Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Site-specific enzymatic introduction of a norbornene modified unnatural base into RNA and application in posttranscriptional labeling

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Supporting Information

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General Methods

Reversed-phase HPLC analysis was performed on an *Agilent 1100* Series HPLC system using an *Nucleodur 100-5*, C18 (*Agilent Technologies*) column. Used eluents are either water (buffer A) and acetonitrile (buffer B, for tetrazine-fluorophore conjugates) or 20 mM triethylammonium acetate (buffer A) and 20 mM ammonium acetate in 50% acetonitrile (buffer B, for triphosphate purification) at a flow rate of 1 ml/min.

NMR-spectra were recorded on an *AM400*, *AM500* and *DRX600* from *Bruker*. The assignment of proton and carbon resonances is based on two-dimensional correlation experiments (COSY, HMQC, HMBC). Chemical shifts (δ) are given in ppm. EI spectra were recorded on *MAT-95XL* mass spectrometer (*Finnigan*). ESI mass spectra were recorded on a *micrOTOF-Q* mass spectrometer from *Bruker Daltonik* in combination with an *Agilent 1200 Series* HPLC system (*Agilent Technologies*) as well as a *HTC esquire* from *Bruker Daltonic* in combination with an *Agilent 1100* Series HPLC system (*Agilent Technologies*) using 10 mM triethylamine/100 mM hexafluoroisopropanol as buffer A and acetonitrile as eluent (buffer B). RNA oligonucleotides were desalted prior to MS using *Zip-tip C18* pipette tips (*Millipore*).

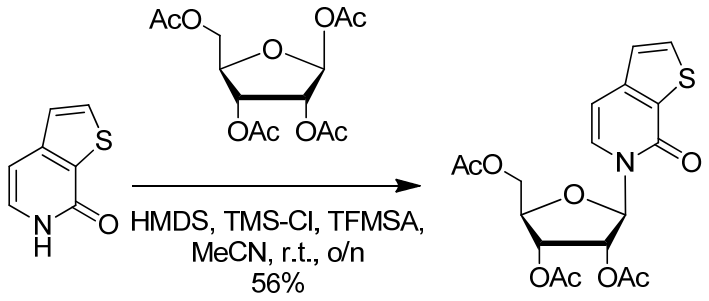
HPLC purification was performed on an *Agilent 1100 Series* HPLC system (*Agilent Technologies*) with an *EC 150/4.6 Nucleodur 100-5 C18eC* from *Macherey-Nagel*.

RNA (at 260 nm) and tetrazine-fluorophore conjugate (at 495 nm, $\varepsilon 495 = 75000\ cm^{-1}$) concentrations were determined using a *Nanodrop* UV-spectrometer *2000c* (*Peqlab*).

For analytical denaturing PAGE (20 %) separation a loading buffer (95% formamide, 20 mM ethylenediaminetetraacetic acid, EDTA) was used. 1x Tris-borate-EDTA buffer (1x TBE) was used as running buffer. Desalting of oligonucleotides was carried out either by *Amicon Ultra-0.5 mL* centrifugal filters (3 K, *Merck Millipore*) or by *ZipTip C18* pipette tips (*Merck Millipore*). Denaturing polyacrylamide gels were stained with SYBR® Safe from *Life Technologies* or ethidium bromide and visualized either by UV illumination using a *Genoplex* gel documentation system (*VWR*) or for fluorescence scans using a *Phosphorimager FLA-3000* (*Fujifilm*).

Syntheses

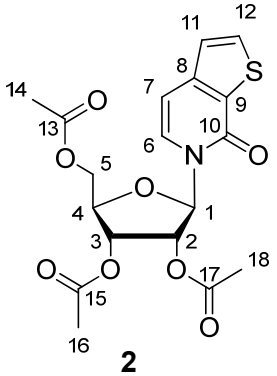
*Synthesis of **2*** - 1'-(2',3',5'-tri-*O*-acetyl-β-D-ribofuranosyl)6*H*-thieno-[2,3-*c*]pyridine-7-one



1 **2**

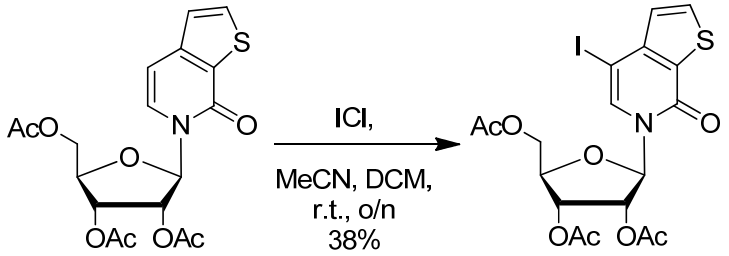
Pyridone derivative **1** (1.61 g, 10.7 mmol, 1.0 eq.) was dissolved in dry MeCN (75 mL) under an atmosphere of Ar. HMDS (2.2 mL, 10.7 mmol, 1.0 eq.) and TMS-Cl (1.4 mL, 10.7 mmol, 1.0 eq.) were added consecutively and the turbid solution was cooled in an ice bath. After dropwise addition of TFMSA (1.1 mL, 12.8 mmol, 1.2 eq.) at 0 °C, the reaction mixture was allowed to warm to room temperature and stirred for 0.5 h before peracylated β-D-ribofuranose (3.39 g, 10.7 mmol, 1.0 eq.) was added. The mixture was stirred at room temperature overnight, diluted with CH_2Cl_2 (200 mL) and washed with saturated aqueous $NaHCO_3$ solution (200 mL). Extraction of the aqueous layer with CH_2Cl_2 (3 x 150 mL), drying over anhydrous $MgSO_4$ and removal of the solvents under reduced pressure gave the crude product which was purified by column chromatography (SiO_2, EtOAc/Cy, 7/4, *v*/*v*, R_f = 0.60) to give **2** (2.46 g, 6.0 mmol, 56%) as a light brown oil of very high viscosity.

^{1}H-NMR ($CDCl_3$, 400 MHz, r.t.): δ [ppm] = 7.71 (d; $^3J_{H\text{-}12/H\text{-}11}$ = 5.1 Hz; 1H; H-12), 7.36 (d; $^3J_{H\text{-}6/H\text{-}7}$ = 7.4 Hz; 1H; H-6), 7.19 (d; $^3J_{H\text{-}11/H\text{-}12}$ = 5.1 Hz; 1H; H-11); 6.69 (d; $^3J_{H\text{-}7/H\text{-}6}$ = 7.4 Hz; 1H; H-7), 6.46 (d; $^3J_{H\text{-}1/H\text{-}2}$ = 4.5 Hz; 1H; H-1), 5.47 (m; 1H; H-2/3), 5.41 (m; 1H; H-3/2), 4.45-4.34 (m; 3H; H-4, H-5), 2.15 (s; 3H; H-14), 2.10 (s; 3H; H-16/18), 2.09 (s; 3H; H-18/16). ^{13}C-NMR ($CDCl_3$, 101 MHz, r.t.): δ [ppm] = 170.41 (C-13), 169.73 (C-15/17), 169.66 (C-17/15), 158.30 (C-10), 145.27 (C-8), 134.39 (C-12), 130.27 (C-9), 127.82 (C-6), 124.39 (C-11), 103.87 (C-7), 87.88 (C-1), 79.59 (C-4), 74.03 (C-3), 70.13 (C-2), 63.21 (C-5), 20.91 (C-14), 20.62 (C-16/18), 20.59 (C-18/16). MS (ESI$^+$, 6 eV): calculated for $[M + Na]^+$ ($C_{18}H_{19}NO_8SNa^+$): 432.0724; found: *m*/*z* = 432.0733.



2

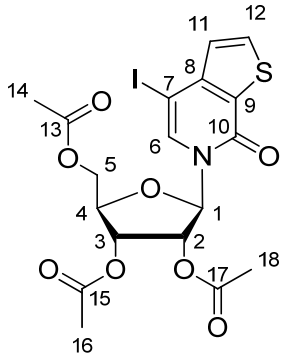
*Synthesis of **3*** - 1'-(2',3',5'-tri-*O*-acetyl-β-D-ribofuranosyl)6*H*-thieno-[2,3-*c*]-4-iodopyridine-7-one



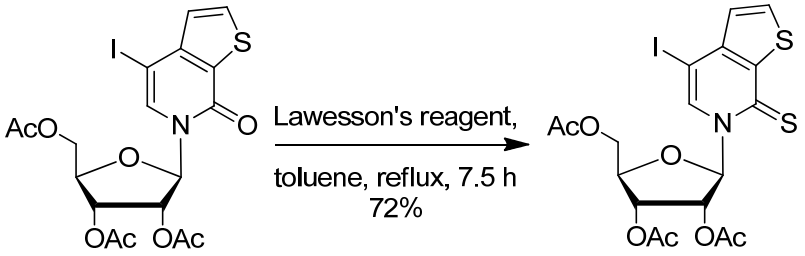
2 **3**

Under an atmosphere of argon, compound **2** (1.00 g, 2.4 mmol, 1.0 eq.) was dissolved in dry MeCN (15 mL) and cooled in an ice bath. Iodine monochloride (1.0 M soln. in CH_2Cl_2, 2.9 mL, 2.9 mmol, 1.2 eq.) was added and the mixture was stirred overnight at room temperature under exclusion of light. The reaction was quenched by the addition of saturated aqueous $NaHCO_3$ and $Na_2S_2O_3$ solution (30 mL each). The aqueous layer was extracted with CH_2Cl_2 (3 x 60 mL), dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. Purification via column chromatography (SiO_2, EtOAc/Cy, 6/4, *v*/*v*, R_f = 0.60) yielded **3** (504 mg, 0.9 mmol, 38%) as a white solid or foam.

^{1}H-NMR ($CDCl_3$, 400 MHz, r.t.): δ [ppm] = 7.75 (d; $^3J_{H\text{-}12/H\text{-}11}$ = 5.2 Hz; 1H; H-12), 7.69 (s; 1H; H-6), 7.20 (d; $^3J_{H\text{-}11/H\text{-}12}$ = 5.2 Hz; 1H; H-11), 6.45 (d; $^3J_{H\text{-}1/H\text{-}2}$ = 4.5 Hz; 1H; H-1), 5.43-5.37 (m; 2H; H-2, H-3), 4.45-4.36 (m; 3H; H-4, H-5), 2.26 (s; 3H; H-14), 2.10 (s; 3H; H-16/18), 2.09 (s; 3H; H-18/16). ^{13}C-NMR ($CDCl_3$, 101 MHz, r.t.): δ [ppm] = 170.41 (C-13), 169.70 (C-15/17), 169.55 (C-17/15), 157.39 (C-10), 147.06 (C-8), 133.93 (C-12), 132.81 (C-6), 129.21 (C-9), 128.27 (C-11), 87.29 (C-1), 79.90 (C-4), 74.23 (C-2), 69.93 (C-3), 64.58 (C-7), 62.89 (C-5), 21.29 (C-14), 20.60 (C-16/18), 20.55 (C-18/16). MS (ESI$^+$, 10 eV): calculated for $[M + Na]^+$ ($C_{18}H_{18}NO_8ISNa^+$): 557.9690; found: *m*/*z* = 557.9703.



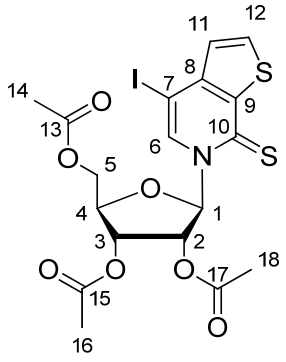
*Synthesis of **4*** - 1'-(2',3',5'-tri-*O*-acetyl-β-D-ribofuranosyl)6*H*-thieno-[2,3-*c*]-4-iodopyridine-7-thione



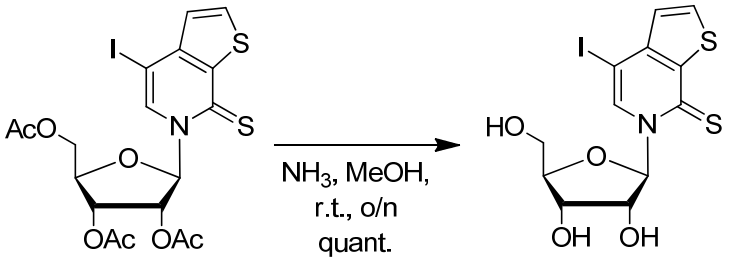
3 **4**

To Lawesson's reagent (397 mg, 1.0 mmol, 1.5 eq.) was added **3** (350 mg, 0.7 mmol, 1.0 eq.) in dry toluene (20 mL) at room temperature under argon. The resulting solution was heated at reflux for 7.5 h, cooled to ambient temperatures and concentrated *in vacuo*. Column chromatography (SiO_2, EtOAc/Cy, 3/7, *v*/*v*, R_f = 0.43) yielded **4** (259 mg, 0.5 mmol, 72%) as a yellow solid.

^{1}H-NMR ($CDCl_3$, 400 MHz, r.t.): δ [ppm] = 8.25 (s; 1H; H-6), 7.85 (d; $^3J_{H\text{-}12/H\text{-}11}$ = 5.3 Hz; 1H; H-12), 7.28 (d; $^3J_{H\text{-}11/H\text{-}12}$ = 5.3 Hz; 1H; H-11), 7.18 (d; $^3J_{H\text{-}1/H\text{-}2}$ = 2.5 Hz; 1H; H-1), 5.56 (dd; $^3J_{H\text{-}2/H\text{-}1}$ = 2.5 Hz, $^3J_{H\text{-}2/H\text{-}3}$ = 5.5 Hz; 1H; H-2), 5.29 (dd; $^3J_{H\text{-}3/H\text{-}4}$ = 7.1 Hz; $^3J_{H\text{-}3/H\text{-}2}$ = 5.5 Hz; 1H; H-3), 4.54-4.46 (m; 3H; H-4, H-5), 2.32 (s; 1H; H-14), 2.16 (s; 3H; H-16/18), 2.07 (s; 3H; H-18/16). ^{13}C-NMR ($CDCl_3$, 101 MHz, r.t.): δ [ppm] = 173.75 (C-10), 170.48 (C-13), 169.60 (C-15/17), 169.22 (C-17/15), 144.45 (C-9/8), 141.18 (C-8/9), 137.94 (C-12), 134.57 (C-6), 128.30 (C-11), 92.00 (C-1), 79.65 (C-4), 74.61 (C-3), 72.03 (C-7), 68.29 (C-2), 61.70 (C-5), 21.57 (C-14), 20.60 (C-16/18), 20.57 (C-18/16). MS (ESI$^+$, 6 eV): calculated for $[M + Na]^+$ ($C_{18}H_{18}NO_7IS_2Na^+$): 573.9462; found: *m*/*z* = 573.9471.



