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Novel C^2-purine position analogs of nitrobenzylmercaptopurine riboside as human equilibrative nucleoside transporter 1 inhibitors

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Abstract—Nucleoside transporter inhibitors have potential therapeutic applications as anticancer, antiviral, cardioprotective, and neuroprotective agents. S^6-(4-nitrobenzyl)mercaptopurine riboside (NBMPR) is a prototype inhibitor of the human equilibrative nucleoside transporter (hENT1), and is a high affinity ligand with a K_d of 0.1–1.0 nM. We have synthesized and flow cytometrically evaluated the binding affinity of a series of novel C^2-purine position substituted analogs of NBMPR at the hENT1. The aim of this research was to understand the substituent requirements at the C^2-purine position of NBMPR. Structure–activity relationships (SAR) indicate that increasing the steric bulk at the C^2-purine position of NBMPR led to a decrease in binding affinity of these ligands at the hENT1. New high affinity inhibitors were identified, with the best compound, 2-fluoro-4-nitrobenzyl mercaptopurine riboside (7), exhibiting a K_i of 2.1 nM. This information, when coupled with the information obtained from other structure–activity relationship studies should prove useful in efforts aimed at modeling the NMBPR and analogs pharmacophore of hENT1 inhibitors.

1. Introduction

In humans, the cellular uptake and efflux of physiological nucleosides and their synthetic analogs is regulated by specialized transport proteins known as nucleoside transporters.[1,2] Nucleoside transporters can be classified into two major classes, namely, bidirectional sodium-independent equilibrative nucleoside transporters (ENTs)[3] and unidirectional sodium dependent concentrative nucleoside transporters (CNTs).[4] In humans, four equilibrative nucleoside transporters[5–8] and six concentrative nucleoside transporters[1,9–13] have been identified to date. Of the four equilibrative nucleoside transporter isoforms, two isoforms, hENT1[5] and hENT2,[6] have been extensively characterized. Prior to the identification of their genes, hENT1 was referred to as *es* (equilibrative inhibitor sensitive) nucleoside transporter and hENT2 was referred to as *ei* (equilibrative inhibitor insensitive) nucleoside transporter.[14] This differentiation was based on their sensitivity to inhibition by S^6-(4-nitrobenzyl)mercaptopurine riboside (NBMPR, **1**). While NBMPR is known to inhibit hENT1 at low nanomolar concentrations, it requires micromolar concentrations to inhibit hENT2.[15] Dipyridamole, a potent non-nucleoside inhibitor, is known to inhibit both hENT1 and hENT2. The other two equilibrative nucleoside transporter isoforms hENT3[7] and hENT4[8] have also been identified recently.

Nucleoside transporter inhibitors have been shown to have a potential for therapeutic application in antimetabolite chemotherapy in cancer,[16,17] viral infections,[18] and AIDS related opportunistic infections such as *Toxoplasma gondii* infection,[19,20] in inflammatory disease,[21] and in heart disease and stroke as cardioprotective[22] and neuroprotective[23] agents, respectively. In humans, the realization of the importance of nucleoside transporters in cellular uptake and efficacy of anticancer and anti-HIV nucleoside analogs has also generated interest in nucleoside transporters. The ubiquitously distributed hENT1 appears to be the most important nucleoside transporter of mammalian tissues and hence it might be a relevant therapeutic target. Toxicity, lack of in vivo efficacy, and/or lack of selectivity have hampered the therapeutic application of the currently

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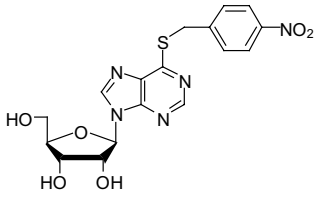
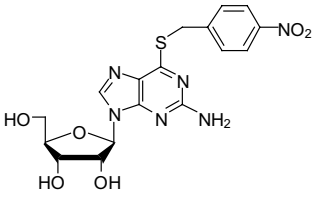
available hENT1 inhibitors. Although NBMPR ($K_i = 0.70 \pm 0.01$ nM) is a potent hENT1 inhibitor, it is not a suitable clinical candidate due to the possible immunosuppressant[24] and mutagenic[25] effects arising from its metabolism. On the other hand, the clinically used agent, dipyridamole, lacks selectivity as in addition to its nucleoside transporter inhibitory effects, it also inhibits phosphodiesterase, and is a stimulator of prostacyclin biosynthesis.[26] Dipyridamole is also highly protein bound to α-1 acid glycoprotein and albumin in plasma,[27,28] a property that greatly limits the concentration of free dipyridamole in blood. This in turn leads to insufficient dipyridamole concentrations for effective nucleoside transporter inhibition at the clinically tolerated dose. Thus there is a need for developing novel hENT1 inhibitors.

Detailed structure–activity relationship (SAR) studies at S^6-benzyl position of NBMPR have been carried out.[29–32] A recent study reported two series of C^8 alkylamine substituted compounds exhibiting binding affinities in the nanomolar range.[33] S^6-(4-nitrobenzyl)thioguanosine riboside (NBTGR, **2**), a prototype of NBMPR possessing an amino substituent on the C^2-purine position, is also a potent inhibitor of hENT1.[29] Although a few C^2-purine position substituted analogs of NBMPR have been reported,[34] a systematic study of this position is largely missing. This paper describes the synthesis and structure–activity relationship (SAR) studies of new C^2-purine position substituted NBMPR analogs as hENT1 ligands. This information will be useful in efforts aimed at modeling the NBMPR and analogs hENT1 inhibitory pharmacophore.

For this study, compounds having different halogen and alkylamino substituents at the C^2-purine position were synthesized and their binding affinities evaluated using a competitive flow cytometric binding assay.[31,32] The effect of bulk at the C^2-purine position was studied by systematically varying alkylamine substituents. A comparison of the inhibitory activities of C^2-purine position substituted analogs of NBMPR indicated that increasing the steric bulk at this position led to a decrease in the binding affinity for hENT1.



NBMPR, **1**



NBTGR, **2**

2. Results and discussion

2.1. Chemistry

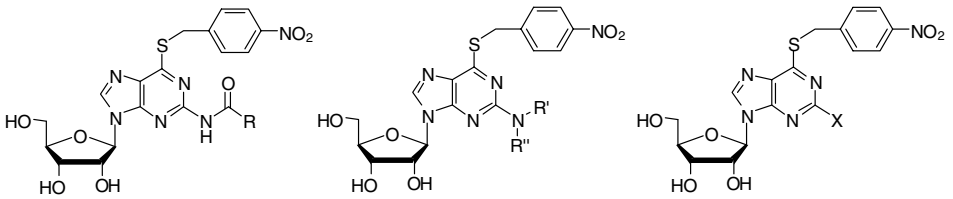
The analogs synthesized for the study are shown in Table 1. The reaction sequence used to synthesize the target compounds is shown in Scheme 1. Guanosine (**3**) was subjected to TBDMS protection[35] using TBDMS-Cl and imidazole to yield compound **4**. Compound **4** was further converted to thioguanosine (**5**) by treatment with Lawesson's reagent.[36] Compound **5** was then reacted with *p*-nitrobenzyl bromide in the presence of potassium carbonate as the base to yield compound **6**, TBDMS protected S^6-(4-nitrobenzyl)thioguanosine.[37] Deprotection of compound **6** using tetrabutylammonium fluoride (TBAF) gave S^6-(4-nitrobenzyl)thioguanosine riboside (NBTGR, **2**). NBTGR was then subjected to diazotization at low temperature in the presence of fluoroboric acid to yield compound **7**, the 2-fluoro analog.[38] Compound **7** was then reacted with appropriate alkyl amines to yield compounds **13–26**.[38,39] TBDMS protected S^6-(4-nitrobenzyl)thioguanosine riboside, compound **6**, was converted to halogenated compounds **9–10** following a diazotization with *tert*-butyl nitrite (TBN).[40] Chlorotrimethylsilane was used as the chlorine source, antimony (III) bromide was the bromine source, and diiodomethane was used as the iodine source for halogenation. The C^2-purine position substituted acetyl and benzoyl derivatives were prepared by reacting compound **6** with the appropriate acid chloride in pyridine to yield compounds **11** and **12**.[37] Compounds **8–12** were subjected to a final deprotection step using TBAF to yield compounds **27–31**. The compounds were characterized using NMR, mass spectroscopy, and HPLC analysis.

