Inorganic Mimics of Ribonucleases and Ribozymes: From Random Cleavage to Sequence-Specific Chemistry to Catalytic Antisense Drugs

Bobby N. Trawick, Andrew T. Daniher, and James K. Bashkin*

Department of Chemistry, Washington University, St. Louis, Missouri 63130

Received July 29, 1997 (Revised Manuscript Received January 2, 1998)

Contents

I. Introduction

Interest in functional mimics of ribozymes and ribonucleases is driven by a variety of scientific and medical goals. After a brief introduction, in which several working definitions, postulates and premises are given, these goals are described and explained. Metal-based strategies for RNA cleavage are then presented and contrasted with other approaches, including biological and purely organic methods. The organic methods are exhaustively discussed in a companion article by Oivanen, Kuusela, and Lönnberg. We describe the evolution of ribozyme mimics from early, nonspecific, ill-defined (but highly active) catalysts that were derived from "free metal ions in buffer", to the advent of well-defined metal complexes that retained RNA cleavage activity.[1,2] Such metal complexes allowed the central premise of this area to be tested and eventually led to proof of the concept that sequence-specific cleavage of RNA can be achieved by synthetic mimics. We explain how the continuing convergence of chemistry, biochemistry, and molecular biology have helped shape research in this area, from the molecular design of early mimics to the choice of analytical methods for the screening of RNA transesterification catalysts. A critical assessment of these analytical approaches is provided.

For the purpose of this review, functional mimics of ribozymes and ribonucleases are defined as "synthetic molecules that cleave RNA in a sequence-directed manner, using biomimetic chemical reactions such as transesterification and hydrolysis". For simplicity, we will use the term "ribozyme mimics" to represent these compounds. The transesterification and hydrolysis reactions are grouped together as "nucleophilic cleavage reactions" for several reasons (Scheme 1). These reactions are closely related by the nucleophilic attack on phosphorus (V) that each employs. The mechanistic connection between these reactions was elegantly unified in a recent paper by Perreault and Anslyn.[3] For transesterification, an alcohol or alkoxide is the nucleophile, while for hydrolysis, water or hydroxide is almost always the nucleophile.[4,5] Furthermore, most inorganic reagents that catalyze RNA transesterification go on to hydrolyze the resulting 2′,3′-cyclic monophosphate to a mixture of 2′- and 3′-phosphate monoesters, as indicated in Scheme 1b.

Describing transesterification and hydrolysis as "nucleophilic cleavage" events also provides convenient language to distinguish them from the oxidative cleavage reactions[6−12] frequently employed in the study of nucleic acids (Scheme 2). We have specifically excluded from this review those reagents that function only by oxidative cleavage mechanisms, including Fenton reagents and bleomycin. Oxidative cleavage agents are discussed elsewhere in this issue in reviews by Pogozelski and Tullius, Burger, and Burrows.

The central postulate of the *chemical approaches* to sequence-specific RNA cleavage was developed in preliminary forms by at least two independent groups in the late 1980s.[13−15] Stein and Cohen focused on imidazole-based approaches in their early published suggestions and later experimental work,[13,16] while Bashkin and co-workers developed a metal-based approach.[17] Others, such as Morrow and Chin and

S0009-2665(96)00422-0 CCC: $30.00
Published on Web 04/15/1998

Bobby N. Trawick was born in Dothan, AL, on September 18, 1973. He was educated at Morehouse College in Atlanta, GA, where he earned a B.S. degree in Chemistry in 1995. He was a Ronald E. McNair Scholar, and his undergraduate research was conducted under the mentorship of Dr. Morris A. Waugh on the synthesis of liquid crystalline materials. During the summer of 1995 he worked with Dr. Patrick J. Lennon at the Monsanto Company in St. Louis, MO, on the synthesis of Mn(II) macrocycles that mimic the enzyme superoxide dismutase. Bobby is currently a Chancellor's Fellow at Washington University in St. Louis, where he works under the direction of Dr. James K. Bashkin on the sequence-specific cleavage of RNA by oligonucleotide conjugates of bipyridine, terpyridine, and zinc(II) macrocycles.

Andrew T. Daniher was born on December 29, 1969, in Chicago, IL. He received his B.S. degree in Chemistry from John Carroll University (Cleveland, OH) in 1992, where he did undergraduate research with Professor Paul R. Challen synthesizing vanadium complexes. He obtained his Ph.D. from Washington University in St. Louis, MO, in 1997 under the supervision of Professor James K. Bashkin. His graduate studies involved the development of terpyridine conjugates of DNA for site-specific cleavage of RNA. Dr. Daniher is currently a Postdoctoral Research Fellow under the direction of Dr. Leonid Beigelman in Organic Chemistry at Ribozyme Pharmaceuticals, Inc., in Boulder, CO.

James K. Bashkin was born in Iowa City in 1958, but spent most of his early years in Tucson, AZ. After attending the University of Arizona for a year, he transferred to the University of California at Irvine. After graduating in 1977, he went to Oxford to do his graduate work with Malcolm L. H. Green, deciding to stay in chemistry rather than join Oxford's Oriental Studies program to pursue a degree in Chinese. He obtained a D.Phil. in organometallic chemistry in 1982, and then moved to R. H. Holm's group at Harvard, where he was an NIH postdoctoral fellow. He then took a position at Monsanto, in the Chemical Sciences Department of Corporate Research. During this time (1985–1991), he developed programs on solid-state reference electrodes, a new green chemistry version of nucleophilic aromatic substitution, and catalytic drugs based on functional mimics of ribozymes. Through the efforts of many co-workers, the green chemistry was recently commercialized in Europe by Flexsys, which is a joint venture between Solutia (formerly Monsanto) and Akzo Nobel. He and co-inventor M. K. Stern shared Monsanto's Thomas and Hochwalt prize for this chemistry. He was appointed to the Editorial Advisory board of *Chemical Reviews* in 1991, cochaired the NSF Organometallic Workshop (1988–1990), and has served on an NSF review panel for SBIR grants. In 1991, he joined the chemistry faculty at Washington University as an Assistant Professor. He has continued to pursue bioorganic and bioinorganic approaches to catalytic drugs, and to study the mechanism of RNA transesterification and hydrolysis. He has recently moved back into the green chemistry arena. He was on the varsity modern pentathlon team at Oxford and continues to run and swim when the opportunity arises, but no longer jumps over fences on horseback. The guitar has supplanted the cello as his musical instrument of choice. His family (Jacob, Samuel, and Shelley) form the most important part of his life.

Komiyama, pursued early work on nucleophilic cleavage of nucleic acids by well-defined metal complexes. Our modern version of the "chemical ribozyme" postulate is that "ribozyme mimics can be constructed by covalently incorporating nucleophilically active RNA cleavage catalysts into DNA oligonucleotides or their analogues". The specificity of these reagents is consequently derived from the Watson−Crick hydrogen bonding of the DNA strand to its complementary RNA sequence (Figure 1), and it exhibits the full specificity of the genetic code. The chemoselectivity of the mimics arises from the relative ease of nucleophilic cleavage of RNA vs DNA.[18,19] This, in turn, is derived from the facile intramolecular nature of the nucleophilic attack that typically drives RNA cleavage (Scheme 1). DNA lacks the 2′-OH functionality and has proved almost completely inert to hydrolysis or transesterification by small molecule catalysts, with a few notable exceptions.[5,20−22] The likelihood for self-destruction of the ribozyme mimic via intramolecular nucleophilic cleavage is therefore minuscule and would be cause for celebration if observed, since DNA hydrolysis is such a difficult reaction to achieve. To our knowledge, no other approach to gene-specific chemistry offers such a remarkable combination of biochemical and chemical selectivity. DNA-based chemical reagents that are designed to attack specific DNA sequences do not provide the same inherent bias against self-destruction that ribozyme mimics offer.

The hope embodied in the central postulate, which has been realized in a number of recent examples from around the world, is that a random RNA cleavage catalyst will retain its activity upon conjugation to a DNA molecule, but that the rate-determining kinetic event will become DNA−RNA strand recognition. Thus, a balance is needed between the on-rate for nucleic acid binding (Figure 1, k_1) and the rate of chemical cleavage (Figure 1, k_2).

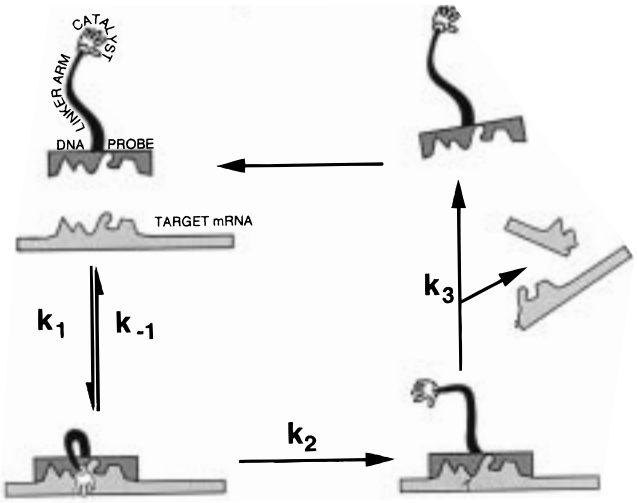
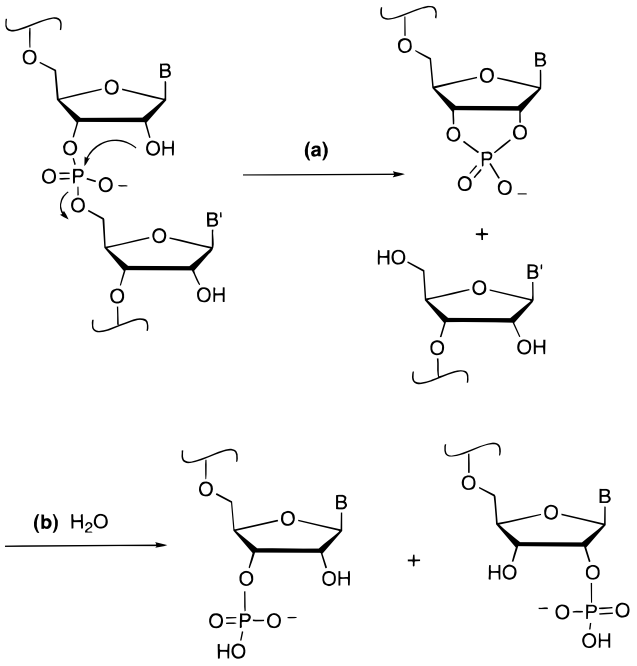


Figure 1. Potential catalytic cycle of a ribozyme mimic. The first step involves recognition and binding of the target mRNA (k_1). In the second step, the RNA target is cleaved in a site-specific manner (k_2). Finally, the product fragments are released (k_3), and the mimic is ready for another cycle.

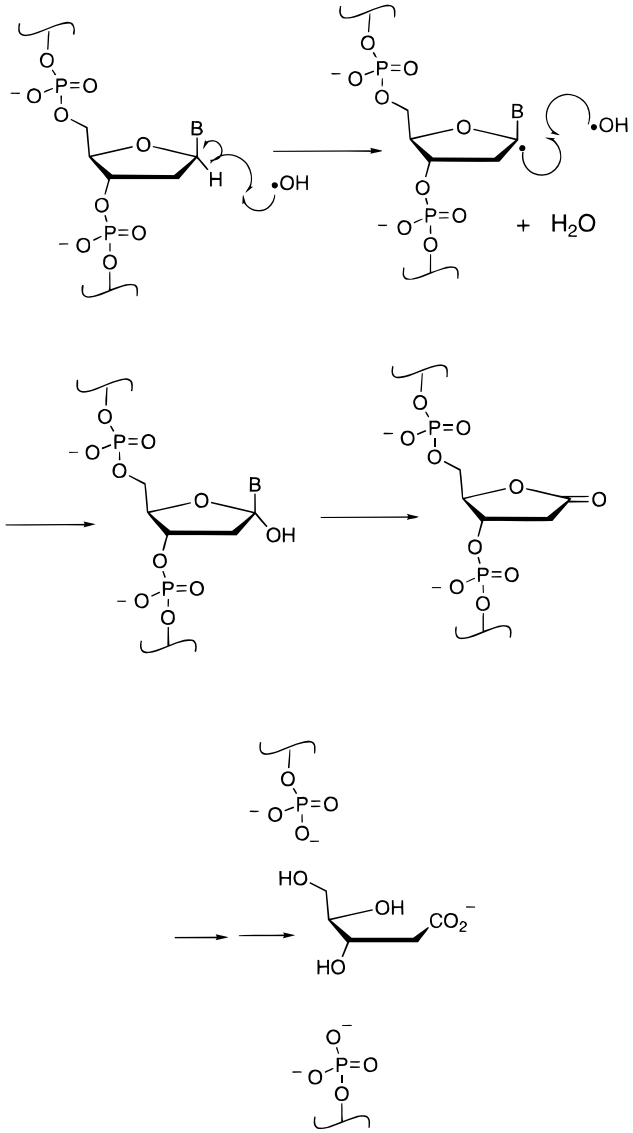
Scheme 1. Nucleophilic Cleavage of RNA: (a) Transesterification with Concommitant Cleavage of the Phosphodiester Backbone and (b) Hydrolysis of the 2′,3′-Cyclic Monophosphate to a Mixture of 2′- and 3′-Monophosphates



Too high a rate of chemical cleavage will result in nonspecific RNA degradation. As will be discussed below, for catalytic turnover to be achieved with ribozyme mimics, the off-rate for release of cleaved RNA fragments by the DNA strand (Figure 1, k_3) is also of fundamental importance.

The process of designing ribozyme mimics has entailed a series of steps. The first step involved the identification of RNA cleavage catalysts that function by transesterification/hydrolysis mechanisms and that are suitable for covalent incorporation into DNA.[1,2,23−27] Second, these catalysts (or catalyst precursors, such as free ligands) were incorporated

Scheme 2. One of the Many Pathways for Oxidative Cleavage of DNA Involves Hydrogen Abstraction at the C-1′ Position of the Ribose Moiety



into DNA in a manner consistent with known cleavage mechanisms and the presumed structure of the RNA−DNA duplex.[17,28−38] This step required the use of preliminary molecular designs that controlled features such as the vectorial approach of the catalyst to the substrate. Third, candidate molecules were tested for sequence-specific cleavage activity, and the structures of mimic−RNA complexes were investigated.[30,33−36,38−41] Finally, test results were fed back into the molecular design process to generate new, optimized, ribozyme mimics.[41−43]

A. Medicinal Motivation

Before reviewing the details of ribozyme mimic development, it is important to examine the reasons why they have generated such an intense interest. The main goal is an efficient, gene-specific approach to chemotherapy.[13,25,40,44,45] The disease targets can be broadly defined as any diseases that involve the production of harmful proteins. This category in-

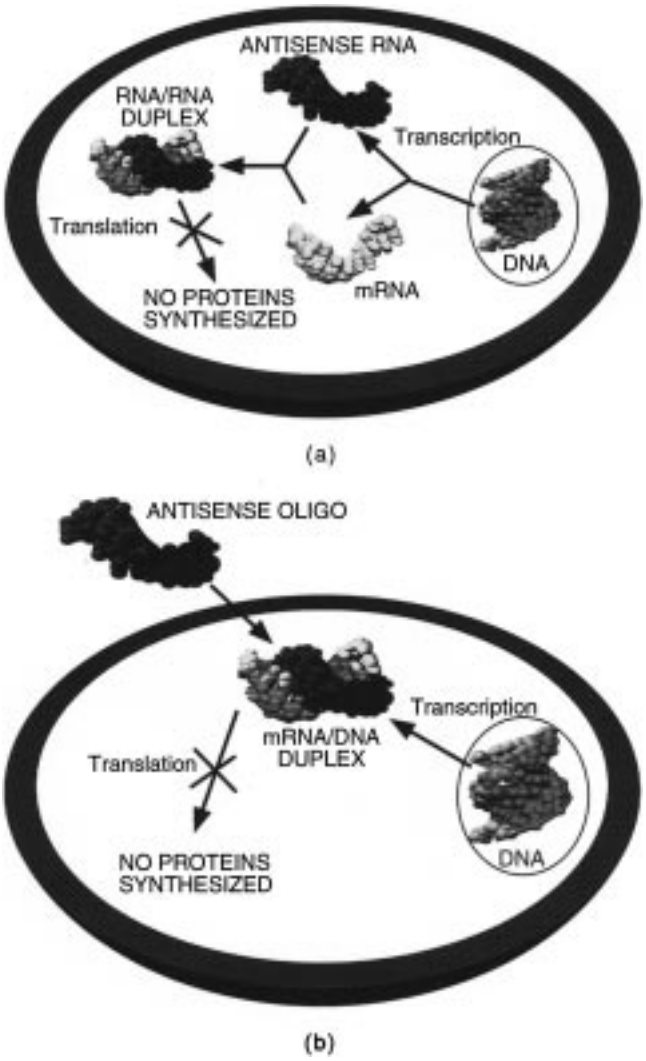


Figure 2. (a) Synthesis of an antisense RNA molecule inside the cell and (b) the chemical version of the antisense technique.

cludes bacterial, viral, and fungal diseases, as well as certain types of cancer. The basis of this approach is the "antisense method" for controlling gene expression (protein synthesis).[46−54] The antisense method is a naturally occurring[55] gene regulation mechanism that relies upon the ability of messenger RNA, which is a single-stranded nucleic acid, to be recognized in a gene-specific manner by complementary nucleic acid molecules. This recognition occurs by the familiar and highly reliable Watson−Crick base pairing that is responsible for the specificity of the genetic code. The inhibitory effect of antisense oligonucleotides was first observed in 1978 by Zamecnik and Stephenson, who used a 13-mer oligonucleotide to inhibit the growth of Rous sarcoma virus in cell culture.[56,57]

In the natural form of this gene regulation method, an antisense RNA molecule is synthesized inside the cell by transcription (Figure 2a). This high molecular weight nucleic acid binds to its target mRNA and prevents the message from being translated into protein at the ribosomes. Observed in both prokaryotes[58] and eukaryotes,[59] antisense regulation can also be introduced artificially via transfection with antisense genes. These processes have no "drug delivery problem" (if we ignore the transfection step), because the antisense reagent is produced inside the cell.

