Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

A cyclopropene-modified nucleotide for site-specific RNA labeling using genetic alphabet expansion transcription

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General Methods

NMR

NMR spectra were obtained using an *Avance dpx 400* or an *Avance III HD 700* from *Bruker*. The assignment of proton and carbon resonances is based on two-dimensional correlation experiments (HMQC, HMBC). Chemical shifts (δ) are given in ppm and spectra are calibrated to the respective deuterated solvent residue signal according to literature values ($CDCl_3$: 7.26 ppm for ^1H and 77.2 ppm for ^{13}C spectra, CD_3OD: 3.31 ppm for ^1H and 49.0 ppm for ^{13}C spectra, D_2O: 4.79 ppm for ^1H spectra).[1] Reported coupling constants are calculated from apparent signal positions in first order approximation.

MS

High resolution ESI mass spectra were recorded on a *Orbitrap XL* mass spectrometer from *Thermo Fisher Scientific* or on a *micrOTOF-Q* mass spectrometer from *Bruker Daltonic* in combination with an *Agilent 1200 Series* HPLC system (*Agilent Technologies*).

LC-MS measurements were performed on an *HTC esquire* from *Bruker Daltonic* in combination with an *Agilent 1100* Series HPLC system (*Agilent Technologies*) using either 10 mM triethylamine/100 mM hexafluoroisopropanol as solvent A employing an *XTerra® MS* (2.1x100 mm, 5 µm) C_{18} column (*Waters*) or a *Zorbax Narrow Bore* (2.1x50 mm, 5 µm) C_{18} column for the analysis of oligonucleotides, or 0.1% (*w*/*v*) ammonium acetate as solvent A using a *Zorbax Narrow Bore* (2.1x50 mm, 5 µm) C_{18} column (*Agilent Technologies*) for the analysis of small molecules. In both cases, solvent B was acetonitrile.

HPLC

HPLC micro purification was performed on an *Agilent 1100 Series* HPLC system (*Agilent Technologies*) with an *EC 150/4.6 Nucleodur 100-5 C18ec* column (*Macherey-Nagel*) using 0.1 M triethylammonium acetate as solvent A and acetonitrile as solvent B.

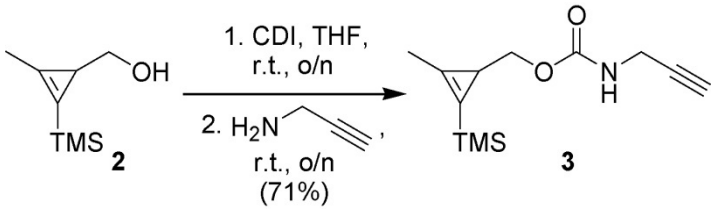
Fluorescence scans

Fluorescence spectra were recorded on an *LS 55* fluorescence spectrometer (*PerkinElmer*) at room temperature using a micro cuvette (QS, 45 µL, 3x3 mm) from *Hellma*.

Chemical Syntheses

TMS-protected methyl cyclopropene alcohol **2** was synthesized according to literature.[2],[3] Tetrazine-fluorophore conjugate **1** as well as unnatural ribonucleoside **4** were generated as previously reported.[4],[5]

*Synthesis of **3** - (2-Methyl-3-(trimethylsilyl)cycloprop-2-en-1-yl)methyl prop-2-yn-1-yl-carbamate*

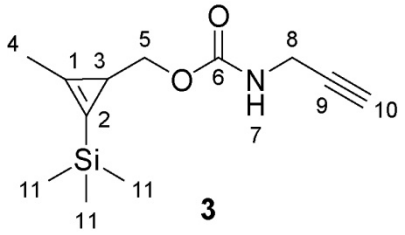


Under argon atmosphere CDI (548 mg, 3.4 mmol, 1.2 eq.) was dissolved in THF (10 mL) and cyclopropenyl alcohol **2** (440 mg, 2.8 mmol, 1.0 eq.) was added dropwise. After stirring overnight at room temperature, propargylamine (0.36 mL, 5.6 mmol, 2.0 eq.) was added and the reaction was agitated overnight. The mixture was diluted with water (25 mL) and the aqueous layer was extracted with Et_2O (3x35 mL). The combined organic layers were dried over anhydrous $MgSO_4$ and the solvent was removed in vacuo to give the crude product. Purification by column chromatography (C_6H_{12}/Et_2O, 3/2, *v*/*v*, R_f = 0.56) yielded **3** (473 mg, 2.0 mmol, 71%) as a colorless oil.

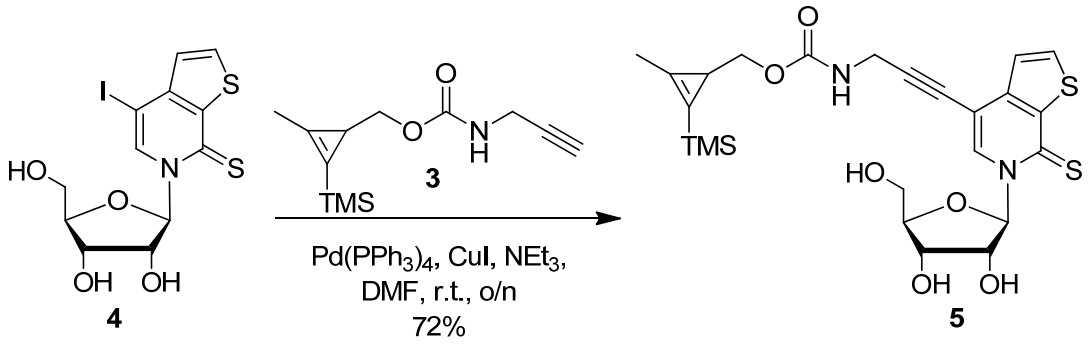
^{1}H-NMR ($CDCl_3$, 400 MHz, r.t.): δ [ppm] = 4.84 (br s; 1H; H-7), 3.98-3.89 (m; 4H; H-5, H-8), 2.22 (t; $^4J_{H\text{-}10/H\text{-}8}$ = 2.5 Hz; 1H; H-10), 2.18 (s; 3H; H-4), 1.53 (t; $^3J_{H\text{-}3/H\text{-}5}$ = 5.3 Hz; 1H; H-3), 0.14 (s; 9H; H-11).

^{13}C-NMR ($CDCl_3$, 101 MHz, r.t.): δ [ppm] = 156.6 (C-6), 134.5 (C-1), 111.1 (C-2), 80.1 (C-9), 74.1 (C-5), 71.5 (C-10), 30.9 (C-8), 18.6 (C-3), 13.3 (C-4), -1.1 (C-11).

HR-MS (ESI^+): calculated for $[M + Na]^+$ ($C_{12}H_{19}NO_2SiNa^+$): 260.1077; found: *m*/*z* = 260.1075.



*Synthesis of **5** - (2-methyl-3-(trimethylsilyl)cycloprop-2-en-1-yl)methyl (β-D-ribofuranos-1'-yl)-7-thioxo-6,7-dihydrothieno[2,3-c]pyridin-4-yl)prop-2-yn-1-yl)carbamate*

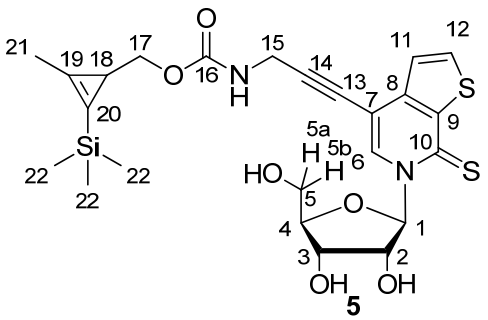


Under an atmosphere of argon, nucleoside **4** (200 mg, 0.48 mmol, 1.0 eq.) and copper(I) iodide (64 mg, 0.34 mmol, 0.7 eq.) were dissolved in dry DMF (30 mL) and the resulting solution was degassed with a stream of argon. Previously degassed NEt_3 (0.20 mL, 1.44 mmol, 3.0 eq.) and a degassed solution of carbamate **3** (170 mg, 0.72 mmol, 1.5 eq.) in dry DMF (10 mL) were added subsequently. After the addition of $Pd(PPh_3)_4$ (56 mg, 0.05 mmol, 0.1 eq.), the mixture was stirred overnight at room temperature under exclusion of light. The solvent was removed in vacuo and the residue was purified by silica gel chromatography (CH_2Cl_2/MeOH, 95/5, *v*/*v*, R_f = 0.44). Nucleoside **5** (185 mg, 0.35 mmol, 72%) was obtained as dark yellow solid.

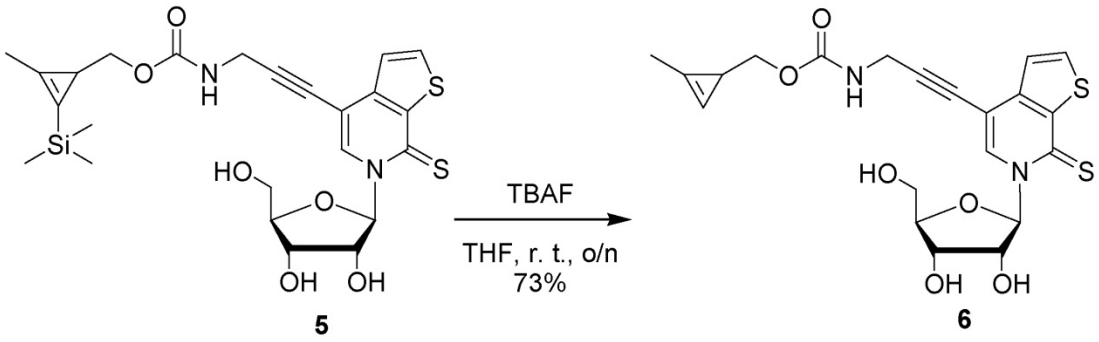
^{1}H-NMR (CD_3OD, 400 MHz, r. t.): δ [ppm] = 8.78 (s; 1H; H-6), 7.99 (d; $^3J_{H\text{-}12/H\text{-}11}$ = 5.3 Hz; 1H; H-12), 7.44 (d; $^3J_{H\text{-}11/H\text{-}12}$ = 5.3 Hz; 1H; H-11), 6.94 (d; $^3J_{H\text{-}1/H\text{-}2}$ = 1.6 Hz; 1H; H-1), 4.26 (dd; $^3J_{H\text{-}2/H\text{-}3}$ = 4.6 Hz; $^3J_{H\text{-}2/H\text{-}1}$ = 1.6 Hz; 1H; H-2), 4.24-4.14 (m; 4H; H-3, H-4, H-15), 4.08 (dd; $^3J_{H\text{-}5a/H\text{-}5b}$ = 12.5 Hz; $^3J_{H\text{-}5a/H\text{-}4}$ = 2.3 Hz; 1H; H-5a), 3.92 (m; 2H, H-17), 3.88 (dd; $^3J_{H\text{-}5b/H\text{-}5a}$ = 12.5 Hz; $^3J_{H\text{-}5b/H\text{-}4}$ = 2.3 Hz; 1H; H-5b), 2.19 (s; 3H; H-21), 1.57 (t; $^3J_{H\text{-}18/H\text{-}17}$ = 5.4 Hz, 1H; H-18), 0.15 (s; 9H; H-22).

