Supporting Information

Identification of Adenosine-to-Inosine RNA Editing with Acrylonitrile Reagents

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1. Synthesis

**General Experimental Information**

Commercial reagents were used as received, and all other reagents were prepared using known literature procedures. Solvents were used as purchased (Fischer Scientific, ACS grade). Reactions were monitored by thin-layer chromatography (TLC) on “TLC Silica gel 60 F254” plates (Merck) and visualized by UV light (254 nm) or by staining with aqueous basic KMnO4. Flash chromatography was performed on silica gel 60 (Geduran® Si 60, 40–63 µm, 230–400 mesh, Merck).

NMR spectroscopic data were recorded on a Bruker Avance III 600 MHz spectrometer utilizing a BBFO (broadband, including fluorine, observe) cryoprobe. Chemical shifts are reported in parts per million (ppm) using the residual non-deuterated solvent resonance for proton measurements and the  as an internal standard (CDCl3: 1H = 7.26 ppm and 13C = 77.16 ppm; CD3OD: 1H = 3.31 ppm and 13C = 49.00 ppm; CD3CN: 1H = 1.94 ppm and 13C = 118.27 ppm). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet, br = broad), coupling constant(s) in Hz, integration.

High resolution mass spectra (HRMS) were recorded on a Waters LCT Premier spectrometer using ESI-TOF (electrospray ionization-time-of-flight) and data are reported in the form of (m/z).

**Preparation of triethylammonium acetate buffer (2 M, Et3N·4AcOH)**

A solution of AcOH (45.80 mL, 800.0 mmol) in H2O (15 mL) was chilled in an ice bath and Et3N (27.90 mL, 200.0 mmol) was added slowly over the course of 10 min in small portions. After warming the mixture to ambient temperature it was diluted with H2O to a total volume of 100 mL.

**Experimental Procedures and Characterization Data for Compounds**

**Preparation of 2-azido-N-methyl-ethanamine hydrobromide (S1)**

The starting material was prepared according to the detailed protocol of Schutte, Weakley, and Tyler1, and the title compound was prepared according to a modified procedure of Carboni, Benalil, and Vaultier.2

\[
\begin{align*}
\text{BrNH} \cdot \text{HBr} & \xrightarrow{\text{NaN}_3, \text{H}_2\text{O}, 80 \, ^\circ\text{C}, 12 \, \text{h}} \text{N}_3\text{NH} \cdot \text{HBr} \\
& \text{54%}
\end{align*}
\]

To a solution of 2-bromo-N-methylethanamine hydrobromide (50.0 g, 228.4 mmol, 1.00 equiv) in H2O (160 mL) was added NaN3 (19.5 g, 300.3 mmol, 1.09 equiv), and the solution was heated to 80 °C for 12 h. The reaction mixture was concentrated at 65 °C (10 mbar) to yield a brown viscous residue. The last traces of H2O were removed by azeotropic concentration from iPrOH (2 × 75 mL). The resulting highly viscous, dark brown residue was treated with iPrOH (250 mL) and heated to 60 °C and then cooled to ambient temperature to precipitate most of the inorganic salts, which were removed by filtration over a fine porous frit. The filter cake was washed with iPrOH (2 × 50 mL). After concentration of the dark brown filtrate to about 150 mL, charcoal (20 g) and acetone (250 mL) were added, which caused further precipitation of minor amounts of inorganic salts. After stirring the suspension for 10 min it was filtered through a frit resulting in a red filtrate. This was concentrated to a highly viscous residue and acetone was added (200 mL). The last traces of inorganic salts were removed via filtration through a cotton plug. To ensure complete transfer, the flask and the filtration setup were washed with acetone (2 × 12.5 mL). The red solution was chilled in the freezer at −21 °C for 24 h. Filtration, washing with ice cold acetone (2 × 30 mL), and drying at 0.1 mbar for 12 h yielded 2-azido-N-methyl-
ethanamine hydrobromide (S1) as slightly orange, hydroscopic plates (22.59 g, 124.8 mmol, 54%).

\(^1\)H NMR (600 MHz, CD\(_3\)CN, 298 K): 6 = 8.63 (brs, 2 H), 3.83 (t, J = 6.0 Hz, 2 H), 3.14 (t, J =6.0 Hz, 2 H) 2.64 (s, 3 H) ppm.

\(^{13}\)C NMR (150 MHz, CD\(_3\)CN, 298 K): 6 = 48.5, 47.6, 33.7 ppm.

