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Supporting Information

# Ribozyme-Catalyzed Late-Stage Functionalization and Fluorogenic Labeling of RNA

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# Supporting Information

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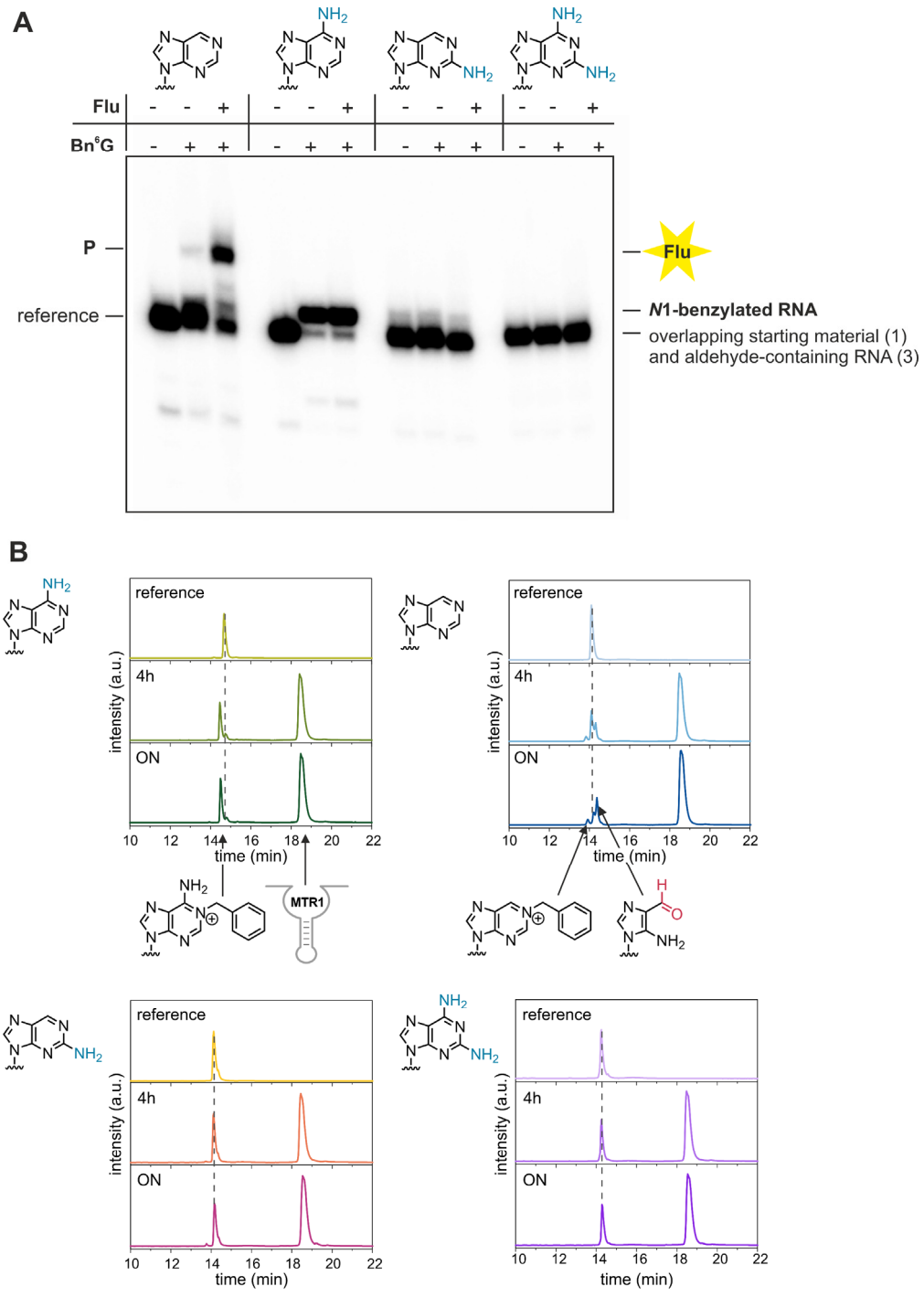



**Figure S1. Reactivity of atomic nebularine mutants. A.** Atomic mutagenesis of the RNA R2. The 5'-32P-labeled RNAs containing either A, Ne, 2AP or DAP at the target site were incubated with MTR1 at 25 °C for 23 h in the presence (+) or absence (-) of $Bn^6G$ followed by labelling with Flu-TSC (+). P = fluorescently labelled reaction product **B**. Anion-exchange HPLC analysis of MTR1-catalyzed reaction of RNA R2 atomic mutants with A, Ne, 2AP or DAP as target sites. N1-benzylated RNA elutes earlier than reference (for A (upper left dataset). Aldehyde-containing RNA elutes later than reference (for Ne, upper right dataset). 2AP and DAP do not show any conversion in PAGE and HPLC analyses.

