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Phosphonated Nucleoside Analogues as Antiviral Agents

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Abstract The present review is focused on the description of synthesis and antiviral activities of both acyclic and carbocyclic nucleoside phosphonates, endowed with an antiviral potential. Despite the outstanding results in antiviral therapy of acyclovir and azidothymidine, a major drawback concerning the use of nucleoside analogues (NA) is the retention of their stability following triphosphorylation within the host cell. The instability of the phosphate forms of NA has been, at least partially, overcome by the introduction of phosphate groups in the molecular structure. This approach gives rise to two main classes of compounds endowed with ascertained or potential antiviral activity, such as acyclic nucleoside phosphonates (ANP) and phosphonated carbocyclic nucleosides (PCN). Regarding ANP, a higher affinity for HIV reverse transcriptase (RT), with respect to NA, and the potent inhibition of HIV and hepatitis B virus (HBV) have been reported

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for some of them. Regarding PCN, some phosphonated cyclopropyl and cyclopentyl carbanucleosides, characterized by the presence of one or more phosphonic groups and by replacement of the endocyclic oxygen atom with a methylene group, showed to be good inhibitors of HBV and HIV infection. Another class of PCN is represented by phosphonated N,O-nucleosides (PCOAN). PCOAN encompass homo phosphonated-, phosphonated- and truncated phosphonated-N,O-nucleosides. Some PCOAN have been shown to directly inhibit RT activity of both murine and human retroviruses and to block HTLV-1 infection in vitro. The flexibility of the phosphonated NA structure suggests the possibility to develop new analogues endowed with antiviral activity towards a broad range of DNA or RNA viruses.

Keywords Acyclic nucleoside phosphonates (ANP), Antivirals, HBV, HCV, HIV, HTLV-1, HSV, Phosphonated carbocyclic nucleoside (PCN), Phosphonated N,O-nucleosides (PCOAN)

Contents

Abbreviations

3TC, lamivudine	(−)-L-3′-thia-2′,3′-dideoxycytidine
ABC, abacavir	Cyclopentenyl-N^6-cyclopropylaminopurine
ACV	Acyclovir
adefovir	PMEA
AdF	(5′S)-5-fluoro-1-isoxazolidin-5-yl-1H-pyrimidine-2,4-dione

ADV	Adefovir dipivoxil
ANP	Acyclic nucleoside phosphonates
APC	Apricitabine
ATL	Adult T-cell leukemia
AZT, zidovudine	3′-Azido-3′-deoxythymidine
BMS986001	Festinavir
CMV	Cytomegalovirus
d4T, stavudine	2′,3′-Didehydro-2′,3′-dideoxythymidine
ddC, zalcitabine	2′3′-Dideoxycytidine
ddI, didanosine	2′3′-Dideoxynosine
ddNMP	Dideoxynucleotide monophosphate
EBV	Epstein–Barr virus
ETC, entecavir	BMS-200475
Fd4AP	5-[(6-Amino-purin-9-yl)-4-fluoro-2,5-dihydro-furan-2-yloxymethyl] phosphonic acid disodium salt (50)
FPMPA	2,6-Diaminopurine
[(−) FTC, emtricitabine]	(−)-L5-fluoro-3′-thia-2′,3′-dideoxycytidine
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDP	Hexadecyloxypropyl
HAM/TSP	HTLV-1 associated-myelopathy/tropical spastic paraparesis
HMCV	Human cytomegalovirus
hOAT1 and hOAT3	Human renal organic anion transporters types 1 and 3
HPMPC, cidofovir	(S)-1-(3-Hydroxy-2-phosphonylmethoxypropyl)cytosine
HSV	Herpes simplex virus
HTLV-1	Human T-cell leukemia/lymphotropic virus type 1
MRP4	Multidrug resistance protein 4
NA	Nucleoside analogues
NNRTI	Non-nucleoside reverse-transcriptase inhibitors
NRTI	Nucleoside reverse-transcriptase inhibitors
ODE	Octadecyloxyethyl
PBMC	Peripheral blood mononuclear cells
PCN	Phosphonated carbocyclic nucleosides
PCOAN	Phosphonated N,O-nucleosides
PMCG	9-[1-(Phosphonomethoxycyclopropyl) methyl] guanine
PMCG	9-[1-(Phosphonomethoxycyclopropyl)methyl]guanine 55
PMEO-DAPy	2,4-Diamino 6-[2-(phosphonomethoxy)ethoxy]pyridine
PMPA	9-(2R,S)-2-phosphonylmethoxypropyl derivatives of adenine
R7128	Mericitabine
TDF	Tenofovir disoproxil fumarate
TPCOAN	Truncated phosphonated N,O-nucleosides 119
VZV	Varicella-zoster virus

1 Introduction

The continuous effort in the development of new antiviral agents is the consequence of the urgent demand for new therapeutic agents in which an improved biological activity against viruses is assisted by a low toxicity towards the host cell. The aim of this review is to summarize the most recent knowledge in literature regarding the synthesis and the biological activity of nucleoside analogues characterized by the presence of one or more phosphonate groups and by a well-proved or a potential antiviral activity. However, suitable reference to the past studies on the structure, the activity and the therapeutic efforts, regarding the wider and prototype class of pharmacologically active antiviral agents represented by the nucleoside analogues (NA), will be also reported.

2 Nucleoside Analogues as Antiviral Agents: An Historical Overview

The term NA refers to compounds in which structural modifications of the heterocyclic bases and/or the sugar moiety of natural nucleosides have been performed (Fig. 1). The development of NA as antivirals achieved a fundamental importance when acyclovir (ACV), an acyclic nucleoside analogue where the sugar unit is lacking, was found to be the first drug able to efficiently counteract herpes simplex virus (HSV) type 1 and 2 infection, in the 1970s. ACV is a guanosine analogue which is activated following phosphorylation promoted firstly by virus thymidine kinase, encoded by UL23 gene of HSV, and successively by cellular kinases (Fig. 2) [1].

In addition, other nucleoside analogues such as the prodrugs valaciclovir, valganciclovir, famciclovir and foscarnet have been clinically used for infections sustained by other components of the herpes virus family, encompassing also varicella-zoster virus (VZV) and cytomegalovirus (CMV) (for a complete review see [1]).

A remarkable input to the research on NA is derived, then, from the urgent need to find a therapeutic approach to withstand human immunodeficiency virus (HIV) infection. In the 1980s, the research on antiretrovirals was highly fruitful and in 1986 the in vitro anti-HIV activity of the prototype antiretroviral drug, 3′-azido-3′-deoxythymidine (AZT, zidovudine), was demonstrated by Mitsuya and Broder [2, 3]. The drug was originally intended to treat cancer, but it was associated with a high side effect profile. In 1987 AZT became the first drug to be approved by the Food and Drug Administration (FDA) for the treatment of AIDS and HIV infection. The designation “nucleoside analogue” was successively adopted for all structurally similar antiviral compounds, derived from the structural similarity of AZT to the building blocks of nucleic acids constituting DNA and RNA. AZT differs from the thymidine by the replacement of the hydroxyl groups in the 3′ position with a $–N_3$

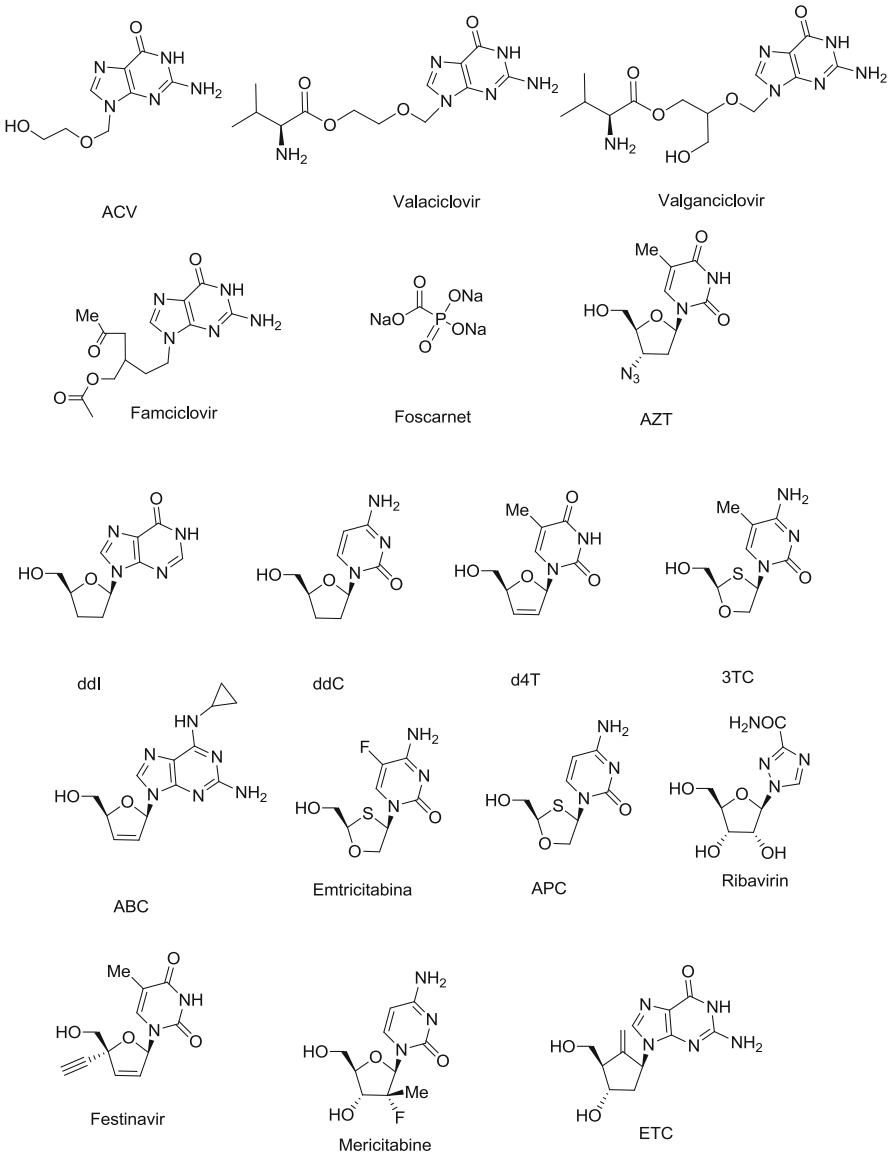


Fig. 1 Molecular structures of representative nucleoside analogues

group, which is unable to form the 5′-3′ phosphodiester linkage, essential for DNA elongation by DNA polymerases, including HIV reverse transcriptase (RT). This effect justifies the definition of AZT and other drugs as belonging to the same class of nucleoside reverse-transcriptase inhibitors (NRTI). The putative target of NRTI is the HIV-1 RT, an error-prone DNA polymerase, biochemically distinct from cell DNA polymerases [4]. In order to be properly incorporated by HIV-RT, NRTI must be

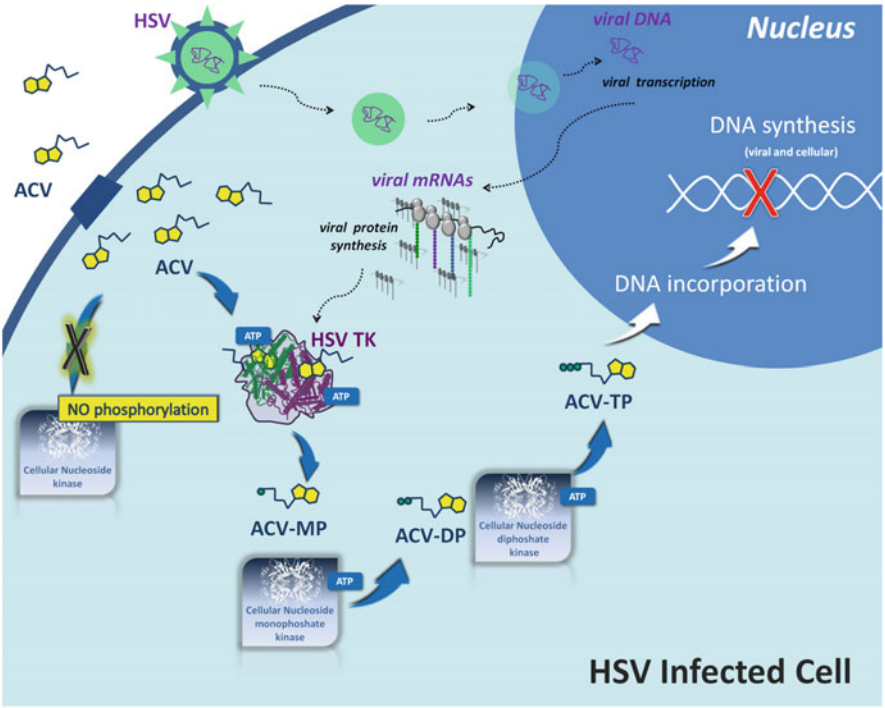


Fig. 2 Phosphorylation steps of acyclovir. The acyclic guanosine analogue acyclovir (ACV) is activated into the triphosphate compound (ACV-TP), in herpes simplex virus (HSV) infected cells, by both viral and cellular kinases. The first phosphorylation step is performed exclusively by HSV thymidine kinase (HSV TK). Successively, acyclovir monophosphate (ACV-MP) is transformed into acyclovir diphosphate (ACV-DP) and ACV-TP by host cell kinases

phosphorylated to their active 5′ triphosphate forms by cellular kinases, and this process might be influenced by the cell cycle phase of the host cell [5]. The 5′-triphosphate forms of NRTI are incorporated into DNA chain as dideoxynucleotide monophosphate (ddNMP) after the removal of the β- and the γ-phosphate groups.