^{13}C-NMR (CD_3OD, 101 MHz, r. t.): δ [ppm] = 174.1 (C-10), 159.1 (C-16), 145.7 (C-9), 140.5 (C-8), 139.2 (C-12), 136.0 (C-19), 135.5 (C-6), 125.0 (C-11), 112.2 (C-20), 105.9 (C-13), 96.4 (C-1), 92.1 (C-14), 85.6 (C-3), 77.2 (C-2), 74.7 (C-17), 69.2 (C-4), 60.7 (C-5), 32.0 (C-15), 19.9 (C-18), 13.2 (C-21), -1.2 (C-22).

HR-MS (ESI$^+$): calculated for $[M + Na]^+$ ($C_{24}H_{31}N_2O_6S_2Si^+$): 535.1387; found: *m*/*z* = 535.1376.



Synthesis of ***6*** (**TPT3^{CP}**) *- (2-Methylcycloprop-2-en-1-yl)methyl (((β-D-ribofuranos-1'-yl)-7-thioxo-6,7-dihydrothieno[2,3-c]pyridin-4-yl)prop-2-yn-1-yl)carbamate*



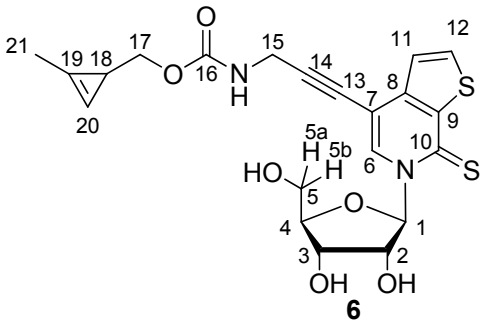
5 **6**

Nucleoside **5** (119 mg, 0.36 mmol, 1.0 eq.) was dissolved in THF (15 mL). Subsequently, tributylammonium fluoride trihydrate (123 mg, 0.39 mmol, 1.1 eq.) was added and the mixture was stirred at room temperature overnight. The solvent was removed in vacuo and the crude product was purified via silica gel chromatography (CH_2Cl_2/MeOH, 95/5, *v*/*v*, R_f = 0.37). Methyl cyclopropene nucleoside **6** was obtained as yellow foam (119 mg, 0.26 mmol, 71%).

^{1}H-NMR (CD_3OD, 400 MHz, r. t.): δ [ppm] = 8.80 (s; 1H; H-6), 8.00 (d; $^3J_{H\text{-}12/H\text{-}11}$ = 5.3 Hz; 1H; H-12), 7.46 (d; $^3J_{H\text{-}11/H\text{-}12}$ = 5.3 Hz; 1H; H-11), 6.95 (d; $^3J_{H\text{-}1/H\text{-}2}$ = 1.5 Hz; 1H; H-1), 6.65 (s; 1H; H-20), 4.25 (dd; $^3J_{H\text{-}2/H\text{-}3}$ = 4.6 Hz; $^3J_{H\text{-}2/H\text{-}1}$ = 1.6 Hz; 1H; H-2), 4.23-4.16 (m; 4H; H-3, H-4, H-15), 4.08 (dd; $^3J_{H\text{-}5a/H\text{-}5b}$ = 12.5 Hz; $^3J_{H\text{-}5a/H\text{-}4}$ = 2.3 Hz; 1H; H-5a), 4.04-3.86 (m; 3H, H-17, H-5b), 2.12 (d; $^3J_{H\text{-}21/H\text{-}20}$ = 1.2 Hz, 3H; H-21), 1.64 (t; $^3J_{H\text{-}18/H\text{-}17}$ = 4.1 Hz, 1H; H-18).

^{13}C-NMR (CD_3OD, 101 MHz, r. t.): δ [ppm] = 174.2 (C-10), 159.1 (C-16), 145.7 (C-9), 140.6 (C-8), 139.3 (C-12), 135.6 (C-6), 125.0 (C-11), 122.2 (C-19), 105.9 (C-13), 102.8 (C-20), 96.4 (C-1), 92.1 (C-14), 85.7 (C-3), 77.3 (C-2), 73.6 (C-17), 69.2 (C-4), 60.7 (C-5), 31.9 (C-15), 18.3 (C-18), 11.6 (C-21).

HR-MS (ESI$^+$): calculated for $[M + Na]^+$ ($C_{21}H_{22}N_2O_6S_2Na^+$): 485.0811; found: *m*/*z* = 485.0810.



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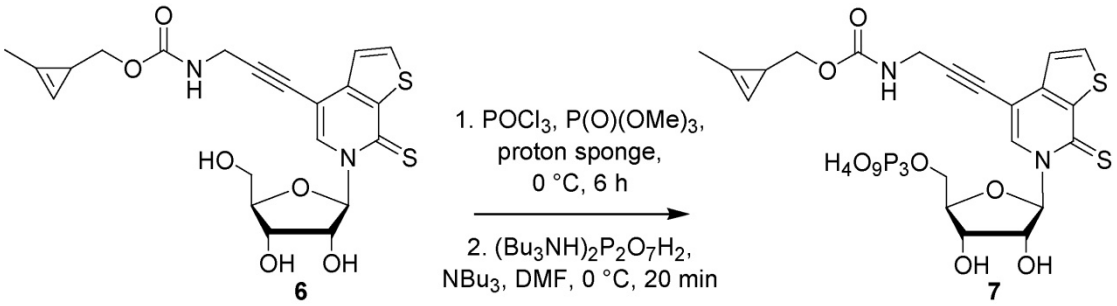
Synthesis of ***7*** (**TPT3^{CP}** TP) - *2-Methylcycloprop-2-en-1-yl)methyl (((β-D-ribofuranos-5'-triphosphate-1'-yl)-7-thioxo-6,7-dihydrothieno[2,3-c]pyridin-4-yl)prop-2-yn-1-yl)carbamate*

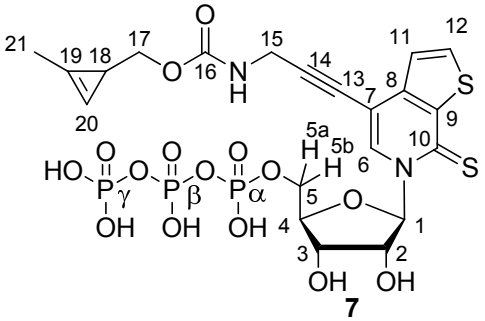


Nucleoside **6** (42 mg, 0.09 mmol, 1.0 eq.) and proton sponge (15 mg, 0.07 mmol, 0.8 eq.) were dried together under high vacuum overnight, dissolved in trimethyl phosphate (0.5 mL, freshly distilled and, stored over 3 Å molecular sieve and under argon) and cooled in an ice bath. $POCl_3$ (25 µL, 0.27 mmol, 3.0 eq., freshly distilled and stored under argon) was added dropwise at 0 °C and the reaction was stirred under ice-cold conditions for 6 h. Then, a solution of bis-tributylammonium pyrophosphate in DMF (0.5 M, 1.0 mL, 0.50 mmol, 5.5 eq., DMF was stored over 3 Å molecular sieve for 12 h) and NBu_3 (0.22 mL, 0.95 mmol, 10.5 eq., stored over 3 Å molecular sieve for 12 h) were added simultaneously under ice-cooling. The mixture was stirred at 0 °C for 20 min and quenched by the addition of TEAB buffer (1.0 M, 7.0 mL, pH = 8.0). Volatiles were evaporated under high vacuum at room temperature and the crude product was purified four times via HPLC (10-40% B in 20 min, flow rate = 1.0 mL min^{-1}). Purified triphosphate **7** was obtained as a yellow solid (11.0 mg, $1.6 \cdot 10^{-2}$ mmol, 17%). Purity and integrity of **7** was confirmed by LC-MS analysis (0-60% B in 20 min, flow rate = 0.4 mL min^{-1}).

^{31}P-NMR (D_2O, 162 MHz, r.t.): δ [ppm] = -10.19 (d; $^2J_{P\text{-}\gamma/P\text{-}\beta}$ = 19.9 Hz, P-γ), -10.77 (d; $^2J_{P\text{-}\alpha/P\text{-}\beta}$ = 20.3 Hz, P-α), -22.58 (t; $^2J_{P\text{-}\beta/P\text{-}\alpha}$, $^2J_{P\text{-}\beta/P\text{-}\gamma}$ = 20.1 Hz, P-β).

^{1}H-NMR (D_2O, 700 MHz, r. t.): δ [ppm] = 8.50 (s; 1H; H-6), 8.13 (d; $^3J_{H\text{-}12/H\text{-}11}$ = 5.4 Hz; 1H; H-12), 7.57 (d; $^3J_{H\text{-}11/H\text{-}12}$ = 4.7 Hz; 1H; H-11), 7.09 (s; 1H; H-1), 6.66 (s; 1H; H-20), 4.50-4.41 (m; 5H; H-2, H-3, H-4, H-17, 4.25 (s; 2H; H-15), 4.10 (br s; 1H; H-5a), 4.00 (br s; 1H, H-5b), 2.11 (s; 3H; H-21), 1.68 (m; 1H; H-18).

