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Original article

Synthesis and antiviral activities of hexadecyloxypropyl prodrugs of acyclic nucleoside phosphonates containing guanine or hypoxanthine and a (*S*)-HPMP or PEE acyclic moiety

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ABSTRACT

Hexadecyloxypropyl esters of acyclic nucleoside phosphonates containing guanine (G) or hypoxanthine (Hx) and a (*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl] [(*S*)-HPMP] or 2-(2-phosphonoethoxy)ethyl (PEE) acyclic moiety have been prepared. The activity of the prodrugs was evaluated *in vitro* against different virus families. Whereas ester derivatives of PEEHx and (*S*)-HPMPHx were antivirally inactive, monoesters of PEEG, and mono- and diesters of (*S*)-HPMPG showed pronounced antiviral activity against vaccinia virus and/or herpesviruses. Monoesters of (*S*)-HPMPG emerged as the most potent and selective derivatives against these DNA viruses. None of the compounds were inhibitory against RNA viruses and retroviruses.

1. Introduction

Acyclic nucleoside phosphonates [1] constitute a group of compounds with a wide range of biological activities including antiviral, cytostatic, antiparasitic and immunomodulatory effects. The compounds with guanine or hypoxanthine as a nucleobase bearing (*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl] [(*S*)-HPMP], or 2-(2-phosphonoethoxy)ethyl (PEE) chain have been reported as potential antimalarial agents [2] capable to inhibit the 6-oxopurine phosphoribosyltransferase [3] of the *Plasmodium* parasite. In order to increase membrane permeability of above mentioned phosphonates, we have masked the ionic character of the drugs using the Hostetler's prodrug strategy [4] and hexadecyloxypropyl esters of 9-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]guanine [(*S*)-HPMPG], 9-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]hypoxanthine [(*S*)-HPMPHx], 9-[2-(phosphonomethoxy)ethyl]guanine (PEEG) and 9-[2-(phosphonomethoxy)ethyl]hypoxanthine (PEEHx) have been prepared. Their activities against *Plasmodium* are the subject of another study [5]. Herein we focus on their antiviral activities against various virus strains. Among the parent drugs, (*S*)-HPMPG has been reported [6] as an active compound against herpes simplex virus (HSV) type 1 and type 2, varicella-zoster virus (VZV), cytomegalovirus (CMV) and vaccinia virus (EC_{50} in the μM range). The octadecyloxyethyl ester of (*S*)-HPMPG, a close analog of prodrug **16** in this study, has been reported [7] to display enhanced antiviral activity compared to the parent drug. Attachment of a highly lipophilic hexadecyloxypropyl moiety increases the cellular permeability as well as antiviral activities of the prodrug, probably due to the greater availability for cellular phosphorylation [4]. Thus strong antiviral activities of hexadecyloxypropyl esters of (*S*)-HPMPG against DNA virus strains can be expected. Also drugs previously found to be weakly active (PEEG) may show more pronounced activity after their conversion to hexadecyloxypropyl esters. Additionally, the structure–activity relationship among various prodrug types (diester, monoester, cyclic monoester) is reported below.

2. Results and discussion

2.1. Chemistry

For alkylation of the phosphonate function with the hexadecyloxypropyl moiety, we considered two approaches using

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PEEG as a model compound. The first is a broadly used methodology [8] involving *in situ* preparation of a bis chloridate followed by subsequent reaction with hexadecyloxypropyl alcohol in the presence of a base. The latter approach [9] involves neutralization of the phosphonate function with tetrabutylammonium hydroxide followed by nucleophilic reaction of the resulting phosphonate salt with hexadecyloxypropyl bromide. However, both methodologies afforded low yields of PEEG esters (<10%) due to the low conversion and the simultaneous alkylation of nucleobase. In order to circumvent these problems,we alkylated the acyclic phosphonate synthon which was subsequently coupled with an appropriate nucleobase. For the preparation of PEEG and PEEHx prodrugs, bis isopropyl phosphonoethoxyethylchloride [10] **1** was transformed to the free phosphonic acid and subsequently to bis chloridate which was treated with hexadecyloxypropyl alcohol in the presence of pyridine and triethylamine. Bis hexadecyloxypropyl ester **2** was isolated in high yield. The nucleophilic substitution reaction of alkylated phosphonate synthon with 6-chloropurine or 2-amino-6-chloropurine in the presence of Cs_2CO_3 as a base followed by acid catalyzed cleavage of the 6-chloro substituent afforded bis hexadecyloxypropyl esters of PEEG or PEEHx. Appropriate monoesters **5** and **6** were prepared by dissolving diesters **3** and **4** in DMF and heating in the presence of a nucleophilic agent (LiN_3) causing removal of one alkyl ester group [11] (Scheme 1).

Similar to the preparation of PEEG and PEEHx prodrugs, the preparation of (*S*)-HPMPG and (*S*)-HPMPHx prodrugs began with the conversion of bis isopropyl phosphonomethyltosylate **7** to bis(hexadecyloxypropyl) phosphonomethyltosylate **8** [12]. The alkylated phosphonate synthon was coupled with pre-prepared 9-(2-hydroxy-3-trityloxypropyl)-6-*O*-benzylpurine **11** or 9-(2-hydroxy-3-trityloxypropyl)-2-amino-6-*O*-benzylpurine **12** in the presence of a base (NaH). Pre-preparation involved nucleophilic opening of the oxirane ring of (*S*)-(−)-glycidol trityl ether with 6-*O*-benzylpurine **9** or 2-amino-6-*O*-benzylpurine **10**. The acid labile protective groups (OBn, OTr) of the resulting adducts were cleaved by the action of HCl. The diesters **13** and **14** were hydrolyzed in a solution of NaOH giving monoesters **15** and **16**. Above mentioned method [11] using milder conditions resulted in incomplete conversion in this case. Intramolecular esterification of **15** and **16** mediated by PyBOP [13] afforded cyclic hexadecyloxypropyl esters **17** and **18** as a mixture of diastereomers (1:1 ratio according to the NMR spectra) (Scheme 2).

2.2. Biological activities

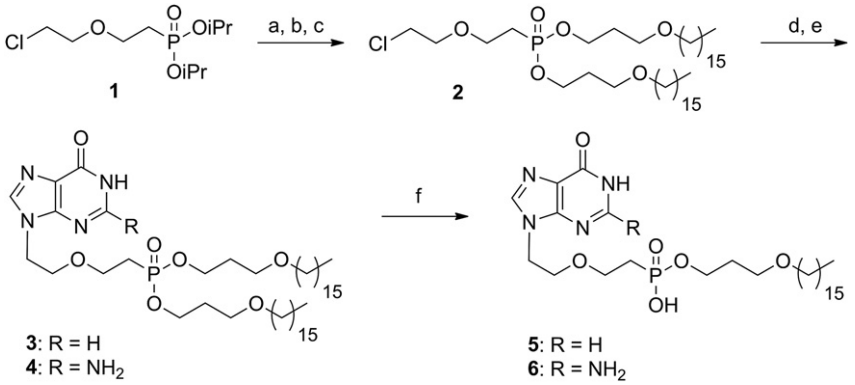
Esterification of acyclic nucleoside phosphonates such as (*S*)-HPMPC and (*S*)-HPMPA with various alkoxyalkyl or alkylglycerol groups has been shown to result in remarkable increases in antiviral activities against herpesviruses and poxviruses [14,15]. The rationale behind this study was to demonstrate whether enhanced lipophilicity of (*S*)-HPMPG, (*S*)-HPMPHx, PEEG and PEEHx correlates with increased antiviral activity. For that purpose, hexadecyloxypropyl diesters, monoesters and cyclic monoesters of these compounds were prepared and their antiviral properties were evaluated *in vitro*.

