SUPPLEMENTARY INFORMATION

Atomic mutagenesis of N^6-methyladenosine reveals distinct recognition modes by human m^6A reader and eraser proteins

Florian Seitz[†], Tina Jungnickel[†], Nicole Kleiber[‡], Jens Kretschmer[‡], Julia Dietzsch[†], Juliane Adelmann[†], Katherine E. Bohnsack[‡], Markus T. Bohnsack[‡,⊥], and Claudia Höbartner[*,†,#].

[†]Institute of Organic Chemistry, University of Würzburg, Am Hubland, 97074 Würzburg, Germany. [‡]Department of Molecular Biology, University Medical Centre Göttingen, Humboldtallee 23, 37073 Göttingen, Germany. [⊥]Göttingen Center for Molecular Biosciences, Georg-August University Göttingen, Justus-von-Liebig-Weg 11, 37077 Göttingen, Germany. [#] Center for Nanosystems Chemistry, University of Würzburg, Theodor-Boveri-Weg, 97074 Würzburg, Germany.

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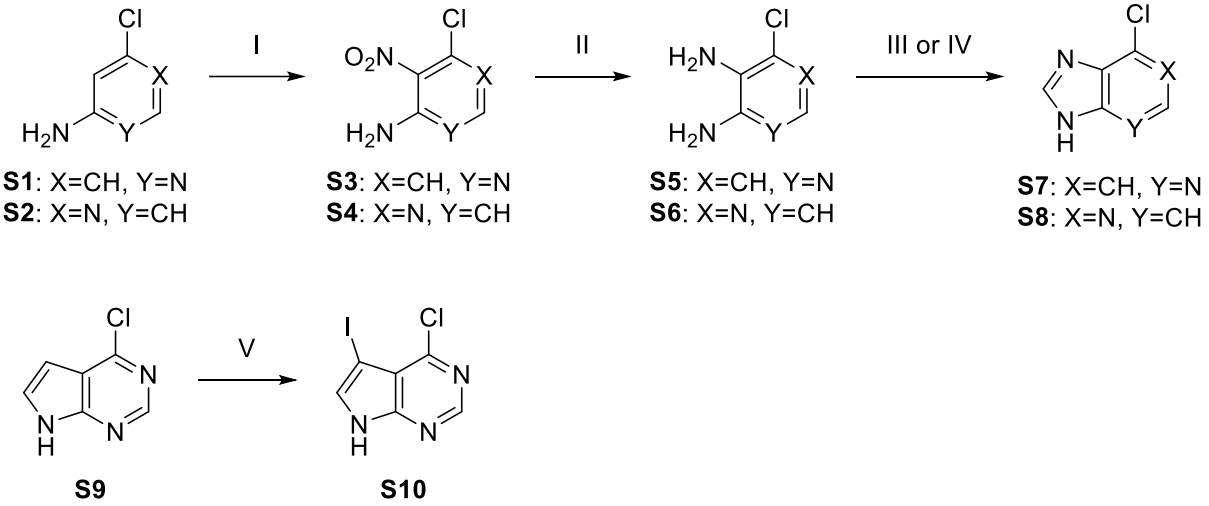
1. Synthesis of nucleosides and phosphoramidite building blocks

1.1. General Methods

Reagents used for synthesis were purchased in 'pro analysis' or 'pro synthesis' quality and used without further purification. Solvents used for synthesis were purchased in 'puriss. over molecular sieves', 'pro analysis' or 'pro synthesis' quality and used without further purification. For column chromatography, solvents in technical quality were purchased and purified by distillation. For solid-phase synthesis, acetonitrile and dichloroethane were dried over molecular sieves. Thin layer chromatography (TLC) was performed on aluminum plates precoated with silica gel 60 F254 (Merck). Substances were detected based on fluorescence quenching at 254 nm. For column chromatography, silica gel 60 (Merck) with a particle size of 40 – 63 µm was used. NMR spectra were recorded using Bruker Avance III (300 MHz), Bruker Avance III (400 MHz), Bruker Avance III HD (400 MHz), Bruker Avance III HD (500 MHz), and Varian Inova (600 MHz) spectrometers. Chemical shifts (δ) are given in ppm and were referenced using the deuterated solvent as internal standard. Data are reported as: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad; Coupling constants (J) are given in Hz. Peaks were assigned based on 2D-spectroscopy (COSY, HSQC, HMBC). High resolution (HR) electrospray ionization (ESI) mass spectra (MS) were recorded on Bruker micrOTOF-Q III and Bruker micrOTOF spectrometers. The detected mass-to-charge ratio (m/z) is given, as well as the calculated monoisotopic mass.

1.2. Experimental methods and compound characterization



S1: X=CH, Y=N
S2: X=N, Y=CH

S3: X=CH, Y=N
S4: X=N, Y=CH

S5: X=CH, Y=N
S6: X=N, Y=CH

S7: X=CH, Y=N
S8: X=N, Y=CH

S9 **S10**

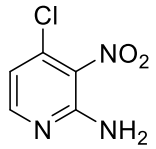
Scheme S1. Synthesis of the nucleobase precursors of 1-, 3-, and 7-deazaadenosine. I) H_2SO_4, HNO_3, 0 °C, 30 min, then rt, 19 h, 39% (for **S3**), 60% (for **S4**); II) NH_4Cl, Zn, MeOH, rt, 30 min, 96% (for **S5**), 84% (for **S6**); III) $(EtO)_3CH$, 110 °C, 3 h, then HCOOH, 110 °C, 2.5 h, 80% (for **S7**); IV) $(MeO)_3CH$, aq. HCl, rt, 16 h, 81% (for **S8**); V) NIS, DMF, rt, 30 min, 80%.

1-Deaza-6-chloropurine (**S7**) and 3-deaza-6-chloropurine (**S8**) were synthesized from 2-amino-4-chloropyridine (**S1**) and 4-amino-2-chloropyridine (**S2**), respectively, by nitration at C3 followed by reduction of the nitro group and cyclization using triethyl orthoformate. 6-Chloro-7-iodo-7-deazapurine (**S10**) was obtained from commercially available 6-chloro-7-deazapurine (**S9**) by treatment with *N*-iodosuccinimide (NIS).

General procedure A: Nitration of amino-chloropyridines

Amino-chloropyridine (5.00 g, 38.9 mmol, 1.00 eq.) was dissolved in concentrated H_2SO_4 (70 mL) at 0 °C. Concentrated HNO_3 (2.60 mL, 62.3 mmol, 1.61 eq.) was added dropwise. The reaction mixture was stirred at 0 °C for 30 min, then allowed to warm to ambient temperature and stirred for 19 h. The mixture was poured onto crushed ice (200 g) and adjusted to pH = 9 with 25% aq. ammonia. The resulting suspension was extracted with EtOAc (6 x 200 mL). The combined organic layers were dried over Na_2SO_4 and the solvent was removed under reduced pressure. The residue was purified by column chromatography.

2-Amino-4-chloro-3-nitropyridine (**S3**)



S3

$C_5H_4ClN_3O_2$

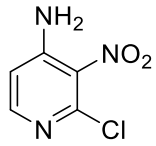
173.56 g·mol^{-1}

2-Amino-4-chloropyridine (**S1**) (5.00 g, 38.9 mmol, 1.00 eq.) was converted according to general procedure A and purified by column chromatography (silica gel, *n*-hexane:EtOAc 3:1) to yield the product **S3** as a yellow solid (2.51 g, 14.5 mmol, 39%). Analytical data agreed with reported values.[1]

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 8.12 (d, *J* = 5.3 Hz, 1H, C6-H), 7.25 (s, 2H, NH_2), 6.86 (d, *J* = 5.3 Hz, 1H, C5-H).
^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6): δ (ppm) = 152.69 (C2), 152.03 (C6), 135.89 (C4), 129.40 (C3), 113.00 (C5).

HR-ESI-MS: *m*/*z* calc. ($C_5H_5ClN_3O_2$ $[M+H]^+$): 174.0065, found: 174.0069.

4-Amino-2-chloro-3-nitropyridine (**S4**)



S4

$C_5H_4ClN_3O_2$

173.56 g·mol^{-1}

4-Amino-2-chloropyridine (**S2**) (5.00 g, 38.9 mmol, 1.00 eq.) was converted according to general procedure A and purified by column chromatography (silica gel, *n*-hexane:EtOAc 1:1) to yield the product **S4** as a yellow solid (4.05 g, 23.3 mmol, 60%). Analytical data agreed with reported values.[2]

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 7.89 (d, *J* = 5.9 Hz, 1H, C6-H), 7.35 (s, 2H, NH_2), 6.81 (d, *J* = 5.9 Hz, 1H, C5-H).

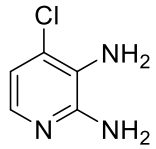
^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6): δ (ppm) = 149.09 (1C, C4), 148.79 (1C, C6), 142.23 (1C, C2), 130.64 (1C, C3), 112.00 (1C, C5).

HR-ESI-MS: *m*/*z* calc. ($C_5H_5ClN_3O_2$ $[M+H]^+$): 174.0065, found: 174.0066.

General procedure B: Reduction of amino-chloro-nitropyridines

Amino-chloro-nitropyridine (2.31 g, 13.3 mmol, 1.00 eq.) and NH_4Cl (7.12 g, 133 mmol, 10.0 eq.) were dissolved in MeOH (270 mL) under inert gas atmosphere and stirred at ambient temperature for 5 min. The mixture was cooled to 0 °C and Zn powder (17.4 g, 266 mmol, 20.0 eq.) was added in portions. The reaction mixture was allowed to warm to ambient temperature and stirred for 30 min. Solids were removed by filtration through a pad of Celite and the filtrate evaporated to dryness. The residue was taken up in EtOAc (200 mL) and water (200 mL) and the aqueous layer was adjusted to pH = 9 by addition of 25% aq. ammonia. The layers were separated, and the aqueous layer was extracted with EtOAc (4 × 200 mL). The combined organic layers were dried over Na_2SO_4 and the solvent was removed under reduced pressure.

2,3-Diamino-4-chloropyridine (**S5**)



S5

$C_5H_6ClN_3$

143.57 g·mol^{-1}

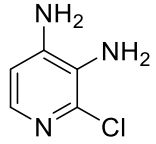
Compound **S3** (2.31 g, 13.3 mmol, 1.00 eq.) was converted according to general procedure B to yield the product **S5** as a light brown solid (1.82 g, 12.7 mmol, 96%). Analytical data agreed with reported values.[3]

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 7.20 (d, *J* = 5.5 Hz, 1H, C6-H), 6.49 (d, *J* = 5.5 Hz, 1H, C5-H), 5.78 (s, 2H, C2-NH_2), 4.90 (s, 2H, C3-NH_2).

^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6): δ (ppm) = 149.22 (1C, C2), 134.85 (1C, C6), 126.31 (1C, C3), 122.24 (1C, C4), 113.20 (1C, C5).

HR-ESI-MS: *m*/*z* calc. ($C_5H_7ClN_3$ $[M+H]^+$): 144.0323, found: 144.0326.

3,4-Diamino-2-chloropyridine (**S6**)



S6

$C_5H_4ClN_3$

143.57 g·mol^{-1}

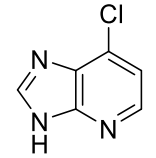
Compound **S4** (3.34 g, 19.2 mmol, 1.00 eq.) was converted according to general procedure B. After workup, the crude product was purified by column chromatography (silica gel, EtOAc) to yield the product **S6** as a light brown solid (2.42 g, 16.8 mmol, 84%).

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 7.28 (d, *J* = 5.1 Hz, 1H, C6-H), 6.43 (d, *J* = 5.1 Hz, 1H, C5-H), 5.76 (s, 2H, C4-NH_2), 4.65 (s, 2H, C3-NH_2).

^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6): δ (ppm) = 142.94 (1C, C4), 137.56 (1C, C6), 135.14 (1C, C2), 126.20 (1C, C3), 108.26 (1C, C5).

HR-ESI-MS: *m*/*z* calc. ($C_5H_7ClN_3$ $[M+H]^+$): 144.0323, found: 144.0324.

6-Chloro-1-deazapurine (**S7**)



S7

$C_6H_4ClN_3$

153.57 g·mol^{-1}

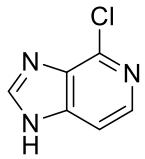
Compound **S5** (1.72 g, 12.0 mmol, 1.00 eq.) was suspended in triethyl orthoformate (25.8 mL, 156 mmol, 13.0 eq.) under inert gas atmosphere. The reaction was stirred at 110 °C for 1 h. The solvent was removed under reduced pressure, and the residue was dissolved in formic acid (38.6 mL, 234 mmol, 19.5 eq.) and stirred at 110 °C for 3 h. The solution was adjusted to pH = 7 by addition of 25% aq. ammonia and extracted with EtOAc (5 × 20 mL). The combined organic layers were dried over Na_2SO_4 and the solvent was removed under reduced pressure to yield the product **S7** as a light brown solid (1.57 g, 10.3 mmol, 86%).

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 13.45 (s, 1H, NH), 8.53 (s, 1H, C8-H), 8.30 (d, *J* = 5.3 Hz, 1H, C2-H), 7.39 (d, *J* = 5.3 Hz, 1H, C1-H).

^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6): δ (ppm) = 144.49 (2C, C2, C8), 117.93 (1C, C1).

HR-ESI-MS: *m*/*z* calc. ($C_6H_5ClN_3$ $[M+H]^+$): 154.0167, found: 154.0171.

6-Chloro-3-deazapurine (**S8**)



S8

$C_6H_4ClN_3$

153.57 g·mol^{-1}

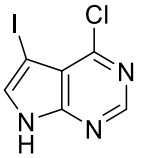
Compound **S6** (245 mg, 1.72 mmol, 1.00 eq.) was suspended in trimethyl orthoformate (4.00 mL, 36.5 mmol, 21.2 eq.) and treated with aq. HCl (37%, 300 µL, 3.62 mmol, 2.10 eq.). The reaction was stirred at ambient temperature for 16 h. The precipitate was collected by filtration, washed with *n*-hexane and dried to yield the product **S8** as a light brown solid (214 mg, 1.39 mmol, 81%).

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 8.75 (s, 1H, C8-H), 8.18 (d, *J* = 5.5 Hz, 1H, C2-H), 7.69 (d, *J* = 5.6 Hz, 1H, C3-H).

^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6): δ (ppm) = 144.69 (1C, C8), 141.19 (1C, C2), 140.65 (1C, C4), 138.94 (1C, C6), 134.08 (1C, C5), 109.00 (1C, C1).

HR-ESI-MS: *m*/*z* calc. ($C_6H_5ClN_3$ $[M+H]^+$): 154.01665, found: 154.01768.

6-Chloro-7-iodo-7-deazapurine (**S10**)



S10

$C_6H_3ClIN_3$

279.47 g·mol^{-1}

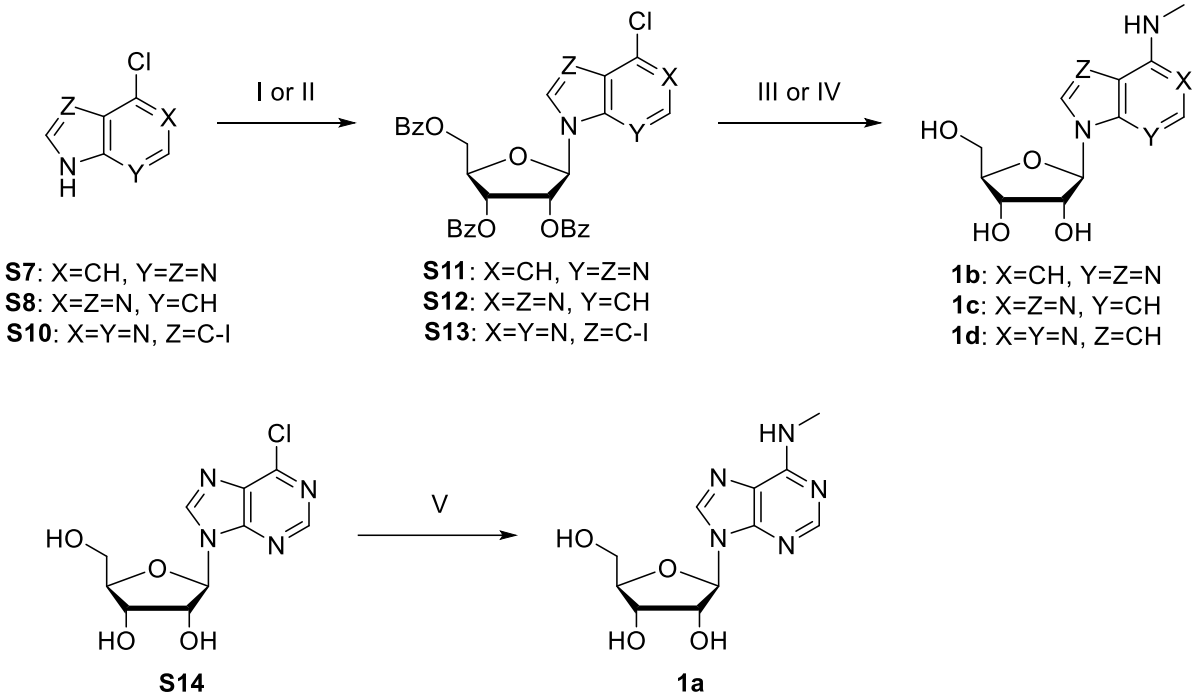
This compound was prepared according to a reported procedure.[4] A solution of 6-chloro-7-deazapurine (**S9**) (1.00 g, 6.52 mmol, 1.00 eq.) in dry DMF (2.5 mL) under inert gas atmosphere was treated with *N*-iodosuccinimide (NIS) (1.61 g, 7.17 mmol, 1.10 eq.). The reaction mixture was stirred at ambient temperature for 30 min before water (1 mL) was added dropwise to the reaction mixture. The precipitate was collected by filtration, washed with water,

and dried to yield the product **S10** as a light brown solid (1.46 g, 5.24 mmol, 80%). Analytical data agreed with reported values.[4]

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 12.95 (s, 1H, NH), 8.59 (s, 1H, C2-H), 7.94 (d, *J* = 2.5 Hz, 1H, C8-H).

^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6): δ (ppm) = 151.53 (1C, C4), 150.74 (1C, C6), 150.51 (1C, C2), 133.90 (1C, C8), 115.79 (1C, C5), 51.75 (1C, C9).

HR-ESI-MS: *m/z* calc. ($C_6H_2ClIN_3$ [M-H]$^-$): 277.89875, found: 277.89934.

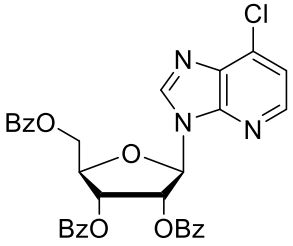


Scheme S2. Synthesis of the N^6-methylated 1-, 3-, and 7-deazaadenosine nucleosides. I) BSA, MeCN, 100 °C, 30 min, then 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose, TMSOTf, 100 °C, 16 h, 94% (for **S11**), 84% (for **S12**); II) BSA, MeCN, rt, 10 min, then 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose, TMSOTf, rt, 15 min, 80 °C, 1 h, 80% (for **S13**); III) $MeNH_2$, EtOH, 125 °C, 24 h, 88% (for **1b**), 88% (for **1c**); IV) *i*-PrMgCl·LiCl, THF, -10 °C, 1 h, then $MeNH_2$, EtOH, 125 °C, 18 h, 86% (for **1d**); V) $MeNH_2$, EtOH, rt, 16 h, 90%.
The chlorinated nucleobases **S7**, **S8**, and **S10** were glycosylated under Vorbrüggen glycosylation conditions, followed by simultaneous debenzoylation and chlorine substitution using $MeNH_2$ in EtOH. For the 7-deaza nucleoside, in between glycosylation and deprotection the iodine at C7 was removed by treatment with *i*-PrMgCl·LiCl followed by aqueous workup. m^6A (**1a**) was synthesized by treatment of commercially available 6-chloropurine riboside (**S14**) with ethanolic methylamine.

General procedure C: Glycosylation under Vorbrüggen conditions

A solution of compound 6-chloro-deazapurine (1.63 g, 10.6 mmol, 1.00 eq.) and *N*,*O*-bis(trimethylsilyl)acetamide (BSA) (6.25 mL, 21.2 mmol, 2.00 eq.) in acetonitrile (70 mL) under inert gas atmosphere was stirred at 100 °C for 30 min. After cooling to ambient temperature, 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (5.35 g, 10.6 mmol, 1.00 eq.) and TMSOTf (3.84 mL, 21.2 mmol, 2.00 eq.) were added. The reaction mixture was stirred at 100 °C for 16 h. It was diluted with EtOAc (70 mL) and washed with sat. aq. NH_4Cl solution (4 × 30 mL). The organic layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The residue was purified by column chromatography.

2',3',5'-Tri-*O*-benzoyl-6-chloro-1-deazapurine-9-β-D-ribofuranoside (**S11**)



S11

$C_{32}H_{24}ClN_3O_7$

598.01 g·mol^{-1}

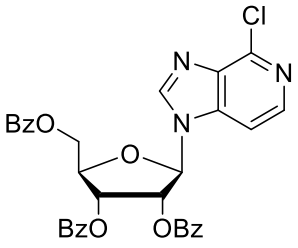
Compound **S7** (1.63 g, 10.6 mmol, 1.00 eq.) was converted according to general procedure C and purified by column chromatography (silica gel, EtOAc) to yield the product **S11** as a light brown solid (5.96 g, 9.97 mmol, 94%).

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 8.83 (s, 1H, C8-H), 8.23 (d, *J* = 5.3 Hz, 1H, C2-H), 8.00–7.93 (m, 4H, benzoyl-H), 7.90–7.86 (m, 2H, benzoyl-H), 7.70–7.62 (m, 4H, benzoyl-H), 7.51 (d, *J* = 5.3 Hz, 1H, C1-H), 7.53–7.42 (m, 5H, benzoyl-H), 6.70 (d, *J* = 4.6 Hz, 1H, C1'-H), 6.57 (dd, *J* = 6.0, 4.6 Hz, 1H, C2'-H), 6.28 (t, *J* = 6.0 Hz, 1H, C3'-H), 4.91–4.86 (m, 1H, 4'-H), 4.79 (dd, *J* = 12.2, 3.5 Hz, 1H, C5'-H^a), 4.66 (dd, *J* = 12.2, 4.7 Hz, 1H, C5'-H^b).

^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6): δ (ppm) = 165.44 (1C, benzoyl-CO), 164.72 (1C, benzoyl-CO), 164.53 (1C, benzoyl-CO), 146.82 (1C, C4), 145.85 (1C, C8), 144.98 (1C, C2), 134.04 (1C, benzoyl-C), 133.95 (1C, benzoyl-C), 133.73 (1C, benzoyl-C), 133.55 (1C, C6), 133.33 (1C, C5), 129.40 (4C, benzoyl-C), 129.31 (2C, benzoyl-C), 129.26 (1C, benzoyl-C), 128.80 (4C, benzoyl-C), 128.74 (2C, benzoyl-C), 128.62 (1C, benzoyl-C), 128.34 (1C, benzoyl-C), 119.29 (1C, C1), 86.84 (1C, C1'), 79.17 (1C, C4'), 72.87 (1C, C2'), 70.68 (1C, C3'), 63.19 (1C, C5').

HR-ESI-MS: *m/z* calc. ($C_{32}H_{25}ClN_3O_7$ [M+H]$^+$): 598.1376, found: 598.1355.

2',3',5'-Tri-*O*-benzoyl-6-chloro-3-deazapurine-9-β-D-ribofuranoside (**S12**)



S12

$C_{32}H_{24}ClN_3O_7$

598.01 g·mol^{-1}

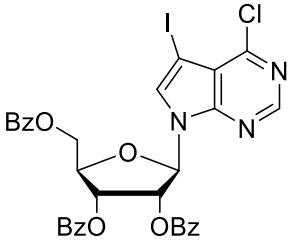
Compound **S8** (1.50 g, 9.77 mmol, 1.00 eq.) was converted according to general procedure C and purified by column chromatography (silica gel, *n*-hexane:EtOAc 1:1) to yield the product **S12** as a light brown solid (4.92 g, 8.24 mmol, 84%).

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 8.26 (s, 1H, C8-H), 8.10–8.06 (m, 2H, benzoyl-H), 8.03 (d, *J* = 5.7 Hz, 1H, C2-H), 8.01–7.97 (m, 2H, benzoyl-H), 7.95–7.91 (m, 2H, benzoyl-H), 7.68–7.52 (m, 4H, benzoyl-H, C3-H), 7.54–7.44 (m, 2H, benzoyl-H), 7.48–7.34 (m, 4H, benzoyl-H), 6.35 (d, *J* = 5.6 Hz, 1H, C1'-H), 5.99 (t, *J* = 5.6 Hz, 1H, C2'-H), 5.94 (dd, *J* = 5.6, 4.5 Hz, 1H, C3'-H), 4.94 (dd, *J* = 12.5, 2.6 Hz, 1H, C5'-H^a), 4.89 (dt, *J* = 4.5, 2.9 Hz, 1H, C4'-H), 4.76 (dd, *J* = 12.5, 3.2 Hz, 1H, C5'-H^b).

$^{13}C\{^1H\}$ NMR (100 MHz, $CDCl_3$): δ (ppm) = 166.21 (1C, benzoyl-CO), 165.43 (1C, benzoyl-CO), 165.14 (1C, benzoyl-CO), 143.58 (1C, C6), 142.32 (1C, C8), 142.17 (1C, C2), 138.78 (1C, C5), 138.50 (1C, C4), 134.29 (1C, benzoyl-C), 134.16 (1C, benzoyl-C), 134.00 (1C, benzoyl-C), 129.95 (4C, benzoyl-C), 129.77 (2C, benzoyl-C), 129.07 (1C, benzoyl-C), 129.03 (2C, benzoyl-C), 128.82 (4C, benzoyl-C), 128.53 (1C, benzoyl-C), 128.10 (1C, benzoyl-C), 106.37 (1C, C3), 88.08 (1C, C1'), 81.23 (1C, C4'), 74.11 (1C, C2'), 71.00 (1C, C3'), 63.33 (1C, C5').

HR-ESI-MS: *m*/*z* calc. ($C_{32}H_{24}ClN_3NaO_7$ $[M+Na]^+$): 620.1195, found: 620.1210.

2',3',5'-Tri-*O*-benzoyl-6-chloro-7-iodo-7-deazapurine-9-β-D-ribofuranoside (**S13**)



S13

$C_{32}H_{23}ClIN_3O_7$

723.90 g·mol^{-1}

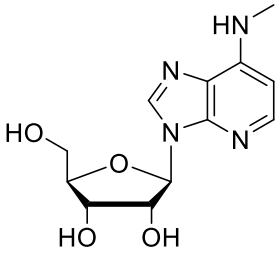
This compound was prepared according to a reported procedure.[5] Compound **S10** (112 mg, 401 µmol, 1.00 eq.) was suspended in dry MeCN (5 mL) under inert gas atmosphere. BSA (110 µL, 450 µmol, 1.12 eq.) was added and the mixture was stirred at ambient temperature for 10 min. 1-*O*-Acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (223 mg, 442 µmol, 1.10 eq.) was added, followed by TMSOTf (80.0 µL, 442 µmol, 1.10 eq.). The reaction mixture was stirred at ambient temperature for 15 min, then at 80 °C for 1 h. The solution was diluted with EtOAc (20 mL) and washed with sat. aq. $NaHCO_3$ solution (10 mL) and brine (10 mL). The organic layer was separated, dried over Na_2SO_4 and the solvent was removed under reduced pressure. The residue was purified by column chromatography (silica gel, *n*-hexane:EtOAc = 3:1), yielding the product **S13** as a colorless foam (232 mg, 320 µmol, 80%).

^1H NMR (400 MHz, $CDCl_3$): δ (ppm) = 8.58 (s, 1H, C2-H), 8.14–8.08 (m, 2H, benzoyl-H), 8.02–7.97 (m, 2H, benzoyl-H), 7.95–7.90 (m, 2H, benzoyl-H), 7.65–7.45 (m, 6H, C8-H, benzoyl-H), 7.45–7.32 (m, 4H, benzoyl-H), 6.67 (d, *J* = 5.4 Hz, 1H, C1'-H), 6.17–6.07 (m, 2H, C2'-H, C3'-H), 4.90 (dd, *J* = 12.3, 3.2 Hz, 1H, C5'-H^a), 4.80 (dt, *J* = 4.4, 3.2 Hz, 1H, C4'-H), 4.68 (dd, *J* = 12.3, 3.6 Hz, 1H, C5'-H^b).

$^{13}C\{^1H\}$ NMR (100 MHz, $CDCl_3$): δ (ppm) = 166.26 (1C, benzoyl-CO), 165.51 (1C, benzoyl-CO), 165.21 (1C, benzoyl-CO), 153.27 (1C, C6), 151.39 (1C, C2), 151.13 (1C, C4), 133.97 (1C, benzoyl-C), 133.93 (1C, benzoyl-C), 133.75 (1C, benzoyl-C), 132.14 (1C, C8), 129.99 (2C, benzoyl-C), 129.97 (2C, benzoyl-C), 129.84 (2C, benzoyl-C), 129.38 (1C, benzoyl-C), 128.98 (2C, benzoyl-C), 128.78 (1C, benzoyl-C), 128.72 (2C, benzoyl-C), 128.67 (2C, benzoyl-C), 128.48 (1C, benzoyl-C), 117.93 (1C, C5), 86.90 (1C, C1'), 80.80 (1C, C4'), 74.28 (1C, C2'), 71.56 (1C, C3'), 63.61 (1C, C5'), 53.83 (1C, C7).

HR-ESI-MS: *m*/*z* calc. ($C_{32}H_{23}ClIN_3NaO_7$ $[M+Na]^+$): 746.01615, found: 746.01704.

N^6-Methyl-1-deazaadenosine (**1b**)



1b

$C_{12}H_{16}N_4O_4$

280.28 g·mol^{-1}

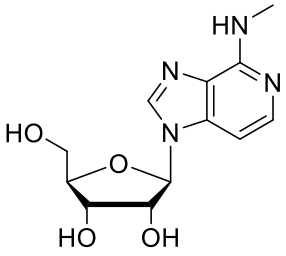
In a sealed pressure tube, a solution of compound **S11** (1.05 g, 1.75 mmol, 1.00 eq.) in $MeNH_2$ (33% in EtOH, 50 mL) was stirred at 125 °C for 24 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography (silica gel, EtOAc:MeOH = 8:1) to yield the product **1b** as a light yellow solid (433 mg, 1.55 mmol, 88%).

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 8.25 (s, 1H, C8-H), 7.88 (d, *J* = 5.7 Hz, 1H, C2-H), 6.94 (q, *J* = 4.9 Hz, 1H, N*H*CH$_3$), 6.31 (d, *J* = 5.7 Hz, 1H, C1-H), 6.02 (dd, *J* = 8.7, 3.6 Hz, 1H, C5'-OH), 5.88 (d, *J* = 6.6 Hz, 1H, C1'-H), 5.38 (d, *J* = 6.4 Hz, 1H, C2'-OH), 5.15 (d, *J* = 4.2 Hz, 1H, C3'-OH), 4.70 (td, *J* = 6.4, 4.9 Hz, 1H, C2'-H), 4.13 (td, *J* = 4.5, 2.4 Hz, 1H, C3'-H), 3.98 (q, *J* = 2.9 Hz, 1H, C4'-H), 3.67 (dt, *J* = 12.2, 3.1 Hz, 1H, C5'-H^a), 3.54 (ddd, *J* = 11.9, 8.3, 3.1 Hz, 1H, C5'-H^b), 2.89 (d, *J* = 4.9 Hz, 3H, NHC*H*$_3$).

^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6): δ (ppm) = 147.58 (1C, C6), 145.75 (1C, C4), 144.74 (1C, C2), 139.77 (1C, C8), 123.76 (1C, C5), 98.52 (1C, C1), 88.54 (1C, C1'), 86.21 (1C, C4'), 72.96 (1C, C2'), 71.08 (1C, C3'), 62.05 (1C, C5'), 29.06 ($NHCH_3$).

HR-ESI-MS: *m*/*z* calc. ($C_{12}H_{17}N_4O_4$ $[M+H]^+$): 281.1244, found: 281.1248.

N^6-Methyl-3-deazaadenosine (**1c**)



1c

$C_{12}H_{16}N_4O_4$

280.28 g·mol^{-1}

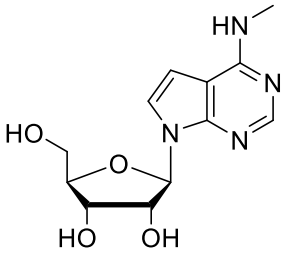
In a sealed pressure tube, a solution of compound **S12** (1.05 g, 1.75 mmol, 1.00 eq.) in $MeNH_2$ (33% in EtOH, 50 mL) was stirred at 125 °C for 20 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography (silica gel, EtOAc:MeOH = 1:0 to 6:4) to yield the product **1c** as a light yellow solid (433 mg, 1.55 mmol, 88%).

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 8.29 (s, 1H, C8-H), 7.73 (d, *J* = 5.9 Hz, 1H, C2-H), 6.92 (d, *J* = 5.9 Hz, 1H, C3-H), 6.78 (s$_{br}$, 1H, N*H*CH$_3$), 5.76 (d, *J* = 6.2 Hz, 1H, C1'-H), 5.50 (d, *J* = 6.5 Hz, 1H, C2'-OH), 5.25 (d, *J* = 4.8 Hz, 1H, C3'-OH), 5.13 (t, *J* = 5.2 Hz, 1H, C3'-OH), 4.30 (q, *J* = 6.0 Hz, 1H, C2'-H), 4.09 (td, *J* = 4.8, 3.1 Hz, 1H, C3'-H), 3.95 (q, *J* = 3.6 Hz, 1H, C4'-H), 3.62 (qdd, *J* = 11.9, 5.2, 3.7 Hz, 2H, C5'-H), 2.93 (d, *J* = 4.8 Hz, 3H, NHC*H*$_3$).

$^{13}C\{^{1}H\}$ NMR (100 MHz, DMSO-d_6): δ (ppm) = 151.92 (1C, C6), 140.15 (1C, C2), 139.76 (1C, C8), 136.90 (1C, C4), 127.26 (1C, C5), 96.94 (1C, C3), 88.68 (1C, C1'), 85.61 (1C, C4'), 74.01 (1C, C2'), 70.12 (1C, C3'), 61.22 (1C, C5'), 27.67 (1C, $NHCH_3$).

HR-ESI-MS: *m*/*z* calc. ($C_{12}H_{17}N_4O_4$ $[M+H]^+$): 281.1244, found: 281.1250.

<u>*N*^{6}-Methyl-7-deazaadenosine (**1d**)</u>



1d

$C_{12}H_{16}N_4O_4$

280.28 g·mol^{-1}

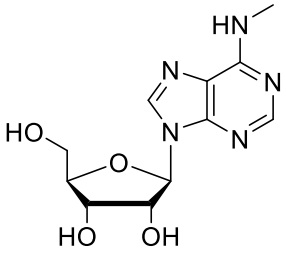
This compound was synthesized by modification of a reported procedure.[6] Compound **S13** (1.90 g, 2.62 mmol, 1.00 eq.) was dissolved in dry THF (10.5 mL) under inert gas atmosphere. The reaction mixture was cooled to -10 °C and *i*-PrMgCl•LiCl (1.30 M in THF, 2.1 mL, 2.73 mmol, 1.04 eq.) was added dropwise. The solution was stirred at this temperature for 1 h and then poured onto a mixture of ice (30 g) and sat. aq. NH_4Cl solution (20 mL). This mixture was extracted with DCM (1 × 50 mL, 3 × 5 mL), the combined organic layers were dried over $MgSO_4$ and the solvent was removed under reduced pressure. The residue was redissolved in $MeNH_2$ (33% in EtOH, 100 mL), transferred to a sealed pressure tube and stirred at 125 °C for 18 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography (silica gel, EtOAc:MeOH = 8:1) to yield the product **1d** as a colorless foam (629 mg, 2.24 mmol, 86%). Analytical data agreed with reported values.[6]

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 8.13 (s, 1H, C2-H), 7.53 (q, *J* = 4.6 Hz, 1H, N*H*CH_3), 7.34 (d, *J* = 3.6 Hz, 1H, C8-H), 6.57 (d, *J* = 3.6 Hz, 1H, C7-H), 5.99 (d, *J* = 6.3 Hz, 1H, C1'-H), 5.33 (dd, *J* = 6.7, 4.7 Hz, 1H, C5'-OH), 5.27 (d, *J* = 6.3 Hz, 1H, C2'-OH), 5.11 (d, *J* = 4.7 Hz, 1H, C3'-OH), 4.42 (q, *J* = 5.9 Hz, 1H, C2'-H), 4.08 (td, *J* = 4.7, 3.0 Hz, 1H, C3'-H), 3.89 (q, *J* = 3.7 Hz, 1H, C4'-H), 3.62 (ddd, *J* = 11.9, 4.7, 3.7 Hz, 1H, C5'-H^a), 3.52 (ddd, *J* = 11.9, 6.7, 3.7 Hz, 1H, C5'-H^b), 2.95 (d, *J* = 4.7 Hz, 3H, NHC*H*$_3$).

$^{13}C\{^{1}H\}$ NMR (100 MHz, DMSO-d_6): δ (ppm) = 156.71 (1C, C6), 151.50 (1C, C2), 149.15 (1C, C4), 122.18 (1C, C8), 103.50 (1C, C5), 99.12 (1C, C7), 87.61 (1C, C1'), 85.10 (1C, C4'), 73.72 (1C, C2'), 70.75 (1C, C3'), 61.87 (1C, C5'), 27.11 (1C, $NHCH_3$).

HR-ESI-MS: *m*/*z* calc. ($C_{12}H_{17}N_4O_4$ $[M+H]^+$): 281.1244, found: 281.1253.

<u>*N*^{6}-Methyladenosine (**1a**)</u>



1a

$C_{11}H_{15}N_5O_4$

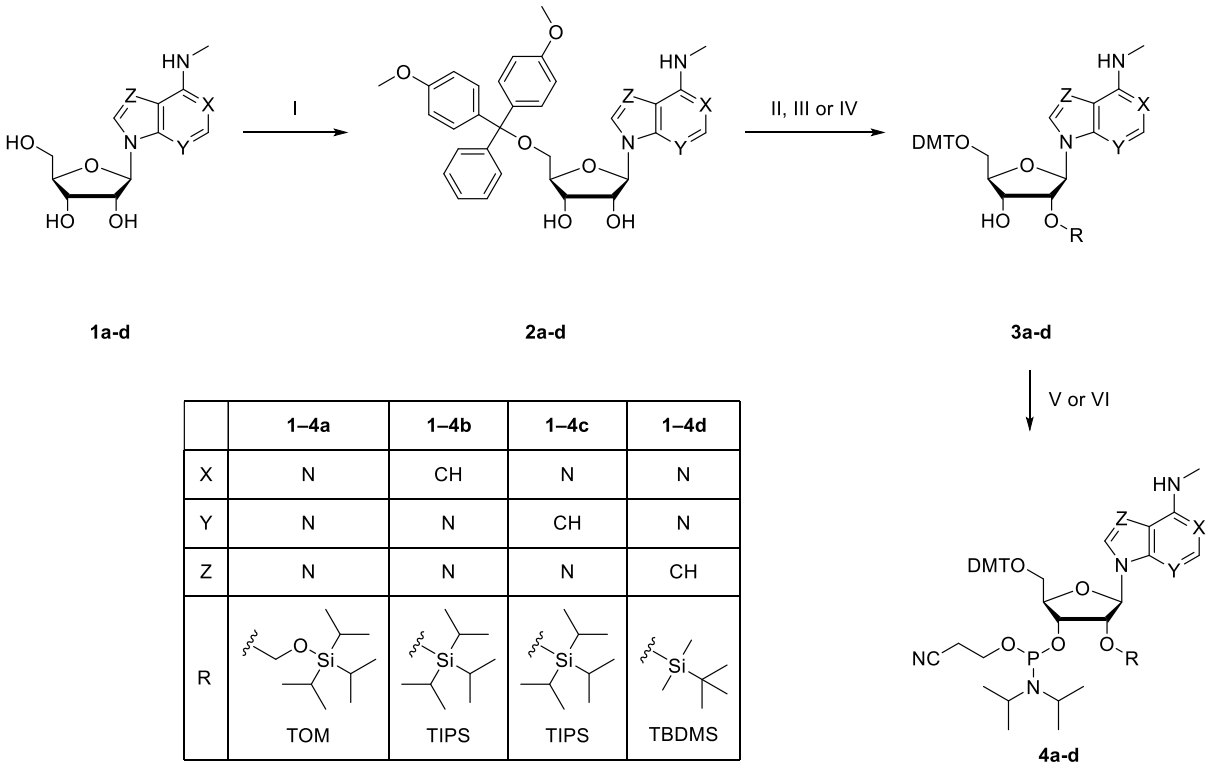
281.27 g·mol^{-1}

Compound **S14** (200 mg, 698 µmol, 1.00 eq.) was treated with $MeNH_2$ (33% in EtOH, 5 mL) and stirred at ambient temperature for 16 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography (silica gel, DCM:MeOH = 9:1) to yield the product **1a** as an off-white solid (178 mg, 633 µmol, 90%). Analytical data agreed with reported values.[7]

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 8.34 (s, 1H, C8-H), 8.23 (s, 1H, C2-H), 7.84 (s, 1H, N*H*CH_3), 5.88 (d, *J* = 6.2 Hz, 1H, C1'-H), 5.45 (dd, *J* = 6.9, 4.8 Hz, 2H, C2'-OH, C5'-OH), 5.20 (d, *J* = 4.6 Hz, 1H, C3'-OH), 4.61 (td, *J* = 6.2, 4.8 Hz, 1H, C2'-H), 4.14 (td, *J* = 4.8, 2.9 Hz, 1H, C3'-H), 3.97 (q, *J* = 3.4 Hz, 1H, C4'-H), 3.72–3.63 (m, 1H, C5'-H^a), 3.60–3.50 (m, 1H, C5'-H^b), 2.95 (s, 3H, NHCH_3).

^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6): δ (ppm) = 155.11 (1C, C6), 152.47 (1C, C2), 148.07 (1C, C4), 139.72 (1C, C8), 119.94 (1C, C5), 87.96 (1C, C1'), 85.96 (1C, C4'), 73.51 (1C, C2'), 70.72 (1C, C3'), 61.72 (1C, C5'), 27.03 (1C, $NHCH_3$).

HR-ESI-MS: *m*/*z* calc. ($C_{11}H_{15}N_5NaO_4$ $[M+Na]^+$): 304.1016, found: 304.1015.



	1–4a	1–4b	1–4c	1–4d
X	N	CH	N	N
Y	N	N	CH	N
Z	N	N	N	CH
R	TOM	TIPS	TIPS	TBDMS

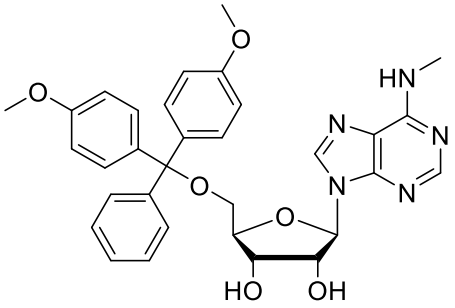
Scheme S3. Synthesis of the phosphoramidite building blocks. I) DMTCl, DMAP, NEt_3, pyridine, rt, 16–23 h, 77% (for **2a**), 88% (for **2b**), 67% (for **2c**); 60% (for **2d**); II) $(t\text{-Bu})_2SnCl_2$, DIPEA, DCE, 70 °C, 15 min, then TOMCl, rt, 30 min, 36% (for **3a**); III) $AgNO_3$, pyridine, rt, 30 min, then TIPSCl, rt, 22 h, 35% (for **3b**), 37% (for **3c**); IV) $AgNO_3$, pyridine, rt, 30 min, then TBDMSCl, rt, 15 h, 35% (for **3d**); V) CEPCl, Me_2NEt, DCM, 2.5–5 h, 75% (for **4a**), 51% (for **4b**), 31% (for **4d**); VI) CEPCl, $i\text{Pr}_2NEt$, pyridine, rt, 5 h, 58% (for **4c**).

The 5'-hydroxy groups of nucleosides **1a–d** were protected with 4,4'-dimethoxytrityl (DMT) groups, followed by protection of the 2'-hydroxy groups as [(triisopropylsilyl)oxy]methyl (TOM), *tert*-butyldimethylsilyl (TBDMS) or triisopropylsilyl (TIPS) ethers. The 2'-5'-protected nucleosides were subsequently converted into the corresponding phosphoramidite building blocks **4a–d** using 2-cyanoethyl-*N*,*N*-diisopropylaminochlorophosphoramidite (CEPCl).

General procedure D: 5'-DMT protection

A solution of N^6-methyladenosine (**1a**) (989 mg, 3.52 mmol, 1.00 eq.) in dry pyridine (25 mL) under inert gas atmosphere was treated with NEt_3 (690 µL, 4.93 mmol, 1.40 eq.) and DMAP (21.2 mg, 174 µmol, 0.05 eq.). The solution was cooled to 0 °C and DMTCl (1.44 g, 4.27 mmol, 1.20 eq.) was added in four portions over 1 h. The reaction mixture was allowed to warm to ambient temperature and stirred for 16 h. The solvent was removed under reduced pressure and the residue was redissolved in DCM (100 mL) and washed with sat. aq. $NaHCO_3$ (3 × 50 mL). The organic layer was separated, dried over Na_2SO_4 and the solvent was removed *in vacuo*. The residue was purified by column chromatography.

5'-*O*-(4,4'-Dimethoxytrityl)-N^6-methyladenosine (**2a**)



2a

$C_{32}H_{33}N_5O_6$

583.65 g·mol^{-1}

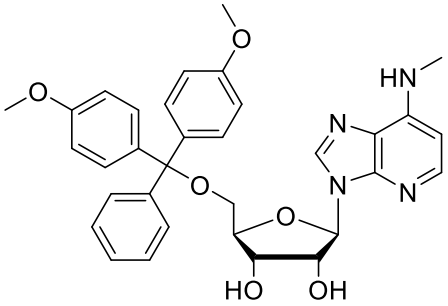
Compound **1a** (989 mg, 3.52 mmol, 1.00 eq.) was converted according to general procedure D and the residue was purified by column chromatography (silica gel, DCM:MeOH = 95:5 + 1% NEt_3) to yield the product **2a** as a light yellow foam (1.58 g, 2.71 mmol, 77%). Analytical data agreed with reported values.[9]

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 8.34 (s, 1H, C2-H), 8.03 (s, 1H, C8-H), 7.29–7.24 (m, 2H, trityl-H), 7.20–7.13 (m, 7H, trityl-H), 6.76–6.69 (m, 4H, trityl-H), 6.04 (s_{br}, 1H, N*H*CH_3), 5.95 (d, *J* = 6.1 Hz, 1H, C1'-H), 4.77 (t, *J* = 5.6 Hz, 1H, C2'-H), 4.44 (td, *J* = 3.4, 1.7 Hz, 1H, C4'-H), 4.38 (dd, *J* = 5.1, 1.7 Hz, 1H, C3'-H), 3.76 (s, 3 H, trityl-OCH_3), 3.75 (s, 3H, trityl-OCH_3), 3.43 (dd, *J* = 10.6, 3.4 Hz, 1H, C5'-H^a), 3.28–3.12 (m, 4H, C5'-H^b, NHC*H*$_3$).

^{13}C{^{1}H} NMR (125 MHz, $CDCl_3$): δ (ppm) = 158.62 (2C, trityl-C), 155.67 (1C, C6), 152.69 (1C, C2), 147.86 (1C, C4), 144.39 (1C, trityl-C), 138.00 (1C, C8), 135.69 (1C, trityl-C), 135.52 (1C, trityl-C), 130.07 (2C, trityl-C), 130.04 (4C, trityl-C), 128.12 (2C, trityl-C), 127.95 (2C, trityl-C), 127.00 (1C, trityl-C), 120.36 (1C, C5), 113.23 (4 C, trityl-C), 91.07 (1C, C1'), 86.63 (1C, trityl-C), 86.61 (1C, C4'), 76.41 (1C, C2'), 73.08 (1C, C3'), 63.73 (1C, C5'), 55.32 (2C, trityl-OCH_3), 27.61 (1 C, NHCH_3).

HR-ESI-MS: *m/z* calc. ($C_{32}H_{33}N_5NaO_6$ [M+Na]$^+$): 606.23230, found: 606.23343.

5'-*O*-(4,4'-Dimethoxytrityl)-N^6-methyl-1-deazaadenosine (**2b**)



2b

$C_{33}H_{34}N_4O_6$

582.66 g·mol^{-1}

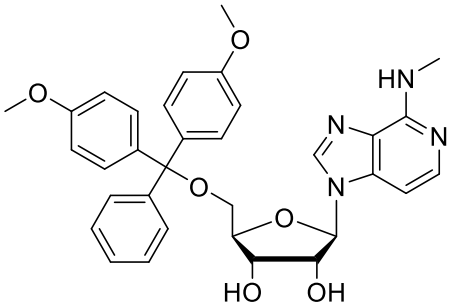
Compound **1b** (548 mg, 1.96 mmol, 1.00 eq.) was converted according to general procedure D and the crude product was purified by column chromatography (DCM:MeOH = 98:2 + 2% NEt_3). to yield the product **2b** as an off-white foam (1.00 g, 1.72 mmol, 88%).

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 8.06 (s, 1H, C8-H), 8.01 (d, *J* = 5.7 Hz, 1H, C2-H), 7.32–7.22 (m, 2H, trityl-H), 7.21–7.14 (m, 7H, trityl-H), 6.75–6.68 (m, 4H, trityl-H), 6.35 (d, *J* = 5.7 Hz, 1H, C1-H), 5.97 (d, *J* = 6.4 Hz, 1H, C1'-H), 5.50 (q, *J* = 5.1 Hz, 1H, N*H*CH_3), 4.72 (dd, *J* = 6.4, 5.0 Hz, 1H, C2'-H), 4.48 (td, *J* = 3.4, 1.2 Hz, 1H, C4'-H), 4.35 (dd, *J* = 5.0, 1.2 Hz, 1H, C3'-H), 3.75 (2 s, 6H, trityl-OCH_3), 3.43 (dd, *J* = 10.4, 3.6 Hz, 1H, C5'-H^a), 3.21 (dd, *J* = 10.5, 3.3 Hz, 1H, C5'-H^b), 3.05 (d, *J* = 5.1 Hz, 3H, NHC*H*$_3$).

^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ (ppm) = 158.58 (1C, trityl-C), 158.56 (1C, trityl-C), 147.96 (1C, C6), 145.91 (1C, C4), 145.17 (1C, C2), 144.42 (1C, trityl-C), 137.64 (1C, C8), 135.83 (1C, trityl-C), 135.62 (1C, trityl-C), 130.10 (2C, trityl-C), 130.05 (2C, trityl-C), 128.18 (2C, trityl-C), 127.95 (1C, trityl-C), 126.95 (1C, trityl-C), 124.05 (1C, C5), 113.23 (2C, trityl-C), 113.22 (2C, trityl-C), 98.33 (1C, C1), 91.31 (1C, C1'), 86.85 (1C, C4'), 86.51 (1C, trityl-C), 76.69 (1C, C2'), 73.28 (1C, C3'), 63.85 (1C, C5'), 55.31 (2C, trityl-OCH_3), 29.50 (1C, $NHCH_3$).

HR-ESI-MS: *m*/*z* calc. ($C_{33}H_{35}N_4O_6$ $[M+H]^+$): 583.2551, found: 583.2556.

5'-*O*-(4,4'-Dimethoxytrityl)-N^6-methyl-3-deazaadenosine (**2c**)



2c

$C_{33}H_{34}N_4O_6$

582.66 g·mol^{-1}

Compound **1c** (730 mg, 2.60 mmol, 1.00 eq.) was converted according to general procedure D and the crude product was purified by column chromatography (DCM:MeOH = 98:2 + 2% NEt_3) to yield the product **2c** as a colorless foam (1.02 g, 1.75 mmol, 67%).

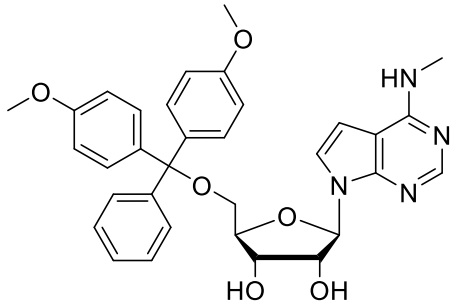
^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 8.18 (s, 1H, C8-H), 7.62 (d, *J* = 5.8 Hz, 1H, C2-H), 7.39–7.33 (m, 2H, trityl-H), 7.31–7.19 (m, 7H, trityl-H), 6.87–6.80 (m, 5H, C3-H, trityl-H), 6.74 (q, *J* = 4.7 Hz, 1H, N*H*CH_3), 5.81 (d, *J* = 5.3 Hz, 1H, C1'-H), 5.61 (d, *J* = 6.1 Hz, 1H, C2'-OH),

5.28 (d, *J* = 5.6 Hz, 1H, C3'-OH), 4.43 (q, *J* = 5.5 Hz, 1H, C2'-H), 4.18 (q, *J* = 5.1 Hz, 1H, C3'-OH), 4.08 (td, *J* = 4.6, 3.1 Hz, 1H, C4'-OH), 3.72 (s, 6H, trityl-OCH_3), 3.26–3.16 (m, 2H, C5'-H), 2.91 (d, *J* = 4.7 Hz, 3H, NHCH_3).

^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6): δ (ppm) = 158.08 (2C, trityl-C), 152.09 (1C, C6), 144.75 (1C, trityl-C), 140.55 (1C, C2), 139.30 (1C, C8), 136.75 (1C, C4), 135.41 (1C, trityl-C), 135.31 (1C, trityl-C), 129.76 (2C, trityl-C), 129.73 (2C, trityl-C), 127.87 (2C, trityl-C), 127.71 (2C, trityl-C), 127.34 (1C, 5), 126.73 (1C, trityl-C), 113.19 (4C, trityl-C), 96.92 (1C, C3), 88.94 (1C, C1'), 85.65 (1C, trityl-C), 83.34 (1C, C4'), 73.43 (1C, C2'), 70.08 (1C, C3'), 63.58 (1C, C5'), 55.03 (2C, trityl-OCH_3), 27.55 (1C, $NHCH_3$).

