Effects of Tubercidin and its 5′-*O*-Methyl Ether on Adenosine Receptors and Mediator Release Functions in Mast Cells

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Tubercidin (7-deazaadenosine, Tu) is a highly cytotoxic nucleoside xenobiotic that, as the nucleoside or nucleotide derivatives, closely mimics the actions of adenosine (or its corresponding nucleotides) in a wide variety of biochemical/biological systems. In light of its acceptance in these test systems as an adenosine (Ado) surrogate, it was postulated that the compound might interact with adenosine receptors. To test this hypothesis, a nonphosphorylatable derivative (5′-*O*-methyl tubercidin, MeTu) was prepared and evaluated in comparison with tubercidin and Ado in a variety of biological systems. In a cell culture assay using Chinese hamster ovary cells, MeTu is approximately one-third as cytotoxic as is Ado and 10^5-fold less cytotoxic than Tu. Both Tu and MeTu inhibited the antigen-stimulated release of β-hexosaminidase from mouse bone marrow derived mast cells *in vitro*, but only Tu was active in the *in vivo* PCA test. The inhibitory effect of MeTu on mast cell mediator release does not appear to involve interaction with adenosine receptors or to be the result of conversion to Tu *per se*.

Introduction

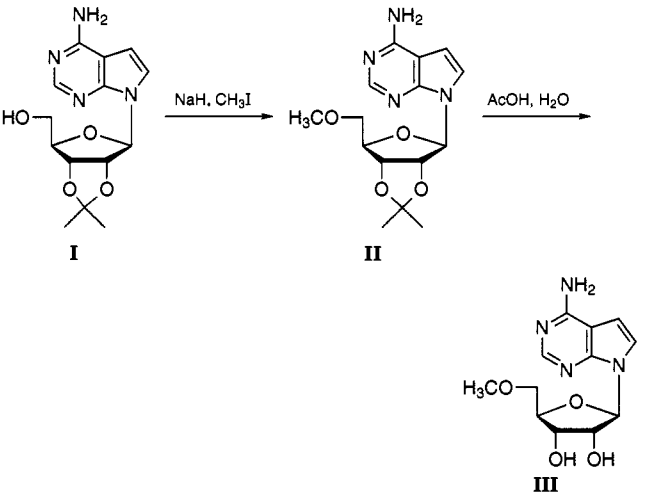
Tubercidin [(7-deazaadenosine (Tu)] is a nucleoside xenobiotic first described by Anzai et al. as an antimycobacterial and cytotoxic agent.[1] In-depth investigations were undertaken with this compound by scientists at the Upjohn Co. and the National Cancer Institute, including clinical evaluation as an antitumor agent.[2–5] Many studies have been carried out on the mode of action of Tu but, to date, none has defined a site of action in mammalian cells. Tu is not deaminated by nucleoside deaminase or cleaved by nucleoside phosphorylase[6] in animal cells. When taken up into mammalian cells, the parent compound is phosphorylated and cannot exit the cell (e.g., retained in a "metabolic trap"). Although Tu, in its fully phosphorylated forms, was not cytotoxic to erythrocytes,[6] this nucleoside (presumably after conversion to the appropriate nucleotides) is highly cytotoxic to dividing animal cells.[2,3] Its cytotoxicity in mammalian cells cannot be reversed by a variety of nucleosides studied,[3] in contrast to a report of reversal in *S. fecalis*.[7]

In light of the many reactions in which Tu, or one of its corresponding nucleotides, substitutes for adenosine (Ado) or adenylic acids in a variety of biochemical reactions,[6,8] it occurred to one of us that an analog or derivative of the compound that could not be phosphorylated in the body should be nontoxic but may well interact effectively at Ado receptor sites. To test this hypothesis, the 5′-*O*-methyl ether of Tu (MeTu) was synthesized and studied for certain biochemical and biological properties, as described below.

Chemistry

In Scheme 1, the synthetic route of 5′-*O*-methyltubercidin (**III**) is depicted. The 2′,3′-*O*-isopropylidene-tubercidin (**I**)[9] was *O*-methylated with methyl iodide, in presence of sodium hydride, in ethylene glycol dimethyl ether to yield 2′,3′-*O*-isopropylidene-5′-*O*-methyltubercidin (**II**). The isopropylidiene group of **II** was removed by heating with acetic acid to yield 5′-*O*-methyltubercidin (**III**).

