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Review

The Diels-Alder reaction and biopolymer catalysis

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Abstract. The Diels-Alder reaction provides a general and facile entry for the synthesis of six-membered cyclic structures. Consequently, it has been used extensively by organic chemists for the construction of complex compounds with pharmaceutical potential. Nature also appears to have utilized this reaction in the biosynthesis of several secondary metabolites. Given its utility, various catalysts have been discovered for the reaction ranging from simple Lewis acidic transition metals to complex catalytic antibodies. More recently, modified RNA has also been shown to be an effective Diels-Alderase with a highly specific active site. The RNA Diels-Alderase activity was also shown to be absolutely dependent on the nature of the base modification and the presence of cupric ion. Together, these results suggest that this RNA Diels-Alderase achieves a portion of its rate acceleration through Lewis acid catalysis, a different mechanistic mode than similar protein Diels-Alderases. The notion that RNA can accelerate reactions through Lewis acid catalysis suggests that modified RNA may serve as a tunable catalytic platform for the creation of structurally diverse compounds using a variety of powerful chemical transformations.

Key words. Diels-Alder reaction; biopolymer catalysis; Diels-Alderase; RNA catalysis; Lewis acid catalysis.

Introduction

Biocatalysis of small molecule chemical transformations is currently of immense interest. Given the exciting discoveries of new natural products with important biological activity and pharmaceutical potential, our research has focused on the development of new RNA biocatalysts for the assembly of novel carbocyclic and heterocyclic products. Recently, we published on the first example of an RNA catalyst capable of orchestrating the Diels-Alder [4 + 2] cycloaddition [1] to form carbocycles [2]. This form of cycloaddition can be implicated in the biosynthesis of several small molecule natural products. The scope and potential of this chemistry for small molecule therapeutic synthesis is substantial, and includes among others the taxane pharmacophore classes. Herein we examine the biopolymer Diels-Alderase catalysts that are either known or implicated, their mechanistic modes of action and prospects for the discovery of new catalysts to assemble complex carbocyclic structures.

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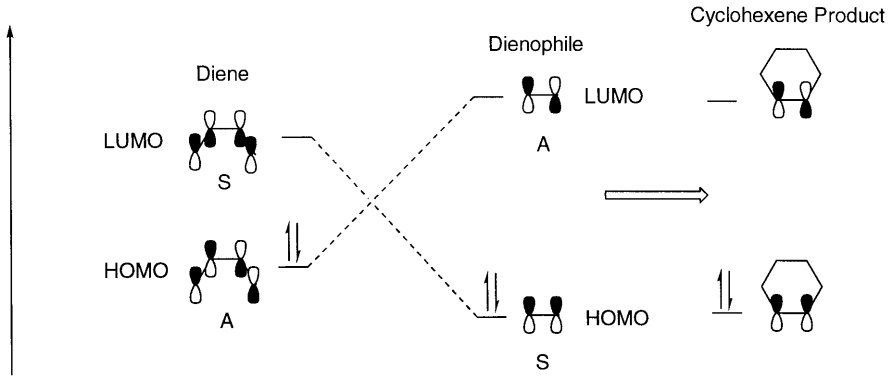
Diels-Alder reaction mechanism

In order to understand the driving forces behind biopolymer Diels-Alderase catalysts, it is useful to examine the mechanistic factors that effect the outcome of this cycloaddition. Two new carbon-carbon bonds and up to four new stereocenters are created in a Diels-Alder cycloaddition reaction, making it one of the most efficient processes for the assembly of carbocycles. Due to its synthetic utility and fundamental chemical interest, the Diels-Alder cycloaddition mechanism has been one