HR-ESI-MS: *m*/*z* calc. ($C_{33}H_{35}N_4O_6$ $[M+H]^+$):: 583.2551, found: 583.2559.

5'-*O*-(4,4'-Dimethoxytrityl)-*N*^{6}-methyl-7-deazaadenosine (**2d**)



2d

$C_{33}H_{34}N_4O_6$

582.66 g·mol^{-1}

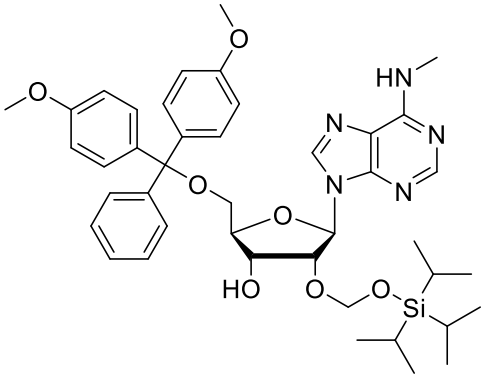
Compound **1d** (593 mg, 2.12 mmol, 1.00 eq.) was converted according to general procedure D with the following modifications: After addition of DMTCl, the reaction was stirred at ambient temperature for 20 h. An additional portion of DMTCl (359 mg, 1.06 mmol, 0.50 eq.) was added and stirring was continued for 3 h before workup. After workup, the crude product was purified by column chromatography (silica gel, DCM:MeOH = 98:2 + 1% NEt_3 to DCM:MeOH = 95:5 + 1% NEt_3) to yield the product **2d** as a light yellow foam (742 mg, 1.27 mmol, 60%).

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 8.25 (s, 1H, C2-H), 7.34 (d, *J* = 3.7 Hz, 1H, C8-H), 7.33–7.27 (m, 2H, trityl-H), 7.24–7.14 (m, 7H, trityl-H), 6.77–6.70 (m, 4H, trityl-H), 6.38 (d, *J* = 3.7 Hz, 1H, C9-H), 6.04 (d, *J* = 6.2 Hz, 1H, C1'-H), 5.17 (s_{br}, 1H, N*H*CH_3), 4.67 (dd, *J* = 6.2, 5.1 Hz, 1H, C2'-H), 4.40 (td, *J* = 3.3, 1.9 Hz, 1H, C4'-H), 4.33 (dd, *J* = 5.2, 1.9 Hz, 1H, C3'-H), 3.76 (2 s, 6H, trityl-$(OCH_3)_2$), 3.47 (dd, *J* = 10.4, 3.3 Hz, 1H, C5'-H^a), 3.21–3.17 (m, 4H, C5'-H^b, NHCH_3).

^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ (ppm) = 158.57 (2C, trityl-C, 157.33 (1C, C6), 151.26 (1C, C2), 148.66 (1C, C4), 144.58 (1C, trityl-C), 135.94 (1C, trityl-C), 135.71 (1C, trityl-C), 130.19 (2C, trityl-C), 130.12 (2C, trityl-C), 128.26 (2C, trityl-C), 127.93 (2C, trityl-C), 126.93 (1C, trityl-C), 122.00 (1C, C8), 113.19 (4C, trityl-C), 103.88 (1C, C5), 98.15 (1C, C7), 91.01 (C1'), 86.50 (1C, trityl-C), 85.85 (1C, C4'), 76.51 (1C, C2'), 73.29 (1C, C3'), 63.80 (1C, C5'), 55.33 (2C, trityl-OCH_3), 28.51 (1C, $NHCH_3$).

HR-ESI-MS: *m*/*z* calc. ($C_{33}H_{35}N_4O_6$ $[M+H]^+$): 583.25511, found: 583.25597.

5'-*O*-(4,4'-Dimethoxytrityl)-2'-*O*-[[(triisopropylsilyl)oxy]methyl]-N^6-methyladenosine (**3a**)



3a

$C_{42}H_{55}N_5O_7Si$

770.02 g·mol^{-1}

2'-TOM protection was performed according to a reported procedure.[9] A solution of compound **2a** (286 mg, 490 µmol, 1.00 eq.) in dry DCE (1.8 mL) under inert gas atmosphere was treated with DIPEA (350 µL, 2.01 mmol, 4.10 eq.) and (*t*-Bu)$_2$SnCl$_2$ (179 mg, 589 µmol, 1.20 eq.). The reaction mixture was stirred at 70 °C for 15 min and then allowed to cool to ambient temperature. TOMCl (131 mg, 588 µmol, 1.20 eq.) was added and the mixture was stirred at rt for 30 min. MeOH (120 µL) was added, the solution was diluted with DCM (50 mL) and washed with sat. aq. $NaHCO_3$ solution (3 × 30 mL). The organic layer was separated, dried and the solvent was removed *in vacuo*. The residue was purified by column chromatography (silica gel, *n*-hexane:EtOAc = 1:9 + 1% NEt_3) to yield the product **3a** as a colorless foam (137 mg, 178 µmol, 36%). Analytical data agreed with reported values.[9]

^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) = 8.33 (s, 1H, C2-H), 7.94 (s, 1H, C8-H), 7.45–7.41 (m, 2H, trityl-H), 7.34–7.30 (m, 4H, trityl-H), 7.27–7.23 (m, 2H, trityl-H), 7.22–7.18 (m, 1H, trityl-H), 6.81–6.77 (m, 4H, trityl-H), 6.15 (d, *J* = 5.3 Hz, 1H, C1'-H), 5.77 (s_{br}, 1H, N*H*CH3), 5.14 (d, *J* = 4.7 Hz, 1H, C2'-OCH$_2^a$), 4.98 (d, *J* = 4.7 Hz, 1H, C2'-OCH$_2^b$), 4.93 (t, *J* = 5.3 Hz, 1H, C2'-H), 4.52 (q, *J* = 4.2 Hz, 1H, C3'-H), 4.27 (q, *J* = 3.9 Hz, 1H, C4'-H), 3.78 (2 s, 6H, (trityl-OCH$_3$)$_2$), 3.49 (dd, *J* = 10.5, 3.6 Hz, 1H, C5'-H^a), 3.38 (dd, *J* = 10.5, 4.2 Hz, 1H, C5'-H^b), 3.19 (s_{br}, 3H, NHC*H*$_3$), 3.09 (s, 1H, C3'-OH), 1.11–1.00 (m, 21H, (C*H*(CH$_3$)$_2$)$_3$, (CH(C*H*$_3$)$_2$)$_3$).

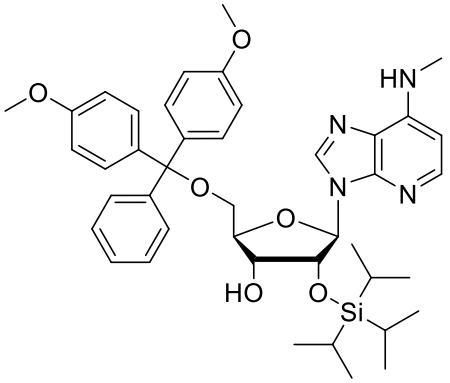
^{13}C{^{1}H} NMR (125 MHz, $CDCl_3$): δ (ppm) = 158.62 (2C, trityl-C), 155.57 (1C, C6), 153.40 (1C, C2), 148.88 (1C, C4), 144.73 (1C, trityl-C), 138.73 (1C, C8), 135.92 (1C, trityl-C), 135.88 (1C, trityl-C), 130.22 (4C, trityl-C), 128.33 (2C, trityl-C), 127.97 (2C, trityl-C), 127.00 (1C, trityl-C), 120.59 (1C, C5), 113.27 (4C, trityl-C), 90.98 (1C, C2'-O*C*H$_2$), 87.13 (1C, C1'), 86.64 (1C, trityl-C), 84.20 (1C, C4'), 82.25 (1C, C2'), 71.02 (1C, C3'), 63.54 (1C, C5'), 55.35 (2C, trityl-OCH$_3$), 27.65 (1C, NHCH$_3$), 17.90 (6C, CH(*C*H$_3$)$_2$), 11.96 (3C, *C*H(CH$_3$)$_2$).

HR-ESI-MS: *m*/*z* calc. ($C_{42}H_{55}N_5NaO_7Si$ [M+Na]$^+$): 792.37630, found: 792.37626.

General procedure E: 2'-TIPS protection

A solution of compound **2b** (750 mg, 1.29 mmol, 1.00 eq.) in dry pyridine (7 mL) under an inert gas atmosphere was treated with $AgNO_3$ (875 mg, 5.15 mmol, 4.00 eq.) and stirred in the dark at ambient temperature for 30 min. TIPSCl (1.10 mL, 5.15 mmol, 4.00 eq.) was added and stirring was continued in the dark for 22 h. The reaction mixture was diluted with DCM (200 mL) and washed with sat. aq. $NaHCO_3$ solution (200 mL). The organic layer was separated, dried over Na_2SO_4 and the solvent was removed *in vacuo*. The residue was purified by column chromatography.

5'-*O*-(4,4'-Dimethoxytrityl)-2'-*O*-(triisopropylsilyl)-*N*^{6}-methyl-1-deazaadenosine (**3b**)



3b

$C_{42}H_{54}N_4O_6Si$

739.00 g·mol^{-1}

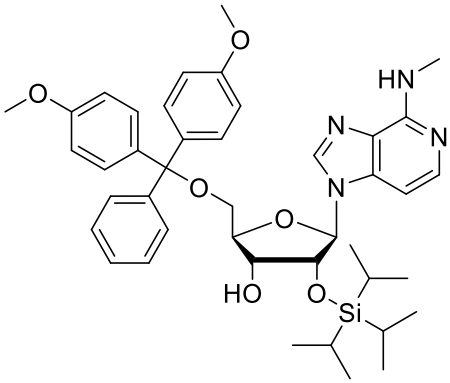
Compound **2b** (750 mg, 1.29 mmol, 1.00 eq.) was converted according to general procedure E and the crude product was purified by column chromatography (*n*-hexane:EtOAc = 2:1 + 1% NEt_3) to yield the product **3b** as a colorless foam (333 mg, 451 µmol, 35%).

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.93–7.89 (m, 2H, C2-H, C8-H), 7.46–7.41 (m, 2H, trityl-H), 7.35–7.30 (m, 4H, trityl-H), 7.29–7.14 (m, 3H, trityl-H), 6.81–6.76 (m, 4H, trityl-H), 6.27 (d, *J* = 5.5 Hz, 1H, C1-H), 6.01 (d, *J* = 5.7 Hz, 1H, C1'-H), 5.32 (dd, *J* = 5.8, 4.0 Hz, 1H, C2'-H), 5.18 (q, *J* = 5.2 Hz, 1H, N*H*CH_3), 4.65 (dd, *J* = 4.1, 2.9 Hz, 1H, C3'-H), 4.27 (ddd, *J* = 5.6, 4.4, 2.8 Hz, 1H, C4'-H), 3.78 (2 s, 6H, trityl-OCH_3), 3.57 (dd, *J* = 10.4, 5.7 Hz, 1H, C5'-H^a), 3.30 (dd, *J* = 10.3, 4.5 Hz, 1H, C5'-H^b), 3.00 (d, *J* = 5.2 Hz, 3H, NHC*H*$_3$), 0.94–0.80 (m, 21H, (C*H*$(CH_3)_2)_3$, CH(C*H*$_3)_2)_3$).

^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ (ppm) = 158.51 (2C, trityl-C), 147.07 (1C, C6), 146.37 (1C, C4), 145.98 (1C, C2), 144.90 (1C, trityl-C), 139.72 (1C, C8), 136.15 (1C, trityl-C), 136.05 (1C, trityl-C), 130.25 (4C, trityl-C), 128.35 (2C, trityl-C), 127.88 (2C, trityl-C), 126.81 (1C, trityl-C), 124.43 (1C, C5), 113.16 (4C, trityl-C), 98.47 (1C, C1), 88.88 (1C, C1'), 86.56 (1C, trityl-C), 84.02 (1C, C4'), 74.73 (1C, C2'), 74.22 (1C, C3'), 63.88 (1C, C5'), 55.32 (2C, trityl-OCH_3), 29.50 (1C, $NHCH_3$), 18.16 (3C, CH(*C*$H_3)_2$), 17.94 (3C, CH(*C*$H_3)_2$), 12.74 (3C, *C*H$(CH_3)_2$).

HR-ESI-MS: *m*/*z* calc. ($C_{42}H_{55}N_4O_6Si$ $[M+H]^+$): 739.3885, found: 739.3901.

5'-*O*-(4,4'-Dimethoxytrityl)-2'-*O*-(triisopropylsilyl)-*N*^{6}-methyl-3-deazaadenosine (**3c**)



3c

$C_{42}H_{54}N_4O_6Si$

739.00 g·mol^{-1}

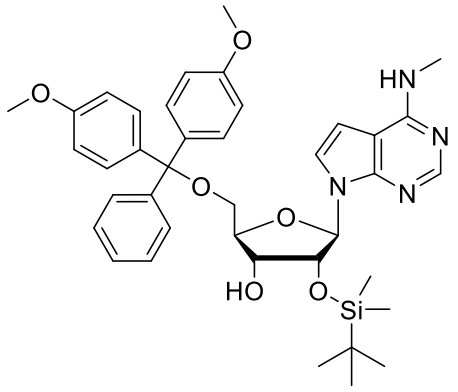
Compound **2c** (678 mg, 1.16 mmol, 1.00 eq.) was converted according to general procedure E and the crude product was purified by column chromatography (*n*-hexane:EtOAc = 1:2 + 1% NEt_3) to yield the product **3c** as a colorless foam (320 mg, 433 µmol, 37%).

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.92 (s, 1H, C8-H), 7.66 (d, *J* = 5.9 Hz, 1H, C2-H), 7.45–7.40 (m, 2H, trityl-H), 7.36–7.20 (m, 7H, trityl-H), 6.84–6.78 (m, 5H, C3-H, trityl-H), 5.78 (d, *J* =6.9 Hz, 1H, C1'-H), 5.49 (s_{br}, 1H, N*H*CH_3), 4.87 (dd, *J* = 6.9, 5.3 Hz, 1H, C2'-H), 4.38 (dd, *J* = 5.3, 2.0 Hz, 1H, C3'-H), 4.27 (q, *J* = 2.6 Hz, 1H, C4'-H), 3.78 (2 s, 6H, trityl-OCH_3), 3.50 (dd, *J* = 10.7, 2.7 Hz, 1H, C5'-H^a), 3.42 (dd, *J* = 10.7, 3.1 Hz, 1H, C5'-H^b), 3.15 (d, *J* = 5.0 Hz, 3H, NHC*H*$_3$), 2.93 (s, 1H, 3'-OH), 1.00–0.77 (m, 21H, C*H*$(CH_3)_2$, CH(C*H*$_3)_2$).

^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ (ppm) = 158.77 (2C, trityl-C), 152.48 (1C, C6), 144.50 (1C, trityl-C), 141.43 (1C, C2), 139.15 (1C, C8), 137.20 (1C, C4), 135.46 (1C, trityl-C), 135.39 (1C, trityl-C), 130.30 (1C, trityl-C), 130.28 (1C, trityl-C), 128.27 (2C, trityl-C), 128.10 (4C, trityl-C), 127.98 (1C, C5), 127.21 (1C, trityl-C), 113.38 (4C, trityl-C), 97.67 (1C, C3), 89.24 (1C, C1'), 87.13 (1C, trityl-C), 84.19 (1C, C4'), 75.86 (1C, C2'), 71.92 (1C, C3'), 63.69 (1C, C5'), 55.36 (2C, trityl-OCH_3), 28.04 (1C, $NHCH_3$), 17.77 (3C, CH(*C*$H_3)_2$), 17.56 (3C, CH(*C*$H_3)_2$), 12.09 (3C, *C*H$(CH_3)_2$).

HR-ESI-MS: *m*/*z* calc. ($C_{42}H_{55}N_4O_6Si$ $[M+H]^+$): 739.3885, found: 739.3889.

5'-*O*-(4,4'-Dimethoxytrityl)-2'-*O*-(*tert*-butyldimethylsilyl)-*N*^{6}-methyl-7-deazaadenosine (**3d**)



3d

$C_{39}H_{48}N_4O_6Si$

696.92 g·mol^{-1}

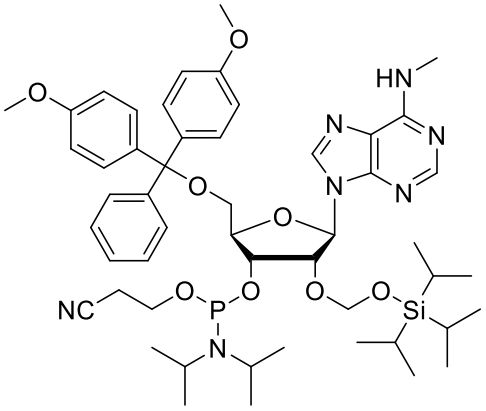
Compound **2d** (617 mg, 1.06 mmol, 1.00 eq.) was dissolved in dry pyridine (6 mL) under inert gas atmosphere. $AgNO_3$ (719 mg, 4.23 mmol, 4.00 eq.) was added and the reaction mixture was stirred in the dark at ambient temperature for 30 min. TBDMSCl (192 mg, 1.27 mmol, 1.20 eq.) was added and the solution was stirred in the dark at ambient temperature for 15 h. EtOAc (250 mL) was added, the resulting suspension was filtered through a pad of Celite and the filtrate was evaporated to dryness. Purification of the residue by column chromatography (silica gel, *n*-hexane:EtOAc 1:3) yielded the product **3d** as a colorless foam (243 mg, 365 µmol, 35%).

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 8.33 (s, 1H, C2-H), 7.50–7.44 (m, 2H, trityl-H), 7.38–7.32 (m, 4H, trityl-H), 7.31–7.20 (m, 4H, C8-H, trityl-H), 6.85–6.78 (m, 4H, trityl-H), 6.34 (d, *J* = 3.7 Hz, 1H, C7-H), 6.31 (d, *J* = 5.9 Hz, 1H, C1'-H), 5.05 (s_{br}, 1H, N*H*CH_3), 4.74 (t, *J* = 5.6 Hz, 1H, C2'-H), 4.29 (dd, *J* = 5.3, 3.1 Hz, 1H, C3'-H), 4.22 (q, *J* = 3.1 Hz, 1H, C4'-H), 3.78 (2 s, 6H, trityl-$(OCH_3)_2$), 3.53 (dd, *J* = 10.5, 3.0 Hz, 1H, C5'-H^a), 3.30 (dd, *J* = 10.5, 3.4 Hz, 1H, C5'-H^b), 3.19 (d, *J* = 5.0 Hz, 3H, NHC*H*$_3$), 2.83 (s_{br}, 1H, 3'-OH), 0.82 (s, 9H, SiC(C*H*$_3)_3$), -0.06 (s, 3H, Si(C*H*$_3)_2$^{a}), -0.19 (s, 3H, Si(C*H*$_3)_2$^{b});

$^{13}C\{^1H\}$ NMR (100 MHz, $CDCl_3$): δ (ppm) = 158.65 (2C, trityl-C), 157.16 (1C, C6), 152.21 (1C, C2), 150.69 (1C, C4), 144.90 (1C, trityl-C), 135.97 (1C, trityl-C), 135.89 (1C, trityl-C), 130.30 (4C, trityl-C), 128.37 (2C, trityl-C), 128.00 (2C, trityl-C), 127.01 (1C, trityl-C), 121.48 (1C, C8), 113.29 (4C trityl-C), 103.60 (1C, C5), 99.21 (1C, C7), 87.34 (1C, C1'), 86.61 (1C, trityl-C), 83.67 (1C, C4'), 76.31 (1C, C2'), 71.77 (1C, C3'), 63.85 (1H, C5'), 55.37 (2C, trityl-OCH_3), 28.50 (1H, $NHCH_3$), 25.73 (3C, $SiC(CH_3)_3$), 18.07 (1C, $SiC(CH_3)_3$), -4.94 (1C, $Si(CH_3)_2$[a]), -5.27(1C, $Si(CH_3)_2$[b]).

HR-ESI-MS: *m*/*z* calc. ($C_{39}H_{49}N_4O_6Si$ $[M+H]^+$): 697.3416, found: 697.3413.

5'-*O*-(4,4'-Dimethoxytrityl)-2'-*O*-[[(triisopropylsilyl)oxy]methyl]-*N*^{6}-methyladenosine-3'-*O*-(2-cyanoethyl)diisopropylphosphoramidite (**4a**)



4a

$C_{51}H_{72}N_7O_8PSi$

970.24 g·mol^{-1}

This compound was synthesized according to a reported procedure.[9] A solution of compound **3a** (250 mg, 325 µmol, 1.00 eq.) in dry DCM (2 mL) under inert gas atmosphere was treated with $EtNMe_2$ (350 µL, 3.23 mmol, 9.95 eq.). CEPCl (110 µL, 493 µmol, 1.52 eq.) was added and the resulting mixture was stirred at ambient temperature for 2.5 h. It was evaporated to dryness and the residue was purified by column chromatography (silica gel, *n*-hexane:EtOAc 3:2 + 1% NEt_3) to yield the product **4a** as a colorless foam (238 mg, 245 µmol, 75%). Analytical data agreed with reported values.[9]

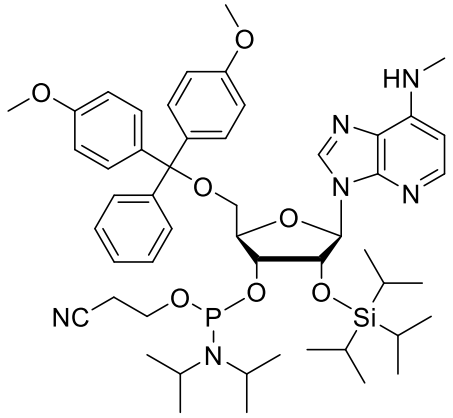
^1H NMR (500 MHz, $CDCl_3$): δ (ppm) = 8.30, 8.28 (2 s, 1H, C2-H), 7.92, 7.90 (2 s, 1H, C8-H), 7.42–7.38 (m, 2H, trityl-H), 7.32–7.15 (m, 7H, trityl-H), 6.81–6.74 (m, 4H, trityl-H), 6.14, 6.11 (2 d, *J* = 5.7 Hz, 1H, C1'-H), 5.72 (s_{br}, 1H, $NHCH_3$), 5.19, 5.16 (2 t, *J* = 5.4 Hz, 1H, C2'-H), 5.00–4.91 (m, 2H, C2'-OCH_2), 4.75–4.65 (m, 1H, C3'-H), 4.37, 4.32 (2 q, *J* = 4.1 Hz, 1H, C4'-H), 3.98–3.84 (m, 1H, $POCH_2$[a]), 3.78 (s, 3H, trityl-OCH_3), 3.77 (s, 3H, trityl-OCH_3), 3.71–3.48 (m, 4H, C5'-H[a], $POCH_2$[b], $N(CH(CH_3)_2)_2$), 3.34–3.29 (m, 1H, C5'-H[b]), 3.19 (s_{br}, 3H, $NHCH_3$), 2.65 (td, *J* = 6.5, 3.8 Hz, 1 H, CH_2[a]CN), 2.37 (t, *J* = 6.6 Hz, CH_2[b]CN), 1.18 (t, *J* = 6.5 Hz, 9H, $N(CH(CH_3)_2)_3$[a,b,c]), 1.07 (d, *J* = 6.8 Hz, 3H, $N(CH(CH_3)_2)_3$[d]), 0.94–0.87 (m, 21H, $Si(CH(CH_3)_2)_3$, $Si(CH(CH_3)_2)_3$).

$^{13}C\{^1H\}$ NMR (125 MHz, $CDCl_3$): δ (ppm) = 158.60, 158.58 (2C, trityl-C), 155.56 (1C, C6), 153.25, 153.23 (1C, C2), 148.95 (1C, C4), 144.76, 144.68 (1C, trityl-C), 139.31, 139.28 (1C, C8), 136.04, 135.95, 135.89 (2C, trityl-C), 130.25, 130.21, 130.18 (4C, trityl-C), 128.42, 128.36 (2C, trityl-C), 127.92 (2C, trityl-C), 126.98, 126.95 (1C, trityl-C), 120.68 (1C, C5), 117.82, 117.53 (1C, CH_2CN), 113.21, 113.20 (4C, trityl-C), 89.75, 89.72, 89.39 (1C, C2'-OCH_2), 87.50, 87.38 (1C, C1'), 86.61, 86.51 (1C, trityl-C), 84.05, 84.03, 83.94, 83.91 (1C, C4'), 77.69, 77.67, 77.04, 76.99 (1C, C2'), 71.93, 71.81, 71.42, 71.29 (1C, C3'), 63.46, 63.06 (1C, C5'), 59.23, 59.10, 58.32, 58.16 (1C, $POCH_2$), 55.35, 55.33 (2C, trityl-OCH_3), 43.54, 43.44, 43.37, 43.27 (2C, $NCH(CH_3)_2$), 27.68 (1C, $NHCH_3$), 24.79, 24.74, 24.72, 24.68 (4C, $NCH(CH_3)_2$), 20.53, 20.43, 20.25, 20.20 (1C, CH_2CN,) 17.83, 17.80 (6C, $CH(CH_3)_2$), 11.95 (3 C, $CH(CH_3)_2$).

$^{31}P\{^{1}H\}$ NMR (202 MHz, $CDCl_3$): δ (ppm) = 150.65, 149.95.

HR-ESI-MS: *m*/*z* calc. ($C_{51}H_{73}N_7O_8PSi$ $[M+H]^+$): 970.50220, found: 970.50210.

5'-*O*-(4,4'-Dimethoxytrityl)-2'-*O*-(triisopropylsilyl)-N^6-methyl-1-deazaadenosine-3'-*O*-(2-cyano-ethyl)diisopropylphosphoramidite (**4b**)



4b

$C_{51}H_{71}N_6O_7PSi$

939.22 g·mol^{-1}

A solution of compound **3b** (88.0 mg, 119 µmol, 1.00 eq.) in DCM (1.4 mL) under an inert gas atmosphere was treated with $EtNMe_2$ (130 µL, 1.19 mmol, 10.0 eq.) and CEPCl (40.0 µL, 179 µmol, 1.51 eq.) and stirred at ambient temperature for 5 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography (*n*-hexane:EtOAc = 2:1 + 1% NEt_3) to yield the product **4b** as a colorless foam (57.0 mg, 60.7 µmol, 51%).

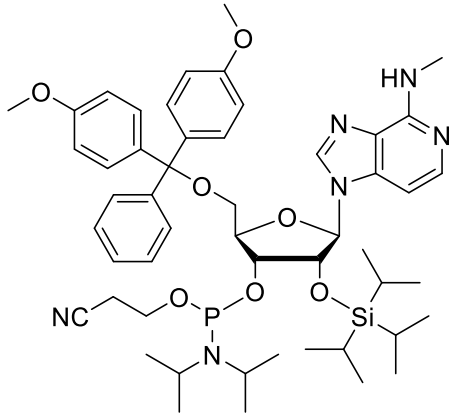
^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.99–7.91 (m, 2H, C2-H, C8-H), 7.50–7.44 (m, 2H, trityl-H), 7.40–7.32 (m, 4H, trityl-H), 7.30–7.15 (m, 3H, trityl-H), 6.83–6.75 (m, 4H, trityl-H), 6.29 (2 d, *J* = 4.8 Hz, 1H, C1-H), 6.04 (2 d, *J* = 6.3 Hz, 1H, C1'-H), 5.38 (2 dd, *J* = 6.3, 4.6 Hz, 1H, C2'-H), 5.28–5.20 (m, 1H, N*H*CH_3), 4.53–4.33 (m, 2H, C3'-H, C4'-H), 4.00–3.81 (m, 1H, $POCH_2^a$), 3.78 (2 s, 6H (trityl-$OCH_3)_2$)), 3.70–3.51 (m, 4H, C5'-H^a, $POCH_2^b$, N(C*H*$(CH_3)_2)_2$), 3.25 (2 dd, *J* = 10.4, 4.3 Hz, 1H, C5'-H^b), 3.01 (2 d, *J* = 5.2 Hz, 3H, NHC*H*$_3$), 2.66–2.59 (m, 1H, CH_2^aCN), 2.30–2.23 (m, 1H, CH_2^bCN), 1.17 (2 d, *J* = 6.8 Hz, 9H, N(CH(C*H*$_3)_2)_2^{a,b,c}$), 1.07 (d, *J* = 6.8 Hz, 3 H, N(CH(C*H*$_3)_2)_2^d$), 0.99–0.74 (m, 21H, Si(C*H*$(CH_3)_2)_3$, Si(CH(C*H*$_3)_2)_3$).

$^{13}C\{^{1}H\}$ NMR (100 MHz, $CDCl_3$): δ (ppm) = 158.55 (2C, trityl-C), 147.14 (1C, C6), 146.54 (1C, C4), 146.08 (1C, C2), 144.83 (1C, trityl-C), 139.26 (1C, C8), 135.99, 135.96 (2C, trityl-C), 130.32, 130.30, 130.27 (4C, trityl-C), 128.47, 128.35 (2C, trityl-C), 127.93 (2C, trityl-C), 126.91, 126.89 (1C, trityl-C), 124.28, 123.98 (1C, C5), 117.77, 117.24 (1C, CH_2*C*N), 113.22 (4C, trityl-C), 98.57 (1C, C1), 88.78, 88.32 (1C, C1'), 86.64, 86.53 (1C, trityl-C), 83.43, 82.97 (1C, C4'), 74.34, 74.16 (1C, C2'), 74.05 (1C, C3'), 63.63, 63.52 (1C, C5'), 58.99, 58.84, 57.79 (1C, $POCH_2$), 55.36, 55.33 (2C, trityl-OCH_3), 43.65, 43.52, 43.21, 43.09 (2C, N*C*H$(CH_3)_2$), 29.51, 29.50 (1C, $NHCH_3$), 24.93, 24.91, 24.85, 24.70, 24.65, 24.59 (4C, NCH(*C*$H_3)_2$), 20.56, 20.49, 20.14, 20.06 (1C, *C*H_2CN), 18.12, 17.84, 17.76 (6C, CH(*C*$H_3)_2$), 12.56, 12.42 (3C, *C*H$(CH_3)_2$).

$^{31}P\{^{1}H\}$ NMR (202 MHz, $CDCl_3$): δ (ppm) = 151.24, 148.58.

HR-ESI-MS: *m*/*z* calc. ($C_{51}H_{71}N_6NaO_7PSi$ $[M+Na]^+$): 961.4783, found: 961.4720.

5'-*O*-(4,4'-Dimethoxytrityl)-2'-*O*-(triisopropylsilyl)-N^6-methyl-3-deazaadenosine-3'-*O*-(2-cyano-ethyl)diisopropylphosphoramidite (**4c**)



4d

$C_{51}H_{71}N_6O_7PSi$

939.22 g·mol^{-1}

A solution of compound **3c** (250 mg, 338 µmol, 1.00 eq.) in dry pyridine (2.5 mL) under an inert gas atmosphere was treated with *i*Pr$_2$NEt (590 µL, 3.38 mmol, 10.0 eq.) and CEPCl (230 µL, 1.01 mmol, 3.00 eq.) and stirred at ambient temperature for 5 h. The mixture was diluted with MeCN (1.3 mL) and concentrated to a volume of ca. 0.6 mL. The solution was diluted with MeCN (0.4 mL) and MTBE (3.1 mL) and washed with water (1.1 mL), water/DMF 1:1 (2 × 2.1 mL), water (1.1 mL) and 13% aq. NaCl solution (1.1 mL). The organic layer was dried over Na_2SO_4 and concentrated to a volume of ca. 0.7 mL. The solution was added dropwise into *n*-heptane (6.3 mL). The volume was reduced to ca. 2 mL and *n*-heptane (4.2 mL) was added. The solvent was removed under reduced pressure and the residue was purified by column chromatography (*n*-hexane:EtOAc = 2:1 + 1% NEt_3) to yield the product **4c** as a colorless foam (185 mg, 197 µmol, 58%).

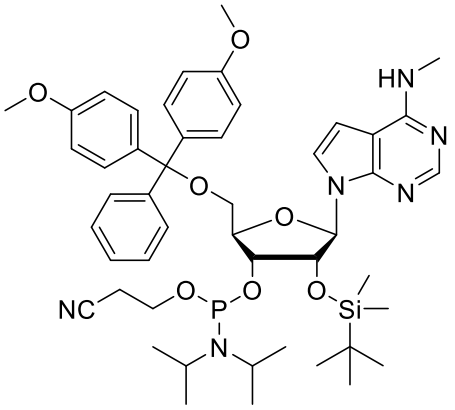
^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.98, 7.95 (2 s, 1H, C8-H), 7.72 (2 d, *J* = 5.9 Hz, 1H, C2-H), 7.48–7.39 (m, 2H, trityl-H), 7.38–7.17 (m, 7H, trityl-H), 6.88–6.77 (m, 5H, C3-H, trityl-H), 5.85, 5.77 (2 d, *J* = 7.5 Hz, 1H, C1'-H), 5.44–5.35 (m, 1H, N*H*CH$_3$), 4.86, 4.79 (2 dd, *J* = 7.5, 4.8 Hz, 1H, C2'-H), 4.50–4.28 (m, 2H, C3'-H, C4'-H), 4.00–3.82 (m, 2H, POCH$_2$), 3.78 (4 s, 6H, (trityl-OCH$_3$)$_2$), 3.71–3.28 (m, 4H, C5'-H, N(C*H*(CH$_3$)$_2$)$_2$), 3.15 (2 d, *J* = 5.1, 3H, NHC*H*$_3$), 2.80–2.55 (m, 2H, CH$_2$CN), 1.31–1.15 (m, 9H, N(CH(C*H*$_3$)$_2$)$_2$[a,b,c]), 1.07 (d, *J* = 6.7 Hz, 3H, N(CH(C*H*$_3$)$_2$)$_2$[d]), 0.97–0.73 (m, 21H, Si(C*H*(CH$_3$)$_2$)$_3$, Si(CH(C*H*$_3$)$_2$)$_3$).

^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ (ppm) = 158.76 (2C, trityl-C), 152.48 (1C, C6), 144.48, 144.35 (1C, trityl-C), 141.42 (1C, C2), 139.09, 139.00 (1C, C8), 137.40 (1C, C4), 135.57, 135.39, 135.28 (2C, trityl-C), 130.34, 130.26 (4C, trityl-C), 128.38, 128.25, 128.09 (4C, trityl-C), 127.99 (1C, C5), 127.21 (1C, trityl-C), 117.63 (1C, CH$_2$*C*N), 113.38 (4C, trityl-C), 97.87, 97.73 (1C, C3), 88.92, 88.75 (1C, C1'), 87.27, 87.12 (1C, trityl-C), 84.55, 83.68, 83.64 (1C, C4'), 75.90, 75.85 (1C, C2'), 73.78, 73.63 (1C, C3'), 63.67, 63.38 (1C, C5'), 58.99, 58.81 (1C, POCH$_2$), 55.36 (2C, trityl-OCH$_3$), 43.80, 43.67, 43.24, 43.12 (2C, N*C*H(CH$_3$)$_2$), 28.00 (1C, NHCH$_3$), 24.90, 24.82, 24.71, 24.64 (4C, NCH(*C*H$_3$)$_2$), 20.58, 20.52 (1C, *C*H$_2$CN), 19.90, 18.03, 17.98, 17.69, 17.68, 17.66, 17.65 (6C, CH(*C*H$_3$)$_2$), 12.46, 12.25 (3C, *C*H(CH$_3$)$_2$).

^{31}P{^{1}H} NMR (202 MHz, $CDCl_3$): δ (ppm) = 152.48, 148.23.

HR-ESI-MS: *m*/*z* calc. ($C_{51}H_{72}N_6O_7PSi$ [M+H]$^+$): 939.4964, found: 939.4936.

5'-*O*-(4,4'-Dimethoxytrityl)-2'-*O*-(*tert*-butyldimethylsilyl)-*N*^{6}-methyl-7-deazaadenosine-3'-*O*-(2-cyanoethyl)diisopropylphosphoramidite (**4d**)



4d

$C_{48}H_{65}N_6O_7PSi$

897.14 g·mol^{-1}

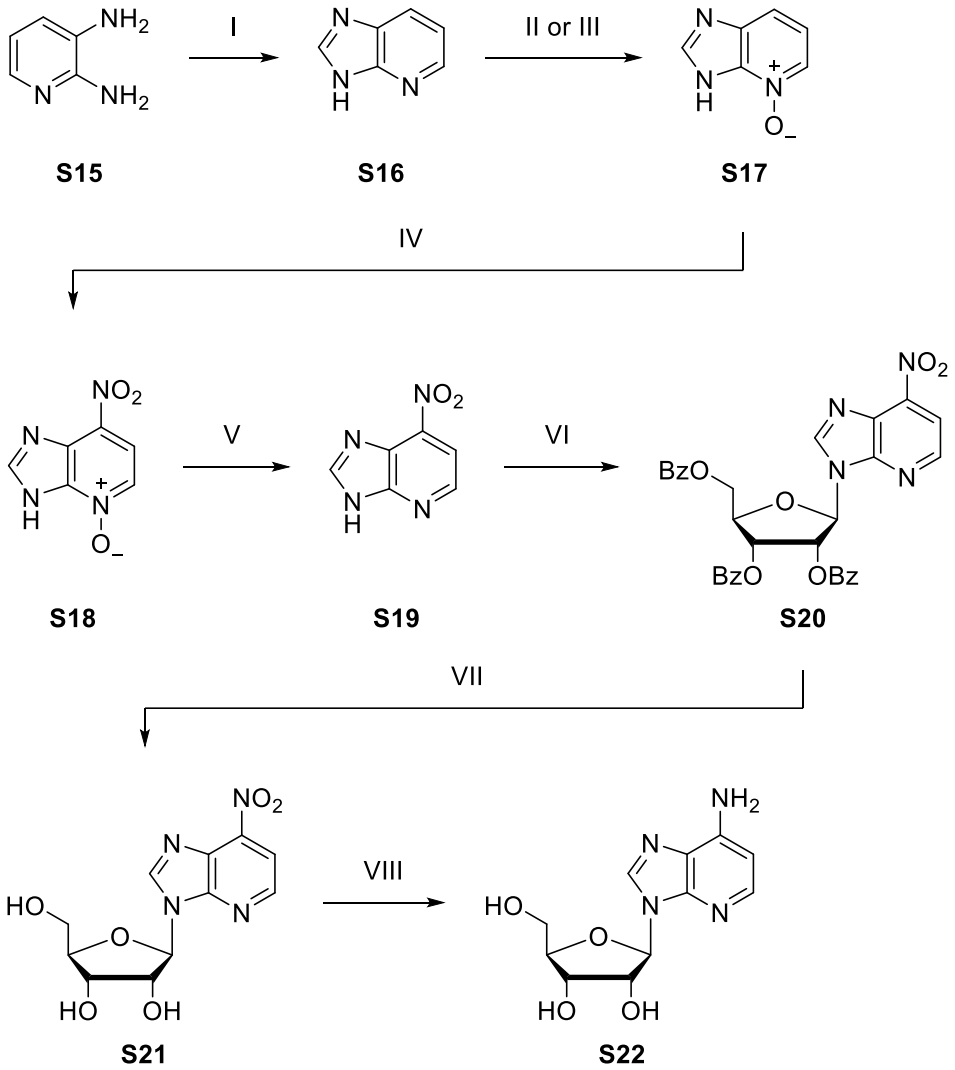
A solution of **3d** (82.0 mg, 118 µmol, 1.00 eq.) in dry DCM (1 mL) under inert gas atmosphere was treated with $EtNMe_2$ (120 µL, 1.11 mmol, 9.41 eq.). CEPCl (30.0 µL, 134 µmol, 1.14 eq.) was added in three portions over 1 h. The resulting mixture was stirred at ambient temperature for 3 h. It was evaporated to dryness and the residue was purified by column chromatography (silica gel, *n*-hexane:EtOAc 1:1 + 1% NEt_3), followed by re-purification of the combined product containing fractions by column chromatography (basic aluminum oxide, *n*-hexane:EtOAc = 1:1 + 1% NEt_3), yielding the product **4d** as a colorless foam (32.4 mg, 36.1 µmol, 31%).

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 8.31, 8.28 (2s, 1H, C2-H), 7.51–7.43 (m, 2H, trityl-H), 7.41–7.31 (m, 4H, trityl-H), 7.32–7.17 (m, 3H, C8-H, trityl-H), 6.86–6.76 (m, 4H, trityl-H), 6.39–6.32 (m, 1H, C9-H), 6.29, 6.26 (2d, *J* = 6.6 Hz, 1H, C1'-H), 5.11 (s_{br}, 1H, N*H*CH_3), 4.85, 4.79 (2dd, *J* = 6.6, 4.7 Hz, 1H, C2'-H), 4.40–4.23 (m, 2H, C3'-H, C4'-H), 4.06–3.83 (m, 1H, $POCH_2^a$), 3.82–3.76 (m, 6H, trityl-$(OCH_3)_2$), 3.70–3.49 (m, 4H, N(C*H*$(CH_3)_2)_2$, C5'-H^a, $POCH_2^b$), 3.28–3.15 (m, 4H, C5'-H^b, NHC*H*$_3$), 2.70–2.64 (m, 1H, CH_2^a-CN), 2.31–2.25 (m, 1H, CH_2^b-CN), 1.21–1.13 (m, 9H, N(CH(C*H*$_3)_2)_2^{a,b,c}$), 1.00 (d, *J* = 6.7 Hz, 3H, N(CH(C*H*$_3)_2)_2^d$), 0.75 (d, *J* = 6.3 Hz, 9H, Si$(CH_3)_2$(C(C*H*$_3)_3$), -0.04, -0.09 (2s, 3H, Si(C*H*$_3)_2^a$(C$(CH_3)_3$), -0.21, -0.23 (2s, 3H, Si(C*H*$_3)_2^b$(C$(CH_3)_3$).

^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ (ppm) = 158.65 (2C, trityl-C), 157.09 (1C, C6), 152.02 (1C, C2), 150.85 (1C, C4), 144.90, 144.79 (1C, trityl-C), 136.07, 135.97, 135.88, 135.82 (2C, trityl-C), 130.37, 130.31, 130.27, 130.25 (4C, trityl-C), 128.47, 128.33 (2C, trityl-C), 128.01, 127.99 (2C, trityl-C), 127.01 (1C, trityl-C), 121.92, 121.87 (1C, C8), 117.95, 117.48 (1C, CH_2*C*N), 113.30, 113.29, 113.26, 113.24 (4C, trityl-C), 103.75 (1C, C5), 99.06 (1C, C9), 87.61, 87.31 (1C, C1'), 86.69, 86.51 (1C, trityl-C), 83.45, 83.30 (1C, C4'), 75.86, 75.33 (1C, C2'), 72.93, 72.78 (1C, C3'), 63.76, 63.52 (1C, C5'), 59.63, 59.03, 57.88, 57.67 (1C, $POCH_2$), 55.38, 55.36 (2C, trityl-OCH_3), 43.54, 43.41, 43.05, 42.92 (2C, N*C*H$(CH_3)_2$), 28.51, 27.40 (1C, NHCH_3), 25.82, 25.80, 25.77 (3C, SiC(*C*H$_3)_3$), 24.91, 24.84, 24.76, 24.70, 24.64 (4C, NCH(*C*H$_3)_2$), 20.65, 20.59, 20.19, 20.12 (1C, *C*H_2CN), 18.16, 18.07 (1C, Si*C*$(CH_3)_3$), -4.64, -4.68 (1C, Si(*C*H$_3)_2^a$), -5.03 (1C, Si(*C*H$_3)_2^b$).

^{31}P{^{1}H} NMR (162 MHz, $CDCl_3$): δ (ppm) = 150.46, 148.71.

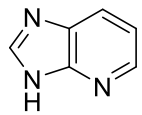
HR-ESI-MS: *m*/*z* calc. ($C_{48}H_{65}N_6NaO_7PSi$ [M+Na]$^+$): 919.43138, found: 919.43085.



Scheme S4. Synthesis of 1-deazaadenosine (**S22**). I) $(EtO)_3CH$, 145 °C, 3 h, then HCOOH, 110 °C, 2 h, 98%; II) *m*-CPBA, $CHCl_3$, rt, 48 h, 90%; III) *m*-CPBA, AcOH, 50 °C, 16 h, 95%; IV) $HNO_3 \cdot NO_2$, TFA, 90 °C, 3 h, 80%; V) PCl_3, MeCN, 0 °C, 90 min, 63%; VI) 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose, $SnCl_4$, MeCN, 16 h, 66%; VII) NH_3, MeOH, rt, 24 h, 86%, VIII) H_2, Pd/C, MeOH, rt, 72 h, 58%.

6-Nitro-1-deazapurine (**S19**) was synthesized from 2,3-diaminopyridine by cyclization with trialkyl orthoformate followed by nitration at C6 using an intermediary *N*-oxide for directing the nitro group to the correct position. **S19** was converted to the corresponding nucleoside **S20**, deprotected and the nitro group was reduced to yield 1-deazaadenosine (**S22**).

1-Deazapurine (**S16**)



S16

$C_6H_5N_3$

119.13 $g \cdot mol^{-1}$

This compound was synthesized according to a reported procedure.[10] 2,3-Diaminopyridine (**S15**) (3.00 g, 27.5 mmol, 1.00 eq.) was suspended in triethyl orthoformate (59.0 mL, 360 mmol, 13.1 eq.) and stirred at 145 °C for 3 h. The solvent was removed under reduced

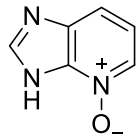
pressure and the residue was redissolved in formic acid (300 mL) and stirred at 110 °C for 2 h. The solvent was removed under reduced pressure, the residue was redissolved in MeOH (90 mL), treated with decolorizing carbon and stirred for 12 h. The suspension was filtered through a pad of Celite, the filtrate was evaporated, and the residue was coevaporated with water (3×) and *n*-hexane (3×) to yield the product **S16** as a brown solid (3.21 g, 26.9 mmol, 98%). Analytical data agreed with reported values.[10]

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 12.92 (s_{br}, 1H, NH), 8.43 (s, 1H, C8-H), 8.35 (dd, *J* = 4.8, 1.5 Hz, 1H, C2-H), 8.01 (dd, *J* = 8.0, 1.5 Hz, 1H, C6-H), 7.23 (dd, *J* = 8.0, 4.8 Hz, 1H, C1-H).

^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6): δ (ppm) = 143.95 (1C, C8), 143.73 (1C, C2), 117.76 (1C, C1).

HR-ESI-MS: *m*/*z* calc. ($C_6H_6N_3$ $[M+H]^+$): 120.05562, found: 120.05663.

1-Deazapurine-3-oxide (**S17**)



S17

$C_6H_5N_3O$

135.13 g·mol^{-1}

Method A (according to a reported procedure):[10]
Compound **S16** (3.21 g, 26.9 mmol, 1.00 eq.) was suspended in $CHCl_3$ (27.4 mL), treated with *m*-CPBA (75%, 13.70 g, 59.5 mmol, 2.21 eq.) and stirred at ambient temperature for 48 h. The precipitate was collected by filtration and purified by column chromatography (silica gel, DCM:MeOH 9:1 to 7:3) to yield the product **S17** as an off-white solid (3.28 g, 24.3 mmol, 90%).

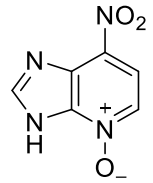
Method B (according to a reported procedure):[11]
A solution of compound **S16** (2.28 g, 19.1 mmol, 1.00 eq.) in acetic acid (100 mL) was treated with *m*-CPBA (75%, 8.19 g, 35.6 mmol, 1.86 eq.) and stirred at 50 °C for 16 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography (silica gel, DCM:MeOH 9:1 to 7:3) to yield the product **S17** as an off-white solid (2.46 g, 18.2 mmol, 95%).

Analytical data agreed with reported values.[10]

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 13.33 (s_{br}, 1H, NH), 8.43 (s, 1H, C8-H), 8.20 (dd, *J* = 6.3, 0.8 Hz, 1H, C2-H), 7.61 (d, *J* = 8.2 Hz, 1H, C6-H), 7.21 (dd, *J* = 8.1, 6.3 Hz, 1H, C1-H).
^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6): δ (ppm) = 143.45 (1C, C8), 132.88 (1C, C2), 118.98 (1C, C1), 112.03 (1C, C6).

HR-ESI-MS: *m*/*z* calc. ($C_6H_5N_3NaO$ $[M+Na]^+$): 158.03248, found: 158.03327.

6-Nitro-1-deazapurine-3-oxide (**S18**)



S18

$C_6H_4N_4O_3$

180.12 g·mol^{-1}

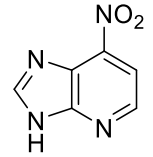
This compound was synthesized according to a reported procedure.[10] Compound **S17** (3.27 g, 24.4 mmol, 1.00 eq.) was dissolved in TFA (25 mL) at 0 °C. Fuming nitric acid (15.3 mL, 366 mmol, 15.0 eq.) was added and the solution was stirred at 90 °C for 3 h. After cooling to ambient temperature, the solution was adjusted to pH = 7 with 25% aq. ammonia. The resulting precipitate was collected by filtration and dried *in vacuo* to yield the product **S18** as a yellow solid (3.52 g, 19.7 mmol, 80%). Analytical data agreed with reported values.[12]

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 14.01 (s_{br}, 1H, NH), 8.61 (s, 1H, C8-H), 8.33 (d, *J* = 7.2 Hz, 1H, C2-H), 8.09 (d, *J* = 7.2 Hz, 1H, C1-H).

^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6): δ (ppm) = 145.82 (1C, C8), 133.45 (1C, C2), 115.19 (1C, C1).

HR-ESI-MS: *m*/*z* calc. ($C_6H_4N_4NaO_3$ [M+Na]$^+$): 203.01756, found: 203.01810.

6-Nitro-1-deazapurine (**S19**)



S19

$C_6H_4N_4O_2$

164.12 g·mol-1

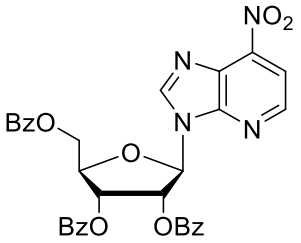
This compound was prepared according to a reported procedure.[12] A solution of compound **S18** (1.20 g, 6.66 mmol, 1.00 eq.) in MeCN (25 mL) under inert gas atmosphere was cooled to 0 °C, treated with PCl_3 (5.28 mL, 60.5 mmol, 9.08 eq.) and stirred at 0 °C for 90 min. The solution was poured onto crushed ice and neutralized by addition of solid Na_2CO_3. The resulting precipitate was collected by filtration and purified by column chromatography (silica gel, DCM:MeOH 9:1) to yield the product **S19** as a reddish yellow solid (695 mg, 4.23 mmol, 63%).

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 13.74 (s, 1H, NH), 8.78 (s, 1H, C8-H), 8.68 (d, *J* = 5.4 Hz, 1H, C2-H), 8.00 (d, *J* = 5.4 Hz, 1H, C1-H).

^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6): δ (ppm) = 148.38 (1C, C8), 144.14 (1C, C2), 110.90 (1C, C1).

HR-ESI-MS: *m*/*z* calc. ($C_6H_4N_4NaO_2$ [M+Na]$^+$): 187.02265, found: 187.02189.

2',3',5'-Tri-*O*-benzoyl-6-nitro-1-deazapurine-9-β-D-ribofuranoside (**S20**)



S20

$C_{32}H_{24}N_4O_9$

608.56 g·mol^{-1}

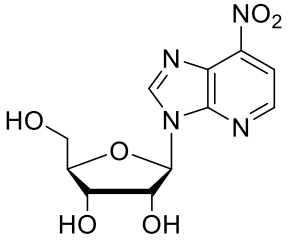
A suspension of compound **S19** (600 mg, 3.66 mmol, 1.00 eq.) and 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (1.85 g, 3.66 mmol, 1.00 eq.) in MeCN (20 mL) under inert gas atmosphere was treated dropwise with $SnCl_4$ (860 µL, 7.32 mmol, 2.00 eq.). The resulting solution was stirred at ambient temperature for 16 h, cooled to 0 °C and neutralized by addition of sat. aq. $NaHCO_3$ solution. The layers were separated and the aqueous layer was extracted with $CHCl_3$ (4 × 20 mL). The combined organic layers were dried over Na_2SO_4, the solvent was removed under reduced pressure, and the residue was purified by column chromatography (silica gel, DCM:MeOH 9:1) to yield the product **S20** as a yellow foam (1.48 g, 2.43 mmol, 66%).

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 8.49 (s, 1H, C8-H), 8.47 (d, *J* = 5.3 Hz, 1H, C2-H), 8.10–8.05 (m, 2H, benzoyl-H), 8.04–8.00 (m, 2H, benzoyl-H), 7.95–7.89 (m, 3H, C1-H, benzoyl-H), 7.63–7.52 (m, 3H, benzoyl-H), 7.48–7.40 (m, 4H, benzoyl-H), 7.40–7.33 (m, 2H, benzoyl-H), 6.54 (d, *J* = 5.0 Hz, 1H, C1'-H), 6.50 (t, *J* = 5.4 Hz, 1H, C2'-H), 6.26 (dd, *J* = 5.7, 5.0 Hz, 1H, C3'-H), 4.93 (dd, *J* = 12.1, 3.3 Hz, 1H, C5'-H^a), 4.90–4.85 (m, 1H, C4'-H), 4.70 (dd, *J* = 12.1, 4.3 Hz, 1H, C5'-H^b).

^{13}C{^{1}H} NMR (100 MHz, $CDCl_3$): δ (ppm) = 166.23 (1C, benzoyl-C), 165.47 (1C, benzoyl-C), 165.26 (1C, benzoyl-C), 150.06 (1C, C4), 147.12 (1C, C8), 145.41 (1C, C2), 144.52 (1C, C6), 134.05 (1C, benzoyl-C), 133.97 (1C, benzoyl-C), 133.66 (1C, benzoyl-C), 129.98 (2C, benzoyl-C), 129.97 (2C, benzoyl-C), 129.88 (2C, benzoyl-C), 129.40 (1C, benzoyl-C), 128.78 (1C, benzoyl-C), 128.77 (2C, benzoyl-C), 128.75 (2C, benzoyl-C), 128.70 (2C, benzoyl-C), 128.40 (1C, benzoyl-C), 128.38 (1C, C5), 113.03 (1C, C1), 87.81 (1C, C1'), 81.06 (1C, C4'), 73.83 (1C, C2'), 71.59 (1C, C3'), 63.55 (1C, C5').

