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Strategies for phosphodiester complexation and cleavage

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Staphylococcal nuclease catalyses the hydrolysis of DNA phosphate linkages with a rate enhancement of the order of 10^{16}. The active site possesses two guanidinium groups and a Ca^{2+}, all of which are proposed to stabilize the formation of the phosphorane intermediate formed along the hydrolysis reaction pathway. In an attempt to achieve a fraction of the natural rate enhancement in aqueous media, several receptors possessing guanidinium groups preorganized and complementary to a phosphodiester have been synthesized. The guanidinium receptor designs were based upon analysis of multiple polyazaclefts used to study the binding of phosphodiesters in non-aqueous media. The spatial relationships of the hydrogen bonding groups with respect to each other are found to be crucial to the strength of binding, with spacers that hold the hydrogen bonding groups at a slightly greater distance than isophthaloyl spacers being optimum. The bis-guanidinium receptors do enhance the cleavage of RNA at low equivalents of the receptors to RNA phosphates. A quantitative assay to measure rates of RNA hydrolysis at 37 °C is briefly discussed.

INTRODUCTION

The hydrolysis of phosphodiester linkages by semi-synthetic enzymes[1] and synthetic artificial enzymes[2] is a current goal in bio-organic chemistry. The research efforts focused in this area are in part driven by possible applications in gene therapy.[3] In addition, such research could address a common weakness of traditional drug and vaccine treatments of viral diseases in which the high mutation rate of viral genomes causes the emergence of drug and vaccine resistant strains.[4] The variant genomic sequences are often the ones that code for surface proteins. Genomic sequences responsible for function necessary for viral replication, however, are thought to be invariant.[4d] Since virtually all viruses must sometime in their life cycle go through RNA intermediates, specific attack on the invariant sequences of viral RNA molecules could be a powerful, effective treatment of viral infection in general.

An approach to such a therapy would be to attach a segment of DNA (or DNA with phosphate replacements) that is complementary to a segment of the targeted mRNA to an RNA hydrolysing catalyst.[3] This sequence specific catalytic approach could be quite powerful since the drug could show numerous turn overs and thus destroy multiple copies of mRNA.[3] Several advances in recent years have made this approach a viable technique within the near future. Computer programs have been developed to predict mRNA intramolecular base-pairing and thus secondary structure, so that anti-sense agents can be directed toward the single stranded regions. In addition, enzymatic manipulations and sequencing of RNA and DNA have become routine laboratory procedures due to the low cost and abundance of molecular biology reagents and enzymes. Finally, DNA phosphate replacements needed to allow transport of the anti-sense agents through cell membranes and to confer nuclease resistance are being researched in many academic and industrial laboratories.[5]

The lynchpin of this approach is to find a catalyst for mRNA cleavage that is active under physiological conditions, and that is attachable to a sequence specific recognition element. A potential catalyst for such an application must be resistant to natural enzymes such as nucleases and peptidases, and should provide hydrolytic cleavage. This paper presents analysis of two natural nucleases and three artificial phosphodiesterases, and summarizes approaches to a new synthetic catalyst design. After describing a possible approach to a catalyst, preliminary work toward achieving the design is described.

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ENZYMATIC EXAMPLES OF NUCLEASES

Staphylococcal nuclease

The mechanism of staphylococcal nuclease (SNase) was first delineated by inspection of a 1.5 Å resolution crystal structure with pdTp bound[6] (Fig. 1). The structure revealed that two waters are ligands to the Ca^{2+}, one water bridges between the carboxylate of Glu-43, and one water bridges to the 5′-phosphoryl-oxygen atom. The thymidine sits in a hydrophobic pocket,[7] but the phosphate is in an exceptionally hydrophilic pocket. The guanidiniums of Arg-35 and Arg-87 both form a pair of hydrogen bonds to the 5′-phosphate, with additional hydrogen bonds to the enzyme polypeptide. Arg-35 hydrogen bonds to the carboxy oxygen of Leu-36 and Val-39, whereas Arg-87 binds to the carboxylate group of Asp-83. All these interactions are highly charged and involve hydrophilic groups.

