INTRODUCTION

Deazanucleosides are needed for atomic mutagenesis studies to explore RNA structure, function, and catalysis. The exchange of a specific nitrogen atom in a nucleobase by carbon can critically affect RNA properties because the hydrogen acceptor (imino, =N−) or hydrogen donor (amido or amino, −NH−) capabilities are impaired at the specific position. This is crucial for base pairing, RNA–protein recognition, RNA–small molecule recognition, and RNA-catalyzed chemical reactions. In particular, atomic mutagenesis lead to our current mechanistic understanding of ribozymes, including the ribosome.

Thus far, diverse deazanucleosides have been utilized for RNA atomic mutagenesis experiments; these are 3-deazacytidine (c3C), 7-deazaadenosine (cA), 3-deazaadenosine (c3A), 1-dezaadenosine (c1A), 3,6,8,9 RNA–protein recognition, 6,8,9 RNA–small molecule recognition, 10 and RNA-catalyzed chemical reactions. 4,11–14 In particular, atomic mutagenesis lead to our current mechanistic understanding of ribozymes, 15–18 including the ribosome.

RESULTS AND DISCUSSION

To date, synthetic routes to 1-deazaguanine nucleoside building blocks for oligonucleotide synthesis have been described for DNA only. 27 DNA containing 1-deaza-2′-deoxyguanosine (c1dG) is unstable toward acids, and this feature has been utilized to generate abasic sites. 28 Access to the naked ribonucleoside 1-deazaguanosine was first reported in the nineteen eighties, 29,30 employing rather harsh nucleosidation reactions involving mercury cyanide and based on Oβ-benzylated 1-deazaguanine, which itself requires stability of RNA double helices. Moreover, we found evidence for Hoogsteen base pair formation of c1G with protonated cytosine in HIV-2 TAR RNA by nuclear magnetic resonance (NMR) spectroscopy. The study is complemented by the crystal structure of a c1G-containing RNA hairpin to shed light on a specific guanine N1–phosphate backbone interaction observed in the wild-type RNA, and finally, we evaluate the crucial role of the guanosine N1 atom in catalysis of phosphodiester cleavage by the twister ribozyme.
laborious multistep synthesis. Later, access to 1-deazaguanosine was demonstrated via 5-amino-1-β-D-ribofuranosylimidazole-4-carboxamide (AICA-riboside) as the key intermediate. We, however, decided to put a direct, more efficient, and unprecedented route from readily available starting materials.

**Synthesis of c¹G Nucleoside.** For c¹G nucleoside 6 and the phosphoramidite precursor 5 (Scheme 1), we started the synthesis from commercially available 6-chloro-1-deazapurine, which was quantitatively transformed to the corresponding 6-iodo-1-deazapurine 1. Following the literature procedure,37 followed by the cleavage of the benzyl group by hydrogenation, and cleavage of the silyl ethers to give 1-phosphorous acids. Then, silyl-Hilbert oxidation of 6-iodo-1-deazapurine was conducted by HSiCl₃ referring to Benaglia and co-workers,38 followed by the reduction of the nitro group using HSiCl₃, followed by the cleavage of the benzyl group by hydrogenation providing nucleoside 7 (Scheme 2). Then, the O₅′ functionality was protected with a (2-nitrophenyl)ethyl (NPE) moiety applying Mitsunobu reaction conditions, followed by protection of the exocyclic NH₂ group using trifluoroacetic anhydride (TFAA), resulting in derivative 8. By the cleavage of the silyl ethers with tetrabutylammonium fluoride (TBAF), triol 9 was quantitatively obtained. Next, the 5′ and 3′ OH groups were simultaneously protected using di-tert-butylsilyl bis(trifluoromethanesulfonate) ([Bu₄Si(OTf)₂]₃8,₉₉ followed by silylation of the 2′-OH group with tert-butylmethylsilyl chloride (TBS-Cl) and subsequent removal of the 5′-O and 3′-O protection clamp with a solution of HF in pyridine to give compound 10. The functionalization of the 5′-O group with 4,4′-dimethoxytrityl chloride was conducted under standard conditions and yielded compound 11. Finally, the phosphoramidite 12 was generated by treatment with 2-cyanoethyl-N,N,N′,N″,N‴-tetraisopropylphosphorodiamidite (CEP[NPr₂]₄) in the presence of 5-benzylthio-1H-tetrazole (BTT). Starting from precursor 5, the target compound 12 was synthesized in six steps, with six chromatographic purifications and an overall yield of 33%; in total, 1.1 g of 12 was obtained in the course of this study.

**Synthesis of c¹G-Modified RNA.** The solid-phase synthesis of RNA with site-specific c¹G modifications was performed using the new building block 12 together with 2′-O-TBS protected A, C, G U phosphoramidites, or alternatively, with 2′-O-[(trisopropylsilyl)oxy]methyl protected (TOM) amidites. The novel building blocks were coupled with

![Scheme 1. Synthesis of c¹G Nucleoside 6 and Phosphoramidite Precursor 5](image)

![Scheme 2. Synthesis of c¹G Phosphoramidite 12](image)
yields higher than 98% according to the trityl assay. The cleavage of the oligonucleotides from the solid support and deprotection were conducted using methyamine/ammonia in water (AMA), followed by treatment with tetra-n-butylammonium fluoride (TBAF) in tetrahydrofuran. Salts were removed by size-exclusion chromatography, and RNAs were purified by anion-exchange chromatography under denaturing conditions (60 to 80 °C column temperature; Figure 1 and Supporting Figure S1). Such a linkage most likely forms during the deprotection of the TOM group where formaldehyde emerges as a byproduct.