The antiviral activity of the different compounds was evaluated against various DNA viruses, including poxviruses [i.e. vaccinia virus (VACV)], herpesviruses [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), and human cytomegalovirus (HCMV) (Table 1), and retroviruses including 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 (VSV), Coxsackie B4 virus, respiratory syncytial virus (RSV), parainfluenza virus type 3, reovirus-1, *Sindbis virus* and Punta Toro virus. None of the prodrugs showed activity against any of the RNA viruses nor retroviruses tested.

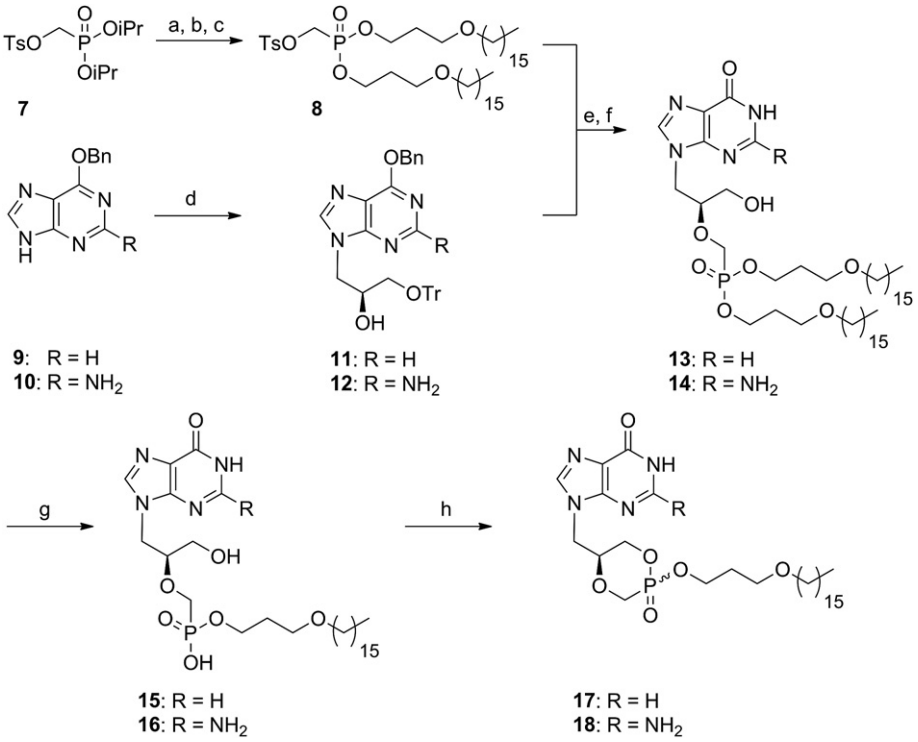
Similar to the parent compound, both the diester and monoester prodrugs of PEEHx (i.e. compounds **3** and **5**) proved inactive against herpesviruses and poxviruses. Although the diester prodrug of PEEG (compound **4**) lacked also activity against these DNA viruses, the monoester derivative (compound **6**) was able to potently inhibit the replication of VZV, HSV and HCMV at EC_{50} values in the range of, respectively, 0.14–0.30 μM, 1.4–2.8 μM, and 0.06–0.13 μM. Since compound **6** inhibited cell growth at a CC_{50} (50% cytostatic concentration) value of 26 μM, selectivity indices (ratio CC_{50} to EC_{50}) ranged from 9 to 433 depending on the nature of the virus. Interestingly, compound **6** was inactive against feline herpesvirus and against VACV.

Among the HPMP prodrugs, attachment of highly lipophilic hexadecyloxypropyl moieties to (*S*)-HPMPHx did not result in an increase of the antiviral activity since compounds **15** (monoester), **13** (diester) and **17** (cyclic monoester) were totally inactive against herpesviruses and poxviruses.

The diester derivative of (*S*)-HPMPG (compound **14**) showed EC_{50} values that were 7-fold (VZV and HCMV), 15- to 40-fold (HSV), 157-fold (feline herpesvirus) and 41-fold (VACV) lower than those for the parent compound. However, the monoester and cyclic monoester derivatives of (*S*)-HPMPG proved more active and selective than the diester derivative. This observation supports previous assumption [4] that the phosphonate double-ester prodrugs penetrate the cell membrane less rapidly than their monoester counterpairs, even in case an ionized phosphonate group is present in the monoester prodrug. Thus, compound **16** (monoester)



Scheme 1. The preparation of prodrugs **3**–**6**. Reagents and conditions: (a) Me_3SiBr, MeCN, r.t., then H_2O; (b) $(COCl)_2$, DMF, CH_2Cl_2, 40 °C; (c) pyridine, 0 °C, then hexadecyloxypropyl alcohol, Et_3N, CH_2Cl_2, −30 °C → 0 °C; (d) 6-chloropurine or 2-amino-6-chloropurine, Cs_2CO_3, DMF, 100 °C; (e) AcOH, H_2O, reflux; (f) LiN_3, DMF, 100 °C.



Scheme 2. The preparation of prodrugs **13**–**18**. The reagents and conditions: (a) Me_3SiBr, MeCN, r.t., then H_2O; (b) $(COCl)_2$, DMF, CH_2Cl_2, 40 °C; (c) pyridine, 0 °C, then hexadecyloxypropyl alcohol, Et_3N, CH_2Cl_2, −30 °C → 0 °C; (d) (*S*)-(−)-glycidol trityl ether, $CsCO_3$, DMF, 100 °C; (e) NaH, DMF, −25 °C → 35 °C; (f) HCl, MeOH, Et_2O, r.t.; (g) NaOH, H_2O, dioxane, r.t.; (h) PyBOP, *i*-Pr_2EtN, DMF, r.t.

and compound **18** (cyclic monoester) inhibited VZV replication with EC_{50} values in the range of 0.001–0.0025 μM for VZV, 0.007–0.09 μM for HSV, and 0.002–0.007 μM for HCMV. While the monoester derivative of (*S*)-HPMPG was inactive against feline herpesvirus, the cyclic monoester derivative was able to inhibit viral replication with an EC_{50} of 0.43 μM. However, compound **16** proved more active than compound **18** against VACV (EC_{50} = 0.011 μM for compound **16** compared to 0.13 μM for compound **18**). As the monoester derivative of (*S*)-HPMPG (compound **16**) proved significantly more cystostatic than the cyclic monoester (compound **18**) with a CC_{50} of 0.72 μM and 19 μM, respectively, SIs were 2- to 66-fold lower for compound **16** compared to compound **18**. Thus, the cyclic monoester of (*S*) HPMPG showed the high selectivity among all the prodrug tested with SIs in the range of 2700–19,000 for VZV, 211–559 for HSV, 3800–9500 for HCMV, and 146 for VACV.

