INTRODUCTION

The discovery of the catalytic properties of ribonucleic acid (RNA) is arguably one of the most important developments in the last quarter-century. The known transformations catalyzed by RNA, though quite limited in comparison to those promoted by proteins, are nonetheless numerous and diverse. RNA has become remarkably simple to synthesize, and large libraries of RNA oligonucleotides can be easily generated and investigated. The techniques of polymerase chain reaction (PCR) and enzymatic forward and reverse transcription make in vitro screening and isolation of active catalysts possible on a level unrivaled by other classes of molecules. The vast array of chemically modified nucleosides, many of them capable of being enzymatically incorporated into oligonucleotides, along with RNA's potential to perform chiral catalysis further contribute to the attractiveness of RNA libraries as an interesting source for catalyst discovery. This report will introduce the design of RNA oligomer libraries, their screening for activity, and the isolation of active RNA catalysts. It will also seek to examine the results that may shed light on the nature of RNA molecules that catalyze several organic reactions, namely the Diels-Alder reaction, the Michael reaction, and the formation of a urea.

IN VITRO SELECTION OF ACTIVE CATALYSTS

One of the major advantages of RNA as a potential catalyst is the ease with which large libraries (>10^12 members) of random oligomers can be made, screened, and amplified in vitro. This selection and amplification process has become known as SELEX (Systematic Evolution of Ligands by Exponential enrichment). While variations and developments of this technology are diverse, a general pattern exists. In catalyst discovery, this process may consist of incubating the RNA library members, often tethered to one of the reactants, with another reactant that is in turn tethered to either a solid phase or an immobilization “handle” such as biotin. In this manner, oligomers that may catalyze the reaction of interest are conveniently isolated from catalytically inactive oligomers. The immobilized putative catalysts are then either cleaved from the solid phase and subsequently amplified or amplified directly on the solid support. Reverse-transcription into a cDNA library, amplification of the library via PCR techniques, and finally transcription to a new RNA library enriched in catalytically active species yield the input for the next round of selection. This process is reiterated using different incubation times,
substrate concentrations, potential cofactors, or other selection pressures to ultimately arrive at a reasonably concise, catalytically active library of oligonucleotides.

**DIELS-ALDER REACTION CATALYZED BY MODIFIED RNA**

The Diels-Alder reaction is one of the most powerful reactions in organic synthesis because it results in the direct formation of two new stereocenters. Moreover, since the reaction is dependent on the orientation of the reactants, RNA appears to be an excellent catalyst candidate.

The first report of carbon-carbon bond formation catalyzed by RNA by Eaton et al. in 1997 was, in fact, a Diels-Alder reaction. This study also revealed the great complexity of RNA catalyst systems. Scheme 1 illustrates the design of the oligonucleotide library (~10^{14} members). A 100-nucleotide random region, flanked by constant regions for PCR primer binding, was covalently tethered to the diene via a long polyethylene glycol (PEG) tether to allow the reactants access to all possible active sites. The maleimide diene was in turn covalently linked to a biotin residue, which allowed active catalysts to be isolated via streptavidin affinity chromatography. The library was thus catalytically enriched by the SELEX process. The use of pyridyl-modified uridine in the place of native uridine was intended to provide sites for Lewis acid/base catalysis during the course of the Diels-Alder reaction.

After 12 rounds of the SELEX process, 46 individual sequences that represented eight different families were isolated. Approximately 80% of the sequences contained the consensus 10-mer UUCUAACGCG. Although computer modeling of representatives from each of these eight families predicted the formation of extended helical regions interrupted by bulge regions, it did not suggest any single tertiary structure in which this consensus motif might reside.

