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Synthesis of Ester Prodrugs of 9-(*S*)-[3-Hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine (HPMPDAP) as Anti-Poxvirus Agents

Marcela Krečmerová,*[,†] Antonín Holý,[†] Graciela Andrei,[‡] Karel Pomeisl,[†] Tomáš Tichý,[†] Petra Břehová,[†] Milena Masojídková,[†] Martin Dračínský,[†] Radek Pohl,[†] Genevieve Laflamme,[§] Lieve Naesens,[‡] Hon Hui,[§] Tomas Cihlar,[§] Johan Neyts,[‡] Erik De Clercq,[‡] Jan Balzarini,[‡] and Robert Snoeck[‡]

[†]*Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, v.v.i., Gilead Sciences and IOCB Research Centre, Flemingovo nám. 2, CZ-166 10, Prague 6, Czech Republic,* [‡]*Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium, and* [§]*Gilead Sciences, Inc., 333 Lakeside Drive, Foster City, California 94404*

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9-(*S*)-[3-Hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine (HPMPDAP) and its cyclic form were selected for further evaluation as potential drug candidates against poxvirus infections. To increase bioavailability of these compounds, synthesis of their structurally diverse ester prodrugs was carried out: alkoxyalkyl (hexadecyloxypropyl, octadecyloxyethyl, hexadecyloxyethyl), pivaloyloxymethyl (POM), 2,2,2-trifluoroethyl, butylsalicylyl, and prodrugs based on peptidomimetics. Most HPMPDAP prodrugs were synthesized in the form of monoesters as well as the corresponding cyclic phosphonate esters. The activity was evaluated not only against vaccinia virus but also against different herpes viruses. The most potent and active prodrugs against vaccinia virus were the alkoxyalkyl ester derivatives of HPMPDAP, with 50% effective concentrations 400−600-fold lower than those of the parent compound. Prodrugs based on peptidomimetics, the 2,2,2-trifluoroethyl, the POM, and the butylsalicylyl derivatives, were able to inhibit vaccinia virus replication at 50% effective concentrations that were equivalent or ∼10-fold lower than those observed for the parent compounds.

Introduction

The family of Poxviridae represents a group of enveloped, double-stranded DNA viruses with unique morphology and cytoplasmic site of replication.[1] They are very large (about 300 × 200 nm) and brick-shaped. Poxvirus-associated diseases are a serious problem for human health as well as veterinary medicine. The most dangerous human pathogen of this group is a member of the *Orthopoxvirus* genus: variola virus (VARV),[a] the causative agent of smallpox. Smallpox was one of the main causes of morbidity and mortality until recent times. In the 20th century alone, smallpox deaths worldwide numbered in the millions. In 1980, after an intensive program of immunization with a related but relatively nonpathogenic virus, i.e., vaccinia virus (VACV), the WHO declared the disease eradicated. The last naturally occurring outbreak of smallpox was in Somalia in 1977. After the disease was eliminated from the world, routine vaccination against smallpox among the general public was stopped because it was no longer necessary for prevention. By 1983, all known stocks of VARV were kept in two WHO collaborating centers: the U.S. Center for Disease Control and Prevention (CDC) in Atlanta and the Russian State Research Center of Virology and Biotechnology in Novosibirsk. However, in the aftermath of the events of September 2001, there is increasing concern that undeclared stocks of VARV might exist and that they might be used as a bioterrorist weapon. The discontinuation of routine vaccination against smallpox has rendered the human population more susceptible to this disease. Moreover, VARV is highly stable and retains its infectivity for relatively long periods of time outside the host. For all of these reasons, special attention has been paid to precautions for dealing with a smallpox outbreak. On the other hand, VACV vaccination is supposed to cause several complications, especially among immunocompromised patients. Therefore, there are increased demands for anti-poxvirus effective drugs to treat acute infections. Despite of progressive investigation in this area, and the number of compounds in preclinical and/or early clinical stage of development, no FDA approved drugs are currently available on the market.[2,3] Only three drugs have been used in the clinic for the treatment of complications associated with vaccination against smallpox.[4] These include the acyclic nucleoside analogue cidofovir and its oral prodrug, hexadecyloxypropyl ester (HDP-CDV), which inhibit viral DNA replication and compound ST-246, an orally bioavailable compound that targets orthopoxvirus morphogenesis.[5]

*Corresponding author: phone, +420 220183475; fax, +420 220183560; e-mail, marcela@uochb.cas.cz.

[a]Abbreviations: ANP, acyclic nucleoside phosphonate; bis(POC)-PMPA, bis(isopropyloxycarbonyloxymethyl) ester of 9-(*R*)-[2-(phosphonomethoxy)propyl]adenine; bis(POM)-PMEA, bis(pivaloyloxymethyl) ester of 9-[2-(phosphonomethoxy)ethyl]adenine; DCC, *N*,*N*′-dicyclohexylcarbodiimide; HATU, *O*-7-(azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HCMV, human cytomegalovirus; HDP-CDV, hexadecyloxypropyl ester of cidofovir; HOBt, 1-hydroxybenzotriazole; HPMPA, 1-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine; HPMPC, 1-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine (cidofovir); HPMPDAP, 9-(*S*)-[3-hydroxy-2-(phosphonomethoxy)-propyl]-2,6-diaminopurine; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; iPr, 2-propyl; POM, pivaloyloxymethyl; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; PyBroP, bromotripyrrolidinophosphonium hexafluorophosphate; TEA, triethylammonium; TEAB, triethylammonium hydrogen carbonate; VACV, vaccinia virus; VARV, variola virus.

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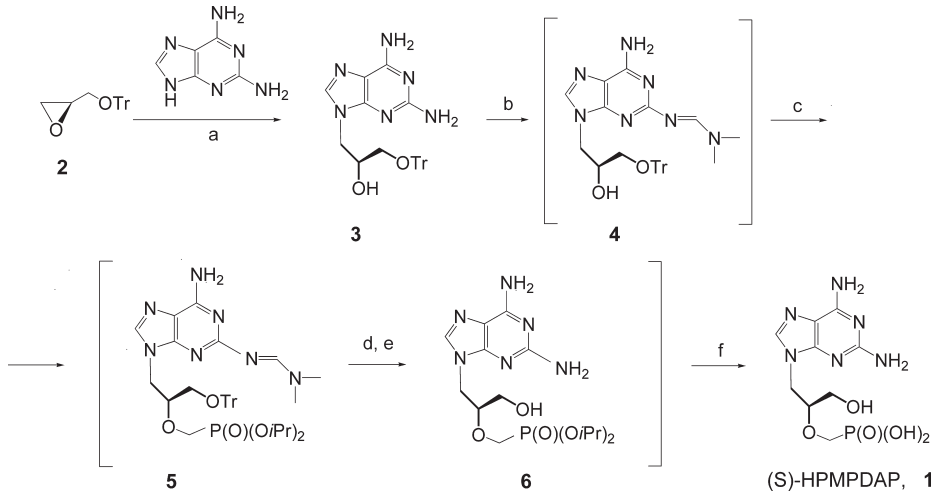
Scheme 1[a]



[a]Conditions: (a) cesium carbonate, DMF, 100 °C; (b) dimethylformamide dimethylacetal; (c) $BrCH_2P(O)(OiPr)_2$, NaH, DMF, room temperature; (d) NH_4OH (H_2O−MeOH); (e) 80% AcOH, reflux; (f) $(CH_3)_3SiBr$, CH_3CN, room temperature.

Until recently, the search for small molecule compounds with anti-orthopoxvirus activity was focused on the area of nucleoside analogues using VACV as a model system for testing. However, new classes of molecules able to selectively inhibit orthopoxvirus replication have been recently described.[6] Several nucleoside analogues have been shown to inhibit VACV replication *in vitro*, and according to their mechanism of action they can be divided into several categories: *S*-adenosylhomocysteine hydrolase inhibitors, inosine monophosphate dehydrogenase inhibitors (ribavirin), orotidine 5′-monophosphate decarboxylase inhibitors, cytidine triphosphate synthetase inhibitors, thymidylate synthase inhibitors, nucleoside analogues, and acyclic nucleoside phosphonates targeted at viral DNA synthesis.[7−9]

One of the most promising groups of potent and selective anti-poxvirus agents is acyclic nucleoside phosphonates (ANPs) of the HPMP series,[10] i.e., (*S*)-[3-hydroxy-2-(phosphonomethoxypropyl)] derivatives of diverse nucleobases: cytosine (HPMPC), 5-azacytosine (HPMP-5azaC),[11] adenine (HPMPA), 2,6-diaminopurine (HPMPDAP),[12] 7-deazaadenine (HPMP-7-deazaA), 3-deazaadenine (HPMP-3-deazaA), and 8-azaadenine (HPMP-8-azaA).[13] In a series of "open-ring" analogues (also called the second generation of ANPs), a remarkable anti-poxvirus activity has been described for (*R*)-{2,4-diamino-3-hydroxy-6-[2-(phosphonomethoxy)-propoxy]}pyrimidine, (*R*)-HPMPO-DAPy.[14] Cidofovir, licensed for the treatment of cytomegalovirus retinitis in AIDS patients, is the only approved acyclic nucleoside analogue with anti-poxvirus activity. Currently, it is recommended by the U.S. Centers for Disease Control and Prevention for the treatment of severe adverse effects following smallpox vaccination.[15,16] However, the use of cidofovir is limited partly by renal toxicity and partly by its poor oral bioavailability, implying it can only be administered by intravenous injections. To improve oral bioavailability of ANPs, there is a tendency for their transformation to lipophilic ester prodrugs. In case of cidofovir, so far the most successful drug candidate blocking smallpox virus reproduction is its hexadecyloxypropyl ester (HDP-cidofovir).[17−20] Other ANPs have been subjected to esterification with alkoxyalkyl groups such as HDP or ODE. In the case of HPMPDAP, the ODE derivative has been shown to be a potent and selective inhibitor of herpes virus and orthopoxvirus replication.[21] In spite of that, the necessity of search for other drug candidates remains still timely.