HR-ESI-MS: *m*/*z* calc. ($C_{32}H_{24}N_4NaO_9$ [M+Na]$^+$): 631.14355, found: 631.14125.

6-Nitro-1-deazapurine-9-β-D-ribofuranoside (**S21**)



S21

$C_{11}H_{12}N_4O_6$

296.24 g·mol^{-1}

A suspension of compound **S20** (750 mg, 1.23 mmol, 1.00 eq.) in MeOH (12.4 mL) was treated with NH_3 (7 M in MeOH, 1.80 mL, 12.6 mmol, 10.2 eq.) and stirred at ambient temperature for 24 h. The solvent was removed under reduced pressure and the residue was purified by

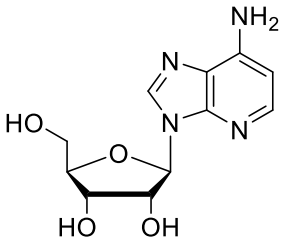
column chromatography (silica gel, EtOAc:MeOH = 8:2) to yield the product **S21** as a light brown solid (314 mg, 1.06 mmol, 86%).

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 9.08 (s, 1H, C8-H), 8.69 (d, *J* = 5.3 Hz, 1H, C2-H), 8.04 (d, *J* = 5.3 Hz, 1H, C1-H), 6.15 (d, *J* = 5.2 Hz, 1H (C1'-H), 5.59 (d, *J* = 5.8 Hz, 1H, C2'-OH), 5.26 (d, *J* = 5.2 Hz, 1H, C3'-OH), 5.12 (t, *J* = 5.5 Hz, 1H, C5'-OH), 4.61 (q, *J* = 5.3 Hz, 1H, C2'-H), 4.20 (q, *J* = 4.9 Hz, 1H, C3'-H), 4.00 (q, *J* = 3.9 Hz, 1H, C4'-H), 3.72 (ddd, *J* = 12.0, 5.2, 4.0 Hz, 1H, C5'-H^{a}), 3.59 (ddd, *J* = 12.1, 5.8, 3.9 Hz, 1H, C5'-H^{b}).

^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6): δ (ppm) = 150.21 (1C, C4), 147.92 (1C, C8), 144.87 (1C, C2), 143.80 (1C, C6), 127.13 (1C, C5), 112.26 (1C, C1), 87.98 (1C, C1'), 85.56 (1C, C4'), 73.94 (1C, C2'), 70.08 (1C, C3'), 61.03 (1C, C5').

HR-ESI-MS: *m*/*z* calc. ($C_{11}H_{12}N_4NaO_6$ $[M+Na]^+$): 319.0649, found: 319.0652.

1-Deazaadenosine (**S22**)



S22

$C_{11}H_{14}N_4O_4$

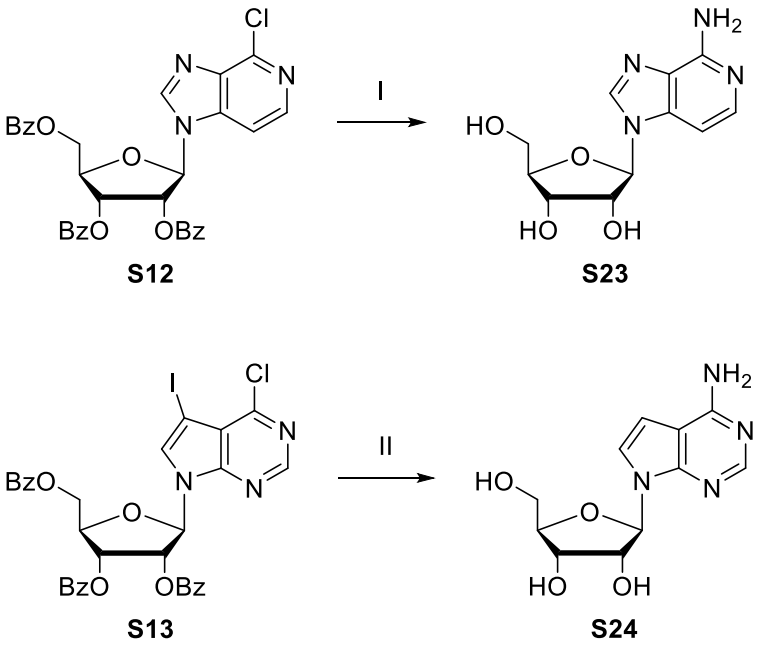
266.26 g·mol^{-1}

This compound was synthesized by modification of a reported procedure.[13] A solution of compound **S21** (250 mg, 844 µmol, 1.00 eq.) in MeOH (40 mL) was treated with 10% Pd/C (125 mg) and stirred under H_2 atmosphere at ambient temperature for 3 d. The suspension was filtered through a pad of Celite and the filtrate was evaporated to dryness. The residue was purified by column chromatography (DCM:MeOH = 9:1) to yield the product **S22** as an off-white solid (131 mg, 492 µmol, 58%). Analytical data agreed with reported values.[14]

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 8.25 (s, 1H, C8-H), 7.77 (d, *J* = 5.6 Hz, 1H, C2-H), 6.50 (s, 2H, NH_2), 6.39 (d, *J* = 5.6 Hz, 1H, C1-H), 6.09 (s_{br}, 1H, C5'-OH), 5.87 (d, *J* = 6.7 Hz, 1H, C1'), 5.39 (d, *J* = 6.5 Hz, 1H, C2'-OH), 5.15 (d, *J* = 4.2 Hz, 1H, C3'-OH), 4.71 (td, *J* = 6.5, 4.9 Hz, 1H, C2'-H), 4.13 (td, *J* = 4.5, 2.3 Hz, 1H, C3'-H), 3.98 (q, *J* = 2.8 Hz, 1H, C4'-H), 3.66 (dd, *J* = 12.3, 3.0 Hz, 1H, C5'-H^{a}), 3.54 (dd, *J* = 12.3, 3.0 Hz, 1H, 5'-H^{b}).

^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6): δ (ppm) = 147.46 (1C, C6), 146.44 (1C, C4), 144.14 (1C, C2), 140.10 (1C, C8), 123.81 (1C, C5), 102.36 (1C, C1), 88.63 (1C, C1'), 86.27 (1C, C4'), 72.87 (1C, C2'), 71.14 (1C, C3'), 62.11 (1C, C5').

HR-ESI-MS: *m*/*z* calc. ($C_{11}H_{15}N_4O_4$[M+H]$^+$): 267.1088, found: 267.1082.



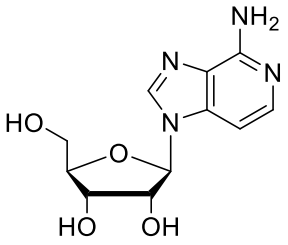
S12 **S23**

S13 **S24**

Scheme S5. Synthesis of 3-deazaadenosine (**S23**) and 7-deazaadenosine (**S24**). I) NH_3, H_2O, 125 °C, 24 h, 88%; II) *i*-PrMgCl•LiCl, THF, -10 °C, 1 h, then NH_3, H_2O, 125 °C, 24 h, 88%.

3-Deazaadenosine (**S23**) and 7-deazaadenosine (**S24**) were synthesized by treatment of the 6-chloro-precursors **S12** and **S13**, respectively, with aqueous ammonia at elevated temperature, which led to simultaneous deprotection of the ribose and introduction of the amino group at C6. For compound **S13**, the iodine at C7 was removed before deprotection, by treatment with *i*-PrMgCl•LiCl followed by aqueous workup.

3-Deazaadenosine (**S23**)



S23

$C_{11}H_{14}N_4O_4$

266.26 g·mol^{-1}

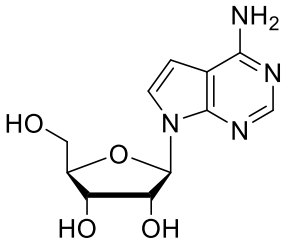
In a sealed pressure tube, a solution of compound **S12** (500 mg, 836 µmol, 1.00 eq.) in NH_3 (25% in H_2O, 50 mL) was stirred at 125 °C for 24 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography (silica gel, EtOAc:MeOH = 8:2) to yield the product **S23** as an off-white solid (197 mg, 740 µmol, 88%).

^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 8.29 (s, 1H, C8-H), 7.66 (d, *J* = 5.8 Hz, 1H, C2-H), 6.91 (d, *J* = 5.8 Hz, 1H, C3-H), 6.18 (s, 2H, NH_2), 5.75 (d, *J* = 6.2 Hz, 1H, C1'-H), 5.49 (d, *J* = 6.4 Hz, 1H, C2'-OH), 5.24 (d, *J* = 4.7 Hz, 1H, C3'-OH), 5.10 (t, *J* = 5.2 Hz, 1H, C5'-OH), 4.32 (q, *J* = 5.8 Hz, 1H, C2'-H), 4.09 (q, *J* = 4.1 Hz, 1H, C3'-H), 3.95 (q, *J* = 3.6 Hz, 1H, C4'-H), 3.70–3.55 (m, 2H, C5'-H).

$^{13}C\{^1H\}$ NMR (100 MHz, DMSO-d_6): δ (ppm) = 152.45 (1C, C6), 140.61 (1C, C2), 139.97 (1C, C8), 137.59 (1C, C4), 126.92 (1C, C5), 97.38 (1C, C3), 88.65 (1C, C1'), 85.55 (1C, C4'), 73.86 (1C, C2'), 70.12 (1C, C3'), 61.25 (1C, C5').

HR-ESI-MS: *m*/*z* calc. ($C_{11}H_{14}NaN_4O_4$ $[M+Na]^+$): 289.0907, found: 289.0905.

7-Deazaadenosine (**S24**)



S24

$C_{11}H_{14}N_4O_4$

266.26 g·mol^{-1}

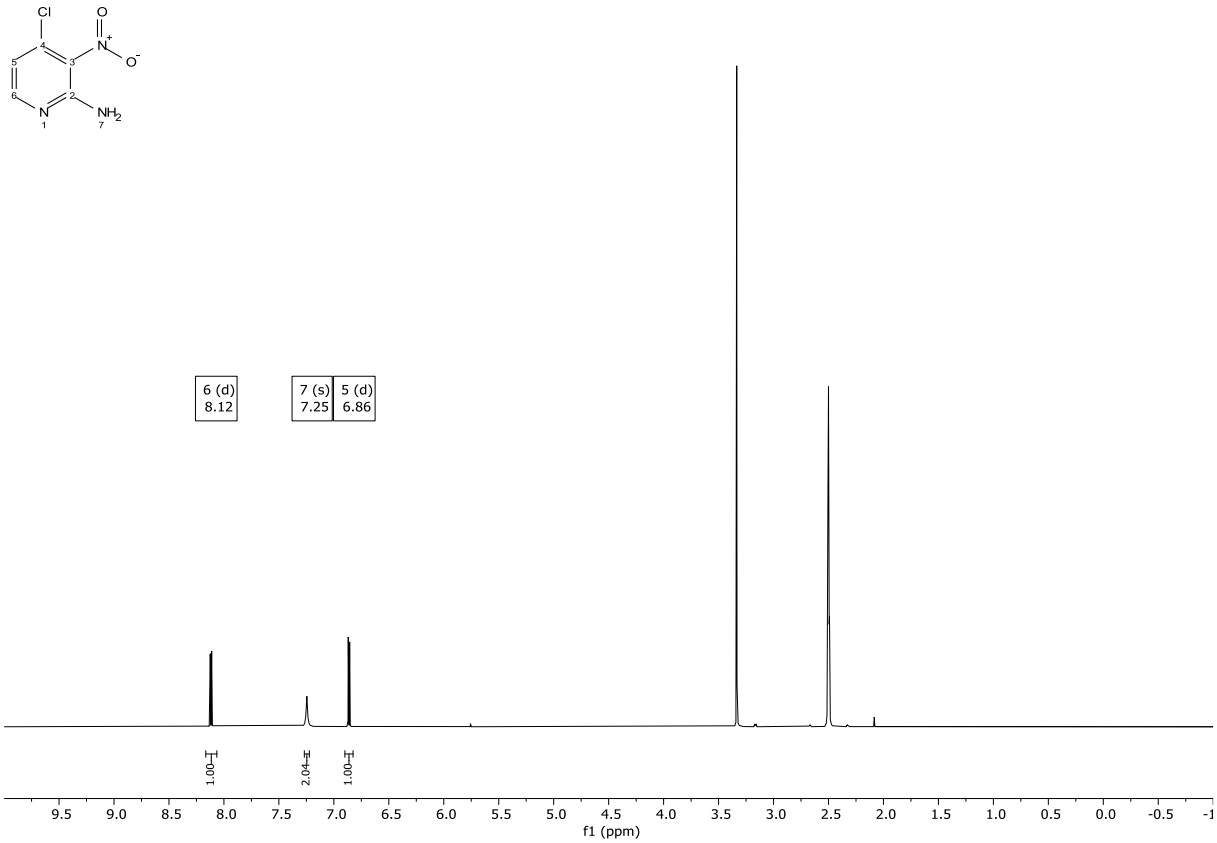
This compound was synthesized by modification of a reported procedure.[6] Compound **S13** (750 mg, 1.04 mmol, 1.00 eq.) was dissolved in dry THF (4 mL) under inert gas atmosphere. The reaction mixture was cooled to -10 °C and *i*-PrMgCl•LiCl (1.30 M in THF, 930 µL, 1.21 mmol, 1.10 eq.) was added dropwise. The solution was stirred at this temperature for 1 h and then poured onto a mixture of ice (10 g) and sat. aq. NH_4Cl solution (10 mL). This mixture was extracted with DCM (3 × 20 mL), the combined organic layers were dried over $MgSO_4$ and the solvent was removed under reduced pressure. The residue was redissolved in NH_3 (25% in H_2O, 50 mL), transferred to a sealed pressure tube and stirred at 125 °C for 24 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography (silica gel, EtOAc:MeOH = 8:1) to yield the product **S24** as an off-white solid (244 mg, 916 µmol, 88%).

^1H NMR (400 MHz, DMSO-d_6): δ (ppm) = 8.04 (s, 1H, C2-H), 7.34 (d, *J* = 3.7 Hz, 1H, C8-H), 7.05 (s, 2H, NH_2), 6.58 (d, *J* = 3.7 Hz, 1H, C7-H), 5.98 (d, *J* = 6.3 Hz, 1H, C1'-H), 5.34 (dd, *J* = 6.6, 4.7 Hz, 1H, C5'-OH), 5.27 (d, *J* = 6.5 Hz, 1H, C2'-OH), 5.11 (d, *J* = 4.7 Hz, 1H, C3'-OH), 4.42 (q, *J* = 6.1 Hz, 1H, C2'-H), 4.07 (td, *J* = 4.7, 3.0 Hz, 1H, C3'-H), 3.89 (q, *J* = 3.6 Hz, 1H, C4'-H), 3.62 (dt, *J* = 12.0, 4.3 Hz, 1H, C5'-H^a), 3.52 (ddd, *J* = 12.0, 6.6, 3.8 Hz, 1H, C5'-H^b).

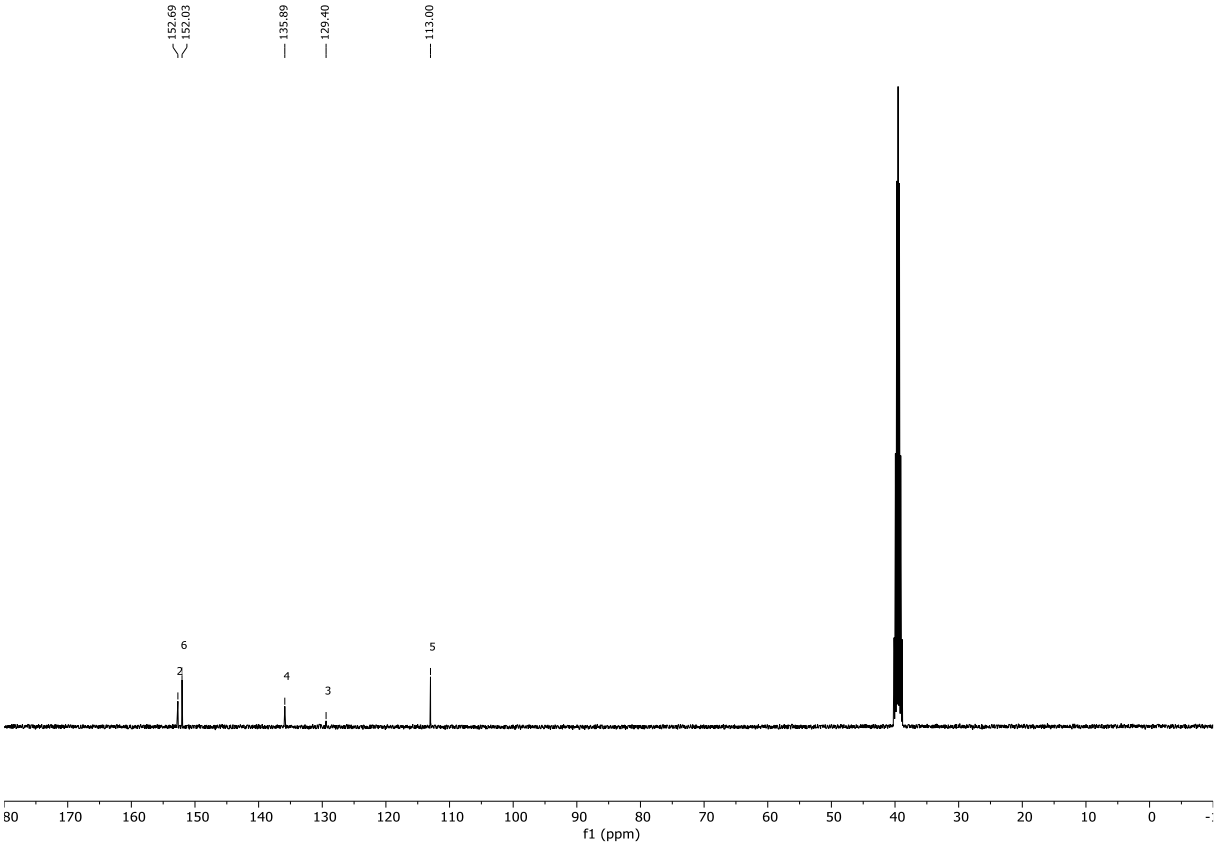
$^{13}C\{^1H\}$ NMR (100 MHz, DMSO-d_6): δ (ppm) = 157.59 (1C, C6), 151.58 (1C, C2), 149.93 (1C, C4), 122.35 (1C, C8), 103.13 (1C, C5), 99.55 (1C, C7), 87.61 (1C, C1'), 85.11 (1C, C4'), 73.68 (1C, C2'), 70.77 (1C, C3'), 61.89 (1C, C5').

HR-ESI-MS: *m*/*z* calc. ($C_{11}H_{15}N_4O_4$ $[M+H]^+$): 267.1088, found: 267.1085.

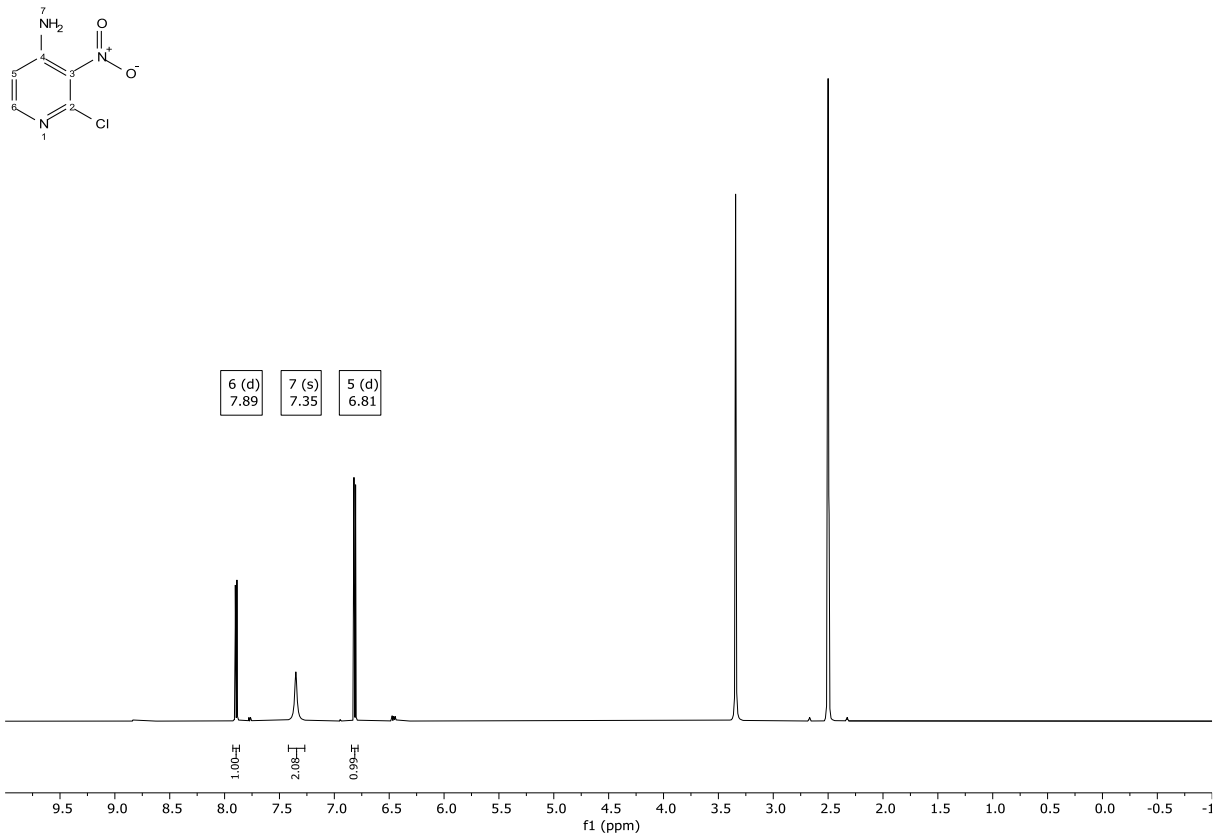
1.3. NMR spectra



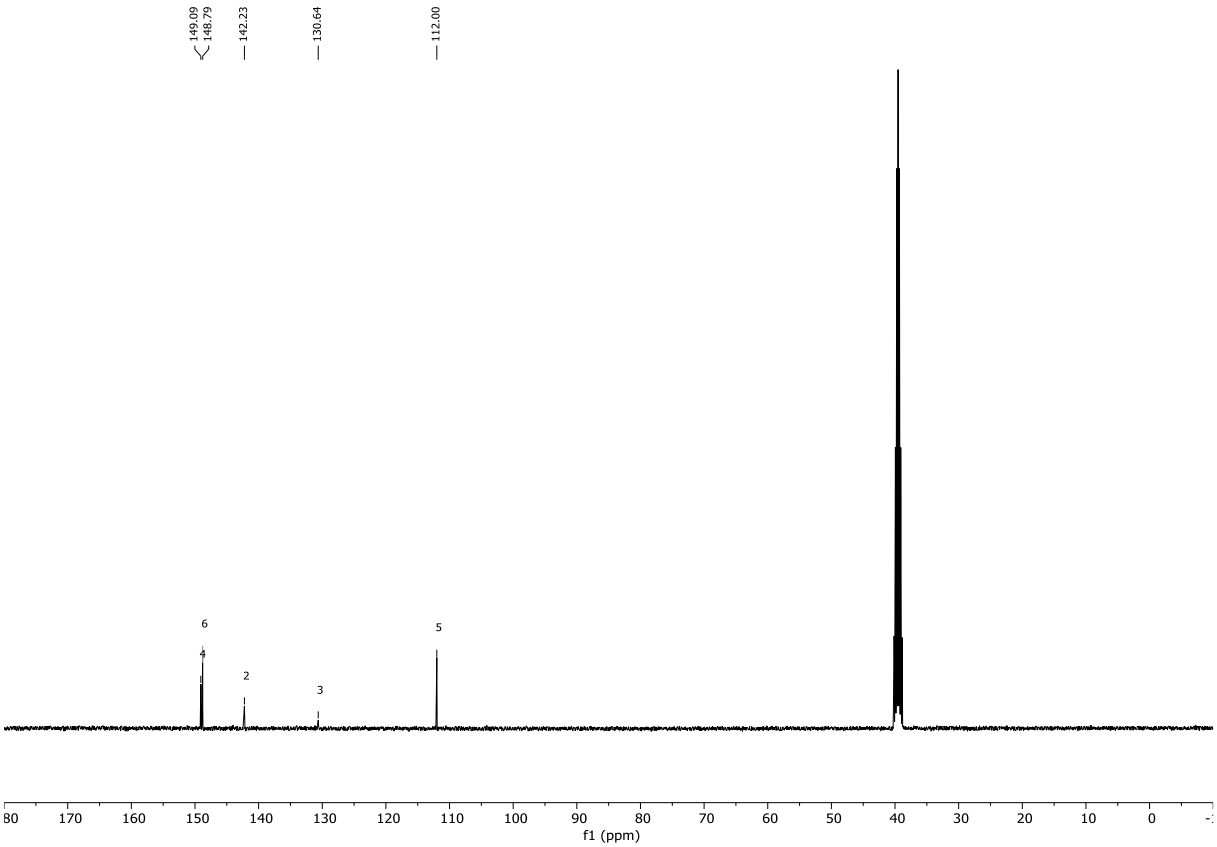
^{1}H-NMR (400 MHz, DMSO-d_6) of compound **S3**.



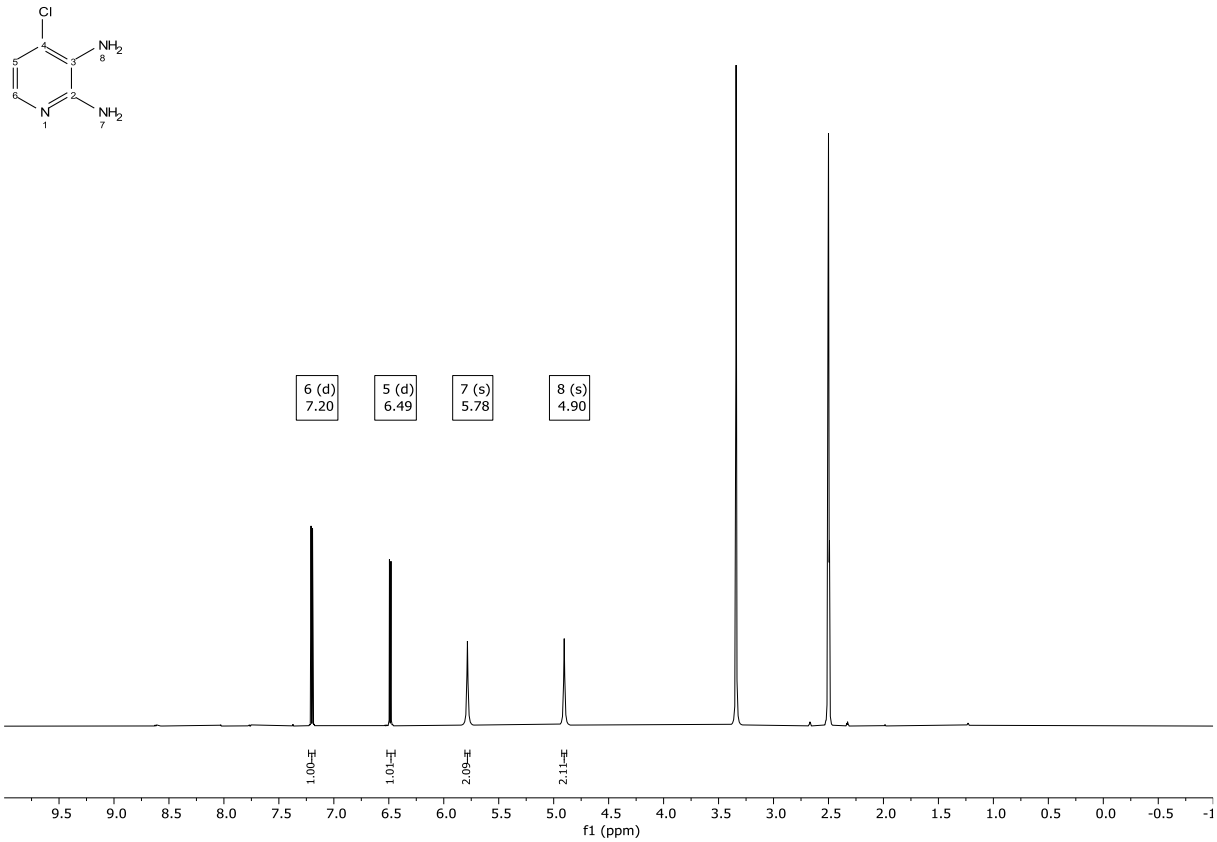
^{13}C{^{1}H}-NMR (100 MHz, DMSO-d_6) of compound **S3**.



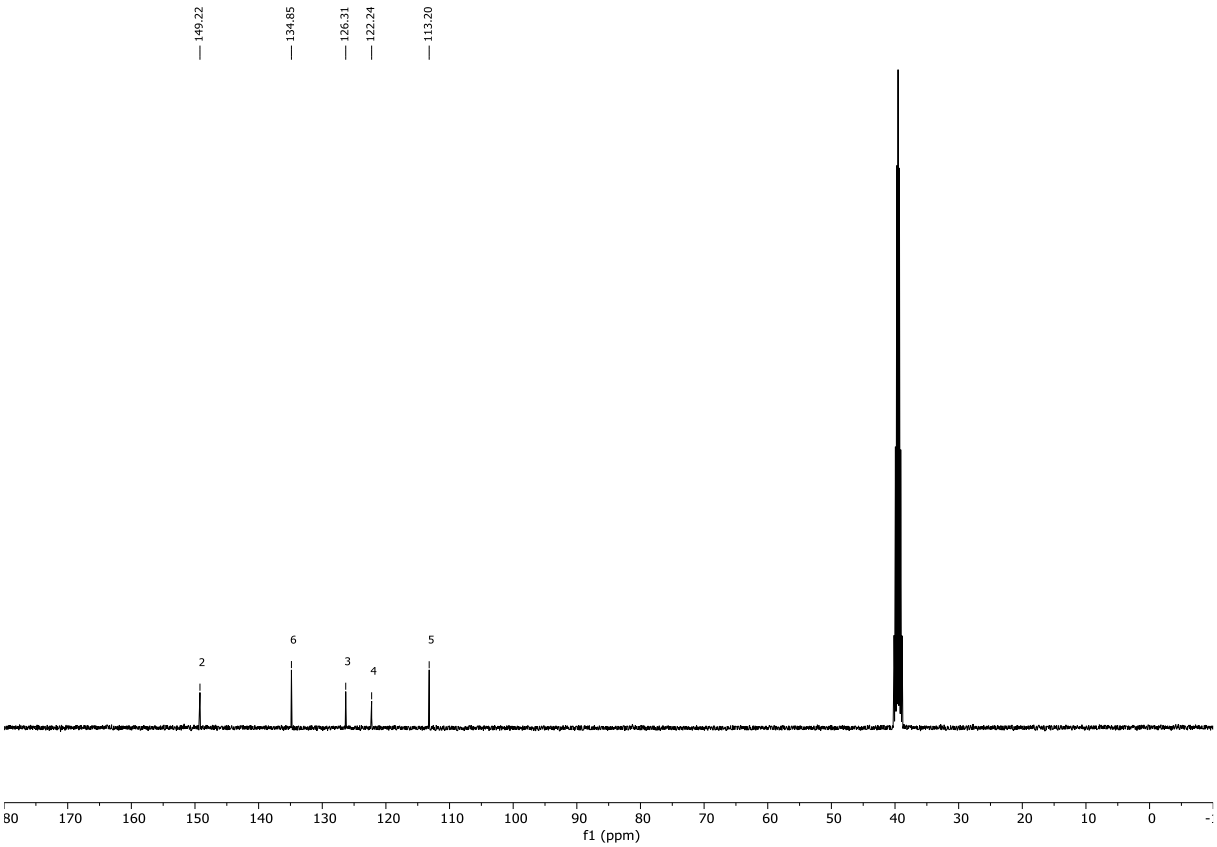
^{1}H-NMR (400 MHz, DMSO-d_6) of compound **S4**.



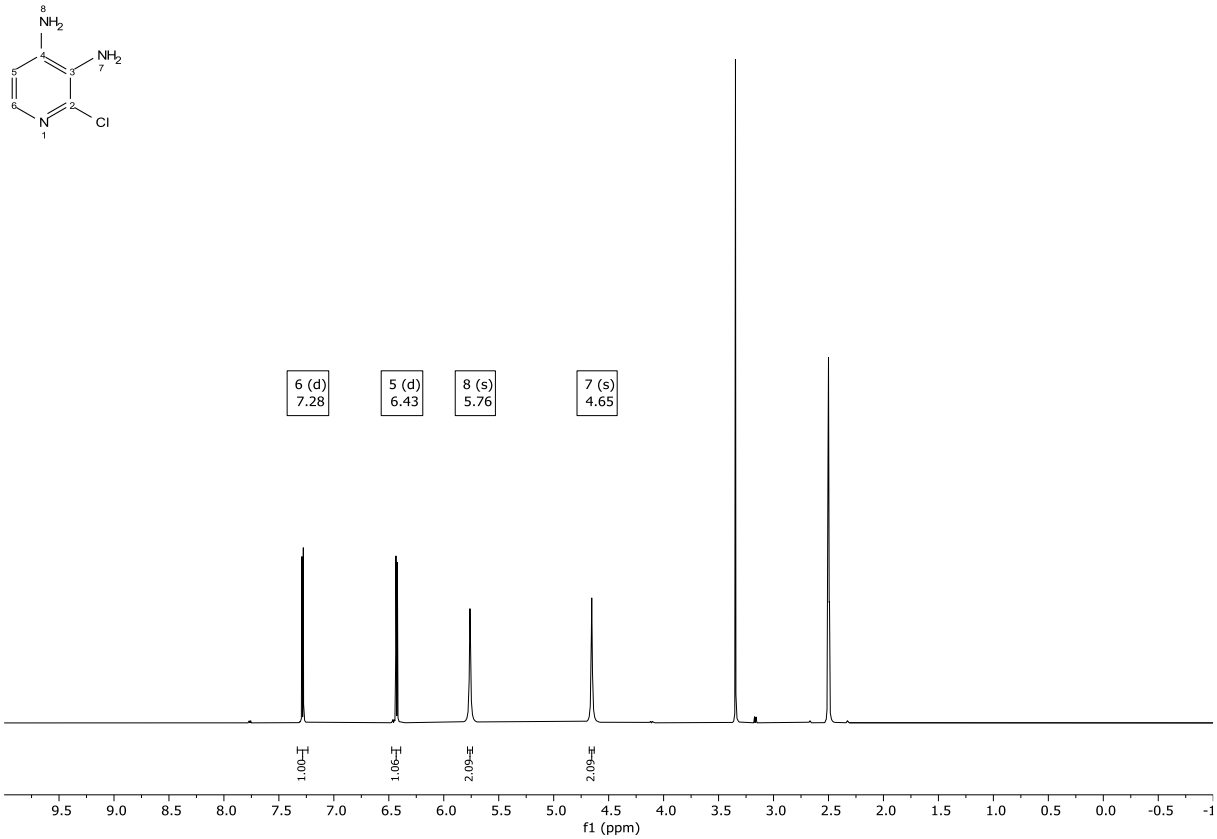
^{13}C{^{1}H}-NMR (100 MHz, DMSO-d_6) of compound **S4**.



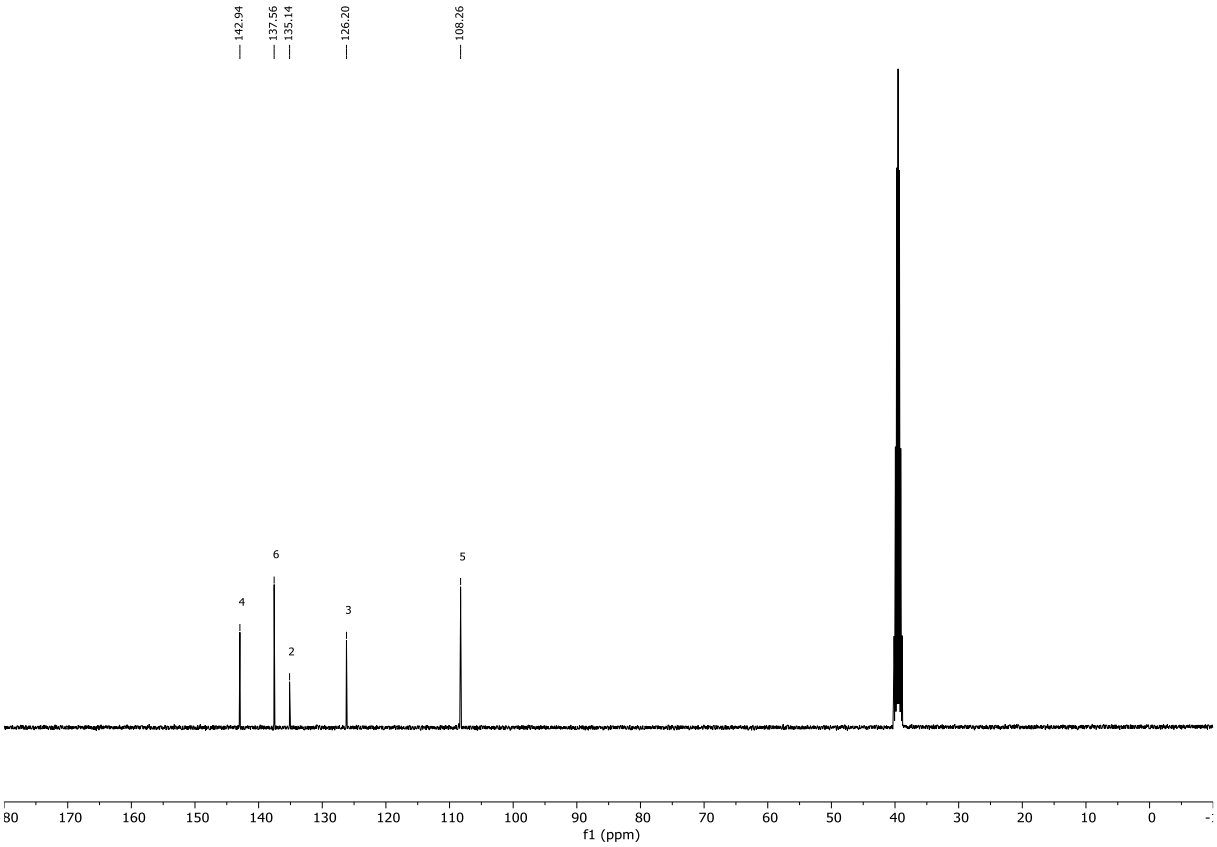
^{1}H-NMR (400 MHz, DMSO-d_6) of compound **S5**.



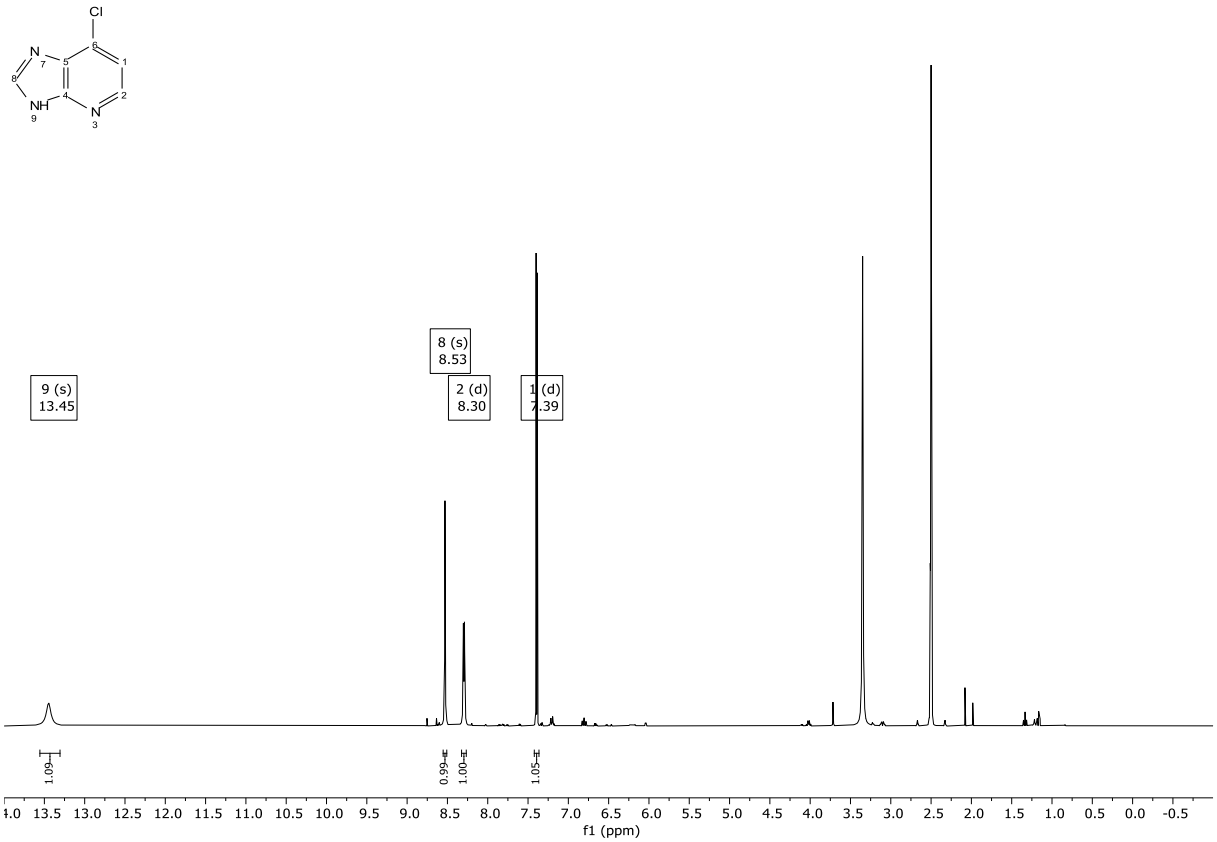
^{13}C{^{1}H}-NMR (100 MHz, DMSO-d_6) of compound **S5**.



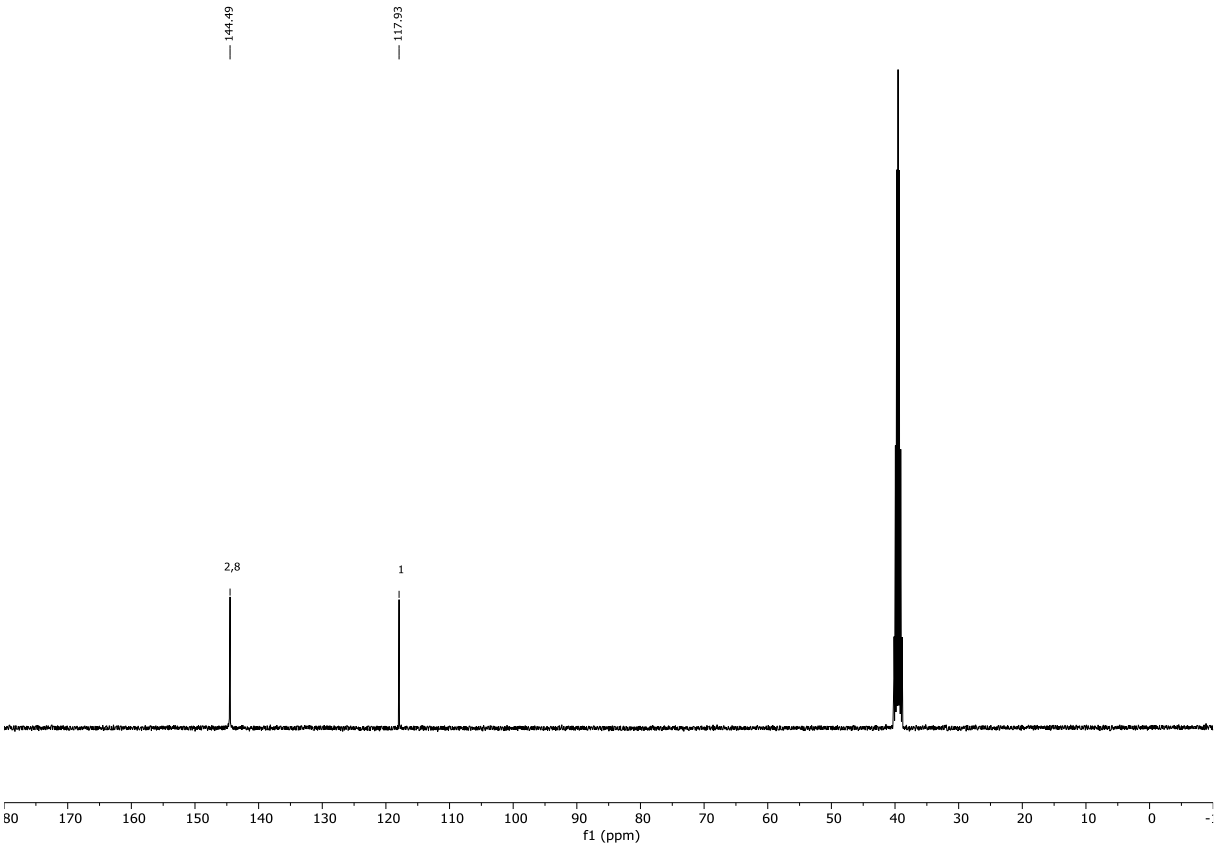
^{1}H-NMR (100 MHz, DMSO-d_6) of compound **S6**.



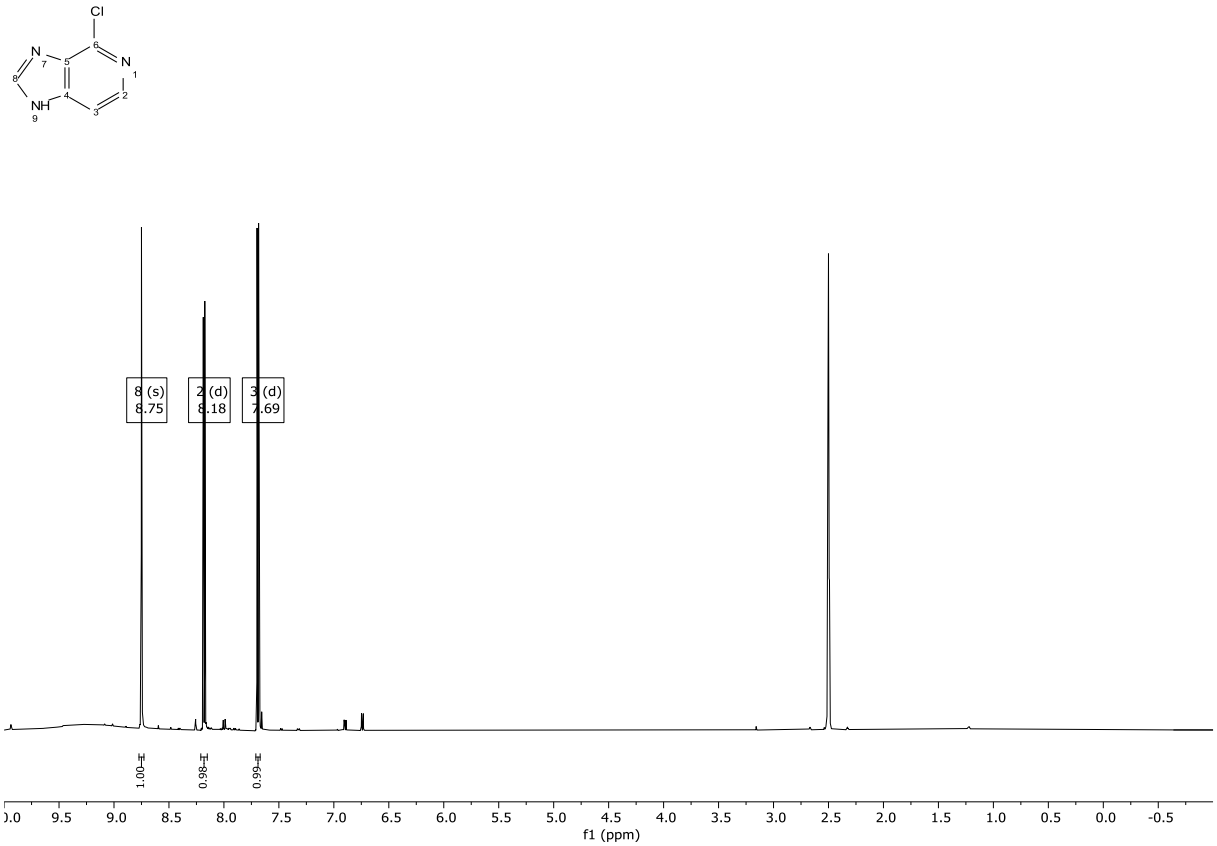
^{13}C{^{1}H}-NMR (100 MHz, DMSO-d_6) of compound **S6**.



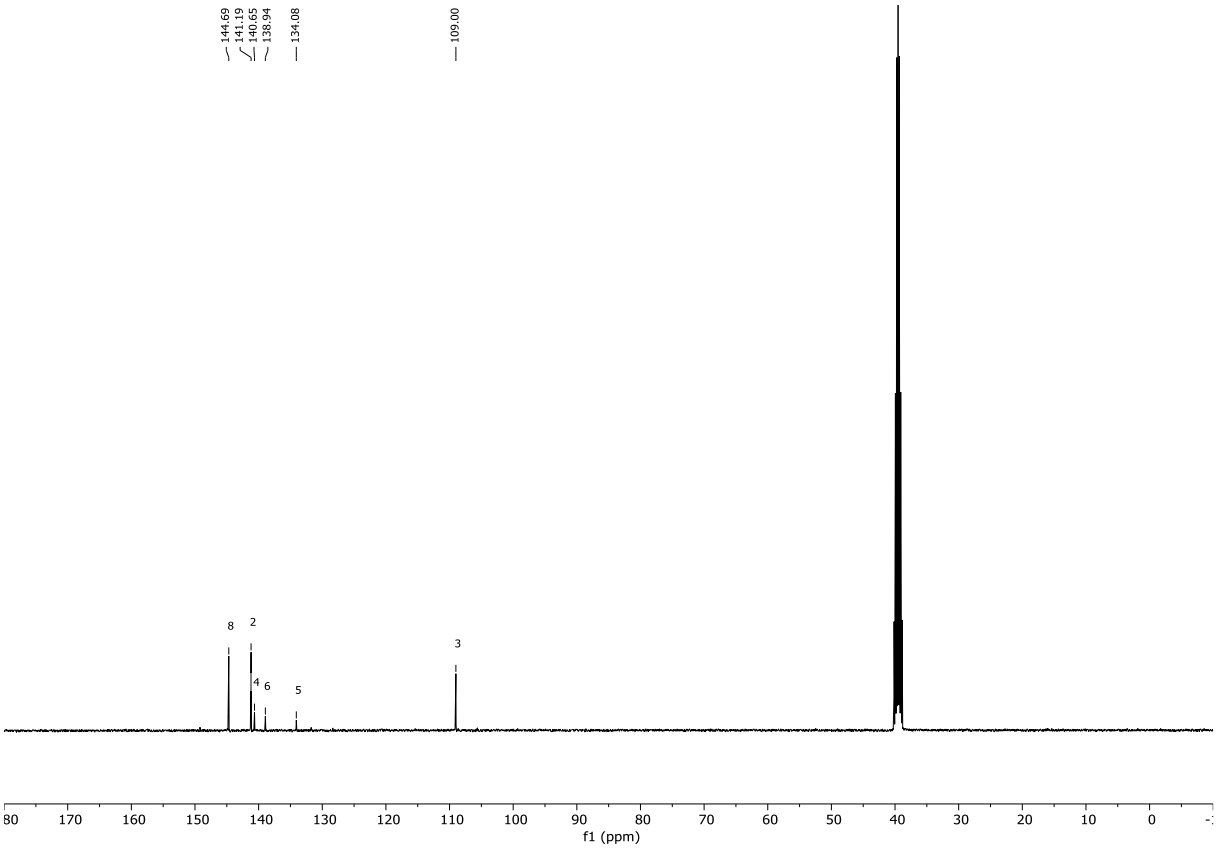
^{1}H-NMR (100 MHz, DMSO-d_6) of compound **S7**.



