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# Xanthine-based acyclic nucleoside phosphonates with potent antiviral activity against varicella-zoster virus and human cytomegalovirus

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## Abstract

While noncanonic xanthine nucleotides XMP/dXMP play an important role in balancing and maintaining intracellular purine nucleotide pool as well as in potential mutagenesis, surprisingly, acyclic nucleoside phosphonates bearing a xanthine nucleobase have not been studied so far for their antiviral properties. Herein, we report the synthesis of a series of xanthine-based acyclic nucleoside phosphonates and evaluation of their activity against a wide range of DNA and RNA viruses. Two acyclic nucleoside phosphonates within the series, namely 9-[2-(phosphonomethoxy)ethyl]xanthine (PMEX) and 9-[3-hydroxy-2-(phosphonomethoxy)propyl]xanthine (HPMPX), were shown to possess activity against several human herpesviruses. The most potent compound was PMEX, a xanthine analogue of adefovir (PMEA). PMEX exhibited a single digit μM activity against VZV ($EC_{50} = 2.6\,\mu M$, $TK^+$ Oka strain) and HCMV ($EC_{50} = 8.5\,\mu M$, Davis strain), while its hexadecyloxypropyl monoester derivative was active against HSV-1 and HSV-2 ($EC_{50}$ values between 1.8 and 4.0 μM). In contrast to acyclovir, PMEX remained active against the $TK^-$ VZV 07–1 strain with $EC_{50} = 4.58\,\mu M$. PMEX was suggested to act as an inhibitor of viral DNA polymerase and represents the first reported xanthine-based acyclic nucleoside phosphonate with potent antiviral properties.

## Keywords

Acyclic nucleoside phosphonates, xanthine, PMEX, antiviral, HCMV, VZV

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## Introduction

The concentration and ratio of purine nucleotides and deoxynucleotides in the nucleotide pool is highly regulated in order to maintain the proper function and genetic stability of mammalian cells.[1] Imbalances in (deoxy)nucleotide pool may have mutagenic consequences[2] and may lead to various diseases, such as combined immunodeficiency (loss of purine nucleoside phosphorylase (PNP) function),[3] hyperuricemia (loss of hypoxanthine-guanine phosphoribosyltransferase (HGPRT) function)[4] or cancer (uncontrollable activity of inosine-5′-monophosphate dehydrogenase, IMPDH).[5]

Xanthosine monophosphate (XMP, **1**, Figure 1) is an important intermediate in the *de novo* synthesis of guanine nucleotides and its concentration is essential for the maintenance of guanine nucleotide pool,[6]

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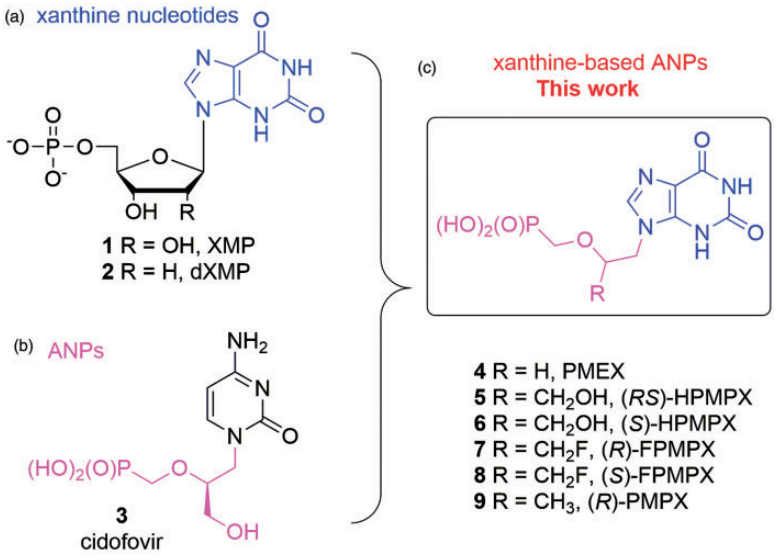



**Figure 1.** (a) Xanthine-based nucleotides; (b) cidofovir (an example of acyclic nucleoside phosphonate, ANP); (c) target xanthine-based ANPs.

where XMP serves as a substrate for guanosine monophosphate synthase,[7] that produces guanosine monophosphate (GMP). XMP is formed either from inosine monophosphate (IMP) by IMPDH or *via* salvage pathway using hypoxanthine or xanthine phosphoribosyltransferase. The XMP level is regulated by 5′-nucleotidase that hydrolyzes XMP to xanthosine.

In contrast, the corresponding deoxyribonucleotide analogues, dXMP (**2**, Figure 1) and dXTP, are catabolic products of dGMP and dGTP enzymatic hydrolysis, or can be formed by defective purine nucleotide metabolism (involving deaminase enzymes),[8] or by chemical hydrolysis[9] of dGMP/dGTP *via* $NO_x$-mediated nitrosative stress.[10] These processes can lead to a substantial incorporation of xanthine nucleotides into DNA and/or RNA,[8] and subsequently to RNA miscoding and mutagenesis.[11] Moreover, deaminated nucleotides can interfere with RNA editing[12] and with functions of noncoding RNAs.[13]

Under cell physiological homeostasis, the concentration and ratio of potentially mutagenic nucleotide intermediates, such as (d)IDP/(d)ITP/(d)XTP, is maintained by housekeeping enzymes,[14] especially those from nudix family such as ITPases/XTPpases,[15,16] NUDT[16] or ITPA,[17] that can hydrolyze corresponding nucleoside di- or triphosphates. The main function of housekeeping enzymes is to prevent or minimize the incorporation of noncanonical nucleotides into DNA/RNA. Unfortunately, the literature on housekeeping enzymes hydrolyzing dXDP/dXTP has been quite rare up to date.

Herpesviruses[18] are DNA-containing enveloped viruses from large *Herpesviridae* family and include herpes simplex virus (HSV), varicella-zoster virus (VZV), cytomegalovirus (CMV), and Epstein-Barr virus (EBV). Although current anti-herpetic therapy uses powerful antivirotics such as nucleoside analogues (acyclovir (ACV), penciclovir, vidarabine, and ganciclovir (GCV)), acyclic nucleoside phosphonate (ANP) cidofovir (CDV) (**3**, Figure 1),[19] or diphosphate mimic foscarnet,[20] many drug insensitive viruses have been identified in the clinics. The origin of virus resistance for HSV, VZV or CMV comes mostly from treatment using DNA polymerase inhibitors, such as ACV and GCV, where various alterations in the viral thymidine kinase gene [*UL23* (HSV) and *ORF36* (VZV)], protein kinase [*UL97* (CMV)] and/or viral DNA polymerase gene [*UL30 (HSV)*, *ORF28* (VZV) and *UL54* (CMV)] may occur.[21–23] As recent literature has shown,[24] the presence of resistant herpesviruses should be considered seriously not only in the case of immunocompromised individuals. Evidently, there is an urgent need for novel potent anti-herpetic agents with high barrier of resistance development.

ANPs,[25] mimics of natural nucleotides (avoiding the first phosphorylation step), represent a potent group of antiviral agents. ANPs are converted inside the cells to their diphosphates (ANPpp) that target DNA polymerase – viral and/or cellular.[19] These nucleoside triphosphate analogues act as competitive inhibitors and/or alternative substrates of the respective enzymes, in the later case leading to termination of DNA chain elongation.[19] Although some ANPs derived from xanthine

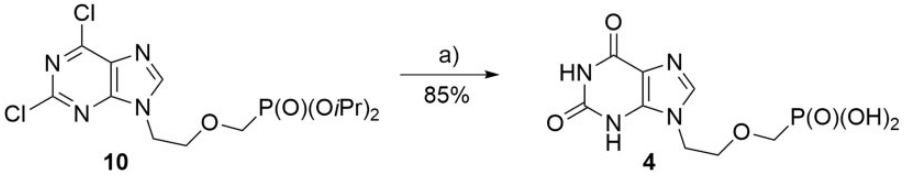
were studied before as potential antiviral agents (namely the 9-[3-fluoro-2-(phosphonomethoxy)propyl] derivative, FPMPX),[26] the general lack of interest in such compounds was probably caused by their relatively complicated synthesis, since simple alkylation of xanthine base was expected to give a mixture of several regioisomers as well as polyalkylated products. Recently, we have reported[27] a simple and high-yielding synthesis of xanthine ANPs exploiting the MW-assisted hydrolysis of the corresponding 2,6-dichloropurine derivatives. Here, we report the synthesis and antiviral evaluation of a series of xanthine-based ANPs (compounds **4**–**9**, Figure 1), designed as non-hydrolyzable analogues of dXMP/XMP.

