**The RNA methyltransferase METTL8 installs $m^3C_{32}$ in mitochondrial $tRNAs^{Thr/Ser(UCN)}$ to optimise tRNA structure and mitochondrial translation**

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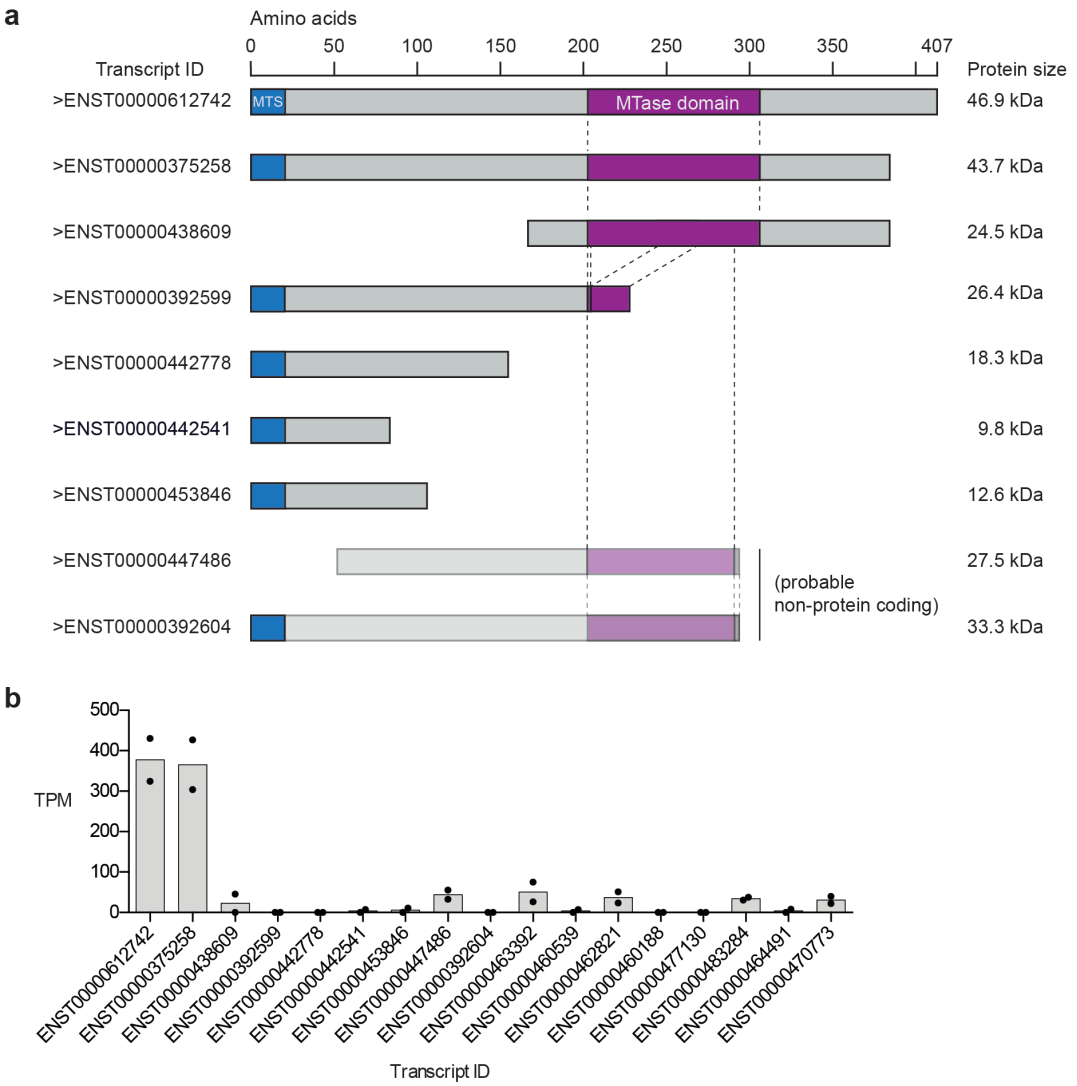
**SUPPLEMENTARY MATERIAL**

## SUPPLEMENTARY RESULTS

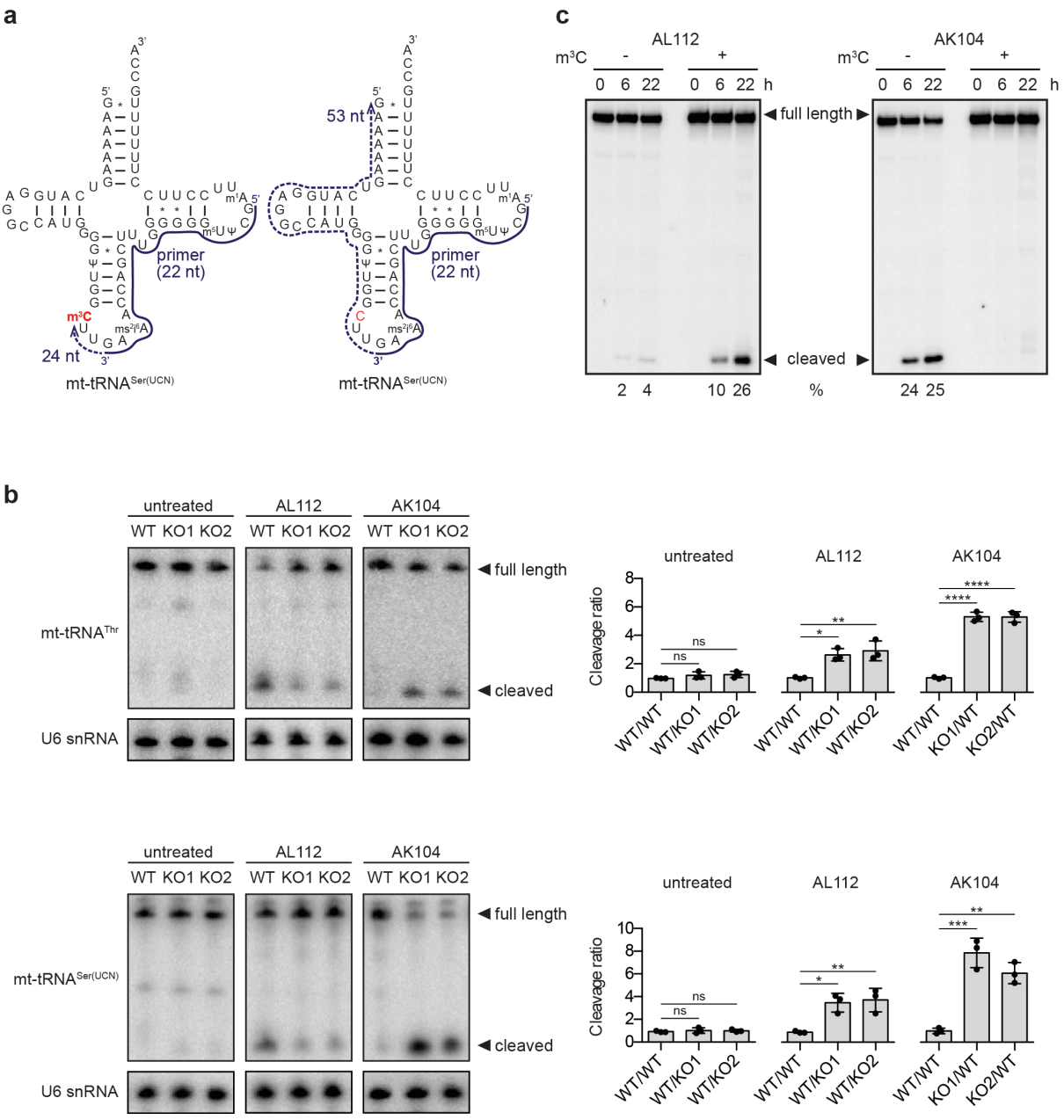
### Supplementary Note 1. Depletion of SARS2 does not strongly affect $m^3C_{32}$ levels in mt-$tRNA^{Ser(UCN)}$ and does not significantly stimulate *in vitro* methylation by METTL8.

The yeast seryl-tRNA synthetase promotes methylation of $tRNA^{Ser}$ by Trm140 and the human mitochondrial seryl-tRNA synthetase SARS2 is present in METTL8-containing complexes[1,2]. Therefore, the requirement of SARS2 for the METTL8-mediated methylation of $C_{32}$ of mt-$tRNA^{Ser(UCN)}$ was investigated. Small RNAs from control cells and those transiently depleted of SARS2, or METTL8 for comparison, by RNAi (Supplementary Figs. 6a and b) were subjected to primer extension to monitor $m^3C_{32}$ levels in mt-$tRNA^{Thr/Ser(UCN)}$. Depletion of METTL8 lead to significantly increased read-through of the $m^3C_{32}$ modification detected in cells treated with control siRNAs, demonstrating that even transient depletion of the enzyme required for $C_{32}$ methylation leads to detectable changes in $m^3C_{32}$ levels in mt-$tRNA^{Thr/Ser(UCN)}$ (Supplementary Fig. 6c). In contrast, decreasing the levels of SARS2 only very mildly affected the amount of $m^3C_{32}$ detected in mt-$tRNA^{Ser(UCN)}$ (Supplementary Fig. 6d). SARS2 interacts with mt-$tRNA^{Ser}$ via the TΨC and D loops[3] but *in vitro* methylation assays on *in vitro* transcribed full-length mt-$tRNA^{Ser(UCN)}$ revealed no methylation by $His_{10}$-SARS2 and almost no stimulation of $His_{14}$-MBP-METTL8-mediated methylation by the presence of $His_{10}$-SARS2 (Supplementary Fig. 6e). Although these transcripts lack $(ms^2)i^6A_{37}$, *in vitro* methylation of $tRNA^{Arg(CCU/UCU)}$ by METTL2A/B, which is likely also stimulated by the presence of a modified $A_{37}$, is enabled by the DALRD3 cofactor even in the absence of the $t^6A_{37}$[4]. However, the presence of SARS2 does not overcome the requirement for $(ms^2)i^6A_{37}$ for METTL8-mediated methylation of $C_{32}$ in the case of mt-$tRNA^{Ser(UCN)}$. In line with the finding that $His_{14}$-MBP-METTL8 alone could fully restore $m^3C_{32}$ in mt-$tRNA^{Ser(UCN)}$ (Figs. 3e and f), these data suggest that SARS2 is largely dispensable for $C_{32}$ methylation of mt-$tRNA^{Ser(UCN)}$.

## SUPPLEMENTARY FIGURES

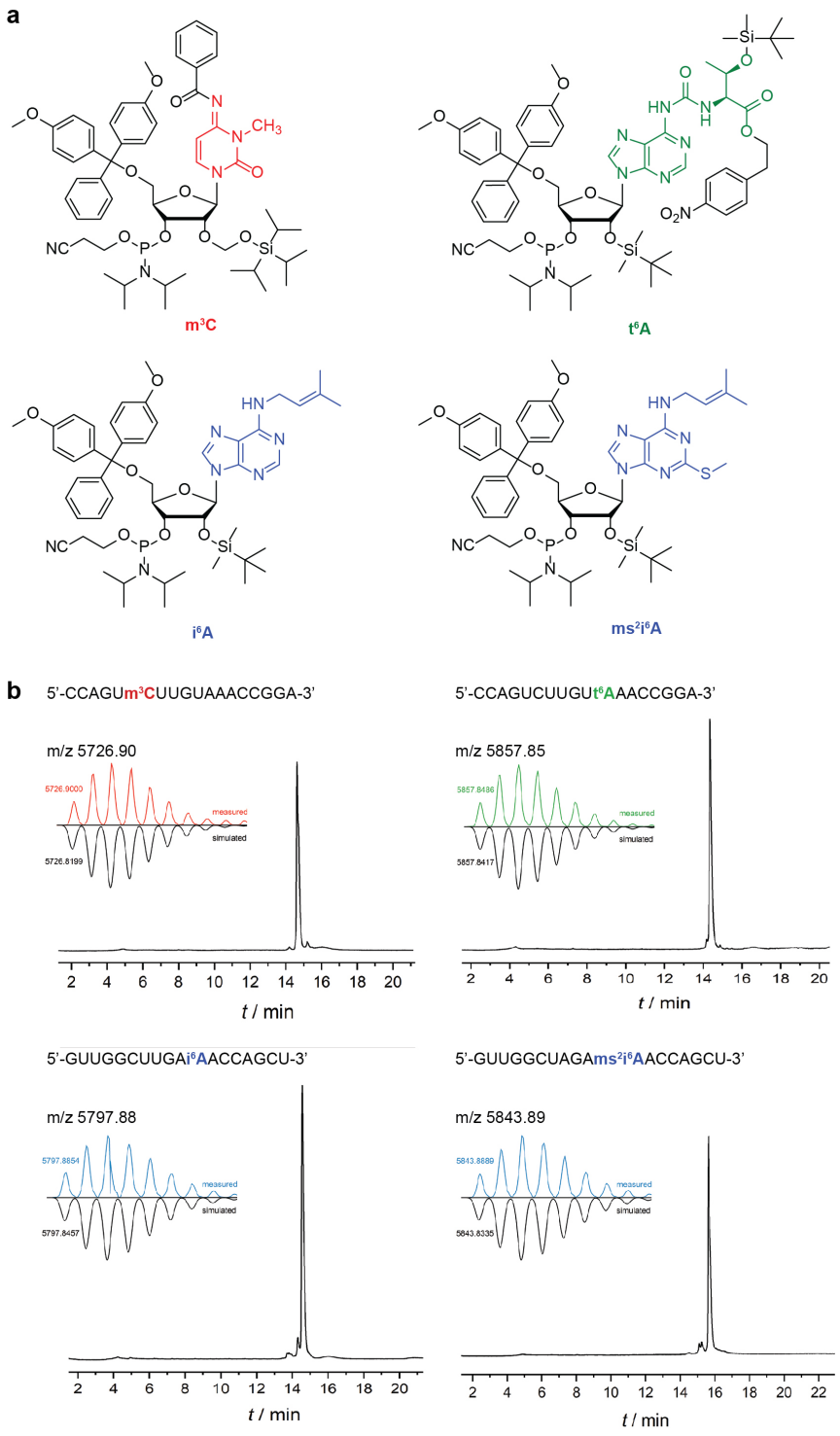



**Supplementary Figure 1. METTL8 isoforms expressed in HEK293 cells contain the mitochondrial targeting signal. a** Schematic view of the proteins encoded by the METTL8 isoforms annotated at the ENSEMBL database according the human genome version GHRch38. Only the main predicted variants are shown. **b** Total RNA from HEK293 cells was subjected to RNA-seq. The abundance of different METTL8 transcripts is represented in transcript per million (TPM) for METTL8. Results from n=2 independent datasets are presented as mean ± standard deviation. Source data are provided as a Source Data file.

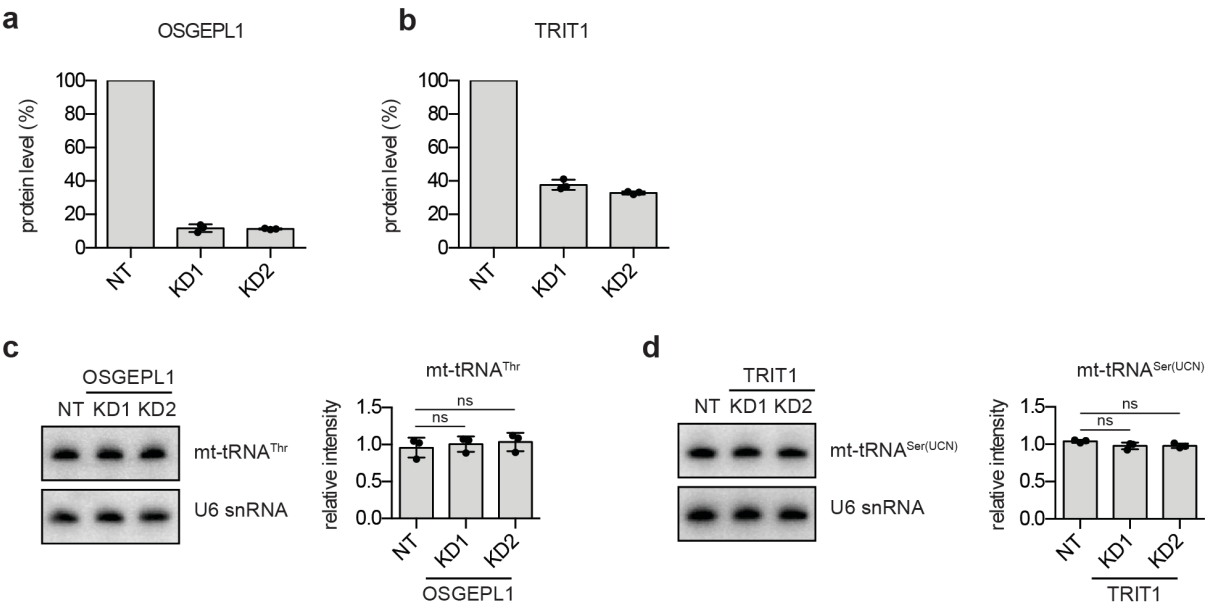



**Supplementary Figure 2. Analysis of $m^3C_{32}$ in mt-tRNA$^{Thr/Ser(UCN)}$. a** Schematic views of $m^3C_{32}$- and $C_{32}$-containing mt-tRNA$^{Ser(UCN)}$ are shown with the binding site of the 22 nucleotide (nt) primer used for primer extension analyses indicated by a solid purple line. Expected primer extension products are indicated by dashed lines and their lengths indicated in nt. **b** Small RNAs extracted from WT and METTL8 KO cells were either left untreated (left panel), subjected to cleavage with an $m^3C$ modification-specific deoxyribozyme (AL112; middle panel) or a deoxyribozyme that cleaves unmodified RNA (AK104; right panel) and formation of

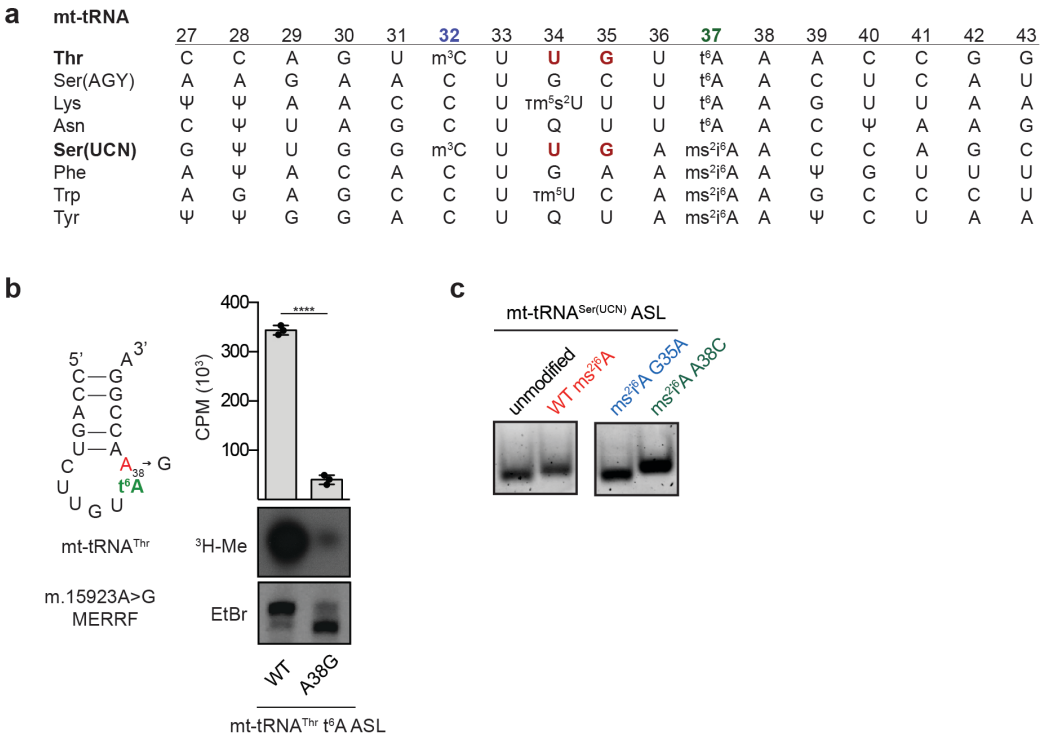
cleavage fragments was analysed by northern blotting. Black arrows indicate the full length and the cleaved mt-tRNA$^{Thr}$ (top) or mt-tRNA$^{Ser(UCN)}$ (bottom). Cleavage ratios were calculated between WT and WT or WT and KO using data from three independent experiments. Values are shown as mean ± standard deviation (error bar) and individual data points are represented by dots. Statistical analysis was done using one-way ANOVA ($F=1.579$, non-significant for untreated m-tRNA$^{Thr}$; $F=14.07$ and $p<0.01$ for mt-tRNA$^{Thr}$ fragments cleaved by AL112; $F=214.1$ and $p<0.0001$ for mt-tRNA$^{Thr}$ fragments cleaved by AK104; $F=0.2932$, non-significant for untreated mt-tRNA$^{Ser(UCN)}$; $F=12.43$ and $p<0.01$ for mt-tRNA$^{Ser(UCN)}$ fragments cleaved by AL112; $F=43.97$ and $p<0.001$ for mt-tRNA$^{Ser(UCN)}$ fragments cleaved by AK104) and significance was calculated using Tukey's multiple comparisons test. **c** Synthetic mt-tRNA$^{Ser(UCN)}$ was treated with deoxyribozymes AL112 and AK104 in a single experiment performed as in (**b**). Source data and p values are provided as a Source Data file.



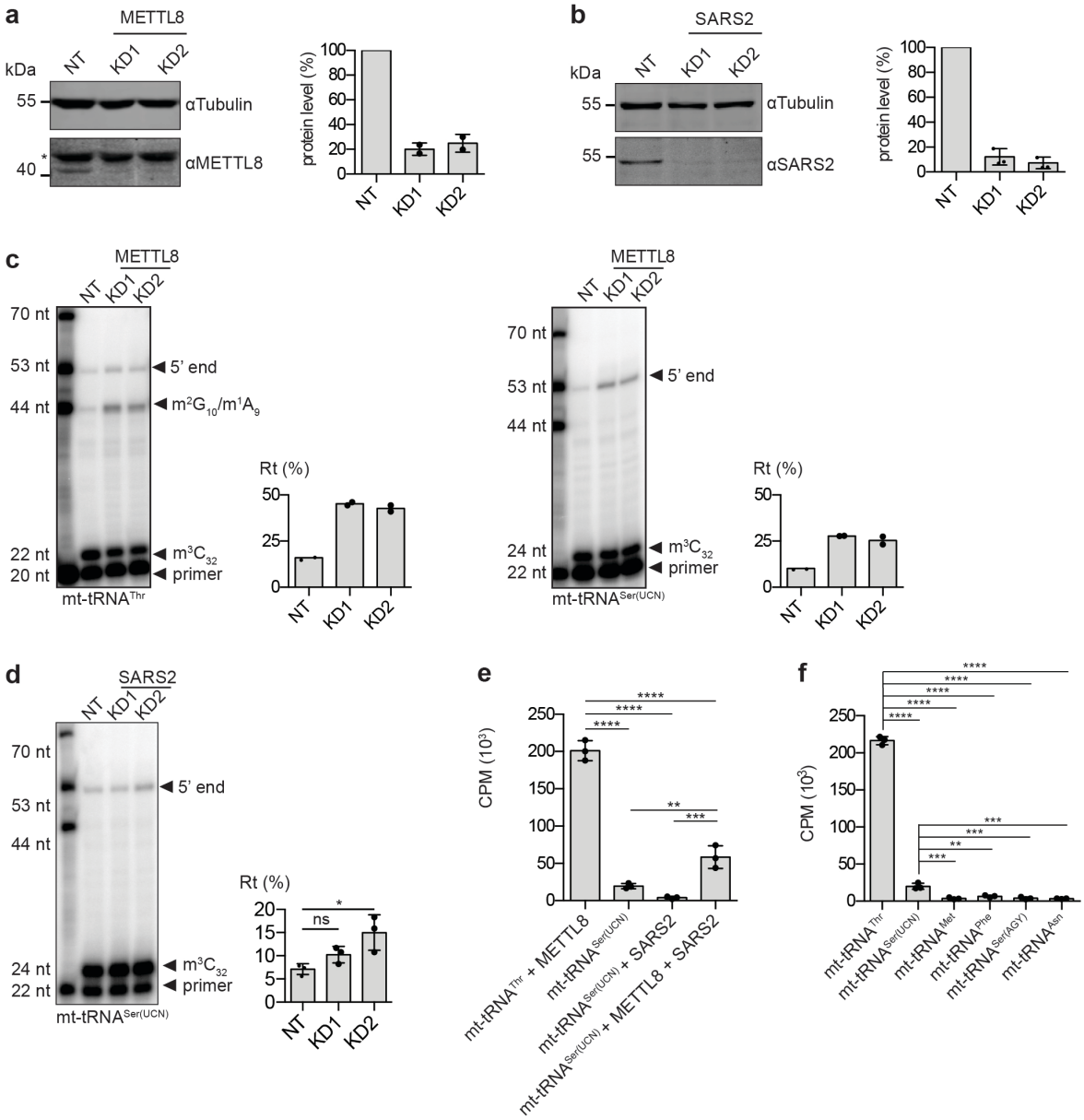

**Supplementary Figure 3. Solid-phase synthesis of ASL RNA oligonucleotides. a** Phosphoramidite building blocks used in this study for the incorporation of $m^3C$, $t^6A$, $i^6A$ and $ms^2i^6A$ (synthetic schemes are in Supplementary Figs. 9 and 10). **b** Exemplary anion exchange HPLC and HR-ESI-MS profiles for PAGE-purified RNA oligonucleotides containing the modified nucleotides. Source data are provided as a Source Data file.




**Supplementary Figure 4. RNAi-mediated depletion of OSGEPL1 and TRIT1. a,b** HEK293 cells were transfected with non-target siRNAs (NT) or two different siRNAs against OSGEPL1 (**a**) or TRIT1 (**b**). Protein extracted from siRNA-treated cells was analysed by western blotting and representative images are shown in Fig. 4d. Signal intensities were quantified in n=3 independent experiments and are shown as mean ± standard deviation. **c,d** Total RNAs from cells transfected with non-target siRNAs (NT) or two different siRNAs (KD1 and KD2) against OSGEPL1 (c) or TRIT1 (d) were separated by denaturing PAGE and mt-tRNA$^{Thr/Ser(UCN)}$ and the U6 snRNA were detected using northern blotting. Representative images of three independent experiments are shown (left). Hybridization signals were quantified and normalised signal intensities from n=3 independent experiments are shown as mean ± standard deviation (right). Statistical analysis was performed using one-way ANOVA (F=0.3128, not significant (c) and F=3.437, not significant (d)) and significance calculated using Tukey's multiple comparisons test. p-values for data in panels c and d are given in source data; ns = not significant. Source data and p values are provided as a Source Data file.

