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A semi-rigid isoindoline-derived nitroxide spin label for RNA†

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A new isoindoline-derived benzimidazole nitroxide spin label, **ImUm**, was synthesized and incorporated into RNA oligoribonucleotides. **ImUm** is the first example of a conformationally unambiguous spin label for RNA, in which the nitroxide N–O bond lies on the same axis as the single bond used to attach the rigid isoindoline-based spin label to a uridine base. This results in minimal displacement of the nitroxide upon rotation of this single bond, which is a useful property for a label to be used for distance measurements. Continuous-wave (CW) EPR measurements of RNA duplexes containing **ImUm** indicate a restricted rotation around this single bond, presumably due to an intramolecular hydrogen bond between the benzimidazole N–H and O4 of the uracil. Orientation-selective pulsed electron–electron double resonance (PELDOR, also called double electron–electron resonance, or DEER) distance measurements between two spin labels in two RNA duplexes showed in one case a strong orientation dependence, further confirming the restricted motion of the spin labels in RNA duplexes.

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Introduction

RNA is a ubiquitous family of biopolymers that have multiple vital roles in the coding, decoding, regulation and expression of genes.^{1–3} The understanding of RNA, DNA and protein function, including their interactions with other molecules, requires knowledge of their respective molecular structures and conformational dynamics. EPR spectroscopy is a useful technique to extract such information.^{4–10} It requires small amounts of material and can be used to study biopolymers under biologically relevant conditions. Since RNA is diamagnetic, paramagnetic groups, or spin labels, must be incorporated at specific sites, a technique referred to as site-directed spin labeling (SDSL).^{11–14} Apart from a recent report of non-covalent and site-specific spin-labeling of RNA, based on binding of a spin-labeled guanine to an abasic site in duplex RNA,¹⁵ there are two strategies that have been used to incorporate spin labels into RNA using covalent bonds. One is *via* post-synthetic labeling of pre-functionalized sites.^{16–23} The other covalent-labeling approach is the phosphoramidite method, in which the spin label is incorporated into the desired oligomer

during the oligonucleotide synthesis.²⁴ This approach has the advantage that spin labels with limited flexibility can be incorporated into the oligoribonucleotides. One such label is **Çm**,²⁴ a ribonucleotide derivative of the rigid spin label **Ç**.^{25–28} (Fig. 1), which has been used to probe mono- and bimolecular RNA structures and measurements of distances and orientation within RNA.²⁹

We have previously reported the conformationally restricted isoindoline-derived benzimidazole spin label **ImU** (Fig. 1) for DNA, which showed EPR-based spectroscopic properties similar to that of the rigid spin label **Ç**.³⁰ The restricted mobility was believed to arise, at least in part, from an intramolecular hydrogen bond between the NH of the benzimidazole and O4 of the uracil base.³⁰ **ImU** has been used for distance measurements in duplex DNAs and showed a strong

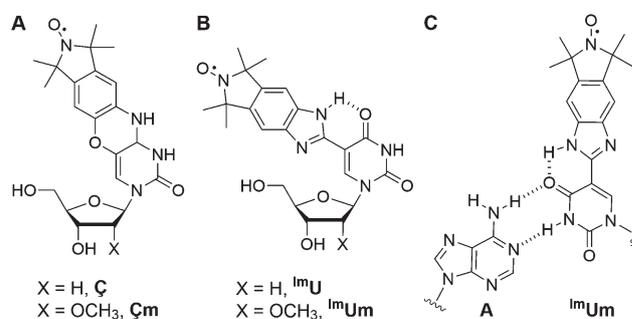


Fig. 1 **Ç** and **Çm** (A), **ImU** and **ImUm** (B), and base-pairing of **ImUm** with adenine (C).

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orientation dependence, another indication of limited mobility.³¹ Aside from the orientation dependence of ¹³C-U, the single bond that connects the benzimidazole moiety to the base lies on an axis that runs through the N–O bond of the nitroxide (Fig. 1), which makes ¹³C-U useful for precise distance measurements.

Herein, we report the synthesis of ¹³C-Um, a nitroxide derivative of uridine for RNA spin labeling. The phosphoramidite of ¹³C-Um was prepared and incorporated into different RNA structural contexts by solid-phase synthesis. The new label was well tolerated in the A-form helices when paired with adenosine (Fig. 1), as judged by its small effect on the thermodynamic stability of the labeled RNAs. ¹³C-Um showed very limited mobility in duplex RNA, indicating that rotation around the single bond, linking the spin label to the uracil, is indeed restricted. CW-EPR spectroscopy was used to show that the ¹³C-Um was able to report on the local environment of the labeling site. PELDOR distance measurements on RNA duplexes using ¹³C-Um were in close agreement with distances derived from molecular modeling. Furthermore, PELDOR measurements of ¹³C-Um-labeled RNA duplexes showed orientation dependence, further confirming the limited motion of this spin label in RNA duplexes.

Results and discussion

Synthesis and incorporation of ¹³C-Um into RNA

The synthesis of ¹³C-Um (Scheme 1) began with iodination of 2'-O-methyluridine (Um).³² The 3'- and 5'-hydroxyl groups of **1** were protected by TBDMS to give **2**,³³ which was treated with vinyl acetate in the presence of Pd(OAc)₂, PPh₃ to yield the 5-vinyl functionalized compound **3** in good yield.³³ Compound **3** was