Supporting Table S1). The molecular weights of the purified RNAs were confirmed by liquid chromatography (LC) electrospray-ionization (ESI) mass spectrometry (MS). The sequences of c1G containing RNAs synthesized in the course of this study are listed in Supporting Table S1. Notably, HPLC analysis of the crude deprotected c1G containing RNAs displayed a second product that was migrating slower, in particular, when TOM amidites were used. Isolation of this product and mass spectrometric analysis using a high-resolution Fourier-transform ion cyclotron resonance (FT ICR) spectrometer suggested RNA dimers that were cross-linked between two c1G nucleosides by a CH2 bridge, most likely between their exocyclic amino groups (for details, see Supporting Information).

Base Pairing Stability of c1G-Modified RNA. In principle, for the nucleobase of c1G, tautomeric forms and distinct rotamers have to be considered. An earlier study reported the energy differences of 9-methyl-1-deazaguanine tautomers and rotamers estimated by ab initio calculations. It was found that the c1G tautomer/syn-rotamer that we show in Figure 2A is the most stable one, followed by the anti-rotamer with 6-OH providing H-donor properties at the Watson Crick face, being 4.7 kcal/mol less stable. Importantly, the N3–H pyridone tautomer is 20.4 kcal/mol less stable.27 c1G is expected to impair Watson–Crick pairing because the central N1–H of G is replaced by C–H, thereby depriving the capability for the formation of strong hydrogen bonds (Figure 2A). The design of the RNA double helices investigated is shown in Figure 2B. The first motif (Type I) represents a bimolecular duplex of nine base pairs with a single c1G modification in the center. The second motif (Type II) is a hairpin with a GCAA loop (extrastable GNRA) and c1G residing in the center of its short stem. The third RNA motif (Type III and III′) consists of palindromic RNA of eight base pairs and equivalent purine–pyrimidine stacking patterns with the c1Gs either directly stacked toward each other, or separated by two standard base pairs. The type III/III′ design is very sensitive for the impact arising from a modification on base pairing. With only two or three regular Watson–Crick base pairs next to the modification, the nucleation of such duplexes can become significantly hindered.42,43 Thus, these RNA palindromes are anticipated to significantly respond to the c1G modification reflected in changes of the thermodynamic pairing parameters (T_m, ΔG, ΔH, ΔS).

Figure 2. Thermodynamic analysis of base pairing of c1G-modified RNAs. (A) Chemical structure of Watson Crick G−C base pair juxtaposed to a hypothetic c1G−C pair. (B) Sequence design in cartoon presentation to highlight stacking interactions (purine−purine and purine−pyrimidine interstrand stacking indicated in orange). (C) Overlay of UV-melting profiles of type I RNA with c1G-N mismatches (N = A, G, or U). (D) Overlay of UV-melting profile of type II RNA hairpin with c1G in stem and loop, respectively. Conditions: c(RNA) = 12 μM; 10 mM Na2HPO4, 150 mM NaCl, pH 7.0.

Figure 1. Characterization of c1G-modified RNA synthesized by standard RNA solid-phase synthesis using c1G building block 12. Anion-exchange HPLC traces (top) of purified 8 nt RNA (A), 27 nt RNA (B), and 27 nt RNA (C), and corresponding LC-ESI mass spectra (bottom). Asterisks indicate RNA dimers cross-linked via a CH2 moiety between two c1Gs (for detailed mass spectrometric analysis, see Supporting Figure S1). HPLC conditions: Dionex DNAPac column (4 × 250 mm), 80 °C (or as indicated), 1 mL min⁻¹, 0–60% buffer B in 45 min; buffer A: Tris−HCl (25 mM), 10 mM NaClO₄, pH 8.0, 20% acetonitrile; buffer B: Tris−HCl (25 mM), 600 mM NaClO₄, pH 8.0, 20% acetonitrile. For LC-ESI MS conditions, see the Supporting Information.

Table 1. Melting Temperatures of Type I RNAs.

| RNA Structure | T_m (°C) |
|---------------|----------|
| 8 nt RNA (A)  | 84.1     |
| 27 nt RNA (B) | 85.7     |
| 27 nt RNA (C) | 87.1     |

Table 2. Melting Temperatures of Type II RNA Hairpins.

| RNA Structure | T_m (°C) |
|---------------|----------|
| 8 nt RNA (A)  | 84.1     |
| 27 nt RNA (B) | 85.7     |
| 27 nt RNA (C) | 87.1     |
Table 1. Thermodynamic Parameters of c1G-Modified RNA (and Unmodified References) Obtained by UV Melting Profile Analysis

