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RNA Hydrolysis by Heterocyclic Amidines and Guanidines: Parameters Affecting Reactivity

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In memory of Klaus Hafner

2-Aminobenzimidazole **10**, although a weak catalyst in the monomeric state, is a successful building block for effective artificial ribonucleases. In an effort to identify new building blocks with improved catalytic potential, RNA cleavage by a variety of heterocyclic amidines and guanidines has been studied. In addition to pK_a values and steric effects, the energy difference between tautomeric forms seems to be another important parameter for catalysis. This information is available from quantum chemical calculations on higher levels, but semiempirical methods are sufficient to get a first estimate. According to this assumption, imidazoimidazol **18**, characterized by isoenergetic tautomeric forms, is superior to 2-aminoimidazol **6**, the best candidate among the simple compounds. By far the largest effects are seen with 2-aminoperimidine **24**, which rapidly cleaves RNA even in the micromolar concentration range. The impressive reactivity, however, is related to a tendency of compound **24** to form polycationic aggregates which are the actual catalysts.

Introduction

Guanidines are important functional groups for the molecular recognition of phosphorylated compounds, both in nature[1] and in host–guest chemistry.[2] Phosphoric acid diesters, forming anions at physiological pH, are protected against nucleophilic attack by their negative charge. As a result, they are practically inert in the absence of powerful catalysts.[3] While nature has met the challenge of developing highly effective phosphoryl transfer enzymes,[4] artificial systems are still far away from this level of sophistication.[5–10] Thus, from the perspective of biomimetic chemistry, creating synthetic phosphoryl transfer catalysts is a challenging task.[11]

Bis- and oligoguanidines related to **1** (Figure 1) have been shown not only to bind phosphates but also to accelerate nucleophilic displacement reactions quite effectively.[5,6] Catalysts of this type have been applied already to manipulate ribonucleic acids.[12] Hydrolytic cleavage of RNA in most cases

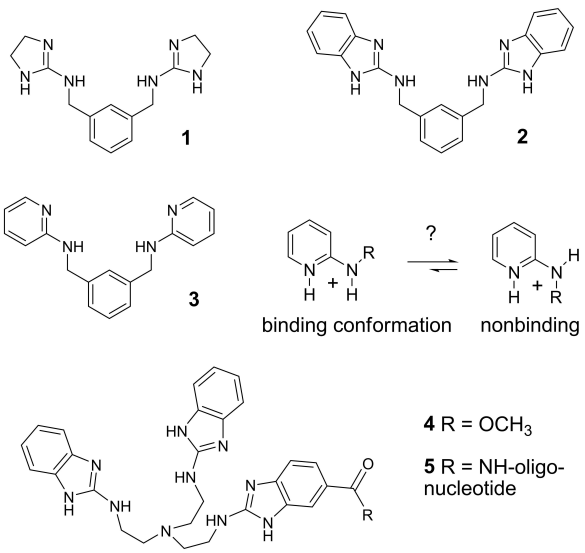

Figure 1. Compounds tested previously as RNA cleaving catalysts.

results from nucleophilic attack of the 2′ hydroxy group forming a 2′,3′ cyclic phosphate and a free 5′ OH of the second fragment.[11] The intramolecular nature of this attack accounts for the large rate increase when compared to analogous intermolecular reactions. The unmodified bisguanidine **1**, however, failed as a catalyst for RNA cleavage.[13] The role of guanidines in such reactions may be threefold: as a general base to deprotonate 2′ OH, as an electrophile/general acid to stabilize the pentavalent transition structure at the phosphorus atom[6t] and as a general acid for leaving group protonation (5′ OH). With pK_a values around 14, normal guanidines will stay protonated at pH 7 and, in consequence, behave as poor

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general acids or bases in neutral aqueous solution. Better catalysts, therefore, can be expected from guanidine analogs with pK_a values shifted towards neutrality. Consistent with that idea, acyl guanidines with structures similar to **1** have been reported to cleave RNA and RNA models more effectively.[5n,o] An alternative approach to lower the pK_a values of amidines and guanidines is to incorporate them into heterocyclic structures (Figure 1).

We previously investigated derivatives of 2-aminobenzimidazoles (**2**, p$K_a \approx 7.0$) and 2-aminopyridines (**3**, p$K_a \approx 6.5$). Compound **2** is active and finally led us to the development of the tris(2-aminobenzimidazole) **4**.[14] Conjugates **5** of this molecule with DNA and PNA cleave complementary RNA strands with high sequence specificity and substrate half-lives in the range of 3.5–20 h.[12,15] Surprisingly, compound **3** failed as RNA cleaving catalyst, in spite of similar pK_a values. We discussed the lack of rotational symmetry around the bond connecting the heterocycle and the exocyclic nitrogen as a possible reason:[14b] Binding to phosphate ions requires two parallel NH groups of the protonated heterocycle to form hydrogen bonds. Unfavorable conformational equilibria thus may prevent substrate binding and catalysis (Figure 1).

