

Efficient new ribozyme mimics: direct mapping of molecular design principles from small molecules to macromolecular, biomimetic catalysts

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ABSTRACT

Dramatic improvements in ribozyme mimics have been achieved by employing the principles of small molecule catalysis to the design of macromolecular, biomimetic reagents. Ribozyme mimics derived from the ligand 2,9-dimethylphenanthroline (neocuproine) show at least 30-fold improvements in efficiency at sequence-specific RNA cleavage when compared with analogous *o*-phenanthroline- and terpyridine-derived reagents. The suppression of hydroxide-bridged dimers and the greater activation of coordinated water by Cu(II) neocuproine (compared with the *o*-phenanthroline and terpyridine complexes) better allow Cu(II) to reach its catalytic potential as a biomimetic RNA cleavage agent. This work demonstrates the direct mapping of molecular design principles from small-molecule cleavage to macromolecular cleavage events, generating enhanced biomimetic, sequence-specific RNA cleavage agents.

INTRODUCTION

The development of artificial nucleases (1–8) is an area of great interest in chemistry and biology today. Ribozyme mimics (see below) form one class of such biomimetic reagents (9–14). They employ a DNA (or DNA analog) strand for molecular recognition and an RNA cleavage agent for activity (typically a metal complex that catalyzes the transesterification and/or hydrolysis of RNA). Therefore, these molecules are functional mimics of ribozymes in which the large catalytic domain of the natural ribozyme has been replaced by a small molecule catalyst, and where the requirement for a properly folded tertiary structure has been eliminated. As such, ribozyme mimics effectively decouple binding and catalytic domains, and allow fundamental investigations of these separate functions.

The first example of a wholly synthetic ribozyme mimic was reported by our group (15) (Fig. 1A), with nearly simultaneous important contributions from other groups in the area (16–19). There has been continued cross-fertilization of this field as

important discoveries have been reported by academic and industrial groups (9,20–24). We have been particularly concerned with developing general principles that govern cleavage efficiency, and how and where cleavage is directed by specific molecular designs (i.e. attack via the major or minor grooves; Fig. 1) (25).

Our initial ribozyme mimic was constructed by the covalent incorporation of Cu(II)–terpyridine [Cu(II)–terpy, a well-known (26–32) RNA transesterification/hydrolysis agent] into a DNA 17mer via attachment at the C-5 position of 2'-deoxyuridine (15). The cleavage efficiency of Cu-terpy-based ribozyme mimics was later improved by attaching terpy to serinol, a 'minimal nucleotide replacement' (24,33,34). Serinol removes one Watson–Crick base pair and increases local flexibility at the site of cleavage, which may enhance the formation of the phosphorane geometries required for nucleophilic cleavage of phosphodiester.

Dramatic improvements in ribozyme mimics have been achieved by employing the principles of small molecule catalysis to the design of macromolecular, biomimetic reagents. Ribozyme mimics derived from the ligand 2,9-dimethylphenanthroline (neocuproine) show at least 30-fold improvements in efficiency at sequence-specific RNA cleavage when compared with analogous *o*-phen- and terpy-derived reagents. These results were demonstrated with a 28mer RNA target, which allowed ready identification of the cleavage products as true transesterification products, and with a 159mer fragment of the HIV *gag* gene mRNA, which demonstrated the precise specificity of cleavage in the presence of many competing sites.

MATERIALS AND METHODS

Compound 2 (serinol–neocuproine phosphoramidite building block)

Neocuproine was nitrated (8) with fuming HNO₃ and H₂SO₄ in 26% yield, then reduced to the amino compound (35) with NH₂NH₂ and Pd/C (81%). The free amine was reacted with succinic anhydride to give the amide-acid in 59% yield. EDC-HCl coupling of the amide-acid to the serinol proceeded in 63% yield. Subsequent DMT protection (51% yield) and phos-

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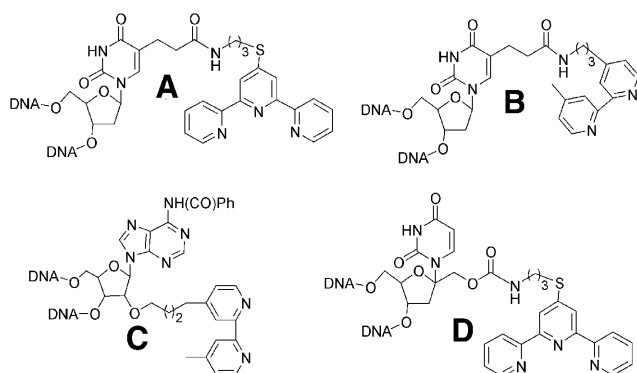


