A new water soluble copper N-heterocyclic carbene complex delivers mild O\textsuperscript{6}G-selective RNA alkylation

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Synthesis

General Experimental Information
All reagents and solvents used were of analytical grade. Buffers were prepared with ultrapure water (M Merck Millipore, EMD Millipore Corporation, Billerica, MA, USA water system, resistivity 18.2 MOhm). All chemicals were purchased from Sigma-Aldrich, Alfa Aesar or Acros and used as received. Butynediazoacetamide[1](BDA) was synthesized before in the lab.

Mononucleotide used in copper sulfate alkylation experiments were obtained from Sigma-Aldrich. Purification was carried out on a preparative Shimadzu UFLC system with a Gemini-NX 5 μm C18 21.2 x 250 mm (Phenomenex) column using 100 mM triethylammonium acetate (TEAA pH 7.25)/acetonitrile (MeCN) gradients as mobile phase. Elution was carried out at a flow rate of 20 mL/min monitored at 254 nm.

Alkylation reactions were analyzed on an analytical Agilent 1100 LC system with clarity 5μOligo XT 150 x 4.6 mm column using 50 mM NH₄OAc/MeCN gradient. Elution was carried out at a flow rate of 1 mL/min.

Method A: 0-16 % MeCN in 18 min, 16-80 % MeCN in 5 min, 80 % MeCN for 3 min with peak detection at 254 nm.

Method B: 0-16 % MeCN in 18 min, 16-80 % MeCN in 5 min, 80 % MeCN for 3 min with peak detection at 254 nm at 55 °C.

Method C: 0-12 % MeCN in 4 min, 12-90 % MeCN in 0.5 min, 90 % for 1 min. Aqueous product fractions were freeze dried on a Christ Alpha 2-4 LDplus flask lyophilizer at 0.1 mbar. ESI MS spectra were measured on a Bruker Esquire3000plus mass spectrometer by direct injection in positive polarity of the ion trap detector.

Polyacrylamide gel electrophoresis (PAGE): PAGE was prepared in 6-8 % gel (29:1 w/w acrylamide/bisacrylamide) in 1X TBE. The electrophoretic samples were prepared by mixing 15-30 ng of RNAs with 6 uL in 50 % (v/v) formamide gel-loading buffer. 12 uL microliters from the resulting mixtures were then applied on the gel without further pre-treatment. The gels were run at 120 V until the Bromophenol blue migrates to approximately 1 cm from the end of the gel. The gels were then washed briefly with deionized water and soaked with SYBR® Gold nucleic acid gel stain for 20 min, washed briefly and visualized using BioRadChemiDoc MP system equipped with Image Lab 5.0 software.
**Synthetic Scheme**

**Scheme S1:**

- a) HBr, 150 °C, 11 h, 51 %;  
- b) NaN₃, 75 °C, 15 h, 60 %;  
- c) chloroacetyl chloride, Et₃N, DCM, 15 h rt, 63 %.

**Scheme S2:**

- a) triethylorthoformate, acetic acid reflux overnight 160 °C, 31% ;  
- b) 2-Chloro-N,N-methyleneazidoacetamide, KI, Et₃N, DMF 100 °C overnight, 62 %;  
- c) triflic anhydride, 2,6-lutidine, -78 °C, 37 %;  
- d) Ag₂O, LiCl, THF;  
- e) CuCl, 19 %.

**Scheme S3:**

- a) propargyl chloride, toluene, rt (64%), b) 1, CuSO₄, sodiumascorbate, DMSO, H₂O, 25-50°C (49%)
### Chemical Synthesis and Analytical Data

**2-bromo-N-methyl-ethanamine (M2):** A 250 mL round-bottom flask was charged with a stir bar and 50 mL (150 mmol) of HBr (48 % w/w). The solution was cooled to 4 °C in an ice bath, and 8 mL (100 mmol) of ice cold 2-(methylamino) ethanol was added dropwise with stirring. The resulting mixture was then attached to a distillation apparatus that had a 2 in, fractionating column built into the distillation head, and the solution was brought to reflux by heating in an oil bath. As the bath temperature neared 150 °C, the head temperature reached 100 °C and distillation of H$_2$O began. The distillation was allowed to continue slowly (9-11 h) until 8-10 mL of distillate was collected. During this time, distillation was performed for 1 h, and then the oil bath was turned down to the point that reflux continued but distillation ceased for 30 min. (The distillation was stopped (but reflux continued) whenever the head temperature rose above 105 °C or dropped significantly below 100 °C, indicating an absence of water.). The distillation/reflux process was repeated four times. When the solution no longer produced any distillate at a head temperature of 100 °C, the bath temperature was increased, the head temperature rose to 123 °C, and distillation of crude HBr began. Failure to distill off the remaining crude HBr at the conclusion of the synthesis inhibited the precipitation of the final product upon addition of cold acetone. The HBr distillation was continued for approximately 2 h, after which time the solution was allowed to cool. After the solution had cooled to 60 °C, it was slowly poured with stirring into a 500 mL beaker containing 200 mL of ice cold acetone, whereupon the desired white product precipitated from solution. This heterogeneous solution was capped and placed in the freezer (-20 °C) overnight. The white precipitate was then vacuum filtered through a sintered glass funnel, washed three times with 100 mL aliquots of ice cold acetone, and dried in the funnel, yielding of white crystalline product. (11.2 g, 51%).

$^1$H-NMR (400 MHz, CDCl$_3$): δ /ppm: 9.21 (s, 2H, NH in salt form), 3.83 (t, J = 8 Hz, 2H, CH$_2$), 3.51 - 3.46 (m, 2H, CH$_2$), 2.82 (t, J = 6 Hz, 3H, CH$_3$); $^{13}$C-NMR (100 MHz, CDCl$_3$): 50.82, 33.87, 24.95; HRMS (ESI): C$_3$H$_8$NBr [M+H]$^+$ Calculated: 137.9913; observed: 137.9911.
2-azido-N-methyl-ethanamine (M3): N-methylamino-1-ethylbromide hydrobromide (5 g, 23 mmol) and sodium azide (2.9 g, 46 mmol) were dissolved in water (1 mL mmol⁻¹) and heated at 75 °C for 15 h. The reaction mixture was cooled in an ice bath and NaOH (4 g) was added. The solution phase separated and the organic phase was removed. The aqueous phase was extracted with diethyl ether twice. The organic layers were combined, dried with MgSO₄, and concentrated to obtain an oil (1.3 g, 57 %).

\[ \delta / ppm \ 7.26 (s, 1H, NH), 3.41 (ddd, 2H, CH₂), 2.74 (tt, \ J = 5.7 \text{ Hz}, 2H, CH₂), 2.43 (s, 3H, CH₃) \]; \[ ^{13}C \text{-NMR (100 MHz, CDCl}_3) : 51.17, 50.48, 36.10 \]; HRMS (ESI): C₃H₈N₄ [M+H]⁺ Calculated: 101.0822; observed: 101.0032.

