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Characterization of an RNA Active Site: Interactions between a Diels−Alderase Ribozyme and Its Substrates and Products

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Abstract: Ribozymes have recently been shown to catalyze the stereoselective formation of carbon−carbon bonds between small organic molecules. The interactions of these Diels−Alderase ribozymes with their substrates and products have now been elucidated by chemical substitution analysis by using 44 different, systematically varied analogues. RNA−diene interaction is governed by stacking interactions, while hydrogen bonding and metal ion coordination appear to be less important. The diene has to be an anthracene derivative, and substituents at defined positions are permitted, thereby shedding light on the geometry of the binding site. The dienophile must be a five-membered maleimidyl ring with an unsubstituted reactive double bond, and a hydrophobic side chain makes a major contribution to RNA binding. The ribozyme distinguishes between different enantiomers of chiral substrates and accelerates cycloadditions with both enantio- and diastereoselectivity. The stereochemistry of the reaction is controlled by RNA−diene interactions. The RNA interacts strongly and stereoselectively with the cycloaddition products, requiring several structural features to be present. Taken together, the results highlight the intricacy of ribozyme active sites which can control chemical reaction pathways based on minute differences in substrate stereochemistry and substitution pattern.

Introduction

Ribozymes are RNA molecules with catalytic properties. The mechanisms by which they accelerate chemical reactions have received much attention in recent years, and several of the naturally occurring ribozymes have been studied in greater detail.[1] These ribozymes catalyze RNA-modifying reactions in which ribozyme−substrate interactions are governed by nucleobase-pairing interactions. Little is known, however, about how ribozymes accelerate reactions at nonnucleotide substrates.[2] Although recent studies on ribosomal peptide bond formation present an active site composed of RNA, compared to the wealth of information available for protein enzymes, our knowledge about the design principles of RNA active sites is very limited.[3]

Our lab has recently described the isolation of RNA molecules that catalyze the formation of carbon−carbon bonds between a tethered anthracene diene and a biotinylated maleimide dienophile by [4 + 2] cycloaddition (Diels−Alder reaction, Figure 1A).[4] This reaction type is one of the most important C−C bond forming processes available to organic chemists, and there is currently much interest in developing catalytic methods for improving its rate and selectivity.[5] The majority of the RNA sequences selected contained a small secondary structure motif (Figure 1B), and a 49-mer oligoribonucleotide containing this motif was found to accelerate the cycloaddition reaction in a truly bimolecular fashion, with the two substrates free in solution, and without any tethering or biotinylation (Figure 1C).[6] Catalysis proceeds with multiple turnover and high enantioselectivity, and a synthetic mirror image of the ribozyme composed of unnatural L-nucleotides was found to have the opposite stereoselectivity.[6]

These features significantly increase the utility of nucleic acid catalysts, which is of prime interest from both a biochemical and a synthetic perspective.[7] The elucidation of the mechanisms by which ribozymes solve these tasks as well as their structural prerequisites may provide for a deeper understanding of RNA catalysis at the molecular level. Due to the small size of the ribozymes and the exhaustively studied chemistry of Diels−

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(3) (a) Ban, N.; Nissen, P.; Hansen, J.; Moore, P. B.; Steitz, T. A. *Science* **2000**, *289*, 905. (b) Muth, G. W.; Ortoleva-Donnelly, L.; Strobel, S. A. *Science* **2000**, *289*, 947. (c) Tarasow, T. M.; Tarasow, S. L.; Tu, C.; Kellogg, E.; Eaton, B. E. *J. Am. Chem. Soc.* **1999**, *121*, 3614. (d) Tarasow, T. M.; Tarasow, S. L.; Eaton, B. E. *J. Am. Chem. Soc.* **2000**, *122*, 1015.
(4) Seelig, B.; Jäschke, A. *Chem. Biol.* **1999**, *6*, 167.
(5) (a) Pindur, U.; Lutz, G.; Otto, C. *Chem. Rev.* **1993**, *93*, 741. (b) Gouverneur, V. E.; Houk, K. N.; de Pascual-Teresa, B.; Beno, B.; Janda, K. D.; Lerner, R. A. *Science* **1993**, *262*, 204. (c) Sauer, J.; Sustmann, R. *Angew. Chem., Int. Ed. Engl.* **1980**, *19*, 779.
(6) Seelig, B.; Keiper, S.; Stuhlmann, F.; Jäschke, A. *Angew. Chem., Int. Ed.* **2000**, *39*, 4576.
(7) (a) Jäschke, A.; Frauendorf, C.; Hausch, F. *Synlett* **1999**, 825. (b) Jäschke, A. *Curr. Opin. Struct. Biol.* **2001**, *11*, 321. (c) Famulok, M.; Jenne, A. *Top. Curr. Chem.* **1999**, *202*, 101.