| #  | sequence (5′ → 3′) | $T_m$ [°C] | $\Delta T_m$ | $\Delta G^\circ_{25K}$ [kcal mol$^{-1}$] | $\Delta H^\circ$ [kcal mol$^{-1}$] | $\Delta S^\circ$ [cal mol$^{-1}$ K$^{-1}$] |
|----|------------------|-------------|-------------|-----------------|-----------------|-----------------|
| I  | GGCAAGGCC / GCCUCUCGCC | 66.7 | -16.5 ± 0.4 | -97.9 ± 4.6 | -212 ± 14 |
| la | GGCAAGGCC / GCCUCUCGCC | 50.9 | -15.8 | -131.1 ± 0.9 | -79.9 ± 7.7 | -224 ± 33 |
| lb | GGCAAGGCC / GCCUCUCGCC | 45.0 | -21.8 | -11.4 ± 0.2 | -73.6 ± 3.9 | -209 ± 12 |
| lc | GGCAAGGCC / GCCUCUCGCC | 43.5 | -23.2 | -10.9 ± 0.3 | -70.9 ± 5.2 | -201 ± 16 |
| Id | GGCAAGGCC / GCCUCUCGCC | 40.6 | -26.1 | -10.2 ± 0.1 | -69.7 ± 3.5 | -200 ± 11 |
| II | GAAGG-GCAAA-CUUCG (hairpin) | 72.7 | -6.6 ± 0.1 | -49.8 ± 0.8 | -145 ± 3 |
| IIa | GAAGG-GCAAA-CUUCG (hairpin) | 44.8 | -27.9 | -2.8 ± 0.4 | -48.5 ± 3.2 | -153 ± 9 |
| IIb | GAAGG-GCAAA-CUUCG (hairpin) | 63.1 | -9.6 | -6.2 ± 0.2 | -55.0 ± 2.7 | -165 ± 8 |
| IIc | GGUGGACCC (palindrome) | 58.3 | -13.2 ± 0.9 | -64.6 ± 8.6 | -172 ± 26 |
| IIla | GGUGGACCC (palindrome) | 22.4 | -35.9 | -6.3 ± 0.2 | -55.0 ± 2.7 | -164 ± 9 |
| IIlb | GGUGGACCC (palindrome) | 60.7 | -14.5 ± 1.1 | -72.3 ± 9.5 | -194 ± 28 |
| IIlc | GGUGGACCC (palindrome) | 24.1 | -36.5 | -6.6 ± 0.2 | -58.4 ± 1.1 | -174 ± 4 |

$^a$Buffer: 10 mM Na$_2$HPO$_4$, 150 mM NaCl, pH 7.0. $T_m$ values are listed at a concentration of 12 μM RNA. The estimated errors of UV-spectroscopically determined $T_m$ values are ±0.2 °C. $\Delta H$ and $\Delta S$ values were obtained by van’t Hoff analysis according to refs 44, 45. Errors for $\Delta H$ and $\Delta S$ arising from noninfinite cooperativity of two-state transitions and from the assumption of a temperature-independent enthalpy, are typically 10–15%. Additional error is introduced when free energies are extrapolated far from melting transitions; errors for $\Delta G$ are typically 3–5%. We note that for the biphasic profiles of Ib and Iib, the $T_m$ values and the errors were calculated for the second melting transition (between 30 and 85 °C).

The thermodynamic data we obtained for the three RNA systems by UV-spectroscopic melting profile measurements are summarized in Table 1 (for the corresponding melting profiles, see the Supporting Figures S2 to S11).$^{44,45}$ The native type I RNA I melts at 66.7 °C (Figure 2C). 1-Deazaguanine opposite of cytosine (Ia) destabilizes the duplex by 15.8 °C. Destabilization is even more pronounced if U, G, and A are the mismatch partner (−21.8 °C for Ib, −23.2 °C for Ic, and −26.1 °C for Id, respectively). For the hairpin RNA (Type II), c1G opposite of C (IIa) also decreases the melting temperature compared to the native hairpin II (by −27.9 °C) (Figure 2D). We note that duplex Ia shows a second melting transition at lower temperature, around 18 °C (Figure 2C). This may arise from a higher order structure (e.g., triplex formation) that we were not able to characterize in detail.

To further elucidate the impact of c1G on base pairing, we investigated the short palindromic RNAs that are particularly sensitive to double helix nucleation as mentioned above.$^{45,43}$ Indeed, for c1G, the destabilization in both palindromic RNAs was large, reflected in −35.9/−36.5 °C reduced $T_m$ values (IIa and IIa), accounting for −17.9/−18.3 °C destabilization per single modification which is higher compared to the destabilization that we observed for a single cG–C base pair in the bimolecular 9 bp duplex Ia with four regular − and hence nucleation-supportive − Watson–Crick base pairs at both 5′ and 3′ directions to the modification site.

Finally, we mention that the replacement of G in the synG•A Hoogsteen base pair of a GNRA loop in hairpin Iib was tolerated with significantly less decrease in stability (Figure 2D). This is reasonable because the G-N1-H atom is not involved in H-bonding in the synG•A Hoogsteen pair. Of note, we observe a second low melting transition for Iib, which may arise from competitive formation of a mismatched duplex.

**Acid–Base Properties of 1-Deazaguanine.** To understand base-pairing and catalytic properties of nucleobases in functional RNA, knowledge about their acid–base properties is crucial.$^{7,25}$ To quantify the acid–base properties of c1G, we conducted pH-dependent UV-spectroscopic titration experiments. Figure 3 shows an overlay of spectra for the c1G nucleobase that were used for pK$_a$ determinations. A value of 3.93 ± 0.07 (pK$_a$ 1) was obtained, attributed to the protonation of N7 (Supporting Figure S12). The second value of 9.10 ± 0.10 (pK$_a$ 2) was attributed to the deprotonation of the 6-OH group. The pK$_a$ values are thus comparable to the ones of guanine, which range from 9.2 to 9.6 (pK$_a$ 1, N1–H) and 3.2 to 3.3 (pK$_a$ 2, N7), respectively.