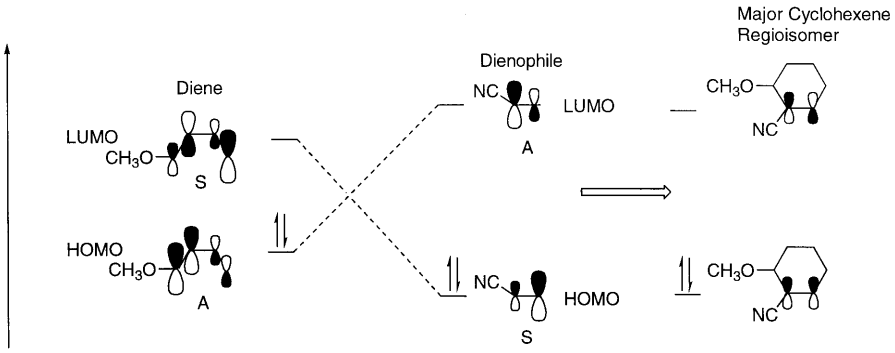
of the most studied and debated reactions of all time [3]. The most accepted version of the mechanism has carbon-carbon bond formation occurring by concerted mixing of the diene and dienophile π-orbitals, as depicted in Scheme 1. For clarity only the HOMO π-electrons are shown in Scheme 1. The alkene or dienophile reacts with a diene to form a six-membered cyclohexene. In effect, two π-bonds in the substrates are converted into two sigma bonds in the six-membered ring. Because six π-electrons are involved in this cycloaddition, it has been viewed as proceeding through an aromatic transition state, which is energetically more favored than a nonconcerted reaction path [4].

The dienophile typically contains π-electron withdrawing groups, such as esters, amides, ketones and nitriles. Although the reaction of alkenes not bearing these electron withdrawing groups can be made to react under harsh reaction conditions, the effect of the electron withdrawing substituents is to lower the energy of the lowest unoccupied molecular π-orbitals (LUMO) of the dienophile, thereby increasing the orbital mixing with the diene highest occupied molecular orbital (HOMO) and facilitating the cycloaddition reaction. Similarly, electron donating groups attached to the diene can raise the energy of the diene HOMO, thereby improving orbital interaction with the LUMO of the dienophile.

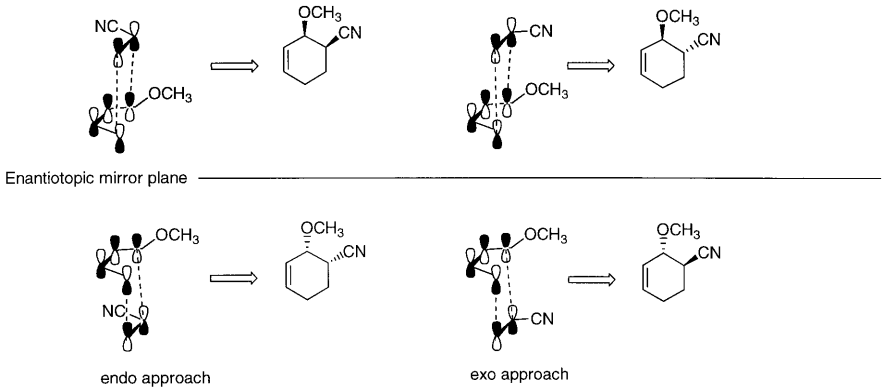
Much of the debate around the Diels-Alder reaction concerns whether the overall process is concerted. Simple symmetry arguments put forth decades ago seem consistent with the overall topology of the reaction when both the diene and dienophile are symmetric [5]. It now appears that in this case both C-C bonds are formed to the same extent simultaneously in a synchronous fashion [4]. When the dienophile or diene are not symmetric in their substitution with activating groups, it appears that some degree of polarization occurs at the transition state but that both bonds are formed to varying degrees at a single transition state



Scheme 1.



Scheme 2.



Scheme 3.

with no intermediates. In the case of unsymmetrical dienes and dienophiles, the best alignment of polarized dienophile LUMO and diene HOMO gives the predominant regioisomer product, as depicted in Scheme 2.

The stereocenters formed in this cycloaddition result from either of two reaction topologies for bringing two π-systems into orbital overlap, one where the electron withdrawing groups of the dienophile are endo to the diene and one where these groups are exo. For dienes or dienophiles not containing other chiral centers, there are two enantiotopic endo and exo transition states leading to four stereoisomers overall (Scheme 3). Of course, four other stereoisomers are possible for the set of regioisomers that places the diene and dienophile substituents in a 1,3 position in the product. It should be noted that the energy differences that drive the formation of different stereo- and regioisomers is typically small and that, when affected by a catalyst, the Diels-Alder reaction can give products that are not favored in the spontaneous reaction (vide infra).

