radioactivity per sample were then loaded onto a 0,4-mm denaturing polyacrylamide gel (8% 19:1 acrylamide/bisacrylamide, 50% urea), and electrophoresed with TBE buffer (100 mM Tris base, 89 mM boric acid, 2 mM EDTA) at 1400 V for 2–3 h. After electrophoresis the gels were exposed to Kodak X-Omat RP film with intensifying screen at −70 °C. Analyses of the autoradiograms demonstrated that the sequences of the DNAs used in this study matched the published sequences[47] of pBR322 plasmid and ϕX174 RF I DNA.

(47) pBR322 plasmid: (a) Sutcliffe, J. G. *Nucleic Acids Res.* **1978**, *5*, 2721. (b) Sutcliffe, J. G. *Cold Spring Harbor Symp. Quant. Biol.* **1979**, *43*, 77. ϕX174 RF I DNA: Sanger, F.; Coulson, A. R.; Friedmann, T.; Air, G. M.; Barrell, B. G.; Brown, N. L.; Fiddes, J. C.; Hutchison, C. A.; Slocombe, P. M.; Smith, M. *J. Mol. Biol.* **1978**, *125*, 225.

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Toward Chemical Ribonucleases. 2. Synthesis and Characterization of Nucleoside–Bipyridine Conjugates. Hydrolytic Cleavage of RNA by Their Copper(II) Complexes

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Contribution from the Monsanto Company, St. Louis, Missouri 63167. *Received June 8, 1990*

Abstract: As part of our program to develop chemical ribonucleases that cleave RNA by phosphodiester hydrolysis, a systematic study of covalently linked nucleoside-2,2'-bipyridine (bpy) conjugates is described. 2′-Deoxythymidine was attached at both its 3′- and 5′-positions to bpy derivatives by using phosphoramidite chemistry, yielding after deprotection ammonium 2′-deoxythymidine 3′-[4-(4′-methyl-2,2′-bipyridin-4-yl)butyl phosphate] (**8**) and triethylammonium 2′-deoxythymidine 5′-[4-[4′-methyl-2,2′-bipyridin-4-yl]butyl phosphate] (**11**). 2′-Deoxyuridine was attached to a modified bpy via derivatization of the uracil ring at C-5, giving 5-[3-[[2-[[4-(4′-methyl-2,2′-bipyridin-4-yl)-1-oxobutyl]amino]ethyl]amino]-3-oxopropyl]-2′-deoxyuridine (**16**). These conjugates and the intermediate bpy derivatives were fully characterized by mass spectrometry and ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy. The ability of the bpy moieties to bind Cu(II) was demonstrated spectroscopically. The copper(II) complexes of **8**, **11**, and **16** were shown to hydrolyze RNA at 37 °C and neutral pH. The difference in reactivity of **8**, **11**, and **16** provides the basis for optimizing the activity of hydrolytic chemical nucleases.

Introduction

Oligonucleotides covalently linked to metal complexes have been employed in a variety of studies that capitalize on the selective binding ability of DNA and the properties of metal complexes. Thus, oligonucleotides can be directed in a Watson–Crick fashion toward complementary, single-stranded nucleic acids, or via triple-helix formation toward double-stranded DNA targets.[1] The properties that metal complexes can provide include reactivity, i.e., oxidative cleavage behavior,[2] and fluorescence, for labeling purposes.[3] Among the most elegant examples in this area are the "chemical nucleases", which cleave nucleic acids in a sequence-directed manner, and which are composed of a single-stranded oligonucleotide linked to a redox-active metal complex such as Cu(II)(o-phenanthroline)$_2$,[4] Fe^{II}EDTA,[5] or iron porphyrins.[6] Cleavage is thought to be effected by metal-bound or free hydroxyl radicals.

Cleavage of DNA or RNA via hydrolysis of the phosphodiester backbone would have distinct advantages over its oxidative counterpart. Hydrolysis would not require redox cofactors to mediate the chemistry nor would highly reactive oxene or oxy radical species be generated. In addition, hydrolytic manipulation of nucleic acid polymers would generate fragments that are chemically competent for ligation to other oligonucleotides by routine enzymatic reactions. Accordingly, there has been considerable interest in developing DNA and RNA hydrolysis catalysts and in probing the mechanism of metal-catalyzed hydrolysis of phosphate esters. Most of these studies have used activated p-nitrophenyl phosphate esters or phosphate anhydrides as substrates.[7] However, there are examples of metal-promoted hy-

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(4) (a) Sigman, D. S. *Acc. Chem. Res.* **1986**, *19*, 180–186. (b) Chen, C.-H. B.; Sigman, D. S. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 7147–7151. (c) Chen, C.-H. B.; Sigman, D. S. *J. Am. Chem. Soc.* **1988**, *110*, 6570–6572. (d) Chu, B. C. F.; Orgel, L. E. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 963–967.
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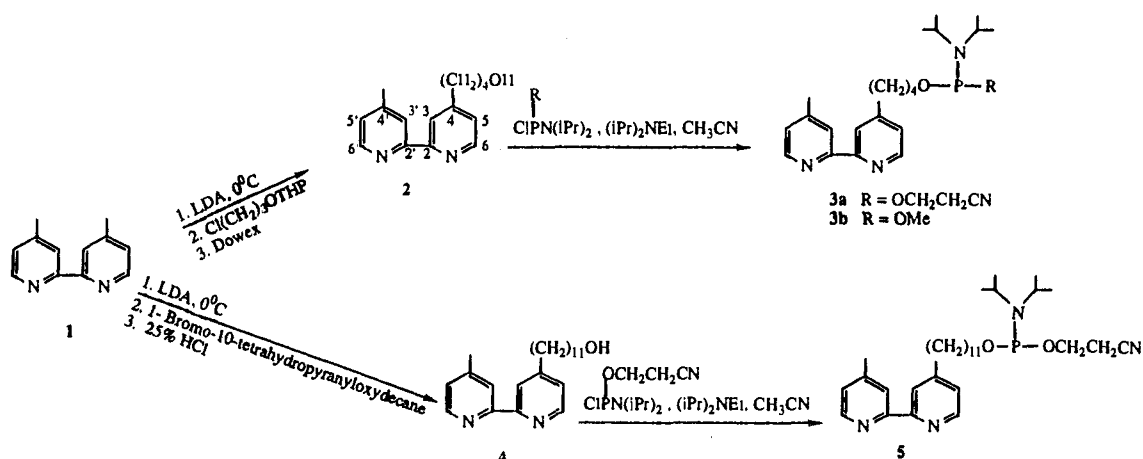
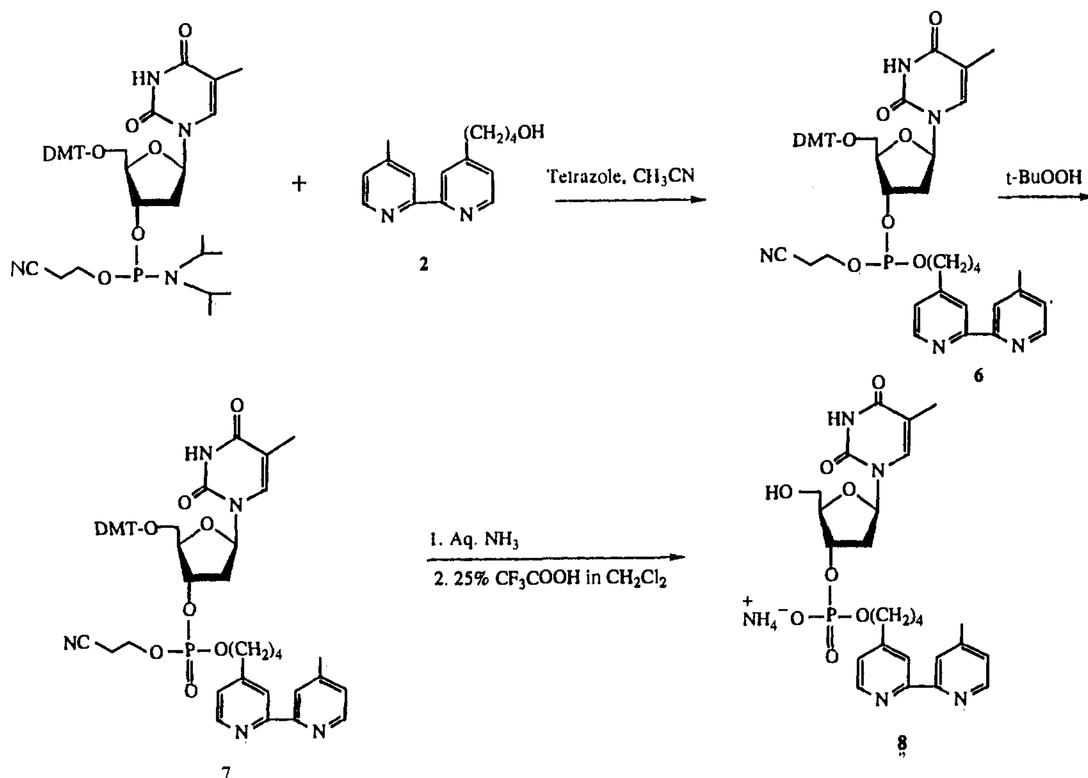
Scheme I



Scheme II



drolysis of unactivated phosphate esters such as those found in DNA and RNA. Metal-activated hydrolytic cleavage of DNA has been reported by Barton,[8a] and tetraamine complexes of Co(III) have been shown to promote the hydrolysis of adenosine monophosphates.[8] In addition, many divalent metal cations are known to catalyze the hydrolysis of RNA.[9]

We have recently reported[10] the first examples of hydrolytic cleavage of RNA oligomers by characterized metal complexes. Among the active species described was $Cu(bpy)^{2+}$ (bpy = 2,2′-bipyridyl). As part of our program to prepare chemical ribonucleases that operate by a hydrolytic mechanism,[11] we wished to develop a series of routes to oligonucleotides with pendant RNA hydrolysis agents. The present study describes the preparation and characterization of nucleosides and nucleotides with attached bpy ligands and the hydrolysis of RNA by their Cu(II) complexes. In order to determine an optimum structure for the chemical ribonucleases reported here, the chemistry was developed to allow the attachment of bpy at both the 3′- and 5′-termini of nucleotides, as well as at C-5 of 2′-deoxyuridine. In particular, phosphoramidite chemistry reminiscent of DNA synthesis protocols was used to attach bpy derivatives to thymidine via 5′- and 3′-phosphodiester bonds, and Bergstrom's modification[12] of the Heck reaction[13] was used to prepare the 5-substituted 2′-deoxyuridine derivative. All novel compounds were extensively characterized by ^1H and ^{13}C NMR and mass spectrometry.

