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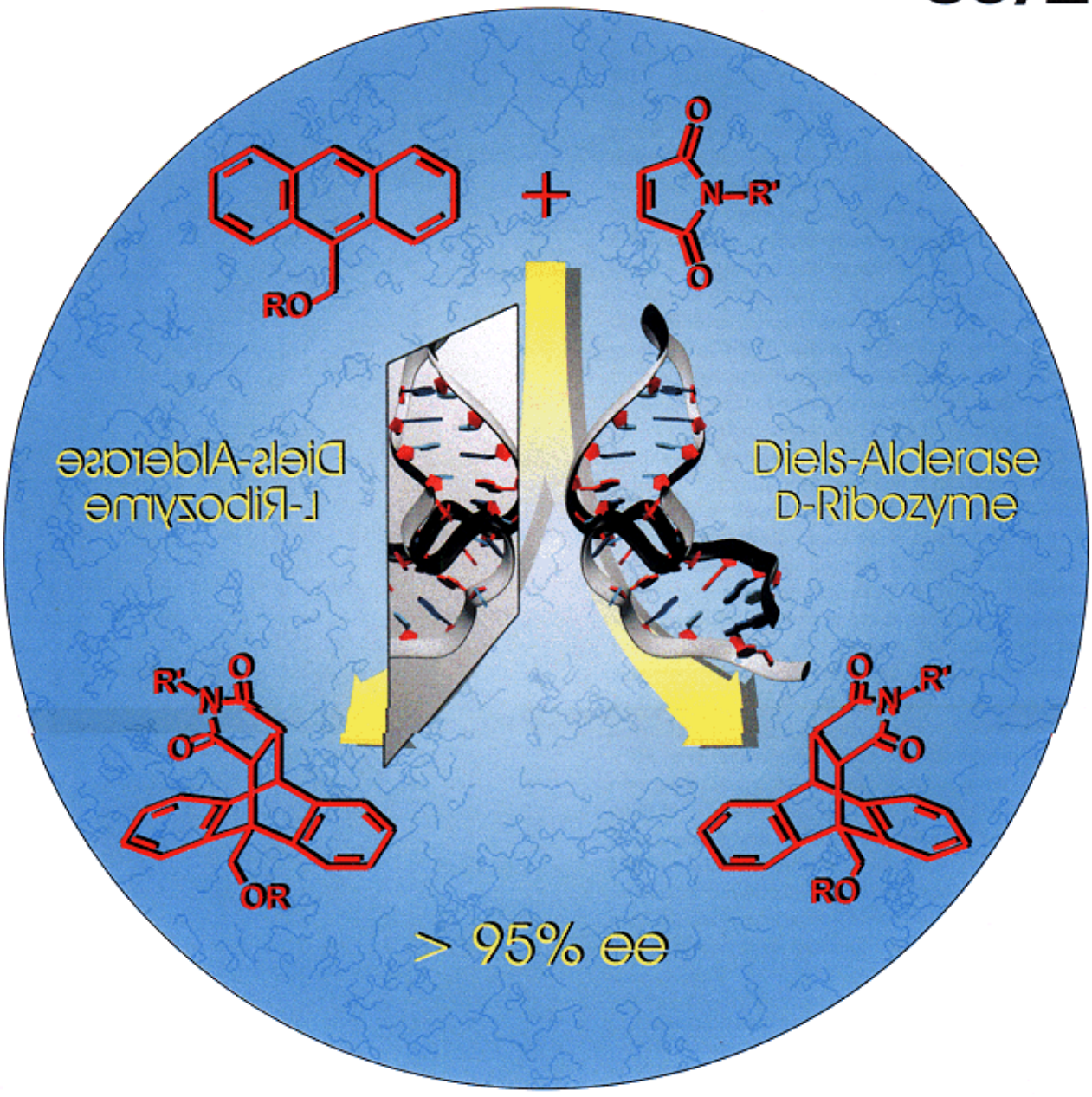

Index Issue

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Experimental Section

All reagents were analytical grade. Stock solutions of ascorbic acid (H_2Asc, 1.0×10^{-3} M) and copper(II) sulfate (4.0×10^{-6} M), each containing H_2SO_4 (1.2×10^{-4} M) and Na_2SO_4 (0.08 M), were prepared using doubly distilled water. Solutions containing ascorbic acid were prepared immediately before use.