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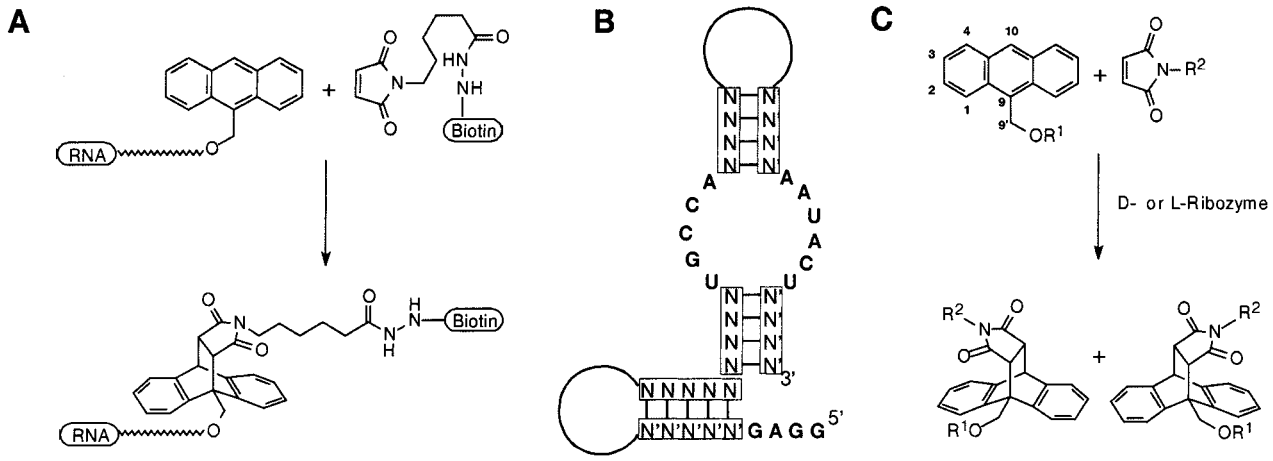

Figure 1. RNA-catalyzed Diels−Alder reactions. (A) Ribozymes were selected from a combinatorial library of random-sequence RNA-tether-anthracene conjugates by reaction with biotin maleimide and subsequent isolation of biotinylated molecules. (B) Secondary structure motif shared by most sequences. N = any nucleotide; N′ = nucleotide complementary to N. (C) Bimolecular cycloaddition catalyzed by D- or L-ribozymes. R^1 = H or larger; R^2 = ethyl or larger.

Alder reactions,[8] this system appears to be ideally suited for structural and mechanistic analysis. Toward this end, we investigated here the interaction of the 49-mer D- and L-ribozymes with substrate and product analogues systematically varied at individual positions. The results provide a first insight into principles of substrate recognition, and into the molecular determinants of stereodifferentiation.

Results

To investigate the importance of individual structural features for ribozyme catalysis, a series of anthracene and maleimide derivatives were synthesized and tested for conversion by the ribozyme. As the poor solubility of most substrates and products prevented full kinetic analysis, all compounds were assayed at a fixed set of standard conditions (100 μM diene, 500 μM dienophile, 7 μM ribozyme), and from the measured initial rates, apparent second-order rate constants of the catalyzed (k_{cat}) and uncatalyzed (k_{uncat}) reactions were determined. The comparatively high ribozyme concentration was required for the precise measurement of initial rates. Alternatively, these derivatives as well as various Diels−Alder product analogues were tested as potential inhibitors by adding appropriate concentrations to the ribozyme-catalyzed standard reactions. It should be noted that substitutions often have tremendous effects on the rates of the uncatalyzed background reactions, too,[9] so both absolute reaction velocities and rate accelerations (k_{cat}/k_{uncat}) have to be considered.

RNA−Diene Interactions. The ribozymes were previously selected from a combinatorial RNA conjugate library, in which each RNA molecule was tethered to the anthracene diene by a poly(ethylene glycol) chain attached via a hydroxymethylene bridge in the 9-position (Figure 1).[4] As 9-hydroxymethylanthracene **1** was found to be accepted by the ribozyme even better than anthracene-hexa (ethylene glycol) **2**, participation of large parts of the tether in substrate recognition can be ruled out.[6]

The diene has to contain three linearly annellated rings. Neither 1-hydroxymethyl-cyclohexa-1,3-diene **3** nor hydroxymethylated naphthalene **4**[10] are substrates of the ribozyme-catalyzed reaction (Table 1).

To map the dimensions of a putative anthracene binding pocket, we introduced methyl or hydroxymethyl substituents at the five distinct positions on the periphery of the anthracene system. In those cases where the methylated 9-hydroxymethylanthracenes were too sparingly water soluble, a hexa (ethylene glycol) (HEG) unit was attached to the 9′-hydroxyl group to improve the water solubility. Substituents in the 1-, 4-, and 10-positions did not impede the action of the ribozyme. Although converted slower than the parent compounds **1** and **2**, rate accelerations for compounds **5**, **6**, and **7** were significant. Substitutions in the 2- and 3-positions (compound **8**), however, almost completely abolished activity while having only marginal influence on the background reaction. Thus, in a binding pocket, there appears to be enough space in front and behind the anthracene to tolerate substitutions while there is a tight fit at the sides.

