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**SUPPORTING INFORMATION**

# A Cationic Morpholino Antisense Oligomer Conjugate: Synthesis, Cellular Uptake and Inhibition of *Gli1* in Hedgehog Signaling Pathway**

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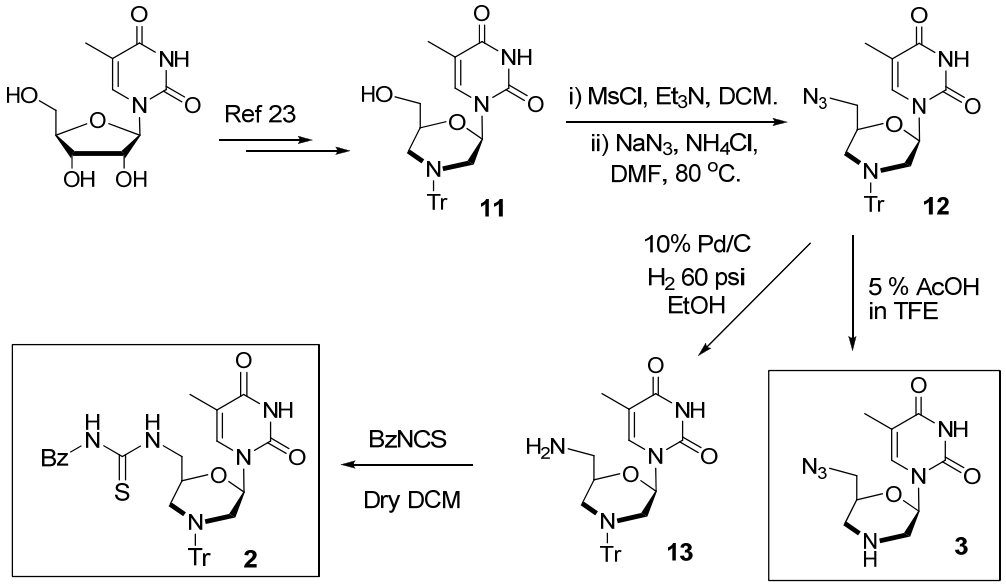
## General Information

All reagents were purchased from commercial sources and used without further purification, unless otherwise mentioned. Solvents were purified and dried according to recommended procedures.[1] All reactions were carried out in oven-dried glassware under argon atmosphere using anhydrous solvents unless otherwise mentioned. TLC was monitored on aluminum backed Silica Gel 60 (F254) 0.25mm plates (Merck) using methanol and dichloromethane mixtures. Compounds were visualized using UV light (254 nm) and staining with a solution of cerium ammonium molybdate and ninhydrin. Column chromatography was performed on silica gel (100-200 mesh). The column was treated with 0.5% triethyamine in DCM prior to purify tritylated compounds. Preparative TLC were carried out using Merck GF 254 silica gel on glass support. Melting points are uncorrected. $^{1}H$ and $^{13}C$ NMR spectra were recorded in Brucker NMR machine at 500 MHz and 125 MHz, respectively using $CDCl_3$ or DMSO-$d_6$ as solvent. Chemical shifts (δ) are given in ppm relative to the solvent residual peak or TMS as internal standard. The following abbreviations are used for multiplicity of NMR signals: s = singlet, d = doublet, t = triplet, sept = septet, m = multiplet, br = broad signal. For cell culture, reagents from Invitrogen were used. Gli1-morpholino was obtained from Gene Tools LLC. Cell culture dishes were obtained from BD Falcon. The fluorescent microscopic images were obtained in a Leica DM 3000 microscope. The CD spectra were recorded in a JASCO J-815 CD spectrometer. The spectra were smoothened and plotted using OriginPro 6.1. Luminescence was measured by integration over 10s in a Thermo Luminoskan Ascent using Promega Bright Glow assay kit. The antibodies were obtained from Cell Signalling and Thermo.

