A New Class of Pentacoordinate Ribonuclease Inhibitors: Synthesis, Characterization, and Inhibition Studies of Ribonucleoside and Anhydropentofuranose Oxorhenium(V) Complexes

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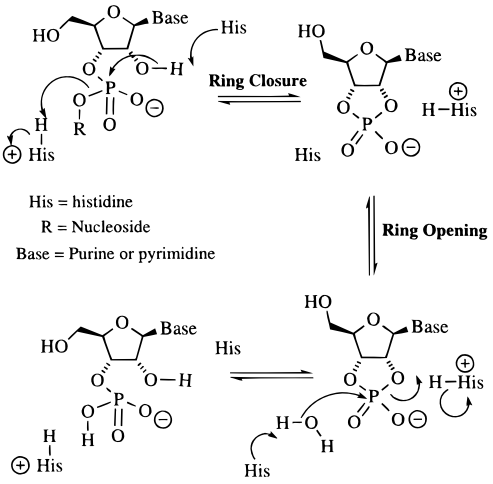
Abstract: This article describes the synthesis, characterization, and kinetic studies of a new class of stable ribonuclease (RNase) inhibitors. Herein the first synthesis of oxorhenium(V) complexes of diaminonucleosides and diaminoaldopentofuranoses is reported. Stability studies have shown that the complexes are stable between pH 3 and 13 at temperatures of 25−80 °C for 24 h with no occurrence of ligand exchange or epimerization at the Re═O core. Kinetic studies show that both the *syn* (**1a**) and *anti* (**1b**) diastereoisomers of oxorhenium(V) complexes of 9-(2′,3′-diamino-2′,3′-dideoxy-*β*-D-ribofuranosyl)adenine (**13**) are excellent inhibitors of the purine specific ribonuclease, RNase U_2. Dixon and Lineweaver−Burke analyses suggest that the inhibition is competitive, with inhibitory constants (K_is) of 15 and 93 μM, respectively. These complexes are of equivalent inhibitory potency to previously reported nucleoside vanadium alkoxide inhibitors with the added advantage of being completely stable in aqueous solution. The oxorhenium(V) complexes of 2,5-anhydro-3,4-diamino-3,4-dieoxy-D-ribitol (**2a-Re**) and (**2b-Re**) display much poorer inhibition (K_i > 2 mM), highlighting the critical nature of the purine base for successful inhibition.

Introduction

Ribonuclease U_2 (EC 3.1.27.4) secreted by the smut fungus *Ustilago sphaerogena*[1,2] is an endoribonuclease which catalyzes the hydrolysis of RNA, specifically at the 3′-phosphodiester bond of adenylic acid, via a two-step mechanism very similar to that of RNases A and T_1.[3−8] Usher[9,10] and co-workers have shown that both steps of the RNase catalyzed reaction follow an in-line associative mechanism with phosphorus existing in a theoretical pentacoordinate trigonal-bipyramidal geometry through each of the transition states (TS) (Scheme 1). Although Usher did not identify the amino acid residues in the binding site responsible for catalysis, these have since been inferred from a number of substrate modification experiments and published structures of RNase−inhibitor complexes.[11−16]

Scheme 1



Pentacoordinate oxyphosphoranes are generally unstable; therefore, other elements that exist as five-coordinate species have been developed as models of the postulated TS for inhibition and mechanistic characterization of RNase activity. From as early as 1973, complexes of uridine with oxovanadium-(IV) and vanadium(V) ions have been shown to be "potent" inhibitors of RNases (K_i 8−12 μM).[17] At the time it was suggested by the authors and later validated by X-ray crystallography that the vanadate was mimicking the pentacoordinate TS for phosphodiester hydrolysis.[18,19] However, vanadium alkoxides are very unstable and undergo rapid ligand exchange

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in aqueous solution making a direct structural analysis of the inhibitory species and its kinetic parameters very complex.[20−24]

In a recent strategy, we have shown that a diastereoisomeric mixture of a ribonucleoside oxotechnetium(V) chelate inhibits RNase U_2, heralding the first water-stable pentacoordinate inhibitor of a RNase.[25] Modeling shows that the technetium exists in a distorted TBP geometry, suggesting that the inhibitor is indeed mimicking the putative transition state for RNA hydrolysis. However, because of the radioactivity associated with technetium and the inherent difficulties of both purifying and analyzing its diastereomers on a preparative scale, we have sought alternative stable, pentacoordinate metal chelates for RNase inhibition.

Rhenium, in common with its congener technetium occupies a central position in the d-block elements and exists in a wide variety of oxidation states and coordination numbers. A number of bifunctional ligands have been developed for Re coordination and these metallocomplexes have found use as radioimaging agents or for the labeling of monoclonal antibodies (radioimmunoassay).[26−32] A number of structural studies have shown that in all cases the Re atom is coordinated to five atoms (N, N, S, S, and O for $Re-N_2S_2$ complexes, or N, N, N, S, and O for $Re-N_3S$ complexes) in a distorted square-pyramidal geometry.[33−36] This paper describes the synthesis, characterization, solution properties, and RNase U_2 inhibition of novel N_2S_2 (diamide dimercaptide) oxorhenium(V) complexes of ribonucleosides and anydropentofuranoses (Figure 1).

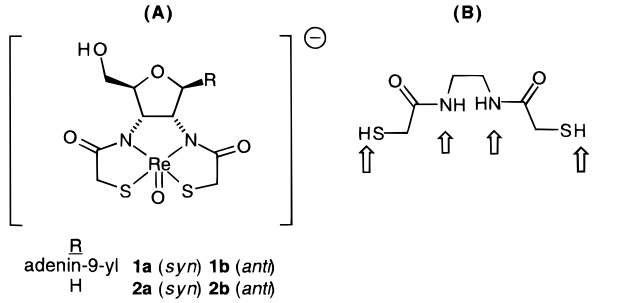
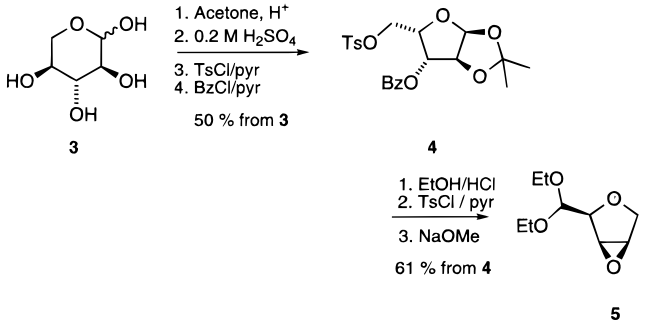


Figure 1. (A) The complexes synthesized as a novel class of pentacoordinate inhibitors for RNase U_2, based on the ribonucleoside adenosine (**1a** and **1b**) and a modified anhydroribitol (**2a** and **2b**). B shows the DADT ligand with the four deprotonation sites which generates the pentacoordinate, negatively charged oxorhenium(V) complexes.