In our approach, several ANPs of the (*S*)-HPMP series (HPMPA, HPMPC, HPMP-azaC, 3- and 7-deaza-HPMPA, HPMPDAP), (*R*)-HPMPO-DAPy, and the corresponding cyclic phosphonates were subjected to detailed in vitro and in vivo investigations. The selected drug candidate had to fulfill the following requirements: high inhibitory activity against poxviruses including drug-resistant virus mutants, low cytotoxicity, low potential for in vivo nephrotoxicity, and sufficient metabolic stability. Based on these criteria, (*S*)-HPMPDAP and its cyclic form were selected for further evaluation[22] including the synthesis and characterization of their structurally diverse ester prodrugs. These esters were subjected to a comparative study concerning their antiviral activity and selectivity.

Chemistry

The originally described synthesis of 9-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine (HPMPDAP, **1**) is an eight-step process based on nucleophilic opening of an oxirane ring in (*R*)-glycidol butyrate with 2,6-diaminopurine and introduction of a phosphonomethyl residue using diisopropyl tosyloxymethylphosphonate and is accompanied by several protection−deprotection steps with an overall yield of 11% of the final (*S*)-HPMPDAP (calculated to starting 2,6-diaminopurine).[12] For our purpose of further development of the compound including prodrugs for animal experiments, first of all we needed to optimize the synthesis of (*S*)-HPMPDAP.

The new approach is described in Scheme 1. At present, synthetic approaches started by nucleophilic opening of (2*S*)-2-[(trityloxy)methyl]oxirane (**2**) with nucleobases belong to the most utilized methodology in preparations of (*S*)-HPMP derivatives. In this case, the overall yield of **1** was 40% (calculated to starting 2,6-diaminopurine); most reaction steps can be performed without isolation and purification of intermediates (intermediates **4**−**6**). The method is appropriate especially for large-scale syntheses.

For an introduction of alkoxyalkyl groups, the starting **1** was transformed to its cyclic form **7** prepared from **1** in quantitative yield by its heating with *N*,*N*′-dicyclohexylcarbodiimide and *N*,*N*′-dicyclohexyl-4-morpholinecarboxamidine

in DMF. Alkylation of **7** with alkoxyalkyl bromides was performed via its tetrabutylammonium salt; the resulting alkoxyalkyl esters of cHPMPDAP **8** were used directly for anti-poxvirus screening or cleaved to the corresponding monoesters **9** (Scheme 2).

With respect to a generally high bioavailability and optimal pharmacokinetic properties, neutral diesters of HPMPDAP, e.g., bis(POM) derivatives, are expected to be optimal prodrugs. However, as we found, transformation to such type of esters is appropriate only for phosphonates lacking a free hydroxyl group in an aliphatic side chain, e.g., PMEA, which is transformed easily to bis(POM)PMEA[23] or (*R*)-PMPA (tenofovir) clinically used in the form of its bis(isopropyloxycarbonyloxymethyl) ester (bis(POC)-PMPA).[24] Unfortunately, this procedure is not possible to use in HPMP series where phosphonic acid diesters are not sufficiently chemically stable due to the presence of a neighboring hydroxyl function.

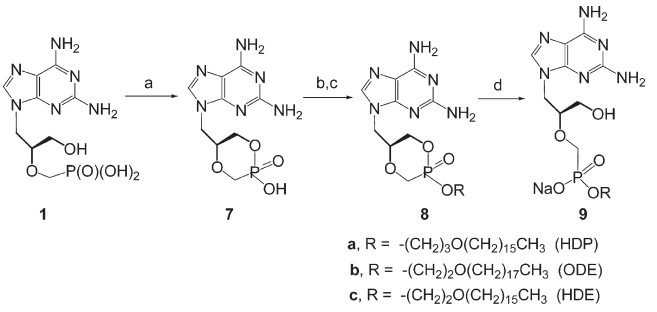
In the case of (*S*)-HPMPDAP, we successfully synthesized two types of POM esters: POM monoester **10** and POM ester of cyclic (*S*)-HPMPDAP **11** (Scheme 3). Transformation of **1** to the corresponding POM monoester was performed by the action of chloromethyl pivalate in DMF in the presence of *N*,*N*′-dicyclohexyl-4-morpholinecarboxamidine. Reaction conditions were optimized so as no cyclization or formation of decomposition products occurred. This procedure was found to be useful also for preparation of other POM HPMP monoesters (e.g., HPMPC).

Transformation of cyclic (*S*)-HPMPDAP to POM ester **11** was carried out by the treatment of its tetrabutylammonium salt with chloromethyl pivalate in refluxing dioxane.

The ratio of diastereoisomers in **11** was 6:1 in favor of the less polar *trans*-isomer; separation of diastereoisomers was performed by HPLC technique. Diastereoisomers were distinguished by their characteristic ^{31}P chemical shifts and by comparing H,H, H,P, and C3′,P coupling constants in chair conformation of the cyclic phosphonate esters that have characteristic values for each diastereoisomer. Determination of the relative configuration of cyclic phosphonate esters from NMR spectra (H,H-ROESY or comparison of ^{31}P (^{1}H dec) NMR spectra) is explained in detail in ref 25. Generally, ^{31}P nucleus in less polar *trans*-diastereoisomers resonates at higher magnetic field than in polar minor *cis*-diastereoisomers. Structures of both diastereoisomers are outlined in Figure 1. Structures of other diastereoisomeric cyclic phosphonates (**8a**−**c**) are analogous. Concerning absolute configuration, all *trans*-isomers have (*S*,*R*) configuration, *cis*-isomers have (*S*,*S*) configuration.

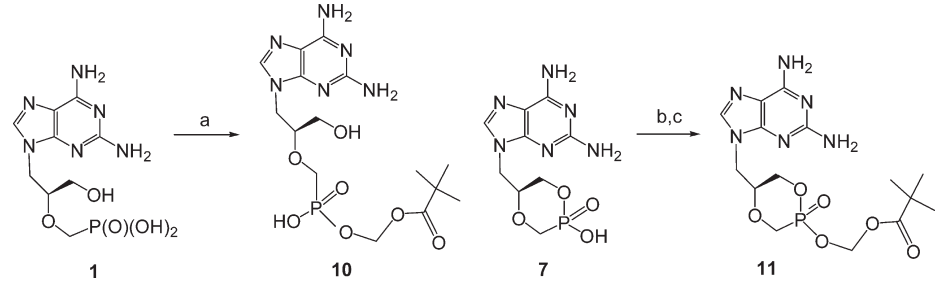
Another type of appropriate prodrug form for phosphonic acid based compounds is 2,2,2-trifluoroethyl esters. These esters are successfully used for example in development of effective anti-HBV agents, 2-amino-6-arylthio-9-[2-(phosphonomethoxy)ethyl]purines.[26,27] These phosphonates were transformed to the corresponding bis(2,2,2-trifluoroethyl) esters which were then partially hydrolyzed to the corresponding monoesters in plasma and liver. In our case of HPMPDAP, we made an effort to synthesize all three possible types of trifluoroethyl ester prodrugs: bis(2,2,2-trifluoroethyl) ester, a corresponding monoester, and 2,2,2-trifluoroethyl ester of cyclic HPMPDAP. Esterification of (*S*)-HPMPDAP and its cyclic form was performed with trifluoroethanol in the presence of ethyldiisopropylamine (Hunig's base) after activation of a phosphonic acid residue with some hexafluorophosphate condensation reagents. Such reagents, e.g., HATU, PyBOP, or PyBroP [bromotripyrrolidinophosphonium hexafluorophosphate], are currently used especially as coupling reagents in peptide chemistry.[28] Transformation of (*S*)-HPMPDAP and its cyclic form to the corresponding trifluoroethyl esters is outlined in Scheme 4. Esterification of **1** using PyBroP as coupling reagent afforded exclusively 2,2,2-trifluoroethyl monoester **12**; no formation of bis(trifluoroethyl) ester or ester of cyclic phosphonate was observed. Analogously, esterification of cyclic form **7** was performed successfully with trifluoroethanol after activation of a phosphonic acid residue with HATU, i.e., *O*-7-(azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate. The resulting cyclic phosphonate **13** was isolated and tested as a diastereoisomeric mixture. We also tried to find some alternative approaches to **13**, but these experiments were mostly unsuccessful (e.g., reaction of tetrabutylammonium salt of **7** with 2,2,2-trifluoroethyl iodide under conditions described for **8** or transformation of **7** to corresponding chloridate by the action of oxalyl chloride followed by reaction with trifluoroethanol).

Scheme 2[a]



[a]Conditions: (a) DCC, *N*,*N*-dicyclohexyl-4-morpholinecarboxamidine, DMF, 100 °C; (b) tetrabutylammonium hydroxide; (c) alkoxyalkyl bromide, DMF, 100−120 °C; (d) 2 M NaOH, 80 °C, 1 h.

Scheme 3[a]



[a]Conditions: (a) chloromethyl pivalate, *N*,*N*-dicyclohexyl-4-morpholinecarboxamidine, DMF, room temperature, 24 h; (b) tetrabutylammonium hydroxide; (c) chloromethyl pivalate, dioxane, reflux 3 h.

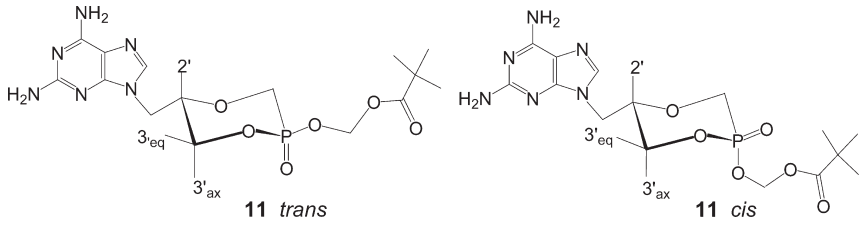
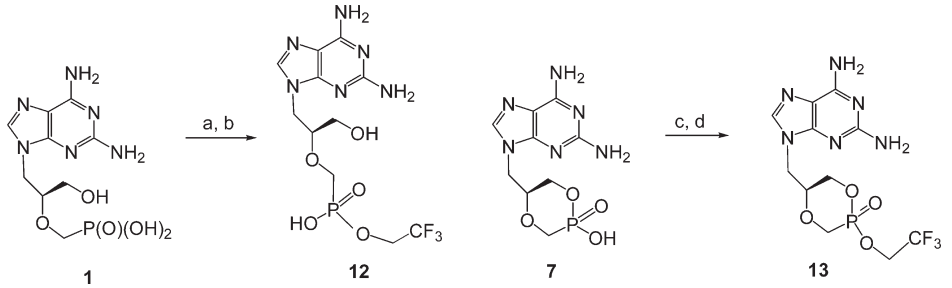


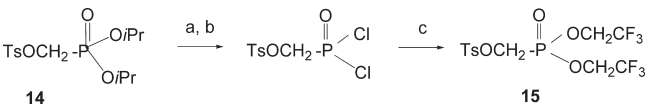
Figure 1

Scheme 4[a]



[a]Conditions: (a) PyBroP, DMF, ethyldiisopropylamine, room temperature; (b) CF_3CH_2OH, room temperature → 90 °C in 3 h; (c) HATU, ethyldiisopropylamine, room temperature; (d) CF_3CH_2OH, room temperature → 90 °C, 3 h.