## Synthetic procedures

**Scheme S1. Synthetic scheme for monomers 2 and 3.**




**7′-Azido-*N*-trityl morpholinothymidine (12).**

Mesyl chloride (96 µL, 1.24 mmol) was added dropwise to an ice-cold solution of **11** (500 mg, 1.03 mmol) and $Et_3N$ (0.25 mL, 1.80 mmol) in dry DCM (5 mL) under Ar atmosphere, and allowed to stir for 2 h at room temperature. The reaction was quenched with water and extracted with EtOAc (2 × 25 mL). The combined organic layers were washed with water, sat. $NaHCO_3$, brine and dried over $Na_2SO_4$. The solvent was removed under reduced pressure to obtain a white mass which was used in the next step without further purification.

To a solution of the mesyl-derivative in dry DMF (3 mL) were added sodium azide (201 mg, 3.1 mmol) and ammonium chloride (220 mg, 4.1 mmol) and heated at 80 °C for 3 h. The solvent was removed under reduced pressure and diluted with EtOAc (40 mL). The organic layer was washed with water (4 × 25 mL), brine, dried over $Na_2SO_4$ and concentrated. Upon purification over silica gel (elution with 20-40 % ethyl acetate in pet. ether), the title product **12** (424.3 mg, 81% over two steps) was obtained as a white solid. mp 118 – 120 °C; IR (KBr): $\nu_{max}$ 2102, 1694 $cm^{-1}$. $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 1.43 (t, 1H, $J$ = 10.5 Hz), 1.55 (t, 1H, $J$ = 11.3 Hz), 1.83 (s, 3H), 3.08 (t, 1H, $J$ = 12.0 Hz), 3.22 (dd, 1H, $J$ = 13.3, 5.8 Hz), 3.35-3.39 (m, 2H), 4.30-4.33 (m, 1H ), 6.14 (dd, 1H, $J$ = 9.8, 2.3 Hz), 7.03 (s, 1H), 7.20 (t, 3H, $J$ = 7.0 Hz), 7.31 (t, 6H, $J$ = 7.5 Hz ), 7.46 (br s, 6H), 8.19 (br s, 1H); $^{13}C$ NMR (125 MHz, $CDCl_3$) $\delta$ 12.5, 49.8, 52.7, 75.9, 77.4, 80.9, 110.9, 126.8, 128.1, 129.3, 135.3, 149.8, 163.4; HRMS (ESI) $m/z$ $[M + Na]^+$, calcd for $C_{29}H_{28}N_6O_3Na$ 531.2121, found 531.2120.

**7´-Azido- morpholinothymidine (3).**

Prepared from **12** by deprotection with 5 % acetic acid in trifluoroethanol (TFE) and used without purification as described in case of synthesis of **4** below.