Scheme 2



Results and Discussion

Synthesis of Rhenium Inhibitors (2a and 2b). The synthetic route comprised of three main parts. First, a *de novo* construction of the 1,4-anhydro-2,3-diamino-2,3-dideoxy-D-ribitol ring; second, incorporation of the complete DADT ligand system; and third, metal insertion. The initial steps from L-xylose (**3**) via the L-*arabino* tosylate **4** led to the reported *lyxo* D-epoxide **5** (Scheme 2).[37]

Opening of the epoxide **5** with lithium azide gives an isomeric mixture of azido alcohols which were isolated as their respective acetates (**6a**-*arabino*) and (**6b**-*xylo*) in good yields, 32% and 47%, respectively (Scheme 3).

Progression to the bis azide **7**, which has the nitrogen heteroatoms of the DADT ligand inserted with the correct regio- and stereochemistry is accomplished equally well with both isomers **6a** and **6b**. Methanolysis of either **6a** or **6b** gives the corresponding azido alcohol (**8**). Epimerization of the alcohol **8** is achieved by activation as its trifluoromethanesulfonate ester followed by displacement with sodium azide and 18-crown-6. The *ribo* bis azide **7**, following catalytic hydrogenation, is acylated immediately with chloroacetyl chloride to give the *ribo* bis(chloroacetamido) derivative **9** in 90% overall yield for the two steps. The intermediate diamine **7a** was not isolated due to its high polarity and tendency to oxidation. Treatment of **9** with sodium thiobenzoate in refluxing ethanol now inserts the masked thiol components of the DADT ligand and gives the bis thioester **10** in excellent yields (94%). Because of the well-known propensity of thiols to oxidation, metal insertion involves a prior deprotection of the thioesters under basic conditions followed by an immediate treatment of the crude dithiol **11** with trichlorobis(triphenylphosphine)rhenium(V) oxide[38] in ethanol by the method described by O'Neill[39] and co-workers. The metal insertion reaction was found to be very base sensitive, if the thioesters of **10** were deprotected with a mild base such as

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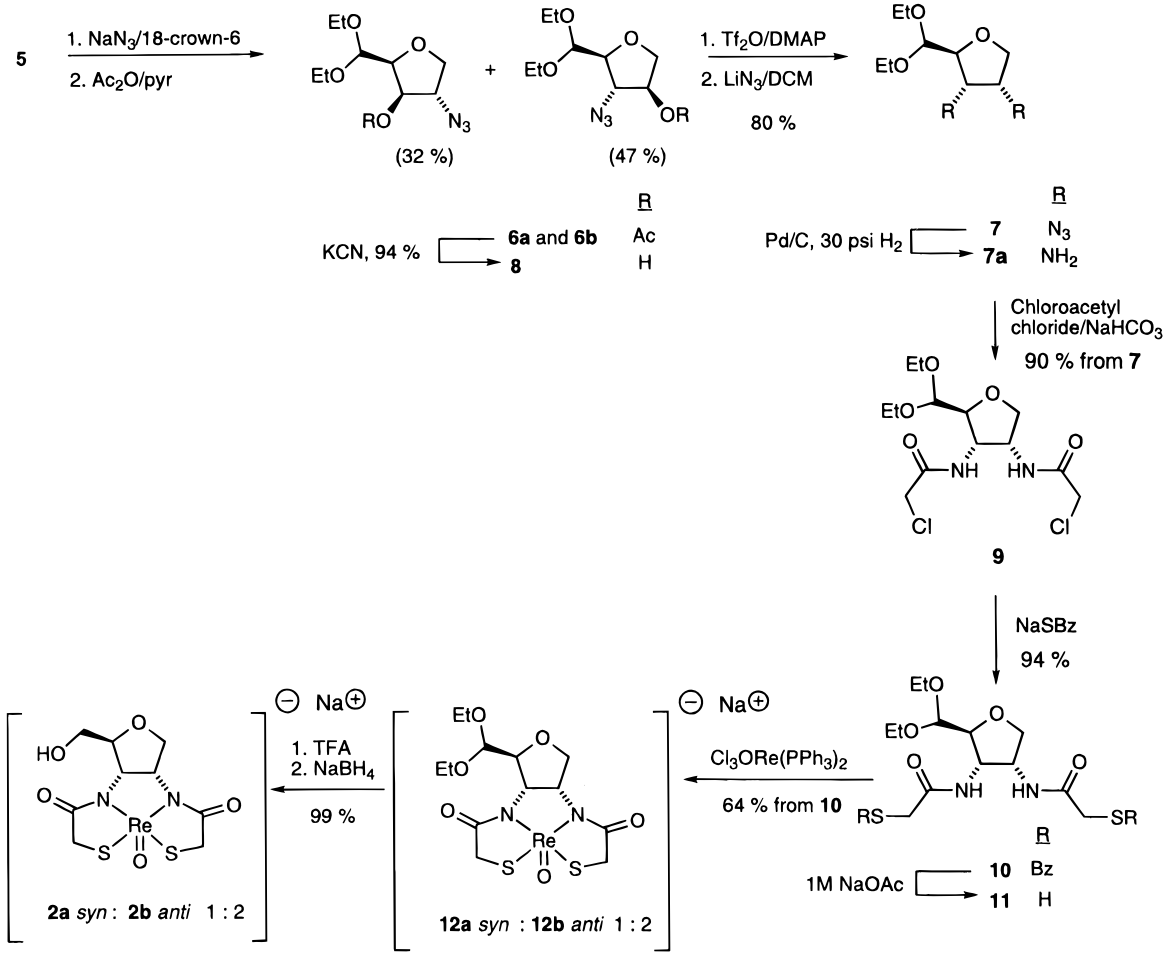
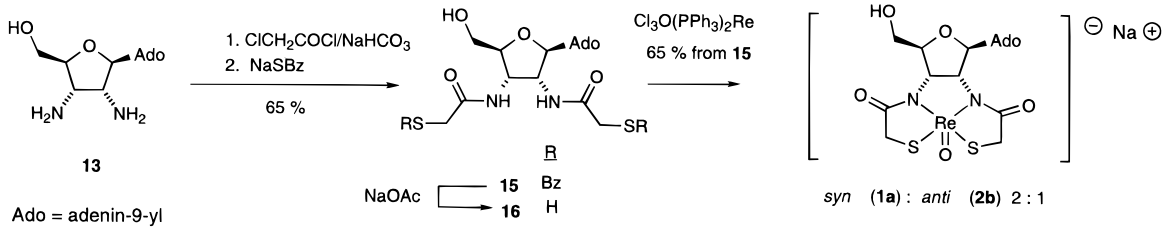
Scheme 3



Scheme 4



sodium acetate, the metal insertion process yielded a higher ratio of *anti*/*syn* than if aqueous sodium hydroxide was employed. By deprotection with sodium acetate a mixture of *syn* (**12a**)/*anti* (**12b**) in the ratio 1:2 was obtained. Acetal hydrolysis with TFA and reduction of the resultant aldehyde with sodium borohydride then gave the required Re−anhydroribitol complexes **2a** and **2b**.