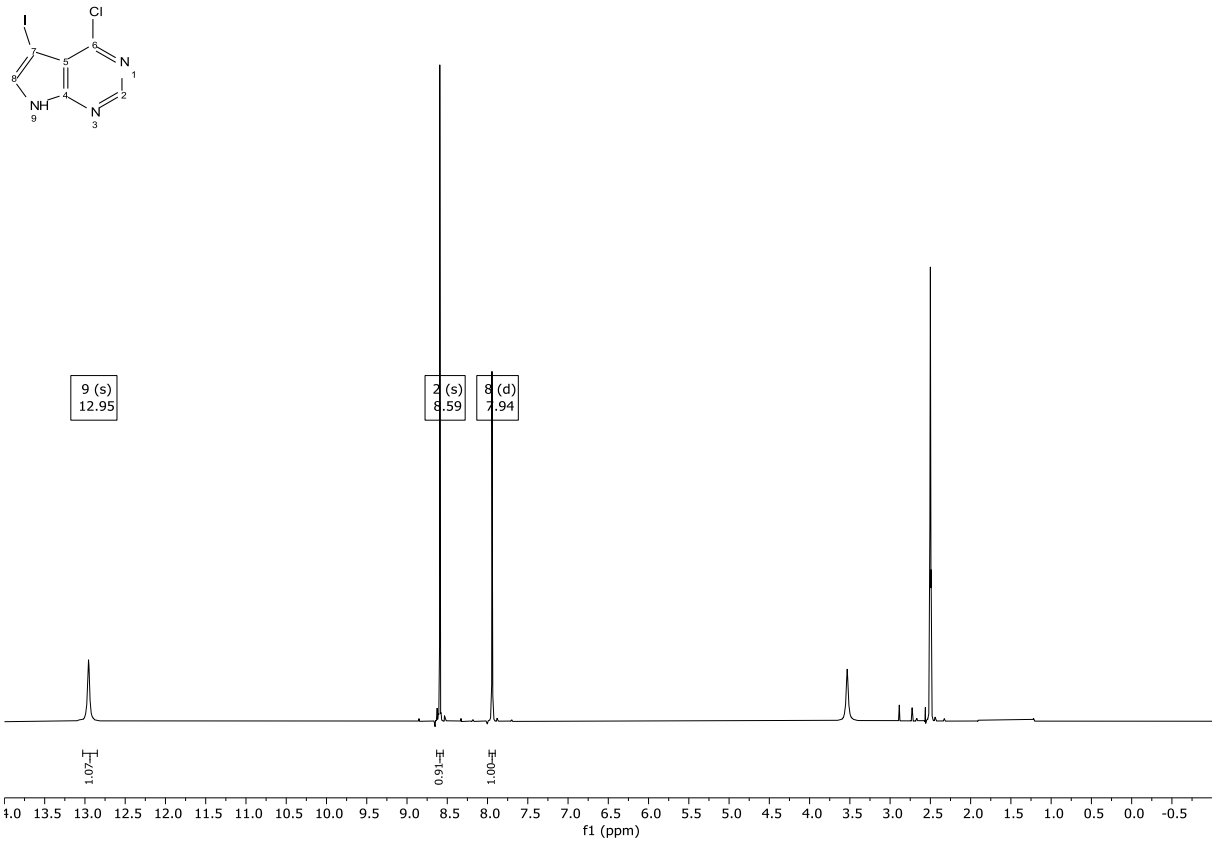
^{13}C{^{1}H}-NMR (100 MHz, DMSO-d_6) of compound **S7**.



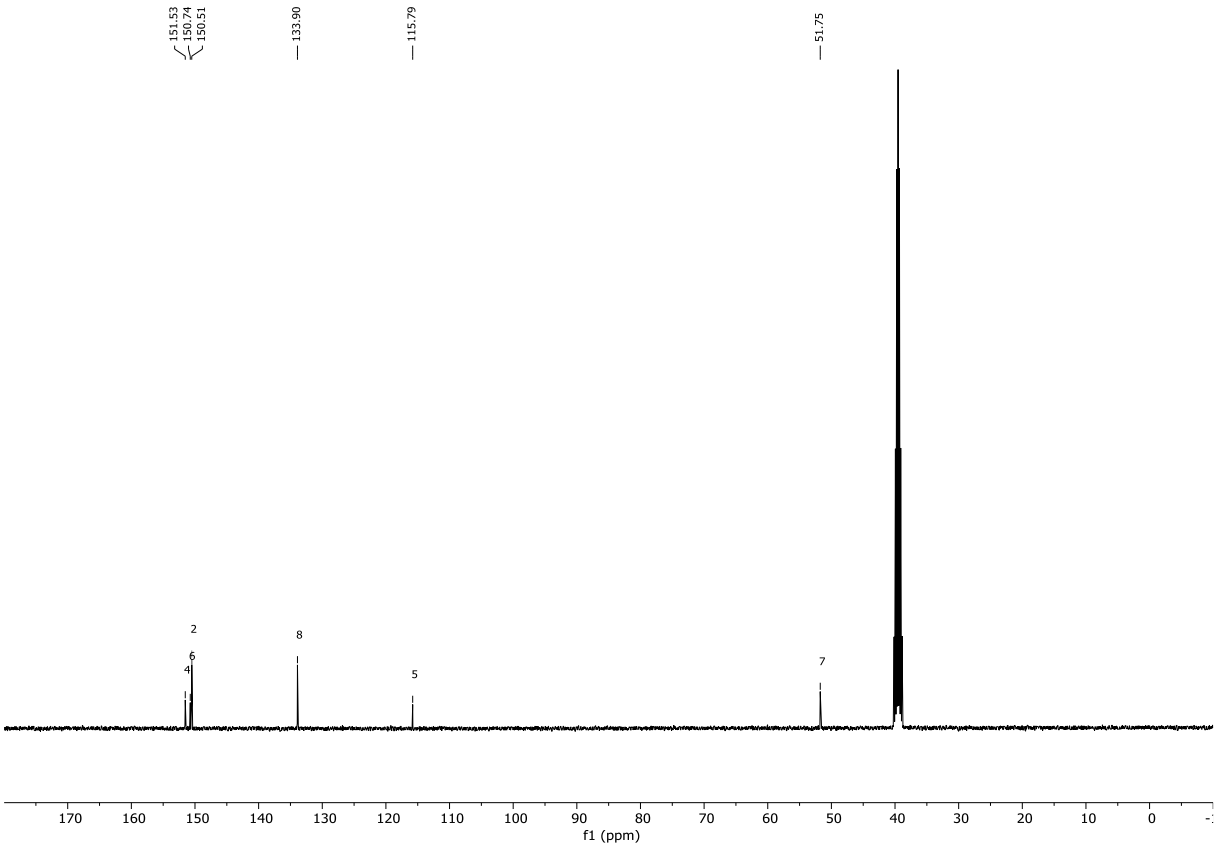
^{1}H-NMR (100 MHz, DMSO-d$_6$) of compound **S8**.



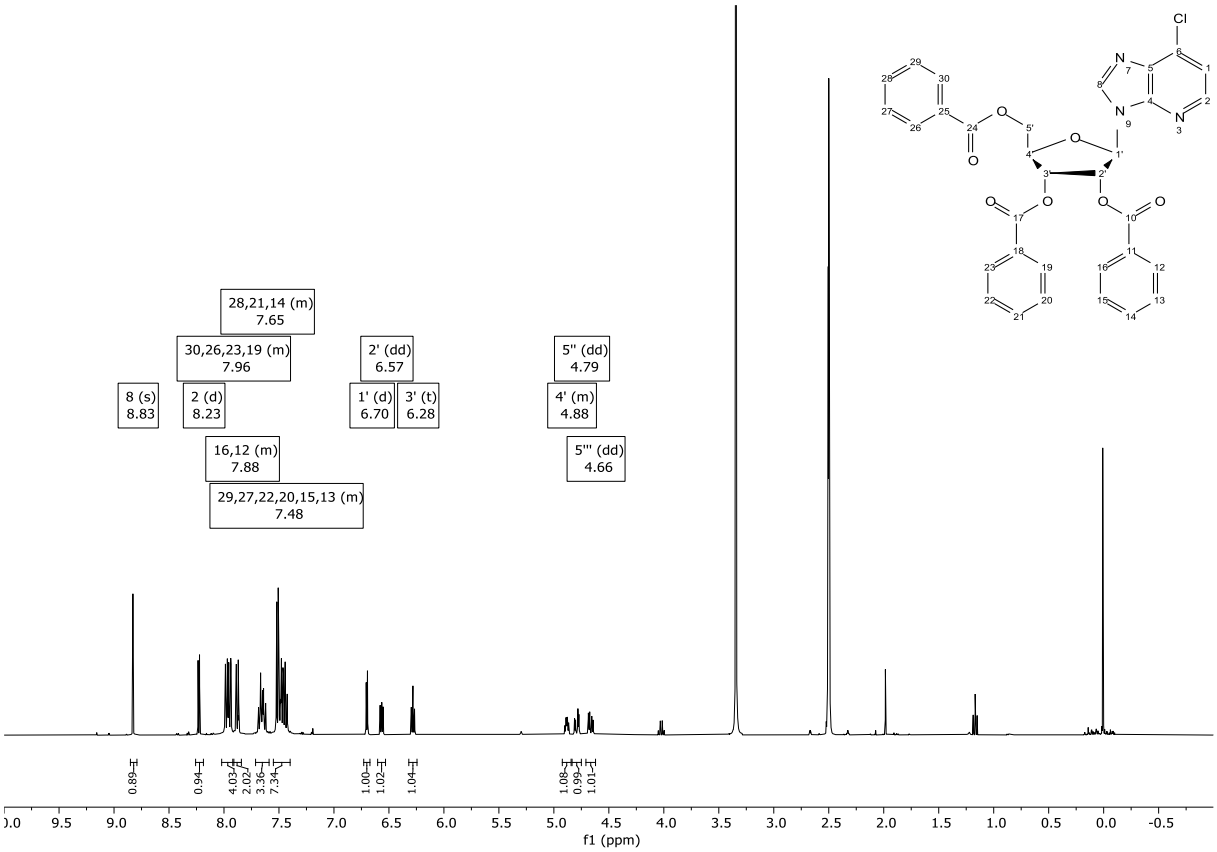
^{13}C{^{1}H}-NMR (100 MHz, DMSO-d$_6$) of compound **S8**.



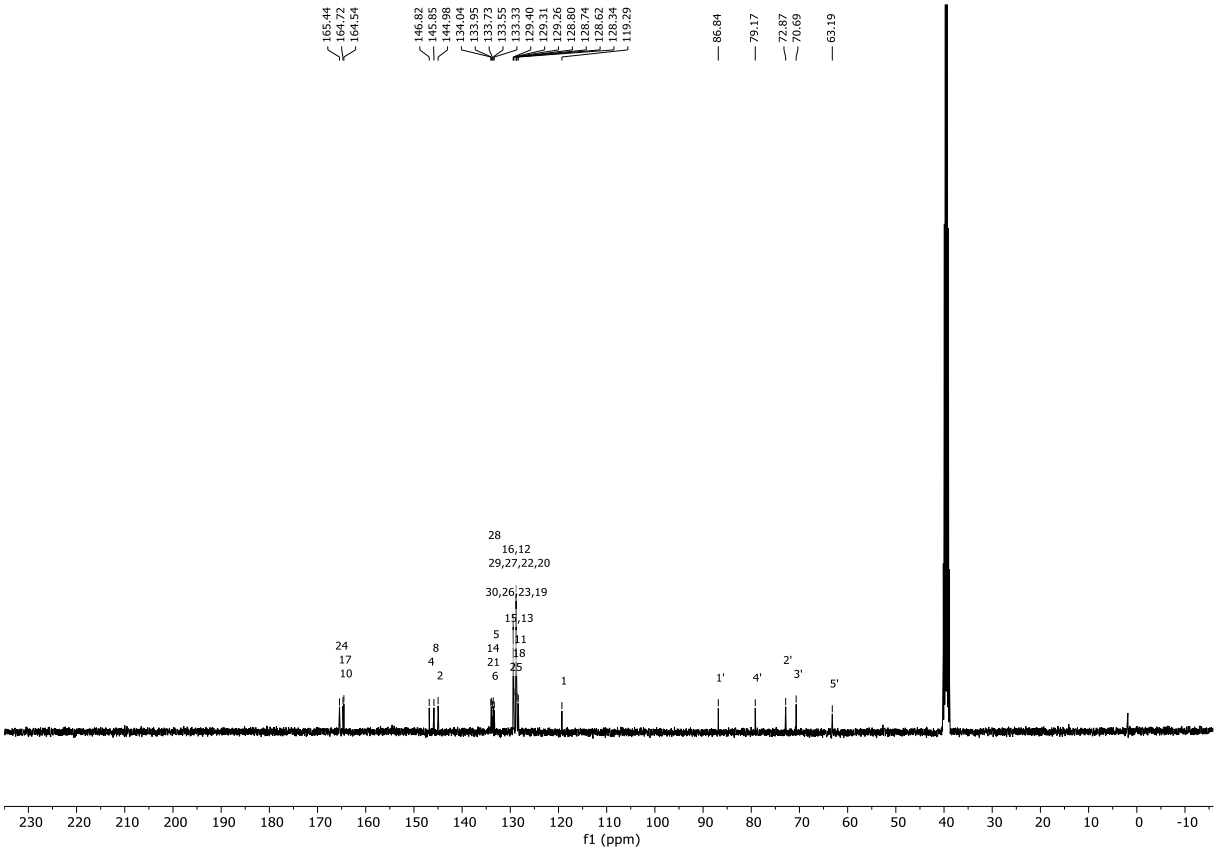
^{1}H-NMR (400 MHz, DMSO-d_6) of compound **S10**.



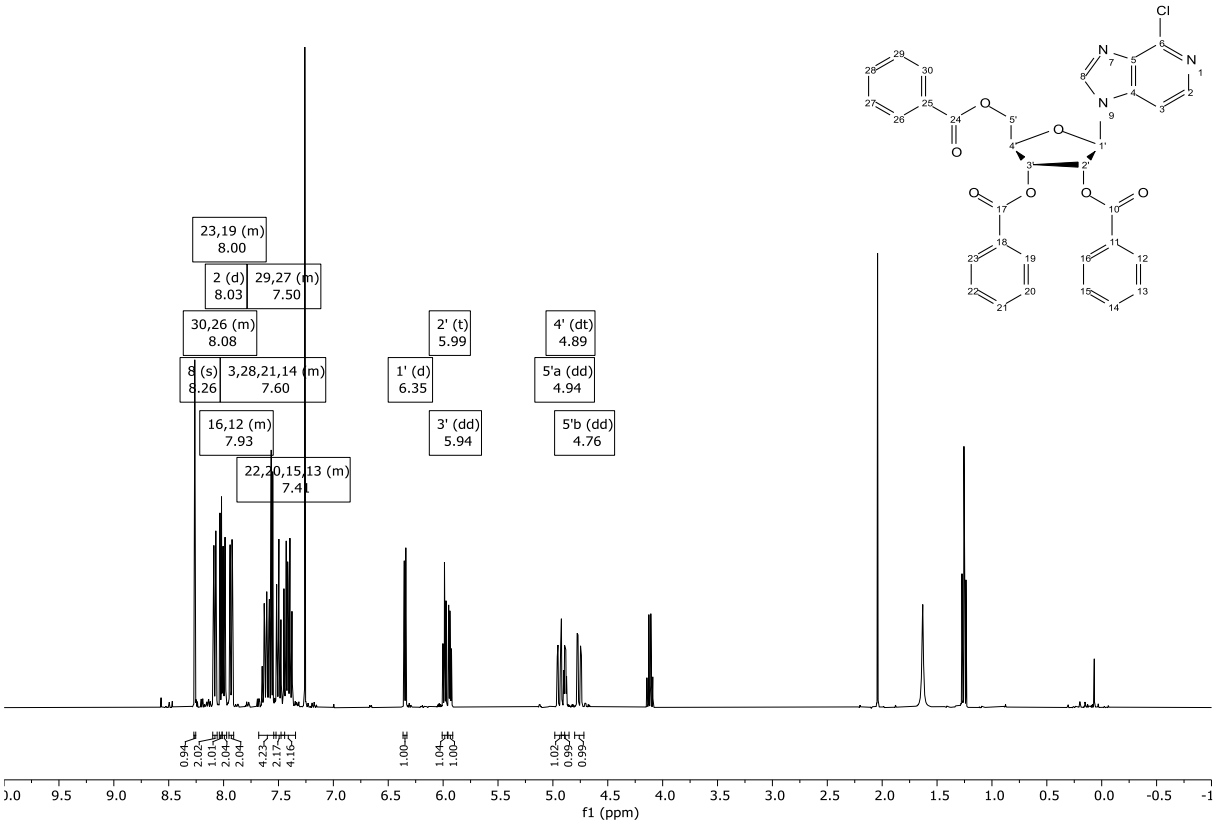
^{13}C{^{1}H}-NMR (100 MHz, DMSO-d_6) of compound **S10**.



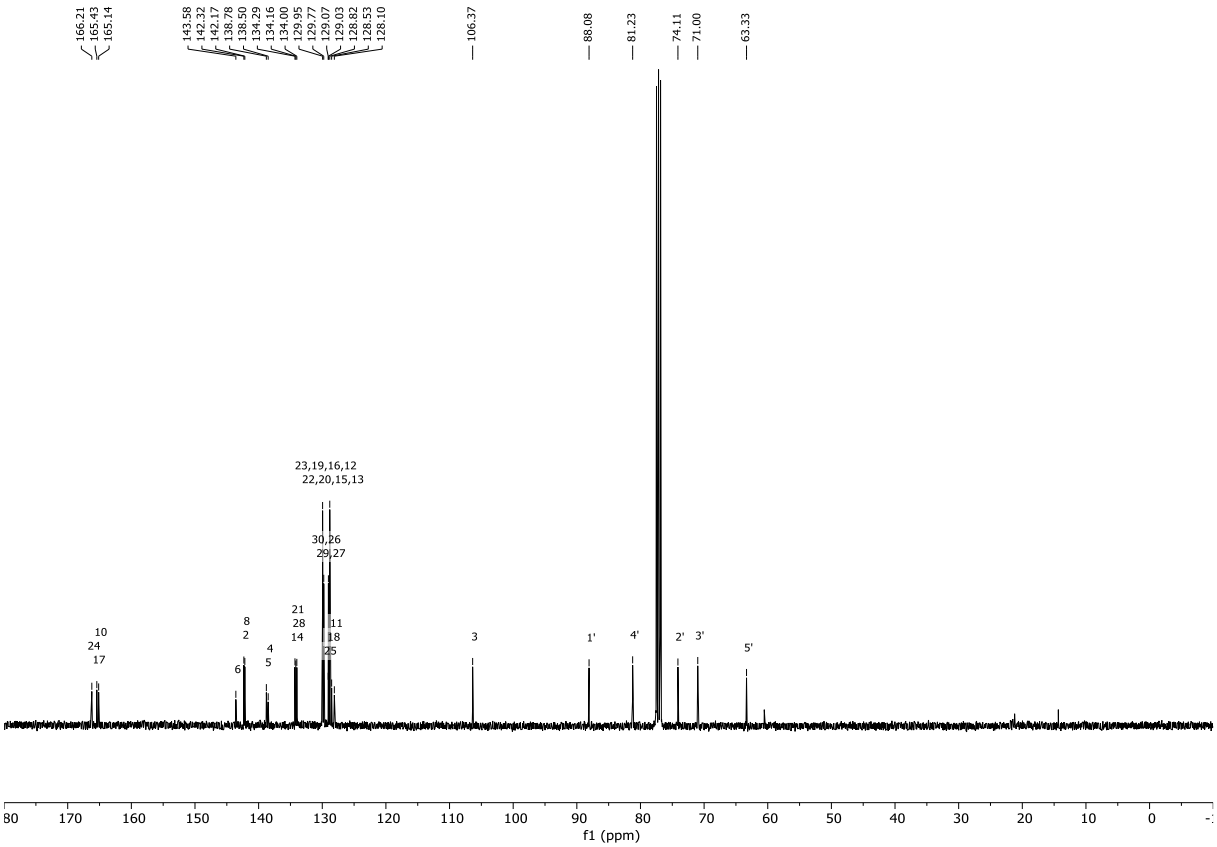
^{1}H-NMR (400 MHz, DMSO-d$_6$) of compound **S11**.



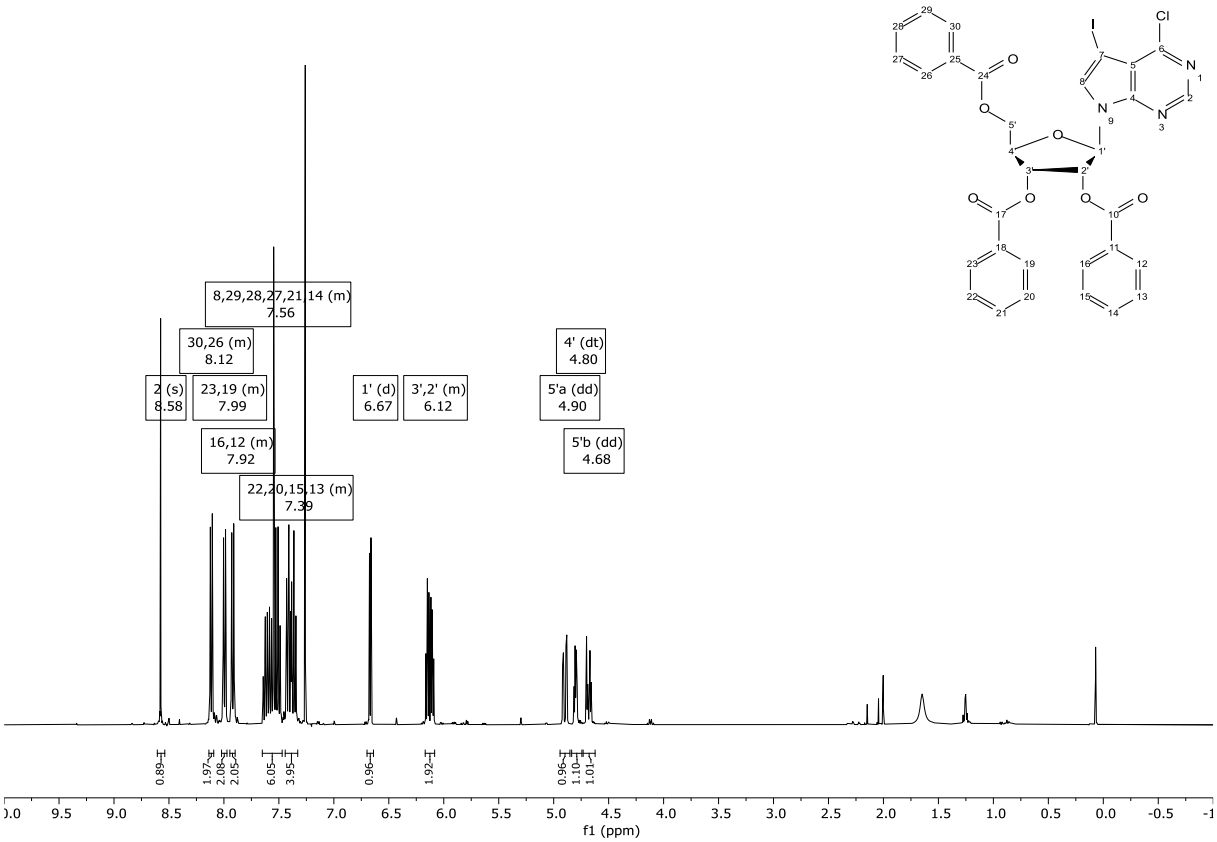
^{13}C{^{1}H}-NMR (100 MHz, DMSO-d$_6$) of compound **S11**.



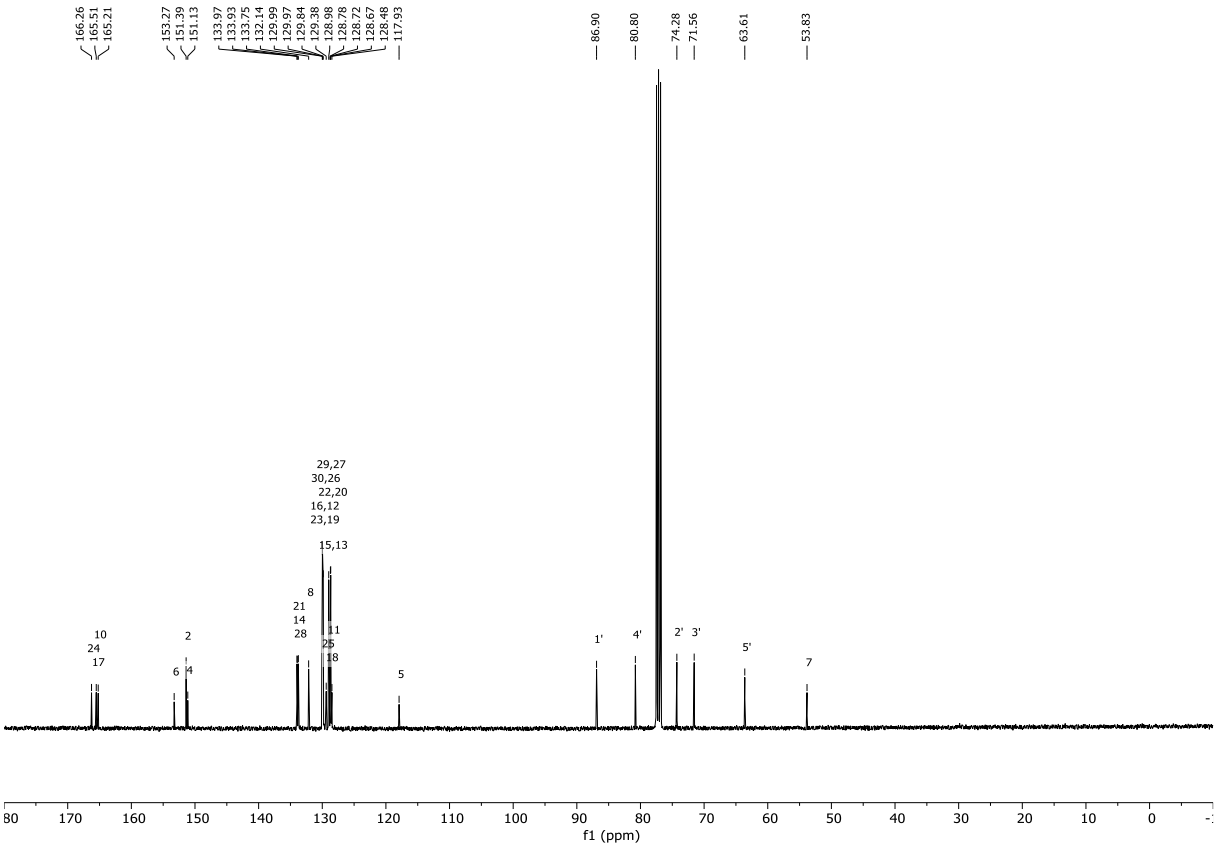
^{1}H-NMR (400 MHz, $CDCl_3$) of compound **S12**.



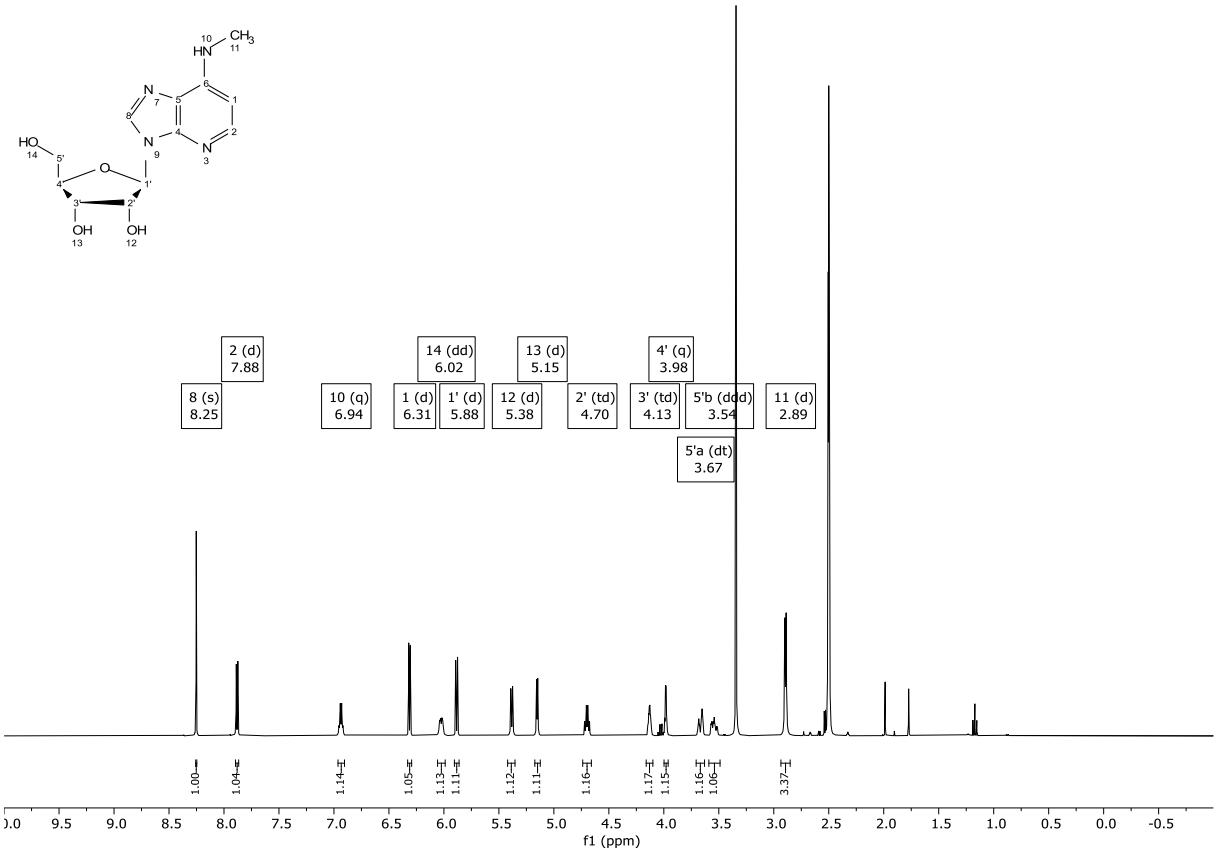
^{13}C{^{1}H}-NMR (100 MHz, $CDCl_3$) of compound **S12**.



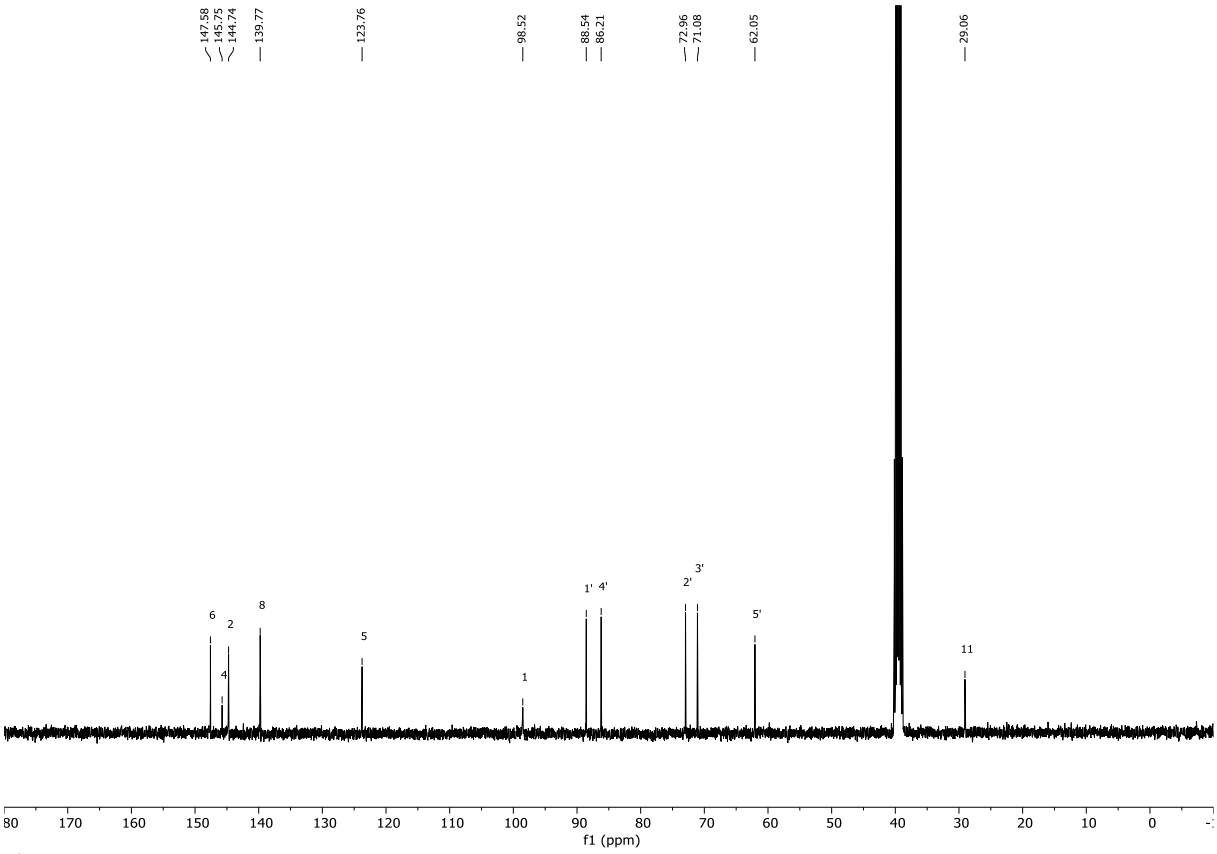
^{1}H-NMR (400 MHz, $CDCl_3$) of compound **S13**.



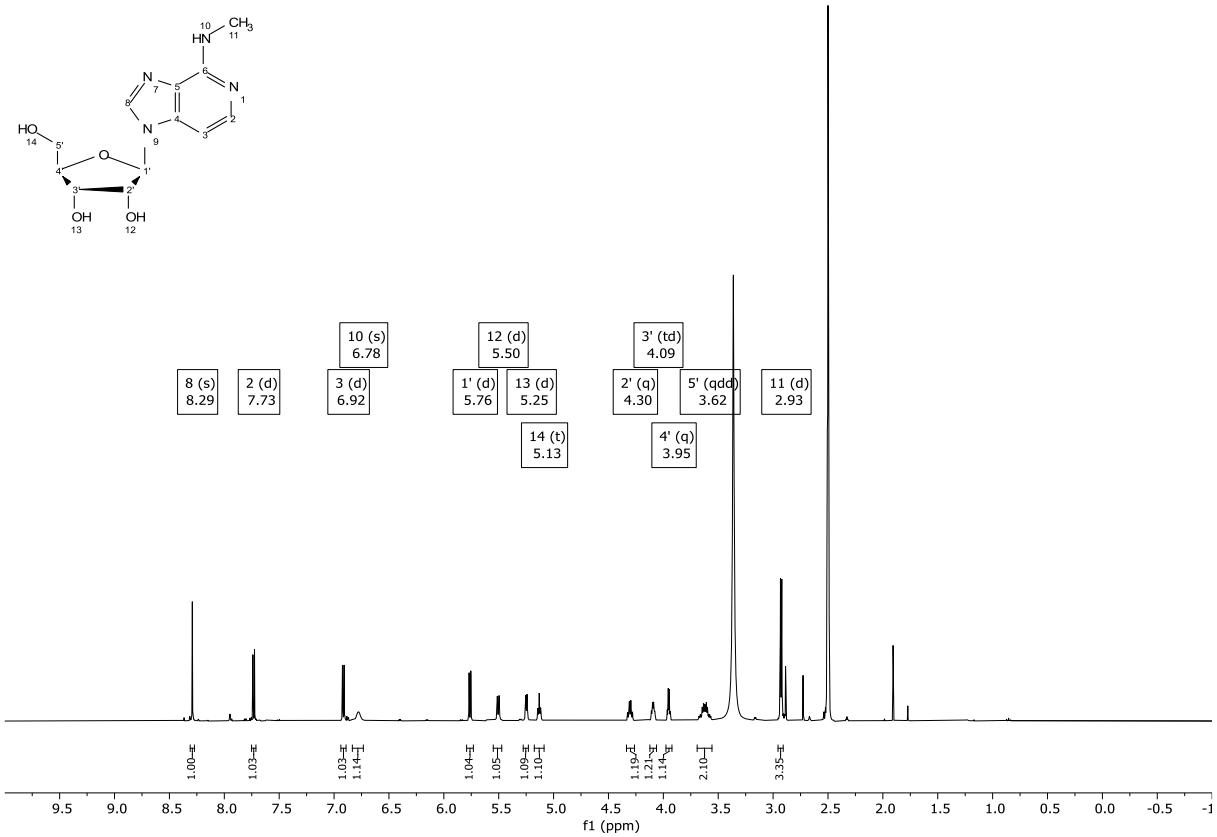
^{13}C{^{1}H}-NMR (100 MHz, $CDCl_3$) of compound **S13**.



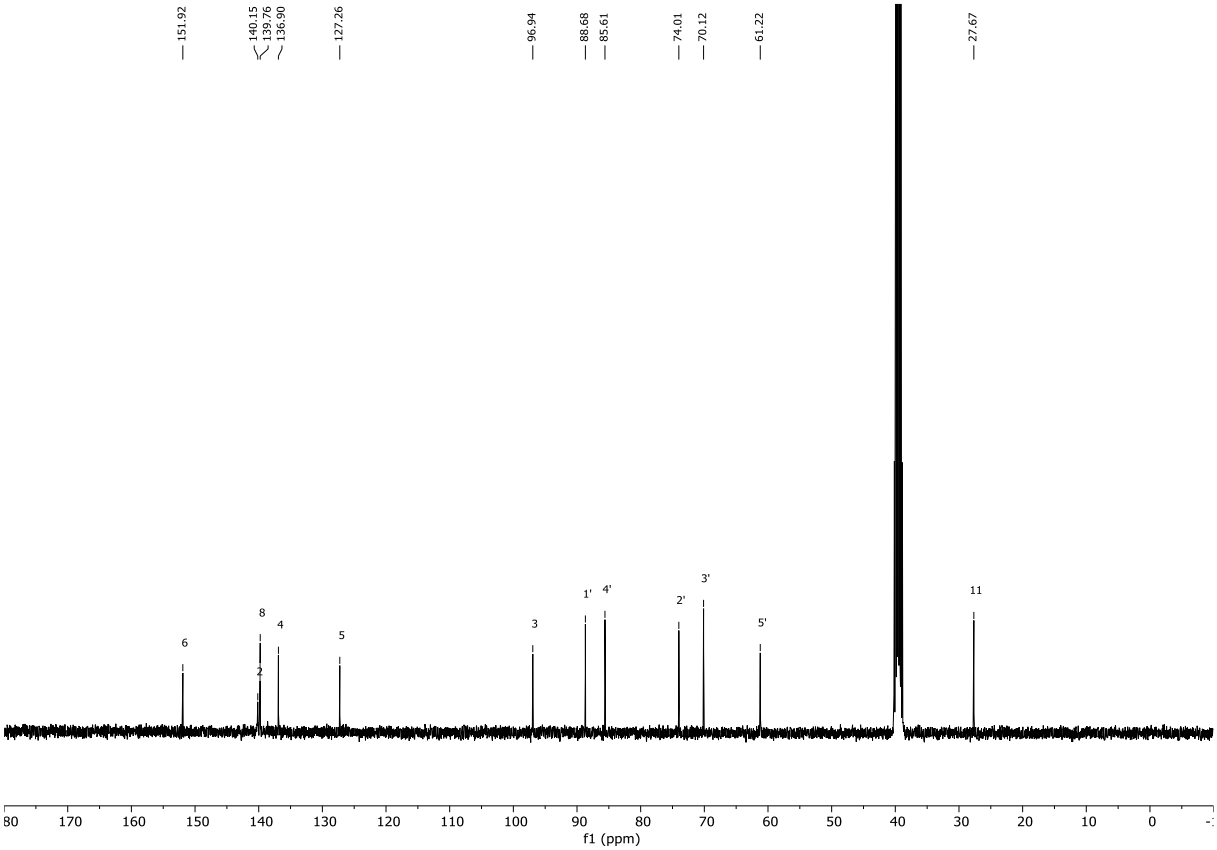
^{1}H-NMR (400 MHz, DMSO-d_6) of compound **1b**.



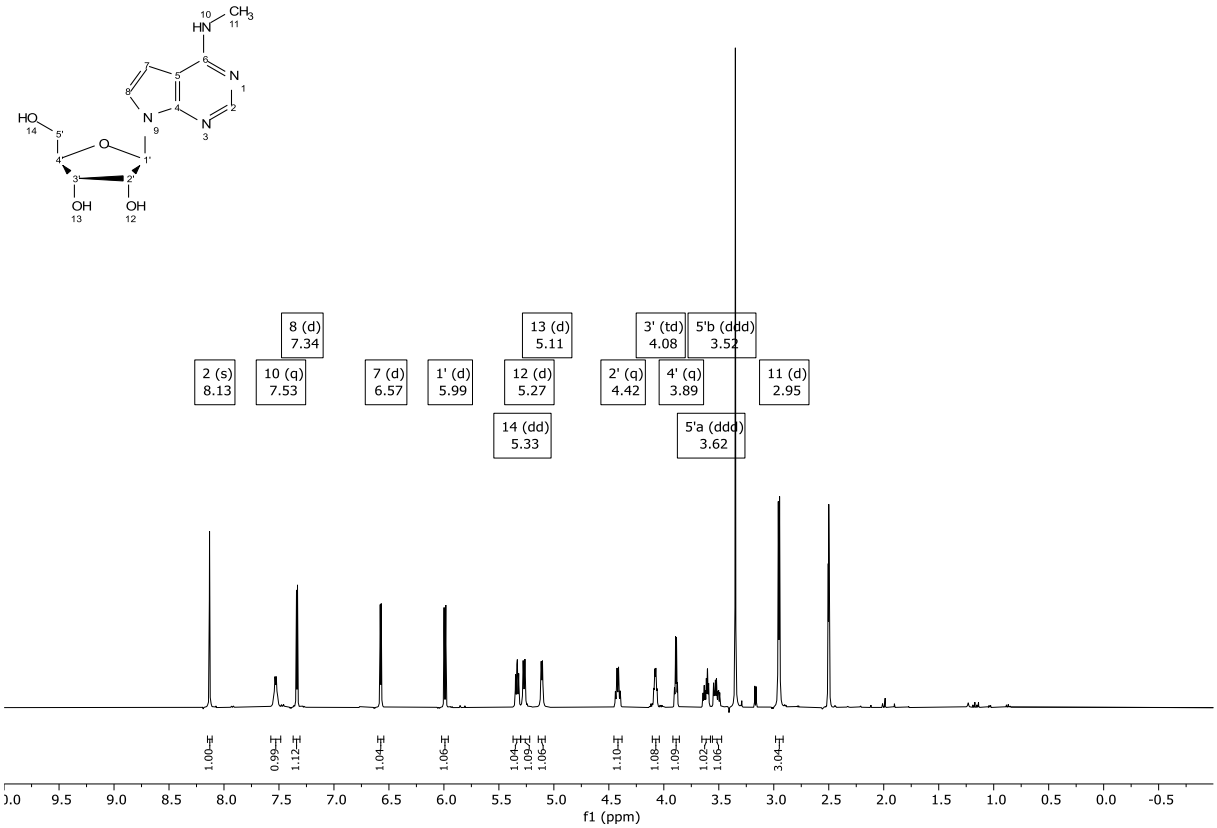
^{13}C{^{1}H}-NMR (100 MHz, DMSO-d_6) of compound **1b**.



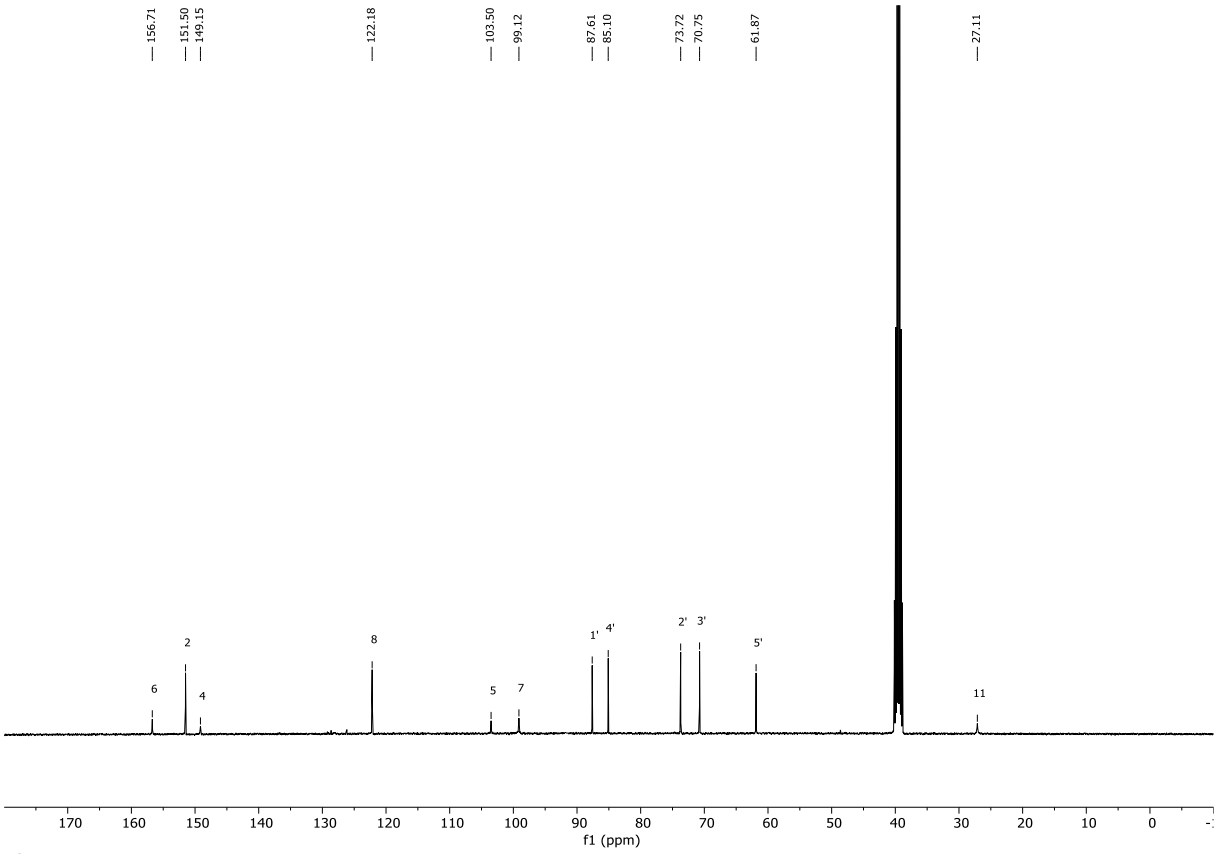
^{1}H-NMR (400 MHz, DMSO-d$_6$) of compound **1c**.



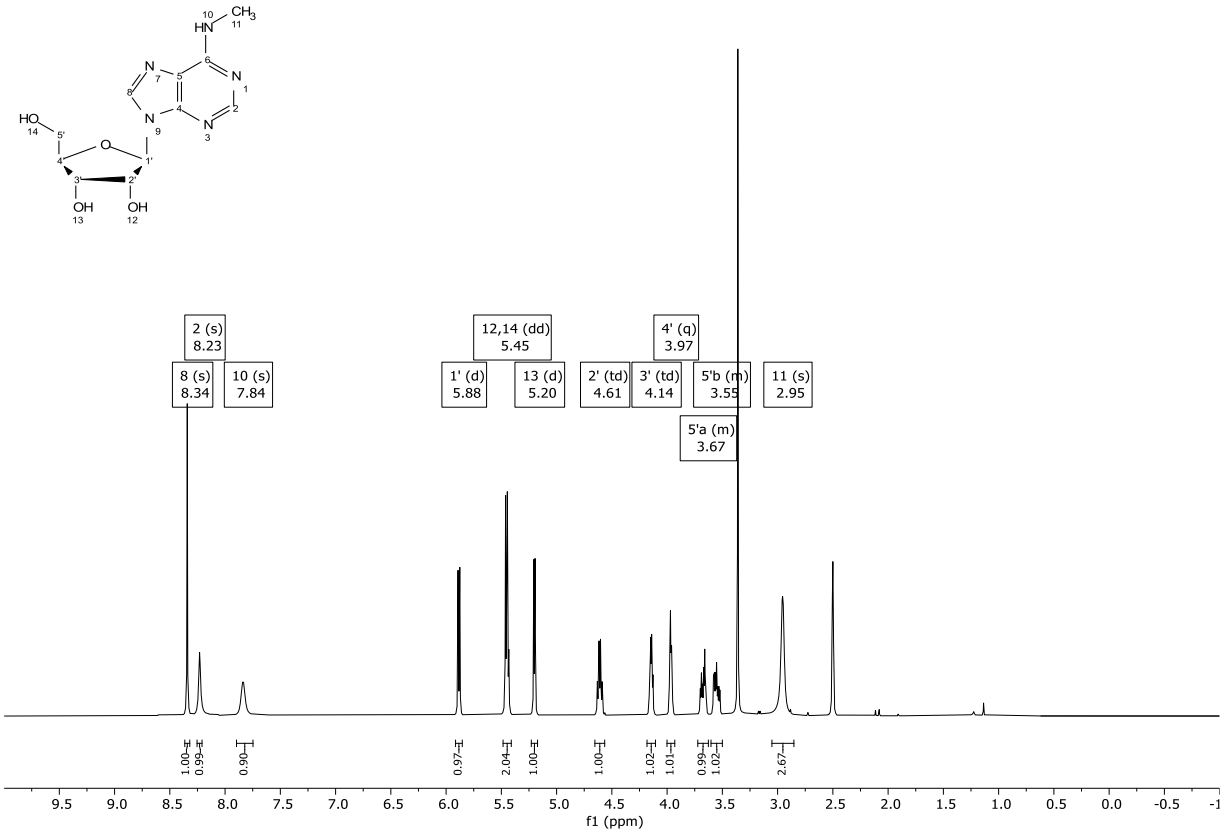
^{13}C{^{1}H}-NMR (100 MHz, DMSO-d$_6$) of compound **1c**.



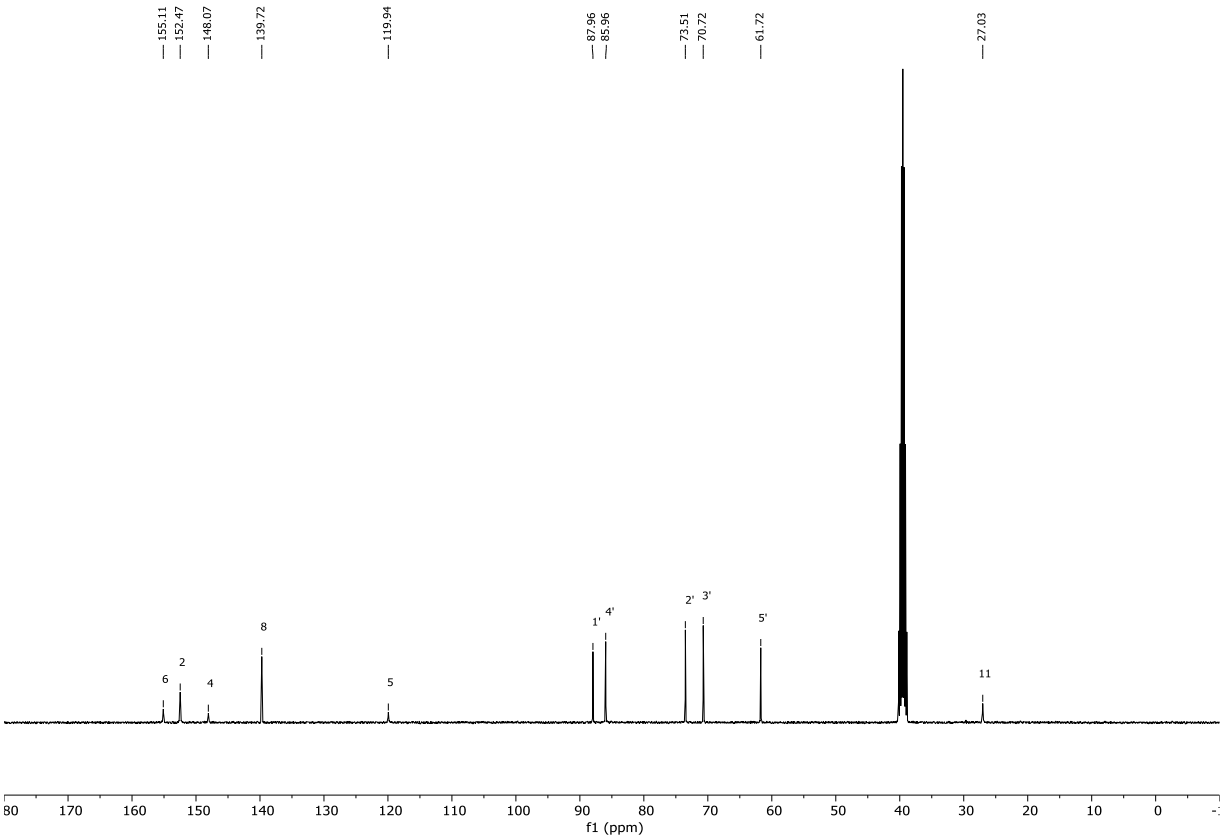
^{1}H-NMR (400 MHz, DMSO-d$_6$) of compound **1d**.



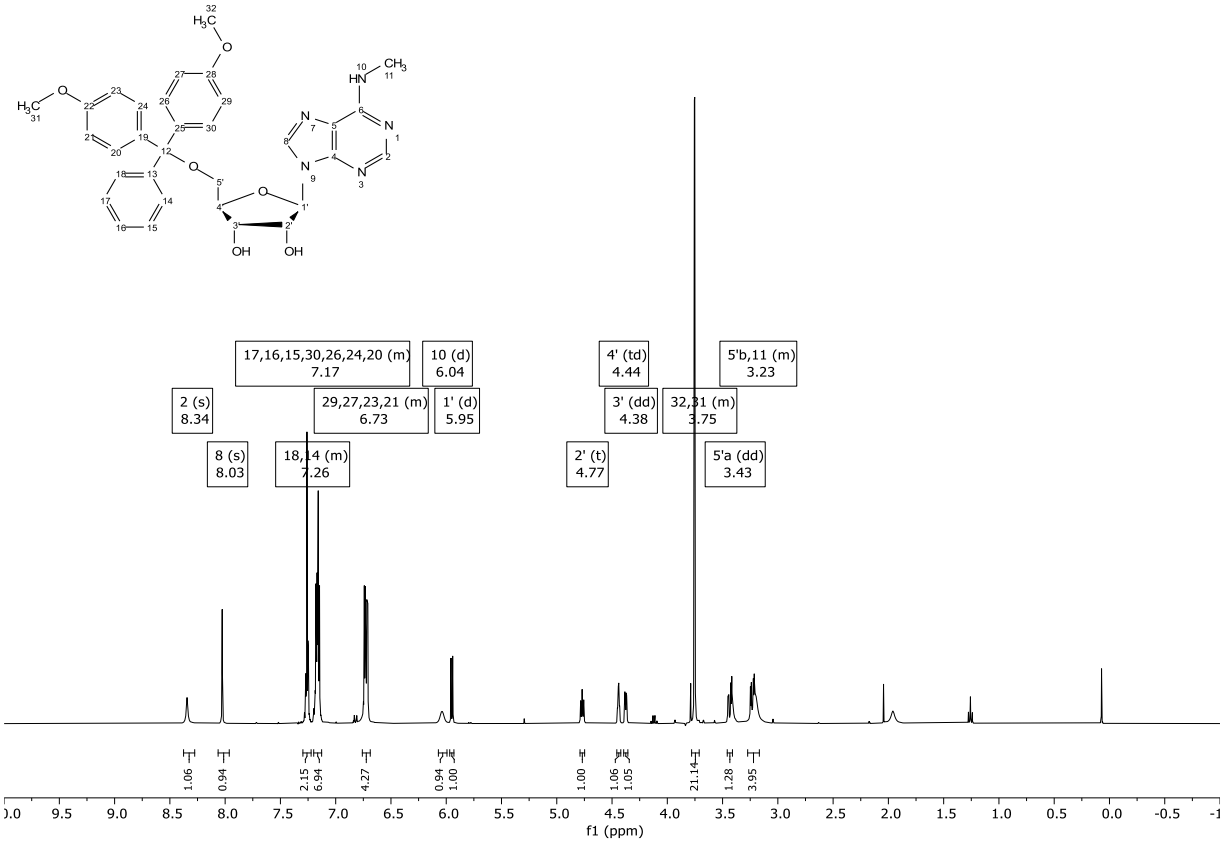
^{13}C{^{1}H}-NMR (100 MHz, DMSO-d$_6$) of compound **1d**.



