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1-Deazainosine-impact on RNA structure and role in exploring ribozyme catalysis

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Synthetic RNAs bearing deazapurine nucleobases are powerful probes for dissecting RNA-catalyzed reactions by atomic mutagenesis. Here we systematically characterize RNA containing 1-deazainosine (c^1I) and compare it with inosine (I). We first report the synthesis of a suitably protected c^1I phosphoramidite and its incorporation into RNA by solid-phase synthesis. We then provide a comprehensive thermodynamic analysis of base-pair stability from UV-melting experiments, showing that c^1I -C pairs are less stable than the corresponding I-C pairs. Although a two-hydrogen-bond Hoogsteen interaction between c^1I and protonated C is conceivable, NMR spectroscopy indicates that c^1I -C predominantly adopts a Watson-Crick-like geometry with a single hydrogen bond. These pairs are accommodated within RNA duplexes without disrupting neighboring base pairing. We also use c^1I to probe poly(I:C) motifs that mimic viral double-stranded RNA, assessing how strand length governs duplex *versus* hairpin formation. Finally, atomic mutagenesis of the twister ribozyme with c^1I supports the hypothesis that an active-site guanine participates directly in phosphodiester-bond cleavage. Together, these results clarify how deazapurines modulate nucleic-acid properties and provide guidance for their use in atomic mutagenesis to interrogate RNA catalysis.

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Introduction

Dezanucleoside modifications are useful for atomic mutagenesis studies to explore RNA structure, function, and reactivity.^{1–5} Thereby, the replacement of a nitrogen atom in a nucleobase by carbon influences the properties of RNA because the hydrogen acceptor capability of an imino functionality (=N-) or the hydrogen donor capability of an amido or amino functionality (-NH-) becomes impaired at the specific position.^{6–8} This is crucial for base pairing,^{8,9} RNA-protein recognition,^{3,6,8,9} RNA-small molecule recognition,¹⁰ and RNA catalysis.^{4,11–14} Atomic mutagenesis has contributed to our mechanistic understanding of ribozymes,^{15–18} including the ribosome.^{19–22} So far, the selection of dezanucleosides that is available for RNA atomic mutagenesis experiments include 3-deazacytidine (c^3C),^{14,23,24} 7-deazaadenosine (c^7A),^{4,12–14,16,17} 3-deazaadenosine (c^3A),^{15,23} 1-deazaadenosine (c^1A),^{12,13,15,23} 1,3-dideazaadenosine,¹⁴ 7-deazaguanosine (c^7G),^{14,16} 3-deazaguanosine (c^3G),^{25,26} 1-deazaguanosine (c^1G),²⁷ and 1,3-dideazaguanosine.²⁸ Another modification that usefully adds to the dezanucleoside toolbox is 1-deazainosine (c^1I). This modification is particularly powerful to probe the involvement of guanine in transition state stabilization of self-cleaving

ribozymes (see Fig. 1). However, there is only one report in the literature describing the application of a c^1I -modified variant of the pistol ribozyme.²⁹ Information on the chemical

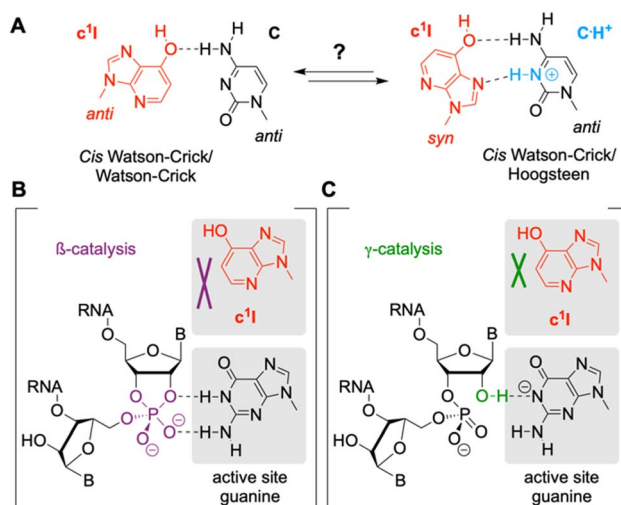


Fig. 1 (A) Possible pairing patterns for 1-deazainosine with cytidine (c^1I -C) in RNA: Watson-Crick vs. Hoogsteen. (B) The cleavage reaction of nucleolytic ribozymes can involve a pentavalent phosphorane transition state that is stabilized by interactions with an active-site guanine (β -catalysis). Access to c^1I allows evaluation of these interactions in functional assays. (C) Same as B but for deprotonation of the attacking 2'-OH (γ -catalysis).

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synthesis and biophysical properties of c^1I RNA has been lacking so far.

In the present work, we report a robust synthesis of an appropriately protected c^1I phosphoramidite building block and its incorporation into oligoribonucleotides by solid-phase synthesis. Furthermore, we describe the impact of c^1I on the thermodynamic stability of RNA double helices, determined by UV spectroscopic melting experiments. In addition, we describe the pairing mode of c^1I with cytidine in short structured RNA motifs (Fig. 1A), based on investigations by nuclear magnetic resonance (NMR) spectroscopy. The study continues with an analysis of how c^1I modifications impact the secondary structure of poly(I:C) RNA motifs. Poly(I:C) RNA is a synthetic analog of double-stranded RNA that is commonly used as a molecular mimic of viral infection. Finally, we challenge the hypothesis that a guanine residue in the active site is involved in the catalysis of phosphodiester cleavage in the twister ribozyme (Fig. 1B and C). We do so by demonstrating the complementary and unique utility of c^1I as an atomic mutagenesis probe for this functional RNA.