Figure 1. Examples of the catalytic cores of several ribozyme mimics. (A) First example of a wholly synthetic ribozyme mimic, a major-groove-directed terpy conjugate. (B) A major-groove-directed bipyridine conjugate. (C) A minor-groove-directed bipyridine conjugate. (D) A minor-groove-directed terpy conjugate.

phoramidation (82% yield) gave the desired building block 2 (Fig. 2).

Compound 4 (serinol–phenanthroline phosphoramidite building block)

Phenanthroline was nitrated (36) in fuming H_2SO_4 and HNO_3 in 46% yield, then reduced in a similar fashion (35) with NH_2NH_2 and Pd/C in 85% yield. Reaction with succinic anhydride (72%), EDC-HCl coupling (60%), DMT protection (64%) and phosphoramidation (73%) yielded the building block 4 (Fig. 2).

Compound 5 (serinol–6,6''-dimethyl-terpy phosphoramidite building block)

6-Methyl-2-pyridinecarboxaldehyde was oxidized with sodium chlorite and hydrogen peroxide in phosphate buffer (37) (100% yield). The corresponding acid was then esterified with $SOCl_2$ and ethanol (100% yield). The ester derivative was converted to the trione (38) in a 40% yield, and cyclization with NH_4OAc proceeded in 76% yield. Chlorination with $POCl_3$ and PCl_5 produced the 4'-chloro-6,6''-terpy derivative (33%). Nucleophilic aromatic substitution for 4-hydroxybutyric acid yielded the free acid (88%). EDC-HCl coupling to serinol (73%), DMT protection (62%) and phosphoramidation (76%) yielded the building block 5 (Fig. 2).

Preparation of the RNA

The 159mer RNA substrate was synthesized by runoff transcription using Ambion's MEGAshortscript™ T7 kit and 5'-end labeled with Ambion's KinaseMAX™ kit. The 28mer substrate was purchased (Oligos Etc.) and 5'-end labeled in a similar fashion. Both were purified by excision from a high-resolution polyacrylamide gel.

RESULTS

Observations which led to the molecular design employed

This report represents the next major enhancement to our ribozyme designs, which came about through the influence of four factors. (i) The pioneering work of Sigman and co-workers (8) on the oxidative cleavage of DNA by phenanthroline and its

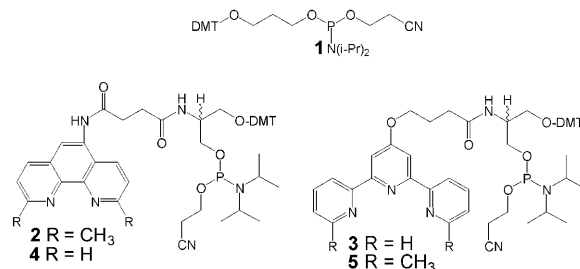


Figure 2. Phosphoramidite building blocks 1–5 used to prepare probes 1–5.

derivatives. (ii) Solution-state dimerization of the ribozyme mimics that contain an embedded terpyridine residue was observed using direct-infusion, negative-electrospray mass spectroscopy (Fig. 1). These $\mu-OH$ bridged dimers are notorious in Cu-terpyridine chemistry; furthermore, they are inactive at RNA transesterification. (iii) Ribozyme mimics constructed containing multiple terpyridine moieties were completely inactive for RNA transesterification. It is suspected that an inactive intramolecular-Cu(II) dimer was being formed (J.K.Bashkin, E.I.Frolova, A.T.Daniher, B.N.Trawick, unpublished results, see Fig. 3). (iv) Linkletter and Chin (27) reported greatly enhanced RNA transesterification by Cu(II) complexes when they employed the neocuproine ligand that suppressed the formation of Cu(II) dimers $[(bipy) Cu (\mu-OH)_2 Cu (bipy)]^{2+}$ by steric interactions.

