

Supporting Information

The Synthesis and Application of a Diazirine-modified Uridine Analogue for Identifying RNA-Protein Interactions

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1. General procedures:

All reagents were purchased from Sigma Aldrich, Fluka, Acros, Apollo Scientific or ABCR at the highest quality available. All anhydrous reactions were performed under Ar and in dried glassware. Anhydrous solvents were obtained by filtration through activated alumina (THF, CH₂Cl₂, MeCN, Et₂O) or purchased in crown capped bottles (pyridine, dioxane, and DMF). 5'-*O*-(4,4'-dimethoxytrityl)-5-iodouridine (**3**) was synthesized following a literature procedure.¹

Column chromatography was performed on silica gel (Silicycle) with an average particle size of 40 µm. All solvents for column chromatography were of technical grade and distilled prior to use. Thin-layer chromatography (TLC) was performed on silica gel plates (Macherey–Nagel, 0.25 mm, UV₂₅₄). Visualisation was achieved under UV light and/or by dipping in staining solution: Cerium (IV) sulfate (2.1 g), phosphomolybdic acid (4.2 g), conc. H₂SO₄ (2.5 mL), H₂O (180 mL); or KMnO₄ (1.5 g), K₂CO₃ (10.0 g), 10% NaOH (1.25 mL), H₂O (200 mL) followed by heating with a heat gun.

NMR spectra were recorded on a Bruker DRX 400 or a Bruker AC 300 spectrometer (400 or 300 MHz for ¹H, 101 or 75.5 MHz for ¹³C, 121.4 MHz for ³¹P, and 376.5 MHz for ¹⁹F) in CDCl₃, CD₃OD or D₂O at ambient temperature and all spectra were referenced to the signals of the corresponding solvent. . Chemical shifts are given in ppm (δ scale) and coupling constants (*J*) in Hz. Signal assignments are based on DEPT and on ¹H–¹H and ¹H–¹³C correlation experiments (COSY/HSQC). Data are reported as follows: chemical shifts, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), integration, and coupling constants (*J* in Hz).

High resolution electrospray ionisation (ESI) mass spectra (MS, *m/z*) were recorded on an Applied Biosystems Sciex QSTAR Pulsar or Thermo Scientific LTQ Orbitrap XL instrument. Where ESI was unsuitable to detect the desired mass, matrix-assisted laser desorption/ionisation (MALDI-TOF) was applied using Bruker Daltonics Autoflex III instrument with a smartbeam Nd:YAG-laser (355 nm).

HPLC was performed on either an Äkta purifier (GE-Healthcare) or an Äkta basic 10/100 (Amersham Pharmacia Biotech System) on either a diethylaminoethyl (DEAE)-HPLC anion exchange column (DNAPAC PA200, 4 x 250 mm analytical column, with pre-column both

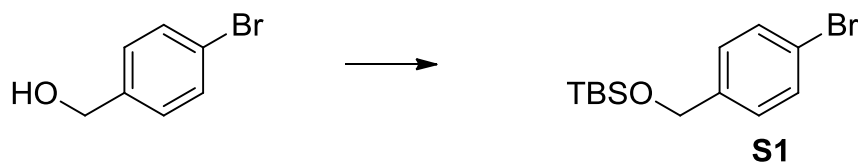
from Dionex) or semi-preparative Phenomenex Jupiter semi-preparative reversed phase (RP)-HPLC column (5 μ C18 300Å) column.

UV-melting curves were recorded on a Varian Cary 100 Bio UV–VIS spectrophotometer. Absorbance was monitored at 260 nm and the heating rate was set to 0.5°C min⁻¹. A cooling–heating–cooling cycle in the temperature range 15–80°C was applied. T_m values were obtained from the maximum of the first derivative curves using the Varian WinUV software and reported as the average of at least three ramps (standard deviation reported as error). To avoid evaporation of the solution, the sample solutions were covered with a layer of dimethylpolysiloxane. All measurements were carried out in NaCl (150 mM)/NaH₂PO₄ (10 mM) pH 7 buffer with duplex concentration of 1 μ M. The absorbance values were converted to hyperchromicity and normalized before plotting.

T7 RNA polymerase was purchased from either Promega or New England Biolabs. Single-stranded DNA binding protein (SSB) and RNasin[®] were purchased from Promega. Natural NTPs were purchased from Invitrogen. Thermostable inorganic pyrophosphatase (TIPP), 100 mM MgSO₄ solution, Trypsin and T4 RNA ligase were purchased from New England Biolabs. All enzymes and proteins were used in the buffers supplied by the manufacturers and according to manufacturer's instructions. 5'-³²P–cytidine-3',5'-bisphosphate (pCp) and α -³²P-adenosine 5'-triphosphate (α -³²P-ATP) were purchased from Hartmann analytic. Crosslinking was achieved by irradiation with Lumos 43 (Atlas Photonics) at 375 nm. Streptavidin coated magnetic beads were obtained from New England Biolabs. Fluorescence was detected with FLA-3000 phosphorimager (Fujifilm). Radioactivity was detected using a Storm 820 phosphorimager with the ImageQuant software (both from GE Healthcare) or ImageJ software (National Institutes of Health).

2. Synthetic procedures and characterizations:

((4-bromobenzyl)oxy)(*tert*-butyl)dimethylsilane (**S1**)



To a stirring solution of 4-bromobenzylalcohol (5.00 g, 26.7 mmol) in DMF (10 mL) was added imidazole (5.46 g, 80.2 mmol) and TBDMS-Cl (6.02 g, 40.1 mmol) and was stirred at room temperature for 15 hours. The reaction mixture was then diluted with distilled water (50 mL), transferred to a separating funnel and extracted with diethyl ether (2x 150 mL). The organic extracts were combined, washed with brine, dried over MgSO_4 , filtered to remove the drying agent and reduced to dryness *in vacuo*. The residue was dissolved in hexanes and purified by column chromatography (0-50% EtOAc in hexanes) to yield 7.58 g (95%) of compound **S1** as a colorless liquid.

Analytical data for compound **S1**

TLC (CH_2Cl_2) $R_f = 0.70$

^1H NMR (300 MHz, CDCl_3) δ 7.44 (d, $J = 8.4$ Hz, 2H, H-arom.), 7.19 (d, $J = 8.5$ Hz, 2H, H-arom.), 4.68 (s, 2H, CH₂), 0.94 (s, 9H, CH₃-*t*-butyl-Si), 0.09 (s, 6H, CH₃-Si).

^{13}C NMR (75 MHz, CDCl_3) δ 140.7, 131.5, 127.9, 120.8, 64.5, 26.1, 18.6, -5.1.

MALDI-TOF⁺MS calculated for $\text{C}_{13}\text{H}_{21}\text{BrOSi}$ ($[\text{M}+\text{H}]^+$): 301.0623, found: 300.835.

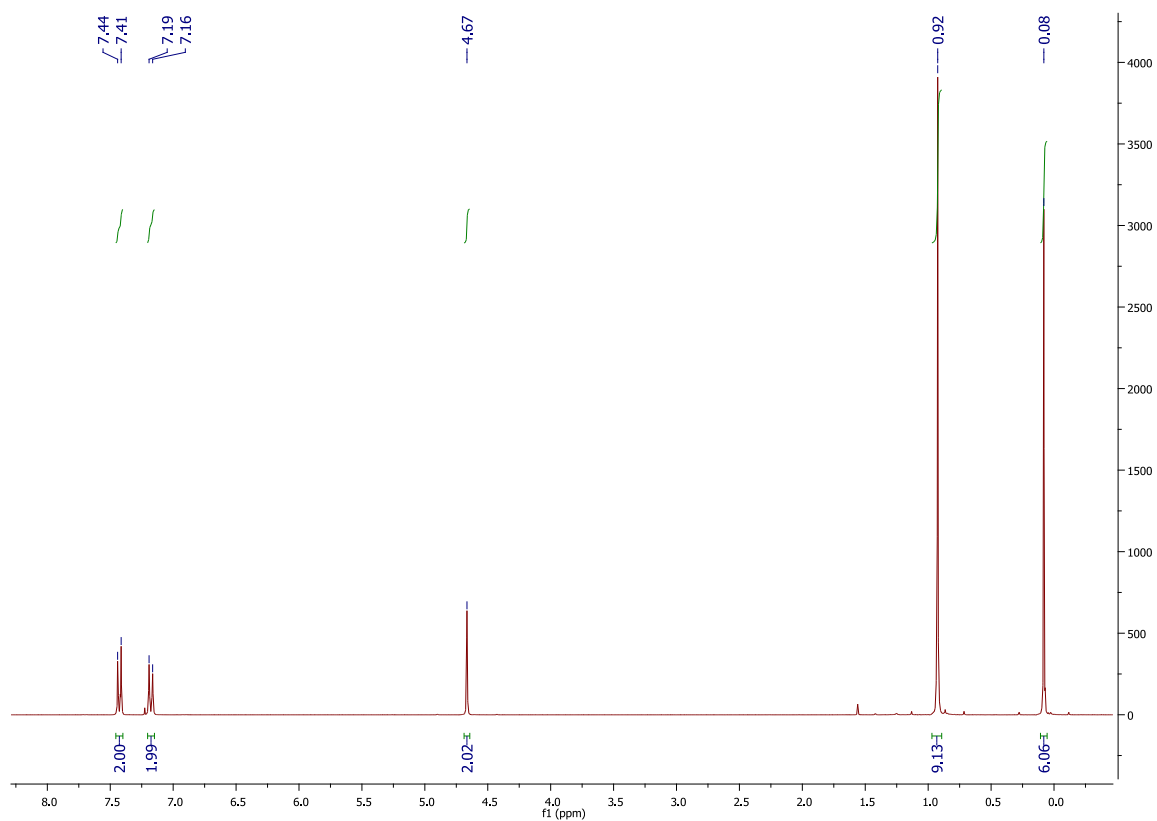


Figure S1. ¹H NMR of compound S1

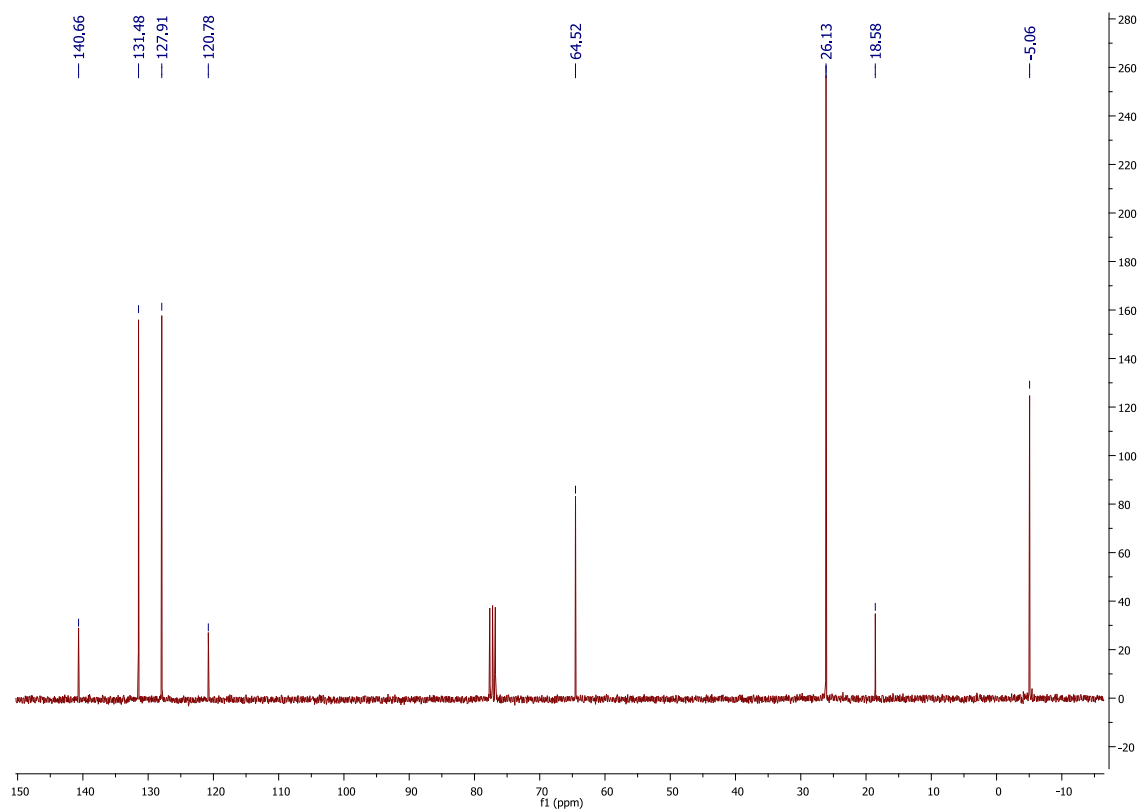
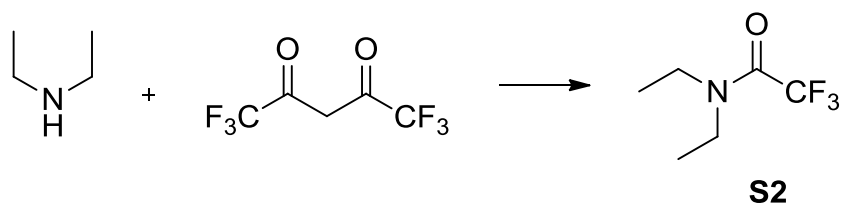


Figure S2. ¹³C NMR of compound S1

***N,N*-diethyl-2,2,2-trifluoroacetamide² (S2)**



Diethylamine (24.7 mL, 238.1 mmol) was dissolved in Et₂O (100 mL) and cooled to -78°C. Trifluoroacetic anhydride (16.8 mL, 119.1 mmol) was added dropwise over 1 hour (temperature was maintained below -50°C). The reaction mixture was stirred for 10 minutes and then warmed to room temperature slowly. The reaction mixture was diluted with distilled water (25 mL) and separated repeatedly until the aqueous layer was neutral. The organic layer was then dried over MgSO₄, filtered, and reduced to dryness *in vacuo* to yield 18.1 g (93%) of compound **S2** as a colorless liquid. Carried through to the next step without further purification.

Analytical data for compound **S2**

¹H NMR (300 MHz, CDCl₃) δ 3.45 – 3.33 (dd, 4H, CH₂), 1.16 (dt, *J* = 10.3, 7.1 Hz, 6H, CH₃).

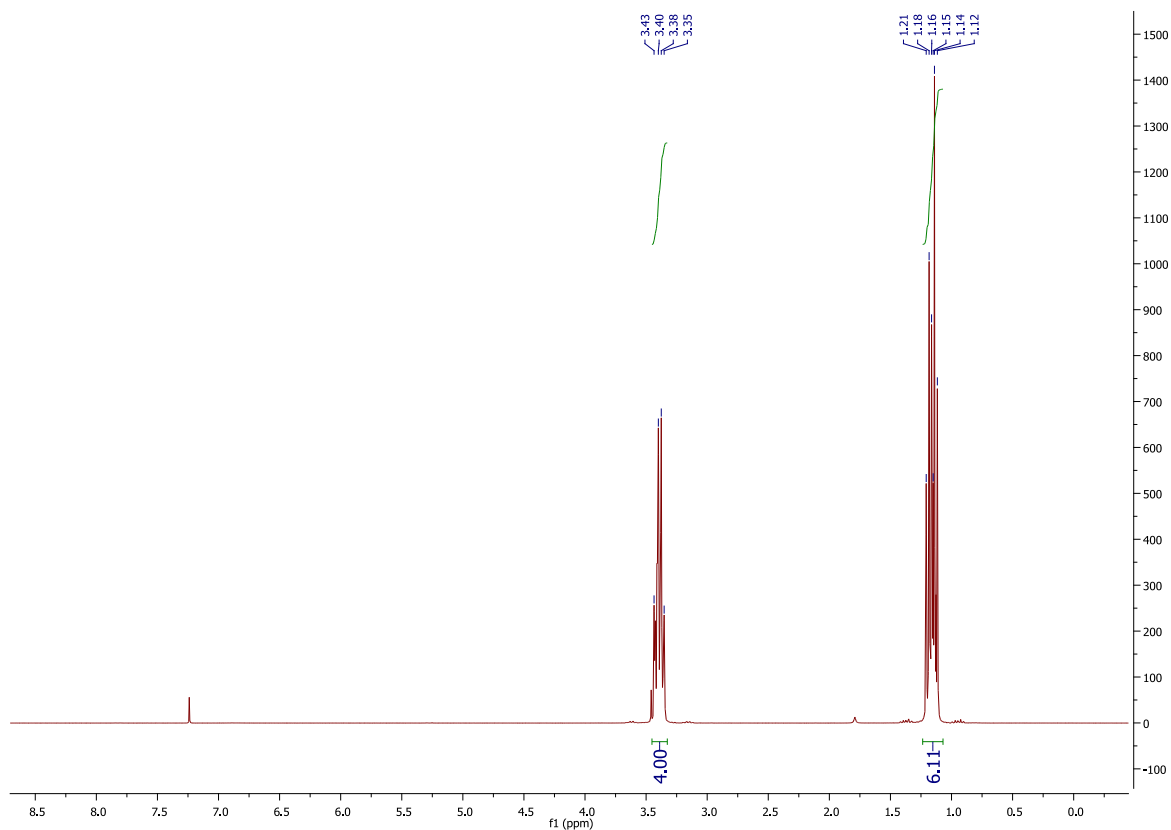
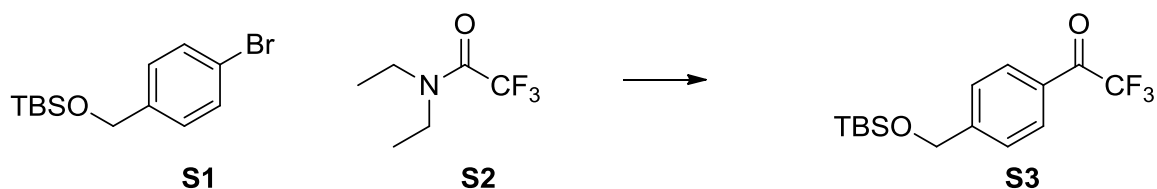


Figure S3. ^1H NMR of compound **S2**.

1-(4-(((*tert*-butyldimethylsilyl)oxy)methyl)phenyl)-2,2,2-trifluoroethanone³ (S3**)**



To a stirring solution of compound **S1** (8.00 g, 26.7 mmol) in dry THF (150 mL) at -78°C under Ar in flame dried glassware was added *n*-BuLi (20 mL, 1.6 M in hexanes, 32.0 mmol) dropwise over 15 minutes. The resulting mixture was stirred at -78°C for 2 hours. Compound **S2** (7.00 mL, 40.1 mmol) was dissolved in dry THF (18 mL) and the resulting solution was added to the reaction mixture dropwise over 15 minutes. The reaction mixture was maintained at -78°C for a further 2 hours. 10% NH_4Cl solution (60 mL) was then added and the reaction mixture was allowed to warm slowly to room temperature before dilution with Et_2O (200 mL). The organic phase was washed with further 10% NH_4Cl solution (2x 30 mL) and brine (50 mL). The organic layer was then dried over MgSO_4 , filtered and reduced to

dryness *in vacuo*. The residue was dissolved in hexanes/EtOAc 9:1 and purified by column chromatography (hexanes/EtOAc 9:1) to yield 7.55 g (89%) of compound **S3** as a colorless liquid.

Analytical data for compound **S3**

TLC (CH₂Cl₂) R_f = 0.73

¹H NMR (300 MHz, CDCl₃) δ 8.04 (d, J = 7.7 Hz, 2H, H-arom.), 7.49 (d, J = 8.7 Hz, 2H, H-arom.), 4.82 (s, 2H, CH₂), 0.94 (s, 9H, CH₃-*t*-butyl-Si), 0.11 (s, 6H, CH₃-Si).

¹³C NMR (75 MHz, CDCl₃) δ 180.2 (d, J = 34.9 Hz, CO₂CF₃), 150.3, 130.5, 130.4, 128.8, 127.9, 126.4, 117.0 (d, J = 291.4 Hz), 64.5, 26.2-26.1, 18.6, -5.2.

¹⁹F NMR (376 MHz, CDCl₃) δ -71.4.

ESI⁺-HRMS calculated for C₁₅H₂₁F₃O₂Si ([M+H]⁺): 319.1336, found: 319.1331.

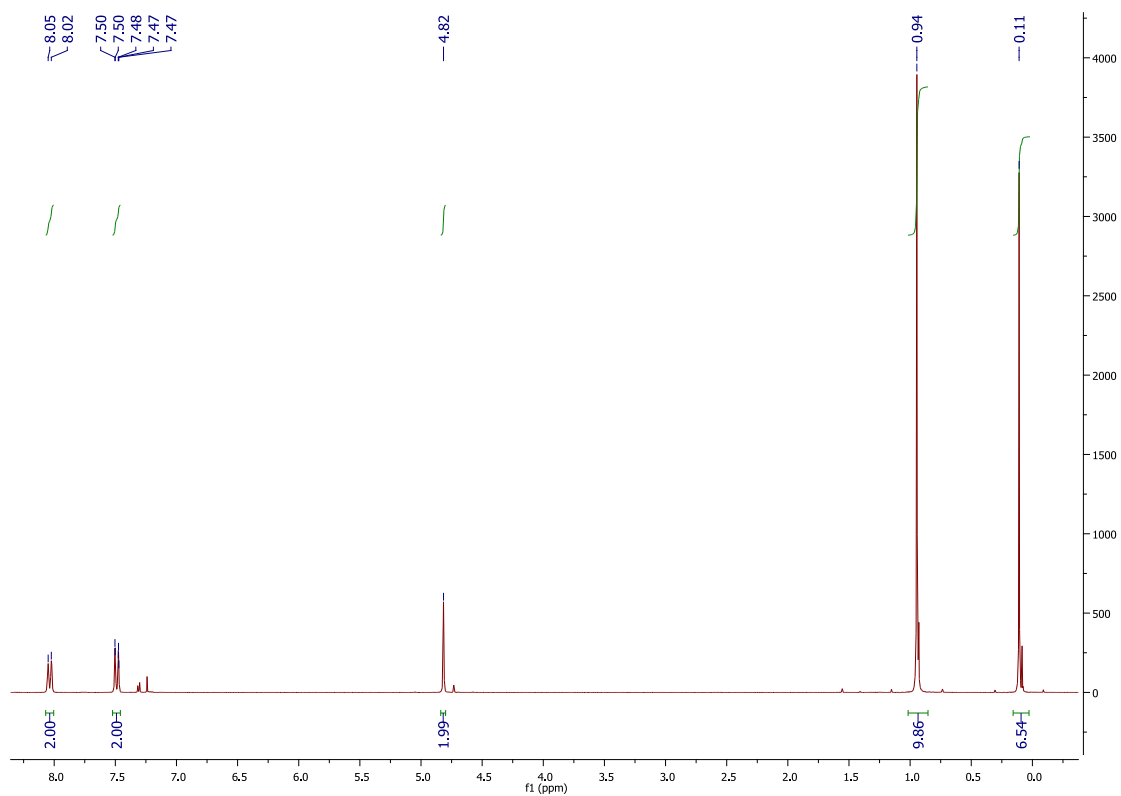


Figure S4. ¹H NMR of compound S3.

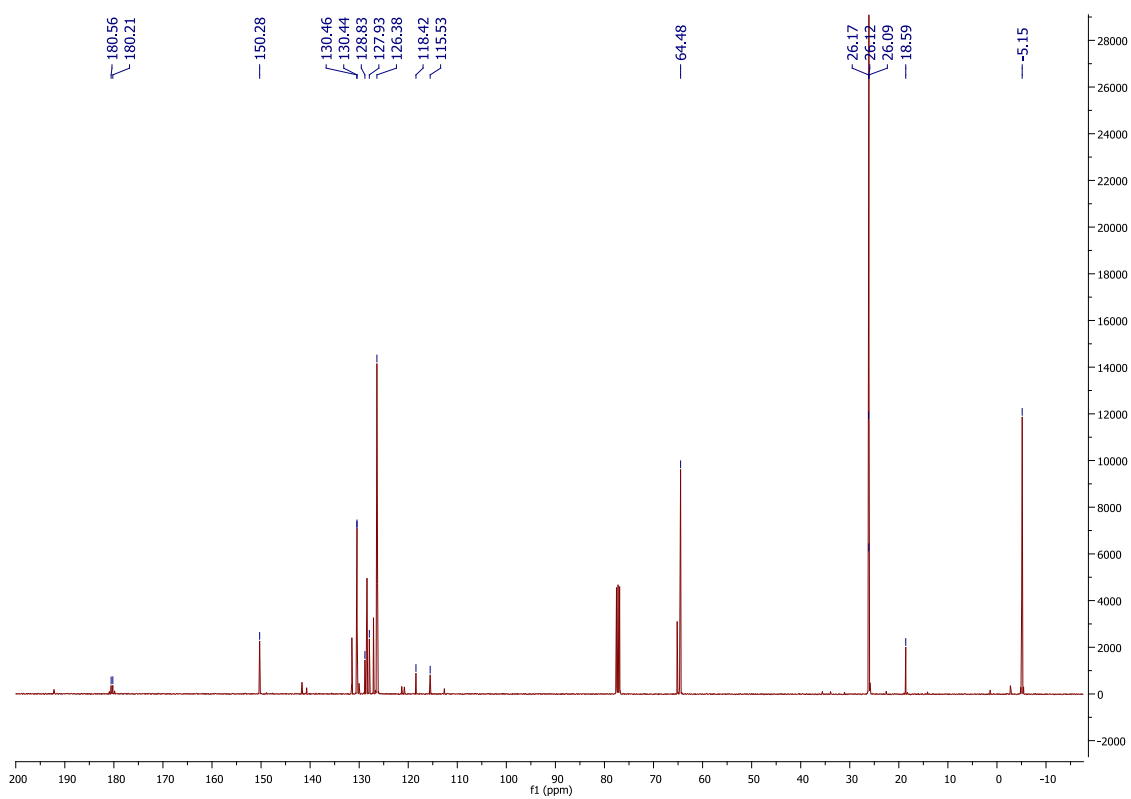


Figure S5. ¹³C NMR of compound S3.