The overwhelming number of Diels-Alder cycloadditions occur via an endo versus an exo transition state. It should be noted that this preference for the endo reaction topology produces the energetically less favorable cyclohexenes, indicating that this cycloaddition is under kinetic control since the exo transition state would yield the thermodynamic cyclohexene products. In organic solvents the bias for the endo transition state is caused by secondary orbital overlap of the π-electrons of the electron withdrawing group(s) of the dienophile with the diene [6]. In aqueous solutions the endo/exo transition state preference is even more exaggerated, due to what has been termed the hydrophobic effect.

Hydrophobic effect in the Diels-Alder reaction

For several decades it has been appreciated that the rate of the Diels-Alder reaction can be greatly accelerated when performed in aqueous solution [7]. Initially, it was suspected that aggregation of the hydrophobic diene and dienophile increased the effective concentration of reactants and thereby increased [4 + 2] cycloaddition rates [8, 9]. More recently, the polarity of the solvent and even specific hydrogen bonds between water molecules and hetero atoms of the dienophile have been proposed to be important contributors to accelerating the Diels-Alder reaction in aqueous solution [10]. No definitive experimental results exist as to the precise origin of the Diels-Alder rate acceleration in aqueous solution; however, if specific hydrogen bonding contacts can be important, it would seem reasonable that biopolymers such as proteins or RNA could serve as a useful scaffold on which suitable hydrogen bonds could be made to the diene or dienophile substrates. Indeed, as discussed later, catalytic antibody Diels-Alderases appear to have taken advantage of this mechanistic mode.

Lewis acid catalysts

From our current understanding of this reaction, it is reasonable to propose that catalysts might involve metal complexes that could coordinate to the dienophile hetero atoms and lower the energy of its LUMO or coordinate to the diene and raise its HOMO. The vast majority of known small molecule catalysts operate by coordination to the dienophile. A number of main group and transition metals have been extensively used as Lewis acids to accelerate Diels-Alder reactions. Examples include, aluminum, boron, gal-

lium, titanium, tin and copper. These metal ion catalysts can also play an important role in alignment of the cycloaddition substrates, thereby altering the regio- and stereochemical outcome of the reaction relative to the thermal process [11]. More recently, stereocontrol of the reaction using chiral Lewis acids has been shown to give enantiospecific product formation for a limited number of substrates [12–16]. Some examples of the chiral small molecule Lewis acid Diels-Alder catalysts are shown in figure 1.

Fine-tuning the metal center to attain just the right balance of Lewis acidity and ability to coordinate has occupied the research interests of prominent chemists for years. With the logic behind Lewis acid catalysis of the Diels-Alder reaction seeming inescapable, it is surprising that there are no known examples of metalloprotein Diels-Alderases. One possible explanation might be that the functional groups inherent to proteins may not be able to supply the appropriate coordination environment for the main group or transition metal ions. In contrast, our work on in vitro selection of functionally modified RNA catalysts has provided numerous examples of metallo-RNA Diels-Alderases (vide infra).

Protein Diels-Alderases: from natural products sources

Several secondary metabolites from widely varying organisms have been proposed to be biosynthesized via the Diels-Alder reaction [17–19]. Possible biosynthetic Diels-Alder products include heliocide H[1] (*Gossypium hirsutum*) [20], alflabene (*Alpinia flabellata*) [21], carpanone (*Cinnamomum* sp.) [22], mevinolin (*Aspergillus terreus*) [23], nargenicin (*Nocardie argentinensis*) [24, 25] and secodaphniphylline (*Daphniphyllum* sp.) [26] (fig. 2). A significant portion of the metabolites thought to be a result of a [4 + 2] cycloaddition have been isolated as racemates, indicating that the Diels-Alder reaction occurred spontaneously. In other proposed pathways the reactants contain established stereocenters that could account for stereospecificity observed in the spontaneous product formation. However, compelling evidence from a few notable examples implicate Diels-Alderase enzymes to be involved in secondary metabolite biosynthesis.

Isolation of optically active Diels-Alder type adducts, such as kuwanon H and J, from mulberry (*Morus alba*) tree extracts [19] and from feeding experiments with *M. alba* cell cultures [27, 28] suggests the presence of a Diels-Alderase enzyme since both the diene and dienophile are achiral (fig. 3). The fact that both endo and exo, enantio-enriched cycloaddition products are observed for different substrates even suggests that there may be more than one Diels-Alderase present in Mulberry.