N-(2-azidoethyl)-2-chloro-N-methyl-acetamide (M4): N-(2-azidoethyl)-2-chloro-N-methyl-acetamide was prepared by the amidation of 2-azido-N-methyl-ethanamine with chloroacetyl chloride in the presence of TEA. A 250 mL round-bottom flask was charged with 2-azido-N-methyl-ethanamine (1.3 g, 13 mmol), TEA (2.1 mL, 15.1 mmol), and dry CH₂Cl₂ (20 mL). The reaction mixture was cooled to 0 °C in an ice-water bath, and 2-chloropropionyl chloride (1.21 mL, 15.1 mmol) in 30 mL of CH₂Cl₂ was added dropwise over a period of 1 h under magnetic stirring. After the addition was completed, the reaction mixture was stirred at 0 °C for 1 h and then at room temperature for 15 h. After filtration and removing all the solvents, the residues were further purified by a silica gel column chromatography using CH₂Cl₂/petroleum ether (3:1 v/v) to afford colorless liquid (1.5 g, 65 %). \[ ^{1}H \text{-NMR (400 MHz, CDCl}_3) : \delta / ppm \ 4.09 (s, 2H, CH₂), 3.58 - 3.53 (m, 6H, CH₂), 3.19 (s, 3H, CH₃) \]; \[ ^{13}C \text{-NMR (100 MHz, CDCl}_3): 166.80, 48.34, 41.33, 37.21, 33.79 \]; HRMS (ESI): C₅H₉ClN₄O [M+Na]⁺ Calculated: 199.0357; observed: 199.0360.

N,N’-bis(2,6-diisopropylphenyl)formamidine (M6): In a distillation apparatus, a mixture of M5 (9.43 mL, 50 mmol), triethylorthoformate (4.15 mL, 25 mmol) and glacial acetic acid (0.285 mL, 5 mmol) was heated at 160 °C overnight and 7-8 mL of ethanol was distilled out during the reaction. The crystalline mass obtained upon cooling was partitioned between diethyl ether (250 mL) and aqueous sodium carbonate (10 %, 30 mL). The ether layer was separated containing microcrystalline solid and filtered over sintered funnel washed 2-3 times with diethyl ether to get pure colorless microcrystalline solid. (2.87 g, 31 %).

\[ ^{1}H \text{ NMR (400 MHz, C}_6D_6) : \delta / ppm \ 10.84 (s, 1H, CH of amidine), 7.10 - 7.04 (m, 6H, Ph), 3.46 - 3.39 (m, 4H, CH of iPr), 1.13 (d, \ J = 6.9 \text{ Hz}, 24H, CH₃ of iPr); ^{13}C \text{ NMR (100 MHz, C}_6D_6) \delta 155.15, 146.74, 145.96, 143.66, 140.65, 138.79, 133.97, 125.73, 123.30, 28.17, 23.58, 22.94 \]; HRMS (ESI): C₂₅H₃₆N₂C [M+H]⁺ Calculated: 365.2951; observed : 365.2957.

N-(2-azidoethyl)-2-(N’-[E]-(2,6-diisopropylphenyl)iminomethyl)-2,6-diisopropyl-anilino-N-methyl-acetamide (M7): To a solution of N,N’-di(2,6-diisopropylphenyl)formamidine (2.4 g, 6.5 mmol) and KI (107 mg, 0.65 mmol in DMF (10 mL), N-(2-azidoethyl)-2-chloro-N-methyl-acetamide (1.5 g, 8.5 mmol ) and triethylamine (1.3 mL, 9.7 mmol) were added
respectively at room temperature. The resulting mixture was stirred at 100 °C overnight. After cooling down to room temperature, water (20 mL) was added to the solution and the mixture was extracted with ether (3×40 mL) and the organic layers were combined and washed again with water (30 mL) and brine (30 mL). After drying over Na₂SO₄, the solution was filtered and evaporated under reduced pressure. The crude residue was purified by column chromatography (SiO₂, Hexane/EtOAc : 6/1) to afford a white solid (2 g, 60%). 

\[ ^1H-NMR \ (400 \text{ MHz}, \text{CDCl}_3) : \delta/\text{ppm} \ 7.32 \ (t, \ J= 8.0 \text{ Hz}, 1H, \text{Ph}), 7.23 \ (s, 1H, \text{Ph}), 7.19 \ (d, \ J= 7.7 \text{ Hz}, 2H, \text{Ph}), 7.08 \ (d, \ J= 7.4 \text{ Hz}, 2H, \text{Ph}), 7.00 \ (t, \ J= 8.0 \text{ Hz}, 1H, \text{Ph}), 4.42 \ (s, 2H), 3.67 – 3.55 \ (m, 4H, \text{CH of iPr}), 3.54 \ (t, \ J= 5.7 \text{ Hz}, 2H, \text{CH}_2), 3.46 \ (t, \ J= 5.7 \text{ Hz}, 2H, \text{CH}_2), 3.22 \ (s, 3H, \text{CH}_3), 1.17 \ (d, \ J= 4 \text{ Hz}, 24H, \text{CH}_3 of iPr) \] 

\[ ^13C-NMR \ (100 \text{ MHz}, \text{CDCl}_3) : 167.97, 151.05, 150.93, 148.43, 148.36, 146.80, 139.98, 139.23, 128.31, 124.45, 51.25, 50.88, 49.69, 49.16, 48.37, 36.96, 27.97, 27.59, 25.04, 24.52, 23.73 \] 

HRMS (ESI): C\textsubscript{30}H\textsubscript{44}N\textsubscript{6}O \ [M+H]\textsuperscript{+} Calculated.: 505.3649; observed: 505.3656.

\textbf{N-(2-azidoethyl)-1,3-bis(2,6-diisopropylphenyl)-N-methyl-imidazol-1-ium-4-amine; trifluoromethanesulfonate (4):} 2,6-lutidine (0.370 mL, 3.2 mmol) was added at room temperature to a solution of compound 7 (1.026 g, 2 mmol) in CH\textsubscript{2}Cl\textsubscript{2} (10 mL) and the solution was cooled down to -78°C. At this temperature, triflic anhydride (0.403 mL, 2.4 mmol) was added dropwise. The mixture was then allowed to warm up to room temperature overnight. A saturated solution of NaHCO\textsubscript{3} (30 mL) was added to the resulting solution and the biphasic mixture was stirred for another 30 minutes. The organic phase was separated and washed again with NaHCO\textsubscript{3} (2×30 mL), dried over Na\textsubscript{2}SO\textsubscript{4} and evaporated under reduced pressure to give a crude product. The crude residue was purified by column chromatography (SiO₂, DCM/MeOH : 9.5/0.5) to afford a white solid (2 g, 60%). 

