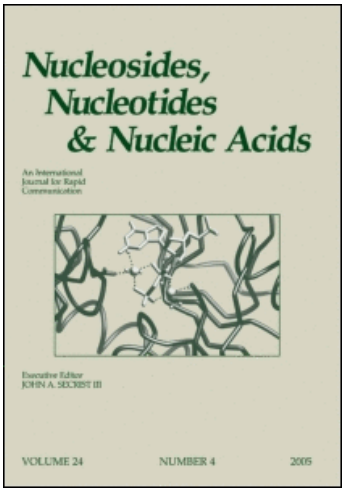

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Synthesis, ^{18}F-Radiolabelling and Biological Evaluations of C-6 Alkylated Pyrimidine Nucleoside Analogues

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ABSTRACT

Synthesis of pyrimidine derivatives with a side-chain attached to the C-6 of pyrimidine ring (**6–14**) is reported. Target compounds **8** and **12** were subjected to in vitro phosphorylation tests, determination of their binding affinities to herpes simplex virus (HSV-1) thymidine kinase (TK) and catalytic turnover constants. Fluorinated pyrimidine derivative **12** (40 μM) exhibited better binding affinity for HSV-1 TK than acyclovir (ACV, 170 μM) and ganciclovir (GCV, 48 μM). Catalytic turnover constant (k_{cat}) of **12** (0.08 s^{-1}) was close to the k_{cat} values of ACV (0.10 s^{-1}) and GCV (0.10 s^{-1}). Furthermore, compounds **8** and **12** showed no cytotoxic effects in HSV-1 TK-transduced and non-transduced cell lines. Besides, compounds **8** and **12** did not exhibit antiviral or cytostatic activities against several viruses and malignant tumor

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cell lines that were evaluated. The new fluorinated pyrimidine derivative **16** that is phosphorylated by HSV-1 TK could be developed as non-toxic PET-tracer molecule. Thus, ^{18}F labelling of the precursor **14** was performed by nucleophilic substitution using [^{18}F] tetrabutylammonium fluoride as the fluorinating reagent.

Key Words: Pyrimidine nucleoside analogues; ^{18}F-radiolabelling; HSV-1 TK; Phosphorylation; Binding affinity.

INTRODUCTION

Acyclic nucleoside analogues are known to be potent anti-herpesvirus agents.[1] Among these agents, acyclovir and ganciclovir were reported to be efficient antiviral agents with low host toxicity,[2,3] ganciclovir being the more active against herpes simplex virus type 1 (HSV-1) and 2 (HSV-2).[4,5] Acyclovir and ganciclovir are quite selective as compared to the other nucleoside antiviral agents.[2] The selectivity of these acyclic nucleoside analogues is due, in part, to the fact that they are phosphorylated only in virus-infected cells, where a virus specific thymidine kinase of low substrate specificity converts the nucleoside analogues to their monophosphate derivatives.[5] The monophosphates are converted to diphosphates and then to the corresponding triphosphates by cellular enzymes.[4] The triphosphates prevent viral replication by inhibition of the viral DNA polymerase. Fluoro derivatives of ganciclovir and penciclovir, *i.e.* 9-[(3-fluoro-1-hydroxy-2-propoxy)methyl]guanine and 9-(4-fluoro-3-hydroxymethylbutyl)guanine were evaluated as tracers for non-invasive positron emission tomography (PET) imaging of herpes simplex virus type 1 thymidine kinase (HSV-1 TK) gene expression.[6–9]

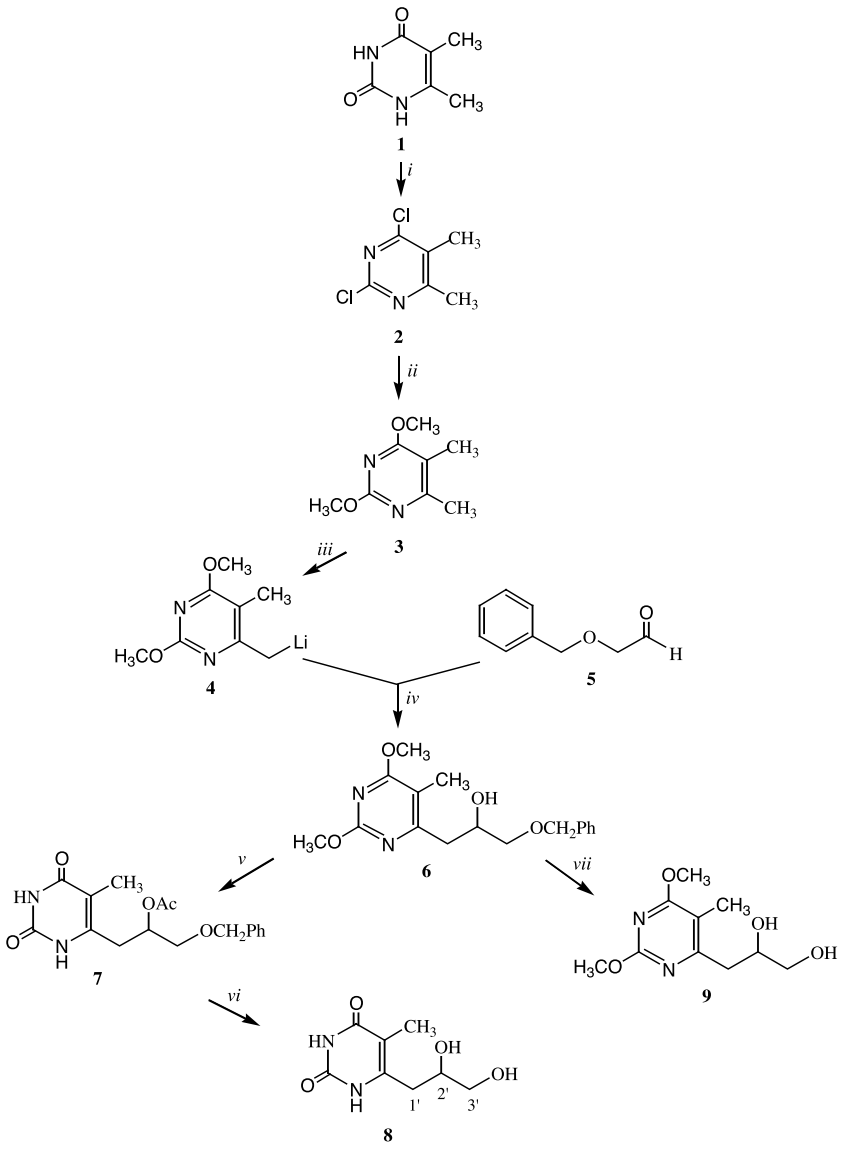
Previous studies have shown that C-6 alkylated pyrimidine derivatives, *i.e.* 6-hydroxypropylthymine[10] and 6-(3-hydroxy-2-hydroxymethylpropyl)thymine, have good binding affinities for HSV-1 TK and no cytotoxic effects. Furthermore, the crystal structures of these two compounds in complex with HSV-1 TK (refined to 2.2 Å) were solved and confirmed to their excellent substrate-binding affinities.[11] These findings led us to synthesize new C-6 alkylated pyrimidine derivatives which might find application as new non-toxic PET-tracer molecules that are specifically and efficiently phosphorylated by the HSV-1 TK.