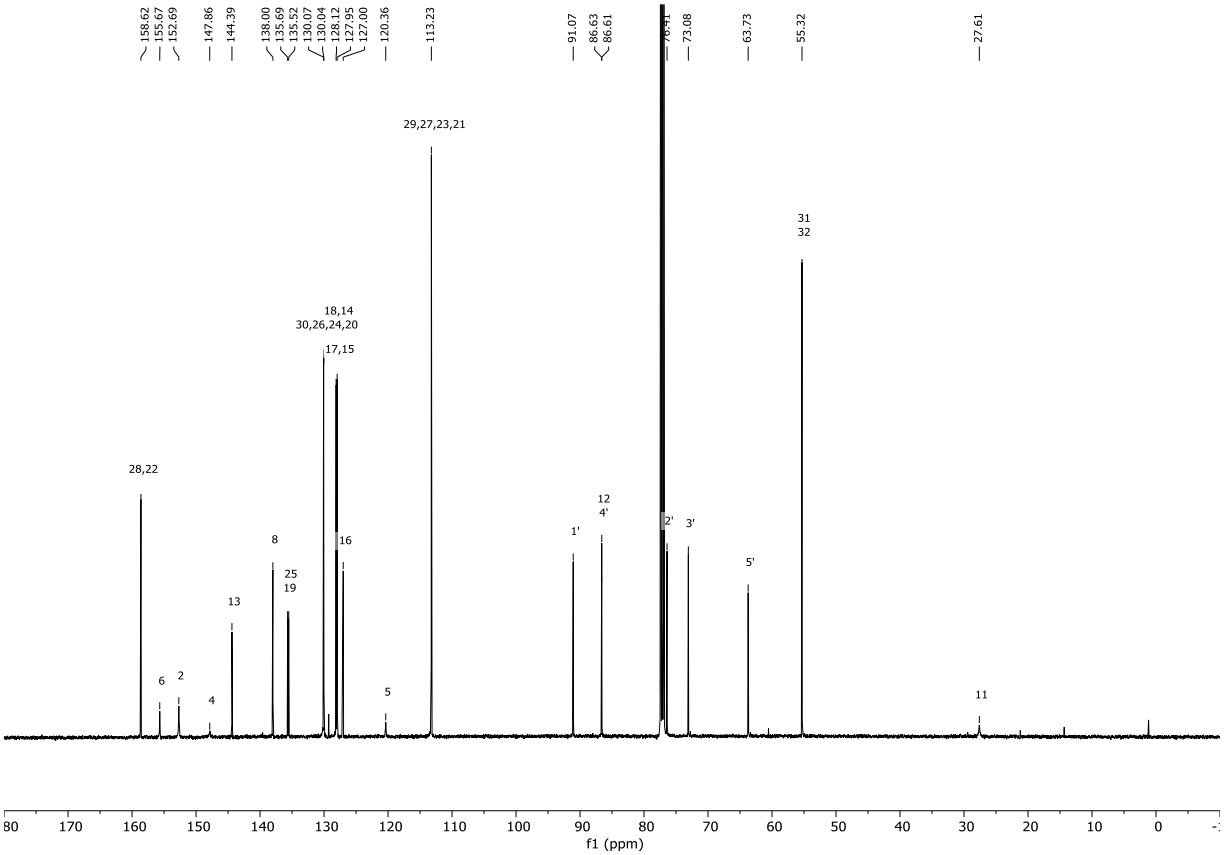
^{1}H-NMR (400 MHz, DMSO-d$_6$) of compound **1a**.



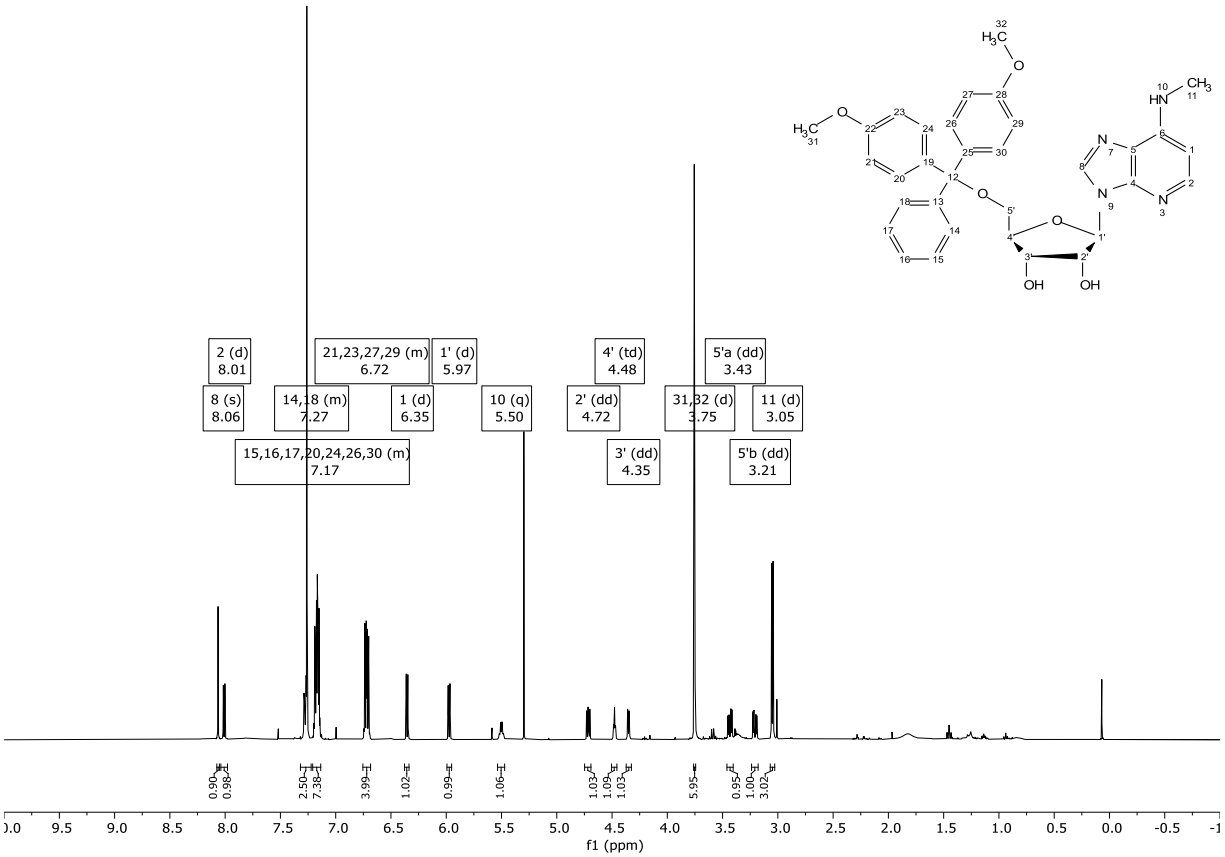
^{13}C{^{1}H}-NMR (100 MHz, DMSO-d$_6$) of compound **1a**.



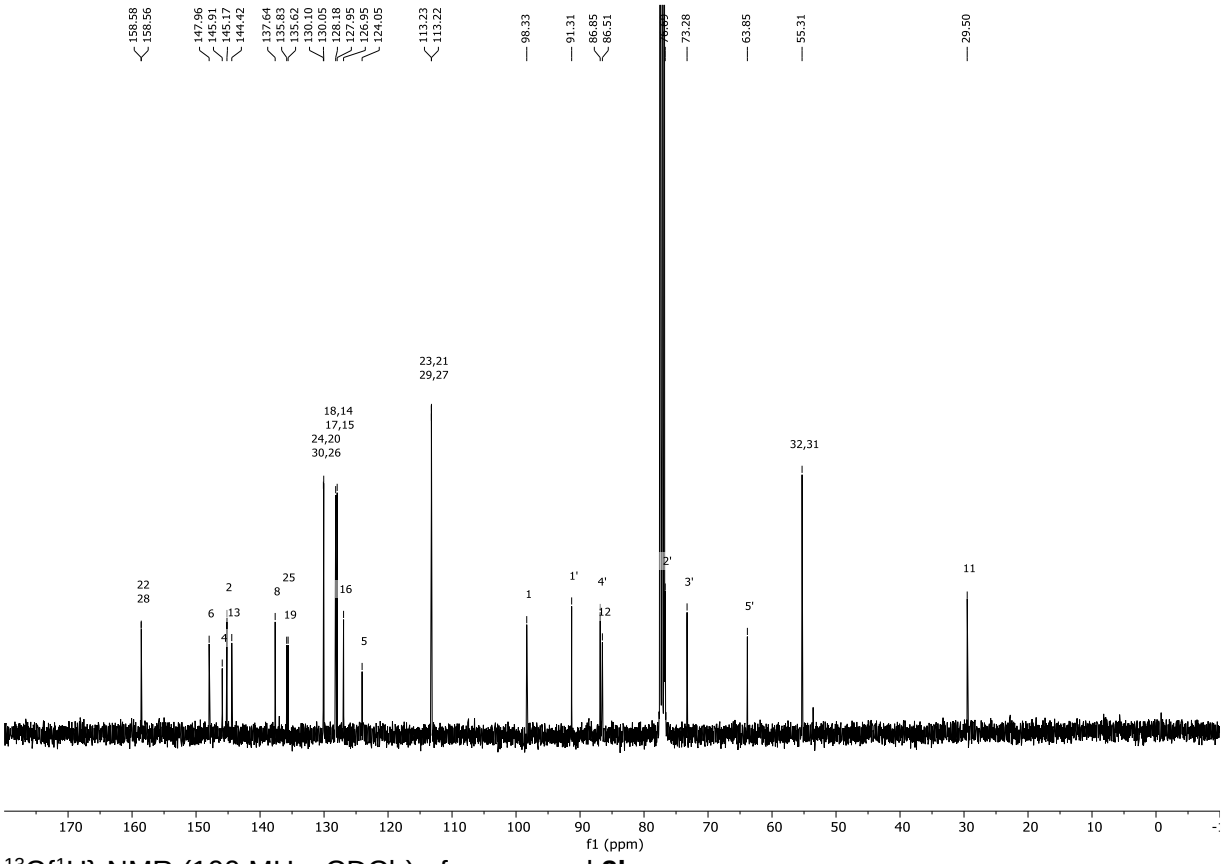
^{1}H-NMR (400 MHz, $CDCl_3$) of compound **2a**.



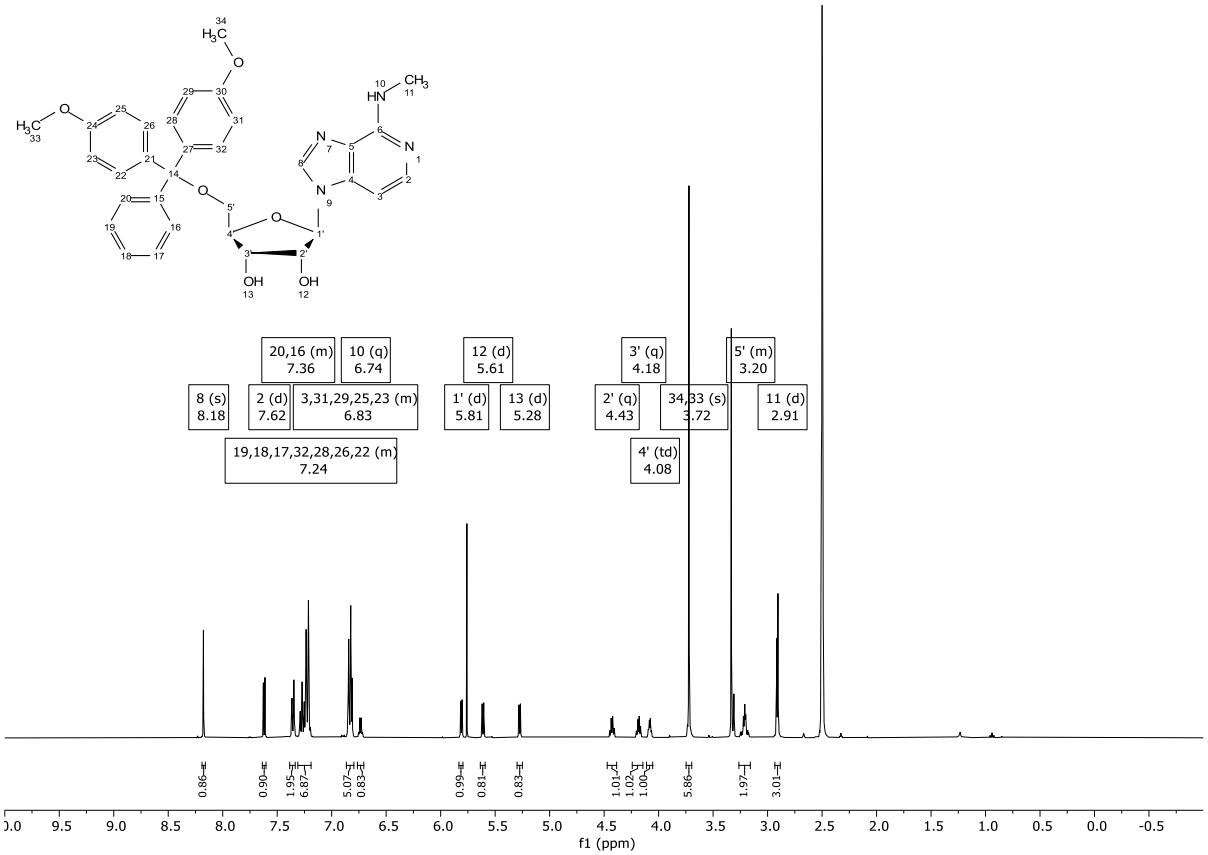
^{13}C{^{1}H}-NMR (125 MHz, $CDCl_3$) of compound **2a**.



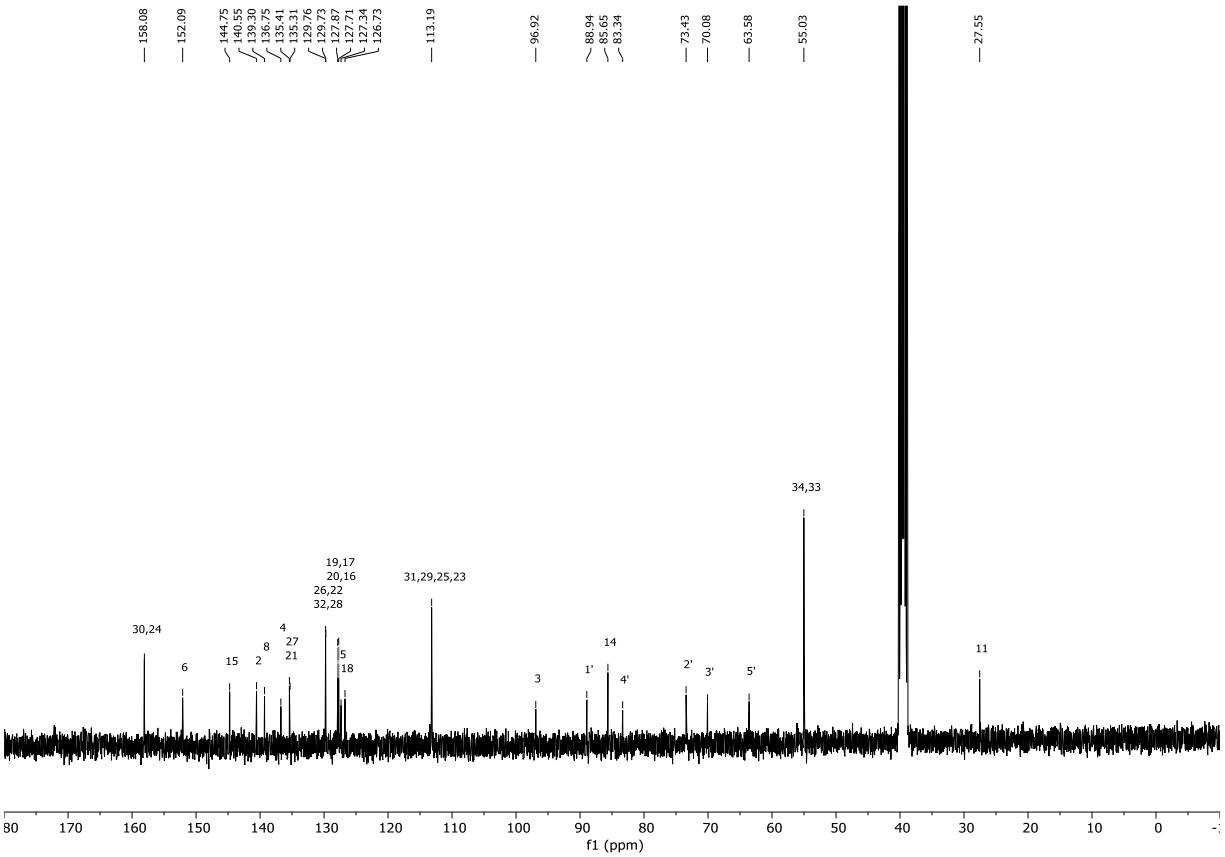
^{1}H-NMR (400 MHz, $CDCl_3$) of compound **2b**.



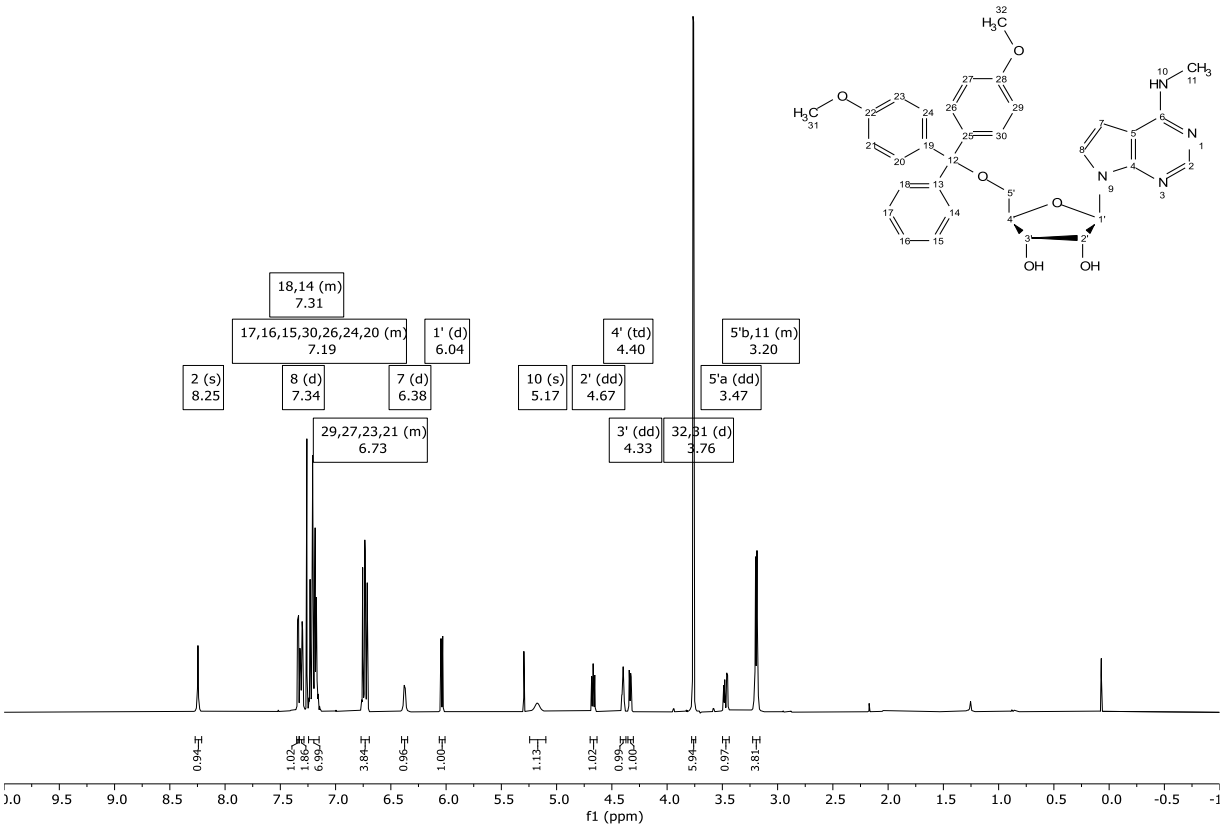
^{13}C{^{1}H}-NMR (100 MHz, $CDCl_3$) of compound **2b**.



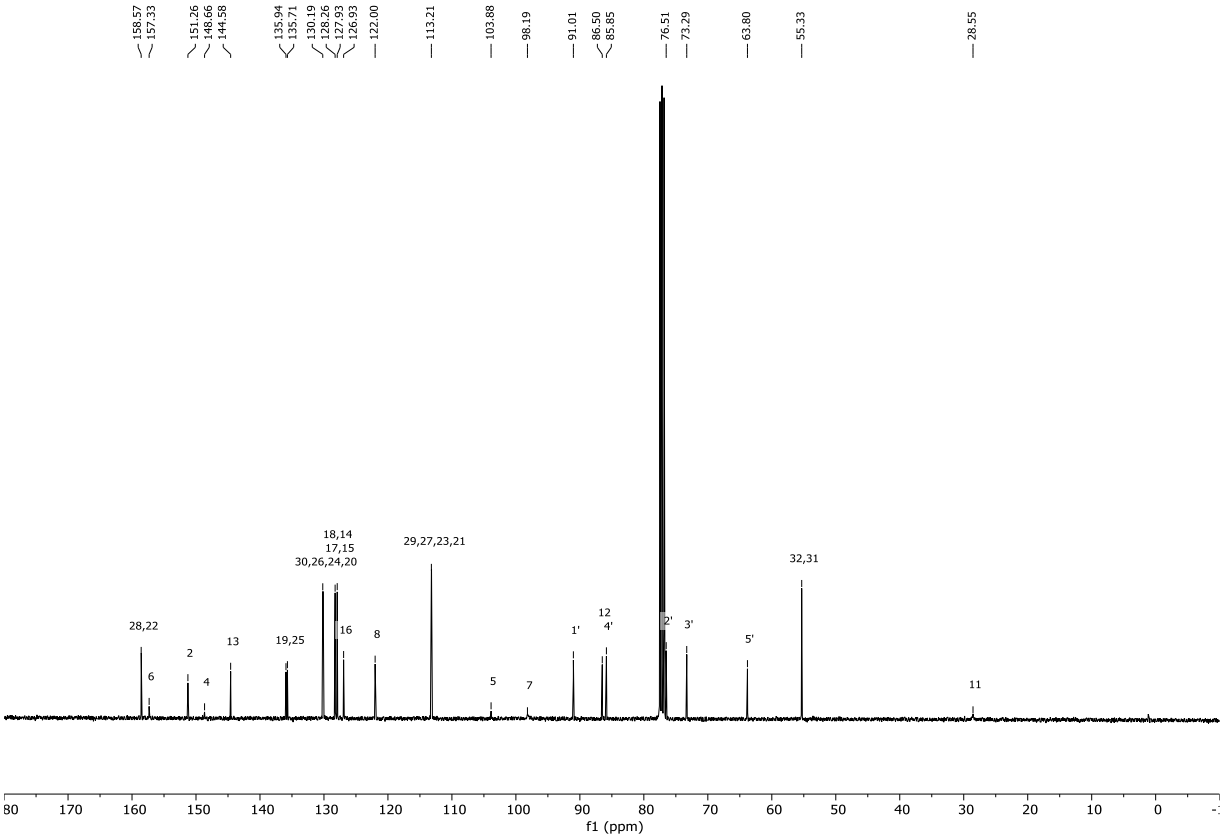
^{1}H-NMR (400 MHz, DMSO-d$_6$) of compound **2c**.



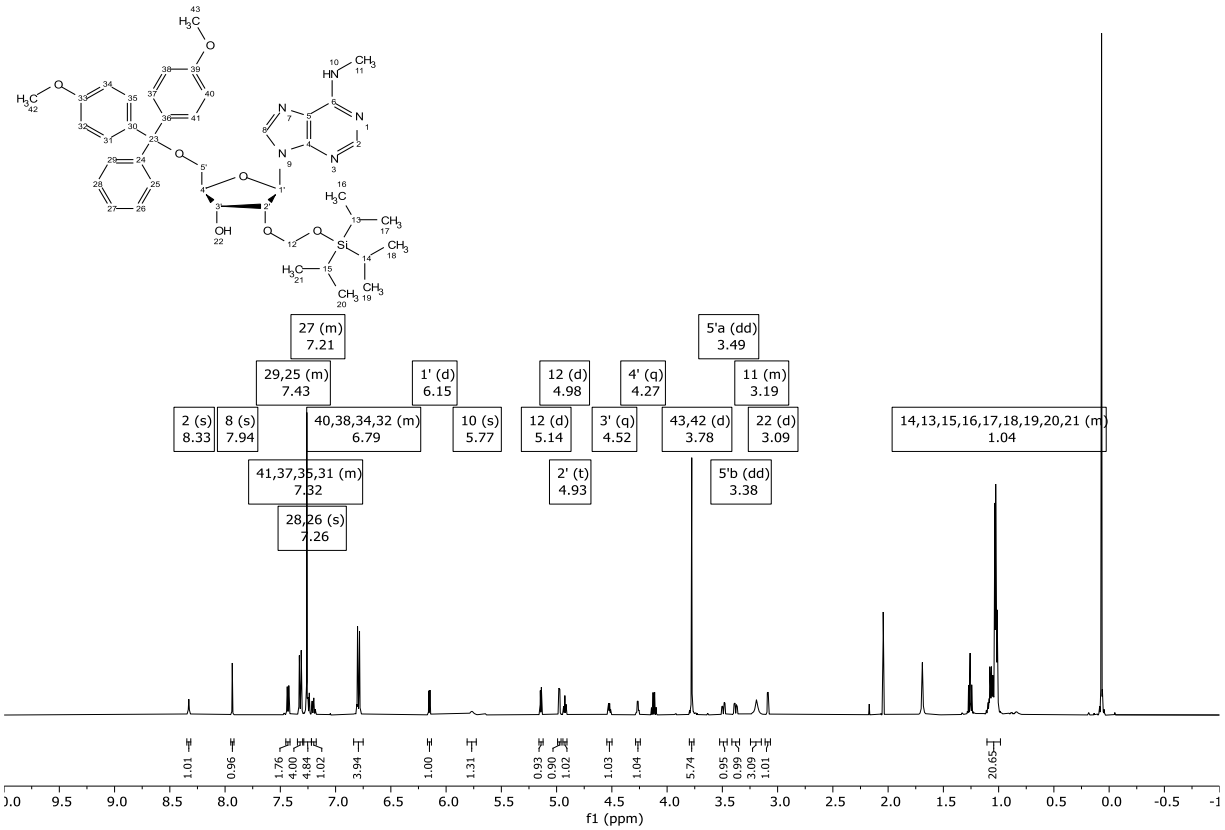
^{13}C{^{1}H}-NMR (100 MHz, DMSO-d$_6$) of compound **2c**.



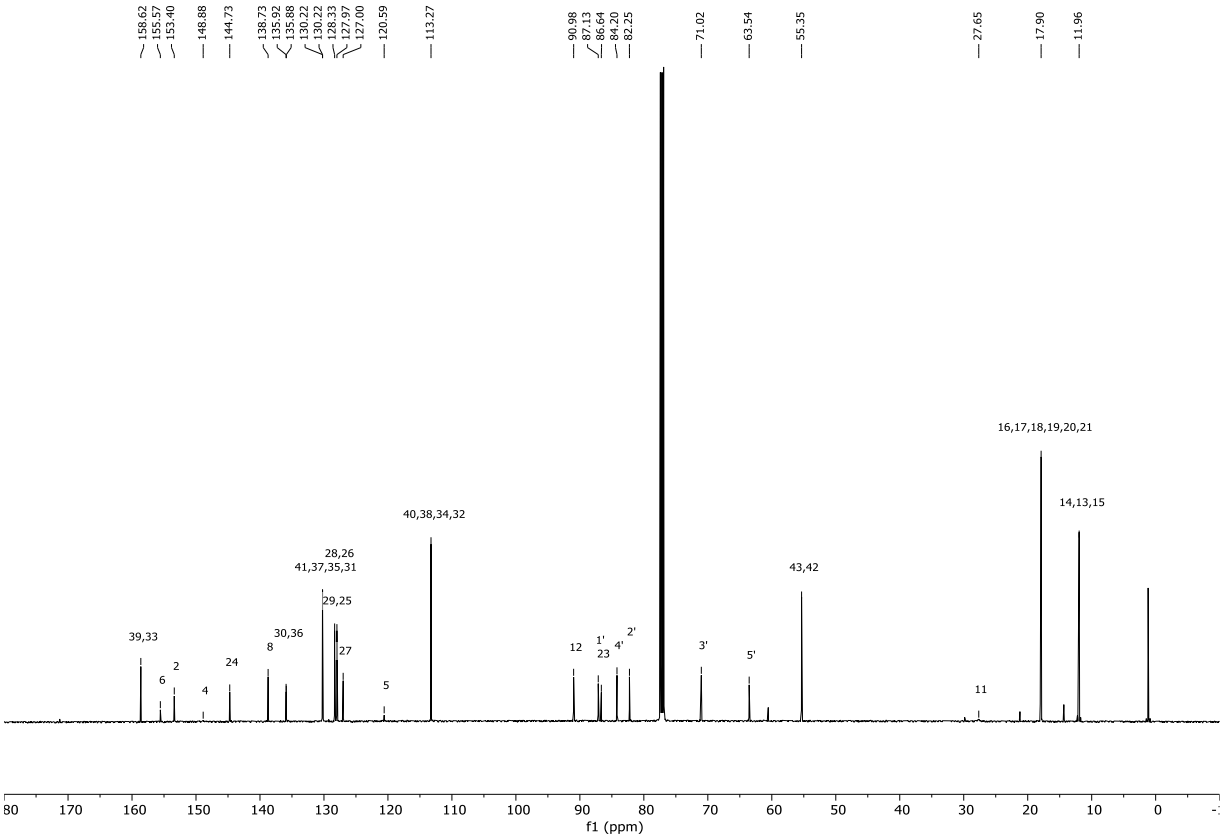
^{1}H-NMR (400 MHz, $CDCl_3$) of compound **2d**.



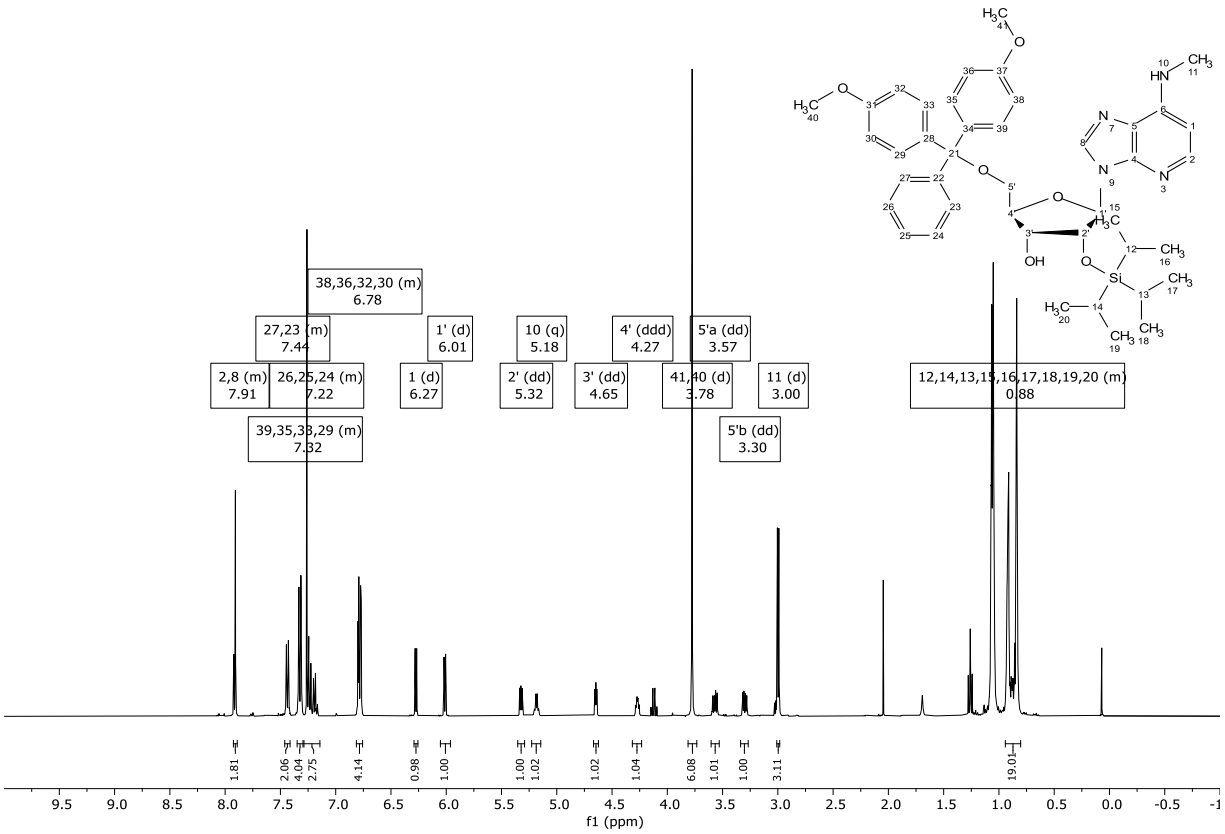
^{13}C{^{1}H}-NMR (100 MHz, $CDCl_3$) of compound **2d**.



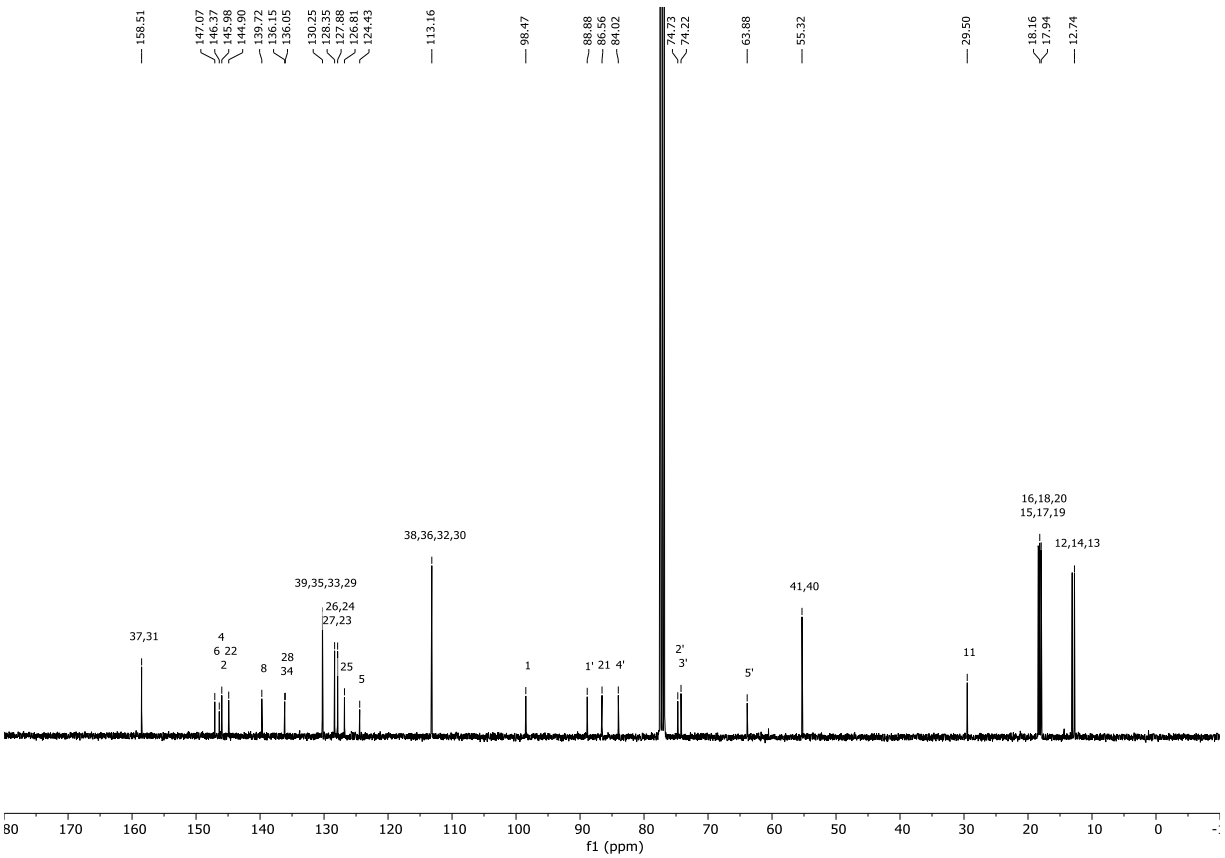
^{1}H-NMR (500 MHz, $CDCl_3$) of compound **3a**.



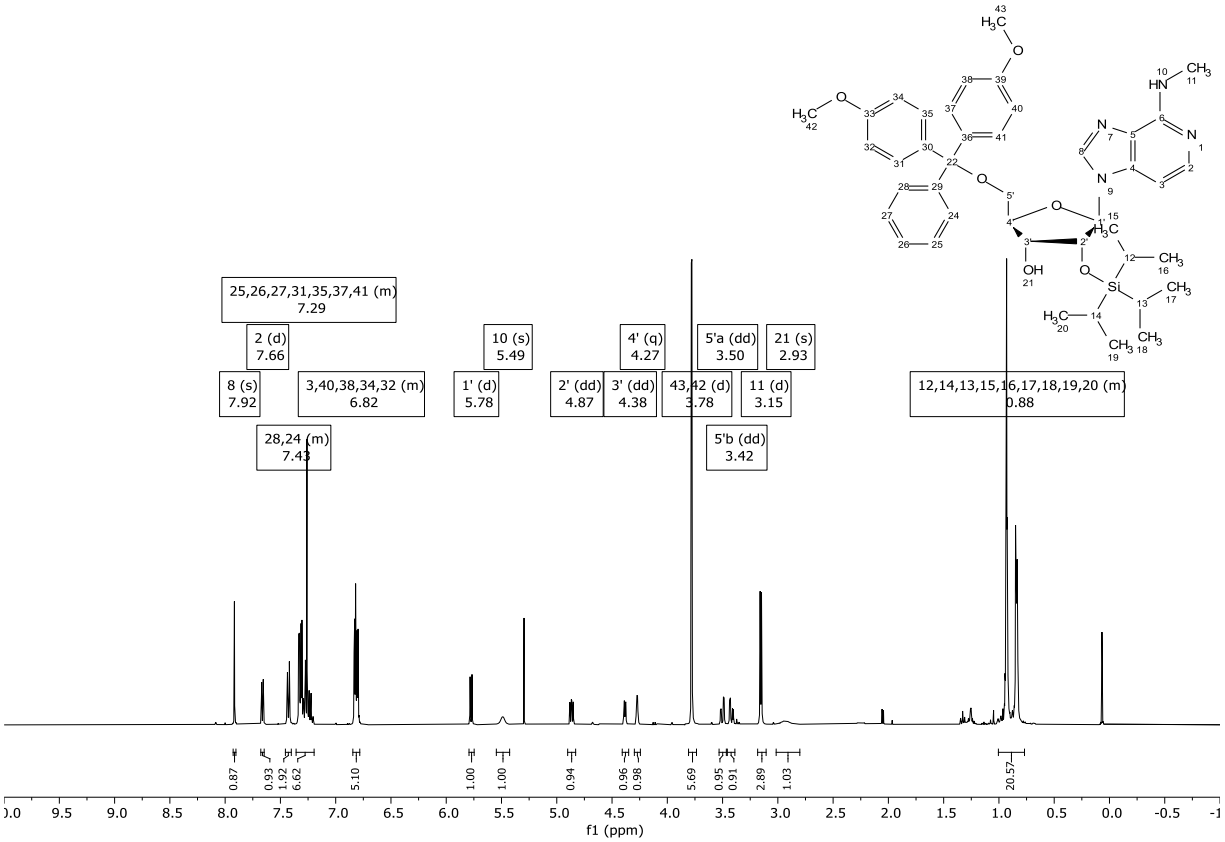
^{13}C{^{1}H}-NMR (125 MHz, $CDCl_3$) of compound **3a**.



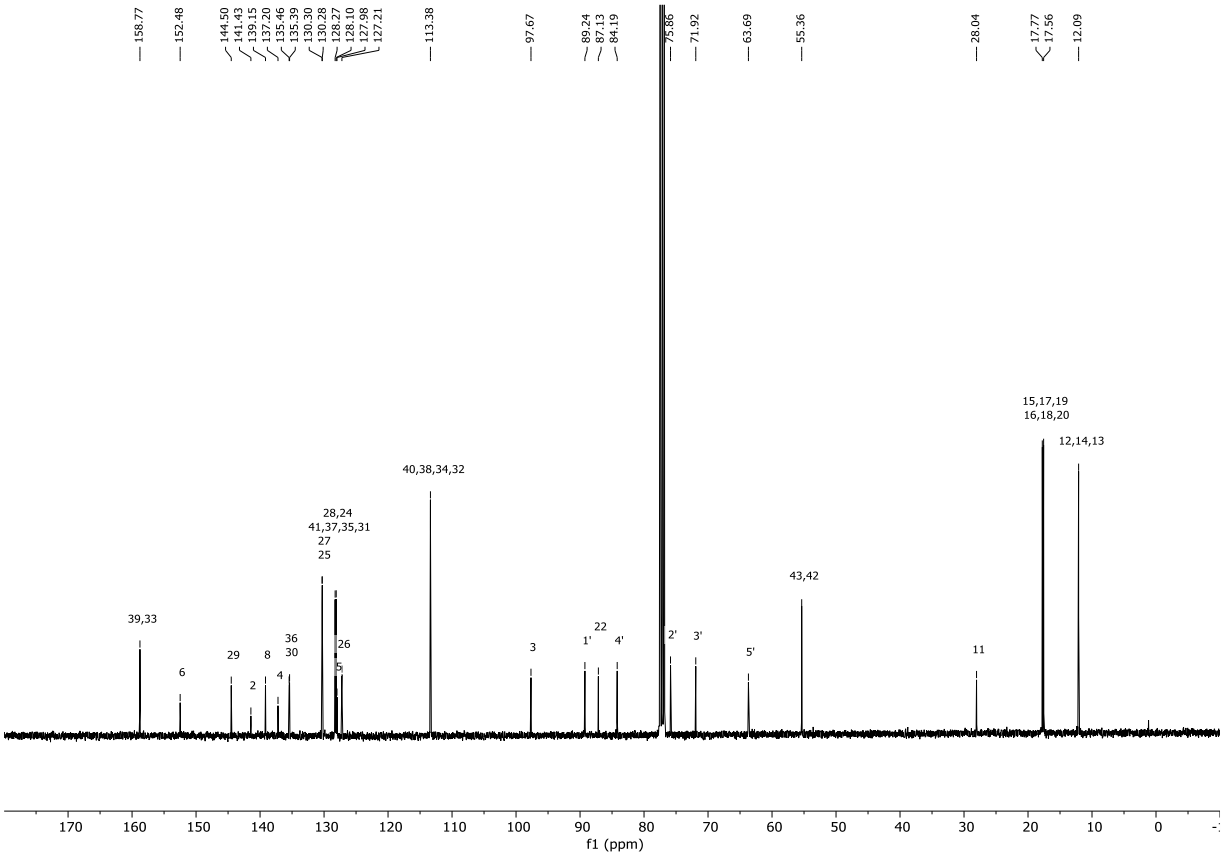
^{1}H-NMR (400 MHz, $CDCl_3$) of compound **3b**.



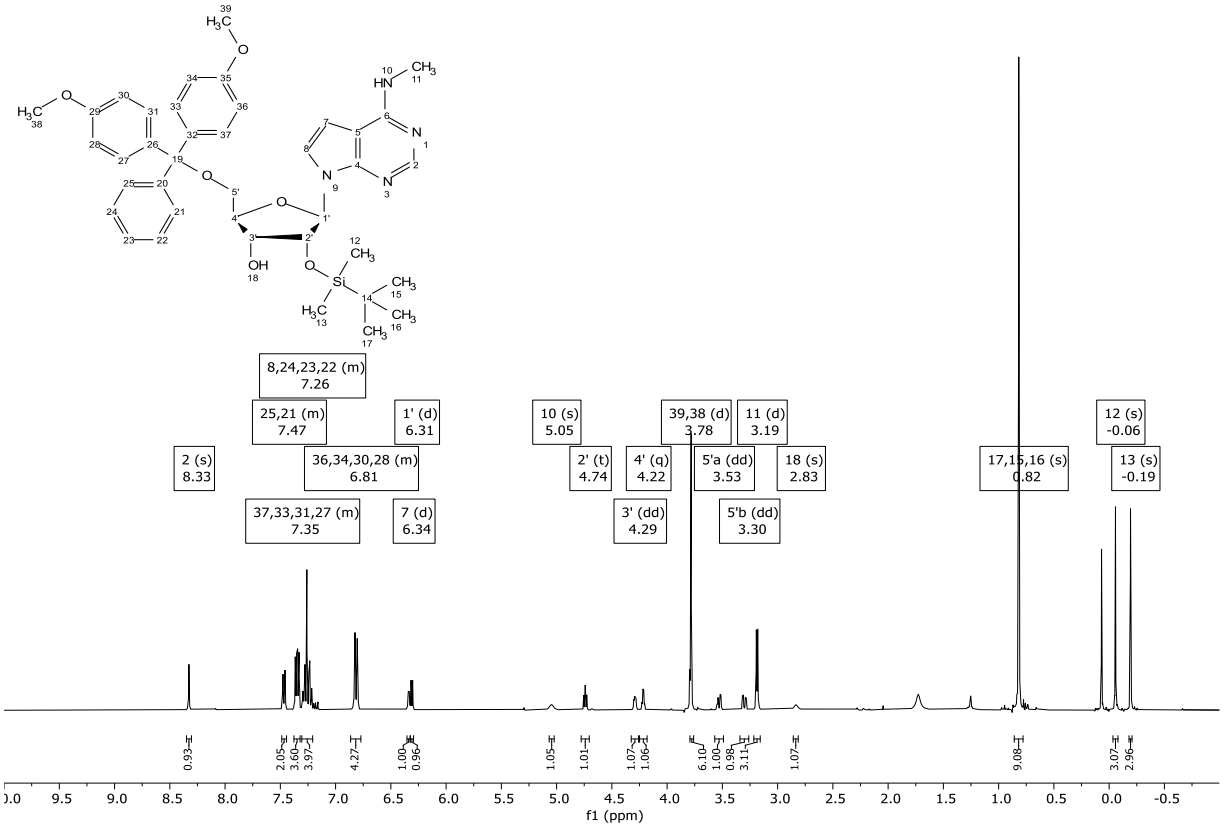
^{13}C{^{1}H}-NMR (100 MHz, $CDCl_3$) of compound **3b**.



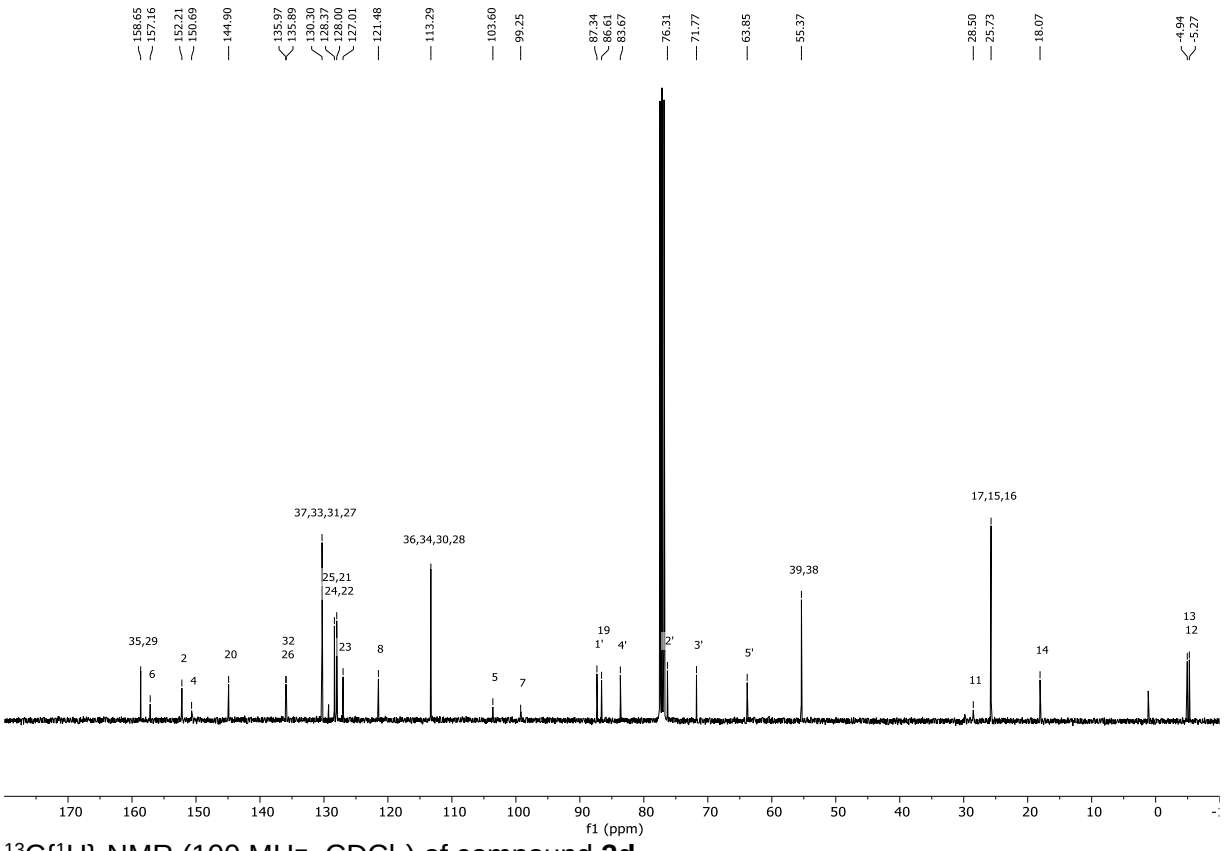
^{1}H-NMR (400 MHz, $CDCl_3$) of compound **3c**.



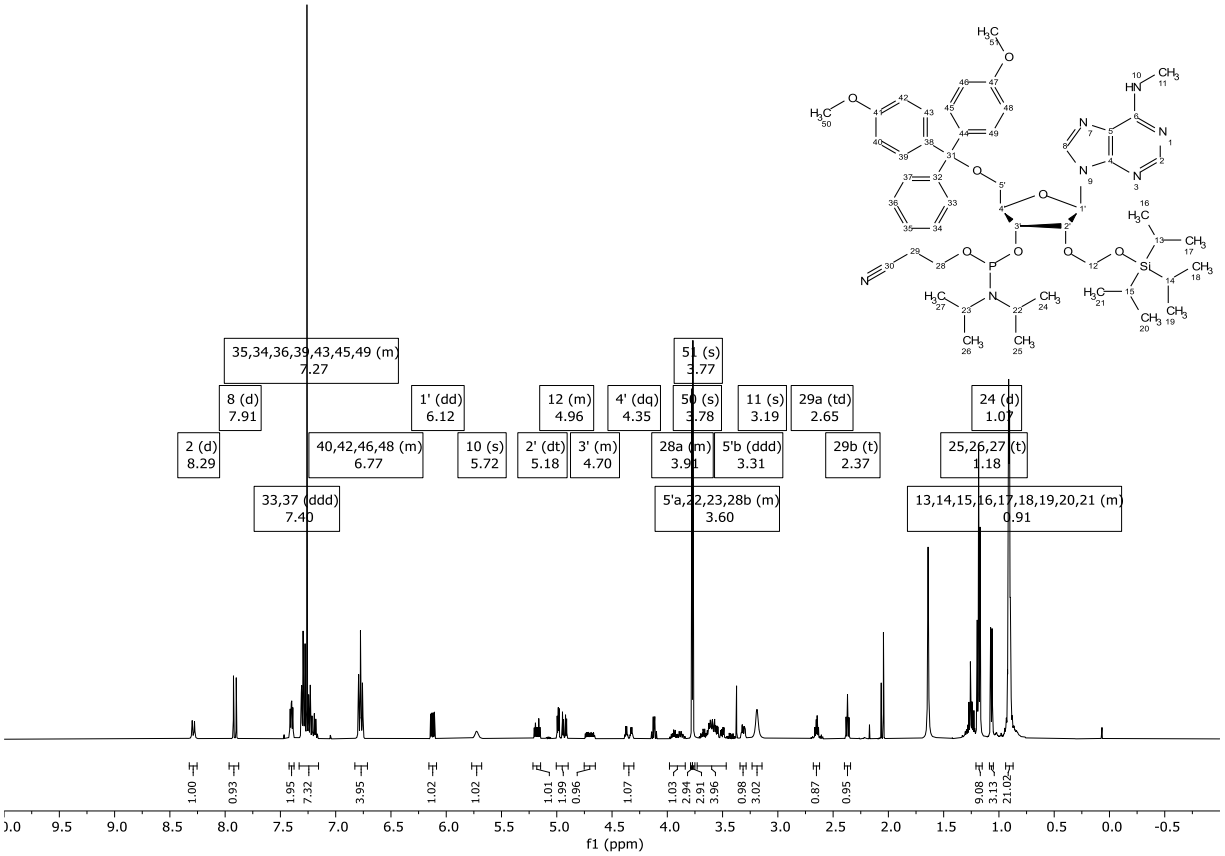
^{13}C{^{1}H}-NMR (100 MHz, $CDCl_3$) of compound **3c**.