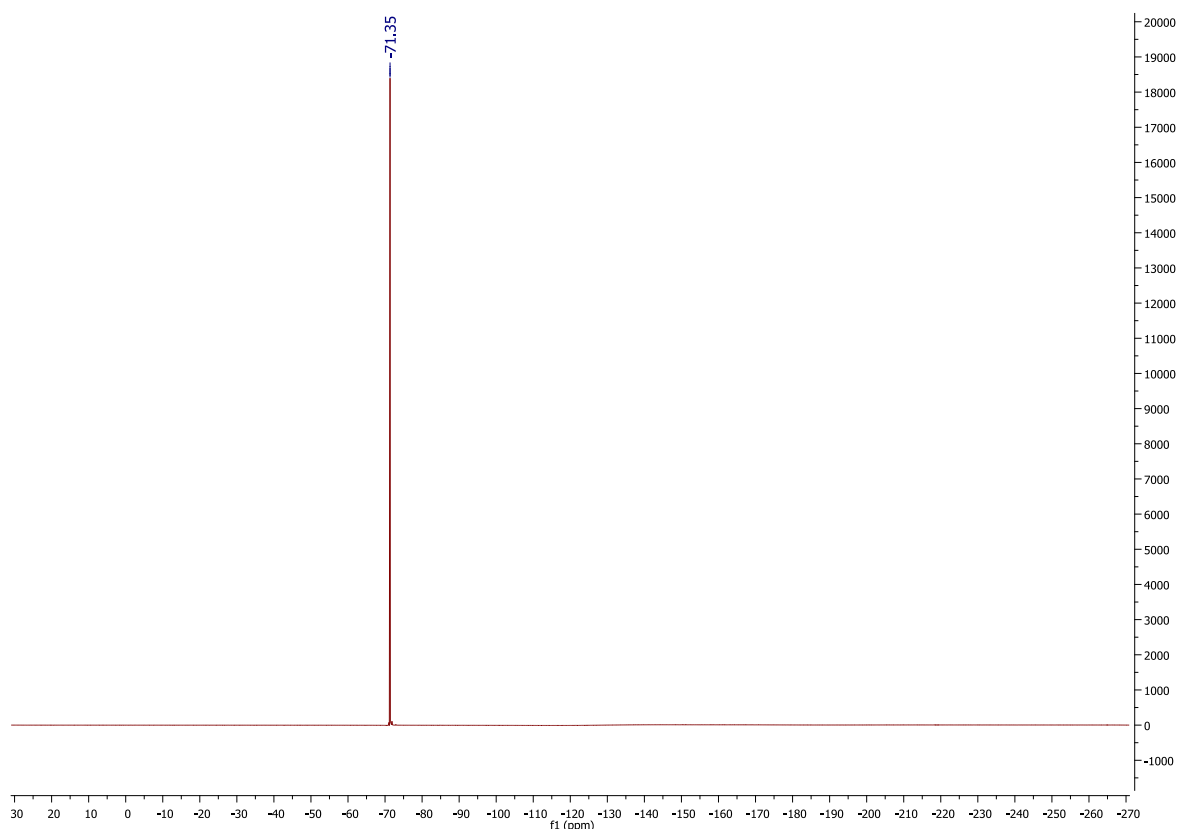
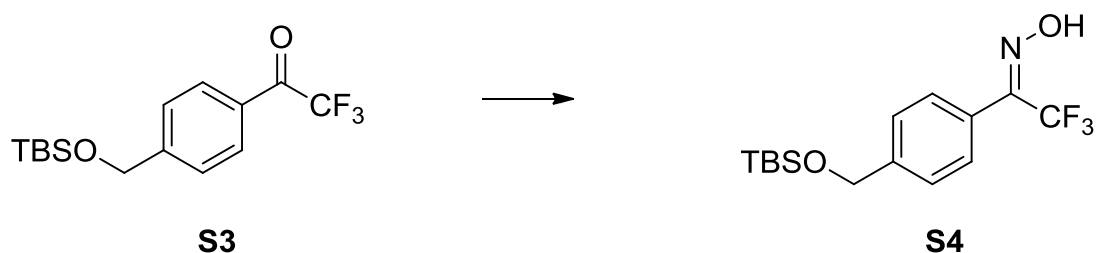


Figure S6. ^{19}F NMR of compound **S3**.

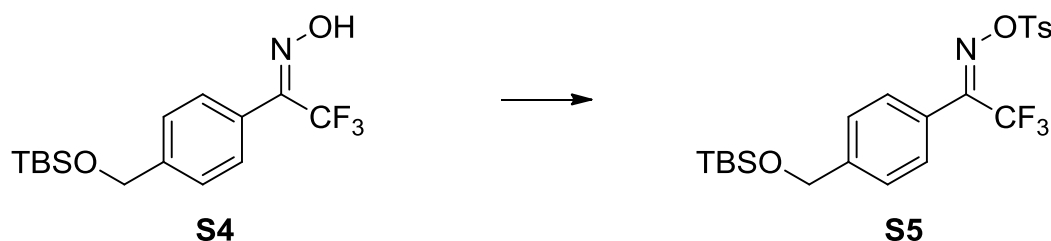
1-(4-(((*tert*-butyldimethylsilyl)oxy)methyl)phenyl)-2,2,2-trifluoroethanone oxime (S4**)**



To a stirring solution of compound **S3** (7.55 g, 23.7 mmol) in EtOH (40 mL) was added dry pyridine (65 mL) and hydroxylamine hydrochloride (1.82 g, 26.1 mmol). The resulting mixture was heated to 60°C for 3 hours. The reaction mixture was then diluted with Et₂O (150 mL) and washed with distilled H₂O (2x 30 mL) and brine (50 mL). The organic layer was then dried over MgSO₄, filtered and reduced to dryness *in vacuo* to yield 6.98 g (88%) of compound **S4** as a colorless liquid. Carried through to the next step without further purification.

TLC (CH₂Cl₂) R_f = 0.40 and 0.34 for each isomer of the product. The ratio of both *E* and *Z* isomers was estimated to be 1:1 by TLC.

1-(4-(((*tert*-butyldimethylsilyl)oxy)methyl)phenyl)-2,2,2-trifluoroethanone *O*-tosyl oxime (S5)



To a stirring solution of compound **S4** (6.98 g, 21.0 mmol) in dry pyridine (40 mL) was added *p*-toluenesulfonyl chloride (5.59 g, 29.4 mmol) at room temperature. The reaction mixture was stirred under Ar for 72 hours. TLC analysis showed remaining starting material, so a further 0.25 equivalents of *p*-toluenesulfonyl chloride (1.00 g, 5.2 mmol) were added and stirred for a further hour. The reaction mixture was diluted with Et₂O (150 mL) and washed with distilled H₂O (2x 50 mL), brine (50 mL), dried over MgSO₄, filtered and reduced to dryness *in vacuo*. The residue was dissolved in CH₂Cl₂/hexanes 1:1 and purified by column chromatography (33-50% CH₂Cl₂ in hexanes) and reduced to dryness *in vacuo* to yield 4.10 g (40%) of compound **S5** as a colorless liquid.

Analytical data for compound **S5**

TLC (CH₂Cl₂/hexanes 1:2) R_f = 0.41

¹H NMR (300 MHz, CDCl₃) δ 7.87 (d, *J* = 8.3 Hz, 2H, H-arom.), 7.38 (t, *J* = 7.3 Hz, 6H, H-arom.), 4.76 (s, 2H, CH₂), 2.46 (s, 3H, CH₃-*p*-tosyl), 0.94 (s, 9H, CH₃-*t*-butyl-Si), 0.11, 0.10 (s, 3H, CH₃-Si).

¹³C NMR (75 MHz, CDCl₃) δ 154.4, 154.0, 146.3, 145.8, 131.5, 130.1, 129.5, 129.4, 129.0, 128.7, 126.2, 123.2, 119.9 (d, *J* = 277.7 Hz), 64.5, 26.1, 22.0, 18.6, -5.1.

¹⁹F NMR (376 MHz, CDCl₃) δ -71.4.

ESI⁺-HRMS calculated for C₂₂H₂₈F₃NO₄SSi ([M+H]⁺): 488.1533, found: 488.1534.

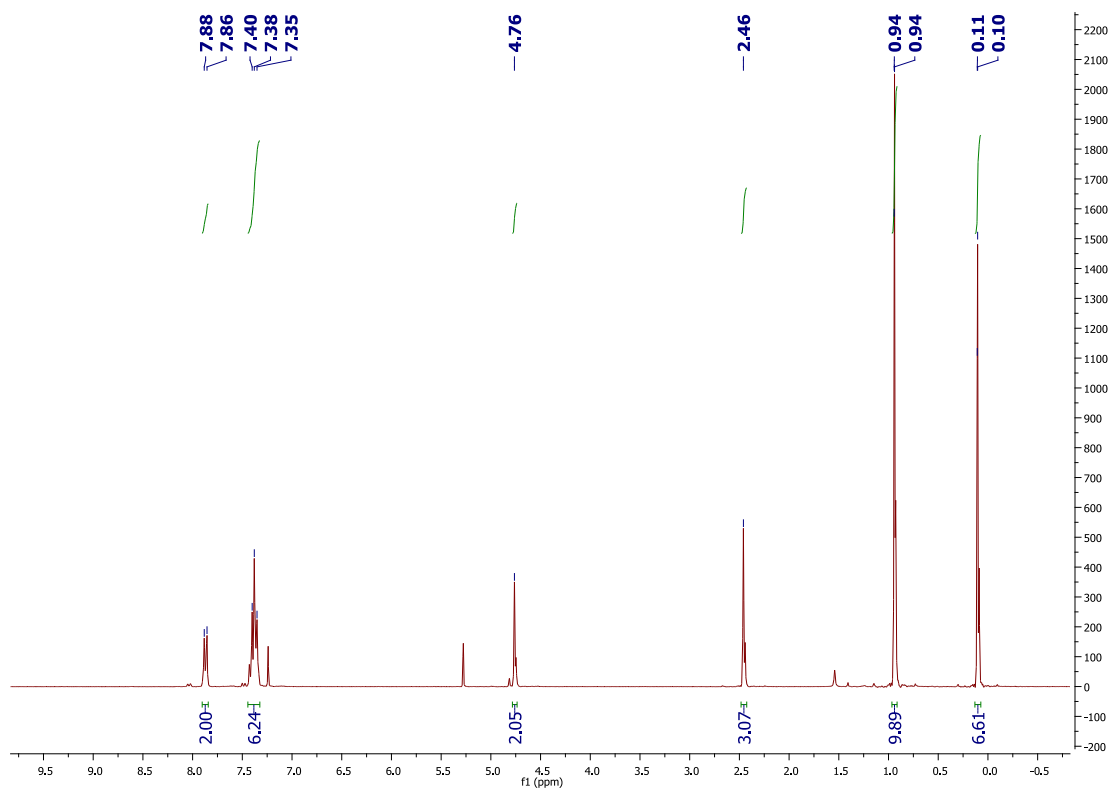


Figure S7. ¹H NMR of compound S5.

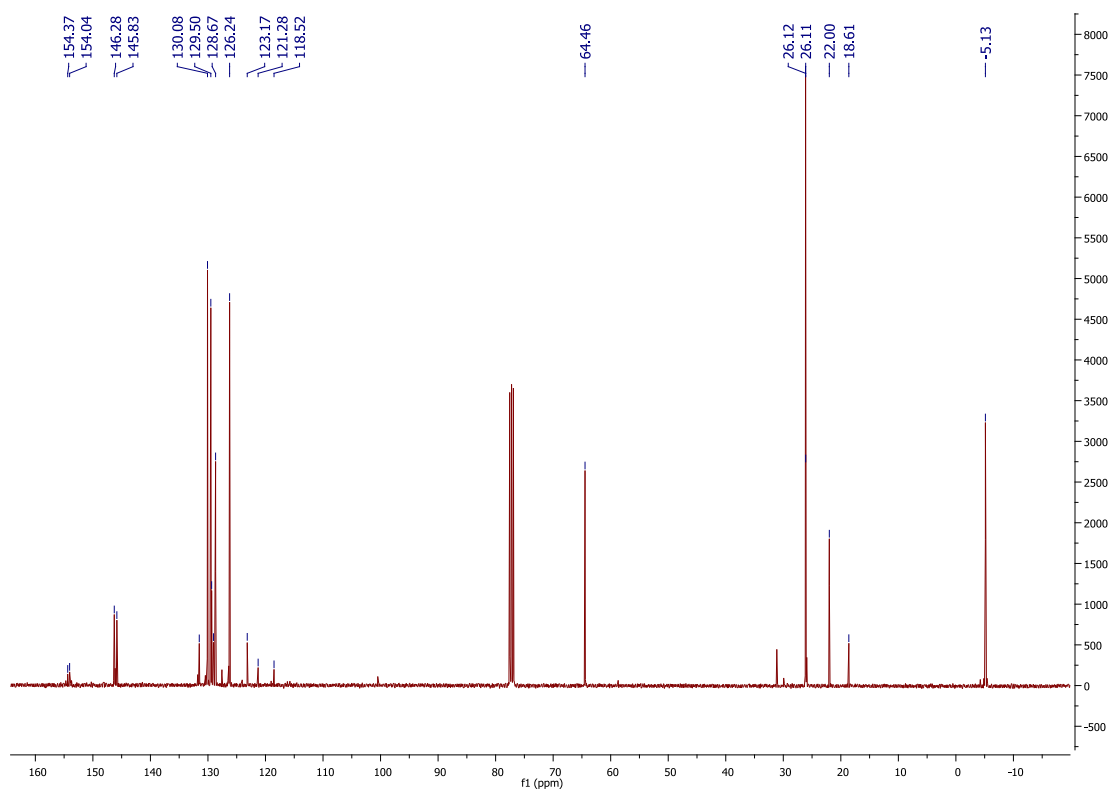


Figure S8. ¹³C NMR of compound S5.

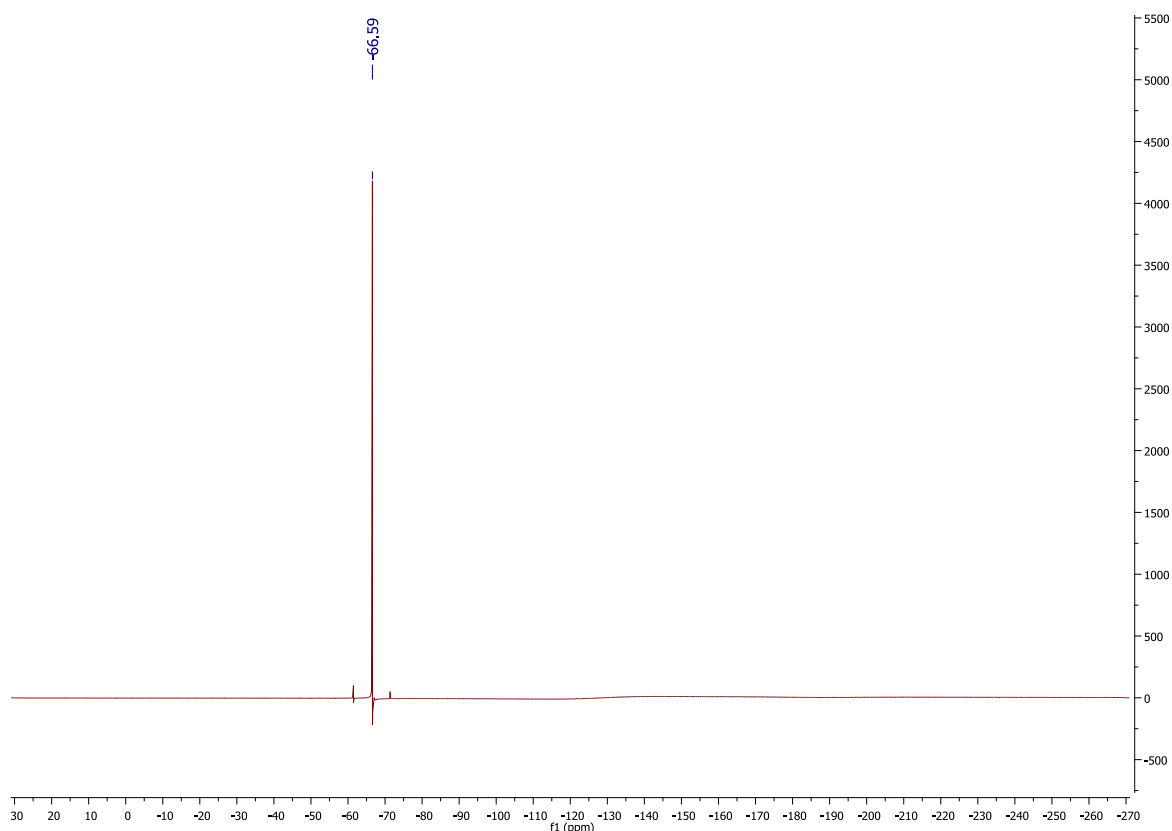
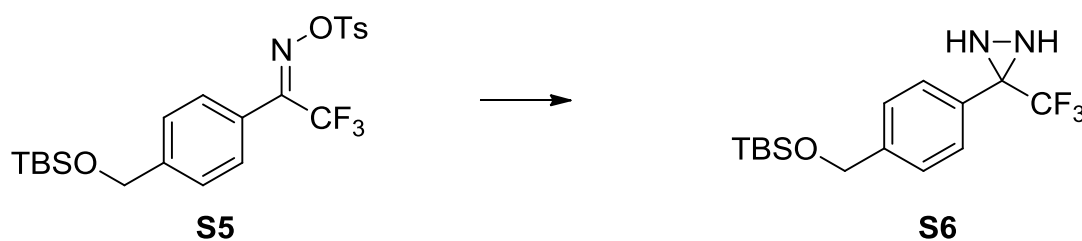


Figure S9. ^{19}F NMR of compound **S5**.

3-(4-(((*tert*-butyldimethylsilyl)oxy)methyl)phenyl)-3-(trifluoromethyl)diaziridine (S6**)**



NH_3 gas was condensed by streaming the gas into a two-necked round-bottom flask submerged in dry ice/acetone, fitted with a cold-finger filled with dry ice/acetone and with a CaCl_2 drying tube.

To a solution of compound **S5** (2.99 g, 6.1 mmol) in dry Et_2O (15 mL) at -78°C in a high pressure reaction vessel was added liquid ammonia gas (20 mL) and sealed. The reaction vessel was allowed to warm to room temperature overnight before re-cooling to -78°C to allow safe removal of the lid. The excess ammonia evaporated upon warming to room temperature and the resulting liquid was filtered to remove the white precipitate, which was

washed with further Et₂O (2x 40 mL). The filtrate was reduced to dryness *in vacuo* to yield 1.93 g (95%) of compound **S6** as a colorless liquid.

Analytical data for compound **S6**

TLC (hexanes/EtOAc 9:1) $R_f = 0.54$

¹H NMR (300 MHz, CDCl₃) δ 7.56 (d, $J = 8.1$ Hz, 2H, H-arom.), 7.36 (d, $J = 8.5$ Hz, 2H, H-arom.), 4.74 (s, 2H, CH₂), 2.76 (d, $J = 8.5$ Hz, 1H, NH), 2.19 (d, $J = 8.7$ Hz, 1H) NH, 0.93 (s, 9H, CH₃-*t*-butyl-Si), 0.09 (s, 6H, CH₃-Si).

¹³C NMR (75 MHz, CDCl₃) δ 144.0, 130.4, 128.2, 126.4, 123.8 (d, $J = 278.1$ Hz), 117.7, 64.6, 26.1, 18.6, -5.1.

¹⁹F NMR (376 MHz, CDCl₃) δ -75.6.

ESI⁺-HRMS calculated for C₁₅H₂₃F₃N₂OSi ([M+H]⁺): 333.1605, found: 333.1606.

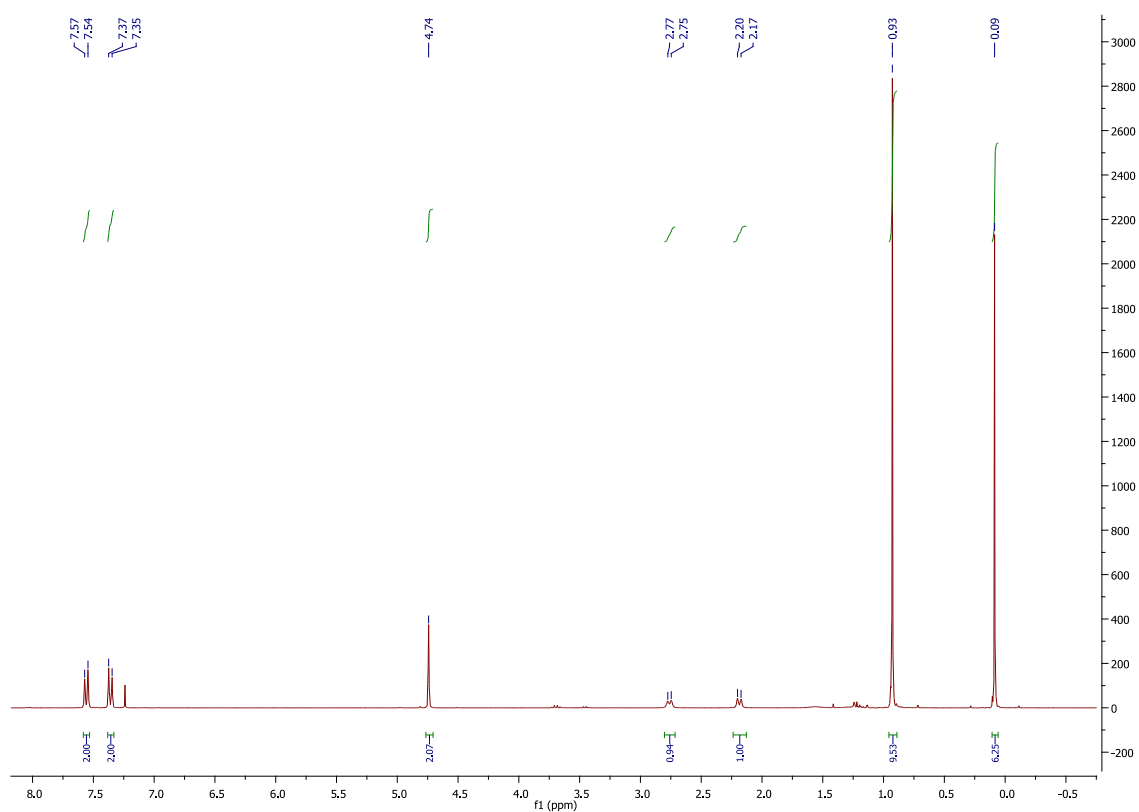


Figure S10. ¹H NMR of Compound **S6**.