In this paper, we describe the synthesis of novel pyrimidine derivatives containing 2,3-dihydroxypropyl (**8**) and 2-fluoro-3-hydroxypropyl (**12**) side-chains attached to C-6 of the pyrimidine moiety, the evaluation of their cytotoxic properties, binding affinities to HSV-1 TK and kinetic turnover constants. Furthermore, we report the labelling of the nucleoside precursors with protected functional groups in the side-chain with ^{18}F.

RESULTS AND DISCUSSION

Chemistry

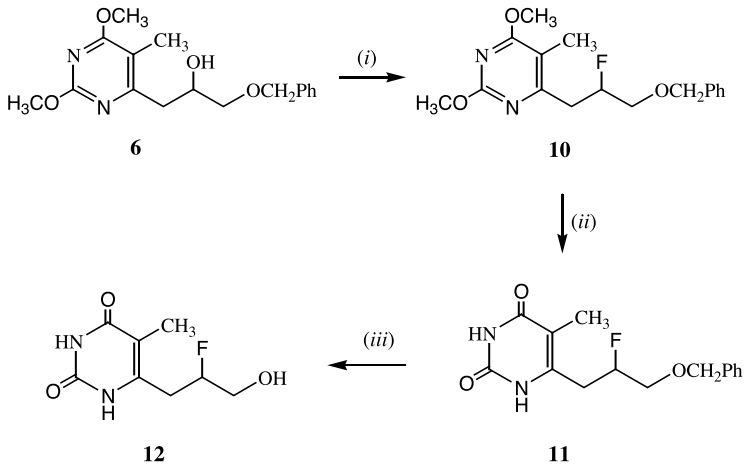
The pyrimidine derivatives containing an acyclic side-chain attached to the C-6 of the pyrimidine ring (**6–14**) were synthesized (Schemes 1 and 2).



Scheme 1. Reagents and conditions: (*i*) $POCl_3$, reflux; (*ii*) $NaOCH_3$, MeOH, r.t.; (*iii*) *n*-BuLi, THF; (*iv*) −50°C, acetic acid; (*v*) AcCl, H_2O; (*vi*) 1. BCl_3, CH_2Cl_2, 2. $NaHCO_3$; (*vii*) BCl_3, CH_2Cl_2.

Chlorination of 2,4-dihydroxy-5,6-dimethylpyrimidine (**1**) with phosphoryl chloride afforded 2,4-dichloro-5,6-dimethylpyrimidine (**2**). Compound **2** was then treated with sodium methoxide in methanol to give 2,4-dimethoxy-5,6-dimethylpyrimidine (**3**) according to the procedure described in the literature.[12]

The key intermediate, 6-(3-benzyloxy-2-hydroxypropyl)-2,4-dimethoxy-5-methyl-pyrimidine (**6**) was prepared *via* an addition reaction of the lithiated 2,4-dimethoxy-5,

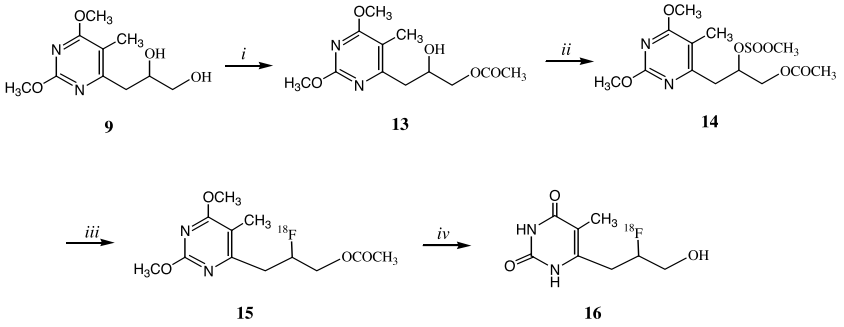


Scheme 2. Reagents and conditions: (*i*) DAST, CH_2Cl_2; (*ii*) AcCl, H_2O; (*iii*) BCl_3, CH_2Cl_2.