All studies were conducted in a CSTR thermostatically controlled at (298.0 ± 0.1) K. Details of the experimental setup are presented elsewhere.[4–7] The state of the system was monitored by measuring the platinum-electrode potential; a Ag/AgCl electrode was used as the reference electrode. Signals were amplified electronically and recorded by a desk computer as a function of time with time steps of 0.1 s. As a consequence of the amplification of the signal the potential is given in arbitrary units.

CSTR experiments have been performed at different flow rates and noise intensities. Solutions of inflow reagents were pumped into the reactor by a precise two-channel piston pump. Syringe 1 contained H_2Asc (1.0×10^{-3} M), H_2SO_4 (1.2×10^{-4} M), and Na_2SO_4 (0.08 M); syringe 2 contained copper(II) sulfate (4.0×10^{-6} M), H_2SO_4 (1.2×10^{-4} M), and Na_2SO_4 (0.08 M). Correspondingly, the reactor concentrations after mixing but before any reaction were: $[H_2Asc]_0 = 5.0 \times 10^{-4}$ M; $[Cu^{2+}]_0 = 2.0 \times 10^{-6}$ M; $[H_2SO_4]_0 = 1.2 \times 10^{-4}$ M; $[Na_2SO_4]_0 = 0.08$ M.

Perturbation of the reaction has been performed according to Equation (1) by using a 16 bit D/A (digital/analogue) converter.

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Enantioselective Ribozyme Catalysis of a Bimolecular Cycloaddition Reaction**

Burckhard Seelig, Sonja Keiper, Friedrich Stuhlmann, and Andres Jäschke*

There is currently a wide-spread interest in the development of new catalytic methods for regio- and stereoselective syntheses. Various approaches aim at the utilization and improvement of synthetic compounds, surfaces, and biomolecules as stereoselective catalysts or synthetic auxiliaries.[1–4] Combinatorial strategies have developed into powerful tools in catalysis research, allowing the isolation of potential catalysts from vast libraries by either screening or iterative deconvolution.[5, 6]

Ribozymes are RNA molecules with catalytic properties. While the chemistry of the naturally occurring ribozymes is restricted to cleavage and joining reactions at internucleotide bonds, artificial ribozymes isolated from synthetic combinatorial libraries have been shown to accelerate the chemical steps in a broad range of reactions, including carbon–carbon and peptide bond formation.[7–10] These ribozymes, however, behave unlike typical chemical catalysts or protein enzymes in that they require at least one of the reactants to be RNA or to be covalently tethered to RNA. Except for the formation of dinucleotides from activated mononucleotides,[11] there is no example of ribozymes accelerating bond-forming reactions between two small organic substrates, a central reaction type in organic synthesis and the basis of all anabolic biochemical pathways. Also, although RNA molecules can exquisitely distinguish between enantiomers when binding target molecules,[12, 13] enantioselective bond formation by ribozymes has not yet been described.

The Diels–Alder reaction is one of the most important carbon–carbon bond-forming processes available to organic chemists. The reaction creates two carbon–carbon bonds and up to four new stereocenters. There is currently much interest in developing catalytic methods for improving its rate and selectivity because, for many applications, the synthetic value of the Diels–Alder reaction relies on the degree of stereocontrol that can be exercised.[14–16]

We have recently described the isolation of Diels-Alderase ribozymes from a combinatorial RNA library that accelerate up to 20000-fold the carbon–carbon bond formation between anthracene, which is covalently tethered to the ribozyme, and a biotinylated maleimide (Scheme 1A).[17] 90% of the active sequences contained a small common structural motif (Scheme 1B), and a synthetic 49-mer oligoribonucleotide containing all elements of this motif was shown to be catalytically