Scheme 5[a]

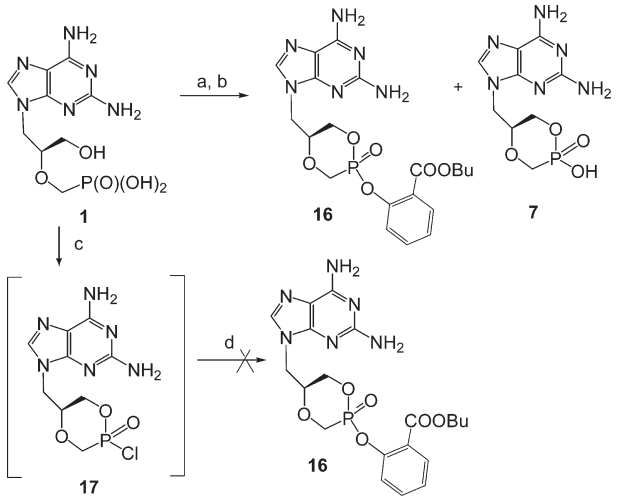


[a]Conditions: (a) $(CH_3)_3SiBr$, CH_3CN, room temperature; (b) oxalyl chloride, dichloromethane (+DMF); (c) CF_3CH_2OH, dichloromethane, Et_3N.

Some attempts to bis(trifluoroethyl) esters were also studied. Regarding the fact that direct esterification of **1** with trifluoroethanol afforded monoester **12** only, we decided to work out preparation of bis(trifluoroethyl) tosyloxymethylphosphonate (**15**) as alkylating reagent for direct phosphonylation of **3** or **4**, respectively. So far, it revealed that such reactions would require special (milder) conditions compared to an analogous process with diisopropyl tosyloxymethylphosphonate (**14**) often used for preparation of ANPs. Such reactions are usually carried out in strongly alkaline conditions[12] (sodium hydride), generally not appropriate for fluorinated compounds. Utilization of synthon **15** for preparations of bis(trifluoroethyl) esters is currently intensively studied. Its synthesis from diisopropyl tosyloxymethylphosphonate is described in Scheme 5.

Another type of phosphonate ester prodrugs is aryl esters: phenyl and its substituted analogues. In a study in PME series (adefovir, anti-hepatitis B agent), these esters [e.g., bis(2-ethoxyphenyl)PMEA] revealed very good oral bioavailability together with sufficient stability in intestinal homogenate and plasma and enzymatic degradation to the parent compound in liver homogenate.[29,30] In the HPMP series aryl esters were developed for the cyclic form of HPMPC (cidofovir). Besides simple phenyl ester and its substituted analogues a large series of alkyl salicylate esters was also developed as a very promising group of prodrugs with a high bioavailability.[31,32] Variations of alkyls on a carboxylic function allow resulting modification of lipophilicity, solubility, and enzymatic stability of the prodrug. Physicochemical properties and stability of salicylates of cyclic HPMPC are strongly dependent on

Scheme 6[a]



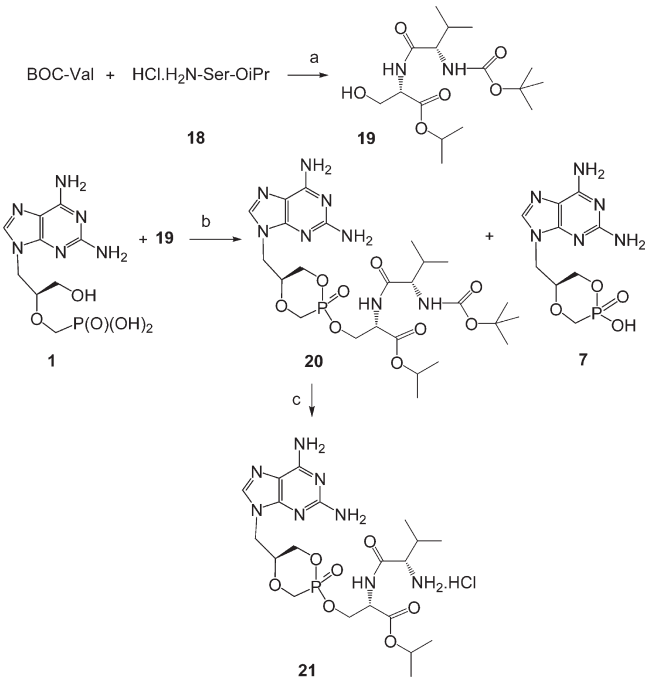
[a]Conditions: (a) PyBOP, DMF, ethyldiisopropylamine, sonication, room temperature; (b) butyl salicylate, room temperature in 6 days, sonication; (c) $[CH(Cl)=N^+(Me)_2]Cl^-$, DMF; room temperature → 100 °C; (d) sodium salt of butyl salicylate, room temperature → 100 °C.

stereochemistry on a phosphorus atom: *trans* (equatorial) isomers displayed poor chemical stability; for further biological evaluation *cis*-isomers were preferable. As to oral bioavailability, the best results were found in the case of the butylsalicylyl ester.[32] This type of salicylyl ester was also selected for preparation of another structural type of HPMPDAP prodrug, compound **16**. Unfortunately, synthesis of **16** according to an original procedure described for cidofovir[31] (reaction of free HPMP derivative with Vilsmeier reagent followed by the action of sodium salt of butyl salicylate) repeatedly failed. As expected, activation and subsequent cyclization of a phosphonate moiety into the intermediate **17** otherwise successfully proceeded (Scheme 6). However, reactivity of thus formed phosphonate with salicylate sodium salt was probably insufficient as observed by TLC monitoring

of the reaction mixture. Therefore, we developed an alternative procedure using PyBOP as a more suitable mild coupling agent (see Scheme 6). In the presence of Hunig's base and butyl salicylate, the phosphonate **1** primarily cyclized into **7**. After a few days of stirring at room temperature and sonication of the reaction mixture, a small quantity of the favored cyclic butyl salicylyl ester **16** was finally observed and separated while the unreacted cyclic HPMPDAP (**7**) could be recovered for further utilization.

Special types of prodrugs represent esters of amino acids, peptides, and peptidomimetics. Their development was initiated with the aim to find prodrugs releasing in target tissue an active compound together with a completely nontoxic and natural accompanying material as byproduct. One of the first examples of such approach was the development of valacyclovir, an L-valine ester of acyclovir.[33,34] Development of orally active peptide drugs is limited by their unfavorable physicochemical properties (size, charge, hydrogen bond formation) preventing them from permeation through a cell membrane as well as their instability toward enzymes present in intestinal mucosa. However, appropriate structural modifications of peptides to form peptidomimetics open the way to circumvent the metabolic enzymes. Modifications increasing the hydrophobicity but simultaneously decreasing the hydrogen-bonding potential are promising strategies for improving the oral bioavailability of peptide drugs.[35] In the field of ANPs, successful utilization of peptidomimetics was described by McKenna's group for design of prodrugs of cyclic cidofovir. The original strategy, based on ethylene glycol-linked amino acid conjugates, was replaced later by esterification of cCDV with a side-chain hydroxyl group of some serine-containing dipeptides.[36,37] Promising pharmacokinetic properties were found especially in the case of Val-Ser dipeptide with a valine carboxyl function esterified by an isopropyl group: Val-Ser-COOiPr cHPMPC.[36] Therefore, this peptidomimetic group was selected finally for esterification of HPMPDAP to prepare additional types of HPMPDAP prodrugs for anti-poxvirus screening. The synthesis was performed employing above-described conditions using PyBOP, Hunig's base, and protected Val-Ser block possessing a free hydroxyl group. The dipeptide moiety was prepared from Boc-L-valine and L-serine isopropyl ester hydrochloride using carbodiimide-mediated coupling. The Boc group of the conjugate **19** was then cleaved by the action of hydrogen chloride in dioxane (Scheme 7).

Scheme 7[a]



[a] Conditions: (a) EDC, HOBt, Et_3N, CH_2Cl_2, room temperature; (b) PyBOP, iPr_2EtN, DMF, room temperature in 2 days, sonication; (c) HCl/dioxane, room temperature.

Biological Activity

The antiviral activity of the different compounds was evaluated against various DNA viruses, including poxviruses [i.e., vaccinia virus (VACV)] and herpes viruses [i.e., herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), thymidine kinase-deficient HSV-1 (acyclovir-resistant, ACV^r), varicella-zoster virus (VZV), human cytomegalovirus (HCMV), and human herpes virus 6 (HHV-6)] (Tables 1 and 2). Some of the compounds were also evaluated against retroviruses [i.e., human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2)]. All compounds were also examined against several RNA viruses, including vesicular stomatitis virus, Coxsackie B4, respiratory syncytial virus (RSV), parainfluenza virus type 3, reovirus-1, Sindbis virus, and Punta Toro virus. None of the compounds showed activity against any of the RNA viruses tested at nontoxic concentrations (data not shown).

(*S*)-HPMPDAP and its cyclic form inhibited VACV replication with EC_{50} values that were ~10-fold lower than those observed for (*S*)-HPMPC (cidofovir), while they did not affect cell morphology or growth of HEL cells measured as respectively the minimum cytotoxic concentration (MCC) or the 50% cytostatic concentration (CC_{50}), up to a concentration of >314 μM ((*S*)-HPMPDAP) or >333 μM (cyclic-(*S*)-HPMPDAP), the highest concentrations tested (Table 1). In the case of HSV-1, HSV-1 ACV^r, HSV-2, and VZV, EC_{50} values for (*S*)-HPMPC, (*S*)-HPMPDAP, and its cyclic form were similar (Table 2). In contrast, (*S*)-HPMPC was more inhibitory toward HCMV than (*S*)-HPMPDAP or its cyclic form (Table 1).