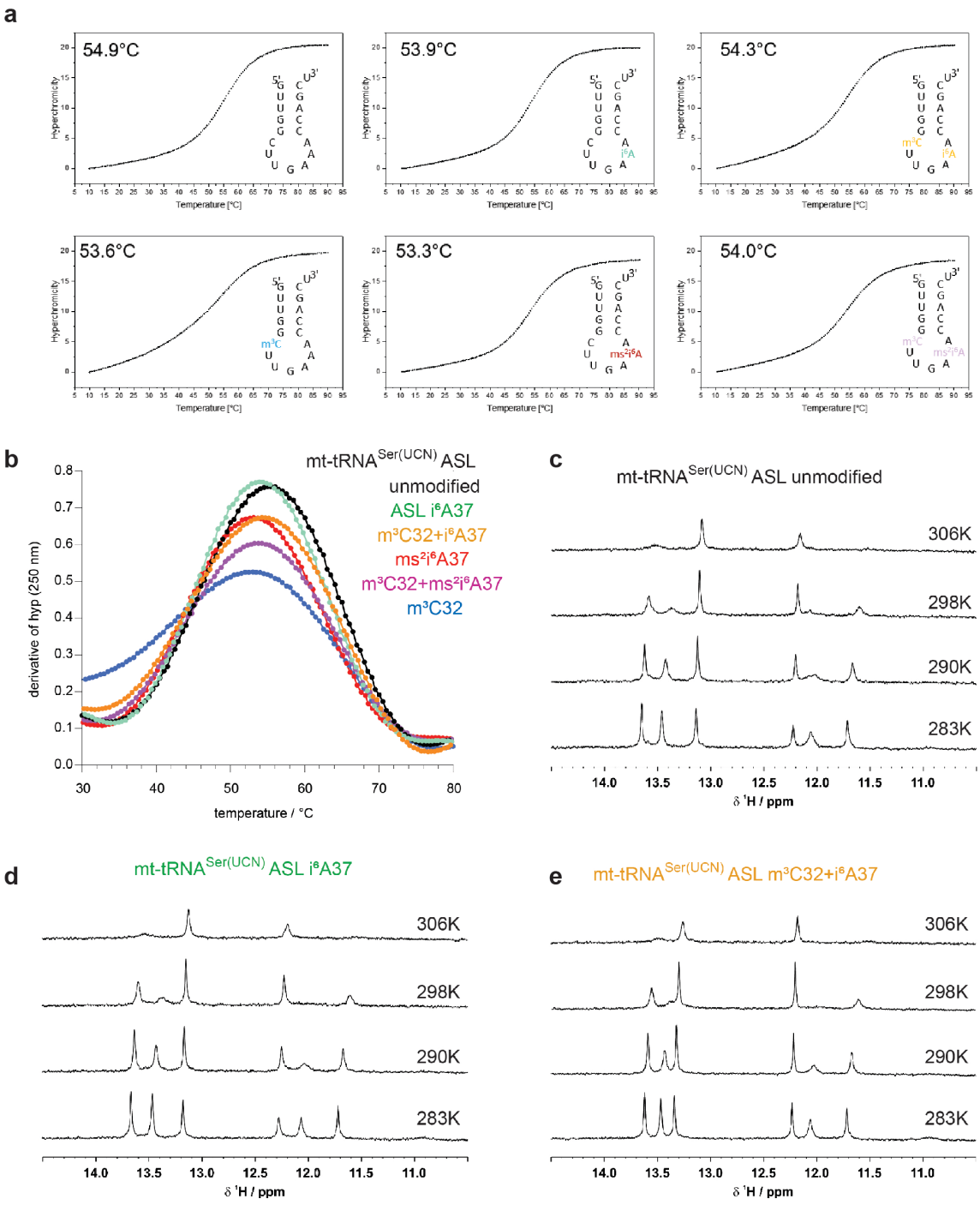



**Supplementary Figure 5. Sequences of mt-tRNAs containing $C_{32}$ and modified $A_{37}$. a** Nucleotide sequences of the anticodon stem loops of mt-tRNAs containing cytidine at position 32 and a modified adenosine at position 37 are shown. Assigned nucleotide numbers (given above) are based on the secondary structure of the mt-RNAs according to standard tRNA nomenclature. Nucleotides unique to mt-tRNA$^{Thr/Ser(UCN)}$ are highlighted in red. **b** An *in vitro* methylation assay was performed using $t^6A_{37}$-containing mt-tRNA$^{Thr}$ ASLs containing A38 (WT) or G38 with $His_{14}$-MBP-METTL8 and [$^3$H]-SAM. Tritium incorporated into the RNA was measured by scintillation counting. Bar plots show mean counts per minute (CPM) of n=3 independent experiments ± standard deviation. Statistical analysis was performed using a two-tailed unpaired Student's t-test (****p<0.0001). RNA was separated by denaturing PAGE, stained with ethidium bromide (EtBr) and labelled RNAs ($^3$H-Me) were detected by autoradiography. Representative image from three independent experiments. **c** Synthetic mt-mt-tRNA$^{Ser(UCN)}$ ASLs with/without the modifications and nucleotide substitutions indicated were separated by native PAGE, and detected using sybr gold staining. A single representative experiment is shown. Source data are provided as a Source Data file.



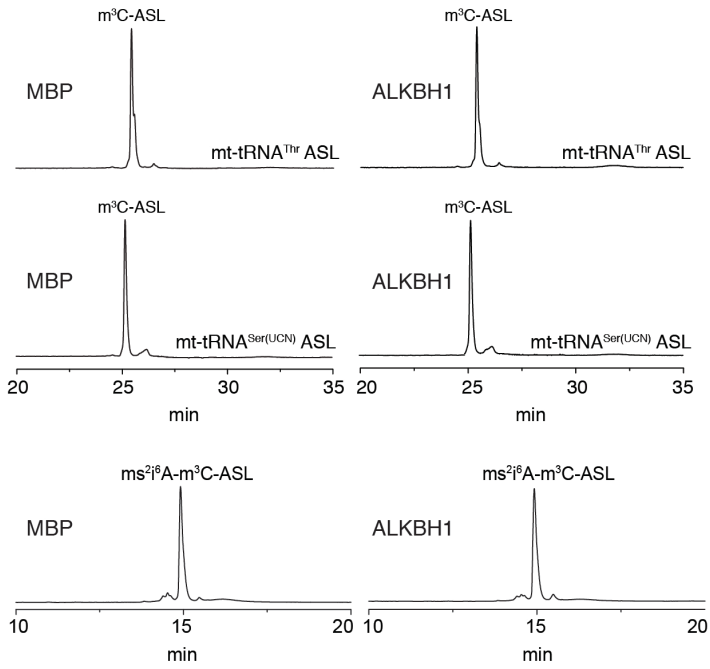

**Supplementary Figure 6. SARS2 is not strictly required for $m^3C_{32}$ formation in mt-tRNA$^{Thr/Ser(UCN)}$. a,b** HEK293 cells were transfected with non-target siRNAs (NT), or those targeting METTL8 (**a**) or SARS2 (**b**). 72 h after transfection, proteins were extracted, separated by SDS-PAGE and analysed by western blotting using the indicated antibodies. Three independent experiments were performed and representative images are shown (left). Quantified protein levels in n=3 experiments are shown as mean ± standard deviation (right). Asterisk indicates a non-specific cross-reaction of the METTL8 antibody. **c,d** Primer extension was performed on small RNA extracted from siRNA-treated cells as in (**a,b**) using [$^{32}$P]-labelled probes hybridising to mt-tRNA$^{Thr}$ (**c**, left panel) and mt-tRNA$^{Ser(UCN)}$ (**c**, right panel and

**d**). Products were separated by denaturing PAGE alongside [$^{32}$P]-labelled DNA oligonucleotides of the indicated sizes and detected using a phosphorimager. Arrows depict non-extended primer and primer extension stops. Signal intensities of the extension products were quantified and the percentage of read-through (Rt) of $m^3C_{32}$ is given as a percentage of the total extension signal. Data from n=2 (**c**) or n=3 (**d**) independent experiments are shown as mean ± standard deviation (error bars) and individual data points are indicated by dots. For (**d**) statistical analysis was performed using one-way ANOVA (F=7.359, p<0.05) and significance was calculated using Tukey's multiple comparisons test (*p<0.05, and ns = not significant). **e** *In vitro* transcribed mt-tRNA$^{Thr/Ser(UCN)}$ was incubated with purified $His_{14}$-MBP-METTL8 in the presence or absence of $His_{10}$-SARS2 and [$^3$H]SAM or $His_{10}$-SARS2 and [$^3$H]SAM alone. Tritium incorporated into the RNA was measured by scintillation counting. Bar plots show counts per minute (CPM) of three independent methylation experiments, error bars represent mean ± standard deviation and individual data points are indicated by dots. Statistical analysis was performed using one-way ANOVA (F=225.8; p<0.0001) and significance was calculated using Tukey's multiple comparisons test (**p<0.01, ***p<0.001, ****p<0.0001). **f** *In vitro* transcribed mt-tRNAs (indicated) were incubated with purified $His_{14}$-MBP-METTL8 and [$^3$H]-SAM. Tritium incorporated into the RNA was measured by scintillation counting. Bar plots show counts per minute (CPM) of n=3 independent methylation experiments, error bars represent mean ± standard deviation and individual data points are indicated by dots. Statistical analysis was performed using one-way ANOVA (F=2458; p<0.0001) and significance was calculated using Tukey's multiple comparisons test (**p<0.01, ***p<0.001, ****p<0.0001). Source data and p values are provided as a Source Data file.

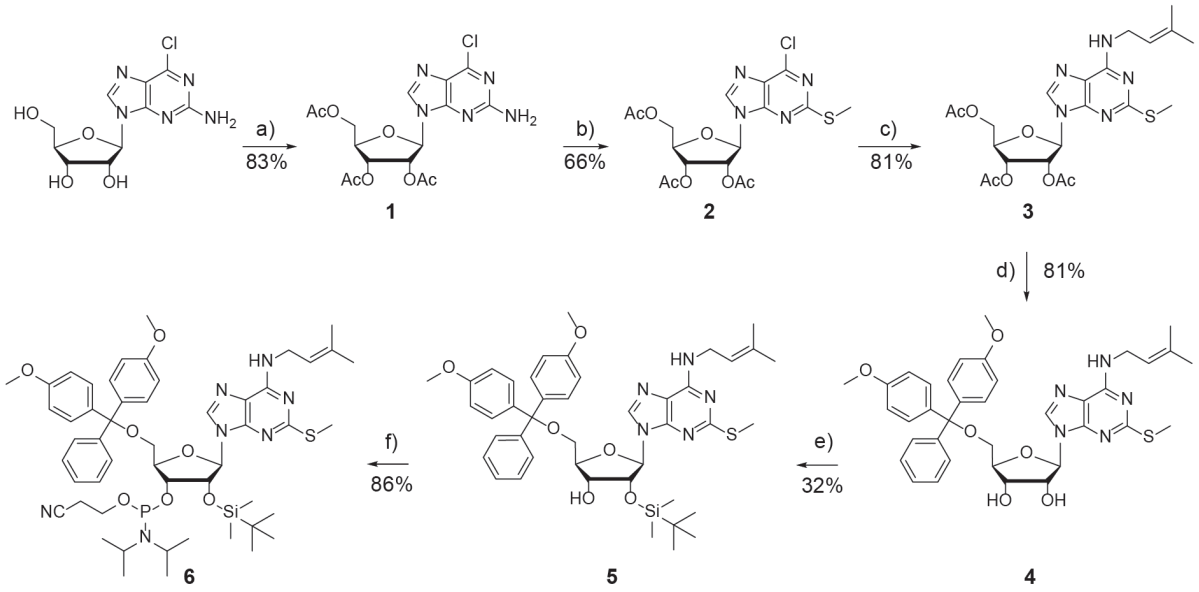



**Supplementary Figure 7. UV thermal melting and $^{1}$H NMR spectroscopy of mt-tRNA$^{Ser(UCN)}$ ASLs. a** Hyperchromicity at 250 nm plotted versus temperature for unmodified ASL (black), three singly modified ASLs containing $m^3C_{32}$ (blue), $i^6A_{37}$ (green) or $ms^2i^6A_{37}$ (red), and two double-modified ASLs containing $m^3C_{32}$ and $i^6A_{37}$ (orange) or $m^3C_{32}$ and $ms^2i^6A_{37}$ (magenta). Conditions: 2 μM RNA in 10 mM sodium phosphate buffer pH 7.0, 100 mM NaCl.

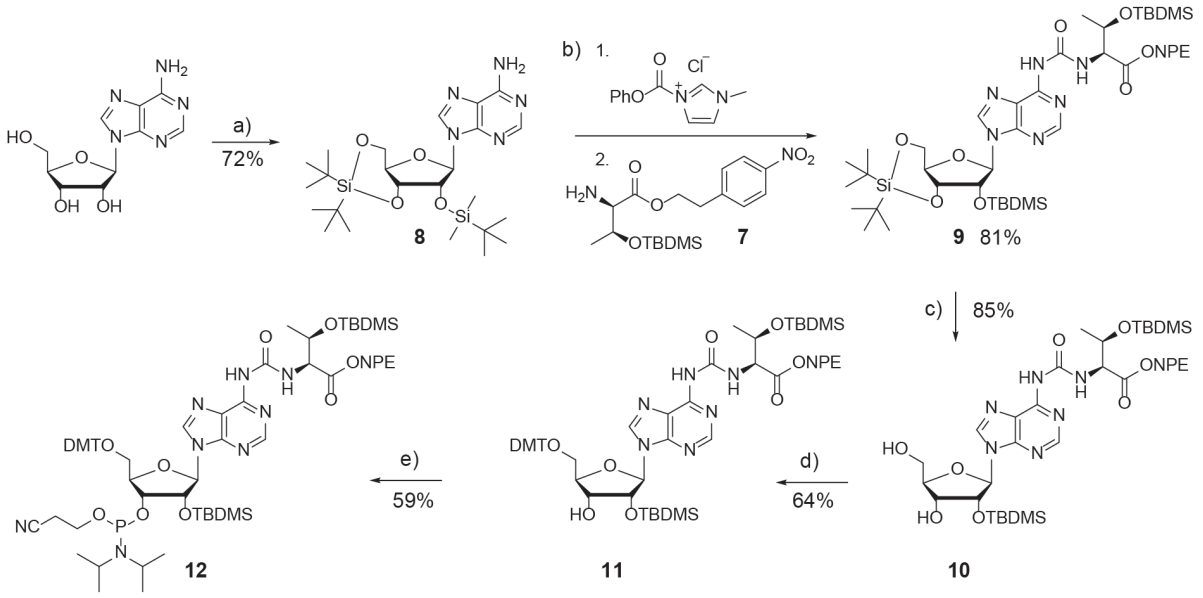
Five heating and cooling ramps were measured and showed fully reversible melting profiles. One representative heating ramp is shown. **b** Overlay of derivative of hyperchromicity curves. Colour code as in (**a**). The maximum of the curve corresponds to the melting temperature. **c-e** Imino proton region of $^{1}H$ NMR spectra recorded at 10°C, 17°C, 25°C and 33°C with 200 µM RNA in 10 mM sodium phosphate buffer pH 7.0, 100 mM NaCl in $H_2O/D_2O$ 9/1 at 600 MHz. (**c**) Unmodified mt-tRNA$^{Ser(UCN)}$ ASL, (**d**) mt-tRNA$^{Ser(UCN)}$ ASL containing $i^6A_{37}$, (**e**) mt-tRNA$^{Ser(UCN)}$ ASL containing $m^3C_{32}$ and $i^6A_{37}$. NMR samples were prepared once, and repeatedly measured at 298K after heating and cooling. Source data are provided as a Source Data file.




**Supplementary Figure 8. Demethylation of $m^3C$-containing RNAs by ALKBH1.** $m^3C_{32}$-containing ASLs of mt-tRNA$^{Thr/Ser(UCN)}$ were treated with $His_{14}$-MBP or $His_{10}$-ALKBH1 and the resultant RNAs were analysed by reverse phase high performance liquid chromatography. Two experiments were performed and data from a single experiment are shown. Source data are provided as a Source Data file.




**Supplementary Figure 9. Synthesis of ms$^2$i$^6$A phosphoramidite. a** $Ac_2O$, DMAP, $NEt_3$, in MeCN. **b** dimethyl disulfide, isoamyl nitrite in MeCN, 60 °C. **c** Isopentenyl amine·HCl, $NEt_3$, in pyridine. **d** 1. MeOH, $NEt_3$, 2. DMT-Cl in pyridine. **e** TBDMS-Cl, $AgNO_3$ in THF/pyridine (1:1). **f** $Me_2NEt$, CEP-Cl in DCM. Source data are provided as a Source Data file.




**Supplementary Figure 10. Synthesis of $t^6A$ phosphoramidite. a** 1. DTBS triflate in DMF, 0°C, 2. imidazole, TBDMS-Cl in DMF, 60°C. **b** 1. in DCM, 2. $NEt_3$ in DCM. **c** $Py\cdot(HF)_n$ in pyridine. **d** DMT-Cl in pyridine. **e** CEP-Cl, DIPEA in DCM. Source data are provided as a Source Data file.

## SUPPLEMENTARY TABLES

**Supplementary Table 1. Expression analysis of *METTL8* isoforms in HEK293 cells**

| Transcript ID | HEK293_1 Raw | HEK293_1 Norm. | HEK293_2 Raw | HEK293_2 Norm. | Mean Raw | Mean Norm. |
|---|---|---|---|---|---|---|
| ENST00000612742 | 324 | 343 | 430 | 399 | 377 | 371 |
| ENST00000375258 | 427 | 451 | 314 | 282 | 365 | 367 |
| ENST00000438609 | 0 | 0 | 45 | 42 | 23 | 21 |
| ENST00000392599 | 0 | 0 | 0 | 0 | 0 | 0 |
| ENST00000442778 | 0 | 0 | 0 | 0 | 0 | 0 |
| ENST00000442541 | 0 | 0 | 8 | 7 | 4 | 4 |
| ENST00000453846 | 0 | 0 | 11 | 10 | 6 | 5 |
| ENST00000447486 | 56 | 59 | 33 | 31 | 44 | 45 |
| ENST00000392604 | 0 | 0 | 0 | 0 | 0 | 0 |
| ENST00000463392 | 75 | 80 | 26 | 24 | 51 | 52 |
| ENST00000460539 | 0 | 0 | 8 | 7 | 4 | 4 |
| ENST00000462821 | 51 | 54 | 24 | 22 | 37 | 38 |
| ENST00000460188 | 0 | 0 | 0 | 0 | 0 | 0 |
| ENST00000477130 | 0 | 0 | 0 | 0 | 0 | 0 |
| ENST00000483284 | 31 | 33 | 38 | 35 | 34 | 34 |
| ENST00000464491 | 8 | 8 | 0 | 0 | 4 | 4 |
| ENST00000470773 | 40 | 42 | 22 | 21 | 31 | 31 |

**Supplementary Table 2. Overview of numbers of sequencing reads deriving from different classes of RNAs in the $His_6$-2xFLAG and METTL8-$His_6$-2xFLAG datasets**

| RNA class | $His_6$-2xFLAG Raw counts | $His_6$-2xFLAG Normalised counts | METTL8-$His_6$-2xFLAG Raw counts | METTL8-$His_6$-2xFLAG Normalised counts |
|---|---|---|---|---|
| pseudogenes | 5525 | 938 | 113172 | 268182 |
| tRNA | 39785 | 6753 | 46547 | 110302 |
| lncRNA | 57151 | 9701 | 37850 | 89693 |
| mRNA | 62465 | 10602 | 160466 | 380254 |
| other | 102815 | 17451 | 20844 | 49394 |
| mt-RNAs | 15639 | 2654 | 930049 | 2203925 |
| mt-mRNA | 1125 | 191 | 72829 | 172582 |
| mt-rRNA | 4750 | 806 | 148642 | 352235 |
| mt-tRNA | 9764 | 1657 | 708578 | 1679108 |

**Supplementary Table 3. Numbers of sequencing reads mapping to different mt-tRNA genes in the $His_6$-2xFLAG and METTL8-$His_6$-2xFLAG datasets**

| mt-tRNA gene | $His_6$-2xFLAG Raw counts | $His_6$-2xFLAG Normalised counts | METTL8-$His_6$-2xFLAG Raw counts | METTL8-$His_6$-2xFLAG Normalised counts |
|---|---|---|---|---|
| mt-tRNA$^{Thr}$ | 13 | 22 | 4316 | 10228 |
| mt-tRNA$^{Ser(UCN)}$ | 4 | 7 | 1117 | 2647 |
| mt-tRNA$^{Ile}$ | 55 | 93 | 11983 | 28396 |
| mt-tRNA$^{Ala}$ | 27 | 46 | 5135 | 12168 |
| mt-tRNA$^{His}$ | 121 | 205 | 20921 | 49576 |
| mt-tRNA$^{Lys}$ | 177 | 300 | 26900 | 63745 |
| mt-tRNA$^{Leu(CUN)}$ | 257 | 436 | 28972 | 68654 |
| mt-tRNA$^{Pro}$ | 541 | 918 | 58464 | 138541 |
| mt-tRNA$^{Asp}$ | 193 | 328 | 19469 | 46135 |
| mt-tRNA$^{Val}$ | 1042 | 1769 | 100383 | 237876 |
| mt-tRNA$^{Gly}$ | 228 | 387 | 21160 | 50143 |
| mt-tRNA$^{Leu(UUR)}$ | 148 | 251 | 13527 | 32055 |
| mt-tRNA$^{Asn}$ | 292 | 496 | 26510 | 62820 |
| mt-tRNA$^{Arg}$ | 212 | 360 | 15757 | 37339 |
| mt-tRNA$^{Met}$ | 965 | 1638 | 62958 | 149191 |
| mt-tRNA$^{Glu}$ | 1198 | 2033 | 77252 | 183063 |
| mt-tRNA$^{Tyr}$ | 163 | 277 | 9798 | 23218 |
| mt-tRNA$^{Gln}$ | 64 | 109 | 3811 | 9031 |
| mt-tRNA$^{Trp}$ | 70 | 119 | 4102 | 9720 |
| mt-tRNA$^{Cys}$ | 541 | 918 | 31100 | 73697 |
| mt-tRNA$^{Ser(AGY)}$ | 1859 | 3155 | 98142 | 232566 |
| mt-tRNA$^{Phe}$ | 1594 | 2706 | 66801 | 158297 |

**Supplementary Table 4. Numbers of sequencing reads mapping to each nucleotide of the genes encoding mt-tRNA$^{Thr}$ in the $His_6$-2xFLAG and METTL8-$His_6$-2xFLAG datasets**

| Nucleotide position in mt-tRNA$^{Thr}$ | $His_6$-2xFLAG Raw counts | $His_6$-2xFLAG Normalised counts | METTL8-$His_6$-2xFLAG Raw counts | METTL8-$His_6$-2xFLAG Normalised counts |
|---|---|---|---|---|
| G1 | 1 | 0.2 | 1146 | 2716 |
| U2 | 1 | 0.2 | 1157 | 2742 |
| C3 | 1 | 0.2 | 1162 | 2754 |
| C4 | 1 | 0.2 | 1169 | 2770 |
| U5 | 1 | 0.2 | 1175 | 2784 |

| | | | | |
|---|---|---|---|---|
| U6 | 1 | 0.2 | 1184 | 2806 |
| G7 | 1 | 0.2 | 1188 | 2815 |
| U8 | 1 | 0.2 | 1196 | 2834 |
| A9 | 1 | 0.2 | 1211 | 2870 |
| G10 | 1 | 0.2 | 1220 | 2891 |
| U11 | 1 | 0.2 | 1243 | 2946 |
| A12 | 1 | 0.2 | 1273 | 3017 |
| U13 | 1 | 0.2 | 1353 | 3206 |
| A14 | 1 | 0.2 | 1423 | 3372 |
| A15 | 1 | 0.2 | 1471 | 3486 |
| A16 | 1 | 0.2 | 1919 | 4547 |
| C17 | 6 | 1.0 | 2775 | 6576 |
| U18 | 10 | 1.7 | 3529 | 8363 |
| A19 | 12 | 2.0 | 3636 | 8616 |
| A20 | 12 | 2.0 | 3666 | 8687 |
| U21 | 13 | 2.2 | 3803 | 9012 |
| A22 | 13 | 2.2 | 3821 | 9055 |
| C23 | 13 | 2.2 | 3863 | 9154 |
| A24 | 13 | 2.2 | 3856 | 9138 |
| C25 | 13 | 2.2 | 3839 | 9097 |
| C26 | 13 | 2.2 | 3821 | 9055 |
| A27 | 13 | 2.2 | 3769 | 8931 |
| G28 | 13 | 2.2 | 3510 | 8318 |
| U29 | 13 | 2.2 | 3268 | 7744 |
| C30 | 13 | 2.2 | 3593 | 8514 |
| U31 | 13 | 2.2 | 3679 | 8718 |
| U32 | 13 | 2.2 | 3790 | 8981 |
| G33 | 13 | 2.2 | 3804 | 9014 |
| U34 | 13 | 2.2 | 3813 | 9036 |
| A35 | 13 | 2.2 | 3849 | 9121 |
| A36 | 13 | 2.2 | 3862 | 9152 |
| A37 | 13 | 2.2 | 3872 | 9175 |
| C38 | 13 | 2.2 | 3872 | 9175 |
| C39 | 13 | 2.2 | 3826 | 9066 |
| G40 | 13 | 2.2 | 3824 | 9062 |
| G41 | 13 | 2.2 | 3165 | 7500 |
| A42 | 12 | 2.0 | 3156 | 7479 |
| G43 | 12 | 2.0 | 3145 | 7453 |
| A44 | 12 | 2.0 | 3139 | 7438 |
| U45 | 11 | 1.9 | 3144 | 7450 |
| G46 | 11 | 1.9 | 3141 | 7443 |

| | | | | |
|---|---|---|---|---|
| A47 | 11 | 1.9 | 3133 | 7424 |
| A48 | 11 | 1.9 | 3123 | 7401 |
| A49 | 11 | 1.9 | 3121 | 7396 |
| A50 | 11 | 1.9 | 3116 | 7384 |
| C51 | 11 | 1.9 | 3093 | 7329 |
| C52 | 11 | 1.9 | 3066 | 7265 |
| U53 | 11 | 1.9 | 3052 | 7232 |
| U54 | 11 | 1.9 | 3036 | 7194 |
| U55 | 11 | 1.9 | 3014 | 7142 |
| U56 | 11 | 1.9 | 2931 | 6946 |
| U57 | 11 | 1.9 | 2871 | 6803 |
| C58 | 11 | 1.9 | 2812 | 6664 |
| C59 | 11 | 1.9 | 1620 | 3839 |
| A60 | 7 | 1.2 | 685 | 1623 |
| A61 | 7 | 1.2 | 675 | 1600 |
| G62 | 7 | 1.2 | 656 | 1555 |
| G63 | 1 | 0.2 | 438 | 1038 |
| A64 | 0 | 0 | 419 | 993 |
| C65 | 0 | 0 | 0 | 0 |

