Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 5754–5760
Bioorganic & Medicinal Chemistry

Synthesis and biochemical application of 2′-*O*-methyl-3′-thioguanosine as a probe to explore group I intron catalysis

Jun Lu, Nan-Sheng Li, Raghuvir N. Sengupta and Joseph A. Piccirilli*

Howard Hughes Medical Institute, Departments of Biochemistry & Molecular Biology, and Chemistry, The University of Chicago, 929 East 57th Street, Chicago, IL 60637, USA

Received 8 February 2008; accepted 24 March 2008
Available online 27 March 2008

Abstract—Oligonucleotides containing 3′-*S*-phosphorothiolate linkages provide valuable analogues for exploring the catalytic mechanisms of enzymes and ribozymes, both to identify catalytic metal ions and to probe hydrogen-bonding interactions. Here, we have synthesized 2′-*O*-methyl-3′-thioguanosine to test a possible hydrogen-bonding interaction in the *Tetrahymena* ribozyme reaction. We developed an efficient method for the synthesis of 2′-*O*-methyl-3′-thioguanosine phosphoramidite in eight steps starting from 2′-*O*-methyl-N^2-(isobutyryl) guanosine with 10.4% overall yield. Following incorporation into oligonucleotides using solid-phase synthesis, we used this new analogue to investigate whether the 3′-oxygen of the guanosine cofactor in the *Tetrahymena* ribozyme reaction serves as an acceptor for the hydrogen bond donated by the adjacent 2′-hydroxyl group. We show that regardless of whether the guanosine cofactor bears a 3′-oxygen or 3′-sulfur leaving group, replacing the adjacent 2′-hydroxyl group with a 2′-methoxy group incurs the same energetic penalty, providing evidence against an interaction. These results indicate that the hydrogen bond donated by the guanosine 2′-hydroxyl group contributes to catalytic function in a manner distinct from the U_{-1} 2′-hydroxyl group.

1. Introduction

Oligonucleotides containing 3′-*S*-phosphorothiolate linkages (Fig. 1), in which a sulfur atom replaces the 3′-bridging oxygen atom of the phosphodiester linkage, provide valuable analogues for studying RNA structure and function.[1–14] Oligodeoxynucleotides containing this linkage have been used to investigate the resolution of the Holliday junction by RuvC, DNA repair by *Escherichia coli* DNA T:G mismatch endonuclease, and the nucleolytic activity of *E. coli* DNA polymerase I and the restriction endonuclease EcoRV.[1,4,9–11,14,15] Phosphorothiolate linkages have also yielded fundamental insights into the catalytic mechanisms of RNA enzymes and RNA splicing machineries, revealing the participation of metal ions and hydrogen bonds in their catalytic function.[2,3,5,7,8,16–21] With respect to the self-splicing group I intron, which has served as a model system for studying the chemistry of RNA splicing and the principles of phosphoryl transfer reactions, crystallographic and biochemical analyses, including studies that

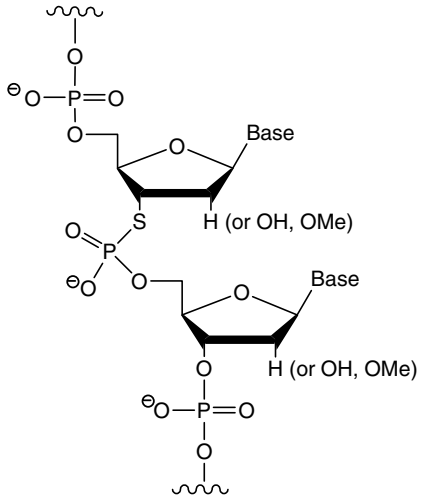


Figure 1. A 3′-*S*-phosphorothiolate linkage.

Keywords: Group I intron; RNA catalysis; Thionucleosides; Atomic mutation cycle; 3′-*S*-Phosphorothioamidite.
* Corresponding author. Fax: +1 773 7020271; e-mail: jpicciri@uchicago.edu

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.03.061

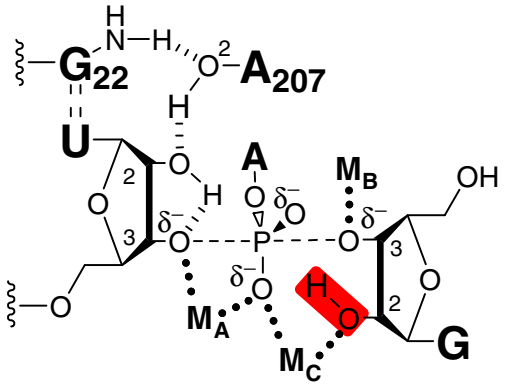


Figure 2. Transition state model of phosphoryl transfer catalyzed by the *Tetrahymena* group I ribozyme. M_A, M_B, and M_C represent the catalytic metal ions in the active site. The red rectangle highlights the 2′-hydroxyl group of the guanosine nucleophile under investigation. Hatched lines indicate putative hydrogen bonds, and dots symbolize metal ion coordination. Figure taken by permission from Ref. 27.

utilize phosphorothiolates, have revealed a complex network of interactions within the active site of this ribozyme (Fig. 2).[22–24] Developing new methods to identify and characterize these interaction networks remains imperative to understanding catalysis by this and other RNAs.

