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## A SELECTIVE SEARCH FOR BIOLOGICALLY ACTIVE TRIPARTATE NUCLEOSIDE PRODRUGS: II

### BİYOLOJİK AKTİVİTESİ OLAN ÜÇ KISIMLI NÜKLEOSİT PRODRUG'LARI İÇİN SEÇİCİ BİR TARAMA: II

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#### ABSTRACT

*In the previous review, among the bipartate and tripartate prodrug approaches, it was only explained bipartate prodrugs. In this review, it was explained the tripartate prodrug approach and recent progress in the design and synthesis tripartate prodrug nucleosides. It was determined that the tripartate approach applied to pronucleotides appears to be very effective in vitro, but toxicity and solubility, as well as synthetic methodology issues should be taken into consideration.*

**Key words:** Nucleosides, Tripartate approaches, Syntheses

#### ÖZET

*Bir önceki derlemede, bipartat ve tripartat önilaç yaklaşımları arasından sadece bipartat önilaç yaklaşımı açıklanmıştır. Bu derlemede, tripartate önilaç yaklaşımı ve tripartat önilaç nükleositlerin tasarım ve sentezlerindeki son gelişmeler açıklanmıştır. Tripartat yaklaşımının, önilaçlara uygulandığının in vitro olarak oldukça etkili olduğu, fakat sentetik metodoloji yanısıra toksisite ve çözünürlüğün de göz önünde bulundurulması gerektiği tespit edilmiştir.*

**Anahtar kelimeler:** Nükleositler, Tripartat yaklaşım, Sentezler.

## INTRODUCTION

Failure of the bipartate drug approach can be associated with the instability of the linkage between carrier and drug or electronic and steric properties of the prodrug as a whole hindering enzymatic cleavage. In either case, the tripartate prodrug approach may overcome these complications by placing a spacer between the carrier and the drug so that the enzymatic cleavage occurs between carrier and spacer instead of between carrier and drug (Figure 1). Once the bond linking carrier and spacer is cleaved, the remaining bond connecting spacer and drug undergoes spontaneous hydrolysis under physiological conditions releasing the drug (1).

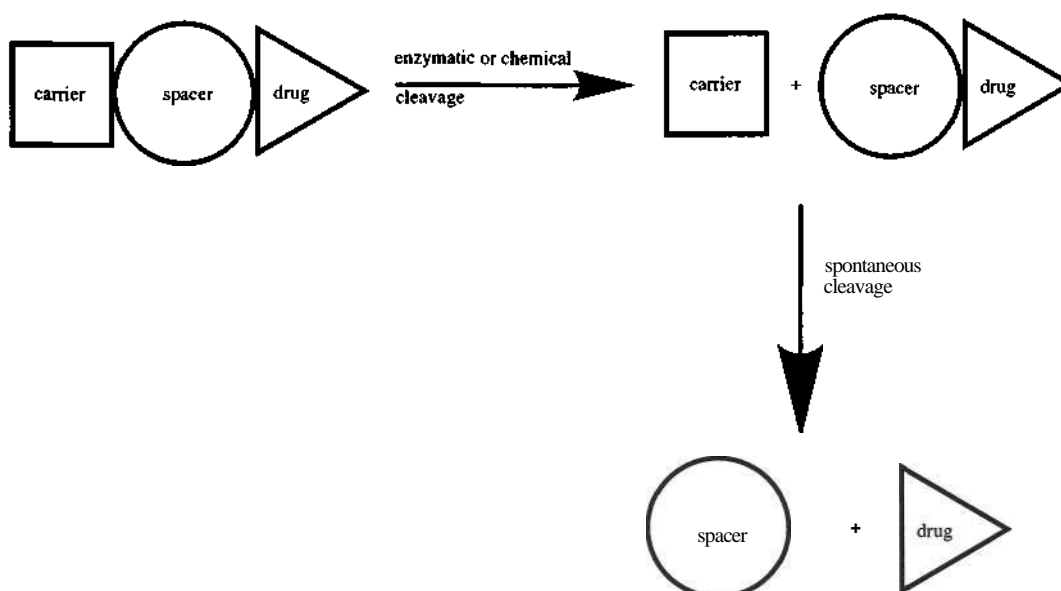


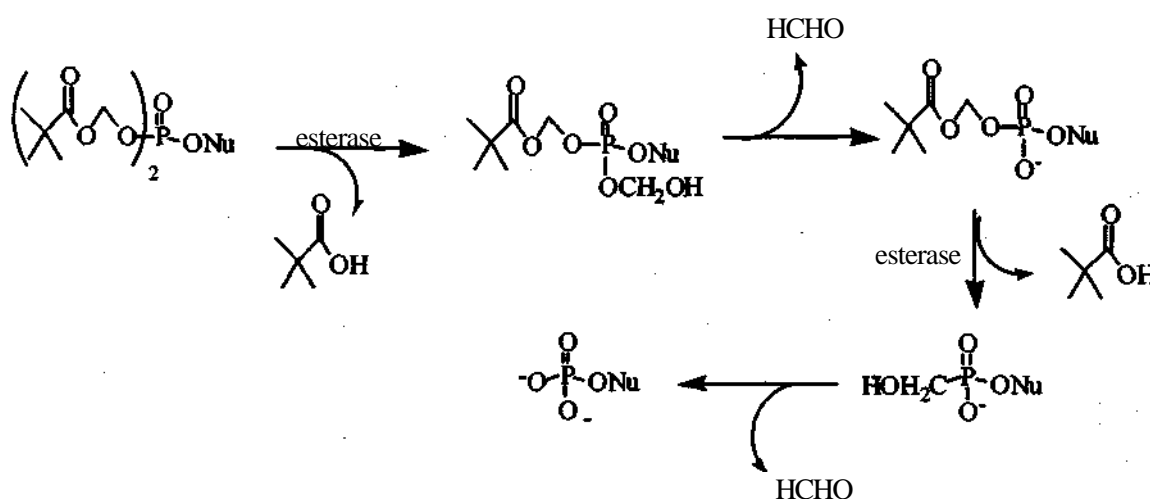
Figure 1. **Tripartate Prodrug**

### Tripartate Prodrug Approach Applied to Phosphotriester

#### Bis-POM and POC Pronucleotides

The approach of using a double ester as a prodrug was first used to improve the bioavailability of marketed  $\beta$ -lactam antibiotics and non-steroidal anti-inflammatory agents (2). In this approach, the diester undergoes enzymatic cleavage, releasing the unstable hydroxyalkyl ester which spontaneously disengages releasing the parent compound (Figure 2). The bulkiness