The mechanism, as proposed by inspection of the crystal structure and theoretical calculations by Warshel,[8] involves binding of the phosphodiester in a highly positively charged microenvironment presented by arginines and the Ca^{2+}. A general base Glu-43 assisted nucleophilic attack of a calcium bound water on the 5′-phosphate in line with the 5′-C–O–P bond forms a trigonal bipyramidal phosphorane intermediate. The ionic bond between the Ca^{2+} and the phosphate, and the four hydrogen bonds from the two arginines to the phosphate, are postulated to assist the neutralization of the second developing negative charge on the phosphate as the nucleophile is delivered. The charge neutralization and bond polarization both play a role in lowering the transition state energy for formation of the phosphorane intermediate. The expulsion of the leaving group is thought to require a proton for assistance such that an alcohol departs instead of an alkoxide which is unstable at near neutral pH.

The arginines are preorganized in the active site by hydrogen bonds to exceptionally hydrophilic groups such as amide carbonyl oxygens and carboxylates. Therefore, the guanidinium side chains are well solvated by interactions with hydrophilic residues in the enzyme active site. In addition, the Ca^{2+} is well solvated by ligation to a bound phosphate, a water, and two carboxylates. The cavity possesses a total 4+ positive charge in the immediate co-ordination sphere of the phosphate (if we discount the carboxylates ligating the Ca^{2+} and the general base).

Single site mutants prepared by Mildvan have nicely shown the role of the active site residues.[9] A change of the arginines to glycine is dramatic. A glycine switch of Arg-35 finds that the substrate is bound only weakly, but a similar switch of Arg-87 has little effect on binding. Conversely, mutation of each Arg to Gly reduces k_{cat} by factors >35,000. Mildvan postulates a mechanism in which Arg-87 interacts only with the trigonal biopyramidal transition state rather than with the substrate.[10] Through several single and double site directed mutants, Mildvan has broken down the 10^{16} fold rate enhancement imparted by SNase. The metal imparts a 10^4 fold enhancement,[10] the transition state stabilization of arginines a $>10^4$ enhancement, the catalysis by the bound water a $>10^3$ enhancement, and finally, general base catalysis imparts a 10^4 enhancement. Each of these large rate enhancements derives from preorganization of Lewis and Bronstead hydrophilic sites convergent around the phospho linkage.

Alkaline phosphatase

The active site of alkaline phosphatase has several key amino acids, among these Asp-101, Ser-102 and Arg-166, along with the essential two Zn^{2+} and one Mg^{2+} (Fig. 2). A crystal structure has yielded insight into the probable mechanism.[11] The three metals are close in space. The Zn^{2+}–Zn^{2+} distance is only 3.94 Å,

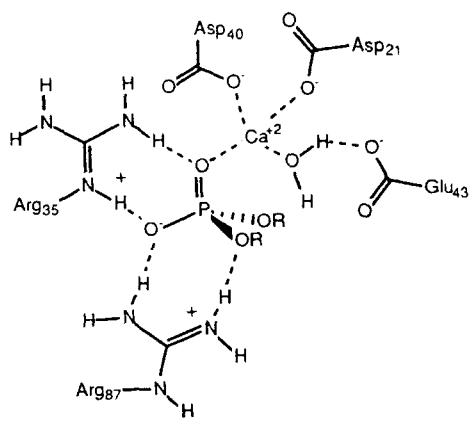

Figure 1 Schematic representation of staphylococcal nuclease active site.