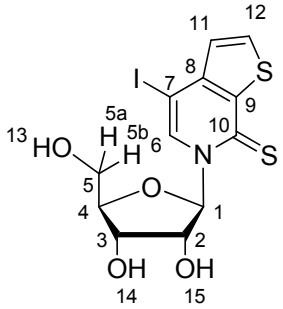
*Synthesis of **5*** - 1'-(β-D-ribofuranosyl) 6*H*-thieno-[2,3-*c*]-4-iodo-pyridine-7-thione



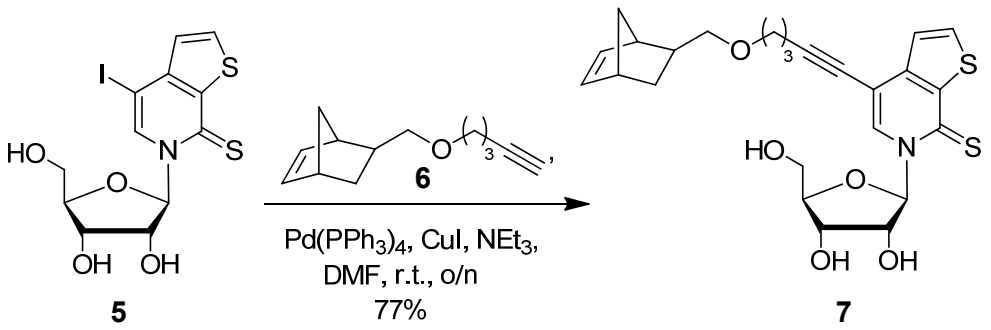
4 **5**

To **4** (667 mg, 1.2 mmol, 1.0 eq.) was added a solution of NH_3 in MeOH (*c* = 7.0 M, 20 mL, 140 mmol, 117 eq.) at room temperature under argon. The resulting solution was set aside overnight. Volatile components were evaporated and the residue was purified by column chromatography (SiO_2, CH_2Cl_2/MeOH, 95/5-90/10, *v*/*v*, R_f = 0.29 for 95/5) to give **5** (493 mg, 1.2 mmol, quant.) as a light yellow solid.

^{1}H-NMR (DMSO-d_6, 400 MHz, r.t.): δ [ppm] = 9.01 (s; 1H; H-6), 8.25 (d; $^3J_{H\text{-}12/H\text{-}11}$ = 5.4 Hz; 1H; H-12), 7.33 (d; $^3J_{H\text{-}11/H\text{-}12}$ = 5.4 Hz; 1H; H-11), 6.81 (d; $^3J_{H\text{-}1/H\text{-}2}$ = 1.2 Hz; 1H; H-1), 5.54 (d; $^3J_{H\text{-}15/H\text{-}2}$ = 4.6 Hz; 1H; H-15), 5.49 (t; 1H; $^3J_{H\text{-}13/H\text{-}5}$ = 4.4 Hz; H-13), 5.07 (d; $^3J_{H\text{-}14/H\text{-}3}$ = 5.5 Hz; 1H; H-14), 4.13-4.03 (m; 3H; H-2, H-3, H-4), 3.89 (ddd; $^2J_{H\text{-}5a/H\text{-}5b}$ = 12.0 Hz; $^3J_{H\text{-}5a/H\text{-}4}$ = 4.5 Hz; $^4J_{H\text{-}5a/H\text{-}3}$ = 2.0 Hz; 1H; H-5a/5b), 3.68 (ddd; $^2J_{H\text{-}5b/H\text{-}5a}$ = 12.0 Hz; $^3J_{H\text{-}5b/H\text{-}4}$ = 4.0 Hz; $^4J_{H\text{-}5a/H\text{-}3}$ = 1.3 Hz; 1H; H-5b/5a). ^{13}C-NMR (DMSO-d_6, 101 MHz, r.t.): δ [ppm] = 171.82 (C-10), 142.94 (C-9/8), 141.20 (C-8/9), 139.25 (C-12), 136.68 (C-6), 127.78 (C-11), 94.33 (C-1), 84.23 (C-2), 75.54 (C-3), 72.52 (C-7), 67.68 (C-4), 58.68 (C-5). MS (ESI$^+$, 6 eV): calculated for $[M + Na]^+$ ($C_{12}H_{12}NO_4IS_2Na^+$): 447.9145; found: *m*/*z* = 447.9139.

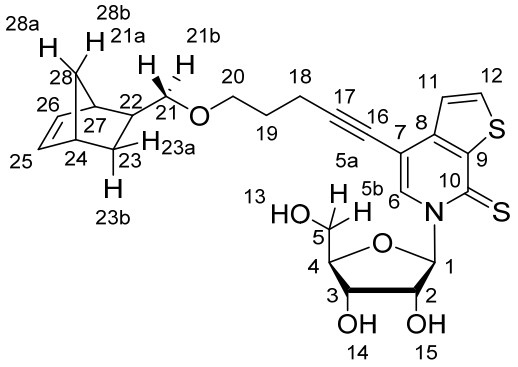


Synthesis of **7** - 1'-(β-D-Ribofuranosyl)6*H*-thieno-[2,3-*c*]-4-[5-(5-norbornene-2-*exo*-methoxy)-pent-1-ynyl]pyridine-7-thione

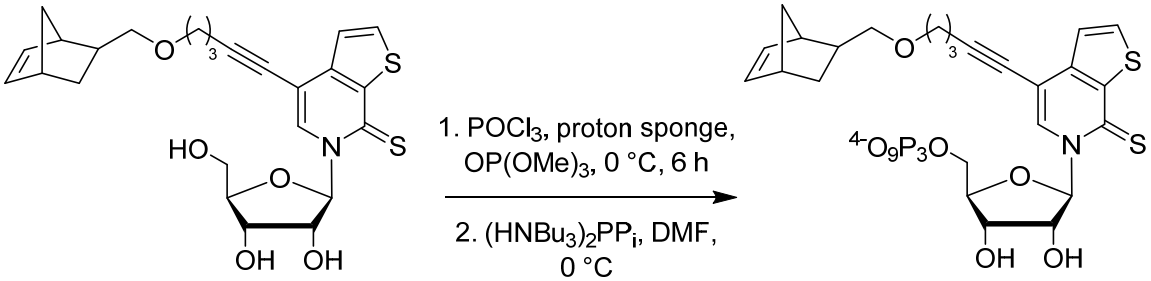


Under argon atmosphere, ribonucleoside **5** (100 mg, 0.24 mmol, 1.0 eq.) and CuI (32 mg, 0.17 mmol, 0.7 eq.) were dissolved in dry DMF (20 mL). NEt_3 (distilled and stored under Ar, 0.07 mL, 0.48 mmol, 2.0 eq.) was added, and the solution was degassed with a stream of argon for 15 min. Subsequently, $Pd(PPh_3)_4$ (23 mg, 0.02 mmol, 10 mol%) and a degassed solution of norbornene derivative **6** (67 mg, 0.35 mmol, 1.5 eq.) in dry DMF (5 mL) were added. The resulting reaction mixture was stirred over night at room temperature under exclusion of light. After *in vacuo* concentration, the residue was chromatographed (SiO_2, CH_2Cl_2/MeOH, 95/5, *v*/*v*, R_f = 0.43) and **7** (90 mg, 0.18 mmol, 77%) was obtained as a yellow to brownish solid.

^{1}H-NMR (MeOD, 500 MHz, r.t.): δ [ppm] = 8.77 (s; 1H; H-6), 7.97 (d; $^3J_{H\text{-}12/H\text{-}11}$ = 5.3 Hz; 1H; H-12), 7.39 (d; $^3J_{H\text{-}11/H\text{-}12}$ = 5.3 Hz; 1H; H-11), 6.94 (s; 1H; H-1), 6.05 (dd; $^3J_{H\text{-}25/H\text{-}26}$ = 5.6 Hz; $^3J_{H\text{-}25/H\text{-}24}$ = 2.8 Hz; 1H; H-25/26), 6.03 (dd, $^3J_{H\text{-}26/H\text{-}25}$ = 5.6 Hz; $^3J_{H\text{-}26/H\text{-}27}$ = 2.8 Hz; 1H; H-26/25), 4.27-4.21 (m; 2H; H-2, H-3), 4.18 (dt; $^3J_{H\text{-}4/H\text{-}3}$ = 7.6 Hz; $^3J_{H\text{-}4/H\text{-}5}$ = 2.0 Hz; 1H; H-4), 4.08 (dd; $^2J_{H\text{-}5a/H\text{-}5b}$ = 12.5 Hz; $^3J_{H\text{-}5a/H\text{-}4}$ = 2.0 Hz; 1H; H-5a/5b), 3.88 (dd; $^2J_{H\text{-}5b/H\text{-}5a}$ = 12.5 Hz; $^3J_{H\text{-}5b/H\text{-}4}$ = 2.0 Hz; 1H; H-5b/5a), 3.65-3.56 (m; 2H; H-20), 3.49 (m; 1H; H-21a), 3.37 (m; 1H; H-21b), 2.75 (s; 1H; H-27/24), 2.70 (s; 1H; H-24/27), 2.59 (t; $^3J_{H\text{-}18/H\text{-}19}$ = 7.1 Hz; 2H; H-18), 1.92-1.86 (m; 2H; H-19), 1.69-1.62 (m; 1H; H-22), 1.31 (d; $^2J_{H\text{-}28a/H\text{-}28b}$ = 8.3 Hz; 1H; H-28a/28b), 1.27 (d; $^2J_{H\text{-}28b/H\text{-}28a}$ = 8.3 Hz; 1H; H-28b/28a), 1.20 (m; 1H; H-23a/23b), 1.15-1.10 (m; 1H; H-23b/23a). ^{13}C-NMR (MeOD, 126 MHz, r.t.): δ [ppm] = 173.14 (C-10), 145.55 (C-9), 140.98 (C-8), 139.15 (C-12), 137.67 (C-25/26), 137.50 (C-26/25), 135.16 (C-6), 124.92 (C-11), 107.12 (C-7), 96.41 (C-1), 95.83 (C-16/17), 85.62 (C-4), 77.27 (C-3/2), 76.68 (C-21), 75.65 (C-17/16), 70.45 (C-20), 69.14 (C-3/2), 60.54 (C-5), 45.85 (C-28), 44.93 (C-24/27), 42.73 (C-27/24), 40.10 (C-22), 30.60 (C-23), 29.86 (C-19), 17.14 (C-18). MS (ESI^+, 6 eV): calculated for $[M + Na]^+$ ($C_{25}H_{29}NO_5S_2Na^+$): 510.1379; found: *m*/*z* = 510.1373.



Synthesis and purification of ***8*** - 1'-(β-D-Ribofuranosyl)6*H*-thieno-[2,3-*c*]-4-[5-(5-norbornene-2-*exo*-methoxy)-pent-1-ynyl]pyridine-7-thione-5'-triphosphate



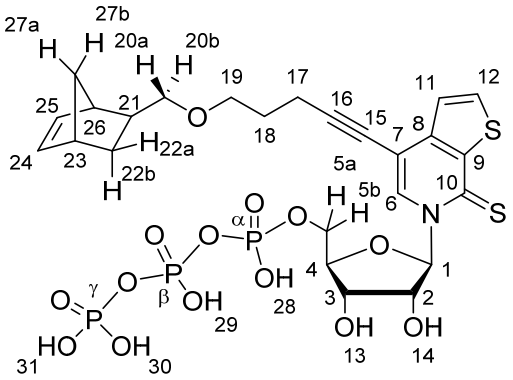
7 **8**

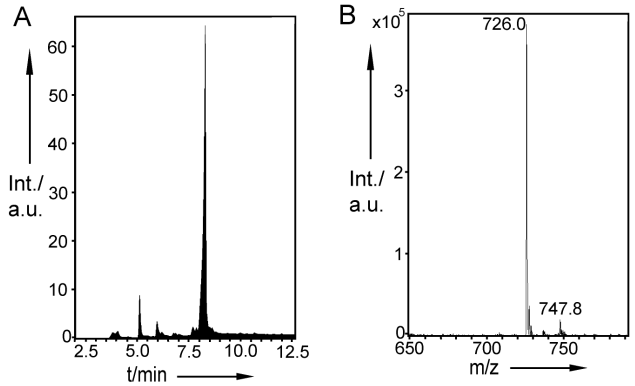
Nucleoside **7** (42 mg, 86 µmol, 1.0 eq.) and proton sponge (18 mg, 86 µmol, 1.0 eq.) were dried under high vacuum for 12 h. Under an atmosphere of argon, trimethyl phosphate (500 µL) was added and the solution was cooled in an ice bath. $POCl_3$ (freshly distilled and stored under argon, 20 µL, 215 µmol, 2.5 eq.) was added dropwise at 0 °C. The mixture was stirred for 6 h at 0 °C, subsequently tributylamine (225 µL, 946 µmol, 1.0 eq.) and a solution of bis-tributylammonium pyrophosphate in DMF (0.5 M, 946 µL, 473 µmol, 5.5 eq.) were added simultaneously. After 15 min of stirring under ice-cold condition, the reaction was quenched by the addition of TEAB (1.0 M, 6.3 mL). Extraction with EtOAc (20 mL) and evaporation of the aqueous layer at room temperature gave the crude product. The crude product was dissolved in approximately 16 mL water and transferred into centrifuge tubes. After the addition of sodium chloride solution (3.0 M, 1.8 mL) and absolute ethanol (approximately 54 mL) the tubes were shaken vigorously. The solution was cooled to -80 °C for one hour and afterwards centrifuged for 40 min at 3200 rpm. The supernatant was removed and after after freeze drying the crude product was yielded as a sodium salt pellet. These pellets can be stored at -20 °C before the final purification via HPLC using a gradient of 20 mM triethylammonium acetate (buffer A) and 20 mM ammonium acetate in 50 % acetonitrile (buffer B). After purification, 10.2 mg (14.1 µmol, 15 %) of **8** was yielded as yellow oil.

