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Site-Specific Cleavage of RNA by a Metal-Free Artificial Nuclease Attached to Antisense Oligonucleotides

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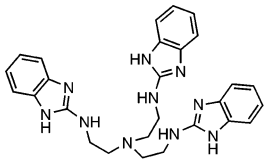
Abstract: RNA cleaving tris(2-aminobenzimidazoles) have been attached to DNA oligonucleotides via disulfide or amide bonds. The resulting conjugates are effective organocatalytic nucleases showing substrate and site selectivity as well as saturation kinetics. The benzimidazole conjugates also degrade enantiomeric RNA. This observation rules out contamination effects as an alternative explanation of RNA degradation. The pH dependency shows that the catalyst is most active in the deprotonated state. Typical half-lifes of RNA substrates are in the range of 12−17 h. Thus, conjugates of tris(2-aminobenzimidazoles) can compete with the majority of metal-dependent artificial nucleases.

Tris(2-aminobenzimidazoles) **1** and **2** (Chart 1) have been recently identified as powerful cleavers of ribonucleic acids.[1] The reaction involves nucleophilic attack by the 2′-hydroxy groups at phosphorus and the formation of 2′,3′-cyclic phosphates. Thus, catalysts **1** and **2** are completely specific for RNA and do not hydrolyze DNA.[1] Unfortunately, tris(2-aminobenzimidazoles) tend to aggregate in a pH-dependent way, thereby preventing deeper mechanistic insight from pH-rate correlations and similar experiments. For future applications, it is also required to determine the catalytic efficiency in the nonaggregated state. This issue has not yet been fully resolved. Here we describe the conjugation of catalyst **2** with a series of DNA oligonucleotides. The resulting conjugates **4**−**8** efficiently cleave complementary RNA strands at submicromolar concentrations in the expected positions. They do not aggregate and allow to complete the characterization of benzimidazole derived RNA cleavers.

A standard procedure to synthesize DNA conjugates involves acylation of amino linkers by active esters in aqueous buffer. When applied to the charged benzimidazoles **2** or **3**, however, complete precipitation of the oligonucleotide prevented coupling. To avoid the aggregation of polyionic species, we used two conjugation methods utilizing protected DNA still bound to the solid support (Scheme 1). In the first method, a trityl-protected mercapto linker was attached to the 5′-end as a phosphoramidite building block (**14**). After removal of trityl, the support was incubated with compound **15**. A disulfide linkage of sufficient stability to survive the subsequent deprotection steps was formed (for details, see Supporting Information).[2] Fractions of 60−70% of conjugated versus nonconjugated strands could be typically obtained. Pure conjugates **4** and **6**−**8** were finally isolated by polyacrylamide gel electrophoresis and characterized by mass spectroscopy. Alternatively, we attached a monomethoxytrityl-modified amino linker to the DNA strand that was deprotected

Chart 1. Sequences of Oligonucleotides **4**−**13**. For Complete Linker Structures, See Scheme 1



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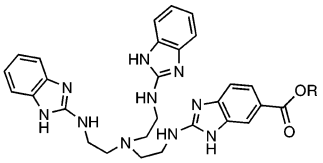

2 R = CH_3 **3** R = H

conjugates with catalyst **2**:

$^{3'}$GATCGGCTGACGGCT$^{5'}$-linker(1)-catalyst **4**

$^{3'}$GATCGGCTGACGGCT$^{5'}$-linker(2)-catalyst **5**

$^{3'}$GATCGGCTGACGGCTAG$^{5'}$-linker(1)-catalyst **6**

$^{3'}$GATCGGCTGACGGCTAGAGC$^{5'}$-linker(1)-catalyst **7**

$^{3'}$ TATGGAACAGTCCTC$^{5'}$-linker(1)-catalyst **8**

RNA substrates **9** - **11** :

Cy5-T_{10}-ribo($^{5'}$CUAGCCGACUGCCGAUCUCGCUGACUGAC)-$T_4{}^{3'}$ 9

Cy5-$^{5'}$CTAGCCGACTGCCGA-*ent*-ribo(UCUCGCUGACUGAC)-$T_4{}^{3'}$ 10

Cy5-T_{10}-ribo($^{5'}$AUACCUUGUCAGGAGAAGAGAGGCCGUUA)-$T_4{}^{3'}$ 11

accessory oligonucleotides:

Cy5-TTTTTTTTTTTTTTTTTTTTU $^{3'}$ 12

TGTGGAATTGTGAGCGGATA $^{3'}$ 13

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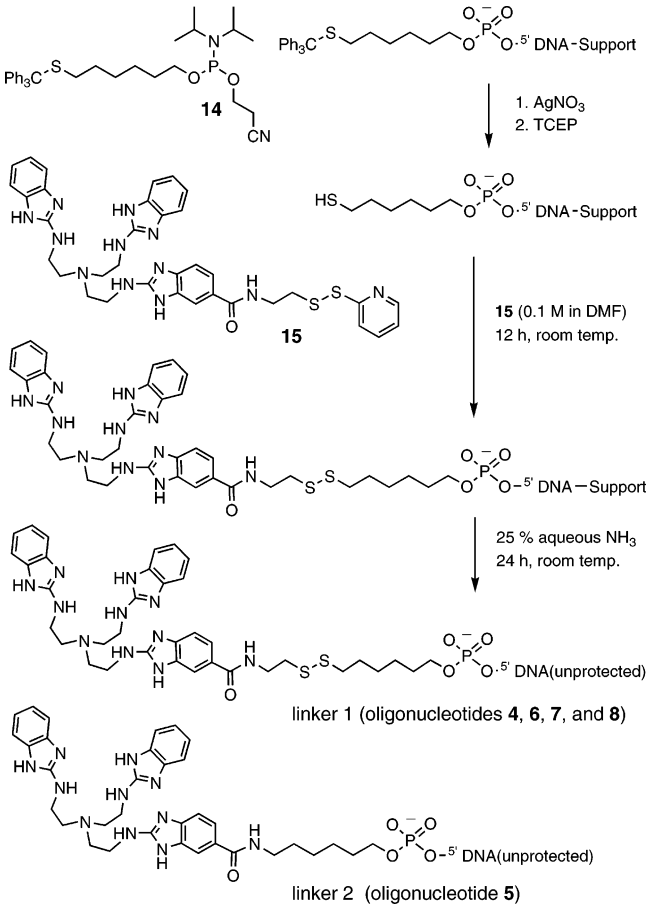
(1) Scheffer, U.; Strick, A.; Ludwig, V.; Peter, S.; Kalden, E.; Göbel, M. W. *J. Am. Chem. Soc.* **2005**, *127*, 2211−2217.

Scheme 1. Synthesis of Oligonucleotide Conjugates



and coupled with acid **3** in the presence of DIC and HOBt. The resulting peptide bond of conjugate **5** was stable under the conditions of DNA deprotection (coupling yield ~ 70%).

All cleavage experiments were run with the RNA substrates **9**−**11**. The fluorescent label allows convenient detection and quantification of products by gel electrophoresis in a DNA sequencer. Note that the sequence of oligonucleotide **10** is analogous to that of **9**. However, it is a hybrid formed of DNA (natural configuration) with an embedded part of enantiomeric RNA.

To exclude the existence of molecular aggregates of aminobenzimidazole conjugates with noncognate oligonucleotides, the dye-labeled DNA **12** (25 nM, diluted with 175 nM unlabeled DNA **13**) was studied by fluorescence correlation spectroscopy (FCS).[1,3] The diffusion time around 150 μs at 24 °C is consistent with the size of **12**. Upon addition of conjugates **4**−**8** (1.5 μM), no change in diffusion times and no signs of aggregation could be observed. Substrate **9** (200 nM) was then mixed with complementary conjugates **4**−**7** (1.5 μM), again without producing significant effects. The gain in molecular mass by hybridization was not expected to be sufficient to influence the diffusion time of **9**. Further experiments with substrate **11** and conjugate **8** confirmed the general view that higher aggregates of oligonucleotides beyond the stage of hybridization will not occur in the experiments shown below.

