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TETRAHEDRON

Facile Synthesis of 5'-Deoxy- and 2',5'-Dideoxy-6-thiopurine Nucleosides by Nucleoside Phosphorylases

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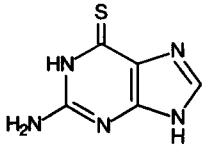
Abstract : 5'-Deoxy-6-thioguanosine, 2',5'-dideoxy-6-thioguanosine, 5'-deoxy-6-mercaptopurine riboside and 2',5'-dideoxy-6-mercaptopurine riboside were synthesized enzymatically from thiopurine bases and corresponding ribosyl donors using nucleoside phosphorylase. This is the first report of trans-5'-deoxyribosylation to thiopurine bases by nucleoside phosphorylase. 5'-Deoxy-6-thioguanosine selectively blocked the growth of *v-ras*-transformed human bronchial epithelial cells. In addition, the *in vitro* antitumor cytotoxicity data for 5'-deoxy- and 2',5'-dideoxy-6-thiopurine nucleosides were comparable to those for the corresponding thiopurine bases.

INTRODUCTION

6-Thioguanine (6TGua, **1a**) and 6-mercaptopurine (6MPur, **1b**) have been known as two of the oldest antineoplastic drugs presently in clinical use[1,2] since their introduction by Elion and Hitchings in 1950s.[3] However, the clinical utility of these thiopurine bases has been limited by poor drug bioavailability, metabolic deactivation, development of resistance, dose limiting toxicity and bone marrow supression.[4]



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1b

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Considerable efforts have been made to prepare other novel nucleoside analogs by traditional chemical methods to improve the antitumor efficacy. 5'-Deoxy-6-thioguanosine (**3b**) was shown to display moderate *in vivo* antitumor activity against L1210 murine leukemia.[5] However, the overall yield for chemical synthesis of **3b** from 2',3'-isopropylideneguanosine is less than 6%.[6] A more efficient synthesis of this compound has thus become essential to its further preclinical studies.

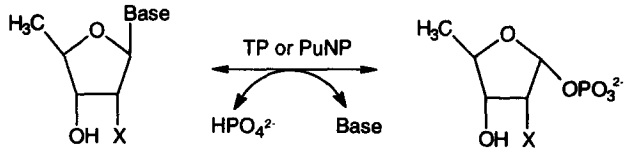
Nucleoside phosphorylases[7] are widely distributed in nature. In recent years, several laboratories have demonstrated synthesis of novel deoxynucleosides by those phosphorylases as a promising alternative to chemical synthesis. A series of nucleoside *N*-transfer reactions *via* phosphate esters of sugar, such as transribosylation,[8] trans-2'-deoxyribosylation,[9] transarabinosylation,[10] trans-aminoribosylation,[11] and trans-2',3'-dideoxyribosylation,[12-13] and trans-α-*L*-2',3'-dideoxyribosylation[14] by nucleoside phosphorylases were described. Trans-2',5'-dideoxyribosylation was recently reported by us[15] and Reja *et. al.*[16] ,respectively. In addition, 2,6-dichloropurine 2'-deoxyriboside[17] and virazole (ribavirin, 1-(β-D-ribofuranosyl)-1,2,4-triazole-3-carboxamide)[18] were also prepared by nucleoside *N*-transfer reactions of phosphorylases.

Here, we report a facile enzymatic synthesis of 2',5'-dideoxy- and 5'-deoxy-6-thiopurine nucleosides using phosphorylases. To our knowledge, this is the first report of trans-5'-deoxyribosylation to thiopurine bases, and trans-2',5'-dideoxyribosylation to thioguanine.