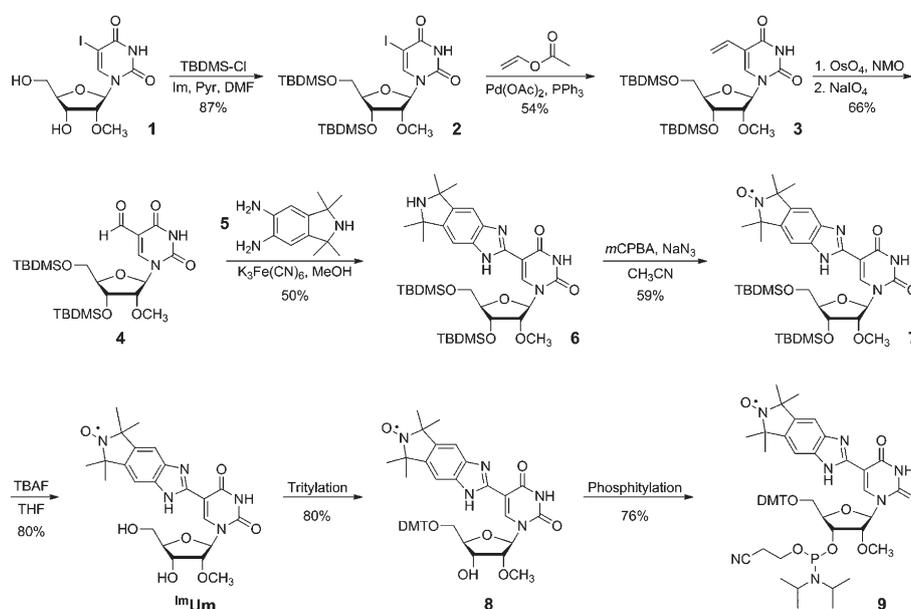
subjected to dihydroxylation using OsO₄ and *N*-methylmorpholine *N*-oxide (NMO) to obtain the corresponding diol,³⁴ which was further treated with NaIO₄ to give the desired aldehyde **4** in good yield.³⁵ Aldehyde **4** was treated with diamino-tetramethylisoindoline **5** and K₃Fe(CN)₆, yielding the imidazole derivative **6**. Compound **6** was subjected to *m*-CPBA-mediated oxidation in the presence of NaN₃,³⁰ which presumably adds transiently to the 6-position of the pyrimidine base,³⁶ generating compound **7**. The TBDMS protecting groups of **7** were removed using TBAF to afford the nucleoside ¹³C-Um, followed by tritylation and phosphitylation to give phosphoramidite **9** in good yield.

Syntheses and purification of RNA oligonucleotides containing ¹³C-Um

¹³C-Um was incorporated into RNA oligoribonucleotides by solid phase synthesis using the previously reported protocol, with a slight modification.^{24,26} Upon completion of the synthesis, the oligoribonucleotides were cleaved from the solid support and the nucleobases and phosphodiester were deprotected in a 1 : 1 mixture of 33% aqueous NH₃ and 8 M MeNH₂ in EtOH. The 2'-*O*-TBDMS groups were removed by treatment with Et₃N·3HF, after which water was added and the oligoribonucleotides precipitated with *n*-butanol. The crude oligoribonucleotides were purified by DPAGE and quantified using UV absorbance spectroscopy.

The effect of ¹³C-Um on RNA duplex stability

The incorporation of ¹³C-Um into RNA was first demonstrated by synthesis of the 14-mer oligoribonucleotide 5'-(CACGA¹³C-Um-GCGAGGUC). After annealing to a complementary RNA, a thermal denaturation experiment of the duplex 5'-(CACGA¹³C-UmGCGAGGUC)-5'-(GACCUCGCAUCGUG) (**II**) showed a



Scheme 1 Synthesis of the nucleoside ¹³C-Um and its corresponding phosphoramidite (**9**).

Table 1 Sequences and T_M s of RNA hairpins and duplexes

RNA	Sequence	T_M	ΔT_M
I	5' CACGAUGCGAGGUC GUGCUACGCUCCAG	73.4 ± 0.6	
II	5' CACGA ^{Im} UmGCGAGGUC GUGCU A CGCUCCAG	67.3 ± 0.3	-6.1
III	A GUCGACAGUA G CAGCUG ^{5'}	85.2 ± 1.0	
IV	A GUCG A CAGUA G CAGC ^{Im} UmG ^{5'}	84.7 ± 0.3	-0.5
V	C GUCGAC U CAGCUGUCAU ^{5'}	76.0 ± 0.5	
VI	C GUCGAC U CAGCUG ^{Im} UmCAU ^{5'}	79.7 ± 0.3	+3.7
VII	5' GUCGACGGAAGUCGACAGUA CAGCUGCCUUCAGCUGUCAU	74.5 ± 1.0	
VIII	5' G ^{Im} UmCGACGGAAGUCGACAGUA C A GCUGCCUUCAGCUGUCAU	73.8 ± 0.8	-0.7
IX	5' GUCGACGGAAGUCGAC A GUA CAGCUGCCUUCAGCUG ^{Im} UmCAU	77.2 ± 0.8	+2.7

4 mM duplex in 10 mM sodium phosphate, 100 mM NaCl, 0.1 mM Na₂EDTA, pH 7.0. T_M is the melting temperature and ΔT_M is the difference in T_M between unmodified and modified duplexes.

single melting transition, lowering the melting temperature (T_M) by 6.1 °C compared to the unmodified duplex (I) (Table 1 and Fig. S1A†). We have previously observed a lowering in T_M by 4.0 °C when ^{Im}U was incorporated into the center of a 14-mer DNA.³⁰ CD spectra of the ^{Im}Um-modified and unmodified oligoribonucleotides were almost identical, confirming that ^{Im}Um is well tolerated in an A-form RNA helix (Fig. S1B†).

The spin-label ^{Im}Um was also incorporated into the stem region of an RNA hairpin 5'-(G^{Im}UmCGACGGAAGUCGACAGUA) (IV), which contains a six base-pair helix and a stable GGAA tetraloop. In hairpin IV, the location of the label is close to the end of the stem and as expected, it showed only a minor effect on the T_M (-0.5 °C), compared to the corresponding unmodified hairpin (III) (Table 1 and Fig. S1C†). ^{Im}Um-modified hairpin IV was annealed to its complementary RNA strand, 3'-(CAGCUGCCUUGAGCUGUCAU), yielding duplex VIII, thus placing the label close to the end of the duplex and resulting in only minor destabilization (-0.7 °C) (Table 1 and Fig. S1E†). ^{Im}Um was also incorporated into the overhang region of the oligoribonucleotide hairpin VI, where it stabilized the hairpin by 3.7 °C, compared to the unmodified hairpin V (Table 1 and Fig. S1D†). When hairpin VI was annealed to its complementary strand, the resulting duplex (IX) was stabilized by 2.7 °C (Table 1 and Fig. S1F†), relative to the unmodified sequence (VII).

