two catalysts can be easily enlarged because a great variety of rylene dyes covering the whole visible spectra is available.[9] Our rapid testing and comparison of supported metallocene catalysts under identical experimental conditions is suitable for other heterogeneous polymerization processes. In order to create larger libraries of heterogeneous catalysts, on-line methods for the detection and characterization of the particles will have to be developed and applied.

Experimental Section

Procedure for supporting and labeling zirconocenes: All experiments were performed under argon by using standard Schlenk techniques. Catalyst **B**: SiO$_2$/MAO (2.50 g, 25 % MAO on silica, TA 02794/HL, Witco GmbH, Bergkamen, Germany) was suspended in toluene (100 mL) followed by addition of **4** (7 mg) in toluene (50 mL) at room temperature and stirred for 2 h. Subsequently, a solution of dye **1** (60 mg) in toluene (50 mL) was added dropwise and the mixture was stirred for 1 h. The almost colorless solution was removed by filtration and the resulting silica/MAO/zirconocene/dye catalyst system was washed twice with toluene and dried in vacuum. Catalysts **C – E** were prepared in a similar way.

Polymerization: General procedure: Two catalysts were mixed and used simultaneously in a single vessel for the polymerization of ethylene. Isobutane (400 mL) and triisobutylaluminum (TIBA)(5 mL; 10 % in n-hexane) were introduced into a Büchi stainless steel reactor (1 L) equipped with a magnetic stirrer and saturated with ethylene (standard conditions: 40 bar, 70 °C). The mixture of catalysts was introduced into the reactor using a pressure gate. After 90 min the polymerization was stopped by releasing the pressure.[10]

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[10] All polymerization experiments presented herein were repeated at least two times to prove the reproducibility.

An Allosteric Ribozyme Regulated by Doxycyline**

Nicolas Piganeau, Andreas Jenne, Vincent Thuillier,* and Michael Famulok*

Specific ligand-binding nucleic acid molecules (aptamers) can be isolated by searching the "shape-space" of vast combinatorial nucleic acid libraries for functional sequences in vitro.[1–3] Selection strategies for the isolation of aptamers usually involve incubation of a complex library of nucleic acids with the immobilized target molecule or the immobilization of a preformed target/nucleic acid complex.

A considerable number of RNA aptamers that bind a variety of targets with high affinity and specificity have been isolated and extensively characterized. Detailed structural investigations of many aptamer/ligand complexes have established that complex formation in most cases is accompanied by extensive conformational changes only in the presence of the cognate ligand by adaptive binding events.[4–11] This property of small-molecule-binding aptamers allowed the rational design of artificial allosteric ribozymes inhibited or activated by a ligand that is bound to an aptamer which has been positioned in immediate proximity to a ribozyme sequence.[12–15]

We have applied a novel in vitro selection strategy based on allosteric inhibition of a hammerhead ribozyme fused to a

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randomized RNA library by low concentrations of the antibiotic doxycycline **1**. After 16 rounds of selection, ribozymes were isolated with inhibition constants as low as 20nM and a remarkable specificity for **1**. Doxycycline **1** was

Doxycycline **1**

Tetracycline **2**
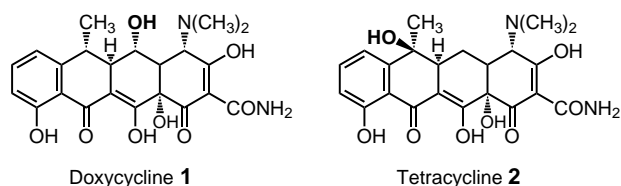
chosen as the "switch molecule" because it represents a cell-permeable small molecule with low toxicity for higher eucaryotes. Thereby, allosteric ribozymes that respond to **1** may potentially be used for the development of conditional gene expression systems based on the cleavage of mRNAs by the inserted hammerhead ribozyme depending on whether or not **1** is present in the cell.

The design of the randomized library is shown in Figure 1 a. Previously, allosteric hammerhead ribozymes have been rationally designed by fusing an aptamer RNA onto helix II.[16, 17] Because helix II is important for the activity of the hammerhead ribozyme,[18] we have chosen this site for positioning the randomized sequence. To obtain a library of active ribozymes the first two base pairs of this helix were maintained. The course of the selection is shown in Figure 1 c. After five cycles of selection an enrichment of the pool for doxycycline-inhibited ribozymes was detected. However, a control selection cycle in the absence of **1** revealed that the selected pool was only poorly responding to the ligand; in the presence of doxycycline **1**, 14 % of the RNA was eluted in selection step 4 (Figure 1 b), whereas 10 % of the RNA was eluted in the negative control without **1**.