\[ ^1H-NMR \ (400 \text{ MHz}, \text{CDCl}_3) : \delta/\text{ppm} \ 8.57 \ (d, \ J= 4 \text{ Hz}, 1H, \text{CH of imidazole ring}), 7.61 \ (t, \ J= 7.8 \text{ Hz}, 1H, \text{pCH of Ph}), 7.54 \ (t, \ J= 7.2 \text{ Hz}, 1H, \text{pCH of Ph}), 7.38 \ (d, \ J= 7.8 \text{ Hz}, 2H, \text{mCH of Ph}), 7.32 \ (d, \ J= 7.9 \text{ Hz}, 2H, \text{mCH of Ph}), 7.14 \ (d, \ J= 1.9 \text{ Hz}, 1H, \text{CH of Imidazole ring}), 3.27 \ (t, \ J= 5.8 \text{ Hz}, 2H, \text{CH}_2 of azide linker), 3.05 \ (t, \ J= 5.8 \text{ Hz}, 2H, \text{CH}_2 of azide linker), 2.81 \ (s, 3H, \text{CH}_3), 2.49 \ (dt, \ J= 13.6, 6.9 \text{ Hz}, 4H, \text{CH of iPr}), 1.33 \ (d, \ J= 6.8 \text{ Hz}, 6H, \text{CH}_3 of iPr), 1.29 \ (d, \ J= 6.8 \text{ Hz}, 6H, \text{CH}_3 of iPr), 1.20 \ (dd, \ J= 9.5, 6.9 \text{ Hz}, 12H, \text{CH}_3 of iPr) \] 

\[ ^13C-NMR \ (100 \text{ MHz}, \text{CDCl}_3) : 145.50, 144.98, 136.97, 133.25, 132.48, 132.03, 130.08, 127.94, 125.32, 124.45, 51.25, 50.88, 49.69, 49.16, 48.37, 36.96, 27.97, 27.59, 25.04, 24.52, 23.73 \] 

HRMS (ESI): C\textsubscript{30}H\textsubscript{44}N\textsubscript{6}O \ [M+H]\textsuperscript{+} Calculated.: 505.3649; observed: 505.3656.

\textbf{[4-[2-azidoethyl(methyl)amino]-1,3-bis(2,6-diisopropylphenyl)imidazol-2-ylidene]-chloro-copper (1):} Imidizolium salt 4 (191 mg, 0.392 mmol) was taken in dry RBF followed by LiCl (83 mg, 1.9 mmol) then dry THF 7 mL was added through syringe under nitrogen atmosphere. Ag\textsubscript{2}O (64 mg, 0.23 mmol) was added immediately into the RBF and condenser was covered with aluminum foil. Reaction mixture stirred 15 min at RM temperature then 2 h at 70 °C. The solvent was removed under vacuum and the residue was suspended in CH\textsubscript{2}Cl\textsubscript{2} (ca. 20 mL), then filtered through a plug of Al\textsubscript{2}O\textsubscript{3} (Brockmann’s I) and washed with CH\textsubscript{2}Cl\textsubscript{2}
until all the complex was eluted (checked by TLC). The solvent was concentrated to 1 mL and CuCl (46.5 mg, 0.47 mmol) was added as a solid and the mixture was stirred overnight at 35°C. The reaction mixture was filtered through a plug of Al₂O₃ (Brockmann’s I) and washed with CH₂Cl₂ until all the complex was eluted dried over Na₂SO₄ and evaporated to yield crude complex. The crude complex dissolved in DCM and precipitated with pentane (35 mg, 16 %).

$^1$H-NMR (400 MHz, CDCl₃): δ/ppm 7.53 (t, $J = 8.0$ Hz, 1H, pCH of Ph), 7.46 (t, $J = 8.0$ Hz, 1H, pCH of Ph), 7.31 (d, $J = 8.0$ Hz, 2H, mCH of Ph), 7.27 (d, $J = 8.0$ Hz, 2H, mCH of Ph), 6.56 (s, 1H, CH of imidazole ring), 5.30 (s, DCM solvent peak) 2.92 (t, $J = 4$ Hz, 2H, CH₂ of azide linker), 2.84 (t, $J = 4$ Hz, 2H, CH₂ of azide linker), 2.71 (s, 3H, CH₃), 2.67 – 2.60 (m, 2H, CH of iPr), 2.56 - 2.49 (m, 2H, CH of iPr), 1.32 (dd, $J = 8.0$ Hz, 12H, CH₃ of iPr); $^{13}$C-NMR (100 MHz, CDCl₃): δ 146.05, 145.62, 130.72, 130.39, 124.61, 124.16, 108.61, 53.65, 49.08, 40.84, 29.01, 28.70, 26.05, 22.56; HRMS (ESI): C₃₀H₄₂CuN₆ [M+ CH₃CN]⁺ Calculated: 590.3027; observed: 590.3032.

**Ammonium functionalized terminal alkyne (M9):** A solution of N,N-dimethylaminoethanol (1.00 g, 11 mM) in toluene (1.5 mL) was added dropwise to a solution of propargylchloride (70 % w/w in toluene, 1.25 mL, 11 mmol) in toluene (1.5 mL). The reaction was stirred for one day at room temperature. The obtained precipitate was filtered and washed with pentane to yield M9 as white solid (1.17 g, 64 %).

$^1$H-NMR (400 MHz, Methanol-d₄) δ/ppm: 4.43 (d, $J = 4$ Hz, 2H, CH₂), 4.05 – 3.99 (m, 2H, CH₂), 3.62 – 3.57 (m, 2H, CH₂), 3.55 (t, $J = 2.5$ Hz, 1H, H of OH), 3.27 (s, 6H, CH₃), 3.33 (s, 1H of alkyne) HRMS (ESI): C₇H₁₃NO [M+1]⁺ Calculated.: 128.1070, Observed: 128.1071.

**Ammonium functionalized in the skeleton (6):** According to the general procedure compound 4 (53.8 mg, 99.0 µM), alkyne M9 (15.4 mg, 94.1 µM), CuSO₄ (24.7 mg, 99.0 µM), sodium ascorbate (19.6 mg, 99.0 µM) were reacted and purified to yield 6 as white solid (36.7 mg, 49 %). $^1$H-NMR (400 MHz, MeOD) δ/ppm: 8.02 (s, 1H, CH of triazole ring), 7.59 (t, $J = 8$ Hz, 1H, pCH of Ph), 7.51 (t, $J = 8$ Hz, 1H, pCH of Ph), 7.41 (d, $J = 8$ Hz, 2H, mCH of Ph), 7.36 (d, $J = 8$ Hz, 2H, mCH of Ph), 7.21 (s, 1H, CH of imidazole ring), 4.71 (s, 2H, CH₂), 4.26 (t, $J = 6.7$ Hz, 2H, CH₂), 4.19 – 4.02 (m, 2H, CH₂), 3.37 - 3.75 (m, 2H, CH₂), 3.14 (s, 6H, CH₃), 2.73 (s, 3H, CH₃), 2.72 – 2.64 (m, 2H, CH of iPr), 2.56 (d, $J = 6.7$ Hz, 2H, CH of iPr), 1.34 – 1.21 (m, 24H, CH₃ of iPr); $^{13}$C-NMR (100 MHz, MeOD) δ 147.54, 147.09, 146.59, 136.56, 131.90, 131.49, 125.82, 125.23, 112.30, 66.39, 60.54, 57.02, 55.96, 51.82, 41.14, 30.20, 29.91, 26.64, 25.30, 24.36, 23.09. HRMS (ESI): C₃₇H₅₆ClCuN₇O⁺ Calculated: 712.3525, Observed: 712.3542.

