Antiviral Oligo- and Polyribonucleotides Containing Selected Triazolo[2,3-*a*]purines[‖]

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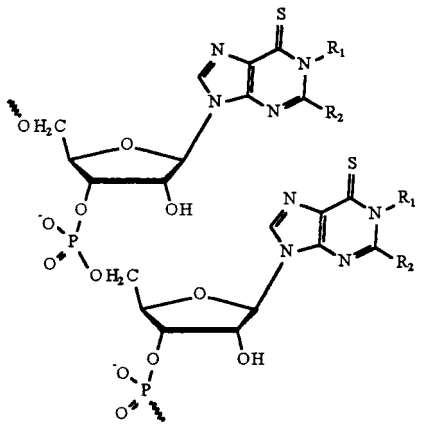
Several amphipathic (hydrophobic base, hydrophilic backbone) polyribonucleotides have recently been shown to have potent antiviral activity against HIV and human cytomegalovirus (HCMV). The working hypothesis developed during these studies was that the ability to form an ordered, non-hydrogen-bonded array in solution was an important criterion for activity. To explore further the role of structure and molecular size on the inhibition of virus replication, one new polynucleotide and two 32-mer oligonucleotides based on the triazolo[2,3-*a*]purine ring system have now been prepared. High-molecular-weight polynucleotide **4a** (PTPR) and sulfur-containing 32-mer **5b** (TTPR) were moderately active against HIV but showed greater potency against HDMV than ganciclovir. Both **4a** and **5b** gave clear evidence of cooperative melting behavior, whereas inactive 32-mer **5a** showed no such behavior.

Introduction

Human acquired immunodeficiency syndrome (AIDS) is an expanding pandemic for which no curative therapy is available. Current estimates indicate that over 20 million adults are alive and infected with HIV or have AIDS, and the World Health Organization projects that 40 million HIV-positive individuals will be alive in the year 2000.[1] At present, only two loci in the HIV replication cycle, reverse transcriptase and viral protease, are targeted by approved drugs. The former target is inhibited by nucleoside analogues such as AZT,[2] ddC,[3] ddI,[4] 3TC,[5] and D4T.[6] The recent advent of nonnucleoside reverse transcriptase inhibitors and protease inhibitors has given rise to combination protocols which markedly reduce viral load and, in some cases at least, lead to the generation of undetectable viral loads in patients.[6] However, resistance to monotherapy and combination therapy is developing,[7,8] and it remains very clear that new drugs having unique structures and targets are urgently needed.

Human cytomegalovirus (HCMV) infections are widespread in AIDS patients and lead to substantial mortality and morbidity. Systemic (visceral) manifestations of HCMV are usually managed with ganciclovir, cidofover, and foscarnet, at great expense and with considerable toxicity.[9] CMV retinitis is a leading cause of blindness in AIDS patients and is poorly controlled by systemic therapy; intraocular therapy has met with some success but remains risky and somewhat controversial.[10] New drugs having increased potency, novel mechanisms of action, and, importantly, equipotent activity against HIV and CMV would be a useful step forward in treating both AIDS and opportunistic cytomegalovirus infections simultaneously.

Chart 1. Prototype Polynucleotides



For a number of years, this laboratory has been involved in studies on the synthesis and biological activity of novel polynucleotides, especially those containing thiopurines. These studies led to the demonstration that poly(1-methyl-6-thioguanylic acid) (PMTG, **1**) and poly(1-methyl-6-thioinosinic acid) (PMTI, **2**) (Chart 1) are potent inhibitors of HIV[11]and HCMV replication and cytopathicity. This observation has prompted further studies designed to explore the parameters responsible for antiviral activity in order to elucidate the mechanism of action and to optimize the therapeutic potential of this class of compounds.

The working hypothesis on the structural basis of antiviral activity has been that amphipathic character (hydrophobic base/hydrophilic backbone) and the ability to form a highly ordered, non-hydrogen-bonded array in solution are prerequisites for antiviral activity. As a test of this hypothesis, the synthesis and biological evaluation of poly(1-amino-6-thioinosinic acid) (PATI,

[‖] This paper is dedicated to our colleague and friend Prof. Dr. Gottfried (Friedl) Heinisch on the occasion of his 60th birthday.
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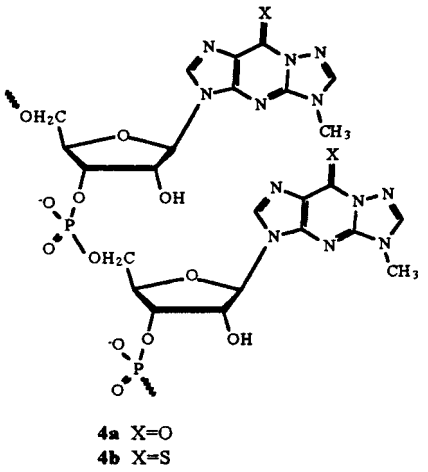
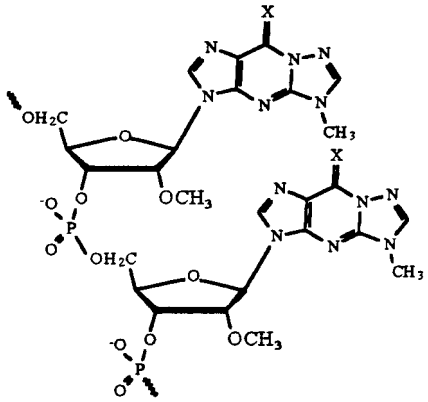
Chart 2. Poly- and Oligonucleotides Containing Triazolo[2,3-*a*]purines