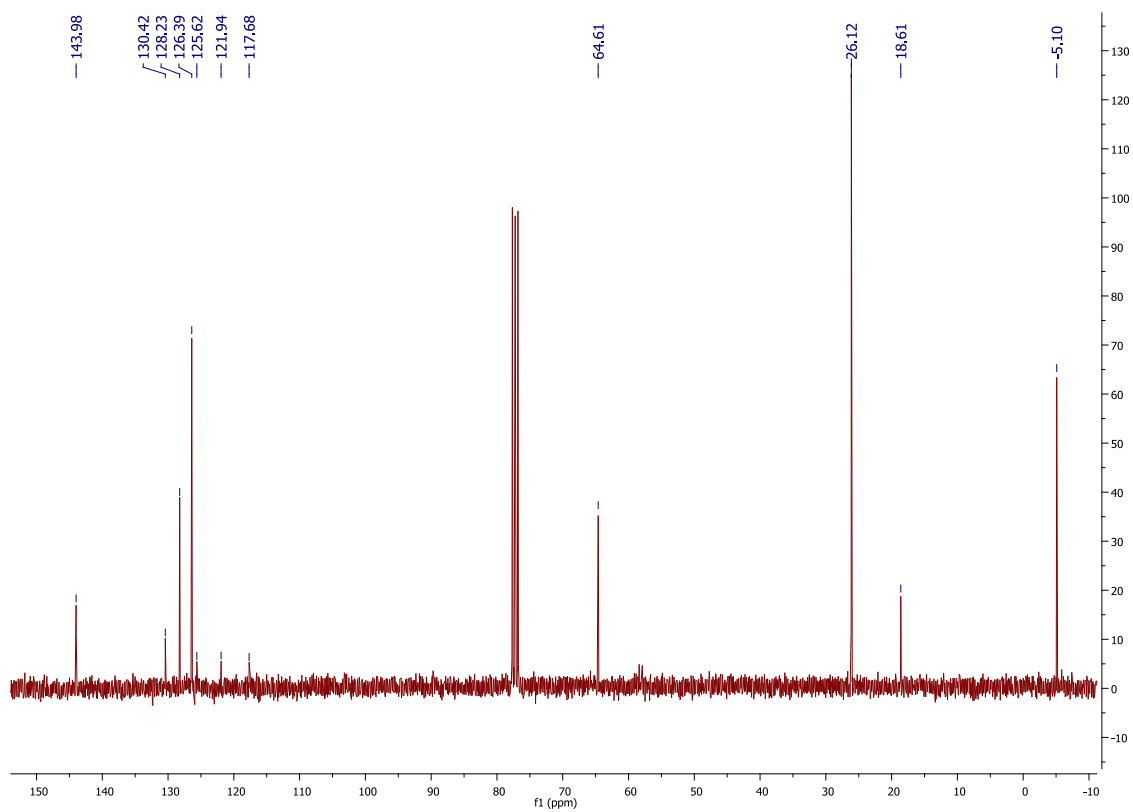


Figure S11. ¹³C NMR of Compound S6.

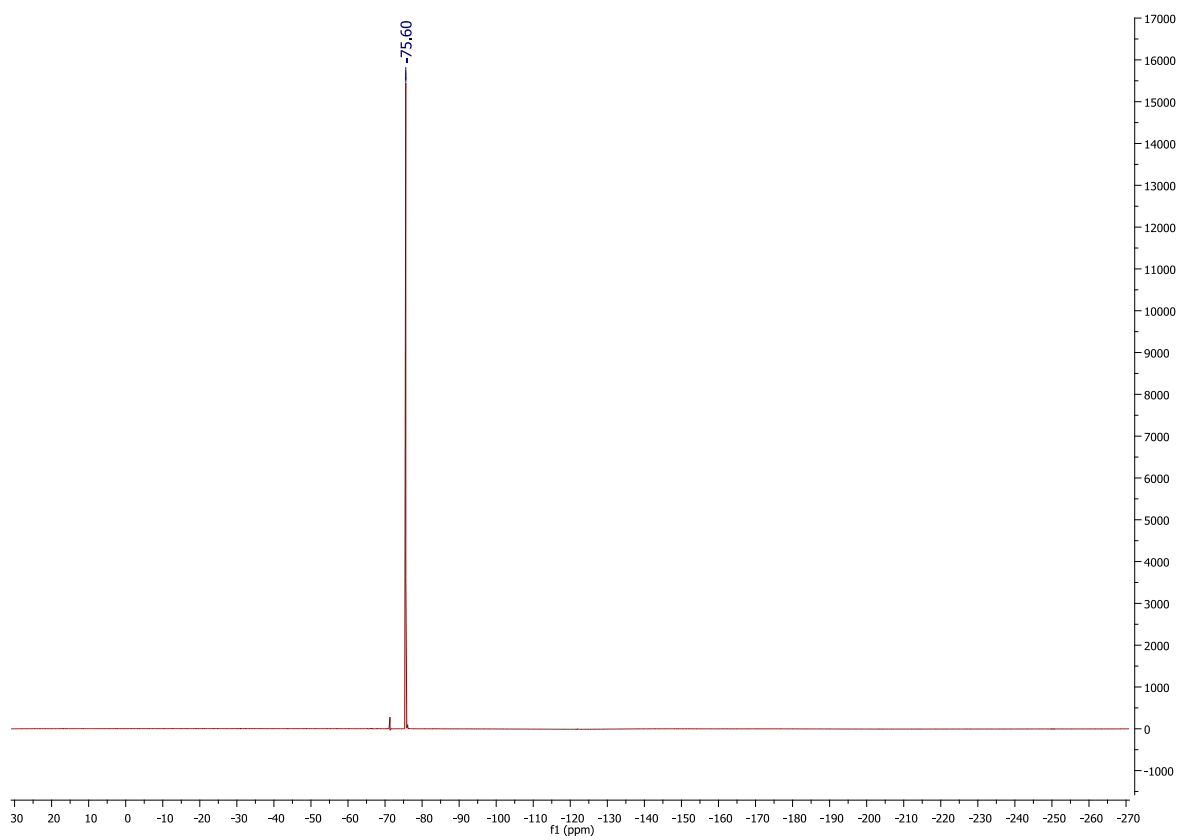
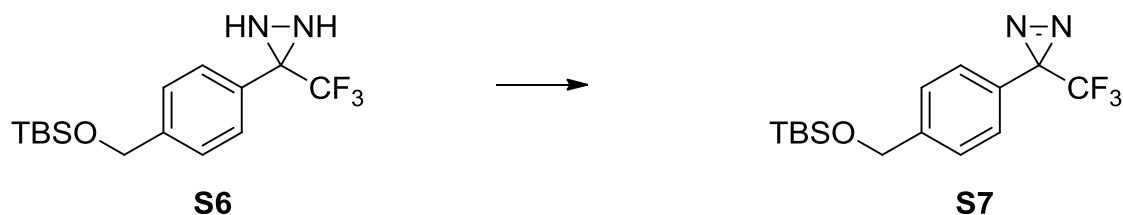


Figure S12. ¹⁹F NMR of Compound S6.

3-(4-(((*tert*-butyldimethylsilyl)oxy)methyl)phenyl)-3-(trifluoromethyl)-3*H*-diazirine (**S7**)



Fresh Ag₂O was prepared before direct use in the reaction. To a stirring solution of AgNO₃ (17.00 g, 0.1 mol) in distilled H₂O (100 mL) at reflux was added 1M NaOH solution (100 mL) dropwise. The brown precipitate was collected by filtration, washed with distilled H₂O (3x 50 mL), acetone (5x 50 mL) and Et₂O (5x 50 mL) with agitation to ensure removal of all remaining H₂O. The resulting brown powder was used immediately.

To a stirring solution of compound **S6** (3.02 g, 9.1 mmol) in dry Et₂O (60 mL) was added the Ag₂O as described above, and was stirred at room temperature for 4 hours in the dark. The reaction mixture was then filtered over celite, washed with further Et₂O (100 mL) and reduced to dryness *in vacuo* to yield 2.86 g (95%) of compound **S7** as a pale yellow liquid.

Analytical data for compound **S7**

TLC (hexanes/EtOAc 9:1) *R_f* = 0.89

¹H NMR (300 MHz, CDCl₃) δ 7.34 (d, *J* = 8.3 Hz, 2H, H-arom.), 7.15 (d, *J* = 8.2 Hz, 2H, H-arom.), 4.73 (s, 2H, CH₂), 0.94 – 0.90 (m, 9H, CH₃-*t*-butyl-Si), 0.08 (s, 6H, CH₃-Si).

¹³C NMR (101 MHz, CDCl₃) δ 143.6, 128.4, 126.6, 126.4, 126.5, 124.2, 64.5, 26.1, 18.6, -5.1.

¹⁹F NMR (376 MHz, CDCl₃) δ -65.3.

ESI⁺MS calculated for C₁₅H₂₁F₃NOSi ([M+Na]⁺): 353.1273, found: 353.27. Exact mass could not be determined, but all other analytical data are in full agreement with the literature.⁴

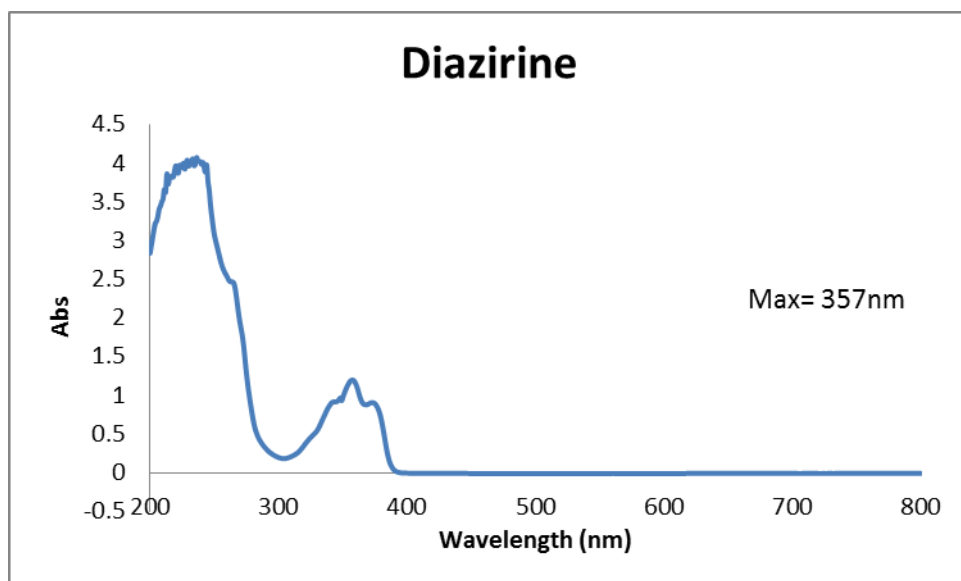


Figure S13. UV absorbance spectrum of compound **S7**.

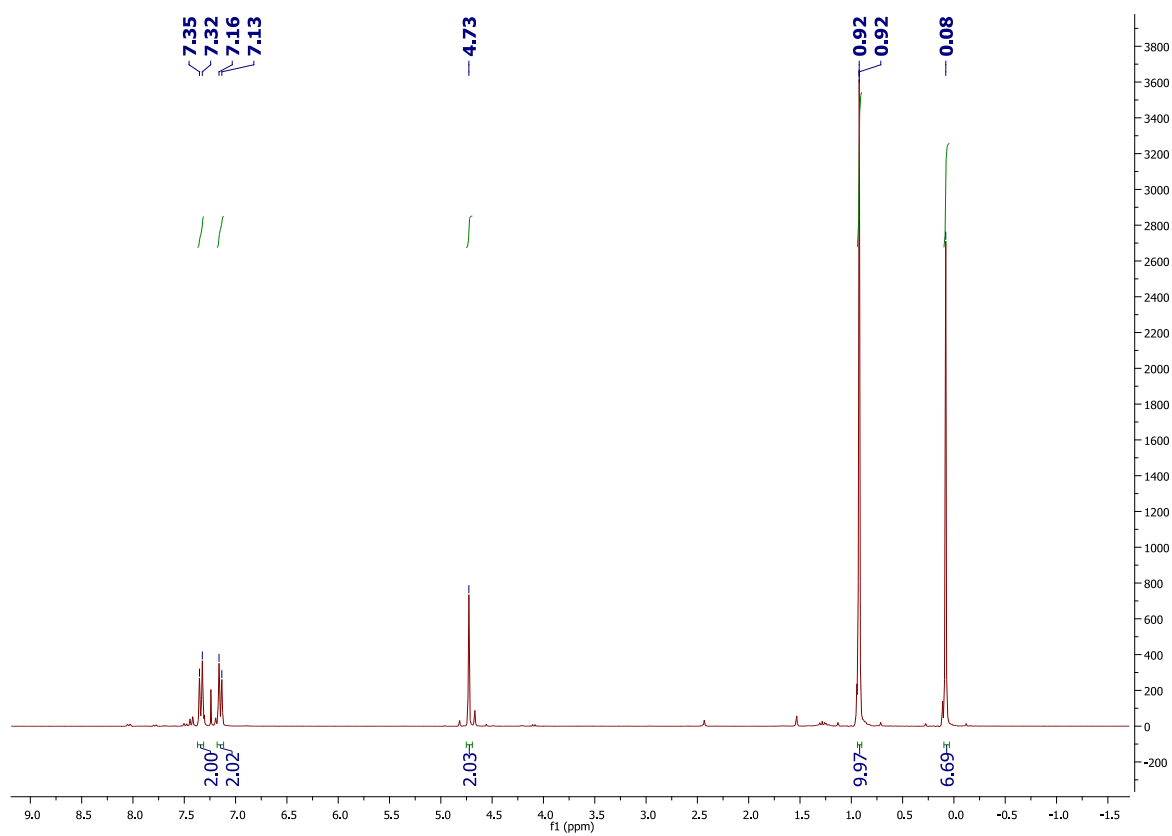


Figure S14. ¹H NMR of compound **S7**.

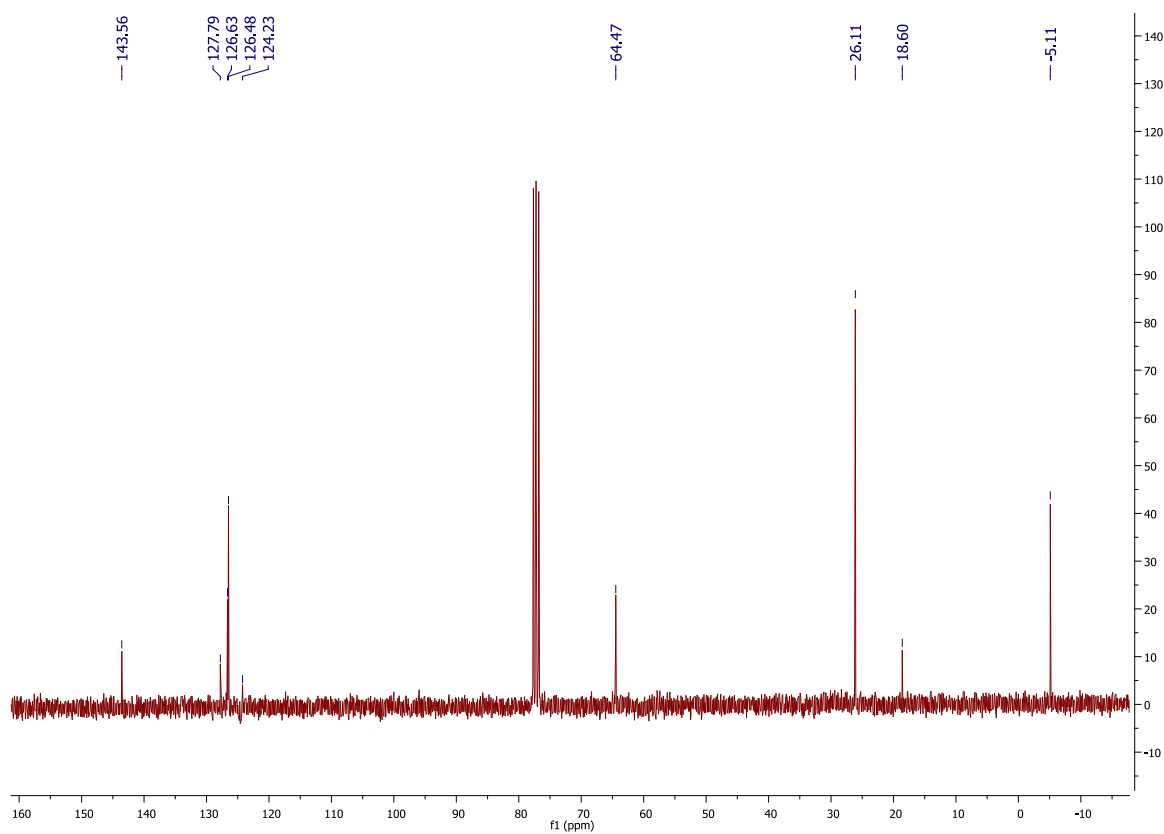


Figure S15. ¹³C NMR of compound S7.

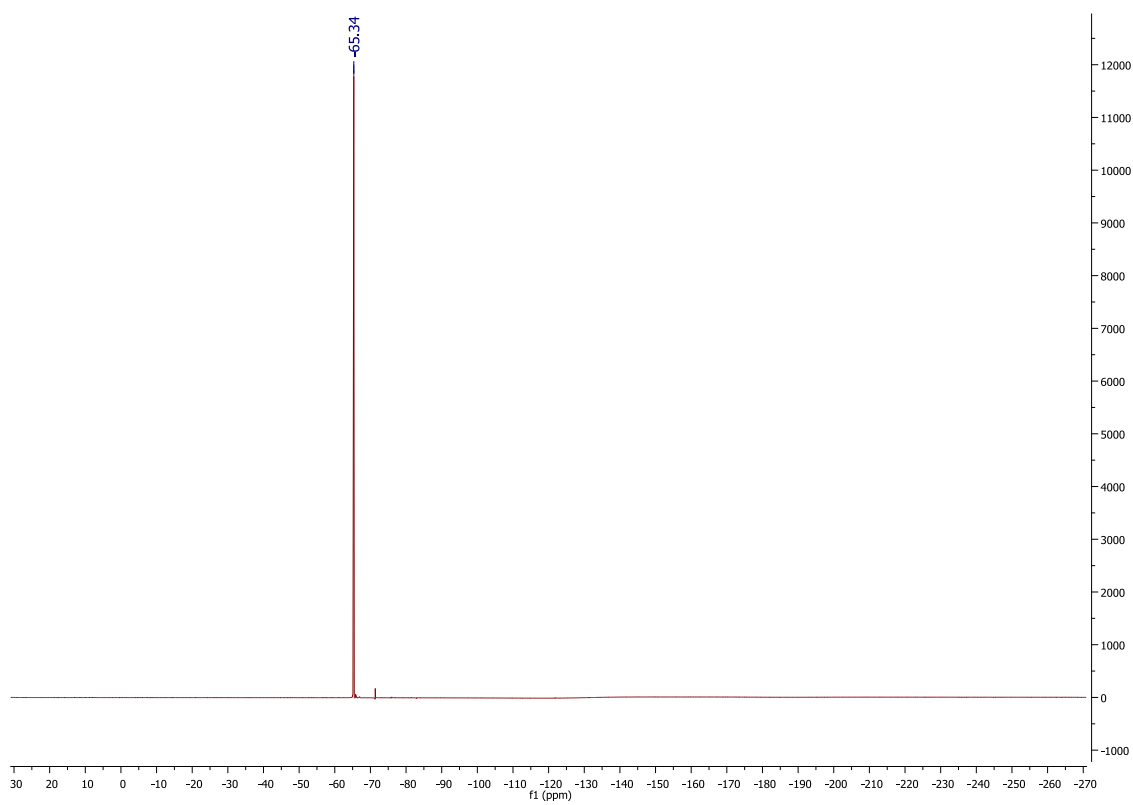
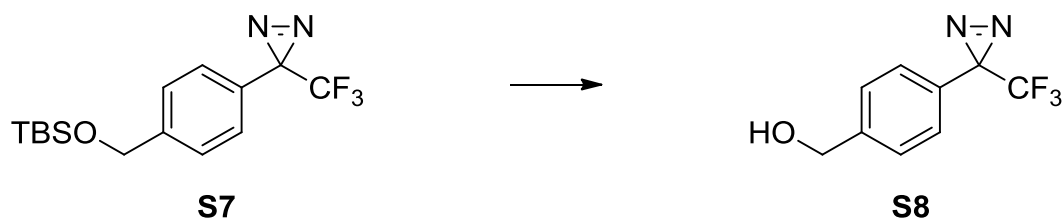


Figure S16. ¹⁹F NMR of compound S7.

(4-(3-(trifluoromethyl)-3H-diazirin-3-yl)phenyl)methanol (S8)



To a stirring solution of compound **S7** (2.86 g, 8.7 mmol) in MeOH (20 mL) at 0°C was added conc. HCl (2 mL) dropwise over 5 minutes. The resulting solution was stirred for 30 minutes before quenching with sat. NaHCO₃ solution (20 mL). The reaction mixture was diluted with Et₂O (100 mL) and washed with further sat. NaHCO₃ solution (20 mL) and brine (20 mL). resulting organic layer was then dried over MgSO₄, filtered and reduced to dryness *in vacuo* to yield 2.94 g of compound **S8** as a pale yellow solution. Carried through to the next step without further purification.

Analytical data for compound **S8**

TLC (CH₂Cl₂) *R_f* = 0.38

¹H NMR (300 MHz, CDCl₃) δ 7.38 (d, *J* = 8.6 Hz, 2H, CH-arom.), 7.17 (d, *J* = 8.1 Hz, 2H, CH-arom.), 4.70 (s, 2H, CH₂).

¹³C NMR (101 MHz, CDCl₃) δ 142.8, 128.7, 127.4, 126.9, 124.2 (d, *J* = 274.7 Hz), 64.7.

¹⁹F NMR (376 MHz, CDCl₃) δ -65.31.

ESI⁺-HRMS calculated for C₉H₇F₃N₂O ([M+H]⁺): 217.0589, found: 217.0467.

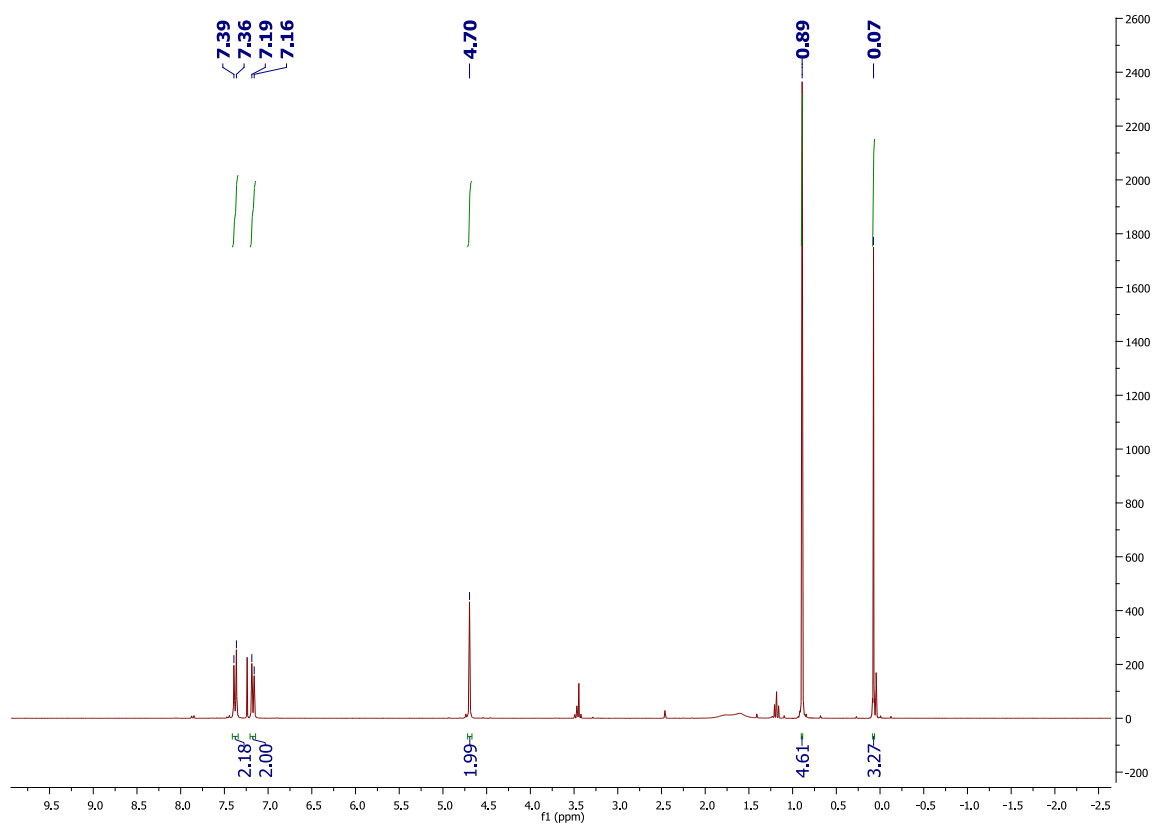


Figure S17. ¹H NMR of compound **S8**.