**Synthesis of 3-azidopropan-1-amine (M10):** Sodium Azide (4.4 g, 67.8 mmol) was added to a mixture of 3-bromopropan-1-amine:hydrobromide (5.00 g, 22.4 mmol) in 20 mL Water. The mixture was stirred at 80 °C for 22 h and allowed to cool at room temperature. A solution of KOH (8.42 g, 150 mmol) in 10 mL Water was added dropwise to the mixture which was cooled at 0 °C. The crude product was extracted with dichloromethane (3x 75 mL) and the organic layer was washed with brine (150 mL). The extracted organic layer was dried over Na₂SO₄, filtered and evaporated with rotational evaporation (25 °C, max 350 mbar) to yield a
light yellow oil (1.9 g, 87 %); 1H NMR δ/ppm (400 MHz, CDCl₃) 3.38 (t, J= 6.7 Hz, 2H, CH₂), 2.81 (t, J= 6.9 Hz, 2H, CH₂), 1.74 (q, J= 6.8 Hz, 2H, CH₂), 1.15 (s, 2H, NH₂).

Synthesis of N-(3-azidopropyl)-2-bromo-acetamide (M11): Sodium Hydroxide (880 mg, 22 mmol) was dissolved in Water (2.5 mL) and added to 5 mL DCM in a 10 mL round-bottom flask. 3-azidopropan-1-amine (1.10 g, 4.11 mmol) was added to the aqueous layer and the mixture was cooled to 0°C. Bromoacetyl bromide (2.87 mL, 33 mmol) was added to the DCM layer and the bilayer mixture was stirred vigorously for 16 hours at room temperature. The aqueous layer was extracted with DCM (3x 50 mL). The combined organic layers were washed with 50 mM Na₂CO₃ (3x 50 mL). The organic layer was then dried over MgSO₄. The mixture was filtered and evaporated rotary evaporator to yield colorless / light yellow oil. (17 g, 89 %). 1H NMR (400 MHz, CDCl₃): δ/ppm 6.70 (sbr, 1H, NH), 3.89 (s, 2H, CH₂), 3.41 (m, 4H, CH₂), 1.83 (q, J= 6.6 Hz, 2H, CH₂). Analytical data is in agreement with the literature[2].

Synthesis of the N-(3-azidopropyl)-2-diazo-acetamide (DZ1): To a solution of N-(3-azidopropyl)-2-bromo-acetamide (5, 600 mg, 2.71 mmol, 1 eq) in THF (10 mL), N, N'-ditosylhydrazine (1, 85 g, 5.43 mmol, 2 eq) was added. The reaction mixture was cooled to 0°C and DBU (1, 75 mL, 13.6 mmol, 5 eq) was added and the mixture was stirred for 20 min at room temperature. Saturated NaHCO₃ (30 mL) was added while precipitation of white solid was observed. The mixture was centrifuged at 4400 rpm for 10 min. The supernatant was extracted with Et₂O (3x 30 mL). The combined organic layers were dried over Na₂SO₄, filtered, concentrated and the residue was purified by chromatography column on Si60 in CH₂Cl₂ and MeOH (prior to purification, the column was neutralized by making the silica gel in a CH₂Cl₂ eluent containing 5 % of Triethylamine) to yield a yellow oil (163 mg, 36 %). 1H NMR (400 MHz, CDCl₃) δ/ppm: 5.15 (sbr, 1H, NH), 4.71 (s, 1H, CH), 3.40 – 3.38 (m, 4H, CH₂), 1.81 (q, J= 6,5 Hz, CH₂); 13C NMR (100 MHz, CDCl₃) 168.4, 48.2, 46.1, 36.2, 28.6; (ESI-MS): C₅H₈N₆NaO⁺ Calculated.: 191.1, Observed: 190.1. All data agrees with the literature[3].

Synthesis of the O⁶-methyl oxy)-N-(but-3-yn-1-yl)acetamide guanosine (S12):

To a solution of CuSO₄ (6.4 mg, 0.02 mM) in H₂O (degassed), MES buffer stock solution (30mL, 13mM), Guanosine disodium monophosphate (50 mg, 0.13 mM), Butynediazo acetamide DZ2 (0.17 mg, 1.3 mM) and ascorbate (25 mg, 0.13 mM) were added and the reaction mixture was stirred at room temperature for 1 h. The reaction mixture was lyophilized and the residue was purified by preparative RP-HPLC using Method A and B. The corresponding product fractions were combined and lyophilized (19 mg, 31 %).1H NMR (500 MHz, DMSO-d₆) δ 8.20(s, 1H, CH of guanidine), 8.14 (d, J = 5.9 Hz, 1H, NH of amide), 8.43 (s, 2H, NH₂ of guanidine), 5.80 (d, J = 5.9 Hz, 1H, CH(1) of ribose), 4.88 (d, J = 2.7 Hz, 2H, CH₂ of O'alkyl), 4.49 (t, J = 5.5 Hz, 1H, CH(3) of ribose), 4.12 (dd, J = 5.0, 3.4 Hz, 1H, CH(2) of ribose), 3.91 (d, J = 3.7 Hz, 1H, CH(4) of ribose), 3.65 (dd, J = 12.0, 4.1 Hz, 1H, CH(5) of ribose), 3.55 (dd, J = 11.9, 4.0 Hz, 1H, CH(5) of ribose), 3.24 (td, J = 7.2, 5.8 Hz, 2H, CH₂ of alkyne), 2.84 (s, 1H, CH of alkyne ), 2.33 (td, J = 7.2, 2.7 Hz, 2H, CH₂ of alkyne); 13C NMR (126 MHz, DMSO-d₆) δ 167.80 , 159.97, 155.02 , 138.76 , 114.37 , 87.10 , 85.75 , 82.59 ,
73.95, 72.64, 70.87, 64.17, 61.89, 38.08, 19.11; HRMS (ESI): C_{16}H_{19}N_{6}O_{9}PH[M-], Calculated: 471.1035, Observed: 471.1044.

**X-ray Crystal structure determination**

X-ray diffraction structure successfully determine for complex 1. Single crystals for X-ray diffraction were grown by slow solvent evaporation method (2 mg of complex 1 was dissolved in DCM, few drops of cyclohexane were added before sealing the tube with perforated cap). Crystallographic data was collected from single crystal samples. Collections were performed using a Bruker KappaAPEX and a STOE IPDS2 diffractometer.

**General procedure for Cu(I)-catalyzed alkylation**

Typically 20 μL of total reaction volume contained 1 mM of CuSO₄, 5 mM of Guanosine monophosphate, 100 mM MES buffer, 50 mM DZ1/DZ2. Stock solutions of each compound were prepared and combined in an Eppendorf tube in that order. The reactions were monitored by injection of 1 μL of the reaction mixture into RP-HPLC using Method A and B. The conversion of guanosine monophosphate was directly calculated from the corresponding peak areas in the HPLC traces. The peaks were separated using Method A and B (6 μL injection of reaction mixture), collected and analyzed by UPLC/MS.