of the terminal alkyl determines the rate at which enzymatic cleavage occurs. Recently this approach has been applied to facilitate the intracellular delivery of monophosphates such as 2', 3'-dideoxy-2', 3'-didehydrouridine monophosphate (ddUMP) (3), 3'-azido-2',3'-dideoxythymidine monophosphate (AZTMP) (4), 5-fluoro-2'-deoxyuridine monophosphate (5-FdUMP) (5), (*R*)-9-(2-phosphono-methoxypropyl)adenine (PMPA) (6) and its diamino analogue (PMPDAP) as well as [9-(2-phosphonylmethoxyethyl)adenine] PMEA (7).



**Figure 2.** Decomposition of bis-POM pro-nucleotides

PMEA has a broad spectrum of antiviral activity, which includes retroviruses, hepadnaviruses, and herpesviruses (7,8). In phase I/II clinical trials, it appears to be a promising anti-HIV candidate (9). Nevertheless, the possibility of PMEA becoming an orally administered drug is limited by its poor bioavailability as shown in monkeys (<1%) (10) and rats (7-11%) (11,12). Its limited bioavailability is due to the negative charge of the phosphonate functionality at physiological pH.

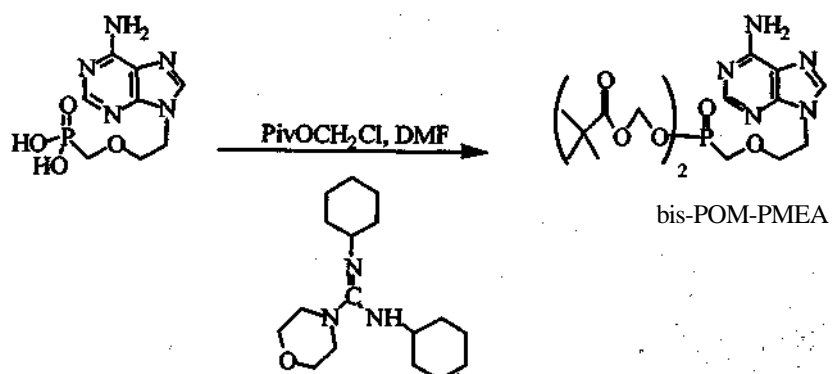
Srinivas *et al.* (6) explored the approach of acyloxyalkyl ester pro-nucleotides as a means of masking the phosphonate negative charges of PMEA, thus forming a more lipophilic derivative with the capacity of crossing the gastrointestinal wall and releasing the parent

compound in the plasma (Scheme 1). Preliminary *in vitro* studies showed that bis-POM-PMEA provided a 100-fold intracellular increase of PMEA (7). *In vitro* studies also showed that bis-pivaloyloxymethyl-PMEA (bis-POM-PMEA) had comparable activity to that of PMEA against human immunodeficiency virus type 1 (HIV-1) infected CEM cells and human cytomegalovirus (HCMV) infected MRC-5 cells. Bis-POM-PMEA was substantially more potent than PMEA against herpes simplex virus (HSV) types 1 and 2 infected Vero cells. Its persistence of HSV-2 inhibition, 20 times longer than that of the parent compound, correlates well with the reported enhanced cellular uptake (13). Bis-POM-PMEA also effected the growth of CEM cells. This inhibition is mostly cytostatic, rather than cytotoxic. At 0.4  $\mu\text{M}$ , the prodrug severely retarded the growth rate. Surprisingly, the growth of CEM cells was completely suppressed at 2  $\mu\text{M}$  concentration of bis-POM-PMEA, which may result from the liberation of two equivalents of formaldehyde and pivalic acid (Figure 3). Furthermore, *in vivo* bis-POM-PMEA demonstrated a 2-fold and 5-fold enhancement in bioavailability in rats (14) and monkeys (7), respectively. At a single 500 mg dose of bis-(POM)-PMEA, oral bioavailability was greater than 40% in clinical trials involving well fed subjects (14).

The synthesis of bis-POM-PMEA was carried out by reacting PMEA with chloromethylpivalate in the presence of the bulky base N,N'-dicyclohexyl-morpholine carboxamidine in 32% yield (Scheme 1). Bis-POM-PMEA has also been synthesized in lower yields by condensing various salts of PMEA with either chloromethyl pivalate or iodomethyl pivalate (15).

Bis-(isopropylloxycarbonyloxymethyl) (bis-POC) pronucleotides are a modification of the bis-POM pronucleotides designed to reduce the cytostatic effect which may be caused by the release of pivalic acid (Scheme 2). Bis-POC pronucleotides are composed of a carbonate diester that undergoes esterase-catalyzed cleavage of the isopropyl ester to yield two equivalents of 2-propanol and formaldehyde (Figure 3). The bis-POC approach was applied to the anti-HIV agent (*R*)-9-(2-phosphono-methoxypropyl)adenine PMPA (16,17). PMPA was reported to completely prevent simian immunodeficiency virus (SIV) infection in monkeys even as late as 24 h after inoculation occurred (18). PMPA showed efficacy without significant toxicity in long-term treatment (13 months) of SIV-infected newborn monkeys (19). Furthermore, PMPA exhibited an effect against chronic SIV infection in monkeys (20). PMPA was also found to be

active against acute and chronic feline immunodeficiency virus (FIV) infections in cats (21). In phase I/II clinical trials, PMPA exhibited a 1.1 log reduction in HIV RNA levels after administration of only eight doses (22). Nevertheless, PMPA displayed low bioavailability in animals.



