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 (EC₅₀ = 2.6 μM, TK⁺ Oka strain) and HCMV (EC₅₀ = 8.5 μM, Davis strain), while its hexadecyloxypropyl monoester derivative was active against HSV-1 and HSV-2 (EC₅₀ values between 1.8 and 4.0 μM). In contrast to acyclovir, PMEX remained active against the TK⁻ VZV 07–1 strain with EC₅₀ = 4.58 μM. PMEX was suggested to act as an inhibitor of viral DNA polymerase and represents the first reported xanthine-based acyclic nucleoside phosphate 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 consequences² 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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where XMP serves as a substrate for guanosine monophosphate synthase,\textsuperscript{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),\textsuperscript{8} or by chemical hydrolysis\textsuperscript{9} of dGMP/dGTP via NO\textsubscript{x}-mediated nitrosative stress.\textsuperscript{10} These processes can lead to a substantial incorporation of xanthine nucleotides into DNA and/or RNA,\textsuperscript{8} and subsequently to RNA miscoding and mutagenesis.\textsuperscript{11} Moreover, deaminated nucleotides can interfere with RNA editing\textsuperscript{12} and with functions of noncoding RNAs.\textsuperscript{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,\textsuperscript{14} especially those from nudix family such as ITPases/XTPases,\textsuperscript{15,16} NUDT\textsuperscript{16} or ITPA,\textsuperscript{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\textsuperscript{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 antiviriotics such as nucleoside analogues (acyclovir (ACV), penciclovir, vidarabine, and ganciclovir (GCV)), acyclic nucleoside phosphonate (ANP) cidofovir (CDV) (3, Figure 1),\textsuperscript{19} or diphosphate mimic foscarnet,\textsuperscript{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.\textsuperscript{21–23} As recent literature has shown,\textsuperscript{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,\textsuperscript{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.\textsuperscript{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.\textsuperscript{19} Although some ANPs derived from xanthine
were studied before as potential antiviral agents (namely the 9-[3-fluoro-2-(phosphonomethoxy)propyl] derivative, FPMPX), 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 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), has been reported by our group earlier. The microwave-assisted hydrolysis of 2,6-dichloropurine derivative 10 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 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, N6-cyclopropylaminopurine derivative 17 (Scheme 3) was prepared in a 75% yield from the corresponding N6-cyclopropyl-2,6-diaminopurine derivative 16 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.](image1)

![Scheme 2. Preparation of xanthine-based ANPs 5–9. Reaction conditions: (a) isoamylnitrite, 80% aq. AcOH, 25 °C, 16 h.](image2)

![Scheme 3. Synthesis of compound 17. Reaction conditions: (a) isoamylnitrite, 80% aq. AcOH, 25 °C, 16 h.](image3)
pound GS-9219 (an acyclic nucleotide analogue with potent antineoplastic activity),36 and abacavir (a carboxylic 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 19 (Scheme 4) bearing a perfluorinated-C12 chain was prepared by the same procedure in a 30% yield. Finally, the bisamide 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 morpholide intermediate40 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'), 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, allovudine, 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 EC50, 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 (EC50 = 2.62 μM (PMEX) versus 3.42 μM (ACV)). In contrast to ACV, compound 4 remained active against the TK– VZV 07–1 strain (EC50 = 4.58 μM). PMEX also inhibited the

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**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) iPr-L-PheAla.HCl, pyridine, Et3N, Aldrithiol-2, triphenylphosphine, 70 °C, 72 h.