Our data show that enhanced lipophilicity of (*S*)-HPMPG resulted in increased antiviral activity and selectivity *in vitro* against herpesviruses and poxviruses. The significant gain in antiviral activity and selectivity for compound **18** compared to the parent compound can be explained in a similar way as for other ANPs, i.e. increased cellular uptake of the ester deriatives, more rapid conversion to the diphosphate active forms and also metabolic stability of the ester derivatives. Even compounds, such as **6** and **16**, that are charged in physiological conditions are able to penetrate the cell membrane since they presented significant antiviral activity. Antimicrobial activities (*Bordetella pertusis*, *Bacillus anthracis*) of the prodrugs will be also tested.

3. Conclusion

Hexadecyloxypropyl diesters, monoesters and cyclic monoesters of (*S*)-HPMPG, (*S*)-HPMPHx, PEEG and PEEHx have been prepared and their antiviral properties were evaluated *in vitro*. None of the hexadecyloxypropyl esters of (*S*)-HPMPHx nor PEEHx proved active against herpesviruses and poxviruses. Although the hexadecyloxypropyl diester of PEEG was antivirally inactive, the monoester derivative gained activity against herpesviruses. Attachment of highly lipophilic hexadecyloxypropyl moieties to (*S*)-HPMPG resulted in improvement of its antiviral activity against herpesviruses. The cyclic monoester derivative of (*S*)-HPMPG emerged as the most selective antiviral compound.

4. Experimental

Unless stated otherwise, solvents were evaporated at 40 °C and compounds were dried at 100 Pa. The purification of the products by reverse phase HPLC was performed on a Waters Delta 600 instrument with a Waters 2487 Dual λ Absorbance Detector using Luna Phenomenex® C-18 preparative columns (10 μm, 21 × 250 mm, flow 12 ml/min); the elution conditions are given in the text. The column chromatography was performed on 60 μm silica gel (Fluka). The ^1H and ^{13}C NMR spectra were measured in $CDCl_3$, D_2O or DMSO-d_6 on a Bruker Avance II 600 spectrometer (^1H at 600 MHz, ^{13}C at 151 MHz) or Bruker Avance II 500 spectrometer (^1H at 500 MHz, ^{13}C at 125.7 MHz). The spectra were referenced to TMS or dioxane (δ 3.75 and 67.19) as internal standards or to the residual solvent signal (δ 2.50 and 39.7 for DMSO). Mass spectra were measured by the ESI technique using LCQ Fleet or LTQ Orbitrap XL (Thermo Fisher Scientific). Most of the chemicals and ion-exchange resins were purchased from Sigma–Aldrich (Czech Republic). The general numbering for the assignment of the NMR signals is depicted below (Fig. 1).

4.1. General method A

Bis isopropyl phosphonoethoxyethylchloride (**1**) (2.58 g, 10.0 mmol) or bis isopropyl phosphonomethyltosylate (**7**) (3.50 g, 10.0 mmol) in anhydrous acetonitrile (130 ml) was treated with

Table 1
Antiviral and cytotoxic/static activity of the compounds against herpesviruses and poxviruses in cell culture.

Compounds	Antiviral activity: EC_{50} (μM) in HEL (human embryonic lung) fibroblasts except for feline herpesvirus and selectivity indexes (in bold italics)											Cytotoxicity in HEL cells (μM)	
	VZV				HSV-1	HSV-2	HSV-1 TK^-	Cytomegalovirus		Feline herpesvirus[a]	Vaccinia virus	Cell morphology	Cell growth
	TK^+ strains		TK^- strains										
	YS	Oka	07/01.	YS/R	KOS	G	KOS ACV^r	AD-169	Davis		Lederle	MCC	CC_{50}
3	N.D.	>23.4	>23.4	N.D.	>117	>117	>117	>23.4	>23.4	>23.4	>117	≥117	117
4	N.D.	>23	>23	N.D.	>115	>115	>115	>23	>23	>115	>115	≥23	>115
5	N.D.	>35	>35	N.D.	>35	>35	>35	>35	>35	>35	>35	175	78
6	0.19	0.14 ± 0.04	0.30 ± 0.13	N.D.	1.37 ± 0	1.9 ± 0.7	2.8 ± 0.4	0.13 ± 0.05	0.06 ± 0.01	>171	≥34	≥34	26 ± 12
	137	***186***	***87***		***19***	***14***	***9***	***200***	***433***		***<1***		
13	N.D.	>23	>4.6	N.D.	>115	>115	>115	>23	>23	>115	>115	≥23	>115
14	1.2 ± 0.4	3.9 ± 1.9	1.4 ± 0.7	2.0 ± 2.4	11.9 ± 2.4	23.2 ± 21.6	30.5 ± 11.2	11.9	10.1	>113	44.7 ± 8.8	≥22.6	>113
	>94	***>29***	***>81***	***>57***	***>9***	***>5***	***>4***	***>9***	***>11***		***>2.5***		
15	N.D.	>6.8	4-Aug	N.D.	>6.8	>6.8	>6.8	>34	15.2	>34	>6.8	≥34	66 ± 46
16	0.0025	0.002	0.0017	N.D.	0.008 ± 0.004	0.007 ± 0.002	0.012 ± 0.007	0.0066	0.0022	>1.33	0.011 ± 0.001	≥1.3	0.72 ± 0.27
	288	***360***	***423***		***90***	***103***	***60***	***109***	***327***		***65***		
17	N.D.	>1.4	>1.4	N.D.	>7	>7	>7	>7	>7	>176	>7	≥7	>176
18	0.001 ± 0.001	0.007 ± 0.010	0.005 ± 0.007	0.005 ± 0.007	0.034 ± 0	0.08 ± 0.08	0.09 ± 0.02	0.005 ± 0.005	0.002 ± 0.002	0.43 ± 0.36	0.13 ± 0.06	≥171.3	19 ± 14
	19,000	***2700***	***3800***	***3800***	***559***	***238***	***211***	***3800***	***9500***	***398***	***146***		
(*S*)-HPMPG	N.D.	0.56 ± 0.13	0.21 ± 0.15	N.D.	0.78 ± 0.22	0.78 ± 0.22	0.78 ± 0.22	1.8 ± 1.1	1.5 ± 0.6	0.72 ± 0.50	1.1 ± 0.22	>100	39 ± 7
		70	***186***		***50***	***50***	***50***	***22***	***26***	***157***	***35***		
(*S*)-cHPMPG	N.D.	0.1 ± 0.1	0.23 ± 0.10	N.D.	0.66 ± 0.47	0.66 ± 0	0.50 ± 0.23	1.5 ± 0	1.6 ± 0	0.90 ± 0.40	1.0 ± 0.5	>100	17 ± 10
		170	***74***		***26***	***26***	***34***	***11***	***11***	***96***	***17***		
Brivudin	0.006 ± 0.004	0.01 ± 0.01	53 ± 67	≥120 ± 42	0.038 ± 0.034	105 ± 32	≥183 ± 103	N.D.	N.D.	N.D.	15 ± 17	>300	347 ± 132
Acyclovir	2.84 ± 2.36	1.64 ± 0.67	54 ± 58	37 ± 7	0.53 ± 0.43	0.38 ± 0.27	≥212 ± 59	N.D.	N.D.	N.D.	>1000	>444	918 ± 578
Ganciclovir	N.D.	N.D.	N.D.	N.D.	0.027 ± 0.005	0.029 ± 0.014	14.7 ± 8.3	9.1 ± 3.0	5.9 ± 1.7	1.15 ± 0.35	≥ 93 ± 17	>394	231 ± 97
Cidofovir	N.D.	N.D.	N.D.	N.D.	0.93 ± 0.45	1.33 ± 0.89	1.75 ± 0.42	1.37 ± 0.51	0.92 ± 0.25	N.D.	14.0 ± 6.2	>317	241 ± 260