**Supplementary Table 5. Numbers of sequencing reads mapping to each nucleotide of the genes encoding mt-tRNA$^{Ser(UCN)}$ in the His$_6$-2xFLAG and METTL8-His$_6$-2xFLAG datasets**

| Nucleotide position in mt-tRNA$^{Ser(CUN)}$ | His$_6$-2xFLAG Raw counts | His$_6$-2xFLAG Normalised counts | METTL8-His$_6$-2xFLAG Raw counts | METTL8-His$_6$-2xFLAG Normalised counts |
|---|---|---|---|---|
| G1 | 0 | 0 | 0 | 0 |
| A2 | 0 | 0 | 707 | 1675 |
| A3 | 0 | 0 | 707 | 1675 |
| A4 | 0 | 0 | 716 | 1697 |
| A5 | 0 | 0 | 719 | 1704 |
| A6 | 0 | 0 | 730 | 1730 |
| G7 | 0 | 0 | 738 | 1749 |
| U8 | 0 | 0 | 751 | 1780 |
| C9 | 0 | 0 | 758 | 1796 |
| A10 | 0 | 0 | 764 | 1810 |
| U11 | 0 | 0 | 778 | 1844 |
| G12 | 0 | 0 | 792 | 1877 |
| G13 | 0 | 0 | 798 | 1891 |

| | | | | |
|---|---|---|---|---|
| A14 | 0 | 0 | 800 | 1896 |
| G15 | 0 | 0 | 801 | 1898 |
| G16 | 0 | 0 | 801 | 1898 |
| C17 | 1 | 0.2 | 801 | 1898 |
| C18 | 1 | 0.2 | 835 | 1979 |
| A19 | 1 | 0.2 | 848 | 2009 |
| U20 | 1 | 0.2 | 858 | 2033 |
| G21 | 1 | 0.2 | 866 | 2052 |
| G22 | 1 | 0.2 | 870 | 2062 |
| G23 | 1 | 0.2 | 861 | 2040 |
| G24 | 1 | 0.2 | 854 | 2024 |
| U25 | 1 | 0.2 | 837 | 1983 |
| U26 | 1 | 0.2 | 818 | 1938 |
| G27 | 1 | 0.2 | 792 | 1877 |
| G28 | 1 | 0.2 | 690 | 1635 |
| C29 | 1 | 0.2 | 636 | 1507 |
| U30 | 1 | 0.2 | 567 | 1344 |
| U31 | 1 | 0.2 | 627 | 1486 |
| G32 | 3 | 0.5 | 652 | 1545 |
| A33 | 3 | 0.5 | 676 | 1602 |
| A34 | 3 | 0.5 | 690 | 1635 |
| A35 | 4 | 0.7 | 690 | 1635 |
| C36 | 4 | 0.7 | 704 | 1668 |
| C37 | 4 | 0.7 | 704 | 1668 |
| A38 | 4 | 0.7 | 705 | 1671 |
| G39 | 4 | 0.7 | 690 | 1635 |
| C40 | 4 | 0.7 | 700 | 1659 |
| U41 | 4 | 0.7 | 702 | 1664 |
| U42 | 4 | 0.7 | 712 | 1687 |
| U43 | 4 | 0.7 | 725 | 1718 |
| G44 | 4 | 0.7 | 709 | 1680 |
| G45 | 4 | 0.7 | 388 | 919 |
| G46 | 4 | 0.7 | 376 | 891 |
| G47 | 4 | 0.7 | 375 | 889 |
| G48 | 4 | 0.7 | 371 | 879 |
| G49 | 4 | 0.7 | 358 | 848 |
| U50 | 4 | 0.7 | 342 | 810 |
| U51 | 4 | 0.7 | 337 | 799 |
| C52 | 4 | 0.7 | 316 | 749 |
| G53 | 4 | 0.7 | 313 | 742 |
| A54 | 4 | 0.7 | 301 | 713 |

| | | | | |
|---|---|---|---|---|
| U55 | 4 | 0.7 | 286 | 678 |
| U56 | 4 | 0.7 | 282 | 668 |
| C57 | 4 | 0.7 | 279 | 661 |
| C58 | 4 | 0.7 | 276 | 654 |
| U59 | 4 | 0.7 | 276 | 654 |
| U60 | 3 | 0.5 | 274 | 649 |
| C61 | 3 | 0.5 | 242 | 573 |
| C62 | 2 | 0.3 | 229 | 543 |
| U63 | 2 | 0.3 | 214 | 507 |
| U64 | 2 | 0.3 | 204 | 483 |
| U65 | 2 | 0.3 | 188 | 446 |
| U66 | 2 | 0.3 | 157 | 372 |
| U67 | 1 | 0.2 | 145 | 344 |
| U68 | 1 | 0.2 | 131 | 310 |
| G69 | 1 | 0.2 | 129 | 306 |

**Supplementary Table 6. DNA oligonucleotides used in this study**

| Name | | Sequence (5'-3') | Application |
|---|---|---|---|
| 6558 | Mettl8_iso1_NcoI_fwd | AAAAAACCATGGCCATGAATATGATTTGGAGAAATTCCATTTC | Cloning into recombinant expression vector |
| 6559 | Mettl8_iso1_Sal1stop_rev | AAAAAAGTCGACTCAGTCTTGTGAAAGGAGTGTAG | Cloning into recombinant expression vector |
| 6560 | Mettl8_iso1_KozakHindIII_fwd | AAAAAAAGCTTGCCACCATGAATATGATTTGGAGAAATTCCATTTC | Cloning into recombinant expression vector |
| 6561 | Mettl8_iso1_NheI_rev | AAAAAAGCTAGCTGAGTCTTGTGAAAGGAGTGTAGATAC | Cloning into recombinant expression vector |
| 7319 | METTL8_fw_HindIII | ATATATAAGCTTGCCACCATGAATATGATTTGGAGAAATTCCATTTCTTGTCTAAGG | Cloning into pcDNA5cFLAG vector |
| 7320 | METTL8_rv_NheI | ATATATGCTAGCGTCTTGTGAAAGGAGTGTAGATACC | Cloning into pcDNA5cFLAG vector |
| 8219 | METTL8_D230A_antisense | GTCTTATCATATCTTCCATAGGCTCGAAATAACAGCATTCCCC | Site-directed mutagenesis |
| 8220 | METTL8_D230A_sense | CACAGCTCCAGAAGCAAAAGCACAACAATACAGAAAGGA | Site-directed mutagenesis |
| 8221 | METTL8_D309A_antisense | GGGGAATGCTGTTATTTCGAGCCTATGGAAGATATGATAAGAC | Site-directed mutagenesis |

| | | | |
|---|---|---|---|
| 8222 | METTL8_D309A_sense | TCCTTTCTGTATTGTTGTGCTTTTGCTTCTGGAGCTGTG | Site-directed mutagenesis |
| 8770 | METTL8_sgRNA5_sense | CACCGTGTGTTCAAAAACTTTGGCT | Cloning of single guide RNA sequence |
| 8771 | METTL8_sgRNA5_antisense | AAACAGCCAAAGTTTTTGAACACAC | Cloning of single guide RNA sequence |
| 8939 | METTL8_sg5_Surveyor_F | GGCCCCACCTAGGAAAGAAT | PCR for cleavage-detection assay |
| 8940 | METTL8_sg5_Surveyor_R | CTAGGAAAGGTGCCACACAGA | PCR for cleavage-detection assay |
| 9203 | pcDNA-METTL8_del8-25 | CTTGCCACCATGAATATGCAAAGTGGTTACCACCCA | Cloning |
| 9204 | pcDNA-METTL8_del8-25-antisense | TGGGTGGTAACCACTTTGCATATTCATGGTGGCAAG | Cloning |
| 9213 | METTL8_sg5_F | TAGGAAGGGCCTGCAAGAAC | PCR for detection of gDNA cleavage |
| 9214 | METTL8_sg5_F | TGTGCATTCTCAGATTGAAAACCT | PCR for detection of gDNA cleavage |
| 9232 | pcDNA-METTL8-del25-HindIII-F | ATATATAAGCTTATGCAAAGTGGTTACCACCCAGTG | Subloning METTL8 without MTS |
| 9235 | MTTT_primer_extension_C32 | GGTTTTCATCTCCGGTTTAC | Primer extension for $m^3C_{32}$ |
| 9236 | MTTS(UCN)_primer_extension_C32 | CGAACCCCCCAAAGCTGGTTTC | Primer extension for $m^3C_{32}$ |
| 8108 | MTTS(UCN)_oligo1 | AAAAACCATGGTTAATACGACTCACTATAGGGGAAAAAGTCATGGAGGCCATGGGGT | Cloning |
| 8109 | MTTS(UCN)_oligo2 | AAGGAATCGAACCCCCCAAAGCTGGTTTCAAGCCAACCCCATGGCCTCCATGAC | Cloning/Northern blot probe for mt-tRNA$^{Ser(UCN)}$ |
| 8110 | MTTS(UCN)_oligo3 | GGGGTTCGATTCCTTCCTTTTTTGCCATGAGACCAAGCTTAAAAA | Cloning |
| 8111 | MTTT_oligo1 | AAAAACCATGGTTAATACGACTCACTATAGGGGTCCTTGTAGTATAAACTAATACAC | Cloning |
| 8112 | MTTT_oligo2 | TTGGAAAAAGGTTTTCATCTCCGGTTTACAAGACTGGTGTATTAGTTTATACTA | Cloning/Northern blot probe for mt-tRNA$^{Thr}$ |
| 8113 | MTTT_oligo3 | TGAAAACCTTTTTCCAAGGACACCATGAGACCAAGCTTAAAAA | Cloning |

| | | | |
|---|---|---|---|
| 8993 | MTTT_C32T_oligo2 | TTGGAAAAAGGTTTTCATCTCCGGTTTACAAAACTGGTGTATTAGTTTATACTA | Cloning |
| 8994 | MTTT_T34A_oligo2 | TTGGAAAAAGGTTTTCATCTCCGGTTTACTAGACTGGTGTATTAGTTTATACTA | Cloning |
| 8995 | MTTT_G35A_oligo2 | TTGGAAAAAGGTTTTCATCTCCGGTTTAGAAGACTGGTGTATTAGTTTATACTA | Cloning |
| 8996 | MTTT_T36C_oligo2 | TTGGAAAAAGGTTTTCATCTCCGGTTTGCAAGACTGGTGTATTAGTTTATACTA | Cloning |
| 7129 | U6_additional U_rev | TATGGAACGCTTCACGAATTTGCGTGTCATCCTTGCGC | Northern blot for U6 |
| 9524 | MTTI | ACTTTGATAGAGTAAATAATAG | Northern blot for mt-tRNA$^{Ile}$ |
| 9408 | MTTT_CCA | TGGTGTCCTTGGAAAAAGGTT | *In vitro* transcription PCR template generation |
| 9409 | MTTN_CCA | TGGCTAGACCAATGGGACTTA | *In vitro* transcription PCR template generation |
| 9410 | MTTM_CCA | TGGTAGTACGGGAAGGGTATA | *In vitro* transcription PCR template generation |
| 9411 | MTTSUCN_CCA | TGGCAAAAAAGGAAGGAATCG | *In vitro* transcription PCR template generation |
| 9412 | MTTF_CCA | TGGTGTTTATGGGGTGATGTG | *In vitro* transcription PCR template generation |
| 9413 | MTTSAGY_CCA | TGGTGAGAAAGCCATGTTGTT | *In vitro* transcription PCR template generation |
| 8987 | MTTN oligo 1 | AAAAACCATGGTTAATACGACTCACTATAGGGTAGATTGAAGCCAGTTGATTAGGG | Cloning |
| 8988 | MTTN oligo 2 | TGGGACTTAAACCCACAAACACTTAGTTAACAGCTAAGCACCCTAATCAACTGGCT | Cloning |
| 8989 | MTTN oligo 3 | GGGTTTAAGTCCCATTGGTCTACCATGAGACCAAGCTTAAAAA | Cloning |
| 9239 | MTTN_UGUA_thr_oligo2 | TGGGACTTAAACCCACAAACACTTAGTTACAAGCTAAGCACCCTAATCAACTGGCT | Cloning |
| 9240 | MTTN_ASL_thr_oligo2 | TGGGACTTAAACCCACAAACACCGGTTTACAAGACTGGCACCCTAATCAACTGGCT | Cloning |
| 9415 | MTTT_oligo2_A38G | TTGGAAAAAGGTTTTCATCTCCGGTCTACAAGACTGGTGTATTAGTTTATACTA | Cloning |
| 9416 | MTTT_oligo2_T33A | TTGGAAAAAGGTTTTCATCTCCGGTTTACATGACTGGTGTATTAGTTTATACTA | Cloning |
| 8422 | MTTS2_oligo1 | AAAAACCATGGTTAATACGACTCACTATAGGGGAGAAAGCTCACAAGAACTGCTA | Cloning |

| | | | |
|---|---|---|---|
| 8423 | MTTS2_oligo2 | CCATGTTGTTAGACATGGGGG CATGAGTTAGCAGTTCTTG | Cloning |
| 8424 | MTTS2_oligo3 | ATGTCTAACAACATGGCTTTCT CACCATGAGACCAAGCTTAAA AA | Cloning |
| 4965 | MT-TM-Oligo1 | AAAAACCATGGTTGAATACGAC TCACTATAGGGAGTAAGGTCA GCTAAATAAGCTATC | Cloning |
| 4966 | MT-TM-Oligo2 | GGGAAGGGTATAACCAACATT TTCGGGGTATGGGCCCGATAG CTTATTTAGCTGACC | Cloning |
| 4967 | MT-TM-Oligo3 | TGTTGGTTATACCCTTCCCGTA CTACCATGAGACCAAGCTTAAA AA | Cloning |
| 6681 | MTTF_NcoI-T7_oligo1 | AAAAACCATGGTTGAATACGAC TCACTATAGGGTTTATGTAGCT TAC | Cloning |
| 6682 | MTTF_oligo2 | GTGATGTGAGCCCGTCTAAAC ATTTTCAGTGTATTGCTTTGAG GAGGTAAGCTACATAAACCCT ATAG | Cloning |
| 6683 | MTTF_BsaI-HindIII_oligo3 | TAGACGGGCTCACATCACCCC ATAAACACCATGAGACCAAGC TTAAAAA | Cloning |
| 9684 | AL112_MTTT_D55 | TCCGGTTTACAAGGTTGCGGT AGCGCCTGGTGCTGGTGTATT | RNA-cleaving deoxyribozyme for $m^3C_{32}$ on mt-tRNA$^{Thr}$ |
| 9685 | AL112_MTTS(UCN) | AGCTGGTTTCAAGGTTGCGGT AGCGCCTGGTGCAACCCCATG GCCT | RNA-cleaving deoxyribozyme for $m^3C_{32}$ on mt-tRNA$^{SerUCN}$ |
| 9704 | AK104_MTTT_D54 | TCCGGTTTACAAGGGTGCCGG GTGAGCGTTATCTGGTGTATT | deoxyribozyme for cleaving unmodified mt-tRNA$^{Thr}$ |
| 9705 | AK104_MTTS(UCN) | AGCTGGTTTCAAGGGTGCCGG GTGAGCGTTATCAACCCCATG GCCT | deoxyribozyme for cleaving unmodified mt-tRNA$^{Ser(UCN)}$ |
| 9726 | MTTT_disruptor | TTTATACTACAAGGAC | Disruptor oligo for mt-tRNA$^{Thr}$ |
| 9727 | MTTS(UCN)_disruptor | CCTCCATGACTTTTTC | Disruptor oligo for mt-tRNA$^{Ser(UCN)}$ |
| 9695 | MTTT_NB_5end | CTGGTGTATTAGTTTATACTAC AAGGAC | Northern blot probe |
| 9696 | MTTS(UCN)_NB_5end | CAACCCCATGGCCTCCATGAC TTTTTC | Northern blot probe |

**Supplementary Table 7. Plasmids used in this study**

| Name | Description | Application |
|---|---|---|
| pMB1386 | A101-H14-MBP-METTL8 | Recombinant protein expression in bacteria |

| | | |
|---|---|---|
| pMB1547 | pcDNA5-FRT-TO-METTL8-cGFP | Generation of human cell line |
| pMB1548 | pcDNA5-FRT-TO-METTL8-cFlagPrcHis | Generation of human cell line |
| pMB1710 | A101-H14-MBP-METTL8-D230A | Recombinant protein expression in bacteria |
| pMB1711 | A101-H14-MBP-METTL8-D309A | Recombinant protein expression in bacteria |
| pMB1858 | pcDNA5-FRT-TO-METTL8-21-407-cFLAG | Generation of human cell line |
| pMB1859 | pcDNA5-FRT-TO-METTL8-21-407-cGFP | Generation of human cell line |
| pMB1906 | px459-METTL8-Cas9-sgRNA5-non-lenti-CRISPR | Generation of CRISPR knockout cell line |
| pMB1714 | A1-T7-mt-tRNAThr | *In vitro* transcription |
| pMB1712 | A1-T7-mt-tRNA$^{Ser(UCN)}$ | *In vitro* transcription |
| pMB1871 | A1-T7-mt-tRNA$^{Thr}$-C32T | *In vitro* transcription |
| pMB1872 | A1-T7-mt-tRNA$^{Thr}$-T34A | *In vitro* transcription |
| pMB1873 | A1-T7-mt-tRNA$^{Thr}$-G35A | *In vitro* transcription |
| pMB1878 | A1-T7-mt-tRNA$^{Asn}$-mt-tRNA$^{Thr}$-anticodon loop | *In vitro* transcription |
| pMB1879 | A1-T7-mt-tRNA$^{Asn}$-mt-tRNA$^{Thr}$-ASL | *In vitro* transcription |
| pMB1880 | A1-MTTT-A38G | *In vitro* transcription |
| pMB1881 | A1-MTTT-A33G | *In vitro* transcription |
| pMB1864 | A8-H10-SARS2 | Recombinant protein expression in bacteria |
| pMB575 | A101 | Recombinant protein expression in bacteria |

| | | |
|---|---|---|
| pMB1851 | A8-H10-ALKBH1 | Recombinant protein expression in bacteria |

**Supplementary Table 8. Antibodies used in this study**

| Name | Source | Dilution |
|---|---|---|
| Anti-METTL8 | Sigma (HPA035421) | 1:1000 |
| Anti-αTubulin | Sigma (T6199) | 1:60000 |
| Anti-FLAG M2 | Sigma (F3165) | 1:30000 |
| IRDye® 680LT Donkey anti-Mouse IgG | LI-COR Biosciences (P/N: 926-68022) | 1:10000 |
| IRDye® 800CW Donkey anti-Rabbit IgG | LI-COR Biosciences (P/N: 925-32213) | 1:10000 |
| IRDye® 800CW Donkey anti-Mouse IgG | LI-COR Biosciences (P/N: 926-32212) | 1:10000 |
| Anti-OSGEPL1 | Invitrogen (PA5-98987) | 1:1000 |
| Anti-TRIT1 | Sigma (AV39298) | 1:1000 |
| Anti-SARS2 | Abcam (ab229227) | 1:1000 |
| Anti-TIM44 | Proteintech (13859-1-AP) | 1:1000 |
| Anti-TIM23 | gift from Prof. Peter Rehling | 1:1000 |
| Anti-uL3m | Proteintech (16584-1- AP) | 1:1000 |
| Anti-uS14m | Proteintech (16301-1-AP) | 1:5000 |
| Anti-MFN2 | Proteintech (12186-1-AP) | 1:1000 |
| Anti-FLAG | Sigma (F3165) | 1:30000 |
| Anti-mL44 | Proteintech (16394-1-AP) | 1:20000 |

**Supplementary Table 9. siRNAs used in this study**

| Name | | Sequence (5'-3') |
|---|---|---|
| si240 | siMETTL8_1 | GAGAGAAUCAUCAUGGGAUdTdT |
| si241 | siMETTL8_1 | UCGCCGCUUACAAGUCAAUdTdT |
| si246 | siSARS2_1 | CGAUCAUCUCGACAUGGCAdTdT |
| si247 | siSARS2_2 | GGCUGUGGGAUGACACCAAdTdT |
| si2 | siNT | CGUACGCGGAAUACUUCGAdTdT |

| | | |
|---|---|---|
| si253 | siTRIT1-1 | UUGUUGGUGAUGAUGUCUAGGdTdT |
| si254 | siTRIT1-2 | UUGACAAGAACUUUCCAGAGCdTdT |
| si255 | siOSGEPL1-1 | UAUAGAAGUUACUUGCGACACdTdT |
| si256 | siOSGEPL1-2 | AAUUGCUGAGAGGUCACUUGGdTdT |

**Supplementary Table 10. RNA oligonucleotides used in this study**

| Name | | Sequence (5'-3') | Application |
|---|---|---|---|
| R420_Flu | Unstructured RNA | GUAAAAGUCGUAACAAAGGU_Flu | Fluorescence anisotropy |
| R599 | mt-tRNA$^{Ser(UCN)}$ ASL | GUUGGCUUGAAACCAGCU | *In vitro* methylation assay |
| R600 | mt-tRNA$^{Thr}$ ASL | CCAGUCUUGUAAACCGGA | *In vitro* methylation assay |
| R622 | mt-tRNA$^{Ser(UCN)}$ $i^6A_{37}$ ASL | GUUGGCUUGA($i^6$A)ACCAGCU | *In vitro* methylation assay |
| R785_Flu | Unstructured RNA | GACAUACUGACCCUUCAAAUA_Flu | Fluorescence anisotropy |
| R816 | mt-tRNA$^{Ser(UCN)}$ $ms^2i^6A_{37}$ ASL | GUUGGCUUGA($ms^2i^6$A)ACCAGCU | *In vitro* methylation assay |
| R817 | mt-tRNA$^{Phe}$ $ms^2i^6A_{37}$ ASL | AUACACUGAA($ms^2i^6$A)AUGUUUA | *In vitro* methylation assay |
| R824 | mt-tRNA$^{Ser(UCN)}$ $m^3C_{32}$ $i^6A_{37}$ ASL | GUUGG($m^3$C)UUGA($ms^2i^6$A)ACCAGCU | *In vitro* methylation assay |

| | | | |
|---|---|---|---|
| R825 | mt-tRNA$^{Ser(UCN)}$ $m^3C_{32}$ $ms^2i^6A_{37}$ ASL | GUUGG($m^3$C)UUGA($ms^2i^6$A)ACCAGCU | *In vitro* (de-)methylation assay |
| R836 | mt-tRNA$^{Thr}$ $t^6A_{37}$ | CCAGUCUUGU($t^6$A)AACCGGA | *In vitro* methylation assay |
| R856 | mt-tRNA$^{Ser(UCN)}$ $ms^2i^6A_{37}$ U34A ASL | GUUGGCUAGA($ms^2i^6$A)ACCAGCU | *In vitro* methylation assay |
| R857 | mt-tRNA$^{Ser(UCN)}$ $ms^2i^6A_{37}$ G35A ASL | GUUGGCUUAA($ms^2i^6$A)ACCAGCU | *In vitro* methylation assay |
| R858 | mt-tRNA$^{Ser(UCN)}$ $ms^2i^6A_{37}$ A38C ASL | GUUGGCUUGA($ms^2i^6$A)CCCAGCU | *In vitro* methylation assay |
| R859 | mt-tRNA$^{Ser(UCN)}$ $ms^2i^6A_{37}$ anticodon on mt-tRNA$^{Phe}$ stem | AUACACUUGA($ms^2i^6$A)AUGUUUA | *In vitro* methylation assay |
| R860 | mt-tRNA$^{Thr}$ $t^6A_{37}$ A38G | CCAGUCUUGU($t^6$A)GACCGGA | *In vitro* methylation assay |
| R861 | mt-tRNA$^{Ser(AGY)}$ $t^6A_{37}$ | AAGAACUGCU($t^6$A)ACUCAUG | *In vitro* methylation assay |
| R599_Flu | mt-tRNA$^{Ser(UCN)}$ ASL | GUUGGCUUGAAACCAGCU_Flu | Fluorescence anisotropy |
| R377_Flu | mt-tRNA$^{Met}$ ASL | UCGGGCCCAUACCCCGA_Flu | Fluorescence anisotropy |
| R883_Flu | mt-tRNA$^{Ser(UCN)}$ $i^6$A ASL | GUUGGCUUGA($i^6$A)ACCAGCU | Fluorescence anisotropy |
| R884_Flu | mt-tRNA$^{Ser(UCN)}$ $m^3$C $i^6$A ASL | GUUGG($m^3$C)UUGA($i^6$A)ACCAGCU | Fluorescence anisotropy |
| D2717_Flu | random DNA sequence | GATGTGCAGCTGCACATC_Flu | Fluorescence anisotropy |

| | | | |
|---|---|---|---|
| R604 | mt-tRNA$^{Ser(UCN)}$ m$^3$C ASL | GUUGG(m$^3$C)UUGAAACCAGCU | *In vitro* demethylation assays |
| R605 | mt-tRNA$^{Thr}$ m$^3$C ASL | CCAGU(m$^3$C)UUGUAAACCGGA | *In vitro* (de-)methylation assays |