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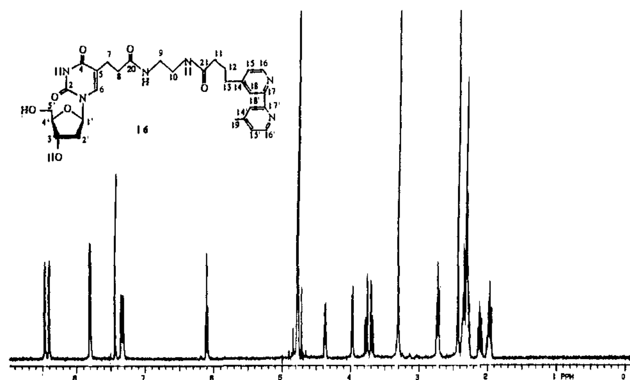

Figure 1. Compound **16** characterized by proton NMR. Spectra were recorded on a Varian VXR 400 spectrometer operating at 399.9 MHz. Spectra were accumulated by using a 10-μs pulse width, 5999-Hz sweep width, 2.50-s acquisition time, 1.0-s recycle delay, and zero filling with 3K points. The presaturation method of solvent suppression was used to decrease the size of the residual HOD peak. A drop of DCl was added to improve the solubility of **16**.

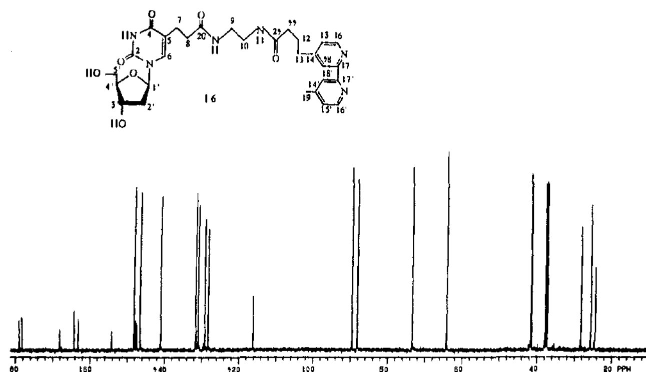

Figure 2. Compound **16** characterized by carbon NMR. Spectra were recorded on a Varian VXR 400 spectrometer operating at 399.9 MHz (proton). Spectra were accumulated by using a 9-μs pulse width, 25 kHz sweep width, 0.6-s acquisition time, and Fourier transformed after application of a 1.0-Hz line-broadening function. A drop of DCl was added to improve the solubility of **16**.

Results

A. Synthesis and Characterization of Bpy–Thymidine Derivatives. A variety of side-chain derivatives of 2,2′-bipyridine are available. We chose to base our syntheses on 4,4′-dimethyl-2,2′-bipyridine (**1**) because its monolithiation with LDA in THF gives a versatile substrate for electrophiles and provides modified, unsymmetrical bipyridines.[14] An additional advantage of **1** is that it allows the construction of a linker arm para to the pyridine nitrogen; as molecular models show, this helps eliminate interactions between a coordinated metal and any functional groups that occur in the linker arm (such as phosphate). A functionalized side chain was introduced by the reaction of lithiated **1** with 2-(3-chloropropoxy)tetrahydropyran, followed by treatment with Dowex 50 W to yield 4-(4′-hydroxybutyl)-4′-methyl-2,2′-bipyridine (**2**). This alcohol, for which an alternative synthesis was recently published,[15] was employed as a precursor to phosphoramidite reagents. Thus, 4-(4′-methyl-2,2′-bipyridin-4-yl)butyl β-cyanoethyl *N,N*-diisopropylphosphoramidite (**3a**) and 4-(4′-methyl-2,2′-bipyridin-4-yl)butyl methyl *N,N*-diisopropylphosphoramidite (**3b**) were prepared by phosphorylation of **2** with the appropriate reagent, as shown in Scheme I.

Lithiated **1**, on treatment with 1-bromo-10-(tetrahydropyranyloxy)decene in THF at 0 °C, followed by acid hydrolysis, yielded 4′-methyl-4-(11-hydroxyundecyl)-2,2′-bipyridine (**4**). Phosphorylation of **4** with 3-cyanoethyl chloro-*N,N*-diisopropylphosphoramidite gave 4-(4-methyl-2,2′-bipyridin-4-yl)undecyl β-cyanoethyl *N,N*-diisopropylphosphoramidite (**5**).

5′-*O*-DMT-2′-deoxythymidine 3′-[β-cyanoethyl *N,N*-diisopropylphosphoramidite] (DMT = bis(4-methoxyphenyl)phenylmethyl) was coupled with alcohol **2** in the presence of tetrazole to give the intermediate **6** with the bipyridine ligand attached at the 3′-position. Attempted oxidation of **6** by the conventional automated DNA synthesis protocol,[16] using I_2/THF/water, led to extensive hydrolysis. However, oxidation of **6** with *tert*-butyl hydroperoxide[17] gave the phosphotriester **7**. Deprotection of **7** sequentially with ammonia and acid yielded **8** (Scheme II).

Similarly, 3′-*O*-acetylthymidine was coupled with phosphoramidite **3b** in the presence of tetrazole, yielding the intermediate **9** with a bipyridine ligand at the 5′-position. Oxidation of **9** with *tert*-butyl hydroperoxide gave the phosphotriester **10**. Deprotection of **7** with NaOMe and thiophenol gave **11** (Scheme III).

As shown in Scheme IV, in order to introduce bpy at the 5-position of 2′-deoxyuridine, the active ester **12** was prepared by dicyclohexylcarbodiimide (DCC) coupling of *p*-nitrophenol to a known carboxylic acid.[15] Preparation of the analogous NHS ester is described in the Experimental Section; an impure preparation of the related compound succinimidyl-4-carboxy-4′-methyl-2,2′-bipyridine was reported earlier, with no spectroscopic data provided.[18] Reaction of ester **12** with the substituted 2′-deoxyuridine **14** resulted in the 5′-DMT-protected nucleoside **15** via amide bond formation. Acid deprotection led to nucleoside **16**, whose ^1H and ^{13}C NMR spectra are shown in Figures 1 and 2.

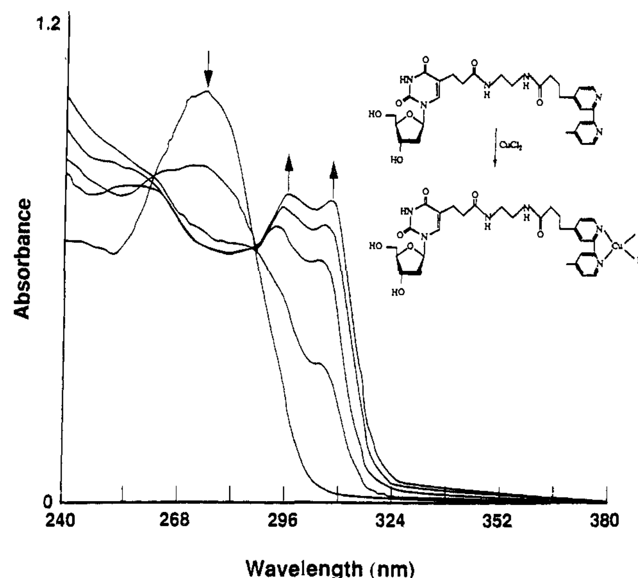

Figure 3. Electronic absorption spectra of the titration of **16** with $CuCl_2$. The concentration of **16** was 5.34×10^{-5} M in 20 mM HEPES buffer (pH 7.1). $CuCl_2$ was added in 0.25-equiv portions until formation of the **16**-Cu(II) complex was complete (λ_{max} = 300, 312 nm).

Ligand–nucleoside conjugates, complex phosphoramidites, and nucleotide derivatives such as those reported here have rarely been fully characterized in the literature. In the present study, compounds **2**, **3a**, **3b**, **4**, **5**, **7**, **8**, **10–13**, **15**, and **16** were fully characterized by 1-D and 2-D NMR (^1H, ^{13}C, and ^{31}P) and mass spectral studies. Complete NMR assignments are provided in the Experimental Section. Assignments were obtained through

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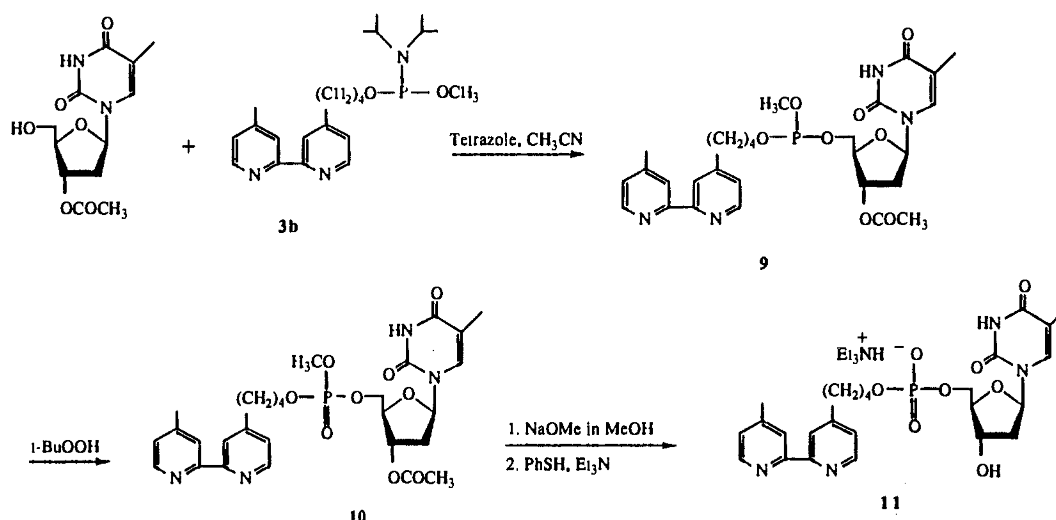
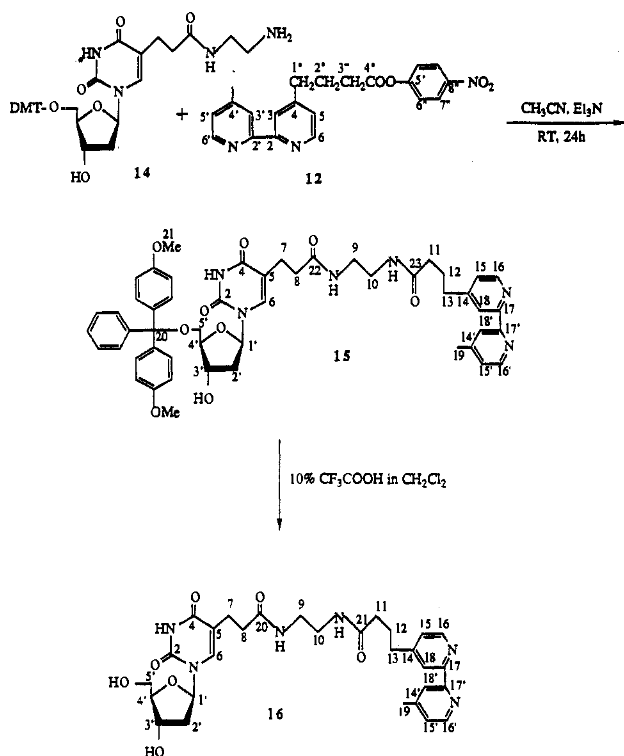
(19) Reference deleted in proof.