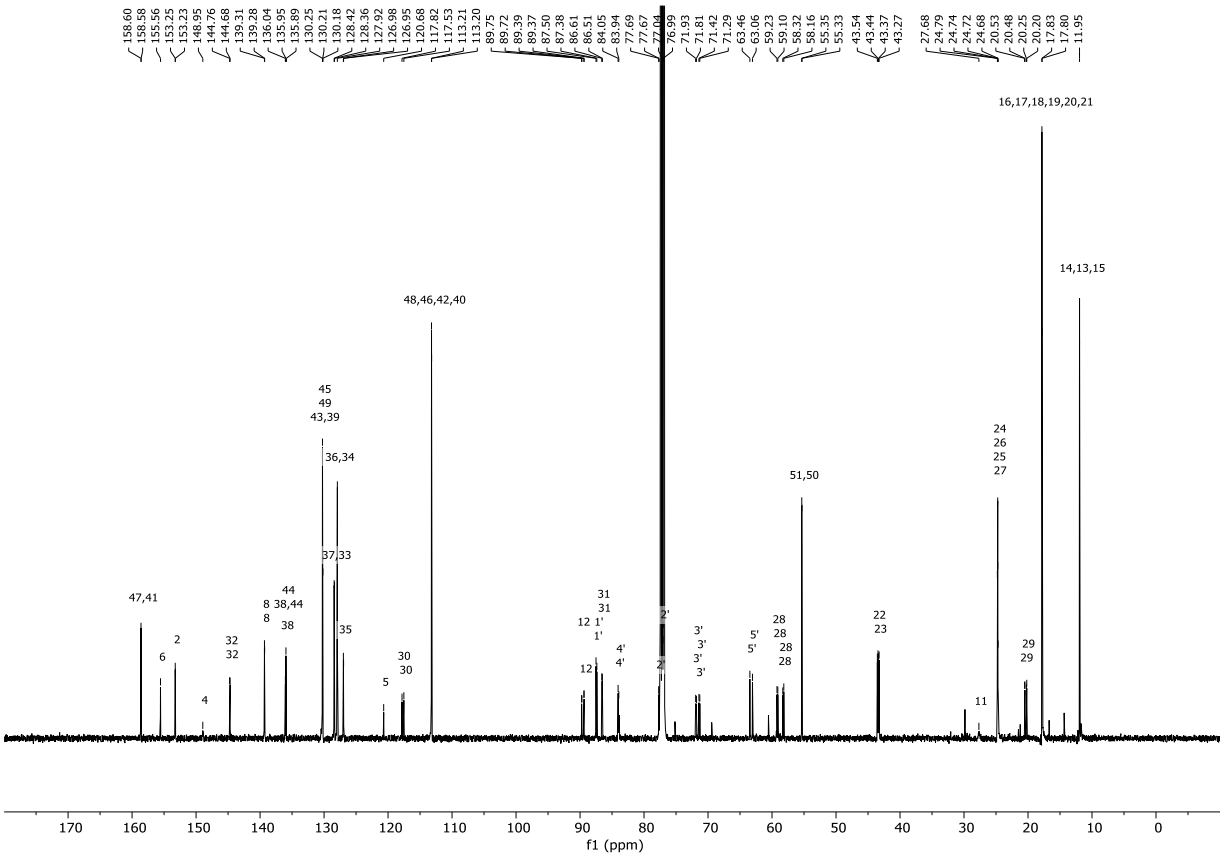
^{1}H-NMR (400 MHz, $CDCl_3$) of compound **3d**.



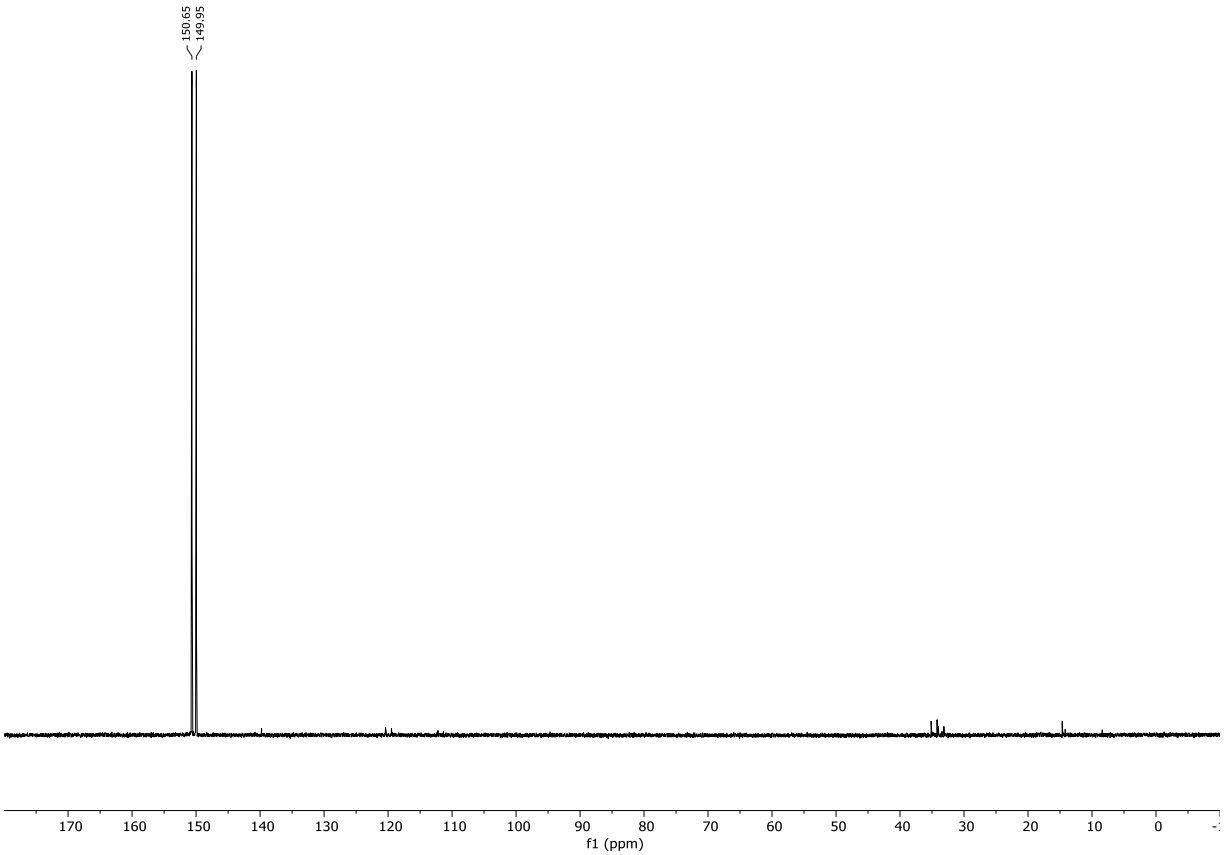
^{13}C{^{1}H}-NMR (100 MHz, $CDCl_3$) of compound **3d**.



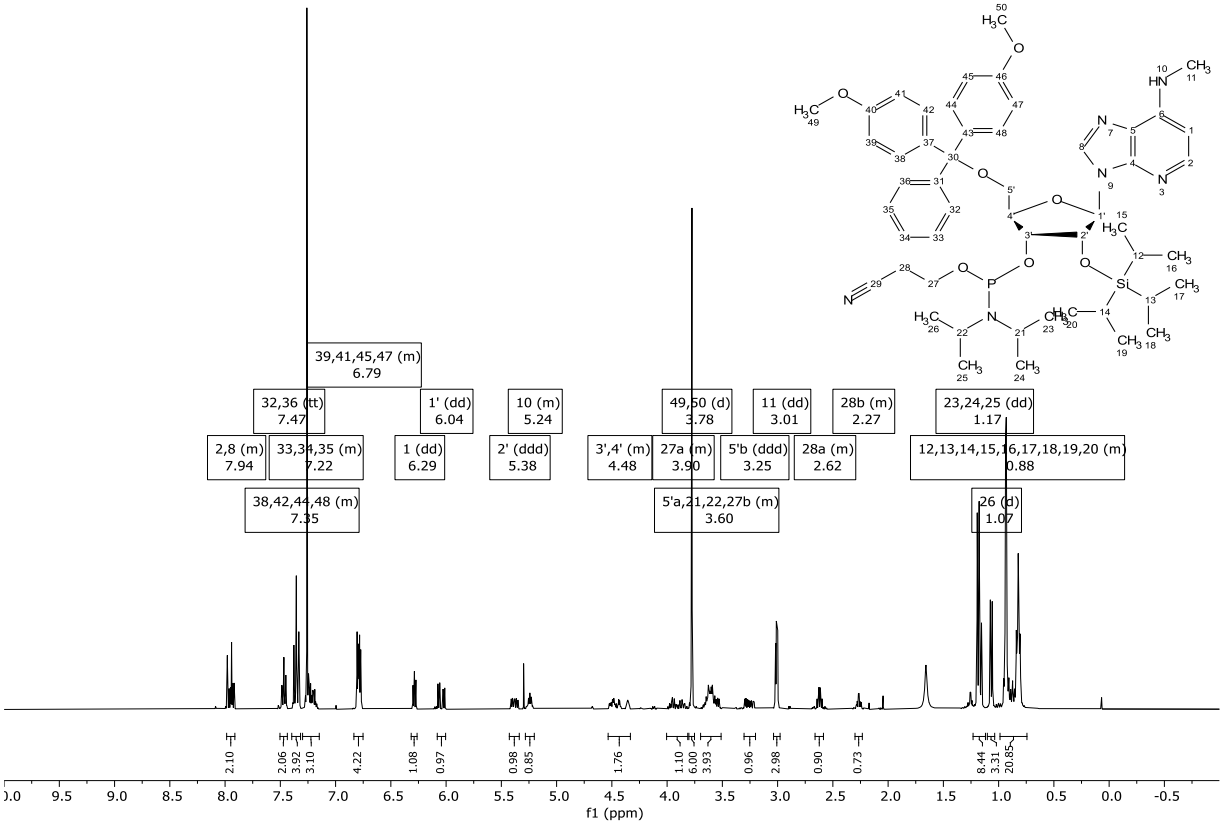
^{1}H-NMR (500 MHz, $CDCl_3$) of compound **4a**.



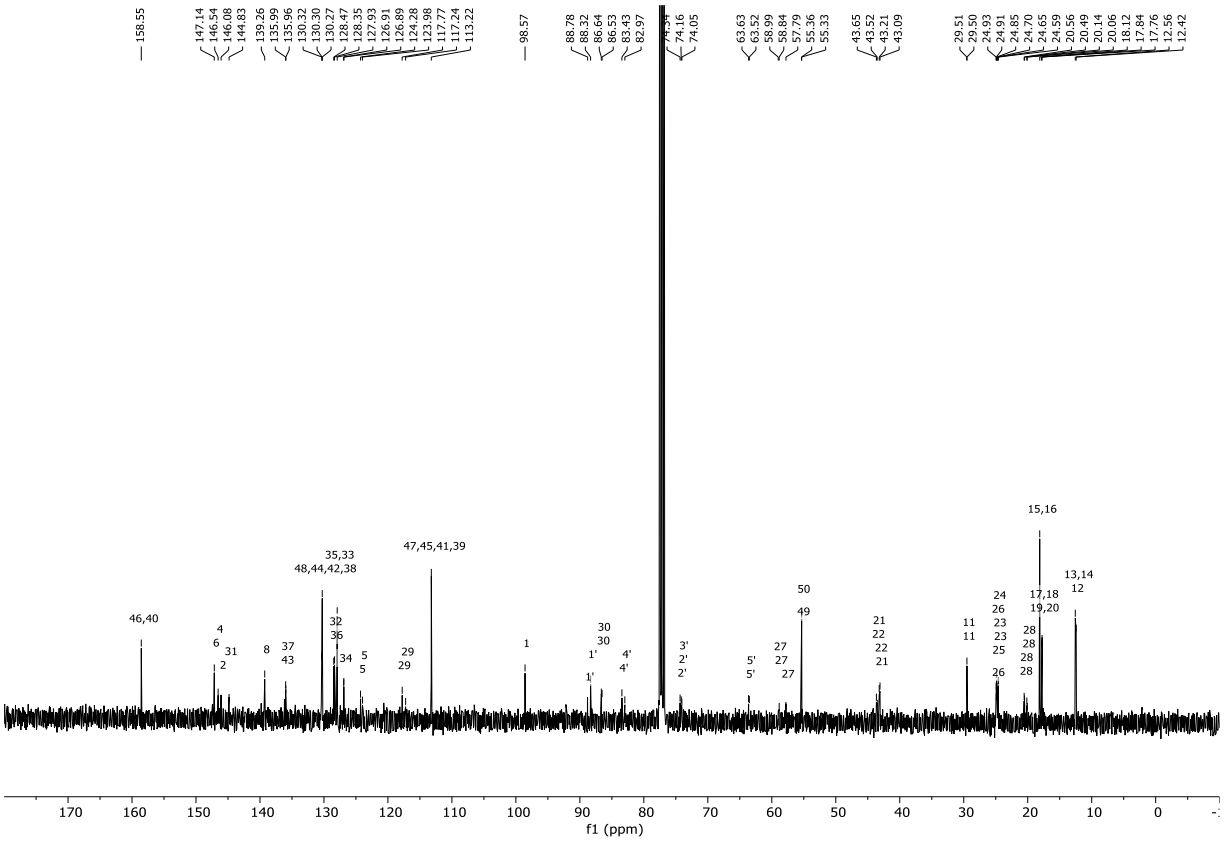
^{13}C{^{1}H}-NMR (125 MHz, $CDCl_3$) of compound **4a**.



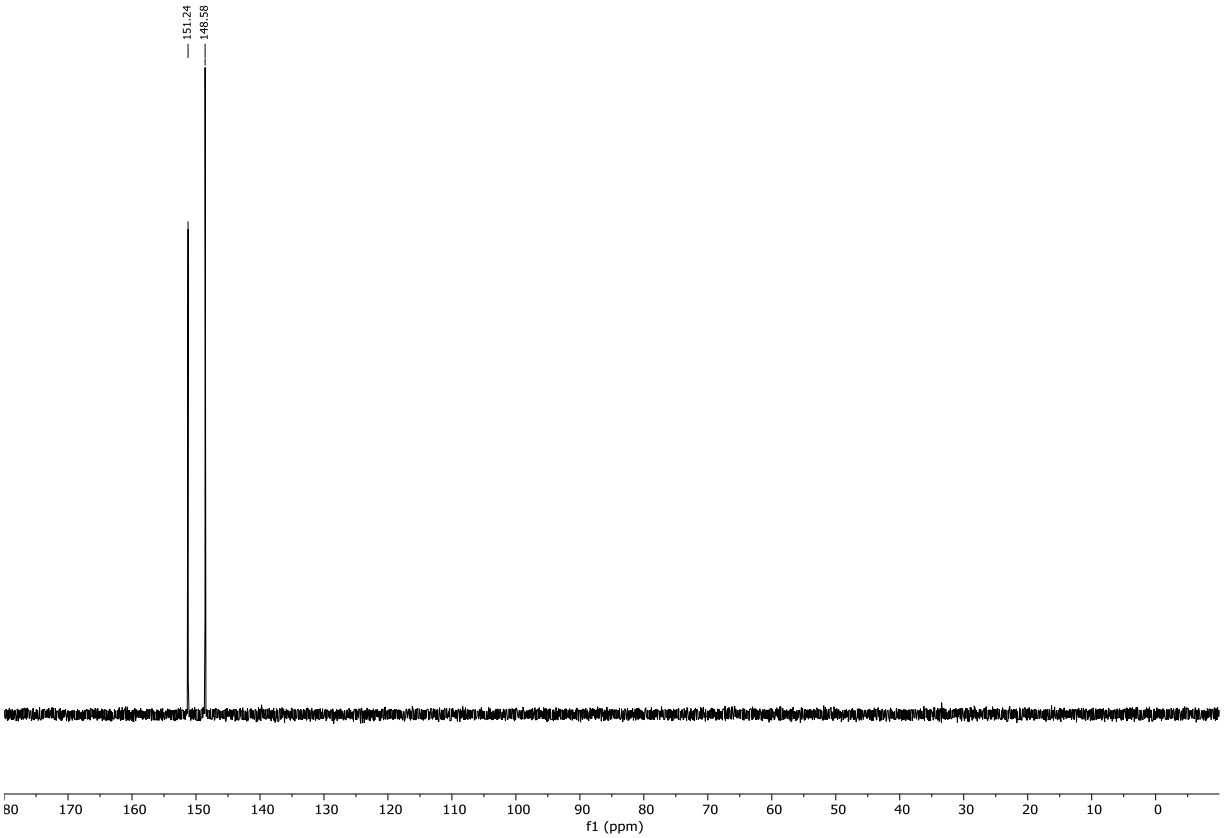
$^{31}P\{^{1}H\}$-NMR (202 MHz, $CDCl_3$) of compound **4a**.



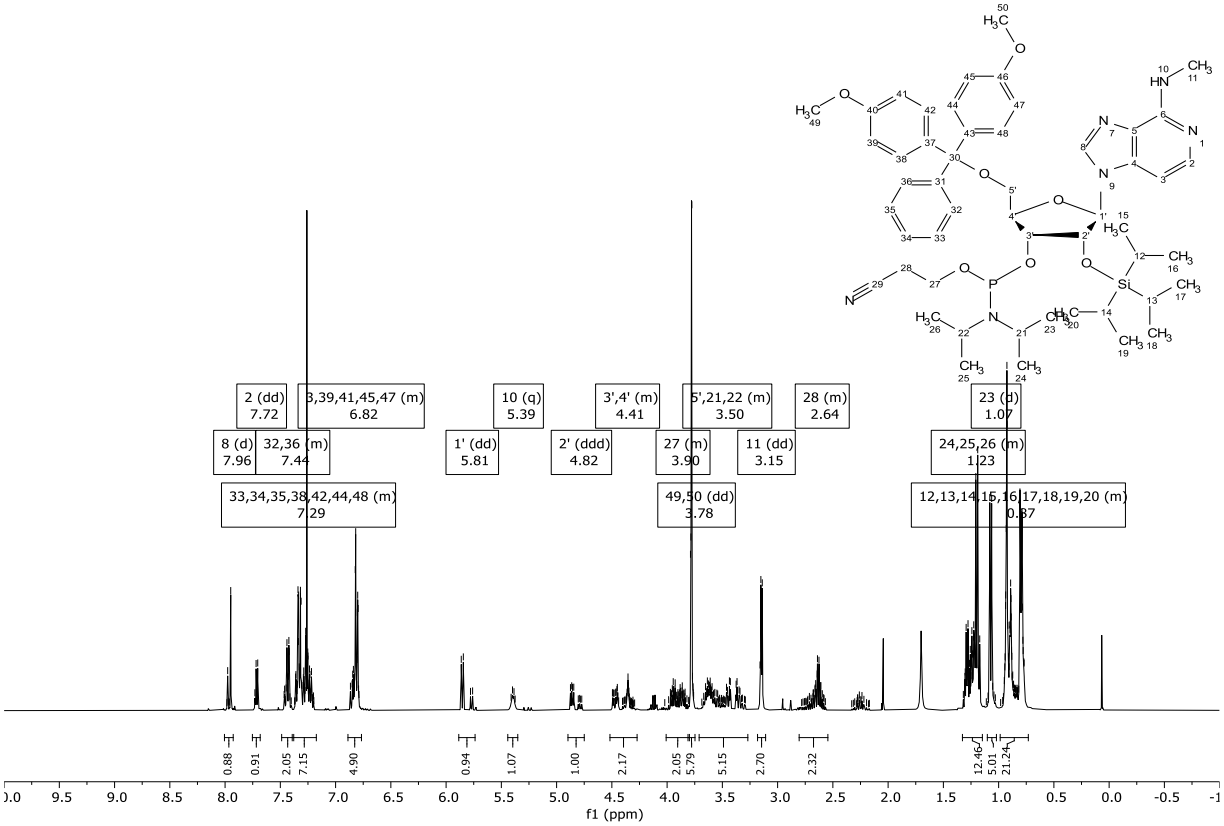
^{1}H-NMR (400 MHz, $CDCl_3$) of compound **4b**.



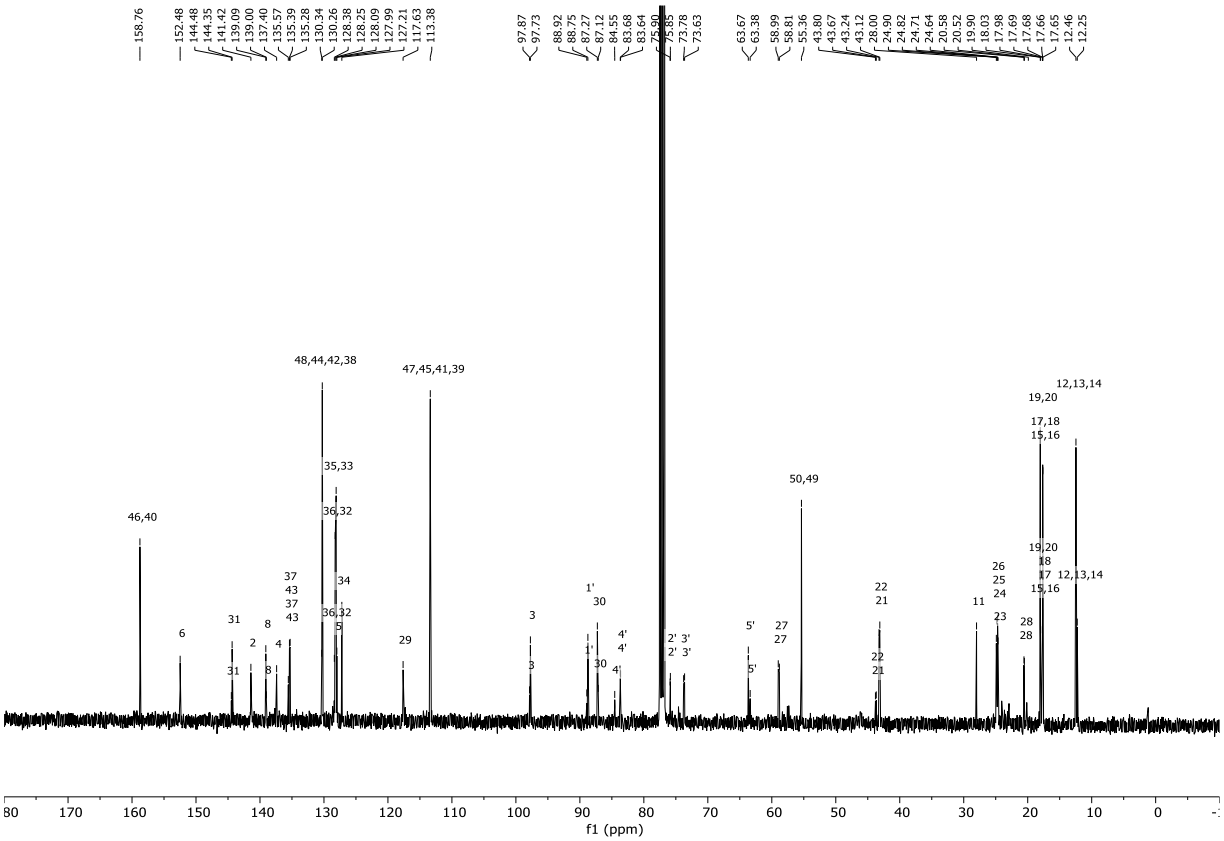
$^{13}C\{^{1}H\}$-NMR (100 MHz, $CDCl_3$) of compound **4b**.



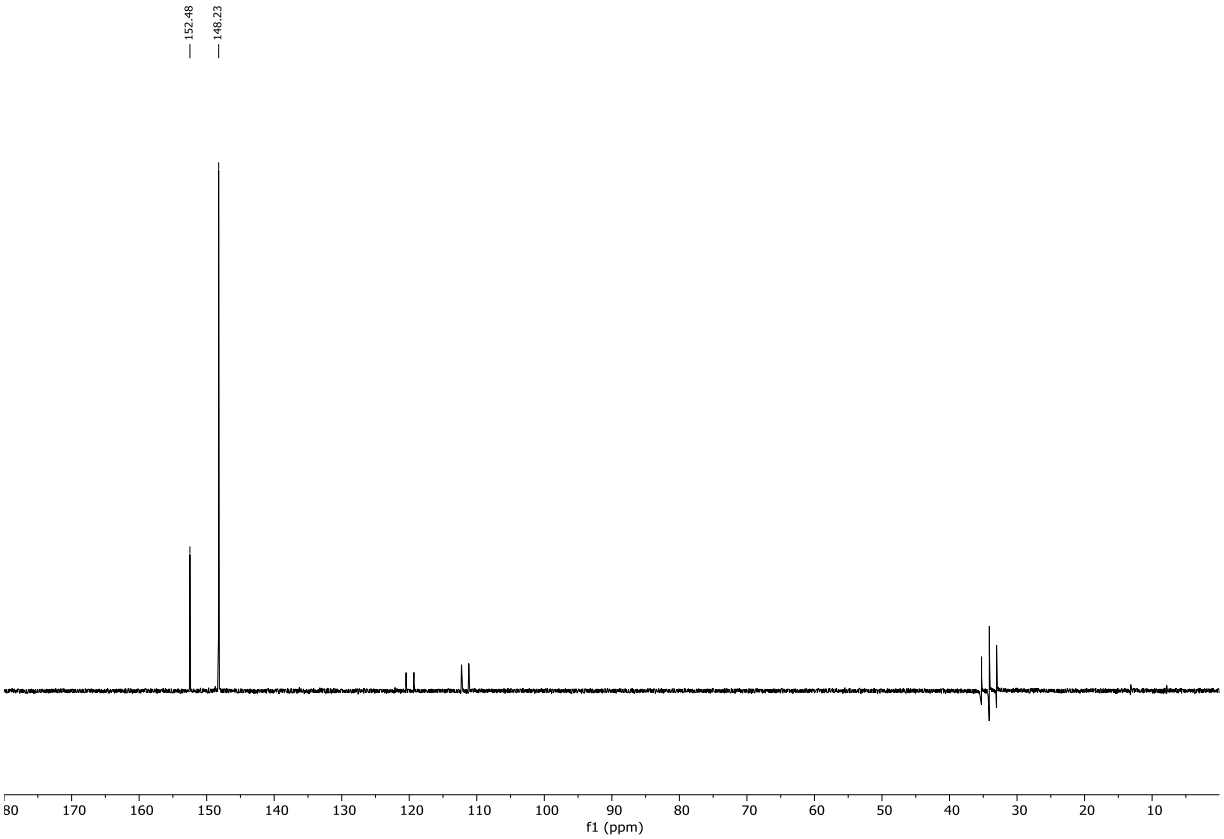
$^{31}P\{^{1}H\}$-NMR (162 MHz, $CDCl_3$) of compound **4b**.



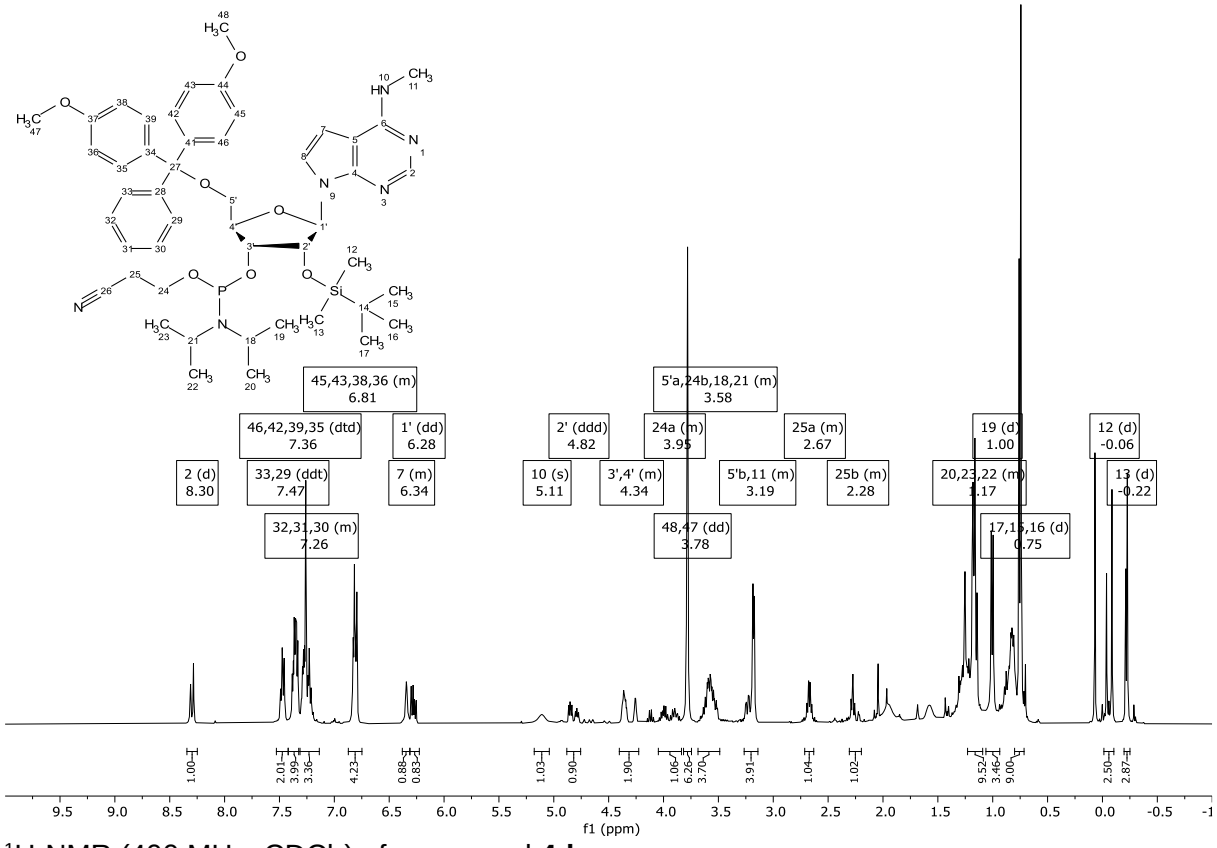
^{1}H-NMR (400 MHz, $CDCl_3$) of compound **4c**.



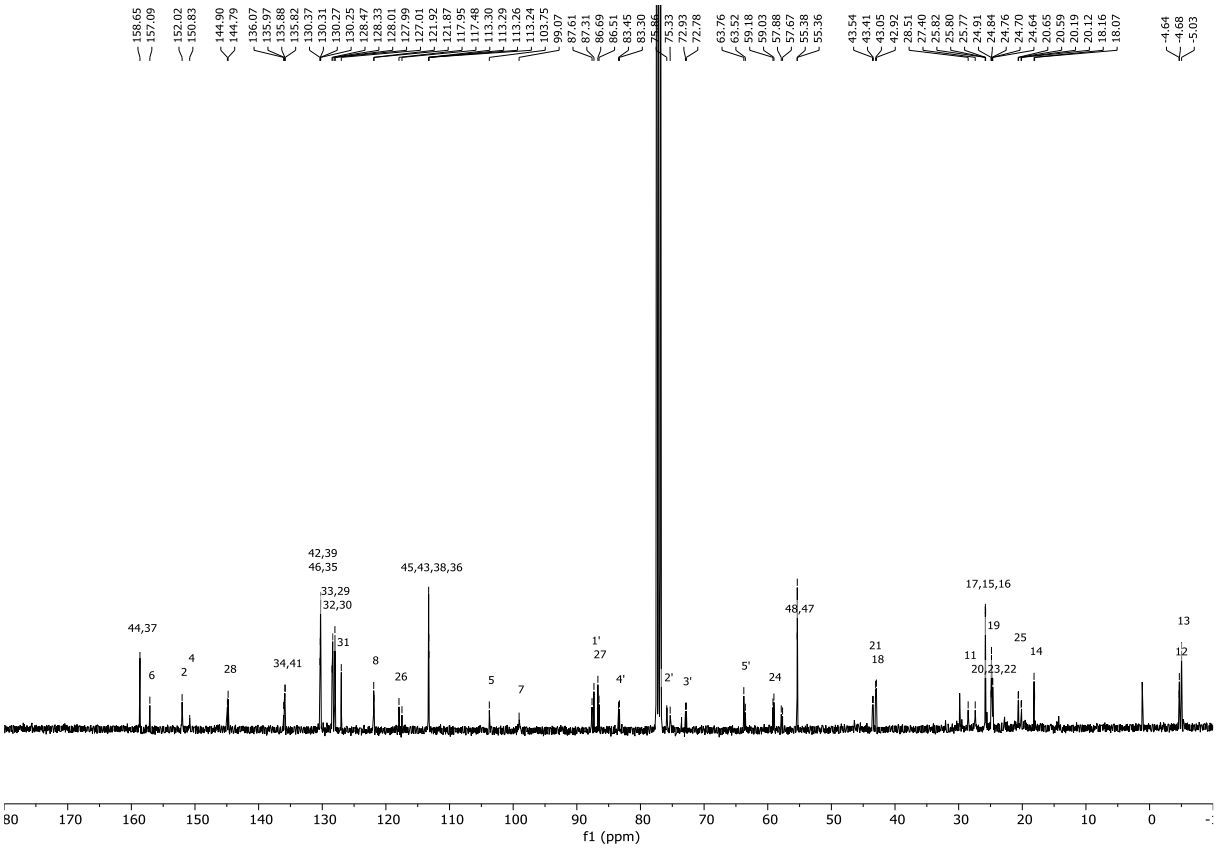
^{13}C{^{1}H}-NMR (100 MHz, $CDCl_3$) of compound **4c**.



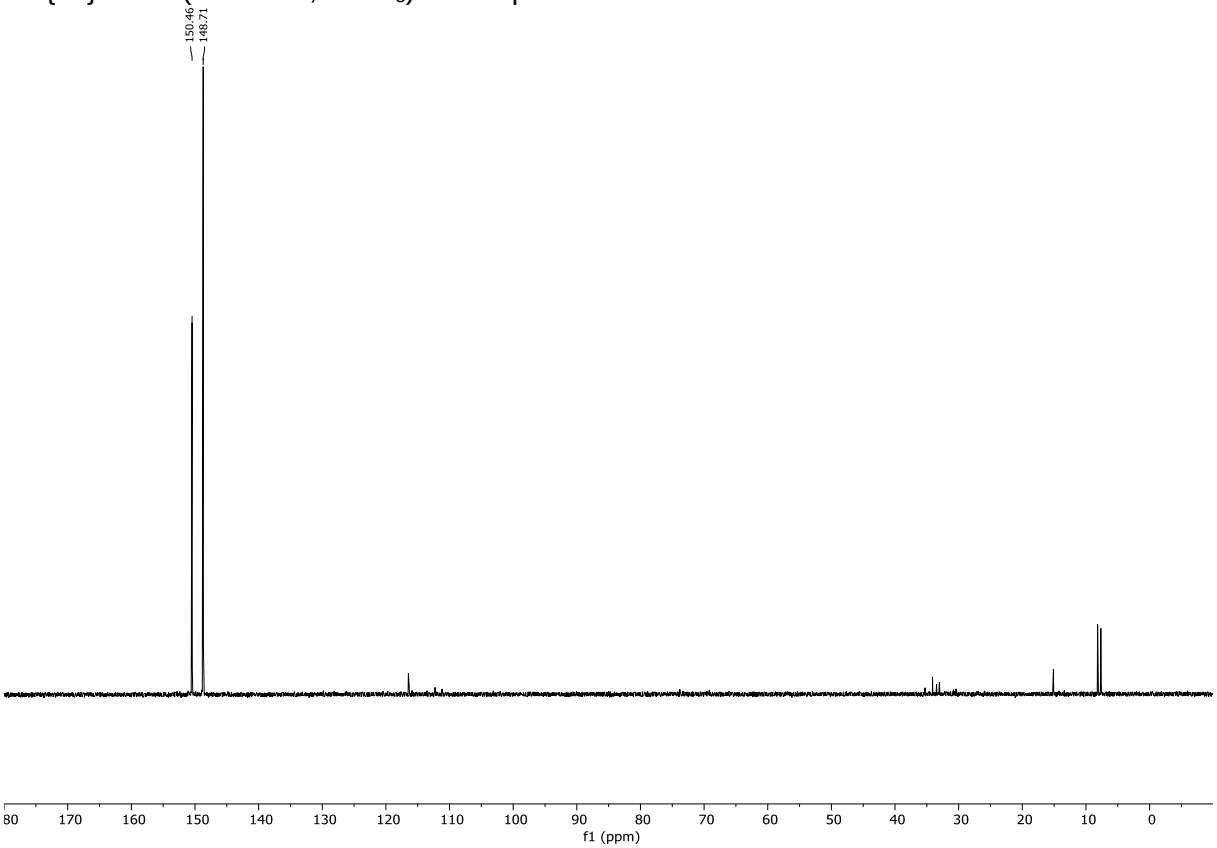
$^{31}P\{^{1}H\}$-NMR (162 MHz, $CDCl_3$) of compound **4c**.



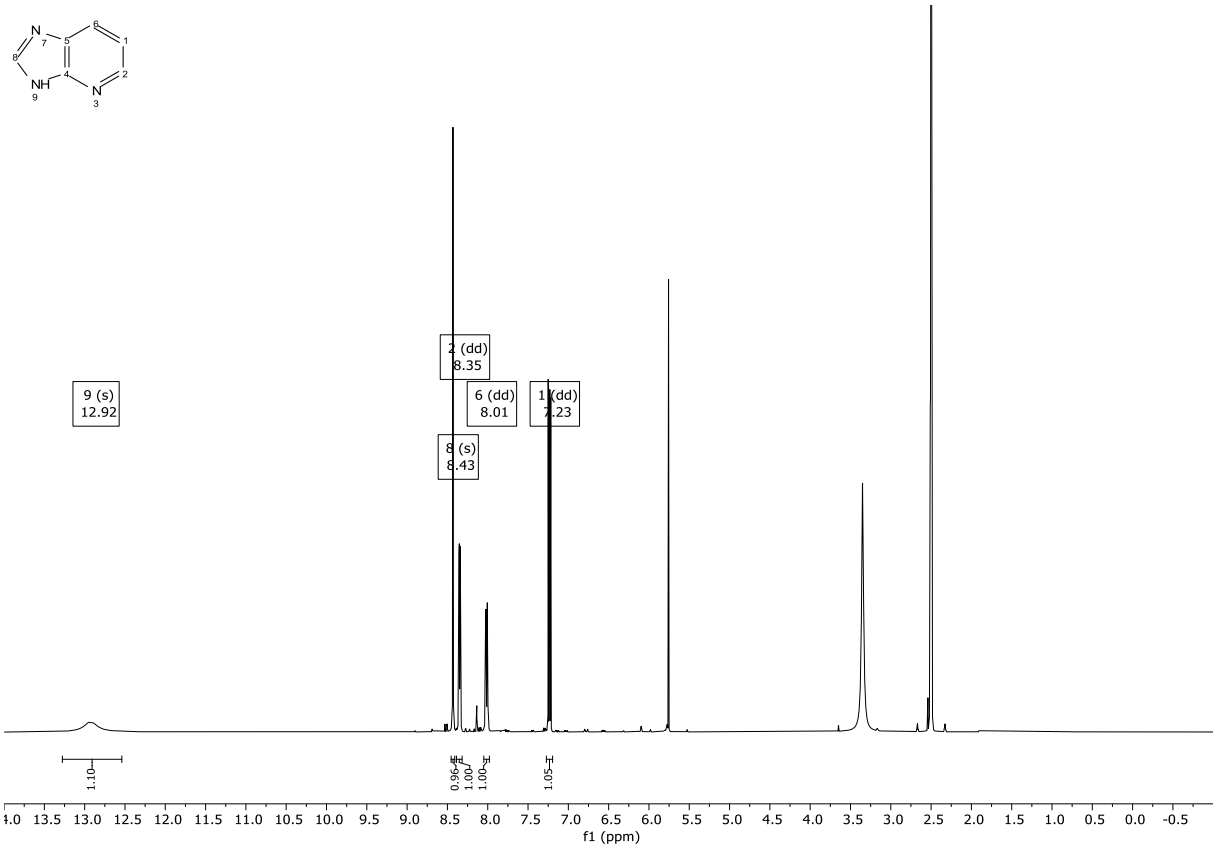
^{1}H-NMR (400 MHz, $CDCl_3$) of compound **4d**.



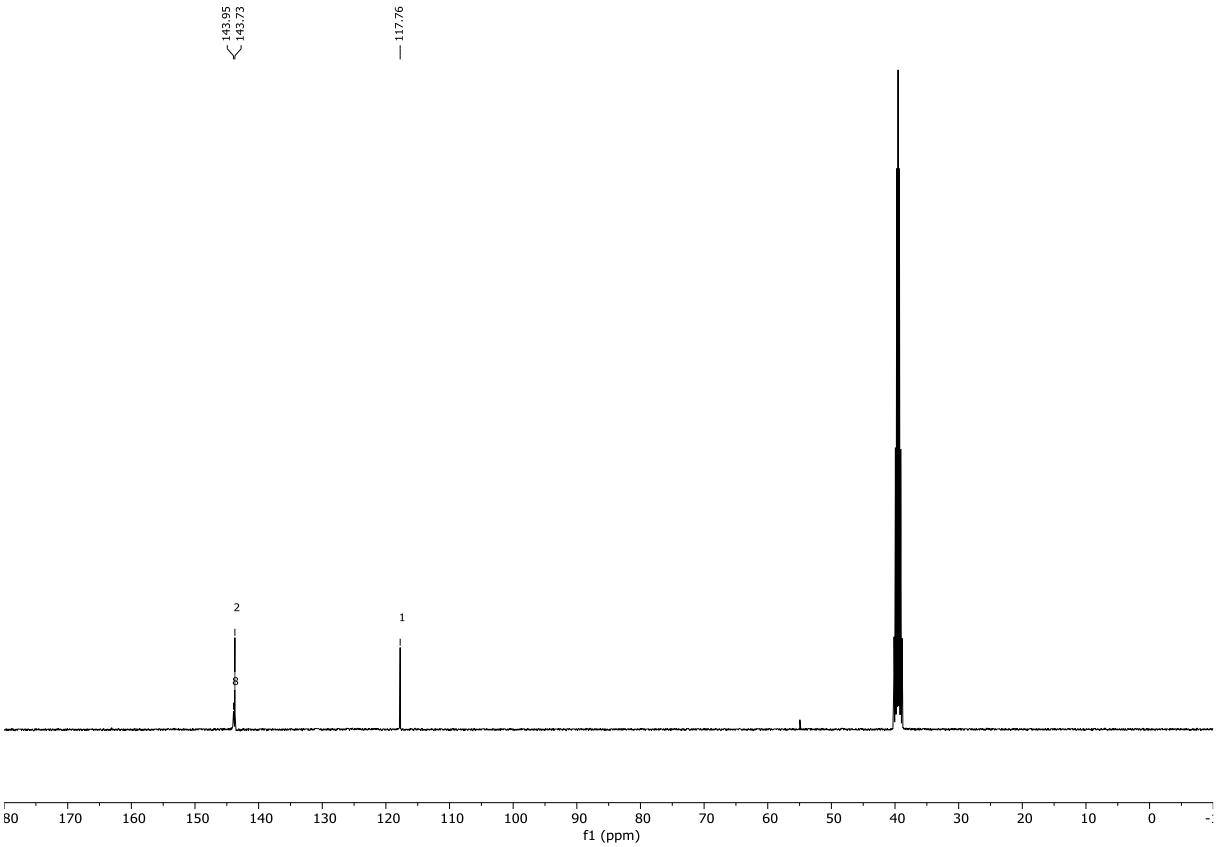
$^{13}C\{^{1}H\}$-NMR (100 MHz, $CDCl_3$) of compound **4d**.



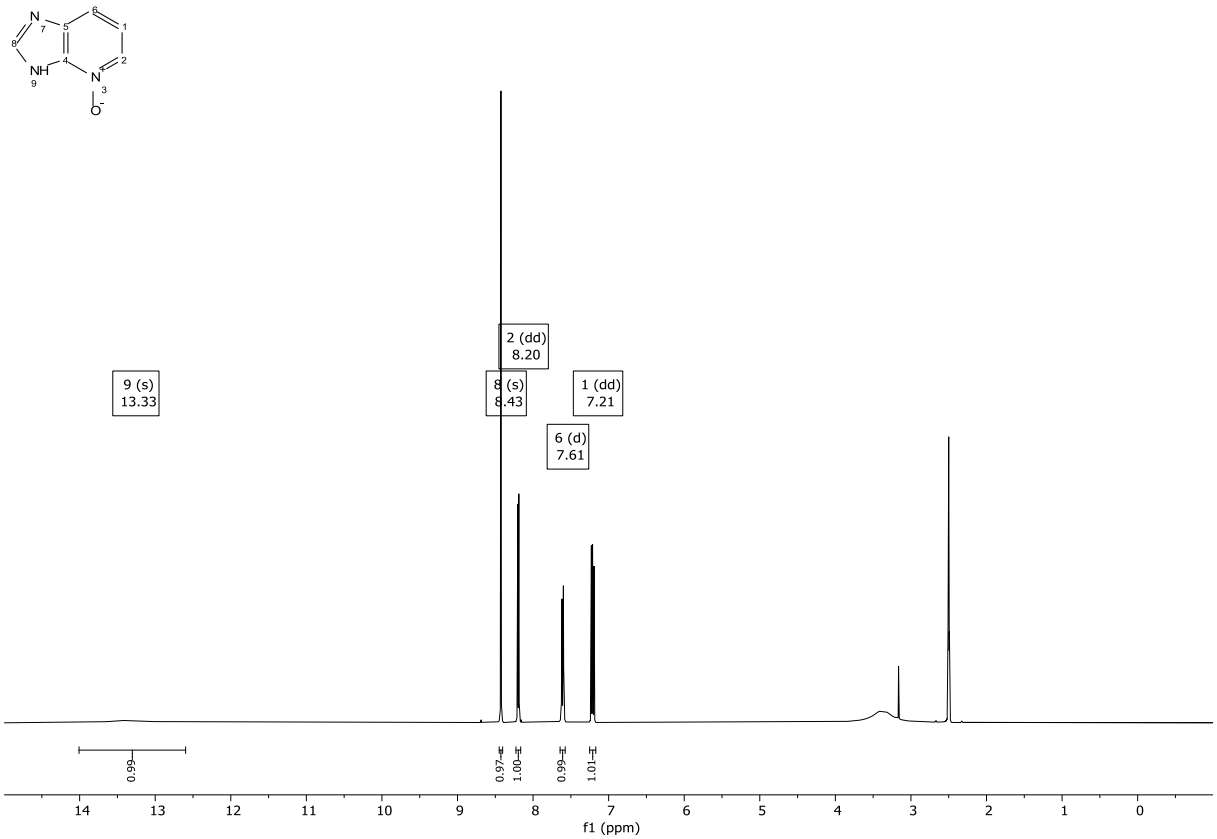
$^{31}P\{^{1}H\}$-NMR (162 MHz, $CDCl_3$) of compound **4d**.



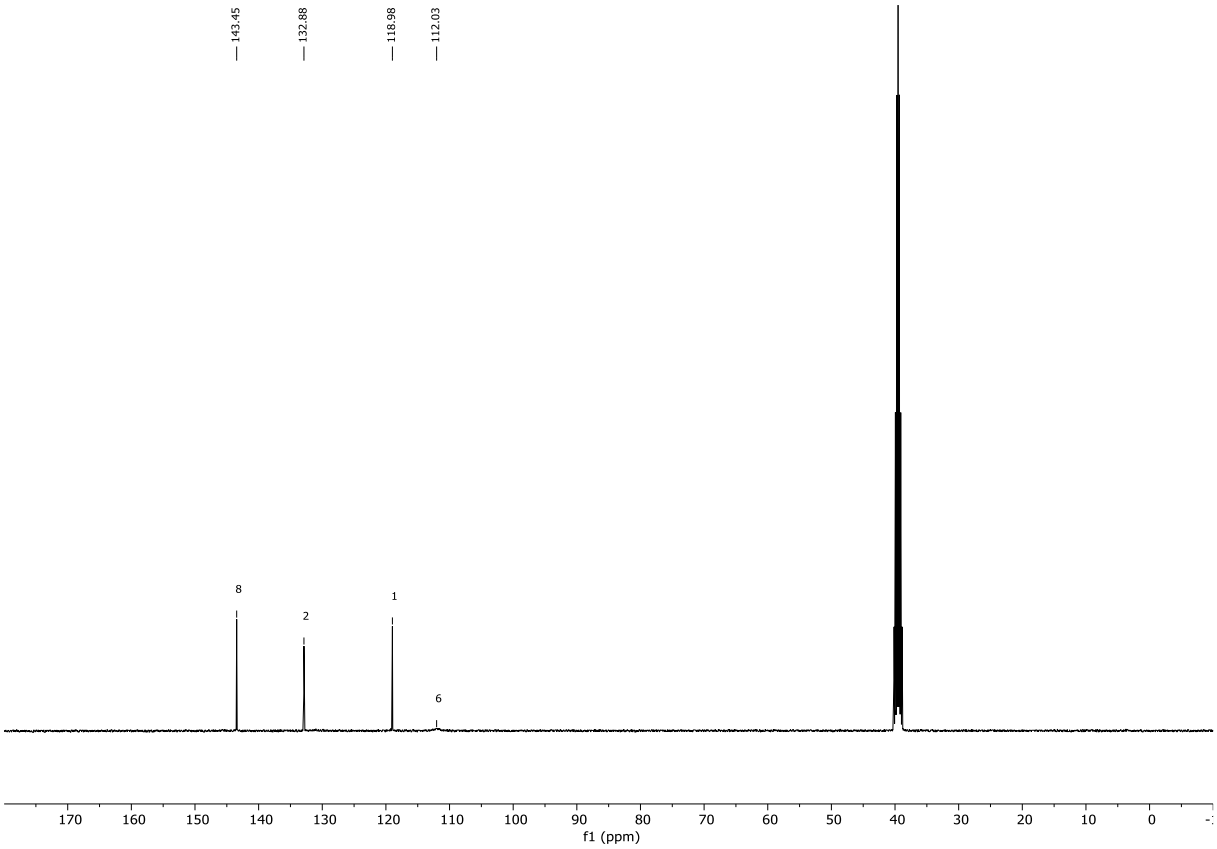
^{1}H-NMR (400 MHz, DMSO-d_6) of compound **S16**.



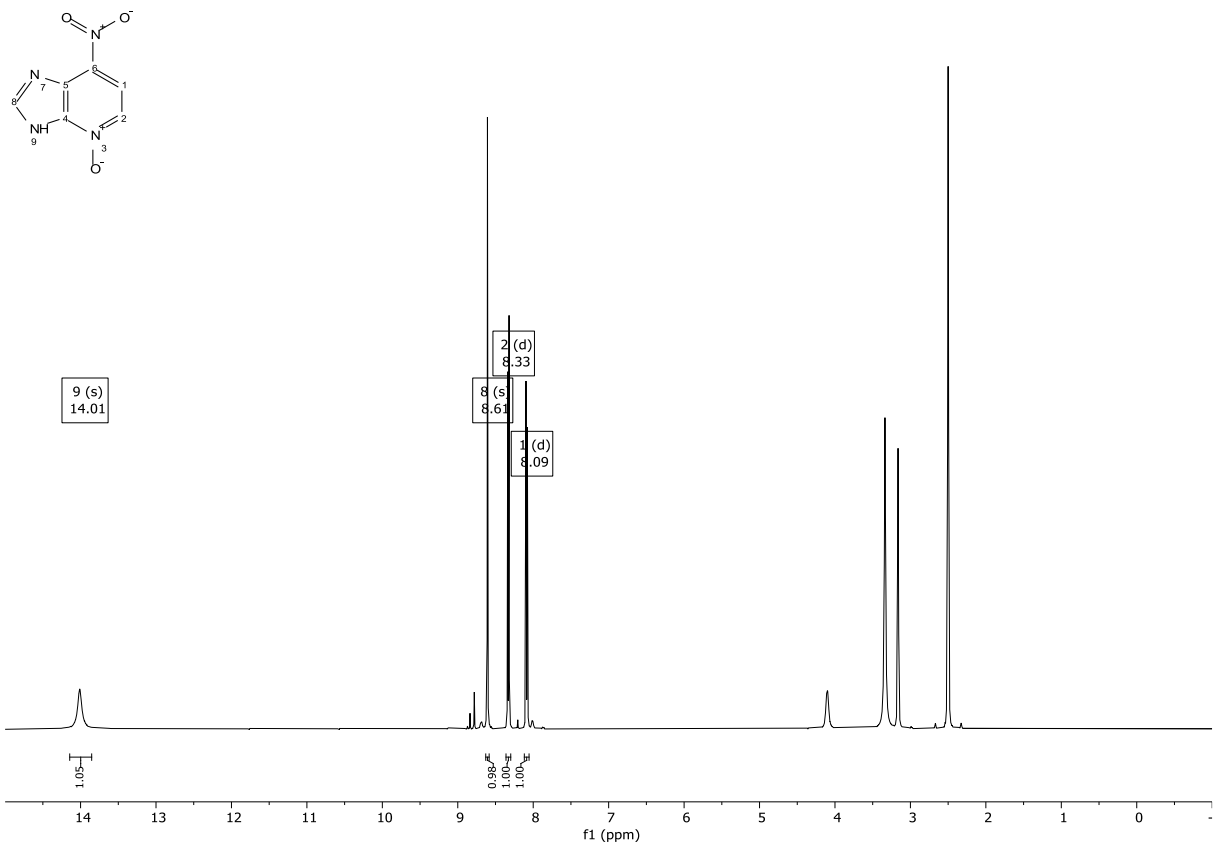
^{13}C{^{1}H}-NMR (100 MHz, DMSO-d_6) of compound **S16**.