Assignment of the oxorhenium(V) diastereoisomers either as *syn* or *anti* is primarily based on the relative infrared stretches of the Re═O bond. Both complexes exhibit a strong absorbance in the characteristic stretching frequency of a Re═O bond (900−100 cm^{-1}).[40] It is known that for a number of different oxorhenium(V) complexes, the two isomers show different infrared stretching frequencies, the *syn* epimer resonating at a lower frequency than the corresponding *anti* isomer.[41,42] Thus **2a** was assigned as the *syn* epimer with an Re═O stretching frequency of 943 cm^{-1} and **2b** denoted as the *anti* isomer with a Re═O frequency of 960 cm^{-1}.

Synthesis of Rhenium Inhibitors (1a and 1b). The starting point for construction of the DADS ligand is 2,3-diamino-2,3-dideoxyadenosine which we synthesized in seven steps, using a modification of our procedure from 9-(β-D-arabinofuranosyl)-adenine (Scheme 4).[43]

Chloroacetylation of **13** and thiobenzylation of the resultant bis(chloroacetamide) intermediate **14** using the same procedure described for **9** and **10**, respectively, gave the fully protected DADT ligand **15**. Incorporation of the rhenium core was much less sensitive to base strength for **16** than for **11**, *vide supra*. By incubating in sodium hydroxide, 100% of the *syn* isomer was produced. Whereas following incubation in sodium acetate (1 M) a 20:1 ratio of *syn*/*anti* was produced (ratios determined by HPLC). This high preference for the *syn* isomer (**1a**) in this case may well be due to steric hindrance during the Re═O

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Table 1. Comparative Inhibition Constants[a] (K_i) and Binding Energies[b] (ΔG) of Oxorhenium(V) Complexes **1a**, **1b**, **2a**, and **2b** with RNase U_2

inhibitor	K_i, μM	ΔG, kcal mol^{-1}
1a (*syn*)	15.7 ± 2	7.1
1b (*anti*)	90 ± 9	6.0
2a (*syn*)	>2000[c]	<4.0
2b (*anti*)	>2000[c]	<4.0
adenine	no inhibition[d]	

[a] K_i values were determined by averaging the results of at least duplicate runs in citrate buffer (pH 3.5, 16.5 mM), with urea (3.5 M) and EDTA (0.85 mM) at 323 K. [b] Binding energies were calculated from $\Delta G = -RT \ln (K_i)$. [c] Problems of solubility meant that an accurate determination of K_i for this inhibitor was unfeasible. [d] Poor solubility beyond a 5 mM assay concentration precluded a K_i determination.

insertion. The adenine ring sits *trans* to the Re═O core during generation of the *syn* isomer, but *syn* for the *anti* isomer. Assignment of the two isomers *syn* (**1a**) and *anti* (**1b**) was based, primarily, on the relative infrared stretching frequencies of the Re═O core, *vide supra*.

Stability Studies. An important component of the strategy for use of the DADT-based rhenium chelates was their known stability in aqueous solution. In accordance with previous reports, the ribonucleoside rhenium complexes (**1a**, **1b**, **2a**, and **2b**) were all stable between pH 3 and 13 at temperatures of 25−80 °C for 24 h.[44−46] No epimerization occurred at the rhenium cores (assayed by HPLC) which allowed for an accurate comparison of the inhibitory effect of each of the epimers in the RNase assay, *vide infra*.

The pH optimum of RNase U_2 is around 4.0,[7] and a concern was that the purine complexes **1a** and **1b** by being linked to the organometallic may be susceptible to acid-catalyzed depurination during the kinetic assay. However, no decomposition was detected either during the stability or inhibition studies.

Kinetic and Inhibition Studies. The enzyme-catalyzed and background transphosphorylation of the dinucleotide ApA and individual inhibition constants for the Re complexes (**1a**, **1b**, **2a**, **2b**), adenosine, and adenine were followed at pH 3.5 (sodium citrate) and 323 K by monitoring the increase in adenosine concentration by HPLC (Table 1). Data was analyzed using initial rates (1−2% of the reaction) and each data point is an average of at least two determinations. Due to the poor solubility of **1a** and **1b** in water, the assay included 4% DMSO as a cosolvent. The kinetic parameters, K_m (Michaelis−Menten constant) and k_{cat} (catalytic rate constant) of RNase U_2 with ApA were redetermined using our assay conditions and found to be 4.5 mM and 3 min^{-1} respectively. The value for K_m is an order of magnitude greater than previously reported while the k_{cat} is in general accord with literature precedence.[7] The apparent elevation of the K_m may be a result of two factors. First, the binding affinity of RNase U_2 for its substrates is known to be perturbed by increasing the percentage of certain organic solvents in acetate buffer.[47] Second, the dinucleotide ApA is only poorly water soluble and the original K_m (234 ± 35 μM) was determined by fitting experimental data obtained from only 10−80 μM of substrate.[7] The addition of DMSO confers increased solubility on the dinucleotide, and our estimation of K_m was based on substrate concentrations up to 2 mM.

All the rhenium complexes assayed were inhibitors of RNase U_2-catalyzed depolymerization of RNA. Figure 2 shows representative Dixon[48] plots for the rhenium complexes **1a** and **1b**, suggesting that the inhibition of RNase by these complexes is competitive in nature. This result was further supported by the fact that Lineweaver−Burke analyses gave similar K_i values and there was no effect on V_{max} with increase in inhibitor concentration.

It is generally accepted that the geometry of phosphorus in the transition state for both of the steps in RNA depolymerization is trigonal bipyramidal (TBP),[9,10] with the negative charge around phosphorus being stabilized by key histidine and lysine residues in the binding site. However there is also much evidence to support a geometry of a distorted trigonal bipyramid tending toward square pyramidal (SP).[49] Theoretical calculations by Holmes[50,51] and co-workers in the presence or absence of amino acid residues, to allow a detailed study of the enzymatic mechanism and catalytic residues, indicate a pentacoordinate transition state for the enzyme-catalyzed reaction, with geometry approaching SP. The O(2′)−P−O(5′) bond angle is 162.2° in the minimum-energy pentacoordinate transition state, which represents a 50% distortion of TBP geometry along the Berry pseudorotation coordinate toward a SP structure. These theoretical analyses are supported by X-ray crystallography data for RNase A complexed with uridine−vanadate which show the core vanadium atom existing in a distorted trigonal-bipyramidal geometry with the axial planes O2′−V−O7 bond angle being 156° rather than the theoretical 180°.[19,23]

Re complexes of diamide-dimercaptide (DADT)-based ligands exist either in a pure or distorted SP geometry.[34,35,52] The Re═O bond is axial and the rhenium classically adopts a position approximately 0.72 Å above the basal plane of the N,N,S,S ligands. There is a formal negative charge associated with these complexes which resides in a delocalized manner around the ligand sphere. Therefore these complexes seemed a good starting point for development of a new class of RNase inhibitors. The inhibition studies support our concept, with K_is of the complexes **1a** and **1b** being 15 and 93 μM respectively (Table 1), which for comparison is the same order of magnitude as the inhibition by vanadate complexes reported by Leinhard[17] for RNase A. If we assume that the K_m is a true dissociation constant, then the purine-based Re complexes bind a factor of 50−300 times stronger to RNase U_2 than does the substrate, which is a critical aspect of a potential TS analog. While at present, we do not have any X-ray diffraction analyses of these Re complexes, there is little reason to doubt that they exist as distorted SP complexes. Therefore, our results add further weight to the theory of a distorted TBP/SP transition state in the RNase mechanism. However, there are a number of reservations that makes further extrapolation of these complexes' mechanistic role limited. First, we have no direct structural information about these complexes which makes their precise orientation in the RNase U_2 binding site only speculative. In addition, the necessary DADT ligand structure around the Re core, which is not a component of vanadate inhibitors, may be too bulky to occupy other RNase binding sites meaning that these complexes may not serve as general RNase inhibitors. Work in our laboratory is underway to answer these questions.