6-dimethylpyrimidine (**4**) with benzyloxyacetaldehyde (**5**),[13] according to an analogous procedure described in the literature.[14] Attempts to hydrolyze the 2- and 4-methoxy groups of **6** in methanolic hydrogen chloride or a mixture of aqueous sodium hydroxide were unsuccessful. However, treatment of **6** with acetyl chloride, containing several drops of water, effected a conversion to the 2,4-dihydroxypyrimidine derivative **7**. Debenzylation of **7** was achieved by using boron trichloride in dichloromethane, while deacetylation was accomplished with sodium hydrogen carbonate to produce 6-(2, 3-dihydroxypropyl)pyrimidine-2,4-dione (**8**, Scheme 1).

Fluorination: Compound **10** was obtained by the fluorination of **6** using diethylaminosulfur trifluoride (DAST) as the fluorinating reagent (Scheme 2).[15]

Deprotection of the methoxy groups in **10** and, in the next step, the benzyloxy group in **11** were carried out with acetyl chloride and boron trichloride, respectively, to give the target compound **12** (Scheme 2).



Scheme 3. Reagents and conditions: (*i*) AcCl/CH_2Cl_2; (*ii*) CH_3SO_2Cl/pyridine; (*iii*) [^{18}F]Bu_4NF/DMSO; (*iv*) HCl, H_2O.

Radiochemistry

Preparation of the precursor **14** for ^{18}F labelling is outlined in Scheme 3. To avoid intramolecular cyclization and formation of conformationally constrained carbon-bridged pyrimidine nucleosides,[16] a precursor with protected carbonyl and hydroxyl groups was synthesized.

The primary hydroxyl group in **9** was protected with an acetyl group (**13**), while the secondary hydroxyl group was converted to the mesylate **14**. The radiosynthesis of the ^{18}F-labelled compound, ^{18}F-HHT was performed *via* a two-step reaction sequence. The first step, a classical nucleophilic substitution on a mesyl leaving, was accomplished using [^{18}F]-tetrabutylammonium fluoride as the fluorinating agent in DMSO. The second step involving the removal of the protecting groups was achieved byhydrolysis with aqueous HCl. The radiochemical yield was rather low and did not exceed 1%. A probable reason might be the instability of the precursor under basic conditions.

The structures of the compounds **2**, **3**, and **6–14** were confirmed by analyses of their ^1H and ^{13}C NMR spectra. The ^1H NMR spectroscopic data of the **2**, **3**, and **6–14** are displayed in Table 1.

The chemical shifts and coupling constants are consistent with the proposed structures. The ^{13}C NMR data are given in the Experimental Section. Presence of the fluorine atom in **10–12** was confirmed in ^{13}C NMR spectra which show characteristic fluorine-carbon interactions with one-bond (at C-2′) and two-bonds (at C-1′ and C-3′) coupling constants of *ca.* 175 Hz and *ca*. 22 Hz, respectively.[17] The chemical shift assignment of novel compounds agrees with these for the related acyclic pyrimidine nucleoside analogues.[15,16]

Biological Evaluations of Compound 8 and Its Fluorinated Derivative 12

Phosphorylation Pattern

To explore the substrate acceptance of HSV-1 TK, new compounds with a 2, 3-dihydroxypropyl (**8**) or 2-fluoro-3-hydroxypropyl side-chain (**12**) were subjected to in vitro phosphorylation tests and determination of binding affinity with HSV-1 TK.

Phosphorylation of compounds **8** and **12** was monitored by the formation of their monophosphorylated derivatives using DAD (Diode Array Detector) HPLC. Comparison of the phosphorylation patterns of compound **8** with HSV-1 TK and without HSV-1 TK showed, besides ATP [retention time (RT)=16.9 min] and **8** (RT=4.8 min), the formation of two new compounds, the ADP and monophosphate derivative of **8** (Fig. 1). The decrease of the peak intensity of compound **8**, while phosphorylation occurred, was also observed. Similar to that, monitoring of phosphorylation of **12** revealed the formation of ADP (RT=5.4 min) and a decrease of the peak intensity for compound **12** (RT=7.4 min, Fig. 1). In the chromatogram, peak of monophosphorylated compound **12** together with ATP peak had retention time of 12.7 min.

The results of the analyses clearly showed the formation of new peaks corresponding to the monophosphate derivatives of **8** (RT=7.1 min) and **12** (RT=12.9 min). Thus, this suggests that compounds **8** and **12** are indeed substrates for HSV-1 TK.

Table 1. Chemical shifts (δ, ppm)[a] and H–H coupling constants (J, Hz) in ^{1}H NMR spectra of compounds **2**, **3**, **6–14**.

