*Original Articles*

# SYNTHESIS AND *IN VITRO* ANTIVIRAL STUDIES OF *BIS* (PIVALOYLOXYMETHYL) ESTER DERIVATIVE OF 9-{((PHOSPHONOMETHYL) AZIRIDIN-1-YL)METHYL} ADENINE (PMAMA) AND ANALOGUES

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إن المشتق الإستري بز (بيفالويلوكسي ميثيل) لمركب 9- [((فوسفونوميثيل) إزيريدين- ١- يل) ميثيل] أدينين (bis(POM)PMAMA)(المركب 1) هو نظير مادة أديفوفير ديبيفوكسيل ومادة تينوفوفير ديزوبروكسيل. وقد تم تحضير المركبات 2، و3، و 4 بنفس طريقة تحضيرالمركب 1. وتم اختبارها معملياً ضد عدد كبير من الفيروسات، وخصوصاً ضد الفيروس المناعي البشري - 1 (HIV-1)، وفيروسات التهاب الكبد الوبائي.

Bis(pivaloyloxymethyl) ester derivative of 9-{((Phosphonomethyl)aziridin-1-yl)methyl}adenine, (bis(POM)PMAMA) (**1**), is an analogue of adefovir dipivoxil and tenofovir disoproxil. Compounds **2**, **3**, and **4** were prepared in similar way to **1**.They were tested *in vitro* against a wide range of viruses, in particularly against HIV-1 and hepatitis viruses.

***Key words:*** Adefovir dipivoxil, Tenofovir disoproxil, HIV.

## Introduction

Adefovir, 9-(2-Phosphonomethoxyethyl) adenine (PMEA)) and Tenofovir, (9-(*R*)-(2-Phosphono-methoxypropyl)adenine (PMPA)) are the prototypes of acyclic nucleoside phosphonates (ANPs) with antiviral activity against a wide range of DNA viruses and retroviruses, including the human immunodeficiency virus (HIV). Adefovir dipivoxil (Hepsera®) and Tenofovir disoproxil fumarate (Viread®) are ANPs prodrugs of adefovir and Tenofovir, respectively. They were recently approved for the treatment of chronic hepatitis B and HIV infection, respectively (1-6).

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Numerous ANPs were synthesized and several structure-activity relationship (SAR) studies have been reported (Figure 1). (*S*)-9-(3-Hydroxy-2-(phosphonomethoxy)propyl)adenine, (*S*-HPMPA), an acyclic nucleotide analogue reported by Holy and De Clercq (7), is a representative of this class which possesses broad spectrum antiviral activity. (*S*) -1-(3 - Hydroxy - 2 - phosphonomethoxy -propyl) cytosine (HPMPC, Cidofovir) (Vistide®) has been approved and now is an antiviral agent for the treatment of CMV-retinitis in AIDS patients (Figure 1)(8). Adefovir (9-(2-Phosphonomethoxyethyl) adenine, (PMEA)) and Tenofovir (9-(*R*)-(2-Phosphono-methoxypropyl) adenine, (PMPA)) (Figure.1) are the prototypes of acyclic nucleoside phosphonates (ANPs) with antiviral activity against a wide range of DNA viruses and retroviruses, including the human immunodeficiency virus (HIV) (9,10).

Several compounds were synthesized and tested for *in vitro* antiviral activity, such as (*R*)-PMPG (11), (*R*)-8-aza-PMPG (12) and aziridinyl and aminophosphonates (a new class of ANPs) (13, 14)

(Figure.2) in the attempt to prepare potent antiviral agents.

