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SYNTHESIS AND COMPARATIVE CYTOSTATIC ACTIVITY OF THE NEW N-7 ACYCLIC PURINE NUCLEOSIDE ANALOGUES WITH NATURAL N-9 REGIOISOMERS

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Abstract - The synthesis of the purine derivatives alkylated at N-7 (**2a**) and N-9 (**2b**) with 2-acetoxyethoxymethyl side chain, and chemical transformations of keto to chloro (**5a**), chloro to thio (**6a**) at C-6, and amino to fluoro (**7a**) at C-2 position of the purine ring were described. Structures of compounds were elucidated by analysis of their ^{1}H and ^{13}C NMR spectra, MS spectra and elemental analyses. N-7 Regioisomers (**2a-7a**) were evaluated for their cytostatic activities and their inhibitory effects were compared with those of the corresponding N-9 isomers. The 2-aminopurin-6-thione derivative (**6a**) showed the highest cytostatic activity, particularly against murine leukemia (L1210).

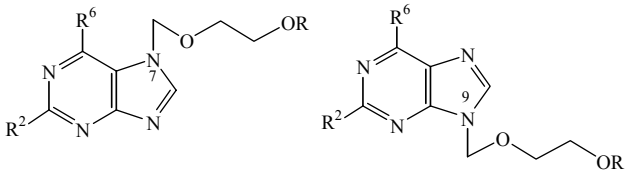
INTRODUCTION

N-9 Acyclic analogues of the natural purine nucleosides have been extensively investigated for their biological activity, especially as anticancer and antiviral agents.[1-3] Thus, acyclovir, ganciclovir and

penciclovir are the therapeutic compounds of choice to interfere with severe herpes virus infections. These compounds act as fraudulent substrates of herpes simplex virus 1 thymidine kinase (HSV TK),[4-6] blocking virus replication by interference with the viral DNA synthesis. Moreover, acyclic nucleoside analogues have been used in combination with suicide enzymes in gene therapy of cancer[7-9] and AIDS.[10] We have found that the N-9 substituted 2-aminopurin-6-thione derivative with a 2-acetoxyethoxymethyl side chain exhibited marked selectivity in its cytostatic activity, particularly against the murine mammary carcinoma FM3A cell line.[11]

N-7 Acyclic nucleoside analogues have been studied less frequently,[12, 13] often in context with N-7/N-9-glycosyl transfer.[14] In comparison with adenine, guanine displays a strikingly different behavior in alkylation and glycosylation reactions. The alkylation step of guanine is not a regiospecific condensation reaction and afforded a mixture of N-9 and N-7 regioisomers in 1:1 ratio.[15] This fact and the very low solubility of guanine in most solvents render the substitution of guanine an unattractive task.

The main purpose of this study was to evaluate the cytostatic activity of the N-7 acyclic 2,6-disubstituted purine derivatives (**2a-7a**, Figure 1) in comparison with the corresponding inhibitory effect of their N-9 regioisomers[11] (**4b-6b**).