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**Scheme 1. RNA catalyzed Diels-Alder reaction with tethered substrates.**

**Figure 1.** Nucleobases used in Diels-Alderases.
One of the most active sequences, when studied further, showed a modest rate acceleration of 800 over the uncatalyzed reaction. Interestingly, catalytic activity was found to be strictly dependent on the Cu\(^{2+}\) ion, one of a variety of transition metal salts with which the substrates were incubated. The authors propose that the cuprate ion dependence supports a Lewis acid catalyzed mechanism for the cycloaddition. When the pyridyl-modified uridine 5 was replaced with structures 4 and 6-8, no rate enhancement was observed, suggesting that the pyridyl unit plays an essential role. Inhibition studies utilizing product-like analogs 9-12 suggest that catalysis by the chosen RNA oligomer takes place within a specific active site. The addition of product-like functionality, as in 10-12, indeed results in heightened inhibition of the cycloaddition reaction (Table 1).

A series of alternate biotin-substituted dienophiles 13-16 were synthesized and observed to react at a fraction of the rate of the target substrate 2 with the chosen catalytic RNA (Table 2). Thus, the selected catalytic sequence appears to be substrate specific with respect to the dienophile. This result is further supported by the observation that the corresponding uncatalyzed reactions of each of the respective modified maleimides all have rates that differ by only a factor of two. The surprisingly sluggish reactivity of dienes and dienophiles with reactivity towards the Diels-Alder cycloaddition similar to dienophile 2 strongly supports the existence of a catalytic substrate-binding pocket in the RNA catalyst. Eaton and coworkers later reported similar results\(^7,8\) when testing representative sequences of each of the eight families: Not only do each of the eight sequences show similar strict cuprate ion dependence and specificity for dienophile 2 over dienophiles 13-16, they also show strong inhibition when incubated with product-like analog 9.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibitor</th>
<th>(K_{app} , (\mu M))</th>
</tr>
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<tbody>
<tr>
<td>9</td>
<td><img src="image" alt="Compound 9" /></td>
<td>32.5 ± 2.6</td>
</tr>
<tr>
<td>10</td>
<td><img src="image" alt="Compound 10" /></td>
<td>77.4 ± 5.3</td>
</tr>
<tr>
<td>11</td>
<td><img src="image" alt="Compound 11" /></td>
<td>1093 ± 150</td>
</tr>
<tr>
<td>12</td>
<td><img src="image" alt="Compound 12" /></td>
<td>2300 ± 480</td>
</tr>
</tbody>
</table>

**Table 1. Inhibition study to probe a possible Diels-Alder active site**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dienophile</th>
<th>Relative Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td><img src="image" alt="Dienophile 2" /></td>
<td>250</td>
</tr>
<tr>
<td>13</td>
<td><img src="image" alt="Dienophile 13" /></td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td><img src="image" alt="Dienophile 14" /></td>
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<tr>
<td>15</td>
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<td>10</td>
</tr>
<tr>
<td>16</td>
<td><img src="image" alt="Dienophile 16" /></td>
<td>15.1</td>
</tr>
</tbody>
</table>

**Table 2. Dienophile study of Diels-Alder catalyst.**
Jäschke et al. devised a method to isolate active Diels-Alder catalysts by tethering the RNA to anthracene via a shorter flexible PEG chain, then incubating this with maleimide covalently tethered to biotin in an aqueous solution containing several cofactors, Lewis acids, metal ions, a dipeptide compound, and a dipyridyl compound. The RNA segment consisted of 120 random nucleotides flanked by two constant 20-nucleotide segments for primer binding. The PEG chain was long enough to give the tethered substrate access to all possible binding sites on the RNA oligomer, and the biotin-tethered maleimide allowed for the recovery of active catalysts via streptavidin agarose affinity chromatography. After several rounds of SELEX, the most catalytically active oligomers were isolated and amplified via PCR techniques. Forty-two unique sequences were isolated and categorized into eight sequence families. The 13 most active sequences showed rate enhancements of more than 15,000, and as high as 18,500, over the background reaction, and showed the homologies indicated in Figure 2. The homologies were probed to investigate the minimal structure of the active catalysts. Thus sequence 17 was structurally modified to identify the maximally stable, structurally compact catalytic moiety: Unpaired bases from helical regions and non-homologous bases were eliminated, the random loops were changed to UUCG sequences known to stabilize loops, and Helix A was modified to coaxially stack on Helix B. Modified sequence 18 of only 49 nucleotides was found to accelerate the Diels-Alder reaction twice as well as the parent sequence 17, and 32,000 times faster than the uncatalyzed reaction. The activity of RNA catalyst 18 was found to be dependent solely on the concentration of Mg$^{2+}$. The binding pocket of RNA 18 was found to be highly substrate specific, as well; three potential inhibitors similar to substructures of the original biotin maleimide substrate failed to inhibit the reaction.