**Supplementary Table 11. High resolution electrospray ionisation mass spectrometric (HR-ESI-MS) characterization of RNA oligonucleotides**

| 5'-sequence-3' [a] | formula | Mass calc. | Mass meas. |
|---|---|---|---|
| GUUGG **C**U *UGA* **A**A CCAGCU | $C_{171}H_{212}N_{67}O_{125}P_{17}$ | 5729.7839 | 5729.8758 |
| GUUGG **X**U *UGA* **A**A CCAGCU | $C_{172}H_{214}N_{67}O_{125}P_{17}$ | 5743.7988 | 5743.8787 |
| GUUGG **C**U *UGA* **Y**A CCAGCU | $C_{176}H_{220}N_{67}O_{125}P_{17}$ | 5797.8457 | 5797.8854 |
| GUUGG **C**U *UGA* **Z**A CCAGCU | $C_{177}H_{222}N_{67}O_{125}P_{17}S$ | 5843.8335 | 5843.8889 |
| GUUGG **X**U *UGA* **Y**A CCAGCU | $C_{177}H_{222}N_{67}O_{125}P_{17}$ | 5811.8614 | 5811.8803 |
| GUUGG **X**U *UGA* **Z**A CCAGCU | $C_{168}H_{212}N_{62}O_{118}P_{16}S$ | 5512.8017 | 5512.8268 |
| GUUGG **X**U *AGA* **Z**A CCAGCU | $C_{178}H_{223}N_{70}O_{123}P_{17}S$ | 5866.8607 | 5866.8864 |
| GUUGG **C**U *UAA* **Z**A CCAGCU | $C_{177}H_{222}N_{67}O_{124}P_{17}S$ | 5827.8386 | 5827.8558 |
| GUUGG **C**U *UGA* **Z**C CCAGCU | $C_{176}H_{222}N_{65}O_{126}P_{17}S$ | 5819.8222 | 5819.8388 |
| AUACA **C**U *UGA* **Z**A UGUUU | $C_{177}H_{220}N_{65}O_{124}P_{17}S$ | 5797.8168 | 5797.8251 |
| CCAGU **C**U *UGU* **A**A ACCGGA | $C_{171}H_{213}N_{68}O_{123}P_{17}$ | 5712.8048 | 5712.8906 |
| CCAGU **X**U *UGU* **A**A ACCGGA | $C_{172}H_{215}N_{68}O_{123}P_{17}$ | 5726.8199 | 5726.9000 |
| CCAGU **C**U *UGU* **V**A ACCGGA | $C_{176}H_{220}N_{69}O_{127}P_{17}$ | 5857.8417 | 5857.8486 |
| CCAGU **X**U *UGU* **V**A ACCGGA | $C_{177}H_{222}N_{69}O_{127}P_{17}$ | 5871.8574 | 5871.8324 |
| CCAGU **C**U *UGU* **V**G ACCGG | $C_{176}H_{220}N_{69}O_{128}P_{17}$ | 5873.8366 | 5873.8465 |
| AAGAA **C**U *GCU* **V**A CUCAU | $C_{177}H_{220}N71O_{125}P_{17}$ | 5865.8509 | 5865.8580 |
| Flu-GUUGG **X**U *UGA* **A**A CCAGCU | $C_{207}H_{249}N_{71}O_{134}P_{18}$ | 6430.0129 | 6430.0024 |
| Flu-GUUGG **C**U *UGA* **Y**A CCAGCU | $C_{206}H_{247}N_{71}O_{134}P_{18}$ | 6415.9973 | 6416.0217 |
| Flu-GUUGG **X**U *UGA* **Y**A CCAGCU | $C_{207}H_{249}N_{71}O_{134}P_{18}$ | 6430.0129 | 6430.0024 |

| | | | |
|---|---|---|---|
| Flu-GATGTGCAGCTGCACATC | $C_{205}H_{248}N_{72}O_{115}P_{18}$ | 6115.1048 | 6115.1236 |

[a] X = $m^3C$, Y = $i^6A$, Z = $ms^2i^6A$, V = $t^6A$, Flu = fluorescein connected via triazol linker

## SUPPLEMENTARY METHODS

### *RNA-seq analysis*

Total RNA was isolated from two independent HEK293 cultures cells using TRI reagent (Sigma) according to the manufacturer's instructions. After DNase-treatment using TURBO DNase (Ambion) for 15 min at 37°C, RNAs were re-purified using the RNA Clean and Concentrator kit (Zymo Research) following the manufacturer's instructions. Ribosomal RNA was depleted and the RNAs subjected to TruSeq total RNA library preparation (Illumina). Sequencing was carried out on HiSeq 4000 (Illumina).

FASTQ files were quality checked with FastQC (version 0.11.9) and all data utilized for further analysis had a Phred score >33. The sequencing reads were first mapped to the rDNA sequence (Genbank: U13369.1) using STAR[5] (version 2.7.0d) using the following criteria:

--genomeLoad NoSharedMemory --runThreadN 6

--sjdbGTFfile $CRACpy/Database/RibosomeFasta/U133369.gtf \

--readFilesCommand zcat

--outSAMattributes All

--outFileNamePrefix \

--outSAMtype BAM SortedByCoordinate

--outReadsUnmapped Fastx

All mapping reads were discarded from downstream analyses and the unmapped reads were then re-mapped to the human genome ensemble (version GRCh38.92) using the following conditions:

--limitBAMsortRAM 21470000000 \

--genomeLoad NoSharedMemory \

--sjdbGTFfile --sjdbOverhang 50 \

--outSAMattributes All \

--outSAMtype BAM SortedByCoordinate \

--quantMode TranscriptomeSAM \

--outSAMstrandField intronMotif \

--outReadsUnmapped Fastx

Expression of different METTL8 isoforms was estimated from the transcriptomeSAM output of STAR using RSEM (version 1.3.3)[6] (rsem-calculate-expression --bam --no-bam-output). An isoform matrix was generated using (rsem-generate-data-matrix) and the EB-Seq package (R version 3.6.3, EB-Seq version 1.2.0) was run using default settings (rsem-run-ebseq --ngvector).

***Bioinformatic analysis of CRAC data***

Barcode removal was performed using pyBarcode Filter (version 1.4.5) (pyBarcodeFilter.py -f -b barcode).

3' adaptor removal and quality filtering was performed using Flexbar (version 3.5.0)[7] (flexbar', '-r', './input/fastq/METTL8_U1811R.fastq.gz', '-t' './output/210723_1012/METTL8_U1811R/flexbar/METTL8_U1811R_N21', '-q', 'TAIL', '-qf', u'i1.8', '-qt', '13', '-u', '0', '-m', '21', '-n', '8', '-at', 'RIGHT', '-z', 'GZ', '-o', '-ao', '2', '-ae', '0.1', '-as', u'TGGAATTCTCGGGTGCCAAGG'].

Identical sequencing reads containing the same UMI were collapsed using pyFastqDuplicateRemover (version 1.4.5) (pyFastqDuplicateRemover.py -f -o).

Sequences were aligned to the human genome (version GRCh38.p13 Ensembl release 104) using STAR (version 2.7.0d):

--genomeDir
./Database/GRCh38.29.primary_assembly.genome_no5SrRNA+rRNA/STARIndex/
--readFilesIn
./output/210714_1417/ASCC3_n401end_U2001R/flexbar/ASCC3_n401end_U2001R_C21.fasta.gz
--readFilesCommand 'gunzip -c'
--outFileNamePrefix
./output/210714_1417/ASCC3_n401end_U2001R/STAR_Aligner/ASCC3_n401end_U2001R_C21
--runThreadN 8

--genomeLoad LoadAndKeep

--quantMode TranscriptomeSAM

--limitBAMsortRAM 21470000000

--outFilterMultimapNmax 20

--outFilterMismatchNmax 4

--outSAMmultNmax 1

--scoreDelOpen -2

--scoreDelBase -2

--outFilterMismatchNoverReadLmax 0.04

--outSAMtype BAM SortedByCoordinate

--outReadsUnmapped Fastx

--outMultimapperOrder Random

--outFilterType BySJout

--alignSJoverhangMin 8

--alignSJDBoverhangMin 1

--outSJfilterOverhangMin -1 -1 -1 -1

--outSJfilterCountTotalMin -1 -1 -1 -1

--outSAMattributes NH HI AS MD nM

--alignEndsType EndToEnd)

Aligned reads were counted using featureCounts (version 1.6.3)[8] (featureCounts -a -s 1 -T 8 -f -t gene -O -M –largestOverlap). The EdgeR package (version 3.34.1)[9] was used for TMM normalization and to calculate fold-changes using default settings. For data visualisation aligned reads were processed using bedtools (version 2.27.1)[10] (bedtools genomecov -bga -strand -split) separated by strand.

Nucleotide substitutions within mapped reads were counted using a custom Python (version 2.7) script:

```
#!/usr/bin/env python2.7
import subprocess
import re
import gzip
import csv
import os
import itertools
import traceback
import sys
import os
from optparse import OptionParser
parser = OptionParser()
parser.add_option("-B", "--BAM", dest="Bam",help="Folder containg Mochifiles with CRAC hits", metavar="Folder",default=None)
parser.add_option("-o","--output_file",dest="output_file",help="otuput folder folder",metavar="Folder",default=None)
(options, args) = parser.parse_args()
BAM= str(options.Bam)
Output=str(options.output_file)
def Mutation_counter(input_file):
    """returns a 1-based dictionary of mutations
        key: ChromosomeStrandPosition
        value: absolute number of mutations (deletions and substitutions)
    """
    outlist = []
    samtools_in = subprocess.Popen(["samtools", "view",input_file], stdout=subprocess.PIPE, bufsize=1)
    for line in samtools_in.stdout:
```

```
    line = line.rstrip("\n").split("\t")
    for row in line:
            chr_read = line[2]
            read_start = int(line[3]) - 1
            if row.startswith('MD:Z:'):
                MD_tag = row.split(':')[-1]
                split_MD = re.findall('\d+|\D+', MD_tag)
                split_MD = split_MD[:-1]
                positions = []
                pos = read_start
                for y in split_MD:
                    if y.isdigit():
                        pos += int(y)
                    elif y.startswith('^'):
                        for b in range(1, len(y)):
                            positions.append(pos + b)
                        pos += len(y) - 1
                    else:
                        for b in range(1, len(y) + 1):
                            positions.append(pos + b)
                        pos += len(y)
                if line[1] == '16':
                else:
                    outlist.extend([chr_read + '+' + str(x) for x in positions])
out_dict={}
print "making dict"
for x in outlist:
    if x in out_dict: out_dict[x]+= 1
```

```
        else: out_dict[x] = 1
    print "finished dict"
    return out_dict
mutation_dictionary= Mutation_counter(BAM)
outlist = []
for key, value in mutation_dictionary.iteritems():
    if "+" in key:
        chrom = key.split('+')[0]
        pos = key.split('+')[-1]
        outlist.append([chrom, int(pos) - 1, int(pos), float(value)]) # normalisation is done here

    elif '-' in key:
        chrom = key.split('-')[0]
        pos = key.split('-')[-1]
        outlist.append([chrom, int(pos) - 1, int(pos), -float(value)]) # normalisation is done here,
negative values are reverse strand
outlist.sort(key = lambda x: (x[0],int(x[1])))
writetable(outlist, Output)
```

***Deoxyribozyme-catalyzed RNA cleavage***

For deoxyribozyme-catalyzed RNA cleavage reactions[11], 500 ng of small RNAs extracted from wild-type and METTL8 KO cell lines were incubated with 10 µM deoxyribozyme and 10 µM disruptor oligonucleotides complementary to the 5' end of the acceptor stem of the corresponding mt-tRNA (Supplementary Table 6) in 50 mM Tris pH 7.5 and 150 mM NaCl in 9 µl. The reactions were denatured at 95 °C for 4 min, cooled down to 25°C at 0.1°C/s and then supplemented with $MgCl_2$ to a final concentration of 40 mM. The cleavage reactions were incubated at 37 °C for 18 h and quenched with 2x RNA loading dye. 35 ng of the small RNAs were separated in a denaturing 10 % polyacrylamide gel and the cleavage was analyzed by

northern blotting with [$^{32}$P]-labelled DNA oligonucleotides antisense to the expected 5' cleavage fragment formed (Supplementary Table 6). Cleavage ratios between WT and WT or WT and METTL8 KOs were calculated for each panel by diving signal intensities of the cleavage fragments after normalising to the signal intensity of the U6 snRNA.

***Cell counting by analytical flow cytometry***

Cell numbers were estimated in R using the following code:

```
library(flowCore)
fileNames <-Sys.glob("*P1.fcs")
Counting <- data.frame(fileNames,counts=seq(1:125))
for(i in 1:length(fileNames)){
  file<-read.FCS(fileNames[i])
  file<-exprs(file)
  file<-as.data.frame(file)
  length(file[,1])
  Counting$counts[i] <- length(file[,1])
```

***UV thermal melting***

UV thermal melting curves were recorded on a Varian Cary 100 spectrometer equipped with a Peltier temperature controller, in quartz cuvettes with 1 cm path lengths, at a heating rate of 0.5°C/min, for two heating and two cooling ramps in a temperature interval of 10–90°C, monitored at 250, 260 and 280 nm. The samples contained RNA (2 μM) in 10 mM sodium phosphate buffer (pH 7.0) with 100 mM NaCl. The melting temperatures were obtained from the inflection point of the hyperchromicity curves, by fitting a two-state transition model.

***In vitro demethylation assays***

For analysis of demethylation activity by high-pressure liquid chromatography (HPLC), 20 μM of synthetic anticodon stem loops containing an $m^3C$ modification (Supplementary Table 10) were incubated with 3 μM $His_{10}$-ALKBH1 or $His_{14}$-MBP in the presence of 1x demethylation buffer (100 μM $Fe(NH_4)_2(SO_4)_2$, 100 μM α-ketoglutarate and 1 unit/ml Ribolock (ThermoFisher scientific) for 1 h at 22°C. The oligonucleotides were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with ethanol. The pellets were resuspended in 10 μL of water, and 50 pmol were used for analysis by anion exchange HPLC at 60°C in an Äkta Purifier device (GE Healthcare Life Sciences) with UV detection set at 260 nm. The oligonucleotides were eluted using a gradient of 0% to 48% Dionex buffer B (25 mM Tris-HCl pH 8; 6 M urea and 0.5 M $NaClO_4$) in 12 column volumes with Dionex buffer A (25 mM Tris-HCl pH 8 and 6 M urea) in Dionex DNAPac PA200 columns (4×250 mm). Elution was monitored by UV absorbance at 260 nm.

## SYNTHETIC METHODS

### *General Information*

All reactions were performed under inert nitrogen atmosphere and the obtained products were stored at -20°C. The chemicals used for the reactions were purchased 'Pro analysis'- or 'For synthesis' quality and used without additional purification. Solvents (technical quality) were distilled prior to use and dry solvents (dichloromethane, acetonitrile, THF, DMF) were obtained from solvent purification system (SPS). Methanol was pre-dried over molecular sieves and further dried with magnesium turnings. Column chromatography was performed on silica gel (Kieselgel 60, Merck, particle size of 0.040-0.063). Reaction progress was monitored by TLC (Alugram® aluminium sheets, Machery-Nagel, UV visualization, 254 nm). NMR spectra were recorded on a Bruker Avance III HD 400 spectrometer. Chemical shifts ($\delta$) were measured relative to the residual solvent signals as internal standards (in ppm; $CDCl_3$: $^1H$ = 7.26, $^{13}C$ = 77.16, DMSO: $^1H$ = 2.50, $^{13}C$ = 39.52). For compounds with DMT-protecting group, silver stabilized $CDCl_3$ was used and filtered over basic aluminium oxide prior to use. Multiplicities are given as: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. Coupling constants (*J*) are given in Hz. High-resolution (HR) electrospray ionization (ESI) mass spectra (MS) were recorded on a Bruker micrOTOF-Q III spectrometer. The detected mass-to-charge ratio (*m/z*) is given and compared to the calculated monoisotopic mass.

### *Experimental procedures for synthesis of ms$^2$i$^6$A phosphoramidite*

2',3',5'-*O*-Triacetyl-6-choro-2-aminopurine riboside (**1**)

2-Amino-6-chloropurine riboside (2.00 g, 6.63 mmol) was suspended in dry acetonitrile (25 mL) under nitrogen atomsphere. After adding DMAP (81.0 mg, 663 µmol, 0.1 eq) and $NEt_3$ (5.51 mL, 39.8 mmol, 6 eq), the mixture was cooled to 0°C. Then acetic anhydride (1.87 mL, 19.8 mmol, 2.99 eq) was added dropwise and the mixture was stirred at room temperature for 3 h. The solvent was evaporated under reduced pressure, followed by recrystallization of the crude product from isopropanol (~ 35 mL). The product was obtained as a colourless solid (2.33 g, 5.45 mmol, 82%).

TLC (in DCM:MeOH =10:1): $R_f$ = 0.63.

**$^1$H-NMR** ($CDCl_3$, 400 MHz): $\delta$ (ppm) = 2.08 (s, 3H, acetyl), 2.10 (s, 3H, acetyl), 2.14 (s, 3H, acetyl), 4.41 – 4.34 (m, 1H, 5'-H), 4.47 – 4.42 (m, 2H, 4'-H, 5'-H), 5.22 (s, 2H, $NH_2$), 5.74 (t, *J* = 5.0 Hz, 1H, 3'-H), 5.95 (t, *J* = 5.1 Hz, 1H, 2'-H), 6.01 (d, *J* = 4.9 Hz, 1H, 1'-H), 7.87 (s, 1H, 8-H).

**$^{13}$C-NMR** ($CDCl_3$, 100 MHz): $\delta$ (ppm) = 20.56 (C-26), 20.67 (C-28), 20.84 (C-22), 63.04 (C-5'), 70.58 (C-3'), 72.84 (C-2'), 80.12 (C-4'), 86.71 (C-1'), 125.89 ($C_q$-5), 140.83 (C-8), 151.97 ($C_q$-2), 153.19 ($C_q$-4), 159.21 ($C_q$-6), 169.49 ($C_q$-24), 169.71 ($C_q$-27), 170.62 ($C_q$-21).

**HR-MS** ($ESI^+$): Exact mass calculated for $C_{16}H_{18}ClN_5NaO_7$ $[M+Na]^+$: 450.0788, found: 450.0798.

2',3',5'-*O*-Triacetyl-2-methylthio-6-chloropurine riboside (**2**)

2',3',5'-*O*-Triacetyl-6-methylthiopurine riboside (1.30 g, 3.04 mmol, **1**) was dissolved in 20 mL of anhydrous acetonitrile under nitrogen atmosphere. Then dimethyl disulfide (2.70 mL, 30.4 mmol) and isopentyl nitrite (817 µL, 6.08 mmol) were added and the mixture was heated to 60 °C. After 2 h the reaction mixture was concentrated to ~ 5 mL. The residue was diluted with DCM and washed with a saturated aqueous $NaHCO_3$ solution. The organic phase was dried over $Na_2SO_4$ and the solvent was removed under reduced pressure. The residual was purified by column chromatography (DCM → DCM:MeOH = 99:1 → 98:2) to yield **2** as a colourless foam (922 mg, 2.01 mmol, 66%).

**$^1$H-NMR** ($CDCl_3$, 400 MHz): $\delta$ (ppm) = 2.09 (s, 3H, acetyl), 2.11 (s, 3H, acetyl), 2.15 (s, 3H, acetyl), 2.64 (s, 3H, S-$CH_3$), 4.32 (dd, *J* = 5.1, 13.0 Hz, 1H, H-5'b), 4.42 – 4.47 (m, 2H, H-4', H-5'a), 5.65 (t, *J* = 5.5 Hz, 1H, 3'), 5.99 (dd, *J* = 4.6, 5.5 Hz, 1H, H-2'), 6.11 (d, *J* = 4.6 Hz, 1H, H-1'), 8.09 (s, 1H, H-8).

**$^{13}$C-NMR** ($CDCl_3$, 100 MHz): $\delta$ (ppm) = 15.01 (S-$CH_3$), 20.55 (acetyl), 20.65 (acetyl), 20.87 (acetyl), 62.85 (C-5'), 70.27 (C-3'), 73.07 (C-2'), 80.17 (C-4'), 87.19 (C-1'), 129.26 (C-5), 142.29 (C-8), 151.46 (C-6), 152.08 (C-4), 167.54 (C-2), 169.46 (C=O), 169.64 (C=O), 170.43 (C=O).

**HR-MS** ($ESI^+$): Exact mass calculated for $C_{17}H_{19}ClN_4NaO_7S$ $[M+Na]^+$: 481.0561, found: 481.0574.

2',3',5'-*O*-triacetyl-$N^6$-isopentenyl-2-thiomethyl-adenosine (**3**)

2',3',5'-*O*-triacetyl-2-methylthio-6-chloropurine riboside (**2**, 200 mg, 436 µmol) was dissolved in anhydrous pyridine (5 mL) under nitrogen atmosphere. After addition of $NEt_3$ (846 µL, 6.10 mmol) and isopentenyl amine hydrochloride (159 mg, 1.31 mmol) the reaction mixture was stirred at room temperature for 17 h. The solvent was evaporated under reduced pressure. The residue was re-dissolved in DCM and washed with 5% citric acid, brine and saturated $NaHCO_3$, respectively. The organic phase was dried over $Na_2SO_4$. The crude product was purified by column chromatography (DCM → DCM:MeOH = 99:1) to yield **3** as a colourless foam (179 mg, 353 µmol, 81%).

**$^1$H-NMR** ($CDCl_3$, 400 MHz): $\delta$ (ppm) = 1.73 (d, $J$ = 1.3 Hz, 3H, $C_q$-$CH_3$ ), 1.74 (d, $J$ = 1.3 Hz, 3H, $C_q$-$CH_3$ ), 2.08 (s, 3H, acetyl), 2.09 (s, 3H, acetyl), 2.13 (s, 3H, acetyl), 2.57 (s, 3H, S-$CH_3$), 4.17 (s, br, 2H, NH-$CH_2$), 4.29 (dd, $J$ = 4.6, 12.0 Hz, 1H, H-5'), 4.39 (ddd, $J$ = 3.4, 4.6, 5.5 Hz, 1H, H-4'), 4.45 (dd, $J$ = 3.4, 12.0 Hz, 1H, H-5'), 5.33 (ddq, $J$ = 1.4, 5.8, 8.6 Hz, 1H, CH=$C_q$), 5.61 (s, 1H, NH), 5.72 (t, $J$ = 5.5 Hz, 1H, H-3'), 6.01 (dd, $J$ = 4.6, 5.5 Hz, 1H, H-2'), 6.04 (d, $J$ = 4.6 Hz, 1H, H-1'), 7.71 (s, 1H, 8-H).

**$^{13}$C-NMR** ($CDCl_3$, 100 MHz): $\delta$ (ppm) = 14.76 (S-$CH_3$), 18.15 (C-35, $CH_3$), 20.59, 20.68, 20.88 (acetyl, $CH_3$), 25.83 (C-34, $CH_3$), 38.60 ($CH_2$), 63.11 (C-5'), 70.45 (C-3'), 73.15 (C-2'), 79.84 (C-4'), 86.85 (C-1'), 118.02 (C-5), 120.22 (C-32, *C*H=$C_q$), 137.30 (C-8), 149.46 (C-4), 153.92 (C-6), 166.82 (C-2), 169.51, 169.64, 170.59 (acetyl, C=O).

**HR-MS** ($ESI^+$): Exact mass calculated for $C_{22}H_{29}N_5NaO_7S$ $[M+Na]^+$: 530.1686, found: 530.1675.

$N^6$-Isopentenyl-5'-*O*-(4,4'-dimethoxytrityl)-2-thiomethyl-adenosine (**4**)

Compound **3** (170 mg, 335 µmol) was dissolved in MeOH (4.5 mL) and triethyl amine (500 µL) and stirred for 48 h at room temperature. After evaporation of the solvent under reduced

pressure the residue was co-evaporated with pyridine. The residue was re-dissolved in anhydrous pyridine (3 mL) under nitrogen atmosphere and 4,4'-dimethoxytriyl chloride (DMT-Cl, 136 mg, 402 µmol) was added in three portions within 1 h. After stirring for an additional hour at room temperature MeOH (3 mL) was added to stop the reaction. The solvent was removed under reduced pressure. Then the residue was re-dissolved with DCM and washed with 5% citric acid, water and saturated $NaHCO_3$. The crude product was purified by column chromatography (DCM + 1% $NEt_3$ → DCM:MeOH = 99:1) to yield **4** as a colourless foam (186 mg, 272 µmol, 81%).

**$^1$H-NMR** ($CDCl_3$, 400 MHz): $\delta$ (ppm) = 1.73 – 1.76 (m, 6H, 2x $C_q$-$CH_3$), 2.54 (s, 3H, S-$CH_3$), 3.16 (d, *J* = 1.5 Hz, 1H, C3'-OH), 3.22 (dd, *J* = 3.5, 10.5 Hz, 1H, H-5'b), 3.41 (dd, *J* = 3.5, 10.5 Hz, 1H, H-5'a), 3.77 (d, *J* = 1.6 Hz, 6H, $OCH_3$), 4.19 (s, br, 2H, NH-$CH_2$), 4.36 (dt, *J* = 1.6, 5.2 Hz, 1H, H-3'), 4.42 (td, *J* = 1.6, 3.5 Hz, 1H, H-4'), 4.71 – 4.77 (m, 1H, H-2'), 5.35 (dddd, *J* = 1.4, 2.9, 5.7, 8.6 Hz, 1H, CH=$C_q$), 5.84 (s, br, 1H, NH), 5.89 (d, *J* = 6.2 Hz, 1H, H-1'), 6.53 (s, br, 1H, C2'-OH) 6.71 – 6.78 (m, 4H, DMT), 7.13 – 7.22 (m, 7H, DMT), 7.24 – 7.29 (m, 2H, DMT), 7.88 (s, 1H, H-8).

**$^{13}$C-NMR** ($CDCl_3$, 100 MHz): $\delta$ (ppm) = 14.65 (S-$CH_3$), 18.16 (C-47, $CH_3$), 25.84 (C-46, $CH_3$), 38.63 (NH-$CH_2$), 55.33 ($OCH_3$, DMT), 63.78 (C5'), 73.12 (C-3'), 76.40 (C2'), 86.56, 86.63 (C4'; $C_q$, DMT), 90.96 (C1'), 113.26 (DMT), 117.81 (C5), 120.08 (*C*H=$C_q$), 127.02 (DMT), 127.99 (DMT), 128.15 (DMT), 130.03, 130.11 (DMT), 135.53, 135.74 ($C_q$, DMT), 136.78 (C8), 137.15 (CH=*C*$_q$), 144.37 ($C_q$, DMT), 148.86 (C4), 153.86 (C6) 158.64 ($C_q$-O, DMT), 165.97 (C2).