2a-7a **2b-7b**

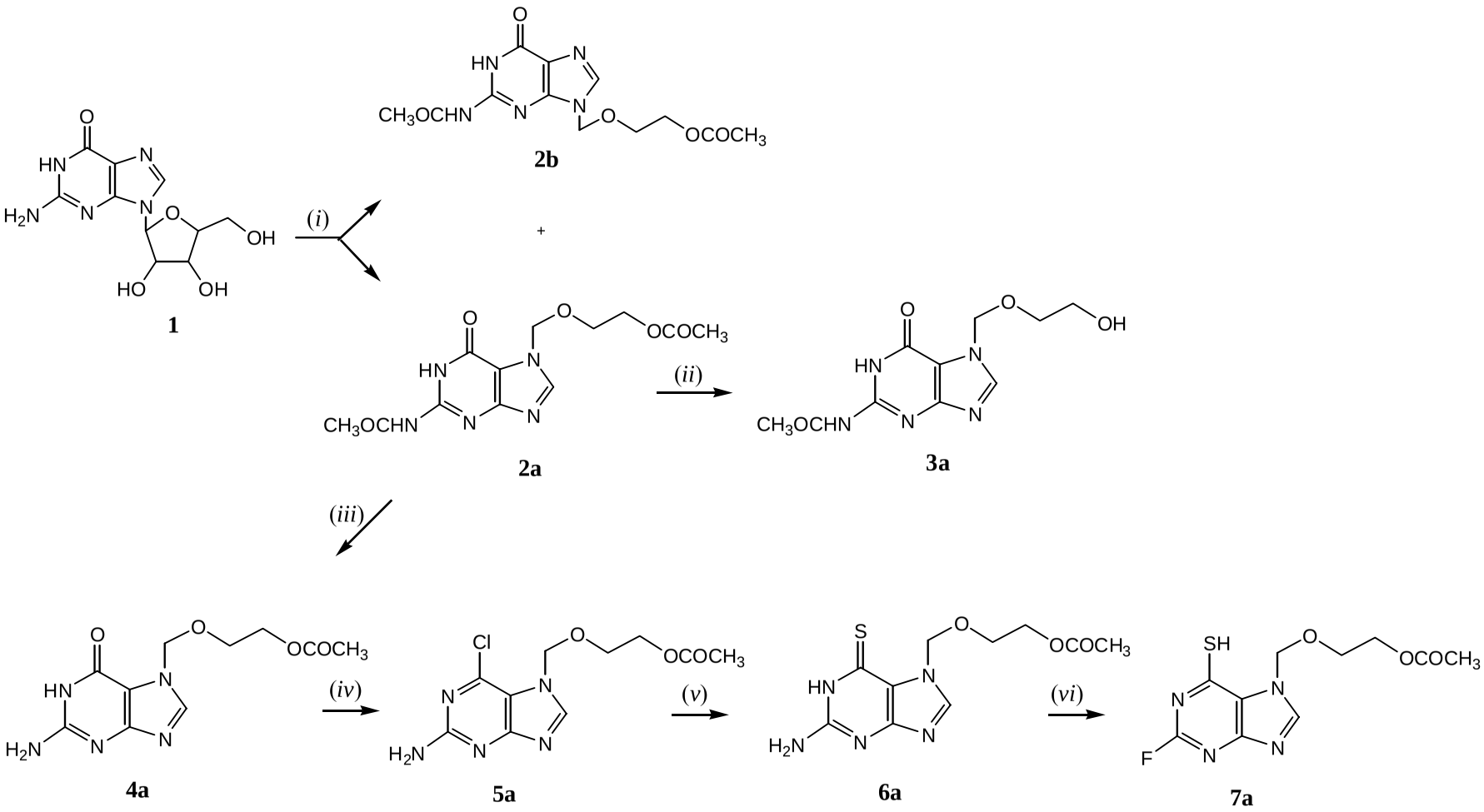
N - 7	*N - 9*	R^2	R^6	*R*
2a	**2b**[11]	$NHCOCH_3$	OH	$COCH_3$
3a	**3b**[11]	$NHCOCH_3$	OH	H
4a	**4b**[11]	NH_2	OH	$COCH_3$
5a	**5b**[11]	NH_2	Cl	$COCH_3$
6a	**6b**[11]	NH_2	SH	$COCH_3$
7a	**7b**[11]	F	SH	$COCH_3$

Figure 1.

RESULTS AND DISCUSSION

CHEMISTRY

A mixture of N-7 (**2a**) and N-9 (**2b**) acyclovir derivatives with acetylated hydroxyl and amino groups was synthesized by transpurination reaction of natural guanosine (**1**) with 2-acetoxyethoxymethyl acetate in the presence of acetic anhydride and catalytic amount of an acid (Scheme 1).[16]



Scheme 1. Reagents and conditions: (*i*) 2-acetoxyethoxymethyl acetate, acetic anhydride, *p*-toluenesulfonic acid monohydrate; (*ii*) sodium, methanol, hydrogen chloride, 24 h: (*iii*) triethylamine, 96% ethanol, 25 h; (*iv*) $POCl_3$, tetraethylammonium chloride, *N*,*N*-diethylaniline, acetonitrile, 10 min; (*v*) thiourea, dry ethanol, 1 h; (*vi*) HF/pyridine, *tert*-butyl nitrite, 10 min.

