Electronic Supplementary Material (ESI) for ChemComm.

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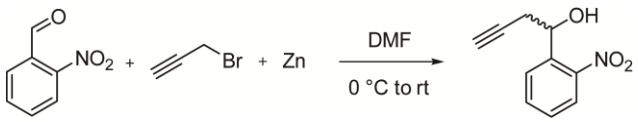
1. General Experimental procedures

Milli-Q water was treated with 0.1% DEPC overnight and was autoclaved before usage. All reagents were obtained from commercial sources and used without further purification. Reactions were performed under an argon atmosphere. Technical grade solvents were used for column chromatography.

2. Chemical synthesis

1-(2-Nitrophenyl)but-3-yn-1-ol (**2**):



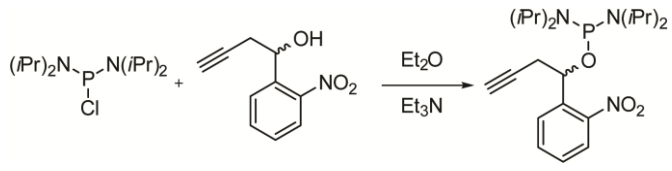
Zinc-powder (1.73 g, 26.5 mmol, 2 eq) was suspended in 5 mL HCl (10%) and the mixture was stirred at room temperature for 1 hour. The solid was filtered, washed with water, acetone and Et_2O and dried under reduced pressure for 1 hour. After resuspending the zinc in 30 mL DMF, the mixture was cooled to 0 °C. Propargyl bromide solution (80 wt. % in toluene, 2.21 mL, 19.9 mmol, 1.5 eq) was added and the mixture was stirred for 1 hour at 0 °C followed by addition of 2-nitrobenzaldehyde (2.00 g, 13.2 mmol, 1 eq). The ice bath was removed and the mixture was stirred at room temperature until the starting material was consumed completely (1 hour). 30 mL saturated NH_4Cl solution was added, the mixture was stirred for 20 minutes and concentrated in vacuum. The residue was extracted with DCM/H_2O. The organic layer was concentrated under reduced pressure and purified *via* column chromatography (cyclohexane:ethyl acetate = 2:1, R_f = 0.45). The solvent was removed under reduced pressure to give **2** as a brown oil (2.51 g, 99%).

^{1}H-NMR (300 MHz, DMSO-d_6): δ [ppm] = 7.92-7.83 (m, 2H), 7.76-7.71 (m, 1H), 7.55-7.50 (m, 1H), 5.88 (d, 1H), 5.20 (m, 1H), 2.78 (t, 1H), 2.79-2.53z (m, 1H).

^{13}C-NMR (75 MHz, DMSO-d_6): δ [ppm] = 147.7, 138.7, 133.1, 128.6, 128.5, 123.7, 81.0, 72.9, 66.3, 28.1.

MALDI-HRMS m/z: $[M+H]^+$ calculated for $C_{10}H_9NO_3$ 192.06552, found 192.06537 (Δm = 0.0015, error 0.8 ppm).

1-(2-Nitrophenyl)but-3-yn-1-yl-*N,N,N',N'*-tetraisopropylphorphoramidite (**3**):



Bis(diisopropylamino)chlorophosphine (390 mg, 1.46 mmol, 1 eq) and Et_3N (296 mg, 2.92 mmol, 2 eq)

were suspended in 6 mL dry Et_2O. Alcohol **2** (307 mg, 1.6 mmol, 1.1 eq) was added stepwise over 10 minutes under continuous stirring at room temperature. After 16 hours, Et_2O was removed by flushing the system with argon at room temperature. The crude product was purified *via* column chromatography (dichlormethane:ethyl acetate = 50:1 → 10:1 , 1% Et_3N, R_f = 0.1). The solvent was removed under reduced pressure at 35 °C to give **3** as yellow powder (392 mg, 64%).

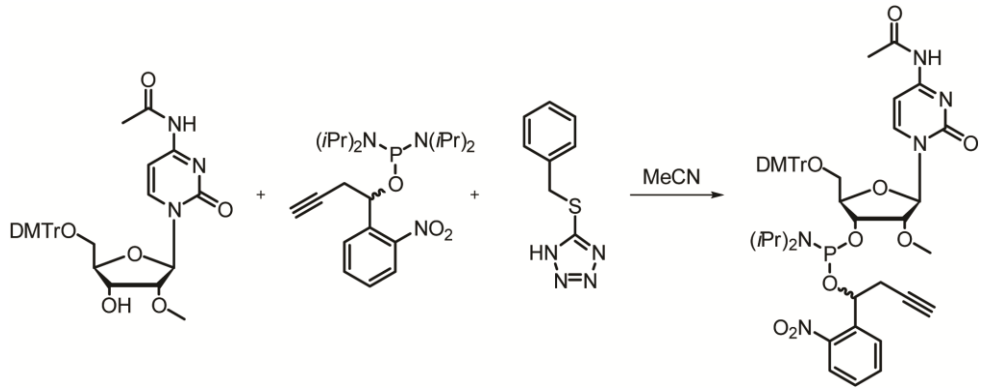
^{1}H-NMR (300 MHz, DMSO-d_6): δ [ppm] = 7.98-7.96 (m, 1H), 7.90-7.88 (m, 1H), 7.81-7.77 (m, 1H), 7.58-7.54 (m, 1H), 5.30-5.25 (m, 1H), 3.59-3.42 (m, 4H), 2.84-2.67 (m, 3H), 1.16-1.07 (m, 18H), 0.97-0.95 (m, 6H).

^{13}C-NMR (120 MHz, DMSO-d_6): δ [ppm] = 147.38, 137.19, 133.26, 129.43, 128.83, 123.99, 79.82, 73.41, 68.10, 67.92, 44.41, 44.34, 44.28, 44.21, 27.82, 24.05, 23.99, 23.95, 23.91, 23.82, 23.79, 23.70 ppm.

^{31}P-NMR (121 MHz, DMSO-d_6): δ [ppm] = 112.7.

MALDI-HRMS m/z: $[M+H]^+$ calculated for $C_{22}H_{36}N_3O_3$ 422.25671, found 422.25662 (Δm = 0.0009, error 0.2 ppm).

*N*4-Acetyl-*O*-(4,4-Dimethoxytrityl)-2'-*O*-methylcytidin-3'-*O*-[*O*-(1-(2-nitrophenyl)but-3-in-1-yl)]-*N*,*N*'-diisopropylphosphoramidite (**4**):



Compound **3** (525 mg, 1.25 mmol, 1.5 eq) and 5-(benzylthio)-1*H*-tetrazol (3 M in MeCN, 64 mg, 332 µmol, 0.4 eq) were dissolved in 5 mL dry MeCN in a microwave vessel. The DMT-protected 2'OMe-C^{Ac} nucleoside (500 mg, 831 µmol, 1 eq) was dissolved in 2 mL dry MeCN and added slowly to the microwave vessel under continuous stirring. The vessel was flushed with argon and the mixture was reacted in the microwave for 4 hours at 40 °C. After cooling to room temperature the mixture was directly purified *via* column chromatography (dichlormethane:acetone = 4:1, 3% Et_3N, R_f = 0.67). For column chromatography, p.a. quality solvents were used. The solvent was removed under reduced pressure at 35 °C to give **4** as a white foam (560 mg, 73%).

