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Biochemical Detection of Cytidine Protonation within RNA

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Abstract: Perturbation of active site functional group pK_as is an important strategy employed by protein enzymes to achieve catalysis. There is increasing evidence to indicate that RNAs also utilize functional group pK_a perturbation for folding and reactivity. One of the best candidates for a functionally relevant pK_a perturbation is the N3 of C (pK_a 4.2), which could be sufficiently raised to allow protonation near physiological pH. Here we report the synthesis and use of a series of α-phosphorothioate tagged cytidine analogues whose altered N3 pK_as make it possible to efficiently detect functionally relevant protonation events by nucleotide analogue interference mapping. 6-Azacytidine (n^6CαS) and 5-fluorocytidine (f^5CαS) both have enhanced acidity at the N3 position (pK_a 2.6 and 2.3, respectively) but leave the hydrogen bonding face of C otherwise unaffected. In contrast, pseudoisocytidine (ΨiCαS) is a charge neutral analogue that mimics the hydrogen bonding character of protonated C. To test the utility of these analogues, we characterized the $C300^+$-G97-C277 mutant form of the *Tetrahymena* group I intron, which is predicted to require C300 protonation for ribozyme folding and reactivity. At neutral to alkaline pHs, C300 was the only site of n^6CαS and f^5CαS interference within the intron, yet both interferences were rescued at acidic pH. Furthermore, ΨiCαS substitution at C300 resulted in enhanced activity at alkaline pHs, consistent with the presence of an N3 proton under the pH conditions studied. Interference mapping with these analogues provides an efficient and sensitive means to identify every site within an RNA where cytidine protonation is important for RNA function and may make it possible to identify C's that participate in general acid/base catalysis within ribozyme active sites.

Introduction

Protein enzymes efficiently catalyze a vast array of biochemical reactions. They are especially well suited to promote reactions involving general acid or general base catalysis under physiological pH conditions because they possess ionizable functional groups with near neutral pK_as. Furthermore, the pK_as of individual functional groups can be perturbed by the microenvironment of the amino acid side chains within the protein's three-dimensional structure.[1−3] Such functional group perturbations are utilized by enzymes to promote catalysis through substrate activation and transition state stabilization.[1]

Like proteins, large RNAs can also adopt complex three-dimensional structures and catalyze chemical reactions.[4] However, none of the functional groups on the planar base residues or phosphate ribose backbone have a pK_a near neutral pH. Therefore, perturbation is required in order for an RNA functional group to achieve such a pK_a. The two groups whose

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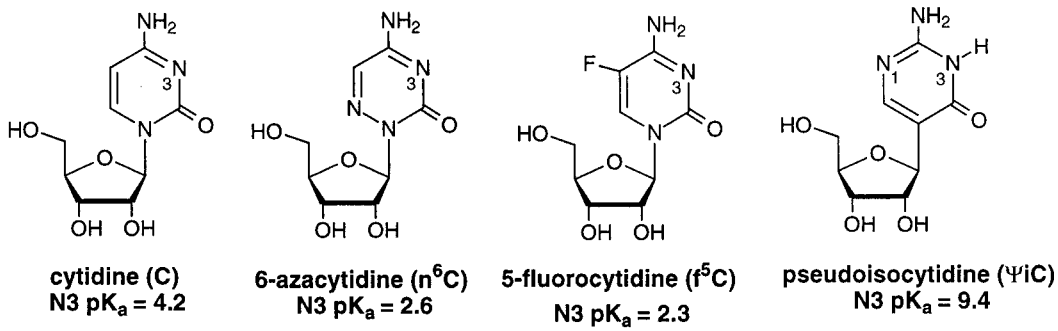

Figure 1. Cytidine, 6-azacytidine, 5-fluorocytidine, and pseudoisocytidine. The unperturbed pK_a of the N3 imino group is specified for all nucleosides.

unperturbed pK_as are closest to neutrality are the imino groups of the heterocyclic rings, specifically the N1 of adenosine (A) with a pK_a of 3.5 and the N3 of cytidine (C) with a pK_a of 4.2.[5] Several structural and biochemical studies have demonstrated that pK_a perturbation at these two positions can occur and that the magnitude of the effect is sufficient to allow protonation near physiological pH.[6−8]

The importance of pK_a perturbation to RNA structure is evident from the involvement of base protonation in several noncanonical base pairing interactions. For example, A^+-C, A^+-G, A^+-A^+, C^+-C, and Hoogsteen C^+-G pairs are all stabilized by base protonation.[6a,b,7,8] Such pK_a perturbations have been postulated to lead to stronger hydrogen bonding, and the accompanying change in dipole moment of the protonated A has been proposed to favor stacking interactions.[6e] Base pairing involving pK_a perturbed nucleosides may also mark a region of structural transition within RNAs.[9]

Perturbation of base pK_as also appears to be a potentially important catalytic mechanism in RNA enzymes. Originally, these biological catalysts were regarded exclusively as metalloenzymes, and their catalytic potential was thought to rest solely in their ability to position catalytic metal ions within their active sites. The discovery that several ribozymes are functional in the absence of divalent metal ions has cast doubt upon the strictly metalloenzyme model and caused several ribozyme catalytic mechanisms to be reconsidered.[10] Models are now emerging that invoke pK_a perturbation in general acid/general base mechanisms of RNA catalysis.[6g−i] Based on crystallographic studies, Doudna and co-workers proposed a model for catalysis by the hepatitis delta virus genomic ribozyme wherein the N3 of a critical C residue, C75, acts as a general base to deprotonate the 2′-OH nucleophile.[6g] The model was subsequently modified by Bevilacqua and co-workers,[6i] who provided compelling evidence that the N3 of C75 acts as a general acid in the transition state to stabilize the negative charge generated on the 5′-OH leaving group. Such a role can only be accomplished, however, if the C75 N3 pK_a is perturbed toward neutrality, which has not yet been directly confirmed. Although this is the best characterized example, pK_a perturbation of active site nucleotides has been observed in other RNA enzymes, including the hairpin ribozyme,[6d] the leadzyme,[6c] and most recently the ribosome,[11] though the catalytic significance of these pK_a perturbed sites has not been demonstrated.

Several approaches have proven useful for determining pK_a values of nucleic acid bases, including titermetry, modification−protection assays, UV and fluorescence spectroscopy, and NMR.[6a,c,e,8] Two-dimensional NMR experiments on isotopically labeled oligonucleotides have been used to estimate that the pK_a of the A N1 is approximately 6.6 in the context of an A^+-C pair, which corresponds to a shift of more than 3 pH units.[6a] Similar experiments have identified an adenine base with a pK_a of 6.5 near the active site of the leadzyme.[6c,e] However, these methods are limited in their scope. Complications arising from solvent exchange make the direct detection by NMR of protonation events very difficult,[6c] and large RNAs are not amenable to NMR methods. Most importantly, the functional roles such pK_a perturbations might play in RNA activity are not identified from such analyses. Thus, the development of a facile means to demonstrate the functional importance of nucleotide pK_a perturbation is essential to our understanding of RNA biochemistry.