## Chemistry

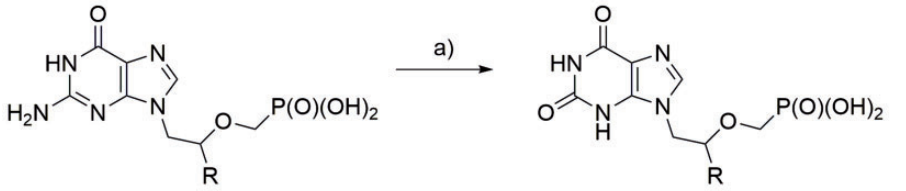
The synthesis of 9-[2-(phosphonomethoxy)ethyl]xanthine (PMEX, **4**, Scheme 1), a xanthine analogue of the well-known antiviral agent adefovir (PMEA),[28] has been reported by our group earlier.[27] The microwave-assisted hydrolysis of 2,6-dichloropurine derivative **10**[29] in aqueous HCl afforded the desired xanthine compound **4** in a 85% yield.

For the synthesis of other target ANPs, compounds **5**–**9** (Scheme 2), previously reported[26,30–34] guanine containing ANPs **11**–**15**, have been exploited as a starting material. Standard diazotization of compounds **11**–**15** followed by 2-hydroxy-dediazoniation afforded the desired xanthine-based ANPs **5**–**9** in moderate to good yields (36–82%).

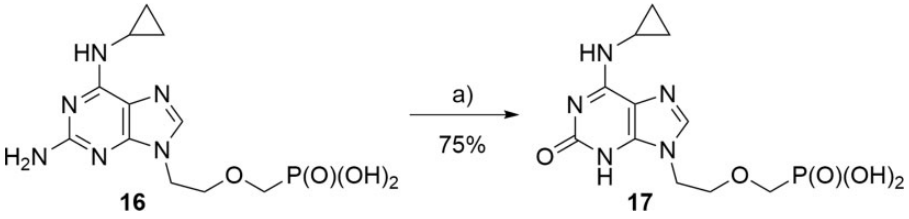
Since PMEX (**4**) exhibited promising antiviral properties, we decided to prepare several PMEX prodrugs in order to improve the compound permeability which might be limited for this negatively charged compound. At first, $N^6$-cyclopropylaminopurine derivative **17** (Scheme 3) was prepared in a 75% yield from the corresponding $N^6$-cyclopropyl-2,6-diaminopurine derivative **16**,[35] using the above mentioned diazotization/2-hydroxy-dediazoniation procedure. Compound **17** was expected to be enzymatically converted (deaminated) to PMEX in an analogy to com-



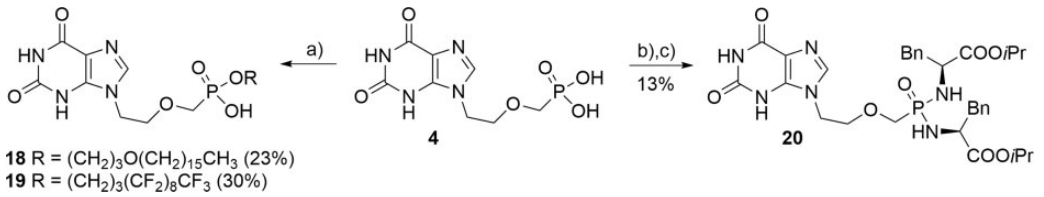

**Scheme 1.** Preparation of PMEX (**4**). Reaction conditions: (a) 1 M aq. HCl, MW-assisted heating, 140 °C, 20 min.



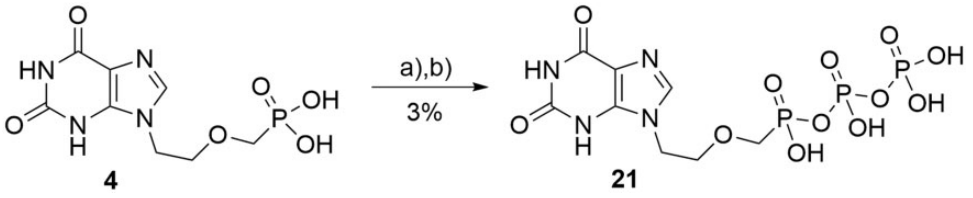

**Scheme 2.** Preparation of xanthine-based ANPs **5**–**9**. Reaction conditions: (a) isoamylnitrite, 80% aq. AcOH, 25 °C, 16 h.




**Scheme 3.** Synthesis of compound **17**. Reaction conditions: (a) isoamylnitrite, 80% aq. AcOH, 25 °C, 16 h.




**Scheme 4.** Synthesis of PMEX prodrugs **18**–**20**. Reaction conditions: (a) corresponding alcohol, pyridine, DCC, 100 °C, 16 h; (b) TMSBr, 25 °C, 16 h; (c) *i*Pr-L-PheAla.HCl, pyridine, $Et_3N$, Aldrithiol-2, triphenylphosphine, 70 °C, 72 h.




**Scheme 5.** Synthesis of PMEX diphosphate **21**. Reaction conditions: (a) morpholine, DCC, *t*-BuOH, $H_2O$, 105 °C, 16 h; (b) pyrophosphate (0.5 M in DMF), DMSO, 25 °C.

pound GS-9219 (an acyclic nucleotide analogue with potent antineoplastic activity),[36] and abacavir (a carbocyclic nucleoside used for the treatment of HIV infection).[37]

Next, PMEX hexadecyloxypropyl (HDP) monoester **18** (Scheme 4), a prodrug approach developed by Hostetler *et al*. as a mimic of natural lipids,[38] was prepared from PMEX (**4**) and hexadecyloxypropyl alcohol *via* DCC-mediated coupling in a 23% yield. Similarly, phosphonate ester **19** (Scheme 4) bearing a perfluorinated-C12 chain was prepared by the same procedure in a 30% yield. Finally, the bisamidate prodrug **20** (Scheme 4) was obtained in a 13% yield starting from PMEX (**4**) and isopropyl ester of L-phenylalanine using the previously described procedure developed in our lab.[39]

In order to confirm the expected mode of action of PMEX (**4**), i.e. viral DNA polymerase inhibition, the corresponding phosphonodiphosphate **21** (Scheme 5), as an analogue of natural nucleoside triphosphate, was also prepared. The two-step synthesis *via* a morpholidate intermediate[40] afforded, after the HPLC purification, the desired triphosphate mimic **21** in a low (3%) yield.