ESI-HRMS: calc. for C\(_6\)H\(_{18}\)BrN\(_8\) [2M + HBr]: m/z = 281.0838, found m/z =281.0831.

Preparation of 2-((2-azidoethyl)(methyl)amino)methylacrylonitrile (Atol\(_N_3\), 1)

A solution of acetic acid (4.24 mL, 74.2 mmol, 2.97 equiv) in water (6 mL) was chilled in an ice bath and Et\(_3\)N (5.14 mL, 37.08 mmol, 1.48 equiv) was added. After addition of 2-azido-N-methylethanamine hydrobromide (4.662 g, 25.75 mmol, 1.03 equiv) or 2-azido-N-methylethanamine (2.578 g, 25.75 mmol, 54%), the pH of the solution was adjusted to pH = 4.5 with acetic acid and the solution was allowed to warm to room temperature. Then cyanoacetic acid (2.127 g, 25.0 mmol, 1.00 equiv) was added followed by addition of formalin (3.91 mL, 37% in H\(_2\)O, 52.3 mmol, 2.10 equiv). The reaction vessel was capped with a bubbler and stirred for 2 h until gas evolution ceased. Then, the reaction mixture was then chilled in an ice bath, layered with Et\(_2\)O (75 mL) and basified with 3 M KOH (60 mL, 180 mmol). After separation of the phases the aqueous phase was extracted with Et\(_2\)O (2 × 75 mL). The combined organic phases were washed with brine and dried over MgSO\(_4\). After careful removal of the solvent the crude product was purified by column chromatography (17.5% EtOAc/hexanes) to obtain the acrylonitrile (Atol\(_N_3\), 1) as colorless oil (2.941 g, 17.8 mmol, 71%).

\(_R_f = 0.19\) (17.5% EtOAc/hexanes).

\(^1\)H NMR (600 MHz, CDCl\(_3\), 298 K): 6 = 5.94 (s, 1 H), 5.89 (s, 1 H), 3.27 (t, J = 6.0 Hz, 2 H), 3.15 (s, 2 H), 2.60 (t, J =6.0 Hz, 2H) 2.25 (s, 3 H) ppm.

\(^{13}\)C NMR (150 MHz, CDCl\(_3\), 298 K): 6 = 132.1, 120.8, 117.9, 60.2, 55.6, 48.6, 41.3 ppm.

ESI-HRMS: calc. for C\(_7\)H\(_{11}\)N\(_5\)Na [M + Na]: m/z = 188.0912, found m/z =188.0911.

Preparation of N-methyl-propargylamine hydrochloride (S2)

N-Boc-N-methyl-propargylamine (680 mg, 4.02 mmol) was dissolved in CH\(_2\)Cl\(_2\) (5 mL) and a solution of HCl in 1,4-dioxane (3 mL, 4 M in dioxane) was added. The reaction mixture was stirred for 24 h or until the starting material was completely consumed (TLC, 10% EtOAc/hexanes, \(_R_f = 0.32\)). The reaction mixture was concentrated to about 2 mL, CHCl\(_3\) (10 mL) was added and then all volatiles were completely removed. After drying for 1 h at 0.1 mbar the hydrochloride salt (S2) was obtained as colorless plates (424 mg, 4.02 mmol, quant.).

\(^1\)H NMR (600 MHz, MeOD, 298 K): 6 = 3.93 (d, J = 2.3 Hz, 2 H), 3.23 (t, J = 2.3 Hz, 1 H), 2.77 (s, 3 H) ppm.

\(^{13}\)C NMR (150 MHz, MeOD, 298 K): 6 = 79.3, 74.4, 38.7, 32.6 ppm.

ESI-HRMS: calc. for C\(_4\)H\(_8\)N [M + H]: m/z = 70.0657, found m/z =70.0656.
Preparation of 2-((methyl(prop-2-ynyl)amino)methyl)acrylonitrile (Atol_yne, 2)