We previously devised an atomic mutation cycle (AMC) to determine whether a RNA 2′-hydroxyl group donates a functionally important hydrogen bond (Fig. 3).[25–27] AMC analysis requires the construction of three analogues bearing modifications at the 2′-position: $-OCH_3$, $-NH_2$, and $-NHCH_3$. When the energetic penalty for the 2′-OH to 2′-OCH_3 substitution ($\Delta\Delta G_{OH \rightarrow OCH_3}$) exceeds that for the 2′-NH_2 to 2′-$NHCH_3$ substitution ($\Delta\Delta G_{NH_2 \rightarrow NHCH3}$), we attribute the difference to the absence of the hydrogen atom ($\Delta G_{H\ removal}$) from the 2′-OCH_3 analogue. When the absence of the hydrogen atom engenders an energetic penalty ($\Delta G_{H\ removal} > 0$), we infer that the 2′-hydroxyl

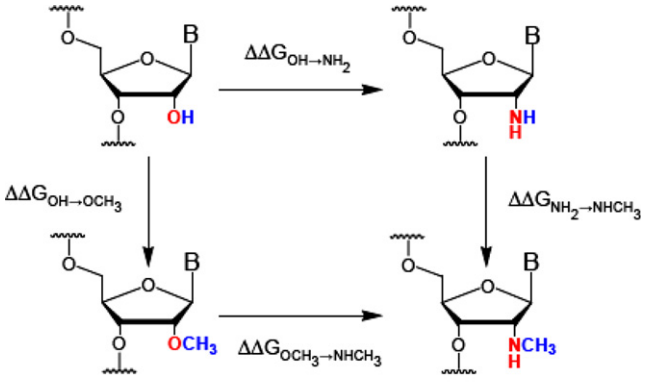


Figure 3. Atomic mutation cycle analysis of an RNA 2′-hydroxyl group.

group donates a hydrogen bond. We have used this strategy with success to investigate the 2′-hydroxyl groups along the reaction coordinate in the *Tetrahymena* ribozyme reaction. The cleavage site uridine (U_{-1}) and the guanosine nucleophile 2′-hydroxyl groups appear to donate hydrogen bonds in the transition state of the reaction, yielding $\Delta G_{H\ removal}$ values of 2.5 and 3.5 kcal/mol, respectively.[27]

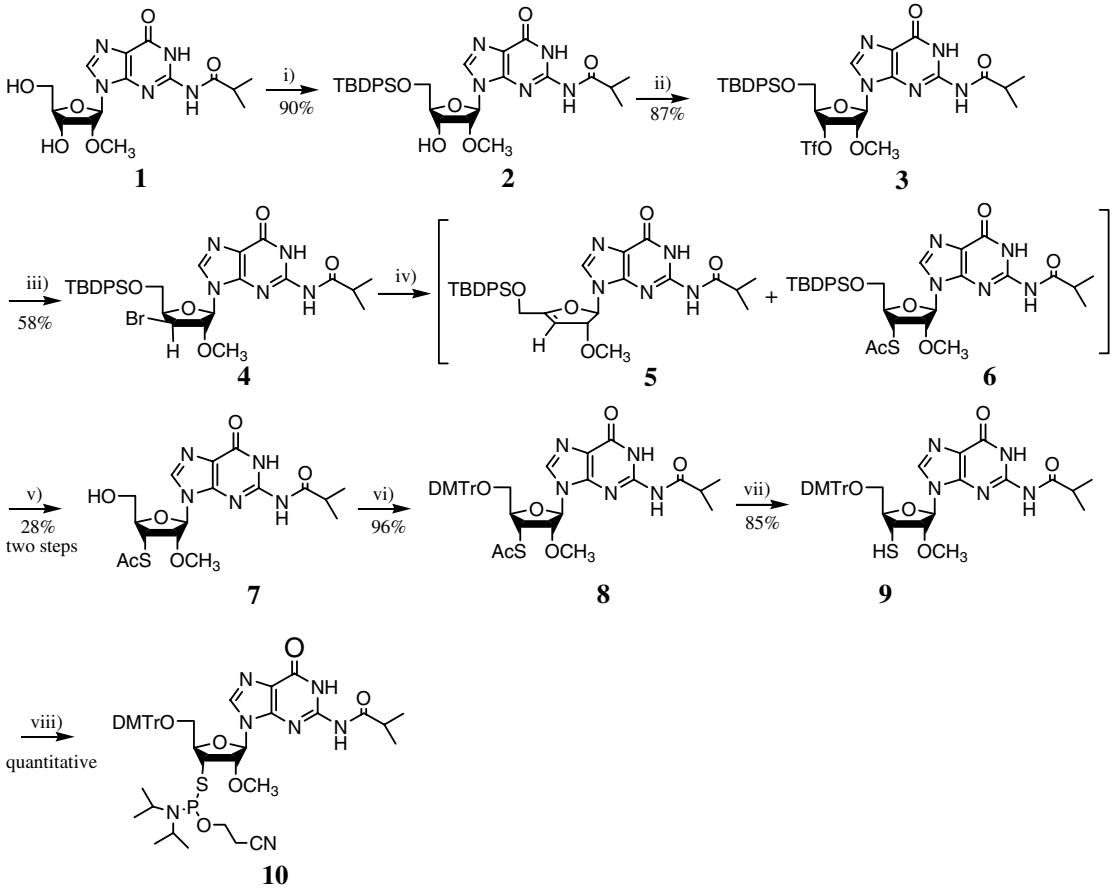
As the U_{-1} 2′-hydroxyl group donates a hydrogen bond to the adjacent 3′-oxygen atom in the transition state of the ribozyme reaction (Fig. 2), we wanted to test whether the analogous interaction involving the guanosine 2′-hydroxyl group also occurs.[28] AMC analysis also offers a strategy to identify the corresponding hydrogen bond acceptor. Mutations that disrupt a hydrogen bond interaction between the 2′-hydroxyl group and a potential acceptor should attenuate the cost of methoxy substitution ($\Delta\Delta G_{OH \rightarrow OCH_3}$) and, therefore, decrease the value of $\Delta G_{H\ removal}$, as the ribozyme bearing the natural nucleotide no longer gains a catalytic advantage from hydrogen bond donation by the 2′-hydroxyl group.[27] To test whether the guanosine nucleophile 2′-hydroxyl group donates a hydrogen bond to the adjacent 3′-oxygen during the reaction, we plan to perform AMC analysis in the context of a 3′-sulfur atom. Sulfur is a weak hydrogen bond acceptor compared to oxygen.[28–33] Therefore, if the guanosine 2′-hydroxyl group donates a hydrogen bond to the 3′-oxygen during the reaction, $\Delta\Delta G_{OH \rightarrow OCH_3}$ and $\Delta G_{H\ removal}$ should decrease in a 3′-sulfur background. To conduct AMC analysis, the four 3′-thioguanosine analogues corresponding to those in Figure 3 must be prepared. As a starting point for this analysis, here we describe the synthesis of one of these analogues and its corresponding phosphoramidite, 2′-methoxy-3′-thioguanosine.[†] Following solid-phase oligonucleotide synthesis, we measured the reactivity of this analogue in the *Tetrahymena* ribozyme reaction and found that the energetic cost of methoxy substitution ($\Delta\Delta G_{OH \rightarrow OCH_3}$) remains the same regardless of the identity of 3′-atom (oxygen/sulfur). These results provide evidence against a hydrogen bond interaction between the 2′-hydroxyl group and the 3′-oxygen atom of the guanosine nucleophile.