The synthesized 2-position NBMPR analogs were studied using a competitive flow cytometric binding assay with the human chronic myelogenous leukemia, K562 cell line as the source of hENT1. The hENT1 specific fluorescent probe, 5-(SAENTA)-*X*8-fluorescein,[41] was used as the ligand for the competitive binding assay.[42] The compounds exhibited a wide range of binding affinities at the hENT1 transporter as measured by their ability to displace 5-(SAENTA)-*X*8-fluorescein. The dose-dependent inhibitory data are depicted in Figure 1 and the K_i values are presented in Table 2.

2.2. Structure–activity relationship (SAR)

Within the series of straight chain C^2-alkylamine analogs, compound **13** possessing the smallest substituent, a methylamino group, had the lowest K_i (24 nM) whereas compound **20** with a long pentylamino substituent displayed the highest K_i value (357 nM), a 14-fold difference in binding affinities. A sudden, 8-fold decrease in binding affinity is seen when the alkyl chain length is increased from ethylamino, compound **15** (K_i = 24 nM), to *n*-propylamino, compound **16** (K_i = 193 nM). The affinity for the transporter decreases with increasing length of the alkyl chain, indicating that an increase in bulk at the C^2-purine position is detrimental to the binding affinity at the hENT1, with regard to alkylamino groups. This is different from the situation at the C^8-position where elongation of the alkylamine chain, in C^8-alkylamine-substituted S^6-(benzyl)mercaptopurine riboside and C^8-alkylamine-substituted S^6-(4-nitrobenzyl)mercaptopurine, led to an improvement in binding affinity at the hENT1.[33]

Table 1. C^2-purine position analogs synthesized and studied using flow cytometry



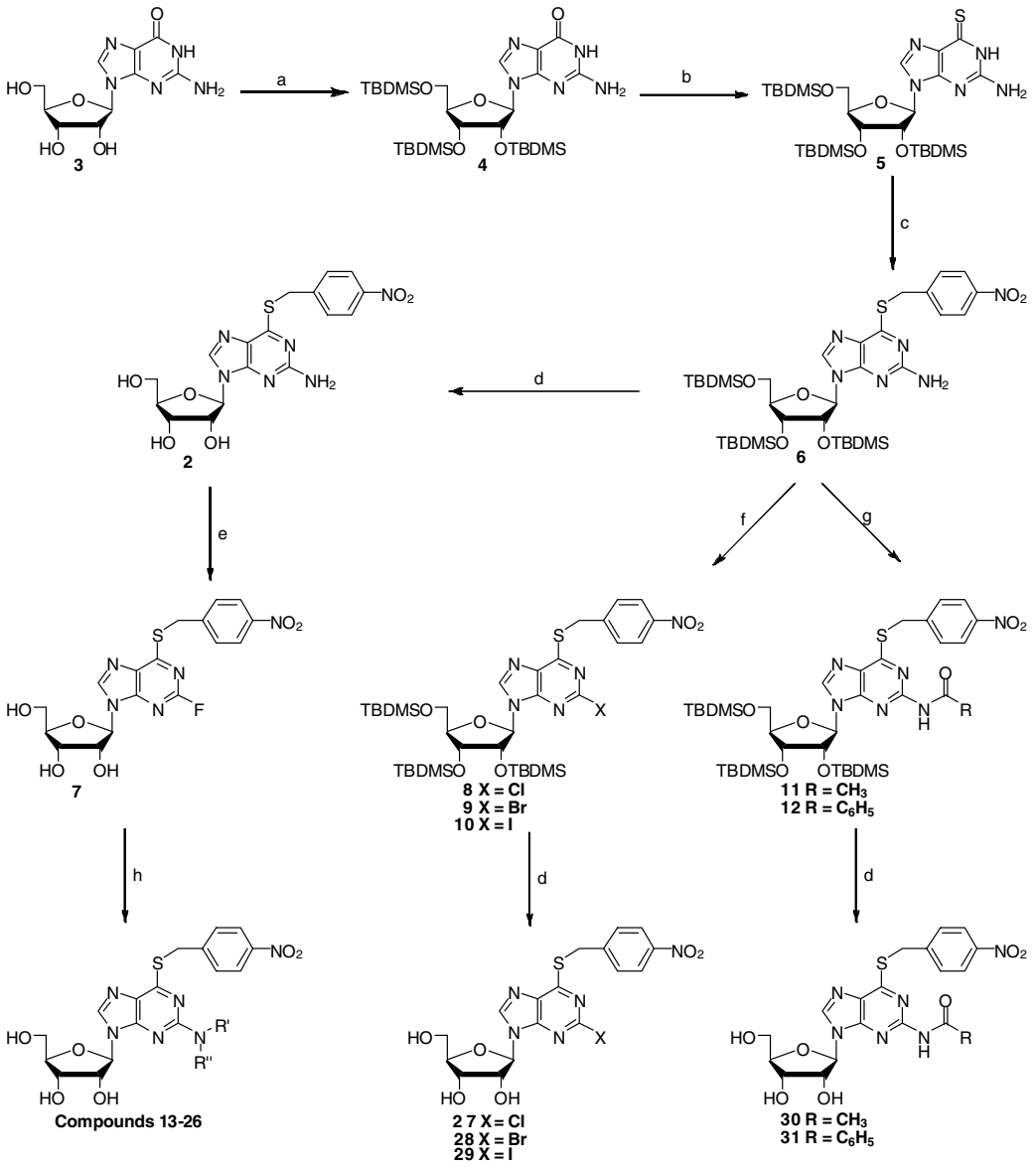
Compound	R	R′	R″	X
2 (NBTGR)	—	–H	–H	—
7	—	—	—	–F
13	—	$-CH_3$	–H	—
14	—	$-CH_3$	$-CH_3$	—
15	—	$-CH_2CH_3$	–H	—
16	—	$-CH_2CH_2CH_3$	–H	—
17	—	–Cyclopropyl	–H	—
18	—	$CH_2CH_2CH_2CH_3$	–H	—
19	—	–Cyclobutyl	–H	—
20	—	$CH_2CH_2CH_2CH_2CH_3$	–H	—
21	—	–Cyclopentyl	–H	—
22	—	–Cyclohexyl	–H	—
23	—	$-CH_2C_6H_5$	–H	—
24	—	$-CH_2CH_2C_6H_5$	–H	—
25	—	$-CH_2CH_2OH$	–H	—
26	—	CH_2CH_2OH	$-CH_2CH_2OH$	—
27	—	—	—	–Cl
28	—	—	—	–Br
29	—	—	—	–I
30	$-CH_3$	—	—	—
31	$-C_6H_5$	—	—	—

Within the series of constrained C^2-alkylamine analogs synthesized, a similar pattern is observed. Compound **17** (K_i = 25 nM), possessing the smallest ring, cyclopropylamino substituent, binds 16-fold better than compound **22** (K_i = 400 nM), possessing the largest substituent, cyclohexylamino. Also compound **19** (K_i = 33 nM), possessing a cyclobutylamino substituent, binds better than compound **21** (K_i = 125), possessing a cyclopentylamino substituent. Thus, increasing the ring size and thereby the bulk led to a decrease in binding affinity. It was also observed that, in each case, the ring constrained analog binds better at the hENT1 than its corresponding straight chain compound. For example, compound **17** (K_i = 25 nM), possessing a cyclopropylamino substituent, binds better than compound **16** (K_i = 195 nM), possessing an *n*-propylamino substituent. Another example of the detrimental effect of bulk on binding is shown by compound **30** (K_i = 34 nM), possessing an acetamido substituent, binding about 10-fold better than compound **31** (K_i = 315 nM), possessing a benzamido substituent. Increasing the bulk from a methylamino substituent, compound **13** (K_i = 24 nM), to a dimethylamino substituent, compound **14** (K_i = 429 nM), resulted in a drastic 17-fold decrease in binding affinity. NBTGR (compound **2**, K_i = 1.1 nM), with an amino substituent in turn has a 22-fold higher binding affinity than the methylamino substituted compound. A similar trend is also seen in the case of the monoethanolamino substituent (compound **25** K_i = 86 nM) and the diethanolamino substituent (compound **26** K_i = 243 nM), where the improvement in binding affinity of the monoethanolamino substituent over the diethanolamino substituent is 3-fold.