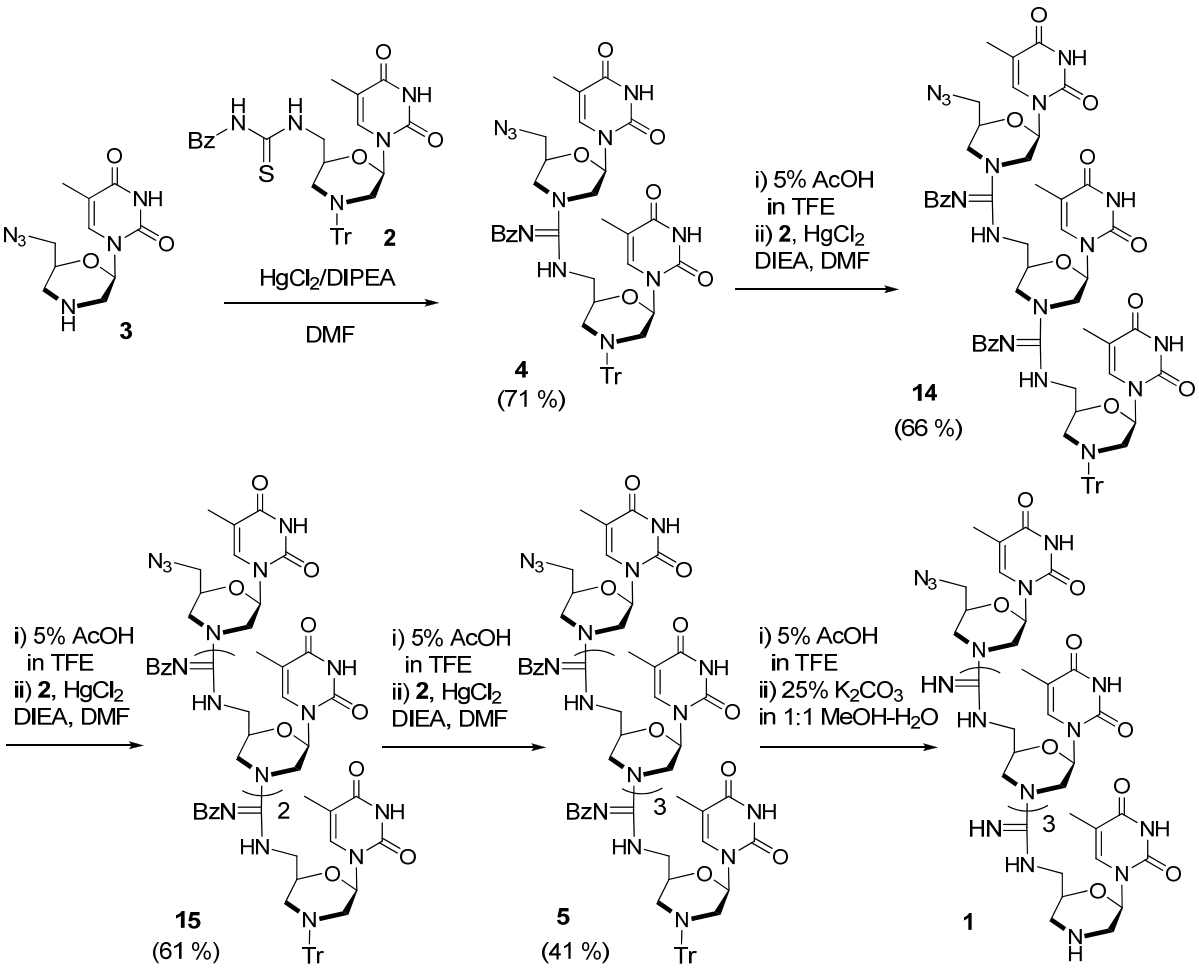
**7´-Amino-*N*-trityl morpholinothymidine (13).**

A solution of the azide nucleoside 1**2** (400 mg, 0.79 mmol) in presence of 10% Pd-C (30 mg) in ethanol was reduced under 60 psi pressure of $H_2$ in a parr apparatus for 8 hr. After completion of the reaction, the catalyst was filtered through a pad of celite and the solvent was removed under reduced pressure. The crude was purified through silica gel (1% TEA treatment of column, then elution with 10% MeOH-DCM, 100-200 mess silica) to afford the title product **13** as a white solid (335 mg, 88 %). IR (KBr): $\nu_{max}$ 3377, 1694 $cm^{-1}$. $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 1.34-1.43 (m, 2H), 2.66-2.76 (m, 2H), 3.07 (d, 1H, *J* = 12.0 Hz), 3.33 (d, 1H, *J* = 10.0 Hz), 4.10-4.14 (m, 1H ), 6.13 (dd, 1H, *J* = 9.8, 2.3 Hz), 6.99 (s, 1H), 7.18 (t, 3H, *J* = 7.0 Hz), 7.29 (t, 6H, *J* = 7.5 Hz ), 7.47 (br s, 6H); $^{13}C$ NMR (125 MHz, $CDCl_3$) $\delta$ 12.5, 44.6, 50.3, 52.2, 53.5, 78.8, 80.7, 110.6, 126.6, 128.0, 129.4, 135.6, 150.0, 163.7; HRMS (ESI) *m/z* $[M + Na]^+$, calcd for $C_{29}H_{30}N_4O_3Na$ 505.2216, found 505.2218.