Scheme 1



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Results and Discussion

The first experiment undertaken with MeTu had the objective of demonstrating lack of cytotoxicity, since that property is a *sine qua non* if the compound is to be used in an intact animal for the treatment of nonfatal diseases. Such an experiment is complicated by the fact that Ado, *per se*, is also cytotoxic to certain mammalian cells in culture.[10] Accordingly, Ado and Tu were compared with MeTu in Chinese hamster ovary (CHO) cells in the presence and absence of an S-9 microsome fraction, and the data are presented in Table 1.

Confirming earlier work with human KB cells,[10] Ado was shown to have demonstrable cytotoxicity against CHO cells at concentrations of 1136 and 114 μM in the absence of microsomes but was noncytotoxic, even at

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Table 1. *In Vitro* Cytotoxicities of Ado, MeTu, and Tu[a]

Percent Inhibition of Cell Growth[a]								
Ado			MeTu			Tu		
μM	−S-9	+S-9[b]	μM	−S-9	+S-9	μM	−S-9	+S-9
1136	43	7	360	37	99	114×10^{-4}	100	100
114	36	0	37	30	36	11.4×10^{-4}	92	80
11	19	0	3.7	12	2	1.1×10^{-4}	16	9
1.1	22	8	0.4	0	8	0.1×10^{-4}	10	4
0.17	0	0.04	0	20	0.01×10^{-4}	9	11	

[a] Assay conducted with Chinese hamster ovary cells, clone K1 subclone BH4, designated CHO-K1-BH4,[11,12] using Pharmakon Res. Intl., Inc. protocol 314-CYT/CHO. Relative cytotoxicity was determined after 5 h exposure to the test article, followed by an overnight recovery period in the absence of the compound. Variability in the numbers presented in the table falls between 10% and 20%. [b] Plus or minus S-9 microsome fraction.

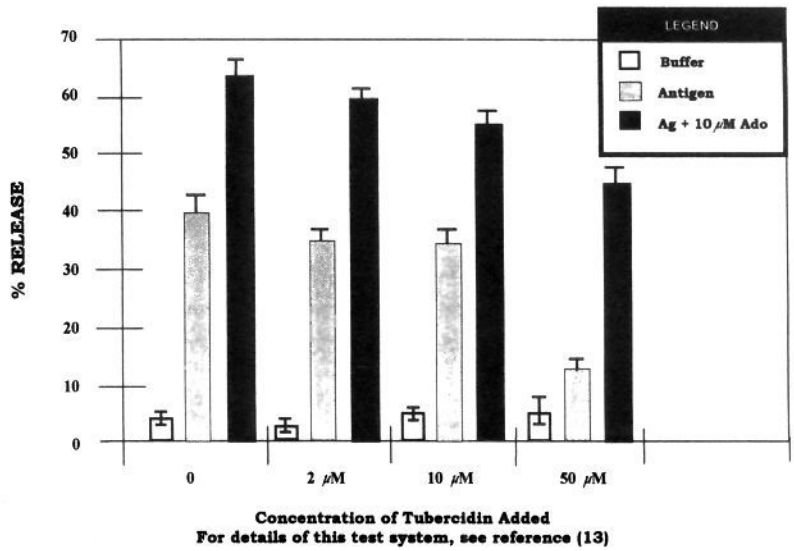

Figure 1. Effect of Tu and Ado on β-hexosaminidase release from mouse bone marrow mast cells. Left to right: buffer, antigen, Ag + 10 μM Ado.

these high concentrations, in the presence of the S-9 fraction. Our conclusion for the lack of activity in the presence of the microsomal fraction is that Ado is almost certainly deaminated by adenosine deaminase and/or cleaved by nucleoside phosphorylase. The high cytotoxicity observed with Tu at 0.01 and 0.001 μM confirms that reported in previous studies,[2,3] and it is evident that S-9 microsomes have no effect on this nucleoside. In the absence of exogenous microsomes, MeTu is approximately three times as cytotoxic as is Ado and 10^5-fold less cytotoxic than Tu. On the other hand, marked cytotoxicity was observed with 360 μM MeTu in the presence of the S-9 fraction. The latter result was unexpected since MeTu cannot be phosphorylated and, at the other concentrations studied, its cytotoxicity is much more comparable to that seen with Ado. De-*O*-alkylation of the order of 0.001%, which would convert MeTu to Tu, could explain the level of cytotoxicity observed at this high concentration of MeTu. Since the cytotoxicity at 37 μM MeTu is not increased in the presence of the S-9 microsome fraction and since the pattern of inhibition of hexoseaminidase release from mast cells with MeTu (Figure 2) is distinct from that observed with Tu (Figure 1), we conclude that MeTu is not active by virtue of bioconversion to Tu.