HR-MS (ESI$^-$): calculated for $[M - H]^-$ ($C_{21}H_{25}N_2O_{15}P_3S_2^-$): 700.9836; found: *m*/*z* = 700.9843.



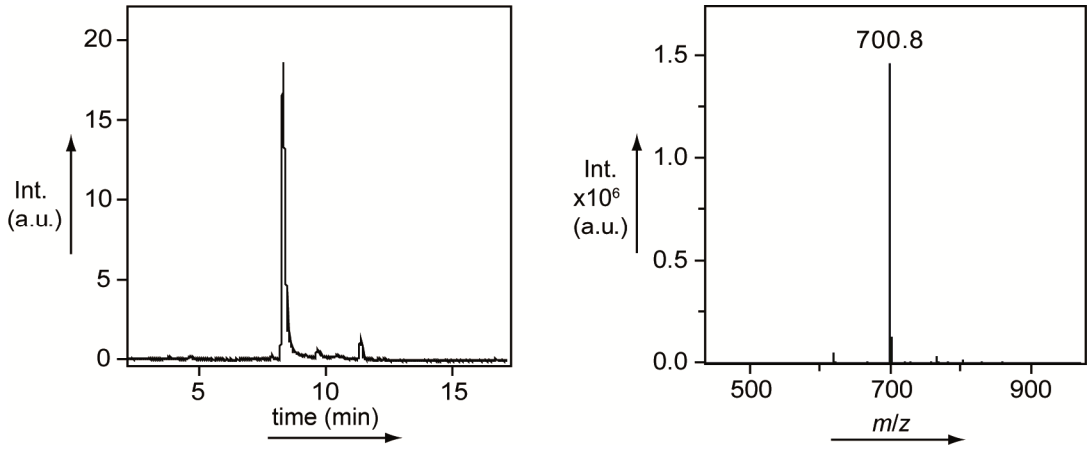
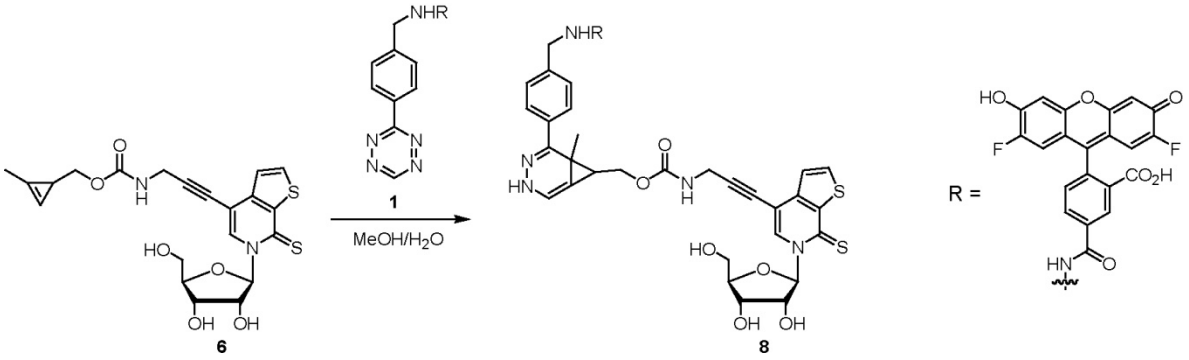


Figure S1. LC-MS analysis of compound **7** (**TPT3^{CP}** TP): HPL-chromatogram (left) at 260 nm and ESI mass spectrum of the peak eluting at a retention time of 8 min (right) (ESI$^-$ calculated for $[M - H]^-$ ($C_{21}H_{25}N_2O_{15}P_3S_2^-$): 700.98).

*Fluorescence measurements of tetrazine-fluorophore conjugate **1** upon incubation with unnatural nucleosides **4** and **6***



For fluorescence measurements, a solution of cyclopropene-modified nucleoside **6** (60 µM in H_2O/MeOH, 4/1, *v/v,* 15 µL) was added to an aqueous solution of tetrazine-fluorophore conjugate **1** (20 µM, 15 µL) in a 45 µL micro cuvette and directly subjected to iterative fluorescence scans ($\lambda_{excitation}$ = 495 nm, $\lambda_{emission}$ = 450-600 nm, scan rate = 120 nm s^{-1}, slit = 5, measurement start every 83 s, 15 repetitions). For negative control, a mixture of tetrazine-fluorophore conjugate **1** (20 µM in H_2O, 15 µL) and iodized nucleoside **4** (60 µM in 4/1 *v/v* H_2O/MeOH, 15 µL) was fluorescence scanned in triplicate ($\lambda_{excitation}$ = 495 nm, $\lambda_{emission}$ = 450-600 nm, scan rate = 120 nm s^{-1}, slit = 5) after an incubation time of 1 h at room temperature.

*Tetrazine iEDDA reactions with cyclopropene-labeled RNA transcripts **RNA^{CP}** and **tRNA^{CP}***

Tetrazine-fluorophore conjugate **1** (1.0 eq., 130 pmol for reaction with **RNA^{CP}**; 3.0 eq., 150 pmol for reaction with **tRNA^{CP}**) was added to cyclopropene-modified RNA transcripts (5 µL of crude transcription reactions, 130 pmol of purified **RNA^{CP}** transcript or 50 pmol of

purified **tRNA^{CP}** transcript) and incubated at room temperature for 1 h under exclusion of light prior to sample preparation for 20% PAGE analysis.

LC-MS analysis of RNA oligonucleotides was performed after overnight incubation with a sevenfold excess of tetrazine-fluorophore conjugate **1**. Elution was carried out with 3% acetonitrile (B) for 9 min followed by a gradient from 3% to 40% B in 11 min at a flow rate of 0.2 mL min^{-1}.

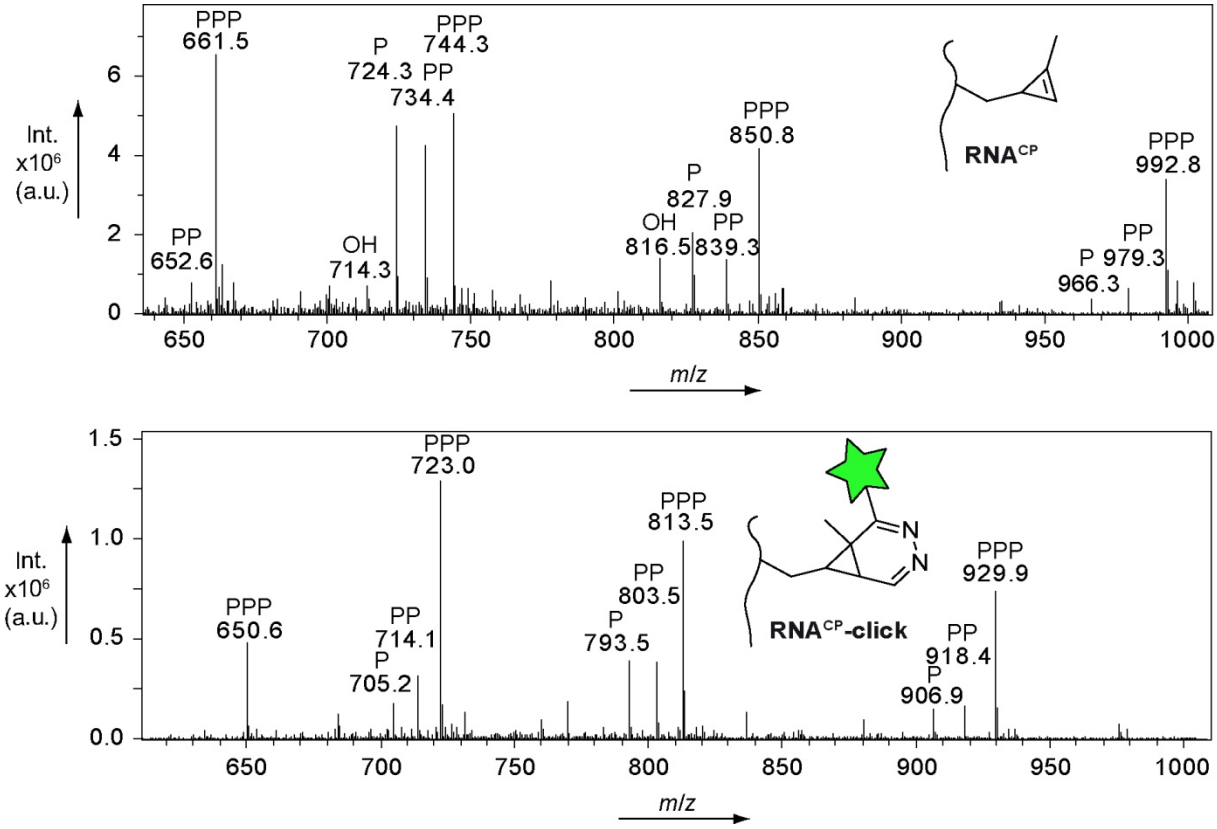


Figure S2.

Raw ESI$^-$ traces of cyclopropene-modified **RNA^{CP}** (5'-GGGAAUCCCGAG-**TPT3^{CP}**-AGUG-3') with assigned peaks for 5'-OH, mono- (P), di- (PP) and triphosphate (PPP) before (upper image) and after (lower image) reaction with tetrazine-fluorophore conjugate **1** (*m*/*z* calcd. for **RNA^{CP}**: 5'-OH = 5722.3, 5'-phosphate = 5802.3, 5'-diphosphate: 5882.3, 5'-triphosphate: 5962.3; *m*/*z* calcd. for **RNA^{CP}-click**: 5'-phosphate = 6356.2, 5'-diphosphate: 6436.2, 5'-triphosphate: 6516.2).