In this alkylation reaction two regioisomers were obtained in an approximate ratio of 1:1. In order to perform chemical transformations at C-2 and C-6 positions of the purine ring in N-7 acyclic purine nucleosides, selective *N*- and *O*-deacetylations of **2a** were performed.[17] *O*-Deacetylated 2-acetylaminopurin-6-one derivative (**3a**) was prepared with sodium methoxide in methanol (Scheme 1). Selective *N*-deacetylation of **2a** was carried out using triethylamine as a base in ethanol to give **4a**. Chlorination of **4a** using an excess of phosphoryl chloride, *N*,*N*-diethylaniline and tetraethylammonium chloride in anhydrous acetonitrile gave 2-amino-6-chloropurine derivative (**5a**).[11] Transformation of 6-chloro to 6-thio group in **6a** was performed using thiourea in dry ethanol in the presence of catalytic amounts of formic acid.[18] The fluorine in 2-position of the purine ring of **7a** was introduced *via* Baltz-Schiemann reaction using 60% (w/w) HF/pyridine and *tert*-butyl nitrite in non-aqueous media.[11]

^{1}H AND ^{13}C NMR SPECTRA

The assignment of ^{1}H and ^{13}C NMR spectra of **2a-7a** was performed on the basis of chemical shifts, substituent induced chemical shifts, signal intensities, magnitude and multiplicity of H-H and C-F coupling constants. The ^{1}H NMR spectral data of N-7 (**2a-7a**) and N-9 (**2b**) acyclic purine derivatives are displayed in Table 1.

Table 1. ^{1}H NMR Chemical Shifts (δ/ppm)[a] and H-H Coupling Constants (J/Hz)[b] of Compounds (**2a-7a**) and (**2b**) (*cf.* Scheme 1).

Compd	H-8	NH	1'	3'	4'	$OCOCH_3$	$NHCOCH_3$
2a	8.36 (s, 1H)	12.16 (br, 1H) 11.61 (br, 1H)	5.68 (s, 2H)	4.06 (t, 2H) *J* = 4.5 Hz	3.71 (t, 2H) *J* = 4.5 Hz	1.94 (s, 3H)	2.16 (s, 3H)
2b	8.13 (s, 1H)	12.05 (br, 1H) 11.79 (br, 1H)	5.47 (s, 2H)	4.06 (t, 2H) *J* = 4.3 Hz	3.68(t, 2H) *J* = 4.4 Hz	1.94 (s, 3H)	2.16 (s, 3H)
3a[c]	8.36 (s, 1H)	11.63 (s, 1H)	5.76 (s, 2H)	3.49 (m, 4H)		/	2.16 (s, 3H)
4a	8.03 (s, 1H)	10.70 (s, 1H)	5.60 (s, 2H)	4.07 (s, 2H)	3.09 (s, 2H)	1.94 (s, 3H)	/
5a[d]	8.55 (s, 1H)	/	5.63 (s, 2H)	4.02 (m, 2H)	3.61 (m, 2H)	1.87 (s, 3H)	/
6a	8.36 (s, 1H)	12.07 (br, 1H)	5.99 (s, 2H)	4.04 (s, 2H)	3.72 (s, 2H)	1.93 (s, 3H)	/
7a	8.96 (s, 1H)	/	5.92 (s, 2H)	4.09 (m, 2H)	3.30 (s, 2H)	1.84 (s, 3H)	/

[a] DMSO-d_6 as solvent; chemical shifts referred to TMS. Multiplicity of coupling and number of protons are given in parentheses. [b] Digital resolution ±0.28 Hz. [c] Signal for –OH: 4.70 ppm (br, 1H). [d] Signal for $–NH_2$: 6.69 ppm (br, 2H).

