Precise control of RNA cleavage by ribozyme mimics

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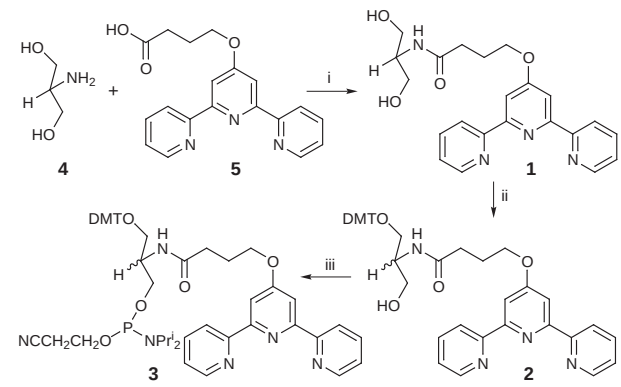
A highly-modified DNA building block, lacking both sugar and base moieties, is synthesized and incorporated into oligonucleotides to form functional mimics of ribozymes.

We demonstrate here that second-generation ribozyme mimics based on a serinol–terpyridine reagent, when incorporated into a DNA oligonucleotide, cleave their target RNA in a sequence-specific manner. Furthermore, the position of cleavage within the target sequence was precisely controlled by the location of the terpyridine within the oligonucleotide. These second-generation mimics function with greatly improved efficiency over our previous terpyridine reagents.[1,2] We attribute the improved cleavage to increased flexibility of the target RNA strand that results when serinol replaces a nucleotide in the DNA sequence: this eliminates a DNA/RNA base-pair near the cleavage site and may lower the barrier for phosphorane formation.

Functional ribozyme mimics consist of an oligonucleotide for molecular recognition and an attached RNA cleavage (transesterification) agent.[3,4] These ribozyme mimics are designed to extend the antisense approach to translation arrest by creating a catalytic cycle independent of any enzyme-mediated RNA cleavage. The antisense method is a gene-specific technique for blocking protein synthesis that inhibits the translation of mRNA into proteins.[5]

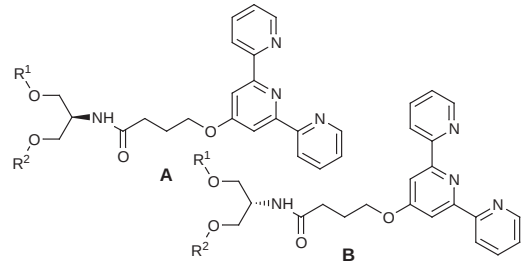
Our group previously reported the first wholly synthetic ribozyme mimic,[1] which was comprised of a 17-mer DNA probe with a pendant terpyridyl (terpy) complex of Cu^{II} incorporated in DNA *via* a thymidine derivative.[2] Aqueous (terpy)Cu^{II} is a known RNA transesterification and hydrolysis agent.[6–8]

We wished to improve cleavage efficiency and the ease of preparation of ribozyme mimics. Here we report greatly improved RNA cleavage and much simpler synthetic routes. The new reagents (Scheme 1) are conjugates of serinol and terpyridine. Serinol, a reduced form of serine, mimics the spacing of the sugar backbone of DNA; it and related compounds have previously been used as building blocks for abasic DNA sites.[9,10] An abasic DNA site eliminates one Watson–Crick base pair in a duplex and increases the conformational flexibility of the double-stranded region. The RNA in an RNA/DNA duplex is relatively inert towards cleavage when compared to its single-stranded form, perhaps because the duplex is a more conformationally rigid structure than the single strand.[11,12] Incorporation of the serinol residue into a DNA sequence allows formation of a duplex with the complementary RNA strand, and increases the flexibility of the RNA in the region opposite the serinol. This flexible RNA region should more readily form the pentacoordinate phosphorane required for transesterification, and should therefore undergo enhanced cleavage compared to a perfectly base-paired sequence.[9]



Scheme 1 *Reagents and conditions*: i, EDC, DMF, room temp., 30 h, 45.5%; ii, Py, Et_3N, DMT-Cl, 30%; iii, Et_3N, CH_2Cl_2, 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, room temp. 20 min, 47%

Derivatizing serinol in an unsymmetrical fashion gave stereoisomers **A** and **B**. Fukui suggested that related compounds, derivatives of the 2*R*,3*R* isomer of L-threoninol, preferentially target the major groove upon incorporation into DNA.[13] Since free rotation can occur in the serinol backbone, both **A** and **B** should be able to reach either groove. This paper describes work on a mixture of stereoisomers **A** and **B**.