Nucleoside analogues that indirectly affect the pK_a of a functional group by altering the electronic state of the heterocyclic ring could provide valuable biochemical probes for identifying sites with functionally relevant perturbations in their pK_a. For example, cytosine derivatives with enhanced basicity at N3, such as carbocyclic 5-methyl-2′-deoxycytidine and 2-amino pyridine C-nucleosides, have been shown to increase the stability of oligonucleotide triple helices.[12] Furthermore, improved binding of G−C base pairs has been seen with analogues that mimic protonated C without the accompanying positive charge.[13] By contrast, two C analogues that significantly reduce the ionizability of the N3 imino group are 6-azacytidine (n^{6}C) and 5-fluorocytidine (f^{5}C), which have pK_as of 2.6 and 2.3, respectively (Figure 1). Despite the dramatic pK_a effects, both analogues leave the hydrogen bonding face of C otherwise

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unaffected.[14,15] A third analogue, pseudoisocytidine (ΨiC), is a charge neutral C derivative that mimics the hydrogen bonding character of protonated C.[13a,b] Analysis of RNAs containing such substitutions could reveal the importance of base ionization in RNA function.

Site specific incorporation of such nucleotide analogues into an RNA could make it possible to quantitatively address the effects of pK_a perturbation, but it is an extremely laborious process, especially if the RNA is large and the desired site of substitution is not adjacent to one end. As a qualitative alternative to such studies, nucleotide analogue interference mapping (NAIM) provides a high throughput approach that makes it possible to simultaneously, yet individually, monitor the effect of incorporating the pK_a modified nucleotide at each C within an RNA.[16] The sites where analogue incorporation are detrimental to RNA function can be readily detected in four steps. First, α-phosphorothioate tagged versions of the C analogues are randomly incorporated at a low frequency into an RNA by in vitro RNA transcription, second, the functional RNAs are separated from the total RNA library by using a selection assay, third, the RNAs are treated with iodine to break the phosphothioate linkages at the sites of analogue incorporation and fourth, the positions of analogue interference are revealed by denaturing polyacrylamide gel electrophoresis and scoring for the disappearance of bands within the active RNA fraction.

We set out to demonstrate that utilization of the N3 pK_a perturbed C-analogues in a NAIM assay could provide a simple method to simultaneously identify all positions within an RNA where C N3 protonation is important for activity. We report the analysis of the C300$^+$-G97-C277 triple mutant form of the L-21 G414 *Tetrahymena* group I ribozyme.[17] The C$^+$-G-C triple is predicted to be an example of a noncatalytic pK_a perturbation, where Hoogsteen pairing promotes protonation of the C300 N3 for hydrogen bonding to the G97 N7 (Figure 2b).[7] The triple forms part of a network of interactions that are critical for aligning the P1 substrate helix into the enzyme's active site.[17] In the presence of an oligonucleotide substrate the L-21 G414 RNA performs a 3′-exon ligation reaction that is analogous to the reverse of the second step of intron splicing (Figure 2a).[18] When the substrate is 3′-end-labeled, the active ribozymes become radioactively tagged, which readily distinguishes them from the less active RNAs in the population. Thus, this intron provides an ideal system to demonstrate the utility of the pK_a perturbed nucleotide analogues in a NAIM assay.

Here we report the synthesis of the 5′-*O*-(1-thio)triphosphates of n^{6}C **4a**, f ^{5}C **4b**, and ΨiC **4c** and their efficient incorporation into RNA by T7 RNA polymerase. We find that while almost every position in the group I intron is monitored by NAIM, these analogues only show effects on ribozyme activity when incorporated at C300, where a perturbed N3 pK_a is expected to be important for RNA folding. The interferences can be rescued in a pH-dependent manner consistent with an ionization specific effect. The specificity of the interference effects observed with these N3 pK_a perturbed C analogues makes them ideal probes for identifying sites where C protonation is crucial to function in other RNAs.

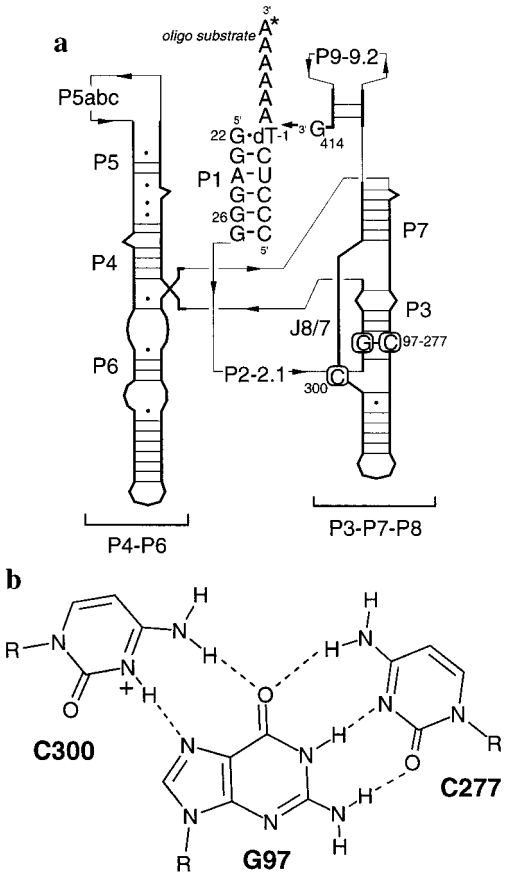

Figure 2. (a) Schematic representation of the C300$^+$-G97-C277 form of the L-21 G414 *Tetrahymena* group I intron secondary structure.[17] Heavy lines and letters indicate nucleic acid sequences, while thin lines and arrows trace the connections between sequence elements. Important paired (P) and joining (J) regions are labeled. Nucleotides in regions P2−P2.1, P5abc, and P9−P9.2 have been omitted for clarity but are present within the RNA characterized in this study. The ribozyme is shown bound to the 3′-end-labeled (*) oligonucleotide substrate CCCUCdTAAAAA*. The last nucleotide of the intron, G414, nucleophilically attacks the 5′-splice site (arrow), which transfers AAAAA* onto the 3′-end of the intron and labels the active introns within the ribozyme population.[16b,18] Formation of the C300$^+$-G97-C277 triple (circles) is critical for the reaction to proceed efficiently.[17] C300 also forms an important 2′-OH to 2′-OH tertiary contact with G26 in the P1 helix.[32] (b) Diagram of the predicted C300$^+$-G97-C277 base triple within the mutant form of the *Tetrahymena* group I intron. Note that the N3 position of C300 is expected to be protonated and that the proton participates in hydrogen bonding to the N7 of G97.[7,17]

Results and Discussion

Synthesis. n^{6}C **1a** and f ^{5}C **1b** were synthesized from their uridine derivatives via C4-triazolyl intermediates.[19] Attempts made to synthesize the 5′-*O*-(1-thio)triphosphates from unprotected nucleosides according to the general procedure described

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by Arabshahi and Frey[20] were unsuccessful. A modification of the procedure in which the initial thiophosphorylation step was performed in a reaction mixture containing 0.5 equiv of trioctylamine, 1.2 equiv of collidine, and $PSCl_3$ in triethyl phosphate at 0 °C for 30 min followed by warming to room temperature within 15 min gave about 20−25% of n^{6}CTPαS **4a**. However, only a trace amount of f^{5}CTPαS **4b** was obtained and no ΨiCTPαS **4c** and CTPαS **4d** were formed by using this protocol.