The most pronounced differences in N-7 (**2a**) and N-9 (**2b**) regioisomers were found for the proton signal (H-8) of the purine skeleton and *N*-methylene protons (CH_2-1') in the alkyl residue. Thus, in N-7

substituted compound (**2a**) H-8 and CH_2-1' are more deshielded (8.36 and 5.68 ppm) than corresponding protons (8.13 and 5.47 ppm) in N-9 isomer (**2b**). ^{1}H NMR spectra of 7-(2-acetoxyethoxymethyl)purine derivatives (**4a-7a**) show, besides H-8 and NH_2 or NH protons of the purine skeleton, the CH_2-1', CH_2-3' and CH_2-4' protons of side chain. The chemical shifts of the methylene protons are in the order $\delta(CH_2\text{-}1') > \delta(CH_2\text{-}3') > \delta(CH_2\text{-}4')$, corresponding to enhanced shielding with increasing distance of the methylene protons from the purine π-system. Generally, the ^{13}C NMR spectra of **2a-7a** show five signals in the aromatic region and five to seven signals in the aliphatic region (EXPERIMENTAL). In the purine skeleton the significant change of ^{13}C chemical shift between N-7 and N-9 isomers exists for C-8 and C-4 atoms. The C-8 and C-4 in **2a** are deshielded for 5 and 3.6 ppm with respect to the corresponding C-atoms in **2b**. Out of the purine skeleton the most pronounced difference in chemical shift between N-7 and N-9 derivatives was observed for *N*-methylene carbon (C-1') of the acyclic residue. This carbon is also deshielded in N-7 isomer with respect to that in N-9 isomer for 2.5 ppm. Introduction of fluorine atom at 2 position in the purin-6-thione derivative (**7a**) causes a change to the tautomeric thiol form. Thus, C-6 is deshielded for 6.3 ppm in **6a** with respect to that in **7a**. The magnitude of one-bond (209 Hz) and two-bond (17 Hz) C-F couplings at C-2, C-6 and C-4 are in accord with those found for related fluorinated derivatives.[11]

BIOLOGICAL RESULTS

Cytostatic Activity - Compounds (**2a-7a**) were evaluated for their activities against malignant tumor cell lines: murine leukemia (L1210) and human T-lymphocyte (Molt4/C8, CEM), colon carcinoma (SW620), laryngeal carcinoma (Hep2), pancreatic carcinoma (MiaPaCa2), cervical carcinoma (HeLa), as well as normal human fibroblasts (WI38) (Table 2). The cytostatic activities of N-7 acyclic purine regioisomers (**2a-7a**) were compared with cytostatic effects of the corresponding N-9 regioisomers (**4b-6b**).

Compounds (**2a-4a**) and (**7a**) exhibited no cytostatic activities against all tested tumor cell lines. 2-Amino-6-chloropurine containing acetoxyethoxymethyl side chain (**5a**) showed moderate and selective cytostatic activity against L1210 (IC_{50}: 87 μg/mL), Molt4/C8 (IC_{50}: 72 μg/mL), and CEM (IC_{50}: 68 μg/mL), and no cytotoxic effect on normal human fibroblasts. Its N-9 isomer (**5b**) exhibited better inhibitory effects on these cells, and on human SW620 and Hep2 cells. Among the N-7 acyclic purine regioisomers, the 2-aminopurin-6-thione derivative (**6a**) showed the highest cytostatic effect. This compound had a rather marked inhibitory effect against murine L1210 cells (IC_{50}: 7.8 μg/mL) and moderate activity against human Molt4/C8 (IC_{50}: 20 μg/mL) and CEM (IC_{50}: 19 μg/mL) cells. The corresponding N-9 regioisomer (**6b**) had somewhat lower cytostatic activity. Furthermore, the regioisomers N-7 (**6a**) and N-9 (**6b**) did not exhibit a cytotoxic effect on normal fibroblasts.

Table 2. Inhibitory Effects of N-7 (**2a-7a**) and N-9 (**4b-6b**) Acyclic Purine Regioisomers on the Growth of Malignant Tumor Cell Lines and Diploid Fibroblasts (WI38).

	IC_{50}[a](μg/mL)							
Compd	L1210	Molt4/C8	CEM	SW620	Hep2	MiaPaCa2	HeLa	WI38
2a	>200	>200	>200	>100	>100	>100	>100	>100
3a	>200	>200	>200	>100	>100	>100	>100	>100
4a	≥200	≥200	≥200	N.D.	N.D.	N.D.	N.D.	N.D.
5a	87±4	72±26	68±5	>100	>100	>100	>100	>100
6a	7.8±3.8	20±3	19±1	>100	>100	>100	>100	>100
7a	>200	>200	>200	>100	>100	>100	>100	>100
4b[11]	>500	>500	>500	>500	>500	>500	>500	>500
5b[11]	3.2±1.2	8.9±0.5	7.5±1.3	4.2	4.9	>500	5.0	>500
6b[11]	40±1	35±6	37±10	>500	>500	>500	>500	>500

[a] 50% inhinitory concentration, or compound concentration required to inhibit tumor cell proliferation by 50%.