Even more compelling is the evidence for a Diels-Alderase in the fungus *Alternaria solani*. Results from feeding experiments using late-stage intermediates sup-

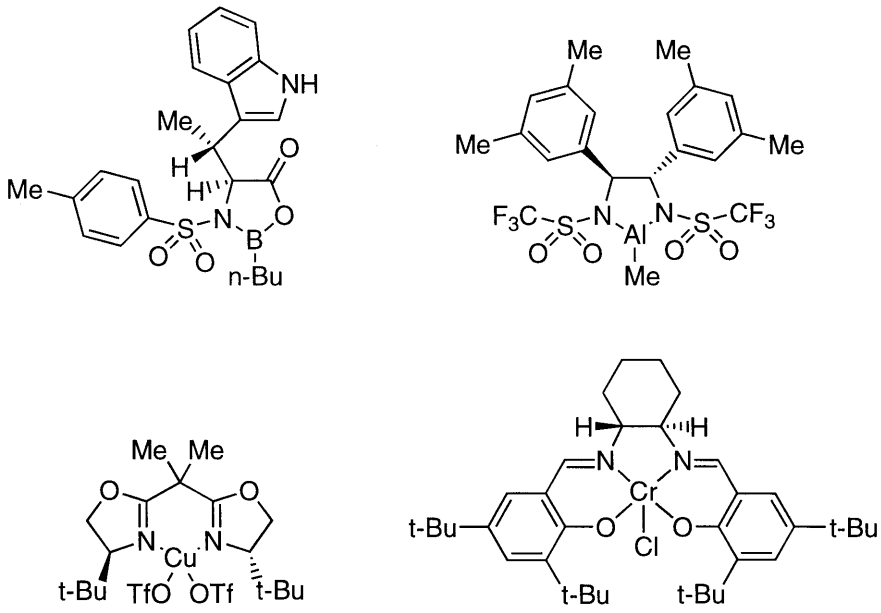


Figure 1. Recent examples of enantioselective chiral Lewis acid catalysts for the Diels-Alder and hetero Diels-Alder cycloadditions.

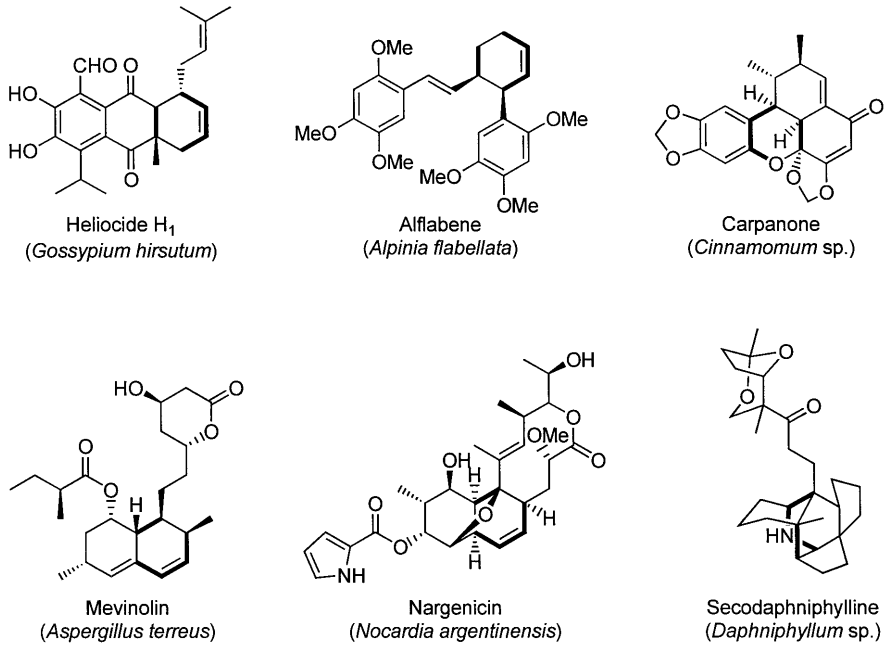


Figure 2. Secondary metabolites thought to be biosynthesized via Diels-Alder cycloadditions. Diene and dienophile components are shown in bold. Note, carpanone and secodaphniphyllum are thought to undergo hetero Diels-Alder reactions.