The aziridinyl acyclic nucleoside phosphonates, in the free acid form, PMAMG, PMAMA, PMAMC, and PMAMT (Figure 2) showed no *in vitro* antiviral or antimicrobial activity (13). The lack of biological activity might be attributed to the replacement of the oxygen with nitrogen in the phosphonate side-chain or to the aziridinyl system. In the attempt to study the above mentioned hypothesis, investigations were conducted in two different directions; one was based upon the opening of the aziridinyl system in order to study the effect of the rigidity on the activity, and the second one was in the synthesis of the corresponding prodrugs to study the effect of the amino phosphornate moiety. Therefore, the acyclic nucleoside phosphonates in which the acyclic side chain has linear acyclic amino phosphonate moiety, compounds (**I**, **II**, **III**) **(**Figure. 2) were synthesized (14) and tested *in vitro* showed no antiviral activity (unpublished data). The absence of the antiviral activity observed from aziridinyl and amino phosphonates derivatives, may suggest the influence of the N-alkyl-α-aminophosphonate on the activity and reinforce the idea of masking the hydroxyl groups by lipophilic substituents. Indeed, one of the most major problems of ANPs is their low intracellular concentration, which affects negatively their activity, due to the formation of negative charges of the hydroxyl groups of the phosphonic moiety at physiologic pH which increase their polarities and drastically decrease their cellular membrane penetration. Prodrugs of ANPs were designed and synthesized to circumvent this problem by masking the hydroxyl groups of phosphonic acid moiety with neutral substituents form more lipophylic derivative capable of crossing the cellular membrane (15). For this purpose, *bis* (pivaloyloxymethyl)-PMEA)(POM-PMEA, adefovir dipivoxil, Hepsera®) (16, 17) (Figure 3), was synthesized and currently approved for the treatment of chronic hepatitis (18-20) (Figure 2). In addition, the isopropyloxy carbonyloxymethyl ester ofPMPA was synthesized(Tenofovir disoproxil fumarate, Viread®) and now is approved for the treatment of HIV infection (22)(Figure 3). Tenofovir disoproxil and Adefovir dipivoxil are converted *in vivo*, after diester hydrolysis to Tenofovir (PMPA) and Adefovir (PMEA), respectively, which then phosphorrylated byAMPkinase(23)and subsequently by cellular nucleoside diphosphate kinase (24) to their corresponding diphosphate active metabolites. The hydrophilicity problem is strongly pronounced in the case of the N-alkyl-α-aminophosphonates because they will be found under zwitter ions form at physiologic pH and so drastically enhancing their polarities which pushed us to think about the synthesis of their corresponding prodrugs. In this work, we report the synthesis of the lipophylic diester form, (*bis* (POM) PMAMA) (**1**), of PMAMA, as analogue of tenofovir disoproxil and adefovir dipivoxil and its analogue compounds **2**, **3**, and **4**.

## Materials and Method

*Chemistry:*

Infrared spectra were recorded on FT/IR JASCO 300 E. $^{1}H$ and $^{13}C$ magnetic resonance spectra were recorded on a Bruker Avance DPX 300 spectrometer and determined at 300 and 75 MHz, respectively. All spectra were determined in $CDCl_3$, DMSO-$d_6$, and chemical shifts are reported in δ units (ppm) relative to tetramethylsilane as an internal standard. All exchangeable protons were confirmed by the addition of $D_2O$. Silica gel 60 (Merck) (70-230 mesh) was used for column chromatography.

The synthesis of **1**, **2, 3,** and **4** was conducted, similar to the published procedure (16, 17) without using the hindered base; N,N`-dicyclohexylmorpholinecarboxamidine, by treatment of PMAMA, PMAMG, PMAMT and PMAMC with *n*-butylamine followed by addition of chloromethyl pivalate in anhydrous DMF at room temperature for 36 h as shown in **scheme 1**. PMAMA, PMAMG, PMAMT and PMAMC were prepared as reported in the published procedure (13).

**9 - { ( ( D*itert*butylcarboxyoxymethylphosphono - methyl)aziridin-1-yl)methyl}adenine (*bis* (POM) PMAMA) (1).** To a solution of 9-{(((Phosphono)methyl)-aziridin-1-yl)methyl}adenine (PMAMA) (1 g, 3.51 mmol) in anhydrous DMF (50 mL) under a nitrogen atmosphere, n-butylamine (0.085 g, 7.02 mmol). Chloromethylpivalte (1.058 g, 7.02 mmol) was then added to the resulted solution and the mixture of reaction was stirred at room temperature for 5 h and then filtered. The filtrate was distilled

under reduced pressure and the residue was chromatographed on silica gel using (Cyclohexane: Ethylacetate, 94:6). Compound **1** was obtained pure as yellow oil (15 %). TLC (Cyclohexane: Ethylacetate, 90:10): *R*f 0.63. IR (Neat): v max 3460 (br.), 2950, 1750, 1300, 1150, 1050 $cm^{-1}$. $^1H$ NMR ($CDCl_3$): δ 0.9-1.4 (m, 18 H, 2x$C(CH_3)_3$), 1.5 (m, 2 H, $CH_2N$), 1.57-2.7 (m, 2 H, $CH_2P$), 3.24 (m, 1 H, CHN), 4.18-4.27 (m, 6 H, $NCH_2$ and 2 x $OCH_2O$), 5.6 (br, s, $NH_2$), 7.53 (s, 1 H, H-8), 7.71 (s, 1 H, H-2). $^{13}C$ NMR ($CDCl_3$): δ 167.94, 132.69, 131.07, 129, 68.36, 38.96, 29.8 (d, $CH_2P$), 27.82, 23.97, 14.23, 11.16. Anal. Calcd. For $C_{21}H_{33}N_6O_7P$: C 49.22, H 6.49, N 16.4. Found: C 49.15, H 6.55, N 16.34.