For this reason we turned to the salicyl phosphoramidite approach, described by Ludwig and Eckstein,[21] for the synthesis of the target thiotriphosphates. This route requires nucleoside starting materials with protection of the base and the 2′- and 3′-OH groups of the sugar. However, the low yields and multiple purification steps accompanying the syntheses of these protected nucleosides precluded the use of the standard nucleoside protecting groups. For this application, the ideal protecting group is one that permits a quantitative and rapid protection of the exocyclic amine and the 2′- and 3′-OH groups in a single step, as well as being a group that can be easily deprotected under reaction conditions that are compatible with the thiotriphosphate bonds. *N*,*N*-Dialkylformamide dialkylacetals meet these conditions and their reaction with nucleosides has been described.[22] Recently, Jones and co-workers reported the use of 2′,3′-*O*-dimethylaminomethylene protected nucleoside to effect a 5′-selective tritylation reaction despite the inherent instability of 2′,3′-*O*-dimethylaminomethylene group under such conditions.[23]

Encouraged by this report, we investigated the use of such protected nucleosides as starting materials in the synthesis of 5′-*O*-(1-thio)triphosphates. The reaction of *N*,*N*-dimethylformamide dimethylacetal with nucleosides **1b**−**d**, using the general procedures described by Jones and co-workers,[23] gave protected nucleosides **2b**−**d** in quantitative yield. After solvent evaporation, crude **2b**−**d** were subjected to the salicyl phosphoramidite chemistry following the Ludwig and Eckstein protocol[21] to give crude 1-thiocyclotriphosphates **3b**−**d**. Aqueous hydrolysis of **3b**−**d** led to the formation of 5′-*O*-(1-thio)-triphosphates with a concomitant deprotection of the 2′,3′-*O*-dimethylaminomethylene group. The exocyclic amidine group was deprotected by treatment with NH_4OAc in NH_4OH[24] to give **4b**−**d** (Figure 3), which were isolated in relatively good yields by chromatography on DEAE-Sephadex. All 5′-*O*-(1-thio)-triphosphates synthesized were characterized by ^{31}P NMR, UV, and electrospray MS.

Incorporation of C nucleotide analogues by T7 RNA polymerase. To be used in a nucleotide analogue interference mapping (NAIM) assay, nucleotide analogues must be uniformly and accurately incorporated into large RNA transcripts by enzymatic runoff transcription in vitro. It is known that halogenated nucleosides are readily incorporated into DNA and RNA when cells are grown in media containing these nucleosides or their bases and some of them have been directly employed in in vitro transcription reactions.[25] Although n^{6}C triphosphate has not been tested in transcription, the related pyrimidine nucleotide 6-azauridine triphosphate has been shown to inhibit *E. coli* RNA polymerase.[26] The incorporation of ΨiC into mammalian DNA and RNA has been inferred by detection of radioactivity in DNA and RNA components of cells grown in the presence of radioactive ΨiC.[27] However, no report exists on the direct use of ΨiC triphosphate as a substrate for RNA polymerase in vitro.

The C nucleotide analogues f^{5}CTPαS, n^{6}CTPαS, and ΨiCTPαS were individually tested both for their ability to sustain transcription in the absence of CTP and for specific and uniform transcriptional incorporation in the presence of CTP. Plasmid DNA pUCL-21G414, which has a T7 RNA polymerase promoter, was digested with EarI for use as a template in runoff transcription reactions. Not unexpectedly, we found that f^5-CTPαS could completely replace the parent nucleotide cytidine without a noticeable loss in transcription efficiency (data not shown). Similarly, n^{6}CTPαS was also able to support transcription in the absence of cytidine, which is in contrast to that reported for the uridine derivative.[26] The fidelity of analogue incorporation was determined by iodine sequencing of the 5′-end radiolabeled RNA transcripts. The RNA cleavage pattern was compared to that of an RNA containing CαS. Within the limits of detection (∼0.5% infidelity), both analogues were incorporated exclusively at C's and the extent of incorporation at each position was essentially equivalent to that of CαS (data not shown).

The third nucleotide used in this study, ΨiC, is a C-linked nucleoside that exists as an equilibrium mixture of two tautomeric forms in aqueous solution (Figure 4).[28] NMR experiments and theoretical calculations have shown that the N3−H tautomer is favored over the N1−H form by about 2−9 kcal mol^{-1}, depending on the solvent polarity.[29] The N3−H tautomer creates an "unnatural" donor−donor−acceptor hydrogen bonding face for base pairing, which might have a deleterious effect on the ability of this analogue to be recognized by the polymerase during transcription. Despite this possibility, ΨiCTPαS served as an efficient substrate for T7 polymerase leading to the formation of full-length RNA transcripts at a level comparable to that of CαS. Iodine sequencing of 5′-end-labeled RNA transcripts containing either 5% or 100% incorporation of ΨiCαS and CαS confirmed that ΨiCαS is incorporated exclusively at C's in the transcript and that the extent of incorporation at individual positions is the same as CαS (Figure 5). The high efficiency and fidelity of ΨiCαS incorporation suggests that pairing to the G in the template shifts the tautomeric equilibrium toward the less stable N1-H form (Figure 4), which creates a hydrogen bonding pattern similar to that of cytidine. Such a tautomeric equilibrium shift has been observed in degenerate nucleosides and oligonucleotides containing these nucleosides.[30]

Approximately 5% incorporation of the phosphorothioate tagged analogues is sufficient to conduct a NAIM analysis for an RNA the size of the group I intron (approximately 400 nucleotide). Nucleotide analogue to CTP ratios needed to achieve this incorporation level were determined for the three