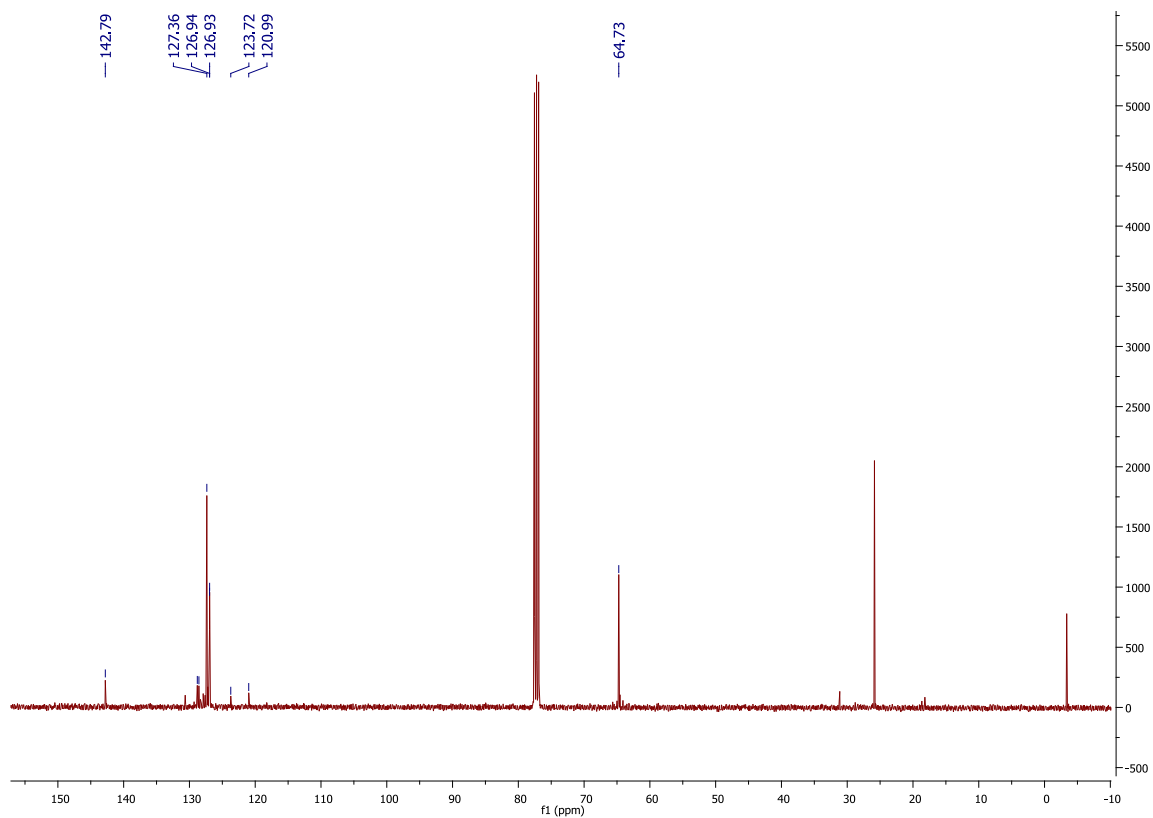


Figure S18. ¹³C NMR of compound **S8**.

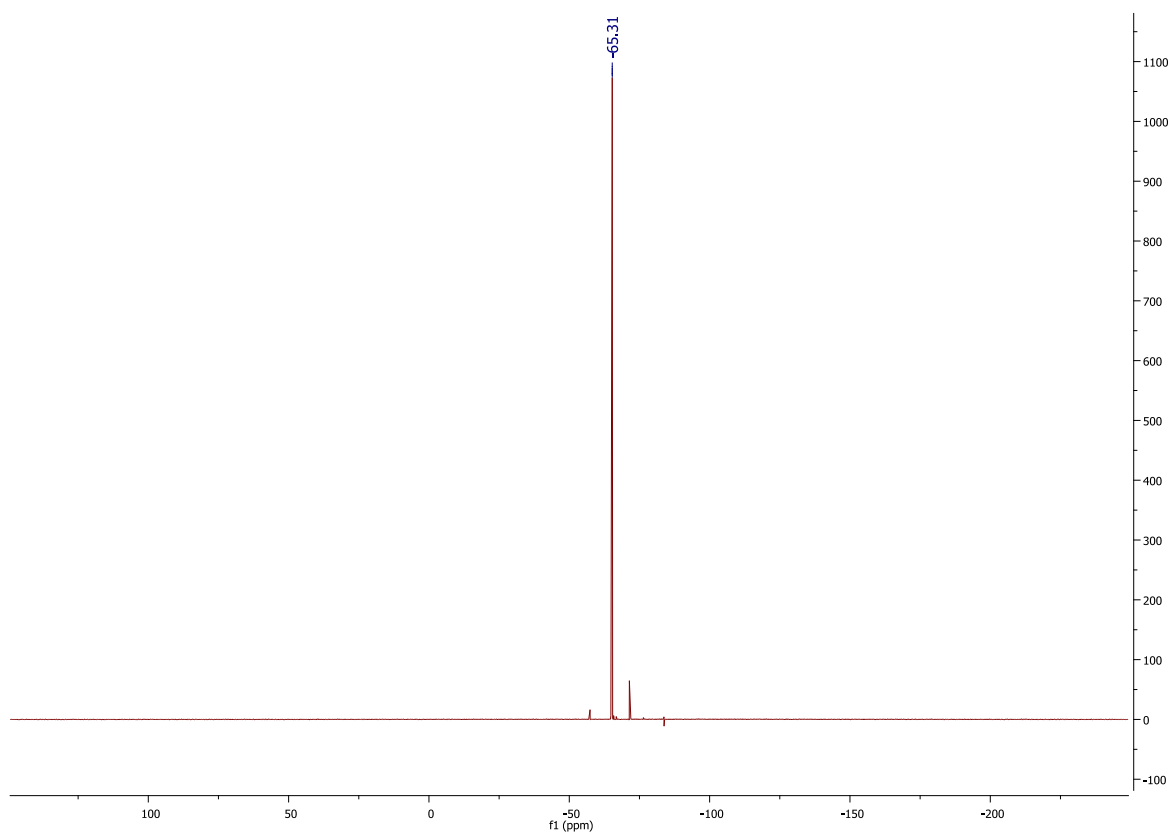
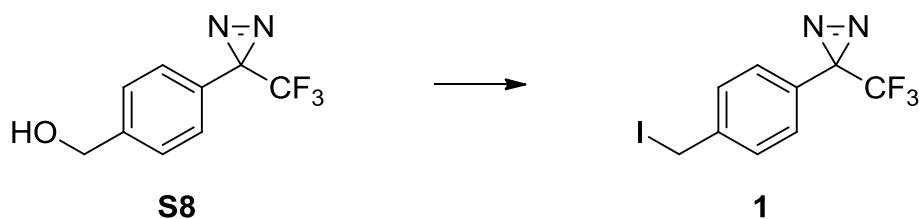


Figure S19. ^{19}F NMR of compound **S8**.

3-(4-(iodomethyl)phenyl)-3-(trifluoromethyl)-3H-diazirine (1)



To a stirring solution of compound **S8** (1.87 g, 8.7 mmol) in dry MeCN (20 mL) in the dark was added methyltriphenoxyposphonium iodide (6.32g, 13.9 mmol) and the reaction mixture was stirred at room temperature for 4.5 hours. A 10% Na_2SO_3 solution (20 mL) was added to quench the reaction and remove excess I_2 . The reaction mixture was then diluted with Et_2O (100mL) and was washed with further 10% Na_2SO_3 solution (10 mL), followed by brine (10 mL). The resulting organic layer was then dried over MgSO_4 , filtered and reduced to dryness *in vacuo*. The residue was dissolved in hexanes/ CH_2Cl_2 7:3 and purified by column chromatography (hexanes/ CH_2Cl_2 7:3) to yield 2.74 g (97%) of a pale yellow liquid.

Even though traces of impurities were detected, compound **1** was used in the next step without further purification.

Analytical data for compound **1**

TLC (hexanes/ CH_2Cl_2 9:1) $R_f = 0.62$

^1H NMR (300 MHz, CDCl_3) δ 7.38 (d, $J = 8.4$ Hz, 2H, H-arom.), 7.09 (d, $J = 8.2$ Hz, 2H, H-arom.), 4.40 (s, 2H, CH₂).

^{13}C NMR (101 MHz, CDCl_3) δ 141.3, 129.4, 129.0 (d, $J = 8.8$ Hz) 128.9, 127.2, 127.1, 122.3 (d, $J = 274.7$ Hz), 3.8.

^{19}F NMR (376 MHz, CDCl_3) δ -65.2.

ESI⁺HRMS calculated for $\text{C}_9\text{H}_6\text{F}_3\text{IN}_2$ ($[\text{M}+\text{H}]^+$) 325.9528. Exact mass could not be determined, but all other analytical data are in full agreement with the literature.⁴

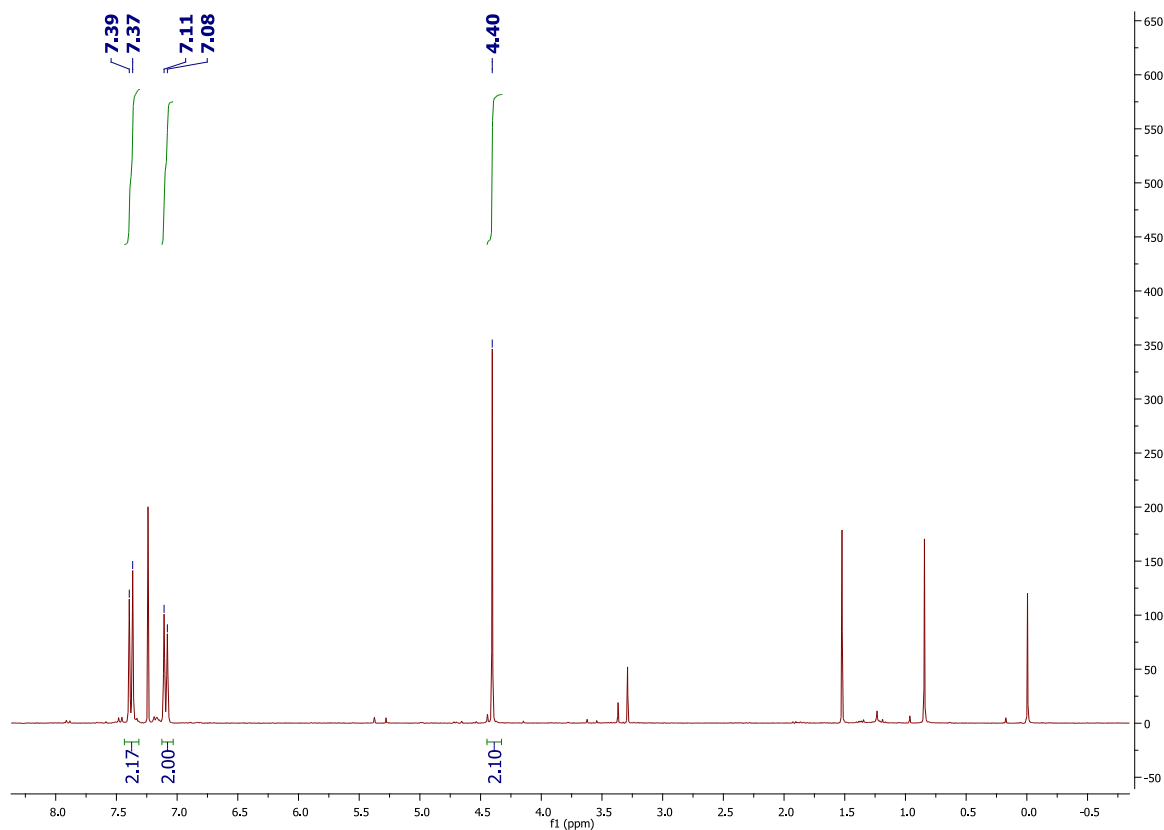


Figure S20. ^1H NMR of compound **1**.

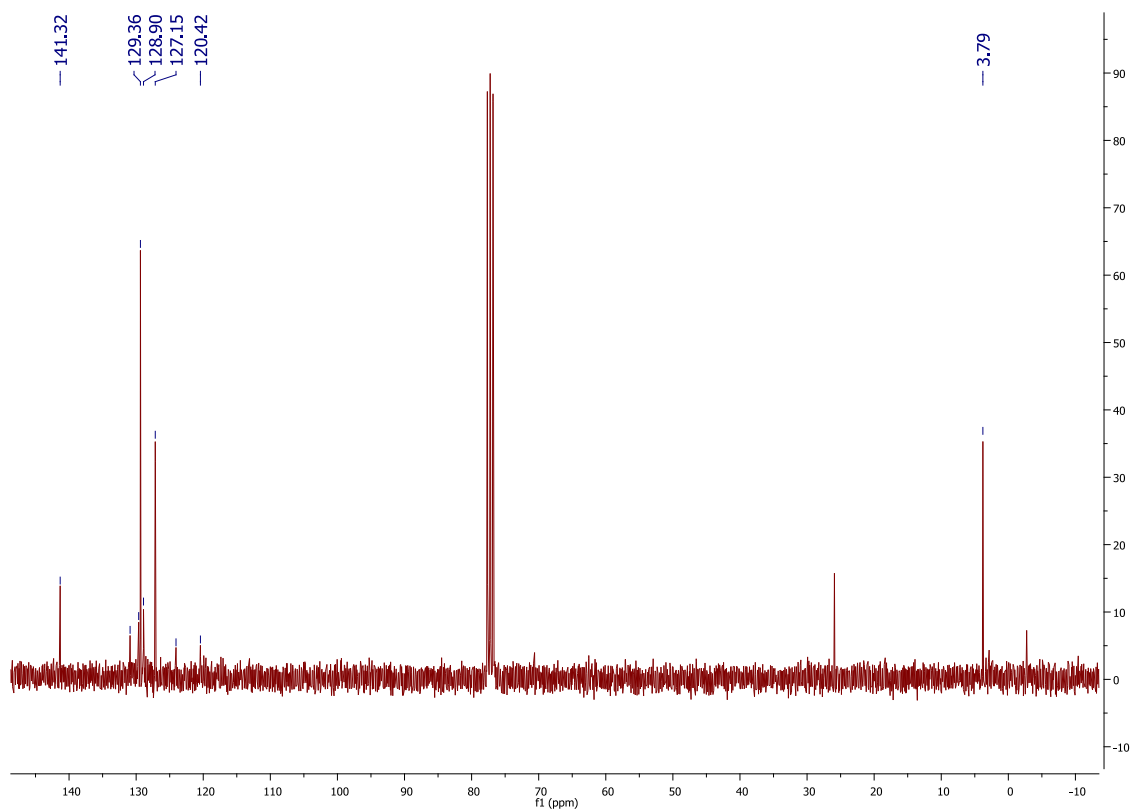


Figure S21. ^{13}C NMR of compound **1**.

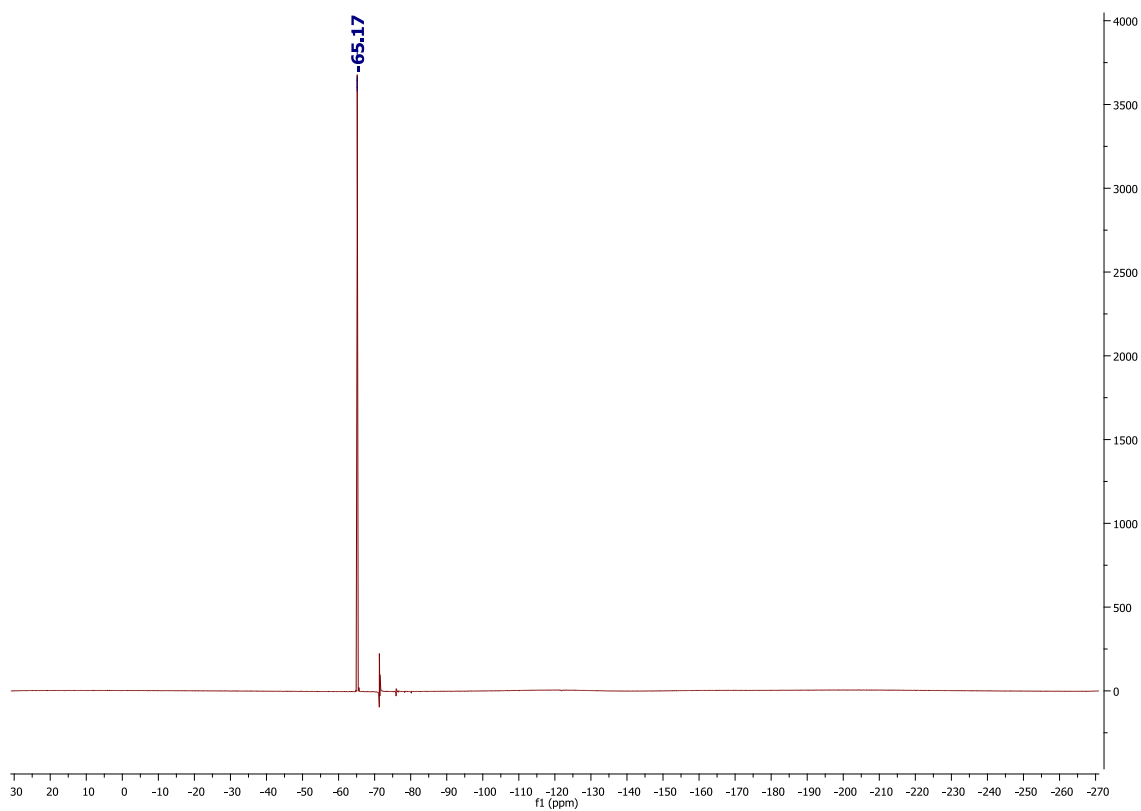
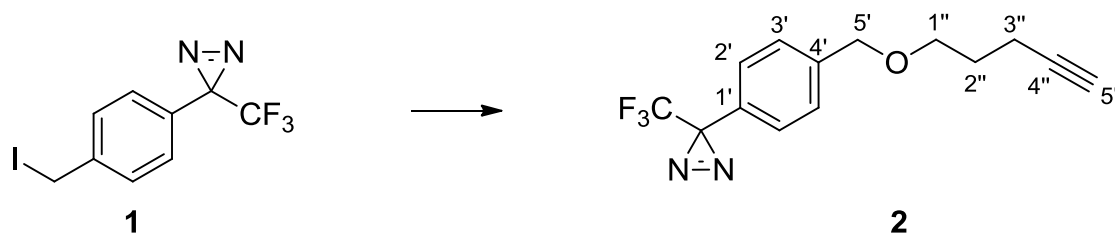


Figure S22. ^{19}F NMR of compound **1**.

3-(4-((pent-4-yn-1-yloxy)methyl)phenyl)-3-(trifluoromethyl)-3H-diazirine (**2**)



To a stirring solution of 4-pentyn-1-ol (0.44 mL, 4.8 mmol) in dry THF (4 mL) at 0°C under Ar was added NaH (128 mg, 60% dispersion in mineral oil, 3.2 mmol) portionwise over 5 minutes. The resulting suspension was stirred until the formation of bubbles ceased. Compound **1** (520mg, 1.6 mmol) in dry THF (1 mL) was then added dropwise and the reaction mixture stirred for 2 hours. The reaction was quenched with MeOH (5 mL), diluted with Et₂O (50mL), washed with distilled H₂O (20 mL), brine (20 mL). The resulting organic layer was dried over MgSO₄, filtered and reduced to dryness *in vacuo*. The residue was dissolved in hexanes/CH₂Cl₂ 8:2 and purified by column chromatography (hexanes/CH₂Cl₂ 8:2) to yield 309 mg (68%) of compound **2** as a pale yellow liquid.

Analytical data for compound **2**

TLC (hexanes/CH₂Cl₂ 9:1) *R_f* = 0.43

¹H NMR (300 MHz, CDCl₃) δ 7.35 (d, *J* = 8.6 Hz, 2H, H-arom.), 7.16 (d, *J* = 8.1 Hz, 2H, H-arom.), 4.50 (s, 2H, H5'), 3.55 (t, *J* = 6.1 Hz, 2H, H1''), 2.30 (td, *J* = 7.0, 2.6 Hz, 2H, H3''), 1.92 (t, *J* = 2.7 Hz, 1H, H5''), 1.86 – 1.75 (m, 2H, H2'').

¹³C NMR (101 MHz, CDCl₃) δ 140.7, 128.5, 128.0, 126.8, 126.8, 122.4 (d, *J* = 274.6 Hz), 84.0, 72.4, 69.1, 68.8, 28.8, 15.5.

¹⁹F NMR (376 MHz, CDCl₃) δ -65.3.

ESI⁺-HRMS calculated for C₁₃H₁₄F₃N₂O ([M+Na]⁺): 305.0872, found: 305.0875.

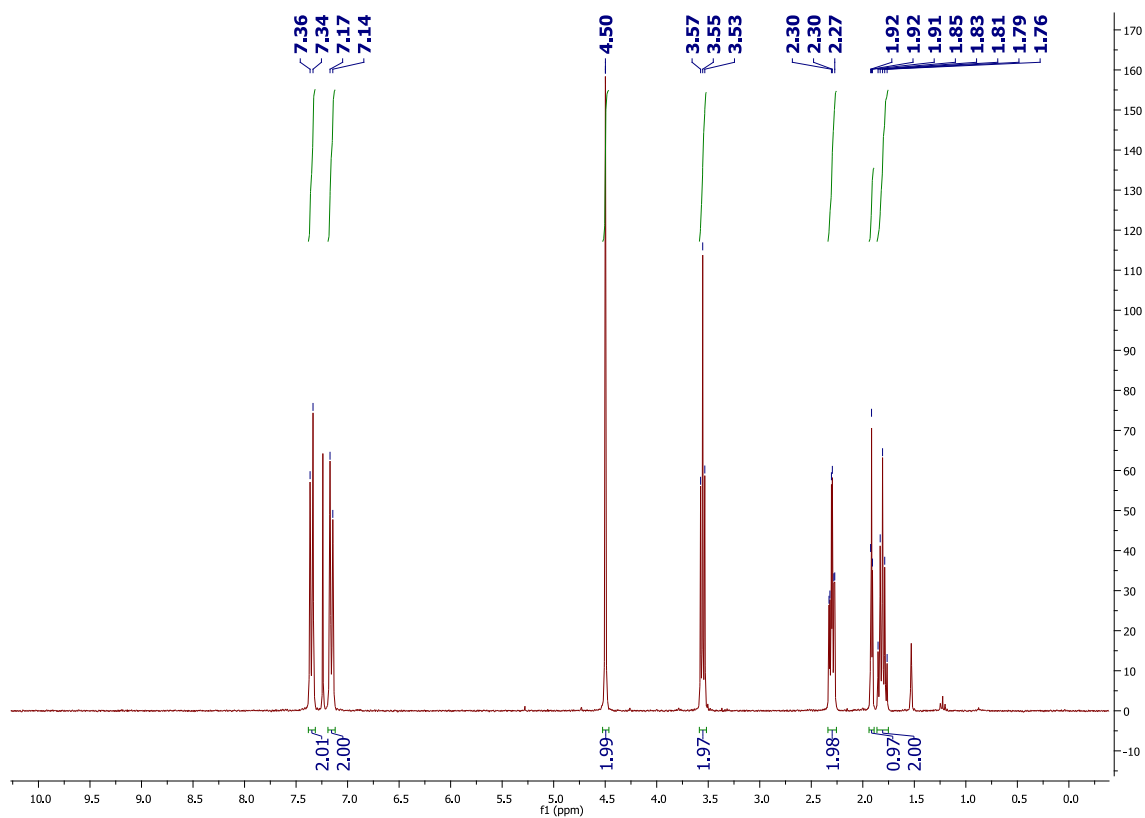


Figure S23. ¹H NMR of compound 2.

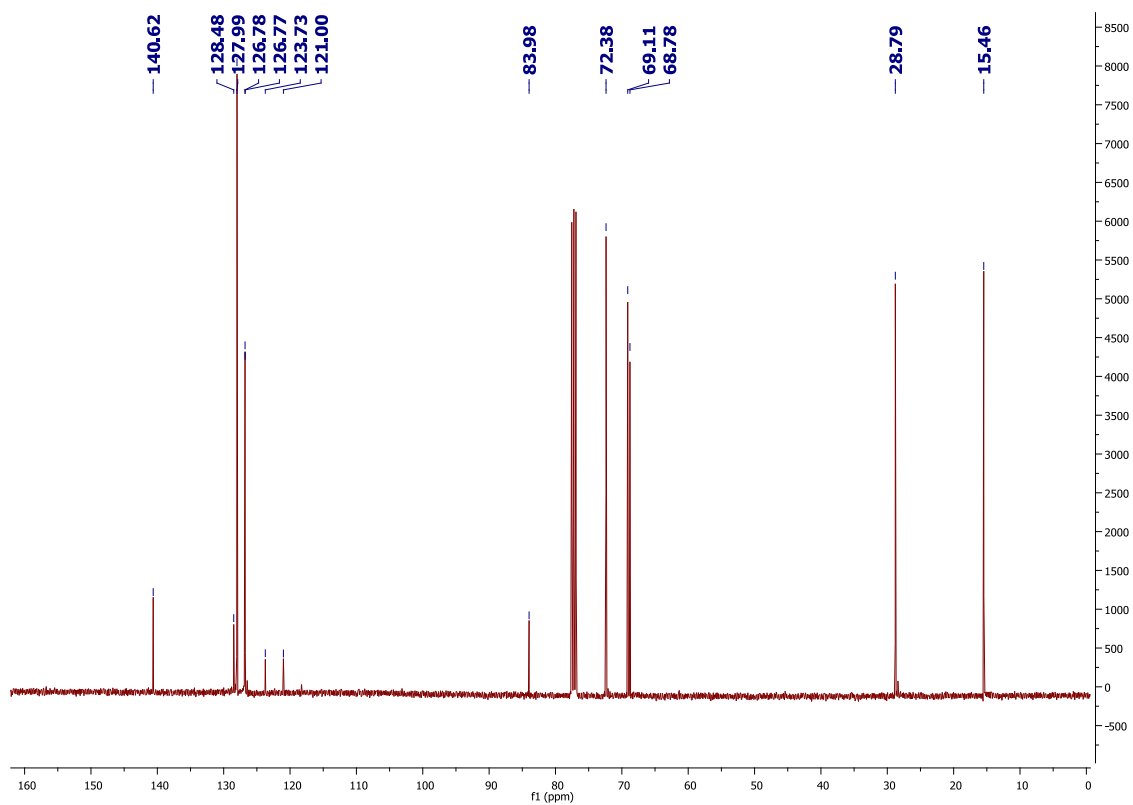


Figure S24. ¹³C NMR of compound 2.