The decrease in binding affinity with increasing substituent bulk at the C^2-purine position was also observed somewhat within the halogen-substituted compound series where, the smallest halogen substituent fluoro (compound **7**, K_i = 2.1 nM) exhibits almost a 5-fold higher binding affinity than the chloro compound (**27**, K_i = 11 nM), bromo compound (**28**, K_i = 11 nM), and the iodo compound (**29**, K_i = 11 nM) substituents. In general, electron withdrawing halogen substituents were well tolerated than electron donating alkylamine substituents. The lack of differences between the three larger halogen substituents, Cl, Br, and I, suggests that the effect of bulk is not the only structural parameter determining binding affinity at the C^8-position in the case of the halogen substituents. The detrimental effect of bulk may be compensated for by the increasing lipophilicity of the halogen.

3. Conclusion

Through this novel series of C^2-purine position substituted analogs of NBMPR it has been recognized that steric interactions appear to play an important role in determining the binding of C^2-purine position NBMPR analogs at the hENT1. Increasing the steric bulk at this position leads to a loss in inhibitory activity indicating a limited bulk tolerance at this site. This information will



Scheme 1. Reagents and conditions: (a) TBDMS-Cl/imidazole/DMF (yield 70%); (b) Lawesson's reagent/toluene/100 °C (yield 89%); (c) *p*-nitrobenzyl bromide/K_2CO_3/DMF (yield 75%); (d) TBAF/THF (yield ~70%); (e) aq fluoroboric acid/$NaNO_2$/−5 °C (yield 39%); (f) for X = Cl, TBN/TMS-Cl/CH_2Cl_2/0 °C (yield 20%); for X = Br, TBN/$SbBr_3$/CH_2Br_2/−10 °C (yield 18%); for X = I, TBN/CH_2I_2/80 °C (yield 26%); (g) RCOCl/pyridine (yield 90%); (h) R′-NH-R″/methanol/rt (yield ~95%).

be combined with the information from analysis of conformationally restricted analogs[42] to aid in our pharmacophore modeling of hENT1 inhibitors. The study has also identified new compounds with substantial affinity for hENT1 that may serve as new leads for developing hENT1 inhibitor-based therapeutics.

4. Experimental

Thin layer chromatography (TLC) was conducted using silica gel GHLF-250 microns plates (Analtech). Compounds were visualized by UV light or 5% H_2SO_4 in EtOH spraying reagent. The ^1H NMR spectra were recorded either on a Bruker ARX 300 MHz NMR or a Varian Unity Inova 500 MHz instrument. All NMR were recorded in DMSO unless otherwise specified. Flash column chromatography was performed on Fisher Silica gel (170–400 mesh). Melting points of final products were determined using a Fisher-Johns Melting Point Apparatus and are reported uncorrected. Mass spectra were obtained on a Bruker-HP Esquire-LC mass spectrometer. HPLC analysis of final products was carried out by a linear gradient elution using acetonitrile/water (0.1% TFA). A reverse phase Luna 5μ C-18 (2) Phenomenex column, of dimensions 2.00×150 mm was used for HPLC analysis. HPLC analysis was conducted using a flow rate of 1.0 ml/min and UV detection at a wavelength of 254 nm. All solvents were purchased from Fisher Scientific and reagents were purchased from Aldrich and were used without further purification.

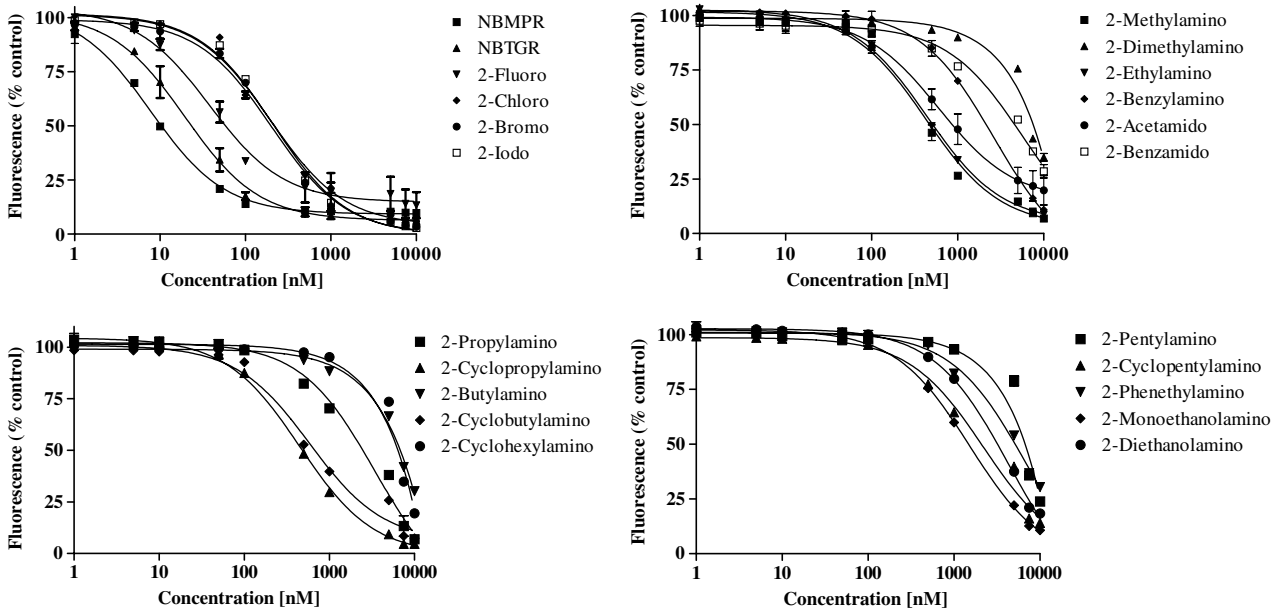


Figure 1. Equilibrium displacement of SAENTA-fluorescein ligand by new C^2-purine position analogs of NBMPR in K562 cells. Cells were incubated with 30 nM SAENTA-*X*8-fluorescein in the presence or absence of inhibitor for 45 min at room temperature and analyzed by flow cytometry (FACSCalibur®). Data were collected on 5000 cells per sample, and mean channel numbers were used as a measure of fluorescence output from ligands.

Table 2. Binding affinities (K_i values) of C^2-purine position analogs determined using flow cytometry

Compound	2-Position substituent	K_i[a] (nM)
1 (NBMPR)	–H	0.70 ± 0.01
2 (NBTGR)	Amino	1.1 ± 0.21
7	Fluoro	2.1 ± 0.10
13	Methylamino	24.0 ± 0.15
14	Dimethylamino	429 ± 0.84
15	Ethylamino	24 ± 0.05
16	Propylamino	193 ± 0.51
17	Cyclopropylamino	25 ± 0.06
18	Butylamino	325 ± 0.51
19	Cyclobutylamino	33 ± 0.15
20	Pentylamino	357 ± 0.97
21	Cyclopentylamino	125 ± 0.29
22	Cyclohexylamino	400 ± 0.97
23	Benzylamino	247 ± 0.58
24	Phenethylamino	394 ± 0.01
25	Ethanolamino	86 ± 0.04
26	Diethanolamino	243 ± 0.30
27	Chloro	11 ± 0.01
28	Bromo	11 ± 0.08
29	Iodo	11 ± 0.07
30	Acetamido	34 ± 0.16
31	Benzamido	315 ± 0.72

[a] Flow cytometrically determined K_i values of C^2-purine position analogs for inhibition of SAENTA-fluorescein binding to the hENT1 in K562 cells (SAENTA-fluorescein, $K_d = 1.8 \pm 0.6$ nM).[42] The results are presented as K_i values ± SEM from two separate determinations.

4.1. 2′,3′,5′-*O*-(*tert*-Butyldimethylsilyloxy)guanosine (4)

To a solution of guanosine (1.0 g, 3.53 mmol) and imidazole (2.42 g, 35.54 mmol) in 9 ml of anhydrous DMF was added 2.67 g (17.71 mmol) of *tert*-butyldimethylsilyl chloride (TBDMS-Cl). The reaction mixture was stirred at room temperature under argon for 26 h. The resulting mixture was poured into ethyl acetate–water. The organic layer was dried over anhydrous Na_2SO_4 and evaporated. Column chromatographic separation using methanol/CH_2Cl_2 (0.4:9.6) gave 1.5 g of compound **4** as a white solid.