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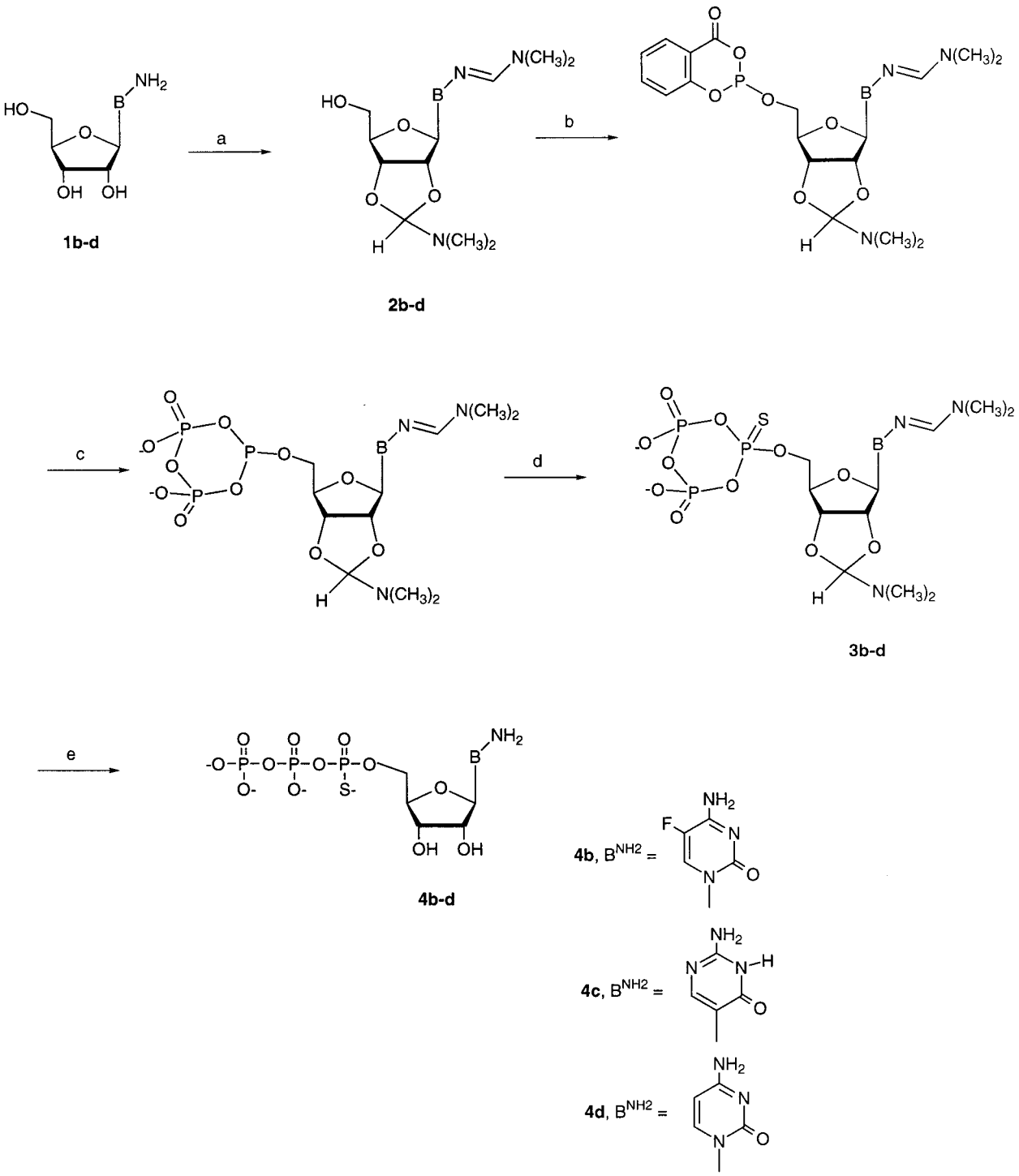

Figure 3. Silacyl phosphoramidite approach to nucleoside 5′-*O*-(1-thio)triphosphates: (a) *N*,*N*-Dimethylformamide dimethylacetal, pyridine, rt, 15 h; (b) 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one, dioxane, pyridine, rt, 10 min; (c) $P_2O_7^{4-}$, DMF/tri-*n*-butylamine, rt, 10 min; (d) sublimed sulfur, rt, 10 min; (e) (i) H_2O, rt, 30 min; (ii) NH_4OH, NH_4OAc, 50 °C, 30 min; (iii) triethylammonium bicarbonate (50 mM, pH = 8.0), rt, overnight.

C derivatives as described previously, and the concentrations are shown in Table 1.[31]

Interference Mapping Analysis with pK_a Perturbed C Analogues. The C300$^+$-G97-C277 mutant form of the L-21 G414 *Tetrahymena* group I intron was used as a model system to determine if NAIM with the C analogue series can detect an N3 protonation event.[17] Ribozymes containing a random distribution of one of the three nucleotides analogues or CαS were reacted with the 3′-labeled substrate CCCUCdTAAAAA for an amount of time sufficient to give 40% ligation. The reactions were quenched in formamide, iodine was added to cleave the phosphorothioate linkages, and the cleavage products were resolved by denaturing polyacrylamide gel electrophoresis. Autoradiography of the sequencing gel was used to reveal gaps in the sequencing ladder corresponding to sites of analogue interference.

Our initial experiments were performed on the wildtype (U300-A97-U277) form of the L-21 G414 ribozyme at pH 8.0. In the context of this ribozyme, no sites of n^6CαS interference

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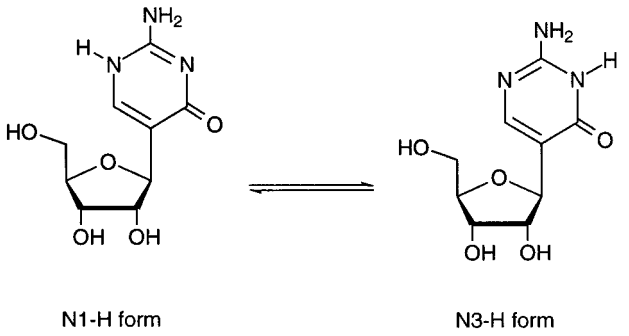

Figure 4. Tautomerism in pseudoisocytidine. Results from NMR experiments and theoretical calculation have shown that the tautomeric equilibrium constant is highly dependent on the polarity of the solvent with the N3−H tautomer being favored.[29] The N1−H tautomer has a hydrogen bonding pattern similar to the parent C while the N3−H tautomer presents an "unnatural" donor−donor−acceptor hydrogen bonding pattern. Evidence from runoff transcription and sequencing suggested that a template assisted equilibrium shift may favor the formation of the N1−H tautomer for recognition by the polymerase. Interference mapping also showed that the N1−H tautomer is favored in G-C base pairings, while the N3−H tautomer is used in the Hoogsteen C in C^+-G-C base triple.

