CUSTOM-MADE CATALYSIS: RNA AS A PROMISING CATALYST IN ORGANIC SYNTHESIS

Reported by Jonathan M. McKay October 9, 2003

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.[1] 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,[2] 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 (*S*ystematic *E*volution of *L*igands by *Ex*ponential enrichment).[3] While variations and developments of this technology are diverse,[4] 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,

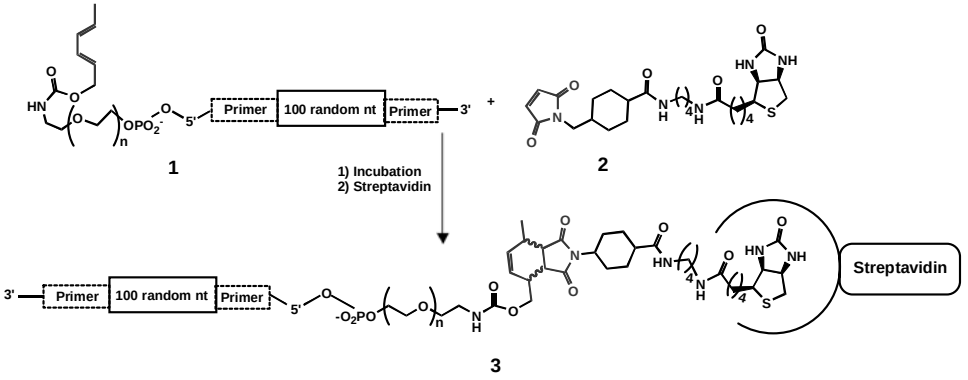
substrate concentrations, potential cofactors, or other selection pressures to ultimately arrive at a reasonably concise, catalytically active library of oligonucleotides.

DIELS-ALDER REACTION CATALYZED BY MODIFIED RNA

The Diels-Alder reaction is one of the most powerful reactions in organic synthesis[5] 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[6] 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 **5** (Figure 1) in the place of native uridine (**4**) was intended to provide sites for Lewis acid/base catalysis during the course of the Diels-Alder reaction.

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



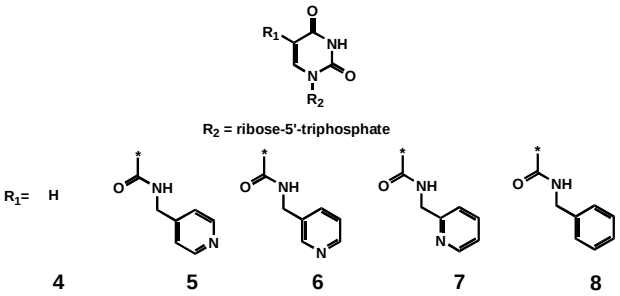
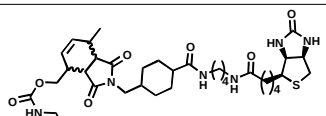
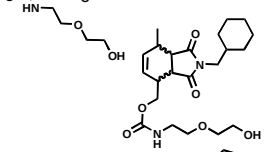
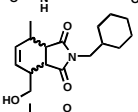
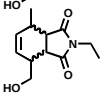


Figure 1. Nucleobases used in Diels-Alderases.

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.

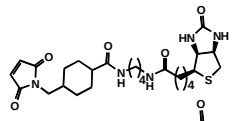
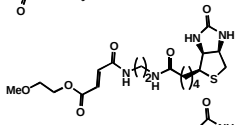
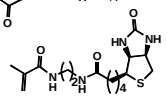
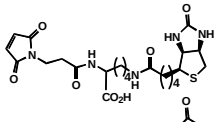
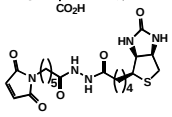
One of the most active sequences, when studied further, showed a modest rate acceleration of 800 over the uncatalyzed reaction. Interestingly, catalytic activity was found to be strictly dependent on the Cu^{2+} ion, one of a variety of transition metal salts with which the substrates were incubated. 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).

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

Compound	Inhibitor	K_{iapp} (μM)
9		32.5 ± 2.6
10		77.4 ±5.3
11		1093 ± 150
12		2300 ± 480

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 2. Dienophile study of Diels-Alder catalyst.

Compound	Dienophile	Relative Rate
2		**250**
13		**1**
14		**1**
15		**10**
16		**15.1**

DIELS-ALDER REACTION CATALYZED BY UNMODIFIED RNA

Jäschke et al. devised a method to isolate active Diels-Alder catalysts[9] 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.