The carbon in the 9′-position can be branched. Branching leads to an attenuation of the rate of conversion, but both (racemic) anthracene ethanol **9** and double-substituted **10** were accepted as substrates (vide infra for details on stereochemistry).

To probe the role of the 9′-oxygen (the one that connected the diene to the poly(ethylene glycol) tether in the selection), it was substituted by S, NH, and CH_2, respectively. Both sulfur (**11**) and even carbon (**12**) substitution led to compounds that were almost as good substrates as the parent ether compound **2**. Displacement by amine nitrogen gave inactive compounds **13** and **14**. The secondary amine **14** is likely to be protonated in the reaction buffer (pH 7.4) and the resulting positive charge likely deactivates the anthracene. Equally inactive as a substrate was carboxylic acid **15**, probably due to the electron-withdrawing force of the carboxylic acid group.

Compound **16** in which the hydrogen bond donor capability of the 9′-oxygen was attenuated by esterification was efficiently converted by the ribozyme. Taken together, these data implied that this oxygen is involved in neither metal ion coordination nor H-bonding. These results prompted us to completely remove

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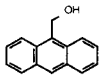
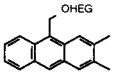
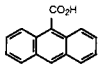
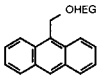
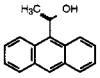
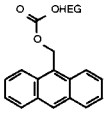
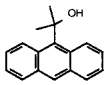
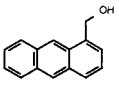
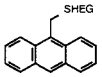
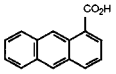
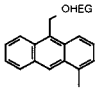
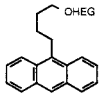
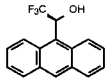
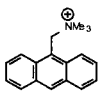
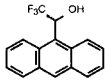
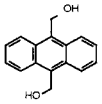
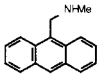
(9) Mielert, A.; Braig, C.; Sauer, J.; Martelli, J.; Sustmann, R. *Liebigs Ann. Chem.* **1980**, 954.
(10) Naphthalene undergoes Diels−Alder reactions under forcing conditions: (a) Kloetzel, M. C.; Herzog, T. *J. Am. Chem. Soc.* **1950**, *72*, 1991. (b) Breton, G. W.; Newton, K. A. *J. Org. Chem.* **2000**, *65*, 2863.

Table 1. Apparent Second Order Rate Constants [M^{-1} s^{-1}] of Potential Dienes with *N*-Pentylmaleimide **21**, with and without Catalysis by the D-49nt Ribozyme[a]

	k_{cat}	k_{uncat}	$\frac{k_{cat}}{k_{uncat}}$		k_{cat}	k_{uncat}	$\frac{k_{cat}}{k_{uncat}}$		k_{cat}	k_{uncat}	$\frac{k_{cat}}{k_{uncat}}$
1 	3.3*	0.023*	**143**	**8** 	0.046*	0.022*	**2**	**15** 	<0.003	n.d.	-
2 	1.1	0.012	**92**	**9** 	2.0	0.08	**25**	**16** 	1.3	0.010	**130**
3 	0.003*	n.d.	-	**10** 	>30*	3*	**>10**	**17** 	1.8	<0.003*	**>600**
4 	<0.003	n.d.	-	**11** 	2.9	0.05	**58**	**18** 	0.9	<0.003	**>300**
5 	0.6	0.012	**50**	**12** 	3.5	0.11	**32**	**19** 	0.13*	0.003*	**43**
6 	12*	0.5*	**24**	**13** 	<0.003	n.d.	-	**20** 	0.03*	0.003*	**10**
7 	14	0.8	**18**	**14** 	<0.003	n.d.	-				

[a] For conditions, see the Experimental Section. Analysis was done by UV/vis-spectroscopy (standard) or HPLC (*). Diastereomeric excesses (de) of thermal Diels−Alder reactions of **19** and **20** were 30%, while the catalyzed reactions proceeded with >95% de for **19** and 60% de for **20**. HEG refers to $(C_2H_4O)_6H$, HEGO, OHEG, or SHEG to the respective ether or thioether compounds.

the substituent in the 9-position. While unsubstituted anthracene is insoluble in the reaction buffer, the hydroxymethyl group could be moved to the 1-position to give compound **17**, which is readily converted by the ribozyme. Whereas the rate of the catalyzed reaction differs only slightly from that of reference compound **1**, the rate of the thermal background reaction is significantly reduced due to the removal of the electron-donating hydroxymethyl group from the 9-position. Therefore, the rate acceleration by the ribozyme is much higher, and while a carboxyl group in the 9-position is not tolerated (**15**), 1-carboxyanthracene **18** is accepted by the catalyst.

To investigate the possibility that for some dienes only the chemical step is not accelerated while they are still bound by the catalyst, competition experiments were carried out. 9-Carboxyanthracene **15** was found to inhibit the ribozyme-catalyzed reaction of **2** and pentylmaleimide **21** with an IC_{50} of 370 μM, suggesting that it competes for the diene binding site.[11] For compounds **4** and **14**, no inhibition was detected, which may be due to their poor water solubilities of 250 and 100 μM, respectively.