In this study it was of interest to quantify the enzyme interactions and hence binding contribution of the purine base,

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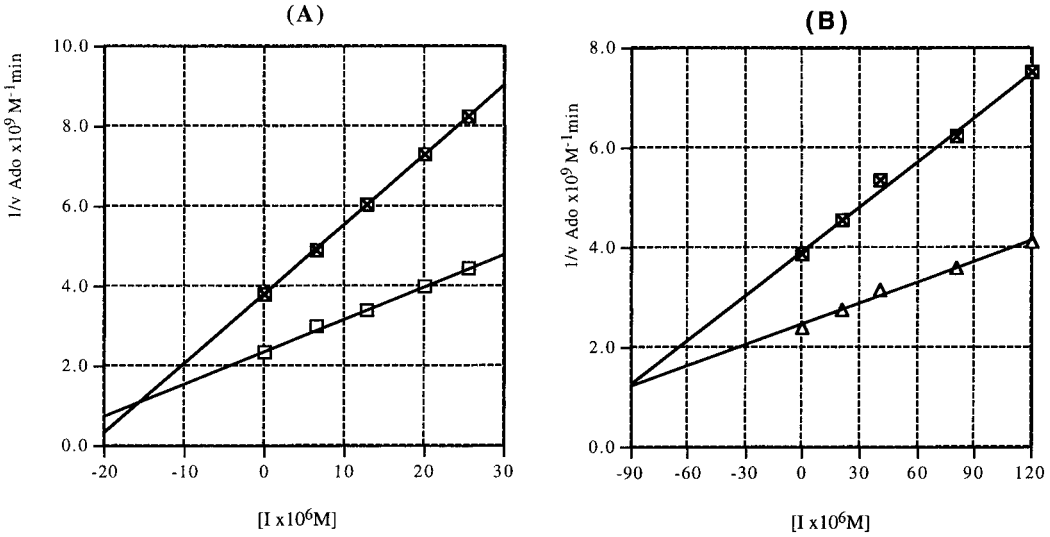

Figure 2. Dixon plots of the reciprocal reaction velocity *vs* inhibitor concentration showing the inhibitory effects of (A) **1a** *syn* and (B) **1b** *anti* in the hydrolysis of ApA by RNase U_2. Each point is the average of at least duplicate determinations.

the ribose ring, and the rhenium complex. A valid approach for assessing the binding contributions of different nucleoside moieties is to determine the ΔG^0 values for binding of appropriate ligand pairs.[53] The values of $\Delta\Delta G°$ obtained from the comparison do not only reflect the intrinsic binding free energy change of the specific ligand sites, but also include other coupled free energy and entropy terms.[54] This necessarily means that the energy of binding of the purine rhenium complexes **1a** and **1b** will be greater than the sum of the binding energies of the anhydropentofuranose inhibitors **2a** and **2b** and the base adenine. The kinetic studies support this view as there is a large potentiation in binding involving the purine base and the rhenium complexed with the ribose ring (Table 1). The Re complexes with a β-adenin-9-yl group (**1a** and **1b**) have $\Delta\Delta G°$ of $>2-3$ kcal mol^{-1} over the anhydropentofuranose inhibitors **2a** and **2b**. A more complete thermodynamic analysis is not possible because the low solubility of adenine meant that an accurate determination of its K_i was unfeasible. What is apparent however, is the importance of the purine base to the inhibition by these TS analogs. Quantification of the binding energy contribution in this case is not possible, but a recent study has shown for RNase A as much as 50% of the binding energy in the transition state is supplied by pyrimidine recognition by threonine, valine, and phenylalanine active-site residues.[24]

Concluding Discussion

In this study we have developed a new class of stable pentacoordinate oxometal complexes based on rhenium(V) with diaminonucleoside or diaminoaldopentofuranose ligands. The diaminonucleoside-based inhibitors **1a** and **1b** are excellent competitive inhibitors of a purine specific RNase. The geometry of these complexes is believed to be distorted TBP/SP which supports the rational of a distorted TBP transition state for RNA hydrolysis.

The ribonucleoside oxorhenium(V) are as potent as previously described vanadate−nucleoside complexes and other metallo−nucleoside complexes.[55] However, their much greater aqueous stability and decreased susceptibility to ligand exchange means that structural characterization of the oxorhenium(V) complexes is much more straightforward.

The most exciting feature of these oxorhenium(V)-based inhibitors is their potential generality. Our synthetic route is applicable for all nucleoside bases, and therefore rhenium complexes containing all four nucleoside bases can be now readily synthesized and their inhibition of a range of RNases and other phosphodiesterases assessed.

Finally, these complexes offer the first real strategy, as TS analogs for phosphodiester hydrolysis, which would be useful for catalytic antibody generation. Their distorted TBP geometry, and anionic charge coupled with aqueous stability makes them ideal candidates for haptens that may generate phosphodiesterase catalytic antibodies.

Experimental Section

General Procedures. Unless otherwise stated, all reactions were performed under an inert atmosphere, with dry reagents and solvents and flame-dried glassware. Analytical thin-layer chromatography (TLC) was performed using 0.25 mm silica gel coated Kieselgel 60 F_{254} plates. Visualization of the chromatogram was by UV absorbance, methanolic sulfuric acid, aqueous potassium permanganate, iodine, and *p*-anisaldehyde. Liquid chromatography was performed using compressed air (flash chromatography) with the indicated solvent system and silica gel 60 (230−400 mesh). Preparative TLC was performed using Merck 1 mm coated silica gel Kieselgel 60 F_{254} plates. Infrared spectra were recorded on a Nicolet FTIR spectrometer and are reported as wavenumbers (cm^{-1}). ^{1}H NMR spectra were recorded on either a Bruker AMX-300 or a Bruker AMX-500 spectrometer. Chemical shifts are reported in parts per million (ppm) on the δ scale from an internal standard. ^{13}C NMR (proton decoupled) spectra were recorded on a Bruker AMX-500 spectrometer at 125.77 MHz. High-resolution mass spectra (HRMS) were recorded on a VG ZAB-VSE mass spectrometer. High-performance liquid chromatography (HPLC) was performed either with a Hitachi D7000 series machine equipped with an Adsorbosphere HS RP-C18 analytical column, or a Rainin preparative HPLC instrument with a VYDAC RP-C18 90 Å pharmaceutical semipreparative column.