From the discovery of AZT, a number of NRTI have been designed, sharing structural similarities with each other and mimicking endogenous nucleosides, such as 2′3′-dideoxynosine (ddI, didanosine), 2′3′-dideoxycytidine (ddC, zalcitabine), 2′, 3′-didehydro-2′,3′-dideoxythymidine (d4T, stavudine), and (−)-L-3′-thia-2′, 3′-dideoxycytidine (3TC, lamivudine), cyclopentenyl-N^6-cyclopropylaminopurine (ABC, abacavir), (−)-L-5-fuoro-3′-thia-2′,3′-dideoxycitidine [(−) FTC, emtricitabine] [6]. NRTI are used also in combination among them, trizivir (ABC, AZT, 3TC) or combivir (AZT, 3TC), or in combination with non-nucleoside reverse-transcriptase inhibitors (NNRTI). More recently, a novel NA, apricitabine (APC), a deoxycytidine analogue, was approved for HIV −1 infection treatment. Its structure is similar to that of

lamivudine, but APC was found to be active versus HIV strain bearing mutation M184 that confers resistance to lamivudine and emtricitabine [7, 8]. Even more recently, festinavir (BMS986001), a thymidine analogue similar to stavudine, was found to be less toxic [9].

A special mention in the chronology of NA deserves also ribavirin, one of the most used ribonucleoside analogue used in therapy. The mechanism of action of ribavirin is not unique and is not entirely clear. However, it is generally assumed that the antiviral activity of ribavirin is due to its competition with the guanosine triphosphate intracellular pool. Ribavirin is efficacious towards both DNA viruses (herpesviruses, adenoviruses, and poxviruses) and RNA viruses (HIV, influenza A virus, hepatitis C virus, respiratory syncytial virus) [10]. The combination of ribavirin with pegylated-interferon α is the backbone treatment in patients with hepatitis C virus (HCV) infection, reaching a sustained virological response in 75% of the treated individuals with genotypes 2 or 3. Other NA compounds, the synthesis of which and first evidence for antiviral activity date back at least 15 years ago, only recently have been demonstrated to be endowed with an actual therapeutic efficacy. The BMS-200475 (ETC, entecavir), a ribonucleoside analogue, was used for hepatitis B virus (HBV) infection. It is a cyclopentyl guanine, with an exo carbon-carbon double bond, that has been shown to be an efficacious and selective inhibitor of HBV polymerase in cultured liver cells [11]. Inhibition occurred following triphosphorylation of the compound in mammalian cells by cellular enzymes [12]. Thus, ETC acts as a structural terminator, presumably by introducing what is called "structural distortion" to preclude the enzyme from optimal interaction with the 3′ end of the growing DNA chain. More recent studies have shown that ETC is effective in both NA-naive and, at least partially, NA-experienced chronic hepatitis B patients [13].

The development of new therapies for the treatment of HCV infection is another intensive area of research for NA. In particular, high effort has been pursued in the research on inhibitors of one of the most important target in HCV, i.e., NS5B RNA-dependent RNA polymerase. A number of NA are in the pipeline such as mericitabine (R7128), which is the prodrug of PSI-6130, under clinical development for HCV chronic infection [14]. It is a ribonucleoside, (2′*R*)-2′-deoxy-2′-fluoro-2′-methylcytidine, which upon phosphorylation into 5′ triphosphate form inhibits HCV NS5B, acting as non-obligate chain terminator. Moreover, compounds in which the oxygen atom of the ribose unit is replaced by alternative heteroatoms, such as azaribonucleosides, have been reported to be endowed with antiviral activity in the cell-based HCV replicon assay [15].

In addition to HIV, HBV and HCV infections, NA were found to have some activity also towards infections sustained by other viruses. For example, a prodrug of the ribonucleoside analogue, 2′-fluoro-2′-deoxy-uridine and their corresponding phosphoramidates, was recently found to be efficacious in influenza virus infection [16].

Although treatments with NA have remarkably changed the course of virus infections, and particularly of HIV infection, the side effects of NA unavoidably influence the response to therapy. The toxicity of NA varies for each analogue and

relies on different markers of damage and tissue specificity [17]. Experimental models revealed that mitochondria were a major target of NA toxicity, showing disruption of a broad range of mitochondrial functions. Moreover, not less important toxic effects were also reported [18–22]. A complex double-edged, both inducing and inhibitory, regulatory action on apoptosis controlling genes has been recently reported for AZT [23].

Besides the toxicity, another major drawback of antiviral therapy with NA is the development of resistance. In the case of HIV infection, HIV-RT is highly prone to accumulate errors due to its lack of proofreading properties, thus generating mutated viruses [24]. Accumulation of one or more mutations in HIV-RT could lead to resistance of the virus to drug treatment. Two are the main mechanisms underlying the resistance to anti-HIV NA. One is the decreased incorporation of the triphosphorylated forms of NA into the growing DNA chain in comparison with normal dNTP or the decreased binding of the same activated forms to RT in competition with natural dNTP. Both effects are defined as discrimination effect.

The second mechanism of resistance is associated with the increased excision of NA from the DNA chain due to a process opposite to polymerization, defined as pyrophosphorolysis.

The outcome of resistance mutations was greatly favored by monotherapy and antagonized by combination therapy. However, despite the important results achieved by the latter, it still contributes to a number of mutations that accelerate the outcome of multi-drug-resistant HIV strains. In addition, also suboptimal therapy might select resistant virus strains.

In the following sections of this review we will focus the attention on the synthesis and the biological activities of acyclic nucleoside phosphonates (ANP), and cyclopropyl, cyclopentyl, and cyclohexyl carbanucleoside phosphonates, endowed with an antiviral potential, paying particular attention to compounds that appeared in literature in the last decade.

3 Acyclic Nucleoside Phosphonates

One of the metabolic drawbacks of NA is the retention of their stability following the triphosphorylation inside the host cell. To overcome the instability of triphosphate NA, several strategies have been proposed to increase their resistance towards the phosphohydrolase or to ensure a more efficient phosphorylation within the target cells. These approaches have led to the design of new classes of nucleotide prodrugs. One of the first described family of nucleotide analogues is represented by the ANP, which contain a phosphonate group linked to the acyclic chain. The development of ANP represents one of the remarkable progresses in the research on antiviral agents. In the phosphonated form these compounds are able to bypass the initial enzymatic phosphorylation step, undergoing only two phosphorylation steps in the host cell and showing resistance to pyrophosphorolysis [25]. In addition, ANP were found to have a higher affinity for RT with respect to NA, acting as potent inhibitors of infections sustained by both hepatitis and immunodeficiency

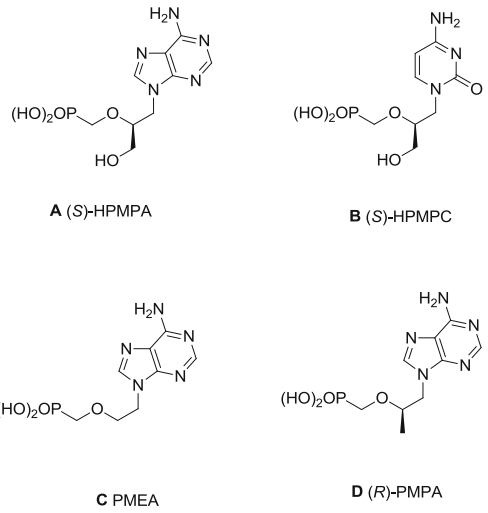


Fig. 3 Acyclic nucleoside phosphonates

viruses. ANP are converted by kinases into the corresponding diphosphorylated phosphonates and interfere with nucleic acid biosynthesis acting as DNA chain terminators.

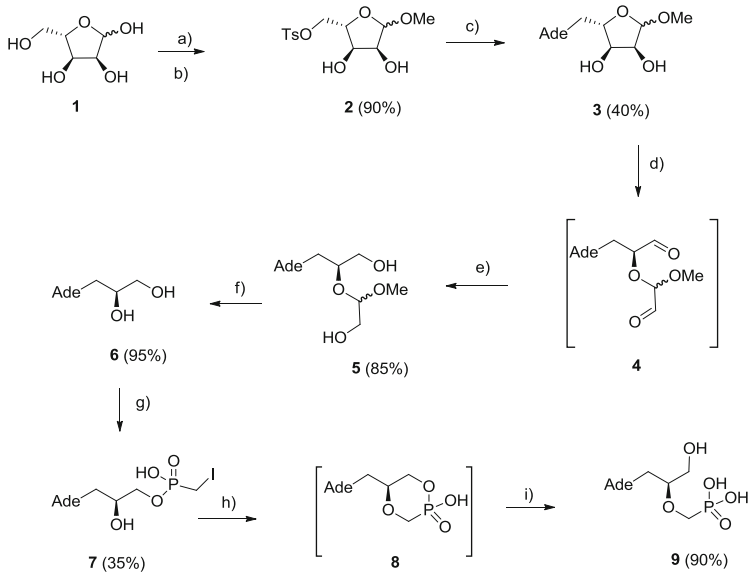
The structure of four ANP prototypes, designed with letters from **A** to **D**, is reported in Fig. 3.

3.1 Compounds of Structure A

The (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine, (*S*)-HPMPA, opened the era of ANP [26–28]. HPMPA is endowed with a strong activity against a variety of DNA viruses, including HSV-1, HSV-2, VZV, CMV, poxviruses, adenoviruses, and HBV. A recent synthetic approach towards (*S*)-HPMPA and its enantiomer starts from L(+)- or D(−)-ribose, respectively [29]. L(+)-ribose **1** was converted into its 5-tosyl derivative **2** and coupled with adenine, to afford the L-9-(*α*,*β*-1-methyl-5-ribofuranosyl)adenine **3**. This product was oxidized to dialdehyde **4** with sodium periodate, reduced with $NaBH_4$ to give **5**, which by treatment with formic acid afforded the diol **6**. The subsequent reaction with iodomethylphosphonic acid, with the formation of **7**, is followed by an intramolecular cyclization to **8**, from which **9** has been isolated, after treatment with water (Scheme 1).

The corresponding enantiomer (*R*)-HPMPA was similarly obtained from D(−)-ribose.

The activity against *CMV* and adenovirus is markedly increased when **9** is transformed into the hexadecyloxypropyl (HDP) **15** or octadecyloxyethyl (ODE) **16** esters. Noteworthy, while (*S*)-HPMPA is virtually inactive against HIV-1, HDP, and ODE, esters are active in nanomolar range and show also activity against HCV replication with EC_{50} of about 1 μM.



Scheme 1 Reagents and conditions: (*a*) AcCl/MeOH, RT, 16 h; (*b*) TsCl/Py, 4°C, 16 h; (*c*) adenine, NaH, DMF, 80°C, 5 days; (*d*) $NaIO_4$, 4°C, 1 h; (*e*) $NaBH_4$, 4°C, 3 h; (*f*) H^+, 6 h; (*g*) $ICH_2PO(OH)_2$/DCC/Py, 16 h; (*h*) NaH/DMF, 6 h, H_2O, 3 h; *i*) glacial AcOH, 3 h

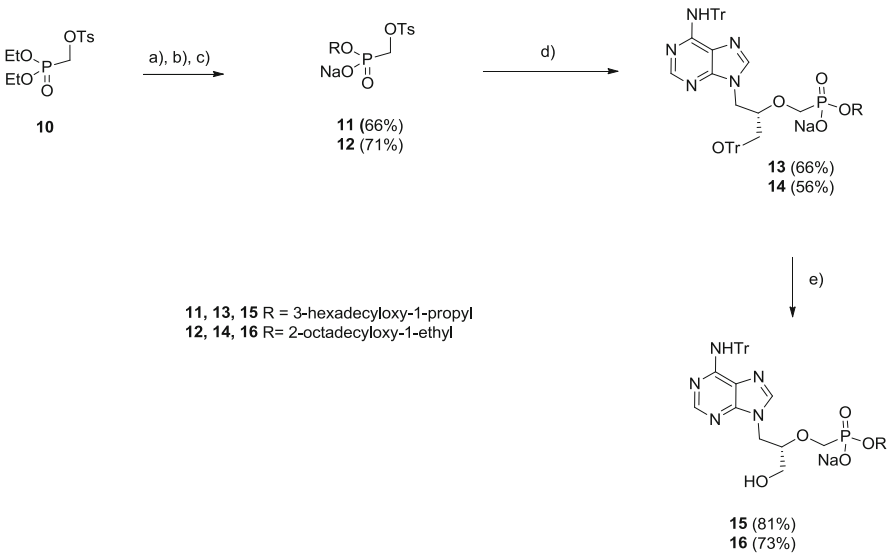
The synthesis of ODE-(*S*)-HPMPA and HDP-(*S*)-HPMPA has been described by Hostetler and Beadle et al. [30] and it is reported in Scheme 2.