4a X=O
4b X=S

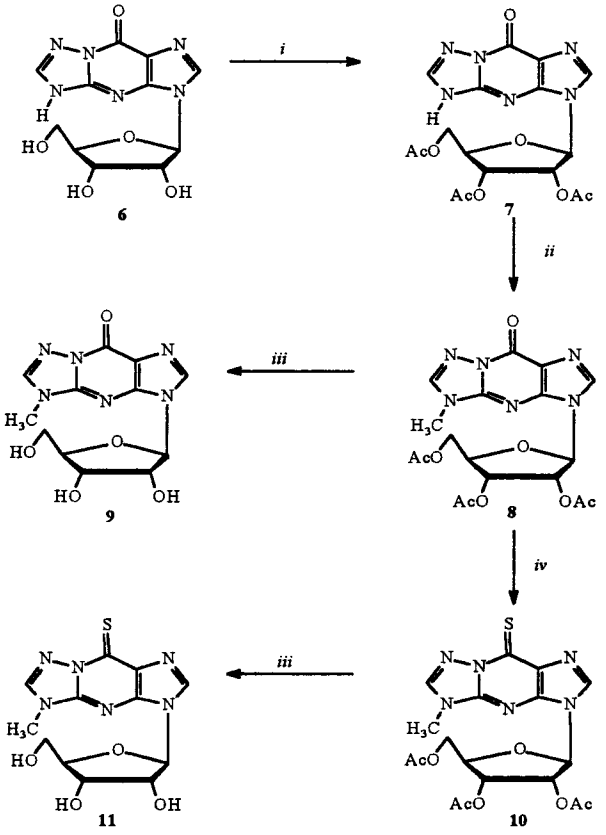


5a X=O
5b X=S

3; Chart 1) was recently reported.[12] This polymer, which has a much more hydrophilic base than PMTI, was completely devoid of solution secondary structure and antiviral activity or cytotoxicity.[12] Since increased hydrophilicity in the base abolished secondary structure and antiviral activity, the next logical step in testing the hypothesis was to enhance the hydrophobicity of the base while retaining a normal phosphodiester backbone. It was reasoned that extending the purine hydrophobic, planar surface into a triazolopurine should provide enhanced opportunity for stacking interactions and facilitate formation of an ordered array in solution. An additional important consideration entered into this design process. It was previously shown[11] that decreased chain length of PMTI to an average chain length of approximately 36 bases resulted in a compound which exhibited no secondary structure and had no biological activity. It was anticipated that increasing the planar surface of the bases might provide shorter compounds which would still retain the desired properties. If this turned out to be true, it would open up the area of oligonucleotide chemical synthesis as a source of antiviral oligonucleotides, obviating the requirement that nucleoside diphosphates be substrates for polynucleotide phosphorylase.

In this paper, the synthesis and characterization of polynucleotide **4a** and oligonucleotides **5a** and **5b** (Chart 2) are described. Activity against HIV and against human cytomegalovirus (HCMV) are also reported.

Scheme 1[a]



[a] (i) Ac_2O, pyridine; (ii) CH_3I, K_2CO_3, DMF; (iii) NH_3, MeOH; (iv) Lawesson's reagent.

Results and Discussion

Chemistry. The key starting material for the requisite polynucleotide and oligonucleotide precursors was *5H*(*7H*)-9-oxo-3-(*β*-D-ribofuranosyl)-1,2,4-triazolo[2,3-*a*]-purine (**6**), which was prepared as previously described[13] by cyclization of 1-aminoguanosine using phosphoryl chloride in DMF.

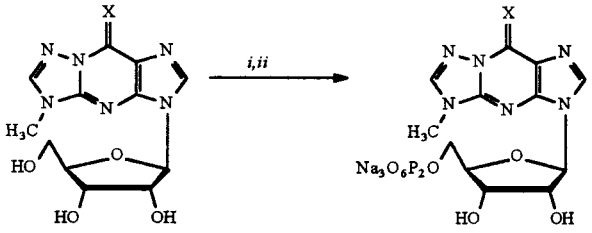
Synthesis of Polynucleotide Precursors. Somewhat surprisingly, nucleoside **6** was found to be quite acidic. The pK_a, as measured by UV, was approximately 4.5 for ionization of the triazolo ring proton, compared to about 10 for triazole itself.[14] Obviously, the nucleoside base would be fully anionic at pH 9; hence, diphosphates of this and related compounds would undoubtedly be very poor substrates for polynucleotide phosphorylase, which has a pH optimum at about 9. It was decided to explore the regioselectivity of methylation of the triazolopurine in order to determine whether N5 could be selectively alkylated. Because **6** was essentially insoluble in DMF, the nucleoside was first converted to its tri-*O*-acetyl derivative **7** using acetic anhydride in pyridine (Scheme 1). Although isolation of this compound was difficult because of the acidity of the heterocyclic base, it was possible to isolate the tri-*O*-acetyl derivative by precipitation from an aqueous solution at pH 4.5. Using the methylation procedure applied to a derivative of the Y nucleoside by Ueda,[15] in which alkylation is carried out by methyl iodide in dimethylformamide in the presence of potassium carbonate, only a single *N*-methyl derivative was obtained. The structure of this compound was unequivocally

Table 1. Selected ^{1}H and ^{13}C NMR Chemical Shifts for Compound **8**[a]

position	δ ^{1}H	δ ^{13}C
2	8.18	138.5
3a		148.4
4a		147.1
6	8.80	143.2
9a		118.0
CH_3	3.72	18.5
H1′	6.20	86.1

[a] Chemical shift data and HETCOR experiments were performed in DMSO-d_6 using a Varian Unity 500-MHz spectrometer.

Scheme 2[a]



[a] (i) $POCl_3$, $P(OEt)_3$; (ii) tris(tetrabutylammonium)pyrophosphate, carbonyl diimidazole.

