Copper carbenes alkylate guanine chemoselectively through a substrate directed reaction

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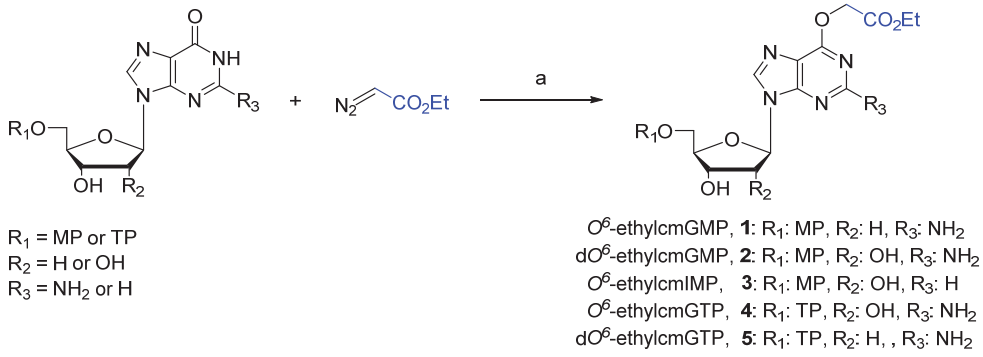
1 Synthesis

1.1 General Experimental Information

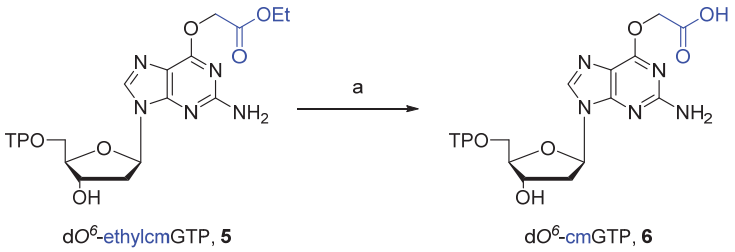
All reagents and solvents used were of analytical grade. Buffers were prepared with ultrapure water. All chemicals were purchased from Sigma-Aldrich, Alfa Aesar or Acros and used as received. Unlabeled dNTPs were obtained from Invitrogen and [γ-^{32}P]ATP was purchased from PerkinElmer Life Sciences. KTqM747K DNA polymerase was purchased from myPOLS Biotec. Oligonucleotides used in primer extension experiments were obtained from VbC Biotech. Solid-phase oligonucleotide synthesis was carried out on an Expedite 8909 nucleic acid synthesis system (PerCeptiveBiosystems) on 1µmol CPG columns using standard phosphoramidite chemistry with 0.3 M 5-benzylthio-1-H-tetrazole as activator. Purification was carried out on a preparative Shimadzu UFLC system with a Gemini-NX 5 µm C18 21.2 x 250 mm (Phenomenex) column using 100 mM triethylammonium acetate (TEAA pH 7.25)/acetonitrile (MeCN) gradients as mobile phase. Elution was carried out at a flow rate of 20 mL/min monitored at 254 nm using **Method A:** 0-15 % MeCN in 30 min, 15-95 % MeCN in 10 min, 95 % MeCN for 5 min for short ssDNA and **Method B**: 0-12% MeCN in 25 min, 12-16 % MeCN in 10 min, 16-95 % MeCN in 5 min, 95 % MeCN for 5 min for longer ssDNA. Alkylation and de-alkylation reactions were analyzed on an analytical Shimadzu UFLC system or Agilent 1100 LC system with an Eclipse XDB-C8 4.6 x 150 mm (Agilent) column using 100 mM TEAA/MeCN gradient. Elution was carried out at a flow rate of 1 mL/min using **Method C**: 0-16 % MeCN in 18 min, 16-80 % MeCN in 5 min, 80 % MeCN for 3 min with peak detection at 254 nm. Purification was carried out on the preperative Shimadzu UFLC system as described above using **Method D**: 0-16 % MeCN in 20 min, 16-20 % MeCN in 5 min, 20-95 % in 1 min, 95 % MeCN for 5 min. Aqueous product fractions were freeze dried on a Christ Alpha 2-4 LDplus flask lyophilizer at 0.1 mbar. ^{1}H, ^{13}C and ^{31}P-NMR, HMQC and HMBC spectra were acquired on a BrukerAvance (400, 500 or 600 MHz proton frequency) spectrometer at 298.15 K. Chemical shifts relative to TMS were referenced to the solvent's residual peak and are reported in ppm. ESI MS spectra were measured on a Bruker Esquire3000plus mass spectrometer by direct injection in positive polarity of the ion trap detector.

Polyacrylamide gel electrophoresis (PAGE): PAGE was done in 12.5 % gel (29:1 w/w acrylamide/bisacrylamide) in 1×TBE (89 mMTris-borate, 2 mM EDTA) with Orange G, Bromophenol blue and XylenCynol FF as tracer dyes. The electrophoretic samples were prepared by mixing 5 μL of sample with 2.5 μL 4×TBE in 40 % (v/v) glycerol. Five microliters from the resulting mixtures were then applied on the gel without further pre-treatment. Trace dye mixtures were applied on separate wells. The gels were run at 30 V/cm until the Orange G migrates to approximately 1 cm from the end of the gel. The gels were then washed briefly with deionized water and soaked with Coomassie Brilliant Blue R-250 for 1 h, destained and visualized using Bio-RadChemiDoc MP system equipped with Image Lab 5.0 software.

1.2 Synthetic Schemes



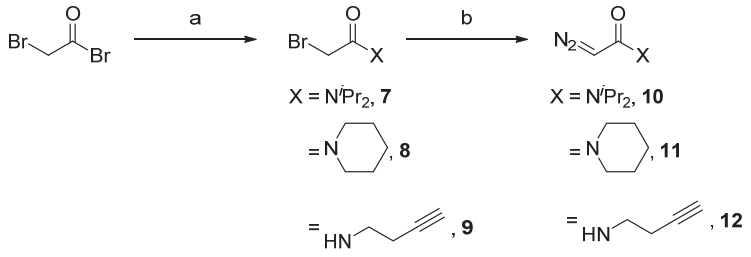
Scheme S1: Synthesis of O^6 modified GMP **1**, dGMP **2**, IMP **3**, dGTP **4** and GTP **5** using Cu(I) carbene chemistry. a) 20 mol% Cu(I), MES buffer pH 6, H_2O, 25°C, 1 h, **1**: 92 %, **2**: 86 %, **3**: 81 %, **4**: 73 %, **5**: 65 %.



Scheme S2: Synthesis of dO^6-cmGTP **6**. a) LiOH, 1 h, 25°C, 57 %.

Synthesis of Diazoacetamides (DAA)

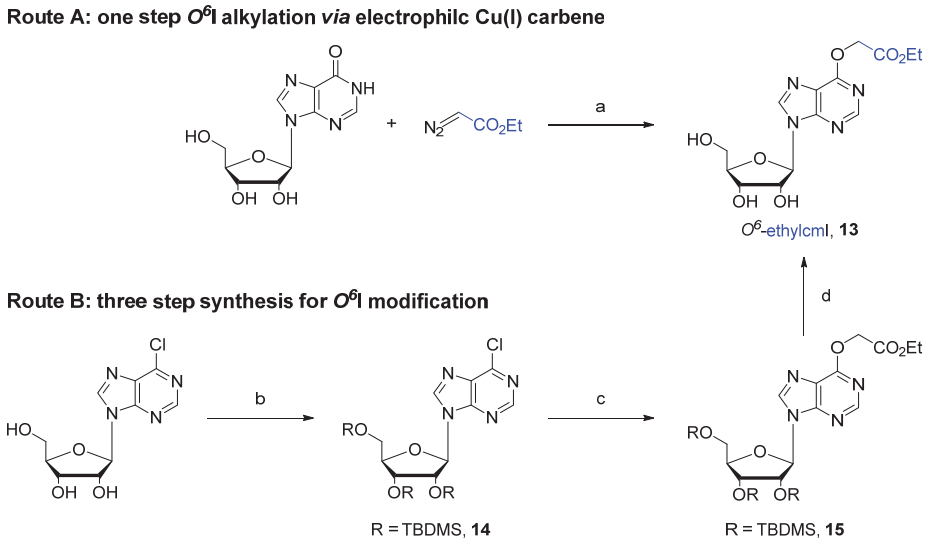
Both DAAs were synthesized according to an adapted procedure by Fukuyama *et al.*[1] Reaction of commercial bromoacetyl bromide (BrAcBr) with the secondary amines (piperidine or *N,N*-diisopropylamine) and treatment with *N,N*-ditosylhydrazine[1] in the presence of DBU yielded the final DAAs (Scheme S3).



Scheme S3: Synthesis of DAAs. a) *N,N*-diisopropylamine/piperidine, CH_2Cl_2, -60°C to 25°C, 30 min, **7**: 31%, **8**: 52%, **9**: 75%. b) *N,N*-ditosylhydrazine, DBU, THF, 0°C-25°C, 20 min, **10** and **11**: 60%, **12**: 34%.

1.3 Chemoslectivity of O^6 alkylation

To verify the alkylation site a different route was chosen for the synthesis of O^6-ethylcarboxymethylinosine (O^6-ethylcmI). HMBC correlations of the synthesized O^6-ethylcmI **13** *via* Cu(I) carbene chemistry (route A, Scheme S4) already suggested that the O^6 position is targeted (Figure S1). In a different strategy (route B, Scheme S4) the O^6 modified inosine **13** was synthesized starting from commercial 6-chloropurine riboside. Protection with *tert*-Butyldimethylsilyl (TBDMS) at the 2`, 3`and 5`-OH, followed by substitution of the chloride with ethylglycolate and final deprotection yielded O^6-ethylcmI **13** in three steps. Comparison of the structural data identical NMR spectra and HPLC traces provided unequivocal verification of the structure.



Scheme S4: Verification of O^6 modification: Synthesis of O^6 alkylated Inosine **13** in one step using Cu(I) carbene chemistry (route A). a) 20 mol % Cu(I), MES buffer pH 6, 6 % (v/v) DMSO, 25°C, 5 h, 60 %. Different synthetic strategy for synthesis of O^6-I alkylated Inosine **13** (route B). b) TBDMS-Cl, imidazole, DMF, 25°C, o.n., 77 %. c) Ethylglycolate, NaH, THF, reflux, 4 h, 48 %. c) Pyridine · HF, pyridine, 25°C, 16 h, 99%.

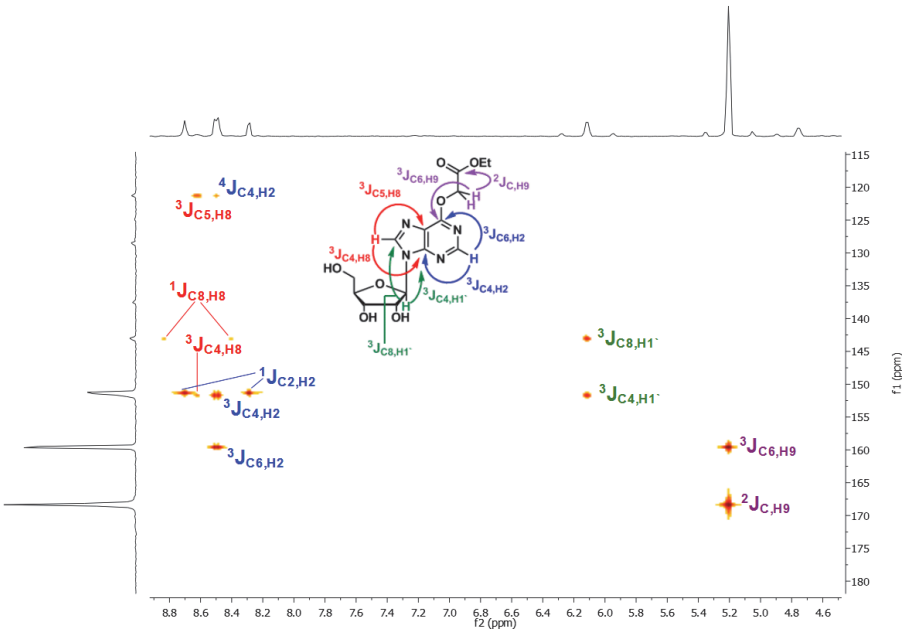


Figure S1: Important HMBC correlations of *O*^{6}-ethylcmI **13** synthesized *via* route A.

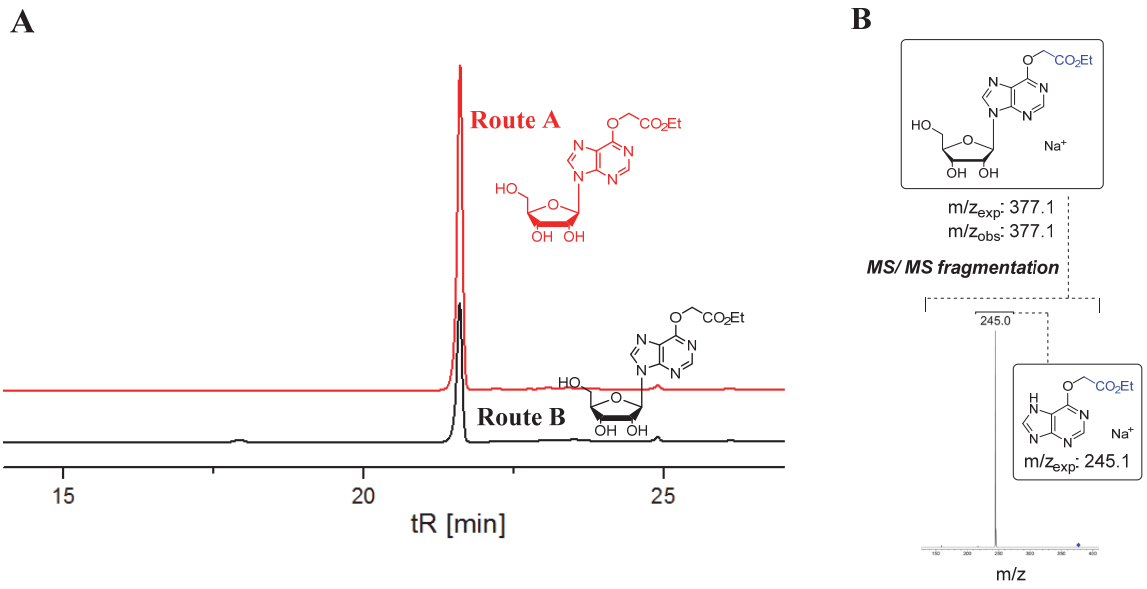


Figure S2: A HPLC analysis of *O*^{6}-ethylcmI **13** synthesized *via* route A (red) and route B (black) using Method C. **B** ESI-MS/MS analysis of *O*^{6}-ethylcmI **13** synthesized *via* route A.

1.4 Alkylation of MP/TP and MS/MS analysis

General Procedure for Cu(I)-catalyzed MP and TP alkylation using EDA (small scale)

Typically 20 µL of total reaction volume contained 1 mM of $CuSO_4$, 5 mM of MP/TP, 100 mM MES buffer, 50 mM EDA. Stock solutions of each compound were prepared and combined in an Eppendorf tube in that order. The reactions were monitored by injection of 1 µL of the reaction mixture into RP-HPLC using Method C. The conversion of MP/TP`s were directly calculated from the corresponding peak areas in the HPLC traces (Table 1). The peaks were separated using Method C (6 µL injection of reaction mixture), collected and analyzed by ESI-MS/MS.

Stock solutions: $CuSO_4$: 5 mM in H_2O; MP/TP: 50 mM in H_2O; MES buffer: 500 mM in H_2O, pH 6

EDA: 250 mM in DMSO; ascorbate: 100 mM in H_2O

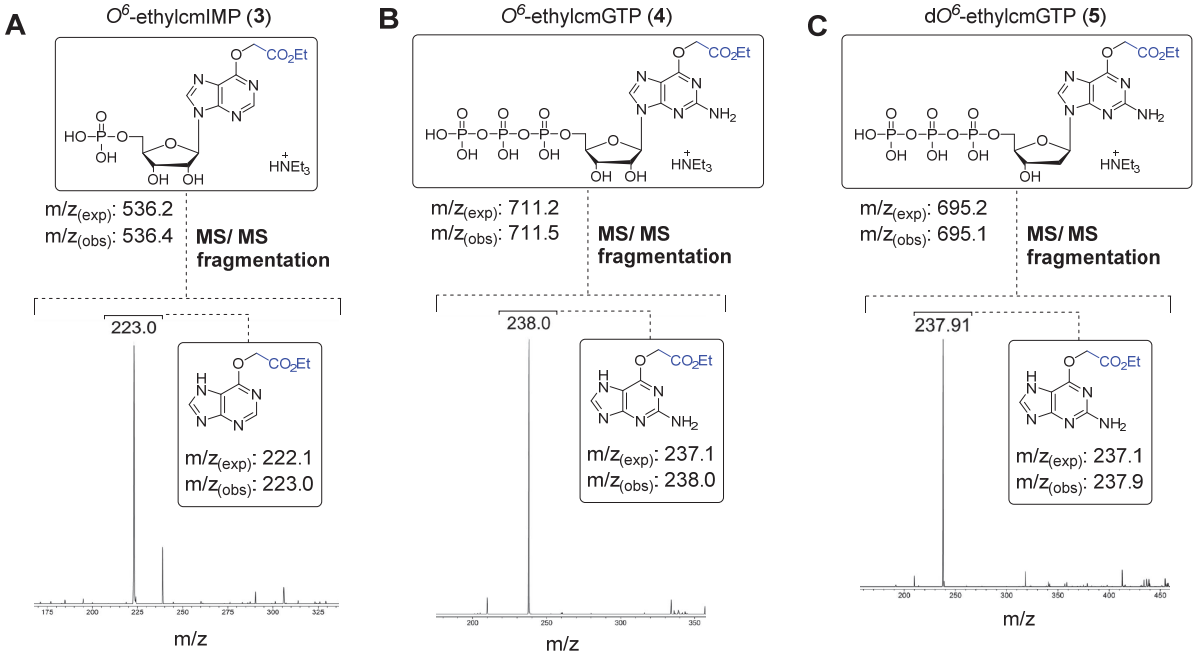


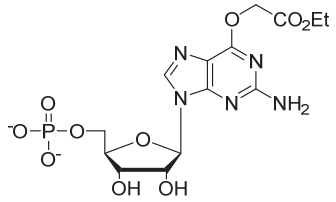
Figure S3: MS-MS analysis of alkylation products following the general procedure. **A** O^6-ethylcmIMP **3**. **B** O^6-ethylcmGTP **4**. **C** dO^6-ethylcmGTP **5**.

1.5 Chemical Synthesis and Analytical Data

General Procedure for Cu(I)-catalyzed MP and TP alkylation using EDA (big scale)

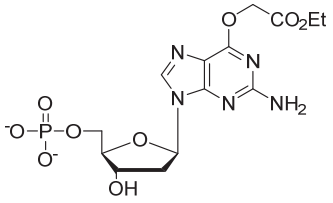
To a solution of $CuSO_4$ (1 mM) in H_2O (degassed), MES buffer stock solution (1 mM), MP/TP (5 mM), EDA (50 mM) and ascorbate (5 mM) were added and the reaction mixture was stirred at room temperature for 1 h. The reaction mixture was lyophilized and the residue was purified by preparative RP-HPLC using Method D. The corresponding product fractions were combined and lyophilized. The residue was dissolved in H_2O (50 mM) and $NaClO_4$ (15 eq.) was added. The mixture was stirred for 16 h at room temperature followed by addition of acetone (400 % v/v). The mixture was centrifuged at 4400 g for 3 min. The supernatant was carefully discarded and the pellet was dissolved in ultra pure H_2O and lyophilized to give the final modified MP/TP.

***O*^{6}-ethylcmGMP (1)**



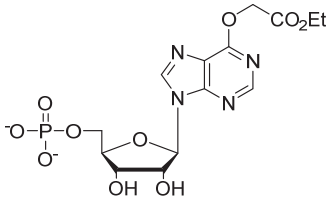
According to the general procedure $CuSO_4$ (4.78 mg, 19.2 µmol), GMP (50.0 mg, 95.8 µmol), EDA (116 µL, 958 µmol), ascorbate (18.98 mg, 95.8 µmol) in H_2O (24.7 mL) and MES buffer stock solution (50.0 µL) were reacted and purified to yield **1** as white solid (45.7 mg, 92 %.). ^{1}H-NMR (500 MHz, MeOD) δ/ppm: 8.32 (s, 1H), 5.97 (d, *J* = 5.8 Hz, 1H), 5.06 (s, 2H), 4.77 (t, *J* = 5.4 Hz, 1H), 4.49 (dd, *J* = 5.1, 3.1 Hz, 1H), 4.24 (q, *J* = 7.1 Hz, 2H), 4.21 (s, 1H), 4.12 – 4.04 (m, 1H), 1.27 (s, 3H). ^{13}C-chemical shifts were extracted from 2D HMQC and HMBC spectra (500 MHz, MeOD) δ/ppm: 168.82, 159.63, 154.34, 139.34, 113.68, 87.81, 84.92, 74.21, 71.14, 63.67, 61.96, 60.91, 13.08. ^{31}P-NMR (202 MHz, MeOD) δ/ppm: 4.89 (s, 1P). HRMS (ESI): $C_{14}H_{19}N_5Na_2O_{10}P^+$ *calcd.*: 494.0659, *found*: 494.0668.

d*O*^{6}-ethylcmGMP (2)



According to the general procedure $CuSO_4$ (5.62 mg, 22.5 µmol), dGMP (50.0 mg, 112 µmol), EDA (136 µL, 1.12 mmol) and ascorbate (22.3 mg, 112 µmol) in H_2O (25.5 mL) and MES buffer stock solution (51.0 µL) were reacted to yield **2** as white solid (41.8 mg, 86 %.). ^{1}H-NMR (500 MHz, MeOD) δ/ppm: 8.27 (s, 1H), 6.34 (t, *J* = 6.7 Hz, 1H), 5.04 (s, 2H), 4.71 (s, br, 1H), 4.22 (q, *J* = 7.1 Hz, 2H), 4.10 (s, br, 1H), 4.01 (s, br, 2H), 2.84 (dt, *J* = 13.2, 6.5 Hz, 1H), 2.38 (ddd, *J* = 13.2, 6.0, 3.1 Hz, 1H), 1.25 (t, *J* = 7.1 Hz, 2H). ^{13}C-chemical shifts were extracted from 2D HMQC and HMBC spectra (500 MHz, MeOD) δ/ppm: 168.81, 159.59, 153.94, 139.15, 86.98, 83.79, 71.84, 63.88, 61.91, 60.79, 38.98, 13.02. ^{31}P-NMR (202 MHz, MeOD) δ/ppm: 5.05 (s, 1P). HRMS (ESI): $C_{14}H_{19}N_5Na_2O_9P^+$ *calcd.*: 478.0710, *found*: 478.0716.