3.2 *Compounds of Structure B*

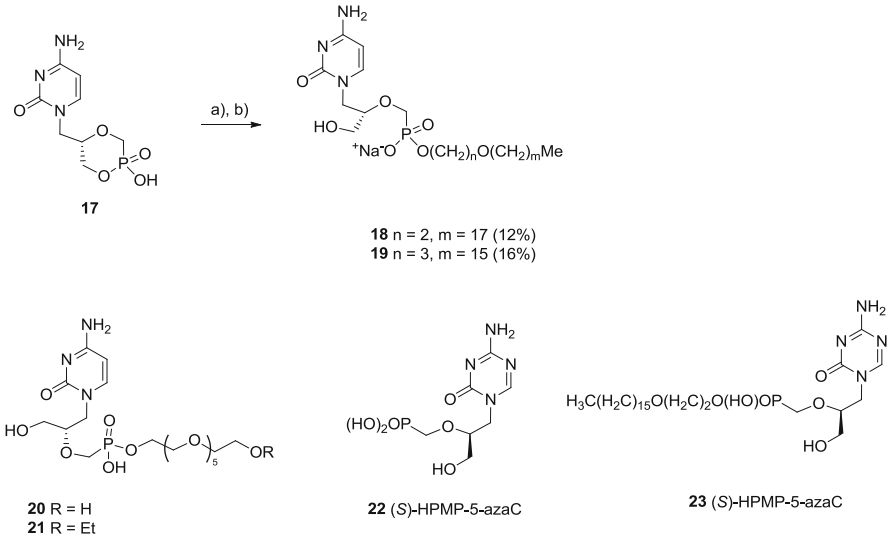
(*S*)-1-(3-Hydroxy-2-phosphonylmethoxypropyl)cytosine B (HPMPC, cidofovir) was firstly reported in 1987 [31]. The antiviral activity spectrum of cidofovir is similar to that of HPMPA: compound is active against virtually all DNA viruses, including polyoma-, papilloma-, adeno-, herpes- and poxviruses [32].

Similarly to (*S*)-HPMPA, alkoxyalkyl esters of cidofovir have been designed as prodrugs, in order to increase the oral bioavailability and reduce the toxicity of cidofovir. The ODE and HDP derivatives **18** and **19** are the most interesting compounds: in particular, pharmacokinetic and safety studies are currently being developed in humans for the use of novel lipid conjugate of **18** in the prevention of dsDNA viral infections [33].

Two new prodrugs of (*S*)-HPMPC, **20** and **21**, have been recently synthesized [34]. These compounds inhibit the in vitro replication of different herpesviruses and poxvirus vaccinia virus with an activity equivalent to that of cidofovir. The

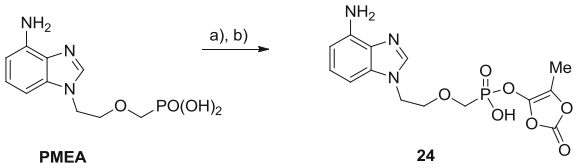


Scheme 2 Reagents and conditions: (*a*) TMSBr, CH_2Cl_2; (*b*) oxalyl chloride, N,N-DMF (0.5 mL), 0°C, 5.5 h; (*c*) 3-hexadecyloxy-1-propanol or 2-octadecyloxy-1-ethanol, Et_2O, Py, 3 h, aq $NaHCO_3$, 1 h; (*d*) (*S*)-9-[3-trityloxy-2-hydroxypropyl]-N^6-trityladenine, NaH, Et_3N (solvent), 50°C, 12 h; (*e*) 80% aq AcOH, 60°C, 1 h



Scheme 3 Reagents and conditions: (*a*) ODE-Br, ODP-Br, or HDP-Br, N,N-DMF, 80°C, 6 h; (*b*) 0.5 M NaOH, 1.5 h, AcOH

synthetic scheme towards these prodrugs starts from the cyclic form of cidofovir **17**, which undergoes a nucleophilic substitution by the corresponding haloderivative (Scheme 3) [35].



Scheme 4 Reagents and conditions: (*a*) Bu_4NOH, MEOH; (*b*) (5-methyl-2-oxo-1,3-dioxolen-4-yl)methylbromide, DMF

Acyclic nucleoside **22**, the (*S*)-HPMP-5-azaC, is characterized by the replacement of pyrimidine ring by a triazine system. This compound shows, against DNA viruses, an activity comparable to that of the parent compound [32]. The insertion of an alkoxyalkyl group, as in its hexadecyloxyethyl (HDE) ester **23**, leads to the enhancement of the antiviral activity against infections sensitive to cidofovir, in particular CMV, HSV, HPV, adeno- and poxivirus infections [36].

3.3 Compounds of Structure C

Amphiphilic prodrugs of PMEA (adefovir) have been prepared by its conversion into alkoxyalkyl esters (Scheme 4): the (5-methyl-2-oxo-1,3-dioxolen-4-yl) methyl ester of PMEA **24** showed significant activities against HIV and herpesviruses [34].

3.4 Compounds of Structure D

Holy and De Clercq have described in 1991 the 9-(2*R*,*S*)-2-phosphonylmethoxypropyl derivatives of adenine (PMPA) and 2,6-diaminopurine (FPMPA) as potent and selective antiretroviral agents [37]. Later, they showed that the anti-HIV activity of this class of compounds resided in (*R*)-enantiomers (Fig. 4) [38].

(*R*)-PMPA (the well-known tenofovir) has been selected as a drug and actually plays a key role in the control of HIV infections [39].

A reported kilogram-scale synthesis of tenofovir (*R*)-PMPA develops in a three-step sequence [40]: (1) condensation of adenine with (*R*)-propylene carbonate **25**; (2) alkylation of the corresponding (*R*)-9-(2-hydroxypropyl)adenine **26** with diethyl *p*-toluenesulfonyloxymethanephosphonate using lithium *t*-butoxide and (3) hydrolysis of ester **27** with bromotrimethylsilane (Scheme 5).

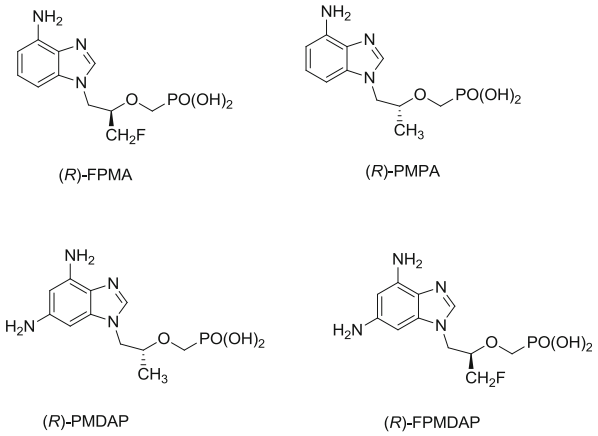
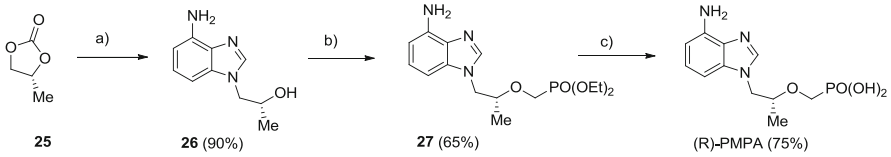


Fig. 4 Structures of (*R*)-FPMA, (*R*)-PMPA, (*R*)-PMPDAP, and (*R*)-FPMDAP



Scheme 5 Reagents and conditions: (*a*) adenine, NaOH, DMF, 140°C, 20 h; *t*-BuOLi, diethyl *p*-toluenesulfonyloxymethanephosphonate, 35°C, 1 h, aq AcOH; (*c*) TMSBr, MeCN

3.5 2,4-Diaminopyrimidine Nucleosides

DAPys, the so-called 2,4-diaminopyrimidine derivatives, constitute a novel class of ANP where the disubstituted pyrimidine ring is linked, through an oxygen bridge, to either the 2-phosphonylmethoxyethyl or 2-phosphonomethoxypropyl moiety (Fig. 5).

The synthesis of 2,4-diamino 6-[2-(phosphonomethoxy)ethoxy]pyridine (PMEO-DAPy) **28** is reported in Scheme 6 [41]. The reaction of diisopropyl 2-chloroethoxymethyphosphonate **33** with 2,4-diamino-6-hydroxypyrimidine leads to a mixture of diesters **34** and **35** in low yields. Compound **34** was isolated and transformed into the free phosphonic acid **28** (78.3%).

The synthesis of 5-Br-PMEO-DAPy **29** and 5-Me-PMEO-DAPy **30** is described in Scheme 7 [42]. Compound **29** has been prepared starting from derivative **34**, intermediate in the synthesis of PMEO-DAPy, by reaction with bromine followed by reaction with TMSBr, and hydrolysis. 5-Me-PMEO-DAPy **30** has been synthesized by coupling reaction of **36** performed with Me_3Al and $Pd(PPh_3)_4$ and subsequent hydrolysis of **37** and **38**.

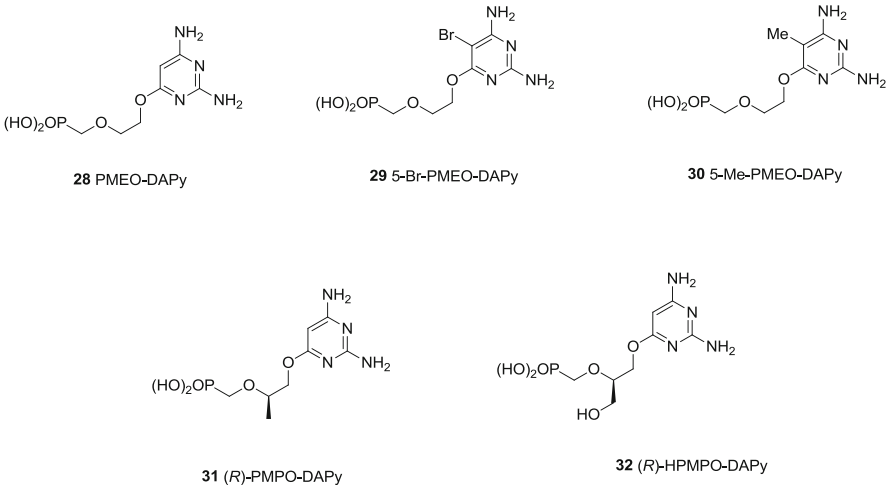
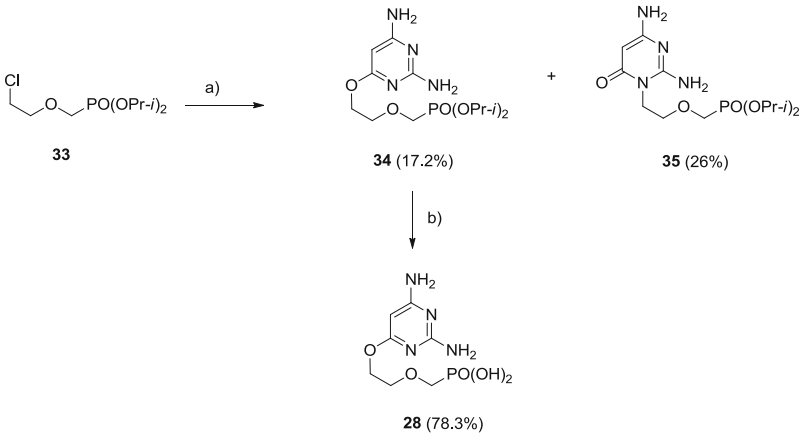


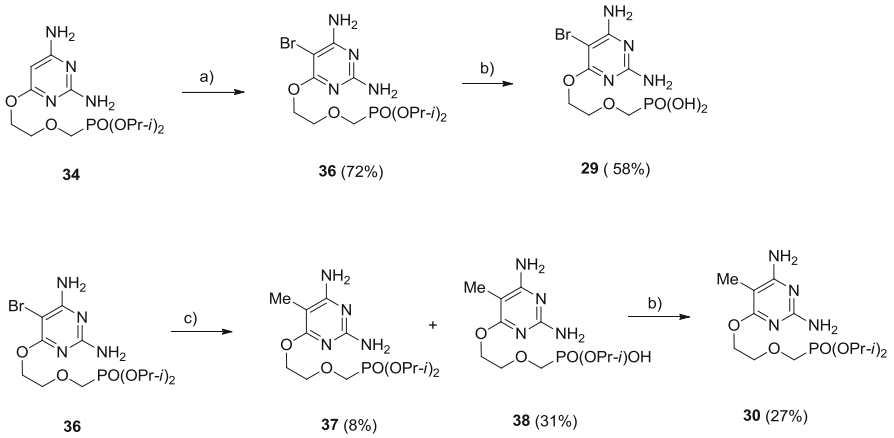
Fig. 5 2,4 Diaminopyridine nucleosides



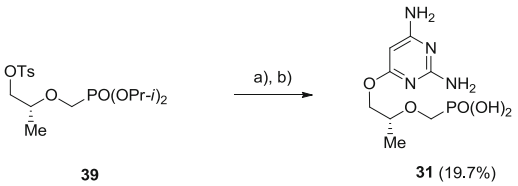
Scheme 6 Reagents and conditions: (*a*) 2,4-diammino-6-hydroxypyrimidine, Cs_2CO_3, DMF, 80°C, 30 min then 100°C 16 h; (*b*) TMSBr, MeCN 14 h, RT, aqueous NH_3

(*R*)-PMPO-DAPy **31** has been synthesized starting from the (*R*)-2-(diisopropyl-phosphoryl)-methoxypropyl tosylate **39** (Scheme 8). (*R*)-PMPO-DAPy has showed pronounced antiherpes and anti-retroviral activity comparable to that of **28**, whereas the (*S*) enantiomer was virtually devoid of antiviral activity.