Scheme 1. Synthesis of bis-POM-PMEA

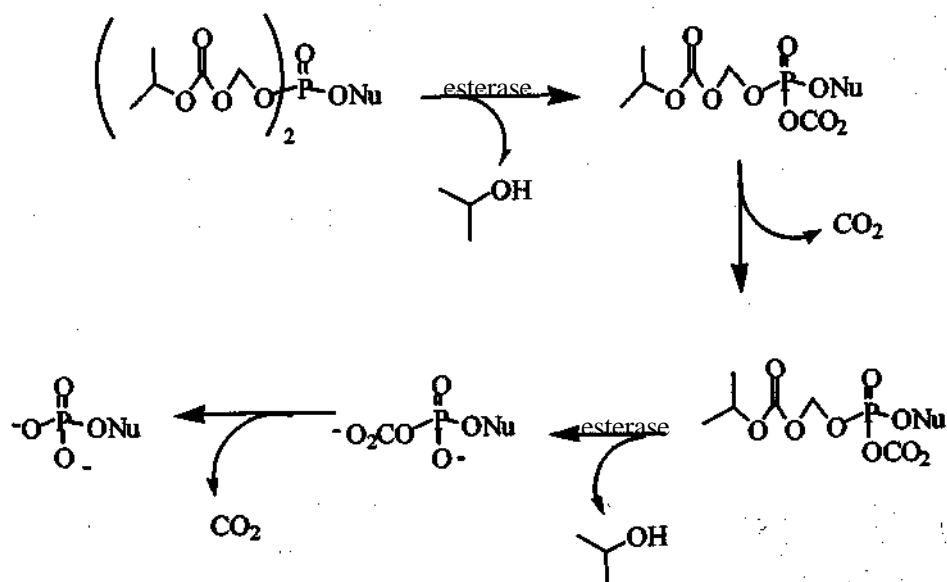
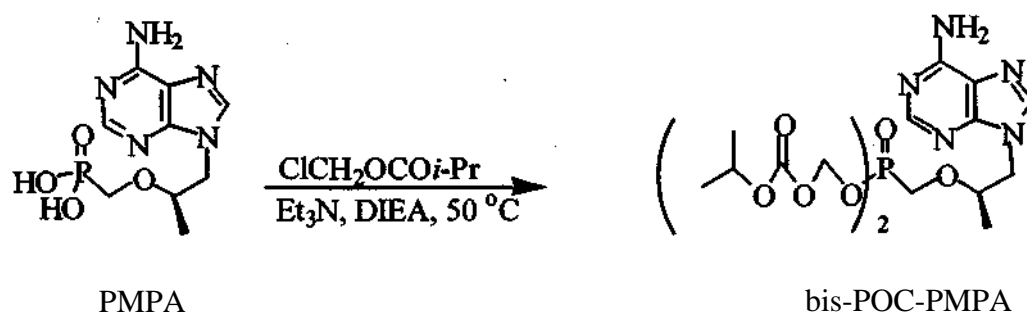


Figure 3. Decomposition of bis-POC pronucleotides

In an effort to improve the oral absorption of PMPA, Arimilli *et al.* (22) synthesized various acyloxyalkyl esters of PMPA. The most promising was the bis-isopropoxyloxycarbonyloxymethyl ester derivative (bis-POC), based upon its stability ( $t_{1/2} > 150$  and 8, 20.5 h at pH 2.2 and 7.4, plasma, respectively), log P (1.3), efficacy, and low toxicity (23). *In vitro* studies of the metabolism of radiolabeled PMPA showed that it was hydrolyzed to PMPA and subsequently underwent phosphorylation to the mono and diphosphate derivatives (24). In addition, bis-POC-PMPA showed an oral bioavailability of 30% in dogs with minimal toxicity in repeated 5-day dosing of 60 mg/kg/day (25).

In the synthesis of bis-POC-PMPA, isopropylchloromethyl carbonate (24) was synthesized by adding pyridine to a cold ethereal solution of chloromethyl chloroformate and 2-propanol. The carbonate was then reacted with PMPA in dimethylformamide (DMF) in the presence of triethylamine (TEA) or diisopropylethylamine (DIPEA) at 50 °C for 20 h (Scheme 2).