^{1}H-NMR (400 MHz, DMSO-d_6): δ [ppm] = 10.98-10.91 (m, 1H), 8.39-8.26 (m, 1H), 7.98-7.87 (m, 1H), 7.81-7.72 (m, 1H), 7.61-7.24 (m, 10H), 7.18-7.16 (m, 1H), 6.99-6.84 (m, 5H), 5.46-5.30 (m, 1H), 4.23-3.87 (m, 2H), 3.74 (t, 6H), 3.59-3.12 (m, 8H), 2.81-2.67 (m, 2H), 2.38-2.33 (m, 1H), 2.13-2.08 (m, 4H), 1.16-0.61 (m, 12H).

^{13}C-NMR (120 MHz, CD_3CN): δ [ppm] = 164.2, 159.8, 148.6, 146.7, 141.3, 141.1, 136.4, 134.5, 131.2, 130.7, 130.3, 130.0, 129.1, 128.1, 125.1, 114.2, 102.5, 89.2, 89.0, 88.7, 88.5, 84.1, 83.0, 81.0, 72.4, 71.6, 69.4, 69.2, 62.8, 62.6, 59.3, 59.3, 56.0, 44.2, 30.9, 29.1, 25.0, 24.4.

^{31}P-NMR (162 MHz, DMSO-d_6): δ [ppm] = 150.5, 150.3, 148.7, 148.6.

ESI-HRMS m/z: $[M+H]^+$ calculated for $C_{50}H_{58}N_4O_7P$ 922.37867, found 922.37830 (Δm = 0.00037, error 0.4 ppm).

3. Solid-phase synthesis

The following oligonucleotides were synthesised:

Sequence	mass calc. [Da]	mass found [Da]
MB1 5'- QGCGCAACAAF$_2$ACAACUUACUACCUCAGCGCF$_1$ -3'	11588.4	11586.3
MB2 5'- QCGCGCAACAAF$_2$ACAACUUACUACCUCAGCGCGF$_1$ -3'	12266.9	12265.4
MB3 5'- QUAUAUCAACAAF$_2$ACAACUUACUACCUCAGAUAUAF$_1$ -3'	12870.2	12869.5
MB4 5'- QCACGAAACAAF$_2$ACAACUUACUACCUCAUCGUGF$_1$ -3'	12236.8	12233.4
MB1b 5'- QQGCGCAACAAUACAACUUACUACCUCAGCGCF$_1$ -3'	11730.5	11727.3
ptMB1b_1 5'- QQGCGCAACAAUA**4**AACUUACUA**4**CUCAGCGCF$_1$ -3'	12272.6	12273.2
ptMB1b_2 5'- QQGCGCAA**4**AAUACAACUUA**4**UACCUCAGCGCF$_1$ -3'	12272.6	12276.4
ptMB3 5'- QUAUAU**4**AACAAF$_2$ACAACUUACUACCU**4**AGAUAUAF$_1$ -3'	13416.3	13414.6

A = 2'OMe-Pac-A-CE Phosphoramidite (*LinkTech*)

C = 2'OMe-Ac-C-CE Phosphoramidite (*Linktech*)

G = 2'OMe-*i*Pr-Pac-G-CE Phosphoramidite (*LinkTech*)

U = 2'OMe-U-CE Phosphoramidite (*LinkTech*)

Q = BBQ-650®-(DMT)-CE-Phosphoramidite (*LinkTech*)

F_1 = Cyanine 5 CPG (*GLEN Research*)

F_2 = Fluorescein-dT Phosphoramidite (*GLEN Research*)

4 = *N*4-Acetyl-*O*-(4,4-Dimethoxytrityl)-2'-*O*-methylcytidin-3'-*O*-[*O*-(1-(2-nitrophenyl)but-3-in-1- yl)]-*N*,*N*'-diisopropylphosphoramidite (compound **4**)

Solid-phase synthesis was performed on an *ABI392* and an *Expedite 8900* instrument. For all synthesised oligonucleotides, Pac_2O was used as capping reagent and BTT (0.3 M) (*emp Biotech*) was used as activator. Coupling time for A, C, G, U was 6 minutes, for Q, F_2 and compound **4** 15 minutes. Synthesis was performed in DMTr-Off mode. The cyanoethyl groups were removed by washing the columns with 20% diethylamine (*emp Biotech*) for 10 minutes, followed by diluting with MeCN, Argon and drying in vacuum. Cleavage from the solid-phase was done with aqueous ammonia (32%) (*Merck*) for 4 hours at room temperature. After spin filtration, the solvent was removed at 4 °C using a vacuum concentrator (*SpeedVac™*, *Thermo Fischer*).

The DMTr-Off oligonucleotides were purified by RP-HPLC on an *Agilent 1200* equipped with a *waters XBridge BEH C18 OBD* column (300 Å, 5 µm, 19x250 mm, 4 mL/min, 60 °C). As solvents 400 mM hexafluoroisopropanol (*fluorochem*), 16.3 mM Et_3N (*Merck*), pH 8.3 and MeOH (*Fluka*) were used with a gradient from 5% to 100% MeOH in 30 minutes. After separation, the solvent was evaporated in a vacuum concentrator at 4 °C.

4. Predicted secondary structures

Secondary structure prediction was done with *mfold* (http://unafold.rna.albany.edu/?q=mfold) using the default parameters. For MB4 two possible secondary structures were found that both have a stem-loop conformation.

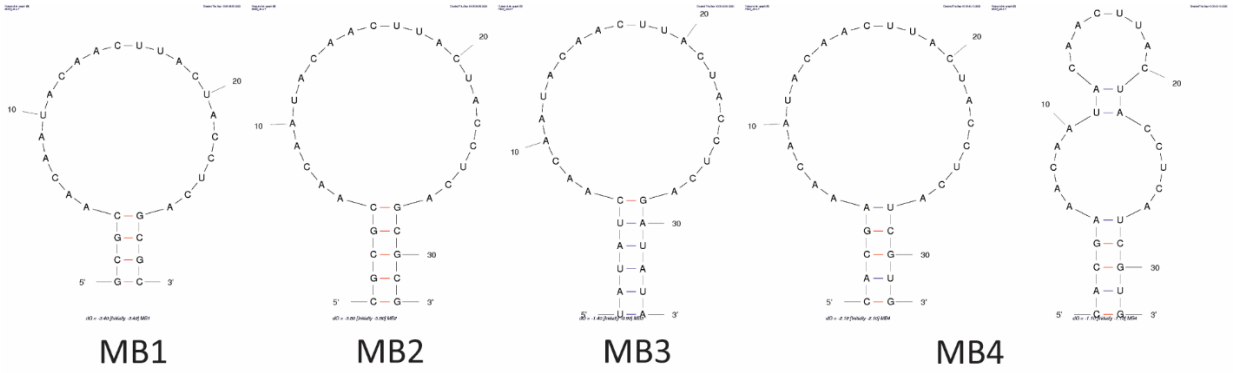


Figure S1: Predicted secondary structures of MB1-MB4.

5. Circularisation of photolabile-protected oligonucleotides

General procedure: 5 nmol of photolabile protected oligonucleotide were dissolved in a mixture of 25 µL DEPC-water and 17 µL DMSO. 5 µL (5 nmol, 1 eq) of a solution of 2-((1,3-diazidopropan-2-yl)oxy)acetic acid in DMSO (c = 1 mM) was added. In a second reaction tube 19 mg CuI (0.1 mmol) was suspended in 1 mL DMSO:*t*BuOH (3:1). The suspension was centrifuged and 1.5 µL supernatant was mixed with 1.5 µL of a 1 M solution of TBTA (1.5 mmol, 300 eq) in DMSO:*t*BuOH (3:1). The resulting Cu(I)-TBTA solution was added to the oligonucleotide solution and the mixture was reacted for 4 hours at 50 °C and 1000 rpm. 150 µL DEPC-water was added and the crude product was purified *via* RP-HPLC on an *Agilent 1200* equipped with an *Xbridge BEH C18 OBD* (300 Å, 3.5 µm, 4.6x250 mm, 0.8 mL/min, 25 °C). As solvents 400 mM hexafluoroisopropanol, 16.3 mM Et_3N, pH 8.3 and MeOH were used with a gradient from 5% MeOH to 100% MeOH in 50 minutes.

