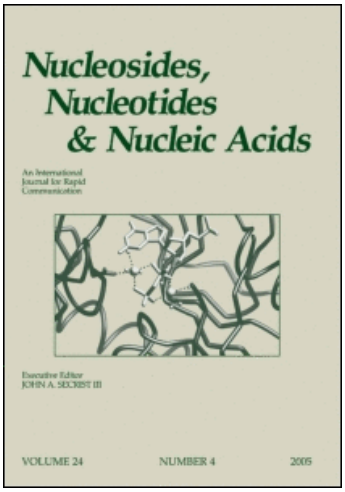

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Solution NMR Conformational Analysis of the Potent Equilibrative Sensitive (*ES*) Nucleoside Transporter Inhibitor, S^6-(4-Nitrobenzyl)mercaptopurine Riboside (NBMPR)

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SOLUTION NMR CONFORMATIONAL ANALYSIS OF THE POTENT EQUILIBRATIVE SENSITIVE (*ES*) NUCLEOSIDE TRANSPORTER INHIBITOR, S^6-(4-NITROBENZYL)MERCAPTOPURINE RIBOSIDE (NBMPR)

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Abstract. High resolution NMR analysis involving one-dimensional (1-D) ^{1}H and nuclear Overhauser (NOE) difference spectroscopy was applied to solutions of NBMPR in DMSO-d_6. Coupling constants were obtained at different temperatures between 285 and 353 K, and used to analyze the rotamer preferences about the C-4'-C-5' bond. The results revealed a rotamer distribution about the χ tortion angle that favors the *high-anti* range, a preponderance of the γ^+ rotamer (at ~64 %) with respect to the γ torsion angle, and a higher population of the south (**S**) conformer, which was favored by as little as the 4 % to as much as 31 % over the north (**N**) conformer as calculated by the program PSEUROT 6.2. The high-*anti* glycosidic torsion orientation appears to be the major conformational difference between the solution structure of NBMPR determined in this study and the structure previously observed in the solid state.

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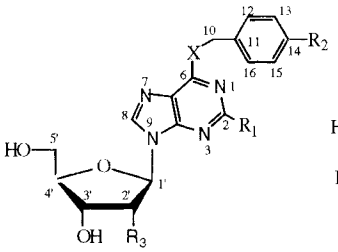
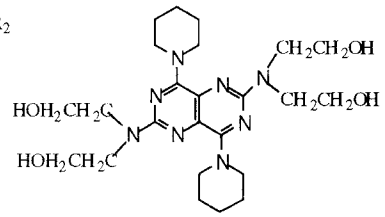
INTRODUCTION

Mammalian cells take up physiological nucleosides such as adenosine, and many of their derivatives by means of integral plasma membrane glycoproteins known as nucleoside transporters. There exist subtypes of both equilibrative (facilitated diffusion) and concentrative (sodium ion-coupled) nucleoside transporters (1,2). Two equilibrative transporters with a similar broad range of substrate specificity have been identified and designated as *es* (*e*quilibrative inhibitor-*s*ensitive) and *ei* (*e*quilibrative inhibitor-*i*nsensitive) based on their sensitivity to inhibition by S^6-(4-nitrobenzyl)mercaptopurine riboside (NBMPR) **1**, and its congeners such as S^6-(4-nitrobenzyl)thioguanosine (NBTGR) **2**, and N^6-(4-nitrobenzyldeoxyadenosine (NBdAdo) **3**, and the non-nucleoside inhibitor dipyridamole (**4**).





1, X = S, R_1 = H, R_2 = NO_2, R_3 = OH
2, X = S, R_1 = NH_2, R_2 = NO_2, R_3 = OH
3, X = NH, R_1 = H, R_2 = NO_2, R_3 = H

4

The *es* transporter is by far the major nucleoside transporter of most mammalian cells examined to date, and can be distinguished from the *ei* transporter by its high sensitivity to inhibition by low nanomolar concentrations of NBMPR. Inhibition of the *es* transporter by NBMPR results from specific high-affinity binding (K_d 0.1-1.0 nM). Of the five Na^+-dependent nucleoside transporters identified (2), only *cs*/N5 is sensitive to inhibition by potent *es* transporter inhibitors like NBMPR. There is considerable current interest in nucleoside transport (NT) inhibitors as potential therapeutic agents in heart disease and stroke, and as part of combination chemotherapy regimens to fight cancer and viral (HIV) infections (3). Unfortunately, translation of experimental success to the clinic has been hampered by the poor pharmacological profiles of current NT inhibitors, which calls for the design and discovery of novel inhibitors.