^{1}H-NMR (D_2O, 400 MHz, r.t.): *δ* [ppm] = 8.41 (s; 1H; H-6), 8.14 (d; $^{3}J_{H\text{-}12/H\text{-}11}$ = 5.3 Hz; 1H; H-12), 7.62 (d; $^{3}J_{H\text{-}11/H\text{-}12}$ = 5.3 Hz; 1H; H-11), 7.16 (d; $^{3}J_{H\text{-}1/H\text{-}2}$ = 2.1 Hz; 1H; H-1), 6.06 (dd; $^{3}J_{H\text{-}24/H\text{-}25}$ = 5.4 Hz; $^{3}J_{H\text{-}24/H\text{-}23}$ = 2.9 Hz; 1H; H-24/25), 5.99 (dd, $^{3}J_{H\text{-}25/H\text{-}24}$ = 5.5 Hz; $^{3}J_{H\text{-}25/H\text{-}26}$ = 2.6 Hz; 1H; H-25/24), 4.50 -4.40 (m; 5H; H-2, H-3, H-4, H-5a, H-5b), 3.82-3.70 (m; 2H; H-19), 3.49 (m; 1H; H-20a/20b), 3.37 (td; $^{2}J_{H\text{-}20b/H\text{-}20a}$ = 10.0 Hz; $^{3}J_{H\text{-}20b/H\text{-}21}$ = 1.7 Hz; 1H; H-20b/20a), 2.75 (s; 1H; H-26/23), 2.67 (t; $^{3}J_{H\text{-}17/H\text{-}18}$ = 6.8 Hz; 2H; H-17), 2.66 (s; 1H; H-23/26), 1.97 (p; $^{3}J_{H\text{-}18/H\text{-}17}$ = 6.6 Hz; $^{3}J_{H\text{-}18/H\text{-}19}$ = 6.6 Hz; 2H; H-18), 1.64-1.57 (m; 1H; H-21), 1.21-1.08 (m; 4H; H-27a, H-27b, H-22a, H-22b).

^{31}P-NMR (D_2O, 162 MHz, r.t.): *δ* [ppm] = -10.19 (d, $J_{P\text{-}\gamma/P\text{-}\beta}$ = 20.0 Hz, Pγ), -10.73 (d, $J_{P\text{-}\alpha/P\text{-}\beta}$ = 20.4 Hz, Pα), -22.55 (t, $J_{P\text{-}\beta/P\text{-}\alpha}$, $J_{P\text{-}\beta/P\text{-}\gamma}$ = 20.1 Hz, Pβ).

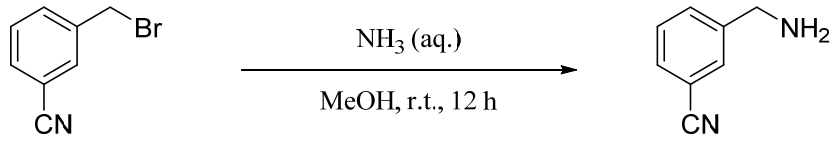
HR-MS (ESI$^-$, 5 eV): calculated for $[C_{25}H_{29}NNaO_{14}P_3S_2]^{2-}$: 373.5075; found: *m/z* = 373.5044.





*LC-MS data of triphosphate **8**. A*. HPL-chromatogram of **8** at 260 nm; B.ESI-MS spectrum of the peak eluting at a retention time of 8 min (ESI$^-$: calculated for $[C_{25}H_{31}NO_{14}P_3S_2]^-$: 726.0; found: *m*/*z* = 726.0.

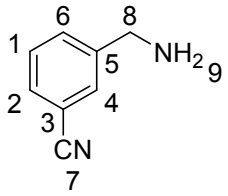
*Synthesis of **13** - 3-(Aminomethyl)benzonitrile.*



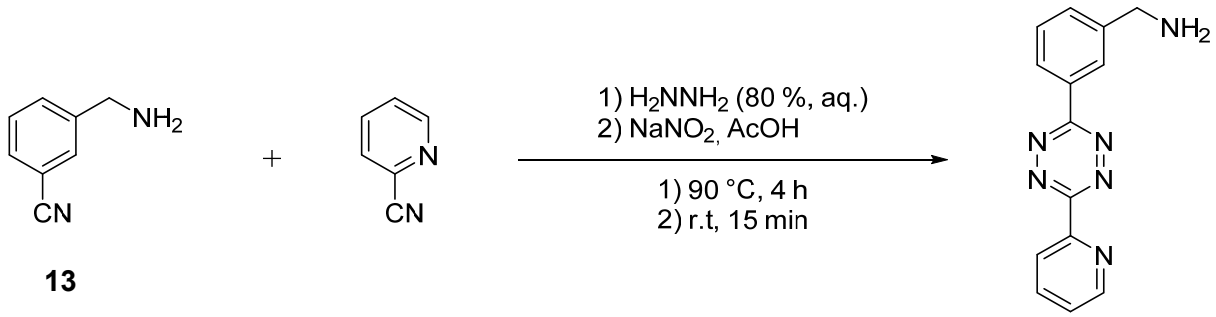
12 **13**

3-(Bromomethyl)benzonitrile **12** (2.10 g, 10.7 mmol) was solved in methanol (20 mL) and concentrated ammonia (40 mL) was added. After stirring over night at room temperature the solvent was evaporated and the crude product was washed twice with dichloromethane. The residue was dried in vacuo to afford a colourless solid (SiO_2, MeOH/DCM, 1/7, *v/v*, Rf = 0.3) of **13** (1.01 g, 71 % yield).

^{1}H-NMR (DMSO-d_6, 400 MHz, r.t.): δ [ppm] = 8.37 (br s; 2H; H-9), 7.98 (s; 1H; H-4), 7.85 (m; 2H; H-1, H-2), 7.66 (d; $^2J_{H\text{-}6/H\text{-}1}$ = 7.9 Hz; 1H; H-6), 4.13 (s; 2H; H-8). ^{13}C-NMR (DMSO-d_6, 75 MHz, r.t.): *δ* [ppm] = 135.6 (C-5), 134.1 (C-6), 132.8 (C-4), 132.2 (C-2), 129.9 (C-1), 118.5 (C-7), 111.4 (C-3), 41.4 (C-8). EI-MS (70 eV, m/z) $[M]^+$·calcd for $C_8H_8N_2$: 132.1, found 132.1.



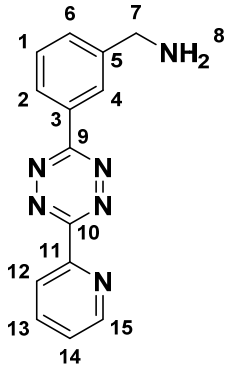
*Synthesis of **14** – (3-(6-(Pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)phenyl)methanamine.*



13 **14**

2-Pyridinecarbonitrile (790 mg, 7.58 mmol, 1.0 eq) and **13** (1.00 g, 7.57 mmol, 1.0 eq) were solved in hydrazine (80 %, 5.0 mL) and stirred for four hours at 90 °C. After cooling down to room temperature, the orange precipitate was filtered off, washed with ice-cold water and dried *in vacuo* and afterwards solved in glacial acetic acid (6.0 mL). As oxidizing agent, sodium nitrite (522 mg, 7.57 mmol, 1.0 eq) was added and the reaction mixture was stirred for 15 minutes at room temperature. The solvent was evaporated under reduced pressure

and the oxidized crude product dried *in vacuo*. Purification via column chromatography (SiO_2, CH_2Cl_2/MeOH, 85/15, *v*/*v*, R_f = 0.2) gave **14** (14.0 mg, 0.05 mmol, 7 %) as a pink solid.
^{1}H-NMR (DMSO-d_6, 400 MHz, r.t.): δ [ppm] = 8.93 (d, $^3J_{H\text{-}15/H\text{-}14}$ = 4.0 Hz, 1H, H-15), 8.63 (s, 1H, H-4), 8.58 (d, $^3J_{H\text{-}12/H\text{-}13}$ = 7.8 Hz, 1H, H-12), 8.47 (d, $^3J_{H\text{-}2/H\text{-}1}$ = 7.8 Hz, 1H, H-2), 8.16 (td, $^3J_{H\text{-}13/H\text{-}14}$ = $^3J_{H\text{-}13/H12}$ = 7.8 Hz, $^4J_{H\text{-}13/H\text{-}15}$ = 1.7 Hz, 1H, H-13), 7.78 – 7.66 (m, 3H, H-1, H-6, H-14), 4.02 (s, 2H, H-7). ^{13}C-NMR (DMSO-d_6, 75 MHz, r.t.): *δ* [ppm] = 163.5 (C-9, C-10), 163.3 (C-9, C10), 150.6 (C-15), 150.2 (C-11), 141.8 (C-3), 137.8 (C-13), 132.4 (C-14/5), 131.7 (C-5/14), 129.5 (C-1), 127.2 (C-4), 126.6 (C-2), 126.6 (C-6), 124.0 (C-12), 44.1 (C-7).
HR-MS (ESI$^+$, 8 eV): calculated for $[C_{14}H_{13}N_6]^+$: 265.1096; found: *m/z* = 265.1201.



*General procedure for the synthesis of tetrazine-fluorophore conjugates **9-11***

The tetrazine, in general 0.1 mg and Oregon Green® 488 carboxylic acid succinimidyl ester, 5-isomer (0.13 mg, 0.25 µmol) or biotin *N*-hydroxysuccinimide ester (0.09 mg, 0.25 µmol) were dissolved in 50 µL dimethylsulfoxide (anhydrous) and 1.50 µl triethylamine. The solution was stirred in the dark at room temperature. The reaction progress was monitored by LC-MS. The product was purified by reverse phase HPLC using a gradient from 15 % to 80 % acetonitrile (HPLC grade) in water (MilliQ). The identity and purity of the conjugates were confirmed by LC-MS (Figure S1)
The 'turn-on' fluorescence of tetrazine-fluorophore conjugate **11** was determined on reaction with 5-norbornene-2-methanol in H_2O using 5 µM tetrazine, 100 fold excess of 5-norbornene-2-methanol and 40 min reaction time.

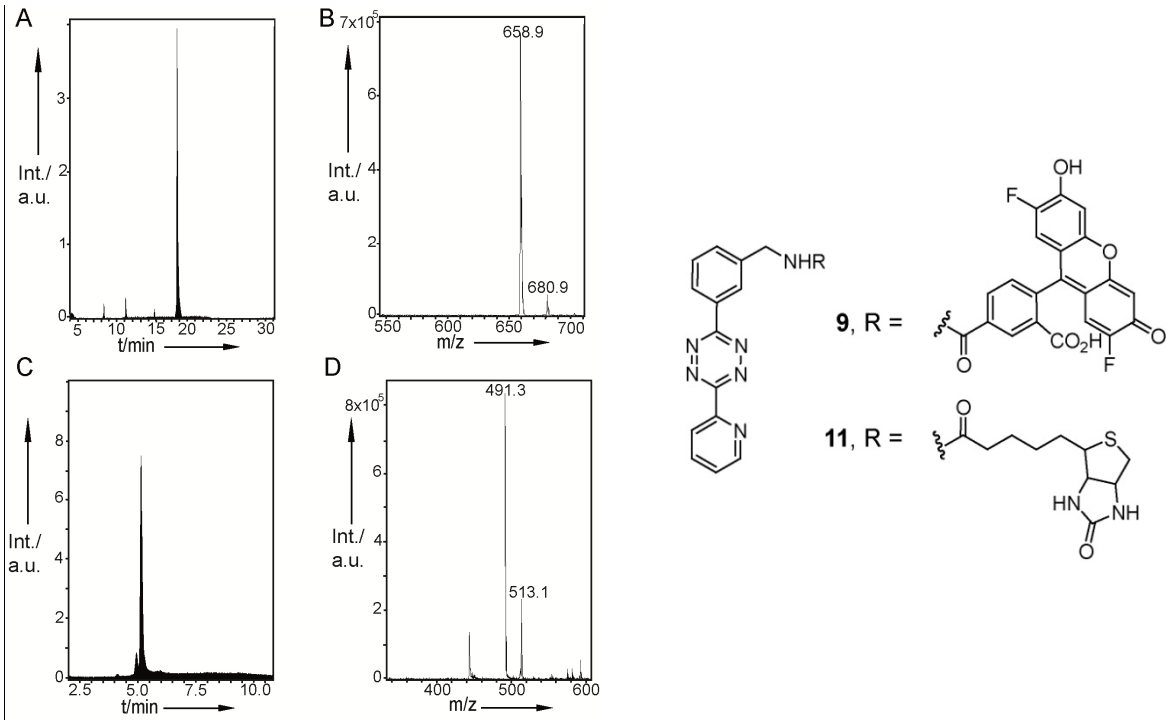


Figure S3. LC-MS data of the synthesized tetrazine-fluorophore conjugates **9** and **11** . a) left panel: HPL-chromatogram of purified conjugate **9** at 260nm; right panel: ESI-MS spectrum of the peak eluting at a retention time of 19 min (m/z_{calcd} $[M+H]^+$ = 659.1, $[M+Na]^+$ = 681.1); b) left panel: HPL-chromatogram of purified conjugate **11** at 260nm; right panel: ESI-MS spectrum of the peak eluting at a retention time of 5.5 min (m/z $[M+H]^+$ = 491.2, $[M+Na]^+$ = 513.2).

Biochemical Experiments

Preparation of DNA templates for T7 transcription

The dNAM phosphoramidite suitably protected for oligonucleotide synthesis was purchased from *Berry & Associates Inc.*, USA. Solid phase synthesis and purification of unmodified oligonucleotides and modified oligonucleotides DNA-1N and DNA-2N was performed in 200 nmol scale by *Ella Biotech*, Germany.

List of DNA primers and templates

A. *Primer for T7 transcription*

TK-1: 5'-TAA TAC GAC TCA CTA TA-3'
TK-2:
5'-TAA TAC GAC TCA CTA TAG GAG ATC TTC CAC GAG GAT TCC CGT CAC AG-3'

B. *Template strands containing X = dNAM*

DNA-1N:
5'-CTG TGA CGG GAA TCC TCG TGG A**X**G ATC TCC TAT AGT GAG TCG TAT TA-3'
DNA-2N:
5'-CTG TG**X**CGG GAA TCC TCG TGG A**X**G ATC TCC TAT AGT GAG TCG TAT TA-3'

C. *Unmodified template control strand*

DNA-0N:
5'-CTG TGA CGG GAA TCC TCG TGG ACG ATC TCC TAT AGT GAG TCG TAT TA-3'

D. *Primers for reverse transcription*

RT-1: 5'-CTG TGA CGG GAA TCC-3'
RT-2: 5'-CTG TGA CGG GAA TCC TCG TGG A-3'

General procedure for T7 transcription and RNA purification.

In vitro RNA transcription was performed on a 100 µl scale with a final concentration of 25 mM $MgCl_2$, 40 mM Tris-HCl (pH = 7.9), and 3 µM DNA primer and template. Primer and template were annealed in $MgCl_2$ and Tris-HCl by slow cooling from 95°C to 37°C. A final concentration of 2.5 mM natural NTPs, 0 or 1 mM triphosphate **8**, 5 mM DTT, 1.24 µl RNAsin (40 U/µl, *Promega*), inorganic pyrophosphatase (2 U/µl, *New England Biolabs*) and 5 µl T7-RNA polymerase (20 U/µl, *homemade*) were added to the annealed template construct. The transcription reaction was incubated at 37 °C for 5 hours. For DNase I digestion, 5 µl DNase I (2 U/µl, *New England Biolabs*) were added and incubated at 37°C for 30 min.