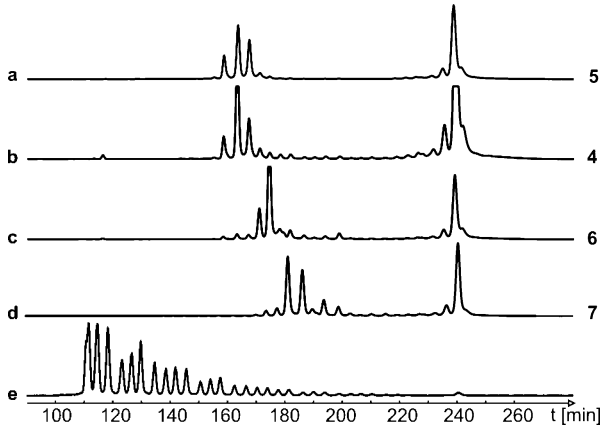


Figure 1. Cleavage of RNA **9** (150 nM) induced by conjugates **4**−**7** at concentrations of 1.5 μM (50 mM Tris-HCl, pH 8.0, 37 °C, 20 h). Lane e: hydrolysis pattern of substrate **9** (Na_2CO_3).

Table 1. Cleavage of RNA **9** by Conjugates **4**−**7** and Cleavage of RNA **11** by Conjugate **8**. Substrate Degradation within 20 h (%) as a Function of pH[a]

pH	control[b]	5[c]	5[d]	4	6	7	control[e]	8
6.0	<1	20.8	8.7	25.3	18.8	29.3	<1	18.8
7.0	<1	43.4	35.6	46.9	41.1	54.4	<1	41.1
8.0	<1	55.3	51	68.8	54.8	69.1	<1	60.4
9.0	<5		46.7			66.9	<5	66.9

[a] Conditions: 150 nM substrate, 1.5 μM conjugate, 50 mM Tris-HCl, 37 °C. [b] Control with substrate **9** at constant ionic strength (buffer + 100 mM NaCl), background at $t = 0$ was subtracted. [c] Experiment without addition of further salt. [d] Analogous experiment at constant ionic strength (buffer + 100 mM NaCl). [e] Control with substrate **11** at constant ionic strength (buffer + 100 mM NaCl), background at $t = 0$ was subtracted.

When substrate **9**[4] was incubated with the 15mer conjugate **5** (1.5 μM) for 20 h, strong cleavage occurred that could be assigned to nucleotides 13, 14, and 15 by comparison with the hydrolysis pattern of **9** (Figure 1, lanes a and e). Almost identical results were obtained in analogous experiments using the disulfide conjugate **4** (lane b). In contrast, the preferred cleavage sites of the 17mer conjugate **6** and 20mer **7** are nucleotides 17 and 19/20, respectively (lanes c and d). The reaction site thus correlates with the position of the benzimidazole moiety in each substrate−catalyst duplex. The preferential formation of 14mer fragments in the reactions of **4** and **5** may be a consequence of fraying due to the weak terminal A−T base pair. No significant turnover could be observed when **4** (150 nM) was incubated with a 10-fold excess of substrate **9**. This is a common observation seen with most RNA cleaving DNA conjugates when the catalyst is attached to either the 5′- or 3′-ends. Addition of EDTA (1 mM) does not change the kinetics and cleavage patterns of these reactions. A mechanistic role of contaminating metal ions bound to the benzimidazole moiety therefore can be ruled out.

The influence of pH on catalyst efficiency was studied next (see Table 1). Since ionic interactions might play a major role in the benzimidazole phosphate interaction, experiments initially were conducted in Tris-HCl buffer (50 mM) without adding inert salts. Upon variation of pH under such conditions, ionic strength is not constant. Critical experiments therefore were repeated in the presence of 100 mM NaCl. Cleavage yield increases with pH and levels off at pH 8. At high ionic strength,

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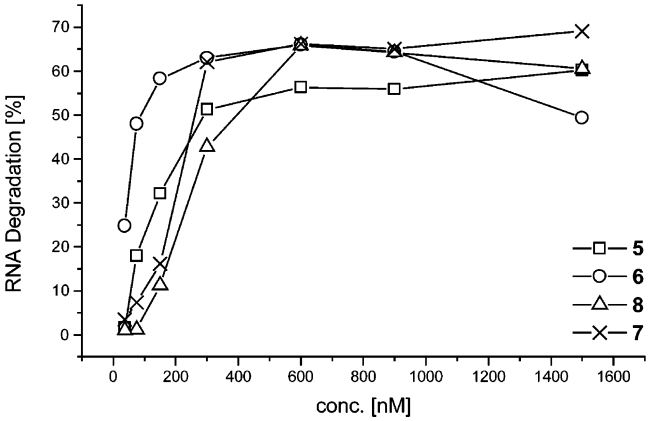

Figure 2. Cleavage of RNA substrates **9** and **11** as a function of conjugate concentrations (150 nM **9** or **11**, 20−1500 nM of conjugates, 50 mM Tris-HCl, pH 8.0, incubation for 20 h). Data points are connected by lines for the sake of clarity.