2. Results and discussion

2.1. Synthesis of 2′-*O*-methyl-3′-thioguanosine phosphoramidite

In 1999, Matulic-Adamic reported the synthesis of 3′-thioguanosine and its 3′-phosphorothioamidite starting from guanosine.[34] The reaction of 5′-N^2-protected guanosine with 2-acetoxyisobutyryl bromide afforded the 3′-β-bromo-derivative stereoselectively, which was converted to 3′-*S*-acyl ribofuranosyl intermediates. The *S*-acylated nucleosides were then converted in seven steps

[†] The preparation of 3′-thioguanosine was previously described.[34,43] The syntheses of 2′-amino-3′-thioguanosine and 2′-methylamino-3′-thioguanosine are yet to be published.



Scheme 1. Reagents and conditions: (i) $^{t}BuPh_2SiCl$, Py, rt, 20 h; (ii) CF_3SO_2Cl, DMAP, CH_2Cl_2 0 °C, 2 h; (iii) NaBr, CH_3COCH_3, reflux, 3 h; (iv) KSAc, DMF, 60 °C, 24 h; (v) $Bu_4NF{\cdot}3H_2O$, AcOH, THF, rt, 36 h; (vi) $(MeO)_2TrCl$, Py, rt, 24 h; (vii) Guanidine hydrochloride/NaOMe (4:1), $MeOH/CH_2Cl_2$ (1:1), rt, 30 min; (viii) $(^{i}Pr)_2NP(Cl)OC_2H_4CN$, $^{i}Pr_2NEt$, 1-methyl-1*H*-imidazole, rt, 1 h.

to the 3′-*S*-phosphorothioamidite in 13% overall yield from guanosine. The corresponding 2′-*O*-methyl-3′-thioguanosine phosphoramidite has not been reported. We adapted Matulic-Adamic's synthetic route to the synthesis of 2′-*O*-methyl-3′-thioguanosine phosphoramidite **10**.

Our synthesis begins with the commercially available 2′-*O*-methyl-N^2-isobutyrylguanosine **1** (Scheme 1). Reaction of **1** with (*tert*-butyl)chlorodiphenylsilyl ($^{t}BuPh_2SiCl$) gave the corresponding 5′-silyl derivative **2** in 90% yield. In the presence of 4-(dimethylamino)pyridine (DMAP), the treatment of compound **2** with trifluoromethanesulfonyl chloride in CH_2Cl_2 at 0 °C gave the 3′-triflate derivative **3** in 83% yield.[35] Subsequent S_N2 substitution with NaBr in acetone afforded the desired 3′-bromo derivative **4** in 58% yield. The treatment of **4** with KSAc in DMF at 60 °C yielded a mixture of the desired 3′-*S*-acetyl derivative **6** and the 3′,4′-unsaturated derivative **5**, which forms via a competing elimination reaction.[34] According to the ^1H NMR spectra, **6** and **5** form in a 9:5 ratio. These two compounds could not be separated by silica gel chromatography. We treated the mixture of **5** and **6** with $Bu_4NF{\cdot}3H_2O$ and AcOH in THF and subsequently isolated the desired 5′-deprotected derivative **7** in 28% yield (two steps). During the deprotection, the 3′,4′-unsaturated derivative decomposed and therefore could not be isolated. The protection of **7** as the 5′-*O*-dimethoxytrityl ether was performed in dry pyridine for 24 h by using excess 4,4′-dimethoxytritylchloride (DMTrCl, 3 equiv), giving 5′-DMTr protected compound **8** in 96% yield.

To prepare 3′-phosphorothioamidite **10**, the selective removal of the 3′-*S*-acetyl group of compound **8** must occur without the loss of the N^2-isobutyryl group. A solution of NaOMe and guanidine hydrochloride in methanol proved to be effective.[36] The treatment of **8** with a 4:1 mixture of guanidine hydrochloride and 25% NaOMe in methanol produced 3′-SH derivative **9** in 85% yield. Phosphitylation of compound **9** under standard conditions gave quantitative conversion to 3′-phosphorothioamidite **10**. We subsequently incorporated the 2′-*O*-methyl-3′-thioguanosine phosphoramidite into the oligonucleotide sequence, $CUCG_{3'S,2'OCH_3}A$, as previously described.[43] Based on trityl yield data, coupling efficiencies throughout oligonucleotide synthesis were excellent (data not shown).

