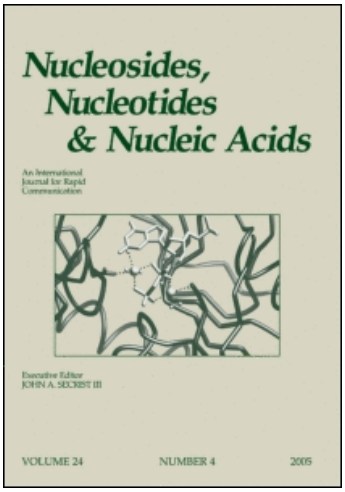

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THE ROLE OF HYDROPHOBIC VERSUS HYDROPHILIC BASE CHARACTER IN THE ANTI-HIV ACTIVITY OF PURINE-CONTAINING POLYRIBONUCLEOTIDES

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ABSTRACT

In contrast to the highly amphiphilic poly(1-methyl-6-thioinosinic acid), a potent anti-HIV agent, poly(1-amino-6-thioinosinic acid) (PATI) lacks the unique melting behavior characteristic of the amphiphilic polymers and is completely devoid of anti-HIV activity. This is consistent with the hypothesis that amphiphilic character and the ability to form an ordered secondary structure in solution are prerequisites for potent anti-HIV activity of single-stranded polynucleotides.

INTRODUCTION

Human acquired immunodeficiency syndrome (AIDS) has become a major pandemic. The World Health Organization currently estimates there are about 16.9 million human immunodeficiency virus (HIV)-infected adults worldwide. The Centers for Disease Control in the United States estimate that a half million AIDS cases have occurred in the United States since the disease was recognized, and some 70,000 new cases were reported in the United States in 1994-1995, the last year for which full data are available.[1]

Many compounds of diverse structure and mechanism of action are in preclinical study or clinical trial. The five approved dideoxynucleoside analogs, AZT (3'-azido-3'-deoxythymidine), ddI (2',3'-dideoxyinosine), ddC (2',3'-dideoxycytidine), d4T (2',3'-didehydro-2'-3'-dideoxythymidine), and 3TC (2',3'-dideoxy-3'-thiacytidine), which act primarily as inhibitors of reverse transcriptase, have recently been joined by several protease inhibitors of which three, indinivar, ritonavir, and saquinavir have now been approved by the FDA for use in the United States.

Combination chemotherapy of AIDS is currently receiving great attention as a possible means of enhancing efficacy, decreasing resistance development and reducing toxicity.[2]

Numerous presentations at the recent XIth International Conference on AIDS in Vancouver revealed striking results using combinations of reverse transcriptase and protease inhibitors in reducing viral burden in patients.[3] However, it is much too early to call these dramatic results a cure, and one must always keep in mind the possibility of multiple drug resistance which may develop with long-term therapeutic application.[4] The topic of antiviral therapy for human immunodeficiency virus infections has been thoroughly reviewed by DeClercq.[5]

This laboratory has been involved for several years in attempting to design and synthesize novel polynucleotides having anti-HIV properties. Nucleic acid-based antiviral compounds may be divided into three classes: (a) "gene therapy" which includes antisense oligodeoxynucleotides, ribozymes, immunogens, and others,[6] (b) inducers of interferon and activators of the 2,5-A system such as ampligen, poly(I)·(C_{12}U),[7] and poly(ICLC),[8] and (c) "antitemplates" or oligo and polynucleotides inhibiting replication of HIV by substituting for the normal template in the reverse transcriptase (RT) binding cleft.[9,10] It is the last of these categories that has been the primary focus of attention in this laboratory, since the demonstration by Chan that poly(1-methyl-6-thioinosinic acid), PMTI, was a potent inhibitor of RT and viral replication from a variety of retroviruses.[11]

Earlier demonstrations show that poly(1-methyl-6-thioguanylic acid) (**1**, PMTG) and poly(1-methyl-6-thioinosinic acid) (**2**, PMTI) (Fig.1) were potent inhibitors of HIV replication and cytopathicity and acted in synergy with a variety of other reverse transcriptase inhibitors.[10] These findings require further exploration of the parameters which are responsible for such activity. It has been the working hypothesis that amphiphilic character (hydrophobic base/ hydrophilic backbone) and the ability to form a highly ordered, non-hydrogen bonded array in solution are prerequisites for antiviral activity. The present report describes the synthesis, characterization and biological evaluation of a polynucleotide designed to provide a further test of that hypothesis.