**X-Ray Structure Analysis of a c1G-Modified RNA.** To shed further light on the structural impact of c1G in RNA, we
aimed at a high-resolution X-ray crystallographic analysis. We utilized the 27 nt fragment of the *E. coli* 23S rRNA sarcin/ricin loop (SRL), which is a frequently applied crystallization scaffold (Figure 4A). For the replacement of G by c1G, we tested three different positions, including nucleotide G2669 which forms a Watson–Crick base pair with C2651 in the regular A-form double helical region, G2659 which forms a Hoogsteen pair with A2662 in the loop, and G2655 which interacts with the phosphate of G2664 in a bidentate fashion (Figure 4B, C). From these three c1G-modified RNAs, only c1G2655-modified SRL RNA provided crystals that diffracted to atomic resolution (0.9 Å) (Supplementary Table S2). X-ray structure determination demonstrated that the c1G nucleobase is well defined in the electron density maps for the c1G-modified RNA (Figure 4D, E). The c1G-modified RNA structure superimposed with the unmodified RNA displayed a root-mean-square deviation (rmsd) of 0.09 Å (within the errors on coordinates of 0.09 Å). Direct comparison of the base triples U2656-A2665-G2655 (Figure 4C) and U2656-A2665-c1G2655 (Figure 4D) reveals that with the weakening (or loss) of the G2655 N1−H⋯O−P H-bond, c1G slightly opens up by retaining the H-bond between c1G2655 2-NH2 and O4 of U2656.

A comparison of the melting profiles of wild-type and c1G modified SRL hairpins indicated a modest weakening of the fold (Supporting Figure S13).

Taken together, our crystallization experiments imply that c1G does not significantly affect an RNA fold as long as it is not replacing G in a Watson–Crick base pair. The weakening (or loss) of an H-bond to the phosphate backbone seems better tolerated and allowed crystallization and structure solution.

**Base Pairing Mode Switched by c1G.** In A-form RNA, Hoogsteen (HG) base pairs are energetically disfavored relative to Watson–Crick (WC) pairs. With 1-deazaguanosine in our hands, however, we were wondering if stable HG pairing might become favorable. We thereby focused on the human immunodeficiency virus type 2 (HIV-2) transactivation response element (TAR) RNA, where a G26-C39 WC bp is adjacent to a dinucleotide bulge (Figure 5A). It was demonstrated earlier that upon replacement of G26 by 1-methylguanosine (m1G26), the formation of a HG base pair with C39 occurs (Figure 5B, C).49 While in this case N1-methylacytation represents a sensitive block at the Watson Crick face, we intended to test the hypothesis if a simple shape-complementary modification (such as c1G) is sufficient to switch the pairing mode (Figure 5B, C). Indeed, our NMR spectroscopic analysis of HIV-2 TAR RNA containing c1G26 revealed that the HG base pair c1G26(syn)-C39H+ forms in a comparable manner. Characteristically, we observed a downfield shifted imino proton at ∼15 ppm (Figure 5D, Supporting Figure S14) and downfield shifted amino protons (Figure 5E) of C39H+ that is hydrogen-bonded to syn c1G26. Furthermore, a strong intra-nucleotide H1′−H8 NOE cross-peak (Figure 5F) is consistent with the syn conformation of the c1G26 base.

**Active-Site c1G Impedes Twister Ribozyme Cleavage.** Deazanucleobase-modified RNAs are frequently applied in atomic mutagenesis studies of ribozymes to shed light on the mechanism of the chemical reactions they catalyze.10,18,24,30 In particular, atomic mutagenesis experiments led to an in-depth understanding of general acid–base catalysis of small nucleolytic ribozymes that cleave their phosphodiester backbone, revealing the functionally crucial imino groups of purines and pyrimidines in the active site. For instance, this concerns the twister ribozyme51 where proton transfer from the (protonated) N3 of a conserved adenine (A6) at the cleavage site to the 5′-O leaving group significantly contributes to reaction catalysis; the replacement of this adenine by c1A or c1c5A rendered the twister ribozyme inactive.15,52 Another example is a phosphodiester cleavage by the pistol ribozyme.53 Replacements of active site purines by c1A, c1A, and c1G revealed the key residue – a highly conserved guanine (G33) – that serves as inner sphere coordination site for a hydrated Mg2+ ion, thereby likely providing a 1st shell water molecule as general acid for protonation of the 5′-O leaving group in the course of the reaction.16,17

---

**Figure 4. X-ray structure of c1G-modified RNA at 0.9 Å resolution.**

(A) Secondary structure of the *E. coli* sarcin/ricin stem-loop (SRL) RNA used for crystallization. The position for c1G nucleotide replacement is highlighted in red. (B) Chemical structure of G2655 interacting with the phosphate between G2664 and A2665 based on the crystal structure of native RNA PDB ID 3DVZ (top) and comparison to the c1G2655 interaction in the same RNA. (C) View on the base triple U2656-A2665-G2655 (PDB ID 3DVZ). (D) View on the base triple U2656-A2665-c1G2655 (PDB ID 7QP2). (E) 2Foobs − Fcalc electron density map contoured at 1.5 σ level showing the c1G2655 containing triple (PDB ID 7QP2). Numbers are distances in Angström (Å).
Access to c1G-modified RNA now allows evaluation of active site guanines that are suspected to be involved in reaction catalysis via their Watson−Crick face. To exemplify this, we picked the three-way junctional twister ribozyme, for which several structures of precatalytic states were solved by X-ray crystallography and structural dynamics elucidated by smFRET imaging.