The ester prodrugs of (*S*)-HPMPDAP, i.e., hexadecyloxypropyl (compound **9a**), octadecyloxyethyl (compound **9b**), and hexadecyloxyethyl (compound **9c**) derivatives, emerged as the most potent and selective compounds against VACV with EC_{50} values consistently in the range of 0.00074−0.0012 μM (Table 1). Although the ester prodrugs of (*S*)-HPMPDAP proved more toxic than the parent compound, either for HEL cell morphology or HEL cell growth (except for compound **9a** that had a $CC_{50} \geq 161$ μM), they were more selective than (*S*)-HPMPDAP, with selectivity indices (ratio = CC_{50}/EC_{50}) higher than 10000 compared to >625 for (*S*)-HPMPDAP. The ester prodrugs of the cyclic form of (*S*)-HPMPDAP [i.e., compounds **8a**, **8b** (*cis* and *trans*), and **8c** (*cis* and *trans*)] inhibited VACV replication with EC_{50} values that were 10-fold (compound **8b**-*trans*), 48-fold (compound **8a**), 100-fold (compound **8b**-*cis*), and 100−200-fold (compound **8c**-*cis* and -*trans*) lower than that of cyclic (*S*)-HPMPDAP (Table 1). The EC_{50}'s obtained for the alkoxyalkyl ester prodrugs of cyclic (*S*)-HPMPDAP were about 3−70-fold higher against vaccinia virus than the corresponding ester prodrugs of (*S*)-HPMPDAP, and they had selectivity indices of 1200−8000. In a previous study, Valieaeva and collaborators reported the antiviral activity of different ODE esters of (*S*)-3-hydroxy-2-(phosphonomethoxy)propyl nucleosides against herpes

Table 1. Antiviral and Cytotoxic Activity of the Compounds against Vaccinia Virus (VACV) and β-Herpes Viruses in Cell Culture

		antiviral activity: EC_{50} (μM)[a]				cytotoxicity (μM)		
		poxvirus	HCMV		HHV-6	cell morphology (MCC)[b]		cell growth (CC_{50})[c]
compd		vaccinia (VACV) (HEL)	AD-169 (HEL)	Davis (HEL)	HHV-6A (HSB-2)	HEL	HSB-2	HEL
8a	HDP, cyclic (diastereoisomeric mixture)	0.011 ± 0.12	0.034 ± 0.017	0.021 ± 0.019	0.16	6.9	8.6	13.7
8b (*trans*)	ODE, cyclic (*trans*-isomer)	0.052 ± 0.067	0.015 ± 0.010	0.0034	> 42 ± 0	6.7	0.5	129
8b (*cis*)	ODE, cyclic (*cis*-isomer)	0.005 ± 0.009	0.027 ± 0.010	0.008 ± 0.011	> 42 ± 0	6.7	0.5	15.1
8c (*trans*)	HDE, cyclic (*trans*-isomer)	0.0035 ± 0.0032	0.015 ± 0.004	0.008 ± 0.006	> 44 ± 0	35.2	0.5	28.1
8c (*cis*)	HDE, cyclic (*cis*-isomer)	0.0026 ± 0.0026	0.011 ± 0.007	0.005 ± 0.002	> 44 ± 0	35.2	0.5	30
9a	HDP, monoester	0.00074 ± 0.00058	0.021 ± 0.018	0.0037 ± 0.0016	10.3 ± 5.6	≥ 6.4	40	≥ 161
9b	ODE, monoester	0.00074 ± 0.00039	0.041 ± 0.013	0.0094 ± 0.0043	34.6 ± 8.3	≥ 6.3	≥ 39.3	18.84
9c	HDE, monoester	0.0012 ± 0.0013	0.0076 ± 0.0059	0.0021 ± 0.0018	> 41 ± 0	33	0.5	23
10	POM, monoester	1.63 ± 1.50	12.9 ± 7.1	11.6 ± 6.3	30.6 ± 14.1	> 227	227	≥ 227
11	POM, cyclic (diastereoisomeric mixture)	0.183 ± 0.037	6.94 ± 3.26	6.80 ± 0.74	7.63 ± 6.01	≥ 231	62.5	≥ 201
11 (*trans*)	POM, cyclic (*trans*-isomer)	0.37 ± 0.19	14.0 ± 3.4	15.5 ± 1.3	14.6 ± 12.3	≥ 231	64.7	> 222
12	trifluoroethyl, monoester	7.95 ± 2.30	44.0 ± 26.7	54.2 ± 18.7	> 62.0 ± 0	> 250	> 62	> 250
13	trifluoroethyl, cyclic (diastereoisomeric mixture)	2.08 ± 1.58	19.1 ± 6.2	24.4 ± 5.0	177	> 239	> 239	≥ 219
16	butylsalicylyl ester, cyclic (diastereoisomeric mixture)	2.30 ± 1.48	11.5 ± 6.8	6.8 ± 2.5	> 205	103	≥ 205	168
21 (*trans*)	peptidomimetics, cyclic (*trans*-isomer)	2.02 ± 1.22	12.0 ± 2.8	7.1	> 177	≥ 177	177	159
21 (*cis*)	peptidomimetics, cyclic (*cis*-isomer)	2.62 ± 1.31	15.2 ± 8.5	22.1 ± 18.8	> 177	> 177	> 177	148
	(*S*)-HPMPDAP	0.50 ± 0.19	11.2 ± 6.4	6.4 ± 0.2	17.6 ± 3.7	> 314	126	> 314
	cyclic-(*S*)-HPMPDAP	0.53 ± 0.20	15.0 ± 2.4	10.0 ± 0.47	ND[d]	> 333	ND	> 333
	(*S*)-HPMPC	7.3 ± 4.3	1.14 ± 0.19	0.79 ± 0	ND	≥ 317	ND	511
	ganciclovir	ND	9.4 ± 2.7	10.2 ± 3.5	ND	≥ 392	ND	1077

[a] Effective concentration (expressed in μM) required to reduce virus-induced cytopathicity by 50%. [b] Minimum cytotoxic concentration (expresed in μM) that causes a microscopically detectable alteration of cell morphology. [c] Cytotoxic concentration (expressed in μM) required to reduce cell growth by 50%. [d] Not determined.

Table 2. Antiviral and Cytotoxic Activity of the Compounds against α-Herpes Viruses in Cell Culture

		antiviral activity: EC_{50} (μM)[a]					cytotoxicity (μM)	
		HSV-1		HSV-2	VZV		cell morphology (MCC)[b]	cell growth (CC_{50})[c]
compd		KOS (HEL)	KOS ACV (HEL)	G strain (HEL)	OKA (HEL)	07/1 (HEL)	HEL	HEL
8a	HDP, cyclic (diastereoisomeric mixture)	0.031 ± 0.038	0.053 ± 0.058	0.053 ± 0.079	0.012 ± 0.002	0.0017 ± 0.0017	6.9	13.7
8b (*trans*)	ODE, cyclic (*trans*-isomer)	0.030 ± 0.028	0.017 ± 0.012	0.037 ± 0.027	0.045 ± 0.057	0.010 ± 0.013	6.7	129
8b (*cis*)	ODE, cyclic (*cis*-isomer)	0.013 ± 0.020	0.007 ± 0.008	0.011 ± 0.016	0.0047 ± 0.0008	0.0007 ± 0.0005	6.7	15.1
8c (*trans*)	HDE, cyclic (*trans*-isomer)	0.026 ± 0.033	0.017 ± 0.015	0.026 ± 0.032	0.004 ± 0.0005	0.0007 ± 0.0005	35.2	28.1
8c (*cis*)	HDE, cyclic (*cis*-isomer)	0.010 ± 0.009	0.007 ± 0.006	0.008 ± 0.009	0.0018 ± 0.0002	0.00018 ± 0	35.2	30
9a	HDP, monoester	0.0045 ± 0.0064	0.0027 ± 0.0035	0.0071 ± 0.011	0.0027 ± 0.0008	0.00021 ± 0.00008	≥6.4	>161
9b	ODE, monoester	0.0008 ± 0.00044	0.00085 ± 0.00083	0.0022 ± 0.0019	0.0013 ± 0.0009	0.00008 ± 0.00003	≥6.3	18.8
9c	HDE, monoester	0.0036 ± 0.0041	0.0023 ± 0.0018	0.0039 ± 0.0041	0.0014 ± 0.0001	0.00008 ± 0.00003	33	23
10	POM, monoester	2.76 ± 1.29	2.95 ± 2.68	3.15 ± 1.74	0.79 ± 0.77	0.34 ± 0.41	>227	≥227
11	POM, cyclic (diastereoisomeric mixture)	2.22 ± 1.64	2.41 ± 2.15	1.34 ± 0.67	0.30 ± 0.12	0.093 ± 0.046	≥231	≥201
11 (*trans*)	POM, cyclic (*trans*-isomer)	1.83 ± 0.95	3.33 ± 2.36	1.78 ± 0.60	0.72 ± 0.02	0.16 ± 0.02	≥231	>222
12	trifluoroethyl, monoester	5.60 ± 4.55	5.0 ± 2.6	10.7 ± 8.5	2.27 ± 1.05	0.43 ± 0.05	>250	>250
13	trifluoroethyl, cyclic (diastereoisomeric mixture)	6.57 ± 4.23	4.78 ± 0	1.91 ± 0	0.62 ± 0.57	0.17 ± 0.10	>239	≥219
16	butylsalicylyl ester, cyclic (diastereoisomeric mixture)	5.36 ± 4.09	3.10 ± 1.13	3.76 ± 0.57	0.53 ± 0.25	0.12 ± 0.10	103	168
21 (*trans*)	peptidomimetics, cyclic (*trans*-isomer)	5.58 ± 2.12	3.52 ± 0	2.30 ± 1.26	0.73 ± 0.60	0.20 ± 0.12	≥177	159
21 (*cis*)	peptidomimetics, cyclic (*cis*-isomer)	7.97 ± 6.27	7.1 ± 0	1.42 ± 0	1.06 ± 0.71	0.25 ± 0.20	>177	148
	(*S*)-HPMPDAP	2.86 ± 1.76	1.35 ± 0.88	2.83 ± 1.35	0.22 ± 7.68	0.0082 ± 0.0025	>314	>314
	cyclic-(*S*)-HPMPDAP	3.60 ± 1.77	3.60 ± 4.90	4.33 ± 4.03	0.37 ± 0.53	0.77 ± 0.043	>333	>333
	(*S*)-HPMPC	1.62 ± 1.08	1.37 ± 0.98	2.19 ± 1.46	0.13 ± 0.13	0.11 ± 0.09	≥317	511
	acyclovir	0.16 ± 0.05	125 ± 73	0.30 ± 0.09	3.69 ± 1.69	60.4 ± 36.4	>444	1576
	brivudin	0.030 ± 0.009	>150 ± 0	75.3 ± 49.8	0.016 ± 0.010	137 ± 115	>300	339