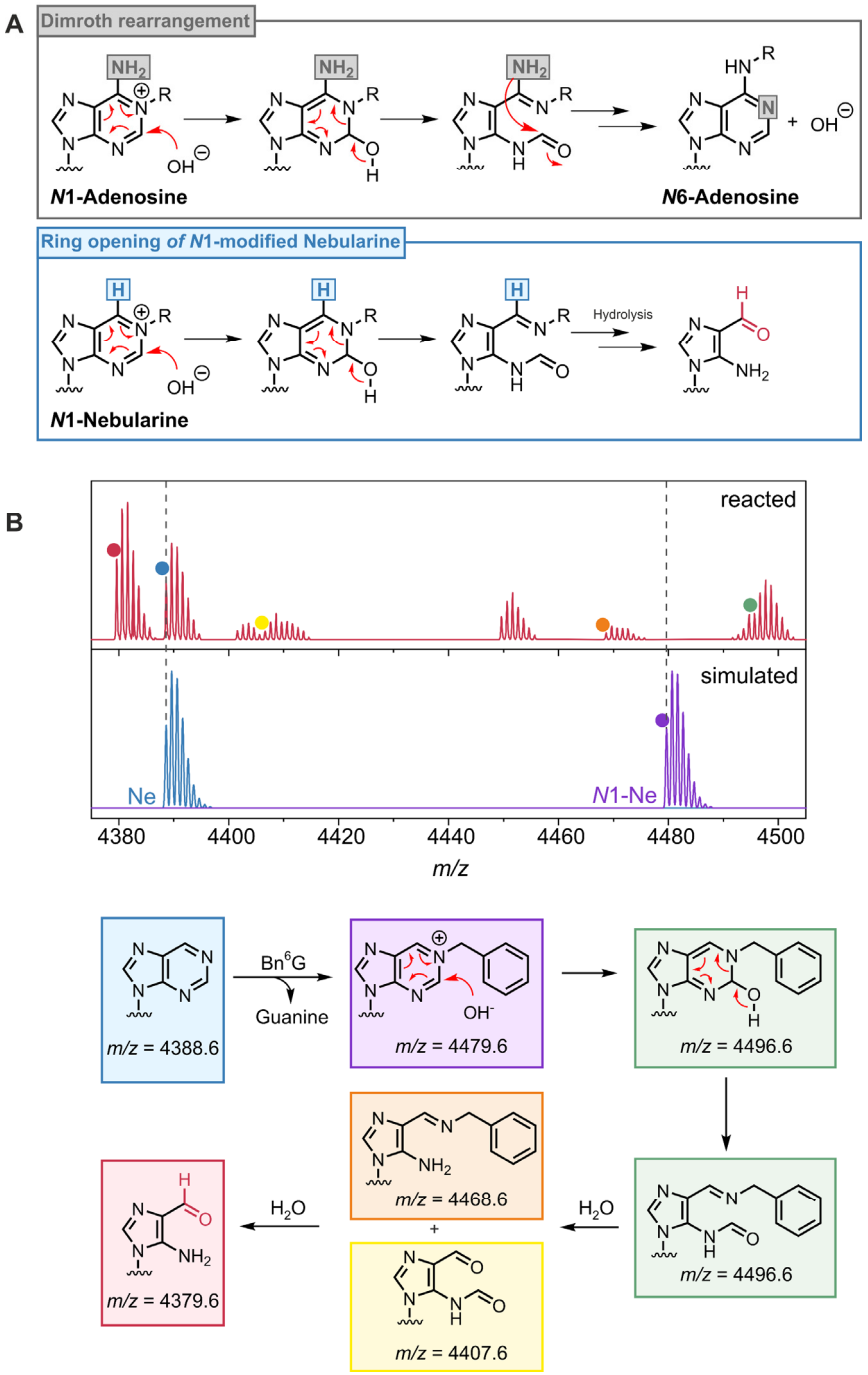



**Figure S2. Mechanism of the MTR1 ribozyme-catalyzed aminoimidazole aldehyde generation. A** Reaction mechanism of the Dimroth rearrangement of $N^1$- to $N^6$-modified adenosine. The analogous reaction of $N^1$-modified nebularine results in a ring-opened intermediate, which is hydrolyzed. **B** Deconvoluted HR-ESI-MS spectrum of the isolated MTR1 + $Bn^6G$ treated Ne-RNA R1 (top) and the simulated references of respective unmodified or $N^1$-benzylated Ne-RNA R1 (bottom) together with the proposed reaction mechanism derived thereof. Note: C2 is expected to be more electrophilic than C6. However, in an alternative mechanism, the first attack of $H_2O$ (or $OH^-$) could also occur at C6 instead of C2, which would lead to isomeric intermediates with same mass, and result in the same final product.

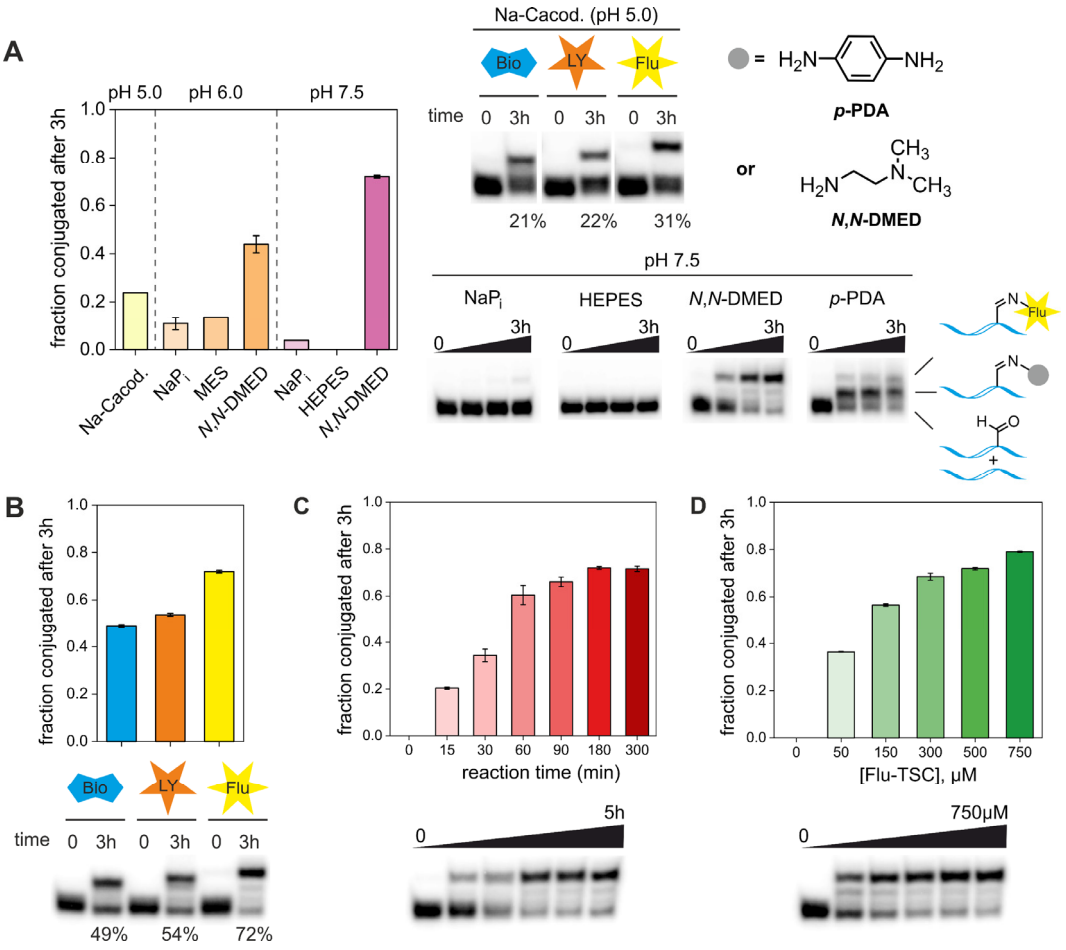



**Figure S3. Optimization of the aldehyde labeling reaction. A** Aldehyde functionalization using various buffers with different pH. Ne-RNA R1 was treated with MTR1 + Bn$^6$G followed by reaction with Flu-TSC for 3 h at 37°C. **B** Aldehyde functionalization on Ne-RNA R1 treated with MTR1 + Bn$^6$G using Flu-TSC in *N,N*-DMED (pH 7.5) for 3 h at 37°C. **C** Time dependency of the aldehyde functionalization in Ne-RNA R1 in *N,N*-DMED (pH 7.5) using 500 µM Flu-TSC with incubation at 37°C. **D** Flu-TSC concentration dependency of the aldehyde functionalization in Ne-RNA R1 in *N,N*-DMED (pH 7.5) with 3 h incubation at 37°C.

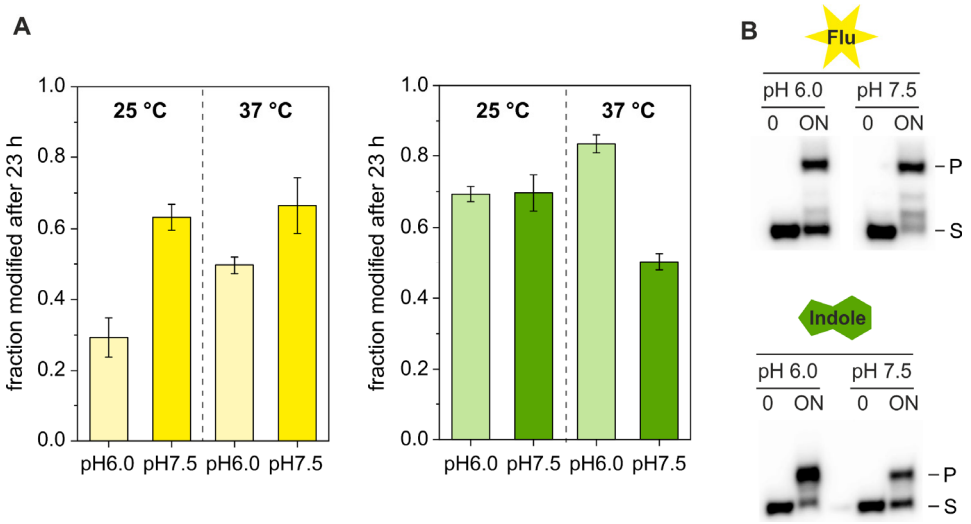



**Figure S4. One-pot modification reactions. A** Analysis of one-pot Ne-modification reactions in Ne-RNA R1 using Flu-TSC (yellow) or **4** (green). Reactions were conducted for 23 h at 25°C or 37°C using *N,N*-DMED (pH 6.0/pH7.5) for Flu-TSC or MES (pH 6.0)/Tris (pH 7.5) for **4**. **B** Representative gel images from one-pot reactions performed at 37 °C. P, product; S, substrate. ON = over night, here 23 h.