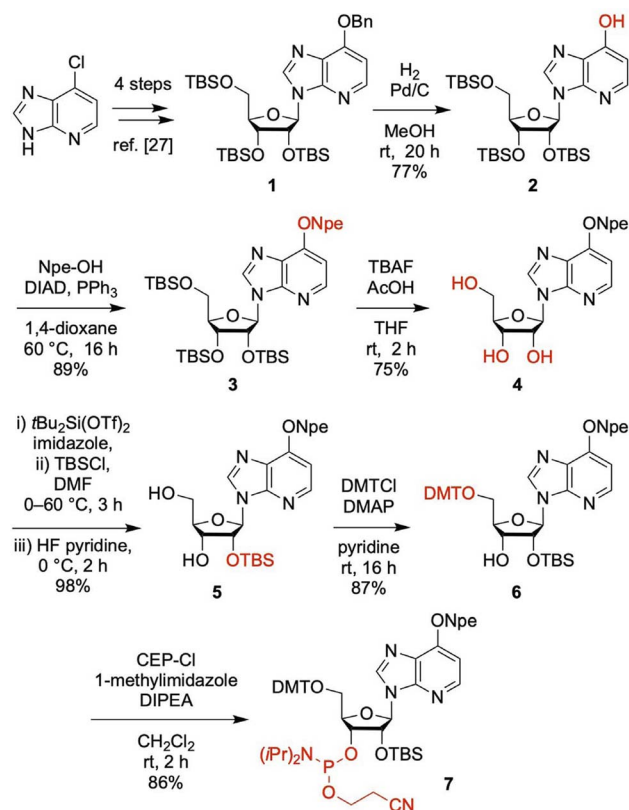
Results and discussion

Synthesis of 1-deazainosine (c^1I) phosphoramidite

The synthetic route to c^1I phosphoramidite made use of a precursor that we introduced recently for the synthesis of 1-deazaguanosine (c^1G) phosphoramidite.²⁷ Starting from commercially available 6-chloro-1-deazapurine and 1,2,3,5-tetraacetyl- β -D-ribofuranose, the O^6 -benzyloxy-1-deazapurine nucleoside **1** was readily obtained in four steps (Scheme 1).²⁷ Then, the benzyl protection was removed under hydrogenation conditions to release the hydroxyl group at C6. Under Mitsunobu conditions using triphenylphosphine, diisopropyl azodicarboxylate (DIAD) and 2-(*p*-nitrophenyl)ethanol (Npe-OH), the C6-OH of compound **2** was protected with a *p*-nitrophenylethyl (Npe) group to provide nucleoside **3**. Subsequent deprotection of the *tert*-butyldimethylsilyl (TBS) groups with tetra-*n*-butylammonium fluoride (TBAF) afforded the triol **4**. Using the Beigelmann approach,³⁰ the 3' and 5' OH groups were first reacted with di-*tert*-butylsilyl bis(trifluoromethanesulfonate) followed by selective protection of the 2'-OH group using TBS-Cl. Removal of the silyl clamp of the 3' and 5'-OH groups with pyridine buffered hydrofluoric acid afforded the selectively 2'-*O*-TBS protected nucleoside **5**. The 5'-OH was protected using 4,4'-dimethoxytrityl chloride (DMT-Cl) under standard condition to give compound **6**. Finally, the phosphoramidite was synthesized under basic conditions using 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (CEP-Cl), 1-methylimidazole and diisopropylethylamine (DIPEA) to give the target compound **7**. Starting from the commercially available 6-chloro-1-deazapurine, the c^1I -phosphoramidite **7** was synthesized in ten steps, with nine chromatographic purifications and an overall yield of 17%.

Synthesis of c^1I -modified RNA

RNAs with site-specific c^1I modifications were synthesized on solid-phase using the new building block **7** together with 2'-*O*-



Scheme 1 Synthesis of 1-deazainosine (c^1I) phosphoramidite. Reaction conditions and yields as indicated. *N,N*-Diisopropylethylamine, DIPEA; (2-nitrophenyl)ethyl, NPE; diisopropyl azodicarboxylate, DIAD; trifluoroacetic anhydride, TFAA; trifluoroacetyl, Tfa; tetra-*n*-butylammonium fluoride, TBAF; *tert*-butyldimethylsilyl, TBS; 4,4'-dimethoxytrityl chloride, DMT; 4-(dimethylamino)pyridine, DMAP; cyanoethyl, CE.

TBS protected A, C, G U phosphoramidites, or alternatively, with 2'-*O*-[(triisopropylsilyloxy)methyl protected (TOM) amidites.^{31,32} The novel building block was coupled with yields higher than 98% according to the trityl assay. The cleavage of the oligonucleotides from the solid support and deprotection were conducted using methylamine/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 (80 °C column temperature; SI Fig. 1 and Table S1). The molecular weights of the purified RNAs were confirmed by liquid chromatography (LC) electrospray-ionization (ESI) mass spectrometry (MS). The sequences of c^1I containing RNAs synthesized in the course of this study are listed in SI Table S1 and (see also SI Fig. 1B)

Impact of c^1I on RNA base pairing stability

In RNA double helices, inosine can pair with cytidine (I-C) in a typical *cis*-Watson-Crick geometry,³³ and the pairing strength of I-C is comparable to a standard adenosine-uridine (A-U) pair.³⁴ The replacement of inosine by c^1I is expected to impair



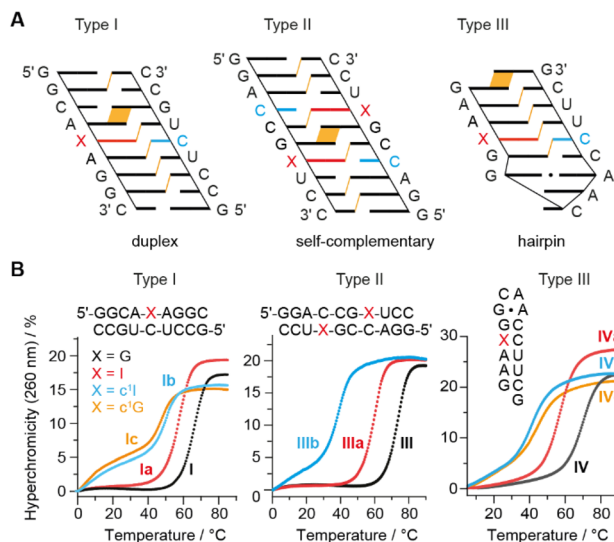


Fig. 2 UV spectroscopic RNA melting study. (A) Sequence design for the thermodynamic analysis of RNA double helices containing c^1 -C base pairs (types I, II and III). The cartoon presentations highlight inter-strand stacking interactions (in orange). (B) Exemplary UV melting profiles of c^1 I modified RNAs and reference systems. For conditions see Table 1 and SI.

pairing strength because the N1-H of I is replaced by C-H, thereby depriving the capability for the formation of a strong hydrogen bond with N3 of C (Fig. 1A). To investigate the thermodynamic impact of c^1 I in more detail we designed different

types of RNA double helices as shown in Fig. 2A. The first motif (Type I) represents a bimolecular duplex of nine base pairs with the target base interaction in the duplex center. The second RNA motif (Type II) consists of a palindromic RNA of ten base pairs with two target interactions separated by two standard base pairs. The type II 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 substantially hindered.^{35,36} Thus, these RNA palindromes are anticipated to strongly respond to a base modification reflected in changes of the thermodynamic pairing parameters (T_m , ΔG , ΔH , ΔS). The third RNA motif (Type III) is a hairpin with a GCAA loop (extra stable GNRA) and the modification residing in the center of its short stem.