## Biology

The synthesized xanthine-based ANPs (compounds **4**–**9**) were evaluated for inhibitory activity against a wide range of DNA and RNA viruses: in human embryonic lung (HEL) cells (herpes simplex virus-1 (KOS strain), herpes simplex virus-2 (G strain), thymidine kinase deficient (ACV resistant) herpes simplex virus-1 ($TK^-$ KOS $ACV^r$), vaccinia virus, vesicular stomatitis virus, human cytomegalovirus (HCMV) (AD-169 strain and Davis strains), VZV ($TK^+$ VZV strain and $TK^-$ VZV strains)), in HeLa cell cultures (vesicular stomatitis virus, Coxsackie virus B4 and respiratory syncytial virus (RSV)), in Vero cell cultures (para-influenza-3 virus, reovirus-1, Sindbis virus, Coxsackie virus B4, Punta Toro virus, yellow fever virus), in CrFK cell cultures (feline corona virus (FIPV)), and in MDCK cell cultures (influenza A virus (H1N1 and H3N2 subtypes) and influenza B virus). GCV, CDV, ACV, brivudin (BVDU), zalcitabine, zanamivir, alovudine, amantadine, rimantadine, ribavirin, dextran sulfate (molecular weight 10000, DS-10000), mycophenolic acid, Hippeastrum hybrid agglutinin (HHA), and Urtica dioica agglutinin (UDA) were used as the reference compounds. The antiviral activity was expressed as the $EC_{50}$, i.e. compound concentration required to decrease virus plaque formation (VZV) or virus-induced cytopathogenicity (other viruses) by 50%. While none of the compounds showed any activity against RNA viruses, compounds **4**, **5** and **6** were able to inhibit the replication of herpesviruses (Tables 1 and 2). PMEX (**4**) emerged as the most active compound against VZV and HCMV, being as active as the reference drug ACV against the $TK^+$ Oka strain ($EC_{50} = 2.62\,\mu M$ (PMEX) *versus* $3.42\,\mu M$ (ACV)). In contrast to ACV, compound **4** remained active against the $TK^-$ VZV 07–1 strain ($EC_{50} = 4.58\,\mu M$). PMEX also inhibited the

**Table 1.** Activity of compounds **4**–**9** against varicella-zoster virus (VZV) and human cytomegalovirus (HCMV) in human embryonic lung (HEL) cells.

| | Antiviral activity $EC_{50}$ (μM)[a] | | | | Cytotoxicity (μM) | |
|---|---|---|---|---|---|---|
| | $TK^+$ VZV | $TK^-$ VZV | HCMV | | | |
| Compound | OKA strain | 07-1 strain | AD-169 strain | Davis strain | Cell morphology (MCC)[b] | Cell growth ($CC_{50}$)[c] |
| **4** PMEX | 2.62 ± 1.19 | 4.58 ± 2.59 | 10.5 ± 3.9 | 8.5 ± 2.0 | >345 | 111 ± 71 |
| **5** (*RS*)-HPMPX | 30.9 ± 12.3 | 27.1 ± 9.8 | 86.9 ± 21.1 | 48.6 ± 24.2 | ≥300 | ≥300 |
| **6** (*S*)-HPMPX | 22.7 ± 6.8 | 17.1 ± 6.4 | 80.2 ± 36.0 | 43.0 ± 33.9 | >300 | >300 |
| **7** (*R*)-FPMPX | >313 | >313 | >313 | ND | >313 | ND[d] |
| **8** (*S*)-FPMPX | >329 | >329 | >329 | ND | >329 | ND |
| **9** (*R*)-PMPX | >100 | >100 | >100 | >100 | >100 | ND |
| Acyclovir | 3.42 ± 2.25 | 115 ± 68 | ND | ND | >440 | >440 |
| Brivudin | 0.019 ± 0.013 | 116 ± 57 | ND | ND | >300 | 309 ± 213 |
| Ganciclovir | ND | ND | 6.13 ± 2.38 | 4.83 ± 1.88 | >350 | ≥445 ± 204 |
| Cidofovir | ND | ND | 0.86 ± 0.37 | 0.92 ± 0.34 | >300 | 263 ± 171 |

[a]Effective concentration required to reduce virus plaque formation (VZV) or viral-induced cytopathic effect (HCMV) by 50%. Virus input was 20 (VZV) or 100 PFU (HCMV) plaque forming units (PFU).
[b]Minimum cytotoxic concentration that causes a microscopically detectable alteration of cell morphology.
[c]Cytostatic concentration required reducing cell growth by 50%.
[d]Not determined.

**Table 2.** Activity of compounds **4**–**9** against herpes simplex virus 1 and 2 (HSV-1 and HSV-2), thymidine kinase deficient ($TK^-$) HSV-1 and vaccinia virus in human embryonic lung (HEL) cells.

| | Antiviral activity $EC_{50}$ (μM)[a] | | | | Cytotoxicity (μM) |
|---|---|---|---|---|---|
| Compound | HSV-1 | HSV-2 | HSV-1 $TK^-$ | Vaccinia virus | Cell morphology (MCC)[b] |
| **4** PMEX | 21.5 ± 7.6 | 25.6 ± 14.8 | 23.7 ± 14.0 | >345 ± 0 | >345 |
| **5** (*RS*)-HPMPX | 39 ± 27 | 20 ± 0 | 38 ± 0 | 39 ± 27 | >100 |
| **6** (*S*)-HPMPX | 33 ± 18 | 16 ± 6 | 20 ± 0 | 39 ± 27 | >100 |
| **7** (*R*)-FPMPX | >313 | >313 | >313 | >313 | >313 |
| **8** (*S*)-FPMPX | >329 | >329 | >329 | >329 | >329 |
| **9** (*R*)-PMPX | >100 | >100 | >100 | >100 | >100 |
| Acyclovir | 0.27 ± 0.12 | 0.14 ± 0.10 | 10 ± 0 | >250 | >250 |
| Brivudin | 0.031 ± 0.020 | 112 ± 37 | 32.4 ± 24.1 | 8.26 ± 9.62 | >250 |
| Ganciclovir | 0.028 ± 0.015 | 0.032 ± 0.004 | 1.47 ± 1.60 | >100 | >100 |
| Cidofovir | 1.50 ± 0.50 | 1.54 ± 1.46 | 1.92 ± 1.31 | 22.0 ± 19.9 | >250 |

[a]Effective concentration required to reduce virus plaque formation (VZV) or viral-induced cytopathic effect (HCMV) by 50%. Virus input was 20 (VZV) or 100 PFU (HCMV) plaque forming units (PFU).
[b]Minimum cytotoxic concentration that causes a microscopically detectable alteration of cell morphology.

replication of HCMV with $EC_{50}$ values of the same order of magnitude as the reference anti-HCMV drug GCV, while PMEX had a 50% cytostatic concentration of 111 μM for HEL cells. Thus, compound **4** was not only potent but also selective as the calculated selectivity indices (ratio $CC_{50}/EC_{50}$) were of 24 and 42 (VZV 07–1 and Oka strains, respectively) and of 11 and 13 (HCMV AD-169 and Davis strains, respectively). Compounds **5** and **6** were, respectively, 6 to 12 folds and 3 to 8 folds less active than PMEX against VZV and HCMV. However, compounds **4**, **5** and **6** inhibited the replication of HSV-1, HSV-2 and TK-HSV-1 at equivalent $EC_{50}$ values in the range of 16 to 39 μM. PMEX (**4**) lacked activity against vaccinia virus, while HPMPX compounds (both *S*-isomer **6** and racemic mixture **5**) were weak inhibitors of this poxvirus ($EC_{50} = 39$ μM, Table 2).

ANPs, including PMEX, are polar compounds, showing severely limited bioavailability. To increase the likelihood of good cell wall permeability, several different prodrug approaches were tested for PMEX (Tables 3 and 4). Compound **17** was completely

**Table 3.** Evaluation of prodrug compounds **18**–**20** against VZV and CMV in HEL cells.

| | Antiviral activity $EC_{50}$ (μM)[a] | | | | Cytotoxicity (μM) | |
|---|---|---|---|---|---|---|
| | $TK^+$ VZV | $TK^-$ VZV | HCMV | | | |
| Compound | OKA strain | 07-1 strain | AD-169 strain | Davis strain | Cell morphology (MCC)[b] | Cell growth ($CC_{50}$)[c] |
| 17 | >100 | >100 | >100 | >100 | >100 | ND[d] |
| 18 | 0.10 ± 0.05 | 0.64 ± 0.77 | 0.20 ± 0.28 | 0.05 ± 0.05 | 100 | 10 ± 0 |
| 19 | >20 | >100 | 63 | 55 | ≥100 | ND |
| 20 | 22 | 26.5 | 63 ± 19 | 6.2 ± 6.8 | >100 | 100 ± 0 |
| Acyclovir | 2.23 ± 2.16 | 98 ± 61 | ND | ND | >440 | >440 |
| Brivudin | 0.019 ± 0.010 | 19.6 ± 23.0 | ND | ND | ≥300 | 168 ± 41 |
| Ganciclovir | ND | ND | 10.0 ± 10.2 | 5.5 ± 4.4 | >350 | ≥317 ± 98 |
| Cidofovir | 0.92 ± 0.38 | 0.78 ± 0.32 | ND | ND | >300 | 170 ± 61 |

[a]Effective concentration required to reduce virus plaque formation (VZV) or viral-induced cytopathic effect (HCMV) by 50%. Virus input was 20 (VZV) or 100 PFU (HCMV) plaque forming units (PFU).
[b]Minimum cytotoxic concentration that causes a microscopically detectable alteration of cell morphology.
[c]Cytostatic concentration required reducing cell growth by 50%.
[d]Not determined.