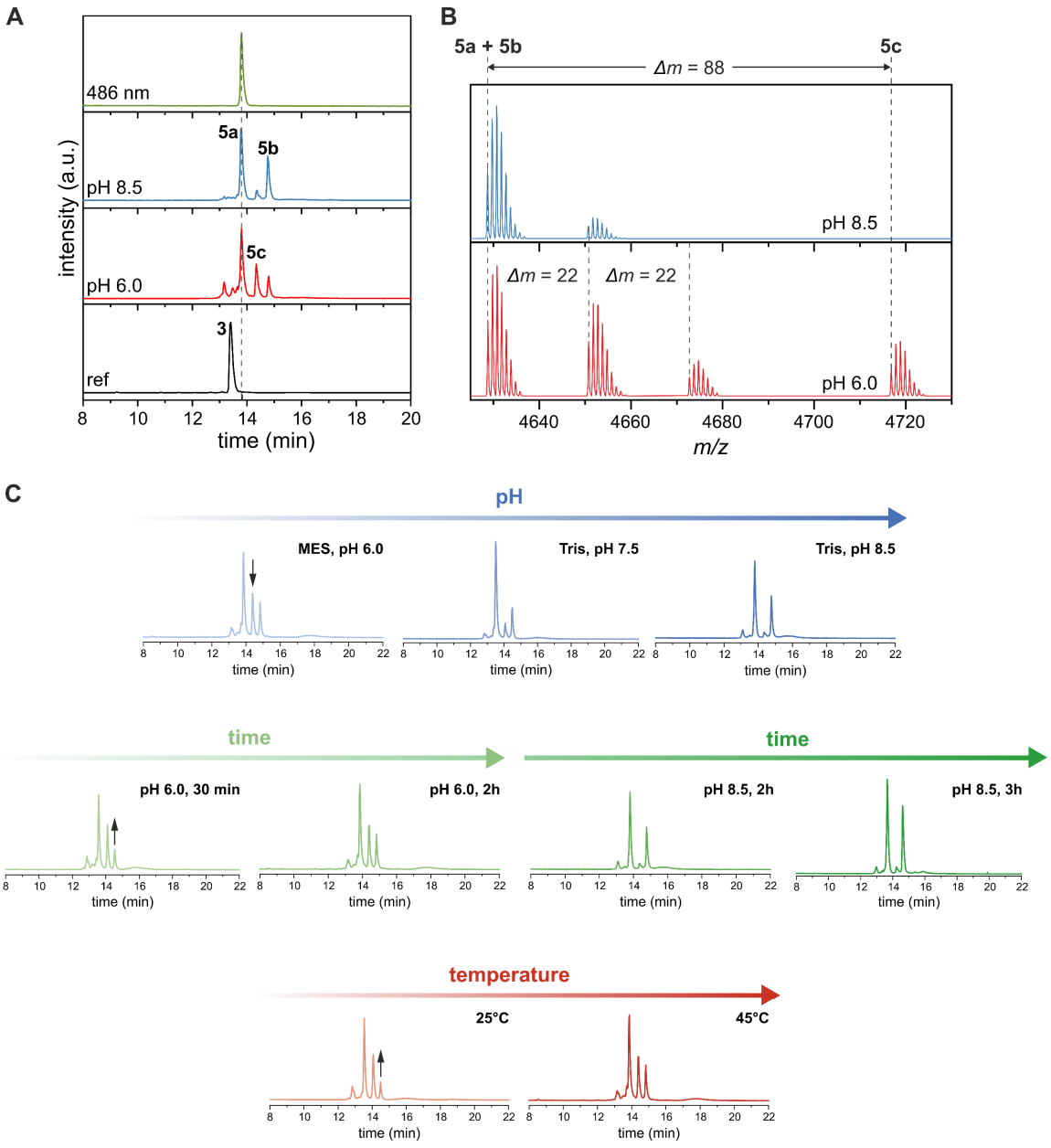



**Figure S5. Optimization of the synthesis of chromophore 5 on RNA. A** Anion-exchange HPLC analysis of the generation of chromophore **5a** in Ne-RNA R1 with formation of side products **5b** and **5c** conducted at pH 6.0 (red trace, monitored at 260 nm) or at pH 8.5 (blue trace, monitore at 260 nm, and green trace at 486 nm ($\varepsilon^{486\,nm} \sim 6x10^4\,L.mol^{-1}.cm^{-1}$)). Unmodified reference R1 monitored at 260 nm shown in black. **B** Deconvoluted HR-ESI-MS spectra of the isolated products for the Ne-RNA R1 reaction that was conducted at pH 8.5 (top, blue trace) or pH 6.0 (bottom, red trace). Δm = 22 denotes $Na^+$-adducts. **C** Anion-exchange HPLC analysis of PAGE-purified modification products. Ne-RNA R1 was first reacted overnight with MTR1 in the presence of $Bn^6G$ followed by reaction with **4** under different conditions, as indicated by variation of pH, reaction time or temperature. The standard set of conditions were (unless otherwise mentioned): 85 µM substrate RNA in 25 mM MES (pH 6.0), 100 mM KCl and 75 mM **4** at 45 °C for 2h.