**Scheme 5.** Synthesis of PMEX diphosphate 21. Reaction conditions: (a) morpholine, DCC, t-BuOH, H2O, 105 °C, 16 h; (b) pyrophosphate (0.5 M in DMF), DMSO, 25 °C.
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 1.** Activity of compounds 4–9 against varicella-zoster virus (VZV) and human cytomegalovirus (HCMV) in human embryonic lung (HEL) cells.

| Compound | Antiviral activity EC$_{50}$ (μM)$^a$ | Cytotoxicity (μM) |
|----------|-------------------------------|-------------------|
|           | TK$^+$ VZV | TK$^-$ VZV | HCMV | Cell morphology MCC$^b$ | Cell growth CC$_{50}$$^c$ |
| PMEX 4   | OKA strain 2.62 ± 1.19 | 07-1 strain 4.58 ± 2.59 | AD-169 strain 10.5 ± 3.9 | Davis strain 8.5 ± 2.0 | >345 | 111 ± 71 |
| (RS)-HPMPX 5 | 30.9 ± 12.3 | 7.1 ± 9.8 | 86.9 ± 21.1 | 48.6 ± 24.2 | >300 | >300 |
| (S)-HPMPX 6 | 22.7 ± 6.8 | 17.1 ± 6.4 | 80.2 ± 36.0 | 43.0 ± 33.9 | >300 | >300 |
| (R)-FPMPX 7 | >313 | >313 | >313 | ND | >300 | >300 |
| (S)-FPMPX 8 | >329 | >329 | >329 | ND | >300 | >300 |
| (R)-PPMPX 9 | >100 | >100 | >100 | >100 | >100 | >100 |
| 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.

| Compound | Antiviral activity EC$_{50}$ (μM)$^a$ | Cytotoxicity (μM) |
|----------|-------------------------------|-------------------|
|           | HSV-I | HSV-2 | HSV-I TK$^+$ | Vaccinia virus | Cell morphology MCC$^b$ |
| PMEX 4   | 21.5 ± 7.6 | 25.6 ± 14.8 | 23.7 ± 14.0 | >345 ± 0 | >345 |
| (RS)-HPMPX 5 | 39 ± 27 | 20 ± 0 | 38 ± 0 | 39 ± 27 | >100 |
| (S)-HPMPX 6 | 33 ± 18 | 16 ± 6 | 20 ± 0 | 39 ± 27 | >100 |
| (R)-FPMPX 7 | >313 | >313 | >313 | >313 | >313 |
| (S)-FPMPX 8 | >329 | >329 | >329 | >329 | >329 |
| (R)-PPMPX 9 | >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.
inactive in all assays. Phosphonate ester 19 and biamidate prodrug 20 did not display any potent antiviral activity which might be explained by insufficient prodrug activation in these cells. The HDP-MEX prodrug 18 proved to be 7 to 26 folds (VZV) or 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 (a and b) DNA polymerases (Table 5). The inhibition of ACV triphosphate and the pyrophosphate analogue of fostecarnet (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 µM). 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

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

| Compound | Antiviral activity EC$_{50}$ (µM)$^a$ | Cytotoxicity (µM) |
|----------|----------------------------------|------------------|
|          | TK$^+$ VZV | TK$^-$ VZV | HCMV | Cell morphology (MCC)$^b$ | Cell growth (CC$_{50}$)$^c$ |
| 17       | OKA strain | >100 | >100 | >100 | >100 |
| 18       | 0.10 ± 0.05 | 0.05 ± 0.05 | 0.20 ± 0.28 | 0.05 ± 0.05 | >100 |
| 19       | >20 | >100 | 63 | 55 | 100 |
| 20       | 22 | 26.5 | 63 ± 19 | 6.2 ± 6.8 | >440 |
| Acyclovir | 2.23 ± 2.16 | 98 ± 61 | ND | ND | >440 |
| Brivudin  | 0.019 ± 0.010 | 19.6 ± 23.0 | ND | ND | >350 |
| Ganciclovir | ND | ND | 10.0 ± 10.2 | 5.5 ± 4.4 | >300 |
| Cidofovir | 0.92 ± 0.38 | 0.78 ± 0.32 | ND | ND | 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.

| Compound | Antiviral activity EC$_{50}$ (µM)$^a$ | Cytotoxicity (µM) |
|----------|----------------------------------|------------------|
|          | 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 | 50 | 16.4 ± 17.8 | >250 |
| Ganciclovir | 0.020 ± 0.014 | 0.045 ± 0.049 | 0.90 ± 0.14 | >100 | >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.
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).