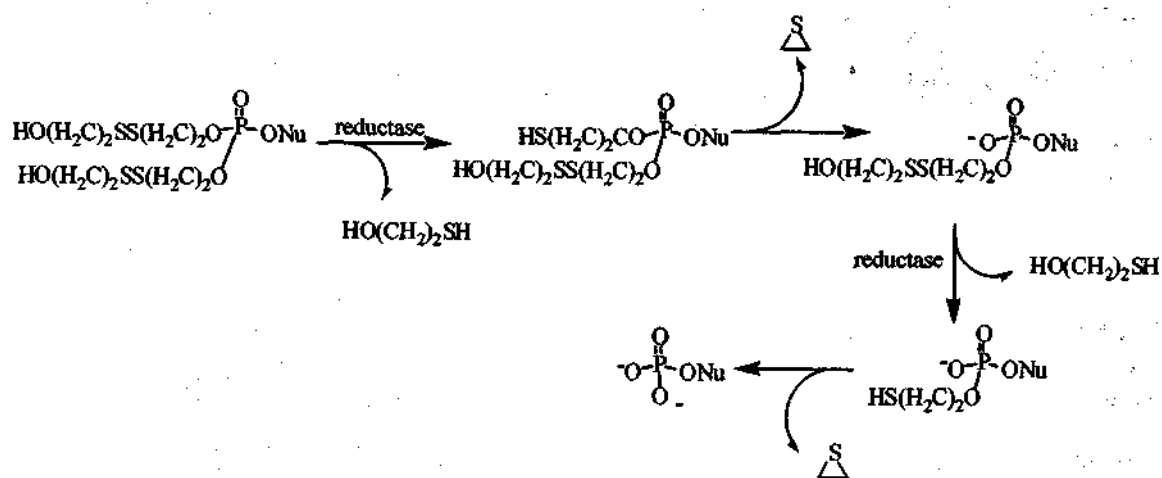


Scheme 2. Synthesis of bis-POC-PMPA

### Bis-SDTE and SATE Pronucleotides

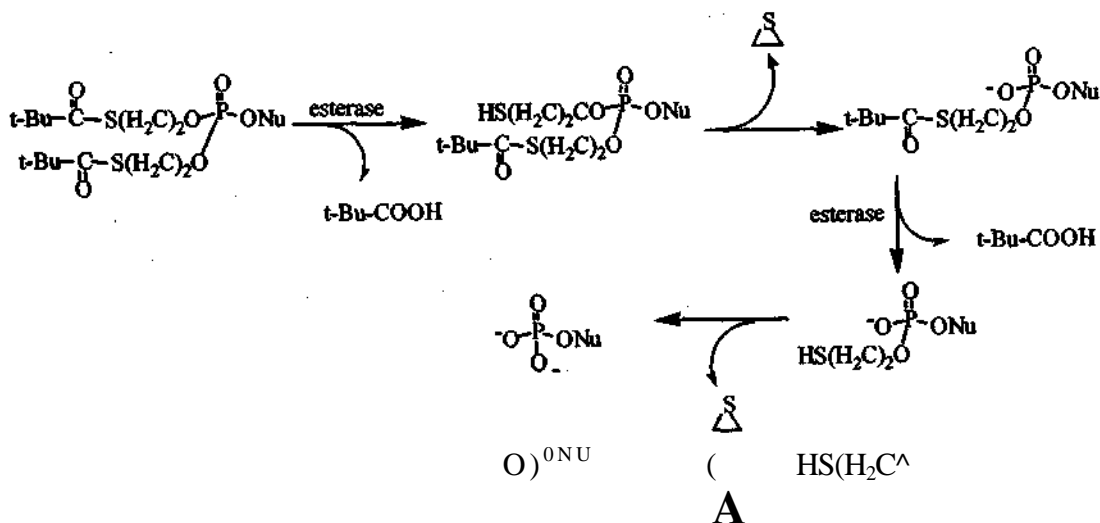
In an effort to improve the pharmacokinetics of some nucleotides, Imbach *et al.* (25,26) designed enzymatically activated pronucleotides, bis-[5-(2-hydroxyethylsulfidyl)-2-thioethyl]- (bis-SDTE) and bis-[S-acyl-2-thioethyl] (bis-SATE). The Bis-SDTE concept was designed to

take advantage of the concentration of reductase within the cytosol to liberate the nucleotide (Figure 4). The thioethyl phosphotriester, formed after reductive cleavage of the disulfide bond, spontaneously decomposes to the phosphodiester releasing episulfide. The phosphodiester then undergoes an identical sequence of enzymatic activation steps to yield the nucleotide. Both bis-SDTE triesters of 2', 3'-dideoxy-2',3'-didehydrouridine monophosphate (d4UMP) and 3'-azido-2',3'-dideoxythymidine monophosphate (AZTMP) were found to be cleaved in cell extracts 30-fold faster than in culture medium (26). The bis-SDTE concept failed to improve the antitumor efficacy of 5-fluoro-2',3'-dideoxyuridine (5-FdU) (27) while bis-SDTE-PMEA (28) exhibited enhanced antiviral activity. Limited success of the bis-SDTE approach is due to chemical instability as well as metabolism in serum.



**Figure 4.** Decomposition of bis-SDTE pronucleotides

The bis-SATE concept, similar to the bis-POM approach, requires esterase-mediated activation to aid in nucleotide delivery (Figure 5) (29).

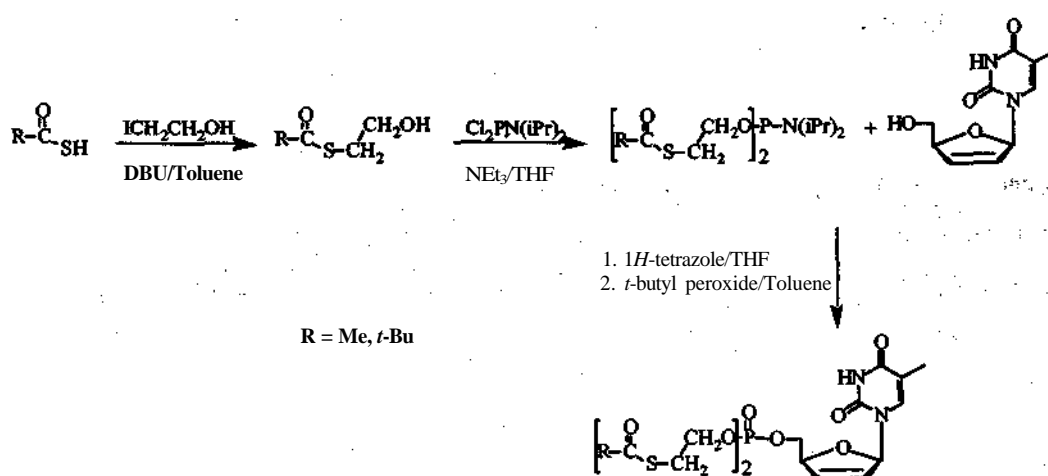


**Figure 5.** Decomposition of bis-SATE pronucleotides

The esterase cleaves the thioester to form the thioethyl phosphonate triester that spontaneously decomposes to episulfide and the phosphonate diester, which undergoes the same sequence of enzymatic activation ultimately releasing two equivalents of episulfide. It has been shown that the addition of SDTE and SATE moieties cause cytotoxicities comparable to that of the parent nucleosides in human bone marrow cells (30).