**HR-MS** ($ESI^+$): Exact mass calculated for $C_{37}H_{42}N_5O_6S$ $[M+H]^+$: 684.2856, found: 684.2862.

*N*$^6$-Isopentenyl-5'-*O*-(4,4'-dimethoxytrityl)-2'-*O*-(tert-butyldimethylsilyl)-2-thiomethyl-adenosine (**5**)

Compound **4** (170 mg, 249 µmol) was dissolved in anhydrous THF/pyridine (1:1, 3 mL) under nitrogen atmosphere followed by the addition of $AgNO_3$ (63.3 mg, 373 µmol) and TBDMS-Cl (41.2 mg, 273 µmol). The reaction was stirred at room temperature for 20 h. After addition of MeOH (1 mL), the suspension was filtered over celite. The solvent of the filtrate was removed

under reduced pressure. The residue was purified by column chromatography (hexane:EtOAc = 5:2 + 1% $NEt_3$ → 2:1) to obtain **5** as a colourless foam (63.5 mg, 79.6 µmol, 32%).

**$^1$H-NMR** ($CDCl_3$, 400 MHz): $\delta$ (ppm) = -0.10 (s, 3H, Si-$CH_3$), -0.00 (s, 3H, Si-$CH_3$), 0.86 (s, 9H, Si(C ($CH_3$)$_3$)), 1.72 – 1.76 (m, 6H, $C_q$($CH_3$)$_2$), 2.45 (s, 3H, S-$CH_3$), 2.45 (d, *J* = 4.8 Hz, 1H, C3'-OH), 3.40 (dd, *J* = 4.3, 10.5 Hz, 1H, H-5'b), 3.46 (dd, *J* = 3.4, 10.5 Hz, 1H, H-5'a), 3.78 (s, 6H, $OCH_3$), 4.12 – 4.24 (m, 3H, H-4', $CH_2$), 4.34 (q, *J* = 4.8 Hz, 1H, H-3'), 4.89 (t, *J* = 4.9 Hz, 1H, H-2'), 5.35 (tdt, *J* = 1.4, 2.9, 7.2 Hz, 1H, CH=$C_q$), 5.62 (s, br, 1H, NH), 5.94 – 5.99 (d, *J* = 4.9 Hz, 1H, H-1'), 6.77 – 6.83 (m, 4H, DMT), 7.17 – 7.23 (m, 1H, DMT), 7.22 – 7.31 (m, 2H, DMT), 7.29 – 7.34 (m, 4H, DMT), 7.39 – 7.45 (m, 2H, DMT), 7.81 (s, 1H, H-8).

**$^{13}$C-NMR** ($CDCl_3$, 100 MHz): $\delta$ (ppm) = -5.11, -4.70 (Si-$CH_3$), 14.58 (S-$CH_3$), 18.15 (C-24, $CH_3$), 25.74, (Si(C($CH_3$)$_3$)), 25.85 (C-23, $CH_3$), 38.61 (NH-$CH_2$), 55.35 ($OCH_3$), 63.71 (C5'), 71.63 (C3'), 75.75 (C2'), 83.91 (C4'), 86.69 ($C_q$, DMT), 88.27 (C-1'), 113.33 (DMT), 117.76 (C-5), 120.33 (*C*H=$C_q$), 127.06 (DMT), 128.04 (DMT), 128.26 (DMT), 130.22 (DMT), 135.73, 135.79 ($C_q$, DMT), 136.91 (CH=*C*$_q$), 137.33 (C-8), 144.71 ($C_q$, DMT), 149.98 (C-4), 158.66 ($C_q$-O, DMT), 166.20 (C-2).

**HR-MS** (ESI$^+$): Exact mass calculated for $C_{43}H_{55}N_5NaO_6SSi$ $[M+Na]^+$: 820.3540, found: 820.3537.

<u>*N*$^6$-Isopentenyl-5'-*O*-(4,4'-dimethoxytrityl)-2'-O-(tert-butyldimethylsilyl)-2-thiomethyl adenosine 3' cyanoethyl *N*,*N*-diisopropyl phosphoramidite (**6**)</u>

Compound **5** (105 mg, 132 µmol) was dissolved in anhydrous DCM (1 mL) under nitrogen atmosphere. $Me_2NEt$ (142 µL, 1.32 mmol) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (CEP-Cl) (46.7 mg, 197 µmol) were added and the resulting mixture was stirred at room temperature for 2h. After evaporation of the solvent under reduced pressure, the crude residue was purified by column chromatography (hexane:EtOAc = 3:1 + 1% $NEt_3$) to obtain **6** as a colourless foam (114 mg, 114 µmol, 86%).

**$^1$H-NMR** ($CDCl_3$, 400 MHz): $\delta$ (ppm) = -0.17 (s, 5H, Si-$CH_3$), -0.05 (s, 3H, Si-$CH_3$), -0.03 (s, 2H, Si-$CH_3$), 0.76 – 0.82 (m, 14H, Si(C($CH_3$)$_3$)), 1.02 – 1.19 (m, 20H, 4x $CH_3$, N(CH($CH_3$)$_2$),

1.73 – 1.74 (m, 6H, $C_q(CH_3)_2$), 1.74 – 1.75 (m, 5H, $C_q(CH_3)_2$), 2.27 – 2.33 (m, 1H, 25'', $POCH_2$), 2.42 (s, 2H, $SCH_3$), 2.44 (s, 3H, $SCH_3$), 2.56 – 2.72 (m, 2H, $POCH_2$), 3.33 (dt, *J* = 4.4, 10.4 Hz, 2H, H-5'a), 3.46 (dd, *J* = 3.7, 10.4 Hz, 1H, H-5'b), 3.50 – 3.55 (m, H-5'a, H-5'b), 3.56 – 3.73 (m, 5H, $N(CH(CH_3)_2)$, 3.77 – 3.78 (m, 7H, $OCH_3$), 3.78 (s, 3H, $OCH_3$), 3.82 – 3.91 (m, 1H, $CH_2CN$), 3.91 – 4.00 (m, 1H, $CH_2CN$), 4.19 (s, 3H, NH-$CH_2$), 4.28 – 4.32 (m, 1H, H-3', H-4'), 4.32 – 4.36 (m, 1H, H-4'), 4.36 – 4.42 (m, 1H, H-3'), 4.89 – 4.96 (m, 2H, H-2'), 5.32 – 5.37 (m, 2H, $CH=C_q$), 5.56 (s, br, 2H, NH), 5.98 (d, *J* = 5.8 Hz, 1H, H-1'), 6.00 – 6.03 (d, *J* = 6.2 Hz, 1H, H-1'), 6.77 – 6.84 (m, 7H, DMT), 7.17 – 7.23 (m, 1H, DMT), 7.21– 7.29 (m, 2H, DMT), 7.29 – 7.37 (m, 7H, DMT), 7.40 – 7.46 (m, 4H, DMT), 7.84 (s, 1H, H-8), 7.87 (s, 1H, H-8).

**$^{13}$C-NMR** ($CDCl_3$, 100 MHz): *δ* (ppm) = -5.04, 1.02 (Si-$CH_3$), 14.28, 14.35 ($SCH_3$), 17.98, 18.00 (C-66, $CH_3$), 20.39, 20.45 ($POCH_2$), 24.60, 24.68 ($N(CH(CH_3)_2)$), 25.61, 25.63, 25.64, 25.70 ($Si(C(CH_3)_3)$), 42.89, 43.02 (($N(CH(CH_3)_2)$), 55.20 ($OCH_3$), 58.83 ($CH_2CN$), 63.44, 63.59 (C-5'), 72.99 (C-4'), 75.48 (C-2'), 83.43 (C-3'), 86.51, 86.64 ($C_q$, DMT), 87.55 (C-1'), 113.15, 113.18, 113.20 (DMT), 117.51, 117.58 (C-5, CN), 120.22 ($CH=C_q$), 126.93 (DMT), 127.90 (DMT), 128.09 (DMT), 130.07, 130.12 (DMT), 135.48, 135.51 ($C_q$, DMT), 136.74 ($CH=C_q$), 137.27 (C-8), 144.50, 144.61 ($C_q$, DMT), 149.77, 149.97 (C-4), 158.53 ($C_q$-O, DMT), 165.94 (C-2).

**$^{31}$P-NMR** ($CDCl_3$, 162 MHz): *δ* (ppm) = 149.08, 150.64

**HR-MS** ($ESI^+$): Exact mass calculated for $C_{52}H_{72}N_7NaO_7PSSi$ $[M+Na]^+$: 1020.4619, found: 1020.4613.

***Experimental procedures for synthesis of t$^6$A phosphoramidite***

L-Threonine-O-(tert-butyldimethylsilyl)-2-(4-Nitrophenyl)ethyl ester (**7**)

L-Threonine (500 mg, 4.20 mmol, 1.0 eq), 2-(4-nitrophenyl) ethanol (2.11 g, 12.6 mmol, 3.0 eq) and TsOH (2.40 g, 12.6 mmol, 3.0 eq) were refluxed in toluene (20 mL) for four days using a *Dean-Stark* apparatus. After cooling down to room temperature, $Et_2O$ was added and the upper layer removed to get the oily residue. Further purification with column chromatography ($SiO_2$, DCM/MeOH 11:1) gave the product as a yellow oil (1.28 g, 2.92 mmol, 70 %).

**$^1$H-NMR** (DMSO-*d*6, 400 MHz): $\delta$ (ppm) = 1.13 (d, *J* = 6.5 Hz, 3H, $CH_3$), 2.29 (s, 3H, $CH_3$, OTs), 3.10 (t, *J* = 6.3 Hz, 2H, O-$CH_2$-C$H_2$), 3.92 (q, *J* = 5.0 Hz, 1H, NH2-C*H*), 4.05 (qd, *J* = 6.5, 3.8 Hz, 1H, O-CH), 4.50 – 4.38 (m, 2H, O-$CH_2$), 7.08-7.14 (m, 2H, OTs), 7.49 – 7.42 (m, 2H, OTs), 7.63 – 7.57 (m, 2H, bz), 8.25 – 8.13 (m, 5H, $NH_3^+$, bz).

L-Threonine-2-(4-Nitrophenyl)ethyl ester (785 mg, 1.79 mmol, 1.0 eq.) was dissolved in dry pyridine (15 mL) and imidazole (366 mg, 5.37 mmol, 3.0 eq) and TBDMS-Cl (809 mg, 5.37 mmol, 3.0 eq) were added. The reaction mixture was stirred overnight at room temperature and then diluted with DCM (80 mL). The organic phase was washed with sat. $NaHCO_3$ solution (2 x 80 mL), water (80 mL) and brine (80 mL) and then dried over $Na_2SO_4$. The solvents were removed *in vacuo* and the product purified with column chromatography ($SiO_2$, 1.5 % MeOH in DCM) to give the protected amino acid **7** as a yellow oil (505 mg, 1.32 mmol, 74 %).

The analytical data were consistent with previously published data.[3]

**$^1$H-NMR** ($CDCl_3$, 400 MHz): $\delta$ (ppm) = 8.21 – 8.14 (m, 2H, C$H_{npe}$), 7.44 – 7.36 (m, 2H, C$H_{npe}$), 4.43 (dt, *J* = 11.0, 6.8 Hz, 1H, O-C$H_2$), 4.31 – 4.24 (m, 1H, O-C$H_2$), 4.21 (qd, *J* = 6.3, 2.3 Hz, 1H, $CH_3$-C*H*OR), 3.28 (d, *J* = 2.8 Hz, 1H, C*H*-$NH_2$), 3.08 (t, *J* = 6.8 Hz, 2H, C$H_2$-Phen), 1.23 (d, *J* = 6.3 Hz, 3H, thr-C$H_3$), 0.82 (s, 9H, Si-C(C$H_3$)$_3$), 0.01 (s, 3H, Si-C$H_3$), -0.08 (s, 3H, Si-C$H_3$).

**$^{13}$C-NMR** ($CDCl_3$, 100 MHz): $\delta$ (ppm) = 174.16 (*C*=O), 147.10 (4-$C_{npe}$-$NO_2$), 145.57 (1-$C_{npe}$), 129.92 ($C$H$_{npe}$), 123.96 ($C$H$_{npe}$), 69.63 ($CH_3$-*C*HOR), 64.63 (O-*C*$H_2$), 60.86 (*C*H-$NH_2$), 35.02 (*C*$H_2$-Phen), 25.75 (Si-C(*C*$H_3$)$_3$), 21.02 (thr-*C*$H_3$), 18.00 (Si-*C*($CH_3$)$_3$), -4.15 (Si-*C*$H_3$), -5.11 (Si-*C*$H_3$).

**HR-MS** ($ESI^+$): Exact mass calculated for $C_{18}H_{31}N_2O_5Si$ $[M+H]^+$: 383.1997, found: 383.2007.

<u>1-*N*-methyl-3-phenoxycarbonyl-imidazolium chloride</u>

Phenyl chloroformate (1.50 mL, 11.9 mmol, 1.0 eq.) was dissolved in dry DCM. *N*-Methyl-imidazole (947 µL, 11.9 mmol, 1.0 eq.) was added at 0 °C and the reaction stirred for 2 h at room temperature. The precipitate was collected by filtration, washed with DCM and dried to give the product as a colourless solid (2.78 g, 11.7 mmol, 98 %).

**$^1$H-NMR** (DMSO-*d*6, 400 MHz): *δ* (ppm) = 10.23 (td, *J* = 1.6, 0.9 Hz, 1H, 2-C*H*), 8.42 – 8.34 (m, 1H, 5-C*H*), 7.99 (dd, *J* = 2.2, 1.5 Hz, 1H, 4-C*H*), 7.61 – 7.54 (m, 2H, 3'-C*H*), 7.49 – 7.43 (m, 3H, 2'-C*H*, 4'-C*H*), 4.01 (d, *J* = 0.8 Hz, 3H, N-CH3).

**HR-MS** (ESI$^+$): Exact mass calculated for $C_{11}H_{11}N_2O_2$ [M+H]$^+$: 203.0815, found: 203.0822.

2'-*O*-(tert-butyldimethylsilyl)-3'-*O*,5'-*O*-di-tert-butylsilylene adenosine (**8**)

To a suspension of adenosine (1.00 g, 3.74 mmol) in DMF (10 mL) di-*tert*-butylsilyl ditriflate (1.20 eq, 1.46 mL, 4.49 mmol) was added dropwise at 0 °C. The solution was stirred at 0 °C for 45 min. After addition of imidazole (5.00 eq, 1.27 g, 18.7 mmol) the reaction mixture was allowed to reach room temperature over 30 min. TBDMS-Cl (1.20 eq, 0.68 g, 4.49 mmol) was added and then the mixture was heated to 60 °C overnight. After cooling to room temperature, the reaction mixture was diluted with EtOAc (40 mL) and washed with water (2 x 50 mL) and brine (60 mL). The organic layer was dried over $Na_2SO_4$ and evaporated. Column Chromatography ($SiO_2$, EtOAc/Hexan 1:1) gave the protected adenosine **8** as a colourless solid (1.40 g, 2.68 mmol, 72%).

**$^1$H-NMR** ($CDCl_3$, 400 MHz): *δ* (ppm) = 8.32 (s, 1H, 2-C*H*), 7.83 (s, 1H, 8-C*H*), 5.91 (s, 1H, 1'-C*H*), 5.89 – 5.82 (brs, 2H, N$H_2$), 4.62 (d, *J* = 4.7 Hz, 1H, 2'-C*H*), 4.54 (dd, *J* = 9.5, 4.7 Hz, 1H, 3'-C*H*), 4.51 – 4.46 (m, 1H, 5'-C$H_2$), 4.21 (td, *J* = 10.1, 5.1 Hz, 1H, 4'-C*H*), 4.03 (dd, *J* = 10.5, 9.1 Hz, 1H, 5'-C$H_2$), 1.08 (s, 9H, Si-C(C$H_3$)$_3$), 1.04 (s, 9H, Si-C(C$H_3$)$_3$), 0.93 (s, 9H, TBDMS-Si-C(C$H_3$)$_3$), 0.16 (s, 3H, Si-C$H_3$), 0.14 (s, 3H, Si-C$H_3$).

**$^{13}$C-NMR** ($CDCl_3$, 100 MHz): *δ* (ppm) = 155.44 (6-*C*), 152.98 (2-*C*H), 149.41 (4-*C*), 139.05 (8-*C*H), 120.45 (5-*C*), 92.59 (1'-*C*H), 75.95 (3'-*C*H), 75.59 (2'-*C*H), 74.82 (4'-*C*H), 67.95 (5'-*C*$H_2$), 27.63 (Si-C(*C*$H_3$)$_3$), 27.16 (Si-C(*C*$H_3$)$_3$), 26.03 (TBDMS-Si-C(*C*$H_3$)$_3$), 22.89 (Si-*C*(CH$_3$)$_3$), 20.49 (Si-*C*(CH$_3$)$_3$), 18.45 (TBDMS-Si-*C*(CH$_3$)$_3$), -4.16 (TBDMS-Si-*C*H$_3$), -4.85 (TBDMS-Si-*C*H$_3$).

**HR-MS** (ESI$^+$): Exact mass calculated for $C_{24}H_{44}N_5O_4Si_2$ [M+H]$^+$: 522.2932, found: 522.2928.

*N*$^6$-(O-*tert*-butyldimethylsilyl-2-(4-nitrophenylethyl)-N-L-threoninyl)carbamoyl-2'-*O*-(*tert*-butyldimethylsilyl)-3'-*O*,5'-*O*-di-*tert*-butylsilylene adenosine (**9**)

The silyl-protected adenosine derivative **8** (334 mg, 0.64 mmol) was dissolved in dry DCM (8 mL). After addition of 1-*N*-methyl-3-phenoxycarbonyl-imidazolium chloride (306 mg, 1.28 mmol) the reaction was stirred for 2 h. Then, a solution of O-TBDMS-protected threonine 4-nitrophenylethyl ester (490 mg, 1.28 mmol) and $NEt_3$ (130 mg, 1.28 mmol) in dry DCM (8 mL) were added and the mixture was stirred at room temperature overnight. After addition of saturated $NaHCO_3$ solution (25 mL) the mixture was extracted with DCM (3 x 20 mL). The combined organic phases were dried over $Na_2SO_4$ and the solvents removed *in vacuo*. Further purification with column chromatography ($SiO_2$, EtOAc/Hexan 3:4) gave **9** as a colourless foam (479 mg, 520 µmol, 81 %).

**$^1$H-NMR** ($CDCl_3$, 400 MHz): $\delta$ (ppm) = 9.93 (d, *J* = 9.0 Hz, 1H, thr-N*H*), 8.42 (s, 1H, 2-C*H*), 8.06 (s, 1H, 8-C*H*), 7.98 – 7.91 (m, 2H, C$H_{npe}$), 7.37 – 7.30 (m, 2H, C$H_{npe}$), 5.98 (s, 1H, 1'-C*H*), 4.66 (d, *J* = 4.6 Hz, 1H, 2'-C*H*), 4.58 – 4.47 (m, 4H, 3'-C*H*, 5'-C$H_2$, thr-NH-C*H*, thr-O-C*H*), 4.38 (td, *J* = 6.6, 2.5 Hz, 2H, O-C$H_2$), 4.25 (d, *J* = 5.0 Hz, 1H, 4'-C*H*), 4.06 (d, *J* = 9.2 Hz, 1H, 5'-C$H_2$), 3.03 (t, *J* = 6.5 Hz, 2H, O-CH$_2$-C$H_2$), 1.25 (d, *J* = 6.2 Hz, 3H, thr-C$H_3$), 1.09 (s, 9H, Si-C(C$H_3$)$_3$), 1.05 (s, 9H, Si-C(C$H_3$)$_3$), 0.95 (s, 9H, Si-C(C$H_3$)$_3$), 0.90 (s, 9H, Si-C(C$H_3$)$_3$), 0.19 (s, 3H, Si-C$H_3$), 0.17 (s, 3H, Si-C$H_3$), 0.07 (s, 3H, Si-C$H_3$), -0.03 (s, 3H, Si-C$H_3$).

**$^{13}$C-NMR** ($CDCl_3$, 100 MHz): $\delta$ (ppm) = 170.94 (thr-*C*OOR), 154.33 (NH-*C*ONH), 150.14 (6-*C*), 149.68 (4-*C*), 146.78 (*C*$_{npe}$), 145.64 (*C*$_{npe}$), 141.11 (8-*C*H), 129.87 (*C*H$_{npe}$), 123.64 (*C*H$_{npe}$), 120.85 (5-*C*), 92.67 (1'-*C*H), 75.93 (3'-*C*H), 75.64 (2'-*C*H), 74.96 (4'-*C*H), 68.72 (thr-O*C*H), 67.91 (5'-*C*H$_2$), 64.67 (O-*C*H$_2$), 59.76 (thr-NH-*C*H), 34.92 (O-CH$_2$-*C*H$_2$), 27.61 (Si-C(*C*H$_3$)$_3$), 27.16 (Si-C(*C*H$_3$)$_3$), 26.04 (Si-C(*C*H$_3$)$_3$), 25.66 (Si-C(*C*H$_3$)$_3$), 22.89 (Si-*C*(CH$_3$)$_3$), 21.24 (thr-*C*H$_3$), 20.50 (Si-*C*(CH$_3$)$_3$), 18.45 (Si-*C*(CH$_3$)$_3$), 17.95 (Si-*C*(CH$_3$)$_3$), -4.15 (Si-*C*H$_3$), -4.86 (Si-*C*H$_3$), -5.27 (Si-*C*H$_3$).

**HR-MS** (ESI$^+$): Exact mass calculated for $C_{43}H_{71}N_7NaO_{10}Si_3$ [M+Na]$^+$: 952.4468, found: 952.4458.

<u>*N*$^6$-(O-*tert*-butyldimethylsilyl-2-(4-nitrophenylethyl)-*N*-L-threoninyl)carbamoyl-2'-*O*-(*tert*-butyldimethylsilyl) adenosine (**10**)</u>

Compound **9** (250 mg, 270 µmol) was dissolved in dry DCM (5 mL). After addition of pyridine (300 µL) the solution was cooled to 0 °C and HF-pyridine (70% HF, 30% pyridine, 44.0 µL) was added. The reaction mixture was stirred for 2 h at 0 °C and then quenched with sat. $NaHCO_3$ solution (5 mL). The aqueous phase was extracted with DCM (3 x 10 mL), then the combined organic phases were washed with water (30 mL), dried over $Na_2SO_4$ and evaporated. Column Chromatography ($SiO_2$, DCM/MeOH 11:1) gave the product **10** as a colourless foam (180 mg, 230 µmol, 85 %).

**$^1$H-NMR** ($CDCl_3$, 400 MHz): $\delta$ (ppm) = 9.88 (d, *J* = 9.1 Hz, 1H, thr-N*H*), 8.50 (s, 1H, 2-C*H*), 8.40 (s, 1H, N*H*), 8.14 (s, 1H, 8-C*H*), 8.12 – 8.06 (m, 2H, C$H_{npe}$), 7.41 – 7.33 (m, 2H, C$H_{npe}$), 5.86 (d, *J* = 6.9 Hz, 1H, 1'-C*H*), 5.08 (dd, *J* = 7.0, 4.7 Hz, 1H, 2'-C*H*), 4.58 (dd, *J* = 9.1, 1.7 Hz, 1H, thr-NH-C*H*), 4.53 – 4.42 (m, 2H, thr-O-C*H*, O-C$H_2$), 4.41 – 4.36 (m, 2H, 3'-C*H*, 4'-C*H*), 4.30 (dt, *J* = 11.0, 6.8 Hz, 1H, O-C$H_2$), 3.98 (dd, *J* = 12.9, 1.7 Hz, 1H, 5'-C$H_2$), 3.78 (dd, *J* = 12.9, 1.6 Hz, 1H, 5'-C$H_2$), 3.07 (t, *J* = 6.7 Hz, 2H, O-$CH_2$-C$H_2$), 1.24 (d, *J* = 6.2 Hz, 3H, thr-C$H_3$), 0.89 (s, 9H, Si-C(C$H_3$)$_3$), 0.82 (s, 9H, Si-C(C$H_3$)$_3$), 0.05 (s, 3H, Si-C$H_3$), -0.06 (s, 3H, Si-C$H_3$), -0.14 (s, 3H, Si-C$H_3$), -0.34 (s, 3H, Si-C$H_3$).

**$^{13}$C-NMR** ($CDCl_3$, 100 MHz): $\delta$ (ppm) = 170.84 (thr-*C*OOR), 153.94 (NH-*C*ONH), 151.24 (6-*C*), 150.87 (2-*C*H), 149.25 (4-*C*), 147.02 (*C*$_{npe}$), 145.53 (*C*$_{npe}$), 142.85 (8-*C*H), 129.97 (*C*H$_{npe}$), 123.87 (*C*H$_{npe}$), 121.69 (5-*C*), 91.48 (1'-*C*H), 87.61 (3'-*C*H), 74.87 (2'-*C*H), 72.69 (4'-*C*H), 68.74 (thr-O*C*H), 65.06 (O-*C*$H_2$), 63.24 (5'-*C*$H_2$), 59.69 (thr-NH-*C*H), 34.98 (O-$CH_2$-*C*$H_2$), 25.65 (Si-C(*C*$H_3$)$_3$), 21.25 (thr-*C*$H_3$), 17.97 (Si-*C*(CH$_3$)$_3$), 17.95 (Si-*C*(CH$_3$)$_3$), -4.10 (Si-*C*$H_3$), -5.15 (Si-*C*$H_3$), -5.20 (Si-*C*$H_3$), -5.29 (Si-*C*$H_3$).

**HR-MS** (ESI$^+$): Exact mass calculated for $C_{35}H_{55}N_7NaO_{10}Si_2$ $[M+Na]^+$: 812.3441, found: 812.3447.

5'-*O*-(4,4'-Dimethoxyrtriyl)-*N*$^6$-(O-*tert*-butyldimethylsilyl-2-(4-nitrophenylethyl)-*N*-L-threoninyl)carbamoyl-2'-*O*-(*tert*-butyldimethylsilyl) adenosine (**11**)

To a solution of **10** (325 mg, 411 µmol) in pyridine (6 mL) 4,4,'-dimethoxytrityl chloride (DMT-Cl, 227 mg, 617 µmol) was added and the resulting mixture was stirred overnight at room

temperature. After removing the volatiles *in vacuo* the compound was purified with column chromatography ($SiO_2$, 1. DCM + 1 % $NEt_3$, 2. Toluene/EtOAc 3:2 + 1 % $NEt_3$) to give **11** as a colourless foam (290 mg, 265 µmol, 64%).