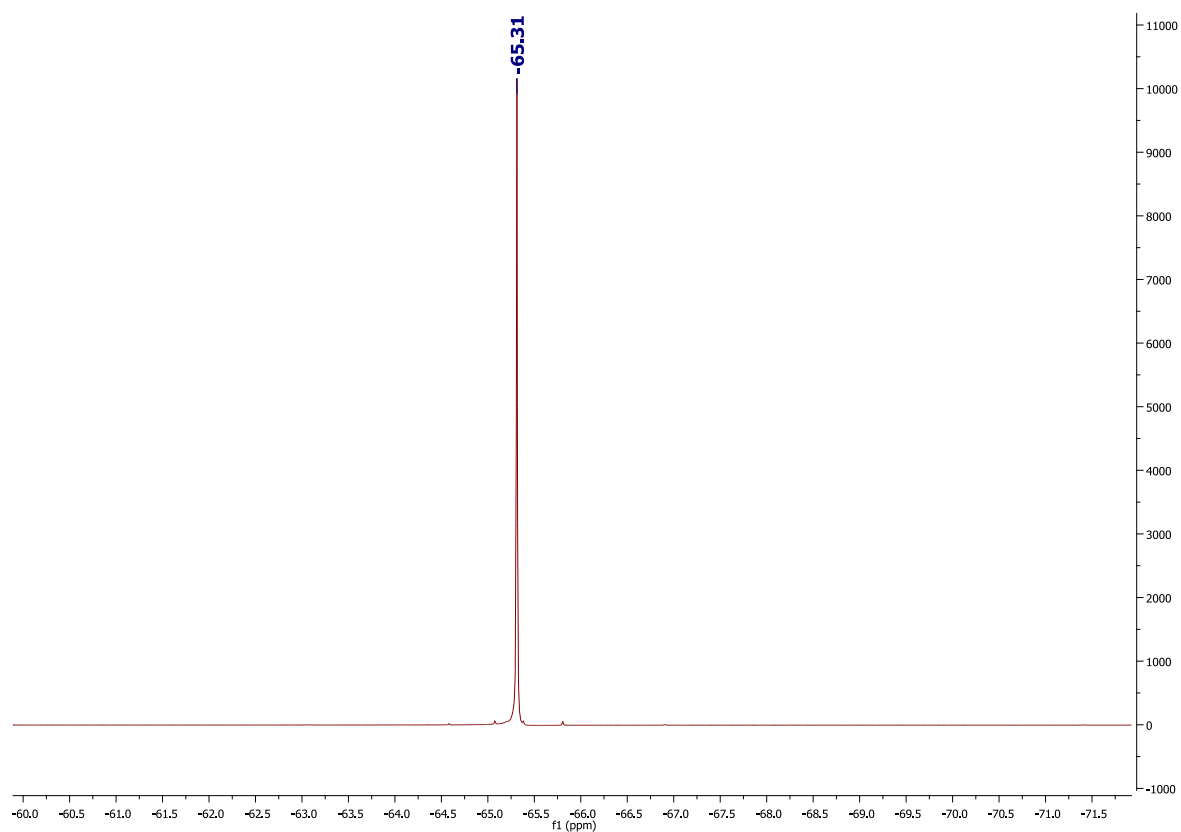
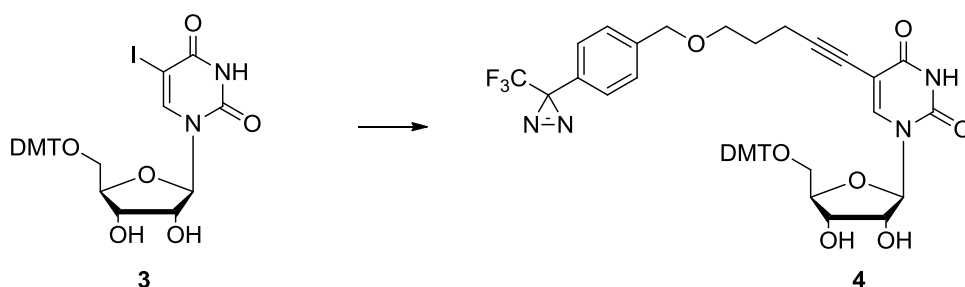


Figure S25. ^{19}F NMR of compound **2**.

5'-O-(4,4'-dimethoxytrityl)-5-(pent-1''-yn-5''-oxy-(methyl-phenyl-10''-(trifluoromethyl)-3*H*-diazirin)-1''-yl)-uridine (4**)**



A solution of compounds **3** (1.15g . 1.71 mmol) and **2** (724 mg, 2.57 mmol) in dry DMF (4 mL) was degassed at -78°C and kept under Ar. CuI (65 mg, 0.34 mmol), Et_3N (0.45 mL, 3.42 mmol) and $\text{Pd}(\text{PPh}_3)_2\text{Cl}_2$ (120 mg, 0.17 mmol) were added in turn and the reaction mixture was stirred at room temperature in the dark for 18 hours. The reaction mixture was then

diluted with EtOAc (50 mL) and washed with NaHCO₃ (2x 10 mL), brine (10 mL), dried over MgSO₄, filtered and reduced to dryness *in vacuo*. The residue was dissolved in CH₂Cl₂ and purified by column chromatography (0-3% MeOH in CH₂Cl₂) and to yield 1.19 g (84%) of compound **4** as a pale orange foam.

Analytical data for compound **4**

TLC (CH₂Cl₂/MeOH 95:5) *R_f* = 0.41

¹H NMR (300 MHz, CDCl₃) δ 8.05 (s, 1H, H6), 7.40 (d, *J* = 7.3 Hz, 2H, H-arom.), 7.34 – 7.07 (m, 11H, H-arom.), 6.80 (d, *J* = 8.1 Hz, 4H, H-arom.), 5.84 (d, *J* = 3.8 Hz, 1H, H1'), 4.43 – 4.31 (m, 4H, H6'', H2' and H3'), 4.22 (s, 1H, H4'), 3.72 (d, *J* = 0.4 Hz, 6H, CH3-*O*-phenyl), 3.47 – 3.25 (m, 4H, H5' and H5''), 2.17 (m, 2H, H3''), 1.51 (p, *J* = 6.7 Hz, 2H, H4'').

¹³C NMR (101 MHz, CDCl₃) δ 162.6, 158.8, 150.6, 144.7, 141.8, 140.7, 135.8, 135.7, 130.2, 130.2, 128.3, 128.2, 128.1, 127.9, 127.1, 126.7, 122.4 (d, *J* = 274.7 Hz), 113.5, 101.3, 95.0, 91.2, 87.2, 84.9, 76.1, 72.1, 71.1, 70.9, 69.3, 62.8, 55.4, 28.5, 16.5.

¹⁹F NMR (376 MHz, CDCl₃) δ -65.3.

ESI⁺-HRMS calculated for C₄₄H₄₁F₃N₄O₉ ([M+Na]⁺): 849.2718, found: 849.2697.

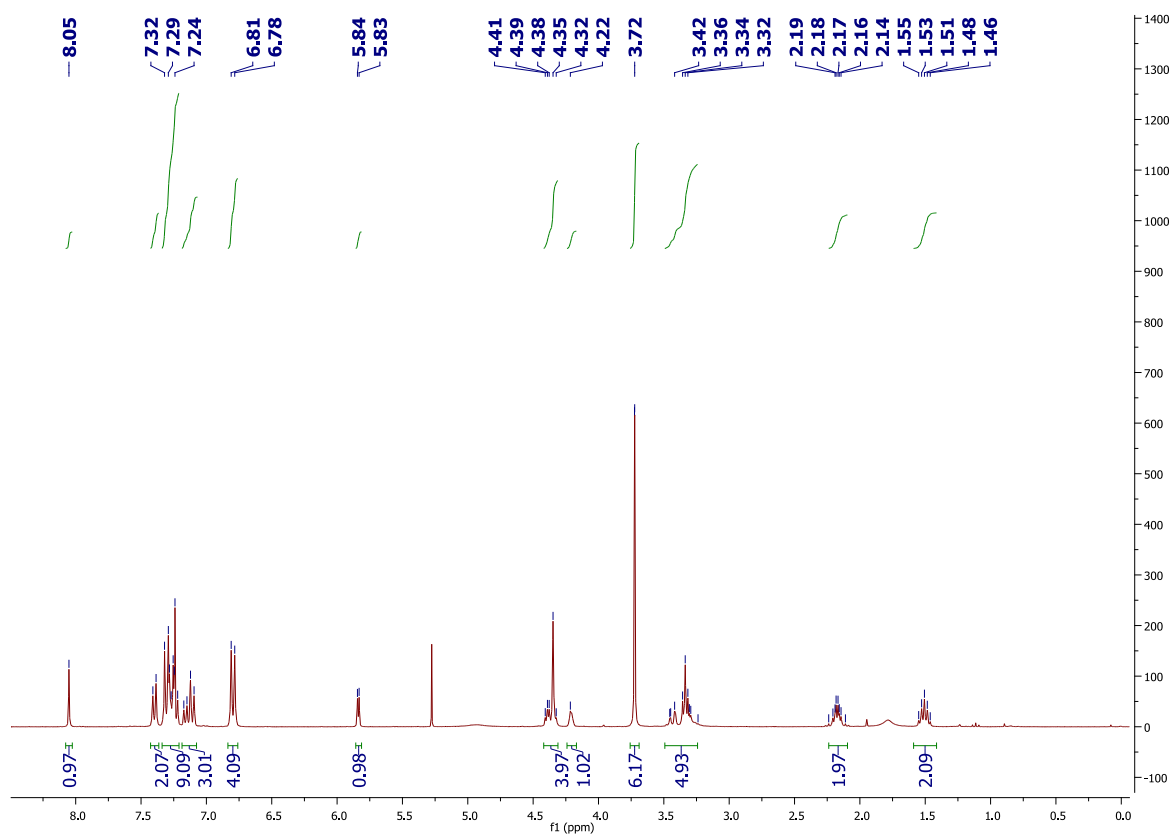


Figure S26. ¹H NMR of compound 4.

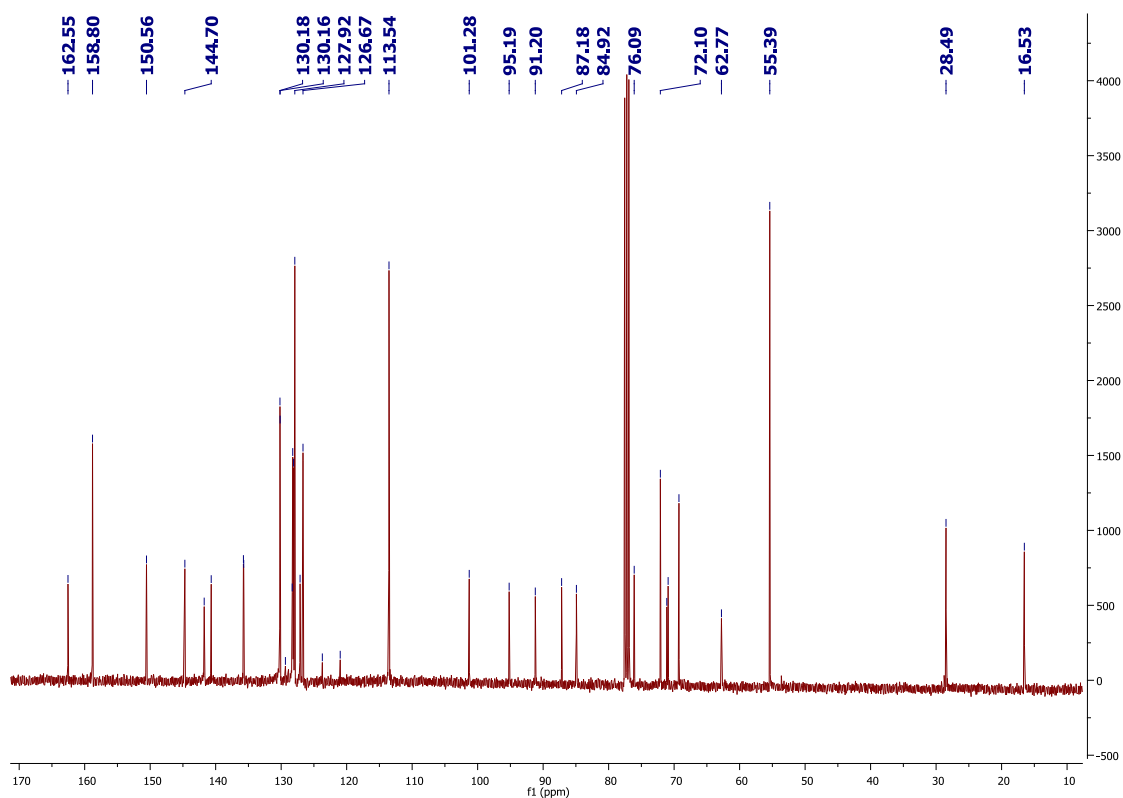


Figure S27. ¹³C NMR of compound 4.

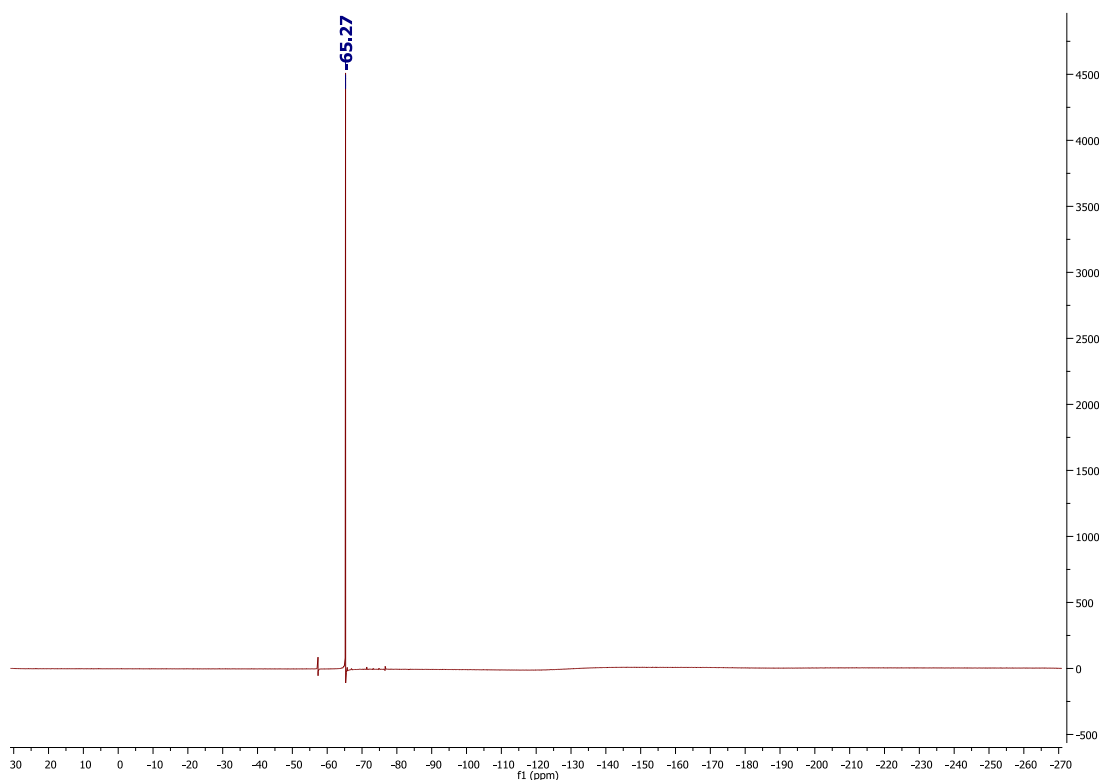
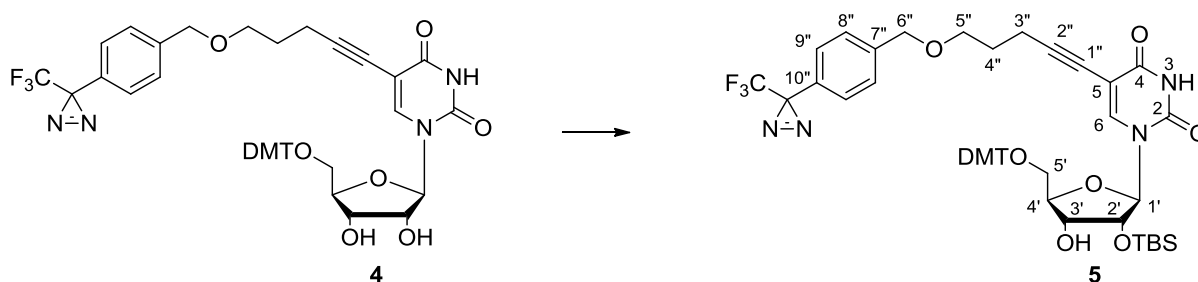


Figure S28. ^{19}F NMR of compound **4**.

2'-O-(*t*-butyldimethylsilyl))-5'-O-(4,4'-dimethoxytrityl)-5-(pent-1''-yn-5''-oxy-(methyl-phenyl-10''-(trifluoromethyl)-3*H*-diazirin)-1''-yl)-uridine (5**)**



To a stirring solution of compound **4** (286 mg, 0.35 mmol) in dry THF (3 mL) in the dark, was added pyridine (0.1 mL, 1.28 mmol) and silver nitrate (71 mg, 0.42 mmol). The solution was stirred at room temperature until the silver nitrate dissolved (~20 minutes). TBDMS-Cl (68 mg, 0.45 mmol) was then added and the reaction mixture stirred for 26 hours at room temperature. The reaction mixture was diluted with EtOAc (30 mL), washed with sat. NaHCO_3 (10 mL), brine (10 mL). The resulting organic layer was dried over MgSO_4 , filtered

and reduced to dryness *in vacuo*. The residue was dissolved in hexanes/EtOAc 8:2 and purified by column chromatography (hexanes/EtOAc 8:2) to yield 234 mg (72%) of compound **5** as a colorless foam.

Analytical data for compound **5**

TLC (CH₂Cl₂/MeOH 99:1) *R_f* = 0.43

¹H NMR (300 MHz, CDCl₃) δ 8.09 (s, 1H, H3), 8.02 (s, 1H, H6), 7.41 (d, *J* = 7.3 Hz, 2H, H-arom.), 7.34 – 7.09 (m, 11H, H-arom.), 6.80 (d, *J* = 8.3 Hz, 4H, H-arom.), 5.98 (d, *J* = 5.5 Hz, 1H, H1'), 4.47 (t, *J* = 5.1 Hz, 1H, H2'), 4.32 (s, 2H, H6''), 4.21 (s, 1H, H3'), 4.13 (d, *J* = 6.3 Hz, 1H, H4'), 3.74 (d, *J* = 1.6 Hz, 6H, CH₃-*O*-phenyl), 3.47 (d, *J* = 11.1 Hz, 1H, H5'), 3.29 (dd, *J* = 13.4, 7.3 Hz, 3H, H5' and H5''), 2.66 (d, *J* = 3.6 Hz, 1H, 3'OH), 2.09 (dd, *J* = 7.3, 5.5 Hz, 2H, H3''), 1.40 (t, *J* = 6.6 Hz, 2H, H4''), 0.89 (s, 9H, CH₃-*t*-butyl-Si), 0.10 (s, 6H, CH₃-Si).

¹³C NMR (101 MHz, CDCl₃) δ 161.7, 158.9, 149.5, 144.7, 141.6, 140.7, 135.6, 135.6, 130.2, 130.2, 128.3, 128.3, 128.1, 127.9, 127.2, 126.7, 122.4 (d, *J* = 274.7 Hz), 113.6, 101.6, 95.0, 88.4, 87.5, 84.3, 76.6, 72.1, 71.7, 70.9, 69.3, 63.4, 55.42, 28.4, 25.81, 18.19, 16.50, -4.57, -4.97.

¹⁹F NMR (376 MHz, CDCl₃) δ -65.3.

ESI⁺-HRMS calculated for C₅₀H₅₅F₃N₄O₉Si ([M+Na]⁺): 963.3583, found: 963.3583.

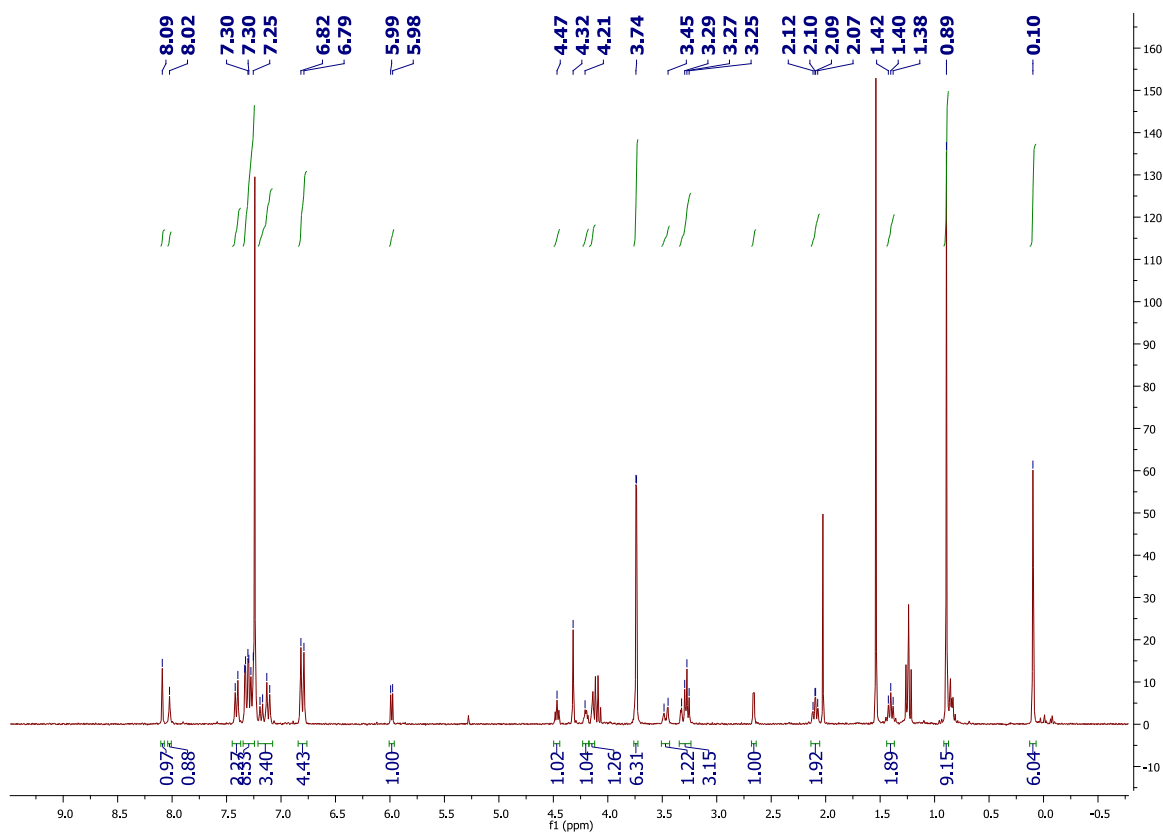


Figure S29. ¹H NMR of compound 5.

