Innately water soluble isatoic anhydrides with modulated reactivities for RNA SHAPE analysis

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Determination of 1M7 water solubility.

A 2 mM standard solution of 1,4-dioxane in D_2O was prepared by dilution of 0.17 µL 1,4-dioxane to 1.0 mL final volume with D_2O. This solution was equilibrated to 25 °C. 1 mL was added to an Eppendorf tube containing ~15 mg 1M7. The tube was vortexed vigorously for 30 s and the resulting suspension was filtered through a 0.2 µM syringe filter into an NMR tube and run on a 500 MHz JOEL EC500 NMR for 16 scans with 20 s relaxation. The integration values of 1M7 was set by the N-methyl group of the isatoic anhydride at 3.77 ppm. 1,4-dioxane peak at 3.64 ppm (8H) integrated ~22.5 times high. The concentration of 1M7 was therefore determined to be ≈ 0.1 mM.

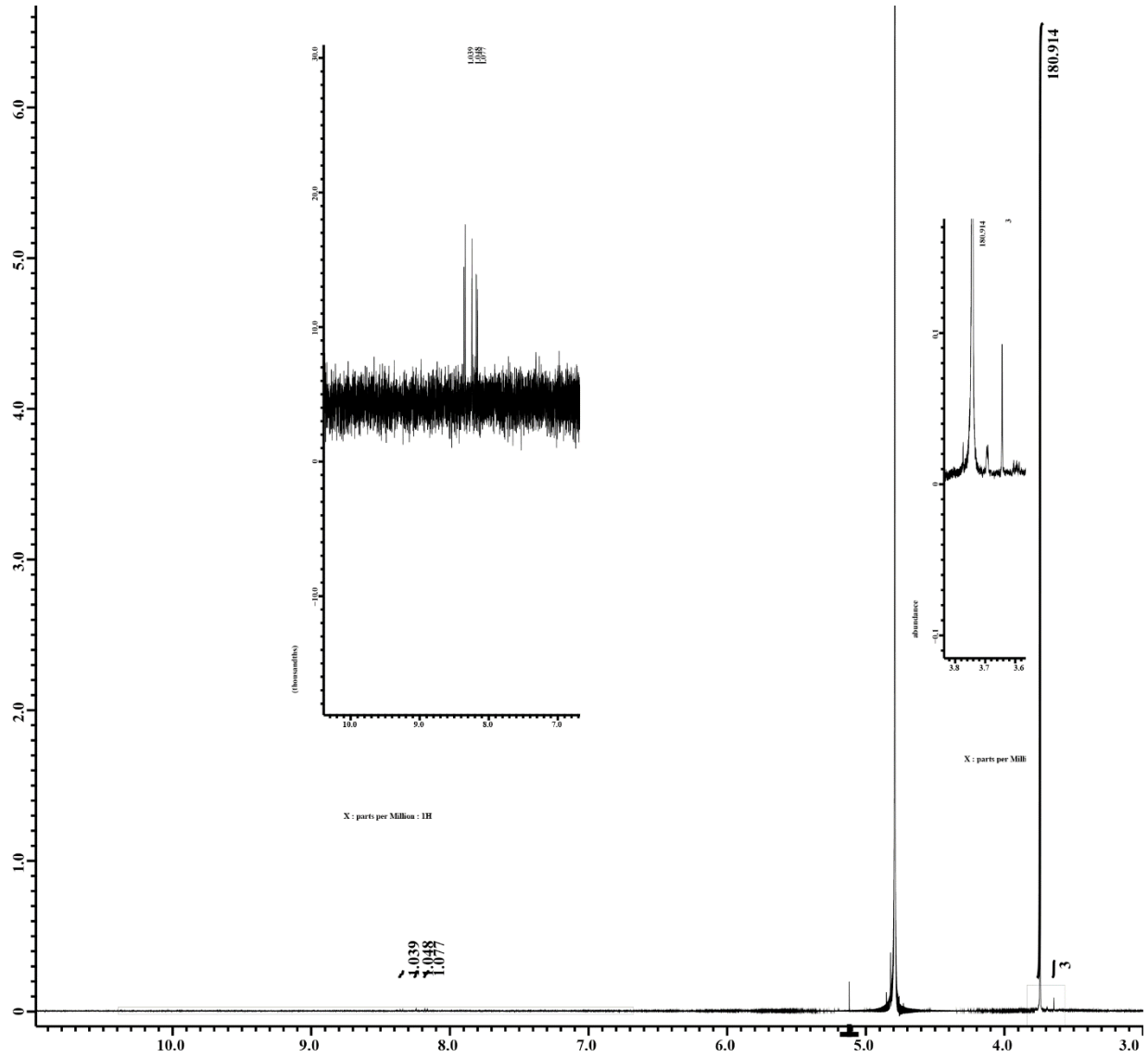
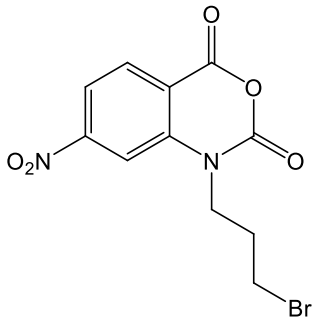
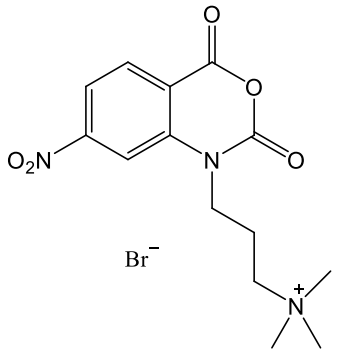