Scheme III



Scheme IV



extensive 1- and 2-D ^{1}H, ^{13}C, and ^{31}P NMR studies, and the spectra resulting from these studies are provided in the supplementary material.

Titration of Bpy-Nucleosides and Nucleotides with $CuCl_2$. Confirmation that the bpy ligand attached to the thymidine nucleotides and uridine nucleoside is capable of coordinating Cu(II) was provided by the titration of these compounds with aqueous $CuCl_2$. Shown in Figure 3 are the changes in the visible spectrum associated with the titration of 5-substituted 2′-deoxyuridine **16**. The addition of $CuCl_2$ causes the band at 276 nm to decrease with concomitant increase in absorbances at 300 and 312 nm. These changes occur with an isosbestic point at 289 nm and are characteristic of coordination of Cu^{2+} to bipyridine.[20] Similar spectral changes were observed for the titration of both 3′- and 5′-bpy-thymidine, **8** and **11**. When thymidine 3′-monophosphate, thymidine 5′-monophosphate, and uridine were titrated with $CuCl_2$, there were no changes in the visible spectrum between 240 and 380 nm.

(20) Titration of bipyridine with $CuCl_2$ generated a spectra nearly identical (λ_{max} = 302, 312 nm) with the one shown in Figure 3.

Table I. Extent of $Poly(A)_{12-18}$ Hydrolysis by Copper(II) Complexes[a]

ligand	[ligand], μM	[$CuCl_2$], μM	% substrate hydrolyzed (±5%)[b]
bpy	157	157	78
bpy	157	0	0
8	157	157	21
8	157	0	0
11	157	157	21
11	157	0	0
16[c]	157	157	65
16	157	157	88
16	157	0	7
		157[c]	35
		157	54
bpy + EDTA	157	157	0
8 + EDTA	157	157	0
11 + EDTA	157	157	0
16 + EDTA	157	157	0

[a] All reactions were run at 37 °C for 48 h except where noted. EDTA was present at 500 μM where noted. [b] The percent cleavage reported is the average of two reactions. [c] Reactions run for 24 h.

B. Cleavage of RNA by Cu(II) Complexes of 8, 11, 16, and Bipyridine. The Cu(II) complexes of **8**, **11**, **16**, and bipyridine were incubated with the RNA homopolymer $poly(A)_{12-18}$ at 37 °C at pH = 7.1 for 24 or 48 h. A typical example of the ion-exchange HPLC analysis of these reactions is shown in Figure 4. The data contained in Table I demonstrates that all of the Cu(II) complexes described here are capable of hydrolyzing RNA oligomers, albeit with varying degrees of activity. Control reactions run in the absence of Cu(II) but in the presence of ligand showed no RNA degradation. In the case of the control reaction containing **16**, a small amount of hydrolysis was observed (Figure 4). The addition of EDTA to reaction mixtures containing both ligand and Cu(II) resulted in the complete inhibition of hydrolysis. Accordingly, we conclude that ribonuclease contamination is not responsible for the observed cleavage of RNA oligomers.[21]

A comparison of the reactivity of $Cu(bpy)^{2+}$ with DNA and RNA was made. Thus, $Cu(bpy)^{2+}$ was reacted with $poly(dA)_{12-18}$ and $poly(A)_{12-18}$ under identical conditions. Figure 5 contains the HPLC analysis of these reactions. After 48 h, the RNA is extensively degraded, but the DNA substrate was not cleaved by $Cu(bpy)^{2+}$. Adenosine 2′,3′-cyclic monophosphate (2′,3′-cyclic AMP) was identified by reverse-phase HPLC as a major product

(21) Ribonucleases are inhibited by a variety of metal cations and EDTA is known stimulate ribonuclease activity by chelation of metal cations: Uchida, T.; Egami, F. *The Enzymes*, 3rd ed.; Academic Press: New York, 1971; Vol III, p 205.

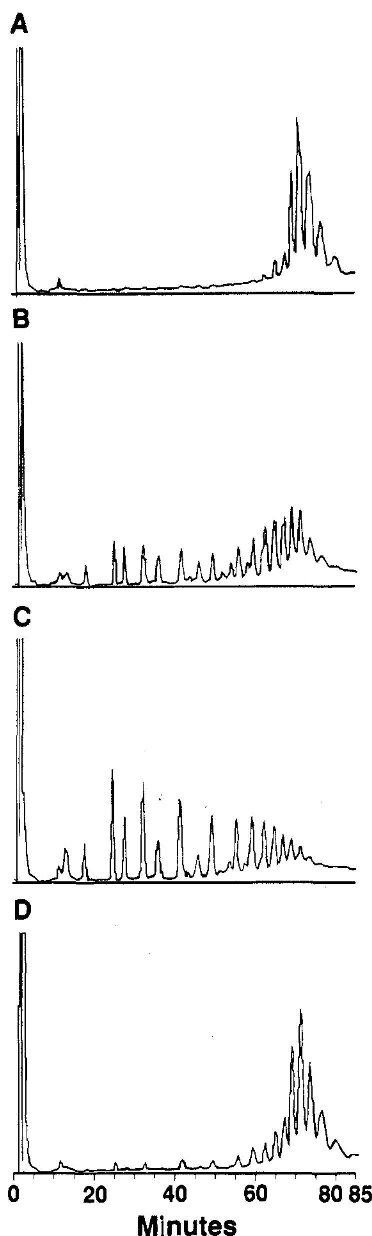


Figure 4. Reaction of the Cu(II) complex of **16** with $poly(A)_{12-18}$ at 37 °C; $[CuCl_2]$ = 157 μM, [**16**] = 157 μM, $[poly(A)_{12-18}]$ = 63 μM. (A) t = 0 h; (B) t = 24 h; (C) t = 48 h; (D) control reaction, **16** in the absences of Cu(II), t = 48 h.

of the reaction between $Cu(bpy)^{2+}$ and $poly(A)_{12-18}$.

Discussion

We recently reported[10] that a variety of transition-metal complexes are capable of hydrolyzing RNA, and $Cu(bpy)^{2+}$ was one of the active species described. In this study we have developed the chemistry to attach the bpy ligand to nucleosides at three distinct positions. The synthetic procedures and novel reagents described are amenable to the preparation of oligonucleotide–bpy conjugates by standard solid-phase protocols,[16] so that $Cu(bpy)^{2+}$ could be delivered to an oligonucleotide to a specific RNA sequence. For example, one can envision using phosphoramidite **3b** to link bpy to the 5′-end of an oligonucleotide by solid-phase synthesis. The active esters **12** and **13** may also be suitable for coupling to oligonucleotides with pendant amino groups. By taking advantage of the 3′, 5′, and uridine C-5 sites of attachment, and by varying the length and conformational flexibility of the linker arms, we have in hand the chemical tools to probe optimal structures for RNA hydrolysis agents.

There are particular advantages to each site of attachment for the bpy ligand, especially with respect to extending this chemistry

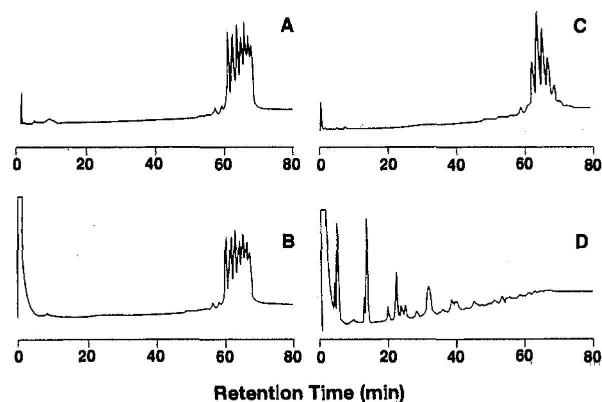


Figure 5. Ion-exchange HPLC analysis of the reaction of $Cu(bpy)^{2+}$ with $poly(dA)_{12-18}$ and $poly(A)_{12-18}$. (A) $poly(dA)_{12-18}$ control reaction; (B) $poly(dA)_{12-18}$ plus $Cu(bpy)^{2+}$; (C) $poly(A)_{12-18}$ control reaction; (D) $poly(A)_{12-18}$ plus $Cu(bpy)^{2+}$.

to oligonucleotide-directed RNA hydrolysis, in which an oligonucleotide would hybridize to a complementary sequence of single-stranded RNA and deliver a hydrolytically active metal complex in a sequence-directed manner. Molecular models of DNA–RNA duplexes with A-type helices[22] reveal that a metal complex pendant from the 5′-position of the oligonucleotide probe would reach across the major groove to the target RNA stand. In contrast, 3′-linkage would deliver the cleavage agent across the minor groove. Attaching a hydrolysis agent at C-5 of 2′-deoxyuridine allows its incorporation at any position in an oligonucleotide sequence; as in the case of 5′-linkage, the pendant group will fall in the major groove when the oligonucleotide is hybridized to an RNA strand. Unlike the 3′- and 5′-derivatives, the C-5-modified oligonucleotide will have both ends available for enzymatic transformations such as ligation.

Hydrolysis of RNA by Cu(bpy) Nucleotide and Nucleoside Conjugates. Several methods were described that allow the attachment of bipyridine to nucleosides and nucleotides at either the base or sugar. The electronic absorption spectra of the titration of these derivatives with $CuCl_2$ confirms that **8**, **11**, and **16** form bipyridine–copper(II) complexes.