\[
\text{\begin{align*}
\text{\text{N-Methyl-propargylamine hydrochloride (218 mg, 2.07 mmol, 1.03 equiv) or N-methyl-propargylamine (143 mg, 2.07 mmol, 1.03 equiv) was dissolved in H}_2\text{O (2 mL) and triethylammonium acetate buffer (1.24 mL, 2 M, Et}_3\text{N-4AcOH in H}_2\text{O, 2.49 mmol, 1.24 equiv) was added. If necessary the pH was adjusted to pH = 4.5 by addition of either AcOH or Et}_3\text{N. Then cyanoacetic acid (171 mg, 2.01 mmol, 1.00 equiv) was added followed by addition of formalin (315 µL, 37% in H}_2\text{O, 4.23 mmol, 2.10 equiv). The reaction vessel was capped with a bubbler and stirred for 2 h until gas evolution ceased. Then, the reaction mixture was chilled in an ice bath, layered with Et}_2\text{O (25 mL) and basified with 1 N KOH (13 mL, 13 mmol). After separation of the phases the aqueous phase was extracted with Et}_2\text{O (2 × 20 mL). The combined organic phases were washed with brine and dried over MgSO}_4. After careful removal of the solvent, the crude product was purified by column chromatography (SiO}_2, 15% - 20% Et}_2\text{O/pentane) to obtain the acrylonitrile Atol-ynes (2) as colorless oil (146 mg, 1.09 mmol, 54%).}
\end{align*}}
\]

\[R_f = 0.21 \text{ (17.5\% Et}_2\text{O/pentane).}\]

\[\text{^1H NMR (600 MHz, CDCl}_3, 298 K): \delta = 5.93 \text{ (s, 1 H), 5.88 (s, 1 H), 3.32 (d, } J = 2.0 \text{ Hz, 2 H), 3.13 \text{ (s, 2 H), 2.26 (s, 3 H), 2.22 (t, } J = 2.0 \text{ Hz, 1 H) ppm.}\]

ESI-HRMS: calc. for C}_8\text{H}_11\text{N}_2\text{ [M + H]}^+: m/z = 135.0922, found m/z = 135.0924.

Preparation of biotin probe (Atol_biotin, 3)

\[\text{A bright green solution of CuSO}_4\cdot 5\text{H}_2\text{O (21.6 mg, 86.5 µmol, 0.20 equiv) in DMSO (600 µL) was treated with DABCO (19.4 mg, 173 µmol, 0.40 equiv) and sodium ascorbate (34.3 mg, 173 µmol, 0.40 equiv), and stirred for 15 min. To the now dark greenish solution was added AcOH (9.9 µL, 173 µmol, 0.40 equiv) followed by N-(prop-2-ynyl)biotinamide (121.6 mg, 432.6 µmol, 1.00 equiv). After stirring for 5 min, azide Atol}_N}_3 \text{ (1, 75.7 mg, 458.5 µmol, 1.06 equiv) was added to the now brownish reaction mixture and it was stirred for further 14 h. The reaction mixture was then directly transferred to a flash column with the aid of DMSO washes (2 × 70 µL) and purified via elution with MeOH/CHCl}_3 \text{ (SiO}_2, 5\% -> 12.5\%). Removal of all volatiles furnished the title compound as off-white foam (60.2 mg, 135 µmol, 31%).}
\]

\[R_f = 0.20 \text{ (10\% MeOH/ CHCl}_3)\].

\[\text{^1H NMR (600 MHz, MeOD, 298 K): } \delta = 8.21 \text{ (t, } J = 4.9 \text{ Hz, 1 H), 7.92 (s, 1 H), 7.90 (s, 2 H), 5.99 (s, 1 H), 5.91 (s, 1 H), 4.50 (m, 3 H), 4.43 (m, 2 H), 4.30 (dd, } J = 7.7, 4.4 \text{ Hz, 1 H), 3.20 (m, 3 H), 2.94 (dd, } J = 12.7, 4.8 \text{ Hz, 1 H), 2.89 (t, } J = 5.8 \text{ Hz, 2 H), 2.71 (d, } J = 12.7 \text{ Hz, 1 H), 2.31 (s, 3 H), 2.23 (t, } J = 7.3 \text{ Hz, 2 H), 1.65 (m, 4 H), 1.43 (m, 2 H) ppm.}\]

\[\text{^13C NMR (150 MHz, MeOD, 298 K): } \delta = 175.8, 166.0, 145.8, 133.7, 125.1, 122.6, 119.2, 79.5, 63.3, 61.6, 61.4, 57.3, 56.9, 41.6, 41.1, 36.6, 35.6, 29.7, 29.4, 26.6 ppm.}\]