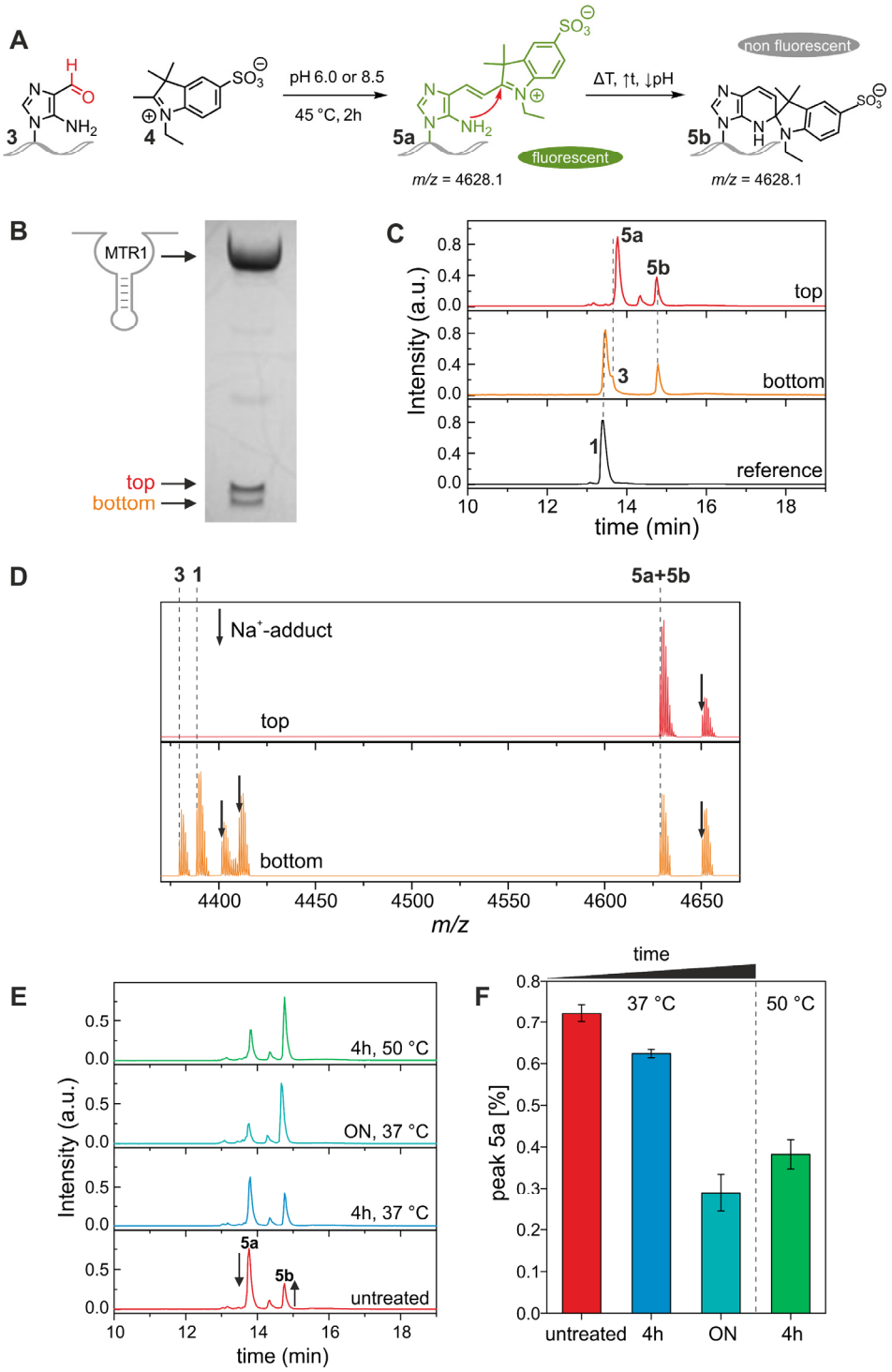



**Figure S6. Conversion of chromophore 5a to the isomeric spiro compound 5b. A** Proposed reaction mechanism for the generation of the isomeric non-fluorescent spiro compound **5b** that is favored under elevated temperatures (ΔT), longer reaction times (↑t,) and lower pH (↓pH). **B** Purification of MTR1 + Bn$^6$G treated Ne-RNA R1 reacted with **4** on denaturing PAGE. **C** Anion-exchange HPLC analysis of RNA fractions isolated from **B** (all traced monitored at 260 nm). **D** Deconvoluted HR-ESI-MS spectrum of RNA fractions isolated from **B**. Arrows denote sodium adducts. **E** Monitoring the conversion of **5a** to **5b** in the "top" RNA fraction from **B** using anion-exchange HPLC (260 nm). The RNA was incubated at pH 8 under elevated temperatures followed by analysis with anion-exchange HPLC. **F** Analysis of the peak areas corresponding to **5a** and **5b** from **E**.

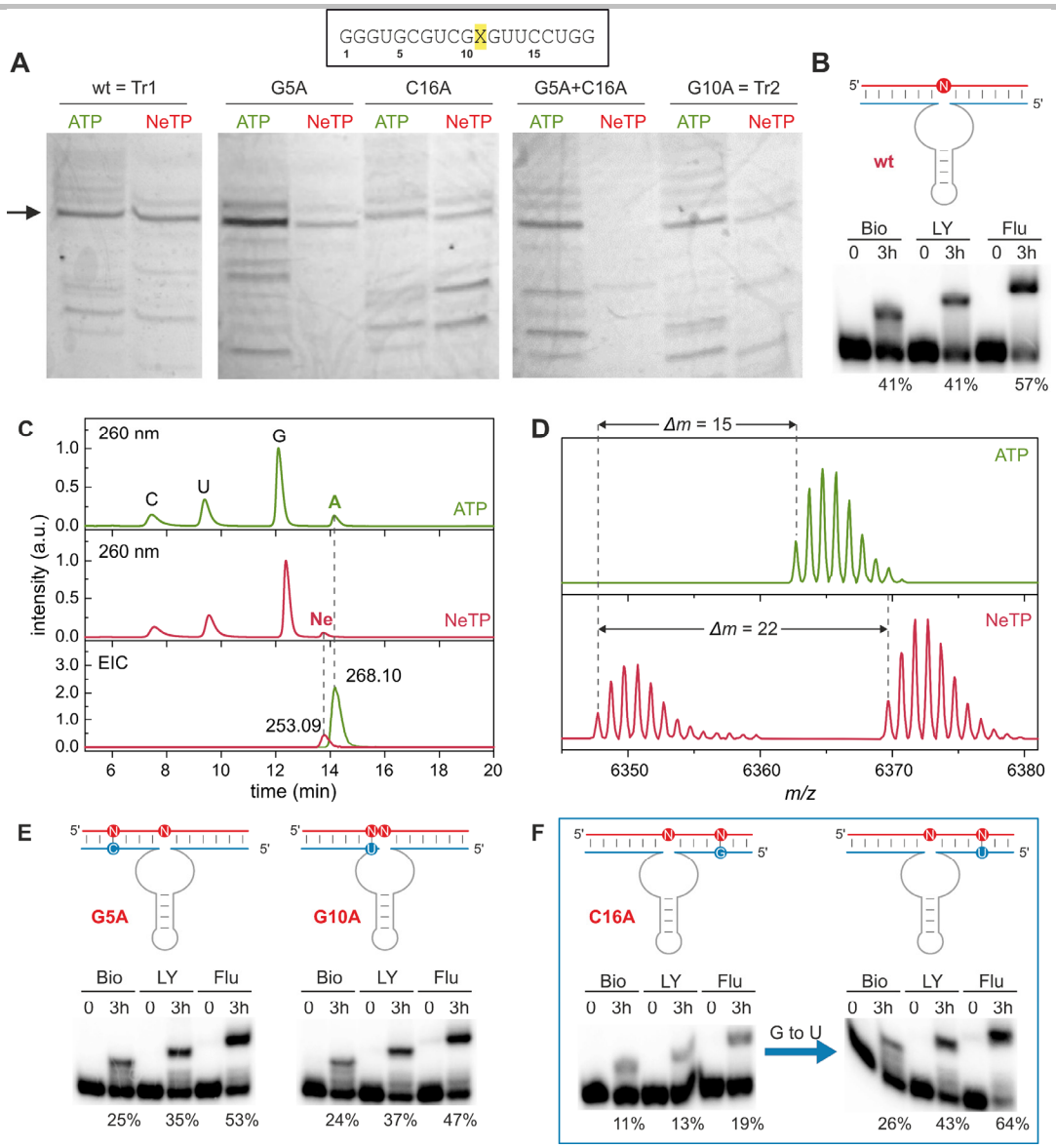



**Figure S7. In vitro transcription using NeTP and modification of the transcripts. A** Purification of a 19-mer substrate RNA Tr1 and its point mutants transcribed using ATP or NeTP. X denotes incorporation site of A or Ne in the wild type (wt) RNA Tr1. Arrow indicates the desired product. **B** Aldehyde functionalization of MTR1 + Bn$^6$G Ne-transcript Tr1 using Bio-hyd. (Bio), LY-CH (LY) or Flu-TSC (Flu). **C** LC-MS analysis of digested unreacted ATP and NeTP wt transcript Tr1. UV traces at 260 nm and EICs (*m/z* 253.09 ± 0.01; corresponding to Ne and *m/z* 268.10 ± 0.01 corresponding to A) are shown. **D** Deconvoluted HR-ESI-MS spectra of wt ATP and NeTP Tr1 transcripts, containing a single adenosine or nebularine, respectively. As expected, the Ne-Tr1 is lighter by 15 mass units. Δm = 22 denotes $Na^+$ adduct. **E** Aldehyde functionalization of MTR1 + Bn$^6$G treated Ne-Tr1 and its point mutants with Bio-hyd, LY-CH or Flu-TSC. Circles denote Ne incorporation sites (N; red) and the respective base-pairing partner in the ribozymes binding arm (blue). **F** Aldehyde functionalization of MTR1 + Bn$^6$G treated Ne-Tr2 (= G10A mutant) with Bio-hyd, LY-CH or Flu-TSC. The G to U point mutation in the ribozymes binding arm restored the labelling efficiency.