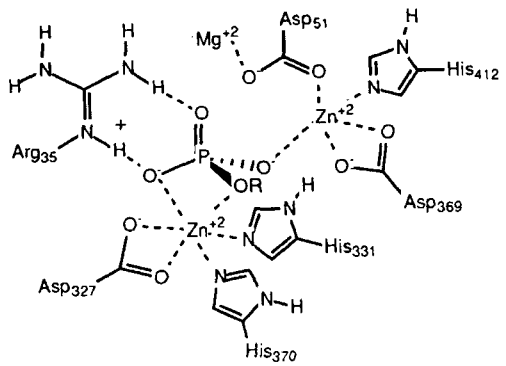

Figure 2 Schematic representation of alkaline phosphatase active site.

and the distance from the Mg^{2+} to the zincs is 4.88 and 7.09 Å. Each zinc atom contacts different phosphate oxygens of the substrate. The other two oxygens are tightly held by two of the amino functions of the guanidinium group of Arg-166. The arginine is oriented in its position by hydrophilic residues such as a hydrogen bond to Asp-101 and a water molecule that is hydrogen bound to Asp-153. The phosphate is also hydrogen bound to the amide hydrogen of Ser-102 and two water molecules, each of which is a ligand to a Mg^{2+} and a Zn^{2+}. The two Zn^{2+}s and the O–P–O group are nearly planar. Nearly perpendicular to this plane is the plane of the other O–P–O segment of the phosphate that is hydrogen bound via a two point contact to the side chain of Arg-166. The metals are ligated by waters, carboxylates and imidazoles. The phosphate substrate is proposed to be closely associated with all three metal atoms. It bridges the two zinc atoms and is hydrogen bound to a water that is co-ordinated to the Mg^{2+}. The arginine acts to orient the phosphate in the cavity in order to direct the best co-ordination geometry of the phosphate oxygens around the Zn^{2+}. This creates a cavity with a nominal charge of 5+ (if the Zn^{2+} ligands are discounted) directly co-ordinated to the phosphate.

All current data supports a mechanism involving a phosphoryl serine intermediate which is then hydrolysed. It is generally accepted that each step in the hydrolysis mechanism proceeds by an in-line attack with inversion of configuration at phosphorus each time, as is well precedented.[12] The Arg-166 orients the phosphorane intermediate in the plane of the Zn atoms. This arrangement allows the required motion of a tetrahedral phosphomonoester to a trigonal bipyramidal phosphorane. Apparently, no Bronsted acid is bound to the leaving group to assist in its departure by protonation; instead, it is proposed that the leaving group is transferred to a Zn^{2+} to make a Zn–alkoxide bond, similar to a Zn–hydroxide bond often postulated for enzymes such as carboxypeptidase.[13] The leaving group may then pick up a proton from another Zn-bound water, releasing the product and producing a Zn-bound hydroxide. Finally, this hydroxide can displace the serine group to finish the hydrolysis.

Several features of SNase and alkaline phosphatase are incorporated into our synthetic artificial receptors. First, we intend to bind the phosphate in a micro-environment that possesses a significantly higher positive charge than the hydrogen bonds and dipoles of pure water. In order to hold the phosphate in a geometry that can be used to direct general bases and electrophilic metals to the phosphate, we intend to use guanidiniums to orient the phosphate by two point hydrogen bonding. In addition, by binding the guanidiniums to the phosphates, the guanidiniums can potentially act as general acids to perform proton transfers.

EXAMINING SYNTHETIC ARTIFICIAL NUCLEASES

The binding site of a typical hydrolytic enzyme has several different components. There is the site for catalysis in which the catalytic functional groups are positioned to stabilize the transition state, and there are the peripheral sites for binding of the substrate that allows for specificity. In hydrolytic reactions, the site that performs the catalysis either on an amide or a phosphodiester can be thought of as separate from the contacts made to the substrate that impart specificity. Typically, the specificity in peptidases is imparted by specific hydrogen bonds to amino acid side chains and peptide linkages, or hydrophobic pockets for lipophilic side chains.[14] Similarly, specificity in phosphodiesterases results from binding sites removed from the enzyme active site.[15] These binding sites can be quite hydrophobic depending upon the specificity required. The micro-environments presented to the substrates in these specific binding sites are often of a very different nature than that found in the actual cleavage site. Proteins can form multiple microenvironments from exceptionally hydrophilic to hydrophobic depending upon which amino acid side chains converge in a pocket.