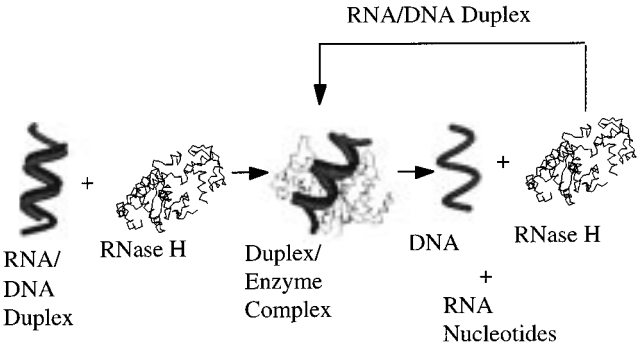


Figure 3. The RNase H pathway for catalytic destruction of specific mRNA with antisense DNA probes. RNase H and the DNA probe are free to bind and destroy another RNA target molecule after the original RNA strand has been digested into nucleotides.

The chemical version of the antisense technique employs antisense sequences that are synthesized outside the cell, such as on a DNA synthesizer (Figure 2b). For several reasons, including enhanced cellular uptake and lower cost, synthetic antisense molecules are much lower in molecular weight (typically 17−20 nucleotides long) than the full-length gene transcripts employed in the natural antisense method.[60−64] The mechanism of action of the short, synthetic antisense sequences does not necessarily parallel the natural antisense mechanism, which involves sequestering the target mRNA by binding it to a complementary RNA strand. Instead of merely binding to specific mRNA sequences, DNA drugs act in concert with the cellular enzyme Ribonuclease H (RNase H) to destroy the target mRNA.[65−67] RNase H is known to digest the RNA strand of DNA−RNA duplexes, and it provides a catalytic pathway for antisense DNA drugs as illustrated in Figure 3.

Given the apparent specificity and catalytic nature of the chemical antisense approach, it may be surprising that antisense drugs are not yet in active use. Several clinical trials are now in progress,[62,63,68−71] but a number of difficulties remain to be overcome. Examples of such difficulties include: (1) drug delivery across cell membranes,[72−74] (2) drug delivery to the correct intracellular regions,[75] (3) in vivo stability of DNA drugs to nuclease enzymes and other biochemical degradation agents, and (4) cost of the reagents. Primarily, these problems result in low efficiency of the method by requiring larger doses of DNA, which can increase side effects.

Many advances have been made in drug-delivery techniques and in the chemistry of DNA analogues[76,77] that retain Watson−Crick base-pairing ability while offering improved delivery and stability properties.[78] However, the primary impasse to using such analogues is that the enzyme RNase H is highly specific about which nucleic acids it will accept as templates for RNA digestion:[79−83] it is a DNA-dependent RNA hydrolysis catalyst. Thus, RNA does not promote RNase H-mediated RNA cleavage, nor do most DNA analogues. Therefore, the dilemma arises that chemical modifications designed to enhance the stability and uptake of DNA drugs actually

block the catalytic pathway shown in Figure 3. A stoichiometric antisense method is much less attractive than its catalytic counterpart, and this is where ribozyme mimics are designed to play the vital role of recovering catalytic activity when DNA analogues are used. The ribozyme mimics deliver their own catalytic function to the target RNA and do not depend on RNase H.

Can natural ribozymes be used for this strategy? Perhaps they *can* be used successfully to combat disease, and several major efforts are devoted to this approach.[44,84,85] One possible advantage of natural ribozymes is that they may be expressed in vivo; however, there may be a number of advantages to using synthetic mimics rather than their natural counterparts. Natural ribozymes require complex tertiary structures that are only achieved when *natural* RNA residues are incorporated at key positions.[86−92] Thus, active ribozymes are not fully amenable to replacing nucleotides with DNA or RNA analogues for enhanced stability in vivo. In addition, the molecular weights of natural ribozymes are usually much greater than the molecular weights of the functional mimics described in this review, which may give the mimics a competitive advantage for drug delivery. Furthermore, the binding and catalytic domains of natural ribozymes are not necessarily separable, so these properties may not be optimized independently. Ribozyme mimics, however, do contain separate recognition and cleavage domains (Figure 1), so they may allow independent optimization of substrate binding, product release, and chemical cleavage steps. The same arguments may also hold true in comparing ribozyme mimics to the highly efficient RNA-cleaving DNA enzyme that was recently discovered by Santoro and Joyce using in vitro selection.[93] None of these arguments should discourage interest in either natural or synthetic reagents. Instead, they provide challenges and opportunities for continued research and development. Experimental results and ingenuity will ultimately determine if natural ribozymes or synthetic mimics succeed or fail in the clinic.

B. Tools for Probing Ribozyme Mechanisms

Although much important information has been gathered about the kinetics, solution structures, and solid-state structures of natural ribozymes,[94] many unanswered questions remain about the intimate molecular mechanisms of ribozyme action. For example, the precise role of metal ions in the chemical cleavage steps is unestablished. As we will describe, ribozyme mimics allow a wide variety of metal complexes to be introduced at precise locations in RNA−DNA duplexes. The use of well-defined complexes permits coordination geometries to be fixed or controlled. The pK_as of coordinated water molecules can be adjusted by changing the identity of the metal and its ligands. Furthermore, as mimics progress through second- and third-generation designs, pairs of metals (or larger numbers) will be positioned at precise relative and absolute locations in the complex formed between a mimic and its RNA substrate. This level of chemical control should allow many mechanistic questions to be answered about the number of metals involved in catalysis and the specific steps that they perform.

C. Tools for Molecular Biology

The rise of modern molecular biology has been partly fueled by the availability of the natural DNA cleavage catalysts known as restriction enzymes.[95] These enzymes recognize specific DNA sequences, usually 4−6 base pairs in length, and cut the DNA in a highly specific manner. The counterparts of restriction enzymes are ligation enzymes, which reassemble DNA fragments in processes coupled to nucleotide triphosphate hydrolysis. The combination of restriction and ligation enzymes allows the dismantling and reconstruction of genes through cutting and stitching reactions that occur at precisely specified sites in large macromolecules. However, the ability to specify only a 4−6 base sequence typically results in multiple cleavage sites in a genome, on plasmids, and in other large DNA substrates. Ribozyme mimics allow cleavage specificity to be tuned simply by changing the length and content of the DNA sequence employed. Unique cleavage sites, or particularly low frequency cleavage sites, could be targeted. Such tools would allow molecular biologists to exert an entirely new level of control over splicing of nucleic acids, albeit at the RNA level. Coupled with methods such as reverse-transcriptase PCR,[96] this could greatly streamline present cloning procedures, assist in DNA mapping and sequencing, and open up entirely new experimental procedures.

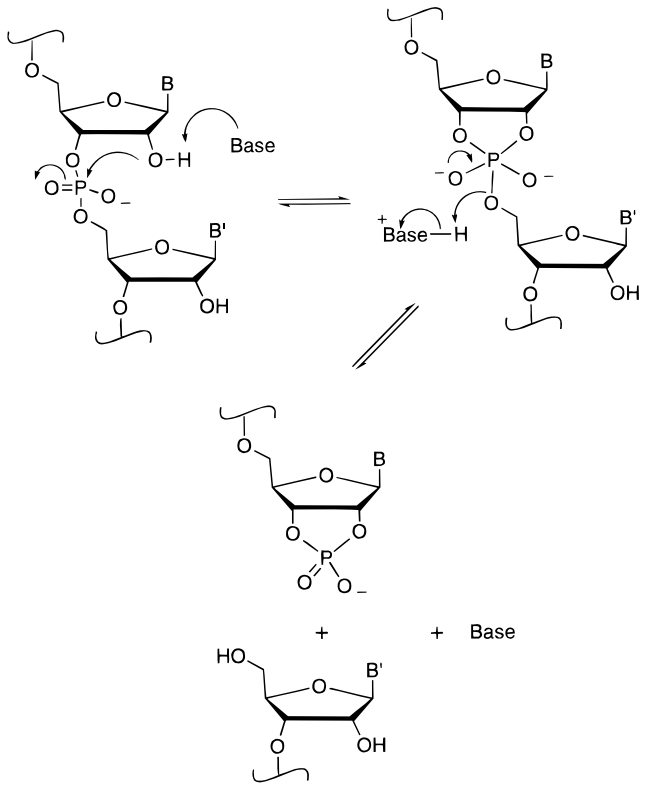
II. The Chemistry of RNA Cleavage

A. Catalysts and Their Mechanisms

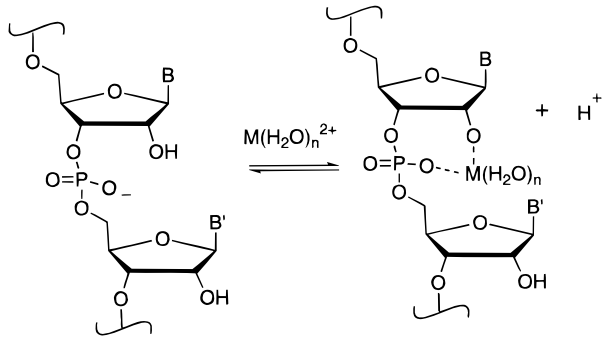
One remarkable feature of RNA transesterification and hydrolysis is that these reactions are catalyzed by a truly astonishing range of species that span almost the entire Periodic Table. Protons, hydroxide, amines and other nitrogen derivatives, Mg(II), Ca(II), Fe(III), Ni(II), Cu(II), Zn(II), Pb(II), trivalent lanthanides, and UO_2^{2+} [97] and Th salts[98] are just some of the species known to cleave RNA through nucleophilic paths. Of course, enzymes and ribozymes must be added to the list. Most of these individual catalyst types have been reviewed recently,[99−106] or are addressed elsewhere in this issue. Although detailed kinetic analysis is sometimes available, the mechanisms of many RNA cleavage reactions remain unestablished or in dispute.[107−113] Certain features (delineated below) are generally applicable to RNA transesterification. However, for any given combination of a particular catalyst and a particular RNA substrate, the order of events, the order of the reaction, the simultaneous or stepwise nature of the processes, and importance of each individual step can vary dramatically. We have chosen to represent certain fundamental steps with dianionic phosphorane intermediates for simplicity, although, as will be pointed out later, charge stabilization is undoubtedly required.

Transesterification of RNA normally requires participation of the 2′-OH as a nucleophile[114] (except for

Scheme 3



Scheme 4



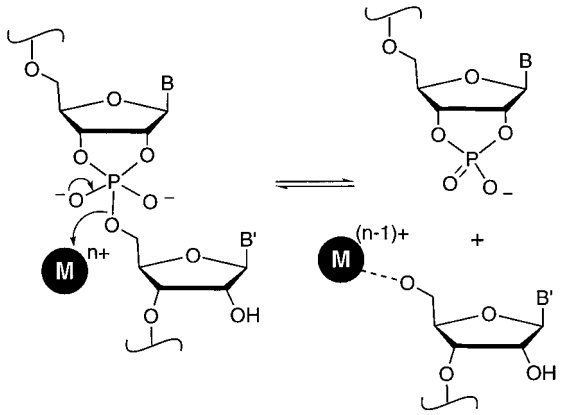
some ribozymes, which employ other nucleophilic hydroxyls). During the reaction, the 2′-OH must be deprotonated, and the leaving 5′-alkoxy group must be protonated. Both steps can be accomplished by one round of base catalysis, for example by imidazole (Im) or a metal hydroxide (M^+-OH), as illustrated in Scheme 3.

It is possible that deprotonation of the nucleophile may occur by prior coordination of a metal ion to the 2′-OH, since this would lower the important pK_a.[115−117] The effective nucleophile would then be a metal alkoxide (Scheme 4).

It is also possible that protonation of the leaving group is of secondary importance to stabilization of the departing alkoxide by a metal ion (Scheme 5).

In general terms, the nucleophile derived from the 2′-OH must attack a tetrahedral phosphodiester, generating either a phosphorane intermediate or a shorter-lived activated complex. Phosphorane intermediates may undergo pseudorotation. Nucleophilic attack on an anionic phosphodiester is notoriously unfavorable from an electrostatic perspective.[19] Therefore, some means of preventing a large negative

Scheme 5



charge from building up in the activated complex is typically necessary. Protons or Lewis acids may provide this charge stabilization.

The number of metal ions involved in metal-mediated RNA cleavage is a matter of some interest and dispute, whether the reaction is performed by enzymes, ribozymes or small metal complexes. To design the most effective ribozyme mimics, it is of vital importance to know how many metal ions must be delivered to the substrate. Given experimental limitations such as the solubility of the catalysts, substrates, and catalyst−substrate complexes, it is not always possible to determine the order of the reaction in an unambiguous manner. Kinetic accidents may contribute to this confusion.[118] The critical assessment of analytical methods provided in the next section may assist in choosing the best method for a given catalyst.

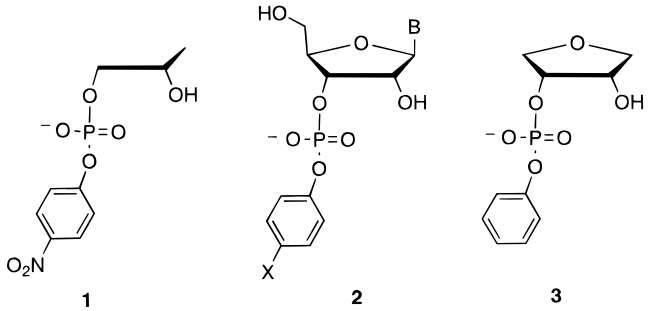
B. Assays for RNA Cleavage

The literature on assays for measuring ribonuclease activity is extensive. The substrates employed range from simple RNA model compounds to more complex ribopolymers, and the specific physical properties that are measured vary widely with the actual substrate under investigation. Assays for ribonuclease activity have been reviewed elsewhere;[119,120] however, many significant advances have been made since that time. This is partly due to the interest in developing improved techniques for screening of RNA cleavage catalysts and the desire to study quantitatively the transesterification and hydrolysis of RNA by various reagents.

1. Models for RNA (Both Good and Bad)

Extrapolation from mechanistic studies on model phosphate ester compounds to RNA has been common.[121−147] This is partly due to the intrinsic simplicity and convenience of many models relative to true RNA substrates, which have a multiplicity of metal ion binding sites and many possible sites for hydrolytic cleavage. Discussed below are selected examples of compounds used to model the transesterification and hydrolysis of RNA. These compounds are designed to mimic the dialkyl phosphate esters that form the anionic backbone of RNA.[148−150] The most frequently used models are those that contain

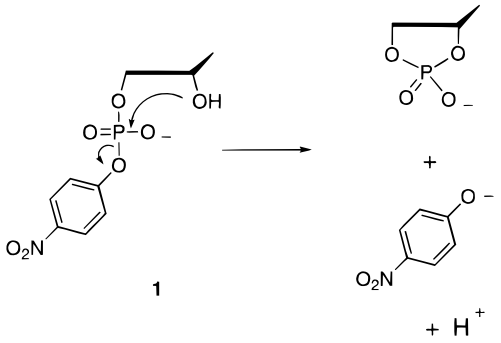
good leaving groups, such as **1**−**3**, which, upon transesterification, release aryloxides that are amenable to study by UV−vis spectrophotometry.



1 **2** **3**

In a typical experiment with **1** as substrate, the cleavage is followed by monitoring the increase in the visible absorbance at 400 nm caused by the release of 4-nitrophenolate ion (Scheme 6).