SYNTHESIS AND CHARACTERIZATION

The synthesis (Scheme 1) of 1-amino-6-thioinosine (**6**) proceeded smoothly from adenosine (**4**) as previously described.[12] It was originally hoped that the Yoshikawa procedure[13] would be suitable for direct phosphorylation of **6** because the highly acidic medium in which the reaction is run might protect the N-amino group by protonation. However, phosphorylation was found to proceed on the amino group under Yoshikawa conditions. Protection of the amino group with the dimethylaminomethylene function[14] followed by 5'-pyrophosphate synthesis according to Poulter[15] proceeded smoothly to give compound **9**.

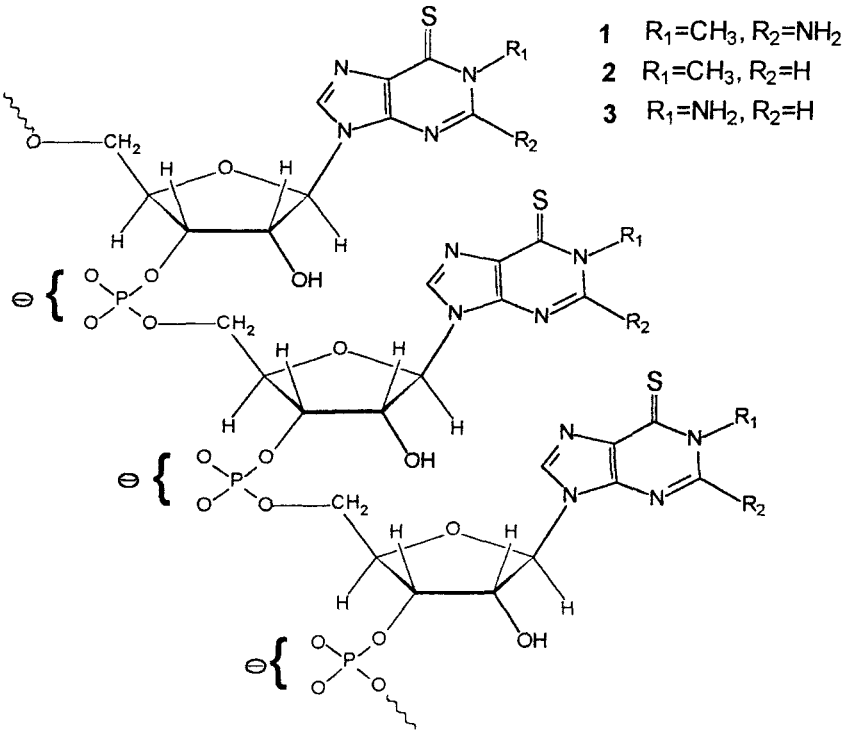
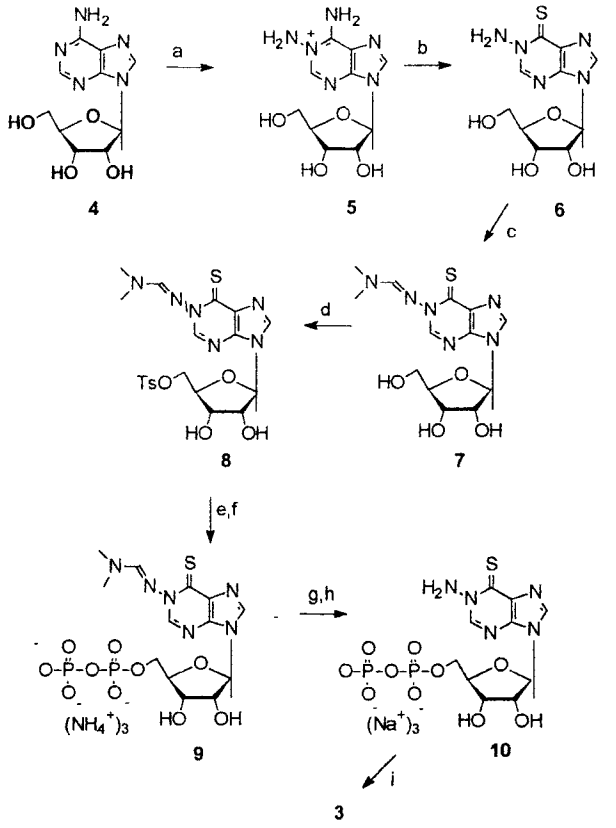