Our conclusion that the cleavage of RNA by the copper(II) complexes of **8**, **11**, **16**, and bipyridine proceeds via a hydrolytic mechanism and not oxidatively is supported by several experimental observations. The precautions taken to exclude ribonuclease contamination are described in the Experimental Section. Of particular importance is the inability of $Cu(bpy)^{2+}$ to degrade $poly(dA)_{12-18}$ while it extensively cleaves $poly(A)_{12-18}$ under identical reaction conditions.[23] It is known that RNA is more susceptible to hydrolysis than DNA, due to the presence of the 2′-OH.[24] However, there are literature reports that both RNA and DNA are oxidatively cleaved by 1,10-phenanthroline–copper(II) ($Cu(phen)_2^{2+}$) at similar rates.[25] Consequently, one would expect to see extensive cleavage of $poly(dA)_{12-18}$ and $poly(A)_{12-18}$ by $Cu(bpy)^{2+}$ if an oxidative mechanism were operative. In contrast, if the reaction were hydrolytic in nature, only $poly(A)_{12-18}$ would be cleaved. On the basis of the results presented in Figure 5, it is clear that the $Cu(bpy)^{2+}$-promoted cleavage of $poly(A)_{12-18}$ is hydrolytic.

Several other lines of evidence support the conclusion that the $Cu(bpy)^{2+}$ nucleoside and nucleotide conjugates hydrolyze RNA. The detection of 2′,3′-cyclic AMP as a product of the reactions between $Cu(bpy)^{2+}$ and $poly(A)_{12-18}$ is indicative of a hydrolytic

(22) Shakked, Z.; Kennard, O. In *Biological Macromolecules and Assemblies*; Jurnak, F. A., McPherson, A., Eds.; Wiley: New York, 1985; Vol. 2, Chapter 1.

(23) Both $Cu(bpy)^{2+}$ and $Cu(trpy)^{2+}$ have been shown to be ineffective at oxidatively cleaving DNA in the presence of thiol and O_2: Graham, D. R.; Marshall, L. E.; Reich, K. A.; Sigman, D. S. *J. Am. Chem. Soc.* **1980**, *102*, 5419–5421.

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cleavage mechanism. 2′,3′-Cyclic phosphates are produced in the hydrolysis of RNA by bovine pancreatic ribonuclease A.[26] In addition, imidazole buffers have been shown to cleave uridylyl-(3′,5′)uridine [(3′,5′)-UpU] to uridine 2′,3′-cyclic monophosphate and uridine.[27] Furthermore, we have recently reported[10] that HPLC traces of the hydrolysis of poly(A)$_{12-18}$ by the potentially redox active complexes Cu(2,2′:6,2″-terpyridine)$^{2+}$ and Cu(bpy)$^{2+}$ are nearly identical with those found for reactions with Zn(II) complexes such as that of 7-(*N*-methyl)-2,12-dimethyl-3,7,11,17-tetraazabicyclo[11.3.1]heptadeca-1(17),2,11,13,15-pentaene. However, when poly(A)$_{12-18}$ was reacted with Cu(phen)$_2^{2+}$ under conditions known to favor oxidative degradation of RNA, the anion-exchange HPLC trace of the reaction, and therefore the nature and distribution of the products, was entirely different.[25] Accordingly, we conclude from these results that the Cu(II) complexes of bpy, **8**, **11**, and **16** degrade poly(A)$_{12-18}$ by hydrolyzing its phosphodiester backbone, and that the mechanism of this reaction may be similar to those proposed for ribonuclease A and imidazole-catalyzed hydrolysis of RNA.[27]

The difference in the hydrolytic activity of the copper(II) complexes of **8**, **11**, and **16** is striking (Table I). That free Cu(bpy)$^{2+}$ is the most active leads us to suggest that linking bipyridine to nucleosides or nucleotides imposes certain steric constraints that attenuate the reactivity of Cu(bpy)$^{2+}$ toward RNA hydrolysis. The position of bipyridine attachment, length of linker arm, and overall charge of the complex influence the activity of these complexes. It is noteworthy that the Cu(II) complex of **16** is the most active, and it contains a linker arm that is longer than those incorporated into **8** and **11**. It is clear from the literature that the orientation of catalytic groups involved in phosphodiester hydrolysis is critical and can dramatically influence the rate of the reaction.[28] Thus, tethering a metal complex to a nucleoside, nucleotide, or oligonucleotide with a linker that allows for the proper approach to the targeted phosphodiester will be fundamental in the design of an efficient artificial ribonuclease.

Conclusions

Synthetic schemes for attaching bpy ligands to nucleosides were developed, providing the methodology for the attachment of bpy at both the 3′- and 5′-ends, and in the middle of, an oligonucleotide chain. First, 4-hydroxyalkyl derivatives of bpy were linked to thymidine at both the 3′- and 5′-positions by using phosphoramidite methods. These reactions essentially followed standard DNA synthesis protocols, except that the step oxidizing P(III) to P(V) had to be carried out under nonaqueous conditions, to avoid hydrolytic decomposition of the bpy–phosphate esters. The bpy ligand was also attached to 2′-deoxyuridine via the introduction of a linker arm at the C-5 position of the pyrimidine ring. The resulting nucleoside–bpy conjugates were fully characterized by NMR and mass spectral methods, they were shown to coordinate Cu(II) through the bpy moiety, and their Cu(II) complexes were shown to hydrolyze RNA oligomers. Since the linking techniques employed are amenable to automated DNA synthesis, and the linker arms can be varied in length and conformational flexibility, we are in a position to optimize the *oligo*nucleotide-directed hydrolysis of RNA by metal complexes.[29]

Experimental Section

General Procedures. Melting points were taken on a Melt-Temp apparatus equipped with a calibrated thermometer. Nuclear magnetic resonance spectra (^{1}H, ^{13}C, and ^{31}P) were recorded on Varian XL-200, VXR-300, or VXR-400 spectrometers. High-resolution mass spectra were recorded on a 40-250T spectrometer. Electronic absorption spectra were measured on a Beckman DU-70 spectrophotometer. Elemental analyses were determined by Galbraith Laboratories, Knoxville, TN. Thin-layer chromatography was performed on Baker-Flex silica gel IB2-F or Baker-Flex aluminum oxide IBF plates; spots were visualized by irradiation with UV light (254 nm). Column chromatography was performed on silica gel (Merck SG-60, 230–240 mesh) or neutral alumina (Brockman activity I, 80–200 mesh). Compounds **8**, **11**, and **16** were purified by RP HPLC using a linear ternary gradient flowing at 1.5 mL/min. Solvent A (0.1 M (Et$_3$NH)OAc) was kept constant while solvent B (MeCN) and solvent C (H$_2$O) were varied.

Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl prior to use. 2-(3-Chloropropoxy)tetrahydropyran (Alfred Baker Chemicals) was dissolved in diethyl ether and refluxed with Norit decolorizing charcoal (~1% by weight). After 20 min the solution was filtered through Celite, dried over 4-Å molecular sieves, and concentrated in vacuo. The residue was fractionally distilled under reduced pressure to yield pure compound (bp 60–85 °C at 22 mmHg). 1-Bromo-10-(tetrahydropyranyloxy)decane (Lancaster synthesis Ltd.), 4,4′-dimethyl-2,2′-bipyridine (Aldrich); *tert*-butyl hydroperoxide 3 M solution in 2,2,4,4-tetramethylpentane (Aldrich); tetrazole (Aldrich), diisopropylethylamine (Aldrich), chloro-(β-cyanoethoxy)-(*N*,*N*-diisopropylamino)phosphine (ABN), (chloromethoxy)-(*N*,*N*-diisopropylamino)phosphine (ABN), 3′-*O*-acetyl-2′-deoxythymidine (Sigma), 5′-*O*-DMT-2′-deoxythymidine 3′-[β-cyanoethyl *N*,*N*-diisopropylphosphoramidite] (Pharmacia), and (*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid) HEPES (Sigma) were used without further purification.

4′-Methyl-4-(4-hydroxybutyl)-2,2′-bipyridine (2). To a cooled solution (0 °C) of 4,4′-dimethyl-2,2′-bipyridine (10 g, 54.2 mmol) dissolved in dry THF (1000 mL) was added dropwise LDA (6.36 g, 59.6 mmol) in THF (300 mL). The resulting dark brown mixture was stirred for 1 h and purified 2-(3-chloropropoxy)tetrahydropyran (14.5 g, 81.3 mmol) in THF (50 mL) was added. The reaction mixture was allowed to warm to room temperature and stirred overnight; it was then quenched with water (5 mL), filtered through Celite, and concentrated to yield crude 4′-methyl-4-(4-*O*-THP-butyl)-2,2′-bipyridine. The compound was purified by column chromatography on TLC grade alumina (J. T. Baker Chemical Co.) and eluted with 5% EtOAc in petroleum ether. The product was dissolved in 50% CH$_2$Cl$_2$ in MeOH and refluxed with Dowex 50 W for 48 h. The resin was filtered off and washed with 50% CH$_2$Cl$_2$ in MeOH (2 × 50 mL). The combined organic layers were concentrated in vacuo, redissolved in CH$_2$Cl$_2$ (100 mL), and washed with saturated NaHCO$_3$ (2 × 25 mL). The organic fraction was dried over Na$_2$SO$_4$ and concentrated in vacuo to yield **2**: 7.7 g, 31.9 mmol, 59% yield; mp 33–34 °C (lit.[15] mp 32–35 °C); ^{1}H NMR (CDCl$_3$) δ 3.6 (t, 2 H, OC*H*$_2$(CH$_2$)$_3$, J_{HH} = 6.3 Hz), 1.6 (m, 2 H, OCH$_2$C*H*$_2$(CH$_2$)$_2$), 1.75 (m, 2 H, O(CH$_2$)$_2$C*H*$_2$CH$_2$), 2.7 (t, 2 H, O(CH$_2$)$_3$C*H*$_2$, J_{HH} = 7.5 Hz), 8.5 (m, 2 H, H6, H6′), 7.1 (m, 2 H, H5, H5′), 8.2 (br, 2 H, H3, H3′), 2.4 (s, 3 H, 4′-CH$_3$), 3.1 (br s, 1 H, OH ex); ^{13}C NMR (CDCl$_3$) ppm 62.7 (O*C*H$_2$(CH$_2$)$_3$), 32.7 (OCH$_2$*C*H$_2$(CH$_2$)$_2$), 27.0 (O(CH$_2$)$_2$*C*H$_2$CH$_2$), 35.6 (O(CH$_2$)$_3$*C*H$_2$), 156.4 (2′C), 156.5 (2C), 121.9 (3′C), 122.7 (3C), 148.7 (4′C), 153.0 (4C), 124.4 (5′C), 125.2 (5C), 149.3 (6′C), 149.5 (6C), 21.7 (4′-CH$_3$); MS *m*/*z* 243 (M + H). Anal. Calcd for C$_{15}$H$_{18}$N$_2$O: C, 74.36; H, 7.49; N, 11.56. Found: C, 74.09; H, 7.43; N, 11.39.