| Compound             | 21 (PMEXpp) | ACV-TP | PFA pyrophosphate |
|----------------------|-------------|--------|-------------------|
|                      | IC$_{50}$ (µM)$^a$ | IC$_{50}$ (µM)$^a$ | IC$_{50}$ (µM)$^a$ |
| HCMV DNA pol         |              |        |                   |
| dGTP$^b$             | >100        | 0.77 ± 0.04 | 7.0 ± 0.7 |
| dTTP$^b$             | >100        | 82 ± 26   | 9.4 ± 6.0 |
| VZV DNA pol          |              |        |                   |
| dGTP$^b$             | 7.4 ± 2.7   | ND$^c$  | 0.18 ± 0.01 |
| dTTP$^b$             |             | ND      | 56.0 ± 12.0 |
| DNA pol α            |              |        |                   |
| dGTP$^b$             | >100        | ND      | ND |
| dTTP$^b$             | >100        | ND      | ND |
| DNA pol β            |              |        |                   |
| dGTP$^b$             | >100        | ND      | ND |
| dTTP$^b$             | >100        | ND      | 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 μm, 250 × 21.2 mm, flow 10 ml/min preparative column).

**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 isoamyl nitrite (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 mixture gave 4 (1.2 g, 85%) as a white solid. The analytical data are in an agreement with the published data. \(^{27}\)

\[
\text{(RS)-}((1-(2,6-Dioxo-1,2,3,6-tetrahydro-9H-purin-9-yl)-3-hydroxypropan-2-yl)oxy)methylphosphonic acid (5), (RS)-HPMPX.}
\]

Treatment of 11 (223 mg, 0.70 mmol) by Method A afforded 5 (160 mg, 71%) as a white solid. \(^{1}\)H NMR (D₂O + NaOD): \(\delta\) 7.84 (s, 1H, H-8), 4.21 (dd, \(J_{gem} = 14.7\) Hz, \(J_{1a-2} = 4.9\) Hz, 1H, H-1a), 4.15 (dd, \(J_{gem} = 14.7\) Hz, \(J_{1b-2} = 6.1\) Hz, 1H, H-1b), 3.80 (m, 1H, H-2'), 3.71 (dd, \(J_{gem} = 12.5\) Hz, \(J_{3a-2} = 3.7\) Hz, 1H, H-3'a), 3.55 (dd, \(J_{gem} = 12.3\) Hz, \(J_{4a-P} = 8.8\) Hz, 1H, H-4'a), 3.49 (dd, \(J_{gem} = 12.3\) Hz, \(J_{4b-P} = 9.6\) Hz, 1H, H-4'b), 3.47 (dd, \(J_{gem} = 12.5\) Hz, \(J_{3b-P} = 5.4\) Hz, 1H, H-3'b). \(^{13}\)C NMR (D₂O + 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, \(J_{C-O-C-P} = 10.8\) Hz, C-2'), 68.66 (d, \(J_{C-O-C-P} = 150.7\) Hz, C-4'), 60.98 (C-3'), 43.59 (C-1'). MS-ESI⁻ m/z (%) 319 (100, M-H⁻). HRMS(ESI) m/z (C₉H₁₃N₄O₇P) [M-H⁻]: calc 319.0449; found 319.0447.

\[
\text{(S)-(}((1-(2,6-Dioxo-1,2,3,6-tetrahydro-9H-purin-9-yl)ethoxy)methyl)phosphonic acid (6), (S)-HPMPX.}
\]

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 (C₉H₁₃N₄O₇P) [M-H⁻]: calc 319.0449; found 319.0448. [\(\alpha\)]\text{D}^\text{20} = −4.7 (c = 0.254 g/100 ml, H₂O).

\[
\text{(2-(2,6-Dioxo-1,2,3,6-tetrahydro-9H-purin-9-yl)ethoxy)methylphosphonic acid (4), PMEX.}
\]