Yield 70%; mp 258–260 °C; MS: (ESI, Pos) *m*/*z* 648.6 $[(M+23)]^+$; ^{1}H NMR (500 MHz, DMSO) δ 10.630 (1H, s), 7.891 (1H, s), 6.465 (2H, br s, disappeared on D_2O exchange), 5.744 (1H, d, $J = 7.0$ Hz), 4.589 (1H, dd, $J = 4.5$, 2.5 Hz), 4.171 (1H, d, $J = 3.5$ Hz), 3.953 (1H, t, $J = 3.5$ Hz), 3.777 (2H, m), 0.910, 0.907, and 0.731 (each s, 3× 9H), 0.121, 0.100, 0.094 (m, 18H).

4.2. 2′,3′,5′-*O*-(*tert*-Butyldimethylsilyloxy)-6-thioguanosine (5)

Lawesson's reagent (316.25 mg, 0.78 mmol) was added to a solution of 2′,3′,5′-*O*-(*tert*-butyldimethylsilyloxy)guanosine (300 mg, 0.47 mmol) in 10 ml toluene and the mixture was stirred at 100 °C for 2 h, at which time the TLC in 5% methanol in CH_2Cl_2 indicated the formation of compound **5**, and the consumption of starting material. Concentration followed by flash chromatography using methanol/CH_2Cl_2 (0.5:9.5) afforded 274 mg of compound **5** as a white solid.

Yield 89%; mp 275–277 °C; MS: (ESI, Pos) *m*/*z* 664.5 $[(M+23)]^+$; ^{1}H NMR (500 MHz, DMSO) δ 12.260 (1H, s), 8.363 (1H, s), 7.101 (2H, br s, disappeared on D_2O exchange), 6.017 (1H, d, $J = 7.0$ Hz), 4.861 (1H, m), 4.454 (1H, m), 4.245 (1H, m), 4.072 (2H, m), 1.188, 1.186, and 1.014 (each s, 3× 9H), 0.398, 0.385, 0.377 (m, 18H).

4.3. 2′,3′,5′-*O*-(*tert*-Butyldimethylsilyloxy)-2-amino-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (6)

To a solution of compound **5** (3.21 g, 5 mmol) in 30 ml of anhydrous DMF was added *p*-nitro benzyl bromide (1.296 g, 5.99 mmol) and potassium carbonate (6 g, 43.41 mmol). The mixture was stirred overnight at room temperature. The mixture was evaporated in vacuo at a temperature below 45 °C to remove the DMF. The residue was dissolved in water and extracted with ethyl acetate. The combined organic layer was washed once with brine, dried over Na_2SO_4, and evaporated in vacuo. Column chromatography using ethyl acetate/hexanes (2:8) gave 2.9 g of compound **6** as a yellow solid.

Yield 75%; mp 85–87 °C; MS: (ESI, Pos) *m*/*z* 799.5 $[(M+23)]^+$; ^{1}H NMR (300 MHz, DMSO) δ 8.139 (3H, s), 7.781 (2H, d, J = 8.7 Hz), 6.676 (2H, s, disappeared on D_2O exchange), 5.820 (1H, d, J = 6.9 Hz), 4.967 (1H, m), 4.650 (2H, d, J = 5.6 Hz), 4.205 (1H, m), 3.958 (1H, m), 3.786 (2H, m), 0.913, 0.886, and 0.695 (each s, 3× 9H), 0.123, 0.103, 0.079 (m, 18H).

4.4. 2′,3′,5′-*O*-(*tert*-Butyldimethylsilyloxy)-2-chloro-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (8)

A solution of *tert*-butylnitrite (0.8 ml, 6.73 mmol) in 5 ml CH_2Cl_2 was cooled to −10 °C while being stirred. Chlorotrimethylsilane (0.4 ml, 3.16 mmol) was added dropwise to this stirred solution followed by dropwise addition of a solution of compound **6** (150 mg, 0.193 mmol) in 5 ml CH_2Cl_2. The reaction mixture was stirred for 2 h at −10 °C, diluted with CH_2Cl_2, and extracted twice with water followed by saturated aqueous $NaHCO_3$. The organic layer was separated, dried over Na_2SO_4, and evaporated under reduced pressure. The resulting yellow oil was subjected to column chromatography using ethyl acetate/hexanes (2:8), which yielded 30.74 mg of compound **8** as a yellow solid.

Yield 20%; mp 97–99 °C; MS: (ESI, Pos) *m*/*z* 818.5 $[(M+23)]^+$; ^{1}H NMR (300 MHz, DMSO) δ 8.687 (1H, s), 8.177 (2H, d, J = 8.7 Hz), 7.762 (2H, d, J = 8.7 Hz), 5.912 (1H, d, J = 4.8 Hz), 4.852 (1H, t, J = 3.9 Hz), 4.737 (2H, d, J = 4.8 Hz), 4.443 (1H, t, J = 3.8 Hz), 3.990 (1H, m), 3.849 (2H, m), 0.909, 0.797, and 0.746 (each s, 3× 9H), 0.131, 0.111, 0.032 (m, 18H).

4.5. 2′,3′,5′-*O*-(*tert*-Butyldimethylsilyloxy)-2-bromo-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (9)

To a flask containing compound **6** (300 mg, 0.38 mmol) was added antimony (III) bromide (190 mg, 0.52 mmol), and the flask was then purged with argon. Anhydrous methylene bromide (10 ml), cooled to 0 °C, was added to the reaction flask which was cooled to 0 °C, as well. After the mixture was further cooled to −10 °C, *tert*-butylnitrite (0.2 ml, 1.68 mmol) was added slowly via a syringe. After completion of the nitrite addition, the reaction mixture was stirred for 1 h at −10 °C and then poured into 25 ml of a mixture of crushed ice, water, and 1 g of sodium bicarbonate. The reaction product was filtered and the filtrate was extracted with methylene chloride. The combined organic extract was dried over sodium sulfate. After filtration the solvent was removed in vacuo, and yellow oil was obtained. Flash chromatography using ethyl acetate/hexanes (2:8) as the eluent yielded 58.4 mg of compound **9** as a yellow solid.

Yield 18%; mp 82–84 °C; MS: (ESI, Pos) *m*/*z* 864.4 $[(M+23)]^+$; ^{1}H NMR (300 MHz, DMSO) δ 8.664 (1H, s), 8.178 (2H, d, J = 8.7 Hz), 7.769 (2H, d, J = 8.7 Hz), 5.908 (1H, d, J = 4.5 Hz), 4.859 (1H, t, J = 4.2 Hz), 4.724 (2H, d, J = 5.4 Hz), 4.426 (1H, t, J = 3.6 Hz), 3.987 (1H, m), 3.843 (2H, m), 0.909, 0.805, and 0.749 (each s, 3× 9H), 0.133, 0.112, 0.040 (m, 18H).

4.6. 2′,3′,5′-*O*-(*tert*-Butyldimethylsilyloxy)-2-iodo-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (10)

To a solution of compound **6** (300 mg, 0.38 mmol) in 0.5 ml CH_3CN and 2 ml CH_2I_2 was added *tert*-butylnitrite (0.16 ml, 1.34 mmol), and the mixture was stirred at 80 °C for 3 h. The reaction mixture was directly purified by silica gel column chromatography using ethyl acetate/hexanes (3:7) as the eluent, to give 89.12 mg of compound **10** as a yellow solid.

Yield 26%; mp 109–111 °C; MS: (ESI, Pos) *m*/*z* 910.4 $[(M+23)]^+$; ^{1}H NMR (300 MHz, DMSO) δ 8.586 (1H, s), 8.170 (2H, d, J = 8.7 Hz), 7.768 (2H, d, J = 8.7 Hz), 5.891 (1H, d, J = 5.1 Hz), 4.890 (1H, t, J = 4.5 Hz), 4.697 (2H, d, J = 6.0 Hz), 4.393 (1H, t, J = 3.6 Hz), 3.975 (1H, m), 3.829 (2H, m), 0.907, 0.820, and 0.741 (each s, 3× 9H), 0.134, 0.111, 0.053 (m, 18H).

4.7. 2′,3′,5′-*O*-(*tert*-Butyldimethylsilyloxy)-2-benzamido-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (12)

Benzoyl chloride (0.12 ml, 1 mmol) was added dropwise to a solution of compound **6** (300 mg, 0.38 mmol) in 5 ml of anhydrous pyridine at 0 °C. The mixture was stirred at room temperature for 4 h. Most of the pyridine was removed in vacuo. The resulting mixture was poured into CH_2Cl_2–H_2O and the organic layer was washed with $CuSO_4$ solution to remove traces of pyridine. The organic layer was dried over anhydrous Na_2SO_4 and filtered. The solvent was removed in vacuo. The compound was separated by flash column chromatography using ethyl acetate/hexanes (2:8) as the eluent, to yield 306 mg of compound **12** as a yellow solid.

