JOC The Journal of Organic Chemistry

Article

pubs.acs.org/joc

Nucleoside-(5′→P) Methylenebisphosphonodithioate Analogues: Synthesis and Chemical Properties

Diana Meltzer,[†,∥] Yael Nadel,[†,∥] Joanna Lecka,[‡,§] Aviran Amir,[†] Jean Sévigny,[‡,§] and Bilha Fischer*[,†]

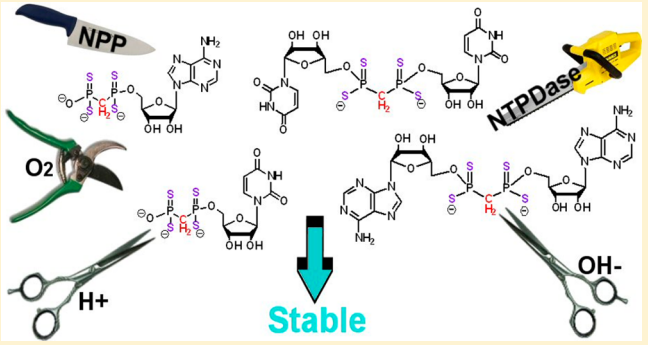
[†]Department of Chemistry, Bar-Ilan University, Ramat-Gan 52900, Israel

[‡]Département de microbiologie-infectiologie et d'immunologie, Faculté de Médecine, Université Laval, Quebec City, Québec, Canada

[§]Centre de recherche du CHU de Québec, Quebec City, Québec, Canada

Supporting Information

ABSTRACT: Nucleoside-(5′→P) methylenebisphosphonodithioate analogues are bioisosteres of natural nucleotides. The potential therapeutic applications of these analogues are limited by their relative instability. With a view toward improving their chemical and metabolic stability as well as their affinity toward zinc ions, we developed a novel nucleotide scaffold, nucleoside-5′-tetrathiobisphosphonate. We synthesized P1-(uridine/adenosine-5′)-methylenebisphosphonodithioate, **2** and **3**, and P1,P2-di(uridine/adenosine-5′)-methylenebisphosphonodithioate, **4** and **5**. Using ^{1}H and ^{31}P NMR-monitored Zn^{2+}/Mg^{2+} titrations, we found that **5** coordinated Zn^{2+} by both N7 nitrogen atoms and both dithiophosphonate moieties, whereas **3** coordinated Zn^{2+} by an N7 nitrogen atom and P_{β}. Both **3** and **5** did not coordinate Mg^{2+} ions. ^{31}P NMR-monitored kinetic studies showed that **3** was more stable at pD 1.5 than **5**, with $t_{1/2}$ of 44 versus 9 h, respectively, and at pD 11 both showed no degradation for at least 2 weeks. However, **5** was more stable than **3** under an air-oxidizing atmosphere, with $t_{1/2}$ of at least 3 days versus 14 h, respectively. Analogues **3** and **5** were highly stable to NPP1,3 and NTPDase1,2,3,8 hydrolysis (0−7%). However, they were found to be poor ectonucleotidase inhibitors. Although **3** and **5** did not prove to be effective inhibitors of zinc-containing NPP1/3, which is involved in the pathology of osteoarthritis and diabetes, they may be promising zinc chelators for the treatment of other health disorders involving an excess of zinc ions.



1. INTRODUCTION

Nucleoside-(5′→P) methylenebisphosphonodithioate analogues, **1**, are bioisosters of natural nucleotides. The sulfur modification introduces important physicochemical properties resulting from the different steric requirements of the sulfur atom (P−S vs P−O bond length), the different affinity toward metal ions ("soft" sulfur vs "hard" oxygen), and the unsymmetrical negative-charge distribution.[1] Since their discovery, nucleoside-*O*-phosphorothioates[2,3] as well as their cyclic[4,5] and oligomeric[6,7] derivatives have become an increasingly important class of nucleotide analogues, possessing potentially useful biological activities.[8] For instance, phosphorothioate analogues of oligonucleotides have a potential use in antisense therapy and were also suggested for use as facilitators in the delivery of biologically active siRNA to mammalian cells.[9−12] Indeed, a phosphorothioate antisense agent has been approved for clinical use to treat cytomegalovirus-induced retinitis.[13] In addition, a second-generation antisense oligonucleotide was approved for the treatment of homozygous familial hypercholesterolemia.[14]

In addition, nucleoside-(5′→P) methylenebisphosphonodithioate analogues were suggested as potential biocompatible metal-ion chelators.[15] We recently found that a terminal thiophosphate moiety enhances the stability of a nucleotide−Zn^{2+} complex. Thus, ADP-β-S and GDP-β-S complexes with Zn^{2+} were ∼0.7 log units more stable than the parent nucleotide complexes.[16]

Furthermore, we have recently shown that the replacement of four oxygen atoms in methylenediphosphonic acid (MDP) with sulfur atoms increased the stability constant of a MDP complex with Zn(II) by ∼1.7 log units (log K = 10.84),[17] implying that nucleotide analogues such as **2**−**5**, bearing the methylenediphosphonotetrathioate (MDPT) moiety, could be good Zn^{2+} chelators as well. Therefore, nucleoside 5′-tetrathiobisphosphonate analogues may be potential therapeutic agents for various health disorders involving an excess of Zn^{2+} ions. Indeed, chelators with high affinity to borderline or soft metal-ions have been suggested as a treatment for neurodegenerative diseases, such as Alzheimer's and Wilson's disease, triggered by an excess of Cu(II) or Zn(II).[18]

Another potential application of compounds **2**−**5** may be the treatment of bone diseases[19] by the inhibition of overexpressed nucleotide pyrophosphatase/phosphodiesterase-1 (NPP1).

Received: May 2, 2013
Published: July 29, 2013

   dx.doi.org/10.1021/jo400931n | J. Org. Chem. 2013, 78, 8320−8329

Overexpression of NPP1 results in the overproduction of extracellular pyrophosphate from extracellular ATP and the deposition of the pathological mineral calcium pyrophosphate dihydrate in cartilage. The catalytic site of NPP1 contains Zn^{2+} ions that may be chelated by nucleoside-(5′→P) methylenebisphosphonodithioate better than the endogenous substrate, ATP.[20] In addition, inhibition of NPP1 may be a potential treatment for type 2 diabetes, where the insulin receptor was found to be damaged because of overexpression of NPP1.[21−25]

Nucleoside phosphonodithioate analogues undergo hydrolysis under mild acidic conditions,[26] whereas the bisphosphonate structure provides enhanced chemical stability as well as metabolic stability.[27] Hence, here we report on the introduction of a methylenebisphosphonodithioate moiety into nucleotide-based compounds. We synthesized both mono- and diadenine or uracil nucleotide analogues bearing this unit, compounds **2**−**5**. Because the rate of the substitution of water oxygen for sulfur in thiomonophosphate anions such as $PO_2S_2^{3-}$, POS_3^{3-}, and PS_4^{3-} is remarkably fast,[28] we evaluated the stability of analogues **3** and **5** under acidic or basic conditions as well as to air oxidation. In addition, we explored the affinity of the new analogues to zinc ions and characterized the Zn^{2+} coordination sites of compounds **3** and **5** as well as their selectivity to Zn^{2+} versus Mg^{2+}. Furthermore, we evaluated the resistance of **3** and **5** to enzymatic hydrolysis by NPP1,3 and nucleoside triphosphate diphosphohydrolase-1 (NTPDase1), -2, -3, and -8 as well as the NPP and NTPDase inhibition potential of these analogues.

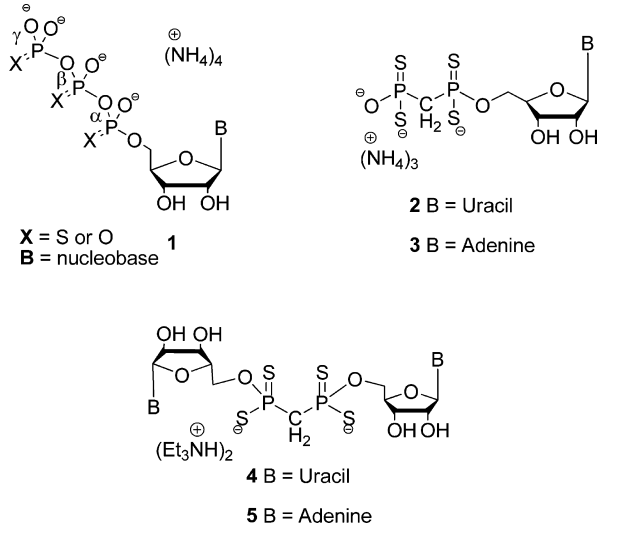


Figure 1. Nucleoside-(5′→P) methylenebisphosphonodithioate analogues.