In order to study models for phosphodiester catalysis in enzymatic systems, low dielectric solvents are often used to enhance complexation between an artificial enzyme and a substrate. This is usually required because many artificial enzymes do not possess the multiple binding sites of a natural enzyme that allow for complexation in aqueous media. The low dielectric aprotic solvent cannot compete for the hydrogen bonding sites and thus recognition between the artificial enzyme and substrate based upon only a few hydrogen bonds is enhanced. Studying catalysis in low dielectric solvents is thus often justified as a model for studying enzymatic active sites. The comparison, however, may not be valid for hydrolytic enzymatic microenvironments where the binding site is not very hydrophobic.

Hamilton has developed an elegant ensemble of acetonitrile solvent, lutidine general base, and catalyst **1** (Fig. 3) which enhances the transesterification of **2** with a rate enhancement over background approaching 1000 fold.[16] The dicationic form of **2** was found to be necessary since the control compound **3** enhanced the reaction by only 2.5 fold under similar conditions.

Breslow has described the use of cyclodextrin-

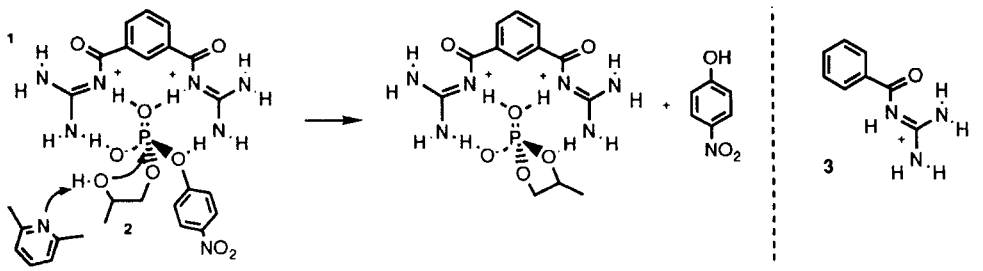

Figure 3 Bis-guanidinium catalyst **1** for transesterification of **2**.

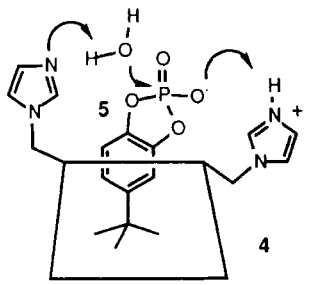

Figure 4 Bis-imidazole-β-cyclodextrin catalyst for ring opening of **5**.

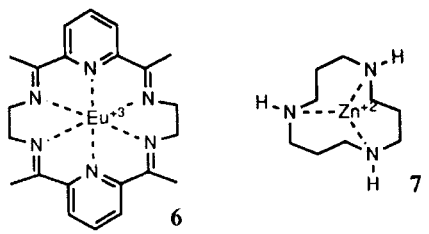

Figure 5 Metal catalysts for RNA hydrolysis.

bis(imidazole) compounds **4** as catalysts for the hydrolysis of *t*-butylcatechol cyclic phosphate **5**[17]. The reaction exhibited a bell-shaped profile for the plot of V_{max} vs. pH. The A,B isomer of the bis-imidazole catalyst was found to be best due to a more complementary arrangement of the general acid and base at the active site for achieving the transition state.[18] A simultaneous bifunctional acid–base mechanism was supported by proton inventory studies. A Michaelis–Menton kinetic plot revealed that this artificial enzyme is only 230 times slower at hydrolysing **5** than ribonuclease A is at hydrolysing cytidine-2,3-cyclic phosphate. The rate enhancement takes place in water with the phosphate group protruding into the aqueous solution. The imidazoles or phosphates are not shielded or desolvated by hydrophobic surfaces, and the β-cyclodextrin serves as a recognition unit to bind the *t*-butylcatechol group so as to place the phosphates in proximity to the imidazoles. This is schematically shown in Figure 4.