# SUPPORTING INFORMATION

## Experimental Procedures

### Materials and equipment

All standard chemicals and solvents including urea, acrylamide/bisacrylamide stock solution, buffer reagents and phenol/chloroform/isoamyl alcohol were purchased from commercial suppliers and used without further purification. Water used for all experiments was purified with a Milli-Q-unit of Millipore. Snake venom phosphodiesterase (SVPD) was purchased from Merck. Bacterial alkaline phosphatase (BAP) was from Invitrogen. RNase T1, T4 Polynucleotide kinase (PNK), Calf Intestinal Alkaline Phosphatase (CIAP) and Klenow exo$^-$ were from ThermoFisher Scientific. Lucifer Yellow-carbohydrazide, Fluorescein-thiosemicarbazide and Biotin-hydrazide were purchased from Sigma. 1-Ethyl-2,3,3-trimethylindolenium-5-sulfonate (**4**) was from Toronto Research Chemicals. Nebularine nucleoside was purchased from Carbosynth. $\gamma$-$^{32}$P-ATP was from Hartmann Analytic GmbH.

Unmodified DNA oligonucleotides were purchased from Microsynth and purified on denaturing PAGE (10-20% acrylamide) prior to use. Modified and unmodified short RNA oligonucleotides were synthesized in house using solid phase synthesis with standard phosphoramidite chemistry. Phosphoramidites for incorporation of unmodified and modified nucleosides were purchased from ChemGenes. NTPs for transcription were purchased from Jena biosciences. T7 RNA polymerase was prepared as previously described.[1]

Fluorescence gel images were recorded using a ChemiDoc MP with epi illumination using blue LEDs (emission filters 530/28) from BioRad. Gels containing radioactive oligonucleotides were exposed to phosphor storage screens and imaged using a Typhoon Phosphorimager from Cytiva. Fluorescent measurements were performed on a Jasco FP-8300 spectrofluorometer. UV-vis spectra were recorded on an Agilent Cary 3500 spectrophotometer with an Agilent Cary UV-Vis Multicell Peltier.

NMR spectra were recorded on a Bruker Avance III HD 400 spectrometer. Chemical shifts were measured relative to the solvent signal ($^1$H-NMR: 4.79 ppm for $D_2O$). Signal multiplicities are denoted as s (singlet), d (doublet), t (triplet) and m (multiplet). Processing of the raw data was performed with Topspin 3.5.

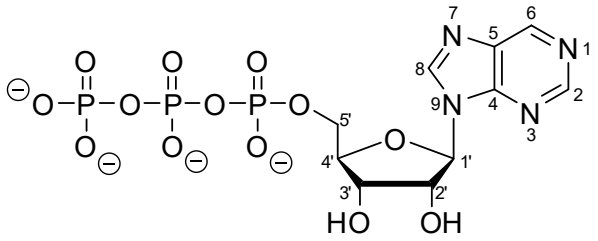
HR-ESI-MS spectra of the oligonucleotides were recorded on a Bruker micrOTOF-Q III spectrometer in negative ion mode upon direct injection of a 3 µM RNA solution in 44% ACN (v/v) containing 44 mM hexafluoroisopropanol (HFIP) and 3.8 mM triethylamine (TEA), using capillary voltage 3 kV, end plate voltage 2.5 kV, nitrogen nebulizer pressure 0.4 bar, dry gas flow 4 L/min, dry temperature 200 °C, and recorded in the range *m*/*z* 200 - 4000. Data evaluation and ion deconvolution were performed by Data analysis software DA 4.2 (Bruker Daltonics).

### 1. RNA synthesis

#### a. Solid phase synthesis

Short RNA oligonucleotides were prepared by solid-phase synthesis using phosphoramidite chemistry with 2′-*O*-TOM-protecting groups. The RNAs were deprotected with a 1:1 mixture of aqueous ammonia and methylamine (AMA) at 37 °C for 4–6 h, followed by 1 M tetrabutylammonium fluoride in THF at 25 °C for 12–16 h. The RNAs were desalted and purified by denaturing PAGE, extracted, and EtOH-precipitated. The RNA purity was confirmed by anion-exchange HPLC (Dionex DNAPac PA200, 2 × 250 mm at 60 °C; solvent A: 25 mM Tris-HCl (pH 8.0) and 6 M urea; solvent B: 25 mM Tris-HCl (pH 8.0), 6 M urea and 0.5 M $NaClO_4$; linear gradient: 0–40% solvent B, with a slope of 4% solvent B per column volume). The identity of the RNAs was confirmed by HR-ESI-MS (micrOTOF-Q III, negative ion mode, direct injection). The oligonucleotide sequences are summarized in Supplementary Table 1 together with their respective measured and calculated molecular weights and anion-exchange HPLC chromatograms.

### b. Synthesis of Nebularine-triphosphate (NeTP)



#### i. Bis(tributylammonium)pyrophosphate

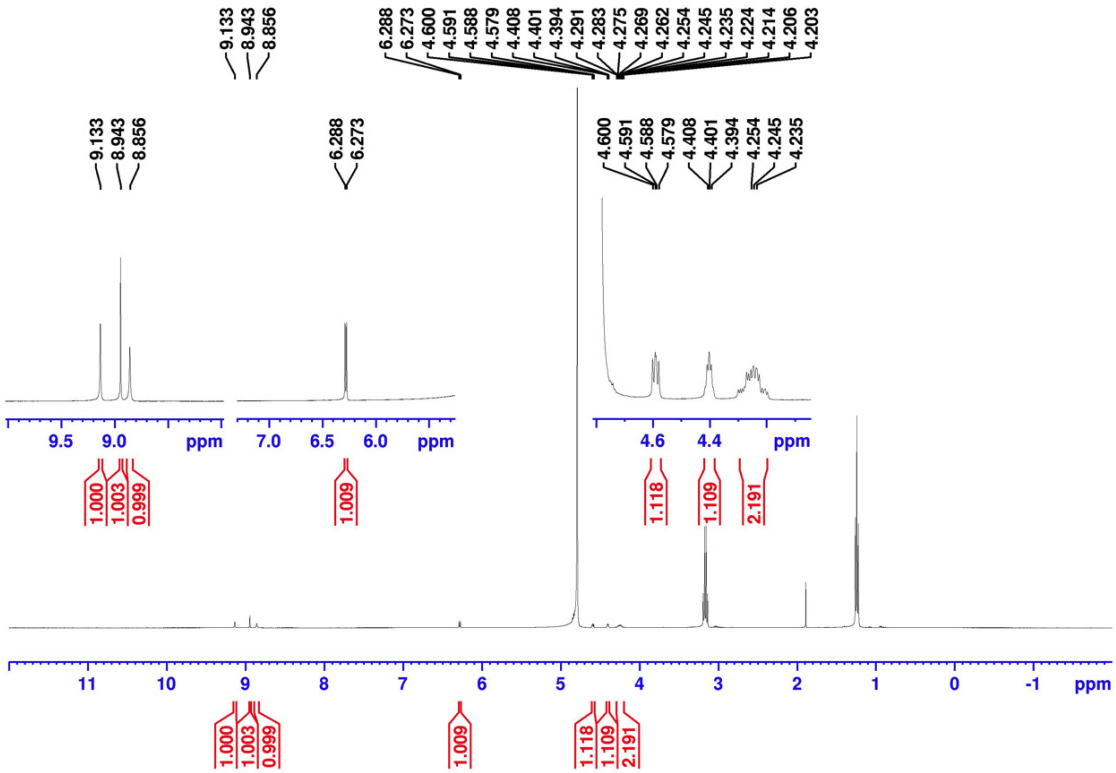
Ion exchange resin Dowex ($H^+$) (15 g) was filled in a column and washed multiple times with methanol until the eluate was colorless followed by washing with nanopure water. Tetrasodium pyrophosphate·10 $H_2O$ (1.12 g, 2.51 mmol, 1.0 eq.) was dissolved in 10 mL nanopure water, added to the resin, and incubated at ambient temperature for 10 min. The pyrophosphate was eluted with nanopure water until a pH of 7.0 was reached. The solution was then adjusted to pH 3.5 using 40 % aq. Tetrabutylammonium hydroxide. The clear liquid was then lyophilized to yield a colorless solid.

