Supporting Information

Polyacetate and Polycarbonate RNA: Acylating Reagents and Properties

Maryam Habibian,[§] Willem A. Velema,[§] Anna M. Kietrys, Yoshiyuki Onishi and Eric T. Kool[*]
Department of Chemistry, Stanford University, Stanford, CA 94305, USA
[§]These authors contributed equally to this manuscript
[*]To whom correspondence should be addressed: kool@stanford.edu

Table of contents

General procedure for acylating RNA

Acetylimidazole, acetyl coenzyme A sodium salt and lithium potassium acetylphosphate were purchased from Sigma Aldrich. Methyl imidazole carbamate (MIC) was prepared as previously described.[1] DMSO stock solutions of acetylimidazole were prepared in advance and were stored at -20 °C. The use of fresh bottle of acetylimidazole in preparing the stock is recommended. See "Structure Mapping" below for an alternative preparation.

For obtaining polyacetate RNA the following procedures were used:

Medium level acylation: To 5 μL of an aqueous solution of RNA was added 5 μL of 3 M DMSO solution acetylimidazole, giving final concentrations of 6 μM RNA and 1.5 M of the acylating reagent. This mixture was incubated for 2 h at 37 °C.

High level acylation: To 2.5 μL of an aqueous solution of RNA was added 7.5 μL of 4 M DMSO solution acetylimidazole, giving final concentrations of 6 μM RNA and 3 M of the acylating reagent. This mixture was incubated for 2 h at 37 °C.

For obtaining polycarbonate RNA the following procedure was used:

Medium level acylation: To 9 μL of an aqueous solution of RNA was added 1 μL of 1M DMSO solution of acylating reagent 2, giving final concentrations of 10 μM RNA and 100 mM acylating reagent. This was incubated for 16 h at room temperature in when 1 μL of 1M DMSO solution of acylating reagent 2 was added and the resulting solution incubated for another 24 h.

High level acylation: The same procedure for obtaining medium level acylation was used with two additional rounds of 24 h incubation with acylating reagent **2**.

To the resulting reaction mixtures was added 1 μL 3 M sodium acetate buffer pH = 5.2, 1 μL of glycogen (10 mg/mL) and and 36 μL EtOH and the mixture was cooled at -80 °C for 1 h. The resulting suspension was centrifuged at 14,800 rpm for 60 min and the supernatant was removed. The solids were washed with 10 μL 70% EtOH and centrifuged at 14,800 rpm for 5 min. The supernatant was removed and the RNA was dried to air for 10 min and redissolved in RNAse free biological grade water at the desired concentration. RNA concentration was determined with a Nanodrop One microvolume UV-VIS spectrophotometer. RNA samples were stored at -20 °C.

Sequences of oligonucleotides used

Oligonucleotides were purchased from PAN facility, Stanford School of Medicine and purified with GlenPak.

RNA 1: 5'OH – AUC CUG CCG ACU ACG CCA MW = 5647
RNA 2: 5'Cy5 – AUC CUG CCG ACU ACG CCA MW = 6181
DNA 1: 5'OH – ATC CTG CCG ACT ACG CCA MW = 5402
DNA complement: 5'OH – TGG CGT AGT CGG CAG GAT
Molecular Beacon: 5'6FAM – CGC GGG CGT AGT CGG CAG GAC GCG – 3'Dabsyl

Half-life measurement

Half-lives were determined by NMR spectroscopy by dissolving the compounds in 25 mM phosphate buffer prepared with deuterium oxide and 2.5% DMSO. Compound hydrolysis was observed over time.