The dramatic success achieved by Linkletter and Chin (27) is indicated by the pseudo-first-order rate constants for the cleavage of RNA dimer ApA obtained with Cu(II)-terpy, Cu(II)-bipyridine and Cu(II)-neocuproine: $1.9 \times 10^{-5} s^{-1}$, $1.9 \times 10^{-7} s^{-1}$ and $3.9 \times 10^{-3} s^{-1}$, respectively. The Cu(II)-neocuproine complex cleaved RNA between two and four orders of magnitude faster than the closely related terpy and bipy complexes.

Although results derived from RNA dimers are not always directly applicable to the cleavage of polymeric RNA substrates (i.e. mRNA) (31), we believed that a strong correlation could be made between Chin's creative results with Cu-mediated ApA cleavage (27) and our observations with macromolecular ribozyme mimics. Here we report the direct mapping of the optimization of small-molecule catalysts onto a macromolecular scaffold. We approached the problem by preparing ribozyme mimics based on terpy, 6,6''-dimethyl-terpy (dimethyl-terpy), *o*-phen and 2,9-dimethyl-*o*-phen (neocuproine). We also used a control sequence with no pendant ligand, but containing an abasic site.

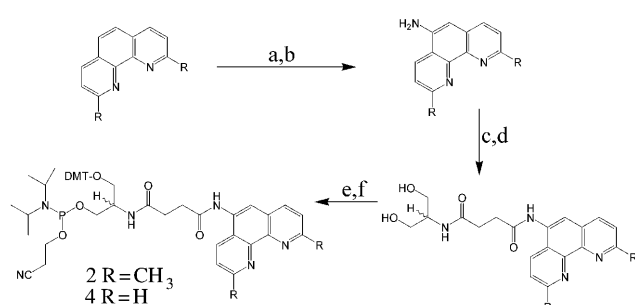
Synthesis of phosphoramidite building blocks

Probe 1, which was synthesized from the commercially available phosphoramidite building block 1, is a control probe to test for any site-specific cleavage derived solely from the presence of an abasic site (33). Phosphoramidite 3 is the previously reported serinol-terpyridine building block and was prepared as before (Fig. 2) (33). Three novel phosphoramidite building blocks derived from serinol (phosphoramidites 2, 4 and 5) were prepared. The syntheses of compounds 2 and 4 are shown in Scheme 1, and synthesis of phosphoramidite 5 is detailed in Scheme 2.

Table 1. Sequence-specific cleavage of 28mer and 159mer RNA targets by ribozyme mimics derived from Cu(II) and probes 1–5

Probe no.	Cleavage % (28mer; 15 h, 37°C)	Cleavage site (28mer)	Cleavage % (159mer; 10 h, 37°C)	Cleavage site (159mer)
1	0	n/a	0	n/a
2	65	G ¹⁵ , A ¹⁶	80	G ¹⁰⁹ , A ¹⁰⁸
3	14	A ¹⁶	8	A ¹⁰⁸
4	5	G ¹⁵ , A ¹⁶	3	G ¹⁰⁹ , A ¹⁰⁸
5	0	n/a	0	n/a

Note that probes 1 and 5 are inactive.



Scheme 1. Synthesis of *o*-phen- and neocuproine-derived phosphoramidite building blocks. Phenanthroline (R = H). (a) fum. H₂SO₄, HNO₃, 170°C, 1 h, 46%. (b) NH₂NH₂, Pd/C, EtOH, reflux, 5 h, 85%. (c) Succinic acid, pyridine, room temperature, 12 h, 72%. (d) EDC-HCl, serinol, DMF, room temperature, 12 h, 60%. (e) DMT-Cl, pyridine, room temperature, 12 h, 64%. (f) 2-Cyanoethyl *N,N*-diisopropylphosphoramidite, room temperature, 1 h, 73%. Neocuproine (R = CH₃). (a) H₂SO₄, fum. HNO₃, reflux, 5 h. (b) NH₂NH₂, Pd/C, EtOH, reflux, 5 h, 26% yield two steps. (c) Succinic acid, pyridine, room temperature, 12 h, 59% yield. (d) EDC-HCl, serinol, DMF, room temperature, 12 h. (e) DMT-Cl, pyridine, room temperature, 12 h, 49% yield two steps. (f) 2-Cyanoethyl *N,N*-diisopropylphosphoramidite, room temperature, 1 h, 82% yield.

