Sydnone-modified nucleosides as versatile tools for bioorthogonal post-synthetic functionalization of antisense oligonucleotides

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Supporting Information

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I. Abbreviations in the manuscript and the supporting information

Aq., Aqueous
ASO, Antisense oligonucleotide
BCN, Bicyclo[6.1.0]nonyne
BTT, 5- (Benzylthio)-1 H -tetrazole
CNE, Cyanoethyl
COMBO, carboxymethylmonobenzocyclooctyne
CPG, Controlled pore glass
CuAAC, Copper(I)-catalyzed alkyne-azide cycloaddition
DBCO, Dibenzocyclooctyne
DIBO, Dibenzocyclooctynol
DIFO, Difluorocyclootyne
DIPEA, *N,N*-Diisopropylethylamine
DMF, Dimethylformamide
DMSO, Dimethylsulfoxide
DMT, Dimethoxytrityl
HRMS, High resolution mass spectrometry
iEDDA, Inverse electron-demand Diels-Alder cycloaddition
LC/MS, Liquid chromatography-tandem mass spectrometry
NMR, Nuclear magnetic resonance
rt, Room temperature
SPAAC, Strain-promoted alkyne-azide cycloaddition
SPS, Solid-phase synthesis
SPSAC, Strain-promoted sydnone-alkyne cycloaddition
TBTU, 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TCO, *trans*-cyclooctene
TEAA, Triethylammonium acetate
TLC, Thin layer chromatography
TMS (NMR), Tetramethylsilane
TMS, Trimethylsilyl

II. Chemical synthesis

All air-sensitive reactions were carried out under a slight positive pressure of argon. All solvents were purchased as reagent grade and used as received. Chemicals and reagents were purchased and used without further purification. Nucleosides **1**, **6**, **7** [36,37] and phenyl-sydnone acid **2** [38,39] were synthesized following reported procedures. TLC (Silica Gel 60 F254) were visualized under UV (254 nm). Purifications were carried out using silica gel chromatography (40-63 µm particle size) as the stationary phase. Reaction monitoring was performed by LC/MS chromatography using BEH-C18 columns either in buffer conditions (A = NH_4HCO_3 0.02 M buffer, B = acetonitrile) using a Waters Acquity UPLC QDA detector (positive or negative mode ESI) or in acidic conditions (A = HCOOH 0.025 M solution in water, B = HCOOH 0.025 M solution in acetonitrile/water) using a Waters Acquity UPLC H-Class SQD detector (positive or negative mode ESI). NMR spectra were recorded on Bruker AVIII-HD 400 or AVIII 500 spectrometers (Karlsruhe, Germany). All ^{13}C and ^{31}P spectra were recorded with proton decoupling during acquisition. Chemical shifts (in ppm) were determined relative to TMS at 0 ppm, or relative to the residual undeuterated solvent signal. Coupling constants in hertz (Hz) were measured from one-dimensional spectra. Various 2D technique experiments were used to establish the structures and to assign signals. Unless otherwise stated, all NMR experiments were performed in deuterated solvents (Eurisotop, Gif-sur-Yvette, France). High-resolution mass spectra (positive or negative mode ESI) were performed on a Thermo Scientific™ Orbitrap Velos Pro Mass Spectrometer coupled with a Thermo Scientific™ Vanquish chromatography.

Synthesis of phosphoramidite 4

5′-O-(4,4′-Dimethoxytrityl)-2′-O-(2((ethoxycarbamoyl)phenyl)-1,2,3-oxadiazol-3-ium-5-olate)-5-methyluridine (**3**).

N-(4-carboxyphenyl)sydnone **2** (200 mg, 0.97 mmol, 1 equiv), TBTU (342 mg, 1.07 mmol, 1.1 equiv), compound 1 (585 mg, 0.97 mmol, 1 equiv) were dissolved in DMF (12 mL). DIPEA (253 µL, 1.45 mmol, 1.5 equiv) was then added. The mixture was stirred at rt for 2 hours. After completion, the solvent was removed under vacuum and the crude product was purified by flash silica gel chromatography (eluted from 1.5 to 5% MeOH in CH_2Cl_2, 1% NH_4OH) to give **3** as an amorphous white solid (503 mg, 66% yield). ^1H NMR (500 MHz, DMSO-*d6*): δ 11.33 (s, 1H), 8.72 (t, J = 5.6 Hz, 1H), 8.07 (d, J = 8.9 Hz, 2H), 8.03 (d, J = 8.9 Hz, 2H), 7.84 (s, 1H), 7.46 (d, J = 1.0 Hz, 1H), 7.39 (dd, J = 7.6 Hz, J = 1.5 Hz, 2H), 7.32 (t, J = 7.6 Hz, 2H), 7.26 (d, J = 8.9 Hz, 4H), 7.22 - 7.25 (m, 1H), 6.90 (d, J = 8.9 Hz, 4H), 5.87 (d, J = 4.4 Hz, 1H), 5.21 (d, J = 6.6 Hz, 1H), 4.26 (dt, J = 6.6 Hz,

[36] J. A. Richardson, M. Gerowska, M. Shelbourne, D. French and T. Brown, *Chembiochem*, 2010, **11**, 2530.
[37] M. Shelbourne, T. Brown, A. H. El-Sagheer and T. Brown, *Chem. Commun.*, 2012, **48**, 11184.
[38] L. Plougastel, O. Koniev, S. Specklin, E. Decuypere, C. Créminon, D.-A. Buisson, A. Wagner, S. Kolodych and F. Taran, *Chem. Commun.*, 2014, **50**, 9376.
[39] C. Favre, L. de Cremoux, J. Badaut and F. Friscourt, *J. Org. Chem.*, 2018, **83**, 2058.

J = 5.5 Hz, 1H), 4.09 (dd, J = 5.5 Hz, J = 4.4 Hz, 1H), 3.97 - 4.02 (m, 1H), 3.78 (t, J = 5.6 Hz, 2H), 3.73 (s, 6H), 3.51 (q, J = 5.6 Hz, 2H), 3.27 (dd, J = 11.0 Hz, J = 4.3 Hz, 1H), 3.21 (dd, J = 11.0 Hz, J = 2.4 Hz, 1H), 1.38 (d, J = 1.0 Hz, 3H); ^{13}C NMR (125 MHz, DMSO-*d6*) δ 168.3, 164.8, 163.6, 158.1, 150.3, 144.5, 137.7, 136.0, 135.4, 135.3, 135.1, 129.6, 128.8, 127.8, 127.6, 126.7, 121.4, 113.2, 109.4, 95.0, 86.7, 85.8, 82.8, 80.8, 68.7, 68.3, 63.0, 55.0, 39.1, 11.6; HRMS (ESI) calcd for $C_{42}H_{41}N_5O_{11}$ $[M-H]^-$: 790.2729, found 790.2716.

5′-O-(4,4′-Dimethoxytrityl)-2′-O-(2((ethoxycarbamoyl)phenyl)-1,2,3-oxadiazol-3-ium-5-olate)-5-methyluridine-3′-O-(2-cyanoethyl-*N,N*-diisopropyl) phosphoramidite (**4**).

In a sealed tube under argon, was added compound **3** (495 mg, 0.63 mmol, 1 equiv), dissolved in CH_2Cl_2 (9.5 mL). 2-Cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphorodiamidite (377 mg, 1.25 mmol, 2 equiv) and diisopropylammonium tetrazolide (0.107 g, 0.63 mmol, 1 equiv) were then added and the solution was stirred at rt for 3 hours. After completion, the solvent was removed under vacuum and the crude product was purified by flash silica gel chromatography (eluted from 1 to 4% MeOH in CH_2Cl_2, 1% NH_4OH) to give **4** as a light yellow solid as a mixture of diastereoisomers (64/36) (460 mg, 74% yield). NMR analyses were performed on the mixture (64/36) of diastereoisomers: ^{1}H NMR (500 MHz, DMSO-*d6*): δ 11.32 (br s, 2H), 8.76 (t, J = 5.5 Hz, 1H), 8.73 (t, J = 5.5 Hz, 1H), 8.05 - 8.11 (m, 4H), 7.99 - 8.05 (m, 4H), 7.83 (s, 1H), 7.83 (s, 1H), 7.50 (d, J = 1.0 Hz, 1H), 7.49 (d, J = 0.9 Hz, 1H), 7.41 (br dd, J = 7.6 Hz, J = 1.5 Hz, 2H), 7.39 (dd, J = 7.6 Hz, J = 1.5 Hz, 2H), 7.29 - 7.35 (m, 4H), 7.22 - 7.29 (m, 10H), 6.87 - 6.92 (m, 8H), 5.89 (d, J = 5.0 Hz, 1H), 5.88 (d, J = 5.0 Hz, 1H), 4.45 (dt, J = 10.0 Hz, J = 5.0 Hz, 1H), 4.41 (dt, J = 11.0 Hz, J = 5.0 Hz, 1H), 4.25 (t, J = 5.0 Hz, 1H), 4.24 (t, J = 5.0 Hz, 1H), 4.14 - 4.18 (m, 1H), 4.07 - 4.13 (m, 1H), 3.75 - 3.88 (m, 4H), 3.74 (s, 6H), 3.73 - 3.82 (m, 2H), 3.73 (s, 6H), 3.56 - 3.69 (m, 2H), 3.46 - 3.61 (m, 4H), 3.37 - 3.54 (m, 4H), 3.21 – 3.37 (m, 4H), 2.78 (td, J = 6.0 Hz, J = 1.0 Hz, 2H), 2.59 (td, J = 6.0 Hz, J = 3.0 Hz, 2H), 1.36 (d, J = 1.0 Hz, 3H), 1.32 (d, J = 1.0 Hz, 3H), 1.10 (d, J = 7.0 Hz, 18H), 1.09 (d, J = 7.0 Hz, 9H), 0.96 (d, J = 6.9 Hz, 6H); ^{13}C NMR (125 MHz, DMSO-*d6*) δ 168.3, 164.7, 164.6, 163.5, 158.1, 150.4, 150.3, 144.4, 137.5, 136.0, 135.4, 135.2, 135.1, 135.0, 134.9, 129.7, 128.8, 127.8, 127.6, 126.8, 121.4, 118.9, 118.6, 113.2, 109.7, 109.5, 95.0, 87.0, 86.7, 86.0, 82.1, 82.0, 79.6, 70.3, 70.2, 68.5, 68.2, 62.7, 62.3, 58.5, 58.4, 57.9, 57.8, 55.0, 42.6, 42.5, 42.4, 39.1, 24.3, 24.2, 24.1, 19.8, 19.7, 19.6, 11.5; ^{31}P NMR (162 MHz, DMSO-*d6*) δ 149.2 (1P, s), 148.7 (1P, s); HRMS (ESI) mass calcd for $C_{51}H_{58}N_7O_{12}P$ $[M+H]^+$: 992.3954, found 992.3967.