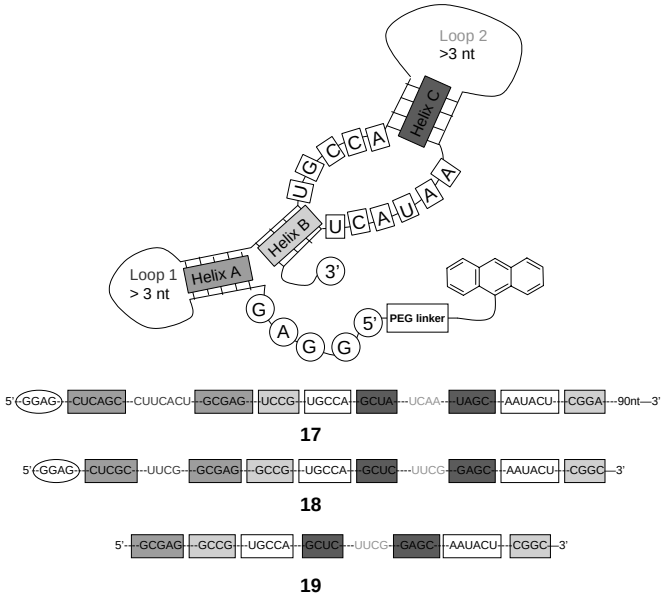


Figure 2. Structure and homologies of catalytically active Diels-Alderases

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

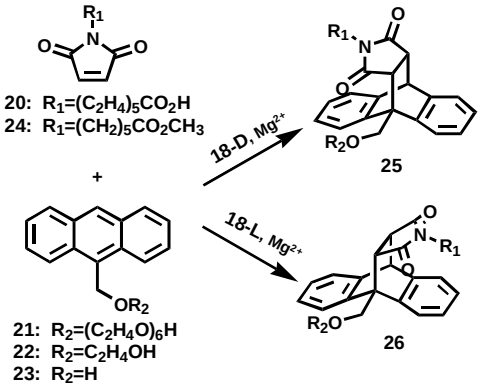
anthracene and the biotinylated maleimide. However, no significant rate acceleration over the background reaction occurred. If as few as four complementary nucleotides were appended to both the 5' terminus of the RNA and the end of the PEG tether, catalytic activity similar to that of the covalently 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 beeen 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).

Scheme 2. Stereoselective Diels-Alder Reaction



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 $(CH_2CH_2O)_6H$ (**21**) to C_2H_4OH (**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

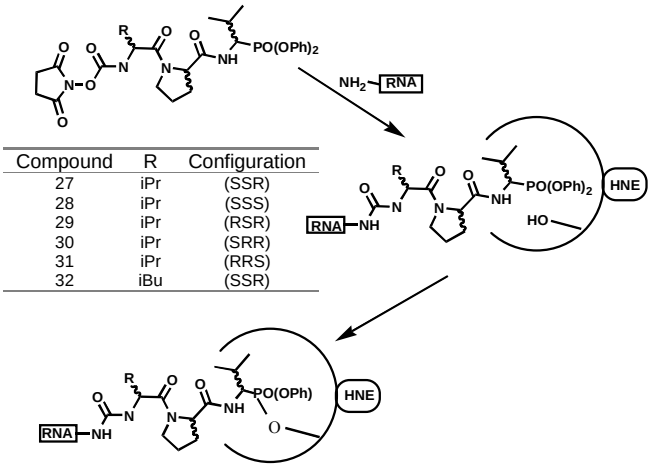
The active site of the above RNA catalyst was probed by measuring the rates and stereochemical outcomes of the reaction with modified dienes and maleimides.[11] With respect to the dienophile, the enzyme responded sensitively to the general shape of the unsubstituted α,β-unsaturated maleimide, and accommodated 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,[12] 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.[13] 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.

Scheme 3. Selection of stereoselective RNA ureases.



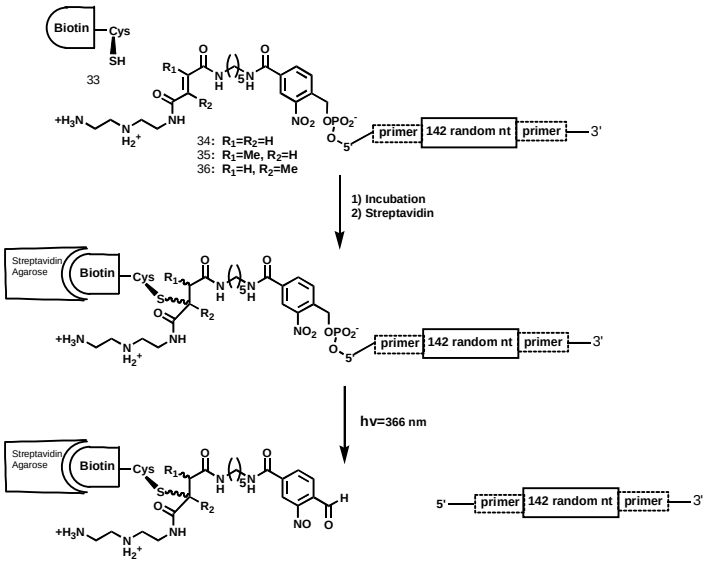
Compound	R	Configuration
27	iPr	(SSR)
28	iPr	(SSS)
29	iPr	(RSR)
30	iPr	(SRR)
31	iPr	(RRS)
32	iBu	(SSR)

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), t**he 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[14] and cellular biology.[15] Sengle et al. have discovered that unmodified RNA can catalyze the Michael reaction[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 *in vitro* selection cycles needed to arrive at the final ten catalytic sequences (from a library of 2.0×10^{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 3×10^5, 4.9×10^4, and 1.8×10^4 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.

Scheme 4. Isolation of RNA Michaelase via photocleavable linker.



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.

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