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.
(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. ¹H NMR (DMSO-d₆): δ 10.84 (bs, 1H, 1 or 3), 7.72 (s, 1H, 8), 4.62 (dd, J₁₂,₁₃ = 47.4 Hz, J₂₁₁ = 10.5 Hz, J₃₁₂ = 3.0 Hz, H-3b), 4.42 (dd, J₃₂₃ = 47.2 Hz, J₄₂₃ = 10.5 Hz, J₅₂₃ = 4.2 Hz, H-3a), 4.27 (dd, J₆₂₃ = 14.9 Hz, J₇₂₃ = 4.3 Hz, H-1b), 4.20 (dd, J₈₂₃ = 14.8 Hz, J₉₂₃ = 7.4 Hz, H-1a), 3.95 (bddd, 1H, J₁₂,₁₃ = 24.1 Hz, J₂₋₁₋₁ = 7.4 Hz, J₁₋₁₋₁ = 4.2 Hz, J₂₋₁₋₁ = 3.0 Hz, H-2), 3.67 (dd, J₇₂₃ = 13.5 Hz, J₈₋₁₋₁ = 9.0 Hz, H-4b), 3.57 (dd, J₈₋₁₋₁ = 13.5 Hz, J₉₋₁₋₁ = 9.0 Hz, H-4a); ¹³C NMR (DMSO-d₆): δ 157.95 (C-6), 151.00 (C-2), 140.80 (C-4), 137.75 (C-8), 114.89 (C-5), 82.26 (d, J₈₋₁₋₁ = 169.9 Hz, C-3'), 77.77 (dd, J₁₂,₁₃ = 18.4 Hz, J₂₋₁₋₁ = 10.3 Hz, C-2'), 65.96 (d, J₁₋₁₋₁ = 160.3 Hz, C-4'), 44.38 (d, J₁₋₁₋₁ = 8.4 Hz, C-1'). HRMS(ESI) m/z (C₇₁₉₁₄₂₂₂₆₆₇O₉P) [M-H]⁻: calc 321.0478; found 321.0472. [α]D²⁰ = + 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. [α]D²⁰ = - 4.2 (c = 0.0238 g/100 ml).