**7´-(Benzoylthiourea)-*N*-trityl morpholinothymidine (2).**

To a solution of **13** (180 mg, 0.373 mmol) in dry DCM (8 mL) was added a solution of benzoyl isothiocyanate (50 μL, 0.373 mmol) in DCM (1 mL) dropwise at 0 $^oC$, under Ar atmosphere. The reaction was left to stir for 30 min and the solvent was evaporated under reduced pressure. The crude was purified through $SiO_2$ (0-5 % MeOH-DCM) column chromatography to obtain the title product **2** as a yellowish white solid (195 mg, 81 %). IR (KBr): $\nu_{max}$ 1691 $cm^{-1}$. $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 1.46 (t, 1H, *J* = 10.5 Hz), 1.54 (t, 1H, *J* = 11.3 Hz), 1.91 (s, 3H), 3.20 (d, 1H, *J* = 12.0 Hz), 3.41 (d, 1H, *J* = 10.0 Hz), 3.74-3.76 (m, 1H), 3.90-3.98 (m, 1H ), 4.50-4.52 (m, 1H), 6.20 (dd, 1H, *J* = 9.5, 2.0 Hz), 7.20 (s, 1H), 7.20 (t, 3H, *J* = 7.5 Hz), 7.33 (t, 6H, *J* = 7.5 Hz ), 7.49 (br s, 6H), 7.55 (t, 2H, *J* = 7.8 Hz), 7.66 (t, 1H, *J* = 7.3 Hz), 7.83 (d, 2H, *J* = 8.0 Hz), 8.19 (br s, 1H), 9.05 (s, 1H), 10.91 (s, 1H); $^{13}C$ NMR (125 MHz, $CDCl_3$) $\delta$ 12.5, 47.8, 50.1, 52.0, 74.6, 76.9, 80.7, 110.8, 126.7, 127.5, 128.1, 129.3, 131.8, 133.8, 135.7, 149.7, 163.3, 166.8, 180.6; HRMS (ESI) *m/z* $[M + Na]^+$, calcd for $C_{37}H_{35}N_5O_4SNa$ 668.2308, found 668.2307.

**Scheme S2. Solution phase synthesis of guanidinium-liked thyminyl morpholino pentamer.**



**7´-Azido-thymidyl benzoylated-GMO dimer (4).**

The azide functionalized morpholino monomer **12** (60 mg, 0.118 mmol) was detritylated with 5 % AcOH-TFE for 3 hr. The solvent was evaporated off and the residue was washed with 5% EtOH-Pet Ether twice to remove the trityl portion. The thiourea monomer **2** (75 mg, 0.116 mmol) was then added and the mixture was dissolved in dry DMF (500 μL). To this solution, dry DIPEA (0.30 mmol, 51 μL) was added and cooled to 0 $^{o}$C. Following, a solution of $HgCl_2$ (35 mg, 0.130 mmol) in dry DMF (250 μL) was added dropwise under Ar atmosphere. Instant cloudy white precipitation appeared. The reaction was left to stir for 2 h, diluted with 1:1 MeOH-DCM and filtered through a pad of celite. The solvent was evaporated and the residue was purified through silica gel (5-10 % MeOH in DCM) to afford the dimer **4** as a white solid (73 mg, 71 %). $^{1}$H NMR (500 MHz, $CDCl_3$) $\delta$ 1.47-1.53 (m, 2H), 1.88 (s, 3H), 1.93 (s, 3H), 2.82 (t, 1H, *J* = 11.5 Hz), 2.88-2.96 (m, 1H), 3.09 (t, 1H, *J* = 11.5 Hz), 3.23 (d, 2H, *J* = 11.5 Hz), 3.34 (d, 1H, *J* = 9.5 Hz), 3.57 (d, 2H, *J* = 10.5 Hz), 3.87 (d, 1H, *J* = 13.0 Hz), 4.05-4.10 (m, 2H), 5.86 (d, 1H, *J* = 9.0 Hz), 6.26 (d, 1H, *J* = 9.0 Hz), 6.70-6.71 (m, 3H), 7.18 (t, 6H, *J* = 7.3 Hz), 7.28 (s, 2H), 7.31-7.44 (m, 7H), 7.50-7.53 (m, 2H), 8.06 (d, 2H, *J* = 7.5 Hz), 9.23 (br s, 1H), 9.56 (br s, 1H); $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 12.3, 12.6, 47.2, 48.1, 49.0, 50.0, 51.3, 52.1, 53.5, 54.6, 73.8, 74.6, 75.3, 77.0, 79.6, 80.1, 111.1, 111.8, 126.6, 127.9, 129.1, 129.4, 131.6, 134.3, 136.6, 137.5, 150.1, 150.2, 163.4, 163.9, 164.3; HRMS (ESI) *m/z* $[M + Na]^+$, calcd for $C_{47}H_{47}N_{11}O_7Na$ 900.3558, found 900.3558.

