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9-[2-(*R*)-(Phosphonomethoxy)propyl]-2,6-diaminopurine (*R*)-PMPDAP and its prodrugs: Optimized preparation, including identification of by-products formed, and antiviral evaluation in vitro



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ABSTRACT

New large-scale synthetic approach to antiretroviral agent 9-[2-(*R*)-(phosphonomethoxy)propyl]-2,6-diaminopurine, (*R*)-PMPDAP, was developed. Reaction of (*R*)-propanediol carbonate with 2,6-diaminopurine afforded exclusively (*R*)-9-(2-hydroxypropyl)-2,6-diaminopurine which was subsequently used for introduction of a phosphonomethyl residue using $\text{TsOCH}_2\text{P}(\text{O})(\text{OiPr})_2$ or $\text{BrCH}_2\text{P}(\text{O})(\text{OiPr})_2$ followed by deprotection of ester groups. All minor ingredients and by-products formed during the process were identified and further studied. The final product was obtained in high yield and its high enantiomeric purity (>99%) was confirmed by chiral capillary electrophoretic analysis using β -cyclodextrin as a chiral selector. Antiretroviral activity data of (*R*)-PMPDAP and its diverse prodrugs against HIV and FIV were investigated. Akin to (*R*)-PMPDAP, both prodrugs inhibit FIV replication in a selective manner. Compared to the parent molecule, the amidate prodrug was 10-fold less active against FIV in cell culture, whereas the alkoxyalkyl ester prodrug was 200-fold more potent in inhibiting FIV replication in vitro.

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1. Introduction

9-[2-(*R*)-(Phosphonomethoxy)propyl]adenine (PMPA, tenofovir, Fig. 1), a compound discovered originally by Holý et al.^{1,2} was approved by the FDA in 2001 as tenofovir disoproxil fumarate^{3,4} for the treatment of HIV infection. This nucleotide reverse transcriptase inhibitor (NRTI) can be used by itself (Viread™) or in combination with other antiretroviral agents (Truvada™, Atripla™). The highly active antiretroviral therapy (HAART) based on treatment using combinations of several antiretroviral drugs is now preferable and over the past decade it has led to significant declines in HIV-associated morbidity and mortality.⁵ In 2008, the same compound, tenofovir disoproxil fumarate was also approved for the treatment of hepatitis B as a drug operating on inhibitory effects towards HBV polymerase.^{6,7} At present, tenofovir has become the most commercially successful drug for the treatment of HIV and HBV infections.

Surprisingly, the 2-amino congener of tenofovir, 9-[2-(*R*)-(phosphonomethoxy)propyl]-2,6-diaminopurine ((*R*)-PMPDAP), prepared by Holý almost 20 years ago has not been thus far sufficiently studied, despite the fact that it is 10-fold more potent against HIV-1 compared to PMPA in vitro and in vivo.^{8,9} On the other hand, initiated by the development of HIV drug resistance, there is a necessity to continue the search for new antiretroviral, especially anti-HIV, compounds. This resistance has developed despite the number of available drugs on the market or the effective Global Strategy for HIV Drug Resistance Prevention worked out by WHO.¹⁰ In this work, we describe an optimized effective methodology for a large scale-preparation of (*R*)-PMPDAP, a promising future drug candidate for the treatment of HIV and/or other retroviral infections. Eventual clinical development of the compound can be supported also by our recent progress in the development of prodrugs. Preparation of prodrugs can improve bioavailability of the parent compound as well as pharmacological properties in general.

Moreover, (*R*)-PMPDAP, similar to many other acyclic nucleoside phosphonates (ANPs) is effective not only against human (retro)viral infections but it is also a compound of great significance for

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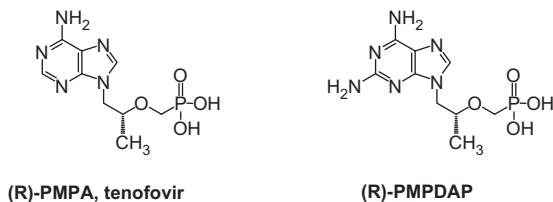


Figure 1. Structures of (R)-PMPA (tenofovir) and (R)-PMPDAP.

veterinary medicine. It proved to be a very potent inhibitor of FIV (feline immunodeficiency virus) infections in cats.¹¹ For this purpose it is currently in clinical development.

2. Results and discussion

Till lately, two synthetic approaches to 9-[2-(R)-(phosphonomethoxy)propyl]-2,6-diaminopurine were described. The first method is the stepwise approach starting from the isobutyl ester of D-(–)-lactic acid. It can be converted to the tetrahydropyranyl derivative, reduced to (R)-2-(2-tetrahydropyranyloxy)propanol and tosylated. Synthesis of (R)-PMPDAP is based on alkylation of 2,6-diaminopurine with thus obtained (R)-2-(2-tetrahydropyranyloxy)propyl tosylate followed by removal of 2-tetrahydropyranyl and etherification of the free hydroxy group with diisopropyl tosyloxymethanephosphonate.^{1a} This process was also patented.^{1b} However, the total yield of the key intermediate, 9-(R)-(2-hydroxypropyl)-2,6-diaminopurine, is only 38% and the introduction of a phosphonomethyl residue followed by deprotection of ester groups via transsilylation reaction with bromotrimethylsilane and hydrolysis proceeds in the total yield 47%. The second method is based on condensation of 2,6-diaminopurine with the appropriate chiral synthon, that is, (R)-2-[bis(2-propyl)phosphonomethoxy]propyl-*p*-toluenesulfonate [(R)-PMP-synthon], the compound prepared from (R)-1-benzyloxy-2-propanol by a multi-step process involving chloromethylation, Arbuzov reaction with triisopropyl phosphite, catalytic hydrogenation and final tosylation.² This method is appropriate for laboratory scale syntheses.

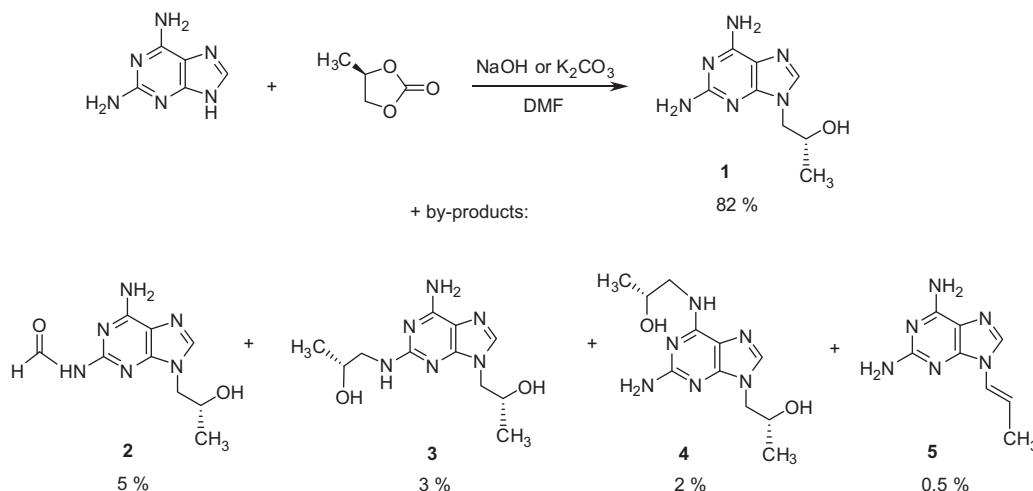
In this work, we describe a new approach to (R)-PMPDAP utilizing nucleophilic reaction of 2,6-diaminopurine with (R)-propanediol carbonate. The advantage of this starting material is its commercial accessibility in high optical purity together with its better reactivity and higher yields compared to above mentioned (R)-2-(2-tetrahydropyranyloxy)propyl tosylate.¹ Despite the fact

that (R)-propanediol carbonate is involved in industrial synthesis of tenofovir,¹² not many synthetic details using other nucleobases are described. A short communication was published regarding its use for synthesis of (R)-PMPA but this work does not contain any experimental details.¹³ Recently, another more detailed paper on the industrial synthesis of tenofovir was issued but description of by-products in this synthesis is focused mostly on the final synthetic step, that is, preparation of the prodrug form (tenofovir disoproxil) and to analytical methods for their quantification.¹⁴