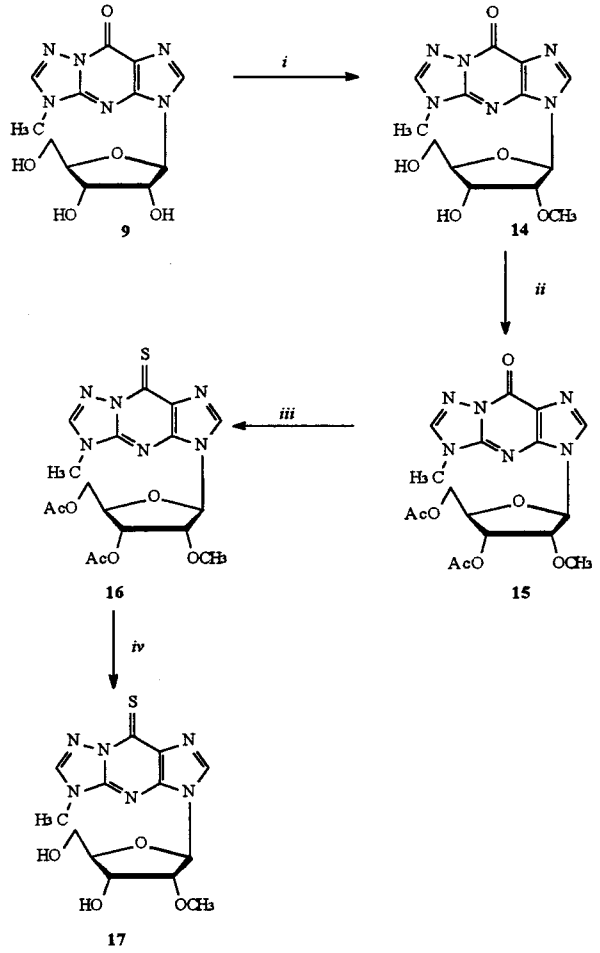
established as the N5-methyl on the basis of HMQC and HMBC experiments.

Chemical shift assignments for carbons and protons used in defining the position of the methyl group are given in Table 1. The aromatic protons attached to C2 and C6 resonated at lowest field, δ 8.18 and 8.80. Heteronuclear multiple quantum correlation (HMQC) spectroscopy revealed that the proton resonating at higher field (δ 8.18) was coupled to a carbon resonating at δ 138.5 and the δ 8.80 proton to the δ 143.2 carbon. The carbon shift at δ 138.5 was assigned to C2 by virtue of a heteronuclear multiple bond correlation (HMBC) experiment which demonstrated three-bond coupling with the proton at H1′(δ 6.20). The methyl protons (δ 3.72) were strongly three-bond-coupled to C6 (δ 143.3) and quaternary carbon C4a (δ 147.1); conversely, H6 was coupled only to C4a and the methyl group. These data conclusively establish the sole site of methylation to be N5.

Treatment of protected nucleoside **8** with Lawesson's reagent afforded the 9-thione **10** (Scheme 1). Deacylation of compounds **8** and **10** using methanolic ammonia provided the unprotected nucleosides **9** and **11**. Both nucleosides **9** and **11** were converted to their respective 5′-diphosphates **12** and **13** using the phosphorylation procedure of Yoshikawa[16] and the diphosphate synthesis of Hoard and Ott[17] (Scheme 2).

Synthesis of Oligonucleotide Precursors. Nucleoside **9** was 2′(3′)-*O*-methylated using the approach of Ts'o and colleagues[18] employing methyl iodide and sodium hydride in DMF. Both the 5,2′-*O*-dimethyl and 5,3′-*O*-dimethyl isomers were isolated from the reaction mixture by silica gel column chromatography. The requisite 5,2′-*O*-dimethyl derivative **14** was separated by crystallization from the mixture in an overall yield of 34%. The usual COSY NMR proof of substitution site, in which one may "walk" from H1′ to H2′ to H3′ to 3′-OH, failed in this case because of complete magnetic

Scheme 3[a]

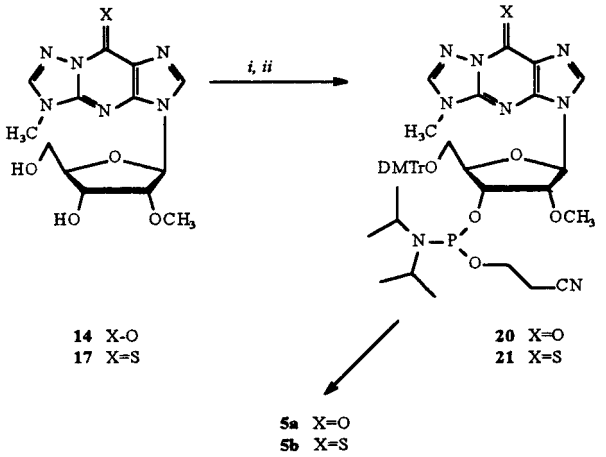


[a] (i) CH_3I, DMF, NaH; (ii) Ac_2O, pyridine; (iii) Lawesson's reagent, DMF; (iv) NH_3, MeOH.

equivalence of the 2′- and 3′-protons, even at 500 MHz. The structure of **14** was confirmed using 2D NOESY spectroscopy; only in the presumed 2′-isomer did the *O*-methyl group show a cross-peak with H1′ as would be expected only if substitution were in the 2′-position. Final structure proof was achieved by the smooth conversion of **14** to its 3′,5′-(tetraisopropyldisiloxane) derivative using the Markiewicz reagent as applied by Robins.[19] Such a conversion cannot be accomplished with the 3′-*O*-methyl derivative. Compound **14** was acetylated, and its 3′,5′-di-*O*-acetyl derivative **15** was converted to the 9-thione **16** in 76% yield. Deacylation of **16** afforded compound **17**. Nucleosides **14** and **17** were converted to their respective 5′-*O*-dimethoxytrityl derivatives **18** and **19** and then to phosphoramidites **20** and **21** in the usual way.[20]

Polymerization of 5′-Diphosphates. Polymerization of the 5′-diphosphate **12** (X = O) was undertaken using *Micrococcus luteus* polynucleotide phosphorylase as previously described[11,12] to give PTPR (**4a**) in about 25% isolated yield (Scheme 3). The average molecular weight, as estimated by size exclusion HPLC,[11] was of the order of 40 000−50 000. Enzymatic digestion of PTPR using a mixture of venom phosphodiesterase and alkaline phosphatase gave only the starting nucleoside **9**, thus attesting to the homogeneity of the polynucleotide. The polymer exhibited fairly cooperative melting behavior with a T_m of 24 °C.