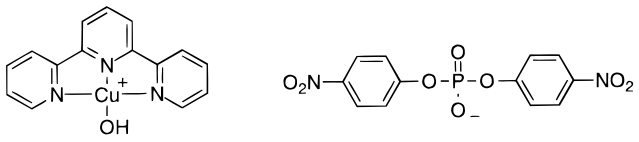
Scheme 6. Cleavage of 1 by Intramolecular Nucleophilic Attack to Produce 4-Nitrophenolate Ion



1

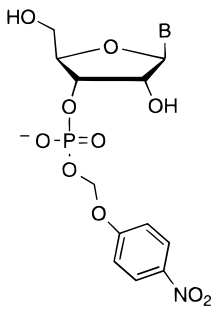
In addition to ease of analysis, the principal advantage of using RNA analogues with good leaving groups is that they usually exhibit enhanced cleavage rates. By comparison, at neutral pH, transesterification of true RNA substrates is relatively slow, which makes it difficult to follow the progress of the reaction. Furthermore, the cleavage of activated models gives compounds that can be readily identified by UV−vis spectrophotometry, high-performance liquid chromatography (HPLC), and ^{31}P NMR spectroscopy.

It is of primary importance to mention, however, that despite the simplicity and convenience offered by activated phosphodiesters, there are a number of drawbacks associated with their use. First, direct parallels to the transesterification and hydrolysis of true RNA substrates cannot be unequivocally drawn, because true RNA possesses additional metal ion binding sites on its nitrogenous bases and phosphates which may be important from a mechanistic standpoint. Second, the stability of the phosphorane is changed dramatically by the presence of an electron-withdrawing group and a good leaving group, and this may have a significant effect on the mechanism and final product distribution. Both of these aspects have been recently reviewed with respect to acid/base catalysis.[3] It is clear, however, that activated substrates are generally poor models for RNA. An interesting example of the dichotomy that exists between RNA models and true RNA substrates is found in the study of copper(II) terpyridine (Cuterpy) **4** as an RNA cleavage catalyst. It was originally and correctly reported[151,152] that Cuterpy failed to promote the hydrolysis of bis(*p*-nitrophenyl) phosphate (BPNPP) **5**; however, it was later discovered[24,153−157] that Cuterpy is an effective catalyst for both the transesterification and hydrolysis of RNA dimers and oligomers and for the hydrolysis of adenosine 2′,3′-cyclic-monophosphate (cAMP). Cuterpy actually inhibits hydrolysis of **5** and may form a stable complex with this substrate.[154]



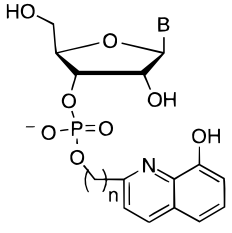
4 **5**

Kirby's group used the RNA model **6,** which was based on a *p*-nitrophenoxy methyl ester, to study the transesterification of RNA.[108] It was estimated that the pK_a of the leaving group was approximately 11, offering a much closer fit to the value of 14.8 for UpU than do other activated phosphodiesters. Upon transesterification, Kirby's substrate rapidly decomposes to give formaldehyde and *p*-nitrophenolate ion, and this allows the reaction to be followed by UV−vis spectrophotometry.



6

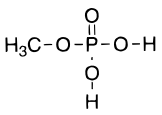
Bruice et al. reported the synthesis and use of 8-hydroxyquinoline-based model substrates such as **7** to examine the influence of various metal ions on RNA transesterification.[142,147,158] The 8-hydroxyquinoline moieties were designed to coordinate a metal and to place the metal at a controlled distance relative to the set of functional groups including the phosphodiester backbone, the leaving group oxygen, and the nucleophilic 2′-hydroxyl group. The cleavage reactions were monitored by HPLC, except in the case of La^{3+}, where the rate was so fast that stopped-flow spectrophotometry had to be used.



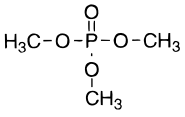
7

Phosphotriesters, which are hydrolyzed about 10^5−10^6 times more rapidly than phosphodiesters under

similar conditions, have also received noteworthy attention.[159,160] It is expected that the mechanism of hydrolysis of a phosphotriester should parallel that of a neutral phosphodiester. The alkyl group of the phosphotriester presumably replaces the proton in the neutral phosphodiester. Studies by Kosonen and Lönnberg have further supported this analogy.[160] Contrastingly, a recent communication by Floriàn and Warshel questioned this concept.[161] In their study, ab initio calculations were used to generate free-energy profiles for the OH^- attack at methyl dihydrogen phosphate **8** vs trimethyl phosphate **9** in solution. Interestingly, their calculations revealed that the $\Delta G^{\ddagger}$ for OH^- attack at methyl phosphate was significantly lower than that for the trimethyl phosphate. This result directly contradicts the assumption that the alkyl group of a phosphotriester mimics the proton of a neutral phosphodiester.



8



9

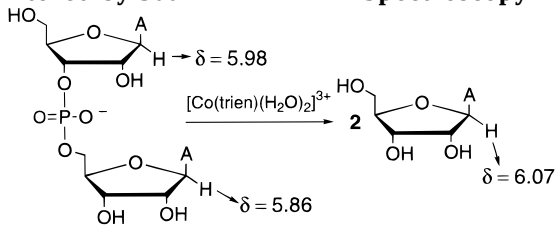
In summary, RNA models have provided a wealth of knowledge with respect to the mechanisms of phosphate ester hydrolysis; however, much caution should be taken when extrapolating results from model studies to RNA. Such extrapolation can lead to incorrect predictions for true RNA substrates, and it is essential that model studies not be used as the sole basis for the selection of RNA cleavage catalysts.

2. Dimeric Assays

In recent years, dinucleoside mono- and diphosphates such as ApA and ApUp have come to the forefront as substrates for mechanistic and kinetic studies of RNA cleavage.[2,24−26,107,109,112−114,162−177] It has been pointed out that dimers resemble normal RNA polymers more closely than do phosphodiesters with good leaving groups. In fact, in a recent study by Kuusela and Lönnberg, the pH−rate profiles for the pH-dependent transesterification of poly U and 3′,5′-UpU were quite similar, so one may infer that the mechanisms are similar.[178] It should be noted that this result was obtained in the absence of metal ions. Work by the groups of Eichhorn,[179] Inoue,[176] and Lönnberg[180] on the hydrolysis of RNA by metal ions has revealed that dimers undergo slow nucleophilic cleavage compared to longer RNA molecules (tetramers and above).

In addition to being true nucleoside phosphodiesters, RNA dimers are convenient substrates for studying RNA hydrolysis. There is only one possible site of cleavage, and the products can be easily separated and identified by HPLC methods. In an interesting example reported by Matsumoto and Komiyama, the cleavage of ApA by triethylenetetraaminecobalt(III) to two adenosine molecules was followed by 500-MHz NMR spectroscopy (Scheme 7).[174] This was achieved by monitoring the signals for the C1′ protons of ApA. The signal for the C1′ proton of the 5′-adenosine appears at $\delta = 5.98$, while that of the 3′-adenosine occurs at $\delta = 5.86$. Upon

Scheme 7. Cleavage of ApA by $[Co(trien)(H_2O)_2]^{3+}$: The Change in Shift of the C1′ Proton Was Monitored by 500-MHz ^{1}H NMR Spectroscopy



transesterification and hydrolysis, the decrease in these signals was accompanied by a stoichiometric increase in the C1′ signal for free adenosine at $\delta = 6.07$. The rate constant evaluated by NMR spectroscopy was further confirmed by HPLC methods.

In a study of the cleavage of dinucleosides and dinucleotides by La^{3+}, Pb^{2+}, and Zn^{2+}, Shelton and Morrow monitored the reactions by measuring the quantity of inorganic phosphate produced.[26] The assay involved treatment of the products with acid to open up the initially produced 2′,3′-cyclic phosphate, followed by alkaline phosphatase digestion to remove the terminal phosphates of the 2′- and 3′-monophosphates. The amount of inorganic phosphate was then determined by treating the samples with an ascorbic acid−molybdate solution to form a reduced phosphorus−molybdate complex. Quantification was performed by spectrophotometry. Measurement of inorganic phosphate production was also used in some of the early studies of RNA cleavage by Eichhorn and Butzow.[181,182]

Despite the potential advantages of using dinucleotides over activated substrates, we believe that they too are not quite the ideal substrates for investigations of RNA cleavage. Dinucleotides lack the polymeric and polyanionic character of the longer, more biologically relevant polyribonucleotides which represent the ultimate targets for most RNA-based drug therapies. Significant differences have also been observed in the chemical reactivity of metal ions and complexes toward dimeric and polymeric RNA. Work by Lönnberg's group on the transesterification/hydrolysis of UpU and poly-U by metal ions revealed a 5- to 20-fold rate enhancement for cleavage of poly-U over UpU.[180] Even greater differences have been seen for Cuterpy-catalyzed cleavage of ApA (very slow)[24,156] and $(rA)_{12-18}$ (much faster).[1]

Considerable differences in reactivity have also been observed among dinucleotides. Butzow and Eichhorn compared the hydrolysis of ApA and ApAp (which differ only by the presence of a 3′-phosphate terminus on ApAp) by Zn^{2+}.[179] It was found that ApAp was hydrolyzed about 2 orders of magnitude more rapidly than ApA, thus implicating the participation of neighboring phosphate groups in the metal ion promoted hydrolysis of RNA. This phenomenon was later investigated by Kuusela et al.[183,184] by comparing the reactivity of NpNpNp(3′) and NpNpNp(2′) to that of NpNpN in the presence of Zn^{2+}. They observed that the 3′-monophosphate group accelerates the cleavage of both of the phosphodiester bonds in the trimer, but the 2′-monophosphate group accelerates only the hydrolysis of the bond Np−

NpNp(2′) and not the bond NpNp−Np(2′). They also showed, by using chimeric DNA−RNA oligomers, that the rate-accelerating effect of the terminal monophosphate is also extended further in the chain[184] and that this influence is intramolecular.[183]

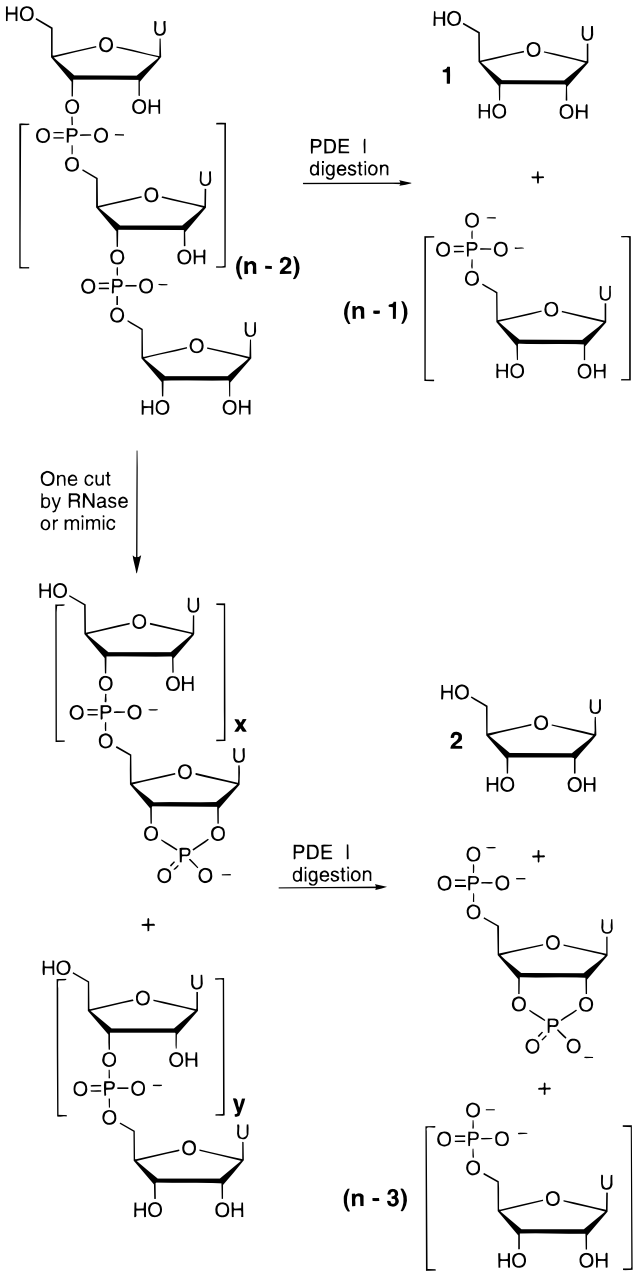
3. Polymeric Assays

Classically, spectrophotometric assays have been used for the quantitative determination of RNase activity on ribopolymers. One of the earliest and most widely used assays is that of Kunitz.[185] This method takes advantage of the hyperchromic shift that occurs in the ultraviolet spectrum of polymeric RNA upon degradation by RNases. The rate of change in the absorption of ultraviolet light as a function of time can thus be measured, and RNase activity can be conveniently determined. Another method, developed by Anfinsen and colleagues,[186] involves precipitation of longer oligonucleotides with uranyl acetate and perchloric acid following treatment of polymeric RNA with RNase. The extent of RNA degradation is then determined by measuring the absorbance of "soluble nucleotide". The explanation for this change in solubility is that the long RNA polymers are degraded into smaller, more soluble oligoribonucleotides and mononucleotides by the RNase. The extent of degradation can thus be followed by allowing the reaction to proceed for varying periods of time, precipitating the high molecular weight material with uranyl acetate, and measuring the absorbance of soluble product in the supernatant.

To develop a quantitative assay for the kinetic analysis of polyribonucleotide cleavage by ribonucleases and ribonuclease mimics, Breslow and colleagues[187] used a variation of an earlier procedure reported by Stevens and Hilmoe.[188] As shown in Scheme 8, the assay involved end-group analysis of the cleavage products from polyuridylic acid (poly U). A 2′,3′-cyclic phosphate was generated by each nucleophilic cleavage event on one side of the cut, and a 5′-OH was generated on the other side. The cyclic phosphates could be subsequently hydrolyzed to the corresponding 3′-monophosphates. Treatment of the cleavage products with phosphodiesterase I (PDE I) cleaved the 3′ O−P bonds to give 5′-monophosphates; however, the residue with the 5′-OH at the cut site was converted to a simple uridine nucleoside. The residue on the other side of the cut became a nucleoside diphosphate. Thus, each cut of poly U by a ribonuclease or a mimic produced an additional unphosphorylated 5′-end, which resulted in an additional molecule of uridine after PDE I treatment. The increase in the concentration of uridine as a function of time could then be followed by HPLC analysis, and rate constants could accordingly be calculated. Lönnberg and co-workers later reported the use of this assay in a quantitative study of RNA hydrolysis by metal ions and complexes.[180]

Smith and Anslyn proposed the use of radioactive end labeling to determine the rates of hydrolysis of polymeric substrates by synthetic RNase mimics.[189] In their study, incubation of the RNA with the mimic generated various cleavage products with 5′-OH

Scheme 8. Phosphodiesterase I Assay for Determining the Kinetics of RNA Cleavage: Each Cleavage Event Produces an Additional Unphosphorylated 5′-Terminus, Leading to an Additional Uridine Nucleoside after Phoshodiesterase I Treatment (from adapted ref 187)



termini. These hydroxyl termini were radioactively labeled with [γ-^{32}P] ATP, using T4 polynucleotide kinase. Unincorporated label was then removed by paper chromatography, and scintillation counting was used to quantify the cleavage results. The advantage of this method was the lack of need for UV-detected HPLC techniques, which generally require long reaction times in order for peak intensity changes to register on a chromatogram. The problem associated with the end-labeling method, however, was the effect that RNase mimics exerted on the labeling reactions. The mimics diminished the labeling efficiencies to various extents, so standard curves

had to be generated for the labeling reactions in the presence of each individual mimic.

Stopped-flow kinetic methods constitute another alternative for studying the rates of polymeric RNA degradation by ribonucleases. The stoichiometric dependence of *Escherichia coli* ribonuclease H activity on magnesium activation was determined by Cowan and colleagues using such an assay.[190] RNase H degrades the RNA moiety in DNA−RNA hybrids, and by using photomultiplier detection to monitor the hyperchromic effect of an $(A{\cdot}dT)_{20}$ hybrid substrate, initial cleavage velocities (v_0) were determined. Plots of the variation of v_0 with substrate concentration and Mg^{2+} concentration led to the conclusion that only one equivalent of metal ion is kinetically important for RNase H activity.

Lönnberg and Kuusela reported an innovative assay for following both the hydrolysis and isomerization of the internucleosidic phosphodiester bonds of polyribonucleotides.[178] This method resembles the phosphodiesterase I method of Breslow; however, the pyrimidine-specific endonuclease RNase A is used to cleave the unreacted starting material. Digestion of an intact pyrimidine polymer with RNase A yields 3′-mononucleotides. However, if the RNA polymer has been cleaved previously in a nonenzymatic fashion (which produces terminal 2′- and 3′-phosphomonoester groups in a constant ratio), subsequent digestion with RNase A produces additional 2′-mononucleotides. The amount of 2′-mononucleotide can then be determined by HPLC, and the number of phosphodiester bonds cleaved nonenzymatically may be determined. In the event that isomerization of the poly U to give 2′,5′-phosphodiester linkages has occurred, treatment with RNase A yields uridylyl-(2′,5′)uridine 2′- or 3′-monophosphate. These products can also be detected by HPLC, and this allows for the rate of isomerization to be estimated.