2. RESULTS AND DISCUSSION

2.1. Synthesis. To synthesize analogues **2**−**5**, we applied methylene-bis(1,3,2-dithiaphospholane-2-sulfide), **6**, of which the preparation was recently reported.[29] Compound **6** was prepared from bis-methylene(phosphonicdichloride) that was treated with 1,2-ethanedithiol and 10 mol % $AlCl_3$ in $CHBr_3$. Recently, we have shown that primary alcohols react successfully with compound **6** to yield *O*,*O*′-diester-methylenediphosphonotetrathioate analogues in good yields.[29] Here, on the basis of this finding, we synthesized mono- and dinucleotide derivatives of methylenediphosphonotetrathioic acid (MDPT), as shown in Schemes 1 and 2. Compounds **9** and **10** were obtained from **7** and **8**, respectively, in a one-pot reaction. First, 2′,3′-methoxymethylidene uridine **7** (or 2′,3′-methoxymethylidene adenosine **8**) was treated with **6** in the presence of molecular sieves in DCM for 24 h. Then, 1 equiv of DBU in DCM was added dropwise over 1 h. The reaction progress was monitored by ^{31}P NMR. Doublets at 100.1 and 90.4 ppm indicated the immediate formation of intermediate **9** (or **10**), which was also indicated by the cloudy reaction mixture turning immediately clear. Without isolating the product, 3-hydroxypropionitrile (6 equiv) and DBU (1 equiv) were added to the reaction flask at 45 °C. ^{31}P NMR indicated the formation of **11** (showing doublets at 104.8 and 104.5 ppm). The workup of the reaction included filtration of the molecular sieves and evaporation of the solvent. This one-pot synthesis is highly moisture sensitive; thus, the use of molecular sieves in this process is necessary. Steps a and b were performed in one pot because attempts to isolate products **9** and **10** on a reverse-phase column resulted in hydrolytic ring-opening of the thiophospholane ring, as indicated by MS analysis and ^{31}P NMR (signals at 105 and 67 ppm).

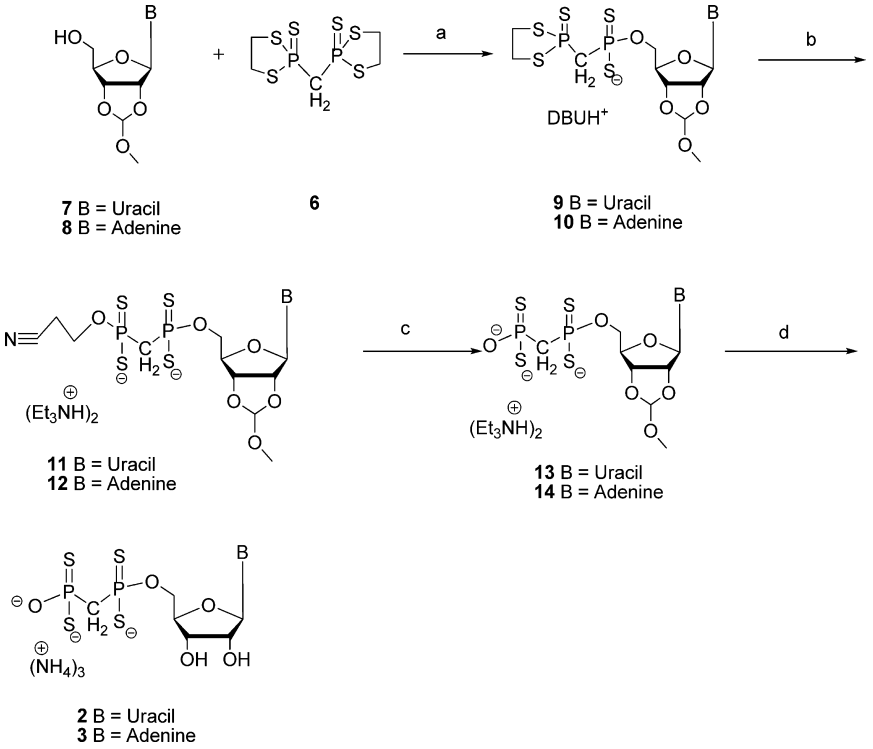
Products **11** and **12** were separated on a silica gel column, applying $CHCl_3$/MeOH (85:15) as the eluent. Further purification was performed by medium-pressure chromatography on a reverse-phase column using 1 M triethylammonium acetate buffer (TEAA) (pH 7)/CH_3CN (78:22) as the eluent. Products **11** and **12** were obtained in 37 and 28% yields, respectively. Products **13** and **14** were obtained upon treatment with *t*-BuO$^-$K$^+$ in THF, resulting in *β*-elimination. The formed acrylonitrile was scavenged with ethanethiol as products **13** and **14** precipitated from the reaction mixture together with EtSK salt. The solvent was removed by decantation, and the solid residue was dissolved in water and titrated with 10% HCl followed by the addition of 24% NH_4OH for the removal of the methoxymethylidene protecting groups. The residue was subjected to Sephadex DEAE ion-exchange chromatography to yield desired products **2** and **3** at 45 and 55% yields, respectively (from **11** and **12**, respectively).

This new synthesis route was further applied to obtain corresponding dinucleotide derivatives **4** and **5**. Intermediates **15** and **16** were synthesized from the reactions of **6** with **7** and **8**, respectively. Because of the high moisture sensitivity of this reaction step, compounds **7** (or **8**) and **6** were stirred overnight together with molecular sieves in dry acetonitrile. Next, DBU (2.1 equiv) was added at 60 °C, and the completion of the reaction was monitored by ^{31}P NMR. After 2 h, desired intermediate **15** (or **16**) was obtained. The workup and the removal of the protecting group were performed as mentioned above for analogues **2** and **3**. The final purification of compounds **4** and **5** was carried out by HPLC on a reverse-phase column by applying 1 M TEAA (pH 7)/CH_3CN as the eluent. Analogues **4** and **5** were obtained at 30 and 36% yields, respectively.

2.2. NMR-Monitored Zn^{2+}/Mg^{2+} Titrations of Analogues 3 and 5: General Methods. As mentioned above, Zn^{2+} chelators are highly desirable as therapeutic agents for diseases in which pathology involves Zn^{2+}. Analogues **2**−**5**, bearing four sulfur atoms, are potential Zn^{2+} chelators on the basis of the thiophilic character of Zn^{2+}.[20] Yet, in a physiological medium there is an excess of alkali and alkali earth metal ions. For instance, the extracellular as well as intracellular concentration of free Mg^{2+} is between 0.5 and 1.3 mM.[30]

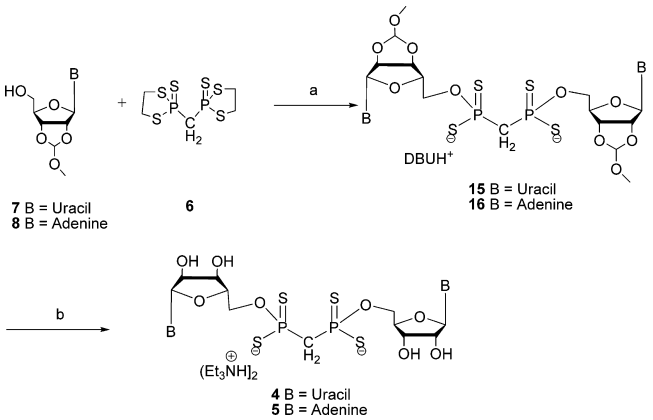
dx.doi.org/10.1021/jo400931n | J. Org. Chem. 2013, 78, 8320−8329

Scheme 1. Synthesis of P1-(Uridine/adenosine-5′)-methylenebisphosphonodithioate Analogues 2 and 3[a]



[a]Reagents and conditions: (a) DBU, DCM, 1 h; (b) DBU, 3-hydroxypropionitrile, 45 °C, 30 min; (c) ethanethiol/THF (v/v), potassium tert butoxide, rt, 2 h; (d) (1) 10% HCl, pH 2.3, rt, 3 h; (2) 24% NH_4OH, pH 9, rt, 45 min. Compounds **2** and **3** were obtained in 45 and 55% yield, respectively.

Scheme 2. Synthesis of P1,P2-Di(uridine/adenosine-5′)-methylenebisphosphonodithioate Analogues 4 and 5[a]



[a]Reagents and conditions: (a) DBU, CH_3CN, 60 °C, 2 h and (b) (1) 10% HCl, pH 2.3, rt, 3 h; (2) 24% NH_4OH, pH 9, rt, 45 min. Compounds **4** and **5** were obtained in 30 and 36% yields, respectively.

Therefore, chelators exhibiting high zinc-ion selectivity are required.[31] For this purpose, we performed a preliminary evaluation of the Zn^{2+} versus Mg^{2+} selectivity of analogues **3** and **5**.

To explore the Zn^{2+}/Mg^{2+} coordination by analogues **3** and **5**, we performed Zn^{2+}/Mg^{2+} titrations monitored by ^1H and ^{31}P NMR spectroscopy. NMR spectra indicating Zn^{2+} binding to analogues **3** and **5** are shown in Figures 2−5. We studied purine rather than pyrimidine nucleotides because of the presence of an imidazole moiety in the former, which preferably coordinates borderline Lewis acids such as Zn^{2+}.[32] The shift in the NMR signals as well as their line broadening indicates Zn^{2+} coordination to several atoms in analogues **3** and **5**.

Solutions of analogues **3** and **5** were titrated by 0.1−10 equiv of Zn^{2+}/Mg^{2+} and monitored by ^1H and ^{31}P NMR at 400 or 600 and 160 or 243 MHz, respectively. Relatively low nucleotide concentrations were used (2−5 mM) to avoid intermolecular base stacking. The titration was performed with 0.15−0.35 M Zn^{2+}/Mg^{2+} solutions in D_2O at pD 7.4 and 300 K. Chemical shifts (δ_H and δ_P) were measured at different Zn^{2+}/Mg^{2+} concentrations.