Diastereoselectivity. Since the substituent in the 9-position was known to determine the stereochemistry in the RNA-catalyzed reactions with achiral dienes,[6] we now investigated the interaction of the ribozymes with chiral dienes, specifically with those branched at the 9′-position, where compounds **9** and **10** showed attenuated conversion. The ribozyme differentiates between the two enantiomers of Pirkle's alcohol, *R*-(**19**)- and *S*-(**20**)-2,2,2-trifluoroethyl-1-(9-anthracenyl)ethanol. The rate accelerations for **19** and **20** are 21% and 90%, respectively, of that obtained with reference compound **1**,[12] thus the two proton positions at the 9′-carbon are not equivalent. In the course of these reactions, product diastereomers are created. While the

(11) k_m values were found as 370 μM for **2** and 8 mM for **34** with a k_{cat} of 21 min^{-1} (ref 6).

(12) As **19** and **20** were measured with dienophile **34** for solubility reasons, reference compound **1** also had to be measured with **34**. In that case, k_{cat} = 1.57 M^{-1} s^{-1}, k_{uncat} 0.033 M^{-1} s^{-1}, and k_{cat}/k_{uncat} = 48.

thermal reaction shows a moderate diastereoselectivity (30%), the ribozyme produces the isomers with 60% de (**20**) and >95% de (**19**). As expected, the mirror-image L-ribozyme shows opposite preferences (>95% de for **20**, ~60% de for **19**).

RNA−Dienophile Interactions. In the in vitro selection, biotin maleimide (Figure 1) was used as a dienophile, and small *N*-alkyl maleimides such as **21** were found to be efficiently converted by the ribozyme. We now dissected the role of individual atomic positions in RNA−dienophile interactions (Table 2).

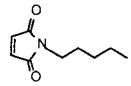
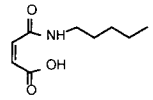
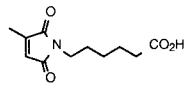
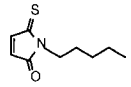
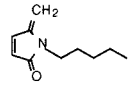
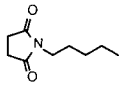
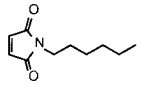
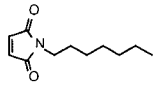
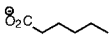
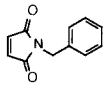
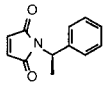
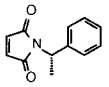
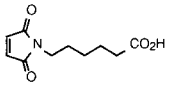
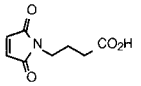
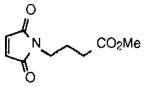
The dienophile has to be a five-membered maleimidyl ring system. No acceleration could be detected for *N*-pentyl maleamic acid **22** in which the maleimidyl ring is opened by formally hydrolyzing a C−N bond. Substituents at the reactive double bond are not permitted. *N*-(5-Carboxy)pentylcitraconimide **23**, carrying an additional methyl group at the double bond, showed no rate acceleration in the presence of ribozyme. One of the carbonyl oxygens can be exchanged for sulfur, and the respective compound **24** is converted very efficiently. If one of the carbonyl oxygens is replaced by a methylene group (**25**), no Diels−Alder reaction can be observed, neither catalyzed nor uncatalyzed (which is not surprising, since **25** is not known to act as a dienophile[13]).

The olefin double bond appears to be essential not only for the chemistry of the reaction but also for recognition by the ribozyme. A 100-fold excess of *N*-pentyl succinimide **26** having a single bond in place of the maleimide double bond does not cause any inhibition to the reactions of **21** and **2**, indicating that it does not compete for the dienophile binding site. Compounds **22** and **25** were also found to be not inhibiting.

We next focused on the role of the side chain. The minimum length for recognition was found previously to be ethyl; for H and Me, rates were indistinguishable from the respective uncatalyzed reaction.[6] Increasing the size of the alkyl chain improves acceptance by the ribozyme; the maximum rate is reached at pentyl (**21**). Hexyl (**27**) and heptyl maleimide (**28**) were also quickly converted by the ribozyme, while the rates of the background reactions display a remarkable acceleration in water that has received much attention as the "hydrophobic effect".[14] For mechanistic reasons, it appears to be more meaningful to compare the ribozyme-catalyzed rates with the background rates measured in 100% ethanol, i.e., under conditions where this effect is negligible.[15] In that case, background rates are slow and indistinguishable for the three maleimides (<0.003 M^{-1} s^{-1}), and the formal rate accelerations are comparable.

Despite the observed importance of the maleimide side chain, caproic acid **29** did not inhibit the reaction at a 100-fold excess. Dienophiles with aromatic side chains are also accepted by the ribozyme. *N*-Benzylmaleimide **30** was as efficiently converted as many alkyl-substituted maleimides. Maleimides branched at the α carbon (the one in the side chain next to the imide N) were not accepted as substrates. Neither the achiral branched aliphatic *N*-isopropylmaleimide **31** nor the chiral branched aromatic compounds **32** and **33** showed a rate acceleration.