6. Sample preparation for *in vivo* use

For use in living cells, remaining HPLC buffer ions had to be removed. Therefore, the oligonucleotides were dissolved in 0.3 M NaOAc (*Merck*) (10 µL per 1 nmol RNA). EtOH (*Fluka*, prechilled to -20 °C, 40 µL per 1 nmol RNA) was added. The mixture was cooled to -20 °C for at least 6 hours. The precipitant was

pelletized by centrifugation at 4 °C, 20000 g for 20 minutes. The residue was redissolved in 0.3 M NaOAc and the precipitation steps were repeated 3 times.

To remove sodium ions, the oligonucleotides were desalted using a 1k cut-off membrane filter (*Microsep Advance Centrifugal Devices with Omega Membrane 1K*, *PALL*). Before adding the oligonucleotides, each filter was washed 5 times with DEPC water at 15000 g, 15 °C for 20 minutes. The desalting step was repeated 3 times.

7. Fluorescence measurements *in vitro*

miR-98 was obtained from *biomers*. *In vitro* fluorescence measurements were performed on a Plate Reader Infinite M-200 Pro (*Tecan*) on 96-well plates (*Cornwell*, flat bottom, black, polystyrene) at 37 °C. For each measurement 50 pmol of molecular beacon was dissolved in brain buffer (*Cold Spring Harbor Protocols*, final concentration: 135 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4) and incubated with 75 pmol miR-98 (final volume : 100 µL). Cyanine-5 was excited with 630 nm. Fluorescence was measured at 670 nm. Every experiment was repeated 3-5 times.

Beyond fluorescence studies of **ptMB3** shown in Fig. 4, we wanted to investigate whether it is possible to inhibit target hybridisation by using other photo-tether positions. Additionally, we wanted to test if adding a second quencher to the stem would reduce the background fluorescence.[22] Therefore, we synthesised **MB1b** (as reference) and the respective photo-tethered probes **ptMB1b_1** and **ptMB1b_2** (Suppl. Fig. S2). To simplify synthesis and purification, these probes did not contain a tracking fluorophore. The background fluorescence of **MB1b** was reduced by 50% compared to **MB1**. But also the maximum signal intensity after miR-98 addition decreased notably. With this result, we decided to not use a second quencher for further experiments with fully labeled ptMBs. The background fluorescence of **ptMB1b_1** and **ptMB1b_2** was comparable to **MB1b**. After miR-98 addition, the hybridisation intensity of the two photo-tethered probes was slightly higher. This is likely a result of flexibility of 3' or 5' end. While **ptMB3** is fully rigid, **ptMB1b_1** and **ptMB1b_2** are able to interact with target RNA at flexible ends of the loop. Due to partial binding, a marginal interaction between MB and target could cause a signal increase. Nevertheless, after photo-activation with 365 nm, the fluorescence intensity of **ptMB1b_1** and **ptMB1b_2** increased notably in presence of miR-98. This indicates that the MB functionality can be restored with light. The overall fluorescence measurements in Fig. 4 and Suppl. Fig. S2 show, that photo-tethers can be applied at different positions of the loop to successfully prevent hybridisation between ptMB and miR-98. However, the optimal photo-tether positions seem to be close to the stem, to provide full loop rigidity.

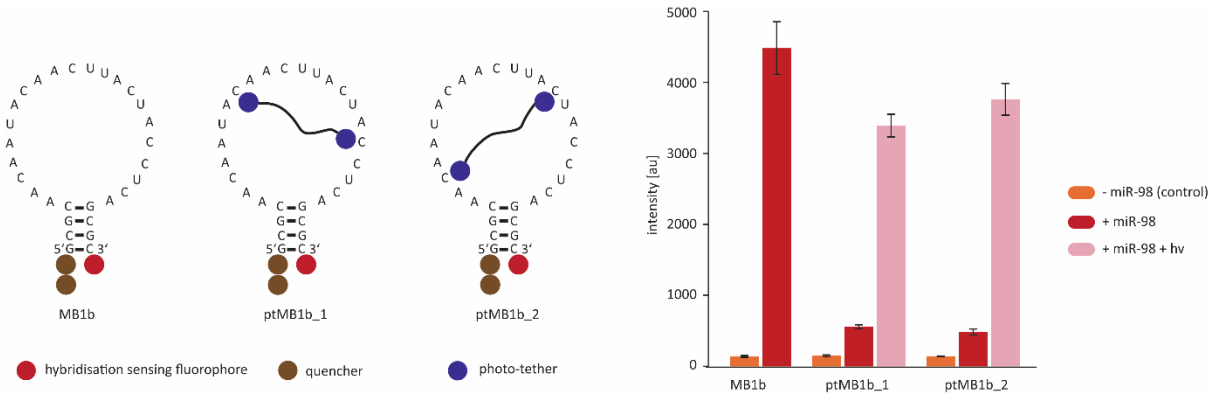


Figure S2: Fluorescence measurements of **MB1b**, **ptMB1b_1** and **ptMB1b_2** at 37 °C in brain buffer (50 pmol, 100 µL, 500 nM). Irradiation was performed with a 365 nm LED for 8 minutes (350 mA).

Negative control experiments of **MB1-MB4** and miR-181a:

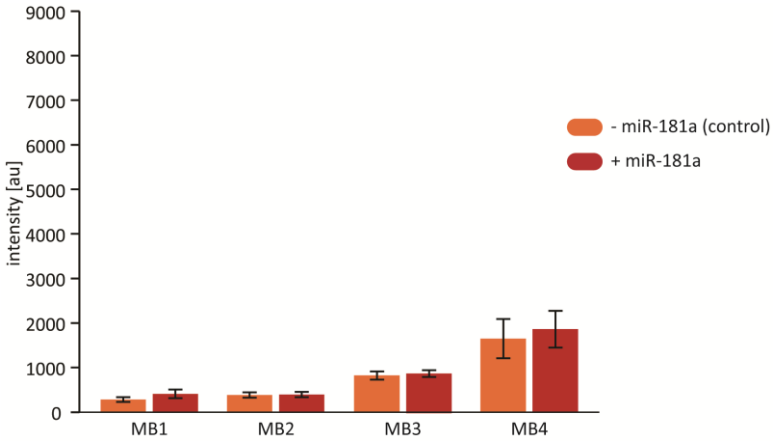


Figure S3: Fluorescence measurements of **MB1-MB4** and negative control miR-181a at 37 °C in brain buffer (50 pmol, 100 µL, 500 nM).

8. Transfection of anti-miR MBs

Hippocampi from postnatal day 0-1 rat pups of either sex were dissected and dissociated with papain (*Sigma*). For live-cell imaging, 30K neurons were plated onto a poly(D-lysine)-coated glass-bottom Petri dish (*Mattek*). Hippocampal neurons were maintained at 37 °C and 5% CO_2 in growth medium consisting of Neurobasal-A (*Gibco*) supplemented with B27 (*Gibco*) and GlutaMax-I (*Gibco*).