**Table 4.** Evaluation of prodrug compounds **18**–**20** on HSV and vaccinia virus in HEL cells.

| | Antiviral activity $EC_{50}$ (μM)[a] | | | | Cytotoxicity (μM) |
|---|---|---|---|---|---|
| Compound | HSV-1 | HSV-2 | HSV-1 $TK^-$ | Vaccinia virus | Cell morphology (MCC)[b] |
| 17 | >100 | >100 | >100 | >100 | >100 |
| 18 | 1.8 | 4.0 | 2 | >100 | >100 |
| 19 | >100 | >100 | >100 | >100 | >100 |
| 20 | >100 | >100 | >100 | >100 | >100 |
| Acyclovir | 0.50 ± 0.14 | 0.50 ± 0.14 | 3.25 ± 1.77 | >250 | >250 |
| Brivudin | 0.05 ± 0.05 | ≥250 ± 0 | 50 | 16.4 ± 17.8 | >250 |
| Ganciclovir | 0.020 ± 0.014 | 0.045 ± 0.049 | 0.90 ± 0.14 | >100 ± 0 | >100 |

[a]Effective concentration required to reduce virus plaque formation (VZV) or viral-induced cytopathic effect (HCMV) by 50%. Virus input was 20 (VZV) or 100 PFU (HCMV) plaque forming units (PFU).
[b]Minimum cytotoxic concentration that causes a microscopically detectable alteration of cell morphology.

inactive in all assays. Phosphonate ester **19** and biamidate prodrug **20** did not display any potent antiherpesvirus activity which might be explained by insufficient prodrug activation in these cells. The HDP-PMEX prodrug **18** proved to be 7 to 26 folds (VZV), 52 to 170 folds (HCMV) and 6 to 12 folds (HSV) more active compound than the parent compound **4**, although a concomitant increase in its cytostatic activity of 11-fold was also observed indicating successful increase in the cell uptake.

In order to determine whether the DNA polymerase was the actual target of action of PMEX (**4**), the compound was evaluated against well-characterized HSV-1 mutant viruses. Alike all ANPs, PMEX remained active against viruses bearing mutations in the viral TK (Figure 2). Importantly, an increase in the $EC_{50}$ of PMEX of the same magnitude as that measured for the ANP adefovir[28] was found for DNA polymerase mutant viruses indicating that the target of action of the active form of compound **4** (i.e. PMEXpp) is the herpesvirus DNA polymerase.

The inhibitory activity of the diphosphate form of PMEX (PMEXpp, **21**) was evaluated in an enzymatic assay against herpes (VZV and HCMV) DNA polymerases compared to cellular (α and β) DNA polymerases (Table 5). The inhibition of ACV triphosphate and the pyrophosphate analogue of foscarnet (PFA) were determined in this study for comparison. Compound **21** was not inhibitory towards cellular DNA polymerases, while it inhibited VZV DNA polymerase when dGTP was used as the competitive radiolabeled substrate ($IC_{50} = 7.4\,\mu M$). However, no activity at the highest concentration of **21** (100 μM) could be detected against HCMV DNA polymerase when either dGTP or dTTP were used as the competitive radiolabeled substrates. These data suggest that compound **21** is a poor inhibitor of HCMV DNA polymerase or that the compound

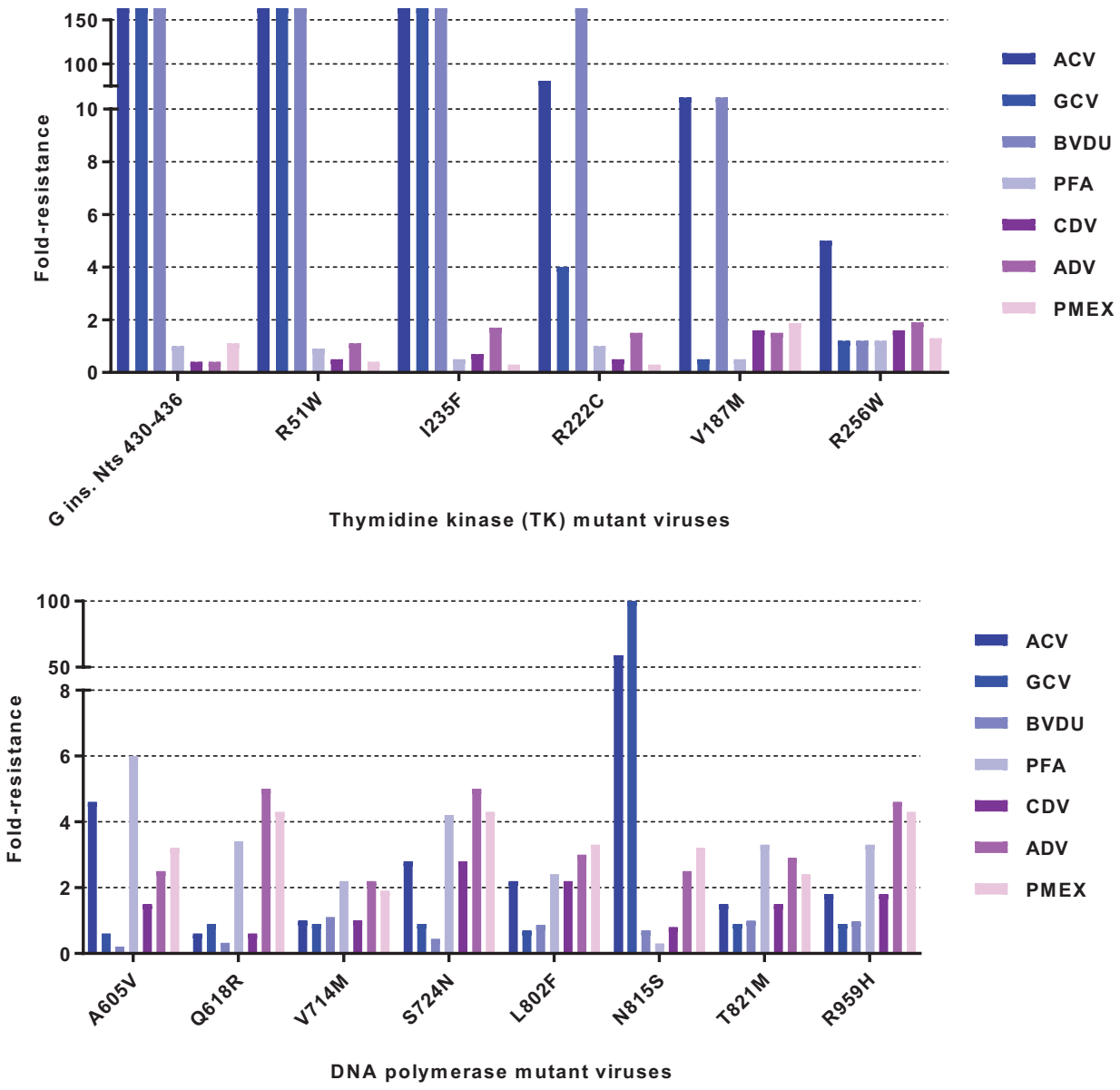



**Figure 2.** Activity of compound **4** against several thymidine kinase and DNA polymerase HSV-1 mutants in HEL cells. Fold-resistance was calculated as the ratio $EC_{50}$ mutant virus/$EC_{50}$ wild-type Kos strain.