RESULTS AND DISCUSSION

Enzymatic Synthesis. The deoxyribosyl transfer reaction catalyzed by nucleoside phosphorylase has been shown to involve the formation and utilization of deoxyribose-1-phosphate. Accordingly, the method for the enzymatic synthesis of 2',5'-dideoxyribonucleoside or 5'-deoxyribonucleoside analogs involves a pair of closely coupled enzymatic reactions. The first reaction (Scheme 1) is the phosphorolysis of a 2',5'-dideoxy-

Scheme 1

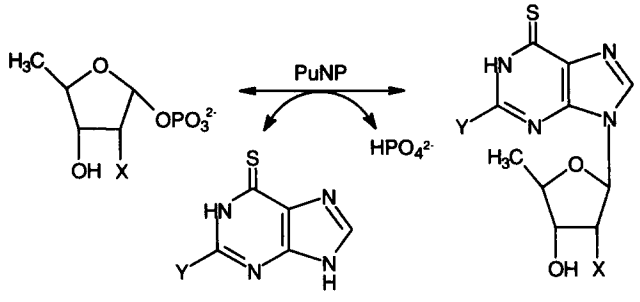


2

	Base	X	Enzyme
2a	thymine	H	TP
2b	hypoxanthine	OH	PuNP

thymidine (**2a**) catalyzed by thymidine phosphorylase (TP) to form 2,5-dideoxyribose-1-phosphate or the phosphorolysis of a 5'-deoxyinosine (**2b**) catalyzed by purine nucleoside phosphorylase (PuNP) to form 5-deoxyribose-1-phosphate. The second reaction (Scheme 2) is the synthesis of the desired product from the 2,5-dideoxyribose-1-phosphate ester or 5-deoxyribose-1-phosphate ester generated in the first reaction and 6-thioguanine (**1a**, Y=NH_2) or 6-mercaptopurine (**1b**, Y=H) by purine nucleoside phosphorylase. The net result of these coupled enzymatic reactions is the transfer of a 2',5'-dideoxyribosyl moiety from a pyrimidine nucleoside (**2a**) or a 5'-deoxyribosyl moiety from a purine nucleoside (**2b**) to one of 6-thiopurine bases (**1a** and **1b**).

Scheme 2

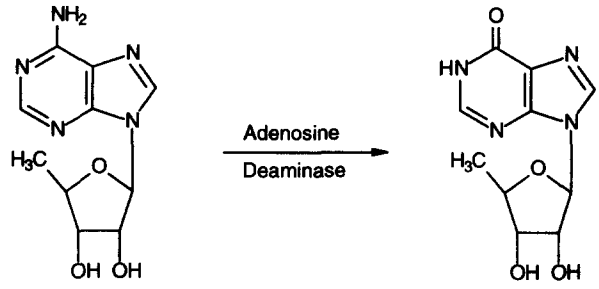


1 3

	X	Y
3a	H	NH_2
3b	OH	NH_2
3c	H	H
3d	OH	H

The formation of the desired products was sequentially monitored by HPLC. The identities of all HPLC peaks except the new product peak were confirmed by comparing their retention times to those of the authentic standard compounds. Furthermore, the assignment of the new peak corresponding to the product was initially made by comparing the complete UV spectrum of the new peak with that of the standard base using a photodiode-array detector. The structures of the isolated products were finally confirmed by 500MHz ^{1}H NMR and high resolution FAB-mass spectral analyses.

Purine nucleoside phosphorylase prefers hypoxanthine base to adenine base as a substrate. Thus, for the synthesis of 5'-deoxy-6-thiopurine nucleosides, 5'-deoxyinosine (**2b**) was used as a 5'-deoxyribosyl donor. We utilized the commercially available 5'-deoxyadenosine to generate 5'-deoxyinosine by adenosine deaminase.



5'-Deoxyadenosine 5'-Deoxyinosine

An organic solvent was added into the enzymatic reaction medium because of the limited solubility of the thiopurine bases in aqueous solution. In case of 6-mercaptopurine (**1b**), the enzyme-catalyzed reaction was carried out in a phosphate buffer solution containing 20% dimethylformamide. The preparative HPLC separation was thus performed with a mobile phase consisting of 1% dimethylformamide and 10-15% CH_3CN for improving the solubility of the desired products in order to enhance the reaction efficiency.

In summary, this enzymatic synthesis of 5'-deoxy- and 2',5'-dideoxy-6-thiopurine nucleosides doesn't involve tedious protection and deprotection processes, and produces only the β-isomer. It clearly demonstrates the advantages of stereospecificity and simplicity of enzymatic reactions. The described approach will be further applied to the synthesis of other prodrug analogs of nucleoside antimetabolites as potential antitumor and antiviral agents.