The thermodynamic data we obtained for the three RNA systems by UV-spectroscopic melting profile measurements are illustrated in Fig. 2B and summarized in Table 1 (for the corresponding melting profiles, see the SI Fig. S2 to S5).³⁷⁻³⁹ The type I RNA **I** melts at 67.7 °C (Fig. 2B). When the central guanosine of the G-C base pair (three hydrogen bonds) is replaced by inosine (I-C, two hydrogen bonds), the duplex (**Ia**) is destabilized by 9.3 °C. This is about the same extent of destabilization as observed for the replacement of G-C by A-U ($\Delta T = -8.2$ °C, $\Delta G = 1.8$ kcal mol⁻¹).⁴⁰ Placing c^1 I opposite of cytosine (**Ib**) destabilizes the duplex further by 10.3 °C ($\Delta G = 2.5$ kcal mol⁻¹) which is consistent with the formal reduction of hydrogen bonds in the Watson-Crick mode to just one. We

Table 1 Thermodynamic parameters of modified RNAs (and references) obtained by UV melting profile analysis^a

#	RNA sequence ^a	T_m [°C]	ΔG^0 [kcal mol ⁻¹] ^b	ΔH^0 [kcal mol ⁻¹] ^b	ΔS^0 [cal mol ⁻¹ K ⁻¹] ^b
I	5' GGCAGAGGC 3' CCGUCUCCG	67.7	-17.2 ± 0.3	-84.0 ± 2.0	-224 ± 6
Ia	5' GGCAIAGGC 3' CCGUCUCCG	58.4	-15.1 ± 0.5	-83.3 ± 8.2	-228 ± 20
Ib	5' GGCAC ¹ IAGGC 3' CCGUCUCCG	48.1	-12.6 ± 0.8	-81.2 ± 8.2	-230 ± 25
Ic^c	5' GGCAC ¹ GAGGC 3' CCGUCUCCG	50.9	-13.1 ± 0.9	-79.9 ± 7.7	-224 ± 23
II	5' GGCUAGCC	60.3	-15.4 ± 0.3	-81.7 ± 2.8	-223 ± 10
IIa	5' GGCUAICC	42.1	-10.3 ± 0.1	-65.1 ± 3.4	-184 ± 11
IIb^d	5' GGCUAAC ¹ ICC ^d	21.9	-6.2 ± 0.6	-50.2 ± 8.4	-148 ± 26
III	5' GGACCGGUCC	74.7	-18.7 ± 0.9	-83.9 ± 6.3	-219 ± 18
IIIa	5' GGACCGIUCC	60.2	-14.4 ± 0.1	-72.7 ± 0.3	-195 ± 1
IIIb	5' GGACCGC ¹ IUCC	42.2	-11.4 ± 0.9	-93.4 ± 7.2	-274 ± 21
IV	5' GAAGG-GCAA-CCUUCG	71.0	-7.5 ± 0.2	-57.9 ± 1.8	-169 ± 5
IVa	5' GAAIG-GCAA-CCUUCG	57.4	-4.9 ± 0.1	-50.7 ± 0.9	-154 ± 3
IVb	5' GAAc ¹ IG-GCAA-CCUUCG	41.3	-2.7 ± 0.6	-41.8 ± 8.8	-131 ± 29
IVc^c	5' GAAc ¹ GG-GCAAACCUUCG ^c	44.8	-2.5 ± 0.3	-38.0 ± 4.5	-119 ± 14
V	5' I ₁₅ C ₁₅	50.4	-5.2 ± 0.4	-57.3 ± 3.3	-174 ± 10
Va	5' I ₇ C ₇	28.5	-7.8 ± 0.2	-88.4 ± 2.3	-271 ± 7
Vb	5' I ₆ c ¹ IC ₇	9.9	-3.7 ± 0.3	-56.8 ± 4.6	-178 ± 15

^a Buffer: 10 mM Na₂HPO₄, 150 mM NaCl, pH 7.0. T_m values are listed at a concentration of 12 μM RNA (calculated from concentration dependent measurements; see 1/ T_m vs. ln c plots in the SI). The estimated errors of UV-spectroscopically determined T_m values are ±0.2 °C. ΔH and ΔS values were obtained by van't Hoff analysis according to refs (37-39). Errors for ΔH and ΔS , 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 ΔG are typically 3-5%. ^b At 298 K. ^c Thermodynamic data of c^1 G reference RNAs were taken from our earlier study, ref. 27. ^d This RNA avoids a direct C- c^1 I interaction by frameshifting; see main text and Fig. 5.



further compared the c^1I -C containing duplex to the previously investigated c^1G -C containing duplex and found that latter is only 2.8 °C more stable ($\Delta G = -0.5$ kcal mol⁻¹).²⁷

The same trend of thermodynamic stabilities for inosine and 1-deazainosine modifications was also found in type II palindromic RNAs **II** and **III** (see e.g., **III** to **IIIc**; G replaced by I results in: $\Delta T = -7.3$ °C per bp, $\Delta G = 2.15$ kcal mol⁻¹ per bp; I replaced by c^1I results in: $\Delta T = -9.0$ °C per bp, $\Delta G = 1.5$ kcal mol⁻¹ per bp) and type III hairpin RNA (**IV** to **IVc**; G replaced by I results in: $\Delta T = -13.5$ °C, $\Delta G = 4.0$ kcal mol⁻¹; I replaced by c^1I : $\Delta T = -16.0$ °C, $\Delta G = 3.0$ kcal mol⁻¹; c^1I replaced by c^1G results in: $\Delta T = 3.1$ °C per bp, $\Delta G = -0.3$ kcal mol⁻¹ per bp) (Fig. 2B and Table 1).

At this point, we mention that we measured the hairpin system (**IV**, **IVa**, and **IVb**) at different NaCl concentrations. As expected, the melting temperature increased with increasing concentrations of NaCl (SI Fig. S6). We found no indication for a change of the c^1I -C pairing mode within the NaCl concentration range measured.

The thermodynamic data obtained in this study is consistent with earlier studies on the thermodynamics of I-C containing oligoribonucleotides that focused on the determination of nearest neighbor parameters.³⁴ Our data is also consistent with a comparative study analyzing the loss of one hydrogen bond in A-U versus P-U (purine-uridine) base pairs⁴¹ that resembles the situation of I-C versus c^1I -C containing RNA, although the extent of destabilization was higher for I-C (two H-bonds) vs. c^1I -C (one H-bonds) (**Ia** vs. **Ib**: $\Delta\Delta G_{37}^{\circ} = 2.6$ kcal mol⁻¹) compared to A-U (two H-bonds) vs. P-U (one H-bonds) (see first entry in Table 2 in ref. 41: $\Delta\Delta G_{37}^{\circ} = 1.85$ kcal mol⁻¹).