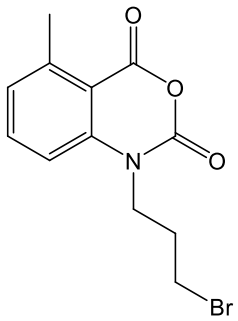
Figure S1: NMR determination of 1M7 water solubility 25° C in D_2O, picture in picture images shown to demonstrate clean 1M7 and relative integrations of aromatic protons.



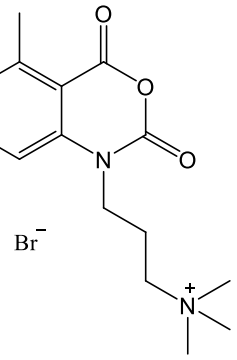
3a. A dry Schlenk flask equipped with a stir bar and flushed with N_2 was charged with NaH (0.06 g, 2.3 mmol) and to this, dry DMF was added (10 mL). 6-nitroisatoic anhydride (300 mg, 1.4 mmol) was added to the solution slowly and then subsequently rinsed down the glass with dry DMF. To the reaction mixture, 1-bromo-3-chloropropane (1.0 eq, 1.4 mmol) was added in one portion. The reaction stirred at RT overnight. The solution was then concentrated under reduced pressure, the crude residue was dissolved with DCM and washed 6x with ice cold DI H_2O. The organic layer was dried overnight with Na_2SO_4, filtered, and concentrated under reduced pressure to afford a bright orange powder. (160 mg, 34%) $C_{11}H_9BrN_2O_5$ (329.11), ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.30 (d, 1H), 8.21 (t, 1H), 8.09 (d, 1H), 4.23 (t, 2H), 3.83 (t, 2H), 2.1 (t, 2H) ppm. ^{13}C NMR (300 MHz, DMSO-d_6): δ 157.9, 152.5, 147.3, 141.7, 131.3, 117.6, 117.1, 109.6, 41.5, 41.3, 26.5 ppm. MS (EI): Calc.: 327.97 (M); Found: 328.0 (M) m/z.



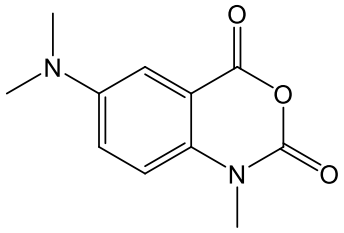
4a. To a 1.5 mL vial flushed with N_2 was added **3a** (20 mg, 61 µmol) in 250 µL dry DMF. To this was added 2.5 eq 2M trimethylamine in THF (54 µL, 0.152 mmol). The vial is sealed and placed at RT overnight. Ethyl ether was added to triturate product from DMF. Vial is washed 5x with ethyl ether, then the residual solvent is removed under reduced pressure. Product was isolated as bright yellow solid. (2 mg, 8%). $C_{14}H_{18}BrN_3O_5$ (387.04), ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.30 (d, 1H), 8.16 (s, 1H), 8.09 (d, 1H), 4.24 (t, 2H), 3.40 (m, 2H), 3.06 (s, 9H) 2.14 (t, 2H) ppm. ^{13}C NMR (500 MHz, DMSO-d_6): δ 157.9, 152.8, 147.5, 141.9, 131.6, 117.9, 116.9, 109.8, 62.6, 52.3, 41.8, 20.5 ppm. HRMS (ESI): Calc: 308.1246 (M^+); Found: 308.1226 (M^+) m/z.