Compd.	5-CH_3	CH_3	2-OCH_3 3-OCH_3	C$_6$H$_5$	CH_2Ph	1′-CH_2	2′-CH_2	3′-CH_2	OH	1-NH 3-NH
6	2.0 (3H, s)	–	3.82 (3H, s) 3.88 (3H, s)	7.36–7.25 (5H, m)	4.49 (2H, s)	2.73 (2H) *J*=6.5 (d)	4.11 (1H, m)	3.41 (2H) *J*=5.4 (d)	4.86 (1H) *J*=5.3 (d)	–
7	1.74 (3H, s)	1.95 (3H, s)	–	7.36–7.32 (5H, m)	4.51 (2H, s)	2.76 (2H, m)	5.26 (1H, m)	3.55 (2H) *J*=4.8 (d)	–	10.97 (1H, s) 10.62 (1H, s)
8	1.74 (3H, s)	–	–	–	–	2.42 (2H, m)	3.72 (1H, m)	3.42 (2H, m)	4.86 (1H) *J*=5.2 (d) 4.7 (1H) *J*=5.7 (t)	10.91 (1H, s) 10.36 (1H, s)
9	2.01 (3H, s)	–	3.83 (3H, s) 3.88 (3H, s)	–	–	2.68 (2H, m)	3.9 (1H, m)	3.35 (2H, m)	4.63 (2H, m)	–
10	2.0 (3H, s)	–	3.82 (3H, s) 3.89 (3H, s)	7.38–7.28 (5H, m)	4.54 (2H, s)	3.01 (2H, m)	5.08 (1H, m)	3.69 (2H, m)	–	–
11	1.70 (3H, s)	–	–	7.36–7.26 (5H, m)	4.51 (2H, s)	2.76 (2H, m)	4.81 (1H, m)	3.59 (2H, m)	–	11.00 (1H, s) 10.60 (1H, s)
12	1.74 (3H, s)	–	–	–	–	2.73 (2H, m)	4.70 (1H, m)	3.52 (2H, m)	5.11 (1H, m)	11.02 (1H, s) 10.62 (1H, s)
13	2.0 (3H, s)	2.01 (3H, s)	3.83 (3H,s) 3.88 (3H, s)	–	–	2.75 (2H, m)	4.15 (1H, m)	3.95 (2H, m)	5.05 (1H) *J*=5.4 (d)	–
14	2.02 (3H, s)	2.05 (3H, s) 3.09 (3H, s)	3.86 (3H,s) 3.90 (3H, s)	–	–	3.15 (2H, m)	5.28 (1H, m)	4.29 (2H, m)	–	–

(*cf.* Schemes 1-3).

[a]DMSO-d_6 solutions. Multiplicity of couplings and number of protons are given in parentheses: s, singlet; d, doublet; m, complex multiplet. Chemical shifts for **2**: 2.26 (s, 3H), 5-CH_3 and 2.47 ppm (s, 3H), 6-CH_3. Chemical shifts for **3**: 1.98 (s, 3H), 5-CH_3; 2.30 (s, 3H), 6-CH_3; 3.83 and 3.87 ppm (s, 3H), 2-OCH_3, 3-OCH_3.

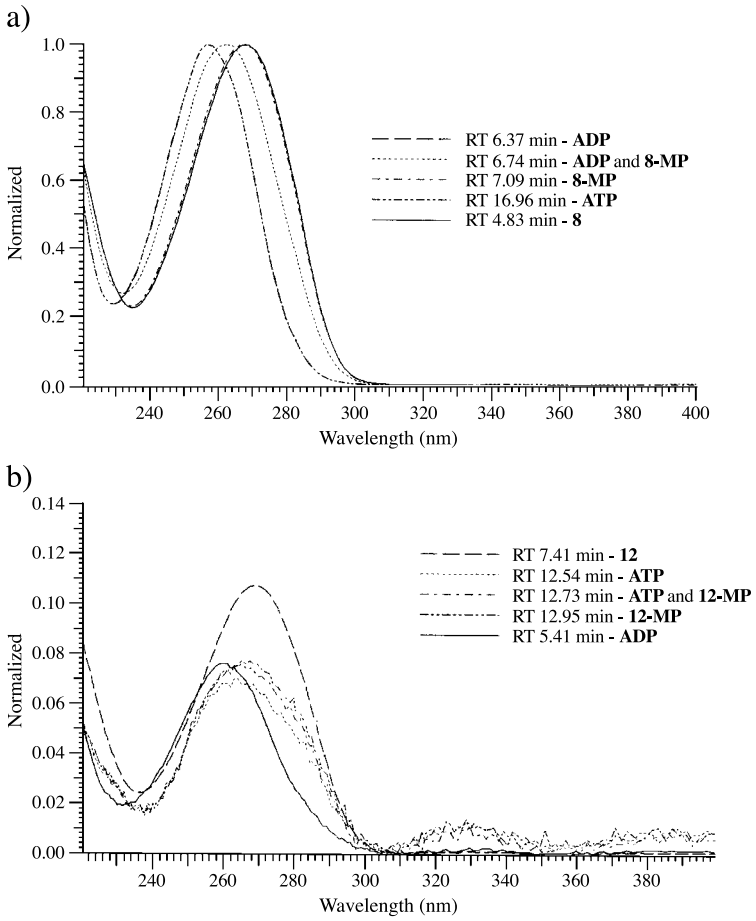


Figure 1. Chromatogram analyses of **8** (a) and **12** (b) with retention times (RT).

Binding Affinity Assays

To evaluate the binding affinities of compounds **8** and **12**, competition studies were performed using radioactively labelled thymidine and the compounds **8** and **12** as competitive ligands. Initial velocity (V_i) was determined by monitoring the increase of radioactively labelled dTMP over time in the presence of various concentrations of the compounds. The affinities of **8** and **12** were calculated as K_i values (Table 2).

Comparison of the binding affinities for **8** and **12** showed that the 2-fluorinated pyrimidine derivative **12** had a better affinity (K_i=39.54 μM) for the HSV-1 TK than its dihydroxy compound **8** (K_i=62.35 μM). It is interesting to note that **12** exhibited better affinity for HSV-1 TK than acyclovir (ACV) and ganciclovir (GCV) (Table 2).

A Spectrophotometric Assay for Quantitative Determination of k_{cat} of HSV-1 TK

The transfer of the γ-phosphate of ATP to the substrate, as catalysed by HSV-1 TK represents the first and limiting step of coupled reaction. The last step of the cascade,

Table 2. Binding affinities (K_i) and catalytic turnover constants (k_{cat}) of the compounds **8** and **12** with HSV-1 TK.