Therefore, we hypothesized that parasital ribozymes were coselected in the course of the selection. These fold into different conformations, some of which are cleavage-competent and others are inactive. During the first incubation (step 2, Figure 1 b), these ribozymes would only partly be eluted because a fraction of them cannot be cleaved due to misfolding. During denaturation/renaturation between steps 2 and 3 (Figure 1 b) some of these ribozymes would refold, again only partly in an active conformation, which would unintentionally be eluted together with the doxycycline-inhibited species after the second incubation (step 3, Figure 1 b). As a consequence, doxycycline-independent ("parasital") ribozymes would be coselected. To eliminate these species, we modified the selection procedure from cycle 6 onwards by interrupting the first incubation (step 2, Figure 1 b) by repetitive washing steps under denaturation/renaturation conditions. Indeed, after cycle 7 the enriched pool showed much lower parasital activity (10 % elution in step 4 in presence of **1**, 0.5 % without **1**). After this cycle the selection pressure was gradually increased by reducing the ligand concentration and the incubation time in step 2 (Figure 1 b,c). After cycles 10 and 13, the complexity of the enriched pool was increased by mutagenic PCR.[19, 20] In parallel, the selection stringency was also increased to
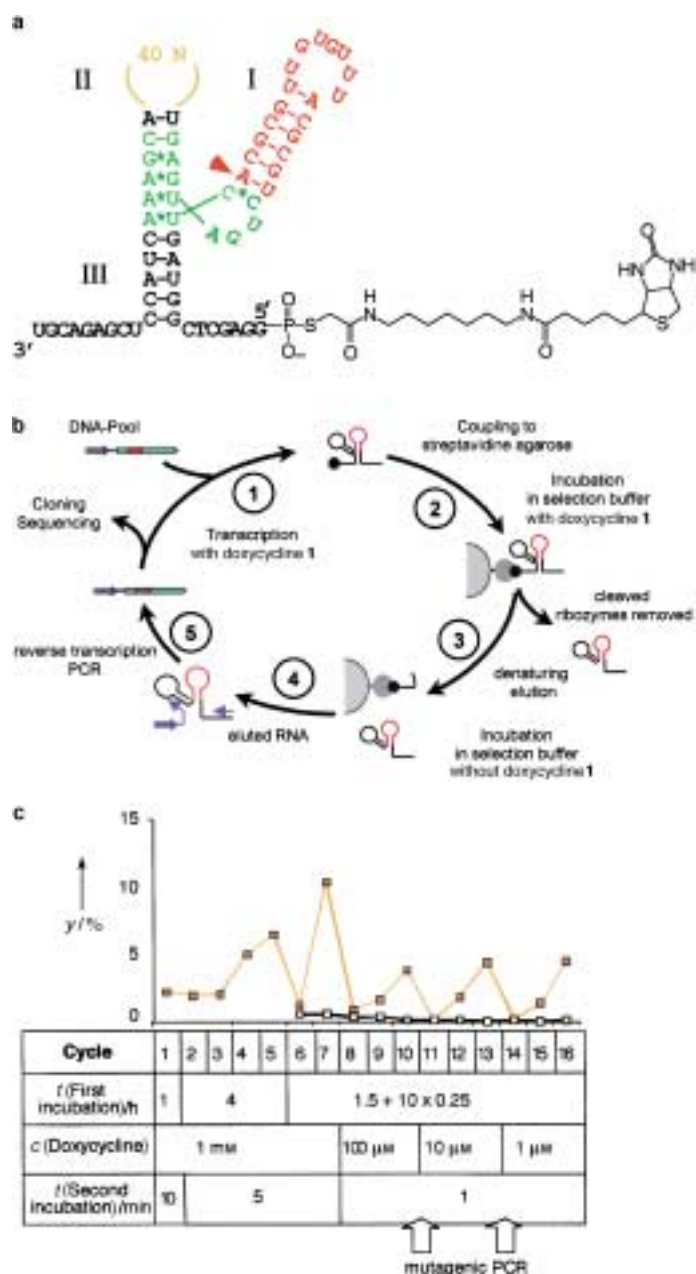