Yield 90%; mp 92–94 °C; MS: (ESI, Pos) *m*/*z* 903.5 $[(M+23)]^+$; ^{1}H NMR (300 MHz, DMSO) δ 11.012 (1H, s, NH-2, disappeared on D_2O exchange), 8.593 (1H, s), 8.146 (2H, d, J = 8.7 Hz), 7.979 (2H, d, J = 6.9 Hz), 7.820 (2H, d, J = 8.7 Hz), 7.581 (3H, m), 5.913 (1H, d, J = 6.0 Hz), 5.277 (2H, d, J = 3.6 Hz), 4.812 (2H, d, J = 4.5 Hz), 4.295 (1H, m), 4.016 (1H, m), 3.836 (2H, m), 0.915, 0.794, and 0.688 (each s, 3× 9H), 0.133, 0.113, 0.049 (m, 18H).

4.8. 2′,3′,5′-*O*-(*tert*-Butyldimethylsilyloxy)-2-acetamido-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (11)

Acetyl chloride (0.07 ml, 1 mmol) was added dropwise to a solution of compound **6** (300 mg, 0.38 mmol) in 5 ml of anhydrous pyridine at 0 °C. The mixture was stirred at room temperature for 4 h. Most of the pyridine was removed in vacuo. The resulting mixture was poured into CH_2Cl_2–H_2O and the organic layer was washed with $CuSO_4$ solution to remove traces of pyridine. The organic layer was dried over anhydrous Na_2SO_4 and filtered. The solvent was removed in vacuo. The compound was separated by flash column chromatography using ethyl acetate/hexanes (2:8) as the eluent, to yield 291 mg of compound **11** as a yellow solid.

Yield 92%; mp 83–85 °C; MS: (ESI, Pos) *m*/*z* 841.4 $[(M+23)]^+$; ^{1}H NMR (300 MHz, DMSO) δ 10.641 (1H, s, disappeared on D_2O exchange), 8.527 (1H, s), 8.153 (2H, d, J = 8.7 Hz), 7.814 (2H, d, J = 8.7 Hz), 5.905 (1H, d, J = 6.0 Hz), 5.143 (1H, m), 4.803 (2H, d, J = 4.6 Hz), 4.258 (1H, m), 4.006 (1H, m), 3.876 (2H, m), 2.205 (3H, s), 0.964, 0.866, and 0.664 (each s, 3× 9H), 0.178, 0.136, 0.070 (m, 18H).

4.9. 2-Acetamido-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (30)

To a solution of compound **11** (291 mg, 0.35 mmol) in 10 ml of anhydrous THF was added 270 mg (1.03 mmol) of tetrabutylammonium fluoride. The solution was stirred at room temperature for 4 h. Concentration of the reaction mixture followed by flash chromatography using methanol/CH_2Cl_2 (1:9) afforded 89.6 mg of compound **30** as a yellow solid.

Yield 53%; mp 95–97 °C; MS: (ESI, Pos) *m*/*z* 499.2 $[(M+23)]^+$; ^{1}H NMR (500 MHz, DMSO) δ 10.672 (1H, s, disappeared on D_2O exchange), 8.624 (1H, s), 8.154 (2H, d, J = 8.5 Hz), 7.828 (2H, d, J = 8.5 Hz), 5.895 (1H, d, J = 5.5 Hz), 5.495 (1H, d, J = 6.0 Hz, disappeared on D_2O exchange), 5.212 (1H, d, J = 4.6 Hz, disappeared on D_2O exchange), 5.207 (1H, t, J = 5.4 Hz, disappeared on D_2O exchange), 4.830 (2H, d, J = 7.5 Hz), 4.571 (1H, dd, J = 11.0, 5.5 Hz), 4.172 (1H, dd, J = 8.5, 4.5 Hz), 3.926 (1H, dd, J = 8.0, 4.0 Hz), 3.599 (2H, m), 2.234 (3H, s). HPLC (retention time: 8.74 min, purity: 97.90%).

4.10. 2-Benzamido-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (31)

To a solution of compound **12** (306 mg, 0.34 mmol) in 10 ml of anhydrous THF was added 280 mg (1.07 mmol) of tetrabutylammonium fluoride. The solution was stirred at room temperature for 4 h. Concentration of the reaction mixture followed by flash chromatography using methanol/CH_2Cl_2 (1:9) afforded 98.7 mg of compound **31** as a dark yellow solid.

Yield 52%; mp 115–117 °C; MS: (ESI, Pos) *m*/*z* 561.3 $[(M+23)]^+$; ^{1}H NMR (500 MHz, DMSO) δ 11.127 (1H, s, disappeared on D_2O exchange), 8.654 (1H, s), 8.194 (2H, d, J = 9.1 Hz), 8.804 (2H, d, J = 8.5 Hz), 7.876 (2H, d, J = 8.8 Hz), 7.681 (1H, t, J = 7.4 Hz), 7.600 (2H, t, J = 7.8 Hz), 6.001 (1H, d, J = 5.8 Hz), 5.553 (1H, d, J = 5.8 Hz, disappeared on D_2O exchange), 5.260 (1H, d, J = 4.7 Hz, disappeared on D_2O exchange), 5.029 (1H, t, J = 5.6 Hz, disappeared on D_2O exchange), 4.852 (2H, d, J = 7.5 Hz), 4.667 (1H, dd, J = 11.0, 5.5 Hz), 4.122 (1H, dd, J = 9.0, 3.5 Hz), 3.984 (1H, dd, J = 9.0, 3.5 Hz), 3.699 (2H, m). HPLC (retention time: 10.20 min, purity: 97.64%).

4.11. 2-Amino-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (2)

To a solution of compound **6** (300 mg, 0.385 mmol) in 10 ml of anhydrous THF was added 290 mg (1.11 mmol) of tetrabutylammonium fluoride. The solution was stirred at room temperature for 4 h. Concentration of the reaction mixture followed by flash chromatography using methanol/CH_2Cl_2 (0.5:9.5) afforded 83.8 mg of compound **2** as a yellow solid.

Yield 50%; mp 203–205 °C; MS: (ESI, Pos) *m*/*z* 457.1 $[(M+23)]^+$; ^{1}H NMR (300 MHz, DMSO) δ 8.188 (1H, s), 8.147 (2H, d, J = 8.4 Hz), 7.780 (2H, d, J = 8.4 Hz), 6.680 (2H, s, disappeared on D_2O exchange), 5.775 (1H, d, J = 5.7 Hz), 5.408 (1H, d, J = 5.7 Hz, disappeared on D_2O exchange), 5.138 (1H, d, J = 4.5 Hz, disappeared on D_2O exchange), 5.047 (1H, t, J = 5.4 Hz, disappeared on D_2O exchange), 4.658 (2H, s), 4.459 (1H, m), 4.105 (1H, m), 3.886 (1H, m), 3.562 (2H, m). HPLC (retention time: 9.00 min, purity: 98.27%).

4.12. 2-Fluoro-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (7)

Finely powdered compound **2** (2.08 g, 4.78 mmol) was added to 14 ml of 48% aqueous fluoroboric acid at 0 °C and the mixture then was cooled to −5 °C. A solution of sodium nitrite (785 mg, 11.37 mmol) in 3 ml of water was slowly added over a period of 1.25 h while the temperature was maintained at −5 °C. The resulting solution was stirred at −5 °C for an additional 15 min. and then diluted with 2 ml. of ice water. A gummy solid formed which coagulated upon dropwise addition of 28% ammonium hydroxide. The acidic aqueous portion then was decanted and discarded. The gum was immediately mixed with 2 ml of ice water and pulverized 0–5 °C with 28% ammonium hydroxide. The solid was filtered and dried in vacuo to give the crude product. Column chromatography using methanol/CH_2Cl_2 (1:9) gave 527 mg of compound **7** as a light yellow solid.