2.2. *Tetrahymena* ribozyme reactions with substrates bearing guanosine ($G_{2'OH}$), 2′-*O*-methylguanosine ($G_{2'OCH_3}$), 3′-thioguanosine ($G_{3'S,2'OH}$), and 2′-*O*-methyl-3′-thioguanosine ($G_{3'S,2'OCH_3}$)

Following oligonucleotide synthesis and deprotection, we determined the effect of the 3′-sulfur atom on 2′-methoxy

substitution in the *Tetrahymena* ribozyme reaction. We utilized a reaction that mimics the second step of group I intron self-splicing in which an oligonucleotide product (CCCUCU) cleaves a specific phosphodiester bond of CUCGA, a 3′-splice site analogue (Eq. 1).[37–41]

$$\mathrm{CCCUCU} + \mathrm{CUCG_pA} \xrightarrow{\mathrm{E}} \mathrm{CCCUCU_pA} + \mathrm{CUGG} \quad (1)$$

We measured the rates of cleavage for $\mathrm{CUCG_{2'OH}A}$ and $\mathrm{CUCG_{2'OCH_3}A}$ to obtain the cost of 2′-methoxy substitution in the context of a 3′-oxygen atom. Under conditions where chemistry is rate-limiting, 2′-methoxy substitution has a 6.9 kcal/mol (8.8×10^4-fold) effect on reactivity ($\Delta\Delta G_{\mathrm{OH} \rightarrow \mathrm{OCH_3}}$) (Fig. 4). This effect remains similar in the context of the 3′-sulfur atom: under the same conditions $\mathrm{CUCG_{3'S,2'OCH_3}A}$ reacts 3.1×10^4-fold slower than does $\mathrm{CUCG_{3'S,2'OH}A}$, indicating that the replacement of the 2′-OH group with a 2′-OCH_3 group imposes a 6.2 kcal/mol effect on reactivity ($\Delta\Delta G_{\mathrm{OH} \rightarrow \mathrm{OCH_3}}$) (Fig. 4). If the 2′-hydroxyl group were donating a hydrogen bond to the adjacent 3′-oxygen, sulfur substitution at the 3′-position would be expected to disrupt this interaction and therefore diminish the catalytic advantage of the 2′-OH group over the 2′-OCH_3 group. However, as sulfur substitution only has a ~3-fold (~0.7 kcal/mol) effect on $\Delta\Delta G_{\mathrm{OH} \rightarrow \mathrm{OCH_3}}$, our results provide evidence against this interaction, suggesting that another neighboring group must accept the hydrogen bond from the 2′-hydroxyl group.

3. Conclusions

We established an efficient method for the synthesis of 2′-*O*-methyl-3′-thioguanosine phosphoramidite **10** in 10.4% overall yield starting from 2′-*O*-methyl-N^2-isobutyrylguanosine. Using solid-phase methods, we achieved efficient incorporation of the 2′-*O*-methyl-3′-thioguanosine phosphoramidite into RNA. We have also utilized this analogue to investigate a potential hydrogen bond interaction in the *Tetrahymena* ribozyme reaction. Our results provide evidence against a model in which the guanosine 3′-oxygen accepts a hydrogen bond from the adjacent 2′-hydroxyl group. Thus, the hydrogen bond donated by the guanosine 2′-hydroxyl group contributes to catalytic function in a manner distinct from the U_{-1} 2′-hydroxyl group. Further analysis will be required to identify its hydrogen-bonding partner.

4. Experimental

4.1. Chemistry

All reactions were carried out under a positive pressure of Ar in anhydrous solvents. Commercially available reagents and anhydrous solvents were used without further purification. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a Bruker 500 or Bruker 400 MHz NMR spectrometer. ^{1}H chemical shifts are reported in δ (ppm) relative to tetramethylsilane and ^{13}C chemical shifts δ (ppm) relative to the solvent used. High resolution mass spectra were obtained from the Department of Chemistry, University of California at Riverside, on the VG-ZAB instrument.

4.1.1. 5′-*O*-(*tert*-Butyl)diphenylsilyl-2′-*O*-methyl-N^2-isobutyrylguanosine (2). To a stirred solution of 2′-*O*-methyl-N^2-isobutyrylguanosine **1** (2.02 g, 5.49 mmol) in pyridine (20 mL), (*tert*-butyl)diphenylsilyl chloride (2.25 mL, 8.23 mmol) was added under Ar. The mixture was stirred at room temperature for 20 h, then quenched with MeOH (4 mL), and evaporated to a syrup. The residue was dissolved in $CHCl_3$ (40 mL) and washed with H_2O. The organic layer was dried over anhydrous sodium sulfate. The sodium sulfate was filtered off, and the solvent was removed by evaporation under vacuum. The residue was purified by silica gel chromatography, eluting with 2% methanol in chloroform, to give the product as a white foam: 2.99 g (90% yield). ^{1}H NMR ($CDCl_3$): δ 12.16 (s, 1H), 9.10 (s, 1H), 7.97 (s, 1H), 7.65–7.33 (m, 10H), 5.94 (d, 1H, J = 5.0 Hz), 4.57 (t, 1H, J = 5.0 Hz), 4.17 (t, 1H, J = 5.0 Hz), 4.13 (m, 1H), 3.98 (dd, 1H, J = 2.5, 11.5 Hz), 3.85 (dd, 1H, J = 3.5, 11.5 Hz), 3.43 (s, 3H), 2.67 (m, 1H), 1.22 (d, 6H, J = 6.5 Hz), 1.06 (s, 9H). ^{13}C NMR ($CDCl_3$): δ 178.9, 155.5, 148.1, 147.8, 137.1, 135.6, 135.4, 132.6, 132.4, 130.0, 129.9, 127.9, 127.8, 121.4, 85.7, 85.1, 84.1, 69.3, 63.5, 58.8, 36.3, 26.9, 19.2, 19.0, 18.9. HRMS (FAB^+): *m*/*z* calcd for $C_{31}H_{40}N_5O_6Si$ [M+H$^+$]: 606.2748; found: 606.2760.