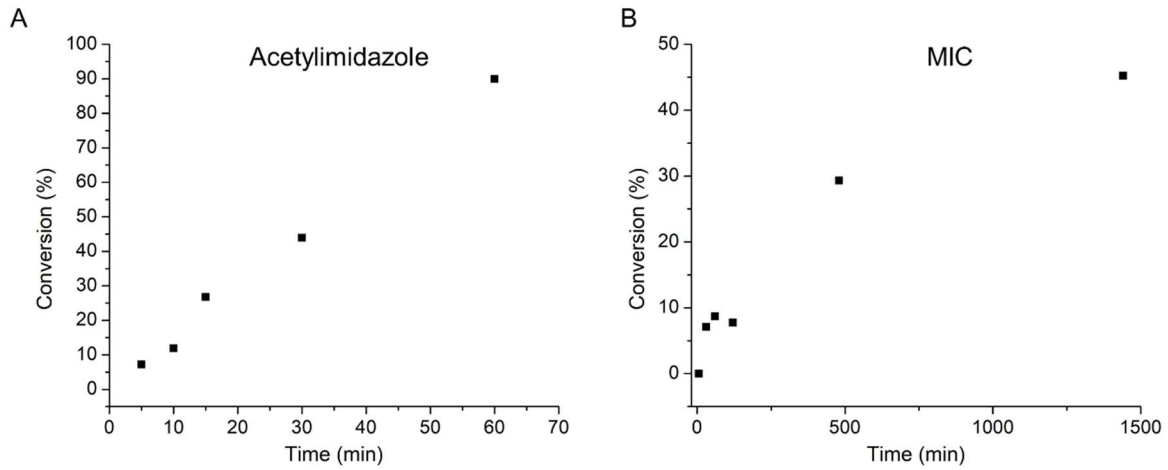


Figure S1. Half-life determination of acetylimidazole (A) and MIC (B) in 25 mM phosphate buffer. 50% substrate conversion is reached after ~ 30 min for acetylimidazole and after ~24 h for MIC.

MALDI-TOF analysis of acylated RNA

Mass spectra were acquired on a Bruker MALDI Microflex LRF instrument using an AnchorChip target and 3-hydroxypicolinic acid as matrix with addition of diammonium hydrogen citrate and TFA.

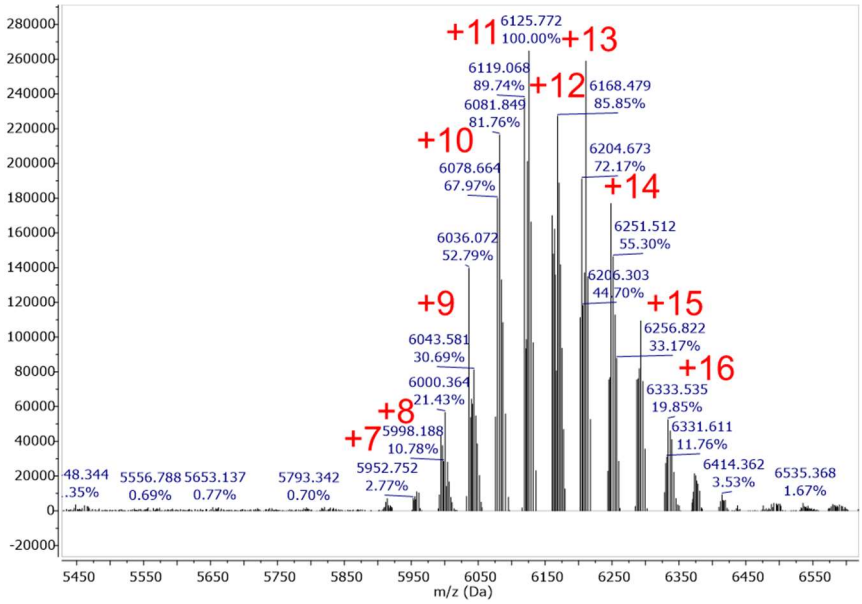


Figure S2. Mass spectrum of high acetylation levels of RNA 1 with AcIm. Number of adducts for each peak is shown in red. Up to 17 acetyl groups were observed.