*2.2.1. NMR-Monitored Zn^{2+} Titrations of Analogues **3** and **5**.* The addition of 0.1 equiv of Zn^{2+} to compound **5** caused line broadening and an upfield shift of the dithiophosphonate signal (Figure 2). Line broadening is a result of the dynamic equilibrium between the free ligand (e.g., compound **5**) and the Zn^{2+}−ligand complex. The singlet at 103 ppm corresponds to the free ligand, and the emerging singlet at 100.5 ppm corresponds to the Zn^{2+}−ligand complex. After the addition of 0.5 equiv of Zn^{2+}, only one singlet at 100.5 ppm is observed. Line sharpening indicates that there is no free ligand, and all molecules of **5** are engaged in a Zn^{2+} complex. These results are consistent with the common tetrahedral geometry of zinc complexes in which two molecules of **5** form a complex with one zinc ion. The addition of up to 10 equiv of Zn^{2+} resulted in no change in the ^{31}P NMR spectrum.

Previously, we reported that the addition of 10 equiv of Zn^{2+} to $AP_3(\beta\text{-S})A$ resulted in the downfield Pβ chemical shift by ∼3 ppm, and the $\Delta\delta$ of Pα in $AP_3(\beta\text{-S})A$ was 0.67 ppm.[16] The minute shifts of the Pα and Pβ signals imply that the major coordination site in the phosphate chain of $AP_3(\beta\text{-S})A$ is Pβ. The 3 ppm shift of the compound **5** dithiophosphonate group

dx.doi.org/10.1021/jo400931n | J. Org. Chem. 2013, 78, 8320−8329

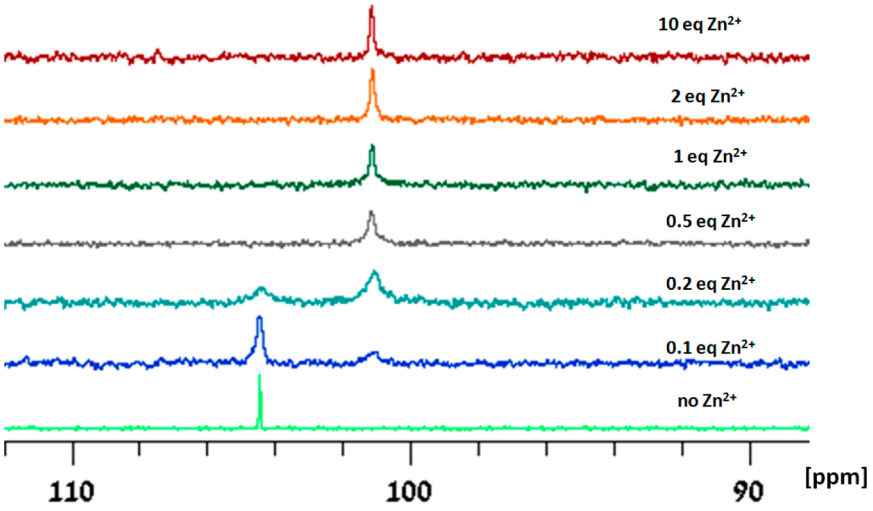


Figure 2. Titration of 3 mM **5** in D_2O at pD 7.38 with Zn^{2+}. ^{31}P NMR spectra were measured at 162 MHz and 300 K.

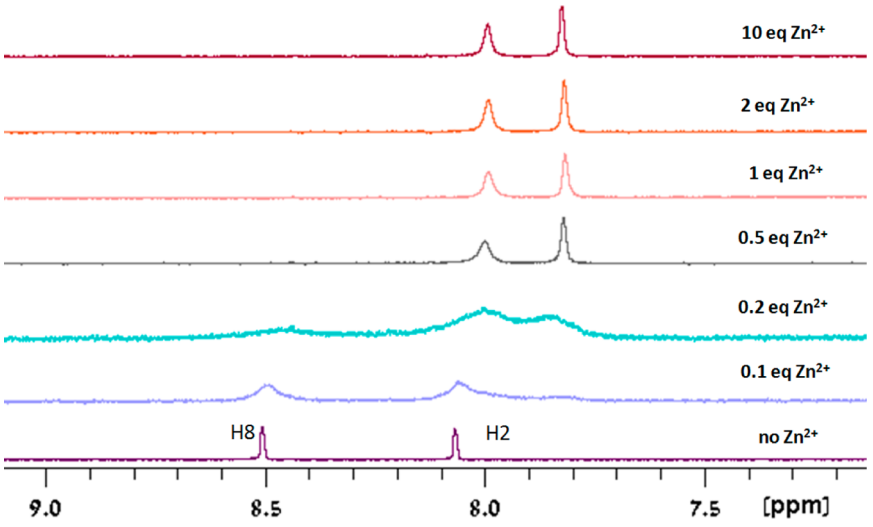


Figure 3. Titration of 3 mM **5** in D_2O at pD 7.38 with Zn^{2+}. ^{1}H NMR spectra were measured at 400 MHz and 300 K.

resulting from Zn^{2+} binding is consistent with that of Pβ of AP_3(β-S)A.

The ^{1}H NMR-monitored Zn^{2+} titration of compound **5** (Figure 3) exhibited similar characteristics to those of compound **3** (Figure S1). After the addition of only 0.1/0.2 equiv of Zn^{2+}, a shift in the signals and line broadening was evident for analogue **5**. H8 was shifted upfield by 0.5 ppm, whereas H2 was shifted by 0.2 ppm. The shift of the H8 signal implies that N7 is a coordination site of Zn^{2+}, whereas the small upfield shift of H2 in the presence of zinc ions possibly results from stacking interactions.[33]

Data from the ^{1}H NMR-monitored Zn^{2+} titrations of compound **3** show line broadening and upfield shifts of H2 and H8 (Figure S1). The upfield shift of the H8 signal by 0.3 ppm implies that N7 is a coordination site of Zn^{2+}, as was also found for **5**.

The addition of Zn^{2+} to a solution of compound **3** resulted in upfield shifts in both the ^{31}P and ^{1}H NMR spectra. Upon the addition of 0.2 equiv of Zn^{2+}, both the free **3** and Zn^{2+}–**3** complex were observed. When 0.5 equiv of Zn^{2+} were added, all of the **3** molecules were engaged in the zinc complex, and the P_β signal shifted 38 ppm upfield (Figure S2). The large shift of ~40 ppm indicates that the dithiophosphonate has a high affinity to Zn^{2+} and is involved in metal-ion binding, as was shown for terminal thiophosphate analogues ATP-γ-S, ADP-β-S, and GDP-β-S.[16]

*2.2.2. NMR-Monitored Mg^{2+} Titrations of Analogues **3** and **5**.* Unlike Zn^{2+} binding to analogues **3** and **5**, no shift in the signals was observed in both the ^{31}P NMR and ^{1}H NMR of compound **5** upon Mg^{2+} titration. The same observation was made also for compound **3**. The lack of shift in the NMR signals indicates no binding of magnesium ions, emphasizing the selectivity of methylenebisphosphonodithioate analogues for Zn^{2+} ions.

2.3. Evaluation of the Chemical Stability of Analogues 3 and 5. To evaluate the chemical stability of the newly synthesized nucleoside-(5′→P) methylenebisphosphonodithioate analogues, we measured the time-dependent reduction of the percentage of analogues **3** and **5** under basic and acidic conditions as well as due to air oxidation by ^{31}P NMR.

The evaluation of the stability of **3** at pD 1.5 was conducted by ^{31}P NMR for 4 days. In the course of the experiment, new signals emerged in the ^{31}P NMR spectra at 104.4, 92.3, 89.2, 86.1, 67.8 ppm (see the assignment of the signals below), and the percentage of the starting material was obtained from the ratio between the integration of the starting material and all

dx.doi.org/10.1021/jo400931n | J. Org. Chem. 2013, 78, 8320–8329

signals in the spectrum. Compound **3** was relatively stable under this drastic condition, with a calculated half-life of 44 h (Figure S3). Mass spectrum (ESI-QTOF negative) analysis of freeze-dried **3** after 4 days at pD 1.5 revealed the fragmentation products shown in Figure 4.

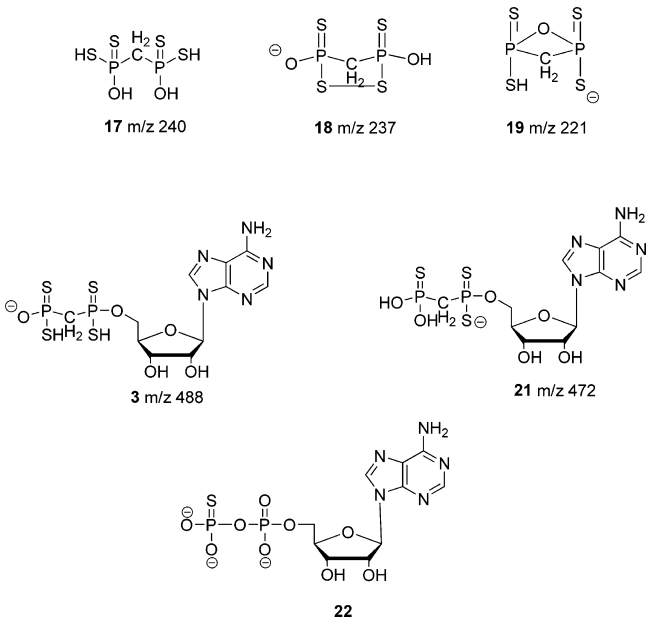


Figure 4. Mass spectrometric data for **3** subjected to hydrolysis at pD 1.5 for 4 days.