4.1.2. 5′-*O*-(*tert*-Butyl)diphenylsilyl-2′-*O*-methyl-3′-*O*-trifluorosulfonyl-N^2-isobutyryl guanosine (3). To a solution of **2** (606 mg, 1.0 mmol) and DMAP (244 mg, 2 mmol) in dry MeCN (6 mL), CF_3SO_2Cl (0.16 mL, 1.5 mmol) was added at 0 °C under Ar. After the mixture was stirred at 0 °C for 2 h, the reaction was quenched with ice water (5 mL) and the mixture was stirred for 15 min. The reaction mixture was diluted with CH_2Cl_2 (20 mL). The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (2× 30 mL). The organic layers were combined and washed with H_2O and brine and subsequently dried over anhydrous sodium sulfate. The sodium sulfate was filtered off, and the solvent was removed by evaporation under vacuum. The residue was purified by silica gel chromatography, eluting with 2% methanol in chloroform, to give the product as a white foam: 644 mg (87% yield). ^{1}H NMR ($CDCl_3$): δ 12.02 (s, 1H), 8.38 (s, 1H), 7.83 (s, 1H), 7.63–7.36 (m, 10H), 5.90 (d, 1H, J = 6.5 Hz), 5.75 (t, 1H, J = 4.0 Hz), 4.59 (t, 1H, J = 5.5 Hz), 4.38 (m, 1H), 3.96 (dd, 1H, J = 3.0, 12.0 Hz), 3.84 (dd, 1H, J = 3.0, 12.0 Hz), 3.42 (s, 3H), 2.54 (m, 1H), 1.24 (d, 6H, J = 6.5 Hz), 1.04 (s, 9H). ^{13}C NMR ($CDCl_3$): δ 178.3, 155.2, 148.1, 147.7, 137.0, 135.4, 135.3, 131.9, 131.8, 130.3, 130.2, 128.0, 121.7, 84.9, 83.2, 81.9, 81.3, 77.3, 77.0, 62.3, 59.4, 36.5, 26.7, 19.1, 18.9, 18.8. HRMS (FAB^+): *m*/*z* calcd for $C_{32}H_{39}N_5O_8F_3SiS$, [M+H$^+$]: 738.2210; found: 738.2235.

4.1.3. 5′-*O*-(*tert*-Butyl)diphenylsilyl-2′-*O*-methyl-3′-β-bromo-N^2-isobutyryl-guanosine (4). A mixture of **3** (1.56 g, 2.12 mmol) and NaBr (0.87 g, 8.47 mmol) in acetone (20 mL) was heated under reflux for 3 h under Ar. The solvent was removed under reduced pressure,

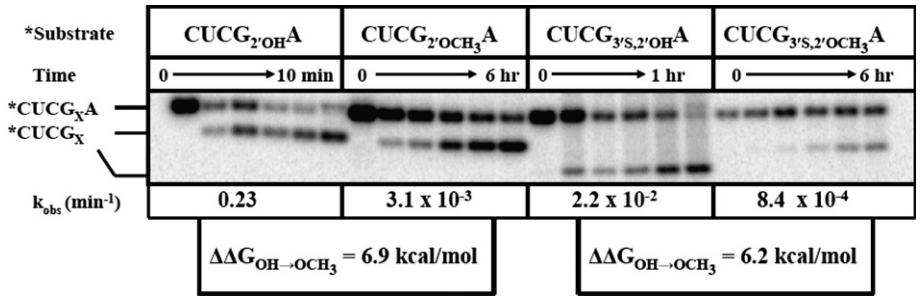


Figure 4. Ribozyme reactions with $CUCG_{2'OH}A$, $CUCG_{2'OCH_3}A$, $CUCG_{3'S,2'OH}A$ and $CUCG_{3'S,2'OCH_3}A$. Reactions took place in the presence of saturating ribozyme and product to form the EP complex. $CUCG_XA$ substrates were 5′-^{32}P-labeled (denoted by the asterisk). For ribozyme reactions with the phosphorothiolate substrates, we observed that $CUCG_{3'SH,2'OCH_3}$ migrated slower than $CUCG_{3'SH,2'OH}$. To confirm the presence of the free thiol, we treated the ribozyme-catalyzed products with iodoacetamide, an alkylating sulfhydryl reagent (data not shown). Both $CUCG_{3'SH,2'OH}$ and $CUCG_{3'SH,2'OCH_3}$ were susceptible to iodoacetamide modification and, upon alkylation, comigrated (data not shown).

and the residue was purified by silica gel chromatography, eluting with 1–3% methanol in chloroform, to give the product as a white foam: 0.822 g (58% yield). ^{1}H NMR ($CDCl_3$): δ 12.14 (s, 1H), 8.95 (s, 1H), 7.97 (s, 1H), 7.70–7.37 (m, 10H), 5.84 (d, 1H, J = 1.5 Hz), 4.41 (d, 1H, J = 3.5 Hz), 4.36 (m, 2H), 4.02 (dd, 1H, J = 5.5, 10.5 Hz), 3.96 (dd, 1H, J = 6.0, 10.5 Hz), 3.47 (s, 3H), 2.66 (m, 1H), 1.23 (d, 6H, J = 5.5 Hz), 1.07 (s, 9H). ^{13}C NMR ($CDCl_3$): δ 178.6, 155.6, 147.8, 147.7, 137.3, 135.5, 135.4, 132.8, 132.7, 130.0, 129.9, 127.8, 127.7, 121.2, 92.2, 88.8, 81.3, 64.5, 58.4, 50.2, 36.4, 26.8, 19.2, 19.0, 18.9. HRMS (FAB^+): *m/z* calcd for $C_{31}H_{39}N_5O_5F_3SiBr$ [M+H$^+$]: 668.1898; found: 668.1913.