All of this work demonstrates that the assays employed for the quantitative study of polymeric RNA hydrolysis are quite complex and time-consuming. Numerous steps are involved in the analyses, increasing the chances for error, but heroic efforts have yielded valuable results.

4. Chimeric Assays

McLaughlin's group reported an elegant assay in which the cleavage of RNA by metal ions was *accelerated* at a single site by a single phosphorothioate RNA nucleotide incorporated into the DNA backbone (Figure 4).[191]

The products of cleavage by a variety of divalent metal ions were a 5′-thiol-containing 6-mer DNA fragment and a 2′,3′-cyclic phosphate-containing 8-mer DNA fragment. Thus, only two products were formed, which allowed kinetic analyses to be performed with the use of high-resolution gel electrophoresis in conjunction with phosphorimaging techniques. The relative cleavage rate enhancements for the metal ions corresponded well with Pearson's HSAB principle, suggesting that the cleavage mechanism involves coordination of the metal to the 5′-mercapto leaving group. This assay should prove invaluable as a tool for the direct comparison of cleavage efficiencies of a variety of catalysts toward

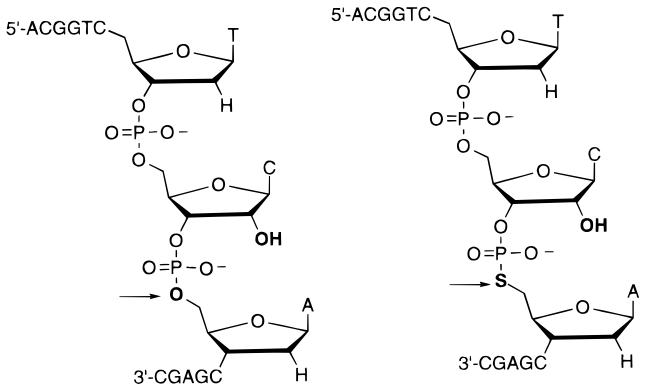


Figure 4. Chimeric substrates used by McLaughlin et al. to study hydrolysis of RNA by ribozymes and metal ions.

polyribonucleotides and has already been applied to ribozyme studies.[192]

In contemporaneous studies, Jenkins et al.[155,193] reported the use of chimeric DNA−RNA molecules, also containing an RNA nucleotide embedded in DNA sequences, as substrates for studying the transesterification of RNA. The substrates, termed embedded RNA (embRNA), display the simplicity of dinucleotide substrates while possessing the multiple phosphate and nucleobase metal-binding sites found in polyribonucleotides. In addition, the DNA residues provide an internal check for oxidative cleavage. In fact, an internal check for any kind of DNA cleavage is built into the embRNA assay, and none was found under the reported conditions. Furthermore, the cleavage products were found to be identical for hydroxide, RNase One and Cu(II)terpy, providing yet another of the many confirmations that Cu(II)terpy functions by a nucleophilic pathway to cleave RNA. In an illustrative example of this assay, the cleavage of an embRNA is shown in Scheme 9.

The image of an electrophoresis gel (Figure 5) illustrates the clean products obtained when $T_{11}UT_7$ is cleaved by enzymatic and inorganic catalysts. These data are well-suited to kinetic analysis on a Phosphorimager.

Several important conclusions derived from the embedded RNA assays are worthy of mention. The use of dinucleotide RNA fragments as substrates for the screening of RNA cleavage agents and mechanistic studies is widespread, but this practice does not accurately predict the relative abilities of metal complexes to cleave polyribonucleotide substrates. EmbRNA substrates offer the simplicity of dinucleotides while maintaining the polymeric and polyanionic character of true RNA. They also provide internal checks for any type of oxidative cleavage. Therefore, embRNA assays appear to be among the best substrates for routine studies of RNA transesterification, and future use of such assays should provide a great deal of insight into the kinetics and mechanistic details of nucleophilic RNA cleavage.

Kuusela et al. have also used chimeric substrates in the study of the metal ion catalyzed hydrolysis of RNA.[184] They investigated the Zn^{2+}-promoted hydrolysis of the 5′-terminal ribonucleoside phosphodiester bond in chimeric substrates such as those in Figure 6. They found that the phosphodiester bond of the RNA in the trimeric and longer oligonucle-

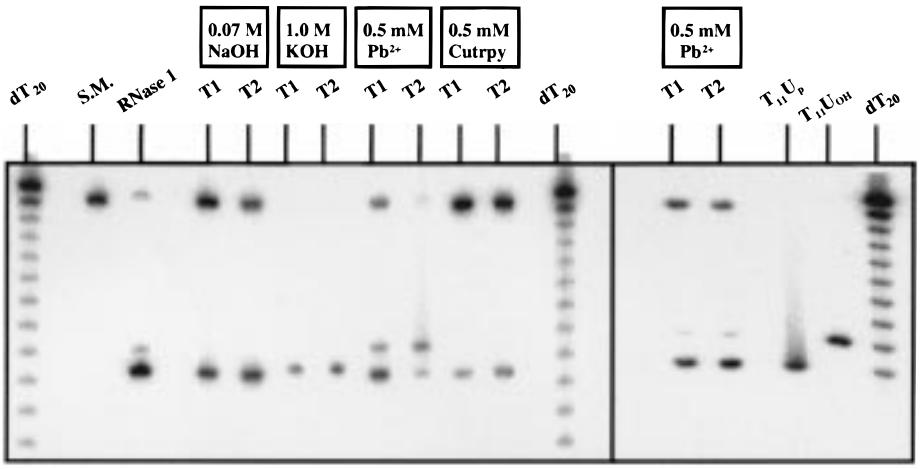


Figure 5. Image of an electrophoresis gel showing the clean products obtained by hydrolytic cleavage of $T_{11}UT_7$. (Note: the extra band in the lane for Pb^{2+}-catalyzed hydrolysis is the result of complete dephosphorylation, as indicated by the authors).

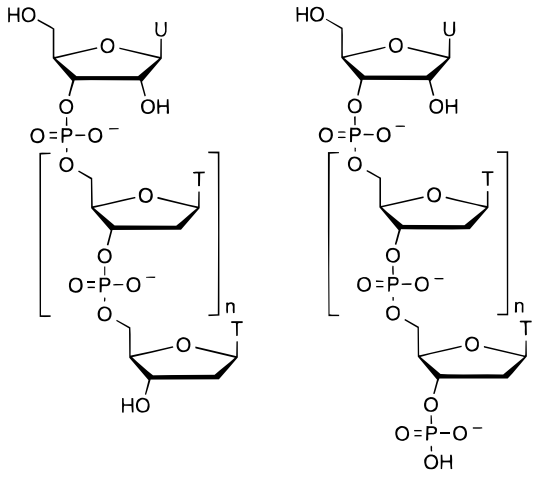


Figure 6. Chimeric substrates used by Kuusela et al.

otides lacking a terminal phosphate group was hydrolyzed about 10 times faster than the same bond in a dinucleoside monophosphate. Addition of a terminal phosphate group to the longer oligonucleotides resulted in a further 10-fold increase in reactivity. This assay has proved a valuable tool in determining the origin of the rate-accelerating effect exhibited by terminal monophosphate groups on RNA cleavage by metal ions.

III. Sequence-Specific Nucleophilic RNA Cleavage

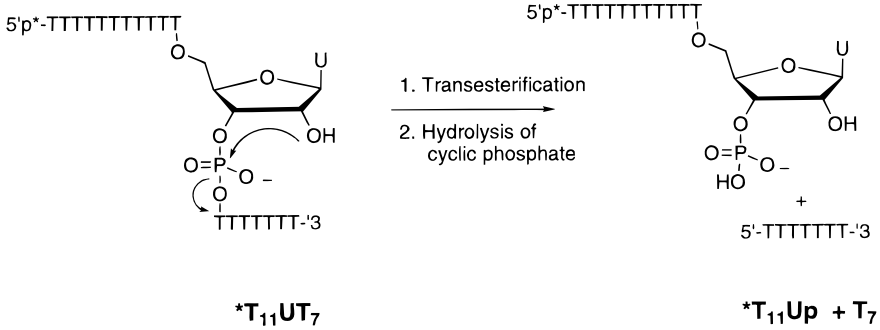
A. Key Design Features

It is by now generally understood that certain design features are critical to the development of effective ribozyme mimics. One of the most heavily emphasized requirements is that of "cleavage within the DNA−RNA duplex region".[30,39−43,194,195] This design element is *essential for catalytic turnover* because the DNA−RNA binding constant is strongly length-dependent. Cleavage within the duplex greatly reduces the binding constant between the ribozyme mimic and its RNA target, which allows the product fragments to be released. This is illustrated in Figure 7, along with the alternative design which places the cleavage agent at the end of the duplex region. Reagents for external cleavage are usually simpler to prepare than reagents for internal cleavage. However, external cleavage results in complete product inhibition, because the DNA−RNA binding constant is not decreased by the cleavage event, so the "catalyst" is entombed in an RNA strand.

Groove delivery is another important consideration for the design of ribozyme mimics. Figure 8a shows the structure of the familiar B-form DNA−DNA helix juxtaposed with the A-form helix, adopted by DNA−RNA helices. Ribozyme mimics can be designed to attack their target RNA across either or both of the two grooves present in the A-form helix. These choices are illustrated in Figure 8b.

Groove placement is governed by the site where the catalyst is attached (see the **bold** bonds in Figure 9). Substituents at C_5 of thymidine (T) lie in the major groove, whereas substituents at the C_1' (or C_2') sugar positions are directed into the minor groove. Delivery of two metal complexes can mimic the

Scheme 9. Cleavage of an embRNA Substrate (* = radioactive ^{32}P label)



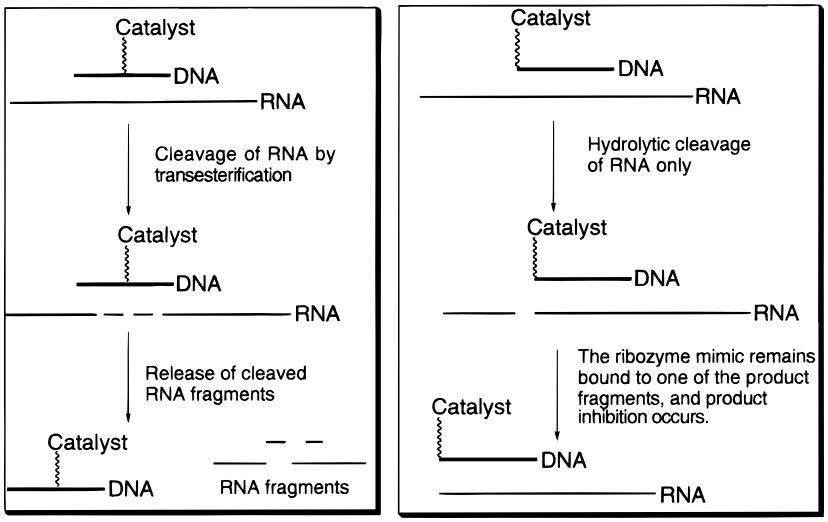


Figure 7. Cleavage of RNA withing the duplex region vs cleavage outside the duplex region.

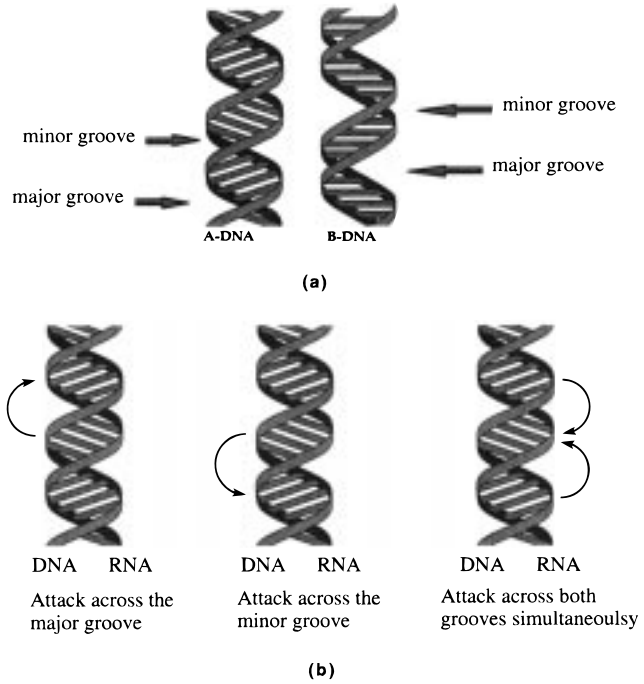


Figure 8. The various modes of catalyst delivery to the RNA target. Delivery can be made across the major groove, the minor groove, or both grooves simultaneously.

dimeric cooperativity of the bimetallic sites in purple acid phosphatase.[196]

The length of the linker arm between the DNA probe and the pendant RNA cleavage catalyst is also of major importance. Molecular modeling is a useful method for estimating the optimum linker arm length for delivering the catalyst to the RNA target. The groove across which the delivery is made is a major factor in this determination. Delivery across the major groove will typically require a longer linker arm than delivery across the smaller minor groove. Early designs have used flexible linkers, sacrificing precision to achieve activity. It is important to remember that a canonical A-form helix may be dramatically distorted by a pendant cationic metal complex. Therefore, structural information on mimic−substrate complexes is needed before precisely optimized linker arms can be designed.

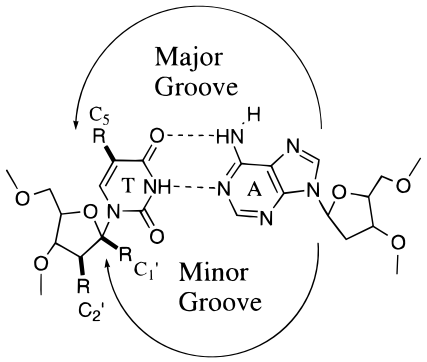


Figure 9. Possible sites for catalyst attachment without directly interfering with the Watson−Crick hydrogen bonding of the nucleobases.

B. Preparation and Behavior of Sequence-Specific RNA Cleavage Agents

1. Hybrid Enzymes

The first reported examples of artificial site-specific cleaving agents for RNA were created by the covalent attachment of nuclease enzymes to deoxyoligonucleotides. Through Watson−Crick base pairing interactions, the deoxyoligonucleotides delivered the relatively nonspecific enzymes to defined sites on the target RNA molecules. Zuckermann and Schultz[197] attached a mutant staphylococcal nuclease (Lys-116 to Cys-116) to the 3′-end of a deoxyoligonucleotide (14-mer) via a disulfide linkage (Figure 10). Treatment of a 59-mer single-stranded RNA target with this construct (in the presence of Ca^{2+}) resulted in cleavage over a 3- to 5-nucleotide region directly adjacent to the hybridization site. Unfortunately, the reagent's specificity decreased with increasing reaction times, possibly as a result of self-cleavage of the DNA−enzyme construct. Staphylococcal nuclease hydrolyzes the phosphodiester bonds of both single-stranded RNA and DNA, which means that it can also destroy the DNA probe to which it is attached.

As a possible solution to this problem, the same group coupled RNase S (a subtilisin digested version of RNase A) to a deoxyoligonucleotide (14-mer) to create a sequence-specific ribonuclease (Figure 11).[198]

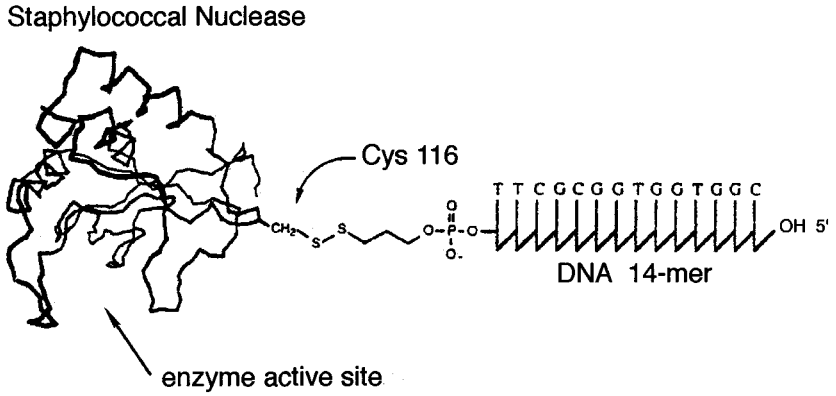


Figure 10. A hybrid staphylococcal nuclease for sequence-specific RNA cleavage (adapted from ref 197).