**9 - { ( ( D*itert*butylcarboxyoxymethylphosphono - methyl) -aziridin-1-yl)methyl}guanine (*bis* (POM) PMAMG) (2).** The synthetic procedure is similar to that mentioned for compound **1** starting with 9-{ ( ( (Phosphono) methyl) – aziridin -1-yl)methyl} guanine (PMAMG). TLC (Cyclohexane: Ethylacetate, 70:30): *R*f 0.40. $^1H$ NMR (DMSO-*d*6): δ 1.23-1.5 (m, 18 H, 2x$C(CH_3)_3$), 1.48 (d, *J* = 6.2 Hz, 1H, $CH_2N$), 1.7 (d, *J* = 3.1 Hz, 1H, $CH_2N$), 1.97 (m,1H, CHN), 2.6 (dd, *J* = 8.7, 12.7 Hz, 1H, $CH_2P$), 2.8 (dd, *J* = 9.2, 13.3 Hz, 1H, $CH_2P$), 3.8 (dd, *J* = 3.8, 5.6 Hz, 1H, $NCH_2$), 3.95 (dd, *J* = 3.9, 5.7 Hz, 1H, $NCH_2$), 4.3 (m, 4 H, 2 x $OCH_2O$), 6.5 (br s, 2H, $NH_2$), 7.6 (s, 1H, H-8), 10.8 (br s, 1H, NH). $^{13}C$ NMR (DMSO-$d_6$): δ 170.5, 157.6, 153.7, 151.6, 138.9, 116.6, 72.0, 71.9, 62.2 (d, *J* = 163.2 Hz), 52.1, 42.3, 40.4, 24.2, 24.1, 23.95, 23.90, 15.83, 12.25. Anal. Calcd. For $C_{21}H_{33}N_6O_8P$: C 47.27, H 6.24, N 15.89. Found: C 47.15, H 6.35, N 16.0.

**1 - { ( ( D*itert*butylcarboxyoxymethylphosphono-methyl) aziridin -1-yl)methyl}thymine (*bis* (POM) PMAMT) (3).** The synthetic procedure is similar to that mentioned for compound **1** starting with 1-{ ( ( ( Phosphono) methyl)-aziridin-1-yl) methyl} thymine (PMAMT). Compound **3** was obtained pure as colorless oil (25 %). TLC (Cyclohexane: Ethylacetate, 90:10): *R*f 0.7 $^1H$ NMR ($CDCl_3$): δ 0.95-1.3 (m, 18 H, 2x$C(CH_3)_3$), 1.48 (d, *J* = 6.4 Hz, 1H, $CH_2N$), 1.65 (m,1H, CH-N), 1.76 (d, *J* = 3.4 Hz, 1H, $CH_2N$), 1.90 (s, 3H, $CH_3$), 2.5 (dd, *J* = 8.9, 13.1 Hz, 1H, $CH_2P$), 2.65 (dd, *J* = 9.1, 13.3 Hz, 1H, $CH_2P$), 3.85 (dd, *J* = 3.9, 5.7 Hz, 1H, $NCH_2$), 4.0 (dd, *J* = 4.1, 6.0 Hz, 1H, $NCH_2$), 4.27 (m, 4H, 2x$OCH_2O$), 7.2 ( s, 1H, H-6), 8.7 ( s, 1H, NH). Anal. Calcd. For $C_{21}H_{34}N_3O_9P$: C 50.11, H 6.75, N 8.34. Found: C 50.25, H 6.65, N 8.21.