EC_{50}: 50% effective concentration or compound concentration required to reduce virus induced cytopathic effect by 50%.
CC_{50}: 50% cystostatic concentration or compound concentration required to reduce cell growth by 50%.
MCC: minimum cytotoxic concentration or the compound concentration that caused a microscopically detectable alteration of cell morphology.
Selectivity index (SI): ratio CC_{50} to EC_{50}.
N.D.: not done.
[a] Assays performed in CRFK (Crandell-Rees feline kidney) cells.

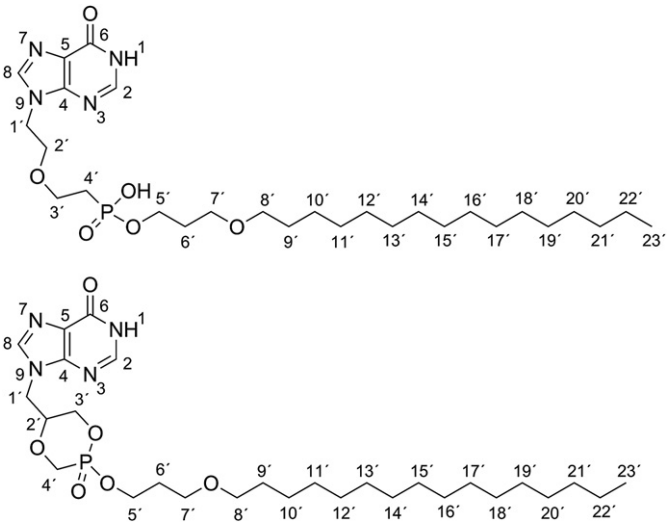


Fig. 1. The general numbering for the assignment of the NMR signals.

bromotrimethylsilane (13 ml) overnight at room temperature. The mixture was then evaporated and codistilled with acetonitrile (3 × 25 mL), water (2 × 25 ml), ethanol (25 mL) and toluene (3 × 25 mL). The syrupy residue was dissolved in dichloromethane (50 ml) then DMF (0.1 ml) and oxalyl chloride (6.0 ml, 68.8 mmol) were added. The solution was gently refluxed (2 h), evaporated to dryness, and redissolved in dichloromethane (20 ml). The solution was cooled down to 0 °C and treated slowly with pyridine (1.6 ml). The mixture was added to a cooled (−30 °C) solution of hexadecyloxypropanol (6.01 g, 20 mmol) in dichloromethane (100 ml) and triethylamine (8.7 ml). The mixture was allowed to reach 0 °C and kept at this temperature for 3 h. The reaction mixture was evaporated, codistilled with toluene (3 × 50 ml) and the residue was chromatographed on a silica gel column (400 g) in EtOAc/hexane (1:2 → 1:1).

4.1.1. Bis(hexadecyloxypropyl)phosphonoethoxyethylchloride (**2**)

Yield 6.50 g of syrup (88%). The crude product was used without further purification procedures. ESI-MS, *m*/*z*: 753.6 (53) $[M + H]^+$, 775.6 (100) $[M + Na]^+$, 791.5 (22) $[M + K]^+$, 812.7 (28); ESI-HRMS calcd for $C_{42}H_{87}O_6ClP$ 753.59233, found: 753.59185 $[M + H]^+$.

4.1.2. Bis(hexadecyloxypropyl)phosphonomethyltosylate (**8**)

Prepared similarly according to the published procedure [12]. Yield 6.94 g of syrup (83%). Identity was verified using authentic sample on TLC, R_f ~0.3 (30% EtOAc/hexane).

4.2. General method B

The mixture of 6-chloropurine or 2-amino-6-chloropurine (1.5 mmol, 232 or 254 mg, respectively), bis (hexadecyloxypropyl) phosphonoethoxyethylchloride (754 mg, 1.0 mmol), Cs_2CO_3 (326 mg, 1.0 mmol) and DMF (10 ml) was heated to 100 °C for 6 h (reaction with aminochloropurine) or 16 h (reaction with chloropurine). The mixture was evaporated and the residue was chromatographed on a silica gel column (50 g) in 6% MeOH/$CHCl_3$. The crude product was refluxed in 80% aqueous acetic acid for 2 h, then concentrated and chromatographed on a silica gel column (30 g) in 7% MeOH/$CHCl_3$.

4.2.1. Bis 3-(hexadecyloxypropyl) ester of 9-[2-(phosphonomethoxy)ethyl]hypoxanthine (**3**)

Yield 285 mg (33%). Crystallization from hexane. Mp: 88–91 °C. Anal. Calcd for $C_{47}H_{89}N_4O_7P$: C, 66.16; H, 10.51; N, 6.57; P, 3.63. Found: C, 65.98; H, 10.77; N, 6.39; P, 3.51. ESI-MS, *m*/*z*: 853.7 (100) $[M + H]^+$, 875.6 (98) $[M + Na]^+$, 891.6 (21) $[M + K]^+$. ESI-HRMS calcd for $C_{47}H_{90}N_4O_7P$ 853.6542, found: 853.6543 $[M + H]^+$; calcd for $C_{47}H_{89}N_4O_7PNa$ 875.6361, found: 875.6358 $[M + Na]^+$. ^1H NMR ($CDCl_3$, ppm) δ: 0.88 (t, 6H, $J_{23'-22'} = 7.0$), 1.20–1.33 (m, 52H, H-10′ to H-22′), 1.54 (m, 4H, H-9′), 1.91 (p, 4H, $J_{6'-5'} = J_{6'-7'} = 6.3$, H-6′), 2.08 (dt, 2H, $J_{4'-P} = 18.7$, $J_{4'-3'} = 7.3$, H-4′), 3.38 (t, 4H, $J_{8'-9'} = 6.7$, H-8′), 3.48 (t, 4H, $J_{7'-6'} = 6.1$, H-7′), 3.71 (m, 2H, H-3′), 3.81 (m, 2H, H-2′), 4.11 (m, 4H, H-5′), 4.44 (m, 2H, H-1′), 8.02 (s, H-2), 8.48 (s, H-8), 12.64 (br s, NH). ^{13}C NMR ($CDCl_3$, ppm) δ: 13.88 (C-23′), 22.39 (C-22′), 25.87 (C-10′), 26.18 (d, $J_{4'-P} = 139.8$, C-4′), 29.06, 29.24, 29.33, 29.35, 29.39, 29.40, 29.43 (C-9′, C-11′ to C-20′), 30.56 (d, $J_{6'-P} = 6.3$, C-6′), 31.62 (C-21′), 43.93 (C-1′), 62.75 (d, $J_{5'-P} = 6.4$, C-5′), 64.87 (C-3′), 66.21 (C-7′), 68.24 (C-2′), 70.91 (C-8′), 121.90 (C-5), 140.07 (C-8), 145.84 (C-2), 147.76 (C-4), 156.09 (C-6).