4-(4′-Methyl-2,2′-bipyridin-4-yl)butyl β-Cyanoethyl *N*,*N*-Diisopropylphosphoramidite (3a). Chloro(β-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine (0.418 g, 2.1 mmol) was dissolved in MeCN (10 mL) in a H-shaped Schlenk flask fitted with a filter frit. Diisopropylethylamine (0.520 mL, 4.0 mmol) was added and the mixture stirred at room temperature for 10 min. 4′-Methyl-4-(4-hydroxybutyl)-2,2′-bipyridine (0.484 g, 2.0 mmol) dissolved in MeCN (10 mL) was added, and stirring was continued for 30 min. The mixture was then filtered through the frit to remove the amine hydrochloride. The solid was washed with MeCN (2 × 10 mL) and concentrated to yield **3a** (0.804 g, 1.82 mmol, 91% yield). Column chromatography on silica gel or alumina led to decomposition of the product by hydrolysis, so unpurified **3a** was used for subsequent reactions: ^{1}H NMR (CD$_3$CN) δ 1.1 (2 d, 12 H, (C*H*$_3$)$_2$CH, J_{HH} = 4.7 Hz), 3.5 (m, 2 H, (CH$_3$)$_2$C*H*), 3.7 (m, 2 H, OC*H*$_2$CH$_2$CN), 2.6 (t, 2 H, OCH$_2$C*H*$_2$CN, J_{HH} = 5.9 Hz), 3.6 (m, 2 H, OC*H*$_2$(CH$_2$)$_3$, 1.6 (m, 2 H, OCH$_2$C*H*$_2$(CH$_2$)$_2$), 1.7 (m, 2 H, O(CH$_2$)$_2$C*H*$_2$CH$_2$), 2.7 (t, 2 H, O(CH$_2$)$_3$C*H*$_2$, J_{HH} = 7.7 Hz), 8.2 (m, 2 H, H3, H3′), 7.2 (m, 2 H, H5, H5′), 8.5 (m, 2 H, H6, H6′), 2.4 (s, 3 H, 4′-CH$_3$); ^{13}C NMR (CD$_3$CN) ppm 25.0 ((CH$_3$)$_2$CH, J_{PC} = 7.2 Hz), 43.8 ((CH$_3$)$_2$*C*H, J_{PC} = 12.3 Hz), 59.3 (O*C*H$_2$CH$_2$CN, J_{PC} = 18.9 Hz), 21.0 (OCH$_2$CH$_2$CN, J_{PC} = 6.8 Hz), 118.3 (OCH$_2$CH$_2$*C*N), 64.0 (O*C*H$_2$(CH$_2$)$_3$), J_{PC} = 17.1 Hz), 31.5 (OCH$_2$*C*H$_2$(CH$_2$)$_2$, J_{PC} = 7.1 Hz), 27.0 (O(CH$_2$)$_2$*C*H$_2$CH$_2$), 35.5 (O(CH$_2$)$_3$*C*H$_2$), 156.0 (2′C), 156.1 (2C), 121.7 (3′C), 122.5 (3C), 149.3 (4′C), 153.5 (4C), 125.0 (5′C), 125.7 (5C), 149.8 (6′C), 150.0 (6C), 21.3 (4′-*C*H$_3$); ^{31}P NMR (CD$_3$CN) ppm 148.5 (s); MS *m*/*z* 449 (M + Li), 396 (M + Li − CH$_2$CH$_2$CN).

4-(4′-Methyl-2,2′-bipyridin-4-yl)butyl Methyl *N*,*N*-Diisopropylphosphoramidite (3b). The procedure to synthesize **3b** was the same as reported for **3a** except THF was used instead of CH$_3$CN as the solvent for the reaction due to low solubility of chloromethoxy-*N*,*N*-diiso-

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propylaminophosphine in CH_3CN. Compound **3b** (85% yield) was purified on a Chromatotron with an alumina plate using CH_2Cl_2/EtOAc/Et_3N (7:2:1) as an eluant: ^1H NMR (CD_2Cl_2) δ 1.1 (2 d, 12 H, $(CH_3)_2CH$, J_{HH} = 6.6 Hz), 3.5 (m, 2 H, $(CH_3)_2CH$), 3.3 (2′ s, 3 H, OMe), 3.6 (m, 2 H, $OCH_2(CH_2)_3$), 1.6 (m, 2 H, $OCH_2CH_2(CH_2)_2$), 1.7 (m, 2 H, $O(CH_2)_2CH_2CH_2$), 2.7 (t, 2 H, $O(CH_2)_3CH_2$, J_{HH} = 7.7 Hz), 8.25 (m, 2 H, H3, H3′), 7.1 (m, 2 H, H5, H5′), 8.45 (m, 2 H, H6, H6′), 2.35 (s, 3 H, 4′-CH_3); ^{13}C NMR (CD_2Cl_2) ppm 25.0 ($(CH_3)_2CH$, J_{PC} = 7.3 Hz), 43.1 ($(CH_3)_2CH$, J_{PC} = 12.2 Hz), 50.8 (OMe, J_{PC} = 17.3 Hz), 63.8 ($OCH_2(CH_2)_3$, J_{PC} = 18.0 Hz), 31.7 ($OCH_2CH_2(CH_2)_2$, J_{PC} = 7.3 Hz), 27.7 ($O(CH_2)_2CH_2CH_2$), 35.5 ($O(CH_2)_3CH_2$), 156.4 (2′C), 156.5 (2C), 121.4 (3′C), 122.1 (3C), 148.4 (4′C), 152.8 (4C), 124.3 (5′C), 124.9 (5C), 149.2 (6′C), 149.3 (6C), 21.1 (4′-CH_3); ^{31}P NMR (CD_2Cl_2) ppm 148.3 (s); MS m/z 404 (M + H); 305 (M + H − N-(iPr)$_2$).

4′-Methyl-4-(11-hydroxyundecyl)-2,2′-bipyridine (4). 4,4′-Dimethyl-2,2′-bipyridine (8.84 g, 48.0 mmol) was dissolved in dry THF (1000 mL) at 0 °C and LDA (5.68 g, 53.0 mmol) in THF (50 mL) was added dropwise. The resulting dark brown mixture was stirred for 1 h and 1-bromo-10-(tetrahydropyranyloxy)decane (17.0 g, 53.0 mmol) in THF (50 mL) was added. The reaction mixture was allowed to warm to room temperature and was stirred overnight. The reaction was quenched with water (5 mL), filtered through Celite, and concentrated in vacuo to yield crude, THP-protected **4**. The residue was dissolved in THF (100 mL) and treated with 25% HCl solution (100 mL) for 1 h. This reaction mixture was concentrated, dissolved in CH_2Cl_2 (200 mL), and washed with saturated $NaHCO_3$ (2 × 50 mL). The organic fraction was dried over Na_2SO_4 and concentrated in vacuo. The residue was chromatographed on a Chromatotron (Harrison Research) using an alumina plate (Analtech) and the desired compound **4** eluted with 5% MeOH in CH_2Cl_2: 10.38 g, 30.5 mmol, 63.5% yield; mp 82–85 °C recrystallized from $CHCl_3$/petroleum ether; ^1H NMR ($CDCl_3$) δ 3.6 (m, 2 H, OCH_2), 1.65 (m, 2 H, OCH_2CH_2), 1.52 (m, 2 H, $O(CH_2)_2CH_2$), 1.3 (br s, 14 H, $O(CH_2)_3(CH_2)_7$), 2.7 (t, 2 H, $O(CH_2)_{10}CH_2$, J_{HH} = 7.5 Hz), 8.25 (m, 2 H, H3, H3′), 7.15 (m, 2 H, H5, H5′), 8.55 (m, 2 H, H6, H6′), 2.4 (s, 3 H, 4′-CH_3), 2.35 (t, 1 H, OH ex); ^{13}C NMR ($CDCl_3$) ppm 62.8 (OCH_2), 32.8 (OCH_2CH_2), 25.8 ($O(CH_2)_2CH_2$), 35.5 ($O(CH_2)_{10}CH_2$), 158.0 (2′C, 2C), 121.4 (3′C), 122.1 (3C), 148.2 (4′C), 153.0 (4C), 124.0 (5′C), 124.6 (5C), 148.7 (6′C), 148.9 (6C), 21.2 (4′-CH_3); MS m/z 341 (M + H). Anal. Calcd for $C_{22}H_{32}N_2O$: C, 77.6; H, 9.47; N, 8.23. Found: C, 77.3; H, 9.44; N, 7.84.

4-(4′-Methyl-2,2′-bipyridin-4-yl)undecyl β-Cyanoethyl *N,N*-Diisopropylphosphoramidite (5). Chloro(β-cyanoethoxy)(N,N-diisopropylamino)phosphine (0.71 g, 3.0 mmol) was dissolved in MeCN (15 mL) in an H-shaped Schlenk flask fitted with a filter frit. Diisopropylethylamine (0.775 mL, 6.0 mmol) was added and the mixture stirred at room temperature for 10 min. A solution of **4** (1.0 g, 3.0 mmol) in MeCN (15 mL) was added and the mixture was stirred for 60 min. The mixture was filtered through the frit to remove the amine hydrochloride. The solid hydrochloride was washed with MeCN (2 × 15 mL), and combined MeCN fractions were concentrated to yield **5** (80% pure by ^{31}P NMR). Column chromatography on silica gel or alumina led to decomposition of the product, so unpurified **5** was used for subsequent reactions: ^1H NMR (CD_3CN) δ 1.15 (2 d, 12 H, $(CH_3)_2CH$), 3.55 (m, 2 H, $(CH_3)_2CH$), 3.7 (m, 2 H, OCH_2CH_2CN), 2.6 (m, 2 H, OCH_2CH_2CN), 3.6 (m, 2 H, $OCH_2(CH_2)_3$), 1.6 (m, 4 H, $OCH_2(CH_2)_2$), 1.3 (br s, 14 H, $O(CH_2)_3(CH_2)_7$), 2.7 (m, 2 H, $O(CH_2)_{10}CH_2$), 8.2 (br s, 2 H, H3, H3′), 7.2 (m, 2 H, H5, H5′), 8.5 (m, 2 H, H6, H6′), 2.4 (s, 3 H, 4′-CH_3); ^{13}C NMR (CD_3CN) ppm 25.2 ($(CH_3)_2CH$, J_{PC} = 6.8 Hz), 43.8 ($(CH_3)_2CH$, J_{PC} = 12.2 Hz), 59.3 (OCH_2CH_2CN, J_{PC} = 18.6 Hz), 21.2 (OCH_2CH_2CN, J_{PC} = 7.1 Hz), OCH_2CH_2CN, 119.4; 64.5 ($OCH_2(CH_2)_3$, J_{PC} = 17.1 Hz), 32.2 ($OCH_2CH_2(CH_2)_9$, J_{PC} = 7.1 Hz), 26.9 ($O(CH_2)_9CH_2CH_2$), 36.2 ($O(CH_2)_{10}CH_2$), 156.9 (2′C, 2C), 121.8 (3′C), 122.5 (3C), 148.9 (4′C), 153.5 (4C), 125.0 (5′C), 125.7 (5C), 149.9 (6′C), 150.0 (6C), 21.6 (4′-CH_3); ^{31}P NMR (CD_3CN) ppm 143.7 (s).