In the mass spectrum of an acidic mixture of **3**, we observed a signal for the M_w of **3**, m/z 488, and additional signals corresponding to hydrolysis products (Figure 4). The combination of the mass analysis with the ^{31}P NMR data for the above mixture revealed that the ^{31}P NMR signals at 104.4 and 67.8 ppm correspond to asymmetric hydrolysis product **21**, m/z 472. A molecular weight of 239 m/z and ^{31}P NMR shift at 92.3 ppm revealed the presence of MDPT **17**. In addition, the ^{31}P NMR signal at 86.1 ppm can be correlated with the formation of oxidized MDPT product **18** (237 m/z), in accordance with our previous findings.[17] The singlet at 89.2 ppm corresponds to compound **19**, formed by an intramolecular nucleophilic attack and the loss of a water molecule. Four-membered-ring heterocyclic compounds such as **19** were reported before, and the typical ^{31}P NMR signal at ∼90 ppm we found here for **19** is in accordance with previous findings.[34]

Compound **3** was found to be highly stable under basic conditions (pD 11). After two weeks, the ^{31}P NMR spectrum was identical to that of the starting material, without any indication of decomposition. This phenomenon is due to the repulsion between the negative charges of the tetrathiobisphosphonate moiety in compound **3** and OH^- ions.

Compound **3** exhibited limited stability under air-oxidizing conditions on the basis of ^{31}P NMR measurements of **3** in an open rotating NMR tube. The half-life of **3** under these conditions was 14 h (Figure S4). MS and ^{31}P NMR analysis of the freeze-dried sample of **3** after 3 days indicated the formation of an intramolecularly oxidized product. New asymmetric centers formed after the oxidation of **3** were observed as complex multiplets in the ^{31}P NMR spectrum (∼105 and ∼65 ppm). Apparently, compound **3** is prone to oxidation and was oxidized spontaneously without treatment with strong oxidatizing agents, as reported for guanosine 5′-*O*-(1,2-dithiotriphosphate) analogue **23**, which was oxidized with I_2 to give analogue **24** (Figure 5). The latter was formed by an intramolecular oxidation to yield a disulfide bond and the subsequent hydrolysis of $P_{\alpha,\beta}$ phosphodiester bond.[35] Intramolecular nucleophilic attack and the formation of a disulfide bond are less likely to occur under basic conditions at pD 11.

Dinucleotide analogue **5** exhibited a half-life of 9 h under pD 1.5. The combination of mass analysis and ^{31}P NMR data for **5** subjected to acidic media for 2 days revealed that **5** underwent decomposition, first to mononucleotide **3**. ^{31}P NMR showed two indicative doublets at 105 and 91 ppm (Figure 6) that correspond to the chemical shifts of product **3**. Then, MDPT **17** was formed, as indicated by a singlet at 92 ppm.

Analogue **5** was highly stable under air-oxidizing conditions in an open NMR tube for 3 days. No change in the amount of **5** was observed. The dinucleotide scaffold increased the resistance to oxidation and the formation of a disulfide bond, as compared to corresponding mononucleotide **3**.

At pD 11, **5** was completely stable even after 2 weeks. The stability of dinucleotide **5** was identical to that of mononucleotide **3** under these conditions.

Moreover, ^{1}H NMR indicated that the bridging methylene hydrogen atoms of analogue **5** are exchangeable because the methylene typical triplet signal had broadened and the integration of this peak decreased. The exchange of the hydrogen atoms with deuterium at pD 11 implies the acidity of the phosphonate methylene group.

2.4. Evaluation of the Hydrolysis of Analogues 3, 5, and 22 by Human Ectonucleotidases. Two major families of ectonucleotidases, NPPs and NTPDases, stop nucleotide signaling via P2X and P2Y receptors by nucleotide hydrolysis.[36,37]

Although ecto-NTPDases comprise a family of Ca^{2+}/Mg^{2+}-dependent ectonucleotidases (ecto-NTPDase 1−8) that hydrolyze the terminal phosphoroester bond of extracellular nucleotides[38] to nucleoside 5′-mono or diphosphate and Pi, NPPs are Zn^{2+}-dependent ecto-enzymes that hydrolyze extracellular nucleotide $P_{\alpha-\beta}$ bonds to nucleoside 5′-monophosphate and the remaining pyrophosphate (or phosphate).[19]

We expected analogues **2**−**5**, bearing four sulfur atoms, to make good chelators of the NPP1 catalytic Zn^{2+} ions.[20] Furthermore, the presence of an imidazole moiety in analogues

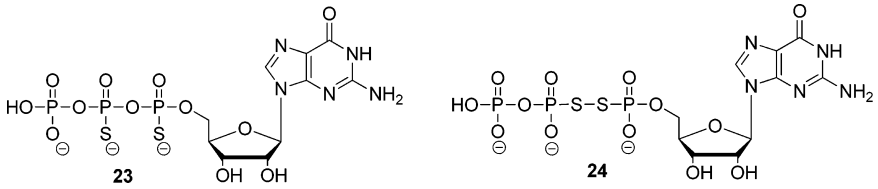


Figure 5. Guanosine 5′-*O*-(1,2-dithiotriphosphate) **23.** Analogue **24** formed from **23** upon I_2/H_2O treatment.

dx.doi.org/10.1021/jo400931n | J. Org. Chem. 2013, 78, 8320−8329

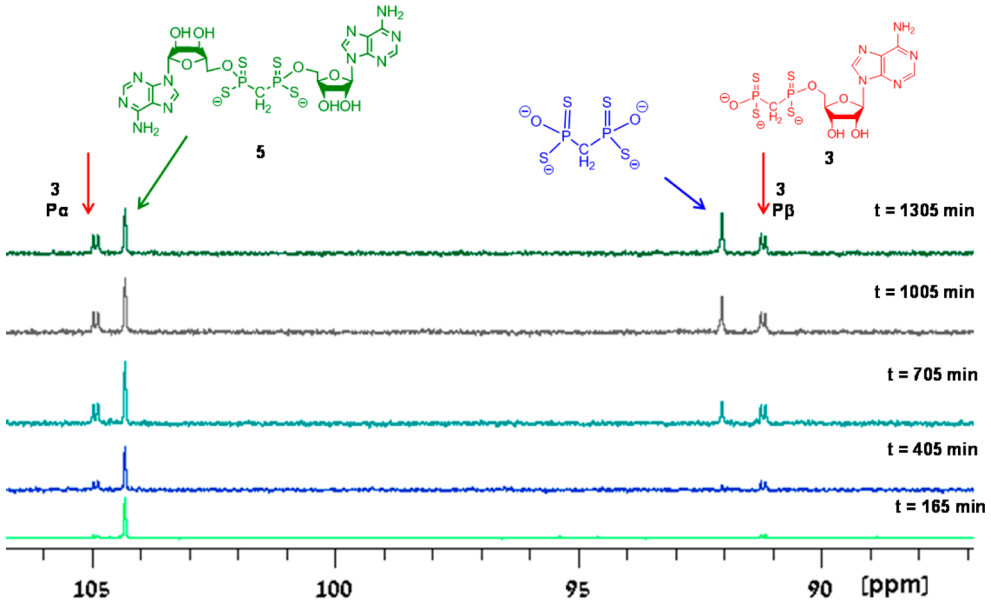


Figure 6. ^{31}P NMR spectra of **5** at pD 1.5. ^{31}P NMR spectra were measured at 243 MHz and 300 K.

3 and **5**, which coordinates Zn^{2+} ions preferentially, may also help to inhibit Zn^{2+}-dependent NPP1 activity.[32,39] Hence, we first studied the resistance of adenosine methylenebisphosphonodithioate analogues **3** and **5** to undergo hydrolysis by the ectonucleotidases. Because uracil nucleotides are hydrolyzed ca. at $^{1}/_{5}$ of the rate of adenine nucleotides,[40] we have not tested analogues **2** and **4**.

The resistance of compound **3** to hydrolysis by NPP1,3 and NTPDase1,2,3,8 was compared with ADP-*β*-S, **22**, to evaluate the effect of the methylene group and extra sulfur atoms on the metabolic stability.

Because members of the NPP family have an alkaline pH optimum,[39] the hydrolysis of analogues **3** and **5** was measured at pH 8.5. A human NPP1 or NPP3 preparation was added to the incubation buffer at 37 °C, and the reaction was started by the addition of a nucleotide analogue (**3**, **5**, and **22**) and terminated after 1 to 2 h by the addition of perchloric acid. The nucleotide hydrolysis products were separated and quantified by HPLC. The concentrations of the reactants and products were determined from the relative areas for their absorbance maxima peaks.

The 1 M pechloric acid that was used to terminate the enzymatic reaction may lead to the partial degradation of the nucleotide analogues. Therefore, the percentage of degradation for each analogue resulting from the acidic treatment was assessed in the absence of enzyme, and this value was subtracted from the percentage of analogue degradation in the presence of the enzyme.

NPP hydrolyzes nucleoside 5′-diphosphate or dinucleoside 5′,5″-diphosphate (e.g., **3**, **5**, **22**) to NMP and inorganic phosphate or nucleotide products. The identity of the degradation products was determined by comparing their retention times to those of relevant controls.