**Table 5.** Inhibition of viral and cellular DNA polymerases (pol) by compound **21** (PMEXpp) compared to the triphosphate form of the nucleoside analogue acyclovir (ACV-TP) and the pyrophosphate analogue of foscarnet (PFA).

| | $IC_{50}$ (μM)[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | HCMV DNA pol | | VZV DNA pol | DNA pol α | | DNA pol β | |
| Compound | dGTP[b] | dTTP[b] | dTTP[b] | dGTP[b] | dTTP[b] | dGTP[b] | dTTP[b] |
| **21** (PMEXpp) | >100 | >100 | 7.4 ± 2.7 | >100 | >100 | >100 | >100 |
| ACV-TP | 0.77 ± 0.04 | 82 ± 26 | ND[c] | ND | ND | ND | ND |
| PFA pyrophosphate | 7.0 ± 0.7 | 9.4 ± 6.0 | 0.18 ± 0.01 | 56.0 ± 12.0 | ND | >100 | ND |

[a]50% inhibitory concentration or compound concentration required to inhibit the polymerase-catalyzed DNA synthesis by 50%.
[b]Enzyme reaction in the presence of calf thymus DNA and radiolabeled [$^3$H]dGTP or [$^3$H]dTTP.
[c]Not determined.

may require the HCMV DNA polymerase interact with other proteins of the replication complex to be active. It is also possible that PMEX or its metabolite targets another viral enzyme. To confirm that the compound targets the viral DNA polymerase, PMEX-resistant herpesviruses should be selected under the pressure of PMEX and characterized both genotypically and phenotypically.

## Experimental part

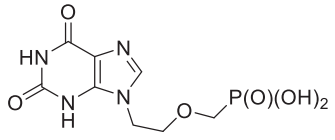
### Methods

Starting compounds and other chemicals were purchased from commercial suppliers or prepared according to the published procedures. Solvents were dried by standard procedures. Solvents were evaporated at 40 °C/2 kPa. Analytical TLC was performed on plates of Kieselgel 60 F 254 (Merck). Column chromatography was performed on silica gel 230–400 mesh, 60 Å (Merck). Reverse phase HPLC separation was performed on a Waters Delta 600 instrument with a Waters 486 Tunable Absorbance Detector using column Phenomenex Gemini C18 (10 $\mu$m, 250 × 21.2 mm, flow 10 ml/min preparative column). NMR spectra were recorded on Bruker Avance 500 ($^1$H at 500 MHz, $^{13}$C at 125.8 MHz, $^{31}$P at 202.4 MHz,) spectrometer with TMS or 1,4-dioxane (3.75 ppm for $^1$H, 67.19 ppm for $^{13}$C NMR) as internal standard or referenced to the residual solvent signal. HR MS spectra were taken on a LTQ Orbitrap XL spectrometer. The purity of the tested compounds was determined by HPLC ($H_2O$-$CH_3CN$, linear gradient) and was higher than 95%.

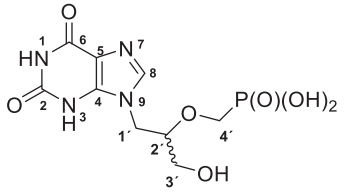
### Method A. General procedure for diazotization/2-hydroxy-dediazoniation of guanine-based starting compounds

Guanine-based phosphonate (0.5 mmol) was dissolved in 80% AcOH (20 mL) and excess of isoamylnitrite (2.0 mL) was added. The reaction mixture was stirred at 20 °C for 16 h. Volatiles were evaporated, and the residue was co-evaporated with water (3 × 10 mL) and evaporated to dryness. The crude product was dissolved in a small amount of water and purified by preparative HPLC in 0.1 M TEAB buffer using gradient water/methanol (from 98/2 to 20/80).

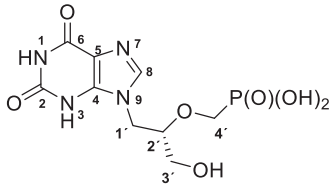
*((2–(2,6-Dioxo-1,2,3,6-tetrahydro-9H-purin-9-yl)ethoxy) methyl)phosphonic acid (4), PMEX.* Synthesis of compound **4** was performed from compound **10** (2.0 g, 4.86 mmol), using previously described procedure.[27] Microwave-assisted heating (130 °C, 20 min) of compound **10** in aqueous HCl (1.0 M), followed by solvent removal and precipitation form a water/methanol mixture gave **4** (1.2 g, 85%) as a white solid. The analytical data are in an agreement with the published data.[27]



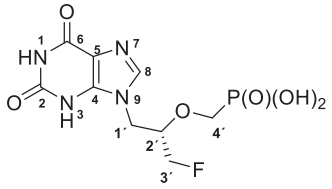
*(RS)-(((1–(2,6-Dioxo-1,2,3,6-tetrahydro-9H-purin-9-yl)-3-hydroxypropan-2-yl)oxy)methyl)phosphonic acid (5), (RS)-HPMPX.* Treatment of **11** (223 mg, 0.70 mmol) by Method A afforded **5** (160 mg, 71%) as a white solid. $^1$H NMR ($D_2O$ + NaOD) $\delta$: 7.84 (s, 1H, H-8), 4.21 (dd, $J_{gem}$ = 14.7 Hz, $J_{1'a\text{-}2'}$ = 4.9 Hz, 1H, H-1′a), 4.15 (dd, $J_{gem}$ = 14.7 Hz, $J_{1'b\text{-}2'}$ = 6.1 Hz, 1H, H-1′b), 3.80 (m, 1H, H-2′), 3.71 (dd, $J_{gem}$ = 12.5 Hz, $J_{3'a\text{-}2'}$ = 3.7 Hz, 1H, H-3′a), 3.55 (dd, $J_{gem}$ = 12.3 Hz, $J_{4'a\text{-}P}$ = 8.8 Hz, 1H, H-4′a), 3.49 (dd, $J_{gem}$ = 12.3 Hz, $J_{4'b\text{-}P}$ = 9.6 Hz, 1H, H-4′b), 3.47 (dd, $J_{gem}$ = 12.5 Hz, $J_{3'b\text{-}2'}$ = 5.4 Hz, 1H, H-3′b). $^{13}$C NMR ($D_2O$ + NaOD): $\delta$ 161.88 (C-6), 160.57 (C-2), 154.36 (C-4), 140.89 (C-8), 114.98 (C-5), 80.60 (d, $J_{C\text{-}O\text{-}C\text{-}P}$ = 10.8 Hz, C-2′), 68.66 (d, $J_{C\text{-}P}$ = 150.7 Hz, C-4′), 60.98 (C-3′), 43.59 (C-1′). MS-ESI$^-$ $m/z$ (%): 319 (100, M-H$^+$). HRMS(ESI$^-$) m/z ($C_9H_{13}N_4O_7P$) [M-H]$^-$: calcd 319.0449; found 319.0447.



*(S)-(((1–(2,6-Dioxo-1,2,3,6-tetrahydro-9H-purin-9-yl)-3-hydroxypropan-2-yl)oxy)methyl) phosphonic acid (6), (S)-HPMPX.* Treatment of **12** (154 mg, 0.48 mmol) by Method A gave **6** (55 mg, 36%) as a white solid. $^1$H NMR and $^{13}$C NMR spectra are identical to those of compound **5**. MS-ESI$^-$ $m/z$ (%): 319 (100, M-H$^+$). HRMS(ESI$^-$) m/z ($C_9H_{13}N_4O_7P$) [M-H]$^-$: calcd 319.0449; found 319.0448. $[\alpha]^{20}_D$ = –4.7 (c = 0.254 g/100 ml, $H_2O$).