**Stock solutions**: CuSO₄: 5 mM in H₂O; GMP: 50 mM in H₂O; MES buffer: 500 mM in H₂O, pH 6 DZ1/DZ2/DZ3: 250 mM in DMSO; ascorbate: 100 mM in H₂O

**Stock solutions**: NHC-Cu 5mM in H₂O/DMSO; GMP: 50 mM in H₂O; MES buffer: 500 mM in H₂O, pH 6 DZ1/DZ2/DZ3: 250 mM in DMSO

\[ \text{O} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{O} \]

\[ \text{N} \quad \text{N} \quad \text{N} \quad \text{N} \quad \text{N} \]

\[ \text{Cu (I) 20 Mol %, MES buffer pH=6, RT, 2h} \]

**Scheme S5**: Alkylation at O\(^{6}\)-GMP using different acetamide
Cu(I)-catalyzed RNA O\textsuperscript{6}-G modification

General procedure for alkylation of transcriptome RNA, mRNA and oligo ssRNA: Typically 10 μL of total reaction volume contained 5 μM of ssRNA, 15 mM MES [pH 6.0; pH adjusted by addition of 1 N NaOH] buffer and water were mixed and heated at 90 °C for 3 min and subsequently snap-cooled on ice., 10 or 100 equiv. of NHC-Cu or CuSO\textsubscript{4}/ascorbate and 5 mM diazoacetatamide (stock in DMSO) were added and reaction mixture was purged with argon before sealing the tube. Stock solutions of each compound was prepared before. The reaction was stirred for 3 h at 25 ºC and monitored by injecting 1 μL of the reaction mixture into RP-HPLC using Method C for oligo RNA. Transcriptome RNA and mRNA further treated with either fluorescent tag or desthiobiotin via click reaction and analyzed by gel electrophoresis. The product purification was carried out by ethanol precipitation, Zymo Clean & Concentrator-5 (R1015, Zymo Research) columns and HPLC. The peaks were separated using Method C (10 μL injection of reaction mixture).

Scheme S6: Alkylation at O\textsuperscript{6}-G of RNA using different acetamide; Scope: Transcriptomic RNA, mRNA, ssRNA and guanosine monophosphate

Table S1. Alkylation reaction on mRNA at different concentration of CuSO\textsubscript{4} and NHC-Cu.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Reaction A</th>
<th>Reaction B</th>
<th>Reaction C</th>
<th>Reaction D</th>
<th>Reaction E</th>
</tr>
</thead>
<tbody>
<tr>
<td>290 nt mRNA</td>
<td>1 eq</td>
<td>1 eq</td>
<td>1 eq</td>
<td>1 eq</td>
<td>1 eq</td>
</tr>
<tr>
<td>MES buffer (pH 6)</td>
<td>3000 eq</td>
<td>3000 eq</td>
<td>3000 eq</td>
<td>3000 eq</td>
<td>3000 eq</td>
</tr>
<tr>
<td>DZ1</td>
<td>1000 eq</td>
<td>1000 eq</td>
<td>1000 eq</td>
<td>1000 eq</td>
<td>1000 eq</td>
</tr>
<tr>
<td>CuSO\textsubscript{4}</td>
<td>-</td>
<td>10 eq</td>
<td>-</td>
<td>100 eq</td>
<td>-</td>
</tr>
<tr>
<td>Sodium Ascorbate</td>
<td>-</td>
<td>10 eq</td>
<td>-</td>
<td>100 eq</td>
<td>-</td>
</tr>
<tr>
<td>Cu-NHC 1</td>
<td>-</td>
<td>-</td>
<td>10 eq</td>
<td>-</td>
<td>100 eq</td>
</tr>
</tbody>
</table>

* eq = equivalent (See PAGE image FigureS2)
Cy5 labelling
The separated ssRNA was mixed at a final concentration of 100 µM with Cy5-DBCO (100 µM final concentration). Half of the reaction volume was water, the other half was DMSO. Incubated at 30 °C for 4 h. ssRNA was recovered via ethanol precipitation and separated on analytical HPLC using method A. HPLC traces are given in Figure S17 and S18.

![Scheme S7: Stain promoted click with O6-G modified ssRNA.](image)

Desthiobiotin Tag
Alkylated RNA was purified through precipitation technique using sodium acetate (3M) and ethanol. Derivatization of desthiobiotin on RNA was done using Cu-AAC reaction. Reaction was performed at 22 °C for 2 h in 50 µL of reaction volume containing desthiobiotin (1 mM), copper sulfate (4 mM) and sodium ascorbate (4 mM). Desthiobiotin tagged RNA was precipitated upon adding 5 µL of sodium acetate (3M) and three volumes of ethanol. Alkylation and desthiobiotin tag on RNA were confirmed by enzyme digestion using benzonase, snake venom diesterase and alkaline phosphatase upon enrichment. UPLC traces are given in Figure S10 and S16.

![Scheme S8. Derivatization of desthiobiotin on RNA using Cu-AAC reaction.](image)
Single nucleotide analysis: enzyme digestion

0.5 µg of modified RNA was used and mixed with 2 µL of a digestion buffer containing benzonase, snake venom diesterase and alkaline phosphatase according to the protocol published by Quinlivan and Gregory [4]. Digestion overnight at 37°C. The following UPLC analysis showed the canonical nucleotides, was well as modified G.

Scheme S9. UPLC/MS analysis shows modified O6-G upon RNA digestion

Synthesis of standard guanosine-desthiobiotin

RNA enrichment upon modification

Before final purification of RNA using streptavidin pull down, RNA was fragmented incubating at 95 °C for 5 min in fragmenting buffer. Then, purified via Zymo Clean & Concentrator-5 (R1015, Zymo Research) columns. The concentrated sample was then subjected to streptavidin pull down assay as following:

Add 100 µL of streptavidin magnetic beads (NEB) in 1.5 mL tube.
After removing solution beads were washed with washing buffer 3 times.
In 50 µL RNA solution add 5X wash buffer mix and incubate for 30 min at rt.
Incubate the tube on the magnetic rack for 1 min to allow the magnetic beads to settle to magnet side of the tube.
Collect the supernatant in another tube.
Mix the magnetic beads in the elution buffer containing 1 mM solution of biotin.
Incubate a tube at 50 °C for 10 min.
Incubate the tube on the magnetic rack again for 1 min to allow the magnetic beads to settle to magnet side of the tube.
Collect the supernatant in separate tube.
Re-disperse magnetic beads in elution buffer and repeat points 7-9.
Enriched RNA used for library preparation.
Western blot technique and RNA digestion followed UHPLC/MS analysis confirms enrichment of modified RNA (see Figures S3, S10 and S16).

**Buffer solutions**

*Table S2.* A list of all buffer solutions used for the procedures described herein.