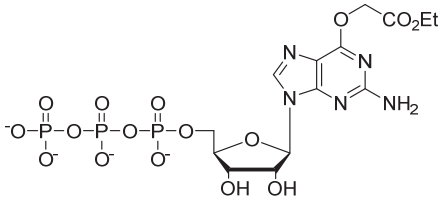
d*O*^{6}-ethylcmIMP (3)



According to the general procedure $CuSO_4$ (4.73 mg, 18.9 µmol), IMP (50.2 mg, 94.7 µmol), EDA (115 µL, 947 µmol), ascorbate (18.7 mg, 94.7 µmol) in H_2O (25.4 mL) and MES buffer stock solution (51.0 µL) were reacted to yield **3** as white solid (33.1 mg, 81 %.). ^{1}H-NMR (500 MHz, MeOD) δ/ppm: 8.88 (s, 1H), 8.47 (s, 1H), 6.21 (d, *J* = 5.6 Hz, 1H), 5.17 (s, 2H), 4.78 (t, *J* = 5.1 Hz, 1H), 4.49 (s_{br}, 1H), 4.29 – 4.17 (m, 3H), 4.07 (s_{br}, 2H), 1.25 (t, *J* = 7.1 Hz, 3H). ^{13}C-chemical shifts were extracted from 2D HMQC and HMBC spectra (500 MHz, MeOD) δ/ppm: 168.51, 159.10, 152.44, 151.45, 143.06, 87.92, 85.39, 75.16, 71.23,

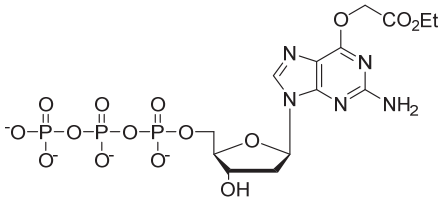
63.64, 62.61, 61.0, 13.03. ^{31}P-NMR (202 MHz, MeOD) δ/ppm: 4.96 (s, 1P). HRMS (ESI): $C_{14}H_{19}N_4NaO_{10}P^+$ *calcd.*: 457.0731, *found*: 457.0730.

O^6-ethylcmGTP (4)



According to the general procedure $CuSO_4$ (6.15 mg, 24.6 µmol), GTP (70.0 mg, 118 µmol), EDA (143 µL, 1.18 mmol), ascorbate (23.5 mg, 118 µmol) in H_2O (26.7 mL) and MES buffer stock solution (54.0 µL) were reacted to yield **4** as white solid (53.2 mg, 73 %). ^{1}H-NMR, peak broadening caused by stacking of compound **4** in D_2O (400 MHz, D_2O) δ/ppm: 8.40 (s_{br}, 1H), 6.11 (s_{br}, 1H), 5.14 (s_{br}, 2H), 4.56 (s_{br}, 1H), 4.37 (s_{br}, 1H), 4.36 – 4.08 (m, 5H), 1.24 (t, *J* = 6.6 Hz, 3H). ^{13}C-chemical shifts extracted from 2D HMQC and HMBC spectra, X carbon signals could not be detected due to the peak broadening in the ^{1}H-NMR (600 MHz, D_2O) δ/ppm: ^{31}P-NMR (243 MHz, D_2O) δ/ppm: -5.39 (s_{br}, 1P), -10.49 (s_{br}, 1P)), -19.39 (s_{br}, 1P). HRMS (ESI): $C_{20}H_{38}N_6O_{16}P_3^+$ *calcd.*: 711.1552, *found*: 711.1551.

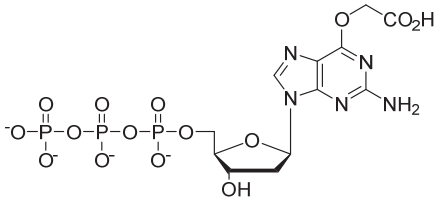
dO^6-ethylcmGTP (5)



According to the general procedure $CuSO_4$ (5.24 mg, 20.9 µmol), dGTP (70.0 mg, 101 µmol), EDA (122 µL, 1.01 mmol), ascorbate (20.0 mg, 101 µmol) in H_2O (22.9 mL) and MES buffer stock solution (46.0 µL) were reacted to yield **5** as white solid (39 mg, 65 %). ^{1}H-NMR (600 MHz, D_2O) δ/ppm: 8.28 (s, 1H), 6.41 (t, *J* = 6.8 Hz, 1H), 5.12 (s, 1H), 5.11 (s, 1H), 4.83 (dt, *J* = 7.0, 3.6 Hz, 1H), 4.32 – 4.27 (m, 3H), 4.24 (ddd,

J = 10.4, 6.2, 4.1 Hz, 1H), 4.21 – 4.15 (m, 1H), 2.83 (dt, *J* = 13.8, 6.8 Hz, 1H), 2.54 (ddd, *J* = 14.1, 6.3, 3.6 Hz, 1H), 1.26 (t, *J* = 7.1 Hz, 3H). ^{13}C-chemical shifts were extracted from 2D HMQC and HMBC spectra (600 MHz, D_2O) δ/ppm: 171.02, 159.71, 153.64, 139.38, 114.1, 85.72, 83.43, 70.84, 65.27, 63.26, 62.74, 38.50, 13.22. ^{31}P-NMR (243 MHz, D_2O) δ/ppm: -6.74 (s, 1P), -10.9 (d, *J* = 19.4 Hz, 1P), -22.04 (s, 1P). HRMS (ESI): $C_{14}H_{19}N_5Na_4O_{15}P_3^+$ *calcd.*: 681.9676, *found*:. 681.9685.

d*O*^{6}-cmGTP (6)



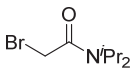
d*O*^{6}ECMGTP **5** (15.3 mg, 22.5 µmol) was placed in an Eppendorf tube (0.5 mL) dissolved in 1M LiOH (33.7 µL, 33.7 µmol) and stirred at 25°C. The reaction mixture was monitored by RP-HPLC using method C. After 1 h full conversion of the starting material was observed. The mixture was freeze dried and purified by preperative RP-HPLC using method D. The corresponding fraction were combined and lyophilized. The residue was dissolved in H_2O (600 µL) and $NaClO_4$ (41.1 mg, 338 µmol,) was added. The mixture was stirred for 5 h at room temperature and then acetone (4 mL) was added. The obtained suspension was centrifuged at 4400 g for 2 min. The supernatant was carefully discarded. The pellet was re-dissolved in ultra pure H_2O and lyophilized to yield **6** as a white solid (12.8 mg, 57%). ^{1}H-NMR (400 MHz, D_2O) δ/ppm: 8.35 (s_{br}, 1H), 6.40 (d, *J* = 7.7 Hz, 1H), 4.83 (s, 2H), 4.33 – 4.06 (m, 3H), 2.81 (dd, *J* = 14.3, 7.3 Hz, 1H), 2.56 (d, *J* = 10.2 Hz, 1H). ^{13}C-chemical shifts were extracted from 2D HMQC and HMBC spectra (600 MHz, D_2O) δ/ppm: 176.20, 160.56, 138.94, 85.42, 83.21, 70.83, 65.14, 63.34, 38.52. ^{31}P-NMR (243 MHz, D_2O) δ/ppm: -5.29 (s, 1P), -10.42 (s, 1P), -19.09 (s, 1P). HRMS (ESI): $C_{18}H_{33}N_6NaO_{15}P_3^+$ *calcd.*: 689.1109, *found*:. 689.1107.

General Procedure: Synthesis of Diazoacetamides

A: To a solution of bromacetyl bromide (BrAcBr) in dry CH_2Cl_2, secondary amine (freshly filtered over neutral aluminium oxide) was added at -60°C. The cooling bath was removed and the reaction mixture was stirred for an additional 1hour. Water was added and the reaction mixture was extracted with EtOAc. The solvent was removed under reduced pressure and the residue purified by flash chromatography on Si_{60} in cyclohexane and EtOAc.

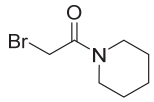
B: To a solution of bromoacetamide derivative in THF, *N,N*-ditosylhydrazine (TsNHNHTs) was added. The reaction mixture was cooled to 0°C and DBU was added and the mixture was stirred for 20 min at room temperature. Saturated $NaHCO_3$ was added while precipitation was observed. The mixture was centrifuged at 4400 g for 2 min. The supernatant was extracted with Et_2O (3 x 10 mL). The combined organic layers were dried over Na_2SO_4, filtered and the residue purified by flash chromatography on Si_{60} in CH_2Cl_2 and MeOH (prior to purification, the column was neutralized with two column volumes CH_2Cl_2 containing 5 % NEt_3).

2-bromo-*N,N*-diisopropylacetamide (7)



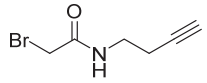
According to the general procedure A BrAcBr (100 µL, 1.15 mmol) and *N,N*-diisopropylamine (324 µL, 2.30 mmol) were reacted to yield **7** as colorless liquid (133 mg, 52 %). The analytical data were in agreement with literature.[2] ^{1}H-NMR (500 MHz, $CDCl_3$) δ/ppm: 3.96 (dt, *J* = 13.2, 6.7 Hz, 1H), 3.82 (s, 2H), 3.43 (p, *J* = 7.2, 6.6 Hz, 1H), 1.38 (d, *J* = 6.9 Hz, 6H), 1.25 (d, *J* = 6.7 Hz, 6H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ/ppm: 165.57, 50.62, 46.47, 28.71, 20.78, 20.20.

2-bromo-1-(piperidin-1-yl)ethan-1-one (8)



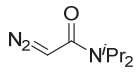
According to the general procedure A BrAcBr (100 µL, 1.15 mmol) and piperidine (228 µL, 2.30 mmol) were reacted to yield **8** as colorless liquid (74.5 mg, 31 %). The analytical data were in agreement with the literature.[3] ^{1}H-NMR (500 MHz, $CDCl_3$) δ/ppm: 3.86 (s, 2H), 3.58 – 3.52 (m, 2H), 3.50 – 3.40 (m, 2H), 1.65 (p, *J* = 2.8 Hz, 4H), 1.56 (t, *J* = 5.6 Hz, 2H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ/ppm: 166.15, 48.05, 43.39, 26.31, 26.28, 25.49, 24.39.

2-bromo-N-(but-3-yn-1-yl)acetamide (9)



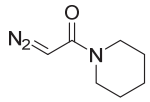
According to the general procedure A BrAcBr (252 µL, 2.90 mmol) and 1-amino-3-butyne (250 µL, 2.90 mmol) were reacted to yield **9** as colorless liquid (415 mg, 75 %). The analytical data were in agreement with the literature.[3] ^{1}H-NMR (250 MHz, $CDCl_3$) δ 6.84 (s, 1H), 3.91 (s, 1H), 3.46 (q, J = 6.3 Hz, 1H), 2.45 (td, J = 6.4, 2.6 Hz, 1H), 2.05 (t, J = 2.6 Hz, 1H).

2-diazo-*N,N*-diisopropylacetamide (10)



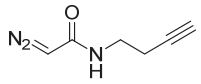
According to the general procedure B 7 (123.3 mg, 555 µmol), TsNHNHTs (378 mg, 1.11 mmol) and DBU (414 µL, 2.78 mmol) were reacted to yield **10** as yellow liquid (56.0 mg, 60 %). ^{1}H-NMR (500 MHz, $CDCl_3$) δ/ppm: 4.94 (s, 1H), 3.65 (p, *J* = 7.2 Hz, 2H), 1.28 (s, 12H). ^{13}C-NMR (101 MHz, $CDCl_3$) δ/ppm: 164.68, 47.83, 47.10, 21.25. HRMS (ESI): $C_8H_{15}N_3NaO^+$ *calcd*.: 192.1107, found: 192.1110.

2-diazo-1-(piperidin-1-yl)ethan-1-one (11)



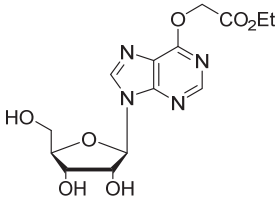
According to the general procedure B **8** (66.3 mg, 322 µmol), TsNHNHTs (219 mg, 643 µmol) and DBU (240 µL, 1.61 mmol) were reacted to yield **11** as yellow waxy solid (29.5 mg, 60%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ/ppm 1H NMR (400 MHz, CDCl3) δ/ppm: 4.99 (s_{br}, 2H), 3.35 (s_{br}, 4H), 1.67 – 1.62 (m, 2H), 1.58 – 1.51 (m, 4H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ/ppm: 164.34, 59.87, 48.39, 46.31,43.40,25.88,24.47. HRMS (ESI): $C_7H_{12}N_3O^+$ *calcd.*: 154.0975, *found*: 154.0976.

2-diazo-1-(piperidin-1-yl)ethan-1-one (12)



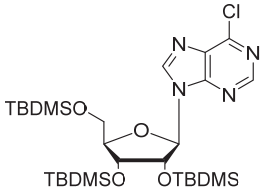
According to the general procedure B **9** (414 mg, 2.18 mmol), TsNHNHTs (1.48 g, 4.36 mmol) and DBU (1.63 mL, 10.9 mmol) were reacted to yield **12** as yellow waxy solid (102 mg, 34%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ/ppm 1H NMR (400 MHz, CDCl3) δ/ppm: 5.42 (s_{br}, 1H), 4.75 (s, 1H), 3.45 (q, J = 6.2 Hz, 2H), 2.42 (td, J = 6.3, 2.6 Hz, 2H), 2.01 (s, 1H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ/ppm: 165.84, 81.60, 70.19, 47.34, 38.62, 19.95. LRMS (ESI): $C_6H_7N_3NaO^+$ *calcd.*: 160.0, *found*: 159.8.

O^6-ethylcmI (13) (*via* route A, Scheme S4)



To a solution of $CuSO_4$ (27.9 mg, 112 µmol) in H_2O (94 mL) and MES buffer (10.8 mL), inosine (150 mg, 559 µmol, dissolved in 7 mL DMSO), EDA (676 µL, 5.59 mmol) and ascorbate (111 mg, 559 µmol) were added and the reaction mixture stirred at room temperature. Analysis of the reaction mixture after 5 h via RP-HPLC showed 66% conversion of inosine. The reaction was stopped, lyophilized and taken up in CH_2Cl_2 while a precipitate was observed. The resulting suspension was centrifuged at 4400 g for 5 min followed by lyophilizing of the supernatant. The residue was purified by flash chromatography on Si_{60} in CH_2Cl_2 and MeOH. Residual MeOH sticking to the product was removed by adding H_2O and lyophilization to obtain **13** as white solid (119 mg, 60 %). ^{1}H-NMR (400 MHz, MeOD) δ/ppm: 8.59 (s, 1H), 8.48 (s, 1H), 6.10 (d, J = 5.8 Hz, 1H), 5.19 (s, 2H), 4.74 (t, J = 5.4 Hz, 1H), 4.36 (dd, J = 5.1, 3.3 Hz, 1H), 4.22 (q, J = 7.1 Hz, 2H), 4.17 (q, J = 3.1 Hz, 1H), 3.91 – 3.3.88 (m, 1H), 3.78 – 3.753 (m, 1H), 1.26 (t, J = 7.1 Hz, 3H). ^{13}C-NMR (101 MHz, MeOD) δ/ppm: 169.76, 160.99, 153.06, 152.70, 144.35, 122.65, 91.01, 87.79, 75.73, 72.29, 64.05, 63.12, 62.44, 14.40. HRMS (ESI): $C_{14}H_{18}N_4NaO_7^+$ *calcd.*: 377.1068, *found*: 377.1073.

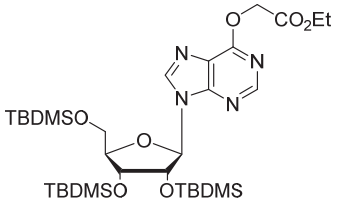
2`, 3`, 5` (TBDMS) 6-chloropurine riboside (14)



To a solution of 6-chloropurine riboside (108 mg, 376 µmol) in dry DMF (1.5 mL), imidazole (256 mg, 3.76 mmol) and TBDMS-Cl (283 mg, 1.88 mmol) were added and the reaction stirred at room temperature

for 22 h. The reaction was diluted with CH_2Cl_2 (5 mL) and H_2O (5 mL). The water layer was extensively extracted CH_2Cl_2 (5 x 5 mL) and the combined organics were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography on Si_{60} in cyclohexane/EtOAc to obtain **14** as white solid (182 mg, 289 µmol, 77 %). ^{1}H-NMR (250 MHz, $CDCl_3$) δ/ppm: 8.8 (s, 1H), 8.51 (s, 1H), 6.08 (d, *J* = 4.6 Hz, 1H), 4.54 (t, *J* = 4.5 Hz, 1H), 4.27 (t, *J* = 4.0 Hz, 2H), 4.12 (dd, *J* = 6.1, 3.2 Hz, 1H), 3.98 (dd, *J* = 11.5, 3.5 Hz, 1H), 3.76 (dt, *J* = 11.6, 2.2 Hz, 1H), 0.91 (s, 9H), 0.88 (s, 9H), 0.74 (s, 9H), 0.08 (m, 12H), -0.07 (s, 3H), -0.28 (s, 3H). ^{13}C-NMR (101 MHz, $CDCl_3$) δ/ppm: 152.08, 151.65, 151.14, 144.21, 132.23, 88.73, 85.87, 76.54, 71.96, 62.54, 26.26, 25.97, 25,76, 18.71, 18.22, 17.97, -4.24, -4.54,-4.91, -5.19. HRMS (ESI): $C_{28}H_{53}ClN_4NaO_4Si_3^+$ *calcd.*: 651.2955, *found*: 651.2956.

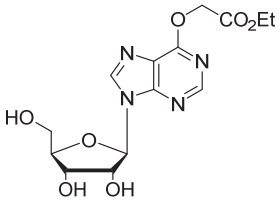
2`, 3`, 5` (TBDMS) *O*^{6}-ethylcmI (15)



NaH (15.9 mg, 397 µmol, 2.50 eq.) was placed in a pressure tube and THF (1.5 mL) was added. Then ethyl glycolate (82.7 mg, 794 µmol) was added and the reaction mixture stirred for 30 min at room temperature. Then chloropurine riboside derivative **14** (100 mg, 159 µmol) was added and the reaction heated to reflux for 4 h. The solvent was removed under reduced pressure and the residue was purified by flash chromatography on Si_{60} cyclohexane/EtOAc to obtain **15** as white solid (53.0 mg, 76.0 µmol, 48 %). ^{1}H-NMR (500 MHz, $CDCl_3$) δ/ppm: 8.47 (s, 1H), 8.39 (s, 1H), 6.08 (d, *J* = 4.7 Hz, 1H), 5.12 (s, 1H), 5.10 (s, 1H), 4.57 (t, *J* = 4.4 Hz, 1H), 4.32 (t, *J* = 4.2 Hz, 1H), 4.23 (q, *J* = 7.1 Hz, 2H), 4.14 (td, *J* = 3.9, 2.5 Hz, 1H), 4.04 (dd, *J* = 11.4, 3.7 Hz, 1H), 3.79 (dd, *J* = 11.5, 2.6 Hz, 1H), 1.25 (m, 2H), 0.95 (s, 9H), 0.92 (s, 9H), 0.80 (s, 9H), 0.14 (s, 3H), 0.13 (s, 3H), 0.10 (s, 3H), 0.09 (s, 3H), -0.03 (s, 3H), -0.19 (s, 3H). ^{13}C-NMR (101 MHz, $CDCl_3$) δ/ppm: 168.19, 159.52, 152.25, 151.8, 141.64, 121.47, 88.80, 85.41, 76.39, 71.69,

63.14, 62.42, 61.45, 26.27, 25.99, 25.82, 18.72, 18.22, 18.00, 14.25, -4.21, -4.57, -4.60, -4.84, -5.18, -5.25. HRMS (ESI): $C_{32}H_{60}N_4NaO_7Si_3^+$ *calcd.*: 719.3662, *found*: 719.3672.

O^6-ethylcmI (13) (*via* route B, Scheme S4)



The TBDMS protected inosine derivative (26.0 mg, 37.0 µmol) was co-evaporated with pyridine (2 mL), dissolved in pyridine (0.5 mL) followed by dropwise addition of hydrogen fluoride pyridine (73.4 µL, 530 µmol) and the reaction mixture was stirred at room temperature for 15 h. The solvent was removed under reduced pressure and the residue purified by flash chromatography Si_{60} CH_2Cl_2/ MeOH. Residual pyridine was co-evaporated with toluene then H_2O was added and the residue was lyophilized to obtain **13** as yellowish solid (13.2 mg, 37.0 µmol, 99%.). ^{1}H-NMR (500 MHz, MeOD) δ/ppm: 8.59 (s, 1H), 8.48 (s, 1H), 6.10 (d, *J* = 5.8 Hz, 1H), 5.19 (s, 2H), 4.74 (t, *J* = 5.5 Hz, 1H), 4.36 (dd, J = 5.1, 3.3 Hz, 1H), 4.23 (q, *J* = 7.1 Hz, 2H), 4.17 (q, *J* = 3.1 Hz, 1H), 3.90 (dd, *J* = 12.4, 2.8 Hz, 1H), 3.77 (m, 1H), 1.26 (t, *J* = 7.1 Hz, 3H). ^{13}C-NMR (101 MHz, MeOD) δ/ppm: 169.76, 160.99, 153.06, 152.7, 144.35, 122.65, 91.01, 87.79, 75.73, 72.29, 64.05, 63.12, 62.44, 14.40. HRMS (ESI): $C_{14}H_{18}N_4NaO_7^+$ *calcd.*: 377.1068, *found*: 377.1071.