Tu and MeTu were next evaluated as possible agonists or antagonists at the Ado receptor. The release of β-hexosaminidase, a granule-associated mediator released in parallel with histamine upon antigen challenge, is the end point measured.[13] Mast cells possess at least two types of cell surface adenosine receptors, the A_{2a} coupled to adenylate cyclase activation, and the A_{2b} coupled to adenylate cyclase activation and/or phosphatidyl inositol metabolism. A_3 receptors have also been reported to be present on these cells. Which of these receptor subtypes is responsible for the augmentation of mast cell degranulation by adenosine is under investigation. Under the experimental conditions used, Tu (2−50 μM) was not cytotoxic, as assessed by spontaneous release of β-hexosaminidase into the medium (Figure 1). At a concentration of 50 μM Tu, this compound induced an approximately 60% inhibition of β-hexosaminidase release ($p < 0.002$). The ability of 10 μM Ado to significantly enhance antigen-stimulated β-hexosaminidase release remained intact at Tu concentrations up to and including 50 μM. Therefore, it is concluded that Tu, at a concentration of 50 μM, inhibits mast cell preformed, granule-associated mediator release but does not obviously inhibit the ability of Ado to enhance mediator release. This would suggest that Tu does not compete at the mast cell Ado receptors involved in the mast cell secretory response because, if it did, no stimulation would be expected to result from the addition of Ado. The apparent decrease in Ado stimulation is, in our experience, a secondary effect that results from the inhibitory effect on antigen stimulation.

In similar experiments performed with MeTu, this nucleoside derivative inhibited antigen-induced mast cell β-hexosaminidase release in a dose-related fashion, with borderline significance at a concentration of 10 μM

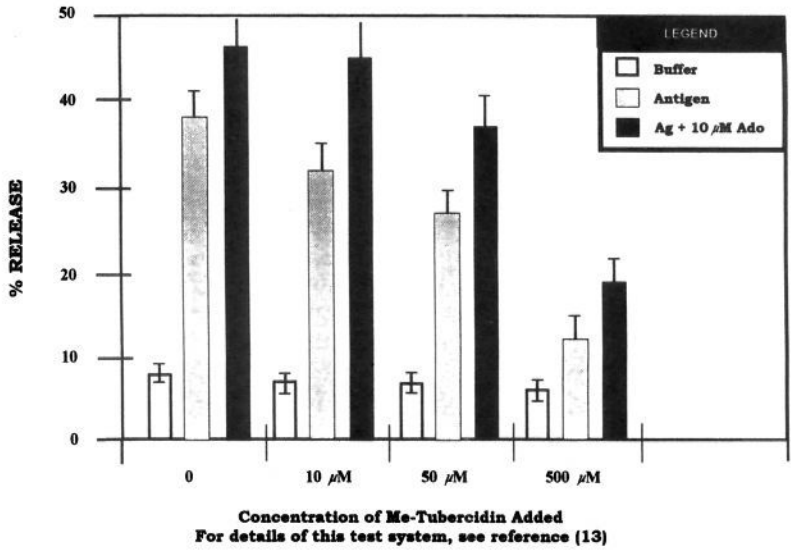

Figure 2. Effect of MeTu on β-hexosaminidase release from mouse bone marrow mast cells. Left to right: buffer, antigen, Ag + 10 μM Ado.

Table 2. Activity of MeTu and Tu in the PCA Mouse Test

injected at site	grade[a]	"p" [b]
saline	0	–
antigen (Ag)	1.74 ± 0.88	–
Ag + Tu	1.17 ± 0.94	<0.05
Ag + MeTu	1.75 ± 1.22	>0.05

[a] 0 = no bluing; grade 1 < 5 mm bluing; grade L = 5–10 mm; grade 3 > 10 mm. [b] p values are compared to antigen alone; p value Tu vs MeTu < 0.02. See ref 14 for details of the PCA test.

($p = 0.06$) and clear inhibition at 50 μM ($p < 0.005$) and 500 μM ($p < 0.005$) (Figure 2). Ado retained its ability to augment mediator release, even in the presence of 500 μM MeTu, suggesting (as with Tu) that this agent is not an agonist or antagonist of the mast cell Ado receptor associated with degranulation.