Antitumor Cytotoxicity. 6-Thioguanine and 6-mercaptopurine are currently used for the treatment of acute leukemia and chronic myelogenous leukemia. It usually manifests bone marrow toxicity, hepatotoxicity and hyperuricemia. 6-Thioguanine or 6-mercaptopurine competes with guanine or hypoxanthine for hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and is converted to 6-thioguanosine monophosphate and 6-thioinosine monophosphate, respectively, which then interferes the synthesis of purine nucleotides of most cells.[18] 5'-Deoxy- or 2',5'-dideoxy-6-thiopurine nucleosides would not be expected to exhibit cytotoxicity by itself toward normal cells due to the absence of the 5'-hydroxyl group. However, it has been demonstrated that nucleoside phosphorylase activity in tumor cells is significantly higher than that of normal cells.[19] Therefore, an intracellular activation to 6-thiopurine bases by purine nucleoside phosphorylase in tumor cells could be achieved to exert antitumor effect because the degree of activation of 5'-deoxy- or 2',5'-dideoxy-6-thiopurine nucleosides to the corresponding bases in tumor tissue will be higher. This selective activation could result in better efficacy and lower toxicity. *In vitro* antitumor cytotoxicity of several

thiopurine bases and nucleosides were tested against human tumor cells (Table 1). The cytotoxicity of 5'-deoxy- (**3d**) or 2',5'-dideoxy-6-mercaptopurine riboside (**3c**) is comparable to that of 6-mercaptopurine. 5'-Deoxy- (**3b**) and 2',5'-dideoxythioguanosine (**3a**) appears slightly less cytotoxic than 6-thioguanine. However, the *in vitro* cytotoxicity profile against a limited number of human tumor cells doesn't provide much information on selectivity. We have thus recently devised a new bioassay to measure selective cytotoxicity.

Table 1. Antiproliferative Activity Against Human Tumor Cells (GI_{50} : μM)[a]

	A-549	MCF-7	HT-29
3a	22	14	5.5
3b	12	11	3.3
6-thioguanine	16	3.1	0.087
6-thioguanosine	5.6	5.8	0.27
3c	100	74	18
3d	160	81	36
6-mercaptopurine	170	110	23
6-mercaptopurine riboside	83	50	4.4
adriamycin	0.010	0.58	0.012

[a] The target cells were A-549 (human lung carcinoma), MCF-7 (human breast adenocarcinoma) and HT-29 (human colon adenocarcinoma) cells. These assays were carried out at the Purdue Cancer Center Cytotoxicity Laboratory, following the microculture tetrazolium assay method.

Selective Cytotoxicity. Mutated *ras* proto-oncogenes have been detected in a large number of human malignancies.[20,21] Selective growth inhibition of *ras*-transformed cells in culture may thus provide an estimate of selective cytotoxicity against tumor cells. Inhibition of the growth of *ras*-transformed human bronchial epithelial cells versus that of normal human bronchial epithelial cells by 5'-deoxy- or 2',5'-dideoxy-6-thioguanosine were compared (Table 2). 5'-Deoxy-6-thioguanosine selectively blocked the growth of *v-ras*-transformed human bronchial epithelial cells. Half-maximal inhibition of cell growth (GI_{50}) occurred at a concentration of 0.71 μM in contrast to an GI_{50} value of 16 μM in normal human bronchial epithelial cells. A similar selective cytotoxicity index was also obtained for 6-thioguanine, suggesting that *ras*-transformed cells

may render highly efficient conversion of 5'-deoxynucleoside into its base. Additional study to demonstrate the induction of phosphorylase gene by activated *ras* will be useful. Our preliminary results indicate that 5'-deoxy-6-thioguanosine exhibits highly selective cytotoxicity against human tumor cells with an activated *ras* oncogene and further *in vivo* evaluation of this compound is desirable.

Table 2. Selective Cytotoxicity Test Against Human Bronchial Epithelial Cells (GI_{50} : μM).