This observation might be rationalized by the fact that c^1I has an altered heterocyclic core (compared to I) with reduced

stacking propensity, in contrast to P whose heterocyclic core remains unchanged (compared to A).

Notably, for c^1I opposite to C, a *trans* Watson-Crick Hoogsteen base pair geometry with a N3-protonated C is conceivable as an alternative to the Watson-Crick pairing mode (Fig. 1). For c^1I , the Hoogsteen pairing mode even seems to be advantageous because of the formation of two hydrogen bonds instead of only one (Fig. 1A). To find evidence for this potential pairing alternative, we performed pH-dependent UV melting experiments as shown in Fig. 3. However, since the observed T_m values showed only little dependence on the pH of the melting buffer (2 to 3 °C), the involvement of a protonated C species in base pairing seems unlikely; if a protonated C species were involved, a pronounced increase in T_m with decreasing pH is expected (~6 to 8 °C), as recently observed for pairing of c^1c^3G to protonated C,²⁸ or for xanthosine pairing to protonated cytosine.⁴⁰

Further evidence against a Hoogsteen-like pairing mode of c^1I -C, and in favor of a Watson-Crick-like geometry, originates from NMR spectroscopy as described below.

NMR spectroscopy of inosine and 1-deazainosine containing RNA

Watson-Crick base-pairs are easily detectable by ¹H-NMR spectroscopy. The signals for hydrogen-bonded protons ('imino protons') directly reflect the formation of double helices within folded RNA.⁴¹⁻⁴³ The chemical shifts of these signals are characteristic for A-U (>13.5 ppm) and C-G (~11.5-13.5 ppm), and the linewidths reflect proton exchange with the solvent. The chemical shifts of imino protons are sensitive to chemical modifications, in particular, if the modification concerns the nucleobase.

Comparative imino proton ¹H NMR spectra of the hairpin 5'-GAAGG-GCAA-CCUUCG **IV** and the corresponding I-C, and c^1I -C modified counterparts, **IVa** and **IVb**, are depicted in Fig. 4. They show that I-C forms a well-defined interaction at room temperature, characterized by a resonance that was shifted from 12.70 ppm (G4-C in **IV**) to 15.45 ppm (I4-C in **IVa**). All other standard Watson-Crick base pairs retained their imino proton chemical shifts (Fig. 4A, left panel; and SI Fig. 7A), indicating that an I-C pair integrates well into an A-form RNA double helix, with little effect on the neighborhood. This is consistent with previous studies reported in the literature that found that I and C can form base pairs that are isosteric to standard (*cis*) Watson-Crick pairs.^{44,45}

The behavior was different for hairpin **IVb** which contained a c^1I opposite to C. At room temperature, the pronounced thermodynamic destabilization is reflected in a substantial broadening of all imino proton resonances in the stem, although their chemical shift values were essentially unaffected (SI Fig. 7A). Only when the temperature was reduced to 5 °C (Fig. 4B), sharp signals were detected for all Watson-Crick base pairs and the characteristic G-A pair in the hairpin loop.^{46,47} No other resonances that would directly suggest a hydrogen-bonded c^1I -C4 base pair (e.g. Hoogsteen pair involving

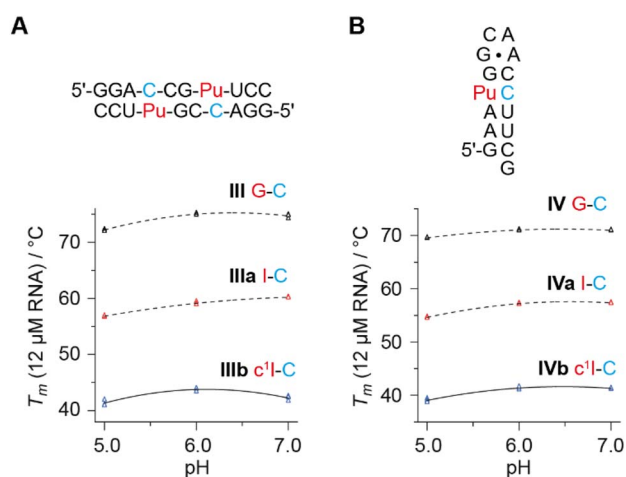


Fig. 3 pH dependent UV-melting analysis of c^1I modified RNAs and reference systems. (A) T_m vs. pH plot for palindromic RNAs **III** (G-C), **IIIa** (I-C) and **IIIb** (c^1I -C). (B) T_m vs. pH plot for hairpin RNAs **IV** (G-C), **IVa** (I-C) and **IVb** (c^1I -C). For the palindromic RNAs, data points were calculated for an RNA duplex concentration of 12 μ M from $1/T_m$ vs. $\ln c$ plots; see the SI). T_m values for the hairpins were confirmed to be concentration independent. Individual data points (open triangles) ($n = 3$ independent experiments). Buffer: 10 mM Na₂HPO₄, 150 mM NaCl, pH as indicated.



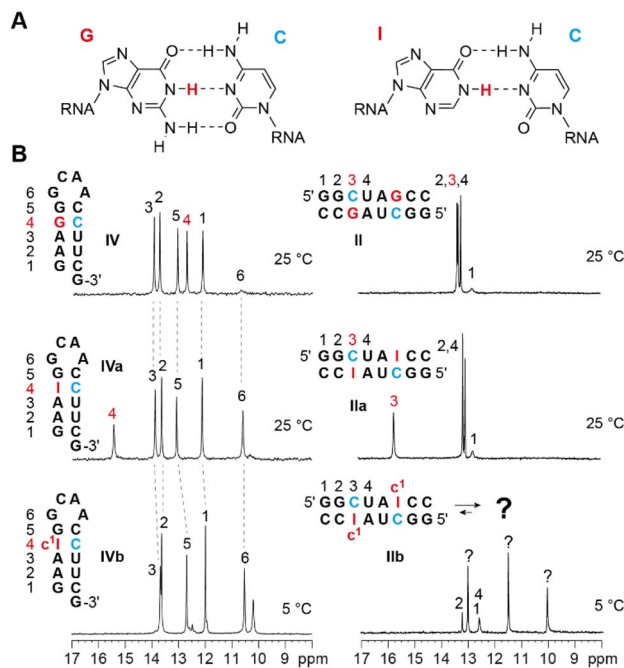


Fig. 4 Comparative ¹H-NMR imino proton spectroscopy of I and c¹I modified RNA. (A) Structures of G–C and I–C Watson–Crick base pairs (top). (B) ¹H NMR spectra of RNA hairpins IV, IVa and IVb (left) and self-complementary RNA duplexes II, IIa and IIb (right) with either G, I or c¹I (red color), recorded at 25 °C and 5 °C, respectively. Note that IIb gives a significantly distinct imino proton pattern compared to the references II and IIa, while this is not the case for IVb when compared to IV and IVa. Conditions: c(RNA) = 0.5 mM, 25 mM NaCl, 15 mM sodium phosphate buffer, H₂O/D₂O 9/1, pH 6.5. Numbers refer to full base pairs (and not to single nucleotides in the RNA sequence).

a protonated C; see Fig. 1A) were observed in the imino proton region.