#### ii. Nebularine-5'-triphospate (NeTP) sodium salt

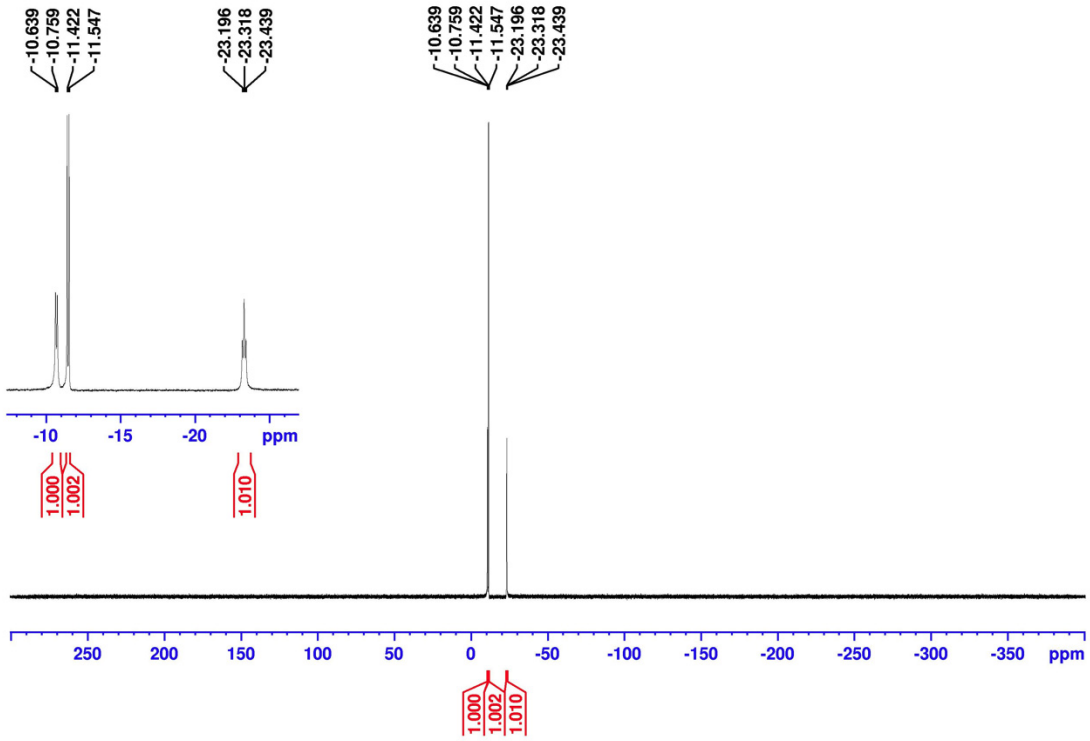
Synthesis of nebularine-5'-triphosphate (NeTP) was previously reported, and here performed following a general established procedure for nucleoside triphosphates.[2] Nebularine (25 mg, 0.10 mmol) and proton sponge (28.4 mg, 0.13 mmol, 1.3 eq.) were dissolved in trimethyl phosphate (1.5 mL). After cooling to -20 °C, freshly distilled $POCl_3$ (14 µL, 0.15 mmol, 1.5 eq.) was added and the reaction mixture was stirred at -15 °C to -20 °C for 3h. A solution of bis(tributylammonium)pyrophosphate in dry DMF (1 M, 0.60 mL) and tri-*n*-butylamine (0.15 mL, 0.63 mmol, 6.3 eq.) were added. After stirring for 1.5 h at -15 °C to -20 °C, the reaction was quenched by the addition of triethylammonium bicarbonate (TEAB) buffer (1M, pH 7.5, 12 mL), warmed to room temperature and washed three times with ethyl acetate. The aqueous layer was lyophilized, and the crude product was purified using anion-exchange chromatography (DEAE Sephadex A-25 column, solvent A: 0.1 M TEAB, pH 7.5; solvent B: 1 M TEAB, pH 7.5; linear gradient: 0–100 % solvent B in 40 min)

The product-containing fraction was lyophilized and further purified by reversed-phase HPLC using triethylammonium acetate (TEAA) buffer (Nucleosil, 8x250 mm, C18 column at 30 °C; solvent A: 100 mM TEAA (pH = 7.0); solvent B: 100 mM TEAA (pH 7.0) in 90% ACN; linear gradient: 0.1-10 % solvent B in 5 CV, 2 mL/min).

The obtained triethylammonium salt was converted to the sodium salt by precipitation from 0.3 ml NaOAc (3M) and 0.9 mL ice cold abs. EtOH. After 30 min centrifugation at 4 °C, the product was obtained as a white solid, dissolved in nanopure water and the concentration was determined with UV absorption spectroscopy using $\varepsilon^{245}$ = 7000 $M^{-1}cm^{-1}$.[3] The yield was 7.0 µmol (7%).




**$^{1}$H** NMR (400 MHz, $D_2O$) δ(ppm) = 9.13 (s, 1H, 6-H), 8.94 (s, 1H, 2-H), 8.86 (s, 1H, 8-H), 6.28 (d, *J* = 5.9 Hz, 1H, 1'-H), 4.59 (dd, *J* = 5.1, 3.5 Hz, 1H, 3'-H), 4.40 (p, *J* = 2.9 Hz, 1H, 4'-H), 4.25 (qdd, *J* = 11.7, 5.3, 3.0 Hz, 2H, 5'-H).




**$^{31}$P** NMR (162 MHz, $D_2O$) δ(ppm) = -10.70 (d, *J* = 19.7 Hz), -11.48 (d, *J* = 20.2 Hz), -23.32 (t, *J* = 20.0 Hz).

### c. *In vitro* transcription and enzymatic incorporation of nebularine

MTR1 ribozymes with varying binding arms, short unmodified und Ne containing substrate RNAs as well as the tRNA were prepared by *in vitro* transcription with T7 RNA polymerase from synthetic DNA templates (Microsynth), as described previously.[4] The reactions contained 1 µM DNA template, 4 mM NTPs, 30 mM $MgCl_2$, 2 mM spermidine and 10 mM DTT. The template for tRNA transcription was assembled from two ssDNA oligonucleotides using Klenow exo$^-$. For Ne incorporation, ATP was substituted by an equal amount of NeTP. The sequences of the transcripts are summarized in Supplementary Table 2.

### d. 5'-$^{32}$P-labelling of oligonucleotides

5'-Radiolabeling reactions were performed by incubation of 100 pmol RNA in 10 µL 1x PNK buffer (50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine) in the presence of 5 µCi $\gamma$-$^{32}$P-ATP and 5 U T4 PNK for 1h at 37 °C. The reaction was quenched by addition of loading dye followed by PAGE-purification.

*In vitro* transcripts were dephosphorylated beforehand by incubation of 100 pmol RNA transcript in 10 µL 1x CIAP buffer (50 mM Tris-HCl (pH 8.5), 0.1 mM EDTA) and 1 U CIAP for 1 h at 37 °C followed by PCI-extraction and EtOH-precipitation.

## 2. Analytical scale aldehyde generation and functionalization

### a. Generation of the aminoimidazole aldehyde (3)

Generation of the aldehyde nucleoside **3** for further functionalization was performed using 10 pmol 5′-$^{32}$P-labelled Ne-containing RNA and 100 pmol MTR1 ribozyme, that were folded (3 min at 95 °C then 10 min at 25 °C) in reaction buffer (120 mM KCl, 5 mM NaCl and 50 mM MES (pH 6.0)). 100 µM substrate Bn$^6$G and 40 mM $MgCl_2$ were added to a final concentration of 10 µL. After overnight incubation at 25 °C, the reaction was EtOH-precipitated and 1/8 of the reaction (1.25 pmol modified RNA substrate with 12.5 pmol MTR1) was submitted to the aldehyde functionalization reaction.