**7´-Azido-thyminyl benzoylated-GMO trimer (14), tetramer (15), pentamer (5).**

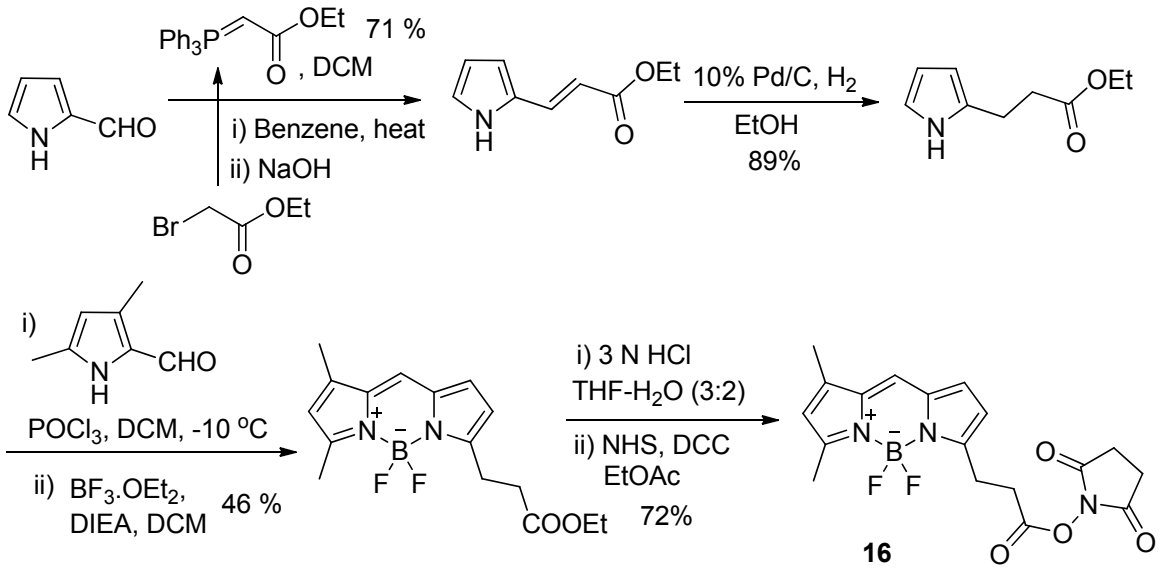
The synthesis is similar to that of **4**. A cyclic two step procedure (Scheme S2) was adopted. The starting oligomer was detritylated and then coupled with the incoming monomer **2,** as done in case of **4**. After reaction, the product was purified through silica gel and characterized by HRMS. The yields for each step are: 66 % for **14**; 61 % for **15**; 41 % for **5**.

| Compound | Mol Formula $(M+Na)^+$ | Calculated Mass | Observed mass |
|---|---|---|---|
| Trimer (**14**) | $C_{65}H_{66}N_{16}O_{11}Na$ | 1269.4995 | 1269.4993 |
| Tetramer (**15**) | $C_{83}H_{85}N_{21}O_{15}Na$ | 1638.6432 | 1638.6226 |
| Pentamer (**5**) | $C_{101}H_{104}N_{26}O_{19}Na$ | 2007.7869 | 2007.2675 |