Antiviral Activity - Compounds (**2a-7a**) were evaluated against herpes simplex virus-1 (HSV-1), herpes simplex virus-2 (HSV-2), vaccinia virus, vesicular stomatitis virus, parainfluenza-3 virus, reovirus-1, Sinbis virus, Coxsackie virus B_4, Punta Toro virus, respiratory syncytial virus, HIV-1 and HIV-2. All compounds were antiviraly inactive at subtoxic concentrations.

CONCLUSIONS

N-7 (**2a**) and N-9 (**2b**) acyclic purine derivatives were synthesized by a transpurination reaction of natural guanosine with 2-acetoxyethoxymethyl acetate in the ratio 1:1.

The 2-amino-6-chloropurine derivative (**5a**) was prepared by chlorination of the carbonyl group at C-6 position of the purine ring in **4a**. Transformation of 6-chloro to 6-thio group (**6a**) was performed using thiourea, and in a subsequent step fluorination *via* Baltz-Schiemann reaction gave 2-fluoropurin-6-thiol derivative (**7a**) as the final product.

N-7 Regioisomers (**2a-7a**) were evaluated for their cytostatic activities and their inhibitory effects were compared with those of corresponding N-9 isomers. 2-Aminopurin-6-thione derivative (**6a**) showed the highest cytostatic activity, particularly against the murine leukemia (L1210, IC_{50}: 7.8 μg/mL). Furthermore, this compound did not exhibit cytotoxic activity against normal fibroblasts (WI 38).

EXPERIMENTAL

General - Melting points (uncorrected) were determined with Kofler micro hot stage (Rechert, Wien). Precoated 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) Kemika; glass column was slurry-packed under gravity. Solvent systems used for the TLC and CLC were CH_2Cl_2 and CH_3OH: 9:1 (S_1), 13:1 (S_2) and 12:1 (S_3). UV spectra were measured on a Varian Cary 50 UV/VIS spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on a Varian Gemini 300 Spectrometer, operating at 75.46 MHz for the ^{13}C resonance. The samples were dissolved in DMSO-d_6 and measured in 5 mm tubes. The ^1H and ^{13}C NMR chemical shifts values (δ) are expressed in ppm referred to TMS and coupling constants (*J*) in Hz.

2-Acetylamino-7-(2-acetoxyethoxymethyl)purin-6-one (2a) and 2-acetylamino-9-(2-acetoxyethoxymethyl)purine-6-one (2b).[16] The mixture of guanosine (8 g; 28.2 mmol), toluene-4-sulfonic acid monohydrate (0.539 g; 2.82 mmol), 2-acetoxyethoxymethyl acetate (15.3 mL; 56.4 mmol) and acetic anhydride (26.7 mL; 0.282 mol) was heated at 100 °C for 20 h. The solvents were removed under reduced pressure and oily residue was purified by column chromatography with S_1 as eluent. After recrystallization from ethyl acetate compounds (**2a**, 1.743 g; 20 %) and (**2b**, 1.894 g; 22 %) were obtained in the ratio 1:1.

2a: mp = 163-166 °C; UV (methanol) λ_{max} 264, 220 (log ε 4.37, 4.46); ^{13}C NMR (DMSO-d_6) δ: 173.49 (NHC=O), 170.31 (OC=O), 157.52 (C-6), 152.58 (C-4), 147.34 (C-2), 145.20 (C-8), 121.23 (C-5), 75.02 (C-1'), 66.47 (C-3'), 62.83 (C-4'), 23.81 ($NHCOCH_3$), 20.63 ($OCOCH_3$).

2b: mp = 168-171 °C; ^{13}C NMR (DMSO-d_6) δ: 173.71 (NHC=O), 170.40 (OC=O), 155.03 (C-6), 149.0 (C-4), 148.26 (C-2), 140.21 (C-8), 120.28 (C-5), 72.55 (C-1'), 66.81 (C-3'), 62.82 (C-4'), 23.90 ($NHCOCH_3$), 20.67 ($OCOCH_3$).