Scheme 4[a]



[a] (i) DMTRCl, pyridine; (ii) β-cyanoethyl tetraisopropylphosphorodiamidite, diisopropylammonium tetrazolide, CH_3CN.

Despite modifications in the source of polynucleotide phosphorylase, divalent counterion, pH, and temperature, no conditions could be found under which 5′-diphosphate **13** (X = S) was a substrate for the enzyme, emphasizing once more the importance of examining oligonucleotides prepared by chemical synthesis.

Oligonucleotide Synthesis. Oligonucleotides **5a** and **5b** were synthesized from their respective 5′-*O*-(dimethoxytrityl)-3′-*O*-phosphoramidites **20** and **21** (Scheme 4) on an automated DNA synthesizer as 32-mers having a single thymidine residue on the 3′-terminus. The crude mixtures were analyzed by SAX HPLC using a 60-min linear gradient elution with 1 mM KH_2PO_4 in formamide/water (6:4) (pH 6.3) and saturated KH_2PO_4 in formamide/water (6:4) (pH 6.3). Purification was performed on a gel filtration column using Sephadex 50-80 with aqueous elution. Purity of the compounds was then checked by SAX HPLC using the same phosphate buffer system. Both oligomers exhibited fairly cooperative melting transitions at 19.0 °C for **8a** and 32.6 °C for **8b.** This significant difference in melting temperature emphasizes the important role of hydrophobicity/polarizability of sulfur compared to oxygen in stabilizing hydrophobic stacking interactions of single-stranded oligonucleotides.

Biological Results. The polynucleotide **4a** and the oligonucleotides **5a** and **5b** were tested for anti-HIV activity as previously described.[11] As noted above, the polynucleotide **4a** was found to be active against HIV III_B in CEM-SS cells with an IC_{50} of ~0.4 μM and was nontoxic (Table 2). The oligomer containing the 9-thiotriazolopurine **5b** was active with an IC_{50} of 1−2 μM in the same system and was nontoxic up to 100 μg/mL, whereas the 9-oxo oligomer **5a** was devoid of activity or cytoxicity.

Perhaps the most striking and unexpected observations in this study relate to activity against human cytomegalovirus (HCMV). Antiviral activity was determined in the laboratories of Drs. Robert Sidwell and John Huffman, Utah State University, using a plaque reduction assay with strain AD-169 of HCMV in MRC-5 cells as previously described.[21] As shown in Table 2, compounds **2**, **4a**, and **5b** are approximately equipotent inhibitors of HCMV and HIV and are somewhat more potent against HCMV than the positive control ganciclovir. Since HCMV is a DNA virus of the herpes class and has no reverse transcriptase, elucidation of the mechanism of this potent inhibition must await further investigation. Regardless of the mechanism of action, however, the ability of these amphipathic oligo- and polyribonucleotides to inhibit both HIV and HCMV opens an exciting new area of therapeutic investigation.

Table 2. Virus Inhibition and Cytotoxicity Values (μM)[a]

	HIV			HCMV[b]		
	IC_{50}	CD_{50}	TI	IC_{50}	CD_{50}	TI
PMTI (**2**)	0.08[c]	>1.0[c]	>12.5	0.15	>3.2	>21
PTPR (**4a**)	1.0	>2.5	>2.5	0.43	>7.9	>18
TPR (**5a**)	>7			6.7	>22.8	>3.4
TTPR (**5b**)	1.7	>7	>4.1	2.8	>22.3	>7.9
DDC	0.0015	>0.1	>67			
ganciclovir				6.3	>125	>20

[a] See text for cell lines, virus strains, and assay conditions; see ref 11 (HIV) and ref 21 (HCMV). [b] HCMV studies were performed by Drs. John Huffman and Robert Sidwell at the Institute for Antiviral Research, Utah State University, Logan, UT. [c] As previously reported.[11]

Experimental Section

^1H and ^{31}P NMR spectra were recorded with an IBM AF 200-MHz FT-NMR or a Varian Unity 500-MHz spectrometer. UV spectra and melting curves were recorded with a Hewlett-Packard 8452A diode array spectrophotometer equipped with a Peltier variable temperature device; circular dichroism spectra were obtained using a Jasco 700A CD spectropolarimeter equipped for variable temperature studies. FAB and EI mass spectra were recorded with a MAT 95 spectrometer. MALDI mass spectra were obtained using a Per Septive Biosystems MALDI-TOF mass spectrometer Voyager − DE STR Bio Spectrometry workstation. Electrospray mass measurements were conducted using a Fisons Instrument Quattro II mass spectrometer in a solvent of 0.4 M 1,1,1,3,3,3-hexafluoro-2-propanol adjusted to pH 7 with triethylamine, and methanol. Whatman plates, precoated with silica gel 60 containing fluorescent indicator F_{254}, were used for thin-layer chromatography, and silica gel 60 (Mallinckrodt SilicAR, 60−200 mesh) was employed for column chromatography.

HPLC experiments were carried out on a Hitachi L6200 pump equipped with an L3000 photodiode array. Reverse-phase chromatography utilized Rainin Microsorb-MV C8 and C18 columns. Purity determinations were made by peak integration of the HPLC traces using 50 mM KH_2PO_4, pH 5 (solvent A), MeOH (solvent B), 50 mM $(NH_4)HCO_3$, pH 7.0 (solvent C), and/or 40% aqueous CH_3CN (solvent D). For strong anion-exchange chromatography, a Partisil 10 SAX WCS analytical column was employed. Size separations were carried out using a BioRad SEC 125 column. The synthesis of polynucleotides was performed using *M. luteus* polynucleotide phosphorylase obtained from Sigma.