[a] Effective concentration (expressed in μM) required to reduce virus-induced cytopathicity by 50%. [b] Minimum cytotoxic concentration (expresed in μM) that causes a microscopically detectable alteration of cell morphology. [c] Cytotoxic concentration (expressed in μM) required to reduce cell growth by 50%.

virus and orthopoxviruses, emerging ODE-(*S*)-HPMPDAP as the most selective compound (selectivity index = 3500) against VACV.[38] In this study, the authors reported EC_{50} values for ODE-(*S*)-HPMPDAP of 0.02, 0.03, and 21.2 μM, for respectively VACV, cowpox virus, and ectromelia virus, resulting in selectivity indices in the range of 100−3500 [SI = 104 (ectromelia virus), 2330 (cowpox virus), and 3500 (VACV). Assays for VACV and cowpox virus were performed in human foreskin fibroblasts while for ectromelia virus they were done in the monkey cells (i.e., BSC-1 cells).

The alkoxyalkyl ester prodrugs of (*S*)-HPMPDAP and its cyclic form proved also remarkably potent and selective against HSV-1, HSV-2, HSV-1 ACV^r, VZV, and HCMV, with EC_{50} values in the range of 0.007−0.0008 μM (Tables 1 and 2). Lower activities of the alkoxyalkyl ester prodrugs of cyclic (*S*)-HPMPDAP than of the corresponding alkoxyalkyl ester prodrugs of (*S*)-HPMPDAP were also observed against HSV and VZV (Table 2). The alkoxyalkyl ester prodrugs also showed potent activity against HCMV, with EC_{50} values at least 225-fold lower than those for the corresponding parent compound (Table 1). The alkoxyalkyl ester prodrugs proved rather toxic against the lymphoblast HSB-2 cells, and only compounds **8a** and **9a**, the HDP derivatives of (*S*)-HPMPDAP and cyclic (*S*)-HPMPDAP, respectively, showed selective activity against HHV-6 at nontoxic concentrations (Table 1).

Although the POM (compounds **10** and **11**-*cis* and -*trans*), 2,2,2-trifluoroethyl (compounds **12** and **13**), and butylsalicylyl (compound **16**) derivatives, and prodrugs based on peptidomimetics (compound **21**-*cis* and -*trans*), proved less active than the alkoxyalkyl ester prodrugs, they appeared to be less cytotoxic and cytostatic (Tables 1 and 2). In contrast to alkoxyalkyl ester prodrugs that affected cell morphology in the concentration range of 0.5−40 μM (HSB-2 cells) and 6−40 μM (HEL cells), the POM, 2,2,2-trifluoroethyl, butylsalicylyl, and peptidomimetic prodrugs showed MCC values of ≥60 μM (HSB-2 cells) and ≥100 μM (HEL cells). The POM derivate of cyclic (*S*)-HPMPDAP (compound **11**), either the diastereoisomeric mixture or the *trans*-isomer, was able to inhibit VACV replication with EC_{50} values similar to those observed with the parent compounds, while compounds **10**, **12**, **13**, and **21** showed EC_{50} values 4−16-fold higher than the parent compounds (Table 1). The POM, 2,2,2-trifluoroethyl, and butylsalicylyl derivatives and prodrugs based on peptidomimetics proved also active against HSV, VZV, and HCMV, with EC_{50}'s similar or 10-fold higher than those of the parent drugs (Tables 1 and 2). Compounds **10** and **11** also displayed potent and selective activity against HHV-6 (Table 1). The EC_{50} values and the selectivity indices for these compounds against HHV-6 were equivalent to those observed for (*S*)-HPMPDAP.

Several compounds showed activity against HIV-1 and HIV-2 although with somewhat narrow selectivity (i.e., **11** (EC_{50}, 7.4−17.6 μM; CC_{50}, 95 μM), **11** (*trans*) (EC_{50}, 30−32 μM; CC_{50}, 109 μM), and **21** (*cis*) (EC_{50}, 70−138 μM; CC_{50}, > 177 μM)) at subtoxic concentrations. It was generally assumed that HPMP derivatives were unable to inhibit HIV in cell culture. However, recent reports have shown that ODE-(S)-HPMPA and ODE-(*S*)-HPMPDAP can inhibit HIV replication in vitro.[39,40] Probably the increased uptake of prodrugs into the target cells may eventually release higher levels of the active metabolites than those that can be achieved by administration of parent drug, resulting in inhibition of the HIV reverse transcriptase and/or incorporation into the viral DNA. It should be noted, however, that the activities of POM derivatives of (*S*)-HPMPDAP and of its cyclic form are substantially weaker compared to similar prodrugs of other acyclic nucleoside phosphonates such as adefovir (i.e., adefovir dipivoxil) and tenofovir (i.e., tenofovir disoproxil fumarate). On the other hand, the HDP ester of tenofovir (CMX-157), reported to be 267-fold more active than tenofovir against HIV-1 and HIV-2,[41] appears to be significantly more active than the current compounds **11**, **11** (*trans*), and **21**.

Conclusion

In conclusion, a series of ester prodrugs of (*S*)-HPMPDAP (**1**) was synthesized for further evaluation against a variety of viruses, in particular VACV, and a general methodology for their preparation was worked out. Besides standard synthetic approaches, special attention was paid also to utilization of hexafluorophosphate coupling reagents for the synthesis of some ANP ester prodrugs. The most active prodrugs against VACV, HSV, VZV, and HCMV were the alkoxyalkyl ester derivatives of HPMPDAP and its cyclic form. Prodrugs based on peptidomimetics, the 2,2,2-trifluoroethyl, the butylsalicylyl, and the POM prodrugs were able to inhibit VACV replication with 50% inhibitory concentrations that were equivalent or ∼10-fold higher than those observed for the parent compounds. The different prodrugs displayed potent and selective activity against different herpesviruses (i.e., HSV, VZV, and HCMV), although only the POM and HDP ester prodrugs were able to selectively inhibit the replication of HHV-6. The 2,2,2-trifluoroethyl, the butylsalicylyl, the pivaloyloxymethyl, and peptidomimetic prodrugs were less potent but also less toxic than the alkoxyalkyl ester produgs, as measured by alteration of cell morphology or cell growth. In conclusion, the alkoxyalkyl and POM prodrugs of both HPMPDAP and its cyclic form are promising candidates for further exploration as candidates for anti-poxvirus therapy.

Experimental Section

Unless stated otherwise, solvents were evaporated at 40 °C/2 kPa, and compounds were dried at 13 Pa. Purification of products (except **16**) by reverse-phase HPLC technique was performed on a Waters Delta 600 instrument with a Waters 2487 dual λ absorbance detector using preparative columns, Luna Phenomenex C-18 (10 μm, 21 × 250 mm, flow 12 mL/min). HPLC purification of **16** was performed on a column 300 × 50 mm; RP C_{18} TESEC; flow rate 45 mL/min. Solvent systems are given in the text. Column chromatography was performed on silica gel 60 μm (Fluka). TLC was performed on silica gel 60 F_{254} plates (Merck KGaA, Darmstadt, Germany). ^{1}H and ^{13}C NMR spectra were measured on a Bruker Avance 600 spectrometer (^{1}H at 600 MHz, ^{13}C at 151 MHz) or Bruker Avance 500 spectrometer (^{1}H at 500 MHz, ^{13}C at 125.7 MHz). ^{31}P NMR spectra were measured on a Bruker Avance 500 spectrometer (202.3 MHz) in $CDCl_3$ using H_3PO_4 as external standard. Mass spectra were measured on a ZAB-EQ (VG Analytical) spectrometer using FAB (ionization with xenon, accelerating voltage 8 kV, glycerol matrix) or by ESI technique. The purity of compounds was ≥95%, and it was determined from elemental analyses (except hygroscopic materials **20** and **21** which purity was determined by HPLC technique using above-mentioned Waters Delta 600 instrument, an analytical column, Nova Pack C18, 3.9 × 150 mm, with flow rate 1 mL/min and solvent system methanol−phosphate buffer).

Materials and Solvents. Most of the chemicals and ion-exchange resins (Dowex 1X2−400) were purchased from Sigma-Aldrich (Czech Republic). (2*S*)-2-[(trityloxy)methyl]oxirane (**2**)

was purchased from DAISO Co. Ltd. (Japan). Dimethylformamide and dioxane were dried by distillation from CaH_2 (DMF in vacuo) and stored over molecular sieves, 4 Å (DMF), or sodium (dioxane). Alkoxyalkyl bromides were prepared from the corresponding alcohols by the action of carbon tetrabromide and triphenylphosphine.[18a,42]