Over a 2 h period, compounds **3**, **5**, and **22** were not metabolized at all by NPP1, yet **22** was significantly hydrolyzed by NPP3 at 25% (Table 1).

Analogues **3**, **5**, and **22** modified with dithiophosphonate and thiophosphate groups, respectively, were relatively stable both toward NPP1,3 and NTPDases hydrolysis. Apparently, the terminal thiophosphate group in **22** and the bis-(dithiophosphonate) group in **3** and **5** conferred stability to the hydrolysis of the $P_{\alpha-\beta}$ phosphodiester bond.

Table 1. Hydrolysis of Nucleoside-5′-thiophosphate 22 and Bis(dithiophosphonate) Analogues 3 and 5 by Human Ectonucleotidases

	relative hydrolysis (% ± SD of ADP or AP_2A hydrolysis)[a]		
human ectonucleotidase	**3**	**5**	**22**
NPP1	ND	ND	ND
NPP3	ND	ND	25 ± 2.8
NTPDase1	1 ± 0.2	ND	ND
NTPDase2	1 ± 0.2	ND	ND
NTPDase3	ND	2 ± 0.1	2 ± 0.1
NTPDase8	7 ± 1.0	3 ± 0.1	14 ± 1.0

[a]ND = not detected. ADP and AP_2A were used as the 100% hydrolysis control.

Compounds **3**, **5**, and **22**, which were found to be nonsubstrates of human ectonucleotidases, were further evaluated as inhibitors of those enzymes.

The human ectonucleotidases inhibition assays were performed with a 100 μM final concentration of both the inhibitor (analogues **3** or **5**) and substrate (thymidine 5′-(p-nitrophenyl) monophosphate (pnp-TMP) or ATP).

For studying NPPs inhibition, analogue **3**, **5**, or **22** along with pnp-TMP or ATP were added to the enzyme and incubated for 15 min, and the reaction was stopped with perchloric acid. Enzyme activity was determined by the release of *p*-nitrophenol from *p*-nitrophenyl phosphate measured with a UV−vis spectrophotometer at 405 nm[41] or by the release of AMP from ATP by analytical HPLC. The inhibition precentage was calculated versus hydrolysis of pnp-TMP or ATP by the enzyme.

NTPDases inhibition was studied by the addition of analogue **3**, **5**, or **22** and ATP to the enzyme and stopping the reaction after 1 to 2 h with a malachite green solution. The released inorganic phosphate or ADP was measured at 630 nm[42] or by analytical HPLC, respectively. The inhibition percentage was calculated versus ATP hydrolysis.

dx.doi.org/10.1021/jo400931n | J. Org. Chem. 2013, 78, 8320−8329

At 100 μM, analogue **3** inhibited NPP1 by 4%, whereas NPP3 was inhibited by 7%. In contrast, NTPDase1, 2, and 8 were inhibited by 54, 42, and 49%, respectively, whereas NTPDase3 was not inhibited at all (Figure 7A).

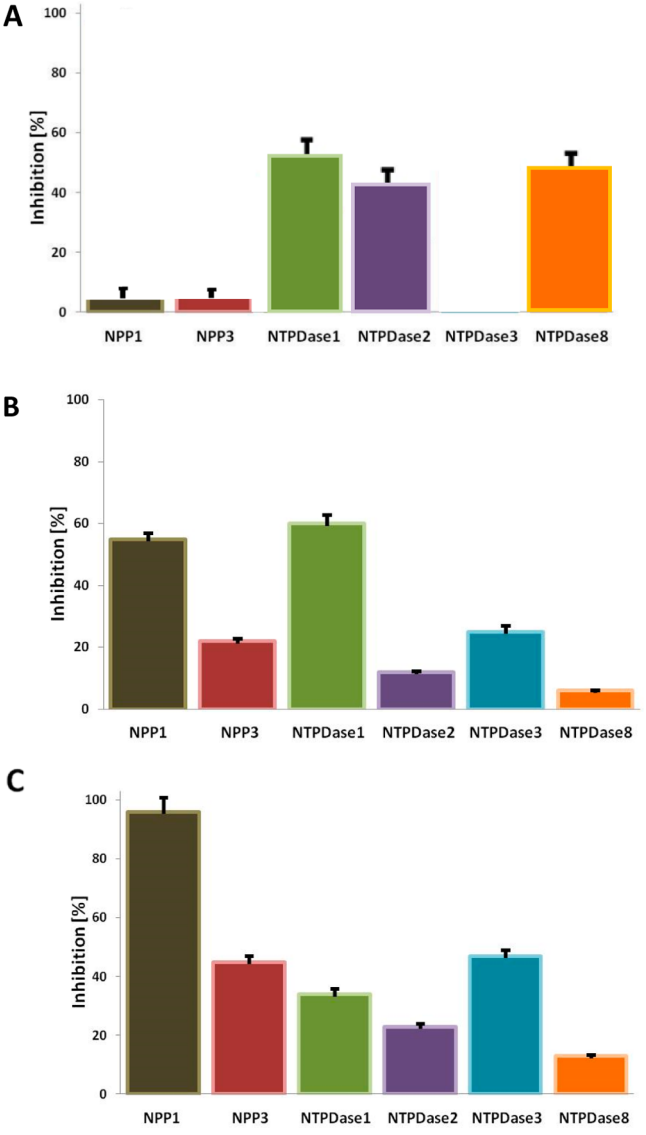


Figure 7. (A) Analogue **3** inhibition of ATP hydrolysis by NPP1,3 and NTPDase1,2,3,8. (B) Analogue **5** inhibition of pnp-TMP and ATP hydrolysis by NPP1,3 and NTPDase1,2,3,8, respectively. (C) Analogue **22** inhibition of pnp-TMP and ATP hydrolysis by NPP1,3 and NTPDase1,2,3,8, respectively.

Analogue **5** inhibited pnp-TMP hydrolysis by NPP1 and NPP3 by ~60 and 20%, respectively. Likewise, analogue **5** inhibited the hydrolysis of ATP by NTPDase1 by ~60% and inhibited NTPDase2,3,8 to a lesser extent (5–20%; Figure 7B).

Hence, analogues **3** and **5** are neither potent nor selective ectonucleotidase inhibitors, unlike compound **22** that at 100 μM inhibited NPP1 by 95%, whereas NPP3 and NTPDase1,2,3,8 were less than 50% inhibited (Figure 7C).

3. CONCLUSIONS

We have successfully synthesized novel nucleotide and dinucleotide analogues containing a methylenebisphosphonodithioate moiety. Compounds **3** and **5** were shown to be good Zn^{2+} chelators that coordinate Zn^{2+} by the thiophosphonate moiety in addition to the adenine N7 nitrogen atom. Furthermore, these compounds are highly selective to Zn^{2+} versus Mg^{2+} ions.

The replacement of the P–O–P bond in natural nucleotides with a P–C–P bond in compounds **3** and **5** enhanced the chemical and metabolic stability of those compounds. Analogues **3** and **5** were found to be stable toward both NPP and NTPDase hydrolysis. Likewise, both **3** and **5** were found to be highly stable under basic conditions (pD 11). Interestingly, compound **3** was more stable to acidic hydrolysis (pD 1.5) than compound **5**, with a half-life of 44 versus 9 h, respectively. However, dinucleotide derivative **5** was far more stable to air oxidation than corresponding mononucleotide **3**. This may be due to the higher rigidity of the dinucleotide structure not allowing the formation of an additional five-membered ring on the bridging phosphonate moiety. The introduction of the methylenebisphosphonodithioate moiety resulted in significantly increased hydrolytic stability of the nucleotide analogues as compared to thiomonophosphate anions (e.g., $PO_2S_2^{3-}$, POS_3^{3-}, and PS_4^{3-}).[28] Although analogue **3** as well as MDPT **17** have a tendency to undergo air oxidation (half-life of 14 h for **3** and 33% oxidation after 27 h for MDPT), they both were relatively stable in acidic (half-life of 3 days for MDPT at pD 1.9 and half-life of 44 h for **3** at pD 1.5) and basic media (half-life of 3.5 days for MDPT at pD 12.4 and no decomposition for 2 weeks for **3** at pD 11). The remarkable metabolic stability of analogues **3** and **5** is attributed to the methylene bridge in the methylenebisphosphonodithioate unit.

Analogues **3** and **5** inhibited NTPDase1,2,8 to some extent (20–60% inhibition), although this was probably not through the catalytic Ca^{2+}/Mg^{2+} ions that coordinate better with an oxygen atom rather than a sulfur atom, but rather through sulfur atom H bonding with neighboring H-donors in the NTPDase catalytic site.[43]

Surprisingly, analogue **3** was a poor Zn^{2+}-containing NPP1/3 inhibitor (4–7% inhibition), whereas dinucleotide methylenebisphosphonodithioate analogue **5** was a more potent NPP1 inhibitor (~60% inhibition), although it was not selective because it inhibited NTPDase1 as well.

We present a new synthesis route to novel nucleoside-(5′→P) methylenebisphosphonodithioate analogues. These analogues were found to be water-soluble and selective chelators of zinc ions. In addition, they are biocompatible, highly stable under basic pH, and relatively stable under highly acidic conditions and air-oxidizing conditions. Although analogues **3** and **5** did not prove to be effective inhibitors of Zn^{2+}-containing NPP1,3, which is involved in osteoarthritis and diabetes pathologies, they still may be promising Zn^{2+} chelators for the treatment of other health disorders involving an excess of Zn^{2+} ions.

