched acyclic uracil derivatives WSP 1320, 1319, 1320 and lead compound WSP 871

A second interesting result came from the two unsaturated acyclic derivatives **72** and **69** (entries **ii** & **iv** respectively in Table 8-6). These two compounds gave a better inhibitory effect compared to the corresponding analogue WSP 1000 with a

simple aliphatic chain (synthesised by other members of the group, Figure 8-7). The *Ki* value for the simple aliphatic compound was about 2 times higher than *E*-unsaturated **72** (*Ki* = 0.61 μM), whilst the *Z*-isomer **69** had a comparable *Ki* value to the aliphatic analogue WSP 1000. The most significant effect produced by the presence of the double bond is on the selectivity; the *E* regioisomer **72** had comparable selectivity to the WSP 1000 (S >1000 and >617, respectively), but much higher than the *Z* isomer **69**, which had almost completely lost the selectivity between the two forms of the enzyme (S = 4.3). In the *in vitro* assays the two unsaturated compounds had comparable values of IC_{50} around 3.5-3.6 μM, still in the same range as the corresponding aliphatic compound (IC_{50} 4.9 μM).

The presence of the double bond slightly increased the enzyme affinity and the *in vitro* activity for the two compounds **69** and **72**, although the most remarkable effect was obtained by the *trans*-derivative WSP 1317 on the selectivity index.

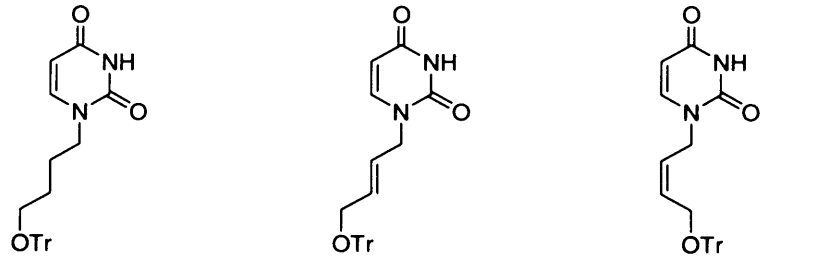


Figure 8-7 Comparison of **72** and **69** with the aliphatic analogue WSP 1000

The last group of acyclic compounds, the β-branched acyclic derivatives **106**, **108** and **107** (entries **iii, vi & viii** in Table 8-6) are illustrated in Figure 8-8.

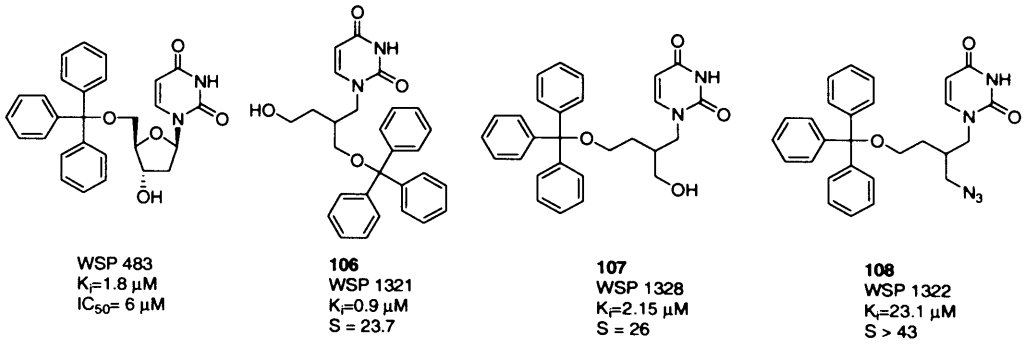
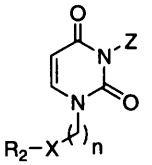


Figure 8-8 β-branched derivatives **106, 107** and **108** with the lead WSP 483

Compound **106** (entry **iii** in Table 8-6) with the trityl group on the pseudo 2'-position had the best inhibition constant (*Ki*=0.9) followed by the derivative **107** (entry **vi**) with the trityl in the pseudo 5'-position (*Ki*=2.15 μM). The azido compound **108** (entry **viii**), instead, showed a poor inhibitory effect on the enzyme (*Ki*=23.1 μM). The selectivity values were similar to those obtained for the γ-branched acyclic (in the range of 20-40). However this group seemed to more active in the *in vitro* parasite's growth inhibition compared to the lead WSP 483. The IC_{50} obtained were between 0.64 and 1.85 μM, with the lowest for the azide **108**. Although, the higher *in vitro* activity of this compound could be attributed to the increased cytotoxicity as reported in the Table 8-6.

8.1.3 Acyclic uracil derivatives and dialkylated uracil derivatives



	Cmpd	Enzyme K_i	In vitro IC_{50}	Selectivity	Toxicity	X	Z	n	R_2
i	**40**	3.76	0.38	>265	46.6	O		5	Ph_3Si
ii	**39f**	4.29	2.15	>233	39.13	NH		5	Trityl
iii	**30a**	73.2	>18	1	192	O		3	TBDMS
iv	**30c**	86.5	7.5	3.62	34.4	O		3	Trityl
v	**31c**	262.1	0.5	0.58	>126	O	$-(CH_2)_3OTr$	3	Trityl
vi	**39e**	>1000	1.1	-	23.4	O		5	Trityl
vii	**39c**	>1000	19.5	-	>454	O		5	OH
viii	**51**	>1000	>14	-	>252	-	$-(CH_2)_3PO_3H_2$	3	$-PO_3H_2$

Table 8-7 Biological results for the aliphatic acyclic series d against *P. falciparum*

This series of compounds has extensively been analysed in the group, since several analogues have been prepared by other people in the group[123,124]. Some of them are reported in Table 8-8, and will be discussed together with the compounds submitted by the author and indicated in Table 8-7.

The research was focused on the evaluation of:

- The effect of the chain length; chain of 3, 4, 5 and 6 carbon atoms has been examined.

- effect of the terminator moiety; bulky lipophilic group (trityl, triphenylsilyl and TBDMS) were investigated
- effect of the heteroatom between the aliphatic chain and the terminator; O and NH were analysed

	cmpd	Enzyme K_i	In vitro IC_{50}	Selectivity	Toxicity	X	Z	n	R_2
ix	120	1.56	0.58	16	70.2	O		3	Ph_3Si
x	121	2.24	0.36	>446	-	O		4	Ph_3Si
xi	40	3.76	0.38	>265	46.6	O		5	Ph_3Si
xii	122	1.69	0.43	226	75.6	O		6	Ph_3Si
xiii	123	>1000	0.9	-	89.4	O		-	Ph_3Si
xiv	124	0.2	4.4	6.75	107.4	NH		3	Trityl
xv	30c	86.5	7.5	3.62	34.4	O		3	Trityl
xvi	125	0.9	3.8	>1111	33.4	NH		4	Trityl
xvii	126	1.62	4.9	>617	42.4	O		4	Trityl
xviii	39f	4.29	2.15	>233	39.13	NH		5	Trityl
xix	39e	>1000	1.1	-	23.4	O		5	Trityl
xx	127	1.84	1.1	>543	40.3	NH		6	Trityl
xxi	128	9.58	2.3	49.7	43.8	O		6	Trityl
xxii	129	0.67	0.9	25.3	70	O		1(O)2	Trityl
xxiii	31c	262.1	0.5	0.58	>126	O	$-(CH_2)_3OTr$	3	Trityl
xxiv	130	>1000	0.01	>1000	>126	NH	$-(CH_2)_3NHTr$	3	Trityl
xxv	131	>1000	1.8	>1000	108.5	NH	$-(CH_2)_6NHTr$	6	Trityl
xxvi	132	73.2	>18	1.01	192	O		3	TBDMS
xxvii	133	43.7	62.2	1.43	>299	O		1(O)2	TBDMS
xxviii	134	8.47	3.9	>118	18	O		6	TBDPS

Table 8-8 Other acyclic uracil derivatives synthesised and screened in the group against *P. falciparum* dUTPase and in vitro against *P. falciparum* strain.

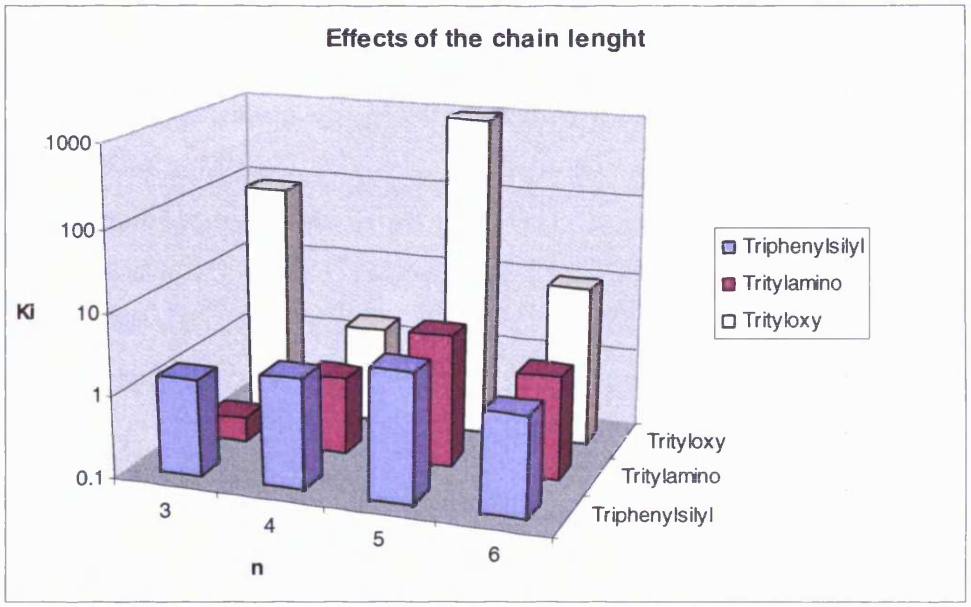


Figure 8-9 Effect of the chain length (n) in the enzyme assay for the three series of acyclic uracil derivatives

The effect produced by the different carbon chains tested varied and depend on the terminal group present in the molecule. The Figure 8-9 shows the variation of the *Ki* values for the compounds divided in three series (triphenylsilyl, triphenylamino and trityloxy) in relation to the extention of the carbon chain (n).

The best enzyme inhibition was obtained for compound **124** with a 3-carbon chain and a tritylamino group at the end of it (entry **xiv** in Table 8-8), with a *Ki* of 0.2 μM. Increasing the length of the spacer chain, the enzyme inhibition decreased with *Ki* = 0.9 μM for the 4-carbon chain **125** (entry **xvi**, Table 8-8) and *Ki* = 4.29 μM for the C-5 (compound **39f**, entry **xviii**, Table 8-8). The activity increases again for analogue **127** with a C6 chain with a *Ki* = 1.84 μM (entry **xx**, Table 8-8). This tendency can be observed in the chart were the tritylamino series is represented in purple colour.

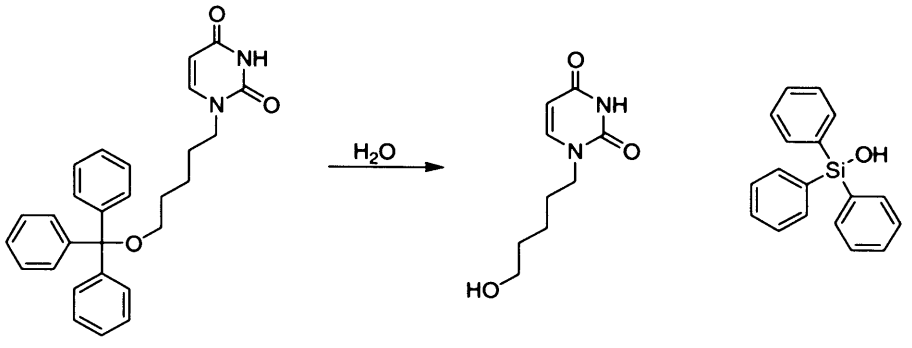
The same sort of trend could be found for the triphenylsilyl derivatives (entries **ix**, **x**, **xi**, **xii**, Table 8-8) with a maximum inhibition of the enzyme for the C-3 derivatives (*Ki* = 1.56 μM). The trend is also shown in the graph with the blue series.

Very different results were obtained for the trityloxy series, (entries **iv** in table Table 8-7, and **xvii**, **xix**, **xxi** in Table 8-8), where the best *Ki* (1.62 μM) was obtained for the C4 derivative **126**. The C5 analogue (compound **39e**, entry **xix**),

instead, gave an inhibition constant higher than the maximum concentration used for the biological assays (1000 μM). This result was unexpected and unexplainable, since the assay was repeated two times, confirming the inactivity.

Polyaromatic groups (like the triphenylmethyl) are required in order to obtain significant enzyme inhibition. Therefore compounds with hydrophilic polar groups like the dialkylated uracil phosphonate **51** (entry **viii**, Table 8-7) and the uracil alcohol **39c** (entry **vii**, Table 8-7) were completely inactive in the enzyme assays. Only the triphenylmethyl, triphenylsilyl and *tert*-butyldiphenylsilyl derivatives had *K*is values below 100 μM.

From the *in vitro* point of view it appeared that the triphenylsilyl derivatives (entries **ix-xii**, Table 8-8) have a higher inhibitory effect on the parasite growth, with IC_{50} values in the range of 0.5-1 μM, compared to the analogues with a trityl group (Figure 8-10). This result was further investigated, since the triphenylsilyl group could undergo a cleavage in the cellular environment releasing the corresponding uracil alcohol and triphenylsilanol. The triphenylsilanol (entry **xiii**, Table 8-8) was tested *in vitro* and showed an IC_{50} of 0.9 μM, thus confirming that the activity of the triphenylsilyl derivatives probably arises both from the release of the silanol inside the parasite' s cell and the compound action on the enzyme (Scheme 8-1).



40
WSP 1228
Ki= 2.24 μM
IC_{50} = 0.36 μM

39c
WSP 1208
Ki >1mM
IC_{50} = 19.5 μM

123
WSP 1211
Ki >1mM
IC_{50} = 0.9 μM

Scheme 8-1 Hydrolysis of compound **40** to release the alcohol intermediate **39c** and the triphenylsilanol **123**

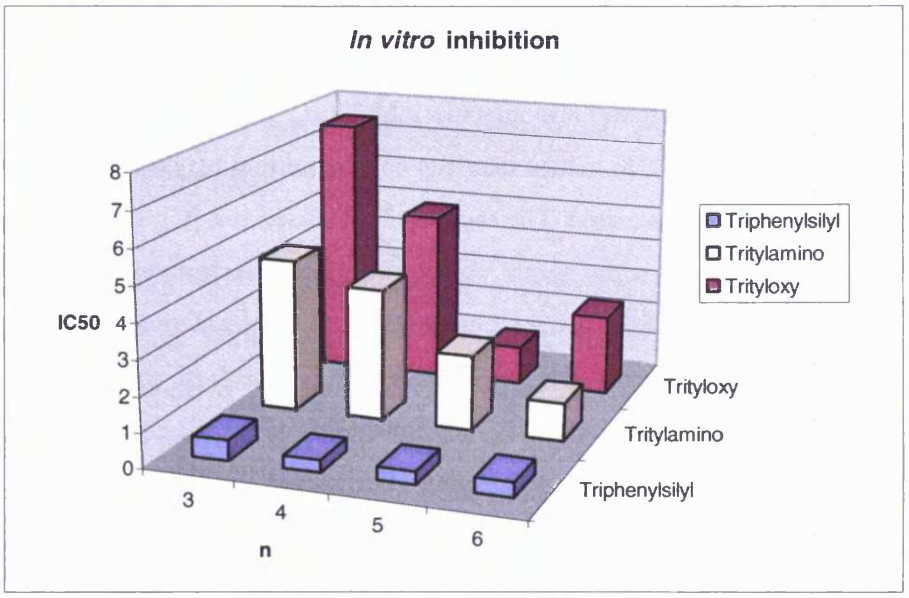


Figure 8-10 Effect of the chain length (n) and the terminator groups on the *in vitro* inhibition

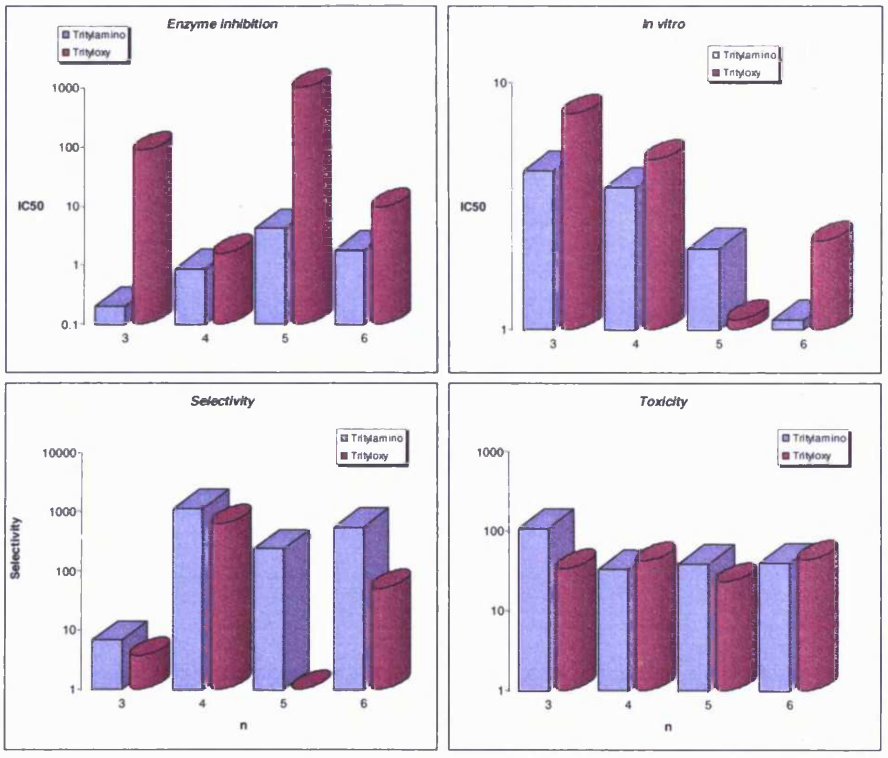


Figure 8-11 Effects of chain length (n) and heteroatom (tritylamino or trityloxy) on enzyme inhibition (top left), *in vitro* inhibition (top right), selectivity (bottom left) and toxicity (bottom right).

The effect of the heteroatom X on enzyme inhibition and parasite growth inhibition is presented in Figure 8-10 and in Figure 8-11. The charts in Figure

8-11 compare the tritylamino derivatives (entries **xiv**, **xvi**, **xviii** and **xx** in Table 8-8) with the corresponding trityloxy (entries **xv**, **xvii**, **xix** and **xxi** in Table 8-8).

The first chart compares the enzyme inhibition. The tritylamino compounds (blue series) have lower *Ki* values than the corresponding trityloxy (purple series), for all the chain lengths tested. The second chart shows the IC_{50} values against the intact parasite for the same series. Again the amino derivatives showed higher activity, with the exception of the C5 derivatives; this latter derivative (entry **xix** in Table 8-8) had an IC_{50} of 1.1 μM against the 2.15 μM of the corresponding triylamino (entry **xviii** in Table 8-8). The third and the fourth charts compare the selectivity and the toxicity. Again the amino derivatives have better selectivity than the oxy derivatives, for all the different chain lengths. No appreciable differences could be highlighted for the cytotoxicity, since all the compounds have similar values.

The chart in Figure 8-12 shows all the *Ki*s and the IC_{50} for all the acyclic trityloxy, tritylamino and triphenysilyl derivatives. It compares the effect of the chain lengths and of the terminator groups.

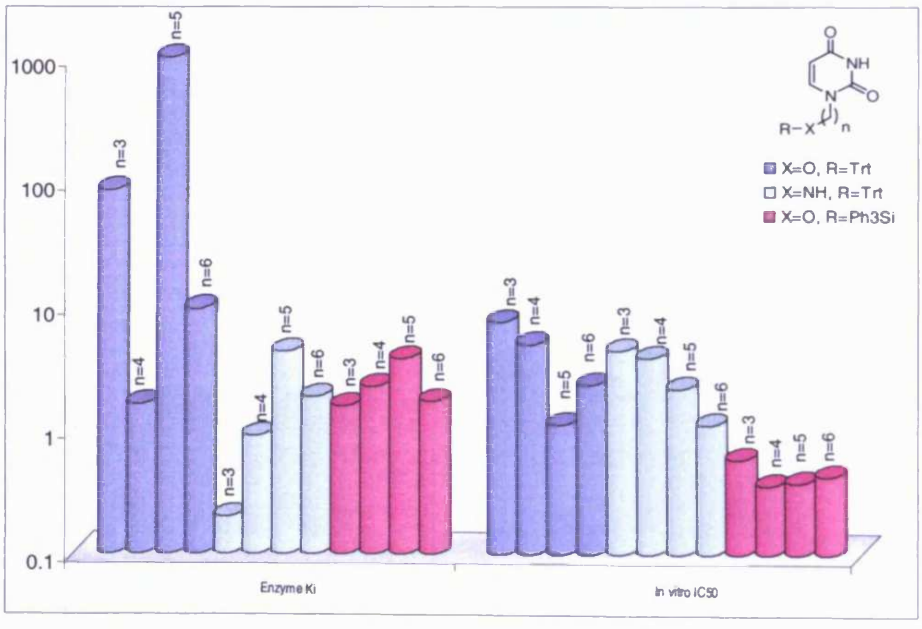
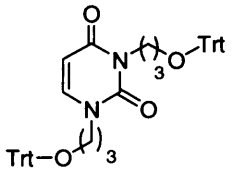


Figure 8-12 Comparison of *Ki* and IC_{50} for the three series of acyclic uracil derivatives

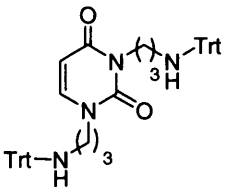
- There is correlation between the results in the two biological assays.

- No particular trend can be highlighted due to the effect of the chain length.
- Triphenylsilyl derivatives (pink group) had the overall best results in the *in vitro* assay.

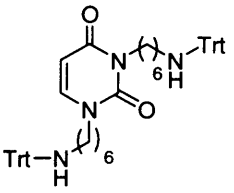
Together with the series of acyclic compounds some dialkylated uracil derivatives (entries **xxiii, xiv, xv** in Table 8-8, Figure 8-13) were tested. The structures are reported in Figure 8-13. As expected these compounds were poor inhibitors of the enzyme dUTPase, since this enzyme required the presence of the uracil ring in the substrate and a modified uracil would not bind strongly in the enzyme active site. Surprisingly the same compounds showed a strong inhibition of the parasites growth, with IC_{50} values in the sub-μM range; compound **130** showed an IC_{50} value of 10 nM.



31c
WSP 960
Ki = 262 μM
IC_{50} = 0.5 μM



130
WSP 1231
Ki > 1mM
IC_{50}=0.01μM

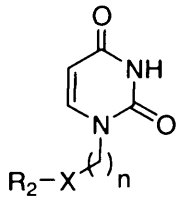


131
WSP 1242
Ki> 1mM
IC_{50} = 1.8 μM

Figure 8-13 Dialkylated uracil derivatives tested

However, due to their poor enzyme inhibition, these structures should act on a different biological pathway of the parasite rather than targeting the enzyme dUTPase. Unfortunately with this project was not possible to investigate further this kind of compound, although from the obtained results it would be worthwhile to try understanding their mechanism of action.

8.1.4 Acyclic phosphates and phosphonates



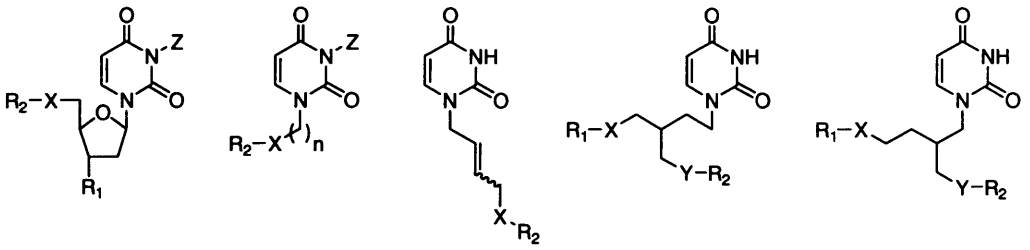
	Cmpd	Enzyme K_i	In vitro IC_{50}	Toxicity	X	n	R_2
i	**42b**	>1000	>18	>340	O	4	$-OPO_3H_2$
ii	**42c**	>1000	>18	>323	O	5	$-OPO_3H_2$
iii	**44a**	>1000	>22	>408	-	2	$-PO_3H_2$
iv	**42a**	>1000	>20	>360	O	3	$-OPO_3H_2$
v	**44b**	>1000	>21	>384	-	3	$-PO_3H_2$
vi	**43a**	>1000	>18	>325	-	2	$-PO_3Et_2$
vii	**43b**	>1000	>17	>310	-	3	$-PO_3Et_2$
viii	**42d**	>1000	>17	>308	O	6	$-OPO_3H_2$
ix	**49a**	>1000	>20	>362	-	4	$-PO_3H_2$
x	**49b**	>1000	147	>343	-	5	$-PO_3H_2$

Table 8-9 Biological results for the acyclic phosphonate/phosphates series. All the values are in μM.

The biological results for the series of acyclic phosphonates and phosphates tested against the *P. falciparum* dUTPase and *P. falciparum* strain are reported in Table 8-1.

Unfortunately this series of compound was inactive in both the assays. This can be considered as a further confirmation of the necessity of a presence of bulky lipophilic group in the molecule for inhibition of the *P. falciparum* dUTPase.

8.1.5 Overview of results



A B C D E

	Structure	Cmpd	Enzyme K_i	In vitro IC_{50}	X	Y	Z	n	R_1	R_2
i	D	102	0.24	3.17	O	NH		-	H	Trityl
ii	C	72	0.61	3.65	O			4 (*E*)	-	Trityl
iii	E	106	0.9	1.85	O	O		-	H	Trityl
iv	C	69	1.2	3.46	O			4 (*Z*)	-	Trityl
v	A	56	1.27	0.988	O	NH_2		-	-NH	Trityl
vi	D	101	1.33	2.84	O	NH		-	Ac	Trityl
vii	A	57a	1.43	2.15	O	NH		-	-NHAc	Trityl
viii	A	64	2.11	3.05	O			-	CH_2OTritly	H
ix	E	107	2.15	1.15	O	O		-	Trityl	H
x	D	97	2.48	0.89	O	NH		-	Trityl	Boc
xi	B	40	3.76	0.38	O	-		5	-	Ph_3Si
xii	A	57c	4.22	1.31	O	NH		-	$-NHSO_2CH_3$	Trityl
xiii	B	39f	4.29	2.15	NH	-		5	-	Trityl
xiv	E	108	23.1	0.64	O	N_3		-	Trityl	
xv	A	53	25.5	0.73	O	O		-	OSO_2CH_3	Trityl
xvi	A	55	48.1	1.34	O	-		-	$-N_3$	Trityl
xvii	B	30a	73.2	>18	O	-		3	-	TBDMS
xviii	B	30c	86.5	7.5	O	-		3	-	Trityl
xix	A	3a	206.3	9.8	O	O		-	OH	OBz
xx	B	31c	262.1	0.5	O	-	$-(CH_2)_3OTr$	3	-	Trityl

Table 8-10 *Ki*s and selectivity indexes for the best results inhibitors of the *P. falciparum* dUTPase. All the values are in μM.

The Table 8-10 presents 20 of the compounds prepared by the author in order of potency of enzyme inhibition. The aim of the table is to give an overview of all

the biological data collected, with the different series presented separately in the previous sections.

- The branched and the unsaturated acyclic uracil derivatives gave the strongest inhibition of the enzyme (entries **i-iv**, Table 8-10) .
- The replacement of the OH function in 3' position of the 2'-deoxyuridine slightly increased the biological activity, but at the same time increased the cytotoxicity of this class of derivatives (entries **v-viii**, Table 8-10).
- The acyclic derivatives with simple aliphatic chain and triphenylsilyl groups as terminator strongly inhibited the parasite growth *in vitro*, although their mechanism of action does not seem to be related only to the inhibition of the enzyme dUTPase (entry **xi**, Table 8-10).

8.2 Leishmania results

The three lead compounds against *L. major* dUTPase were the three 2'-deoxythymidine-5'-propylphosphates provided by Medivir AB, (Figure 8-14).

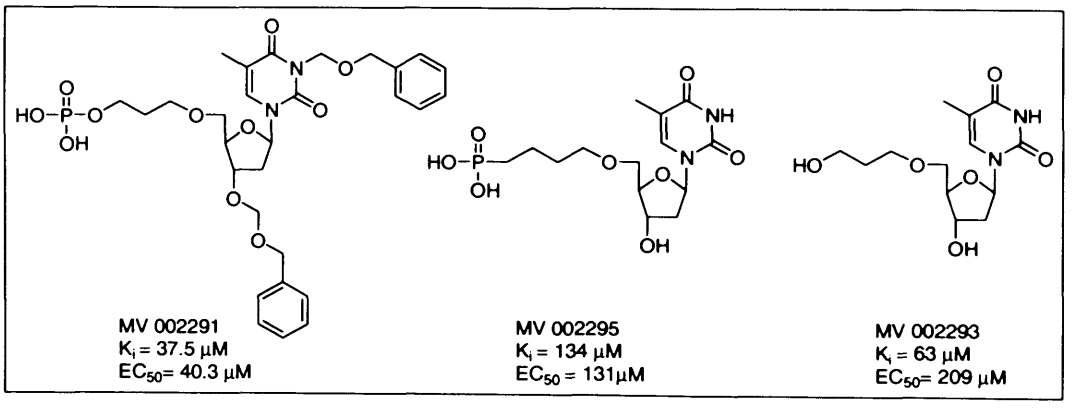
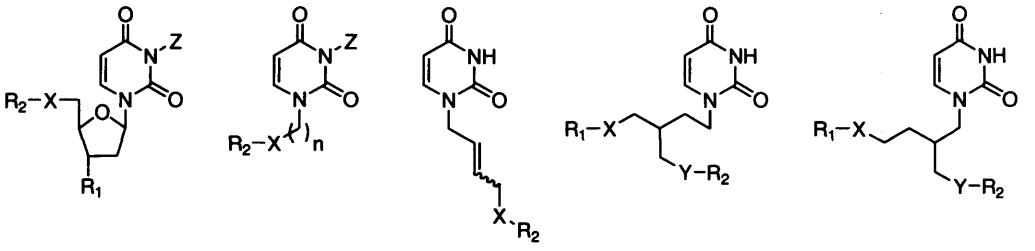


Figure 8-14 lead compounds against *L. major*

The series will be described in the same way as the previous section. A first summary of biological data is reported in Table 8-11. The list is sorted by IC_{50} values from the most to the less active.



A B C D E

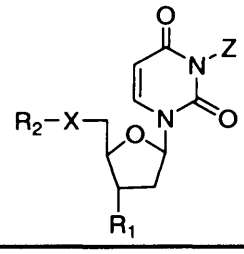
	Structure	Cmpd	Enzyme K_i	In vitro IC_{50}	Toxicity	X	Y	Z	n	R_1	R_2
i	A	55		1.87	25.8	O	N_3	H	-	-	Trityl
ii	C	69		2.13	38.7	O		H	4 (*Z*)		Trityl
iii	D	97		2.6	14.7	O	NH	H		Trityl	Boc
iv	E	108		2.9	18.4	O	N_3	H		Trityl	
v	A	64		4.51	28.6	O		H		CH_2OTritly	OH
vi	C	72		4.81	33.9	O		H	4 (*E*)		Trityl
vii	B	39e	>1000	5	23.4	O	-	H	5	-	Trityl
viii	A	53		5.46	37.7	O	O	H	-	OSO_2CH_3	Trityl
ix	E	106		6.86	57.1	O	O	H		H	Trityl
x	A	60		6.97	20.8	O		H		CH_2OH	Trityl
xi	A	57c		7.3	18.4	O	NH	H	-	$-SO_2CH_3$	Trityl
xii	E	107		8.42	32.6	O	O	H		Trityl	H
xiii	A	57e		8.99	44.17	O	NH	H	-	Ac	Trityl
xiv	A	11		9	>314	O	-	H		H	$H_2O_3PO(CH_2)_3$
xv	D	102		10.13	55.4	O	NH	H		H	Trityl
xvi	D	101		11.38	37.3	O	NH	H		Ac	Trityl
xvii	B	40	-	12	46.6	O	-	H	5	-	Ph_3Si
xviii	B	39f	-	17.5	39.13	NH	-	H	5	-	Trityl
xix	A	54		20.11	54.6	O	-*-	H	-*-	-*-	Trityl
xx	C	80a		20.2	206.7	O		H	3 (*E*)		$MeCO_2$-
xxi	A	56		27.9	3.02	O	NH_2	H	-	-	Trityl
xxii	A	30a	>1000	95.9	192	O	-	H	3	-	TBDMS

Table 8-11 Most active compounds against *L. major* dUTPase, together with the *in vitro* inhibition of axenic amastigote cultures of *L. donovani*. *: anhydronucleoside **54**.

Table 8-11 shows an overview of the data available for the compounds sent. It is not an inclusive list and includes only the best 22 results.

The results are quite promising as the obtained IC_{50} values are in the same range or lower (1.9-28 μM) than those described for the lead compounds (40.3 μM, 83 μM and 131 μM respectively).

8.2.1 2'-deoxyuridine analogues series



	Cmpd	Enzyme K_i	In vitro IC_{50}	Toxicity	X	Z	R_1	R_2
i	55	n.a.	1.87	25.8	O		N_3	Trityl
ii	64	n.a.	4.51	28.6	O		$-CH_2OTr$	H
iii	53	n.a.	5.46	37.7	O		OSO_2CH_3	Trityl
iv	60	n.a.	6.97	20.8	O		$-CH_2OH$	Trityl
v	57c	n.a.	7.3	18.4	O		$-NHSO_2CH_3$	Trityl
vi	57e	n.a.	8.99	44.17	O		-NHAc	Trityl
vii	11	n.a.	9	>314	O		H	$H_2O_3PO(CH_2)_3$-
viii	54	n.a.	20.11	54.6	O		-*-	Trityl
ix	56	n.a.	27.9	3.02	O		NH_2	Trityl
x	3e	>1000	-	95.7	O		-OBz	Bz
xi	5b	>1000	-	-	O	Bom	-OBom	H
xii	3d	>1000	-	34	O		-OBz	H
xiii	3g	>1000	-	11	O		OTBDMS	TBDMS
xiv	5a	>1000	-	124	O	Bn	-OBn	H
xv	3a	>1000	>90	-	O		-OH	Bz

Table 8-12 Most active 2'-deoxyuridine analogues *in vitro* against *L. donovani*. N.A.: not available. *: anhydronucleoside **54**

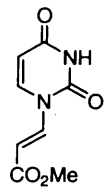
The analysis of the 2'-deoxyuridine analogues shows that the most active is the 3'-azido derivative **55** (entry **i** in Table 8-12), with IC_{50} of 1.87 μM. Unfortunately the enzyme assays are not available yet, therefore it is not possible to conclude if the activity for this and all the other compounds submitted is due to their inhibitory action on the *L. major* dUTPase.

The presence of bulky lipophilic group, like the triphenylmethyl, seems to help the activity as the first ten compounds in Table 8-12 have a trityl moiety in their structures, either on the oxygen in 5' position (entries **i, iii, iv, v, vi, viii** and **ix**) or on the 3' position (entry **ii**). The only structure without a trityl group is the direct analogue of the lead MV002293.

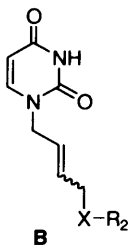
The 2'-deoxyuridine analogue **11** (entry **vii**) with a hydroxypropyl chain in the 5'-position had an IC_{50} of 9 μM *in vitro* against *L. donovani*, whilst the lead MV002293 had an IC_{50} of 131 μM. The two compounds differ only in the nucleobase (uracil for **11**, thymidine for MV002293), thus confirming that the enzyme is selective toward the base. By simple replacement of the thymidine with uracil increased the IC_{50} value nearly 15 times. However the two data are not directly comparable since the lead compound MV002293 was tested against the amastigote form of the parasite within the macrophage; whereas compound **11** was tested against an axenic (without the macrophage) culture of the amastigote form *L. donovani*, and therefore more susceptible to the effect of the drug.

It is difficult to understand the effect of the substituent in 3'-position. The overall effect could be that bulky groups (OTr, mesylate, sulphonamide) are preferred to a small polar group (OH, NH_2), even though the best result obtained is with the azide which could be considered as a small polar group, but again no further conclusions can be advanced because of the absence of enzyme data.

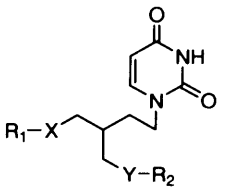
8.2.2 Branched and unsaturated acyclic uracil series



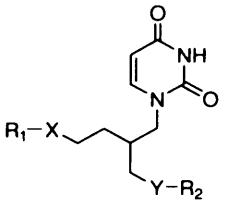
A
80a



B
69
72



C
101
97
102



D
106
108
107

	Structure	Cmpd	Enzyme K_i	In vitro IC_{50}	Toxicity	X	Y	n	R_1	R_2
i	B	69	n.a.	2.13	38.7	O		4 (*Z*)		Trityl
ii	C	97	n.a.	2.6	14.7	O	NH		Trityl	Boc
iii	D	108	n.a.	2.9	18.4	O	N_3		Trityl	
iv	B	72	n.a.	4.81	33.9	O		4 (*E*)		Trityl
v	D	106	n.a.	6.86	57.1	O	O		H	Trityl
vi	D	107	n.a.	8.42	32.6	O	O		Trityl	H
vii	C	102	n.a.	10.13	55.4	O	NH		H	Trityl
viii	C	101	n.a.	11.38	37.3	O	NH		Ac	Trityl
ix	A	80a	n.a.	20.2	206.7	O		3 (*E*)		$MeCO_2$-

Table 8-13 *In vitro* results for unsaturated and branched acyclic uracil derivatives against *L. donovani*

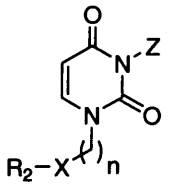
All the branched and unsaturated derivatives had a quite good activity with IC_{50} values between 2.1 and 20 μM.

The best inhibitor is the compound **69** 1-((Z)-4-(trityloxy)but-2-enyl)uracil) with IC_{50} of 2.13 μM. The configuration of the double bond was not as important as for *P. falciparum* results. The second isomer with *E* configuration (entry **iv**) reported an IC_{50} 2 times higher than the *Z* isomer, therefore not a very significant difference between the two isomers.

The N-Boc protected trityloxy derivative **97** (entry **ii** in Table 8-13) inhibited the parasite's growth with IC_{50} of 2.6 μM. This compound can mimic the corresponding 3'-amino-2'-deoxyuridine (entry **ix** in Table 8-12) which had IC_{50} 10 fold higher. The reason of this increased activity could be attributed either to the rigidity of the cyclic structure orienting the substituents in sub-optimal positions or to the presence of the Boc group in the acyclic structure which could help in the penetration inside the parasite.

A second interesting result comes from the azide derivative **108** (entry **iii** in Table 8-13), which showed an IC_{50} factor of 2.9 μM. This value is in the same range as that obtained for the 3'-azido-uridine derivative **55** (entry **i** in Table 8-12), thus confirming that the azide group is effective in the inhibition of Leishmania growth.

8.2.3 Aliphatic acyclic uracil series



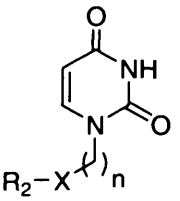
		WSP	Enzyme K_i	In vitro IC_{50}	Toxicity	X	Z	n	R_2
1	**39e**	1142	>1000	5	23.4	O		5	Trityl
2	**40**	1228	-	12	46.6	O		5	Ph_3Si
3	**39f**	1224	-	17.5	39.13	NH		5	Trityl
4	**30a**	962	>1000	95.9	192	O		3	TBDMS
5	**31c**	960	>1000	-	>126	O	$-(CH_2)_3OTr$	3	Trityl
6	**30c**	961	>1000	-	34.4	O		3	Trityl
7	**39c**	1208	-	>151	>454	O		5	H

Table 8-14 Results for the aliphatic acyclic uracil derivatives against *L. major* dUTPase and in vitro against *L. donovani*

This series of compound showed a different behaviour compared to the results obtained for the parasite *P. falciparum.*

The only data available on the enzyme inhibition showed that this class of molecules is not acting on the enzyme dUTPase (all the *Ki* values are higher than 1mM), consequently the activity observed in the parasite growth inhibition is due to a different mechanism of action.

8.2.4 Acyclic phosphate and phosphonate uracil series



		WSP	Enzyme K_i	In vitro IC_{50}	Toxicity	X	n	R_2
1	42d	1225		>102	>308	O	6	$-OPO_3H_2$
2	43b	1151	>1000	>103	>310	-	3	$-PO_3Et_2$
3	42c	1144	>1000	>107	>323	O	5	$-OPO_3H_2$
4	43a	1150	>1000	>108	>325	-	2	$-PO_3Et_2$
5	42b	1143	>1000	>113	>340	O	4	$-OPO_3H_2$
6	49b	1227		>114	>343	-	5	$-PO_3H_2$
7	42a	1146	>1000	>119	>360	O	3	$-OPO_3H_2$
8	49a	1226		>120	>362	-	4	$-PO_3H_2$
9	44b	1147	>1000	>128	>384	-	3	$-PO_3H_2$
10	44a	1145	>1000	>136	>408	-	2	$-PO_3H_2$
11	51	1148	>1000	>84	>252	-	3	$-PO_3H_2$

Table 8-15 Results for the aliphatic phosphonates and phosphates uracil derivatives against both *L. major* dUTPase and in vitro against *L. donovani*

As for the *P. falciparum* assays in the case of *L. major* dUTPase the series of acyclic phosphates and phosphonates was found inactive in both biological tests.

Chapter 9 Microbiology

9.1 Pathogenic bacteria

Bacteria are single-celled micro-organisms that lack a nuclear membrane (prokaryotic cells), are metabolically active and divide by binary fission. Medically they can be a major cause of disease. Bacteria that cause disease are called pathogenic bacteria. The diseases caused by bacteria are diverse and include food poisoning, tooth ache, anthrax and even certain forms of cancer[125].

Superficially, bacteria appear to be relatively simple forms of life; in fact they are sophisticated and highly adaptable. Many bacteria multiply at a rapid rate, and different species can utilize an enormous variety of hydrocarbon substrates, including phenol, rubber and petroleum. These organisms exist widely in both parasitic and free-living forms. Because they are ubiquitous and have a remarkable capacity to adapt to changing environments by selection of spontaneous mutants, the importance of bacteria in every field of medicine cannot be overstated.

Major advances in bacteriology over the last century have resulted in the development of many effective vaccines (e.g. pneumococcal polysaccharide vaccine, diphtheria toxoid, and tetanus toxoid) as well as of other vaccines (e.g., cholera, typhoid, and plague vaccines) that are less effective or have side effects. Another major advance was the discovery of antibiotics. These antimicrobial substances have not eradicated bacterial diseases, but they are powerful therapeutic tools. Their efficacy is reduced by the emergence of antibiotic resistant bacteria, now an important medical management problem. In reality, improvements in sanitation and water purification have a greater effect on the incidence of bacterial infections in a community than does the availability of antibiotics or bacterial vaccines. Nevertheless, many serious bacterial diseases remain[126] and anti-biotics are very important in the treatment of these diseases.

9.2 Aims and objectives

The objective of this small project of work was to investigate the antimicrobial activity of some compounds from the chemical library available in the Welsh School of Pharmacy of Cardiff University. The compounds were designed and synthesised as potential inhibitors of dUTPase enzyme. As shown in the earlier chapters the enzyme dUTPase is widespread in nature and also found in bacteria. Therefore inhibitors of bacterial dUTPases might have eventual application as antibacterial agents.

9.3 Antimicrobial evaluation

The screening to evaluate the antimicrobial activity was performed by measuring the MIC (Minimum Inhibitory Concentration) values for the compound library. The minimum inhibitory concentration is defined as the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a micro-organism after overnight incubation. MICs are used by diagnostic laboratories, to confirm resistance, but most often as a research tool to determine the *in vitro* activity of new antimicrobial substances[127].

Several methods are approved and recommended by different committees such as the National Committee for Clinical Laboratory Standards (NCCLS) or the British Society for Antimicrobial Chemotherapy (BSAC) for the *in vitro* susceptibility test of chemical entities against micro-organism (bacteria, yeast and fungi). One of these methods, the Broth Microdilution Method (BMD)[128] was chosen as reference protocol for this project. The procedure applied for the screening project was then based on the BMD method, although some modifications were introduced.

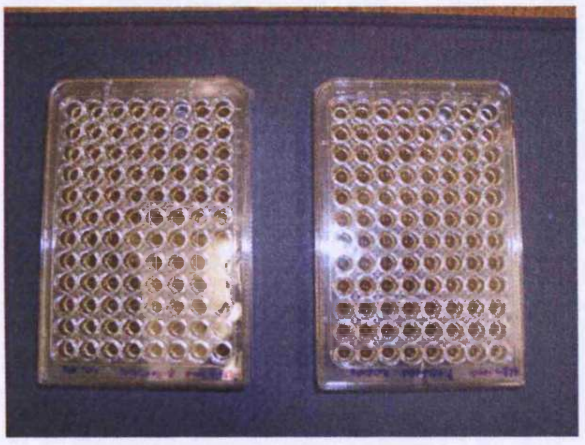


Figure 9-1 Picture of the 96 well plates used for the screening with the BMD procedure

With the BMD method several concentrations of a chemical compound (dilutions) are tested by overnight incubation with suspensions of standard bacterial strains, using a 96 well plates (Figure 9-1).

The effects produced by the different concentrations of compounds on the bacteria strains are evaluated by comparison of the bacterial growth with reference controls made of

(i) Bacterial strains & nutrition broth,

(ii) Bacterial strain & nutrition broth & solvent (DMSO),

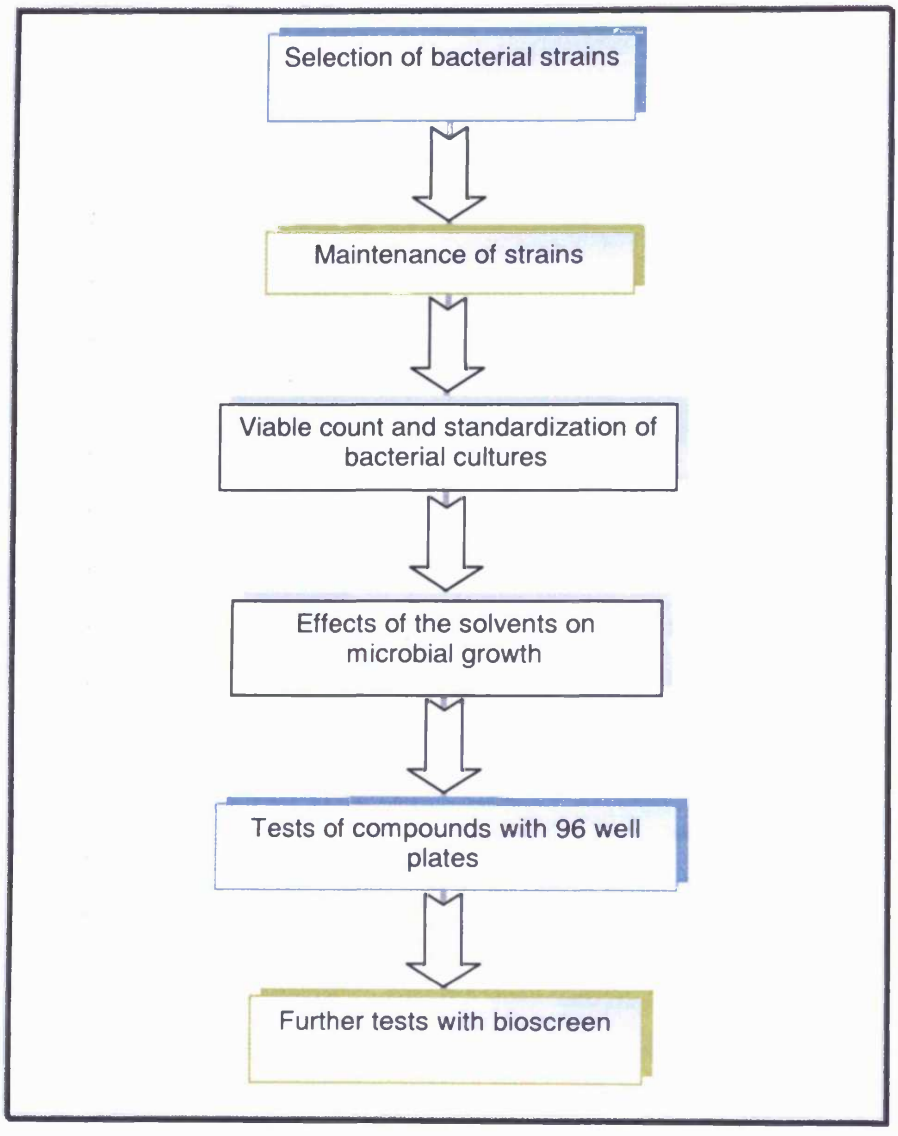
(iii) Nutrition broth alone,

(iv) Nutrition broth & solvent.

The first two are the positive controls which should show the normal growth of the bacteria in the conditions used for the tests. The remaining two are the negative controls which do not show any bacterial growth.

The lowest concentration of a compound which inhibits the growth is considered as the MIC value for the corresponding compound.

In Scheme 9-1 is reported a flow chart depicting all the steps followed in this microbiology project, in order to achieve the proposed aims.



Scheme 9-1 Flow chart of microbiology work done to the compounds

9.4 Bacterial strains

Five standard strains were selected to carry out the screening, according to the NCCLS these were:

- *Staphylococcus aureus.*
- *Enterococcus faecalis*
- *Pseudomonas aeruginosa.*
- *Proteus vulgaris*
- *Escherichia coli*

The first two are Gram-positive bacteria and the remaining three are Gram-negative. The Figure 9-2 and Table 9-1 summarize the main differences between Gram+ and Gram – bacteria.

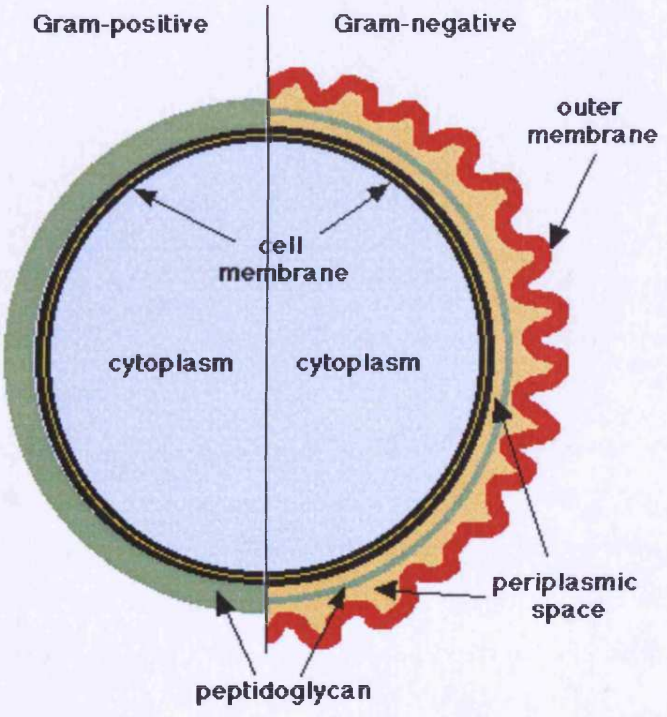


Figure 9-2 Schematic representation of the difference between Gram-positive and Gram-negative bacteria

Gram-positive	Gram-negative
Thicker cellular wall made of peptidogycan	Very thin cellular wall
Presence of teichoic acids in the cellular membrane	Absence of teichoic acid in the cellular membrane
Absence of outer membrane	Outer membrane made of lipid, protein and lipopolysaccaride, with porin protein that create pores
Absence of periplasim space between the inner membrane and the cellular wall	Large periplasmic space between inner membrane, cellular wall and outer membrane. This space contains many specialized proteins.

Table 9-1 Differences between Gram+ and Gram- bacteria

Due to the presence of the outer membrane, the Gram-negative bacteria are generally more resistant to adverse conditions and to antimicrobial agents than the Gram-positive.

9.5 Maintenance of strains

The bacterial strains were maintained by growing them with the appropriate medium (Tryptone Soya Broth TBS or Tryptone Soya Agar TSA). The strains were first incubated in a suspension of nutrient broth by incubation at 36 °C for 16 hours. The bacterial suspension was then transferred onto plates with agar as nutrient medium. The suspension was spread on the plates using a sterilised (by flame) loop. The transfer from bacterial suspension to plate culture was done in order to isolate single colonies of each bacteria strain. The plates were incubated at 36 °C at the same temperature for 16 hours.

After the incubation period, a single colony for each strain was taken from the plates and used to inoculate vials containing nutrient broth. The vials were incubated overnight under constant shaking, to generate the bacterial suspensions that were used for the viable count and the antimicrobial screening.

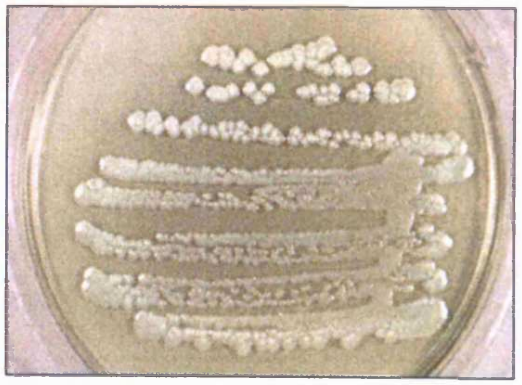


Figure 9-3 Pictures of *P. aeruginosa* strain on TSA agar plate

9.6 Viable count and standardization

The standard conditions required for antimicrobial screening with the BMD method are restricted to the use of bacterial suspensions having 10^5-10^6 cells per ml, or colony forming units (cfu). This required the standardization of the cell cultures in order to know the bacterial density for each suspension.

The number of bacterial cells in a suspension can be measured by turbidimetry. Bacterial suspensions absorb at an appropriate wavelength of electromagnetic radiation (500 nm) and the absorption is directly related to the number of cells present in the suspension. This technique, known as optical density measurement, requires the drawing of a chart which reports the relationship between absorbance and number of cell per unit of volume (standardization curve). Once this chart is obtained it is possible to control the number of cell of bacterial suspension by simple dilution to adjust the absorbance to the values corresponding to the desired cfu, according to the calibration curve, (10^5-10^6 cfu).

The procedure used to obtain the required data for the standardization curve is illustrated in Figure 9-4.

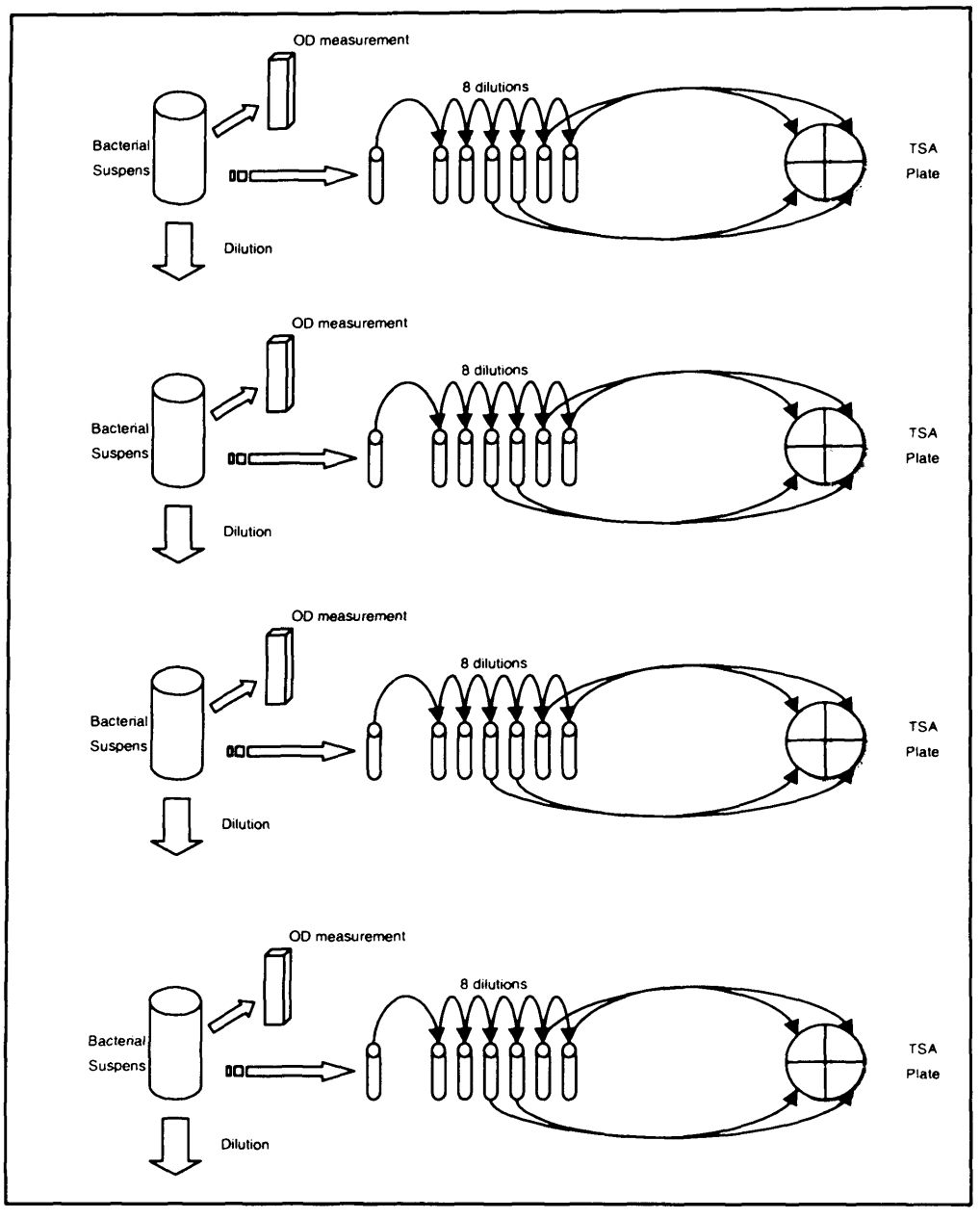


Figure 9-4 Schematic representation of the drop counting method used for the construction of the standardization curve for each bacterial strain

After the optical density measurement (OD) the microbial suspensions were diluted 8 times, inoculated in TS agar (TSA) plates and incubated overnight (first row in Figure 9-4). The main suspension was then diluted once and the steps previously described were applied again (second row in Figure 9-4). These operations were repeated two further times (third and fourth rows), and two further optical density values were recorded after two dilutions. By counting the number of colonies developed after incubation in each TSA plate, it was possible

to correlate the OD values recorded for the mother suspension with the number of cells present in it.