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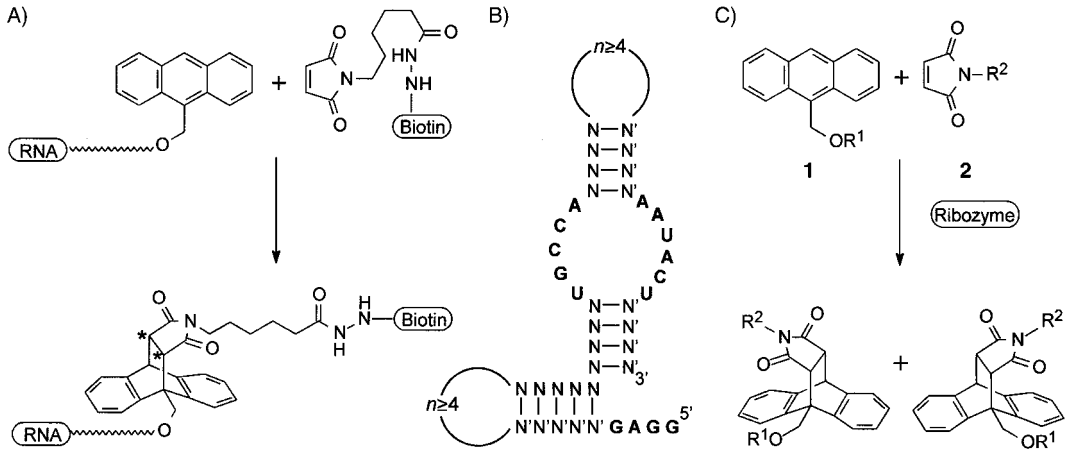


Scheme 1. RNA-catalyzed Diels–Alder reactions. A) Previously selected ribozymes accelerate the reaction between a tethered anthracene and biotin maleimide.[17] B) Minimum motif shared by most of the selected Diels-Alderase ribozymes. For the experiments shown in (A), anthracene was tethered through the 5′ terminus of the ribozyme, while the experiments in (C) were done without tethering. N = any nucleotide, N′ = nucleotide complementary to N. C) True catalysis by ribozymes in the bimolecular Diels–Alder reaction of **1** and **2**. R^1 is either H (**1a**) or $(C_2H_4O)_6$-H (**1b**); for R^2, see Table 1 and Figure 2.

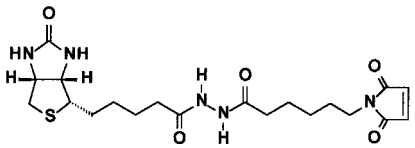
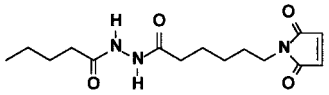
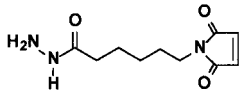
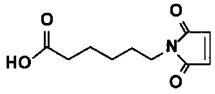
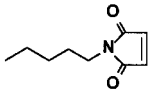
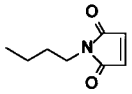
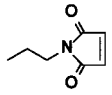
active towards a covalently tethered anthracene. We now report the action of Diels-Alderase ribozymes as true catalysts for a reaction involving two small, organic substrate molecules in solution (Scheme 1C).

The reaction of different anthracene derivatives with biotin maleimide was studied by UV spectroscopy in the absence and presence of the 49-mer ribozyme in aqueous buffer. Covalent tethering of anthracene to the RNA was found not to be required. 9-(Hydroxymethyl)anthracene (**1a**, $R^1 = H$) is the minimum diene which is efficiently converted into the respective Diels–Alder product (Scheme 1C). Analysis of a series of fragments and analogs of biotin maleimide revealed that the biotinyl residue can be removed with little loss of activity (Table 1). The maleimidyl group, in combination with an alkyl chain, appears to be the structural feature required for recognition by the minimum 49-mer ribozyme. Maleimides as small as *N*-ethylmaleimide were accepted as substrates. The highest rate acceleration was measured for *N*-pentylmaleimide, which reacted significantly faster than biotin maleimide. Thus, the 49-mer ribozyme accelerates a bond-forming reaction between two small organic molecules.

The kinetics of the ribozyme-catalyzed reactions were determined by measuring the differences in the initial rates between the catalyzed and the background reactions. For solubility reasons, anthracene hexaethylene glycol **1** ($R^1 = (C_2H_4O)_6$-H) and maleimidocaproic acid (**2**, $R^2 = (CH_2)_5COOH$) were used for these measurements. Initial velocities were determined by monitoring the decrease of the anthracene absorbance at 365 nm over approximately 5% conversion. Catalysis was examined as a completely random bireactant system.