To shed further light on the c¹I–C base interactions in a double helix, we first investigated a short palindromic duplex of eight base pairs (GGCUAGCC, II), in which G3 was replaced by I and c¹I, respectively. For the inosine containing duplex IIa we observed again a resonance shift of almost 3 ppm that was attributed to the formation of two I–C base pairs (Fig. 4B). For the c¹I containing duplex IIb, however, we observed a distinct resonance pattern that, according to ¹H,¹H-NOESY and ¹H,¹⁵N HSQC NMR spectra, suggested the formation of G–U wobble base pairs (SI Fig. 7B). This hypothesis was confirmed by using U4-¹⁵N3 and C3-¹⁵N3 labelled palindromes, respectively, for ¹H,¹⁵N-coupled NMR experiments (Fig. 5). The c¹I–C mismatches destabilize the fully paired duplex to such an extent that the formation of a frameshifted duplex (of four base pairs: 2 G–C, 2 G–U) with 3' overhangs becomes thermodynamically favorable (Fig. 5 and SI Fig. 7B).

To learn more about the actual base pairing mode of c¹I–C, we examined the 10 nt palindromic duplex (GGACCGGUCC, III) where we expected no escape possibility for the intended c¹I replacement. RNA III exhibited the anticipated five imino proton resonances in the ¹H NMR spectrum (Fig. 6A). When G7 (III) was substituted with I7 (IIIa), the characteristic imino proton shift of nearly 3 ppm was evident (Fig. 6A). Finally,

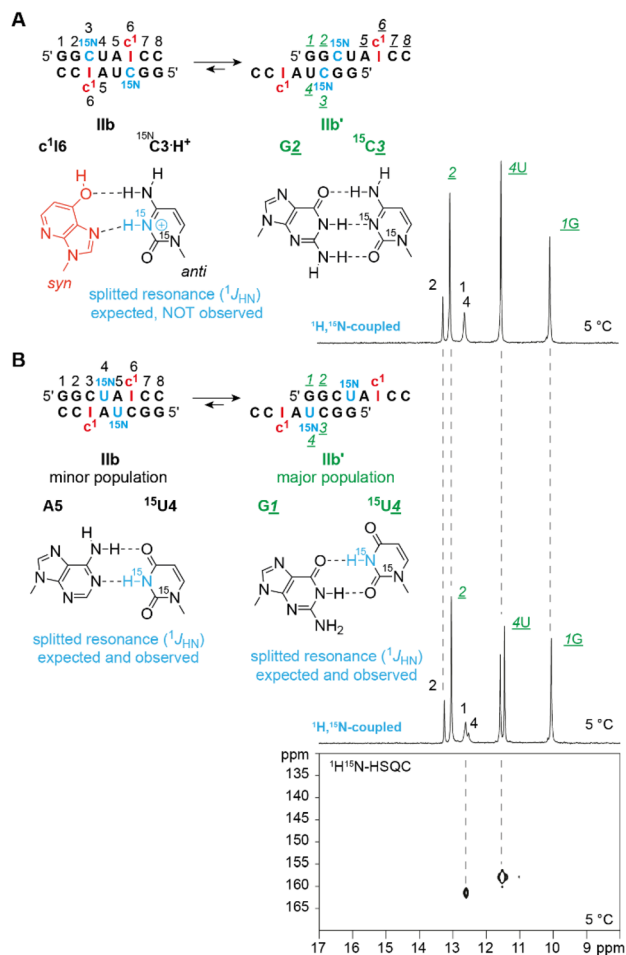


Fig. 5 The palindrome GGCUAc¹ICC prefers a 4-base pair (IIb') over an 8-base pair (IIb) register in a temperature dependent equilibrium (also see the SI Fig. 7A). Evidence comes from an ¹H,¹⁵N coupled ¹H-NMR spectroscopic comparison of (A) GG¹⁵Nc¹UAc¹ICC with a single ¹⁵N labelled cytosine and (B) GGC¹⁵NUAc¹ICC with a single ¹⁵N labelled uridine. For ¹H,¹H-NOESY and ¹H,¹⁵N-HSQC NMR spectra see the SI. Conditions: c(RNA) = 0.5 mM, 25 mM NaCl, 15 mM sodium phosphate buffer, H₂O/D₂O 9/1, pH 6.5. Numbers refer to nucleotides in the RNA sequence.

replacing I7 with c¹I7 (IIIb) resulted in the loss of the corresponding imino signal while all other resonances were retained (Fig. 6A). This is consistent with the notion that the base pairs around the c¹I–C mismatch were unaffected and fully formed, even at 25 °C.

At this point, we mention that we measured pH dependent NMR spectra of the palindrome IVb to reveal potential protonation effects on cytosine. However, even at pH 5.2, we found no indication of a protonated cytosine involved in a Hoogsteen base pair (Fig. 1A, SI, S8).

Furthermore, we hypothesized that c¹I–C in Watson–Crick geometry would result in a “sequential walk” of imino protons (accessible by ¹H,¹H-NOESY spectroscopy), evidenced by a potential correlation between the c¹I7–C1H resonance and the neighboring G6–N1H and U8–N3H imino resonances. There could also be an interbase NOE between c¹I–C1H and C4–NH₂