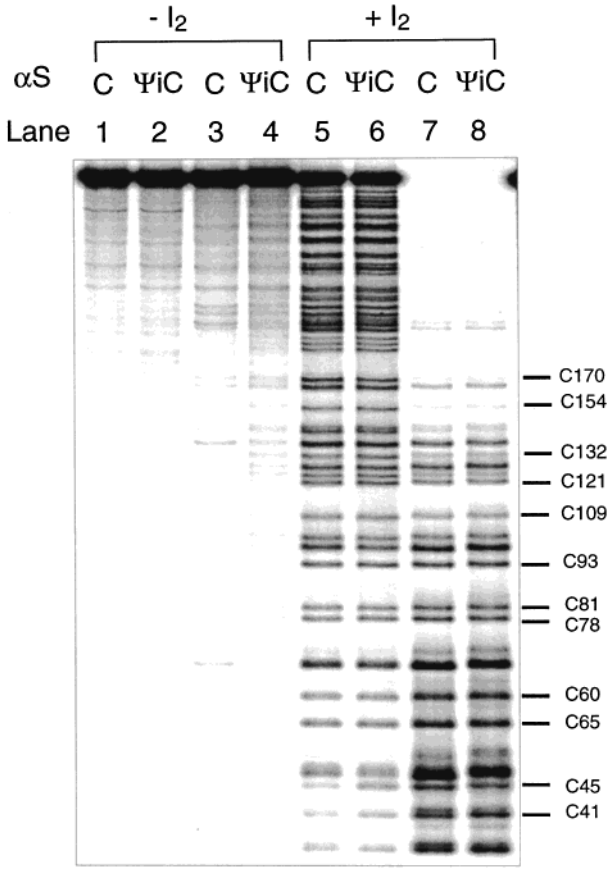

Figure 5. Iodine sequencing of RNA transcripts show that pseudoisocytidine is incorporated as C. Shown in lanes 1−4 are the noniodine controls, while lanes 5 and 6 represent 5% (see Table 1 for transcription conditions) and lanes 7 and 8 the 100% level of incorporation of CαS and ΨiCαS, respectively.

were observed. This includes an analysis of 58 C's predicted to be involved in Watson−Crick base pairs and 15 C's expected to be in noncanonical pairings or single-stranded regions. None of these C's are predicted to be in noncanonical pairs wherein they would be protonated in the folded RNA. The lack of n^6CαS interference at these 73 sites demonstrates that the addition of the N6 nitrogen and the perturbation of the N3 pK_a does not broadly disrupt RNA duplex formation or nonspecifically perturb RNA structure.

Table 1. Transcription Conditions for Incorporating Nucleotide Analogue into the C300$^+$-G97-C277 RNA at Approximately a 5% Level of Incorporation[a]

analogue (diastereomers)	analogue concn, mM	CTP concn, mM
CαS	0.075	1.0
n^6CαS	1.0	0.5
f^5CαS	1.0	1.0
ΨiCαS	0.10	1.0

[a] Transcription reactions contain the specified amount of nucleotide analogue (diastereomeric mixtures) and CTP, as well as 1 mM ATP, GTP, and UTP in transcription buffer containing 40 mM Tris-HCl, pH 7.5, 15 mM $MgCl_2$, 10 mM DTT, 4 mM spermidine, 0.05% Triton X-100, 0.1 μg/μL DNA and 0.08 μg/μL T7 RNA polymerase. The reactions are incubated at 37 °C for 3 h.

The interference experiment was repeated using the C300$^+$-G97-C277 mutant form of the ribozyme at pH 8.0. A single n^6CαS interference was observed at C300, the site predicted to be protonated within the triple (Figure 6a, lane 2). Although this interference is likely to result from disruption of the critical C^+-G-C triple due to the lower n^6CαS pK_a at the N3 position, an alternative possibility is that the RNA is folded into an inactive RNA conformation due to interaction with the N6 imino group. These two possibilities can be distinguished by the pH dependence of the interference. If it is caused by an N3 pK_a effect, then a reduction in the reaction pH should restore protonation and eliminate or significantly reduce interference; whereas, the interference would not be pH dependent if it results from a conformational effect. The interference experiment was repeated at pHs from 8.0 to 5.0 in order to test for the pH dependence of the n^6CαS interference. Consistent with a pK_a specific effect, the extent of C300 interference was progressively reduced at lower pHs, to the point where interference was completely eliminated at pH 5.0 (Figure 6a, compare lanes 2 and 5, Figure 7a, shaded bars).

Another approach to demonstrate a pK_a specific effect is to utilize a second analogue with a comparable pK_a perturbation and show that it has a similar interference pattern. For this reason we prepared f^5CαS, which also has enhanced acidity at the N3 position but does not include an extra N6 imino group. Consistent with a functionally important pK_a perturbation at C300, interference with f^5CαS closely matched that of n^6CαS. Incorporation at C300 caused inhibition at pH 8.0, while activity was almost fully restored at pH 5.0 (Figure 6b, compare lanes 2 and 5). The degree of pH rescue for f^5CαS was not quite as complete as that of n^6CαS (Figure 7b, shaded bars), which is consistent with the fact that the pK_a of f^5CαS is slightly lower than n^6CαS and is therefore more difficult to protonate.

It is possible that the pH rescue of these analogues could result from a nonspecific conformational change in the RNA that is pH dependent. To exclude this possibility, we tested for interference across the same pH range using analogues that are indicative of a native conformation in the same region of the molecule, but whose interference is unlikely to be pH dependent. Like n^6CαS and f^5CαS, the incorporation of 2′-deoxycytidine derivative (dCαS) shows interference at C300, but in this case deletion of the C300 2′-OH disrupts a tertiary contact between C300 and the P1 substrate helix instead of the P7 helix.[32] Because this contact is mediated by hydrogen bonding between 2′-OH groups, it should not be pH dependent. Consistently

(32) Szewczak, A. A.; Ortoleva-Donnelly, L.; Ryder, S. P.; Moncoeur, E.; Strobel, S. A. *Nature Struct. Biol.* **1998**, *5*, 1037−1042.