4.2.2. Bis 3-(hexadecyloxypropyl) ester of 9-[2-(phosphonomethoxy)ethyl]guanine (**4**)

Yield 460 mg (52%). Crystallization from hexane. Mp 130–132 °C. Anal. Calcd for $C_{47}H_{90}N_5O_7P$: C, 65.02; H, 10.45; N, 8.07; P, 3.57. Found: C, 64.69; H, 10.37; N, 7.94; P, 3.48. ESI-MS, *m*/*z*: 868.7 (100) $[M + H]^+$, 890.6 (77) $[M + Na]^+$, 906.6 (20) $[M + K]^+$. ESI-HRMS calcd for $C_{47}H_{91}N_5O_7P$ 868.6651, found: 853.6650 $[M + H]^+$; calcd for $C_{47}H_{90}N_5O_7PNa$ 890.6470, found: 890.6464 $[M + Na]^+$. ^1H NMR ($CDCl_3$, ppm) δ: 0.88 (t, 6H, $J_{23'-22'} = 7.1$), 1.19–1.35 (m, 52H, H-10′ to H-22′), 1.54 (m, 4H, H-9′), 1.92 (p, 4H, $J_{6'-5'} = J_{6'-7'} = 6.3$, H-6′), 2.15 (m, 2H, H-4′), 3.38 (m, 4H, H-8′), 3.49 (m, 4H, H-7′), 3.75–3.92 (m, 4H, H-2′, H-3′), 4.14 (m, 4H, H-5′), 4.37 (m, 2H, H-1′), 6.85 (br s, 2H, NH_2), 7.96 (br s, H-8), 12.05 (br s, NH). ^{13}C NMR ($CDCl_3$, ppm) δ: 14.10 (C-23′), 22.67 (C-22′), 26.16 (C-10′), 26.38 (d, $J_{4'-P} = 140.7$, C-4′), 29.35, 29.53, 29.63, 29.65, 29.68, 29.69, 29.71 (C-9′, C-11′ to C-20′), 30.83 (d, $J_{6'-P} = 6.3$, C-6′), 31.91 (C-21′), 43.22 (C-1′), 63.25 (d, $J_{5'-P} = 6.6$, C-5′), 64.98 (d, $J_{3'-P} = 2.6$, C-3′), 66.49 (C-7′), 68.90 (C-2′), 71.22 (C-8′), 115.93 (C-5), 138.04 (C-8), 151.03 (C-4), 154.30 (C-2), 158.05 (C-6).

4.3. General method C

The mixture of **3** or **4** (0.2 mmol, 170 mg or 174 mg respectively) and LiN_3 (1.6 mmol, 78 mg) in DMF (2 ml) was heated to 100 °C for 16 h and then evaporated. The syrupy residue was chromatographed on a silica gel column in MeOH/$CHCl_3$ (20% → 40%). The combined fractions containing product were evaporated and dissolved in hot water (2 ml, drop of 10% aqueous ammonia was added for complete dissolution). The solution was neutralized with aqueous hydrochloric acid to pH ≈ 3. Crystallized product was filtered off and washed with water.

4.3.1. 3-(Hexadecyloxypropyl) ester of 9-[2-(phosphonomethoxy) ethyl]hypoxanthine (**5**)

Yield: 73 mg (64%). Mp 90–92 °C. Anal. Calcd for $C_{28}H_{52}N_4O_{6.5}P$ (hemihydrate): C, 58.01; H, 9.04; N, 9.66; P, 5.34. Found: C, 58.19; H, 9.04; N, 9.41; P, 5.14. ESI-MS, *m*/*z*: 571.4 (89) $[M + H]^+$, 593.4 (100) $[M + Na]^+$, 609.3 (62) $[M + K]^+$, 615.3 (40). ESI-HRMS calcd for $C_{28}H_{52}N_4O_5P$ 571.3619, found: 571.3617 $[M + H]^+$; calcd for $C_{28}H_{51}N_4O_6PNa$ 593.3438, found: 593.3434 $[M + Na]^+$. ^1H NMR (D_2O + NaOD, ppm) δ: 0.90 (t, 3H, $J_{23'-22'} = 7.1$), 1.24–1.35 (m, 26H, H-10′ to H-22′), 1.53 (m, 2H, H-9′), 1.79–1.87 (m, 4H, H-4′, H-6′), 3.37 (t, 2H, $J_{8'-9'} = 6.9$, H-8′), 3.47 (t, 2H, $J_{7'-6'} = 6.5$, H-7′), 3.60 (m, 2H, H-3′), 3.72 (t, 2H, $J_{2'-1'} = 5.1$, H-2′), 3.83 (q, 2H, $J_{5'-6} = J_{5'-P} = 6.6$, H-5′), 4.19 (br t, 2H, $J_{1'-2'} = 5.1$, H-1′), 7.88 (s, H-8), 8.01 (s, H-2). ^{13}C NMR (D_2O + NaOD, ppm) δ: 14.57 (C-23′), 23.31 (C-22′), 26.65 (C-10′), 27.79 (d, $J_{4'-P} = 131.5$, C-4′), 29.98, 30.15, 30.27, 30.48, 30.52, 30.56, 30.59, 30.61 (C-9′, C-11′ to C-20′), 31.14 (d, $J_{6'-P} = 6.3$, C-6′), 32.64 (C-21′), 43.79 (C-1′), 61.89 (d, $J_{5'-P} = 5.6$, C-5′), 66.83 (C-3′), 67.95 (C-7′), 68.68 (C-2′), 71.53 (C-8′), 123.60 (C-5), 140.96 (C-8), 150.22 (C-4), 153.96 (C-2), 168.13 (C-6).