CW-EPR analyses of ^{Im}Um in duplexes and hairpins

To evaluate the mobility of ^{Im}Um in RNA, we recorded the CW-EPR spectra of the nucleoside ^{Im}Um, the ^{Im}Um-labeled

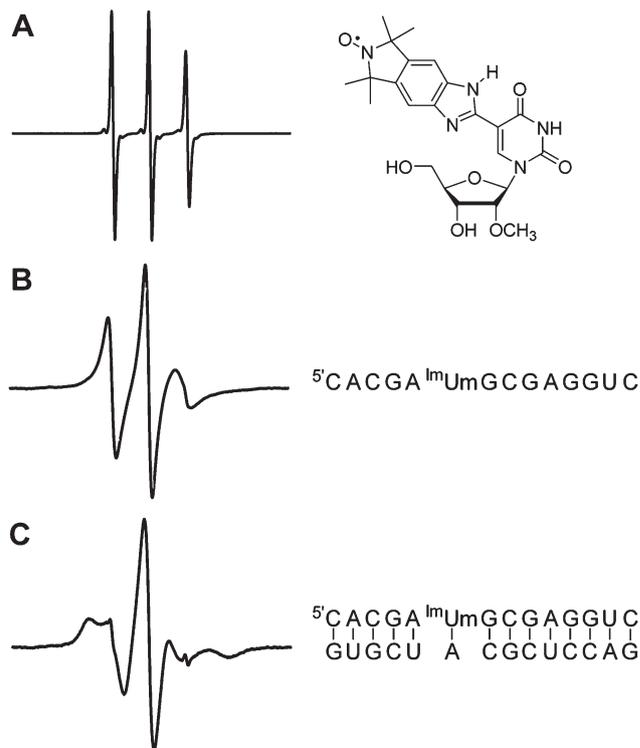


Fig. 2 EPR spectra of ^{Im}Um (A), the 14-mer RNA single strand 5'-(CACGA^{Im}UmGCGAGGUC) (B) and the duplex 5'-(CACGA^{Im}UmGCGAGGUC)-3'-(GACCUCGCAUCGUG) (II) (C). EPR spectra were recorded at 20 °C in a phosphate buffer (10 mM, pH 7.0) containing NaCl (100 mM) and Na₂EDTA (0.1 mM).

14-mer RNA single strand and the corresponding 14-mer RNA duplex II (Fig. 2). The nucleoside showed three sharp lines (Fig. 2A) that broadened upon incorporation into the 14-mer oligoribonucleotide 5'-(CACGA^{Im}UmGCGAGGUC) (Fig. 2B). Upon annealing to its complementary strand 3'-(GACCUCGCAUCGUG), the CW-EPR spectrum showed a splitting of the high- and low-field components (Fig. 2C), similar to the previously reported rigid spin labels Ç²⁵ and Ç^m,²⁴ as well as to the semi-rigid ^{Im}U.³⁰ Such broadening is characteristic for the slow-motion regime of nitroxide radicals.²⁴

To investigate the mobility of the ^{Im}Um in different structural contexts, it was incorporated into RNA hairpins and duplexes (Fig. 3). The ^{Im}Um-labeled hairpins IV (Fig. 3A) and VI (Fig. 3B) exhibited broadened CW-EPR spectra, compared to single strands. However, spectral line-broadening of hairpin VI is less pronounced than hairpin IV, since the label is located in the overhang region of hairpin VI. The ^{Im}Um-labeled duplexes VIII and IX showed additional broadening of the spectra, compared to the hairpins. This is presumably due to the increased size of the RNA and the associated slower rotational correlation times, as has been observed with the rigid spin label Ç^m.²⁴ The EPR spectrum of IX at 20 °C is slightly broader than for duplex VII (Fig. 3), presumably because the spin label in the latter is closer to the duplex end, where base pairs are more dynamic.^{37,38}

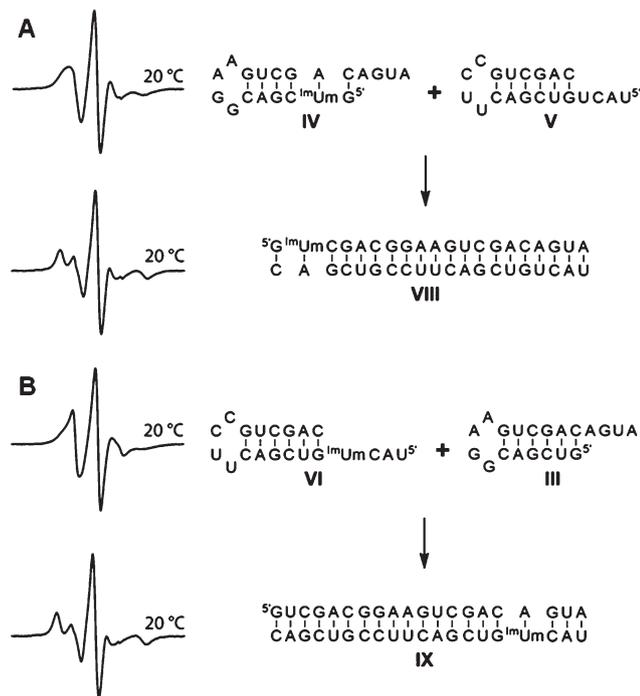


Fig. 3 EPR spectrum of the hairpin IV and the spectrum after annealing IV to the complementary hairpin V to form duplex VIII (A). EPR spectrum of the hairpin VI and the spectrum after annealing V with the complementary hairpin III to form duplex IX (B). EPR spectra were recorded at 20 °C in 10 mM phosphate buffer (pH 7.0) containing NaCl (100 mM) and Na₂EDTA (0.1 mM).