7-(2-Hydroxyethoxymethyl)-2-acetylaminopurin-6-one (3a). In a cooled flask containing distilled methanol (4.1 mL) sodium (0.074 g; 3.24 mmol) was added slowly with exclusion of moisture. After sodium had been dissolved, compound (**2a**, 0.500 g; 1.62 mmol) was added to reaction mixture. After 15 min 1.4 M HCl (2.5 mL) was added. The reaction mixture was stirred overnight at rt. The white precipitate was collected by filtration and recrystallized from 96% ethanol. The resulting **3a** was obtained (117 mg; 27%; mp 198-200 °C). UV (methanol) λ_{max} 264, 220, 206 (log ε 3.81, 3.51, 4.28); ^{13}C NMR (DMSO-d_6) δ: 173.54 (C=O), 157.53 (C-6), 152.60 (C-4), 147.35 (C-2), 145.21 (C-8), 111.26 (C-5), 75.35 (C-1'), 70.44 (C-3'), 59.99 (C-4'), 23.86 (CH_3); MS (70 eV) m/z: 267 [M^+]; Anal. Calcd for

$C_{10}H_{13}N_5O_4$: C, 44.94; H, 4.90; N, 26.21. Found: C, 45.07; H, 4.89; N, 26.11.

7-(2-Acetoxyethoxymethyl)-2-aminopurin-6-one (4a). A suspension of **2a** (0.767 g; 2.48 mmol) and 1M solution of triethylamine in 95% ethanol (9.92 mL) was heated under reflux for 25 h. The mixture was cooled to rt and evaporated to dryness. The obtained crystals of **4a** were washed well with ethanol and filtered off (505 mg; 76%; mp 250-253 °C). UV (methanol) λ_{max} 287, 245, 213 (log ε 3.80, 3.78, 4.24); MS (70 eV) m/z: 267 [M^+]; Anal. Calcd for $C_{10}H_{13}N_5O_4$: C, 44.94; H, 4.90; N, 26.21. Found: C, 44.90; H, 4.91; N, 26.08.

7-(2-Acetoxyethoxymethyl)-2-amino-6-chloropurine (5a). Previously dried **4a** (0.586 g; 2.19 mmol) and tetraethylammonium chloride (0.578 g; 3.50 mmol) were dissolved in dry acetonitrile (6 mL) with exclusion of moisture. *N*,*N*-Diethylaniline (0.35 mL; 2.19 mmol) and phosporyl chloride (1.22 mL; 13.14 mmol) were added. The reaction mixture was heated at reflux for 10 min. The solvent was evaporated to dryness at the reduced pressure (bath temperature bellow 45 °C). The resulting oil was dissolved in cold water (20 mL) and kept in ice bath. The pH value of the solution was kept between pH 6 and pH 6.5 by adding a saturated solution of $NaHCO_3$. The aqueous solution was extracted with dichloromethane (4 x 15 mL). The combined organic phases were dried over Na_2SO_4, evaporated in vacuo and eluted by column chromatography with S_2 as eluent. Fractions containing the product were combined and evaporated to give an oily material, which was recrystallized from 2-propanol to give **5a** (62 mg; 10%; mp 149-151 °C). UV (methanol) λ_{max} 322, 219 (log ε 3.84, 4.45); ^{13}C NMR (DMSO-d_6) δ: 170.53 (C=O), 164.99 (C-2), 150.30 (C-6), 160.62 (C-4), 143.25 (C-8), 114.86 (C-5), 75.43 (C-1'), 66.17 (C-3'), 63.03 (C-4'), 20.83 (CH_3); MS (70 eV) m/z: 285 [M^+]; Anal. Calcd for $C_{10}H_{12}N_5O_3Cl$: C, 42.04; H, 4.23; N, 24.51. Found: C, 42.17; H, 4.23; N, 24.53.