Synthesis of phosphoramidite 9

5-{3-[4-(propynylcarbamoyl)phenyl]oxadiazol-3-ium-5-olate}-5′-O-(4,4′-dimethoxytrityl)-2′-deoxyuridine (**8**).

N-(4-carboxyphenyl)sydnone **2** (41 mg, 0.197 mmol, 1 equiv), TBTU (70 mg, 0.197 mmol, 1 equiv), compound **7** (115 mg, 0.197 mmol, 1 equiv) were dissolved in DMF (2.3 mL). DIPEA (51.3 µL, 0.295 mmol, 1.5 equiv) was then added. The mixture was stirred at rt overnight. The solvent was removed under vacuum and the crude product was purified by flash silica gel chromatography (eluted from 1 to 8% MeOH in CH_2Cl_2, 0.1% NH_4OH) to give **8** as a yellow solid (114 mg, 75% yield). ^{1}H NMR (500 MHz, DMSO-*d6*): δ 11.67 (br s, 1H), 9.20 (t, J = 5.3 Hz, 1H), 8.13 (d, J = 8.5 Hz, 2H), 8.06 (d, J = 8.5 Hz, 2H), 7.92 (s, 1H), 7.86 (s, 1H), 7.40 (d, J = 8.0 Hz, 2H), 7.25 - 7.33 (m, 6H),

7.20 (t, J = 8.0 Hz, 1H), 6.85 - 6.92 (m, 4H), 6.10 (t, J = 6.6 Hz), 5.32 (d, J = 4.5 Hz, 1H), 4.23 - 4.30 (m, 1H), 4.20 (dd, J = 18.0 Hz, J = 5.3 Hz, 1H), 4.16 (dd, J = 18.0 Hz, J = 5.3 Hz, 1H), 3.89 - 3.95 (m, 1H), 3.72 (s, 6H), 3.26 (dd, J = 10.5 Hz, J = 5.6 Hz, 1H), 3.08 (dd, J = 10.5 Hz, J = 2.4 Hz, 1H), 2.24 - 2.31 (m, 1H), 2.13 - 2.23 (m, 1H); ^{13}C NMR (125 MHz, DMSO-*d6*) δ 168.4, 164.3, 161.6, 158.1, 149.3, 144.8, 143.4, 137.1, 136.3, 135.7, 135.2, 129.8, 129.6, 129.1, 127.9, 127.5, 126.6, 121.6, 113.2, 98.2, 95.1, 89.2, 85.8, 85.8, 85.1, 74.3, 70.5, 63.8, 55.0, 39.3, 29.5; HRMS (ESI) calcd for $C_{42}H_{37}N_5O_{10}$ $[M+H]^+$: 772.2613, found 772.2626.

5-{3-[4-(propynylcarbamoyl)phenyl]oxadiazol-3-ium-5-olate}-5′-O-(4,4′-dimethoxytrityl)-2′-deoxyuridine-3′-O-(2-cyanoethyl-*N,N*-diisopropyl) phosphoramidite (**9**).

In a sealed tube under argon, was added compound **8** (81 mg, 0.105 mmol, 1 equiv), dissolved in CH_2Cl_2 (1.6 mL). 2-Cyanoethyl-*N,N,N′,N′*-tetraisopropyl-phosphorodiamidite (63 mg, 0.21 mmol, 2 equiv) and diisopropylammonium tetrazolide (18 mg, 0.105 mmol, 1 equiv) were then successively added. The solution was stirred at rt for 3 hours. The solvent was then removed under vacuum and the crude product was purified by flash silica gel chromatography (eluted from 1 to 5% MeOH in CH_2Cl_2, 1% NH_4OH) to give **9** as a light yellow solid as a mixture of diastereoisomers (51/49) (53 mg, 51% yield). NMR analyses were performed on the mixture (51/49) of diastereoisomers: ^{1}H NMR (500 MHz DMSO-*d6*) δ 11.66 (br s, 2H), 9.22 (t, J = 5.5 Hz, 1H), 9.21 (t, J = 5.5 Hz, 1H), 8.13 (2d, J = 8.5 Hz, 4H), 8.06 (2d, J = 8.5 Hz, 4H), 7.97 (s, 1H), 7.96 (s, 1H), 7.86 (s, 2H), 7.40 (d, J = 8 Hz, 2H), 7.39 (d, J = 8 Hz, 2H), 7.24 - 7.34 (m, 12H), 7.17 - 7.23 (m, 2H), 6.85 - 6.91 (m, 8H), 6.10 (t, J = 6.5 Hz, 1H), 6.08 (t, J = 6.5 Hz, 1H), 4.40 - 4.55 (m, 2H), 4.22 (d, J = 5.3 Hz, 2H), 4.19 (d, J = 5.5 Hz, 2H), 4.07 (q, J = 3.6 Hz, 1H), 3.98 - 4.04 (m, 1H), 3.72 (br s, 12H), 3.59 - 3.78 (m, 4H), 3.45 - 3.58 (m, 4H), 3.26 - 3.30 (m, 2H), 3.19 (dd, J = 10.5 Hz, J = 4 Hz, 1H), 3.13 (dd, J = 10.5 Hz, J = 3.6 Hz, 1H), 2.75 (t, J = 5.9 Hz, 2H), 2.64 (t, J = 5.9 Hz, 2H), 2.26 - 2.47 (m, 4H), 0.95 - 1.15 (m, 24H); ^{13}C NMR (125 MHz, DMSO-*d6*) δ 168.4, 164.2, 161.6, 158.1, 144.6, 137.0, 136.3, 135.5, 135.0, 129.6, 129.1, 127.8, 127.5, 126.6, 121.5, 118.9, 113.1, 98.3, 89.2, 85.9, 85.8, 85.2, 58.2, 54.9, 42.6, 29.4, 24.2, 19.8; ^{31}P NMR (162 MHz, DMSO-*d6*) δ 147.6 (1P, s), 147.2 (1P, s); MS (ESI) mass calcd for $C_{51}H_{54}N_7O_{11}P$ $[M-H]^-$: 970.4, found 970.5.

Synthesis of phosphoramidite 12

3-[4-(3-hydroxypropylcarbamoyl)phenyl]oxadiazol-3-ium-5-olate (**11**).

N-(4-carboxyphenyl)sydnone **2** (100 mg, 0.49 mmol, 1 equiv), TBTU (234 mg, 0.73 mmol, 1.5 equiv), 3-amino-1-propanol (55.7 µL, 0.73 mmol, 1.5 equiv) were dissolved in DMF (2 mL). DIPEA (0.169 mL, 0.97 mmol, 2 equiv) was then added. The mixture was stirred at rt for 20 hours. The solvent was removed under vacuum and the crude compound was purified by an inverse phase purification under ammonium bicarbonate buffer conditions (0 to 17% of CH_3CN) to give **11** after freeze-drying as a yellow solid (95 mg, 74% yield). ^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.71 (br t, J = 5.0 Hz, 1H), 8.11 (d, J = 8.0 Hz, 2H), 8.05 (d, J = 8.0 Hz, 2H), 7.85 (br s, 1H), 4.48 (t, J = 5.0 Hz, 1H), 3.48 (td, J = 6.5 Hz, J = 5.0 Hz, 2H), 3.35 (td, J = 6.5 Hz, J = 5.5 Hz, 2H), 1.70 (quin, J = 6.5 Hz, 2H); ^{13}C NMR (125 MHz, DMSO-*d6*) δ 168.4, 164.6, 137.9, 136.1, 128.9, 121.5, 95.1, 58.5, 36.8, 32.3; HRMS (ESI) mass calcd for $C_{12}H_{13}N_3O_4$ $[M+H]^+$: 264.0979, found 264.0978.

5′-O-(4,4′-Dimethoxytrityl)-3′-[(diisopropylamino)-3-[4-(3-

propoxycarbamoyl)phenyl]oxadiazol-3-ium-5-olate]phosphanyl]oxy-thymidine (**12**).