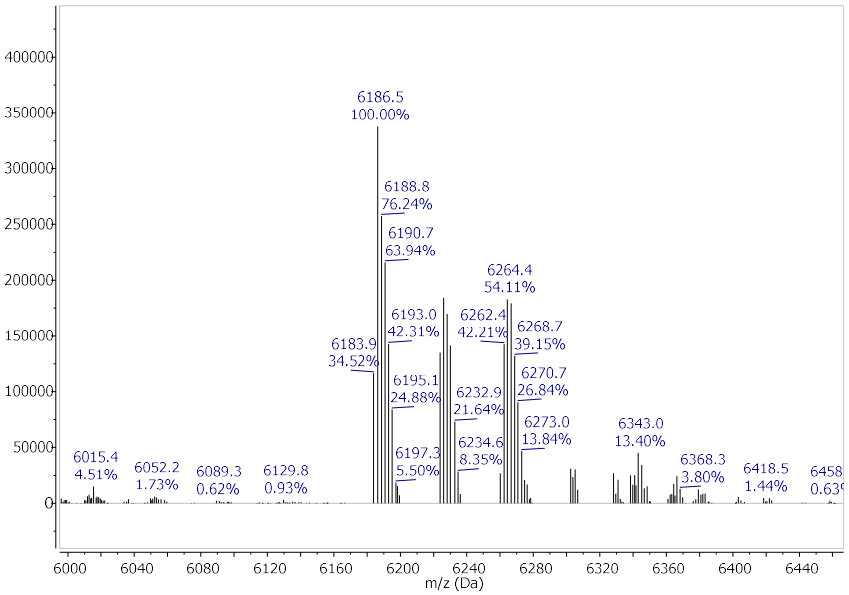


Figure S3. Control mass spectral analysis of unreacted RNA **2**. Calculated mass of RNA 2 is 6181.

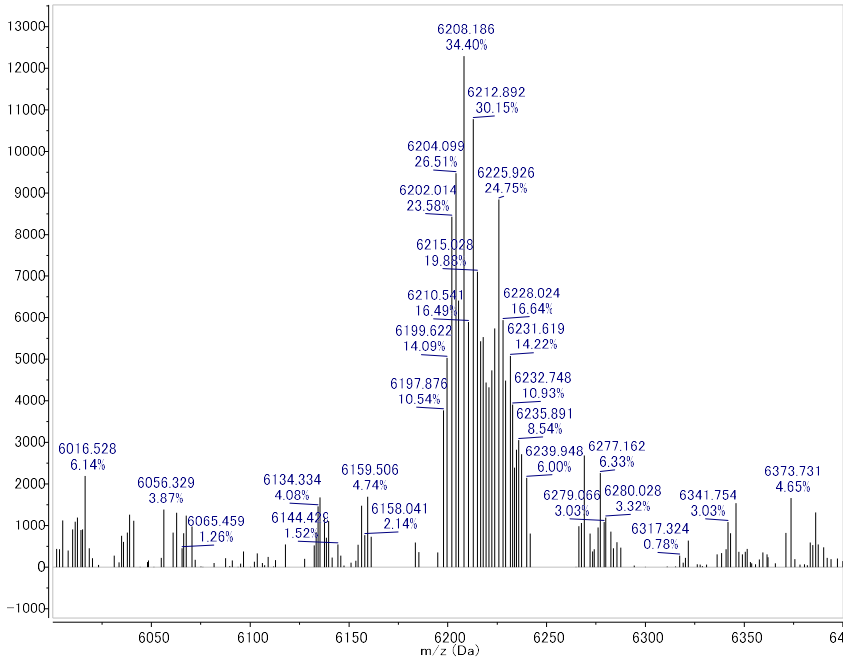


Figure S4. Mass spectral analysis of attempted acylation of RNA **2** with acetylCoA. 6 μM of RNA **2** was incubated with 100 μM acetylCoA solution in 100 mM HEPES buffer, pH 7.5, 6 mM $MgCl_2$, 100 mM NaCl, for 4 hours at 37 °C and sequentially purified by precipitation as described above. No significant acetylation was observed under these conditions. Calculated mass of RNA 2 is 6181.