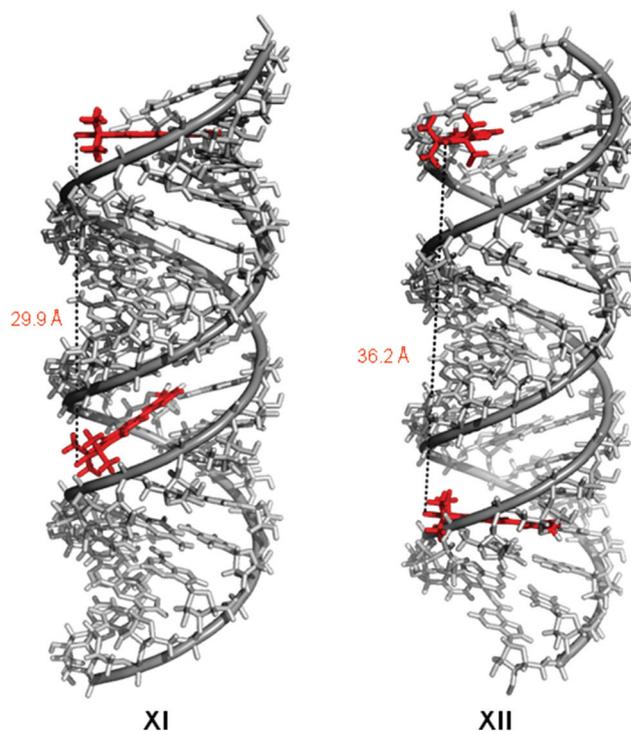


Fig. 4 Spartan-derived molecular models of RNA duplexes containing two ¹HU spin labels, placed either nine base-pairs apart (duplex XI, 5'-(G¹HU)MCGACGGAAG¹HU)MCGACAGUA)-3'-(CAGCUGCCUUGAGCUGUCAU), left) or 13 base-pairs apart (duplex XII, 5'-(G¹HU)MCGACGGAAGUCGACAGUA)-3'-(CAGCUGCCUUGAGCUG¹HU)CAU), right).

Distance measurements of ¹HU-labeled RNAs by PELDOR

Two doubly ¹HU-labeled RNA duplexes were prepared for distance measurements, one containing nine base pairs (XI) and the other fourteen base pairs (XII) between the spin labels (Fig. 4). Both the distances between the two spin-label pairs and their relative orientations were different in these duplexes. Orientation selective PELDOR²⁷ was performed at X-Band frequencies in which the pump frequency coincided with the cavity resonance maximum as well as the maximum in spectral density of the nitroxide spectrum. The detection was performed with different frequency offsets on the high frequency side, relative to the pump frequency. For both RNAs, the PELDOR time traces of each offset were different from each other (Fig. 5). This is a strong indication of orientation dependence resulting from limited mobility of the spin labels within the duplexes. The orientation dependence is very clear, in particular for RNA XII. However, in the case of RNA XI, the orientation dependence is not as clear, but the differences between the time traces are evident. For each duplex, the different time traces were added to get an average time trace, which removes the orientation effects. DeerAnalysis³⁹ of these time traces yielded the distance distribution shown in Fig. 5. The measured distances between the spin labels in RNAs XI (32.5 Å) and XII (38.5 Å) was close to that of the modelled distances (29.9 Å and 36.2 Å, respectively).

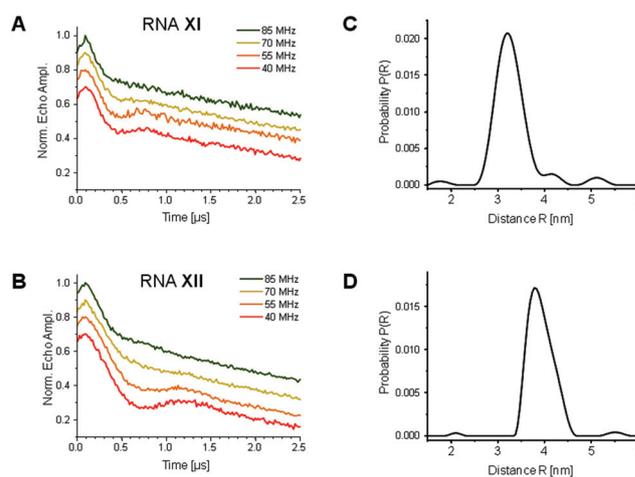


Fig. 5 PELDOR time traces at different frequency offsets for duplex XI 5'-(G¹HU)MCGACGGAAG¹HU)MCGACAGUA)-3'-(CAGCUGCCUUGAGCUGUCAU) (A) and duplex XII 5'-(G¹HU)MCGACGGAAGUCGACAGUA)-3'-(CAGCUGCCUUGAGCUG¹HU)CAU) (B). Evaluation of interspin distances in doubly spin-labeled RNA duplexes from average over all PELDOR time traces by DeerAnalysis³⁹ for duplex XI (C) and duplex XII (D).

Conclusions

The new spin label ¹³Um, a derivative of uridine, was prepared for RNA spin-labeling. It was incorporated into different RNA structural contexts by solid-phase oligonucleotide synthesis. The spin-labeled RNAs were analyzed by thermal denaturation experiments, CD- and EPR-spectroscopy. The new label ¹³Um was fairly well tolerated in A-form helices, as judged by thermal denaturation experiments. CW-EPR spectra of ¹³Um-labeled RNA duplexes showed limited mobility, indicating that rotation around the single bond, linking the spin label to the uracil, is restricted. ¹³Um also reported on its local environment by CW-EPR and provided information on the transition from RNA hairpins to duplexes. Distances between pairs of ¹³Um spin labels in RNA duplexes, measured by PELDOR, were in close agreement with distances derived from molecular models. The PELDOR measurements showed a strong orientation-dependence, in particular for RNA XII, indicating limited motion of the spin label. Therefore, ¹³Um is a promising RNA spin label for extracting accurate distances and obtaining information about orientations using PELDOR. Such information gives insights into both structure and conformational dynamics of the RNA under investigation.