**$^1$H-NMR** ($CDCl_3$, 400 MHz): $\delta$ (ppm) = 9.97 (d, *J* = 9.1 Hz, 1H, thr-N*H*), 8.41 (s, 1H, 2-C*H*), 8.18 (s, 1H, 8-C*H*), 8.03 – 7.99 (m, 2H, C$H_{npe}$), 7.98 (s, 1H, N*H*), 7.46 – 7.42 (m, 2H, DMT-C*H*), 7.36 – 7.31 (m, 6H, C$H_{npe}$, DMT-C*H*), 7.31 – 7.18 (m, 3H, DMT-C*H*), 6.84 – 6.78 (m, 4H, DMT-C*H*), 6.08 (d, *J* = 4.9 Hz, 1H, 1'-C*H*), 4.97 (t, *J* = 5.0 Hz, 1H, 2'-C*H*), 4.57 (dd, *J* = 9.1, 1.6 Hz, 1H, thr-NH-C*H*), 4.51 (qd, *J* = 6.2, 1.6 Hz, 1H, thr-O-C*H*), 4.44 – 4.30 (m, 3H, 3'-C*H*, O-C$H_2$), 4.26 (q, *J* = 3.6 Hz, 1H, 4'-C*H*), 3.78 (d, *J* = 1.4 Hz, 6H, trityl-OC$H_3$), 3.54 (dd, *J* = 10.7, 3.0 Hz, 1H, 5'-C*H2*), 3.41 (dd, *J* = 10.7, 3.9 Hz, 1H, 5'-C$H_2$), 3.04 (t, *J* = 6.6 Hz, 2H, O-$CH_2$-C$H_2$), 2.67 (d, *J* = 4.7 Hz, 1H, 3'-O*H*), 1.27 (d, *J* = 6.3 Hz, 3H, thr-C$H_3$), 0.88 (s, 9H, Si-C(C$H_3$)$_3$), 0.86 (s, 9H, Si-C(C$H_3$)$_3$), 0.06 (s, 3H, Si-C$H_3$), 0.03 (s, 3H, Si-C$H_3$), -0.05 (s, 3H, Si-C$H_3$), -0.08 (s, 3H, Si-C$H_3$).

**$^{13}$C-NMR** ($CDCl_3$, 100 MHz): $\delta$ (ppm) = 171.00 (thr-*C*OOR), 158.72 (DMT-$C_q$-OCH3), 154.25 (NH-*C*ONH), 151.36 (2-*C*H), 150.38 (4-*C*), 150.21 (6-*C*), 146.89 ($C_{npe}$), 145.58 ($C_{npe}$), 144.69 (DMT-$C_q$), 141.54 (8-*C*H), 135.70 (DMT-$C_q$), 135.66 (DMT-$C_q$), 130.24 (DMT-*C*H), 130.22 (DMT-*C*H), 129.88 (*C*H$_{npe}$), 128.26 (DMT-*C*H), 128.05 (DMT-*C*H), 127.12 (DMT-*C*H), 123.74 (3''-*C*H), 120.96 (5-*C*), 113.34 (DMT-*C*H), 88.71 (1'-*C*H), 86.85 (DMT-O*C*), 84.32 (4'-*C*H), 75.98 (2'-*C*H), 71.53 (3'-*C*H), 68.74 (thr-O*C*H), 64.83 (O-*C*$H_2$), 63.43 (5'-*C*$H_2$), 59.70 (thr-NH-*C*H), 55.36 (DMT-O*C*$H_3$), 34.94 (O-CH2-*C*H2), 25.72 (Si-C(*C*$H_3$)$_3$), 25.62 (Si-C(*C*$H_3$)$_3$), 21.28 (thr-*C*$H_3$), 18.05 (Si-*C*(CH$_3$)$_3$), 17.92 (Si-*C*(CH$_3$)$_3$), -4.18 (Si-$_{CH3}$), -4.74 (Si-*C*$H_3$), -5.03 (Si-*C*$H_3$), -5.29 (Si-*C*$H_3$).

**HR-MS** (ESI$^+$): Exact mass calculated for $C_{56}H_{73}N_7NaO_{12}Si_2$ $[M+Na]^+$: 1114.4753, found: 1114.4745.

5'-*O*-(4,4'-Dimethoxyrtrityl)-$N^6$-(O-*tert*-butyldimethylsilyl-2-(4-nitrophenylethyl)-*N*-L-threoninyl)carbamoyl-2'-*O*-(*tert*-butyldimethylsilyl) adenosine 3' cyanoethyl *N*,*N*-diisopropyl phosphoramidite (**12**)

The 5'-DMT-2'-TBDMS-protected nucleoside **11** (90.0 mg, 82.0 µmol) was dissolved in anhydrous DCM (1.5 mL) and Hünig's Base (56.0 µL, 328 µmol) was added dropwise. After addition of 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (CEP-Cl) (49.0 mg, 205 µmol) the reaction was stirred at room temperature for 4 h. The solvent was removed and the product purified by column chromatography ($SiO_2$, hexane/EtOAc 2:1 + 1 % $NEt_3$) to obtain **12** as a colourless foam (62.0 mg, 48.0 µmol, 59 %).

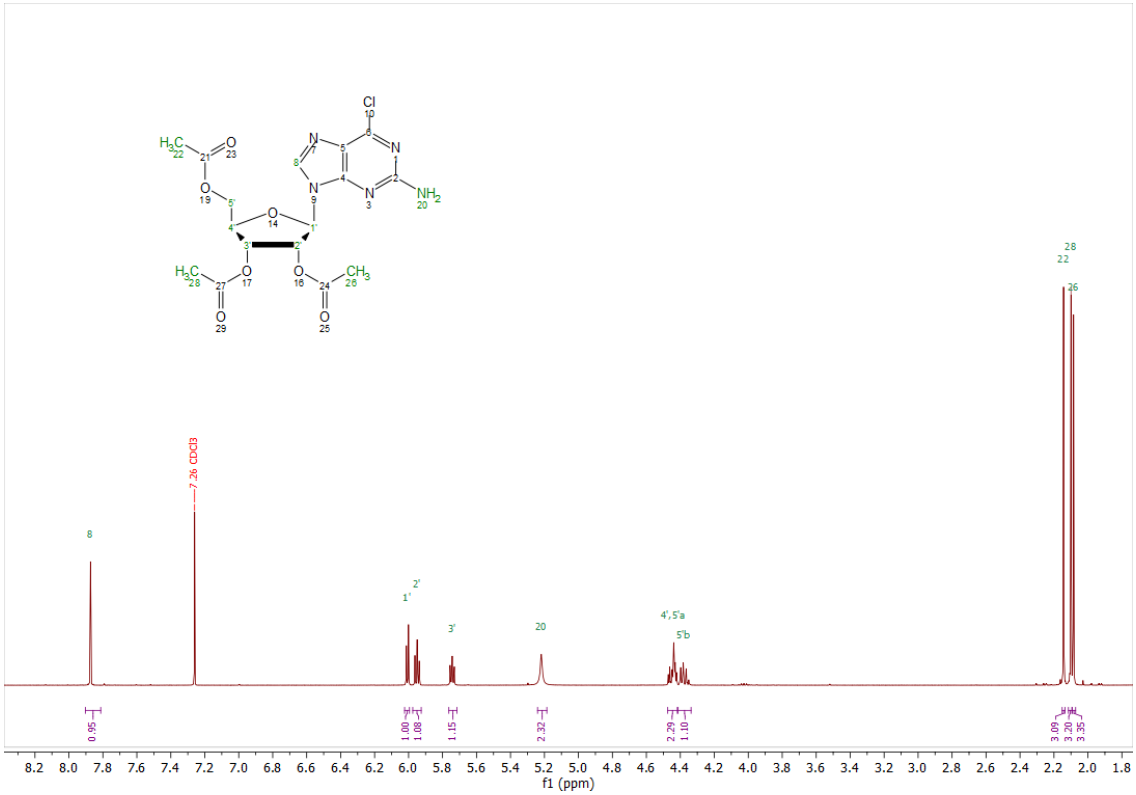
**$^1$H-NMR** ($CDCl_3$, 400 MHz): *δ* (ppm) = 9.98 (dd, J = 9.1, 7.3 Hz, 1H, thr-NH), 8.40 (d, J = 5.0 Hz, 1H, 2-CH), 8.20 (d, J = 13.5 Hz, 1H, 8-CH), 8.08 (d, J = 1.5 Hz, 1H), 8.04 – 7.99 (m, 3H, $CH_{npe}$), 7.46 (ddd, J = 7.7, 6.4, 1.4 Hz, 2H, DMT-CH), 7.38 – 7.30 (m, 6H, $CH_{npe}$, DMT-CH), 7.30 – 7.19 (m, 3H, DMT-CH), 6.85 – 6.78 (m, 4H, DMT-CH), 6.08 (dd, J = 21.1, 5.8 Hz, 1H, 1'-CH), 5.00 (dd, J = 5.9, 4.6 Hz, 1H, 2'-CH), 4.56 (dd, J = 9.0, 1.6 Hz, 1H, thr-NH-CH), 4.55 – 4.46 (m, 1H, thr-O-CH), 4.47 – 4.37 (m, 2H, 3'-CH, O-$CH_2$), 4.37 – 4.27 (m, 2H, 4'-CH, O-CH2), 4.00 – 3.83 (m, 1H, PO-$CH_2$), 3.78 (dd, J = 2.1, 1.4 Hz, 6H, DMT-$OCH_3$), 3.69 – 3.51 (m, 4H, PO-$CH_2$, 5'-CH2, $NCH(CH_3)_2$), 3.33 (td, J = 10.5, 3.8 Hz, 1H, 5'-CH2), 3.04 (t, J = 6.6 Hz, 2H, O-$CH_2$-$CH_2$), 2.65 (tt, J = 6.3, 2.9 Hz, 1H, PO-$CH_2$-$CH_2$), 2.31 (td, J = 6.5, 2.5 Hz, 1H, PO-$CH_2$-$CH_2$), 1.28 – 1.24 (m, 6H, thr-$CH_3$, $NCH(CH_3)_2$), 1.20 – 1.15 (m, 6H, $NCH(CH_3)_2$), 1.04 (d, J = 6.8 Hz, 3H, $NCH(CH_3)_2$), 0.88 (d, J = 3.5 Hz, 9H, Si-$C(CH_3)_3$), 0.78 (d, J = 1.1 Hz, 9H, Si-$C(CH_3)_3$), 0.05 (d, J = 2.5 Hz, 3H, Si-$CH_3$), -0.01 (d, J = 10.8 Hz, 3H, Si-$CH_3$), -0.06 (d, J = 1.7 Hz, 3H, Si-$CH_3$), -0.16 (d, J = 8.4 Hz, 3H, Si-$CH_3$).

**$^{13}$C-NMR** ($CDCl_3$, 100 MHz): *δ* (ppm) = 171.04 (thr-COOR), 158.72 (DMT-$C_q$-$OCH_3$), 154.20 (NH-CONH), 151.17 (2-CH), 150.40 (6-C), 150.17 (4-C), 146.92 ($C_{npe}$), 145.58 ($C_{npe}$), 144.56 (DMT-$C_q$), 141.58 (8-CH), 130.25 (DMT-CH), 129.89 ($CH_{npe}$), 128.38 (DMT-CH), 128.27 (DMT-CH), 128.07 (DMT-CH), 127.14 (DMT-CH), 123.77 ($CH_{npe}$), 120.77 (5-C), 113.35 (DMT-CH), 88.21 (1'-CH), 86.95 (DMT-OC), 84.05 (4'-CH), 74.89 (2'-CH), 73.17 (3'-CH), 68.62 (thr-OCH), 64.87 (O-$CH_2$), 63.22 (5'-$CH_2$), 59.71 (thr-NH-CH), 58.85 (PO-$CH_2$), 55.37 (DMT-$OCH_3$), 42.88 ($NCH(CH_3)_2$), 34.95 (O-$CH_2$-$CH_2$), 25.73 (Si-$C(CH_3)_3$), 25.62 (Si-$C(CH_3)_3$), 24.85 ($NCH(CH_3)_2$), 24.76 ($NCH(CH_3)_2$), 21.28 (thr-$CH_3$), 20.04 (PO-$CH_2$-$CH_2$), 18.07 (Si-$C(CH_3)_3$), 17.92 (Si-$C(CH_3)_3$), -4.18 (Si-$CH_3$), -4.69 (Si-$CH_3$), -4.93 (Si-$CH_3$), -5.28 (Si-$CH_3$).

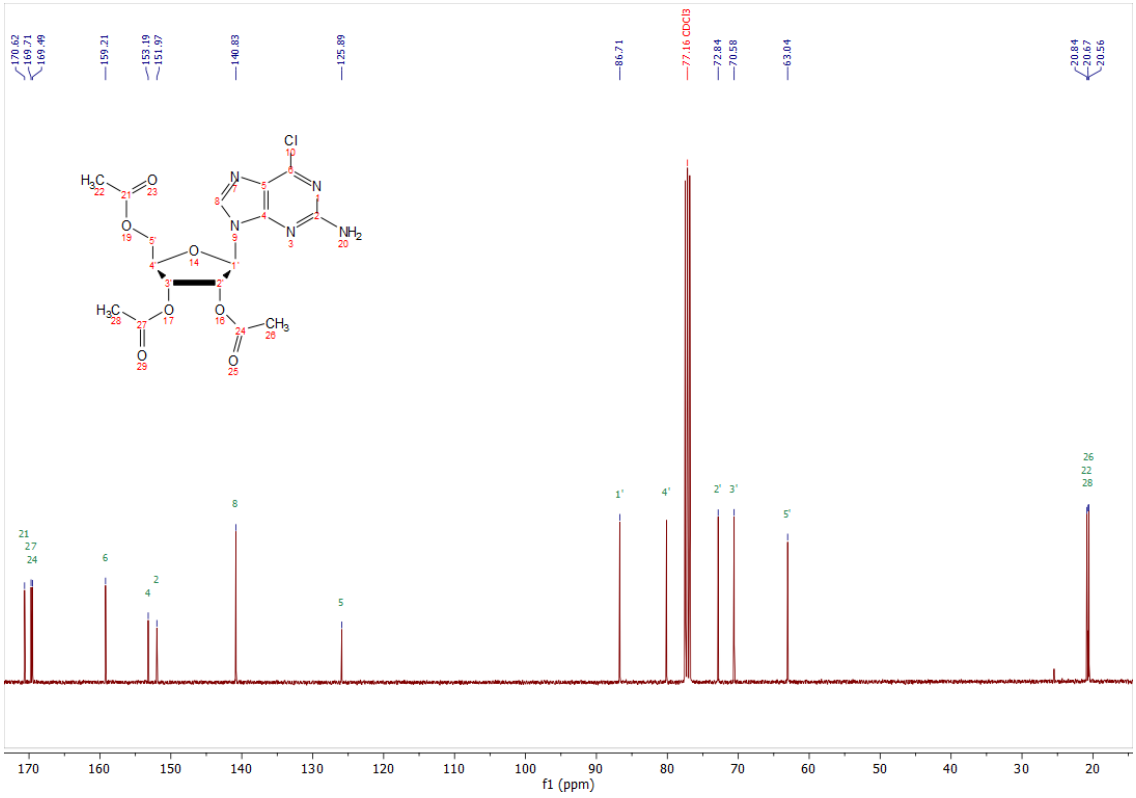
**$^{31}$P-NMR** ($CDCl_3$, 162 MHz): $\delta$ (ppm) = 149.09, 150.77.

**HR-MS** ($ESI^+$): Exact mass calculated for $C_{65}H_{90}N_9NaO_{13}PSi_2$ $[M+Na]^+$: 1314.5826, found: 1314.5848.

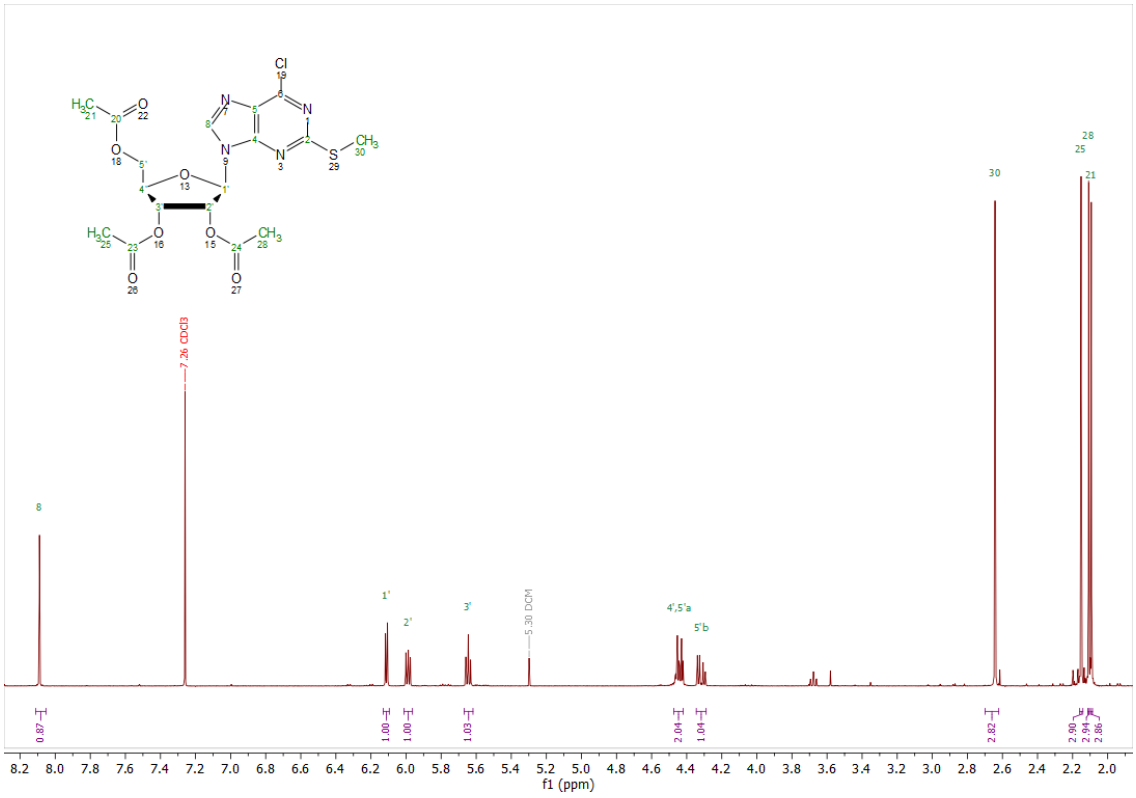
***NMR spectra***



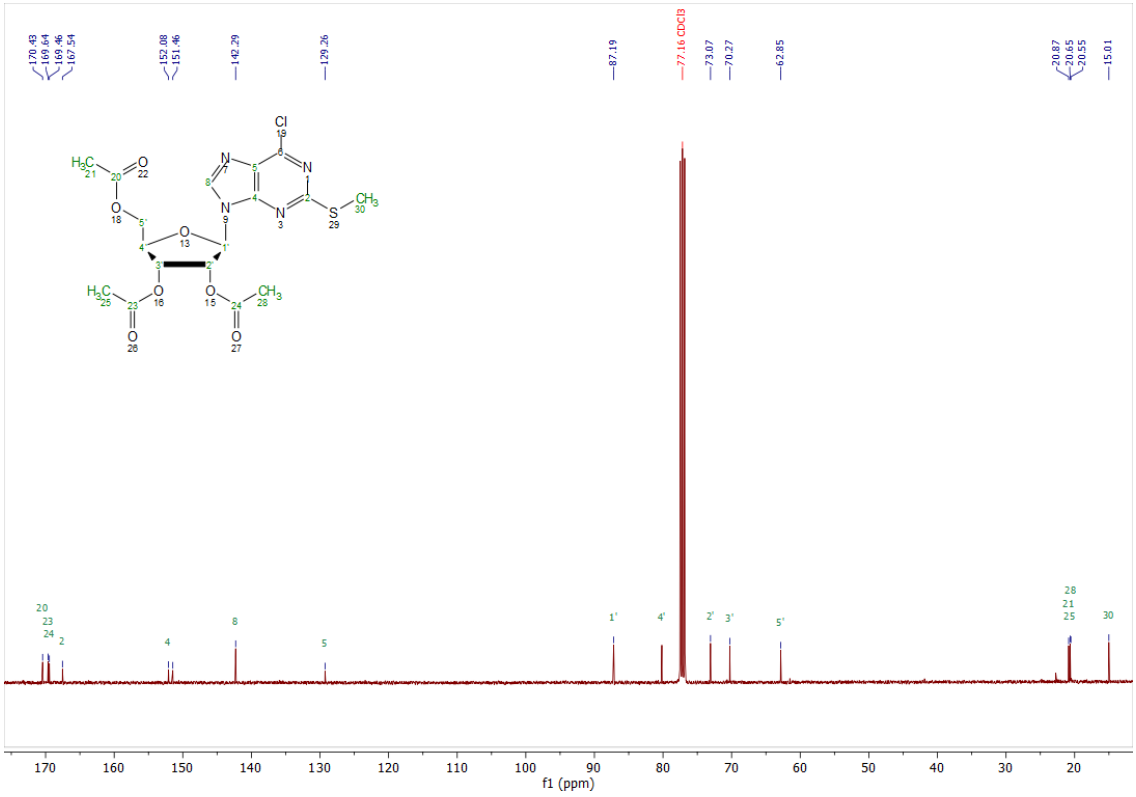

$^{1}$H-NMR (400 MHz, $CDCl_3$) of compound **1**.



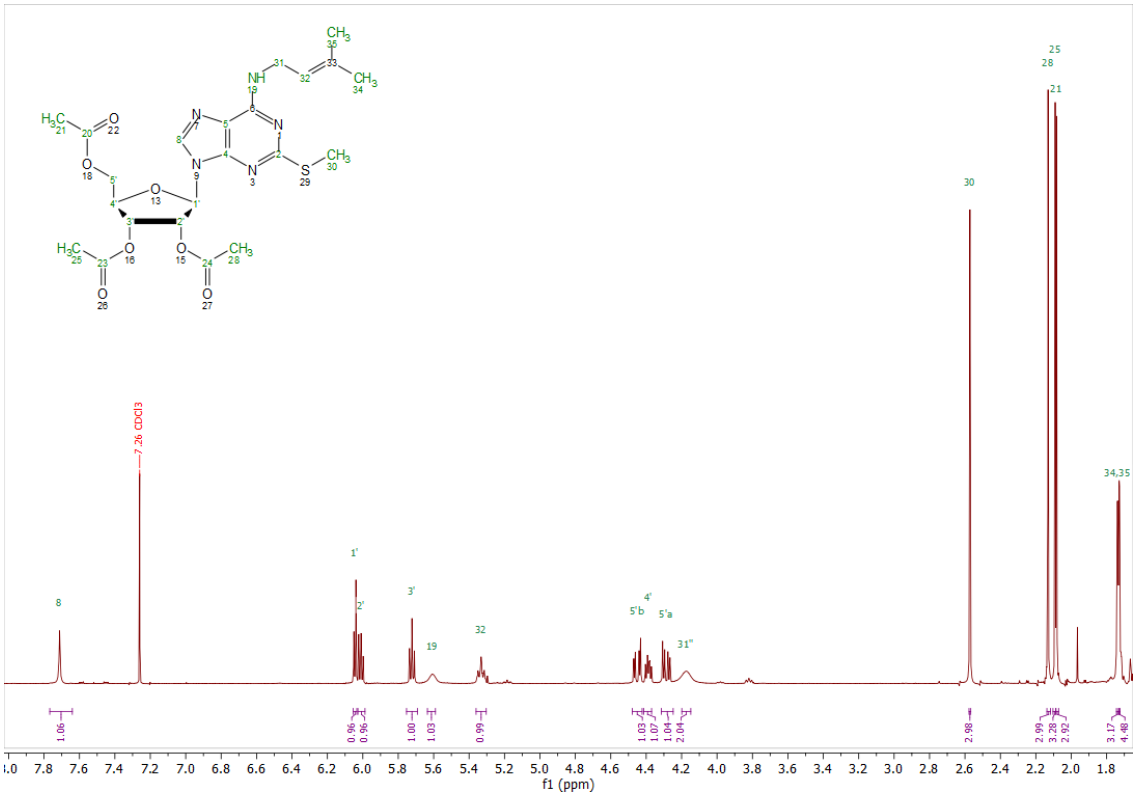

$^{13}$C-NMR (100 MHz, $CDCl_3$) of compound **1**.



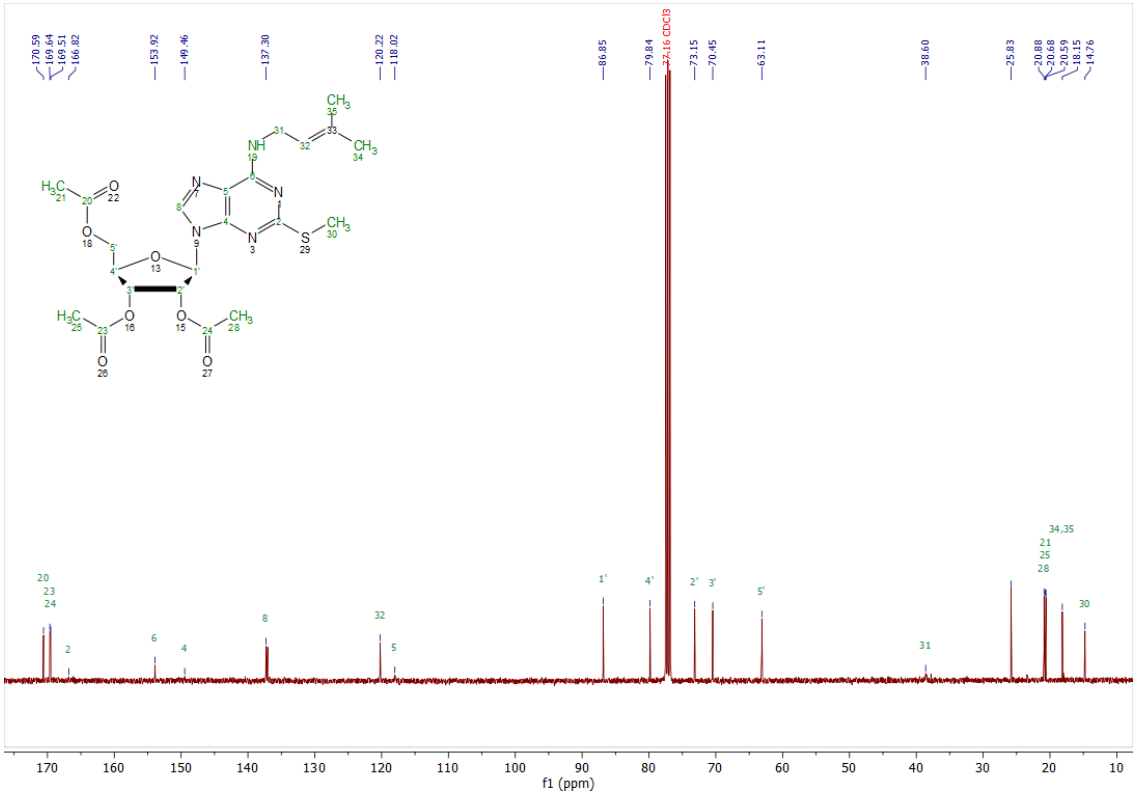

$^{1}$H-NMR (400 MHz, $CDCl_3$) of compound **2**.