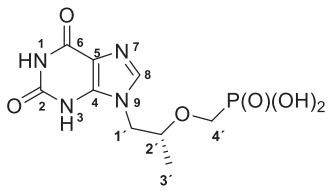


*(R)-(((1–(2,6-Dioxo-1,2,3,6-tetrahydro-9H-purin-9-yl)-3-fluoropropan-2-yl)oxy)methyl)phosphonic acid (7), (R)-FPMPX.* Treatment of **13** (150 mg, 0.47 mmol) by Method A gave **7** (123 mg, 82%) as a white solid. $^1$H NMR (DMSO-$d_6$): $\delta$ 10.84 (bs, 1H, 1 or 3), 7.72 (s, 1H, 8), 4.62 (ddd, $J_{3'b\text{-}F} = 47.4$ Hz, $J_{gem} = 10.5$ Hz, $J_{3'b\text{-}2'} = 3.0$ Hz, H-3′b), 4.42 (ddd, $J_{3'a\text{-}F} = 47.2$ Hz, $J_{gem} = 10.5$ Hz, $J_{3'a\text{-}2'} = 4.2$ Hz, H-3′a), 4.27 (dd, $J_{gem} = 14.9$ Hz, $J_{1'b\text{-}2'} = 4.3$ Hz, H-1′b), 4.20 (dd, $J_{gem} = 14.8$ Hz, $J_{1'a\text{-}2'} = 7.4$ Hz, H-1′a), 3.95 (bddtd, 1H, $J_{2'\text{-}F} = 24.1$ Hz, $J_{2'\text{-}1'a} = 7.4$ Hz, $J_{2'\text{-}1'b} = J_{2'\text{-}3'a} = 4.2$ Hz, $J_{2'\text{-}3'b} = 3.0$ Hz, H-2′), 3.67 (dd, $J_{gem} = 13.5$ Hz, $J_{4'b\text{-}P} = 9.0$ Hz, H-4′b), 3.57 (dd, $J_{gem} = 13.5$ Hz, $J_{4'a\text{-}P} = 9.0$ Hz, H-4′a); $^{13}$C NMR (DMSO-$d_6$): $\delta$ 157.95 (C-6), 151.00 (C-2), 140.80 (C-4), 137.75 (C-8), 114.89 (C-5), 82.26 (d, $J_{3'\text{-}F} = 169.9$ Hz, C-3′), 77.77 (dd, $J_{2'\text{-}F} = 18.4$ Hz, $J_{2'\text{-}P} = 10.3$ Hz, C-2′), 65.96 (d, $J_{4'\text{-}P} = 160.3$ Hz, C-4′), 44.38 (d, $J_{1'\text{-}F} = 8.4$ Hz, C-1′). HRMS(ESI$^-$) m/z ($C_9H_{12}FN_4O_6P$) [M-H]$^-$: calcd 321.0478; found 321.0472. $[\alpha]^{20}_D = +6.2$ (c = 0.194 g/100 ml).

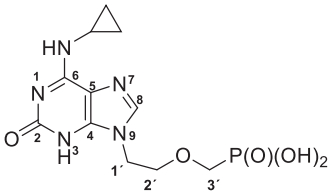


*(S)-(((1–(2,6-Dioxo-1,2,3,6-tetrahydro-9H-purin-9-yl)-3-fluoropropan-2-yl)oxy)methyl)phosphonic acid (8), (S)-FPMPX.* Treatment of **14** (150 mg, 0.47 mmol) by Method A gave **8** (73 mg, 49%) as a white solid. The analytical data are identical to compound **7**. $[\alpha]^{20}_D$ =- 4.2 (c = 0.0238 g/100 mL).

*(R)-(((1–(2,6-Dioxo-1,2,3,6-tetrahydro-9H-purin-9-yl)propan-2-yl)oxy)methyl)phosphonic acid (9), (R)-PMPX.* Treatment of **15** (250 mg, 0.82 mmol) by Method A gave **9** (129 mg, 51%) as a white solid. $^1$H NMR (DMSO-$d_6$): $\delta$ 10.65 (bs, 1H, 1 or 3), 7.61 (s, 1H, 8), 4.27 (dd, $J_{gem} = 14.5$ Hz, $J_{1'a\text{-}2'} = 4.6$ Hz, H-1′b), 4.13 (dd, $J_{gem} = 14.5$ Hz, $J_{1'b\text{-}2'} = 4.5$ Hz, H-1′a), 3.86 (m, 1H, H-2′), 3.54 (dd, $J_{gem} = 13.5$ Hz, $J_{4'b\text{-}P} = 9.0$ Hz, H-4′b), 3.45 (dd, $J_{gem} = 13.6$ Hz, $J_{4'a\text{-}P} = 6.8$ Hz, H-4′a), 0.99 (d, 3H, $J_{3'\text{-}2'} = 6.4$ Hz, H-3′). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 158.24 (C-6), 151.22 (C-2), 141.57 (C-4), 138.04 (C-8), 115.38 (C-5), 74.64 (d, $J_{2'\text{-}P} = 6.7$ Hz, C-2′), 48.64 (C-1′), 17.45 (C-3′), C-4′- not observed. HRMS(ESI$^-$) m/z ($C_9H_{13}N_4O_6P$) [M-H]$^-$: calcd 303.0573; found 303.0499. $[\alpha]^{20}_D = +4.7$ (c = 0.189 g/100 ml).

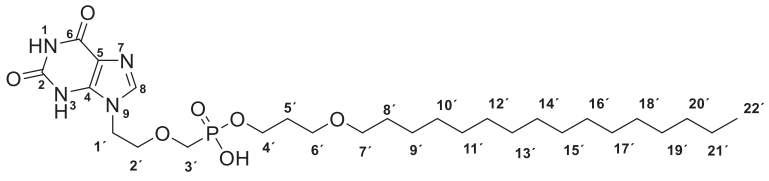


*((2–(6-(Cyclopropylamino)-2-oxo-2,3-dihydro-9H-purin-9-yl)ethoxy)methyl)phosphonic acid (17).* Treatment of **16** (200 mg, 0.61 mmol) by Method A gave **17** (149 mg, 75%) as a white solid. $^1$H NMR (DMSO-$d_6$): $\delta$ 8.67 (bs, 1H, N*H*-CH), 7.80 (s, 1H, H-8), 4.15 (t, 2H, $J_{1'\text{-}2'} = 5.2$ Hz, H-1′), 3.78 (t, 2H, $J_{2'\text{-}1'} = 5.2$ Hz, H-2′), 3.56 (d, 2H, $J_{3'\text{-}P} = 8.4$ Hz, H-3′), 2.98 (bs, 1H, NH-C*H*), 0.77 (m, 2H, $CH_2$-cPr), 0.66 (m, 2H, $CH_2$-cPr). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 155.73 (C-6), 153.45 (C-2), 149.9 (C-4), 139.40 (C-8), 110.37 (C-5), 70.30 (d, $J_{2'\text{-}P} = 9.7$ Hz, C-2′), 67.0 (d, $J_{3'\text{-}P} = 158.8$ Hz, C-3′), 42.76 (C-1′), 24.2 (CH-NH), 7.02 ($CH_2$-cypr). HRMS(ESI$^-$) m/z ($C_{11}H_{16}N_5O_5P$) [M-H]$^-$: calcd. 328.0889; found 328.0816.