<table>
<thead>
<tr>
<th>Name</th>
<th>Conc.</th>
<th>Recipe</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSC</td>
<td>20X</td>
<td>3 M NaCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300 mM citric acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH adjusted with NaOH to 7.0</td>
</tr>
<tr>
<td>Beads Binding Buffer / Beads Washing Buffer</td>
<td>5X</td>
<td>2.5 M NaCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 mM Tris-HCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 mM EDTA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH adjusted to 7.5</td>
</tr>
<tr>
<td>Beads Low Salt Buffer</td>
<td>5X</td>
<td>0.75 M NaCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 mM Tris-HCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 mM EDTA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH adjusted to 7.5</td>
</tr>
<tr>
<td>Beads Biotin Elution Buffer</td>
<td>5X</td>
<td>50 mM Tris-HCl,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 mM EDTA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH adjusted to 7.5</td>
</tr>
</tbody>
</table>
**Fragmentation**

For each sample, 3 μg of the isolated RNA were taken and the volume set to 5 μL. If the resuspended RNA concentration was less than 3 μg/μL, the volume was adjusted accordingly by drying the sample on a SpeedVac (Eppendorf Concentrator 5301). The RNA was then mixed with 10 μL of the fragmentation buffer (50 mM sodium carbonate at pH 9.2 with 1 mM EDTA) and heated for 20 minutes at 90 °C. The RNA was cooled to 0 °C and washed with Zymo Clean & Concentrator-5 (R1015, Zymo Research) columns using their standard protocol, elution in 10 μL of water.

**End healing and 5’ phosphorylation**

The RNA fragments have been mixed with 2 μL PNK Buffer A (Thermo Scientific), 2 μL ATP (10 mM), 1.5 μL T4 PNK (Thermo Scientific, EK0032), 0.5 RiboLock (Thermo Scientific, EO0381) and 4 μL water. The reaction was done for 2 h at 37 °C. Purification with Zymo Clean&Concentrator-5 columns using the standard protocol and elution in 10 μL water.

**3’ Adapter ligation**

The 3’ adapter ligation was done with the Universal miRNA Cloning Linker (S1315, NEB) and T4 RNA ligase 2, truncated (M0242S, NEB). The reaction was set up with:

- 10X reaction buffer 2.0 μL
- linker 0.5 μg/μL 1.0 μL
- PEG8000 2.0 μL
- Ligase (200 U/μL) 1.0 μL
- RiboLock 0.5 μL

The volume was adjusted to 20 μL. The reaction was done overnight at +4 °C and the reaction has been cleaned with RNeasy MinElute Cleanup Kit (Qiagen, Cat. 74204) using their standard protocol. RNA has been eluted with 10 μL water.

**5’ adapter ligation**

The 5’ adapter ligation was done with the stop adapter (100 μM, see chapter 7) and T4 RNA Ligase 1 (NEB, M0204S) with:

- 10X reaction buffer 3.0 μL
- Adapter (100 μM) 1.0 μL
- ATP (NEB) 1.5 μL
- PEG8000 3.0 μL
- T4 RNA Ligase 1 1.0 μL
- RiboLock 0.5 μL

The volume has been adjusted to 30 μL. The reaction was done overnight at +14 °C in a Bioer Mixing Block MB-102 without shaking. Reaction workup with RNeasy MinElute Cleanup Kit using their standard protocol. Elution in 27 μL of water.

**Positive enrichment**

The adapter ligated RNA has been mixed with 2 μL of SSC Buffer (20X) and 1 μL of the fishing probe A (10 μM, see chapter 7). This mixture was heated to 98°C for 3 minutes in the Bioer Mixing Block which was then shut off to anneal the probe to the sequence by slowing letting it cool down to room temperature (4 hours waiting period in total).
The RNA was then mixed with 6 μL of binding buffer (5X) and mixed with washed Streptavidin beads (100 μL beads originally, NEB, S1420S). The mixture was incubated at room temperature for 30 minutes and the beads were washed twice with binding buffer and once with cold low salt buffer to get rid of unbound RNA. Then, the beads were mixed with elution buffer, heated to 98 °C for 2 minutes and quickly put on a magnet to release the target RNA. The elution buffer was removed, and the RNA was recovered from it with ethanol precipitation and redissolved in 10 μL of water.

Reverse Transcription
For reverse transcription, the starting material has been mixed with 1 μL of reverse transcription-primer (2 μM), 1 μL of water, and 1 μL of dNTPs (10 mM, Sigma-Aldrich or Solis BioDyne). The mixture was heated to 65 °C for 5 minutes and placed on ice for > 1 minute. Then, the following was added:
- 5X first strand buffer 4 μL
- 0.1 M DTT 1 μL
- Ribolock 1 μL
- SSIII 1 μL
The mixture was gently mixed and then incubated for 60 min at 50 °C, and then the reaction was inactivated at 70 °C for 15 min.

Gel isolation
The reverse transcription (RT) reaction mixture was separated on a PAGE gel (6 %, 19:1 Acrylamide/Bisacrylamide with TBE buffer). The gel was then stained with SYBR gold for 5 minutes and illuminated with blue light. Bands between ca 150 and 350 bp have been cut out and put into Eppendorf tubes. The gel portions were soaked in TE buffer (1 mL) with 0.5 M NaCl added and the gel has been crushed. Incubated over night at room temperature.

The gel suspension was put in a centrifuge and the liquid removed. Additional 0.5 mL of TE buffer have been added and vortexed, the suspension was centrifuged, and the liquid removed again. To the combined, removed liquid, 1 M NaOH was added to a final concentration of 0.1 M to destroy RNA. The liquid was heated for 20 min at 90 °C. Then, the same amount of a 1 M HCl solution was added to neutralize the buffer. Precipitation of cDNA was done by adding the same volume of isopropanol. The tube was incubated at -20 °C for 2 h for precipitation. The isolated cDNA pellet was not washed, only dried and redissolved in 10 μL of water.

Cyclisation
The cDNA was cyclized using CircLigase II (Epicentre) using the standard protocol including Betaine. The reaction mixture was incubated for 2 h at 60 °C and inactivated for 10 min at 80 °C. The reaction mixture was used directly in the negative enrichment.

Negative enrichment
To the cyclisation reaction mixture, 7 μL of water, 2 μL of SSC buffer (20X) and 1 μL of fishing probe B (see Chapter 7) were added. This mixture was heated to 98°C for 3 minutes in the Bioer Mixing Block which was then shut off to anneal the probe to the sequence by slowing letting it cool down to room temperature (4 hours waiting period in total).

The RNA was then mixed with 6 μL of binding buffer (5X) and mixed with washed Streptavidin beads (100 μL beads originally, NEB, S1420S). The mixture was incubated at room temperature for 30
minutes. The flow-through was then isolated, since the beads should only bind circular cDNA with the stop-adapter on it. The flow-through-mixture was then directly used for PCR.