Since Tu and its 5′-methyl ether appear to inhibit mast cell mediator release in a noncytotoxic manner, experiments were carried out to determine whether either or both of these compounds were able to inhibit the passive cutaneous anaphylaxis (PCA) reaction *in vivo*.[14] Briefly, mice were sensitized intradermally with DNP–IgE or saline control, followed 24 h later by the subcutaneous injection of saline, Tu or MeTu. Five minutes after these subcutaneous injections, a mixture of DNP–HSA antigen and Evan's blue dye were infused into the mouse tail vein. Assessment of the local bluing reaction, an extravasation of Evans blue dye indicative of increased vascular permeability ("bluing"), was determined 5 min after the intravenous infusion and graded as detailed in Table 2. A pilot study was performed in mice to determine whether or not either of the compounds would show bluing by virtue of cytotoxicity or, perhaps, some other local mechanism. When 25 μL of a 50 μM solution of Tu or 25 μL of a 500 μM solution of MeTu were injected subcutaneously, neither compound alone caused any bluing, which was readily observed at the positive IgE injection site. In a second experiment, 24 mice were sensitized intradermally with anti DNP–IgE, followed 24 h later by subcutaneous injection of saline, Tu, or MeTu and intravenous injection with DNP–HSA antigen and antigen. The data are shown in Table 2. Using the paired t test, Tu induces a significant decrease in bluing compared to antigen alone ($p < 0.05$) but MeTu did not ($p = 0.28$). The difference between Tu and MeTu was statistically significant ($p < 0.02$).

It is interesting and, perhaps, somewhat surprising that Tu *per se* showed no evidence of Ado receptor effects in the systems reported herein in light of its demonstrated ability to mimic Ado in a variety of biochemical test systems, including hexokinase, myokinase, PEP carboxykinase, RNA polymerase, DNA polymerase, AA-tRNA synthetases, myosin ATPase, terminal -CCA tRNA pyrophosphorylase, and adenosine kinase.[8] Notable exceptions to Tu functioning as a surrogate for Ado are its lack of deamination by Ado deaminase or cleavage by nucleoside phosphorylase[6] and its inability, as the triphosphate nucleotide, to replace ATP as an energy source for the aminoacylation of tRNA.[15]

In light of the ability of Tu to function in place of Ado in so many biochemical systems[8] and as a substitute for Ado in maintaining erythrocyte viability *in vivo*,[6] it was postulated that Tu might interact very effectively with Ado receptors. All available evidence suggests that Tu, like most other cytotoxic nucleosides, exerts its cytotoxicity only after conversion to its 5′-phosphate.[3] In light of the severe toxicity observed with Tu in animals and humans,[3–5] one could not hope to use this compound as an Ado receptor blocker (or agonist), other than in life-threatening disease. MeTu was selected as an exemplary derivative of Tu that could not be phosphorylated but that would retain major chemical equivalence with Tu *per se*. To our knowledge, this ether has not been reported in the literature, in spite of considerable synthetic activity over the years on Tu, *per se*.[16–19] MeTu was synthesized to provide a derivative of Tu that would, hopefully, not be converted metabolically to Tu but that was postulated to interact at Ado receptors. The data presented here show that neither Tu nor MeTu inhibits the mast cell Ado receptor function involving augmented mediator release although effects on other adenosine receptor-mediated pathways cannot be ruled out. However, these compounds inhibit mast cell mediator release from mouse bone marrow cells *in vitro* by an unknown mechanism not involving Ado receptors.

Furthermore, Tu, but not MeTu, showed inhibitory activity in the PCA test in mice. This provides additional *in vivo* support for the antiallergic activity of Tu demonstrated in the *in vitro* mast cell secretory assays. MeTu showed activity only on the inhibition of β-hexoseaminidase release from mast cells but was not active in the *in vivo* PCA test.

Experimental Section

Melting points were measured on a Thomas-Hoover capillary melting point apparatus. The ^{1}H NMR spectra were recorded on a Varian Gemini 200 MHz spectrometer. The chemical shifts are reported in parts per million relative to tetramethylsilane as an internal standard. The TLC analysis were run on E. Merck precoated silica gel 60F-254 plates of 0.25 mm thickness. Compounds of interest were detected either by ultraviolet lamp (Spectroline, 254 nm) or by staining with iodine.