Experimental section

All commercially available reagents were purchased from Sigma-Aldrich or Acros Organics and used without further purification. 2'-O-Methyluridine was purchased from Rasayan Inc. USA. All moisture or air sensitive reactions were performed in flame-dried glasswares under a positive pressure of nitrogen. Solvents were distilled and stored over activated 4 Å molecular sieves under nitrogen. Water was purified on a Milli-Q water purification system. Analytical thin layer chromatography (TLC) was performed on silica gel glass plates (Silicycle, ultra-pure silica gel, 60 Å, F₂₅₄), TLC visualisation was performed with UV light. Flash-column chromatography was performed on silica gel (Silicycle, 230–400 mesh, 60 Å). ¹H-Spectra were recorded using deuterated solvents as internal standards on a Bruker Advance 400 spectrometer and are reported in ppm. Residual proton signals from the deuterated solvents were used as references [D₂O (4.81 ppm), d₆-DMSO (2.50 ppm), CDCl₃ (7.26 ppm), for ¹H spectra]. ¹³C NMR chemical shifts are reported in reference to undeuterated residual solvent (CDCl₃ (77.0 ppm), d₆-DMSO (39.43 ppm)). ³¹P NMR chemical shifts were reported relative to 85% H₃PO₄ as an external standard. Commercial grade CDCl₃ was passed over basic alumina shortly before use. Mass spectrometric analyses of all organic compounds were performed on an ESI-HRMS (Bruker, microTOF-Q) in positive or negative mode.

Compound 1

To a solution of 2'-O-methyluridine (5.0 g, 0.019 mol) in CH₃CN (18 mL) was added iodine (2.94 g, 0.012 mol) and ammonium cerium(IV) nitrate (5.30 g, 0.0097 mol). The result-

ing solution was heated at 80 °C for 1 h, followed by cooling to 22 °C. The solid was filtered, washed with H₂O (100 mL) and CH₃CN (50 mL), and dried *in vacuo* to yield compound 1 as a white solid (6.4 g, 86%). ¹H NMR (d₆-DMSO): δ 11.69 (s, 1H), 8.53 (s, 1H), 5.79 (d, *J* = 3.9 Hz, 1H), 5.30 (s, 1H), 5.12 (d, *J* = 6.3 Hz, 1H), 4.11 (d, *J* = 5.5 Hz, 1H), 3.86 (d, *J* = 5.5 Hz, 1H), 3.79 (d, *J* = 4.4 Hz, 1H), 3.70 (ddd, *J* = 12.0, 4.6, 2.8 Hz, 1H), 3.57 (ddd, *J* = 12.1, 4.3, 2.4 Hz, 1H), 3.38 (s, 3H). ¹³C NMR (d₆-DMSO): δ 160.46, 150.09, 144.82, 86.55, 84.76, 83.02, 69.31, 67.79, 60.25, 59.64, 57.57. HRMS-ESI: *m/z* calcd for C₁₀H₁₂IN₂O₆ (M – H)[–] 382.9746, found 382.9741.

Compound 2

To a solution of 2'-O-methyl-5-iodouridine (6.4 g, 0.017 mol) in DMF (19.2 mL) and pyridine (19.2 mL) was added *tert*-butyldimethylsilyl chloride (7.53 g, 0.050 mol) and imidazole (3.40 g, 0.050 mol). After stirring at 22 °C for 16 h, the solvent was removed under reduced pressure and the residue was dissolved in EtOAc (200 mL) and H₂O (100 mL) was added. The EtOAc layer was separated and washed with H₂O (2 × 100 mL). The organic layer was dried over Na₂SO₄, filtered and the solvent evaporated *in vacuo*. The crude product was purified by flash column chromatography (silica gel) using a gradient elution (EtOAc : pet. ether; 10 : 90 to 15 : 85) to give compound 2 as a white solid (8.9 g, 87% yield). ¹H NMR (CDCl₃): δ 8.64 (s, 1H), 8.06 (s, 1H), 5.99 (d, *J* = 4.5 Hz, 1H), 4.23 (t, *J* = 4.8 Hz, 1H), 4.09–4.01 (m, 1H), 3.97 (dd, *J* = 11.7, 1.8 Hz, 1H), 3.76 (dd, *J* = 11.7, 1.8 Hz, 1H), 3.67 (t, *J* = 4.7 Hz, 1H), 3.46 (s, 3H), 0.98 (s, 9H), 0.91 (s, 9H), 0.19 (d, *J* = 1.6 Hz, 6H), 0.10 (d, *J* = 7.9 Hz, 6H). ¹³C NMR (CDCl₃): δ 159.93, 149.99, 144.40, 87.50, 85.65, 84.15, 69.84, 69.00, 62.26, 58.52, 26.56, 25.92, 18.91, 18.35, –4.38, –4.55, –4.71, –4.81. HRMS-ESI: *m/z* calcd for C₂₂H₄₀IN₂O₆Si₂ (M – H)[–] 611.1475, found 611.1443.

Compound 3

Palladium(II) acetate (74 mg, 0.33 mmol), PPh₃ (154 mg, 0.590 mmol) and anhydrous triethylamine (3.64 mL, 26.11 mmol) were combined in anhydrous DMF (10.8 mL) and stirred at 64 °C until an intense red colour developed. A solution of compound 5 (2 g, 3.26 mmol) and vinyl acetate (15.17 g, 176.25 mmol) in anhydrous DMF (16.50 mL) was added and the stirring maintained at 64 °C for 17 h. The resulting precipitate of palladium was removed by filtration, the filtrate evaporated to dryness and H₂O (75 mL) was added to the residue. The aqueous mixture was extracted with CH₂Cl₂ (3 × 100 mL). The organic layer was dried over Na₂SO₄, filtered, the solvent evaporated *in vacuo* and the crude product was purified by flash column chromatography (silica gel) using a gradient elution (CH₂Cl₂ : pet. ether; 5 : 95 to 10 : 90) to give compound 3 as a white solid (903 mg, 54% yield). ¹H NMR (CDCl₃): δ 8.64 (s, 1H), 8.06 (s, 1H), 5.99 (d, *J* = 4.5 Hz, 1H), 4.23 (t, *J* = 4.8 Hz, 1H), 4.09–4.01 (m, 1H), 3.97 (dd, *J* = 11.7, 1.8 Hz, 1H), 3.76 (dd, *J* = 11.7, 1.8 Hz, 1H), 3.67 (t, *J* = 4.7 Hz, 1H), 3.46 (s, 3H), 0.98 (s, 9H), 0.91 (s, 9H), 0.19 (d, *J* = 1.6 Hz, 6H), 0.10 (d, *J* = 7.9 Hz, 6H). ¹³C NMR (CDCl₃): δ ¹³C NMR (CDCl₃) δ 161.91, 159.99, 150.03, 149.56, 144.39, 137.03, 128.18,

117.13, 112.96, 87.49, 85.64, 85.22, 84.15, 83.91, 69.80, 69.03, 62.28, 58.52, 58.46, 26.56, 26.30, 25.93, 18.91, 18.82, 18.37, -4.36, -4.57, -4.71, -4.82, -4.98, -5.10. HRMS-ESI: m/z calcd for $C_{24}H_{43}N_2O_6Si_2$ ($M - H$)⁻ 511.2665, found 511.2643.