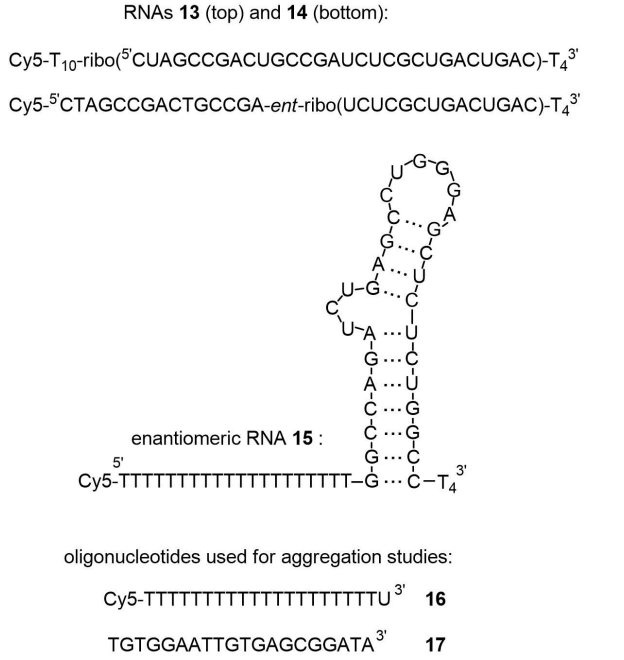

Figure 3. RNA substrates **13**–**15** and accessory oligonucleotides **16**–**17**. The RNA part of **15** is built from enantiomeric nucleotides.

Results and Discussion

To test the conformation hypothesis, we have investigated the RNA cleaving potential of some simple heterocycles (**6**–**12**, Figure 2), all containing the structural element of amidines or guanidines. Conformations unfavorable for catalysis as in the case of **3** do not exist in compounds **6**–**12**. Cleavage experiments look simple, but should be interpreted with caution:

The dye labeled RNA **13** (Figure 3) was incubated for 20 h at 37 °C and pH 7 with test compounds in concentrations of 10 mM. Afterwards, fluorescently labeled RNA fragments were separated with an ALFexpress II sequencer and the corresponding signals integrated as reported before.[14,15] Such reaction mixtures are far from being ideal solutions. Instead they can be heavily aggregated, depending on the nature of the heterocycle (see below). Standard loading buffers may precipitate the sample when it is transferred to the separation gel thus preventing analysis. In addition, many runs showed increased cleavage between pyrimidines and adenosines, a pattern typical for minor contaminations with natural RNases. Exact quantification of weak cleavage activities is hardly possible under such conditions. Therefore, all data shown in Table 1 were finally obtained from assays using enantiomeric *L*-RNA **14**,[15a] known to be stable against natural RNases. Achiral compounds **6**–**12**, in contrast, are unable to distinguish between both enantiomeric forms.[14a] A freshly purified sample of enantio RNA **14** still contained detectable traces of cleavage fragments in the 14mer ribo part, adding up to 0.9% of the total peak area. When incubated at pH 7 for 20 h at 37 °C the combined area of fragments rose to 1.03 – 1.06%. This value was unchanged in the presence of compounds **7**–**9** or **11** (10 mM) or by addition of DMSO (20%). Increased cleavage was only seen with compounds **10**, **12** and in particular with 2-aminoimidazole **6**.

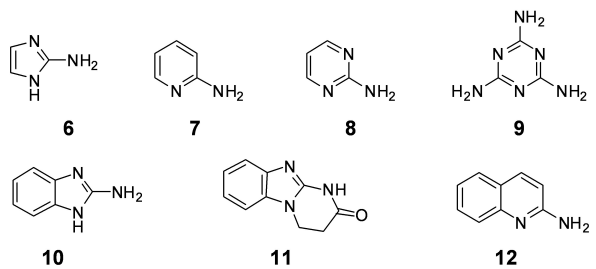

Figure 2. Structures of compounds **6**–**12**.

Table 1. Catalytic potential, pK_a and $\Delta_r H°_{298}$ of compounds **6**–**12**.

	pH	cleavage[a]	pK_a	$\Delta_r H°_{298}$[g]
6×0.5 H_2SO_4	7.0	3.1 ± 0.5%	8.5[c]	40.6 kJ mol^{-1}
	6.1	0.8 ± 0.2%		
7[b]	7.0	–	6.7[d]	71.0 kJ mol^{-1}
8[b]	7.0	–	3.4[d]	65.1 kJ mol^{-1}
9[b]	7.0	–	5.2[e]	63.4 kJ mol^{-1}
	6.1	–		
	5.1	< 0.1%		
10[b]	7.0	0.3 ± 0.1%	7.5[e]	15.7 kJ mol^{-1}
11[b]	7.0	–	3.7[f]	34.9 kJ mol^{-1}
12[b]	7.0	0.2 ± 0.1%	7.3[e]	43.7 kJ mol^{-1}

[a] 150 nM of **14**, 10 mM of heterocycle, 37 °C, 20 h. Total RNA cleavage after subtraction of background reaction. All experiments were run at least in triplicate. [b] 20% DMSO was added to the buffer to keep the heterocycles in solution. [c] Ref. [17]. [d] Ref. [16]. [e] Ref. [18]. [f] Determined as described in ref. [14b]. [g] Energy difference between tautomers, calculated on the semiempirical AM1 level.