Yield 25.16%; mp 92–94 °C; MS: (ESI, Pos) *m*/*z* 460.1 $[(M+23)]^+$; ^{1}H NMR (300 MHz, DMSO) δ 8.736 (1H, s), 8.193 (2H, d, J = 8.7 Hz), 7.760 (2H, d, J = 9.0 Hz), 5.890 (1H, d, J = 5.4 Hz), 5.554 (1H, d, J = 5.7 Hz, disappeared on D_2O exchange), 5.250 (1H, d, J = 5.1 Hz, disappeared on D_2O exchange), 5.049 (1H, t, J = 5.4 Hz, disappeared on D_2O exchange), 4.765 (2 H, s), 4.512 (1H, m), 4.154 (1H, m), 3.960 (1H, m), 3.624 (2H, m). HPLC (retention time: 9.94 min, purity: 99.39%).

4.13. 2-Methylamino-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (13)

To a solution of compound **7** (30 mg, 0.069 mmol) in 10 ml of methanol was added 1 ml of a 2.0 M solution of methylamine in methanol. The solution was stirred at room temperature for 30 min and then was evaporated to oil in vacuo. The compound was separated using flash column chromatography using methanol/ethyl acetate (1:9) as the eluent, to give 28.6 mg of compound **13** as a yellow solid.

Yield 93%; mp 112–114 °C; MS: (ESI, Pos) *m*/*z* 449.1 $[(M+23)]^+$; ^{1}H NMR (500 MHz, DMSO) δ 8.168 (3H, m), 7.763 (2H, d, J = 8.7 Hz), 7.162 (1H, br s, disappeared on D_2O exchange), 5.801 (1H, d, J = 5.8 Hz), 5.402 (1H, d, J = 6.2 Hz, disappeared on D_2O exchange), 5.167 (1H, d, J = 4.6 Hz, disappeared on D_2O exchange), 4.959 (1H, br s, disappeared on D_2O exchange), 4.769 (2H, s), 4.684 (1H, br s), 4.159 (1H, m), 3.905 (1H, m), 3.592 (2H, m), 2.857 (3H, d, J = 4.6 Hz). HPLC (retention time: 9.41 min, purity: 99.35%).

4.14. 2-Dimethylamino-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (14)

To a solution of compound **7** (30 mg, 0.069 mmol) in 10 ml of methanol was added 1 ml of a 2.0 M solution of dimethylamine in THF. The solution was stirred at room temperature for 30 min and then was evaporated to oil in vacuo. The compound was separated using flash column chromatography using methanol/ethyl acetate (1:9) as the eluent, to give 29.1 mg of compound **14** as a yellow solid.

Yield 92%; mp 192–194 °C; MS: (ESI, Pos) *m*/*z* 485.2 $[(M+23)]^+$; ^{1}H NMR (300 MHz, DMSO) δ 8.209 (1H, s), 8.180 (2H, d, J = 8.7 Hz), 7.720 (2H, d, J = 8.4 Hz), 5.807 (1H, d, J = 5.7 Hz), 5.410 (1H, d, J = 6.0 Hz, disappeared on D_2O exchange), 5.189 (1H, d, J = 5.1 Hz, disappeared on D_2O exchange), 4.902 (1H, t, J = 5.7 Hz, disappeared on D_2O exchange), 4.717 (2H, s), 4.624 (1H, m), 4.156 (1H, m), 3.892 (1H, m), 3.576 (2H, m), 3.146 (6H, s). HPLC (retention time: 9.97 min, purity: 98.19%).

4.15. 2-Ethylamino-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (15)

To a solution of compound **7** (30 mg, 0.069 mmol) in 10 ml of methanol was added 1 ml of a 2.0 M solution of ethylamine in THF. The solution was stirred at room temperature overnight and then was evaporated to an oil in vacuo. The compound was separated using flash column chromatography using methanol/ethyl acetate (1:9) as the eluent, to give 29.8 mg of compound **15** as a yellow solid.

Yield 94%; mp 173–175 °C; MS: (ESI, Pos) *m*/*z* 485.2 $[(M+23)]^+$; ^{1}H NMR (500 MHz, DMSO) δ 8.170 (3H, m), 7.753 (2H, d, J = 8.5 Hz), 7.197 (1H, br s, disappeared on D_2O exchange), 5.786 (1H, d, J = 5.9 Hz), 5.418 (1H, d, J = 6.1 Hz, disappeared on D_2O exchange), 5.179 (1H, d, J = 4.6 Hz, disappeared on D_2O exchange), 4.973 (1H, br s, disappeared on D_2O exchange), 4.689 (2H, s), 4.658 (1H, br s), 4.140 (1H, m), 3.895 (1H, m), 3.583 (2H, m), 3.296 (2H, m), 1.117 (3H, m). HPLC (retention time: 9.84 min, purity: 99.51%).

4.16. 2-Propylamino-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (16)

To a solution of compound **7** (30 mg, 0.069 mmol) in 5 ml of methanol was added 0.1 ml (1.21 mmol) of propylamine. The solution was stirred for 1 day at 45 °C and then was evaporated to oil in vacuo. The compound was separated using flash column chromatography using methanol/ethyl acetate (1:9) as the eluent, to give 30 mg of compound **16** as a yellow solid.

Yield 92%; mp 93–95 °C; MS: (ESI, Pos) *m*/*z* 499.1 $[(M+23)]^+$; ^{1}H NMR (300 MHz, DMSO) δ 8.163 (3H, m), 7.746 (2H, d, J = 8.4 Hz), 7.215 (1H, br s, disappeared on D_2O exchange), 5.775 (1H, d, J = 5.7 Hz), 5.402 (1H, d, J = 6.0 Hz, disappeared on D_2O exchange), 5.152 (1H, d, J = 4.8 Hz, disappeared on D_2O exchange), 4.953 (1H, br s, disappeared on D_2O exchange), 4.683 (2H, s), 4.581 (1H, br s), 4.135 (1H, m), 3.895 (1H, m), 3.577 (2H, m), 3.253 (2H, m), 1.540 (2H, m), 0.881 (3H, t, J = 6.6 Hz). HPLC (retention time: 10.29 min, purity: 95.19%).

4.17. 2-Cyclopropylamino-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (17)

To a solution of compound **7** (30 mg, 0.069 mmol) in 5 ml of methanol was added 0.1 ml (1.42 mmol) of cyclopropylamine. The solution was stirred for 1 day at 45 °C and then was evaporated to oil in vacuo. The compound was separated using flash column chromatography using methanol/ethyl acetate (1:9) as the eluent, to give 29.6 mg of compound **17** as a yellow solid.

Yield 91%; mp 184–186 °C; MS: (ESI, Pos) *m*/*z* 497.3 $[(M+23)]^+$; ^{1}H NMR (500 MHz, DMSO) δ 8.182 (3H, m), 7.775 (2H, d, J = 5.4 Hz), 7.455 (1H, br s, disappeared on D_2O exchange), 5.804 (1H, d, J = 6.0 Hz), 5.427 (1H, d, J = 6.0 Hz, disappeared on D_2O exchange), 5.179 (1H, d, J = 5.0 Hz, disappeared on D_2O exchange), 4.946 (1H, t, J = 5.7 Hz, disappeared on D_2O exchange), 4.717 (2H, s), 4.625 (1H, br s), 4.192 (1H, br s), 3.902 (1H, m), 3.605 (2H, m), 2.755 (1H, m), 0.710 (2H, m), 0.525 (2H, m). HPLC (retention time: 10.00 min, purity: 97.27%).

4.18. 2-Butylamino-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (18)

To a solution of compound **7** (30 mg, 0.069 mmol) in 5 ml of methanol was added 0.15 ml (1.51 mmol) of *n*-butylamine. The solution was stirred for 1 day at 45 °C and then was evaporated to oil in vacuo. The compound was separated using flash column chromatography using methanol/ethyl acetate (1:9) as the eluent, to give 30.2 mg of compound **18** as a yellow solid.

Yield 90%; mp 87–89 °C; MS: (ESI, Pos) *m/z* 513.3 $[(M+23)]^+$; ^{1}H NMR (300 MHz, DMSO) δ 8.162 (3H, m), 7.742 (2H, d, J = 8.7 Hz), 7.186 (1H, br s, disappeared on D_2O exchange), 5.774 (1H, d, J = 6.0 Hz), 5.398 (1H, d, J = 6.0 Hz, disappeared on D_2O exchange), 5.146 (1H, d, J = 4.8 Hz, disappeared on D_2O exchange), 4.953 (1H, br s, disappeared on D_2O exchange), 4.686 (2H, s), 4.576 (1H, br s), 4.132 (1H, m), 3.887 (1H, m), 3.576 (2H, m), 3.270 (2H, m), 1.502 (2H, m), 1.319 (2H, m), 0.869 (3H, t, J = 6.0 Hz). HPLC (retention time: 10.79 min, purity: 97.55%).