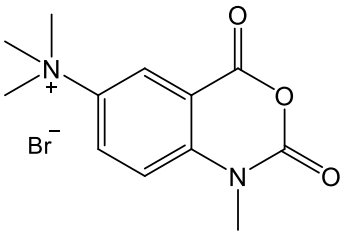
3b. A dry Schlenk flask equipped with a stir bar and flushed with N_2 was charged with NaH (0.06 g, 2.3 mmol) and to this, dry DMF was added (10 mL). 6-methylisatoic anhydride (300 mg, 1.7 mmol) was added to the solution slowly and then subsequently rinsed down the glass with dry DMF (10 mL). To the reaction mixture, 1-bromo-3-chloropropane (1.0 eq, 1.7 mmol) was added in one portion. The reaction stirred at RT overnight. The solution was then concentrated under reduced pressure, the crude residue was dissolved with DCM and washed 6x with ice cold DI H_2O. The organic layer was dried overnight with Na_2SO_4, filtered, and concentrated under reduced pressure. Product was isolated as a light brown solid (1.1 g, 51%). $C_{12}H_{12}BrNO_3$ (298.14), ^{1}H NMR (500 MHz, DMSO): δ 7.69 (t, 1H), 7.35 (d, 1H), 7.18 (d, 1H), 4.06 (t, 2H), 3.37 (t, 2H), 2.64 (s, 3H), 2.15 (p, 1H). ^{13}C NMR (300 MHz, DMSO): δ 157.9, 141.8, 143.2, 142.4, 135.9, 126.4, 112.5, 110.4, 42.2, 30.5, 22.3, 3.73 ppm. MS (EI): Calc. 297.0; Found: 297.1 (M) m/z



4b. To a 1.5 mL vial flushed with N_2 was added **3b** (168 mg, 56 µmol) in 1000 µL dry acetone. To this was added roughly 2.5 eq 2M trimethylamine in THF (54 µL, 0.152 mmol). The vial is sealed and placed at RT overnight. Vial is washed 5x with ethyl ether then the residual solvent is removed under reduced pressure Final product isolated as a light brown powder (119 mg, 59%). $C_{15}H_{21}BrN_2O_3$ (356.07), ^{1}H NMR (500 MHz, DMSO): δ 7.72 (t, 1H), 7.42 (d, 1H), 7.22 (d, 1H), 4.11 (t, 2H), 3.49 (m, 2H), 3.04 (s, 9H), 2.66 (s, 3H), 2.09 (m, 9H) ppm. ^{13}C NMR (500 MHz, DMSO): δ 157.9, 147.9, 143.4, 142.2, 136.1, 126.6, 112.7, 110.31, 62.6, 52.2, 41.7, 22.3, 20.6, ppm. HRMS (ESI): Calc: 277.1552 (M^+); Found: 277.1544 (M^+) m/z



5. A dry Schlenk flask equipped with a stir bar, flushed with N_2, was charged with NaH (0.06 g, 2.3 mmol) and to this, dry DMF was added (10 mL). 5-dimethylaminoisatoic anhydride (300 mg, 1.5 mmol) was added to the solution slowly and then subsequently rinsed down the glass with dry DMF (2-3 mL). To the reaction mixture, CH_3I (1.0 eq, 1.5 mmol) was added in one portion. The reaction stirred at RT overnight. The solution was then concentrated under reduced pressure, the crude residue was dissolved with DCM and washed 6x with an ice cold solution of saturated sodium bicarbonate. The organic layer was dried with Na_2SO_4, filtered, and concentrated under reduced pressure to afford a faint green solid. (207 mg, 65%). $C_{11}H_{12}N_2O_3$, ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.31 (d, 2H), 7.14 (t, 1H), 3.42 (s, 3H), 2.94 (s, 6H) ppm. ^{13}C NMR (500 MHz, DMSO-d_6): δ 159.9, 148.1, 147.3, 147.3, 133.3, 122.7, 116.4, 110.1, 40.9, 32.1 ppm. HRMS (EI): Calc. 221.0926; Found: 221.0917 (MH^+) m/z.