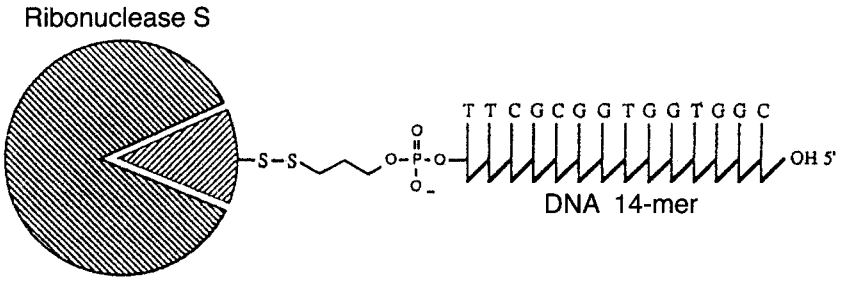


Figure 11. A hybrid ribonuclease S (adapted from ref 198).

In contrast to the hybrid staphylococcal nuclease, this hybrid was incapable of self-cleavage because RNase S is specific for RNA only. As was expected, the oligonucleotide delivered the enzymatic activity to a specific site on its 62-mer RNA target. Cleavage occurred at one pyrimidine-purine site adjacent to the site of hybridization. Unfortunately, cleavage efficiency and specificity decreased at elevated temperatures (>37 °C), and the authors attributed this to the probable dissociation of the RNase S adduct into its two protein fragment components.

Kanaya and colleagues[199] later created a hybrid enzyme by covalently linking *E. coli* RNase H to the 5′ terminus of a 9-mer deoxyoligonucleotide (Figure 12). This was accomplished using site-directed mutagenesis to substitute a cysteine residue for Glu[135] in a mutant form of the enzyme. The free cysteine residue allowed for coupling of the enzyme to a maleimide group, which was attached at the 5′-end of the 9-mer deoxyoligonucleotide via a flexible tether. The target for this conjugate was a synthetic 9-mer single-stranded RNA, and sequence-specific cleavage was achieved between the fifth and sixth residues of the target. An important property of this particular hybrid is that it exhibited 7- and 4-fold decreases in its K_m and k_{cat} values respectively in comparison to the unmodified enzyme, indicating that catalytic turnover was achieved. This property can best be explained by the fact that the cleavage event must occur within the duplex region (since RNase H only cleaves RNA in DNA−RNA duplexes), and this must lower the binding affinity enough to allow for the dissociation of the cleavage products from the hybrid and free it for another cycle. Subsequent melting temperature studies performed on the DNA 9-mer and the RNA cleavage products indicated that a dramatic decrease in the stability of the DNA−RNA duplex would occur after cleavage

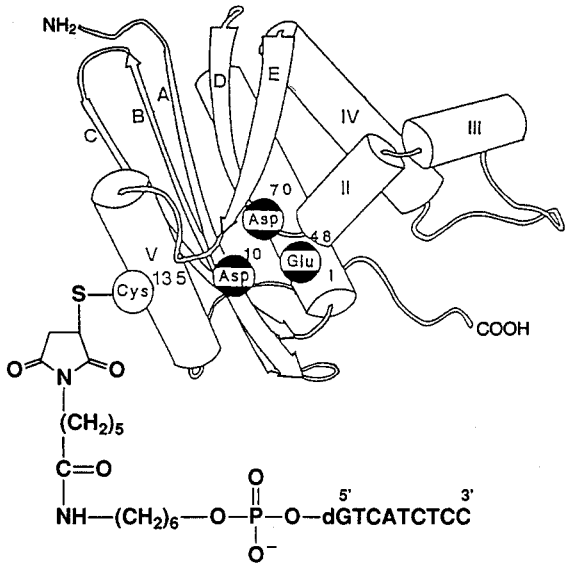


Figure 12. A hybrid RNase H. (Reprinted from ref 199b. Copyright 1994 American Chemical Society.)

by RNase H. The same group later reported the preparation of a series of the same hybrid in which the length of the linker arm was varied (18, 24, and 27 Å). The hybrid with the 27-Å linker arm cleaved a 22-mer RNA target at almost exclusively one position.

2. Ribozyme Mimics Based on Metal Complexes

While hybrid enzymes exhibit highly specific and efficient cleavage of their RNA targets, their large molecular weights and difficulty of preparation probably preclude their use in practical applications. Recently, we and several other groups have covalently attached metal complexes to oligonucleotides to form ribozyme mimics. In 1994, the first example

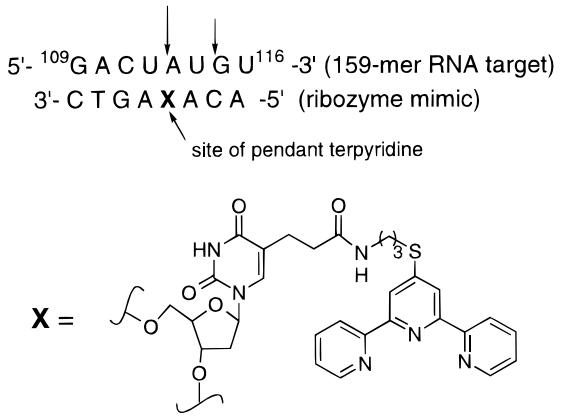
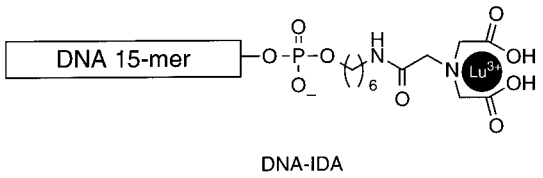


Figure 13. The first wholly synthetic, functional mimic of a ribozyme. The arrows represent Cu(terpy)OH^+ reaching across the major groove to cleave the target RNA.



DNA-IDA

Figure 14. A DNA 15-mer functionalized at the 5′-end with an iminodiacetate residue.

of a *wholly synthetic*, functional mimic of a ribozyme was reported.[30] This mimic consisted of a 17-mer DNA oligonucleotide with a covalently attached terpyridine (terpy) ligand at C-5 of an internal uracil residue (Figure 13). The mimic was synthesized (via solid-phase DNA chemistry) using a modified DNA building block. The target was a 159-mer RNA sequence derived from the *gag*-mRNA of HIV, and sequence-specific cleavage was observed at physiological pH (7.5) in the presence of $CuCl_2$. The cleavage was located at two positions within the duplex region opposite the modified base. The efficiency of cleavage was 11% at 37 °C and 18−25% at 45 °C over a period of 72 h, with a probe concentration of 5 μM and a RNA target concentration of $\sim 10^{-3}$ μM. These results were pivotal in that they *proved the concept* that ribozyme mimics can be constructed by covalently linking RNA transesterification catalysts to DNA. Thus, the complex catalytic region of a natural ribozyme was replaced with a small molecule catalyst.

Shortly thereafter, other examples of sequence-specific RNA cleavage agents based on metal complexes were reported. Matsumura et al.[34] prepared a 15-mer deoxyoligonucleotide which was functionalized at the 5′-end with a lanthanide-complexing iminodiacetate residue (DNA−IDA) (Figure 14). This conjugate was synthesized using a postsynthetic strategy in which a DNA 15-mer with an amino group at its 5′-end was reacted with the 4-nitrophenyl ester of the metal-complexing moiety. In the presence of various lanthanide ions (i.e., Lu(III), Th(III), and Eu(III)), the modified oligo cleaved a synthetic 39-mer RNA target outside the duplex region, opposite the metal complex. The efficiency of cleavage was 7.3% after 4 h and 17% after 8 h. Reactions were performed at 37 °C and pH 8, and the concentrations

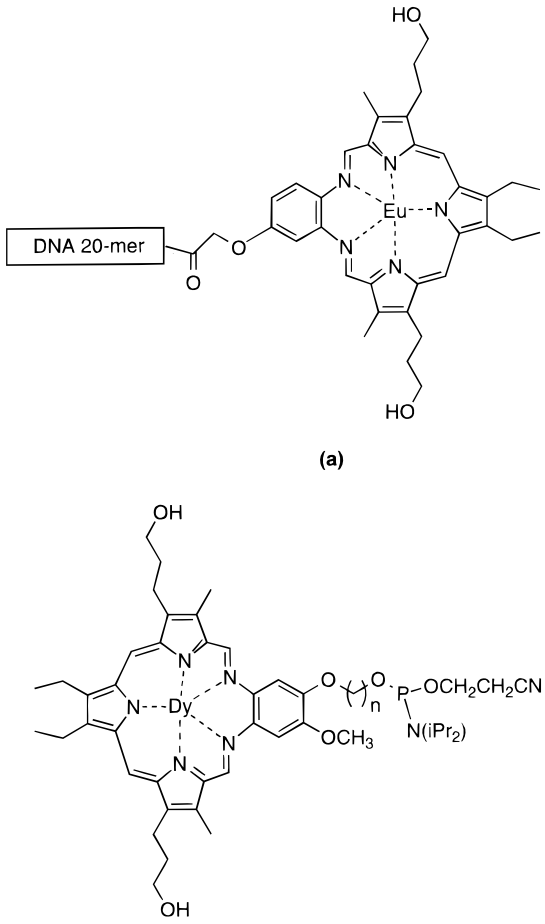


Figure 15. (a) Eu(III) texaphyrin conjugate used for sequence-specific RNA cleavage and (b) Dy(III) texaphyrin phosphoramidite used as a reagent for the automated synthesis of ribozyme mimics.

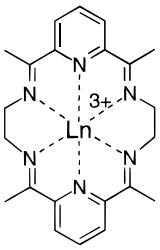
of DNA-IDA and RNA target were 10 and 0.3 μM, respectively.

Magda et al.[200] synthesized ribozyme mimics by attaching Eu(III), chelated by a monoanionic, pentadentate texaphyrin ligand (EuTx), to 20-mer DNA probes. Their postsynthetic modification strategy involved the synthesis of DNA oligonucleotides containing alkylamine groups either at C-5 of an internal thymine residue or at a 5′-terminal phosphate. This was followed by treatment of the deoxyoligonucleotides with the europium(III)-texaphyrin carboxylic acid to effect amide coupling to the alkylamine groups (Figure 15a). The conjugate with the texaphyrin complex attached at the 5′-end of the DNA strand site specifically cleaved a chemically synthesized 30-mer RNA target near the expected location. Approximately 30% cleavage was observed after 24 h at 37 °C and pH 7.5 in a reaction containing 2.5 nM probe and ∼1 nM RNA target. In contrast, no cleavage was observed with the internally modified deoxyoligonucleotides. A major contribution from this work was the use of the stable preformed Eu(III) complex. Whereas other approaches required the addition of free metal ion cofactors for the cleavage event to take place, this method allowed the cleavage reaction to occur independently of such cofactors. This may prove to be extremely important

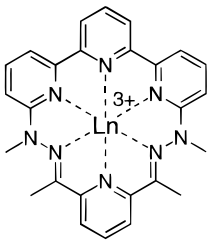
when considering the use of ribozyme mimics for in vivo applications in the presence of competing protein ligands and other bioavailable metals.

More recently, Magda reported a variation of this approach in which the synthesis of similar cleavage agents was accomplished using a dysprosium(III) texaphyrin phosphoramidite as an auxiliary reagent on a commercial DNA synthesizer (Figure 15b).[201] The objective was to avoid the need for solution-phase conjugation of the preformed metal complex and postsynthetic addition of a metal cation to a DNA-bound ligand. The complex was attached at the 5′-end of a 20-mer deoxyoligonucleotide during the course of automated DNA synthesis, and sequence-specific cleavage of a complementary 36-mer RNA target was achieved. A series of structural variants of this conjugate was constructed, and one mimic cleaved ~80% of the RNA target after 6 h at 37 °C and pH 7.5. The concentrations of conjugate and RNA target were 50 nM and 2.0 nM, respectively.

Much success has been achieved by Hall et al.[42,202] with the terpyridine-derived, lanthanide macrocyclic complexes linked covalently to deoxyoligonucleotides. Previous work by Morrow et al. revealed that related, hexadentate Schiff base macrocyclic lanthanide complexes **10** were efficient RNA transesterification catalysts.[25] Unfortunately, these complexes were susceptible to hydrolytic decomposition in aqueous solution, and this prompted the synthesis of the terpyridine derived macrocyclic complexes **11** with hydrazone-type linkages in place of the imine linkages for added stability.



10



11

Europium(III) complexes of this type were linked to a 5′-hexylamino-derivatized deoxyoligonucleotide (20-mer) by using postsynthetic strategies, and the resulting conjugates (Figure 16) were tested for their ability to effect the specific cleavage of a synthetic 29-mer RNA target. Analysis revealed near-quantitative cleavage (88%) of the target RNA strand at almost exclusively one site outside of the duplex region after incubation at 37 °C and pH 7.4 for 16 h. Reactions contained a RNA target concentration of ~10 to 50 nM and a probe concentration of 400 nM.

Lönnberg and co-workers [203] developed a sequence-specific RNA cleavage agent by tethering a histamine group to the 3′-terminus of a 10-mer deoxyoligonucleotide (Figure 17). This was accomplished by attaching an ester function to the deoxyoligonucleotide during chain assembly. Treatment of the deoxyoligonucleotide with the appropriate primary amine afforded the desired conjugate. Site-specific cleavage of a synthetic 16-mer RNA target was observed only in the presence of zinc(II) ion. The extent of cleavage

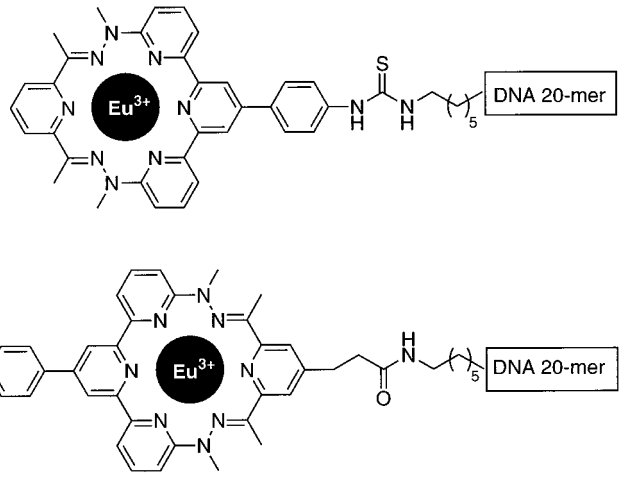


Figure 16. DNA conjugates based on 11.

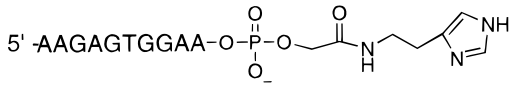
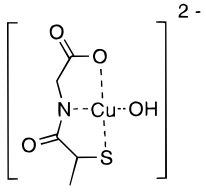


Figure 17. A ribozyme mimic with a histamine at the 5′-end of the DNA probe.

was determined to be ~2−5% after 19 h at room temperature in a reaction containing 2.5 μM deoxyoligonucleotide conjugate, 50 mM Zn^{2+}, and 0.5 μM RNA target at pH 7.0. Cleavage was achieved with only the 3′-end modified probe and not with probes containing the histamine at the 5′-end or with 1′-modified deoxyoligonucleotides containing the histamine at an internal duplex position.

A novel approach by Baker and co-workers targeted the 5′-cap of messenger RNA.[204] It is known that the mRNAs and pre-mRNAs of eukaryotes and most of their viruses possess a N7-methylated guanosine (m^7G) residue attached to their 5′-termini via a triphosphate linkage. These caps have been associated with several important functions, such as the transport of mRNA from the nucleus to the cytoplasm, protection of mRNA from nuclease attack, pre-mRNA splicing, and attachment of the 40S ribosomal unit to the mRNA for protein synthesis. Thus, the authors assessed that decapitation of a specific mRNA may provide an effective means for specific protein downregulation due to loss of mRNA function. Previously, Baker used free Cu(II) complexes in solution to cleave the cap region.[205] The RNA decapitating agent used in this study was the peptide derivative *N*-(2-mercaptopropionyl)glycine **12**, which coordinates copper(II) in solution via the thiol, amide, and carboxylate groups.



12

For incorporation into a deoxyoligonucleotide, a glutamate analogue of **12**, *N*-(2-mercaptoacetyl)-glutamate was used. Two equivalents of this ana-

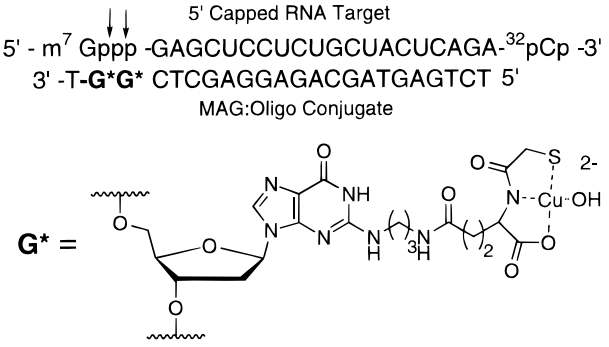


Figure 18. Decapitation of an RNA target by the MAG: Oligonucleotide conjugate (from adapted ref 204).