4.19. 2-Cyclobutylamino-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (19)

To a solution of compound **7** (30 mg, 0.069 mmol) in 5 ml of methanol was added 0.15 ml (1.75 mmol) of cyclobutylamine. The solution was stirred for 1 day at 45 °C and then was evaporated to oil in vacuo. The compound was separated using flash column chromatography using methanol/ethyl acetate (1:9) as the eluent, to give 29.4 mg of compound **19** as a yellow solid.

Yield 88%; mp 116–118 °C; MS: (ESI, Pos) *m/z* 511.3 $[(M+23)]^+$; ^{1}H NMR (500 MHz, DMSO) δ 8.188 (3H, m), 8.760 (2H, d, J = 8.5 Hz), 7.508 (1H, br s, disappeared on D_2O exchange), 5.793 (1H, d, J = 6.0 Hz), 5.432 (1H, d, J = 6.0 Hz, disappeared on D_2O exchange), 5.200 (1H, m, disappeared on D_2O exchange), 4.980 (1H, m, disappeared on D_2O exchange), 4.712 (2H, s), 4.576 (1H, br m), 4.371 (1H, br m), 4.147 (1H, m), 3.904 (1H, m), 3.597 (2H, m), 2.239 (2H, m), 1.989 (2H, m), 1.674 (2H, m). HPLC (retention time: 10.53 min, purity: 96.95%).

4.20. 2-Pentylamino-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (20)

To a solution of compound **7** (30 mg, 0.069 mmol) in 5 ml of methanol was added 0.15 ml (1.29 mmol) of *n*-pentylamine. The solution was stirred for 2 days at 45 °C and then was evaporated to oil in vacuo. The compound was separated using flash column chromatography using methanol/ethyl acetate (1:9) as the eluent, to give 30.4 mg of compound **20** as a yellow solid.

Yield 88%; mp 82–84 °C; MS: (ESI, Pos) *m/z* 527.3 $[(M+23)]^+$; ^{1}H NMR (500 MHz, DMSO) δ 8.169 (3H, m), 7.748 (2H, d, J = 8.4 Hz), 7.213 (1H, br s, disappeared on D_2O exchange), 5.785 (1H, d, J = 6.0 Hz), 5.420 (1H, d, J = 6.0 Hz, disappeared on D_2O exchange), 5.171 (1H, d, J = 4.5 Hz, disappeared on D_2O exchange), 4.974 (1H, br s, disappeared on D_2O exchange), 4.692 (2H, s), 4.605 (1H, m), 4.143 (1H, m), 3.903 (1H, m), 3.588 (2H, m), 3.279 (2H, m), 1.822 (2H, br s), 1.276 (4H, m), 0.864 (3H, br s). HPLC (retention time: 11.30 min, purity: 98.35%).

4.21. 2-Cyclopentylamino-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (21)

To a solution of compound **7** (30 mg, 0.069 mmol) in 5 ml of methanol was added 0.15 ml (1.46 mmol) of cyclopentylamine. The solution was stirred for 2 days at 45 °C and then was evaporated to oil in vacuo. The compound was separated using flash column chromatography using methanol/ethyl acetate (1:9) as the eluent, to give 29.6 mg of compound **21** as a yellow solid.

Yield 86%; mp 102–104 °C; MS: (ESI, Pos) *m/z* 525.3 $[(M+23)]^+$; ^{1}H NMR (300 MHz, DMSO) δ 8.168 (3H, m), 7.740 (2H, d, J = 9.0 Hz), 7.149 (1H, br s, disappeared on D_2O exchange), 5.779 (1H, d, J = 5.7 Hz), 5.406 (1H, d, J = 6.3 Hz, disappeared on D_2O exchange), 5.159 (1H, d, J = 4.8 Hz, disappeared on D_2O exchange), 4.948 (1H, m, disappeared on D_2O exchange), 4.703 (2H, s), 4.586 (1H, m), 4.153 (2H, m), 3.883 (1H, m), 3.539 (2H, m), 1.871 (2H, m), 1.683 (2H, m), 1.517 (4H, m). HPLC (retention time: 10.88 min, purity: 98.19%).

4.22. 2-Cyclohexylamino-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (22)

To a solution of compound **7** (30 mg, 0.069 mmol) in 5 ml of methanol was added 0.2 ml (1.74 mmol) of cyclohexylamine. The solution was stirred for 2 days at 45 °C and then was evaporated to oil in vacuo. The compound was separated using flash column chromatography using methanol/ethyl acetate (1:9) as the eluent, to give 30.8 mg of compound **22** as a yellow solid.

Yield 87%; mp 78–80 °C; MS: (ESI, Pos) *m/z* 539.2 $[(M+23)]^+$; ^{1}H NMR (500 MHz, DMSO) δ 8.193 (3H, m), 7.760 (2H, m), 7.252 (1H, br s, disappeared on D_2O exchange), 5.792 (1H, d, J = 5.5 Hz), 5.453 (1H, d, J = 6.0 Hz, disappeared on D_2O exchange), 5.184 (1H, d, J = 4.5 Hz, disappeared on D_2O exchange), 4.946 (1H, m, disappeared on D_2O exchange), 4.727 (2H, s), 4.604 (1H, m), 4.172 (1H, br s), 3.913 (1H, m, J = 4.0, 8.5 Hz), 3.663 (1H, m), 3.563 (2H, m), 1.893 (2H, m), 1.724 (2H, m), 1.614 (1H, m), 1.296 (4H, m), 1.178 (1H, m). HPLC (retention time: 11.26 min, purity: 99.12%).

4.23. 2-Benzylamino-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (23)

To a solution of compound **7** (30 mg, 0.069 mmol) in 5 ml of methanol was added 0.15 ml (1.37 mmol) of benzylamine. The solution was stirred at room temperature for 30 min and then was evaporated to oil in vacuo. The compound was separated using flash column chromatography using methanol/ethyl acetate (1:9) as the eluent, to give 31.5 mg of compound **23** as a yellow solid.

Yield 88%; mp 82–84 °C; MS: (ESI, Pos) *m/z* 547.3 $[(M+23)]^+$; ^{1}H NMR (300 MHz, DMSO) δ 8.197 (1H, s), 8.045 (2H, d, J = 8.6 Hz), 7.760 (2H, d, J = 8.7 Hz), 7.298 (6H, m, 1H disappeared on D_2O exchange), 5.784 (1H, d, J = 6.0 Hz), 5.398 (1H, d, J = 6.0 Hz, disappeared on D_2O exchange), 5.155 (1H, d, J = 5.1 Hz, disappeared on D_2O exchange), 4.969 (1H, m, disappeared on D_2O exchange), 4.541 (5H, m), 4.117 (1H, m), 3.886 (1H, m), 3.555 (2H, m). HPLC (retention time: 10.80 min, purity: 98.60%).

4.24. 2-Phenethylamino-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (24)

To a solution of compound **7** (30 mg, 0.069 mmol) in 5 ml of methanol was added 0.2 ml (1.59 mmol) of phenethylamine. The solution was stirred overnight at 45 °C and then was evaporated to oil in vacuo. The compound was separated using flash column chromatography using methanol/ethyl acetate (1:9) as the eluent, to give 31.7 mg of compound **24** as a yellow solid.

Yield 86%; mp 195–197 °C; MS: (ESI, Pos) *m*/*z* 561.3 $[(M+23)]^+$; ^{1}H NMR (300 MHz, DMSO) δ 8.145 (3H, m), 7.731 (2H, d, J = 7.8 Hz), 7.229 (6H, m, 1H disappeared on D_2O exchange), 5.801 (1H, d, J = 6.0 Hz), 5.422 (1H, d, J = 6.0 Hz, disappeared on D_2O exchange), 5.159 (1H, d, J = 4.8 Hz, disappeared on D_2O exchange), 4.969 (1H, m, disappeared on D_2O exchange), 4.682 (2H, s), 4.588 (1H, m), 4.137 (1H, m), 3.897 (1H, m), 3.596 (2H, m), 3.507 (2H, m), 2.856 (2H, m). HPLC (retention time: 11.15 min, purity: 98.11%).