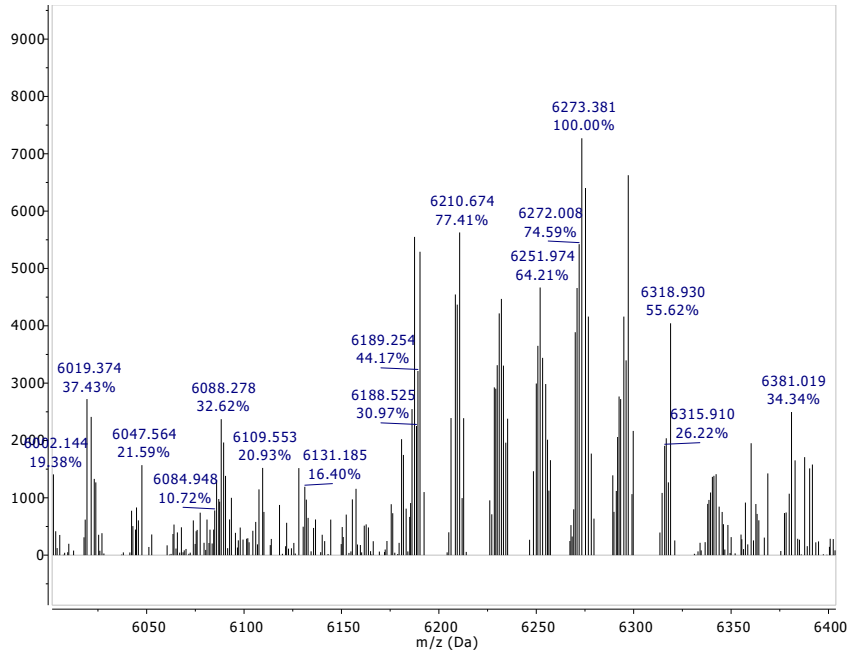


Figure S5. Mass spectral analysis of attempted acylation of RNA **2** with acetylphosphate. 10 μM of RNA **2** was incubated with 100 mM acetylphosphate in water for 16 hours at room temperature and purified by precipitation as described above. No significant acetylation was observed under these conditions. Calculated mass of RNA 2 is 6181.

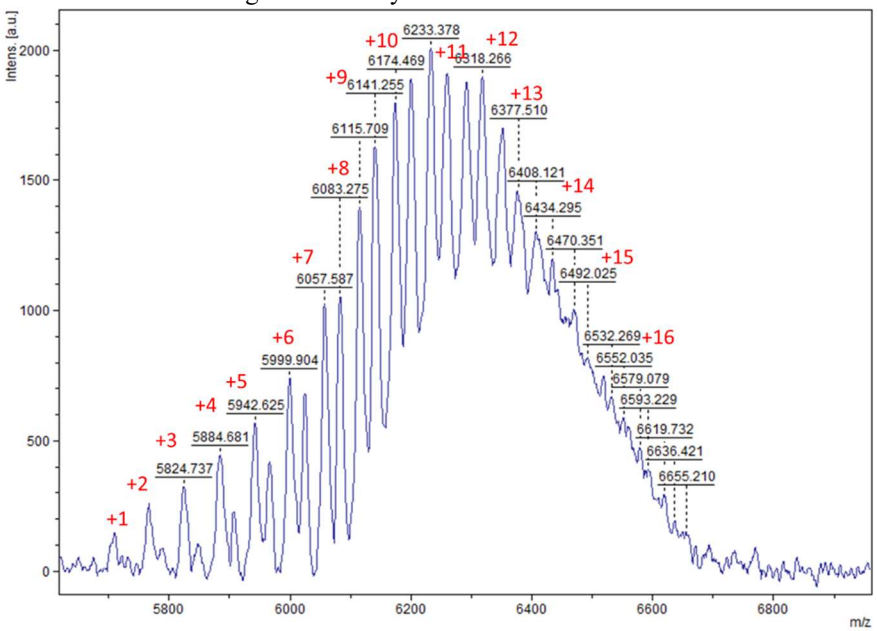


Figure S6. Mass spectrum of high acylation levels of RNA **1** with compound **2** MIC. Number of adducts is shown in red for each peak. Up to 16 acetyl groups were observed.