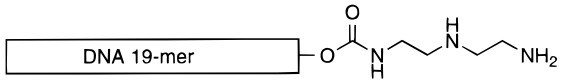


Figure 19. An ethylenediamine−DNA hybrid.

logue were attached to the 3′-end of a 20-mer antisense deoxyoligonucleotide equipped with two N2-propylamine deoxyguanosine residues near its 3′-end using a post-conjugation strategy (Figure 18). Subsequent metalation of the MAG-containing deoxyoligonucleotide with copper(II) provided the active RNA decapitating conjugate. A complementary synthetic 20-mer RNA strand possessing a 5′- m^7G cap was used as the target, and 20% decapitation was observed after 120 h at 37 °C and pH 7.4. Reactions contained 10 μM of the antisense deoxyoligonucleotide conjugate.

3. Ribozyme Mimics Based on Organic Catalysts

Synthetic ribonucleases have also been developed by the covalent attachment of organic RNA transesterification catalysts to deoxyoligonucleotides. These reagents are covered extensively by Oivanan, Kuusela, and Lönnberg's accompanying article, but brief reference is made here to certain relevant advances. This work started with the ideas reported by Cohen[13] and with subsequent papers by the Bashkin group on the synthesis of DNA building blocks containing pendant organic catalysts.[28,29] Komiyama and coworkers were the first to report successful sequence-specific RNA cleavage using this method.[206] Their mimic consisted of a urethane-linked ethylenediamine at the 5′-end of a 19-mer DNA probe complementary to A44-A62 of $tRNA^{Phe}$ (Figure 19). It was prepared by the treatment of a solid-supported DNA 19-mer with 1,1′-carbonyldiimidazole, followed by diethylenetriamine. This artificial enzyme site specifically cleaved about 10% of the $tRNA^{Phe}$ after 4 h at 50 °C. Cleavage occurred just outside the duplex region adjacent to the ethylenediamine residue. Reactions consisted of 0.1 mM probe, 1 μM $tRNA^{Phe}$, and 1 mM EDTA at pH 8.0. The EDTA was added to the reaction mixture to sequester any metal ions and rule out the participation of metal ions in the cleavage reaction. Site-specific cleavage of a linear 30-mer oligoribonucleotide was also achieved using the same construct.[33] The extent of cleavage was ~10% after 4 h at pH 8.0 and 50 °C. The concentrations of conjugate and RNA were 100 and 1.0 μM, respectively.

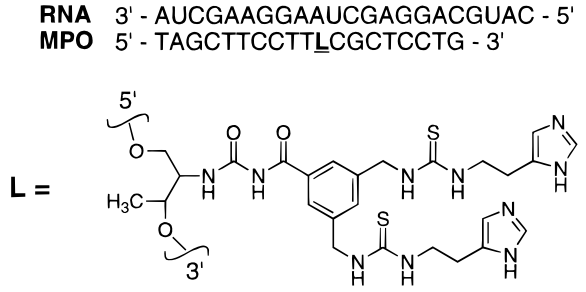


Figure 20. Structure of the modified MPO conjugate containing a nonnucleotide linker and its RNA target.

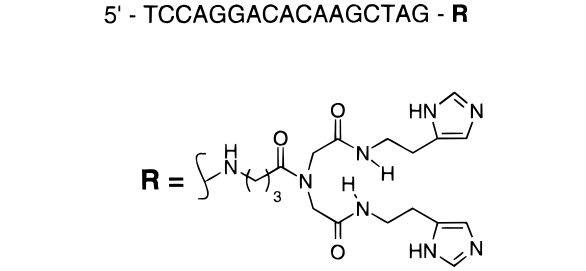


Figure 21. Oligonucleotide conjugate containing a diamidazole construct attached at the 3′-end.

Reynolds et al. made an imidazole-based synthetic ribonuclease.[38] Recognizing that single-stranded RNA is more susceptible to nucleophilic cleavage than duplexed RNA (and also the importance of cleavage within the duplex region for release of the cleaving agent), they engineered into their probe a nonnucleotide-linker with an attached imidazole group in place of one of the complementary bases (Figure 20). Using this technique, the authors hoped to introduce added flexibility into the opposite RNA strand, thereby allowing it to adopt a conformation more favorable for transesterification. Two of their probes yielded site-specific cleavage of a 22-mer RNA strand within the duplex region. Less than 10% cleavage was observed after 5 days at 25 °C and pH 7.2, with a final probe concentration of 75 μM. EDTA (1.0 mM) was also added to each reaction to scavenge metals, thus ruling out the participation of metal contaminants in the reaction.

Quite recently, Vlassov et al.[37] obtained remarkable site-specific cleavage for an organic-based synthetic ribonuclease. They made deoxyoligonucleotide derivatives conjugated with moieties containing two histamine residues that mimic the catalytic active site of RNase A. Deoxyoligonucleotides with the modifications at either the 3′- or 5′-end, separately or together, site specifically cleaved $tRNA^{Phe}$. Maximum cleavage (60%) of the target was attained after 8 h at 37 °C (pH 7.0). Two probes were used. The longer of the two probes with the modification at the 5′-end gave the most efficient cleavage (Figure 21). The cleavage pattern corresponded to regions on the RNA just opposite the histamine groups, outside the duplex region. An additional cleavage site was found away from the expected targeted region. Apparently this site is close, in a three-dimensional sense, to the targeted cleavage region after folding. To show that the cleavage was not due to metals binding to the histamines, all solutions contained 1 mM EDTA.

IV. Recent Ideas for the Design of Ribozyme Mimics

A. Creating RNA Bulges and Loops

One of the central ideas behind ribozyme mimic development is that following DNA−RNA hybrid formation, the attached RNA transesterification catalyst will be able to cleave the RNA in the duplex region. While most of the studies of RNA cleavage by metal ions and complexes have been performed on single-stranded RNA, Morrow's group set out to test the abilities of metal complexes to cleave RNA in DNA−RNA hybrids.[207] When a short DNA oligonucleotide was annealed to a segment of t-RNA^{Phe}, the complementary RNA sequence was protected from cleavage by Eu^{3+} and La^{3+} hexadentate Schiff base macrocycles, which are efficient catalysts for the transesterification of single-stranded RNA. Thus, they concluded that, in the design of ribozyme mimics, it may be more advantageous to place the catalyst at the end of the DNA strand (proximate to single-stranded RNA) rather than in the center where the RNA is duplexed by DNA and inert to cleavage. This is a reasonable conclusion, but it does not take into account any distortion of the DNA−RNA duplex that might be caused by a covalently attached metal complex. There is also a dramatic increase in the local concentration of catalyst at the target site when the catalyst is linked to a DNA probe, and this should be taken into consideration as well. In fact, cleavage in the DNA−RNA duplex region was demonstrated with the ribozyme mimic based on Cuterpy as described previously. Work by Burstyn's group[208] on the transesterification of RNA hairpin structures by a copper(II) macrocycle has also shown that the results with lanthanide complexes are not necessarily true for complexes of Cu(II). Burstyn observed cleavage in both the single- and double-stranded regions of the hairpin structure. While the cleavage in the double-stranded regions was not as intense as that for the single-stranded regions, it was clearly observed. One explanation for the inertness of duplexed RNA to lanthanide complexes, but not to copper complexes, is that the lanthanides are much larger than copper. Thus, they may be less capable of accessing the regions within the duplex necessary to promote the transesterification reaction. One must also consider the possibility of disruption of the duplexed region by interaction of the RNA nitrogenous bases with the added metal ion. Unwinding and rewinding of double-stranded nucleic acid structures by Cu(II) was shown by Eichhorn many years ago.[209]

RNA bulges in DNA−RNA duplexes have been shown to be susceptible to cleavage by metal complexes.[195] Presumably, the bulged RNA is more flexible (or more strained) than the duplexed RNA and can achieve a conformation that is suitable for cleavage by transesterification. One such conformation allows an in-line attack by the 2′-OH on the phosphodiester linkage which permits a concerted mechanism rather than sequential addition−elimination steps. On the basis of the results with RNA bulges, the group of Häner and colleagues designed deoxyoligonucleotides bearing lanthanide complexes

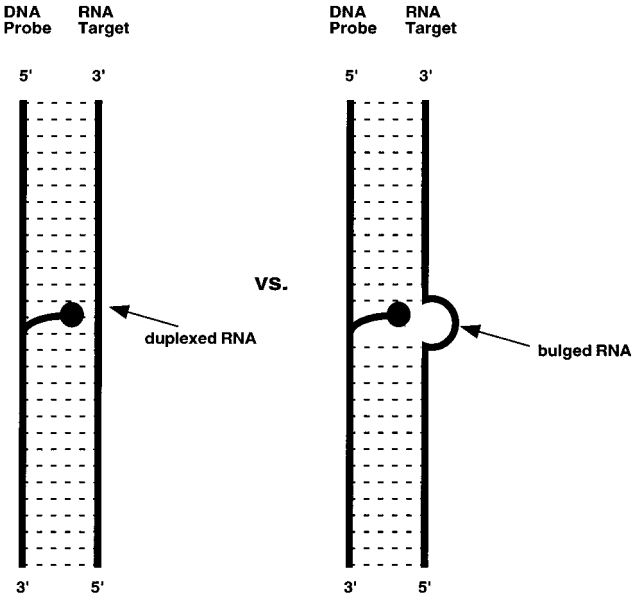


Figure 22. Cleavage of RNA at the bulge in a DNA−RNA duplex.

directed at cleavage within the duplex region (Figure 22).[42] The modified DNA probes were directed to *partially* complementary RNA targets, which would form bulges in the RNA strand of the resultant DNA−RNA duplex. The deoxyoligonucleotides that formed duplexes with bulged RNA residues cleaved 92% of their targets, whereas those that formed a perfect duplex cleaved only 7%. Cleavage assays were performed at 37 °C for 16 h at pH 7.4. The probe concentration was 0.6 μM, and the RNA target concentration was 10−50 nM. It was also found that cleavage was more efficient when the reagent was delivered through the minor groove (61−92%) than through the major groove (21%) of the RNA−DNA duplex.

B. Minimal Nucleotide Replacement

Another approach to overcoming the conformational rigidity asssociated with DNA−RNA duplexes involves eliminating a Watson−Crick base pair within the duplex, thereby increasing the local flexibility of the helix. This concept was introduced by Reynolds et al.[38] with their imidazole-based ribozyme mimic as described previously, and several groups have recently reported interesting variations of this approach. Komiyama and co-workers positioned various oligoamines (catalysts for RNA hydrolysis) between two DNA strands via a nonnucleoside monomer (Figure 23).[210] Site-specific cleavage of a 28-mer RNA target was achieved within the duplex region; however, the cleavage efficiency was only 3% after 16 h at 37 °C and pH 7.5. The concentration of RNA target in the reaction mixture was 0.2 mM, and that of the conjugate was 20 mM.

Magda et al. created a ribozyme mimic by replacing an internal nucleotide with an asymmetric branching glyceryl linker and coupling the resulting 18-mer DNA oligomer to a dysprosium(III) texaphyrin phosphoramidite (Figure 24b).[43] The mimic cleaved a 36-mer RNA target in a sequence-specific manner directly across from the site of the modification in the DNA probe. The extent of cleavage is of particu-

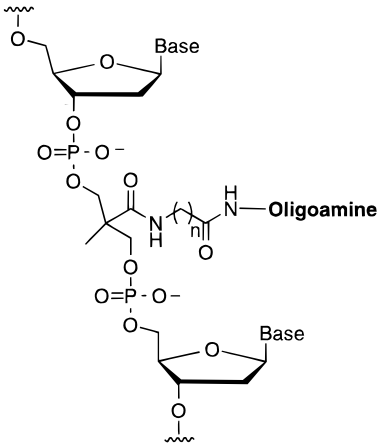
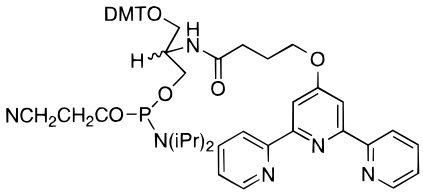


Figure 23. Modified oligonucleotides consisting of oligoamines interpositioned between two DNA oligomers.

lar interest in this study. In reactions containing 50 nM DyTx−DNA conjugate and a 10-fold excess (500nM) of RNA target at pH 7.5 and 37 °C, 67% of the total RNA was cleaved after 24 h. This corresponds to cleavage of 335 nM of the RNA, which is 6.7 times the concentration of the DyTx−DNA conjugate in the reaction. The above data provide evidence that catalytic turnover was indeed achieved by this ribozyme mimic, which represents a *major discovery*.

Daniher et al. also recently reported an interesting example of the "minimal nucleotide replacement" approach.[41] The nucleotide replacement was based on serinol (a reduced form of serine), which lacks the ribose and nucleobase moieties, but possesses the correct spacing between hydroxyl groups to mimic a nucleotide (Figure 24c). Serinol has previously been used as a DNA building block, usually to introduce abasic sites and intercalators in double-stranded DNA. Solution NMR studies have documented an increased conformational flexibility in duplex DNA induced by serinol reagents. The amino group of serinol offers a convenient point of attachment for many catalytic groups. Phosphoramidite **13** was prepared (as a mixture of stereoisomers) in four steps from hydroxybutyric acid, serinol, and 6′-chloroterpyridine. Ribozyme mimics synthesized with **13** provided greatly improved reactivity and exceptional control over the site of RNA cleavage with respect to the previous mimic based on Cu(II)terpy.



13

The gel electrophoresis image presented in Figure 25 shows how, by introducing (serinol-terpy) at four different positions in a 17-mer DNA probe, the target 159-mer HIV mRNA sequence can be cleaved at precisely controlled sites. The ribozyme mimics used for this experiment had serinol incorporated successively at the 5′-end, at position 6, at position 9, and at position 11 of the 17-mer. The cleavage sites followed the location of the terpy reagent in the duplex (see the arrows in Figure 25). The site of cleavage is not biased by sequence or tertiary structure. Cleavage reactions consisted of 5 μM probe, 10 nM RNA target, and 10 μM $CuCl_2$ at pH 7.5 and 45 °C. The extent of cleavage was ∼84% after 72 h.

V. Comparison of Systems

Table 1 lists the results achieved with the various metal- and organic-based ribozyme mimics to date. Direct comparisons are made difficult by the fact that different conditions were employed in each study. It is evident, however, that the systems based on lanthanide metal complexes have achieved the most success thus far with respect to the extent of cleav-

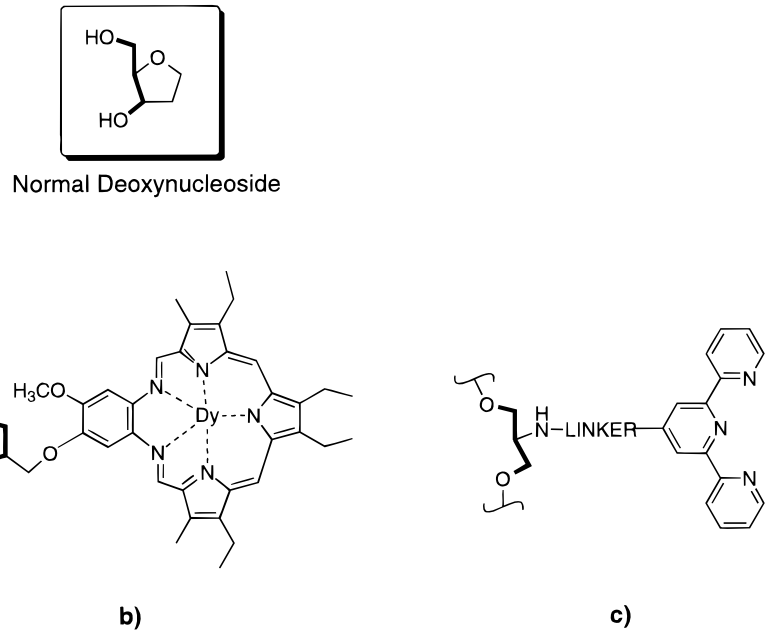


Figure 24. (a) Reynolds et al.,[38] (b) Magda et al.,[43] and (c) Daniher et al.[41]

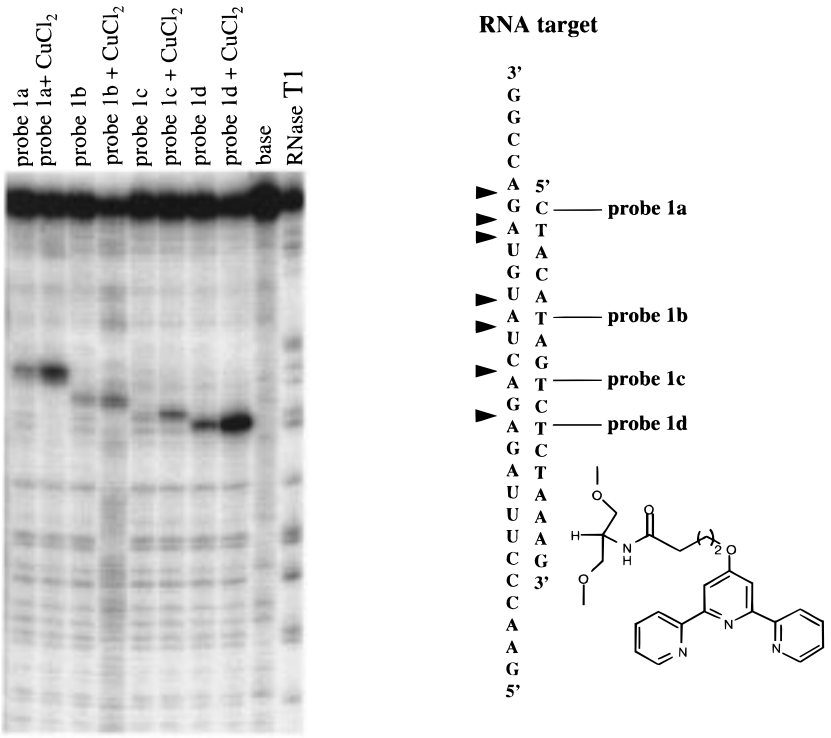


Figure 25. Image of an electrophoresis gel showing the cleavage of a 159-mer RNA sequence with serinol−terpyridine probes. The cleavage sites follow the change in location of the catalyst within the probe.