2,5:3,4-Dianhydro-D-lyxose Diethyl Acetal (5). Sodium methoxide (684 mg, 11.8 mmol) in methanol (50 mL) was added dropwise to a stirring solution of 2,5-anhydro-3-*O*-benzoyl-1,2-*O*-methylethylidene-4-*O*-(*p*-toluenesulfonyl)-L-xylose diethyl acetal (**4**) (5.0 g, 10.7 mmol) in methanol (120 mL) which had been cooled on ice. The reaction mixture was allowed to warm to room temperature and was stirred for 8 h. The volatiles were removed *in vacuo*, and the residue, dissolved in ethyl acetate (EtOAc) (100 mL), was washed with water (3 × 50 mL), 5% citric acid (2 × 50 mL), saturated aqueous sodium bicarbonate ($NaHCO_3$) (3 × 50 mL), and brine (2 × 50 mL). The combined organic fractions were then dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to give a tan oil which was purified by silica gel chromatography

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(25% EtOAc/hexane). This gave the title compound as a colorless oil (1.5 g, 74.6%): $R_f = 0.3$ (50% EtOAc/hexane); ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.53 (d, *J* 7.5 Hz, 1H, H1), 4.08 (d, J^2 10.7 Hz, 1H, H5a), 3.83 (dd, *J* 2.0 Hz, 1H, H2), 3.80−2.60 (m, 7H, H3, H4, H5b, 2 × CH_3CH_2O), 1.26 (t, *J* 7.1 Hz, 3H, C*H*$_3$CH$_2$O), 1.25 (t, *J* 7.1 Hz, 3H, C*H*$_3$CH$_2$O); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ 101.52, 78.07, 67.91, 63.85, 62.32, 56.47, 55.95, 15.26, 15.11; LRFABMS (*m*/*z*)$^+$ 211 (M + Na)$^+$.

4-*O*-Acetyl-2,5-anhydro-3-azido-3-deoxy-D-arabinose Diethyl Acetal (6a) and 3-*O*-Acetyl-2,5-anhydro-4-azido-4-deoxy-D-xylose Diethyl Acetal (6b). NaN_3 (7.5 g, 115.00 mmol) was added to a stirred solution of the epoxide **5** (4.74 g, 25.18 mmol) in DMF (100 mL), and the mixture was heated under reflux for 8 h. The DMF was removed *in vacuo*, and the brown residue was dissolved in ethyl acetate and washed sequentially with water, aqueous $NaHCO_3$, and brine. The combined organic fractions were then dried over Na_2SO_4. Following filtration of the drying agent, evaporation of the EtOAc *in vacuo* gave a crude mixture of diastereomeric azido alcohols. This mixture was stirred overnight in acetic anhydride (15 mL, 125 mmol) and pyridine (15 mL). The pyridine was then removed *in vacuo* to yield a brown residue which was dissolved in DCM (200 mL) and washed with water, aqueous $NaHCO_3$ and brine, and the combined organic fractions were dried over Na_2SO_4 and evaporated to dryness. The two title diastereomeric acetates were isolated following silica gel purification (EtOAc/petroleum ether 1:10) as pale yellow oils (**6a**, 2.20 g, 32%, **6b**, 3.22 g, 47%) in a ratio of *arabino*/*xylo* (2:3), **6a**-*arabino*: $R_f = 0.5$ (EtOAc/petroleum ether 1:10); ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.99 (ddd, *J* 2.5 Hz, *J* 5.1 Hz, *J* 2.6 Hz, 1H, H4), 4.56 (d, *J* 5.3 Hz, 1H, H1), 4.1 (dd, *J* 5.2 Hz, *J* 2.5 Hz, 1H, H2), 3.98 (dd, 1H, H5a), 3.92 (dd, 1H, 5b), 3.84−3.70 (m, 3H, H3, CH_3CH_2O), 3.68−3.58 (m, 2H, CH_3CH_2O), 2.10 (s, 3H, COC*H*$_3$), 1.25 (t, *J* 7.1 Hz, 3H, C*H*$_3$CH$_2$O), 1.23 (t, *J* 7.1 Hz, 3H, C*H*$_3$CH$_2$O); LRFABMS (*m*/*z*)$^+$ 296 (M + Na)$^+$, 228 (M − OEt)$^+$, M − OEt − HOAc)$^+$. **6b**-*xylo*; $R_f = 0.3$ (EtOAc/petroleum ether 1:10); ^{1}H NMR (300 MHz, $CDCl_3$) v 5.25 (dd, *J* 4.9 Hz, *J* 1.3 Hz, 1H, H3), 4.66 (d, *J* 7.5 Hz, 1H, H1), 4.21 (dd, J^2 9.9 Hz, *J* 5.6 Hz, 1H, H5a), 4.09 (dd, *J* 4.9 Hz, 1H, H2), 4.08 (dd, *J* 4.2 Hz, 1H, H5b), 3.78−3.61 (m, 3H, H4, CH_3CH_2O), 3.55−3.45 (m, 2H, CH_3CH_2O), 2.11 (s, 3H, COC*H*$_3$), 1.26 (t, *J* 7.1 Hz, 3H, C*H*$_3$CH$_2$O), 1.17 (t, *J* 7.1 Hz, 3H, C*H*$_3$CH$_2$O); LRFABMS (*m*/*z*)$^+$ 296 (M + Na)$^+$, 228 (M − OEt)$^+$.

2,5-Anhydro-4-azido-4-deoxy-D-xylose Diethyl Acetal (8). The azidoacetate (3.25 g, 11.91 mmol) was dissolved in MeOH (70 mL) and cooled on ice. To this stirred solution was added sodium methoxide (90 mg, 0.60 mmol) in one portion. The reaction mixture was then allowed to warm to room temperature and stirred for 3 h. The volatiles were then removed *in vacuo*, and the crude residue was purified by silica gel chromatography (EtOAc/petroleum ether 1:10). This yielded the title compound as a colorless oil (2.60 g, 94%): $R_f = 0.1$ (EtOAc/petroleum ether 1:10); ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.77 (d, *J* 4.6 Hz, 1H, H1), 4.37 (ddd, *J* 3.5 Hz, *J* 3.5 Hz, *J* 1.5 Hz, 1H, H3), 4.24 (dd, J^2 9.8 Hz, *J* 4.7 Hz, 1H, H5a), 4.05 (ddt, 1H, *J* 1.9 Hz, H4), 4.00 (dd, 1H, H2), 3.82 (dd, 1H, H5b), 3.85−3.74 (m, 2H, CH_3CH_2O), 3.72−3.56 (m, 2H, CH_3CH_2O), 1.60 (s, 1H, O*H*), 1.26 (t, *J* 7.1 Hz, 3H, C*H*$_3$CH$_2$O), 1.24 (t, *J* 7.1 Hz, 3H, C*H*$_3$CH$_2$O); LRFABMS (*m*/*z*)$^+$ 254 (M + Na)$^+$, 232 (M + H)$^+$.