5*H*(7*H*)-9-Oxo-3-(β-D-ribofuranosyl)-1,2,4-triazolo[2,3-*a*]purine (6). This compound was prepared from 1-aminoguanosine as previously described.[5]

2′,3′,5-Tri-*O*-acetyl-5*H*(7*H*)-9-oxo-3-(β-D-ribofuranosyl)-1,2,4-triazolo[2,3-*a*]purine (7). Compound **6** (13 g, 42 μmol) was added to a mixture of acetic anhydride (126 mL) and pyridine (169 mL) and stirred for 2 h at room temperature. The clear solution was evaporated, and the residue was repeatedly coevaporated with toluene until the odor of pyridine was no longer present. The residue was partially dissolved in cold water, and the pH of the suspension was adjusted from nearly 2 to 4.5 by slow addition of ammonium hydroxide; then the suspension was cooled at 4 °C overnight. The precipitate was filtered, washed with ice water and then cold ethanol, and then dried under vacuum to give 17 g of **7** (93%). ^1H NMR (DMSO-d_6): δ 8.73 (s, 1, H6); 8.19 (s, 1, H2); 6.14 (d, 1, H1′); 5.90 (t, 1, H2′); 5.53 (t, 1, H3′); 4.34 (m, 3, H4′ and H5′,5″); 2.1 (s, 3, $COCH_3$); 2.03 (2s, 6, $COCH_3$). EI HRMS (M − HOAc)$^+$:

calcd 374.09748, obsd 374.09808. Purity was >99% as judged by HPLC (C18) using A:B (75:25) and C8 using C:D (75:25).

2′,3′,5′-Tri-*O*-acetyl-5-methyl-9-oxo-3-(β-D-ribofuranosyl)-1,2,4-triazolo[2,3-*a*]purine (8). To a solution of **7** (8.68 g, 20 mmol) in dimethylformamide (100 mL) were added powdered potassium carbonate (4.15 g, 30 mmol) and methyl iodide (4.26 g, 30 mmol). The suspension was stirred for 18 h at room temperature and passed through a layer of Celite. The white precipitate was carefully washed with dimethylformamide until the filtrate exhibited no UV absorption. The filtrate was then evaporated to an oil, and the product was purified by chromatography using a silica gel column eluted by a gradient of methanol in chloroform (0−5%). The pure fractions were pooled and evaporated to give 6.27 g of **8** (69%). ^{1}H NMR (DMSO-d_6): δ 8.8 (s, 1, H6); 8.16 (s, 1, H2); 6.18 (d, 1, H1′); 5.93 (t, 1, H2′); 5.85 (t, 1, H3′); 4.33−4.24 (m, 3, H4′, H5′, H5″), 3.71 (s, 3, NCH_3); 2.11, 2.06, 1.95 (s, 3 each, $COCH_3$). FAB HRMS (MH^+): calcd 449.14209, obsd 449.14148. Purity was >99% as judged by HPLC (C18) using A:B (75:25) and C8 using C:D (60:40).

5-Methyl-9-oxo-3-(β-D-ribofuranosyl)-1,2,4-triazolo[2,3-*a*]purine (9). Compound **8** (4.48 g, 10 mmol) was placed in a stainless steel bomb and treated with methanolic ammonia saturated at −20 °C (200 mL). The mixture was allowed to stand at room temperature for 18 h. The ammonia was vented, and the contents were transferred into a beaker and allowed to stand. White crystals were then collected by filtration and washed with cold MeOH. Yield: 2.97 g, 92%. ^{1}H NMR (DMSO-d_6): δ 8.8 (s, 1, H6); 8.2 (s, 1, H2); 5.87 (d, 1, H1′); 5.46 (d, 1, OH); 5.24 (d, 1, OH); 5.02 (t, 1, OH); 4.55 (m, 1, H2′); 4.14 (m, 1, H3′); 3.94 (m, 1, H4′); 3.63−3.59 (m, 5, H5′, H5″ and NCH_3). FAB HRMS (MH^+): calcd 323.11039, obsd 323.10898. Purity was >99% as judged by HPLC (C18) using A:B (90:10) and C8 using C:D (90:10).

2′,3′,5′-Tri-*O*-acetyl-5-methyl-3-(β-D-ribofuranosyl)-1,2,4-triazolo[2,3-*a*]purine-9-thione(10). Compound **8** (6 g, 13.4 mmol) was dissolved in anhydrous DME (50 mL) under argon. Lawesson's reagent (3.30 g, 8.57 mmol) was added to the solution. The suspension was refluxed for 1 h at which time TLC in $CHCl_3$/CH_3OH (9:1) indicated that all starting material had been consumed. The mixture was allowed to cool and was poured into a beaker containing water (250 mL), and brine (50 mL) was added. The milky aqueous solution was extracted with $CHCl_3$ (6 × 100 mL). The chloroform extract was washed with water (100 mL) and dried over sodium sulfate, and the dried extract was concentrated in vacuo to give the crude product. It was purified by silica gel chromatography using 2% CH_3OH in chloroform as eluent to give 3.59 g of **10** (58%). ^{1}H NMR (DMSO-d_6): δ 8.8 (s, 1, H6); 8.2 (s, 1, H2); 5.87 (d, 1, H1′); 5.46 (d, 1, OH); 5.24 (d, 1, OH); 5.02 (t, 1, OH); 4.55 (m, 1, H2′); 4.14 (m, 1, H3′); 3.94 (m, 1, H4′); 3.63−3.59 (m, 5, H5′, H5" and NCH_3). FAB HRMS (MH^+): calcd 465.11924, obsd 465.12088. Purity was 94−96% as judged by HPLC (C18) using A:B (65:35) and C8 using C:D (60:40).