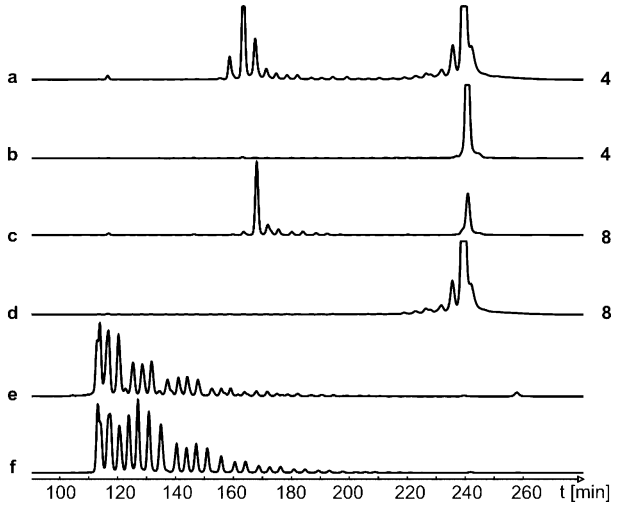

Figure 3. Substrate specificity of conjugates **4** and **8** (150 nM substrate, 1.5 μM catalyst, 50 mM Tris-HCl, pH 8.0, incubation for 20 h). Lane a: conjugate **4** and substrate **9**. Lane b: conjugate **4** and substrate **11**. Lane c: conjugate **8** and substrate **11**. Lane d: conjugate **8** and substrate **9**. Lanes e and f: hydrolysis patterns of **9** and **11** (Na_2CO_3).

a slight drop at pH 9 was observed. Since the pK_a of related benzimidazoles comes close to 7, the catalyst should be mostly deprotonated under such conditions. Thus, general base catalysis must be an important mechanistic aspect.

As shown in Figure 2, RNA cleavage by conjugates **5**−**8** obeys saturation kinetics. Full activity is reached at concentrations of 200−300 nM. This, together with the positional selectivity discussed above, clearly demonstrates that cleavage is mediated by hybridization of catalyst and substrate. Increasing ionic strength results in duplex stabilization. Accordingly, in 50 mM buffer containing 100 mM NaCl, saturation is reached at slightly lower concentration of conjugate (see Figure S6). Concentrations of 1.5 μM are far above the saturation value under either low or high salt conditions. The results in Table 1, therefore, should not be affected my minor pH-dependent changes of duplex stabilities.

An important requirement for artificial nucleases is substrate specificity. Figure 3 shows a cross reactivity experiment with two nonhomologous RNA substrates **9** and **11** challenged by the conjugates **4** and **8**. Cleavage fully depends on complementarity. While both catalysts transform their cognate substrates

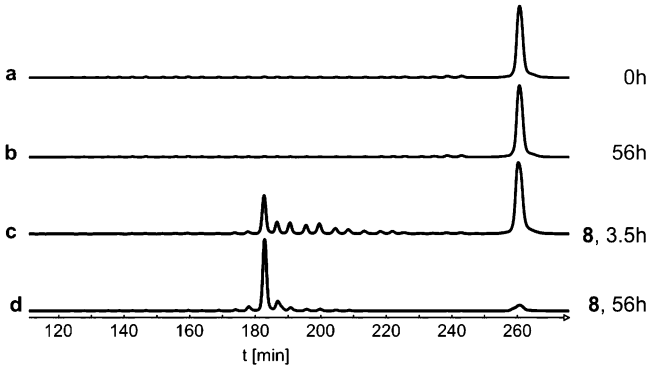

Figure 4. Time-dependent cleavage of RNA **11** by conjugate **8**. Conditions: 150 nM substrate, 0 or 1.5 μM conjugate, 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 37 °C. (a) Control without incubation. (b) Control after 56 h. (c and d) Cleavage of **11** after 3.5 and 56 h.