Compd.	K_i [μM]	k_{cat} [s^{-1}]	Specificity[a]
8	62.35 ± 5.6	0.04 ± 0.01	0.00036
12	39.54 ± 3.5	0.08 ± 0.01	0.00115
dT[18,19]	0.2	0.35	1
ACV[18,19]	170	0.10	0.00034
GCV[18,20]	48	0.10	0.0012
PCV[21,22]	1.5	0.045	0.025

[a]The specificity is defined as [k_{cat}(ligand)/K_i(ligand)/ k_{cat}(dT)/K_M(dT)].

namely the consumption of the cofactor NADH by L-lactate dehydrogenase and formation of NAD^+, corresponds in an equimolar way to the initial phosphorylation of the HSV-1 TK substrate and leads to a decrease of absorbance at 340 nm. This method has been applied and further developed in a previous study[22] to characterize the catalytic turnover constant (k_{cat}) of several substrates of HSV-1 TK. This simple in vitro method is based on the correlation between the k_{cat} and the decrease of absorbance at 340 nm per μg of HSV-1 TK ($\Delta abs_{340nm} min^{-1}$ μg^{-1}). Deoxythymidine (dT), acyclovir (ACV), ganciclovir (GCV) and penciclovir (PCV) were chosen as reference compounds because their k_{cat} values have been reported in the literature. The following k_{cat} values were used for dT, ACV, GCV and PCV: 0.35 s^{-1}, 0.10 s^{-1}, 0.10 s^{-1} and 0.045 s^{-1}, respectively.

The $\Delta abs_{340nm} min^{-1}$ μg^{-1} values obtained for **8** and **12** were introduced in the calibration curve equation and their k_{cat} were calculated as described in the literature (Table 2).[21] The presence of compounds **8** and **12** induced a clear decrease in absorption, indicating that both compounds are substrates for HSV-1 TK.

The calculated k_{cat} value for **8** was 0.04 s^{-1} (Table 2). For the 2-fluorinated compound **12**, k_{cat} value was 0.08 s^{-1}, which was close to that of ACV and GCV (0.10 s^{-1}) and higher than that for PCV (0.045 s^{-1}). The specificity of HSV-1 TK for the fluorinated derivative **12** was three-fold higher than that for **8**. Furthermore, **12** and GCV had almost the same specificity to HSV-1 TK, while comparison of **12** with ACV showed that HSV-1 TK was three times more specific for **12** than for ACV.

Antiviral and Antitumor Evaluations

The compounds **6**, **8**, **9**, **11** and **12** were evaluated against HSV-1 (strain KOS), HSV-1 (TK-deficient strain KOS), HSV-2 (strain G), vaccinia virus and vesicular stomatitis virus. The compounds were also evaluated for their cytostatic activity against malignant tumor cell lines: murine leukemia (L1210) and human T-lymphocyte (Molt4/C8 and CEM) cells. No specific antiviral or antitumor activities were observed against any of the evaluated viruses or tumor cell lines. Target compounds **8** and **12** did not exhibit any cytotoxic effects (minimum cytotoxic concentration > 400 μg/ml).

Functional Assay

Cytotoxicity assays in HSV-1 TK-transduced and non-transduced osteosarcoma (MG63) and non-small lung cancer cells (H122 NSCLC) showed that both compounds **8** and **12** were not cytotoxic at the tested concentration of 200 μg/ml.

CONCLUSIONS

Novel pyrimidine derivatives with a 2,3-dihydroxypropyl (**8**) or 2-fluoro-3-hydroxypropyl (**12**) side-chain were synthesized. The key intermediate **6** for the preparation of **8** and **12** was obtained by introduction of the C-6 side-chain of pyrimidine ring *via* an addition reaction of the lithiated pyrimidine derivative (**4**) with benzyoxyacetaldehyde (**5**).

To explore the substrate acceptance of HSV-1 TK, compounds **8** and **12** were subjected to in vitro kinetic evaluations and determination of catalytic turnover constants. Comparison of binding affinities of fluorinated pyrimidine derivative **12** and ACV and GCV showed that **12** had an affinity of 40 μM which is better than that of ACV (170 μM) and GCV (48 μM). The catalytic turnover constant (k_{cat}) of **12** (0.08 s^{-1}) was close to those of ACV (0.10 s^{-1}) and GCV (0.10 s^{-1}). Compounds **8** and **12** did not exhibit any cytotoxic effects (minimum cytotoxic concentration > 400 μg/ml). No specific antiviral or antitumor activities were observed against any of the evaluated viruses or tumor cell lines.

The non-toxic (unlabelled) fluorinated compound **12** can be efficiently phosphorylated by HSV-1 TK and thus fulfils the necessary requirements for application as a tracer molecule for non-invasive TK imaging with PET. ^{18}F labelling of the precursor **14** was performed, although the radiochemical yield of ^{18}F incorporation into **14** was very low.

EXPERIMENTAL SECTION

General Methods

Melting points were determined with Kofler micro hot-stage (Reichert, Wien) and are uncorrected. Precoated Merck silica gel 60F-254 plates were used for thin layer chromatography (**TLC**) and the spots were detected under UV light (254 nm). Column chromatography (**CLC**) was performed using silica gel (0.063–0.2 mm, Merck); glass column was slurry-packed under gravity. The electron impact mass spectra were recorded with a Fisous Trio-2000 spectrometer with ionising energy 70 eV at the Paul Scherrer Institute. Elemental analyses were performed in the Central Analytic Service, Ruđer Boškovi Institute, Zagreb. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX-300, operating at 75.46 MHz for the ^{13}C resonance. The samples were dissolved in DMSO-d_6 and measured in 5 mm NMR tubes. The ^{1}H and ^{13}C NMR chemical shift values (δ) are expressed in ppm referred to TMS and coupling constants (***J***) in Hz.