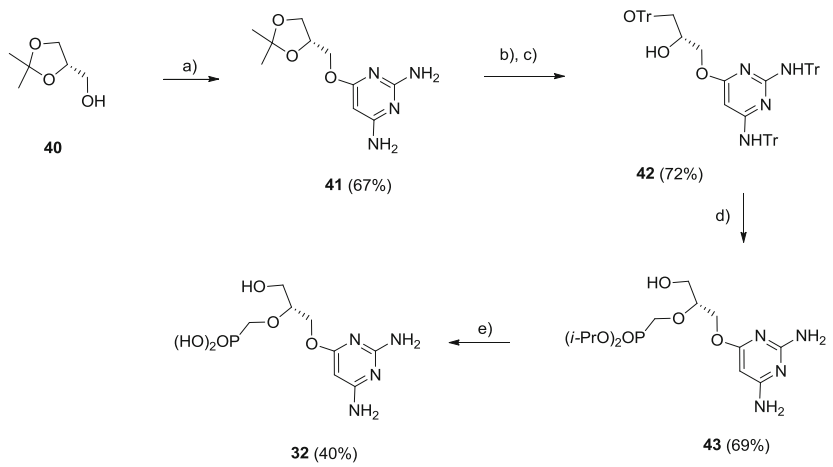
Finally, compound **32** has been prepared according to Scheme 9 in five steps, involving a sequence of reactions that starts from compound **40** [43].



Scheme 7 Reagents and conditions: (*a*) Br_2, DMF/CCl_4, Cs_2CO_3, DMF, 80°C, 30 min then 100°C 16 h; (*b*) TMSBr, MeCN 14 h, RT, aqueous NH_3; (*c*) $AlMe_3$, $Pd(Ph_3P)_4$, THF



Scheme 8 Reagents and conditions: (*a*) 2,4-diammino-6-hydroxypyrimidine, DBU, DMF, 90–100°C, 24 h; (*b*) TMSBr, MeCN 14 h, RT, aqueous NH_3



Scheme 9 Reagents and conditions: (*a*) NaH, THF, 2,4-diamino-6-chloropyrimidine, 50°C, 12 h; (*b*) H_2SO_4, RT 12 h; (*c*) trityl chloride, DMAP, Py, 50°C, 15 h; (*d*) diisopropyl *p*-toluenesulfonyloxymethylphosphonate, NaH, 3d, RT; (*e*) TMSBr, MeCN, 12 h

Table 1 Antiviral activity of acyclic nucleoside phosphonates

Compound	Structural analogy	Molecular targets	Virus	References
Class				
representative compounds				
HPMPA (*S*)-*HPMPA*	Adenine analogues	Viral DNA polymerase, HBV-RT	Herpesviruses, poxviruses, adenovirus, HBV	[26, 30]
HPMPC *Cidofovir*	Cytosine analogues	Viral DNA polymerase	Herpesviruses, poxviruses, adenovirus, polyomavirus, papillomavirus	[31, 32, 34–36, 44]
PMEA *Adefovir*	Adenine analogues	Viral DNA polymerase, HIV-RT	Herpesviruses, HIV	[34, 45–47]
PMPA *Tenofovir*	Adenine analogues	HIV-RT, HBV-RT, HTLV-RT	HIV, HBV, HTLV-1	[37–40, 48–52]
DAPy *PMEO-DAPy* (*R*) - *HPMPOyDAPy*	Pyrimidine analogues	HIV-RT, HBV-RT, viral DNA polymerase	HIV-1, HIV-2, HBV, herpesviruses, poxviruses, adenovirus, papillomavirus	[41–43, 59–62]

3.6 *Highlights on Antiviral Activity and Toxicity of ANP*

Currently, three ANP are in clinical use (Table 1). Cidofovir was the first ANP to be approved in 1996 by FDA for CMV infection in HIV patients and is also in use for HPV infection [44]. Adefovir dipivoxil (ADV) is an orally bioavailable prodrug, chemically defined as a nucleotide analogue of adenosine monophosphate. It was previously used in HIV infection, but it was discontinued because of toxic effects in 1999. In 2002 FDA approved its use for HBV infection in hepatitis B e antigen (HBeAg)-positive as well as hepatitis B e antigen-negative patients [45]. The results of a phase III study, encompassing 500 individuals with chronic HBeAg-positive infection, treated with ADV versus placebo, showed that the ADV groups had greater histologic improvement, decreased viral load and higher rates of HBeAg seroconversion [46]. A study published in 2004 demonstrated that adding ADV to lamivudine treatment in individual resistant to lamivudine gave better results in the clearance of HBeAg and in the decreasing of viral load, in comparison with switching to monotherapy with ADV in lamivudine-resistant individuals [47]. ADV was well tolerated and the most serious side effect was ascribed mainly to nephrotoxicity, taking place more often in individual suffering of kidney dysfunction. Tenofovir is a 9-[2-(*R*)-(phosphonomethoxy) propyl] adenine which requires, similarly to other ANP, two phosphorylation steps by cellular kinases to be activated. Its spectrum of antiviral activity includes HIV and hepadnaviruses [39, 48]. In addition, it has been also shown that tenofovir was able to protect peripheral blood mononuclear cells from healthy donors against human T-cell leukemia/lymphotropic virus type 1 (HTLV-1) infection in vitro [49]. However,

the tenofovir prodrug, tenofovir isoproxil fumarate (TDF), when used as a monotherapy, was unable to inhibit viral replication in HTLV-1-infected patients affected by tropical spastic paraparesis [50]. Conversely, when adopted in HIV infection in combination therapy, TDF caused a significant decline in plasma HIV-1 RNA levels in randomized, double-blind placebo-controlled clinical trials in HIV-1 infected individuals, including those with nucleoside resistance mutations [51, 52]. TDF use was then approved by FDA in a single-tablet formulation with emtricitabine (Truvada) in 2004 or with efavirenz and emtricitabine (Atripla) in 2006. Tenofovir, like other ANP, provides a long-term antiviral response, leads poorly to the emergence of drug resistance and is well tolerated in vivo, in HIV-infected patients [51, 52], although alteration of renal proximal tubules has been reported [53].

ANP are endowed with a relative limited toxicity. At the cellular level, the limited toxicity of tenofovir seems due to a low capability to produce mitochondrial dysfunction. This seems to be related to the poor capacity of tenofovir to act as a substrate for human cellular and mitochondrial α, β, γ and ε DNA polymerases in vitro [54]. It has been shown that tenofovir has limited effects in vitro on the proliferation of renal proximal tubule epithelial cellsand erythroid and myeloid progenitors [55]. Conversely, in vivo studies demonstrated that acyclic nucleotides are prone to accumulate in kidney, since they are secreted through glomerular filtration and tubular secretion [56]. Accumulation of ANP in renal proximal tubules has been shown to be regulated by human renal organic anion transporters types 1 and 3 (hOAT1 and hOAT3) [57]. On the other hand, multidrug resistance protein 4 (MRP4) counteracts the action of the transporter by reducing accumulation of ANP in tubular cells [58]. Thus modulation of ANP transporter plays a critical role for accumulation of the drugs within basal membrane of proximal tubule epithelial cells and related toxicity.

Regarding the antiviral activity of 2,4-diaminopyrimidine nucleosides, it has been pointed out that 6-[2-(phosphonomethoxy)ethoxy]pyrimidines bearing amino group concomitantly on both C-2 and C-4, or an amino on C-2 and an OH group on C-4, exhibited antiviral activity [59]. Some of these compounds have shown promising antiviral activity. The prototypes PMEO-DAPy 28, 5-Br-PMEO-DAPy 29, 5-Me-PMEO-DAPy 30, and (*R*)-PMPO-DAPy **31** have shown activity against different HIV-1 isolates and HIV-1 mutant strains, HIV-2 and also HBV, while (*R*)-HPMPO-DAPy **32** has shown activity against herpes-, adeno, pox- and papillomaviruses (Fig. 5) [42, 43, 59–62]. In particular Balzarini et al. in 2002 reported that the prototypes 6-PMEO 2,4-diaminopyrimidine and 6-PMPO 2,4-diaminopyrimidine derivatives showed a potent activity against HIV replication, in CEM and MT-4 cells as well as in primary culture of peripheral blood mononuclear cells and that they were endowed with a limited cytotoxicity. Further studies have shown how 5-substituted PMEO-pyrimidine derivatives, methyl, bromo, formyl, cyano, chloro, were endowed with an anti-HIV-1 and anti-HIV-2 antiretroviral activity in CEM cells, comparable with that of adefovir and tenofovir. In particular PMEO-5-Me-DAPy exhibited an EC_{50} of 0.06 μg/mL, 25 times lower than that showed by ADV (EC_{50} 1.5 μg/mL). In addition, it has to be underlined that all the 5-substituted PMEO-pyrimidine

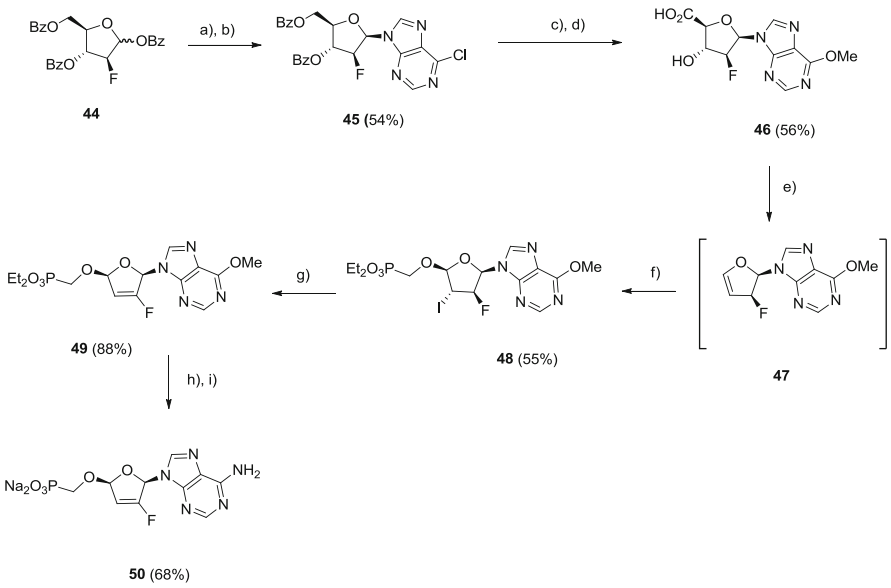
derivatives were not toxic in cell culture except for PMEO-5-Me-DAPy with a CC_{50} of 3.4 μg/mL, i.e., a value that was in any case 5 and 37 times lower than that of adefovir and tenofovir, respectively [61].