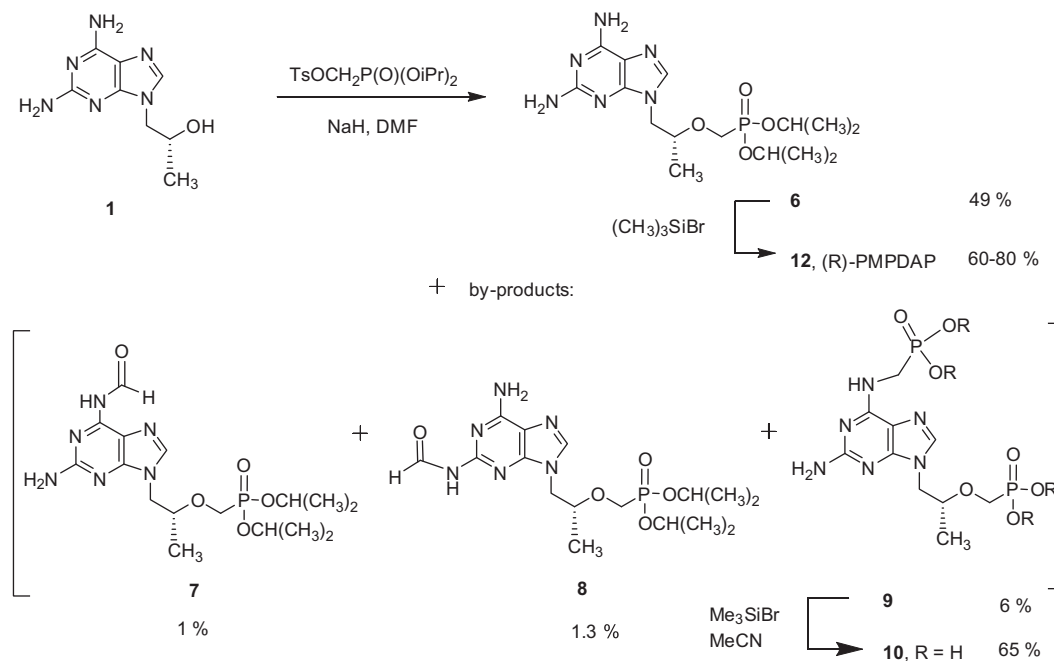
In our case, base catalyzed reaction of (R)-propanediol carbonate with 2,6-diaminopurine proceeded to form the desired (R)-9-(2-hydroxypropyl)-2,6-diaminopurine (**1**) in high yield (above 80%) and regioselectivity to the position N-9 (Scheme 1). As a side effect, formylation of one of the amino groups occurs, always to the N² position. The formylated by-product **2** can be easily transformed to **1** in aqueous alkali. Other by-products, bis(hydroxyalkyl) derivatives **3** and **4** and the elimination product **5** are formed after longer reaction time; their total quantity in the mixture is very low (<3%) and they are easily removed.

Introduction of a phosphonomethyl residue was performed using diisopropyl tosyloxymethanephosphonate or diisopropyl bromomethanephosphonate.¹⁵ The first possibility does not require any protection of amino groups. Reaction performed under basic conditions in dimethylformamide afforded the desired phosphonate ester **6** accompanied by a small amount of N² and N⁶ formyl derivatives, bis(phosphonomethyl) derivative **9** and also by a small amount of compound **5** as in previous step (Scheme 2). Both formylated products **7** and **8** could be transformed by basic hydrolysis to the desired intermediate **6** which was subsequently deprotected to the final product, (R)-PMPDAP by transsilylation reaction with bromotrimethylsilane followed by hydrolysis.^{1,2} The yield of this final deprotection step leading to (R)-PMPDAP is in a range 60–80% which is in agreement with previously published data (79%).²

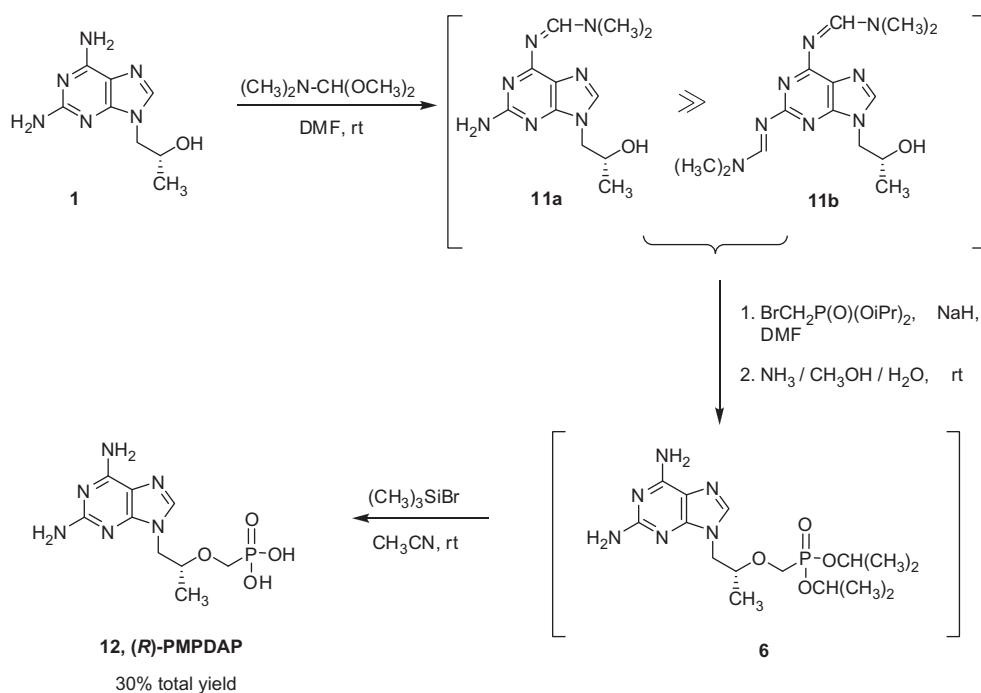
An alternative method of preparation of phosphonomethyl derivatives is based on utilization of diisopropyl bromomethanephosphonate. This approach usually requires protection of amino groups. In our case, we selected a protection step with dimethylformamide dimethyl acetal. This reaction leads preferentially to mono-substituted product **11a** (Scheme 3). Its further reaction with diisopropyl bromomethanephosphonate can be realized without necessity to isolate intermediates. This fact belongs to the main advantages of this process. Its particular disadvantages include complicated monitoring of the reaction course due to the



Scheme 1. The course of nucleophilic opening of (R)-1,2-propanediol carbonate with 2,6-diaminopurine.



Scheme 2. Reaction of unprotected intermediate (**1**) with $\text{TsOCH}_2\text{P}(\text{O})(\text{OiPr})_2$.

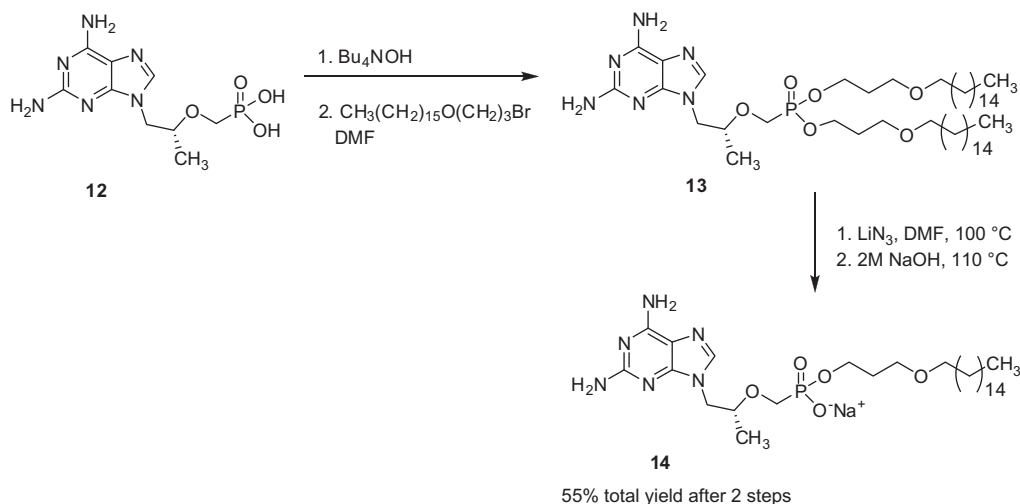


Scheme 3. Preparation of (R)-PMPDAP via protection of **1** with dimethylaminomethylene groups.

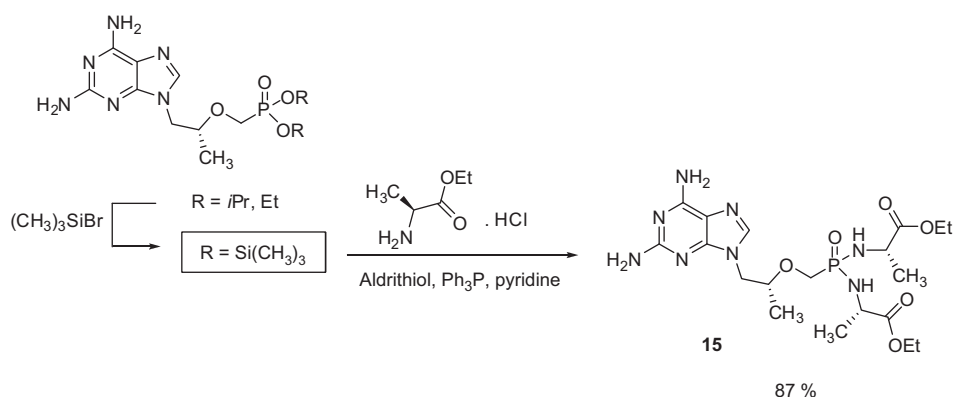
presence of decomposition products originating from dimethylaminomethylene compounds and also a lower yield.