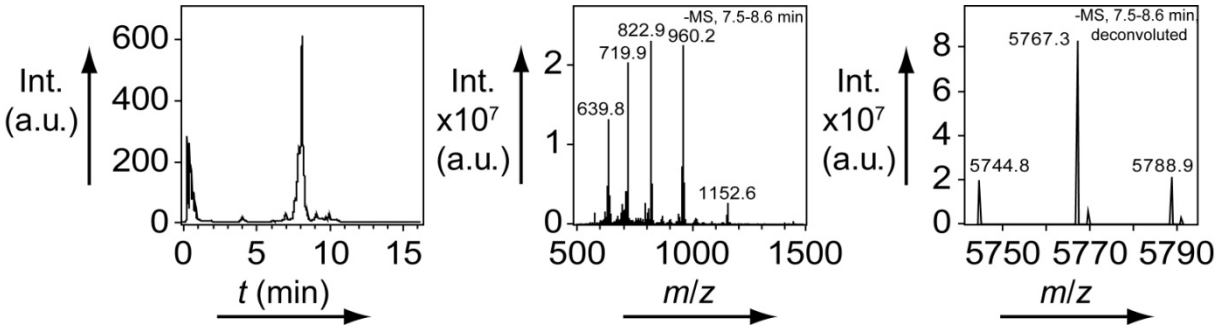


Figure S3.

LC-MS analysis of crude **RNAc** transcriptions with **TPT3^{CP}** TP added to the reaction. The sample (50 µL) was desalted via *illustra^{TM} Microspin^{TM} G-25* column (*GE Healthcare*), and 8 µL of the resulting solution were subjected for analysis. Left: UV trace; middle: raw ESI$^-$ traces; right: deconvoluted ESI$^-$ data, *m*/*z* calcd. for **RNA^C** (5'-GGGAAUCCCGAGUAGUG-3'): 5'-triphosphate: 5745.3, 5'-triphosphate sodium adduct: 5767.3, 5'-triphosphate disodium adduct: 5789.3. No unspecific incorporation of triphosphate **7** is observed.

Biochemical Experiments

Preparation of DNA templates for T7 transcription

The d**NaM** phosphoramidite, suitably protected for oligonucleotide synthesis, was purchased from *Berry & Associates Inc.*, USA. Solid phase syntheses and purification of modified oligonucleotides **DNA^{NaM}** and **tDNA^{NaM}** were performed in 200 nmol scale by *Ella Biotech*, Germany.

Primers and unmodified DNA oligonucleotides were synthesized and purified by *Biomers*, Germany.

List of DNA primers and templates

A. *Primer for T7 transcription*

5'-ATA ATA CGA CTC ACT ATA GGG-3'

B. *Template strands containing* **X** = d**NaM**

DNA^{NaM}

5'-CAC T**X**C TCG GGA TTC CCT ATA GTG AGT CGT ATT AT-3'

tDNA^{NaM}

5'-TGG TCC GGC GGG CCG GAT TTG AAC CAG CGC CAT GCG GAT T**X**A GAG TCC GCC GTT CTG CCC TGC TGA ACT ACC GCC GGT ATA GTG AGT CGT ATT ATC-3'

C. *Template strands containing canonical nucleobases*

DNAc

5'-CAC TAC TCG GGA TTC CCT ATA GTG AGT CGT ATT AT-3'

tDNAc

5'-TGG TCC GGC GGG CCG GAT TTG AAC CAG CGC CAT GCG GAT TAA GAG TCC GCC GTT CTG CCC TGC TGA ACT ACC GCC GGT ATA GTG AGT CGT ATT ATC-3'

tDNA_RV

5'-GAT AAT ACG ACT CAC TAT ACC GGC GGT AGT TCA GCA GGG CAG AAC GGC GGA CTC TAA ATC CGC ATG GCG CTG GTT CAA ATC CGG CCC GCC GGA CCA-3'

List of resulting RNA transcripts

5'-GGG AAU CCC GAG **X**AG UG-3'

X = **TPT3^{CP}**: **RNA^{CP}**; **X** = U: **RNAc**

5'-CCG GCG GUA GUU CAG CAG GGC AGA ACG GCG GAC UCU **X**AA UCC GCA UGG CGC UGG UUC AAA UCC GGC CCG CCG GAC CA-3'

X = **TPT3^{CP}**: **tRNA^{CP}**; **X** = U: **tRNAc**

PAGE

For analytical (ca. 5 mL gel volume) and preparative (ca. 50 mL gel volume) 20% denaturing PAGE separation, a solution of formamide/8.3 M urea (95/5, *v*/*v*) supplemented with 20 mM ethylenediaminetetraacetic acid (EDTA) was used as loading buffer in equal ratio with the sample volume. Samples were heated to 95 °C for 2 min prior to gel loading. Analytical gels were run at 300 V for 1 h, preparative gels were run at 400 V for 4 h.

1x Tris-borate-EDTA buffer (1x TBE) was employed as running buffer. Analytical polyacrylamide gels were stained with SybrSafe® (*Life Technologies)* and visualized either by UV illumination using a *Genoplex* gel documentation system (*VWR*) or by fluorescence scanning using a *Phosphorimager FLA-3000* (*Fujifilm*) with an excitation wavelength of $\lambda_{excitation}$ = 473 nm and an emission wavelength of $\lambda_{emission}$ = 520 nm.

T7 transcription

In vitro RNA transcription was performed on a 100 µL scale with a 3 µM final concentration of DNA primer and template in 25 mM $MgCl_2$, 40 mM Tris-HCl (pH = 7.9). Primer and template were annealed in by slow cooling (short sequences: 15 min, long templates: o/n) from 95 °C to room temperature. A final concentration of 2.5 mM of each natural NTP, 0 or 1 mM triphosphate **7**, 5 mM DTT, 1.24 µL RNAsin (40 U μL^{-1}, *Promega*), 0.2 µL inorganic pyrophosphatase (2 U μL^{-1}, *New England Biolabs*) and 5 µL T7 RNA polymerase (20 U μL^{-1}, *homemade*) were added to the annealed template construct. The transcription reaction was incubated at 37 °C for 5 h. For DNase I digestion, 2 µL DNase I (10 U μL^{-1}, *Roche Diagnostics GmbH*) and 8 µL 10x DNase I incubation buffer (*Roche Diagnostics GmbH*) were added and incubated at 37 °C for 30 min.

RNA purification

Purification of RNA transcripts was performed using preparative denaturing gel electrophoresis. Oligonucleotides were visualized by UV shadowing after PAGE separation, excised from the gel, eluted into ammonium acetate (8 M, 170 µL) using an electroelution chamber (*selfmade*, 150 V, 1 h) and precipitated from ethanol (1.3-1.5 mL, o/n, -20 °C). Samples were centrifuged (1 h, 13000 rpm, 4 °C), the supernatant was removed, and the RNA pellet was washed with EtOH (70%, *v*/*v*, 200 µL). After lyophilization, pellets were redissolved in water (20 µL).

Determination of transcription yields

Transcription reactions were DNase I digested and purified as stated above. Absorption at 260 nm (A_{260}) was determined using a *Nanodrop* UV-spectrometer *2000c* (*Peqlab*).

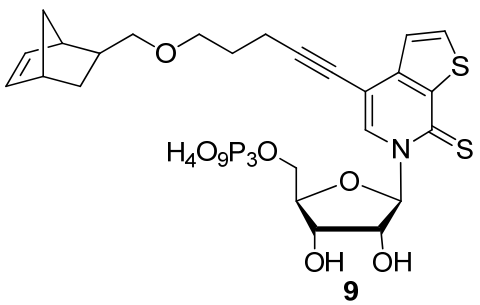
Concentrations were obtained from the A_{260} value and software-assisted calculation employing the unmodified sequences (*http://biotools.nubic.northwestern.edu/OligoCalc.html*).

Table S1. Obtained yields for purified transcripts of unmodified **RNAc** and **RNA^{CP}**.

template	c [µM]	n [nmol] per 100 µL TK	normalized
RNAc	489	4.9	1.00
RNA^{CP}	157	1.1	0.22

Table S2. Comparison of transcription yields incorporating **TPT3^{CP}** TP (**7**) and previously published **TPT3^{NOR}** TP (**9**)[5] employing the **DNA^{NaM}** template. A_{260} values are averaged from four measurements. Concentrations were calculated for the sequence 5'-GGGAAUCCCGAGUAGUG-3' using the *Oligonucleotide Properties Calculator* at http://biotools.nubic.northwestern.edu/OligoCalc.html. Amount of RNA obtained (n [nmol]) corresponds to 36 µL transcription reactions, DNA and reagent concentrations as stated above (see section *T7 Transcription*).

	TPT3^{CP} TP		**TPT3^{NOR} TP**	
	30 min	5 h	30 min	5 h
A_{260} (1)	2.013	5.845	1.083	7.778
A_{260} (2)	1.244	9.086	0.636	2.898
A_{260} (3)	2.340	5.718	1.126	3.618
A_{260} (average)	1.87 ± 0.56	6.88 ± 1.91	0.95 ± 0.27	4.76 ± 2.63
c [µM]	**8.72** ± 2.61	**32.08** ± 8.90	**4.43** ± 1.26	**22.19** ± 12.26
n [nmol]	**0.17** ± 0.054	**0.64** ± 0.18	**0.09** ± 0.03	**0.44** ± 0.25
normalized	**100%**	**100%**	**53%**	**69%**



9

TPT3^{NOR} TP

PAGE images

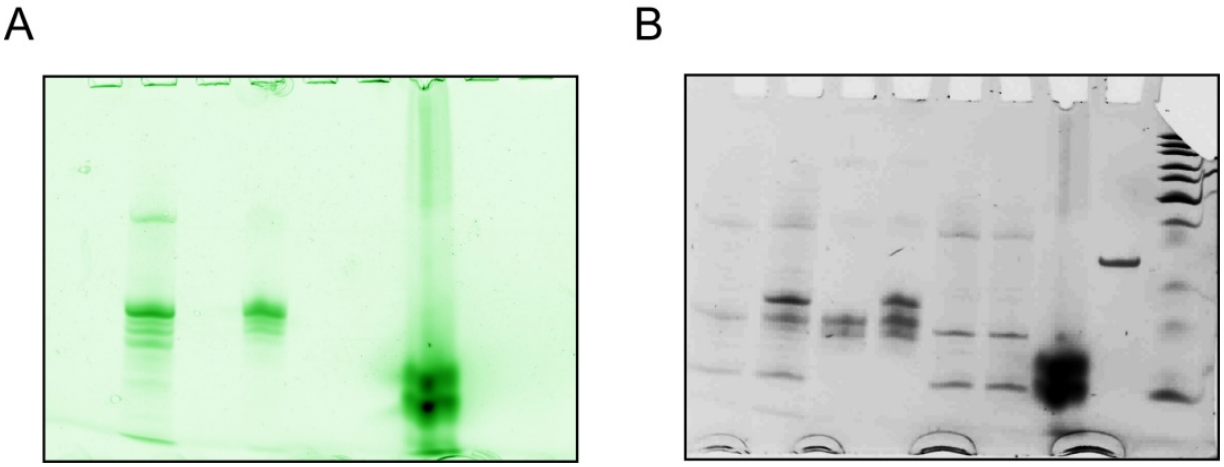


Figure S4. Complete lanes both 20% denaturing polyacrylamide gels as shown in Figure 2 B and D. Samples (4-6 µL) were diluted with 20 µL H_2O, desalted via *illustra*^{TM} *Microspin*^{TM} *G-25* columns (*GE Healthcare*), and concentrated by freeze-drying (ca. 30 min) prior to preparation for gel loading; A. fluorescence scan **RNA**^{CP}; B. SybrSafe® stain **RNA**^{CP}; Marker: *Thermo Scientific GeneRuler* Ultra Low Range DNA Ladder.