The first step was carried out according to Van Boom protocol.[40] In a dried sealed tube equipped with a magnetic stir bar and a septum, protected thymidine **10** (100 mg, 0.18 mmol, 1 equiv) was dissolved under argon in anhydrous 1,4-dioxane (0.92 mL). To this solution, Et_3N (40 µL, 0.28 mmol, 1.5 equiv) and bis(*N,N*-diisopropylamino) chlorophosphine (59 mg, 0.22 mmol, 1.2 equiv) were added. The reaction mixture was stirred under argon at rt for 30 minutes. The reaction was monitored by ^{31}P NMR. Removal of the $Et_3N.HCl$ salts by filtration and concentration of the filtrate in vacuo afforded the crude phosphorodiamidite that was used in the next step without purification. Phosphorodiamidite intermediate (118 mg, 0.15 mmol, 1.4 equiv) was dissolved in CH_2Cl_2 (1.25 mL). Compound **11** (29 mg, 0.11 mmol, 1 equiv) and a 0.25 M solution of 5-ethylthiotetrazole in CH_3CN (0.43 mL, 0.11 mmol, 1 equiv) were successively added. The reaction was stirred at rt for 30 minutes and the solvent was removed in vacuo. The crude compound was purified by an inverse phase purification under ammonium bicarbonate buffer conditions (60 to 85% of CH_3CN) to give two diastereoisomers of **12** after freeze-drying (without delay). Both diastereoisomers were combined to give **12** as an amorphous white solid as a mixture of diastereoisomers (55/45) (48 mg, 49% yield). ^1H NMR (500 MHz, DMSO-*d6*) δ 11.33 (br s, 2H), 8.72 (t, J = 5.5 Hz, 1H), 8.69 (t, J = 5.5 Hz, 1H), 8.09 (d, J = 8.1 Hz, 2H), 8.08 (d, J = 8.2 Hz, 2H), 8.03 (d, J = 8.2 Hz, 4H), 7.85 (s, 1H), 7.84 (s, 1H), 7.54 (s, 1H), 7.52 (s, 1H), 7.38 (d, J = 7.6 Hz, 4H), 7.29 (t, J = 7.6 Hz, 4H), 7.20 - 7.27 (m, 10H), 6.85 - 6.90 (m, 8H), 6.2 (t, J = 6.5 Hz, 1H), 6.19 (t, J = 6.5 Hz, 1H), 4.48 - 4.58 (m, 2H), 4.04 - 4.07 (m, 1H), 3.97 - 4.01 (m, 1H), 3.72 (2s, 12H), 3.44 - 3.68 (m, 8H), 3.21 – 3.41 (m, 8H), 2.22 - 2.43 (m, 4H), 1.82 (quin, J = 6.7 Hz, 2H), 1.73 (quin, J = 6.7 Hz, 2H), 1.49 (br s, 3H), 1.46 (br s, 3H), 0.95 – 1.14 (m, 24H); ^{13}C NMR (125 MHz, DMSO-*d6*) δ 168.4, 164.6, 164.5, 163.6, 158.2, 150.3, 144.6, 137.8, 136.1, 135.8, 135.6, 135.3, 135.2, 135.1, 129.7, 128.9, 127.8, 127.7, 127.6, 126.8, 121.4, 113.2, 109.7, 109.6, 85.9, 84.6, 84.2, 83.9, 72.9, 72.8, 72.6, 72.5, 63.4, 63.2, 60.9, 60.8, 60.7, 55.0, 42.5, 42.4, 38.4, 36.5, 30.7, 24.4, 24.3, 24.2, 11.7; ^{31}P NMR (162 MHz, DMSO-*d6*) δ 146.5 (1P, s), 146.3 (1P, s); HRMS (ESI) mass calcd for $C_{49}H_{57}N_6O_{11}P$ $[M-H]^-$: 935.3750, found 935.3723.

III. Antisense oligonucleotide synthesis and characterization

1. Antisense oligonucleotide synthesis

ASO syntheses were carried out by Eurogentec on a K&A H-8 SE DNA/RNA synthesizer. Phosphoramidites and reagents were purchased from either Glen Research, Merck or Biosolve Chimie. The synthesis of ASOs was performed at a 1 µM scale using a CUTAG universal CPG support (1000 Å) from Merck. Phosphoramidite building blocks were incorporated according to the following specifications: 2'- MOE amidites (A(Bz), G(iBu), and 5meC(Bz); DNA amidites (A(Bz), G(dmf), and 5meC(Bz)).

Standard coupling conditions were applied using 0.25 M benzylthiotetrazole (BTT) in acetonitrile as the activator, with coupling times of 1 minute for DNA, 4 minutes for MOE,

[40] J. E. Marugg, A. Burik, M. Tromp, G. A. van der Marel and J. H. van Boom, *Tetrahedron Lett.*, 1986, **27**, 2271.

and 15 minutes for sydnone-modified amidites. An exception was made for the P-Syd-ASO synthesis, where tetrazole (0.45 M in acetonitrile) was utilized as the activator.

Deprotection and cleavage from the solid support were conducted in a two-step process: initial treatment with diethylamine (10% in acetonitrile), followed by concentrated aqueous ammonia (NH_4OH 28%) for 24 hours at room temperature. The oligonucleotides were purified in DMT-on mode. The DMT group was subsequently cleaved during the purification process using a 2% trifluoroacetic acid (TFA) solution.

After cleavage, the oligonucleotides were purified on a semi-preparative reversed-phase HPLC system (X-Bridge-BEH C18-OBD (10 mm x 50 mm, 130 Å, 5 µm), A =TEAA 0.1 M buffer, B = acetonitrile, C =TFA 2%, D = H_2O). The purified oligonucleotide strands were quantified photometrically using a Spectra max ABS plus spectrometer. High-resolution mass spectra (positive or negative mode ESI) were performed on a Thermo Scientific™ Orbitrap Velos Pro Mass Spectrometer coupled with a Thermo Scientific™ Vanquish chromatography.

2. AF546-ASOs synthesis

AF546-DBCO was purchased from JENA BIOSCIENCE.
65-85 µM solution of Syd-ASOs (1 eq) in 0.1 M phosphate buffer pH 7.5 was treated with AF546-DBCO (2.5-3.25 eq) for 5-6 h in a Thermomixer at 37 °C, 600 rpm. Monitoring of click reactions were performed using a Kromasil C18 column (2.5 µm particle size, pore size 100 Å, L × I.D. 100 mm × 2.1 mm) in buffer conditions (A = NH_4HCO_3 0.02 M buffer, B = acetonitrile) with a gradient (0 to 40 %B, 20 min) using an Agilent LC 1260 coupled with a 6130 single quadrupole (positive or negative mode ESI). After completion of the reaction, the labeled ASOs were purified in buffer conditions using a 10 min gradient from 0 to 30%B on Kromasil C18 column (5 µm particle size, pore size 100 Å, L × I.D. 250 mm × 4.6 mm) in buffer conditions (A = NH_4HCO_3 0.02 M buffer, B = acetonitrile) using an Agilent LC 1260 Infinity. The expected fractions were freeze-dried and analyzed.

3. Characterizations of oligonucleotides

High-resolution mass spectra (positive or negative mode ESI) were performed on a Thermo Scientific™ Orbitrap Velos Pro Mass Spectrometer coupled with a Thermo Scientific™ Vanquish chromatography.

a. HRMS of ASOs

Oligonucleotides	Sequences	Formula	Mass calculated	Mass found
Ref-ASO	**T*G*C*C*T***T*T*A*G*G*A*T*T*C*T***A*G*A*C*A**	$C_{230}H_{316}N_{71}O_{121}P_{19}S_{19}$	$[M-15H+9Na]^{6-}$: 1232.6400 $[M-16H+7Na]^{9-}$: 816.5394 $[M-14H+5Na]^{9-}$: 811.6545	$[M-15H+9Na]^{6-}$: 1232.6338 $[M-16H+7Na]^{9-}$: 816.5376 $[M-14H+5Na]^{9-}$: 811.6522
Int-2'-Syd-ASO	**T*G*C*C*T**$^{(2'\text{-Syd})}$***T*T*A*G*G*A*T*T*C*T*A*G*A*C*A**	$C_{238}H_{319}N_{74}O_{123}P_{19}S_{19}$	$[M-6H]^{6-}$: 1228.5042 $[M-7H]^{7-}$: 1052.8597 $[M-8H]^{8-}$: 921.1263	$[M-6H]^{6-}$: 1228.5076 $[M-7H]^{7-}$: 1052.8608 $[M-8H]^{8-}$: 921.1214
Ext-2'-Syd-ASO	**T**$^{(2'\text{-Syd})}$***G*C*C*T*T*T*A*G*G*A*T*T*C*T*A*G*A*C*A**	$C_{238}H_{319}N_{74}O_{123}P_{19}S_{19}$	$[M-9H]^{9-}$: 818.6671 $[M-9H+2Na]^{7-}$: 1059.1403 $[M-12H+3Na]^{9-}$: 825.9944	$[M-9H]^{9-}$: 818.6676 $[M-9H+2Na]^{7-}$: 1059.1398 $[M-12H+3Na]^{9-}$: 825.9941
5-Syd-ASO	**T*G*C*C*T*T*T*A*G*G*A***T$^{(5\text{-Syd})}$***T*C*T*A*G*A*C*A**	$C_{241}H_{321}N_{74}O_{124}P_{19}S_{19}$	$[M-18H+12Na]^{6-}$: 1281.4699 $[M-16H+8Na]^{8-}$: 949.8596 $[M-19H+9Na]^{10-}$: 761.8844	$[M-18H+12Na]^{6-}$: 1281.4673 $[M-16H+8Na]^{8-}$: 949.8581 $[M-19H+9Na]^{10-}$: 761.8865
P-Syd-ASO	**T*G*C*C*T*T*T*A*G*G*A***T$^{(P\text{-Syd})}$***T*C*T*A*G*A*C*A**	$C_{242}H_{327}N_{74}O_{124}P_{19}S_{19}$	$[M-6H]^{6-}$: 1240.5138 $[M-7H]^{7-}$: 1063.1537 $[M-8H]^{8-}$: 930.1335	$[M-6H]^{6-}$: 1240.5114 $[M-7H]^{7-}$: 1063.1516 $[M-8H]^{8-}$: 930.1323