Similarly as all other ANPs, (R)-PMPDAP has a highly polar character due to the presence of a free phosphonic acid function and, hence, unfavorable pharmacological properties can be expected. A phosphonate group subjected to the physiological pH deprotonates and an ionized drug molecule is mostly impermeant to the cellular membrane unless pinocytosis is involved. In addition, a phosphonate based drug can be thus absorbed by gastrointestinal tract in a limited scope which disqualifies the drug for oral applica-

tion. In order to achieve oral bioavailability and intracellular delivery of ANPs, their transformation to appropriate prodrug form is highly advisable.¹⁶ In our case, we decided to transform (R)-PMPDAP to two structurally different types of prodrugs: an alkoxyalkyl (hexadecyloxypropyl) monoester and an amidate prodrug. The first type was synthesized by reaction of tetrabutylammonium salt of (R)-PMPDAP and hexadecyloxypropyl bromide in DMF . The reaction afforded neutral diester **13**, which was subsequently transformed in alkaline conditions to hexadecyloxypropyl monoester **14** (Scheme 4). The amidate prodrug **15** was prepared according



Scheme 4. Preparation of hexadecyloxypropyl esters of (*R*)-PMPDAP.



Scheme 5. Example of preparation amidate prodrugs of (*R*)-PMPDAP.

to a procedure developed originally in our laboratory:¹⁷ an appropriate phosphonate ester (ethyl or diisopropyl) is treated with bromotrimethylsilane, followed by reaction with amino acid ester hydrochloride in a presence of triphenyl phosphine and bis(2-pyridinyl)-disulfide (Aldrithiol) under basic conditions (Scheme 5).

Methodology of preparation of (*R*)-PMPDAP utilizing (*R*)-propanediol carbonate enabled achievement of the final product of extraordinary optical purity. Despite the diverse manners of introduction of a phosphonomethyl group or work up of reaction mixtures, an enantiomeric purity of (*R*)-PMPDAP was always higher than 99%. Chiral analysis of the product was performed by capillary electrophoresis (CE) with β -cyclodextrin (β -CD) chiral selector.¹⁸ Fast (ca. 11 min) separation of (*R*)- and (*S*)-enantiomers of PMPDAP was achieved by CE using 30 mM sodium tetraborate, adjusted by NaOH to pH 10.0 as background electrolyte (running buffer) and 20 mg/mL β -CD as chiral selector, see Figure 2a. Average enantiomeric purity degree of the analyzed samples (three batches, A–C) is given in Table 1. High enantiomeric purity of the synthesized (*R*)-PMPDAP, batch B, is shown in Figure 2b.

3. Biological activity

Comparisons of anti-HIV and anti-FIV activities of (*R*)-PMPDAP and its prodrugs are given in Table 2.

Akin to (*R*)-PMPDAP, both prodrugs inhibit FIV replication in a selective manner. Compared to the parent molecule, the amidate

prodrug **15** is 10-fold less active against FIV in cell culture, whereas the alkoxyalkyl ester prodrug is 200-fold more potent in inhibiting FIV replication in vitro.

Besides this, the HDP monoester **14** also reveals surprising activity against selected RNA viruses (Coxsackie B4: EC₅₀ 9 μ g/mL, Punta Toro virus: EC₅₀ 12 μ g/mL) in Vero cells but more investigation in this field is still needed. No activity was found against most DNA viruses (HSV-1, HSV-2, Feline herpesvirus, VV and HCMV) except varicella zoster virus, where the compound reveals strong activity against both TK⁺VZV (OKA) (EC₅₀ <0.032 μ g/mL) and TK⁻ VZV (07-1) (EC₅₀ 0.058 μ g/mL) at non-toxic concentrations.

New structures isolated as by-products—disubstituted diaminopurine derivatives **3,4** and bis(phosphonate) derivative **10**—were subjected to a detailed antiviral screening in cell cultures. Unfortunately, these compounds did not show any antiviral activity (EC₅₀ >100 μ g/mL) towards the following viruses: HIV-1, HIV-2 in CEM cells, HCMV, HSV-1, HSV-2, vaccinia virus and vesicular stomatitis virus (VSV) in HeL cells, Feline herpes virus and Feline corona virus (FIPV) in CRFK cells, VSV, Coxsackie virus B4 and respiratory syncytial virus (RSV) in HeLa cells, Parainfluenza 3-virus, Reovirus-1, Sindbis virus Coxsackie B4 virus and Punta Toro virus in MDCK cells. No cytotoxicity was observed towards above mentioned cell lines concerning minimum cytotoxicity concentration required to cause a microscopically detectable alteration of normal cell morphology (MCC >100 μ g/mL) or required to reduce virus-induced cytopathogenicity by 50% (CC₅₀ >100 μ g/mL).

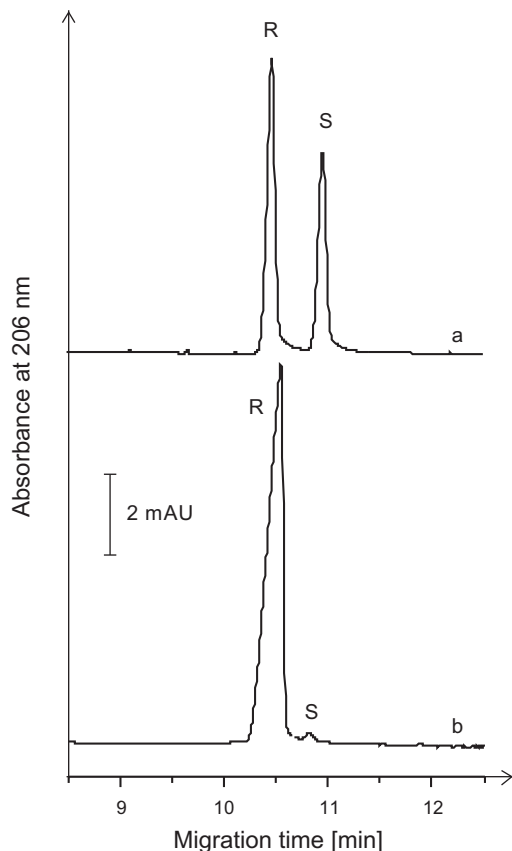


Figure 2. Separation of (*R*)- and (*S*)-enantiomers of PMPDAP; (a) and determination of enantiomeric purity of (*R*)-PMPDAP, batch B; (b) by capillary electrophoresis using β -cyclodextrin as chiral selector. For experimental conditions, see the Section 5.14.

Table 1
Enantiomeric purity of three batches (A,B,C) of (*R*)-PMPDAP

Sample	Enantiomeric purity (%)	Content of <i>S</i> -isomer (%)
(<i>R</i>)-PMPDAP, A	99.1	0.9
(<i>R</i>)-PMPDAP, B	99.2	0.8
(<i>R</i>)-PMPDAP, C	99.1	0.9

4. Conclusions

Synthesis of acyclic nucleoside phosphonates of (*R*)-[2-(phosphonomethoxy)propyl] type (PMP derivatives) based on utilization of (*R*)-propanediol carbonate was described in all details including isolation, characterization and antiviral and cytotoxicity screening of by-products formed during single reaction steps. As proven in the case of antiretrovirally active (*R*)-PMPDAP, synthesis based on utilization of (*R*)-propanediol carbonate is easier, gives higher yields compared to the originally described procedures,^{1,2} and the final product is isolated in extraordinary high optical purity. The described methodology is applicable especially for large-scale syntheses of (*R*)-PMPDAP. Excellent antiretroviral activity data and selectivity indices of (*R*)-PMPDAP and its prodrugs in vitro give an impetus for the future in vivo experiments. Further investigation of these compounds as drug candidates against HIV-1 in human medicine and other antiretroviral infections such as FIV in veterinary medicine is thus warranted.