*4.3.2. 3-(Hexadecyloxypropyl) ester of 9-[2-(phosphonomethoxy) ethyl]guanine (**6**)*

Yield: 63 mg (54%). Mp 243–247 °C. Anal. Calcd for $C_{28}H_{53}N_5O_{6.5}P$ (hemihydrate): C, 56.55; H, 8.98; N, 11.78; P, 5.21. Found: C, 56.92; H, 9.31; N, 11.42; P, 4.95. ESI-MS, *m*/*z*: 586.4 (97) $[M + H]^+$, 608.4 (100) $[M + Na]^+$, 630.3 (51), 652.3 (32). ESI-HRMS calcd for $C_{28}H_{53}N_5O_6P$ 586.3728, found: 586.3726 $[M + H]^+$; calcd for $C_{28}H_{52}N_5O_6PNa$ 608.3547, found: 608.3542 $[M + Na]^+$. ^{1}H NMR (D_2O + NaOD, ppm) δ: 0.87 (t, 3H, $J_{23'-22'} = 7.0$, H-23′), 1.22–1.31 (m, 26H, H-10′ to H-22′), 1.54 (m, 2H, H-9′), 1.84–1.91 (m, 4H, H-4′, H-6′), 3.39 (t, 2H, $J_{8'-9'} = 7.0$, H-8′), 3.50 (t, 2H, $J_{7'-6'} = 6.6$, H-7′), 3.64 (m, 2H, H-3′), 3.72 (br t, 2H, $J_{2'-1'} = 5.1$, H-2′), 3.87 (q, 2H, $J_{5'-6'} = J_{5'-P} = 6.5$, H-5′), 4.10 (br t, 2H, $J_{1'-2'} = 5.1$, H-1′), 7.64 (s, 1H, H-8). ^{13}C NMR (D_2O + NaOD, ppm) δ: 14.59 (C-23′), 23.22 (C-22′), 26.68 (C-10′), 27.80 (d, $J_{4'-P} = 132.1$, C-4′), 30.01, 30.17, 30.29, 30.49, 30.54, 30.58, 30.60, 30.63 (C-9′, C-11′ to C-20′), 31.15 (d, $J_{6'-P} = 6.3$, C-6′), 32.65 (C-21′), 43.38 (C-1′), 61.91 (d, $J_{5'-P} = 5.5$, C-5′), 66.79 (C-3′), 67.98 (C-7′), 68.79 (C-2′), 71.56 (C-8′), 117.83 (C-5), 138.91 (C-8), 151.95 (C-4), 160.88 (C-2), 167.82 (C-6).

4.4. General method D

A mixture of 6-*O*-benzylpurine (**9**) or 2-amino-6-*O*-benzylpurine (**10**) (20 mmol, 4.52 g or 4.82 g, respectively), *S*-(−)-glycidol trityl ether (6.33 g, 20 mmol), Cs_2CO_3 (800 mg, 2.5 mmol) in DMF (100 ml) was heated to 100 °C for 10 h. The solution was evaporated and the syrupy residue was chromatographed on a silica gel column (500 g) in EtOAc/toluene (1:3).

*4.4.1. 9-(S)-(2-Hydroxy-3-trityloxypropyl)-6-O-benzylpurine (**11**)*

Yield 4.60 g (42%). Crystallization from toluene. Mp 180 °C. Anal. Calcd for $C_{34}H_{30}N_4O_3$: C, 75.26; H, 5.57; N, 10.33. Found: C, 75.34; H, 5.62; N, 10.15.

*4.4.2. 9-(S)-(2-Hydroxy-3-trityloxypropyl)-2-amino-6-O-benzylpurine (**12**)*

Yield 4.89 g (44%). Crystallization from toluene. Mp 177 °C, Anal. Calcd for $C_{34}H_{31}N_5O_3$: C, 73.23; H, 5.60; N, 12.56. Found: C, 73.15; H, 5.62; N, 12.46.

4.5. General method E

A mixture of **11** or **12** (2.4 mmol, 1.30 g or 1.34 g respectively) and **8** (2.66 g, 3.6 mmol) in anhydrous DMF (20 ml) was treated with NaH (60% suspension in mineral oil, 290 mg, 7.2 mmol) while cooling down (−25 °C). The mixture was stirred at a room temperature overnight and then heated to 35 °C for 8 h. The mixture was then neutralized with acetic acid (0.27 ml, 4.8 mmol) and partitioned between chloroform (100 ml) and aqueous saturated solution of NaCl (100 ml). The aqueous portion was extracted with chloroform again (50 ml). The combined organic portions were dried with $MgSO_4$ evaporated and the syrupy residue was chromatographed on a silica gel column (200 g) in gradient EtOAc/toluene (1:1) → EtOAc. Syrupy trityl derivative was dissolved in Et_2O/MeOH (1:1, 10 ml). The solution was acidified with methanolic solution of hydrogen chloride to pH 2–3 and stirred for 48 h. The product crystallized overnight in a fridge. The mother liquors were concentrated and chromatographed on a silica gel column in methanol/chloroform/hexane (1:5:5).

*4.5.1. Bis 3-(hexadecyloxypropyl) ester of 9-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]hypoxanthine (**13**)*

Yield 1.27 g (61%). Mp 86 °C. Anal. Calcd for $C_{47}H_{89}N_4O_8P$: C, 64.95; H, 10.32; N, 6.45; P, 3.56. Found: C, 64.99; H, 10.42; N, 6.44; P, 3.73; ESI-MS, *m*/*z*: 869.6 (24) $[M + H]^+$, 891.7 (100) $[M + Na]^+$. ESI-HRMS calcd for $C_{47}H_{90}N_4O_8P$ 869.6491, found: 869.6490 $[M + H]^+$; calcd for $C_{47}H_{89}N_4O_8PNa$ 891.6310, found: 891.6311 $[M + Na]^+$. ^{1}H NMR ($CDCl_3$ + MeOD, ppm) δ: 0.89 (t, 6H, $J_{23'-22'} = 7.0$, H-23′), 1.23–1.35 (m, 52H, H-10′ to H-22′), 1.56 (m, 4H, H-9′), 1.92 (m, 4H, H-6′), 3.42 (m, 4H, H-7′), 3.50 (m, 4H, H-8′), 3.61 (dd, 1H, $J_{gem} = 12.3$, $J_{3'-2'} = 4.7$, H-3′b), 3.69 (dd, 1H, $J_{gem} = 12.3$, $J_{3'-2'} = 4.8$, H-3′a), 3.87 (m, 1H, H-2′), 3.89 (dd, 1H, $J_{gem} = 13.8$, $J_{4'-P} = 9.1$, H-4′b), 4.01 (dd, 1H, $J_{gem} = 13.8$, $J_{4'-P} = 8.9$, H-4′a), 4.10–4.18 (m, 4H, H-5′), 4.37 (dd, 1H, $J_{gem} = 14.6$, $J_{1'-2'} = 7.0$, H-1′b), 4.49 (dd, $J_{gem} = 14.6$, $J_{1'-2'} = 4.0$, H-1′a), 8.00 (s, 1H, H-2), 8.04 (s, 1H, H-8). ^{13}C NMR ($CDCl_3$ + MeOD, ppm) δ: 12.97 (C-23′), 21.91 (C-22′), 25.43 (C-10′), 28.63–28.96 (m, C-9′, C-11′ to C-20′), 29.97 (d, $J_{6'-P} = 6.0$, C-6′), 29.98 (d, $J_{6'-P} = 6.0$, C-6′), 31.21 (C-21′), 43.65 (C-1′), 59.64 (C-3′), 62.61 (d, $J_{4'-P} = 167.6$, C-4′), 63.39 (d, $J_{5'-P} = 6.7$, C-5′), 63.55 (d, $J_{5'-P} = 6.6$, C-5′), 65.66, 65.68 (C-7′), 70.48 (C-8′), 80.16 (d, $J_{2'-P} = 11.3$, C-2′), 123.14 (C-5), 140.79 (C-8), 144.61 (C-2), 148.38 (C-4), 156.85 (C-6).