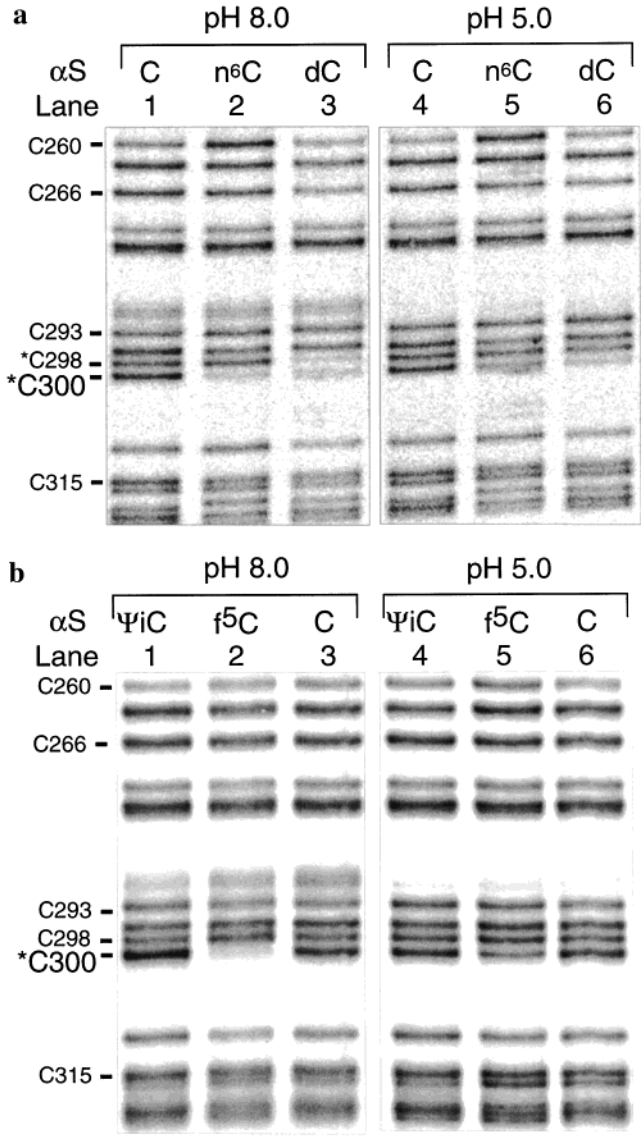

Figure 6. NAIM analysis of the C300^{+}-G97-C277 L-21G414 RNA. (a) Autoradiographs of RNA containing CαS, n^{6}CαS, and dCαS reacted with substrate at pH 8.0 (lanes 1−3) and 5.0 (lanes 4−6). (b) Autoradiographs of RNA containing CαS, f^{5}CαS, and ΨiCαS reacted with substrate at pH 8.0 (lanes 1−3) and pH 5.0 (lanes 4−6). Bands corresponding to specific nucleotide positions are identified to the left with positions of interference marked with asterisks. The analogue that is incorporated into the RNA and the pH of the reaction are specified above the gel.

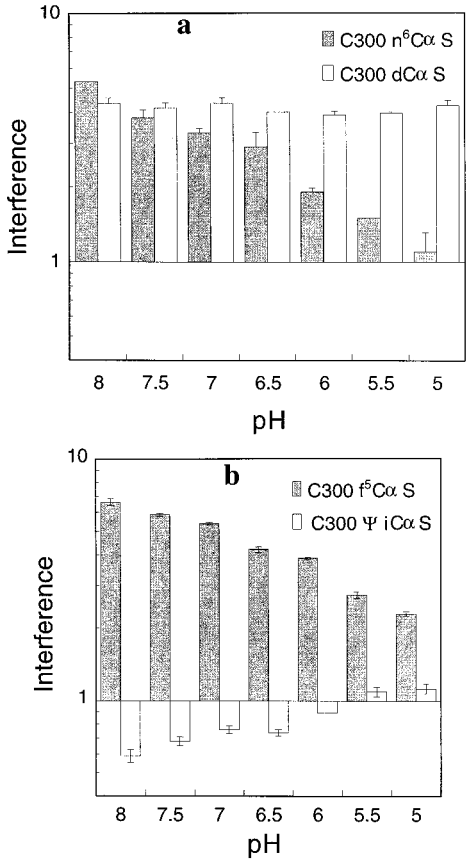

Figure 7. Histograms indicating the effect of analogue incorporation at C300 as a function of pH. (a) n^{6}CαS (shaded bars) and dCαS (open bars). (b) f^{5}CαS (shaded bars) and ΨiCαS (open bars). The magnitude of the interference is calculated as described in the Experimental Section. An interference value of 1 indicates no effect of ribozyme substitution, while values greater than 1 indicate interference and values less than 1 indicate enhancement of RNA activity. Note that in b the interference is plotted on a log scale in order to emphasize the pH dependence of ΨiCαS activity enhancement. All values are calculated from at least two experimental trials and the deviation from the mean is indicated by the error bars.

strong C300 dCαS interference was observed at all pHs tested between 5.0 and 8.0 (Figure 6a, lanes 3 and 6, Figure 7a, open bars). Likewise, AαS interferences at the adjacent nucleotides in J8/7, including A302, A306, and A308, were unaffected by changes in pH (data not shown). The single exception to the pH independence of these alternative interferences is dCαS interference at C298, which was observed at pH 8.0 and rescued at lower pH (Figure 6a). Interference at this site is only observed when the 300•97−277 triple is disrupted, such as occurs in the context of the noncomplementary mutation C300xA97-U277.[17,32] The pH dependence of the dCαS C298 interference most likely results from destabilization of the adjacent C300^{+}-G97-C277 triple at high pH and restoration of triple stability at low pH. Consistent with this interpretation, dCαS C298 interference is not observed in the pH independent U300•A97-U277 wild type form of the intron at any pH.

Enhanced Activity with Pseudoisocytidine Incorporation. Pseudoisocytidine has been shown to stabilize DNA triplexes containing C^{+}-G-C base triples at neutral to slightly basic pHs.[13a,b] Therefore, incorporation of ΨiCαS at C300 is expected to have a stabilizing effect on the C^{+}-G-C base triple if the positive charge associated with C300 protonation does not play a significant role in ribozyme function and the proton does not need to be transferred to a recipient in the course of the ribozyme reaction. Analyses of the ΨiCαS interference pattern shows that all positions in the intron are tolerant of the substitution, including paired regions and single-stranded joiner segments. The single exception was C300, where ΨiCαS showed an effect almost opposite to that of n^{6}CαS and f^{5}CαS. At pH 8.0, ΨiCαS demonstrated an enhancement of band intensity at C300, which suggests that RNAs with ΨiCαS at C300 have a selective advantage over the other RNAs in the population. However, the enhancement was eliminated at pH 5.0 (Figure 6b, compare lanes 1 and 4, Figure 7b, open bars). Under pH conditions where C is less efficiently protonated (pH 8.0), the charge neutral nature of ΨiCαS appears to provide a selective advantage for folding and reactivity. However, at lower pH, this advantage is lost because C can also be efficiently protonated. ΨiCαS enhancement confirms that the second hydrogen bond generated

by C300 protonation plays a significant role in the stability of the $C300^+$-G97-C277 base triple.

Conclusions

We have described a simple and novel methodology to rapidly identify sites of pK_a perturbation critical to activity in RNAs. The protocol relies on the use phosphothioate tagged nucleoside analogues with perturbed pK_as in a NAIM assay. A one-pot synthesis involving a transient protection of the 2′,3′-OH and the exocyclic amino group was used to prepare the nucleoside thiotriphosphates used in this investigation. These analogues are shown to serve as efficient substrates for T7 RNA polymerase.