The synthesis of **3** is shown in Scheme 1. Serinol, a *meso* compound, possesses the same spacing between alcohols (three carbons) as a normal deoxynucleoside. Serinol **4** was coupled to the terpyridine (terpy) acid **5** with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), giving **1** (45.5%). Subsequent 4,4′-dimethoxytrilyl (DMT) protection of one of the primary alcohols of **1** gave **2** in 30% yield. Phosphitylation of **2** resulted in the desired phosphoramidite **3** as a mixture of stereoisomers (47%).

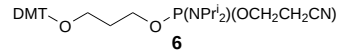
Several 17-mer probes designed to target a 159-mer fragment of the HIV *gag* gene mRNA were prepared *via* automated DNA synthesis, using **3** and/or standard nucleoside phosphoramidites. The RNA sequence is shown below, with the 17-mer recognition region underlined.

5′-$^{1(775)}$GGAGAA^{6} AUUUAUAAAA^{16} GAUGGAUAAU^{26} CCUGGGAUUA^{36}
AAUAAAAUAG^{46} UAAGAAUGUA^{56} UAGCCCUACC^{66} CAGCAUUCUG^{76}
GACAUAAGAC^{86} AAGGACCAAA^{96} GGAAC<u>CUUUA</u>^{106} <u>GAGACUAUGU</u>^{116}
<u>AGA</u>CCGGUUC^{126} UAUAAAACUC^{136} UAAGAGCCGA^{146} GCAAGCUUCA^{156}
CAG$^{159(933)}$-3′

The 17-mer DNA probe sequences, in 5′ to 3′ orientations with **X** indicating the serinol–terpy residue, are:

1a 5′-CTACATAGTCTCTAAAG-3′ **1b** **X**TACATAGTCTCTAAAG
1c CTACA**X**AGTCTCTAAAG **1d** CTACATAG**X**CTCTAAAG
1e CTACATAGTC**X**CTAAAG

Derivatives **1b**–**e** of probe **1** differ in the location of the serinol–terpy residue, but all bind to the same region of the RNA target. Control probes for **1b**–**e** [named **1(b**–**e)-ctrl**] were also prepared using **6** (Glen Research) in place of serinol–terpy reagent **3**.



6

These probes explicitly test for any enhanced cleavage activity that flexible, abasic reagents might confer on the target RNA.

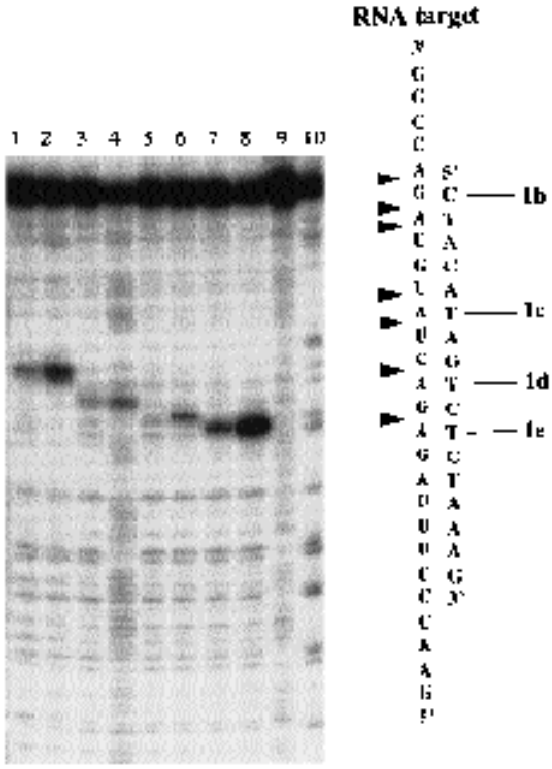


Fig. 1 Site-specific RNA cleavage with serinol–terpyridine probes. Lane 1: **1b**. Lane 2: **1b** + $CuCl_2$. Lane 3: **1c**. Lane 4: **1c** + $CuCl_2$. Lane 5: **1d**. Lane 6: **1d** + $CuCl_2$. Lane 7: **1e**. Lane 8: **1e** + $CuCl_2$. Lane 9: base. Lane 10: RNase T1. Reactions were carried out at 45 °C in a total volume of 10 μl containing the following: 0.1 M $NaClO_4$, 10 mM HEPES (pH 7.5), *ca* 10^{-9} M RNA target, 5 μM probe and 10 μM $CuCl_2$. The probes and $CuCl_2$ were premixed. The reactions were stopped after 24 h with EDTA.