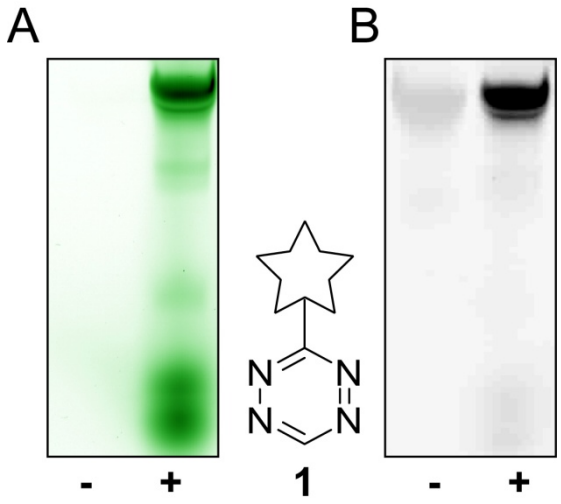


Figure S5. iEDDA reaction of **1** with purified **tRNA**^{CP}: 20% denaturing polyacrylamide gel showing purified **tRNA**^{CP}, reacted with 5 eq. tetrazine **1**; A. fluorescence scan; B. SybrSafe® stain.

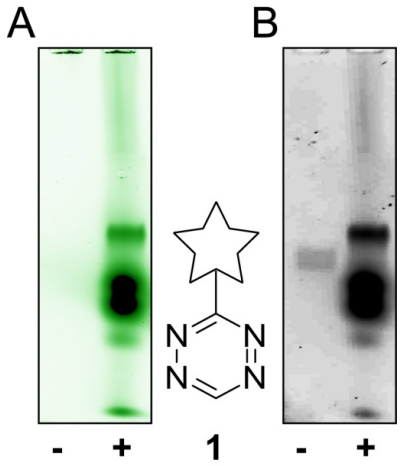


Figure S6. iEDDA reaction of purified **RNA**^{CP} with an excess of 5 eq. tetrazine **1**: 20% denaturing polyacrylamide gel; A. fluorescence scan; B. SybrSafe® stain. No educt band is detected after the reaction, indicating, that the reaction proceeds quantitatively.

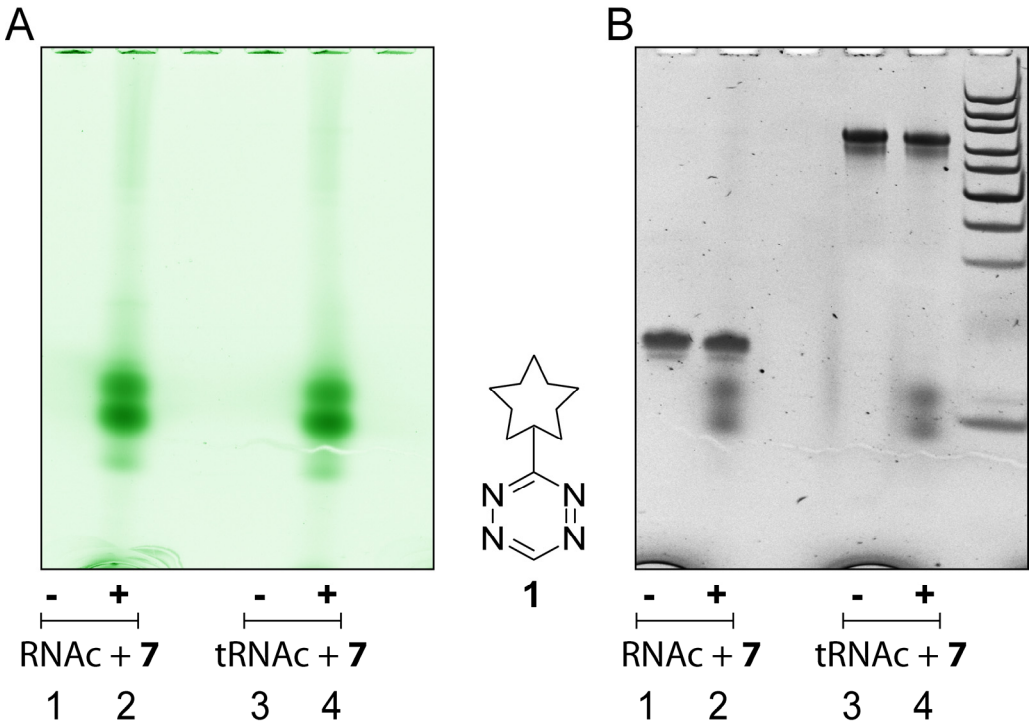
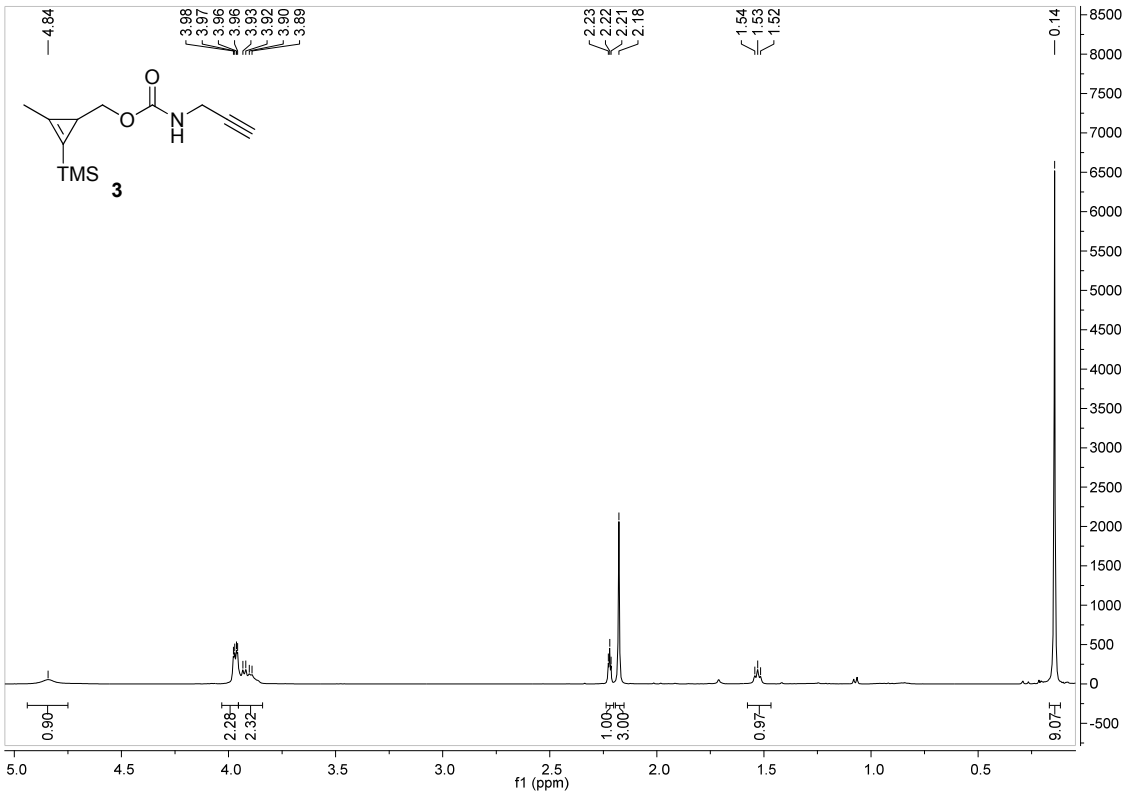


Figure S7. Denaturing PAGE analysis of control transcription reactions using the unmodified, natural-base DNA templates in the presence of **TPT3**^{CP} TP (**7**) for in vitro transcription. The resulting **RNAc** and **tRNAc** transcripts were incubated with 4 eq. tetrazine **1**. The left image shows the fluorescence scan of the gel (excitation 473 nm, emission 520 nm). The right image shows the same gel after staining with SybrSafe®. Lane 1: Transcription reaction using **DNAc** as template and adding **7** to the transcription reaction (**RNAc** product); Lane 2: **DNAc** as template, triphosphate **7** added, **RNAc** product, incubated with tetrazine **1**. Lane 3: **tDNAc** as template, triphosphate **7** added, **tRNAc** product. Lane 4: **tDNAc** as template, triphosphate **7** added, **tRNAc** product, incubated with tetrazine **1**.