5′-*O*-[Bis(4-methoxyphenyl)phenylmethyl]-2′-deoxythymidine 3′-[4-(4′-Methyl-2,2′-bipyridin-4-yl)butyl β-cyanoethyl phosphate] (7). 5′-O-[Bis(4-methoxyphenyl)phenylmethyl]-2′-deoxythymidin-3′-yl β-cyanoethyl N,N-diisopropylphosphoramidite (0.4 g, 0.537 mmol) was dissolved in anhydrous CH_3CN (5 mL) under N_2. Tetrazole (0.112 g, 1.61 mmol) was added, and the resulting mixture was stirred at room temperature for 15 min. A solution of **2** (0.130 g, 0.540 mmol) dissolved in anhydrous CH_3CN (5 mL) was added, and after 1 h, the mixture was concentrated to yield a glass. The glass was dissolved in CD_3CN for ^{31}P NMR, which showed the expected two singlets at 140 ppm, indicating the presence of diastereoisomers of **6**. The glass was dissolved in CH_2Cl_2 (3 mL), cooled to 0 °C in an ice bath, and t-BuOOH in 2,2,4,4-tetramethylpentane (0.643 mL, 1.93 mmol) was added. After 20 min, the mixture was concentrated in vacuo to yield a glass. The desired product **7** (0.382 g, 0.432 mmol, 80.4% yield) was eluted from an alumina column (neutral) by using 5% MeOH in CH_2Cl_2: ^1H NMR ($CDCl_3$) δ 6.4 (m, 1 H, H1′), 2.55 (m, 2 H, H2′), 5.1 (m, 1 H, H3′), 4.3 (m, 1 H, H4′), 3.5 (m, 2 H, H5′), 1.4 (s, 3 H, T-CH_3), 2.4 (s, 3 H, 4′-CH_3), 4.2 (m, 2 H, OCH_2CH_2CN), 2.7 (m, 2 H, OCH_2CH_2CN), 4.2 (m, 2 H, $OCH_2(CH_2)_3$), 1.75 (m, 2 H, $OCH_2CH_2(CH_2)_2$), 1.75 (m, 2 H, $O(CH_2)_2CH_2CH_2$), 2.7 (m, 2 H, $O(CH_2)_3CH_2$), 8.25 (m, 2 H, H3, H3′), 7.1 (m, 2 H, H5, H5′), 8.5 (m, 2 H, H6, H6′), 3.8 (s, 3 H, OCH_3); ^{13}C NMR ($CDCl_3$) ppm 85.0 (1′C), 39.6 (2′C), 79.6 (3′C), 85.0 (4′C), 63.8 (5′C), 87.8 (Ph_3C), 112.2 (5C), 135.7 (6C), 12.2 (T-CH_3), 21.7 (4′-CH_3), 55.8 (OMe), 164.2 (4CO), 150.9 (2CO), 62.6 (OCH_2CH_2CN), 20.6 (OCH_2CH_2CN), 116.8 (OCH_2CH_2CN), 68.9 ($OCH_2(CH_2)_3$), 30.2 ($OCH_2CH_2(CH_2)_2$), 26.7 ($O(CH_2)_2CH_2CH_2$), 35.3 ($O(CH_2)_3CH_2$), 156.4 (2′C), 156.9 (2C), 121.8 (3′C), 122.6 (3C), 148.8 (4′C), 152.2 (4C), 124.3 (5′C), 125.3 (5C), 149.4 (6′C), 149.7 (6C); ^{31}P NMR ($CDCl_3$) ppm −2.2 (2 s's, diastereoisomers); MS m/z 892 (M + Li), 839 (M + Li − CH_2CH_2CN).

Ammonium 2′-Deoxythymidine 3′-[4-(4′-Methyl-2,2′-bipyridin-4-yl)butyl phosphate] (8). Compound **7** (0.382 g, 0.432 mmol) was dissolved in aqueous NH_3 (10 mL) and left to stir at room temperature for 6 h. The mixture was concentrated by using EtOH to remove the water, and the residue was treated with 25% CF_3COOH in CH_2Cl_2 (5 mL) for 15 min. After the volatile components were removed, the residue was dissolved in water (10 mL) and the aqueous layer was washed with ether (2 × 5 mL) and CH_2Cl_2 (2 × 5 mL). The aqueous layer was concentrated to yield the desired deprotected nucleoside **8**: 0.192 g, 0.354 mmol, 82% yield: ^1H NMR (D_2O) δ 6.0 (t, 1 H, H1′), 2.2 (m, 2 H, H2′), 4.7 (m, 1 H, H3′), 4.1 (m, 1 H, H4′), 3.75 (m, 2 H, H5′), 7.4 (s, 1 H, H6), 1.7 (s, 3 H, T-CH_3), 2.35 (s, 3 H, 4′-CH_3), 3.95 (m, 2 H, $OCH_2(CH_2)_3$), 1.75 (m, 2 H, $OCH_2CH_2(CH_2)_2$), 1.75 (m, 2 H, $O(CH_2)_2CH_2CH_2$), 2.7 (t, 2 H, $O(CH_2)_3CH_2$), 7.7 (2 s's, 2 H, H3, H3′), 7.25 (m, 2 H, H5, H5′), 8.35 (m, 2 H, H6, H6′); ^{13}C NMR (D_2O) ppm 88.2 (1′C), 40.9 (J_{PC} = 3.6 Hz, 2′C), 78.4 (J_{PC} = 5.0 Hz, 3′C), 89.2 (J_{PC} = 5.5 Hz, 4′C), 64.5 (5′C), 114.4 (5C), 140.3 (6C), 14.8 (T-CH_3), 23.9 (4′-CH_3), 169.2 (4CO), 154.3 (2CO), 69.3 (J_{PC} = 5.9 Hz, $OCH_2(CH_2)_3$), 32.5 (J_{PC} = 6.6 Hz, $OCH_2CH_2(CH_2)_2$), 28.9 ($O(CH_2)_2CH_2CH_2$), 37.5 ($O(CH_2)_3CH_2$), 156.3 (2′C), 156.5 (2C), 125.1 (3′C), 125.9 (3C), 154.3 (4′C), 158.1 (4C), 128.3 (5′C), 128.9 (5C), 151.1 (6′C), 151.4 (6C), ^{31}P NMR (D_2O) ppm 0.35 (s); MS m/z 569 (M + Na), 547 (M + H); exact mass found 552.45386; calcd for $C_{25}H_{30}N_4O_8Li$ 552.45261.

3′-*O*-Acetyl-2′-deoxythymidine 5′-[4-(4′-Methyl-2,2′-bipyridin-4-yl)butyl methyl phosphate] (10). To **3b** (0.101 g, 0.25 mmol) dissolved in THF (1 mL) was added tetrazole (0.021 g, 0.3 mmol), and the mixture was stirred at room temperature for 10 min. 3′-O-Acetyl-2′-deoxythymidine (0.071 g, 0.25 mmol) dissolved in CH_2Cl_2 (1 mL) was added to the reaction mixture and stirred for 60 min. The mixture was filtered to remove the tetrazole. The solid was washed with MeCN (5 mL) and CH_2Cl_2 (5 mL) and concentrated to yield a glass **9**. The glass was dissolved in MeOH (1 mL) and cooled to 0 °C, and *tert*-butyl hydroperoxide 3 M solution in 2,2,4,4-tetramethylpentane (0.3 mL, 0.9 mmol) was added to the stirred reaction mixture. After 15 min, the ice bath was removed and the mixture was stirred at room temperature for 20 min. The mixture was concentrated to a glass and flash chromatographed on an alumina column (TLC grade). The desired compound **10** (0.071 g, 0.118 mmol, 47% yield) was eluted with a gradient of CH_2Cl_2 to 10% MeOH in CH_2Cl_2: ^1H NMR ($CDCl_3$) δ 6.35 (m, 1 H, H1′), 2.25 (m, 2 H, H2′), 5.25 (m, 1 H, H3′), 4.1 (m, 1 H, H4′), 4.3 (m, 2 H, H5′), 1.9 (s, 3 H, T-CH_3), 2.45 (s, 3 H, 4′-CH_3), 2.1 (s, 3 H, $OCOCH_3$), 3.8 (2 d, 3 H, OMe), 4.3 (m, 2 H, $OCH_2(CH_2)_3$), 1.8 (m, 2 H, $OCH_2CH_2(CH_2)_2$), 1.8 (m, 2 H, $O(CH_2)_2CH_2CH_2$), 2.75 (m, 2 H, $O(CH_2)_3CH_2$), 8.2 (m, 2 H, H3, H3′), 7.1 (m, 2 H, H5, H5′), 8.5 (m, 2 H, H6, H6′); ^{13}C NMR ($CDCl_3$) ppm 84.5 (1′C), 37.2 (2′C), 74.4 (3′C), 82.8 (4′C), 67.9 (5′C), 111.8 (5C), 134.9 (6C), 12.4 (T-CH_3), 21.2 (4′-CH_3), 163.7 (4CO), 150.6 (2CO), 55.5 (OMe), 67.1 ($OCH_2(CH_2)_3$), 29.8 ($OCH_2CH_2(CH_2)_2$), 26.2 ($O(CH_2)_2CH_2CH_2$), 34.8 ($O(CH_2)_3CH_2$), 155.9 (2′C), 156.3 (2C), 121.2 (3′C), 122.1 (3C), 148.2 (4′C), 151.5 (4C), 123.8 (5′C), 124.7 (5C), 148.9 (6′C), 149.1 (6C), 20.8 ($COCH_3$), 170.4 ($COCH_3$); ^{31}P NMR ($CDCl_3$) ppm 0.9 (2 s's, diastereoisomers); MS m/z 609 (M + Li), 603 (M + H); exact mass found 609.52572, calcd for $C_{28}H_{35}N_4O_9Li$ 609.52531.