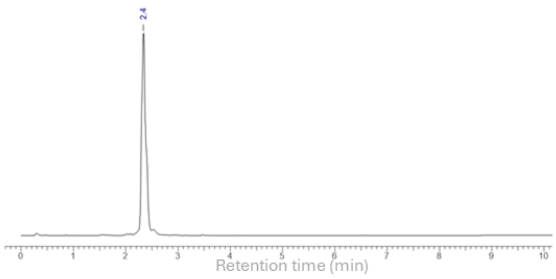
b. HRMS of AF546-ASOs

Oligonucleotides	Sequences	Formula	Mass calculated	Mass found
Int-2'-AF546-ASO	**T*G*C*C*T**$^{(2'\text{-AF546})}$***T*T*A*G*G*A*T*T*C*T*A*G*A*C*A**	$C_{289}H_{366}Cl_3N_{78}O_{132}P_{19}S_{22}$	$[M-17H+10Na]^{7-}$: 1235.7135 $[M-17H+9Na]^{8-}$: 1078.3757 $[M-17H+8Na]^{9-}$: 956.0018	$[M-17H+10Na]^{7-}$: 1235.7078 $[M-17H+9Na]^{8-}$: 1078.3697 $[M-17H+8Na]^{9-}$: 955.9991
Ext-2'-AF546-ASO	**T**$^{(2'\text{-AF546})}$***G*C*C*T*T*T*A*G*G*A*T*T*C*T*A*G*A*C*A**	$C_{289}H_{366}Cl_3N_{78}O_{132}P_{19}S_{22}$	$[M-17H+10Na]^{7-}$: 1235.7135 $[M-17H+9Na]^{8-}$: 1078.3757 $[M-17H+8Na]^{9-}$: 956.0018	$[M-17H+10Na]^{7-}$: 1235.7118 $[M-17H+9Na]^{8-}$: 1078.3708 $[M-17H+8Na]^{9-}$: 955.9979
5-AF546-ASO	**T*G*C*C*T*T*T*A*G*G*A***T$^{(5\text{-AF546})}$***T*C*T*A*G*A*C*A**	$C_{292}H_{368}Cl_3N_{78}O_{133}P_{19}S_{22}$	$[M-19H+12Na]^{7-}$: 1249.7099 $[M-19H+11Na]^{8-}$: 1090.6225 $[M-17H+7Na]^{10-}$: 863.5037	$[M-19H+12Na]^{7-}$: 1249.7093 $[M-19H+11Na]^{8-}$: 1090.6182 $[M-17H+7Na]^{10-}$: 863.5017
P-AF546-ASO	**T*G*C*C*T*T*T*A*G*G*A***T$^{(P\text{-AF546})}$***T*C*T*A*G*A*C*A**	$C_{293}H_{374}Cl_3N_{78}O_{133}P_{19}S_{22}$	$[M-14H+7Na]^{7-}$: 1236.5866 $[M-13H+5Na]^{8-}$: 1076.3919 $[M-14H+5Na]^{9-}$: 956.6809	$[M-14H+7Na]^{7-}$: 1236.5879 $[M-13H+5Na]^{8-}$: 1076.3911 $[M-14H+5Na]^{9-}$: 956.6802

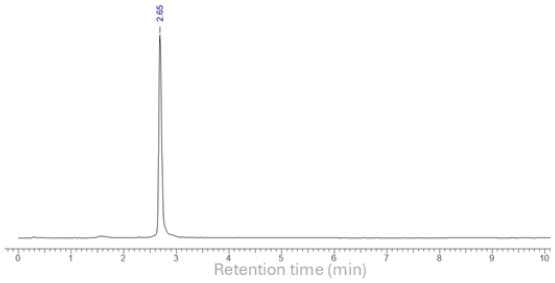
c. LC analysis of AF546-ASOs

Analysis of final labelled oligonucleotides was performed on ACQUITY BEH C18 column (1.7 µm particle size, pore size 130 Å, L × I.D. 50 mm × 2.1mm) in buffer conditions (A = NH_4HCO_3 0.02 M buffer in H_2O/CH_3CN 98/2 (v/v), B = NH_4HCO_3 0.02 M buffer in H_2O/CH_3CN 20/80 (v/v)) using a Waters UPLC I-Class.

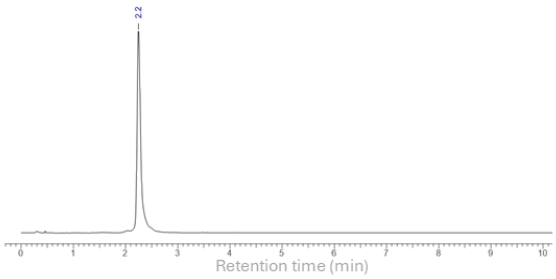
LC of **Int-2'-AF546-ASO**, gradient from 0 to 100%B, 10 min



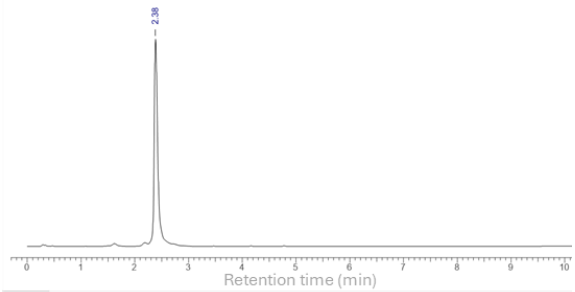
LC of **Ext-2'-AF546-ASO**, gradient from 0 to 100%B, 10 min



LC of **5-AF546-ASO**, gradient from 0 to 100%B, 10 min



LC of **P-AF546-ASO**, gradient from 0 to 100%B, 10 min



IV. Cell culture

U-87 MG is a glioblastoma cell line, and HeLa is an epithelial cell line, both cultured in ATCC EMEM supplemented with 10% FBS (Eurobio) and 1% Penicillin-Streptomycin (Life Technologies). These commercially available cell lines were obtained from ATCC. Cell subculturing was performed weekly using DPBS without calcium/magnesium and TrypLE (Gibco). They were incubated at 37 °C with 5% CO_2 and 80% relative humidity (RH) and maintained under 80% confluency.

V. ASO in vitro activity assay by RT-qPCR

Syd-ASOs and AF546-ASOs were loaded into 384-well plates (Revvity) using Opti-MEMTM as a diluting solvent, with or without LipofectamineTM RNAiMAX transfection reagent (both from Life Technologies). After 5 min of incubation at room temperature for lipoplex formation, 2.500 U-87 MG cells were added in each well. Cells with the different ASO conditions were subsequently incubated at 37 °C. After 48 h of treatment, RT-qPCR was performed to assess Malat1 lncRNA level. The two-step RT-qPCR Cell-to-CtTM Fast Advanced kit from Invitrogen was used. After cell medium removal and a DPBS (without calcium nor magnesium) wash at 4 °C, cell lysis was done directly in culture wells. 5.5 µL of lysate were transferred in a PCR plate containing RT Master Mix for RT in a Veriti thermal cycler with the following program: 37 °C for 30 min, 95 °C for 5 min, and a 4 °C hold. 2 µL of cDNA were transferred in a qPCR plate containing qPCR Master Mix and two TaqMan Assays (one assay VIC for GAPDH reference gene Hs99999905_m1 and one assay FAM for Malat1 target gene Hs00273907_s1, both from Invitrogen) for qPCR in a QuanstudioTM Flex 7 with the following program: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 sec, and 60 °C for 1 min. Raw data were exported from Thermo Scientific QuantStudioTM software to Microsoft Excel. Target RNA expression levels were quantified using the 2-DDCT formula, a calibrator untreated condition, and a GAPDH reference gene for normalization. The results were expressed as a percentage of expression with Fold Change*100, where,

$$\text{Fold change} = 2^{-\Delta\Delta Ct} = 2^{-[(Ct_{\text{target, sample}} - Ct_{\text{reference, sample}}) - (Ct_{\text{target, calibrator}} - Ct_{\text{reference, calibrator}})]}$$

Raw Ct values were exported to an excel for Fold change calculation following the 2-DDCT formula. IC_{50} was calculated using Graphpad Prism with a non-linear regression model.