Cultured neurons (DIV 3-4 weeks) were transfected with anti-miR-98 MBs (20 pmol) using either Lipofectamine Messenger Max (*Thermofisher*) according to manufacturer's protocol. In brief, 20 pmol anti-miR-98 were incubated with 4 µl Lipofectamine Messenger-Max in 200 µl culture medium for 30 min at room temperature. Transfection mix was then added to neurons and live-cell imaging was performed 2 – 4 h post-transfection.

9. Photoactivation and live-cell imaging

In vitro irradiation was performed with a *Thorlabs* LED driver DC2100 equipped with a *Thorlabs* M365L2 LED (365 nm, 350 mA) on the 96-well plates.

Neurons were imaged in buffer (120 mM NaCl, 3 mM KCl, 10 mM D-Glucose, 10 mM HEPES) supplemented with essential amino acids (Gibco), B27 (Gibco) and GlutaMax-I (Gibco). Live-cell imaging was performed in airy scan modus using a Zeiss LSM880 confocal laser fluorescence microscope system. The incubator was set to 37 °C and a 40x water immersion objective were used. Photoactivation was performed using a multi-photon laser set-up at 355 nm with either 0.18 mW laser power. Images were acquired every 5 sec for 250 sec or 300 sec from a single plane. Following the acquisition of the pre-photo-activation fluorescence images ("baseline") for the first 10 frames (50 sec), consecutive photo-activation pulses were given starting from the 11st frame and repeated every five frames (8 pulses in total).

10. NMR and mass spectra

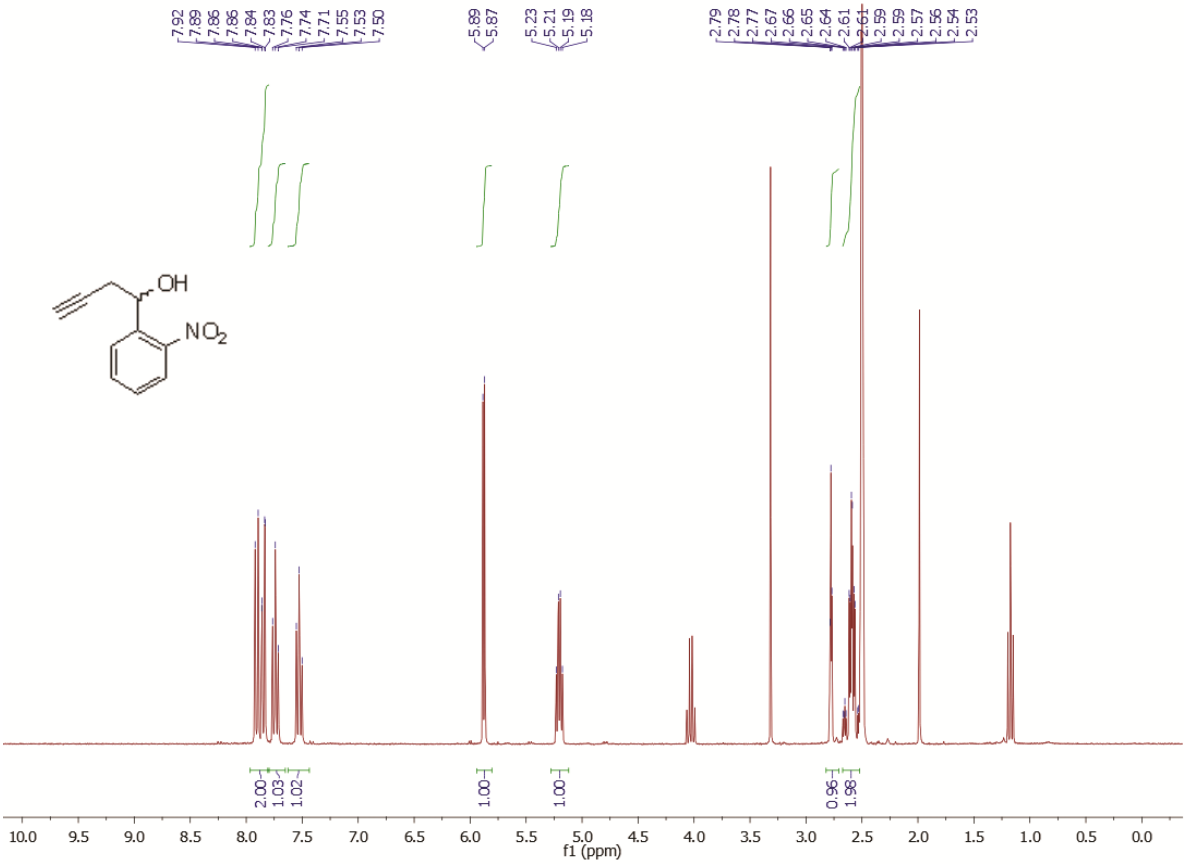


Figure S4: ^{1}H-NMR spectrum of compound **2** in DMSO-d_6. Residual EtOAc signals visible.

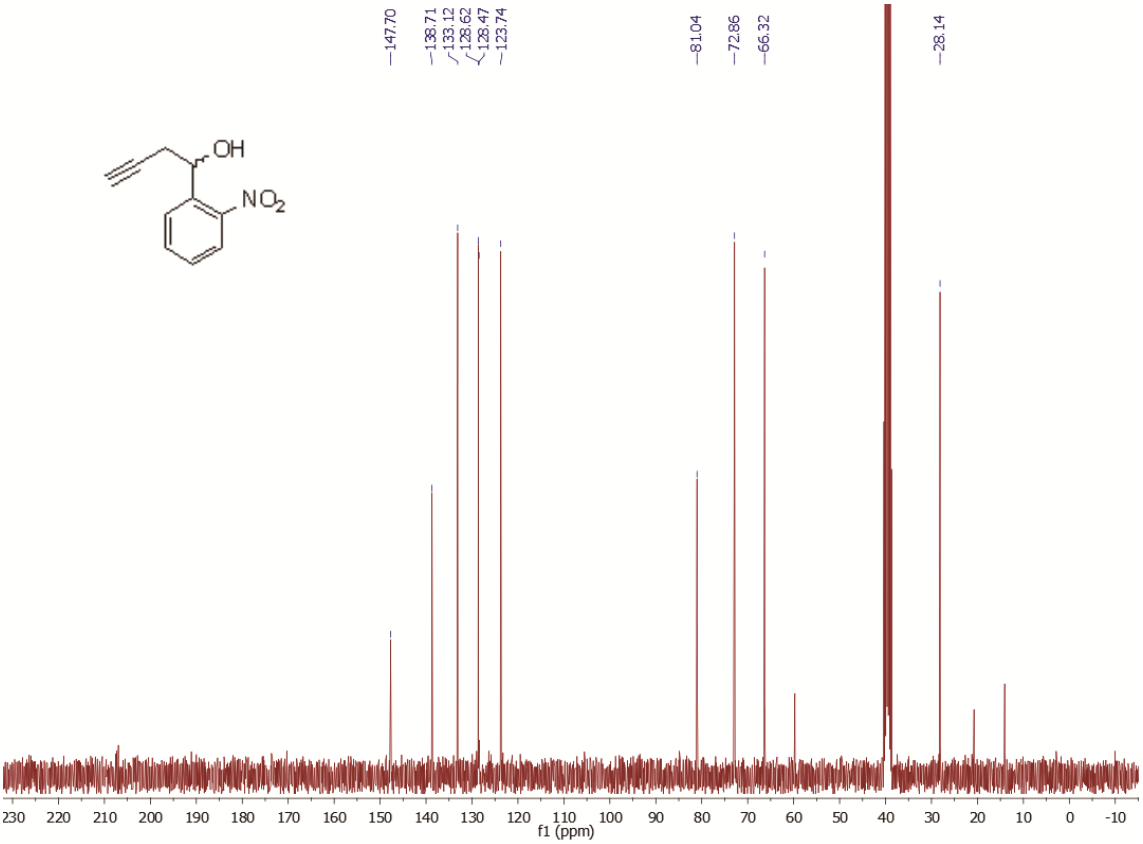


Figure S5: ^{13}C-NMR spectrum of compound **2** in DMSO-d_6. Residual EtOAc signals visible.