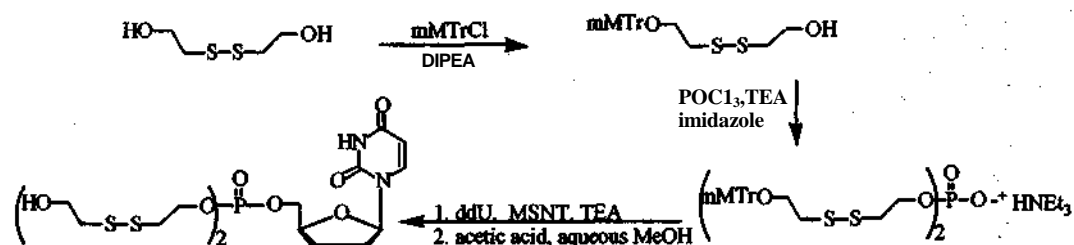
Among the various bis-SATE side chains synthesized, bis-*t*-buSATE-AZTMP was the most stable in culture medium and cell extract (27). Its stability was mostly due to the bulkiness of *t*-butyl residue, which prevents rapid cleavage. The stability of *t*-buSATE moiety was also demonstrated in the case of the *t*-buSATE derivative of 2',3'-dideoxy-3'-oxoadenosine (isoddA) (31). The bis-SATE concept has been proven to be successful in the cases of 2',3'-didehydro-2',3'-dideoxythymidine monophosphate (d4TMP) (32), AZTMP (29) and 2', 3'-didehydro-2',3'-dideoxyadenosine monophosphate (d4AMP) (33), which were active against HIV infected TK- CEM cells (thymidine kinase negative CEM cells). In addition to enhanced activity, the prodrug of d4A was more stable than d4A itself against acid catalyzed depurination (34). For many bis-SATE pronucleotides, there was a decrease in activity against HIV infected TK-CEM cells.

Thioesters of bis-SATE pronucleotides of d4T were synthesized by reaction of thiocarboxylic acids with 2-iodoethanol, followed by condensation with *N*, *N*-diisopropylphosphorodichloridite in tetrahydrofuran in the presence of triethylamine to yield the corresponding phosphoramidites. These were coupled with d4T in the presence of 1*H*-tetrazole and oxidized *in situ* with 3-chloroperoxybenzoic acid to obtain the bis-SATE phosphotriester (Scheme 3).



**Scheme 3.** Synthesis of SATE derivatives of d4TMP

For the synthesis of the bis-DTE phosphotriester of 2',3'-dideoxyuridine (ddU) (35), dithiodiethanol phosphodiester was synthesized by protecting dithiodiethanol with monomethoxytrityl chloride in the presence of diisopropylethanolamine followed by phosphorylation using phosphoryloxy chloride, imidazole and triethylamine. The dithiodiethanol phosphodiester was condensed with ddU in triethylamine in the presence of 1-mesitylene-2-sulfonyl-3-nitro-1,2,4-triazole. Following treatment with acetic acid and aqueous methanol gave bis-DTE ddU (Scheme 4).

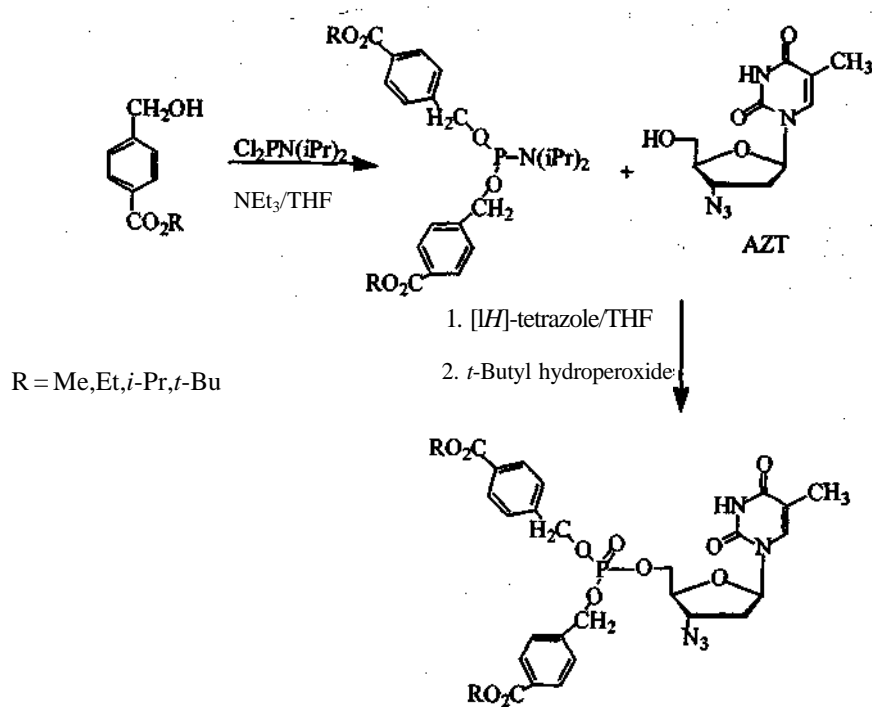


**Scheme 4.** Synthesis of DTE pronucleotides of ddU



monophosphate in the presence of porcine liver esterase. Like with DTE pronucleotides, the bis-acyloxybenzyl approach is limited by very short half-lives of the prodrugs. In addition, these prodrugs are too lipophilic (log P values range from 1 to 4) for systemic administration (40). *In vivo*, the bis(4-acyloxybenzyl) derivatives of ACVMP exhibited no significant toxic side effects at concentrations up to 100 mg/kg of body weight (40).