9-(*S*)-[3-Hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine (1). Cesium carbonate (1.3 g) was added to a stirred mixture of 2,6-diaminopurine (8.46 g, 56.3 mmol) and (*S*)-tritylglycidol (**2**, 17.8 g, 56.3 mmol) in DMF (110 mL) at 100 °C, and the heating was continued for additional 8 h. The mixture was evaporated and the residue coevaporated with toluene and chromatographed on a column of silica gel (900 mL) in system chloroform−methanol (9:1) to give 24 g (91%) of compound **3** as a yellowish foam. This intermediate was dissolved in chloroform (130 mL) and treated with dimethylformamide dimethylacetal (50 mL) at 25 °C for 24 h, and the solution was then evaporated and dried in vacuo. DMF (200 mL) was added, followed by 60% oil suspension of NaH (4 g, 100 mmol) and $BrCH_2P(O)(OiPr)_2$ (14.8 g, 57 mmol). After being stirred at 25 °C for 24 h, the mixture was neutralized with acetic acid to pH 7 and evaporated and the residue coevaporated with toluene. A mixture of methanol (250 mL) and 25% aqueous ammonia (65 mL) was added and the solution set aside for 24 h at room temperature and evaporated. The residue was refluxed with 80% acetic acid (270 mL) for 1 h, then evaporated, and partitioned between water (800 mL) and ether (400 mL). An aqueous layer was concentrated to approximately 200 mL and applied onto a column of Dowex 50 (H^+ form, 300 mL) and elution performed by aqueous ammonia (1 L), followed by water (1 L) and 2.5% aqueous ammonia. Product containing ammonia fractions was evaporated and dried in vacuo. Acetonitrile (250 mL) was added, followed by bromotrimethylsilane (40 mL), and the mixture was stirred in the dark for 24 h. After evaporation, the mixture of 50% aqueous ethanol and triethylamine (20:1) was added to neutral reaction, the mixture evaporated, and the residue crystallized from water. The solid product **1** was filtered off, washed with water, followed by acetone and ether, and dried in vacuo. Mother liquors were desalted on Dowex 50 (H^+ form, 150 mL) as described above; UV absorbing ammonia fractions were evaporated, and the residue was dissolved in water and applied onto a column of Dowex 1 (acetate form, 100 mL). Elution was performed with a linear gradient of acetic acid (0−1 M, 2 L), and compound **1** was eluted with 0.5 M acetic acid. Appropriate fractions were evaporated and coevaporated with water (3 × 50 mL), and the product was crystallized from water. Overall yield: 7 g (39%). Spectral data are in agreement with literature.[12]

9-{[(5*S*)-2-Hydroxy-2-oxido-1,4,2-dioxaphosphinan-5-yl]methyl}-2,6-diaminopurine (7). A mixture of **1** (4.8 g, 15 mmol), *N*,*N*′-dicyclohexyl-4-morpholinecarboxamidine (4.7 g, 16 mmol), and DCC (3.5 g, 17 mmol) in DMF (50 mL) was heated at 100 °C for 3 h. After being cooled to room temperature, the solid was filtered off and washed with water, and combined filtrates were applied onto a column of Dowex 1 (acetate form, 150 mL). Elution was performed with methanol (2 L), followed by water (1 L) and a linear gradient of acetic acid (0−0.5 M, 2 L). Elution of **7** started at 0.2 M AcOH. Product containing fractions were evaporated, and the residue was coevaporated with water (3 × 100 mL) and finally recrystallized from water. Yield: 3.2 g (71%) of *trans*-isomer. White crystals. Mp >297 °C (dec). ^{1}H NMR (500.0 MHz, D_2O): 3.75 (dd, 1H, J_{gem} = 14.1, $J_{H,P}$ = 2.2, CH_aH_bP), 3.99 (dd, 1H, J_{gem} = 14.1, $J_{H,P}$ = 8.8, CH_aH_bP), 4.12 (m, 3H, H-2′, H-3′), 4.23 and 4.32 (2 × m, 2 × 1H, H-1′), 7.78 (s, 1H, H-8). ESIMS: 323 $(M + Na)^+$ (10), 301.1 $(MH)^+$. HRMS (ESI): for $C_9H_{14}N_6O_4P$ $(MH)^+$ calculated, 301.0809; found, 301.0816.

Transformation of 7 to Cyclic Phosphonate Esters. General Procedure. One molar methanolic tetrabutylammonium hydroxide (3 mL, 3 mmol) was added to a solution of **7** (900 mg, 3.0 mmol) in absolute methanol (100 mL) and the mixture stirred for 10 min in an ultrasound bath and evaporated. The residue was coevaporated with toluene (50 mL), dissolved in appropriate solvent (20 mL, DMF for alkoxyalkyl esters or dioxane for POM esters), and stirred with alkoxyalkyl bromide (8 mmol) at 100−115 °C for 3 h or refluxed with chloromethyl pivalate (5 mmol) for 3 h (TLC control). The reaction mixture was cooled to room temperature, diluted with methanol (2 mL), and evaporated. The residue was chromatographed on a column of silica gel (50 mL) in system chloroform−methanol (85:15). To remove the rest of tetrabutylammonium salts, final purification of products was performed by crystallization or by HPLC technique.

3-(Hexadecyloxy)propyl Ester of 9-{[(5*S*)-2-Hydroxy-2-oxido-1,4,2-dioxaphosphinan-5-yl]methyl}-2,6-diaminopurine (8a). Crystallization from acetone afforded 600 mg (33%) of **8a** as a diastereoisomeric mixture (*trans*:*cis*, 1.34:1.0). Product contained in mother liquors was additionally purified by preparative reverse-phase HPLC: gradient 50−100% methanol in 25 min eluted impurities and tetrabutylammonium salts, elution with 100% methanol afforded **8a**-*trans* (150 mg), followed by **8a**-*cis* (50 mg). Overall yield: 800 mg (46%) of a white solid. ^{1}H NMR (500.0 MHz, $CDCl_3$) for *trans*-isomer: 0.87 (t, 3H, J_{vic} = 7.0, CH_3), 1.28 (m, 26H, CH_2), 1.54 (m, 2H, CH_2), 1.97 (p, 2H, J_{vic} = 6.2, CH_2), 3.38 (m, 2H, OCH_2), 3.48 (t, 2H, J_{vic} = 6.1, OCH_2), 3.91 (dd, J_{gem} = 14.8, $J_{H,P}$ = 0.5, CH_aH_bP), 3.99 (dd, 1H, J_{gem} = 14.8, $J_{H,P}$ = 6.9, CH_aH_bP), 4.08 (m, 1H, H-2′), 4.28−4.13 (m, 4H, H-3′ and P-OCH_2), 4.38 (m, 2H, H-1′), 5.10 (br s, 2H, NH_2-2), 6.10 (br s, 2H, NH_2-6), 7.58 (s, 1H, H-8). ^{1}H NMR (500.0 MHz, $CDCl_3$) for *cis*-isomer: 0.88 (t, 3H, J_{vic} = 7.0, CH_3), 1.26 (m, 26H, CH_2), 1.54 (m, 2H, CH_2), 1.94 (p, 2H, J_{vic} = 6.2, CH_2), 3.38 (t, 2H, J_{vic} = 6.7, OCH_2), 3.48 (t, 2H, J_{vic} = 6.1, OCH_2), 3.86 (dd, 1H, J_{gem} = 14.3, $J_{P,H}$ = 3.3, CH_aCH_bP), 4.12 (dd, 1H, J_{gem} = 14.3, $J_{P,H}$ = 0.9, CH_aCH_bP), 4.13 (m, 1H, H-2′), 4.28−4.18 (m, 4H, H-3′ and P-OCH_2), 4.42 (m, 2H, H-1′), 4.76 (br s, 2H, NH_2), 5.53 (br s, 2H, NH_2), 7.56 (s, 1H, H-8). ^{31}P (^{1}H dec) NMR (202.3 MHz, $CDCl_3$): 11.08 (*trans*), 12.79 (*cis*). Anal. ($C_{28}H_{51}N_6O_5P$) C, H, N, P. FABMS: 583 $(MH)^+$ (65). HRMS (FAB): for $C_{28}H_{52}N_6O_5P$ (MH^+) calculated, 583.3737; found, 583.3746.

2-(Octadecyloxy)ethyl Ester of 9-{[(5*S*)-2-Hydroxy-2-oxido-1,4,2-dioxaphosphinan-5-yl]methyl}-2,6-diaminopurine (8b). Overall yield: 800 mg (45%, *trans*:*cis*, 3:1). Separation of diastereoisomers by column chromatography afforded 600 mg (34%) of **8b**-*trans* (mp 100−104 °C, methanol) and 200 mg (11%) of **8b**-*cis* (mp 110−114 °C, methanol). Anal. ($C_{29}H_{53}N_6O_5P$) C, H, N, P. ESIMS(−): 595 $(M - H)^-$ (100). HRMS (+ESI): for $C_{29}H_{54}N_6O_5P$ $(MH)^+$ calculated, 597.3888; found, 597.3876.

2-(Hexadecyloxy)ethyl Ester of 9-{[(5*S*)-2-Hydroxy-2-oxido-1,4,2-dioxaphosphinan-5-yl]methyl}-2,6-diaminopurine (8c). Yield after column chromatography: 882 mg (52%) of a diastereoisomeric mixture (*trans*:*cis*, 3:1), amorphous solid. Separation of diastereoisomers was performed by HPLC technique using gradient water−MeOH 50−100% in 40 min, followed by 100% MeOH. Elution with 100% MeOH afforded 420 mg (25%) of **8c**-*trans* followed by 190 mg (11%) of **8c**-*cis* (amorphous solid). Anal. ($C_{27}H_{49}N_6O_5P$) C, H, N, P. ESIMS: 569.4 $(MH)^+$ (100). HRMS (ESI): for $C_{27}H_{50}N_6O_5P$ $(MH)^+$ calculated, 569.3575; found, 569.3560.

Pivaloyloxymethyl Ester of 9-{[(5*S*)-2-Hydroxy-2-oxido-1,4,2-dioxaphosphinan-5-yl]methyl}-2,6-diaminopurine (11). Yield after chromatography: 534 mg (43%) of a white foam. Final purification and separation of diastereoisomers were performed by preparative reverse-phase HPLC using gradient 20−100% MeOH: **11** (*trans*:*cis* 6:1) was eluted with 95−100% MeOH (200 mg), followed by pure **11**-*cis* eluted with 100% MeOH (70 mg). ^{1}H NMR (500.0 MHz, $CDCl_3$) for *trans*-isomer, 7.48 (s, 1H, H-8), 5.72 and 5.69 (2 × dd, 2H, $J_{P,CH}$ = 5.3, J_{gem} = 12.2, OCH_2O), 5.60 (br s, 1H, NH_2), 4.81 (br s, 1H, NH_2), 4.41−4.13 (m, 4H, NCH_2, OCH_2), 4.07 (m, 1H, H-2′), 3.97 (dd, 1H,

J_{gem} = 14.4, $J_{P,CHax}$ = 7.1, PCH_{ax}), 3.92 (br d, 1H, J_{gem} = 14.4, PCH_{eq}), 1.21 (s, 9H, *t*-Bu). ^{1}H NMR (500.0 MHz, $CDCl_3$) for *cis*-isomer: 7.50 (s, 1H, H-8), 6.28 (br s, 1H, NH_2), 5.74 (dd, 1H, $J_{P,CH}$ = 5.6, J_{gem} = 12.3, OCH_2O), 5.60 (dd, 1H, $J_{P,CH}$ = 5.1, OCH_2O), 5.13 (br s, 1H, NH_2), 4.45−5.03 (m, 5H, H-1′, OCH_2, H-2′), 3.94 (br d, 1H, $J_{P,CHax} \leq 1.0$, J_{gem} = 14.4, PCH_{ax}), 3.85 (dd, 1H, J_{gem} = 14.4, $J_{P,CHeq}$ = 3.2, PCH_{eq}), 1.19 (s, 9H, *t*-Bu). Anal. ($C_{15}H_{23}N_6O_6$ P.H_2O) C, H, N, P. ESIMS: 850.5 (2M + Na)$^+$ (41), 437.0 (M + Na)$^+$ (10), 414.9 (6) $(MH)^+$. HRMS (ESI): for $C_{15}H_{23}N_6O_6Na$ P $(M + Na)^+$ calculated, 437.1314; found, 437.1305.