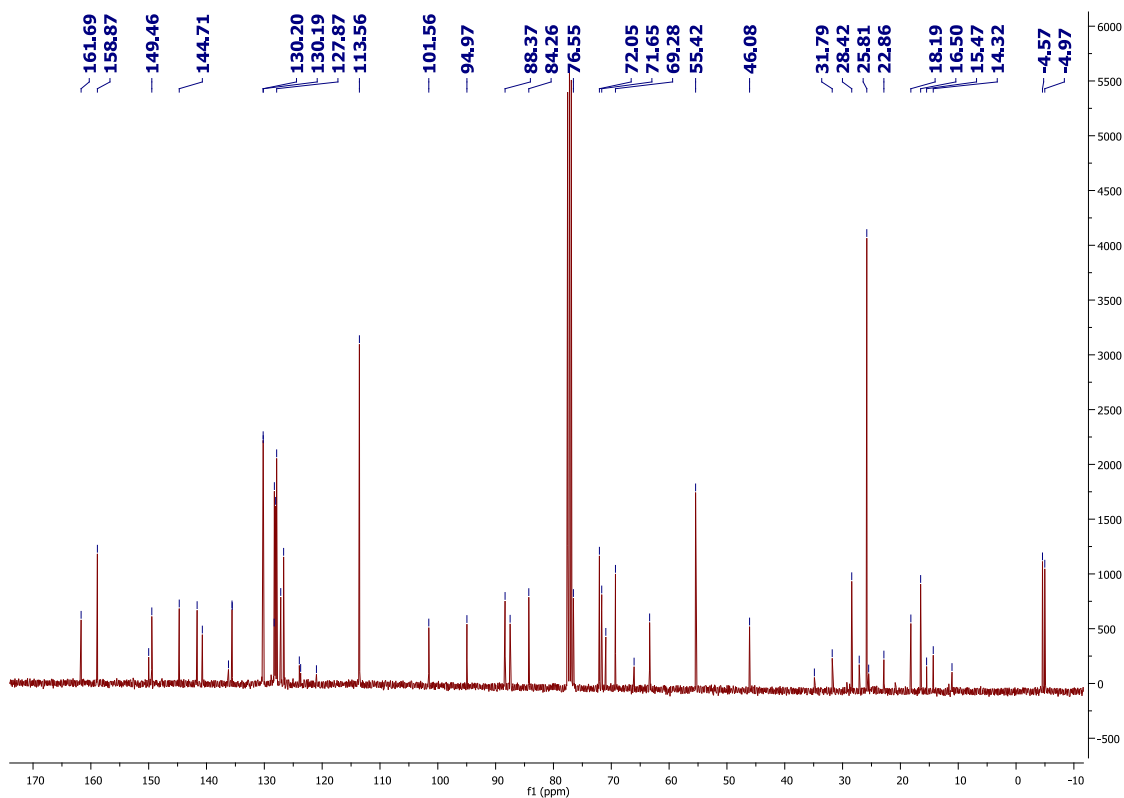


Figure S30. ¹³C NMR of compound 5.

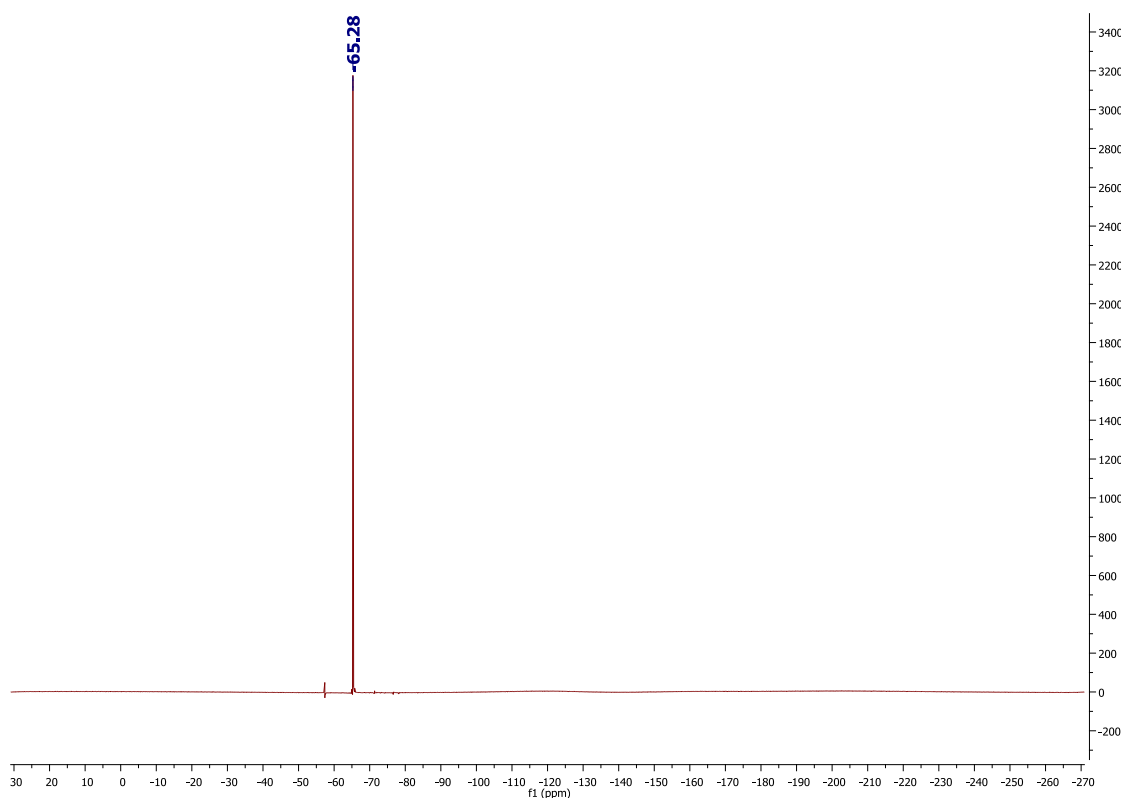
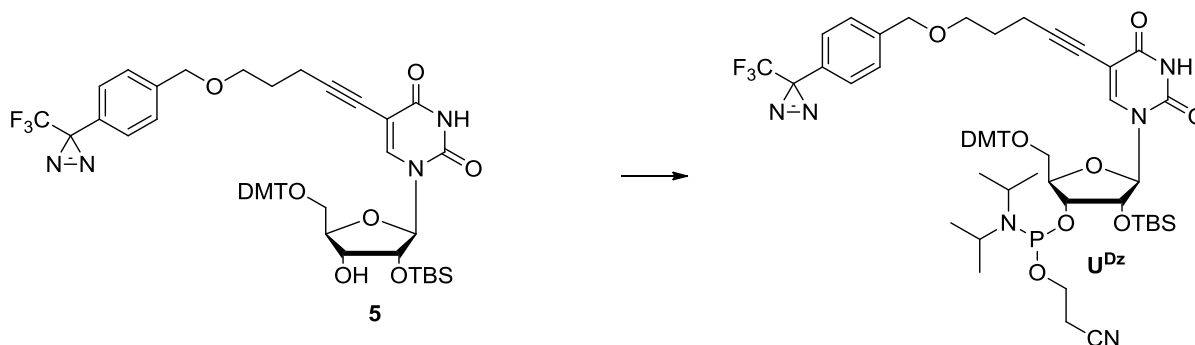


Figure S31. ^{19}F NMR of compound **5**.

2'-O-(*t*-butyldimethylsilyl))-3'-O-(2-cyanoethoxy)diisopropylaminophosphyno-5'-O-(4,4'-dimethoxytrityl)-5-(pent-1"-yn-5"-oxy-(methyl-phenyl-10"- (trifluoromethyl)-3*H*-diazirin)-1"-yl)-uridine (U^{Dz})



To a stirring solution of nucleoside **5** (230 mg, 0.24 mmol) in dry THF (2.5 mL) were added DIPEA (0.26 mL, 1.47 mmol) and CEP-Cl (0.21 mL, 0.73 mmol). The reaction mixture was stirred at room temperature under Ar for 1 hour. The reaction mixture was then diluted with CH_2Cl_2 (20 mL) and washed with sat. NaHCO_3 solution (10 mL) and brine (10 mL). The organic layer was then dried over MgSO_4 , filtered and reduced to dryness *in vacuo*. The

residue was dissolved in hexanes/EtOAc 1:1 and purified by column chromatography (hexanes/EtOAc 1:1). In order to remove the remaining impurities (as determined by ^1H and ^{31}P NMR), the residue was dissolved in CH_2Cl_2 and re-purified by column chromatography (0-2% MeOH in CH_2Cl_2) and reduced to dryness *in vacuo* to yield 208 mg (78%) of U^{Dz} as a colourless foam.

Analytical data for U^{Dz}

TLC ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 98:2) $R_f = 0.93$

^1H NMR (400 MHz, CDCl_3) δ 8.15, 8.10 (2s, 1H, $\underline{\text{H}}_6$), 7.54 – 7.06 (m, 13H, $\underline{\text{H}}$ -arom.), 6.81 (dd, $J = 8.6, 7.2$ Hz, 4H, $\underline{\text{H}}$ -arom.), 6.07 (d, $J = 7.0$ Hz, 0.5H, $\underline{\text{H}}_1$), 5.97 (d, $J = 6.2$ Hz, 0.5H, $\underline{\text{H}}_1$), 4.60 – 4.41 (m, 1H, $\underline{\text{H}}_2'$), 4.38 – 4.23 (m, 2H, $\underline{\text{H}}_6''$), 4.20 – 4.11 (m, 1H, $\underline{\text{H}}_3'$), 3.89 (m, 1H, $\underline{\text{H}}_7'$), 3.76 – 3.71 (m, 6H, $\underline{\text{CH}}_3\text{-O-phenyl}$), 3.63 – 3.40 (m, 4H, $\underline{\text{H}}_7'$, H_{10}' and $\underline{\text{H}}_5'$), 3.32 – 3.18 (m, 3H, $\underline{\text{H}}_5''$ and $\underline{\text{H}}_5'$), 2.77 – 2.21 (m, 2H, $\underline{\text{H}}_8'$), 2.17 – 2.03 (m, 2H, $\underline{\text{H}}_3''$), 1.38 (m, 2H, $\underline{\text{H}}_4''$), 1.22 – 1.08 (m, 9H, $\underline{\text{H}}_{11}'$), 0.98 (d, $J = 6.8$ Hz, 3H, $\underline{\text{H}}_{11}'$), 0.86 (m, 9H, $\underline{\text{CH}}_3\text{-}t\text{-butyl-Si}$), 0.08- -0.02 (m, 6H, $\underline{\text{CH}}_3\text{-Si}$).

^{13}C NMR (101 MHz, CDCl_3) δ 161.6, 161.5, 158.9, 149.5, 149.4, 144.7, 144.7, 141.9, 141.8, 140.8, 135.8, 135.6, 135.5, 130.4, 130.3, 130.2, 128.3, 128.2, 128.0, 127.9, 127.2, 127.2, 126.7, 117.7 (d, $J = 45.7$ Hz), 113.6, 113.5, 101.6, 101.3, 94.8, 88.2, 87.8, 87.6, 87.5, 84.3, 84.3, 76.4, 75.7, 73.7, 73.6, 73.0, 72.1, 72.0, 71.1, 71.0, 69.3, 69.3, 63.7, 63.2, 59.3, 59.2, 57.8, 57.6, 55.4, 43.7, 43.6, 43.2, 43.1, 28.5, 28.4, 26.0, 25.9, 25.0, 24.9, 24.8, 20.8, 20.7, 20.4, 20.3, 18.3, 18.2, 16.5, 16.5, -4.40, -4.54, -4.63, -4.67.

^{19}F NMR (376 MHz, CDCl_3) δ -65.3.

^{31}P NMR (122 MHz, CDCl_3) δ 151.1, 149.2.

ESI $^+$ -HRMS calculated for $\text{C}_{59}\text{H}_{72}\text{F}_3\text{N}_6\text{O}_{10}\text{PSi}$ ($[\text{M}+\text{H}]^+$): 1141.4842, found: 1141.4872.

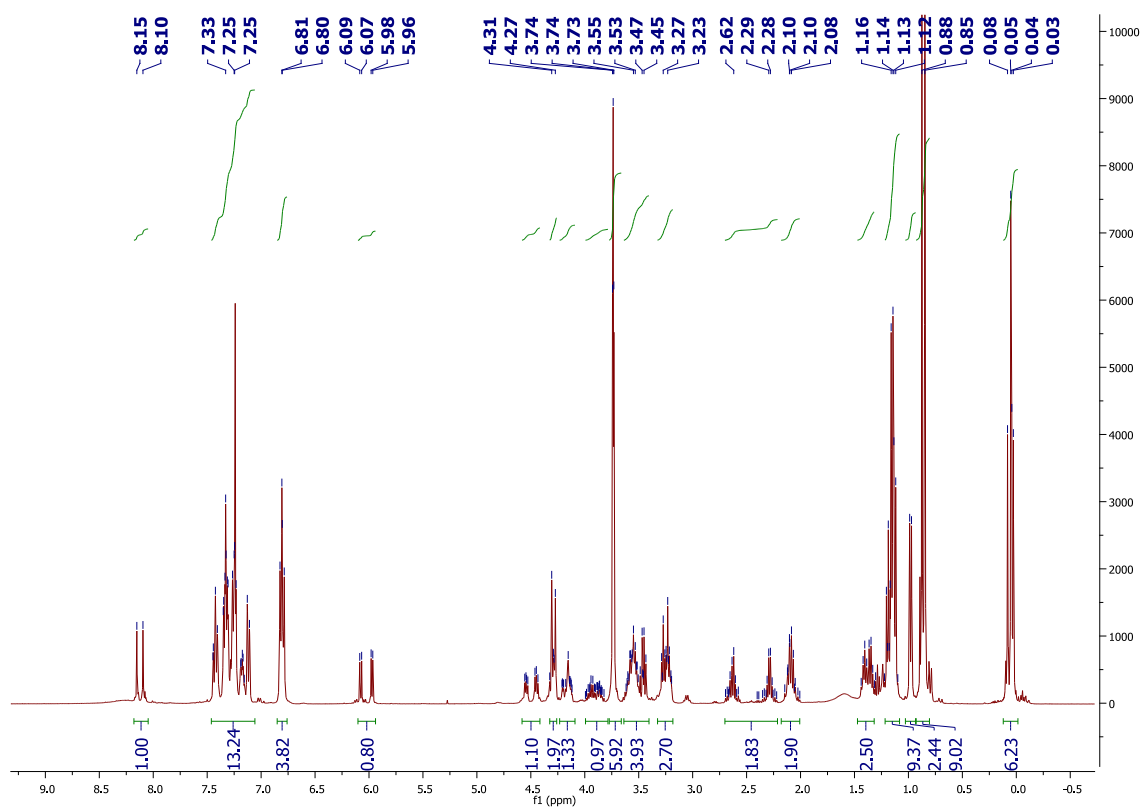


Figure S32. ^1H NMR of U^{Dz} .

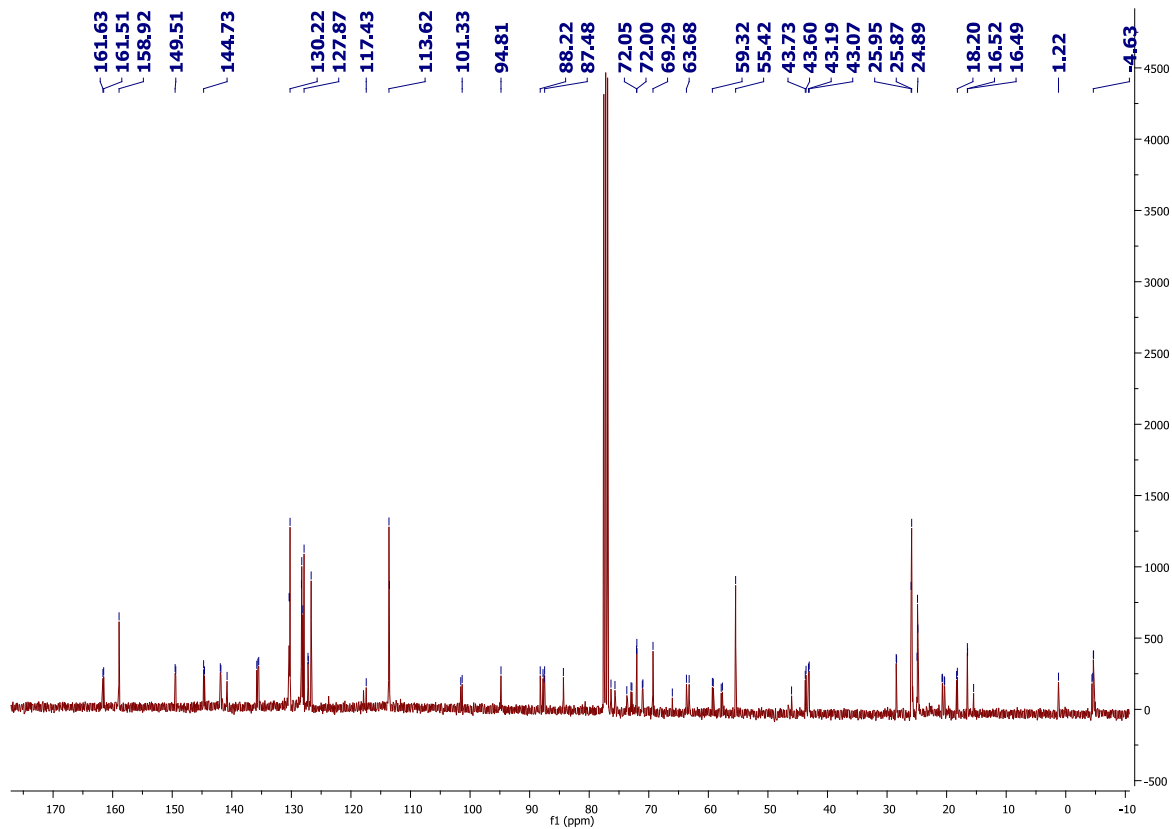


Figure S33. ^{13}C NMR of U^{Dz} .

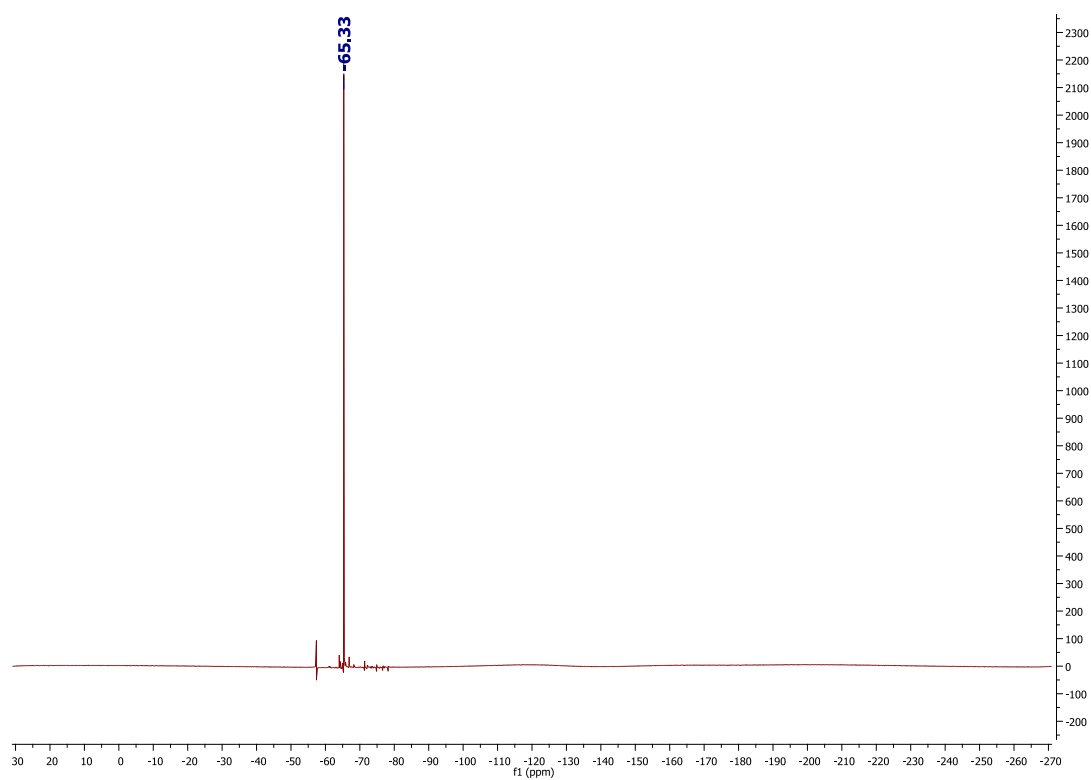


Figure S34. ^{19}F NMR of U^{Dz} .

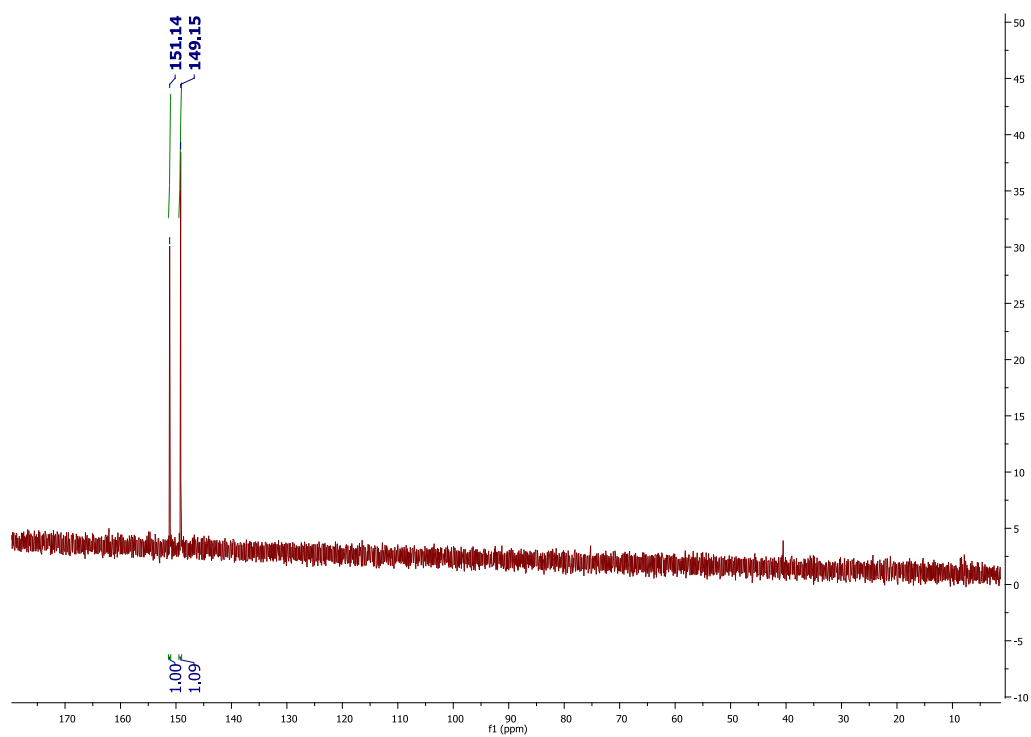
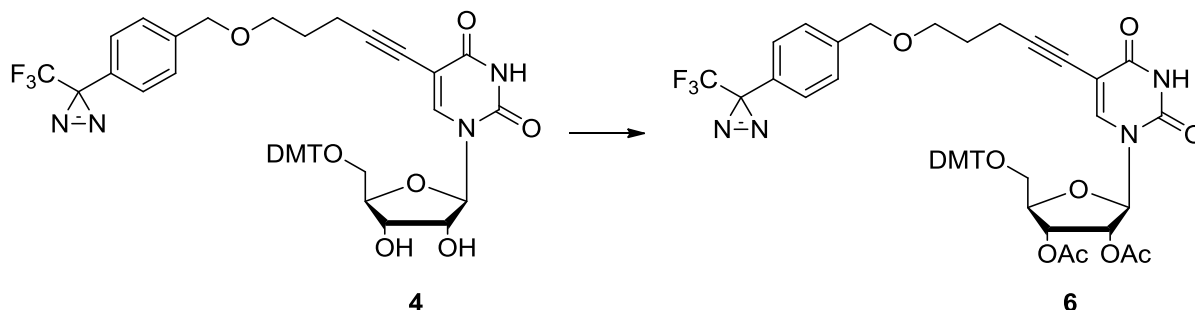


Figure S35. ^{31}P NMR of U^{Dz} .

Bis-2',3'-O-(acetyl)-5'-O-(4,4'-dimethoxytrityl)-5-(pent-1''-yn-5''-oxy-(methyl-phenyl-10''-(trifluoromethyl)-3*H*-diazirin)-1''-yl)-uridine (6)



To a stirring solution of Compound **4** (400 mg, 0.48 mmol) at 0°C in dry pyridine (10 mL) was added DMAP (6 mg, 0.05 mmol) and Ac₂O (0.14 mL, 1.45 mmol). The reaction mixture was allowed to warm to room temperature and then stirred for 2 hours. The reaction mixture was quenched with sat. NaHCO₃ solution (10 mL) and then diluted with CH₂Cl₂ (100 mL), washed with further sat. NaHCO₃ solution (2x 10 mL), brine (10 mL). The organic layer was dried over MgSO₄, filtered and reduced to dryness *in vacuo*. The residue was dissolved in CH₂Cl₂ and purified by column chromatography (0-2% MeOH in CH₂Cl₂), to yield 315 mg (71%) of compound **6** as a pale orange foam.

Analytical data for compound **6**

TLC (CH₂Cl₂/MeOH 96:4) *R_f* = 0.62

¹H NMR (300 MHz, CDCl₃) δ 7.92 (s, 1H, H₆), 7.49 – 7.06 (m, 13H, H-arom.), 6.82 (dd, *J* = 8.8, 1.2 Hz, 4H, H-arom.), 6.23 (d, *J* = 7.2 Hz, 1H, H_{1'}), 5.69 – 5.61 (m, 1H, H_{2'}), 5.53 (dd, *J* = 5.4, 2.1 Hz, 1H, H_{3'}), 4.35 (s, 2H, H_{6''}), 4.19 (d, *J* = 2.1 Hz, 1H, H_{4'}), 3.73 (d, *J* = 1.1 Hz, 6H, CH₃-O-phenyl), 3.35 (dd, *J* = 14.1, 8.0 Hz, 4H, H_{5'} and H_{5''}), 2.19 (t, *J* = 7.2 Hz, 2H, H_{3''}), 2.10, 2.08 (2s, 6H, CH₃CO), 1.62 – 1.44 (m, 2H, H_{4''}).