2,4-Dichloro-5,6-dimethylpyrimidine (2).[12] The mixture of 2,4-dihydroxy-5,6-dimethylpyrimidine (**1**) (5 g, 3.57 mmol) and $POCl_3$ (10 ml, 109.24 mmol) was heated under reflux for 1 h. Excess of $POCl_3$ was then removed under reduced pressure and the residue was added to ice, washed with ether and dried over sodium sulphate. The crude product was recrystallized from ethanol to give **2** (3.883 g, 60.2 %, mp 70–71°C); MS m/z 177 (M^+); ^{13}C NMR (DMSO) δ 155.24 (C-2), 171.48 (C-3), 127.74 (C-5), 160.54 (C-6), 22.83 (CH_3), 14.40 (CH_3).

2,4-Dimethoxy-5,6-dimethylpyrimidine (3).[12] To a solution of sodium (571 mg, 24.83 mmol) in methanol (14 ml) was added **2** (2 g, 11.17 mmol). The reaction mixture was refluxed for 3 h. The solvent was evaporated and water was added to dissolve NaCl.

The oily layer was extracted with dichloromethane, dried over sodium sulphate and concentrated under reduced pressure. The residue was kept in refrigerator and gave colourless crystals of **3** (1.748 g, 93.6 %, mp 39–40°C); MS m/z 169 (M^+); ^{13}C NMR (DMSO) δ 162.10 (C-2), 168.67 (C-3), 107.25 (C-5), 165.67 (C-6), 53.96 (OCH_3), 53.74 (OCH_3), 21.46 (CH_3), 9.67 (CH_3).

6-(3-Benzyloxy-2-hydroxypropyl)-2,4-dimethoxy-5-methylpyrimidine (6). *N*-Butyllithium (1.6 M, 2.2 ml, 3.5 mmol) was added dropwise to a solution of **3** (500 mg, 2.94 mmol) in anhydrous tetrahydrofuran (7 ml) at −70°C under argon atmosphere. The temperature was warmed to −55°C and the solution was stirred for 30 min. Benzyloxyacetaldehyde (**5**)[13] (530 mg, 3.53 mmol) dissolved in tetrahydrofuran was added dropwise to the solution and the stirring was continued for 2 h. The solution was neutralized by the addition of acetic acid and the temperature was raised to room temperature. The solvent was removed under reduced pressure and the residue was partitioned between dichloromethane and water. Organic layer was dried and the solvent was removed. The crude product was purified by column chromatography (*n*-hexane:ethyl acetate - 4:1) providing oily compound **6** (593 mg, 63 %); MS m/z 318 (M^+); ^{13}C NMR (DMSO) δ 162.10 (C-2), 169.02 (C-4), 108.39 (C-5), 166.58 (C-6), 138.51–127.36 (C_6H_5), 53.97 (OCH_3), 53.82 (OCH_3), 38.54 (C-1′), 68.83 (C-2′), 74.08 (C-3′), 72.21 (CH_2Ph), 9.79 (CH_3). Anal. Calcd for $C_{17}H_{22}N_2O_4$: C, 64.13; H 6.97; N, 8.80. Found: C, 63.96; H 6.98; N, 8.77.

6-(2-Acetoxy-3-benzyloxypropyl)-5-methyl-(1*H*,3*H*)-pyrimidine-2,4-dione (7). A solution of **6** (354 mg) in acetyl chloride (5 ml) was refluxed for 10 h. Few drops of water were added and the mixture was stirred at room temperature overnight. The solution was evaporated under reduced pressure. The residue was applied to column chromatography (CH_2Cl_2:MeOH - 30:1) to give **7** (193 mg, 52 %); MS m/z 332 (M^+); ^{13}C NMR (DMSO) δ 169.66 (C=O), 150.75 (C-2), 164.80 (C-4), 106.18 (C-5), 146.51 (C-6), 138.1–127.49 (C_6H_5), 31.57 (C-1′), 70.25 (C-2′), 70.51 (C-3′), 72.06 (CH_2Ph), 20.67 (CH_3), 9.75 (CH_3).

6-(2,3-Dihydroxypropyl)-5-methyl-(1*H*,3*H*)-pyrimidine-2,4-dione (8). A solution of **7** (110 mg, 0.329 mmol) in dichloromethane (8 ml) was cooled to −75°C. Boron trichloride (1M, 1.65 ml, 1.65 mmol) was added via syringe under argon atmosphere. The mixture was stirred at −75°C for 2 h and then the temperature was raised to −40°C. A solution of MeOH/CH_2Cl_2 (1:1, 10 ml) was added and the cooling bath was removed. The solution was neutralized with $NaHCO_3$ and stirred for an additional 30 min. After filtration, the solvent was removed under reduced pressure. Water (2 ml) was added to the residue and the solvent was evaporated. The crude product was applied to column chromatography (CH_2Cl_2:MeOH - 7:1) to afford **8** (35 mg, 53 %, mp 186–187°C); MS m/z 200 (M^+); ^{13}C NMR (DMSO) δ 150.81 (C-2), 164.73 (C-4), 105.24 (C-5), 149.08 (C-6), 34.58 (C-1′), 70.08 (C-2′), 65.59 (C-3′), 9.82 (CH_3). Anal. Calcd for $C_8H_{12}N_2O_4$: C, 48.0; H 6.04; N, 13.99. Found: C, 48.12; H 5.98; N, 13.95.