Morrow has recently reported an example of a metal complex **6** that shows catalytic behaviour in RNA transesterification at 37 °C and neutral pH (Fig. 5).[19] The complex was shown to transesterify ApUp with rate constants near 0.14 h^{-1}. This is one of the most efficient metal complexes to promote transesterifications of RNA. In addition, Morrow reports that **7** is one of the most efficient Zn^{2+} macrocyclic complexes for promoting transesterification.[20] Morrow's metal complexes may promote hydrolysis by a bifunctional mechanism in which the metal acts as an electrophile, but also as a base with a metal-bound hydroxide to deliver the 2'-OH. Morrow has good evidence for this bifunctional mechanism with **7**.[20] The metal complexes catalyze the formation of the transition state which is normally impeded in neutral water since there are no electrophiles, and low concentrations of ^{-}OH.

OUR DESIGN STRATEGY

We have been exploring a strategy of binding phosphodiesters to cavities which mimic the active site of SNase.[21] The goal is to complex the phosphodiester between two guanidinium-like moieties such that the guanidiniums may play the role of electrophilic catalysts to supply protons to quench developing negative charges and to protonate leaving groups.

Figure 6 shows a schematic representation of the four-point hydrogen bonding design that each receptor affords. In order to complex the phosphodiesters in highly aqueous media, we first concentrated on optimizing the placement of the guanidinium groups so as to be highly preorganized and complementary to a phosphodiester. In order to accomplish the fine tuning of the positioning of the hydrogen bonding groups to best complement a phosphodiester, we initially examined complexation in low dielectric solvents such as chloroform and acetonitrile. These solvents favour complex formation, and thus are useful to delineate subtle differences in spatial arrangements

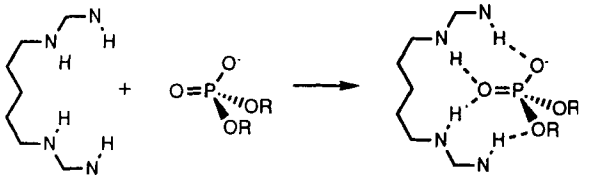

Figure 6 Schematic representation four-point hydrogen bonding design of receptor.

of non-covalent bonding groups. Receptor **8** shows a lower binding constant for dibenzylphosphoric acid than receptor **9** by 1.15 kcal/mol (Fig. 7*a*). Likewise, receptor **10** binds diphenylphosphate with a binding constant similar to receptor **11** in the range of 10^4 M^{-1},[22] even though **11** has no charge pairing (Fig. 7*b*). The receptors **9** and **11** possess more open cavities that compounds **8** and **10**. The increased binding is thus attributed partly to receptors **9** and **11** accommodating the phosphodiester with an optimum binding arrangement. Molecular mechanics suggest that the cavity of **8** does not allow hydrogen bonds formed between receptor and phosphate to be nearly linear. A similar situation was recently found for barbituate receptors due to slightly too tight a cavity.[23] In contrast, if the cavities of **8** and **10** are opened up to yield compounds **9** and **11**, molecular mechanics reveal four linear hydrogen bonds. The synthetic complexity of receptors **8** and **9** allowed for altering the spacer to achieve such an optimized host-guest complementarity.

This lesson from low dielectric aprotic solvents was directly transferred to aqueous media. We required a spacer for two guanidinium groups which placed them further apart and at a larger angle with respect to each other than is possible with a isophthyloyl like spacer. We tested this hypothesis by comparing receptors **12** and **13** (Fig. 7*c*), but found similar complexation constants with dibenzylphosphate. With the optimization of the receptors completed, we turned our attention to RNA cleavage catalysis.