2,5-Anhydro-3,4-azido-3,4-dideoxy-D-ribose Diethyl Acetal (7). Trifluoromethanesulfonic anhydride (733 mg, 2.6 mmol) was added dropwise, over a 10 min period, to a cold (0 °C) stirred solution of the alcohol **8** (0.5 g, 2.16 mmol) and (*N*,*N*-dimethylamino)pyridine (DMAP) (792 mg, 6.48 mmol) in DCM (20 mL). After 15 min the reaction mixture was poured into a cold 1% acetic acid solution (20 mL). The mixture was partitioned and the organic layer washed with cold $NaHCO_3$ (3 × 20 mL) and brine (1 × 20 mL). The combined organic fractions were then dried over Na_2SO_4, filtered, and dried *in vacuo*, to give the crude triflate (850 mg, 90%) which was used without further purification. The triflate was dissolved in DMF (6 mL) and LiN_3 (217 mg, 4.35 mmol) was added in one portion. The reaction mixture was stirred at room temperature for 4 h and then diluted with EtOAc (50 mL). The mixture was then washed with water (3 × 100 mL), aqueous $NaHCO_3$ (2 × 50 mL), and brine (2 × 50 mL). The combined organic fractions were then dried and evaporated as described for **4**. The crude residue was purified by silica gel chromatography (EtOAc/petroleum ether 1:10) to give the title compound as a colorless oil (532 mg, 80%): $R_f = 0.4$ (EtOAc/petroleum ether 1:10); ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.54 (d, *J* 3.5 Hz, 1H, H1), 4.33 (dd, *J* 5.6 Hz, *J* 5.6 Hz, 1H, H3), 4.11 (ddd, *J* 5.0 Hz, *I* 4.1 Hz, 1H, H4), 4.03 (dd, 1H, J^2 9.5 Hz, H5a), 4.00 (dd, 1H, H2), 3.81 (dd, 1H, H5b) 3.86−3.71 (m, 2H, CH_3CH_2O), 3.66−3.53 (m, 2H, CH_3CH_2O), 1.26 (t, *J* 7.1 Hz, 3H, C*H*$_3$-CH$_2$O), 1.24 (t, *J* 7.1 Hz, 3H, C*H*$_3$CH$_2$O); LRFABMS (*m*/*z*)$^+$ 279 (M + Na)$^+$, 211 (M − OEt)$^+$.

2,5-Anhydro-3,4-bis(2-chloroacetamido)-3,4-dideoxy-D-ribose Diethyl Acetal (9). Palladium on carbon (50 mg) was added to a solution of the diazide **7** (479 mg, 1.87 mmol) in MeOH (5 mL). The reaction mixture was then shaken on a Parr hydrogenator under 30 psi of H_2. After 2 h, the reaction mixture was filtered through Celite and the solvent was removed *in vacuo* to give the diamine **7a** as a colorless oil (332 mg, 82%): $R_f = 0.2$ (isopropyl alcohol/ethanol/water 5:3:2); ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.48 (d, *J* 5.3 Hz, 1H, H1), 4.02 (dd, 1H, J^2 9.2 Hz, *J* 4.8 Hz, H5a), 3.86−3.69 (m, 2H, CH_3CH_2O), 3.66−3.55 (m, 4H, H5b, CH_3CH_2O), 3.47−3.42 (m, 1H), 3.35 (dd, 1H, *J* 6.0 Hz, *J* 6.7 Hz, 1H), 1.23 (t, *J* 7.1 Hz, 3H, C*H*$_3$CH$_2$O), 1.24 (t, *J* 7.1 Hz, 3H, C*H*$_3$CH$_2$O); LRFABMS (*m*/*z*)$^+$ 205 (M + H)$^+$, 113 (M − 2OEt)$^+$. A solution of the diamine **7a** (317 mg, 1.55 mmol) in DCM (5 mL) was added dropwise to a cold (0 °C), vigorously stirred, suspension of $NaHCO_3$ (1.3 g, 15.5 mmol) and chloroacetyl chloride (876 mg, 7.8 mmol) in DCM (10 mL). TLC analysis (EtOAc/petroleum ether 1:10) showed the reaction was complete after 2 h. The reaction mixture was diluted with DCM (20 mL), poured onto ice water, and partitioned. The organic layer was repeatedly washed with water (3 × 50 mL), aqueous $NaHCO_3$ (2 × 50 mL), and brine (2 × 50 mL). The combined organic fractions were then dried and evaporated as described above for **4**. The residue was purified by silica gel chromatography (EtOAc/petroleum ether 1:7) to give the title compound as a white solid, which crystallized from EtOAc/Ether to give needles (431 mg, 90%); $R_f = 0.3$ (EtOAc/petroleum ether 5:1); mp 194−198 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.94 (bs, 1H, N*H*), 6.86 (bs, 1H, N*H*), 4.69 (dddd, *J* 7.0 Hz, *J* 5.8 Hz, *J* 4.7 Hz, *J* 6.5 Hz, 1H, H4), 4.55 (d, *J* 4.4 Hz, 1H, H1), 4.42 (ddd, *J* 7.0 Hz, *J* 7.0 Hz, H3), 4.25 (dd, J^2 9.5 Hz, 1H, H5a), 4.05 (s, 4H, 2 × C*H*$_2$Cl), 3.97 (dd, 1H, H2), 3.88 (dd, 1H, H5b), 3.84−3.72 (m, 2H, CH_3CH_2O), 3.66−3.55 (m, 2H, CH_3CH_2O), 1.24 (t, *J* 7.1 Hz, 3H, C*H*$_3$CH$_2$O), 1.22 (t, *J* 7.1 Hz, 3H, C*H*$_3$CH$_2$O); 13C NMR (125.77 MHz, DMSO-d_6) δ 165.94, 165.61, 102.30, 81.07, 70.07, 63.44, 62.19, 51.79, 51.09, 42.63, 15.31, 15.26; LRFABMS (*m*/*z*)$^+$ 357/359 (M + H)$^+$, 313/311 (M − OEt)$^+$; HRFABMS calcd for $C_{13}H_{22}N_2O_5Cl_2$ 3570984, obsd 357.0977.

2,5-Anhydro-3,4-bis[(benzoylthio)acetamido]-3,4-dideoxy-D-ribose Diethyl Acetal (10). A freshly prepared solution of sodium thiobenzoate in ethanol [3.13 mL (0.84 M), 2.62 mmol)] was added dropwise to a stirred solution of **9** (447 mg, 1.19 mmol) in ethanol (20 mL). The reaction mixture was heated under reflux for 2 h. After cooling, the volatiles were removed *in vacuo*, and the residue was dissolved in DCM (50 mL), and partitioned with water (50 mL). The organic layer was then washed with aqueous $NaHCO_3$ (3 × 50 mL) and brine (2 × 50 mL). The combined organic fractions were dried and evaporated as described for **4**. This gave a yellow solid which was purified by silica gel chromatography to yield the title compound as a white solid (491 mg, 74%): $R_f = 0.8$ (EtOAc/methanol 20:1); mp 208−210 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 7.95 (m, 4H, Ar-H), 7.62 (m, 2H, Ar-H), 7.48 (m, 4H, Ar-H), 4.61−4.56 (m, 2H, H3, H4), 4.50 (d, *J* 4.7 Hz, 1H, H1), 4.13 (dd, J^2 9.0 Hz, *J* 5.7 Hz, H5a), 3.90 (dd, *J* 5.6 Hz, 1H, H2), 3.84 (s, 2H, COC*H*$_2$S), 3.82 (s, 2H, COC*H*$_2$S), 3.78−3.66 (m, 3H, H5b, CH_3CH_2O), 3.65−3.52 (m, 2H, CH_3CH_2O), 1.17 (t, *J* 7.1 Hz, 3H, C*H*$_3$CH$_2$O), 1.16 (t, *J* 7.1 Hz, 3H, C*H*$_3$CH$_2$O); ^{13}C NMR (125.77 MHz, DMSO-d_6) δ 192.82, 191.65, 166.42, 165.98, 153.21, 150.15, 140.21, 136.05, 135.95, 133.15, 133.02, 102.35, 86.21, 84.29, 81.01, 79.98, 51.82, 51.15, 51.09, 42.48, 15.30, 15.29; LRFABMS (*m*/*z*)$^+$ 583 (M + Na)$^+$, 561 (M + H)$^+$, 515 (M − OEt)$^+$; HRFABMS calcd for $C_{27}H_{32}N_2O_7S_2$ 561.1729, obsd 561.1717.