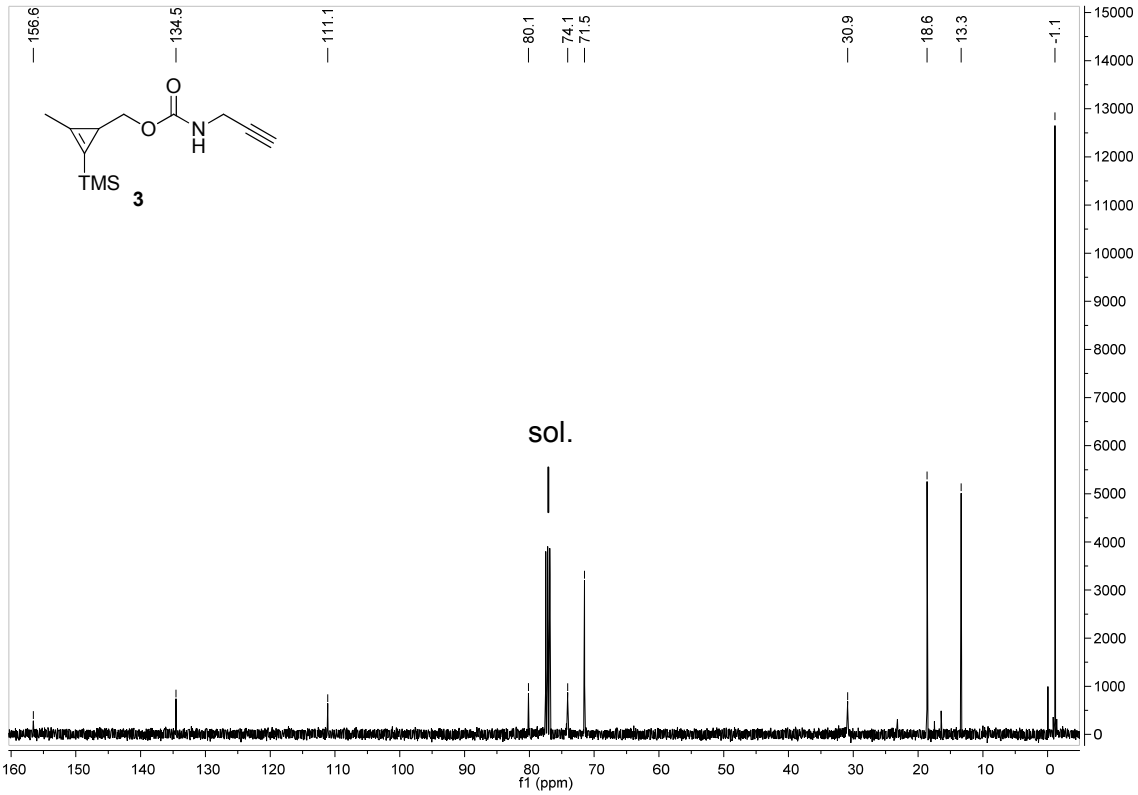
Spectra

NMR spectra

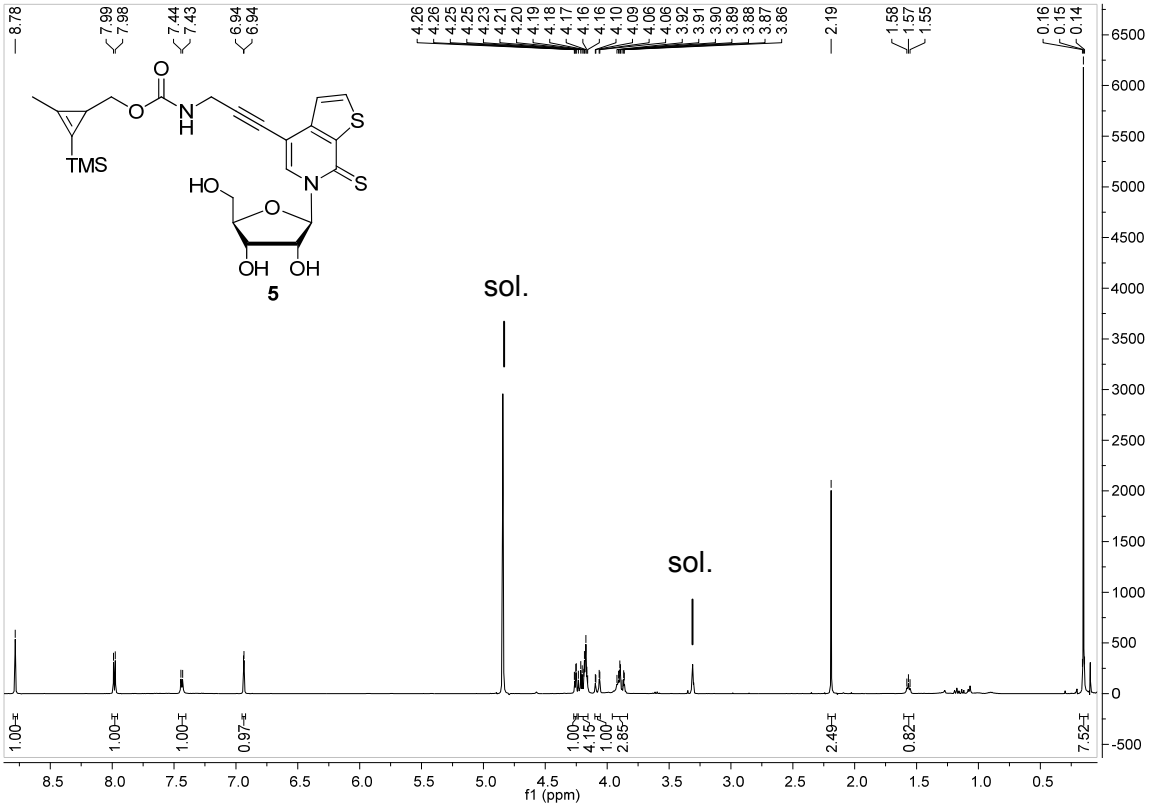
^{1}H-NMR spectrum of compound **3** ($CDCl_3$, 400 MHz, r.t.).



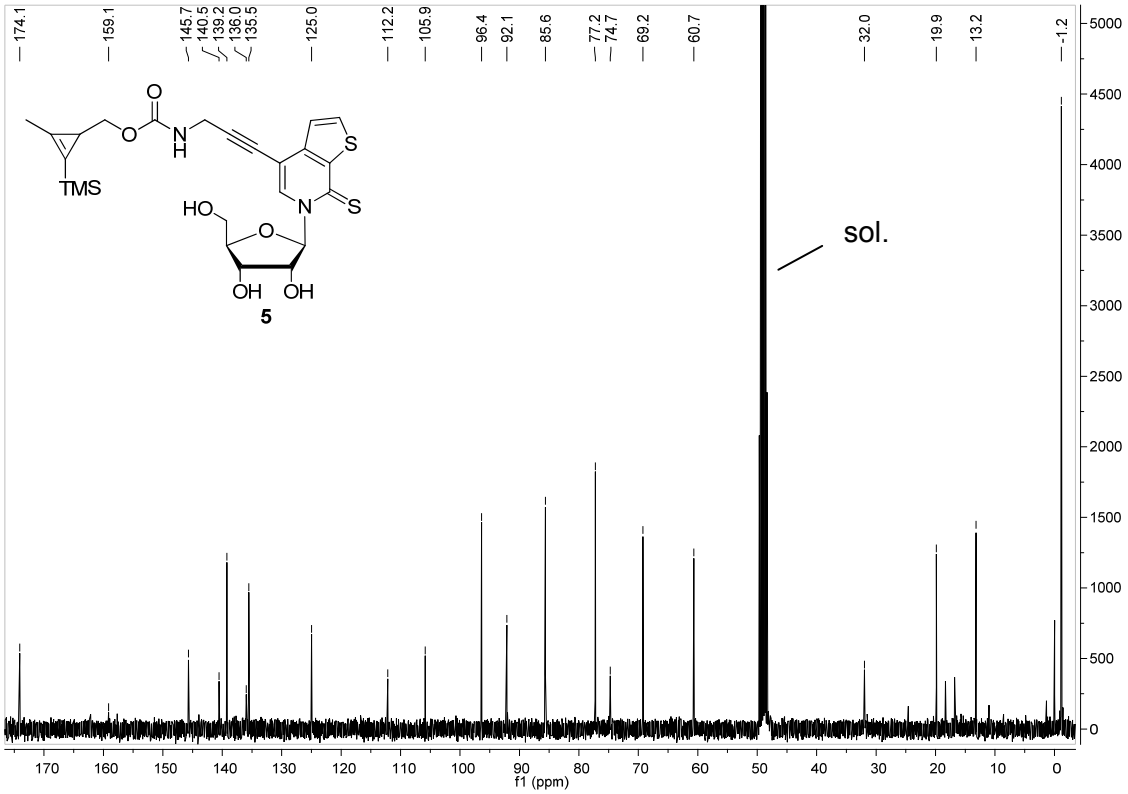
^{13}C-NMR spectrum of compound **3** ($CDCl_3$, 101 MHz, r.t.).



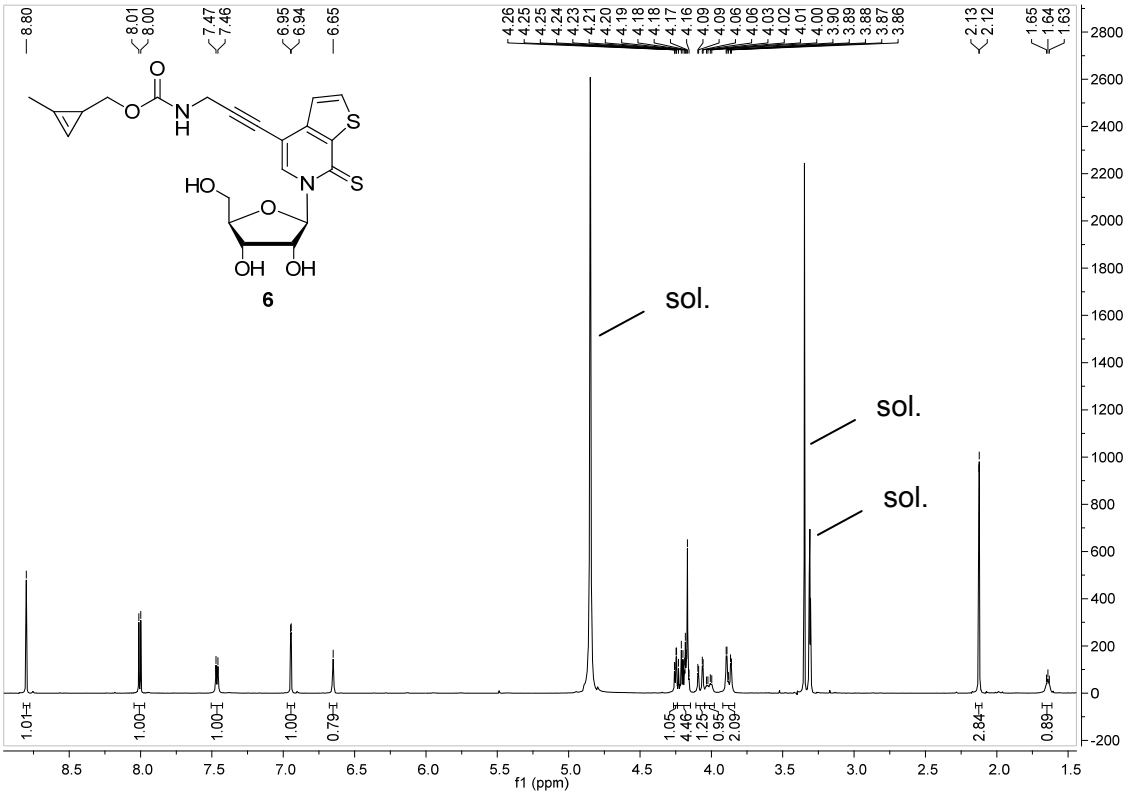
^{1}H-NMR spectrum of compound **5** (CD_3OD, 400 MHz, r. t.).