3.7 Resistance to ANP

ANP are associated with few resistance mutations in viral polymerases, differently from NA. Some of them deserve to be described. The primary mutation in HIV-RT conferring resistance to tenofovir is the K65R, which is sheared with some NA such as ddI, abacavir and emtricitabine. This mutation decreased the sensitivity of HIV viral isolates to tenofovir in vitro [63]. Moreover, also HIV-RT from patients not responding to therapy with tenofovir exhibited the K65R mutation [64]. Studies about the processivity of tenofovir on wild type and mutant HIV strains, respectively, revealed that K65R mutation containing viruses were unable to bind or incorporate tenofovir [65]. Another mutation which equally seems to confer resistance in HIV-RT to ANP is the K70E. This mutation was first identified following treatment with adefovir [66]. After stopping the therapy with ADV in HIV infection, the resistance mutation disappeared. The re-emergence of the K70E resistance mutation was observed in patients receiving treatment with ABC, 3TC and TDF [67]. Molecular studies revealed that K70E mutation confers resistance to tenofovir and NRTI by preventing the positioning of the NRTI-TP in the active center of HIV-RT, which results in increased discrimination process, due to a failure of the catalytic efficiency of polymerase [68]. A few studies have investigated the resistance to pyrimidine analogue phosphonates. Recently, it was reported that acyclic pyrimidine nucleoside phosphonate analogues, presenting an amino group at C-2 of the pyrimidine ring together with an amino group at C-4, maintained activity against HIV laboratory strains bearing L100I, K103N, Y181C, and Y188H mutations in the RT [43].This activity was maintained also versus three clinical isolates either untreated, HIV-1/L1S, or bearing NRTI-specific mutations, HIV-1/L6S, or bearing mutation to PMEA, HIV-1/L6S/PMEA [43].

3.8 GS-9148: New Promising Phosphonated Nucleoside

Recently, the synthesis of GS-9148 (phosphonomethoxy-2-fluoro-2,3-dideoxydide-hydroadenosine **50**; Fd4AP) [69], a novel nucleotide HIV reverse-transcriptase inhibitor, has been reported. GS-9148 is an adenosine derivative equipped with a 2′,3′-dihydrofuran ring containing a 2′-fluoro group. This compound was prepared starting from commercially available 2-fluoro-1,3,5-tri-*O*-benzoyl-D-arabinofuranose **44**, in nine steps, according to Scheme 10. Thus, fluorosugar **44** was transformed into **45**, by reaction with HBr and addition of 6-chloropurine sodium salt. The chloropurine derivative **45** was converted into 6-methoxy acid **46** by hydrolysis



Scheme 10 Reagents and conditions: (*a*) HBr/Ac, CH_2Cl_2; (*b*) NaH, 6-chloropurine, MeCN; (*c*) K_2CO_3, MeOH; (*d*) Jones' reagent, acetone; (*e*) DIAD, Ph_3P, CH_2Cl_2; (*f*) $HOCH_2PO_3Et_2$, IBr, CH_2Cl_2; (*g*) AcOH, MeOH, NaClO; (*h*) NH_4OH aq, Δ; *i*) 2,6-Lutidine, TMSBr, MeCN

and selective Jones oxidation. This acid led to compound **48** via the glycal intermediate **47**, by Mitsunobu activation followed by treatment with IBr and diethyl hydroxymethylphosphonate. **48** was transformed into unsaturated derivative **49** using buffered sodium hypochlorite oxidation of the iodide, followed by elimination. Finally, the target **50** was obtained from **49** by reaction with NH_4OH followed by hydrolysis with trimethylsilylbromide and $NaHCO_3$ treatment.

Molecular studies revealed that GS-9148 exhibited 5–10 times less affinity than cidofovir, adefovir and tenofovir for hOAT1 and 60–100 times lower transport efficiency by the transporter [70]. The poor affinity for the transporter, demonstrated in vitro, very likely is the cause of a lower accumulation of GS-9148 in kidney of dog observed in vivo in comparison with the other ANP [70]. Although GS-9148 was found less toxic than tenofovir, its oral bioavailability needed to be improved. The phosphoamidate prodrug of GS-9148, GS-9131, was optimized to increase the delivery of GS-9148-DP into lymphoid cells by 200-folds relative to that of GS-9148 [71]. Further studies have demonstrated that GS-9131 inhibited HIV replication in CD4+ primary cells with EC_{50} (3.7 nM) lower than that of tenofovir diphosphate (EC_{50} 1.5 μM) [72]. Therefore, given the pharmacokinetic properties of GS-9131 and its ability to load lymphoid cells with long intracellular half-life and to be efficiently phosphorylated, it can be considered as a potential candidate for HIV antiretroviral therapy. In addition, it has been reported that GS-9148 selected a unique HIV resistance mutation. Biochemical studies have shown that the HIV-1 mutant RT

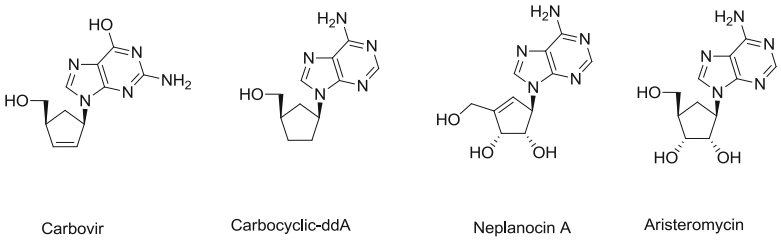


Fig. 6 Relevant carbocyclic nucleosides

Q151L exhibited resistance to GS-9148 by blocking its incorporation in the DNA growing chain. This was found to be peculiar for GS-9148 since the Q151L resistance mutation did not affect the incorporation of other adenosine analogues such as tenofovir [41].

4 Phosphonated Carbocyclic Nucleosides (PCN)

Carbocyclic nucleosides, the class of compounds by which phosphonated carbocyclic nucleosides derive, are structural analogues of natural and synthetic nucleosides, characterized by the replacement of the endocyclic oxygen atom with a methylene group. Due to the absence of the labile glycosidic bond, these analogues are chemically and enzymatically more stable than the natural nucleosides. Figure 6 reports some members of this family: carbovir and carbocyclic-ddA, naturally occurring carbo-nucleosides, show a good activity against HIV. Natural neplanocin A and aristeromycin, and other synthetic derivatives, exhibit powerful antitumor and antiviral activities [73].

4.1 Phosphonated Cyclopropyl Nucleosides

Phosphonated cyclopropyl nucleosides, reported here, can be classified in different groups depending on the relative position of nucleobase and phosphonic group (Fig. 7).

4.1.1 Compounds of Structure I

The 9-[1-(phosphonomethoxycyclopropyl)methyl]guanine **55** (PMCG), containing a cyclopropyl moiety at the 2′-position, is endowed with a selective anti-HBV activity ($EC_{50} = 0.5$ μM). The synthetic route involves, as the key step, a titanium-mediated Kulinkovich cyclopropanation and develops in seven steps starting from

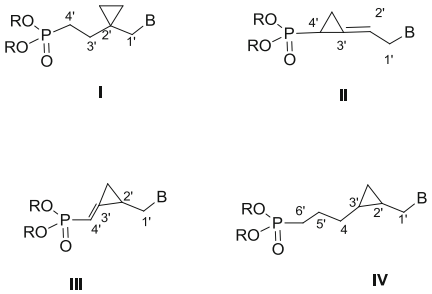
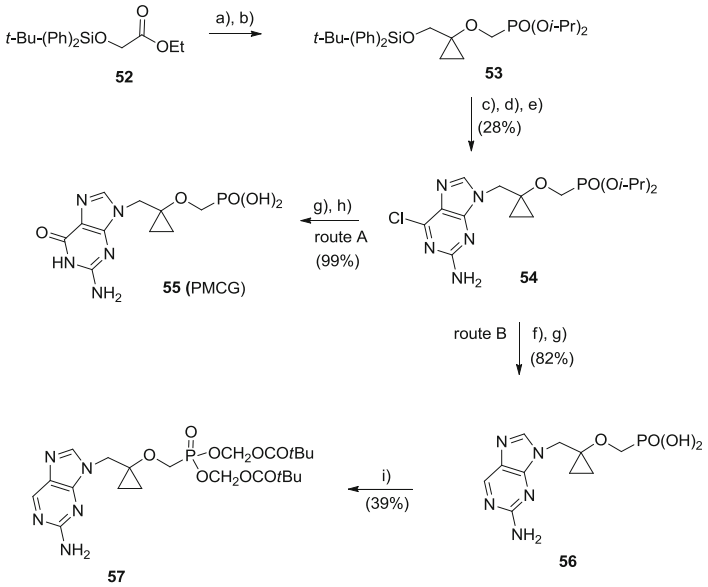


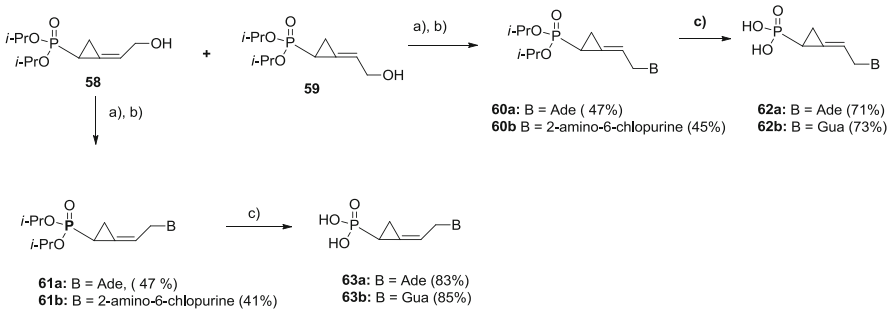
Fig. 7 Phosphonated cyclopropyl nucleosides



Scheme 11 Reagents and conditions: (*a*) CH_3CH_2MgBr, $Ti(O\textit{i}\text{-}Pr)_4$ (0.25 equiv), 10 h, THF 0−25°C; (*b*) $BrCH_2P(O)(O\textit{i}\text{-}Pr)_2$, LiI (cat.), *t*-BuOLi, DMF, THF, 60°C, 4 h; (*c*) NH_4F, MeOH, reflux, 10 h; (*d*) MsCl, TEA, MDC, 0−25°C; (*e*) 6-chloroguanine, NaH, DMF, 80°C, 4 h; (*f*) H_2, 5% Pd/C, THF, 1 atm, 18 h; (*g*) TMSBr, MDC, reflux, 18 h; (*h*) 2 N HCl, reflux, 6 h; *i*) chloromethyl pivalate, TEA, 1-methyl-2-pyrrolidinone, 25°C, 48 h

ethyl 2-acetate **51**. Its conversion into the cyclopropanol derivative **52** (80% yield), by reaction with ethylmagnesium bromide and titanium (IV) isopropoxide, is followed by the reaction with diisopropylbromomethylphosphonate to give **53**, from which the target phosphonated **55** was obtained (Scheme 11) [74].

Hydrogenation of **54**, followed by hydrolysis and etherification with chloromethyl pivalate (39% yield), afforded the orally available Dipivoxil **57** (Scheme 11 route b).



Scheme 12 Reagents and conditions: (*a*) Ph_3P, CBr_4, CH_2Cl_2; (*b*) adenine or 2-amino-6-chloropurine; (*c*) HCl

4.1.2 Compounds of Structure II

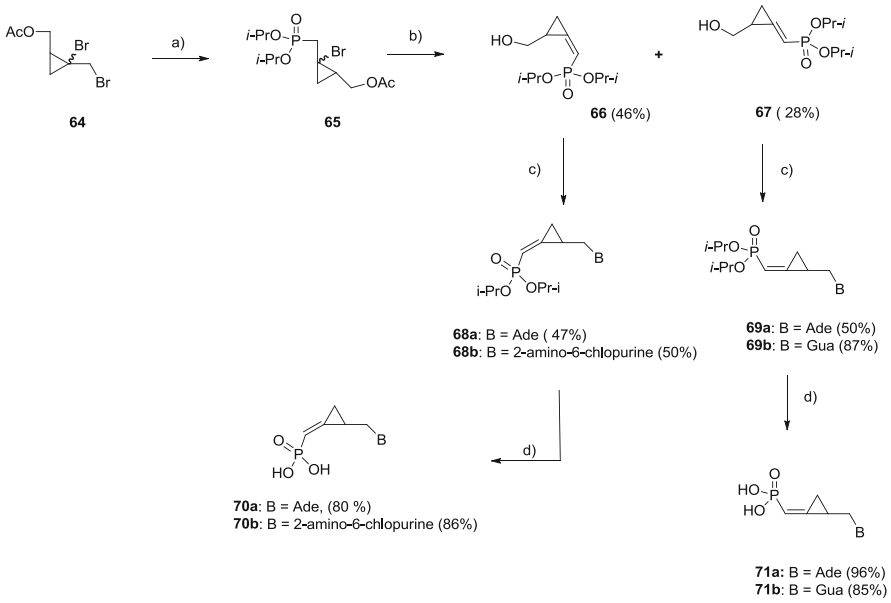
An easy entry towards this class of compounds is reported in Scheme 12. In particular, diisopropyl (*Z*)- and (*E*)-(2-hydroxyethylidene)-1-cyclopropylphosphonate **58** and **59** were converted into the corresponding nucleotides **60** and **61** utilizing an Appel reaction, followed by nucleophilic amination with adenine or 2-amino-6-chloropurine. The subsequent acidic hydrolysis led to adenine and guanine analogues **62** and **63** [75] (Scheme 12). Phosphonates **61a** and **61b** are potent inhibitors of Epstein–Barr virus (EBV) replication.