6-(2,3-Dihydroxypropyl)-2,4-dimethoxy-5-methylpyrimidine (9). The compound **6** (500 mg, 1.57 mmol) was treated according to the procedure analogous to that for the preparation of **8**. The crude product was submitted to column chromatography (CH_2Cl_2:MeOH - 10:1) to give oily product **9** (240 mg, 67 %); MS m/z 228 (M^+); ^{13}C

NMR (DMSO) δ 162.12 (C-2), 169.02 (C-4), 108.39 (C-5), 167.17 (C-6), 54.0 (O*C*H$_3$), 53.83 (O*C*H$_3$), 38.34 (C-1′), 71.09 (C-2′), 65.64 (C-3′), 9.84 (*C*H$_3$). Anal. Calcd for $C_{10}H_{16}N_2O_4$: C, 52.62; H 7.07; N, 12.27. Found: C, 52.50; H 7.09; N, 12.23.

6-(3-Benzyloxy-2-fluoropropyl)-2,4-dimethoxy-5-methylpyrimidine (10). Compound **6** (369 mg, 1.159 mmol) was dissolved in anhydrous dichloromethane (4 ml). Diethylaminosulfur trifluoride (DAST, 0.22 ml, 1.679 mmol) was then added dropwise with stirring at room temperature under argon atmosphere. After 50 min the reaction mixture was partitioned between dichloromethane and aqueous sodium bicarbonate. The organic layer was concentrated and purified by silica column chromatography (*n*-hexane:ethyl acetate - 4:1) to give oily **10** (126 mg, 34 %); MS m/z 320 (M^+); ^{13}C NMR (DMSO) δ 164.51 (C-2), 169.63 (C-4), 108.96 (C-5), 169.63 (C-6), 135.66–127.99 (*C*$_6$H$_5$), 54.52 (O*C*H$_3$), 54.42 (O*C*H$_3$), 35.35 (C-1′, *J*=23 Hz), 91.93 (C-2′, *J*=172 Hz), 71.55 (C-3′, *J*=20 Hz), 72.75 (*C*H$_2$Ph), 9.55 (*C*H$_3$). Anal. Calcd for $C_{17}H_{21}FN_2O_3$: C, 63.74; H 6.61; N, 8.74. Found: C, 63.81; H 6.59; N, 8.72.

6-(3-Benzyloxy-2-fluoropropyl)-5-methyl-(1*H*,3*H*)-pyrimidine-2,4-dione (11). The compound **11** (113 mg, 0.353 mmol) was treated according to the procedure analogous to that for the preparation of **7**. Crude product **11** was purified by column chromatography (CH_2Cl_2:MeOH - 30:1) afforded white crystals of **11** (43 mg, 42 %, mp 132–132.5°C); MS m/z 292 (M^+); ^{13}C NMR (DMSO) δ 151.21 (C-2), 165.17 (C-4), 106.73 (C-5), 146.54 (C-6), 135.66–128.02 (*C*$_6$H$_5$), 32.45 (C-1′, *J*=22 Hz), 91.15 (C-2′, *J*=175 Hz), 71.32 (C-3′, *J*=20 Hz), 72.75 (*C*H$_2$Ph), 10.15 (*C*H$_3$). Anal. Calcd for $C_{15}H_{17}FN_2O_3$: C, 61.63; H 5.86; N, 9.58. Found: C, 61.51; H 5.87; N, 9.56.

6-(2-Fluoro-3-hydroxypropyl)-5-methyl-(1*H*,3*H*)-pyrimidine-2,4-dione (12). The compound **11** (71 mg, 0.243 mmol) was treated according to a procedure that was analogous to that for the preparation of **8**. The residue was submitted to column chromatography (CH_2Cl_2:MeOH - 10:1) to afford crystals of **12** (26 mg, 53 %, mp 151–152°C); MS m/z 202 (M^+); ^{13}C NMR (DMSO) δ 150.78 (C-2), 164.71 (C-4), 106.10 (C-5), 146.54 (C-6), 31.9 (C-1′, *J*=22 Hz), 91.49 (C-2′, *J*=174 Hz), 62.67 (C-3′, *J*=21 Hz), 9.70 (*C*H$_3$). Anal. Calcd for $C_8H_{11}FN_2O_3$: C, 47.52; H 5.48; N, 13.86. Found: C, 47.40; H 5.49; N, 13.82.

6-(3-Acetoxy-2-hydroxypropyl)-2,4-dimethoxy-5-methylpyrimidine (13). A solution of **9** (202 mg, 0.885 mmol) and acetyl chloride (0.063 ml, 0.887 mmol) in dichloromethane (2ml) was stirred at room temperature for 30 min. The solvent was evaporated under reduced pressure. Column chromatography (CH_2Cl_2:MeOH - 20:1) of the residue gave **13** (109 mg, 46 %). MS m/z 270 (M^+).

6-(3-Acetoxy-2-mesylpropyl)-2,4-dimethoxy-5-methylpyrimidine (14). To compound **13** (86 mg, 0.318 mmol) dissolved in anhydrous pyridine (1.5 ml), methanesulfonyl chloride (0.074 ml, 0.954 mmol) was added under argon atmosphere. The solution was kept on ice for 1 h. Ice water was added to the mixture. After extraction with dihloromethane, column chromatography was performed to give oily **14** (86 mg, 81 %). MS m/z 348 (M^+). Anal. Calcd for $C_{13}H_{20}N_2O_7S$: C, 44.82; H 5.79; N, 8.04. Found: C, 44.75; H 5.80; N, 8.01.