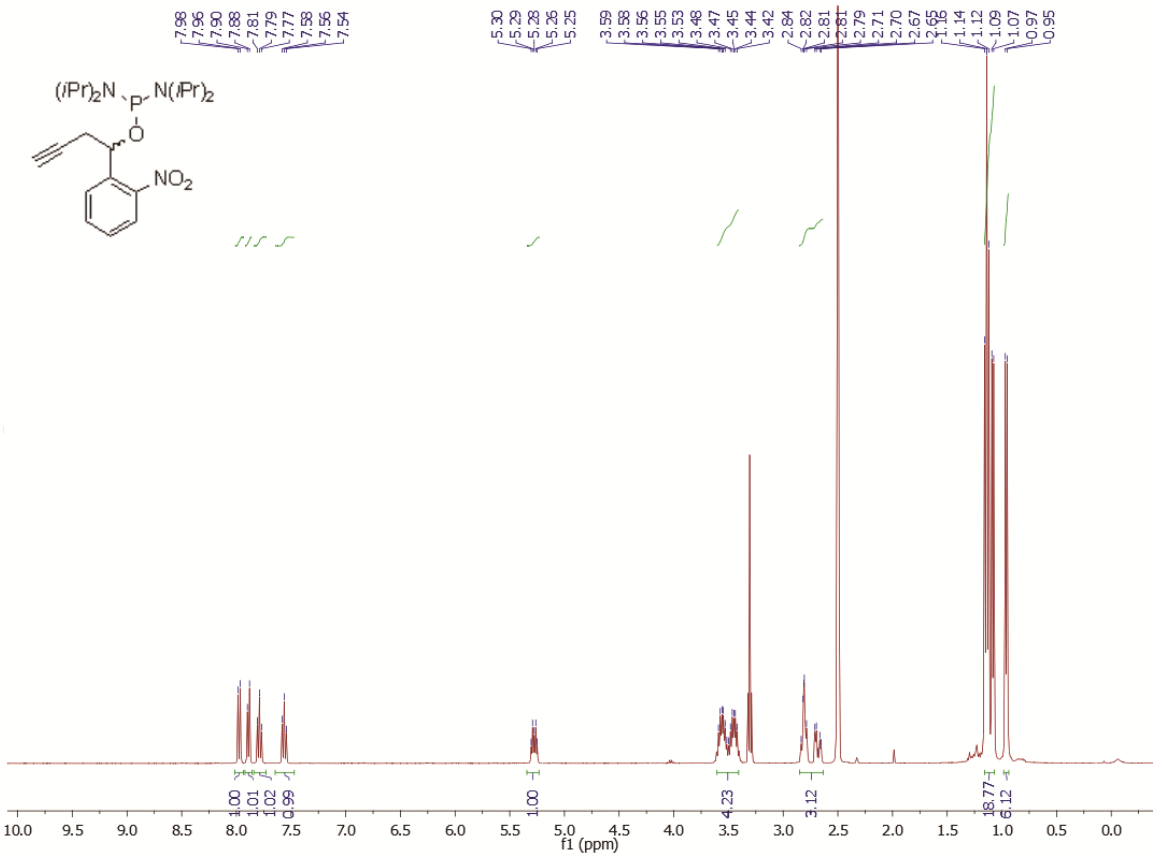


Figure S6: ^{1}H-NMR spectrum of compound **3** in DMSO-d_6.

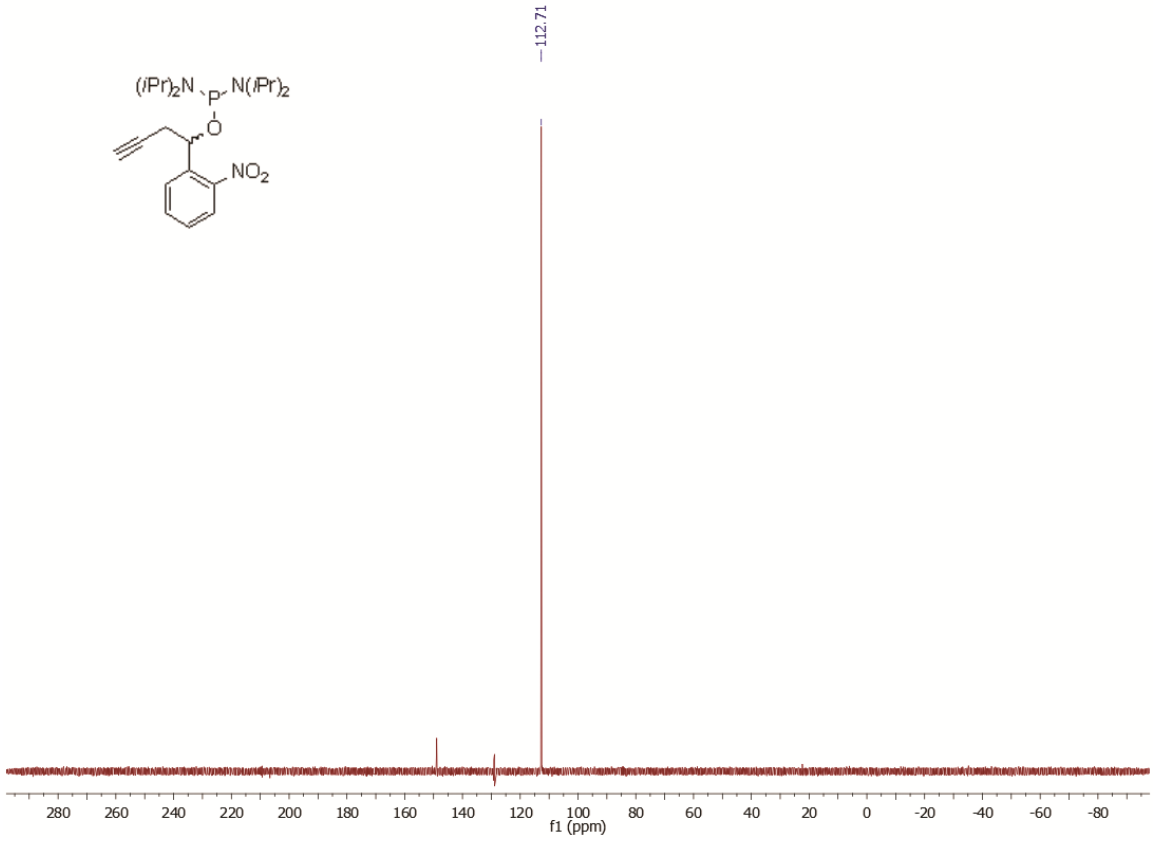


Figure S7: ^{31}P-NMR spectrum of compound **3** in DMSO-d_6.