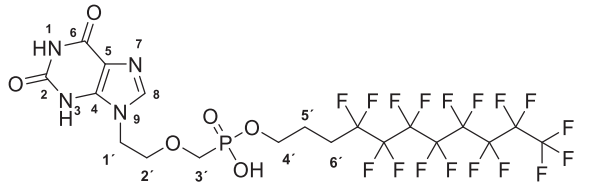


*3-(Hexadecyloxy)propyl hydrogen ((2–(2,6-dioxo-1,2,3,6-tetrahydro-9H-purin-9-yl)ethoxy)methyl)phosphonate (18).* A suspension of **4** (100 mg, 0.31 mmol) and 3-(hexadecyloxy)propan-1-ol (124 mg, 0.41 mmol) in anhydrous pyridine (10 mL) was preheated to 100 °C. Dicyclohexylcarbodiimide (142 mg, 0.69 mmol) in anhydrous pyridine (3 ml) was added and the reaction mixture was stirred at 100 °C for 16 h. Solvent was evaporated and the crude product was purified using silica gel chromatography ($CHCl_3$/MeOH, 0–50%) to give **18** (46 mg, 23%) as a white amorphous solid. $^1$H NMR ($CD_3OD$): $\delta$ 7.69 (s, 1H, H-8), 4.18 (m, 2H, H-1′), 3.91 (q, 2H, $J_{4'\text{-}5'} = J_{4'\text{-}P} = 6.4$ Hz, H-4′), 3.80 (m, 2H, H-2′), 3.61 (d, 2H, $J_{3'\text{-}P} = 8.9$ Hz, H-3′), 3.46 (t, 2H, $J_{6'\text{-}5'} = 6.5$ Hz, H-6′), 3.36 (t, 2H, $J_{7'\text{-}8'} = 6.6$ Hz, H-7′), 1.79 (p, 2H, $J_{5'\text{-}4'} = J_{5'\text{-}6'} = 6.4$ Hz, H-5′), 1.52 (m, 2H, H-8′), 1.34–1.27 (m, 26H, H-9′–21′), 0.90 (t, 3H, $J_{22'\text{-}21'} = 7.0$ Hz, H-22′). $^{13}$C NMR ($CD_3OD$): $\delta$ 161.79 (C-6), 154.0 (C-4), 139.66 (C-8), 115.98 (C-5), 72.24 (d, $J_{2'\text{-}P} = 12.2$ Hz, C-2′), 72.04 (C-7′), 68.48 (C-6′), 68.02 (d, $J_{3'\text{-}P} = 158.5$ Hz, C-3′), 63.10 (d, $J_{4'\text{-}}$

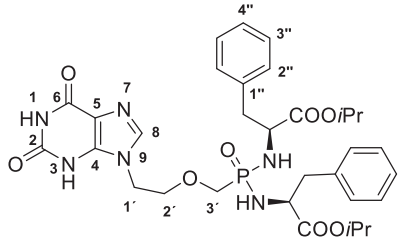
$_P = 5.6$ Hz, C-4′), 44.35 (C-1′), 33.07 (C-20′), 32.40 (d, $J_{5'\text{-}P} = 6.2$ Hz, C-5′), 30.82–30.46 (m, C-8′, 10′–19′), 27.30 (C-9′), 23.73 (C-21′), 14.43 (C-22′). HRMS ($ESI^+$) m/z ($C_{27}H_{49}N_4NaO_7P$) $[M+Na]^+$: calcd. 595.3237; found 595.32314.



*4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,11-Heptadecafluoroundecyl hydrogen ((2–(2,6-dioxo-1,2,3,6-tetrahydro-9H-purin-9-yl) ethoxy)methyl)phosphonate (19).* A suspension of **4** (100 mg, 0.31 mmol) and 4,4,5,5,6,6,7,7,8,8,9,9,10, 10,11,11,11-heptadecafluoroundecan-1-ol (198 mg, 0.41 mmol) in anhydrous pyridine (10 mL) was preheated to 100 °C. Dicyclohexylcarbodiimide (142 mg, 0.69 mmol) in anhydrous pyridine (3 ml) was added and the reaction mixture was stirred at 100 °C for 16 h. Solvent was evaporated and the crude product was purified using silica gel chromatography ($CHCl_3$/MeOH, 0–50%) to give **19** (78 mg, 30%) as a white amorphous solid. $^1$H NMR ($CD_3OD$): $\delta$ 7.69 (s, H-8), 4.19 (m, 2H, H-1′), 3.91 (q, 2H, $J_{4'\text{-}5'} = J_{4'\text{-}P} = 6.1$ Hz, H-4′), 3.81 (m, 2H, H-2′), 3.63 (d, 2H, $J_{3'\text{-}P} = 8.9$ Hz, H-3′), 2.28 (m, 2H, H-6′), 1.84 (m, 2H, H-5′). $^{13}$C NMR ($CD_3OD$): $\delta$ 161.73 (C-6), 153.1 (C-4), 139.58 (C-8), 116.02 (C-5), 72.28 (d, $J_{2'\text{-}P} = 12.2$ Hz, C-2′), 68.21 (d, $J_{3'\text{-}P} = 158.8$ Hz, C-3′), 64.60 (d, $J_{4'\text{-}P} = 5.5$ Hz, C-4′), 44.40 (C-1′), 28.68 (t, $J_{6'\text{-}F} = 21.8$ Hz, C-6′), 23.20 (C-5′). $^{19}$F NMR ($CD_3OD$, ref. $C_6F_6$ –163 ppm): $\delta$ –123.7 (m, 2F), –120.83 (m, 2F), –120.17 (m, 2F), –119.34 (m, 2F), –119.13 (m, 2F), –111.75 (m, 2F), –78.81 (m, 3F). HRMS($ESI^+$) m/z ($C_{19}H_{16}F_{17}N_4NaO_6P$) $[M+Na]^+$: calcd. 773,0434; found 773.0429.

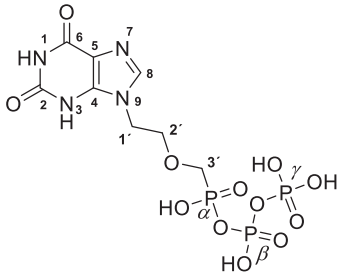


*Bis(L-phenylalanine isopropyl ester) ((2–(2,6-dioxo-1,2,3,6-tetrahydro-9H-purin-9-yl)ethoxy)methyl)phosphonate (20).* TMSBr (330 µL) was added to the mixture of **4** (117 mg, 0.33 mmol) in dry pyridine (4 ml) and DMF (1 ml). The reaction mixture was stirred at 25 °C for 16 h. Volatiles were removed and the moisture-sensitive product was permanently kept under argon. Solid isopropyl ester L-phenylalanine hydrochloride (330 mg, 1.4 mmol) was added to the silylated intermediate under argon, followed by dry pyridine (5 ml) and dry $Et_3N$ (660 µl, 4.7 mmol). The mixture was preheated to 70 °C and freshly prepared solution of aldrithiol-2 (0.45 g, 2.0 mmol) and triphenylphosphine (0.54 g, 2.0 mmol) in pyridine (4 ml) was added. The resulting mixture was stirred at 70 °C for 72 h. Reaction mixture was evaporated *in vacuo* and the residue was purified by column chromatography (0–100% MeOH in a mixture of Hexane:EtOAc, 6:4) followed by C18 reversed phase column chromatography (0–100% MeOH in water) to give **20** (30 mg, 13%) as an amorphous white solid. $^1$H NMR (DMSO-$d_6$): $\delta$ 9.93 (bs, 1H, NH), 7.50 (s, 1H, H-8), 7.27–7.09 (m, 10H, H-2'',3'',4''), 4.86–4.74 (m, 2H, CH-*i*Pr), 4.45 (m, 1H, CH-*NH*), 4.19 (m, 1H, CH-*NH*), 4.03 (m, 2H, H-1'), 3.95 (m, 1H, *CH*-NH), 3.88 (m, 1H, *CH*-NH), 3.57 (m, 2H, H-2'), 3.30–3.20 (m, 2H, H-3'), 2.90–2.73 (m, 4H, $CH_2$Ph); 1.16, 1.11, 1.06 a 1.01 (4 × d, 12H, $J_{CH3,CH} = 6.3$ Hz, $CH_3$). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 172.49 and 172.35 (m, COO), 158.90 (C-6), 154.20 (C-2), 146.20 (C-4), 137.31 and 137.24 (C-1''), 136.63 (C-8), 129.66 (C-2''), 128.28 and 128.24 (C-3''), 126.66 and 126.61 (C-4''), 115.03 (C-5), 70.65 (d, $J_{2'\text{-}P} = 10.6$ Hz, C-2'), 68.15 and 68.01 (CH-*i*Pr), 67.61 (d, $J_{C\text{-}P} = 134.0$ Hz, C-3′), 54.25 and 54.04 (NH-*CH*), 43.10 (C-1'), 39.90 ($CH_2$Ph), 21.69, 21.63, 21.56 and 21.49 ($CH_3$-*i*Pr). HRMS($ESI^+$) m/z ($C_{32}H_{42}N_6O_8P$) $[M+H]^+$: calcd. 669.2798; found 669.2799.