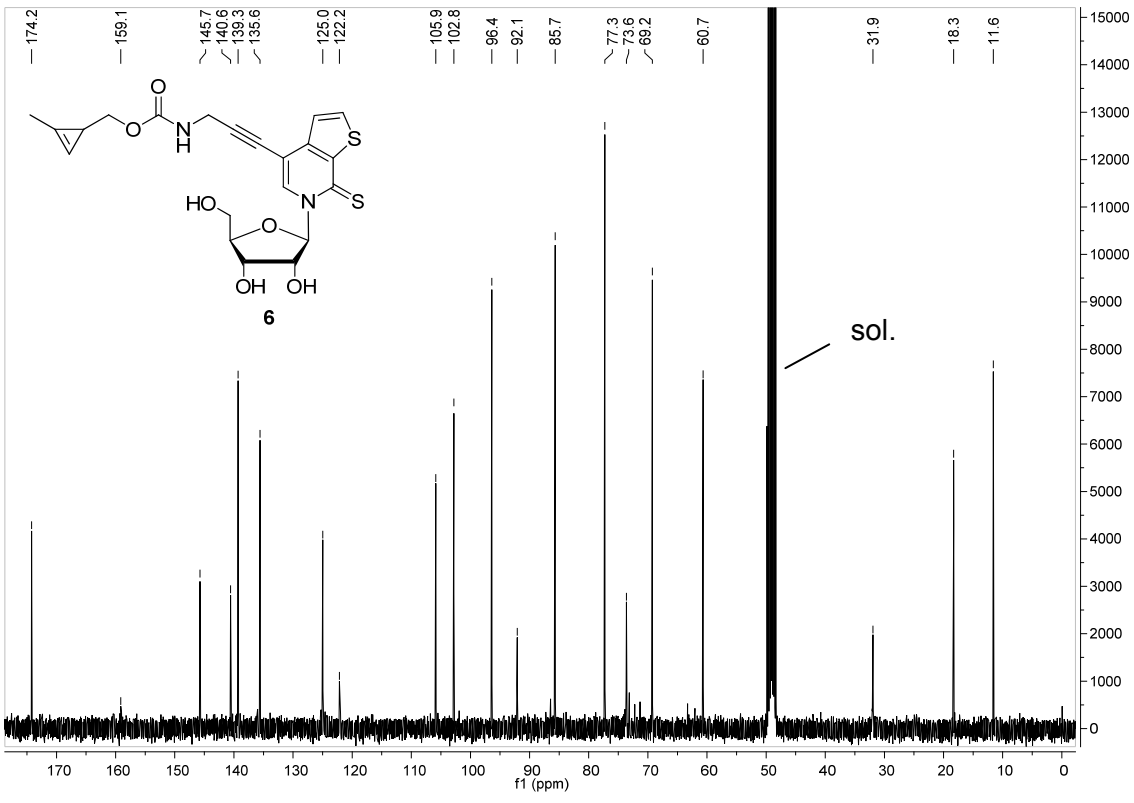
^{13}C-NMR spectrum of compound **5** (CD_3OD, 101 MHz, r. t.).



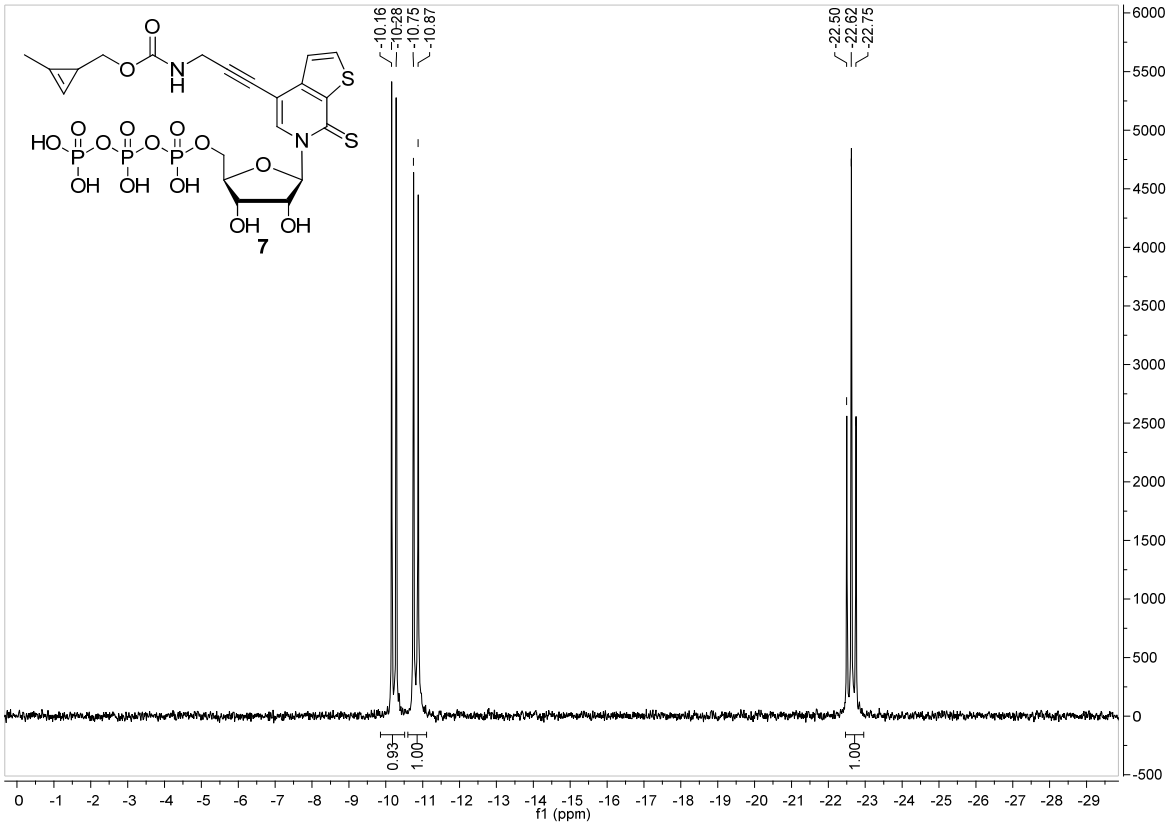
^{1}H-NMR spectrum of compound **6** (**TPT3^{CP}**) (CD_3OD, 400 MHz, r. t.).



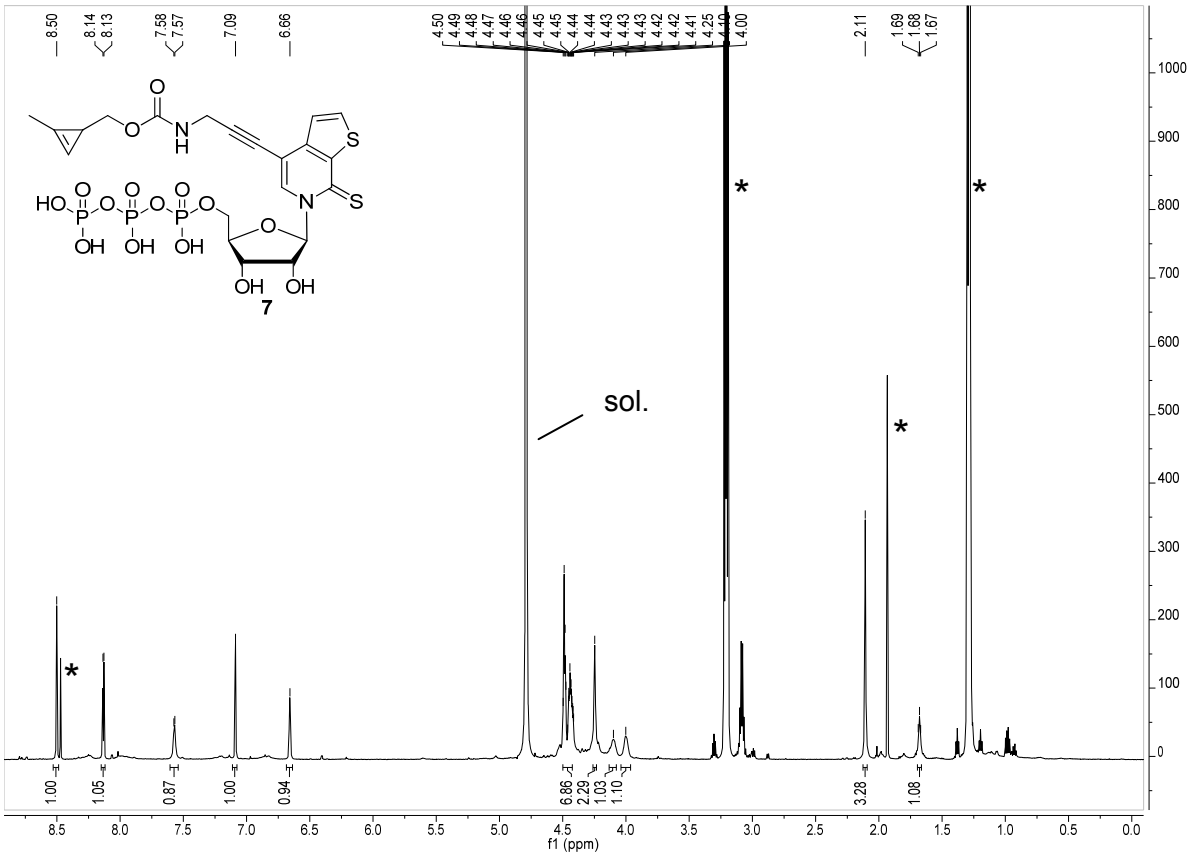
^{13}C-NMR spectrum of compound **6** (**TPT3^{CP}**) (CD_3OD, 101 MHz, r. t.).