Compound 4

Compound 3 (3.72 g, 0.00725 mol), *N*-methylmorpholine-*N*-oxide (2.13 g, 0.0181 mol), and OsO_4 (0.0276 g, 0.000109 mol) were stirred in a solution of acetone- H_2O -*t*-BuOH (4:1:1, 30 mL) at 22 °C for 22 h. The reaction mixture was partitioned between EtOAc (300 mL) and H_2O (150 mL). The aqueous layer was extracted with EtOAc (2 × 100 mL). The combined organic layers were dried over Na_2SO_4 , filtered, the solvent evaporated *in vacuo* and the crude product was purified by flash column chromatography (silica gel) using a gradient elution (MeOH:CH₂Cl₂; 0:100 to 2:98) to give compound 4A, the diol intermediate, as a white solid (3.17 g, 80% yield). ¹H NMR (CDCl₃): δ 9.07 (s, 1H), 7.65 (d, $J = 7.9$ Hz, 1H), 6.07–5.89 (m, 1H), 4.77–4.44 (m, 1H), 4.23 (dd, $J = 10.0, 5.0$ Hz, 1H), 4.07–3.97 (m, 1H), 3.91 (ddd, $J = 11.5, 5.5, 2.8$ Hz, 1H), 3.87–3.79 (m, 1H), 3.79–3.63 (m, 3H), 3.46 (dd, $J = 14.5, 2.8$ Hz, 3H), 1.11–0.75 (m, 18H), 0.27 to -0.05 (m, 12H). ¹³C NMR (CDCl₃): δ 163.58, 163.45, 149.92, 149.87, 138.32, 138.19, 113.76, 87.84, 87.71, 85.43, 83.46, 83.37, 70.20, 70.07, 69.98, 69.73, 65.79, 62.70, 62.56, 58.48, 58.45, 26.27, 25.93, 18.77, 18.36, -4.40, -4.43, -4.55, -5.06, -5.10, -5.13. HRMS-ESI: calcd for $C_{24}H_{46}N_2O_8Si_2Na$ ($M + Na$)⁺ 569.2685, found 569.2688.

To a solution of 4A (790.0 mg, 1.444 mmol) in CH₂Cl₂ (50 mL) and H_2O (5 mL) at 22 °C was added $NaIO_4$ (4.015 g, 18.77 mmol) and the resulting mixture was stirred vigorously for 16 h. The reaction was diluted with H_2O (25 mL) and extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were dried over Na_2SO_4 , filtered and the solvent evaporated *in vacuo*. The crude product was purified by flash column chromatography (silica gel) using a gradient elution (CH₂Cl₂:pet. ether; 50:50 to 100:0) to give compound 4 as a white solid (610 mg, 82% yield). ¹H NMR (CDCl₃): δ 10.01 (s, 1H), 8.90 (s, 1H), 8.48 (s, 1H), 6.03 (d, $J = 4.6$ Hz, 1H), 4.25 (t, $J = 4.7$ Hz, 1H), 4.08 (dt, $J = 4.6, 2.4$ Hz, 1H), 3.96 (dd, $J = 11.7, 2.3$ Hz, 1H), 3.86–3.69 (m, 2H), 3.46 (s, 3H), 0.92 (t, $J = 6.9$ Hz, 19H), 0.13 (dd, $J = 23.0, 6.1$ Hz, 12H). ¹³C NMR (CDCl₃): δ 185.69, 161.74, 149.40, 145.65, 111.81, 88.08, 86.18, 84.24, 69.91, 62.31, 58.57, 26.34, 25.91, 18.82, 18.33, -4.41, -4.52, -5.21, -5.22. HRMS-ESI: m/z calcd for $C_{23}H_{42}N_2O_7Si_2Na$ ($M + Na$)⁺ 537.2423, found 537.2432.

Compound 6

To a solution of compound 4 (876.0 mg, 1.701 mmol) and 1,1,3,3-tetramethylisindoline-5,6-diamine (5) (349.0 mg, 1.701 mmol) in MeOH (9 mL) was added $K_3Fe(CN)_6$ (672.0 mg, 2.041 mmol) and the reaction stirred for 16 h at 22 °C. The reaction mixture was concentrated *in vacuo* and the crude product was purified by flash column chromatography (silica gel) using a gradient elution (CH₂Cl₂:MeOH; 98:02 to 90:10) to give compound 6 as a yellow solid (590.0 mg, 50% yield). ¹H

NMR (*d*₆-DMSO): δ 12.04 (s, 1H), 8.59 (s, 1H), 7.29 (s, 1H), 7.20 (s, 1H), 5.93 (d, $J = 4.4$ Hz, 1H), 4.28 (t, $J = 5.0$ Hz, 1H), 4.04–3.75 (m, 4H), 3.39 (d, $J = 5.0$ Hz, 3H), 1.39 (s, 13H), 0.89 (d, $J = 4.6$ Hz, 19H), 0.13 (dd, $J = 17.6, 2.4$ Hz, 12H). ¹³C NMR (*d*₆-DMSO): δ 161.83, 149.56, 145.62, 143.70, 143.45, 142.45, 139.88, 134.09, 109.73, 104.78, 104.12, 87.23, 84.69, 82.16, 69.66, 62.25, 61.74, 61.60, 57.69, 45.67, 32.59, 32.56, 30.64, 25.96, 25.58, 18.09, 17.76, 11.49, -4.79, -5.01, -5.37, -5.38. HRMS-ESI: m/z calcd for $C_{35}H_{58}N_5O_6Si_2$ ($M + H$)⁺ 700.3920, found 700.3952.