The data were used to plot the calibration curve as shown in Figure 9-5.

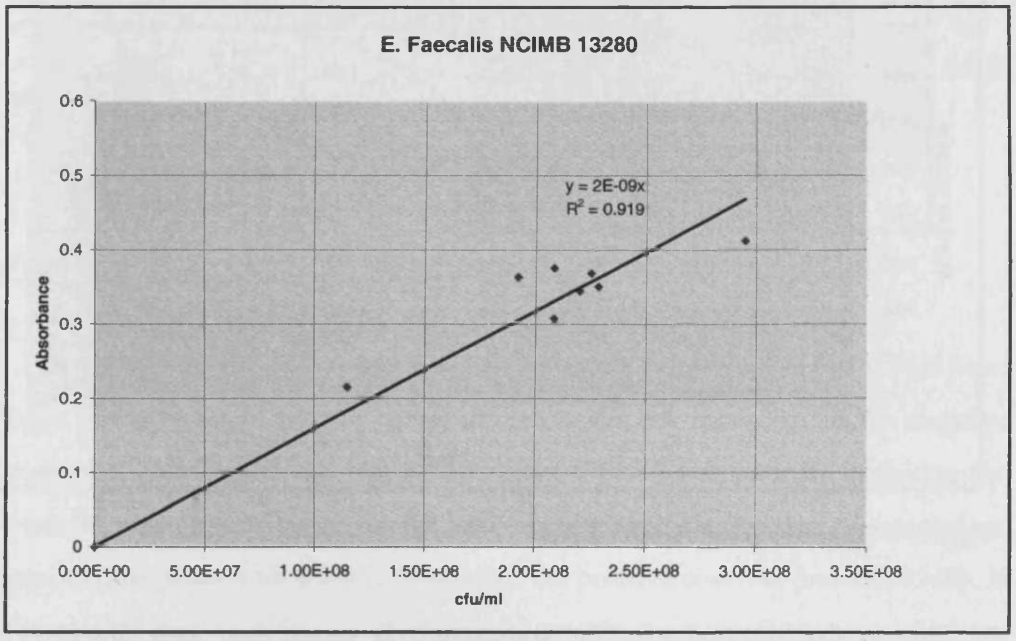


Figure 9-5 Calibration curve for the *E. faecalis* strains, the other curves are reported in appendix

9.7 Effect of the solvent on bacterial growth

The bacterial growth over time can be plotted as cell number over time. This is called a growth curve, Figure 9-6. The growth curve can be divided in 3 main phases: the lag phase, the log phase and the stationary phase.

The lag phase is the first part of the curve, when the number of bacterial cell is not increasing significantly. During this phase the bacteria are adapting their metabolism to the growth medium and they are multiplying slowly.

The second phase is the log or exponential phase, which correspond to the part with increased slope in the chart. During this phase the bacteria start to multiply very rapidly and the number of cells is increasing exponentially.

The third phase, the stationary phase, is the last part corresponding to the plateau value. During this phase the bacterial culture reached the maximum number of cell; but now no increment is observed due to the increased production of toxic substances, by the bacteria themselves, which slows down the bacterial metabolism and the division of the cells.

Consequently growth curves are "S" shaped with different slopes of log phase depending on the bacterial strain and its metabolism.

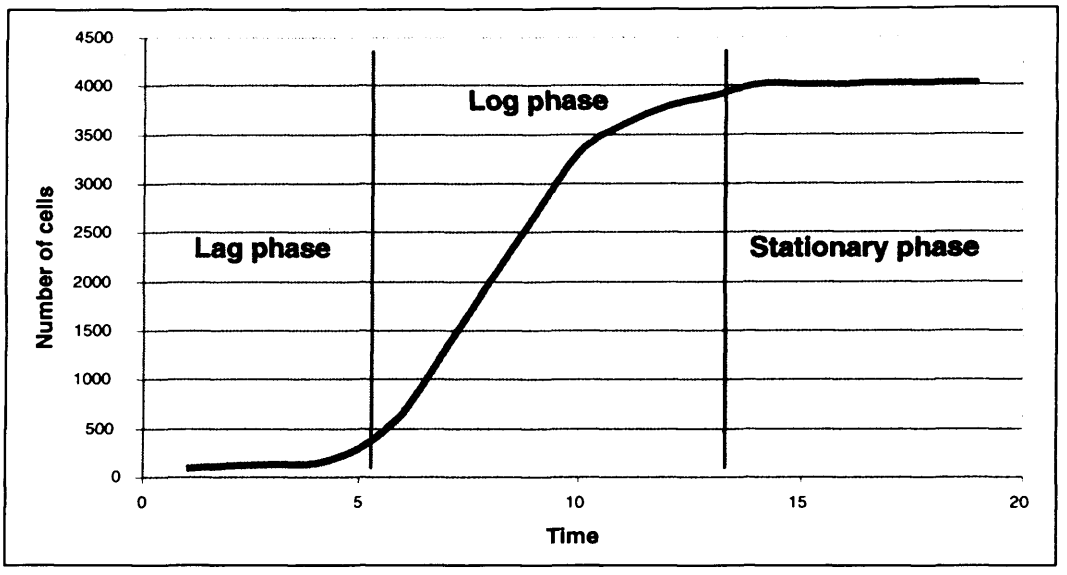


Figure 9-6 Shape of a generic growth curve

Due to the insolubility of the compounds in water, they were dissolved in DMSO; therefore it was required a first study to understand the effect of the solvent (DMSO) on the bacterial growth. This was done to identify the maximum concentration of dimethylsulfoxide tolerated by the bacteria strains, in order to avoid false antimicrobial activity attributed to the compound, but actually due to a too high concentration of DMSO in the bacterial suspension.

This preliminary study was done by recording the bacterial growth curves of the five strains in the presence of different concentrations of DMSO during the 16 hours of incubation. The template of the 96 well plate used for this experiment is illustrated in Figure 9-7.

Six different concentrations of DMSO were tested against the five bacterial strains in duplicate (rows A, B, C, D, E and F in the template illustrated in Figure 9-7). The control used in this test were suspensions of the bacteria with only nutrient broth (rows G and H), the highest concentration of DMSO used (row I, L in columns 1, 2), a solution of nutrient broth & DMSO (rows I, L, columns 3, 4) and a solution of only nutrient broth (rows I, L, columns 4, 5).

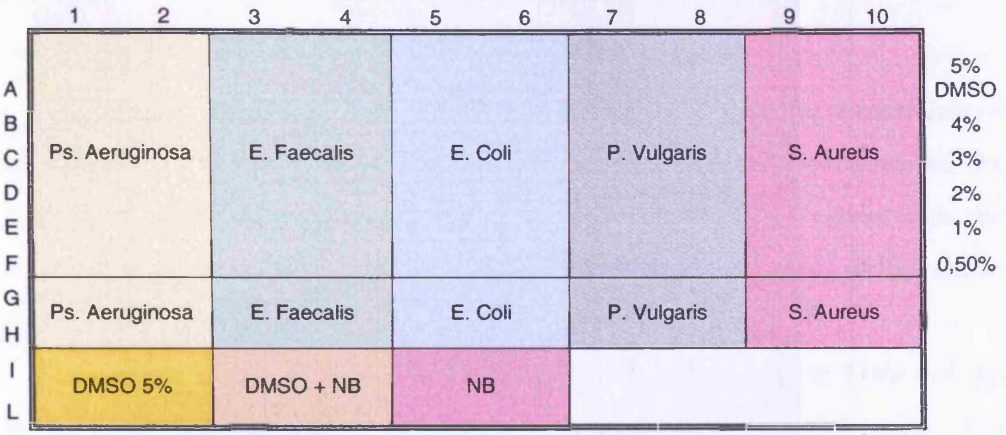


Figure 9-7 Template of the 96 well plate used to test different concentration of DMSO

If the solvent was totally inhibiting the bacterial growth a flat line would have been observed in the growth curve, similar to the one recorded for the negative controls (DMSO+NB and NB alone). If the DMSO was partially inhibiting the bacteria, the growth curve would have shown an increased lag phase and an exponential phase with a lower slope than the positive controls (bacteria+NB). If the solvent had no effect on the bacterial growth, the normal curve would have been recorded.

The results for the test are reported in Figure 9-8. It is clearly visible that none of the six concentrations of DMSO inhibited the bacterial growth; since no significant changes in the bacterial growth curves could be found between the cultures in the presence of DMSO and the controls cultures with only nutrient broth. Only the highest tested concentration of DMSO (5%) slightly reduced the log phase in the bacterial growth, but this effect was not significant enough to consider such concentration toxic for the bacteria; however it was decided to use a concentration of maximum 2.5% of DMSO to carry on with the tests.

Therefore the use of DMSO as solvent was compatible with the test protocols to evaluate the MIC values.

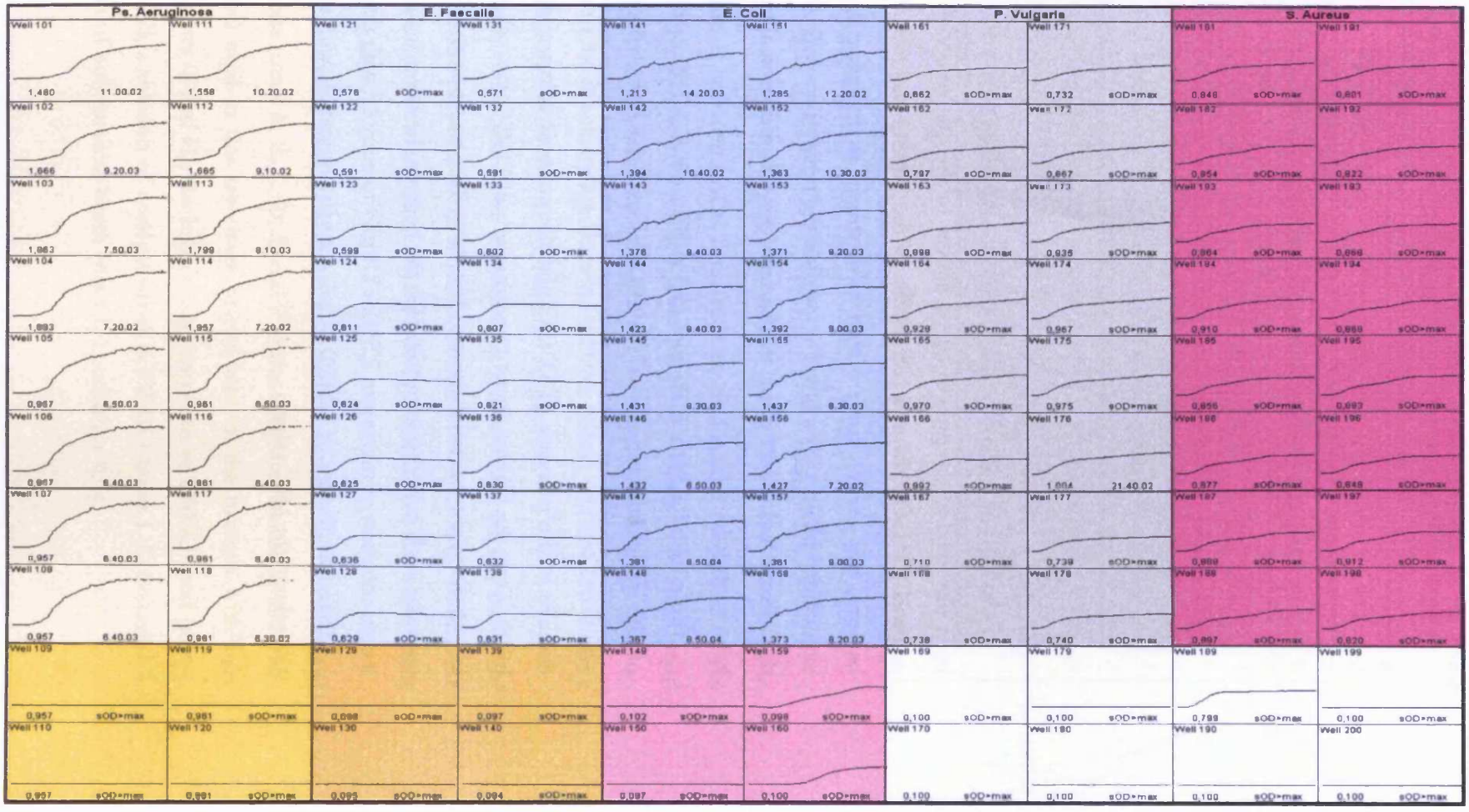


Figure 9-8 Bacterial growth curves in the presence of decreasing DMSO concentrations, for a complete description of the table refer to the template in Figure 9-7

9.8 Antimicrobial screening

The 96 well plates were used to evaluate the MIC values for the compounds of the chemical library. These were obtained by measuring the absorbance of the culture after 16 hours of incubation and comparing the OD values with the controls: positive (NB and micro-organism) and negative (NB alone, NB+DMSO, DMSO alone).

A template for the 96 well plate used for this test is illustrated in Table 9-2. To avoid contamination a single plate was used for each bacterial strain. Ten compounds in 5 different concentrations were tested in duplicate against one bacterial strain with one 96 well plate.

The wells in the first two columns were filled with the five controls NB, DMSO, NB+DMSO, micro-organism+DMSO+NB and micro-organism+NB. The first three are the negative controls the latter two are the positive.

The remaining ten columns were used to test the bacterial suspension in the presence of several concentrations of the compounds. Each compound was tested in duplicate. For example the wells 3A and 4A had the same compound in the same concentration; the following well in the same columns (the wells 3B and 4B) had the same compound with a diluted concentration of that present in the two previous wells.

<table>
<tr><th></th><th colspan="2">1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
<tr><td>A</td><td colspan="2" rowspan="3">NB
DMSO
NB+DMSO</td><td rowspan="4"></td><td rowspan="4"></td><td rowspan="4"></td><td rowspan="4"></td><td rowspan="4"></td><td rowspan="4"></td><td rowspan="4"></td><td rowspan="4"></td><td rowspan="4"></td></tr>
<tr><td>B</td></tr>
<tr><td>C</td></tr>
<tr><td>D</td><td colspan="2" rowspan="3">µorg+DMSO+NB</td></tr>
<tr><td>E</td><td rowspan="4"></td><td rowspan="4"></td><td rowspan="4"></td><td rowspan="4"></td><td rowspan="4"></td><td rowspan="4"></td><td rowspan="4"></td><td rowspan="4"></td><td rowspan="4"></td></tr>
<tr><td>F</td></tr>
<tr><td>G</td><td colspan="2" rowspan="2">µorg + NB</td></tr>
<tr><td>H</td></tr>
</table>

Table 9-2 Template for the 96 well plate used for the MIC determination

Once ready the plates were incubated overnight. When antimicrobial activity was observed the optical density measured for the well containing a certain concentration of the drug, was similar to the values measured for the negative controls. The lowest concentration of compound which inhibits the bacterial

growth is the corresponding MIC for the compound. When no antimicrobial activity was observed, the bacteria in the well were growing without difficulty, and then the OD values were similar to the positive controls. If a compound did not have any antimicrobial activity in the concentrations used for the test, it was assumed that its MIC values would have been higher than the concentrations used in the screening. All the detailed procedures and experiment are reported in the experimental section.

9.9 Results and Discussion

9.9.1 Bacterial growth and OD500

Standardisation for all the bacterial strains was performed; *Escherichia coli* NCIMB 8879, *Enterococcus faecalis* NCIMB 13280, *Staphylococcus aureus* NCTC 6571, *Proteus vulgaris* NCIMB 8067 and *Pseudomonas aeruginosa* NCTC 15442. Plots which relate the OD_{500} measurements with the number of viable cells (CFU/ml) per millilitre of suspension (cfu/ml) for all the strains used are shown in appendix.

For subsequent experiments (i.e. bacterial growth and MICs) the bacterial inoculum was adjusted to the appropriate concentration (approx. 10^6 CFU/ml) using these standard plots.

The effect of the solvent (DMSO) was investigated also with the 96 well plates reading the final absorbances after the 16 hours of incubation, and comparing them with the values obtained for the controls.

	1	2	3	4	5	6	7	8	9	10	11	12	DMSO
A	0.241	0.24	1.484	1.587	0.698	0.9	1.666	1.353	1.193	1.171	0.597	0.64	5%
B	0.24	0.249	1.628	1.73	0.765	0.993	1.385	1.379	1.152	1.185	0.574	0.652	4%
C	0.247	0.251	1.733	1.81	0.627	1.028	1.405	1.396	1.087	1.049	0.568	0.646	3%
D	0.248	0.248	1.781	1.869	0.899	1.044	1.422	1.487	1.297	1.252	0.649	0.716	2%
E	0.031	0.032	1.869	1.94	0.81	1.046	1.445	1.446	1.229	1.226	0.736	0.811	1%
F	0.027	0.027	1.866	1.961	0.816	1.04	1.428	1.438	1.273	1.227	0.757	0.792	0,50%
G			1.965		0.824	1.065	1.423	1.425	1.021	1.009	0.739	0.813	0%
H					0.813	1.01	1.463	1.461	1.129	1.081	0.903	1.076	0%

Table 9-3 Optical density measurements of the growth of bacteria in the presence of various concentrations of DMSO, after 16 hours of incubation at 37°C. Bacterial species as follows: columns 3,4 *Ps. aeruginosa*; columns 5,6 *E. faecalis*; colums 7,8 *E. coli*; columns 9,10 *P. vulgaris*; columns 11,12 *S. aureus*

	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.234	0.235	1.836	1.898	0.753	0.927	1.431	1.433	1.206	1.24	0.694	0.729	2,50%
B	0.241	0.236	1.872	1.937	0.793	0.975	1.453	1.464	1.271	1.276	0.657	0.784	2,50%
C	0.223	0.239	1.831	1.94	0.837	1.024	1.448	1.473	1.279	1.27	0.675	0.705	3%
D	0,235	0.240	1.782	1.867	0.828	1.029	1.430	1.428	1.254	1.278	0.622	0.723	2%
E	0.033	0.031	1.876	1.915	0.814	1.001	1.453	1.461	1.258	1.279	0.631	0.679	1%
F	0.03	0.027	1.871	1.981	0.831	1.012	1.481	1.499	1.279	1.287	0.639	0.68	0,50%
G			1.872	1.966	0.825	1.01	1.585	1.577	1.001	1.023	0.694	0.697	0%
H			1.936	1.994	0.84	1.021	1.601	1.595	1.032	1.06	0.762	0.737	0%

Table 9-4 Optical density measurements of the growth of bacteria in the presence of various concentrations of DMSO, after 16 hours of incubation at 37°C. Bacterial species as follows: columns 3,4 *Ps. aeruginosa*; columns 5,6 *E. faecalis*; colums 7,8 *E. coli*; columns 9,10 *P. vulgaris*; columns 11,12 *S. aureus*

In Table 9-3 and Table 9-4 the effects of different concentration of DMSO on OD_{500} are presented. In columns 3-12 the final values of OD_{500} had no differences from the values recorded for the controls, rows G-H, columns 3-12. Every bacterial strain is indicated with a different colour in the table.

These results confirmed the data reported with the bacterial growth curve, that a concentration up to 5% v/v of DMSO was not toxic for the bacterial strains

tested. Nevertheless, a final concentration of 1.25% v/v DMSO was used for subsequent MIC and bacterial growth experiments.

9.9.2 MIC results

A summary of results for all the compounds against all the micro-organisms tested is presented in Table 9-5.

	Micro-organism				
WSP	*S. aureus*	*P. vulgaris*	*E. faecalis*	*E. coli*	*Ps. aeruginosa*
483	>163	>163	>163	>163	>163
485	>194.2	>194.2	>194.2	>194.2	>194.2
487	>205.4	>205.4	>205.4	>205.4	>205.4
665	>155.4	>155.4	>155.4	>155.4	>155.4
658	>164.8	>164.8	>164.8	>164.8	>164.8
659	>236.4	>236.4	>236.4	>236.4	>236.4
660	>414.9	>414.9	>414.9	>414.9	>414.9
661	>292.4	>292.4	>292.4	>292.4	>292.4
662	>611.5	>611.5	>611.5	>611.5	>611.5
663	>524.6	>524.6	>524.6	>524.6	>524.6
666	>219.7	>219.7	>219.7	>219.7	>219.7
667	>254.7	>254.7	>254.7	>254.7	>254.7
669	>359	>359	>359	>359	>359
668	>473.8	>473.8	>473.8	>473.8	>473.8
680	>216.6	>216.6	>216.6	>216.6	>216.6
704	>127	>127	>127	>127	>127
788	>1002.9	>1002.9	>1002.9	>1002.9	>1002.9
784	>507.4	>507.4	>507.4	>507.4	>507.4
785	>340.8	>340.8	>340.8	>340.8	>340.8
786	>219.1	>219.1	>219.1	>219.1	>219.1
787	>398.8	>398.8	>398.8	>398.8	>398.8
789	>163.3	>163.3	>163.3	>163.3	>163.3
791	>298.6	>298.6	>298.6	>298.6	>298.6
804	>407,1	>407,1	>407,1	>407,1	>407,1
826	>264	>264	>264	>264	>264
828	>436.6	>436.6	>436.6	>436.6	>436.6
829	>332	>332	>332	>332	>332
830	>403.3	>403.3	>403.3	>403.3	>403.3
872	>410.9	>410.9	>410.9	>410.9	>410.9
873	>532.2	>532.2	>532.2	>532.2	>532.2
874	>757.6	>757.6	>757.6	>757.6	>757.6

875	>432.3	>432.3	>432.3	>432.3	>432.3
929	>250	>250	>250	>250	>250
931	>174.5	>174.5	>174.5	>174.5	>174.5
932	>187.5	>187.5	>187.5	>187.5	>187.5
665	>190.5	>190.5	>190.5	>190.5	>190.5
865	>218.5	>218.5	>218.5	>218.5	>218.5
866	>178.3	>178.3	89.0	>178.3	>178.3
867	99.00	>795.8	398.00	>795.8	>795.8
868	>426.4	>426.4	>426.4	>426.4	>426.4
869	>157.7	>157.7	>157.7	>157.7	>157.7
870	>168.3	>168.3	>168.3	>168.3	>168.3
1000	>137.7	>137.7	>137.7	>137.7	>137.7
1001	>115.8	>115.8	>115.8	>115.8	>115.8
1002	>212.4	>212.4	>212.4	>212.4	>212.4
1003	>196.8	>196.8	>196.8	>196.8	>196.8
1004	>79.7	>79.7	>79.7	>79.7	>79.7
1005	98.0	>98	24.0	>98	>98
484	>154.1	>154.1	>154.1	>154.1	>154.1
486	>125.6	>125.6	>125.6	>125.6	>125.6
706	>129.9	>129.9	>129.9	>129.9	>129.9

Table 9-5 MIC values for all the tested compounds in μM. The results highlighted in red are those for the compound which showed antibacterial activity

Optical density measurements (OD_{500}), after incubation at 37 °C, showed no difference between the control wells (i.e. bacteria+TSB+DMSO 1.25% v/v) and the wells containing decreasing concentrations of all the tested compounds for *Ps. aeruginosa*, *P. vulgaris* and *E. coli*. Therefore, as shown in Table 9-5 all the screened compounds had MIC values higher than the maximum concentration used in the tests against this Gram-negative bacterial strains.

Some activity was observed with the two Gram-positive strains *S. aureus* and *E. faecalis*. Against these two strains three out of 51 compounds had MIC values within the tested concentrations, as reported with the red values in Table 9-5.

The compound WSP 867 had an MIC value of 99 μM (44 μg/ml) and 398 μM (178 μg/ml) against *S. aureus* and *E. faecalis* respectively. In addition, WSP 866 had an MIC of 89 μM (40 μg/ml) against *E. faecalis*; WSP 1005 was found active against the same strain with MIC of 24 μM (11 μg/ml), whilst it had a MIC of 98 μM against *S. aureus*.

The structures and some chemical properties of these compounds are shown in Table 9-6.

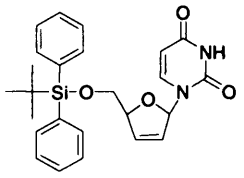
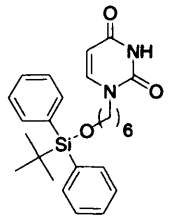
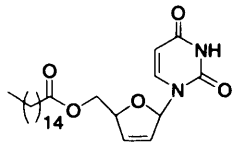
Structure	Name	C log P	Mol. Weight
	867	5.1	448.6
	1005	5.7	450.6
	866	6.8	434.45

Table 9-6 Structure, molecular weight and C log P for the active compounds

To complete and verify these results, the three compounds underwent further investigation with the Bioscreen Microbiological Growth C Analyser, which can monitor the bacterial growth during 16 hours of incubation, in order to obtain more accurate MIC values, or to confirm the value obtained with the BMD method.

In this study four decreasing concentrations of each compound were tested with the sensitive stains (*S. aureus* and *E. faecalis*). The bacterial growth was monitored and compared with the same controls used in the previous tests.

9.9.3 Effect of compounds on bacterial growth

The effects of the active or "potentially" active compounds on bacterial growth are shown in next charts.

The activity of WSP 866 was confirmed against both *E. faecalis* and *S. aureus*. (Figure 9-9 and Figure 9-10 respectively). Bioscreen experiment clearly showed a severe disruption of bacterial growth over a 16 hours period.

The growth of *E. faecalis* was totally inhibited with all four concentrations tested. As shown in Figure 9-9 no bacterial growth was observed with the four concentrations tested, 178 μM (dark blue line), 89 μM (magenta), 44 μM (yellow) and 22 μM (cyan). They produced flat growth curves, completely different from the positive controls (light blue and purple lines), thus in agreement with the previous assays the MIC for WSP 866 was found to be 22 μM.

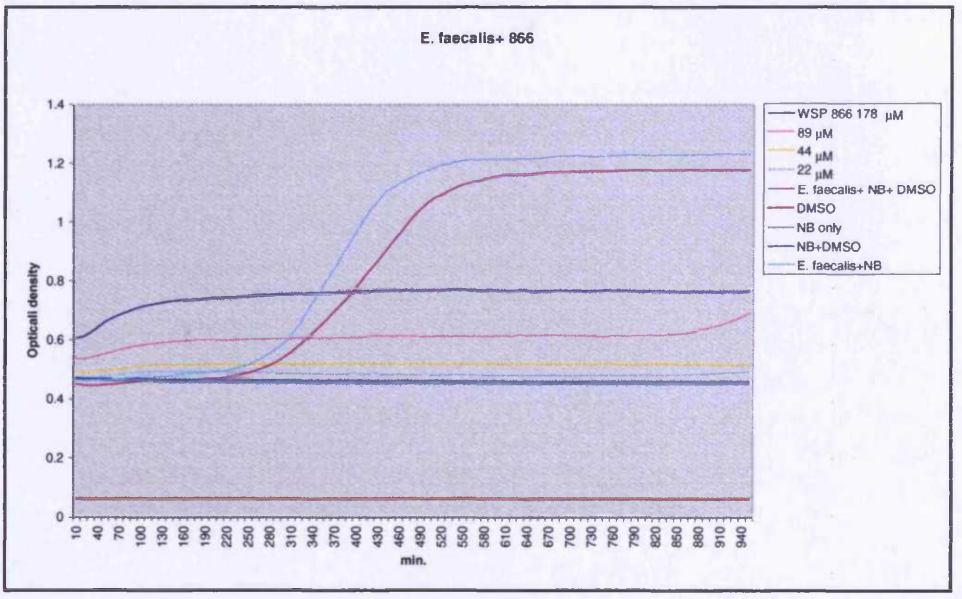


Figure 9-9 Bioscreen results for the compound WSP 866 against *E. faecalis*

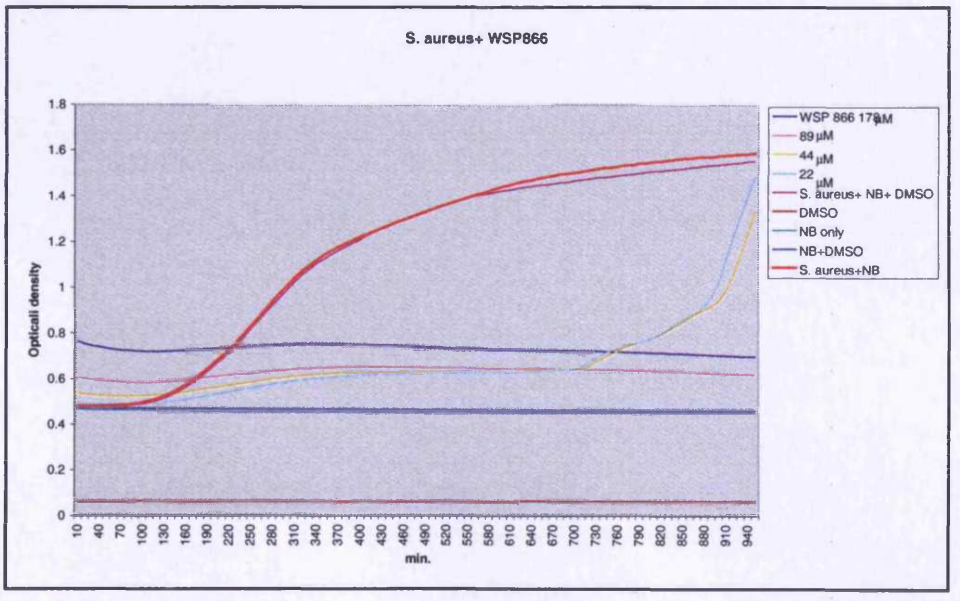


Figure 9-10 Bioscreen results for the compound WSP 866 against *S. aureus*

The same compound WSP 866 was also active against *S. aureus* with MIC of 89 μM, as shown in Figure 9-10. The concentrations of 178 μM (blue line) and 89 μM (pink line) did not show any bacterial growth for all the 16 hours of incubation; whilst the two more diluted concentrations 44 μM and 22 μM (yellow and cyan lines) only slowed the microbial growth. The chart shows that after 14 hours *circa* the micro-organism reached the exponential phase of the growth. Thus indicates that the concentration of compound was not enough active to stop completely the bacteria metabolism.

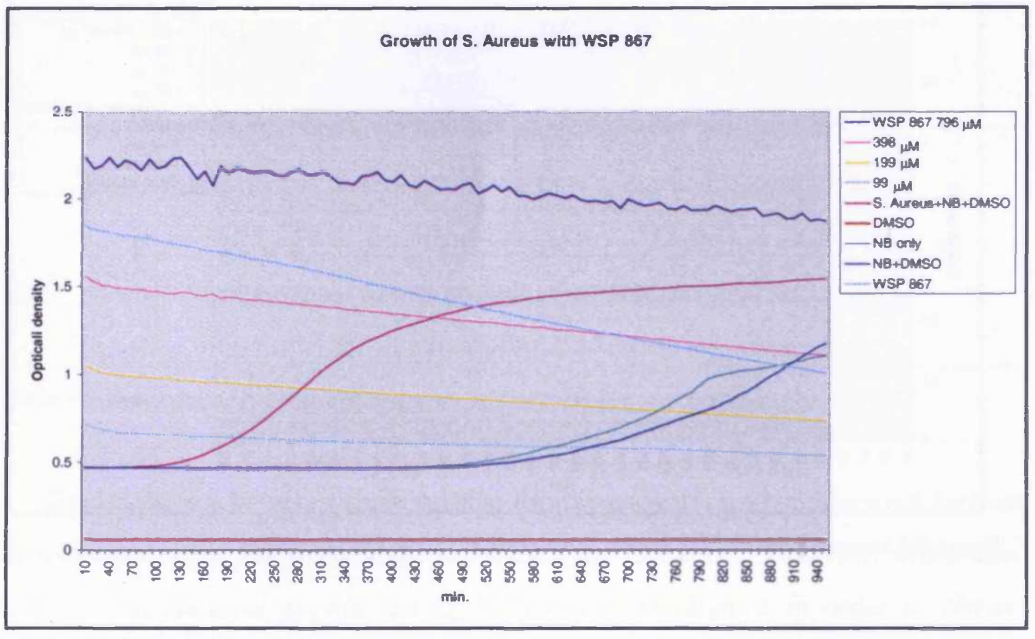


Figure 9-11 Bioscreen results for the compound WSP 867 against *S. aureus*

Problems were encountered during the monitoring of the OD_{500} for the compound WSP 867 with the *S. aureus* strain (Figure 9-11). In this case the compound precipitated in the suspension and interfered with the measuring of the absorbance values. For this reason the lines for the micro-organism in the presence of the compound (dark blue, magenta, yellow and cyan) had higher OD_{500} values than the controls. However for the lower concentrations tested (i.e. 199 μM and 99μM) the decreasing values of absorbance could indicate an inhibitory effect against the bacteria *S. aureus*. In this case the lowest concentration that inhibited the growth was 99 μM, and it was chosen as the MIC value, although this result has to be taken with caution.

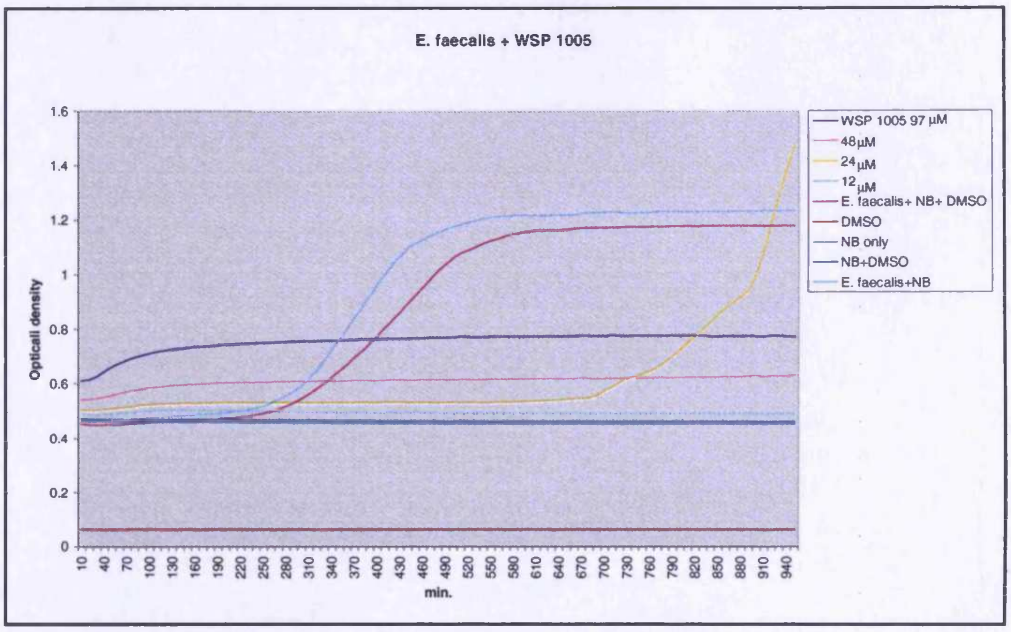


Figure 9-12 Bioscreen results for the bacterial growth of *E. faecalis* in the presence of WSP 1005

Compound WSP 1005 was studied for its effect on the growth of *E. faecalis* and *S. aureus* (Figure 9-12 and Figure 9-13).

This confirmed the reported activity against the *E. faecalis* strain (Figure 9-12). The compound clearly affected the growth of the micro-organism since the final OD_{500} measurement was much lower than that of the control for three out the four tested concentrations. The OD_{500} readings corresponding to treatment with WSP1005 24 µM (yellow line) increased sharply after 10h of incubation to higher values compared to the controls, indicating a possible contamination; therefore this result was not completely reliable. However the lowest concentration tested (cyan line) did not show any bacterial growth for all the 16 hours, indicating that the MIC for this compound is 12 µM.

Unfortunately the same WSP 1005 did not show any activity against *S. aureus* (Figure 9-13). Due to the small differences in the growth curve reported in the graph it is not possible to conclude that the compound can affect the growth of *S. aureus*. Therefore without inhibition of the bacterial growth, the MIC value for this compound is higher than the concentrations used for this test.

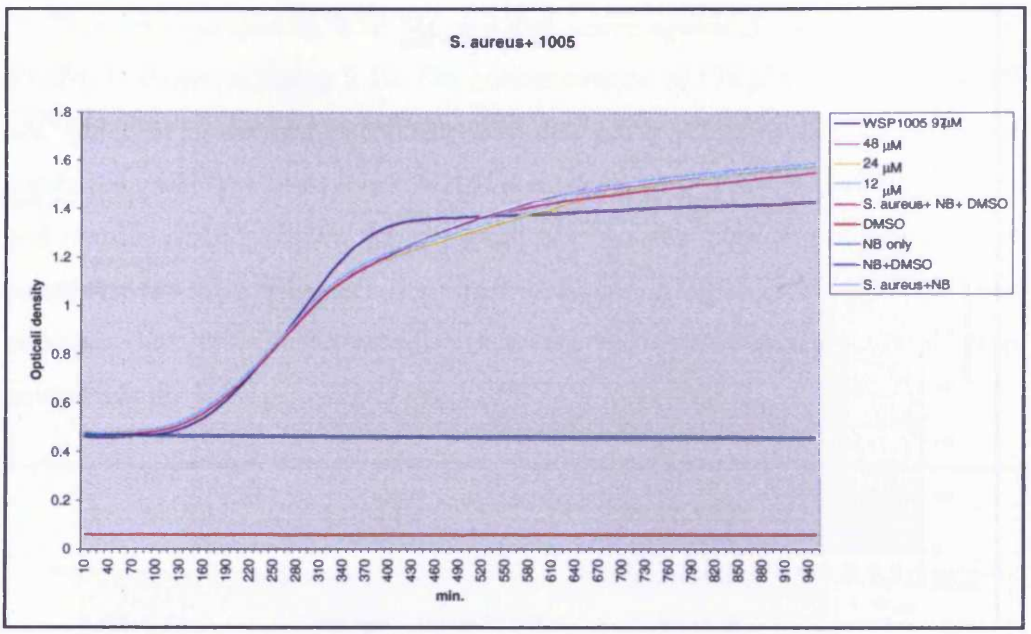


Figure 9-13 Bioscreen results for WSP 1005 against *S. aureus*

Some compounds that decreased the final OD_{500} (from MIC data) after 16h incubation at 37°C were also tested with the Bioscreen Microbiological Growth C Analyser.

Unfortunately the growth curves obtained in those tests did not show any kind of antibacterial activity.

9.9.4 Antibacterial activity of WSP 484, 486, 706 (insoluble compounds)

Compounds WSP 484, 486, 706 were completely insoluble in the mixture DMSO/Water and were tested with a different technique. Since the precipitation of the compound in the bacterial suspension would interfere with the optical density measurement. The use of an alternative procedure was required, in order to evaluate the bacterial growth without measuring the absorbance.

These compounds were added to the bacterial suspension, and incubated in vials with the micro-organism for 16 hours. After the incubation the suspensions were filtered through a membrane filters. The filters were then incubated in TS agar plate for further 16 hours. The bacterial growth was evaluated measuring the dimension of the spot generated on the surface of the filters by the bacterial

growth. The spots were compared with those obtained for the positive controls (only bacterial strains & nutrient broth) in order to understand if the presence of the compounds in the media had affected the normal bacterial growth.

The antibacterial activity of the insoluble compounds against all bacterial strains tested is shown in Table 9-7. None of the three compounds showed an antibacterial activity against the selection of test organisms, since bacterial growth was observed on the membrane filter after incubation. However, these results have to be taken with caution, since the lack of activity might be due to a poor solubility of the compounds, although the bacterial inoculums were incubated under constant agitation.

	Micro-organism				
	S. aureus	*P. vulgaris*	*E. faecalis*	*E. Coli*	*Ps. aeruginosa*
control	++	++	++	++	++
WSP 484	++	++	++	++	++
WSP 486	++	++	++	++	++
WSP 706	+	++	++	++	++

Table 9-7 Antibacterial activity of WSP 484, 486, 706. "++": normal growth; "+": slightly lower growth; "-": no growth

9.9.5 Conclusion

44 out of 69 compounds were tested for their antibacterial activity against five bacterial strains. Only three of them, WSP866, WSP867 and WSP1005 showed potential activity against the Gram-positive micro-organisms. Although an MIC was not always obtained, these compounds disrupted the growth of these micro-organisms. None of the compounds showed activity against the Gram-negative bacteria.

However, from this first screening it is not possible elucidate the mechanism of action of the compounds and particularly to ascertain whether the lack of activity, especially against the Gram-negative bacteria, was due to a poor penetration (uptake) of the compounds inside the micro-organism or to the lack

of activity at the target site (dUTPase) or capacity of the bacteria to overcome the effect produced by the compounds.

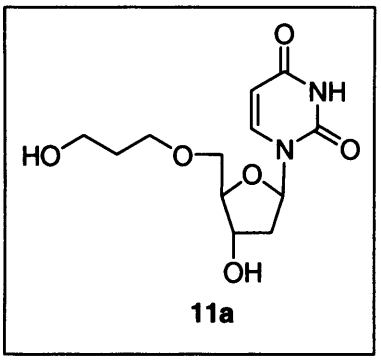
Chapter 10 Conclusions and Future Work

10.1 Conclusions

10.1.1 Chemistry

The synthesis of almost all the proposed target molecules has been achieved; six different classes of nucleosides analogues were prepared:

- Problems were encountered in the synthesis of the phosphonate/phosphate derivatives, only one of the proposed structures was obtained, this required a multistep synthesis with the use of orthogonal protecting groups and Williamson etherification (Chapter 4).



11a

Figure 10-1 2'-deoxyuridine analogue synthesised

- Some acyclic uracil derivatives were prepared using either a direct coupling of the unprotected uracil base with some aliphatic bromo alcohols or by a Mitsunobu coupling of the 3-*N*-benzoylated uracil with monoprotected diols (Section 5.1.1Chapter 5).

- Acyclic uracil phosphate and phosphonates were prepared using Mitsunobu coupling and a phosphorylation step with *N,N*-diisopropyl phosphoramidite (Section 5.1.1 and Section 5.1.2).

- 3-modified analogues of 2'-deoxyuridine were synthesised (Chapter 6).

- Unsaturated acyclic uracil derivatives were obtained again *via* the Mitsunobu coupling between protected uracil and unsaturated monoprotected diols (Section 7.2).

- Branched acyclic uracil derivatives were prepared starting from some intermediates received from Medivir. These were obtained following a multistep synthesis with orthogonal protecting groups (Section 7.3).

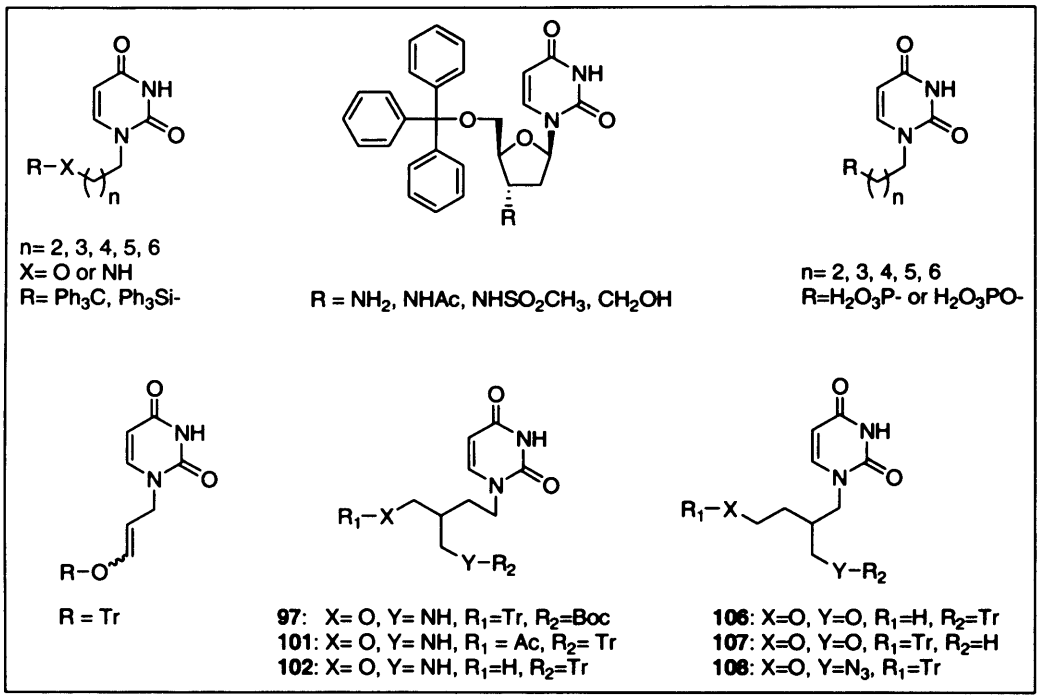


Figure 10-2 Summary of the main classes of compound synthesised

10.1.2 Biological Evaluation

The synthesised compounds had good inhibitory effect on *P. falciparum* dUTPase. In particular some of them had *Ki* values in the sub-μM range (Figure 10-3).

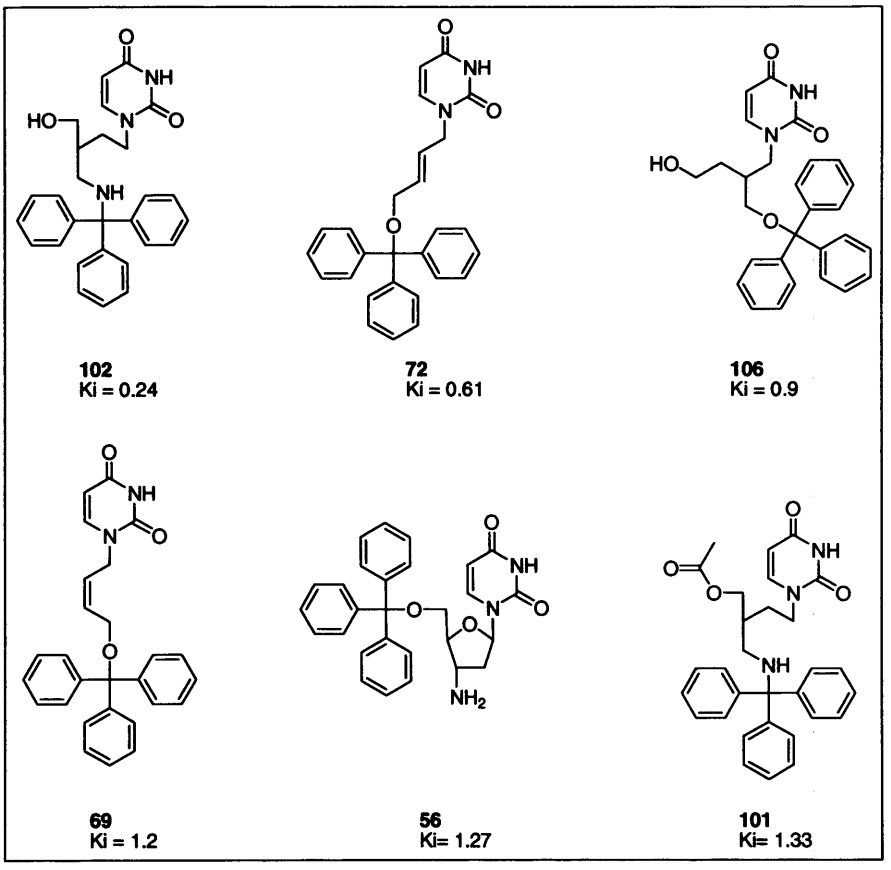


Figure 10-3 Best inhibitors of *P. falciparum* dUTPase. All the values are in μM

The compounds also inhibited the *in vitro* growth of the parasite, confirming the validity of the biological target dUTPase (Figure 10-4); they showed good selectivity indexes proving that a selective inhibition of the parasitic enzyme is achievable.

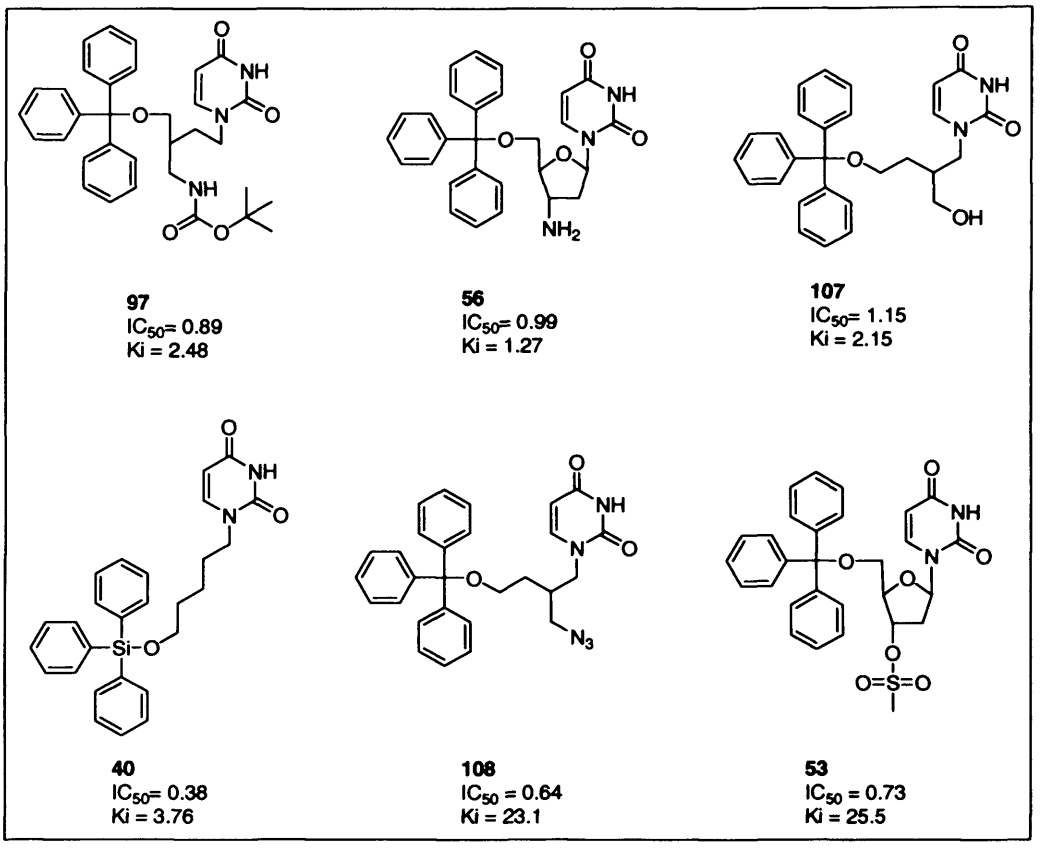


Figure 10-4 Most remarkable *in vitro* inhibitors of *P. falciparum* parasite. All the values are in μM

Some very interesting results emerged from the biological evaluation, two compounds were very potent inhibitors of the *P. falciparum* parasite, but were inactive against the enzyme dUTPase (Figure 10-5). Therefore the compounds are acting on a different biological target rather than inhibiting the enzyme dUTPase.

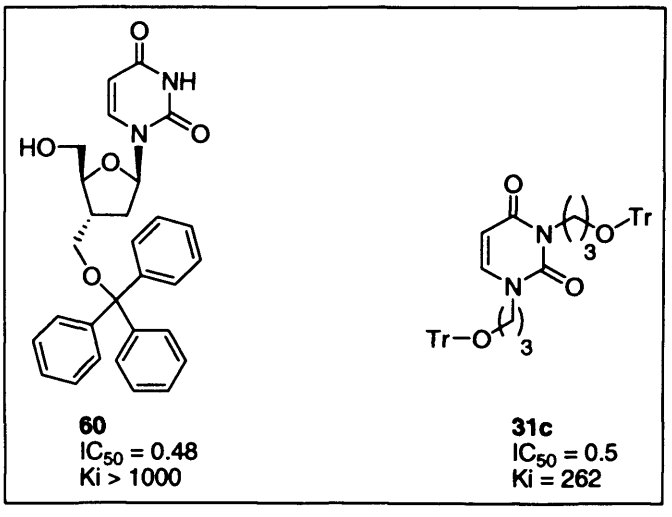


Figure 10-5 The two *in vitro* inhibitors of *P. falciparum*

Encouraging results were also obtained against *L. donovani* parasite. Some compound had inhibitory effects on the parasite growth, but this results need to be completed with the enzyme inhibition data.

Other compounds previously synthesised in the group were screened against five bacteria strains, in order to investigate potential application as antimicrobial agents. From the screening only three compounds were active against two Gram-positive bacteria strains. These compounds had MIC_{50} values in the range of 50-100 μM (Chapter 9); unfortunately none of the tested compound was active against Gram-negative bacteria.

10.2 Future Work

10.2.1 Inhibition of Dimeric dUTPase

If some more time was available the research could be progressed. This could be focused in the synthesis of different phosphate bioisosteres such as dicarboxylic acids, heterocycles or hydroxy malonic acid.

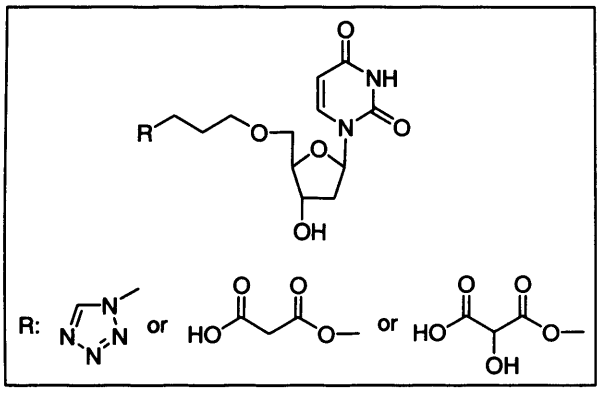


Figure 10-6 Bioisosteres of the phosphate group

10.2.2 Inhibition of Trimeric dUTPase

Inhibition of the *P. falciparum* enzyme appears to require a trityl moiety and uracil moiety. Therefore a possible way to develop more potent inhibitors would be make modifications to the 3' position of the nucleoside analogues on the branched chain/acyclic compounds.

10.2.3 Mode of Action / Proteomics Studies

A chemical proteomic approach[129] could be used for a further study of the inhibitors of *P. falciparum* **60** and **31c** (Figure 10-5), in order to identify their mechanism of action. These could be achieved by linking these compounds or some analogues of them to a tag moiety such as biotin (Figure 10-7). This kind of probes can be then used to isolate the biological target from a mixture of enzymes by affinity chromatography[130].

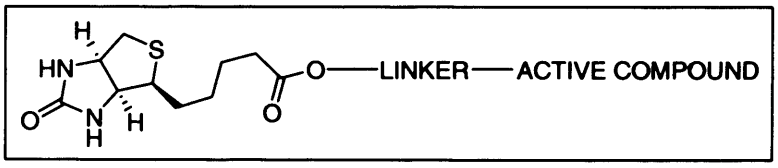


Figure 10-7 General structure of a biotin probe

Chapter 11 Experimental

11.1 Instrumentation and conventions

All ^{1}H, ^{13}C and ^{31}P NMR spectra were performed either with a Bruker Avance DPX 300 spectrometer at 300 MHz; 75 MHz and 121 MHz respectively or with a Bruker Avance DPX 500 at 500 MHz, 125 MHz and 200 MHz, respectively. Each resonance signal was reported to the following convention: chemical shifts (δ) in ppm (part per million); coupling constants *J* in Hertz (Hz). Signal splitting patterns are described as singlet (s), broad singlet (bs), doublet (d), triplet (t), quartet (q), multiplet (m) or combination thereof.

Low resolution mass spectra were acquired either with a Platform II mass spectrometer (Micromass) from Fision or with a Bruker MicroTof spectrometer.

High resolution mass spectrometry was performed respectively on a Waters ZQ4000 and Finningan MAT 95XP at the EPSRC National Mass Spectrometry Service Centre, University of Wales, Swansea.

IR analysis was performed using a FT-IR Perkin-Elmer 1600 series spectrophotometer.

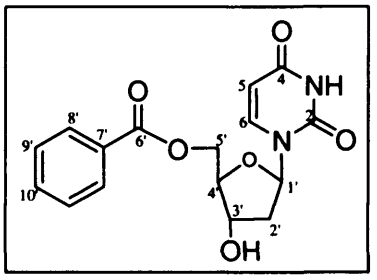
The analytical TLC were performed on Silica gel 60 F_{254} plates (*Merck*); all the chromatographic purifications were made using Silica gel 60 (*Fluka*) or using Isolute disposable columns with a Jones Chromatography Flash Master II apparatus.

Microwave reactions were carried out using an Initiator™ Microwave Synthesizer apparatus from Biotage.

Melting points were recorded using a Gallenkamp melting point apparatus.