	TBE[a]	HBE[a]	Selective Cytotoxicity Index[b]
3a	1.9×10^{-2}	2.2×10^{-1}	12
3b	7.1×10^{-1}	1.6×10^{1}	23
6-thioguanine	5.9×10^{-2}	1.2×10^{0}	20
adriamycin	6.0×10^{0}	5.0×10^{0}	1

[a] Human bronchial epithelial cells (HBE) were cultured from tissue specimens obtained during biopsy. Transformed bronchial epithelial cells (TBE) were isolated from HBE cells transfected with plasmid H1 carrying the *v-Ha-ras* oncogene via protoplast fusion.[22] GI_{50} for tested compounds were obtained from the dose-responses of HBE and TBE cells in a proliferation assay.[23]

[b] Selective cytotoxicity index : GI_{50} (HBE)/GI_{50} (TBE)

EXPERIMENTAL SECTION

General. All reagents and solvents were of analytical reagent quality. 2',5'-Dideoxythymidine, 5'-deoxyadenosine, 6-thioguanine, 6-mercaptopurine, purine nucleoside phosphorylase (EC 2.4.2.1.), thymidine phosphorylase (EC 2.4.2.4.) and adenosine deaminase (EC 3.5.4.4.) were purchased from Sigma Chemical Co. The melting point is uncorrected. ^{1}H NMR spectra were obtained at ambient temperature at 500 MHz with a Varian VXR-5000 spectrometer. The DMSO-d_5 peak in the NMR solvent was used as the internal reference for all the ^{1}H NMR spectra and was referenced at 2.49 ppm relative to TMS. The fast atom bombardment (FAB) mass spectral data were obtained using DTT/DTE (dithiothreitol : dithioerythritol = 3 : 1) as the sample matrix on a Kratos MS-50 sector mass spectrometer. Analytical HPLC utilized an Alltech Econosphere RP-C18 (150 x 4.6 mm, 3 μm) column and eluted with a linear gradient of 4-10% CH_3CN in 50 mM $HCOONH_4$ at 1 mL/min for 5 min. Preparative HPLC was done on an Alltech Econosphere RP-C18 (250 x 22.5 mm, 10

μm) column and eluted isocratically with a mobile phase of 10-15% CH_3CN and 1% dimethylformamide in water at 5 mL/min. The HPLC samples were filtered through Centrifree Filters (Amicon Division, W.R. Grace & Co.) to prevent column contamination by high molecular weight impurities like proteins. The mobile phase were prepared, filtered through 0.2 μm millipore filters and degassed prior to use.

2',5'-Dideoxy-6-thioguanosine (3a). A suspension of 100 mg (0.6 mmol) of 6-thioguanine (**1a**) and 50 mg (0.23 mmol) of 2',5'-dideoxythymidine (**2a**) in 150 mL of 10 mM sodium phosphate buffer, pH 7.4 was prepared. To this suspension 100 units of thymidine phosphorylase and 100 units of purine nucleoside phosphorylase were added. After 24 h of reaction with stirring at 37 °C, the reaction mixture was heated to 90 °C and held for 5 min to stop the reaction. After cooling to room temperature, the mixture was evaporated to dryness under reduced pressure. The powder was redissolved in 10 mL of dimethylformamide (DMF) and was loaded on preparative HPLC column by consecutive injection of 1.0 mL each. Preparative HPLC was eluted isocratically with a mobile phase of 10% CH_3CN and 1% DMF in water at 3 mL/min. The fractions corresponding to the 2',5'-dideoxy-6-thioguanosine were collected, combined and evaporated under reduced pressure, and the remainder of the fluid was finally lyophilized, yielding 29 mg (0.11 mmol, 48 %) of 2',5'-dideoxy-6-thioguanosine (**3a**) as white powder. : mp >295°C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.042 (H_8, s, 1H), δ 6.046 ($H_{1'}$, t, 1H, $^3J_{1',2'_\alpha} = {}^3J_{1',2'_\beta}$ = 6.7 Hz), δ 4.100 ($H_{3'}$, dt, 1H, $^3J_{3',2'_\beta}$ = 6.7 Hz, $^3J_{3',2'_\alpha} = {}^3J_{3',4'}$ = 4.0 Hz), δ 3.851 ($H_{4'}$, qd, 1H, $^3J_{4',5'}$ = 6.4 Hz, $^3J_{4',3'}$ = 4.0 Hz), δ 2.622 ($H_{2'_\beta}$, dt, 1H, $^2J_{2'_\beta,2'_\alpha}$ = 13.3 Hz, $^3J_{2'_\beta,1'} = {}^3J_{2'_\beta,3'}$ = 6.7 Hz), δ 2.196 ($H_{2'_\alpha}$, ddd, 1H, $^2J_{2'_\alpha,2'_\beta}$ = 13.3 Hz, $^3J_{2'_\alpha,1'}$ = 6.7 Hz, $^3J_{2'_\alpha,3'}$ = 4.0 Hz), δ 1.226 ($H_{5'}$, d, 3H, $^3J_{5',4'}$ = 6.4 Hz) ; FAB-MS m/z (relative intensity) 268.0865 (268.0868, calcd. for $C_{10}H_{14}N_5SO_2$; MH^+, 77) and 168 (BH^+, 100).