VI. ASO in vitro cell imaging assay

Solutions of 1 µM of Syd-ASOs and AF546-ASOs were loaded in PhenoPlate 384-well plates with clear plastic bottoms from Revvity using OptiMEM as a diluting solvent and, 2,500 U-87 MG cells were then added. At various treatment timepoint, wells were fixed using PFA 4% and stained with the following cellular dyes: DAPI for the nuclei and CellMaskTM DeepRed for the plasma membrane. The stained plates were read at 60× water-immersion magnification using a CellVoyagerTM 7000 system, a spinning disk confocal microscope from Yokogawa, composed of four lasers (405, 488, 561, and 640 nm) and four filters (445/45, 525/50, 400/37, and 679/29 nm). An arbitrary confocal plane was initially selected for each cell line using the CellVoyagerTM software's autofocus mode and subsequently adjusted manually by the experimenter. We aimed

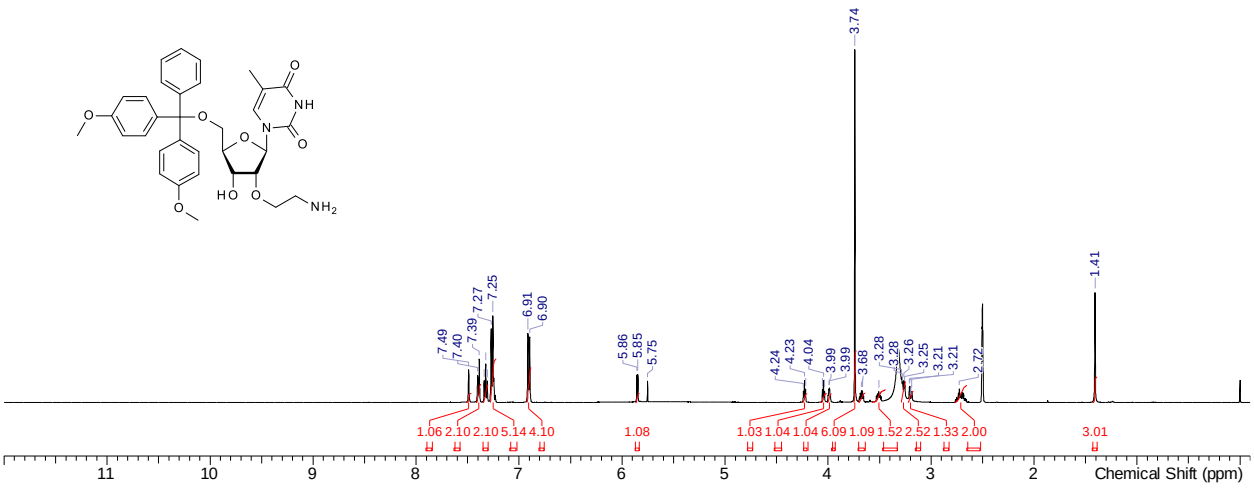
to choose the clearest and most fluorescent plane. For each acquisition, every condition was in technical triplicates, and 21 fields were acquired per well. Raw images in MRF/TIF format were then imported into the Columbus Software from Revvity for analysis. Analysis includes the subcellular compartment detection based on the following definitions: (i) the nuclei as the DAPI-stained region and (ii) the cytoplasm as the CellMask-stained region. Next, ASO spots were detected and quantified in each compartment. The analysis results were then imported to Microsoft Excel.

VII. In-cell SPSAC reaction for ASO Functionalization

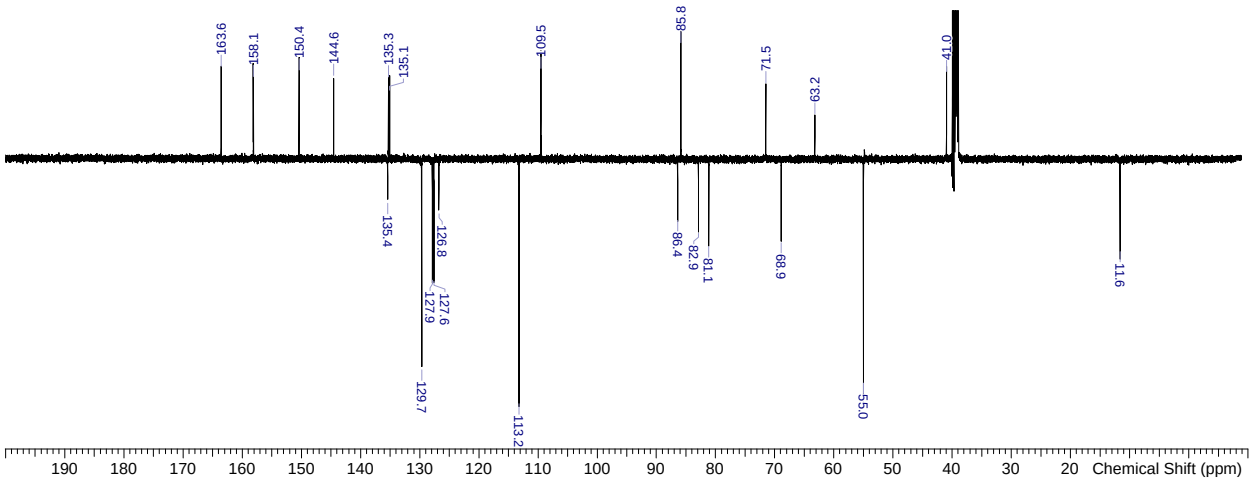
Solution of 1 µM of 5-Syd-ASO was loaded into 96-well plates using OptiMEMTM as a diluting solvent, with or without ScreenFect A (ScreenFect). After 5 min of incubation, 10.000 HeLa cells were added. Cells with the different ASO conditions were subsequently incubated at 37 °C. After 48 hours of incubation, cells were fixed with PFA 4% and washed with a solution of 50 mM glycine/50 mM ammonium chloride in PBS with a 5 min soak. Finally, 100 µM (100 equiv) of DBCO-AF546 in DPBS were added and incubated for 3 hours at 37 °C. After a DPBS wash, cells were stained with DAPI and CellMask and imaged at 20x using the CellVoyagerTM 7000 system.

VIII. NMR spectra

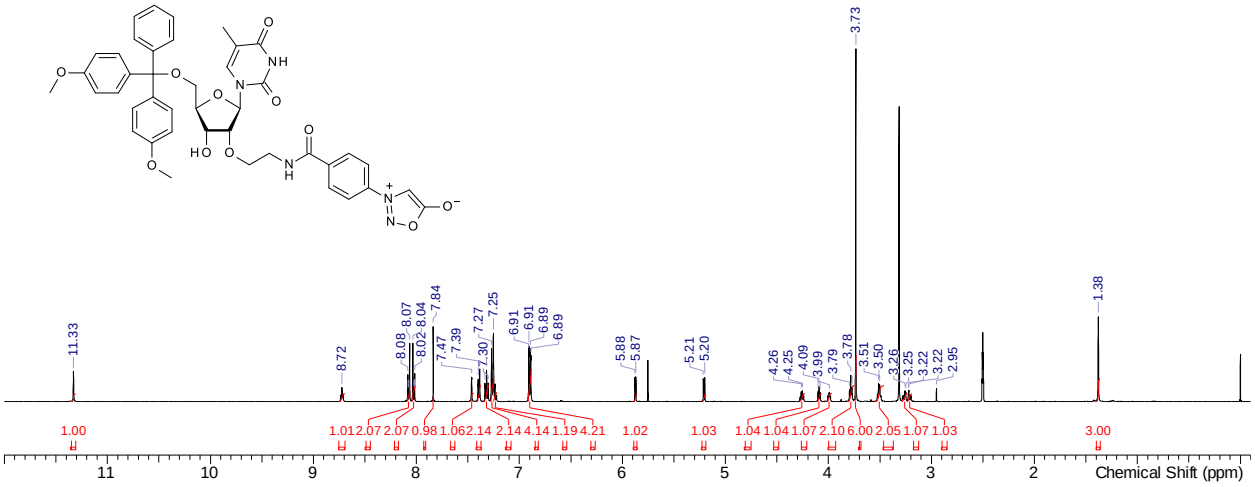
Copy of ^{1}H NMR spectrum of compound **1** (500 MHz, DMSO-*d6*)



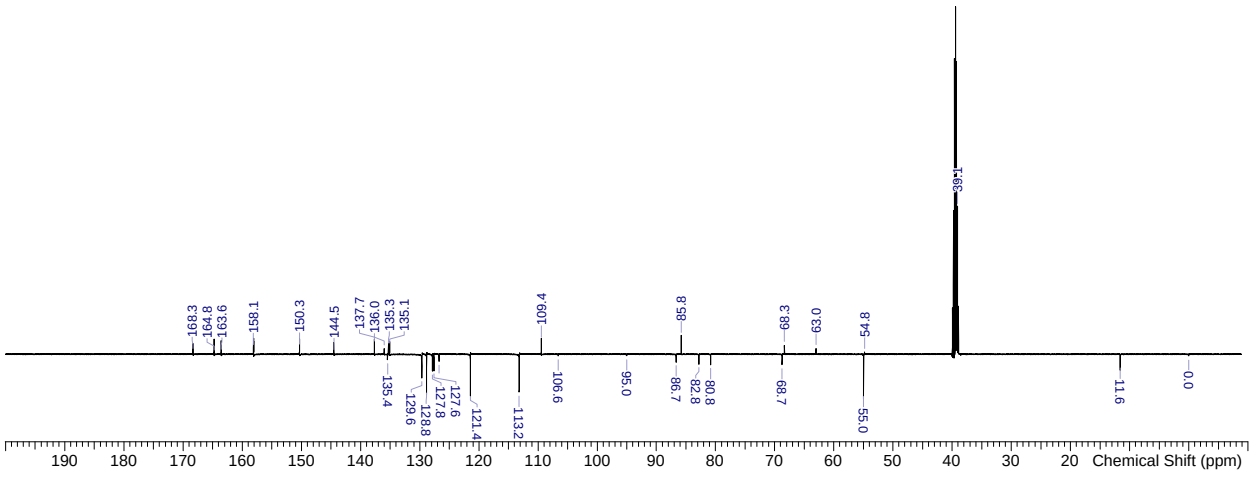
Copy of ^{13}C (JMod) NMR spectrum of compound **1** (125 MHz, DMSO-*d6*)