7-(2-Acetoxyethoxymethyl)-2-aminopurin-6-thione (6a). To a suspension of **5a** (720 mg; 2.68 mmol) in dry ethanol (24 mL), thiourea (760 mg; 9.94 mmol) was added followed by 3 drops of formic acid. The mixture was heated under reflux for 1 h and then allowed to cool to rt. The white precipitate was collected by filtration and washed with cold dry ethanol to give pure **6a** (450 mg; 63%; mp 216-218 °C). UV (methanol) λ_{max} 352, 206 (log ε 3.82, 4.20); ^{13}C NMR (DMSO-d_6) δ: 170.66 (C=O), 169.27 (C-6), 157.47 (C-2), 153.74 (C-4), 149.17 (C-8), 119.31 (C-5), 74.67 (C-1'), 66.17 (C-3'), 63.30 (C-4'), 21.04 (CH_3); MS (70 eV) m/z: 283 [M^+]; Anal. Calcd for $C_{10}H_{13}N_5O_3S$: C, 42.39; H, 4.63; N, 24.72. Found: C, 42.35; H, 4.62; N, 24.77.

7-(2-Acetoxyethoxymethyl)-2-fluoropurin-6-thiol (7a). HF (70%) in pyridine (w/w), in a HF-resistant flask was cooled at -50 °C by dry ice/acetone bath. The solution was then diluted to 60% (w/w; 6.3 mL)

by careful addition of dry pyridine. The compound (**6a**) (208 mg; 0.73 mmol) was added to the flask and the temperature of the bath was allowed to rise to -30 °C. Then, *tert*-butyl nitrite (0.13 mL; 1.09 mmol) was added to the stirred mixture and stirring was continued for 10 min with exclusion of moisture. The solution was rapidly poured into 100 g of crushed ice/H_2O. The aqueous mixture was extracted with 5 x 30 mL of CH_2Cl_2 and the combined organic phase was washed with 3 x 10 mL of H_2O and 5% $NaHCO_3/H_2O$ to pH~7, then dried over Na_2SO_4, filtered off and the solvent evaporated in vacuo. The oily residue was purified by column chromatography (solvent system S_3). Fractions containing the product were combined and evaporated to give an oily material. The crude oily product was recrystallized from 2-propanol to give analytically pure **7a** (122 mg; 58%; mp 166-168 °C). UV (methanol) λ_{max} 295, 217 (log ε 3.78, 4.30); ^{13}C NMR (DMSO-d_6) δ: 170.14 (C=O), 157.61 (d, C-2, J = 209.0 Hz), 163.00 (d, J = 16.7 Hz, C-6), 152.62 (d, J = 17 Hz, C-4) , 152.21 (C-8), 121.71 (C-5), 76.42 (C-19), 66.35 (C-3'), 62.63 (C-4'), 20.43 (CH_3); MS (70 eV) m/z: 286 [M^+]; Anal. Calcd for $C_{10}H_{11}N_4O_3FS$: C, 41.95; H, 3.87; N, 19.57. Found: C, 41.99; H, 3.86; N, 19.65.

Materials for Biological Tests. Cell Culturing. Biological evaluation of the compounds (**2a-7a**) was performed to test potential antitumor activity. In this study, the effects of different concentrations of each compound on proliferation of tumor and normal cell lines were examined. The cells (colon carcinoma, SW620; laryngeal carcinoma, Hep2; pancreatic carcinoma, MiaPaCa2; cervical carcinoma, HeLa; and human normal fibroblasts, WI38) were seeded in 96-well plates at a concentration of $3x10^4$ /mL in D-MEM, supplemented with 10 % FBS and glutamine (2 mM) and grown in a humidified atmosphere with 5% CO_2. At 24 h later, the test compounds were added at a final concentration of 10^{-6}, 10^{-5} and 10^{-4} M. After 72 h of treatment, the number of the cells was determined by the MTT test.[19] The compounds were dissolved in DMSO as a 10^{-1} M solution and diluted with a medium to the appropriate concentration. The final concentration of DMSO was less than 0.1%, *i.e.* it did not influence cell growth. Control cells were grown in D-MEM without any addition.

Antitumor activity against L1210 (murine leukemia), Molt4/C8 and CEM (human T-lymphocytes) cell lines was measured essentially as originally described for the mouse leukemia (L1210) cell line.[20]

Each test was performed in triplicate in three individual experiments. The results are expressed as IC_{50}, the concentration necessary for 50% inhibition. Each result is a mean value from three separate experiments.

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