### b. Attachment of aldehyde reactive probes

To attach different probes, the MTR1-reacted RNA was mixed with 25 mM *N,N*-dimethylethylenediamine (*N,N*-DMED, adjusted to pH 7.5 with HCl) and 500 µM reagent (Fluorescein-thiosemicarbazide, Biotin-hydrazide or Lucifer Yellow-carbohydrazide), followed by 37 °C incubation for 3 h.

### c. Synthesis of chromophore 5 on the RNA

Generation of the chromophore **5** within RNAwas performed by mixing the MTR1-treated RNA with 25 mM Tris (pH 8.5), 100 mM KCl and 75 mM of commercially available 1-ethyl-2,3,3-trimethyliodoleninium-5-sulfonate (**4**, dissolved in 0.1 M NaOH). The reaction mixture was incubated at 45°C for 2h.

For both functionalization reactions, aliquots of 1µL were taken at timepoint 0 and after completion of the reaction and quenched by immediate addition of loading dye (4 µl) and freezing in liquid nitrogen. Half of each time-point sample was resolved on denaturing PAGE (20 %) and the band intensities were quantified using phosphorimaging.

### d. Conversion of chromophore 5a to isomer 5b

125 pmol PAGE purified RNA modified with Indole **4** were incubated in 15 µL 25 mM Tris (pH 8.0) at 37 °C or 50 °C for 4 h or 23 h followed by immediate analysis using anion-exchange HPLC.

### 3. One-pot aldehyde generation and functionalization reactions

For one-pot modification reactions, 5 pmol of 5′-$^{32}$P-labelled Ne-containing RNA and 50 pmol of the respective MTR1 ribozyme were annealed (3 min at 95 °C then 10 min at 25 °C). 100 µM Bn$^6$G and 40 mM $MgCl_2$ were added. For fluorescein modification, the sample was mixed with 25 mM *N,N*-DMED (pH 6.0/7.5) and 500 µM Flu-TSC. For generation of chromophore **5**, 25 mM MES (pH 6.0) or Tris (pH 7.5), 100 mM KCl and 75 mM **4** were used instead. The reactions had a final volume of 5 µL and were incubated at 25 °C or 37 °C.

1 µL aliquots were taken at timepoint 0 and after overnight incubation and quenched by immediate addition of loading dye (4 µL). Half of each time-point sample was resolved on denaturing PAGE (20 %) and the band intensities were quantified using phosphorimaging.

### 4. Preparative scale generation of the aldehyde (3)

For generation of the aldehyde **3** in RNA, 1 nmol Ne-containing oligonucleotides were mixed with 1.2 nmol of the respective MTR1 ribozyme in reaction buffer (120 mM KCl, 5 mM NaCl and 50 mM MES (pH 6.0)). After annealing (3 min at 95 °C then 10 min at 25 °C) 100 µM substrate Bn$^6$G and 40 mM $MgCl_2$ were added to a final reaction volume of 20 µL. After overnight incubation at 25 °C the RNA was EtOH-precipitated and separated from the ribozyme and *N*1-modified RNA on denaturing PAGE. The isolated RNA was then submitted for ESI-MS measurements. The calculated and observed masses are summarized in Supplementary Table 3.

### 5. Two-step preparative scale generation of chromophore 5

Generation of the aldehyde **3** in RNA was performed by mixing 3 nmol Ne-containing RNA and 3.4 nmol of the respective MTR1 ribozyme in reaction buffer (120 mM KCl, 5 mM NaCl and 50 mM MES (pH 6.0)). After annealing (3 min at 95 °C then 10 min at 25 °C) 100 µM substrate Bn$^6$G and 40 mM $MgCl_2$ were added to a final reaction volume of 60 µL. After overnight incubation at 25 °C the RNA was EtOH-precipitated and dissolved in 25 mM Tris (pH 8.5), 100 mM KCl and 75 mM **4** (in 0.1 M NaOH) to a final reaction volume of 40 µL. The reaction was incubated at 45 °C for 2h followed by EtOH-precipitation and PAGE purification. The isolated RNA was submitted for ESI-MS measurements. The calculated and observed masses are summarized in Supplementary Table 3.

### 6. Analysis of modified RNA using alkaline hydrolysis and RNase T1 digestion

RNase T1 digestion was performed by incubating 90 IPS 5'-$^{32}$P-RNA at 37 °C for 30 sec in 5 µL 50 mM Tris-HCl (pH 7.5) using 5U RNase T1. For alkaline hydrolysis, 300 IPS $^{32}$P-RNA were incubated in 10 µL NaOH (25 mM) at 95 °C for 5 min. The reactions were quenched by immediate addition of loading dye and placing on ice.

### 7. Chromatographic analysis of modified RNA

#### a. High-performance liquid chromatography

90 pmol substrate RNA were annealed with 100 pmol of the respective MTR1 ribozyme (3 min at 95 °C the 10 min at 25 °C) in reaction buffer (120mM KCl, 5mM NaCl, 50mM MES, pH 6.0). 100 µM substrate Bn$^6$G and 40 mM $MgCl_2$ were added to a final volume of 10 µL and the reaction was incubated overnight at 25 °C. The sample was analyzed by anion-exchange HPLC on a Dionex DNAPac PA200 column, 2 × 250 mm, at 60 °C with UV detection at 260 nm. Solvent A was 25 mM Tris-HCl (pH 8.0) and 6 M urea, and solvent B was 25 mM Tris-HCl (pH 8.0), 6 M urea and 0.5 M $NaClO_4$, with a linear gradient of 0–48% solvent B in 12 column volumes.

For anion exchange HPLC analysis of the generation of chromophore **5**, the sample was prepared according to section 5 and 100 pmol of the PAGE purified RNA were injected.

### b. Digestion and LC-MS analysis

For LC-MS analysis, 450 pmol of the RNA were digested at 37 °C for 20 h using 12 U bacterial alkaline phosphatase (BAP) and 1 U snake venom phosphodiesterase (SVPD) in 50 µL 40 mM Tris-HCl (pH 7.5) containing 20 mM $MgCl_2$. The sample was then brought to 100 µL with $H_2O$ and extracted twice using an equal volume of chloroform. The aqueous layer was lyophilized and analyzed by LC-MS using an RP-18 column (Synergi, 4-µm Fusion-RP C18 80 Å, 250 × 2 mm; Phenomenex) at 25 °C with aqueous mobile phase A (5 mM $NH_4OAc$, pH 5.3) and organic mobile phase B (100 % ACN). The flow rate was 0.2 ml/min with a gradient of 0–5 % B in 15 min, followed by 5–70 % B in 30 min. The micrOTOF-Q III with an ESI ion source was operated in positive-ion mode, with capillary voltage of 4.5 kV, end plate offset of 500 V, nitrogen nebulizer pressure 1.4 bar, dry gas flow 9 l/min and dry temperature 200 °C. The data were analyzed with Data Analysis software DA 4.2 (Bruker Daltonics).

## 8. Spectroscopic analysis of modified RNA

### a. UV-vis spectroscopy

UV-vis measurements were conducted in a total volume of 100 µL $H_2O$ containing 500 pmol RNA modified with **4** that was freshly prepared in a two-step preparative scale reaction as described in section 4. The spectra were measured in Hellma Quartz Suprasil cuvettes (10 mm path length) at ambient temperature using the following conditions: 200 - 550 nm measurement range, 2 nm spectral bandwidth, 3000 nm/min scan speed, 1 nm data interval, 20 ms averaging time.