5'-Deoxy-6-thioguanosine (3b). A solution of 55 mg (0.22 mmol) of 5'-deoxyadenosine in 15 mL of 50 mM sodium phosphate buffer, pH 7.4 was prepared. To this solution 1000 units of adenosine deaminase was added. After 20 h of reaction with stirring at room temperature, 110 mg (0.66 mmol) of 6-thioguanine (**1a**) in 250 mL of double distilled water and 1000 units of nucleoside phosphorylase in 5 mL of double distilled water were added sequentially. After 7 days of reaction at 37 °C, the reaction mixture was heated to 90 °C and held for 5 min to stop the reaction. After cooling to room temperature, the mixture was lyophilized. The powder was resuspended in 20 mL of double distilled water and separated by preparative HPLC column in a manner similar to the above-mentioned procedure for **3a**. The solvent was removed by lyophilization and 28 mg (0.10 mmol, 45 %) of 5'-deoxy-6-thioguanosine (**3b**) was isolated as white powder. : mp 242 °C dec. (lit.[6] mp 246°C dec.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.065 (H_8, s, 1H), δ 5.632 ($H_{1'}$, d, 1H, $^3J_{1',2'}$ = 5.2 Hz), δ 4.460 ($H_{2'}$, t, 1H, $^3J_{2',1'} = {}^3J_{2',3'}$ = 5.2 Hz), δ 3.911 ($H_{4'}$, qd, 1H, $^3J_{4',5'}$ = 6.3 Hz, $^3J_{4',3'}$ = 5.2 Hz), δ 3.858

($H_{3'}$, t, 1H, $^3J_{3',4'} = {}^3J_{3',2'} = 5.2$ Hz), δ 1.269 ($H_{5'}$, d, 3H, $^3J_{5',4'} = 6.3$ Hz) ; FAB-MS m/z (relative intensity) 284.0811 (284.0817, calcd. for $C_{10}H_{14}N_5SO_3$; MH^+, 79) and 168 (BH^+, 100).