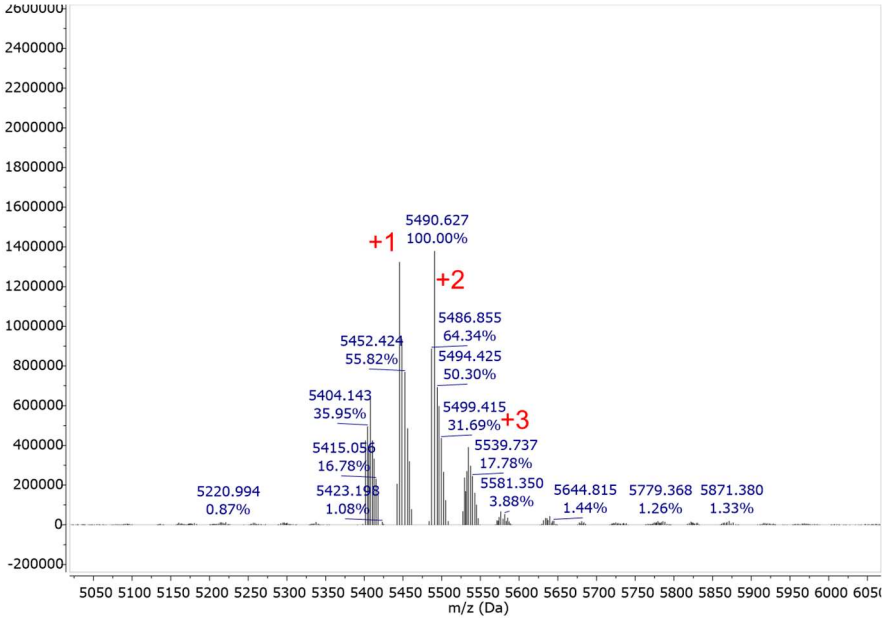


Figure S7. Mass spectrum of acylation levels of DNA **1** with compound **2** MIC using the high acylation conditions (see above). Number of adducts is shown in red for each peak. Up to 3 acetyl groups were observed, but a significant amount of unmodified DNA remained, indicating that acetylation at the 5'OH and 3'OH and/or exocyclic amines is less favored than acetylation at the 2'OH of RNA.

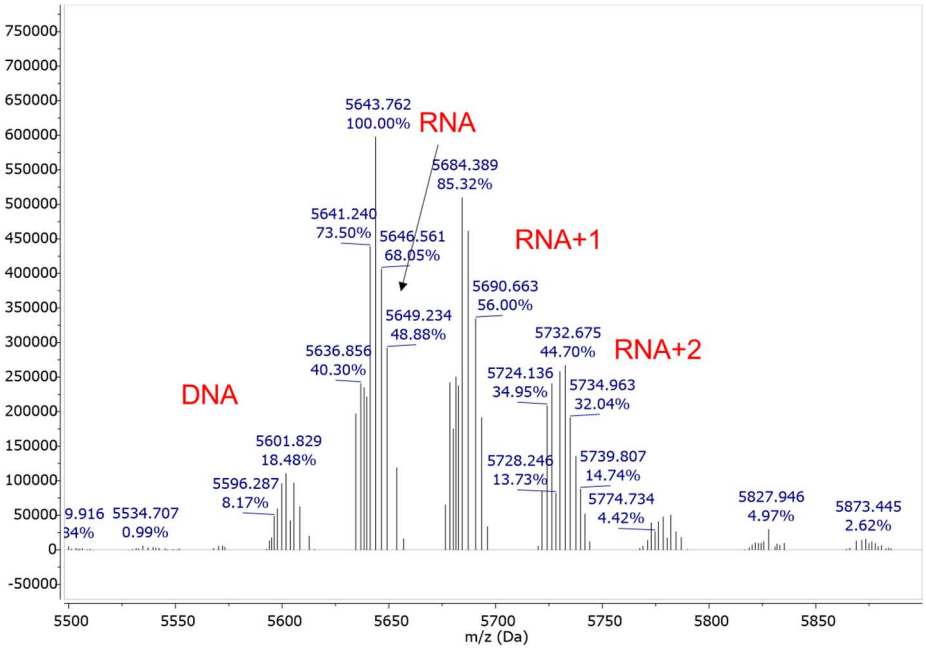


Figure S8. Mass spectrum of acylation levels of duplex RNA **1** and complementary DNA with compound **2** MIC using the high acylation conditions (see above). Up to 2 acetyl groups were observed, far fewer than single stranded RNA, which showed up to 16 acetyl groups.

Molecular Beacon experiments

The molecular beacon experiments were conducted as described.[2] In short, to 100 µL of 100 nM molecular beacon in 5 mM Phosphate buffer pH = 7.4, 1 mM $MgCl_2$, 140 mM KCl was added 1.0 µL of 1.2 µM acylated RNA 1 for AcIm and RNA 2 for MIC and the fluorescence was measured using a Thermo Fluoroskan Ascent FL microplate reader, λ_{ex}= 485 nm, λ_{em}= 538 nm at room temperature. The fluorescence intensity at t = 10 min was normalized to the positive control (unfunctionalized RNA) after subtraction of the negative control (no RNA).