As a model system, we explored a version of the L-21 G414 *Tetrahymena* group I intron in which formation of the $C300^+$-G97-C277 base triple between J8/7 and P7 is known to be critical to intron folding.[17] Incorporation of $n^6C\alpha S$ and $f^5C\alpha S$ at C300 interferes with ribozyme function at slightly basic to neutral pHs, but the effects can be rescued at slightly acidic pHs. Incorporation of ΨiCαS showed an opposite result, leading to an enhancement of activity at slightly basic to neutral pHs and a wild-type like function at slightly acidic pHs. $n^6C\alpha S$, $f^5C\alpha S$, and ΨiCαS incorporation at all other sites within this intron did not affect RNA function at any pH tested.

RNA pK_a perturbation can play both structural and catalytic roles in RNA function, and these three analogues may make it possible to distinguish between such affects. pK_a perturbed nucleotides that participate in a structural role, such as in the C^+-G-C triple studied here, have the additional proton locked into a hydrogen bonding interaction.[6e] However, in the case of a catalytically relevant pK_a perturbation, the nucleotide undergoes proton transfer as part of the chemical rate acceleration.[6g−i] Due to the reduced pK_a, n^6C and f^5C can probe in a nondiscriminatory fashion for structurally and catalytically important protonated C's within RNAs. However, ΨiC mimics a protonated C, yet the unperturbed pK_a of ΨiC is 9.4,[29] which makes it difficult for the N3 to participate in proton transfer. Even if the pK_a is perturbed, it is likely to be perturbed in the wrong direction because it is being placed into the same chemical microenvironment as C. Consequently, ΨiC could serve as a useful probe to distinguish between the structural and catalytic roles of pK_a perturbation. We have shown that in a structural context ΨiC causes enhancement of activity, and we predict that for those few cases where the C has a catalytic role as a general acid or general base, the ΨiC would instead cause interference under the same reaction conditions.

These results highlight the utility of these three nucleoside analogues as biochemical probes to identify and characterize sites of cytidine protonation that are important for RNA function. In addition to being a functional assay, this interference based approach is not limited by RNA size and other physical parameters such as solvent exchange, which may hinder direct detection by previously described procedures. Most importantly, all the C's in an RNA can be rapidly screened for such pK_a perturbations and the effect on RNA function directly determined. This approach should therefore find general utility in deciphering the mechanistic roles of pK_a perturbation in a wide range of RNAs and other functional oligonucleotides.

Experimental Section

The 2′-ortho ester protected oligonucleotide substrate CCCUCdTAAAAA was purchased from Dharmacon Research and deprotected according to manufacturer protocol.[33] 5′-*O*-(1-thio)adenosine triphosphate (ATPαS) and 5′-*O*-(1-thio)-2′-deoxycytidine (2′-dCTPαS) were purchased from Amersham (Piscataway, NJ). Pseudoisocytidine was obtained from Berry & Associates, Inc. (Ann Arbor, MI). Nucleoside triphosphates and all molecular biology grade reagents were from Sigma. All other chemical reagents were purchased from Aldrich and were used without further purification. Nanopure water was obtained from a Millipore ultra water purification system.

Thin-layer chromatography was carried out on Analtech silica gel plates (60 F_{254}) and the spots were visually detected with UV light. Column chromatography was performed on 200−400 mesh silica gel. NMR spectra were recorded on GE Omega-300 Instrument at 300 MHz (^{1}H NMR) and at 121 MHz (^{31}P NMR). All ^{31}P spectra were referenced against 85% H_3PO_4 external standard. UV spectra were determined on Cary 50 Bio UV/vis spectrophotometer (Varian). Electrospray mass spectral analyses (exact mass, negative ion mode) were performed on a Q-Tof model mass spectrometer (Micromass), a quadrupole/time-of-flight instrument with ATP as an internal standard and 50% acetonitrile in 20 mM triethylammonium acetate as solvent.

Preparation of Ribozymes. Transcription of the $C300^+$-G97-C277 *Tetrahymena* group I intron RNA was performed using the EarI digested plasmid pUC L-21G414 3x.[17] Nucleotide analogues were randomly incorporated into the RNA transcripts through T7 RNA polymerase transcription. Ribozymes containing CαS, dCαS, and AαS were prepared for interference controls as previously described.[17,32] CαS, $n^6C\alpha S$, $f^5C\alpha S$, and ΨiCαS were incorporated into RNA transcripts as specified in Table 1. The RNA transcripts were purified by 6% polyacrylamide gel electrophoresis, eluted at 4 °C overnight into 10 mM tris-HCl, 100 mM NaCl, and 0.1 mM EDTA pH 7.5, precipitated with ethanol, redissolved in 10 mM tris-HCl, and 0.1 mM EDTA pH 7.5, and stored at −20 °C.

Radiolabeling. Substrate oligonucleotide CCCUC(dT)AAAAA was 3′-end-labeled with 5′-^{32}P pCp by incubating with T4 RNA ligase at 16 °C overnight.[17] Labeled substrate was purified on 20% native gel and eluted into TE. The C300+G97-C277 RNA transcripts were incubated with calf intestine alkaline phosphatase and 5′-end-labeled with [γ-^{32}P]ATP using polynucleotide kinase. The 5′-labeled RNAs were purified on 6% PAGE and eluted into 2% SDS for 6 h. The SDS solution was phenol−chloroform extracted and the RNAs were precipitated with ethanol and redissolved in formamide loading buffer. The RNAs were cleaved by adding $^1/_{10}$ volume of 50 mM I_2 in EtOH and incubating at 90 °C for 2 min and the cleavage products resolved by denaturing 6% polyacrylamide gel electrophoresis. These unselected RNAs served to control for the extent of analogue incorporation at each position.

NAIM. Interference experiments were performed by adapting previously described procedures.[16b] HEPES (50 mM) was used for experiments performed between pH 7.0 and 8.0, while MES (25 mM) was used for experiments between pH 5.0 and 6.5. To obtain the same extent of ligation at all pHs investigated, kinetic experiments were carried out to determine the time required to obtain 40% ligation. Incubation times of 1 min were sufficient for experiments at pH 7.5 and 8.0, while 1.5, 2, 6, 12, and 15 min were necessary for experiments at pH 7.0, 6.5, 6.0, 5.5, and 5.0, respectively. Reported pH values are at 25 °C.