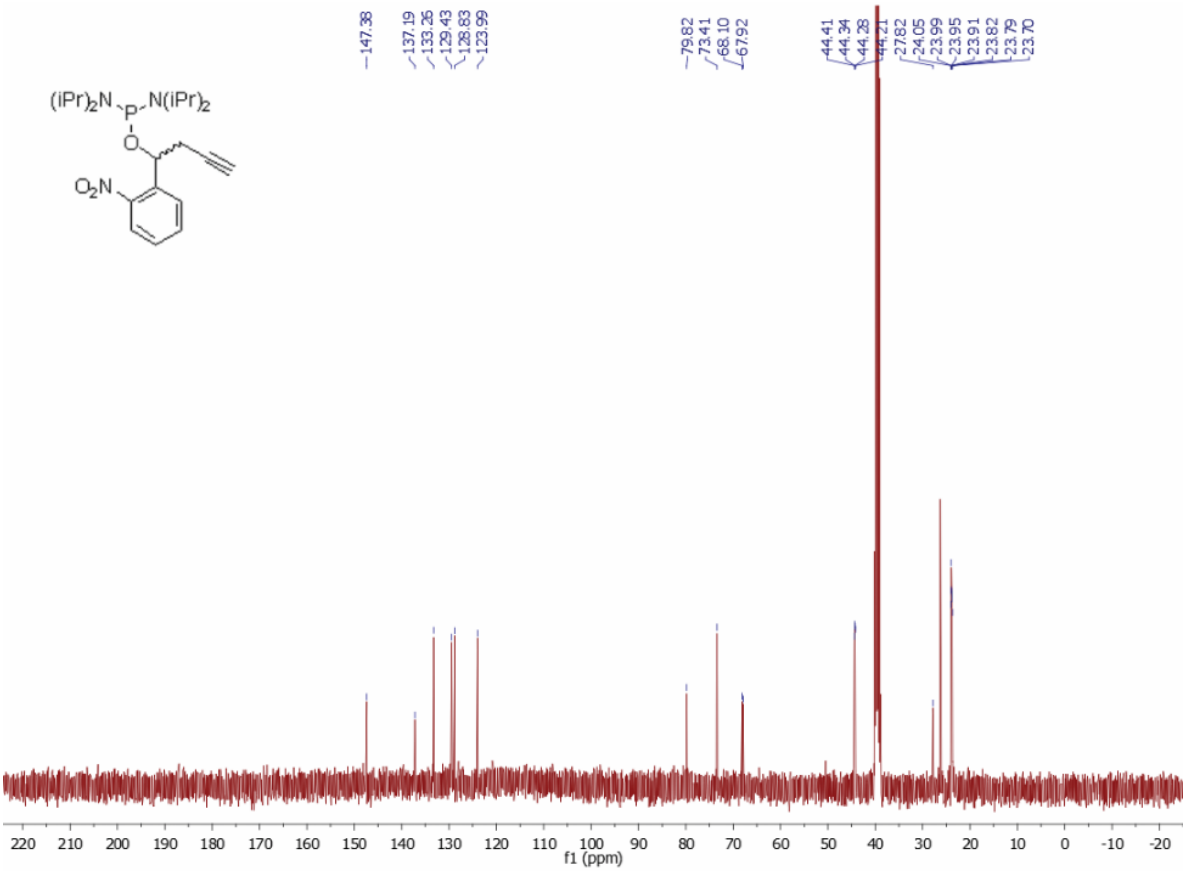


Figure S8: ^{13}C-NMR spectrum of compound **3** in DMSO-d_6. Residual cyclohexane signal visible.

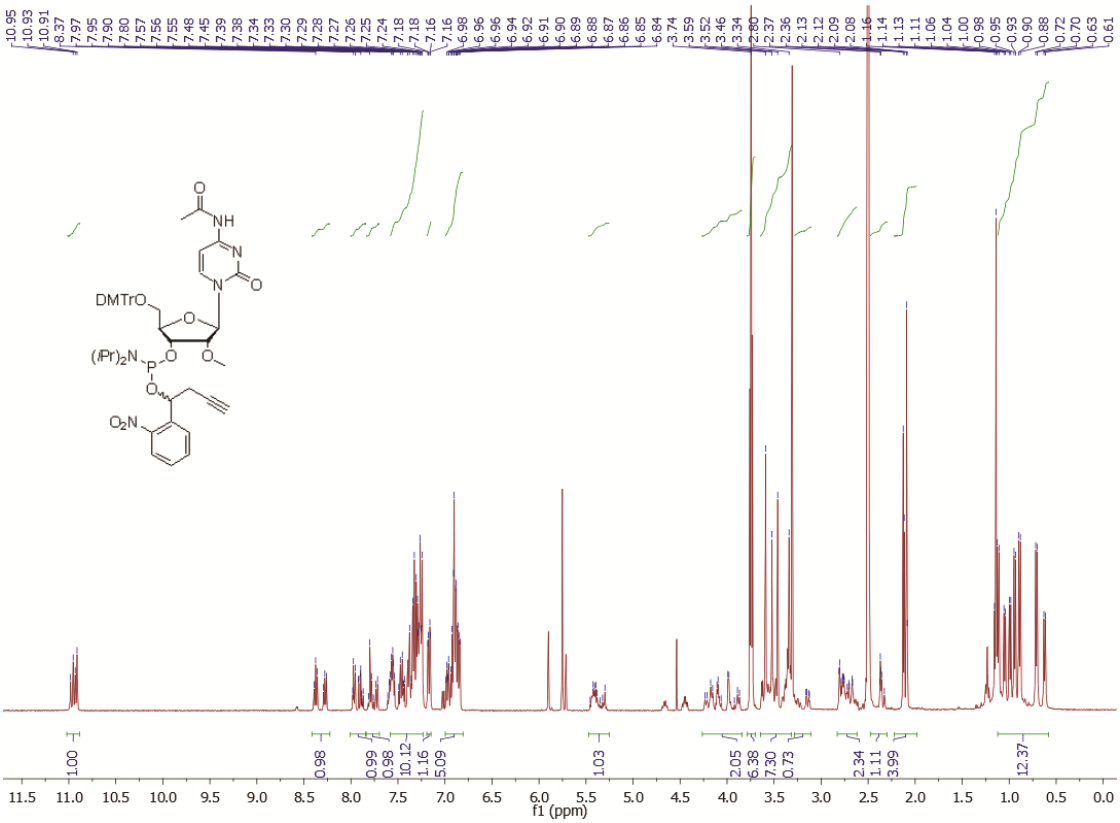


Figure S9: ^{1}H-NMR spectrum of compound **4** in DMSO-d_6.