Figure 1

The protected nucleoside diphosphate **9** was not a substrate for polynucleotide phosphorylase under a variety of conditions. Therefore, the dimethylaminomethylene group was removed and the resulting diphosphate converted to the sodium salt (Na^+ is the monovalent cation of choice in carrying out polynucleotide phosphorylase-catalyzed polymerizations). Compound **10** was smoothly polymerized to provide poly(1-amino-6-thioinosinic acid), PATI, in about 20% isolated yield after removal of protein and exhaustive dialysis. A technique previously described[10] was used to estimate molecular size. Size-exclusion HPLC estimation using a BioRad SEC-125 column provided an estimated average molecular weight of 40,000 to 50,000. As is always the case with polynucleotide phosphorylase-catalyzed enzymatic polymerizations, the product was relatively polydisperse.

PMTI demonstrated a remarkably cooperative melting transition with a very high degree of hyperchromicity and a melting temperature of about 12°C[10,16] (Fig. 2). Similarly, the circular dichroism spectra for PMTI demonstrated dramatic conformational changes over the temperature range of 5 to 25°C (Fig. 3A).



a. Dinitrophenylhydroxylamine, DMF, 37°,; b. H_2S, aq. pyridine; c. $(CH_3)_2NCH(OCH_3)_2$, DMF; d. TsCl/pyridine; e. $[(nBu_4)N]_3HP_2O_7$; f. Dowex AG 50W-X8 NH_4^+; g. 29% aq. ammonia; h. Dowex AG 50W X8 Na^+; i. polynucleotide phosphorylase

SCHEME 1

In sharp contrast, PATI showed no cooperative transition in either the UV (Fig. 2) or CD (Fig. 3B) compared to the marked transitions exhibited for PMTI (Fig. 2, 3A). Thus, it is clear that conversion of the hydrophobic 1-methyl-6-mercaptopurine base to the much more hydrophilic 1-amino-6-mercaptopurine base resulted in the loss of secondary structure across the accessible temperature range and lends support to the view that the unusual helical stacking interaction observed for PMTI results from hydrophobic stacking/water exclusion effects and not from features inherent in the purine-6-thione moiety.