Compound 7

To a suspension of 6 (270.0 mg, 0.39 mmol) in CH₃CN (18 mL) was added NaN_3 (100.0 mg, 1.54 mmol) and the reaction stirred at 22 °C. After 30 min, *m*CPBA (133.0 mg, 0.77 mmol) was added, the reaction mixture stirred for 3 h and concentrated *in vacuo*. The residue was purified by silica gel column chromatography using a gradient elution (CH₂Cl₂:MeOH; 100:0 to 98:2) to give compound 7 as a yellow solid (162.0 mg, 59% yield). ¹H NMR (CDCl₃): δ 11.45 (br s), 9.90 (br s), 8.92 (br s), 8.08 (br s), 7.98 (br s), 7.26 (br s), 6.13 (br s), 4.37 (br s), 4.11 (br s), 4.00 (br s), 3.97 (br s), 3.86 (br s), 3.46 (br s), 2.18 (br s), 2.13 (br s), 1.04 (br s), 0.93 (br s), 0.89 (br s), 0.21 (br s), 0.19 (br s), 0.14 (br s), 0.13 (br s). ¹³C NMR (CDCl₃): δ 170.34, 162.59, 149.30, 146.90, 141.72, 136.68, 136.01, 133.89, 131.40, 129.73, 129.15, 127.73, 104.30, 88.24, 86.11, 83.15, 77.55, 77.23, 76.91, 73.48, 69.94, 62.49, 58.17, 31.52, 29.54, 26.03, 25.91, 25.74, 25.63, 25.56, 18.35, 17.97, 8.29, -4.79, -4.82, -5.34, -5.45. HRMS-ESI: m/z calcd for $C_{35}H_{57}N_5O_7Si_2$ ($M + H$)⁺ 715.3791, found 715.3776.

¹³U_m

To a solution of compound 7 (320 mg, 0.4475 mmol) in THF (32 mL) was added *tert*-butyl ammonium fluoride (1 M in THF, 1.03 mL, 0.985 mmol) and the solution was stirred at 22 °C for 16 h. The solvent was removed under reduced pressure to give a sticky reddish oil. The crude product was purified by flash column chromatography (silica gel) using a gradient elution (CH₂Cl₂:MeOH; 100:0 to 95:5) to give compound ¹³U_m as a yellow solid (175 mg, 80% yield). ¹H NMR (*d*₆-DMSO): δ 12.45 (br s), 12.00 (br s), 8.77 (br s), 7.90 (br s), 7.71 (br s), 7.54 (br s), 6.00 (br s), 5.74 (br s), 5.22 (br s), 4.17 (br s), 3.95 (br s), 3.74 (br s), 3.65 (br s), 3.42 (br s), 3.32 (br s), 3.17 (br s). ¹³C NMR (*d*₆-DMSO): δ 161.86, 149.30, 132.60, 130.54, 128.65, 127.76, 86.68, 85.14, 82.82, 68.27, 60.53, 57.57, 55.17. HRMS-ESI: m/z calcd for $C_{23}H_{29}N_5O_7$ ($M + H$)⁺ 487.2061, found 487.2068.

Compound 8

¹³U_m (150.0 mg, 0.31 mmol), DMTCl (189.0 mg, 0.56 mmol) and DMAP (4.0 mg, 0.032 mmol) were weighed into a round bottom flask and kept *in vacuo* for 12 h. Pyridine (2.0 mL) was added and the solution was stirred at 22 °C for 3 h, after which MeOH (100 μL) was added. The solvent was removed *in vacuo* and the residue was purified by flash column chromatography (silica gel) using a gradient of (CH₂Cl₂:MeOH; 100:0 to

97:2.5 + 0.5% Et₃N); the column was prepared in 0.5% Et₃N in CH₂Cl₂. Compound **8** was obtained as a yellow solid (195.0 mg, 80.0%). ¹H NMR (CDCl₃): δ 11.32 (br s), 8.91 (br s), 8.69 (br s), 7.56 (br s), 7.49 (br s), 6.78 (br s), 6.09 (br s), 5.33 (br s), 4.50 (br s), 4.50 (br s), 4.20 (br s), 4.13 (br s), 3.71 (br s), 3.60 (br s), 2.98 (br s). ¹³C NMR (CDCl₃): δ 161.97, 157.71, 157.66, 149.08, 148.58, 143.83, 141.07, 135.35, 135.15, 129.52, 129.41, 127.59, 127.11, 126.05, 112.41, 88.12, 86.13, 83.36, 82.81, 68.44, 61.75, 58.59, 54.88, 45.07, 8.81. HRMS-ESI: *m/z* calcd for C₄₄H₄₉N₅O₇ (M + H)⁺ 789.3368, found 789.3396.

Compound 9

Diisopropyl ammonium tetrazolide (33.0 mg, 0.19 mmol) and compound **8** (100.0 mg, 0.13 mmol) were dissolved in pyridine (2 mL), the solvent evaporated *in vacuo* and residue kept under vacuum for 17 h. CH₂Cl₂ (3.0 mL) and 2-cyanoethyl *N,N,N',N'*-tetraisopropyl phosphoramidite (115.0 mg, 0.38 mmol) were added. The reaction mixture was stirred at 22 °C for 16 h, diluted with CH₂Cl₂ (10 mL) and washed successively with saturated aq. NaHCO₃ (3 × 10 mL) and saturated aq. NaCl (2 × 10 mL). The organic layer was dried over anhydrous Na₂SO₄, filtered and concentrated *in vacuo*. The crude solid was purified by precipitation by first dissolving it in CH₂Cl₂ (0.5 mL), followed by addition of pet. ether (50 mL). The liquid was decanted and the operation repeated three times to furnish phosphoramidite **9** as a pale yellow solid (95 mg, 76%). ¹H NMR (CDCl₃): δ 11.29 (br s), 9.02 (br s), 8.95 (br s), 7.61 (br s), 7.53 (br s), 6.82 (br s), 6.19, 6.10 (br s), 4.70 (br s), 4.54 (br s), 4.39 (br s), 4.25 (br s), 3.66 (br s), 3.54 (br s), 2.71 (br s), 2.45 (br s), 1.47 (br s), 1.35 (br s), 1.24 (br s), 1.13 (br s). ¹³P NMR (CDCl₃): δ 150.73. HRMS-ESI: *m/z*: calcd for C₅₃H₆₄N₇O₁₀P (M + H)⁺ 989.4446, found 989.4411.