2-Aminopyridine **7**, although the pK_a (6.7[16]) comes close to the ideal value of 7, did not show noticeable RNA cleavage. This observation clearly disproves our initial attempt to explain the catalytic incompetence of compound **3**. The lacking activity of compounds **8** and **11** at pH 7 results from their unfavorable pK_a. However, melamine **9** remained almost inactive even at pH 6.1 and 5.1. Matching the buffer pH and the pK_a of the catalyst is important: When aminoimidazole **6** was tested at pH 6.1, the amount of RNA cleavage dropped from 3.1% to 0.8%.

An alternative view on catalytic efficiencies starts to ask for the protonation state of the reacting phosphate esters during RNA hydrolysis. Computational and experimental methods have determined values of 8–9 as first pK_a and approximately 14 as second pK_a of equatorial phosphorane hydroxy groups.[19] It is thus reasonable to consider protonation of dianionic phosphoranes in the pH region of 7–8: Breslow suggested a proton transfer from 2′-OH to the phosphorane to occur in the mechanism of RNase A.[20] Detailed QM/MM simulations have supported this idea and assigned the role of the proton shuttle to His12.[21] A mechanism proposed by Cleland for the pH-independent nonenzymatic cleavage of RNA assumes a water molecule acting simultaneously as a general acid and base to transfer a proton from 2′-OH to the pentavalent phosphorane.[22] While computational studies have emphasized the importance of this proton transfer step,[23a] the role of water as a proton shuttle has been supported by some[23b] but not all authors.[23c] However, as Lönnberg pointed out, it may well be that heterocyclic guanidine analogs function as proton shuttles.[6r,11c,d] Proton inventory studies have shown that even weakly acidic guanidinium ions can contribute to catalysis by protonation of the phosphorane.[5j] Heterocyclic guanidines acting as proton shuttles would be tautomerized to form an exocyclic imine as shown in Figure 4. This mechanism is feasible in such cases only when the energy difference of both tautomers is not too large. Fast semiempirical calculations are sufficient to get a first estimate of the energy (heat of formation) differences of tautomers (Table 1; see below for a comparison with more advanced methods) and to find a weak correlation with the catalytic potential of the heterocycles: Smaller values (15.7–43.7 kJ mol^{-1}) are obtained for the active compounds **6**, **10** and **12**, larger energy differences (63.4–71.0 kJ mol^{-1}) for compounds **7**–**9**. Compound **11**,[24] however, has a smaller energy difference than **6** and **12** but is nevertheless inactive due to its unfavorable pK_a. Furthermore, the sterically less hindered aminoimidazole **6** is a much better catalyst when compared to the benzimidazole analog **10**, in spite of its less favorable $\Delta_r H^\circ_{298}$ value. This shows that the energy difference alone cannot predict the catalytic potential but may be one criterion in addition to pK_a values, steric effects and the tendency of compounds to aggregate.

To further test the tautomer hypothesis, the catalytic potential of guanidine analogs which are expected to have a low or even zero energy difference was investigated (Figure 5, Table 2). Imidazoimidazole **18**[25] exists in structurally identical tautomeric forms. This property and the pK_a value of 7.4 made **18** an interesting candidate that turned out to be even twice as active as aminoimidazole **6**. Unfortunately, the compound slowly degraded in aqueous buffer. All our attempts to attach side chains for conjugation with oligonucleotides failed due to the low stability of the intermediates. In contrast, no degradation was observed with compounds **19**[25b,26] and **20**–**23** (Figure 5). The energy difference between tautomeric forms of compound **20**,[27] **21** and **23** is also zero due to symmetry. The benzene rings, however, shifted the pK_a to unfavorable values, increased steric hindrance and also reduced solubilities in water. As a consequence, even at pH 5 and in the presence of

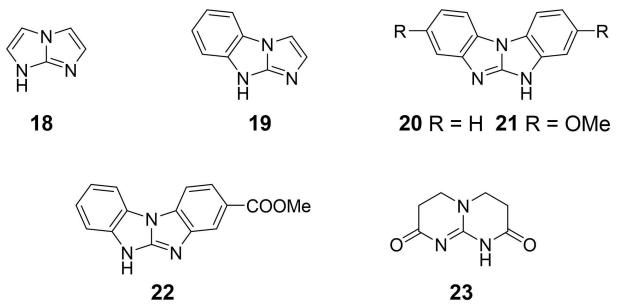

Figure 5. Guanidine analogs **18**–**23**.