Figure 1. Selection of allosteric hammerhead ribozymes that are inhibited by doxycycline **1**. a) Secondary structure of the transcripts from the initial pool. Helix II was shortened to two bases pairs, and the loop II was replaced with a 40 nucleotide random region. Green: nucleotides of the catalytic center of the hammerhead ribozyme (HHR). Red arrow: cleavage site. b) Schematic representation of the selection procedure. For details see Experimental Section. Step 1: Transcription of the DNA library by using T7 RNA polymerase in the presence of guanosine monophosphothioate (GMPS) and 1.0 mM **1** (red: radomized region; blue arrow: T7 promoter); separation of uncleaved from cleaved RNA transcripts by denaturing polyacylamide gel electrophoresis and chemical biotinylation of the 5′-sulfanyl group (black filled circle). Step 2: Coupling to streptavidine agarose and incubation with **1**. Step 3: Washing under denaturing conditions to remove **1** and cleaved ribozyme products; incubation of the remaining immobilized RNA without **1** in the selection buffer. Step 4: Elution of the cleaved ribozymes. Step 5: Reverse transcription and PCR amplification; blue arrows: primer 3 and 4. c) Percentages of eluted radioactivity y after the second incubation step. Yellow: in the presence of **1**; black: control experiment. The table below the graph relates each selection cycle to the respective selection conditions. Arrows indicate selection cycles amplified under mutagenic PCR conditions. For details see text.

promote the enrichment of allosteric ribozymes with improved values of K_i and catalytic rate.

The activity of the selected pools from cycles 10, 13, and 16 was monitored by time–course experiments with different concentrations of the inhibitor. The sensitivity of the pool ribozymes for doxycycline **1** increased continously (Figure 2); from cycle 16 the pool was completely inhibited in the presence of 1 μM **1**. The selected pools were therefore cloned and sequenced after cycles 10, 13, and 16 (Figure 2b). We obtained eight different sequence classes from which one representative member from each was chosen for further analysis. Kinetic analyses were performed in the presence or absence of 1 μM **1** (Figure 2b). The values of k_{obs} and the factor of inhibition with respect to the unselected pool are also shown in Figure 2b. At this concentration, no allosteric inhibitory effect was found for a sequence member from cycle 10 which is not surprising as this cycle was performed at 100 μM of **1**. Clones selected under higher stringency exhibited clear allosteric effects with differences in the k_{obs} values ranging from threefold to nearly 50-fold. Interestingly, selected ribozymes showed a decreased catalytic activity in the absence of the regulatory drug. Their mean k_{obs} values are around 0.3 min^{-1} which is roughly 10-fold slower than a corresponding "wild-type" hammerhead ribozyme that does not contain additional sequences fused to helix II and for which a value of k_{obs} around 3.0 min^{-1} was determined (data not shown).

The four clones showing the best inhibition values were chosen for a determination of their inhibition constants (K_i)

(Table 1). The clones exhibited impressive sensitivity for **1** with values of K_i ranging from 20 to 70 nM. These values belong to the highest affinities found for RNA/small molecule interactions until now.[21] To investigate the specificity of these

Table 1. K_i values for **1** and **2** as well as rate constants of the noninhibited reaction, and maximal levels of inhibition.

Clone	k_{obs} (uninhibited) [min^{-1}]	K_i(**1**) [nM]	Maximal inhibition factor	K_i(**2**) [nM]
1D13-01	0.13 ± 0.02	70 ± 20	11	$> 1\,000\,000$
1D16-05	0.40 ± 0.02	20 ± 5	49	100 ± 20
1D16-06	0.13 ± 0.02	25 ± 3	12	100 ± 20
1D16-13	0.09 ± 0.01	50 ± 30	12	$> 1\,000\,000$

clones we determined the K_i value in the presence of tetracycline **2**, which differs from **1** only in the substitution pattern of a single OH group (Table 1). At 1.0 mM **2**, no allosteric effect was observed for clones D13-01 and D16-13, corresponding to at least a 10 000-fold discrimination between **1** and **2** (Table 1). The two other clones tested showed moderate selectivity (four- to fivefold). These data suggest that clones D13-01 and D16-13 might form a different binding pocket for **1** than clones D16-05 and D16-06.