Purification of RNA transcripts was performed using denaturing gel electrophoresis. Oligonucleotides were visualized by UV shadowing after gel electrophoresis, excised from the gel, eluted using an electroelution chamber (*selfmade*) and precipitated from ethanol. Purity and identity were confirmed by reverse-phase HPLC-ESI MS using 10 mM triethylamine/100 mM hexafluoroisopropanol as buffer A and acetonitrile as eluent (buffer B).

Table S1. *ESI-MS data of transcribed oligonucleotides RNA-Nor1, RNA-Nor2, RNA-C. 5'-OH, mono, di- and triphosphate RNA strands are detected*

RNA	sequence	$m/z_{calculated}$	$m/z_{measured}$
RNA-Nor1	5'-GGA GAU C(Nor-UB)U CCA CGA GGA UUC CCG UCA CAG-3'	5'-OH: 9830.8 Mono-P: 9910.8 Di-P: 9990.8 Tri-P: 10070.8	5'-OH: 9831.5 Mono-P: 9911.0 Di-P: 9990.8 Tri-P: 10070.0
RNA-Nor2	5'-GGA GAU C(Nor-UB)U CCA CGA GGA UUC CCG (Nor-UB)CA CAG-3'	5'-OH: 10073.9 Mono-P: 10153.8 Di-P: 10233.8 Tri-P: 10313.8	5'-OH: 10074.9 Mono-P: 10155.1 Di-P: 10235.1 Tri-P 10314.4
RNA-C	5'-GGA GAU CGU CCA CGA GGA UUC CCG UCA CAG-3'	Mono-P: 9706.9 Di-P: 9786.8 Tri-P:9866.8	Mono-P: 9706.7 Di-P: 9786.7 Tri-P: 9866.2

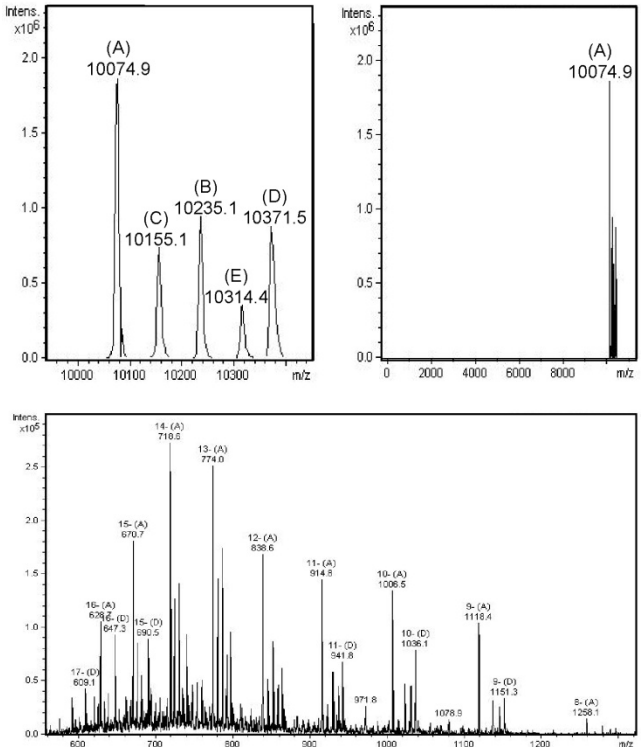


Figure S1. *ESI-MS data of transcript RNA-Nor2. Upper image: deconvoluted spectrum. Lower image: raw spectrum with assigned peaks. 5'-OH, mono, di- and triphosphate RNA strands are detected (*m/z_{calcd}*: 5'-OH: 10073.9, Mono-P: 10153.8, Di-P: 10233.8, Tri-P: 10313.8).*

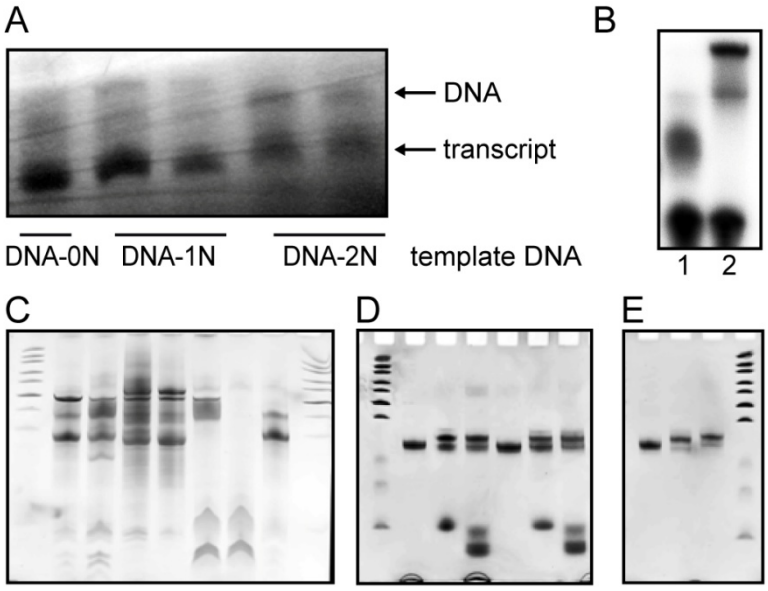


Figure S2. *Preparative gel electrophoresis for purification of transcribed RNA and complete PAA-gels depicted in Fig. 1 and 2.* A. Preparative denaturing 20 % PAA-gel electrophoresis for purification of transcribed RNA-Nor1, RNA-Nor2 and RNA-C. The RNA was visualized by UV-shadowing. B-D. B. Reverse Transcription reaction using RNA-Nor1 (lane 1) and RNA-C (lane 2) as template and a 15mer DNA primer. The reverse transcription reaction was stopped after 30 min. C-E. Complete gels depicted in Figure 1C (C), Figure 2C (D) and Figure 2 D (E).

Reverse transcription of RNA-Nor1

1.5 pmol DNA Primer RT-1 was labeled with [γ-^{32}P]-ATP (*PerkinElmer*) and T4 polynucleotide kinase (*New England Biolabs*) and annealed to 10 pmol RNA-Nor1 in annealing buffer (50 mM HEPES pH 7.0 and 100 mM KCl) by slow cooling from 95°C to 42°C. An extension buffer was added to a final concentration of 130mM Tris pH 8.0, 10 mM $MgCl_2$ and 10 mM DTT containing 0.75 mM dNTPs and 1 µl AMV reverse Transcriptase (10 U/µl, *Promega*). The reaction was incubated at 42°C for 45 min. The transcriptase was heat inactivated at 70°C for 10 min. Subsequently, 1 µl RNase H was added and incubated at 37°C for 20 min followed by heat inactivation at 70°C for 10 min. The reaction was fractionated by denaturing 20% PAGE and analyzed using a *Phosphorimager FLA-3000* (*Fujifilm*).

*General method for the cycloaddition reaction of tetrazines **9-11** with transcribed RNA*

20 pmol of RNA-Nor1, RNA-Nor2 or RNA-C in H_2O were incubated with a threefold excess of the corresponding tetrazine-fluorophore conjugate **9**, **10** or **11** for 1 h at room temperature. The reaction was directly analyzed by denaturing 20% PAGE. For ESI-MS analysis, the

crude product was desalted and the excess of the tetrazine-fluorophore conjugates was removed using *ZipTip C18* pipette tips (*Merck Millipore*).

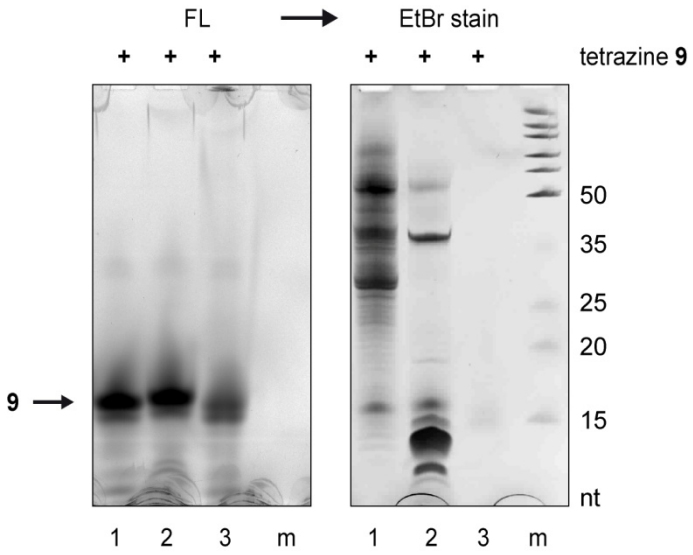


Figure S4. *PAGE analysis of unpurified control transcription reactions (5 µl) incubated with 60 pmol tetrazine* ***9****.* The left image shows the fluorescence scan of the gel (excitation 473 nm, emission 520 nm). The right image shows the same gel after staining with ethidium bromide. Lane 1: transcription reaction using DNA-0N as template (RNA-C product); Lane 2: transcription reaction with template DNA-1N without adding unnatural triphosphate 8. No full length RNA product is detected; Lane 3: tetrazine **9**.

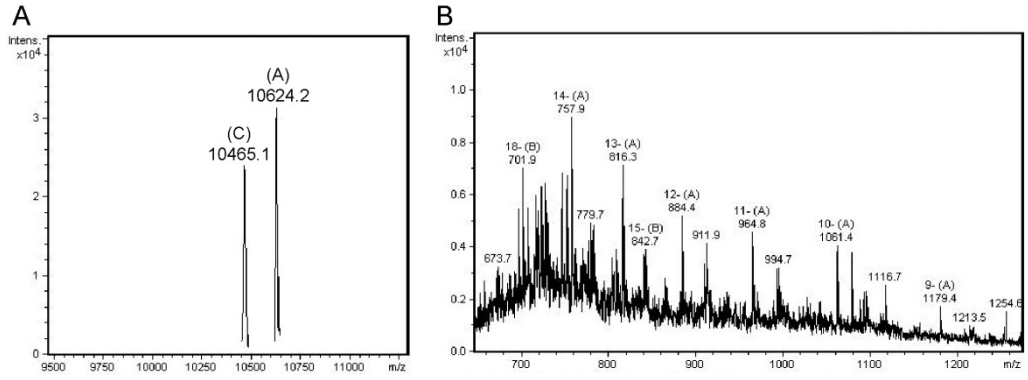


Figure S5. *ESI-MS data of transcript RNA-Nor1 after reaction with tetrazine* ***9****.* A. Deconvoluted spectrum: 5'-OH and diphosphate RNA strands are detected (m/z_{calcd}: 5'-OH-click: 10461.0, Di-P-click: 10630.9). B. raw spectrum with assigned peaks.

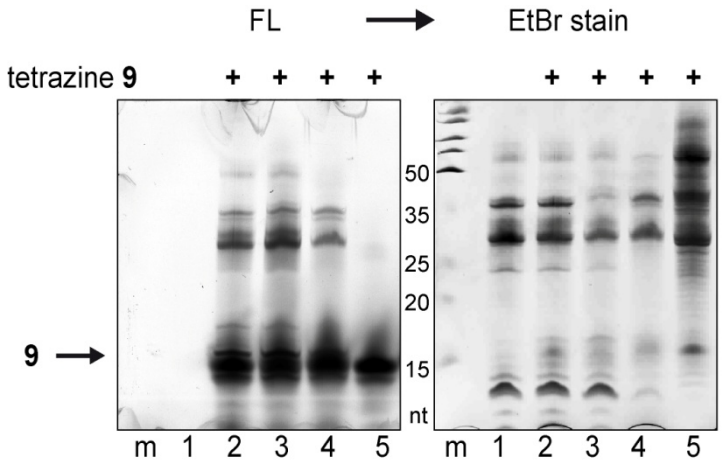


Figure S6. *Denaturing PAGE analysis of the iEDDA reaction of unpurified RNA-Nor1 transcripts with tetrazine* ***9****. For this, 5 µl of the transcription reaction were incubated with 60 pmol tetrazine conjugate* ***9****.* The left image shows the fluorescence scan of the gel (excitation 473 nm, emission 520 nm). The right image shows the same gel after staining with ethidium bromide. Lane 1: transcription reaction DNA-2N as template, **8**, no tetrazine **9** added; Lane 2: DNA-2N template, **8**, RNA-Nor2 product, **9**. The fluorescent click products are clearly visible. Two distinct fluorescent bands are detected. Most likely these bands arise from secondary structures in the RNA transcript which could not denatured completely. In contrast, only one band is detected during preparative gel purification of the transcript (Fig. S2) and we cannot observe this structure for purified RNA transcripts; Lane 3: DNA-2N template, **8**, RNA-Nor2 product, **9** after DNase I digestion; Lane 4: Lane 2: DNA-1N template, **8**, RNA-Nor1 product, **9**; Lane 5: DNA-0N template, RNA-C product, **9**.

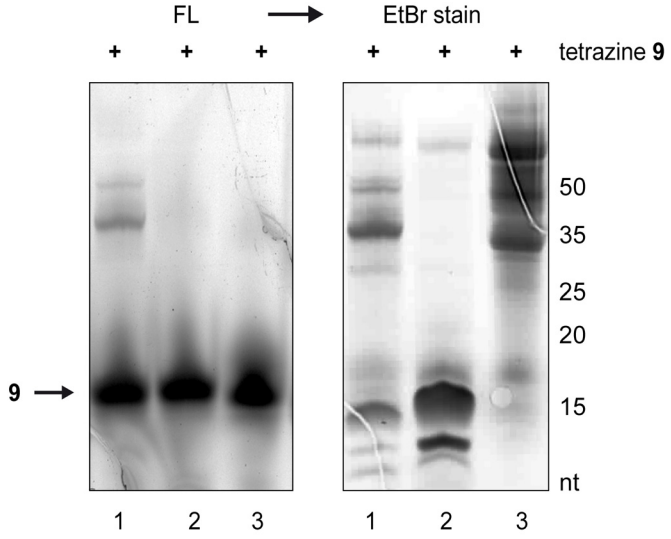


Figure S7. *Denaturing PAGE analysis of the iEDDA reaction of unpurified transcripts with tetrazine* ***9****.* The left image shows the fluorescence scan of the gel (excitation 473 nm, emission 520 nm). The right image shows the same gel after staining with ethidium bromide. Lane 1: transcription reaction using DNA-2N as template, **8**, RNA-Nor2 product, **9**; Lane 2:

DNA-2N template, **9**, no unnatural triphosphate **8** added; Lane 3: DNA-0N template, triphosphate **8** added, RNA-C product, **9**. No fluorescent band is detected in lane 3 confirming that triphosphate **8** is not unspecifically incorporated into transcripts.