Table 1. Cleavage Results of Metal and Organic-Based Ribozyme Mimics

Figure	probe length	target length	% cleavage	pH	*T* (°C)	*t* (h)	ref(s)
13	17-mer	159-mer	25	7.5	45	72	30
14	15-mer	39-mer	17	8.0	37	8	34
15a	20-mer	30-mer	30	7.5	37	24	200
15b	20-mer	36-mer	80	7.5	37	6	201
16	20-mer	29-mer	88	7.4	37	16	202
17	10-mer	16-mer	5	7.0	25	19	203
18	20-mer	20-mer	20	7.4	37	120	204
19	19-mer	76-mer, 30-mer	10	8.0	50	4	33, 206
20	19-mer[a]	22-mer	<10	7.2	25	600	38
21	17-mer	76-mer	60	7.0	37	8	37
22	29-mer	31-mer[b]	90	7.4	37	16	42
23	21-mer	28-mer	3	7.5	37	16	210
24b	18-mer[a]	36-mer	67[c]	7.5	37	24	43
24c	17-mer[a]	159-mer	84	7.4	45	72	41

[a] One nucleotide replaced by a nonnucleoside linker. [b] Bulged RNA residues. [c] Catalytic turnover achieved; 6.7 turnovers observed.

age. It also appears that systems involving unpaired nucleotides provide the most favorable results. As the table indicates, only one instance of catalytic turnover has been reported. While this represents a major advancement in the field, improvements in the catalytic rate are much needed, since the observed first-order catalytic rate constant for the mimic is approximately 10- to 1000-fold lower than those reported for hammerhead ribozymes.

VI. Concluding Remarks

The past 10 years have seen ribozyme mimics progress from the initial concept, through demonstration of the concept, to testing of advanced designs. Continued progress in this area offers great promise for gene-specific chemotherapy. Progress will rely on a number of factors: improved catalysts are needed, and they will undoubtedly be identified with the help of relevant assays that test the cleavage of true, polymeric RNA substrates. Better understanding of the catalytic mechanisms is needed. Structural information about the complex formed between ribozyme mimics and their target RNA sequences is sorely lacking. We must continue to elucidate key features required for cleavage of RNA within duplexes. At the same time, enhanced ribozyme mimics must be constructed using DNA analogues to allow for activity in vivo. The diversity of research groups whose work appears in this review and in this thematic issue augers well for the continued and rapid advancement of this field.

VII. Acknowledgments

The authors thank our co-workers, Todd A. Osiek (cover art) and William C. Putnam, for assistance with illustrations. We are also grateful to Harri Lönnberg and Heather Bigott for careful reading of this manuscript. This work was supported in part

by the NSF Grant CHE-9318581. Acknowledgment is also made to the donors of the Petroleum Research Fund, administered by the ACS, for partial support of this work. B.N. Trawick is a Chancellor's Fellow at Washington University (1995−2000).

VIII. References

(1) Stern, M. K.; Bashkin, J. K.; Sall, E. D. *J. Am. Chem. Soc.* **1990**, *112*, 5357.
(2) Shelton, V. M.; Morrow, J. R. *Inorg. Chem.* **1991**, *30*, 4295−99.
(3) Perreault, D. M.; Anslyn, E. V. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 433−50.
(4) Kimball, A. S.; Lee, J.; Jayaram, M.; Tullius, T. D. *Biochemistry* **1993**, *32*, 4698−701.
(5) Takasaki, B. K.; Chin, J. *J. Am. Chem. Soc.* **1994**, *116*, 1121−22.
(6) Sigman, D. S.; Mazumder, A.; Perrin, D. M. *Chem. Rev.* **1993**, *93*, 2295−316.
(7) Meijler, M. M.; Zelenko, O.; Sigman, D. S. *J. Am. Chem. Soc.* **1997**, *119*, 1135−6.
(8) Tullius, T. D. *Compr. Supramol. Chem.* **1996**, *5*, 317−42.
(9) Churchill, M. E. A.; Hayes, J. J.; Tullius, T. D. *Biochemistry* **1990**, *29*, 6043−50.
(10) Tullius, T. D.; Dombroski, B. A.; Churchill, M. E. A.; Kam, L. *Methods Enzymol.* **1987**, *155*, 537−58.
(11) McLachlan, G. A.; Muller, J. G.; Rokita, S. E.; Burrows, C. J. *Inorg. Chim. Acta* **1996**, *251*, 193−9.
(12) Muller, J. G.; Zheng, P.; Rokita, S. E.; Burrows, C. J. *J. Am. Chem. Soc.* **1996**, *118*, 2320−5.
(13) *Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression*; Cohen, J. S., Ed.; CRC Press: Boca Raton, 1989.
(14) Bashkin, J. K.; Stern, M. K.; Modak, A. S. European Patent EP533753, World Patent WO9119730 **1990**, applied for.
(15) Bashkin, J. K.; Modak, A. S.; Stern, M. K. 1990 (patent application filed 11/1/90).
(16) Stein, C. A.; Cohen, J. S. *Cancer Res.* **1988**, *48*, 2659−68.
(17) Modak, A. S.; Gard, J. K.; Merriman, M. C.; Winkeler, K. A.; Bashkin, J. K.; Stern, M. K. *J. Am. Chem. Soc.* **1991**, *113*, 283.
(18) Butzow, J. J.; Eichhorn, G. L. *Nature* **1975**, *254*, 358−9.
(19) Westheimer, F. H. *Science* **1987**, *235*, 1173−78.
(20) Basile, L. A.; Raphael, A. L.; Barton, J. K. *J. Am. Chem. Soc.* **1987**, *109*, 7550.
(21) Schnaith, L. M. T.; Hanson, R. S.; Que, L., Jr. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 569−73.
(22) Komiyama, M.; Kodama, T.; Takeada, N.; Sumaoka, j.; Shiiba, T.; Matsumoto, Y.; Yashiro, M. *J. Biochem.* **1994**, *115*, 809−10.
(23) Breslow, R.; Huang, D.-L. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 4080−83.
(24) Linkletter, B.; Chin, J. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 472−4.
(25) Morrow, J. R.; Buttrey, L. A.; Shelton, V. M.; Berback, K. A. *J. Am. Chem. Soc.* **1992**, *114*, 1903−5.
(26) Morrow, J. R.; Shelton, V. M. *New J. Chem.* **1994**, *18*, 371−5.
(27) Morrow, J. R. *Met. Ions Biol. Syst.* **1996**, *33*, 561−92.
(28) Bashkin, J. K.; Gard, J. K.; Modak, A. S. *J. Org. Chem.* **1990**, *55*, 5125.
(29) Bashkin, J. K.; McBeath, R. J.; Modak, A. S.; Sample, K. R.; Wise, W. B. *J. Org. Chem.* **1991**, *56*, 3168.
(30) Bashkin, J. K.; Frolova, E. I.; Sampath, U. *J. Am. Chem. Soc.* **1994**, *116*, 5981−2.
(31) Bashkin, J. K.; Sondhi, S. M.; Sampath, U.; d'Avignon, D. A.; Modak, A. S. *New J. Chem.* **1994**, *18*, 305−16.
(32) Bashkin, J. K.; Xie, J.; Daniher, A. T.; Sampath, U.; Kao, J. L.-F. *J. Org. Chem.* **1996**, *61*, 2314−21.
(33) Komiyama, M.; Inokawa, T.; Yoshinari, K. *J. Chem. Soc., Chem. Commun.* **1995**, 77−8.
(34) Matsumura, K.; Endo, M.; Komiyama, M. *J. Chem. Soc., Chem. Commun.* **1994**, 2019−20.
(35) Wang, G.; Bergstrom, D. E. *Tetrahedron Lett.* **1993**, *34*, 6725−8.
(36) Bergstrom, D. E.; Zhang, P. *Tetrahedron Lett.* **1991**, *32*, 6485−8.
(37) Vlassov, V.; Abramova, T.; Godovikova, T.; Giege, R.; Silnikov, V. *Antisense Nucleic Acid Drug Dev.* **1997**, *7*, 39−42.
(38) Reynolds, M. A.; Beck, T. A.; Say, P. B.; Schwartz, D. A.; Dwyer, B. P.; Daily, W. J.; Vaghefi, M. M.; Metzler, M. D.; Klem, R. E.; Arnold, L. J., Jr. *Nucleic Acids Res.* **1996**, *24*, 760−5.
(39) Bashkin, J. K.; Sampath, U. S.; Frolova, E. I. *Appl. Biochem. Biotechnol.* **1995**, *54*, 43−56.
(40) Bashkin, J. K.; Xie, J.; Daniher, A. T.; Jenkins, L. A.; Yeh, G. C. In *DNA and RNA Cleavers and Chemotherapy of Cancer and Viral Diseases*; Meunier, B., Ed.; NATO ASI Series, Series C, 479; Kluwer: The Netherlands, 1996.
(41) Daniher, A. T.; Bashkin, J. K. *Chem. Commun.* **1998**, in press.
(42) Hall, J.; Huesken, D.; Haener, R. *Nucleic Acids Res.* **1996**, *24*, 3522−26.
(43) Magda, D.; Wright, M.; Crofts, S.; Lin, A.; Sessler, J. L. *J. Am. Chem. Soc.* **1997**, *119*, 6947−8.
(44) Cech, T. R. *JAMA* **1988**, *260*, 3030−4.
(45) Haner, R.; Hall, J. *Antisense and Nucleic Acid Drug Dev.* **1997**, *7*, 423−30.
(46) Uhlmann, E.; Peyman, A. *Chem. Rev.* **1990**, *90*, 543−84.
(47) Altmann, K.-H.; Dean, N. M.; Fabbro, D.; Freier, S. M.; Geiger, T.; Haener, R.; Huesken, D.; Martin, P.; Monia, B. P.; et al. *Chimia* **1996**, *50*, 168−76.
(48) De Mesmaeker, A.; Haener, R.; Martin, P.; Moser, H. E. *Acc. Chem. Res.* **1995**, *28*, 366−74.
(49) Seliger, H.; Krist, B.; Roesch, H.; Roesch, R.; Rueck, A.; Steiner, R.; Gupta, K. C.; Ortigao, J. F. R. *Prog. Biotechnol.* **1994**, *9*, 681−4.
(50) Crooke, S. T. *Cancer Ther.* **1997**, 299−336.
(51) Iversen, P. L.; Bayever, E. *Clin. Gene Anal. Manipulation* **1996**, 372−90.
(52) Nicot, A.; Pfaff, D. W. *J. Neurosci. Methods* **1997**, *71*, 45−53.
(53) Oberbauer, R. *Wien. Klin. Wochenschr.* **1997**, *109*, 40−6.
(54) Uhlmann, E. *Prog. Biotechnol.* **1994**, *9*, 667−73.
(55) Inouye, M. *Gene* **1998**, *72*, 25−34.
(56) Stephenson, M. L.; Zamecnik, P. C. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 285−8.
(57) Zamecnik, P. C.; Stephenson, M. L. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 280−4.
(58) Simons, R. W.; Kleckner, N. *Cell* **1983**, *34*, 683.
(59) Heywood, S. M. *Nucleic Acids Res.* **1986**, *14*, 6771.
(60) Akhtar, S.; Agrawal, S. *Trends Pharmacol. Sci* **1997**, *18*, 12−7.
(61) Iversen, P. L.; Copple, B. L.; Tewary, H. K. *Antisense Ther.* **1996**.
(62) Zhang, R.; Lu, Z.; Zhang, X.; Zhao, H.; Diasio, R. B.; Liu, T.; Jiang, Z.; Agrawal, S. *Clin. Chem.* **1995**, *41*, 836−43.
(63) Agrawal, S.; Temsamani, J.; Galbraith, W.; Tang, J. *Clin. Pharmacokinet.* **1995**, *28*, 7−16.
(64) Agrawal, S.; Temsamani, J.; Tang, J. In *Delivery Strategies Antisense Oligonucleotide Ther.*; Akhtar, S., Ed.; CRC: Boca Raton, 1995.
(65) Uhlmann, E.; Peyman, A. *Chem. Rev.* **1990**, *90*, 543−84.
(66) Ogawa, T.; Okazaki, T. *Mol. Gen. Genet.* **1984**, *193*, 231−7.
(67) Toulme, J. J.; Boiziau, C.; Larrouy, B.; Frank, P.; Albert, S.; Ahmadi, R. In *DNA and RNA Cleavers and Chemotherapy of Cancer and Viral Diseases*; Meunier, B., Ed.; Kluwer: The Netherlands, 1996; Vol. 479.
(68) de Feyter, R.; Young, M.; Schroeder, K.; Dennis, E. S.; Gerlach, W. *Mol. Gen. Genet.* **1996**, *250*, 329−38.
(69) Zelphati, O.; Imbach, J. L.; Signoret, N. *Nucleic Acids Res.* **1994**, *22*, 4307−14.
(70) Bishop, M. R.; Iversen, P. L.; Bayever, E.; Sharp, J. G.; Greiner, T. C.; Copple, B. L.; Ruddon, R.; Zon, G.; Spinolo, J.; et, a. *J. Clin. Oncol.* **1996**, *14*, 1320−6.
(71) Matteucci, M. D.; Wagner, R. W. *Nature* **1996**, *384*, 20−2.
(72) Zelphati, O.; Szoka, F. C., Jr. *J. Liposome Res.* **1997**, *7*, 31−49.
(73) Zelphati, O.; Szoka, F. C., Jr. *Proc. Natl. Acad. Sci. U.S.A* **1996**, *93*, 11493−8.
(74) Zelphati, O.; Szoka, F. C., Jr. *J. Controlled Release* **1996**, *41*, 99−119.
(75) Zelphati, O.; Szoka, F. C., Jr. *Pharm. Res.* **1996**, *13*, 1367−72.
(76) *Protocols for Oligonucleotides and Analogues*; Agrawal, S., Ed.; Humana: Totowa, NJ, 1993; Vol. 20.
(77) *Oligonucleotides and Analogues: A Practical Approach*; Eckstein, F., Ed.; Oxford Unicersity Press: Oxford, 1991.
(78) Baum, R. In *Chem. Eng. News*, **1994**, *72*, 21−22.
(79) Imbach, J. L.; Rayner, B.; Morvan, F. *Nucleosides Nucleotides* **1989**, *8*, 627−48.
(80) Monia, B. P.; Lesnik, E. A.; Gonzalez, C.; Lima, W. F.; McGee, D.; Guinosso, C. J.; Kawasaki, A. M.; Cook, P. D.; Freier, S. M. *J. Biol. Chem.* **1993**, *268*, 14514−22.
(81) Davies, J.; Hostomka, Z.; Hostomsky, Z.; Jordan, S.; Matthews, D. *Science* **1991**, *252*, 88−95.
(82) Hostomsky, Z.; Hostomska, Z.; Matthews, D. A. In *Nucleases*; Linn, S. M., Lloyd, R. S., Roberts, R. J., Eds.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor: NY, 1993.
(83) Crooke, S. T.; Lemonidis, K. M.; Neilson, L.; Griffey, R.; Lesnik, E. A.; Monia, B. P. *Biochem. J.* **1995**, *312*, 599−608.
(84) Sarver, N.; Cantin, E. M.; Chang, P. S.; Zaia, J. A.; Ladne, P. A.; Stephens, D. A.; Rossi, J. J. *Science* **1990**, *247*, 1222−25.
(85) Christoffersen, R. E.; Marr, J. J. *J. Med. Chem.* **1995**, *38*, 2023−37.
(86) Eckstein, F. *NATO ASI Ser., Ser. C* **1996**, *479*, 291−4.
(87) Heidenreich, O.; Pieken, W.; Eckstein, F. *FASEB J.* **1993**, *7*, 90−6.
(88) Olsen, D. B.; Benseler, F.; Aurup, H.; Pieken, W. A.; Eckstein, F. *Biochemistry* **1991**, *30*, 9735−41.
(89) Pieken, W. A.; Olsen, D. B.; Aurup, H.; Williams, D. M.; Heidenreich, O.; Benseler, F.; Eckstein, F. *Nucleic Acids Symp. Ser.* **1991**, *24*, 51−3.
(90) Usman, N.; Beigelman, L.; McSwiggen, J. A. *Curr. Opin. Struct. Biol.* **1996**, *6*, 527−33.