Incubation of RNA with receptors **12** and **13**, along

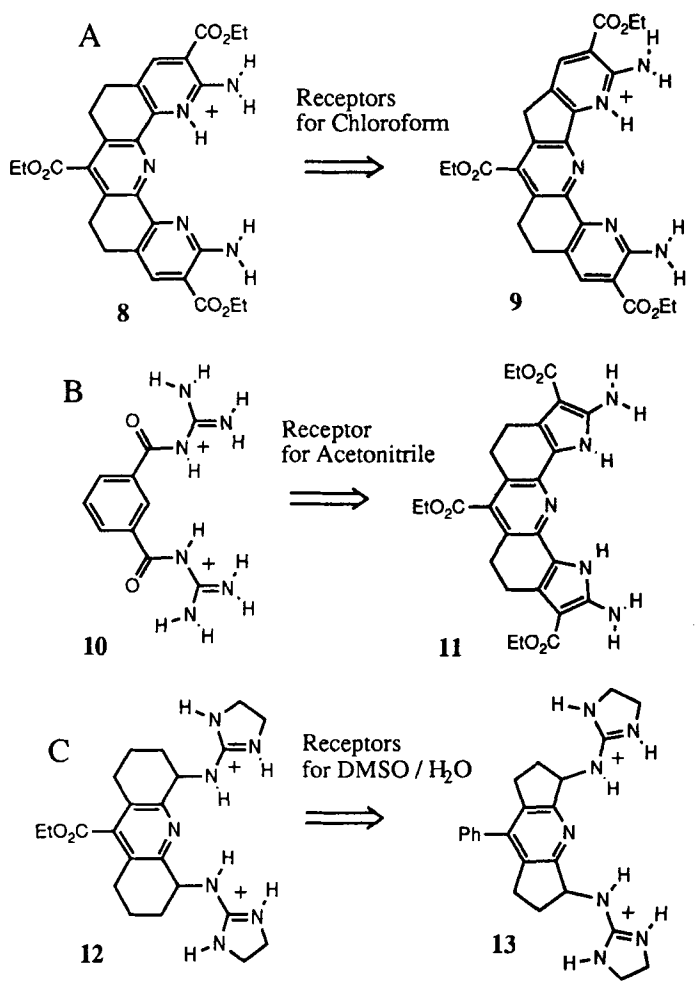

Figure 7 Four-point hydrogen bonding receptors for phospho-diesters. The arrows indicate a change toward a more open cavity.

with 250 mM imidazole buffer, pH 7.0, at 37 °C, was followed by a 5% acrylamide gel electrophoresis that immediately indicated enhanced hydrolysis. It has taken us a considerable amount of time to quantitate our first rate enhancement. This is because no suitable assay existed to allow the measurement of slow RNA and DNA hydrolysis rates accurately at 37 °C. Thus, the cleavage rate was determined by a quantitation assay of our own design. First, plasmid DNA was linearized with a restriction enzyme digestion at a site downstream of a bacteriophage T7 RNA polymerase promoter region. RNA run-off transcription then occurred with the addition of the highly processive T7 RNA polymerase enzyme.[24] The mRNA from that reaction had a 5′-phosphate group that could not be end-labelled by bacteriophage T4 polynucleotide kinase in the forward kinase reaction which catalysed the transfer of the γ-phosphate of ^{32}P-ATP to the 5′-hydroxyl end of RNA.[25] Second, we incubated the artificial nuclease with the mRNA. If transesterification did take place, then additional 5′ ends would be generated. Except this time, the 5′ ends would have hydroxyl groups and therefore would become the correct substrate for 5′ end-labelling with [γ-^{32}P]ATP. Third, we then separated unincorporated ^{32}P-ATP from the radioactively labelled RNA by gel filtration column or by acid precipitation. The RNA sample could then be counted in a scintillation counter to ascertain how many cleavage events occurred. Because the background noise level was so low, this assay was extremely sensitive to even very small numbers of cleavage events. We are currently writing up this assay in a detailed manner for publication.