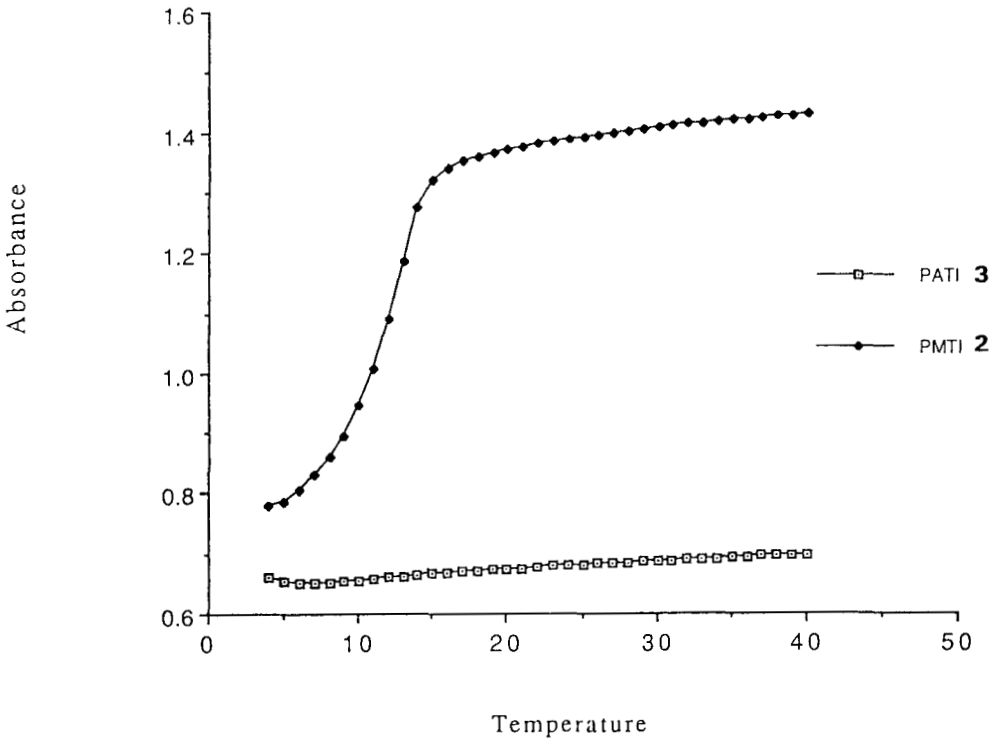


Figure 2. **T_m curves for PMTI and PATI in 0.005 M Mops buffer (pH 7.0).**

As had been previously observed with PMTI and PMTG,[10] enzymatic digestion of the PATI using a mixture of venom phosphodiesterase and alkaline phosphatase gave rise to 1-amino-6-thioinosine as the sole nucleoside product, thus attesting to the homogeneity of the polyribonucleotide.

BIOLOGICAL RESULTS

PATI was evaluated as an inhibitor of HIV-1-induced cytopathicity in CEM-SS cells as previously described.[10] It was completely devoid of antiviral activity or cytotoxicity at the highest dose tested (100 μg/mL, $\sim$2 μM). This lack of activity and cytotoxicity is consistent with, but does not prove, the hypothesis that the ability to form a secondary structure, even with melting temperatures below 37°C, is an important component of the presumed anti-template activity of these compounds in the inhibition of retroviral reverse transcriptase. Additional structure-activity relationship studies required to further substantiate the hypothesis are in progress.