Loop 1 was enzymatically cleaved, separating the PEG-tethered anthracene from the catalyst, and the resultant 35-mer 19 was then tested as a true catalyst by reacting it with PEG-tethered
anthracene and the biotinylated maleimide. However, no significant rate acceleration over the background reaction occurred. If as few as four complementary nucleotides were appended to both the 5' terminus of the RNA and the end of the PEG tether, catalytic activity similar to that of the covalently linked 49-mer was observed, with a turnover of 1.9 every 4 hours.

A STEREOSELECTIVE RNA DIELS-ALDERASE

The field of catalytic asymmetric reactions has attracted considerable attention in recent years; however, only very recently has RNA been reported to catalyze stereoselective reactions.10

When the covalent linker between catalyst 18 and anthracene was removed, 18 was found to act as a true catalyst when incubated with maleimide 20, Mg$^{2+}$, and anthracenes 21-23, only the second report of RNA catalysis between two free-standing small molecules. While untethered 18 exhibits a modest rate acceleration of 1100 and a turnover number of 6 per minute, it was able to perform enantioselective catalysis (Scheme 2).

When 24 and 21 were reacted in the presence of untethered 18, product 25 was formed with an enantiomeric ratio (er) of at least 95:5 over product 26. When catalyst 18 was synthesized from L-nucleotides, it catalyzed the preferential formation of 26, also with an er of more than 95:5. When the R$_2$ group on anthracene was modified from (CH$_2$CH$_2$O)$_6$H (21) to C$_2$H$_4$OH (22) to H (23), the er values dropped precipitously from 19:1 to 2:1 to 3:2, respectively.

CHARACTERIZATION OF A DIELS-ALDERASE ACTIVE SITE

The active site of the above RNA catalyst was probed by measuring the rates and stereochemical outcomes of the reaction with modified dienes and maleimides.11 With respect to the dienophile, the enzyme responded sensitively to the general shape of the unsubstituted $\alpha,\beta$-unsaturated maleimide, and accommodated unbranched, hydrophobic, N-substituted maleimides. With respect to the diene, the steric demands of the RNA catalyst appear more stringent and the ends than at the sides. A pocket seems to accommodate polar groups, and is also sensitive to stereochemical arrangement. The inhibition assays also suggest that the pocket is highly stereoselective as a 25-like analog was at least 100 times more potent an inhibitor than its 26-like enantiomer. The data collectively suggest a highly
discriminating binding pocket, and confirm the ability of RNA to discern between small molecule substrates, a requisite characteristic of a useful organic catalyst.

**UREA BOND FORMING REACTIONS**

Although the amide bond-catalyzing properties of RNA catalysts had already been well established, Eaton and coworkers sought to explore the possibility that an in vitro selection process, with the use of an optically active substrate, could lead to the isolation of a stereoselective RNA catalyst. Incubation of the RNA with two N-hydroxy succimide (NHS) activated tripeptides 27 and 28 (Scheme 3), differing in stereochemistry at only one position, gave the corresponding urea via addition of an exocyclic NH$_2$ of cytidine to the carbamate carbonyl bond. In contrast to the previous isolation of putative biotin-tethered catalysts via streptavidin affinity chromatography, however, Eaton et al. employed the chiral active site of human neutrophile elastase (HNE) to selectively immobilize and isolate RNA conjugates with tripeptide 27. Upon binding of the tripeptide-nucleic acid conjugates by HNE, the C-terminal phosphonate of the peptide is subsequently attacked by the active site serine residue of HNE; thus, only RNA catalyzing the urea bond-forming reaction with peptide 27 is isolated. 5-imidazoluridine replaced uridine in the RNA oligomers.