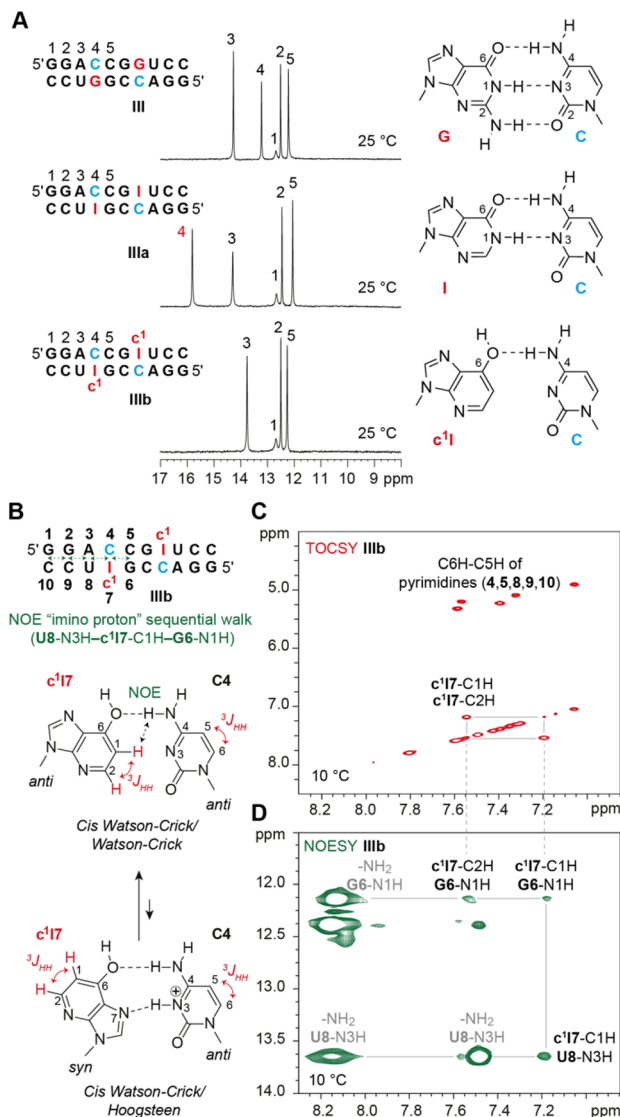


Fig. 6 NMR analysis of a 10 nt I and c^1I modified RNA palindrome. (A) 1H NMR spectra self-complementary RNA duplexes III, IIIa and IIIb (left) with either G, I or c^1I (red color), recorded at 25 °C. Structures of Watson–Crick base pairs of G–C, I–C, and putative isosteric base pairing of c^1I –C (right); numbers refer to base pairs. (B) Approach to validate the prevalent base pair mode of c^1I –C of RNA IIIb; numbers refer to nucleotides in the RNA sequence. In Watson–Crick geometry, the “imino proton” sequential walk ($^1H, ^1H$ -NOESY) should show a correlation between the c^1I7 -C1H to the neighbouring G6-N1H and U8-N3H imino resonances. (C) Assignment of c^1I7 -C1H and c^1I7 -C2H in IIIb by $^1H, ^1H$ -TOCSY experiments. (D) The $^1H, ^1H$ -NOESY spectrum of IIIb indeed shows the anticipated cross peaks for c^1I7 -C in Watson-Crick mode (for complete spectra see the SI Fig. S9 to S11). Conditions: c(RNA) = 0.5 mM, 25 mM NaCl, 15 mM sodium phosphate buffer, H_2O/D_2O 9/1, pH 6.5.

(Fig. 6B). To this end, we first assigned the c^1I7 -C1H and C2H using $^1H, ^1H$ -TOCSY experiments (Fig. 6C). Even more exciting was observing correlations between the c^1I7 -C1H resonance and the neighboring G6-N1H and U8-N3H imino resonances in the $^1H, ^1H$ -NOESY spectrum (Fig. 6D). These results provide good evidence that the c^1I7 -C4 base pair populates a Watson–Crick-

like geometry to a significant extent rather than a Hoogsteen-pair conformation (Fig. 6B). For the latter, the c^1I7 -C1H hydrogen atom is too distant to the imino protons of G6-N1H and U8-N3H to provide pronounced NOEs (Fig. 6B).

Finally, we selected the GAAIG-GCAA-CCUUCG (**IVa**) vs. GAAc¹IG-GCAA-CCUUCG (**IVb**) hairpin system for additional NMR experiments, with the aim for a more detailed NMR structure determination. High quality data spectra were obtained for the I4-C11 RNA **IVa**, (SI Fig. S12), and the NOE, scalar coupling and residual dipolar coupling data was used to determine the solution structure (PDB ID 30SC; SI Fig. S13 and Table S3). For the corresponding c^1I modified RNA **IVb**, the NMR data set strongly suggests that the sequence parts flanking the c^1I4 -C11 pair are in A-form helical arrangement (SI Fig. S14). Unfortunately, for the c^1I4 -C11 base pair in **IVb**, we found a very limited number of NOEs indicating a high flexibility (*i.e.* an exchange process on the intermediate time scale leading to broad resonances) with the two nucleotides c^1I4 and C11 sampling various conformational states leading to an ill-defined structure (SI Fig. S14A). We further could not detect analogous NOE cross-peaks for the c^1I4 -C11 pair to the direct adjacent base pairs in hairpin **IVb**, likely due to increased local dynamics relative to the palindromic duplex **IIIb**.

Poly I:C RNA oligos and impact of c^1I

Polyinosinic:polycytidylic acid, commonly referred to as poly I:C, is a synthetic analog of double-stranded RNA.⁴⁸ It is widely used in scientific research as a molecular mimic of viral infection because dsRNA is a common intermediate in the replication of many viruses.⁴⁹ In immune stimulation, poly I:C is recognized specifically by Toll-like receptor 3 (TLR3), located in endosomes, or by RIG-I-like receptors, such as MDA5, found in the cytoplasm.⁵⁰ Activation of these receptors triggers downstream signaling pathways, leading to the production of type I interferons (*e.g.*, IFN- α and IFN- β) and pro-inflammatory cytokines.⁵¹

To investigate the base pairing properties of poly I:C RNA, we first synthesized the self-complementary model system $I_{15}C_{15}$ (**V**) by solid-phase synthesis and recorded a 1H NMR spectrum (Fig. 7A). It showed a major signal at 14.9 ppm and two minor ones at slightly higher chemical shifts, consistent with the formation of IC base pairs. Further, UV melting experiments gave biphasic sigmoid-shaped melting profiles with a defined T_m value of 50.4 °C. The melting was independent from RNA concentration suggesting a monomolecular process, which is compatible with a hairpin secondary structure of $I_{15}C_{15}$.