**1 - { ( ( D*itert*butylcarboxyoxymethylphosphono - methyl) ) – aziridin – 1 - yl)methyl } cytosine (*bis* (POM) PMAMC) (4).** The synthetic procedure is similar to that mentioned for compound **1** starting with 1-{(((Phosphono)methyl)-aziridin-1-yl)methyl} cytosine (1 g, 3.84 mmol) in anhydrous DMF (50 mL) under a nitrogen atmosphere, n-butylamine (0.093 g, 7.68 mmol) was added and at the resulted solution was then added chloromethylpivalte (1.157 g, 7.68 mmol). The mixture of reaction was stirred at room temperature for 5 h and then filtered. The solvent was removed under reduced vacuum pressure and the residue was chromatographed on silica gel using (Cyclohexane: Ethylacetate, 90:10). Compound **4** was obtained pure as yellow oil (20 %). TLC (Cyclohexane: Ethylacetate, 90:10): *R*f 0.45. $^1H$ NMR ($CDCl_3$): δ 1.0-1.4 (m, 18 H, 2x$C(CH_3)_3$), 1.53 (d, *J* = 6.4 Hz, 1H, $CH_2N$), 1.7 (m,1H, CH-N), 1.8 (d, *J* = 3.4 Hz, 1H, $CH_2N$), 2.52 (dd, *J* = 8.9, 13.1 Hz, 1H, $CH_2P$), 2.75 (dd, *J* = 9.1, 13.3 Hz, 1H, $CH_2P$), 3.6 (dd, *J* = 3.9, 5.7 Hz, 1H, $NCH_2$), 4.18 (dd, *J* = 4.1, 6.0 Hz, 1H, $NCH_2$), 4.37 (m, 4H, 2x$OCH_2O$), 7.2 (d, J = 7.05 Hz, 1H, H-5), 7.45 (d, J = 7.05 Hz, 1H, H-6), 8.7 ( br s, 2H, $NH_2$). Anal. Calcd. For $C_{20}H_{33}N_4O_8P$: C 49.17, H 6.75, N 11.46. Found: C 49.15, H 6.85, N 11.34.

*Biological determination*:

Test compounds were dissolved in DMSO at an initial concentration of 200 μM and then were serially diluted in culture medium. MT-4 cells (grown in RPMI 1640 containing 10% foetal calf serum (FCS), 100 UI/mL penicillin G and 100 μg/mL streptomycin) were used for anti-HIV-1 assays. The 2.2.15 cells line (clonal cells derived from HepG2 cells that were transfected with a plasmid containing (HBV DNA)) grown in DMEM supplemented with 4% foetal calf serum, 100 IU/mL penicillin G and 100 μg/mL streptomycin and 0.5 mM glutamine were used for anti-HBV assays.

*Anti-HIVAssay*:

Activity against HIV-1 (HIV-1, $III_B$ strain, obtained from supernatants of persistently infected

$H9/III_B$ cells.) multiplication in acutely infected cells was based on inhibition of virus-induced cytopathogenicity in MT-4 cells. Briefly, 50 µL of RPMI 10% FCS containing $1x10^4$ cells were added to each well of flat-bottomed microtiter trays containing 50 µL of medium and serial dilutions of test compounds. 20 µL of an HIV-1 suspension containing 100 $CCID_{50}$ were then added. After a 4 day incubation at 37 °C, the number of viable cells was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method. Cytotoxicity of compounds, based on the viability of mock-infected cells as monitored by the MTT method, was evaluated in parallel with their antiviral activity.

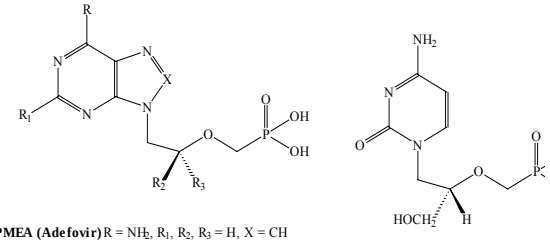
*Anti-HBV Assay*:

As previously described (25), cells were cultured for 11 days in the presence of drug with medium changes every 3 days. At the end of the subsequent 3-day period, an aliquot of the culture medium was harvested and processed to obtain extracellular HBV-DNA by slot blot analysis.

For intracellular HBV DNA analysis cells were lysed (10 mM Tris-HCl pH 7.5, 5mM EDTA, 150 mM NaCl, 1%SDS). Total intracellular DNA was extracted; HBV DNA was digested with HindIII restriction endonuclease, separated by electro-phoreses and transferred to a nylon membrane. Filters from Slot and Southern blot were hybridized with a HBV-specific probe, prepared from a full length HBV DNA genome template excised from plasmid. Quantification was performed on a Personal Molecular Imager FX (Bio-Rad). For each compound the 50% effective concentration ($EC_{50}$) was determined in duplicate 24-well plates by plaque reduction assays. Cell monolayers were infected with 100 PFU/well of virus. Then, serial dilutions of test compounds in medium supplemented with 2% inactivated serum and 0.75% of methyl cellulose were added to the monolayers. Cultures were further incubated at 37°C for 3 days, and then fixed with 50% ethanol and 0.8% Crystal Violet, washed and air-dried. Then plaques were counted.