Table 2. Apparent Second Order Rate Constants [M^{-1} s^{-1}] of Dienophiles[a]

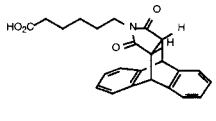
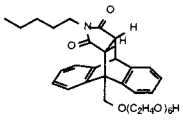
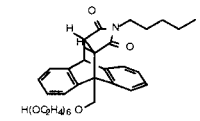
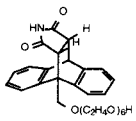
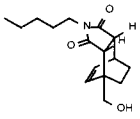
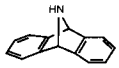
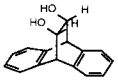
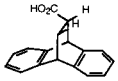
		k_{cat}	k_{uncat}	k_{cat}/k_{uncat}
21		1.1	0.012*	**92**
22		<0.003	<0.003	-
23		<0.003	<0.003	-
24		0.13*	0.002	**65**
25		<0.003	<0.003	-
26		+	+	-
27		0.7	0.035	**20**
28		0.6	0.1	**6**
29		+	+	-
30		0.4	0.015	**27**
31		0.01	n.d.	-
32		0.03	n.d.	-
33		0.03	n.d.	-
34		0.43	0.018	**24**
35		0.07	0.04	**< 2**
36		0.25	0.01	**25**

[a] Diels−Alder reactions with **2** catalyzed by 7 μM 49nt Diels−Alderase ribozyme. Analysis was done by UV/vis spectroscopy (standard) or HPLC (*). Dagger (+): tested only as inhibitors.

The incorporation of charged groups into the chain leads to an attenuation of the rate acceleration. This effect is the more dramatic the closer it happens to the maleimide. Maleimidocaproic acid **34** is efficiently converted (albeit with reduced rate compared to pentyl maleimide **21**), while hardly any acceleration can be measured for γ-maleimidobutyric acid **35**. Esterification

(13) Snieckus, V.; Tse, I. *J. Chem. Soc., Chem. Commun.* **1976**, *1976*, 505.
(14) (a) Breslow, R.; Rideout, D. C. *J. Am. Chem. Soc.* **1980**, *102*, 7816, (b) Breslow, R.; Rizzo, C. J. *J. Am. Chem. Soc.* **1991**, *113*, 4340. (c) Breslow, R.; Zhu, Z. N. *J. Am. Chem. Soc.* **1995**, *117*, 9923. (d) Otto, S.; Engberts, J.; Kwak, J. C. T. *J. Am. Chem. Soc.* **1998**, *120*, 9517.
(15) Meijer, A.; Otto, S.; Engberts, J. *J. Org. Chem.* **1998**, *63*, 8989.

Table 3. Inhibitors of the D-49nt Diels–Alderase[a]

	IC_{50} [μM]		IC_{50} [μM]
 **37**	21	 **38**	11
 **39**	270	 **40**	1300
 **41**	>2000	 **42**	>1000
 **43**	800	 **44**	>2000

[a] Measured at fixed concentrations of diene **2** (100 μM), dienophile **21** (500 μM), and ribozyme (7 μM). For conditions, see the Experimental Section.

of the carboxylate group (**36**), however, restores substrate properties. These findings support the idea of a hydrophobic binding pocket that accommodates a hydrophobic substituent at the maleimide nitrogen quite unspecifically.

RNA–Product Interactions. Due to the product-like nature of the transition state in Diels–Alder cycloadditions, the interactions between product and catalyst may provide valuable mechanistic information.[16] Therefore, the inhibition of the RNA-catalyzed reaction between **2** and **21** by cycloaddition products and analogues was studied (Table 3). With achiral **37**, a strong product inhibition with an IC_{50} of ~20 μM was observed. As bond formation is highly enantioselective, product inhibition should also be stereospecific, showing the same stereochemical preferences. This could be verified by using enantiomers **38** and **39** (>99% ee) carrying the hexa (ethylene glycol) moiety. The *R,R*-product enantiomer **38** that is produced with over 95% ee by the D-ribozyme inhibits about 20-fold more strongly than the *S,S*-product **39**.[17] Conversely, the mirror image ribozyme composed of unnatural L- ribonucleotides is strongly inhibited by **39** (IC_{50} 30 μM) and only weakly by **38** (370 μM).

The fact that the stronger inhibiting enantiomer has essentially the same IC_{50} as achiral **37** again confirms that the substituent in the 9-position of the anthracene does not significantly contribute to the binding energy. If, on the other hand, the pentyl chain at the imide nitrogen is removed, the IC_{50} increases by almost 2 orders of magnitude (compound **40**), confirming the importance of the hydrophobic side chain.