6-(2-[^{18}F]Fluoro-3-hydroxypropyl)-5-methyl-(1*H*,3*H*)-pyrimidine-2,4-dione (16) (^{18}F-HHT). Aqueous H^{18}F (37 GBq) from the cyclotron target was added to 10 µl of tetrabutylammoniumhydroxide (1M solution in MeOH). The solution was heated at 110°C and evaporated to dryness with the aid of a N_2 flow. This process was repeated three times by adding acetonitrile (1 ml). To the residue was added a solution of 8–10 mg of **14** in dry DMSO (1ml), and the reaction mixture was heated at 120°C for 30 min. The reaction was quenched by adding water (7 ml), and the resulting mixture was passed through a Sep-Pak Plus C18 cartridge (Waters). The Sep-Pak cartridge was washed with water (3 ml) and the fluorine-18 labelled intermediate retained on the C18 was eluted with MeCN (5 ml). The MeCN elute was evaporated to dryness under reduced pressure and a flow of N_2. The residue was acidified with 37% HCl (1 ml) and heated for 45 min at 120°C. Upon cooling, the contents were neutralized with 6N NaOH (1 ml). Purification of the crude product was achieved by high-performance liquid chromatography (HPLC) (Column: Bondclone C-18, 300 × 7.8 mm). The mobile phase was water and the flow rate was 6 ml/min. The retention time of ^{18}F-HHT was 11 min.

HPLC Assays for Monitoring the Phosphorylation of Substrates 8 and 12

DAD HPLC (L-7455 Merck) system was based on reverse-phase ion-pair chromatography using a previously published protocol[19] (Column, RP-18; solvent, 0.2 M NaH_2PO_4, 25 mM tetrabutylammonium hydrogen sulphate, 3% methanol; flow 1.1 ml/min; detection, UV 254 nm and diode array). Reactions were carried out in a final volume of 75 µl containing 50 mM Tris, pH 7.2, 5 mM ATP, 5 mM $MgCl_2$, 2 mM substrate and 1–5 µg of HSV-1 thymidine kinase (TK). The reaction was stopped after 1 h at 37°C by a 10-fold dilution in water EDTA-solution (2.5 mM) and freezing at −20°C prior to injection. The formation of the pyrimidine monophosphate was monitored qualitatively. Two different blank reactions (no enzyme or no substrate) were run concurrently to account for the (minimal) ATP independent hydrolysis.

Determination of K_{cat} by Spectrophotometric Assays

K_{cat} values were determined as described in the literature.[22] Reaction mixture with a final volume of 75 µl containing 50 mM Tris buffer pH 7.2, 1 mM 1,4-dithio-DL-threitol, 0.21 mM phosphoenolpyruvate, 2.5 mM $MgCl_2$, 5 mM ATP, 0.18 mM NaDH, 0.8 µg pyruvate kinase, 0.5 µg L-lactate dehydrogenase, and three different concentrations of the substrate (0.2 mM, 0.6 mM, and 1.0 mM) were incubated at 37°C. Two minutes later, 1 to 6 µg of HSV-1 TK was added in order to initiate the reaction. The changes in absorbance at 340 nm corresponding to ADP formation of the TK-dependent reaction at 37°C were monitored during 10 minutes. The data presented are the results of three independent series of measurements performed in triplicate. Control experiments were performed in order to take into account the spontaneous hydrolysis of ATP under the experimental conditions.

Binding Affinity Determination

Kinetic studies measuring the conversion of ^{3}H-labeled dT to dTMP in the presence of various concentrations (0, 50, 200 µM) of **8** and **12** were performed using

the DEAE-cellulose method.[20,23] Reactions were carried out in 30 μl of 50 mM Tris, pH 7.2, 5 mM ATP, 5 mM $MgCl_2$, 1.5 mg/ml BSA. The appropriate amount of enzyme and concentartions of [^{3}H]thymidine were chosen for Michaelis–Menten conditions for initial velocity measurements. The K_i values were determined by a nonlinear fit of the raw data to the equation: $V=V_{max}$ [dT]/{K_m(1 + [compd.]/K_i) + [dT]} for competitive inhibition[24] using the Origin 6.0 software. The calculated values were based on at least three independent experiments.

Functionality Assay

HSV-1 TK-transduced and non-transduced osteosarcoma (MG63) and non-small lung cancer (H125 NSCLC) cell lines were split 4 days before the cytotoxicity assay. Therefore, cells reached maximum speed of growth when the assay was started. The cells were trypsinized in 10 ml of fresh RPMI 1640 medium with 10% foetal calf serum and 1% antibiotic PSF solution. The cell concentration of a 1:1 dilution with trypan blue was determined using a Neubauer's counting chamber. The suspension was diluted with medium to a final concentration of 20000 cells per ml. The cell suspension (2000 cells in 100 μl) were seeded in each well of a 96 well Nunclon™ Surface plate from Nunc™. After 1.5 h incubation at 37°C the cells were attached to the bottom of the wells. Sterile solutions (100 μl) of GCV, **8** and **12** in RPMI 1640 were added to the cells leading to final substrate concentrations of 0, 50, 200, 500, 1000, and 2000 μM. All samples were prepared in triplicate for each cell line. GCV was used as reference control for a cytotoxic drug to HSV-1 TK expressing cells. The wells with no thymidine analogue allow calculating the standard value of 100% of cell growth. The plates were incubated for 4.5 days at 37°C in the presence of 5% CO_2. Viability of the cells was measured using the XTT reagent. The XTT reagent (50 μl) was added to each well. While the plates were incubated at 37°C in the presence of 5% CO_2 the living cells converted the XTT (tetrazolium derivative) by biodegradation to a water-soluble reddish formazan derivative.[25,26] After 9 h incubation the number of cells in each vial was determined by colorimetric UV absorption measurement of the converted XTT with an ELISA reader at a wavelength of 450 nm.

Antitumor Activity Assays

Antitumor activity against L1210 (murine leukemia), Molt4/C8 and CEM (human T-lymphocytes) cell lines were measured essentially as originally described for the mouse leukemia (L1210) cell line.[27]

Antiviral Activity Assays

Antiviral activity against HSV-1, HSV-2, vaccinia virus and vesicular stomatitis virus was determined as described previously.[28]

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