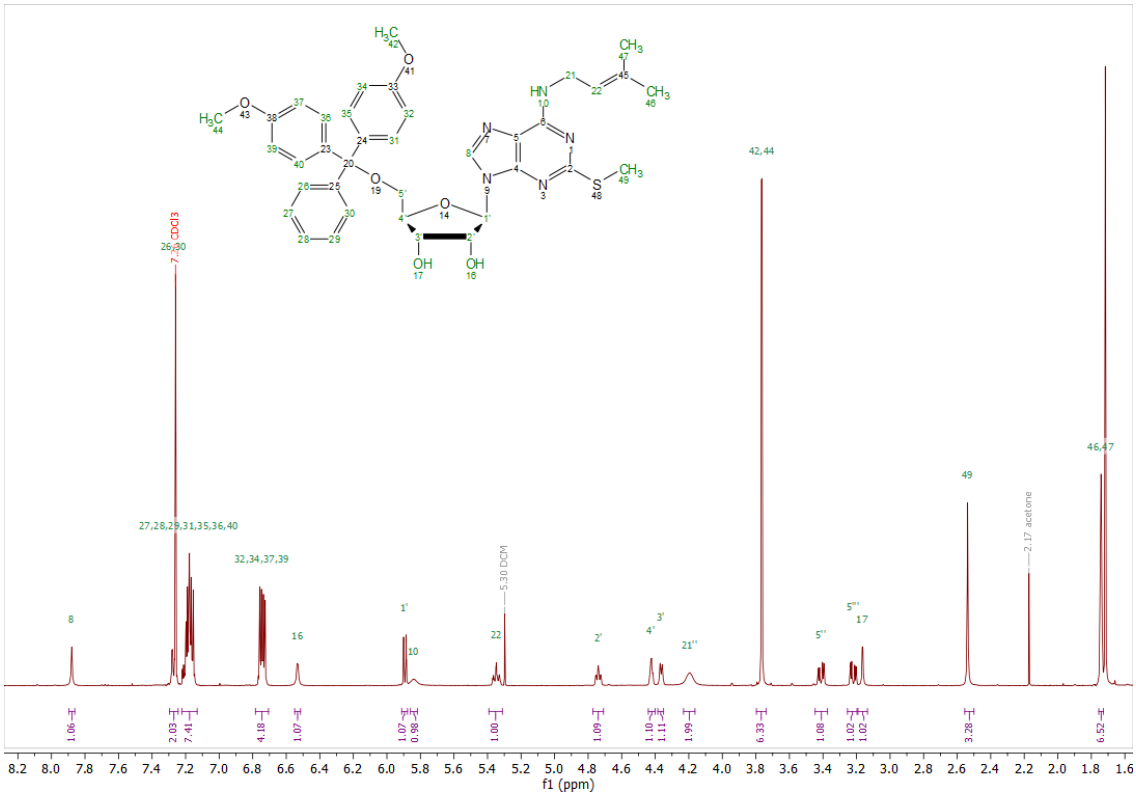

$^{13}$C-NMR (100 MHz, $CDCl_3$) of compound **2**.



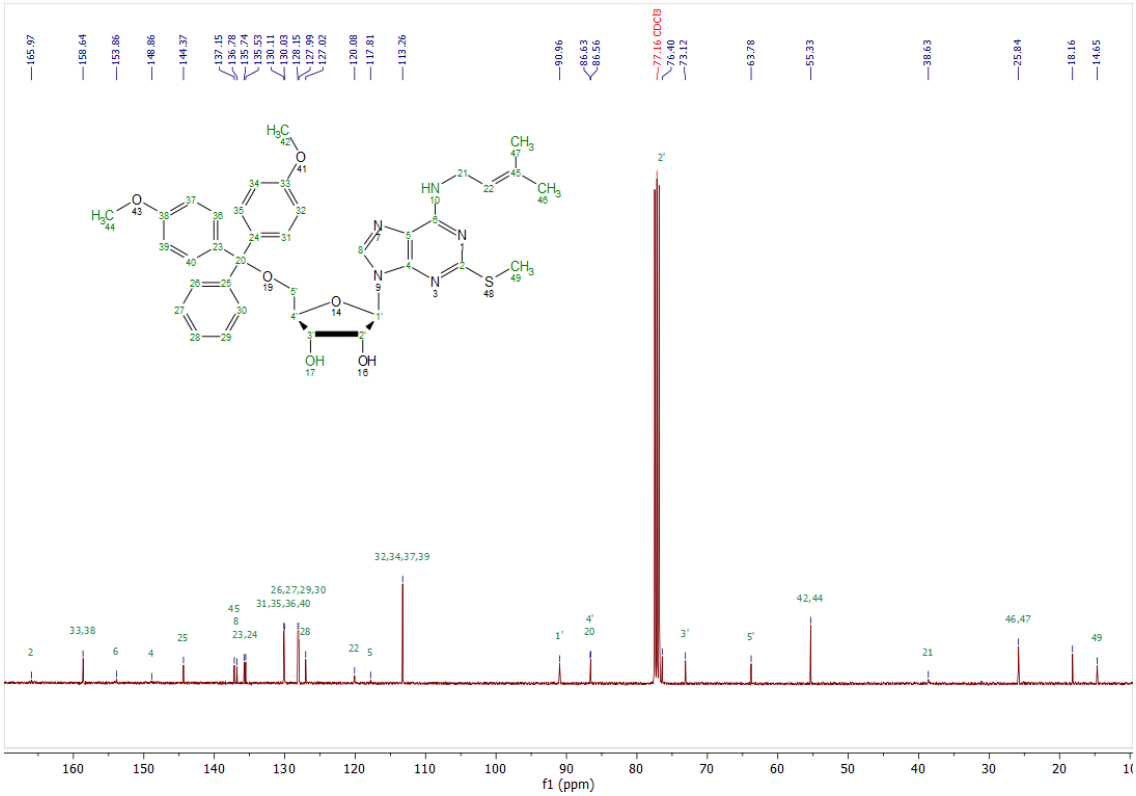

$^{1}$H-NMR (400 MHz, $CDCl_3$) of compound **3**.



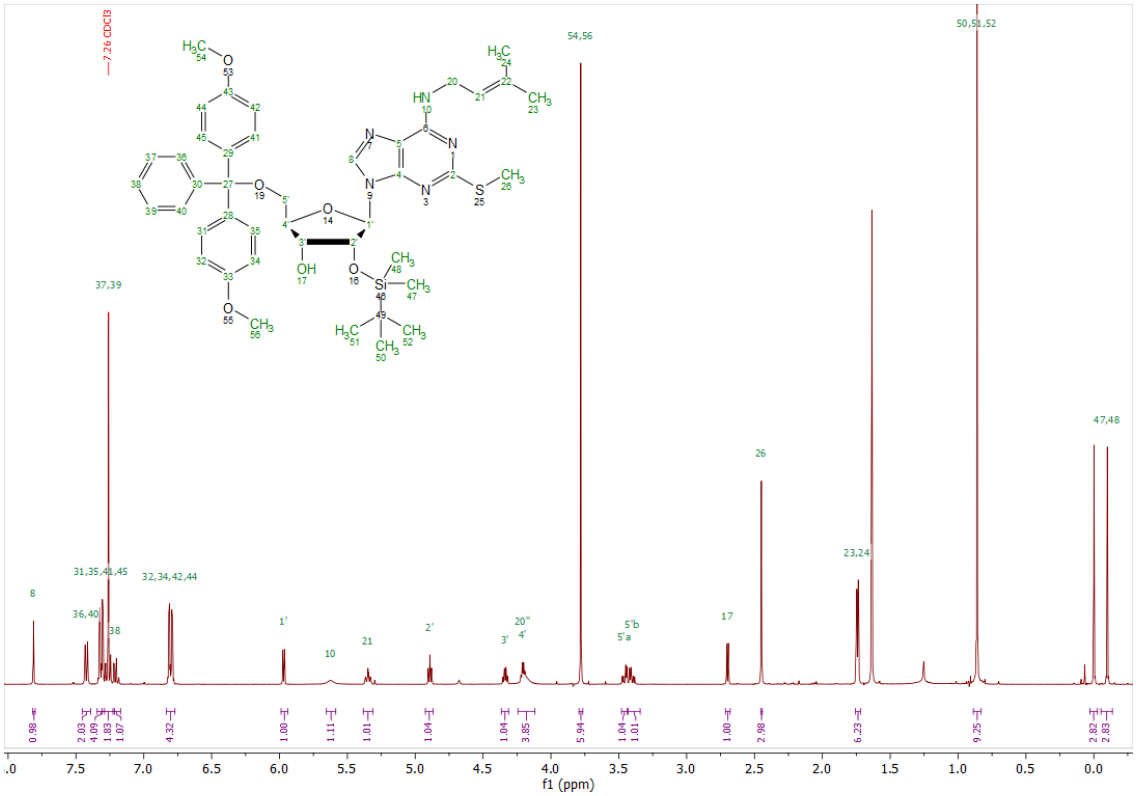

$^{13}$C-NMR (100 MHz, $CDCl_3$) of compound **3**.



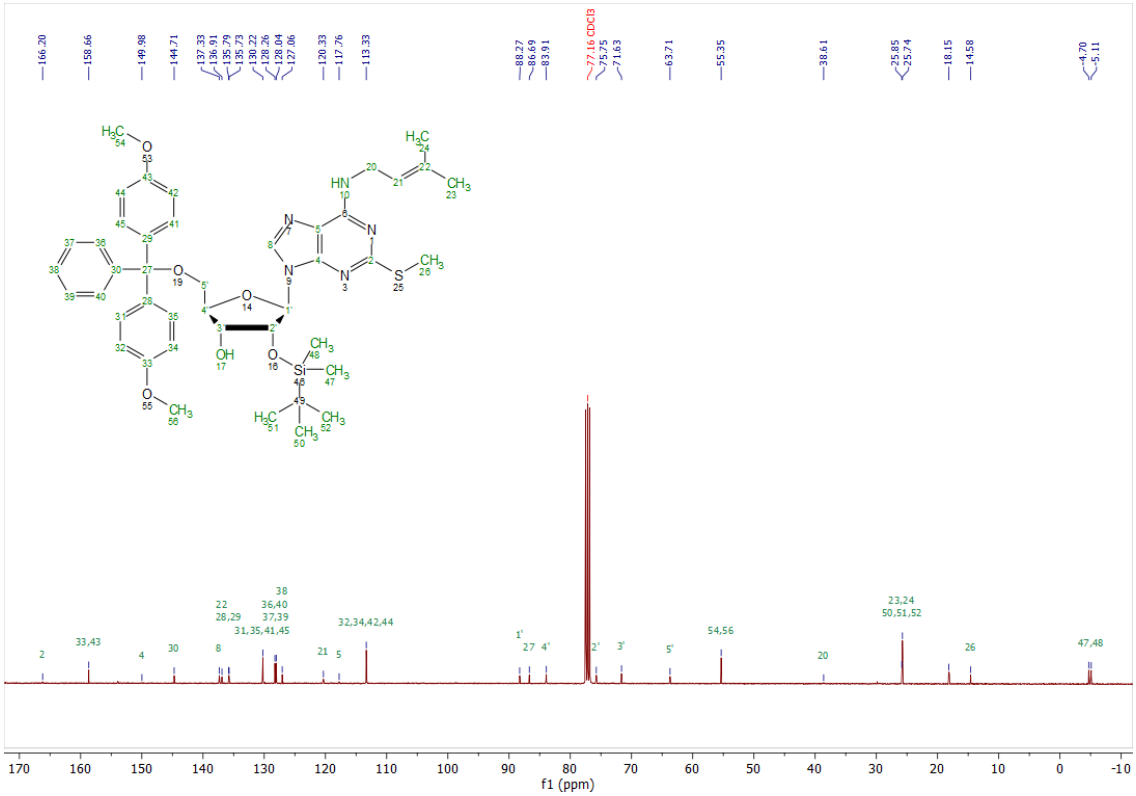

$^{1}$H-NMR (400 MHz, $CDCl_3$) of compound **4**.



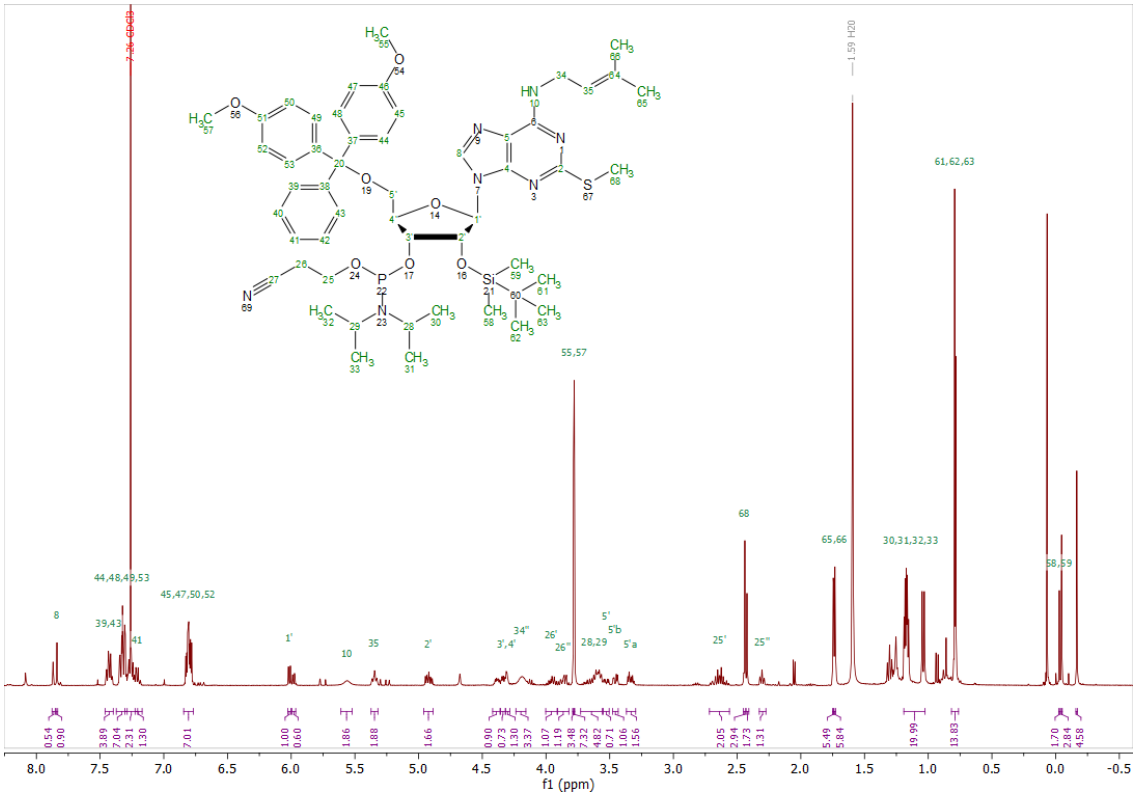

$^{13}$C-NMR (100 MHz, $CDCl_3$) of compound **4**.



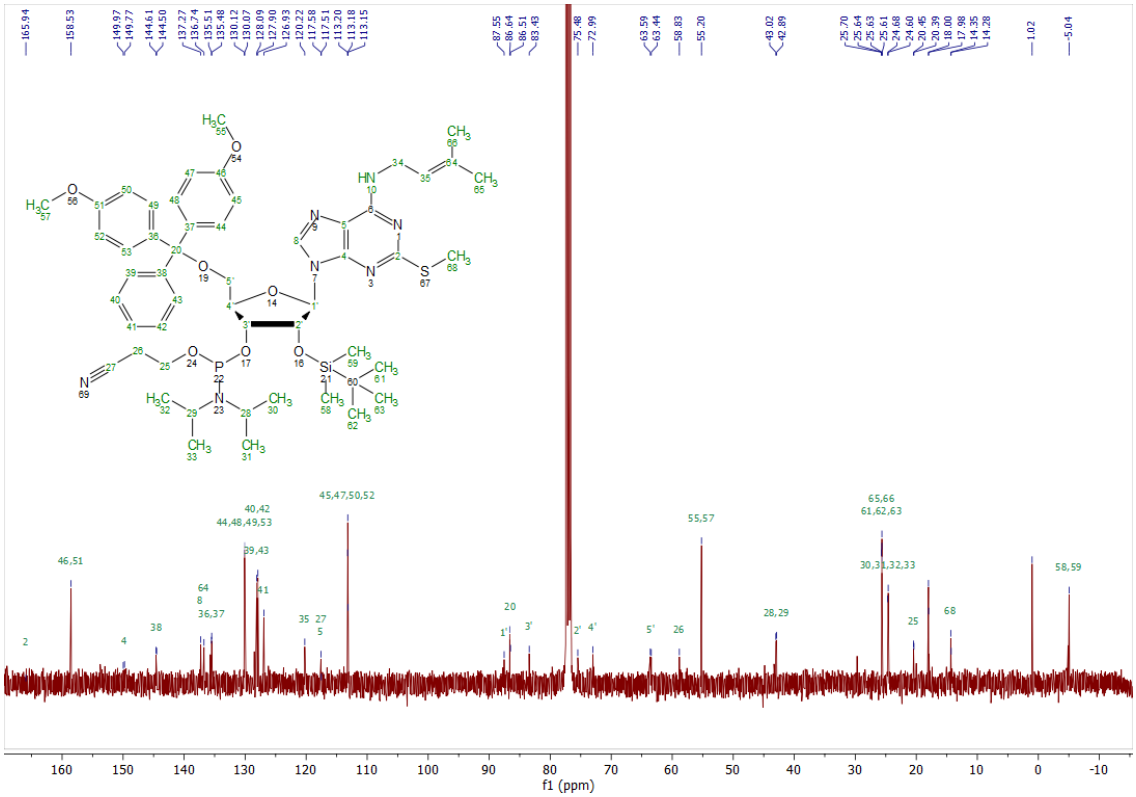

$^{1}$H-NMR (400 MHz, $CDCl_3$) of compound **5**.



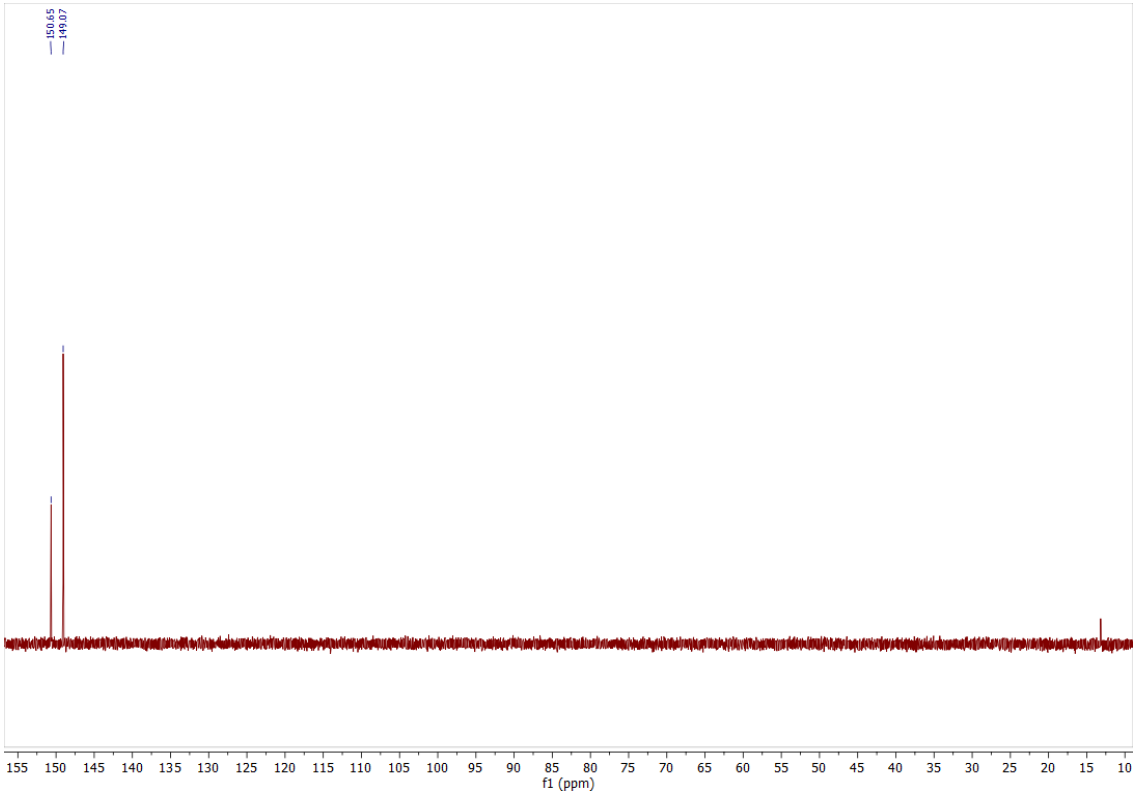

$^{13}$C-NMR (100 MHz, $CDCl_3$) of compound **5**.



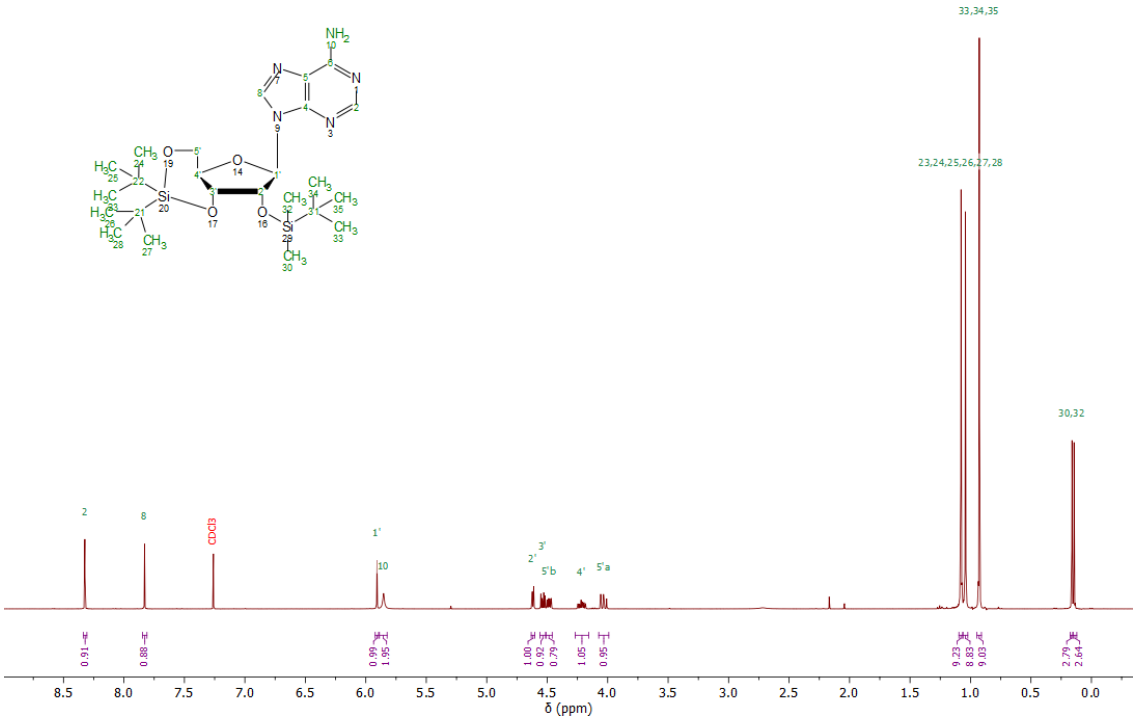

$^{1}$H-NMR (400 MHz, $CDCl_3$) of compound **6**.



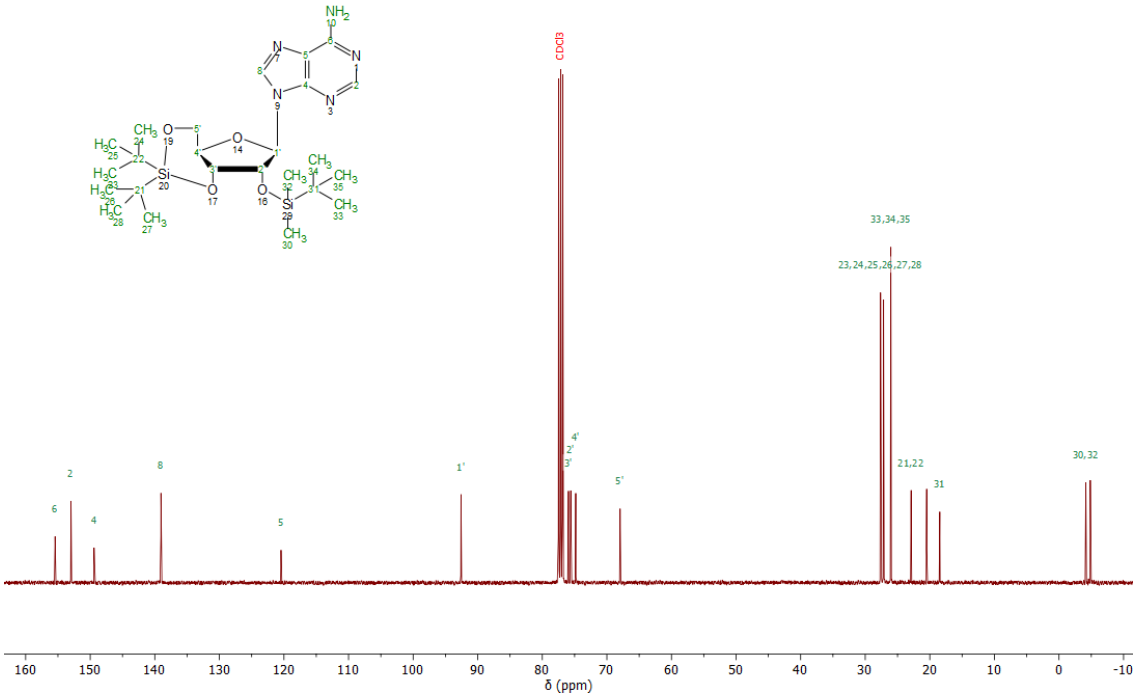

$^{13}$C-NMR (100 MHz, $CDCl_3$) of compound **6**.