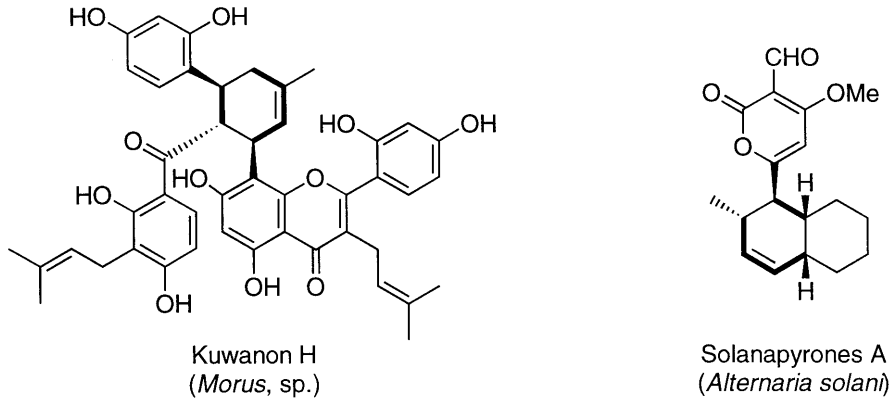


Figure 3. Secondary metabolites for which there are compelling data supporting biosynthesis by naturally occurring Diels-Alderase enzymes. Diene and dienophile components are shown in bold.

port a Diels-Alder reaction in the biosynthesis of solanapyrones in this organism (fig. 3) [29]. In addition, spectroscopic characterization of the products using cell-free extracts showed an enrichment of the exo adduct over the amount that is formed in the spontaneous reaction or in the presence of denatured cell-free extract [30]. Finally, this observed stereospecific exo Diels-Alderase activity has been isolated to a partially purified fraction of cell-free extract [31]. It is interesting to note that this partially purified extract also displays an oxidase activity which precedes the cycloaddition reaction, possibly suggesting that the enzyme did not originally evolve as a Diels-Alderase. The evidence for naturally occurring Diels-Alderases continues to mount, but complete isolation and characterization of members of this class of enzymes remain elusive.

Protein Diels-Alderases: catalytic antibodies

While naturally occurring protein enzymes capable of catalyzing the Diels-Alder reaction have been elusive, the immune system has proven a rich source for unique and interesting Diels-Alderase catalytic antibodies [32–36]. A number of clever approaches have been used to elicit antibodies capable of accelerating various carbocyclic Diels-Alder reactions. Stable transition-state analogs have even been designed to mimic either the endo or exo or even both transition states, generating catalysts that promote a single reaction pathway preferentially over the other possible outcomes [35, 36]. The properties of these catalysts provide a glimpse of what is mechanistically and practically possible for protein Diels-Alderases.

Recent crystallographic data have provided insight into how these abzymes achieve their catalysis and stereospecific product formation. Two reports describe antibody Diels-Alderase active sites as sequestering the transition state analog hapten in a hydrophobic environment where numerous van der Waals contacts and two to three key hydrogen bonds comprise the protein–small molecule interactions [37, 38]. Catalysis of the cycloadditions has been attributed to binding the diene and dienophile in a reactive conformation. However, it has also been suggested from both crystal structures that hydrogen bonds to the dienophile carbonyl, enhanced by the hydrophobic medium, account for a portion of rate acceleration by rendering the dienophile more electron-deficient and more reactive by lowering the energy of its LUMO. This catalytic mechanism is similar to the hydrophobic effect seen in aqueous solvent, where cycloaddition rate enhancement has been attributed to minimization of hydrophobic hydration shells through forced contact of the diene and dienophile and specific hydrogen bonding of water to the electron withdrawing group of the dienophile. It should be noted that antibody Diels-Alderases have yet to take advantage of metal-centered Lewis acid catalysis.

The other biopolymer catalyst: RNA Diels-Alderase

Despite what may be considered limitations, namely a highly charged backbone and only four different monomers, suitably modified RNA has been shown to catalyze a variety of reactions [39, 40]. An important feature of RNA lies within the ability to iteratively select and amplify individual molecules with a desired property from a beginning pool of $>10^{14}$ different sequences. This process of in vitro selection [41, 42] is unmatched when working with proteins. Further, recent chemical advances allow RNA to be modified with a broad range of functionalities, even beyond those found in proteins, that are compatible with in vitro selection techniques and may be adapted to the catalytic requirements of the desired chemical transformation [43–46].