Triethylammonium 2′-Deoxythymidine 5′-[4-(4′-Methyl-2,2′-bipyridin-4-yl)butyl phosphate] (11). To **10** (0.071 g, 0.118 mmol) dissolved in CH_2Cl_2 (3 mL) was added 25% NaOMe in MeOH (0.05 mL, 0.12 mmol), and the reaction was stirred at room temperature. After 15 min, the reaction was quenched with glacial acetic acid (0.06 g, 0.12 mmol). Dichloromethane (50 mL) was added to the mixture and the organic layer washed with saturated $NaHCO_3$ solution (2 × 20 mL) and water (10 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo to a glass (0.064 g, 0.114 mmol, 97% yield). The glass was dissolved in 0.5 mL of thiophenol/dioxane/triethylamine (1:2:2)

(commercial deprotection reagent, Sigma) and left to stir for 90 min. The mixture was concentrated to a glass and the residue dissolved in water (10 mL). The aqueous layer was washed with petroleum ether (2 × 10 mL) to remove traces of thiophenol. Final purification was carried out on an RP C-18 Sep-Pak column, eluting the desired product **11** (0.069 g, 0.106 mmol, 90% yield) with H_2O/CH_3CN (4:1): ^{1}H NMR (D_2O) δ 5.9 (t, 1 H, H1′, J = 6.5 Hz), 1.95 (m, 2 H, H2′), 4.3 (m, 1 H, H3′), 3.9 (m, 1 H, H4′), 3.8 (m, 2 H, H5′), 7.3 (s, 1 H, H6), 1.5 (s, 3 H, T-CH_3), 2.3 (s, 3 H, 4′-CH_3), 3.8 (m, 2 H, $OCH_2(CH_2)_3$), 1.5 (m, 2 H, $OCH_2CH_2(CH_2)_2$), 1.6 (m, 2 H, $O(CH_2)_2CH_2CH_2$), 2.6 (t, 2 H, $O(CH_2)_3CH_2$, J = 7.3 Hz), 7.7 (2 s's, 2 H, H3, H3′), 7.2 (m, 2 H, H5, H5′), 8.3 (m, 2 H, H6, H6′), 1.15 (t, 9 H, $(CH_3CH_2)_3N$), 3.05 (q, 6 H, $(CH_3CH_2)_3N$); ^{13}C NMR (D_2O) ppm 87.2 (1′C), 41.6 (2′C), 73.3 (3′C), 88.0 (J_{PC} = 9.1 Hz, 4′C), 68.7 (J_{PC} = 5.7 Hz, 5′C), 113.7 (5C), 139.4 (6C), 14.2 (T-CH_3), 23.2 (4′-CH_3), 168.4 (4CO), 153.6 (2CO), 67.3 (J_{PC} = 5.0 Hz, $OCH_2(CH_2)_3$), 31.8 (J_{PC} = 6.5 Hz, $OCH_2CH_2(CH_2)_2$), 28.5 ($O(CH_2)_2CH_2CH_2$), 36.8 ($O(CH_2)_3CH_2$), 155.6 (2′C), 155.8 (2C), 124.5 (3′C), 125.4 (3C), 154.2 (4′C), 157.6 (4C), 127.6 (5′C), 128.4 (5C), 150.2 (6′C), 150.7 (6C), 49.3 ($(CH_3CH_2)_3N$), 10.8 ($(CH_3CH_2)_3N$); ^{31}P NMR (D_2O) ppm 1.3 (s); MS m/z 568 (M + Na), 546 (M + H); exact mass found 552.45386, calcd for $C_{25}H_{30}N_4O_8Li$ 552.45261.

4-[3-Carbonyl-3-(*p*-nitrophenoxy)propyl]-4′-methyl-2,2′-bipyridine (12). 4-(3-Carboxypropyl)-4′-methyl-2,2′-bipyridine[15] (1.33 g, 5.2 mmol) and *p*-nitrophenol (1.18 g, 5.72 mmol) were dissolved in CH_2Cl_2 (10 mL) and the mixture was cooled to 0 °C in an ice bath. DCC (0.875 g, 6.24 mmol) was added to this mixture in small portions and the mixture stirred at 0 °C for 30 min. The ice bath was removed and the mixture was allowed to stir at room temperature for 12 h. The urea that precipitated out was filtered off and the filtrate was concentrated. The residue was dissolved in CH_2Cl_2 (100 mL) and washed with water 2 × 50 mL), and the dried organic extract was concentrated. Flash chromatography of the residue on neutral alumina gave the desired ester **12** (1.12 g, 2.97 mmol, 57% yield), which eluted off the column with 50% Et_2O/hexane. The ester was crystallized from CH_2Cl_2/hexane: mp 118–120 °C; ^{1}H NMR ($CDCl_3$) δ 2.9 (t, 2 H, H1″, J = 7.5 Hz), 2.2 (m, 2 H, H2″), 2.7 (t, 2 H, H3″, J = 7.4 Hz), 2.5 (s, 3 H, 4′-Me), 7.3 (d, 2 H, H6″, J = 9.1 Hz), 8.3 (d, 2 H, H7″, J = 9.1 Hz), 8.3 (m, 2 H, H3 & H3′), 7.2 (m, 2 H, H5 and H5′), 8.6 (dd, 2 H, H6 and H6′); ^{13}C NMR ($CDCl_3$) ppm 34.9 (1″C), 25.6 (2″C), 33.9 (3″C), 171.1 (4″C), 122.9 (6″C), 125.7 (7″C), 145.8 (8″C), 21.7 (4′-Me), 155.8, 156.3, 156.9 (5″C, 2C, 2′C), 121.8 (3′C), 122.6 (3C), 148.8 (4′C), 151.3 (4C), 124.4 (5′C), 125.3 (5C), 149.5 (6′C), 149.8 (6C); FABMS m/z 384 (M + Li), 338 (M + Li − NO_2), 217 (M + Li − p-$NO_2C_6H_4OH$); exact mass found 384.1544, calcd for $C_{15}H_{16}N_2O_2Li$ 384.1536. Anal. Calcd for $C_{21}H_{19}N_3O_4$: C, 66.83; H, 5.07; N, 11.13. Found: C, 67.05; H, 5.12; N, 10.98.

4-[3-Carbonyl-3-(succinimidyl)propyl]-4′-methyl-2,2′-bipyridine (13). 4-(3-Carboxypropyl)-4′-methyl-2,2′-bipyridine (1.0 g, 3.9 mmol) and *N*-hydroxysuccinimide (0.494 g, 4.3 mmol) were dissolved in EtOAc (10 mL) and the mixture was cooled to 0 °C in an ice bath. DCC (0.804 g, 3.9 mmol) was added to this mixture in small portions and the mixture stirred at 0 °C for 30 min. The ice bath was removed and the mixture allowed to stir at room temperature for 12 h. The urea, which precipitated out, was filtered off and the filtrate was concentrated. The residue was dissolved in CH_2Cl_2 (100 mL) and was washed with water 50 mL). The dried organic extract was concentrated to yield **13**: 0.827 g, 2.3 mmol, 59% yield); ^{1}H NMR ($CDCl_3$) δ 2.8 (t, 2 H, H1″), 2.1 (m, 2 H, H2″), 2.6 (t, 2 H, H3″, J = 7.4 Hz), 2.4 (s, 3 H, 4′-Me), 8.2 (m, 2 H, H3 and H3′), 7.1 (m, 2 H, H5 and H5′), 8.5 (dd, 2 H, H6 and H6′), 2.8 (s, 4 H, H6″); ^{13}C NMR ($CDCl_3$) ppm 34.5 (1″C), 25.6 (2″C), 30.7 (3″C), 168.7 (4″C), 169.7 (5″C), 26.1 (6″C), 21.6 (4′-Me), 156.3 (2′C), 156.9 (2C), 121.6 (3′C), 122.5 (3C), 148.6 (4′C), 151.2 (4C), 124.5 (5′C), 125.2 (5C), 149.4 (6′C), 149.8 (6C); FABMS m/z 360$^+$ (M + Li); exact mass found 360.31683, calcd for $C_{19}H_{19}N_3O_4Li$ 360.31998.

5′-*O*-[Bis(4-methoxyphenyl)phenylmethyl]-5-[3-[[2-[[4-(4′-methyl-2,2′-bipyridin-4-yl)-1-oxobutyl]amino]ethyl]amino]-3-oxopropyl]-2′-deoxyuridine (15). A solution of 5-[3-[(2-aminoethyl)amino]-3-oxopropyl]-5′-O-DMT-2′-deoxy-uridine[5b] (**14**, 0.322 g, 0.5 mmol) in CH_3CN (5 mL) and Et_3N (0.2 mL) was cooled to 0 °C in an ice bath and **12** (0.566 g, 1.5 mmol) was added to the stirred reaction mixture. After 15 min, the ice bath was removed and the mixture was stirred at room temperature for 24 h. The reaction mixture was diluted with 20 mL of CH_2Cl_2 and water (10 mL). The aqueous layer was washed with CH_2Cl_2 (2 × 20 mL). The dried ($MgSO_4$) organic layer was concentrated and flash chromatographed on a silica gel column, eluting with a gradient of CH_2Cl_2 to 15% EtOH in CH_2Cl_2. The desired product **15** (0.211 g, 0.24 mmol, 48%) was eluted by using 15% EtOH in CH_2Cl_2: ^{1}H NMR ($CDCl_3$) δ 6.3 (t, 1 H, H1′, J = 6.5 Hz), 2.35 (m, 2 H, H2′), 4.45 (m, 1 H, H3′), 4.0 (m, 1 H, H4′), 3.35 (m, 2 H, H5′), 7.45 (s, 1 H, H6), 2.2–2.3 (m, 4 H, H7 and H8), 3.2 (br s, 4 H, H9 and H10), 2.2 (m, 2 H, H11), 1.95 (m, 2 H, H12), 2.6 (m, 2 H, H13), 2.4 (s, 3 H, H19), 8.5 (dt, 2 H, H16 and H16′), 7.1 (m, 2 H, H15 and H15′), 8.15 (br s, 2 H, H18 and H18′), 3.75 (s, 6 H, H21); ^{13}C NMR ($CDCl_3$) ppm 84.9 (1′C), 40.7 (2′C), 72.1 (3′C), 86.3 (4′C), 63.9 (5′C), 164.1 (4CO), 150.7 (2C-O), 113.6 (5C), 137.0 (6C), 23.5 (7C), 35.3 (8C), 39.9 and 40.0 (9C and 10C), 35.5 (11C), 25.9 (12C), 34.7 (13C), 148.4 (14′C), 151.5 (14C), 123.9 (15′C), 124.7 (15C), 148.8 (16′C), 149.1 (16C), 121.5 (18′C), 122.3 (18C), 155.9 (17′C), 156.0 (17C), 21.2 (19C), 86.7 (20C), 55.3 (21C), 173.4 (22C), 173.0 (23C); FABMS m/z 990 (M + Li), 587 (M + Li − DMT).