2′,3′-*O*-Isopropylidene-5′-*O*-methyltubercidin (II). To a solution of 0.61 g (2 mmol) of 2′,3-*O*-isopropylidenetubercidin (**I**) dissolved in 10 mL of ethylene glycol dimethyl ether was added 80 mg (64 mg, 2.6 mmol) of 80% sodium hydride in oil dispersion. Next, 0.15 mL (0.34 g, 2.4 mmol) of methyl iodide was added, followed 40 min later by 1 mL of dimethylformamide. After 1 h, the mixture was concentrated under vacuum, treated with water and chloroform, and separated. The organic layer was dried over anhydrous sodium sulfate and concentrated to an oil. The oil was purified in a silica gel column (200–400 mesh), eluting with 5% methanol in chloroform to give 210 mg (0.66 mmol, 33% yield) of the desired product. NMR ($CDCl_3$): δ 1.37, 1.63 (each 3H, s, isopropyl), 3.38 (3H, s, 5′-OCH_3), 3.60 (2H, m, H-5′$_{a,b}$), 4.35 (1H, m, H-4′), 4.96 (1H, m, H-3′), 5.10 (2H, br s, NH_2′), 5.19 (1H, m, H-2′), 6.11 (1H, d, H-1′, $J_{1',2'}$ = 2.88 Hz), 6.37 (1H, d, H-9, $J_{9,8}$ = 3.7 Hz), 7.17 (1H, d, H-8, $J_{8,9}$ = 3.7 Hz), 8.33 (1H, s, H-2).

5′-*O*-Methyltubercidin (III). A mixture of 200 mg (0.62 mmol) of **II**, 3 mL of glacial acetic acid, and 3 mL of water was heated of 90 °C for 4 h. The solution was concentrated under vacuum at 60 °C and the concentrate triturated with ether and filtered. The solid was washed with ether and dried to give 155 mg (0.45 mmol, 72% yield) of a white solid as an acetic acid salt. The above material (128 mg) was purified on a 100 g silica gel (70–230 mesh) column, eluting with 20% methanol in chloroform. The collected product was concentrated, dissolved in methanol, and filtered through a disposable filter (Supelco 5-8072, 25 mm, 0.45 mm porosity). The filtrate was concentrated and crystallized from methanol–ether to obtain 101 mg of product, mp 161–162 °C. NMR (DMSO-d_6): δ 3.30 (3H, s, 5′-OCH_3), 3.50 (2H, m, H-5′$_{a,b}$), 3.95 (1H, m, H-4′), 4.06 (1H, m, H-3′), 4.34 (1H, m, H-2′), 5.18 (1H, d, 3′-OH, $J_{3',OH}$ = 5.1 Hz), 5.32 (1H, d, 2′-OH, $J_{2',OH}$ = 6.4 Hz), 6.06 (1H, d, H-1′, $J_{1',2'}$ = 5.6 Hz), 6.60 (1H, d, H-9, $J_{9,8}$ = 3.8 Hz), 7.00 (2H, br s, NH_2), 7.28 (1H, d, H-8, $J_{8,9}$ = 3.8 Hz), 8.06 (1H, s, H-2).

Anal. ($C_{12}H_{16}N_4O_4$·0.5 H_2O). Calcd: 49.82; H, 5.92; N, 19.36. Found: C, 49.85; H, 5.58; N, 19.22.