Synthesis of ribozyme mimics

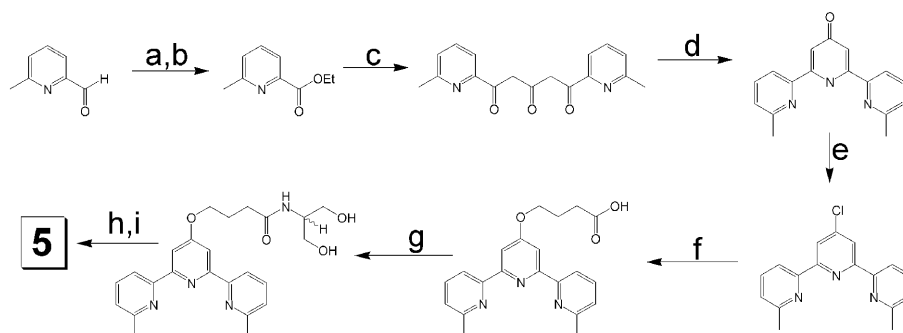
These building blocks were incorporated at the 11th position of a 17mer DNA strand by standard automated DNA synthesis. The family of sequences used is detailed here: 5'-CTA CAT AGT CXC TAA AG-3', where X = three-carbon linker (probe 1) [from phosphoramidite 1]; serinol–neocuproine (probe 2)

[from phosphoramidite 2]; serinol–terpyridine (probe 3) [from phosphoramidite 3]; serinol–phenanthroline (probe 4) [from phosphoramidite 4]; serinol–dimethylterpy (probe 5) [from phosphoramidite 5].

These probes were designed to target a 17mer region that is common to a 28mer RNA and to a 159mer fragment of the HIV *gag* gene mRNA. The 28mer RNA sequence is 5'-AAAC-CAACCCUUUAGAGACUAUGUAGAC-3' (recognition sequence underlined). The full sequence of the 159mer was published previously (15).

RNA cleavage

Figure 4 is a representative 20% polyacrylamide denaturing gel (8 M urea) of the cleavage of the 28mer RNA by the different probes when incubated with copper. Cleavage reactions were conducted with 5'-end labeled RNA at a concentration of ~10⁻⁷ M. Each reaction contained 5 μM probe, 5 μM Cu(SO₄), 0.1 M NaClO₄ and 10 mM HEPES (pH 7.4) in a total reaction volume of 10 μl at 37°C for 15 h. Cleavage of the 159mer (Fig. 5) was carried out under similar conditions, but with an RNA concentration of ~10⁻⁸ M. Two reactions were conducted with each probe: (i) in the absence of added metal and (ii) in the presence of added Cu(II)SO₄. EDTA (10 μM) was added to the control reaction of probe 3 as described previously (33) to remove trace Cu(II) scavenged from the buffers that arises due to the very strong binding of terpy for Cu(II). The extent of site-specific cleavage (for the reaction times noted) in the presence of Cu(II) is reported in Table 1.



Scheme 2. Synthesis of dimethylterpy-derived phosphoramidite building blocks. 6,6''-Dimethyl-terpy: (a) NaClO₂, NaH₂PO₄, H₂O₂, 10°C, 4 h, 100%. (b) SOCl₂, EtOH, reflux, 6 h, 100%. (c) Acetone, NaH, THF, reflux, 6 h, 40%. (d) NH₄OAc, EtOH, reflux, 6 h, 76%. (e) POCl₃, PCl₅, reflux, 12 h, 33%. (f) 4-Hydroxybutyric acid, KOH, DMSO, 55°C, 24 h, 88%. (g) EDC-HCl, HOBt, serinol, DMF, 12 h, room temperature, 73%. (h) DMT-Cl, pyridine, room temperature, 12 h, 62%. (i) 2-Cyanoethyl *N,N*-diisopropylphosphoramidite, CH₂Cl₂, 1 h, room temperature, 76%.

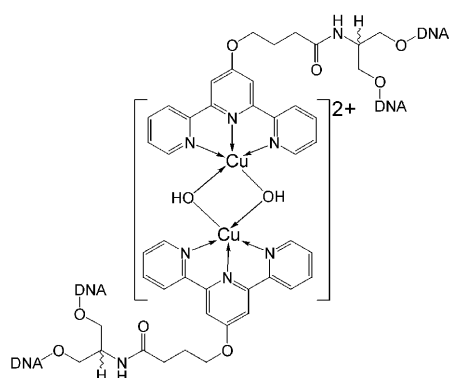


Figure 3. A representation of intermolecular ribozyme mimic dimerization through $[\text{Cu}(\text{II}) (\mu\text{-OH})_2 \text{Cu}(\text{II})]^{2+}$ centers supported by mass spectral studies.