The most active clone D16-05 was chosen to determine a minimal motif for doxycycline binding. For that, we synthesized 16 truncated versions of a cleavage-inactive mutant of this clone and carried out cleavage reactions in the presence of a tenfold molar excess of these constructs with respect to the inhibitor **1**. If truncated constructs act as aptamers they would be able to compete with the allosteric ribozyme for binding to the inhibitor, thereby restoring ribozyme activity. Truncated constructs without aptameric activity cannot act as decoys for **1**. Therefore, in this case, the allosteric ribozyme D16-05 remains inhibited. The result of this experiment is shown in Figure 3a. According to these data, the randomized region alone is not capable of binding to **1**. For an effective competition the presence of helix I of the original hammerhead motif is required in addition to the randomized region. Experiments 9–11 in Figure 3a show that not only the sequence of the loop region in this helix but also that of the paired region appear to be important. Thus, the sequence shown in Figure 3b is likely to represent the minimal motif for the aptameric binding of **1**. According to the algorithm of Turner and Zuker[22] it folds into the secondary structure shown.

The requirement of helix I for inhibition suggests that this helix is an integral part of the doxycycline-

a

b

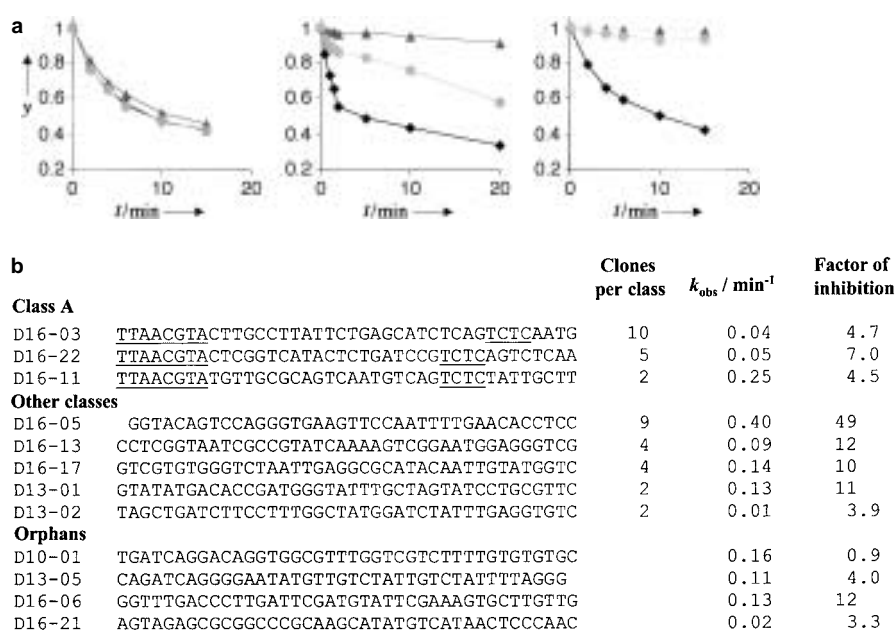|		Clones per class	k_{obs} / min^{-1}	Factor of inhibition
Class A				
D16-03	<u>TTAACGTA</u>CTTGCCTTATTCTGAGCATCTCAGT<u>CTC</u>AATG	10	0.04	4.7
D16-22	<u>TTAACGTA</u>CTCGGTCATACTCTGATCCGT<u>CTC</u>AGTCTCAA	5	0.05	7.0
D16-11	<u>TTAACGTA</u>TGTTGCGCAGTCAATGTCAGT<u>CTC</u>TATTGCTT	2	0.25	4.5
Other classes				
D16-05	GGTACAGTCCAGGGTGAAGTTCCAATTTTGAACACCTCC	9	0.40	49
D16-13	CCTCGGTAATCGCCGTATCAAAAGTCGGAATGGAGGGTCG	4	0.09	12
D16-17	GTCGTGTGGGTCTAATTGAGGCGCATACAATTGTATGGTC	4	0.14	10
D13-01	GTATATGACACCGATGGGTATTTGCTAGTATCCTGCGTTC	2	0.13	11
D13-02	TAGCTGATCTTCCTTTGGCTATGGATCTATTTGAGGTGTC	2	0.01	3.9
Orphans				
D10-01	TGATCAGGACAGGTGGCGTTTGGTCGTCTTTTGTGTGTGC		0.16	0.9
D13-05	CAGATCAGGGGAATATGTTGTCTATTGTCTATTTTAGGG		0.11	4.0
D16-06	GGTTTGACCCTTGATTCGATGTATTCGAAAGTGCTTGTTG		0.13	12
D16-21	AGTAGAGCGCGGCCCGCAAGCATATGTCATAACTCCCAAC		0.02	3.3