Assignments supported by comparison to the corresponding TOCSY spectra (Supporting Figure S10) and ref 52. Conditions: 25 mM NaCl, 10% D2O, pH 5.8.

Figure 6. Atomic mutagenesis of the twister ribozyme: impact of an active site G-to-c1G mutation on activity to elucidate the mechanism of the phosphodiester cleavage. (A) Crystal structure of the twister ribozyme in a precatalytic state (PDB ID 4RGE). Active site highlighted by gray frame. Cleavage site dU5-A6 is colored yellow. (B) Close-up view showing the interaction of guanine-48 with the scissile phosphate; the 2′-OH nucleophile is modeled on U5; distance in Å. (C) Secondary structure of the two-strand ribozyme assembly used for functional cleavage assays. (D) HPLC traces of wild-type G48 (left) and c1G48 modified (right) ribozyme at two time points illustrate that product formation of the c1G-modified ribozyme is significantly impeded under otherwise same reaction conditions. Cleavage rate determination of wild-type G48 (E) and c1G48 (F) ribozymes.

stabilization. Furthermore, several studies propose the hypothesis of concerted general acid−base catalysis for twister in which G48 acts as the general base (Figure 6B).
With c′G in hand, we can now probe whether or not the NH donor of the Watson–Crick face of the suspected G48 is indeed a determinant in reaction catalysis (i.e., β and/or γ-catalysis according to refs 61, 62). Involvement of G48 as general base in phosphodiester cleavage catalysis implies either N1-deprotonation or enol tautomerization or at least hydrogen bonding to activate the attacking 2′-OH nucleophile. Also, stabilization of the pentavalent phosphorane transition state is conceivable as extrapolated from the G48N1–H···O=PPP interaction seen in the crystal structure.54 All these scenarios are severely affected upon the replacement of G48 by c′G; we therefore anticipated that cleavage becomes abolished. We, however, found that cleavage still occurs, albeit with a 275-fold reduction in rate (Figure 6C–F and Supporting Figures S15, S16).

The remaining cleavage activity indicates that the other catalytic determinants (i.e., α- and δ-catalysis according to refs 61 and 62) are sufficient to achieve residual activity of c′G48-modified twister ribozyme. We further note that the 6-OH group of c′G (possessing a comparable pKₐ to N1–H of G) is dislocated in comparison with N1 (in G) and therefore is not likely to be able to efficiently take over its role. However, the 2′-NH₂ group of guanine is present also in c′G, and therefore, this NH₂ group can contribute to stabilization of the phosphorane transition state (together with a remaining weak stabilization originating from a C1–H interaction with phosphorane).

\section*{CONCLUSIONS}

Our study introduces robust syntheses of c′G, the corresponding phosphoramidite, and c′G modified ribozyme. We have verified our synthesis foundation enabled comprehensive analysis of the biophysical properties of such modified RNA, and furthermore, enabled c′G atomic mutagenesis in functional RNA assays. This led to evidence for c′G switching the mode of base pairing from Watson–Crick to Hoogsteen. Moreover, the approach allows direct evaluation of ribozyme mechanistic proposals that claim a catalytic role for guanosines via their N1 position, the central H-donor of their Watson–Crick face. Beyond twister, such guanosines are found in many ribozymes including twister sister, pistol, hatchet, and the most recently discovered RNA methyltransferase ribozymes.18,24,63 Functional atomic mutagenesis relying on c′G RNA modifications will contribute to achieve an in-depth understanding of RNA catalysis of ribozymes that exhibit a much broader reactivity scope than previously anticipated.

\section*{ASSOCIATED CONTENT}

\begin{itemize}
  \item Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.2c01877.

Additional methods information, synthetic procedures and NMR spectra of all compounds, table of all RNAs synthesized and MS data, RNA melting curves, X-ray data collection and crystallographic refinement statistics, NMR spectra, and HPLC cleavage assays of the twister ribozyme (PDF)

\end{itemize}

\section*{AUTHOR INFORMATION}

\subsection*{Corresponding Author}

Ronald Micura – Institute of Organic Chemistry and Center for Molecular Biosciences, University of Innsbruck, Innsbruck 6020, Austria; orcid.org/0000-0003-2661-6105; Email: ronald.micura@uibk.ac.at

\subsection*{Authors}

Raphael Bereiter – Institute of Organic Chemistry and Center for Molecular Biosciences, University of Innsbruck, Innsbruck 6020, Austria

Eva Renard – Architecture et Réactivité de l’ARN - CNRS UPR 9002, Université de Strasbourg, Institut de Biologie Moléculaire et Cellulaire, Strasbourg 67084, France

Kathrin Breuker – Institute of Organic Chemistry and Center for Molecular Biosciences, University of Innsbruck, Innsbruck 6020, Austria

Christoph Kreutz – Institute of Organic Chemistry and Center for Molecular Biosciences, University of Innsbruck, Innsbruck 6020, Austria

Eva Renard – Architecture et Réactivité de l’ARN - CNRS UPR 9002, Université de Strasbourg, Institut de Biologie Moléculaire et Cellulaire, Strasbourg 67084, France

Complete contact information is available at: https://pubs.acs.org/10.1021/jacs.2c01877

\subsection*{Author Contributions}

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

\subsection*{Funding}

This work was supported by the Austrian Science Fund FWF (P27947, P31691, F8011-B to R.M; P32773 to C.K.; P30087 to K.B.), the Austrian Research Promotion Agency FFG [West Austrian BioNMR 858017], and the Wiener Wissenschafts-, Forschungs- und Technologiefonds (WWTF LS17-003). Open Access is funded by the Austrian Science Fund (FWF).