**PCR**

To optimize the amount of PCR cycles for exponential amplification, pilot PCR with 2 μL of circular, stop adapter depleted cDNA was done for all samples for 12, 14, 16, 18 and 20 cycles. For most, ~14 cycles were enough for having enough PCR product. The PCR-Polymerase used was a standard Taq-Polymerase (Sigma, D1806). The primers used were bought from NEB (E7335S for Set 1, E7500S for Set 2, compatible with Illumina sequencing platform). These primers allow platform-compatible multiplexing of samples.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circular cDNA</td>
<td>1 μL</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>1 μL</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>1 μL</td>
</tr>
<tr>
<td>dNTP (10 mM)</td>
<td>1 μL</td>
</tr>
<tr>
<td>Reaction Buffer</td>
<td>5 μL</td>
</tr>
<tr>
<td>DNA Polymerase</td>
<td>0.5 μL</td>
</tr>
</tbody>
</table>

For sequencing, the PCR with the determined amount of cycles was then done in a 50 μL reaction using the same conditions. PCR product was cleaned up with AMPure beads (Ambion) using 90 μL beads for the PCR reaction according to the standard protocol. The beads were eluted in beads elution buffer (40 μL) and submitted to sequencing.

**Sequencing**

Sequencing has been done either at the Genomics Facility Basel, CH; or at the Brigham Young University Utah, US using standard illumina sequencing, either on a HiSeq 2500 (SR50) or on a NextSeq (SR75). Before sequencing, the quality of the libraries was measured by the respective sequencing department using a Agilent Bioanalyzer 2100, and the samples were pooled according to their concentration at 200-400 bp. All samples and their total aligned reads are summarized in Table S3.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sequencing Facility</th>
<th>Read type</th>
<th>Multiplex-Group</th>
<th>Aligned Reads[^a]</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO, in vitro</td>
<td>Basel</td>
<td>SR75</td>
<td>B</td>
<td>16.698456</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7.105669</td>
</tr>
<tr>
<td>Untreated, in vitro</td>
<td>Basel</td>
<td>SR75</td>
<td>B</td>
<td>15.569810</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17.605559</td>
</tr>
<tr>
<td>CuSO₄₉, DR</td>
<td>Utah</td>
<td>SR50</td>
<td>C</td>
<td>0.805352</td>
</tr>
</tbody>
</table>

[^a]: In million reads (Mr) Duplicates naturally have two entries, single sample experiments one

**Adapters**

The adapters used for this paper are summarized in Table S4.

<table>
<thead>
<tr>
<th>Name</th>
<th>Supplier</th>
<th>Sequence[^a]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Universal</td>
<td>NEB</td>
<td>a-pp-CTGTAGGCACCATCAAT–NH₂</td>
</tr>
</tbody>
</table>
### Cloning

<table>
<thead>
<tr>
<th>Linker</th>
<th>Stop</th>
<th>Marker</th>
<th>Microsynt</th>
<th>ATCGTaggcaccugaaa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fishing</td>
<td>Microsynt</td>
<td>h</td>
<td>TTTACGGTCCTACGAT-BiotinTEG</td>
<td></td>
</tr>
<tr>
<td>Probe A</td>
<td>Fishing</td>
<td>Eurofins</td>
<td>Microsynt</td>
<td>BiotinTEG-ATCGTAGGCACCTGAAA</td>
</tr>
<tr>
<td>Probe B</td>
<td>Reverse transcriptio</td>
<td>h</td>
<td>Microsynt</td>
<td>pAGATCGGAAGAGCGTGTCGTGTAGGGAAAGAGTGTAGATCTCGGTG</td>
</tr>
</tbody>
</table>

**[a]** Sequence denoted in 5'->3' direction. Lower case letters denote RNA nucleotides, upper case DNA nucleotides. pp is a 5'-diphosphate-5' bridge. [Sp18] is an 18-atom hexa-ethylene glycol spacer.

### Bioinformatics

The raw reads were first cleaned from their 3’ adapter sequence using cutadapt [5], only using reads > 25 nt. Reads containing the 5’ stop adapter were removed completely from the read pool with cutadapt. Then, STAR [6] was used to align the reads to the human genome (Release Ch38.10 [7]). For STAR, the genome index was generated with a genome annotation file to only allow known splicing junctions. Reads with 1 < n <= 10 alignment possibilities were randomly distributed over the possible locations; reads with more than 10 alignment possibilities were ignored. STAR, in contrast to bowtie1, can also align insertions, deletions and introns. Using bedtools [8], the alignments were intersected with a gene annotation file to cross-reference feature metadata with read positions. From there, we used our own python procedures to add read raw information to the intersection (raw READ and CIGAR string) and to calculate the stop positions, as well as localization of reads, deletions, insertions and the nucleotides on each read on each position.

From this file, we used the algorithm developed by Talkish et al [9] to find significant positions by comparing every position in control and treatment with a Cochran-Mantel-Haenszel test. Positions are then classified according to their odds-ratio:

- Class 1: OR < 2
- Class 2: 2 <= OR < 3
- Class 3: 3 <= OR < 4
- Class 4: 4 <= OR < 5
- Class 5: OR >= 5

The python script collection was based on the original Mod Seeker program that was adjusted to our needs and is available at [https://github.com/Gillingham-Lab/ChemProfileSeq](https://github.com/Gillingham-Lab/ChemProfileSeq).

### Experimental data
Figure S1. Consensus sequence of the reverse transcription stop. Negative positions are upstream of the stop position (=0), positive ones are downstream.

Figure S2. This figure shows the preferred topography of G alkylations with CuSO₄ in comparison to TMS-DAM and Streptozotocin on 18S rRNA (DNA for the latter to have been taken from B. Sauter and D. Gillingham, ChemBioChem 2018, doi:10.1002/cbic.201800235). [10]
Figure S3. Western blot analysis shows enrichment of modified RNA. L1) first flow through after incubation with biotin elution buffer, L2) second flow through after incubation with biotin elution buffer, L3) first flow through after incubation with wash buffer.

Figure S4. PAGE image of alkylation reaction on mRNA at different concentration of Cu-NHC and CuSO₄. PAGE analysis shows partial degradation of mRNA at 100 eq. of CuSO₄. See Table 1 for reactions conditions. mRNA alkylation reactions were purified through commercial RNA clean and concentrator kit. Purified samples were analysed on 8 % non-denaturing PAGE.

L1: low MW ssRNA ladder
L2: mRNA without reaction
L3: Reaction A
L4: Reaction B
L5: Reaction C
L6: Reaction D
L7: Reaction E
Figure S5. HPLC analysis of NHC-Cu catalyzed GMP alkylation using butynediazoacetamide (GMP = guanosine monophosphate).

Figure S6. HPLC analysis of NHC-Cu catalyzed GMP alkylation with propyl diazoazidoacetamide (GMP = guanosine monophosphate).
Figure S7. HPLC analysis of NHC-Cu catalyzed GMP alkylation with DZ3 pipyridyl diazoacetamide (GMP = guanosine monophosphate.).

Figure S8. UPLC analysis of single nucleotides of unmodified RNA after enzyme digestions.
Figure S9. UPLC analysis of chemically synthesized desthiobiotinylated guanosine, (red) UV absorbance at 254 nm and (yellow) extracted ion trace for M/Z 808 Da.