4.1.3 Compounds of Structure III

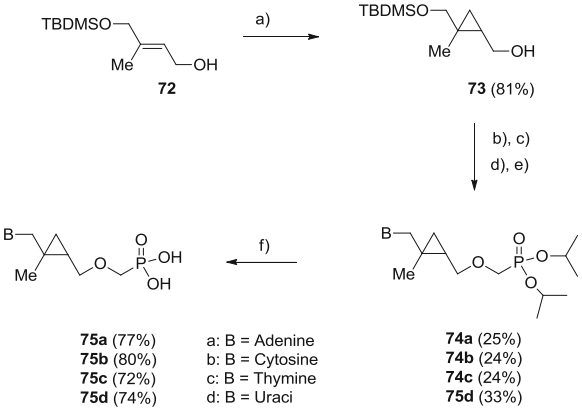
Reversed methylenecyclopropane phosphonated nucleosides **70** and **71** have been synthesized starting from 1-bromomethylcyclopropane 64, which was converted into the bromocyclopropyl phosphonate **65** by Michaelis–Arbuzov reaction. The subsequent β-elimination, followed by nucleobase introduction and hydrolysis, furnishes the target phosphonates **70** and **71** (Scheme 13) [76].

4.1.4 Compounds of Structure IV

Compounds bearing a methyl substitution at the cyclopropyl ring have been synthesized by Kim and coworkers [77], employing the Simmons-Smith reaction as key step. Thus, the allylic alcohol **72** was reacted with $ZnEt_2$ and CH_2I_2 to form the cyclopropyl alcohol **73** that was converted, according to standard procedures, into the phosphonated nucleosides **74a–d** (Scheme 14). The phosphonic acid nucleosides **75a–d**, obtained by hydrolysis of **74a–d**, have been evaluated for their antiviral activities. Unfortunately, all compounds proved to be inactive against HIV-1, HSV-1, HCMV, and CoxB3, except **74b** that shows a low activity against CoxB3 and HIV-1 (EC_{50} 43.5 and 55.7 mg/mL, respectively).

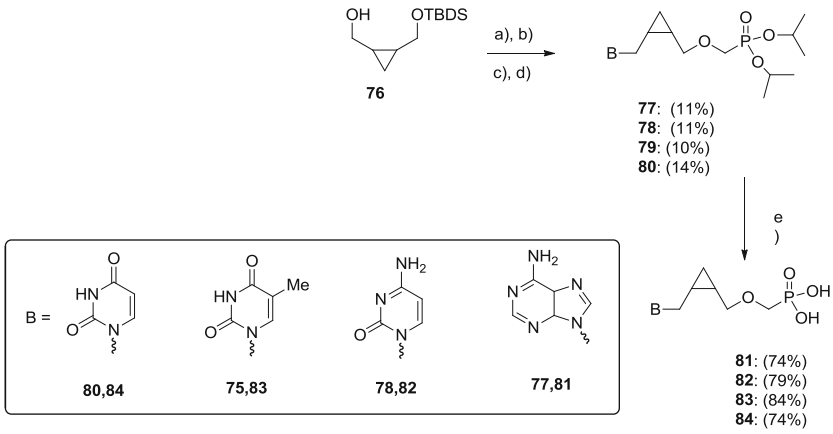


Scheme 13 Reagents and conditions: (*a*) triisopropyl phosphite, 120°C, 48 h; (*b*) NaOH, 40 min., RT; (*c*) Ph_3P, adenine or 2-amino-6-chloropurine, DEAD 0°C, 12 h RT, (*d*) HCl



Scheme 14 Reagents and conditions: (*a*) CH_2I_2, Et_2Zn, CH_2Cl_2; (*b*) diisopropyl bromomethylphosphonate, LiI, *t*-BuOLi,DMF; (*c*) TBAF, THF; (*d*) MsCl, TEA, CH_2Cl_2; (*e*) adenine, cytosine, thymine or uracil, K_2CO_3, 18-C-6, DMF; (*f*) Me_3SiBr, CH_2Cl_2

Hong et al. [78], applying the same methodology above reported [77], have prepared various unsubstituted cyclopropyl phosphonates of structure IV, starting from the *cis*-[2-({[*t*-butyl(dimethyl)silyl]oxy}methyl)cyclopropyl]methanol **76** (Scheme 15). Only the adenine derivative **81** exhibited moderate activity against human cytomegalovirus (HMCV) (EC_{50} = 22.8 μg/mL), with cytotoxic activity to the host cell at a concentration higher than 100 μg/mL.



Scheme 15 Reagents and conditions: (*a*) diisopropyl bromomethylphosphonate, LiI, *t*-BuOLi, DMF; (*b*) TBAF; (*c*) MsCl, TEA, CH_2Cl_2; (*d*) uracil, thymine, cytosine, K_2CO_3, adenine, 18-C-6, DMF; (*e*) Me_3SiBr, CH_2Cl_2

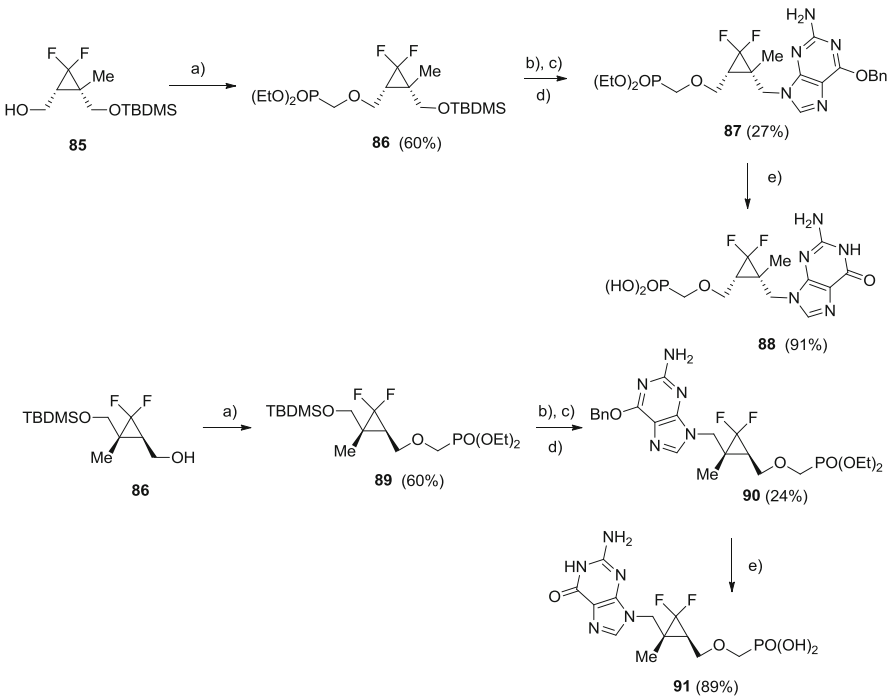
In the same context difluoro cyclopropyl analogues **88** and **91** have been prepared involving, as key intermediates, the difluoro-cyclopropyl alcohols **85** and **86** (Scheme 16) [79]. Phosphonated nucleoside **88** exhibits in vitro anti-HIV-activity similar to that of PMEA ($EC_{50} = 2.4$ μM).

4.2 Phosphonated Cyclopentyl Nucleosides

Starting from commercially available desymmetrized cyclopentanediol **92**, 4′-C-branched carbocyclic nucleoside phosphonates **95** and **96** have been recently synthesized (Scheme 17) [80]. Thus, **92**, by reaction with 6-chloropurine, was converted in **93**, which was deacetylated and oxidized with Dess–Martin reagent to intermediate **94**. **94** was treated with organocerium reagents and transformed into compounds **95** and **96** by reaction with diisopropyl bromomethylphosphonate and ammonia. Furthermore, compound **96** was converted in its diphosphonate **97** and finally in **98**, through Lindlar hydrogenation. The biological assay performed on these compounds have shown that compound **96** is a potent inhibitor of HIV-RT.

In the same context, the synthesis of 4′-ethyl-5′-norcarbocyclic adenosine phosphonic acid analogues **101** and **104** has been reported [81]. The synthetic route starts from the racemic 4-ethyl-4-(4-methoxybenzyloxy)-cyclopent-2-enol **99**, which can be easily converted into the target compounds by methods above reported (Scheme 18).

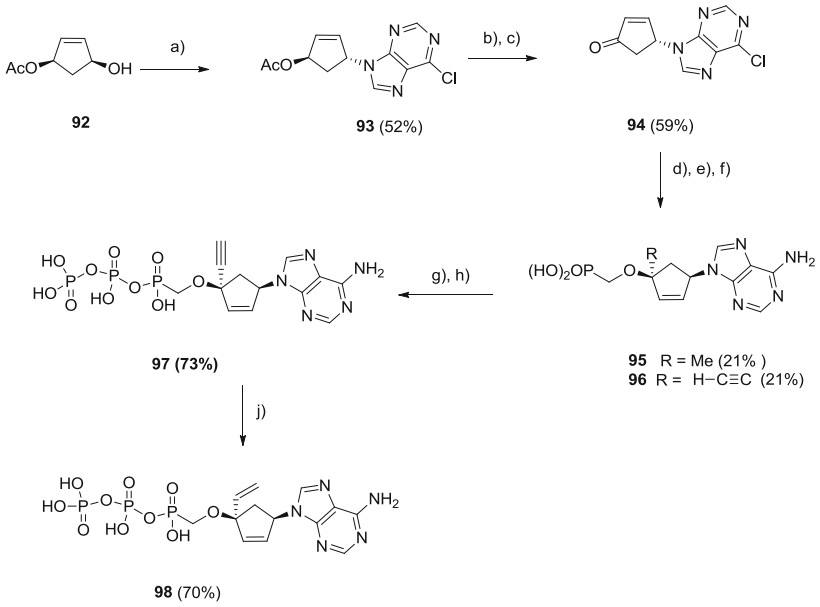
Only derivatives **100** and **101** have shown a moderate antiviral activity against HIV-1 ($EC_{50} = 55$ and 21 μM, respectively), while compounds **102** and **104** did not show any interesting activity.



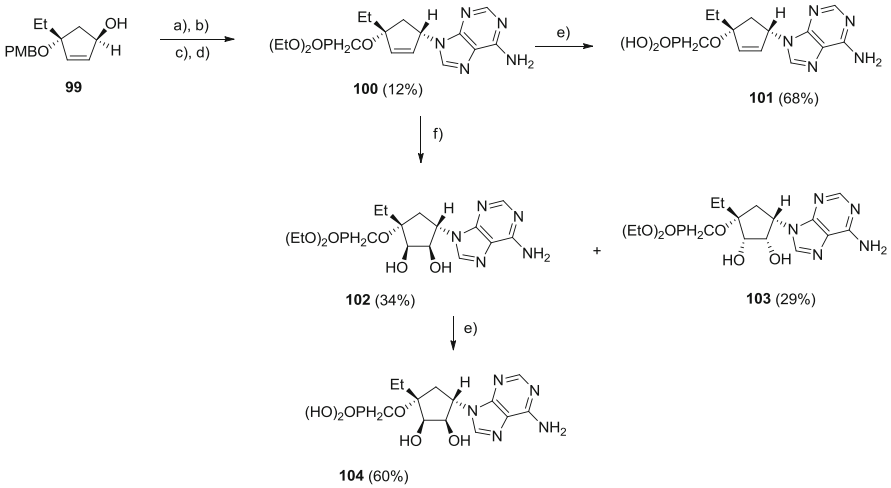
Scheme 16 Reagents and conditions: (*a*) $(EtO)_2POCH_2OTf$, LiO-*t*-Bu, THF; (*b*) TBAF; (*c*) NBS, Ph_3P, CH_2Cl_2; (*d*) 2-amino-6-benzoyloxypurine, K_2CO_3, DMF; (*e*) Me_3SiBr, DMF, RT, aq. HCl, RT, NaOH, H_2O

6′-Fluoro-6′methyl-5′-noradenosine nucleoside phosphonic acid **109** and its SATE (S-acyl-2-thioethyl) prodrug **110** have been prepared from the key fluorinated alcohol **106**, obtained through a selective ring-opening of epoxide **105**. Coupling of **106** with N^6-bis-Boc-adenine under Mitsunobu conditions, followed by phosphonation and deprotection, gave the nucleoside phosphonic acid **109**, from which the final *t*-Bu-SATE prodrug **110** was obtained by reaction with AS-2-hydroxyethyl-2,2-dimethyl-propanethioate, in the presence of 1-(2-mesitylenesulfonyl)-3-nitro-1*H*,1,2,4-triazole (Scheme 19) [82].