EXPERIMENTAL SECTION

4.1. General Methods. Reactions were performed in oven-dried flasks under an N_2 atmosphere. 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) and 3-hydroxypropionitrile were distilled under reduced pressure before use. $CHCl_3$ was distilled over P_2O_5. Purification was performed using flash chromatography on silica gel and C_{18} reverse-phase supports. ^{1}H NMR spectra were obtained using a 200, 400, or 600 MHz spectrometer. ^{13}C NMR spectra were obtained at 50, 100, or 150 MHz. ^{31}P NMR spectra were obtained at 81, 162, or 243 MHz. HRMS spectra were measured on a MALDI-TOF or an ESI-TOF instrument. Purification of the nucleotides was achieved on a liquid chromatography (LC) system with a Sephadex DEAE-A25 column,

dx.doi.org/10.1021/jo400931n | J. Org. Chem. 2013, 78, 8320–8329

which was swelled in 1 M $NaHCO_3$ in the cold for 1 day. IR spectra were recorded on FT−IR spectrometer using a ZnSe crystal for powder samples pressed.

*P1-(Uridine-5′)-methylenebisphosphonodithioate Tris-ammonium Salt, **2**.* To a two necked round-bottomed flask containing molecular sieves, **6** (150 mg, 0.462 mmol), **7** (264.65 mg, 0.924 mmol), and DCM (4.5 mL) were added. The mixture was stirred overnight under a nitrogen atmosphere. Next, a mixture of DBU (0.462 mmol, 0.07 mL) in DCM (4.5 mL) was added dropwise over 1 h. ^{31}P NMR showed the formation of **9** (doublets at 100.10 and 90.45 ppm). 3-Hydroxypropionitrile (2.772 mmol, 0.19 mL) and DBU (0.462 mmol, 0.07 mL) were then added. The reaction mixture was stirred under nitrogen at 45 °C for 30 min. ^{31}P NMR showed the formation of **11** (doublets at 104.8 and 104.5 ppm). The mixture was filtered, and the molecular sieves were washed with DCM. After evaporation of the solvent, **11** was separated on silica column using $CHCl_3$/MeOH (85:15). This fraction was further purified on a reverse phase column using TEAA 1 M (pH 7)/CH_3CN (78:22) as the eluent to give **11** in 37% yield (130 mg). ^1H NMR (Acetone-d_6; 600 MHz): δ 8.42 (d; J = 7.8 Hz; 1H), 6.06−6.07 (m; 2H), 5.77 (d; J = 7.8 Hz; 1H), 5.48 (dd; J = 6.0 Hz; J = 1.8 Hz; 1H), 5.19 (dd; J = 6.0 Hz; J = 3.6 Hz; 1H), 4.43−4.46 (m; 2H), 4.30−4.32 (m; 3H), 3.51 (td; J = 13.2 Hz; J = 1.8 Hz; 2H), 3.27 (s; 3H), 2.88 (td; J = 7.5 Hz; J = 1.8 Hz) ppm. ^{31}P NMR (Acetone-d_6; 81 MHz): δ 105.28 (d; J = 25.8 Hz; P_α), 104.18 (d; J = 25.8 Hz; P_β) ppm. ^{13}C NMR (Acetone-d_6; 151 MHz): δ 163.6, 151.5, 143.6, 119.2, 118.1, 103.3, 90.9, 85.4 (d; J = 9.8 Hz), 84.9, 81.8, 64.6 (d; J = 6.6 Hz), 62.7 (t; J = 60.6 Hz), 60.1 (d; J = 6.5 Hz), 50.9, 19.9 (d; J = 8.6 Hz) ppm. HR MALDI (negative) m/z: calcd for $C_{15}H_{20}N_3O_8P_2S_4$, 559.960; found, 559.957.

Product **11** (130 mg, 0.17 mmol) was dissolved in THF (3 mL) and ethylmercaptane (3 mL). Potassium *tert*-butoxide (57.3 mg, 0.51 mmol) was added in portions. After 2 h, ^{31}P NMR indicated the presence of only starting material in the solution as well as a mixture of starting material and product in the precipitate obtained in the reaction. The solution was treated with an additional portion of potassium *tert*-butoxide (57.3 mg, 0.51 mmol). The solid residue was dissolved again in THF (3 mL) and ethylmercaptane (3 mL). After 1.5 h, ^{31}P NMR showed no starting material in the solution, and desired product **13** was observed in the precipitate. The solvent was removed by decantation, and the solid was dissolved in water and freeze-dried. Product **13** was dissolved in water and titrated with 10% HCl until pH 2.4 was achieved. The mixture was stirred at room temperature for 3 h. Next, 24% NH_4OH was added until reaching pH 9, and the mixture was stirred for 45 min. The mixture was freeze-dried. The residue (100 mg, yield: 90%) was subjected to ion-exchange chromatography (on a DEAE Sephadex column swollen overnight in 1 M $NaHCO_3$ at 4 °C). The product was eluted by applying a gradient of 0−0.5 M (800 mL of each) of ammonium bicarbonate solution, pH 7.6, to obtain **2** in 45% yield (40 mg). ^1H NMR (D_2O; 600 MHz): δ 8.14 (d; J = 7.8 Hz; 1H), 5.95 (d; J = 8.4 Hz; 1H), 5.91 (d; J = 5.4 Hz; 1H), 4.50 (dd; J = 4.8 Hz; J = 4.2; 1H), 4.44 (t; J = 5.4 Hz; 1H), 4.25−4.26 (m; 3H), 3.43 (t, J = 13.2 Hz, PCH_2P, 2H) ppm. ^{31}P NMR (D_2O; 81 MHz): δ 106.26 (d; J = 22.5 Hz; P_α), 78.06 (d; J = 22.5 Hz; P_β) ppm. ^{13}C NMR (D_2O; 151 MHz): δ 166.1, 151.8, 142.4, 102.5, 87.9, 83.5, 73.7, 69.9, 62.9, 60.3 (t; J = 56.3 Hz) ppm. HR MALDI (negative) m/z: calcd for $C_{10}H_{15}N_2O_7P_2S_4$, 464.923; found, 464.920. IR (ZnSe): ν 3094, 1651, 1404, 1264, 1101, 1071, 868, 764, 710, 629 cm^{-1}.

*P1-(Adenosine-5′)-methylenebisphosphonodithioate Tris-ammonium Salt, **3**.* Product **3** was prepared according to the above procedure for the preparation of **2**. Compound **12** was obtained from **8** (285.80 mg, 0.924 mmol) and **6** (150 mg, 0.462 mmol) in 28% yield (100 mg). ^1H NMR (Acetone-d_6; 600 MHz): δ 9.09 (s; 1H), 9.03 (s; 1H), 8.19 (s; 1H), 8.18 (s; 1H), 6.46 (d; J = 3.6 Hz), 6.22 (d; J = 3.6 Hz), 6.16 (s; 1H), 5.98 (s; 1H), 5.45−5.63 (m; 3H), 5.41−5.42 (m; 1H), 4.59−4.62 (m; 1H), 4.53−4.58 (m; 1H), 4.26−4.30 (m; 4H), 3.55−3.62 (m; 4H), 3.42 (s; 3H), 3.27 (s; 3H), 2.87−2.89 (m; 4H) ppm. ^{31}P NMR (Acetone-d_6; 81 MHz): δ 105.35 (d; J = 25.9 Hz; P_α), 104.02 (d; J = 25.9 Hz; P_β) ppm. ^{13}C NMR (Acetone-d_6; 151 MHz): δ 156.5, 156.4, 153.5, 150.3, 141.6, 141.5, 119.6, 119.5, 119.1, 117.8, 90.4, 89.9, 87.1 (d; J = 9.9 Hz), 86.1, 85.5 (d; J = 9.7 Hz), 85.4, 82.8, 82.4, 65.0 (d; J = 6.9 Hz), 64.5 (d; J = 6.8 Hz), 61.5−62.3 (m; PCP), 59.7−59.9 (m; CH_2−O), 52.4, 50.8 ppm. HR MALDI (negative) m/z: calcd for $C_{16}H_{21}N_6O_6P_2S_4$, 582.987; found, 582.987.

After LC separation, **3** was obtained in 55% yield (38 mg). ^1H NMR (D_2O; 600 MHz): δ 8.74 (s; 1H), 8.27 (s; 1H), 6.12 (d; J = 5.4 Hz; 1H), 4.90 (t; J = 5.4 Hz; 1H), 4.68 (dd; J = 4.2 Hz; J = 4.8 Hz; 1H), 4.28−4.44 (m; 3H), 3.47 (t, J = 13.2 Hz, PCH_2P, 2H) ppm. ^{31}P NMR (D_2O; 81 MHz): δ 104.99 (d; J = 21.1 Hz; P_α), 90.59 (d; J = 21.1 Hz; P_β) ppm. ^{13}C NMR (D_2O; 150 MHz): δ 154.4, 151.1, 148.8, 140.9, 118.5, 87.1, 84.0, 74.3, 70.5, 62.9, 57.8 (t; J = 59.0 Hz) ppm. HR MALDI (negative) m/z: calcd for $C_{11}H_{16}N_5O_5P_2S_4$, 487.950; found, 487.952. IR (ZnSe): ν 3110, 1679, 1635, 1416, 1331, 1215, 1069, 945, 894, 779, 711, 625 cm^{-1}.