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References

(1) Anzai, K.; Nakamura, G.; Suzuki, S. A new antibiotic tubercidin. *J. Antibiot., Ser. A* **1957**, *X*, 201–204.
(2) Owen, S. P.; Smith, C. G. Cytotoxicity and Antitumor Properties of the Abnormal Nucleoside Tubercidin. *Cancer Chemother. Rep.* **1964**, *36*, 19–22.
(3) Smith, C. G.; Gray, G. D.; Carlson, R. G.; Hanze, A. R. Biochemical and Biological Studies with Tubercidin (7-Deazaadenosine), 7-Deazainosine and Certain Nucleotide Derivatives of Tubercidin. *Adv. Enzyme Regul.* **1967**, *5*, 123–153.
(4) Bisel, H. F.; Ansfield, F. J.; Mason, J. H.; Wilson, W. L. Clinical Studies with Tubercidin Administered by Direct Intravenous Injection. *Cancer Res.* **1970**, *30*, 76–78.
(5) Grage, T. B.; Rochlin, D. B.; Weiss, A. J.; Wilson, W. L. Clinical Studies with Tubercidin Administered after Absorption into Human Erythrocytes. *Cancer Res.* **1970**, *30*, 79–81.
(6) Smith, C. G.; Reinecke, L. M.; Burch, M. R.; Shefner, A. M.; Muirhead, E. E. Studies on the Uptake of Tubercidin (7-Deazaadenosine) by Blood Cells and its Distribution in Whole Animals. *Cancer Res.* **1970**, *30*, 69–75.
(7) Bloch, A.; Leonard, R. J.; Nichol, C. A. On the mode of action of 7-deaza-adenosine (Tubercidin). *Biochim. Biophys. Acta* **1967**, *138*, 10–25.
(8) Acs, G.; Reich, E.; Mori, M. Biological and Biochemical Properties of the Analog Antibiotic Tubercidin. *Proc. Natl. Acad. Sci.* **1964**, *52*, 493–501.
(9) Pike, J. E.; Slechta, L.; Wiley, P. F. Tubercidin and Related Compounds. *J. Heterocycl. Chem.* **1964**, *1*, 159–161.
(10) Smith, C. G.; Buskirk, H. H.; Lummis, W. L. Nucleic Acids II: Cytotoxicity Studies with Nucleotides and Dinucleoside Phosphates Containing *Ara*-Cytidine. *J. Med. Chem.* **1967**, *10*, 774–776.
(11) Hsie, A. W.; Casciano, D. A.; Couch, D. B.; Krahn, D. F.; O'Neil, J. P.; Whitfield, B. L. The use of Chinese hamster ovary cells to quantify specific locus mutation and to determine mutagenicity of chemicals. A report of the gene-tox program. *Mutat. Res.* **1981**, *86*, 193–214.
(12) O'Neill, J. P.; Brimer, P. A.; Machanoff, R.; Hirsch, G. P.; Hsie, A. W. A quantitative assay of mutation induction at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster ovary cells (CHO/HGPRT system): development and definition of the system. *Mutat. Res.* **1977**, *45*, 91–101.
(13) Schwartz, L. B.; Austen, K. F.; Wasserman, S. I. Immunologic Release of β-Hexosaminidase and β-Glucuronidase From Purified Rat Serosal Mast Cells. *J. Immunol.* **1979**, *123*, 1445–1450.
(14) Mican, J. M.; Arora, N.; Burd, P. R.; Metcalfe, D. D. Passive cutaneous anaphylaxis in mouse skin is associated with local accumulation of interleukin-6 mRNA and immunoreactive interleukin-6 protein. *J. Allergy Clin. Immunol.* **1992**, *90*, 815–824.
(15) Uretsky, S. C.; Acs, G.; Reich, E.; Mori, M.; Altwerger, L. Pyrrolopyrimidine nucleotides and protein synthesis. *J. Biol. Chem.* **1968**, *243*, 306–312.
(16) Hughes, B. G.; Robins, R. K. 2′,5′-Oligoadenylates and Related 2′,5′-Oligonucleotide Analogues. 2. Effect on Cellular Proliferation, Protein Synthesis, and Endoribonuclease Activity. *Biochemistry* **1983**, *22*, 2127–2135.
(17) De Clercq, E.; Balzarini, J.; Madej, D.; Hansske, F.; Robins, M. J. Nucleic Acid Related Compounds. 51. Synthesis and Biological Properties of Sugar-Modified Analogues of the Nucleoside Antibiotics. Tubercidin, Toyocamycin, Sangivamycin, and Formycin. *J. Med. Chem.* **1987**, *30*, 481–486.
(18) Gupta, P. K.; Daunert, S.; Nassiri, M. R.; Wotring, L. L.; Drach, J. C.; Townsend, L. B. Synthesis, Cytotoxicity, and Antiviral Activity of Some Acyclic Analogues of the Pyrrolo[2,3-*d*]pyrimidine Nucleoside Antibiotics Tubercidin, Toyocamycin, and Sangivamycin. *J. Med. Chem.* **1989**, *32*, 402–408.
(19) Townsend, L. B.; Drach, J. C.; Wotring, L. C.; Vittori, S.; Pudlo, J. S.; Swayze, E. E.; Gupta, P.; Maruyama, P.; Saxena, N.; Coleman, L. A.; Westerman, A. C.; Spurr, J. L.; Nassiri, M. R.; Turk, S. R.; Krawczyk, S. H. Design, synthesis, and studies on the structure activity relationships of certain pyrrolo[2,3-d]-pyrimidine nucleosides and structurally related analogs as potential antineoplastic and antiviral agents. *Farmaco* **1991**, *46* (1, Suppl.), 113–139.

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