5-[3-[[2-[[4-(4′-Methyl-2,2′-bipyridin-4-yl)-1-oxobutyl]amino–]ethyl]amino]-3-oxopropyl]-2′-deoxyuridine (16). A solution of nucleoside **15** (0.150 g, 0.17 mmol) in CH_2Cl_2 (5 mL) was treated with 10% CF_3CO-OH in CH_2Cl_2 (5 mL) for 15 min. The mixture was concentrated to a glass and dissolved in water (10 mL). The aqueous layer was washed with CH_2Cl_2 (2 × 10 mL). Compound **16** (0.093 g, 0.16 mmol, 94%) was purified by RP HPLC using a linear ternary gradient flowing at 1.5 mL/min. Solvent A (0.1 M (Et_3NH)OAc) was kept constant while solvent B (MeCN) and solvent C (H_2O) were varied. NMR spectra were run in D_2O with one drop of DCl added to improve the solubility of **16**: ^{1}H NMR (D_2O) δ 6.1 (t, 1 H, H1′), 2.2 (m, 2 H, H2′), 4.35 (m, 1 H, H3′), 3.95 (m, 1 H, H4′), 3.75 (m, 2 H, H5′), 7.55 (s, 1 H, H6), 2.35 (m, 4 H, H7 and H8), 3.3 (s, 4 H, H9 and H10), 2.3 (m, 2 H, H11), 1.95 (m, 2 H, H12), 2.7 (t, 2 H, H13), 2.4 (s, 3 H, H19), 7.3 (dd, 2 H, H15 and H15′), 7.8 (d, 2 H, H18 and H18′), 8.4 (dd, 2 H, H16 and H16′); ^{13}C NMR (D_2O) ppm 88.1 (1′C), 41.7 (2′C), 73.4 (3′C), 89.5 (4′C), 64.2 (5′C), 168.1 (4CO), 154.1 (2CO), 116.1 (5C), 140.9 (6C), 25.7 (7C), 37.3 (8C), 41.6 and 41.5 (9C and 10C), 37.9 (11C), 28.4 (12C), 37.5 (13C), 147.3 (17′C), 147.5 (17C), 130.9 (15′C), 131.6 (15C), 146.5 (16′C), 148.1 (16C), 163.1 (14′C), 164.2 (14C), 128.2 (18′C), 129.1 (18C), 22.2 (19C), 178.5 (20C), 178.1 (21C); FABMS m/z 593 (M + 2Li − H), 587 (M + Li); exact mass found 587.2806, calcd for $C_{29}H_{36}N_6O_7Li$ 587.2851.

Titration of 5-[3-[[2-[[4-(4′-Methyl-2,2′-bipyridin-4′-yl)-1-oxobutyl]amino]ethyl]amino]-3-oxopropyl]-2′-deoxyuridine (16) with $CuCl_2$. A 1-mL aliquot of a 53 μM solution of **16** in 20 mM HEPES buffer (pH 7.1) was placed in a quartz cuvette and aliquots of a 1.78 mM stock solution of $CuCl_2$ in water were added. Changes in the visible spectrum were monitored between the wavelength of 240 and 380 nm. An identical procedure was followed for the titration of **8**, **11**, 2′-deoxythymidine 3′-monophosphate, 2′-deoxythymidine 5′-monophosphate, and 2′-deoxyuridine.

RNA Cleavage Assay. HPLC analysis was performed with a Waters 600 multisolvent delivery system and a 490 programmable multiwavelength detector. Data were acquired on a NEC APC IV advanced personal computer using Waters Maxima 820 software. Extensive precautions were taken to avoid RNase contamination in the hydrolysis reactions. All buffers were made with distilled–deionized water, which was treated with diethyl pyrocarbonate (0.1% v/v). Hydrolysis reactions were run in sterilized polypropylene tubes. The reactions were analyzed on a 7-μm Nucleogen DEAE 60-7 column with the following elution gradient: 0–15 min 25% B, 15–45 min 60% B, 45–60 min 100% B; solvent A, 20 mM KH_2PO_4, 20% acetonitrile pH 5.5; solvent B, solvent A + 1 M KCl. With this system it was possible to determine the area under all the substrate peaks simultaneously. The percent substrate hydrolysis was determined from the ratio of the integration of substrate peak at t = 48 or 24 h and t = 0 h. RNA concentrations refer to the concentration of phosphodiester units.

Hydrolysis of RNA by Cu(II) Complexes of Bpy, 8, 11, and 16. All hydrolysis reactions were run in 20 mM HEPES buffer (pH 7.1) at 37 °C. A stock solution of [poly$(A)_{12-18}$] = 761 μM was prepared by dissolving 10 units of poly$(A)_{12-18}$ (Pharmacia) in 1 mL of 20 mM HEPES buffer. In a typical reaction, the assay solution contained, in a total volume of 1.5 mL, 63 μM poly$(A)_{12-18}$, 157 μM ligand, 157 μM $CuCl_2$, and 20 mM HEPES buffer (pH 7.1). A 200-μL aliquot was removed from the reaction and subjected to HPLC analysis to determine the t = 0 substrate integration. The reaction mixture was incubated at 37 °C for either 24 or 48 h, after which time a second 200-μL aliquot was assayed. Hydrolysis reactions carried out in the presence of EDTA contained EDTA at a total concentration of 500 μM.

Reaction of $Cu(bpy)^{2+}$ with Poly$(dA)_{12-18}$ and Poly$(A)_{12-18}$. A stock solution of poly$(dA)_{12-18}$ (Pharmacia) was prepared by dissolving 25 units of poly$(dA)_{12-18}$ in 1.0 mL of 20 mM HEPES buffer (pH 7.1). The reaction mixture contained, in a total volume of 1.5 mL, 63 μM poly$(dA)_{12-18}$ or poly$(A)_{12-18}$, 157 μM bipyridine, 157 μM $CuCl_2$, and 20 mM HEPES buffer. The solutions were incubated at 37 °C for 48 h, after which time they were assayed by ion-exchange HPLC. Control reactions were run under identical conditions but in the absence of $CuCl_2$.

Identification of 2′,3′-Cyclic AMP. In a total of 1.5 mL, the reaction mixture contained 600 μM poly$(A)_{12-18}$, 157 μM $CuCl_2$, 157 μM bi-

pyridine, and 20 mM HEPES buffer (pH 7.1). The reaction mixture was incubated at 37 °C for 18 h, after which time a 500-μL aliquot was removed and divided into two 250-μL portions. The reactions were analyzed by reverse-phase HPLC using a LiChrospher 100RP-18 Column (5 μm) (EM Science) with a binary solvent system of (A) 20 mM KH_2PO_4 (pH 4.5) and (B) methanol/water (3:2) with a linear elution gradient of 0–50% solvent B over 30 min with a flow rate of 1.0 mL/min. Analysis of a 200-μL sample of one portion of the reaction mixture shows a peak with a retention time of 20.0 min and several peaks with longer retention times. HPLC performed on 25 μL of a 4.0 mM stock solution (20 mM HEPES buffer, pH 7.1) of authentic 2′,3′-cyclic AMP (Sigma) also produced a peak with a retention time of 20.0 min. The second portion of the reaction mixture was spiked with 10 μL of stock 2′,3′-cyclic AMP. HPLC analysis showed an increase in only the peak at retention time of 20 min. Retention times of authentic samples of adenosine 3′-monophosphate, adenosine 5′-monophosphate, adenosine 2′-monophosphate, and adenosine are 18.0, 13.2, 21.9, and 23.6 min, respectively. An identical HPLC protocol was used to identify 2′,3′-cyclic AMP in the hydrolysis of ApAp by polypeptides.[30]

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Supplementary Material Available: 1- and 2-D ^{1}H, ^{13}C, and ^{31}P NMR spectra of the novel compounds (42 pages). Ordering information is given on any current masthead page.

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Catalytic Antibodies with Acyl-Transfer Capabilities: Mechanistic and Kinetic Investigations

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Abstract: Antibodies have been shown to catalyze acyl-transfer reactions. The processes by which they perform such tasks have often been postulated but largely remain unknown. An extended study is presented on three different monoclonal antibodies that catalyze the hydrolysis of an alkyl ester and an aryl amide bond. Antibodies 2H6 and 21H3 catalyze the hydrolysis of an unactivated benzyl ester and show exquisite specificity for substrates with either the *R* or *S* configuration, respectively, while 43C9 catalyzes the cleavage of a *p*-nitroanilide amide bond. New substrates were synthesized and buffer-assisted reactions were employed to determine antibody–substrate fidelity. Oxygen-18 incorporation experiments were performed providing evidence that these antibody-mediated reactions proceed through attack at the acyl carbonyl, and excluding the possibility of an S_N2 displacement mechanism for the ester hydrolysis reaction. A pH–rate profile study in protium and deuterium oxide was performed on antibody 43C9. This revealed an apparent pK_a of ~9 involved in catalysis, but both the presence and absence of a solvent isotope effect in the pH-dependent and -independent regions suggested a multistep reaction pathway may be operative.

The number of chemical transformations catalyzed by antibodies (abzymes) is rapidly increasing. Antibodies have been shown to catalyze acyl-transfer, pericyclic, elimination, and redox reactions among others.[1] Limits to the types of reactions that antibodies can catalyze would be more systematically explored, if our knowledge on how "abzymes" perform their catalytic processes were extended.

We have been engaged in several programs aimed at eliciting antibodies with catalytic capabilities. One such program has been targeted at the development of acyl-transfer abzymes.[2] To date, our main successes have relied on the utilization of transition-state theory in the design of the haptens (antigens) used in the production of these hydrolytic antibodies. Specifically we have utilized tetrahedral phosphorus moieties as haptens to mimic the putative tetrahedral intermediate in the acyl-transfer reactions. While an extensive body of knowledge has been developed as to the manner in which these transition-state analogues inhibit enzymatic reactions, little is known about the complementary molecular surfaces these entities elicit when they are used as haptens to challenge the immune system.

Recently we reported two separate studies of antibodies that catalyze the hydrolysis of either an amide or an ester bond. In our first report we demonstrated that phosphonamidate **2** could induce catalytic antibodies for the hydrolysis of amide **2a** (Figure 1).[2f] In the second communication we studied the propensity of racemic antigen **1** to induce catalytic antibodies with *R* or *S* selectivity for benzyl ester **1a**.[2g] Herein we describe extensions of these studies aimed at elucidating some of the catalytic characteristics of these hydrolytic abzymes, i.e., their substrate specificity, the nature of the reaction pathway, and the source of rate accelerations.

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