RNA has recently been shown to be capable of Diels-Alderase activity that rivals its protein counterparts [2]. The in vitro selection approach shown in Scheme 4 was applied using modified RNA containing 5-(carboxamide-4-pyridylmethyl) uridine triphosphate (UTP) and selected main group and transition metal ions. The pyridine modification in combination with the metal ions were chosen anticipating that unique metal binding sites capable of Lewis acid catalysis could be created. After 12 rounds of in vitro selection, the pool activity had increased 200-fold over the spontaneous rate of cycloaddition. Subsequent cloning and sequencing of the post-round 12 population revealed eight completely unique sequences capable of accelerating the Diels-Alder reaction shown in Scheme 4. One sequence (# 22) was chosen for detailed characterization.

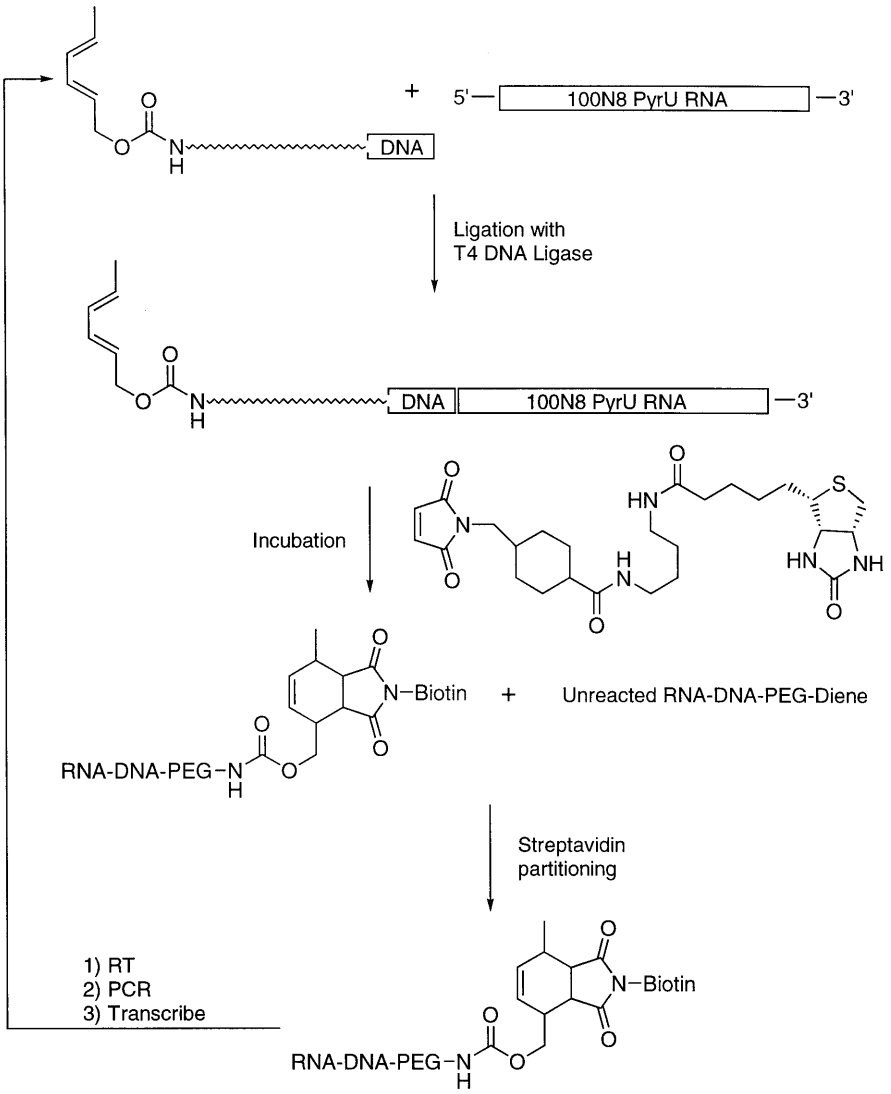
Isolate 22 catalyzes the Diels-Alder cycloaddition depicted in Scheme 4 with an 800-fold rate acceleration over the spontaneous reaction. Because the Diels-Alder reaction proceeds through a product-like transition state, reaction-rate inhibition studies using various product analogs can be revealing with regard to the size of the active site binding pocket. Inhibitor studies have demonstrated that the active site of isolate 22 recognizes functional components in and around the reaction center of both the diene and dienophile (fig. 4) [47]. In addition, experiments using alternative substrates have revealed the active site of isolate 22 to be highly specific, discriminating between electronically identical dienophiles that only differ by the alkyl appendage to the maleimide ring (fig. 4) [47]. The relative Diels-Alder activity is greater than 16-fold higher for maleimide **3** versus either **4** or **5**.