Interestingly, when we investigated the shorter I_7C_7 oligo (**Va**) by UV melting experiments we found a lower, and importantly, concentration-dependent melting point (Fig. 8B), consistent with a bimolecular process (duplex formation). Furthermore, the modification of the central I–C/C–I pairs by c^1I -C/C- c^1I ($I_6c^1IC_7$, **Vb**) did not result in a switch to hairpin structures but only lowered the stability of the duplex. This is supported by the concentration dependence of the melting points and an under lying bimolecular melting process (Fig. 8B). Only for very low concentrations of $I_6c^1IC_7$ (<5 μM), the



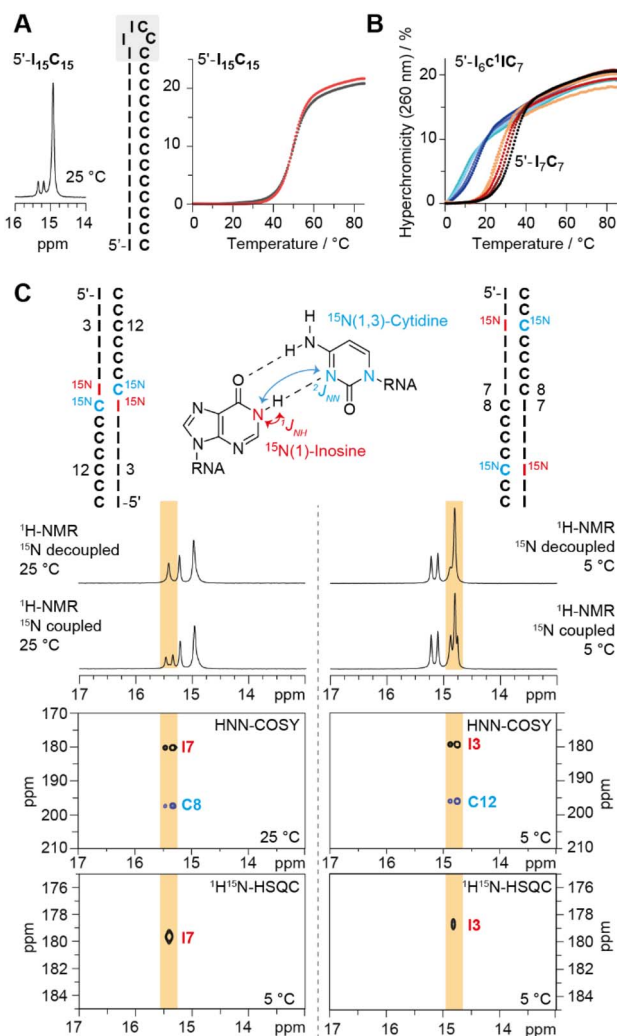


Fig. 7 Characterization of poly-I:C RNA oligos. (A) 5'-I₁₅C₁₅ (V): ¹H-NMR imino proton spectrum, secondary structure, and UV melting profiles at two different RNA concentrations (2 and 12 μM). (B) Concentration-dependent UV-melting profiles of 5'-I₇C₇ (Va) (red bundle) and 5'-I₆c¹C₇ (Vb) (blue bundle). (C) Site-specifically ¹⁵N-labelled 5'-I₇C₇ RNAs (¹⁵N patterns as indicated): ¹H NMR, HNN, and HSQC spectra verify I–C base pair formation in Watson–Crick geometry in strand positions 7,8 and 3,12, respectively.

melting profiles were biphasic, suggesting a hairpin duplex equilibrium.

To better understand the pairing properties of the I₇C₇ strand, we performed NMR experiments with stable isotopically labelled RNA strands. Direct evidence for hydrogen-bonds in a base pair interaction can be obtained from HNN-COSY experiments that were originally introduced by Dingley and Grzesiek.⁵² The size of these cross hydrogen-bond scalar couplings ($^2J_{NN} = 6\text{--}7$ Hz) allows efficient magnetization transfer to correlate chemical shifts in base pairs.⁵² To date, a large number of experiments are available to measure J -couplings across hydrogen bonds in nucleic acids.^{53–61} For weak base pairing interactions, the BEST-sellr experiment (originally introduced by the Sattler group)⁶² provides a large sensitivity improvement for hydrogen-bond correlation experiments by the

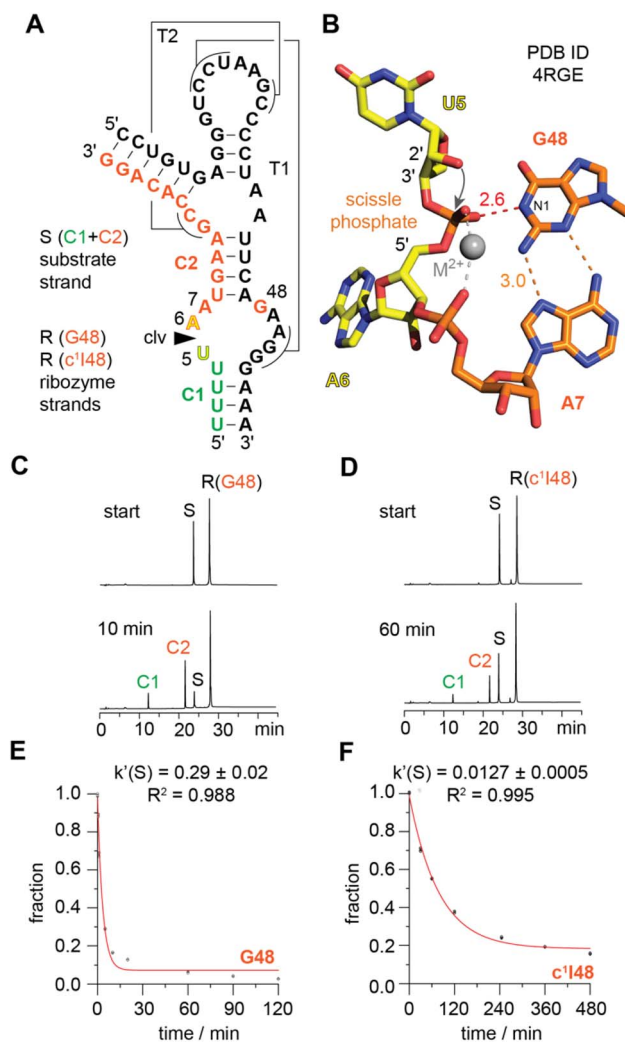


Fig. 8 Atomic mutagenesis of the twister ribozyme: impact of an active site G-to-c¹ mutation on activity to elucidate the mechanism of the phosphodiester cleavage. (A) Secondary structure of the two-strand ribozyme assembly used for functional cleavage assays. (B) X-ray structure of the twister ribozyme (pre-catalytic state; PDB ID 4RGE); interactions of guanine-48 with the scissile phosphate and A7 is shown; the 2'-OH nucleophile is modeled on dU5; distances in Å. HPLC traces of wild-type G48 (D) and c¹I48 modified (D) ribozyme at two time points illustrate that product formation of the c¹I-modified ribozyme is impeded under otherwise same reaction conditions. Cleavage rate determination of wild-type G48 (E) and c¹I48 (F) ribozymes.

optimizations of magnetization transfers in combination with the detection of non-exchangeable protons. This experiment enables the detection of weak and transient A–U base pairs, and we therefore considered it appropriate for studying I–C interactions as well.^{63,64}

We evaluated the formation of the central I–C/C–I pairs of I₇C₇ in solution, using a chemically synthesized, site-specifically labeled RNA with ¹⁵N(1)-I7 and ¹⁵N(1,3)-C8 (Fig. 7C, left panel). If I₇C₇ forms a duplex (and not a hairpin) then these interactions (¹⁵N(1)-I7:¹⁵N(1,3)-C8 base pairs) should be directly detectable. Indeed, in the ¹H,¹⁵N HSQC spectrum of the RNA,



we observed one resonance for I7, with a clear correlation to C8 in the HNN COSY spectrum (Fig. 7C, left panel). This observation suggests that a major population of fully paired, self-complementary duplex exists in solution. These experiments also assigned of the most down field-shifted I-C resonance to I7-C8.