All the reactions were carried out using dry solvents unless otherwise stated. The numbering and the nomenclature system used for the drawn structures in this chapter is to aid NMR interpretation and is not necessarily referring to IUPAC nomenclature.

(3a) 5'-*O*-Benzoyl-2'-deoxyuridine



To a solution of 2'-deoxyuridine (3.00 g, 2.52 mmol) in dry pyridine (50 ml) and DCM (50 ml), under N_2, at 0 °C, was added dropwise a solution of benzoyl chloride (1.52 ml, 2.52 mmol) in DCM (20 ml). The mixture was left under stirring for 1 h and 30 min. After this time water was added (50 ml), then extracted with DCM (2×20 ml). The organic phase was washed with HCl 3M (2×20 ml), and with a saturated solution of $NaHCO_3$ (2×20 ml); the organic layer was then dried with $MgSO_4$ and the solvent evaporated. The residue was purified by flash chromatography, with a mixture of MeOH/Chloroform as eluent, to obtain the desired compound.

White solid, 2.2 g, 51%.

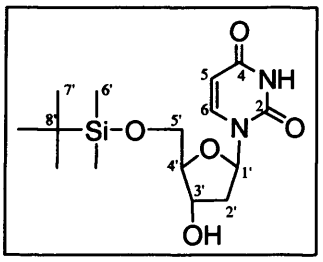
Mp: 165-167 °C. (Lit. 162 °C)

^{1}H-NMR (300 MHz; $(CD_3)_2SO$): δ 11.30 (s, 1H, N-H); 8.00-7.98 (m, 2H, H-8'); 7.73-7.5 (m, 4H, H-6+H-9'+H-10'); 6.20 (t, *J*=6.67 Hz, 1H, H-1'); 5.56-5.52 (m, 2H, H-5+O-H-3'); 4.56- 4.34 (m, 3H, H-5'+H-4'); 4.09-4.05 (m, 1H, H-3'); 2.32-2.17 (m, 2H, H-2').

^{13}C-NMR (75 MHz; $(CD_3)_2SO$): δ 165.9 (C-6'); 163.4 (C-4); 150.7 (C-2); 140.9 (C-6); 133.9 (C-10'); 129.7 (C-7'); 129.6 (C-8'); 129.2 (C-9'); 102.3 (C-5); 84.6 (C-4'); 84 (C-1'); 70.4 (C-3'); 64.8 (C-5'); 39.8 (C-2').

LRMS (ES+): m/z 355.2 $[(M+Na)^+$, 100%].

(3b) 5'-*O-tert*-butyldimethylsilyl-2'-deoxyuridine



A solution of *t*-butyldimethylsilylchloride (2.18 g, 14.46 mmol) and imidazole (1.07 g, 28.92 mmol) in DMF (30 ml) was added slowly (dropwise) to a stirred solution of 2'-deoxyuridine (3 g, 13.15 mmol) in dry DMF (40 ml), with ice-bath cooling at 0 °C, under nitrogen.

After 2 h, H_2O (100 ml) was added and the mixture was extracted with EtOAc (3×100 ml). The combined extracts were washed with saturated $NaHCO_3$ (2×100 ml), dried ($MgSO_4$) and concentrated. The residue was purified by flash chromatography to give the desired compound.

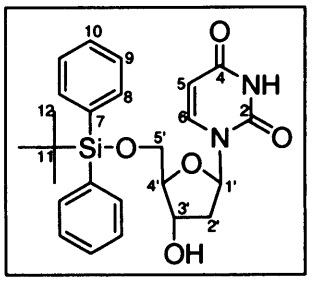
White foam; 3.49 g; 78 %.

R_f: 0.38 in DCM/MeOH 90:10.

^{1}H-NMR (300 MHz; $CDCl_3$): 9.5 (s, 1H, N-H); 8.02 (d, *J*=8.23 Hz, 1H, H-6); 6.45 (t, *J*=6.95 Hz, 1H, H-1'); 5.78 (d, *J*=8.23 Hz, 1H, H-5); 5.50 (bs, 1H, O-H-3'); 4.15 (m, 1H, H-4'); 4.01 (dd, J_1=11.5 Hz, J_2=2.56 Hz, 1H, C*H*H-5'), 3.95 (dd, J_1=11.5 Hz, J_2=2.19 Hz, 1H, CH*H*-5'); 3.06 (d, *J*=5.03 Hz, H-3'); 2.5 (m, 1H, C*H*H-2'); 2.2 (m, 1H, CH*H*-2'); 1.0 (s, 9H, H-7'); 0.2 (s, 6H, H-6').

^{13}C-NMR (75 MHz; $CDCl_3$): δ 163.9 (C-4); 150.9 (C-2); 140.8 (C-6); 102.7 (C-5); 87.9 (C-4'); 85.8 (C-1'); 72.5 (C-3'); 63.7 (C-5'); 42.0 (C-2'); 26.3 (C-8'); 18.8 (C-7'). Ref. [131]

(3c) 5'-*tert*-butyldiphenylsilyl-2'-deoxyuridine



2'-deoxyuridine (3 g; 13.15 mmol) was dissolved in dry DMF (40 ml), under nitrogen at room temperature. The solution was cooled to 0 °C with an ice bath and then a solution of TBDPS-Cl (3.97 g; 14.46 mmol) and imidazole (1.97 g; 28.80 mmol) in DMF (30 ml) was added dropwise over 2 hours time. After addition of the mixture the reaction was left warm to room temperature and stirred for 1 hour and half, then water (100 ml) was added. The aqueous solution was extracted with EtOAc (3×80 ml); the organic phase was washed with a saturated solution of $NaHCO_3$. The solvent was dried with $MgSO_4$ and evaporated under reduced pressure, to give a crude residue which was purified by flash chromatography using a mixture of DCM/Methanol as eluent.

White solid, 5.75 g, 93%.

R_f: 0.46 in DCM/Methanol (90:10)

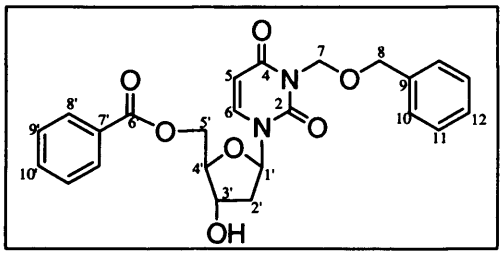
Mp: 67-70 °C.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 7.82 (d, *J*=8.23 Hz, 1H, H-6); 7.69-7.65 (m, 4H, H-8); 7.51-7.41 (m, 6H, H-9+H-10); 6.38 (t, *J*=6.40 Hz, 1H, H-1'); 5.48 (d, *J*=8.23 Hz, 1H, H-5); 4.58 (bs, 1H, H-3'); 4.03-3.99 (m, 2H, H-4'+H-5'); 3.90-3.85 (m, 1H, H-5'); 2.66 (bs, 1H, OH-3'); 2.51-2.43 (m, 1H, C*H*H-2'); 2.27-2.19 (m, 1H, CH*H*-2'); 1.11 (s, 9H, H-12).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 163.7 (C-4); 150.7 (C-2); 140.4 (C-6); 136.0-135.8 (C-8); 133.2-132.7 (C-7); 130.6 (C-10); 128.4-128.5 (C-9); 102.8 (C-5); 87.5 (C-4'); 85.4 (C-1'); 71.8 (C-3'); 64.1 (C-5'); 41.7 (C-2'); 27.4 (C-12); 19.7 (C-11).

LRMS (ES+): m/z 467.2 [$(M+H)^+$, 100%]; m/z 489.2 [(M+Na)+, 84%].

(3h) 5'-*O*-Benzoyl-3-*N*-benzyloxymethyl-2'-deoxyuridine[83]



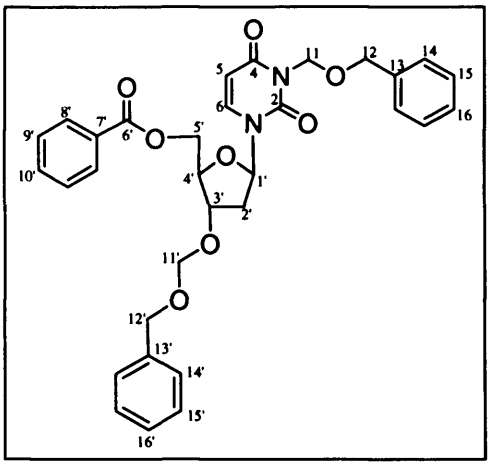
A solution of 5'-*O*-benzoyl-2'-deoxyuridine **3a** (300 mg, 0.90 mmol) and K_2CO_3 (0.45 g) in dry acetone (20 ml) was warmed to 56 °C. After 10 min benzyl chloromethyl ether (0.313 ml, 1.354 mmol) was added. The reaction was left 2 hours at the same temperature and checked by TLC (Hexane/EtOAc 2:1). After 2 hours other benzyl chloromethyl ether was added (0.1 ml); then the mixture was left overnight at room temperature. The suspension was filtered and washed with acetone. The solvent was dried with $MgSO_4$ and evaporated under reduced pressure. The obtained residue was purified by flash chromatography, using MeOH/Chloroform as the eluent.

Yellow oil; 233 mg, 57%.

^{1}H-NMR (300 MHz; $(CD_3)_2SO$): δ 7.95 (d, *J*=7.13 Hz, 2H, H-8'); 7.75-7.60 (m, 2H, H-Ar.); 7.58-7.45 (m, 2H, H-Ar.+H-6); 7.34-7.23 (m, 5H, H-Arom.); 6.19 (t, *J*=6.58 Hz, 1H, H-1'); 5.66 (d; *J*=8.05 Hz, 1H, H-5); 5.58 (bs, 1H, OH-3'); 5.30 (s, 2H, H-7); 4.60 (s, 2H, H-8); 4.55-4.35 (m, 3H, H-3'+H-5'); 4.12 (m, 1H, H-4'); 2.26 (t, *J*=6.2 Hz, 2H, H-2').

^{13}C-NMR (75 MHz; $(CD_3)_2SO$): δ 165 (C-6'); 162 (C-4); 150 (C-2); 140 (C-6); 133 (C-7'+C-9); 126-129 (C-arom.); 101.5 (C-5); 86.0 (C-1'); 82.1 (C-4'); 76.6 (C-3'); 71.4 (C-7); 70 (C-8); 64.8 (C-5'); 40.1 (C-2').

(4a) 5'-*O*-Benzoyl-3-*N*-3'-*O*-*bis*(benzyloxymethyl)-2'-deoxyuridine



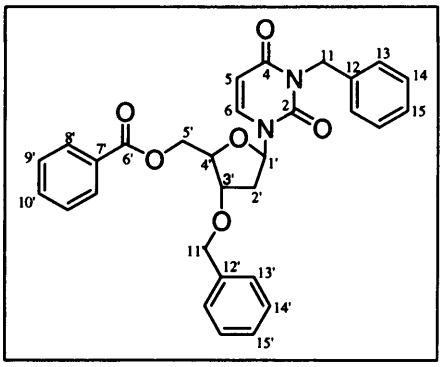
To a solution of 5'-*O*-benzoyl-2'-deoxyuridine **3a** (1.00 g, 3 mmol) in THF (50 ml) and 1,2-dichloroethane (50 ml) was added BomCl (1.62 g, 10.35 mmol) and *N,N*-diisopropylethylamine (1.28 g, 9.93 mmol). The reaction was stirred at 50 °C, under N_2. After 72 hours water and NH_3 were added, stirred for 15 min, then the phases were separated. The organic layer was dried with $MgSO_4$ and evaporated. The residue was purified by flash chromatography using EtOAc/Hexane as eluent.

Colourless oil; 977 mg; 57%.

^{1}H-NMR (300 MHz; $(CD_3)_2SO$): 7.95 (d, *J*=7.68 Hz, 2H, H-8'); 7.80-7.64 (m, 2H, H-6+H-10'); 7.55-7.45 (m, 2H, H-9'); 7.35-7.15 (m, 10H, H-14+H-15+H-16+H-14'+H-15'+H-16'); 6.15 (t, *J*= 6.6 Hz, 1H, H-1'); 5.67 (d, *J*=8.05 Hz, 1H, H-5); 5.30 (s, 2H, H-11); 4.85 (s, 1H, H-11'); 4.65 (s, 4H, H-12+H-12'); 4.58-4.45 (m, 2H, H-3'+H-5'); 4.32 (m, 1H, H-4'), 2.42 (m, 2H, H-2').

^{13}C-NMR (75 MHz; $(CD_3)_2SO$): δ 165.8 (C-6'); 162.0 (C-4); 150.9 (C-2); 140.2 (C-6); 138.4, 138.1 (C-13,C-13'); 133.9 (C-10); 129.6-126.7 (C-arom.); 101.6 (C-5); 94.1 (C-11'); 86.1 (C-1'); 82.1 (C-4'); 76.6 (C-3'); 71.4 (C-11); 70.3 (C-12'); 69.6 (C-12); 64.5 (C-5'); 37.1 (C-2'). Ref.[83]

(4b) 5'-*O*-Benzoyl-3-*N*-3'-*O*-*bis*(benzyl)-2'-deoxyuridine



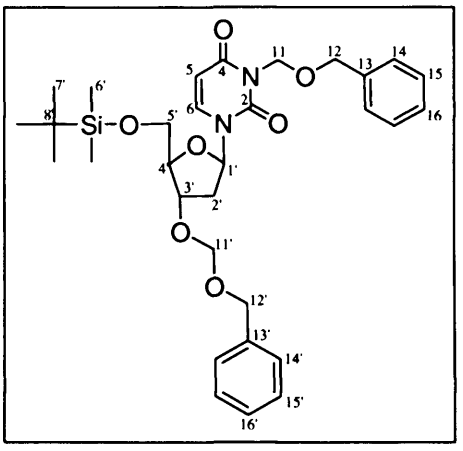
NaH (91 mg, 3.76 mmol) was added to a solution of 5'-*O*-benzoyl-2'-deoxyuridine **3a** (500 mg, 1.5 mmol) in dry DMF (10 ml) under nitrogen at room temperature. One hour later the benzyl bromide (457 µl, 3.45 mmol) was added dropwise to the cooled mixture (0°C). The reaction was then stirred overnight a room temperature. After one day the TLC (EtOAc/Hexane 10:40) showed the complete disappearance of the starting material and the reaction was quenched with water (10 ml). The aqueous solution was extracted with EtOAc (3×10 ml), washed with brine (3×10 ml) and water (2×10 ml). The organic phase was dried over Na_2SO_4 and evaporated under reduced pressure. The residue was purified by chromatography to give the desired compound as white oil. 335 mg; 43%.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 8.09-7.99 (m, 2H, H-8'); 7.68-7.63 (m, 1H, H-10'); 7.56-7.46 (m, 3H, H-9'+H-6); 7.39-7.32 (m, 10H, H-Ar.); 6.33 (t, *J*=6.49 Hz, 1H, H-1'); 5.70 (d, *J*=8.13 Hz, 1H, H-5); 5.16 (d, *J*=13.73 Hz, 1H, C*H*H-11); 5.12 (d, *J*=13.73 Hz, 1H, CH*H*-11) 4.69-4.55 (m, 4H, H-11'+H-4'+H-3'); 4.48-4.44 (m, 1H, C*H*H-5'); 4.30-4.26 (m, 1H, CH*H*-5'); 2.72-2.65 (m, 1H, C*H*H-2'); 2.18-2.08 (m, 1H, CH*H*-2').

^{13}C-NMR (75 MHz; $CDCl_3$): δ 166.5 (C-6'); 162.9 (C-4); 151.1 (C-2); 137.4 (C-6); 137.1 (C-12, C-12'); 134.1 (C-10'); 129.9-128.1 (C-arom.); 102.6 (C-5); 86.8 (C-1'); 83.0 (C-4'); 78.4 (C-3'); 72.2 (C-11'); 63.5 (C-5'); 44.6 (C-11); 38.7 (C-2').

LRMS (ES+): m/z 534.9 [$(M+Na)^+$, 100%].

(4c) 5'-*O*-*tert*-butyldimethysilyl-3-*N*-3'-*O*-*bis*(benzyloxymethyl)-2'-deoxyuridine



A solution of 5'-*O*-*tert*-butyldimethysilyl-2'-deoxyuridine **3b** (1 g, 2.92 mmol), DIPEA (1.28 g, 1.70 ml, 9.94 mmol) and Bom-Cl (1.55 g, 1.37 ml, 9.94 mmol) in 1,2-dichloroethane (15 ml), was warmed to 50 °C, under nitrogen for 12 h.

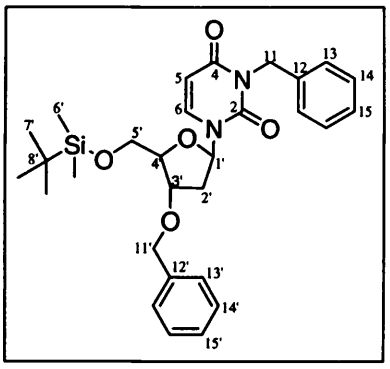
After 12 h water and NH_3 were added, the mixture was stirred for 15 min. The phases were separated. The organic layer dried on Na_2SO_4 and the solvent evaporated under reduced pressure. The residue was then purified by flash chromatography, using a mixture of Hexane and EtOAc as eluent.

Colourless oil; 1.478 g; 87%.

^{1}H-NMR (300 MHz; $CDCl_3$) δ: 7.95 (d, *J*=8.18 Hz, 1H, H-6); 7.48-7.35 (m, 10H, H-Ar.); 6.39 (t, *J*=6.44 Hz; 1H, H-1'); 5.78 (d, *J*=8.18 Hz, 1H, H-5); 4.89 (s; 2H; H-11); 4.78 (s, 2H, H-11'); 4.72 (s, 4H, H-12+H-12'); 4.47-4.43 (m, 1H, H-4'); 4.21-4.18 (m; 1H, H-3'); 3.99 (dd, J_1=11.40 Hz, J_2=2.12 Hz, 1H, C*H*H-5'); 3.82 (dd, J_1=11.40 Hz; J_2=1.62 Hz, 1H, CH*H*-5'); 2.60-2.53 (m, 1H, C*H*H-2'); 2.19-2.10 (m, 1H, CH*H*-2'); 1.02 (s, 9H, H-7'); 0.20 (s; 6H; H-6').

^{13}C-NMR (75 MHz; $CDCl_3$): δ 163.4 (C-4); 151.3 (C-2); 141.5 (C-6); 138.0 (C-13); 137.8 (C-13'); 127-129 (C-arom.); 102.1 (C-5); 94.2 (C-11'); 86.4 (C-4'); 86 (C-1'); 78.1 (C-3'); 72.6 (C-11);70.7 (C-12'); 65.5 (C-12); 63.5 (C-5'); 39.6 (C-2'); 26.3 (C-7'); 18.8 (C-8').

(4d) 5'-*O-tert*-butyldimethylsilyl-3-*N*-3'-*O-bis*(benzyl)-2'-deoxyuridine



NaH (146 mg, 3.65 mmol) was added to a solution of 5'-*O-tert*-butyldimethylsilyl-2'-deoxyuridine **3b** (500 mg, 1.46 mmol) in dry DMF (10 ml), under nitrogen at room temperature. After 30 min benzyl bromide (445 µl, 3.36 mmol) was slowly added to the cooled solution. The reaction was monitored by TLC (EtOAc Hexane 10:40) and after 3 hours, at room temperature, reached completion. The mixture was diluted with EtO_2 (15 ml), and washed with water (3×10 ml). The organic phases were dried on $MgSO_4$, and the solvent was evaporated to give a residue, which was purified by chromatography using a mixture of Hexane/EtOAc as eluent.

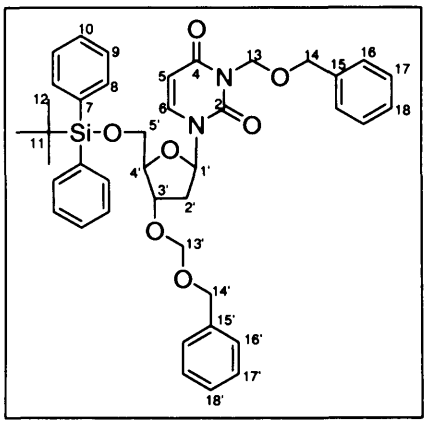
Colourless oil; 392 mg, 51.4%.

^{1}H NMR (300 MHz; $(CD_3)_2SO$): δ 7.83 (d, *J*=8.23 Hz, 2H, H-6); 7.38-7.22 (m, 10H, H-Arom.); 6.20 (t, *J*=7.13 Hz, 1H, H-1'); 5.82 (d, *J*=8.23 Hz, 1H, H-5); 5.00 (s, 2H, H-11); 4.55 (bs, 2H, H-11'); 4.15 (m, 2H, H-3'+H-4'); 3.85-3.70 (m, 2H, H-5'); 2.50-2.40 (m, 1H, C*H*H-2'); 2.25-2.15 (m, 1H, CH*H*-2'); 0.85 (s, 9H, H-7'); 0.04 (s, 6H, H-6').

^{13}C NMR (75 MHz; $(CD_3)_2SO$): δ 163.2 (C-4); 151.3 (C-2); 138.4 (C-6); 137.8 (C-12); 137.3 (C-12'); 129.5-128.0 (C-arom.); 102.2 (C-5); 86.5 (C-1); 85.7 (C-4'); 78.6 (C-3'); 71.9 (C-11'); 63.7 (C-5'); 44.5 (C-11); 38.9 (C-2'); 26.3 (C-7'); 18.7 (C-8').

LRMS (ES+): m/z 545.1 $[(M+Na)^+$, 100%].

(4e) 5'-*O*-*tert*-butyldiphenylsilyl-*N*-3-*O*-3'-*bis*(benzyloxymethoxyl)-2'-deoxyuridine



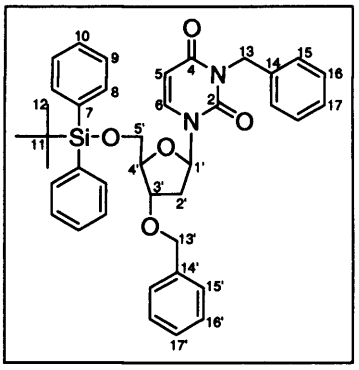
BomCl (6.71 ml; 28.97 mmol) was added to a 3 neck flask containing a solution of compound **3c** (4 g; 8.57 mmol) and DIPEA (5.04 ml; 28.97 mmol) in 1,2-dichloroethane (90 ml). The reaction was heated overnight at 50 °C, under nitrogen. After 16 hours the TLC (EtOAc/Hexane 2:3) showed the complete disappearance of the starting material, and then concentrated NH_3 (10 ml) was added. The mixture was stirred for 15 min, the phases were separated, the organic layer was dried with $MgSO_4$. The solvent was removed under reduced pressure to give a crude residue which was purified by flash chromatography using a gradient of Hexane/EtOAc (90:10) → Hexane/EtOAc (50:50). Colourless oil; 2.92 g. 48%.

$\mathbf{R_f}$: 0.59 in EtOAc/Hexane (40:60).

^{1}H-NMR (300 MHz; $CDCl_3$): δ 7.69 (d, *J*=8.23 Hz, 1H, H-6); 7.66-7.61 (m, 4H, H-8); 7.52-7.32 (m, 16H, H-arom.); 6.37 (t, *J*=6.59 Hz, 1H, H-1'); 5.53 (s, 2H, H-13); 5.50 (d, *J*=8.23 Hz, 1H, H-5); 4.85-4.80 (m, 2H, H-13'); 4.76 (s, 2H, H-14); 4.68-4.62 (m, 2H, H-14'); 4.53-4.49 (m, 1H, H-3'); 4.13-4.10 (m, 1H, H-4'); 4.02 (dd, J_1=11.70 Hz, J_2=2.92 Hz, 1H, C*H*H-5'); 3.84 (dd, J_1=11.52 Hz, J_2=2.56 Hz, 1H, CH*H*-5'); 2.60-2.52 (m, 1H, C*H*H-2'); 2.21-2.11 (m, 1H, CH*H*-2'); 1.14 (s, 9H, H-12).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 163.4 (C-4); 150.8 (C-2); 138.9 (C-6); 138.4, 137.8 (C-15,C-15'); 136.0, 135.8 (C-8,); 133.2, 132.7 (C-7); 130.6, 130.5 (C-10); 128.9-128.1 (C-Ar.); 102.3 (C-5); 94.3 (C-13'); 86.1 (C-4'); 85.7 (C-1'); 76.7 (C-3'); 72.7 (C-13); 70.7 (C-14); 70.4 (C-14'); 64.1 (C-5'); 39.5 (C-2'); 27.4 (C-12'); 19.8 (C-11).

(4f) 5'-*O*-*tert*-butyldiphenylsilyl-3-*N*-3'-*O*-*bis*(benzyl)-2'-deoxyuridine



A suspension of 60% sodium hydride in mineral oil (913 g; 22.82 mmol) was added slowly to a solution of 5'-*tert*-butyldiphenylsilyloxy-2'-deoxyuridine **3c** (4.8 g; 10.37 mmol) in DMF (70 ml), under nitrogen, cooled with ice bath (exothermic reaction). After 5 min from the addition of NaH, benzyl bromide (2.47 ml; 20.74 mmol) was added to the mixture. The reaction was left under stirring at room temperature for about 3 hours, until TLC (EtOAc/Hexane 1:4) showed the disappearance of the starting material. Diethyl ether (70 ml) was added to the reaction, the organic phase was washed with water (2×70 ml), brine (2×70 ml) and dried on $MgSO_4$. The solvent was removed under reduced pressure. The obtained residue (colourless oil) was purified by chromatography using a mixture of EtOAc/Hexane as eluent. White foam; 2.46 g, 37% yield.

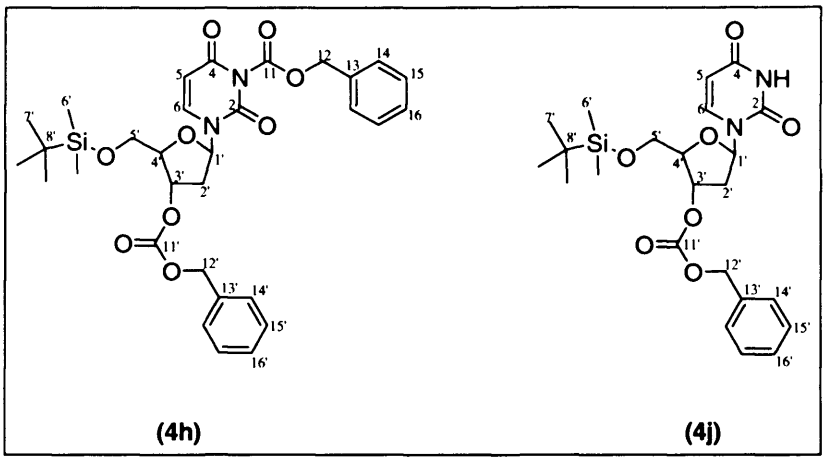
R_f: 0.50 in EtOAc/Hexane (20:80)

^{1}H-NMR (300 MHz; $CDCl_3$): δ 7.81 (d, *J*=8.05 Hz, 1H, H-6); 7.69-7.66 (m, 4H, H-8); 7.56-7.32 (m, 16H, H-arom.); 6.40 (t, *J*=6.23 Hz, 1H, H-1'); 5.59 (d, *J*=8.05 Hz, 1H, H-5); 5.17 (s, 2H, H-13'); 4.61 (d, *J*=11.68 Hz, 1H, C*H*H-13); 4.50 (d, *J*=11.76 Hz, 1H, CH*H*-13); 4.36-4.30 (m, 1H, H-3'); 4.16-4.10 (m, 1H, H-4'); 4.03 (dd, J_1=11.55 Hz, J_2=2.83 Hz, C*H*H-5'); 3.85 (dd, J_1=11.58 Hz, J_2=2.50 Hz, CH*H*-5'); 2.65-2.59 (m, 1H, C*H*H-2'); 2.19-2.10 (m, 1H, CH*H*-2'); 1.14 (s, 9H, H-12).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 163.1 (C-4); 151.3 (C-2); 138.2 (C-6); 137.8-137.3 (C-14); 136.2-136.0 (C-8); 133.2-132.7 (C-7); 130.6-128.0 (C-arom.); 102.4 (C-5); 86.3 (C-4'); 85.5 (C-1'); 78.3 (C-3'); 72.0 (C-13'); 64.2 (C-5'); 44.6 (C-13); 38.9 (C-2'); 27.4 (C-12); 19.7 (C-11).

LRMS (ES+): m/z 669.0 [(M+Na), 90%].

(4h) Attempted synthesis of 5'-*O-tert*-butyldimethylsilyl-3-*N*-3'-*O-bis*(benzyloxycarbonyl) -2'-deoxyuridine



Method A

Benzyl chloroformate (573 mg, 0.480 ml, 3.35 mmol) was added slowly to a solution of 5'-*O-tert*-butyldimethylsilyl-2'-deoxyuridine **3b** (500 mg, 1.46 mmol) and DMAP (357 mg, 2.92 mmol) in dry DCM (10 ml), under nitrogen and in ice bath. After the addition the solution was warmed to room temperature and stirred for 48 hours. The mixture of reaction was washed with water (2×20 ml), with a saturated solution of $NaHCO_3$ (3×20 ml) and HCl 3M (3×20 ml). The organic phase was dried on $MgSO_4$ and evaporated under reduced pressure, to give a compound that was not the desired, but the 3'-monoprotected **4j**. White solid; 295 mg; 42%.

R_f: 0.68 in DCM/MeOH 90:10.

Mp: 152-155 °C.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 9.05 (bs, 1H, NH); 7.97 (d, J=8.17 Hz, 1H, H-6); 7.49-7.43 (m, 10H, H-arom.); 6.47 (dd, J_1=8.62 Hz, J_2=5.55 Hz, 1H, H-1'); 5.79 (dd; J_1=8.17 Hz, J_2=2.08 Hz, 1H, H-5); 5.27-5.26 (bs, 3H, H-12'+H-3'); 4.31-4.30 (bs, 1H, H-4'); 4.01 (dd, J_1=11.42 Hz, J_2=1.97 Hz, 1H, CH*H*-5'); 3.96 (dd, J_1=11.45 Hz, J_2=1.92 Hz, 1H, C*H*H-5'); 2.65 (ddd, J_1=13.92 Hz, J_2=5.53 Hz, J_3=0.89, 1H, C*H*H-2'); 2.24 (ddd, J_1=14.45 Hz, J_2=8.67 Hz, J_3=6.07 Hz, 1H, CH*H*-2'); 0.99 (s, 9H, H-7'); 0.20 (s, 6H, H-6').

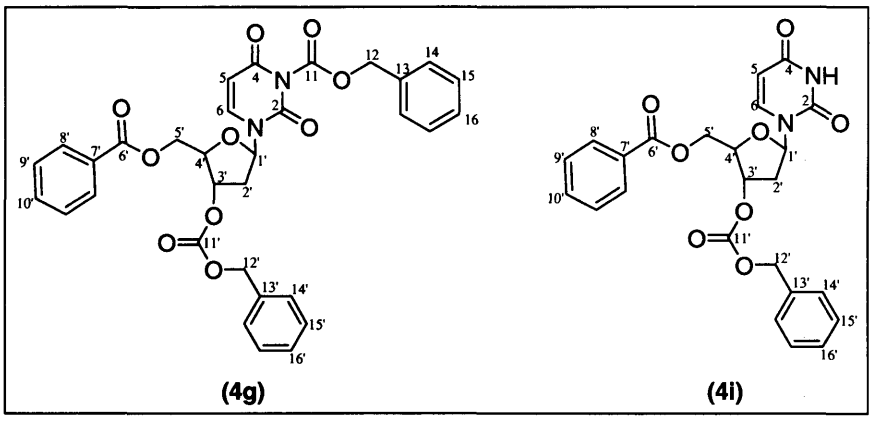
^{13}C-NMR (75 MHz; $CDCl_3$): δ 163.5 (C-4); 154.9 (C-11'); 150.6 (C-2); 140.2 (C-6); 135.1 (C-13'); 129.2-128.8 (C-Arom.); 103.0 (C-5); 85.7 (C-1'); 85.6 (C-4'); 79.3 (C-3'); 70.5 (C-12'); 64.1 (C-5'); 39.0 (C-2'); 26.3 (C-7'); 18.7 (C-8').

LRMS (ES+): m/z 633 [$(M+Na)^+$, 100%].

Method B

Benzyl chloroformate (573 mg, 0.480 ml, 2.86 mmol) was added to a solution of 5'-*O*-*tert*-butyldimethylsilyl-2'-deoxyuridine **3b** (500 mg, 1.43 mmol) and DIPEA (370 mg, 0.498 ml, 2.86 mmol) in 1,2-dichloethane (10 ml), under nitrogen. The mixture was warmed to 50 °C for 24h, then water and NH_3 concentrated were added to the reaction and stirred for 15 min. The organic layers were separated; dried on $MgSO_4$ and evaporated affording a mixture of compounds which was not possible to separate by chromatography.

(4g) Attempted synthesis of 5'-*O*-benzoyl-3-*N*-3'-*O*-*bis*(benzyloxycarbonyl)-2'-deoxyuridine



Method A

Benzyl chloroformate (589 mg, 3.45 mmol) was added to a solution of 5'-*O*-benzoyl-2'-deoxyuridine **3a** (500 mg, 1.50 mmol) and DMAP (367 mg, 3 mmol) in DCM (10 ml) and THF (20 ml), under nitrogen. The reaction was stirred at room temperature for 48 hours, checked by TLC (EtOAc/Hexane 30:20). The mixture was washed with water (1×20 ml), saturated solution of $NaHCO_3$ (3×20 ml) and HCl 3M (3×20 ml). The organic phases were collected, dried on $MgSO_4$, and

solvent was removed under reduced pressure. After chromatographic purification only the monoprotected compound **4i** was isolated.

Method B

NaH (90 mg, 3.75 mmol) was added to a solution of 5'-*O*-benzoyl-2'-deoxyuridine **3a** (500 mg, 1.50 mmol) in dry DMF (10 ml), at room temperature under nitrogen. After 30 min (the solution changed colour from colourless to orange-brown) then benzyl chloroformate (589 mg, 0.492 ml, 3.45 mmol) was added slowly. The mixture was left over night a room temperature, 24 hours later the reaction was diluted with Et_2O (20 ml), washed with water (2×10 ml). The organic phase was dried on $MgSO_4$, and evaporated under reduced pressure affording the same compound **4i** as method A.

R_f: 0.69 in DCM/MeOH 90:10.

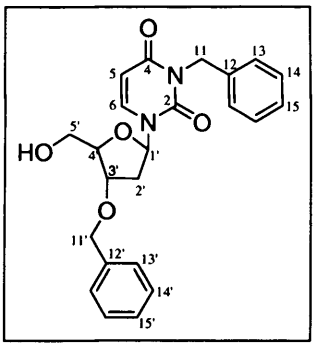
Mp: 66-69 °C.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 8.55 (bs, 1H, NH); 8.05-8.03 (m, 2H, H-9'); 7.70-7.65 (m, 1H, H-10'); 7.55-7.45 (m, 8H, H-6, H-8'+H-14'+H-15'+H-16'); 6.34 (dd, J_1=8.23 Hz, J_2=5.65 Hz, 1H, H-1'); 5.62 (d, J=8.18 Hz, 1H, H-5); 5.38-5.36 (m, 1H, H-3'); 5.25 (s, 2H, H-12'); 4.74 (dd, J_1=12.30 Hz, J_2=3.30 Hz, 1H, C*H*H-5'); 4.66 (dd, J_1=12.26 Hz, J_2=3.55 Hz, 1H, CH*H*-5'); 4.54-4.52 (m, 1H, H-4'); 2.74 (ddd, J_1=14.37 Hz, J_2=5.60 Hz, J_3=1.86 Hz; 1H, C*H*H-2'): 2.34-2.24 (m, 1H, CH*H*-2').

^{13}C-NMR (75 MHz; $CDCl_3$): δ163.2 (C-4); 160.4 (C-11'); 150.4 (C-2); 139.2 (C-6); 134.9 (C-10); 134.1 (C-13'); 129.9-128.8 (C-Arom.); 103.3 (C-5); 85.8 (C-1'); 82.8 (C-4'); 77.9 (C-3'); 70.8 (C-12'); 64.5 (C-5'); 38.5 (C-2').

LRMS (ES+): m/z 499.1 [$(M+Na)^+$, 100%].

(5a) 3-*N*-3'-*O*-*bis*(benzyl)-2'-deoxyuridine



Method A

5'-*O*-*tert*-butyldimethylsilyl-3-*N*-3'-*O*-*bis*(benzyl)-2'-deoxyuridine **4d** (800 mg, 1.53 mmol) was dissolved in THF (10 ml), at room temperature, and TBAF (400 mg, 1.53 mmol) was added. The mixture was left at room temperature until the TLC (EtOAc/Hexane 30:20) showed complete reaction (~ 1 hour). The solvent was removed under reduced pressure and the residue was purified by chromatography (EtOAc/Hexane 50:50 as eluent) to give the required compound as colourless oil (410 mg, 71.4%).

Method B

5'-*O*-benzoyl-3-*N*-3'-*O*-*bis*benzyl-2'-deoxyuridine **4b** (335 mg, 0.65 mmol) was dissolved into a solution of MeONa/MeOH 0.2 M (30 ml). The mixture was stirred at room temperature until the TLC (EtOAc/Hexane 60:40) showed complete reaction, the solution was neutralized with Dowex ion exchange resin (H^+ form), filtrated and the solvent removed under vacuum affording a crude residue, which was purified by chromatography. White solid, 180 mg, 67%.

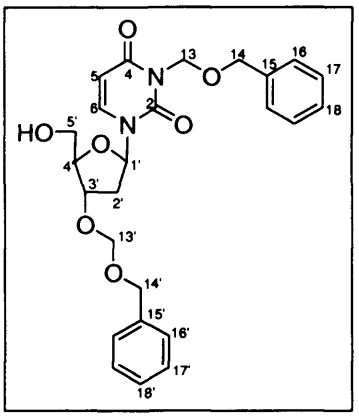
Mp: 122-124 °C.

^{1}H-NMR (300 MHz; $(CD_3)_2SO$): δ 7.97 (d, *J*=8.05 Hz, 1H, H-6); 7.37-7.25 (m, 10H, H-Arom.); 6.20 (t, *J*=6.40 Hz, 1H, H-1'); 5.87 (d, *J*=8.05 Hz, 1H, H-5); 5.15 (t, *J*=4.94 Hz, 1H, OH-5'); 4.98 (s, 2H, H-11'); 4.55 (s, 2H, H-11); 4.20 (bs, 1H, H-3'); 4.07 (bs, 1H, H-4'); 3.60 (bs, 2H, H-5'); 2.40 (m, 1H, C*H*H-2'); 2.20 (m, 1H, CH*H*-2').

^{13}C-NMR (75 MHz; $(CD_3)_2SO$): δ 162.2 (C-4); 151.0 (C-2); 139.6 (C-6); 138.5 (C-12); 137.4 (C-12'); 128-127 (C-Arom.); 101.5 (C-5); 85.7 (C-1'); 85.4 (C-4'); 79.2 (C-3'); 70.4 (C-11'); 62.4 (C-5'); 44.4 (C-11); 39.0 (C-2').

LRMS (ES+): m/z 431.1 $[(M+Na)^+$, 30%].

(5b) 3-*N*-3'-*O*-*bis*(benzyloxymethyl)-2'-deoxyuridine



Method A[83]

A solution of 5'-*O*-benzoyl-3-*N*-3'-*O*-bis(benzyloxymethyl)-2'-deoxyuridine **4a** (910 mg, 1.59 mmol) in MeONa/MeOH 0.2 M was stirred at room temperature for 1 hour. The reaction was monitored by TLC (EtOAc/Hexane 8:2). When the TLC showed the reaction was complete, the mixture was neutralized with Dowex ion exchange resin (H^+ form). The resin was filtered off, the solvent evaporated and the residue was purified by flash chromatography using a mixture of Hexane/EtOAc as eluent, to obtain the desired compound.

White solid; 533 mg, 71 %.

Method B

5'-*O*-*tert*-butyldimethysilyl-3-*N*-3'-*O*-bis(benzyloxymethyl)-2'-deoxyuridine **4b** (1.47 mg, 2.52 mmol) was dissolved in THF (20 ml) at room temperature. TBAF (0.66 g, 2.52 mmol) was added to the solution. The reaction was monitored by TLC (EtOAc/Hexane 50:50) until the disappearance of the starting material was observed (~ 1 hour). The mixture was concentrated to give a residue which was purified by flash chromatography using a mixture of EtOAc/Hexane as eluent.

Colourless oil; 560 mg; 46%.

Method C

Compound **4e** (1.84 g; 2.6 mmol) was dissolved in THF (10 ml) and stirred at room temperature. TBAF (689 mg; 2.6 mmol) was added into the flask and the mixture was left stirring until the disappearance of the starting material was observed by TLC (DCM/MeOH 95:5). After 3 hours the solvent was removed

under reduced pressure, the residue was dissolved in dichloromethane and purified by flash chromatography. White solid, 927 mg, 76%.

R_f: 0.18 in EtOAc/Hexane (60:40).

Mp: 73-75 °C.

^{1}H-NMR (300 MHz; $(CD_3)_2SO$): δ 7.94 (d, *J*=8.23 Hz, 1H, H-6); 7.36-7.28 (m, 10H, H-arom.); 6.18 (t, *J*=6.59 Hz, 1H, H.-1'); 5.81 (d, *J*=8.23 Hz, 1H, H-5); 5.34 (s, 2H, H-13); 5.16 (t, *J*=5.12 Hz, 1H, OH-5'); 4.68-4.82 (m, 2H, H-13'); 4.61-4.56 (m, 4H, H-14+H-14'); 4.41-4.37 (m, 1H, H-3'); 4.03-4.00 (m, 1H, H-4'); 3.63-3.59 (m, 2H, H-5'); 2.42-2.34 (m, 1H, C*H*H-2'); 2.22-2.13 (m, 1H, CH*H*-2').

^{13}C-NMR (75 MHz; $CDCl_3$): δ 163.4 (C-4); 151.4 (C-2); 139.9 (C-6); 138.3, 137.8 (C-15; C-15'); 129.0-127.4 (C-arom.); 102.3 (C-5); 94.4 (C-13'); 87.3 (C-4'); 85.9 (C-1'); 77.1 (C-3'); 72.7 (C-13); 70.8, 70.5 (C-14; C-14'); 62.6 (C-5'); 39.0 (C-2').

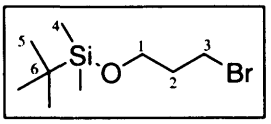
LRMS (ES+): m/z 491.0 [(M+Na), 100%].

HRMS (ES+): found 491.1817; required 491.1789 for $C_{25}H_{28}N_2NaO_7^+$; $[M+Na]^+$.

Microanalysis calculated for $C_{25}H_{28}N_2O_7$ + 0.7 HCl

C 60.78, H 5.86, N 5.67%; found C 60.86, H 5.77, N 5.67%.

(10a) (3-bromopropoxy)*tert*-butyldimethylsilane



A solution of *tert*-butyldimethylsilyl chloride (2.56 g, 16.98 mmol) and imidazole (1.23 g, 18.15 mmol) in dry DMF (10 ml), was cooled to 0 °C under nitrogen, and a solution of 3-bromo-1-propanol (2 g, 14.39 mmol) in DMF (10 ml) was added. The reaction was warmed to room temperature overnight. After 12 hours the mixture was diluted with Et_2O (20 ml), and washed with $NaHCO_3$ (2×20 ml) and HCl 3M (2×20 ml). The organic layer was dried ($MgSO_4$), and the solvent evaporated under reduced pressure to give the desired compound without further purification.

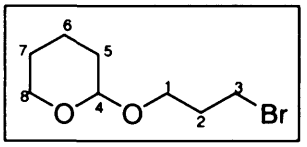
Yellow oil; 3.2 g; 93%.

$\mathbf{R_f}$; 0.78 in Hexane/EtOAc.

^{1}H-NMR (300 MHz; $CDCl_3$) δ: 3.75 (t, *J*=5.67 Hz, 2H, H-1); 3.47 (t, *J*=6.40 Hz, 2H, H-3); 2.00 (q, *J*=6.40 Hz, 2H, H-2); 0.90 (s, 9H, H-5); 0.50 (s, 6H, H-4).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 60.7 (C-1); 35.9 (C-3); 31.0 (C-2); 26.5 (C-5); 18.7 (C-6).

(10b) 2-(3-bromopropoxy)-tetrahydro-2H-pyran



A solution of 3,4-dihydro –α-pyran (3.63 g, 43.17 mmol) in DCM (24 ml) was added dropwise into a well stirred solution of 3-bromo-1-propanol (2 g, 14.37 mmol) and PPTS (80 mg, 32 mmol) in DCM (40 ml). The mixture was stirred at room temperature for 3 hours, then it was washed with brine (3×25 ml), and dried with $MgSO_4$. The evaporation of the solvent gave a crude product which was purified by chromatography, using a mixture of EtOAc/Hexane as eluent, affording the desired compound.

Colourless oil; 2.32 g, 73%.

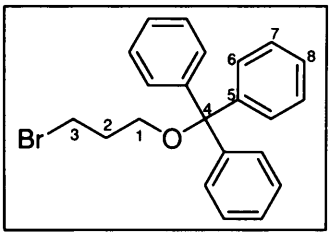
^{1}H-NMR (300 MHz; $CDCl_3$): δ 4.62 (t, *J*=3.8 Hz, 1H, H-4); 3.85 (m, 2H, H-1); 3.55 (m, 4H, H-8+H-3); 2.15 (m, 2H, H-2); 1.90-1.50 (m, 6H, H-5+H-6+H-7).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 99.3 (C-4); 65.3 (C-8); 62.6 (C-1); 33.3 (C-3); 31.1 (C-5); 31.0 (C-7); 25.8 (C-2); 19.9 (C-6).

LRMS (ES+): m/z 222.2 [$(M)^+$, 100%].

Ref.[132]

(10c) (3-bromopropoxy)triphenylmethane[133]



Trityl chloride (2.0 g, 7.19 mmol) was added to a solution of 3-bromo-1-propanol (0.625 ml, 7.19 mmol), DIPEA (2.5 ml, 7.19 mmol), a catalytic amount of DMAP, in THF (30 ml), under nitrogen. The reaction was left at room temperature for 24 hours, and then water (30 ml) was added into the mixture. The solution was extracted with EtOAc (3×10 ml) and the organic phase was washed with HCl 3N (2×10 ml), dried and concentrated under reduced pressure. The obtained residue (light yellow cream) was purified by chromatography to afford the desired compound.

White solid; 900 mg, 33 %.

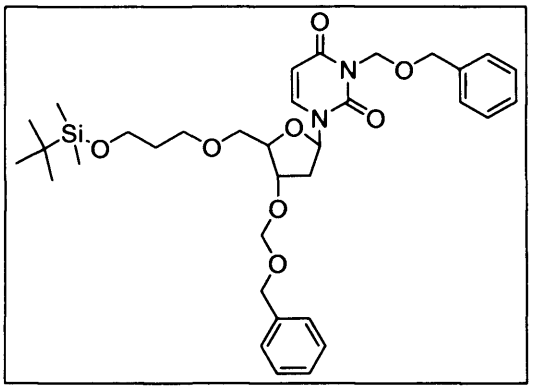
Mp: 80-82 °C.

^{1}H-NMR (300 MHz; $CDCl_3$) δ: 7.52-7.32 (m, 15H, H-Arom.); 3.64 (t, *J*=6.77 Hz, 2H, H-1); 3.27 (t, *J*=5.70 Hz, 2H, H-3); 2.18 (q, *J*=6.22 Hz, 2H, H-2).

^{13}C-NMR (75 MHz; $CDCl_3$) δ: 144.5 (C-5); 129.1-128.2 (C-Arom.); 86.9 (C-4); 61.6 (C-1); 33.9 (C-2); 31.3 (C-3).

LRMS (ES+): m/z 304.2 [$(M-Br)^+$, 80%].

(6b) Attempted synthesis of 5'-*O*-(3-(*tert*-butyldimethylsilyloxy)propyl)-3-*N*-3'-*O*-*bis*(benzyloxymethyl)-2'-deoxyuridine



Method A

NaH (31.8 mg, 0.795 mmol) was added slowly to a solution of 3-*N*-3'-*O*-*bis*(benzyloxymethyl)-2'-deoxyuridine **5b** (250 mg, 0.53 mmol) in dry DMF (10 ml) under nitrogen at room temperature. After 20 min the TBDMS-bromoalcohol **10a** (0.141 g, 0.59 mmol) was added to the mixture. The reaction was stirred at the same temperature (25 °C) for two days, checked by TLC (Hexane/ EtOAc 40:60). After this period water (20 ml) was added and the organic phase extracted with EtOAc (3×20 ml), dried and the solvent evaporated to give a residue made of a complex mixture of compounds. Unfortunately the attempt to separate the mixture by chromatography was unsuccessful.

Method B

NaH (64 mg, 1.6 mmol) was added to a solution of 3-*N*-3'-*O*-*bis*(benzyloxymethyl)-2'-deoxyuridine **5b** (500 mg, 1.07 mmol) in THF (20 ml), under nitrogen at room temperature. After 20 min (the solution changed colour from colourless to orange-brown), TBDMS-bromopropanol **10a** (332 mg, 1.3 mmol) was added. After 1 day water (20 ml) was added, and the mixture was extracted with DCM (3×20 ml), the solvent dried and evaporated to give a residue purified by chromatography without success.

Method C

(3-bromopropoxy)*tert*-butyldimethylsilane **10a** (86 mg, 0.36 mmol) was added to a suspension of K_2CO_3 (83 mg, 0.6 mmol) and 3-*N*-3'-*O*-bis(benzyloxymethyl)-2'-deoxyuridine **5b** (140 mg, 0.3 mmol) in CH_3CN, at room temperature and under nitrogen.The reaction was monitored by TLC (EtOAc/Hexane 60:40), but after 2 days the TLC did not show any new spot.

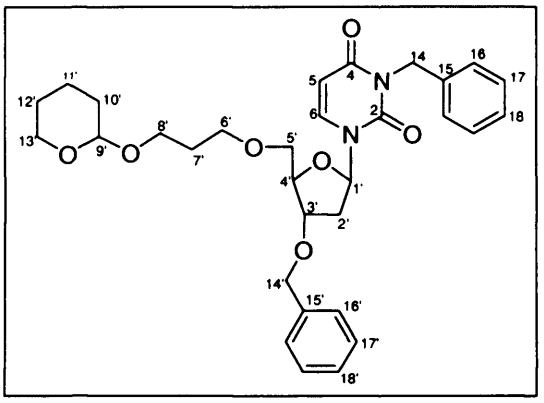
Method D

NaH (23 mg, 0.96 mmol) was added slowly to a solution of 3-*N*-3'-*O*-bis(benzyloxymethyl)-2'-deoxyuridine **5b** (300 mg, 0.64 mmol) and imidazole (catalytic amount) in dry DMF (5 ml). After 30 min (the solution became orange-brown) and the silylated bromopropanol **10a** (230 mg, 0.96) was added to the mixture. The reaction was stirred overnight at room temperature, under nitrogen. After 48 hours the same work-up as the previously described methods (A and B) was followed. The attempt of purification of the obtaianed residue did not succeed, since NMR analysis of the collected fractions did not show the presence of the desired compound.

Method E

(3-bromopropoxy)*tert*-butyldimethylsilane **10a** (127 mg, 0.53 mmol) was added to a solution of 3-*N*-3'-*O*-bis(benzyloxymethyl)-2'-deoxyuridine **5b** (250 mg, 0.53 mmol) and DBU (97 mg, 0.097 ml, 0.64 mmol) in THF (5 ml) at room temperature, under nitrogen. The reaction was sirred at room temperature overnight. After 24 hours TLC (EtOAc/Hexane 4:1) did not show any new spot, the mixture was then heated to 45 ° C. 48 hours later the TLC didn't show new spots, consequently the experiment was abandoned.

(6c) 5'-*O*-(3-(tetrahydro-pyran-2-yloxy)-propyl)-3-*N*-3'-*O*-*bis*(benzyl)-2'-deoxyuridine



Method A

NaH (67 mg, 1.46 mmol) was added to a solution of bis(benzyl)-2'-deoxyuridine **5a** (300 mg, 0.73 mmol) in THF (10 ml), under N_2 at room temperature. After 30 min 3-bromo-tetrahydropyranil-1-propanol **10b** (177 mg, 0.80 mmol) was added. The reaction was stirred at room temperature and monitored by TLC (EtOAc/Hexane 1:1). After 24 hours the TLC showed several new spots, then water (10 ml) was added. The aqueous solution was extracted with EtOAc (3×15 ml). The organic phase was dried with Na_2SO_4 and concentrated to give a residue, which was purified by flash chromatography using a mixture of EtOAc/Hexane as eluent.

Yellow oil; 88 mg, 22%.

Method B

NaH (60 mg, 1.25 mmol) was added to a solution of bis(benzyl)-2'-deoxyuridine **5a** (340 mg, 0.83 mmol) in DMF (10 ml), under N_2 at room temperature. After 30 min 3-bromo-tetrahydropyranil-1-propanol **10b** (222 mg, 1 mmol) was added. The mixture was stirred at room temperature. After 24 hours the reaction was quenched with water (10 ml) and extracted with Et_2O (3×15 ml). The organic phase was washed with H_2O (2×10 ml), dried over sodium sulphate and evaporated to give a residue, which was purified by flash chromatography using a mixture of EtOAc/Hexane as eluent.

81 mg, 18%.

Method C

NaH (70 mg, 1.1 mmol) was added into a solution of bis(benzyl)-2'-deoxyuridine **5a** (300 mg, 0.73 mmol) in THF (10 ml) in the presence of 15-crown-5-ether (0.174 ml, 0.87 mmol). After 30 min 3-bromo-tetrahydropyranil-1-propanol (177 mg, 0.80 mmol) was added with a syringe. The reaction was stirred 24 hours at room temperature. After the addition of water (10 ml), the mixture was extracted with EtOAc (3×15 ml). The organic phase was dried with $MgSO_4$ and the solvent removed under reduced pressure to give the desired compound after chromatographic purification.

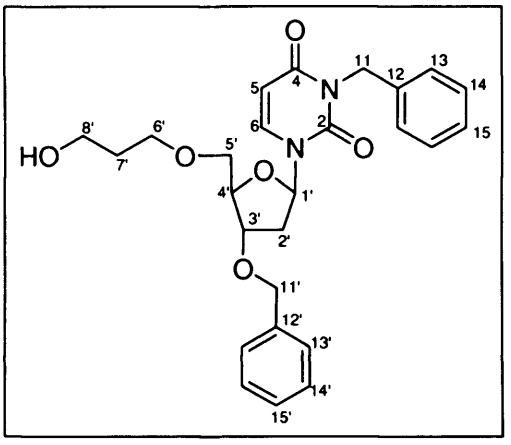
Yellow oil; 174 mg, 43%.

^{1}H-NMR (300 MHz; $CDCl_3$) δ: 7.87 (dd, J_1=8.12 Hz, J_2=2.01 Hz, 1H, H-6); 7.53 (d, J=7.35 Hz, 2H, H-18+H-18'); 7.44-7.32 (m, 8H, H-16+H-17+H-16'+H-17'); 6.39 (t, J=6.57 Hz, 1H, H-1'); 5.86 (dd, J_1=8.12 Hz, J_2=2.80 Hz, 1H; C*H*H-5); 5.16 (s, 2H, H-14); 4.65-4.51 (m, 3H, H-13'+H-9'); 4.27 (s, 2H, H-14'); 3.89-3.76 (m, 3H, H-8'+H-4'); 3.65-3.47 (m, 5H, H-6'+H-5'+H-3'); 2.60-2.53 (m, 1H, C*H*H-2'); 2.16-2.09 (m, 1H, CH*H*-2'); 1.95-1.58 (m, 8H, H-10'+H-12'+H-11'+H-7').

^{13}C-NMR (75 MHz; $CDCl_3$) δ: 163.2 (C-4); 151.4 (C-2); 138.5 (C-6); 138.0 (C-15); 137.3 (C-15'); 129.5-128.0 (C-Arom.); 102.2 (C-5); 99.5 (C-9'); 86.7 (C-1'); 84.5 (C-4'); 79.3 (C-3'); 71.8 (C-14'); 71.2 (C-5'); 69.2 (C-8'); 64.7 (C-6'); 63.0 (C-13'); 44.6 (C-14); 38.7 (C-2'); 31.2 (C-12'); 30.5 (C-10'); 25.8 (C-7'); 20.1 (C-11').

LRMS (ES+): m/z 573.1 [$(M+Na)^+$, 100%]

(7a) 5'-*O*-(3-hydroxypropyl)-3-*N*-3'-*O*-*bis*(benzyl)-2'-deoxyuridine



HCl 3M (0.5 ml) was added dropwise, at room temperature, into a solution of compound **6c** (177 mg, 0.32 mmol) in MeOH (10 ml). The reaction was stirred 30 min and further drops of HCl were added until the disappearance of the starting material was observed by TLC (Hexane/EtOAc 30:70). The solvent was removed under reduced pressure to afford the desired compound without any further purification.

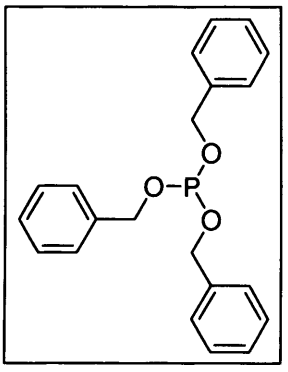
Yellow oil, 134 mg; 89%.