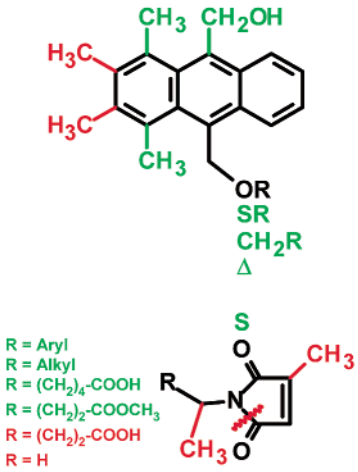


Figure 2. Summarized structural requirements of dienes and dienophiles for acceptance by 49-mer ribozyme. Substitutions shown in green are tolerated, while those in red are deleterious. Δ: removal of the whole substituent.

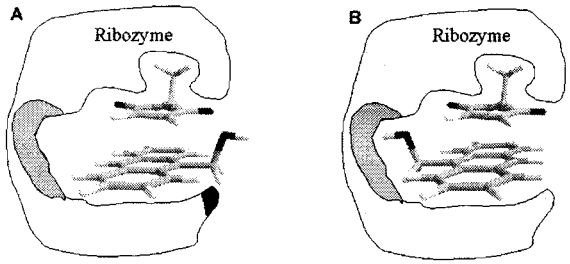


Figure 3. Schematic view of RNA-substrate interactions and the structural basis of stereoselectivity. A: head-first, B: tail-first orientation. (See text for details.)

Removal of the two outer benzene rings leads to product analogue **41**, which is inactive as an inhibitor, demonstrating the critical role of these rings. Imide-bridged analogue **42**[18] shows no inhibition, highlighting the importance of the pyrrolidinedione ring and the size of the bicyclic system. Compound **43** showed a weak inhibition, while **44** had no effect up to the solubility limit.

Discussion

The data obtained show that the ribozyme has strict requirements regarding structural features of both substrates, which are summarized in Figure 2. These data suggest that both reactants are specifically recognized, and in both cases hydrophobic interactions play an important role.[19] Concerning the general chemistry, the ribozyme is restricted to the diene and dienophile systems used in the selection, i.e., anthracene and maleimide, but numerous substitutions are tolerated.

According to the general mechanism of a Diels–Alder reaction, the catalytic pocket must be wide enough to accommodate the two reactants in a stacked, coplanar arrangement (Figure 3). Intercalation of the diene between two base pairs is unlikely, since the dienophile would not get close enough to the reactive positions of the diene.[20] As not a single heteroatom is necessary for recognition of the diene, hydrogen bonding and

(16) Chen, J.; Deng, Q.; Wang, R.; Houk, K. N.; Hilvert, D. *ChemBioChem* **2000**, *1*, 255.
(17) Stereochemistry was assigned based on chromatographic properties in comparison to published compounds with known structure: (a) Riant, O.; Kagan, H. B.; Ricard, L. *Tetrahedron* **1994**, *50*, 4543. (b) Tokioka, K.; Masuda, S.; Fujii, T.; Hata, Y.; Yamamoto, Y. *Tetrahedron-Asym.* **1997**, *8*, 101.
(18) Carpino, L. A.; Padykula, R. E.; Barr, D. E.; Hall, F. H.; Krause, J. G.; Dufresne, R. F.; Thoman, C. J. *J. Org. Chem.* **1988**, *53*, 2565.
(19) (a) Baugh, C.; Grate, D.; Wilson, C. *J. Mol. Biol.* **2000**, *301*, 117. (b) Zimmermann, G. R.; Jenison, R. D.; Wick, C. L.; Simorre, J. P.; Pardi, A. *Nat. Struct. Biol.* **1997**, *4*, 644.
(20) Kumar, C. V.; Punzalan, E. H. A.; Tan, W. B. *Tetrahedron* **2000**, *56*, 7027.

metal ion coordination are unlikely to play an important role in RNA−diene interactions. Therefore, we assume that anthracene is bound primarily by stacking against the hydrophobic surface at one side of the catalytic pocket.[21] This stacking is directional, and the size of the binding cleft is restricted to about 10 Å (three annellated rings) along the long axis of anthracene, as substituents in the 2- and 3-positions are not tolerated. The acceptance of large substituents in the 9-position indicates an opening of the binding site toward this side. This opening appears to be rather narrow and cleft-like, as branching the 9′-carbon leads to a stereospecific reduction of the rate acceleration, compared to much larger unbranched substituents, suggesting disturbing contacts between substituent and RNA (steric clash). Behind the bound anthracene, the binding site accommodates small substituents (CH_3, CH_2OH, $CH_2OCH_2CH_2OH$[6]) in the 10- and 4-positions.

The dienophile is likely to be bound to the opposite side of the binding pocket. The data compiled in Table 2 illustrate the importance of a hydrophobic side chain that is presumably recognized quite unspecifically by a hydrophobic binding pocket. Even benzyl maleimide having rather different steric demands is rapidly converted. Charged groups in the side chain disturb the hydrophobic interaction and thereby lead to a reduced reaction rate. It cannot be ruled out, however, that the different substrate properties of **35** and **36** are due to electrostatic repulsion between the carboxylate and the charged phosphate backbone of the ribozyme at the active site. The data obtained with compounds **22**, **25**, and **26** indicate that recognition of the maleimidyl part cannot be ascribed to an individual atomic position. A five-membered ring with correct heterosubstituents and conjugated π-electron density at the right positions all appear to be important.