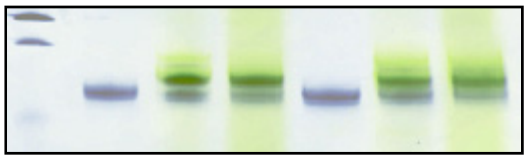
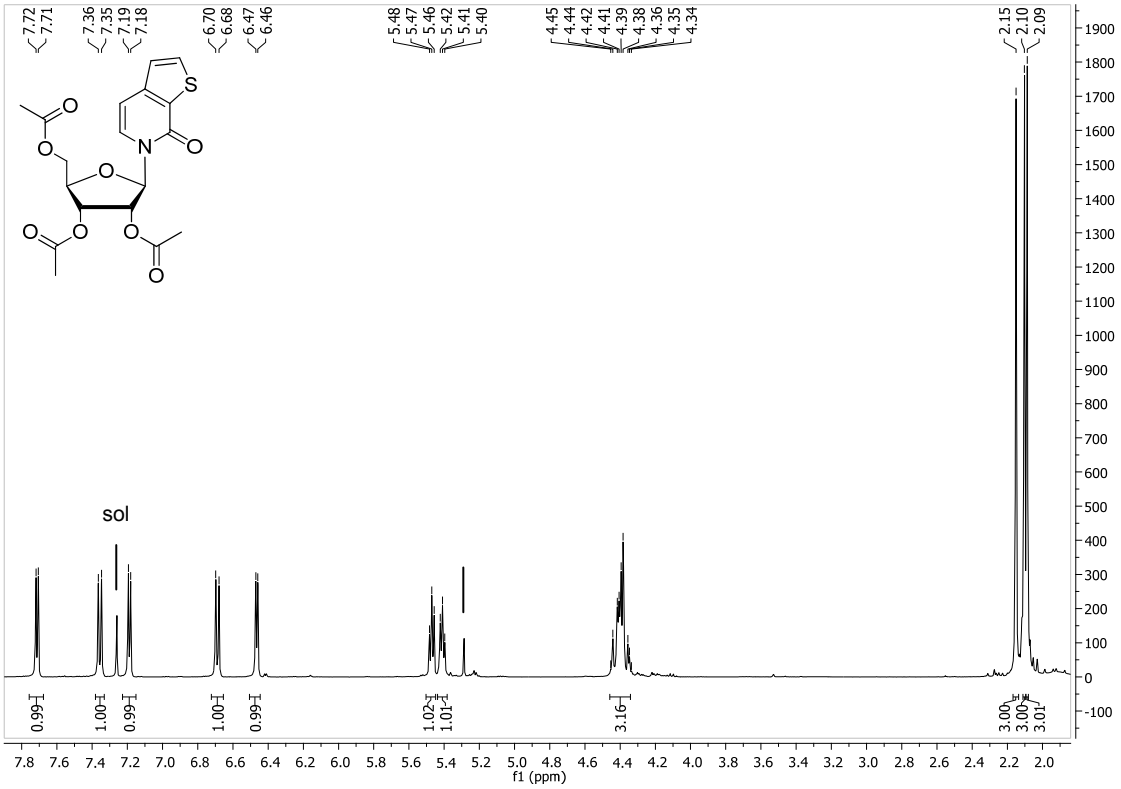


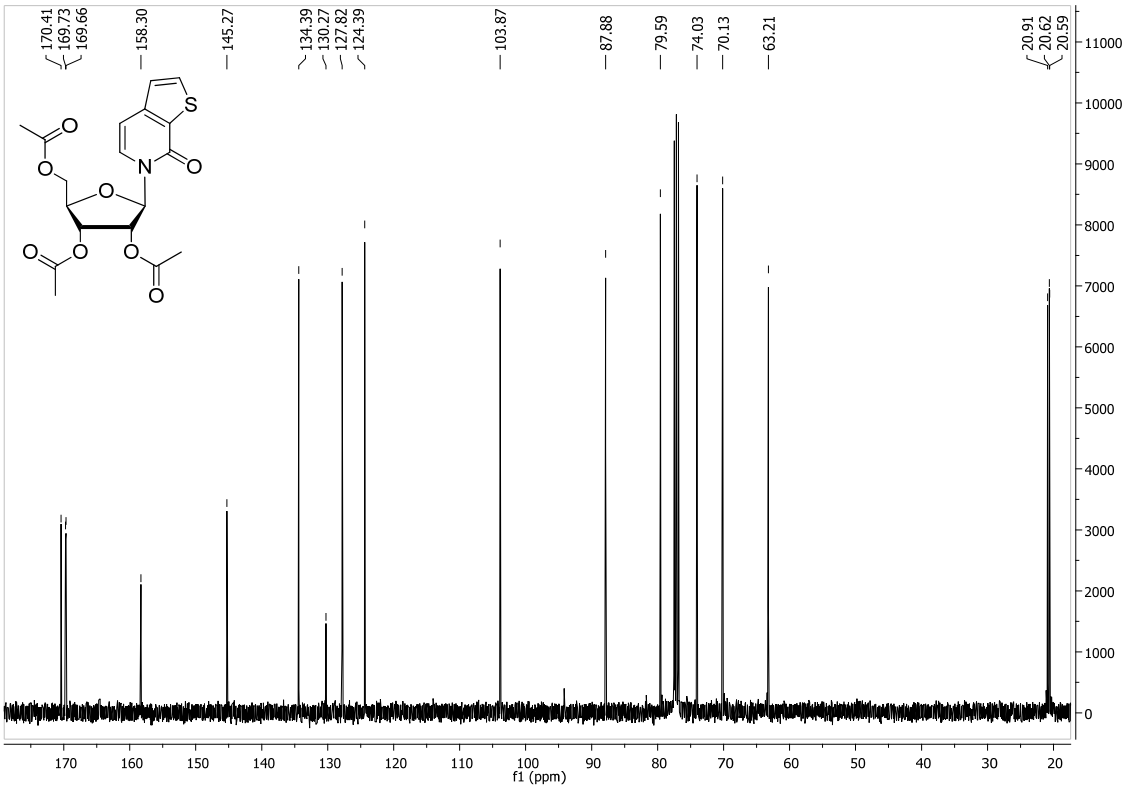
Figure S8. *Overlay of the gel depicted in Fig 2B.* The fluorescence scan is coloured in yellow, the EtBr stain is depicted in blue. Overlaid bands appear green.

Spectra

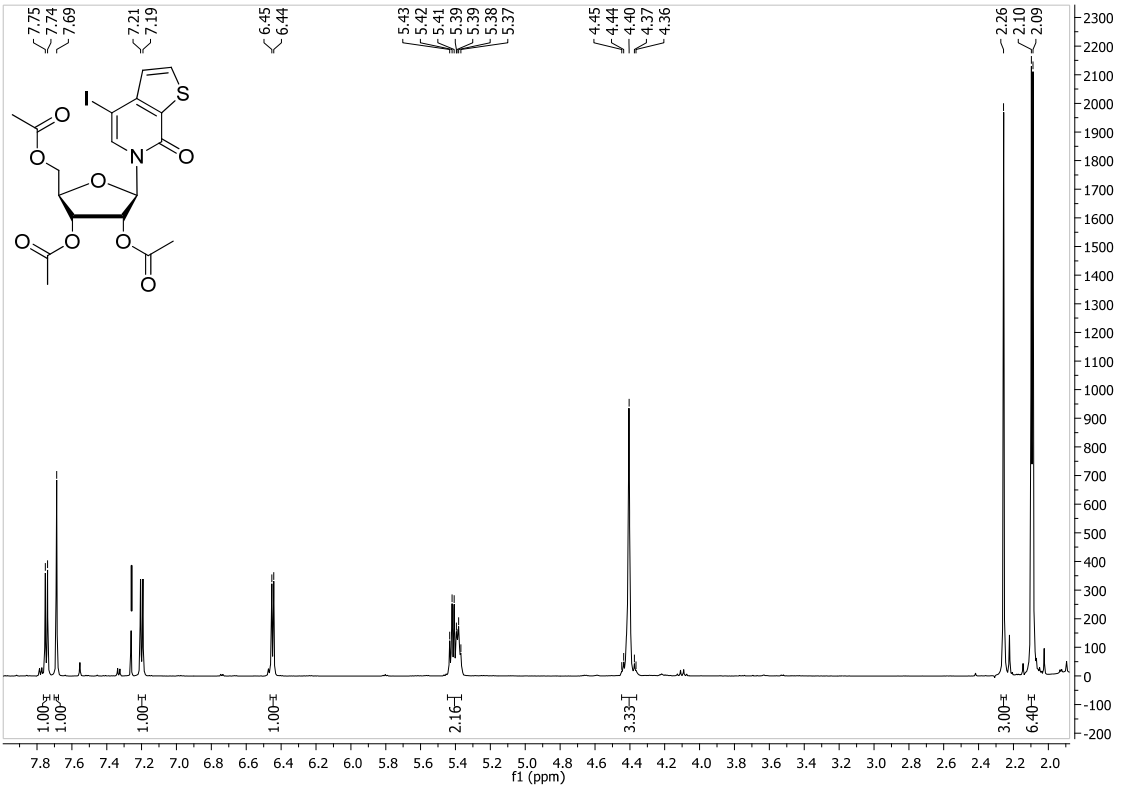
^{1}H-NMR spectrum of compound **2**.



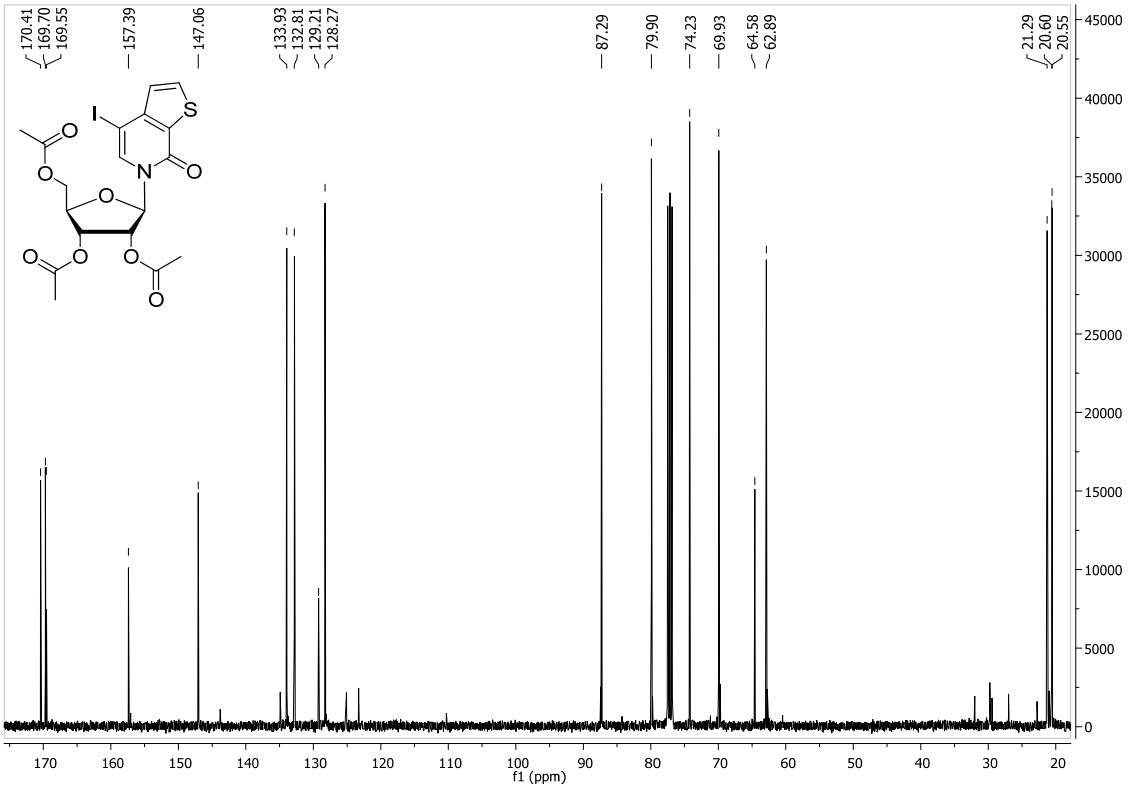
^{13}C-NMR spectrum of compound **2**.



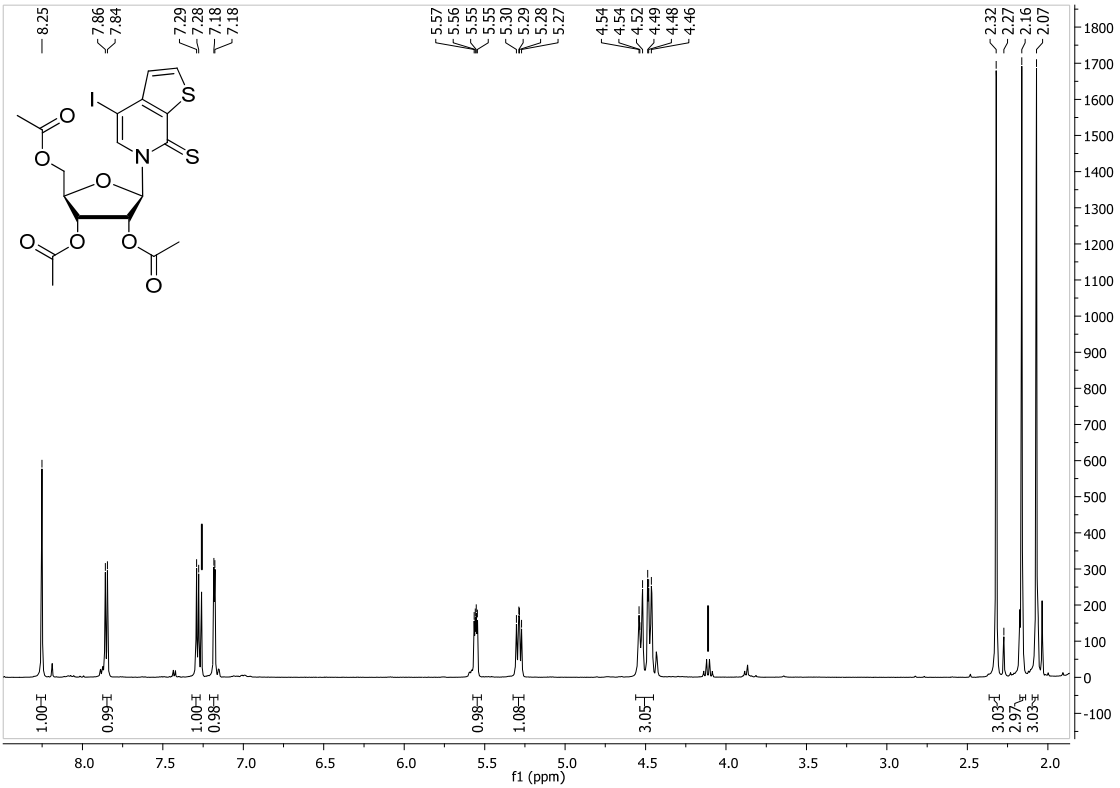
^{1}H-NMR spectrum of compound **3**.