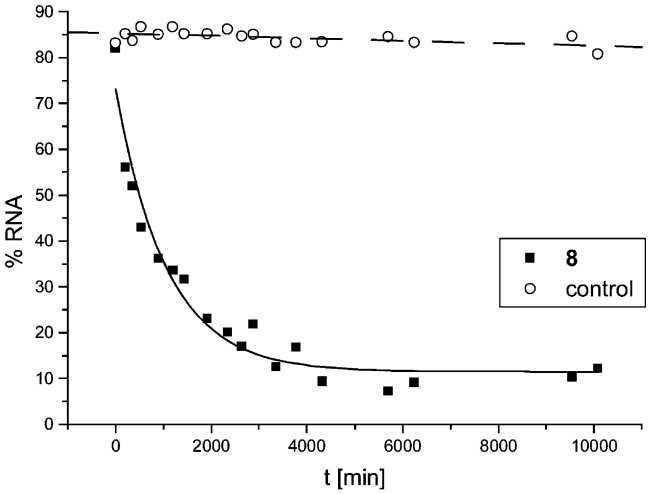

Figure 5. Cleavage kinetics of **11** in the presence and absence of conjugate **8**. Conditions: 150 nM substrate, 0 or 1.5 μM conjugate, 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 37 °C. The solid curve is calculated by assuming first-order kinetics.

(lanes a and c), they do not react with noncomplementary RNA (lanes b and d). All other combinations of substrates **9**−**11** and catalysts **4**−**8** have been tested as well. The results consistently fit into the picture (data not shown).

Cleavage kinetics was studied in detail for the reaction of conjugate **8** with substrate **11** (pH 8, high ionic strength). Without catalyst, the substrate proved almost stable for several days (Figure 4, lane b; Figure 5; Figure S8). In contrast, conjugate **8** cleaves significantly within a few hours. The substrate is almost completely degraded after 56 h. A small percentage, however, does not react even after 1 week (Figure 5). This may result from structural damages in chemically synthesized RNA preventing hybridization with conjugate **8** (e.g., 2′,5′ linkages, residual protective groups, etc.). Interestingly, a more defined cleavage pattern arises after longer incubation times (Figure 4, lane d; see also lane c in Figure 3 and Figure S7). Upon standing, secondary cleavage events may cut off all conformationally mobile ribonucleotides protruding out of the duplex with conjugate **8**.

Figure 5 shows the decay of RNA **11** as a function of time. As in all other experiments, the amount of **11** is calculated by dividing the peak area of **11** by the sum of all detectable peak areas. The regression line demonstrates the high substrate stability in the control reaction ($t_{1/2} \sim 3500$ h). For the cleavage

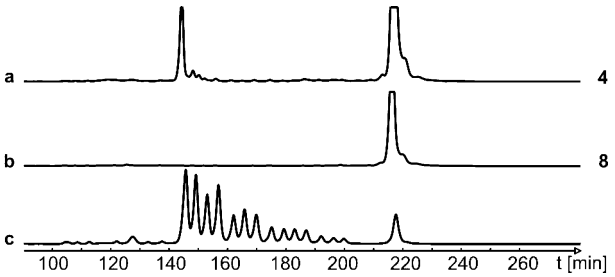

Figure 6. Lane a: conjugate **4** cleaves the *enantio* RNA insert of substrate **10**. Lane b: no cleavage occurs with the noncomplementary conjugate **8**. Conditions: 150 nM substrate, 1.5 μM conjugate, 50 mM Tris-HCl, pH 8.0, 37 °C, 20 h. Lane c: hydrolysis pattern of substrate **10** (Na_2CO_3).

induced by conjugate **8**, a proper fit of experimental and calculated data (solid curve) is obtained by assuming a first-order rate law ($k_1 = 0.056$ h^{-1}, $t_{1/2} = 12.4$ h). Analogous experiments were conducted to characterize the cleavage of substrate **9** by conjugate **5** ($k_1 = 0.042$ h^{-1}, $t_{1/2} = 16.5$ h).