The specific inhibition of the forward reaction by the cycloaddition products supports the assumption of a concerted, typical Diels−Alder reaction pathway with a product-like transition state.[22] The most important structural elements for recognition of the substrates were also found essential in the products. In addition, the size of the newly formed ring appears to be critical. The fact that both the planar anthracene dienes and the bent Diels−Alder products are specifically bound by the ribozyme may be explained by structural rearrangement of the ribozyme. However, bending alone is not sufficient for being an inhibitor, as seen with compounds **42**−**44**. The stereoselectivity of the ribozymes can be explained by the model shown in Figure 3. The maleimide side chain, which contributes significantly to binding, was found to have no effect on stereoselectivity. Most likely, the maleimide is always bound in one fixed orientation with respect to the ribozyme. Stereochemically important is the substituent in the 9-position of anthracene, which can be removed without penalty to the reaction rate. While compound **1** gives only 16% ee, **2** is converted with over 95% ee. We assume that, due to the symmetric nature of anthracene and the observed lack of specific recognition of any substituents, there are two possible binding modes for the anthracene: head-first and tail-first (Figure 3). With a small substituent as in **1**, both modes are roughly equivalent, and both products are observed. With increasing steric demand (**2**, **19**, and **20**), the tail-first orientation becomes disfavored due to collision of the substituent with the backside of the binding pocket (dark gray area in Figure 3). Therefore, only the product of the head-first orientation is observed. In the case of branched substituents, there are also collisions of the substituent with the edges of the binding cleft when bound in the head-first orientation (black edge in Figure 3), which are responsible for the different rates observed in the conversion of enantiomers **19** and **20**.

So far, it is not clear by which mechanism the ribozyme achieves catalysis. One possibility is that the rates are accelerated by just increasing the local concentration of both reactants in a hydrophobic pocket.[23] On the other hand, it is well-known from Lewis-acid-catalyzed Diels−Alder reactions that the energy of the dienophile's LUMO can be lowered by withdrawal of electron density, which could in our system be easily achieved via the maleimide's carbonyl oxygens.[5c,24] This mechanism has indeed been observed in antibody-catalyzed Diels−Alder reactions.[25] Alternatively, one could imagine that the RNA destabilizes the diene by bending toward the transition state, thereby raising the energy of its HOMO.[26] Shape complementarity of the catalytic pocket to the transition state is another catalytic strategy that was found crucially important in antibody catalysis.[16,27] With the present data, it is not possible to judge the relative contributions of these possible strategies.

In summary, we could demonstrate that small unmodified RNA active sites can control chemical reaction pathways based on minute differences in substrate stereochemistry and substitution pattern. These capabilities may be of relevance both for synthetic applications and in the context of the "RNA world" scenario of the origin of life.

Experimental Section

The 49nt RNA (sequence: GGAGCUCGCUUCGGCGAGGCCGUGCCAGCUCUUCGGAGCAAUACUCGGC) was purchased from Dharmacon (Boulder, CO) or IBA-NAPS (Göttingen, Germany). The mirror-image 49nt L-ribozyme was kindly supplied by Noxxon Pharma AG, Berlin. Chemicals were purchased from Sigma Aldrich or Acros and used as received. Syntheses of compounds not previously reported in the literature are described in the Supporting Information. UV spectra were recorded on a Shimadzu UV-2101PC spectrometer. NMR spectra were recorded on Bruker A250 and AMX500 spectrometers.

UV-Spectrometric Determination of Rate Constants. Relative reaction rates were determined by UV/vis-spectroscopy for all compounds except where noted. The absorbance of the respective anthracene derivative was followed for at least 15 min at an appropriate wavelength, usually between 360 and 380 nm. The thermostat temperature was 24 °C. All reactions were conducted in 30 mM Tris-HCl buffer (pH 7.4), 80 mM $MgCl_2$, 10% ethanol, containing 7 μM 49nt ribozyme for the catalyzed reactions. In those cases where the background reactions produced no steady decay curves because of solubility problems, the salts were omitted, which usually resulted in a smooth curve. With use of dienes **1**, **2**, **6**, and **10**, and dienophiles **21** and **34**, rates of