(91) Matulic-Adamic, J.; Karpeisky, A. M.; Gonzales, C.; Burgin, A. B., Jr.; Usman, N.; McSwiggen, J. A.; Beigelman, L. *Collect. Czech. Chem. Commun.* **1996**, *61*, 1, S271−5.
(92) Burgin, A. B., Jr.; Gonzalez, C.; Matulic-Adamic, J.; Karpeisky, A. M.; Usman, N.; McSwiggen, J. A.; Beigelman, L. *Biochemistry* **1996**, *35*, 14090−7.
(93) Santoro, S. W.; Joyce, G. F. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 4262−6.
(94) *Catalytic RNA*; Eckstein, F., Lilley, D. M. J., Eds.; Heidelberg: Springer-Verlag: 1996; Vol. 10.
(95) *Current Protocols in Molecular Biology*; Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., Struhl, K., Eds.; Green Publishing Associates and Wiley-Interscience: New York, 1992; Vol. 1.
(96) *Current Protocols in Molecular Biology*; Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., Struhl, K., Eds.; Green Publishing Associates and Wiley-Interscience: New York, 1992; Vol. 2.
(97) Moss, R. A.; Bracken, K.; Zhang, J. *Chem. Commun.* **1997**, 563−4.
(98) Ihara, T.; Shimura, H.; Ohmori, K.; Takeuchi, J.; Takagi, M. *Chem. Lett.* **1996**, 687−8.
(99) Hannon, C. L.; Anslyn, E. V. *Bioorg. Chem. Front* **1993**, *3*, 193−255.
(100) Bashkin, J. K.; Jenkins, L. A. *Comments Inorg. Chem.* **1994**, *16*, 77−93.
(101) Morrow, J. R. *Adv. Inorg. Biochem.* **1994**, *9*, 41−74.
(102) Morrow, J. R.; Shelton, V. M. *New J. Chem.* **1994**, *18*, 371−5.
(103) Komiyama, M.; Matsumura, K.; Takarada, T. *Tanpakushitsu Kakusan Koso* **1995**, *40*, 911−7.
(104) Morrow, J. R. *Met. Ions Biol. Syst* **1996**, *33*, 561−92.
(105) Breslow, R. *Nato ASI Ser., Ser. E* **1996**, *320*, 113−35.
(106) Kuusela, S.; Lönnberg, H. *Metal Ions in Biological Systems*; Sigel, A., Sigel, H., Eds.; Marcel Dekker: New York, 1996; Vol. 32.
(107) Chandler, A. J.; Kirby, A. J. *J. Chem. Soc., Chem. Commun.* **1992**, *24*, 1769−70.
(108) Kirby, A. J.; Marriott, R. E. *J. Am. Chem. Soc.* **1995**, *117*, 833−4.
(109) Haim, A. *J. Am. Chem. Soc.* **1992**, *114*, 8384−8.
(110) Breslow, R. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1208−11.
(111) Breslow, R. *Acc. Chem. Res.* **1991**, *24*, 317−24.
(112) Breslow, R.; Xu, R. *J. Am. Chem. Soc.* **1993**, *115*, 10705−13.
(113) Breslow, R.; Dong, S. D.; Webb, Y.; Xu, R. *J. Am. Chem. Soc.* **1996**, *118*, 6588−600.
(114) Morrow, J. R.; Aures, K.; Epstein, D. *J. Chem. Soc., Chem. Commun.* **1995**, *23*, 2431−2.
(115) Yeh, G. C.; Beatty, A. M.; Bashkin, J. K. *Inorg. Chem.* **1996**, *35*, 3828−35.
(116) Sawata, S.; Komiyama, M.; Taira, K. *J. Am. Chem. Soc.* **1995**, *117*, 2357−8.
(117) Shiiba, T.; Komiyama, M. *Nucleic Acids Symp. Ser* **1992**, *27*, 39−40.
(118) Breslow, R.; Labelle, M. *J. Am. Chem. Soc.* **1986**, *108*, 2655−9.
(119) Blackburn, P.; Moore, S. *Ribonuclease Assay Review #2*; Academic Press: New York, 1982.
(120) Richards, F. M.; Wycoff, H. W. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York, 1971; Vol. 4.
(121) Chin, J.; Banaszczyk, M. *J. Am. Chem. Soc.* **1989**, *111*, 4103−5.
(122) Gellman, S. H.; Petter, R.; Breslow, R. *J. Am. Chem. Soc.* **1986**, *108*, 2388−94.
(123) Fife, T. H.; Pujari, M. P. *J. Am. Chem. Soc.* **1988**, *110*, 7790−7.
(124) Fife, T. H.; Natarajan, R.; Werner, M. H. *J. Org. Chem.* **1987**, *52*, 740−6.
(125) Morrow, J. R.; Buttrey, L. A.; Berback, K. A. *Inorg. Chem.* **1992**, *31*, 16−20.
(126) Oivanen, M.; Schnell, R.; Pfleiderer, W.; Lönnberg, H. *Nucleosides Nucleotides* **1991**, *10*, 569−70.
(127) Oivanen, M.; Schnell, R.; Pfleiderer, W.; Lönnberg, H. *J. Org. Chem.* **1991**, *56*, 3623−8.
(128) Oivanen, M.; Lönnberg, H. *Acta Chem. Scand.* **1991**, *45*, 968−71.
(129) Koike, T.; Kimura, E. *J. Am. Chem. Soc.* **1991**, *113*, 8935−41.
(130) Jubian, V.; Dixon, R. P.; Hamilton, A. D. *J. Am. Chem. Soc.* **1992**, *114*, 1120−1.
(131) Vance, D. H.; Czarnik, A. W. *J. Am. Chem. Soc.* **1993**, *115*, 12165−6.
(132) Kim, J. H.; Chin, J. *J. Am. Chem. Soc.* **1992**, *114*, 9792−5.
(133) Chin, K. O. A.; Morrow, J. R. *Inorg. Chem.* **1994**, *33*, 5036−41.
(134) Chapman, W. H., Jr.; Breslow, R. *J. Am. Chem. Soc.* **1995**, *117*, 5462−9.
(135) Jubian, V.; Veronese, A.; Dixon, R. P.; Hamilton, A. D. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1237−9.
(136) Karet, G. B.; Kostic, N. M. *Chemtracts* **1997**, *10*, 138−43.
(137) Williams, N.; Chin, J. *J. Chem. Soc., Chem. Commun.* **1996**, *2*, 131−2.
(138) Kalesse, M.; Loos, A. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2063−8.
(139) Breslow, R.; Schmuck, C. *J. Am. Chem. Soc.* **1996**, *118*, 6601−5.
(140) Kim, N.; Suh, J. *J. Org. Chem.* **1994**, *59*, 1561−71.
(141) Shiiba, T.; Komiyama, M. *Tetrahedron Lett.* **1992**, *33*, 5571−4.
(142) Bruice, T. C.; Tsubouchi, A.; Dempcy, R. O.; Olson, L. P. *J. Am. Chem. Soc.* **1996**, *118*, 9867−75.
(143) Gross, R.; Durner, G.; Gobel, M. W. *Liebigs Ann. Chem.* **1994**, 49−58.
(144) Wahnon, D.; Hynes, R. C.; Chin, J. *J. Chem. Soc., Chem. Commun.* **1994**, 1441−2.
(145) Gobel, M. W.; Bats, J. W.; Durner, G. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 207−9.
(146) Wall, M.; Hynes, R.; Chin, J. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1633−5.
(147) Browne, K. A.; Bruice, T. C. *J. Am. Chem. Soc.* **1992**, *114*, 4951−8.
(148) Davis, A. M.; Hall, A. D.; Williams, A. *J. Am. Chem. Soc.* **1988**, *110*, 5105−8.
(149) Usher, D. A.; Richardson, D. I., Jr.; Oakenfull, D. G. *J. Am. Chem. Soc.* **1970**, *92*, 4699−712.
(150) Breslow, R.; Huang, D. L.; Anslyn, E. V. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 1746−50.
(151) Morrow, J. R.; Trogler, W. C. *Inorg. Chem.* **1988**, *27*, 3387−94.
(152) Morrow, J. R.; Trogler, W. C. *Inorg. Chem.* **1992**, *31*, 1544.
(153) Bashkin, J. K.; Jenkins, L. A. *J. Chem. Soc., Dalton Trans.* **1993**, 3631−2.
(154) Jenkins, L. A.; Bashkin, J. K.; Pennock, J. D. *Inorg. Chem.* **1998**, manuscript under revision.
(155) Jenkins, L. A.; Bashkin, J. K.; Autry, M. E. *J. Am. Chem. Soc.* **1996**, *118*, 6822−25.
(156) Jenkins, L. J.; Bashkin, J. K. *Inorg. Chim. Acta* **1997**, *263*, 49−52.
(157) Liu, S.; Hamilton, A. D. *Tetrahedron Lett.* **1997**, *38*, 1107−10.
(158) Dempcy, R. O.; Bruice, T. C. *J. Am. Chem. Soc.* **1994**, *116*, 4511−2.
(159) Varila, J.; Hankamaeki, T.; Oivanen, M.; Koole, L. H. *J. Org. Chem.* **1997**, *62*, 893−8.
(160) Kosonen, M.; Lönnberg, H. *J. Chem. Soc., Perkin Trans. 2* **1995**, 1203−9.
(161) Florian, J.; Warshel, A. *J. Am. Chem. Soc.* **1997**, *119*, 5473−4.
(162) Kuusela, S.; Rantanen, M.; Lönnberg, H. *J. Chem. Soc., Perkin Trans. 2* **1995**, 2269−73.
(163) Kuusela, S.; Lönnberg, H. *Nucleosides Nucleotides* **1996**, *15*, 1669−78.
(164) Yashiro, M.; Ishikubo, A.; Komiyama, M. *J. Chem. Soc., Chem. Commun.* **1997**, *1*, 83−4.
(165) Komiyama, M.; Matsumoto, Y. *Chem. Lett.* **1989**, *5*, 719−22.
(166) Komiyama, M.; Matsumura, K.; Matsumoto, Y. *J. Chem. Soc., Chem. Commun.* **1992**, 640−1.
(167) Anslyn, E.; Breslow, R. *J. Am. Chem. Soc.* **1989**, *111*, 4473−82.
(168) Breslow, R.; Huang, D. L. *J. Am. Chem. Soc.* **1990**, *112*, 9621−3.
(169) Jarvinen, P.; Oivanen, M.; Lönnberg, H. *J. Org. Chem.* **1991**, *56*, 5396−401.
(170) Kuusela, S.; Lönnberg, H. *J. Phys. Org. Chem.* **1993**, *6*, 347−56.
(171) Hurst, P.; Takasaki, B. K.; Chin, J. *J. Am. Chem. Soc.* **1996**, *118*, 9982−83.
(172) Young, M. J.; Chin, J. *J. Am. Chem. Soc.* **1995**, *117*, 10577−8.
(173) Chu, F.; Smith, J.; Lynch, V. M.; Anslyn, E. V. *Inorg. Chem.* **1995**, *34*, 5689−90.
(174) Matsumoto, Y.; Komiyama, M. *J. Chem. Soc., Chem. Commun.* **1990**, 1050−51.
(175) Yashiro, M.; Hattori, M.; Ishikubo, A.; Komiyama, M. *Nucleic Acids Symp. Ser.* **1996**, *35*, 143−4.
(176) Ikenaga, H.; Inoue, Y. *Biochemistry* **1974**, *13*, 577−82.
(177) Breslow, R.; Huang, D. L. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 4080−3.
(178) Kuusela, S.; Lönnberg, H. *J. Chem. Soc., Perkin Trans. 2* **1994**, 2109−13.
(179) Butzow, J. J.; Eichhorn, G. L. *Biochemistry* **1971**, *10*, 2019−27.
(180) Kuusela, S.; Lönnberg, H. *J. Chem. Soc., Perkin Trans. 2* **1994**, 2301−6.
(181) Eichhorn, G.; Butzow, J. J. *Biopolymers* **1965**, *3*, 79−94.
(182) Butzow, J. J.; Eichhorn, G. L. *Biopolymers* **1965**, *3*, 95−107.
(183) Kuusela, S.; Guzaev, A.; Lönnberg, H. *J. Chem. Soc., Perkin Trans. 2* **1996**, 1895−9.
(184) Kuusela, S.; Azhayev, A.; Guzaev, A.; Lönnberg, H. *J. Chem. Soc., Perkin Trans. 2* **1995**, 1197−202.
(185) Kunitz, M. *J. Biol. Chem.* **1946**, *164*, 563−8.
(186) Anfinsen, C. B.; Redfield, R. R.; Choate, W. L.; Page, J.; Carroll, W. *J. Biol. Chem.* **1954**, *207*, 201−10.
(187) Corcoran, R.; Labelle, M.; Czarnik, A. W.; Breslow, R. *Anal. Biochem.* **1985**, *144*, 563−8.
(188) Stevens, A.; Hilmoe, R. J. *J. Biol. Chem.* **1960**, *235*, 3016−22.
(189) Smith, J.; Anslyn, E. V. *Anal. Biochem.* **1994**, *220*, 53−7.
(190) Black, C. B.; Cowan, J. A. *Inorg. Chem.* **1994**, *33*, 5805−8.
(191) Kuimelis, R. G.; McLaughlin, L. W. *Nucleic Acids Res.* **1995**, *23*, 4753−60.

(192) Kuimelis, R. G.; McLaughlin, L. W. *J. Am. Chem. Soc.* **1995**, *117*, 11019−20.
(193) Bashkin, J. K.; Xie, J.; Daniher, A. T.; Jenkins, L. A.; Yeh, G. C. In *DNA and RNA Cleavers and Chemotherapy of Cancer and Viral Diseases*; Meunier, B., Ed.; NATO ASI Series, Series C, 479; Kluwer: The Netherlands, 1996.
(194) Bashkin, J. K. In *Bioinorganic Chemistry of Copper*; Karlin, K. D., Tyeklar, Z., Eds.; Chapman and Hall: New York, 1993.
(195) Huesken, D.; Goodall, G.; Blommers, M. J. J.; Jahnke, W.; Hall, J.; Haener, R.; Moser, H. E. *Biochemistry* **1996**, *35*, 16591−600.
(196) Davis, J. C.; Averill, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 4623−7.
(197) Zuckermann, R. N.; Schultz, P. G. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 1766−70.
(198) Zuckermann, R. N.; Schultz, P. G. *J. Am. Chem. Soc.* **1988**, *110*, 6594−96.
(199) (a) Kanaya, S.; Nakai, C.; Konishi, A.; Inoue, H.; Ohtsuka, E.; Ikehara, M. *J. Biol. Chem.* **1992**, *267*, 8492−8. (b) Uchiyama, Y.; Inoue, H.; Ohtsuka, E.; Nakai, C.; Kanaya, S.; Ueno, Y.; Ikehara, M. *Bioconjugate Chem.* **1994**, *5*(4), 327−32.
(200) Magda, D.; Miller, R. A.; Sessler, J. L.; Iverson, B. L. *J. Am. Chem. Soc.* **1994**, *116*, 7439−40.
(201) Magda, D.; Crofts, S.; Lin, A.; Miles, D.; Wright, M.; Sessler, J. L. *J. Am. Chem. Soc.* **1997**, *119*, 2293−94.
(202) Hall, J.; Huesken, D.; Pieles, U.; Moser, H. E.; Haener, R. *Chem. Biol.* **1994**, 185−90.
(203) Hovinen, J.; Guzaev, A.; Azhayeva, E.; Azhayev, A.; Lönnberg, H. *J. Org. Chem.* **1995**, *60*, 2205−09.
(204) Baker, B. F.; Ramasamy, K.; Kiely, J. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1647−52.
(205) Baker, B. F. *J. Am. Chem. Soc.* **1993**, *115*, 3378−9.
(206) Komiyama, M.; Inokawa, T. *J. Biochem.* **1994**, *116*, 719−20.
(207) Kolasa, K. A.; Morrow, J. R.; Sharma, A. P. *Inorg. Chem.* **1993**, *32*, 3983−4.
(208) Hegg, E. L.; Deal, K. A.; Kiessling, L. L.; Burstyn, J. N. *Inorg. Chem.* **1997**, *36*, 1715−8.
(209) Eichhorn, G. L.; Tarien, E. *Biopolymers* **1967**, *5*, 273−80.
(210) Endo, M.; Azuma, Y.; Saga, Y.; Kuzuya, A.; Kawai, G.; Komiyama, M. *J. Org. Chem.* **1997**, *62*, 846−52.

CR960422K