Having prepared the serinol–terpy probes and appropriate controls, we investigated their inherent RNA cleavage abilities. Fig. 1 shows representative results from one set of experiments in which the cleavage of the target RNA was carried out by **1b–e**. To perform these reactions, a solution of a probe (5 μM) was combined with 5′-end labelled RNA (*ca*. 10^{-3} μM) in 10 mM HEPES buffer (pH 7.5), with 0.1 M $NaClO_4$ to control ionic strength. As indicated in Fig. 1, each experiment was done both with and without added 10 μM $CuCl_2$. Reactions were incubated at 45 °C and analyzed by electrophoresis on a denaturing 6% polyacrylamide gel.

Cleavage resulting from the various serinol–terpy-containing probes occurred at the RNA nucleotides opposite the serinol–terpy groups. As the terpy was moved from one internal position to the next, the cleavage followed in a precise manner (Fig. 1). Cleavage occurred whether the terpy was at an internal or external position of the RNA/DNA duplex. No specific cleavage occurred for the unmodified DNA control probe **1a** or the controls **1(b–e)-ctrl** (data not shown). Thus, the flexible, abasic site generated by **6** did not promote cleavage of the RNA target.

The amount and the location of cleavage depended on the serinol–terpy probe used and the temperature. However, the major determining factor was the primary sequence and location of the catalyst. Cleavage sites were identified by comparison with RNase Tl and base hydrolysis lanes (Fig. 1 and Table 1). Generally, cleavage occurred to the 3′- and 5′-sides of the RNA nucleotide opposite serinol, and spanned from one to three positions. Apparent background cleavage is seen in Fig. 1 in those lanes (1,3,5 and 7) corresponding to reactions with no added Cu^{II}. Additional experiments showed that this background cleavage derives from Cu^{II} ion that is scavenged by the terpy-containing DNA probes during synthesis and purification (data not shown). Treating the probes with EDTA prior to reaction eliminated the background cleavage. Adding Cu^{II} (but not divalent Fe, Mg, Pb or Zn) recovered the cleavage activity of the EDTA-treated ribozyme mimics (data not shown).

Table 1 Cleavage results from sequence-specific reactions of ribozyme mimics with the 159-mer target RNA, in the presence of Cu^{II} ion

		% Total cleavage	
Probe	Cleavage Sites	24 h	72 h
1b	G^{118}, A^{117}, U^{116}	18	48
1c	A^{113}, U^{112}	10	35
1d	A^{110}	10	40
1e	A^{108}	48	84

The synthesis of the serinol–terpyridine building block allows the introduction of cleaving agents anywhere within a DNA probe. This approach provides a much more efficient synthetic means of incorporating terpy into oligonucleotides than the preparation of intact nucleoside derivatives. We observed a marked increase in cleavage efficiency compared to our first-generation ribozyme mimics. Our first mimic cleaved 28% of its target after 3 days at 45 °C. Our best second-generation mimic cleaved 84% of its target after 3 days at 45 °C. The serinol backbone may enhance transesterification because it creates an artificial abasic site when incorporated into an oligonucleotide and duplexed to RNA. The RNA site opposite this modified monomer is not base-paired, so it has greater flexibility. This flexibility should allow the terpy ligand to span both the major and minor grooves in the RNA/DNA duplex, and should allow the RNA to adopt reactive conformations. The serinol–terpyridine ribozyme mimics are consistent with our belief that cleavage must occur within the duplex region in order to free the mimic for catalytic turnover.

This work was supported in part by NSF Grant CHE-9318581. Acknowledgment is also made to the donors of The Petroleum Research Fund, administered by the ACS, for partial support of this research.

Notes and References

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Received in Bloomington, IN, USA, 30th January 1998; 8/00818C