The initial rate of the ribozyme-catalyzed reaction was found to be proportional to the ribozyme concentration. The reaction shows saturation-type kinetics with respect to both substrates (Figure 1A). The secondary double-reciprocal plot shown in Figure 1B gives Michaelis constants of 370 μM for the diene and 8 mM for the dienophile. The calculated maximum rate V_{max} is 150 μM min^{-1}, which corresponds to a

Table 1. Relative rate constants of ribozyme-catalyzed Diels–Alder reactions of **1a** with different maleimides.[a]

Compound	k_{rel}
	1.0
	0.7
	0.7
	0.8
	1.6
	0.7
	0.1
	< 0.1
	0

[a] Conditions: 100 μM **1a**, 500 μM maleimide, and 7 μM 49-mer ribozyme in buffer (composition: 300 mM NaCl, 80 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.4), 10% ethanol). The reaction mixture was transferred into a 7 μL cuvette with a 1 cm light path and the anthracene absorbance at 365 nm was recorded. For the sequence of the 49-mer RNA, see Figure 2.[24] Tris = tris(hydroxymethyl)aminomethane.

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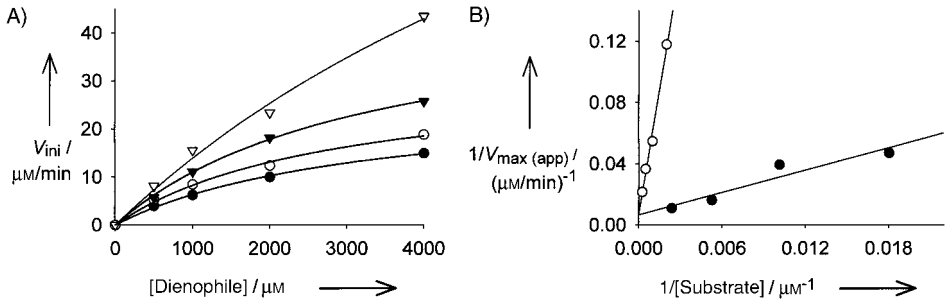

Figure 1. Kinetic characterization of the ribozyme-catalyzed reaction of **1b** with maleimidocaproic acid (**2**, $R^2 = (CH_2)_5COOH$). A) Dependence of the reaction rate on the dienophile concentration at fixed diene concentrations of ● 55, ○ 99, ▼ 190, and ▽ 415 μM. All conditions were as in Table 1, except for the concentrations of ethanol (0%) and DMSO (2%). Initial rates (V_{ini}) were obtained by monitoring the UV absorbance over the first 5% of conversion and calculating the least-square fits of the progress curves. For each diene concentration (at fixed dienophile concentrations), the apparent maximum rate $V_{max(app)}$ was obtained as the intercept with the y axis of the Lineweaver–Burk plot ($1/V_{ini}$ against 1/[dienophile]). B) Plot of the apparent maximum rate values against fixed substrate concentrations (● diene, ○ dienophile) to obtain the true V_{max} value as 1/(the y intercept) and the Michaelis constant (K_m) values as -1/(the x intercept).[25]

k_{cat} of 21 min^{-1} ([ribozyme] = 7 μM). The highest initial rate that was directly measured (at subsaturation concentrations of 415 μM diene and 4 mM dienophile) was 43 μM min^{-1} (Figure 1 A), which corresponds to a catalytic turnover of about six transformations per catalyst molecule per minute. The second-order rate constant for the background reaction, k_{uncat}, was found to be 3.2 M^{-1} min^{-1}. A measure of the entropic gain of this ribozyme-catalyzed reaction can be estimated by the ratio k_{cat}/k_{uncat}. Here, the "effective molarity" was determined to be 6.6 M, and $(k_{cat}/K_{m,dienophile})/k_{uncat}$ gives the lower estimate for the rate acceleration as 1100-fold.[15]

Thus, the ribozyme acts as a true enzyme with fast multiple turnovers. In agreement with earlier results,[17] a reverse reaction cannot be detected, which indicates an irreversible chemical reaction step. Addition of Diels-Alder product, however, inhibits the forward reaction; this suggests that the product competes for the same binding sites as the substrates. The mode of inhibition is currently under investigation.