Sites of analogue interference were mapped by reacting the ribozymes (100 nM) containing $n^6C\alpha S$, $f^5C\alpha S$, ΨiCαS, CαS, dCαS, or AαS with 3′-end-labeled substrate CCCUCdTAAAAA at 50 °C in 3 mM $MgCl_2$ and 1 mM $Mn(OAc)_2$ buffered to the appropriate pH for an amount of time sufficient to yield 40% ligation. The reactions were quenched and iodine sequencing was performed as previously described.[34] Gel peak intensities were quantified by using a Storm PhosphorImager (Molecular Dynamics). Peak intensities for both the parental nucleotide PαS and the analogue δαS were used to calculate interference values (κ) according to the formula

$$\kappa = \mathrm{NF}\,\frac{\text{P}\alpha\text{S ligation}/\delta\alpha\text{S ligation}}{\text{P}\alpha\text{S 5}'\text{ control}/\delta\alpha\text{S 5}'\text{ control}}$$

A κ value of 1 indicated no interference. PαS 5′ control is a 5′-end-

(33) Scaringe, S. A.; Wincott, F. E.; Caruthers, M. H. *J. Am. Chem. Soc.* **1998**, *120*, 11820−11821.

(34) Ortoleva-Donnelly, L.; Szewczak, A. A.; Gutell, R. R.; Strobel, S. A. *RNA* **1998**, *4*, 498−519.

labeled unselected parental nucleotide RNA, while δαS 5′ control is a 5′-end-labeled unselected analogue RNA. NF is a normalization factor for correcting for the loading and ligation differences as previously described.[17]

5′-*O*-(1-Thio)-6-azacytidine Triphosphate (4a). 6-Azacytidine **1a** (0.0215 g, 0.088 mmol) was dissolved in triethyl phosphate (0.5 mL), trioctylamine (0.0195 mL, 0.044 mmol), and collidine (0.0142 mL, 0.106 mmol). The solution was stirred at 0 °C in an ice−water bath. $PSCl_3$ (0.011 mL, 0.106 mmol) was added to the mixture and stirring was continued at 0 °C for 30 min during which a white precipitate formed in the reaction. The reaction was allowed to warm to room temperature within 15 min and excess $PSCl_3$ was removed in vacuo for 5 min. A solution of tetra(tri-*n*-butylammonium) pyrophosphate (0.0973 g, 0.211 mmol) in triethyl phosphate (1 mL) was added and stirring was continued at room temperature for 10 min. The reaction product was precipitated by adding Et_3N (10 mL) and the precipitate was isolated by centrifugation. The precipitate was dissolved in triethylammonium bicarbonate (10 mL, 50 mM, pH 7.0) and the solution was stirred at room temperature overnight. The crude product was purified on DEAE-A25 sephadex column eluting with a gradient from 0.005 to 0.8 M triethylammonium bicarbonate yielding (TEAB) **4a** as a diastereomeric mixture (0.0207 mmol, 24% by UV, HPLC analysis on C18 column eluting with a gradient from 0 to 10% CH_3CN in 0.1 M TEAB revealed a diastereomerically pure mixture). ^{31}P NMR (H_2O, 121 MHz) δ 43.87(m), −10.51 (m), −23.73 (m); UV λ_{max} at 262; HRMS (ES, 50% CH_3CN/20mM TEAA) calcd for $[C_8H_{15}N_4O_{13}P_3S-H]^-$ 498.9491, found 498.9494.

Representative Procedure for the Silacyl Phosphoramidite Approach to Nucleoside 5′-*O*-(1-Thio)triphosphates. 5′-*O*-(1-Thio)-cytidine Triphosphate (4d). Cytidine **1d** (0.100 g, 0.405 mmol) was dried by repeated evaporation in anhydrous pyridine (2 × 5 mL) and then suspended in pyridine (2 mL). *N*,*N*-Dimethylformamide dimethylacetal (0.46 mL, 3.24 mmol) was added to the suspension and the mixture was stirred at room temperature. A homogeneous solution resulted within 3 h and stirring was continued overnight. Excess reagent and solvent were removed in vacuo to give crude amidine protected nucleoside **2d** as a white foam. Crude **2d** was dissolved in anhydrous pyridine (0.5 mL) and the solution was kept under argon. A solution of 1 M 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one in anhydrous dioxane (0.45 mL, 0.45 mmol) was added to the mixture resulting in a white precipitate. Stirring was continued for 10 min after which a solution of 0.5 M tributylammonium pyrophosphate in DMF/tri-*n*-butylamine 3:1 (1.62 mL, 0.81 mmol) was added with vigorous stirring leading to a clear solution. The reaction was stirred for 10 min followed by the addition of sublimed sulfur (0.026 g, 0.81 mmol), and stirring was continued for another 10 min. The reaction was quenched with water (5 mL) within 30 min and solvent was removed in vacuo. The crude product was dissolved in concentrated NH_4OH (15 mL) and NH_4OAc (0.018 g, 0.233 mmol) was added. The resulting mixture was kept at 50 °C for 30 min after which the solvent was evaporated in vacuo. The residue was taken up into triethylammonium bicarbonate (20 mL, 50 mM, pH 8.0) and stirred at room temperature overnight. ^{31}P NMR of the crude product showed that the only phosphate product is the desired 5′-*O*-(1-thio)triphosphate **4d**. However, the crude was purified on DEAE-A25 sephadex column eluting with a gradient from 0.005 to 0.8 M triethylammonium bicarbonate yielding **4d** as a diastereomeric mixture (102.4 mg, 51%). ^{31}P NMR (H_2O, 121 MHz) δ 43.57 (m), −9.29 (m), −23.48 (m); HRMS (ES, 50% CH_3CN/20 mM TEAA) calcd for $[C_9H_{16}N_3O_{13}P_3S-H]^-$ 497.9539, found 497.9540.

5′-*O*-(1-Thio)-5-fluorocytidine Triphosphate (4b). 5′-*O*-(1-Thio)-5-fluorocytidine analogue **4b** was prepared from **1b** as described above. ^{31}P NMR (H_2O, 121 MHz) δ 43.00 (m), −6.04 (m), −23.01 (m); UV λ_{max} at 280; HRMS (ES, 50% CH_3CN/20mM TEAA) calcd for $[C_9H_{15}FN_3O_{13}P_3S-H]^-$ 515.9445, found 515.9452.

5′-*O*-(1-Thio)pseudoisocytidine Triphosphate (4c). 5′-*O*-(1-Thio)-pseudoisocytidine analogue **4c** was prepared from **1c** as described above. ^{31}P NMR (H_2O, 121 MHz) δ 44.13 (m), −7.88 (m), −22.79 (m); UV λ_{max} at 288 (shoulder), 266; HRMS (ES, 50% CH_3CN/20mM TEAA) calcd for $[C_9H_{16}N_3O_{13}P_3S-H]^-$ 497.9539, found 497.9539.

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Supporting Information Available: General experimental details for the synthesis of 6-azacytidine and 5-fluorocytidine, including ^1H NMR and ^{13}C NMR spectral information. This material is available free of charge via the Internet at http://pubs.acs.org.

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