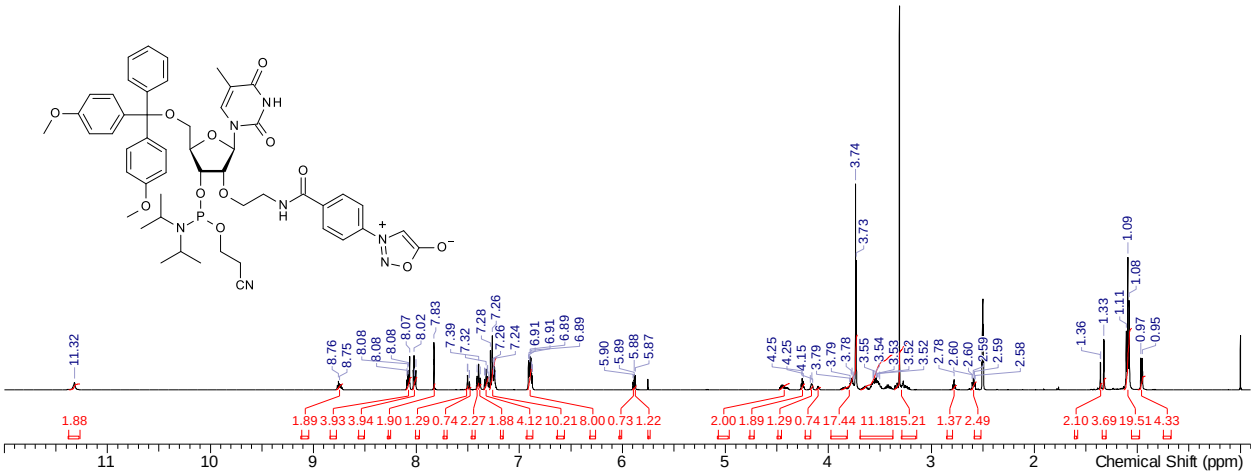
Copy of ^{1}H NMR spectrum of compound **3** (500 MHz, DMSO-*d6*)



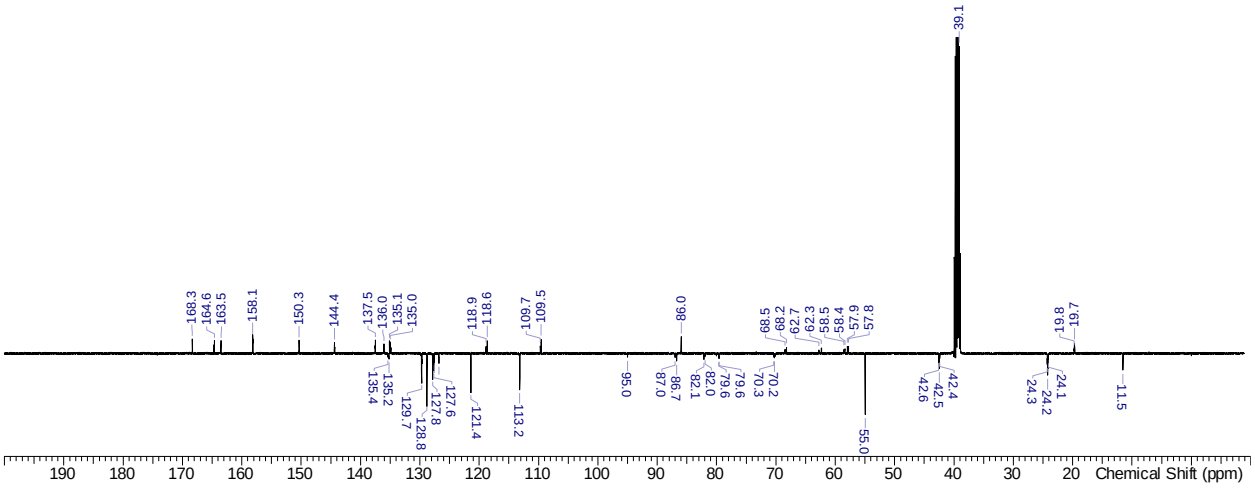
Copy of ^{13}C (JMod) NMR spectrum of compound **3** (125 MHz, DMSO-*d6*)



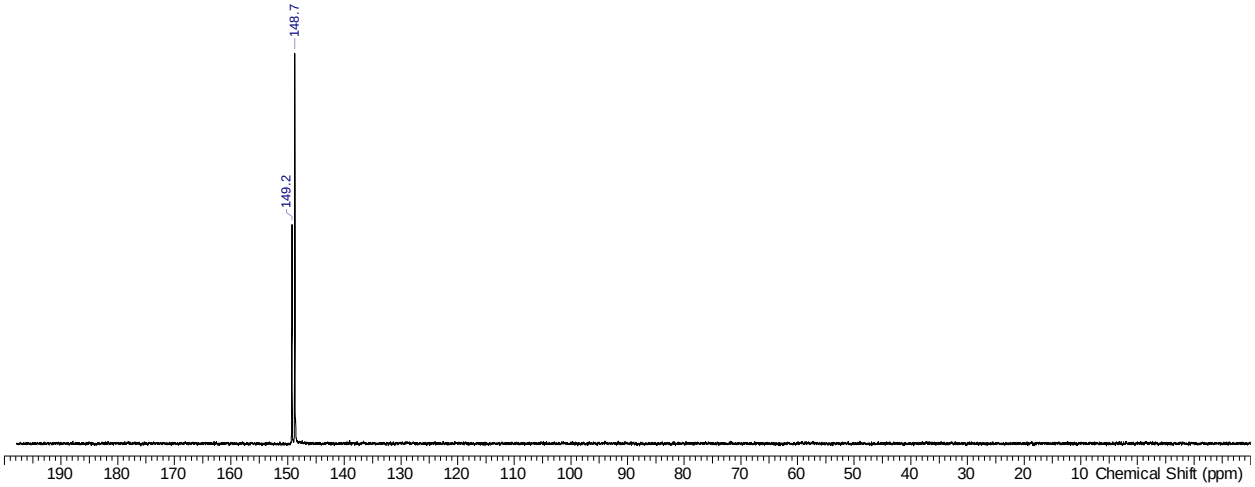
Copy of ^{1}H NMR spectrum of compound **4** (500 MHz, DMSO-*d6*)



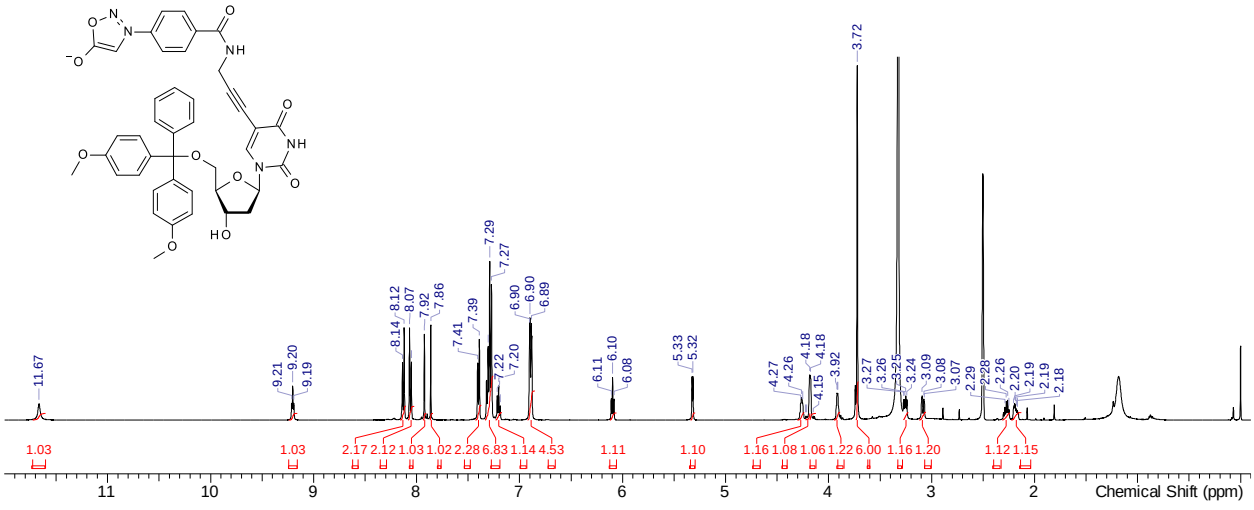
Copy of ^{13}C (JMod) NMR spectrum of compound **4** (125 MHz, DMSO-*d6*)