Transformation of Cyclic Phosphonate Esters to Monoesters. General Procedure. A mixture of **8** (1 mmol) and 2 M NaOH (20 mL) was heated at 80 °C for 1 h, then cooled to room temperature, neutralized with glacial acetic acid to pH 5, and set aside at 4 °C for 12 h. A precipitated product was filtered off, washed with water, followed by acetone and ether, and dried in vacuo. Final purification for in vivo experiments was performed by preparative HPLC technique using gradient 50−100% MeOH (0−18 min), followed by 100% MeOH; monoesters **9** were eluted with 100% MeOH with retention time 20−25 min. Another possibility of purification is column chromatography on silica gel (100 mL) in system ethyl acetate−acetone−ethanol−water (15:3:4:3), followed by crystallization of the product from the same system.

3-(Hexadecyloxy)propyl Ester of 9-(*S*)-[3-Hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine Sodium Salt (9a). Yield: 338 mg (54%) of a white solid. ^{1}H NMR (500.0 MHz, CD_3OD): 7.89 (s, 1H, H-8), 4.64 (m, 1H, H-2′), 4.31 (dd, 1H, $J_{1'a,2'}$ = 4.0, J_{gem} = 14.6, H-1′a), 4.24 (dd, 1H, $J_{1'b,2'}$ = 6.0, H-1′b), 3.93 (m, 2H, OCH_2), 3.69 (dd, 1H, $J_{P,CH}$ = 9.3, J_{gem} = 13.6, PCH_a), 3.66 (dd, 1H, $J_{P,CH}$ = 9.3, PCH_b), 3.64 (dd, 1H, $J_{3'a,2'}$ = 4.2, J_{gem} = 12.4, H-3′a), 3.51 (dd, 1H, $J_{3'b,2'}$ = 4.6, H-3′b), 3.49 (t, 2H, *J* = 6.5, OCH_2), 3.38 (t, 2H, *J* = 6.7, OCH_2), 1.81 (pent, 2H, CH_2), 1.53 (m, 2H, CH_2), 1.30 (m, 26H, CH_2), 0.91 (t, 3H, $J_{CH3,CH2}$ = 7.2, CH_3). Anal. ($C_{28}H_{52}N_6O_6$ NaP) C, H, N, P. FABMS: 645 (M + Na)$^+$ (51), 623 $(MH)^+$ (40). HRMS (FAB): for $C_{28}H_{53}N_6O_6NaP$ (MH^+) calculated, 623.3662; found, 623.3673.

2-(Octadecyloxy)ethyl Ester of 9-(*S*)-[3-Hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine Sodium Salt (9b). Yield: 330 mg (52%) of a white solid. Anal. ($C_{29}H_{55}N_6O_6$ NaP) C, H, N, P. ESIMS: 637 $(MH)^+$ (30), 615 (M − Na + 2H)$^+$ (100). HRMS (QTOF): for $C_{29}H_{55}N_6O_6NaP$ (MH^+) calculated, 637.3818; found, 637.3826.

2-(Hexadecyloxy)ethyl Ester of 9-(*S*)-[3-Hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine Sodium Salt (9c). Yield: 416 mg (68%) of a white solid. Anal. ($C_{27}H_{50}N_6O_6$ NaP) C, H, N, P. ESIMS: 587.4 $(MH)^+$ (100) − phosphonic acid, 609.3 (30) $(MH)^+$ − sodium salt. HRMS (ESI): for $C_{27}H_{51}N_6O_6NaP$ (MH^+) calculated, 609.3500; found, 609.3508.

Pivaloyloxymethyl Ester of 9-(*S*)-[3-Hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine (10). (a) Chloromethyl pivalate (1.3 mL, 9 mmol) was added to a mixture of **1** (600 mg, 1.88 mmol) and *N*,*N*′-dicyclohexyl-4-morpholinecarboxamidine (1.32 g, 2.25 mmol) in DMF (10 mL), and the mixture was stirred for 24 h at 25 °C. Methanol (2 mL) was added, the solution evaporated, and the residue chromatographed on a column of silica gel (150 mL) in system ethyl acetate−acetone−ethanol−water (15:3:4:3). Crystallization from dry ether afforded **5** in yield 350 mg (43%, free monoester). ^{1}H NMR (500.0 MHz, CD_3OD): 7.88 (s, 1H, H-8), 5.54 (dd, 1H, $J_{P,OCH}$ = 5.4, J_{gem} = 12.2, OCH_2O), 5.50 (dd, 1H, $J_{P,OCH}$ = 4.9, OCH_2O), 4.60 (m, 1H, H-2′), 4.28 (dd, 1H, $J_{1'a,2'}$ = 4.1, J_{gem} = 14.5, H-1′a), 4.20 (dd, 1H, $J_{1'b,2'}$ = 6.4, H-1′b), 3.71 (dd, 1H, $J_{P,CHa}$ = 9.3, J_{gem} = 13.0, PCH_a), 3.65 (dd, 1H, $J_{P,CHb}$ = 9.1, PCH_b), 3.60 (dd, 1H, $J_{3'a,2'}$ = 4.6, J_{gem} = 12.4, H-3′a), 3.49 (dd, 1H, $J_{3'b,2'}$ = 4.9, H-3′b), 1.15 (s, 9H, *tert*-butyl). Anal. ($C_{15}H_{25}N_6O_7$ P.1/2H_2O) C, H, N, P. FABMS: 455 (M + Na)$^+$ (0.2), 433 $(MH)^+$ (0.15). HRMS (FAB): for $C_{15}H_{26}N_6O_7P$ (MH^+) calculated, 433.1600; found, 433.1606; for $C_{15}H_{25}N_6O_7NaP$ (M + Na)$^+$ calculated, 455.1420; found, 455.1413.

(b) An alternative purification was performed on a column of DEAE-Sephadex A25 (1.5 × 6 cm) activated with 0.02 M triethylammonium hydrogen carbonate (TEAB) using elution with water (250 mL) followed by a linear gradient of TEAB (0−0.5M, 400 mL). Collected UV absorbing fractions were evaporated at 25 °C and finally purified by preparative HPLC technique using the following conditions: 10% MeOH (0−10 min), gradient 10−100% MeOH (10−25 min). Elution of **10** occurred at concentration 85−95% MeOH. Yield after HPLC: 250 mg (25%, TEA salt).

2,2,2-Trifluoroethyl Ester of 9-(*S*)-[3-Hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine (12). PyBroP (2.8 g, 6 mmol) was added to a suspension of **1** (1 g, 3.14 mmol) in DMF (12 mL) and stirred at 25 °C until dissolution (0.5−1 h). Ethyldiisopropylamine (1.6 mL, 9.0 mmol) was added after 1 h followed by trifluoroethanol (2 mL) and an additional amount of ethyldiisopropylamine (1 mL). The mixture was stirred at 90 °C for 3 h. The reaction course was monitored by TLC in system 2-propanol−25% ammonia−water (7:1:2). In case of insufficient conversion, DCC was added (660 mg, 3.2 mmol) and the heating continued for an additional 1 h. The mixture was evaporated in vacuo and the residue chromatographed on a short column of silica gel (height 4 cm, width 5 cm) in system chloroform−methanol (4:1). The crude product was additionally chromatographed in system ethyl acetate−acetone−ethanol−water (15:3:4:3). Final purification of **12** was performed by preparative HPLC technique using a linear gradient of methanol in water (5−100% MeOH in 60 min); elution of **12** occurred at 40% MeOH. A product containing fraction was evaporated and the pure product lyophilized from water. Yield: 335 mg (27%) of a white solid. Anal. ($C_{11}H_{16}N_6O_5F_3P$) C, H, N, P. ESIMS: 401.1 $(MH)^+$ (15). HRMS (ESI): for $C_{11}H_{17}N_6O_5F_3P$ (MH^+) calculated, 401.0945; found, 401.0946.

2,2,2-Trifluoroethyl Ester of 9-{[(5*S*)-2-Hydroxy-2-oxido-1,4,2-dioxaphosphinan-5-yl]methyl}-2,6-diaminopurine (13). Ethyldiisopropylamine (0.79 mL, 4.5 mmol), followed by HATU (1.7 g, 4.5 mmol), was added under stirring in an ultrasound bath to a suspension of **7** (670 mg, 2.23 mmol) in DMF (10 mL). Trifluoroethanol (4 mL) was added after 15 min and the mixture stirred for 1 h at 25 °C and then heated to 90 °C during 3 h. The resulting dark brown solution was evaporated and the residue chromatographed on a silica gel column (100 mL) in ethyl acetate−acetone−ethanol−water (15:3:4:3) followed by additional chromatography in chloroform−methanol (4:1) to give TLC pure **13** as a yellow foam (450 mg). Final purification of **13** was performed by preparative HPLC using a gradient of MeOH (5−100% MeOH in 40 min, retention time 20 min). Yield: 300 mg (35%) of a diastereoisomeric mixture (white solid). Anal. ($C_{11}H_{14}N_6O_4F_3P.2H_2O$) C, H, N, P. ESIMS: 383.1 $(MH)^+$ (100). HRMS (ESI): for $C_{11}H_{15}N_6O_4F_3P$ (MH^+) calculated, 383.0839; found, 383.0840.