^{1}H-NMR (300 MHz; $CDCl_3$) δ: 7.82 (d, *J*=7.86 Hz, 1H, H-6); 7.58-7.48 (m, 2H, H-15+H-15'); 7.42-7.24 (m, 8H, H-13+H-14+H-13'+H-14'); 6.38 (t, *J*=6.40 Hz, 1H, H-1'); 5.82 (d, *J*=7.68 Hz, 1H, H-5); 5.18 (s, 2H, H-11); 4.64 (d, *J*=11.71 Hz, 1H, C*H*H-11'); 4.55 (d, *J*=11.71 Hz, 1H, CH*H*-11'); 4.26 (bs, 2H, H-5'); 3.80-3.58 (m, 6H, H-3'+H-4'+H-6'+H-8'); 2.63-2.48 (m, 1H, C*H*H-2'); 2.28 (bs, 1H, O-H); 2.16-2.02 (m, 1H, CH*H*-2'); 1.95-1.78 (quintet, *J*=6.03 Hz, 2H, H-7').

^{13}C-NMR (75 MHz; $CDCl_3$) δ: 163.4 (C-4); 151.2 (C-2); 138.6 (C-6); 137.9 (C-12); 137.1 (C-12'); 129.5-128.1 (C-arom.); 102.2 (C-5); 86.8 (C-1'); 84.4 (C-4'); 79.1 (C-3'); 71.9 (C-11'); 71.3 (C-5'); 69.7 (C-8'); 60.7 (C-6'); 44.6 (C-11); 38.7 (C-2'); 32.7 (C-7').

LRMS (ES+): m/z 489.1 [$(M+Na)^+$, 100%].

Tribenzyl phosphite[88]



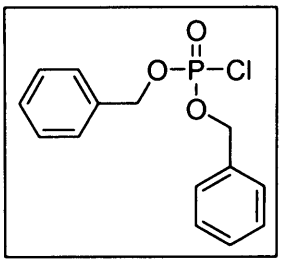
To a vigorously stirred solution of PCl_3 (4.37 ml; 0.05 mol); in anhydrous Et_2O (250 ml) at –10 °C, were successively added triethylamine (21.6 ml; 0.15 mol) in Et_2O (25 ml) and a solution of benzyl alcohol (15.52 ml; 0.15 mol) in Et_2O (25 ml). The resultant mixture was stirred for 30 min at –10 °C and 24 hours at room temperature. The chlorohydrate salt was filtered and washed with Et_2O (4 × 25 ml). The combined filtrate and washing solution were concentrated under reduced pressure to give 18 g of crude material. After dilution with Hexane/Et_2O/TEA (49.5:49.5:1) the product was purified by chromatography using the same solvent as eluent. The title compound was isolated as colourless liquid, and stored in absence of oxygen to avoid its oxidation to tribenzyl phosphate.

^{1}H-NMR (300 MHz; $CDCl_3$) δ: 7.51-7.38 (m; 15H; H-Ar.); 5.00 (d; J=8.05 Hz; 6H; O-C$\underline{H}_2$-Ph)

^{13}C-NMR (75 MHz; $CDCl_3$) δ: 138.7 (d, J=4.70 Hz, C_5H_5-$\underline{C}$-CH_2); 129.2-128.0 (C-Arom); 64.9 (d, J=11.25 Hz, Ph-$\underline{C}H_2$-P).

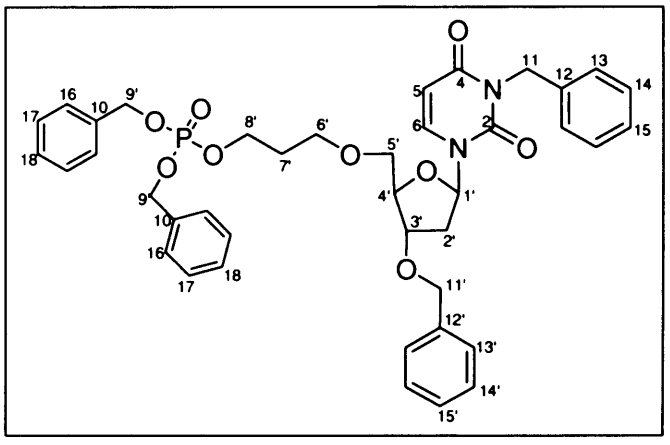
^{31}P-NMR ($CDCl_3$) δ: 140.89 (s).

Dibenzyl chlorophosphite[134]



Sulfuryl chloride (0.032 ml) was added with a syringe to a flask containing a solution of dibenzyl phosphate (0.1 ml) in CCl_4. After 1 hour at room temperature the volatiles were removed *in vacuo* and the crude yellow oil was used without further purification in the next stage.

(8) Dibenzyl ester of 5'-*O*-(phosphonopropyl)-3-*N*-3'-*O*-*bis*(benzyloxymethyl)-2'-deoxyuridine



Method A

Compound **7a** (100 mg; 0.21 mmol) was dissolved in dry pyridine (2 ml) under nitrogen. The dibenzyl chlorophosphite previously prepared in situ (0.15 ml) was added into the clear solution with a syringe. The mixture was stirred 2 h at room temperature. The solvent was removed under reduced pressure and the crude oil (300 mg) was purified by flash chromatography with DCM→DCM/MeOH 95:5 as gradient. The desired compound was obtained from the first fraction of the column chromatography.

Colourless oil, 60 mg, 38%.

Method B

Iodine was added into a solution of tribenzyl phosphite (0.206 g; 0.567 mmol) in DCM (1 ml) at –20°C. After 20 min the clear colourless solution was warmed to room temperature and subsequently added dropwise into another solution of alcohol **7a** (130 mg; 0.27 mmol) in pyridine (0.5 ml) and DCM (0.5 ml) at –20°C. After 1 hour the mixture was warmed to room temperature and stirred for 3 h. The solvent was removed under reduced pressure and the residue was purified by flash chromatography to give the title product

Colourless oil, 49 mg, 25%.

$\mathbf{R_f}$: 0.80 in DCM/MeOH 90:10.

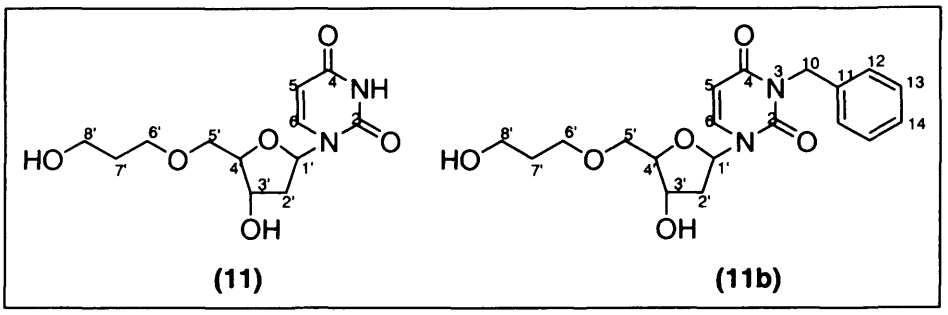
^{1}H-NMR (300 MHz; $CDCl_3$) δ: 7.67 (d, *J*=8.23 Hz, 1H, H-6); 7.50-7.47 (m, 2H, H-18); 7.36-7.26 (m, 18H, H-Arom.); 6.29 (t, *J*=6.22 Hz, 1H, H-1'); 5.77 (d, *J*=8.23 Hz, 1H, H-5); 5.10 (s, 2H, H-11'); 5.05-5.01 (m, 4H, H-9+H-9'); 4.53 (d, *J*=11.71 Hz, 1H, 1 of H-11); 4.46 (d, *J*=11.71 Hz, 1H, 1 of H-11); 4.19-4.13 (m, 2H, H-5'); 4.07-4.00 (m, 2H, H-8'); 3.66-3.62 (m, 1H, H-3'); 3.55-3.44 (m, 3H, H-4'+H-6'); 2.52-2.44 (m, 1H, C*H*H-2'); 2.02-1.94 (m, 1H, CH*H*-2'); 1.88-1.80 (quintet, *J*=5.85 Hz, 2H, H-7').

^{13}C-NMR (75 MHz; $CDCl_3$) δ: 163.0 (C-4); 151.3 (C-2); 138.2 (C-6); 137.9-137.2 (C-12+C-12'); 136.2 (d, J=6.55 Hz, C-10+C-10'); 129.5-128.0 (C-arom.); 102.3 (C-5); 86.6 (C-1'); 84.3 (C-4'); 79.2 (C-3'); 71.8 (C-9 + C-9'); 71.2 (C-11'); 69.8 (d, *J*=5.74 Hz, C-8'); 67.8 (C-5'); 65.0 (d, *J*=6.32 Hz, C-6'); 44.6 (C-11); 38.6 (C-2'); 30.8 (d, *J*=6.32 Hz, C-7').

^{31}P-NMR ($CDCl_3$) δ: 0.59 (s).

LRMS (ES+): m/z 748.9 [$(M+Na)^+$, 100%].

(11a) 5'-*O*-(hydroxylpropyl)-2'-deoxyuridine



Method A

A suspension of compound **7b** (90 mg; 0.17 mmol) and 10% Pd/C (10 mg) in MeOH (5 ml) was stirred at room temperature in a sealed round bottom flask. The air inside the flask was replaced 3 times with nitrogen using a three way tap connected to the vacuum and to a nitrogen balloon, and then the nitrogen was displaced with hydrogen. After 3 h the TLC (DCM/MeOH 90:10) showed a new spot at lower Rf. The suspension was left stirring overnight, and some more fresh catalyst was added. When the TLC showed the disappearance of the starting material (24 h) the reaction was filtered through a Celite pad. The solvent was removed *in vacuo* and the residue was purified by chromatography using DCM/MeOH 95:5 →90:10 as gradient affording compound **11a**.

Off white solid; 39 mg; 80%.

R_f: 0.17 in DCM/MeOH 90:10.

^{1}H-NMR (300 MHz; CD_3OD); δ 7.97 (d, *J*=8.12 Hz, 1H, H-6); 6.27 (t, *J*=6.61 Hz, 1H, H-1'); 5.72 (d, *J*=8.12 Hz, 1H, H-5); 4.40 (q, *J*=3.20 Hz, 1H, H-3'); 4.03 (q, *J*=2.85 Hz, 1H, H-7'); 3.75-3.56 (m, 6H, H-5'+H-7+H-9); 2.35-2.15 (m, 2H, H-2'); 1.82 (quintet, *J*=6.30 Hz, 2H, H-8).

^{13}C-NMR (75 MHz; CD_3OD); δ 166.7 (C-4); 152.6 (C-2); 142.7 (C-6); 102.3 (C-5); 88.1 (C-1'); 87.2 (C-4'); 73.0 (C-7); 72.1 (C-5'); 69.7 (C-9); 41.9 (C-2'); 34.0 (C-8).

LRMS (ES+): m/z 309.0 $[(M+Na)^+$, 100%].

HRMS (ES+): found 304.1507; required 304.1503 $[M+NH_4]^+$.

Method B

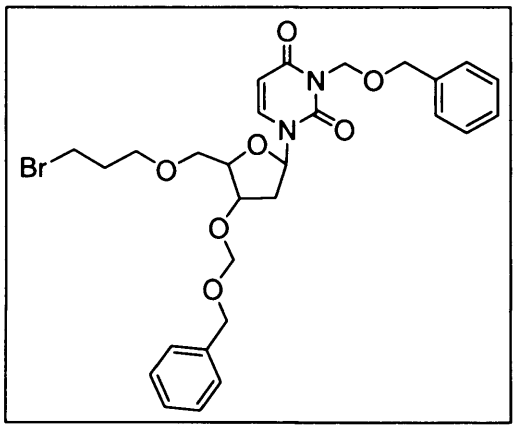
Compound **7a** (100 mg; 0.21 mmol) was dissolved in MeOH (7 ml) and hydrogenated following the same procedure described in the previous method. The mixture was left under stirring overnight. The TLC showed the complete disappearance of the starting material after 16 h. The catalyst was filtered through Celite. All the volatiles were removed *in vacuo*. NMR analysis of the obtained residue, without further purification, proved to be the monobenzylated compound **11b**.

$\mathbf{R_f}$: 0.13 in EtOAc/Hexane (20:80).

^{1}H-NMR (300 MHz; CD_3OD): δ 7.98 (d, *J*=8.05 Hz, 1H, H-6); 7.37-7.24 (m, 5H, H-arom.); 6.29 (t, *J*=6.59 Hz, 1H, H-1'); 5.83 (d, *J*=8.05 Hz, 1H, H-5); 5.07 (s, 2H, H-10); 4.40-4.42 (m, 1H, H-3'); 4.04-4.02 (m, 1H, H-4'); 3.74-3.58 (m, 7H, H-8'+H-6'+H-5'+OH); 2.37-2.29 (m, 1H, C*H*H-2'); 2.22-2.14 (m, 1H, CH*H*-2'); 1.81 (quintet, *J*=6.22 Hz, 2H, H-7').

^{13}C-NMR (75 MHz; CD_3OD): δ 165.3 (C-4); 152.7 (C-2); 141.0 (C-6); 138.6 (C-11); 129.8-128.9 (C-arom.); 102.4 (C-5); 88.2 (C-1'); 79.9 (C-4'); 72.9 (C-3'); 72.1 (C-5'); 69.8 (C-6'); 60.3 (C-8'); 45.5 (C-10); 42.2 (C-2'); 34.1 (C-7').

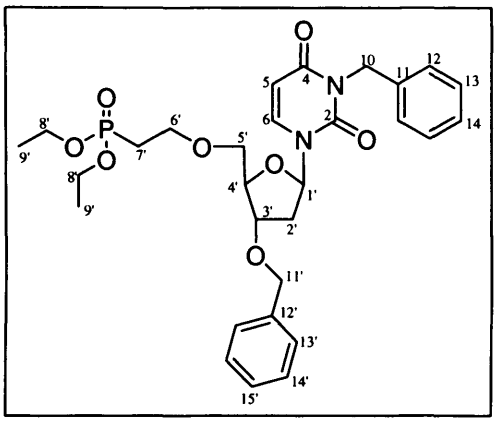
(12) Attempted synthesis of 5'-*O*-(bromopropyl)-3-*N*-3'-*O*-*bis*(benzyloxymethyl)-2'-deoxyuridine



NaH (0.046 g, 1.16 mmol) was added slowly to a stirred solution of 3-*N*-3'-*O*-*bis*(benzyloxymethyl)-2'-deoxyuridine **5b** (271 mg, 0.578 mmol) in DMF (10 ml), at room temperature and under nitrogen. After 30 min 1,3-dibromopropane (0.064 ml, 0.64 mmol) was added to the reaction mixture.

After 72 h 20 ml of water was added, the organic layer extracted with EtOAc (2×100 ml), dried with $MgSO_4$ and the solvent evaporated, to obtain a a complex mixture, which was impossible to purified by chromatography.

(13a) Diethyl ester of 5'-*O*-(phosphonoethyl)-3-*N*-3'-*O*-*bis*(benzyl)-2'-deoxyuridine



NaH (40 mg, 0.91 mmol) was added to a solution of 3-*N*-3'-*O*-*bis*(benzyl)-2'-deoxyuridine **5a** (250 mg, 0.61 mmol) in THF (5 ml). After 15 min, diethyl(2-chloroethyl) phosphonate **16** (0.127 ml, 0.73 mmol) was added to the clear solution, and the mixture was stirred at room temperature, under N_2, for 2 days.

EtOAc (10 ml) was added, the organic phase washed with water (2×10 ml), dried and concentrated under reduced pressure to afford an orange oil which was purified by flash chromatography.

Yellow oil; 180 mg, 51.5 %.

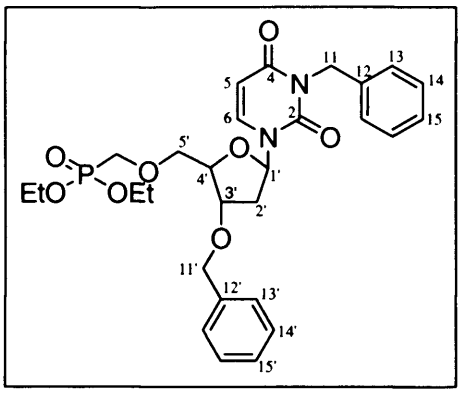
^{1}H-NMR (300 MHz; $CDCl_3$) δ: 7.78 (d, *J*=8.23 Hz, 1H, H-6); 7.52-7.30 (m, 10H, H-Arom.); 6.37 (t, *J*=6.04 Hz, 1H, H-1'); 5.84 (d, *J*=8.23 Hz, 1H, H-5); 5.14 (s, 2H, H-11); 4.60 (d, *J*=11.75 Hz, 1H, 1 of H-11'); 4.51 (d, *J*=11.75 Hz, 1H, 1 of H-11'); 4.24 (bs, 2H, H-3'+H-4'); 4.19-4.09 (m, 4H, H-8+H-8'); 3.83-3.73 (m, 3H, H-6'+C*H*H-5'); 3.61 (dd, J_1=10.43 Hz, J_2=2.19 Hz, 1H, CH*H*-5'); 2.59-2.51 (m, 1H, C*H*H-2'); 2.20-2.05 (m, 3H, CH*H*-2'+ H-7'); 1.35 (t; *J*=7.13 Hz, 6H, H-9+H-9').

^{13}C-NMR (75 MHz; $CDCl_3$) δ: 163.1 (C-4); 151.3 (C-2); 138.6 (C-6); 137.9 (C-12); 137.2 (C-12'); 129.5-128.0 (C-Ar.); 102.3 (C-5); 86.6 (C-1'); 84.2 (C-4'); 79.2 (C-3'); 71.8 (C-11'); 71.2 (C-5'); 65.9 (C-6'); 62.2 (d, *J*=6.32 Hz, C-8'+C-8); 44.6 (C-11); 38.4 (C-2'); 27.4 (d, *J*=140.8 Hz, C-7'); 16.8 (d, *J*=6.32 Hz, C-9').

^{31}P-NMR (121 MHz; $CDCl_3$) δ: 29.06.

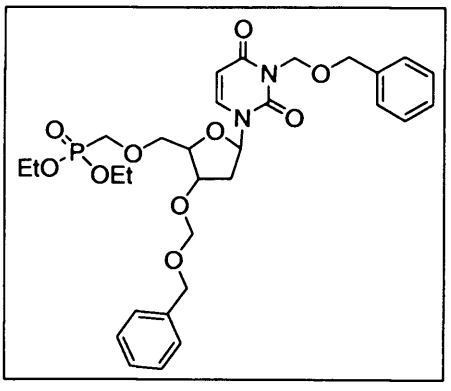
LRMS (ES+): m/z 594.9 [$(M+Na)^+$, 75%].

(13b) Attempted synthesis of diethyl ester of 5'-O-(phosphonomethyl)-3-N-3'-O-bis(benzyl)-2'-deoxyuridine



NaH (20 mg, 0.64 mmol) was added to a solution of 3-*N*-3'-*O*-bis(benzyl)-2'-deoxyuridine **5a** (200 mg, 0.49 mmol) in DMF (2 ml). After 30 min (the solution changed colour from colourless to orange-brown) diethyl (chloromethyl)-phosphonate **16** (91 mg, 0.079 ml, 0.49 mmol) was added into the mixture. After 24 h stirring at room temperature, the TLC (EtOAc/Hexane 6:4) showed the presence of new spots. The mixture was extracted with DCM (2×10 ml), the organic phase was washed with water (2×10 ml), dried and concentrated *in vacuo*. The crude residue was purified by chromatography affording only unreacted starting material was recovered.

(13c) Attempted synthesis of diethyl ester of 5'-O-(phosphonomethyl)-3-N-3'-O-bis(benzyloxymethyl)-2'-deoxyuridine



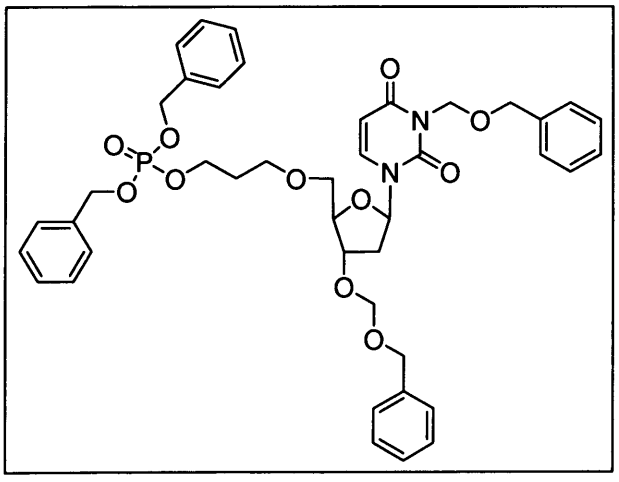
Method A

Diethyl (chloromethyl)-phosphonate **16** (132 mg, 0.114 ml, 0.70 mmol) was added into a solution of 3-*N*-3'-*O*-bis(benzyloxymethyl)-2'-deoxyuridine **5b** (300 mg, 0.64 mmol) and TEA (71 mg, 0.098 ml, 0.70 mmol) in DCM (5ml), under nitrogen, at room temperature. The reaction was followed by TLC (EtOAc/Hexane 60:40), but after 24 h the appearance of any new spot was not observed, and the mass spectra did not show the presence of the desired compound.

Method B

NaH (39 mg, 1.6 mmol) was added to a solution of 3-*N*-3'-*O*-bis(benzyloxymethyl)-2'-deoxyuridine **5b** (500 mg, 1.07 mmol) in DMF (5 ml). After 30 min (the solution changed colour from colourless to orange-brown). Diethyl (chloromethyl)-phosphonate **16** (220 mg, 0.191 ml, 1.1 mmol) was added into the mixture. After 24 h the reaction was quenched with water. The mixture was extracted with DCM (2×10 ml), washed with water (2×10 ml) and concentrated under reduced pressure. Purification of the residue by chromatography afforded only unreacted starting material.

(13d) Attempted synthesis of dibenzyl ester of 5'-*O*-(phosphonopropyl)-3-*N*-3'-*O*-*bis*(benzyloxymethyl)-2'-deoxyuridine



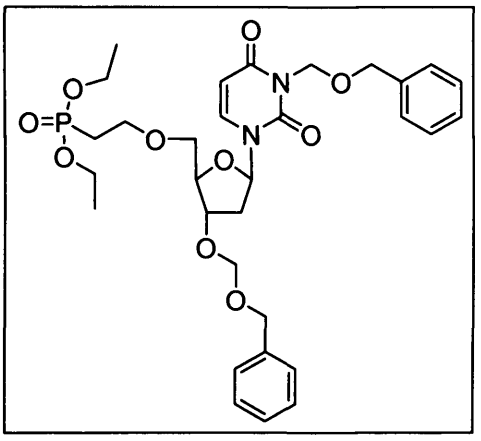
Method A

Compound **5b** (184 mg; 0.39 mmol) was dissolved in dry THF (5 ml) and added to a flask containing a suspension of sodium hydride (24 mg; 0.58 mmol) in THF (2 ml); followed by the addition of 15-crown ether (0.115 ml; 0.58 mmol). Once the suspension changed colour (from white to yellow/orange) a solution of dibenzyl-(3-bromopropyl) phosphonate **29b** (157 mg; 0.39 mmol) in dry THF (10 ml) was added dropwise, at room temperature. The reaction was stirred overnight and monitored by TLC (Hexane/EtOAc 30:20). After 20 h the TLC did not show any presence of starting phosphonate **29b**. The solution was concentrated under reduced pressure, affording a mixture of compounds which was not possible to purify by chromatography due to the small differences in the Rfs of the components of the mixture.

Method B

Compounds **7a** and **29b** were dried at the high vacuum pump for 3 h to remove all the traces of water. The *bis*(benzyloxymethyl)-2'-deoxyuridine **5b** (210 mg; 0.4 mmol) was dissolved in dry THF (8 ml) under nitrogen. Sodium hydride (35 mg; 0.85 mmol) was added slowly to the THF solution under flow of nitrogen. The mixture was stirred at room temperature for 15 min and a change of colour from white to orange was observed. Crown ether (125 µl) was added with a syringe, the reaction was cooled to -78 °C and a solution of phosphonate **29b** (179 mg; 0.4 mmol) in dry THF (2 ml) was added to the suspension. The mixture was stirred under nitrogen, warmed to room temperature and stirred for 20 h. After that time TLC (EtOAc/Hexane 20:30) showed the disappearance of the starting material **29b**, then water (15 ml) was added into the mixture which was stirred for other 2 min and extracted with DCM (2×10 ml). The organic phase was dried over $MgSO_4$ and the solvent evaporated *in vacuo*. Chromatographic purification of the residue afforded a mixture of compound which did not have the NMR properties of the desired compound.

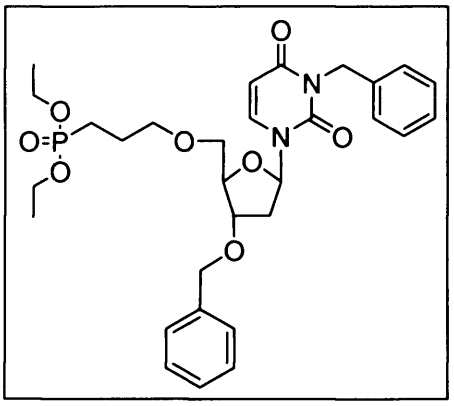
(13f) Attempted synthesis of diethyl ester of 5'-O-(phosphonoethyl)-3-N-3'-O-bis(benzyloxymethyl)-2'-deoxyuridine



Method A

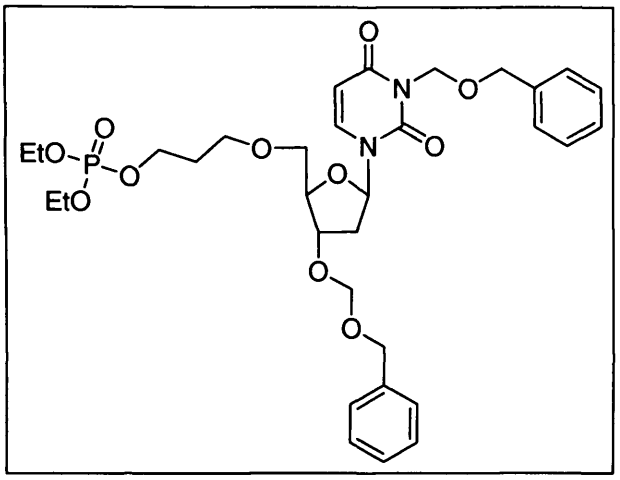
Compound **5b** (174 mg; 0.371 mmol) was dissolved in dry THF (5 ml) and added into a 3 neck flask containing a suspension of sodium hydride (54 mg; 1.35 mmol) under nitrogen. The white suspension was stirred at room temperature until it became orange, then diethyl-2-bromoethyl phosphonate **17** (120 μl; 0.37 mmol) was added to the mixture, with a syringe. The reaction was left under stirring at room temperature overnight, and monitored by TLC (EtOAc/Hexane 30:20). After 20 h EtOAc (10 ml) was added and the organic phase was washed with water, dried and the solvent was removed under reduced pressure. NMR of the collected fractions did not show the presence of the desired compound.

(13g) Attempted synthesis of diethyl ester of 5'-O-(phosphonopropyl)-3-N-3'-O-bis(benzyl)-2'-deoxyuridine



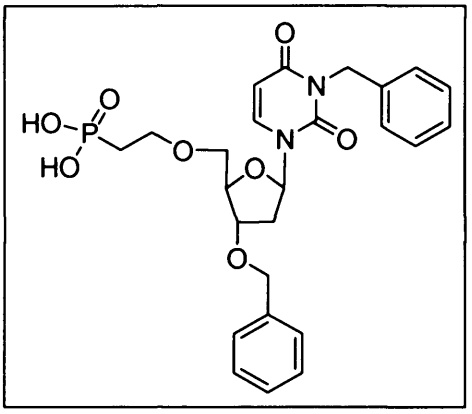
NaH (30 mg; 0.733 mmol) was added to a solution of **5a** (200 mg; 0.489 mmol) in dry THF (5 ml) under nitrogen at room temperature. After 30 min the solution became slightly yellow, crown ether (145 µl; 0.733 mmol) and diethyl (3-bromopropyl) phosphonate **18** (107 µl; 0.538 mmol) were added into the flask. The reaction was monitored by TLC (DCM/Acetone 9:1) and ^{31}P-NMR spectroscopy. The mixture was stirred for 24 h and then water (10 ml) was added. The solution was extracted with EtOAc (3×10 ml), the organic phase was dried with $MgSO_4$ and the solvent was removed under reduced pressure to afford a colourless oil (463 mg). The purification by chromatography failed due to the complexity of the crude mixture.

(13h) Attempted synthesis of diethyl ester of 5'-*O*-(phosphonopropyl)-3-*N*-3'-*O*-*bis*(benzyloxymethyl)-2'-deoxyuridine



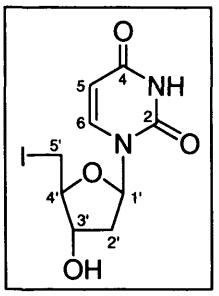
Bis(benzyloxymethyl)-2'-deoxyuridine **5b** (300 mg; 0.64 mg) was dissolved in THF (5 ml) and added into a flask containing sodium hydride (40 mg; 0.96 mmol), under nitrogen. After 15 min, when the suspension changed colour from white to orange, crown ether (190 μl; 0.96 mmol) and diethyl (3-bromopropyl) phosphonate **18** (140 μl; 0.70 mmol) were added to the reaction. The mixture was left stirring 20 h; water (10 ml) was added. The aqueous solution was extracted with EtOAc (3×10 ml), the organic fractions were collected and dried with $MgSO_4$. The solvent was removed under reduced pressure to afford a crude yellow residue which was purified by flash chromatography. Unfortunately NMR analysis of the collected fraction showed that it was a mixture of compounds.

(14) Attempted synthesis of 5'-*O*-phosphonoethyl--3-*N*-3'-*O*-*bis*(benzyl)-2'-deoxyuridine



The diethyl phosphonate **13a** (193 mg; 0.298 mmol) was dissolved in toluene (2 ml). The solvent was removed *in vacuo*, and the residue was re-dissolved in dry DCM (2 ml) under nitrogen. Trimethylsilyl bromide (236 μl; 1.79 mmol) was then added into the solution. The reaction was stirred 24 h at room temperature, then the solvents were removed under reduced pressure and the residue was taken in a mixture EtOH/H_2O 1:1 (5 ml) and stirred at room temperature. After 30 min the solvents were evaporated, and the latter operation was repeated 3 times. The obtained residue was dissolved in a mixture of CH_3CN/H_2O and purified by reverse phase chromatography without success.

(20a) 5'-iodo-2'-deoxyuridine[95]



PPh_3 (785 mg, 3 mmol) was added to a suspension of 2'-deoxyuridine (457 mg, 2 mmol) and iodine (760 mg, 3 mmol) in dioxane (10 ml) at room temperature, under N_2. The mixture was left aver night under stirring at room temperature. Methanol (1 ml) was added. The solvent was evaporated under reduced pressure, the residue was dissolved in EtOAc, washed with water (2×10 ml), and $Na_2S_2O_4$ (10% in water, 2×10 ml). After drying with $MgSO_4$, the solvent was concentred to a syrup which was taken in hot EtOH and filtered. The filtrate was concentrated, Et_2O (1 ml) was added and on cooling the desired compound was crystallized out.

White crystals, 0.496 g, 49%.

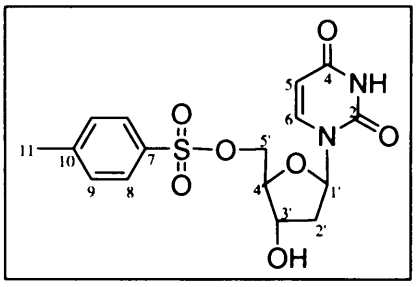
Mp: 146 °C (Lit. 173-174 °C)[95].

^{1}H-NMR (300 MHz; $(CD_3)_2SO$) δ: 11.35 (bs, 1H, N-H); 7.68 (d, *J*=8.05 Hz, 1H, H-6); 6.21 (t, *J*=6.95 Hz, 1H, H-1'); 5.67 (d, *J*=8.05 Hz, 1H, H-5); 5.50 (d, *J*=3.66 Hz, 1H, O-H); 4.19 (s, 1H, H-3'); 3.82-3.79 (m, 1H, H-4'); 3.53-3.48 (m, 1H, C*H*H-5'); 3.41-3.31 (m, 1H, CH*H*-5'); 2.32-2.23 (m, 1H, C*H*H-2'); 2.16-2.08 (m, 1H, CH*H*-2').

^{13}C-NMR (75 MHz; $(CD_3)_2SO$) δ: 163.4 (C-4); 150.8 (C-2); 141.1 (C-6); 102.5 (C-5); 85.8 (C-4'); 84.6 (C-1'); 73.3 (C-3'); 38.5 (C-2'); 8.2 (C-5').

LRMS (ES-): m/z 336.9 m/z [$(M)^-$, 80%]

(20b) 5'-*O*-tosyl-2'-deoxyuridine



A solution of *para*-toluensulfonyl chloride (917 mg, 4.81 mmol) in DCM (5 ml) was added dropwise into a solution of 2'-deoxyuridine (1 g, 4.38 mmol) in dry pyridine (5 ml), under nitrogen, cooled to 0 °C, under vigorous stirring. The reaction was left overnight at room temperature and then the evaporation of the solvent gave a crude residue, which was washed with Et_2O (5×10 ml) and cold water (5×10 ml). The precipitate was filtered off, washed with water and recrystallized from a solution of $CHCl_3$ and MeOH by dilution with petroleum ether.

White solid; 600 mg, 40%.

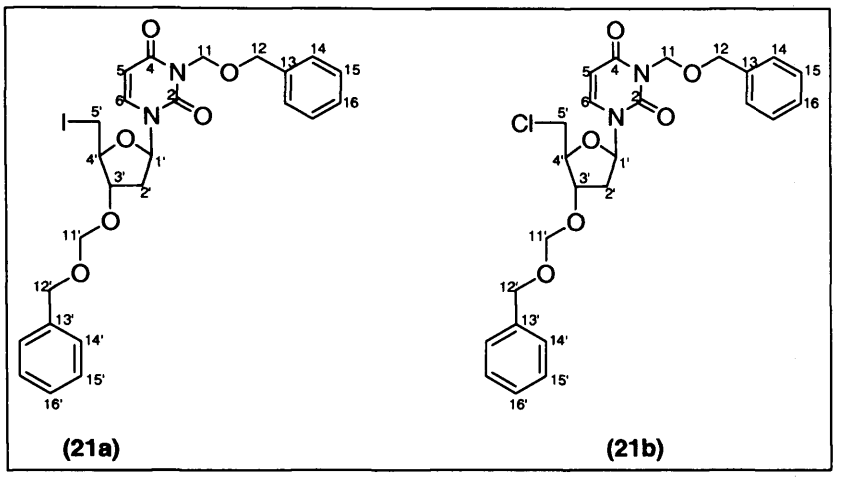
Mp: 151-154 °C (Lit. 154-156 °C)[135]

^{1}H-NMR (300 MHz; $(CD_3)_2SO$): δ 11.35 (s, 1H, NH); 7.80 (d, *J*=8.23 Hz, 2H, H-9), 7.48 (m, 3H, H-6+H-8); 6.12 (t, *J*=6.79 Hz, 1H, H-1'); 5.58 (d, *J*=8.05 Hz, 1H, H-5); 5.47 (bs, 1H, OH-3'); 4.27-4.12 (m, 3H, H-5'+ H-4'); 3.87 (m, 1H, H-3'); 2.43 (s, 3H, H-11), 2.13 (m, 2H, H-2').

^{13}C-NMR (75 MHz; $(CD_3)_2SO$): δ 163.4 (C-4); 150.6 (C-2); 145.5 (C-10); 140.9 (C-6); 132.4 (C-7); 130.8 (C-9); 128.0 (C-8); 102.3 (C-5); 84.7 (C-1'); 83.6 (C-4'); 70.4 (C-5'); 70.2 (C-3'); 39.8 (C-2'), 21.5 (C-11).

LRMS (ES+): m/z 383.1 [$(M+H)^+$, 75%]; m/z 405.0 [$(M+Na)^+$, 100%], m/z 421.0 [$(M+K)^+$, 43%].

(21a) Attempted synthesis of 5'-iodo-3-*N*-3'-*O*-*bis*(benzyloxymethyl)-2',5'-dideoxyuridine



BomCl (0.695 ml, 3 mmol) was added to a solution of 5'-iodourydine **20a** (300 mg, 0.89 mmol) and DIPEA (0.552 ml, 3 mmol) in 1,2-dichloroethane (3 ml) and THF (3 ml). The mixture was warmed at 50 °C overnight under nitrogen. Water and NH_3 conc. were added and the reaction was stirred for another 15 min. at room temperature. The organic phase was concentrated under reduced pressure; and the residue, an yellow oil, was purified by flash chromatography, using a mixture of EtOAc/Hexane as eluent. Low resolution mass of the isolated compound proved it was the 5'-chloro derivative **21b**.

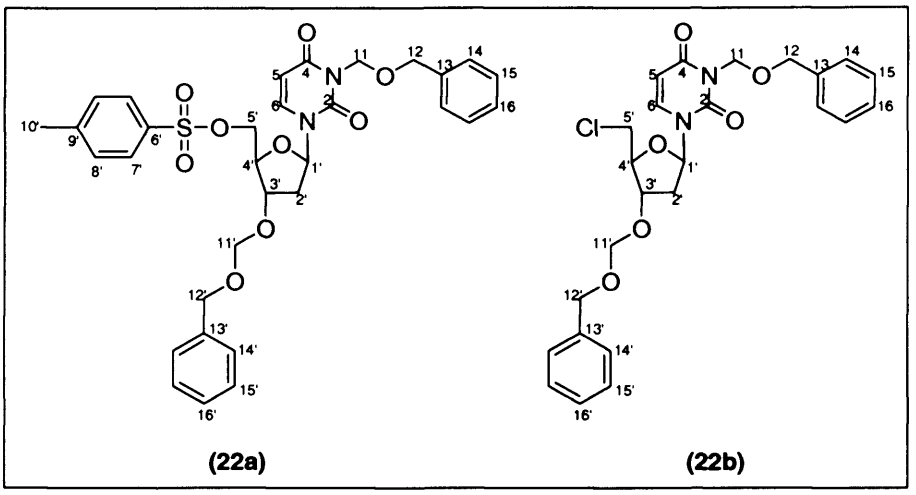
Colourless oil; 170 mg, 33%.

^{1}H-NMR (300 MHz; $CDCl_3$) δ: 7.53 (d, *J*=8.23 Hz, 1H; H-6); 7.37-7.26 (m, 10H, H-Arom.); 6.26 (t, *J*=6.04 Hz, 1H, H-1'); 5.77 (d, *J*=8.23 Hz, 1H, H-5); 5.47 (s, 2H, H-11); 4.81 (s, 2H, H-11'); 4.69 (s, 2H, H-12); 4.63 (s, 2H, H-12'); 4.40-4.37 (m, 1H, H-3'); 4.27-4.23 (m, 1H, H-4'); 3.78 (dd, J_1=12.11 Hz, 3.76 Hz, J_2=, 2H, C*H*H-5'); 3.68 (dd, J_1=12.06 Hz, , J_2=3.27 Hz, 2H, CH*H*-5'); 2.52-2.43 (m; 1H; C*H*H-2'); 2.14-2.04 (m; 1H; CH*H*-2').

^{13}C-NMR (75 MHz; $CDCl_3$) δ: 162.9 (C-4); 151.3 (C-2); 138.5 (C-6); 138.3 (C-13); 137.6 (C-13'); 129.0-128.11 (C-arom.); 102.8 (C-5); 94.5 (C-11'); 86.2 (C-1'); 83.9 (C-4'); 77.7 (C-3'); 72.7 (C-11); 70.7 (C-12', C-12'); 45.2 (C-5'); 38.8 (C-2').

LRMS (ES+): m/z 509.1 [$(M+Na)^+$, 100%], m/z 510.9 [$(M+Na)^+$, 30%].

(22a) 5'-*O*-tosyl-3-*N*-3'-*O*-*bis*(benzyloxymethyl)-2',5'-dideoxyuridine



(22a) (22b)

A solution of 5'-*O*-tosyl-2'-deoxyuridine **20b** (300 mg, 0.75 mmol), DIPEA (346 mg, 0.46 ml, 2.68 mmol) and BomCl (418 mg, 0.62 ml, 2.68 mmol) in 1,2-dichloroethane/THF 1:1 (10 ml), was warmed to 50 °C, under nitrogen over night.

After 24 h water and NH_3 concentrated were added, the mixture was stirred for 15 min. The phases were separated; the organic layer was dried and concentrated. The residue was purified by flash chromatography, using a mixture of Hexane/EtOAc as eluent, to give the desired compound **22a** as yellow oil (110 mg, 25%) and the compound **22b** with the tosylate displaced by the chloride in 5' position (115 mg, 32%).

Compound **22a**:

^{1}H-NMR (300 MHz; $CDCl_3$): δ 7.82 (d, *J*=8.23 Hz, 2H, H-6+H-Arom.); 7.45-7.25 (m, 13H, H-Ar.); 6.28 (t, *J*=6.40 Hz, 1H, H-1'); 7.75 (d, *J*=8.23, 1H, H-5); 5.5 (s, 2H, H-11); 4.85 (s, 2H, H-11'); 4.75 (s, 2H, H-12'); 4.65 (s, 2H, H-12); 4.35 (m, 1H, H-3'); 4.30-4.25 (m, 3H, H-4'+H-5'); 2.48 (m, 4H, H-2'+H-10'); 2.15 (m, 2H, H-2').

^{13}C-NMR (75 MHz; CDC_3): δ 162.9 (C-4); 151.3 (C-2); 146.0 (C-9'); 138.5 (C-6); 138.3 (C-13); 137.7 (C-13'); 132.7 (C-6'); 130-127 (C-Arom.); 102.7 (C-5); 94.5 (C-11'); 86.4 (C-1'); 83.9 (C-4'); 78.0 (C-3'); 72.7 (C-11); 70.7 (C-12); 70.5 (C-12'); 69.0 (C-5'); 38.7 (C-2'); 22.1 (C-10').

LRMS (ES+): m/z 644.8 [(M+Na)$^+$, 70%].

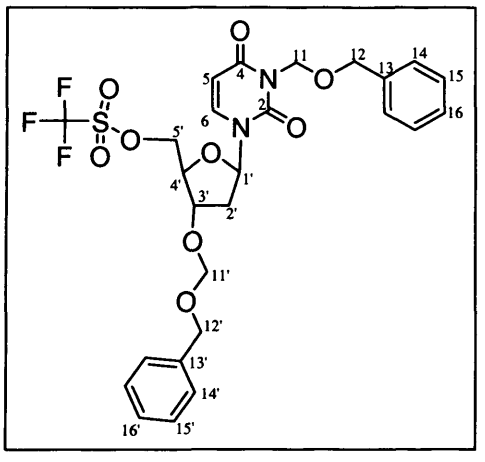
Compound **22b**:

^{1}H-NMR (300 MHz; $CDCl_3$): δ 7.64 (d, *J*=8.19 Hz, 1H, H-6); 7.47-7.35 (m, 10H, H-Arom.); 6.35 (dd, J_1=7.70 Hz, J_2=6.03 Hz, 1H, H-1'); 5.87 (d, *J*=8.19 Hz, 1H, H-5); 5.57 (s, 2H, H-11); 4.90 (s, 2H, H-11'); 4.79 (s, 2H, H-12), 4.72 (s, 2H, H-12'); 4.51-4.47 (m, 1H, H-4'), 4.37-4.33 (m, 1H, H-3'); 3.87 (dd, J_1=12.08 Hz, J_2=3.76 Hz, 1H, C*H*H-5'); 3.78 (dd, J_1=12.09 Hz, J_2=3.30 Hz, 1H, CH*H*-5'); 2.62-2.54 (m, 1H, C*H*H-2'); 2.23-2.13 (m, 1H, CH*H*-2').

^{13}C-NMR (75 MHz; CDC_3): δ 162.9 (C-4); 151.3 (C-2); 138.6 (C-6); 138.3 (C-13); 138.7 (C-13'), 129.0-128.1 (C-Arom.); 102.8 (C-5); 94.5 (C-11'); 86.2 (C-1'); 83.9 (C-4'); 77.7 (C-3'); 72.7 (C-11); 70.7 (C-12); 70.7 (C-12'); 45.2 (C-5'); 38.8 (C-2').

LRMS (ES+): m/z 508.9 [(M+Na)$^+$, 100%]; 509.9 [(M+Na)$^+$, 30%].

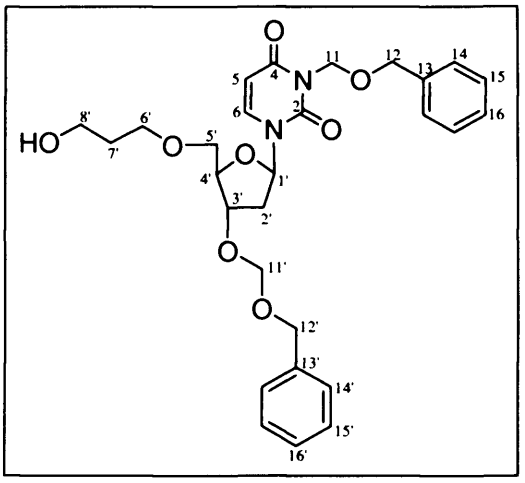
(23) 5'-*O*-(trifluoromethansulfonyl)-3-*N*-3'-*O*-*bis*(benzyloxymethyl)-2'-deoxyuridine



A solution of 3-*N*-3'-*O*-*bis*(benzyloxymethyl)-2'-deoxyuridine **5b** (1g, 2.13 mmol) and *N,N*-diisopropylethylamine (387 mg, 0.51 ml, 2.99 mmol) in DCM (20 ml) was cooled to -78 °C, under nitrogen. Trifluoromethane sulfonic anhydride (845 mg, .5 ml, 2.99 mmol) was added into the mixture, which was warmed to room temperature. TLC (EtOAc/hexane 6:4) showed complete reaction after 1

hour; water was added into solution, separated the phases, dried the organic layer on $MgSO_4$ and evaporated the solvent (co-evaporated with toluene) at max 20 °C. Due to the high reactivity, the compound was directly used for the next step without further purification and characterization.

(24) 5'-*O*-(3-hydroxypropyl)-3-*N*-3'-*O*-*bis*(benzyloxymethyl)-2'-deoxyuridine



Method A

NaH (300 mg, 7.5 mmol) was added to a solution of 1,3-propanediol (3 ml) in dry DMF (20 ml), under nitrogen at room temperature. The mixture was stirred until the effervescence was finished and the solution became clear. Then it was cooled to -50 °C, and the triflate **23** dissolved in THF (5 ml) was added dropwise with a syringe. The solution was left to warm to room temperature and was checked by TLC (EtOAc/hexane 80:20). After the disappearance of the starting material (~ 2 h) water (20 ml) was added to the system, the mixture was extracted with EtOAc (3×15 ml), the organic phases washed with water (3×10 ml) and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the obtained residue was purified by flash chromatography using the mixture EtOAc/Hexane as eluent.

Yellow oil; 540 mg, 48%.

^{1}H-NMR (300 MHz; $(CD_3)_2SO$): δ 7.78 (d, *J*=8.23 Hz, 1H, H-6); 7.40-7.27 (m, 10H, H-Ar.); 6.25 (t, *J*=6.4 Hz, 1H, H-1'); 5.80 (d, *J*=8.23 Hz, 1H, H-5'); 5.32 (s, 2H, H-11); 4.82 (s, 2H, H-11'); 4.60 (s, 4H, H-12+H-12'); 4.35 (m, 1H, H-3'); 4.12

(m, 1H, H-4'); 4.05 (t, *J*=6.59 Hz, 2H, H-6'); 3.65-3.45 (m, 4H, H-8'+H-5'); 2.38 (m, 1H, C*H*H-2'); 2.20 (m, 1H, CH*H*-2'); 1.82 (m, 2H, H-7').

^{13}C-NMR (75 MHz; $(CD_3)_2SO$): δ 162.3 (C-4); 150.9 (C-2); 139.8 (C-6); 138.4 (C-13+C13'); 128-127.6 (C-arom.); 101.4 (C-5); 95.5 (C-11'); 85.9 (C-1'); 83.8 (C-4'); 76.8 (C-3'); 71.4 (C-11); 70.5 (C-12'); 70.3 (C-12); 69.4 (C-6'); 67.6 (C-8'); 61.5 (C-5'); 39.2 (C-2'); 31.8 (C-7').

LRMS (ES+): m/z 548.9 $[(M+Na)^+$, 65%].

Method B

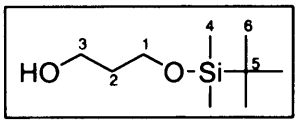
1,3-propanediol (18.3 mg, 0.017 ml, 0.24 mmol) was dissolved in dry THF (2 ml) stirred at room temperature, under nitrogen. NaH (10 mg, 0.2 mmol) was added into the mixture and after 15 min a solution of 5'-*O*-tosyl-2'-deoxyuridine **20b** (100 mg, 0.16 mmol) in THF (2 ml) was added. The reaction was left under stirring at room temperature. When the TLC (EtOAc/Hexane 50:50) showed the disappearance of the starting material, the mixture was quenched with water (5 ml) and extracted with EtOAc (2×10 ml). The organic phases were washed with water (2×10 ml), dried and the solvent was evaporated to give a crude residue, which was purified by chromatography affording the desired compound.

Yellow oil; 60 mg, 70%.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 7.80 (d, *J*=8.05 Hz, 1H, H-6); 7.48-7.32 (m, 10H, H-Ar.); 6.26 (t, *J*=6.40 Hz, 1H, H-1'); 5.80 (d, *J*=8.05 Hz, 1H, H-5); 5.56 (s, 2H, H-11); 4.85 (s, 2H, H-11'); 4.78 (s, 2H, H-12'); 4.68 (s, 2H, H-12); 4.45 (m, 1H, H-3'), 4.25 (m, 1H, H-4'); 3.80-3.58 (m, 6H, H-5'+H-8'+H-6'); 2.63-2.56 (m, 1H, C*H*H-2'); 2.26-2.17 (m, 1H, CH*H*-2'); 1.85 (quintet, *J*=6.04 Hz, 3H, H-7').

^{13}C-NMR (75 MHz; $CDCl_3$): δ 161.7 (C-4); 149.9 (C-2); 137.6 (C-6); 136.9 (C-13'); 136.2 (C-13); 127-126.6 (C-Arom.); 100.7 (C-5); 92.8 (C-11'); 85.1 (C-1'); 83.1 (C-4'); 76.4 (C-3'); 71.2 (C-11); 69.6 (C-12'); 69.2 (C-12); 69.0 (C-6'); 67.4 (C-8'); 59.3 (C-5'); 37.8 (C-2'); 28.7 (C-7').

(26) 3-(*tert*-butyldimethylsilanyloxy)-propan-1-ol



A solution of TBDMS-Cl (1.98 g; 13.14 mmol) and imidazole (1.97 g; 28.9 mmol) in dry DMF (30 ml), was added dropwise into a solution of 1,3-propane diol (2 g; 26.28 mmol) in DMF (40 ml); under N_2 at room temperature. After three h stirring at room temperature, water (70 ml) was added to quench the reaction. The aqueous solution was then extracted with EtOAc (3×100 ml). The organic phase was washed with a saturated solution of $NaHCO_3$ (3×100 ml), dried with $MgSO_4$ and the solvent was removed under reduced pressure. The crude residue (colourless oil) was purified by flash chromatography using the mixture Hexane/EtOAc (1:1) as eluent.

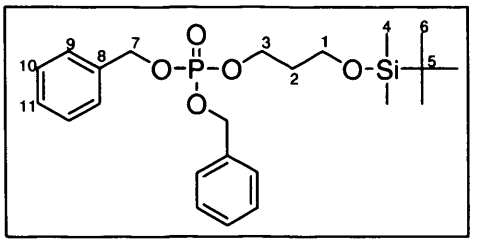
Colourless oil; 1.05 g; 42%.

R_f: 0.77 in Hexane/EtOAc (50:50)

^{1}H-NMR (300 MHz; $CDCl_3$): δ 3.90-3.83 (m, 4H, H-1+H-3); 2.73 (bs, 1H, OH); 1.83 (quintet, *J*=5.67 Hz, 2H, H-2); 0.96 (s, 9H, H-6); 0.13 (s, 6H, H-4).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 63.2 (C-3); 62.7 (C-1); 34.7 (C-2); 26.3 (C-6); 18.6 (C-5).

(27) Phosphoric acid dibenzyl ester 3-(*tert*-butyl-dimethyl-silanyloxy)-propyl ester



Sulfuryl chloride (485 mg; 289 µl; 3.6 mmol) was added to a solution of dibenzyl phosphite (945 mg; 0.814 ml; 3.6 mmol) in CCl_4 (4 ml). The reaction was stirred at room temperature under nitrogen for 2 h. The volatiles were removed under reduced pressure and the residue (light yellow oil) was added with a syringe into a solution containing the protected alcohol **26** (500 mg; 2.62 mmol) and pyridine (829 mg; 4 mmol) in dry DCM (4 ml) cooled with an ice bath, under N_2. The reaction was stirred at 0 °C for 1 hour and then 2 h at room temperature. The solvents were removed under reduced pressure and the crude reside was purified by flash chromatography.

Colourless oil, 100 mg, 8.5%.

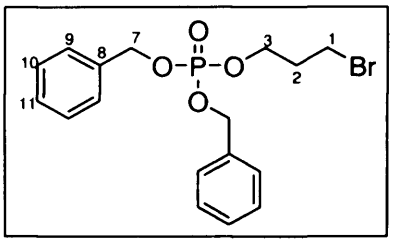
R_f: 0.81 in Hexane/EtOAc 50:50.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 7.34-7.29 (m, 10H, H-arom.); 5.02 (dd, J_1=4.23 Hz; J_2=1.65 Hz; 4H; H-7); 4.09 (q; J=6.57 Hz; 1H; H-3); 3.63 (t, J=6.04 Hz, 2H, H-1); 1.79 (quintet, J=6.04 Hz, 2H, H-2); 0.85 (s, 9H, H-6); 0.00 (s, 6H, H-4).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 136.4 (d, J=6.87 Hz, C-8); 128.9-128.3 (C-arom.); 69.5 (d, J=5.43 Hz, C-7); 65.3 (d, J=5.96 Hz, C-3); 59.3 (C-1); 33.7 (d, J=7.01 Hz, C-2); 26.3 (C-6); 18.7 (C-5); -5.0 (C-4).

^{31}P-NMR (121 MHz; $CDCl_3$): δ 0.56.

(29b) Dibenzyl (3-bromopropyl) phosphate



Iodine (883 mg; 3.48 mmol) was added to a solution of tribenzyl phosphite (1.29 g; 3.65 mmol) in DCM (7 ml), at -20 °C, under nitrogen. After 30 min the brown solution was allowed to reach room temperature and it was subsequently added dropwise into a solution of 3-bromo-1-propanol (250 mg; 0.184 ml; 1.74 mmol) in dry pyridine (550 mg; 0.560 ml; 6.96 mmol) at -50 °C. After 1 hour the solution was warmed to 25 °C and stirred for other 30 min. The precipitate was filtered off and the organic solution was washed with a solution of sodium thiosulphate 5% (2×10 ml) and HCl 0.1 M (2×10 ml). The organic layers were dried with $MgSO_4$ and the solvent was evaporated under reduced pressure to give a yellow oil which was purified by flash chromatography.

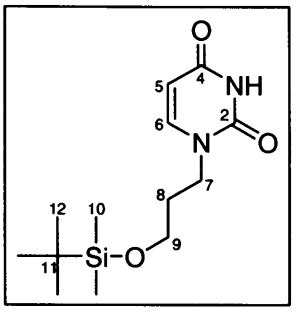
Colourless oil, 173 mg, 26%.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 7.48-7.35 (m, 10H, H-arom.); 5.11 (dd, J_1=8.42 Hz, J_2=3.29 Hz, 4H, H-7); 4.18 (q, J=6.40 Hz, 2H, H-3); 3.59 (t, J=6.22 Hz, 2H, H-1); 2.13-2.01 (m, 2H, H-2).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 136.1 (d, J=6.89 Hz, C-8); 129.2-128.26 (C-arom.); 69.8 (d, J=5.74 Hz, C-7); 64.7 (d, J=5.74 Hz, C-3); 41.0 (C-1); 33.3 (d, J=6.89 Hz, C-2).

^{31}P-NMR (121 MHz; $CDCl_3$): δ 0.37.

(30a) 1-(*tert*-butyldimethylsilyloxypropyl) uracil



Method A

3-bromo-1-tetrabutyldimethylsilylpropanol **10a** (641 mg, 2.68 mmol) was added into a suspension of uracil (300 mg, 2.68 mmol) and Cs_2CO_3 (872 mg, 2.68 mmol) in THF (10 ml), under N_2. The reaction was warmed to 40 °C. After 12 h the TLC did not show the presence of any new spot.

Method B

3-bromo-1-tetrabutyldimethylsilylpropanol **10a** (641 mg, 2.68 mmol) was added into a solution of uracil (300 mg, 2.68 mmol) and Cs_2CO_3 (872 mg, 2.68 mmol) in DMF (10 ml), under N_2. The reaction was warmed to 60 °C for 4 h, until the TLC (EtOAc/Hexane 3:1) showed the complete disappearance of the uracil. Water (10 ml) was added, and the mixture was extracted with EtOAc (3×10 ml). The organic phase was dried and evaporated under reduced pressure. The obtained residue (colourless oil) was purified by flash chromatography using a mixture of EtOAc/Hexane as eluent. Colourless oil; 250 mg, 33%.

M.p.: 120-122 °C.