\subsection*{Notes}

The authors declare no competing financial interest.

\section*{ACKNOWLEDGMENTS}

We thank Marco Oberlechner (University of Innsbruck) for synthetic support. We thank Vincent Olieric for help during data collection at SLS, Villigen. We thank Daniel Fellner and Ulrike Schober (University of Innsbruck) for technical support.

We thank Elisabeth Mairhofer (University of Innsbruck) for discussions.

\section*{REFERENCES}

(1) Polacek, N. Atomic mutagenesis of the ribosome: towards a molecular understanding of translation. Chimia 2013, 67, 322–326.

(2) Hoernes, T. P.; Clementi, N.; Juen, M. A.; Shi, X.; Faserl, K.; Willi, J.; Gasser, C.; Kreutz, C.; Joseph, S.; Lindner, H.; Hüttenhofer, A.; Erlacher, M. D. Atomic mutagenesis of stop codon nucleotides reveals the chemical prerequisites for release factor-mediated peptide release. Proc. Natl. Acad. Sci. U. S. A. 2018, 115, E382–E389.

(3) Hu, T.; Suter, S. R.; Mumbleau, M. M.; Beal, P. A. TLR8 activation and inhibition by guanosine analogs in RNA: Importance of functional groups and chain length. Bioorg. Med. Chem. 2018, 26, 77–83.

(4) Forconi, M.; Benz-Moy, T.; Gleitsman, K. R.; Ruben, E.; Metz, C.; Herschlag, D. Exploring purine N7 interactions via atomic mutagenesis: the group I ribozyme as a case study. RNA 2012, 18, 1222–1229.

(5) Seela, F.; Debela, H.; Usman, N.; Burgin, A.; Beigelman, L. l-Deazaadenosine: synthesis and activity of base-modified hammerhead ribozymes. Nucleic Acids Res. 1998, 26, 1010–1018.
Journal of the American Chemical Society

(6) Kapinos, L. E.; Opschersall, B. P.; Larsen, E.; Sigel, H. Understanding the acid-base properties of adenosine: the intrinsic basicities of N1, N3 and N7. *Chem. – Eur. J.* 2011, 17, 8156–8164.

(7) Krishnamurthy, R. Role of pK(a) of nucleobases in the origins of chemical evolution. *Acc. Chem. Res.* 2012, 45, 2035–2044.

(8) Acharya, P.; Cheruku, P.; Chatterjee, S.; Acharya, S.; Chattopadhyaya, J. Measurement of nucleobase pKa values in model mononucleotides shows RNA-RNA duplexes to be more stable than DNA-DNA duplexes. *J. Am. Chem. Soc.* 2004, 126, 2862–2869.

(9) Bande, O.; Braddick, D.; Agrnello, S.; Jang, M.; Pezo, V.; Schepers, G.; Rozenski, J.; Lescrinier, E.; Marlère, P.; Herdewijn, P. Base pair involving artificial bases stable than DNA-DNA duplexes. *J. Am. Chem. Soc.* 2020, 142, 15128–15133.

(10) Micura, R.; Höbartner, C. Fundamental studies of functional nucleic acids: aptamers, riboswitches, ribozymes and DNAzymes. *Chem. Soc. Rev.* 2020, 49, 7331–7353.

(11) Fuchs, E.; Falschlunger, C.; Micura, R.; Breuker, K. The effect of adenine protonation on RNA phosphodiester backbone bond cleavage elucidated by deaza-nucleobase modifications and mass spectrometry. *Nucleic Acids Res.* 2019, 47, 7223–7234.

(12) Spitale, R. C.; Volpini, R.; Heller, M. G.; Krucinska, J.; Cristalli, G.; Wedekind, J. E. Identification of an imino group indispensable for cleavage by a small ribozyme. *J. Am. Chem. Soc.* 2009, 131, 6093–6095.

(13) Spitale, R. C.; Volpini, R.; Mengillo, M. V.; Krucinska, J.; Cristalli, G.; Wedekind, J. E. Single-atom imino substitutions at A9 and A10 reveal distinct effects on the fold and function of the hairpin ribozyme catalytic core. *Biochemistry* 2009, 48, 7777–7779.

(14) Zheng, L.; Falschlunger, C.; Huang, K.; Mairhofer, E.; Yuan, S.; Wang, J.; Patel, D. J.; Micura, R.; Ren, A. Proc. Natl. Acad. Sci. U. S. A. 2011, 116, 10783–10791.

(15) Kośutíc, M.; Neuner, S.; Ren, A.; Flurí, S.; Wunderlich, C.; Mairhofer, E.; Vušurović, N.; Seikowski, J.; Breuker, K.; Höbartner, C.; Patel, D. J.; Kreutz, C.; Micura, R. A Mini-Twister Variant and Impact of Residues/Cations on the Phosphodiester Cleavage of this Ribozyme Class. *Angew. Chem., Int. Ed.* 2015, 54, 15128–15133.