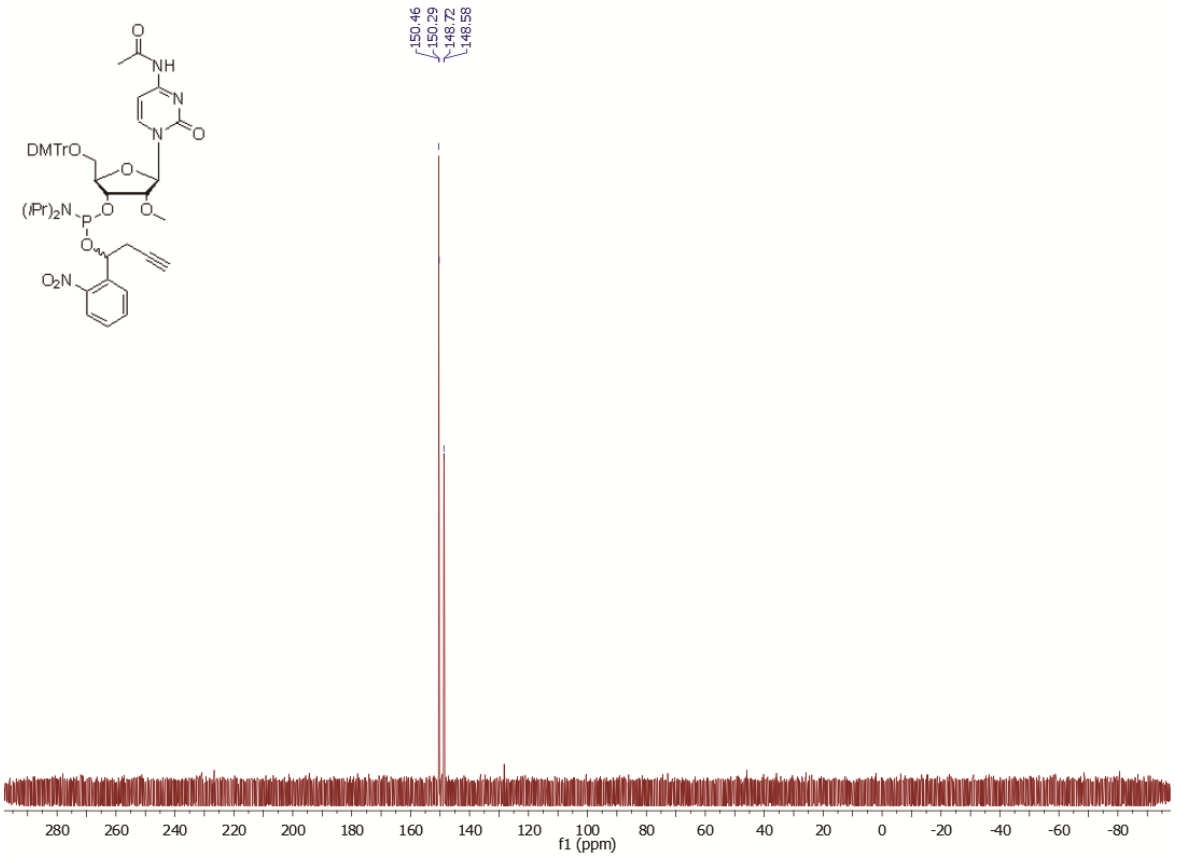


Figure S10: ^{31}P-NMR spectrum of compound **4** in DMSO-d_6.

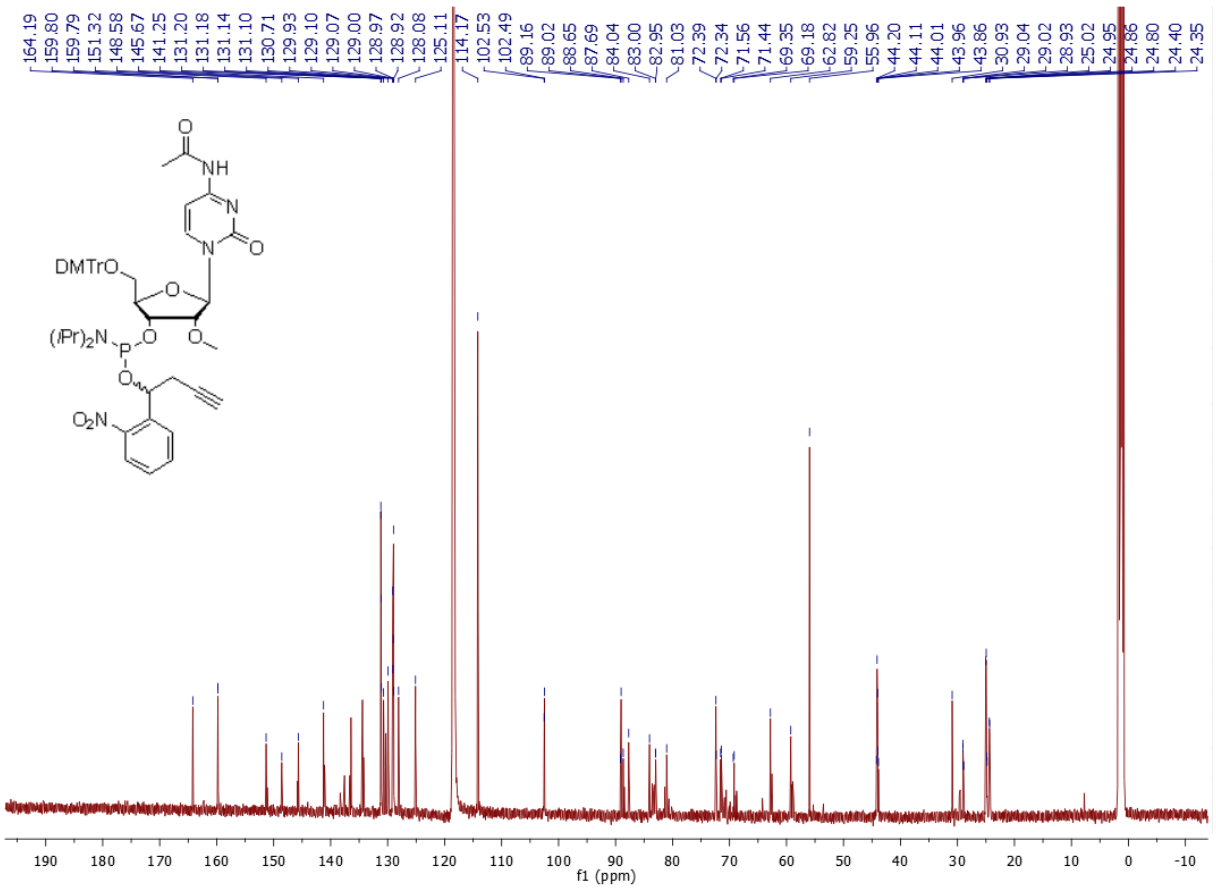


Figure S11: ^{13}C-NMR spectrum of compound **4** in CD_3CN.