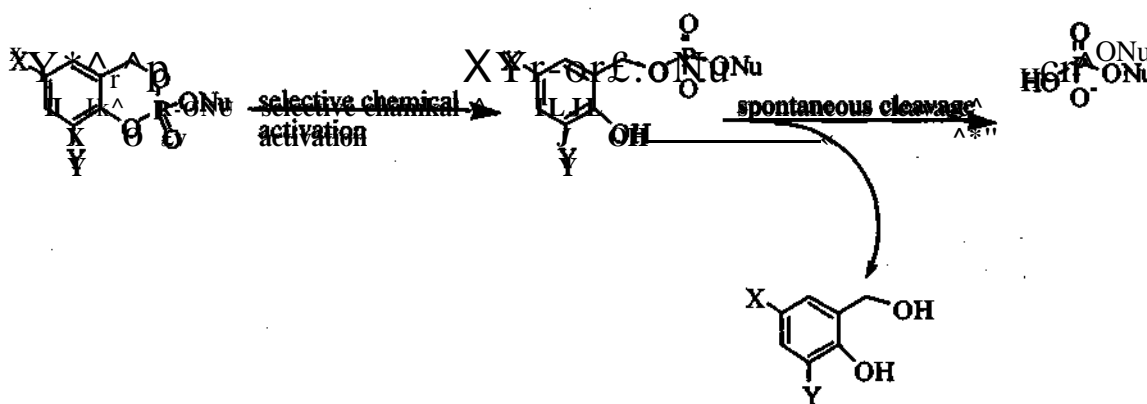
In the synthesis of bis-acyloxybenzyl phosphotriester of AZT, the appropriate 4-acyloxybenzyl alcohol is reacted with *N,N*-diisopropylphosphorochloridate in the presence of triethylamine to yield the corresponding phosphodiester. The phosphodiester is coupled with the AZT in the presence of [1H]-tetrazole and oxidized *in situ* with *m*CPBA to obtain the phosphotriester (Scheme 5) (41).



**Scheme 5.** Synthesis of bis-acyloxybenzyl-derivatives of AZT-MP

### CycloSal-Pronucleotides

The cycloSal-pronucleotide concept involves nucleotide delivery based upon pH-driven selective chemical hydrolysis (Figure 7) (42,43). The tandem cleavage originates with the hydrolysis of a phenyl ester followed by hydrolysis of a benzyl ester of the phosphotriester. This concept is based upon the principle that the selection of phenyl, benzyl and alkyl phosphate esters can influence the hydrolysis steps of the tripartate approach (44). The phenyl ester is cleaved initially because of stabilization caused by delocalization of the negative charge in the aromatic ring yielding the 2-hydroxybenzylphosphodiester. The sequence of hydrolytic steps has been verified by multinuclear NMR spectroscopy and mass spectrometry (45,46). This concept was applied to anti-HIV agents such as d4T (44,46), 5-Furd (45), AZT (47,48), 2',3'-dideoxyadenosine (ddA) (49, 50), d4A (50) and 2'-fluoro-2',3'-dideoxyadenosines (F-ara-ddA and F-ribo-ddA)(51).

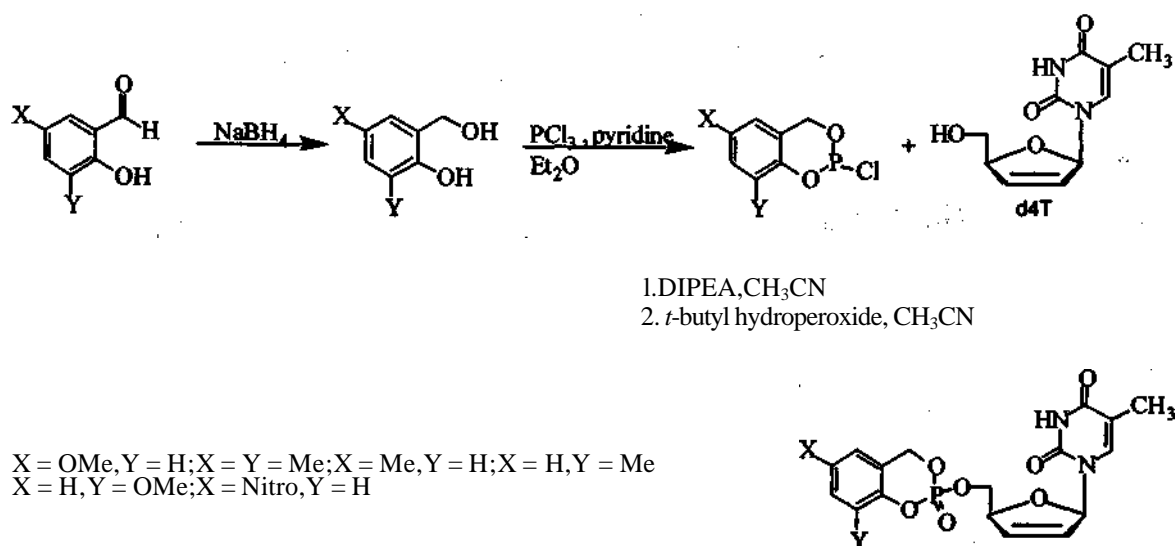


**Figure 7.** Proposed decomposition of cycloSal-pronucleotides

CycloSal-Pronucleotides have been successfully used to deliver mono-phosphates of d4T, ddA, F-ara-ddA and F-ribo-ddA. *In vitro* studies of cycloSal-nucleotides of d4T showed that 3-,5-methyl and 3, 5-dimethyl-cycloSal-d4TMPs had more potent anti-HIV activity than the parent compound (44).

More importantly, the potency of cycloSal-d4TMPs was maintained in thymidine kinase-deficient CEM (TKCEM) cells. The electron donating capacity of the ring substituent influenced the degree of biological activity with the stronger electron-donating group having the greatest potency. CycloSal-ddA was synthesized to circumvent deamination by adenosine deaminase (ADA) and adenosine monophosphate deaminase (AMPDA). Studies with ADA and AMPDA demonstrated that the cycloSal-triesters are not susceptible to enzymatic deamination (49) as reported earlier for 5'-*O*-protected adenosine (52). CycloSal-ddAMPs and cycloSal-d4AMP showed more potency than the respective parent compounds (49). In addition, this increase in potency was accompanied with a higher selectivity index than the parent compounds. CycloSal-derivatives of F-ara-ddA and F-ribo-ddA were stable in the presence of ADA and AMPDA and more potent than the parent compounds (51).