(16) Neuner, S.; Falschlunger, C.; Fuchs, E.; Himmelstoss, M.; Ren, A.; Patel, D. J.; Micura, R. Atom-Specific Mutagenesis Reveals Structural and Catalytic Roles for an Active-Site Adenosine and a nearby Adenine. *Org. Lett.* 2008, 10, 485–488.

(17) Teplova, M.; Falschlunger, C.; Krasheninnikova, O.; Egger, M.; Ren, A.; Patel, D. J.; Micura, R. Role of the 2′-hydroxyl group of A2451. *Nucleic Acids Res.* 2005, 33, 1618–1627.

(18) Erlander, M. D.; Lang, K.; Shankaran, N.; Wotzel, B.; Hüttenhofer, A.; Micura, R.; Mankin, A. S.; Polacek, N. Chemical engineering of the peptidyl transferase center reveals an important role of the 2′-hydroxyl group of A2451. *Nucleic Acids Res.* 2005, 33, 4924–4934.

(19) Polikanov, Y. S.; Steitz, T. A.; Innis, C. A. A proton wire to couple aminocarbamyl tRNA accommodation and peptide-bond formation on the ribosome. *Nat. Struct. Mol. Biol.* 2014, 21, 787–793.

(20) Das, S. R.; Piccirilli, J. A. General acid catalysis by the hepatitis delta virus ribozyme. *Nat. Chem. Biol.* 2005, 1, 45–52.

(21) Lang, K.; Erlander, M. D.; Wilson, D. N.; Micura, R.; Polacek, N. The role of 2′-deoxyguanosine and its application to postsynthetic modification. *Org. Lett.* 2005, 7, 709–712.

(22) Schelling, J.; Salemkirn, C. Deazapurine derivatives XIV. The synthesis of 1-deaza-2′-deoxyguanosine and its application to postsynthetic modification. *Org. Lett.* 2007, 9, 156–159.

(23) Schelling, J.; Salemkirn, C. Deazapurine derivatives III. Disubstituted imidazo[4,5-b]pyridine. A new synthesis of 1-deaza-2′-deoxyguanosine. *Recueil: J. Royal Netherlands Chem. Soc.* 1974, 93, 160–162.

(24) Kojima, N.; Minakawa, N.; Matsuda, A. Nucleosides and Nucleotides. Part 207: Studies in the Chemical Conversion of 4-Carboxamide Group of 5-Amino-1-(2′-deoxyguanosinyl)imidazo[4,5-b]pyrindine-7-one (1-Deazaguanosine) and Related Nucleosides. *J. Heterocycl. Chem.* 1978, 15, 839–847.

(25) Kojima, N.; Sugino, M.; Miikami, A.; Ohtsuka, E.; Komatsu, Y. Generation of an abasic site in an oligonucleotide by using acridine-1-deaza-2′-deoxyguanosine and its application to postsynthetic modification. *Biochemistry* 2002, 41, 973–976.

(26) Deugrén, H.; Wanner, M. J.; Koemen, G. J. Mild and regioselective nitration of 1-deazapurine nucleosides using TBAN/WF. *Tetrahedron Lett.* 2000, 41, 1569–1573.

(27) Deugrén, H.; Wanner, M. J.; Koemen, G. J. Selective nitration of purine nucleosides: synthesis of 2-nitrodeoxynucleosides and 2-nitrodeoxynucleosides. *Tetrahedron Lett.* 2000, 41, 1291–1295.

(28) Wanner, M. J.; Rodenko, B.; Koch, M.; Koemen, G. J. New (1-deaza)purine derivatives via efficient C-2 nitration of the 1-deazapurine ring. *Nucl., Nucl. Acids Res.* 2004, 23, 1313–1320.

(29) Orlandi, M.; Tosi, F.; Bonasignore, M.; Benaglia, M. Metal-Free Reduction of Aromatic and Aliphatic Nitro Compounds to Amines: A HSciCl3-Mediated Reduction of Wide General Applicability. *Org. Lett.* 2015, 17, 3941–3943.

(30) Serebryany, V.; Beigelman, L. An efficient preparation of protected ribonucleosides for phosphoramidite RNA synthesis. *Tetrahedron Lett.* 2002, 43, 1983–1985.

(31) Serebryany, V.; Beigelman, L. Synthesis of 2′-O-Substituted Ribonucleosides. *Nucl. Nucl. Acids Res.* 2003, 22, 1007–1009.

(32) Pitsch, S.; Weiss, P. A.; Jenny, J.; Stuttz, A.; Wu, X. Reliable Chemical Synthesis of Oligoribonucleotides (RNA) with 2′-O-[(Triisopropylsilyl)oxy]methyl (2′-O-tom)-Protected Phosphoramidites. *Helm. Chim. Acta* 2001, 84, 3773–3795.

(33) Wachiowsiu, F.; Høbartner, C. Chemical RNA modifications for studies of RNA structure and dynamics. *ChemBioChem* 2010, 11, 469–480.

(34) Saenger, W. *Principles of Nucleic Acid Structure*; Springer: Berlin, 1984.

(35) Majlessi, M.; Becker, M. M. Formation of the double helix: a mutational study. *Nucleic Acids Res.* 2008, 36, 2981–2989.
(44) Marky, L. A.; Breslauer, K. J. Calculating thermodynamic data for transitions of any molecule from equilibrium melting curves. *Biopolymers* 1987, 26, 1601–1620.