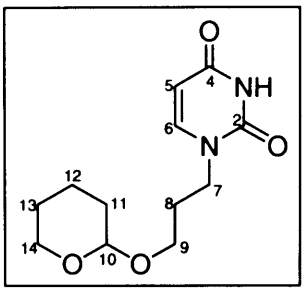
^{1}H-NMR (300 MHz; $CDCl_3$) δ: 9.15 (bs, 1H, N-H); 7.30 (d, *J*=7.87 Hz, 1H, H-6); 5.72 (d, *J*=7.87 Hz, 1H, H-5); 3.91 (t, *J*= 6.58 Hz, 2H, H-9); 3.70 (t, *J*=6.58 Hz, 2H, H-7); 1.94 (quintet, *J*=6.58 Hz, 2H, H-8); 0.95 (s, 9H, H-12); 0.00 (s, 6H, H-10).

^{13}C-NMR (75 MHz; $CDCl_3$) δ: 164.3 (C-4); 151.2 (C-2); 145.8 (C-6); 102.0 (C-5); 59.4 (C-9); 46.6 (C-7); 31.6 (C-8); 26.3 (C-11); 18.6 (C-12); -5.0 (C-10).

LRMS (ES+): m/z 307.2 [$(M+Na)^+$, 100%]

HRMS (ES+): found 285.1630; required 285.1629 for $C_{13}H_{24}N_2SiO_3^+$ $[M+H]^+$.

(30b) 1-(3-(tetrahydro-pyran-2-yloxy)-propyl) uracil



Method A

3-bromo-1-tethrahydropyranyl propanol **10b** (595 mg, 2.68 mmol) was added to a suspension of uracil (250 mg, 2.23 mmol) and potassium carbonate (370 mg, 2.68 mmol) in THF (10 ml). The reaction was heated to 50 °C and monitored by TLC. After 48 h no new spot was observed on TLC, consequently the attempt was abandoned.

Method B

3-bromo-1-tethrahydropyranyl propanol **10b** (595 mg, 2.68 mmol) was added to a suspension of uracil (250 mg, 2.23 mmol) and potassium carbonate (370 mg, 2.68 mmol) in DMF (10 ml). The reaction was stirred at 90 °C under nitrogen. When the TLC showed the complete disappearance of the starting material (48 h circa) the mixture was allowed to cold at room temperature. The aqueous solution was extracted with EtOAc (2×10 ml). The organic phase was washed with water (2×10 ml) and dried with $MgSO_4$. The solvent was removed under vacuum to give a residue which underwent chromatographic purification. Spectroscopic analysis showed it was the 1,3-*N*,*N* dialkylated uracil.

Method C

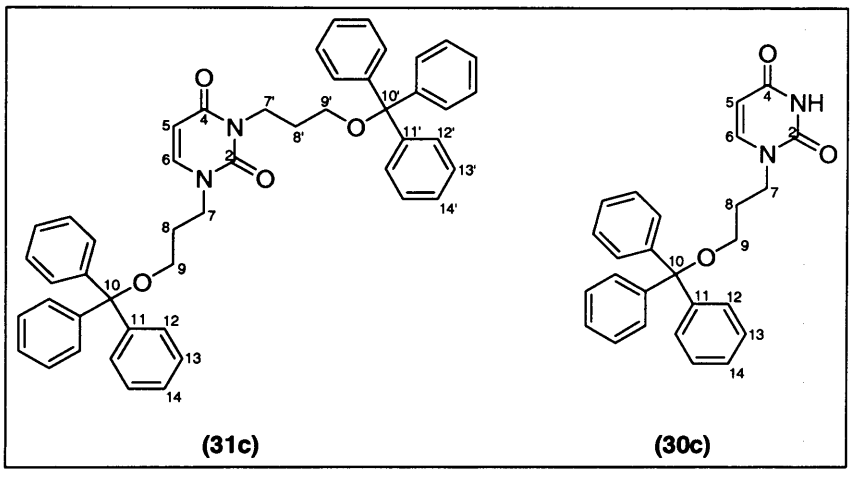
A mixture of 3-bromo-1-*O*-tethrahydropyranyl propanol **10b** (595 mg, 2.68 mmol), caesium carbonate (872 mg, 2.68 mmol) and uracil (300 mg, 2.68 mmol) in DMF (10 ml) was stirred at 40 °C under nitrogen. After 4 h the TLC showed the disappearance of the starting material, then water (10 ml) was added into the suspension. The mixture was extracted with EtOAc (3×15 ml). The organic layer was washed with brine, dried and the solvent was removed under reduced pressure to give a crude residue purified by flash chromatography to afford the desired compound.

Colourless oil, 235 mg, 22%.

^{1}H-NMR (300 MHz; $CDCl_3$) δ: 10.23 (bs, 1H, N-H); 7.27 (d, *J*=7.87 Hz, 1H; H-6); 5.68 (d, *J*= 7.87 Hz, 1H, H-5); 4.53 (s, 1H, H-10); 3.90-3.76 (m, 4H, H-9+H-7); 3.52-3.40 (m, 2H, H-14); 1.99-1.83 (q, *J*=6.10 Hz, 2H, H-8); 1.80-1.67 (m, 2H, 1 of H-11+ 1 of H-12); 1.55-1.50 (m, 4H, H-13+ 1 of H-12+ 1 of H-11).

^{13}C-NMR (75 MHz; $CDCl_3$) δ: 164.9 (C-4); 151.6 (C-2); 145.8 (C-6); 102.1 (C-5); 99.7 (C-10); 64.3 (C-14); 63.3 (C-9); 46.9 (C-7); 31.1 (C-11); 29.1 (C-8); 25.7 (C-13); 20.3 (C-12).

(30c) 1-(3-triphenylmethoxypropyl) uracil



A mixture of 3-bromo-1-*O*-trityl propanol **10c** (400 mg, 1.05 mmol), caesium carbonate (385 mg, 1.05 mmol) and uracil (129 mg, 1.15 mmol) in DMF (10 ml) was heated to 40 °C under nitrogen. After 4 h the TLC showed the disappearance of the starting material, then water (10 ml) was added into the suspension, and extracted with EtOAc (3×15 ml). The organic layer was washed with brine, dried and the solvent was removed under reduced pressure. The crude residue was purified by chromatography affording two main fractions. Compound **30c** was isolated from the second fraction, whilst compound **31c** from the first fraction.

Compound **30c**: white solid, 134 mg, 31%.

Mp: 161-164 °C.

^{1}H-NMR (300 MHz; $CDCl_3$) δ: 8.10 (bs, 1H, N-H); 7.50-7.22 (m, 15H, H-Arom.); 6.95 (d, J=7.87 Hz, 1H, H-6); 5.47 (dd, J_1=7.87 Hz, J_2=2.37 Hz, 1H, H-5); 4.02 (t, J=6.58 Hz, 2H, H-9); 3.22 (t, J=5.67 Hz, 2H, H-7); 2.14-2.02 (m, 2H, H-6).

^{13}C-NMR (75 MHz; $CDCl_3$) δ: 160.0 (C-4); 150.7 (C-2); 148.9 (C-6); 148.7 (C-11); 137.5 (C-11); 128-125 (C-arom.); 101.7 (C-5); 97.2 (C-10); 67.0 (C-9); 48.0 (C-7); 26.5 (C-8).

LRMS (ES+): m/z 435.2 [$(M+Na)^+$, 100%].

HRMS (ES+): found 435.1680; required 435.1679 for $C_{25}H_{24}N_2NaO_3^+$; $[M+Na]^+$.

Microanalysis calculated for $C_{26}H_{24}N_2O_3$:

C 75.71, H 5.86, N 8.79%; found C 75.54, H 5.99, N 6.58%.

Compund **31c**: white solid, 116 mg, 15%.

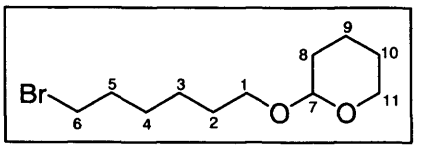
Mp: 70-73 °C.

^{1}H-NMR (500 MHz; $CDCl_3$) δ: 7.45-7.43 (m, 12H, H-12+H-12'); 7.35-7.21 (m, 18H, H-13+H-14+H-15+H-13'+H-14'+H-15'); 6.85 (d, J=8.0 Hz, 1H, H-6); 5.45 (d, J=8.0 Hz, 1H, H-5); 4.00 (t, J=7.0 Hz, 2H, H-7'); 3.88 (t, J=6.50 Hz, 2H, H-7); 3.16-3.12 (m, 4H, H-9+H-9'); 1.99-1.92 (m, 4H, H-8+H-8').

^{13}C-NMR (125 MHz; $CDCl_3$) δ: 162.9 (C-4); 151.2 (C-2); 144.2, 143.7 (C-11, C-11'); 142.7 (C-6); 128.7, 128.5 (C-12, C-12'); 127.9, 127.7 (C-13, C-13'); 126.8 (C-14, C-14'); 100.9 (C-5); 86.7 86.5 (C-10, C-10'); 61.8 (C-9); 59.4 (C-9'); 47.1 (C-7); 39.2 (C-7'); 29.7 (C-8); 29.6 (C-8').

LRMS (ES+): m/z 243.1 [$(Ph_3P)^+$, 100%]; m/z 735.3 [$(M+Na)^+$, 28%]; 751.3 [$(M+K)^+$, 59%].

(33a) 2-(6-Bromo-hexyloxy)-tetrahydro-pyran[136]



A solution of DHP (1.393 g, 16.66 mmol) in dichloromethane (10 ml) was added dropwise into a solution of 6-bromo-1-hexanol (0.722 ml, 5.52 mmol) and PPT (27.74 mg, 0.11 mmol) in DCM (20 ml), under N_2. The solution was stirred over night at room temperature, and then washed with brine (3×25 ml). The organic phase was dried with $MgSO_4$ and concentrated to give a crude product, which was purified by flash chromatography to afford the desired compound.

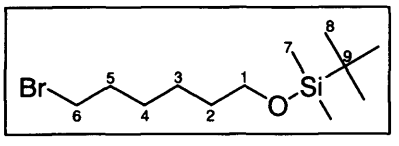
Colourless oil, 900 mg, 61 %.

^{1}H-NMR (300 MHz; $CDCl_3$) δ: 4.64-4.62 (m, 1H, H-7); 4.00-3.86 (m, 1H, C*H*H-1); 3.85-3.75 (m, 1H, CH*H*-1); 3.60-3.40 (m, 4H, H-6+H-11); 1.99-1.40 (m, 14H, H-2+H-3+H-4+H-5+H-8+H-9+H-10).

^{13}C-NMR (75 MHz; $CDCl_3$) δ: 99.3 (C-7); 67.8 (C-1); 62.8 (C-11); 34.3 (C-6); 33.1 (C-5); 31.2 (C-8); 29.9 (C-4); 28.4 (C-2); 27.7 (C-3); 25.9 (C-10); 20.1 (C-9).

LRMS (ES+): m/z 287.1 [$(M+Na)^+$, 100%]

(33b) - (6-Bromo-hexyloxy)-*tert*-butyl-dimethyl-silane[137]



6-bromo-1-hexanol (0.5g, 0.722 ml, 5.52 mmol) was added to a stirred solution of TBDMSCl (981 mg, 6.51 mmol) and imidazole (1.031 g, 4.26 mmol) in DMF (10 ml) under nitrogen. The mixture was stirred over night at room temperature, then the solution was diluted with Et_2O, washed with saturated $NaHCO_3$ and HCl 3M respectively. The organic phase dried and evaporated to give a residue which did not required further purification.

Colourless oil; 1 g, 61%.

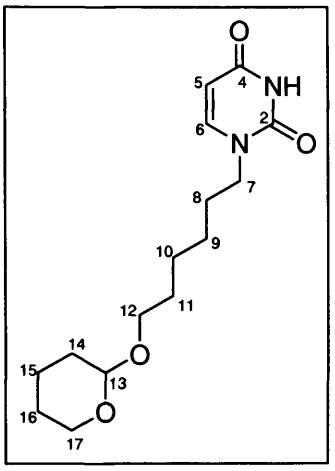
^{1}H-NMR (300 MHz; $CDCl_3$) δ: 3.56 (t, *J*=6.40 Hz, 2H, H-1); 3.48 (t; *J*=6.50 Hz, 2H, H-6); 1.89-1.21 (m, 8H, H-2+H-3+H-4+H-5); 0.95 (s, 9H, H-8); 0.05 (s, 6H, H-7).

^{13}C-NMR (75 MHz; $CDCl_3$) δ: 63.5 (C-1); 33.2 (C-6); 28.4 (C-2); 27.1 (C-5); 26.4 (C-3); 26.0 (C-9); 25.5 (C-4); 18.8 (C-8); -4.8 (C-7).

General procedure A: coupling of uracil base with protected bromo alcohols or other bromo derivatives

The bromo intermediate (1 mmol) was added into a suspension of uracil (1.1 mmol), caesium carbonate (1 mmol) in dry DMF (5 ml) under nitrogen. The reaction was heathed to 40-60 °C according to the reactivity of the bromo substrate. When the TLC showed the complete disappearance of the starting bromo intermediate the reaction was quenched with water (10 ml). The aqueous solution was extracted with EtOAc (3×15 ml). The organic layer was washed with brine (3×10 ml), dried and concentrated under reduced pressure. The crude residue was purified by flash chromatography.

(34a) 1-(6-(tetrahydro-pyran-2-yloxy)-hexyl) uracil



The title compound was prepared following the general procedure **A**, from 6-bromo-1-*O*-tetrahydropyranilpropanol **33a** (265 mg, 1.0 mmol), caesium carbonate (326 mg, 1.0 mmol) and uracil (125 mg, 1.10 mmol) in DMF (5 ml).

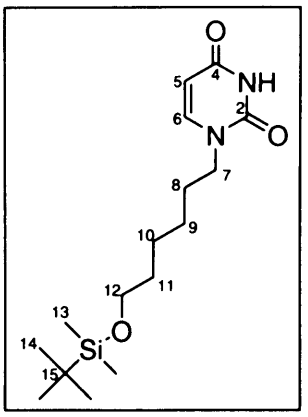
White solid 55 mg, 18.5%.

^{1}H-NMR (300 MHz; $CDCl_3$) δ: 10.3 (s, 1H, N-H); 7.18 (d, *J*=7.87 Hz, H-6); 5.66 (d, *J*=7.87 Hz, 1H, H-5); 4.53 (bs, 1H, H-13); 3.85-3.78 (m, 1H, C*H*H-17); 3.72-3.65 (m, 3H, H-7+CH*H*-17); 3.43-3.38 (m, 1H, C*H*H-12); 3.36-3.30 (m, 1H, CH*H*-12); 1.81-1.34 (m, 14H, H-8+H-9+H-10+H-11+H-14+H-15+H-16).

^{13}C-NMR (75 MHz; $CDCl_3$) δ: 164.8 (C-4); 151.5 (C-2); 145.0 (C-6); 102.4 (C-5); 99.2 (C-13); 67.8 (C-12); 62.7 (C-17); 49.1 (C-7); 31.1 (C-14); 30.0 (C-8); 29.9 (C-16); 29.3 (C-15); 26.6 (C-10); 26.2 (C-10); 20.0 (C-9).

LRMS (ES+): m/z 319.2 [$(M+Na)^+$, 50%].

(34b) 1-(6-(*tert*-butyldimethylsilanyloxy)-hexyl) uracil



The title compound was prepared starting from 6-bromo-1-*O*-tethrabutyldimethylsilyl hexanol **33b** (600 mg, 1.50 mmol), caesium carbonate (585 mg, 1.80 mmol), uracil (200 mg, 1.80 mmol) in DMF (10 ml) and following the general procedure **A**.

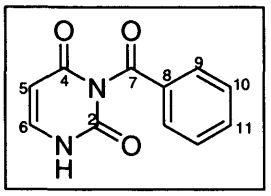
White solid, 85 mg; 17%.

^{1}H-NMR (300 MHz; $CDCl_3$) δ: 9.39 (bs, 1H, N-H); 7.11 (d, J=7.86 Hz, 1H, H-6); 5.66 (dd, J_1=7.86 Hz, J_2=2.19 Hz, 1H, H-5); 3.68 (t, J=7.31 Hz, 2H, H-7); 3.55 (t, J=6.22 Hz, 2H, H-12); 1.70-1.57 (m, 2H, H-11); 1.55-1.43 (m, 2H, H-8); 1.39-1.24 (m, 4H, H-9+H-10); 0.84 (s, 12H, H-14); 0.00 (s, 6H, H-13).

^{13}C-NMR (75 MHz; $CDCl_3$) δ: 164.4 (C-4); 151.3 (C-2); 144.9 (C-6); 102.5 (C-5); 63.3 (C-12); 49.3 (C-7); 45.5 (C-11); 33.0 (C-10); 29.5 (C-9); 26.6 (C-8); 26.4 (C-14); 18.8 (C-15); -4.9 (C-13).

LRMS (ES+): m/z 349.3 [$(M+Na)^+$, 100%].

(35) 3-*N*-benzoyl uracil[100]



Benzoyl chloride (5.2 ml; 44.8 mmol) was added to a stirred suspension of uracil (2.24 g; 20 mmol) in dry acetonitrile (20 ml) and pyridine (8 ml) under nitrogen. After 5 min the suspension became a yellow solution which was left stirring at room temperature overnight. After 12 h the formation of a white precipitate was observed. 24 h later the products were concentrated under reduced pressure and the residue was partitioned between dichloromethane and water (100 ml each). The formation of a white precipitate in the aqueous phase was observed. The organic phase was separated and the solvent evaporated. The residue was taken into a mixture of dioxane (40 ml) and K_2CO_3 0.5 M (20 ml) and stirred for 30 min. The pH was lowered to 5 with glacial acetic acid. The products were concentrated under reduced pressure and then dissolved in $NaHCO_3$ (100 ml). The solution was stirred at room temperature for 1 hour, the precipitate was filtered off, washed with cold water (3×10 ml) and recrystallised from aqueous acetone.

Off-white solid; 2.27 g; 53%.

Mp: 214-217 °C; (Lit. 173.5-175 °C)[100]

R_f: 0.42 in DCM/MeOH (90:10).

^{1}H-NMR (300 MHz; $(CD_3)_2SO$): δ 11.61 (s, 1H, NH); 7.97 (d, *J*=8.23 Hz, 2H, H-9); 7.79 (t, *J*=6.90 Hz, 1H, H-11); 7.68 (d, *J*=8.60 Hz, 1H; H-6); 7.62 (t, *J*=7.68 Hz, 2H, H-10); 5.79 (d, *J*=8-60 Hz, 1H; H-5).

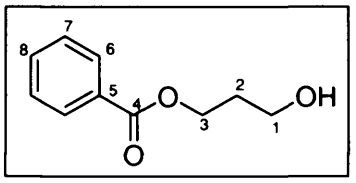
^{13}C-NMR (75 MHz; $(CD_3)_2SO$): δ 170.4 (C-7); 163.3 (C-4); 150.4 (C-2); 143.7 (C-6); 135.8 (C-8); 131.7 (C-11); 130.6 (C-9); 129.8 (C-10); 100.4 (C-5).

LRMS (ES+): m/z 239.1 [(M+Na), 100%].

General procedure B: monobenzoylation of diols[102]

To a solution of diol (1 equivalent) in THF (5 ml) was added a catalytic amount of dimethyltin dichloride (0.01 eq.), solid K_2CO_3 (2.0 eq.) and benzoyl chloride (1.2 eq.), at room temperature. After stirring the mixture at room temperature until the diol was disappeared (checked by TLC), the mixture was poured onto water and the organic portion was extracted with DCM. After evaporation of the solvent, a residue was obtained and purified by flash chromatography using a mixture of DCM/MeOH as eluent.

(37a) 3-hydroxypropyl benzoate[102]



The title compound was synthesised following the general procedure **B** for the monobenzoylation of diols, starting with 1,3-propanediol (546 mg, 7.18 mmol), K_2CO_3 (1.99 g, 14.37 mmol), benzoyl chloride (1.21 g, 8.62 mmol) and dimethyltin dichloride (15 mg, 0.07 mmol).

Colourless oil, 724 mg; 55%.

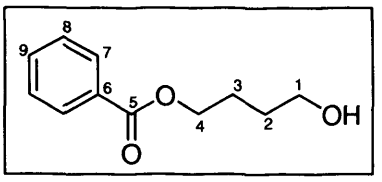
R_f: 0.48 in EtOAc/Hexane 40:60

^{1}H-NMR (300 MHz; $CDCl_3$): δ 8.10 (d, *J*=6.95 Hz, 2H, H-6); 7.63 (t, *J*=7.32 Hz, 1H, H-8); 7.50 (t, *J*=7.68 Hz, 2H, H-7); 4.55 (t, *J*=6.04 Hz, 2H, H-1); 3.84 (t, *J*=6.04 Hz, 2H, H-3); 2.11-2.03 (m, 3H, H-2+OH).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 167.4 (C-4); 133.5 (C-8); 130.5 (C-5); 130.0 (C-6); 128.8 (C-7); 62.2 (C-1); 59.6 (C-3); 32.3 (C-2).

LRMS (ES+): m/z 203.1 [$(M+Na)^+$, 81%].

(37b) 4-hydroxybutyl benzoate[102]



The title compound prepared according to the general procedure **B** for the monobenzoylation of diols, starting from 1,4-butanediol (647 mg, 7.18 mmol), K_2CO_3 (1.99 g, 14.37 mmol), benzoyl chloride (1.21 g, 8.62 mmol) and dimethyltin dichloride (15 mg, 0.07 mmol). Colourless oil, 897 mg; 62%.

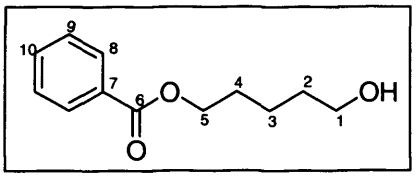
R_f: 0.58 in EtOAc/Hexane 40:60

^{1}H-NMR (300 MHz; $CDCl_3$): δ 8.08 (d, *J*=6.95 Hz, 2H, H-7); 7.60 (t, *J*=7.32 Hz, 1H, H-9); 7.45 (t, *J*=7.32 Hz, 2H, H-8); 4.41 (t, *J*=6.58 Hz, 2H, H-1); 3.77 (t, *J*=6.40 Hz, 2H, H-4); 1.97-1.87 (m, 2H, H-2); 1.82-1.73 (m, 2H, H-3).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 167.2 (C-5); 133.4 (C-9); 130.7 (C-6); 130.0 (C-7); 128.8 (C-8); 65.2 (C-1); 62.7 (C-4); 29.6 (C-2); 25.6 (C-3).

LRMS (ES+): m/z 217.1 [$(M+Na)^+$, 100%].

(37c) 5-hydroxypentyl benzoate[102]



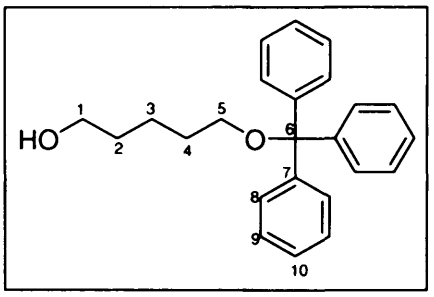
1,5-pentane diol (748 mg, 7.18 mmol) was benzoylated according to the general procedure **B**, with K_2CO_3 (1.99 g, 14.37 mmol), BzCl (1.21 g, 8.62 mmol) and dimethyltin dichloride (15 mg, 0.07 mmol). Colourless oil, 593 mg; 40%

R_f: 0.64 in EtOAc/Hexane 40:60

^{1}H-NMR (300 MHz; $CDCl_3$): δ 8.03 (d, *J*=6.95 Hz, 2H, H-8); 7.55 (t, *J*=7.32 Hz, 1H, H-10); 7.43 (t, *J*=7.31 Hz, 2H; H-9); 4.33 (t, *J*=6.59 Hz, 2H, H-1); 3.67 (t, *J*=6.40 Hz, 2H, H-5); 1.81 (quintet, *J*=7.50 Hz; 2H, H-3); 1.69-1.47 (m, 5H, H-2+H-4+OH).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 166.7 (C-6); 132.8 (C-10); 130.4 (C-7); 129.5 (C-8); 128.3 (C-9); 64.9 (C-1); 62.7 (C-5); 32.3 (C-2); 28.5 (C-4); 22.6 (C-3).

(37e) 5-(trityloxy)pentan-1-ol



A solution of trityl chloride (1.87 g; 6.72 mmol) and triethylamine (937 µl; 6.72 mmol) in dry DCM (25 ml) was added dropwise into a solution of 1,5-pental diol (1 g; 9.60 mmol) in dry DCM (25 ml), at 0 °C, under nitrogen. The mixture was stirred one hour at 0 °C and then it was left to warm to room temperature. The organic solution was washed with HCl 0.1 M (2×10 ml) and brine (2×10 ml). The organic phase was dried with $MgSO_4$ and the solvent was removed under reduced pressure. The crude residue was then purified by column chromatography, affording the desired compound.

Colourless oil; 1.06 g; 32%.

R_f: 0.50 in EtOAc/Hexane 40:60.

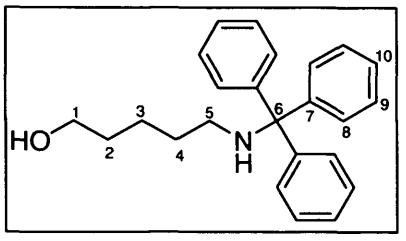
^{1}H-NMR (300 MHz; $CDCl_3$): δ 7.52-7.49 (m, 6H, H-9); 7.39-7.26 (m, 9H, H-8+H-10); 3.68 (bs, 2H, H-1); 3.13 (t, *J*=6.40 Hz, 2H, H-5); 1.72 (quintet, *J*=7.32 Hz, 2H, H-4); 1.65-1.45 (m, 4H, H-2+H-3).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 144.9 (C-7); 129.1 (C-8); 128.1 (C-9); 127.3 (C-10); 86.8 (C-6); 63.9 (C-5); 63.4 (C-1); 33.0 (C-4); 30.2 (C-2); 22.9 (C-3).

LRMS (ES+): m/z 392.2 [$(M+Na)^+$; 100%].

Ref. [138]

(37f) 5-(tritylamino)pentan-1-ol



A solution of trityl chloride (1.08 g; 3.87 mmol) in DCM (10 ml) was added dropwise into a solution of 5-amino-1-pentanol (500 mg; 4.85 mmol) and TEA (504 µl; 3.87 mmol) in DCM (20 ml), under nitrogen at 0 °C. After the addition, the mixture was warmed to 25 °C and stirred another 60 min. The reaction was quenched with water (25 ml), the phases were separated and the organic layer was washed with a saturated solution of $NaHCO_3$ (25 ml). The solvent was dried with $MgSO_4$ and removed under reduced pressure to afford a residue which was purified by column chromatography.

Colourless oil; 327 mg; 32%.

R_f: 0.33 in EtOAc/Hexane 20:30

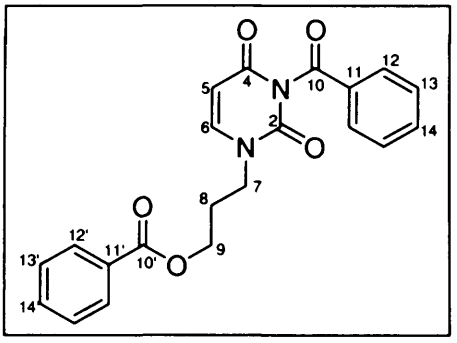
^{1}H-NMR (300 MHz; $(CD_3)_2SO$): δ 7.42-7.39 (m, 6H, H-9); 7.29 (m, 6H, H-8); 7.20-7.15 (m, 3H, H-10); 4.33 (t, *J*=5.49 Hz, 1H, OH); 3.36 (t, *J*=6.22 Hz, 2H, H-1); 1.95 (q, *J*=7.87 Hz, 2H, H-5); 1.51-1.42 (m, 2H, H-2); 1.38-1.25 (m, 4H, H-3+H-4).

General procedure C: Mitsunobu coupling of alcohols with 3-*N*-benzoyluracil[99]

The polymer supported triphenylphosphine (2.5 eq; 3 mmol/g loading) was swelled in dry THF for 15 min; then the corresponding alcohol (1 eq.) and the N^3-benzoyluracil (1.5–2 eq.) were added into the suspension, which was shaken at room temperature under nitrogen. After 15 min a solution of DIAD (2 eq.) in THF was added slowly into the suspension. The reaction was shaken overnight until the disappearance of the alcohol was observed by TLC. The resin was then filtered off and washed with THF (5 ml). The solvent was removed under reduced

pressure to afford a residue which was taken in a mixture of Hexane/EtOAc (50:50). The formed white precipitate (unreacted starting material), if present, was removed and the solution was concentrated to an orange oil which was purified by flash chromatography using a mixture of Hexane/EtOAc (40:60) as eluent.

(38a) 1-(benzoylpropyl)-3-benzoyl uracil[99]



The benzoyl alcohol **37a** (200 mg; 1.11 mmol) and the 3-*N*-benzoyl uracil **35** (480 mg; 2.22 mmol) were coupled using the previously described general procedure **C** for the Mitsunobu coupling.

Colourless oil; 183 mg; 43%.

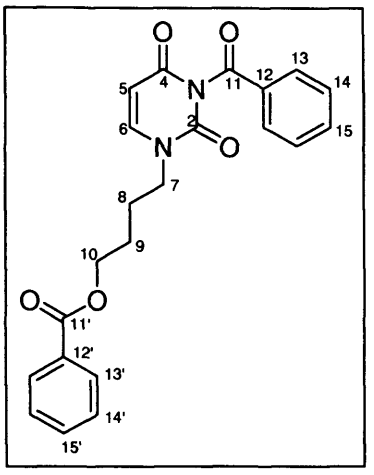
$\mathbf{R_f}$: 0.19 in EtOAc/Hexane 40:60.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 8.04 (d, *J*=7.13 Hz, H-12'); 7.93 (d, *J*=7.13 Hz, 2H, H-12); 7.65 (t, *J*=7.32 Hz, 1H, H-14'); 7.59 (t, *J*=7.32 Hz, 1H, H-14); 7.51-7.43 (m, 4H, H-13'+H-13); 7.33 (d, *J*=7.87 Hz, 1H, H-6); 5.77 (d, *J*=7.87 Hz, 1H, H-5); 4.41 (t, *J*=6.04 Hz, 2H, H-9); 3.94 (t, *J*=6.95 Hz, 2H, H-7); 2.21 (t, *J*=6.22 Hz, 2H, H-8).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 169.3 (C-10'); 166.8 (C-10); 162.8 (C-4); 150.2 (C-2); 144.9 (C-6); 135.6 (C-14'); 133.8 (C-14); 131.8 (C-11'); 130.8 (C-12'); 130.1 (C-11); 130.0 (C-12); 129.7 (C-13'); 129.0 (C-13); 102.6 (C-5); 62.1 (C-9); 47.3 (C-7); 28.5 (C-8).

LRMS (ES+): m/z 401.1 [$(M+Na)^+$; 100%].

(38b) 1-(benzoylbutyl)-3-benzoyl uracil[99]



The title compound was prepared from the benzoyl alcohol **37b** (215 mg; 1.11 mmol) and the 3-*N*-benzoyl uracil **35** (480 mg; 2.22 mmol) following the general procedure **C**.

Colourless oil; 205 mg; 47%.

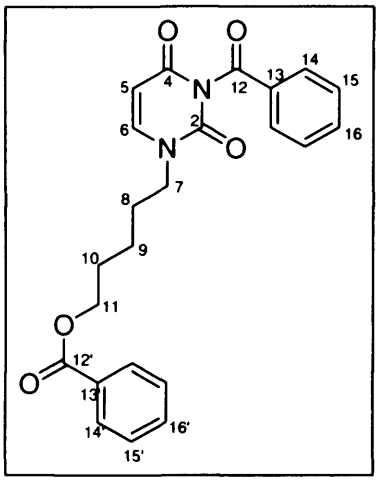
$\mathbf{R_f}$: 0.23 in EtOAc/Hexane 50:50.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 8.00 (d, *J*=6.95 Hz, 2H, H-13'); 7.89 (d, *J*=7.13 Hz, 2H, H-13); 7.62 (t, *J*=7.50 Hz, 1H, H-15'); 7.55 (t, *J*=7.50 Hz, 1H, H-15); 7.49-7.39 (m, 4H, H-14'+H-14); 7.25 (d, *J*=8.05 Hz, 1H, H-6); 5.79 (d, *J*=8.05 Hz, H-5); 4.35 (t, *J*=5.85 Hz, 2H, H-10); 3.82 (t, *J*=7.13 Hz, 2H, H-7); 1.88-1.79 (m, 4H, H-8+H-9).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 169.5 (C-11'); 167.0 (C-11); 162.8 (C-4); 150.2 (C-2); 144.4 (C-6); 135.5 (C-15'); 133.5 (C-15); 131.9 (C-12'); 130.9 (C-13'); 130.4 (C-12); 130.0 (C-13); 129.6 (C-14'); 128.9 (C-14); 102.7 (C-5); 64.3 (C-10); 49.2 (C-7); 26.3 (C-9); 26.2 (C-8).

LRMS (ES+): m/z 415.2 [$(M+Na)^+$; 80%]; m/z 806.9 [$(2M+Na)^+$; 20%].

(38c) 1-(benzoylpentyl)-3-benzoyl uracil[99]



The Benzoyl alcohol **38a** (150 mg; 0.77 mmol) was coupled with the 3-*N*-benzoyl uracil **35** (250 mg; 1.15 mmol) using the previously described Mitsunobu's procedure **C**.

White solid; 127 mg; 41%.

R_f: 0.14 in EtOAc/Hexane 50:50.

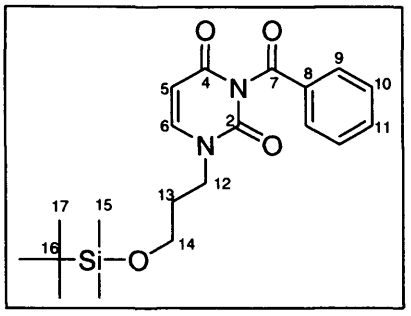
Mp: 110-112 °C.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 8.07 (d, *J*=6.95 Hz, 2H, H-14'); 7.97 (d, *J*=7.13 Hz, 2H, H-14); 7.68 (t, *J*=7.32 Hz, 1H, H-16'); 7.60 (t, *J*=6.95 Hz, 1H, H-16); 7.55-7-45 (m, 4H, H-15'+H-15); 7.31 (d, *J*=7.87 Hz, 1H, H-6); 5.80 (d, *J*=7.87 Hz, 1H, H-5); 4.36 (t, *J*=6.40 Hz, 2H, H-11); 3.81 (t, *J*=7.32 Hz, 2H, H-7); 1.90-1.78 (m, 4H, H-10+H-8); 1.59-1.49 (m, 2H, H-9).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 169.4 (C-12'); 167.0 (C-12); 162.9 (C-4); 150.2 (C-2); 144.7 (C-6); 135.6 (C-16'); 133.5 (C-16); 131.9 (C-13'); 130.8 (C-14'); 130.6 (C-13); 129.9 (C-14); 129.6 (C-15'); 128.9 (C-15); 102.4 (C-5); 64.8 (C-11); 49.4 (C-7); 29.0 (C-10); 28.7 (C-8); 23.4 (C-9).

LRMS (ES+): m/z 429.0 [$(M+Na)^+$; 100%].

(38d) 1-(*tert*-butyldimethylsilyloxypropyl)-3-benzoyl uracil



3-*N*-benzoyl uracil **35** (216 mg, 1 mmol) was coupled with and 3-(*tert*--butyl-dimethyl-silanyloxy)-propan-1-ol **10a** (90.1 mg, 0.5 mmol) following the general Mitsunobu procedure **C**, in the presence of DIAD (202 mg, 1 mmol) and polymer bound triphenylphosphine (416 mg, 1.25 mmol) and THF (4 ml).

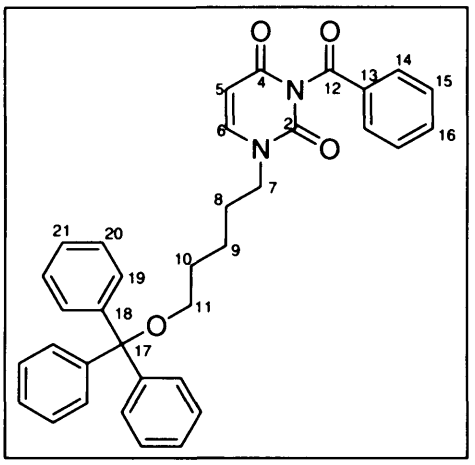
Colourless oil, 81mg; 42%.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 7.97 (t, *J*=7.13 Hz, 2H, H-9); 7.69 (t, *J*=7.32 Hz, 1H, H-11); 7.54 (t, *J*=7.50 Hz, 2H, H-10); 7.37 (d, *J*=8.06 Hz, 1H, H-6); 5.80 (d, *J*=8.06 Hz, H-5); 3.93 (t, *J*=6.58 Hz, 2H, H-14); 3.73 (t, *J*=5.67 Hz, 2H, H-12); 1.95 (quintet, *J*=6.40 Hz, 2H, H-13); 0.96 (s, 9H, H-17); 0.12 (s, 6H, H-15).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 169.3 (C-7); 163.0 (C-4); 150.2 (C-2); 145.5 (C-6); 135.5 (C-11); 131.9 (C-8); 130.8 (C-9); 129.6 (C-10); 101.9 (C-5); 59.5 (C-14); 46.9 (C-12); 31.6 (C-13); 26.3 (C-17); 18.6 (C-16); -4.9 (C-15).

LRMS (ES+): m/z 411.1 [$(M+Na)^+$; 100%].

(38e) 1-(trityloxypentyl)-3-benzoyl uracil



The trityl alcohol **37e** (300 mg; 0.87 mmol) was coupled with the 3-*N*-benzoyl uracil **35** (280 mg; 1.30 mmol) using the Mitsunobu procedure **C**.

White foam; 236 mg; 50%.

R_f: 0.57 in EtOAc/Hexane 50:50.

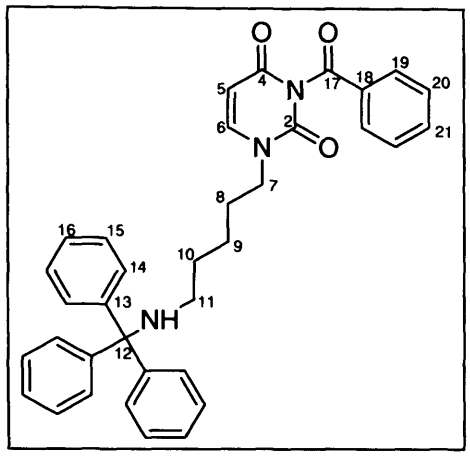
Mp: 62-65 °C.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 7.97 (d, *J*=7.13 Hz, 2H, H-14); 7.69 (t, *J*=7.32 Hz, 1H, H-16); 7.54-7.47 (m, 8H, H-15+H-20); 7.38-7.24 (m, 10H, H-6+H-arom.); 5.83 (d, *J*=7.87 Hz, 1H, H-5); 3.79 (t, *J*=7.32 Hz, 2H, H-7); 3.14 (t, *J*=6.22 Hz, 2H, H-11); 1.80-1.67 (m, 4H, H-8+H-10); 1.55-1.44 (m, 2H, H-9).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 170.9 (C-12); 169.1 (C-4); 155.2 (C-2); 144.7 (C-6); 144.5 (C-18); 135.5 (C-16); 132.0 (C-13); 130.9 (C-14); 129.6 (C-15); 129.1 (C-20); 128.2 (C-19); 127.4 (C-21); 102.4 (C-5); 86.9 (C-17); 63.4 (C-11); 49.5 (C-7); 29.9 (C-10); 29.2 (C-8); 23.7 (C-9).

LRMS (ES+): m/z 566.9 [$(M+Na)^+$; 100%].

(38f) 1-(tritylaminopentyl)-3-benzoyl uracil



The 5-(tritylamino)pentan-1-ol **37f** (300 mg; 0.87 mmol) was coupled with the 3-*N*-benzoyl uracil **35** (280 mg; 1.30 mmol) according to the general Mitsunobu procedure **C**.

White foam; 187 mg; 40%.

R_f: 0.27 in EtOAc/Hexane 50:50.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 7.97 (d, *J*=7.50 Hz, 1H, H-21); 7.55-7.50 (m, 8H, H-arom.); 7.35-7.21 (m, 11H, H-arom+H-6); 5.85 (d, *J*=7.87 Hz, 1H, H-5); 3.78 (t, *J*=7.68 Hz, 2H, H-7); 2.19 (t, *J*=6.22 Hz, 2H, H-11); 1.74 (quintuplet, *J*=6.95 Hz, 2H, H-8); 1.64-1.32 (m, 4H, H-10+H-9).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 171.3 (C-17); 161.5 (C-4); 153.4 (C-2); 146.6 (C-13); 144.8 (C-6); 135.5 (C-18); 130.8-126.7 (C-arom.); 102.5 (C-5); 71.2 (C-12); 48.7 (C-7); 43.7 (C-11); 32.9 (C-8); 29.4 (C-10); 24.7 (C-9).

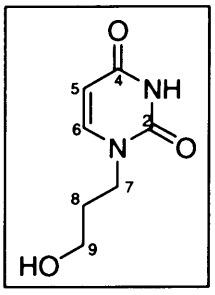
LRMS (ES+): m/z 243.0 $[(Ph_3C)^+$, 100%]; m/z 544.0 $[(M+H)^+$, 40%]; m/z 565.9 $[(M+Na)^+$, 60%].

General procedure D: hydrolysis of benzoyl or acetyl esters with sodium methoxide

The ester was dissolved in a solution of sodium methoxide 0.2M in MeOH. The reaction was stirred at room temperature until the disappearance of the starting esters was observed by TLC. The solution was neutralised with Dowex ion exchange resin. The resin was filtered and washed twice with methanol. The

organic solution was concentrated to a crude residue which was purified by chromatography.

(39a) 1-(3-hydroxypropyl) uracil



Method A

0.5 ml of HCl 3M were added into a solution of the THP protected uracil alcohol **30b** (114 mg; 0.45) in MeOH (5 ml). The mixture was stirred at room temperature until the starting material was completely disappeared (circa 2 h), TLC (DCM/MeOH 90:10). The solvent was removed under reduced pressure and the desired compound was obtained as colourless oil, without necessity of further purification. 70 mg, 92%.

Method B

The dibenzoylpropyl uracil **38a** (183 mg; 0.48 mmol) was deprotected following the general procedure D for the hydrolysis of esters with sodium methoxide. The obtained residue was purified by chromatography using the gradient DCM → DCM/MeOH 99.5:0.5.

White solid; 60 mg; 74%.

R_f: baseline in DCM/MeOH 90:10.

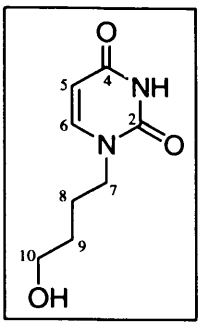
Mp: 124-126 °C.

^{1}H-NMR (300 MHz; CD_3OD): δ 7.59 (d, *J*=7.87 Hz, 1H, H-6); 6.65 (d, *J*=7.87 Hz, 1H, H-5); 3.87 (t, *J*=6.95 Hz, 2H, H-9); 3.61 (t, *J*=6.22 Hz, H-7); 1.89 (t, *J*=6.59 Hz, 2H, H-8).

^{13}C-NMR (75 MHz; CD_3OD): δ 163.7 (C-4); 153.0 (C-2); 148.0 (C-6); 102.5 (C-5); 59.9 (C-9); 47.4 (C-7); 32.8 (C-8).

LRMS (ES+): m/z 192.8 [$(M+Na)^+$; 100%].

(39b) 1-(4-hydroxybutyl) uracil



The dibenzoyl compound **38b** (205 mg; 0.52 mmol) was dissolved in a mixture of dioxane/NaOH 0.1 M 1:1 (8 ml) and stirred at room temperature for 48 h. The solvents were removed *in vacuo* and the obtained residue was purified by flash chromatography using the mixture DCM/MeOH 90:10 as eluent.

Off-white solid; 51 mg; 53%.

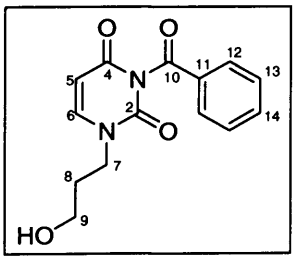
R_f: 0.26 in DCM/MeOH 90:10.

^{1}H-NMR (300 MHz; CD_3OD): δ 7.69 (d, *J*=7.87 Hz, 1H, H-6); 5.75 (d, *J*=7.87 Hz, 1H, H-5); 3.88 (t, *J*=7.32 Hz, 2H, H-10); 3.68 (t, *J*=6.40 Hz, 2H, H-7); 1.89-1.79 (m, 2H, H-9); 1.69-1.60 (m, 2H, H-8).

^{13}C-NMR (75 MHz; CD_3OD): δ 167.2 (C-4); 153.2 (C-2); 147.8 (C-6); 102.6 (C-5); 62.7 (C-10); 49.9 (C-7); 30.8 (C-9); 27.0 (C-8).

LRMS (ES+): m/z 207.0 [$(M+Na)^+$; 100%].

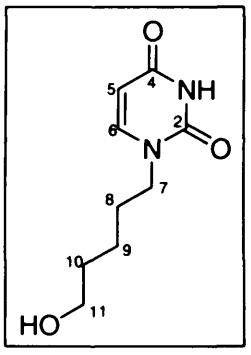
(39d) Attempted synthesis of 3-benzoyl-1-(3-hydroxypropyl) uracil



The compound **38d** (58 mg; 0.15 mmol) was dissolved in THF (5 ml) and stirred at room temperature then TBAF (39 mg; 0.15 mmol) was added to the solution. The reaction was stirred at room temperature until the TLC

(DCM/MeOH 90:10) showed the complete disappearance of the starting material. The solvent was removed under reduced pressure affording an orange residue. The attempt of purification by flash chromatography was unsuccessful due to the small scale of the reaction and the polarity of the mixture.

(39c) 1-(5-hydroxypentyl) uracil



The benzoylated intermediate **38c** (127 mg; 0.31 mmol) was deprotected according to the general procedure **D** for the cleavage of benzoyl group.

Colourless oil; 60 mg; 80%.

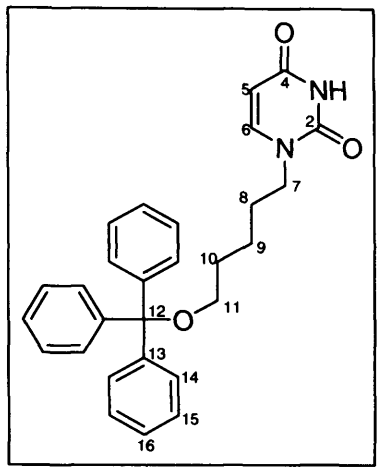
R_f: baseline in DCM/MeOH 90:10.

^{1}H-NMR (300 MHz; CD_3OD): δ 7.60 (d, *J*=7.87 Hz, 1H, H-6); 5.66 (d, *J*=7.87 Hz, 1H, H-5); 3.77 (t, *J*=7.32 Hz, 2H, H-11); 3.56 (t, *J*=6.40 Hz, 2H, H-7); 1.71 (quintet, *J*=7.50 Hz, 2H, H-10); 1.63-1.53 (m, 2H, H-8); 1.46-1-35 (m, 2H, H-9).

^{13}C-NMR (75 MHz; CD_3OD): δ 167.2 (C-4); 153.2 (C-2); 147.8 (C-6); 102.6 (C-5); 63.0 (C-11); 48.9 (C-7); 33.5 (C-10); 30.2 (C-8); 24.2 (C-9).

LRMS (ES+): m/z 221.0 [$(M+Na)^+$; 100%].

(39e) 1-(5-trityloxypentyl) uracil



Compound **38e** (231 mg; 0.42 mmol) was dissolved in a mixture of NaOH 1M and dioxane 1:1 (8 ml) and stirred 48 h at room temperature. Brine (8 ml) was added to the solution, and the mixture was extracted with EtOAc (3×8 ml). The organic phase was dried with $MgSO_4$; the solvent was evaporated and the crude residue was purified by flash chromatography using DCM/MeOH 90:10 as eluent. The title compound was obtained as a colourless oil which precipitated after treatment with a mixture of Water/EtOH 1:1. White solid; 112 mg; 60%.

R_f: 0.64 in DCM/MeOH 90:10.

Mp: 56-59 °C.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 9.13 (bs, 1H, NH); 7.57-7.55 (m, 6H, H-14); 7.46-7.34 (m, 9H, H-15+H-16), 7.21 (d, *J*=7.87 Hz, 1H, H-6); 5.80 (d, *J*=7.87 Hz, 1H, H-5); 3.83 (t, *J*=7.32 Hz, 2H, H-11); 3.22 (t, *J*=6.22 Hz, 2H, H-7); 1.84-1.74 (m, 4H, H-10+H-8); 1.60-1.50 (m, 2H, H-9).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 164.6 (C-4); 151.4 (C-2); 144.9 (C-6); 144.8 (C-13); 129.1 (C-14); 128.4 (C-15); 127.4 (C-16); 102.5 (C-5); 86.9 (C-12); 63.5 (C-11); 49.2 (C-7); 29.9 (C-10); 29.2 (C-8); 23.6 (C-9).

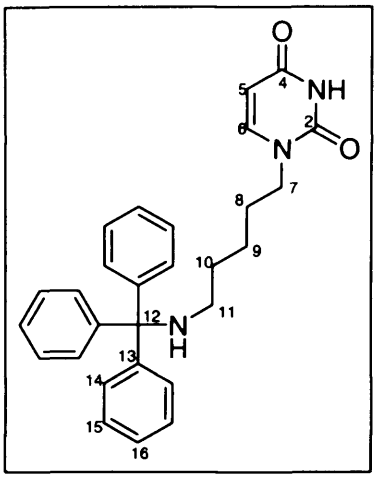
LRMS (ES+): m/z 463.0 $[(M+Na)^+$; 100%].

HRMS (ES+): found 458.2439; required 458.2438 for $C_{28}H_{32}N_3O_3^+$ $[M+NH_4]^+$.

Microanalysis calculated for $C_{28}H_{28}N_2O_3$ + 0.7 HCl

C 72.16, H 6.21, N 6.01%; found C 72.34, H 6.08, N 5.98%.

(39f) 1-(5-tritylaminopentyl) uracil



The title compound was prepared according to the general procedure **D** for the hydrolysis of the benzoyl group; starting from the intermediate **38f** (187 mg; 0.35 mmol). The residue was purified by flash chromatography using DCM/MeOH 90:10 as eluent.

White foam; 240 mg; 52%.

R_f: 0.50 in DCM/MeOH 90:10.

Mp: 65-69 °C.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 8.70 (bs, 1H, N-H); 7.52 (d, *J*=9.88 Hz, 6H; H-14); 7.36-7.22 (m, 9H, H-15+H-16); 7.14 (d, *J*=7.87 Hz, 1H, H-6); 5.73 (d, *J*=7.87 Hz, 1H, H-5); 3.74 (t, *J*=7.32 Hz, 2H, H-7); 2.18 (t, *J*=7.19 Hz, 2H, H-11); 1.75-1.63 (m, 4H, H-8+H-10); 1.49-1.36 (m, 2H, H-9).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 163.8 (C-4); 151.0 (C-2); 146.6 (C-13); 144.8 (C-6); 129.0 (C-14); 128.2 (C-15); 126.7 (C-16); 102.5 (C-5); 71.3 (C-12); 49.2 (C-7); 43.7 (C-11); 30.8 (C-8); 29.5 (C-10); 24.6 (C-9).

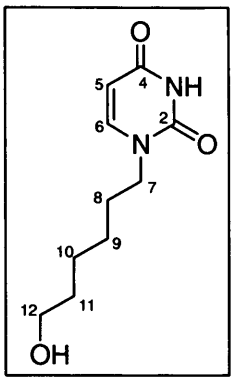
LRMS (ES+): m/z 243.1 [$(Ph_3C)^+$, 100%]; m/z 439.9 [$(M+H)^+$, 50%].

HRMS (ES+): found 440.2333; required 440.2341 for $C_{28}H_{30}N_3O_2^+$, $[M+H]^+$.

Microanalysis calculated for $C_{28}H_{29}N_3O_2$ + 0.3 EtOH

C 75.77, H 6.85, N 9.27%; found C 76.18, H 7.42, N 8.33%.

(39g) 1-(6-hydroxyexyl) uracil



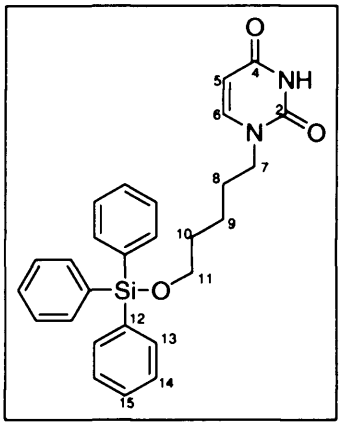
3 drops of HCl 3M were added to a solution of THP protected uracil alcohol **34a** (160 mg; 0.54 mmol) in MeOH (5 ml). The solution was stirred at room temperature until the TLC (EtOAc/Hexane 4:1) showed complete reaction (1 h). The solvent was removed under reduced pressure giving the title product without further purification.

White solid, 94 mg, 82%.

^{1}H-NMR (300 MHz; CD_3OD) δ: 7.51 (d, *J*=7.87 Hz, 1H, H-6); 5.57 (d, *J*=7.87 Hz, 1H, H-5); 3.67 (t, *J*=7.50 Hz, 2H, H-7); 3.47 (t, *J*=6.04 Hz, 2H, H-12); 1.66-1.59 (m, 2H, H-11); 1.51-1.42 (m, 2H, H-8); 1.38-1.21 (m, 4H, H-10+H-9).

^{13}C-NMR (75 MHz; CD_3OD) δ: 167.2 (C-4); 153.2 (C-2); 147.8 (C-6); 102.5 (C-5); 63.1 (C-12); 48.8 (C-7); 33.8 (C-11); 30.3 (C-8); 27.7 (C-10); 26.9 (C-9).

(40) 1-(5-triphenylsilanyloxypentyl) uracil



A solution of triphenylsilyl chloride (81 mg; 0.27 mmol) in dry pyridine (1 ml) was added into a solution of uracil alcohol **39c** (50 mg; 0.25 mmol) in dry pyridine (1 ml) under nitrogen, at room temperature. The reaction was stirred at 25 °C 24 h. The solvent was removed under reduced pressure and the residue (slightly yellow oil) was purified by chromatography using a mixture of DCM/MeOH 99:1 as eluent.

Light orange syrup, 30 mg; 26%.

R_f: 0.69 in DCM/MeOH 95:5.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 9.26 (s, 1H, NH); 7.68-7.65 (m, 6H, H-13); 7.54-7.43 (m, 9H, H-14+H-15); 7.08 (d, *J*=7.87 Hz, 1H, H-6); 5.69 (d, *J*=7.87 Hz, 1H, H-5); 3.87 (t, *J*=6.13 Hz, 2H, H-7); 3.72 (t, *J*=7.30 Hz, 2H, H-11); 1.76-1.66 (m, 4H, H-10+H-8); 1.51-1.43 (m, 2H, H-9).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 164.2 (C-4); 151.2 (C-2); 144.9 (C-6); 136.4 (C-13); 134.4 (C-14); 130.5 (C-15); 128.7 (C-12); 102.4 (C-5); 63.8 (C-11); 49.2 (C-7); 32.2 (C-10); 29.0 (C-8); 23.2 (C-9).

LRMS (ES+): m/z 479.0 [$(M+Na)^+$, 100%].

HRMS (ES+): found 479.1780; required 479.1761 for $C_{27}H_{28}N_2NaSiO_3^+$, $[M+Na]^+$.

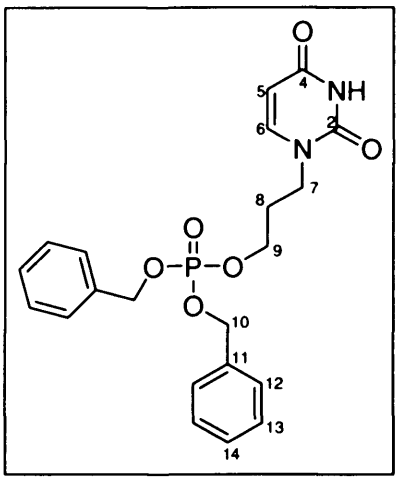
Microanalysis calculated for $C_{27}H_{28}N_2O$ + 0.6 H_2O

C 69.4, H 6.30, N 5.99%; found C 69.79, H 6.26, N 5.45%.

General procedure E: Phosphorylation of uracil alcohols with dibenzyl *N,N*-diisopropylphosphoramidite and tetrazole[102]

Dibenzyl-*N,N*-phosphoramidite (2 mmol) was added into a solution of uracil alcohol (1 mmol) and 1-*H*-tetrazole (3.15 ml of solution 0.45 M in CH_3CN) in dry THF (20 ml), under nitrogen at room temperature. The reactions were stirred two h and then cooled to -40 °C, followed by the slow addition of a solution of *tert*-butyl hydroperoxide 70% in water. The mixture was allowed to warm to room temperature and stirred for 2 h. The slight excess of peroxide was neutralized with a solution of sodium sulphite and the solvents were removed under reduced pressure without heating. The crude residue was purified by chromatography using DCM/MeOH 99.1:0.1 as eluent. Traces of tetrazole, if still present, were removed by washing with acetonitrile.

(41a) dibenzyl 3-(uracil-1-yl)propyl phosphate



Method A

A solution of **39a** (160 mg; 0.94 mmol) in pyridine (2 ml) was cooled to –40 °C and stirred and nitrogen. Dibenzylchlorophosphite in CCl_4 (~363 mg; 1.23 mmol) was prepared *in situ and* added to the mixture. The reaction was warmed to room temperature and stirred for 4 h. The solvents were removed *in vacuo* affording a complex mixture of products which was not possible to purify by chromatography.