[1,4-Anhydro-2,3-dideoxy-2,3-bis(mercaptoacetamido)-D-ribitol]-oxorhenium(V) (2a-*syn* and 2b-*anti*). A. Rhenium Incorporation. The bis(thiobenzoate) **10** (68 mg, 0.12 mmol) was dissolved in a methanolic sodium acetate (1 M) solution (5 mL) and heated under reflux for 1 h. Immediately after cooling of the reaction mixture oxotrichlorobis(triphenylphosphine)rhenium (V) (111 mg, 0.15 mmol)

was added, and the mixture was reheated under reflux for a further 45 min. The solution turns black during the reaction. The solvent was removed *in vacuo*, and the residue was purified by silica gel chromatography (EtOAc/MeOH 5:1), to give a 1:2 *syn*/*anti* mixture which was used without further purification; LRESMS $(m/z)^-$ 551/549 **B. Acetal Hydrolysis and Aldehyde Reduction.** The diastereomeric *syn*/*anti* mixture from above was dissolved in a 50% aqueous TFA solution (3 mL) and stirred overnight at room temperature. Following repeated evaporation of the volatiles *in vacuo*, the crude mixture of aldehydes were dissolved in ethanol (4 mL) and 4 mL of an ethanolic sodium borohydride solution (0.1 M) were added dropwise with stirring. The reaction was stirred at room temperature for 6 h, and then the solvent was removed *in vacuo*. The solid residue was purified by silica gel chromatography (EtOAc/MeOH 3:1) to give the title compound as a 1:2 *syn*/*anti* mixture (40 mg, 98%). The diastereoisomers were separated by preparative HPLC [(65% acetonitrile/35% water (0.1% TFA)]. **2a** *syn*: t_R = 10.51 min; mp 288−292 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 5.00 (m, *J* 6.8 Hz, *J* 5.6 Hz, *J* 5.3 Hz, 1H, H2), 4.92 (dd, *J* 8.4 Hz, 1H, H3), 4.54 (d, J^2 17.9 Hz, 1H, COCH_αS), 4.50 (dd, J^2 9.3 Hz, 1H, H1$_\alpha$), 4.45 (d, J^2 18.3 Hz, 1H, COCH_αS), 4.09 (d, 1H, COCH_βS), 4.07 (d, 1H, COCH_βS), 3.92 (m, J^2 12.5 Hz, *J* 6.5 Hz, 2H, H4, H5a), 3.82 (m, J^2 12.5 Hz, 1H, H5b), 3.73 (dd, 1H, H1$_\beta$); ^{13}C NMR (101 MHz, CD_3OD) δ 192.01, 191.87, 87.10, 74.26, 72.4, 72.08, 62.81, 42.09, 41.92; UV λ_{max} (H_2O) 228 nm, ϵ 8600; IR (KBr) 943 cm^{-1}; LRESMS $(m/z)^-$ 477/479 $(M)^-$. **2b** *anti*: t_R = 10.09 min; mp 294−297 °C; ^{1}H NMR (300 MHz, CD_3COCD_3) δ 4.92 (d, J^2 18.2 Hz, 1H, COCH_βS), 4.90 (m, *J* 6.3 Hz, *J* 6.3 Hz, 1H, H2), 4.73 (dd, *J* 9.1 Hz, *J* 3.9 Hz, 1H, H3), 4.72 (d, J^2 18.0 Hz, 1H, COCH_βS), 4.49 (dd, J^2 9.3 Hz, 1H, H1$_\alpha$), 3.99 (m, *J* 7.6 Hz, *J* 3.9 Hz, 1H, H4), 3.92 (m, 2H, 5a, 5b), 3.89 (d, 1H, COCH_αS), 3.82 (d, 1H, COCH_αS), 3.43 (dd, 1H, H1$_\beta$); ^{13}C NMR (125.77 MHz, CD_3OD) δ 191.25, 190.51, 87.05, 74.27, 73.31, 71.05, 70.66, 44.82, 44.61; UV λ_{max} (H_2O) 230 nm ϵ 8900; IR (KBr) 960 cm^{-1}; LRESMS $(m/z)^-$ 479/477 $(M)^-$.

9-[2′,3′-Bis(chloroacetamido)-2′,3′-dideoxy-β-D-ribofuranosyl]adenine (14). Diaminoadenosine (**13**) (1.7 g, 6.6 mmol) was added in one portion to a stirred suspension of sodium bicarbonate (5.5 g, 65.7 mmol) and chloroacetyl chloride (1.15 mL, 14.5 mmol) in DMF (100 mL) on ice. The reaction mixture was allowed to warm to room temperature and stirred until reaction was complete as judged by TLC (80% DCM/MeOH). The DMF was then evaporated *in vacuo*. The residue was dissolved in methanol (50 mL), silica gel (100 g) was added and then evaporated to dryness *in vacuo*. Silica gel chromatography (95% DCM/MeOH) of the crude residue gave the title compound (1.9 g, 80%) as a white solid: R_f 0.4 (80% DCM/MeOH); mp 220 °C dec; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.55 (d, 1H, *J* 8.6 Hz, N*H*), 8.40 (d, 1H, *J* 9.0 Hz, N*H*), 8.35 (s, 1H, Ar−H), 8.05 (s, 1H, Ar−H), 7.35 (bs, 2H, NH_2), 6.12 (d, 1H, *J* 7.5 Hz, 1H, H1′), 5.40 (t, 1H, *J* 6.4 Hz, O*H*), 5.05 (dd, 1H, J), 4.6; ^{13}C NMR (125.77 MHz, DMSO-d_6) δ 166.42, 166.27, 156.13, 152.67, 149.29, 139.18, 119.01, 85.40, 83.89, 61.91, 54.49, 50.73, 42.71, 42.36; HRFABMS calcd for $C_{14}H_{18}N_7O_4Cl_2$ 418.0755, obsd 418.0762.