(R)-((1-(2,6-Dioxo-1,2,3,6-tetrahydro-9H-purin-9-yl)-3-fluoropropan-2-yl)oxy)methyl)phosphonic acid (9), (R)-FPMPX. Treatment of 15 (250 mg, 0.82 mmol) by Method A gave 9 (129 mg, 51%) as a white solid. ¹H NMR (DMSO-d₆): δ 10.65 (bs, 1H, 1 or 3), 7.61 (s, 1H, 8), 4.27 (dd, J₁₂,₁₃ = 14.5 Hz, J₂₋₁₋₁ = 4.6 Hz, H-1'b), 4.13 (dd, J₁₂,₁₃ = 14.5 Hz, J₂₋₁₋₁ = 4.5 Hz, H-1'a), 3.86 (m, 1H, H-2'), 3.54 (dd, J₁₂,₁₃ = 13.5 Hz, J₂₋₁₋₁ = 9.0 Hz, H-4'b), 3.45 (dd, J₁₂,₁₃ = 13.6 Hz, J₂₋₁₋₁ = 6.8 Hz, H-4'a), 0.99 (d, 3H, J₂₋₁₋₁ = 6.4 Hz, H-3'). ¹³C NMR (DMSO-d₆): δ 158.24 (C-6), 151.22 (C-2), 141.57 (C-4), 138.04 (C-8), 115.38 (C-5), 74.64 (d, J₂₋₁₋₁ = 6.7 Hz, C-2'), 48.64 (C-1'), 17.45 (C-3'), C-4' not observed. HRMS(ESI) m/z (C₇₁₉₁₄₂₂₂₆₆₇O₉P) [M-H]⁻: calc 303.0573; found 303.0499. [α]D²⁰ = + 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. ¹H NMR (DMSO-d₆): δ 8.67 (bs, 1H, NH-CH), 7.80 (s, 1H, H-8), 4.15 (t, 2H, J₁₋₁ = 5.2 Hz, H-1'), 3.78 (t, 2H, J₂₋₁₋₁ = 5.2 Hz, H-2'), 3.56 (d, 2H, J₂₋₁₋₁ = 8.4 Hz, H-3'), 2.98 (bs, 1H, NH-CH), 0.77 (m, 2H, CH₂-CPr), 0.66 (m, 2H, CH₂-CPr). ¹³C NMR (DMSO-d₆): δ 155.73 (C-6), 153.45 (C-2), 149.9 (C-4), 139.40 (C-8), 110.37 (C-5), 70.30 (d, J₂₋₁₋₁ = 9.7 Hz, C-2'), 67.0 (d, J₂₋₁₋₁ = 158.8 Hz, C-3'), 42.76 (C-1'), 24.2 (CH-CH), 7.02 (CH₂-Cpyr). HRMS(ESI) m/z (C₁₁₉₁₆₂₂₆₆₇O₉P) [M-H]⁻: calc 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-(hexadecyloxyl)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 (CHCl₃/MeOH, 0–50%) to give 18 (46 mg, 23%) as a white amorphous solid. ¹H NMR (CD₃OD): δ 7.69 (s, 1H, H-8), 4.18 (m, 2H, H-1'), 3.91 (q, 2H, J₂₋₁₋₁ = 6.4 Hz, H-4'), 3.80 (m, 2H, H-2'), 3.61 (d, 2H, J₂₋₁₋₁ = 8.9 Hz, H-3'), 3.46 (t, 2H, J₂₋₁₋₁ = 6.5 Hz, H-6'), 3.36 (t, 2H, J₂₋₁₋₁ = 6.6 Hz, H-7'), 1.79 (p, 2H, J₂₋₁₋₁ = 6.4 Hz, H-5'), 1.52 (m, 2H, H-8'), 1.34–1.27 (m, 26H, H-6'-21'). ¹³C NMR (CD₃OD): δ 161.79 (C-6), 154.0 (C-4), 139.66 (C-8), 115.98 (C-5), 72.24 (d, J₂₋₁₋₁ = 12.2 Hz, C-2'), 72.04 (C-7'), 68.48 (C-6'), 68.02 (d, J₂₋₁₋₁ = 158.5 Hz, C-3'). 63.10 (d, J₂₋₁₋₁ = 10.6 Hz, H-2').
p = 5.6 Hz, C-4'), 44.35 (C-1'), 33.07 (C-20'), 32.40 (d, J_2'_,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_{4}NaO_{7}P) [M+Na]^+: calcd. 795.3237; found 795.32314.

4,4,5,5,6,6,7,7,8,9,9,10,11,11,11-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 4,4,5,5,6,6,7,7,8,8,9,9,10,11,11,11-heptadecafluoroundecan-1-ol (198 mg, 0.41 mmol) in anhydrous pyridine (10 mL) 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-\textit{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-Pr), 4.45 (m, 1H, CH-NH), 4.19 (m, 1H, CH-NH), 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_2Ph); 1.16, 1.11, 1.06 a 1.01 (4 \times d, 12H, J_{CH,CH} = 6.3 Hz, CH_3). 13^C NMR (DMSO-\textit{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'), 136.33 (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',P} = 10.6 Hz, C-2'), 68.15 and 68.01 (CH-Pr), 67.61 (d, J_{C,P} = 134.0 Hz, C-3'), 54.25 and 54.04 (NH-CH), 43.10 (C-1'), 39.90 (CH_2Ph), 21.69, 21.63, 21.56 and 21.49 (CH_3Pr). HRMS(ESI^+) m/z (C_{32}H_{42}N_{6}O_{8}P) [M+H]^+: calcd. 669.2798; found 669.2799.