The requirements for catalysis by isolate 22 are also highly specific. Diels-Alderase activity is absolutely dependent on the presence of cupric ion, and none of the other metal ions present during the in vitro selection can substitute for copper [30]. This absolute copper dependence and the fact that isostructural metal ions cannot act as substitutes are consistent with the RNA-bound copper forming a Lewis acid center that contributes to the catalysis of the Diels-Alder reaction. Molecular replacement and rearrangement experiments of the modified uridine contained in this Diels-Alderase have further defined the requirements for catalytic activity and provided some indication of what ligands may comprise the Lewis acidic active site. It was reasoned at the beginning of the in vitro selection that the pyridyl modified uridines would provide unique metal ligands in the context of a specific RNA environment. By either replacing the pyridyl nitrogen with a carbon-hydrogen or moving the pyridyl nitrogen around the

aromatic ring relative to the point of attachment to the uridine, Diels-Alderase activity has been demonstrated to be completely dependent on the 4-pyridylmethylcarboxamide modification (**7**, fig. 5; no activity was observed using RNA transcribed with triphosphates **6**, **8** or **9**) [47]. These results suggest that the pyridyl functionality serves as ligands in a highly specific copper ion binding site which, in turn, acts as a Lewis acid in the acceleration of the Diels-Alder reaction. While still lacking structural verification, the proposed copper ion Lewis acid catalytic mechanism for RNA Diels-Alderase activity represents a new mode of Diels-Alder catalysis for biopolymers and highlights the potential differences in selected nucleotide catalysts and their protein counterparts. Clearly, additional functional and structural analysis will provide further insight into how these RNA Diels-Alderases achieve their rate acceleration and how chemical mechanistic principles can be applied to create more efficient and even new RNA cycloaddition catalysts.

Prospects for discovering new RNA catalysts

RNA offers an excellent macromolecular platform from which to create novel Diels-Alderase catalysts [48]. The ability to design in vitro selection strategies for the isolation of catalysts from vast combinatorial RNA pools is a truly powerful characteristic unique to



Scheme 4.