Figure 2. Allosteric inhibition of pools and individual selected clones. a) With the number of selection cycles (10, 13, 16, from left to right), the sensitivity of the library for **1** increases. Cleavage occurred in the selection buffer (SB; 40 mM Tris-HCl, pH 8, 50 mM NaCl, 10 mM spermidine, 8 mM MgCl₂) at 37 °C with or without **1** [c(**1**) = 0 (♦), 1 (●), 10 μM (△)]. Cleavage reactions were initiated by addition of Mg²⁺. b) Sequences of the clones analyzed after the selection. The label (D10, D13, or D16) refers to the selection cycle from which the clone stems. Some clones could be grouped into classes based on sequence homologies. Family A represents a class that contains a short common motif (underlined). The k_{obs} values in the first 3 min of the reaction were used to determine the ratio of the k_{obs} values of the inhibited and the uninhibited reaction, designated as "factor of inhibition".
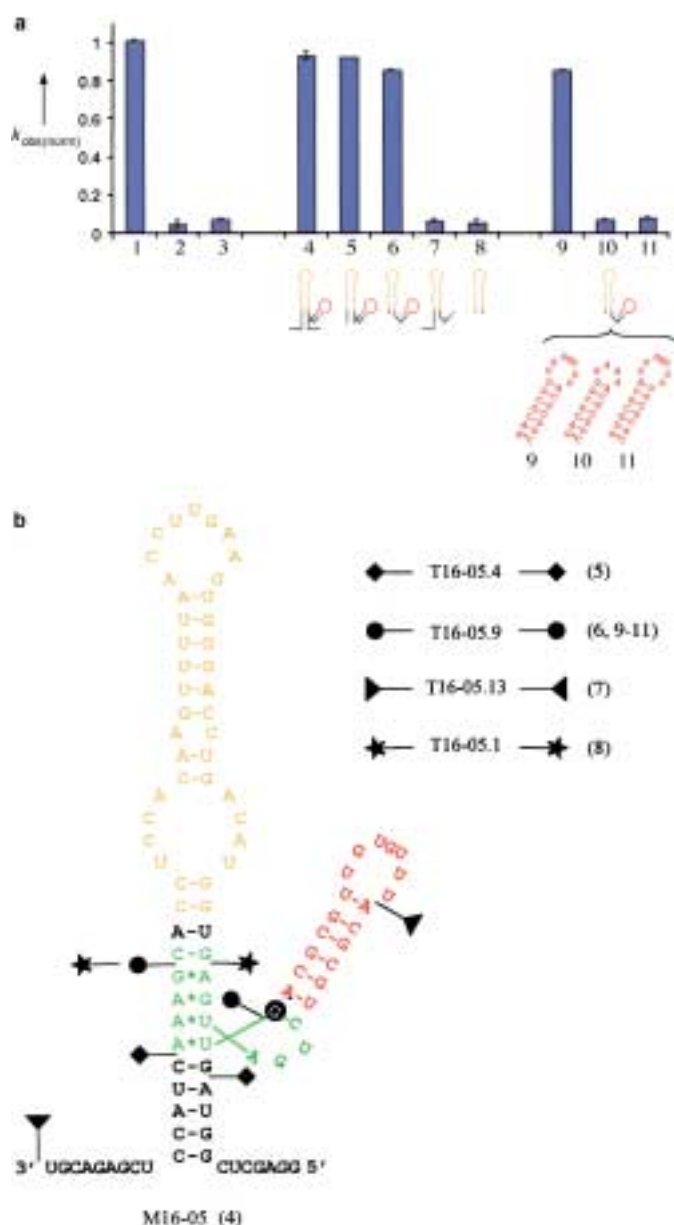
Figure 3. Determination of the minimal sequence of clone D16–05. a) Relative activity $k_{obs(norm)}$ of the cleavage reaction under various conditions (always in the presence of 1 μM tRNA as an unspecific competitor). 1: Cleavage activity of D16–05 (1 nM). 2: Same in the presence of 200 nM **1**. 3: Same as in 2 in the presence of 2.0 μM unselected pool as an unspecific competitor. 4–8: Same as in 2 in the presence of 2.0 μM cleavage inactive point mutant M16–05 (4), construct T16–05.4 (5), construct T16–05.9 (6), construct T16–05.13 (7), construct T16–05.1 (8). 9–11: Same as in 2 in the presence of 2.0 μM T16–05.9 with the depicted variations in helix I. b) Computer-generated secondary structure of the point mutant D16–05. The various marker symbols flank the sequences of the truncated versions tested. To enable T7-transcription two guanosine residues, which are not shown here, were inserted into the 5′-position of the truncated constructs. Orange: The original randomized region; red: constant region from the HHR (helix I); green: catalytic core. The guanosine residue shaded in black shows the point-mutation C→G. The numbers in parentheses refer to the numbering in (a).