Seventeen active clones from a library of 6x10$^{14}$ members were identified, representing four major motifs. Two of the catalysts were subjected to a thorough kinetic study, and exhibited rate enhancements of approximately 10$^6$ over the background reaction. More importantly, however, when RNA catalysts were incubated with tritiated 27 and a variety of potentially competitive substrates (28-32), the reaction with radioactive 27 showed significant inhibition only at very high inhibitor concentrations, even when the structure of the potential inhibitor only minimally differed from the substrate 27 (Figure 3). Thus, it was shown that the SELEX process can be used to develop highly stereoselective RNA catalysts.
MICHAEL REACTION

Conjugate 1,4 additions, known as the Michael reaction, are ubiquitous in both organic chemistry\textsuperscript{14} and cellular biology.\textsuperscript{15} Sengle et al. have discovered that unmodified RNA can catalyze the Michael reaction\textsuperscript{16} between the thiol group of a biotin-tethered cysteine residue of compound 33 and RNA-tethered fumaric diamide 34 (Scheme 4), mimicking a reaction of biological importance. A photocleavable linker was utilized to simplify the RNA isolation after immobilization on a streptavidin affinity column. In this manner the number of \textit{in vitro} selection cycles needed to arrive at the final ten catalytic sequences (from a library of $2.0 \times 10^{15}$ members) was reduced from 13 to 10. The linker also prevented isolation of RNA sequences that bound to the biotinylated cysteine residue via an undesired side reaction with Michael acceptors in the RNA. The system exhibits a dependence on cations for catalytic activity. No one specific ion was required, as both Mg$^{2+}$ and Mn$^{2+}$ displayed similar rate accelerations, the limited metal dependence of the reaction raises the question once again as to whether the reaction is being catalyzed by a Lewis acidic metal ion or whether the cation is simply serving to make an active site available to substrate binding by adding structural rigidity to a potential tertiary structure. Biotin was shown to be a powerful inhibitor of the catalytic activities, but acyl cysteine and cysteine showed no inhibitory effect. Thus, biotin appears to be essential to the recognition process of the substrate. Rate enhancement by the sequence was shown to be dramatic for three different Michael acceptor substrates 34, 35, and 36, measuring $3 \times 10^5$, $4.9 \times 10^4$, and $1.8 \times 10^4$ respectively. The kinetic data are admittedly ambiguous with respect to the both the regioselectivity of the reaction and the nature of the putative catalyst binding site.

CONCLUSION

Although the several successes reported have expanded the field of RNA catalysis, several obstacles must be overcome. First, few reports have shown RNA to act as an untethered catalyst capable of multiple turnover in an organic reaction. This limits both the utility of the reaction and the
techniques available to probe the active site of RNA catalysts. Second, the catalytic repertoire seems to be largely limited by the paucity of innate functionality in native nucleosides, namely four rather similar bases, which is clearly much less than the 21 residues that constitute enzymes. Current efforts are directed toward expanding the functionality available to RNA enzymes, which might then increase the range of reactions and conditions in which RNA catalysts may operate. Third, since the efficiency and specificity of the catalyst depend largely on the in vitro selection process, new methods that limit side reactions, increase the fidelity of the transcription process, or allow for a more thorough analysis of the reaction products must be devised. Finally, greater amounts of more definite information concerning the mechanism and structure of RNA catalysts will allow chemists to design and customize the RNA catalytic library. The practical utility of RNA catalysts as synthetic tools ultimately hinges on the ability of chemists to overcome these challenges. Nonetheless, the development of synthetically useful RNA catalysts should remain an interesting area of research for future chemists with diverse interests.

REFERENCES