4.1.4. 5′-*O*-(*tert*-Butyl)diphenylsilyl-2′-*O*-methyl-3′-deoxy-β-D-glycero-pent-3′-enofuranosyl-N^2-isobutyrylguanosine (5) and 5′-*O*-(*tert*-butyl) diphenyl-silyl-2′-*O*-methyl-3′-*S*-acetyl-N^2-isobutyrylguanosine (6). To a solution of **4** (49 mg, 0.073 mmol) in dry DMF (2 mL), potassium thioacetate (25 mg, 0.22 mmol) was added, and the mixture was stirred at 60 °C for 24 h and evaporated to a syrup. The residue was partitioned between an aqueous $NaHCO_3$ solution/brine (v/v, 1:1) and CH_2Cl_2; the organic layer was dried (Na_2SO_4) and evaporated. The residue was purified by silica gel chromatography; eluting with 1–2% methanol in chloroform, to give a mixture of **5** and **6** (35 mg) (**5/6** 5:9 based on ^{1}H NMR). ^{1}H NMR ($CDCl_3$): δ 12.08 (s, 1H, **5**), 11.91 (s, 1H, **6**), 9.24 (s, 1H, **6**), 8.71 (s, 1H, **5**), 7.99 (s, 1H, **5**), 7.86 (s, 1H, **6**), 7.28–7.70 (m, 20H, **5** + **6**), 6.29 (d, 1H, J = 1.6 Hz, **5**), 5.89 (s, 1H, **6**), 5.63 (m, 1H, **6**), 5.43 (t, 1H, J = 1.1 Hz, **5**), 4.66 (s, 1H, **5**), 4.43 (d, 1H, J = 4.7 Hz, **6**), 4.29 (d, 1H, J = 9.0 Hz, **6**), 4.13 (m, 1H, **5**), 3.86 (dd, 1H, J = 2.1, 11.9 Hz, **6**), 3.71 (dd, 1H, J = 3.5, 11.9 Hz, **6**), 3.50 (s, 3H, **6**), 3.34 (s, 3H, **5**), 2.67 (m, 1H, **5**), 2.61 (m, 1H, **6**), 2.44 (s, 3H, **6**), 1.21–1.27 (m, 12H, **5** + **6**), 1.07 (s, 9H, **5**), 0.88 (s, 9H, **6**).

4.1.5. 3′-*S*-Acetyl-2′-*O*-methyl-N^2-isobutyryl-3′-thioguanosine (7). To the above mixture **5/6** (0.90 g) in THF (15 mL), AcOH (0.40 mL, 6.5 mmol) was added, followed by $Bu_4NF{\cdot}3H_2O$ (0.80 g, 2.6 mmol). The mixture was stirred at room temperature for 24 h, then diluted with CH_2Cl_2, and washed with H_2O and 10% aq $NaHCO_3$. The aqueous layers were extracted with CH_2Cl_2 and the combined organic layers were dried (Na_2SO_4). Following the removal of the solvent by evaporation under vacuum, the resulting residue was purified by silica gel chromatography, eluting with 2–4% methanol in chloroform, to give product **7** as a yellow foam: (137 mg, 28% from **4**). ^{1}H NMR: δ 12.04 (s, 1H), 9.31 (s, 1H), 7.97 (s, 1H), 5.86 (d, 1H, J = 2.0 Hz), 5.06 (dd, 1H, J = 5.5, 9.0 Hz), 4.31 (d, 1H, J = 5.5 Hz), 4.21 (d, 1H, J = 9.0 Hz), 3.97 (d, 1H, J = 13.0 Hz), 3.72 (d, 1H, J = 13.0 Hz), 3.46 (s, 3H), 2.74 (m, 1H), 2.44 (s, 3H), 1.28 (d, 6H, J = 7.0 Hz). ^{13}C NMR ($CDCl_3$): δ 196.2, 179.6, 155.4, 147.8, 147.3, 138.4, 121.2, 88.2, 85.1, 85.0, 60.8, 58.7, 43.4, 36.1, 30.6, 19.0, 18.8. HRMS (FAB^+): *m/z* calcd for $C_{17}H_{24}N_5O_6S$ [M+H$^+$]: 426.1447; found: 426.1430.

4.1.6. 5′-*O*-(4,4′-Dimethoxytrityl)-3′-*S*-acetyl-2′-*O*-Methyl-N^2-isobutyryl-3′-thioguanosine (8). To a solution of **7** (48 mg, 0.11 mmol) in dry pyridine (2 mL), DMTrCl (115 mg, 0.34 mmol) was added. The mixture was stirred at room temperature for 20 h, quenched with MeOH, and evaporated to a syrup which was partitioned between 5% aq $NaHCO_3$ and CH_2Cl_2. The organic layer was washed with brine, dried (Na_2SO_4), and evaporated, and the residue was purified by chromatography (silica gel, gradient 0–2%) MeOH/CH_2Cl_2) with 0.5% Et_3N to give compound **8** (79 mg, 96% yield). ^{1}H NMR: δ 7.88 (s, 1H), 7.15–7.31 (m, 9H), 6.71–6.73 (m, 4H), 5.90 (s, 1H), 5.58 (m, 1H), 4.36 (d, 1H, J = 5.0 Hz), 4.17 (dd, 1H, J = 3.0, 11.0 Hz), 3.76 (s, 6H), 3.47 (s, 3H), 3.45 (m, 1H), 3.15 (m, 1H), 2.55 (m, 1H), 2.35 (s, 3H), 1.18 (d, 3H, J = 7.0 Hz), 1.12 (d, 3H, J = 7.0 Hz). ^{13}C NMR ($CDCl_3$): δ 195.9, 178.7, 158.3, 155.6, 147.4, 147.3, 144.3, 138.7, 135.6, 135.5, 129.9, 128.0, 127.6, 126.7, 121.9, 112.82, 112.80. HRMS (FAB^+): *m/z* calcd for $C_{38}H_{41}N_5O_8NaS$ [M+Na$^+$]: 750.2568; found: 750.2594.

4.1.7. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-methyl-N^2-isobutyryl-3′-thioguanosine (9). To a mixture of guanidine hydrochloride (208 mg, 0.285 mmol), MeOH (5 mL) and $NaOCH_3$ (25% in MeOH, 60 μL) a solution of **8** (20 mg, 0.027 mmol) was added in CH_2Cl_2 (5 mL), and the mixture was stirred at room temperature for 30 min. The reaction mixture was partitioned between H_2O and CH_2Cl_2. The organic layer was dried over anhydrous sodium sulfate. The sodium sulfate was filtered off, and the