¹³C NMR (101 MHz, CDCl₃) δ 170.0, 169.9, 161.9, 158.9, 149.9, 144.3, 141.1, 140.7, 135.5, 135.3, 130.2, 130.2, 128.3, 128.2, 128.1, 127.9, 127.2, 126.6, 122.3 (d, *J* = 274.7 Hz), 113.6, 102.2, 95.0, 87.8, 85.4, 82.7, 73.1, 72.1, 71.7, 70.9, 69.2, 63.1, 55.4, 28.5, 20.8, 20.6, 16.5.

¹⁹F NMR (376 MHz, CDCl₃) δ -65.3.

ESI⁺-HRMS calculated for C₃₄H₃₃IN₂O₁₀ ([M+H]⁺): 911.3110, found: 911.3095.

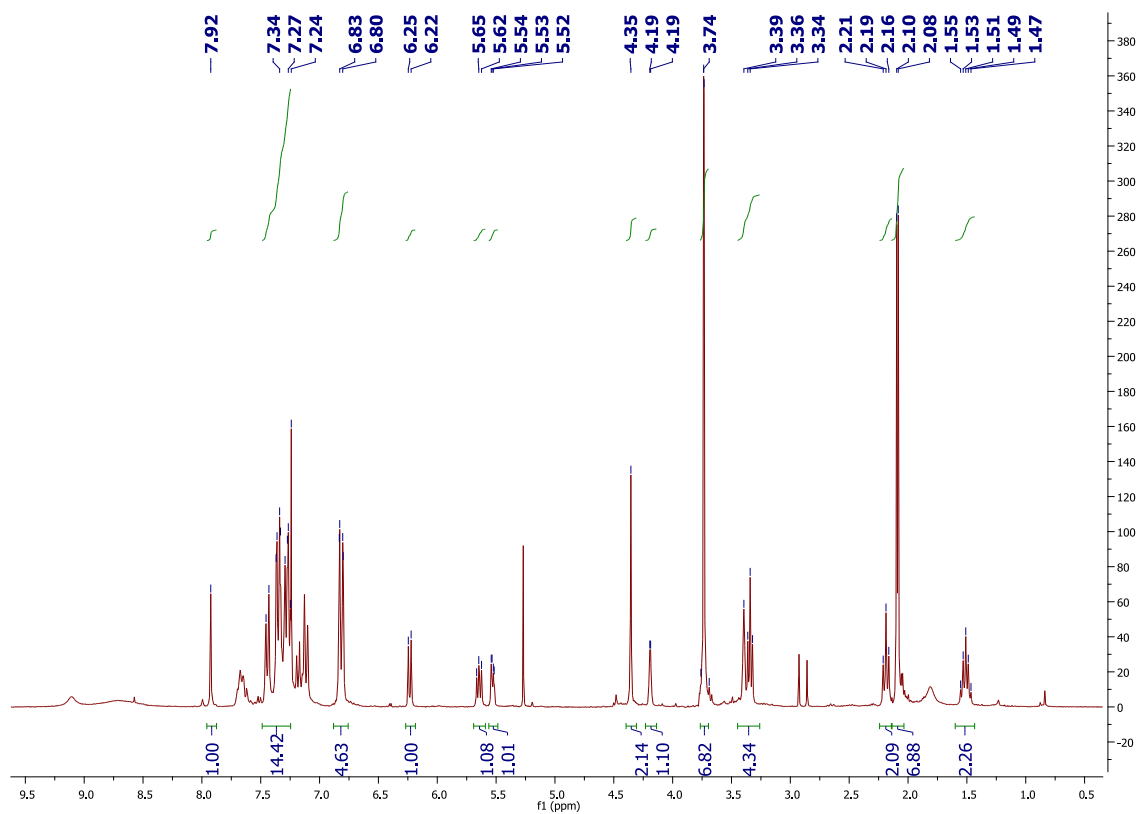


Figure S36. ¹H NMR of compound **6**.

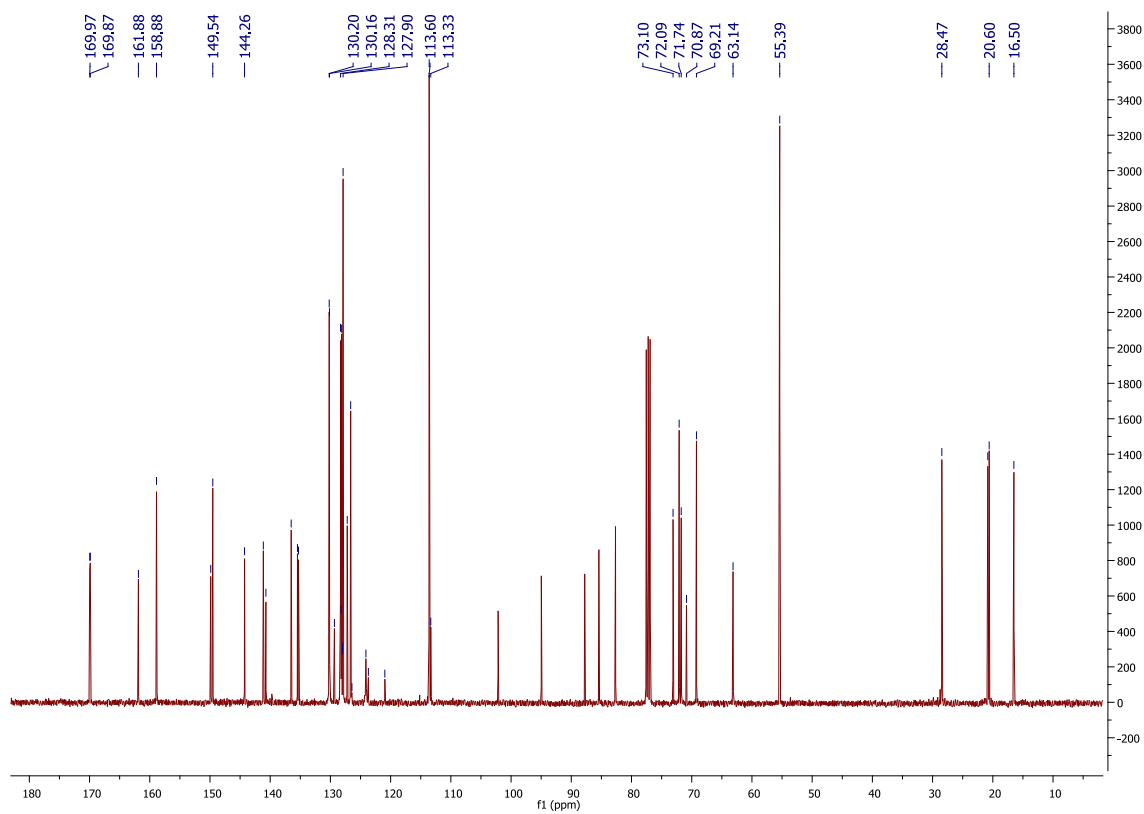


Figure S37. ¹³C NMR of compound **6**.

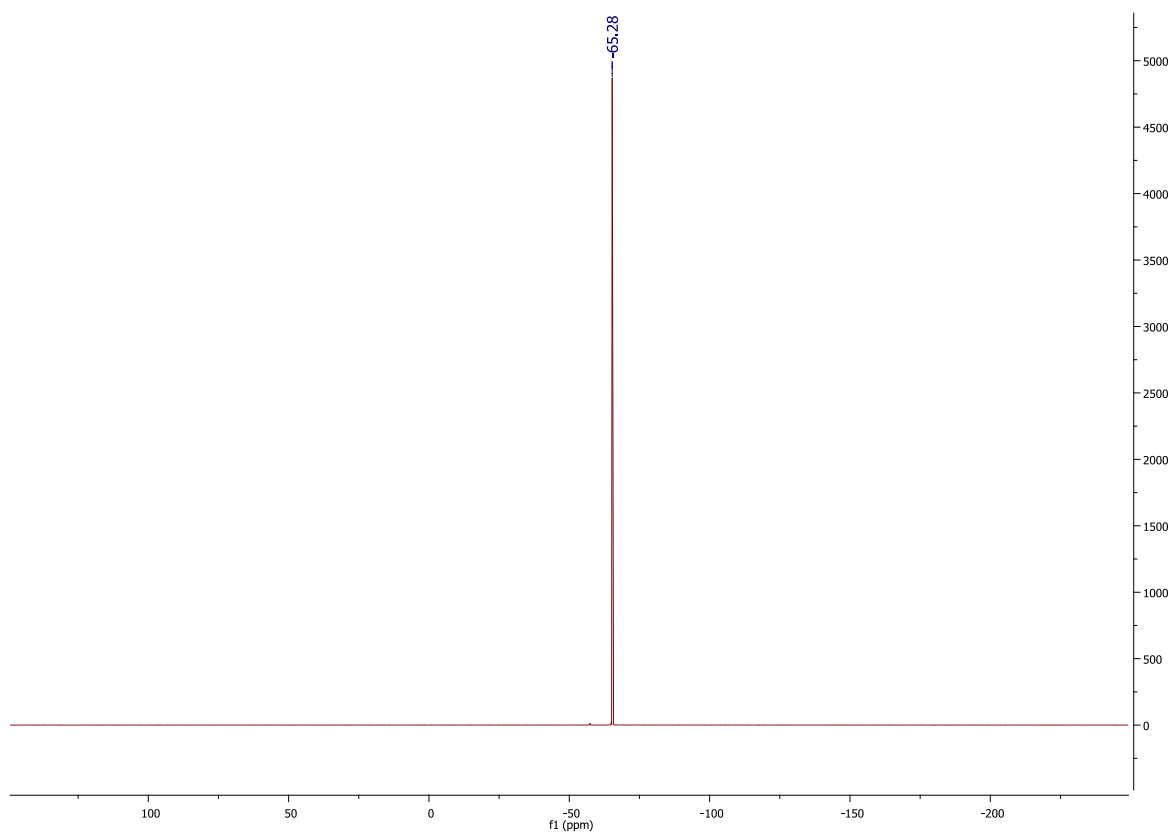
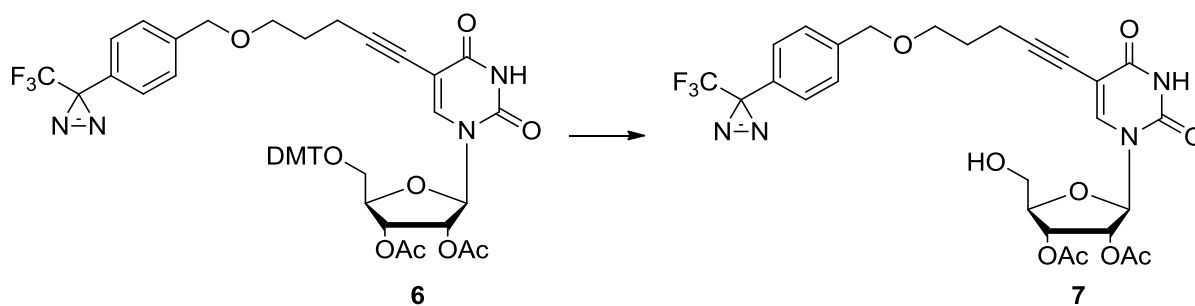


Figure S38. ^{19}F NMR of compound **6**.

Bis-2',3'-O-(acetyl)-5-(pent-1''-yn-5''-oxy-(methyl-phenyl-10''-(trifluoromethyl)-3H-diazirin)-1''-yl)-uridine (7)



To a stirring solution of Compound **7** (315 mg, 0.35 mmol) in CH_2Cl_2 (10 mL) at 0°C was added dichloroacetic acid (1%, 0.1 mL) and stirred for 1.5 hours. TLC analysis showed the presence of remaining starting material (~50%), so further dichloroacetic acid (0.1 mL) was added at 0°C . The reaction mixture was then stirred for 30 minutes at 0°C . The reaction mixture was quenched with MeOH (10 mL) and reduced to dryness *in vacuo*. The residue was dissolved in CH_2Cl_2 (100 mL), washed with distilled H_2O (10 mL), brine (10 mL), dried

over MgSO₄, filtered and reduced to dryness *in vacuo*. The residue was dissolved in CH₂Cl₂ and purified by column chromatography (1-2% MeOH in CH₂Cl₂) to yield 101 mg (48%) of compound **7** as a colorless foam.

Analytical data for compound **7**

TLC (CH₂Cl₂/MeOH 98:2) *R_f* = 0.26

¹H NMR (300 MHz, MeOD) δ 8.24 (s, 1H, H6), 7.45 (d, *J* = 8.5 Hz, 2H, H-arom.), 7.22 (d, *J* = 8.0 Hz, 2H, H-arom.), 6.10 (d, *J* = 5.6 Hz, 1H, H1'), 5.49 (s, 1H, H2'), 5.46 – 5.42 (m, 2H, H3' and H4'), 4.56 (s, 2H, H6''), 4.23 (d, *J* = 2.6 Hz, 1H, 5'OH), 3.80 (m, 2H, H5'), 3.63 (t, *J* = 6.1 Hz, 2H, H5''), 2.51 (t, *J* = 7.0 Hz, 2H, H3''), 2.12, 2.05 (2s, 6H, CH₃CO), 1.86 (m, 2H, H4'').

¹³C NMR (101 MHz, MeOD) δ 171.7, 171.5, 164.4, 151.6, 144.1, 142.6, 129.3, 129.1, 127.7, 127.7, 123.8 (d, *J* = 273.9 Hz), 102.1, 95.0, 88.3, 85.3, 75.0, 73.1, 72.9, 72.9, 70.2, 62.3, 29.8, 20.7, 20.4, 17.1.

¹⁹F NMR (376 MHz, MeOD) δ -67.0.

ESI⁺-HRMS calculated for C₂₇H₂₇F₃N₄O₉ ([M+H]⁺): 609.1803, found: 609.1788.

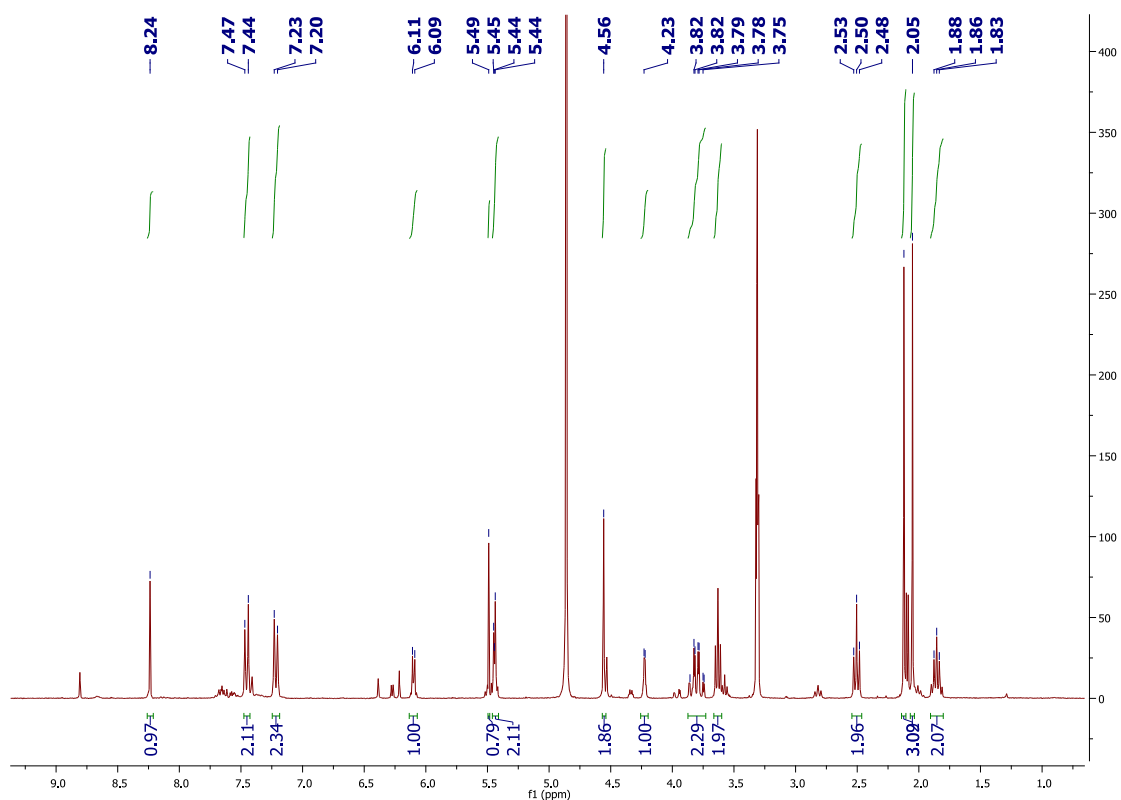


Figure S39. ¹H NMR of compound **7**.

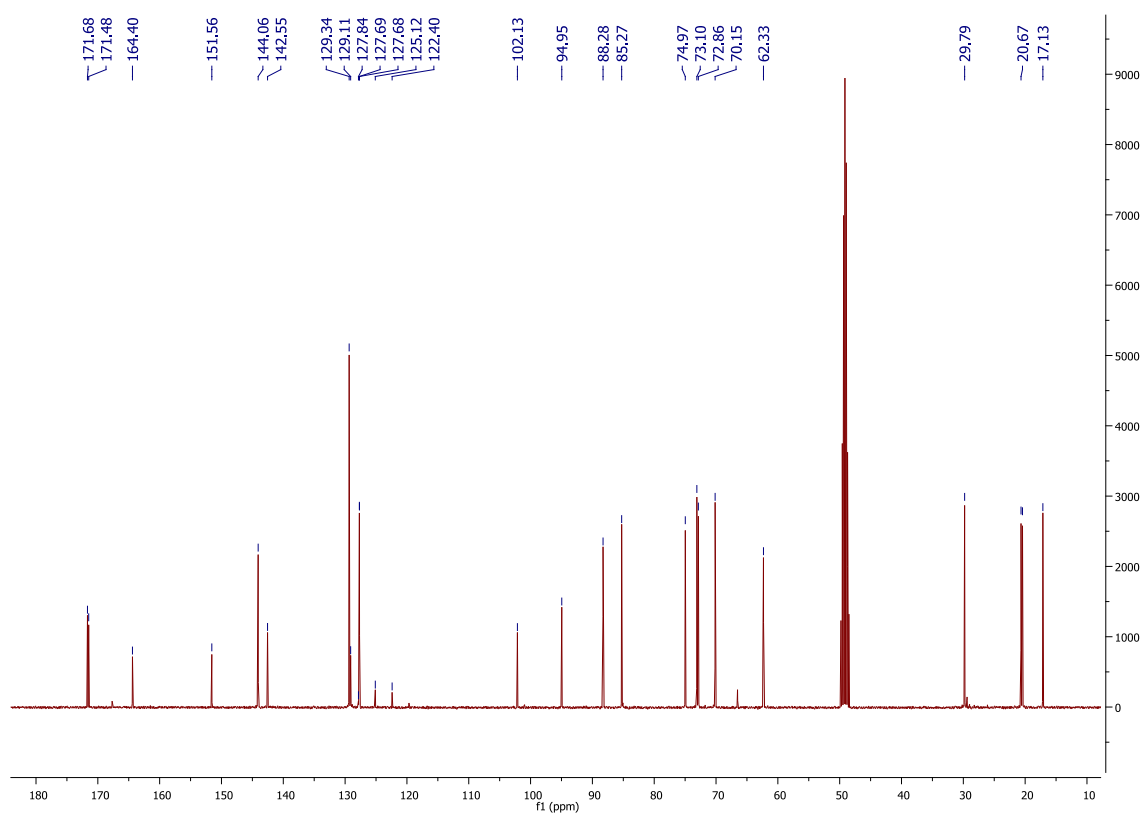


Figure S40. ¹³C NMR of compound **7**.

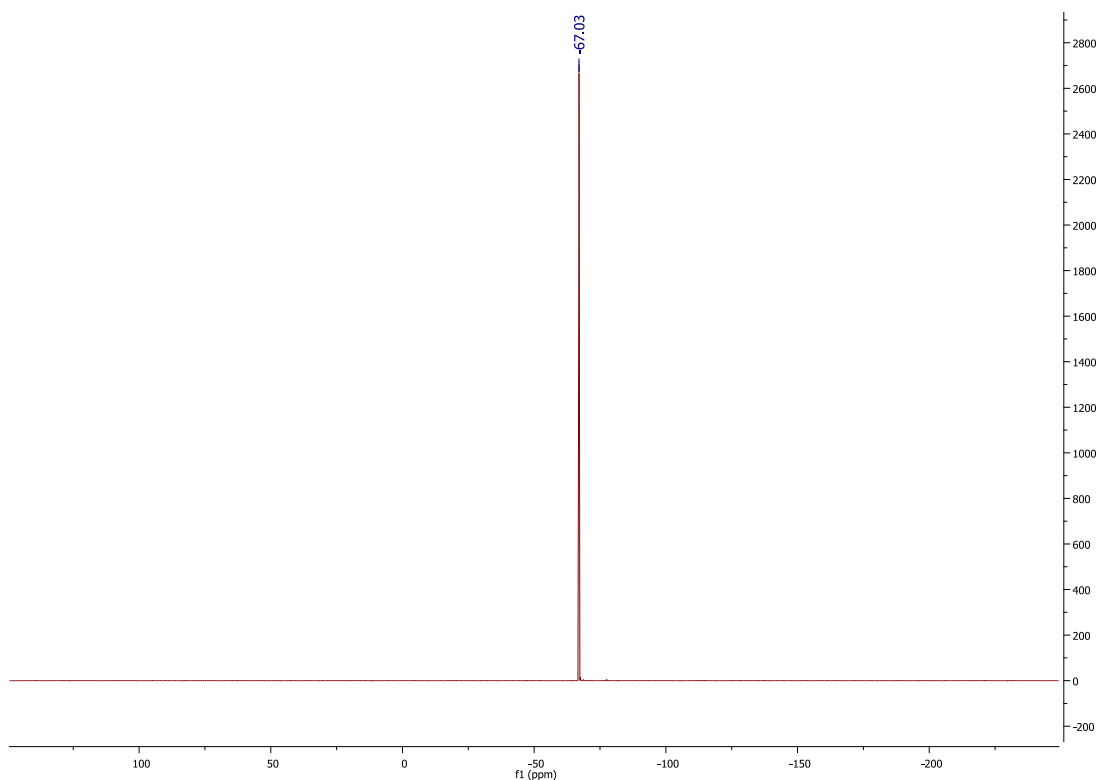
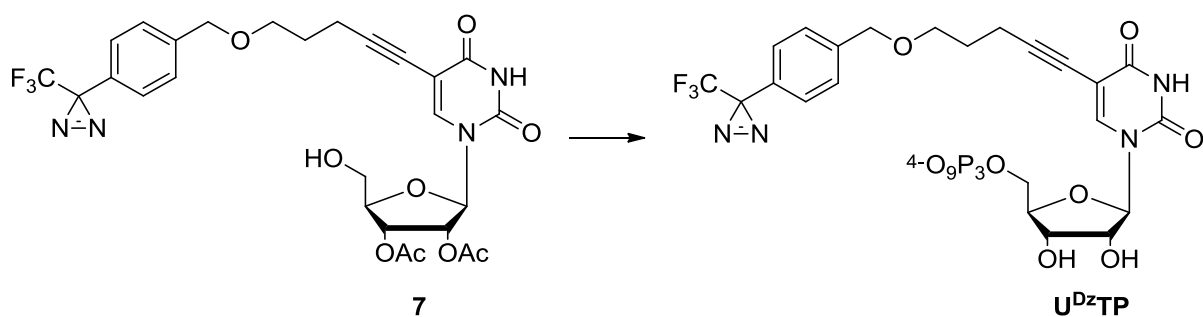


Figure S41. ^{19}F NMR of compound **7**.

5-(pent-1''-yn-5''-oxy-(methyl-phenyl-10''-(trifluoromethyl)-3*H*-diazirin)-1''-yl)-uridine triphosphate ($\text{U}^{\text{Dz}}\text{TP}$)



Compound **7** was co-evaporated with dry pyridine (3x 1 mL) and dried overnight *in vacuo*. Tributylammonium pyrophosphate was dried overnight *in vacuo*. Flasks were removed under Ar. Immediately before use, the hydrolysed outer layer of 2-chloro-1,3,2-benzodioxaphosphorin-4-one was removed.

To a stirring solution of compound **30** (48 mg, 0.08 mmol) in dry pyridine (0.2 mL) and dry 1,4-dioxane (0.4 mL) under Ar was added 2-chloro-1,3,2-benzodioxaphosphorin-4-one (18 mg, 0.09 mmol) and stirred at room temperature for 45 minutes.

Tributylammonium pyrophosphate (56 mg, 0.10 mmol) was dissolved in dry DMF (170 μ L) and distilled tri-*n*-butylamine (60 μ L) and added to the reaction mixture, which was stirred for 40 minutes at room temperature.