6. To a 1.5 mL vial flushed with N_2 was added **5** (100 mg, 0.45 mmol) through a 0.2 µm filter in 750 µL dry acetone. To this was added excess MeBr condensed in 250 µL cold acetone. The vial was sealed and placed at RT overnight. Vial was washed 5x with ethyl ether then the residual solvent is removed under reduced pressure. (65 mg, 50%). $C_{12}H_{15}BrN_2O_3$ (315.17), ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.44 (m, 2H), 7.69 (s, 1H), 3.66 (s, 9H), 3.51 (s, 3H) ppm. ^{13}C NMR (500 MHz, DMSO-d_6): δ 158.1, 147.4, 142.9, 141.9, 129.2, 121.3, 116.7, 112.2, 56.5, 32.1 ppm. HRCMS (ESI): Calc. 235.1083; Found: 235.1071 (M+) m/z

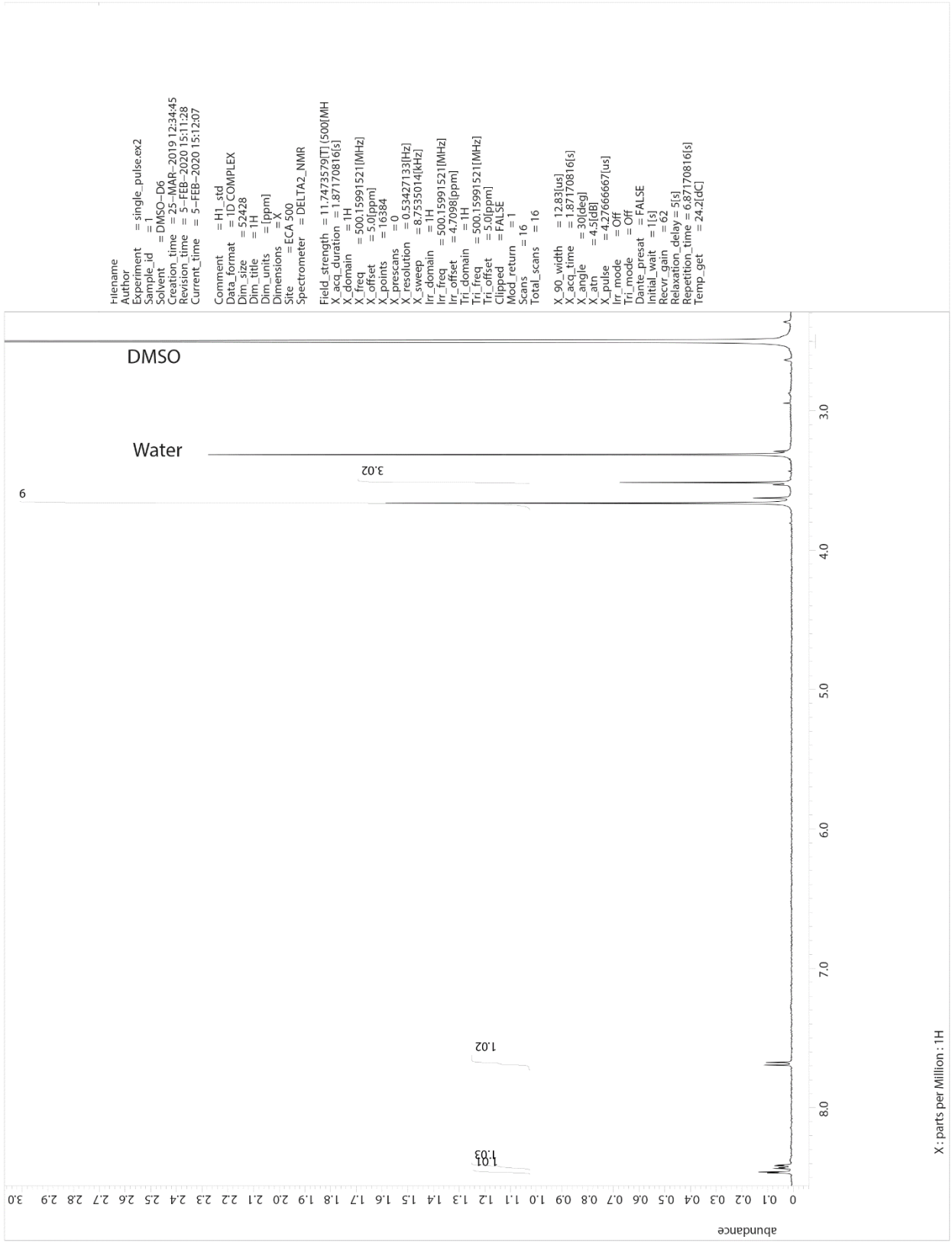


Figure S2: ^{1}H NMR of **6**

PCR amplification and *in vitro* Transcription:

Primary PCR amplification of *Plasmodium falciparum* U3 small nucleolar RNA (snoRNA) was done from genomic DNA of *Plasmodium falciparum* 3D7 strain as described earlier.[1] The resultant PCR amplified DNA of PfU3 snoRNA was *in vitro* Transcribed (IVT) and purified using the MEGAscript and MEGAclear kits (Life Technologies, USA) following the manufacturer's recommendation.

***in vitro* SHAPE modification:**

For SHAPE modification of PfU3snoRNA, 5 µg of *in vitro* transcribed RNA was denatured by heating at 95 °C for 2 min followed by immediate incubation on ice for 3 min. The modification was carried out by incubation of the RNA in 3X SHAPE buffer consisting of 333 mM of NaCl, 333 mM of HEPES and 20 mM of $MgCl_2$ at 37 °C prior to the addition of 10 mM of 1M7, **4b** and **6** for 70 sec. Following incubation, the modified RNA was ethanol precipitated, dissolved in water and subjected to nanodrop analysis.