(45) Petersheim, M.; Turner, D. H. Base-stacking and base-pairing contributions to helix stability: thermodynamics of double-helix formation with CCGG, CCGGp, ACCGGp, CCGGAp, and ACCGGAp. *Biochemistry* 1983, 22, 256–263.

(46) Verdolino, V.; Cammi, R.; Munk, B. H.; Bernhard Schlegel, H. Calculation of pKa values of nucleobases and the guanine oxidation products guanidinohydantoin and spiroiminodihydantoin using density functional theory and a polarizable continuum model. *J. Phys. Chem. B* 2008, 112, 16860–16873.

(47) Oliferic, V.; Rieder, U.; Lang, K.; Serganov, A.; Schulze-Briese, C.; Micura, R.; Dumas, P.; Ennifar, E. A fast selenium derivatization strategy for crystallization and phasing of RNA structures. *RNA* 2009, 15, 707–715.

(48) Correll, C. C.; Wool, I. G.; Munishkin, A. The two faces of the Escherichia coli 23 S rRNA sarcin/ricin domain: the structure at 1.11 Å resolution. *J. Mol. Biol.* 1999, 292, 275–287.

(49) Rangadurai, A.; Zhou, H.; Merriman, D. K.; Meiser, N.; Liu, B.; Shi, H.; Szymbanski, E. S.; Al-Hashimi, H. M. Why are Hoogsteen base pairs energetically disfavored in A-RNA compared to B-DNA? *Nucleic Acids Res.* 2018, 46, 11099–11114.

(50) Kath-Schorr, S.; Wilson, T. J.; Li, N. S.; Lu, J.; Piccirilli, J. A.; Lilley, D. M. J. General acid-base catalysis mediated by nucleobases in the hairpin ribozyme. *J. Am. Chem. Soc.* 2012, 134, 16717–16724.

(51) Roth, A.; Weinberg, Z.; Chen, A. G.; Kim, P. B.; Ames, T. D.; Breake, R. A. A widespread self-cleaving ribozyme class is revealed by bioinformatics. *Nat. Chem. Biol.* 2014, 10, 56–60.}

(52) Gebetsberger, J.; Micura, R. Unwinding the twister ribozyme: from structure to mechanism. *Wiley Interdiscip. Rev. RNA* 2017, 8, No. e1402.

(53) Weinberg, Z.; Kim, P. B.; Chen, T. H.; Li, S.; Harris, K. A.; L ü nse, C. E.; Breake, R. R. New classes of self-cleaving ribozymes revealed by comparative genomics analysis. *Nat. Chem. Biol.* 2015, 11, 606–610.

(54) Ren, A.; Košutić, M.; Rajashankar, K. R.; Frener, M.; Santner, T.; Westhof, E.; Micura, R.; Patel, D. J. In-line alignment and Mg2+ coordination at the cleavage site of the env222 ribozyme. *Nat. Commun.* 2014, 5, 5534.

(55) Liu, Y.; Wilson, T. J.; McPhee, S. A.; Lilley, D. M. J. Crystal structure and mechanistic investigation of the twister ribozyme. *Nat. Chem. Biol.* 2014, 10, 739–744.

(56) Wilson, T. J.; Liu, Y.; Domnick, C.; Kath-Schorr, S.; Lilley, D. M. J. The Novel Chemical Mechanism of the Twister Ribozyme. *J. Am. Chem. Soc.* 2016, 138, 6151–6162.

(57) Gaines, C. S.; York, D. M. Ribozyme Catalysis with a Twist: Active State of the Twister Ribozyme in Solution Predicted from Molecular Simulation. *J. Am. Chem. Soc.* 2016, 138, 3058–3065.

(58) Ucisk, M. N.; Bevilaqua, P. C.; Hammes-Schiffer, S. Molecular Dynamics Study of Twister Ribozyme: Role of Mg(2+) Ions and the Hydrogen-Bonding Network in the Active Site. *Biochemistry* 2016, 55, 3834–3846.

(59) Vusurovic, N.; Altman, R. B.; Terry, D. S.; Micura, R.; Blanchard, S. C. Pseudoknot Formation Seeds the Twister Ribozyme Cleavage Reaction Coordinate. *J. Am. Chem. Soc.* 2017, 139, 8186–8193.

(60) Eiler, D. D.; Wang, J.; Steitz, T. A. Structural basis for the fast self-cleavage reaction catalyzed by the twister ribozyme. *Proc. Natl. Acad. Sci. U. S. A.* 2014, 111, 13028–13033.

(61) Breake, R. R.; Emilsson, G. M.; Lazarev, D.; Nakamura, S.; Puskari, I. J.; Roth, A.; Sudarsan, N. A common speed limit for RNA-cleaving ribozymes and deoxyribozymes. *RNA* 2003, 9, 949–957.

(62) Bevilaqua, P. C.; Harris, M. E.; Piccirilli, J. A.; Gaines, C.; Ganguly, A.; Kostenbader, K.; Ekesan, S.; York, D. M. An Ontology for Facilitating Discussion of Catalytic Strategies of RNA-Cleaving Enzymes. *ACS Chem. Biol.* 2019, 14, 1068–1076.

(63) Jiang, H.; Gao, Y.; Zhang, L.; Chen, D.; Gan, J.; Murchie, A. I. H. The identification and characterization of a selected SAM-dependent methyltransferase ribozyme that is present in natural sequences. *Nat. Catal.* 2021, 4, 872–881.