Conformational analysis of NBMPR and its active analogs will be helpful in attempts to determine the bioactive conformation(s) of this class of *es* transporter inhibitors needed for rational drug design. The X-ray crystal structure of NBMPR has been reported (4), but not its solution structure. The present study used high resolution NMR to analyze

the solution conformation of NBMPR with respect to nucleoside conformational preferences and equilibria.

MATERIALS AND METHODS

NBMPR was purchased from Sigma Chemical Company (St Louis, MO). Deuterated dimethylsulfoxide (DMSO-d_6) was purchased from Cambridge Isotope Labs (Andover, MA). NMR spectroscopy was performed on a 500 MHz Bruker AMX500 instrument (Bruker, Billierica, MA), using an inverse broad-band probe. Samples for 1-dimensional (1-D) proton (^{1}H) and nuclear Overhauser effect (NOE) spectra contained 12 mM NBMPR in DMSO-d_6 or DMSO-d_6 with one drop of deuterium oxide (D_2O) to obtain spectra after exchange of the hydroxyl resonances. Spectra for coupling constant analysis were collected with 64K time domain points, and were zero-filled to 128K. Coupling constants were determined from resolution enhanced (Gaussian broadening, LB = -1, GB = 0.1) 1-D spectra at various temperatures. Coupling constant values were ultimately confirmed by spectral simulation using the program HYPERNMR (Hypercube Inc., Waterloo, Ontario, Canada). Estimated errors in the values obtained from spectral simulations were ± 0.1 Hz. Assignments were made based on standard methods, and the C-5' protons were stereospecifically specified according to Wu *et al.* (5). Equilibrium NOE difference spectra were obtained by subtracting a spectrum with the decoupler off-resonance from the one collected with multiple irradiation of each proton multiplet. This was done in the context of the Bruker pulse program NOEMUL. Irradiations were performed in 50 cycles at 50 msec/cycle with a recycle delay of 4 sec. Heteronuclear multiple quantum coherence (HMQC) spectra were recorded with standard pulse sequences with a refocusing delay optimized for $^1J_{H,C}$ = 140 Hz.

Pseudorotational parameters were calculated from the measured coupling constants by the program PSEUROT 6.2 (DOS version, purchased from Professor Cornelius Altona, University of Leiden, Leiden, The Netherlands) running on a Pentium 90 personal computer (6-8). The program uses measured proton-proton coupling constants to calculate pseudorotational parameters using an improved generalized Karplus equation as modified by Donders *et al.* (9). Standard ribonucleoside parameters were used and no adjustments were made for the nitrobenzylthio group on the purine. Calculations were made with and without corrections for the Barfield effect (8), and the maximum number of iterations was set to 25.

RESULTS AND DISCUSSION

The conformation of nucleosides is usually defined in terms of four parameters: 1) the χ torsion angle which indicates orientation of the base about the glycosidic bond (*syn* if

the N-3 a purine or the O-2 of a pyrimidine base lies over the sugar ring, or *anti* if the 8-H of the purine or C-6 of the pyrimidine base lies over the sugar ring, Figure 1A), 2) the γ torsion angle which indicates the orientation of the 5'-OH with respect to C-3' and O-4' (Figure 1A), which may be γ^+ (above the center of the furanose ring, i. e., between C-3' and O-4'), γ^t (on the O-4' side of the furanose ring), or γ^- (on the C-3' side of the furanose ring), 3) the deviation from planarity of the furanose ring atoms (Figure 1B, 1C and 1D) known as puckering, which may create an envelope or twist conformation, with a particular ring atom being *endo*, i. e., above the plane of the ring or *exo*, i. e., below the plane of the ring, and is represented by the pseudorotational phase angle, *P*, and 4) the amplitude (degree) of puckering, ν_{max}. A ring pucker with C-2'-*endo* and C-3'-*exo* is designated as south (**S**, P_S, Figure 1C) whereas a pucker with C-3'-*endo* and C-2'-*exo* is designated as north (**N**, P_N, Figure 1D). X-ray crystallographic studies (10) of nucleosides have indicated that the furanose ring conformations in the solid state cluster around the southern (C-2'-*endo*/C-3'-*exo*) and northern (C-3'-*endo*/C-2'-*exo*) conformers. A rapid equilibrium exits between these two states in solution (6-8).