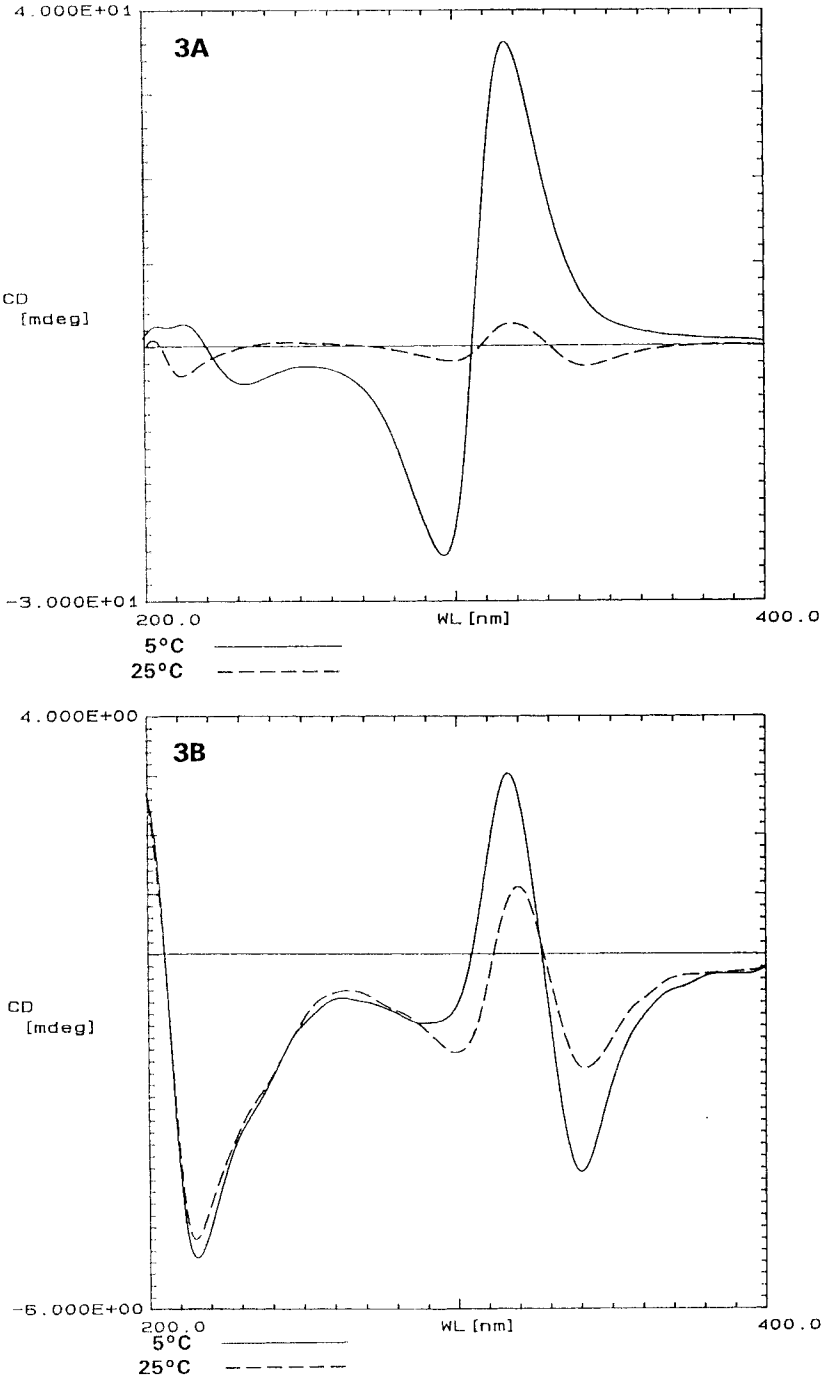


Figure 3. CD spectra of PMTI (A) and PATI (B) at 5°C (———) and 25°C (- - - -) at a concentration of 40 mg/L (~0.1 mM in nucleotide units).

EXPERIMENTAL

^1H and ^{31}P-NMR spectra were recorded with an IBM AF200 MHz FT-NMR 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. Mass spectra were recorded with a MAT 95 spectrometer. Whatman plates, precoated with silica gel 60 containing fluorescent indicator F_{254}, were used for thin-layer chromatography, and silica gel 60 (Mallinckroot SilicAR, 60-200 mesh) was employed for column chromatography.

HPLC experiments were carried out on a Hitachi L6200 pump equipped with an L3000 photo diode array. Reverse-phase chromatography utilized Rainin Microsorb-MV C8 and C18 columns. 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 carried out using *M. luteus* polynucleotide phosphorylase obtained from Midland Certified Reagent Company in Midland, Texas. The enzyme was provided in frozen solution containing 380 IU/mL.

1-N-[(Dimethylamino)methylene]-6-thioinosine (2). A suspension of 1-amino-6-thioinosine (4 g, 10.8 mmoles) in 40 mL of dimethylformamide was shaken with 9.2 mL of dimethylformamide dimethyl acetal at room temperature overnight. The resulting solution was evaporated *in vacuo* (oil pump). The residual syrup was diluted with 100 mL of H_2O, and the solution was freeze dried to give 3.67 g of a yellow tacky solid (96%).