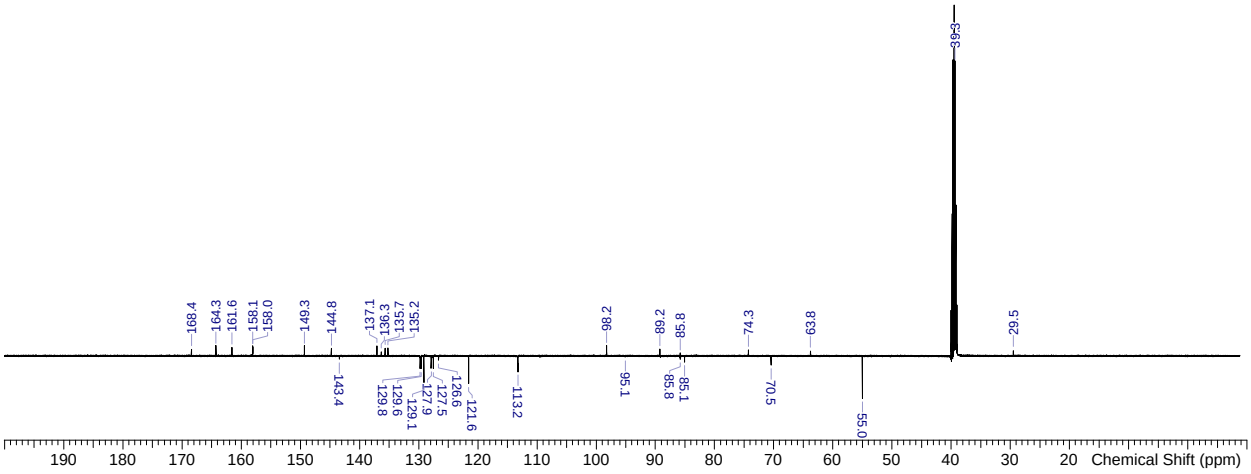
Copy of ^{31}P NMR spectrum of compound **4** (162 MHz, DMSO-*d6*)



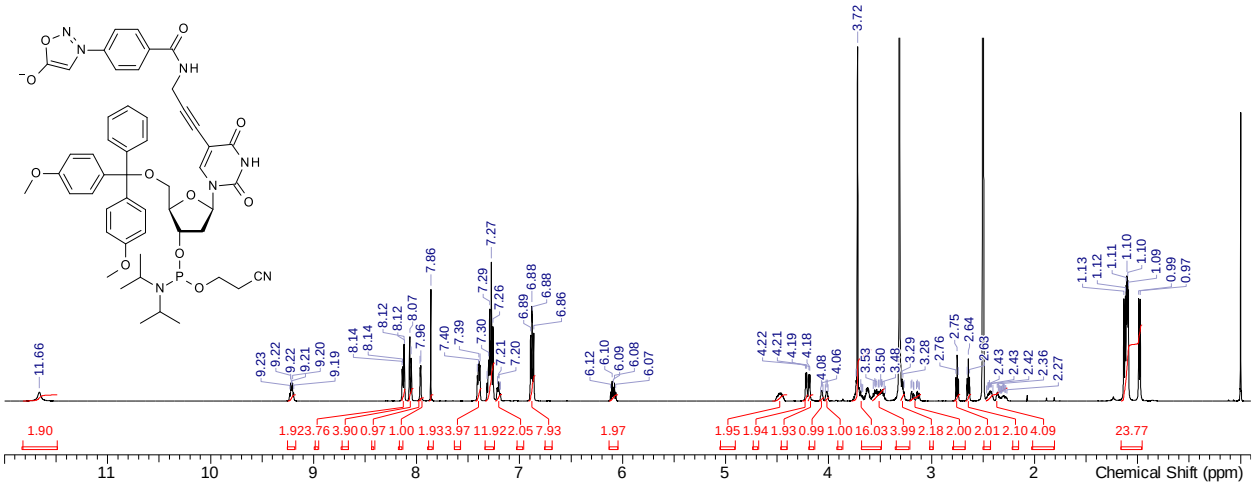
Copy of ^{1}H NMR spectrum of compound **8** (500 MHz, DMSO-*d6*)



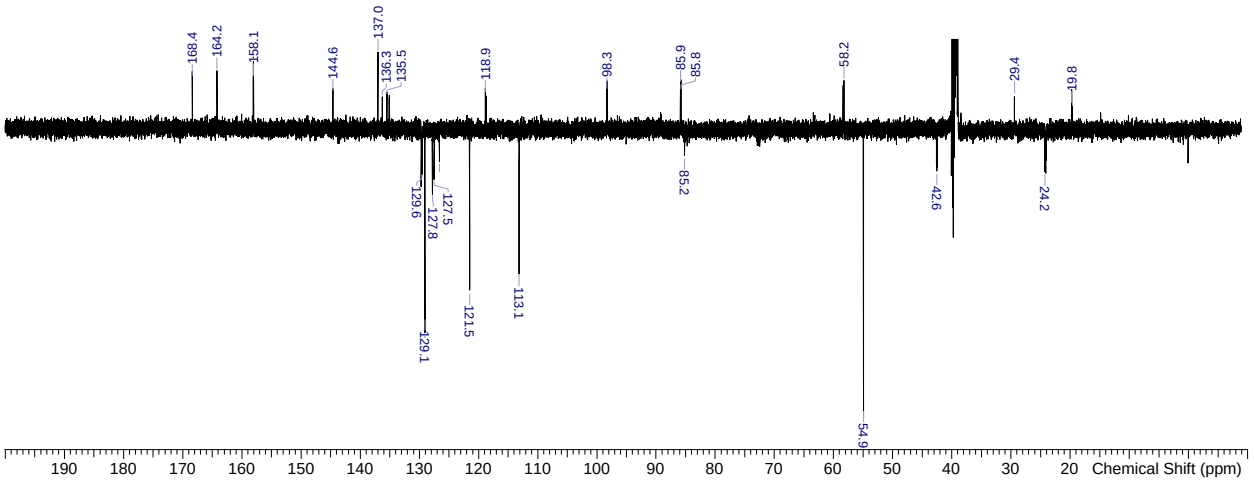
Copy of ^{13}C (JMod) NMR spectrum of compound **8** (125 MHz, DMSO-*d6*)



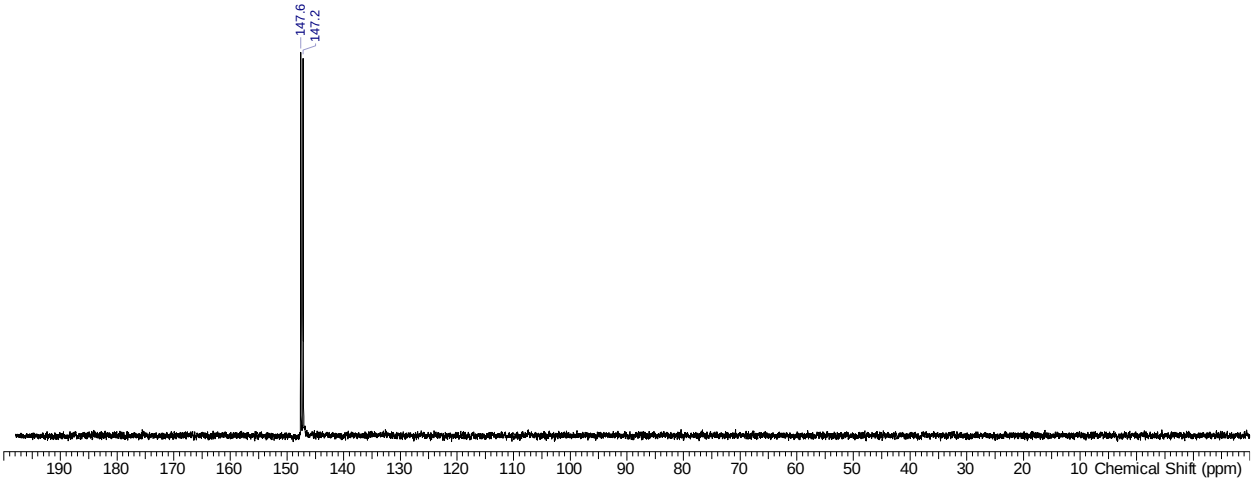
Copy of ^{1}H NMR spectrum of compound **9** (500 MHz, DMSO-*d6*)



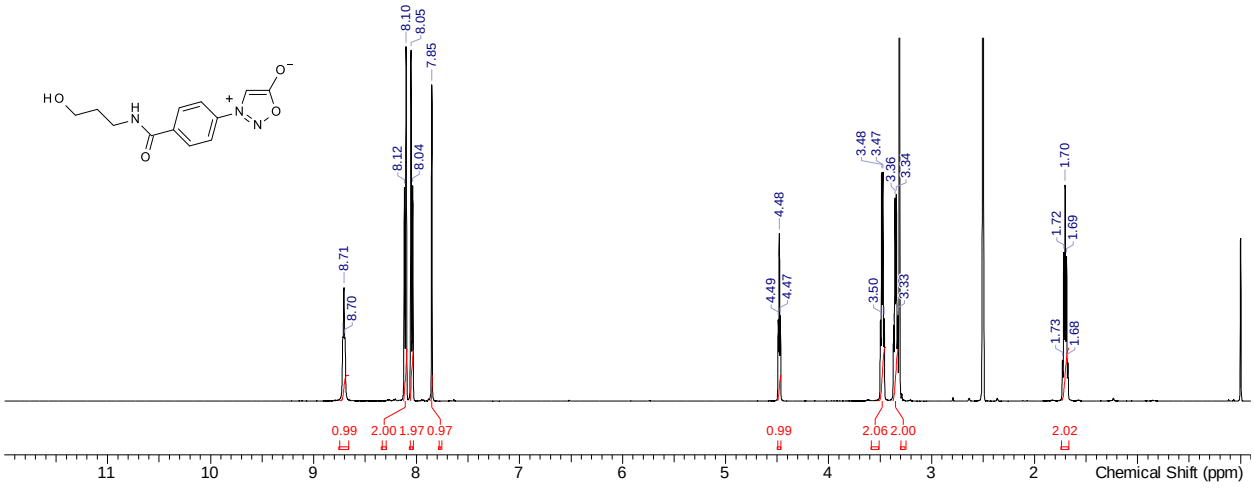
Copy of ^{13}C (JMod) NMR spectrum of compound **9** (125 MHz, DMSO-*d6*)