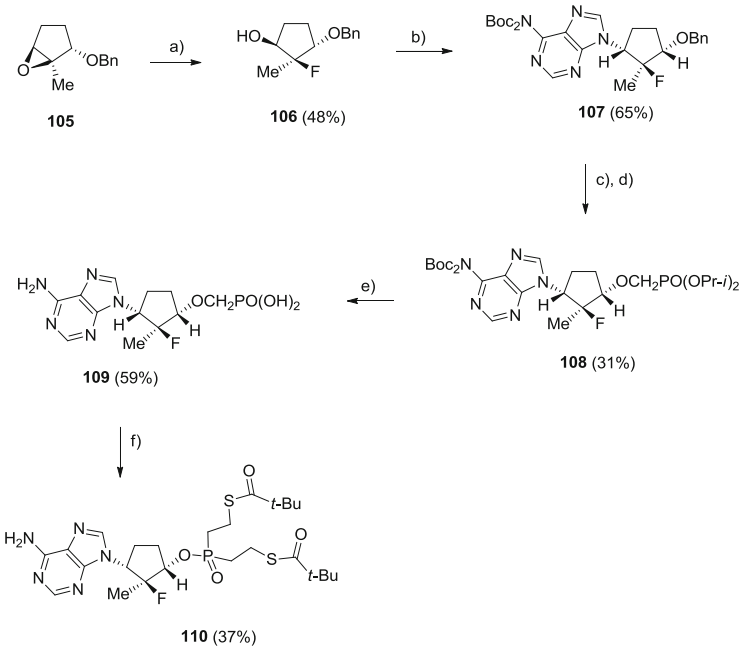
The phosphonic nucleoside analogues **109** and its prodrug **110** showed an antiviral activity against HIV-1 with $EC_{50} = 62$ and 16.7 μM, respectively. The increased anti-HIV activity for the neutral phosphodiester **110** is the result of increased cellular uptake, followed by intracellular release of the parent phosphonic acid.



Scheme 17 Reagents and conditions: (*a*) 6-chloropurine, Ph_3P, DIAD, dioxane; (*b*) $MeOH/NH_3/H_{20}$ 8:1:1; (*c*) Dess-Martin reagent; (*d*) $CeCl_3$, MeMgBr or TMS—≡, THF, −78°C; (*e*) diisopropyl bromomethylphosphonate, *t*-BuOLi, THF, 50°C; (*f*) NH_3, MeOH, 50°C; (*g*) TMSBr, 2,6-lutidine, DMF, 50°C, then MeOH, NH_4OH; (*h*) Et_3NHCO_3, $(lm)_2CO$, tributyl ammonium pyrophosphate; (*j*) Lindlar catalyst, quinoline, H_2, H_2O



Scheme 18 Reagents and conditions: (*a*) DIAD, 6-chloropurine, THF; (*b*) DDQ, CH_2Cl_2/H_2O, RT; (*c*) $(EtO)_2POCH_2OTf$, LiOBu-*t*; (*d*) NH_3/MeOH, 70°C; (*e*) 2,6-lutidine, TMSBr, MeCN; (*f*) OsO_4, NMO,acetone/*t*-$BuOH/H_2O$



Scheme 19 Reagents and conditions: (*a*) $(NH_4)_2SiF_6$, 47% HF, CsF; (*b*) DIAD, Ph_3P, N^6bis-BOC-adenine; (*c*) $Pd(OH)_2$, cyclohexene, MeOH, Δ; (*d*) $(i\text{-}PrO)_2POCH_2Br$, LiI, LiOBu-*t*, DMF, 60°C; (*e*) TMSBr, MeCN, 60°C, 12 h; (*f*) Bu_3N, MeOH, S-2-hydroxyethyl 2,2-dimethylpropanethioate, 1-(2-mesitylenesulfonyl)-3-nitro-1*H*-1,2,4-triazole, RT, 6 h

4.3 Highlights on Antiviral and Biological Activities of Phosphonated Cyclopropyl and Cyclopentyl Nucleosides

Carbovir is the prototype of non-phosphonated carbocyclic nucleosides endowed with antiviral activity (Table 2). The recent importance of carbovir in antiviral therapy is owed to the fact that it is the in vivo relevant anabolite of abacavir. Actually, abacavir is the cyclopropylaminopurine analogue of carbovir. Early studies showed a moderate activity versus HIV infection (EC_{50} 2.4 μM) in peripheral blood mononuclear cells (PBMC), with a cytotoxicity towards PBMC and Vero and CEM cell lines at concentrations higher than 100 μM [72]. The 9-[1-(phosphonomethoxycyclopropyl) methyl] guanine (PMCG) is frequently regarded as an "adefovir-like" nucleotide, able to block HBV infection in HepG22.2.15 cells with a EC_{50} of 0.5 μM and a cytotoxicity 2,000 times higher than that able to inhibit HBV infection. Conversely no activity versus HIV was ever shown [73]. Clinical studies have shown a remarkable HBV DNA suppression in patients treated with 60 mg or higher dose of PMCG over 28 days of therapy [74, 83]. However large clinical studies are needed to confirm these results. Among the phosphonated cyclopentyl nucleosides, the novel carbocyclic nucleoside phosphonate compound **96**, bearing a 4-ethynyl

Table 2 Antiviral activity of phosphonated carboyclic nucleosides

Compound	Structural analogy	Molecular targets	Virus	References
Class				
representative compounds				
Phosphonated cycloproyil nucleosides	Purine and Pyrimidine monophosphate analogues	Viral DNA-polymerase, HIV-RT	HBV, EBV, CoxB3, HIV-1, HMCV	[74–79]
PMCG				
Phosphonated cyclopentil nucleosides	Purine and Pyrimidine mono- di-phosphate analogues	HIV-RT	HIV-1	[80–82]
SATE				
Phosphonated N, O-nucleoside (PCOAN)	Purine and Pyrimidine monophosphate analogues	Retroviral RT	AMV, MLV, HTLV-1, HIV-1	[85–91, 94–98]
Truncated Phosphonated N, O-nucleosides (TPCOAN)				
Phosphonated N, O-Psiconucleosides				

group, exhibited inhibition of HIV infection in MT-2 cells with EC_{50} of 0.13 μM, 2–5 times weaker than that of abacavir. Interestingly, compound **96** inhibited HIV-1 RT activity with an EC_{50} comparable to that of abacavir triphosphate (EC_{50}, 0.13 μM) [79]. The evaluation of the resistance profile indicated a 2.2-fold lower susceptibility to compound **96** of HIV mutant strain bearing K65 mutation. However, this resistance is negatively counterbalanced by the 30 times less susceptibility of strain with M184V mutation, which is the common mutation in treated HIV-positive patients [79]. The phosphonic nucleoside analogue prodrug **110** is potentially an important improvement from the biological point of view, since it counteracts the ionic character of the phosphonic acid which represents a drawback for cell permeability. Esterification of the phosphonic acid with two SATE groups helps the delivery of phosphonate drug into the cells. The antiviral effect of the newly phosphonate **109** versus the prodrug **110** activity was assayed in HIV infection of MT-4 cells. The results revealed that the SATE derivative was four times more active than the parent nucleotide in inhibiting HIV infection, although it showed higher cytotoxic effect versus uninfected cells [81].

4.4 Phosphonated N,O-Nucleosides (PCOAN)

With the aim to bypass the first limiting step of phosphorylation [84], phosphonated N,O-nucleosides, as mimetic of monophosphate nucleosides [85], have been synthesized (Fig. 8). In this context, the groups of Romeo and Chiacchio have synthesized homo phosphonated-, phosphonated- and truncated phosphonated-N,

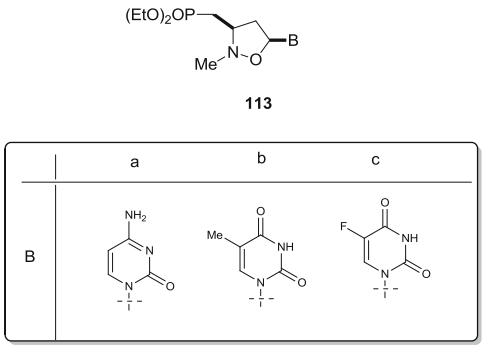
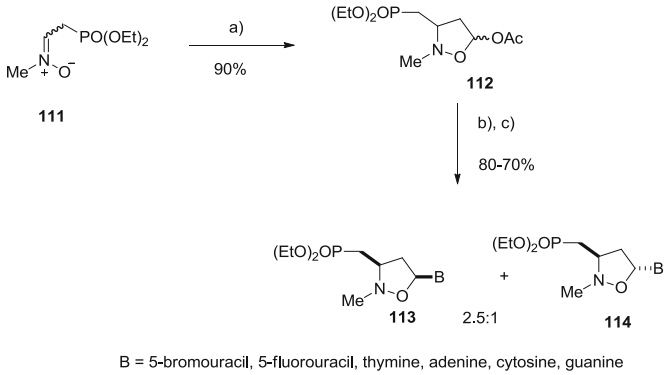


Fig. 8 PCOAN (compounds 113): (**a**) AdC-P; (**b**) AdT-P; (**c**)AdF-P



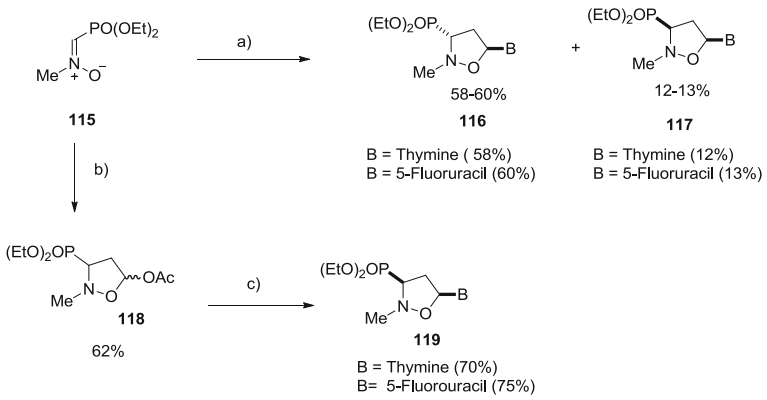
B = 5-bromouracil, 5-fluorouracil, thymine, adenine, cytosine, guanine

Scheme 20 Reagents and conditions: (*a*) vinyl acetate (30 mL), 60°C, 24 h; (*b*) 5-Bromuracil, 5-fluorouracil, thymine, adenine, cytosine or guanine (0.62 mmol), MeCN, bis(trimethylsilyl)-acetamide (BSA) (2.54 mmol), refluxed for 15 min; (*c*)TMSOTf, 55°C, 6 h

O-nucleosides, by exploitation of the 1,3-dipolar cycloaddition methodology [86–88], starting from nitrones containing a phosphonic group [89–91].

The route towards phosphonated carbocyclic 2′-oxa-3′-aza- nucleosides (PCOAN) **113** is described in Scheme 20 [89, 90]. The cycloaddition of phosphonated nitrone **111** with vinyl acetate affords a mixture of *cis*/*trans* isoxazolidines **112**, which were converted, according to the Vorbrüggen nucleosidation, into phosphonated N, O-nucleosides **113** and **114**, containing thymine, 5-fluorouracil, 5-bromouracil, cytosine, adenine and guanine, in 2.5:1 ratio, respectively (Scheme 20).

Cis stereoisomers **113** show low levels of cytotoxicity, assessed by conventional methods to detect viability. Noteworthy, the pyrimidinyl derivatives were endowed with an interesting biological activity [89, 90].



Scheme 21 Reagents and conditions: (*a*) vinyl thymine or vinyl 5-fluoruracil, MeCN, 100 W, 90°C; (*b*) $PhCH_3$, vinyl acetate, 60°C, 40 h; (*c*) thymine or 5-fluorouracil, BSA, TMSOTf, 70°C

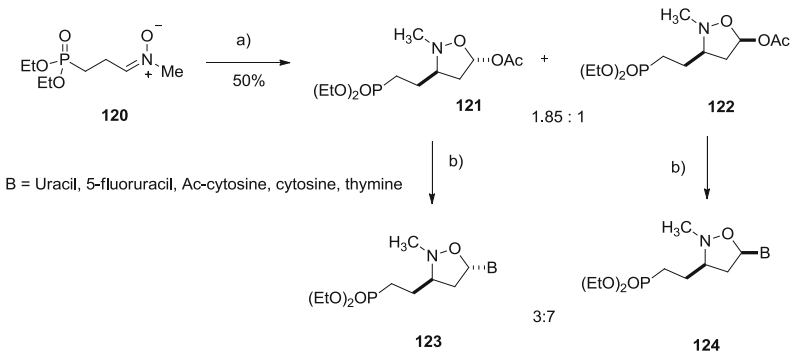
4.5 Truncated Phosphonated N,O-Nucleosides (TPCOAN)

Truncated phosphonated N,O-nucleosides **119** (TPCOAN) [91], containing a diethylphosphonate group directly linked at C3 of the isoxazolidine ring, have been obtained by using the phosphonated nitrone **115** as dipole. The reaction of **115** with vinyl nucleobases was performed under microwave irradiation, with formation of the *trans* nucleosides **116** as main adducts. The corresponding *cis* derivatives **117** were obtained in high yield by a two-step procedure, involving the vinyl acetate cycloaddition, followed by nucleosidation (Scheme 21).