^{31}P-NMR spectrum of compound **7** (**TPT3^{CP}** TP) (D_2O, 162 MHz, r.t.).

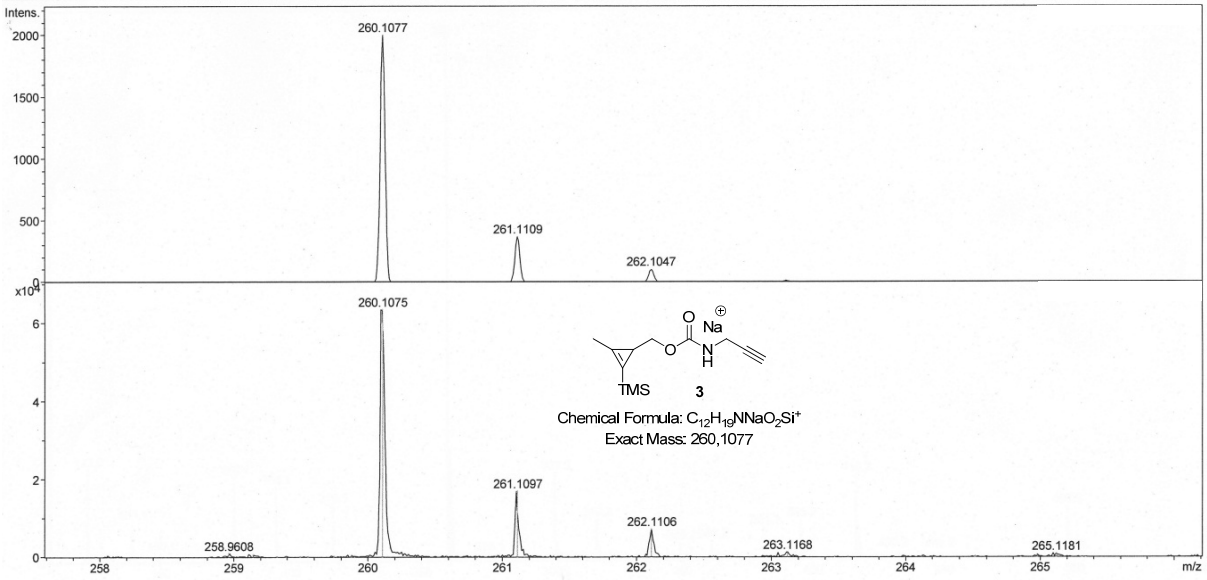


^{1}H-NMR spectrum of compound **7** (**TPT3^{CP}** TP) (D_2O, 700 MHz, r.t.); *$HNEt_3OAc$ traces.

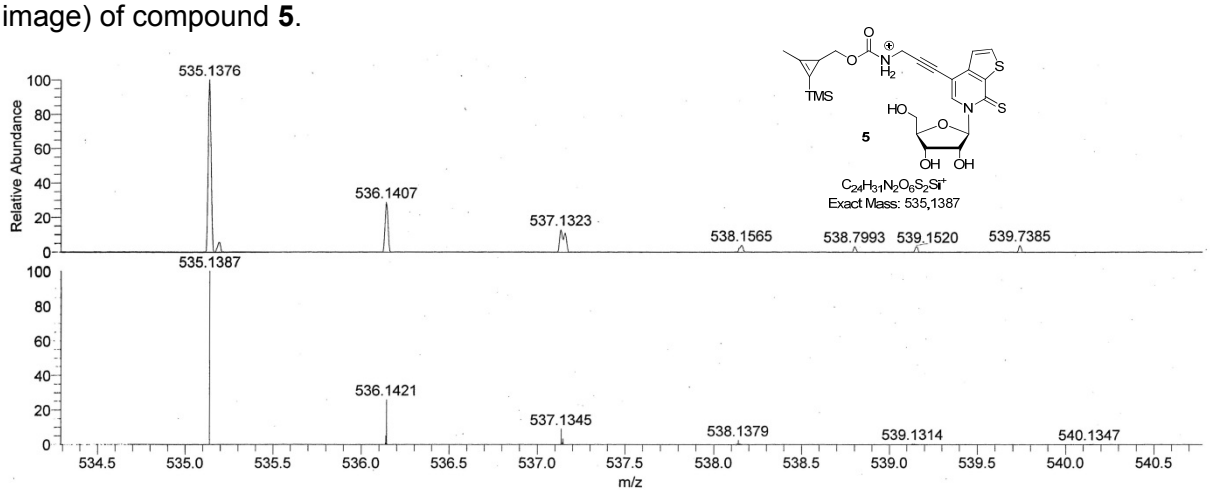


Mass spectra

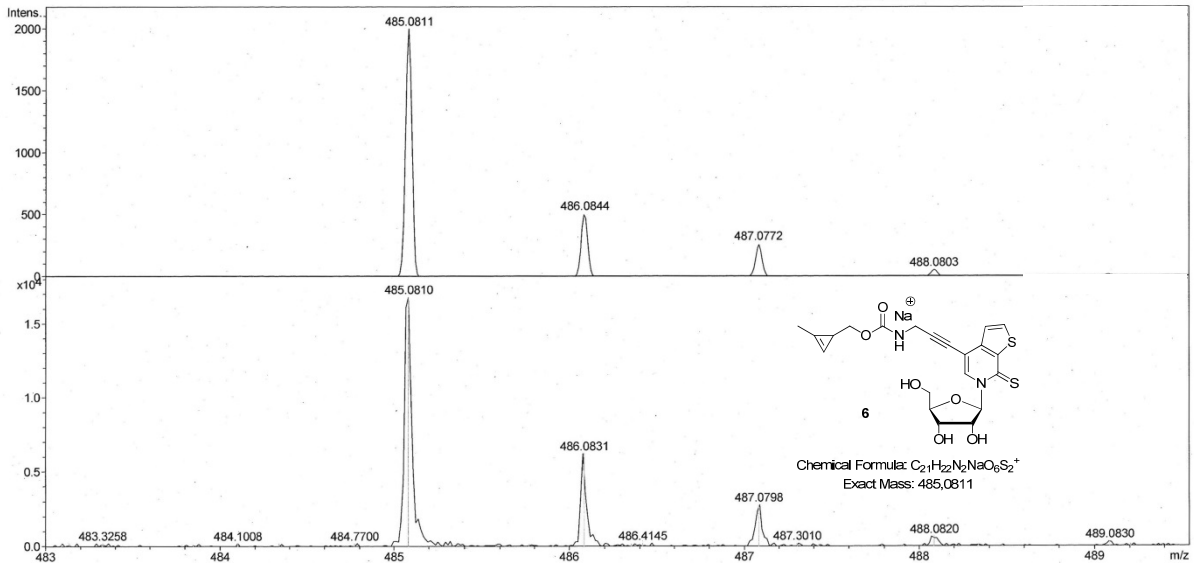
Calculated (upper image) and high resolution ESI$^+$ mass spectrum (lower image) of compound **3**.



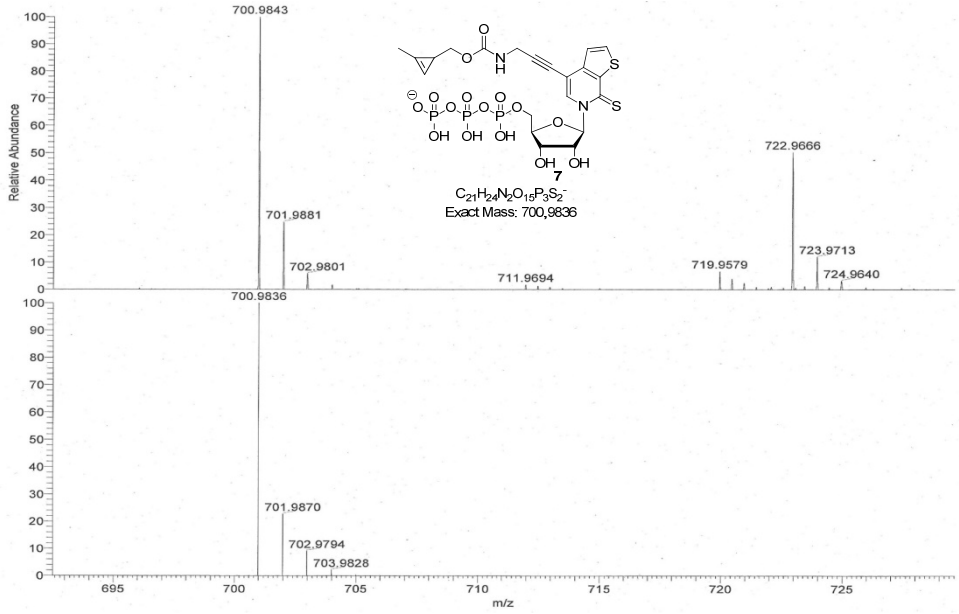
High resolution ESI$^+$ mass spectrum (upper image) and calculated mass spectrum (lower image) of compound **5**.



Calculated (upper image) and high resolution ESI$^+$ mass spectrum (lower image) of compound **6** (**TPT3^{CP}**).



High resolution ESI$^-$ mass spectrum (upper image) and calculated mass spectrum (lower image) of compound **7** (**TPT3^{CP}** TP).



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[5] Domnick, C.; Eggert, F.; Kath-Schorr, S., Site-specific enzymatic introduction of a norbornene modified unnatural base into RNA and application in post-transcriptional labeling, *Chem. Commun.* **2015**, *51*, 8253–8256.