5. Experimental

5.1. General

Unless stated otherwise, solvents were evaporated at 40 °C/ 2 kPa and compounds were dried at 13 Pa. Melting points were determined on a Kofler block and are uncorrected. Analytical TLC was performed on silica gel 60 F₂₅₄ plates (Merck KGaA, Darmstadt, Germany); chromatographic systems are described in text. Column chromatography was performed on silica gel 60 μ m (Fluka). Preparative reverse phase HPLC separations were performed on a Waters Delta 600 instrument with a Waters 2487 Dual λ Absorbance Detector using a column Luna Phenomenex® C-18 (10 μ m, 21 \times 250 mm) or XTerra® Prep, RP18OBD (10 μ m, 19 \times 300 mm), flow 12 ml/min. ¹H and ¹³C NMR spectra were measured on a Bruker Avance 500 spectrometer (¹H at 500 MHz, ¹³C at 125.7 MHz) in D₂O or DMSO-*d*₆ solutions (referenced to sodium 3-(trimethylsilyl)propane-1-sulfonic acid (DSS) or residual solvent signal). The numbering system for assignment of NMR signals is outlined in Figure 3. Mass spectra were measured on LCQ classic instrument using electrospray ionization (ESI). Optical rotations were measured on Autopol IV polarimeter (Rudolph Research Analytical, U.S.A.) at 20 °C, [α]_D values are given in 10⁻¹ deg cm² g⁻¹. Most of chemicals and ion-exchange resins (Dowex 1 \times 2-400, Dowex 50 \times 8) were purchased from Sigma-Aldrich (Czech Republic). (*R*)-1,2-Propane diol carbonate was a product of Chemos. Diisopropyl bromomethanephosphonate¹⁵ and diisopropyl tosyloxymethanephosphonate were synthesized at the Institute. The amide prodrug **15** was prepared according to the procedure developed previously in our laboratory.¹⁷ Reaction details and characterization of the amide product **15** are in agreement with literature.¹⁷

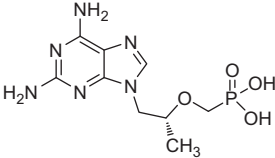
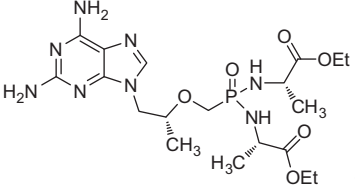
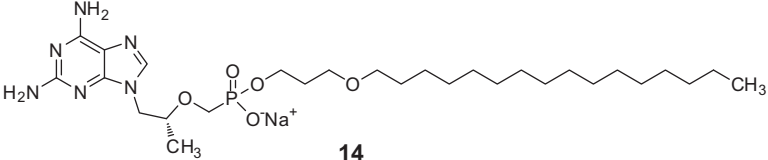
5.2. (*R*)-9-(2-Hydroxypropyl)-2,6-diaminopurine (**1**)

Sodium hydroxide pearls (1 g) were added to a stirred mixture of 2,6-diaminopurine (10 g, 66.6 mmol) and (*R*)-1,2-propanediol carbonate (10 g, 98 mmol) in DMF (250 mL) preheated to 100 °C. The whole mixture was then heated to 120 °C till conversion to **1** is complete (18–24 h). Additional portions of (*R*)-1,2-propanediol carbonate (3 g) and sodium hydroxide (1 g) were added during the reaction (approx. after 8 h). Reaction course was monitored by TLC in system ethyl acetate/acetone/ethanol/water (15:3:4:3): *R_F* (diaminopurine) = 0.29, *R_F* (**1**) = 0.49, *R_F* (by-product **3**) = 0.68. The hot mixture was filtered through a thick glass frit, the filtrate evaporated and the residue coevaporated with toluene (200 mL). The syrupy residue was crystallized from ethanol to afford a crude yellowish product that was additionally recrystallized (from ethanol) to give 9.89 g of **1** as white crystals. Additional portion (1.52 g) was obtained by crystallization from mother liquors. Total yield of **1**: 11.4 g (82%) as white crystals, mp 184–186 °C (literature: 192 °C).¹ HPLC purity 92%. ESIMS, *m/z*: 438.8 (2 M+Na)⁺ (4), 231.1 (M+Na)⁺ (21), 209.1 (MH)⁺ (100). NMR data are in agreement with literature.¹

5.3. (*R*)-*N*-(6-Amino-9-(2-hydroxypropyl)-9H-purin-2-yl)formamide (**2**)

Isolated from mother liquors after crystallization of **1**. The mother liquors were evaporated and chromatographed on a column of silica gel in system ethyl acetate/acetone/ethanol/water (15:3:4:3), the isolated solid (**2**) was crystallized from ethanol to give 800 mg (5%) of white crystals, mp 257 °C. ¹H NMR (DMSO-*d*₆, ppm) δ : 1.06 (d, 3H, *J*_{3',2'} = 6.0, H-3'), 3.92 (dd, 1H, *J*_{1'a,2'} = 8.5, *J*_{gem} = 14.5, H-1'a), 3.98–4.04 (m, 2H, H-1'b, H-2'), 5.00 (br d, 1H,

Table 2
Anti-HIV-1 and anti-FIV activity and cytotoxicity data of (R)-PMPDAP and its prodrugs

Compound	HIV-1 EC ₅₀ (μM)	FIV EC ₅₀ (μM)	CC ₅₀ (μM)	CrFK CC ₅₀ (μM)
 12	0.94	0.12	>100	>500
 15	0.29	1.1	>100	215
 14	0.00224	0.00058	19.8	96

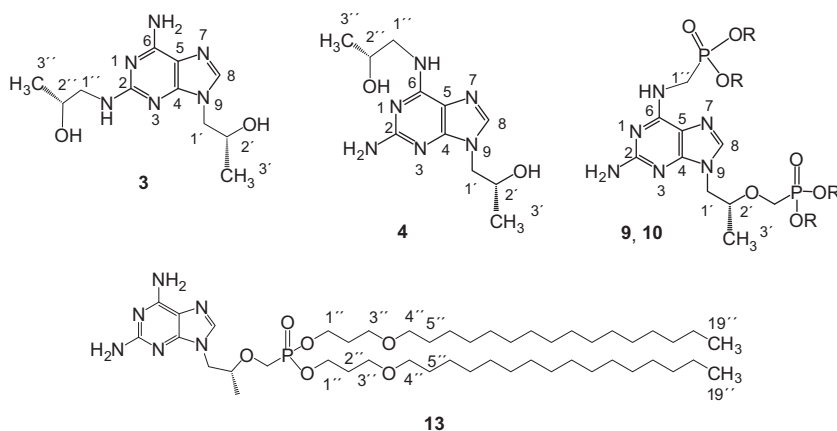


Figure 3. General numbering scheme for assignment of NMR signals.

$J_{\text{OH},2'} = 4.4$, OH), 7.27 (br s, 2H, 6-NH₂), 7.91 (s, 1H, H-8), 9.33 (br d, 1H, $J_{\text{vic}} = 10.3$, CH=O), 10.36 (br d, 1H, $J_{\text{vic}} = 10.3$, NH). ¹³C NMR (DMSO-*d*₆, ppm) δ: 21.10 (C-3'), 50.20 (C-1'), 64.72 (C-2'), 116.22 (C-5), 140.81 (C-8), 150.68 (C-4), 153.06 (C-2), 156.21 (C-6). ESIMS: 259.1 (M+Na)⁺ (25), 237.1 (MH)⁺ (100). HRMS (ESI): For C₉H₁₃N₆O₂ (MH)⁺ calcd 237.1095; found: 237.1095; for C₉H₁₂N₆O₂Na (M+Na)⁺ Calcd 259.0914. Found: 259.0914.

5.4. (R)-1-[6-Amino-2-[(R)-2-(hydroxypropyl)amino]-9H-purin-9-yl]propan-2-ol (3)

Isolated from mother liquors after crystallization of **1** using chromatography on silica gel in system ethyl acetate/acetone/ethanol/water (15:3:4:3). Yield 544 mg (3%), white solid. $[\alpha]_{\text{D}} -49.1$ (c 0.483, CH₃OH). ¹H NMR (DMSO-*d*₆, ppm) δ: 1.03 and 1.06 (2 × d, 2 × 3H, $J_{\text{CH}_3,\text{CH}} = 6.2$, CH₃), 3.13 and 3.25 (2 × m, 2 × 1H, H-1''), 3.77 (m, 1H, H-2''), 3.84 (dd, 1H, $J_{1'a,2'} = 7.0$, $J_{\text{gem}} = 13.8$, H-1'a), 3.91 (dd, 1H, $J_{1'b,2'} = 4.2$, $J_{\text{gem}} = 13.8$, H-1'b), 3.99 (m, 1H, H-2'), 4.84 (br s, 1H, OH), 5.09 (br s, 1H, OH), 6.07 (t, 1H, $J_{\text{NH},1'} = 5.7$, NH), 6.69 (br s, 2H, NH₂), 7.63 (s, 1H, H-8). ¹³C NMR (DMSO-*d*₆, ppm) δ: 21.19 and 21.60 (C-3', C-3''), 49.42 and 50.09 (C-1', C-1''), 64.96 and 66.10 (C-2', C-2''), 113.29 (C-5),

138.52 (C-8), 151.93 (C-4), 156.19 (C-6), 159.82 (C-2). Anal. Calcd for C₁₁H₁₈N₆O₂ · 1/3 H₂O: C, 48.52; H, 6.91; N, 30.86. Found: C, 48.80; H, 6.91; N, 30.59.