Method B

Compound **39a** (70 mg, 0.36 mmol) was phosphorylated according to the general procedure **E** for the phosphorylation of uracil alcohols.

Colourless oil; 124 mg; 80%.

R_f: 0.44 in DCM/MeOH 99.5:0.5.

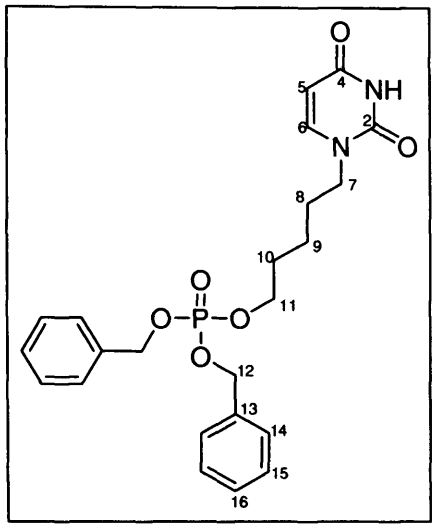
^{1}H-NMR (300 MHz; $CDCl_3$): δ 10.08 (bs, 1H, NH); 7.43 (s, 10H, H-arom.); 7.09 (d, *J*=7.87 Hz, 1H, H-6); 5.61 (d, 7.87 Hz, 1H, H-5); 5.13 (quintet, *J*=8.78 Hz, 4H, H-10); 4.08-4.01 (q, *J*=5.67 Hz, 2H, H-9); 3.76 (t, *J*=6.59 Hz, 2H, H-7); 2.00 (quintet, *J*=6.22 Hz, 2H, H-8).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 164.6 (C-4); 151.4 (C-2); 145.5 (C-6); 136.1 (d, *J*=6.32 Hz, C-11); 129.2 (C-13); 129.1 (C-12); 128.5 (C-14); 102.5 (C-5); 70.0 (d, *J*=5.75 Hz, C-10); 64.6 (d, *J*=5.75 Hz, C-9); 45.9 (C-7); 29.5 (d, *J*=6.32 Hz, C-8).

^{31}P-NMR (121 MHz; $CDCl_3$): δ 0.52.

LRMS (ES+): m/z 452.9 [$(M+Na)^+$; 100%].

(41c) dibenzyl 5-(uracil-1-yl)pentyl phosphate



The title compound was prepared according to the general procedure **E** fot the phosphorilation of alcohols; starting from compound **39c** (60 mg; 0.31 mmol) was treated with dibenzyl phosphoramidite (184 µl, 0.56 mmol), tetrazole (2.5 ml of solution 0.45 M) and a solution of *tert*-butylhydroperoxide 7.78 M (126 µl; 0.98 mmol).

Colourless oil; 94 mg; 66%.

R_f: 0.5 in DCM/MeOH 90:10.

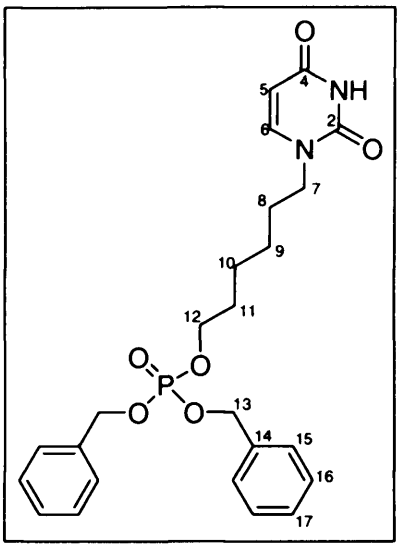
^{1}H-NMR (300 MHz; CD_3OD): δ 7.50 (d, *J*=7.87 Hz, 1H, H-6); 7.36 (bs, 10H, H-arom.); 5.62 (d, *J*=7.87 Hz, 1H, H-5); 5.04 (dd, J_1=8.96 Hz, J_2=1.28 Hz, 4H, H-12); 3.98 (q, *J*=6.40 Hz, 2H, H-11); 3.68 (t, *J*=7.32 Hz, 2H, H-7); 1.68-1.58 (m, 4H, H-8+H-10); 1.37-1.29 (m, 2H, H-9).

^{13}C-NMR (75 MHz; CD_3OD): δ 167.1 (C-4); 153.1 (C-2), 147.6 (C-6); 137.6 (d, *J*=6.32 Hz, C-13); 130.2 (C-14); 130.1 (C-15); 129.6 (C-16); 102.6 (C-5); 71.1 (d, *J*=6.32 Hz, C-12); 69.4 (d, *J*=5.75 Hz, C-10); 49.5 (C-7); 30.0 (d, *J*=6.90 Hz, C-10); 29.7 (C-8); 23.8 (C-9).

^{31}P-NMR (121 MHz; CD_3OD): δ 0.01.

LRMS (ES+): m/z 480.8 [$(M+Na)^+$; 100%]; m/z 496.9 [$(M+K)^+$; 30%].

(41d) dibenzyl 6-(uracil-1-yl)hexyl phosphate



Method A

Iodine (263 mg; 1.09 mmol) was added into a solution of tribenzyl phosphite (385 mg; 1.09 mmol) in DCM (2 ml) at –20 °C. After 20 min the clear colourless solution was allowed to warm at room temperature and subsequently it was added dropwise into another solution of uracil alcohol **39g** (110 mg; 0.52 mmol) in dry pyridine (0.167 ml) and DCM (0.5 ml) at –20 °C. After 1 hour the mixture was let to reach room temperature and stirred for 3 h. The solvents were removed under reduced pressure and the attempt to purify the residue by chromatography did not succeed.

Method B

The uracil alcohol **39d** (112 mg; 0.52 mmol) was reacted with dibenzyl *N,N*-diisopropylphosphoramidite (345 µl; 1.05 mmol); tetrazole 0.45M solution in CH_3CN (4.69 ml; 2.11 mmol); *tert*-butylhydroperoxide (234 µl; 1.82 mmol); according to the general procedure.

Colourless oil; 156 mg; 63%.

R_f: 0.61 in DCM/MeOH 90:10

^{1}H-NMR (300 MHz; $CDCl_3$): δ 10.10 (bs, 1H, NH); 7.39-7.36 (m, 10H, H-arom.); 7.15 (d, *J*=7.87 Hz, 1H, H-6); 5.71 (d, *J*=7.87 Hz, 1H, H-5); 5.13-5.01 (m, 4H, H-13+H-13'); 4.01 (q, *J*=6.47 Hz, 2H, H-12); 3.69 (t, *J*=7.50 Hz, 2H, H-7); 1.71-1.58 (m, 4H, H-10+H-11); 1.42-1.25 (m, 4H, H-8+H-9).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 164.7 (C-4); 151.5 (C-2); 144.9 (C-6); 136.4 (d, *J*=6.89 Hz; C-14); 128.9 (C-15); 128.4 (C-16); 128.3 (C-17); 102.5 (C-5); 69.8 (d, *J*=5.17 Hz, C-13); 68.0 (d, *J*=6.32 Hz, C-12); 49.1 (C-7); 30.3 (d, *J*=6.89 Hz, C-11); 29.2 (C-8); 26.2 (C-10); 25.4 (C-9).

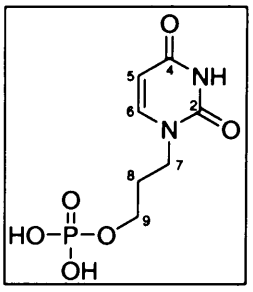
^{31}P-NMR (121 MHz; $CDCl_3$): δ 0.48.

LRMS (ES+): m/z 966.7 [$(2M+Na)^+$; 100%]; m/z 494.8 [$(M+Na)^+$; 90%].

General procedure F: Cleavage of benzyl ester by hydrogenolysis with Pd/C

The benzyl ester (1 eq.) derivative was dissolved in MeOH (10 ml) and placed in a sealed flask. The catalyst 10% Pd/C (5% mmol) was added to the solution. The air inside the flask was replaced with nitrogen three times. A balloon containing hydrogen was connected to the flask using a 3 way tap then the Nitrogen was replaced with the hydrogen. The reaction was stirred at room temperature, under a slight pressure of H_2 and monitored by TLC. When the disappearance of the starting material was observed, the remaining hydrogen was displaced with a flow of N_2, and the catalyst was filtered on a celite pad. The volatiles were removed under reduced pressure affording the pure deprotected compound without presence of any by-products.

(42a) 3-(uracil-1-yl)propyl phosphate



The title compound was obtained according to the general procedure **F** for the cleavage of benzyl esters, starting from compound **41a** (34 mg; 0.08 mmol).

White solid; 20 mg; 98%.

R_f: baseline in DCM/MeOH 90:10.

Mp: 163-166 °C.

^{1}H-NMR (300 MHz; CD_3OD): δ 7.60 (d, *J*=7.68 Hz, 1H, H-6); 5.67 (d, *J*=7.68 H, 1H, H-5); 4.04 (bs, 2H, H-9); 3.90 (t, *J*=6.77 Hz, 2H, H-7); 2.06 (t, *J*=5.67 Hz, 2H, H-8).

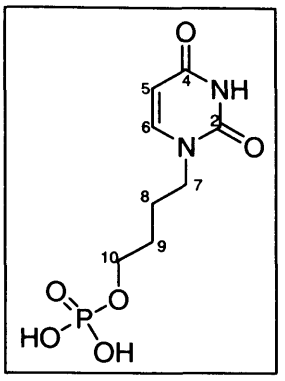
^{13}C-NMR (75 MHz CD_3OD): δ 167.2 (C-4); 153.2 (C-2), 148.0 (C-6); 102.6 (C-5); 65.0 (d, *J*=4.60 Hz, C-9); 47.3 (C-7); 30.8 (d, *J*=6.32 Hz, C-8).

^{31}P-NMR (121 MHz; CD_3OD): δ 1.18.

LRMS (ES-): m/z 249.0 [$(M)^-$; 100%].

HRMS (ES-): found 249.0287; required 249.0282 for $C_7H_{10}N_2O_6P^-$.

(42b) 4-(uracil-1-yl)butyl phosphate



Dibenzylester **41b** (100 mg; 0.22 mmol) was hydrogenated according to the general procedure **F** for the cleavage of benzyl esters.

Off-white solid; 50 mg; 80%.

R_f: baseline in DCM/MeOH 90:10.

Mp: 154-156 °C.

^{1}H-NMR (300 MHz; CD_3OD): δ 7.59 (d, *J*=7.87 Hz, 1H, H-6); 5.64 (d, *J*=7.87 Hz, 1H, H-5); 4.00 (q, *J*=6.22 Hz, 2H, H-10); 3.79 (t, *J*=7.13 Hz, 2H, H-7); 1.83-1.67 (m, 4H, H-8+H-9).

^{13}C-NMR (75 MHz; CD_3OD): δ 165.9 (C-4); 152.0 (C-2); 146.5 (C-6); 101.4 (C-5); 66.2 (d, *J*=5.75 Hz, C-10); 48.4 (C-7); 27.4 (d, *J*=6.89 Hz, C-9); 25.6 (C-8).

^{31}P-NMR (121 MHz; CD_3OD): δ 1.30.

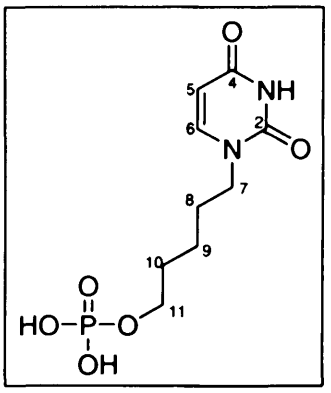
LRMS (ES-): m/z 526.9 [$(2M)^-$; 100%]; m/z 263.2 [$(M)^-$; 45%].

HRMS (ES-): found 249.0287; required 249.0282 for $C_7H_{10}N_2O_6P^-$.

Microanalysis calculated for $C_8H_{13}N_2O_6P$

C 36.37, H 4.96, N 10.60, P 11.72%; found C 36.11, H 4.75, N 10.37, P 10.76%.

(42c) 5-(uracil-1-yl)pentyl phosphate



Compound **41c** (120 mg; 0.26 mmol) was hydrogenated following to the general procedure **F** for the cleavage of dibenzylphosphate esters.

White solid, 65 mg; 90%.

R_f: baseline in DCM/MeOH 90:10.

Mp: 147-151 °C brown liquid.

^{1}H-NMR (300 MHz; CD_3OD): δ 7.60 (d, *J*=7.87 Hz, 1H; H-6); 5.65 (d, *J*=7.87 Hz, 1H, H-5); 4.02 (q, *J*=6.22 Hz, 2H, H-11); 3.79 (t, *J*=7.13 Hz; 2H; H-7); 1.83-1.76 (m, 4H, H-8+H-10); 1.73-1.67 (m, 2H; H-9).

^{13}C-NMR (75 MHz; CD_3OD): δ 167.1 (C-4); 157.2 (C-2); 147.7 (C-6); 102.5 (C-5); 67.7 (d, *J*=6.32 Hz, C-11); 48.5 (C-7); 31.3 (d, *J*=7.47 Hz; C-10); 29.9 (C-8); 23.9 (C-9).

^{31}P-NMR (121 MHz; CD_3OD): δ 1.36.

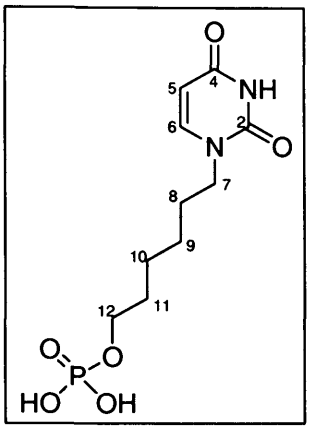
LRMS (ES-): m/z 554.8 [(2M)$^-$, 100%]; m/z 277.2 [(M)$^-$, 65%].

HRMS (ES-): found 277.0594; required 277.0595 for $C_9H_{14}N_2O_6P^-$.

Microanalysis calculated for $C_7H_{11}N_2O_6P$ + 0.5 MeOH + 0.1 CH_3CN

C 34.22, H 4.96, N 10.88, P 11.46%; found C 34.74, H 4.62, N 10.10, P 11.32%.

(42d) 6-(uracil-1-yl)hexyl phosphate



The title compound was obtained following the standard procedure **F** for hydrogenation with 10% Pd/C, starting from compound **41d** (130 mg; 0.275 mmol).

White solid; 78 mg; 97%.

R_f: baseline in DCM/MeOH.

Mp: 184-186 °C.

^{1}H-NMR (300 MHz; CD_3OD): δ 7.58 (d, *J*=7.83 Hz, 1H, H-6); 5.64 (d, *J*=7.83 Hz, 1H, H-5); 3.96 (q, *J*=6.6 Hz, 2H, H-12); 3.74 (t, *J*=7.27 Hz, 2H, H-7); 1.73-1.63 (m, 4H, H-8+H-11); 1.49-1.35 (m, 4H, H-10+H-9).

^{13}C-NMR (75 MHz; CD_3OD): δ 167.2 (C-4); 153.2 (C-2); 147.8 (C-6); 102.5 (C-5); 67.9 (d, *J*=5.58 Hz, C-12); 49.1 (C-7); 31.7 (d, *J*=7.32 Hz, C-11); 30.2 (C-8); 27.4 (C-9); 26.6 (C-10).

^{31}P-NMR (121 MHz; CD_3OD): δ 1.38.

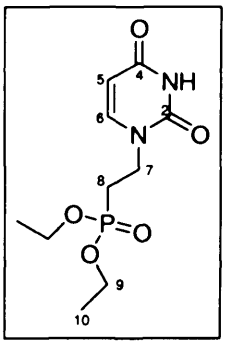
LRMS (ES-): m/z 291.1 [$(M)^-$, 100%]; m/z 582.9 [$(2M)^-$, 15%].

HRMS (ES+): found 293.0902; required 293.0897 for $C_{10}H_{18}N_2O_6P^+$; $[M+H]^+$.

Microanalysis calculated for $C_{10}H_{17}N_2O_6P$

C 41.10, H 5.86, N 9.58, P 10.60%; found C 41.04, H 5.89, N 9.15, P 10.17%.

(43a) diethyl ester of 1-(phosphonoethyl)uracil



A suspension of uracil (200 mg; 1.78 mmol), caesium carbonate (580 mg; 1.78 mmol) and diethyl 2-bromoethylphosphonate **17** (259 µl; 1.48 mmol) in dry DMF (10 ml) was heated to 40 °C, under nitrogen. After three h no new spot on TLC (chloroform/MeOH 90:10) was observed and then the temperature was increased to 50 °C for 5 days. Water (10 ml) was added to the mixture and stirred for 15 min, the suspension became a solution. The aqueous solution was extracted with DCM (5×15 ml), the collected organic phases were washed with brine (3×10 ml) and dried with $MgSO_4$. The solvents were removed under reduced pressure. The obtained residue (colourless oil) was purified by flash chromatography using DCM→DCM/MeOH 90:10 as gradient.

Colourless oil; 105 mg; 38%.

R_f: 0.43 in DCM/MeOH (90:10).

^{1}H-NMR (300 MHz; $CDCl_3$): δ 10.42 (bs, 1H, NH); 7.36 (d, *J*=7.86 Hz, 1H, H-6); 5.80 (d, *J*=7.86 Hz, 1H, H-5); 4.12-3.85 (m, 6H, H-9+H-7); 2.29-2.13 (m, 2H, H-8); 1.30 (t, *J*=7.13 Hz, 6H, H-10).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 164.8 (C-4); 151.4 (C-2); 145.7 (C-6); 102.3 (C-5); 62.5 (d, *J*=6.30 Hz, C-9); 44.6 (d, *J*=4.00 Hz, C-7); 25.2 (d, *J*=140.23 Hz, C-8); 16.7 (d, *J*=6.30 Hz, C-10).

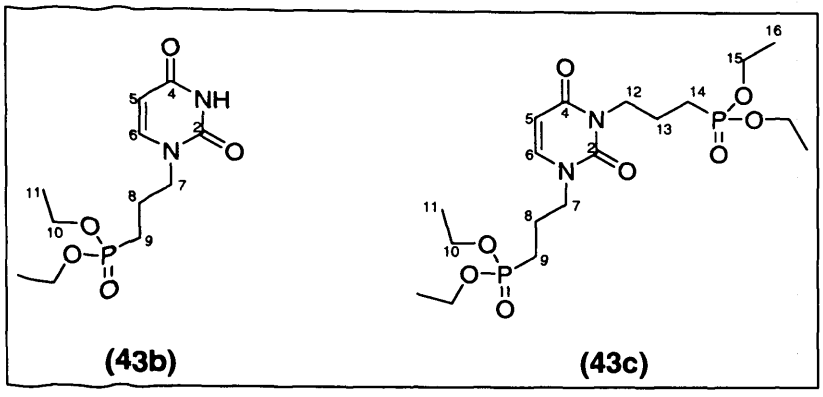
^{31}P-NMR (121 MHz; $CDCl_3$): δ 28.64.

LRMS (ES+): m/z 299.0 [$(M+Na)^+$, 100%].

Microanalysis calculated for $C_{10}H_{17}N_2O_5P$

C 43.48, H 6.20, N 10.14, P 11.21%; found C 43.60, H 6.31, N 9.83, P 10.79%.

(43b) diethyl ester of 1-(phosphonopropyl)uracil



Diethyl(3-bromo-propyl) phosphonate **18** (285 μl; 1.48 mmol) was added with a syringe to a suspension of Uracil (200 mg; 1.78 mmol), Cs_2CO_3 (580 mg; 1.78 mmol) in dry DMF (10 ml) cooled with an ice bath. The reaction was warmed to room temperature and stirred for 12 h. Water (20 ml) was added to the suspension, which became a solution, and stirred for few min. The aqueous solution was extracted with DCM (5×15 ml). The collected organic fractions were washed with brine (1×15 ml), dried with $MgSO_4$ and the solvent was removed under reduced pressure. The crude residue was purified by flash chromatography using DCM→DCM/MeOH 99:1 as gradient affording the two compounds **43b** and **43c**.

Compound **43b:**

Colourless oil; 140 mg; 32%.

R_f: 0.35 in MeOH/DCM 10:90.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 10.41 (s, 1H, NH); 7.31 (d, *J*=7.87 Hz, 1H, H-6); 5.67 (d, *J*=7.87 Hz, 1H, H-5); 4.14-4.07 (m, 4H, H-10); 3.82 (t, *J*=6.95 Hz, 2H, H-7); 2.06-1.91 (m, 2H, H-9); 1.79-1.68 (m, 2H, H-8); 1.29 (t, *J*=7.13 Hz, 6H, H-11).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 164.4 (C-4); 152.9 (C-2); 145.1 (C-6); 102.7 (C-5); 62.3 (d, *J*=6.89 Hz, C-10); 48.9 (d, *J*=14.93 Hz, C-7) 23.7 (d, *J*=142.53 Hz, C-9); 22.7 (d, *J*=142.97, C-14) 22.5 (d, *J*=5.17 Hz, C-8); 21.2 (d, *J*=4.52 Hz, C-13); 16.8 (d, *J*=6.32 Hz, C-11).

^{31}P-NMR (121 MHz; $CDCl_3$): δ 31.66.

LRMS (ES+): m/z 313.1 [$(M+Na)^+$, 100%]; m/z 329.1 [$(M+K)^+$, 20%].

Compound (43c)

R_f: 0.58 in MeOH/DCM 10:90.

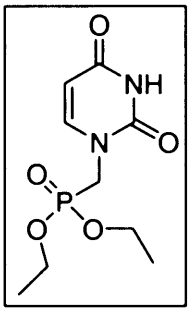
^{1}H-NMR (300 MHz; $CDCl_3$): δ 7.61 (d, *J*=7.87 Hz, 1H, H-6); 5.75 (d, *J*=7.87 Hz, 1H, H-5); 4.17-4.06 (m, 8H, H-10+H-15); 4.00 (t, *J*=6.75 Hz, 2H, H-12); 3.87 (t, *J*=6.96 Hz, 2H, H-7); 2.17-1.98 (m, 4H, H-9+H-14); 2.05-1.79 (m, 4H, H-8+H-13); 1.34 (t, *J*=7.05 Hz, 6H; H-11); 1.33 (t, *J*=7.05 Hz, 6H, H-16).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 163.3 (C-4); 151.7 (C-2); 142.9 (C-6); 102.1 (C-5); 62.2 (d, *J*=6.89 Hz, C-10); 62.0 (d, *J*=6.32 Hz, C-15); 50.0 (d, *J*=13.79 H, C-7); 41.8 (d, *J*=21.26 Hz, C-12); 16.9 (d, *J*=5.75 Hz, C-11+C-16).

^{31}P-NMR (121 MHz; $CDCl_3$): δ 32.43; 31.52.

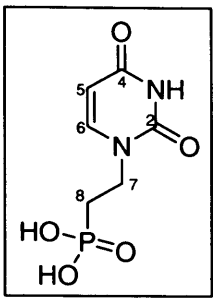
LRMS (ES+): m/z 469.2 [$(M+H)^+$, 100%]; m/z 491.2 [$(M+Na)^+$, 39%].

(43d) Attempted synthesis of diethyl ester of 1-(phosphonomethyl)uracil



Diethylchloromethyl phosphonate (230 µl; 1.48 mmol) was added to a suspension of uracil (200 mg; 1.78 mmol) and Cs_2CO_3 (580 mg; 1.78 mmol) in dry DMF (10 ml). The reaction was warmed to 40 °C, and stirred for 72 h under nitrogen. Water was added to the mixture and stirred for 10 min. The aqueous phase was then extracted several times with EtOAc (10 ml). The organic phases were collected and dried with $MgSO_4$. The solvent was removed under reduced pressure. The residue was subjected to purification by chromatography, but NMR analysis did not show the presence of the desired compound in any of the collected fractions.

(44a) 1-(phosphonoethyl)uracil



Trimethylsilyl bromide (200 μl; 1.7 mmol) was added into a solution of **43a** (95 mg; 0.34 mmol) in dry DCM (2 ml). After the addition a white precipitate was formed which disappeared after 30 min. The mixture was stirred at room temperature and monitored by TLC (DCM/MeOH 90:10). 24 h later the volatiles were removed in vacuo and the residue was dissolved in 2 ml of a mixture of EtOH/water (1:1). The solution was stirred at room temperature for 30 min and then the solvents were evaporated. This latter procedure was repeated three times to give the title compound without necessity of further purification.

Orange solid; 76 mg; 100%.

R_f: baseline in DCM/MeOH 90:10.

Mp: 232-235 °C.

^{1}H-NMR (300 MHz; D_2O): δ 7.54 (d, J=7.87 Hz, 1H, H-6); 5.69 (d, J=7.87 Hz, 1H, H-5); 3.88 (dd, J_1=11.3 Hz, J_2=7.5 Hz, 2H, H-7); 2.06 (dt, J_1=18.3 Hz, J_2=7.8 Hz, 2H, H-8).

^{13}C-NMR (75 MHz; D_2O): δ 167.2 (C-4); 152.3 (C-2); 147.7 (C-6); 101.7 (C-5); 44.5 (C-7); 26.2 (d, J=134.27 Hz, C-8)

^{31}P-NMR (121 MHz; D_2O): δ 23.35.

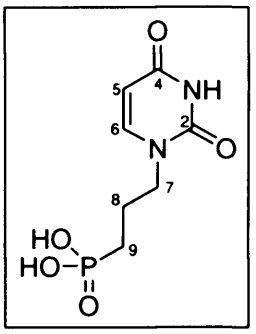
LRMS (ES-): m/z 219.0 [$(M)^-$; 100%].

HRMS (ES-): found 219.0175; required 219.0176 for $C_6H_8N_2O_5P^-$.

Microanalysis calculated for $C_6H_9N_2O_5P$ + 0.3 H_2O

C 31.95, H 4.29, N 12.42, P 13.73%; found C 32.19, H 4.10, N 12.06, P 13.59%.

(44b) 1-(phosphonopropyl)uracil



The title compound was obtained following the same procedure as compound **44a**; starting from compound **43b** (140 mg; 0.48 mmo), trimethyl silyl bromide (318 µl; 2.41 mmol) and DCM (3 ml).

Off white solid; 100 mg; 90%.

R_f: baseline in DCM/MeOH 90:10.

Mp: 212-215 °C decomp.

^{1}H-NMR (300 MHz; D_2O): δ 7.38 (dd, J_1=7.87 Hz, J_2=2.01 Hz, 1H, H-6); 5.53 (dd, J_1=7.87 Hz, J_2=2.01 Hz, 1H, H-5); 3.57 (t, J=7.13 Hz, 2H, H-7); 1.73-1.49 (m, 4H, H-8+H-9).

^{13}C-NMR (75 MHz; D_2O): δ 167.0 (C-4); 152.4 (C-2); 147.5 (C-6); 101.8 (C-5); 49.3 (d, J=19.5 Hz, C-7); 23.4 (d, J=136.8 Hz, C-9); 21.9 (d, J=4.0 Hz, C-8).

^{31}P-NMR (121 MHz; D_2O): δ 31.37.

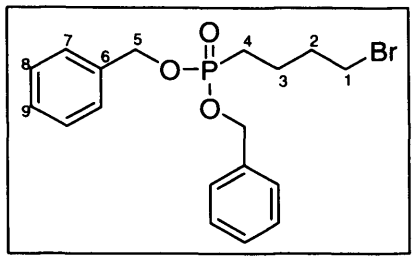
LRMS (ES-): m/z 233.0 [(M)$^-$; 100%]; m/z 467.0 [(2M); 75%]; m/z 700.7 [(3M); 30%].

HRMS (ES-): found 233.0326; required 233.0333 for $C_7H_{11}N_2O_5P$.

Microanalysis calculated for $C_7H_{11}N_2O_5P$ + 0.5 H_2O

C 34.58, H 4.97, N 11.52, P 12.74%; found C 35.06, H 4.89, N 10.85, P 12.27%.

(47a) dibenzyl 4-bromobutylphosphonate



Dibenzyl phosphite (254 µl; 1.15 mmol) was added to a suspension of caesium carbonate (1.124 g; 3.45 mmol) and TBAI (1.274 g, 3.45 mmol) in dry DMF (10 ml) at room temperature, under nitrogen. After stirring the mixture for 60 min, 1,4-dibromobutane (276 µl; 2.31 mmol) was added with a syringe into the flask. The mixture was stirred at 25 °C for 72 h. Water (10 ml) was added to the reaction and stirred for 10 min. The aqueous phase was extracted with EtOAc (3×10 ml). The collected organic fractions were washed with water (1×10 ml) and Brine (2×10 ml). The solvent was dried with $MgSO_4$ and removed under reduced pressure. The crude residue (yellow oil) was purified by column chromatography using a mixture of Hexane/EtOAc 50:50 as eluent.

Colourless oil; 240 mg, 52%.

R_f: 0.21 in Hexane/EtOAc 50:50.

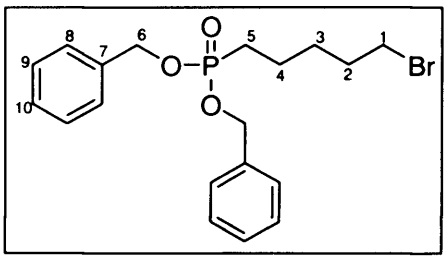
^{1}H-NMR (300 MHz; $CDCl_3$): δ 7.41-7.39 (m, 10H, H-arom.); 5.15-4.98 (m, 4H, H-5); 3.36 (t, *J*=6.59 Hz, 2H, H-1); 1.96-1.87 (m, 2H, H-4); 1.86-1.72 (m, 4H; H-2+H-3).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 136.8 (d, *J*=5.75 H, C-6); 129.1 (C-7); 128.9 (C-8); 128.4 (C-9); 67.6 (d, *J*=6.32 Hz, C-5); 33.5 (C-1); 33.2 (d, *J*=16.7 Hz, C-2); 25.6 (d, *J*=141.4, C-4); 21.5 (d, *J*=5.17 Hz, C-3).

^{31}P-NMR (121 MHz; $CDCl_3$): δ 33.86.

LRMS (ES+): m/z 418.9 [$(M+Na)^+$; 100%]; 466.8 [$(M+Na)^+$; 70%] (iodo derivative).

(47b) dibenzyl 5-bromopentylphosphonate



The title compound was prepared following the same procedure as the previous compound **47a**, starting with 1,5-dibromopentane (315 µl; 2.31 mmol), dibenzyl phosphite (254 µl; 1.15 mmol), caesium carbonate (1.124 g; 3.45 mmol) and TBAI (1.274 g, 3.45 mmol) in dry DMF (10 ml)

Colourless oil; 150 mg; 32%.

R_f: 0.26 in Hexane/EtOAc 50:50.

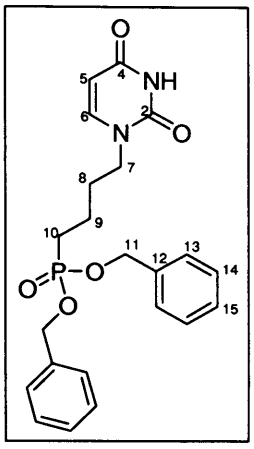
^{1}H-NMR (300 MHz; $CDCl_3$): δ 7.41-7.39 (bs, 10H, H-arom.); 5.15-4.98 (m, 4H, H-6); 3.37 (t, *J*=6.59 Hz, 2H, H-1); 1.87-1.69 (m, 8H, H-5+H-4+H-3+H-2).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 136.8 (d, *J*=5.75 Hz, C-7); 129.0 (C-8); 128.9 (C-9); 128.4 (C-10); 67.5 (d, *J*=6.32 Hz, C-6); 33.8 (C-1); 32.5 (d, *J*=1.72 Hz, C-2); 29.35 (d, *J*=16.7 Hz, C-3); 26.3 (d, *J*=140.8 Hz, C-5); 21.9 (d, *J*=5.17 Hz, C-4).

^{31}P-NMR (121 MHz; $CDCl_3$): δ 34.43.

LRMS (ES+): m/z 432.8 [$(M+Na)^+$; 100%]; 480.9 [$(M+Na)^+$; 60%] (iodo derivative).

(48a) dibenzyl 1-(phosphonobutyl) uracil



Dibenzyl 4-bromobutylphosphonate **47a** (240 mg; 0.60 mmol) was added to a suspension of uracil (100 mg; 90 mmol), Cs_2CO_3 (195 mg; 0.60 mmol) in dry DMF (5 ml), at 0 °C, under nitrogen. The reaction was left to warm to room temperature and stirred for other 72 h. Water (5 ml) was added to the mixture, which became a colourless solution. The aqueous phase was extracted with DCM (3×10 ml). The collected organic fractions were washed with brine (2×10 ml) and dried with $MgSO_4$. After removal of the solvent by reduced pressure, the residue was purified by flash chromatography using the mixture DCM/MeOH 99.5:0.5 as eluent.

Colourless oil; 64 mg; 41%.

R_f: 0.43 in DCM/MeOH 90:10

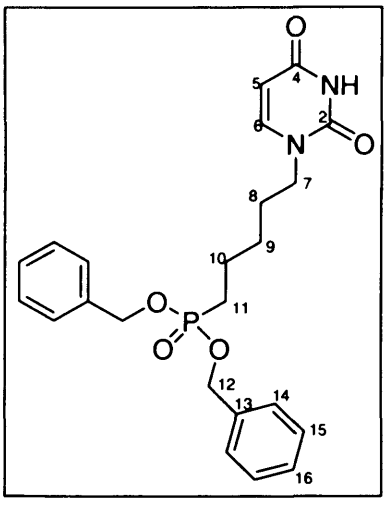
^{1}H-NMR (300 MHz; $CDCl_3$): δ 9.49 (s, 1H, NH); 7.41-7.36 (m, 10H, H-arom.); 7.06 (d, J=7.87 Hz, 1H, H-6); 5.67 (dd, J_1=7.87 Hz, J_2=1.65 Hz, 1H, H-5); 5.14-4.96 (m, 4H, H-11); 3.67 (t, J=7.32 Hz, 2H, H-7); 1.88-1.54 (m, 6H, H-8+H-9+H-10).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 164.4 (C-4); 151.4 (C-2); 144.7 (C-6); 136.75 (d; J=5.75 Hz; C-12); 129.1 (C-14); 128.9 (C-13); 128.5 (C-15); 102.7 (C-5); 67.6 (d, J=6.32 Hz, C-11); 48.5 (C-7); 29.8 (d, J=14.95 Hz, C-8); 25.6 (d, J=140.80 Hz, C-10); 19.8 (d, J=5.17 Hz, C-9)

^{31}P-NMR (121 MHz; $CDCl_3$): δ 33.62.

LRMS (ES+): m/z 450.9 [$(M+Na)^+$; 100%].

(48b) dibenzyl 1-(phosphonopentyl) uracil



The title compound was prepared following the same procedure as the previous compound **48a**, starting with dibenzyl 5-bromopentylphosphonate **47b** (150 mg; 0.36 mmol), uracil (60 mg; 0.54 mmol), Cs_2CO_3 (117 mg; 0.36 mmol) in dry DMF (5 ml).

Colourless oil; 103 mg; 65%.

R_f: 0.50 in DCM/MeOH 90:10.

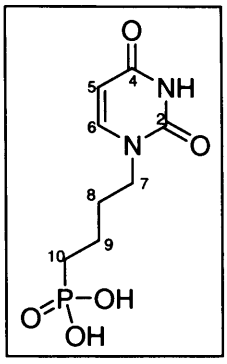
^{1}H-NMR (300 MHz; $CDCl_3$): δ 9.84 (s, 1H, NH); 7.41-7.36 (m, 10H, H-arom.); 7.09 (d, *J*=7.87 Hz, 1H, H-6); 5.71 (d, *J*=7.87 Hz, 1H, H-5); 5.14-4.96 (m, 4H, H-12); 3.68 (t, *J*=7.32 Hz, 2H, H-7); 1.85-1.59 (m, 6H, H-8+H-10+H-11); 1.44-1.30 (m, 2H, H-9).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 164.4 (C-4); 151.3 (C-2); 144.8 (C-6); 136.8 (d, *J*=5.75 Hz, C-13); 129.0 (C-15); 128.9 (C-14), 128.4 (C-16); 102.6 (C-5); 67.5 (d, *J*=6.32 Hz, C-12); 49.0 (C-7), 28.9 (C-8); 27.5 (d, *J*=16.09 Hz, C-9); 26.1 (d, *J*=140.80 Hz, C-11); 22.3 (d, *J*=5.17 Hz, C-10).

^{31}P-NMR (121 MHz; $CDCl_3$): δ 34.25.

LRMS (ES+): m/z 464.9 [$(M+Na)^+$; 100%].

(49a) 1-(phosphonobutyl) uracil



The title compound was prepared using the standard procedure **E** for the cleavage of dibenzyl esters with 10% Pd/C; starting from **48a** (84 mg; 0.19 mmol).

White solid; 48 mg; 97%.

R_f: baseline in DCM/MeOH.

Mp: 188-191 °C.

^{1}H-NMR (300 MHz; CD_3OD): δ 7.60 (d, *J*=7.83 Hz, 1H; H-5); 5.66 (d, *J*=7.83 H, 1H, H-5); 3.79 (t, *J*=6.95 Hz, 2H, H-7); 1.86-1.63 (m, 6H, H-10+H-9+H-8).

^{13}C-NMR (75 MHz; CD_3OD): δ 167.1 (C-4); 153.2 (C-2); 147.6 (C-6); 102.6 (C-5); 49.1 (C-7); 31.1 (d, *J*=16.07 Hz, C-8); 28.0 (d, *J*=140.53 Hz, C-10); 21.4 (d, *J*=4.83 Hz, C-9).

^{31}P-NMR (121 MHz; CD_3OD): δ 30.18.

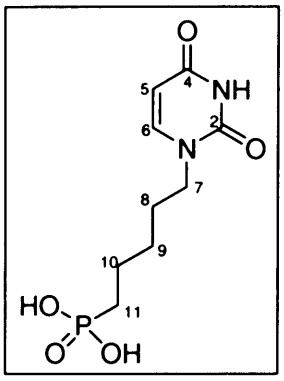
LRMS (ES-): m/z 247.1 [$(M)^-$, 100%]; m/z 494.9 [$(2M)^-$, 35%].

HRMS (ES-): found 247.0492; required 247.0489 for $C_8H_{12}N_2O_5P^-$.

Microanalysis calculated for $C_8H_{13}N_2O_5P$

C 38.72, H 5.28, N 11.28, P 12.48%; found C 38.90, H 5.42, N 10.44, P 11.88%.

(49b) 1-(phosphonopentyl) uracil



Compound **48b** (103 mg; 0.23 mmol), was hydrogenated in the presence of 10% Pd/C following the general procedure **E** for the cleavage of dibenzyl esters.

White solid; 48 mg; 79%.

R_f: baseline in DCM/MeOH.

Mp: 205-209 °C, brown liquid.

^{1}H-NMR (300 MHz; CD_3OD): δ 7.60 (d, *J*=7.84 Hz, 1H, H-6); 5.67 (d, *J*=7.84 Hz, 1H, H-5); 3.77 (t, *J*=7.23 Hz, 2H, H-7); 1.77-1.69 (m, 6H, H-8+H-10+H-11); 1.48-1.46 (m, 2H, H-9).

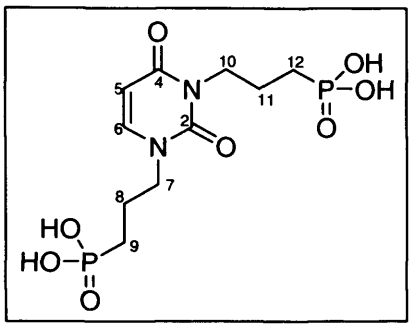
^{13}C-NMR (75 MHz; CD_3OD): δ 167.2 (C-4); 153.2 (C-2); 147.8 (C-6); 102.6 (C-5); 49.1 (C-7); 29.9 (C-8); 28.7 (d, *J*=16.60 Hz, C-9); 28.3 (d, *J*=136.53 Hz, C-11); 24.0 (d, *J*=4.52 Hz, C-10).

^{31}P-NMR (121 MHz; CD_3OD): δ 30.91.

LRMS (ES-): m/z 261.0 [$(M)^-$, 100%]; m/z 522.8 [$(2M)^-$, 25%].

HRMS (ES-): found 261.0636; required 261.0640 for $C_9H_{14}N_2O_5P^-$.

(51) 1,3-*bis*(phosphonopropyl) uracil



The title compound was obtained following the same procedure applied for the synthesis of compound **44a**, starting from the diethyl ester **43c** (55 mg; 0.12 mmol) and trimethyl silyl bromide (154 µl; 1.2 mmol) in DCM (4 ml).

Orange oil; 50 mg; 100%

R_f: baseline in DCM/MeOH 90:10.

^{1}H-NMR (300 MHz; D_2O): δ 7.47 (d, *J*=7.86 Hz, 1H, H-6); 5.73 (d, *J*=7.86 Hz, 1H, H-5); 3.82 (t, *J*=6.04 Hz, 2H, H-7); 3.74 (t, *J*=6.79 Hz, 2H, H-10); 1.89-1.61 (m, 8H, H-8+H-9+H-11+H-12).

^{13}C-NMR (75 MHz; D_2O): δ 166.1 (C-4); 152.7 (C-2); 145.5 (C-6); 101.4 (C-5); 50.5 (d, *J*=19.5 Hz, C-10); 42.0 (d, *J*=19.5 Hz, C-7); 24.1 (d, *J*=135.6 Hz, C-12); 23.6 (d, *J*=136.2 Hz, C-9); 21.9 (d, *J*=4.0 Hz, C-11); 20.6 (d, *J*=2.9 Hz, C-8).

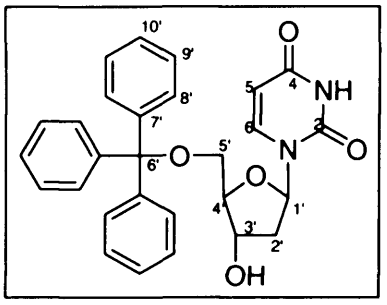
^{31}P-NMR (121 MHz; D_2O): δ 31.8; 31.3.

LRMS (ES-): m/z 355.0 [$(M)^-$; 100%]; m/z 710.7 [$(2M)^-$; 50%].

Microanalysis calculated for $C_{10}H_{18}N_2O_8P_2$ + 1.1 H_2O

C 31.34, H 5.52, N 7.31, P 16.16%; found C 31.57, H 4.77, N 5.90, P 13.84%.

(52) 5'-*O*-trityl-2'-deoxyuridine[74]



2'-deoxyuridine (4.00 g, 17.5 mmol) and triphenylmethyl chloride (5.37 g, 19.25 mmol) were stirred in anhydrous pyridine (70 ml) at 50 °C overnight. Additional triphenylmethyl chloride (1.00 g, 3.59 mmol) was added after 18 h, and the mixture was stirred for a further 4 h at 50 °C. The reaction mixture was then poured into ice-H_2O (300 ml) and stirred vigorously. The precipitate was extracted with EtOAc (3×100 ml). The organic solution was then washed with 0.5 M HCl (4×100 ml), dried with $MgSO_4$ and filtered. The filtrate was then washed further with EtOAc, which was then evaporated, and finally with DCM. Solvent was removed under reduced pressure affording the title compound sufficiently pure for the next step.

White solid; 7.69 g; 93%.

R_f: 0.42 in DCM/MeOH 95:5.

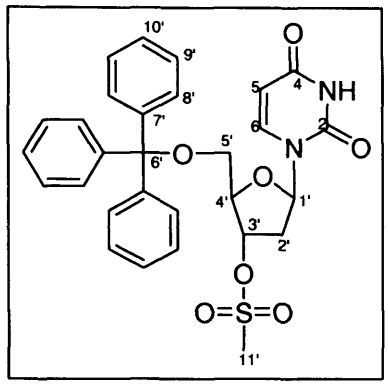
^{1}H-NMR (300 MHz; $CDCl_3$): δ 8.91 (s, 1H, N-H), 7.79 (d, J=8.2 Hz, 1H, H-6), 7.44-7.25 (m, 15H, H-aromatic), 6.34 (t, J=6.3 Hz, 1H, H-1'), 5.41 (dd, J_1=8.13 Hz, J_2=2.03 Hz, 1H, H-5), 4.58 (s, 1H, H-3'), 4.05 (q, J=3.2 Hz, 1H, H-4'), 3.51-3.49 (m, 2H, H-5'), 2.52-2.43 (m, 1H, CH*H*-2'), 2.33-2.24 (m, 1H, C*H*H-2')

^{13}C-NMR (75 MHz; $(CD)_2SO$): δ 163.4 (C-4), 150.7 (C-2), 143.8 (C-7'), 140.8 (C-6), 128.6 (C-8'), 128.3 (C-9'), 128.1 (C-10'), 101.9 (C-5), 86.7 (C-6'), 85.6 (C-1'), 84.5 (C-4'), 70.3 (C-3'), 64.0 (C-5'), 39.6 (C-2')

LRMS (ES+): m/z 492.9 [$(M+Na)^+$, 90%].

HRMS (ES+): Found 493.1728; required 493.1739 for $C_{28}H_{26}N_2O_5Na$ $[M+Na]^+$.

(53) 3'-*O*-mesyl–5'-*O*-trityl–2'-deoxyuridine[139]



Methanesulphonyl chloride (0.173 ml, 2.24 mmol) was added to a solution of 5'-*O*-trityl-2'-deoxyuridine **52** (0.30 g, 0.64 mmol) in anhydrous pyridine (5 ml) with ice-bath cooling. The mixture was stirred for 4 h at room temperature. After this time, ice-water (1 ml) was added; the mixture was stirred for 5 min, then poured into ice-water (30 ml) and filtered. The precipitate was dissolved in $CHCl_3$ (30 ml), the solution was washed with 0.5 M HCl (10 ml) and water (3×10 ml), dried ($MgSO_4$), filtered, and concentrated *in vacuo*.

Yellow/orange solid; 0.29 g; 83%.

R_f: 0.49 in DCM/MeOH 95:5.

Mp: 100-103 °C (Lit. 106-108 °C)[139]

^{1}H-NMR (300 MHz; $CDCl_3$): δ 8.39 (s, 1H, N-H); 7.71 (d, *J*=8.2 Hz, 1H, H-6); 7.41-7.24 (m, 15H, H-aromatic); 6.39-6.33 (m, 1H, H-1'); 5.43-5.38 (m, 2H, H-3'+H-5); 4.34 (q, *J*=2.6 Hz, 1H, H-4'); 3.59-3.50 (m, 2H, H-5'); 3.04 (s, 3H, H-11'); 2.77-2.70 (m, 1H, C*H*H-2'); 2.49-2.39 (m, 1H, CH*H*-2').

^{13}C-NMR (75 MHz; $CDCl_3$): δ 165.2 (C-4); 151.9 (C-2); 143.3 (C-7'); 138.1 (C-6); 129.0 (C-8'); 128.6 (C-9'); 128.4 (C-10'); 103.9 (C-5); 87.5 (C6'); 85.5 (C-1'); 84.3 (C-4'); 79.6 (C-3'), 62.3 (C-5'), 39.3 (C-2'), 39.1 (C-11').

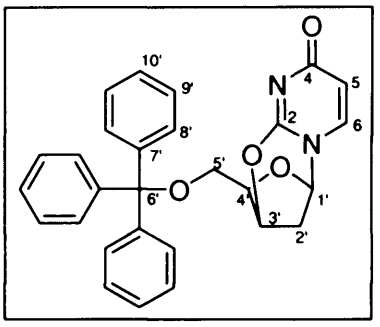
LRMS (ES+): m/z 571.1 [$(M+Na)^+$, 60%].

HRMS (ES+): found 571.1510; required 571.1515 for $C_{29}H_{28}N_2O_7NaS$ $[M+Na]^+$.

Microanalysis calculated for $C_{29}H_{28}N_2O_7S$ + 1.5 HCl + 1 H_2O:

C 56.06, H 5.11, N 4.51, S 5.16% found C 56.48, H 4.59, N 4.43, S 5.33%.

(54) 2, 3'-Anhydro-5'-*O*-trityl-2',3'-dideoxyuridine[140]



DBU (1.00 ml, 7.12 mmol) and 3'-*O*-Mesyl-5'-*O*- trityl-2'-deoxyuridine **53** (3.55 g, 6.48 mmol) were stirred in DCM (25 ml) over 30 h. The mixture was washed with water (2×30 ml), the organic layer was dried ($MgSO_4$), filtered and reduced in vacuo. The residue was then purified by column chromatography (5% $MeOH/CHCl_3$).

White solid; 2.42 g; 82%.

R_f: 0.29 in Chloroform/MeOH 95:5.

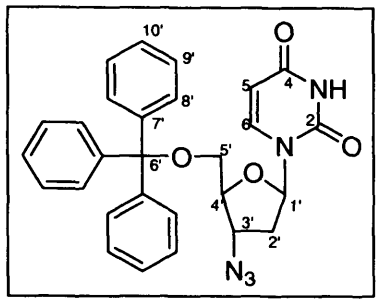
^{1}H-NMR (300 MHz; $CDCl_3$): δ 7.49-7.22 (m, 15H, H-aromatic); 7.11 (d, *J*=7.4 Hz, 1H, H-6); 5.98 (d, *J*=7.4 Hz, 1H, H-5); 5.54 (d, *J*=3.8 Hz, 1H, H-1'); 5.21 (s, 1H, H-3'); 4.35-4.30 (m, 1H, H-4'); 3.51-3.36 (m, 2H, H-5'); 2.67 (d, *J*=12.8 Hz, 1H, C*H*H-2'); 2.46 (d, *J*=12.9, 1H, CH*H*-2').

^{13}C-NMR (75 MHz; $CDCl_3$): δ 161.5 (C-4); 153.9 (C-2); 143.7 (C-7'); 139.3 (C-6); 129.0 (C-8'); 128.4 (C-9'); 127.7 (C-10'); 110.2 (C-5); 88.1 (C-1'); 87.8 (C-6'); 85.1 (C-4'); 77.3 (C-3'); 62.6 (C-5'); 34.2 (C-2').

IR (in KBr) υ cm^{-1}: 3038 (C-H arom.); 1649 (C=O); 1514(NC=O).

LRMS (ES+): m/z 474.9 [$(M+Na)^+$, 100%].

(55) 3'-Azido-5'-*O*-trityl-2',3'-dideoxyuridine



Method A

Lithium fluoride (0.016 g, 0.63 mmol) was suspended in *N,N*-dimethylformamide (1 ml) and heated to 105 °C with stirring. To the stirred suspension was added TMEDA (1.5 ml) followed by azidotrimethylsilane (0.073 g, 0.63 mmol). After stirring for an hour, 2,3′-anhydro-5′-*O*-trityl-2′,3′-dideoxyuridine **54** (0.16 g, 0.35 mmol) dissolved in DMF (0.5 ml) was added, and the reaction was allowed to proceed for 20 h at 110 °C. The mixture was cooled, poured into $CHCl_3$ (12 ml) and filtered through celite. The filtrate was washed with water (2×20 ml), dried ($MgSO_4$), filtered and concentrated. The concentrated mixture was purified by column chromatography (3% MeOH/$CHCl_3$).

Orange solid; 0.098 g; 56.5 %.

Method B

Lithium fluoride (0.145 g, 5.61 mmol) was suspended in DMF (3 ml) and heated to 105 °C with stirring. To the stirred suspension was added TMEDA (5 ml) followed by azidotrimethylsilane (0.645 g, 5.61 mmol). After stirring for an hour, **54** (1.409 g, 3.114 mmol) dissolved in DMF (2 ml) was added, and the reaction was allowed to proceed for 20 h at 110 °C. The mixture was cooled, poured into $CHCl_3$ (110 ml) and filtered through celite. The solvent was removed under reduced pressure and the residue (brown oil) was taken in EtOAc (100 ml). The organic phase was washed with water (4×180 ml), dried ($MgSO_4$), filtered and concentrated. The concentrated mixture was purified by column chromatography (3% MeOH/$CHCl_3$).

Orange solid; 0.996 g; 65 %.

R_f: 0.78 in Chloroform/MeOH 95:5.

Mp: 67-70 °C

^{1}H-NMR (300 MHz; $CDCl_3$): δ 8.41 (s, 1H, N-H); 7.90 (d, *J*=8.2 Hz, 1H, H-6); 7.46-7.32 (m, 15H, H-aromatic); 6.25 (t, *J*=5.3 Hz, 1H, H-1'); 5.44 (dd, J_1=8.1 Hz, J_2=1.9 Hz, 1H, H-5); 4.40 (q, *J*=6.3 Hz, 1H, H-4'); 4.02-3.98 (m, 1H, H-3'); 3.61 (dd, *J*=11.1 Hz, J_2=1.9 Hz, 1H, C*H*H-5'); 3.49 (dd, J_2=11.1 Hz, J_2=2.8, Hz, 1H, CH*H*-5'); 2.61-2.41 (m, 2H, H-2').

^{13}C-NMR (75 MHz; $CDCl_3$):δ 161.4 (C-4); 151.2 (C-2); 143.4 (C-7'); 140.2 (C-6); 129.0 (C-8'); 128.6 (C-9'); 128.0 (C-10'); 102.8 (C-5); 88.2 (C-6'); 85.2 (C-4'); 84.1 (C-1'); 64.0 (C-5'); 59.8 (C-3'); 36.8 (C-2').

IR (in KBr) υ cm^{-1}: 3038 (C-H arom.); 2108 (N=N=N); 1691 (C=O).

LRMS (ES+): m/z 518.0 [$(M+Na)^+$, 60%].

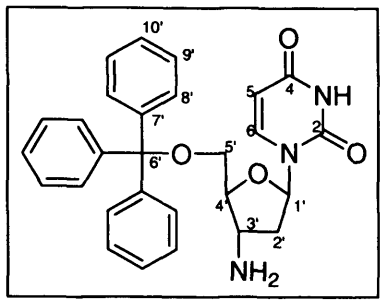
HRMS (ES+): found 518.1824; required 518.1804 for $C_{28}H_{25}N_5O_4Na$ $[M+Na]^+$.

Microanalysis calculated for $C_{28}H_{25}N_{4.5}O_4+0.6\ H_2O$

C 66.87, H 5.33, N 12.53%; found C 66.59, H 4.68, N 12.70%.

Ref.[139]

(56) 3'-amino-5'-*O*-trityl-2',3'-dideoxyuridine



Method A

10% Pd/C catalyst (10 mg, 5% mol) was added to **55** (0.098 g, 0.198 mmol), which was than suspended in methanol (5 ml). Air was removed from the flask by flushing with nitrogen several times. The nitrogen was then removed and replaced with hydrogen. The mixture was stirred for 36 h. After stirring the mixture was filtered through celite. The solvent was evaporated, which left a white solid (0.332 g, impure). Purification was attempted by column chromatography (3% MeOH/$CHCl_3$), but was unsuccessful. From this, 40 mg of compound was recovered, which was starting material.

Method B

Polymer supported triphenylphosphine ($PSPPh_3$) (0.17 g, loaded with 3 mmol PPh_3/g resin) was suspended in anhydrous pyridine (5 ml) and **55** (0.10 g, 0.20 mmol) was added. The suspension was slightly shaken overnight at room temperature. After that time concentrated ammonia (4 ml, 28 %) was added, and left for 6 h. The mixture was filtered and the solvent evaporated. The method was unsuccessful, no compound was recovered from the chromatographic purification.

Method C

5% Pd/C catalyst (40 mg) was added to **55** (0.10 g, 0.20 mmol), and was then suspended in methanol (10 ml). Air was removed from the flask by flushing with nitrogen several times. The nitrogen was then removed and replaced with hydrogen. The mixture was stirred for 6 h, and then filtered through Celite. The solvent was evaporated and the concentrated solution was purified by column chromatography (DCM/MeOH 98:2→90:10), which gave the product **56** (0.052 g, yield 56 %) as a white solid.

Method D

Lindlar's catalyst (20 mg) was added to the 3′-azido-5′-*O*-trityl-2′,3′-dideoxyuridine **55** (0.10 g, 0.20 mmol), and was then suspended in ethanol (5 ml). Air was removed from the flask by flushing with nitrogen several times. The nitrogen was then removed and replaced with hydrogen. The mixture was stirred for 5 h, and then filtered through Celite. Fresh Lindlar's catalyst (20 mg) was added to the filtrate. The flask was flushed with nitrogen and then hydrogen as previously, and the reaction was left stirring for another 3 h. The reaction mixture was filtered through Celite. The solvent was evaporated and the concentrated solution was purified by column chromatography (DCM/MeOH 98:2→90:10).

White solid; 0.065 g; 70%.

Method E

To a solution of **55** (0.10 g, 0.20 mmol) in anhydrous THF (2 mL), triphenyl phosphine (0.063 g, 0.242 mmol) was added, and the reaction mixture was stirred at room temperature overnight. Water (0.04 mL, 2.22 mmol) was then added to the mixture which was then heated at 60 °C for 5 h. After this time, the mixture was allowed to cool and the solvent was evaporated. Purification was carried out by column chromatography (DCM/MeOH 98:2→90:10) which gave the title compound **56** (0.080 g, 84% yield) as a white solid.

R_f: 0.3 in DCM/MeOH 90:10.

Mp: 110-114 °C.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 7.97 (d, *J*=8.2 Hz, 1H, H-6); 7.47-7.27 (m, 15H, H-aromatic); 6.20 (q, *J*=3.3 Hz, 1H, H-1'); 5.41 (d, *J*=8.1 Hz, 1H, H-5); 3.73-3.38 (m, 5H, H-3', H-4', H-5'); 2.44-2.18 (m, 2H, H-2').