The major site of cleavage varies by one base for *o*-phen- and terpy-derived catalysts. For example, with the 28mer RNA substrate, probe 3 cleaves to the 3'-side of A¹⁶, whereas probes 2 and 4 both cleave at the 3'-side of G¹⁵. A parallel variation in the site of cleavage is seen with of the 159mer substrate (Table 1). The cleavage products from the transesterification/hydrolysis reactions carried out by the ribozyme mimics co-migrate with both the NaOH digest and the RNase T₁ digest, as can be observed in Figure 4, a high-resolution gel for the cleavage of the 28mer. RNase T₁ cleaves RNA at G residues, leaving a guanosine-3'-monophosphate terminus and a 5'-OH terminus. Furthermore, the zinc(II) adduct of probe 3 produces the same cleavage product as the Cu(II) derivative (39). Zinc is a metal

that has no accessible oxidative cleavage pathway under our reaction conditions. For every RNA cleavage reaction, background cleavage was found at all of the 5'-UA-3' sites, in keeping with their high natural propensity for scission (17,40,41).

DISCUSSION

The enhancement in activity noted for the presence of alpha methyl groups in the case of the phenanthroline ring system (probe 4 versus probe 2) is not found in the terpy system (probe 3 versus probe 5). The dimethylterpy-derived probes may be too sterically hindered to be active.

The extent of cleavage reported here follows the trends that Linkletter and Chin (27) observed for the parent metal complexes. The ordering of the ribozyme mimics in terms of transesterification efficiency is: probe 2 (neocuproine) >> probe 3 (terpy) > probe 4 (*o*-phen) >>> probe 5 (dimethylterpy-inactive) = probe 1 (abasic control-inactive)

The reactivity trend is strongly influenced by the pK_a values of water coordinated to the copper centers [pK_a values: Cu(neocuproine) 7.0 and Cu(terpy) 8.08]. Cu(II)-neocuproine has a double advantage as a RNA transesterification agent. Under our reaction conditions, the concentration of Cu(II)-OH (the most probable active species) is much greater for neocuproine than for terpy or *o*-phen, and the methyl groups of neocuproine suppress the formation of μ-OH bridged dimers. Examining the speciation of Cu(II)-terpy with respect to the pK_a, a ratio of 17:83 of Cu(II)-OH to Cu(II)-OH₂ is expected, and with Cu(II)-neocuproine a ratio of 72:28 is expected at pH 7.4. Assuming the metal-bound hydroxide is the active species,