5-Methyl-3-(β-D-ribofuranosyl)-1,2,4-triazolo[2,3-*a*]purine-9-thione (11). Compound **10** (2.65 g, 5.57 mmol) was placed in a stainless steel bomb and treated with methanolic ammonia saturated at −20 °C (100 mL). The mixture was allowed to stand at room temperature for 18 h. The ammonia was vented, and the contents were concentrated to one-third volume. The crystals were filtered, washed with methanol, and recrystallized with methanol/water to give compound **11** (1.35 g, 70%). ^{1}H NMR (DMSO-d_6): δ 9.02 (s, 1, H6); 8.4 (s, 1, H2); 5.95 (d, 1, H1′); 5.5 (d, 1, OH); 5.25 (d, 1, OH); 5.02 (t, 1, OH); 4.55 (m, 1, H2′); 4.14 (m, 1, H3′); 3.93 (m, 1, H4′); 3.74 (s, 3, NCH_3); 3.61 (m, 2, H5′, 5”). FAB MS (MH^+): 339. Anal. Calcd: C, 42.60; H, 4.17; N, 24.84; S, 9.48. Obsd: C, 42.54; H, 4.25; N, 24.91; S, 9.23.

The nucleoside-5′-diphosphates **12** and **13** were prepared from their respective nucleosides **9** and **11** according to the phosphorylation procedure of Yoshikawa[16] and the diphosphate synthesis of Hoard and Ott.[17]

5-Methyl-9-oxo-3-(β-D-ribofuranosyl)-1,2,4-triazolo[2.3-*a*]purine-5′-diphosphate (12). Yield: 35.4% (two steps, overall yield, based upon starting nucleoside **9**). ^{1}H NMR (D_2O): 8.42 (s, 1, H6); 8.20 (s, 1, H2); 5.98 (d, 1, H1′); 4.67 (m, 1, H2′); 4.52 (t, 1, H3′); 4.24 (m, 1, H4′); 4.13 (m, 2, H5′,5″); 3.65 (s, 3, NCH_3). ^{31}P NMR (D_2O): −5.5 (d); −9.75 (d). FAB MS: 503 $(M + Na - H)^-$.

5-Methyl-3-(β-D-ribofuranosyl)-1,2,4-triazolo[2,3-*a*]purine-9-thione-5′-diphosphate (13). Yield: 30.7% (two steps, overall yield, based on starting nucleoside **11**). ^{1}H NMR (D_2O): 8.67 (s, 1, H6); 8.42 (s, 1, H2); 6.05 (d, 1, H1′); 4.66 (m, 1, H2′); 4.56 (m, 1,H3′); 4.26 (m, 1, H4′); 4.13 (m, 2, H5′,5″). ^{31}P NMR: −5.43 (d); −9.86 (d). FAB MS (glycerol): 519 $(M + Na - H)^-$.

Poly[5-methyl-9-oxo-3-(β-D-ribofuranosyl)-1,2,4-triazolo-[2,3-*a*]purinylic acid] (PTPR, 4a). A solution made from the following components was incubated for 5 h at 37 °C: Tris·HC1 (pH 9, 2 M), 0.666 mL; $MgCl_2$ (0.1 M), 0.666 mL; 2-mercaptoethanol (0.28 M), 0.666 mL; PNPase (*M. luteus*), 10 mg (∼400−900 units); water, 2.44 mL; diphosphate **12**, 100 mg. After incubation, the reaction mixture was diluted with H_2O, and the pH was adjusted to approximately 7.5 with very dilute aqueous HCl. The solution was then extracted 12 times with 60 mL of chloroform/isoamyl alcohol (5:2) to give a clear aqueous solution. The aqueous layer was dialyzed against 0.1 M HCl for 24 h and then H_2O for 48 h. Lyophilization of the aqueous solution afforded PTPR (**4a**) (28 mg, 28%) as a fluffy solid. UV: λ_{max} 270 nm, ϵ_{max} 8340 (0.1 N HCl).

HPLC analysis using a size exclusion column (BioRad SEC 125), isocratically eluted with an aqueous solution containing NaH_2PO_4 (50 mM), Na_2HPO_4 (50 mM), NaCl (150 mM), and NaN_3 (10 mM), pH 6.8, at a flow rate of 1 mL/min gave a broad peak, retention time: 7.06 min.

Enzymatic Degradation of PTPR (4a). A solution made from venom phosphodiesterase, 150 μL; $MgCl_2$ (0.1 M), 150 μL; Tris·HCl (2 M, pH 9); phosphatase, alkaline, 50 μL; PTPR (**4a**) (2 mg/1 mL of H_2O), 550 μL; HCl (0.1 M), was incubated at 37 °C overnight. After incubation, the solution was diluted to 3 mL with HPLC water and extracted six times with chloroform/isoamyl alcohol (5:2) to give a clear solution. HPLC analysis on reverse-phase C_{18} column isocratically eluted with KH_2PO_4 buffer (50 mM)/MeOH (85:15) gave a peak at retention time: 5.29 min, which corresponds to the starting nucleoside **9** as confirmed by a co-injection experiment.

5,2′-*O*-Dimethyl-9-oxo-3-(β-D-ribofuranosyl)-1,2,4-triazolo[2,3-*a*]purine (14). A solution of **9** (5.796 g, 18 mmol) in 180 mL of dry dimethylformamide was cooled to ice temperature. NaH (600 mg; 24 mmol, 60% dispersion in mineral oil) was washed with benzene (3 × 20 mL) and added to the solution. The reaction mixture was stirred vigorously for 1 h; then methyl iodide (1 mL, ∼16.2 mmol) in 10 mL of dimethylformamide was added in two portions of 5 mL each (at a 45-min interval). The reaction mixture was stirred for 4 h at 0 °C in an ice bath. The dimethylformamide was evaporated in vacuo and the residue dissolved in a minimum amount of methanol and dried on silica gel. The compound was purified by chromatography using a silica gel column eluting with a methanol gradient in chloroform (8−15%). The fractions collected initially below the yellow band contained mostly the 2′-OCH_3 compound (2.52 g). Those fractions that were eluted and collected later (1.94 g) contained a mixture of 2′-OCH_3 and 3′-OCH_3 in a ratio of 2.2:1. Ethanol recrystallization of the first pool of fractions gave 2.05 g of pure 2′-OCH_3 compound **14** (34%, isolated yield). The reaction was repeated on a larger scale starting from 14.5 g of **9** to give 6.12 g of 2′-OCH_3 compound **14**. ^{1}H NMR (D_2O): δ 8.37 (s, 1, H6); 8.00 (s, H, H2); 5.88 (d, 1, H1′); 4.40 (m, 2, H2′ and H3′); 4.07 (m, 1, H4′); 3.76 (m, 2, H5′,5″); 3.59 (s, 3, NCH_3); 3.36 (s, 3, OCH_3). FAB HRMS (MH^+): calcd 337.12604, obsd 337.12880. Purity was >99% as judged by HPLC (C18) using A:B (85:15) and C8 using C:D (60:40).