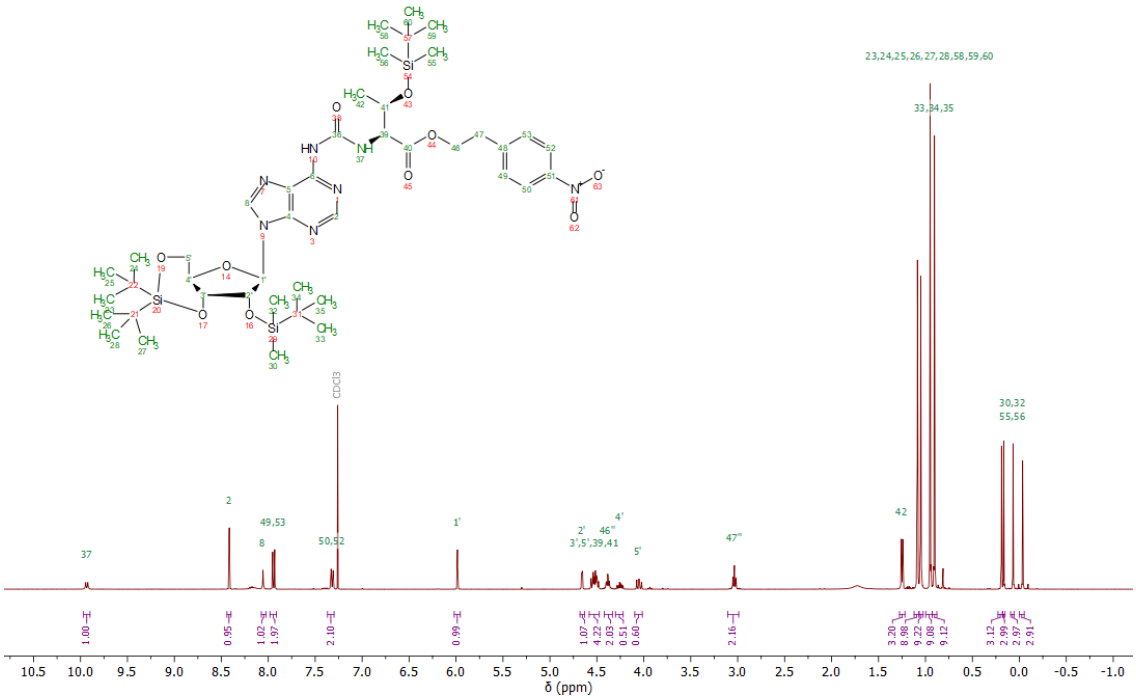

$^{31}$P-NMR (162 MHz, $CDCl_3$) of compound **6**.



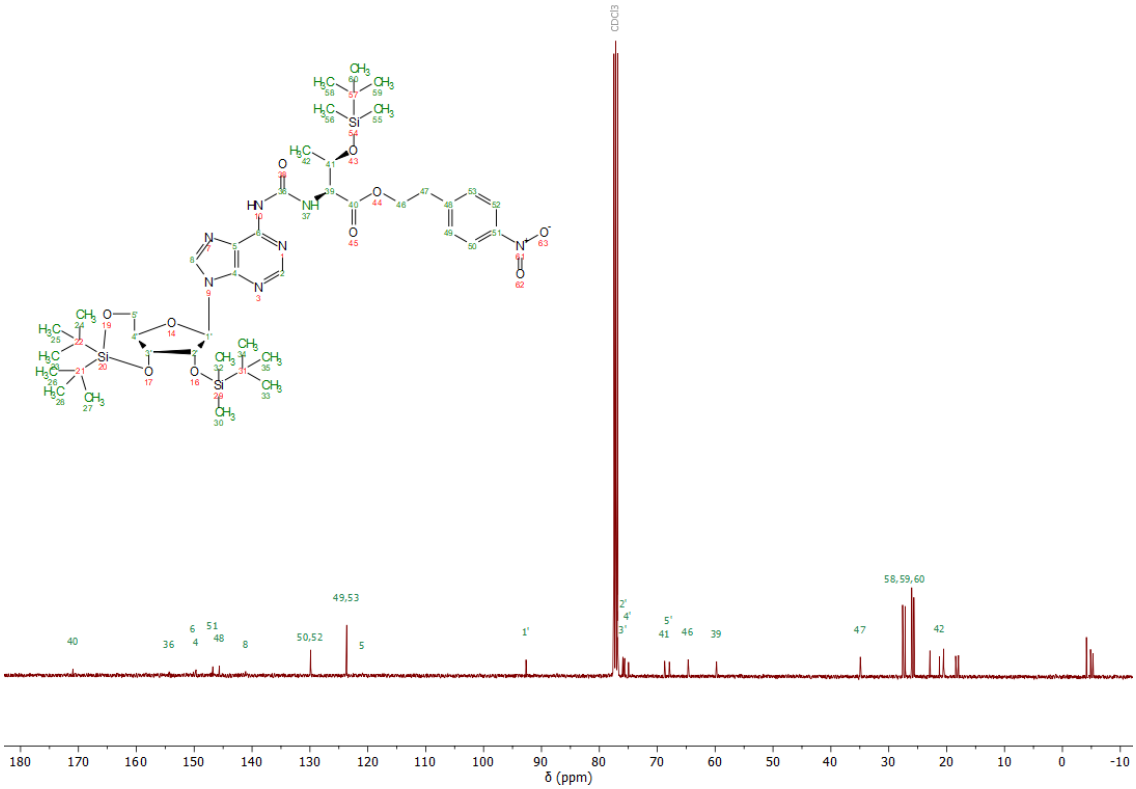

$^{1}$H-NMR (400 MHz, $CDCl_3$) of compound **8**.



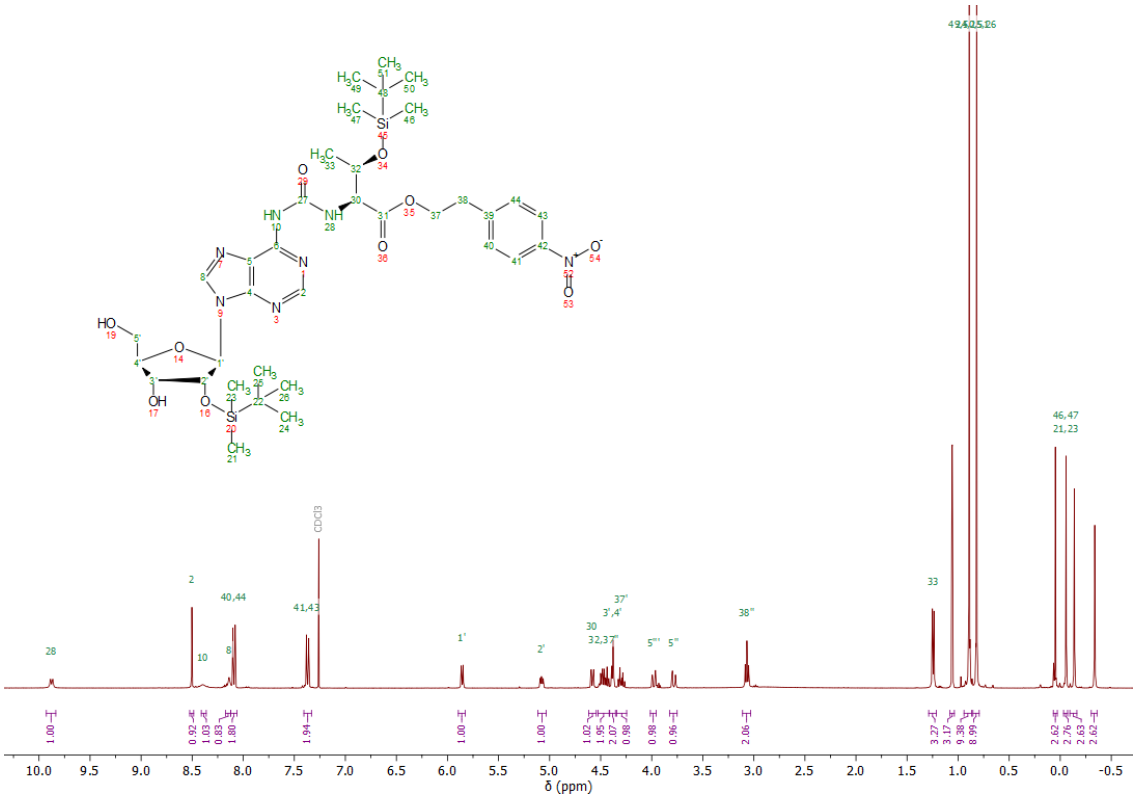

$^{13}$C-NMR (100 MHz, $CDCl_3$) of compound **8**.



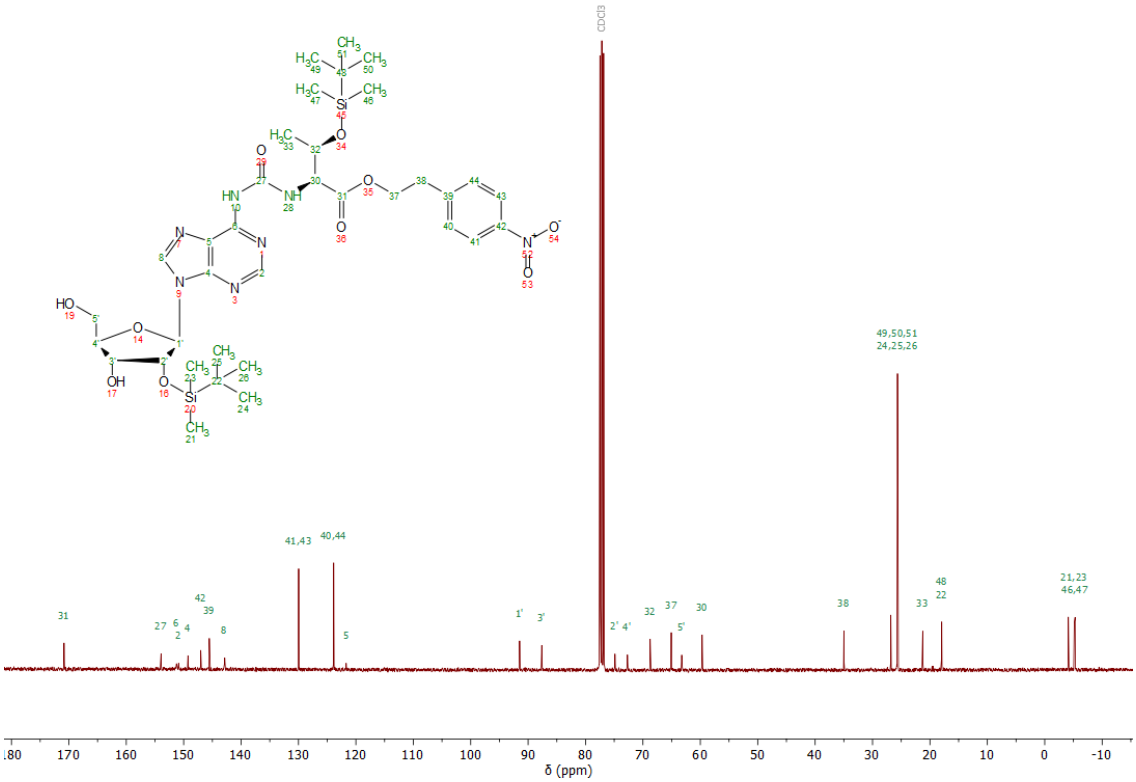

$^{1}$H-NMR (400 MHz, $CDCl_3$) of compound **9**.



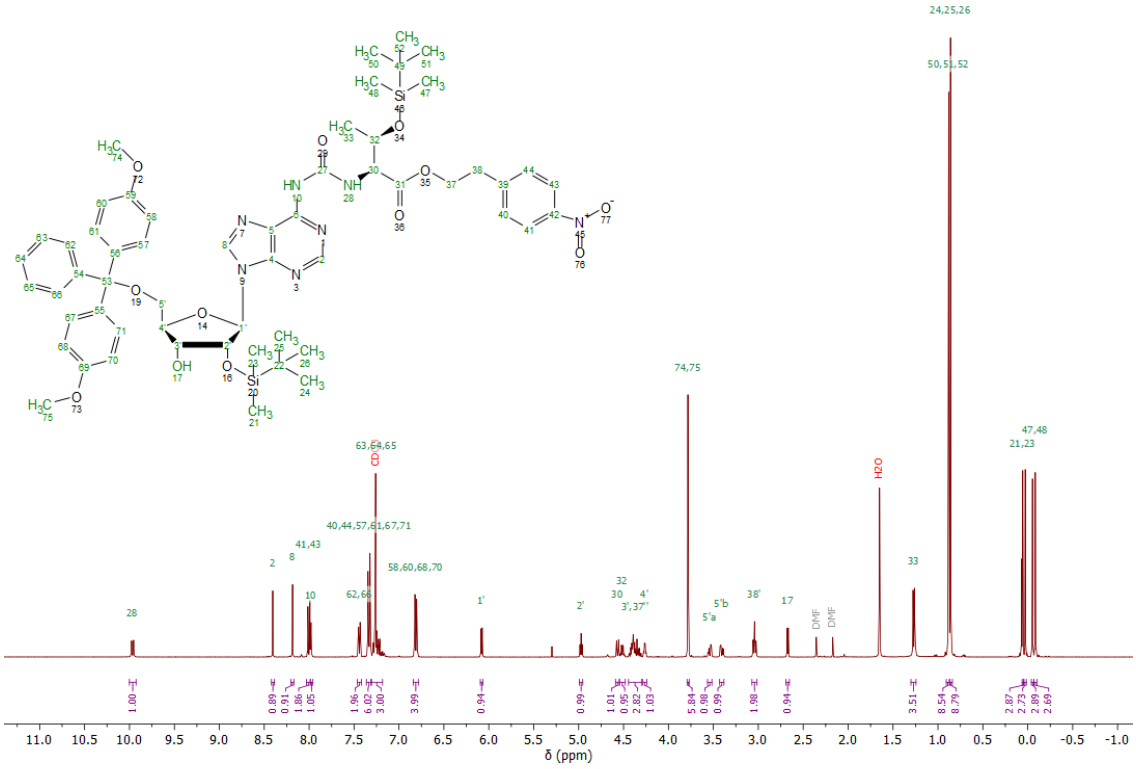

$^{13}$C-NMR (100 MHz, $CDCl_3$) of compound **9**.



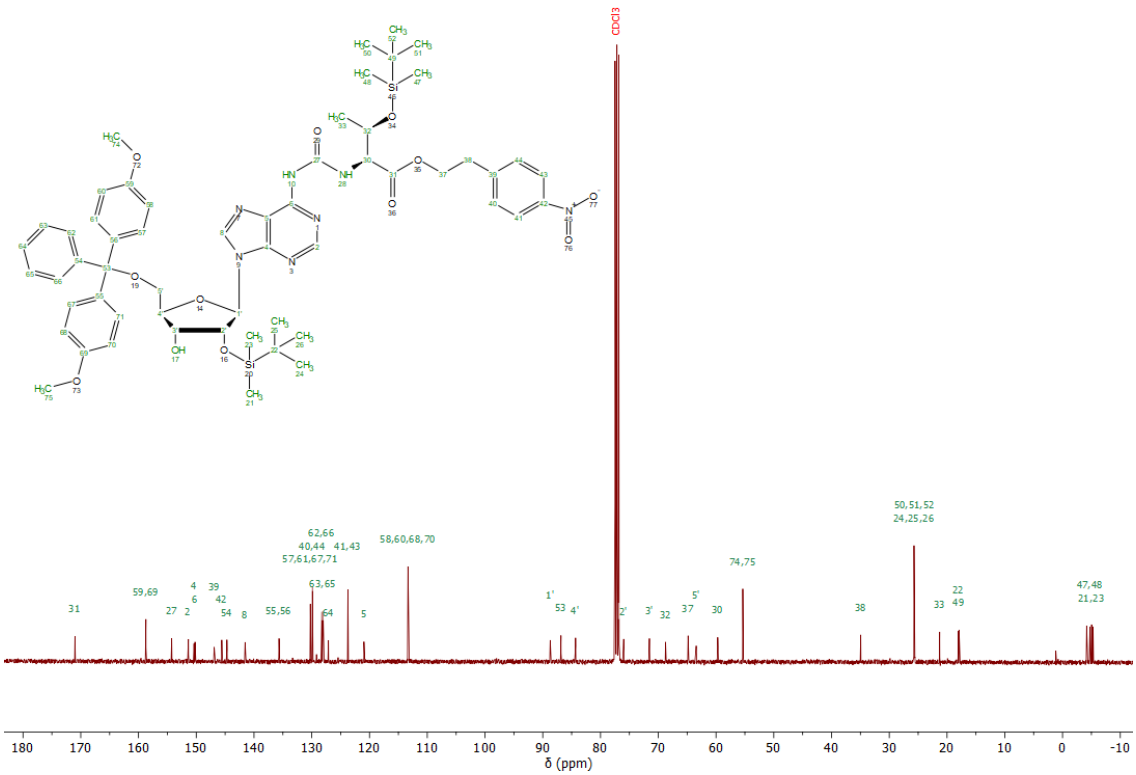

$^{1}$H-NMR (400 MHz, $CDCl_3$) of compound **10**.



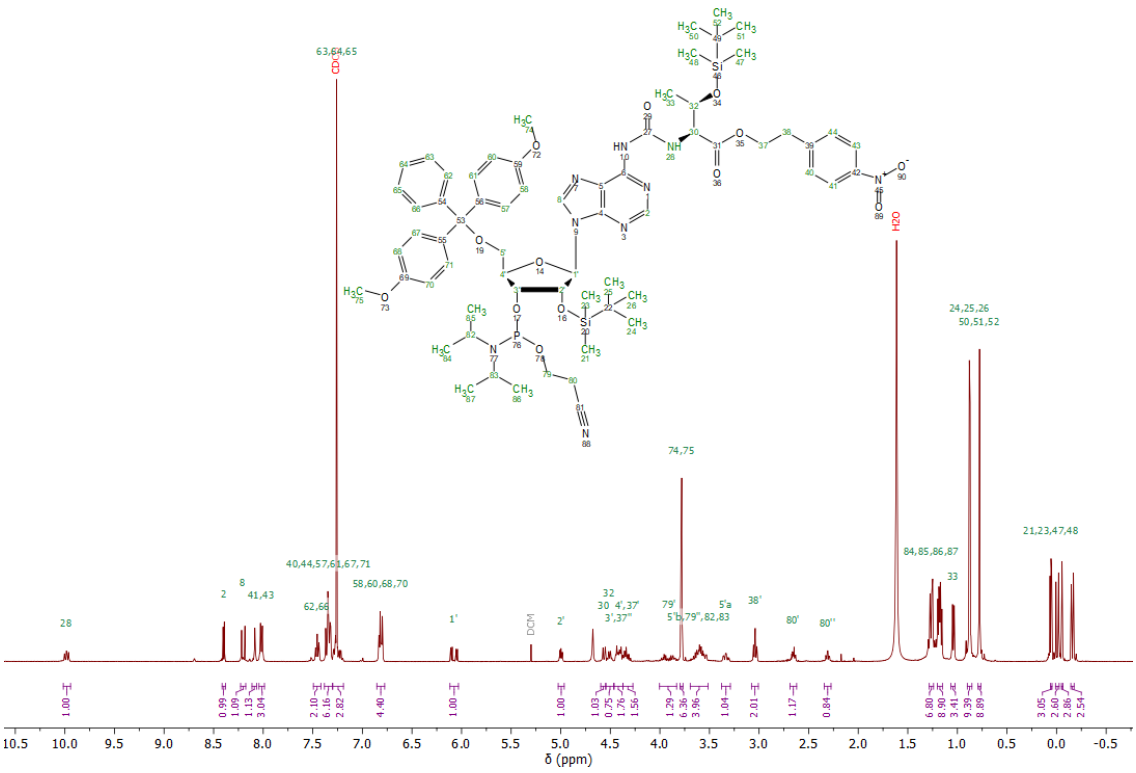

$^{13}$C-NMR (100 MHz, $CDCl_3$) of compound **10**.



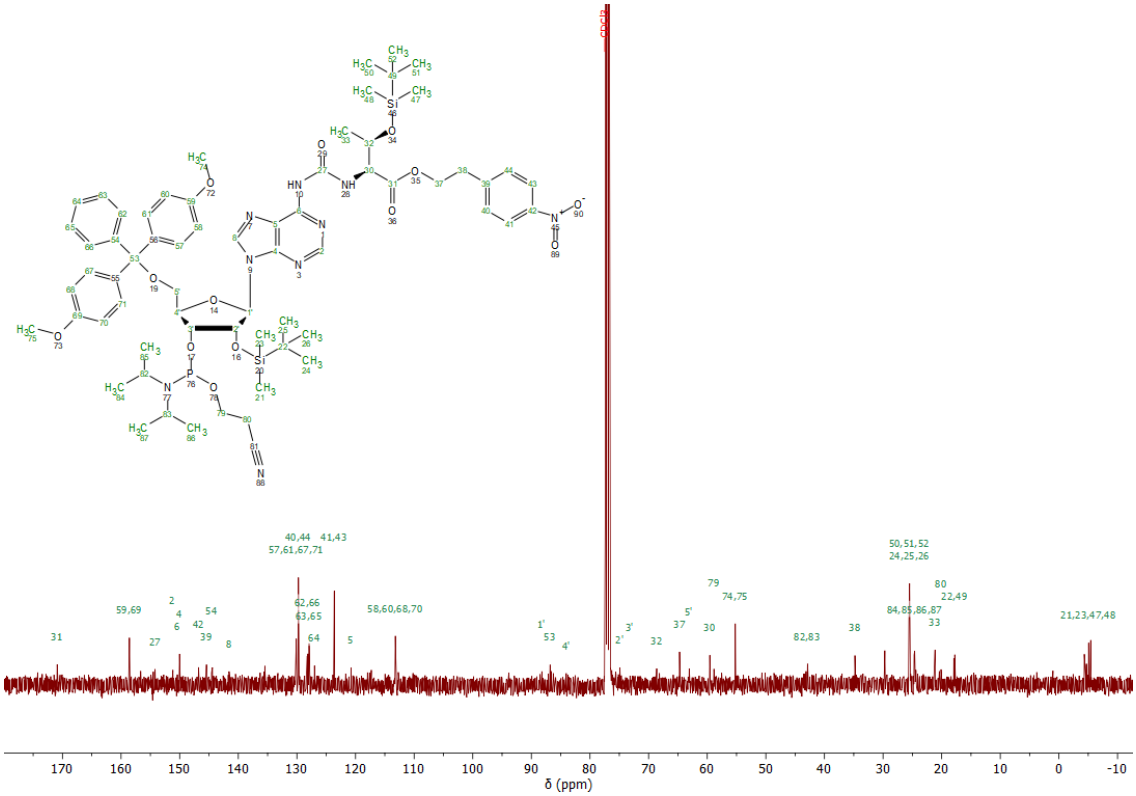

$^{1}$H-NMR (400 MHz, $CDCl_3$) of compound **11**.



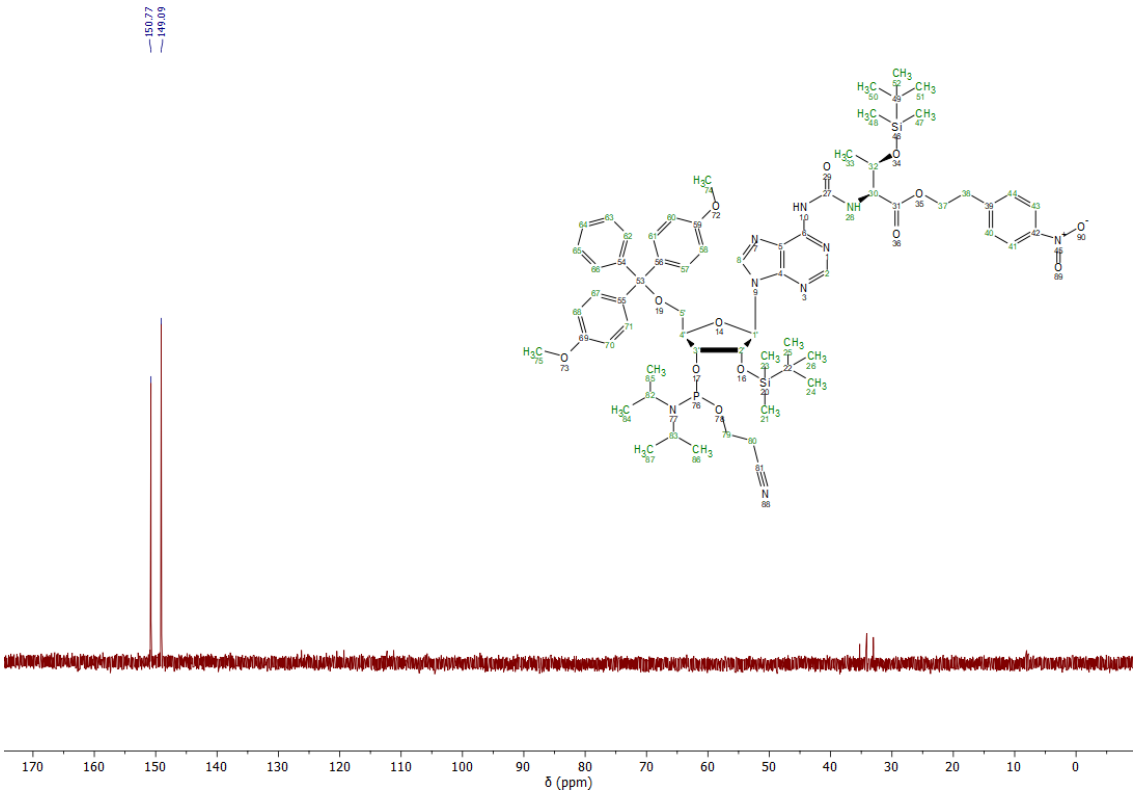

$^{13}$C-NMR (100 MHz, $CDCl_3$) of compound **11**.




$^{1}$H-NMR (400 MHz, $CDCl_3$) of compound **12**.




$^{13}$C-NMR (100 MHz, $CDCl_3$) of compound **12**.




$^{31}$P-NMR (162 MHz, $CDCl_3$) of compound **12**.

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