2 Alkylation of ssDNA catalyzed by Cu(I) carbenes

2.1 Synthesis of ssDNA

Solid-phase DNA synthesis was carried out on 1-µmol CPG columns using standard phosphoramidite chemistry with 0.3 M 5.benzylthio-1-H-tetrazole as activator. The DNA was cleaved from the resin with 32 % (v/v) aqueous ammonia for 2 h at room temperature. Deprotection was carried out at 55°C for 18 h. The

residue was freeze dried and purified as described in Methods A and B. The identity of all ssDNA was confirmed by ESI-MS or MALDI TOF MS.

2.2 Cu(I)-catalyzed ssDNA O^6-G modification using EDA or DAAs

General procedure for ssDNA alkylation

Typically 20 µL of total reaction volume contained 1 mM of $CuSO_4$, 5 mM of ssDNA, 100 mM MES buffer, 50 mM EDA or DAA and 5 mM ascorbate. Stock solutions of each compound were prepared and combined in an Eppendorf tube in that order. The reactions were monitored by injecting 1 µL of the reaction mixture into RP-HPLC using Method C. The conversions of the ssDNA`s were directly calculated from the corresponding peak areas in the HPLC traces. The peaks were separated using Method C (6 µL injection of reaction mixture), collected and analyzed by ESI-MS or UPLC-MS.

Stock solutions: $CuSO_4$: 5 mM in H_2O; MES buffer: 500 mM in H_2O, pH 6; EDA: 250 mM in H_2O containing 10 % (v/v) ^{t}BuOH or DMSO; DAA: 250 mM in DMSO or dioxane; ascorbate: 100 mM in H_2O.

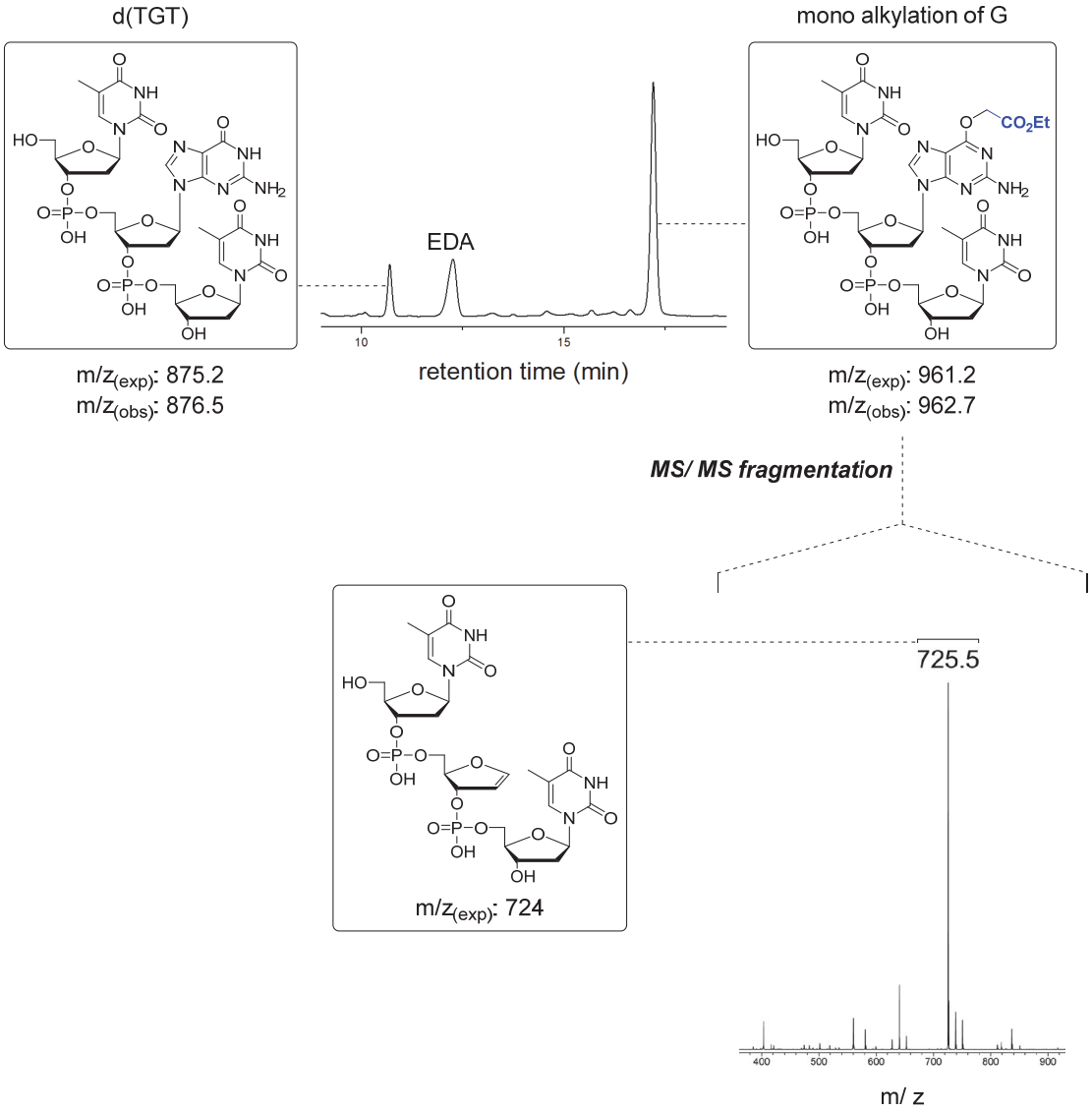


Figure S4: HPLC-MS/MS analysis of O^6-G alkylation of d(TGT) with EDA following the general procedure (conversion see main paper Table 1, Entry 13).

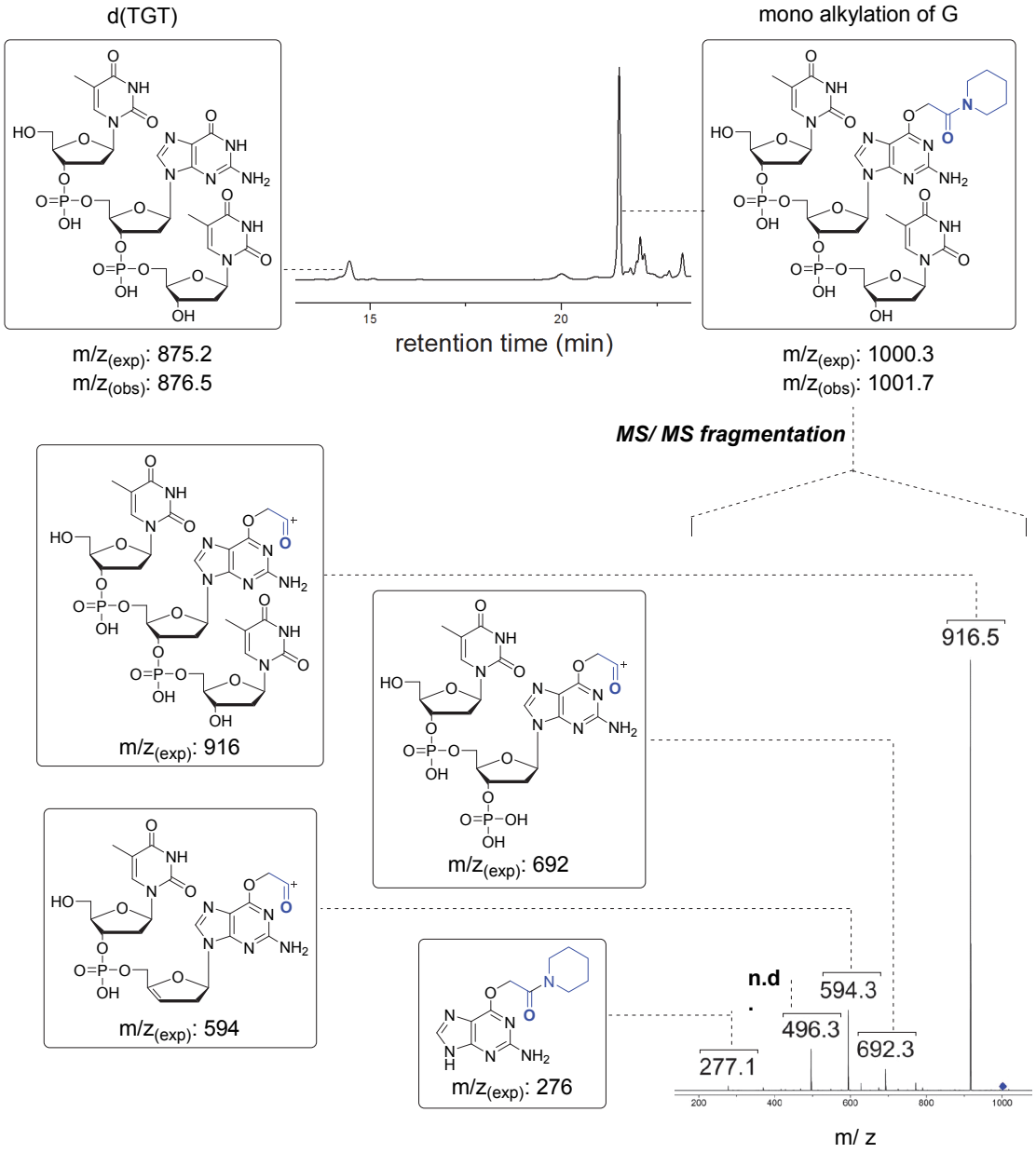


Figure S5: HPLC-MS/MS analysis of O^6-G alkylation of d(TGT) with DAA **10** following the general procedure (conversion see main paper Table 2, Entry 1).

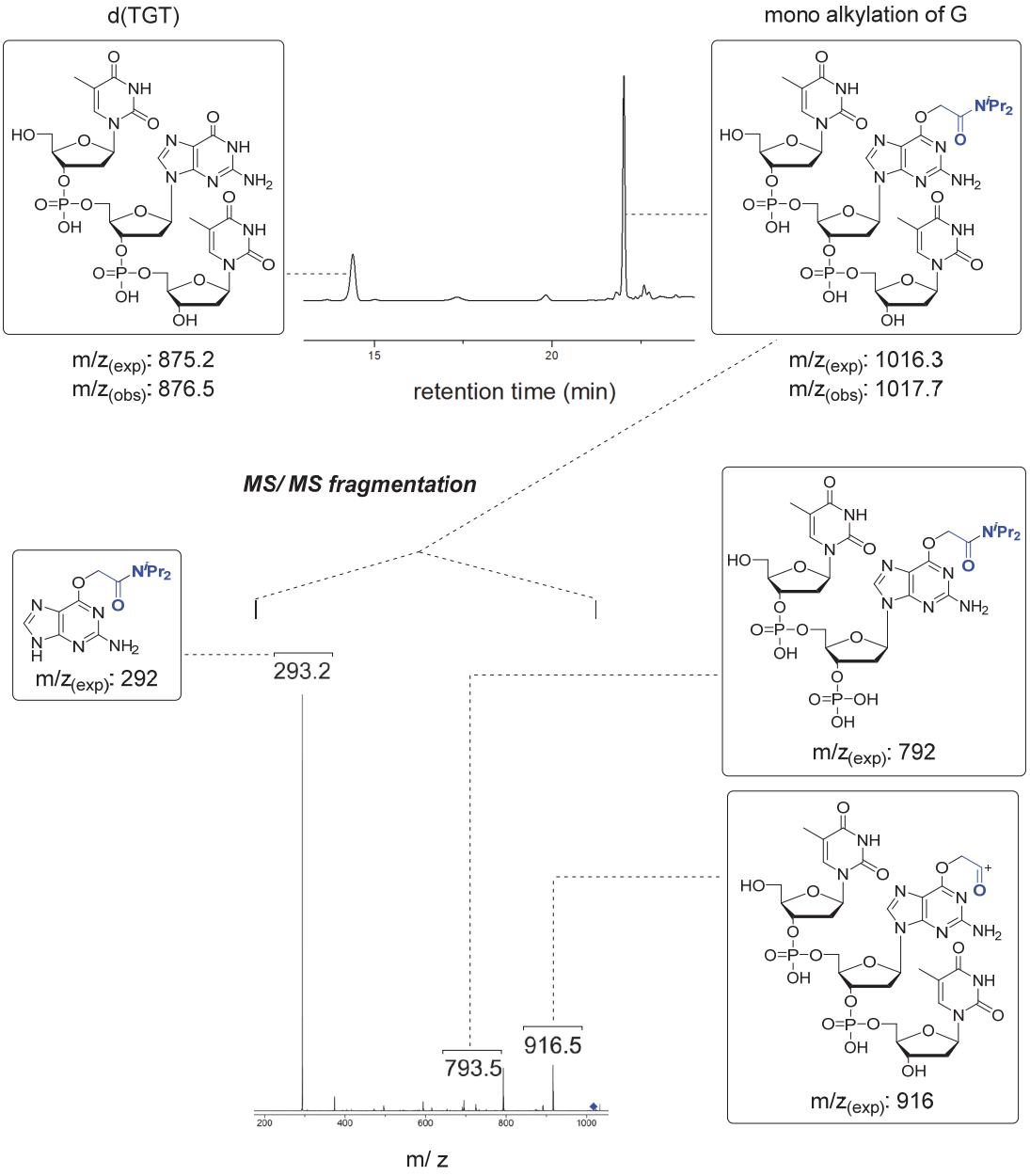


Figure S6: HPLC-MS/MS analysis of O^6-G alkylation of d(TGT) with DAA **9** following the general procedure (conversion see main paper Table 2, Entry 2).

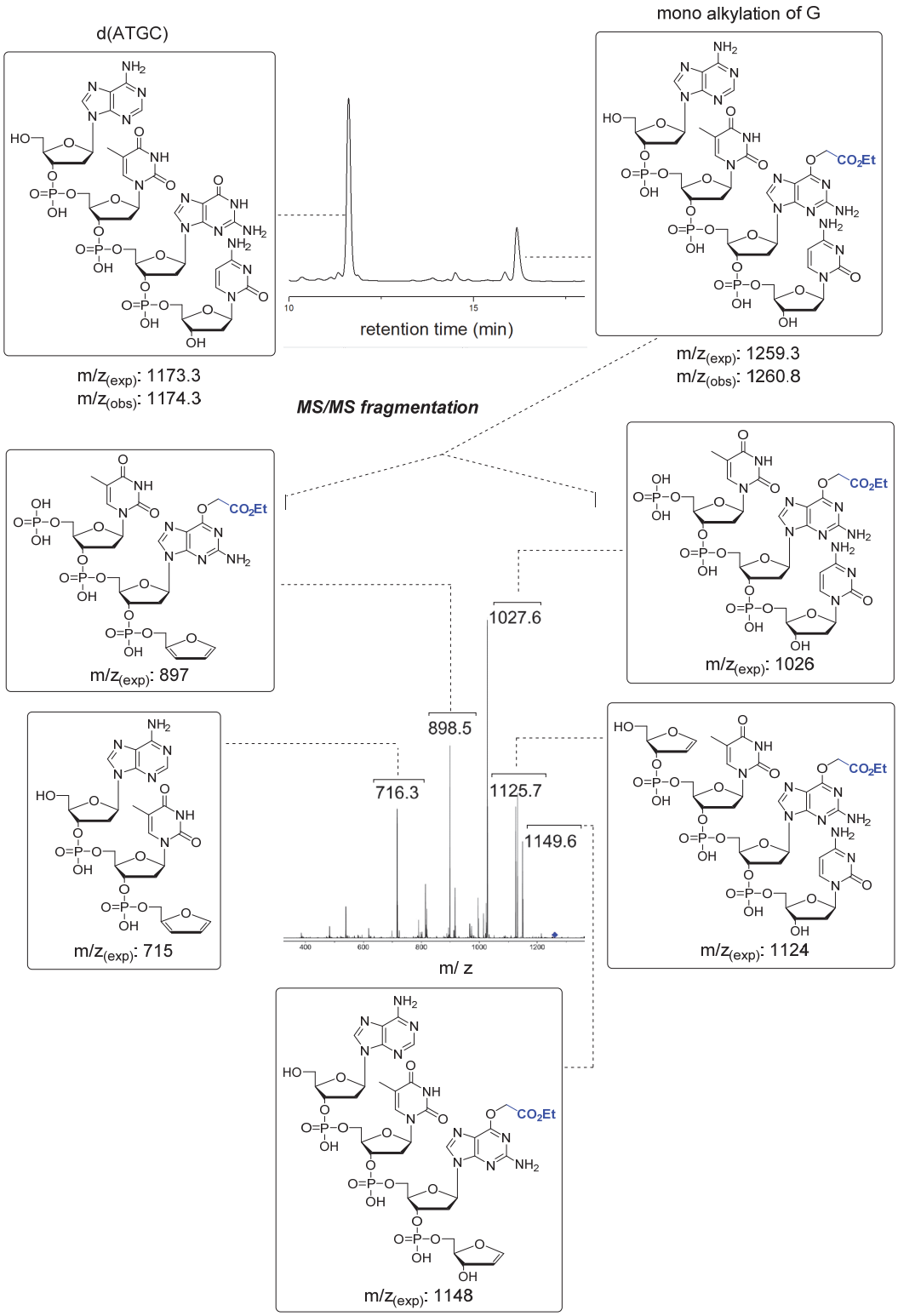


Figure S7: HPLC-MS/MS analysis of O^6-G alkylation reaction of d(ATGC) with EDA following the general procedure (conversion see main paper Table1, Entry15).

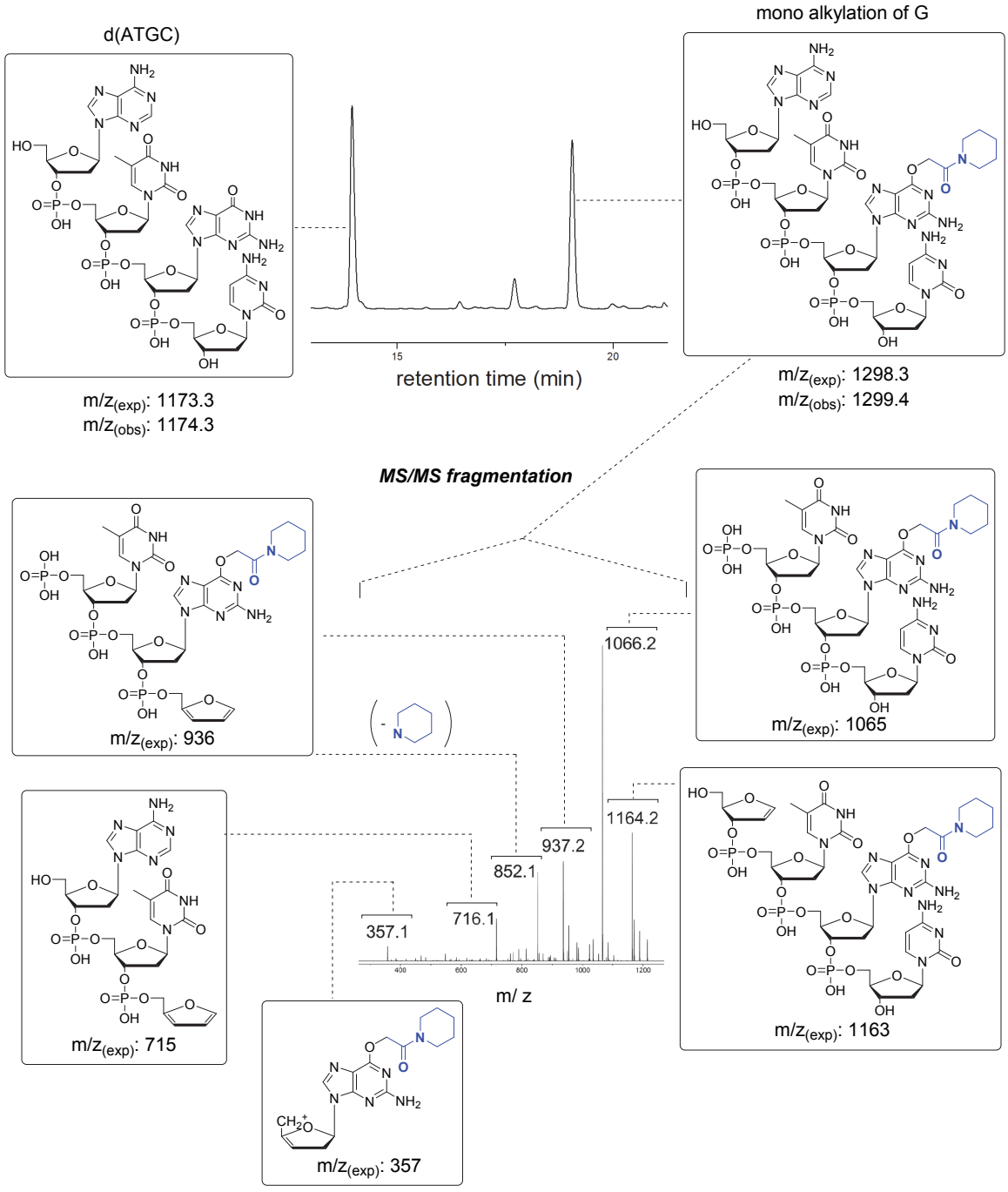


Figure S8: HPLC-MS/MS analysis of O^6-G alkylation reaction of d(ATGC) with DAA **10** following the general procedure (conversion see main paper Table 2, Entry 3).