^{13}C-NMR (75 MHz; $CDCl_3$): δ 163.5 (C-4); 150.5 (C-2); 143.7 (C-7'); 140.7 (C-6); 129.1 (C-8'); 128.5 (C-9'); 127.8 (C-10'); 102.2 (C-5); 87.9 (C-6'); 87.2 (C-4'); 85.2 (C-1'); 62.2 (C-5'); 50.7 (C-3'); 42.7 (C-2').

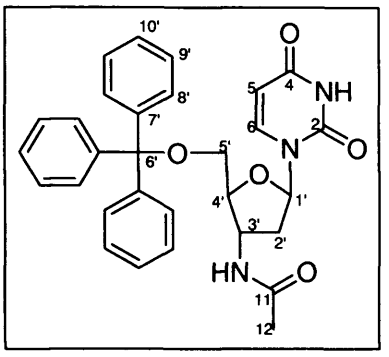
LRMS (ES+): m/z 491.7 [$(M+Na)^+$, 45%]; m/z 243.2 [$(Ph_3C)^+$, 100%].

HRMS (ES+): found 492.1902; required 492.1899 for $C_{28}H_{27}N_3O_4Na$ $[M+Na]^+$.

Microanalysis calculated for $C_{28}H_{27}N_3O_4+0.5\ H_2O$

C 70.28, H 5.90, N 8.78%; found C 70.64, H 5.92, N 8.41%.

(57a) 3'-acetylamino-5'-*O*-trityl-2',3'-dideoxyuridine



Compound **56** (0.10 g, 0.213 mmol) was suspended in DCM (5 ml), and to this was added acetic anhydride (0.047 g, 0.044 ml, 0.469 mmol) and TEA (0.065 ml, 0.469 mmol). The mixture was stirred at room temperature for 3 h. After this time the solvent was evaporated to give a white solid. The product was purified by column chromatography (DCM/MeOH 98:2→94:6), and evaporation of the solvent gave the title compound **57a** as a white solid.

White solid; 0.103 g; 95%.

R_f: 0.45 in DCM/MeOH 90:10.

Mp: 135-140 °C.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 9.82 (s, 1H, N-H); 7.83 (d, *J*=8.2 Hz, 1H, H-6); 7.46-7.28 (m, 15H, H-aromatic); 6.94 (s, 1H, N-H); 6.34 (t, *J*=6.3 Hz, 1H, H-1'); 5.39 (d, *J*=8.1 Hz, 1H, H-5); 4.79-4.72 (m, 1H, H-3'); 4.07 (s, 1H, H-4'); 3.59-3.47 (m, 2H, H-5'); 2.50-2.32 (m, 2H, H-2'); 2.04 (s, 3H, H-12').

^{13}C-NMR (75 MHz; $CDCl_3$): δ 163.6 (C-4); 154.9 (C-2); 143.6 (C-7'); 140.5 (C-6); 129.1 (C-8'); 128.5 (C-9'); 127.9 (C-10'); 103.1 (C-5); 88.1 (C-6'); 87.3 (C-1'); 85.2 (C-4'); 62.1 (C-5'); 50.9 (C-3'); 38.8 (C-2').

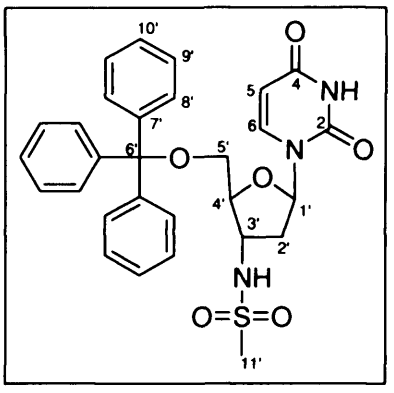
LRMS (ES+): m/z 533.8 [$(M+Na)^+$, 20%].

HRMS (ES+): found 534.2009; required 534.2005 for $C_{30}H_{29}N_3O_5Na$ $[M+Na]^+$.

Microanalysis calculated for $C_{30}H_{29}N_3O_5$+1.0 HCl+1.0 H_2O

C 63.66, H 5.70, N 7.42%; found C 63.20, H 5.15, N 7.11%.

(57c) 3'-Sulfonylamino-5'-*O*-trityl-2',3'-dideoxyuridine



Mesyl chloride (24.5 mg; 0.017 ml; 0.21 mmol) was added with a syringe to a solution of 3'-amino-5'-*O*-trityl-2'-deoxyuridine **56** (92 mg; 0.19 mmol), TEA (0.030 ml; 0.21 mmol) in DCM (5ml) at room temperature, under nitrogen.

The mixture was stirred 1 hour and then the solvent was removed under reduced pressure. The crude residue (yellow oil) was taken in DCM (1 ml) and purified by flash chromatography using DCM/MeOH 90:10 as eluent.

White solid; 55mg; 51%.

R_f: 0.38 in DCM/MeOH 90:10.

Mp: 128-134 °C.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 8.98 (s, 1H, NH); 7.80 (d, *J*=8.18 Hz, 1H, H-6); 7.42-7.34 (m, 15H, H-arom.); 6.45 (t, *J*=6.34 Hz, 1H, H-1'); 6.21 (d, *J*=8.60 Hz, 1H, NH); 5.47 (dd, J_1=10.8 Hz, J_2=1.85 Hz, 1H, H-5); 4.31-4.23 (m, 1H, H-7'); 4.11-4.08 (m, 1H, H-3'); 3.57 (dq; J_1=10.8 Hz, J_2=2.9 Hz, 2H, H-5'); 2.90 (s, 3H, H-11'); 2.48-2.43 (m, 2H, H-2').

^{13}C-NMR (75 MHz; $CDCl_3$): δ 162.7 (C-4); 151.3 (C-2); 143.3 (C-6); 139.7 (C-7'); 129.1 (C-9'); 128.6 (C-10'); 128.0 (C-8'); 103.6 (C-5); 88.7 (C-6'); 85.4 (C-1'); 84.8 (C-4'); 63.0 (C-5'); 53.9 (C-3'); 42.0 (C-11'); 39.7 (C-2').

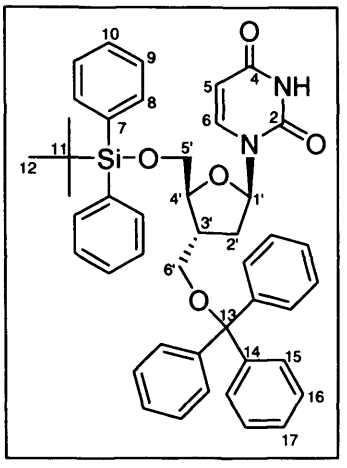
LRMS (ES+): m/z 569.0 [(M+Na), 100%].

HRMS (ES+): found 570.1674; required 570.1669 for $C_{29}H_{29}N_3NaSO_6^+$; $[M+Na]^+$.

Microanalysis calculated for $C_{29}H_{29}N_3O_6S+0.9\ H_2O$:

C 61.78, H 5.51, N 7.45, S 5.69%; found C 61.41, H 5.20, N 7.22, S 6.15%.

(59) 3'-trityloxymethyl -5'-*O-tert*-butyldiphenylsilyl-2',3'-dideoxyuridine



The title compound was prepared according to the general procedure for the tritylation of nucleosides. The starting material **58** (100 mg, 0.21 mmol), trityl chloride (81 mg, 0.29 mmol) and pyridine (5 ml) were used for the reaction.

White solid, 116 mg, 77%.

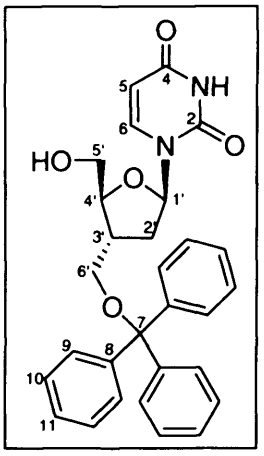
R_f: 0.69 in $CHCl_3$/MeOH 90:10.

^{1}H-NMR (500 MHz; $CDCl_3$): δ 8.05 (bs, 1H, NH); 7.99 (d, J=8.15 Hz, 1H, H-6); 7.41-7.28 (m, 25H, H-arom.); 6.12 (dd, J_1=6.84 Hz, J_2=3.35 Hz, 1H, H-1'); 5.34 (dd, J_1=8.15 Hz, J_2=2.30 Hz, 1H, H-5); 4.09 (dd, J_1=11.82 Hz, J_2=2.22 Hz, 1H, CH*H*-5'); 3.95 (dt, J_1=8.18 Hz, J_2=2.32 Hz, 1H, H-4'); 3.64 (dd, J_1=11.82 Hz, J_2=2.64 Hz, 1H, C*H*H-5'); 3.22 (dd, J_1=9.41 Hz, J_2=5.62 Hz, 1H, CH*H*-6'); 3,09 (dd, J_1=9.45 Hz, J_2=5.44 Hz, 1H, C*H*H-6'); 2.75-2.67 (m, 1H, H-3'); 2.47-2.42 (m, 1H, C*H*H-2'); 2.22-2.17 (m, 1H, CH*H*-2'); 1.09 (s, 9H, H-12).

^{13}C-NMR (125 MHz; $CDCl_3$): δ 163.8 (C-4); 149.9 (C-2); 143.6 (C-14); 140.3 (C-6); 135.5 (C-7); 130.0 (C-8); 128.6 (C-15); 127.9 (C-9); 127.3 (C-10); 127.2 (C-17); 101.6 (C-5); 85.3 (C-1'); 83.9 (C-4'); 63.5 (C-5'); 62.6 (C-6'); 37.4 (C-3'); 36.9 (C-2'); 27.0 (C-12); 15.5 (C-11).

LRMS (ES+): m/z 745.4 [$(M+Na)^+$, 100%].

(60) 3'-trityloxymethyl-2',3'-dideoxyuridine



The 3'-trityloxymethyl intermediate **59** (116 mg; 0.16 mmol) was dissolved in THF (3 ml) and TBAF (50 mg; 0.192 mmol) was added into the solution. The reaction was stirred at room temperature until the TLC ($CHCl_3$/MeOH 90:10) showed complete disappearance of the starting material. The solvent was evaporated and the crude was purified by chromatography.

White solid; 47 mg; 60%.

R_f: 0. 62 in $CHCl_3$/MeOH 90:10.

Mp: 107-110 °C.

^{1}H-NMR (500 MHz; $CDCl_3$): δ 8.34 (bs, 1H, NH): 7.87 (d, *J*=8.16 Hz, 1H, H-6); 7.43 (d, *J*=7.23 Hz, 6H, H-9); 7.34 (d, *J*=7.51 Hz, 6H, H-10); 7.29-7.27 (m, 3H, H-11); 6.06 (dd, J_1=7.03 Hz, J_2=3.48 Hz, 1H, H-1'); 5.70 (dd, J_1=8.16 Hz, J_2=1.96 Hz, 1H, H-5); 4.03-3.99 (m, 1H, C*H*H-5'); 3.94 (dt, J_1=8.29 Hz, J_2=3.14 Hz, 1H, H-4'); 3.82-3.78 (m, 1H, CH*H*-5'); 3.33 (dd, J_1=9.59 Hz, J_2=4.87 Hz, 1H, C*H*H-6'); 3.16 (dd; J_1=7.00 Hz, J_2=9.56 Hz, 1H, CH*H*-6'); 2.56-2.49 (m, 1H, H-3'); 2.31-2.25 (m, 1H, C*H*H-2'); 2.20-2.15 (m, 1H, CH*H*-2'); 1.60 (bs, 1H, OH).

^{13}C-NMR (125 MHz; $CDCl_3$): δ 163.1 (C-4); 150.0 (C-2); 143.5 (C-8); 140.4 (C-6); 128.5 (C-9); 128.0 (C-10); 127.3 (C-11); 101.9 (C-5); 85.8 (C-1'); 84.7 (C-4'); 63.6 (C-6'); 62.9 (C-5'); 38.1 (C-3'); 36.2 (C-2').

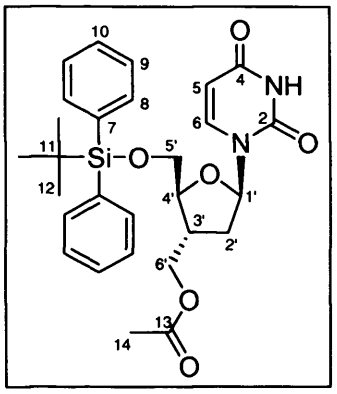
LRMS (ES+): m/z 243.2 [$(Ph_3C)^+$, 100%].

HRMS (ES+): found 502.2342; required 502.2336 $[M+NH_4]^+$.

Microanalysis calculated for $C_{29}H_{28}N_2O_5$+0.8 H_2O

C 69.81, H 5.98, N 5.61%; found C 69.61, H 5.74, N 5.57%.

(61) 3'-acetoxymethyl -5'-*O-tert*-butyldiphenylsilyl-2',3'-dideoxyuridine



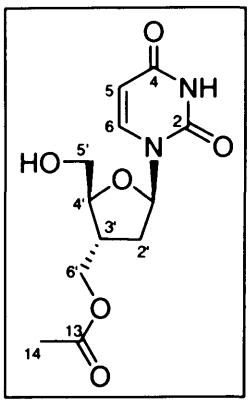
The compound was acetylated in 6' position according to the general procedure for the acetylation of alcohols. It was prepared starting from 5'-O-TBDPS-3'-methylhydroxyl-2'-deoxyuridine **58** (150 mg, 0.31 mmol), acetic anhydride (35 μl, 0.37 mmol) in pyridine (4 ml). After the reaction reached completion, the product was extracted with EtOAc (3×5 ml). The organic phase was dried with $MgSO_4$ and concentrated to give a residue pure enough for the next step.

White foam, 100 mg, 61%.

R_f: 0.75 $CHCl_3$/MeOH 90:10.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 9.81 (bs, 1H, NH); 8.04 (d, *J*=8.13 Hz, 1H, H-6); 7.70 (t, *J*=6.91 Hz, 4H, H-9); 7.54-7.43 (m, 6H, H-8+H-10); 6.19 (dd, J_1=6.69 Hz, J_2=3.35 Hz, 1H, H-1'); 5.43 (d, *J*=8.14 Hz, 1H, H-5); 4.18 (dd, J_1=11.81 Hz, J_2=2.02 Hz, 1H, C*H*H-5'); 4.13-4.02 (m, 2H, H-6'); 3.74 (d, *J*=8.23 Hz, 1H, H-4'); 3.82 (dd, J_1=11.84 Hz, J_2=2.30 Hz, 1H, CH*H*-5'); 2.90-2.77 (m, 1H, H-3'); 2.43-2.22 (m, 2H, H-2'); 2.13 (s, 3H, H-14); 1.06 (s, 9H, H-12).

(62) 3'-acetoxymethyl-2',3'-dideoxyuridine



TBAF (94 mg, 0.35 mmol) was added to a solution of compound **61** (150 mg, 0.29 mmol) in THF (3 ml). The reaction was stirred at room temperature for 3 h, then the solvent was removed *in vacuo* and the residue was purified by chromatography.

Colourless oil, 46 mg, 56 %.

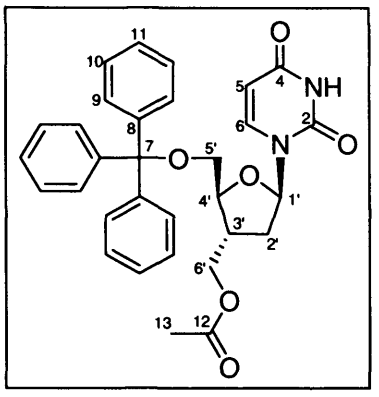
R_f: 0.35 in $CHCl_3$/MeOH 90:10.

^{1}H-NMR (250 MHz; $CDCl_3$): δ 9.54 (bs, 1H, NH); 7.70 (d, J=8.12 Hz, 1H, H-6); 5.87 (dd, J_1=6.38 Hz, J_2=3.20 Hz, 1H, H-1'); 5.49 (d, J=8.09 Hz, 1H, H-5); 3.93-3.56 (m, 5H, H5'+H-4'+H-6'); 2.60-2.40 (m, 1H, H-3'); 2.10-2.02 (m, 2H, H-2'); 1.86 (s, 3H, H-14).

General procedure G: tritylation of alcohol with trityl chloride in pyridine using the Radleys 12 tube carousel

A carousel tube was charged with trityl chloride (1.4 equivalents). The tube was sealed and the air inside was replaced with nitrogen; then a solution of alcohol (1 equivalent) in dry pyridine was added with a syringe. The reaction was stirred 24 h at 70 °C. After disappearance of the starting material, the reaction was cooled to room temperature and poured into a round bottom flask. The solvent was evaporated under reduced pressure. The residue was dissolved in EtOAc and washed with HCl 0.5M (15 ml). The organic phase was dried with $MgSO_4$, concentrated and purified by chromatography.

(63) 3'-acetoxymethyl -5'-*O*-trityloxy-2',3'-dideoxyuridine



The title compound was prepared following the general procedure **G** for the tritylation of alcohols with the radleys carousel. Starting from compound **62** (68 mg, 0.24 mmol). The crude residue was purified by chromatography using $CHCl_3$ →$CHCl_3$/MeOH 95:5 as gradient.

White solid, 80 mg, 95%.

R_f: 0.57 in $CHCl_3$/MeOH 90:10.

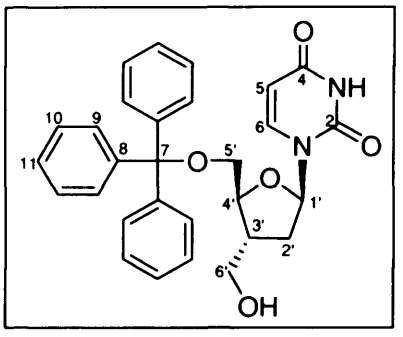
Mp: 74-77 °C.

^{1}H-NMR (500 MHz; $CDCl_3$): δ 8.21 (bs, 1H, NH); 8.05 (d, J=8.16 Hz, 1H, H-6); 7.44-7.42 (m, 6H, H-9); 7.37-7.28 (m, 9H, H-10+H-11); 6.14 (dd, J_1=6.75 Hz, J_2=2.96 Hz, 1H, H-1'); 5.29 (dd, J_1=8.14 Hz, J_2=1.91 Hz, 1H, H-5); 4.09 (dd, J_1=11.34 Hz, J_2=5.96 Hz, 1H, CH*H*-6'); 4.00-3.97 (m, 2H, H-4'+C*H*H-6'); 3.67 (dd, J_1=10.90 Hz, J_2=2.47 Hz, 1H, C*H*H-5'); 3.44 (dd, J_1=10.93 Hz, J_2=3.31 Hz, 1H, CH*H*-5'); 2.87-2.79 (m, 1H, H-3'); 2.36-2.24 (m, 2H, H-2'); 1.94 (s, 3H, H-13).

^{13}C-NMR (125 Hz; $CDCl_3$): δ 173.3 (C-12); 161.7 (C-4); 149.9 (C-2); 143.1 (C-8); 140.3 (C-6); 128.8 (C-9); 128.0 (C-10); 127.5 (C-11); 101.7 (C-5); 85.3 (C-1'); 83.3 (C-4'); 63.9 (C-5'); 62.8 (C-6'); 36.9 (C-2'); 26.6 (C-3'); 20.6 (C-13).

LRMS (ES+): m/z 549.2 [$(M+Na)^+$, 100%].

(64) 3'-hydroxylmethyl -5'-*O*-trityloxy-2',3'-dideoxyuridine



The 5'-*O*-trityl-3'-acetoxymethyl derivative **63** (80 mg; 0.15 mmol) was deprotected following the general procedure **D** for the cleavage of ester with sodium methoxide 0.2M in MeONa. The title compound 64 was obtained after chromatographic purification with $CHCl_3$/MeOH as eluent.

White solid; 42 mg; 57%.

R_f: 0. 59 in $CHCl_3$/MeOH 90:10.

Mp: 106-108 °C.

^{1}H-NMR (500 MHz; $CDCl_3$): δ 8.57 (bs, 1H, NH); 7.99 (d, *J*=8.14 Hz, 1H, H-6); 7.46 (d, *J*=7.28 Hz, 6H, H-9); 7.31 (t, *J*=7.48 Hz, 6H, H-10); 7.31-7.28 (m, 3H, H-11); 6.11 (dd, J_1=6.84 Hz, J_2=3.16 Hz, 1H, H-1'); 5.38 (d, *J*=8.14 Hz, 1H, H-5); 3.99 (dt, J_1=8.31 Hz, J_2=3.15 Hz, 1H, H-4'); 3.66-3.62 (m, 2H, H-6'); 3.59 (dd, J_1=10.42 Hz, J_2=2.97 Hz, 1H, C*H*H-5'); 3.47 (dd, J_1=10.83 Hz, J_2=3.29 Hz, 1H, CH*H*-5'); 2.68-2.61 (m, 1H, H-3'); 2.39-2.33 (m, 1H, C*H*H-2'); 2.24-2.19 (m, 1H, CH*H*-2'); 1.64 (bs, 1H, OH).

^{13}C-NMR (125 MHz; $CDCl_3$): δ 161.2 (C-4); 150.3 (C-2); 143.2 (C-8); 140.4 (C-6); 128.7 (C-9); 128.0 (C-10); 127.4 (C-11); 101.7 (C-5); 85.4 (C-1'); 82.9 (C-4'); 63.4 (C-5'); 62.7 (C-6'); 40.3 (C-3'); 36.5 (C-2').

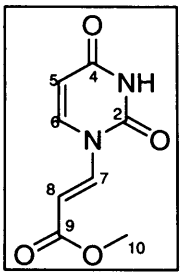
LRMS (ES+): m/z 243.2 [$(Ph_3C)^+$, 100%].

HRMS (ES+): found 502.2328; required 502.2336 $[M+NH_4]^+$.

Microanalysis calculated for $C_{29}H_{28}N_2O_5$+HCl

C 66.85, H 5.98, N 5.38%; found C 66.87, H 5.48, N 5.24%.

(80a) 1-(((*E*)-2-methoxycarbonyl)vinyl) uracil[117]



A 3 neck round bottom flask was charged with uracil (200 mg; 1.78 mmol) and caesium carbonate (290 mg; 0.89 mmol) under N_2, then DMF (20 ml). The white suspension was stirred at room temperature for 15 min and methyl propiolate **81** (0.190 ml; 2.14 mmol) was added dropwise with a syringe. The reaction was stirred at room temperature and the formation of a yellow suspension was observed. After 1 hour TLC (DCM/MeOH 90:10) showed the complete disappearance of the starting material; water (40 ml) was added to quench the reaction. The aqueous solution was extracted with EtOAc (2×20 ml). TLC of the two phases showed the presence of the product in the aqueous layer, consequently the solvent was evaporated and the residue was purified by chromatography using DCM/MeOH 90:10 as eluent.

White solid; 110 mg; 56%.

R_f: 0.71 in DCM/MeOH 90:10.

Mp: 187-190 °C.

^{1}H-NMR (300 MHz; CD_3OD): δ 8.20 (d, *J*=14.7 Hz, 1H, H-7); 7.97 (d, *J*=8.19 Hz, 1H, H-6); 6.20 (d, *J*=14.6 Hz, 1H, H-8); 5.85 (d, *J*=8.15 Hz, 1H, H-5); 3.79 (s, 3H, H-10).

^{13}C-NMR (75 MHz; CD_3OD): δ 168.7 (C-9); 165.6 (C-4); 151.0 (C-2); 140.8 (C-6); 138.9 (C-7); 106.7 (C-8); 105.7 (C-5); 52.7 (C-10).

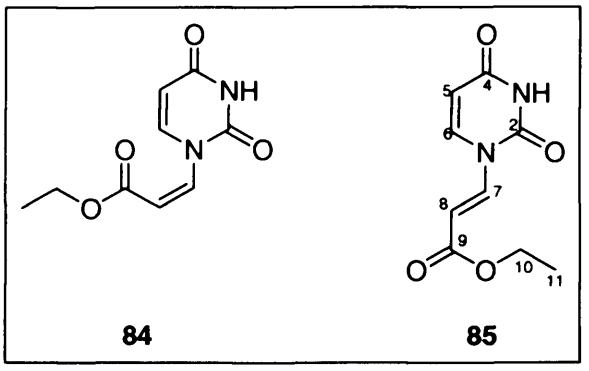
LRMS (ES+): m/z 219 [$(M+Na)^+$, 100%].

HRMS (ES+): found 214.0851; required 214.0822 for $[M+NH_4]^+$;

Microanalysis calculated for $C_8H_8N_2O_8$:

C 49.98, H 4.11, N 14.27%; found C 48.67, H 4.22, N 13.54%.

(84) Attempted synthesis of 1-(((*Z*)-2-ethoxycarbonyl)vinyl) uracil



84 **85**

A 3 neck flask was charged with uracil (200 mg; 1.78 mmol); CuI (34 mg; 0.17 mmol); Cs_2CO_3 (290 mg; 0.89 mmol) and *N,N*-dimethylglycyne (50 mg; 0.37 mmol) and dioxane (5 ml). Ethyl iodoacrylate (0.228 ml; 1.78 mmol) was added to the solution under nitrogen. The reaction was stirred at 60 °C for 72 h. TLC (DCM/MeOH 90:10) showed new spot with R_f between the two starting materials. The mixture was cooled to room temperature; water and MeOH (10 ml) were added. The milky suspension was filtered through a pad of silica. The solvents were removed under reduced pressure and the crude residue (brown oil) was purified by chromatography, first using DCM/MeOH as eluent and then Hexane/EtOAc 50:50→70:30. Unfortunately the only product isolated from the purification was the *E* isomer **85**.

Off white solid; 50 mg; 13%.

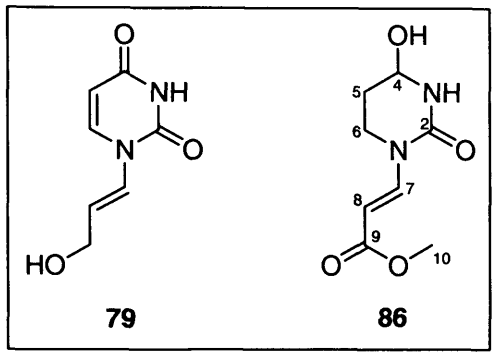
R_f: 0.29 in DCM/MeOH 90:10.

^{1}H-NMR (300 MHz; CD_3OD): δ 8.19 (d, *J*=14.6 Hz, 1H, H-7); 7.97 (d, *J*=8.19 Hz, 1H, H-6); 6.19 (d, *J*=14.6 Hz, 1H, H-8); 5.86 (d, *J*=8.19 Hz, 1H, H-5); 4.25 (q, *J*=7.12 Hz, 2H, H-10); 1.32 (t, *J*=7.13 Hz, 3H, H-11).

^{13}C-NMR (75 MHz; CD_3OD): δ 165.9 (C-9); 162.3 (C-4); 148.9 (C-2); 138.4 (C-6); 137.0 (C-7); 107.2 (C-8); 105.6 (C-5); 61.5 (C-10); 14.7 (C-11).

LRMS (ES+): m/z 233.1 [$(M+Na)^+$, 100%].

(79) Attempted synthesis of 1-((*E*)-3-hydroxyprop-1-enyl) uracil



79 **86**

Method A

$LiAlH_4$ 1M in THF (0.105 ml; 0.105 mmol) was added dropwise with a syringe in a suspension of **80a** (41mg; 0.21 mmol) in THF (2ml) at -78 °C under nitrogen. The reaction was allowed to reach -20 °C and then the suspension became a yellow solution. After 1 at -20 °C MeOH (1ml) was added to quench the reaction, and the formation of a white precipitate was observed. The solid was filtered off; the filtrate was evaporated to afford a crude residue which was purified by chromatography using DMC/MeOH (90:10) as eluent. Unfortunately NMRs analysis proved to be compound **86**, instead of **79**.

White solid; 8 mg; 19%.

R_f: 0.32 in DCM/MeOH 90:10.

^{1}H-NMR (300 MHz; CD_3OD): δ 8.52 (d, *J*=14.3 Hz, 1H, H-7); 5.30 (d, *J*=14.3 Hz, 1H; H-8); 5.02 (t, *J*=2.86 Hz, 1H, H-4); 3.72 (s, 3H, H-10); 3.63-3.58 (m, 2H, H-6); 2.08-2.02 (m, 2H, H-5).

^{13}C-NMR (75 MHz; CD_3OD): δ 170.8 (C-8); 144.0 (C-7); 97.2 (C-8); 73.6 (C-4); 52.1 (C-10); 40.0 (C-6); 29.4 (C-5).

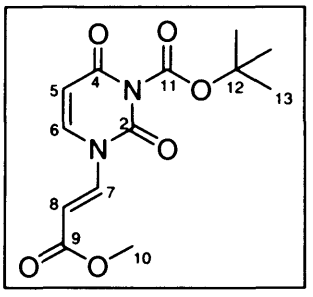
LRMS (ES+):m/z 223.0 [$(M+Na)^+$, 100%].

Method B

The ethyl ester **85** (50 mg, 0.24 mmol) was dissolved in dry THF (2 ml) and cooled to -78 °C under nitrogen. A solution of DIBAL-H 1M in THF (0.48 ml) was added dropwise with a syringe. After the addition the mixture was let to reach 25 °C, and monitored by TLC (Hexane/EtOAc 30:70). After 2 h the reaction was quenched with water (2 ml), the suspension was then filtered through celite. The

filtrate was concentrated under reduced pressure affording a complex mixture of products. Attempt to purify the crude by chromatography was unsuccessful.

(87) 1-(((*E*)-2-methoxycarbonyl)vinyl)-3-(*tert*-butoxycarbonyl) uracil



Boc anhydride (82 mg, 0.37 mmol) was added into a solution of (2*E*)-methyl 3-(uracil-yl) acrylate **80a** (50 mg, 0.25 mmol), TEA (28 mg, 0.28 mmol) and DMAP (5 mg) in THF at room temperature. The reaction was stirred at 25 °C for 3 h. The solution became yellow and a new spot was visible on TLC. The reaction was quenched with water (5 ml); the aqueous solution was then extracted with DCM (3×10 ml). The collected fractions were dried with $MgSO_4$, concentrated to give a yellow solid. The crude solid was dissolved with a mixture of Hexane and EtOAc, adsorbed on silica and purified by chromatography using Hexane/EtOAc 70:30→50:50 as gradient.

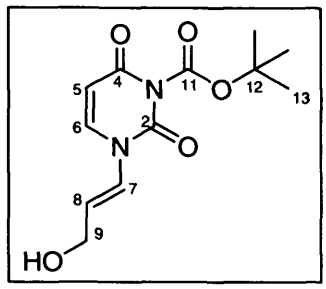
White solid, 58 mg, 86%.

R_f: 0.24 in Hexane/EtOAc 60:40.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 8.21 (d, *J*=14.61 Hz, 1H, H-7); 7.49 (d, *J*=8.32 Hz, 1H, H-6); 6.02 (d, *J*=14.62 Hz, 1H, H-8); 6.00 (d, *J*=8.32 Hz, 1H, H-5); 3.86 (s, 3H, H-10); 1.67 (s, 9H, H-13).

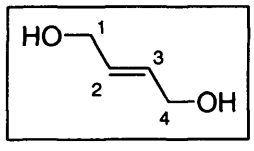
^{13}C-NMR (75 MHz; $CDCl_3$): δ 166.2 (C-9); 159.5 (C-11); 149.2 (C-2); 136.7 (C-6); 137.2 (C-7); 107.2 (C-8); 105.3 (C-5); 88.1 (C-12); 52.5 (C-10); 27.9 (C-13).

(88) Attempted synthesis of 1-((*E*)-3-hydroxyprop-1-enyl)-3-(*tert*-butoxycarbonyl) uracil



A solution 1M of DIBAL-H in THF (0.34 m l, 0.34 mmol) was added slowly with a syringe into a cooled (-78 °C) solution of **87** (50 mg, 0.17 mmol) in THF under nitrogen. The reaction was let to warm to 25 °C and stirred for 24, then a further equivalent of DIBAL-H was added and the mixture was stirred for other 12 h. TLC showed several new spots at lower Rfs. Some Celite and water were added in the solution, the suspension was stirred for few min and the filtered through a Celite pad. The filtrate was concentrated under reduced pressure and the residue was attempted of chromatographic purification. NMR of the collected fraction did not show the presence of the desired compound.

(91) *trans*-2-buten-1,4-diol[119]



2-butyn-1,4-diol **89** (1 g; 11.64 mmol) was dissolved in dry THF (25 ml) under nitrogen. The solution was cooled to -78 °C with a dry-ice/acetone bath. A cold solution of LAH in THF 1M (12.7 ml; 12.7 mmol) was added with a syringe. The reaction was warmed to room temperature in 4 h. The disappearance of the starting alkyne was observed by TLC (Hexane/EtOAc 30:70); then the solution was cooled to -0 °C with an ice bath and the quenched with NaOH 1M, until no gas was developed. The pH was adjusted to 8 with HCl 1M and then silica was

added to the solution. The solvents were removed under reduced pressure and the residue was loaded into a chromatographic column and purified using Hexane/EtOAc 30:70 as eluent.

Colourless oil; 817 mg; 79%.

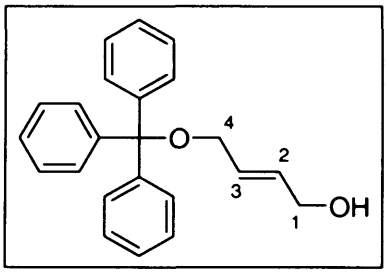
R_f: 0.11 in Hexane/EtOAc 30:70 (PMA).

^{1}H-NMR (300 MHz; CD_3OD): δ 5.83 (bs; 2H; H-2+H-3); 4.07 (d; *J*=3.57 Hz; 4H; H-1+H-4)

^{13}C-NMR (75 MHz; CD_3OD): δ 131.7 (C-2 & C-3); 63.4 (C-1 & C-4)

LRMS (ES+): m/z 111.0 [$(M+Na)^+$, 100%].

(92a) (*E*)-4-(trityloxy)but-2-en-1-ol



A solution of trityl chloride (500 mg; 1.81 mmol), TEA (0.277 ml; 1.99 mmol) and DMAP (8.8 mg; 0.072 mmol) in dry DCM (5 ml) was added with a syringe to a solution of diol **91** (800 mg; 9.07 mmol) in DCM (15 ml). The mixture was stirred at room temperature for 1 hour and 30 min, then more TrCl, TEA and DMAP (0.5 equivalents) were added. The reaction was stirred at the same temperature until TLC (Hexane/EtOAc 50:50) showed complete disappearance of trityl chloride. After 1.5 h water (20 ml) was added and the reaction was stirred for few min, then the phases were separated. The organic layer was washed with water (25 ml) and brine (25 ml). The solvent was dried over $MgSO_4$ and evaporated to afford a crude oil which was purified by flash chromatography using Hexane/EtOAc 50:50 as eluent.

Colourless oil, 637 mg, 71%.

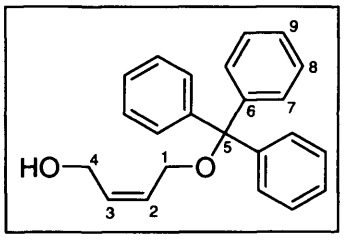
R_f: 0.72 in Hexane/EtOAc 50:50 (UV/PMA)

^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.54-7.51 (m, 6H, H-7); 7.39-7.27 (m, 9H, H-8+H-9); 6.07 (dt, J_1=15.5 Hz, J_2=5.4Hz, 1H, H-3); 5.87 (dt, J_1=15.5 Hz, J_2=4.9 Hz, 1H, H-2); 4.24 (bs, 2H, H-4); 3.71-3.71 (m, 2H, H-1).

^{13}C-NMR (75 MHz, $CDCl_3$): δ 144.6 (C-6); 130.5 (C-3 & C-2); 129.0 (C-8); 128.7 (C-7); 127.4 (C-9); 87.3 (C-5); 64.5 (C-4); 63.8 (C-1).

LRMS (CI+): m/z 331.2 [$(M+H)^+$, 100%].

(92b) (*Z*)-4-trityloxy-but-2-en-1-ol



The title compound was prepared following the same procedure described for the previous compound **92a**. Trityl chloride (557 mg; 2 mmol), TEA (0.306 ml; 2.2 mmol), DMAP (10 mg; 0.08 mmol) and *cis*-2-buten-1,4-diol **90** (1.76 g; 20 mmol) in DCM (10 ml).

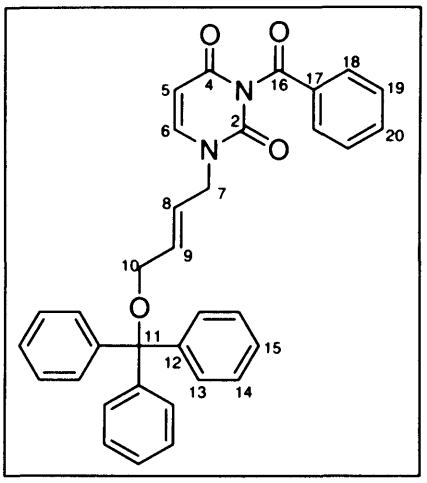
Colourless oil; 563 mg; 81%.

R_f: 0.81 in Hexane/EtOAc 50:50.

^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.53-7.50 (m, 6H, H-8); 7.39-7.27 (m, 9H, H-7+H-9); 5.89-5.74 (m, 2H, H-3+H-2); 4.08 (bs, 2H, H-1); 3.76 (d, J=5.32 Hz, 2H, H-4).

^{13}C-NMR (300 MHz, $CDCl_3$): δ 144.4 (C-9); 131.5 (C-3); 129.3 (C-2); 129.0 (C-8); 128.3 (C-7); 127.5 (C-9); 86.7 (C-5); 60.6 (C-11); 59.3 (C-1).

(93a) 3-benzoyl-1-[(*E*)-4-(trityloxy)-2-butenyl] uracil[99]



The compound was prepared according to the general procedure **C** for the Mitsunobu coupling. Polymer bound triphenylphosphine (0.75 g; 2.25 mmol); 3-*N*-benzoyluracil **35** (290 mg; 1.35 mmol); DIAD (0.354 ml; 1.80 mmol); trityl alcohol **92a** (300 mg; 0.90 mmol) and THF (5 ml).

White foam; 222 mg; 48%.

R_f: 0.55 in Hexane/EtOAc 50:50 (UV/PMA)

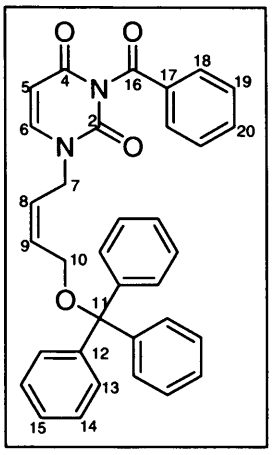
Mp: 77-80 °C.

^{1}H-NMR (300 MHz, CD_3OD): δ 8.00-7.91 (m, 2H, H-18); 7.74-7.66 (m, 2H, H-6+H-20); 7.56-7.43 (m, 8H, H-13+H-19); 7.34-7.25 (m, 9H, H-14+H-15); 5.94-5.79 (m, 3H, H-5+H-8+H-9); 4.40 (d, *J*=5.35 Hz, 2H, H-10); 3.71 (bs, 2H, H-7).

^{13}C-NMR (75 MHz, CD_3OD): δ 174.5 (C-16); 165.1 (C-4); 151.8 (C-2); 147.5 (C-6); 145.9 (C-12); 136.7 (C-17); 134.0 (C-8); 131.8 (C-20); 130.8 (C-19); 130.1 (C-13 & C-18); 129.2 (C-14); 128.6 (C-15); 125.6 (C-9), 102.8 (C-5); 89.2 (C-11); 65.3 (C-10); 51.1 (C-7).

LRMS (CI+): m/z 546.3 [$(M+NH_4)^+$, 100%]; m/z 529.3 [$(M+H)^+$, 45%].

(93b) 3-benzoyl-1-[(*Z*)-4-(trityloxy)-2-butenyl] uracil



The compound was prepared according to the general procedure **C** for the Mitsunobu coupling, frrom 3-*N*-benzoyluracil **35** (290 mg; 1.35 mmol); trityl alcohol **92b** (300 mg; 0.90 mmol) and DIAD (0.354 ml; 1.80 mmol) in THF (2 ml).

White solid; 136 mg; 29%.

Mp: 75-78 °C.

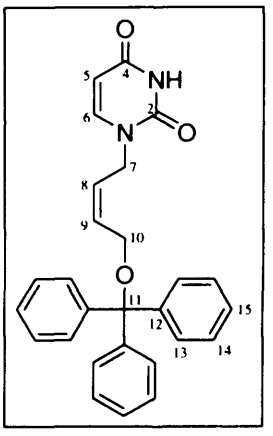
$\mathbf{R_f}$: 0.57 in EtOAc/Hexane 50:50.

^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.97 (d, *J*=7.15 Hz, H-18); 7.70 (t, *J*=7.5 Hz, 1H, H-20); 7.51-7.42 (m, 8H, H-19+H-12); 7.41-7.27 (m, 10H, H-arom+H-6); 6.09 (td, J_1=11.0 Hz, J_2=6.5 Hz, 1H, H-9); 5.75 (d, *J*=8.00 Hz, 1H, H-5); 5.68 (td, J_1=10.9 Hz, J_2=5.7 Hz, 1H, H-8); 4.32 (d, *J*=7.34 Hz, 2H, H-10); 3.81 (d, *J*=6.4 Hz, 2H, H-7).

^{13}C-NMR (300 MHz, $CDCl_3$): δ 162.9 (C-16); 164.0 (C-4); 151.2 (C-2); 144.0 (C-12); 143.9 (C-6); 135.5 (C-20); 132.9 (C-8); 131.9 (C-17); 130.9 (C-18); 129.6 (C-19); 129.0 (C-13); 128.5 (C-14); 127.7 (C-15); 125.9 (C-9); 102.7 (C-5); 87.9 (C-11); 59.8 (C-10); 45.2 (C-7).

LRMS (CI+): m/z 529.3 [$(M+H)^+$, 100%].

(69) 1-[(Z)-4-(trityloxy)-2-butenyl] uracil



The benzoylated intermediate **93b** (50 mg; 0.095 mmol) was deprotected following the general procedure **D** for the cleavage of bezoyl group with sodium methoxide.

White solid; 38 mg; 90%.

R_f: 0.66 in DCM/MeOH 90:10.

Mp: 65-68 °C.

^{1}H-NMR (300 MHz, $CDCl_3$): δ 8.21 (bs, 1H, NH); 7.52-7.49 (m, 6H, H-14); 7.41-7.29 (m, 9H, H-13+H-15); 7.16 (d, *J*=7.95 Hz, 1H, H-6); 6.09-6.01 (m, 1H, H-8); 5.69-5.98 (m, 2H, H-5+H-9); 4.28 (d, *J*=7.40 Hz, 2H, H-10); 3.79 (d, *J*=6.20 Hz, 2H, H-7).

^{13}C-NMR (75 MHz, $CDCl_3$): δ 164.0 (C-4); 151.2 (C-2); 144.2 (C-6); 144.1 (C-12); 132.5 (C-8); 129.0 (C-13); 128.4 (C-14); 127.7 (C-15); 126.1 (C-9); 102.1 (C-5); 87.8 (C-11); 59.9 (C-10); 44.9 (C-7).

LRMS (ES+): m/z 447.2 [$(M+Na)^+$, 100%].

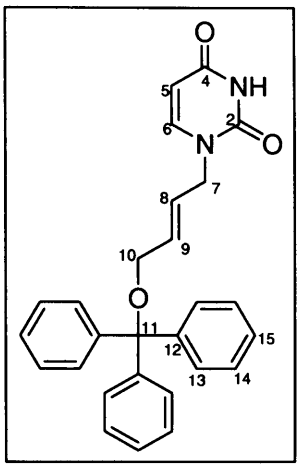
HRMS (ES+): found 447.1680; required 447.1679 $[M+Na]^+$.

Microanalysis calculated for $C_{27}H_{24}N_2O_3$ + 0.4 H_2O

C 75.12, H 5.79, N 6.49%; found C 75.31, H 6.12, N 6.10%.

Ref. [99]

(72) 1-[(*E*)-4-(trityloxy)-2-butenyl] uracil



Intermediate **93a** (50 mg; 0.09 mmol) was deprotected according to the general procedure **D** for the hydrolisys of benzoyl groups.

White solid; 38 mg; 90%.

R_f: 0.51 in $CHCl_3$/MeOH 90:10.

Mp: 70-72 °C.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 9.04 (bs, 1H, NH); 7.49 (d, *J*=7.81 Hz, 6H, H-13); 7.39-7.27 (m, 9H, H-14+H-15); 7.19 (d, *J*=7.90 Hz, 1H, H-6); 5.98-5.83 (m, 2H, H-8+H-9); 5.77 (d, *J*=7.90 Hz, 1H, H-5); 4.42 (d, *J*=5.63 Hz, 2H, H-10); 3.74 (d, *J*=3.92 Hz, 2H, H-7).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 163.9 (C-4); 151.1 (C-2); 144.3 (C-12); 144.1 (C-6); 133.3 (C-8); 129.0 (C-13); 128.3 (C-14); 124.1 (C-15); 124.1 (C-9); 102.9 (C-5); 87.5 (C-11); 64.0 (C-10); 49.6 (C-7).

LRMS (ES+): m/z 447.2 [$(M+Na)^+$, 100%].

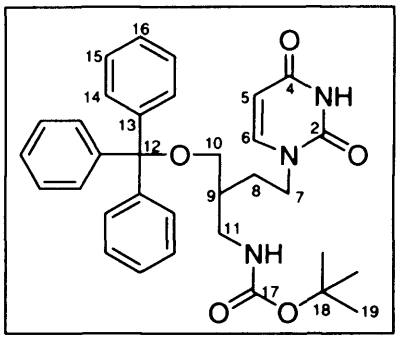
HRMS (ES+): found 442.2128; required 442.2125 [$M+NH_4]^+$.

Microanalysis calculated for $C_{27}H_{24}N_2O_3$+0.2 H_2O:

C 75.75, H 5.74, N 6.54%; found C 75.42, H 5.99, N 6.22%

Ref.[99]

(97) 1-[3-((*tert*-butoxycarbonyl)aminomethyl)-4-(trityloxy)butyl] uracil



A glass tube for microwave was charged with starting material **94** (50 mg; 0.155 mmol), trityl chloride (54 mg; 0.19 mmol) and dry pyridine (2 ml). The tube was sealed with a septum cap. The vial was irradiated with microwaves to reach the temperature of 160 °C for 5 min. The irradiating cycle was repeated 3 times, and then the mixture was poured into a round bottom flask. The solvent was removed under reduced pressure and the crude was purified by chromatography using Hexane/EtOAc 40:60 as eluent.

White foam, 70 mg, 79%

R_f: 0.27 in Hexane/EtOAc 40:60.

Mp: 86-88 °C.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 8.08 (bs, 1H, NH): 7.48 (d, J=7.04 Hz, 6H, H-14); 7.40-7.27 (m, 10H, H-6+H-15+H-16); 5.67 (dd, J_1=7.90 Hz; J_2=2.35 Hz, 1H, H-5); 4.58 (t, J=6.13 Hz, 1H, NHBoc); 3.84-3.76 (m, 2H, H-10); 3.31-3.23 (m, 3H, H-7 + 1 of H-11); 3.10-3.05 (m, 1H, 1 of H-11); 1.85-1.67 (m, 2H, H-8); 1.62-1.53 (m, 1H, H-9); 1.47 (s, 9H, H-19).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 163.8 (C-4); 156.5 (C-17); 150.8 (C-2); 145.0 (C-6); 143.7 (C-13); 128.6 (C-14); 127.8 (C-15); 126.9 (C-16); 101.9 (C-5); 86.8 (C-12); 79.2 (C-18); 64.3 (C-10); 46.8 (C-7); 40.8 (C-11); 36.9 (C-9); 29.7 (C-8); 28.4 (C-19).

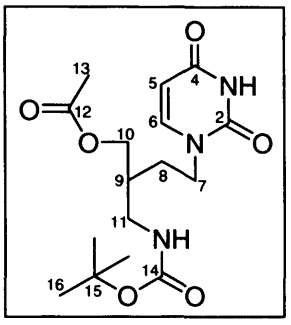
LRMS (CI+): m/z 243.2 [$(Ph_3C)^+$, 100%].

HRMS (ES+): found 498.2384; required 498.2387 $[M+H]^+$.

Microanalysis calculated for $C_{33}H_{37}N_3O_5$+0.2 H_2O

C 70.87, H 6.74, N 7.51%; found C 70.86, H 7.13, N 6.98%.

(99) 1-[3-((*tert*-butoxycarbonyl)aminomethyl)-4-(acetyloxy)-butyl] uracil



Acetic anhydride (0.016 ml, 0.175 mmol) was added with a syringe into a solution of starting material **94** (50 mg, 0.16 mmol) in dry pyridine (2 ml). The reaction was stirred at room temperature until the complete disappearance of the starting material was observed by TLC (Chloroform/MeOH). After 3 h circa, MeOH (2 ml) was added; the solvents were removed under reduced pressure and the crude residue was purified by chromatography using $CHCl_3$/MeOH as eluent.

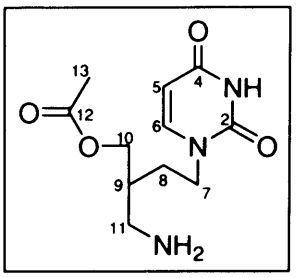
White solid, 50 mg, 88%.

$\mathbf{R_f}$: 0.40 in $CHCl_3$/MeOH 95:5.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 9.73 (bs, 1H, NH); 7.43 (d, *J*=7.81 Hz, 1H, H-6); 5.71 (d, *J*=7.81 Hz, 1H, H-5); 5.12 (t, *J*=5.92 Hz, 1H, N*H*Boc); 4.17-4.00 (m, 2H, H-10); 3.99-3.84 (m, 2H, H-7); 3.34-3.11 (m, 2H, H-11); 2.11 (s, 3H, H-13); 1.90 (bs, 1H, H-9); 1.81-1.57 (m, 2H, H-8); 1.47 (s, 9H, H-16).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 171.7 (C-12); 164.5 (C-4); 156.7 (C-14); 151.4 (C-2); 145.3 (C-6); 102.6 (C-5); 80.0 (C-15); 64.9 (C-10); 46.9 (C-7); 40.1 (C-11); 36.7 (C-8); 28.7 (C-16); 28.2 (C-9); 21.3 (C-13).

(100) 1-[3-(aminomethyl)-4-(acetyloxy)butyl] uracil

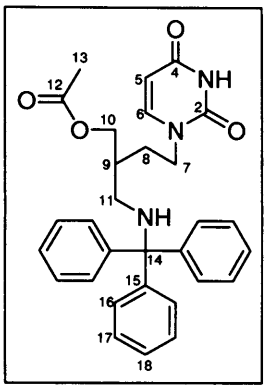


TFA (0.2 ml) was added to a solution of boc-protected intermediate **99** (50 mg, 0.14 mmol) in DCM (2 ml). The reaction was stirred at room temperature for 3 h, and then the solution was concentrated under reduced pressure affording a crude residue which was used for the next step without further purification.

Yellow oil, 30 mg.

^{1}H-NMR (300 MHz; CD_3OD): δ 7.65 (d, *J*=7.83 Hz, 1H, H-6); 5.70 (d, *J*=7.83 Hz, 1H, H-5); 3.89-3.75 (m, 2H, H-10); 3.55-3.54 (m, 1H, 1 of H-7); 3.38-3.26 (m, 3H, 1 of H-7+H-11); 2.09 (s, 3H, H-13); 1.84-1.61 (m, 2H, H-8); 1.32-1.25 (m, 1H, H-9).

(101) 1-[3-(tritylaminomethyl)-4-(acetyloxy)-butyl] uracil



Trityl chloride (128 mg; 0.46 mmol) was added to a solution of compound **100** (100 mg; 0.43 mmol) in dry DCM (5 ml) in the presence of TEA (0.64 ml; 0.46 mmol). The reaction was stirred at room temperature. After six h the TLC (DCM/MeOH 90:10) showed still presence of starting material, then more TrCl (0.5 eq.) and TEA (0.5 eq.) were added. The reaction was left stirring overnight. Water (5 ml) was added and the mixture was stirred other 5 min. The phases were separated and the organic layer was washed with HCl 0.1M (5 ml). The organic solution was dried over $MgSO_4$, concentrated and purified by flash chromatography using DCM → DCM/MeOH 95:5 as gradient. White foam, 150 mg; 70%.

R_f: 0. 60 in $CHCl_3$/MeOH 90:10

Mp: 68-72 °C.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 9.07 (bs, 1H, NH); 7.46 (d, *J*=7.34 Hz, 6H, H-16); 7.30-7.19 (m, 9H, H-17+H-18); 7.02 (d, *J*=7.88 Hz, 1H, H-6); 5.66 (d, *J*=7.88 Hz, 1H, H-5); 4.27 (d, *J*=5.00 Hz, 2H, H-10); 3.68 (m, 2H, H-7); 2.17 (d, *J*=6.02 Hz, 2H, H-11); 2.03 (s, 3H, H-12); 1.83-1.65 (m, 3H, H-9+H-8).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 171.5 (C-12); 1640 (C-4); 151.1 (C-2); 146.2 (C-15); 144.7 (C-6); 129.0 (C-16); 128.3 (C-17); 126.8 (C-18); 102.7 (C-5); 71.3 (C-14); 65.3 (C-10); 47.4 (C-7); 44.6 (C-11); 37.0 (C-9); 29.7 (C-8); 21.3 (C-13).

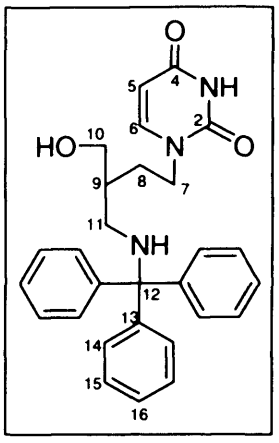
LRMS (CI+): m/z 498.3 [$(M+H)^+$, 100%].

HRMS (ES+): found 498.2384; required 498.2387 $[M+H]^+$.

Microanalysis calculated for $C_{30}H_{31}N_3O_4$+1.5 H_2O

C 68.68, H 6.53, N 8.01%; found C 68.66, H 6.35, N 7.68%.

(102) 1-[3-(tritylaminomethyl)-4-(hydroxy)-butyl] uracil



The title compound was prepared following the general procedure **D** for the ester hydrolisys with sodium methoxide, from compound **101** (110 mg; 0.22 mmol) and 5 ml of MeONa 0.2M in MeOH.

White foam; 100 mg; 99%.

R_f: 0. 66 in $CHCl_3$/MeOH 90:10

Mp: 75-80 °C.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 8.16 (bs, 1H, NH): 7.48-7.41 (m, 6H, H-14); 7.35-7.23 (m, 9H, H-15+H-16); 7.03 (d, *J*=7.91 Hz, 1H, H-6); 5.65 (d, *J*=7.90 Hz, 1H, H-5); 5.26 (bs, 2H, OH+NH); 3.95-3.90 (m, 1H, 1 of H-10); 3.82-3.57 (m, 3H, H-7+ 1 of H-10); 2.48-2.40 (m, 2H, H-11); 1.84 (bs, 1H, H-9); 1.68-1.47 (m, 2H, H-8).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 163.7 (C-4); 150.9 (C-2); 145.1 (C-13); 144.7 (C-6); 128.9 (C-14); 128.5 (C-15); 127.2 (C-16); 102.7 (C-5); 97.8 (C-12); 67.0 (C-10); 48.1 (C-11); 47.5 (C-7); 38.3 (C-9); 29.4 (C-8).

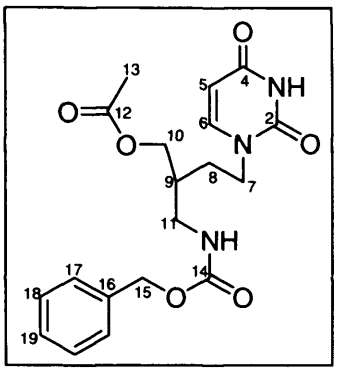
LRMS (CI+): m/z 456.3 [$(M+H)^+$, 100%].

HRMS (ES+): found 456.2283; required 456.2282 [$M+H]^+$.

Microanalysis calculated for $C_{28}H_{29}N_3O_3$+1.0 H_2O+HCl

C 67.12, H 6.24, N 8.39%; found C 67.15, H 5.88, N 8.16%.

(103) 1-[3-((carbobenzyloxyl)aminomethyl)-4-(acetyloxy)butyl] uracil



Benzylchloroformate (0.25 ml, 1.75 mmol) was added in portion to a solution of the intermediate **100** (500 mg, 1.25 mmol) and TEA (0.26 ml, 1.87 mmol) in DCM (8 ml) at 0 °C. After the addition, the reaction was warmed to room temperature and stirred for 3 h. MeOH (10 ml) was added into the flask and the mixture was stirred for few min. The solvents were removed *in vacuo* and the crude residue (yellow oil) was dissolved in DCM, adsorbed on silica and purified by chromatography.

Light yellow oil, 259 mg, 53%.