ESI-HRMS: calc. for C}_20\text{H}_30\text{N}_8\text{NaO}_2\text{S [M + Na]}^+: m/z = 469.2110, found m/z = 469.2122.
NMR spectra of compounds

file: ...
ame/nmr/194/194NaNH_aizel/1/fid excp: <exp30>
transmitter freq.: 600.13401 Hz
RAM width: 39460 points
width: 9615.38 Hz = 16.0255000000 ppm = 0.0505000000 Hz/pt
number of scans: 1

file: ...
ame/nmr/194/194NaNH_aizel/3/fid excp: <exp30>
transmitter freq.: 150.91940 Hz
RAM width: 65536 points
width: 36231.88 Hz = 240.0744 ppm = 0.552855 Hz/pt
number of scans: 32
2. Biological Studies

General biological methods
Buffer salts and NTPs were purchased from commercial sources. All chemical reagents were purchased from commercial sources. Superscript III was purchased from Life technologies. 32P was purchased from PerkinElmer. Gels were imaged on the Typhoon Imager GE Healthcare. Optokinase was purchased from Affymetrix. TGIRTIII reverse transcriptase was purchased from Ingex and HIV reverse transcriptase was ordered as SunscriptTM from Expedeon.

HPLC conditions
Different nucleic analogs were prepared as stocks at 1 M. 2 μl of the analog were incubated with 17.5 μl CE buffer and 0.5 μl of Atol_N3 (pure, liquid, ~5 M) at 70°C for 30 min. 10 μl of the reaction mixture was diluted into 1 mL for HPLC analysis. The elution condition was 0-1 min (5% B solvent), 1-7 min (5-35% B solvent), 7-8 min (35-95% B solvent), 8-9 min (95% B solvent), 9-10 min (95-5% B solvent), 10-11 min (5% B solvent). B solvent is acetonitrile and A solvent is 0.01% TFA in water.

Oligo RNA modification
Commercially available oligo RNA was resuspended in 10 mM Tris and 100 μM EDTA buffer as 1 mM solution. 2 μl oligo RNA was mixed with 7.5 μl CE buffer and incubated with 0.5 μL of Atol_N3 (pure, liquid, ~5 M). The mixture was incubated at 70 °C for 30 min and added 10 vol% NaCl (5 M), 100 vol% isopropanol and 2 μl Glycogen (20 μg/μl). The samples were kept at −80 °C for at least 30 min before being centrifuged at 4 °C for 30 min at max speed.

CuAAC
Click reactions were prepared using 10 μg of RNA, 1 mM Atol_yne, and fresh 4.6 mM THPTA to a final concentration of 1 mM, fresh 10.6 mM NaAsc to a final concentration of 1.77 mM, and 12 mM CuSO4 to a final concentration of 200 μM. The reactions were incubated with shaking at room temperature for 30 min. The reaction can be scaled up and down accordingly. The modified RNA were purified using Zymo RNA clean and concentrator-5 spin column according to the manufacturers instructions, with RNA eluted in 7 μL of nuclease free water.

SPAAC
10 μg RNA was dissolved in 9 μL nuclease free water, 1 μL of DBCO biotin was added and the mixture as incubated at 37°C for 45 min. The modified RNA was purified using Zymo RNA clean and concentrator-5 spin column according to the manufacturers instructions, with RNA eluted in 7 μL of nuclease free water.

HRP-streptavidin dot blotting
Equal amounts of purified RNA or DNA were loaded onto Hybond-N+ membrane (GE Healthcare) and UV-crosslinked to the membrane (Stratalinker UV crosslinker). The membrane was blocked followed by incubation with high sensitivity streptavidin-HRP (Thermo Fisher Scientific), washed twice in a 1:10 solution of blocking buffer for 20 min each and twice in Tris-saline buffer for 5 min each. It was then incubated in SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and imaged on a ChemiDoc MP imaging system (Bio-Rad). The recovered membrane was incubated with
methylene blue stain (0.04% w/v, 0.3 M NaOAc) for 15 min and washed with deionized water several times for loading control.

### 32P end labeling for reverse transcription

200 pmol of primer DNA was phosphorylated according to the manufacturer’s conditions by Affymetrix. The reaction was allowed to proceed for 2 h at 37 °C. Reactions were stopped by the addition of equal amounts of Gel Loading Buffer II (Ambion, Inc.). The reactions were loaded onto a 15% denaturing PAGE gel. The band of interest was visualized by a phosphorimager (Typhoon, GE healthcare). The resulting band was excised and eluted overnight in 300 mM KCl. Resulting solution was EtOH precipitated and dissolved to 8,000 counts per minute (c.p.m.)/μL for further use in reverse transcription.