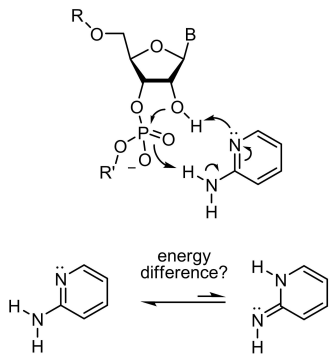

Figure 4. Heterocyclic guanidine analogs, when acting as proton shuttles, are converted into tautomeric forms that may be unfavorable as shown in the case of compound **7**.

Table 2. Catalytic potential, pK_a and $\Delta_r H^\circ_{298}$ of compounds **18**–**23**.

	pH	Cleavage[a]	pK_a[d]	$\Delta_r H^\circ_{298}$[e]
18[b]	7.0	5.9 ± 1 %	7.4	0 kJ mol^{-1}
19[b,c] (20% DMSO)	6.9	< 0.1 %	6.5	17.2 kJ mol^{-1}
	5.9	0.95 ± 0.3 %		
20[c] (40% DMSO)	7.0	–	4.7	0 kJ mol^{-1}
	6.1	–		
	5.0	< 0.1 %		
21[c] (40% DMSO)	7.0	–	4.5	0 kJ mol^{-1}
22[c] (40% DMSO)	7.0	–	3.7	2.8 kJ mol^{-1}
23[b]	7.0	–	low	0 kJ mol^{-1}

[a] 150 nM of **14**, 10 mM of **18**, **19** or **23**, 2 mM of **20**–**22**, 37 °C, 20 h. Total RNA cleavage after subtraction of background reaction. All experiments were run at least in triplicate. [b] The hydrochloride salt was used. [c] DMSO was added to the buffer to increase the solubility of heterocycles. Even then the solutions of **20**–**22** remained cloudy. [d] determined by spectrophotometric titration (Supporting Information). [e] energy difference between tautomers, calculated on the semiempirical AM1 level.

40 % DMSO less than 2 mM of compounds **20**–**22** were soluble. No RNA cleavage was seen at pH 7 or 6.1 and only minor effects at pH 5.0. In contrast, compound **19** is characterized by a pK_a value of 6.5 and tautomers of similar energy. Although hardly active at pH 6.9, it is a good RNA cleaver at pH 5.9 and outperforms compound **10** by a factor of 3. We could not determine the pK_a of compound **23**[28] by our UV-spectroscopic method but the corresponding hydrochloride is a strongly acidic compound outside of the useful pK_a range.

2-Aminoperimidine **24**[29] turned out in our systematic search as a promising candidate with a $\Delta_rH°_{298}$ value of only 20.5 kJ mol^{-1} (Figure 6 and Table 3). In fact, compound **24** appeared to be the most effective catalyst among all monomeric guanidine analogs we have tested so far. At the optimal concentration of 250 μM it almost attained the activity of trisbenzimidazole **4**. Increasing the concentration of **24** into the mM range caused reduced cleavage, presumably by massive aggregation (Figure 7). This result prompted us to take a closer look on **24** and its derivatives **25**–**31**.

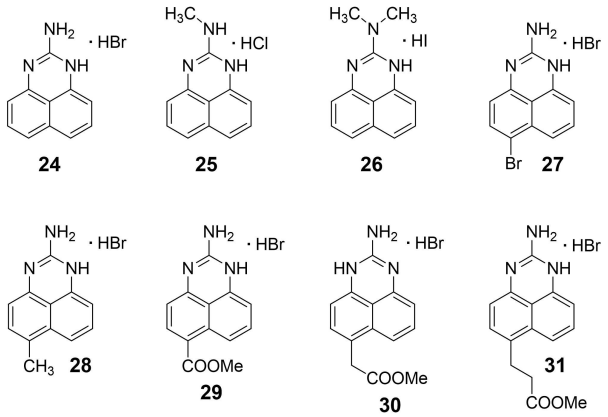

Figure 6. 2-Aminoperimidine **24** and derivatives **25**–**31**.

Table 3. pK_a and $\Delta_rH°_{298}$ values of compounds **24**–**31**.

	24	25	27	28	29	30	31
pK_a[a]	8.1	7.7	7.2	8.1	6.7	7.7	7.9
$\Delta_rH°_{298}$[b]	20.5	3.8	21.5	20.8	21.4	21.3	21.3

[a] Determined by spectrophotometric titration (Supporting Information). [b] energy difference between tautomers in kJ mol^{-1}, calculated on the semiempirical AM1 level.

2-Methylaminoperimidine **25**[30] was active in the same concentration range as **24** but, possibly due to steric hindrance, RNA degradation was much slower. Almost no reaction was seen with 2-dimethylaminoperimidine **26**. To form stable ion pairs with phosphates, protonated guanidine analogs must have two parallel NH groups not available in compound **26**.[30] The structure also does not allow proton shuttling as shown in Figure 4. To synthesize conjugates of 2-aminoperimidines with oligonucleotides, attachment as 2-alkylamino derivatives in analogy to compound **4** would be attractive. The diminished activity of such derivatives, however, motivated us to investigate other linking modes. As expected, for all perimidines **27**–**31** tautomeric forms with similar energy differences were found (Table 3).