Thermal denaturation studies

Thermal denaturation curves were obtained on a Varian Cary 300 Bio UV-Visible Spectrophotometer with temperature controller. Treated and untreated RNAs **1** and **2** were mixed with the complementary DNA strand in 5 mM Phosphate buffer pH = 7.4, 1 mM $MgCl_2$, 140 mM KCl at room temperature. Melting curves were obtained by monitoring the absorbance at 260 nm of the nucleic acid solution while increasing the temperature at a rate of 1 °C/min.

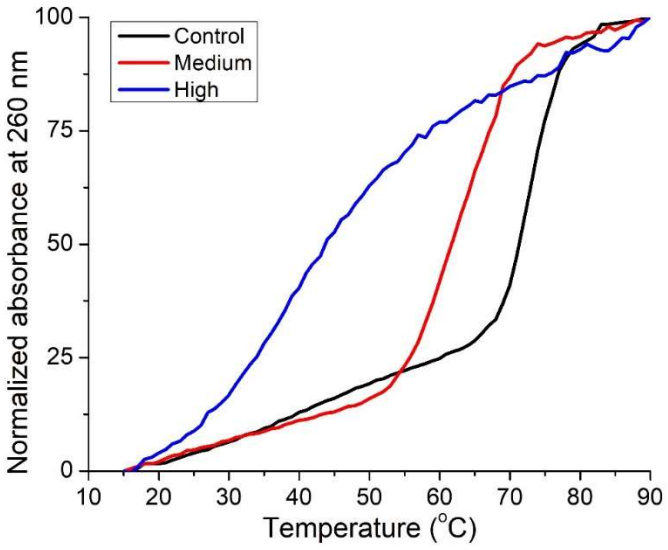


Figure S9. Thermal denaturation curves of unmodified RNA oligomer (black) and medium level acetylated RNA **1** (red) and high level acetylated RNA **1** (blue) with a complementary DNA strand. Note that the red and blue curves represent an average of the modified RNA population having varied numbers and positions of acetyl groups.