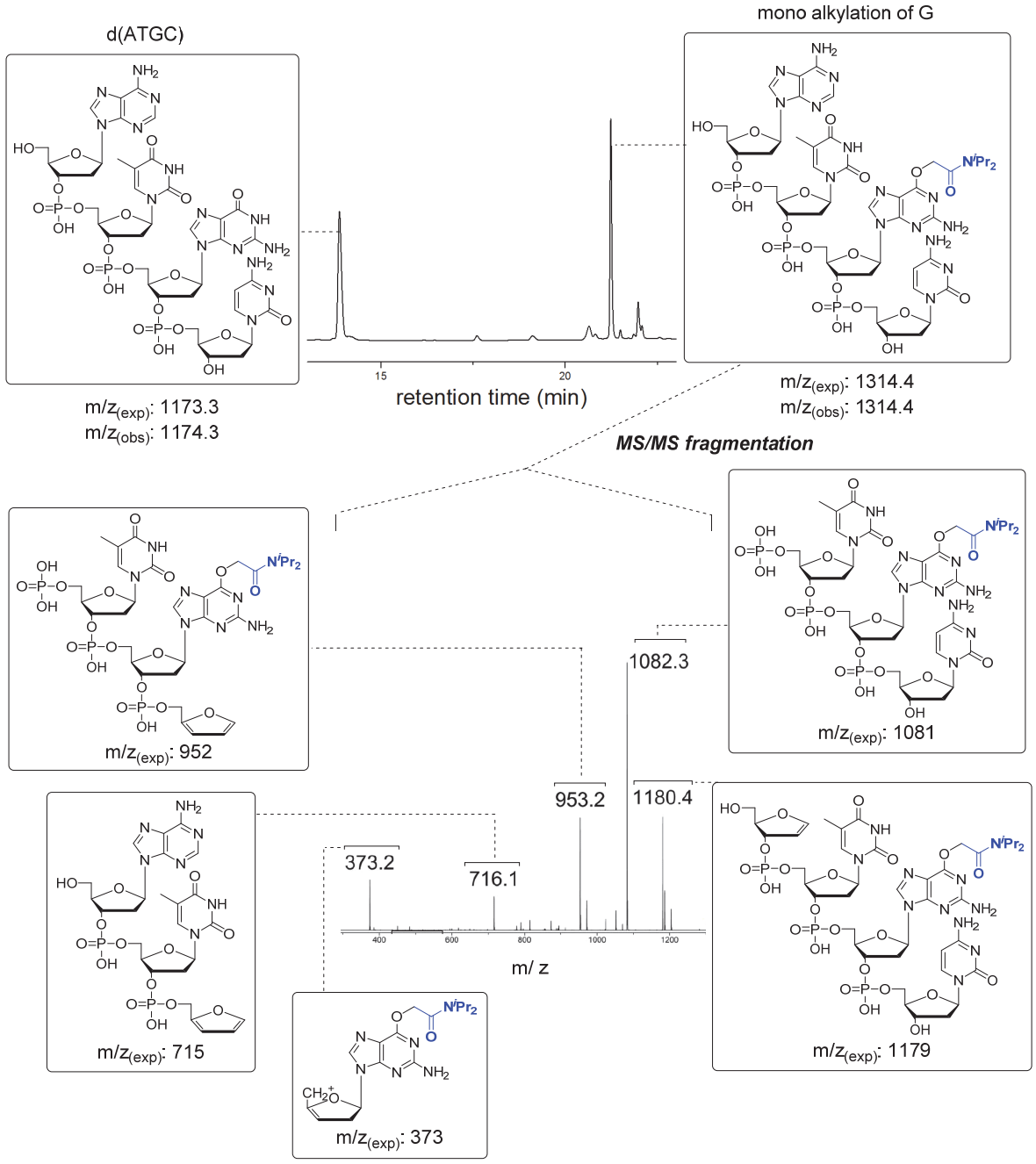


Figure S9: HPLC-MS/MS analysis of O^6-G alkylation reaction of d(ATGC) with DAA **9** following the general procedure (conversion see main paper Table 2, Entry 4).

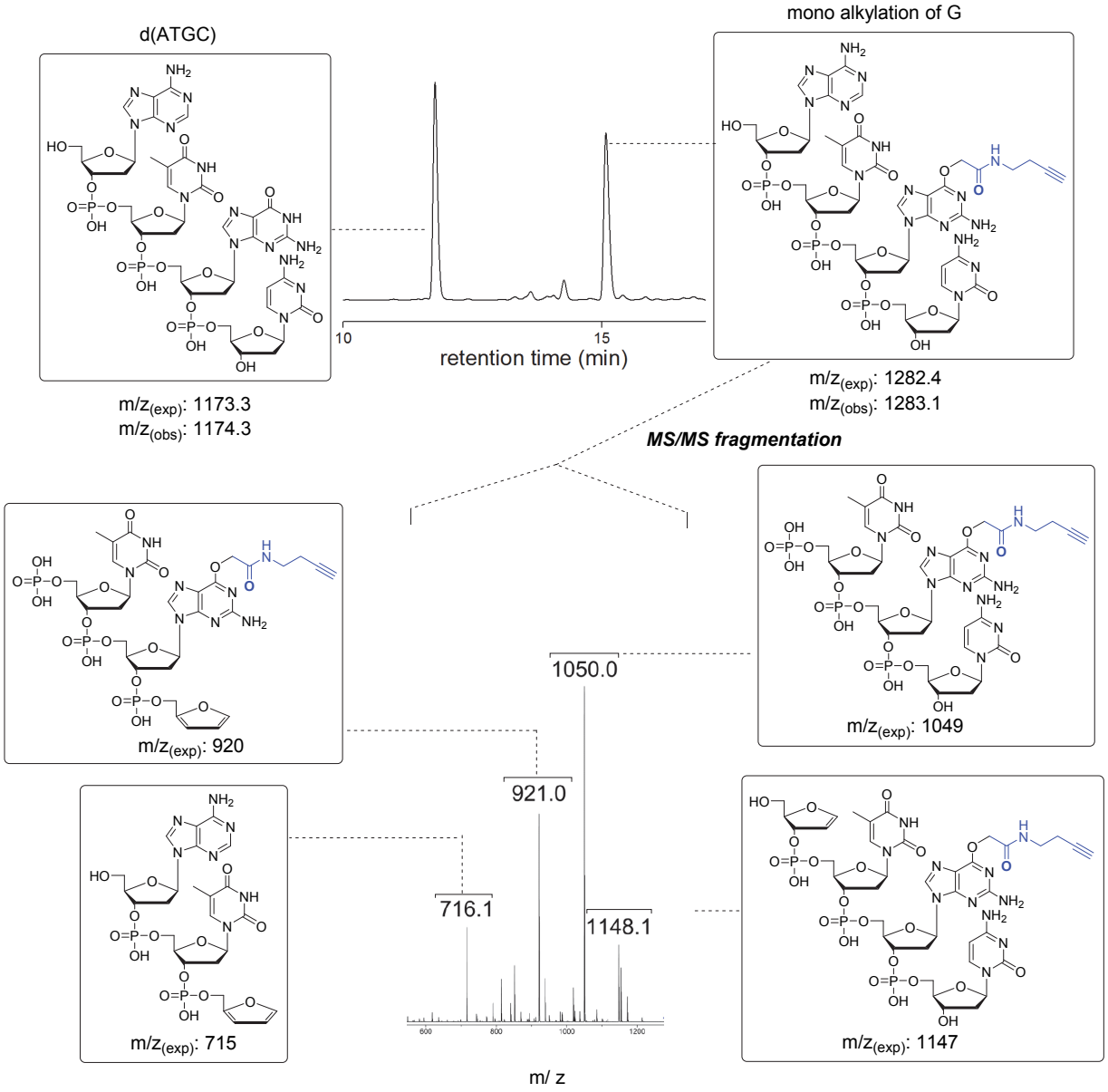


Figure S10: HPLC-MS/MS analysis of O^6-G alkylation reaction of d(ATGC) with DAA **12** following the general procedure (conversion see main paper Table 2, Entry 10).

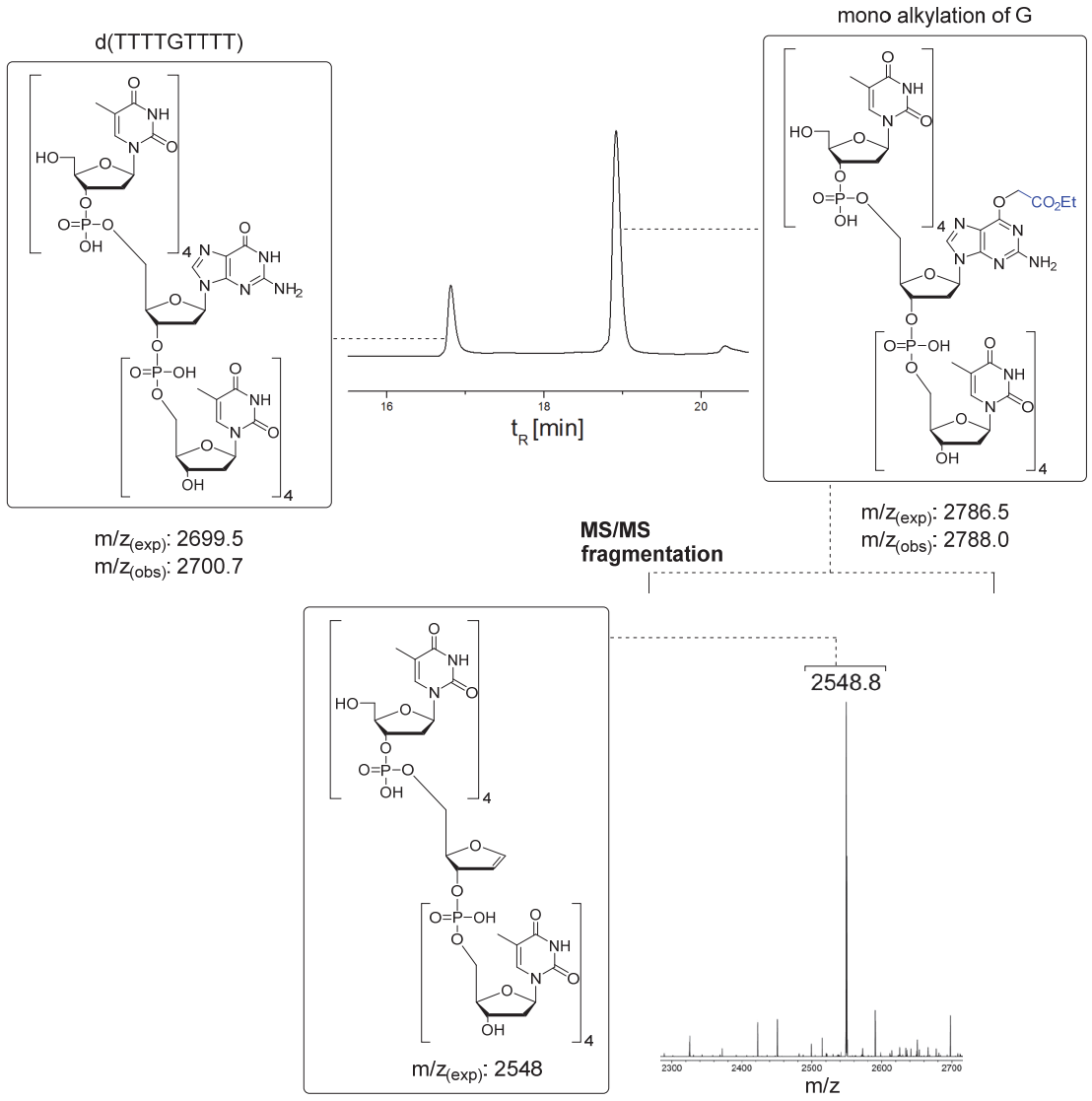


Figure S11: HPLC-MS/MS analysis of O^6-G alkylation reaction of d(TTTTGTTTT) with EDA following the general procedure (conversion see main paper Table 1, Entry 17)

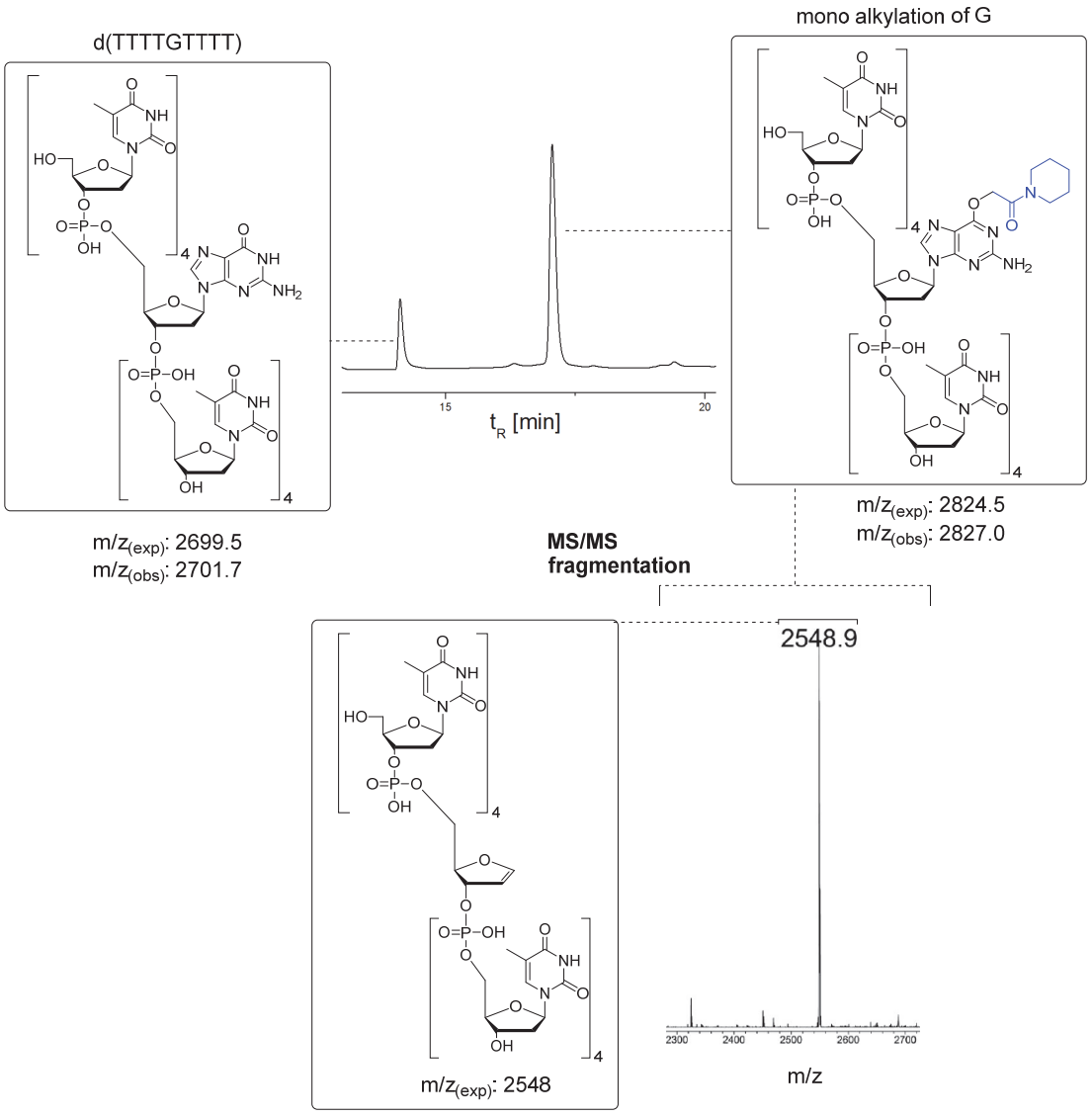


Figure S12: HPLC-MS/MS analysis of O^6-G alkylation reaction of d(TTTTGTTTT) with DAA **10** following the general procedure (conversion see main paper Table 2, Entry 6).

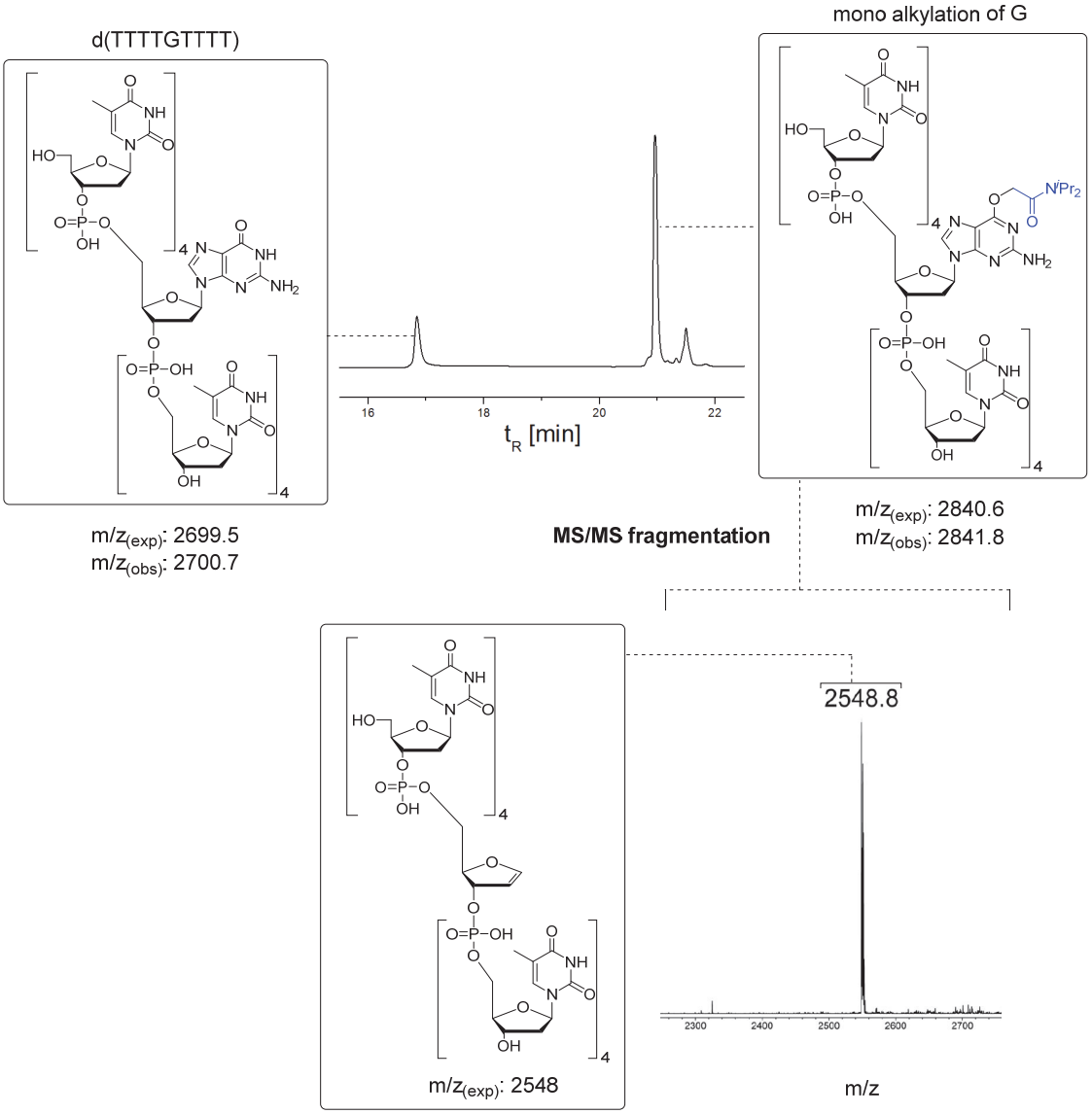


Figure S13: HPLC-MS/MS analysis of O^6-G alkylation reaction of d(TTTTGTTTT) with DAA **11** following the general procedure (conversion see main paper Table 2, Entry 7).

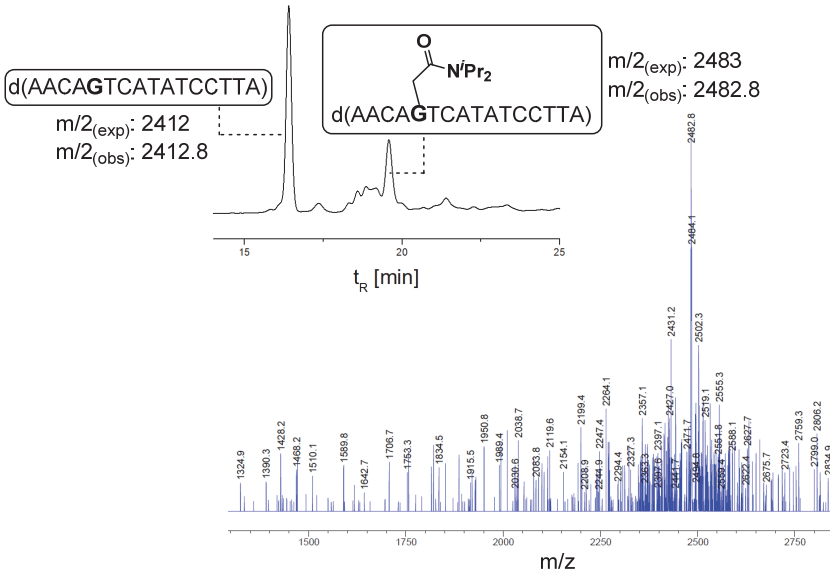


Figure S14: HPLC-UPLC-MS analysis of O^6-G alkylation reaction of d(AACAGTCATATCCTTA) with DAA **11** following the general procedure (conversion see main paper Table 2, Entry 9).