The first reaction tested consists of compound **12** and mRNA, 1.7 kb in length with a concentration of 0.5 μg/ml, in 250 mM imidazole buffer, pH 7.05, at 37 °C. Experiments were performed with compound **12** ranging in concentration from 1.25×10^{-7} to 5.00×10^{-7} M. Samples were taken at hourly intervals. Pseudo-first-order rate constant for the transesterification of the mRNA by 500 nM of compound **12** was calculated to be $1.69 \times 10^{-3}\ h^{-1}$. This gave a 20.7 rate enhancement over background with imidazole alone. The concentration of receptor **12** was 1.36 equivalents of **12** per phosphodiester in the RNA chain. Thus, this enhancement of 20 fold is an extremely good result compared with other catalytic systems based upon amine groups. In the case of arginine, ethylene diamine, and other simple amine/ammonium catalysis,[26] several hundred to thousand fold excesses of the amines are needed to yield observable catalysis. In addition, since we are using 250 mM imidazole buffer as the background, it should be remembered that the background rate we are comparing with is faster than pure water. We need to measure the imidazole catalysis at varying buffer concentrations and extrapolate to zero buffer to have the true enhancement of the catalytic conditions over water. Indeed, the design does enhance the catalysis of RNA, and we intend to quantitate the reaction rates with the other receptors under varying reaction conditions.

Compound **13** is now being analysed for cleavage. Just as with compound **12** qualitative analysis was done first before going on to quantitative analysis. At neutral pH, there was little noticeable hydrolysis over the background of the imidazole buffer. However, as the pH dropped compound **13** showed significant hydrolysis over that of background. This points to acid catalysed cleavage acting in concert with the receptor. Figure 8 shows the autoradiograph of a 5% acrylamide gel indicating rapid cleavage in lanes 4 and 5 with the addition of compound **13**.

SYNTHETIC APPROACHES

The receptors described herein were constructed with either of two different routes.[21,27] Figure 9 shows

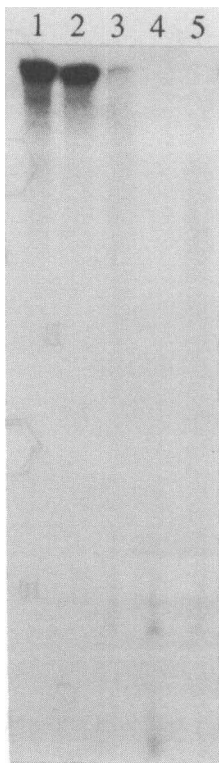

Figure 8 Representative cleavage patterns of ^{32}P-labelled RNA under acidic conditions with compound **13** as shown by an autoradiograph of a 5% denaturing acrylamide gel. Lane 1 is RNA at pH 7.0; lane 2 is RNA and 2.5 mM of compound **13** at pH 7.0; lane 3 is RNA at pH 2.7; lane 4 is RNA and 2.5 mM of compound **13** at pH 2.7; and lane 5 is RNA and 1.25 mM of compound **13** at pH 2.7.