(21) Guckian, K. M.; Schweitzer, B. A.; Ren, R. X. F.; Sheils, C. J.; Tahmassebi, D. C.; Kool, E. T. *J. Am. Chem. Soc.* **2000**, *122*, 2213.
(22) Nakash, M.; Clyde-Watson, Z.; Feeder, N.; Davies, J. E.; Teat, S. J.; Sanders, J. K. M. *J. Am. Chem. Soc.* **2000**, *122*, 5286.
(23) (a) Kang, J.; Rebek, J., Jr. *Nature* **1997**, *385*, 50. (b) Kang, J.; Santamaria, J.; Hilmersson, G.; Rebek, J., Jr. *J. Am. Chem. Soc.* **1998**, *120*, 7389.
(24) (a) Fukui, K. *Acc. Chem. Res.* **1971**, *4*, 57. (b) Woodward, R. B.; Hoffmann, R. *Angew. Chem., Int. Ed. Engl.* **1969**, *8*, 781.
(25) (a) Heine, A.; Stura, E. A.; Yli-Kauhaluoma, J. T.; Gao, C.; Deng, Q.; Beno, B. R.; Houk, K. N.; Janda, K. D.; Wilson, I. A. *Science* **1998**, *279*, 1934. (b) Romesberg, F. E.; Spiller, B.; Schultz, P. G.; Stevens, R. C. *Science* **1998**, *279*, 1929.
(26) Houk, K. N.; Li, Y.; Evanseck, J. D. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 682.
(27) Xu, J.; Deng, Q.; Chen, J.; Houk, K. N.; Bartek, J.; Hilvert, D.; Wilson, I. A. *Science* **1999**, *286*, 2345.

uncatalyzed product formation were also measured by HPLC (vide infra) in both the presence and the absence of salt, and no significant differences were found. Catalyzed reactions were followed in a microcuvette (Hellma, nominal volume 10 μL, 1 cm light path) on a 7 μL scale. Due to a better signal-to-noise ratio, uncatalyzed reactions were recorded in a 50 μL, 1 cm light path cuvette (Hellma). Concentrations of dienes were always 0.1 mM, and of maleimides always 0.5 mM. Therefore, all rate constants are apparent rate constants obtained under these conditions. Assays were performed by adding the organic substrates that were dissolved in ethanol successively to an aqueous solution containing the RNA and the buffer salts. Initial rates were obtained by monitoring the UV absorbance over the first 5% of conversion.

Determination of Rate Constants by HPLC. Reactions were performed on a 70 μL scale. Solutions contained 10% EtOH and were 0.1 mM in diene, 0.5 mM in dienophile, 7 μM in 49nt ribozyme, 80 mM in $MgCl_2$, and 30 mM in Tris-HCl, pH 7.4. For determination of background rate constants, the 49 nt ribozyme was omitted. The temperature was 24 °C. After 1, 2, 5, 15, 30, 60, and 120 min the complete content of the vial was mixed with 20 μL of quenching buffer that consisted of 0.1% mercaptoethanol in 0.1 M triethylammonium acetate pH 7.0 in 45% aqueous acetonitrile. The quenching buffer was shown to immediately deactivate the dienophile while leaving the Diels−Alder products unaffected. The vials were rinsed with 10 μL of ethanol, which was added to the quenched mixture. A 90 μL sample of the resulting 100 μL was injected onto a Beckman Ultrasphere C18 (compounds **3**, **19**, **20**) or Phenomenex Luna C18 column (**1**, **6**, **8**, **24**) eluting with ethanol/water (60/40) at 46 °C oven temperature and 0.6 mL/min (**19**, **20**), acetonitrile/water (40/60) at 1 mL/min (**3**), or 0.1 M triethylammonium acetate in appropriate acetonitrile/water mixtures at 1 mL/min (**1**, **6**, **8**, **24**). Peak areas were converted into molar amounts by comparison with a standard curve generated by injecting nine different amounts between 12.5 pmol and 1 nmol of the respective chemically synthesized Diels−Alder adduct.

Diels−Alderase Inhibition Byproduct Analogues. IC_{50} values were determined by adding the product analogues in acetonitrile solution to UV/vis-assays of diene **2** and dienophile **21** as described above. Conditions were identical with the exception of the organic modifier, which was 5% acetonitrile and 5% ethanol. Concentrations of the inhibitors were between 1 μM and 2 mM in those cases where the solubilities of the compounds permitted such high concentrations. Initial rate values were plotted half-logarithmically vs inhibitor concentration and the IC_{50} values were determined graphically.

Determination of de Values. 100 μM diene **19** and **20**, respectively, were reacted with 500 μM maleimidocaproic acid **34** in 30 mM Tris-HCl buffer pH 7.4, 30 mM $MgCl_2$, 10% ethanol, 7 μM D- or L-ribozyme for 1 h, injected onto a Beckmann Ultrasphere C18 column eluting with 100 mM triethylammonium acetate pH 7.0, 40% acetonitrile isocratic at 46 °C oven temperature and 1 mL/min with UV detection at 230 nm.

Acknowledgment. This work was supported by the Deutsche Forschungsgemeinschaft (Ja 794/3-1) and the Bundesministerium für Bildung und Forschung (BEO 0311861). We thank Noxxon Pharma AG for providing the L-ribozyme, Dr. B. Höhne (Technical University, Berlin) for HRMS measurements, and Drs. B. Seelig (Harvard Medical School) and D. J. Patel (MSKCC, New York) for comments on the manuscript.

Supporting Information Available: Synthesis and analytical characterization of the compounds listed in Tables 1−3 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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