Primers and RNAs used for Reverse transcription:
- reverse primer: AACTATAGTGTCACCTAAAT
- oligo 1 (inosine): UCUCUCCUCACUAUAGUGUCACCUAAAU
- oligo 2 (adenosine): UCACUCUCACUAUAGUGUCACCUAAAU

### Reverse transcription (RT)

32P-end-labeled DNA primers were annealed to modified oligo RNA by incubating 95 °C for 2 min, then at 25 °C for 2 min, and finally 4 °C for 2 min. To the reaction, first strand buffer, DTT, and dNTP’s were added. The reaction was pre-incubated at 52 °C for 1 min, then superscript III (2 units/μL final concentration) was added. Extensions were performed for 15 min. To quench the reaction, 1 μL of 4 M sodium hydroxide was added and allowed to react at 95 °C for 5 min. The resulting complementary DNA (cDNA) was snapped cooled on ice, and ethanol precipitated according to above procedures. Purified cDNA was resuspended in 2 μL of nuclease-free water and 2 μL of Gel Loading Buffer II was added. cDNA products were resolved on 10% denaturing PAGE gel. cDNA extensions were visualized and analyzed.

### RT-qPCR for Biotinylated RNA

A total of 2 μg of human brain total RNA was modified by AtoI_N3 and underwent SPAAC treatment as mentioned above. And then the RNA sample was subjected to cDNA synthesis with random hexamers using the PrimeScript Reverse Transcriptase from Takara. A total of 2 μL out of a 20-μL reaction was diluted with 18 μL of nuclease free water and set aside as INPUT. Dynabeads MyOne Streptavidin C1 (Invitrogen, Cat# 65002) beads were washed three times with bead binding buffer (100 mM Tris, pH 7.0, 1 M NaCl, 10 mM EDTA, 0.2% (v/v) Tween-20), and then added to enrich biotinylated RNA-cDNA hybrids. The mixture was incubated at RT for 30 min on a shaker. Using a DynaMag side magnet to retain the streptavidin beads, the flow-through was removed. The beads were washed four times with 4 M wash buffer (100 mM Tris, pH 7.0, 4 M NaCl, 10 mM EDTA, 0.2% (v/v) Tween-20) at RT, and twice with PBS (1×, pH 7.4). The beads were then treated with RNase H mixture [1× RNase H buffer, 12.5 mM d-biotin (Life Technologies, Cat# B20656), 0.1 U/μL RNase H (New England Biolabs, Cat# M0297S), RNase A/T1 cocktail at 40 ng/μL (Thermal Scientific, Cat# EN0551)] for 30 min at 37 °C on a shaker. Each RNase reaction was added to 1 μL of DMSO and incubated at 95–98 °C for 5 min. Using a DynaMag side magnet to retain the beads, released cDNA was collected and purified using DNA Clean and Concentration columns from Zymo Research (Cat# D4003) according to the manufacturer’s instructions. Purified cDNA was eluted with 20 μL of nuclease free water and set aside as ENRICH. INPUT (2 μL) and ENRICH (2 μL) were subjected to RT-qPCR using a SYBR Advantage qPCR Premix from Takara and Biorad
CFX connect real time system. The fold of enrichment was calculated against a DMSO negative control: $2^{\Delta\Delta Ct} = 2^{(Ct_{ENRICH-DMSO} - Ct_{INPUT-DMSO}) - (Ct_{ENRICH} - Ct_{INPUT})}$.

**Primers used in qPCR analysis**

- DHFR 3’ UTR qPCR forward, 5’- TCTTTTTTTTTTAGACAGCGTTTC-3’; DHFR 3’ UTR qPCR reverse, 5’- AGCCTGAGGCAGGAGAATCG-3’;
- BCKDK qPCR forward, 5’- CTTCACTACTGCTGAGGCCA; BCKDK qPCR reverse, 5’- GTGCATGGGTCTGACTGG-3’.
Figure S1. HPLC traces for adduct formation of 1 with nucleosides. a. Example HPLC trace of reactions between 1 and A and I. b. Relative yield of adduct formation between 1 and each nucleoside.
Figure S2. Dot blot assay for RNA oligo modification condition optimization. Oligo sequences containing inosine or adenosine (Same as Figure 1, main manuscript) were incubated with probe 1. Following incubation RNA was precipitated and biotinylated with Biotin-DBCO for dot-blot analysis.
References