Oligonucleotide synthesis

RNA oligonucleotides were synthesized on an automated ASM800 DNA/RNA synthesizer (Biosset, Novosibirsk, Russia) by using a trityl-off protocol and phosphoramidites with standard protecting groups on a 1.0 mmol scale, using 1000 Å CPG columns. All commercial phosphoramidites, CPG columns, and solutions were purchased from ChemGenes Corporation (Wilmington, MA). ¹²⁵IUm was incorporated into RNA oligonucleotides by solid phase synthesis using the previously reported protocol,²⁴ with a slight modification. The activator 5-(benzylthio)-1*H*-tetrazole that is normally used for RNA synthesis was not suitable for ¹²⁵IUm, as we observed a coupling efficiency of less than 5% (as judged by the change of color during trityl deprotection after coupling of ¹²⁵IUm phosphoramidite) when using this reagent. Therefore, 5-(ethylthio)-1*H*-tetrazole, the activator generally used in our laboratory for DNA synthesis, was used instead. The spin-labeled phosphoramidite was incorporated manually into the oligonucleotides by pausing the synthesizer program after completion of the prior cycle, removing the column from the synthesizer, and running the standard activator solution (200 μL) and a solution of the spin-labeled phosphoramidite (0.05 M, 200 μL) in 1,2-dichloroethane back and forth through the column for 10–12 min.

After manual coupling, the column was remounted on the synthesizer and the synthesis cycle completed. Upon completion of the synthesis, the oligoribonucleotides were cleaved from the solid support and the nucleobases and the phosphodiester deprotected in a 1:1 mixture of conc. aqueous NH₃ and 8 M MeNH₂ in EtOH (2 mL) at 65 °C for 40 min. The supernatant was collected, the beads washed three times with a mixture of EtOH:H₂O (1:1, 300 μL), and the combined washings were dried. The 2'-*O*-TBDMS groups were removed by treatment with a mixture of Et₃N·3HF:DMF (3:1800 μL) at 55 °C for 1.5 h, followed by addition of H₂O (200 μL). This mixture was transferred to a 50 mL Falcon tube and *n*-butanol (40 mL) was added and stored at -20 °C for 12 h, centrifuged and the solvent decanted from the RNA pellet. The crude RNA was subsequently purified by 20% denaturing polyacrylamide gel electrophoresis. The RNA oligonucleotide bands were visualized under UV light, excised from the gel, crushed, and eluted from the gel with a Tris buffer (2 × 10 mL; Tris (10 mM, pH 7.5), NaCl (250 mM), Na₂EDTA (1 mM)). The RNA elutions were filtered through a 0.45 mm cellulose acetate membrane (Whatman) and desalted using a Sep-Pak cartridge (Waters Corporation). The dried oligoribonucleotides were dissolved in sterile H₂O (400 μL) and their final concentrations were calculated according to Beer's law based on UV absorbance of oligoribonucleotides at 260 nm. Extinction coefficients were determined by using the UV WinLab oligoribonucleotide calculator (V2.85.04; PerkinElmer). Molecular weights of oligoribonucleotides were determined by MALDI-TOF analysis (Bruker, Autoflex III) after calibration with an external standard. UV/vis spectra were recorded on a PerkinElmer Lambda 25 UV/vis spectrometer. CD spectra were recorded on a JASCO J-810 spectropolarimeter at 20 °C with path length of 1 mm (Hellma), 10 scans, scanned from 500 to 200 nm with response of 1 s, data pitch of 0.1 nm, and bandwidth of 1.0 nm.

CW-EPR measurements

CW-EPR spectra were recorded on a MiniScope MS200 spectrometer using 100 kHz modulation frequency, 1.0 G modulation amplitude, and 2.0 mW microwave power. Each spectrum was scanned 100–120 times. The temperature was regulated by a Magnettech temperature controller M01 with an error of ±0.5 °C. The sample was prepared by dissolving spin-labeled, single-stranded RNA (2.0 nmol) and its complementary strand (2.4 nmol) in phosphate buffer (10 mM phosphate, 100 mM NaCl, 0.1 mM Na₂EDTA, pH 7.0; 10 mL, oligonucleotide final conc. 200 mM). The resulting sample was annealed by using the following protocol: 90 °C for 2 min, 60 °C for 5 min, 50 °C for 5 min, 40 °C for 5 min, 22 °C for 15 min. The samples (10 μL) were placed in a quartz capillary (BLAUBRAND intraMARK) prior to EPR measurements.

PELDOR measurements

The RNA samples for PELDOR measurement were prepared by annealing 10 nmol of each strand with 10 nmol of its complementary strand in phosphate buffer (100 μL, 10 mM, pH 7.0), NaCl (100 mM), and EDTA (0.1 mM), followed by evaporation

of the water. The annealed and dried samples were dissolved in 20% ethylene glycol/H₂O (100 μ L) before the PELDOR measurements. The dead-time free four-pulse PELDOR sequence was used for all experiments⁴⁰ and carried out on a Bruker Elexsys E580 X-band spectrometer equipped with Flexline MS-3 probe in an Oxford CF935 cryostat and a PELDOR frequency unit. Microwave pulses were amplified by a 1 kW TWT amplifier (ASE 117x). Typical pulse lengths were 32 ns ($\pi/2$ and π) for the probe pulses and 12 ns (π) for the pump pulse. The delay between the first and second probe pulses was varied between 132 and 196 ns in 8 ns steps to reduce contributions from proton modulations. The pulse separation between the second and third probe pulses was 3.0 μ s. The frequency of the pump pulse was fixed to the central maximum of the nitroxide powder spectrum to obtain maximum pumping efficiency. The probe frequency was chosen 40–85 MHz above this frequency. This range corresponds to the smallest frequency offset that avoids a strong pump/probe frequency overlap, and therefore large proton modulation artifacts. The 85 MHz offset is the frequency offset that excites the edge of the nitroxide spectrum. All PELDOR experiments were carried out at 50 K.

Conflicts of interest

There are no conflicts to declare.

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