RNA cleavage experiments were conducted near the pH optimum for each compound: pH 7 for the less basic derivatives **27** and **29** and at pH 8 for compounds **28**, **30**, and **31**. Most perimidine derivatives have activities comparable to those of **24** (Figure 8). Ester **30** and in particular the bromo derivative **27** are distinctly weaker catalysts. The importance of the reaction pH coming close to the pK_a of the catalyst is demonstrated in Figure 9. Methylperimidine **28** (pK_a = 8.1) cleaves faster at pH 8 whereas ester **29** (pK_a = 6.7) works better at pH 7. Bromoperimidine **27** (pK_a = 7.2), a weak catalyst already at pH 7, does not work at all under more basic conditions (Figure 9).

Upon titration with NaOH solution under air, 2-aminoperimidine **24** turns brown in the strongly basic pH region by irreversible oxidation.[31,32] The same effect occurred with most other aminoperimidine derivatives. Although we have not seen

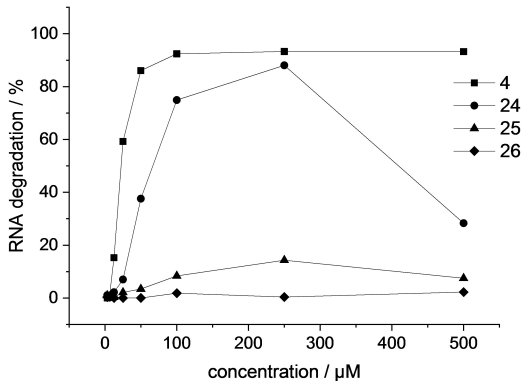

Figure 7. Cleavage of RNA substrate **13** by tris(2-aminobenzimidazole) **4** and 2-aminoperimidines **24**, **25**, and **26** as a function of catalyst concentration. Conditions: 150 nM RNA **13**, 3.13–500 μM cleaver, 50 mM TRIS-HCl, pH 8, 37 °C, 20 h. Data points, determined at least in duplicate, are connected by lines for the sake of clarity.

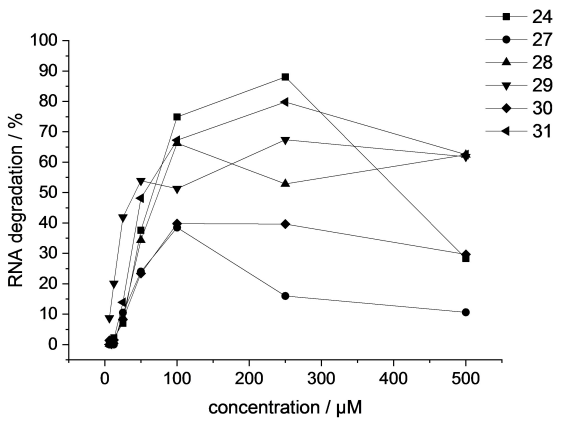

Figure 8. Cleavage of RNA substrate **13** by 2-aminoperimidine derivatives **24** and **27**–**31** as a function of catalyst concentration. Conditions: 150 nM RNA **13**, 3.13–500 μM cleaver, 50 mM TRIS-HCl, pH 7 or 8, 37 °C, 20 h. Data points, determined at least in duplicate, are connected by lines for the sake of clarity.

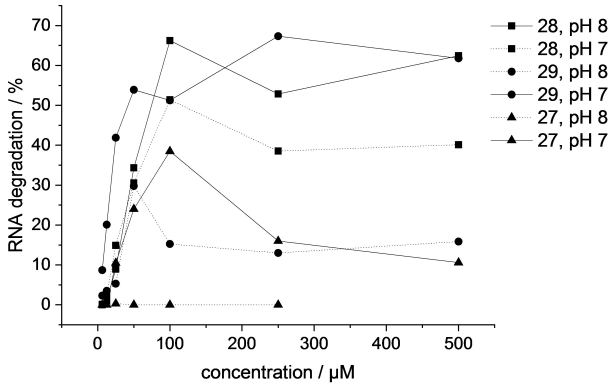

Figure 9. pH-dependent cleavage of RNA substrate **13** by aminoperimidine derivatives **27**, **28**, and **29**. Conditions: 150 nM RNA **13**, 6.25–500 µM catalyst, 50 mM TRIS-HCl pH 7 or 8, 37 °C, 20 h. Data points are connected by lines for the sake of clarity.

problems in the RNA cleavage experiments, oxygen sensitivity may complicate or even prevent the synthesis of oligonucleotide conjugates. A notable exception is ester **29**, protected by its electron withdrawing substituent.