*P1,P2-Di(uridine-5′)-methylenebisphosphonondithioate Bis-triethylammonium Salt, **4**.* To a two necked round-bottomed flask containing molecular sieves, **7** (281.60 mg, 0.984 mmol), **6** (80 mg, 0.246 mmol), and dry acetonitrile (7 mL) were added. The mixture was stirred overnight under a nitrogen atmosphere. DBU (0.520 mmol, 0.08 mL) was then added, and the mixture was stirred at 60 °C for 2 h. ^{31}P NMR showed the formation of desired product **15** (singlet at 105.1 ppm). The mixture was filtered, and the molecular sieves were washed with $CHCl_3$. After evaporation of the solvent, **15** was separated on a silica gel column using $CHCl_3$/MeOH (90:10).

Most of product **15** (73 mg) was dissolved in water and titrated with 10% HCl until pH 2.4 was achieved. The mixture was stirred at room temperature for 3 h. Next, 24% NH_4OH was added until pH 9 was reached, and the mixture was stirred for 45 min. The mixture was freeze-dried. The residue was purified on a reverse-phase column using 1 M TEAA (pH 7)/CH_3CN (92:8) as the eluent to give **4** in 30% yield (65 mg). Final purification was carried out by HPLC using a semipreparative reverse-phase column by applying an isocratic TEAA/CH_3CN 92:8 in 15 min (4 mL/min): t_R 9.36 min. ^{31}P NMR (D_2O; 81 MHz): δ 104.02 (s; 2P) ppm. ^1H NMR (D_2O; 600 MHz): δ 8.15 (d; J = 8.4 Hz; 1H), 5.95−5.98 (m; 2 Hz), 4.49 (dd; J = 4.8 Hz; J = 4.2 Hz; 1H), 4.44 (t; J = 4.8 Hz; 1H), 4.28−4.32 (m; 3H), 3.51 (t; J = 13.8 Hz; PCH_2P; 2H), 3.19 (q; J = 7.2 Hz; 5H), 1.27 (t; J = 7.2 Hz; 8H) ppm. ^{13}C NMR (D_2O; 150 MHz): δ 166.4, 151.9, 142.2, 102.4, 88.4, 83.2 (t; J = 4.8 Hz), 73.9, 69.8, 62.3, 57.4 (t; J = 65 Hz), 46.5, 8.1 ppm. HRMS (ESI) m/z: calcd for $C_{19}H_{25}N_4O_{12}P_2S_4$, 690.983; found, 690.981. IR (ZnSe): ν 3053, 2980, 1673, 1462, 1386, 1266, 1103, 1070, 1034, 988, 935, 891, 787, 715, 629 cm^{-1}.

*P1,P2-Di(adenosine-5′)-methylenebisphosphonondithioate Bis-triethylammonium Salt, **5**.* Compound **5** was prepared according to the same procedure as for **4**. Compound **16** was obtained from **7** (371 mg, 1.19 mmol) and **6** (100 mg, 0.308 mmol). Compound **5** was obtained after the removal of the methoxymetylidene protecting group from intermediate **16** and purification on a reverse-phase column using 1 M TEAA (pH 7)/CH_3CN (93:7) as the eluent to give **5** in 36% yield (100 mg). Final purification was carried out by HPLC using a semipreparative reverse-phase column by applying an isocratic elution with TEAA/CH_3CN 90:10 in 15 min (4 mL/min): t_R 8.35 min. ^1H NMR (D_2O; 600 MHz): δ 8.50 (s; 1H), 8.06 (s: 1H), 6.01 (d; J = 5.4 Hz), 4.61 (t; J = 4.2 Hz; 1H), 4.43−4.47 (m; 1H), 4.28−4.35 (m; 2H), 3.59 (t; J = 14.4 Hz; PCH_2P; 2H), 3.19 (q; J = 7.2 Hz; 5H), 1.27 (t; J = 7.2 Hz; 8H) ppm. ^{31}P NMR (D_2O; 81 MHz): δ 104.44 (s; 2P) ppm. ^{13}C NMR (D_2O; 150 MHz): δ 154.7, 152.5, 148.2, 139.6, 117.6, 87.2, 84.0 (t; J = 5.1 Hz), 75.6, 70.7, 62.2, 57.9 (t; J = 68.7 Hz), 46.5, 8.1 ppm. HRMS (ESI) m/z: calcd for $C_{21}H_{26}N_{10}O_8NaP_2S_4$, 759.019; found, 759.020. IR (ZnSe): ν 3320, 3176, 2979, 1640, 1600, 1574, 1474, 1418, 1331, 1299, 1244, 1210, 1072, 1029, 986, 938, 899, 795, 713, 676, 630 cm^{-1}.

4.2. Evaluation of the Resistance of Analogues 3, 5, and 22 to NPP1 and NPP3 Hydrolysis. The percentage of hydrolysis of the new analogues by human NPP1,3 was evaluated as follows: 67 or 115 μg of human NPP1 or NPP3 extract, respectively, was added to 0.579 mL of the incubation mixture (1 mM $CaCl_2$, 200 mM NaCl, 10 mM KCl, and 100 mM Tris, pH 8.5) and preincubated at 37 °C for 3 min. The reaction was initiated by the addition of 4 mM analogue (0.015 mL). The reaction was stopped after 30 min or 1 h for NPP1 or NPP3, respectively, by adding ice-cold 1 M perchloric acid (0.350 mL). These

dx.doi.org/10.1021/jo400931n | J. Org. Chem. 2013, 78, 8320−8329

samples were centrifuged for 1 min at 10 000*g*. The supernatants were neutralized with 2 M KOH (140 μL) in 4 °C and centrifuged for 1 min at 10 000*g*. The reaction mixture was filtered and freeze-dried.

Each sample was dissolved in HPLC-grade water (200 μL), and 20 μL of sample was injected into an analytical HPLC column (Gemini analytical column (5 μ C18 557 110A; 150 mm × 4.60 mm)). An isocratic elution with 85−97% 100 mM TEAA (pH 7) and 15−3% CH_3CN and a flow rate 1 mL/min was used. The ratio of buffer and CH_3CN depended on the chemical structure of the analogue.

The hydrolysis rates of analogues **3**, **5**, and **22** by NPP1 or NPP3 were determined by measuring the change in the integration of the HPLC peaks for each analogue over time versus control. The percentage of compound degradation was calculated versus control by taking into consideration the degradation of the compounds resulting from the addition of acid to stop the enzymatic reaction. Therefore, each of the samples was compared to a control that was transferred to acid but to which no enzyme was added. The percentage of degradation was calculated from the area under the curve of the nucleoside monophosphate peak after subtraction of the control, which is the amount of the nucleoside monophosphate peak formed because of acidic hydrolysis.

4.3. Evaluation of the Resistance of Analogues 3, 5, and 22 to Hydrolysis by NTPDase1,2,3,8. The percentage of hydrolysis of analogues **3**, **5**, and **22** by human NTPDase1,2,3,8 was evaluated as follows: 2.8 or 4.3 μg of human NTPase1 or NTPDase2 extract, respectively, was added to 0.579 mL of the incubation mixture (10 mM $CaCl_2$ and 160 mM Tris, pH 7.4) and preincubated at 37 °C for 3 min. The reaction was initiated by the addition of 4.24 mM analogue solution (0.012 mL). The reaction was stopped after 1 h by adding ice-cold 1 M perchloric acid (0.350 mL). These samples were centrifuged for 1 min at 10 000*g*. The supernatants were neutralized with 2 M KOH (140 μL) at 4 °C and centrifuged for 1 min at 10 000*g*. The reaction mixture was filtered and freeze-dried.

Each sample was dissolved in HPLC-grade water (200 μL), and a 20 μL sample was injected into an analytical HPLC column (Gemini analytical column (5 μ C 18 557 110A; 150 mm × 4.60 mm)) and eluted using isocratic elution with 78−97% 100 mM TEAA (pH 7) and 22−3% CH_3CN, with a flow rate of 1 mL/min. The ratio of buffer and CH_3CN depended on the chemical structure of the analogue.

The hydrolysis rates of analogues **3**, **5**, and **22** by NTPDase1,2,3,8 were determined by measuring the change in the integration of the HPLC peaks for each analogue over time versus control. The percentage of compound degradation was calculated versus control by taking into consideration the degradation of the compounds resulting from the addition of acid to stop the enzymatic reaction. Therefore, each of the samples was compared to a control that was transferred to acid but to which no enzyme was added. The percentage of degradation was calculated from the area under the curve of the nucleoside monophosphate peak after subtraction of the control, which is the amount of the nucleoside monophosphate peak formed because of acidic hydrolysis.

4.4. Evaluation of the Inhibition of NPP Activity. The evaluation of the effect of analogues **3** and **5** on human NPP1 and 3 activity was carried out either with pnp-TMP or ATP as the substrate.[44] pnp-TMP hydrolysis was carried out at 37 °C in 0.2 mL of the following incubation mixture: 1 mM $CaCl_2$, 130 mM NaCl, 5 mM KCl, and 50 mM Tris, pH 8.5, with or without analogues **3** and **5**. pnp-TMP or ATP and analogues **3** or **5** were all used at a final concentration of 100 μM. Recombinant human NPP1 or NPP3 cell lysates were added to the incubation mixture and preincubated at 37 °C for 3 min. The reaction was initiated by the addition of pnp-TMP or ATP and stopped by the addition of 3% trichloroacetic acid (0.200 mL) and perchloric acid (0.350 mL), respectively. The production of *para*-nitrophenol was measured at 310 nm 15 min after the initiation of the reaction, and the AMP product of ATP hydrolysis was measured by the integration of the absorbance after injection into the HPLC, as mentioned above.