Bis(L-phenylalanine isopropyl ester) ((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 the silylated intermediate under argon, followed by dry pyridine (5 ml) and dry Et_3N (660 \mu l, 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.

13^C NMR (DMSO-\textit{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'), 136.33 (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',P} = 10.6 Hz, C-2'), 68.15 and 68.01 (CH-Pr), 67.61 (d, J_{C,P} = 134.0 Hz, C-3'), 54.25 and 54.04 (NH-CH), 43.10 (C-1'), 39.90 (CH_2Ph), 21.69, 21.63, 21.56 and 21.49 (CH_3Pr). HRMS(ESI^+) m/z (C_{32}H_{42}N_{6}O_{8}P) [M+H]^+: calcd. 669.2798; found 669.2799.
added to a mixture of 4 (360 mg, 1.0 mmol) in t-BuOH/ 
H₂O (35 mL, 2.5/1, v/v) preheated to 100 °C. Then, a  
solution of dicyclohexylcarbodiimide (825 mg,  
4.0 mmol) in t-BuOH/H₂O (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 over- 
night. 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 × 10 mL) and the  
organic layer was dried (Na₂SO₄) and evaporated to  
dryness. Crude morpholidate was used directly for the  
pyrophosphate coupling.

Pyrophosphate coupling: Prepared morpholidate  
(0.2 mmol) was carefully dried over P₂O₅ and treated  
with (NH₄)₂H₃P₂O₇ (0.5 M solution in DMF, 3 ml)  
at room temperature for 48 h. The product was precip-  
itated with diethyl ether (10 mL) and the solid was  
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 evaporated into a sodium  
with (Dowex 50 in Na⁺) cycle. Lyophilisation afforded 21  
(5 mg, 3%) as a white amorphous solid. ¹H NMR  
(D₂O): δ 7.86 (s, 1H, H-8), 4.26 (t, 2H, Jᵥ₋ₓ = 5.1 Hz,  
H-1’), 3.96 (t, 2H, Jᵥ₋ₓ = 5.1 Hz, H-2’), 3.85 (d, 2H, Jᵥ₋ₓ,  
p = 8.3 Hz, H-3’), 3.19 (q, 20H, Jᵥ₋ₓ = 7.3 Hz, Et₃N), 1.27  
t (28H, J = 7.3 Hz, Et₃N). ³¹P NMR (D₂O): δ 10.73 (d,  
J = 26.2 Hz, Pₓ), −6.96 (dm, J = 20.0 Hz, Pₓ), −20.62  
(dd, J = 26.0 Hz, J = 20.3 Hz, Pₓ). HRMS(ESI) m/z  
(C₈H₁₇O₁₂N₄NaP₃) [M-H]: calc. 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'),  
herpes simplex virus type 2 (HSV-2) strain G, VZV  
strain Oka, TK− VZV strain 07−1, human cytomegalo-  
virus (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 anti-  
viral assays were based on inhibition of virus-induced  
cytotoxicity 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 CCID₅₀ of virus (1 CCID₅₀ 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  
various concentrations of the test compounds. Viral  
cytotoxicity 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  
EC₅₀ or compound concentration required reducing  
virus-induced cytotoxicity 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 adefo-  
vir (ADV).

Cytotoxicity of the tested compounds was expressed  
as the minimum cytotoxic concentration or the com-  
pound concentration that caused a macroscopically  
detectable alteration of cell morphology. Alternatively,  
the cytostatic activity of the test com-  
pounds was measured based on the inhibition of cell  
growth. HEL cells were seeded at a rate of 5 × 10⁶ cells/  
well into 96-well microtiter plates and allowed to proli-  
erate 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 CC₅₀, 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 (α and  
β) and viral (VZV and HCMV) DNA polymerases  
were determined as previously described using activat- 
vated calf thymus DNA, 100 μM of each of the three unlabeled dNTPs, and 0.5 μM of the rate limiting tritium-  
abeled 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 con-  
centration 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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