The cleavage pattern induced in the enantiomeric TAR RNA **15**[14] by compound **24** (Figure 10a) and by imidazole buffer (Figure 10b) reveals a striking difference. Probing of the stable stem-loop structure with imidazole shows, as expected, cleavage restricted to the bulge and the loop regions. In contrast, **24** induces hydrolysis in all possible positions. This requires full denaturation of the RNA stem-loop and suggests binding of **15** to polycationic aggregates formed by **24**. At 20 µM, however, the amount of cleavage induced by **24** drops drastically and a pattern similar to Figure 10b occurs. In contrast, the cleavage pattern of compounds **6** and **18** represents the bulge and loop structure of RNA **15** even at the highest concentration of 10 mM.

Direct evidence for aggregates formed from aminoperimidine **24** and oligonucleotides is provided by fluorescence correlation spectroscopy (FCS).[14] Dye labeled DNA **16** (19 nM) when diluted with oligo **17** (131 nM; 150 nM total oligonucleotide concentration as in the cleavage experiments) in buffer free of heterocyclic guanidines shows high mobility consistent with a monomeric state. At concentrations of 250 µM and more, **24** massively raises the diffusion time of **16** indicating the presence of large aggregates. According to Figure 10a, however, aggregation is not absent even at lower concentrations. Aminoimidazole **6**, in contrast, has no impact on substrate mobility up to 10 mM. Some effects are found for compounds **10** and **18**, but only in the absence of cosolvent whereas compound **19** due to its more lipophilic structure causes considerable aggregation above 2 mM.

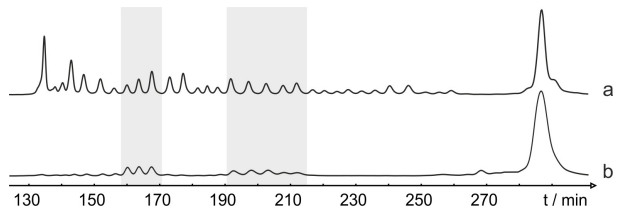

Figure 10. Cleavage pattern of TAR RNA **15** induced by different agents and analyzed with an ALFexpress II sequencer detecting the fluorescence of Cy5-labeled fragments. a) 150 nM RNA **15**, 100 µM 2-aminoperimidine **24**, 50 mM TRIS-HCl pH 8, 37 °C, 20 h. b) 150 nM RNA **15**, 2 M imidazole pH 7, 40 mM NaCl, 500 µM EDTA, 37 °C, 20 h. The non-denaturing imidazole buffer cleaves RNA **15** preferentially in the single-stranded bulge and loop regions (indicated in grey, see Figure 3).

To test how reliable the semi empirical description of the energy difference by AM1 is, more advanced quantum chemical calculations were performed. The comparison is shown in Figure 11. The values for $\Delta_r H^\circ_{298}$ obtained by AM1 have a similar order of magnitude as $\Delta_r G^\circ_{298}$ calculated by the other methods and show overall similar qualitative trends. Due to the semi-empirical nature of AM1, zero point vibrational energies and thermal corrections are already included in the definition of self-consistent field energies, so that we did not attempt to disentangle different contributions and omitted furthermore entropic corrections in directly comparing here $\Delta_r H^\circ$ values from semi-empirical AM1 with $\Delta_r G^\circ$ values from ab initio methods. For the relevant compounds **6**–**12**, **19**, **22**, **24**, **25** and **27**–**31** the mean absolute error (MAE) between AM1 and the hybrid density functional B3LYP is 7.7 kJ mol^{-1} which is similar to the MAE between AM1 and second-order Møller-Plesset perturbation theory with the resolution of identity approximation RI-MP2 (6.9 kJ mol^{-1}) and not much larger than the MAE between B3LYP and RI-MP2 (5.9 kJ mol^{-1}). For the relevant compounds $\Delta_r G^\circ_{298}$ by RI-MP2 is always larger than $\Delta_r G^\circ_{298}$ by B3LYP. $\Delta_r G^\circ_{298}$ based on high level coupled cluster theory with the resolution of identity approximation RI-CCSD(T) is between

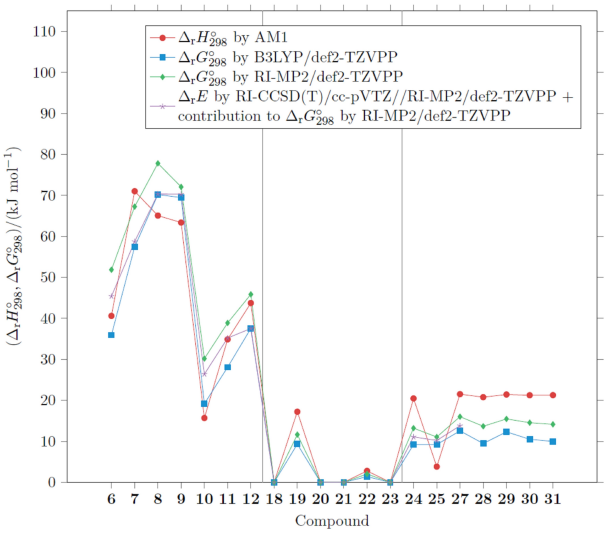

Figure 11. Energy difference between the relevant tautomers of the various compounds calculated by different theoretical methods (RI-CCSD(T) only for **6**–**12**, **23**–**27** and those where it is zero due to symmetry). Data points are connected by lines for the sake of clarity.

the values of B3LYP and RI-MP2. As very accurate energy differences seem not to be important in the classification whether the compound has cleaving abilities or not, AM1 is a fast method to get a first estimate.