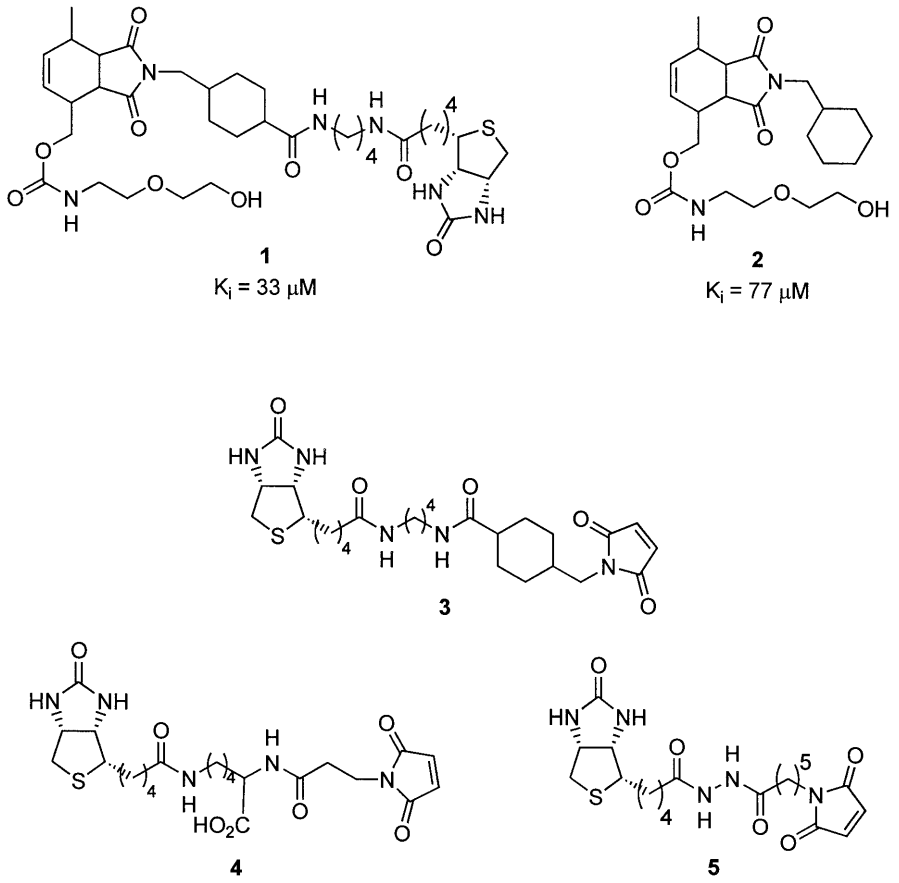


Figure 4. Product analogs **1** and **2** used to probe the size of the RNA Diels-Alderase active site. Maleimide compounds **3**, **4**, and **5** were surveyed as dienophile substrates.

oligonucleotides. These easily created random sequence libraries contain RNA molecules representing an enormous number of discretely folded topologies. The ability to include functionally modified bases further allows this biopolymer catalyst platform to be tailored to the reaction of interest. Recently, enzyme-compatible base modifications were reported for DNA as well, leading the way for modified DNA in vitro selections [49]. Given these remarkable properties of oligonucleotides, RNA and perhaps DNA are likely to prove a rich source of not only new Diels-Alder catalysts but catalysts for a variety of other reactions as well, in particular, reactions such as cycloadditions, where metal coordination to the π-electrons of the substrates (either LUMO or HOMO interactions) appear most desirable in terms of useful biocatalysis.

It is now apparent that RNA Diels-Alderases can take advantage of chemical mechanisms not observed in selected catalytic antibodies. As for selecting new modes of Lewis Acid catalysis, the combination of base modification and diverse metal ions lends itself to a combinatorial selection that has no rival. Clearly a plethora of possible ligand/metal combinations and concomitant coordination geometries are yet to be discovered for the RNA-catalyzed cycloaddition of small molecule reactants.

As more RNA metallo-Diels-Alderases are isolated and characterized from this vast combination of modifications and metals, RNA should be capable of accelerating other reactions that are susceptible to Lewis acid catalysis such as cationic cyclizations, nucleophilic epoxide opening and aldol condensations to name a few. As with the Diels-Alder cycloaddition, these reactions are also valuable synthetic tools for the construction of important pharmacophores. Examples include the formation of various polycyclic structures by cationic cyclization of squalene, synthesis of cyclic ethers found in numerous natural products by in tramolecular epoxide opening, and aldol and Claissen

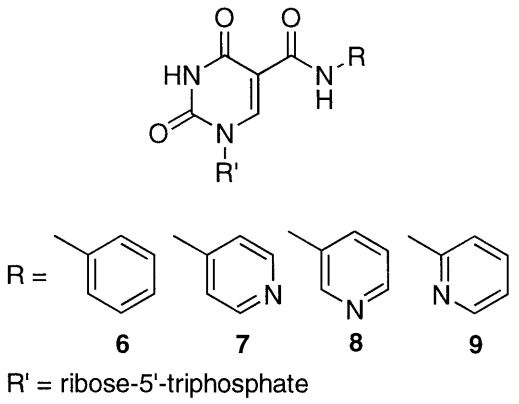


Figure 5. Modified uridine triphosphates used to probe the catalytic requirements of the RNA Diels-Alderase.

condensations such as those used to create the numerous varieties of polyketide secondary metabolites. The addition of these reactions to the scope of RNA-catalyzed chemistries would make this biopolymer an extremely valuable biocatalyst for the assembly of a diversified group of natural product-like molecules [50].

Conclusions

It appears that we are entering a new era in biocatalysis where the attributes of nucleic acid scaffolds are beginning to be appreciated. The streamlined versatile chemistry for the modification of RNA can now provide a means to include functional groups useful for creating catalytic active sites for important cycloaddition reactions such as the Diels-Alder reaction. Vast modified RNA libraries can yield new structures with remarkable substrate specificity and complexity. For example, from the pyridyl molecular replacement and rearrangement experiments, the folded topology of the active site of RNA DA22 appears to involve intricate three-dimensional contacts. In addition, the substrate specificity and product inhibition data on DA22 support a well-defined binding pocket that rivals its antibody counterparts. Fueled by advances in chemistry, biochemistry and molecular biology, the future for highly efficient and specific RNA catalysis is upon us.

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