## Results and Discussion

In attempt to complete the study of the structure activity relationship of the N-alkyl aminophospho-nates type of ANPs class. Compounds **1, 2, 3,** and **4** were evaluated *in vitro* for cytotoxicity and antiviral activity against Human Immunodeficiency Virus type 1 and Hepatitis B Virus. They have shown no cytotoxicity and were resulted inactive against HIV-1 and Hepatitis B Virus. Zidovudine (AZT) was used as a reference drug in the anti HIV assay and Lamivudine (3TC) as a reference drug in the anti HBV, and they both confirmed their activity in the respective assays. The lack of antiviral activity could be explained by the absence of the oxygen atom in the phosphonate side-chain. Indeed, this is might be confirmed because the acyclic amino phosphonates, which are resulted by the opening of the aziridinyl system in attempt to study the effect of rigidity on antiviral activity in precedent work, were demonstrated inactive. Also, when the doubt of the polarity concept was eliminated by the realization of compounds **1, 2, 3,** and **4** more lypophilic; the antiviral inactivity enhances and improves the hypothec of oxygen atom lacking in the phosphonate side chain which may, furthermore, confirm the essentiality of the oxygen presence in the phosphonate side chain on the activity.



**PMEA (Adefovir)** R = $NH_2$, $R_1$, $R_2$, $R_3$ = H, X = CH
**PMPA (Tenofovir)** R = $NH_2$, $R_1$, $R_2$ = H, $R_3$ = $CH_3$, X = CH
**(R)-PMPG** R = OH, R = $NH_2$, $R_2$ = H, $R_3$ = $CH_3$, X = CH
**(R)-8-aza-PMPG** R = OH, R = $NH_2$, $R_2$ = H, $R_3$ = $CH_3$, X = N
**(S)-HPMPA** R = $NH_2$, $R_1$, $R_2$ = H, $R_3$ = $CH_2OH$, X = CH