3′,5′-Di-*O*-acetyl-5,2′-*O*-dimethyl-9-oxo-3-(β-D-ribofuranosyl)-1,2,4-triazolo[2,3-*a*]purine (15). Compound **14** (5.38 g, 16 mmol) was added to a mixture of acetic anhydride (48 mL) and pyridine (64 mL). The reaction mixture was stirred at room temperature for 2 h. The clear solution was evapo-

rated to dryness, and a few pieces of ice were added to destroy excess acetic anhydride. The mixture was evaporated and coevaporated twice with toluene (50 mL). The residue was dissolved in $CHCl_3$ (200 mL), and the chloroform solution was washed with cold 0.1 N aqueous HCl (2 × 50 mL), cold water (5 × 50 mL), cold aqueous 5% $NaHCO_3$ (2 × 50 mL), and cold water (2 × 50 mL). The chloroform extract was dried over Na_2SO_4 and evaporated to give 5.85 g of **15** (87%). ^{1}H NMR (DMSO-d_6): δ 8.8 (s, 1, H6); 8.2 (s, 1, H2); 6.00 (d, 1, H1′); 5.56 (d, 1, H2′); 4.81 (m, 1, H3′); 4.32 (m, 3, H4′ and H5′,5″); 3.68 (s, 3, NCH_3); 3.31 (s, 3, OCH_3); 2.14 and 2.02 (s, 3 each, $COCH_3$). FAB HRMS (MH^+): calcd 421.14717, obsd 437.12444. Purity was >99% as judged by HPLC (C18) using A:B (75:25) and C8 using C:D (60:40).

3′,5′-Di-*O*-acetyl-5,2′-*O*-dimethyl-3-(β-D-ribofuranosyl)-1,2,4-triazolo[2,3-*a*]purine-9-thione (16). Compound **15** (3.00 g, 7.2 mmol) was dissolved in anhydrous DME under argon. Lawesson's reagent (1.78 g) was added to the solution. The suspension was refluxed for 2 h at which time TLC in $CHCl_3$/MeOH (9:1) indicated that the all starting material had been consumed. The mixture was cooled and was poured into a beaker containing 270 mL of water. The milky aqueous solution was extracted with chloroform (3 × 100 mL). The chloroform extract was washed with water (20 mL) and dried over Na_2SO_4. The dried extract was concentrated in vacuo to give the crude product. It was purified by silica gel chromatography using 2% MeOH/$CHCl_3$ as eluent to give 2.39 g of **16** (76%).^{1}H NMR (DMSO-d_6): δ 9.03 (s, 1, H6); 8.44 (s, 1, H2); 6.03 (d, 1, H1′); 5.56 (m, 1, H2′); 4.82 (t, 1, H3′); 4.32 (m, 3, H4′ and H5′, 5″); 3.76 (s, 3, NCH_3); 3.29 (s, 3, OCH_3); 2.14 and 2.01 (s, 3 each, $COCH_3$). FAB HRMS (MH^+): calcd 437.12433, obsd 437.12444. Purity was at least 95% as judged by HPLC (C18) using A:B (65:35) and C8 using C:D (60:40).

5,2′-*O*-Dimethyl-3-(β-D-ribofuranosyl)-1,2,4-triazolo-[2,3-*a*]purine-9-thione (17). Compound **16** (2.20 g, 5.045 mmol) was placed in a stainless steel bomb and heated with methanolic ammonia saturated at −20 °C (100 mL) and the mixture stood at room temperature for 18 h. The ammonia was vented, and the contents were transferred to a beaker and allowed to stand. The white crystals were collected by filtration and washed with cold methanol. Yield of **17**: 1.56 g, 88%. ^{1}H NMR (DMSO-d_6): δ 9.02 (s, 1, H6); 8.45 (s, 1, H2); 6.01 (d, 1, H1′); 5.32 (d, 1, OH); 5.09 (t, 1, OH); 4.32 (m, 2, H2′ and H3′); 3.97 (m, 1, H4′); 3.61 (m, 2, H5′,5″); 3.73 (s, 3, NCH_3), 3.33 (s, 3, OCH_3). FAB HRMS (MH^+): calcd 353.10320, obsd 353.10435. Purity was >99% as judged by HPLC (C18) using A:B (85:15) and C8 using C:D (60:40).