The second, site-specifically labelled RNA of I₇C₇ contained the nucleosides ¹⁵N(1)-I3 and ¹⁵N(1,3)-C12. In a paired duplex, the corresponding base pairs of ¹⁵N(1)-I3:¹⁵N(1,3)-C12 are positioned within the continuous purine-pyrimidine tracks. As expected, the ¹H,¹⁵N HSQC spectrum of the RNA showed one resonance for I3, with a correlation to C12 in the HNN COSY spectrum.

In summary, the above experimentation reveals the intricate conformational interplay of poly I:C RNA pairing for the first time and contributes to a better understanding of the sequence requirements for secondary structure switches of these highly relevant biological RNAs.

Atomic mutagenesis with c¹I to explore ribozyme catalysis

One of the main benefits of c¹I is that it creates new opportunities to evaluate the role of guanines in RNA general acid/base catalysis.²⁹ In particular, c¹I's application in atomic mutagenesis experiments enables investigations of the chemical mechanism of self-cleaving ribozymes. The reaction is thought to pass through a pentavalent phosphorane transition state that can become stabilized by interactions with the Watson-Crick face of a conserved guanine in the active site (β-catalysis) (Fig. 1B).^{29,65,66} However, these guanines may also facilitate deprotonation of the attacking 2'-OH (γ-catalysis) (Fig. 1C) or be involved in both β- and γ-catalysis.⁶⁷

Compared to guanine, the nucleobase c¹I lacks an N1 atom, which is replaced by a carbon atom (Fig. 1). This deletion accounts for a “general base knockout” and consequently eliminates acid/base properties of this position. Additionally, the exocyclic C2-NH₂ is absent, which can play a primary role in β- (Fig. 1B) but not in γ-catalysis (Fig. 1C).

For an exemplary c¹I atomic mutagenesis study, we focus on the well-investigated Twister (Tw) ribozyme (Fig. 8).⁶⁸ Proton transfer from the protonated N3 of a conserved adenine (A6) at the cleavage site to the 5'-O leaving group is the primary factor in catalysis of this ribozyme (δ-catalysis) (Fig. 8B). This was shown previously based on the replacement of this adenine by 3-deazaadenine (c³A) which rendered Twister inactive.^{15,69}

In contrast, the contribution of a conserved active site guanine (G48) remained less clear for a long time. This issue was addressed only very recently with greater precision.^{27,28} When 1-deazaguanosine (c¹G48) was introduced in the active site, the “general base knockout” resulted in a rate reduction of approximately 10².²⁷ When 1,3-dideazaguanosine (c¹c³G48), the resulting rate reduction was approximately 10⁵.²⁸ The latter was attributed to the perturbation of c¹c³G in forming the conserved G48-A7 base pairing interaction (Fig. 8B), in addition to its direct impact on β/γ-catalysis.

We expected the c¹I48 variant of Twister to exhibit a reduced rate, and this expectation was met. However, the extent of the

reduction was rather small (approximately 25-fold) for a mutant belonging to the “general base knockout” family in which N1 is absent (Fig. 1C). Additionally, the absence of exocyclic C2-NH₂ (compared to the native G, and the mutants c¹G and c¹c³G) appears to reduce the likelihood of transition state stabilization (Fig. 1B). To gain a clearer understanding, we re-investigated two additional Twister variants: inosine-48 (I48) and 2-amino-purine (Ap48).^{15,69} We confirmed the previously reported rate reductions of approximately 10² to 10³ for I48 and approximately 10 to 10² for Ap48 (see SI Fig. S15).

One possible explanation for the small rate reduction of c¹I is that c¹I has sufficient space to shift in the pocket, allowing the phenolic oxygen atom to take over the role of acid/base catalysis and transition state stabilization, either in the deprotonated or neutral state. However, this hypothesis needs further clarification in future studies.

Conclusions

Deazanucleoside modifications are powerful tools for atomic mutagenesis because replacing a ring nitrogen with carbon perturbs hydrogen-bond donor/acceptor capabilities, thereby altering RNA base pairing, recognition, and catalysis. Expanding this toolbox, the present study develops a robust synthesis of an appropriately protected 1-deazainosine (c¹I) phosphoramidite and demonstrates its efficient incorporation into RNA by solid-phase methods. Thermodynamic analyses from UV melting reveal that c¹I-C pairs are markedly less stabilizing than the corresponding I-C pairs and can even induce shifts in base-pairing register to avoid the weakened interaction. NMR spectroscopy with selectively ¹⁵N3-labeled pyrimidines indicates that c¹I-C adopts a Watson-Crick-like geometry with a single classical H-bond, rather than the conceivable two-H-bond Hoogsteen pairing with protonated cytidine.

These weakened but structurally compatible pairs can be accommodated within duplex RNA without disrupting adjacent base pairs. Complementary poly(I:C) analyses show length-dependent preferences for duplex *versus* hairpin conformations, refining our understanding of viral dsRNA mimics used in therapeutic contexts. Together, the work fills a key gap by providing the chemistry, structural characterization, and biophysical properties of c¹I-modified RNA. It establishes c¹I as a distinct probe whose single-H-bond pairing modulates helix stability and register. These insights guide atomic mutagenesis designs to dissect RNA catalytic mechanisms with increased precision. Overall, c¹I strengthens the deazanucleoside repertoire and offers predictable, context-dependent impacts on RNA structure and function.

Author contributions

C. M. and R. B. synthesized the c¹I phosphoramidite and carried out oligonucleotide syntheses. C. M. conducted the chemical, biochemical, and biophysical characterization of the modified RNAs. C. M. and C. K. performed the RNA NMR experiments. A. S. conducted RNA crystallization experiments. R. M., C. K., and



E. E. supervised the study. R. M. conceived the project and wrote the manuscript with input from all authors.

Conflicts of interest

CK is an advisor to and holds an ownership interest in Innotope, a company providing RNA SI-labelling products. The remaining authors declare no competing interests.

Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information is available. See DOI: <https://doi.org/10.1039/d6sc04009h>.

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