binding site. If binding-site formation is accompanied by a conformational change of the ribozyme tertiary structure, this would explain the inhibition induced by **1**. Another possible explanation for the requirement of helix I is that the antibiotic

is locking the structure of the ribozyme in a defined conformation thereby reducing the entropic advantage of product formation on the ribozyme which in turn shifts the equilibrium of the reaction towards ligation.[23]

In conclusion, we have applied an in vitro selection scheme based on allosteric regulation, which led to ribozymes that respond to strikingly low concentrations of the target molecule. Some allosteric ribozymes exhibit unexpected specificity for the regulator molecule. Previously, Breaker et al. have applied an analogous selection principle in which allosteric ribozymes that respond to cAMP and cGMP as *effector* molecules were obtained de novo.[24] While being inactive in the absence, ribozyme cleavage only took place in the presence of cAMP or cGMP at 100 μM concentrations. Thereby, a more than 1000-fold allosteric activation of hammerhead cleavage activity was observed. In the present study, selection for allosteric inhibition led to 10–50-fold responses, at nanomolar concentrations of a nontoxic, cell-permeable molecule of low molecular weight. When inserted into the mRNA of a certain target gene, allosteric molecular switches of this type may serve as a valuable tool for the development of tailored conditional gene expression systems that can be controlled by the presence or absence of any kind of small molecule.

Experimental Section

Random pool synthesis: DNA oligonucleotides were synthesized on an Expedite nucleic acid synthesizer and purified on denaturing polyacryl-amide (PA) gels. The pool-primer (5′-CGC GTT GTG TTT ACG CTC CT-G ATG AGT-N$_{40}$-ACG AAA CTA CCT CGA GAC GT) was initially amplified by using primers 1 (5′-CGC GTT GTG TTT ACG CGT CTG ATG) and 4 (5′-ACG TCT CGA GGT AGT TTC GT).[25] Further amplification was performed under the same conditions by using primers 3 (5′-AGC TGG TAC CTA ATA CGA CTC ACT ATA GGA GCT CGG TAG T-CA CGC GTT GTG TTT ACG CGT CTG ATG) and 4. The resulting DNA library with a complexity of 10^{15} different sequences was used as the template for the in vitro transcription with T7-RNA polymerase for the initial selection cycle.