The compound was used for the next step without purification. Material for HRMS analysis was obtained by reverse-phase HPLC using a C8 column eluted with H_2O/40% aqueous CH_3CN 90:10. FABMS (glycerol), m/z 355 (MH^+); ^1H-NMR (DMSO-d_6): 8.54 (1H, s, H2), 8.50 (1H, s, H8); 7.97 (1H, s, N=CH); 5.87, 5.85 (1H, d, H1'); 5.56, 5.53 (1 H, d, OH); 5.25, 5.22 (1H, d, OH); 5.07 (1H, t, OH); 4.48, 4.46 (1H, dd, H2'); 4.13, 4.11 (1H, dd, H3'); 3.94, 3.92 (1H, q, H4'); 3.62 (1H, m, H5'); 3.56 (1H, m, H5"); 3.38 (3H, s, $N(CH_3)_2$). HRMS of MH^+ Calcd. 355.11885. Obsv. 355.12249.

1-N-[(Dimethylamino)methylene]-5'-O-tosyl-6-thioinosine (8). The dry solid **2** (3 g, 8.4 mmoles) was dissolved in 10 mL of dry pyridine and cooled at 0°C using an ice water bath; added drop-wise was a solution of tosyl chloride (1.9 g, 10 mmoles) in 10 mL of dry pyridine over 20 min. The reaction mixture was stirred at 0°C for 16 h at which time TLC in $CHCl_3/CH_3OH$ (9:1) indicated a complete disappearance of starting material.

The reaction was terminated by adding a piece of ice. The reaction mixture was then poured into 100 mL of ice water and extracted three times with 100 mL of chloroform. The

chloroform extracts were washed three times with saturated aqueous sodium bicarbonate, once with 50 mL of H_20, dried over sodium sulfate and evaporated to dryness. The compound was purified by silica gel column chromatography eluted with H_2O/CH_3CN (97:3). The fractions judged pure by TLC were pooled and evaporated to yield 1.09 g of 5'-O-tosyl compound **8** (30%).

A sample for HRMS analysis was obtained by a reverse-phase HPLC, C18 column and eluted with H_2O/CH_3CN 70:30. FABMS (glycerol), m/z 509 (MH^+); ^{1}H-NMR (DMSO-d_6): 8.45 (1H, s, H2); 8.31 (1H, s, H8); 7.97 (1H, s, N=CH); 7.73 (2H, d, phenyl H); 7.37 (2H, d, phenyl H); 5.85, 5.82 (1H, d, H1'); 5.68, 5.65 (1H, d, 2'-OH); 5.48, 5.45 (1H, d, 3'-OH); 4.51, 4.49 (1H, dd, H2'); 4.27 (1H, t, H3'); 4.13 (1H, m, H5'); 4.16 (1H, m, H5"); 3.00 (3H, s, $N(CH_3)_2$); 2.97 (3H, s, $N(CH_3)$. HRMS of MH^+ Calcd. 509.12770. Obsv. 509.12615.

1-N-[(Dimethylamino)methylene]-6-thioinosine-5'-diphosphate (9). The nucleoside tosylate **8** (1.0 g) was dissolved in dry acetonitrile (2 mL) and tris(tetra-n-butylammonium)-hydrogen pyrophosphate (2.22 g) was added to the stirred solution. Progress of the reaction was monitored by HPLC using a SAX column with a gradient elution of 0.006 M KH_2PO_4 buffer (pH 4.0) to 0.6 M KH_2PO_4 buffer (pH 5.0). Upon completion ($\sim$90 h), the reaction mixture was diluted with 10 mL of H_2O and the tetra-n-butylammonium cation was exchanged for ammonium by passing the solution through a Dowex AG50W-X8 column (100-200 mesh) and eluting with deionized water. The eluant was lyophilized, and the resultant solid was dissolved in acetonitrile/ammonium bicarbonate buffer (100 mM) 10:1. The resultant solution was purified by elution on a CF-11 cellulose column first with an acetonitrile/ammonium bicarbonate buffer (100 mM) 10:1, then the same system with a 10:3 ratio.