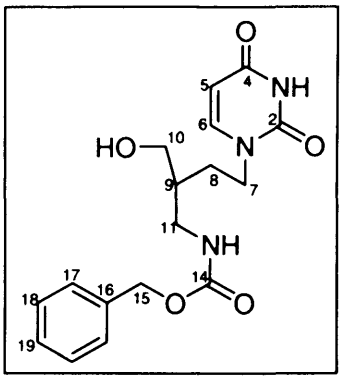
R_f: 0.48 in DCM/MeOH 90:10.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 9.34 (bs, 1H, NH); 7.31-7.27 (m, 5H, H-arom.); 7.18 (d, *J*=7.85 Hz, 1H, H-6); 5.60 (d, *J*=7.85 Hz, 1H, H-5); 5.34 (t, *J*=6.17 Hz, 1H, NH); 5.06 (s, 2H, H-15); 4.09-3.95 (m, 2H, H-10); 3.87-3.72 (m, 2H, H-7); 3.32-3.11 (m, 2H, H-11); 2.03 (s, 3H, H-13); 1.87 (bs, 1H, H-9); 1.71-1.50 (m, 2H, H-8).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 176.3 (C-12); 163.9 (C-4); 157.5 (C-14); 151.2 (C-2); 145.0 (C-6); 136.9 (C-16); 129.0, 128.7, 128.1 (C-17, C-18, C-19); 102.7 (C-5); 67.3 (C-15); 64.8 (C-10); 47.0 (C-7); 40.8 (C-11); 36.7 (C-9); 28.2 (C-8); 21.3 (C-13).

LRMS (EI+): m/z 389.3 [$(M)^+$, 100%].

(104) 1-[3-((carbobenzyloxyl)aminomethyl)-4-(hydroxyl)butyl] uracil



Compound **103** (200 mg, 0.51 mmol) was deprotected following the general procedure **D** for the hydrolysis with a solution of MeONa 0.2 M in MeOH (5 ml).

White foam, 167 mg, 94%.

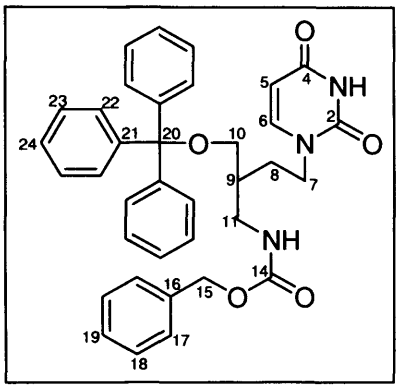
R_f: 0.46 in DCM/MEOH 90:10.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 9.32 (bs, 1H, NH); 7.44-7.37 (m, 5H, H-arom.); 7.24 (d, *J*=7.87 Hz, 1H, H-6); 5.73 (d, *J*=7.87 Hz, 1H, H-5); 5.52 (t, *J*=6.30 Hz, 1H, N*H*Cbz); 5.16 (s, 2H, H-15); 3.93-3.75 (m, 2H, H-7); 3.71-3.66 (m, 1H, C*H*H-10); 3.58-3.52 (m, 1H, CH*H*-10); 3.40-3.28 (m, 2H, H-11); 1.79-1.61 (m, 3H, H-8+H-9).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 164.0 (C-4); 158.2 (C-12); 151.6 (C-2); 144.9 (C-6); 136.8 (C-16); 129.0 (C-17); 128.7 (C-18); 128.5 (C-19); 103.0 (C-5); 67.5 (C-13); 62.8 (C-10); 47.1 (C-7); 41.3 (C-11); 39.0 (C-9); 28.0 (C-8).

LRMS (EI+): m/z 347.3 [$(M)^+$ 100%].

(105) 1-[3-((carbobenzyloxyl)aminomethyl)-4-(trityloxy)butyl] uracil



The title compound was prepared according to the general procedure **G** for the trytilation of alcohols with the Radleys parallel synthesiser, from the Z-amino alcohol **104** (160 mg, 0.46 mmol); trityl chloride (180 mg, 0.64 mmol), and pyridine (5 ml).

White foam, 188 mg, 69%.

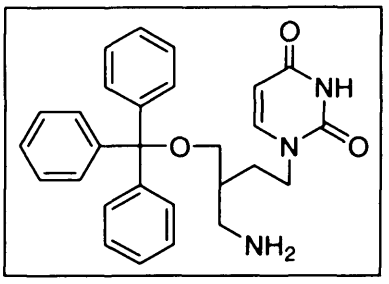
R_f: 0.52 in $CHCl_3$/MeOH 95:5.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 8.44 (bs, 1H, NH); 7.41-7.21 (m, 20H, H-arom.); 7.08 (d, *J*=7.89 Hz, 1H, H-6); 5.56 (d, *J*=7.89 Hz, 1H, H-5); 5,03 (s, 2H, H-15); 4.76 (t, J=5.97 Hz, 1H, N*H*Cbz); 3.79-3.58 (m, 2H, H-10); 3.28 (t, *J*=5.49 Hz, 2H, H-7); 3.21-3.17 (m, 1H, C*H*H-11); 3.04-2.98 (m, 1H, CH*H*-11); 1.81-1.43 (m, 3H, H-9+H-8).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 162.1 (C-4); 160.5 (C-14); 156.5 (C-2); 145.1 (C-6); 144.1 (C-21); 137.0 (C-16); 128.9, 128.4, 128.2, 127.7 (C-arom.); 102.5 (C-5); 87.3 (C-20); 67.1 (C-10); 64.5 (C-15); 47.2 (C-7); 41.8 (C-11); 37.5 (C-8); 28.6 (C-9).

LRMS (ES+): m/z 607.29 [$(M+NH_4)^+$, 100%].

(98) Attempted synthesis of 1-[3-(aminomethyl)-4-(trityloxy)butyl] uracil



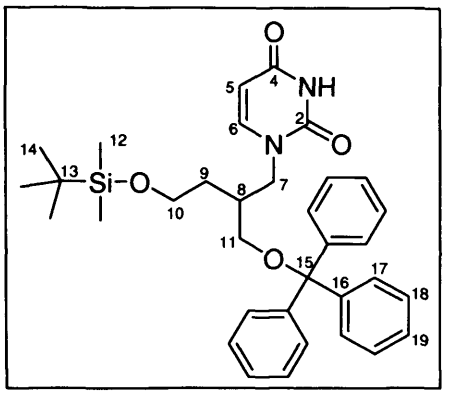
Method A

The Boc-protected intermediate **97** (40 mg, 0.07 mmol) was dissolved in DCM (2 ml). After stirring the colourless solution at room temperature for few min, TFA (0.1 ml) was added with a syringe. The solution changed from colourless to bright yellow and in 30 min circa the complete disappearance of the starting material was observed by TLC ($CHCl_3$/MeOH 90:10). The solution was diluted with more DCM (2 ml). The solvent was evaporated under reduced pressure affording a crude (yellow oil) which after NMR analyis showed the loss of the trityl group.

Method B

The trityl derivative **105** (188 mg, 0.32 mmol) was dissolved in MeOH (5 ml). 5% Pd on charcoal (35 mg) was added to the solution. The air inside the flask was replaced several times with nitrogen, and then the nitrogen was replaced with a balloon of hydrogen. The black suspension was stirred 8 h at room temperature, and TLC showed still presence of starting material. The catalyst was filtered off and replaced with some fresh (50 mg). After 24 h at room temperature the complete disappearance of the starting material was observed on TLC. The suspension was filtered through a Celite pad. The filtrated was concentrated under reduced pressure and the residue was attempted to chromatographic purification. Unfortunately NMR analysis of the products showed it was a mixture of two isomers. All the attempts to separate the two isomers by chromatography were unsuccessful.

(111) 1-(2-(trityloxymethyl)-4-(*tert*-butyldimethylsilyloxy)-butyl) uracil



The title compound was prepared following the general procedure **G**, from trityl chloride (120 mg, 0.42 mmol), starting alcohol **95** (100 mg, 0.30 mmol) in dry pyridine (5 ml). The desired compound was obtained after chromatographic purification using Hexane/EtOAc 50:50 as eluent.

White solid, 130 mg, 76%.

R_f: 0.63 in Hexane/EtOAc 40:60.

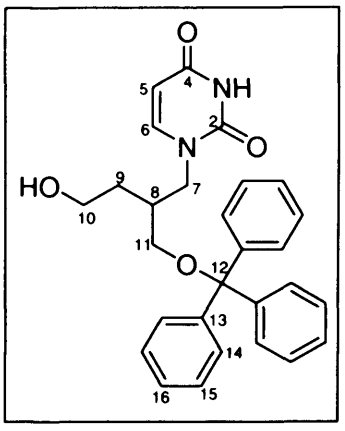
Mp: 79-80 °C.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 8.24 (bs, 1H, NH); 7.39-7.37 (m, 6H, H-17); 7.32-7.21 (m, 9H, H-18+H-19); 6.83 (d, J=7.89 Hz, 1H, H-6); 5.29 (dd, J_1=7.89 Hz, J_2=2.34 Hz, 1H, H-5); 3.92-3.87 (m, 1H, C*H*H-10); 3.84-3.70 (m, 1H, CH*H*-10); 3.70-3.54 (m, 2H, H-7); 3.17-3.12 (m, 1H, 1 of H-11); 3.06-3.00 (m, 1H, 1 of H-11); 1.66-1.65 (m, 2H, H-9); 1.27-1.20 (m, 1H, H-8); 0.85 (s, 9H, H-14); 0.00 (s, 6H, H-12).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 163.6 (C-4); 151.1 (C-2); 145.7 (C-6); 143.9 (C-16); 129.0 (C-17); 128.4 (C-18); 127.7 (C-19); 101.7 (C-5); 87.1 (C-15); 62.7 (C-11); 61.0 (C-10); 50.2 (C-7); 36.8 (C-8); 32,8 (C-9); 29.3 (C-14); 18.7 (C-13); -4.9 (C-12).

LRMS (ES+): m/z 593.3 [$(M+Na)^+$, 100%].

(106) 1-(2-(trityloxymethyl)-4-(hydroxyl)-butyl) uracil



The TBDMS protected intermediate **111** (130 mg, 0.23 mmol) was dissolved in THF (3 ml). TBAF (71 mg, 0.27 mmol) was added into the solution. The reaction was stirred at room temperature until the complete disappearance of the starting material was observed by TLC. The solvent were removed under reduced pressure and the crude residue was purified by flash chromatography.

White solid, 49 mg, 47%.

Mp: 85-88 °C.

^{1}H-NMR (500 MHz; $CDCl_3$): δ 8.23 (bs, 1H, NH); 7.43 (d, *J*=7.64 Hz, 6H, H-14); 7.34 (t, *J*=7.46 Hz, 3H, H-16); 7.30-7.27 (m, 6H, H-15); 6.69 (d, *J*=7.88 Hz, 1H, H-6); 5.32 (d, *J*=7.88 Hz, 1H, H-5); 3.99 (dd, J_1=13.82 Hz, J_2=4.10 Hz; CH*H*-7); 3.84-3.79 (m, 1H, CH*H*-11); 3.76-3.71 (m, 2H, C*H*H-7+C*H*H-7); 3.19-3.14 (m, 2H, H-10); 2.22-2.20 (m, 1H, H-8); 2.14 (bs, 1H, OH); 1.77-1.69 (m, 2H, H-9).

^{13}C-NMR (125 MHz; $CDCl_3$): δ 163.7 (C-4); 151.1 (C-2); 145.3 (C-6); 143.4 (C-13); 128.6 (C-14); 128.0 (C-15); 127.3 (C-16); 101.5 (C-5); 86.7 (C-12); 62.5 (C-10); 60.2 (C-11); 49.4 (C-7); 36.1 (C-8); 32.3 (C-9).

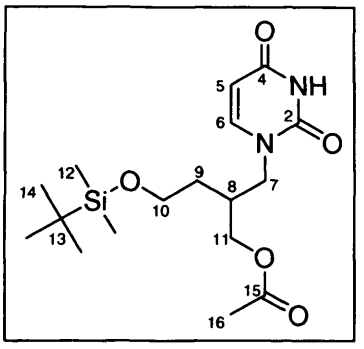
LRMS (ES+): m/z 479.3 [$(M+Na)^+$, 100%].

HRMS (ES+): found 479.1935; required 479.1941 [$M+Na]^+$.

Microanalysis calculated for $C_{28}H_{28}N_2O_4$+1.4 H_2O

C 69.81, H 6.44, N 5.81%; found C 69.82, H 6.09, N 5.49%.

(112) 1-(2-(acetyloxymethyl)-4-(*tert*-butyldimethylsilyloxy)-butyl) uracil



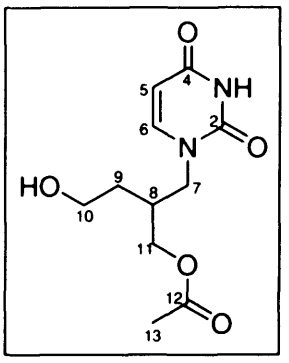
Acetic anhydride (47 μl, 0.55 mmol) was added to a solution of starting alcohol **95** (150 mg, 0.46 mmol) in dry pyridine (4 ml) under nitrogen. The mixture was stirred at room temperature for 3 h. The reaction was diluted with EtOAc (15 ml). The organic solution was washed with HCl 0.5 M (3×10 ml). The phases were separated; the organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The crude residue (colourless oil) was used for the next step without further purification.

Colourless oil, 115 mg, 67%.

R_f: 0.67 in $CHCl_3$/MeOH 90:10.

^{1}H-NMR (500 MHz; $CDCl_3$): δ 8.68 (bs, 1H, NH); 7.13 (d, J=7.92 Hz, 1H, H-6); 5.64 (dd, J_1=7.94 Hz, J_2=2.26 Hz, 1H, H-5); 4.07-3.98 (m, 2H, H-10); 3.77-3.61 (m, 4H, H-7+H-11); 2.33-2.27 (m, 1H, H-8); 2.00 (s, 3H, H-16); 1.58-1.50 (m, 2H, H-9); 0.83 (s, 9H, H-14); 0.00 (s, 6, H-12).

(113a) 1-(2-(acetyloxymethyl)-4-(hydroxy)-butyl) uracil



The title compound was obtained following the same procedure described for the synthesis of compound **106**; starting from the intermediate **112** (150 mg, 0.40 mmol), with TBAF (127 mg, 0.48 mmol) in THF (3 ml).

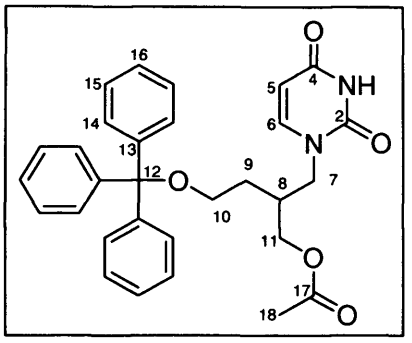
Colourless oil, 83 mg, 78%.

$\mathbf{R_f}$: 0.38 in $CHCl_3$/MeOH 90:10.

^{1}H-NMR (500 MHz; $CDCl_3$): δ 9.70 (bs, 1H, NH); 7.08 (d, J=7.88 Hz, 1H, H-6); 5.54 (d, J=7.88 Hz, 1H, H-5); 3.97 (dd, J_1=11.6 Hz, J_2=4.0 Hz,1H, CH*H*-11); 3.88 (dd, J_1=11.6 Hz, J_2=5.9 Hz, 1H, C*H*H-11); 3.73 (dd, J_1=14.0 Hz, J_2=5.4 Hz, 1H, CH*H*-7); 3.65-3.60 (m, 1H, H-10); 3.58-3.54 (m, 1H, H-10); 3.49 (dd, J_1=14.0 Hz, J_2=8.2 Hz, 1H, C*H*H-7); 2.54-2.18 (m, 1H, H-8); 1.89 (s, 3H, H-13); 1.49-1.38 (m, 2H, H-9).

^{13}C-NMR (125 MHz; $CDCl_3$): δ 171.0 (C-12); 164.0 (C-4); 151.5 (C-2); 145.2 (C-6); 102.2 (C-5); 64.6 (C-10); 59.6 (C-11); 50.5 (C-7); 34.7 (C-8); 31.4 (C-9); 20.9 (C-13).

(114) 1-(2-(acetyloxymethyl)-4-(trityloxy)-butyl) uracil



The title compound was prepared according to the general procedure **G** for the tritylation of alcohols. Starting from compound **113a** (83 mg, 0.32 mmol), trityl chloride (125 mg, 0.45 mmol) and pyridine (5 ml).

White foam, 140 mg, 90%.

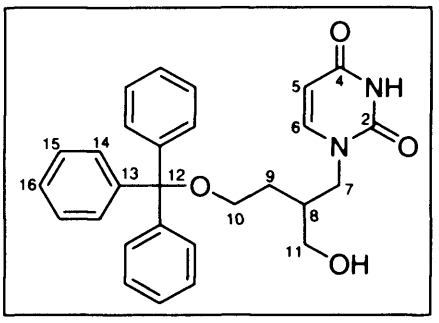
R_f: 0.68 in $CHCl_3$/MeOH 90:10.

^{1}H-NMR (500 MHz; $CDCl_3$): δ 8.23 (bs, 1H, NH); 7.44 (d, J=7.26 Hz, 6H, H-14); 7.36-7.25 (m, 9H, H-16+H-15); 7.06 (d, J=7.92 Hz, 1H, H-6); 5.66 (d, J=7.85 Hz, 1H, H-5); 4.03 (dd, J_1=11.59 Hz, J_2=4.13 Hz, 1H, C*H*H-11); 3.95 (dd, J_1=11.59 Hz, J_2=5.88 Hz, 1H, CH*H*-11); 3.73 (dd, J_1=14.02 Hz, J_2=8.03 Hz, 1H, C*H*H-7); 3.68 (dd, J_1=14.01 Hz, J_2=6.43 Hz, 1H, CH*H*-7); 3.26 (m, 2H, H-10); 2.38-2.33 (m, 1H, H-8); 2.04 (s, 3H, H-18); 1.73-1.63 (m, 2H, H-9).

^{13}C-NMR (125 MHz; $CDCl_3$): δ 171.0 (C-17); 164.0 (C-4); 151.5 (C-2); 145.2 (C-13); 144.5 (C-6); 128.6 (C-14); 127.9 (C-15); 127.1 (C-16); 102.0 (C-5); 64.4 (C-10); 60.7 (C-11); 50.3 (C-7); 35.1 (C-8); 29.3 (C-9); 20.8 (C-18).

LRMS (ES+): m/z 521.4 [$(M+Na)^+$, 100%].

(107) 1-(2-(hydroxymethyl)-4-(trityloxy)-butyl) uracil



The intermediate **114** (140 mg, 0.28 mmol) was de-acetylated according to the general procedure **D** for the cleavage of acetyl esters with MeONa 0.2 Mi in MeOH (5 ml).

White solid, 73 mg, 57%.

R_f: 0.51 in DCM/MeOH 90:10.

Mp: 83-85 °C.

^{1}H-NMR (500 MHz; $CDCl_3$): δ 9.67 (bs, 1H, NH); 7.45 (d, *J*=7.35 Hz, 6H, H-14): 7.33 (d, *J*=7.55 Hz, 6H, H-15); 7.28-7.25 (m, 3H, H-16); 7.12 (d, *J*=7.89 Hz, 1H, H-6); 5.69 (d, *J*=7.87 Hz, 1H, H-5); 3.78 (dd, J_1=14.03 Hz, J_2=8.97 Hz, 1H, C*H*H-7); 3.63 (dd J_1=14.02 Hz, J_2=4.88 Hz, 1H, CH*H*-7); 3.49 (dd, J_1=12.02 Hz, J_2=3.44 Hz, 1H, C*H*H-11); 3.44 (dd, J_1=12.03 Hz, J_2= 4.43 Hz, 1H, CH*H*-11); 3.28-3.21 (m, 2H, H-10); 2.10-2.03 (m, 1H, H-8); 1.77-1.64 (m, 2H, H-9).

^{13}C-NMR (125 MHz; $CDCl_3$): δ 163.8 (C-4); 151.9 (C-2); 145.6 (C-6); 143.9 (C-13); 128.7 (C-14); 127.9 (C-15); 127.3 (C-16); 102.4 (C-5); 87.0 (C-12); 61.1 (C-10); 61.0 (C-11); 49.0 (C-7); 38.3 (C-8); 29.1 (C-9).

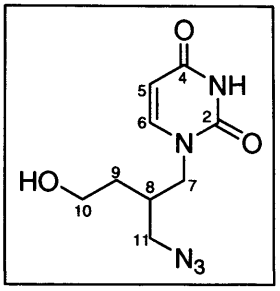
LRMS (ES+): m/z 479.2 [(M+Na)$^+$, 100%].

HRMS (ES+): found 479.1946; required 479.1941 for $C_{28}H_{28}N_2NaO_4^+$; [M+Na]$^+$.

Microanalysis calculated for $C_{28}H_{28}N_2O_4$+1.2 H_2O:

C 70.33, H 6.41, N 5.80%; found C 70.31, H 6.16, N 5.67%.

(115) 1-(2-(azidomethyl)-4-(hydroxy)-butyl) uracil



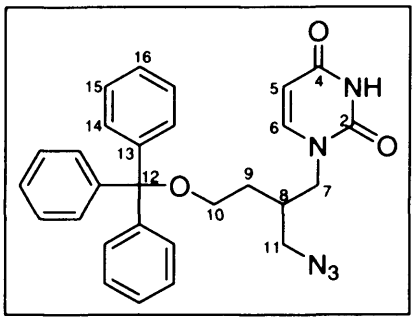
The starting TBDMS-protected alcohol **96** (150 mg, 0.42 mmol) was reacted with TBAF (130 mg, 0.50) in THF (3 ml) according to the procedure previously described for the synthesis of compound **106**.

Colourless oil, 59 mg, 58%.

$\mathbf{R_f}$: 0.40 in $CHCl_3$/MeOH.

^{1}H-NMR (250 MH; $CDCl_3$): δ 9.18 (bs, 1H, NH), 7.18 (d, *J*=7.91 Hz, 1H, H-6); 5.66 (d, *J*=7.88 Hz, 1H, H-5); 3.87-3.32 (m, 6H, H-7+H-10+H-11); 2.32-2.19 (m, 1H, H-8); 1.68-1.52 (m, 2H, H-9).

(108) 1-(2-(azidomethyl)-4-(trityloxy)-butyl) uracil



The azido alcohol **115** (100 mg; 0.42 mmol) was tritylated following the standard procedure **G** with TrCl (163 mg; 0.58 mmol) in dry pyridine (5ml). White solid, 163 mg; 82%.

R_f: 0. 57 in $CHCl_3$/MeOH 90:10.

Mp: 75-80 °C.

^{1}H-NMR (500 MHz; $CDCl_3$): δ 8.52 (bs, 1H, NH): 7.25 (d, *J*=7.34 Hz, 6H, H-14); 7.14 (t, *J*=7.56 Hz, 6H, H-15); 7.09-7.06 (m, 3H, H-16); 6.90 (d, *J*=7.90 Hz, 1H, H-6); 5.49 (dd, J_1=7.90 Hz, J_2=2.00 Hz, 1H, H-5); 3.50 (dd, J_1=6.12 Hz, J_2=13.95 Hz, 1H, CH*H*-11); 3.46-3.42 (m, 1H, C*H*H-11); 3.14-2.96 (m, 4H, H-7+ H-10); 2.11-2.03 (m, 1H, H-8); 1.51 -1.42 (m, 2H, H-9).

^{13}C-NMR (125 MHz; $CDCl_3$): δ 163.3 (C-4); 150.8 (C-2); 144.8 (C-6); 143.9 (C-13); 128.6 (C-14); 127.9 (C-15); 127.1 (C-16); 102.5 (C-5); 60.5 (C-10); 51.9 (C-11); 50.5 (C-7); 36.5 (C-8); 29.6 (C-9).

IR (in KBr) υ cm^{-1}: 3061 (C-H arom.); 2102 (N=N=N); 1593 (C=O); 1449 (C-O).

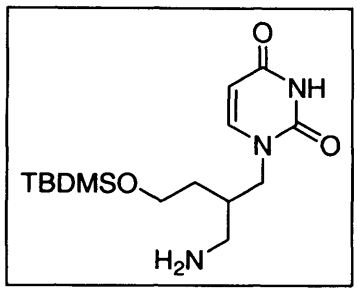
LRMS (ES+): m/z 504.3 [$(M+Na)^+$, 100%]; (ES-): m/z 482.4 [(M) , 100%].

HRMS (ES+): found 504.1997; required 504.2006 $[M+Na]^+$.

Microanalysis calculated for $C_{28}H_{24}N_5O_2$

C 69.84, H 5.65, N 14.54%; found C 69.49, H 5.67, N 13.79%.

(116) Attempted synthesis of 1-(2-(aminomethyl)-4-(*tert*-butyldimethylsilyloxy)-butyl) uracil



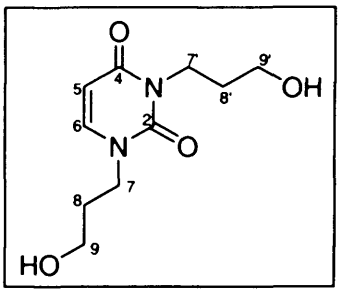
Method A

A round bottom flask was charged with the starting material **96** (150 mg, 0.42 mmol), 5% Pd/C (90 mg) and methanol (5 ml). The flask was sealed and the air inside was replaced with nitrogen several times. A balloon of hydrogen was connected to the flask *via* a three way tap. The nitrogen was replaced with hydrogen and the suspension was stirred at room temperature for 6 h. After such period of time the TLC showed the disappearance of the starting material. The catalyst was filtered through a celite pad. The filtrate was concentrated under reduced pressure and the obtained residue was analysed by NMR spectroscopy and low resolution mass. Unfortunately the spectroscopic analysis did not show the presence of the desired compound in the mixture, therefore no purification was attempted.

Method B

THF (2 ml) was added into a round bottom flask was charged with the starting material **96** (100 mg, 0.28 mmol) and triphenylphosphine (178 mg, 0.68 mmol) under nitrogen. The obtained solution was stirred at room temperature for 12 h. Two new spots were observed on TLC. Water (11μl, 0.62 mmol) was added to the solution and the reaction was warmed to 50 °C for further 6 h. The solvents were removed under reduced pressure and the residue was purified by flash chromatography. NMR analysis of the isolated compound did not have the spectroscopic properties of the desired comopound.

1-N-3-N-*bis*(3-hydroxy-propyl) uracil



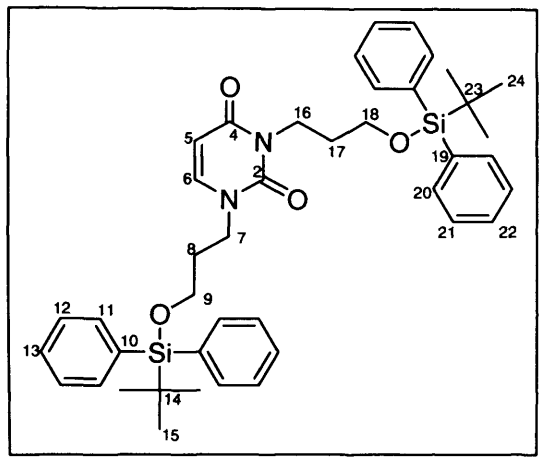
A solution of dialkylated uracil **31b** (140 mg, 0.35 mmol) in MeOH (10 ml) was stirred at room temperature. Three drops of HCl 3M were added to the mixture. The reaction was stirred for 2 h and monitoring the disappearing of the starting material by TLC (EtOAc/Hexane 50:10). The solvent was removed under reduced pressure to give title compound, no further purification was necessary.

White solid, 70 mg; 87%.

^{1}H-NMR (300 MHz; $CDCl_3$) δ: 7.25 (d, *J*=7.68 Hz, 1H, H-6); 5.69 (d, *J*=7.68 Hz, 1H, H-5); 3.99 (t, *J*=6.22 Hz, 2H, H-9); 3.88-3.82 (m, 4H, H-9'+O-H); 3.55 (t, *J*=5.49 Hz, 2H, H-7); 3.45 (t, *J*=5.67 Hz, 2H, H-7'); 1.90-1.73 (m, 4H, H-8+H-8').

^{13}C-NMR (75 MHz; $CDCl_3$) δ: 164.4 (C-4); 152.2 (C-2); 143.9 (C-6); 101.9 (C-5); 59.2 (C-9); 58.7 (C-9'); 47.2 (C-7); 38.3 (C-7'); 31.6 (C-8); 30.7 (C-8').

1-N-3-N-*bis*[(*tert*-butyldiphenylsilyl)propyl] uracil



Tert-butyldiphenylsilyl chloride (141 µl; 1.97 mmol) was added to a solution of 1-N-3-N-*bis*(3-hydroxy-propyl) uracil (150 mg; 0.65 mmol) and Imidazole (104 mg; 1.97 mmol) in dry DMF (10 ml). The reaction was stirred overnight at room temperature under nitrogen. The mixture was extracted with EtOAc (3×10 ml), washed with water (2×10 ml) and brine (2×10 ml). The solvent was dried with $MgSO_4$ and evaporated to afford a residue which was purified by chromatography.

Colourless oil; 47mg; 11%.

R_f: 0.66 in Hexane/EtOAc 50:50.

^{1}H-NMR (300 MHz; $CDCl_3$): δ 7.75-7.69 (m, 8H, H-Arom); 7.51-7.40 (m, 12H, H-arom.); 7.09 (d, *J*=7.68 Hz, 1H, H-6); 5.67 (d, *J*=7.68 Hz, 1H, H-5); 4.12 (t, *J*=7.50 Hz, 2H, H-9); 3.94 (t; *J*=6.59 Hz; 2H; H-7); 3.79 (t, *J*=6.22 Hz, 2H, H-18); 3.75 (t, *J*=5.85 Hz, 2H, H-16); 1.99-1.90 (m, 4H; H-8+H-17); 1.14 (s, 9H, H-15); 1.11 (s, 9H, H-24).

^{13}C-NMR (75 MHz; $CDCl_3$): δ 163.5 (C-4); 151.7 (C-2); 143.1 (C-6); 136.0 (C-11); 135.9 (C-20); 135.2 (C-22); 134.3 (C-10); 133.7 (C-19); 130.3 (C-12); 130.1 (C-21); 128.3 (C-arom.); 128.0 (C-arom.); 101.7 (C-5); 62.5 (C-9); 60.5 (C-18); 47.3 (C-7); 39.3 (C-16); 31.6 (C-8); 31.0 (C-17); 27.4 (C-15); 27.3 (C-24); 19.7 (C-23); 19.6 (C-14).

LRMS (ES+): m/z 726.8 $[(M+Na)^+$; 100%].

HRMS (ES-): found 727.3358; required 727.3367 for $C_{42}H_{52}O_4N_2NaSi^+$.

11.2 Experimental Microbiology – Material and Methods

11.2.1 Antimicrobial compounds – WSP compounds

The WSP (Welsh School of Pharmacy) compounds were resuspended in water (reverse osmosis) or DMSO, (purchased from SIGMA) and tested for activity.

The complete list of tested compounds is reported in Table 11-1.

WSP	Mol. Weight	Mass (mg)	µMol	Solvent
483	470.23	6.44	0.014	DMSO
484	584.79	7.75	0.013	DMSO
485	342.47	5.32	0.016	DMSO
486	574.00	6.20	0.011	DMSO
487	460.00	7.56	0.016	DMSO
665	459.39	5.71	0.012	DMSO
658	478.55	6.31	0.013	DMSO
659	364.29	6.89	0.019	DMSO
660	332.31	11.03	0.033	DMSO
661	331.33	7.75	0.023	DMSO
662	270.24	13.22	0.049	DMSO
663	269.26	11.30	0.042	DMSO
666	382.39	6.72	0.018	DMSO
667	381.40	7.77	0.020	DMSO
669	312.00	8.96	0.029	DMSO
668	311.34	11.80	0.038	DMSO
680	305.30	5.29	0.017	DMSO
704	573.66	6.12	0.011	DMSO
706	563.51	6.15	0.011	DMSO
788	406.32	32.60	0.080	DMSO
784	520.59	21.13	0.041	DMSO
785	405.34	11.05	0.027	DMSO
786	501.43	8.79	0.018	DMSO
787	464.57	14.82	0.032	H_2O/DMSO
789	591.76	7.73	0.013	DMSO
790	421.54	16.76	0.040	DMSO
791	307.28	7.34	0.024	DMSO
804	227.22	7.40	0.033	H_2O/DMSO
826	358.40	7.57	0.021	DMSO
827	400.50	7.04	0.018	DMSO
828	324.40	12.74	0.039	DMSO
829	210.10	5.72	0.027	DMSO
830	289.20	9.33	0.032	DMSO

871	496.50	8.50	0.017	DMSO
872	290.20	9.54	0.033	DMSO
873	300.40	12.79	0.043	DMSO
874	334.30	20.26	0.061	DMSO
875	332.20	11.49	0.035	DMSO
876	702.60	7.60	0.011	DMSO
929	332.00	6.64	0.020	DMSO
930	436.40	7.37	0.017	DMSO
931	468.50	6.54	0.014	DMSO
932	332.00	4.98	0.015	DMSO
933	456.70	5.98	0.013	DMSO
665	459.40	7.00	0.015	DMSO
864	452.51	7.31	0.016	DMSO
865	372.42	6.51	0.017	DMSO
866	448.61	6.40	0.014	DMSO
867	448.60	28.56	0.064	DMSO
868	344.46	11.75	0.034	DMSO
869	472.52	5.96	0.013	DMSO
870	428.49	5.77	0.013	DMSO
946	471.53	4.20	0.009	Methanol
947	488.59	9.60	0.020	Methanol
949	486.60	7.40	0.015	Methanol
950	466.61	8.00	0.017	Methanol
951	384.55	7.20	0.019	Methanol
952	317.35	6.30	0.020	Methanol
953	583.81	5.40	0.009	Methanol
958	389.45	6.10	0.016	Methanol
959	408.46	6.79	0.017	DMSO
960	712.86	7.12	0.010	Chloroform
961	412.49	6.16	0.015	Chloroform
1000	426.52	4.70	0.011	DMSO
1001	442.59	4.10	0.009	DMSO
1002	288.31	4.90	0.017	DMSO
1003	184.20	2.90	0.016	DMSO
1004	454.57	2.90	0.006	DMSO
1005	450.66	3.50	0.008	DMSO

Table 11-1 List of the WSP compounds.

All the compounds were first dissolved in 1 ml of DMSO. 500 µl of compound solution was diluted into a sterile centrifuge tubes with 19.5 ml of sterile water, to give a solution with a final concentration of 2.5% v/v DMSO.

Compounds that were not soluble in those conditions were tested separately.

11.2.2 Media and chemicals

Tryptone Soya Agar (TSA) and Tryptone Soya Broth (TSB) were purchased from Oxoid. HEPES buffer was purchased from SIGMA, and its pH corrected to pH 7.4 with NaOH (0.1 M).

11.2.3 Consumable and instruments

96 well plates were purchased from sterilin and 100 well bioscreen plate purchased from Labsystem. Optical density (OD) measurements were performed with a Pharmacia LKB Novaspec II spectrophotometer and Varian Cary 50 Scan UV-Visible spectrophotometer. Bacterial growth was monitored using the Bioscreen Microbiological Growth C Analyzer (ThermoLabsystems).

11.2.4 Micro-organisms

The five bacterial strains used for the test were: *Escherichia coli* NCIMB 8879, *Enterococcus faecalis* NCIMB 13280, *Staphylococcus aureus* NCTC 6571, *Proteus vulgaris* NCIMB 8067 and *Pseudomonas aeruginosa* NCTC 15442.

11.2.5 Maintenance of strains

Bacteria were inoculated onto TSA plates and incubated at 37°C for 16 h. Inoculated plates were kept at 4°C for 4 days before being renewed. In addition all bacterial strains were re-suspended in HEPES buffer and 20% v/v glycerol and stored at -80°C.

11.2.6 Preparation of broth culture

Fresh broth culture was prepared by inoculating 15 ml of TSB, in sterile McCartney bottles, with 1 bacterial colony and incubated at 37°C under constant agitation for 16 h.

After incubation TSB cultures were centrifuged at approx 2000g and the bacterial pellet resuspended in HEPES buffer.

11.2.7 Viable count and OD_{500} measurements

The washed inoculum was diluted serially to 4 decreasing concentrations with HEPES buffer. The absorbances of the four suspensions were recorded. After measuring the OD_{500} of the suspension, a bacterial aliquot was serially diluted (1 to 10) in HEPES buffer. Three separate drops of 10 µl of the highest dilutions were then plated onto the surface of dried TSA plates as described in Figure 9-4. Plates were then incubated for 16 h at 37°C, and the colonies subsequently counted. Colony count (CFU) was correlated with OD_{500} and plots of absorbance against the number of cells per ml (cfu/ml) were constructed.

11.2.8 Bacterial growth

Bacterial growth was monitored with the Bioscreen Microbiological Growth C Analyzer. Bacterial cultures were incubated in TSB under constant agitation for 16 h at 37°C and OD measurements recorded automatically every 10 min.

Controls included the growth of all bacterial strains in various concentrations of DMSO (5% v/v, 4% v/v, 3% v/v, 2% v/v, 1% v/v, 0.5% v/v respectively). The effect of compounds that showed possible activity against a particular bacterial strain(s) (i.e. during MIC testing) was also monitored using the Bioscreen Microbiological Growth C Analyzer.

11.2.9 Minimum inhibitory concentration

Broth MIC (minimum inhibitory concentration) of all the compounds was determined in 96 well plates (Figure 9-1). A washed bacterial inoculum of approx. 10^6 cfu/ml resuspended in 100 µl of double strength TSB was mixed with 100 µl of compound solution, and incubated at 37 °C for 16 h.

Controls included, non-inoculated TSB, TSB+DMSO (at the highest concentration), DMSO only, and micro-organism and TSB and DMSO (at the highest concentration). A typical 96 well plate template is described in Table 11-2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NB		WSP 1	WSP 1	WSP 2	WSP 2	WSP 3	WSP 3	WSP 4	WSP 4	WSP 5	WSP 5
B	DMSO											
C	NB+DMSO											
D	µorg+DMSO+NB		WSP 6	WSP 6	WSP 7	WSP 7	WSP 8	WSP 8	WSP 9	WSP 9	WSP 10	WSP 10
E												
F												
G	µorg + NB											

Table 11-2 Example of 96 well plate template; the concentrations of the compounds decrease from the side to the centre of the plate; NB= nutrient broth

After 16 h incubation at 37°C under constant agitation, the OD_{540} of inoculated control (in TSB and DMSO) was compared to those of the test wells (i.e. those containing decreasing concentrations of compounds).

Only compounds soluble in a maximum DMSO concentration of 2.5% v/v were tested with this method.

11.3 Effect of compounds on bacterial growth

Compounds that showed some antimicrobial activity as determined with the BMD method or compounds that decreased the overall OD after 16h incubation as compared to the control in TSB and DMSO were tested for their effect of bacterial growth using the Bioscreen Microbiological Growth C Analyzer.

A washed bacterial suspension containing approx. 10^6 cfu/ml was inoculated into the 100 well bioscreen plate with four different concentrations of compounds, using the same controls described for the broth MIC determination. Plates were incubated 16 h at 37°C and the OD recorded every 10 min.

11.4 Antimicrobial activity of insoluble compounds

A spectrophotometric analysis in the visible region of wavelengths (from 200nm to 800nm) was performed with the insoluble compounds using a spectrophotometer Varian Cary 50 Scan, in order to investigate the possible interference with bacterial OD measurements. All compounds that did not

show absorbance around the wavelength of 500 nm were tested with the broth micro dilution method (BMD) as previously described.

The activity of all the other insoluble compounds was tested as follows: washed bacterial suspension was inoculated in 15 ml (final volume) TSB and compound with a concentration of DMSO lower than 5% v/v. After incubation at 37°C overnight, the broth was filtered through a 0.45 μm cellulose nitrate membrane filter (Sartorius). Membrane filters were then placed onto the surface of a dried TSA plate and incubated for a further 16 h at 37°C. After incubation, the absence of microbial growth (i.e. colonies) indicated antimicrobial activity. A decrease in bacterial growth on the filter, as compared to the control, indicated a possible activity.

Controls consisted of TSB and 5% v/v DMSO and TSB alone.

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Enzyme assays

Enzyme purification and inhibition assays

Both recombinant *P. falciparum* and human dUTPases were expressed in *E. coli* BL21 (DE3) cells which had been transformed with the pET11*Pfdut*and pET3*Hudut* (kindly provided by P.O. Nyman, Lund University, Sweden) expression vectors respectively. For dUTPase purification, the same procedure was used for the human and *Plasmodium* enzymes. Cell pellets from a two litre IPTG-induced culture were re-suspended in 40 mL of buffer A (20 mM sodium acetate (pH 5.5), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT) and 20 μM PMSF. The cells were lysed by sonication, and the cell extract was cleared by centrifugation at 15,000 rpm for 45 min. The supernatant was loaded onto a 50 ml phosphocellulose (Whatman P-11) column at 4°C and eluted with a 50 mM – 2 M NaCl gradient in buffer A. The enzyme was then dialyzed against buffer A, prior to gel filtration chromatography on a Superdex 200 HA 10/30 column at 4°C. Purified fractions contained dUTPase of approximately 96 % purity.

Nucleotide hydrolysis was monitored by mixing enzyme and substrate with a rapid kinetic accessory (Hi-Tech Scientific) attached to a spectrophotometer (Cary 50) and connected to a computer for data acquisition and storage. Protons, released through the hydrolysis of nucleotides, were neutralised by a pH indicator in weak buffered medium with similar pK_a and monitored spectrophotometrically at the absorbance peak of the basic form of the indicator. The ratio between the indicator and the buffer concentration was 50:2500 (μM) and the absorbance changes were kept within 0.1 units. The indicator/buffer pair used was bromocresol purple/MES (pH 5.7-6.2, 605 nm). Assay mixes contained 30 nM of *Pf*dUTPase, 50 μM dUTP, 5mM $MgCl_2$ and 2.5 mM DTT, 1.25 mg/ml BSA and 100 mM KCl. V_{max} and K_{Mapp} were calculated by fitting the resulting data to the integrated Michaelis-Menten equation. The apparent K_M values were plotted against inhibitor concentration, and K_i values (Table 4) were obtained according to Equation I.

$$K_{Mapp} = \frac{K_M}{K_i}[I] + K_M \qquad \text{(I)}$$

P. falciparum in vitro assay

Parasite cultures

Two strains of *P.falciparum* are used in this study: The drug sensitive NF54 (an airport strain of unknown origin) and K1 (Thailand, chloroquine and pyrimethamine resistant). The strains are maintained in RPMI-1640 medium with 0.36 mM hypoxanthine supplemented with 25 mM HEPES, 25 mM $NaHC0_3$, neomycin (100 U/ml) and Albumax^R (lipid-rich bovine serum albumin) (GIBCO, Grand Island, NY) (5g/l), together with 5% washed human A+ erythrocytes. All cultures and assays are conducted at 37°C under an atmosphere of 4% $C0_2$, 3% 0_2 and 93% N_2. Cultures are kept in incubation chambers filled with the gas mixture. Subcultures are diluted to a parasitaemia of 0.1-0.5% and the medium changed daily.

Drug sensitivity assays

Antimalarial activity is assessed using an adaptation of the procedures described by Desjardins et al.(Antimicrob. Agents Chemother. 16(6):710-8, 1979), and Matile and Pink (In: Lefkovits, I. and Pernis, B. (Eds.). Immunological Methods. Academic Press, San Diego, pp. 221-234, 1990).

Stock drug solutions are prepared in 100% DMSO (dimethylsulfoxide) at 10 mg/ml, unless otherwise suggested by the supplier, and heated or sonicated if necessary. After use the stocks are kept at –20°C. The compound is further diluted to the appropriate concentration using complete medium without hypoxanthine.

Assays are performed in sterile 96-well microtiter plates, each well containing 200 µl of parasite culture (0.15% parasitemia, 2.5% hematocrit) with or without serial drug solutions. Seven 2-fold dilutions are used covering a range from 5 µg/ml to 0.078 µg/ml. For active compounds the highest concentration is lowered (e.g. to 100 ng/ml), for plant extracts the highest concentration is increased to 50 µg/ml. Each drug is tested in duplicate and repeated once for

active compounds showing an IC_{50} below 0.5 µg/ml. After 48 hours of incubation at 37°C, 0.5 µCi ^{3}H-hypoxanthine is added to each well. Cultures are incubated for a further 24 h before they are harvested onto glass-fiber filters and washed with distilled water. The radioactivity is counted using a Betaplate™ liquid scintillation counter (Wallac, Zurich, Switzerland). The results are recorded as counts per minute (CPM) per well at each drug concentration and expressed as percentage of the untreated controls. From the sigmoidal inhibition curves IC_{50} values are calculated.

Primary screen

K1 strain is used. The compounds are tested at 7 concentrations (5000 to 78 ng/ml). Artemisinin and chloroquine are included as reference drugs.

If the IC_{50} is >5 µg/ml, the compound is classified as inactive

If the IC_{50} is 0.5-5 µg/ml, the compound is classified as moderately active

If the IC_{50} is <0.5 µg/ml, the compound is classified as active and is further evaluated using two strains, K1 and NF54. A new range of concentrations is chosen depending on the IC_{50} determined (e.g. 100 to 1.56 ng/ml) and the assay is carried out 2× independently.

The standard drugs are chloroquine and artemisinin which are run in the same assay. The IC_{50} values for chloroquine are 2.9 ng/ml for NF54 and 48 ng/ml for K1; for artemisinin 1.9 ng/ml for NF54 and 0.8 ng/ml for K1.

Secondary screen

A panel of 14 different strains is available in liquid nitrogen. The strains are of different origin and some show resistances to chloroquine and/or pyrimethamine. If the range of the IC_{50} values for the 14 strains is within a factor 3-5 x then the tested compound is considered not to show cross resistance.

L. donovani in vitro assay

Parasite and cell cultures:

The *Leishmania donovani* strain MHOM/ET/67/L82 is used. The strain is maintained in the hamster. Amastigotes are collected from the spleen of an infected hamster and adapted to axenic culture conditions at 37°C. The medium is a 1:1 mixture of SM medium (Cunningham I., J. Protozool. 24, 325-329, 1977) and SDM-79 medium (Brun, R. & Schönenberger, M., Acta Trop. 36, 289-292, 1979) at pH 5.4 supplemented with 10% heat-inactivated FBS under an atmosphere of 5% CO_2 in air.

Drug sensitivity assays

Stock drug solutions are prepared in 100% DMSO (unless otherwise suggested by the supplier) at 10 mg/ml, and heated or sonicated if necessary. After use the stocks are kept at –20°C. For the assays, the compound is further diluted to the appropriate concentration using complete medium.

Assays are performed in 96-well microtiter plates, each well containing 100 µl of culture medium with 10^5 amastigotes from axecic culture with or without a serial drug dilution. The highest concentration for the test compounds is 90 µg/ml. Seven 3-fold dilutions are used covering a range from 30 µg/ml to 0.041 µg/ml. Each drug is tested in duplicate and each assay is repeated at least once. After 72hours of incubation the plates are inspected under an inverted microscope to assure growth of the controls and sterile conditions.

10µl of Alamar Blue (12.5 mg resazurin dissolved in 1L distilled water) are now added to each well and the plates incubated for another 2 hours. Then the plates are read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wave length of 536 nm and an emission wave length of 588 nm.

Data are analysed using the microplate reader software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA).

Primary screen

The compounds are tested in duplicate at 7 concentrations. Miltefosine is used as the reference drug and shows an IC_{50} value of 0.12 μg/ml.

If the IC_{50} is >3 μg/ml, the compound is classified as inactive

If the IC_{50} is 0.1-3 μg/ml, the compound is classified as moderately active

If the IC_{50} is <0.1 μg/ml, the compound is classified as active

Secondary screen

Active and moderately active compounds are tested in the macrophage assay with intracellular amastigotes in their host cells, murine macrophages.

L. donovani macrophage *in vitro assay*

Parasite and cell cultures:

The *Leishmania.donovani* strain MHOM/ET/67/L82 obtained from Dr. S. Croft, London) is used. The strain is maintained in the Syrian Golden hamster. Amastigotes are collected from the spleen of an infected hamster Amastigotes are grown in axenic culture at 37°C in SM medium (Cunningham I., J. Protozool. 24, 325-329, 1977) at pH 5.4 supplemented with 10% heat-inactivated foetal bovine serum under an atmosphere of 5% $C0_2$ in air.

Primary peritoneal macrophages from NMRI mice are collected 1 day after a macrophage production stimulation with an i.p injection of 2ml of a 2% potato starch suspension (FLUKA, Switzerland) All cultures and assays are done at 37°C under an atmosphere of 5% $C0_2$ in air.

Drug sensitivity assays

Stock drug solutions are prepared in 100% DMSO (unless otherwise suggested by the supplier) at 10 mg/ml, and heated or sonicated if necessary. After use the stocks are kept at –20°C. For the assays, the compound is further diluted in serum-free culture medium and finally to the appropriate concentration in complete medium.

Assays are performed in sterile 16-well chamber slides (LabTek, Nalgene/Nunc Int.) To each well 100 µl of a murine macrophage suspension (4 x 10^5/ml) in RPMI 1640 (containing bicarbonate and HEPES) supplemented with 10% heat inactivated fetal bovine serum is added. After 24 hrs 100 µl of a suspension containing amastigotes (1.2 x 10^6/ml) is added resulting in a 3:1 ratio of amastigotes/macrophages. The amastigotes are harvested from an axenic amastigote culture and suspended in RPMI/FBS. 24 hrs later, the medium containing free amastigotes is removed, washed 1x and replaced by fresh medium containing four 3-fold drug dilutions. In this way 4 compounds can be tested on one 16-well tissue culture slide. Untreated wells serve as controls. Parasite growth

in the presence of the drug is compared to control wells. After 4 days of incubation the culture medium is removed and the slides fixed with methanol for 10 min followed by staining with a 10% Giemsa solution. Infected and non-infected macrophages are counted for the control cultures and the ones exposed to the serial drug dilutions. The infection rates are determined. The results are expressed as % reduction in parasite burden compared to control wells, and the IC_{50} calculated by linear regression analysis.

Primary screen

The compounds are tested in duplicate at 4 concentrations ranging from 9 to 0.3 μg/m. If the IC_{50} is below 0.3 μg/ml then the range is changed to 1 to 0.03 μg/ml. Miltefosine is used as the reference drug and shows an IC_{50} value of 0.325 μg/ml (0.22 – 0.42 μg/ml; n=4)

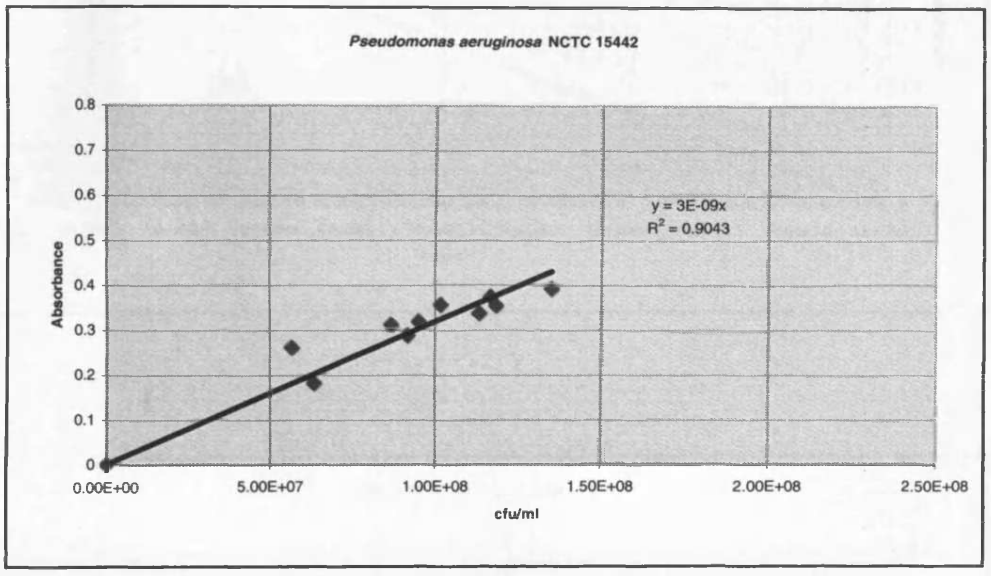
If the IC_{50} is higher than 10 μg/ml, the compound is classified as inactive.

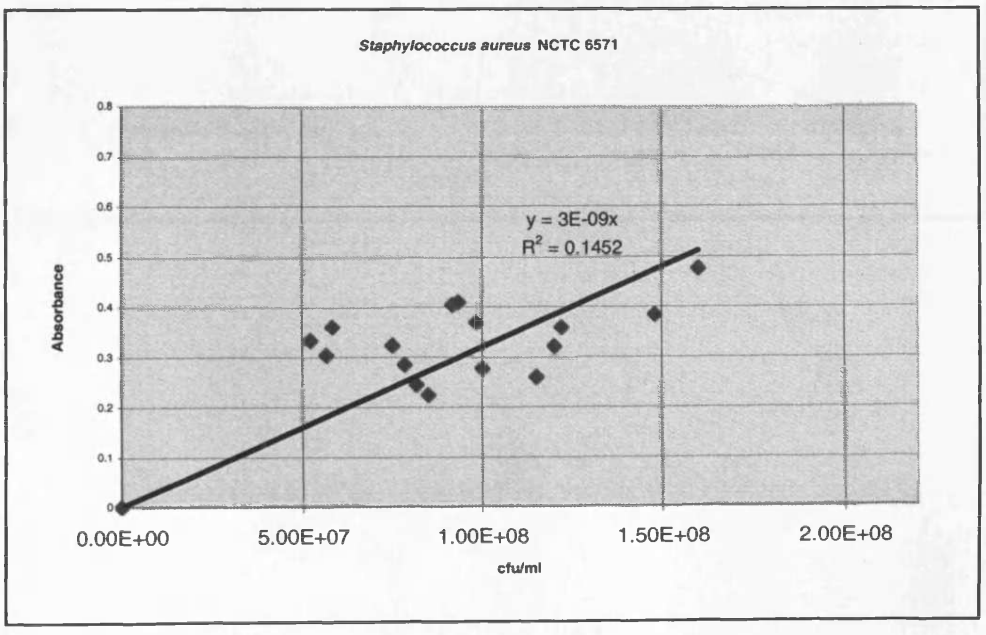
If the IC_{50} is between 2 and 10μg/ml, the compound is classified as moderately active.

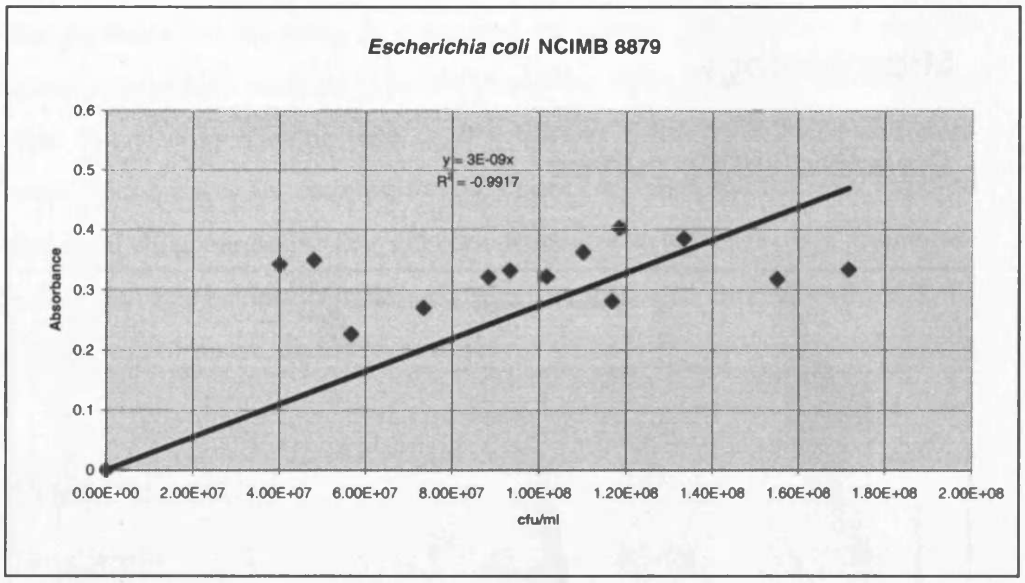
If the IC_{50} is < 2 μg/ml, the compound is classified as active and is further evaluated in a secondary screening.

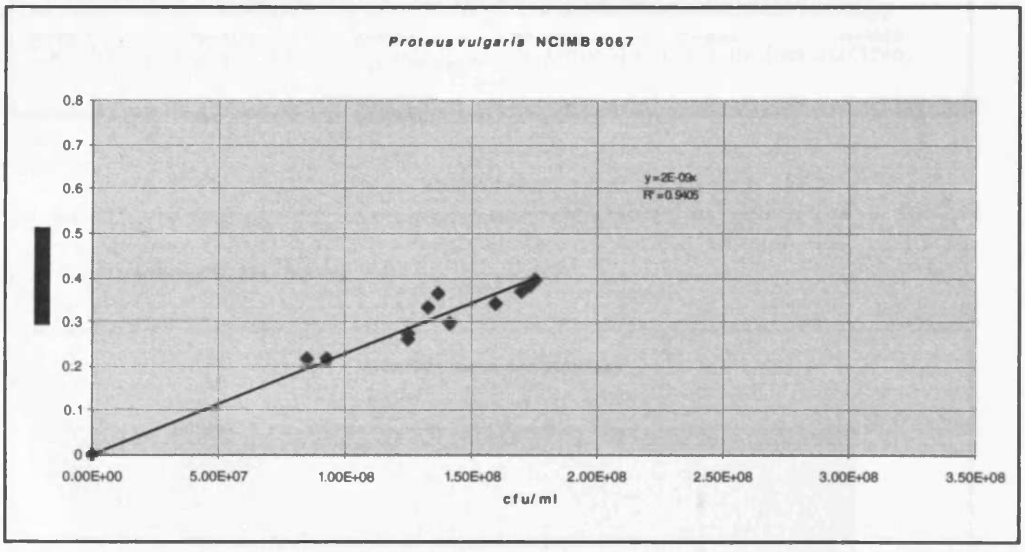
Microbiology

Standardization curves









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