5.5. (R)-1-[2-Amino-6-[(R)-2-hydroxypropyl]amino]-9H-purin-9-yl]propan-2-ol (4)

Isolated from mother liquors after crystallization of **1** using chromatography on silica gel in system ethyl acetate/acetone/ethanol/water (15:3:4:3). Yield 355 mg (3%), white solid. $[\alpha]_{\text{D}} -55.4$ (c 0.464, CH₃OH). ¹H NMR (DMSO-*d*₆, ppm) δ: 1.03 and 1.07 (2 × d, 2 × 3H, $J_{\text{CH}_3,\text{CH}} = 6.2$, CH₃), 3.29 (br s, 1H, H-2''), 3.82 (m, 3H, H-1'', H-1'a), 3.90 (dd, 1H, $J_{1'b,2'} = 4.2$, $J_{\text{gem}} = 13.8$, H-1'b), 3.96 (m, 1H, H-2'), 4.90 (br s, 1H, OH), 5.03 (br s, 1H, OH), 5.82 (br s, 2H, NH₂), 6.88 (br s, 1H, NH), 7.63 (s, 1H, H-8). ¹³C NMR (DMSO-*d*₆, ppm) δ: 21.21 (C-3'), 21.51 (C-3''), 45.50 (C-1''), 50.07 (C-1'), 64.90 (C-2'), 65.84 (C-2''), 113.34 (C-5), 138.34 (C-8), 150.00 (C-4), 155.30 (C-6), 160.32 (C-2). ESIMS, *m/z*: 554.9 (2 M+Na)⁺ (100), 289.1 (M+Na)⁺ (100), 267.2 (MH)⁺ (100). HRMS (ESI): For C₁₁H₁₉N₆O₂ (MH)⁺ calcd 267.1564; found: 267.1570. Anal. Calcd for C₁₁H₁₈N₆O₂ · 2/3 H₂O: C, 47.47; H, 7.00; N, 30.20. Found: C, 47.74; H, 6.98; N, 29.94.

5.6. (E)-9-(Prop-1-en-1-yl)-9H-purine-2,6-diamine (5)

Isolated from mother liquors after crystallization of **1** using chromatography on silica gel in system ethyl acetate/acetone/ethanol/water (15:3:4:3). Yield 63 mg (0.5%), white solid. $^1\text{H NMR}$ (DMSO- d_6 , ppm) δ : 1.77 (dd, 3H, $J_{3',2'} = 6.8$, $J_{3',1'} = 1.7$, H-3'), 5.91 (br s, 2H, NH $_2$), 6.39 (dd, 1H, $J_{2',1'} = 14.4$, $J_{2',3'} = 6.8$, H-2'), 6.79 (br s, 2H, NH $_2$), 6.87 (dd, 1H, $J_{1',2'} = 14.4$, $J_{1',3'} = 1.7$, H-1'), 7.95 (s, 1H, H-8). $^{13}\text{C NMR}$ (DMSO- d_6 , ppm) δ : 18.93 (C-3'), 112.07 (C-2'), 123.74 (C-1'), 113.28 (C-5), 138.14 (C-8), 150.21 (C-4), 155.47 (C-6), 160.65 (C-2). ESIMS, m/z : 189.1 (M) $^-$ (100), HRMS (ESI $^-$): For C $_8$ H $_9$ N $_6$ (M) $^-$ calcd: 189.0889; found: 189.0890. Anal. Calcd for C $_8$ H $_9$ N $_6$: C, 50.52; H, 5.30; N, 44.18. Found: C, 50.64; H, 5.47; N, 44.06.

5.7. Introduction of a phosphonomethyl residue I

5.7.1. Reaction of unprotected intermediate (1) with TsOCH $_2$ P(O)(OiPr) $_2$. Preparation of diisopropyl ester of (R)-9-[2-(phosphonomethoxy)propyl]-2,6-diaminopurine (6)

60% Sodium hydride dispersion (5.6 g, 140 mmol) was added to a stirred mixture of diisopropyl tosyloxymethanephosphonate (24.5 g, 70 mmol) and intermediate **1** (9.85 g, 47.3 mmol) in DMF (250 mL) cooled to -20°C . Reaction temperature was then gradually let warm from -20°C to room temperature and the mixture stirred for 24 h. Acetic acid was added for neutralization to pH 7 and the mixture was evaporated. The residue was chromatographed on a column of silica gel (800 mL) in system chloroform/methanol (9:1). Elution of a complex mixture of by-products (2.5 g, including 7,8,9) occurred first followed by the desired product **6**. Yield: 9 g (49%) of **6** as a yellowish foam. $[\alpha]_D^{25} -46.9$ (c 0.326, CHCl $_3$). NMR data are in agreement with literature.² ESIMS, m/z : 794.7 (2M+Na) $^+$ (73), 409.1 (M+Na) $^+$ (100), 387.1 (96) (MH) $^+$. Anal. Calcd for C $_{15}$ H $_{27}$ N $_4$ O $_6$ P: C, 46.03; H, 7.04; N, 21.75; P, 8.02. Found: C, 46.27; H, 7.02; N, 21.33; P, 7.81.

5.7.2. Separation and identification of by-products 7, 8, 9

A mixture of by-products from the previous process was evaporated and chromatographed on silica gel column in system ethyl acetate/acetone/ethanol/water (18:3:2:2).

The following fractions were obtained:

5.7.3. Diisopropyl ((1R)-2-[2-amino-6-(formylamino)-9H-purin-9-yl]-1-methylethyl)oxy)methylphosphonate (7)

Yield 400 mg (1%) as a colorless oil. R_F 0.38. $^1\text{H NMR}$ (DMSO- d_6 , ppm) δ : 1.10 and 1.12 (2 \times d, 2 \times 3H, $J_{\text{CH}_3,\text{CH}} = 6.2$, CH $_3$), 1.16 (d, 6H, CH $_3$), 1.20 (d, 3H, CH $_3$), 3.67 (dd, 1H, $J_{\text{P,CH}_a} = 9.5$, $J_{\text{gem}} = 13.7$, PCH $_a$), 3.78 (dd, 1H, $J_{\text{P,CH}_b} = 9.1$, $J_{\text{gem}} = 13.7$, PCH $_b$), 3.94 (m, 1H, H-2'), 4.04 (dd, 1H, $J_{1',a,2'} = 7.1$, $J_{\text{gem}} = 14.3$, H-1'a), 4.13 (dd, 1H, $J_{1',b,2'} = 3.6$, $J_{\text{gem}} = 14.3$, H-1'b), 4.50 (m, 2H, P-OCH), 6.38 (br s, 2H, NH $_2$), 7.93 (s, 1H, H-8), 9.86 (br d, 1H, $J_{\text{NH,CH}} = 10.0$, CH=O), 10.80 (br d, 1H, NH). $^{13}\text{C NMR}$ (DMSO- d_6 , ppm) δ : 16.83 (C-3'), 23.74 and 23.82 (2 \times d, $J_{\text{P,C}} = 4.5$, 2 \times CH $_3$), 23.90 and 23.95 (2 \times d, $J_{\text{P,C}} = 3.8$, 2 \times CH $_3$), 46.80 (C-1'), 62.61 (d, $J_{\text{P,C}} = 165.7$, P-C), 70.285 (d, $J_{\text{P,C}} = 6.4$, P-OCH), 70.335 (d, $J_{\text{P,C}} = 6.4$, P-OCH), 75.33 (d, $J_{\text{P,C}} = 12.6$, C-2'), 114.23 (C-5), 141.77 (C-8), 149.81 (C-4), 154.67 (C-6), 160.09 (C-2), 164.51 (NCH=O). ESIMS, m/z : 850.5 (2M+Na) $^+$ (88), 437.1 (M+Na) $^+$ (100), 415.1 (19) (MH) $^+$.