2.3 Click reaction with alkyne O^6-G modified d(ATGC) and azide biotin conjugate

According to the genereal procedure (see section 2.2), d(ATGC) was modified using Cu(I) carbene chemistry and DAA **12.** The alkylation product was purified using Method C and directly used for click reaction with azide biotin conjugate **16**, synthesized by a literature known procedure.[4] d(ATO^6-akyneGC) stock solution (100 µL in 1mM TEAA buffer pH 7.25, 34.8 µM stock solution) was mixed with azide biotin conjugate **16** (53 µL in DMSO, 100 µM stock solution), sodium ascorbate (87 µM in H_2O, 1 mM stock solution), DMSO (28.2 µL) and degassed for 30 seconds. The $CuSO_4$ stock solution (1.40 µL in H_2O, 12.5 mM stock solution) was added (see Figure S15, panel A) and the mixture was stirred at room temperature for 1 h. Analysis of the reaction mixture by RP-HPLC using method C (40 µL injection) showed 81 % conversion of the starting material to the target click product (see Figure S15, panel B).

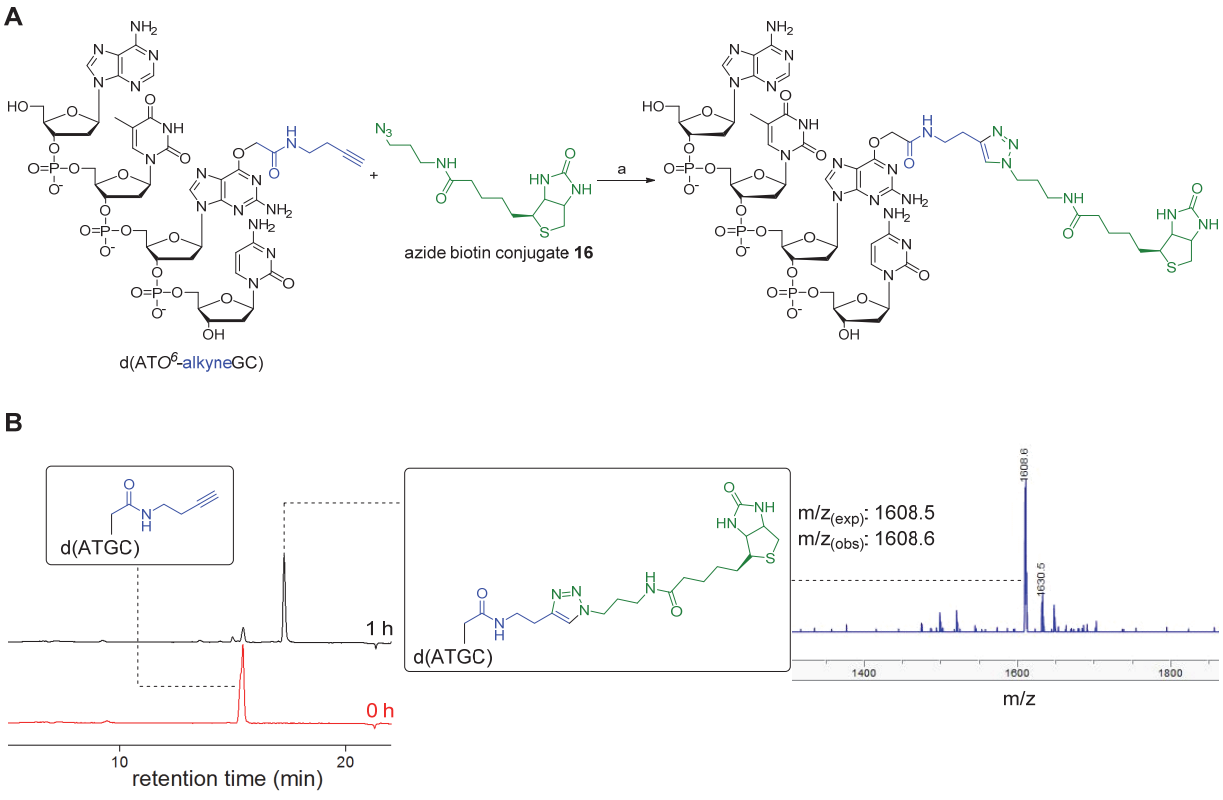
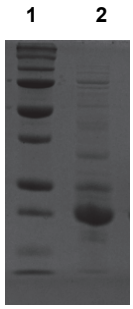


Figure S15: A Click reaction of d(ATO^6-alkyneGC) with biotin azide conjugate **16**. a) 5 eq.$CuSO_4$, 5 eq. sodium ascorbate, DMSO 50 % (v/v), 500 µM TEAA pH 7.25, 25°C, 1 h. **B** HPLC analysis using Method C of click reaction after 0 h = red and 1 h = black.

3 Reparation of O^6-G modified ssDNA 9 mer by hAGT

3.1 Expression and purification of hAGT

hAGT WT carrying an *N*-terminal hexa-histidine-tag was produced in *Escherichia coli* (*E.coli*) BL21 cells were grown in LB medium containing 50 mg/L ampicillin and 34 mg/L chloramphenicol. Protein expression was induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 18°C. After 16 h at 18°C the cells were harvested, re-suspended in lysis buffer (20 mM TrisHCl pH 8 and 300 mM NaCl), lysed by sonication and centrifuged for 20 min at 4°C. The protein was purified by nickelnitrilotriaceticacid affinity (Ni-NTA) by mixing the clear lysate with Ni(II)-NTA agarose (Qiagen) for 20 min at 4°C. The agarose beads were washed with lysis buffer containing 10 mM and 20 mM imidazole. After elution of the protein using 200 mM imidazole the fractions found to contain the hAGT using SDS PAGE (Figure S15) were pooled and precipitated with 72% ammonium sulfate. The pellet was re-dissolved in urea and dialyzed in 50 mM Tris-HCl pH 7.6, 250 mM NaCl and 5 mM DTT and stored at -20°C.

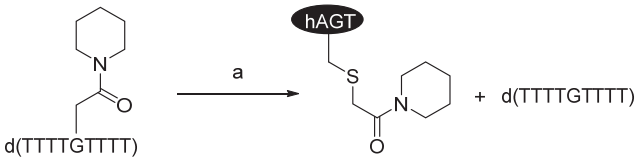


1: Protein ladder 10-250 kDA
2: Elution with 200 mM imidazole

Figure S16: SDS PAGE analysis of the purified hAGT protein.

3.2 De-alkylation of O^6-G modified ssDNA 9-mer

The O^6-G modified ssDNA 9-mer (79 µM) was incubated with hAGT (86 µM) for 4 h at 37°C (Scheme S5). After 0.5 and 4 h aliquots of 30 µL were taken, centrifuged, the supernatant analyzed by RP-HPLC using Method C and the pellet re-dissolved in 0.1 % formic acid containing water and analyzed by HR-MS (ESI).



Scheme S5: De-alkylation of O^6-G modified ssDNA 9-mer by hAGT. a) 50 mM TrisHCl buffer pH 7.6, 250 mM NaCl, 5 mM DTT, 37°C, 4 h.

4 Single primer extension experiment with synthetic dO^6-cmGTP

4.1 Oligonucleotides and DNA sequences

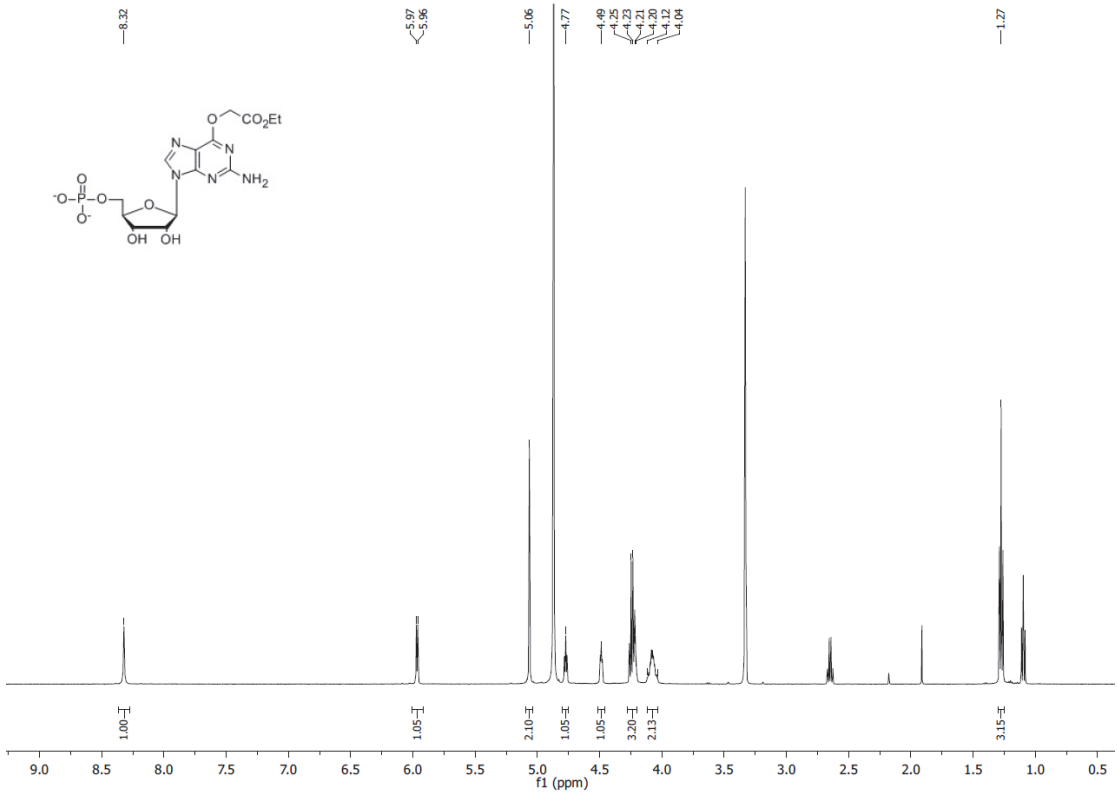
Table S1: Oligonucleotides and DNA sequences for primer extension experiment.

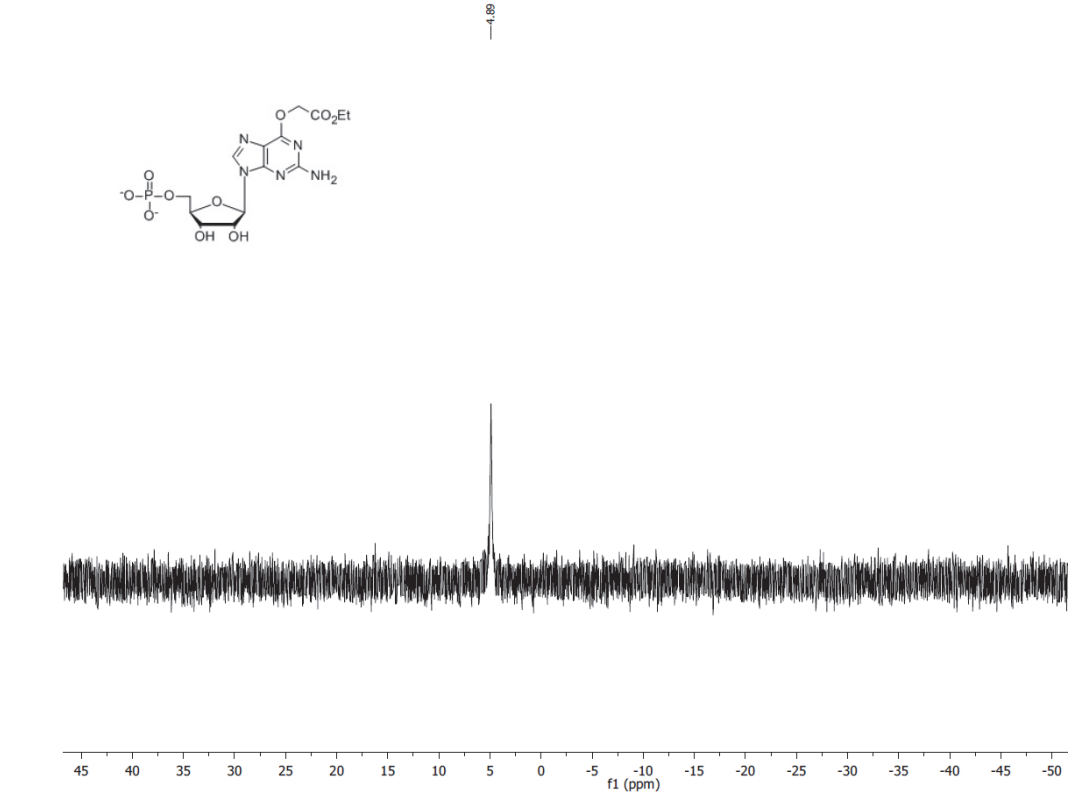
Primer	Sequence (5' - 3')
23-mer	TAA TAC GAC TCA CTA TAG GGA GA
Templates	
28-mer G	ACT C**G**T CTC CCT ATA GTG AGT CGT ATT A
28-mer A	ACT C**A**T CTC CCT ATA GTG AGT CGT ATT A
28-mer T	ACT C**T**T CTC CCT ATA GTG AGT CGT ATT A
28-mer C	ACT C**C**T CTC CCT ATA GTG AGT CGT ATT A

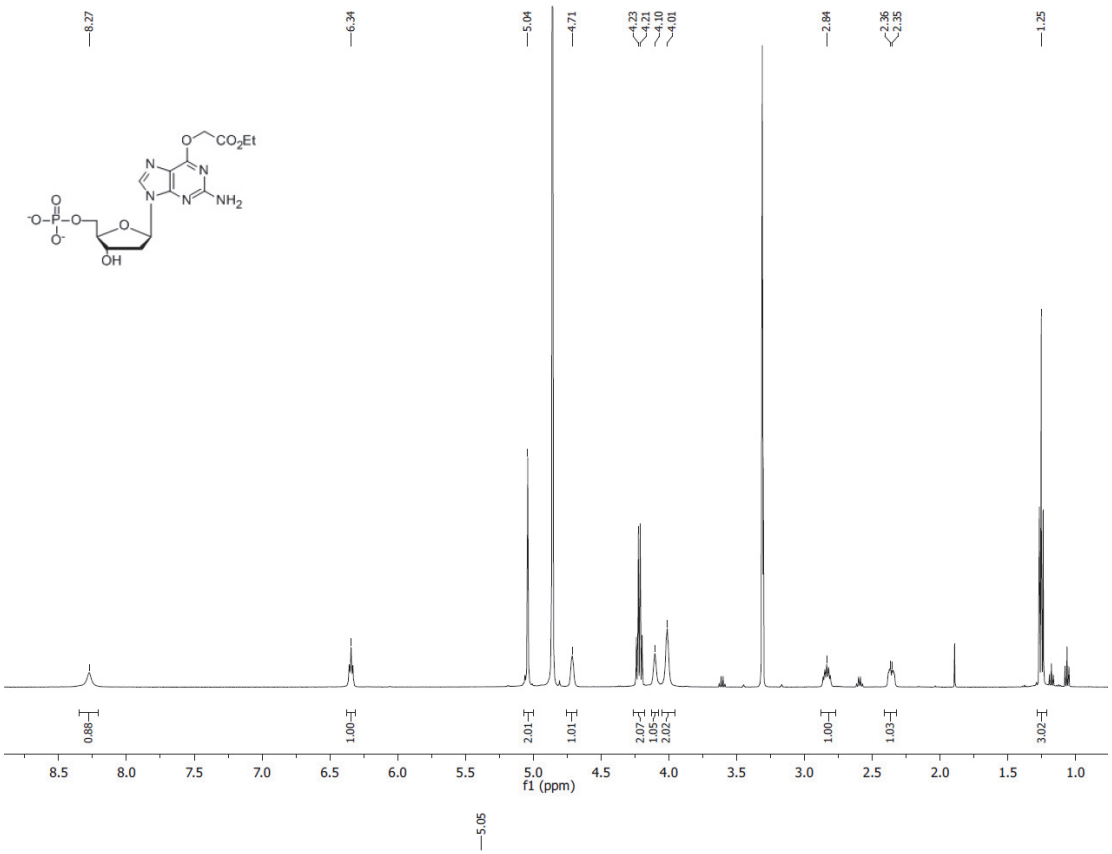
3.2 Primer extension assays

Radioactive labeling of primer strands at their 5'end was performed using T4 polynucleotide kinase (Promega) and [γ-^{32}P]ATP following the manufacturer protocol. 1 µM of primer was annealed with 1.5 µM of corresponding templates at 95°C for 5 minutes followed by a slow cooling over 2 hours. Standard primer extension reactions (10 µL) contained 1x reaction buffer (50 mM Tris HCl pH 9.2, 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.1% Tween 20), 5 nM DNA polymerase, 15 nM DNA (15 nM primer and 22.5 nM template), and 10 µM dNTPs. Reactions were initiated by adding dNTP(s) to the enzyme/DNA mixtures and allowed to react at 55°C for 10 min. Reactions were then quenched by the addition of 10 µL PAGE gel loading buffer (80% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanole FF) and the product mixtures were analyzed by 15% polyacrylamide/7M urea denaturing gels and subjected to autoradiography (Bio-Rad).