**HPMPC [Cidofovir, Vistic**

**Figure 1.** Structural formulas for some ANPs. HPMPC is in clinical uses.

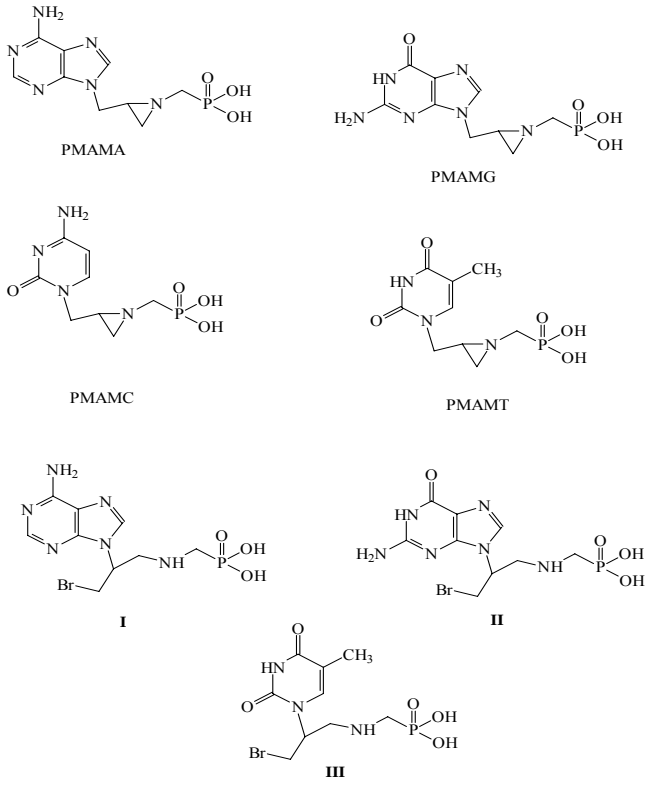



**Figure. 2**: Aziridinyl and acyclic amino phosphonate structures.

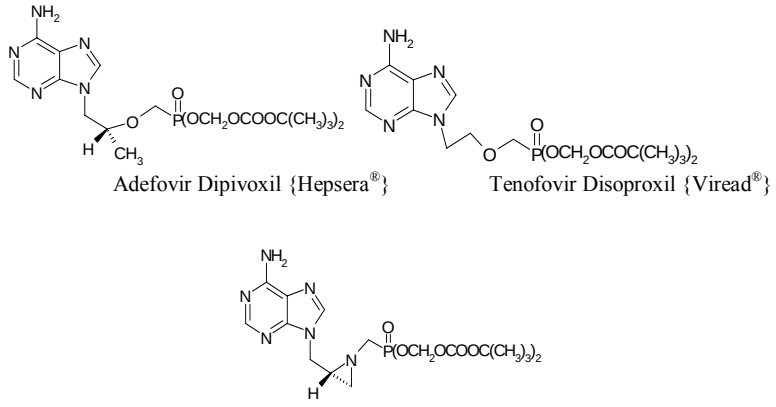
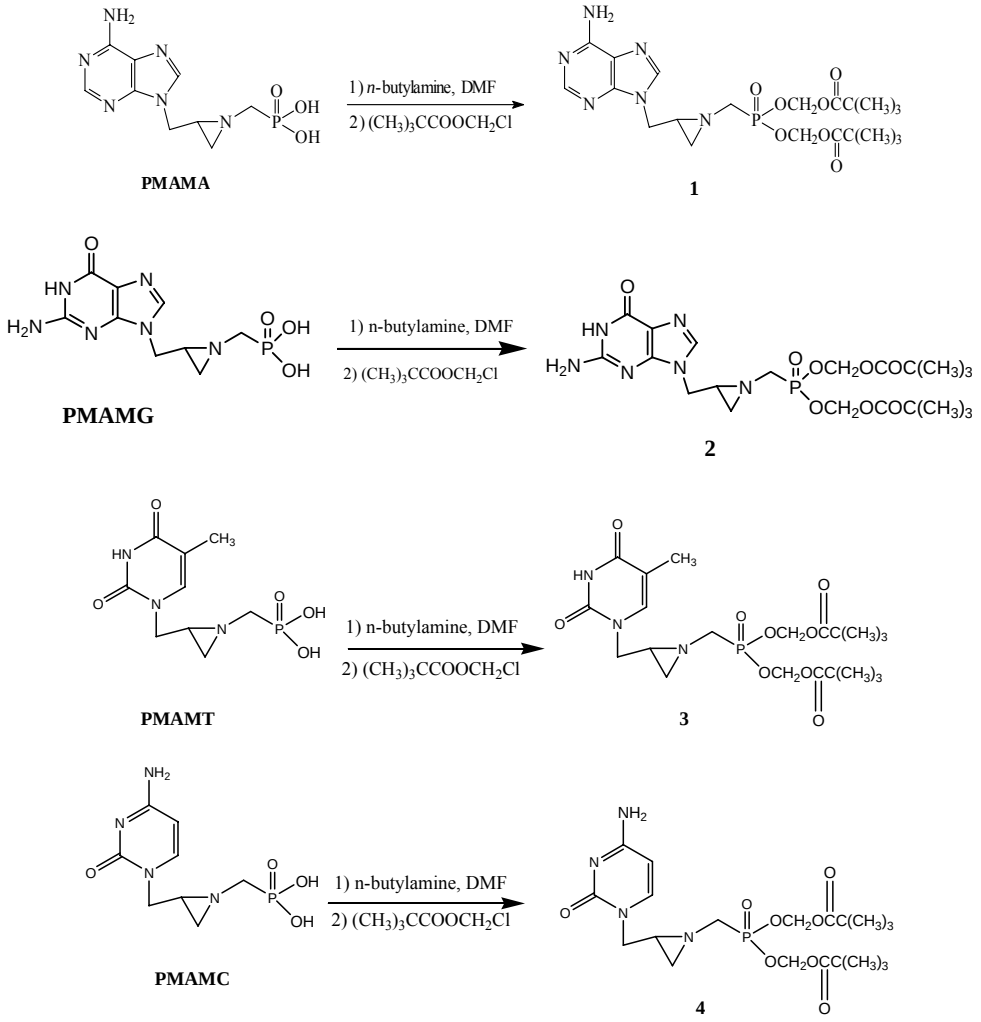



**Figure. 3:** Acyclic nucleoside phosphonate prodrugs. Hepsera and Viread are in clinical uses.




**Scheme 1**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