9-[2′,3′-Bis[(benzoylthio)cetamido]-2′,3′-dideoxy-β-D-ribofuranosyl]adenine (15). Freshly prepared sodium thiobenzoate solution (10.14 mL, 0.25 M, 2.6 mmol) was added dropwise to a stirred solution of the bis(chloroacetamide) **14** (370 mg, 0.88 mmol) in ethanol (50 mL). The reaction mixture was then heated under reflux for 2 h. During cooling, a white solid precipitated from the reaction mixture. This precipitate was filtered and recrystallized (ethanol/ether) to give the title compound as white needles (421 mg, 78%): R_f 0.3 (80% DCM/MeOH); mp 202−204 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.80 (d, 1H, *J* 8.6 Hz, N*H*), 8.65 (d, 1H, *J* 9.0 Hz, N*H*), 8.35 (s, 1H, Ar−H), 8.12 (s, 1H, Ar−H), 7.94 (d, 2H, Ar−H), 7.85 (d, 2H, Ar−H), 7.78 (m, 2H, Ar−H), 7.55 (m, 4H, Ar−H), 7.42 (bs, 2H, NH_2), 6.12 (d, 1H, *J* 7.5 Hz, H$_1$′), 5.45 (bs, 1H, O*H*), 5.05 (dd, 1H, *J* 3.5 Hz, H2′), 4.58 (ddd 1H, *J* 5.6 Hz, H3′), 4.12 (m, 1H, H4′), 3.92 (s, 2H, CH_2Cl), 3.82 (s, 2H, CH_2Cl), 3.66 (dd, 1H, J^2 9.8 Hz, H5a′), 3.55 (dd, 1H, *J* 3.7 Hz, H5b′); ^{13}C NMR (125.77 MHz, DMSO-d_6) δ 190.47, 190.12, 167.28, 167.10, 155.82, 152.26, 149.17, 139.51, 135.96, 135.85, 134.09, 134.04, 129.17, 129.12, 126.88, 118.96, 86.21, 84.27, 61.83, 54.96, 54.51, 50.68, 50.57; HRFABMS calcd for $C_{28}H_{27}N_7O_6S_2$ 622.1543, obsd 622.1557.

[9-[2′,3′-Bis(mercaptoacetamido)-2′,3′-dideoxy-β-D-ribofuranosyl]-adenine]oxorhenium(V) (1a-*syn* and 1b-anti). To a stirred solution of the bis thioester (**15**) (200 mg, 0.32 mmol) in degassed ethanol/water (1:1) (40 mL) was added trichlorobis(triphenylphosphine)-rhenium(V) oxide (400 mg, 0.48 mmol) and aqueous 1 M sodium hydroxide (3.2 mL, 3.2 mmol). The reaction mixture was heated at 80 °C for 20 min. On cooling, a white precipitate of sodium benzoate formed and was filtered. The mother liquor was evaporated *in vacuo* to give a brown solid. Purification by reversed-phase preparative HPLC [22% acetonitrile, 78% water (0.1% TFA)] gave the title compounds as orange powders **1a** *syn* (86 mg, 44%) and **1b** *anti* (44 mg, 23%). **1a** (*syn*): t_R 22.4 min; R_f 0.2 (EtOAC/MeOH 3:1); mp 285−289 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.80 (s, 1H, Ar−H), 8.45 (s, 1H, Ar−H), 6.08 (d, 1H, *J* 3.5 Hz, H1′), 5.15 (m, 2H, H2′, H3′), 4.10 (m, 1H, H4′), 4.04 (d, 1H, J^2 17 Hz, CH_βS), 3.94 (d, 1H, J^2 18.3 Hz, CH_βS), 3.87 (dd, 1H, J^2 11.97 Hz, *J* 2.35 Hz, H5a′), 3.78 (d, 1H, CH_αS), 3.75 (dd, 1H, *J* 5.93 Hz, H5b′), 3.65 (d, 1H, COCH_αS); ^{13}C NMR (125.77 MHz, DMSO-d_6) δ 192.08, 191.74, 150.91, 148.29, 146.16, 142.86, 118.47, 90.17, 88.04, 75.61, 69.91, 62.81, 40.71, 40.18; UV λ_{max} 236 nm, ϵ 21 680; IR (KBr) 950 cm^{-1}; LRESMS $(m/z)^-$ 612/610 $(M)^-$, 477/475 (M − adenine)$^-$. **1b** *anti*: t_R 26.8 min; R_f 0.25 (EtOAC/MeOH 3:1); mp 273−278 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.55 (s, 1H, *J* 8.6 Hz, Ar−H), 8.65 8.32 (s, 1H, Ar−H), 8.12 (s, 1H, Ar−H), 6.12 (d, 1H, *J* 4.3 Hz, H1′), 5.35 (m, 1H, H2′), 5.15 (m, 1H, H3′), 4.10 (dd, 1H, *J* 5.7 Hz, H4′), 4.07 (d, 1H, J^2 16.3 Hz, COCH_αS), 3.86 (d, 1H, J^2 18.4 Hz, COCH_αS), 3.85 (dd, 1H, J^2 11.8 Hz, *J* 2.02 Hz, H5a′), 3.80 (m, 2H, H5b′, COCH_βS), 3.60 (d, 1H, J^2 18.4 Hz, COCH_βS); ^{13}C NMR (125.77 MHz, DMSO-d_6) δ 192.08, 191.95, 154.01, 149.82, 146.72, 128.93, 90.24, 89.52, 72.93, 69.82, 62.83, 40.91, 40.68; UV λ_{max} 238 nm, ϵ 19 670; IR (KBr) 975 cm^{-1}; LRESMS $(m/z)^-$ 612/610 $(M)^-$.

General Kinetic Method. Rigorous precautions were taken to minimize the risk of nonspecific ribonuclease contamination. All glassware was heated at 180 °C for 5 h prior to use. All buffers were prepared daily and were autoclaved prior to use. The urea was purchased as RNase free from Sigma Chemical Co. and used as received.

We used a modification of our previously reported procedure to follow the formation of adenosine and 3′,5′-cyclic adenosine monophosphate from ApA, catalyzed by RNase U_2.[25] The assay mixture comprised of 10 units of sequencing grade RNase U_2, 16.6 mM sodium citrate (pH 4.5), 0.85 mM EDTA, 3.5 M urea, and the desired concentration of substrate and inhibitor (a stock solution prepared in DMSO) to a final volume of 50 μL (4% DMSO). After addition of the substrate the reaction vessels were vortexed and heated in a water bath at 50 ± 0.1 °C.

Data Collection. Sealed tubes, compatible with use in a D-7000 series Hitachi autosampler, were used as reaction vessels. Each tube was taken out of the incubator every 1−2 h for HPLC analysis. Aliquots were removed, and the components were separated on an Alltech Adsorbosphere HS C-18 reversed-phase column at 260 nm, using an isocratic elution system with a flow rate of 1 mL min^{-1}. The mobile phase was 7% acetonitrile/93% ammonium acetate (50 mM, pH 4.5). The observed rates were linear over the time period followed. To obtain each rate, 5 or 6 time points were taken. The substrate (ApA) concentrations ranged from 60 μM to 4 mM for the K_m and k_{cat} determinations. Inhibitor concentrations ranged from 6 μM to 2 mM. Every rate plot had a correlation coefficient of at least 0.985 and was repeated either two or three times. The data was analyzed by using standard nonlinear regression (enzyme kinetics) analysis using the GraFit computer program on a Compaq PC and linear regression analysis for the Dixon plots on a Macintosh computer.

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