5.7.4. Diisopropyl ((1R)-2-[6-amino-2-(formylamino)-9H-purin-9-yl]-1-methylethyl)oxy)methylphosphonate (8)

Yield 520 mg (1.3%) as a colorless oil. R_F 0.32. $^1\text{H NMR}$ (DMSO- d_6 , ppm) δ : 1.09, 1.12, 1.155, 1.165 and 1.19 (5 \times d, 5 \times 3H, $J_{\text{CH}_3,\text{CH}} = 6.2$, CH $_3$), 3.725 (dd, 1H, $J_{\text{P,CH}_a} = 9.5$, $J_{\text{gem}} = 13.7$, PCH $_a$), 3.79 (dd, 1H, $J_{\text{P,CH}_b} = 9.2$, $J_{\text{gem}} = 13.7$, PCH $_b$), 3.96 (m, 1H, H-2'), 4.07 (dd, 1H, $J_{1',a,2'} = 6.6$, $J_{\text{gem}} = 14.4$, H-1'a), 4.17 (dd, 1H,

$J_{1',b,2'} = 3.7$, $J_{\text{gem}} = 14.4$, H-1'b), 4.50 (m, 2H, P-OCH), 7.29 (br s, 2H, NH $_2$), 7.92 (s, 1H, H-8), 9.33 (d, 1H, $J_{\text{NH,CH}} = 10.2$, CH=O), 10.36 (d, 1H, NH). $^{13}\text{C NMR}$ (DMSO- d_6 , ppm) δ : 16.95 (C-3'), 23.75 and 23.81 (2 \times d, $J_{\text{P,C}} = 4.6$, 2 \times CH $_3$), 23.92 and 23.95 (2 \times d, $J_{\text{P,C}} = 4.3$, 2 \times CH $_3$), 46.77 (C-1'), 62.59 (d, $J_{\text{P,C}} = 165.6$, P-C), 70.275 (d, $J_{\text{P,C}} = 6.1$, P-OCH), 70.32 (d, $J_{\text{P,C}} = 6.1$, P-OCH), 75.44 (d, $J_{\text{P,C}} = 12.9$, C-2'), 116.04 (C-5), 140.58 (C-8), 150.74 (C-4), 156.26 (C-6), 153.14 (C-2), 163.76 (NCH=O). ESIMS, m/z : 850.6 (2M+Na) $^+$ (45), 437.0 (M+Na) $^+$ (100), 415.1 (13) (MH) $^+$.

5.7.5. Diisopropyl ((2-amino-9-[(2R)-2-(diisopropoxyphosphorylmethoxy)propyl]-9H-purin-6-yl)amino)methylphosphate (9)

Yield 1.6 g (6%) of white foam. R_F 0.21. $^1\text{H NMR}$ (DMSO- d_6 , ppm) δ : 1.06 (d, 3H, $J_{3',2'} = 6.1$, H-3'), 1.175 and 1.185 (2 \times d, 2 \times 3H, CH $_3$), 3.67 and 3.76 (2 \times dd, 2 \times 2H, $J_{\text{P,CH}} = 9.5$, $J_{\text{gem}} = 13.6$, PCH $_2$), 3.91 (m, 1H, H-2'), 3.95 (br m, 2H, PCH $_2$ -N), 3.98 (dd, 1H, $J_{1',a,2'} = 6.7$, $J_{\text{gem}} = 14.2$, H-1'a), 4.06 (dd, 1H, $J_{1',b,2'} = 3.1$, $J_{\text{gem}} = 14.2$, H-1'b), 4.52 and 4.60 (2 \times m, 2 \times 2H, P-OCH), 5.90 (br s, 2H, NH $_2$), 6.94 (br s, 1H, NH), 7.67 (s, 1H, H-8). $^{13}\text{C NMR}$ (DMSO- d_6 , ppm) δ : 16.91 (C-3'), 23.83, 23.85, 23.96, 23.99 and 24.05 (5 \times d, 8C, $J_{\text{P,C}} = 3.9$, 8 \times CH $_3$), 35.99 (br d, $J_{\text{P,C}} = 155.2$, P-C-N), 46.57 (C-1'), 62.73 (d, $J_{\text{P,C}} = 165.9$, P-C), 70.33 (d, 2C, $J_{\text{P,C}} = 6.2$, P-OCH), 70.37 (d, 2C, $J_{\text{P,C}} = 6.2$, P-OCH), 75.60 (d, $J_{\text{P,C}} = 13.0$, C-2'), 113.27 (C-5), 138.33 (C-8), 150.0 (C-4), 154.31 (C-6), 160.16 (C-2). ESIMS, m/z : 587.1 (M+Na) $^+$ (100), 565.1 (39) (MH) $^+$.

5.8. (R)-9-[2-(Phosphonomethoxy)propyl]-N 6 -phosphonomethyl-2,6-diaminopurine (10)

Bromotrimethylsilane (6.6 mL, 50 mmol) was added to a solution of **9** (1.4 g, 2.5 mmol) in acetonitrile (40 mL) and the solution was set aside in dark for 24 h at room temperature. The mixture was evaporated, the residue coevaporated with acetonitrile and in a form of aqueous solution (10 mL) applied onto a column of Dowex (H $^+$ form, 40 mL). Elution was performed with water (1000 mL; after 500 mL the first portion of compound **10** was eluted). The product was evaporated and then lyophilized from water to give 540 mg (55%) of **10** as a white solid. The column was then eluted with 2.5% aqueous ammonia. UV absorbing fraction was evaporated and applied onto a column of Dowex 1 (acetate form, 50 mL). Elution was performed with water 250 mL followed by a linear gradient of acetic acid (0–1 M, 1 L) and then 3 M formic acid. UV absorbing fraction (eluted by 3 M-HCOOH) was evaporated and purified on reverse phase HPLC using isocratic elution with water. Evaporation of appropriate fractions and lyophilization from water gave additional portion of **10**: 100 mg (10%). Overall yield of **10** was 640 mg (65%). $^1\text{H NMR}$ (D $_2$ O, ppm) δ : 1.17 (d, 3H, $J_{3',2'} = 6.4$, H-3'), 3.56 (dd, 1H, $J_{\text{P,CH}_a} = 10.1$, $J_{\text{gem}} = 12.8$, PCH $_a$), 3.78 (dd, 1H, $J_{\text{P,CH}_b} = 9.8$, $J_{\text{gem}} = 12.8$, PCH $_b$), 3.85 (br d, 2H, $J_{\text{P,CH}} = 11.7$, PCH $_2$ -N), 3.96 (m, 1H, H-2'), 4.15 (dd, 1H, $J_{1',a,2'} = 5.7$, $J_{\text{gem}} = 14.6$, H-1'a), 4.33 (bdd, 1H, $J_{1',b,2'} = 3.0$, $J_{\text{gem}} = 14.6$, H-1'b), 7.97 (s, 1H, H-8). $^{13}\text{C NMR}$ (D $_2$ O, ppm) δ : 15.08 (C-3'), 38.70 (br d, $J_{\text{P,C}} = 155.5$, P-C-N), 49.08 (C-1'), 64.39 (d, $J_{\text{P,C}} = 158.6$, P-C), 76.01 (d, $J_{\text{P,C}} = 13.7$, C-2'), 114.83 (C-5), 140.39 (C-8), 151.83 (C-4), 153.19 (C-6), 160.59 (C-2). +ESIMS, m/z : 397.1 (100) (MH) $^+$. -ESIMS, m/z : 395.1 (54) (M-H) $^-$. -ESI-HRMS Calcd for C $_{10}$ H $_{17}$ O $_7$ N $_6$ P $_2$ 395.0639. Found: 395.0644 (M-H) $^-$.

5.9. Introduction of a phosphonomethyl residue II. Preparation of (R)-9-[2-(phosphonomethoxy)propyl]-2,6-diaminopurine (12) from 1 using protection with dimethylaminomethylene groups

N,N-Dimethylformamide dimethylacetal (40 mL) was added to a suspension of **1** (9.9 g; 47.5 mmol) in DMF (100 mL). The mixture

was stirred at room temperature for 24 h and evaporated. The residue was coevaporated with xylene (200 mL) and dried in vacuo at 25 °C.

The obtained yellow powder is *N'*-[6-amino-9-(2-hydroxypropyl)-9*H*-purin-2-yl]-*N,N*-dimethylimidoforamide (intermediate **11**). ESIMS: 549.0 (2M+Na)⁺ (18), 264.2 (MH)⁺ (100). HRMS (ESI): For C₁₁H₁₈N₇O (MH)⁺ calcd: 264.1567; found: 264.1567.

A suspension of thus obtained **11** and 60% sodium hydride (3.6 g; 90 mmol) in DMF (200 mL) was stirred vigorously for 15 min, then diisopropyl bromomethanephosphonate (13.6 g; 53 mmol) was added and the reaction mixture stirred for 24 h at room temperature. The mixture was then neutralized with acetic acid to pH 7 and evaporated. The residue was mixed with a solution of methanol (200 mL) and 25% aqueous ammonia (60 mL), set aside for 2 days at room temperature and evaporated. The residue was coevaporated with absolute ethanol (150 mL) and dry acetonitrile (150 mL). Acetonitrile (300 mL) was then added, followed by bromotrimethylsilane (64 mL; 480 mmol) and the mixture stirred in dark at room temperature for 2 days. The mixture was evaporated, the residue coevaporated with acetonitrile, followed by methanol. The residue was dissolved in aqueous ethanol and applied onto a column of Dowex 50 (H⁺ form, 400 mL). Elution was performed with water (2 L) followed by 2.5% aqueous ammonia. UV absorbing fractions were collected and evaporated. The residue was dissolved in water and applied onto a column of Dowex 1 (acetate form, 150 mL). Elution with water separated unreacted starting material **1** containing small amount of 2,6-diaminopurine. Its purification was performed by crystallization and subsequent chromatography of mother liquors in system chloroform/methanol

(85:15). This procedure afforded 2.53 g (25.5%) of recovered compound **1**.