2',5'-Dideoxy-6-mercaptopurine Riboside (3c). A solution of 112 mg (0.66 mmol) of 6-mercaptopurine and 50 mg (0.23 mmol) of 2',5'-dideoxythymidine in a mixed solvent containing 20 mL of dimethylformamide and 70 mL of 10 mM sodium phosphate buffer, pH 7.4, was prepared. To this solution 1000 units of thymidine phosphorylase and 1000 units of purine nucleoside phosphorylase were added. After 12 h of reaction with stirring at room temperature, the reaction mixture was heated to 90 °C and held for 5 minutes to stop the reaction. After cooling to room temperature, the mixture was evaporated to dryness under reduced pressure. The residue was loaded on preparative HPLC column by consecutive injection of 1.0 mL each. Preparative HPLC was eluted isocratically with a mobile phase of 10% CH_3CN in 20 mM ammonium formate buffer at 5 mL/min. The fractions corresponding to the 2',5'-dideoxy-6-mercaptopurine riboside were collected, combined and evaporated under reduced pressure, and the remainder of the fluid was finally lyophilized, yielding 19 mg (0.08 mmol, 35 %) of 2',5'-dideoxy-6-mercaptopurine riboside **(3c)** as white powder : mp 186 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.434 (H_2, s, 1H),δ 8.193 (H_8, s, 1H), δ 6.251 ($H_{1'}$, t, 1H, $^3J_{1',2'_\alpha} = {}^3J_{1',2'_\beta} = 6.7$ Hz), δ 4.171 ($H_{3'}$, ddd, 1H, $^3J_{3',2'_\beta} = 6.7$ Hz, $^3J_{3',2'_\alpha} = 4.6$ Hz, $^3J_{3',4'} = 4.0$ Hz), δ 3.895 ($H_{4'}$, qd, 1H, $^3J_{4',5'} = 6.4$ Hz, $^3J_{4',3'} = 4.0$ Hz), δ 2.732 ($H_{2'_\beta}$, dt, 1H, $^2J_{2'_\beta,2'_\alpha} = 13.4$ Hz, $^3J_{2'_\beta,1'} = {}^3J_{2'_\beta,3'} = 6.7$ Hz), δ 2.292 ($H_{2'_\alpha}$, ddd, 1H, $^2J_{2'_\alpha,2'_\beta} = 13.4$ Hz, $^3J_{2'_\alpha,1'} = 6.7$ Hz, $^3J_{2'_\alpha,3'} = 4.6$ Hz), δ 1.244 ($H_{5'}$, d, 3H, $^3J_{5',4'} = 6.4$ Hz) ; FAB-MS m/z (relative intensity) 253.0754 (253.0759, calcd. for $C_{10}H_{13}N_4SO_2$; MH^+, 95) and 153 (BH^+, 100).

5'-Deoxy-6-mercaptopurine Riboside (3d). The procedure described for the synthesis of compound **(3b)** was utilized using 112 mg (0.66 mmol) of 6-mercaptopurine **(1b)** instead of 6-thioguanine **(1a)** as the starting material. The product was purified by preparative HPLC as indicated above. The solvent was removed by lyophilization and 24 mg (0.09 mmol, 41%) of 5'-deoxy-6-mercaptopurine riboside **(3d)** was isolated as white powder. : mp 210°C dec.; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.389 (H_2, s, 1H), δ 8.184 (H_8, s, 1H), δ 5.810 ($H_{1'}$, d, 1H, $^3J_{1',2'} = 5.0$ Hz), δ 4.550 ($H_{2'}$, t, 1H, $^3J_{2',1'} = {}^3J_{2',3'} = 5.0$ Hz), δ 3.962 ($H_{4'}$, qd, 1H, $^3J_{4',5'} = 6.3$ Hz, $^3J_{4',3'} = 5.0$ Hz), δ 3.919 ($H_{3'}$, t, 1H, $^3J_{3',4'} = {}^3J_{3',2'} = 5.0$ Hz), δ 1.290 ($H_{5'}$, d, 3H, $^3J_{5',4'} = 6.3$ Hz) ; FAB-MS m/z (relative intensity) 269.0702 (269.0708, calcd. for $C_{10}H_{13}N_4SO_3$; MH^+, 94) and 153 (BH^+, 100).

Cytotoxicity Assay. Cytotoxicity against three human solid tumor cell lines was assayed by the microculture tetrazolium assay method[24] performed at the Purdue Cancer Center Cytotoxicity Laboratory. The target cells were A-549 (human lung carcinoma), MCF-7 (human breast adenocarcinoma) and HT-29 (human colon adenocarcinoma) cells. Adriamycin was used for antitumor cytotoxicity control. *Ras*-transformed cells

were obtained from transfection of human bronchial epithelial cells (Comparative Human Tissue Network, Columbus, Ohio) with a plasmid containing the *v*-Ha-*ras* oncogene.[22] Selective cytotoxicity testings against normal (HBE) and *ras*-transformed (TBE) human bronchial epithelial cells were carried out according to the method described previously.[23]

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