Solution NMR analyses of NBMPR were performed between 285 K and 353 K to observe temperature effects on proton-proton coupling constants of the ribose moiety with temperature. Only slight changes were observed in the coupling constants over the temperature range studied, indicating only minor fluctuations in the conformational ensemble with increasing temperature. 1-D NOE difference spectroscopy was used to estimate the *syn/anti* distribution about the C1'-N-9 glycosidic bond (χ torsion) based on the method of Rosemeyer *et al.* (11).. The NOE difference spectra revealed moderately strong enhancements of the H-1' and H-2' signals along with a weak enhancement of the H-3' signal of the ribose ring upon irradiation of the purine H-8. The NOE to H-2' was slightly greater in intensity than that to H-1'. This, along with the remaining NOE data suggested that the rotamer distribution about χ is in the high-*anti* range, which is close to the *syn* range (11). In addition, the purine could be in a higher *anti* range with the sugar pucker biased toward the southern pucker. Comparison of the base orientations in the crystal structures of unbound nucleosides and nucleotides (from the Cambridge Structural Database) and protein-bound nucleosides and nucleotides (from the Brookhaven Protein Data Bank) showed a preference for an *anti* or high-*anti* disposition of the base (χ = -90° to -60°) in the unbound state (for both pyrimidine and purine nucleosides). The bound conformations showed an even greater preference for the *anti* base orientation (12).

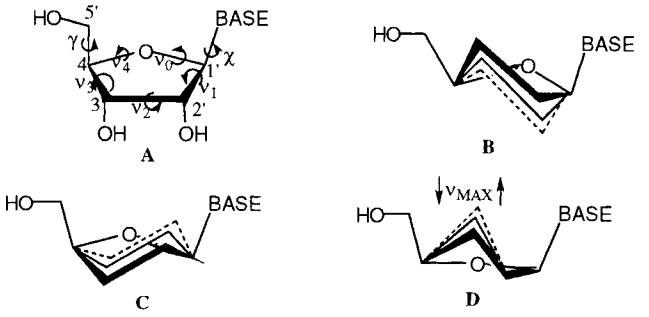


FIG. 1. Nucleoside Conformational Parameters: A) Torsion angles, B) **N** conformers, C)**S** conformers, D) puckering amplitude

The coupling constants calculated and confirmed by spectral simulation for NBMPR are shown in Table 1. At room temperature, coupling constant analysis of the rotamer distribution about the C-4'-C-5' bond (γ torsion angle) revealed that the γ^+ rotamer is populated to a large extent (~64%) with γ at 34% and only 2% for the γ^- rotamer. This rotamer distribution is in accord with previous studies which showed a preponderance of the γ^+ rotamer for unbound nucleosides in the solid state (12).

PSEUROT analysis of the ribose ring system based on the observed coupling constants seemed to indicate nearly an equal distribution of **N** and **S** conformers at room temperature. The coupling constants were calculated with a high degree of accuracy since the RMS deviations of the PSEUROT-calculated and observed values are below 0.1 Hz indicating a well-defined fit between the calculated and observed data. The data from several PSEUROT analyses are presented in Table 2.

The PSEUROT program calculates five parameters, namely, P_N, P_S, ν_N, ν_S and X_N. Several starting parameters were used with different ones held constant (7). The data were calculated under different starting conditions with the southern or northern parameters held constant. These data show that the **S** conformer is slightly favored by as little as 4 % to as much as 31 % over the **N** conformer. The analysis seems to be sensitive to the starting parameters. P values ranged from -28.8° to 14.5° for the **N** conformer and around 128°-140° for the **S** conformer depending on the starting parameters for P and ν_{MAX}. The ν_{MAX} values were lower for the **N** conformer (~14-24°) than for the **S** conformer which showed ν_{MAX} values closer to the average puckering amplitudes for ribonucleosides (~25-45°). The

TABLE 1. Calculated ^{1}H-^{1}H Coupling Constants

Temp. (°K)	$J_{1',2'}$	$J_{2',3'}$	$J_{3',4'}$	$J_{4',5'}$	$J_{4',5''}$
285	5.7	5.4	3.9	3.9	3.90
298	5.6	5.3	3.9	3.9	4.00
313	5.6	5.3	3.9	3.9	4.05
333	5.5	5.3	3.9	3.9	4.05
353	5.5	5.3	4.0	3.9	4.10