solvent was removed by evaporation under vacuum. The residue was purified by silica gel chromatography, eluting with 2% methanol in chloroform with 0.5% Et_3N, to give compound **9** (16 mg, 85%). ^1H NMR ($CDCl_3$): δ 12.26 (s, 1H), 9.59 (s, 1H), 8.04 (s, 1H), 7.17–7.42 (m, 9H), 6.72–6.81 (m, 4H), 5.96 (d, 1H, J = 1.2 Hz), 4.04 (d, 1H, J = 9.5 Hz), 4.00 (d, 1H, J = 4.5 Hz), 3.75 (s, 6H), 3.67 (m, 1H), 3.60 (d, 1H, J = 11.0 Hz), 3.48 (s, 3H), 3.38 (m, 1H), 2.58 (m, 1H), 1.71 (d, 1H, J = 10.0 Hz), 1.13 (d, 6H, J = 5.5 Hz). ^{13}C NMR ($CDCl_3$): δ 179.3, 158.6, 155.7, 147.9, 147.6, 144.3, 137.0, 135.5, 135.4, 130.0, 129.9, 128.0, 127.9, 127.0, 121.6, 113.2, 87.2, 86.5, 86.2, 85.5, 61.0, 58.7, 55.2, 39.0, 36.1, 18.9, 18.8. HRMS (FAB^+): m/z calcd for $C_{36}H_{39}N_5O_7NaS$ [M+Na^+]: 708.2462; found: 708.2495.

4.1.8. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-methyl-N^2-isobutyryl-3′-thioguanosine-3′-*S*-(2-cyanoethyl)-*N*,*N*-diisopropylphoramidothioite (10). To a solution of compound **9** (52 mg, 0.076 mmol) in dry dichloromethane (5 mL) under Ar, were added *N*,*N*-diisopropylethylamine (66 μL, 0.34 mmol), 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (51 μL, 0.34 mmol), and 1-methylimidazole (6.2 μL, 0.076 mmol). The reaction mixture was stirred at room temperature until all the starting material was consumed (1 h). The reaction mixture was quenched with methanol (1 mL) and stirred for 5 min. After the solvent was removed, the residue was purified by silica gel chromatography, eluting with 2% acetone in dichloromethane with 0.5% Et_3N to give the corresponding phosphoramidite **10** (100% conversion). ^{31}P NMR (CD_3CN): δ 167.0, 161.2. MS (FAB^+): m/z calcd for $C_{45}H_{57}N_7O_8PS$ [M+H^+]: 886.4; found: 886.2.

4.1.9. Oligonucleotide synthesis. The oligonucleotide $CUCG_{3'S,2'OCH_3}A$ was synthesized on an Expedite 8900 DNA synthesizer through a modified protocol that was previously reported from our laboratory.[43] Following standard oligonucleotide deprotection conditions (3:1 NH_4OH/EtOH, 4 h; TEA3HF/NMP, 1.5 h), the MALDI mass spectrum of $CUCG_{3'S,2'\ OCH_3}A$ gave the expected peak at 1557 Da, consistent with the calculated molecular weight (1556 Da).

4.2. Ribozyme reactions

Ribozyme was prepared through standard methods.[42] $CUCG_{2'OH}A$ and $CUCG_{2'OCH_3}A$ were purchased from Dharmacon (Lafayette, CO) and deprotected according to the manufacturer's protocol. $CUCG_{3'S,2'OH}A$ was prepared by published procedures.[43] All oligonucleotide substrates were 5′-^{32}P-radiolabeled using [γ-^{32}P]ATP (Perkin-Elmer) and T4 polynucleotide kinase (New England Biolabs) according to the manufacturer's protocols and subsequently purified on a 20% nondenaturing polyacrylamide gel. The band corresponding to the full-length substrate was visualized by autoradiography, excised, and eluted at 4 °C overnight into water. Following elution, the substrate was P/C/I extracted and subsequently ethanol precipitated. Reverse reactions were performed and analyzed essentially as described previously.[17] All ribozyme reactions were performed at 30 °C in 50 mM NaEPPS buffer, pH 8.0, 50 mM $MgCl_2$, 1 μM E, and 2 μM P. To stimulate cleavage of the phosphorothiolate substrates, all reactions were performed in the presence of 10 mM $MnCl_2$. To ensure that chemistry was rate-limiting, reactions with $CUCG_{2'OH}A$ and $CUCG_{3'S,2'OH}A$ were performed with −1d,rP (CCCUCdT), a product analogue with 2′-deoxythymidine at the 3′-terminus. This substitution slows the chemical step while having no effect on other reaction steps.[44] For the corresponding 2′-methoxy analogues, no reaction was observed with −1d,rP. Therefore, reactions were performed in the presence of an all-ribo product (CCCUCU) and the resulting k_{obs} was divided by 1188, the reported differential in reactivity between rP and −1d,rP.[44] Each rate constant represents the average of two independent determinations that vary by less than 25%.

Acknowledgments

J.L. is a Research Associate, N.-S.L. is a Research Specialist, R.N.S. is a Research Technician, and J.A.P. is an Investigator of the Howard Hughes Medical Institute. We thank Q. Dai for assistance with MALDI mass spectrometry and C. Lea for assistance with substrate preparation and kinetic measurements. We thank J. Ye, T. Novak, J. Min De-Bartolo, S. Koo, and Q. Dai for helpful discussions and critical comments on the manuscript.