Subsequent elution of Dowex 1 with a linear gradient of acetic acid (0.2–1 M, 5 L) eluted pure (*R*)-PMPDAP (**12**). The remaining product was released from the ion exchanger by its boiling with water (1 L). All fractions containing **12** were evaporated, the residue coevaporated with water (2 × 250 ml) and crystallized from water. According to the X-ray fluorescence method (Fig. 4), so prepared sample contained about 4 weight percent of Si. These silylated impurities (origin from bromotrimethylsilane) were removed by repeated solid phase extraction of the solid sample of (*R*)-PMPDAP (**12**) by boiling dichloromethane (4 × 50 ml). Yield 4.34 g (30%), white crystals. –ESIMS: 602.6 (2M–H)[–] (7), 301.0 (M–H)[–] (100). HRMS (–ESI): For C₉H₁₄N₆O₄P (M–H)[–] calcd: 301.0814; found: 301.0822. NMR data are in agreement with literature.¹

5.10. Hexadecyloxypropyl ester of (*R*)-9-[2-(phosphonomethoxy)propyl]-2,6-diaminopurine, sodium salt (**14**)

1 M Tetrabutylammonium hydroxide in methanol (6.6 mL) was added to a suspension of **12** (1 g, 3.3 mmol) in methanol (50 mL). Thus formed clear solution was evaporated, the residue coevaporated with toluene (50 mL), dissolved in DMF (50 mL) and then stirred with hexadecyloxypropyl bromide (2.5 g, 7 mmol) at 100 °C for 3 h. After addition of ethanol (5 mL), the mixture was evaporated to ½ volume and heated at 100 °C with lithium azide (260 mg, 5.3 mmol) added in two portions during 15 h. The reaction was completed by evaporation and heating of the residue with

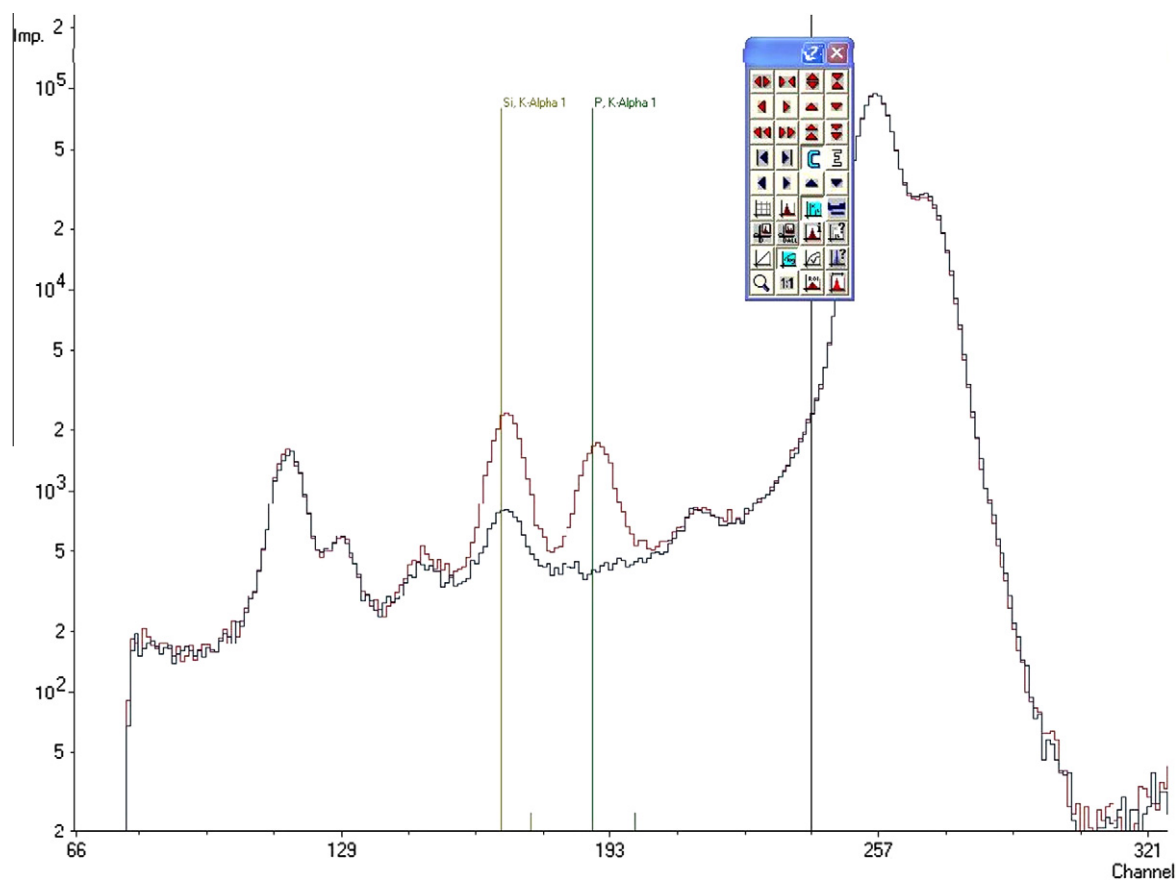


Figure 4. Elemental analysis using X-ray fluorescence method (SPECTRO iQII spectrometer). Pure solvent—blue line, solution of **12**—red line. X-axis frequencies of emitted electron characteristic for each element, Y-axis number of detected electrons (logarithmic scale). Characteristic frequency highlighted: the element phosphorus—green line, the element silicon—yellow line.

2 M NaOH (10 mL) for 2 h at 110 °C. After cooling to room temperature, the mixture was neutralized with acetic acid to pH 6, diluted with water and the product crystallized in refrigerator. The product was collected by suction, washed with water (250 mL) and recrystallized from boiling methanol. Additional recrystallization from ethanol afforded pure product **14** as white crystals in yield 940 mg (49%). Additional portion of **14** was obtained from mother liquors by chromatography on silica gel performed in system ethyl acetate/acetone/ethanol/water, first in combination (18:3:1:1, elution of the rest of diester **13**), followed by the same system in ratio (13:3:4:5) to give additional 120 mg (6%) of **14**. The overall yield of **14** was 1.060 g (55%). $[\alpha]_D -10.8$ (c 0.259, ethanol). $^1\text{H NMR}$ ($\text{D}_2\text{O} + \text{NaOD}$, ppm) δ : 0.82 (bt, 3H, $J = 7.0$, CH_3), 1.05 (bt, 3H, $J_{3,2'} = 6.0$, H-3'), 1.20 (m, 26 H, CH_2), 1.49 (m, 2H, CH_2), 1.83 (m, 2H, CH_2), 3.31 and 3.44 ($2 \times$ m, $2 \times$ 2H, OCH_2), 3.50 and 3.68 ($2 \times$ bdd, $2 \times$ 1H, $J_{\text{P,CH}} = 10.0$, $J_{\text{gem}} = 12.8$, PCH_2), 3.75 (m, 1H, H-2'), 3.89 (m, 2H, OCH_2), 3.90 (m, 1H, H-1'a), 4.10 (br d, 1H, $J'_{1,2'} = 3.0$, $J_{\text{gem}} = 14.0$, H-1'b), 7.76 (s, 1H, H-8). $^{13}\text{C NMR}$ ($\text{D}_2\text{O} + \text{NaOD}$, ppm) δ : 13.59 (CH_3), 15.92 (C-3'), 22.37, 25.89, 29.22, 29.30, 29.54, 27.72 (8C), 30.67 and 31.04 (CH_2), 47.00 (C-1'), 62.07 ($J_{\text{P,C}} = 4.6$, OCH_2), 63.41 (d, $J_{\text{P,C}} = 158.3$, P-C), 67.19 (OCH_2), 70.60 (OCH_2), 75.41 (d, $J_{\text{P,C}} = 12.6$, C-2'), 112.23 (C-5), 139.73 (C-8), 150.89 (C-4), 155.53 (C-6), 159.59 (C-2). –ESIMS, m/z : 583.41 (100) (M–H) $^-$ (free acid, $\text{C}_{28}\text{H}_{53}\text{N}_6\text{O}_5\text{P}$). Anal. Calcd for $\text{C}_{28}\text{H}_{52}\text{N}_6\text{O}_5\text{PNa} \cdot \frac{1}{2} \text{H}_2\text{O}$: C, 54.62; H, 8.68; N, 13.65; P, 5.03. Found: C, 54.63; H, 8.78; N, 13.35; P, 5.20.