In the course of the reaction, two product enantiomers can be generated (Scheme 1 C). RNA is a homochiral polymer and should therefore not only accelerate the reaction but also influence the enantiomeric distribution. To investigate this possibility, the reaction products ($R^1 = (C_2H_4O)_6$-H, $R^2 = (CH_2)_5COOCH_3$) were analyzed with HPLC on a chiral stationary phase (Figure 2). The peaks at 94 min and 102 min represent the two product enantiomers, as confirmed using reference compounds synthesized according to standard procedures. The uncatalyzed reaction yields, as expected, a racemic mixture, while the reaction catalyzed by the 49-mer minimum ribozyme produced the two compounds in a 20:1 ratio according to peak areas, which corresponds to an enantiomeric excess (*ee*) value of 90%. After correction of the peak areas for the uncatalyzed background reaction, an *ee* value of over 95% is obtained. Both compounds have the same molecular weight (by ESI-MS) as that calculated and produce the same UV spectra. The CD spectra, however, are inverted, as is always the case for enantiomers.

If a homochiral catalyst induces enantioselective bond formation, then, by the laws of stereochemistry, the other enantiomer of the catalyst molecule should show exactly the opposite enantioselectivity. While similar experiments with RNA aptamers showed reciprocal binding specificities of the corresponding D- and L-nucleic acids,[18, 19] there is no example of a catalytically active L-nucleic acid to date. In agreement with the assumption, a synthetic 49-mer composed of L-nucleotides, a mirror-image ribozyme, not only showed similar rate acceleration but also the opposite product ratio (1:20) compared to the natural 49-mer D-ribozyme (Figure 2).

The substituent in the 9 position of the diene **1** is an important determinant of the enantioselectivity of the ribozyme-catalyzed reaction. If R^1 is shortened to C_2H_4OH, the enantioselectivity is reduced to 33% *ee*, and the products of the reaction with 9-(hydroxymethyl)anthracene **1a** are obtained with only 16% *ee*. Variations of the substituent at the R^2 position on the maleimide, however, cause less significant changes in enantioselectivity.

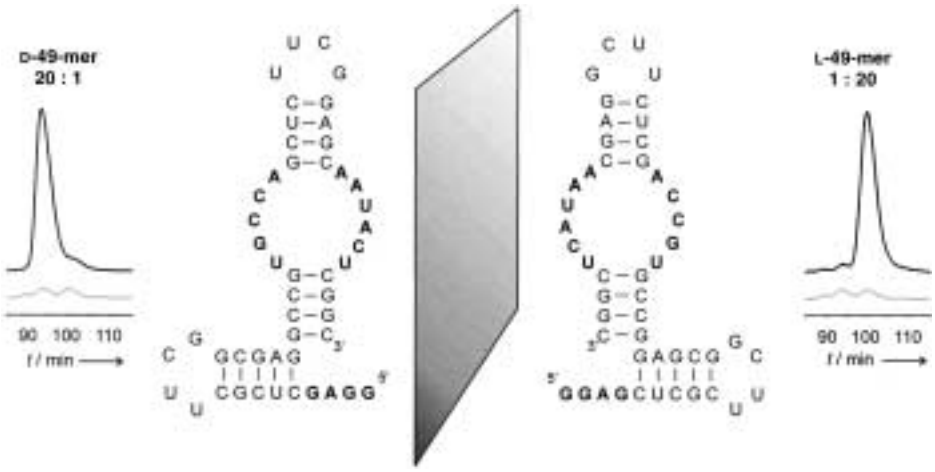

Figure 2. HPLC analysis of Diels–Alder reactions catalyzed by 49-mer D-RNA and the enantiomeric 49-mer L-ribozyme. 1 mM **1b**, 1.5 mM **2** ($R^2 = (CH_2)_5COOCH_3$), and 80 μM ribozyme were mixed for 6 minutes under the conditions given in Table 1. Samples were analyzed on a chiral NEA (R) column (YMC Europe, 250 × 4.6 mm) with water:ethanol (65:35) as the eluent, isocratic at 0.8 mL min^{-1}, with UV detection at 210 nm. The light gray curve in the chromatograms corresponds to the uncatalyzed background reaction that occurs in the absence of ribozyme.