References and notes

1. Curley, J. F.; Joyce, C. M.; Piccirilli, J. A. *J. Am. Chem. Soc.* **1997**, *119*, 12691–12692.
2. Gordon, P. M.; Piccirilli, J. A. *Nat. Struct. Biol.* **2001**, *8*, 893–898.
3. Piccirilli, J. A.; Vyle, J. S.; Caruthers, M. H.; Cech, T. R. *Nature* **1993**, *361*, 85–88.
4. Shah, R.; Cosstick, R.; West, S. C. *EMBO J.* **1997**, *16*, 1464–1472.
5. Sontheimer, E. J.; Sun, S.; Piccirilli, J. A. *Nature* **1997**, *388*, 801–805.
6. Warnecke, J. M.; Sontheimer, E. J.; Piccirilli, J. A.; Hartmann, R. K. *Nucleic Acids Res.* **2000**, *28*, 720–727.
7. Weinstein, L. B.; Jones, B. C.; Cosstick, R.; Cech, T. R. *Nature* **1997**, *388*, 805–808.
8. Gordon, P. M.; Sontheimer, E. J.; Piccirilli, J. A. *RNA* **2000**, *6*, 199–205.
9. Brautigam, C. A.; Sun, S.; Piccirilli, J. A.; Steitz, T. A. *Biochemistry* **1999**, *38*, 696–704.
10. Horton, N. C.; Connolly, B. A.; Perona, J. J. *J. Am. Chem. Soc.* **2000**, *122*, 3314–3324.
11. Vyle, J. S.; Connolly, B. A.; Kemp, D.; Cosstick, R. *Biochemistry* **1992**, *31*, 3012–3018.
12. Beevers, A. P.; Fettes, K. J.; O'Neil, I. A.; Roberts, S. M.; Arnold, J. R.; Cosstick, R.; Fisher, J. *Chem. Commun.* **2002**, 1458–1459.
13. Beevers, A. P. G.; Witch, E. M.; Jones, B. C. N. M.; Cosstick, R.; Arnold, J. R. P.; Fisher, J. *Magn. Reson. Chem.* **1999**, *37*, 814–820.
14. Elliott, S. L.; Brazier, J.; Cosstick, R.; Connolly, B. A. *J. Mol. Biol.* **2005**, *353*, 692–703.
15. Szczelkun, M. D.; Connolly, B. A. *Biochemistry* **1995**, *34*, 10724–10733.
16. Gordon, P. M.; Fong, R.; Piccirilli, J. A. *Chem. Biol.* **2007**, *14*, 607–612.

17. Hougland, J. L.; Kravchuk, A. V.; Herschlag, D.; Piccirilli, J. A. *PLoS Biol.* **2005**, *3*, e277.
18. Shan, S.; Yoshida, A.; Sun, S.; Piccirilli, J. A.; Herschlag, D. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 12299–12304.
19. Sontheimer, E. J.; Gordon, P. M.; Piccirilli, J. A. *Genes Dev.* **1999**, *13*, 1729–1741.
20. Szewczak, A. A.; Kosek, A. B.; Piccirilli, J. A.; Strobel, S. A. *Biochemistry* **2002**, *41*, 2516–2525.
21. Yoshida, A.; Sun, S.; Piccirilli, J. A. *Nat. Struct. Biol.* **1999**, *6*, 318–321.
22. Hougland, J. L., Piccirilli, J. A., Forconi, M., Lee, J., Herschlag, D. In *The RNA World*, 3rd ed.; Gesteland, R. F., Cech, T. R., Atkins, J.F., Eds.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, 2006, pp 133–205.
23. Stahley, M. R.; Strobel, S. A. *Curr. Opin. Struct. Biol.* **2006**, *16*, 319–326.
24. Vicens, Q.; Cech, T. R. *Trends Biochem. Sci.* **2006**, *31*, 41–51.
25. Das, S. R.; Fong, R.; Piccirilli, J. A. *Curr. Opin. Chem. Biol.* **2005**, *9*, 585–593.
26. Gordon, P. M.; Fong, R.; Deb, S. K.; Li, N. S.; Schwans, J. P.; Ye, J. D.; Piccirilli, J. A. *Chem. Biol.* **2004**, *11*, 237–246.
27. (a) Hougland, J. L.; Deb, S. K.; Maric, D.; Piccirilli, J. A. *J. Am. Chem. Soc.* **2004**, *126*, 13578–13579; (b) Hougland, J. L.; Sengupta, R. N.; Dai, Q.; Deb, S. K.; Piccirilli, J. A. **2008**, submitted for publication.
28. Abboud, J. L. M.; Roussel, C.; Gentric, E.; Sraidi, K.; Lauransan, J.; Guiheneuf, G.; Kamlet, M. J.; Taft, R. W. *J. Org. Chem.* **1988**, *53*, 1545–1550.
29. Allen, F. H.; Bird, C. M.; Rowland, R. S.; Raithby, P. R. *Acta Crystallogr., Sect. B-Struct. Sci.* **1997**, *53*, 696–701.
30. Crampton, M. R. *Acidity and Hydrogen Bonding*; John Wiley & Sons: New York, 1974.
31. Meotner, M.; Sieck, L. W. *J. Am. Chem. Soc.* **1986**, *108*, 7525–7529.
32. Platts, J. A.; Howard, S. T.; Bracke, B. R. F. *J. Am. Chem. Soc.* **1996**, *118*, 2726–2733.
33. Yoshida, A.; Shan, S.; Herschlag, D.; Piccirilli, J. A. *Chem. Biol.* **2000**, *7*, 85–96.
34. Matulic-Adamic, J.; Beigelman, L. *Helv. Chim. Acta* **1999**, *82*, 2141–2150.
35. Ozols, A. M.; Azhayev, A. V.; Dyatkina, N. B.; Krayevsky, A. A. *Synthesis* **1980**, 557–559.
36. Ellervik, U.; Magnusson, G. *Tetrahedron Lett.* **1997**, *38*, 1627–1628.
37. Bevilacqua, P. C.; Sugimoto, N.; Turner, D. H. *Biochemistry* **1996**, *35*, 648–658.
38. Karbstein, K.; Carroll, K. S.; Herschlag, D. *Biochemistry* **2002**, *41*, 11171–11183.
39. Mei, R.; Herschlag, D. *Biochemistry* **1996**, *35*, 5796–5809.
40. Woodson, S. A.; Cech, T. R. *Cell* **1989**, *57*, 335–345.
41. Zaug, A. J.; Cech, T. R. *Science* **1986**, *231*, 470–475.
42. Zaug, A. J.; Grosshans, C. A.; Cech, T. R. *Biochemistry* **1988**, *27*, 8924–8931.
43. Sun, S.; Yoshida, A.; Piccirilli, J. A. *RNA* **1997**, *3*, 1352–1363.
44. McConnell, T. S. Ph.D. thesis, University of Colorado, Boulder, CO, 1995.