Trace contaminations by natural ribonucleases are a constant threat to all experiments with artificial RNA cleavers. We reported earlier on the difficulty to rule out false positive effects.[1,5] Substrate specificity (Figure 3) is already a strong argument against contamination. As a further control, we have investigated the cleavage of substrate **10**, an oligonucleotide analogous to **9** that consists of two major parts. A 15mer DNA sequence (natural configuration) allows formation of duplexes with catalysts **4** or **5**. The second part, 14 nucleotides long and prepared from enantiomeric ribonucleotides, is completely resistant against natural ribonucleases.[6] However, it should be degraded by achiral catalysts, such as aminobenzimidazoles **1** and **2**, with the same efficiency as natural RNA.[1] Since duplexes of conjugate **4** with substrates **9** and **10** are not simply enantiomers, similar but not identical cleavage patterns are expected in both experiments. Results are shown in Figure 6. The reaction occurs after nucleotide 16 selectively, the first ribonucleotide placed in close proximity to the benzimidazole moiety (lane a). Note that the DNA part of **10** cannot be degraded. The preferred cleavage sites of the duplex **4•9** (nucleotides 13, 14, and 15) are thus inert in duplex **4•10**. No reaction is seen with the noncomplementary catalyst **8** (lane b).

Discussion

The present study was in part motivated by the difficulties in characterizing the catalytically relevant protonation state of trisbenzimidazole **2**. In contrast to compounds **2** and **3** themselves, all conjugates **4**−**8** did not aggregate with complementary or noncognate oligonucleotides. Under conditions ensuring complete hybridization of substrates, it was thus possible to establish the pH dependency of cleavage rates. Unexpectedly, rates were found to increase with increasing pH, leveling off around pH 8. This suggests that the highest activity is associated with the fully unprotonated state, in contrast to our initial design idea. Tris(aminobenzimidazoles) were considered to combine general base and general acid catalysis together with electrostatic stabilization of pentacoordinated transition states. Since conjugation with the polyanionic DNA may shift the catalyst's pK_a considerably, it seems premature, however, to rule out entirely a participation of the monoprotonated catalyst. Future experiments have to clarify if general base catalysis is sufficient to explain trisbenzimidazole-induced RNA degradation.

The study also demonstrates the high catalytic potency of tris(2-aminobenzimidazoles) in the monomeric form. At concentrations far below the critical aggregation threshold of the nonconjugated catalyst, impressive cleavage rates could be observed. Conjugates **5**−**8** also show saturation kinetics, site and substrate specificity. In typical experiments, 60−70% of the RNA substrate is cleaved within 20 h, corresponding to half-lifes of 12−17 h or first-order rate constants for substrate degradation of 0.042−0.056 h^{-1}. Successful strategies have been developed to achieve multiple substrate turnover with metal complex conjugates.[7] Adopting such strategies seems to be a promising way to further optimize the RNA cleaving potential of tris(2-aminobenzimidazole) **3**.

It is interesting to compare the efficiency of DNA−benzimidazole conjugates **4**−**8** with other oligonucleotide-based artificial nucleases.[2b,4,7−14] Most often used are metal complexes. Among such conjugates, complexes of lanthanide ions are especially successful with typical first-order rates of 0.2−0.3 h^{-1}.[10] Even faster RNA cleavage was recently achieved by the

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concerted action of two Cu(II) terpyridine complexes bound to oligonucleotides ($k_1 = 0.8\ h^{-1}$).[11i] The majority of metal ion conjugates with DNA, however, exhibit reactivities in the range of compounds **4**−**8** or below.[8a] DNA conjugates with metal-free RNA cleavers are in most cases inferior to **4**−**8**.[14] An oligoamine−PNA conjugate forms a notable exception ($k_1 = 0.09\ h^{-1}$).[14d] Very high cleavage rates have been observed in the reaction of oligonucleotide−imidazole conjugates with yeast $tRNA^{Phe}$,[14g,h] a substrate not fully comparable with the linear substrates used in most of the other studies.

Apart from applications in the antisense field, tris(benzimidazole) **3** is a promising building block to prepare a range of novel RNA cleaving conjugates where substrate specificity is achieved by RNA binding peptides and proteins, or even small molecules that exhibit affinity for selected RNA sequences. Conjugates of **3** and such RNA ligands may become valuable tools to identify their binding sites by affinity cleavage.[15,16]

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Supporting Information Available: Experimental details, spectroscopic and analytical data for all new compounds. Figure S1−S5: MALDI spectra of conjugates **4**−**8**. Figure S6: Cleavage of RNA **9** as a function of conjugate concentration. Comparison of high and low salt conditions. Figure S7: Cleavage of RNA **11** by conjugate **8**. Fourteen plots from $t = 0$ to $t = 72$ h. Figure S8: Cleavage of RNA **11** by conjugate **8**. Expanded plots of controls and of the reaction at $t = 56$ h. This material is available free of charge via the Internet at http://pubs.acs.org.

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