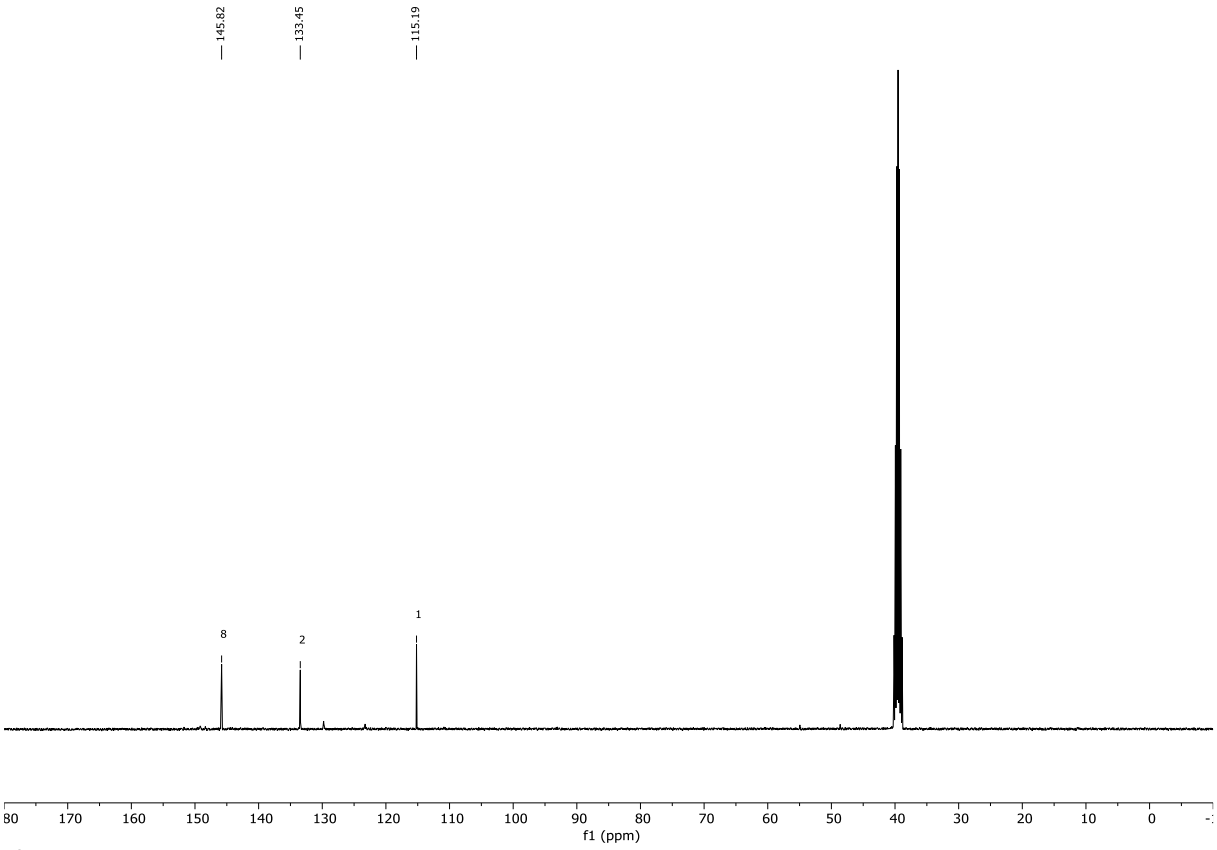
^{1}H-NMR (400 MHz, DMSO-d_6) of compound **S17**.



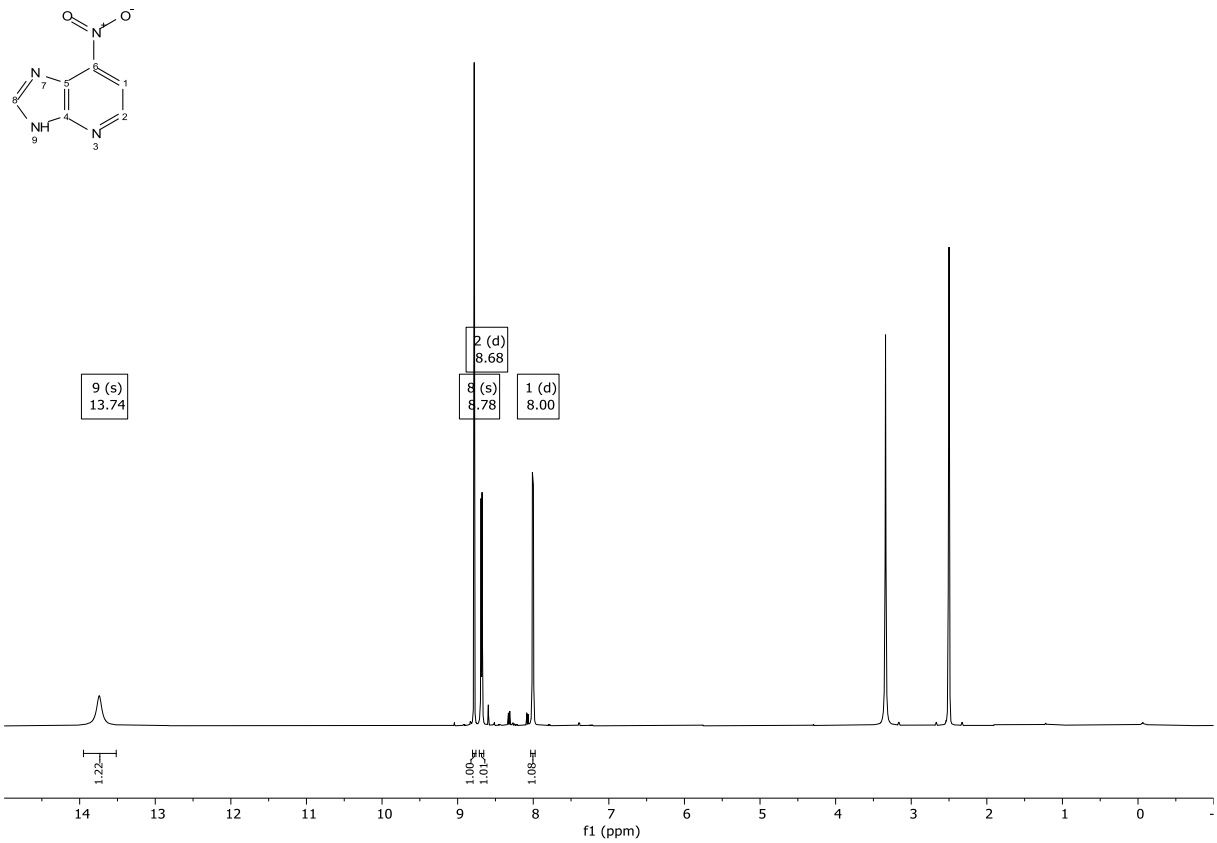
^{13}C{^{1}H}-NMR (100 MHz, DMSO-d_6) of compound **S17**.



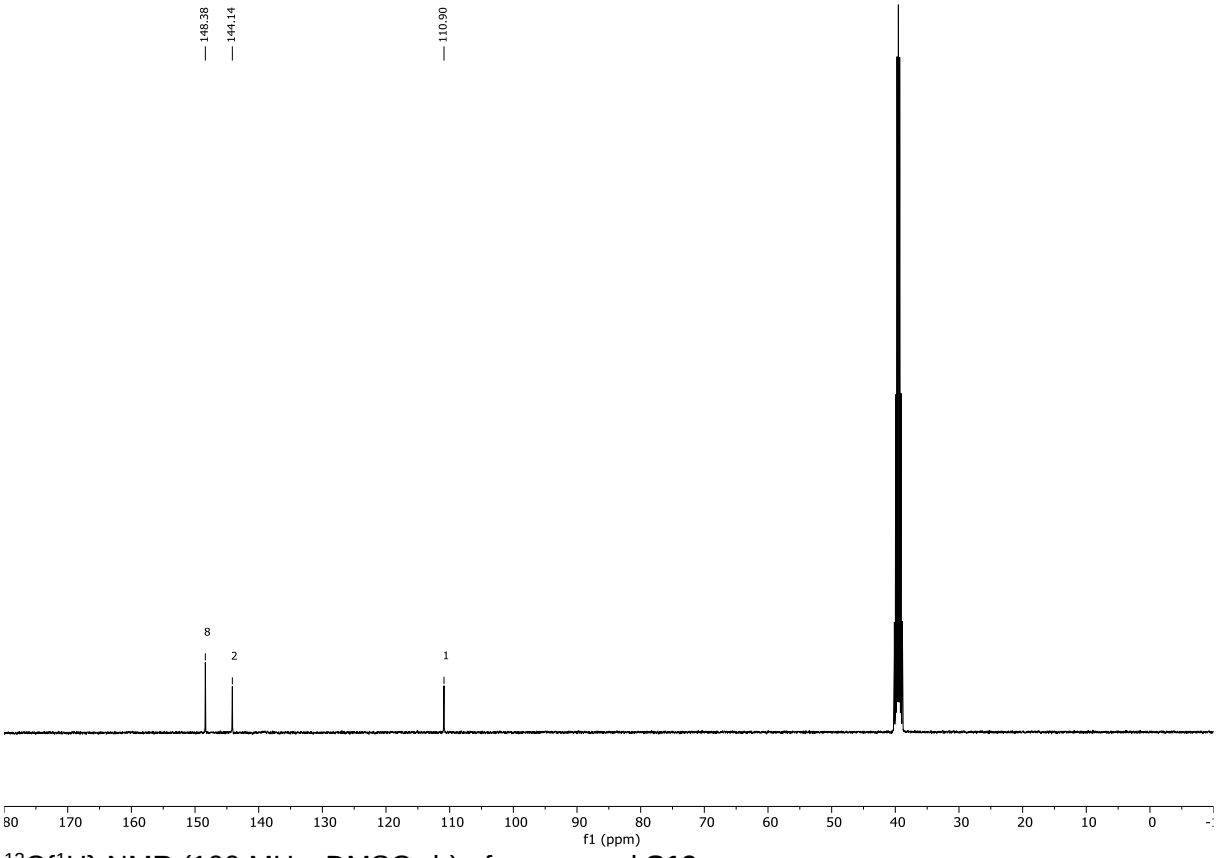
^{1}H-NMR (400 MHz, DMSO-d_6) of compound **S18**.



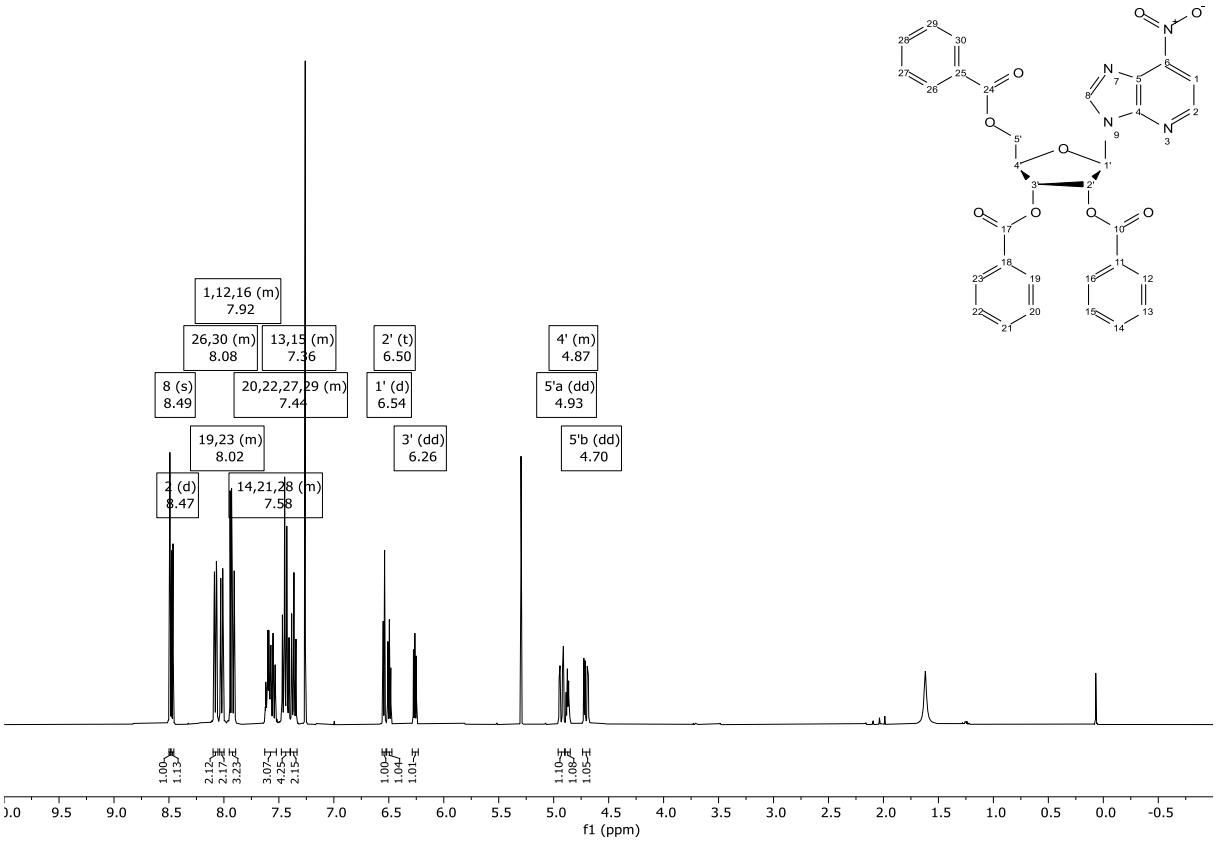
^{13}C{^{1}H}-NMR (100 MHz, DMSO-d_6) of compound **S18**.



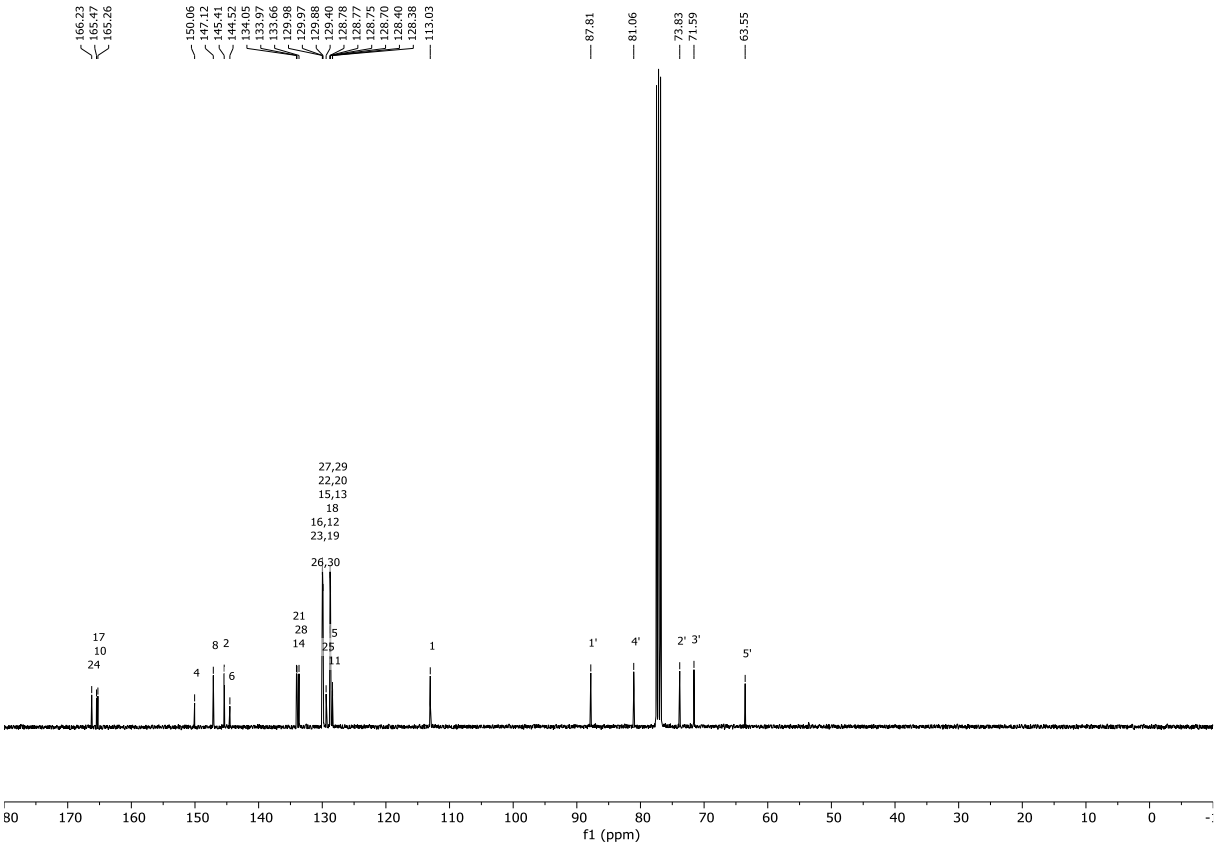
^{1}H-NMR (400 MHz, DMSO-d_6) of compound **S19**.



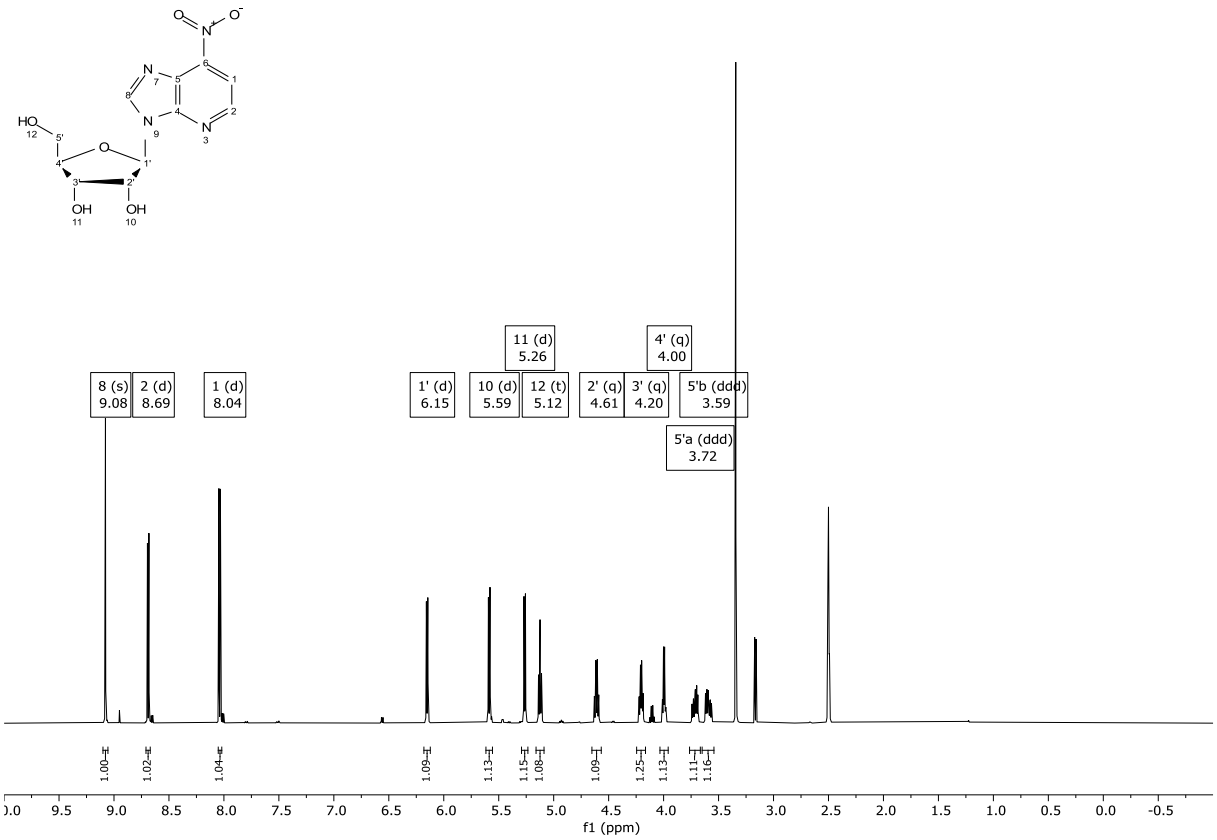
^{13}C{^{1}H}-NMR (100 MHz, DMSO-d_6) of compound **S19**.



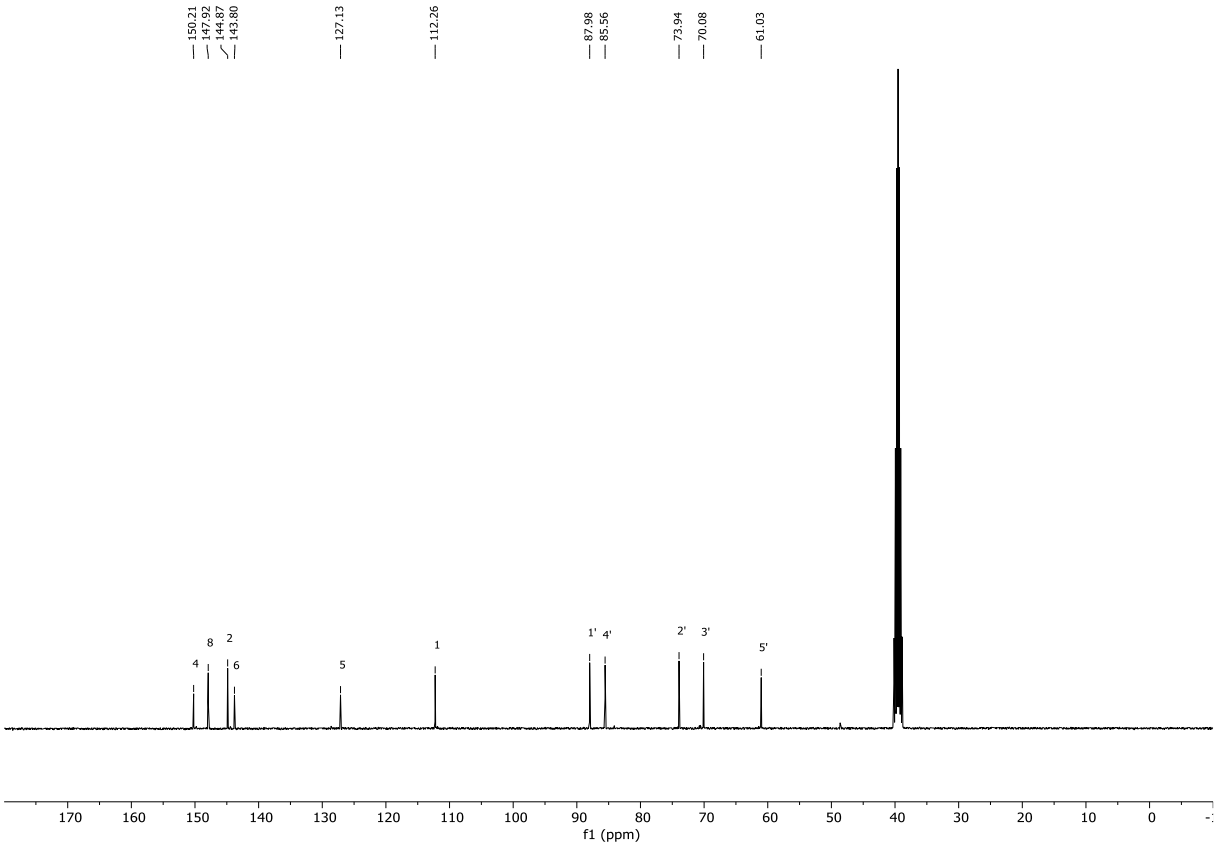
^{1}H-NMR (400 MHz, $CDCl_3$) of compound **S20**.



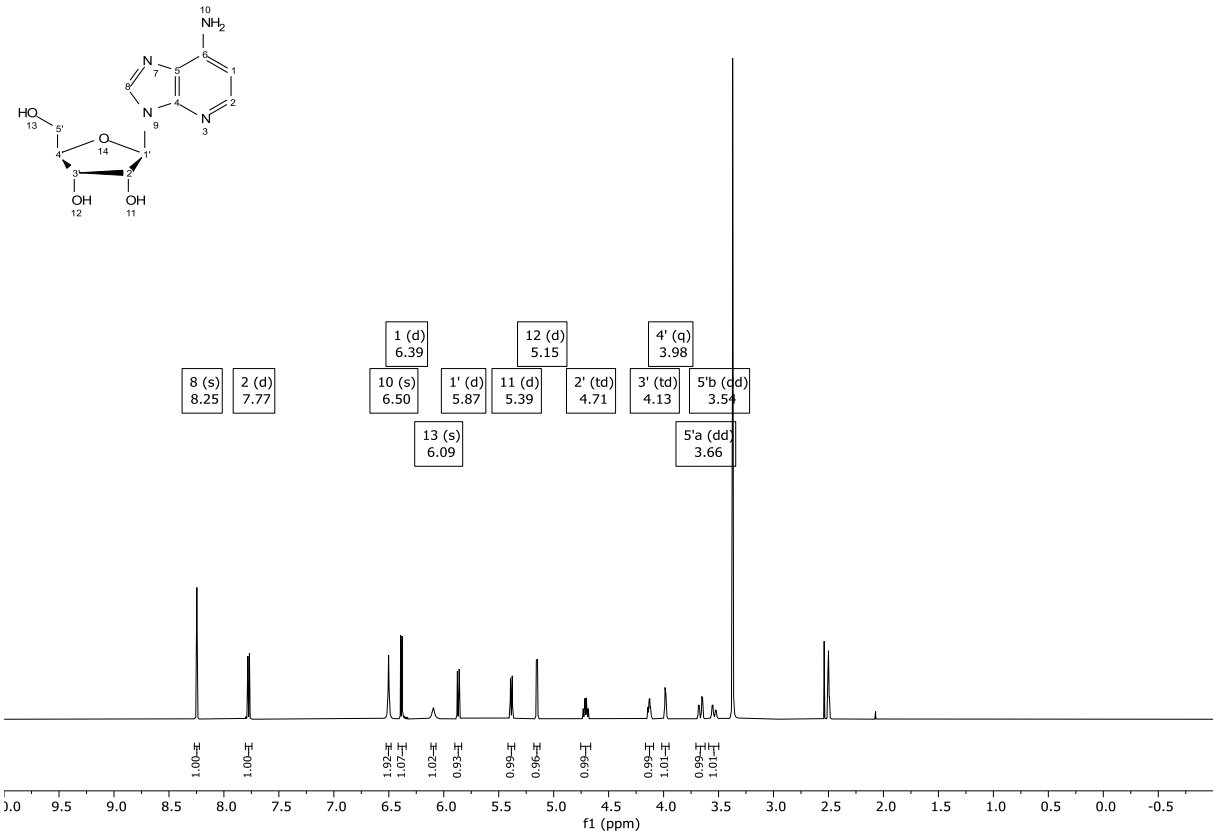
^{13}C{^{1}H}-NMR (100 MHz, $CDCl_3$) of compound **S20**.



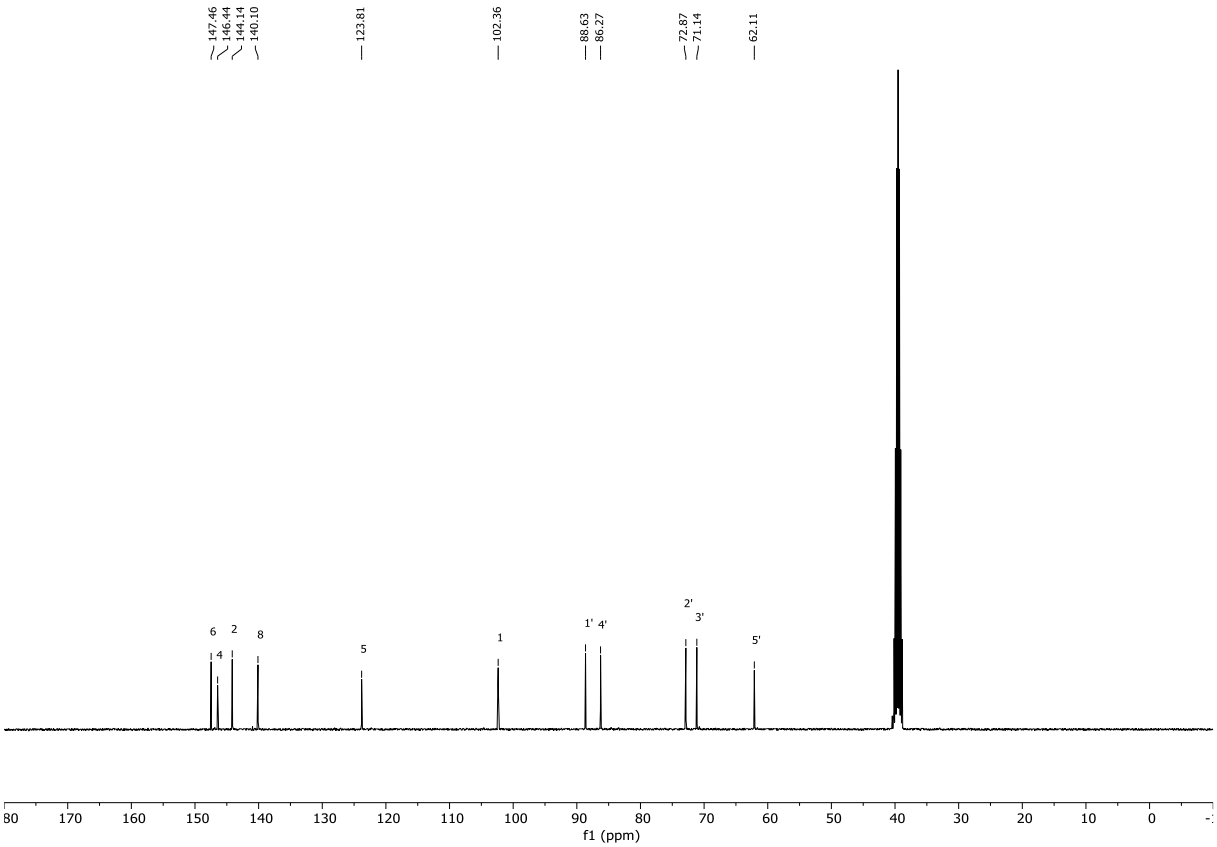
^{1}H-NMR (400 MHz, DMSO-d_6) of compound **S21**.



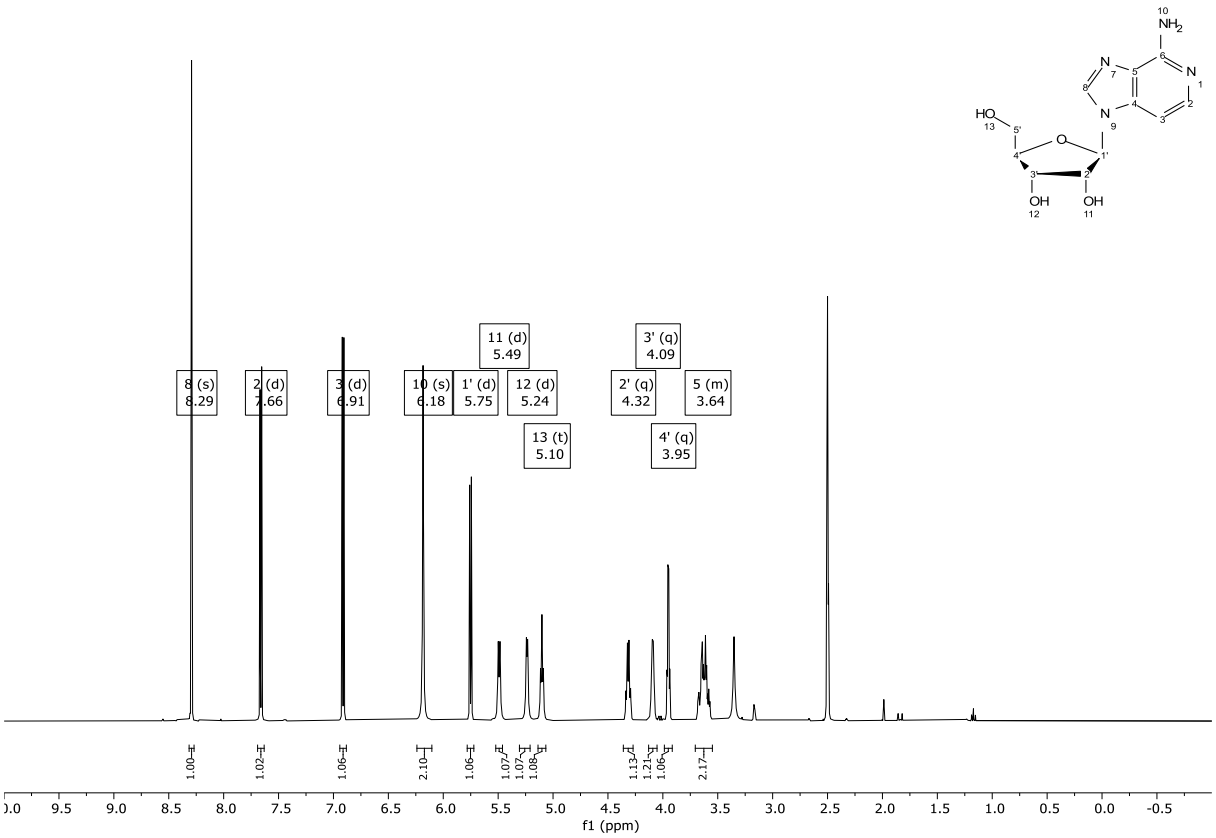
^{13}C{^{1}H}-NMR (100 MHz, DMSO-d_6) of compound **S21**.



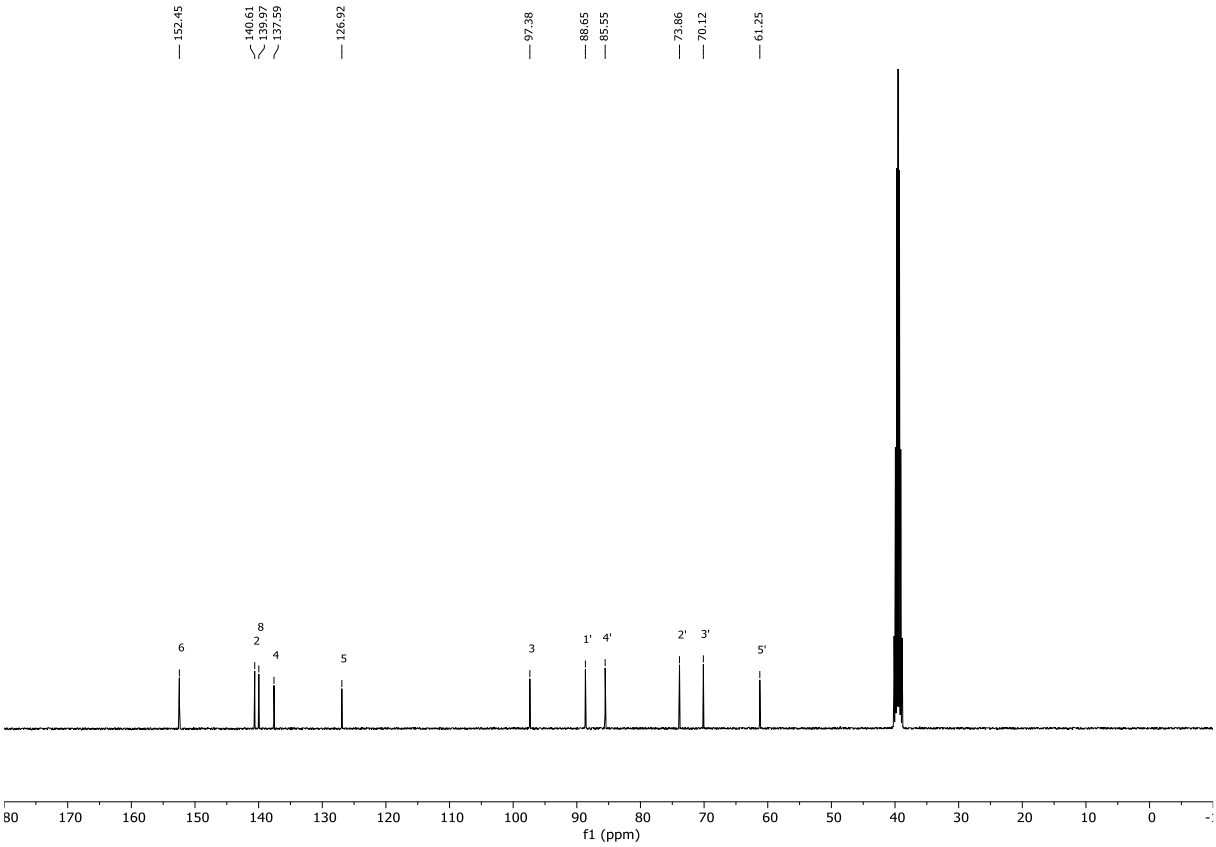
^{1}H-NMR (400 MHz, DMSO-d_6) of compound **S22**.



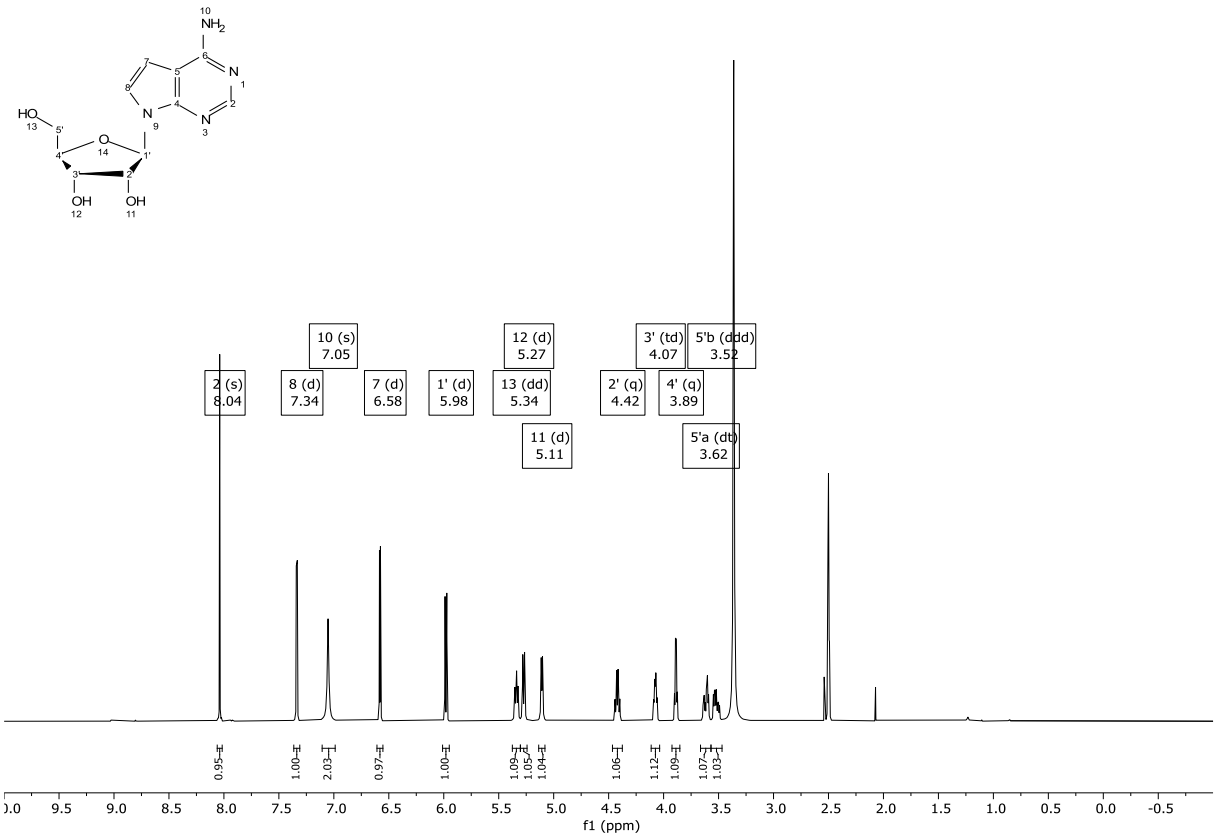
^{13}C{^{1}H}-NMR (100 MHz, DMSO-d_6) of compound **S22**.



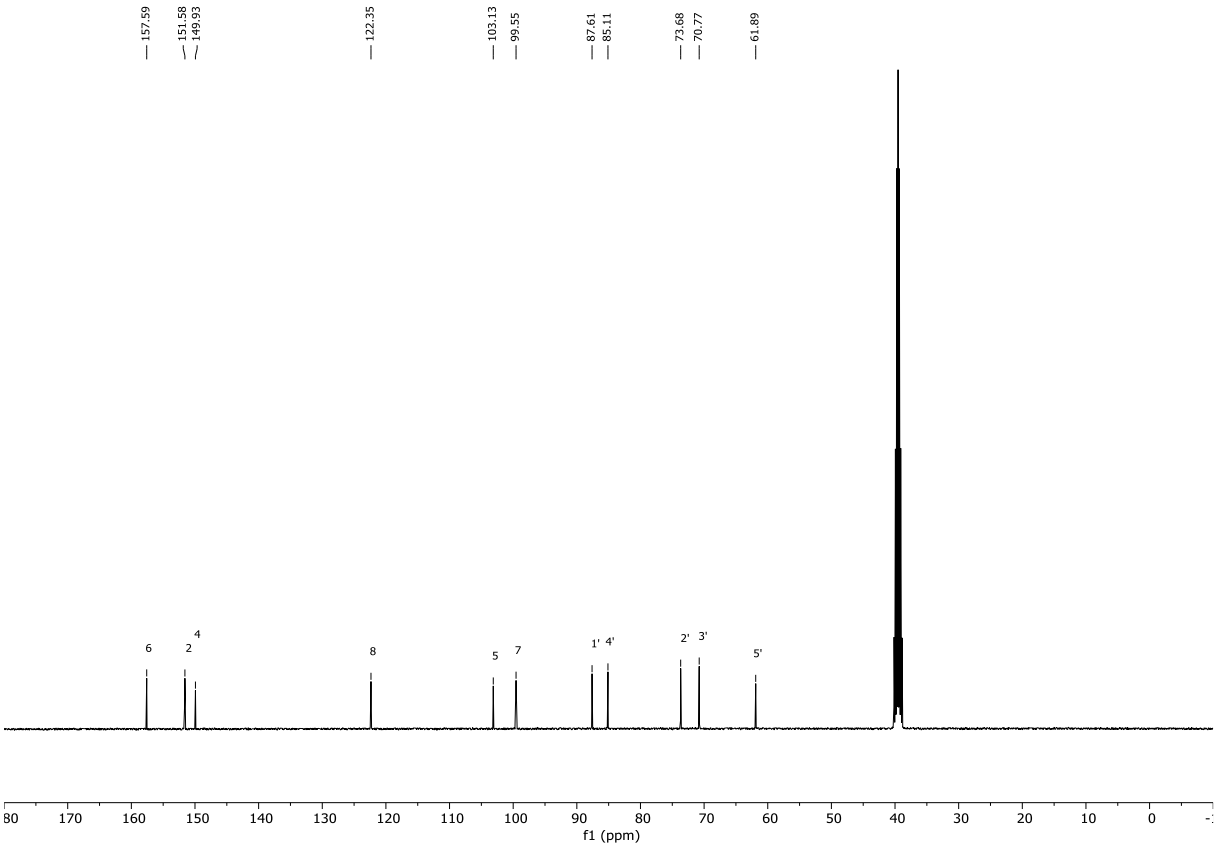
^{1}H-NMR (400 MHz, DMSO-d_6) of compound **S23**.



^{13}C{^{1}H}-NMR (100 MHz, DMSO-d_6) of compound **S23**.



^{1}H-NMR (400 MHz, DMSO-d_6) of compound **S24**.



^{13}C{^{1}H}-NMR (100 MHz, DMSO-d_6) of compound **S24**.

2. UV-Vis spectroscopy

UV-Vis spectra were recorded on a Cary Bio100 UV-Visible Spectrophotometer in cuvettes with path length d = 1 cm. For determination of the molar extinction coefficient ε, 80, 40, 20, 10, 5, and 2.5 µM samples in water were prepared by serial dilution. Their absorption spectra were recorded, and the absorbance A was plotted against the sample concentration c. From the slope of a linear fit ε was calculated according to Lambert-Beer's law. All measurements were performed as triplicates.

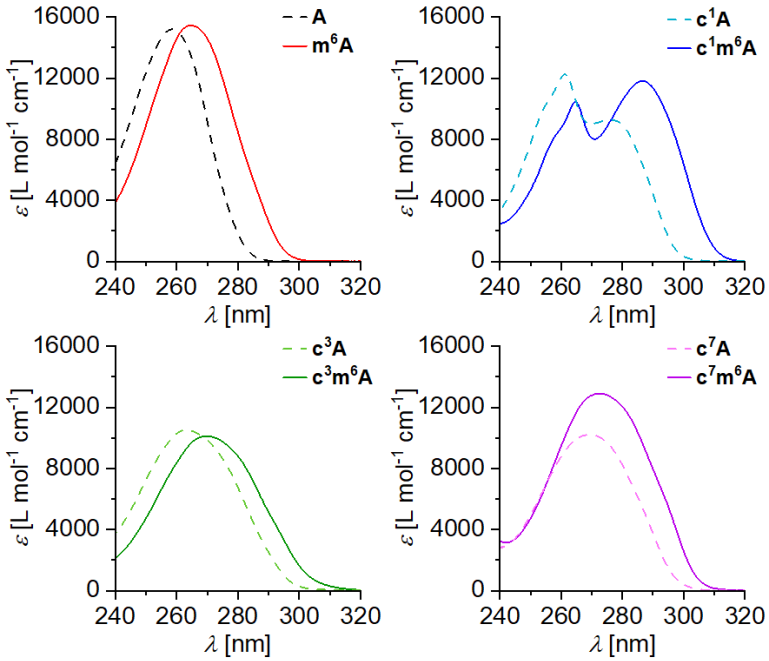


Figure S1. UV-Vis absorption spectra of N^6-methylated and unmethylated (deaza)-adenosine nucleosides. For all adenosine analogs, methylation of the amino group leads to a red-shifted absorption maximum. In contrast to all other variants, c^1A and c^1m^6A display two absorption maxima; the ratio of the two ε values is dependent on the methylation status.

Table S1. Absorption maxima λ_{max} and molar extinction coefficients at the absorption maximum (ε_{max}) and at 260 nm (ε_{260}). Values are given as mean ± s.e.m of triplicates.

Nucleoside	λ_{max} [nm]	ε_{max} [L·mol^{-1}·cm^{-1}]	ε_{260} [L·mol^{-1}·cm^{-1}]
A	258	15180 ± 90	15080 ± 80
m^6A	265	15450 ± 50	14580 ± 40
hm^6A[a)]	264	11530 ± 210	10970 ± 190
c^1A	261 276	12240 ± 130 9300 ± 110	12030 ± 140
c^1m^6A	265 287	10340 ± 200 11810 ± 220	8680 ± 180
c^3A	263	10510 ± 250	10320 ± 240
c^3m^6A	270	10110 ± 230	8510 ± 240
c^7A	269	10220 ± 200	8770 ± 60
c^7m^6A	273	12900 ± 120	9470 ± 110

[a)]Extinction coefficient determined by HPLC: hm^6A was synthesized by incubation of a solution of adenosine (100 µM) and formaldehyde (30 mM) at 60 °C for 4 h.[15] 50 µL of the sample were analyzed by RP-HPLC (Phenomenex Synergi Fusion 2 × 250 mm, flow rate 0.2 mL/min, at 25 °C. Solvent A: Water. Solvent B: MeCN. Gradient: 0–20% solvent B in 40 min). The molar extinction coefficient of hm^6A was calculated from the ratio of the peak areas of hm^6A peak and A peak.

3. Fluorescence spectroscopy

Fluorescence measurements were performed in Quartz SUPRASIL 10×2 mm High Precision Cell cuvettes (Hellma Analytics) at 25 °C, using a Jasco FP8300 spectrofluorometer. Fluorescence excitation and emission spectra were recorded at the respective emission and excitation maxima of the samples. Samples were prepared as 10 mM nucleoside stock solutions in DMSO and diluted with water to a final concentration of 10 µM.

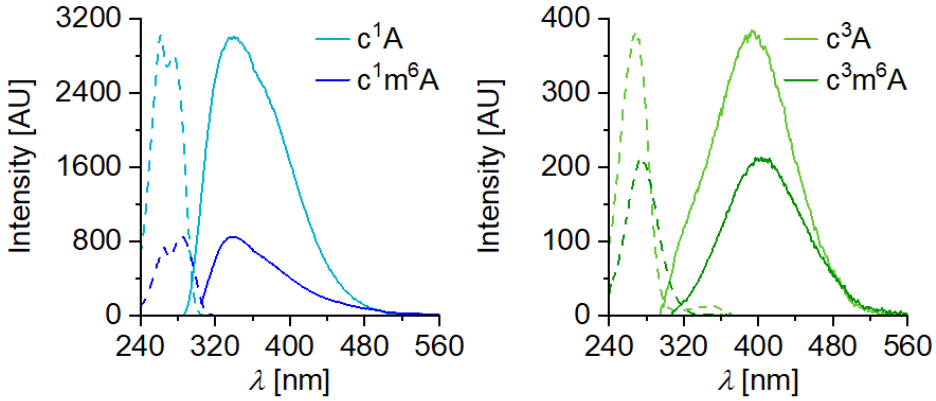


Figure S2. Fluorescence excitation and emission spectra of 1- and 3-deaza (*N*^{6}-methyl)adenosine derivatives. Spectra were recorded at the respective excitation and emission maxima. In contrast to A, m^{6}A and their 7-deaza-analogs, which are non-fluorescent, 1-deaza and 3-deaza nucleosides show fluorescent emission. Fluorescence intensities are strongly influenced by the methylation state of the nucleobase; methylation at *N*^{6} of c^{1}A or c^{3}A reduces the emission intensity by 70% and 45%, respectively.

4. DFT-based geometry optimizations

Molecule geometries were optimized on the level of density functional theory (DFT) with the Orca 5.0 software package[16] using the B3LYP functional[17-19] together with the def2-TZVPPD basis set.[20] To simplify calculations, the ribose was substituted for a methyl group at N9 of the nucleobases. ΔG^0 denotes the calculated standard Gibbs energy between *syn*- and *anti*-conformation of the N^6-methyl group.

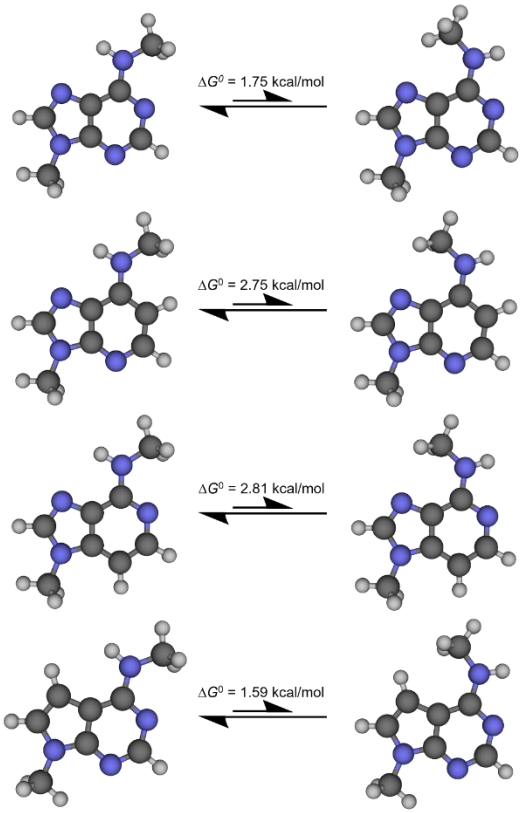


Figure S3. Geometry-optimized structures of the adenosine derivatives used in this study (top to bottom: m^6A, c^1m^6A, c^3m^6A, c^7m^6A), with the N^6-methyl group in either *syn*- (left column) or *anti*-conformation (right column) with respect to N1/C1. ΔG^0 denotes the calculated differences in standard Gibbs free energy between *syn*- and *anti*-conformation. To simplify calculations, the ribose was substituted for a methyl group at N9.

5. Oligonucleotide synthesis and labeling

5.1. RNA solid phase synthesis

RNA oligonucleotides **R1**–**R6** were synthesized by automated solid phase synthesis using a Pharmacia LKB Gene Assembler Plus synthesizer. The oligonucleotides were prepared on a CPG support (0.6 µmol scale) using 2'-*O*-TOM-protected N^6-acetyl-rA, N^4-acetyl-rC, N^2-acetyl-rG, and rU phosphoramidites (100 mM in MeCN). The modified phosphoramidites **4a**–**4d** were used as 100 mM solutions in MeCN (**4a**) or 1,2-dichloroethane (**4b**–**4d**), 5'-hexynyl phosphoramidite was used as a 100 mM solution in MeCN. Coupling was performed using 5-ethylthio-1*H*-tetrazole (ETT, 250 mM in MeCN) as activator, with 4 min coupling time for canonical phosphoramidites and 12 min coupling time for modified phosphoramidites. Detritylation was performed with 3% dichloroacetic acid (DCA) in dichloroethane (DCE). Capping solutions contained 4-dimethylaminopyridine (0.5 M) in MeCN for Cap A, and acetic anhydride/*sym*-collidine/MeCN (2:3:5) for Cap B. Oxidation was performed with iodine (10 mM) in acetonitrile/*sym*-collidine/water (10/1/5). The oligonucleotides were deprotected with 33% $MeNH_2$ in EtOH/water (1:1) at 37 °C for 5 h, followed by 1 M TBAF in THF for 12 h, and purified by denaturing polyacrylamide gel electrophoresis.

5.2. Fluorescent labeling

The 5'-hexynyl-modified oligonucleotides **R1**–**R6** were fluorescently labeled by Cu(I)-catalyzed azide-alkyne cycloaddition with 6-FAM azide. A solution of RNA oligonucleotide (10 nmol) in water (10 µL) was diluted with DMSO/*tert*-BuOH (3:1, 6 µL) and a solution of FAM-azide (50 mM in DMSO, 1.25 µL) was added. A solution of CuBr (100 mM in DMSO/*t*-BuOH 3:1, 1.25 µL) was pre-mixed with a solution of tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA, 100 mM in DMSO/*t*-BuOH 3:1, 2.5 µL) and added to the oligonucleotide solution. The sample was incubated under exclusion of light at 37 °C for 4 h before the reaction was stopped by addition of Stop Solution (80% (v/v) formamide, 50 mM EDTA, 89 mM boric acid, 89 mM TRIS, pH 8.0). The labeled oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis.