5′-(Dimethoxytrityl)-5,2′-*O*-dimethyl-9-oxo-3-(β-D-ribofuranosyl)-1,2,4-triazolo[2,3-*a*]purine (18). Compound **14** (1.53 g, 4.6 mmol) was dried by coevaporation with pyridine (3 × 20 mL), dissolved in 20 mL of anhydrous pyridine, and cooled to 0 °C in an ice bath. Dimethoxytrityl chloride (1.86 g, 5.5 mmol) was added to the above solution, and the reaction was warmed to room temperature. It was stirred for 2 h at which time TLC in 5% MeOH/$CHCl_3$ indicated complete disappearance of **14**. Methanol (3 mL) was added to quench the reaction. The solvent was evaporated under vacuum, and the residue was dissolved in chloroform (35 mL). The chloroform solution was washed with 5% aqueous $NaHCO_3$ (2 × 15 mL) and water (15 mL) and dried over Na_2SO_4. The chloroform solution was evaporated, and the product was purified by silica gel chromatography with a gradient of methanol in chloroform (0−5%) containing triethylamine (0.1%) to prevent degradation of tritylated product. Yield of **18**: 2.26 g, 71%. ^{1}H NMR (DMSO-d_6): δ 8.78 (s, 1, H6); 8.12 (s, 1, H2); 7.36−6.77 (m, 13, Ar); 6.02 (d, 1, H1′); 5.34 (d, 1, OH); 4.36 (m, 2, H2′ and H3′); 4.06 (m, 1, H4′); 3.70 (s, 6, OCH_3); 3.50 (s, 3, NCH_3); 3.30 (s, 3, OCH_3). FAB MS: 639 (MH^+).

5′-(Dimethoxytrityl)-5,2′-*O*-dimethyl-9-oxo-3-(β-D-ribofuranosyl)-1,2,4-triazolo[2,3-*a*]purine-3′-*O*-phosphoramidite (20). Purified acetonitrile (18 mL) and *O*-(β-cyanoethyl) *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (0.56 mL, 1.70 mmol) were added to **18** (1 g, 1.56 mmol) and dried diisopropylammonium tetrazolide (133 mg, 0.78 mmol). The reaction mixture was stirred at room temperature for 18 h at which time TLC in 5% MeOH/$CHCl_3$ indicated complete disappearance of **18**. The reaction mixture was diluted with saturated aqueous $NaHCO_3$ and extracted with dichloromethane (2 × 70 mL). The dichloromethane extract was washed with brine (3 × 20 mL), dried over Na_2SO_4, and evaporated to a foam. The product was purified by flash chromatography using $CHCl_3$/CH_3CN (8:2) as eluent to give **20** (1.05 g, 80%). ^{31}P NMR: δ 151.229 and 151.547.

5′-(Dimethoxytrityl)-5,2′-*O*-dimethyl-3-(β-D-ribofuranosyl)-1,2,4-triazolo[2,3-*a*]purine-9-thione (19). Compound **17** (1.4 g, 3.97 mmol) was dried by coevaporation with pyridine (2 × 15 mL) and was suspended in pyridine (15 mL). Dimethoxytrityl chloride (2.16 g, 6.4 mmol) was added to the above suspension, and the reaction mixture was stirred at room temperature. After 8 h, the suspension turned to a clear solution and the TLC in $CHCl_3$/MeOH (9:1) indicated that all starting material had been consumed. Methanol (3.7 mL) was added to quench the reaction. The solvent was evaporated in vacuo and the residue dissolved in $CHCl_3$ (35 mL). The chloroform solution was washed with 5% aqueous $NaHCO_3$ (2 × 15 mL) and brine (15 mL) and dried over Na_2SO_4. The dried chloroform solution was evaporated, and the product was purified by silica gel column chromatography with a gradient of methanol in chloroform (0−3%) containing triethylamine (0.1%). Yield of **19**: 2.24 g, 86%. ^{1}H NMR: δ 9.01 (s, 1, H6); 8.33 (s, 1, H2); 7.37−6.77 (m, 13, Ar); 6.06 (d, 1, H1′); 5.36 (d, 1, OH); 4.38 (m, 2, H2′ and H3′); 4.08 (m, 1, H4′); 3.70 (s, 6, OCH_3); 3.58 (s, 3, NCH_3); 3.30 (s, 3, OCH_3). FAB MS: 655 (MH^+).

5′-(Dimethoxytrityl)-5,2′-*O*-dimethyl-3-(β-D-ribofuranosyl)-9-thio-1,2,4-triazolo[2,3-*a*]purine-3′-*O*-phosphoramidite-9-thione (21). Purified acetonitrile (27 mL) and *O*-(β-cyanoethyl) *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (0.84 mL) were added to **19** (1.5 g, 2.29 mmol) and dry diisopropylammonium tetrazolide (195 mg). The reaction mixture was stirred at room temperature for 18 h at which time TLC in $CHCl_3$/MeOH (95:5) indicated that the starting material had been consumed. The reaction mixture was diluted with saturated aqueous $NaHCO_3$ and extracted with dichloromethane (2 × 100 mL). The dichloromethane extract was washed with brine (3 × 25 mL) and dried over Na_2SO_4, and the dried solution was evaporated to foam. The product was then purified by flash chromatography using $CHCl_3$/CH_3CN (9:1) as eluent to give **21** (1.52 g, 78%). ^{31}P NMR: δ 151.542 and 151.375.

TPR 32-mer 5a. TPR 32-mer was synthesized from 5′-*O*-DMTr-3′-*O*-phosphoramidite (**20**) on a 1-mmol scale synthesis using an automated DNA synthesizer to afford 10.26 mg of crude **5a**. The purification was performed by gel filtration using a Sephadex 50-80 column and eluting with deionized and sterilized water. Pure fractions as judged by SAX HPLC were pooled and freeze-dried to give 6.35 mg of material **5a**. UV: ϵ_{max} 6800 at 268. MALDI mass spectrum: calcd 12 986, obsd 2 993. Electrospray MS: calcd 12 986, obsd 12 985.

TTPR 32-mer 5b. TTPR 32-mer was synthesized from 5′-*O*-DMTr-3′-*O*-phosphoramidite (**21**) on a 1-mmol scale using an automated DNA synthesizer to afford 7.32 g of crude. The product was purified by gel filtration using a Sephadex 50-80 column to give 4.76 mg of pure TTPR 32-mer **5b**. The purity of the compound was checked by SAX HPLC using the same system as described for **5a**. UV: ϵ_{max} ~6000 at 328. MALDI mass spectrum: calcd 13 500, obsd 13 517.

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