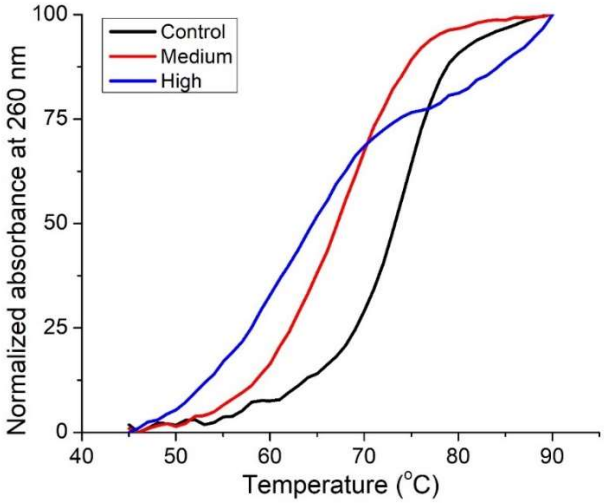
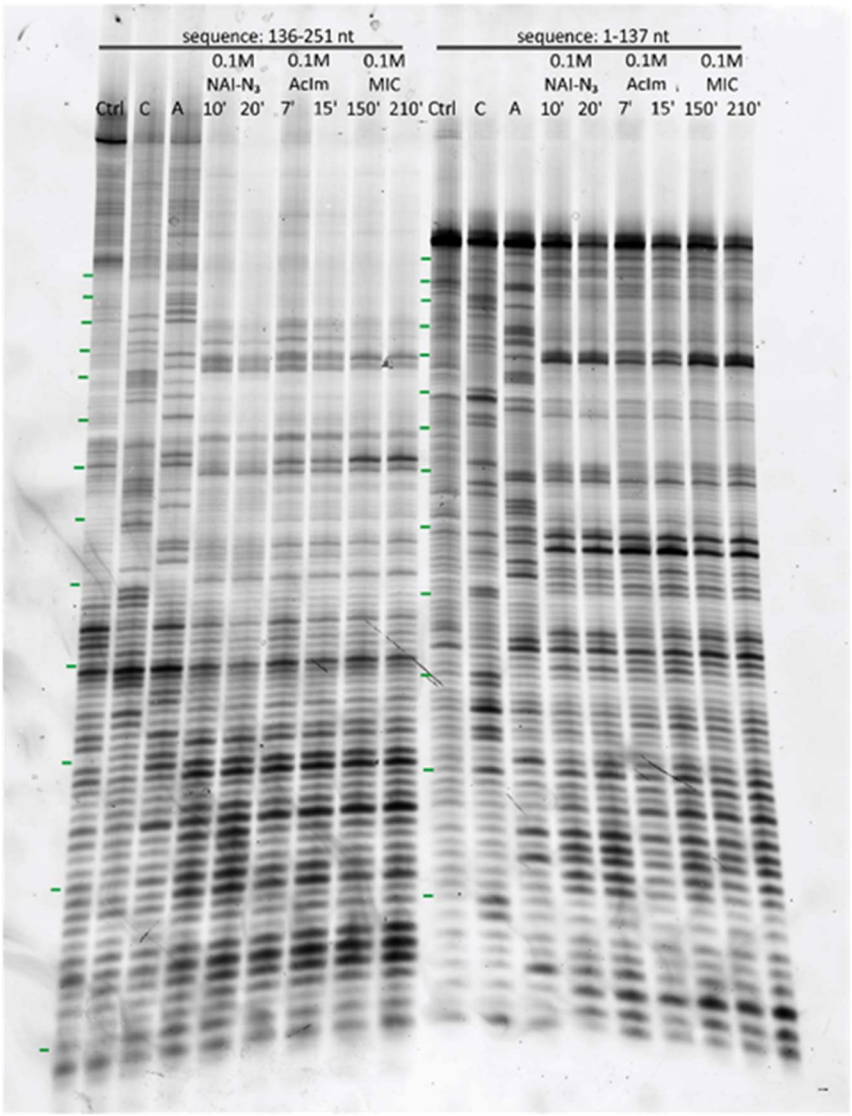


Figure S10. Thermal denaturation curves of unmodified RNA oligomer (black) and medium level methyl carbonate modified RNA **2** (red) and high level methyl carbonate modified RNA **2** (blue) with a complementary DNA strand. Note that the red and blue curves represent an average of the modified RNA population having varied numbers and positions of methyl carbonate groups.