Conclusions

The aim of the present study is to identify heterocyclic amidines and guanidines as building blocks of improved artificial ribonucleases related to conjugate **5** – and to understand why some are better catalysts than others. Parameters we consider relevant are the pK_a values, the steric demand and the hydrophobicity of the compounds: The weakly basic candidates such as **8**, **9**, and **11** all fail in the cleavage assay. In addition, we have found a weak correlation between catalytic activity and the energy difference between amino and imino tautomers (Figure 12). This parameter prompted us to investigate 2-aminoperimidine **24**. At first glance, **24** is the most effective catalyst of this study, a powerful RNA cleaver even in the micromolar concentration range. In contrast, much less cleavage occurs in the presence of 2-aminobenzimidazole **10**, the active component of our previous artificial ribonucleases. A direct comparison, however, is complicated by the fact that RNA cleavage is not catalyzed by a single guanidine. Instead, a concerted interaction of two or more subunits is required. When monomeric building blocks are tested, this cooperativity can result from high initial concentrations or by local enrichment caused by aggregation phenomena. The latter is obviously the case with compound **24**. We assume that in general RNA hydrolysis by monomeric guanidine analogs occurs in non-ideal solutions. Compounds with large, flat and hydrophobic ring systems, therefore, may become active at lower concentrations than small and hydrophilic molecules. On the other hand, when linked together by appropriate frameworks, derivatives of 2-aminobenzimidazole such as **4** and **5** turn into powerful catalysts. Against this background, the cleavage activity of aminoimidazole **6** is quite remarkable - a compound with minimal aggregation potential. It is superior to compound **10**, in spite of a less favorable value of $\Delta_rH^\circ_{298}$. The difference may be attributed to the steric hindrance caused by the benzene ring of **10**. When $\Delta_rH^\circ_{298}$ is reduced to zero by the symmetry of imidazoimidazole **18**, a further increase in reactivity is seen. Although this does not prove the relevance of proton shuttling as depicted in Figure 4, it may be wise to take tautomeric equilibria into account. The effort to predict them by AM1 calculations is insignificant.

It is difficult in general to ascribe changes in reactivity in a strict sense to changes of a single parameter. Thus, a final evaluation of catalysts is done best in form of conjugates with oligonucleotides such as **5**. Nanomolar absolute concentrations then avoid aggregation phenomena whereas high local concentrations of catalyst can lead to fast and specific cleavage of complementary RNA strands. Not every compound is sufficiently stable to synthesize such conjugates, but **6**, **19** and **29** seem to be good starting points for developing the next generation of synthetic RNA cleavers.

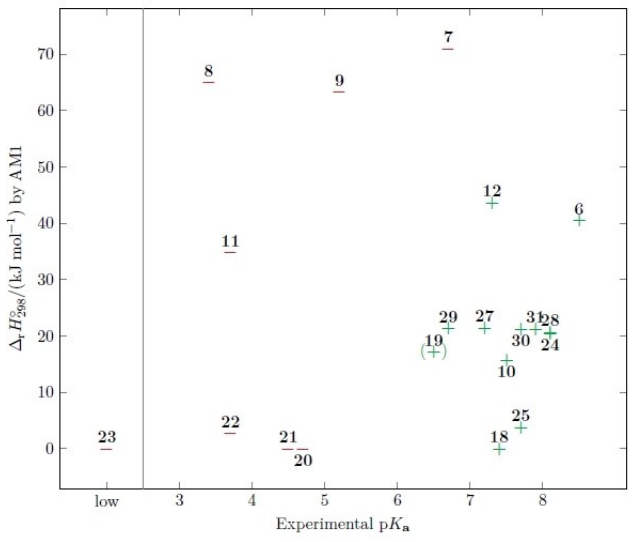

Figure 12. Classification of the cleavage behavior ("-" marks compounds that do not cleave, "+" marks compounds that are active cleavers and "(+)" marks compounds that show only little cleavage at pH 7) of various compounds in dependence of their experimental pK_a and computed $\Delta_rH^\circ_{298}$ values as obtained on the AM1 level (for a variant of this figure with $\Delta_rG^\circ_{298}$ from RI-MP2 see Supporting Information).

Experimental Section

Materials: Commercially available guanidines and amidines were purchased from Acros (**8**), Aldrich (**7**), Alfa Aesar (**6**, **10**), Fluka (**9**, hydrobromide of **24**), and Maybridge (**12**). The remaining compounds were prepared as described in the literature (**11**, **18**, **19**, **23**, **25**–**27**) and in the Supporting Information (**18**–**22** and **28**–**31**). **21**, **22**, and **28**–**31** are new compounds.