cDNA synthesis and RNA-SHAPE analysis by denaturing Polyacrylamide Gel Electrophoresis (PAGE):

1.6 µg of SHAPE modified RNA was denatured by heating at 95 °C for 2 min followed by immediate incubation on ice for 3 min. For Primer extension, 5′ cy5 labelled primer (Table S1) for reverse transcription along with 10 mM dNTPs were mixed with the SHAPE modified RNA and incubated at 65 °C for 5 min. Four dideoxy sequencing reactions using ddNTPs were also performed in parallel using the unmodified PfU3 snoRNA in the similar manner as mentioned above. Following incubation, all the reactions were supplemented with 1X SuperScript II First-strand buffer (50mM Tris-HCl, pH 8.3, 75 mM KCl and 5 mM $MgCl_2$), 10mM DTT and 1 µL of RiboLock RNase inhibitor and incubated at 42 °C for 2 min. After addition of SuperScript II Reverse Transcriptase enzyme, the whole reaction was incubated at 42 °C for 60 min, followed by inactivation at 70 °C for 15 min. The, resultant mixtures of cDNA were precipitated by adding isopropanol and then dissolved in water. cDNA generated from primer extension were mixed with 80% formaldehyde, heated at 80 °C for 3 min and then resolved by 12% denaturing PAGE gel. The gel was scanned using Typhoon phosphorimager (Figure S2). Nucleotide position for each band was identified form dideoxy sequencing lane (Figure S2). Secondary structure of RNA was generated by Mfold[2] and the SHAPE reactivities of accessible nucleotide were mapped on it.