Bis(2,2,2-Trifluoroethyl) Tosyloxymethylphosphonate (15). Bromotrimethylsilane (25 mL, 187 mmol) was added to a solution of **14** (10 g, 28.6 mmol) in acetonitrile (150 mL). After standing for 48 h, the solution was evaporated and the residue coevaporated gradually with solvents in the following order, acetonitrile, water, absolute ethanol, and dichloromethane, and dried in vacuo. DMF (1 mL) and dichloromethane (400 mL) were added, the solution was cooled to 0 °C, and oxalyl chloride (16 mL, 180 mmol) was added dropwise under stirring. The mixture was heated to room temperature and then refluxed for 4 h. The solution was evaporated, the residue dissolved in dichloromethane (300 mL), and pyridine (10 mL) added at 0 °C. The resulting solution was added to a mixture of trifluoroethanol (5 mL, 70 mmol), triethylamine (46 mL), and dichloromethane cooled to −25 °C. The resulting black solution was stirred at −25 °C for 2 h and then 2 days at room temperature and evaporated. The residue was chromatographed on a silica

gel column (1 L) in a gradient of toluene−ethyl acetate (3:1 to 1:1). R_f of **15**: 0.6 in toluene−ethyl acetate (1:1). Yield: 3.5 g (28%) of colorless syrup crystallizing after standing in a refrigerator. Anal. ($C_{12}H_{13}F_6O_6PS$) C, H, F, P, S. ESIMS: 452.91 $(M + Na)^+$ (100), 430.8 $(MH)^+$ (5). HRMS (ESI): for $C_{12}H_{13}F_6O_6PSNa$ $(M + Na)^+$ calculated, 452.9967; found, 452.9963.

Butylsalicyl Ester of 9-{[(5*S*)-2-Hydroxy-2-oxido-1,4,2-dioxaphosphinan-5-yl]methyl}-2,6-diaminopurine (16). A mixture of **1** (2.09 g, 6.96 mmol) and PyBOP (9.01 g, 17.32 mmol) in DMF (240 mL) was sonicated in an ultrasonic bath for 45 min. *N*-Diisopropylethylamine (4.45 g, 34.44 mmol) and butyl salicylate (2.14 g, 11.01 mmol) were added, and the resulting mixture was sonicated for 15 min. The suspension was stirred for 6 days at room temperature as long as the conversion of **1** to **16** proceeded (monitoring of the reaction mixture was performed by TLC in chloroform−methanol 4:1). During this reaction, sonication was repeated several times. The mixture was evaporated in vacuo to dryness, and the residue was chromatographed on a column of silica gel (360 mL) in chloroform−methanol (95:5, followed by 4:1). Fractions containing the mixture of compound **16** (R_f = 0.62) and PyBOP byproducts (R_f = 0.56) were combined and evaporated in vacuo. The residue was subsequently purified by preparative HPLC technique using gradient 40−100% MeOH/H_2O (0−30 min). Product was collected in 80−100% MeOH/H_2O. Final purification was performed by preparative TLC chromatography in chloroform−methanol (5:1). The product **16** (*trans*:*cis* 12:88) was obtained after lyophilization as a white amorphous solid. Yield: 522 mg (15%). Anal. ($C_{20}H_{25}N_6O_6P\cdot 0.6H_2O$) C, H, N, P. ESIMS: 477 $(M + H)^+$. HRMS (ESI): for $C_{20}H_{26}O_6N_6P$ $(M + H)^+$ calculated, 477.1646; found, 477.1646.

L-Serine Isopropyl Ester Hydrochloride (18). Thionyl chloride (120 mL) was added dropwise to cooled (∼−30 °C) 2-propanol (300 mL). Addition rate and cooling should be set up in such a way that maximum temperature of reaction mixture did not violate 0 °C. The solution was stirred an additional 1 h at 0 °C; then L-serine (10 g, 95 mmol) was added. The suspension was kept to reach room temperature. Gas was evolved during several hours. Gas production was controlled by means of subsequent slow heating up to 80 °C (additional several hours). When the mixture was completely dissolved and no gas evolved, 2-propanol was partly distilled off, and the product was crystallized from a distillation residue (∼100 mL). A crude product was crystallized from 2-propanol again. Yield 9.0 g (52%). Anal. ($C_6H_{14}O_3NCl$) C, H, N, Cl.

Isopropyl *tert*-Butyloxycarbonyl-L-valyl-L-serinate (19). Boc-Val-OH (2.09 g, 9.60 mmol), compound **18** (1.76 g, 9.60 mmol), and hydroxybenzotriazole (1.30 g, 9.62 mmol) were suspended in CH_2Cl_2 (30 mL). The mixture was cooled (0 °C), and triethylamine (1.34 mL, 9.61 mmol) and EDC (1.78 mL, 10.0 mmol) were added. The mixture was stirred for 1 h at 0 °C and then overnight at room temperature. The solution was diluted with CH_2Cl_2 and washed with a solution of $NaHCO_3$, citric acid, and $NaHCO_3$ again. The organic layer was dried with $MgSO_4$ and evaporated, and the residue was chromatographed on a silica gel column (250 mL) in hexane−ethyl acetate (3:2 → 2:3). The syrupy product was precipitated from Et_2O−hexane. Yield: 2.73 g (82%). Anal. ($C_{16}H_{30}O_6N_2$) C, H, N.

Isopropyl-(*tert*-butyloxycarbonyl-L-valyl)-L-serin-O^3-yl Ester of 9-{[(5*S*)-2-Hydroxy-2-oxido-1,4,2-dioxaphosphinan-5-yl]methyl}-2,6-diaminopurine (20). A mixture of **1** (1.15 g, 3.62 mmol) and PyBOP (4.81 g, 9.25 mmol) in DMF (120 mL) was sonicated in an ultrasonic bath for 45 min. *N*-Diisopropylethylamine (3.25 mL, 18.55 mmol) and **19** (1.38 g, 3.98 mmol) were added, and the resulting mixture was sonicated for 15 min. The suspension was stirred for 2 days at room temperature as long as the conversion of **1** to **20** proceeded (monitoring of the reaction mixture was performed by TLC in $CHCl_3$−MeOH (5:1), R_f(**20**) ∼ 0.5, two spots close to each other). During this reaction a process of sonication was repeated several times. The mixture was evaporated in vacuo to dryness, and the residue was chromatographed on a column of silica gel (400 mL) in $CHCl_3$−MeOH (9:1). Fractions containing **20**-*cis* or **20**-*trans* contaminated with PyBOP related products were combined and evaporated in vacuo. Fractions containing **20**, mixture of *cis* and *trans*, were chromatographed again. Fractions containing **20**-*cis* or -*trans* were subsequently purified by preparative HPLC technique using gradient 30−100% MeOH/H_2O (0−25 min, 10 mL/min). Product was collected in 75−85% MeOH/H_2O. Final purification was performed by means of crystallization from EtOH−Et_2O. Total yield: 0.48 g (21%). ESIMS: 629.0, 630.0 $[M + H]^+$, 651.1, 652.1 $[M + Na]^+$.

Isopropyl-(L-valyl)-L-serin-O^3-yl Ester of 9-{[(5*S*)-2-Hydroxy-2-oxido-1,4,2-dioxaphosphinan-5-yl]methyl}-2,6-diaminopurine (21). Boc derivative **20**-*cis* or **20**-*trans* (135 mg, 0.215 mmol) was treated with HCl in dioxane (4 M, 1 mL) at 0 °C. Mixture was allowed to reach room temperature 30 min later. Conversion was monitored (TLC in $CHCl_3$−MeOH, 5:1). The mixture was evaporated and codistilled (dioxane) when the starting compound disappeared. Crude product was purified by preparative HPLC technique using gradient 20−60% MeOH/H_2O (0−25 min, 10 mL/min). Product was collected in 35−50% MeOH/H_2O. Yield: 101 mg (83%) of amorphous solid. ESIMS: 528.9, 529.9 $[M + H]^+$, 551.0, 552.1 $[M + Na]^+$. HRMS (ESI): for $C_{20}H_{34}O_7N_8P$ $[M + H]^+$ calculated, 529.2283; found, 529.2288.

Antiviral Activity Assays. The compounds were evaluated against the following viruses: 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) strains Lyons and G, varicella-zoster virus (VZV) strain Oka, TK^- VZV strain 07-1, human cytomegalovirus (HCMV) strains AD-169 and Davis, human herpes virus 6 subtype A (HHV-6A) strain GS, vaccinia virus Lederle strain, human immunodeficiency virus (HIV) type 1 (III_B) and type 2 (ROD), respiratory syncytial virus (RSV) strain Long, vesicular stomatitis virus (VSV), Coxsackie B4, Parainfluenza 3, Reovirus-1, Sindbis, and Punta Toro. The antiviral assays, other than HIV, were based on inhibition of virus-induced cytopathicity or plaque formation in human embryonic lung (HEL) fibroblasts, African green monkey cells (Vero), human epithelial cells (HeLa), or human T-lymphoblasts HSB-2, according to previously established procedures.[16] 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 plaque forming units (PFU). After 1−2 h adsorption period, residual virus was removed, 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 concentration (expressed in micromolar) required for reducing virus-induced cytopathogenicity or viral plaque formation by 50%. The methodology of the anti-HIV assays was as follows: human CEM (∼3×10^5 cells/cm^3) were infected with 100 $CCID_{50}$ of HIV-1 (III_B) or HIV-2 (ROD)/mL and seeded in 200 μL wells of a microtiter plate containing appropriate dilutions of the test compounds. After 4 days of incubation at 37 °C, HIV-induced CEM giant cell formation was examined microscopically.

Cytotoxicity Assays. Cytotoxicity measurements were based on the inhibition of cell growth. HEL cells were seeded at a rate of 5×10^3 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 3 days of incubation at 37 °C, the cell number was determined with a Coulter counter. The cytostatic concentration (expressed in micromolar) was calculated as the CC_{50} or the compound concentration required for reducing cell proliferation by 50% relative to the number of cells in the untreated controls. CC_{50} values were estimated from graphic plots of the number of cells

(percentage of control) as a function of the concentration of the test compounds. Alternatively, cytotoxicity of the test compounds was expressed as the minimum cytotoxic concentration (MCC) or the compound concentration that caused a microscopically detectable alteration of cell morphology.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectral data of compounds, characterization of intermediates in preparation of HMPMDAP, and elemental analyses and HPLC of tested compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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