5.11. Bis(hexadecyloxypropyl) ester of (R)-9-[2-(phosphonomethoxy)propyl]-2,6-diaminopurine (**13**)

$[\alpha]_D -21.0$ (c 0.162, CHCl_3). $^1\text{H NMR}$ (CDCl_3 , ppm) δ : 0.88 (t, 6H, $J_{19,18} = 7.0$, H-19''), 1.20–1.34 (m, 55H, $\text{CH}-\text{CH}_3$, H-6''–18''), 1.54 (m, 4H, H-5''), 1.92 (m, 4H, H-2''), 3.39 (m, 4H, H-4''), 3.48 (m, 4H, H-3''), 3.62 (dd, $J_{\text{P,CHa}} = 9.9$, $J_{\text{gem}} = 13.4$, PCH_a), 3.87 (m, 1H, NCH_2-CH), 3.89 (dd, 1H, $J_{\text{P,CHb}} = 9.1$, $J_{\text{gem}} = 13.4$, PCH_b), 3.99 (m, 1H, NCH_a), 4.12–4.24 (m, 5H, H-1'', NCH_b), 6.16 (br s, 2H, NH_2), 7.80 (br s, 2H, NH_2), 7.93 (br s, 1H, H-8). $^{13}\text{C NMR}$ (CDCl_3 , ppm) δ : 14.11 (C-19''), 16.43 ($\text{CH}-\text{CH}_3$), 22.68 (C-18''), 26.16 (C-6''), 29.35–29.71 (m, C-5'', C-7''–16''), 30.85 (d, $J_{\text{P,C}} = 6.0$, C-2''), 31.92 (C-17''), 47.7 (N- CH_2), 62.58 (d, $J_{\text{P,C}} = 169.7$, P-C), 63.88 (d, $J_{\text{P,C}} = 6.6$, C-1''), 64.01 (d, $J_{\text{P,C}} = 6.5$, C-1''), 66.48 (C-3''), 71.24 (C-4''), 76.19 (N- CH_2-CH), 142.8 (C-8). ESIMS, m/z : 867.7 (100) (MH) $^+$. Anal. Calcd for $\text{C}_{47}\text{H}_{91}\text{N}_6\text{O}_6\text{P}$: C, 65.09; H, 10.58; N, 9.69; P, 3.57. Found: C, 64.89; H, 10.52; N, 9.51; P, 3.77.

5.12. Testing of anti-FIV activity

Compounds for anti-FIV antiviral testing were dissolved in sodium bicarbonate at a concentration of 5 mM. Crandell Reese Feline kidney (CrFK) cells were grown in Dulbecco minimum essential medium (DMEM, Life Technologies) containing 1% sodium bicarbonate (Life Technologies) and 5% fetal calf serum (FCS, Biochrom). In a 96-well plate, a total of 5000 CrFK cells were pre-seeded and incubated for 24 h at 37 °C in a humid atmosphere containing 5% CO_2 . Subsequently, the cells were washed with 100 μL phosphate buffered saline (PBS) containing 50 $\mu\text{g}/\text{mL}$ DEAE-dextran and inoculated with 100 CCID₅₀ FIV in the presence of a serial dilution series of the test compound. Following 2 h of incubation, the supernatant was removed and the cells were washed with PBS. The infected cells were subsequently overlaid with 5% FCS containing DMEM to which a serial dilution series of the compounds was added. Infected cells without compounds were included as positive controls (virus controls) and uninfected cells were included as negative controls (cell controls). The plates were incubated for six days at which time viral supernatant was collected and analyzed by an enzyme-linked immunosorbent

assay (ELISA) for detection FIV p24 antigen. A total of 100 μL supernatant was incubated for 10 min in 0.1% TritonX100 and applied on a maxisorp plate (Nunc) that was pre-coated overnight with monoclonal anti-p24 antibody (11C7C7C1) and blocked with 5% FCS in PBS. Subsequently, the amount of FIV p24 was detected by a monoclonal biotinylated antibody (5E6D11) followed by a colorimetric reaction which is based on extravidin peroxidase (Sigma) and OPD substrate (Sigma). The absorbance was read at a wavelength of 492 nm (OD_{492}). EC_{50} -values were determined as the compound concentration that inhibited 50% of FIV p24 antigen detection as compared with virus controls. Cytotoxicity of the compounds was determined by incubating CrFK cells in the presence of a serial dilution series of the compounds at 37 °C and under humidified conditions. After 3 days of incubation the viability of the cells was measured using the CellTiter 96 Aqueous One Solution Assay (Promega) as per manufacturer's protocol. CC_{50} -values were determined as the compound concentration where 50% of the cells were death as compared with cell controls.

MT-4 cells (HTLV-1 transformed, human T lymphoblastoid cell line) were HTLV transformed enabling them to be highly sensitive to HIV-1 infection. The cell line was chosen from other available CD4+ T cell lines in an effort to develop a sensitive assay to measure potency and cytotoxicity of anti-HIV compounds.

5.13. Testing of anti-HIV activity

To ascertain HIV-1 compound antiviral efficacy, freshly harvested MT-4 cells were collected and pre-infected with HIV-1 IIIIB Virus (Advanced Biotechnologies) at a (low multiplicity of infection, MOI), a pre-determined concentration of virus that yields an EC_{50} for AZT of 20 μM . Infected MT-4 cells were placed in a rotator within a humidified incubator for one hour to initiate and expedite the infection process. The cells were then diluted in assay medium and promptly added to 384 assay plates at a density of 2000 cells per well. The methodology and workflow for compound dilution and addition into 384 well assay plates was the same as used for the CC_{50} assay. Cytopathic effects of HIV infection show up as a compound dose dependent luminescent readout via a Cell Titer Glo assay (measures ATP/cell viability). Non compound treated (unprotected) cells die and exhibit a low luminescent readout. Cells in the presence of drug (5 μM AZT, positive control) are expected to be viable and relatively healthy after the 5-day incubation. A percent luminescent signal is calculated from a given well of a given compound dose based on the negative and positive treated controls. Compound potency data (EC_{50}) are calculated by non-linear regression using Pipeliner Pilot software (Accelrys, San Diego, CA).

5.14. Determination of cytotoxicity

To determine compound cytotoxicity, compounds were tested in a standardized high-throughput 384-well assay format. Each compound was serially diluted 3-fold in 100% DMSO in polypropylene 384-well plates using a Biomek FX Workstation, and 0.4 μL compound added to an assay plate containing 40 μL assay medium. (RPMI 1640 + GlutaMAX™ (Irvine Scientific) supplemented with 10% fetal bovine serum (Fbr s, Hyclone, Logan, UT), 100 Units/mL Penicillin, 100 $\mu\text{g}/\text{mL}$ Streptomycin). Compounds were arranged in a horizontal pattern, with 10 concentrations per compound, and 8 compounds added per plate. Due to low DMSO tolerability, the final DMSO concentration never exceeded 0.5% (v/v). Each assay plate contained 10 μM Puromycin and 0.5% DMSO in assay medium as positive and negative controls respectively. MT-4 cells were added in volumes of 35 μL per well and 2000 cells per well using a Biotek uFlow Workstation (Biotek, Winooski, VT), and the

plates subsequently incubated for 5 days at 37 °C in an incubator set at 5% CO₂ and 90% humidity.

After 5 days, 22 µL Cell Titer Glo (Promega) was added to the assay plates with a Biotek uFlow Workstation. Plates were subsequently placed on a Perkin Elmer Envision Plate Reader for 5 minutes before the luminescence signal was read. CC₅₀ values were calculated from the compound concentration that caused a 50% decrease in luminescence signal, a measure of toxicity, and calculated by non-linear regression using Pipeline Pilot software (Accelrys, San Diego, CA).

5.15. Chiral analysis of (R)-PMPDAP by capillary electrophoresis

Analyses were performed in a commercial P/ACE MDQ capillary electrophoresis (CE) apparatus (Beckman Coulter, Fullerton, CA, USA), equipped with an internally untreated fused silica capillary with outer polyimide coating, total length 395 mm, effective length (from injection end to the detector) 294 mm, I.D./O.D. 50/375 µm (Polymicro Technologies, Phoenix, AR, USA). The analytes were monitored by UV–Vis absorption spectrophotometric photodiode array detector (190–600 nm) at two wavelengths, 206 and 254 nm, respectively. The temperature of capillary liquid coolant was set at 20 °C. Separation voltage was 15 kV.

The samples were injected hydrodynamically, by pressure 20.7 mbar for 5 s. The analytes were dissolved in deionized water at ca. 1 mM concentration.

The analyses were performed in chiral background electrolyte of the following composition: 30 mM sodium tetraborate, adjusted by NaOH to pH 10.0, chiral selector β-cyclodextrin (20 mg/mL). Enantiomeric purity was determined from the ratio of peak areas of (R)- and (S)-isomers as an average value from three experiments, which did not differ more than 3%.

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