*4.5.2. Bis 3-(hexadecyloxypropyl) ester of 9-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]guanine (**14**)*

Yield 0.68 g (32%). Mp 166–168 °C. Anal. Calcd for $C_{47}H_{92}N_5O_9P$ (monohydrate): C, 62.57; H, 10.28; N, 7.76; P, 3.43. Found: C, 62.79; H, 10.18; N, 7.69; P, 3.41. ESI-MS, *m*/*z*: 301.1 (68), 884.6 (61) $[M + H]^+$, 906.7 (100) $[M + Na]^+$. ESI-HRMS calcd for $C_{47}H_{91}N_5O_8P$ 884.6600, found: 884.6606 $[M + H]^+$; calcd for $C_{47}H_{90}N_5O_8PNa$ 906.6419, found: 906.6425 $[M + Na]^+$. ^{1}H NMR ($CDCl_3$ + MeOD, ppm) δ: 0.85 (m, 6H, H-23′), 1.18–1.32 (m, 52H, H-10′ to H-22′), 1.52 (m, 4H, H-9′), 1.89 (m, 4H, H-6′), 3.38 (m, 4H, H-8′), 3.47 (m, 4H, H-7′), 3.56 (m, 2H, H-3′), 3.76 (m, 1H, H-2′), 3.85–3.96 (m, 2H, H-4′), 4.10–4.18 (m, 5H, H-1′b, H-5′), 4.25 (m, 1H, H-1′a), 7.74 (s, 1H, H-8). ^{13}C NMR ($CDCl_3$ + MeOD, ppm) δ: 13.40 (C-23′), 22.17 (C-22′), 25.64 (C-10′), 28.87–29.20 (m, C-9′, C-11′ to C-20′), 30.22 (d, $J_{6'-P} = 5.9$, C-6′), 31.44 (C-21′), 43.07 (C-1′), 59.54 (C-3′), 62.35–63.87 (m, C-4′, C-5′), 65.93 (C-7′), 70.81 (C-8′), 80.66 (d, $J_{2'-P} = 10.9$, C-2′), 115.00 (C-5), 138.30 (C-8), 151.09 (C-4), 153.52 (C-2), 157.01 (C-6).

4.6. General method F

To a solution of **13** or **14** (0.5 mmol, 435 mg or 442 mg, respectively) in dioxane (25 ml) an aqueous solution of NaOH (2M, 0.5 ml, 1.0 mmol) was added and the mixture was stirred for 48 h. The solution was neutralized with hydrochloric acid (1 M, 1.1 ml, 1.1 mmol). Mixture was evaporated, codistilled with toluene and the residue was chromatographed on a silica gel column (20 g) in methanol/chloroform (1:4 → 1:2).

*4.6.1. 3-(Hexadecyloxypropyl) ester of 9-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]hypoxanthine (**15**)*

Yield: 237 mg (81%). Mp 199–202 °C. ESI-MS, *m*/*z*: 585.5 (100) $[M - H]^-$. ESI-HRMS calcd for $C_{28}H_{50}N_4O_7P$ 585.3423, found: 585.3421 $[M - H]^-$. HPLC purity 97.36% (254 nm). ^{1}H NMR (D_2O + NaOD, ppm) δ: 0.88 (t, 3H, $J_{23'-22'} = 7.8$, H-23′), 1.20–1.33 (m, 26H, H-10′ to H-22′), 1.55 (m, 2H, H-9′), 1.86 (m, 2H, H-6′), 3.38–3.74 (m, 8H, H-3′, H-4′, H-7′, H-8′), 3.91 (m, 2H, H-5′), 4.15, 4.24 (m, m, 2H, H-1′), 7.92 (s, 1H, H-2), 8.00 (s, 1H, H-8). ^{13}C NMR (D_2O + NaOD, ppm) δ: 14.47 (C-23′), 23.22 (C-22′), 26.61 (C-10′), 29.97–30.55 (m, C-9′, C-11′ to C-20′), 31.22 (C-6′), 32.54 (C-21′), 44.28 (C-1′), 60.87 (C-3′), 62.80 (d, $J_{5'-P} = 5.3$, C-5′), 65.67 (d, $J_{4'-P} = 158.0$, C-4′), 67.91 (C-7′), 71.52 (C-8′), 80.98 (d, $J_{2'-P} = 11.8$, C-2′), 123.44 (C-5), 141.38 (C-8), 150.39 (C-4), 154.03 (C-2), 168.18 (C-6).

*4.6.2. 3-(Hexadecyloxypropyl) ester of 9-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]guanine (**16**)*

Yield 265 mg (88%). Mp 235–238 °C. Anal. Calcd for $C_{28}H_{54}N_5O_8P$ (monohydrate): C, 54.27; H, 8.78; N, 11.30; P, 5.00. Found: C, 54.16; H,

8.68; N, 11.29; P, 5.25. ESI-MS, *m/z*: 249.0 (22), 600.4 (100) $[M - H]^-$. ESI-HRMS calcd for $C_{28}H_{51}N_5O_7P$ 600.3532, found: 600.3526 $[M - H]^-$. ^1H NMR (D_2O + NaOD, ppm) δ: 0.87 (m, 3H, H-23′), 1.19–1.34 (m, 26H, H-10′ to H-22′), 1.55 (m, 2H, H-9′), 1.87 (m, 2H, H-6′), 3.39–3.72 (m, 8H, H-3′, H-4′, H-7′, H-8′), 3.92 (m, 2H, H-5′), 4.01–4.14 (m, 2H, H-1′), 7.64 (s, 1H, H-8). ^{13}C NMR (D_2O + NaOD, ppm) δ: 14.53 (C-23′), 23.28 (C-22′), 26.66 (C-10′), 30.00–30.63 (m, C-9′, C-11′ to C-20′), 31.22 (d, $J_{6'-P} = 5.3$, C-6′), 32.61 (C-21′), 43.79 (C-1′), 60.70 (C-3′), 62.84 (d, $J_{5'-P} = 5.4$, C-5′), 65.63 (d, $J_{4'-P} = 158.7$, C-4′), 67.95 (C-7′), 71.56 (C-8′), 80.97 (d, $J_{2'-P} = 11.9$, C-2′), 117.80 (C-5), 139.19 (C-8), 152.07 (C-4), 161.43 (C-2), 168.59 (C-6).