4.25. 2-Chloro-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (27)

To a solution of compound **8** (30.7 mg, 0.038 mmol) in 5 ml of anhydrous THF was added 40 mg (0.15 mmol) of tetrabutylammonium fluoride. The solution was stirred at room temperature for 4 h. The product was concentrated by removing the solvent in vacuo. Compound **27**, 8.4 mg, was isolated as a yellow solid using preparative thin layer chromatography using methanol/ethyl acetate (0.5:9.5) as the mobile phase.

Yield 48%; mp 108–110 °C; MS: (ESI, Pos) *m*/*z* 476.0 $[(M+23)]^+$; ^{1}H NMR (300 MHz, DMSO) δ 8.760 (1H, s), 8.188 (2H, d, J = 8.7 Hz), 7.767 (2H, d, J = 8.7 Hz), 5.915 (1H, d, J = 5.4 Hz), 5.558 (1H, d, J = 5.4 Hz, disappeared on D_2O exchange), 5.251 (1H, d, J = 5.1 Hz, disappeared on D_2O exchange), 5.054 (1H, t, J = 5.7 Hz, disappeared on D_2O exchange), 4.751 (2H, s), 4.504 (1H, m), 4.149 (1H, m), 3.992 (1H, m), 3.660 (2H, m). HPLC (retention time: 10.21 min, purity: 93.07%).

4.26. 2-Bromo-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (28)

To a solution of compound **9** (58.4 mg, 0.069 mmol) in 5 ml of anhydrous THF was added 60 mg (0.23 mmol) of tetrabutylammonium fluoride. The solution was stirred at room temperature for 4 h. The product was concentrated by removing the solvent in vacuo. Compound **28**, 15.21 mg, was isolated as a yellow solid using preparative thin layer chromatography using methanol/ethyl acetate (0.5:9.5) as the mobile phase.

Yield 44%; mp 91–93 °C; MS: (ESI, Pos) *m*/*z* 522.0 $[(M+23)]^+$; ^{1}H NMR (300 MHz, DMSO) δ 8.733 (1H, s), 8.185 (2H, d, J = 8.1 Hz), 7.771 (2H, d, J = 8.4 Hz), 5.911 (1H, d, J = 4.8 Hz), 5.547 (1H, d, J = 5.7 Hz, disappeared on D_2O exchange), 5.250 (1H, d, J = 5.4 Hz, disappeared on D_2O exchange), 5.042 (1H, t, J = 5.4 Hz, disappeared on D_2O exchange), 4.734 (2H, s), 4.503 (1H, m), 4.149 (1H, m), 3.960 (1H, m), 3.620 (2H, m). HPLC (retention time: 10.30 min, purity: 97.61%).

4.27. 2-Iodo-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (29)

To a solution of compound **10** (89.1 mg, 0.1 mmol) in 5 ml of anhydrous THF was added 80 mg (0.30 mmol) of tetrabutylammonium fluoride. The solution was stirred at room temperature for 4 h. The solvent was evaporated in vacuo. Compound **29**, 25.1 mg, was isolated as a yellow solid using preparative thin layer chromatography using methanol/ethyl acetate (0.5:9.5) as the mobile phase.

Yield 46%; mp 135–137 °C; MS: (ESI, Pos) *m*/*z* 568.0 $[(M+23)]^+$; ^{1}H NMR (300 MHz, DMSO) δ 8.651 (1H, s), 8.181 (2H, d, J = 8.7 Hz), 7.772 (2H, d, J = 8.7 Hz), 5.897 (1H, d, J = 5.4 Hz), 5.526 (1H, d, J = 6.0 Hz, disappeared on D_2O exchange), 5.252 (1H, d, J = 5.4 Hz, disappeared on D_2O exchange), 5.051 (1H, t, J = 5.1 Hz, disappeared on D_2O exchange), 4.701 (2H, s), 4.508 (1H, m), 4.136 (1H, m), 3.948 (1H, m), 3.657 (2H, m). HPLC (retention time: 10.43 min, purity: 97.45%).

4.28. 2-Monoethanolamino-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (25)

To a solution of compound **7** (30 mg, 0.069 mmol) in 5 ml of methanol was added 0.1 ml (0.97 mmol) of monoethanolamine. The solution was stirred overnight at 45 °C and then was evaporated to oil in vacuo. The compound was separated by flash column chromatography using methanol/ethyl acetate (1:9) as the eluent, to give 31 mg of compound **25** as a yellow solid.

Yield 95%; mp 96–98 °C; MS: (ESI, Pos) *m*/*z* 501.1 $[(M+23)]^+$; ^{1}H NMR (300 MHz, DMSO) δ 8.161 (3H, m), 7.749 (2H, d, J = 8.7 Hz), 7.058 (1H, br s, disappeared on D_2O exchange), 5.779 (1H, d, J = 6.0 Hz), 5.406 (1H, d, J = 6.0 Hz, disappeared on D_2O exchange), 5.168 (1H, d, J = 4.8 Hz, disappeared on D_2O exchange), 4.975 (1H, m, disappeared on D_2O exchange), 4.682 (3H, m, 1H disappeared on D_2O exchange), 4.556 (1H, m), 4.124 (1H, m), 3.890 (1H, m), 3.625 (2H, m), 3.539 (4H, m). HPLC (retention time: 8.66 min, purity: 99.12%).

4.29. 2-Diethanolamino-6-(4-nitrobenzylthio)-9-β-D-ribofuranosylpurine (26)

To a solution of compound **7** (30 mg, 0.069 mmol) in 5 ml of methanol was added 0.1 ml (1.04 mmol) of diethanolamine. The solution was stirred overnight at 45 °C and then was evaporated to oil in vacuo. The compound was separated using flash column chromatography using methanol/ethyl acetate (1:9) as the eluent, to give 33 mg of compound **26** as a yellow solid.

Yield 93%; mp 108–110 °C; MS: (ESI, Pos) *m*/*z* 545.3 $[(M+23)]^+$; ^{1}H NMR (300 MHz, DMSO) δ 8.204 (3H, m), 7.707 (2H, d, J = 8.7 Hz), 5.784 (1H, d, J = 6.0 Hz), 5.410 (1H, d, J = 6.3 Hz, disappeared on

D_2O exchange), 5.195 (1H, d, $J = 4.8$ Hz, disappeared on D_2O exchange), 4.938 (1H, t, $J = 5.1$ Hz, disappeared on D_2O exchange), 4.751 (2H, t, $J = 5.1$ Hz, disappeared on D_2O exchange), 4.688 (2H, s), 4.607 (1H, m), 4.136 (1H, m), 3.893 (1H, m), 3.637 (10H, m). HPLC (retention time: 9.45 min, purity: 97.34%).

4.30. Flow cytometry

The hENT1 binding ability of the compounds was studied using a flow cytometric assay. Human leukemia K562 cells, grown in RPMI 1640 medium, were washed once and resuspended at 1.6×105 cells/mL in phosphate buffered saline at pH 7.4, and incubated with 5-(SAENTA)-*X*8-fluorescein (30 nM) in the presence or absence of varying concentrations of the test compounds at room temperature for 45 min. Flow cytometric measurements of cell associated fluorescence were then performed with a FACSCalibur (Becton Dickinson, San Jose, CA) equipped with a 15mW-argon laser (Molecular Resources Flow Cytometry Facility, University of Tennessee Health Sciences Center). In each assay, 5000 cells were analyzed from suspensions of 5×105 cells/mL. The units of fluorescence were arbitrary channel numbers. Percentage (%) of control (i.e., hENT1 specific fluorescence in the presence of SAENTA-fluorescein without test compounds) was calculated for each sample by the equation below Eq. 1.

$$\% \text{ control} = (SF_s) \cdot 100/(SF_f) \quad (1)$$

where SF_s is the hENT1 specific fluorescence of test samples and SF_f is the hENT1 specific fluorescence of the SAENTA-fluorescein ligand standard in mean channel numbers.

The results obtained were entered in the PRISM program (GraphPad, San Diego, CA) to derive the concentration dependent curves as shown in Figure 1. From these curves, the IC_{50} values were computed and used to calculate inhibition constants (K_i) values from Eq. 2.

$$K_i = IC_{50}/(1 + [L]/K_L) \quad (2)$$

where $[L]$ and K_L are the concentration and the K_d value of SAENTA-fluorescein, respectively.

The K_i values were used to compare the abilities of the new compounds to displace the hENT1 specific ligand 5-(SAENTA)-*X*8-fluorescein, and for that matter their affinity for the hENT1.

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