The fractions judged pure by HPLC and cellulose TLC were pooled and neutralized with CO_2, concentrated, and lyophilized to give 679 mg ammonium salt **9** (67%). FABMS (glycerol), m/z 513 (M-3NH_3-H); ^{1}H-NMR (D_2O): 8.50 (1H, s, H2); 8.45 (1H, s, H8); 6.03, 6.00 (1H, d, H1'); 4.64 (1H, m, H2'); 4.47 (1H, t. H3'); 4.26 (1H, brs, H4'); 4.09 (2H, m., H5', 5H"); 2.97 (3H, s, $N(CH_3)_2$); 2.94, (3H, s, $N(CH_3)_2$. ^{31}P-NMR (D_2O): -7.76 (d, J_{pp}=21.1 Hz); -10.5 (d, J_{pp}=21.1 Hz).

1-Amino-6-thioinosine-5'-diphosphate (10). The protected nucleotide **9** (670 mg) was dissolved in 15 mL of concentrated aqueous ammonia (30%) and the solution was stirred at room temperature. After 71 h, the reaction was completed (checked by HPLC and UV). The reaction mixture was then diluted with 50 mL of H_2O, and the resulting solution was neutralized with CO_2 and freeze dried.

The deprotected nucleotide was purified by linear gradient elution from a DEAE Sephadex anion exchange resin (HCO_3^- form) column with ammonium bicarbonate buffer (0.05-0.5 M), pH 7.8. The pure fractions as judged by SAX HPLC were pooled, neutralized with CO_2 and lyophilized. The ammonium cation was exchanged for sodium by dissolving the solid in H_2O and passing the solution through a Dowex AG50W-X8 column (100-200 mesh) and eluted with deionized water. The eluant was lyophilized to yield 297 mg of deprotected diphosphate **10** (48%). FABMS (glycerol), m/z 458 (M-1); ^{1}H-NMR (D_2O): 8.72 (1H, s, H2); 8.60 (1H, s, H8); 6.12, 6.09 (1H, d, H1'); 4.62 (1H, t, H3'); 4.33 (1H, m, H4'); 4.16 (2H, m, H5', H5"). ^{31}P-NMR (D_2O): -9.38 (d, Jpp=20.5 Hz), -10.77 (d, Jpp=20.5 Hz).

Poly(1-amino-6-thioinosinic acid) (3). A solution was made from:

Tris.HCl (pH 9.0; 2 M)	2.09 mL
$MgCl_2$ (0.1 M)	2.09 mL
2-Mercaptoethanol (0.28 M)	2.09 mL
H_2O	6.03 mL
PNPase (*M. luteus*)	9.6 units/mg (in 7.5 mL buffer containing 0.02 M Tris.HCl, 0.1 M NaCl, 0.001 M 2-mercaptoethanol pH 8.0)
10	250 mg

The solution was incubated for 25 h at 37°C. After incubation, the reaction mixture was diluted with H_2O and the pH was adjusted to approximately 7.25 with very dilute aqueous HCl. The solution was then extracted nine times with 50 mL of chloroform/isoamyl alcohol (5:2) to give a clear aqueous solution. The aqueous layer was dialyzed against 0.1 M NaCl for 24 h and then H_2O for 48 h. Lyophilization of the aqueous solution afforded PATI (**3**) (49.9 mg, 20%) as a fluffy solid, UVλ_{max} 318 nm, ϵ_{max} 13,669 (0.1 N NaCl), s_{20w} ~6.0. HPLC analysis using a size-exclusion column (BioRad SEC 125), isocratically eluted with an aqueous solution containing NaH_2PO_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: 7.85 min.

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