Table S1: Primer used for PfU3 snoRNA cDNA synthesis

Name	Sequence
PfU3 snoRNA RT	5′ cy5- AAG TTC AGC GGA CGC AAG CTC-3′

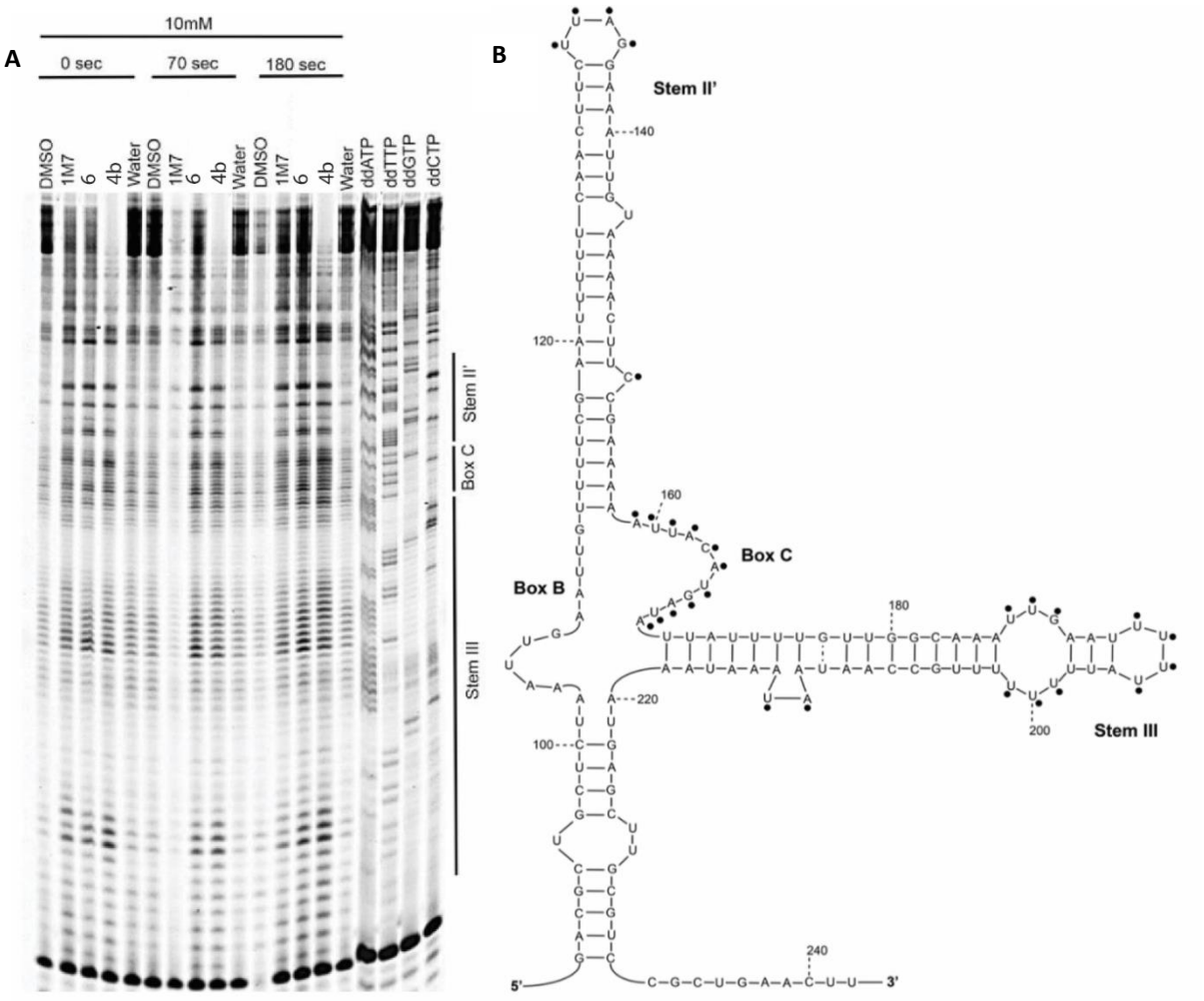


Figure S3: (**A**) cy5 labeled denaturing gel comparing modifications in the synthetic pfU3 snoRNA using 1M7 and **4b** and **6** at 10 mM concentration (**B**) Partial secondary structure of PfU3 snoRNA. Nucleotides modified by the SHAPE electrophiles are indicated by black circles

in vitro Transcription of TbTR catalytic core:

The catalytic core of *T. brucei* telomerase RNA (TbTR) comprising of the Template boundary element (TBE), Template and Template proximal and distal helix regions were synthesized as a G-block oligos from IDT. T7 RNA promoter sequence was added to the 5' end of TBE during the synthesis. The resultant oligos was *in vitro* transcribed (IVT) using the MEGAscript and purified MEGAclear kit (Life Technologies, USA) following the manufacturer's recommendation. The resultant RNA was quantified by nanodrop analysis.

in vitro SHAPE modification:

For SHAPE modification of TbTER catalytic core, 5 µg of *in vitro* transcribed RNA was denatured by heating at 95 °C for 2 min followed by immediate incubation on ice for 3 min. The modification was carried out by incubating the RNA in 3X SHAPE buffer consisting of 333 mM of NaCl, 333 mM of HEPES and 20 mM of $MgCl_2$ at 37 °C prior to the addition of 10 mM of 1M7, **6** and **4b** for 70 sec. As a negative control equivalent amount of DMSO/water was added to the RNA. Following incubation, the modified RNA was ethanol precipitated, dissolved in water and subjected to nanodrop analysis prior to cDNA synthesis.

cDNA synthesis and RNA-SHAPE analysis by denaturing Polyacrylamide Gel Electrophoresis (PAGE):

1.6 µg of SHAPE modified RNA was denatured by heating at 95 °C for 2 min followed by immediate incubation on ice for 3 min. For Primer extension, 5' cy5 labelled primer (Table S2) for reverse transcription along with 10mM dNTPs were mixed with the SHAPE modified RNA and incubated at 65 °C for 5 min. Four dideoxy (dd) sequencing reactions were also performed using the unmodified TbTR catalytic core in the similar manner as mentioned above. Additionally, to the individual sequencing reactions were also added ddATP, ddTTP, ddGTP and ddCTP. Following incubation all the reactions were supplemented with 1X SuperScript II First-strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl and 5 mM MgCl2), 10 mM DTT and 1 µl of RiboLock RNase inhibitor and incubated at 42 °C for 2 min. After addition of SuperScript II Reverse Transcriptase enzyme, the whole reaction was incubated at 42 °C for 60 min, followed by inactivation at 70 °C for 15 min. The, resultant mixtures of cDNA were precipitated by adding isopropanol and then dissolved in water. cDNA generated from primer extension were mixed with 80% formaldehyde, heated at 80 °C for 3 min and then resolved by 12% denaturing PAGE gel. The gel was scanned using Typhoon phosphorimager. Nucleotide position for each band was identified form dideoxy sequencing lane. Secondary structure of TbTR catalytic core was generated by RNAstructure software[3] and the SHAPE reactivities of accessible nucleotide were mapped on it.

Table S2: Primer used for TbTR Catalytic core cDNA synthesis

Name	Sequence
TbTR-Template RT	5′ cy5- TAT TGC ACA CCA ACA GCA CTA -3′
TbTR-Template Proximal helix RT	5′ cy5- CAA TGA TGA CGT GAA GAG GAG GA -3′
TbTR-Template Distal helix RT	5′ cy5- TAA ACA AAC CGG CTA GAG AGG -3′

Signal-to-background ratio calculation:

Signal-to-background ratio (S/B) is the arithmetic mean of intensity of bands for sample lanes (except the dideoxy sequencing reactions and control lanes) to the background. Mean Pixel intensity of randomly chosen three bands for sample lane was calculated using ImageJ software and divided by the mean pixel intensity of background which is the low intensity region present adjacent to each band. Same position bands were used for calculation for each lane.

High Resolution Mass Spectrometry:

High resolution MS data were analyzed on an Orbitrap XL in positive mode at a resolution of 60000, spray voltage of 5 kV, sheath 12, Aux 10, Sweep 0, Capillary Temp 275 °C, Capillary Voltage 32.00 V, Tube Lens of 85.00 V Compounds were dissolved in 1 mL ACN, then 8 µL was diluted in 1:1 H_2O:ACN with 0.1% Formic acid. 2 µL of this solution was injected using an Accela 1250 LC system, at a solvent flowrate of 200 µL/min of 1:1 H_2O:ACN with 0.1% Formic Acid. X-ray data were collected on an Agilent/Oxford Diffraction Gemini A Ultra diffractometer utilizing Mo radiation (λ = 0.71073 Å) at 100 K.

References:

1. Fessler, A. B., Dey, A., Garmon, C. B., Finis, D. S., Saleh, N.-A., Fowler, A. J., Jones, D. S., Chakrabarti, K., and Ogle, C. A. (2018) Water-Soluble Isatoic Anhydrides: A Platform for RNA-SHAPE Analysis and Protein Bioconjugation. *Bioconjugate Chem. 29*, 3196-3202.

2. Zuker, M., (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31 (13), 3406.

3. Reuter, J. S.; Mathews, D. H., RNAstructure: software for RNA secondary structure prediction and analysis. *BMC Bioinform*. **2010,** *11* (1), 129.