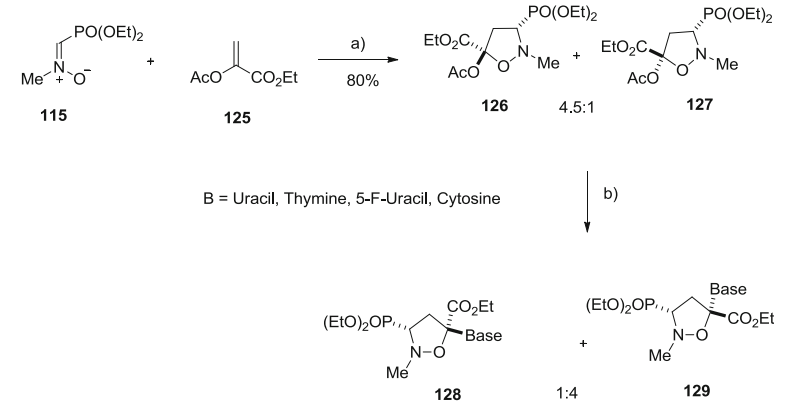
Cis-TPCOAN **119** are able to completely inhibit the RT of Avian Moloney Virus (AMV) and HIV, at concentrations 1 ± 0.1 nM, a level comparable with that of tenofovir (1 nM) and tenfold lower than that of AZT (10 nM). MTS assays indicate a very low toxicity ($CC_{50} > 500$ μM) in comparison with AZT (CC_{50} 12.14 μM) [91].

Homologous derivatives **123** and **124** have been prepared starting from nitrone **120**. Its reaction with vinyl acetate afforded a mixture of *trans*/*cis* adducts (1.85:1 ratio) **121** and **122**, which were then converted into α- and β-anomers **123** and **124**, in a ratio varying from 1:9 (N-acetylcytosine) to 3:7 (thymine and 5-fluorouracil) (Scheme 22). All the products have been evaluated for their ability to inhibit the RT of avian myeloblastosis retrovirus, and no significant activity was observed [92].

A rationalization for the lack of antiviral activity of full-length phosphonated N, O-nucleosides can be supported by the data reported by Sigel [93]. Viral polymerases recognize and use triphosphates nucleosides, complexed with metal ions such as an Mg^{++} and Mn^{++}; the type of complexation determines the reaction pattern of nucleotides. Thus, in the case of phosphonated N,O-nucleosides, the proximity of the N- to P-atom could be discriminant with regard to the biological activity. In full-length nucleotides **124**, the N-atom cannot assure a positive contribute because a 7-membered ring should be formed by chelation, while short length and truncated PCOAN could form 6- six or 5-membered chelates, thus facilitating the bond break between P-α and P-β and allowing the transfer of the nucleotide group with release of pyrophosphate.



Scheme 22 Reagents and conditions: (*a*) vinyl acetate, 100 W, 60°C, 20 min; (*b*) uracil, 5-fluorouracil, Ac-cytosine, cytosine, thymine, BSA, TMSOTf, 70°C, 6 h



Scheme 23 Reagents and conditions: (*a*) THF, reflux, 24 h; (*b*) isoxazolidines **126** and **127**, MeCN, TMSOTf, silylated Thy (overall yield 72%), 5-Fu (overall yield 80%), U (overall yield 71%), Ac-Cy (overall yield 61%), Cy(42% from Ac-Cy)

4.6 Phosphonated N,O-Psiconucleosides

Natural psicofuranosyl nucleosides, characterized by the presence of a hydroxymethyl group at the anomeric carbon atom, are endowed with interesting biological activities. Chiacchio and Romeo [94–96] have designed an easy entry towards the new class of truncated phosphonated N,O-psiconucleosides, starting from the reaction of the phosphonated nitrone **115** with ethyl 2-acetyloxyacrylate **125** [97]. The cycloaddition leads to a mixture of *trans*/*cis* isoxazolidines **126** and **127** (4.5:1 ratio; 80% yield). The subsequent coupling with silylated nucleobases, performed at 70°C, produces, as expected, the *cis*-anomers **129** as almost exclusive compounds (Scheme 23).

Biological tests show that the *cis*-anomers **129** inhibit the RT of AMV, HTLV-1 and HIV. The 5-fluorouracil derivative is the more promising compound, acting on AMLV and on HIV at a concentration of 1 and 10 nM, respectively. The inhibitory activity towards HTLV-1 and HIV was tenfold higher than that of tenofovir and was similar to that of AZT. Moreover, this compound does not show any cytotoxicity according to MTS assay [97].

4.7 Highlights on Antiretroviral and Biological Activities of Phosphonated N,O-Nucleosides

Series of studies were performed by the group directed by Macchi and Mastino to assess the antiviral and biological activities of PCOAN. Actually PCOAN were evaluated for their activity towards both the HTLV-1 RT activity and the HTLV-1 infection in vitro. The ability of the newly synthesized compounds to inhibit RT activity was firstly determined by means of a novel, cell-free assay [98]. This assay measured the effect of PCOAN on the RT activity of commercial avian myeloblastosis virus RT (AMV-RT) and Moloney murine leukemia virus RT (MLV-RT), using, as a template, RNA isolated from stable transfectants expressing constitutively the glycoprotein D of HSV-1. All phosphonates, AdC-P (nucleoside 113 containing cytosine), AdT-P(nucleoside 113 containing thymine) and AdF-P (nucleoside 113 containing 5-fluorouracil) completely inhibited the formation of amplified products at 10 nM concentration, while the (5′S)-5-fluoro-1-isoxazolidin-5-yl-1H-pyrimidine-2,4-dione (AdF), a nucleoside compound showing biological activity, but lacking RT inhibitory activity due to the absence of a hydroxymethyl group in the furanose ring, was actually unable to inhibit RT activity in this assay. PCOAN were then tested to assess their possible cytotoxic effect on lymphoid and monocytoid cells. In particular, the ability of the new compounds to specifically induce apoptosis and to inhibit the cell metabolic activity was assayed. The results indicated that AdC-P and AdT-P did not induce apoptosis or other toxic effects neither in lymphoid cells nor in monocytoid cells. Conversely, AdF-P was shown to cause detectable levels of toxicity [89]. Successively, the activity of PCOAN was tested versus both HTLV-1 RT activity and HTLV-1 infection in vitro [89]. HTLV-1 is an oncogenic human retrovirus endemic in certain areas of the world including Japan and North and South America, where about 5% of the estimated 20 million HTLV-1-infected people develop HTLV-1-associated diseases [99], such as adult T-cell leukemia (ATL), HTLV-1 associated myelopathy/tropic spastic paraparesis (HAM/TSP), or other minor inflammatory diseases. HTLV-1 preferentially infects T lymphocytes with a CD4+ phenotype. In HTLV-1 infection viremia is essentially

a "cytoviremia," since the virus is cell associated. In fact, cell-free virions are rarely infectious, and spreading of the virus in vivo does not require the extracellular release of viral particles. The spread of the virus within an individual host is most commonly recognized as being through cell divisions, and the clonal expansion of infected cells ensures a constant level of viral load. However, recently, it has been highlighted that the HTLV-1 viral load in vivo is also sustained by cell-to-cell contact, involving an horizontal spread of viral particles. Unfortunately, although a number of strategies for therapeutic intervention have been pursued, limited advances have been achieved.

NA have been used for therapy in ATL and in TSP patients. They were used in combination with IFN in ATL or in a different cocktail including AZT, 3TC and TDF in TSP patients. ATL patients exhibited remission for several years during treatment, while TSP patients gave a poor response to NA. Apparently NA utilized in HIV infection provided limited results in HTLV-1 infection. Thus it was worthwhile to investigate whether newly synthesized nucleoside/nucleotide analogues could be useful in HTLV-1 infection. Parallel experiments carried on with AZT, as positive control, revealed that PCOAN inhibited RT activity at a concentration of 10 nM, comparable to that of AZT. The activity of PCOAN was investigated also on HTLV-1 infection in vitro. PCOAN were able to completely inhibit HTLV-1 cell-to-cell transmission in vitro when added in co-treatment, at a concentration of 1 μM, similar to what has been observed for tenofovir and AZT. The inhibition of HTLV-1 infection was ascribed to the block of HTLV-1 RT, as demonstrated in the cell-free HTLV-1 RT inhibition assay.

5 Conclusions

In this review we have tried to summarize the present knowledge concerning the antiviral potential of both old and newly synthesized phosphonated nucleoside analogues (Fig. 9). A high input to continue research in this field is driven from the clinical results in antiviral therapy obtained with the known prototype compounds and from encouraging antiviral effects demonstrated in vitro by newly synthesized compounds. Further substitutions of a number of moieties have shown the flexibility of phosphonated nucleoside analogues structure. This is an additional impulse to design and develop new phosphonated nucleoside-based analogues, endowed with an antiviral activity towards a broader range of DNA or RNA viruses.

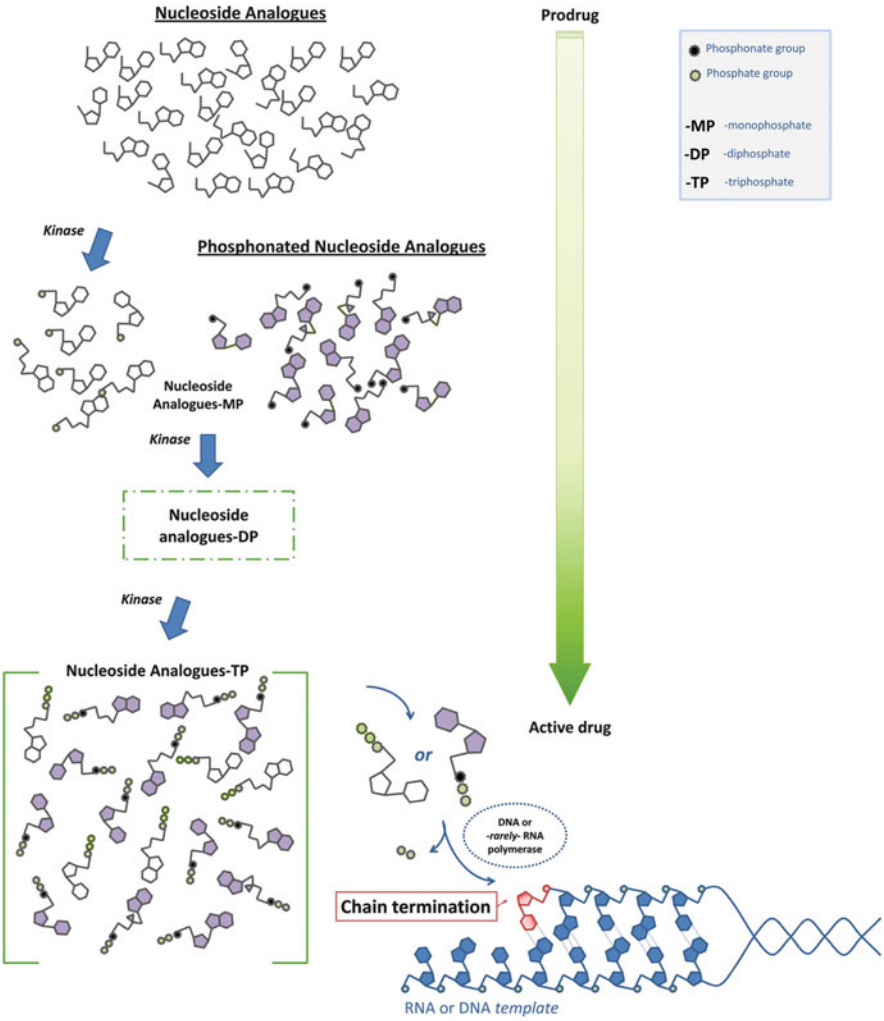


Fig. 9 Transformation from prodrug into active drug of nucleoside analogues and phosphonated nucleoside analogues. The nucleoside analogues are activated into the triphosphate (TP) by three phosphorylation steps performed by cellular kinases. Phosphonated nucleoside analogues can bypass the first phosphorylation steps

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