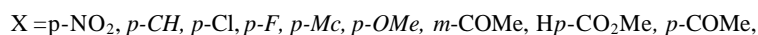
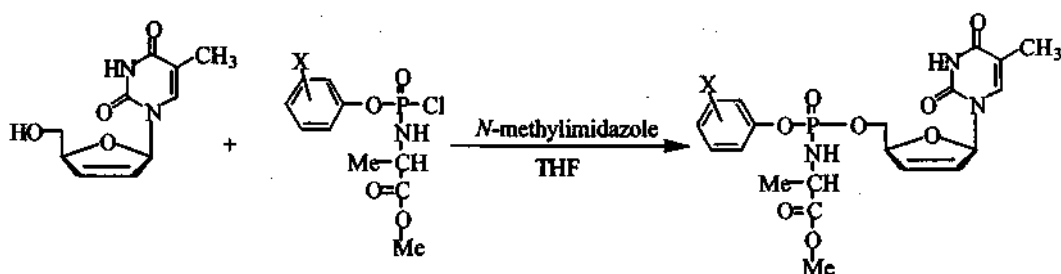
In the synthesis of cycloSal-d4T monophosphates (44), salicyl alcohols were obtained by first reduction of salicylaldehydes or salicylic acids using sodium borohydride or lithium aluminum hydride in 75-90% yields. The salicyl alcohols were reacted with phosphorus trichloride to obtain the cyclic saligenylchlorophosphanes. These were condensed with d4T in the presence of diisopropylethylamine and subsequently oxidized *in situ* by *t*-butylhydroperoxide (TBHP) to obtain the corresponding phosphotriesters in 50-73% yield (Scheme 6).



Scheme 6. Synthesis of cycloSal-d4TMP

### Phosphoramidate and Cyclic Phosphoroamidate Pronucleotides

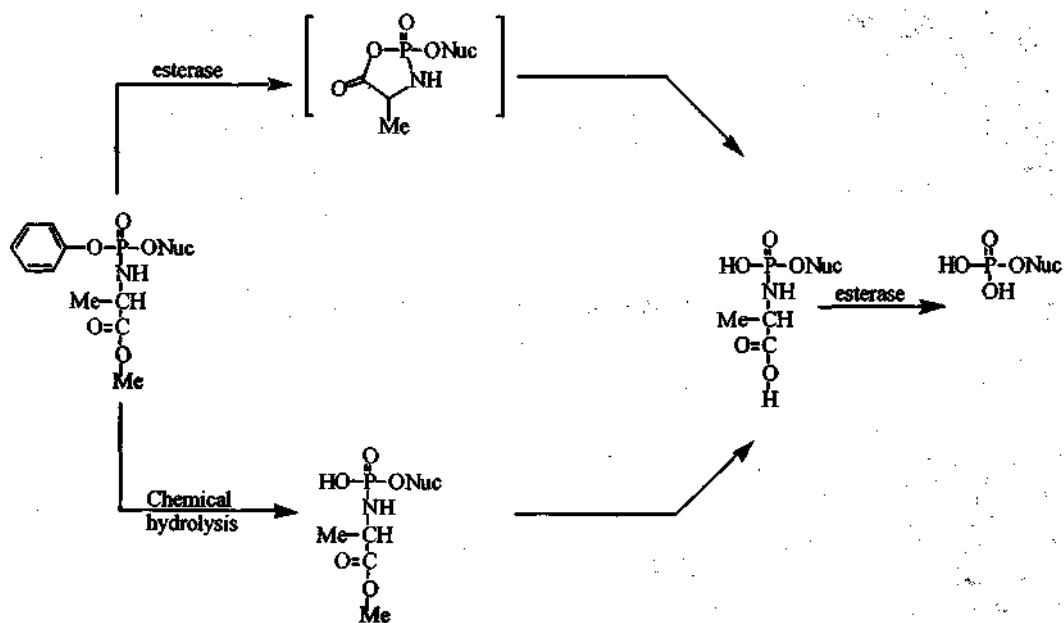
McGuigan *et al.* (53) designed and synthesized phosphoramidate pronucleotides as a mean of circumventing the membrane impermeability of negatively charged nucleotides (Scheme 7). Phosphoramidate derivatives of d4TMP (54-57), 2', 3'-dideoxy-3'-thiacytidine monophosphate (3TCMP) (58), AZTMP (59), (ddAMP) (60) and d4AMP (60,61) have been synthesized in an effort to enhance the delivery of their corresponding monophosphate. Delivery of the nucleotide analogue involves degradation of the prodrug by the liberation of the phenyl group or cleavage of the methyl ester, which ultimately leads to complete unmasking mediated by enzyme or chemical catalysis (Figure 8).



**Scheme 7.** Synthesis of phosphoramidate derivatives of d4T

*In vitro* studies of d4T phosphoramidate showed that the prodrug was potent against HIV-2 infected CEM and TK CEM cells, which supports evidence of efficient intracellular delivery of nucleotide (62). The prodrug of d4T was reported to be active against other retroviruses such as SIV, FIV, Visna Virus and Moloney murine sarcoma virus *in vitro* (63). Among various β-amino acid derivatives synthesized, the L-alanine provided the most efficacy as an anti-viral agent (63), while its enantiomer was 30 times less potent. *In vitro* studies indicated that phosphoramidates of 3TC and AZT provided no benefit as inhibitors of HIV replication (58, 59). In the case of ddA and d4A, the potency of the phosphoramidate prodrugs increased significantly (60). In addition, these phosphoramidates displayed anti-HBV activity equal in

potency to that of 3TC. Both aryloxyphosphoramidate prodrugs displayed a higher selectivity index in comparison with the parent compounds.