In vitro selection: Standard transcription reactions contained the following components: 250 U T7-RNA-polymerase (Stratagene), T7-buffer, 2.5 mM dNTPs, 20 mM GMPS, 20 μCi α^{32}P GTP, RNAsin (50 U), **1** (1 mM), template DNA (2 μM). Transcription was performed for 16 h at 37 °C. The uncleaved RNA transcript was purified by 8 % denaturing polyacryl-amide gel electrophoresis (PAGE) and the 5′-sulfanyl group was sub-sequently derivatized by treatment with *N*-biotin-*N*′-iodoacetylhexame-thylenediamine (200-fold excess) for 90 min at 25 °C. The biotinylation was achieved with an efficiency of 40 %. 5′-Modified RNA was again purified by PAGE and incubated with streptavidine agarose (Pierce) for 30 min at 25 °C. Immobilized RNA was then washed six times alternatively with 1 mL of washing buffer A (WA; 25 mM HEPES pH 7.4, 1M NaCl, 5 mM EDTA), washing buffer B (WB; 3M urea, 5 mM EDTA), and water. It was then incubated in the selection buffer (SB; 40 mM Tris HCl pH 8, 50 mM NaCl, 10 mM spermidine, 8 mM MgCl$_2$) at 37 °C for different times (see Figure 1 c) and in the presence of the respective concentrations of **1**. Cleavage reactions were started by addition of Mg^{2+}. After cycle 5 incubation during the first 2.5 h was interrupted ten times by alternate washes with WB, WA, and water. The second incubation in SB was performed under the same conditions without **1**. The RNA was eluted, extracted with phenol/CHCl$_3$, precipitated with EtOH, and reverse transcribed by using Tth-DNA-polymerase (Boehringer); the resulting cDNA was PCR-amplified with primers 3 and 4. The PCR primers were designed in such a way that the cleaved 5′-end of HHR and the T7 promoter were reintroduced. This DNA was used as a template for T7 transcription for the next round of selection.

Kinetic analysis of the 12 clones: The clones were labeled at the 5′-end with γ-^{32}P-ATP and incubated at 1.0 nM in SB+8 mM MgCl$_2$ at 37 °C. Aliquots were taken at different times, quenched with urea and EDTA on ice, and loaded onto a denaturing PA gel. Bands corresponding to the uncleaved and cleaved fractions were quantified by phosphorImaging (Molecular Dynamics). The k_{obs} values were then determined by using an exponential curve fitting of the measured values of the uncleaved ribozyme fraction during the first 3 min.

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Homoleptic Phosphoraneiminato Complexes of Rare Earth Elements as Initiators for Ring-Opening Polymerization of Lactones[**]

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Phosphoraneiminato complexes of transition metals containing the NPR$_3^-$ group are known in many coordination-chemical variations[1] as a result of varying bonding modes. In complexes with metal ions in low oxidation states (+I, +II) the NPR$_3^-$ group acts as μ_3-N bridging ligand, in medium oxidation states (+III, +IV) preferably as μ_2-N bridging ligand, and in high oxidation states (+V to +VIII) exclusively as terminal ligand. In the latter mode short metal–nitrogen distances and linear or almost stretched M-N-P axes are observed, which can be described with the resonance structures **A** and **B**. As first deduced for imido complexes (NR^{2-})[2]
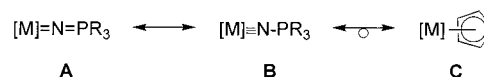
style **B** requires a σ, 2π orbital set for the M≡N bond that proves the NPR$_3^-$ ligand to be ψ-isolobal to the η^5-cyclopentadienide(Cp) ligand **C** [3] with respect to equal charge. Recent ab initio calculations confirm this interpretation.[4]

Since we surprisingly found the bonding mode **A/B** also in the mixed-ligand complexes [Ln$_2$Cp$_3$(NPPh$_3$)$_3$] (Ln = Y, Dy, Er, Yb) containing Ln^{3+} ions,[5] we examined the opportunity to synthesize homoleptic compounds [Ln(NPPh$_3$)$_3$], too, and to compare them with the ψ-isolobal complexes [LnCp$_3^*$] (Cp* = C$_5$Me$_5$) that are known as initiators for ring-opening polymerization (ROP) of lactones.[6–8]

Compounds **1** are formed in almost quantitative yields by reacting the alkaline phosphoraneiminates MNPPh$_3$ (M = Na, K, Rb, Cs)[9] with the anhydrous metal trichlorides or anhydrous metal triflates in THF [Eq. (1)]. Interestingly, only

$$2\ LnCl_3 + 6\ NaNPPh_3 \xrightarrow[-\ NaCl]{} [\{Ln(NPPh_3)_3\}_2] \qquad (1)$$

Ln = La (**1a**), Yb (**1b**)
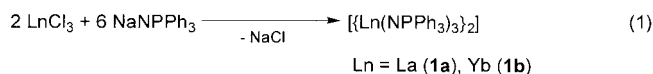
the lanthanum compound crystallizes as a THF-solvate, that is **1a**·2 THF, whereas the ytterbium compound **1b** forms a solvate-free complex due to the ionic radius of the Yb^{3+} ion which is 16 pm smaller than that of the La^{3+} ion.[10]

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