5 NMR Spectra



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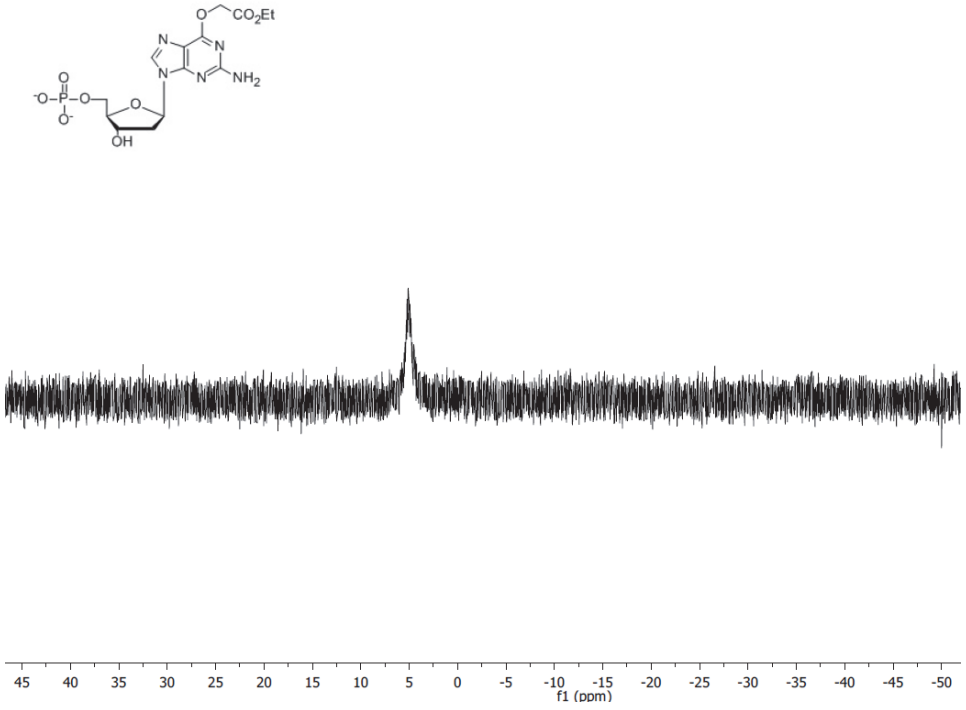
2.01
1.01
2.07
1.05
2.02
1.00
1.03
3.02
f1 (ppm)
5.05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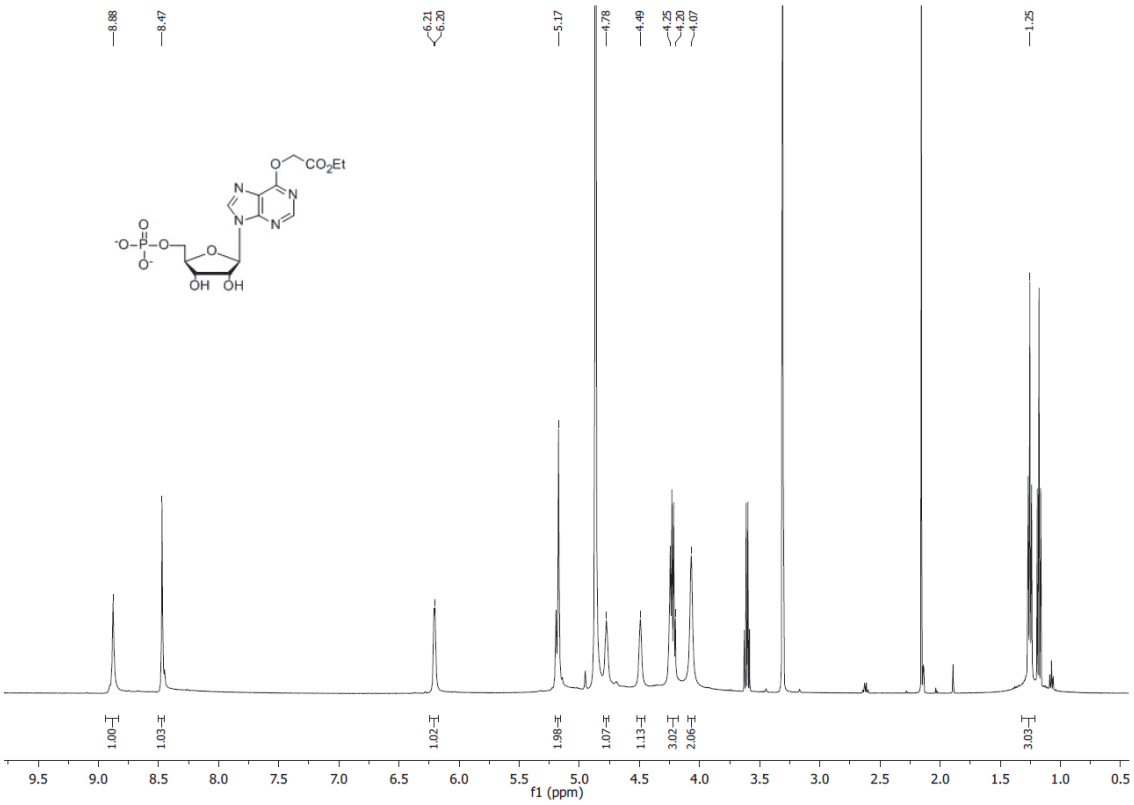
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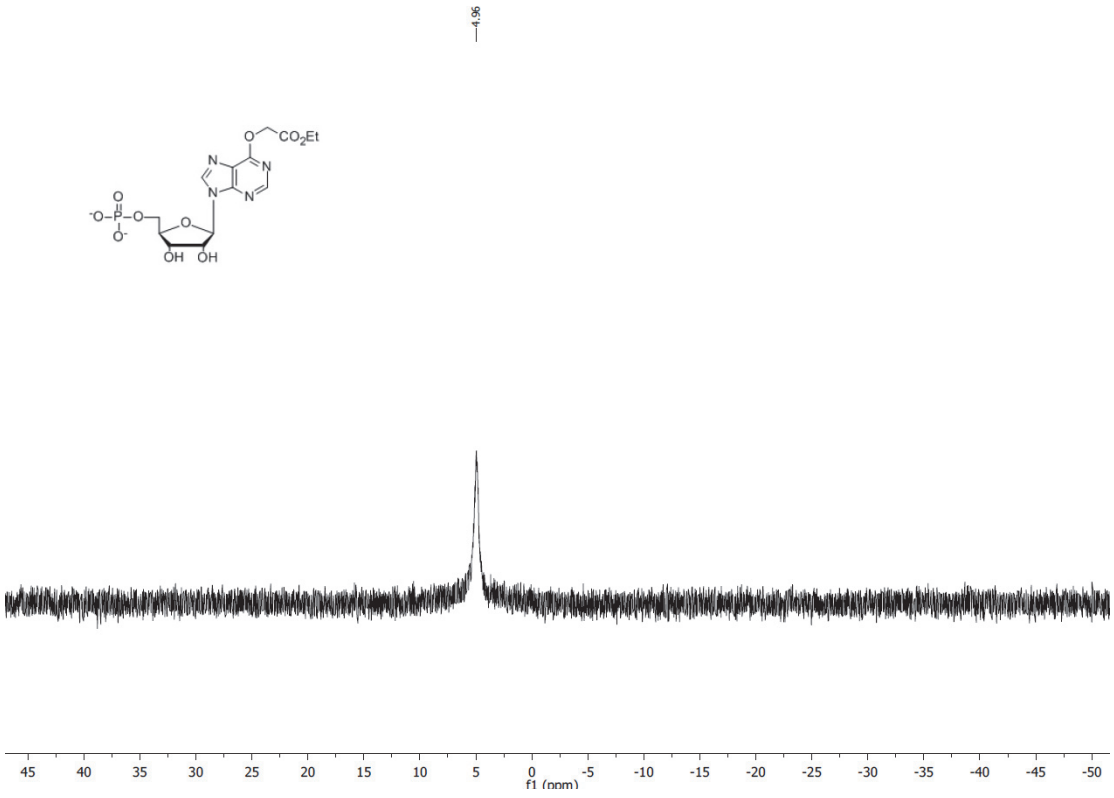
3.03
f1 (ppm)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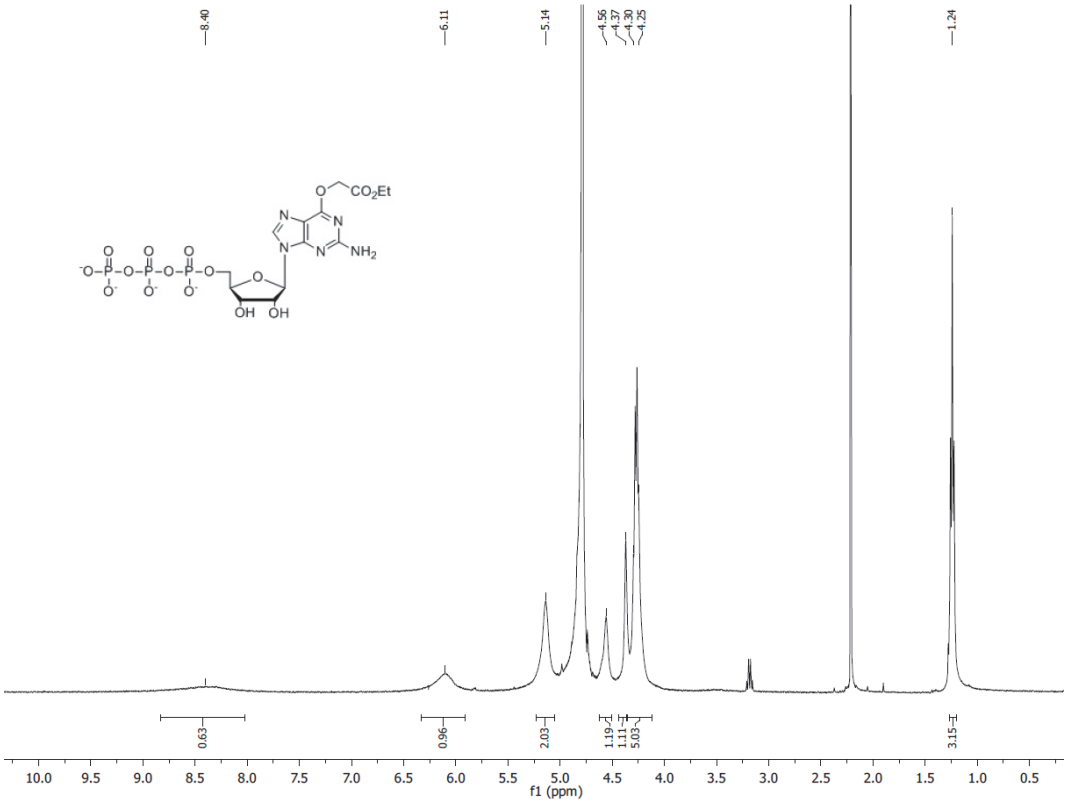
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5.03
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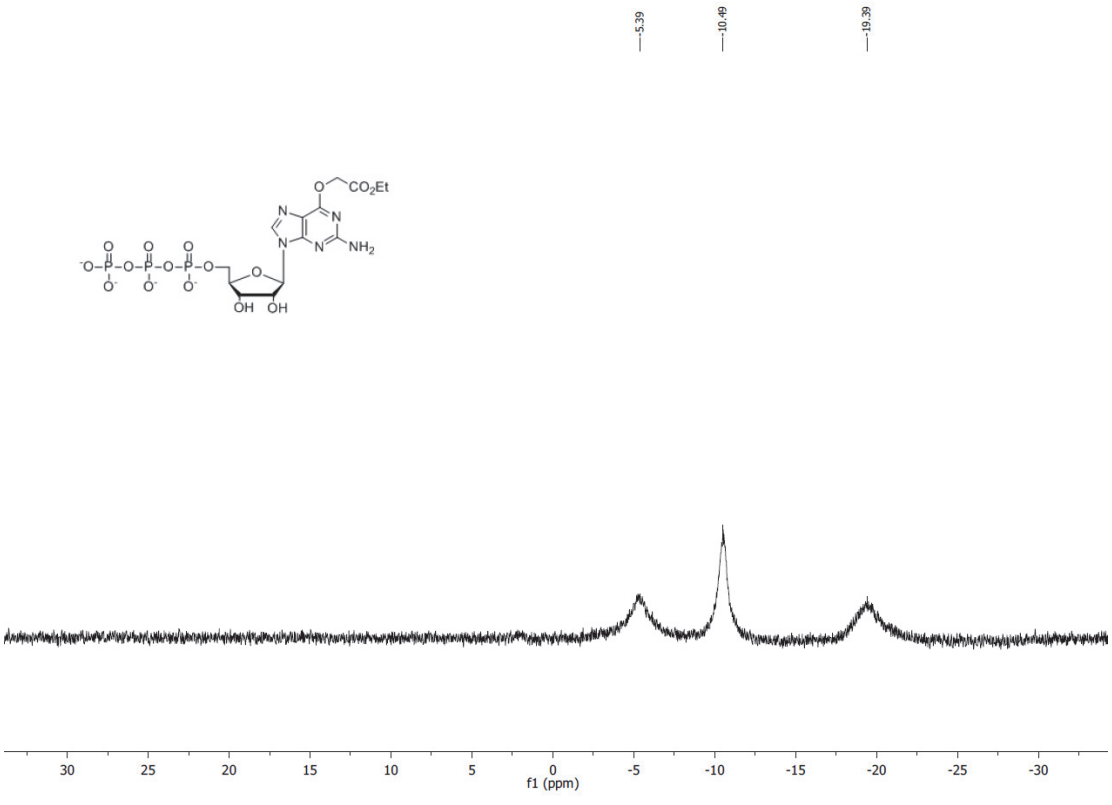
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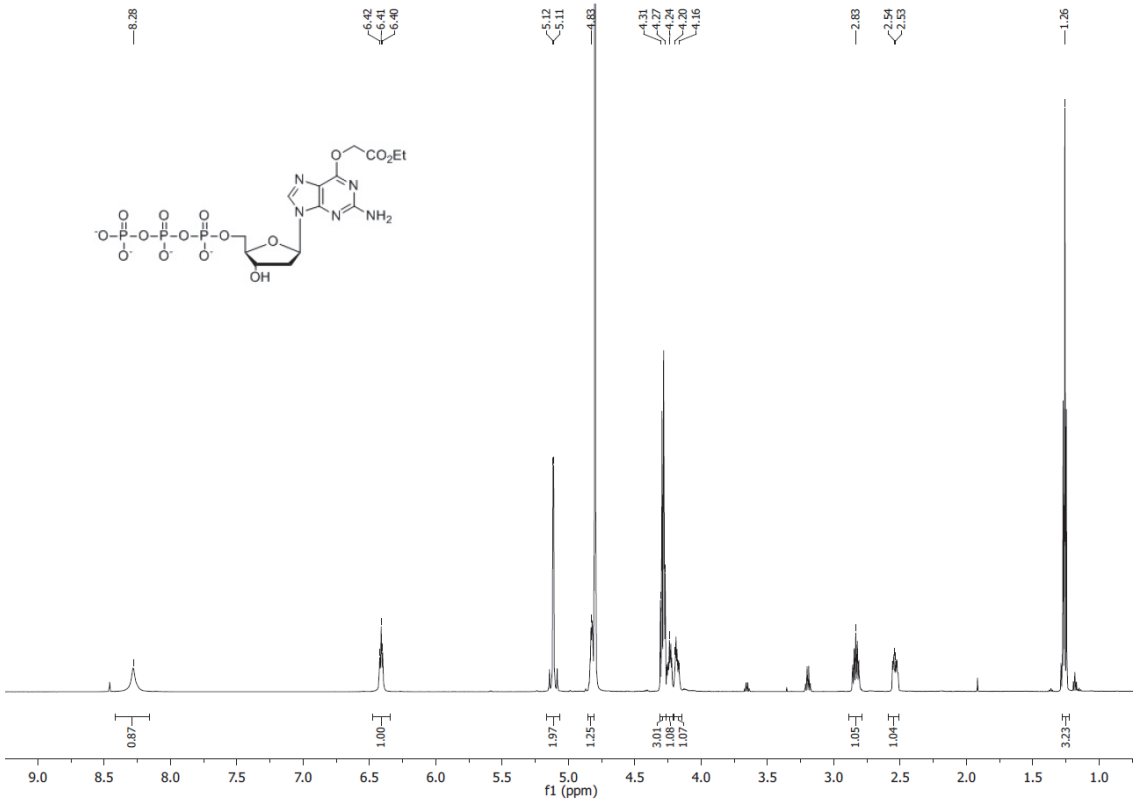
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5.11
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4.24
4.20
4.16
2.83
2.54
2.53
1.26
CO2Et
NH2
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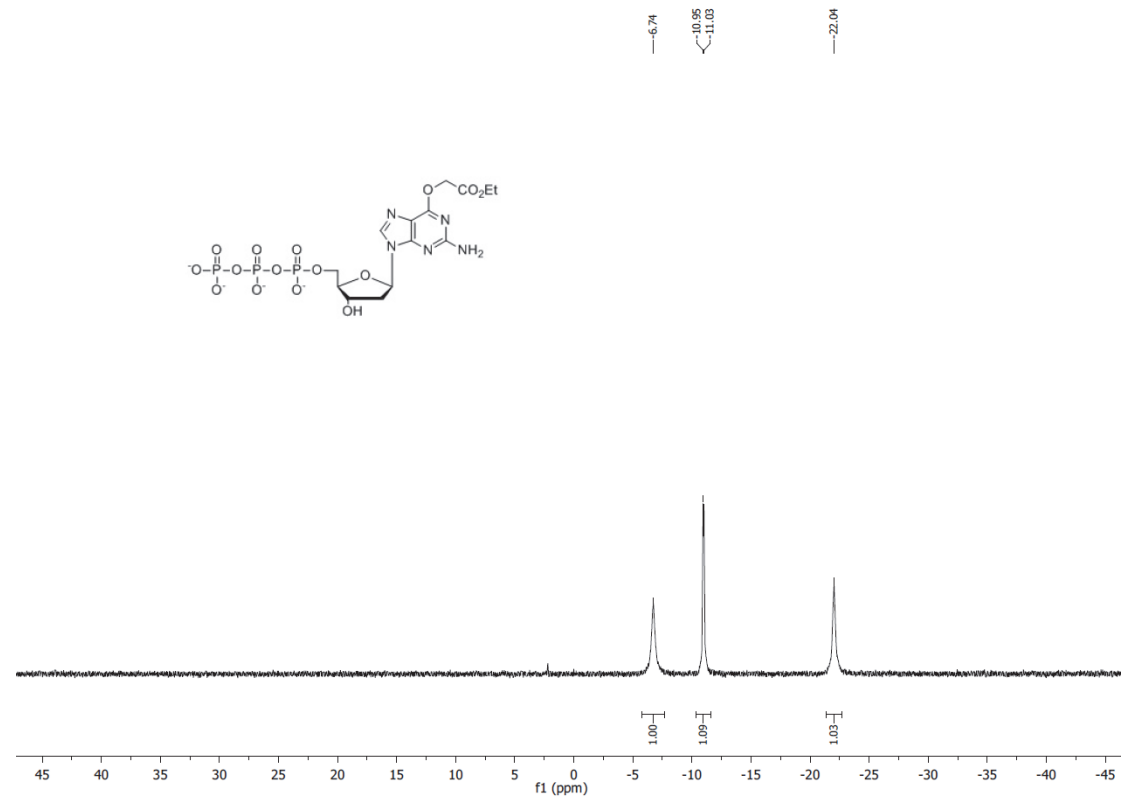
3.23
f1 (ppm)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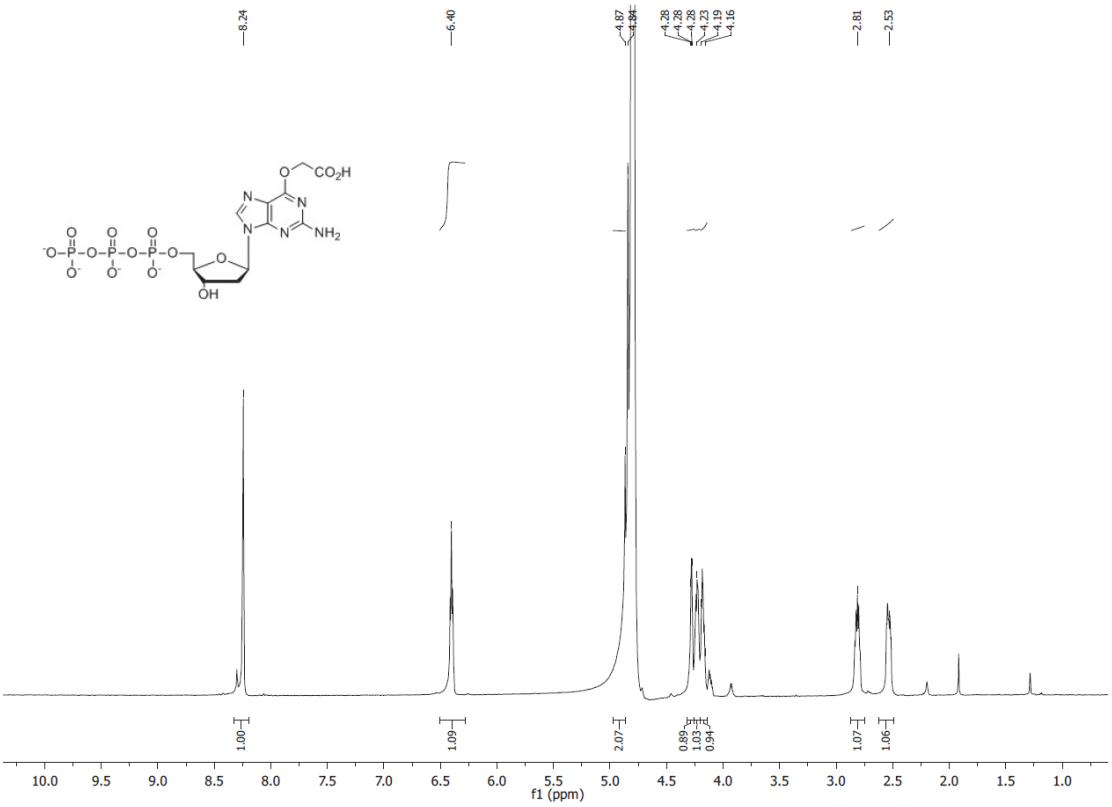
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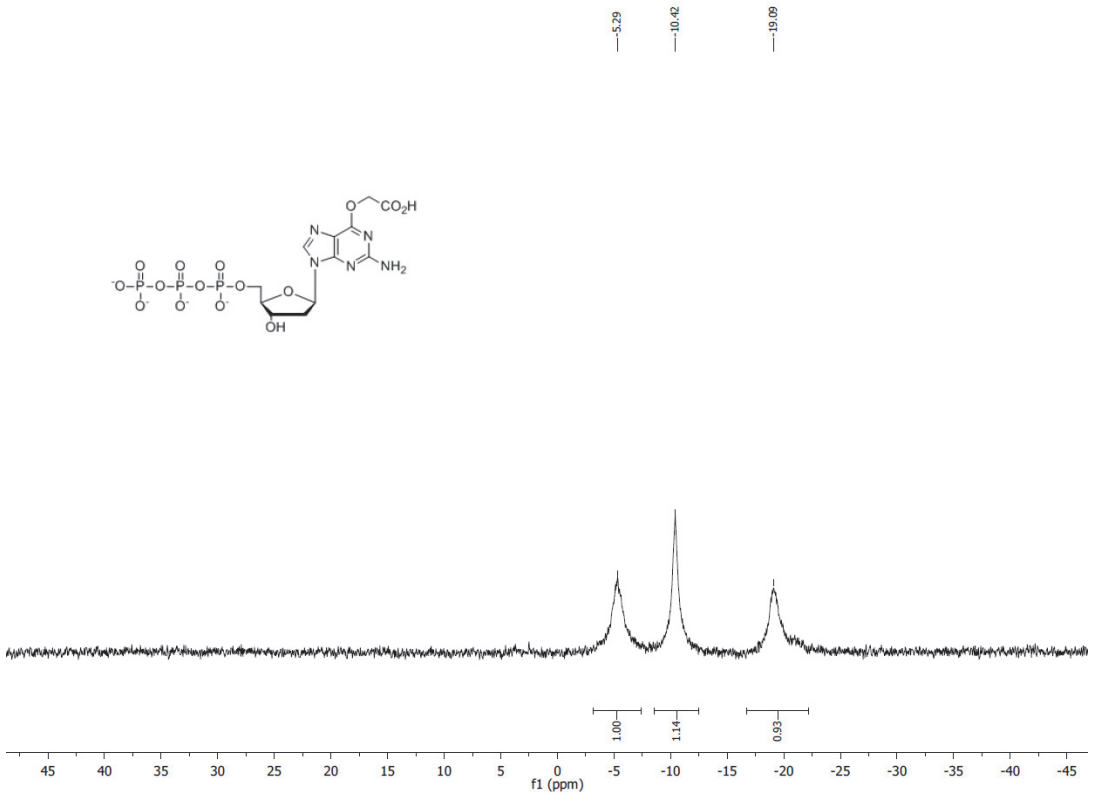
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0.93
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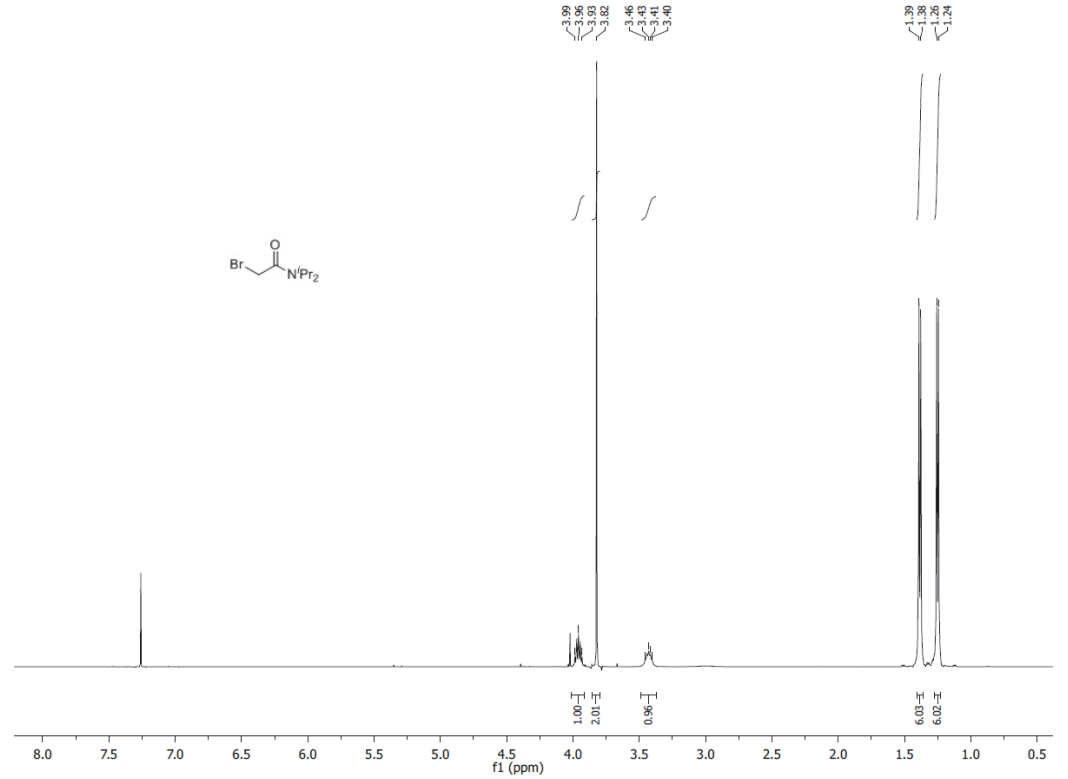
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3.43
3.41
3.40
1.39
1.38
1.26
1.24
Br
O
N^{i}Pr$_2$
1.00
2.01
0.96
6.03
6.02
8.0
7.5
7.0
6.5
6.0
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4.0
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3.0
2.5
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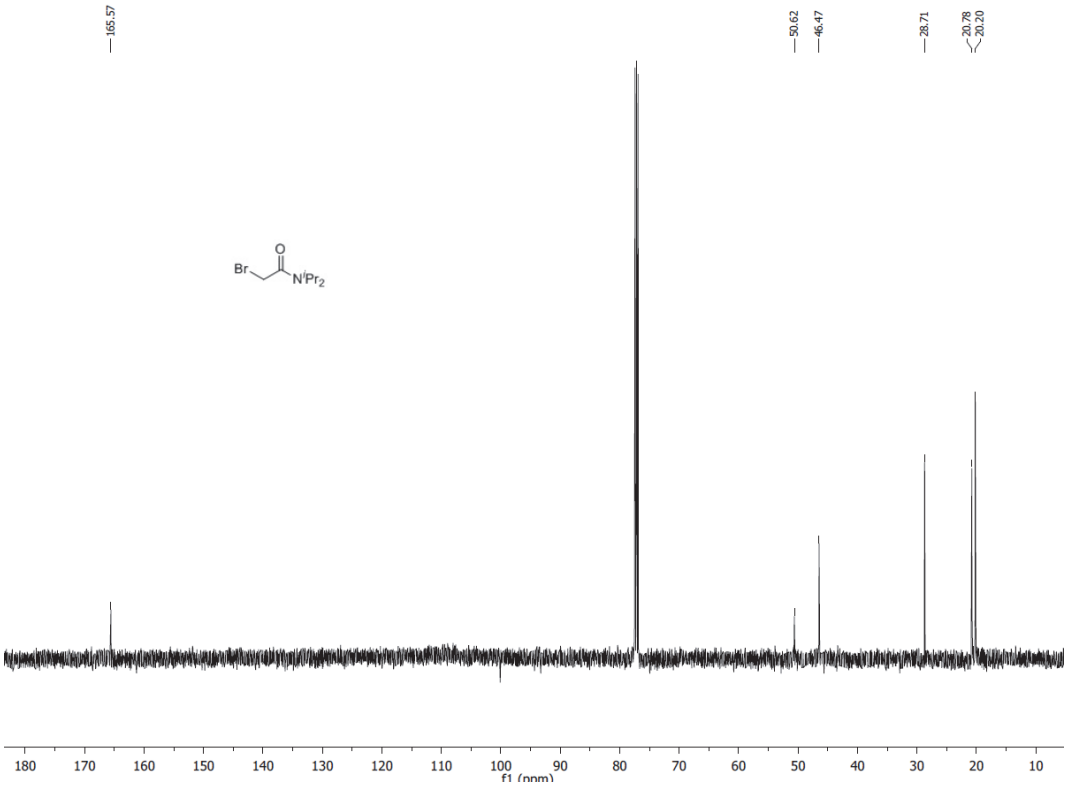
0.5
f1 (ppm)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O