4.5. Evaluation of the Inhibition of NTPDase Activity. The activity of analogue **3** and **5** was measured as described previously[36] in 0.2 mL of incubation medium Tris-Ringer buffer (120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 5 mM glucose, and 80 mM Tris, pH 7.3) at 37 °C with or without analogues **3** or **5** (final concentration 100 μM) and with or without 100 μM ATP as a substrate. NTPDases protein extracts were added to the incubation mixture and preincubated at 37 °C for 3 min. The reaction was initiated by the addition of ATP and analogue **3** or **5** and stopped after 15 min with the addition of malachite green reagent (50 μL). The released inorganic phosphate (Pi) was measured at 630 nm according to Baykov et al.[42]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^1H and ^{31}P NMR monitored titrations of compound **3** with Zn^{2+}, kinetic profiles showing the changes in the percentage of **3** under acidic conditions, pD 1.5, and **3** subjected to air oxidation. Spectral data (^1H, ^{13}C, and ^{31}P NMR) of compounds **2**−**5**, **11**, and **12**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Fax: 972-3-6354907; Tel: 972-3-5318303; E-mail: bilha.fischer@biu.ac.il.

Author Contributions

‖These authors contributed equally to this work

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS:

(TEAA) triethylammonium acetate; (MDP) methylenediphosphonic acid; (MDPT) methylenediphosphonotetrathioate; (E-NTPDases) ectonucleoside triphosphate diphosphohydrolases; (NPPs) nucleotide pyrophosphatase/phosphodiesterases; (pnp-TMP) p-nitrophenyl 5′-thymidine monophosphate; (DBU) 1,8-diazabicyclo[5.4.0]undec-7-ene

■ REFERENCES

(1) Frey, P. A.; Sammons, R. D. *Science* **1985**, *228*, 541−545.
(2) Eckstein, F. *J. Am. Chem. Soc.* **1966**, *88*, 4292−4294.
(3) Tomaszewska, A.; Guga, P.; Stec, W. J. *Chirality* **2011**, *23*, 237−244.
(4) Eckstein, F.; Simonson, L. P.; Baer, H. P. *Biochemistry* **1974**, *13*, 3806−3810.
(5) Baraniak, J.; Lesnikowski, Z. J.; Niewiarowski, W.; Zielinski, W. S.; Stec, W. J. *ACS Symp. Ser.* **1981**, *171*, 77−81.
(6) Eckstein, F.; Gindl, H. *Eur. J. Biochem.* **1970**, *13*, 558−564.
(7) Nukaga, Y.; Yamada, K.; Ogata, T.; Oka, N.; Wada, T. *J. Org. Chem.* **2012**, *77*, 7913−7922.
(8) Ludwig, J.; Eckstein, F. *J. Org. Chem.* **1991**, *56*, 1777−1783.
(9) Tamura, Y.; Tao, M.; Miyano-Kurosaki, N.; Takai, K.; Takaku, H. *Antisense Nucleic Acid Drug Dev.* **2000**, *10*, 87−96.
(10) Kusunoki, A.; Saitou, T.; Miyano-Kurosaki, N.; Takaku, H. *FEBS Lett.* **2001**, *488*, 64−68.
(11) Overhoff, M.; Sczakiel, G. *EMBO Rep.* **2005**, *6*, 1176−1181.
(12) Winkler, J.; Stessl, M.; Amartey, J.; Noe, C. R. *ChemMedChem* **2010**, *5*, 1344−1352.
(13) Agrawal, S.; Kandimalla, E. R. *Nat. Biotechnol.* **2004**, *22*, 1533−1537.
(14) Rosenmayr-Templeton, L. *Ther. Delivery* **2013**, *4*, 429−433.
(15) Sigel, R. K. O.; Song, B.; Sigel, H. *J. Am. Chem. Soc.* **1997**, *119*, 744−755.
(16) Sayer, A. H.; Itzhakov, Y.; Stern, N.; Nadel, Y.; Fischer, B., submitted for publication.
(17) Amir, A.; Sayer, A. H.; Ezra, A.; Fischer, B. *Inorg. Chem.* **2013**, *52*, 3133−3140.

(18) Scott, L. E.; Orvig, C. *Chem. Rev.* **2009**, *109*, 4885−4910.
(19) Stefan, C.; Jansen, S.; Bollen, M. *Trends Biochem. Sci.* **2005**, *30*, 542−550.
(20) Lippard, S. J.; Berg, J. *Principles of Bioinorganic Chemistry*; University Science Books: Mill Valley, CA, 1994.
(21) Maddux, B. A.; Sbraccia, P.; Kumakura, S.; Sasson, S.; Youngren, J.; Fisher, A.; Spencer, S.; Grupe, A.; Henzel, W.; Stewart, T.; Reaven, G. M.; Goldfine, I. D. *Nature* **1995**, *373*, 448−451.
(22) Costanzo, B. V.; Trischitta, V.; Di Paola, R.; Spampinato, D.; Pizzuti, A.; Vigneri, R.; Frittitta, L. *Diabetes* **2001**, *50*, 831−836.
(23) Maddux, B. A.; Goldfine, I. D. *Diabetes* **2000**, *49*, 13−19.
(24) Grupe, A.; Alleman, J.; Goldfine, I. D.; Sadick, M.; Stewart, T. A. *J. Biol. Chem.* **1995**, *270*, 22085−22088.
(25) Belfiore, A.; Costantino, A.; Frasca, F.; Pandini, G.; Mineo, R.; Vigneri, P.; Maddux, B.; Goldfine, I. D.; Vigneri, R. *Mol. Endocrinol.* **1996**, *10*, 1318−1326.
(26) Reese, C. B.; Yan, H. *Tetrahedron Lett.* **2004**, *45*, 2653−2656.
(27) Szajnman, S. H.; Ravaschino, E. L.; Docampo, R.; Rodriguez, J. B. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4685−4690.
(28) Maki, H.; Ueda, Y.; Nariai, H. *J. Phys. Chem. B* **2011**, *115*, 3571−3577.
(29) Amir, A.; Sayer, A. H.; Zagalsky, R.; Shimon, L. J. W.; Fischer, B. *J. Org. Chem.* **2013**, *78*, 270−277.
(30) Clarke, K.; Kashiwaya, Y.; King, M. T.; Gates, D.; Keon, C. A.; Cross, H. R.; Radda, G. K.; Veech, R. L. *J. Biol. Chem.* **1996**, *271*, 21142−21150.
(31) Rodriguez-Rodriguez, C.; Telpoukhovskaia, M.; Orvig, C. *Coord. Chem. Rev.* **2012**, *256*, 2308−2332.
(32) Pearson, R. G. *Chemical Hardness*; Wiley-VCH: New York, 2005; pp 1−27.
(33) Stern, N.; Major, D. T.; Gottlieb, H. E.; Weizman, D.; Fischer, B. *Org. Biomol. Chem.* **2010**, *8*, 4637−4652.
(34) Toyota, K.; Ishikawa, Y.; Shirabe, K.; Yoshifuji, M.; Okada, K.; Hirotsu, K. *Heteroat. Chem.* **1993**, *4*, 279−285.
(35) Ludwig, J.; Eckstein, F. *J. Org. Chem.* **1991**, *56*, 5860−5865.
(36) Kukulski, F.; Levesque, S. A.; Lavoie, E. G.; Lecka, J.; Bigonnesse, F.; Knowles, A. F.; Robson, S. C.; Kirley, T. L.; Sevigny, J. *Purinergic Signalling* **2005**, *1*, 193−204.
(37) Nahum, V.; Tulapurkar, M.; Levesque, S. A.; Sevigny, J.; Reiser, G.; Fischer, B. *J. Med. Chem.* **2006**, *49*, 1980−1990.
(38) Enjyoji, K.; Sevigny, J.; Lin, Y.; Frenette, P. S.; Christie, P. D.; Schulte, A. E. J., II; Imai, M.; Edelberg, J. M.; Rayburn, H.; Lechi, M.; Beeler, D. L.; Csizmadia, E.; Wagner, D. D.; Robson, S. C.; Rosenberg, R. D. *Nature Med.* **1999**, *5*, 1010−1017.
(39) Bobyr, E.; Lassila, J. K.; Wiersma-Koch, H. I.; Fenn, T. D.; Lee, J. J.; Nikolic-Hughes, I.; Hodgson, K. O.; Rees, D. C.; Hedman, B.; Herschlag, D. *J. Mol. Biol.* **2012**, *415*, 102−117.
(40) Nadel, Y. Ph.D. Thesis, Bar-Ilan University, Israel, 2013.
(41) Brandenberger, H.; Hanson, R. *Helv. Chim. Acta* **1953**, *36*, 900−906.
(42) Baikov, A. A.; Evtushenko, O. A.; Avaeva, S. M. *Anal. Biochem.* **1988**, *171*, 266−270.
(43) Gendron, F.-P.; Halbfinger, E.; Fischer, B.; Duval, M.; D'Orleans-Juste, P.; Beaudoin, A. R. *J. Med. Chem.* **2000**, *43*, 2239−2247.
(44) Belli, S. I.; Goding, J. W. *Eur. J. Biochem.* **1994**, *226*, 433−443.