*((2–(2,6-Dioxo-1,2,3,6-tetrahydro-9H-purin-9-yl)ethoxy) methyl)phosphonic diphosphoric anhydride (21).* PMEX morpholidate: Morpholine (0.35 mL, 4.0 mmol) was

added to a mixture of **4** (360 mg, 1.0 mmol) in *t*-BuOH/$H_2O$ (35 mL, 2.5/1, v/v) preheated to 100 °C. Then, a solution of dicyclohexylcarbodiimide (825 mg, 4.0 mmol) in *t*-BuOH/$H_2O$ (48 mL, 5/1, v/v) was added dropwise to the boiling reaction mixture over a period of 1 h. The mixture was heated to 100 °C overnight. After cooling down, solids were filtered off, and the mixture was concentrated up to half of the volume, and diluted with water (200 ml). The aqueous solution was extracted with diethyl ether ($3 \times 10$ mL) and the organic layer was dried ($Na_2SO_4$) and evaporated to dryness. Crude morpholidate was used directly for the pyrophosphate coupling.



Pyrophosphate coupling: Prepared morpholidate (0.2 mmol) was carefully dried over $P_2O_5$ and treated with $(NHBu_3)_2H_2P_2O_7$ (0.5 M solution in DMF, 3 ml) at room temperature for 48 h. The product was precipitated with diethyl ether (10 ml) and the solid was washed with diethyl ether (10 ml). The precipitated product was dissolved in 0.05 M TEAB (4 ml) and purified on a column packed with POROS® 50 HQ (50 ml) with use of a gradient of TEAB in water (0.05–0.5 M). The product was co-evaporated several times with water and converted into a sodium salt form (Dowex 50 in $Na^+$ cycle). Lyophylisation afforded **21** (5 mg, 3%) as a white amorphous solid. $^1$H NMR ($D_2O$): $\delta$ 7.86 (s, 1H, H-8), 4.26 (t, 2H, $J_{1'\text{-}2'} = 5.1$ Hz, H-1′), 3.96 (t, 2H, $J_{2'\text{-}1'} = 5.1$ Hz, H-2′), 3.85 (d, 2H, $J_{3'\text{-}P} = 8.3$ Hz, H-3′), 3.19 (q, 20H, $J = 7.3$ Hz, $Et_3N$), 1.27 (t, 28H, $J = 7.3$ Hz, $Et_3N$). $^{31}$P NMR ($D_2O$): $\delta$ 10.73 (d, $J = 26.2$ Hz, $P_\alpha$), –6.96 (dm, $J = 20.0$ Hz, $P_\gamma$), –20.62 (dd, $J = 26.0$ Hz, $J = 20.3$ Hz, $P_\beta$). HRMS(ESI$^-$) m/z ($C_8H_{11}O_{12}N_4NaP_3$) [M-H]$^-$: calcd. 470.9489; found 470.9488.

*Biological assays.* The compounds were evaluated against different herpesviruses, including herpes simplex virus type 1 (HSV-1) strain KOS, thymidine kinase-deficient (TK–) HSV-1 KOS strain resistant to ACV (ACV$^r$), herpes simplex virus type 2 (HSV-2) strain G, VZV strain Oka, TK– VZV strain 07–1, human cytomegalovirus (HCMV) strains AD-169 and Davis as well as vaccinia virus, adeno virus-2, vesicular stomatitis virus, para-influenza-3 virus, reovirus-1, Sindbis virus, Coxsackie virus B4, Punta Toro virus, RSV, FIPV and influenza A virus subtypes H1N1 (A/PR/8), H3N2 (A/HK/7/87) and influenza B virus (B/HK/5/72). The antiviral assays were based on inhibition of virus-induced cytopathicity or plaque formation in human embryonic lung (HEL) fibroblasts, African green monkey kidney cells (Vero), human epithelial cervix carcinoma cells (HeLa), Crandell-Rees feline kidney cells (CRFK), or Madin Darby canine kidney cells (MDCK). Confluent cell cultures in microtiter 96-well plates were inoculated with 100 $CCID_{50}$ of virus (1 $CCID_{50}$ being the virus dose to infect 50% of the cell cultures) or with 20 PFU, and the cell cultures were incubated in the presence of varying concentrations of the test compounds. Viral cytopathicity or plaque formation (VZV) was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compounds. Antiviral activity was expressed as the $EC_{50}$ or compound concentration required reducing virus-induced cytopathicity or viral plaque formation by 50%.

Compound **4** was evaluated against several TK and DNA polymerase mutants derived from the reference Kos strain by CPE reduction assay using as reference drugs ACV, GCV, BVDU, foscavir, CDV and adefovir (ADV).

Cytotoxicity of the tested compounds was expressed as the minimum cytotoxic concentration or the compound concentration that caused a microscopically detectable alteration of cell morphology. Alternatively, the cytostatic activity of the test compounds was measured based on the inhibition of cell growth. HEL cells were seeded at a rate of $5 \times 10^3$ cells/well into 96-well microtiter plates and allowed to proliferate for 24 h. Then, medium containing different concentrations of the test compounds was added. After three days of incubation at 37°C, the cell number was determined with a Coulter counter. The cytostatic concentration was calculated as the $CC_{50}$, or the compound concentration required reducing cell proliferation by 50% relative to the number of cells in the untreated controls.

The inhibitory effects of PMEXpp on human ($\alpha$ and $\beta$) and viral (VZV and HCMV) DNA polymerases were determined as previously described using activated calf thymus DNA, 100 μM of each of the three unlabeled dNTPs, and 0.5 μM of the rate limiting tritium-labeled dNTP, and serial dilutions of PMEXpp (**21**). Foscarnet pyrophosphate and acyclovir triphosphate (ACV–TP) were included as the reference compound. The 50% inhibitory concentration or compound concentration required to inhibit the polymerase-catalyzed DNA synthesis by 50% was then determined.

## Conclusions

A series of novel ANPs bearing xanthine as a nucleobase was prepared and evaluated for their potential antiviral properties. Two synthetic approaches were exploited for the synthesis of the target compounds: (a) recently developed MW-assisted hydrolysis of 2,6-dichloropurine derivatives and (b) well-established diazotization/2-hydroxy-dediazoniation of the corresponding guanine analogues. All prepared ANPs were tested against a wide range of DNA and RNA viruses. Two compounds exhibited antiviral activity. PMEX (**4**) was active against VZV, HCMV, HSV-1, and HSV-2 (with $EC_{50}$ values between 2.6 and 25.6 μM), while HPMPX (both as *S*-isomer **6** and as a racemic mixture **5**) exhibited moderate to weak activity ($EC_{50}$ in the range of 17–43 μM) against VZV, HCMV, HSV-1, HSV-2, and Vaccinia virus. PMEX (**4**) was the most active compound against VZV in the series ($EC_{50} = 2.62$ μM, $TK^+$ Oka strain) and was equipotent to the reference drug ACV ($EC_{50} = 3.42$ μM). In contrast to ACV, PMEX (**4**), as ANP which activity is independent of the first phosphorylation step, remained active against the $TK^-$ VZV 07–1 strain with $EC_{50} = 4.58$ μM. The hexadecyloxypropyl monoester derivative of PMEX, compound **18**, slightly improved the anti-HSV potency of the parent compound ($EC_{50}$ values between 1.8 and 4.0 μM). Further studies of PMEX (**4**) and of its diphosphate analogue PMEXpp (**21**) suggested that the compound acts as the inhibitor of herpesvirus DNA polymerases (HSV-1 and VZV). This study represents the first report of xanthine containing ANPs with potent antiviral properties and urges further studies of various xanthine nucleotide analogues as potential antiviral agents.

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