Figure S10. UPLC analysis of desthiobiotinylated guanosine of transcriptomic RNA after enzyme digestions, (green) UV absorbance at 254 nm and (blue) extracted ion trace for M/Z 808 Da.
Figure S11. HPLC analysis of chemically synthesized O6-butynecmG (red) and O6-desthiobiotinG (blue) respectively. (GMP = guanosine monophosphate, DTB = desthiobiolin).

Figure S12. UPLC/MS analysis of NHC-Cu catalyzed O6-butynecmG M/Z = 473 Da.
**Figure S13.** UPLC/MS analysis of NHC-Cu catalyzed \(\text{O}^6\)-propylazidocmG M/Z = 504 Da.

**Figure S14.** UPLC/MS analysis of chemically synthesized standard \(\text{O}^\alpha\)-butynecmG M/Z = 473 Da.
Figure S15. UPLC/MS analysis of chemically synthesized standard O\textsuperscript{6}-desthiobiotin\textsuperscript{G} M/Z = 808 Da.

Figure S16. UPLC/MS analysis of O\textsuperscript{6}-desthiobiotin\textsuperscript{G} derived from transcriptomic RNA digestion after RNA modification M/Z = 808 Da. The noise is coming from the protein mixture which used unpurified snake venom phosphodiesterase.
Figure S17. HPLC traces of ssRNA (black line) and after its modification (red 1.5 h and 4 h (partial degradation)) with the Cu(I) catalyzed O6-G alkylation. The cyan line is alkylated RNA after SPAAC at 254 nm, the pink the same HPLC on the FLD (649 nm (ex) / 670 nm (em)) showing the conversion to the triazene. The last two entries (gray and red) show DBCO-Cy5 without RNA.

Figure S18. (black) ssRNA1 starting material reference. (red) ssRNA-alk re-injection after purification via HPLC. (blue) ssRNA-Cy5 re-injection after purification via HPLC. (cyan) FLD trace of ssRNA-Cy5 after purification.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical formula sum</td>
<td>C\textsubscript{30}H\textsubscript{42}ClCuN\textsubscript{6}</td>
</tr>
<tr>
<td>Chemical formula weight</td>
<td>585.71</td>
</tr>
<tr>
<td>Temperature</td>
<td>123</td>
</tr>
<tr>
<td>Crystal system</td>
<td>monoclinic</td>
</tr>
<tr>
<td>Space group</td>
<td>\textit{P 21/n}</td>
</tr>
<tr>
<td>Unit cell dimension</td>
<td></td>
</tr>
<tr>
<td>a/Å</td>
<td>16.6483(5)</td>
</tr>
<tr>
<td>(a^\circ)</td>
<td>90</td>
</tr>
<tr>
<td>b/Å</td>
<td>10.5534(4)</td>
</tr>
<tr>
<td>(b^\circ)</td>
<td>97.541(2)</td>
</tr>
<tr>
<td>c/Å</td>
<td>18.3838(6)</td>
</tr>
<tr>
<td>(c^\circ)</td>
<td>90</td>
</tr>
<tr>
<td>Volume/Å\textsuperscript{3}</td>
<td>3202.03(19)</td>
</tr>
<tr>
<td>z</td>
<td>4</td>
</tr>
<tr>
<td>Calculated density/ Mg/m³</td>
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</tr>
<tr>
<td>absorpt_coefficient_μ</td>
<td>4.325</td>
</tr>
<tr>
<td>F(000)</td>
<td>1240</td>
</tr>
<tr>
<td>crystal size/mm</td>
<td>0.180x 0.080x 0.040</td>
</tr>
<tr>
<td>Θ range data collection</td>
<td>2.93 to 57.649</td>
</tr>
<tr>
<td>Absorption correction</td>
<td>multi-scan</td>
</tr>
<tr>
<td>Data/restraints/parameters</td>
<td>3713/0/343</td>
</tr>
<tr>
<td>Goodness-of-fit on F\textsuperscript{2}</td>
<td>0.9887</td>
</tr>
<tr>
<td>R Indices ([I&gt;1.0\sigma(I)])</td>
<td>R\textsubscript{1} = 0.0883</td>
</tr>
<tr>
<td></td>
<td>(wR\textsubscript{2} = 0.0936)</td>
</tr>
<tr>
<td>R Indices (all data)</td>
<td>R\textsubscript{1} = 0.1541</td>
</tr>
<tr>
<td></td>
<td>(wR = 0.1890)</td>
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<tr>
<td>refine_diff_density</td>
<td>-0.82 / 0.63</td>
</tr>
<tr>
<td>CCDC Number</td>
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</tr>
</tbody>
</table>
Table S6. Characteristic bond distances of 1 in crystal structure

<table>
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**Table S7. Characteristic Bond angles of 1 in crystal structure**

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NMR Spectra:

**Figure S20.** $^1$H NMR spectra of compound M2 in CDCl$_3$.

**Figure S21.** $^{13}$C NMR spectra of compound M2 in CDCl$_3$. 
Figure S22. $^1$H NMR spectra of compound M3 in CDCl$_3$.

Figure S23. $^{13}$C NMR spectra of compound M3 in CDCl$_3$. 
Figure S24. $^1$H NMR spectra of compound M4 in CDCl$_3$

Figure S25. $^{13}$C NMR spectra of compound M4 in CDCl$_3$
Figure S26. $^1$H NMR spectra of compound M6 in C$_6$D$_6$.

Figure S27. $^{13}$C NMR spectra of compound M6 in C$_6$D$_6$ (Benzene-d$_6$).
Figure S28. $^1$H NMR spectra of compound M7 in CDCl$_3$

Figure S29. $^{13}$C NMR spectra of compound M7 in CDCl$_3$
Figure S30. $^1$H NMR spectra of final ligand 4 in CDCl$_3$

Figure S31. $^{13}$C NMR spectra of final ligand 4 in CDCl$_3$
Figure S32. ^1^H NMR spectra of final complex 1 in CDCl$_3$

Figure S33. ^13^C NMR spectra of final complex 1 in CDCl$_3$
Figure S34. $^1$H NMR spectra of final complex M9 in CD$_3$OD (methanol-d$_4$)
Figure S35. $^1$H NMR spectra of final complex 6 in CD$_3$OD (methanol-d$_4$)

Figure S36. $^{13}$C NMR spectra of final complex 6 in MeOD
Figure S37. $^1$H NMR spectra of compound M10 in CDCl$_3$.

Figure S38. $^1$H NMR spectra of compound M11 in CDCl$_3$. 
Figure S39. $^1$H NMR spectra of final compound DZ1 in CDCl$_3$.

Figure S40. $^{13}$C NMR spectra of final compound DZ1 in CDCl$_3$. 
Figure S41. $^1$H NMR spectra of standard compound S12 in DMSO-d$_6$

Figure S42. $^{13}$C NMR spectra of standard compound S12 in DMSO-d$_6$
References

(5) M. Martin, EMBNet.journal 2011, 17, 10-12.
(8) A. R. Quinlan and I. M. Hall, Bioinformatics 2010, 26, 841-842.