### b. Fluorescence emission spectra

Fluorescence emission spectra were recorded using 30 pmol RNA in 12 µL 10 mM Tris (pH 7.5). The RNA was freshly modified with **4** in a two-step preparative scale reaction as described in 4. For measurement with the complementary RNAs, 30 pmol of the RNA modified with 4 were annealed to 30 pmol of its complement (3 min at 95 °C then 10 min at 25 °C). Measurements were performed in Hellma Quartz Suprasil cuvettes (1.5 x 1.5 mm) at 25 °C using the following conditions: 500 - 700 nm measurement range, excitation at 486 nm, 5 nm ex/em bandwidth, 50 ms response, 0.2 nm data interval, 1000 nm/min scan speed, 680 V PMT voltage. The spectra were manually background corrected by subtraction of a spectrum of the respective untreated RNA in pure buffer.

# SUPPORTING INFORMATION

## Supplementary Tables

**Table S1:** RNA oligonucleotides prepared by solid phase synthesis together with calculated and measured masses using HR-ESI-MS. Red denotes the targeted modification site.

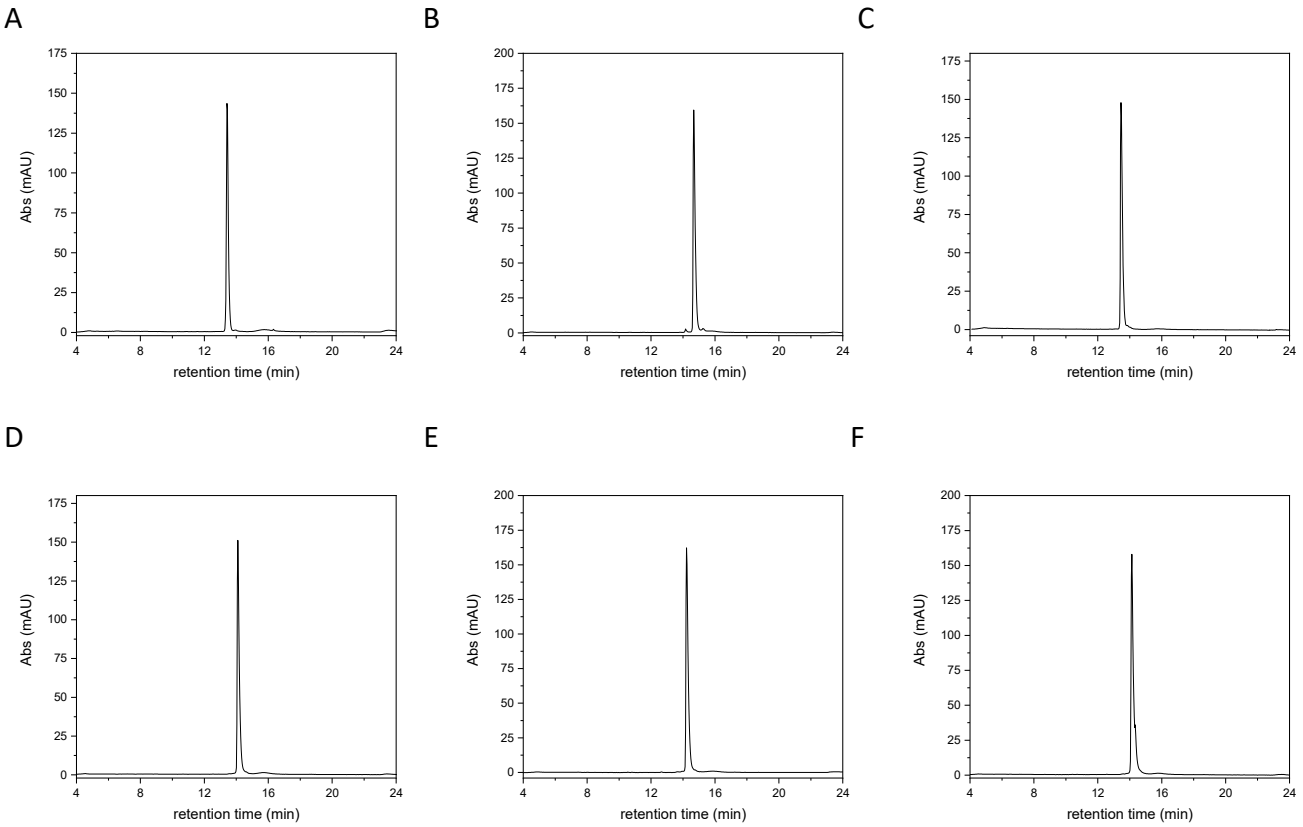
| description | 5'-sequence-3' | Mass (calculated) | Mass (found) |
|---|---|---|---|
| R1_un | CCACUGAGAGCUUC | 4403.62 | 4403.63 |
| R2_un | GGAUAAUACGACUCAC | 5085.74 | 5085.75 |
| R1_Ne | CCACUGNeGAGCUUC | 4388.62 | 4388.63 |
| R2_Ne | GGAUAAUACGNeCUCAC | 5070.73 | 5070.72 |
| R2_DAP | GGAUAAUACGDAPCUCAC | 5100.75 | 5100.74 |
| R2_2AP | GGAUAAUACG2APCUCAC | 5085.74 | 5085.73 |

**Table 2:** RNA oligonucleotides prepared by in vitro transcription. Red denotes the targeted modification site, blue denotes incorporation of A or Ne, green denotes nucleotides placed opposite chromophore **5**. Ribozyme binding arms are underlined.

| description | 5'-sequence-3' |
|---|---|
| Tr1 | GGGUGCGUCGAGUUCCUGG |
| Tr1_G5A | GGGUACGUCGAGUUCCUGG |
| Tr1_C16A | GGGUGCGUCGAGUUCAUGG |
| Tr1_G10A = Tr2 | GGGUGCGUCAAGUUCCUGG |
| Tr1_G5A+C16A | GGGUACGUCGAGUUCAUGG |
| MTR1_R1 | GGAAGCUCUGACCGACCCCCCGAGUUCGCUCGGGGACAACUAGACAUACAGUGG |
| MTR1_R2 | GGUGAGUGACCGACCCCCCGAGUUCGCUCGGGGACAACUAGACAUACGUAUUAUC |
| MTR1_Tr1 | GGCCAGGAACUGACCGACCCCCCGAGUUCGCUCGGGGACAACUAGACAUACGACGCACCC |
| MTR1_Tr1_G5A+C16A | GGCCAUGAACUGACCGACCCCCCGAGUUCGCUCGGGGACAACUAGACATACGACGUACCC |
| MTR1_Tr1_G10A | GGCCAGGAACUGACCGACCCCCCGAGUUCGCUCGGGGACAACUAGACAUAUGACGCACCC |
| U-complement of R1 | GGAAGCUCUCAGUGG |
| A-complement of R1 | GGAAGCUCACAGUGG |
| C-complement of R1 | GGAAGCUCCCAGUGG |
| G-complement of R1 | GGAAGCUCGCAGUGG |
| *T.thermophilus* tRNA-Asp A58 | GGCCCCGUGGUGUAGUUGGUUAACACACCCGCCUGUCACGUGGGAGAUCGCGGGUUCGAGUC CCGUCGGGGCCGCCA |
| MTR1 *T.thermophilus* tRNA-Asp | GGACGGGACUGACCGACCCCCCGAGUUCGCUCGGGGACAACUAGACAUACGAACCC |

**Table S3:** Isolated RNA modification products together with calculated and measured masses. Red denotes the modification (Ald = 5-aminoimidazole-4-carbaldehyde; Hcy = hemicyanine).

| description | 5'-sequence-3' | Mass (calculated) | Mass (found) |
|---|---|---|---|
| R1_Ne_Ald | CCACUGAldGAGCUUC | 4379.62 | 4379.60 |
| R1_Ne_Hcy | CCACUGHcyGAGCUUC | 4628.70 | 4628.71 |




Anion exchange HPLC chromatograms to confirm purity of RNA oligonucleotides prepared by solid-phase synthesis. (A) R1_un, (B) R2_un, (C) R1_Ne, (D) R2_Ne, (E) R2_DAP, (F) R2_2AP. Dionex DNAPAc PA200, 2x250 mm, 60°C, monitored at 260 nm.

## References

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