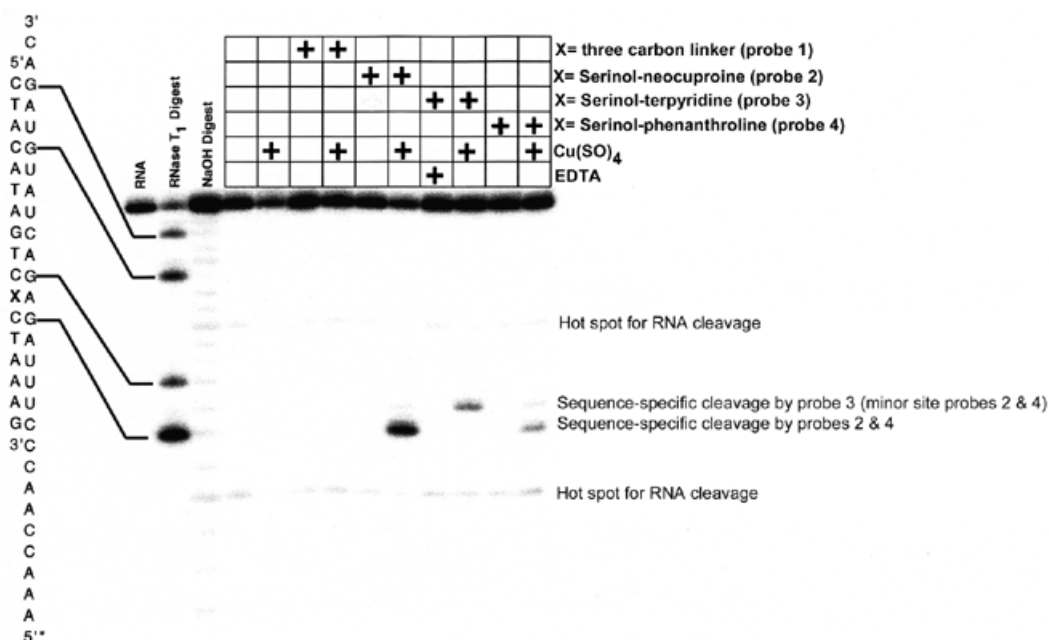


Figure 4. Autoradiogram of a 20% polyacrylamide gel (8 M urea) of the site-specific cleavage of the 5'-end-labeled 28mer RNA (0.1 μM) by ribozyme mimics (reaction conditions: 15 h, 37°C). Lane 1, unreacted RNA starting material; lane 2, ribonuclease T₁ digest (G specific); lane 3, NaOH digest; lanes 4 and 5, reaction with no ribozyme mimic present both in the absence and presence of copper; lanes 6 and 7, treatment with probe 1 [three-carbon linker (5 μM)] both in the absence and presence of copper; lanes 8 and 9, treatment with probe 2 [11.NEO (5 μM)] both in the absence and presence of copper; lanes 10 and 11, treatment with probe 3 [11.ST (5 μM)] both in the absence and presence of copper (EDTA treatment to remove trace contamination of copper); lanes 12 and 13, treatment with probe 4 [11.PHE (5 μM)] both in the absence and presence of copper.

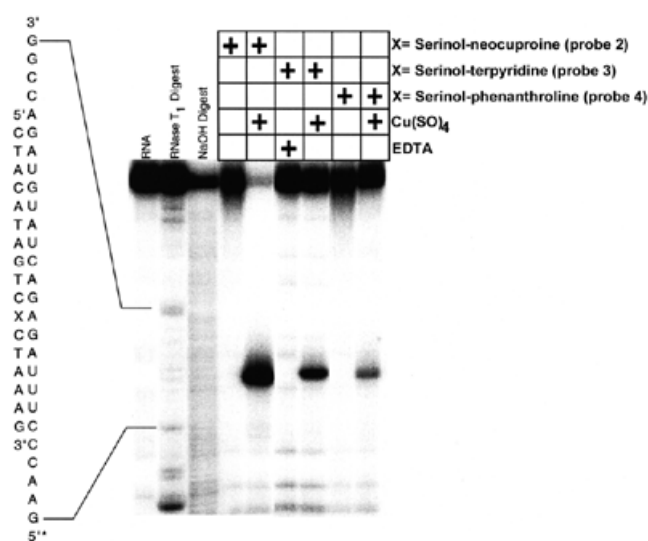


Figure 5. Autoradiogram of 6% polyacrylamide gel (8 M urea) of the site-specific cleavage of the 159mer RNA (0.01 μM) by ribozyme mimics (reaction conditions: 10 h, 37°C). Lane 1, unreacted RNA starting material; lane 2, ribonuclease T₁ digest (G specific); lane 3, NaOH digest; lanes 4 and 5, treatment with probe 2 [11.NEO (5 μM)] both in the absence and presence of copper; lanes 6 and 7, treatment with probe 3 [11.ST (5 μM)] both in the absence and presence of copper (EDTA treatment to remove trace contamination of copper); lanes 8 and 9, treatment with probe 4 [11.PHE (5 μM)] both in the absence and presence of copper.

an enhancement of 4.2-fold should be found. In both the 159mer (10-fold) and the 28mer (4.6-fold) a greater enhancement is observed. These results demonstrate the double advantage of neocuproine.

We conclude that the high concentration of Cu(II)-OH and the suppression of hydroxide-bridged dimers by neocuproine allow Cu(II) to better reach its catalytic potential as an RNA cleavage agent. This work demonstrates the direct mapping of molecular design principles from small-molecule cleavage to macromolecular cleavage events, generating enhanced biomimetic, sequence-specific RNA cleavage agents.

SUPPLEMENTARY MATERIAL

Supplementary Material, including characterization of novel compounds, is available at NAR Online.

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