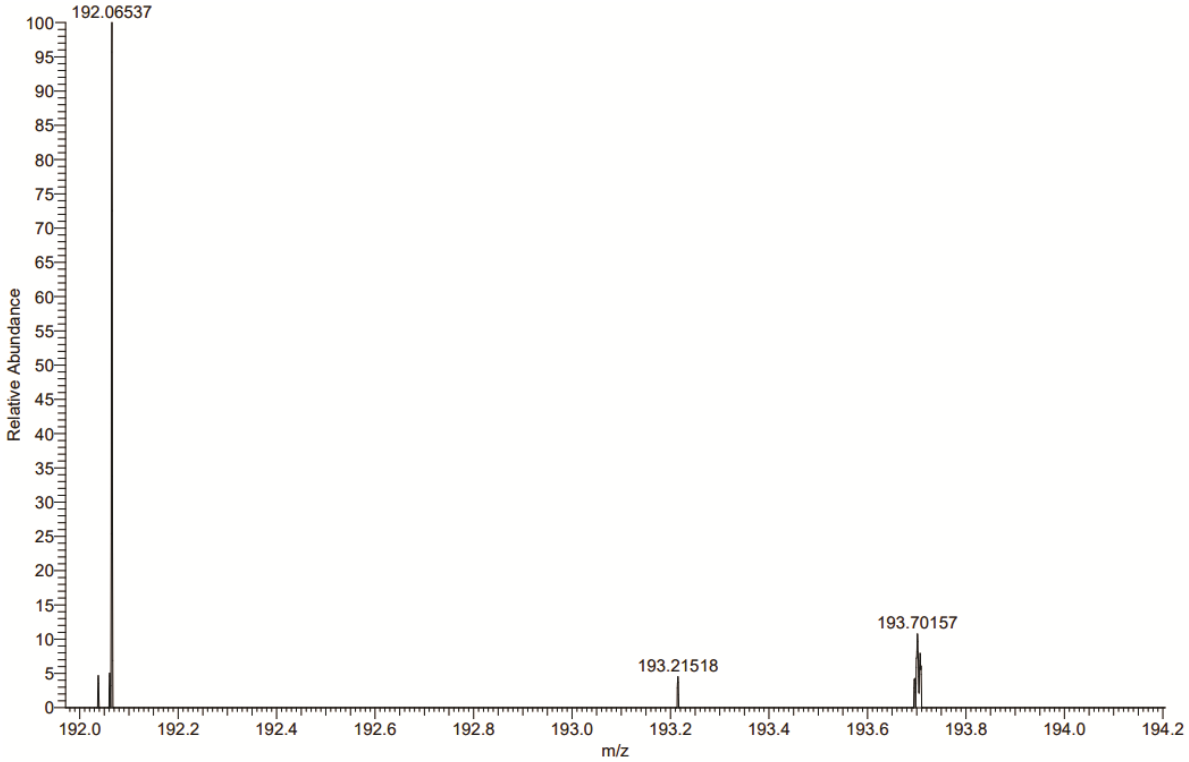


Figure S12: MALDI-HRMS spectrum of compound **2** (calculated $[M+H]^+$ 192.06552).

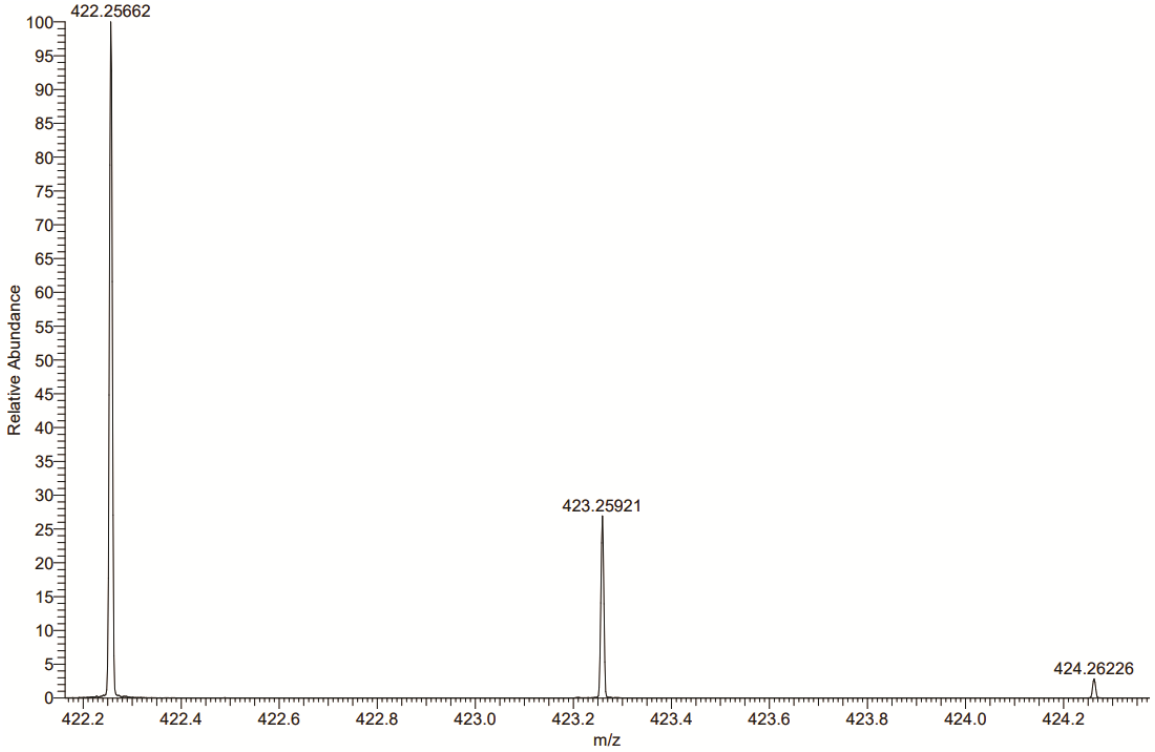


Figure S13: MALDI-HRMS spectrum of compound **3** (calculated $[M+H]^+$ 422.25671).

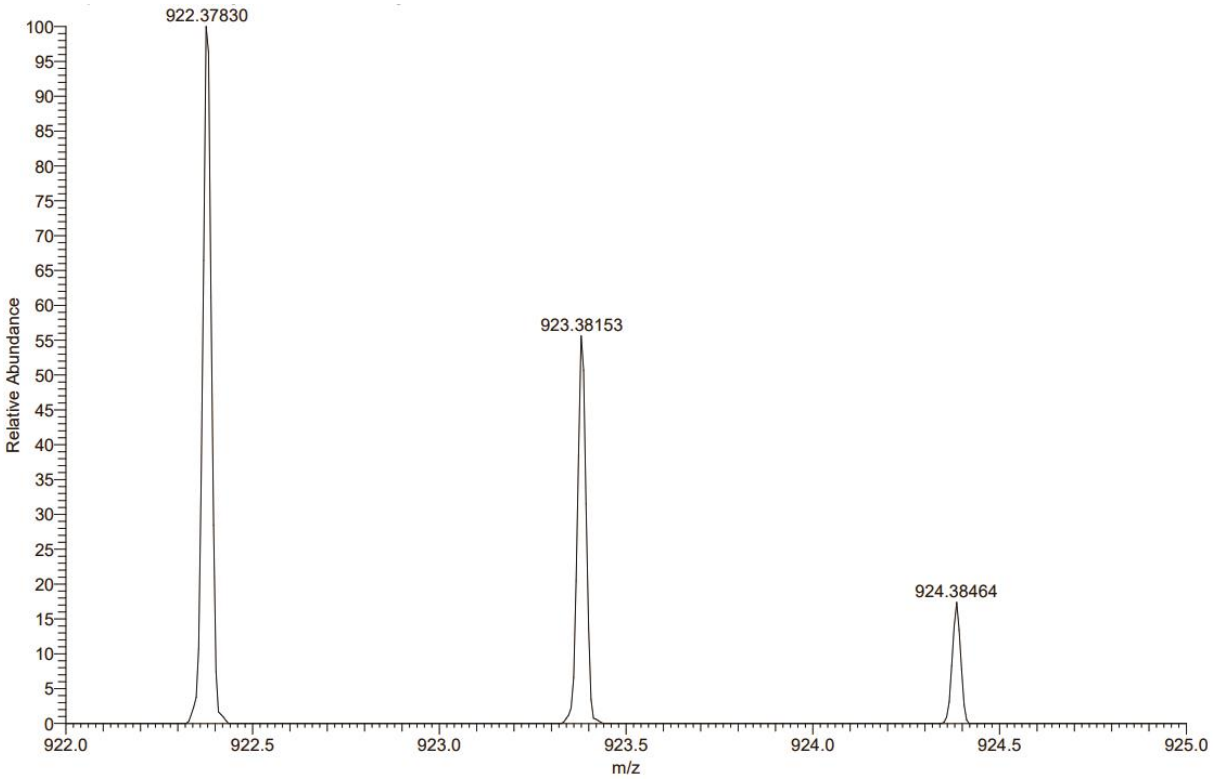


Figure S14: ESI-HRMS spectrum of compound **4** (calculated $[M+H]^+$ 922.37867).

NMR spectra were recorded on (300 MHz, 400 MHz or 500 MHz) *Bruker* instruments. High resolution mass spectra (HRMS) were obtained with a *Thermo Scientific* LTQ Orbitrap XL.