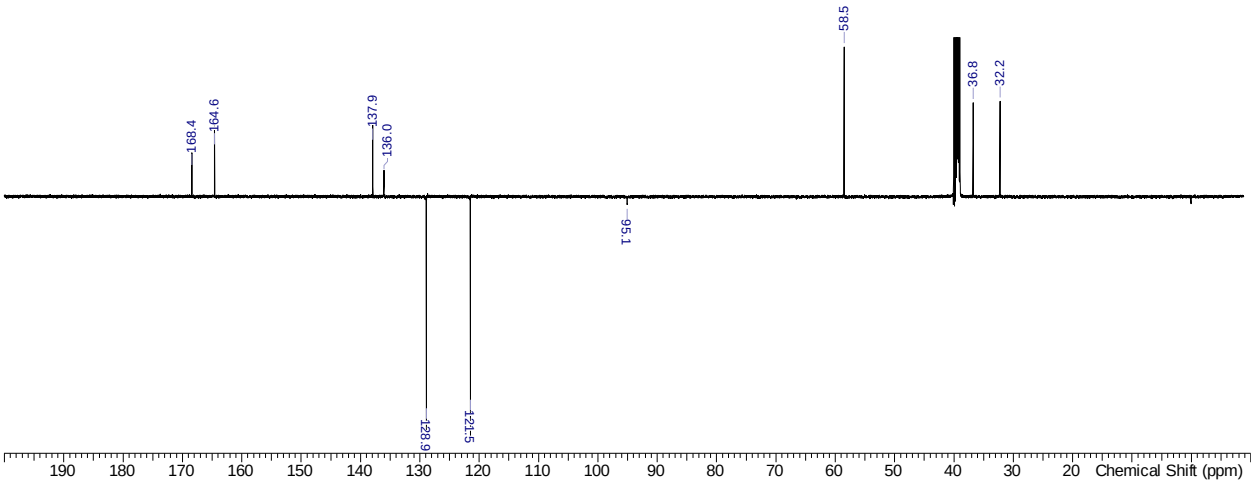
Copy of ^{31}P NMR spectrum of compound **9** (162 MHz, DMSO-*d6*)



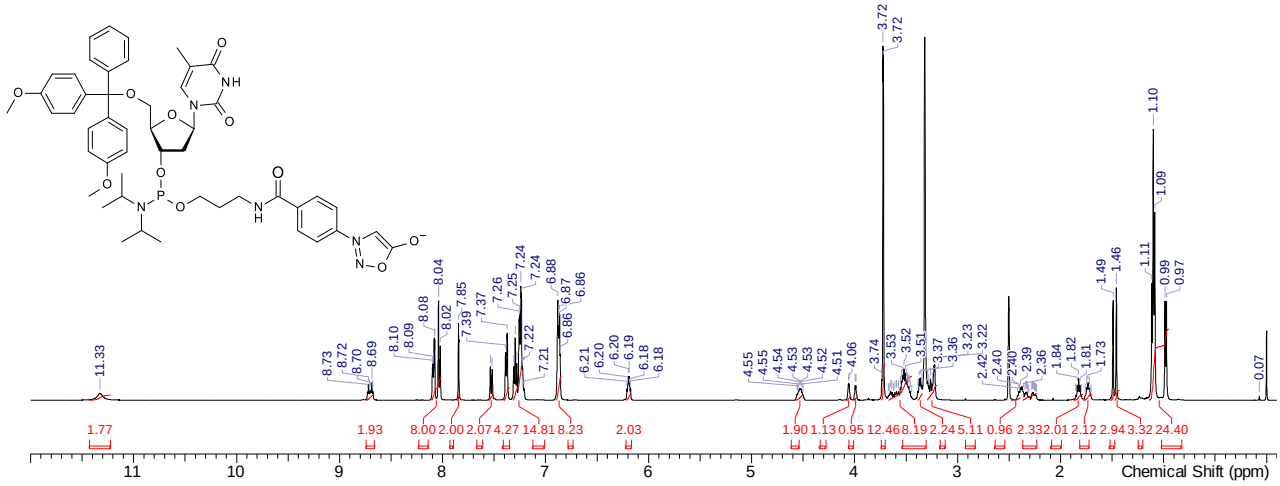
Copy of ^{1}H NMR spectru of compound **11** (500 MHz, DMSO-*d6*)



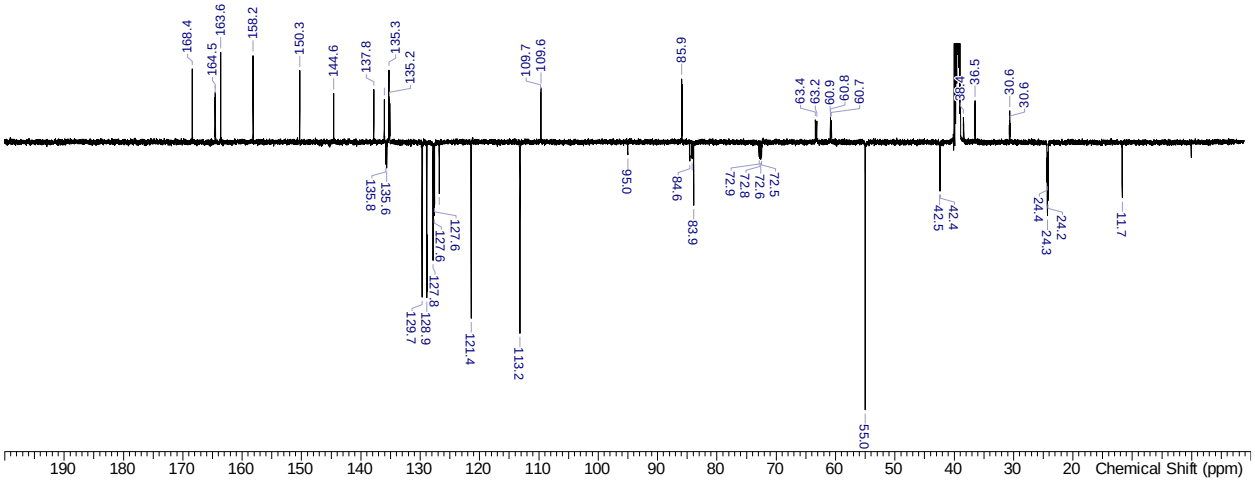
Copy of ^{13}C (JMod) NMR spectrum of compound **11** (125 MHz, DMSO-*d6*)



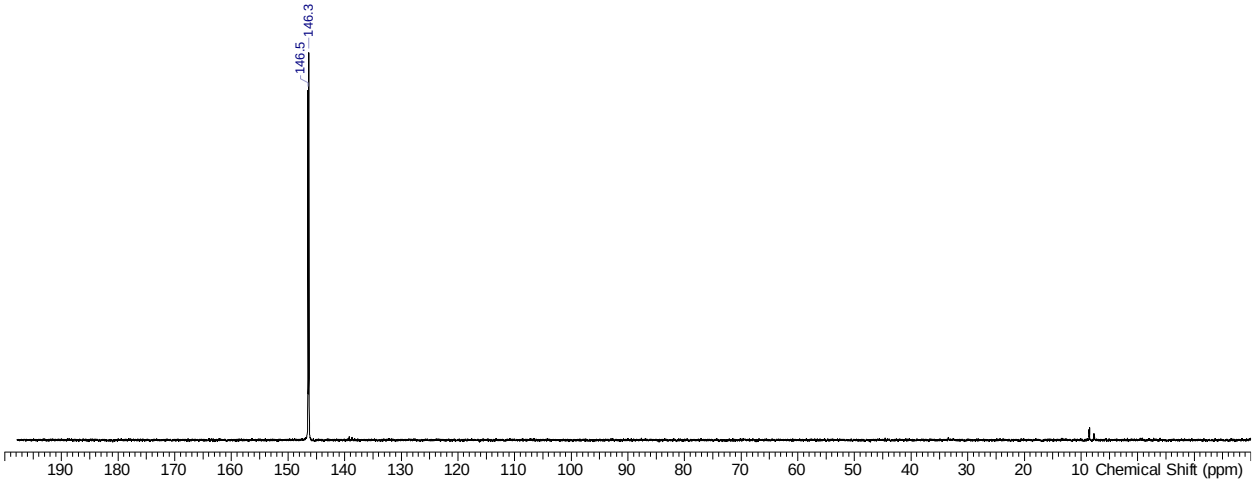
Copy of ^{1}H NMR spectrum of compound **12** (500 MHz, DMSO-*d6*)



Copy of ^{13}C (JMod) NMR spectrum of compound **12** (125 MHz, DMSO-*d6*)



Copy of ^{31}P NMR spectrum of compound **12** (162 MHz, DMSO-*d6*)



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