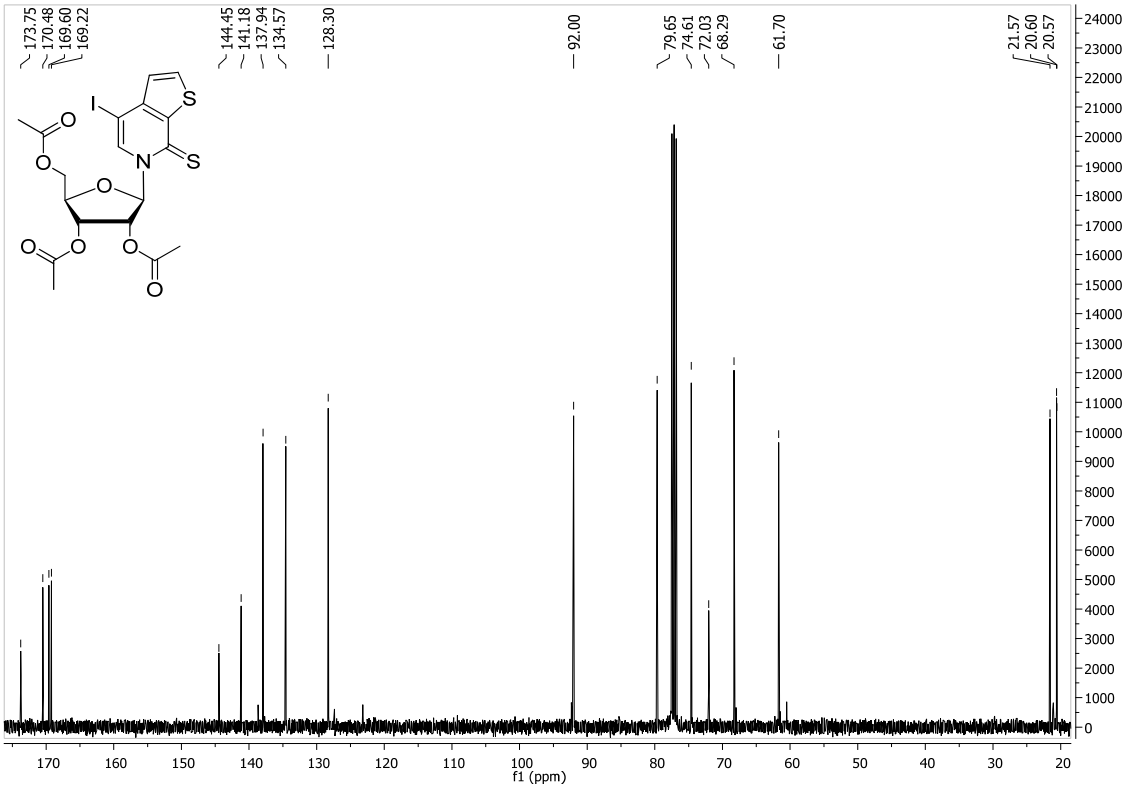
^{13}C-NMR spectrum of compound **3**.



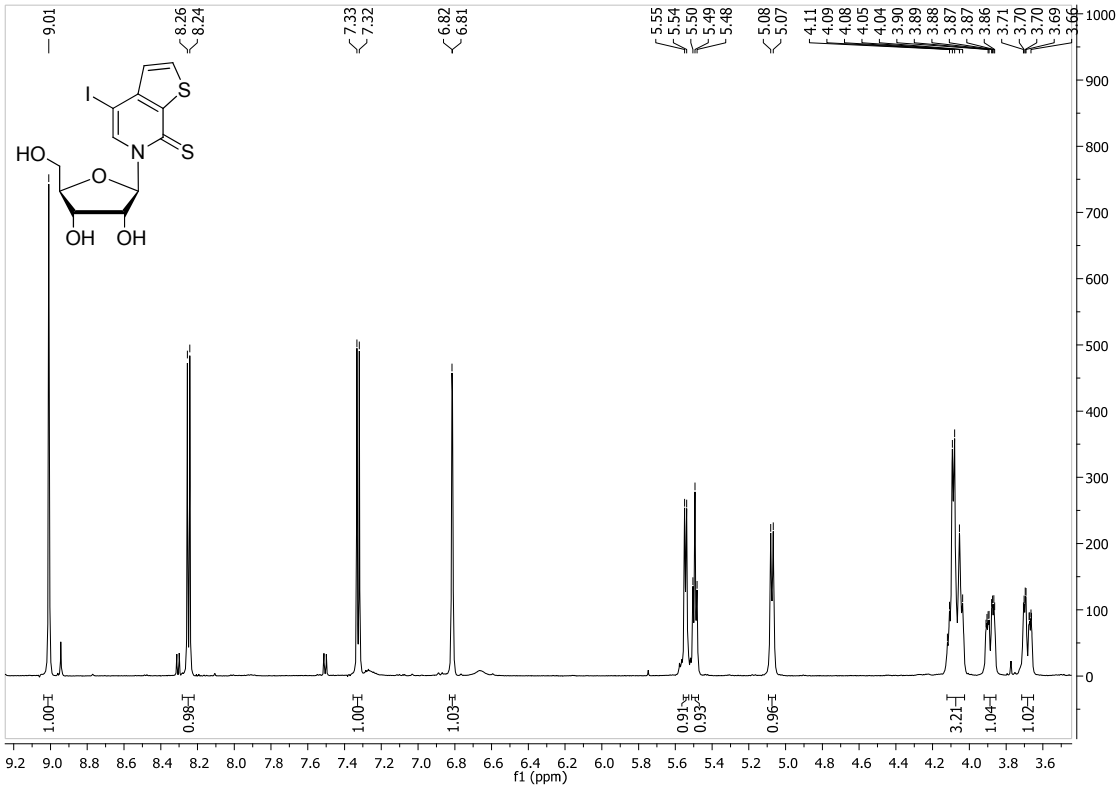
^{1}H-NMR spectrum of compound **4**.



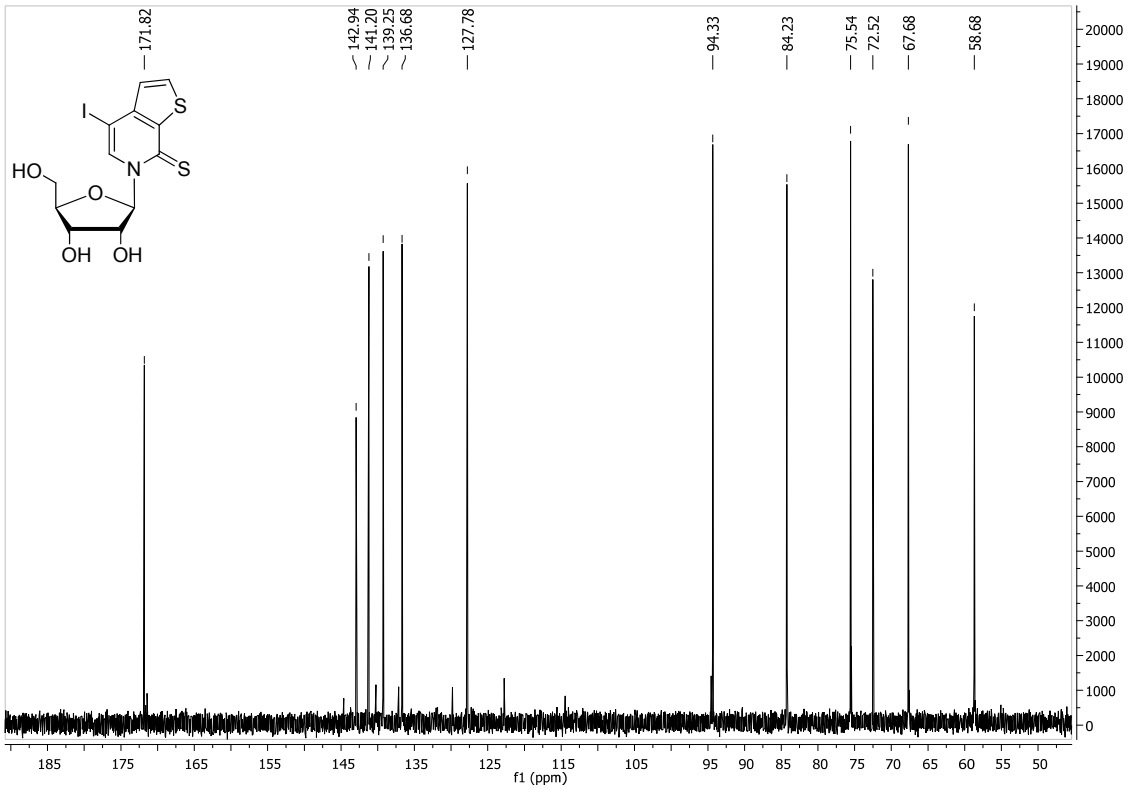
^{13}C-NMR spectrum of compound **4**.



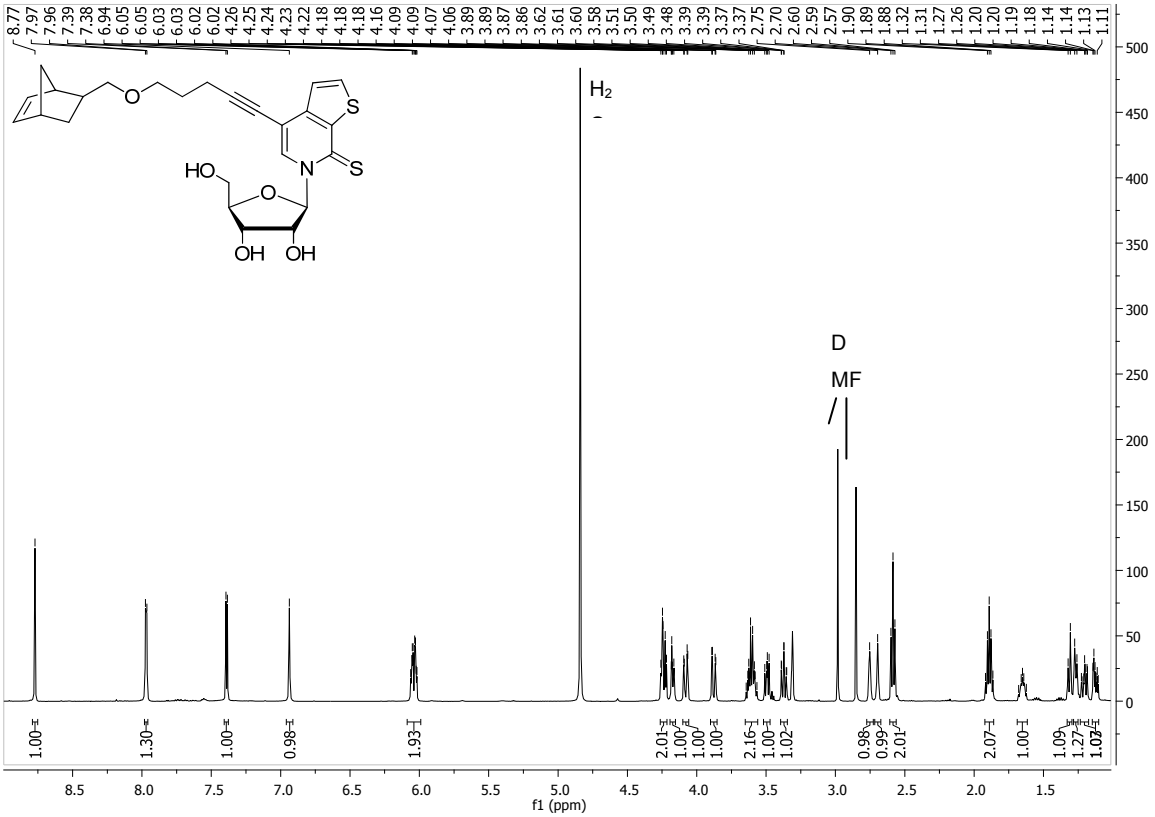
^{1}H-NMR spectrum of compound **5**.



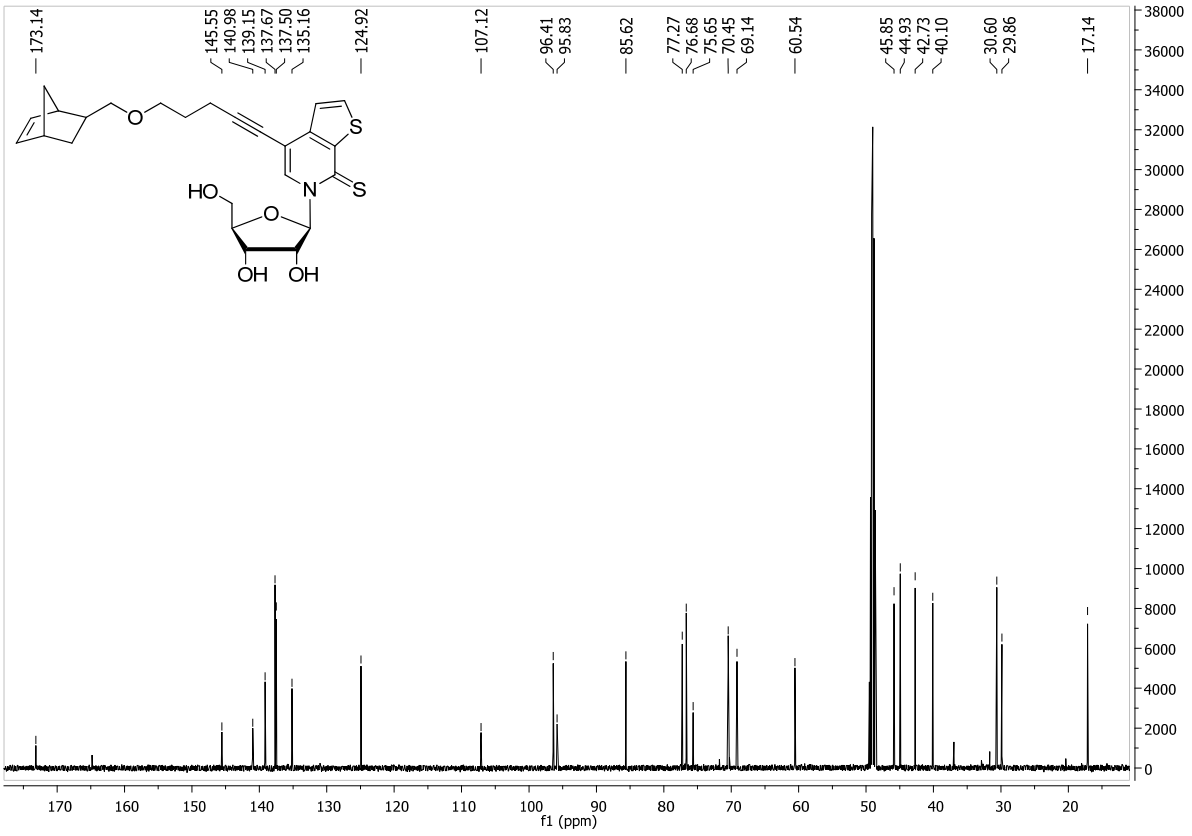
^{13}C-NMR spectrum of compound **5**.