4.7. General method G

Hünig's base (0.4 mmol, 70 μl) was added to a mixture of **15** or **16** (0.2 mmol, 117 mg or 120 mg respectively), PyBOP (0.4 mmol, 208 mg) in DMF (1 ml). The suspension was sonicated until it dissolved and then kept at room temperature overnight. The mixture was evaporated and the syrupy residue was chromatographed on a silica gel column (50 g) in 12% MeOH/$CHCl_3$. The product was crystallized from methanol.

4.7.1. 3-(Hexadecyloxypropyl) ester of 9-{[(5S)-2-hydroxy-2-oxido-1,4,2-dioxaphosphinan-5-yl]methyl}hypoxanthine (**17**)

Yield: 76 mg (67%). Mp 121–180 °C. ESI-MS, *m/z*: 569.3 (48) $[M + H]^+$, 591.4 (100) $[M + Na]^+$, 628.1 (13), 688.9 (16), 808.6 (78), 1159.1 (14) $[2M + Na]^+$. ESI-HRMS calcd for $C_{28}H_{50}N_4O_6P$ 569.34625, found: 569.34645 $[M + H]^+$. HPLC purity 99.23% (254 nm). ^1H NMR ($CDCl_3$ + MeOD, ppm) δ: 0.89 (t, 3H, $J_{23'-22'} = 7.0$, H-23′), 1.24–1.35 (m, 26H, H-10′ to H-22′), 1.56 (m, 2H, H-9′), 1.96 (m, 2H, H-6′), 3.42 (m, 2H, H-8′), 3.52 (m, 2H, H-7′), 3.91–3.98 (m, 1H, H-4′b), 4.17–4.56 (m, 8H, H-1′, H-2′, H-3′, H-4′a, H-5′), 7.99 (s, 0.5H, H-8), 8.02 (s, 1H, H-2), 8.04 (s, 0.5H, H-8). ^{13}C NMR ($CDCl_3$ + MeOD, ppm) δ: 13.00 (C-23′), 21.92 (C-22′), 25.39, 25.44 (C-10′), 28.63–28.96 (m, C-9′, C-11′ to C-20′), 29.84 (d, $J_{6'-P} = 6.1$, C-6′), 29.98 (d, $J_{6'-P} = 5.7$, C-6′), 31.22 (C-21′), 42.36, 42.65 (C-1′), 62.03 (d, $J_{4'-P} = 147.6$, C-4′), 62.34 (d, $J_{4'-P} = 145.1$, C-4′), 62.78 (d, $J_{5'-P} = 6.4$, C-5′), 64.01 (d, $J_{5'-P} = 6.8$, C-5′), 65.48, 65.50 (C-7′), 70.21 (d, $J_{3'-P} = 7.0$, C-3′), 70.47, 70.52 (C-8′), 71.51 (d, $J_{3'-P} = 8.5$, C-3′), 72.96 (d, $J_{2'-P} = 4.9$, C-2′), 73.49 (d, $J_{2'-P} = 5.3$, C-2′), 123.06, 123.16 (C-5), 140.49, 140.56 (C-8), 144.90 (C-2), 148.18 (C-4), 156.77, 156.81 (C-6).

4.7.2. 3-(Hexadecyloxypropyl) ester of 9-{[(5S)-2-hydroxy-2-oxido-1,4,2-dioxaphosphinan-5-yl]methyl}guanine (**18**)

Yield: 70 mg (60%). Mp >300 °C (decomp.). ESI-MS, *m/z*: 505.2 (12), 584.4 (77) $[M+H]^+$, 606.4 (100) $[M+Na]^+$, 628.4 (18). ESI-HRMS calcd for $C_{28}H_{51}N_5O_6P$ 584.35715, found: 584.35689 $[M + H]^+$. HPLC purity 97.40% (254 nm). ^1H NMR ($CDCl_3$ + MeOD, ppm) δ: 0.89 (t, 3H, $J_{23'-22'} = 6.9$, H-23′), 1.23–1.35 (m, 26H, H-10′ to H-22′), 1.56 (m, 2H, H-9′), 1.95 (m, 2H, H-6′), 3.42 (m, 2H, H-8′), 3.52 (m, 2H, H-7′), 3.92–4.00 (m, 1H, H-4′b), 4.09–4.32 (m, 6.5H, H-1′, H-2′, H-3′, H-4′a, H-5′), 4.39–4.53 (m, 1.5H, H-3′), 7.67 (s, 0.5H, H-8), 7.74 (s, 0.5H, H-8). ^{13}C NMR ($CDCl_3$ + MeOD, ppm) δ: 12.78 (C-23′), 21.80 (C-22′), 25.28, 25.35 (C-10′), 28.51–28.85 (m, C-9′, C-11′ to C-20′), 29.73 (d, $J_{6'-P} = 5.9$, C-6′), 29.87 (d, $J_{6'-P} = 5.6$, C-6′), 31.10 (C-21′), 41.53, 41.98 (C-1′), 61.90 (d, $J_{4'-P} = 147.0$, C-4′), 62.15 (d, $J_{4'-P} = 144.8$, C-4′), 62.57 (d, $J_{5'-P} = 6.4$, C-5′), 63.80 (d, $J_{5'-P} = 6.8$, C-5′), 65.33, 65.39 (C-7′), 70.30 (d, $J_{3'-P} = 6.5$, C-3′), 70.31, 70.36 (C-8′), 71.61 (d, $J_{3'-P} = 8.7$, C-3′), 72.74, 73.37 (d, $J_{2'-P} = 5.0$, C-2′), 114.89, 115.03 (C-5), 137.74, 137.97 (C-8), 150.93 (C-4), 153.39 (C-2), 157.03 (C-6).

4.8. 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, feline herpesvirus, vaccinia virus Lederle strain, human immunodeficiency virus (HIV) type 1 (IIIB) and type 2 (ROD), respiratory syncytial virus (RSV) strain Long, vesicular stomatitis virus (VSV), Coxsackie B4, Parainfluenza 3, Reovirus-1, *Sindbis*, Punta Toro, feline coronavirus, influenza A virus subtypes H1N1 and H3N2, and influenza B virus 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), Crandell-Rees feline kidney cells (CRFK), or Madin Darby canine kidney cells (MDCK). Confluent cell cultures in microtiter 96-well plates were inoculated with 100 $CCID_{50}$ of virus (1 CCID50 being the virus dose to infect 50% of the cell cultures) or with 20 plaque forming units (PFU). After a 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 required to reduce virus-induced cytopathogenicity or viral plaque formation by 50%. The methodology of the anti-HIV assays was as follows: human CEM cells ($\sim 3 \times 105$ cells/cm^3) were infected with 100 $CCID_{50}$ of HIV-1(IIIB) or HIV-2(ROD)/ml and seeded in 200-μL wells of a 96-well 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.

4.9. 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 adhere and proliferate for 24 h. Then, medium containing different concentrations of the test compounds was added. After 3 days of further incubation at 37 °C, the cell number was determined with a Coulter counter. The cytostatic concentration was calculated as the CC_{50}, or the compound concentration required reducing cell proliferation by 50% relative to the number of cells in the untreated controls. 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. Selectivity indexes were calculated as the ratio CC_{50} to EC_{50}.

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