TABLE 2. Calculated Pseudorotational Parameters for NBMPR[a]

Run#	P_N	ν_N	P_S	ν_S	X_{st}	X_N	RMS
1	(9.0*)	(36.0*)	140.0(167.0)	26.3(40.0)	0.5	0.28	0.031
2	(9.0*)	(36.0*)	140.0(173.0)	26.3(34.0)	0.5	0.28	0.031
3	10.8 (9.0)	22.2 (36.0)	138.8(167.0)	36.9(40.0)	0.5	0.43	0.032
4	10.7 (9.0)	22.3 (36.0)	138.8(167.0)	36.8(40.0)	0.5	0.43	0.032
5	58.6 (9.0)	19.6 (36.0)	(167.0*)	(40.0*)	0.5	0.59	0.040
6	56.0 (9.0)	22.6 (36.0)	(170.0*)	(36.0*)	0.5	0.54	0.040
7	9.0 (9.0)	20.9 (39.0)	137.2(173.0)	(39.0*)	0.5	0.44	0.032
8	14.5 (9.0)	22.7 (39.0)	140.4(173.0)	35.8(39.0)	0.5	0.42	0.032
9	53.7 (9.0)	41.9 (39.0)	170.8(173.0)	25.9(39.0)	0.8	0.36	0.042
10	(9.0*)	(39.0*)	139.3(173.0)	25.1(39.0)	0.8	0.26	0.031
11	-10.4(9.0)	20.5(39.0)	130.2(173.0)	45.0(39.0)	0.2	0.45	0.032
12	(9.0*)	(39.0*)	139.3(173.0)	25.1(39.0)	0.2	0.26	0.031
13	-28.8(9.0)	(34.0*)	128.1(167.0)	(36.0*)	0.5	0.38	0.028
14[b]	26.5(9.0)	17.8(34.0)	140.3(167.0)	42.4(36.0*)	0.5	0.50	0.037

[a]Calculated with the PSEUROT Program version 6.2. Values in parentheses are starting values for the calculations; starred values are held fixed. X_{st} is the starting value for the mole fraction of the south (**S**) conformer. X_N is the calculated mole fraction of the north (**N**) conformer from each run. RMS values are in Hz. [b]The mole fractions of the first two temperatures were held fixed.

p-nitrothiobenzyl group seems to have little effect on the pseudorotational preference of the furanose. This group appears to be conformationally averaged. The present PSEUROT data are consistent with those obtained on other ribofuranoses in which there is no overwhelming preference for a **N** or **S** pucker (8,13). However it is clear from the coupling constant analysis that the **N** conformation is not well defined for NBMPR in solution as calculated by PSEUROT. The pseudorotational preferences of the furanose ring in NBMPR and analogs may be important for biological activity since recent experimental (14,15) and theoretical (16) studies have suggested a possible correlation between furanose ring conformational preferences and biological potency of some dideoxynucleoside anti-HIV compounds. A study that examined protein-bound conformations of nucleosides and nucleotides in the Brookhaven Protein Databank showed that protein binding distinctly favored a C-2'-*endo* (**S**) conformation of the furanose ring (12).

The X-ray crystallographic data for NBMPR (Figure 2) show a *syn* orientation of the purine about the glycosidic bond torsion (χ), a γ^+ orientation of the 5'-OH , and a C-2'-*endo* envelope furanose pucker (**S**) in the solid state (4). The solution conformational parameters that we have determined by NMR are in general agreement with the X-ray structure. Both the X-ray and NMR data indicate a pseudorotational preference for a C-2'-*endo* furanose pucker (**S**), and a γ^+ orientation of the 5'-OH. The only, and probably minor difference is that the crystal structure exhibits a perfect *syn* orientation around χ, whereas the NMR data indicates a high-*anti*, close to *syn* orientation in solution. The involvement of a hydrogen bond between the 5'-OH and N-3 of the purine in the solid state appears to stabilize it in the *syn* conformation, thereby allowing the purine ring to assume a perfect *syn*-conformation. This stabilizing effect appears to be absent in solution, thereby leaving the purine in the high-*anti*-close-to-*syn*-orientation. The solid state structure of the 2-amino derivative of NBMPR, compound **2** (NBTGR), an equally potent *es* transporter inhibitor, also shows the same conformational parameters as NBMPR in the solid state (17).