Cleavage experiments and aggregation studies: RNA handling and FCS measurements were carried out as described before.[14] Cleavage experiments and fragment analysis were done similar to the described procedures.[14] However, to prevent precipitation of RNA fragments in the presence of heterocycles during incubation or in the gel pockets, some changes were made to the loading buffer and the gel composition (see below).

RNA cleavage assay: 150 nM Cy5-labelled RNA **13**, **14**, or **15** was incubated in a final volume of 10 μL with the indicated cleaver concentration (0.031–10 mM) in a 50 mM Tris-HCl buffer at pH 6.0, 7.0 or 8.0 (checked with a glass electrode and adjusted if required). All cleavage reactions were performed at 37 °C for 20 h.

Polyacrylamide gel electrophoresis: The oligonucleotide fragments were separated by denaturing PAGE (16 % monomer, 8 M urea) on a DNA sequencing device (ALFexpress, Amersham Biosciences). Prior to electrophoresis, 15 μL of loading buffer (8 M urea, 20 mM EDTA and 0.2 % crocein orange in DEPC-treated H_2O) were added to each sample and 10 μL of the sample were loaded on the gel. Following running conditions were chosen: 1500 V (maximum), 60 mA (maximum), 25 W (constant), 60 °C, 2 s sampling interval and 400 min running time. For analysis of the electropherograms, the AlleleLinks 1.01 software package (Amersham Biosciences, Uppsala, Sweden) was used. The peak areas under the curves were added up, and the percentage of degraded RNA was calculated. Multiple

cleavage reactions were disregarded in this system. All data were averaged over a minimum of three experiments.

Computational details: The structures of the different tautomeric forms of the molecules in this study were analyzed with different theoretical methods. Either with AM1[33] or by density functional theory (B3LYP[34,35]/def2-TZVPP[36,37]) or with post-Hartree–Fock methods (RI-MP2[38–40]/def2-TZVPP[36,37] or RI-CCSD(T)/cc-pVTZ[41–44]). In all calculations solvent effects were disregarded.

In the case of AM1 the structures were energy minimized using Gaussian.[45] The convergence criterion for the energy change between two successive cycles was 1 μE_h. Additionally the maximal change in the density matrix was smaller than 10^{-6} and the root mean square (RMS) of its elements was smaller than 10^{-8}. The structure was varied till the maximum force in internal coordinates was below 15 $\mu E_h a_0^{-1}$ and 15 $\mu E_h rad^{-1}$ and the RMS deviation of the force was below 10 $\mu E_h a_0^{-1}$ and 10 $\mu E_h rad^{-1}$.

For B3LYP (with the VWN5 local correlation functional) the structural optimizer of Gaussian[45] was used together with Turbomole[46–48] that provided the energy and gradients. An m4-grid[49,50] was used for numerical integration. The energy was minimized till the change between two successive SCF cycles was below 0.01 μE_h. For the energy minimization of the structures the same convergence criteria for the force were used as in the AM1 case (see previous paragraph).

For structural energy minimization at the RI-MP2/def2-TZVPP level of theory Turbomole[46,47] was used exclusively. In the correlation of electrons, the frozen core approximation was employed. The final energy changes between two successive cycles was 0.01 μE_h and the RMS of the change of the density was below 10^{-5}. Between two successive structure changes, the convergence criterion of the energy difference was 1 μE_h, the maximum gradient change was below 1 $mE_h a_0^{-1}$ and its RMS deviation was below 500 $\mu E_h a_0^{-1}$.

On some structures obtained by RI-MP2/def2-TZVPP single point energies on RI-CCSD(T)/cc-pVTZ level were performed by Turbomole.[46,47] The frozen core approximation was used as well. The same convergence criteria as described in the RI-MP2 case (see previous paragraph) were used.

By calculating the harmonic vibrational frequencies, it was checked that all presented energy optimized structures are minima on the potential energy hypersurface for the particular method used in the optimization. For AM1 and B3LYP this was done analytically;[51] for RI-MP2 numeric harmonic vibrational frequencies were calculated by using central differences of analytically calculated first derivatives.

The thermodynamic corrections for the Gibbs energy G°_{298} at a temperature of 298.15 K and a pressure of 10^5 Pa were computed using the unscaled harmonic vibrational frequencies of the particular method. For RI-CCSD(T)/cc-pVTZ the electronic energy obtained by this method was combined with the thermodynamic corrections obtained at a RI-MP2/def2-TZVPP level of theory.

The energy differences for each compound are calculated by subtracting the energy of the tautomer shown in the Figure 2, Figure 5 and Figure 6 of its corresponding ketimin tautomer. Compound **11**, **18**–**23**, **25** and **26** are secondary ketimines and for them just another secondary ketimin tautomer exists. As expected all energy differences are ≥ 0.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Ab initio calculations · Catalysis · Post-Hartree-Fock methods · RNA cleavage · Semi-empirical calculations

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