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There are currently no data indicating by which mechanism the Diels–Alder reaction is catalyzed by the ribozymes. It is generally agreed that in a Diels–Alder reaction, bond formation occurs by concerted mixing of the diene and dienophile π-orbitals, specifically of the highest occupied molecular orbital (HOMO) of the diene and the lowest unoccupied molecular orbital (LUMO) of the dienophile.[20] The majority of small-molecule (Lewis acid) catalysts operate by coordination to (and withdrawal of electron density from) the dienophile, thereby lowering the energy of its LUMO.[14, 16] For antibody catalysis of a Diels–Alder reaction, both binding of the reactants in a reactive conformation and lowering of the dienophile's LUMO through hydrogen bonding to the dienophile carbonyl oxygen have been discussed.[21, 22] It is conceivable that similar mechanisms are involved in ribozyme catalysis as well. Recent mutation experiments have identified conserved nucleotides in the formally single-stranded regions of the ribozyme (see Scheme 1B); this indicates participation in the formation of the catalytic site. Experiments on the chemical basis of catalysis and on the origin of stereoselectivity using a variety of diene, dienophile, and product analogues are under way.

The results demonstrate that ribozymes can act as catalysts in C–C bond forming reactions. They can recognize two small organic molecules, bring them to reaction, dissociate the product(s), and repeat this cycle several times. Another characteristic of natural enzymes has also been demonstrated for the first time: enantioselective bond formation.

Thus, selective catalysts for synthetically relevant reactions can be generated by in vitro selection from combinatorial nucleic acid libraries. Compared to classical chemical catalysts, they offer the advantages that their development does not require prior knowledge of structural prerequisites and reaction mechanisms or laborious trial and error synthesis. The iterative procedure of in vitro selection, in which active compounds are first enriched and then deconvoluted,[17] allows the handling of up to 10^{16} different compounds in a single test tube, an order that is far beyond the capacities of high-throughput screening. Selective pressure allows the properties of the catalyst to be fine tuned.

The rate accelerations and enantioselectivities of over 95% *ee* described here are similar to those of catalytic antibodies for cycloaddition reactions. Compared to these antibodies, the ribozymes described here are much smaller (molecular weight of 16000 Da) and, most importantly, are amenable to preparative chemical or enzymatic synthesis at reasonable costs. Therefore, we envision the use of in vitro selected oligonucleotides as tailor-made catalysts in organic synthesis.[23]

Enantioselective bond formation is of broad interest beyond synthetic chemistry. Most reactions in biochemistry display a high level of stereocontrol, and the results shown here demonstrate that the level of stereocontrol exercised by a ribozyme can be similar to protein enzymes. This indicates how in a hypothetical "RNA world" ribozymes might have controlled the stereochemistry of chemical reactions.

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[23] A. Jäschke, C. Frauendorf, F. Hausch, *Synlett* **1999**, 825.

[24] For biotin maleimide, 2% of DMSO was added for solubility reasons.
[25] The fitted equations for the secondary plots are $y = 2.4309x + 0.0066$ ($R^2 = 0.903$) for the diene and $y = 54.539x + 0.0066$ ($R^2 = 0.990$) for the dienophile. Despite fairly good regression coefficients, the standard deviations of the intercept with the y axis were in the same range as the mean values (± 0.0060 and ± 0.0045, respectively), rendering the V_{max} values (which are 1/(the y intercept)) and the K_m values ($-$slope/(the y-intercept)) potentially erroneous. To support the obtained kinetic constants, we additionally carried out direct nonlinear least-square regression of the data shown in Figure 1A according to a completely random bi–uni mechanism and obtained a V_{max} value of 140 ± 25 μM min^{-1}, a $K_{m,diene}$ value of 200 ± 35 μM, and a $K_{m,dienophile}$ value of 5.2 ± 1.3 mM with a correlation coefficient of $R^2 = 0.980$.

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