N^{i}Pr$_2$
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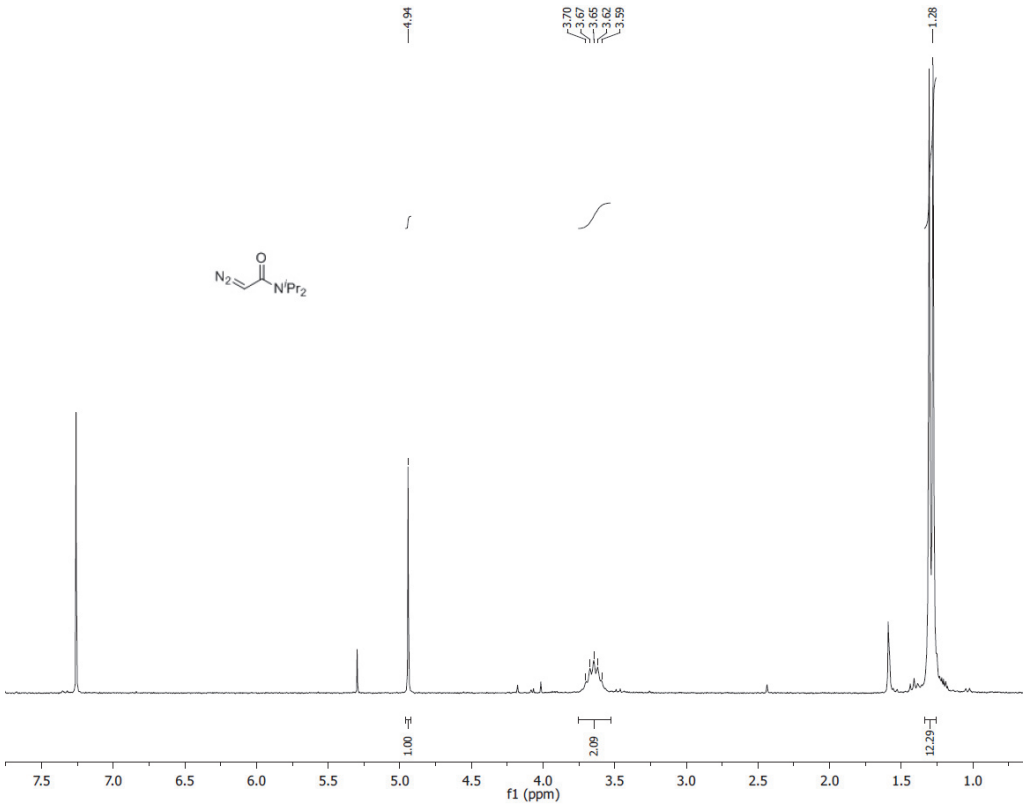
30
20
10
f1 (ppm)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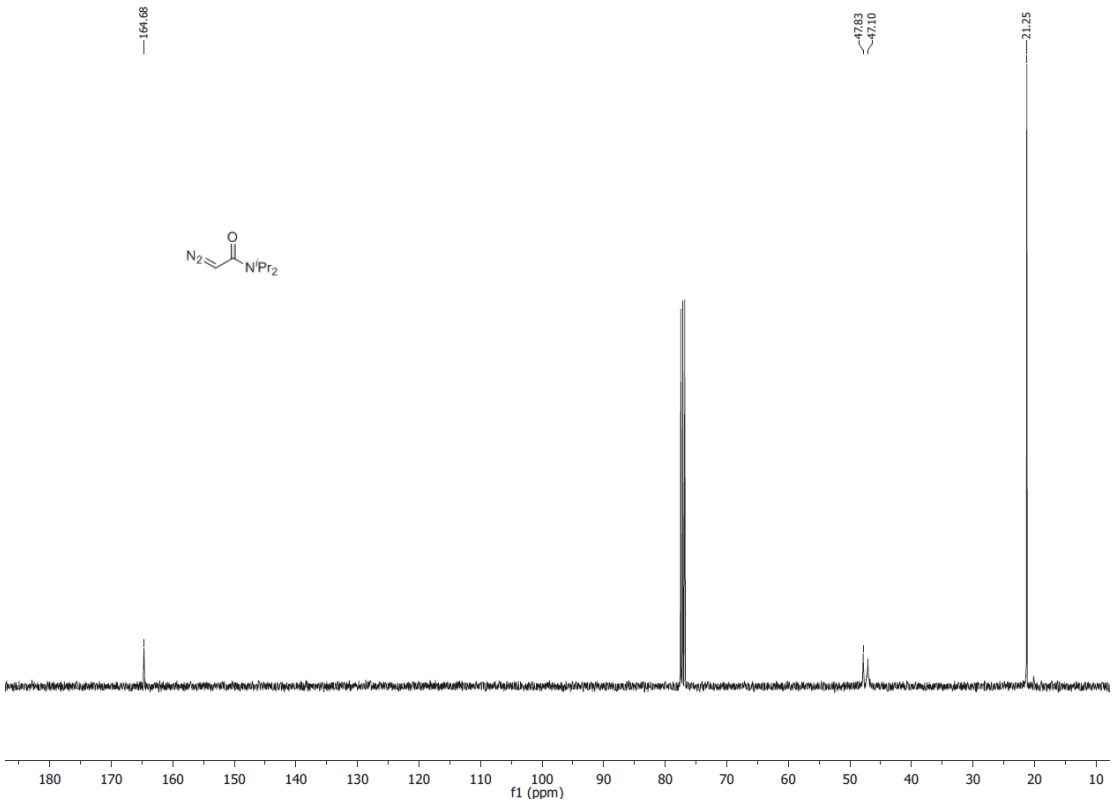
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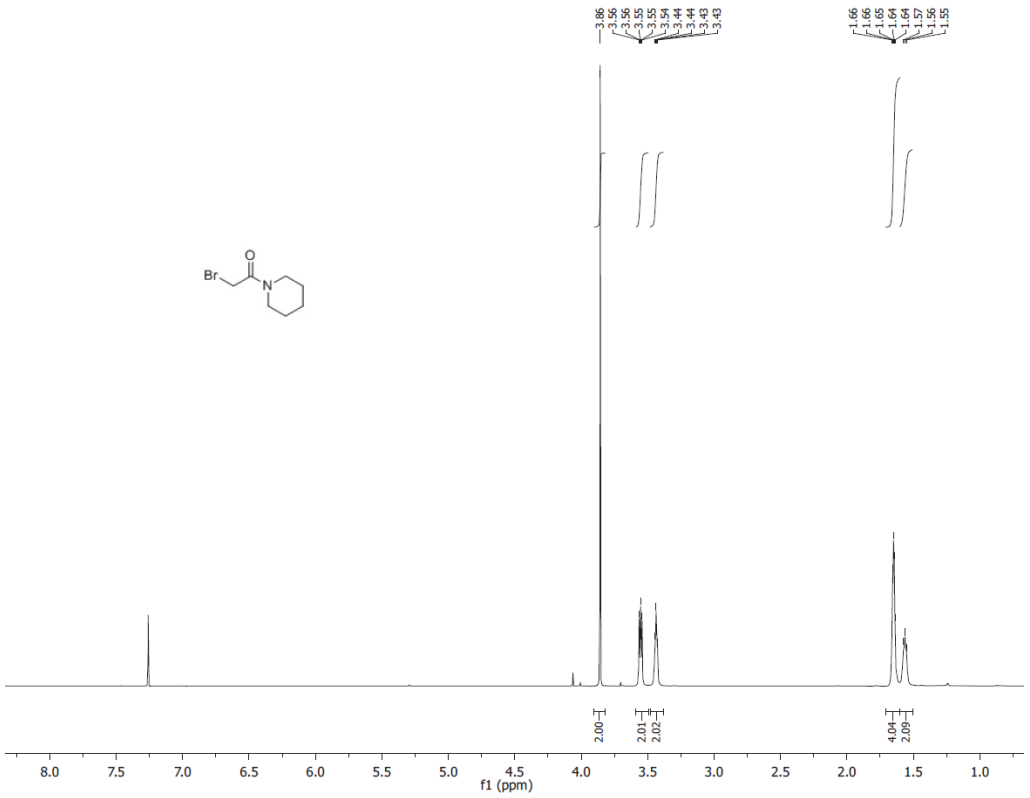
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O
N
2.00
2.01
2.02
4.04
2.09
8.0
7.5
7.0
6.5
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5.5
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4.5
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3.0
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2.0
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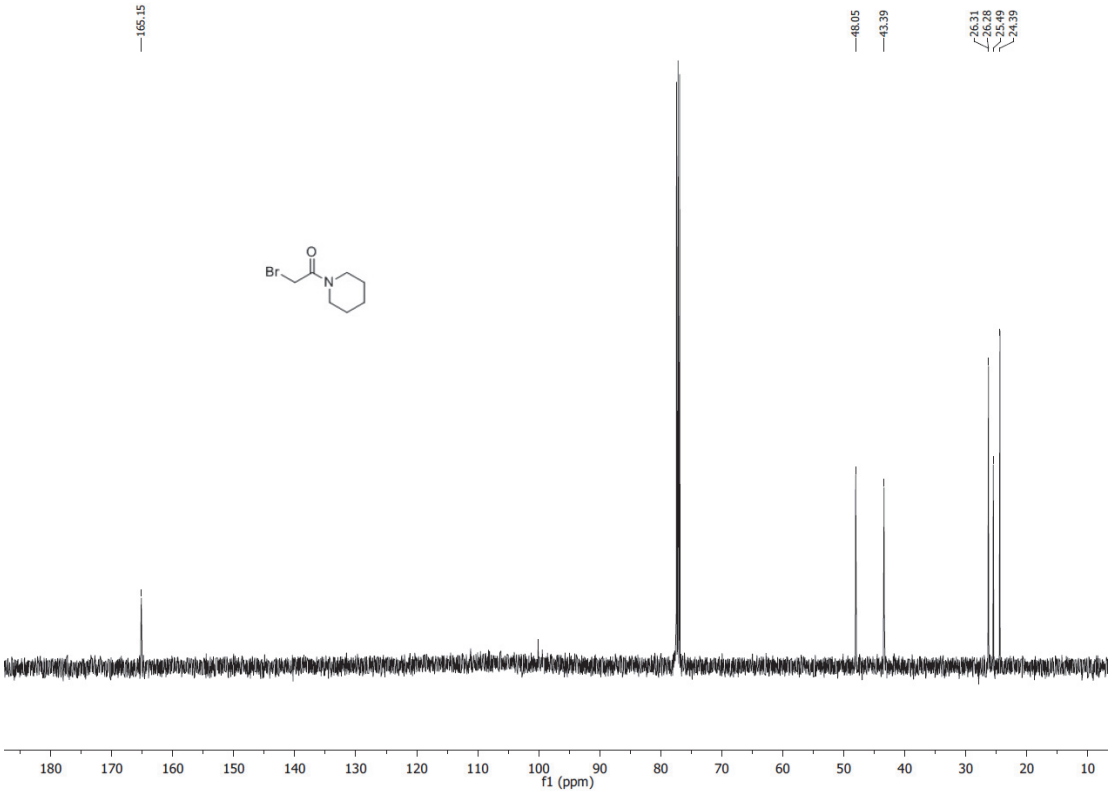
1.0
f1 (ppm)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40
30
20
10
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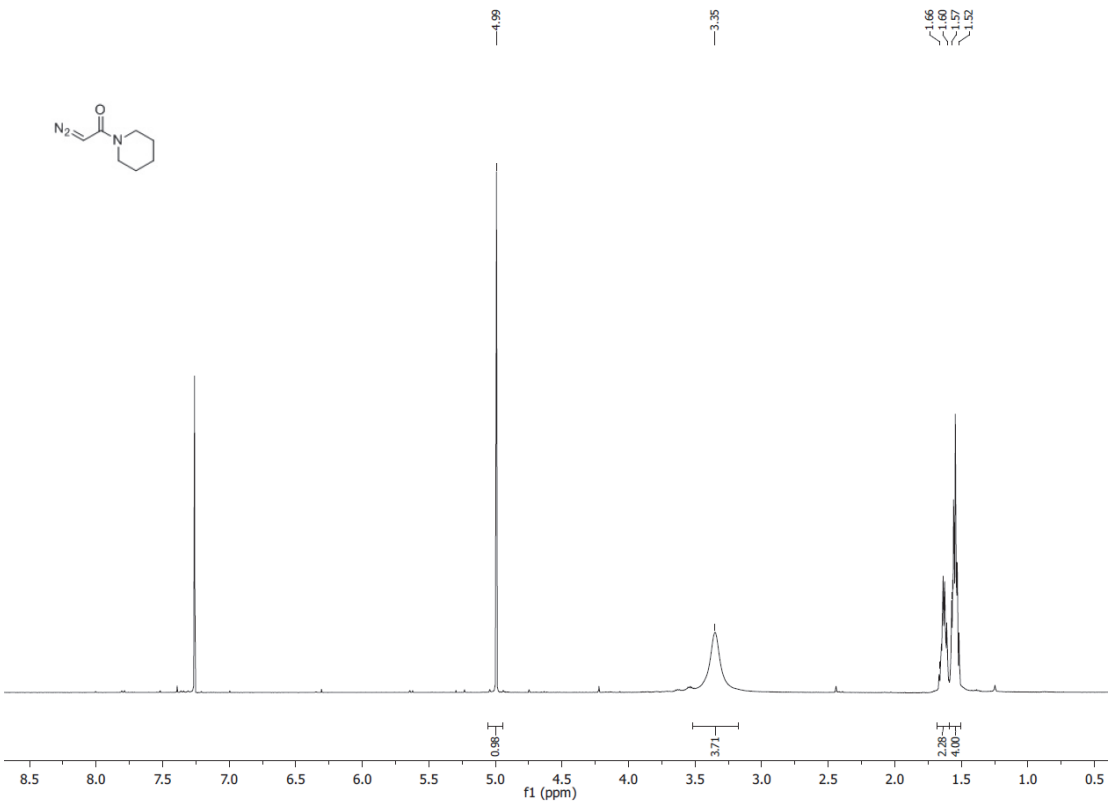
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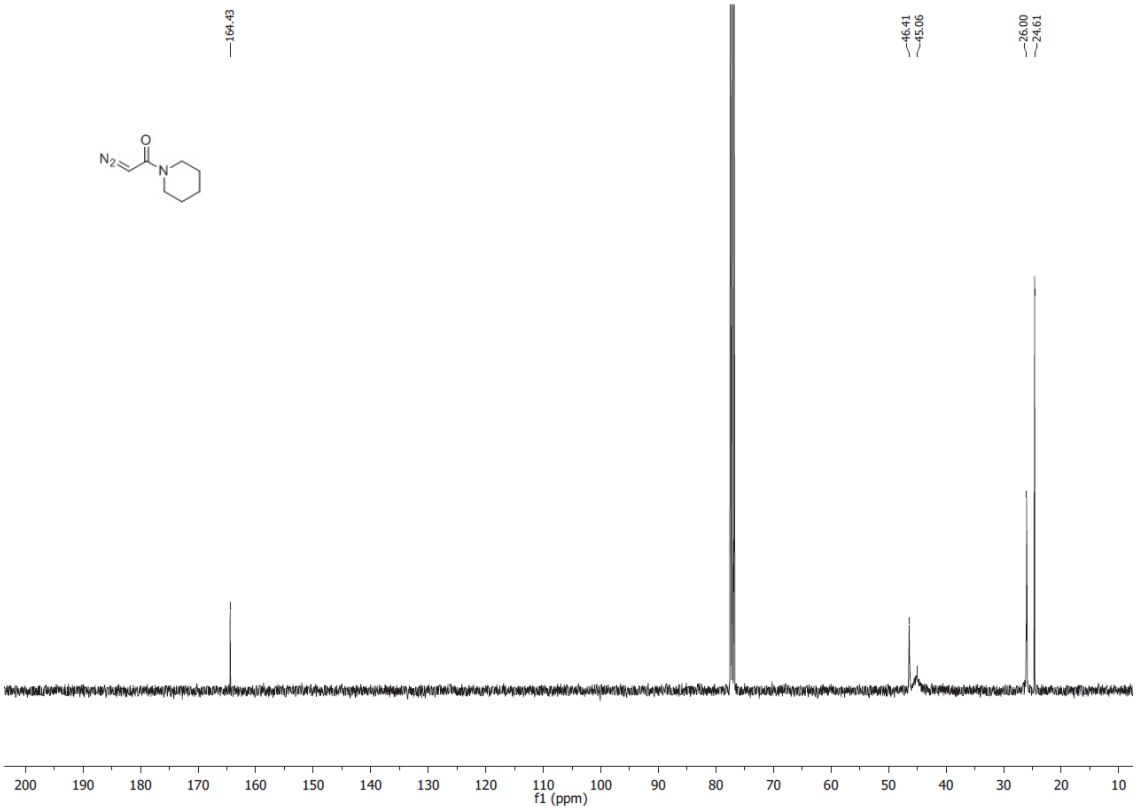
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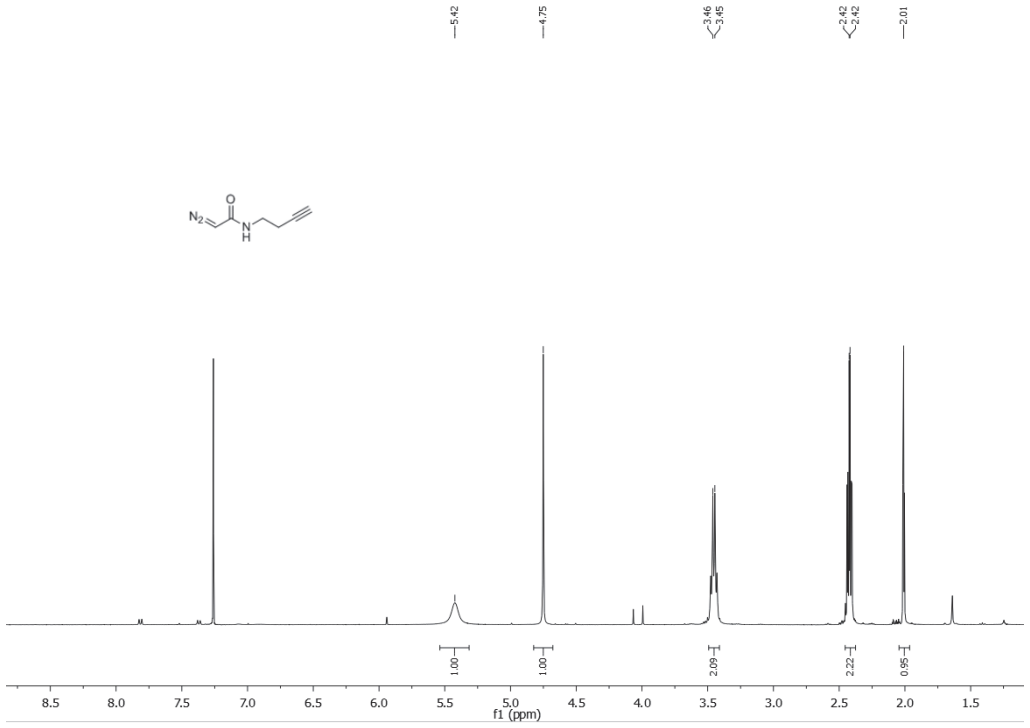
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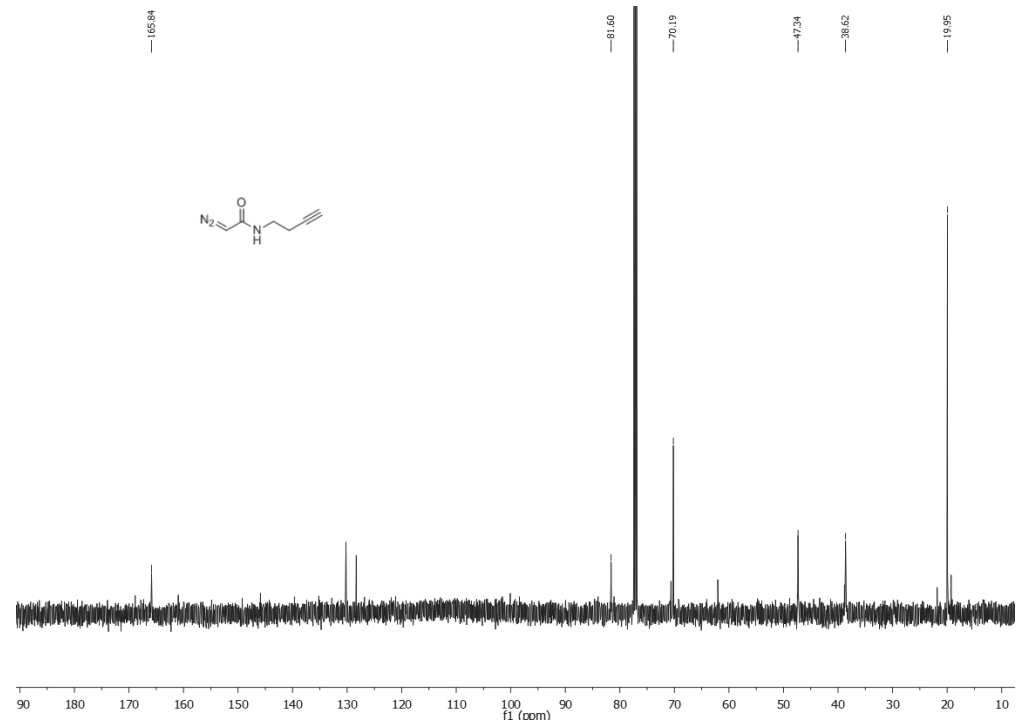
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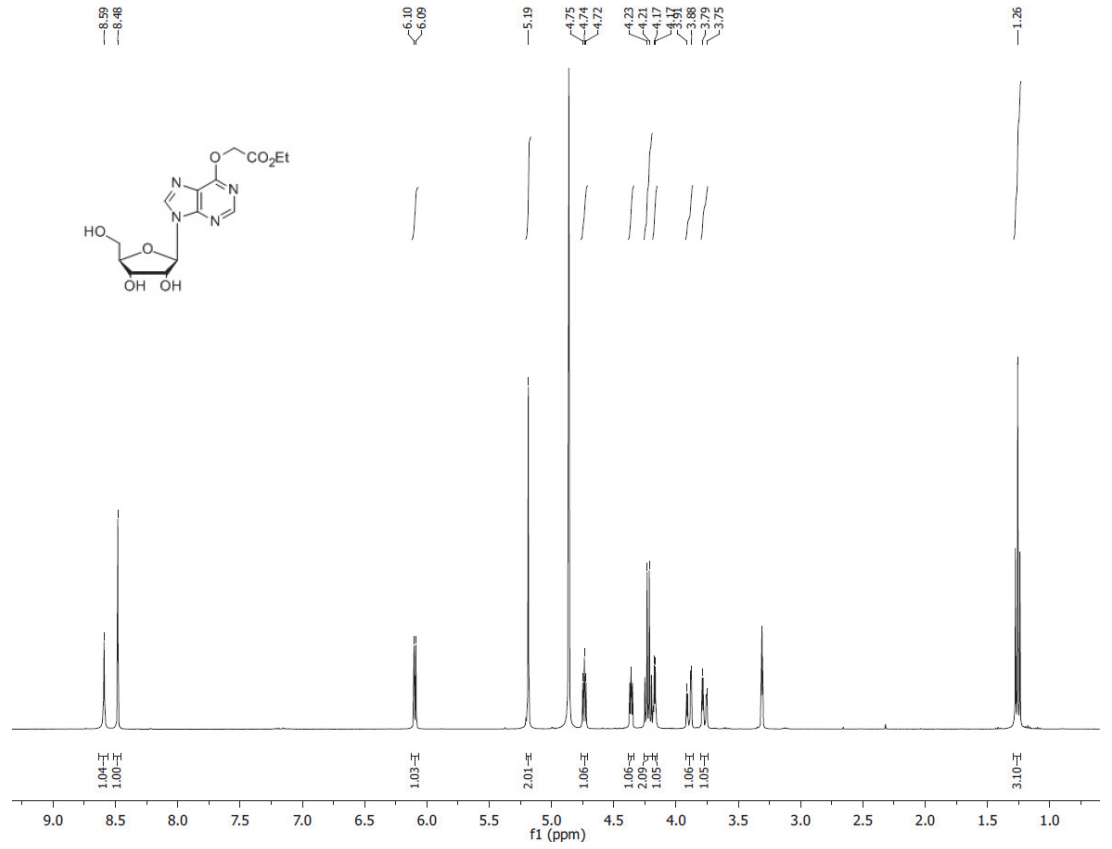
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3.79
3.75
1.26
CO2Et
HO
OH OH
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1.06
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1.06
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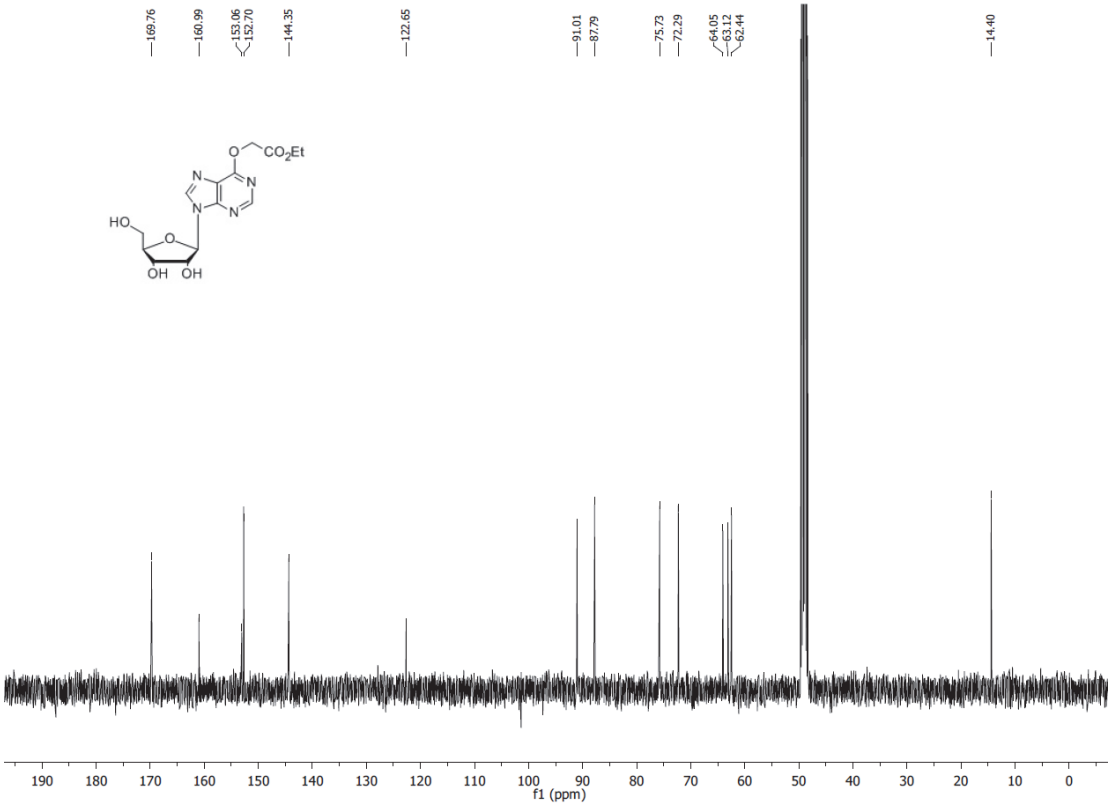
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HO
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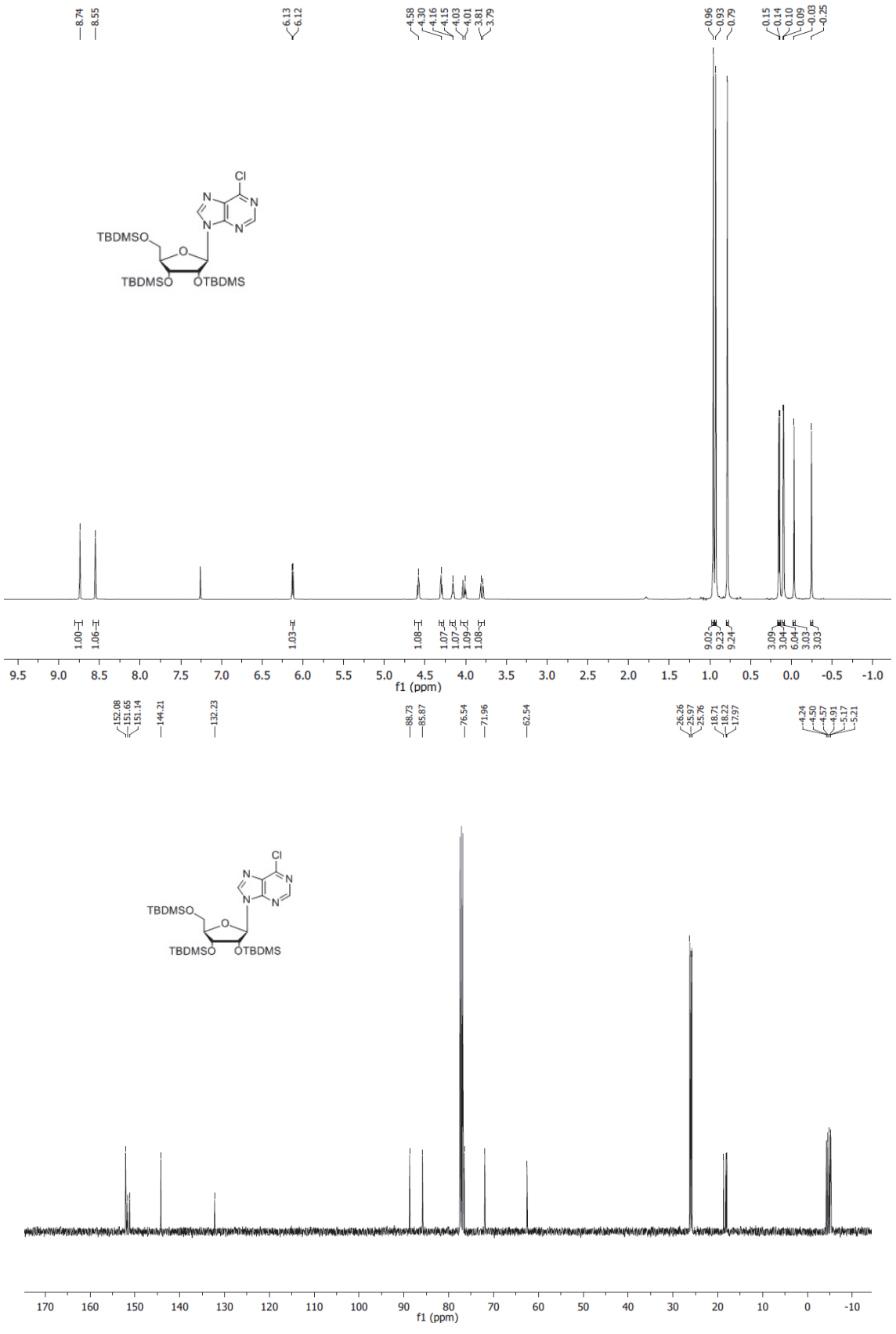