The crystal structure of the deoxyadenosine analog, compound **3** (NBdAdo), another tightly bound *es* transporter ligand (K_d 2.4 nM), on the other hand, shows different nucleoside conformational parameters from those of NBMPR and NBTGR (18), such as, an *anti*-orientation about the χ torsion, an unusual C-1'-*exo* envelope pucker of the furanose ring, and a γ^+ orientation of the 5'-OH (18). NBMPR and NBTGR have crystal structures similar to that of their parent 6-thioinosine in terms of all the major

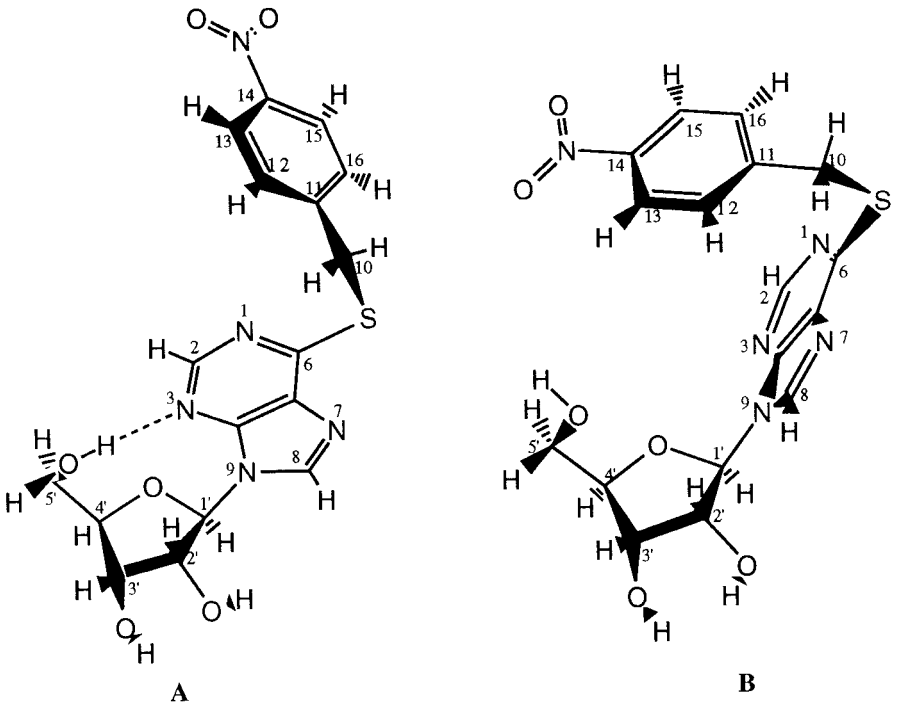


Figure 2. Conformations of NBMPR: Structure **A** is the published X-ray crystallographic structure (10); the broken line shows a H-bond between 5'-OH and N-3. Structure **B** is the major solution conformation calculated from the coupling constant and NOE data, and further minimized with the CHARMM force field in the QUANTA molecular modeling package (Molecular Simulations, San Diego, CA).

conformational parameters (4), whereas NBdAdo, differs from its parent deoxyadenosine by exhibiting a C-1'-*exo* envelope in contrast to the C-3'-*exo* envelope of the parent (18). Except in the case of NBdAdo, the nitrobenzyl group in NBMPR and NBTGR appears to have no influence on the solid state conformation of the parent nucleosides. The replacement of sulfur by nitrogen in NBdAdo significantly shortens the distance between the nitrobenzyl group and the purine ring, possibly limiting conformational flexibility. Part of the reason for the differences may also be that the 2'-deoxyribose ring system of NBdAdo is more sensitive to the effects of the nitrobenzyl group than the ribose ring system in both NBMPR and NBTGR.

This study has provided new information on conformational preferences of NBMPR, showing that the 5'-OH to N-3 hydrogen bonding observed in the crystal

structure is lost in solution in a polar solvent like dimethylsulfoxide. The loss of this hydrogen bonding should result in more conformational flexibity, and make it possible to adopt an *anti*-conformation which has been suggested to be favored over the *syn*-conformation at the *es* transporter permeant binding site (19).

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