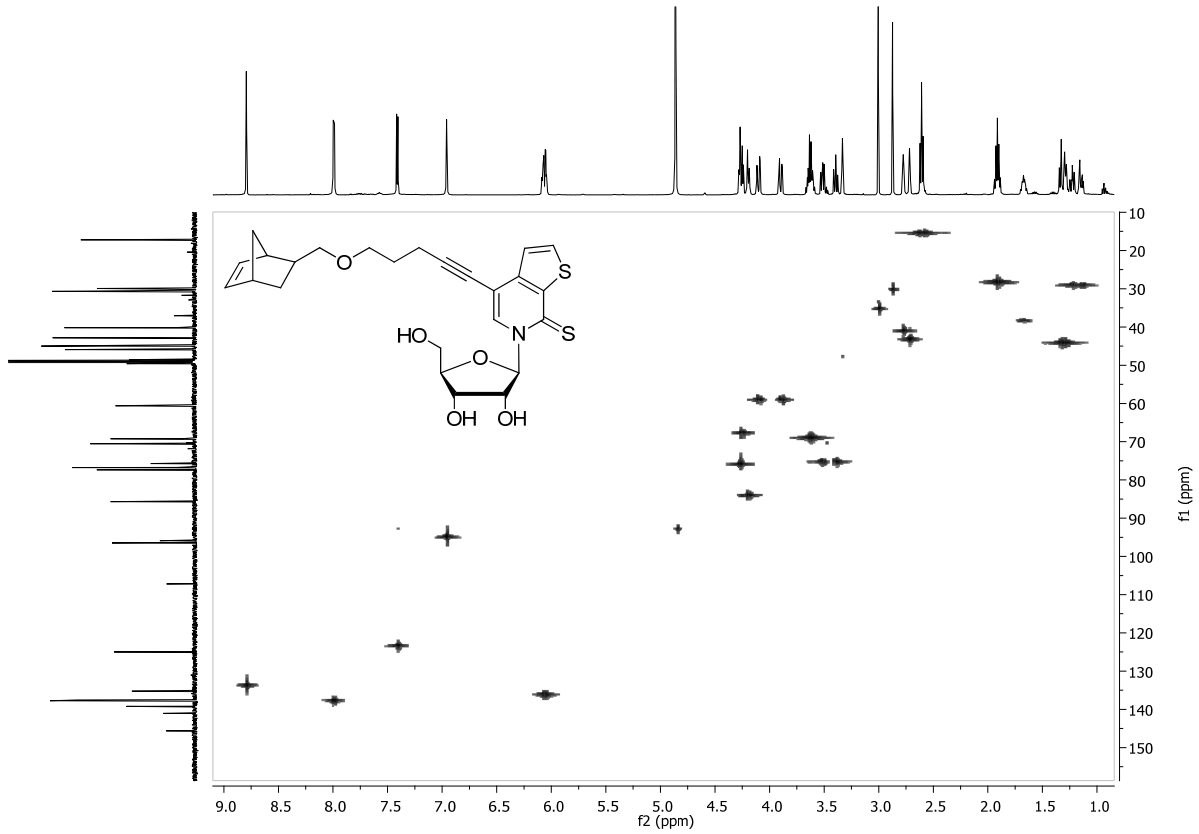
^{1}H-NMR spectrum of compound **7**.



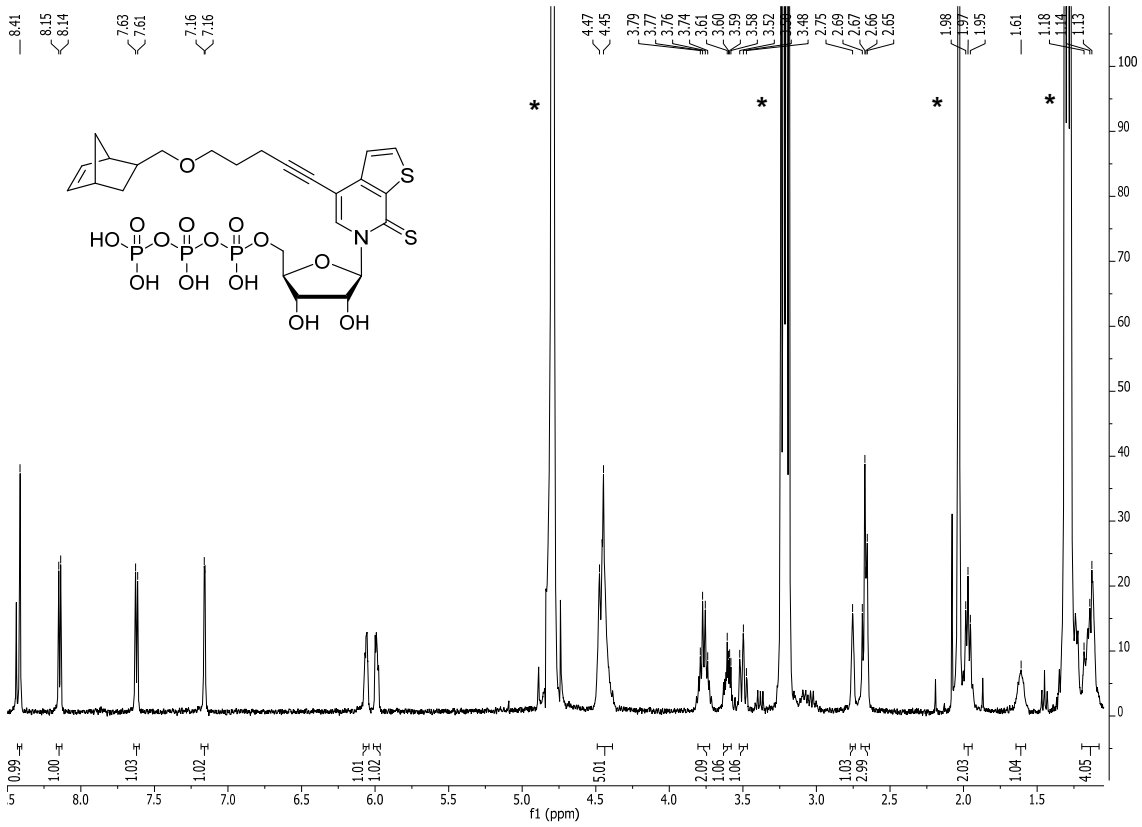
^{13}C-NMR spectrum of compound **7**.



HMQC spectrum of compound **7**.

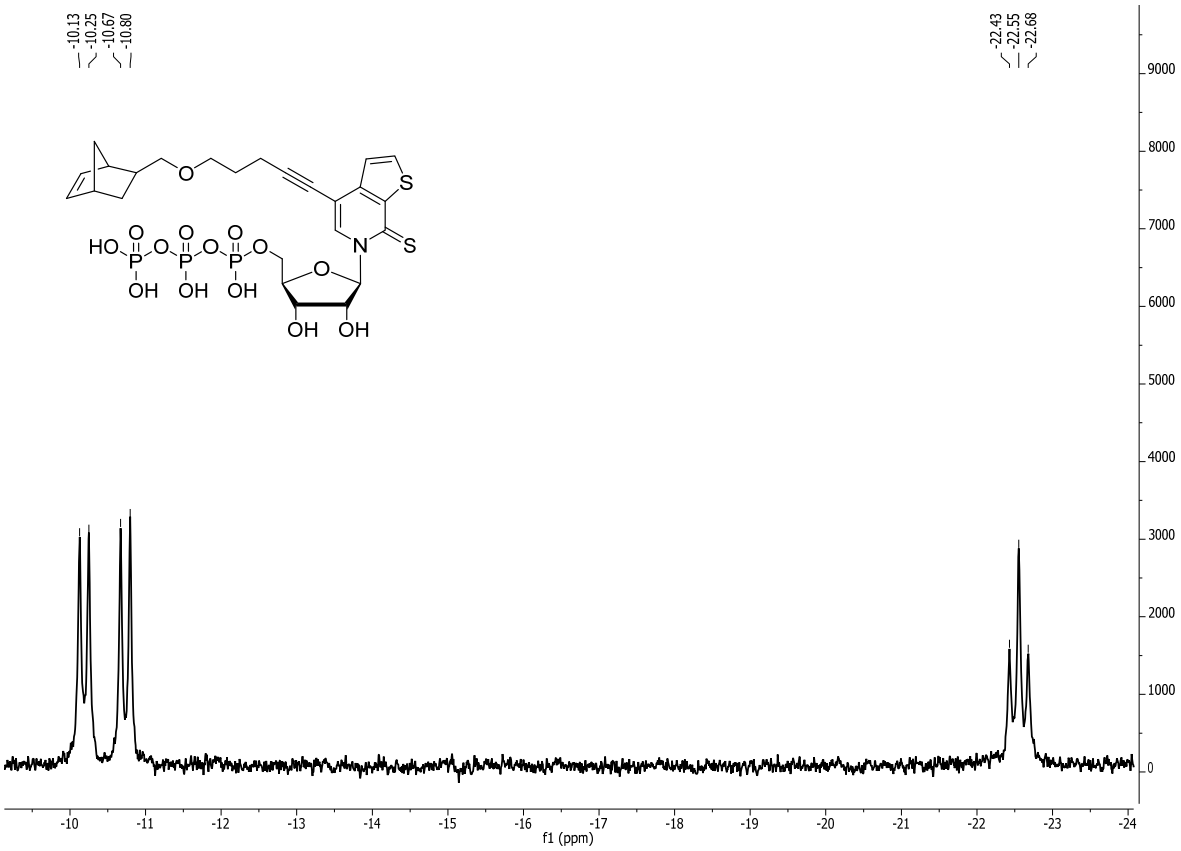


^{1}H-NMR spectrum of compound **8**.

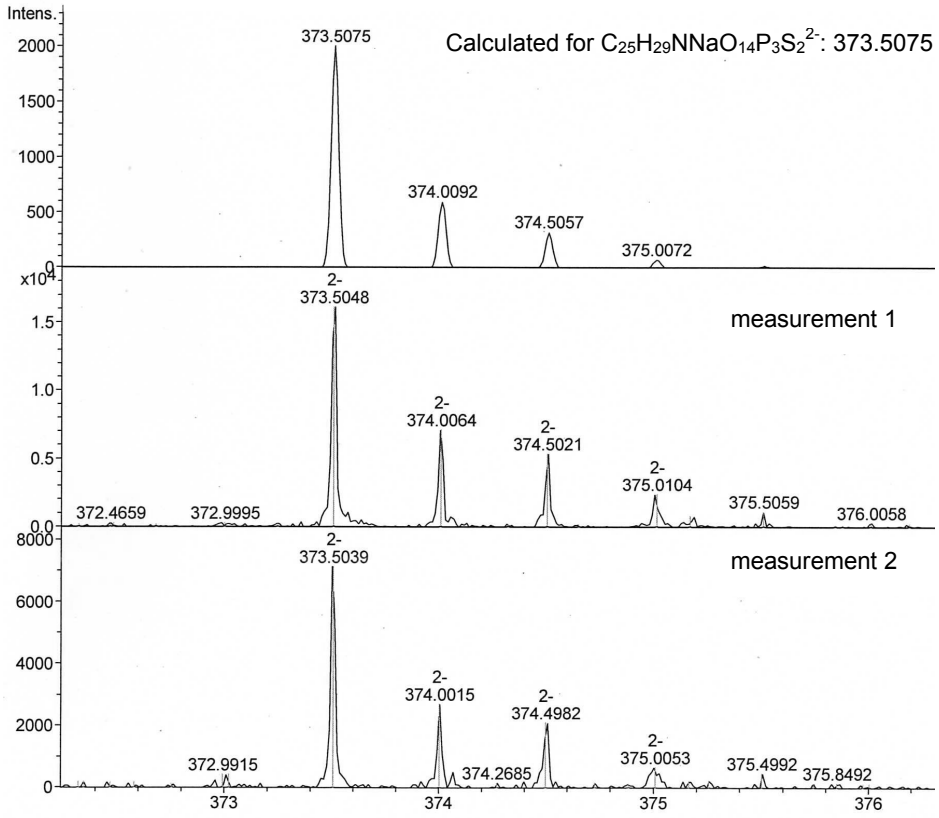


The spectrum contains the marked signals (*) of triethylammonium acetate used in the HPLC purification

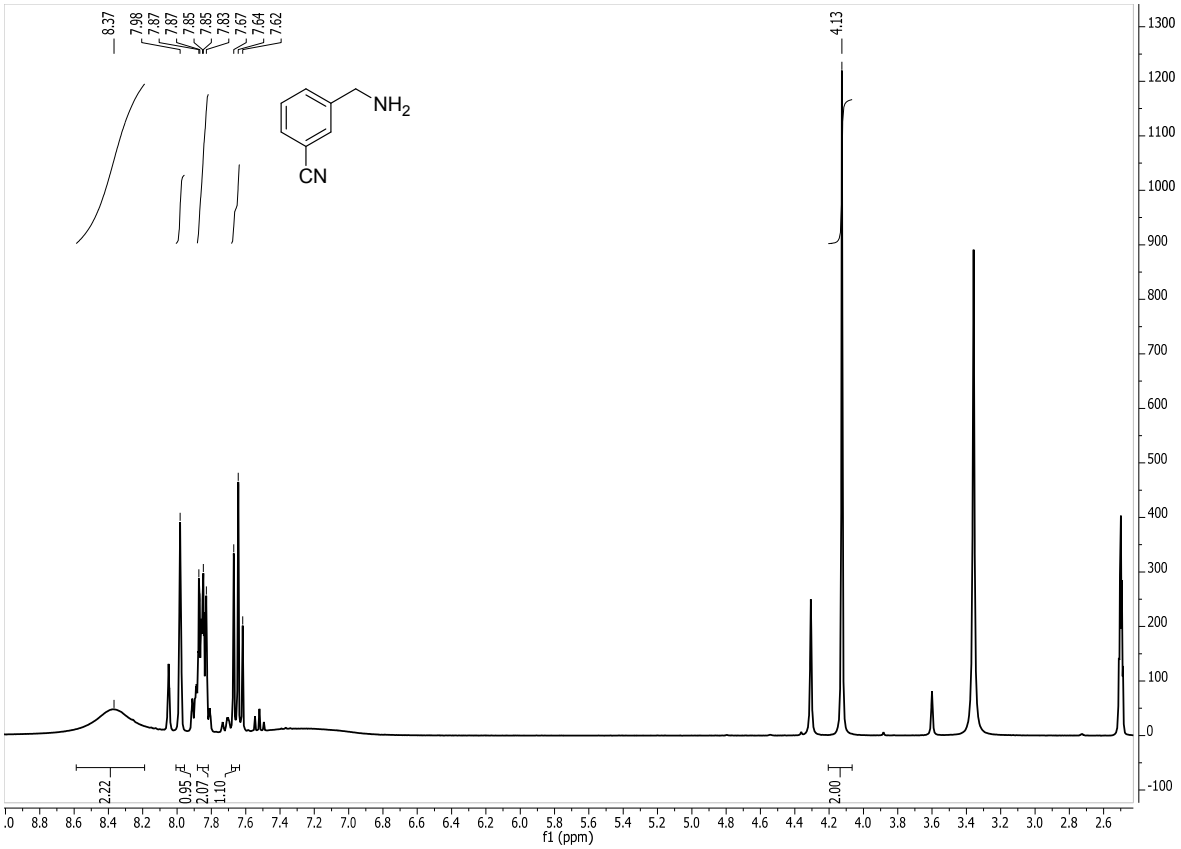
^{31}P-NMR spectrum of compound **8**.