f1 (ppm)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4.01
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0.93
0.79
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0.03
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N
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N
TBDMSO
O
TBDMSO
OTBDMS
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1.03
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1.07
1.07
1.09
1.08
9.02
9.23
9.24
3.09
3.04
6.04
3.03
3.03
f1 (ppm)
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151.14
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132.23
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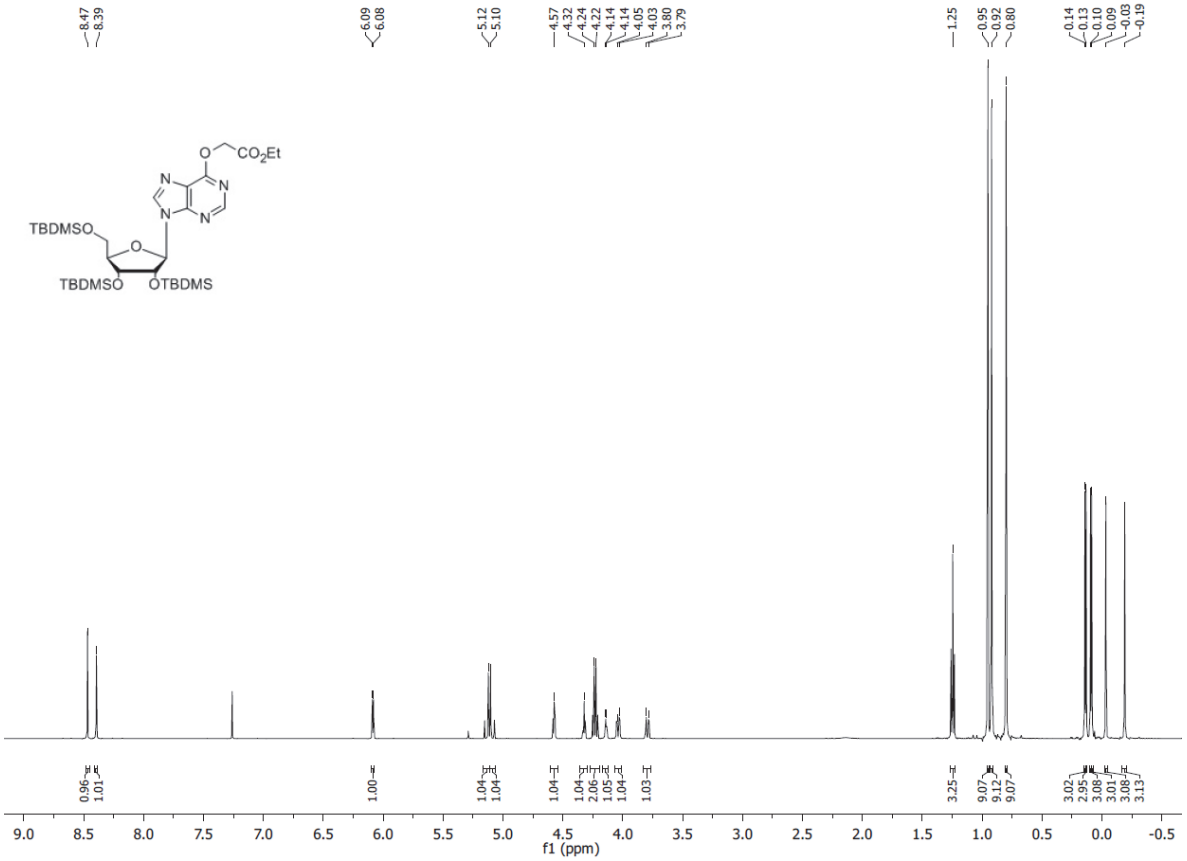
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0.14
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CO2Et
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1.04
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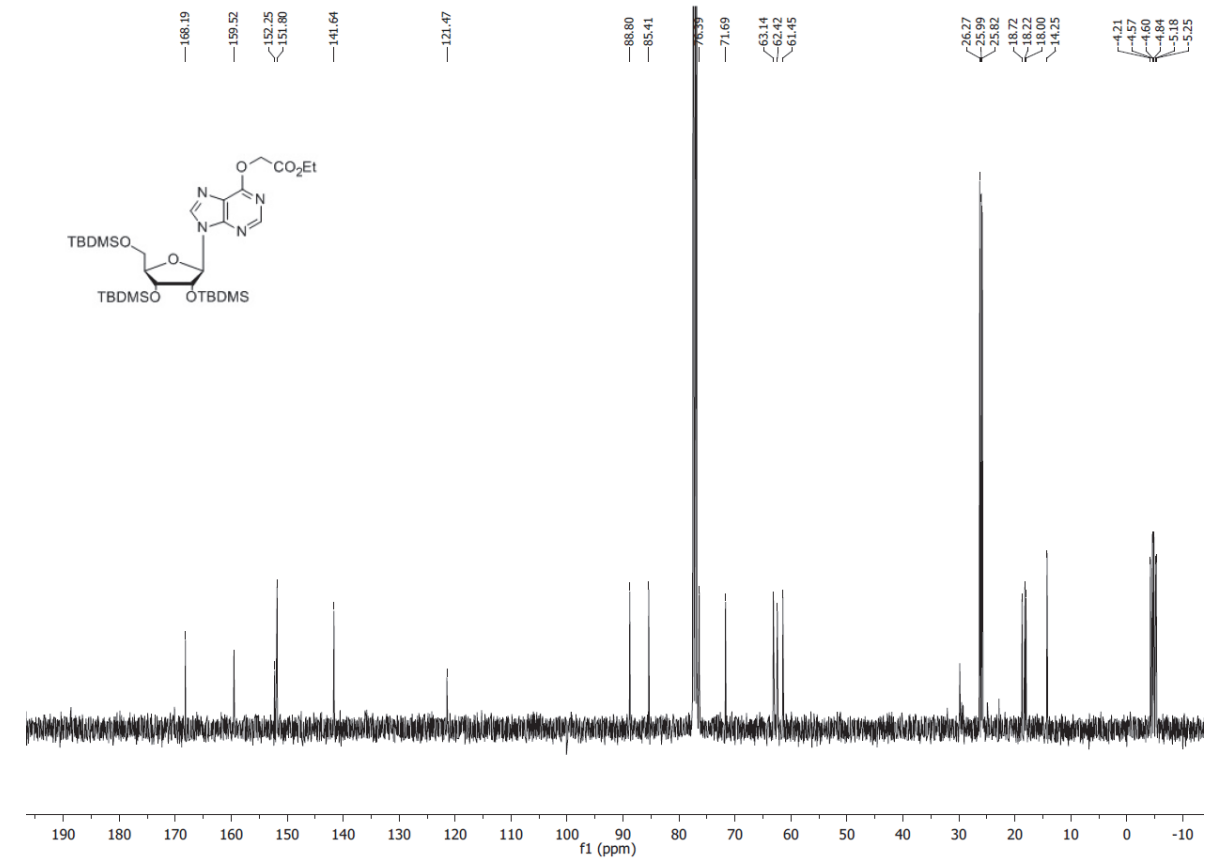
f1 (ppm)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26.27
25.99
25.82
18.72
18.22
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14.25
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-4.57
-4.60
-4.84
-5.18
-5.25
CO2Et
TBDMSO
TBDMSO
OTBDMS
f1 (ppm)

6 References

1: T. Toma, J. Shimokawa, T. Fukuyama, *Org. Lett.* **2007**, *9*, 3195-3197.

2: S. L. Riches, C. Saha, N. F Filgueira, E. Grange, E. M. McGarrigle, V. K. Aggarwal*, J. Am. Cem. Soc* **2010**, 133, 7626-7630.

3: P. Anastasopoulou, G. Kythreoti, T. Cosmidis, C. Pyrkotis, V. R. Nahmias, D. Vourloumis,*Bioorg. Med.Chem. Lett.* **2014**, 24, 1122-1126.

4: Xuejuan Wan, Guoying Zhang, Zhishen Ge, Ravin Naraina, Shiyong Liu, *Chem. Asian J.* **2011**, 6, 2835 – 2845