Structure Mapping (SHAPE) of FMA aptamer *in vitro*

For SHAPE experiments we used a fresh preparation of 2M acetylimidazole in dry DMSO, prepared as follows: Carbonyldiimidazole (162.15mg) in 150uL of dry DMSO was mixed with glacial acetic acid (60.05mg) in 200 uL dry DMSO. The mixture was vortexed for 30 sec. and incubated for 1h at room temperature. RNA was transcribed using MEGAshortscript T7 Transcription Kit (Ambion), according to the manufacturer's protocol. RNA was transcribed from amplified dsDNA sequence: GCUUAUUCUCAGGGCGGGGCGAAAUUCCCCACCGGCGGUAAAUCAACUCAGUUGAAAGCC CGCGAGCGCUUUGGGUGCGAACUCAAAGGACAGCAGAUCCGGUGUAAUUCCGGGGCCGAC GGUUAGAGUCCGGAUGGGAGAGAGUAACGAUUCUGUCGGGCAUGGACCCGCUCACGUUA UUUUGGCUAUAUGCCGCCACUCCUAAGACUGCCCUGAUUCUGGUAACCAUAAUUUUAGUG AGGUUUUUUUACCAUGAAUCAGACGCUAA. 100 pmole of FMN aptamer RNA was heated in folding buffer (0.06 M MOPS, pH 7.5; 0.1 M KCl; 2.5 mM $MgCl_2$) to 96°C for 2 min. and cooled to 37°C (gradient Δ3°C/min.). RNA was incubated at 37°C for 30 min and next to the mixture was added 0.1 vol. of SHAPE reagent stock solution (NAI-N_3, AcIm or MIC) or DMSO (control reaction) and the reaction was incubated for 10 min. at 37°C. Reactions were quenched by addition of 9 volumes of precipitation solution (0.33M NaOAc, pH 5.2, glycogen 0.2 mg/mL) and 30 volumes of ice-cold ethanol. RNA was precipitated overnight at -70˚C, and then centrifuged (21000 RCF) for 40 min at 4˚C. The pellet was washed with 80% ethanol, air dried, and resuspended in 10 µL RNase-free water. 4 pmole of FMN RNA was mixed with 6 pmole RT Primer (Thermo Fisher Scientific, NED- TTAGCGTCTGATTCATGG or NED-CAGAATCGTTACTCTCTCC) and 0.25 µL dNTP mix (10 mM each, Invitrogen), and incubated for 5 min at 65˚C, then immediately chilled on ice for 2 min. To the final volume of 10 µL were added: 2 µL 5x First-Strand Buffer (Invitrogen), 1 µL 0.1 M DTT, 0.5 µL RNaseOUT and 0.25 µL Super Script II (200 U/µl, Invitrogen). The reaction was incubated using the following program: 10 min at 25˚C, 50 min. at 42˚C, and 50 min. at 52˚C. To the reaction was added 10 µL loading dye (95% formamide, 5mM EDTA pH 8, 0.05% Bromophenol blue, 0.05% Xylene cyanol FF), the mixture was denatured for 3 min. at 96˚C, and loaded on a denaturing 8% polyacrylamide gel. Products were separated in a gel in 1x TBE (pH 8.3, Sigma Aldrich), 35mA, ~3.5h. The cDNA gel was visualized by fluorescence imaging (Typhoon, GE Healthcare). SHAPE cDNA bands intensity was measured and normalized to control.

A



B

A - reactivity increase >50%
A - reactivity increase >25%
A - unmapped

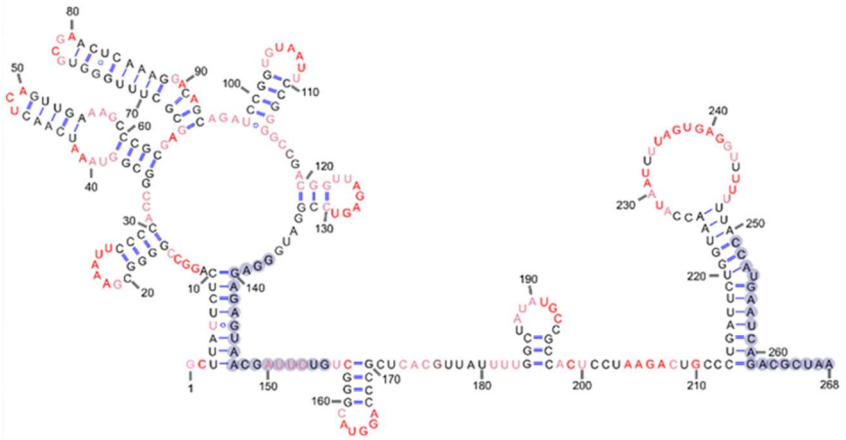


Figure S11. Mapped structure of FMN aptamer using NAI-N_3, AcIm and MIC. (A) The aptamer structure probing patterns generated using a set of SHAPE reagents. From left: Ctrl – FMN RNA; C and A - dideoxy sequencing lanes using ddG and aaT respectively in reverse transcription; DMSO - RNA treated with 10% DMSO; RNA treated with 0.1M NAI-N_3 for 10 and 20 min.; RNA treated with 0.1M AcIm for 7 and 15 min.; RNA treated with 0.1M MIC for 2.5 and 3 h. In the gel the positions of selected bases are indicated on the right by green markers. (B) The secondary structure of FMN RNA with mapped level of reactivity normalized to DMSO sample.

References

(1) Velema, W. A.; Kool, E. T. Water-Soluble Leaving Group Enables Hydrophobic Functionalization of RNA. *Org. Lett.* **2018**, *20* (20), 6587–6590.

(2) Tyagi, S.; Kramer, F. R. Molecular Beacons: Probes That Fluoresce Upon Hybridization. *Nat. Biotechnol.* **1996**, *14*, 303–308.