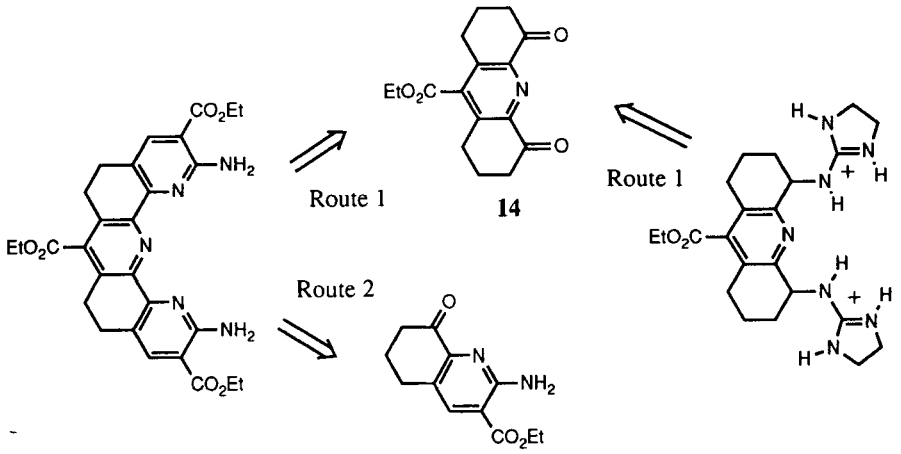

Figure 9 General synthetic approaches.

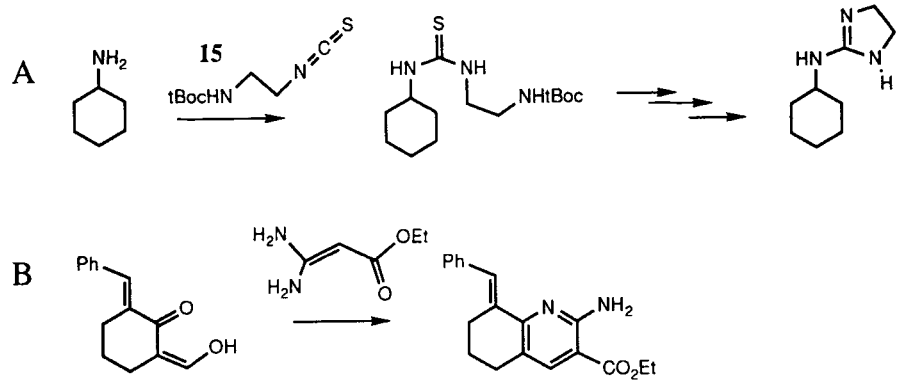

Figure 10 Key synthetic steps in the syntheses of the receptors of Figure 7.

a retrosynthetic analysis of the two approaches. In route 1 we constructed the central pyridine ring and then built outward to attach the desired recognition elements. In route 2 we built two different fragments, and condensed them together to form a non-symmetric receptor. Both of these approaches were successful. Route 1 is typically used for the symmetric receptors such as **8** and **11**, whereas route 2 is required for the non-symmetric receptors such as **9**.

The synthesis of the diketone **14** required for the symmetric approach relied upon condensing benzaldehyde with cyclohexanone[28] (in the synthesis of **13** cyclopentanone[29] was used), to produce a dibenzylidene derivative which was then allowed to react with ozone. The diketone derivatives **14** were transformed to diamines by first forming the bis-oximes and reducing with $TiCl_4/NaBH_4$,[30] or by reductive amination with NH_4OAc and $NaB(CN)H_3$. The amino-imidazoline groups were formed by using the Boc-protected thioisocyanate **15**, which can be readily transformed to imidazoline rings using conventional methods (Figure 10*a*).

The synthesis of the terpyridine receptors **8** and **9** relied upon an annulation reaction involving ethyl-3,3-diamino-2-propenoate with α-formyl ketones (Fig. 10*b*). This reaction constructed the peripheral pyridine rings in either route 1 or 2.

SUMMARY

Several polyazaclefts have been constructed to test the optimum spatial complementarity of four hydrogen bond donors and acceptors for a phosphodiester. The isophthaloyl spacer can be optimized to yield increasingly complementary interactions of hydrogen bonding groups with a phosphodiester by slightly widening the cavity. Cavities that rigidly hold amino-imidazoline groups to be complementary to RNA phosphates do enhance RNA cleavage reactions under a variety of conditions. Future work will involve attaching metals to the receptors to further increase the electrophilic character of the catalysts.

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