5.3. Anion exchange HPLC

The purity of the unlabeled and labeled RNA oligonucleotides was analyzed by anion-exchange HPLC (Dionex DNAPac PA200, 2 × 250 mm, flow rate 0.5 mL/min, at 60 °C. Solvent A: 25 mM Tris-HCl (pH 8.0), 6 M Urea. Solvent B: 25 mM Tris-HCl (pH 8.0), 6 M Urea, 0.5 M $NaClO_4$. Gradient: 0–48% solvent B in 12 CV).

5.4. ESI-MS

The identities of all purified oligonucleotides were confirmed by HR-ESI-MS (Bruker micrOTOF-Q III, negative ion mode, direct injection; monoisotopic masses of the oligonucleotides were obtained by charge deconvolution).

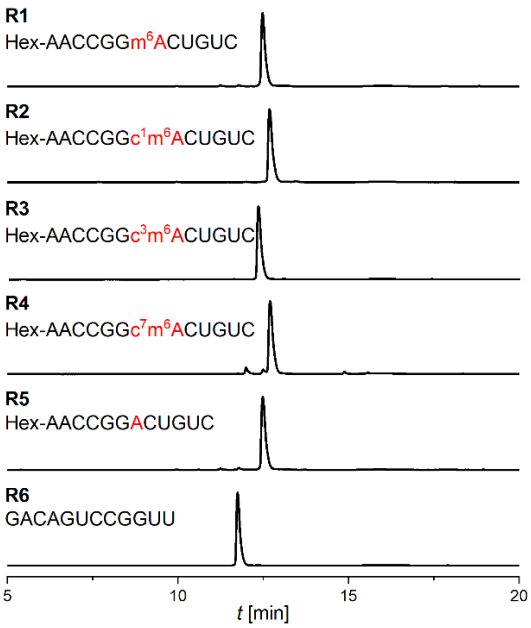


Figure S4. Anion exchange HPLC chromatograms (260 nm) of the RNA oligonucleotides **R1**–**R6**.

Table S2. Sum formulae, calculated and measured monoisotopic masses of the RNA oligonucleotides **R1**–**R6** and the 5'-FAM-labeled oligonucleotides **R1-FAM** – **R5-FAM**.

Oligonucleotide	Sum Formula	Mass calculated [Da]	Mass found [Da]
R1	$C_{121}H_{154}N_{46}O_{84}P_{12}$	3966.60443	3966.58816
R2	$C_{122}H_{155}N_{45}O_{84}P_{12}$	3965.60918	3965.61277
R3	$C_{122}H_{155}N_{45}O_{84}P_{12}$	3965.60918	3965.62937
R4	$C_{122}H_{156}N_{45}O_{84}P_{12}$	3965.60918	3965.57447
R5	$C_{120}H_{152}N_{46}O_{84}P_{12}$	3952.58933	3952.58980
R6	$C_{114}H_{142}N_{45}O_{83}P_{11}$	3809.53932	3809.53574
R1-FAM	$C_{145}H_{172}N_{50}O_{90}P_{12}$	4424.72706	4424.72774
R2-FAM	$C_{146}H_{173}N_{49}O_{90}P_{12}$	4423.73181	4423.73623
R3-FAM	$C_{146}H_{173}N_{49}O_{90}P_{12}$	4423.73181	4423.75013
R4-FAM	$C_{146}H_{173}N_{49}O_{90}P_{12}$	4423.73181	4423.72953
R5-FAM	$C_{144}H_{170}N_{50}O_{90}P_{12}$	4410.71196	4410.71356

6. Thermal UV-Vis melting curves

Thermal UV melting curves of 1, 2, 5, 10, and 20 µM duplexes in phosphate buffer (100 mM NaCl, 10 mM phosphate, pH 7.0) were recorded using an Agilent Cary UV-Vis Multicell Peltier spectrometer. Sample concentrations of 1, 2, and 5 µM were measured in 10 mm semi-micro quartz cuvettes, whereas 10 and 20 µM samples were measured in 1 mm quartz cuvettes. The samples were overlaid with silicon oil to prevent evaporation during heating. Following an initial annealing ramp from 25 to 90 °C, four temperature ramps with a heating/cooling rate of 0.5 °C/min between 10 and 90 °C were recorded at wavelengths of 250, 260, and 280 nm. To determine the melting temperatures, the obtained melting curves were fit to a two-state transition model with upper and lower baselines as previously reported.[21] Thermodynamic parameters were obtained by van't Hoff analysis.

Table S3. Melting temperatures (for 5 µM duplex) and thermodynamic data of the analyzed duplexes. For the melting temperatures, an error of ±0.5 °C is assumed. Melting temperature differences ΔT_m are given relative to the unmodified duplex/m^{6}A-modified duplex, respectively.

Duplex	Modification	$T_{m,\ 5\ \mu M}$ [°C]	ΔT_m [°C]	ΔH [kcal mol^{-1}]	ΔS [cal mol^{-1} K^{-1}]	$\Delta G_{37\ °C}$ [kcal mol^{-1}]
R1/R6	m^{6}A	63.4	-2.8 / 0	-97.4	-263	-15.8
R2/R6	c^{1}m^{6}A	53.9	-12.3 / -9.5	-93.4	-260	-12.9
R3/R6	c^{3}m^{6}A	58.7	-7.5 / -4.7	-94.7	-260	-14.2
R4/R6	c^{7}m^{6}A	62.9	-3.3 / -0.5	-95.5	-258	-15.5
R5/R6	A	66.2	0 / +2.8	-94.6	-253	-16.2

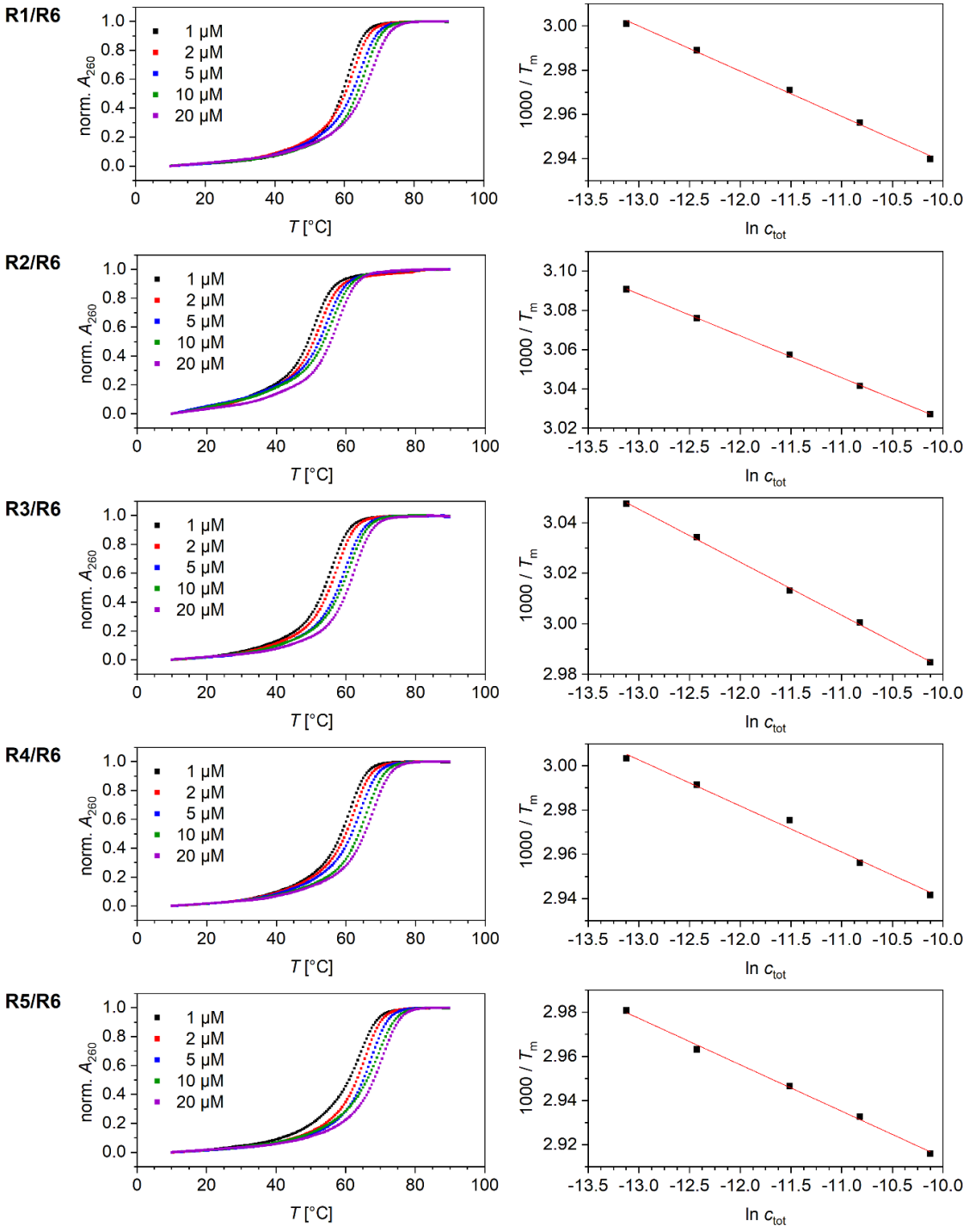


Figure S5. UV-Vis melting curves (260 nm) and van't Hoff plots of the investigated duplexes.

7. pK_a titrations

Samples containing 50 µM nucleoside in 30 mM Britton-Robinson buffer (30 mM AcOH, 30 mM H_3PO_4, 30 mM H_3BO_3) of respective pH were prepared at different pH values in the range between 2 and 10. UV absorption spectra of each sample were recorded in quartz cuvettes (path length: d = 1 cm) using an Agilent Cary Spectrophotometer (spectral bandwidth: 2 nm; averaging time: 20 ms; data interval: 0.5 nm) and blank-corrected by subtraction of absorption spectra of pure buffer in the same cuvettes. Sample absorbances at two different wavelengths, representing A_{HA} (absorbance of the neutral species) and A_{A^-} (absorbance of the anionic species), respectively, were plotted against the pH value. The pK_a value was obtained by fitting the data points according to the Henderson-Hasselbalch equation.

$$A = A_{HA} + \frac{A_{A^-} - A_{HA}}{1 + 10^{pK_a - pH}}$$

All data points were collected as triplicates, pK_a values are given as mean ± s.e.m.

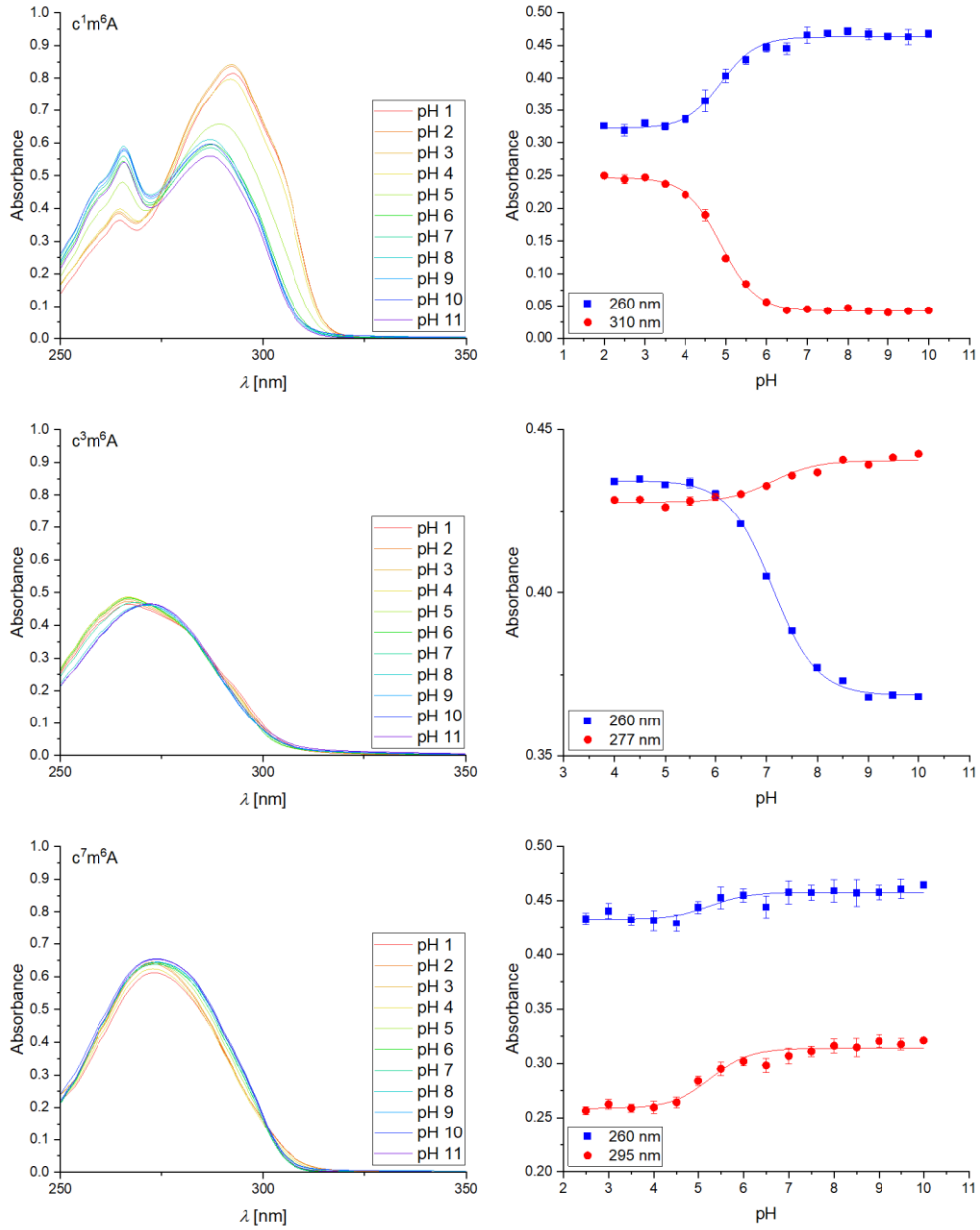


Figure S6. pH-dependent UV-Vis absorption spectra (left) and Henderson-Hasselbalch fits for pK_a determination (right) of c^1m^6A, c^3m^6A and c^7m^6A.

Table S4. pK_a values of c^1m^6A, c^3m^6A and c^7m^6A nucleosides. Values are given as mean ± s.e.m of triplicates.

Nucleoside	pK_a
c^1m^6A	4.88 ± 0.03
c^3m^6A	7.12 ± 0.03
c^7m^6A	5.27 ± 0.05

8. HPLC-MS-based demethylation assays

In a final volume of 10 µL, RNA oligonucleotide (20 µM) and demethylase (4 µM) were incubated in demethylation buffer (300 µM $Fe(NH_4)_2(SO_4)_2$, 300 µM α-ketoglutaric acid, 4 mM ascorbic acid, 100 mM KCl, 2 mM $MgCl_2$, 50 mM HEPES pH 7.0) at 25 °C for 2, 30, or 120 min. The reactions were stopped by snap-freezing in liquid nitrogen. The samples were diluted to a volume of 20 µL and digested with 1 U Nuclease P1 (Sigma Aldrich) and 2 U Shrimp Alkaline Phosphatase (rSAP, New England BioLabs) at 37 °C for 20 min. The reactions were stopped by snap-freezing in liquid nitrogen. The samples were diluted to a total volume of 70 µL and the residual denatured protein was removed by extraction with $CHCl_3$ (2 × 70 µL). The aqueous layer was concentrated under reduced pressure at 35 °C for 20 min to remove residual $CHCl_3$ and adjusted to a final sample volume of 60 µL containing 10 mM NH_4OAc, pH = 5.3.

The samples were analyzed by RP-HPLC (Phenomenex Synergi Fusion, 2 × 250 mm, flow rate 0.2 mL/min, at 25 °C. Solvent A: 10 mM NH_4OAc in H_2O, pH 5.3. Solvent B: MeCN. Gradient: 0–50% solvent B in 25 min.) coupled with ESI-MS (Bruker micrOTOF-Q III). All MS spectra were recorded in positive ion mode in the range *m/z* 50–2500 with the following settings: Capillary voltage 4.5 kV, end plate offset 500 V, nitrogen nebulizer pressure 1.4 bar, dry gas flow 9 L/min, dry temperature 200 °C; Funnel 1 RF was 80 Vpp and Funnel 2 RF 150 Vpp, Hexapole RF 150 Vpp; Quadrupole ion energy 5 eV, Quadrupole low mass 50 *m/z*; Collision energy 10 eV, collision RF 300 Vpp, transfer time 60 µs, pre pulse storage 7 µs. The mass spectrometer was calibrated with sodium formate prior to measurement of the samples. The spectra were recorded with the Compass Software (Bruker Daltonics) containing the OTOF Control 3.4 and Hystar 3.2 software for controlling the MS instrument and the HPLC. For data evaluation the Data Analysis software DA 4.2 (Bruker Daltonics) was used. UV Chromatograms were recorded at 260 nm. Peaks in the UV chromatogram were identified based on the corresponding extracted ion chromatograms (EICs): C: *m/z* = 244.09±0.01; U: *m/z* = 245.08±0.01, c^1A, c^3A, c^7A: *m/z* = 267.11±0.01; A: *m/z* = 268.10±0.01; c^1m^6A, c^3m^6A, c^7m^6A: *m/z* = 281.12±0.01; m^6A: *m/z* = 282.12±0.01; G: *m/z* = 284.10±0.01; c^7hm^6A: *m/z* = 297.12±0.01; hm^6A: *m/z* = 298.11±0.01; Hex-A: *m/z* = 428.13±0.01.

For quantitative analysis, the integrated areas under the UV signals were corrected for the extinction coefficient **ε** of the respective nucleoside. The relative fraction of each species was then calculated so that the sum of methylated, demethylated and (if observed) intermediate species accumulated to 100%.

Exemplary calculation (**R2** treated with ALKBH5 for 2 min, replicate 1):

Nucleoside	c^1m^6A	c^1A
Integrated Area (UV, 260 nm) *A*	$8.20 \cdot 10^{-3}$	$3.16 \cdot 10^{-2}$
ε_{260} [$L \cdot mol^{-1} \cdot cm^{-1}$]	8680	12030
Corrected Area: $A_{corr} = A / \varepsilon_{260}$	$9.45 \cdot 10^{-7}$	$2.63 \cdot 10^{-6}$
Rel. Fraction [%]: $x = 100 \cdot A_{corr} / \Sigma A_{corr}$	26.4	73.6

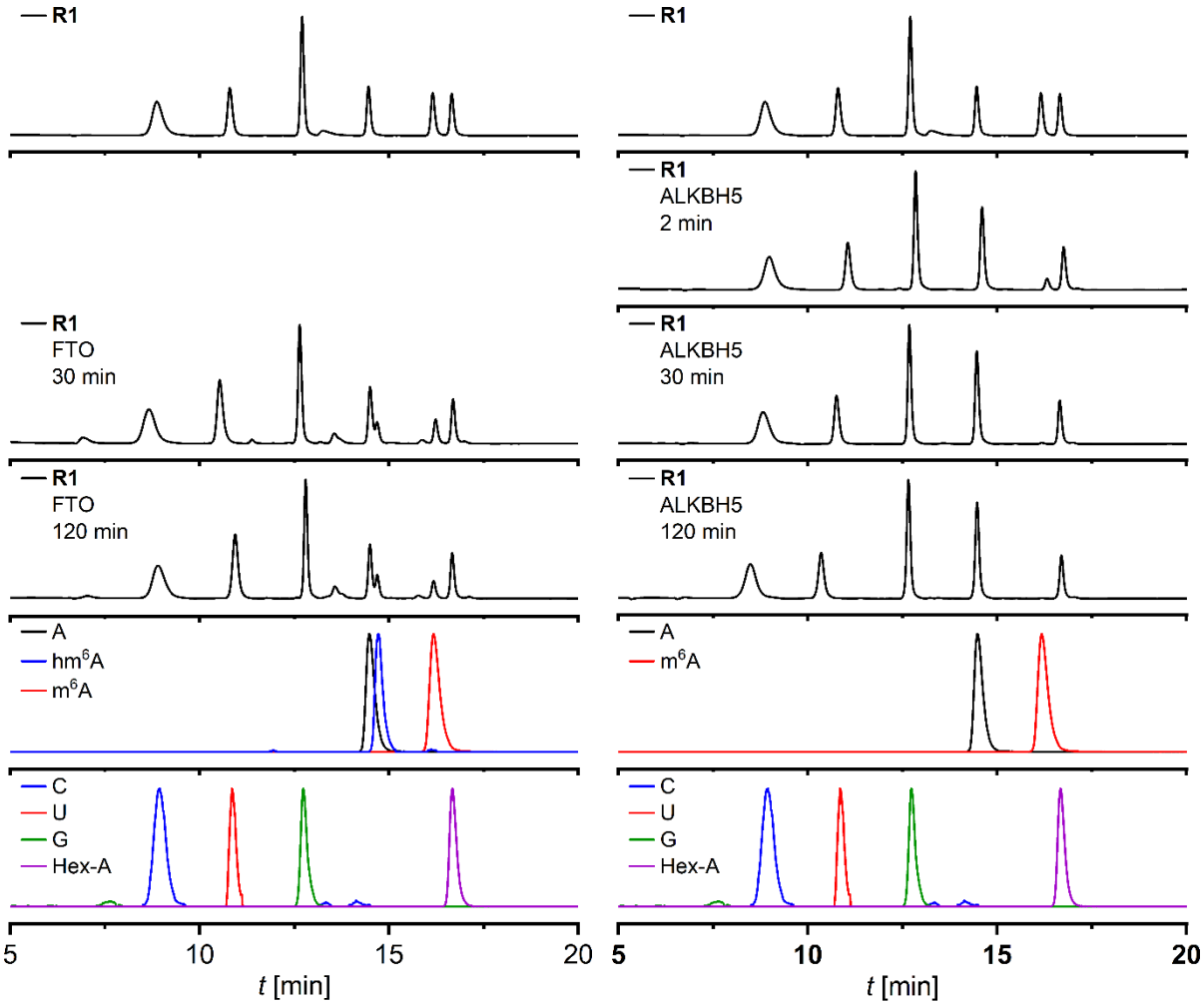


Figure S7. HPLC-MS analyses of digested **R1** oligonucleotide samples. The UV (260 nm) chromatograms of **R1** treated with FTO (left) for 0, 30, or 120 min or ALKBH5 (right) for 0, 2, 30, or 120 min are shown stacked on top of the nucleoside $[M+H]^+$ extracted ion chromatograms. Analyses were performed as duplicates; one replicate is shown.

Table S5. Quantitative analysis of the UV chromatograms shown in Figure S.

Sample	Nucleoside	Replicate 1	Replicate 2	mean ± s.e.m.
R1 FTO, 30 min	A	0.8%	4.9%	2.9±2.9%
	hm^6A	54.9%	47.1%	51.0±5.6%
	m^6A	44.3%	48.1%	46.2±2.7%
R1 FTO, 120 min	A	6.2%	2.7%	4.5±2.5%
	hm^6A	56.8%	59.0%	57.9±1.6%
	m^6A	37.0%	38.3%	37.7±0.9%
R1 ALKBH5, 2 min	A	74.5%	68.2%	71.3±4.5%
	m^6A	25.5%	31.8%	28.7±4.5%
R1 ALKBH5, 30 min	A	97.6%	96.6%	97.1±0.7%
	m^6A	2.4%	3.4%	2.9±0.7%
R1 ALKBH5, 120 min	A	98.5%	98.7%	98.6±0.2%
	m^6A	1.5%	1.3%	1.4±0.2%

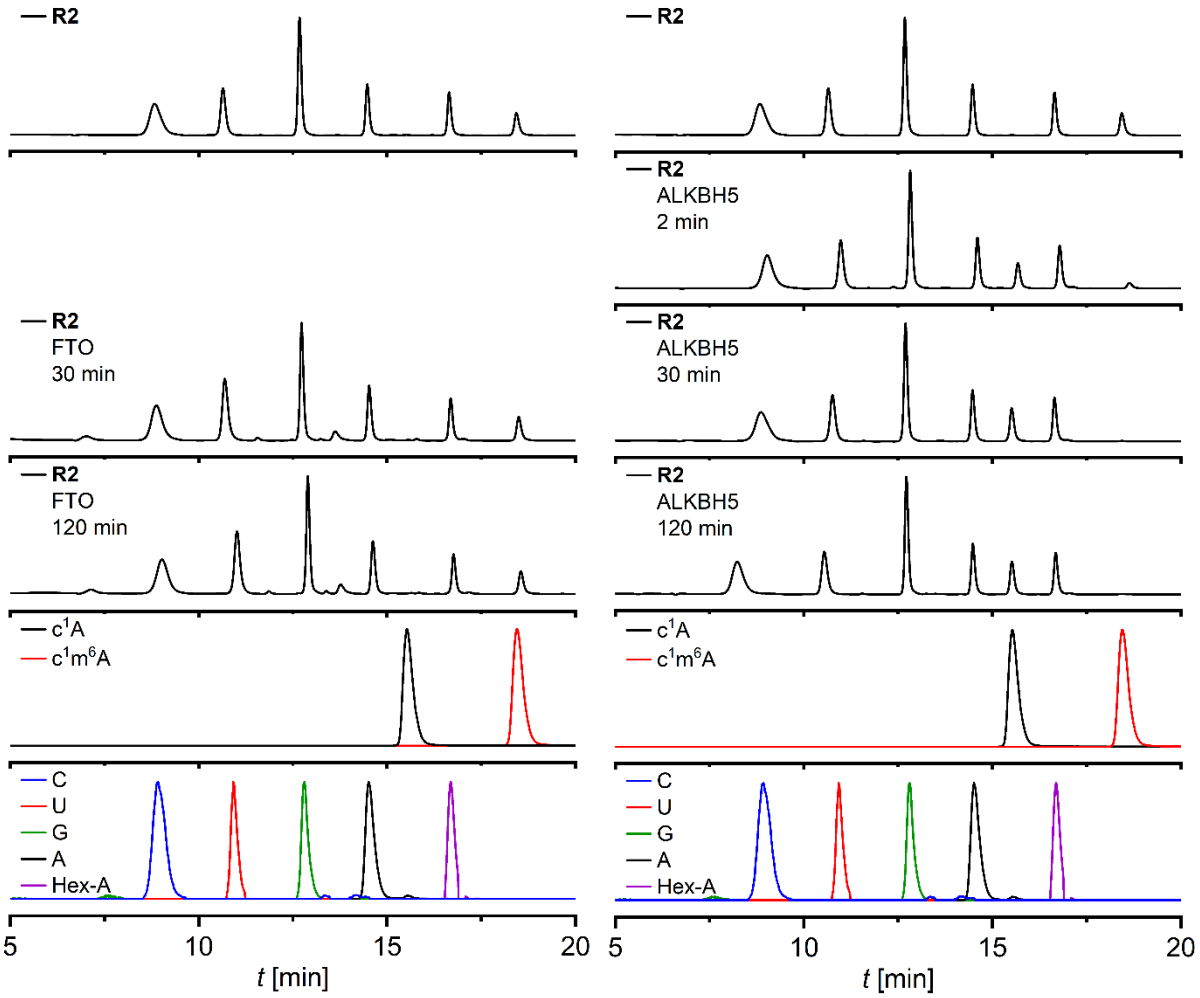


Figure S8. HPLC-MS analyses of digested **R2** oligonucleotide samples. The UV (260 nm) chromatograms of **R2** treated with FTO (left) for 0, 30, or 120 min or ALKBH5 (right) for 0, 2, 30, or 120 min are shown stacked on top of the nucleoside $[M+H]^+$ extracted ion chromatograms. Analyses were performed as duplicates; one replicate is shown.

Table S6. Quantitative analysis of the UV chromatograms shown in Figure S.

Sample	Nucleoside	Replicate 1	Replicate 2	mean ± s.e.m.
R2 FTO, 30 min	c^1A	2.2%	2.3%	2.3±0.1%
	c^1m^6A	97.8%	97.7%	97.7±0.1%
R2 FTO, 120 min	c^1A	1.7%	2.0%	1.8±0.2%
	c^1m^6A	98.3%	98.0%	98.2±0.2%
R2 ALKBH5, 2 min	c^1A	73.6%	70.2%	71.9±2.4%
	c^1m^6A	26.4%	29.8%	28.1±2.4%
R2 ALKBH5, 30 min	c^1A	97.8%	98.2%	98.0±0.3%
	c^1m^6A	2.2%	1.8%	2.0±0.3%
R2 ALKBH5, 120 min	c^1A	96.8%	97.4%	97.1±0.5%
	c^1m^6A	3.2%	2.6%	2.9±0.5%

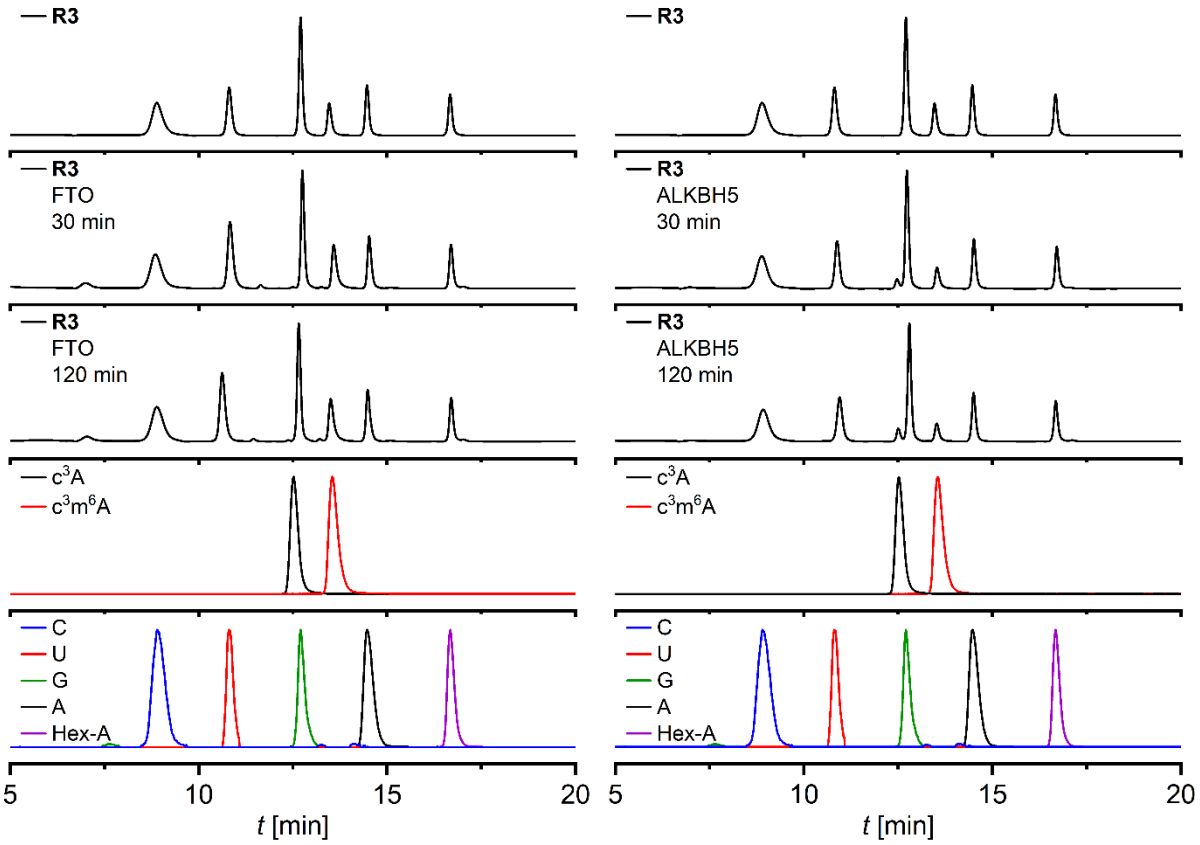


Figure S9. HPLC-MS analyses of digested **R3** oligonucleotide samples. The UV (260 nm) chromatograms of **R3** treated with FTO (left) for 0, 30, or 120 min or ALKBH5 (right) for 0, 30, or 120 min are shown stacked on top of the nucleoside $[M+H]^+$ extracted ion chromatograms. Analyses were performed as duplicates; one replicate is shown.

Table S7. Quantitative analysis of the UV chromatograms shown in Figure S.

Sample	Nucleoside	Replicate 1	Replicate 2	mean ± s.e.m.
R3 FTO, 30 min	c^3A	1.2%	2.1%	1.7±0.6%
	c^3m^6A	98.8%	97.9%	98.3±0.6%
R3 FTO, 120 min	c^3A	1.5%	2.1%	1.8±0.5%
	c^3m^6A	98.5%	97.9%	98.2±0.5%
R3 ALKBH5, 30 min	c^3A	24.5%	23.2%	23.8±0.9%
	c^3m^6A	75.5%	76.8%	76.2±0.9%
R3 ALKBH5, 120 min	c^3A	34.7%	33.8%	34.2±0.7%
	c^3m^6A	65.3%	66.2%	65.8±0.7%

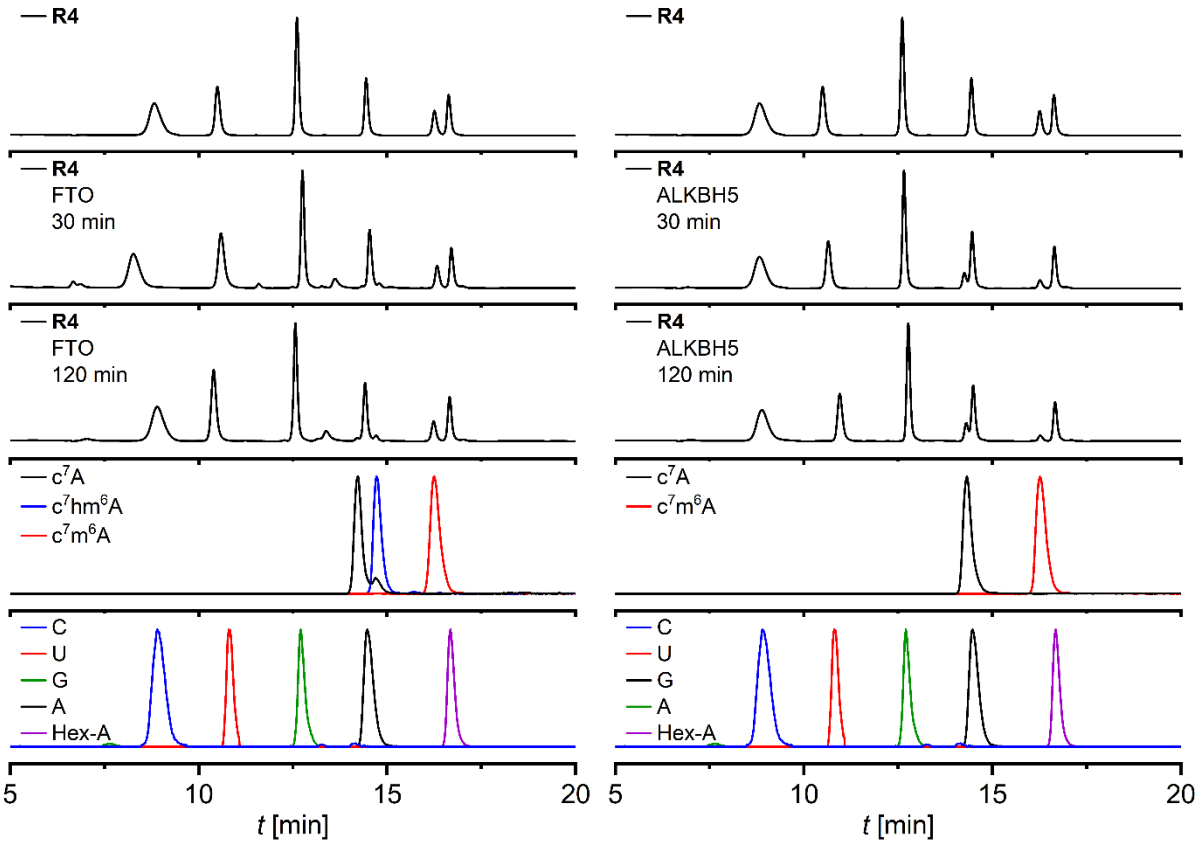


Figure S10. HPLC-MS analyses of digested **R4** oligonucleotide samples. The UV (260 nm) chromatograms of **R4** treated with FTO (left) for 0, 30, or 120 min or ALKBH5 (right) for 0, 30, or 120 min are shown stacked on top of the nucleoside $[M+H]^+$ extracted ion chromatograms. Analyses were performed as duplicates; one replicate is shown.

Table S8. Quantitative analysis of the UV chromatograms shown in Figure S1.

Sample	Nucleoside	Replicate 1	Replicate 2	mean ± s.e.m.
R4 FTO, 30 min	c^7A	5.4%	4.6%	5.0±0.6%
	c^7hm^6A	14.6%	15.8%	15.2±0.9%
	c^7m^6A	80.0%	79.6%	79.8±0.3%
R4 FTO, 120 min	c^7A	9.5%	10.2%	9.8±0.5%
	c^7hm^6A	23.2%	17.3%	20.3±4.2%
	c^7m^6A	67.3%	72.5%	69.9±3.7%
R4 ALKBH5, 30 min	c^7A	60.3%	59.8%	60.1±0.4%
	c^7m^6A	39.7%	40.2%	39.9±0.4%
R4 ALKBH5, 120 min	c^7A	72.1%	73.7%	72.9±1.2%
	c^7m^6A	27.9%	26.3%	27.1±1.2%

9. Electrophoretic mobility shift assays (EMSA)

Electrophoretic mobility shift assays to stabilize and visualize covalent RNA-ALKBH5 adducts were performed in analogy to the protocol previously described by Toh *et al.*[22]

In a final volume of 5 µL, 10 µM of 5'-FAM-labeled oligonucleotide were incubated with 2.5 µM of FTO in a buffer containing 50 mM HEPES pH = 7.0, 300 µM $Fe(NH_4)_2(SO_4)_2$, 300 µM α-ketoglutaric acid, 4 mM ascorbic acid, 100 mM KCl, 2 mM $MgCl_2$ and 19.4% (v/v) glycerol at 25 °C for 30 min. The samples were snap-frozen in liquid N_2 and thawed (3 freeze-thaw cycles) to denature FTO. 1 µL of sample was incubated in a final volume of 5 µL with 5 µM of ALKBH5 in a buffer containing 50 mM HEPES pH 7.0, 300 µM $Fe(NH_4)_2(SO_4)_2$, 300 µM α-ketoglutaric acid, 4 mM ascorbic acid, 100 mM KCl, 2 mM $MgCl_2$ and 19.4% (v/v) glycerol at 25 °C for 1 h. As reference samples, 1 µL of FTO-treated sample was incubated in absence of ALKBH5 under the same conditions. 1.5 µL of sample were mixed with 1.5 µL of loading buffer (60 mM TRIS·HCl pH 6.8, 10% (v/v) glycerol) and analyzed by gel electrophoresis. Oligonucleotide samples which were only treated with FTO were loaded as control samples.

Native polyacrylamide gel electrophoresis was performed using 2-layer gels (stacking layer 8 × 1 mm, 5% acrylamide/bisacrylamide 19:1, 125 mM TRIS·HCl pH 6.8; resolving layer 54 × 1 mm, 15% acrylamide/bisacrylamide 19:1, 375 mM TRIS·HCl pH 8.8) in TRIS-glycine buffer (25 mM TRIS·HCl pH 8.3, 192 mM glycine). Gels were resolved at 200 V, 4 °C for 1 h. Fluorescent bands were detected on a Bio-Rad ChemiDoc MP Imaging System using Blue Epi illumination and 530/28 nm detection filters.

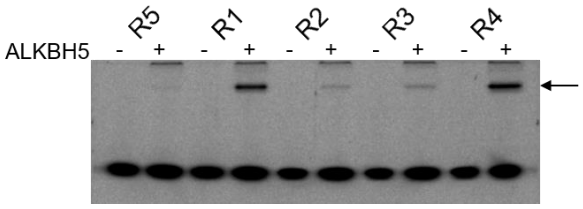


Figure S11. Electrophoretic mobility shift assays. Oligonucleotide samples, pre-treated with FTO were incubated with ALKBH5 in the presence of its native cofactors. Bands corresponding to ALKBH5-RNA adducts are marked with an arrow. Since only **R1** and **R4** are oxidized by FTO, only these two oligonucleotides can form stable covalent adducts with ALKBH5. The band intensity in these lanes is therefore increased compared to **R2**, **R3** and **R5**.

10. Competitive demethylation assay

In a final volume of 10 µL, RNA oligonucleotide **R1** (20 µM) and a competitor oligonucleotide (**R2**–**R5**) were incubated with FTO (4 µM) or ALKBH5 (2 µM) in demethylation buffer (300 µM $Fe(NH_4)_2(SO_4)_2$, 300 µM α-ketoglutaric acid, 4 mM ascorbic acid, 100 mM KCl, 2 mM $MgCl_2$, 50 mM HEPES pH 7.0) at 25 °C for 5 (ALKBH5) or 30 min (FTO). The reactions were stopped by snap-freezing in liquid nitrogen. The demethylase was denatured by heating of the sample to 95 °C for 5 min. The samples were diluted to a volume of 20 µL and digested with 0.2 U Snake Venom Phosphodiesterase (SVPD, Sigma Aldrich) and 2 U Shrimp Alkaline Phosphatase (rSAP, New England BioLabs) at 37 °C for 18 h. The enzymes were denatured by heating of the sample to 95 °C for 5 min. The samples were diluted to a total volume of 70 µL and the residual denatured protein was removed by extraction with $CHCl_3$ (2 × 70 µL). The aqueous layer was concentrated under reduced pressure at 35 °C for 20 min to remove residual $CHCl_3$ and adjusted to a final sample volume of 60 µL containing 10 mM NH_4OAc, pH 5.3.

The digested samples were analyzed and quantified as described in Section 8.

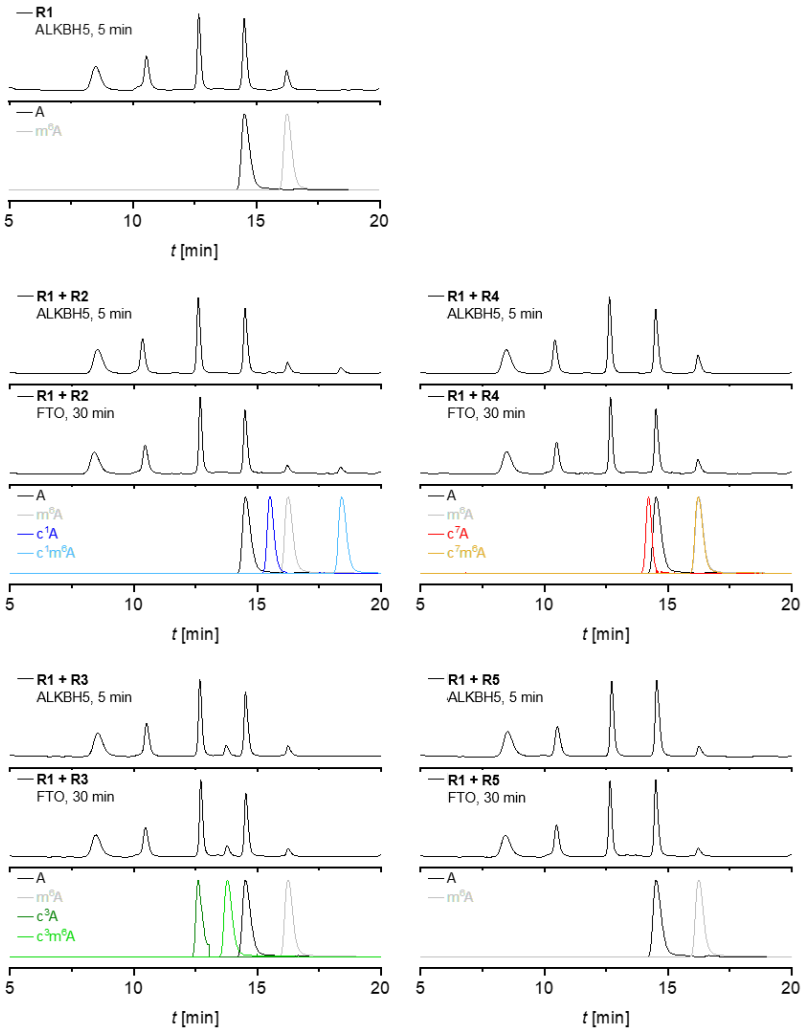


Figure S12. HPLC-MS analyses of the competitive demethylation assays. For each sample, the UV (260 nm) chromatograms are shown stacked on top of the nucleoside $[M+H]^+$ extracted ion chromatograms. Analyses were performed as duplicates; one replicate is shown.

Table S9. Quantitative analysis of the UV chromatograms shown in Figure S12.

Sample	Nucleoside	Replicate 1	Replicate 2	mean ± s.e.m.
R1 ALKBH5, 5 min	A	27.3%	29.6%	28.5±1.7%
	m^6A	72.7%	70.4%	71.5±1.7%
R1 + **R2** FTO, 30 min	A	35.8%	34.3%	35.1±1.1%
	m^6A	64.2%	65.7%	64.9±1.1%
	c^1A	0.0%	0.0%	0.0±0.0%
	c^1m^6A	100.0%	100.0%	100.0±0.0%
R1 + **R2** ALKBH5, 5 min	A	20.0%	15.4%	17.7±3.3%
	m^6A	80.0%	84.6%	82.3±3.3%
	c^1A	13.9%	14.0%	14.0±0.1%
	c^1m^6A	86.1%	86.0%	86.0±0.1%
R1 + **R3** FTO, 30 min	A	38.6%	38.4%	38.5±0.1%
	m^6A	61.4%	61.6%	61.5±0.1%
	c^3A	0.0%	0.0%	0.0±0.0%
	c^3m^6A	100.0%	100.0%	100.0±0.0%

R1 + **R3** ALKBH5, 5 min	A	17.4%	19.5%	18.4±1.6%
	m^6A	82.6%	80.5%	81.6±1.6%
	c^3A	0.0%	0.0%	0.0±0.0%
	c^3m^6A	100.0%	100.0%	100.0±0.0%
R1 + **R4** FTO, 30 min	A	39.8%	39.3%	39.5±0.4%
	m^6A	60.2%	60.7%	60.5±0.4%
	c^7A	10.1%	8.7%	9.4±1.0%
	c^7m^6A	89.9%	91.3%	90.6±1.0%
R1 + **R4** ALKBH5, 5 min	A	16.2%	22.3%	19.3±4.4%
	m^6A	83.8%	77.7%	80.7±4.4%
	c^7A	4.5%	10.5%	7.5±4.3%
	c^7m^6A	95.5%	89.5%	92.5.0±4.3%
R1 + **R5** FTO, 30 min	A	36.7%	34.0%	35.4±1.9%
	m^6A	63.3%	66.0%	64.6±1.9%
R1 + **R5** ALKBH5, 5 min	A	27.3%	29.6%	28.5±1.7%
	m^6A	72.7%	70.4%	71.5±1.7%

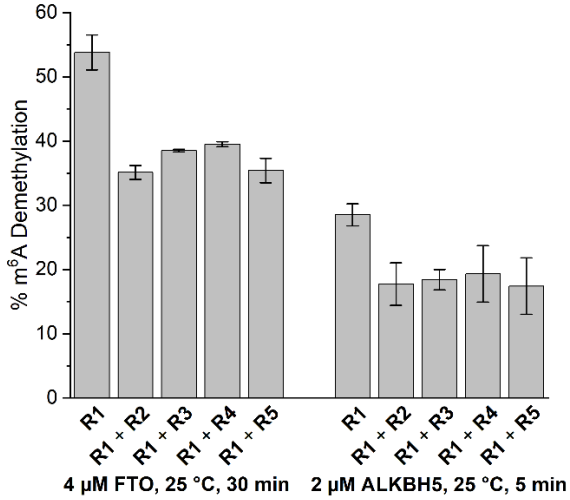


Figure S13. Competitive demethylation of m^6A in oligonucleotide **R1** by FTO or ALKBH5. Graphical representation of the data presented in Table S9. All data points were collected as duplicates and are represented as mean ± s.e.m.

11. Fluorescence anisotropy studies

Fluorescence anisotropy measurements were performed in Quartz SUPRASIL 10×2 and 3×3 mm High Precision Cell cuvettes (Hellma Analytics) at 25 °C, using a Jasco FP8300 spectrofluorometer with manually operated polarization filters. The excitation and emission wavelengths were set to 470 and 520 nm, respectively. Measurements were performed using 20 nM of 6-FAM-labeled RNA in anisotropy buffer (30 mM TRIS pH 7.5, 120 mM NaCl). The samples were incubated with increasing concentrations of protein for 5 min at ambient temperature prior to the respective measurements. Fluorescence anisotropy values *r* were calculated as

$$r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2 \cdot G \cdot I_{VH}}$$

where I_{VV} denotes emission intensity at vertical excitation/vertical emission and I_{VH} emission intensity at vertical excitation/horizontal emission. The grating factor *G*

$$G = \frac{I_{HV}}{I_{HH}}$$

is introduced as a correction factor to account for inherent unequal sensitivities of the spectrometer toward the different polarization planes.

K_D values were obtained from nonlinear fitting of the anisotropy data according to

$$r = r_f + (r_b - r_f) \cdot \frac{[L] + K_D + [R] + \sqrt{([L] + K_D + [R])^2 - 4 \cdot [L] \cdot [R]}}{2 \cdot [L]}$$

Here, r_f and r_b denote the respective anisotropy values of free ligand and bound complex, while [L] and [R] are the total ligand (RNA) and receptor (protein) concentrations in the sample.

12. Protein expression and purification

12.1 m^{6}A demethylases

The CDS of FTO (NM_001080432.3) and the truncated CDS encoding ALKBH5 (residues 66-292) (NM_017758.4) were cloned into a pET28a(+) vector[23] encoding a C-terminal His_6-tag or a pQE80 derivative vector encoding an N-terminal His_{10}-tag,[24] respectively. *Escherichia coli* (*E. coli*) Rosetta 2 (DE3) cells were transformed with the aforementioned plasmids and grown in LB medium (5 g/L yeast extract, 10 g/L NaCl, 10 g/L tryptone) supplemented with chloramphenicol (34 µg/mL) and either kanamycin (50 µg/mL; pET28a) or ampicillin (100 µg/mL; pQE80-derivative) at 37 °C until OD_{600} of 0.6 was reached. Overexpression of recombinant FTO-His_6 and His_{10}-ALKBH5$_{66-292}$ was induced overnight at 18 °C by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cultures were harvested by centrifuging at 4000 × *g* for 20 min at 4 °C and cells were washed once with phosphate-buffered saline (PBS). Cell pellets were resuspended in cold lysis buffer (30 mM phosphate buffer pH 7.3, 300 mM KCl, 10% (v/v) glycerol, 3 mM imidazole, 0.1 mM dithiothreitol (DTT)) and lyzed at 4 °C using an Emulsiflex-C3 (Avestin). The lysates were cleared by centrifuging at 20 000 × *g* for 30 min twice and clear lysates were incubated with cOmplete His-Tag Purification Resin (Roche). The resin was washed twice with wash buffer (30 mM phosphate buffer pH 7.3, 300 mM KCl, 10% (v/v) glycerol, 10 mM imidazole, 0.1 mM DTT) and proteins were eluted with elution buffer (30 mM phosphate buffer pH 7.3, 300 mM KCl, 10% (v/v) glycerol, 200 mM imidazole, 0.1 mM DTT). Fractions containing high protein content were pooled and dialyzed overnight against a buffer containing 30 mM phosphate buffer pH 7.3, 100 mM KCl, 50% (v/v) glycerol, 1 mM DTT and 0.1 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0. Purified proteins were examined using Coomassie Blue-stained SDS-polyacrylamide gels and concentrations were determined using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific).

12.2 YTH domain reader proteins

The sequences encoding the YTH domains of YTHDF2 (NM_016258.3; amino acids 380-579), YTHDC1 (NM_001031732.4; amino acids 344-509) and YTHDC2 (NM_022828.5; amino acids 1277-1430) were cloned into a pQE80-derived vector for expression of proteins with an N-terminal His_{10}-ZZ-tag. *E. coli* BL21-CodonPlus cells were transformed with the appropriate plasmids and grown in LB medium (5 g/L yeast extract, 10 g/L NaCl, 10 g/L tryptone) supplemented with 34 µg/mL chloramphenicol and 100 µg/mL ampicillin at 37 °C to an OD_{600} of 0.5. Overexpression of the recombinant YTH domains was induced overnight at 16 °C by addition of 0.3 mM IPTG. The cultures were centrifuged at 6000 × *g* for 15 min at 4 °C and cells were washed once with PBS. Cell pellets were resuspended in lysis buffer (30 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10% (v/v) glycerol) and lyzed using sonication (Branson Digital Sonifier; 45 % amplitude, 0.7 sec on, 0.3 sec off, 4x 30 pulses with 30 sec pause in between). The lysates were cleared by centrifuging

at 25 000 × *g* for 30 min and clear lysates were passed three times over cOmplete His-Tag Purification Resin (Roche) that had been pre-equilibrate in lysis buffer. The resin was then washed twice with wash buffer (30 mM Tris-HCl pH 7.4, 300 mM NaCl, 1 mM $MgCl_2$, 10% (v/v) glycerol, 15 mM imidazole) and proteins were eluted in 1 ml fractions using elution buffer (30 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 10% (v/v) glycerol, 500 mM imidazole pH 8.0). Fractions with high protein content were pooled and dialyzed overnight against a buffer containing 30 mM Tris-HCl pH 7.0, 120 mM NaCl, 2 mM $MgCl_2$, 20% (v/v) glycerol. Purified proteins were separated by SDS-polyacrylamide gel electrophoresis and detected using Coomassie Blue staining. Protein concentrations were determined using the Pierce Coomassie Plus Bradford assay kit (ThermoFisher Scientific).

13. References

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