Iodine (32 mg, 0.13 mmol) was dissolved in dry pyridine (1 mL) and ultrapure water (20 μ L) and added to the reaction mixture and stirred for 30 minutes.

A 10% solution of Na₂SO₃ in H₂O was then added and the reaction mixture was stirred until colourless. The reaction mixture was then evaporated *in vacuo* (and keeping the temperature below 35°C). The residue was dissolved in ultra-pure water (5 mL) and left to stand for 30 minutes.

Aqueous ammonia (25%, 10 mL) was added and the reaction mixture was stirred at room temperature for 2 hours. Ammonium hydroxide was removed *in vacuo*. The residue was dissolved in D₂O (2 mL) and a ³¹P NMR spectrum of the crude was measured. The sample was split in two, and 2% NaClO₄ in acetone (11 mL) added to each half. The resulting mixture was centrifuged for 25 minutes (5000 rpm). The supernatant was removed and this precipitation procedure was repeated. The residue in each sample was dissolved in ultrapure water (2 mL) and combined. The combined 4 mL were then purified by RP-HPLC (0-20% B in 20 minutes then 20-80% B in 30 minutes using the TEAB buffer system (eluent A: 50 mM TEAB, pH 7.7; eluent B: 50 mM TEAB in CH₃CN–H₂O 1 : 1)) with UV monitoring at 270, 280 and 290 nm. The appropriate fractions were freeze-dried and coevaporated several times with ultrapure water (10 mL). Application of this procedure yielded 18 mg (21%, tris-triethylammonium salt) of triphosphate **U^{Dz}TP** as a colorless foam.

Analytical data for **U^{Dz}TP**

R_t = 43.5 min.

¹H NMR (300 MHz, D₂O) δ 7.78 (s, 1H, H6), 7.41 (d, J = 8.2 Hz, 2H, H-arom.), 7.11 (d, J = 8.0 Hz, 2H, H-arom.), 5.90 (d, J = 5.2 Hz, 1H, H1'), 4.53 (d, J = 5.9 Hz, 2H, H6''), 4.35 – 4.11 (m, 5H, H2', H3', H4' and H5'), 3.61 (dd, J = 11.2, 5.2 Hz, 2H, H5''), 2.41 (dd, J = 12.1, 5.6 Hz, 2H, H3''), 1.76 (dd, J = 12.1, 6.0 Hz, 2H, H4'').

^{31}P NMR (122 MHz, D_2O) δ -10.74 (m, P_γ), -11.56 (d, $J = 18.3$ Hz, P_α), -23.12 (m, P_β).

ESIHRMS calculated for $\text{C}_{23}\text{H}_{24}\text{F}_3\text{N}_4\text{O}_{16}\text{P}_3$ ($[\text{M}+\text{H}]^+$): 763.0436, found: 763.0420.

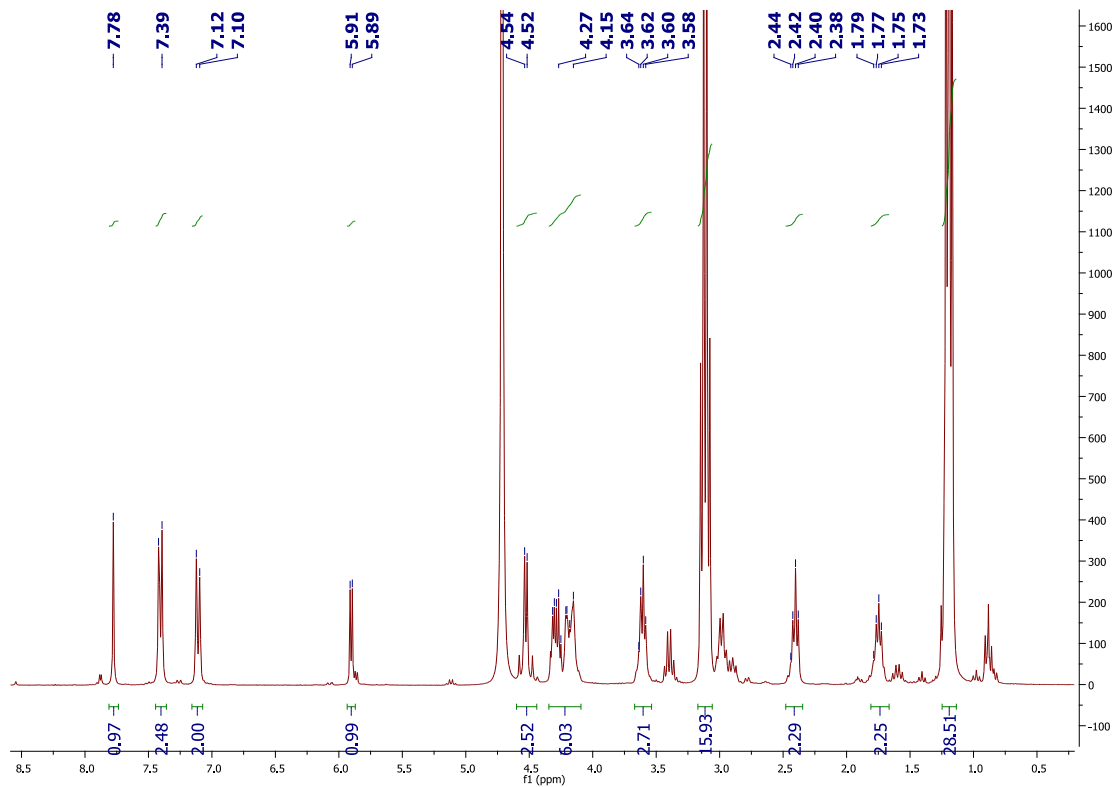


Figure S42. ^1H NMR of $\text{U}^{\text{Dz}}\text{TP}$.

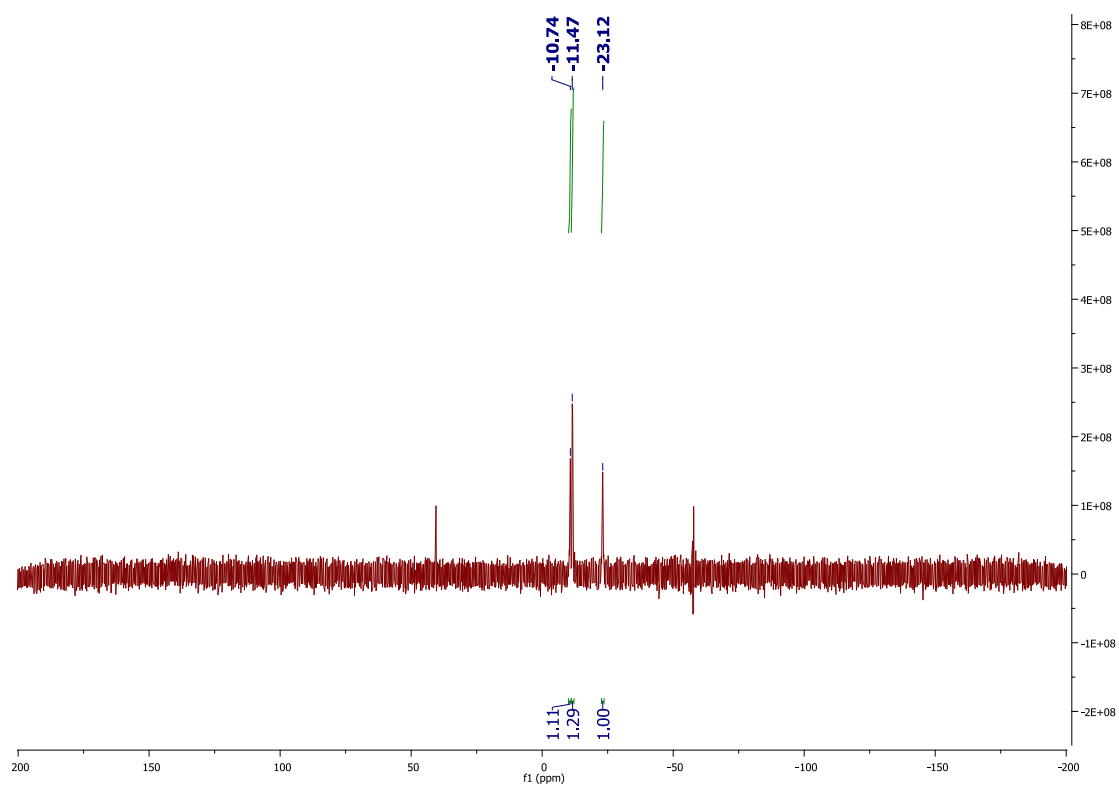


Figure S43. ^{31}P NMR of $\text{U}^{\text{Dz}}\text{TP}$.

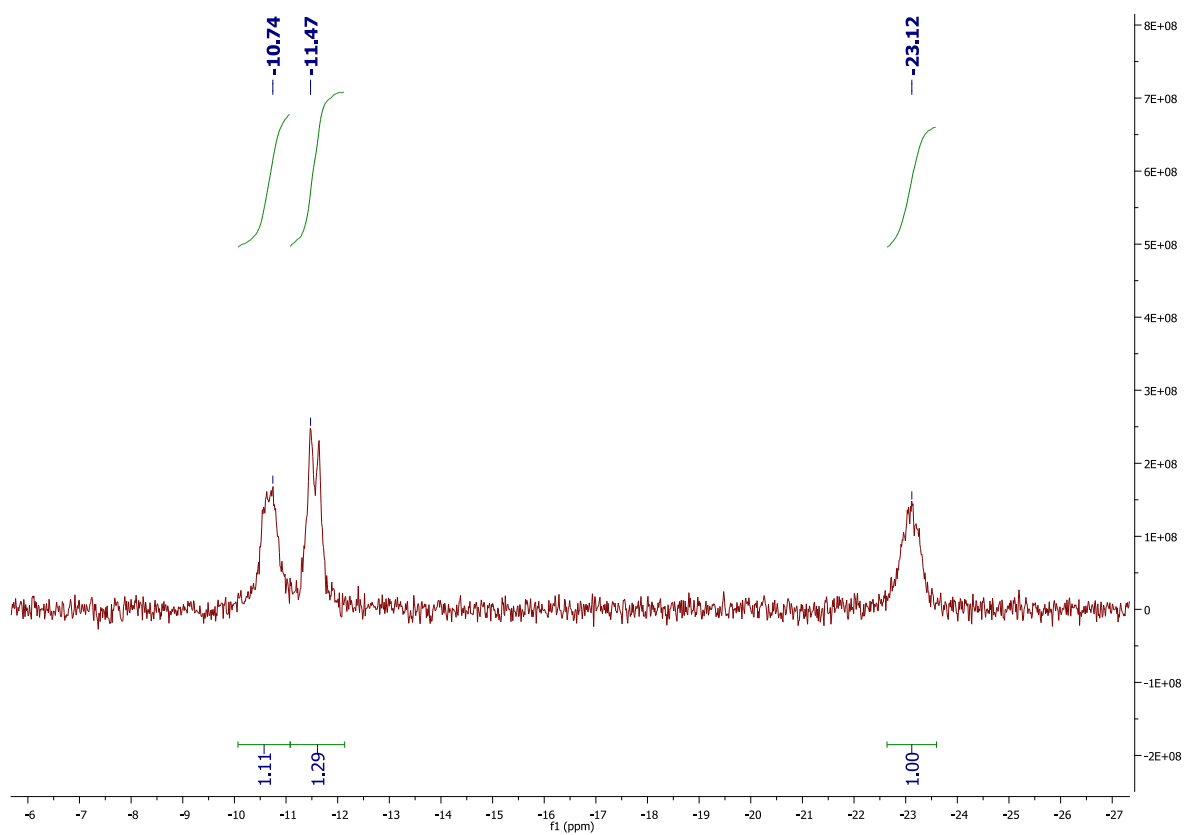


Figure S44. Zoom of ^{31}P NMR spectrum of $\text{U}^{\text{Dz}}\text{TP}$.

3. Oligonucleotide synthesis

Syntheses of oligonucleotides were performed on the 1.3 μmol scale of a LKB Gene Assembler Plus (*Pharmacia*) DNA synthesizer using standard solid-phase phosphoramidite chemistry. Natural RNA nucleoside phosphoramidites were purchased from AZCO Biotech or SAFC.

Oligomers were synthesized using the manufacturer's protocols on controlled pore-glass (CPG) preloaded with DMTr- and base-protected natural nucleoside solid supports (Glen Research). Natural and modified phosphoramidites were both prepared as a 0.1 M solution in CH_3CN . Strictly anhydrous CH_3CN was used and the phosphoramidite solutions were dried on 4 Å activated molecular sieves prior to use. An extended coupling time (12 min) for all phosphoramidite solutions was necessary to achieve acceptable average coupling efficiencies. Coupling yields were calculated from the absorbance at 436 nm of the DMTr⁺ cation (trityl assay). Manual detritylation of monomer U^{Dz} was required to ensure complete deprotection. All oligonucleotides were cleaved and deprotected using standard protocols. Crude oligomers were purified by ion-exchange HPLC. As mobile phases, the following buffers were prepared: (A) 25 mM in ultrapure H_2O , pH 8.0; (B) 25 mM Trizma, 1.25 M NaCl in ultrapure H_2O , pH 8.0. Linear gradients of B in A were used (typically 20–80% B over 30 min), with a 1 mL/min flow rate and detection at 260 nm. Oligonucleotides ON1-4 (Table S1) had a retention time of ~14 min. Purified oligonucleotides were desalted over Sep-Pak cartridges, quantified at 260 nm with a Nanodrop spectrophotometer (Thermo Scientific), and analyzed by ESI-mass spectrometry (see Table S1). Solutions of oligonucleotides in H_2O were then stored at -18°C .

Table S1.^a Sequence and analytical data of sequences ON1-4.

	Sequence (5'→3')	$m/z_{\text{calc.}}$	m/z_{found}
ON1	Biotin-CCUAUGUCACUGAAACUAUCC	6979.04	6982.00
ON2	Biotin-CCUAUGUCACUGAAAC X AUCC	7259.13	7261.10
ON3	Biotin-CCUAUGUCAC X GAAACUAUCC	7259.13	7261.09
ON4	Biotin-CC X AUGUCACUGAAACUAUCC	7259.13	7260.08

^a X indicate the position of the U^{Dz} modifications.

4. Crosslinking and transcription assays:

i) List of Buffers

B1. Irradiation buffer 50 mM Tris-HCl, 10 mM MgCl₂, 10 mM DTT, (pH 7.8).

B2. 5x transcription buffer: 200 mM Tris-HCl, 50 mM NaCl, 50 mM DTT, 30 mM MgCl₂, 10 mM spermidine, 0.25% Tween, (pH 7.9).

B3. Loading buffer: 70% formamide, 50 mM EDTA, bromophenol blue and xylene cyanol FF (each 0.1 %).

ii) Oligonucleotides:

Table S2. Sequence composition of oligonucleotides.

Oligonucleotide	Sequence (shown 5' to 3')
ON1	Biotin-r(CCUAUGUCACUGAAACUAUCC)
ON2	Biotin-r(CCUAUGUCACUGAAACU ^{Dz} AUCC)
ON3	Biotin-r(CCUAUGUCACU ^{Dz} GAAACUAUCC)
ON4	Biotin-r(CCU ^{Dz} AUGUCACUGAAACUAUCC)
ON5	r(GGAUAGUUUCAGUGACAUAGG)
ON6	d(GGATAGTTTCAGTGACATAGG)
T1	d(TAATACGACTCACTATAGGGAGACCATGCACAACCGGAA ACCC)
T1'	d(GGGTTTCCGGTTGTGCATGGTCTCCCTATAGTGAGTCGTA TTA)
T2	d(TAATACGACTCACTATAGGGAGACCAAGCACAACCGGTT TCCC)
T2'	d(GGGAAACCGGTTGTGCTTGGTCTCCCTATAGTGAGTCGTA TTA)
T3	d(TAATACGAGTCACTATAGGGAGACCATGCACTACCGGTA ACCC)
T3'	d(GGGTTACCGGTAGTGCATGGTCTCCCTATAGTGAGTCGTA TTA)
T4	d(TAATACGACTCACTATAGGGAGACCATGCACAACCGGAAAC CCGGGAGACCATGCACTACCGGTAACCC)
T4'	d(GGGTTACCGGTAGTGCATGGTCTCCCGGGTTTCCGGTTGTGC ATGGTCTCCCTATAGTGAGTCGTATTA)

iii) General Procedure for the Protein Crosslinking Assay

Single-stranded binding protein (SSB) (1 µg) and oligonucleotide (3 pmoles of radiolabelled or 50 pmoles of fluorescently labelled) were incubated in **B1** in a total volume of 15 µL in a PCR vial at room temperature for at least 30 minutes prior to irradiation. The sample was irradiated at 375 nm for 30 minutes. **B3** (15 µL) was then added and heated to 95 °C for 5 minutes. The resulting RNA-protein complexes were then resolved by 10% SDS-PAGE at

10W for 2 hours. Radiolabelled samples were exposed on the imaging plate at -18 °C overnight before visualising by phosphorimaging.

iv) **General Procedure for Post-Cross-linking RNA Labelling**

The irradiated samples were EtOH precipitated before being re-suspended in ultrapure H₂O (100 µL) and purified by Millipore spin column (30 kDa cut off), washing with **B1**. The resulting solution was EtOH precipitated again, and re-suspended and incubated in **B1**, T4 RNA ligase I (20 units), ATP (1mM) and 1µM [³²P]pCp with the addition of ultrapure H₂O to a total volume of 30 µL at 4 °C overnight. **B3** (30 µL) was added and heated to 95 °C for 5 minutes. The samples were then resolved on 10% SDS PAGE.

v) **General Procedure for T7 RNA Transcription**

Oligonucleotides used for transcription assays were purchased from Microsynth (Table S2). Oligonucleotides shorter than 50mer were purified by phenol/chloroform extraction followed by EtOH precipitation prior to use. 62mer oligonucleotides T4 and T4' were purified by gel (10% PAGE) before EtOH precipitation and G₁₀ Sephadex spin column before use.

All transcription reactions contained: dsDNA templates (40 pmoles), natural or modified NTPs (0.4 mM each), T7 RNA polymerase (100 units), RNasin[®] (20 units), α-³²P-ATP (10µCi), TIPP (0.6 units) in **B2** with ultrapure H₂O added to reach a total volume of 20 µL.

Oligonucleotides and **B2** were placed in an eppendorf vial and annealed by slow cooling from 90°C to room temperature over one hour. A cocktail containing the appropriate NTPs, H₂O and RNasin[®] was then added to the annealed primer-template duplex. α-³²P-ATP was added before cooling to 0°C. TIPP and T7 RNA polymerase were then added in turn and the reaction mixture was incubated at 37°C for the required reaction time. The reaction was quenched with **B3** (20 µL), heated to 95°C for 20 minutes and resolved on 15-20% denaturing PAGE.

5. Additional Gels:

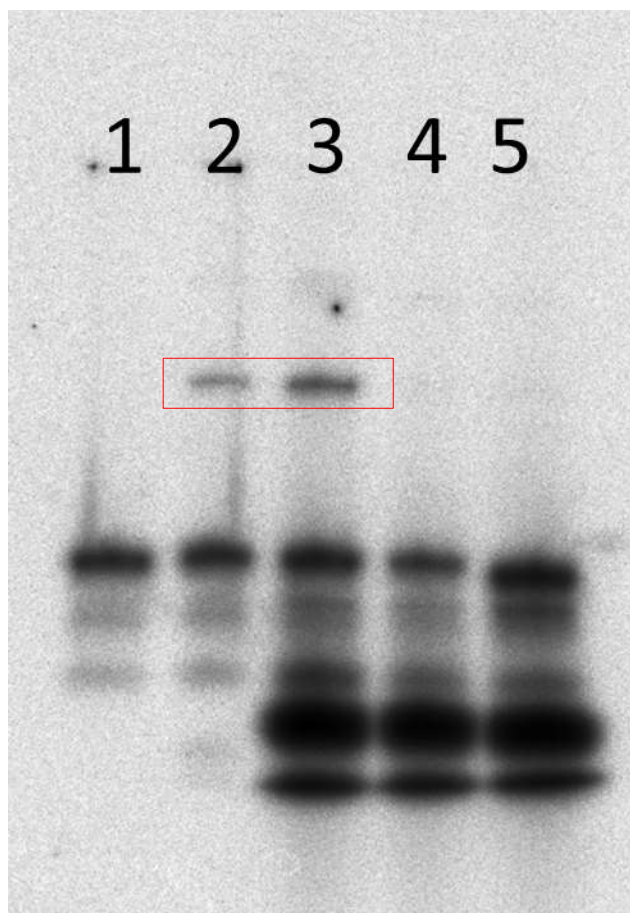


Figure S45. Radioautograph of 10% SDS PAGE of cross-linking of ON4 and SSB with pre- and post-labelling and controls. Lane 1: Pre-labelled ON4. Lane 2: Pre-labelled ON4 + SSB, irradiated. Lane 3: Post-labelled ON4 + SSB, irradiated. Lane 4: Post-labelled ON4 + SSB, non-irradiated. Lane 5: Post-labelled ON1 + SSB, irradiated.

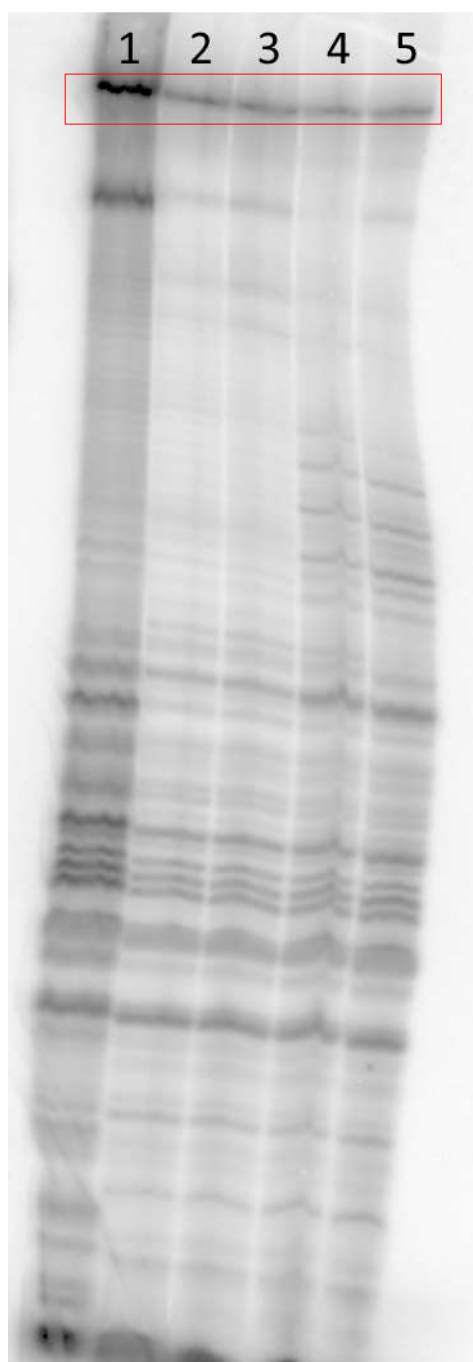


Figure S46. Radioautograph of 20% PAGE of transcription assays with the T4/T4' system with a reaction time of 24-75 hours. Lane 1: Natural control, reaction time 24 hours. Lane 2: $\text{U}^{\text{Dz}}\text{TP}$, reaction time 26 hours. Lane 3: $\text{U}^{\text{Dz}}\text{TP}$, reaction time 44 hours. Lane 4: $\text{U}^{\text{Dz}}\text{TP}$, reaction time 68 hours. Lane 5: $\text{U}^{\text{Dz}}\text{TP}$, reaction time 75 hours. Red highlights full-length transcript. Conditions: 40 pmol of dsDNA template T4/T4', 0.4 mM of natural NTPs, 0.6 mM NTP 31, 100 units of T7 RNA polymerase, 0.6 units of PPIase, 0.5 μL RNasin, and 10 μCi of $\alpha\text{-}^{32}\text{P}\text{-ATP}$ in the transcription optimised buffer composed of 40 mM Tris-HCl (pH 7.9), 6 mM MgCl_2 , 10 mM NaCl, 2 mM spermidine, 10 mM DTT and 0.05% of the polysorbate detergent Tween. Reactions were incubated at 37°C 24-75 hours. Maximum yield of modified RNA compared to natural control was 37%.

6. Cited references:

1. Wahba, A. S.; Esmaeili, A.; Damha, M. J.; Hudson, R. H. E. *Nucleic Acids Res.* **2010**, 38, 1048.
2. Robson, J. H.; Reinhart, J. *J. Am. Chem. Soc.* **1955**, 77, 498.
3. Fishwick, C. W. G.; Sanderson, J. M.; Findlay, J. B. C. *Tetrahedron Lett.* **1994**, 35, 4611.
4. Smith, D. P.; Anderson, J.; Plante, J.; Ashcroft, A. E.; Radford, S. E.; Wilson, A. J.; Parker, M. J. *Chem. Commun.* **2008**, 5728.