**Figure 8.** Decomposition of phosphoramidates

Five and six membered cyclic phosphoramidate derivatives were also used to aid in the delivery of nucleotide analogs (Figure 9). Jones *et al.* (64) found that 5'-O-3"-methyl-1", 3", 2"-oxazaphosphacyclopentan-2"-yl-thymidine 2"-oxide and other five membered cyclic phosphotriesters were very unstable at physiological pH. De Clerq *et al.* (65) synthesized 5'-O-3"-methyl-1",3",2"-oxazaphosphacyclopentan-2"-yl derivatives of acyclovir and (*E*)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) after finding that their hydrolysis rate may be dependent upon the buffer used (Scheme 8). The phosphoramidates of 5-fluoro BVDU were synthesized by Meyers *et al.* (66) and found to be inactive against TK<sup>+</sup> HSV-1 replication in rabbit kidney cells indicating no release of BVDU-monophosphate. Farquhar *et al.* (67) synthesized the more stable six membered cyclic phosphoramidate as means to delivery 5-FdUMP intracellularly (Scheme 9). Both prodrugs were resistant to enzymatic degradation by 5'-nucleotidase, alkaline phosphatase, venom phosphodiesterase and crude snake venom. The dioxaphosphorinanyl derivative of 5-FdUrd (X = O) showed virtually no activity in the mice model study.

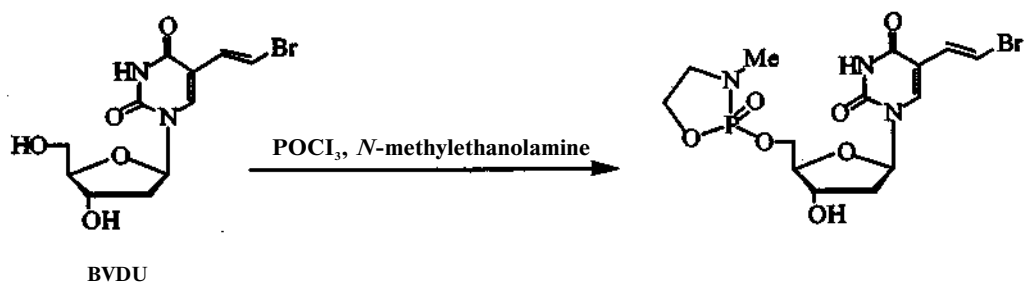
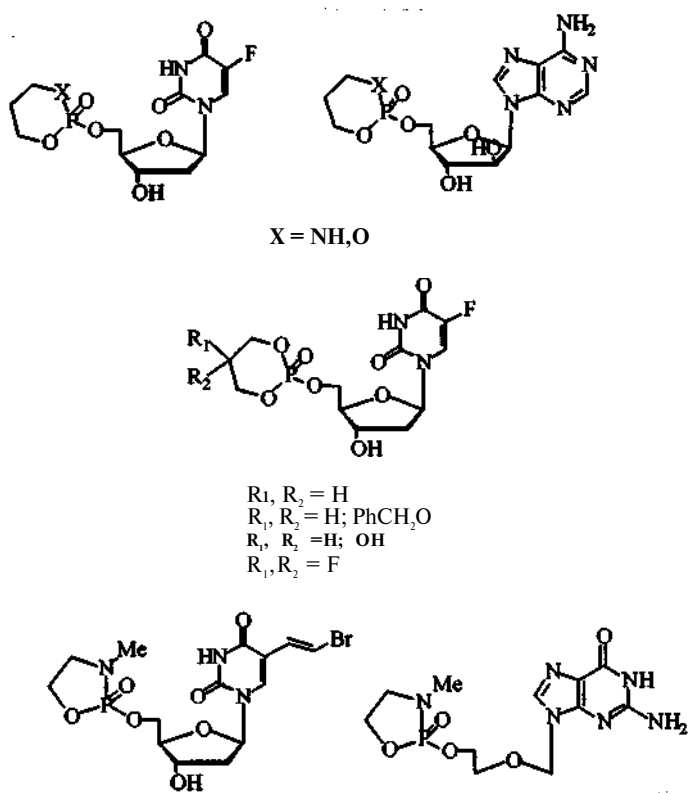
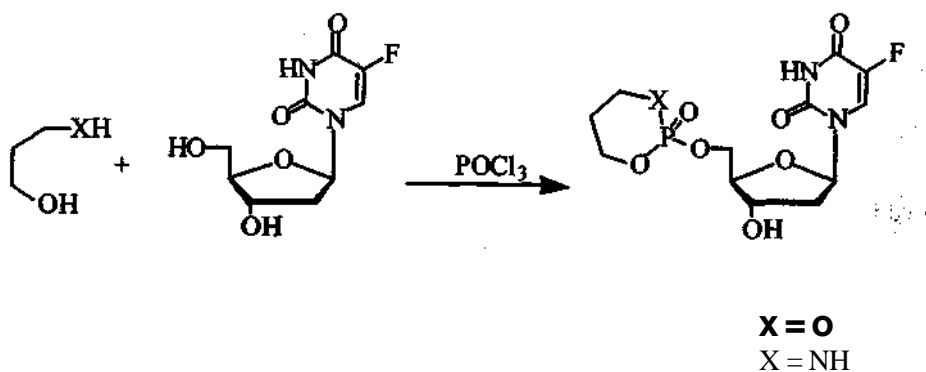


Figure 9. Oxazaphosphacyclic and dioxophosphacyclic nucleoside derivatives



Scheme 8. Synthesis of oxaphosphacyclopentanyl derivative of BVDU



**Scheme 9.** Synthesis of oxaza- and dioxaphosphacyclohexyl derivatives of 5-Furd

### Conclusions

Consequently, tripartate prodrugs provide more potent compounds comparing with bipartate prodrugs. Since tripartate prodrugs overcome the stability of the linkage between carrier and drug. But it is obvious that both prodrugs need further investigation to explain details of mechanism and get more potent prodrugs. These recent findings suggest us that it is inevitable to find new approach to design prodrugs as anti-viral and anti-cancer compounds.

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