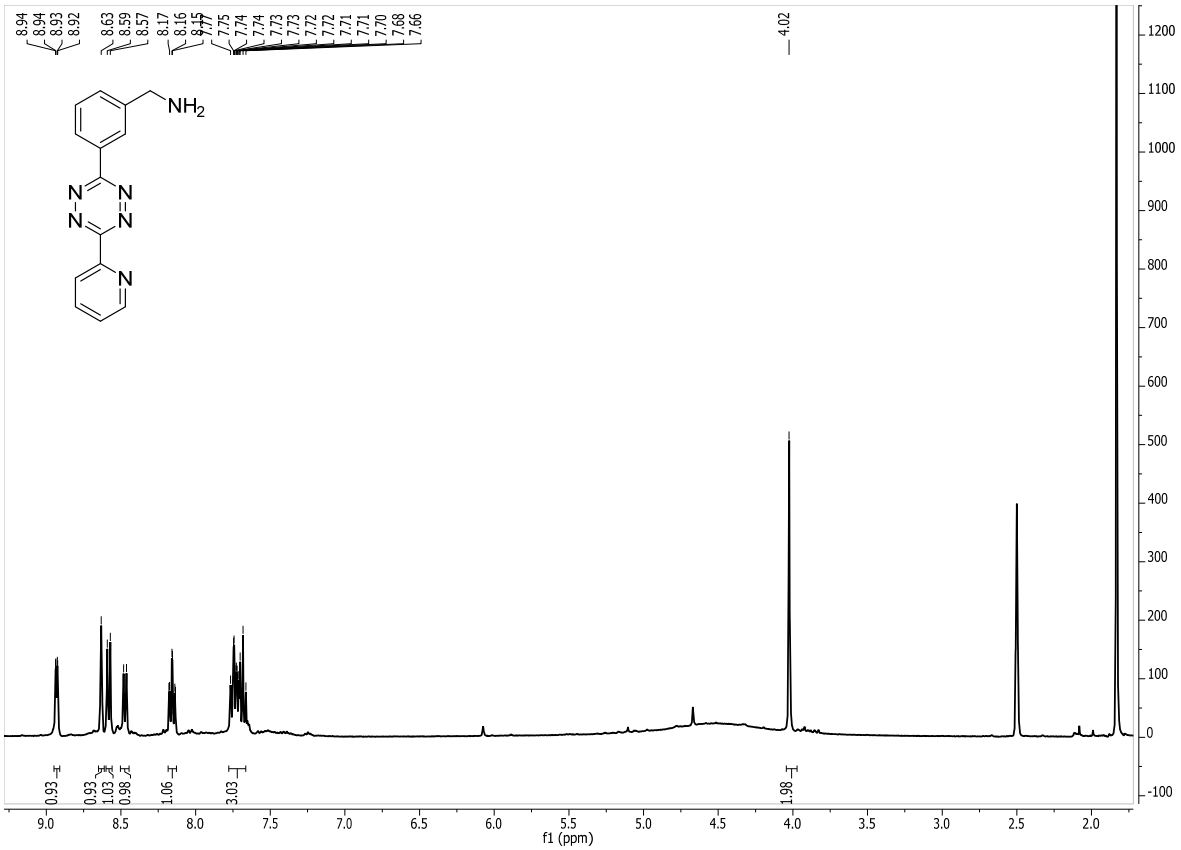
HR-MS of compound **8**.



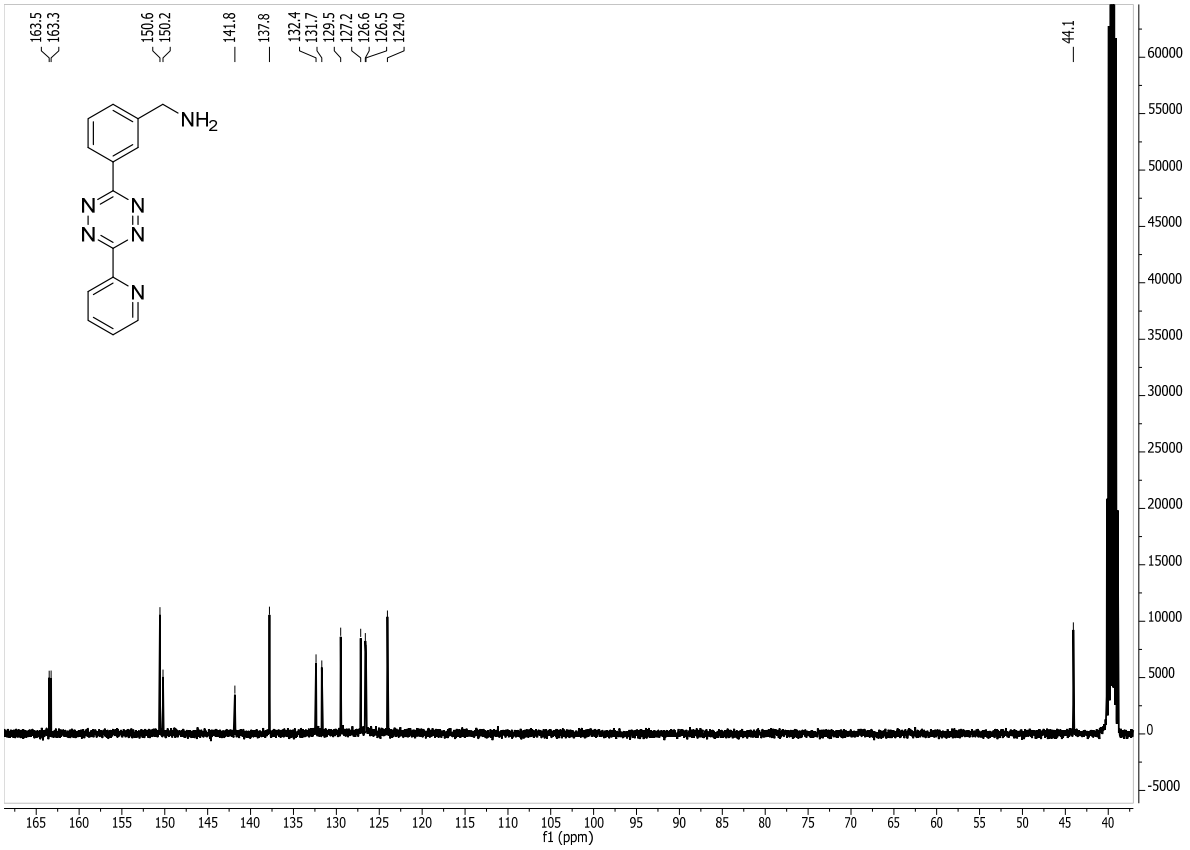
^{1}H-NMR spectrum of compound **13**.



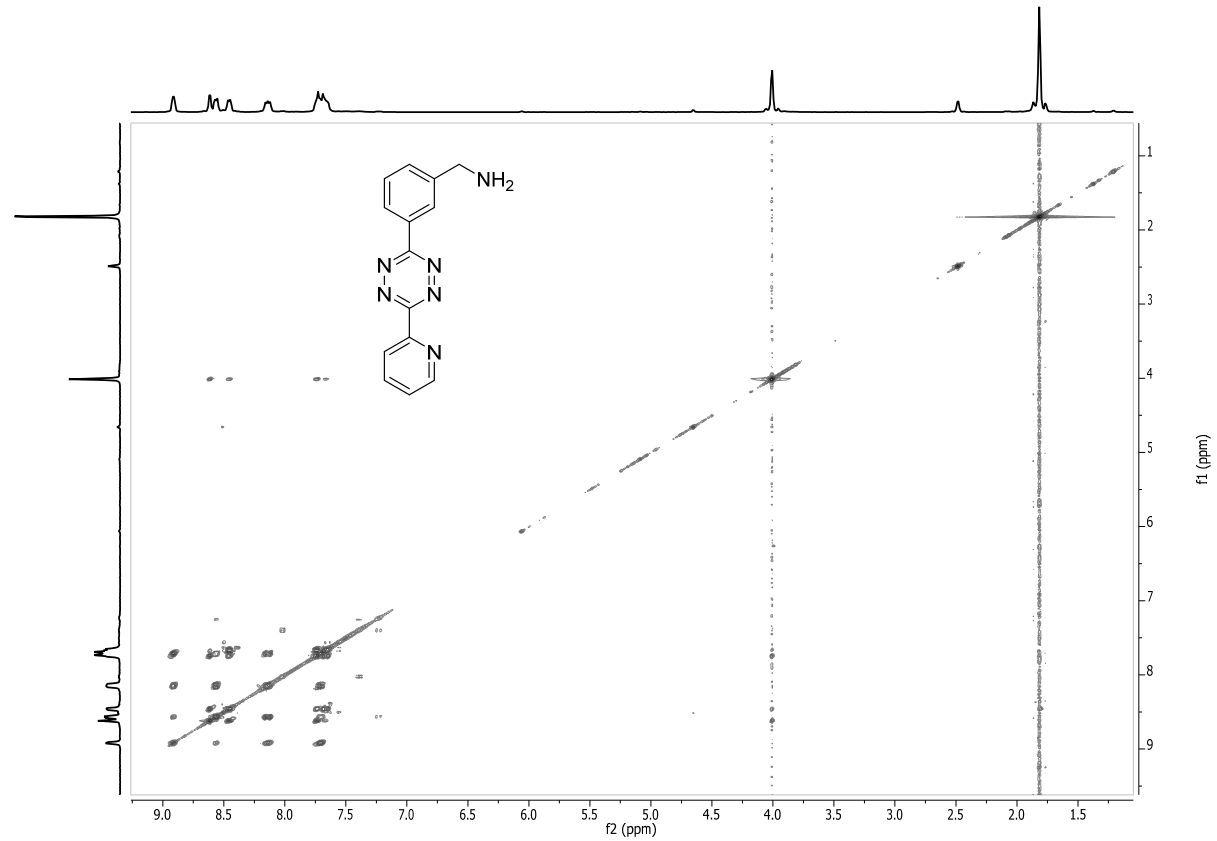
^{1}H-NMR spectrum of compound **14**.



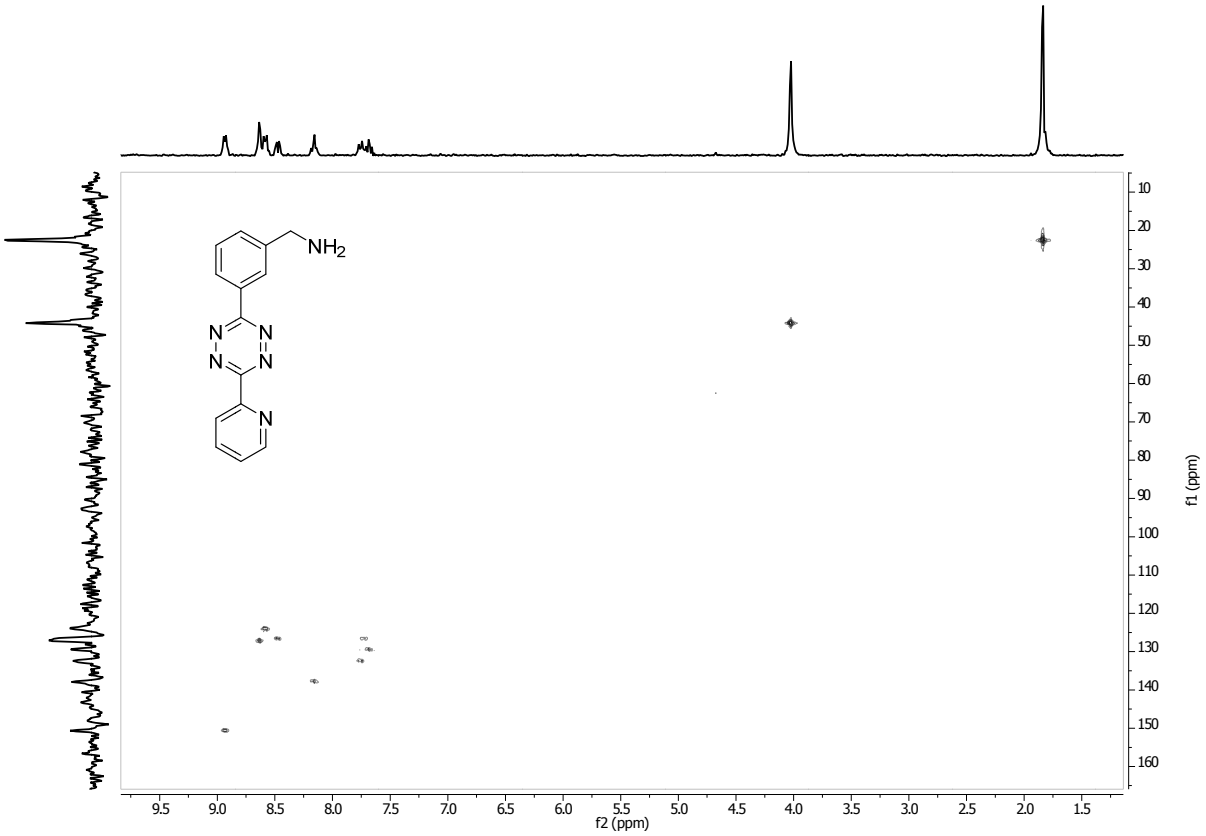
^{13}C-NMR spectrum of compound **14**.



H,H-COSY spectrum of compound **14**.



HMQC spectrum of compound **14**.



HRMS of compound **14**.