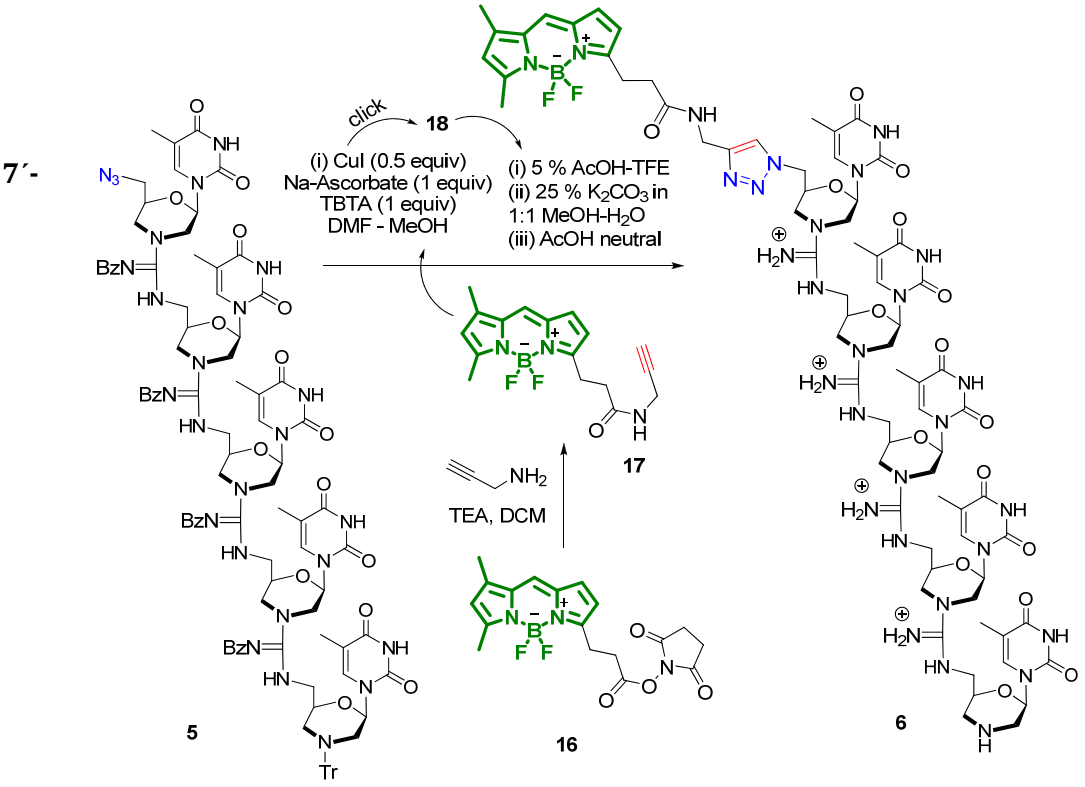
**Guanidinium-linked morpholino pentamer (1)**

Trityl group of **5** (3.2 mg, 1.57 nMol) was deprotected with 5% HOAc in TFE, solvent was removed and the solution of trityl-deprotected **5** in 25 % $K_2CO_3$ in MeOH-$H_2O$ (1:1) was stirred for 12 h at room temperature. The MeOH layer was reduced to half and the aqueous layer was extracted with DCM with vigorous vortexing. The DCM layer was evaporated; the residue was dissolved in $H_2O$ and dialysed with a dialysis bag (MW cut off 650) for 24 hr. The aqueous solution was concentrated to dryness and the residue was washed thrice with ethyl acetate and acetone. The amount of the oligomer was quantified with UV measurement and 68 % yield of **1** was obtained. For CD measurement, the residue was further purified with reverse phase (C18, Merck) column chromatography. HRMS (ESI) *m/z* $[M + 4H]^{4+}$, calcd for $C_{54}H_{78}N_{26}O_{15}^{4+}$ 332.6530, found 333.0684.

**Scheme S3. Modified literature route[2] for the synthesis of the BODIPY –NHS ester 16.**



**Scheme S4. Solution phase synthesis of guanidinium-liked thyminyl morpholino pentamer.**




**BODIPY-thymidyl benzoylated-GMO pentamer (18).**

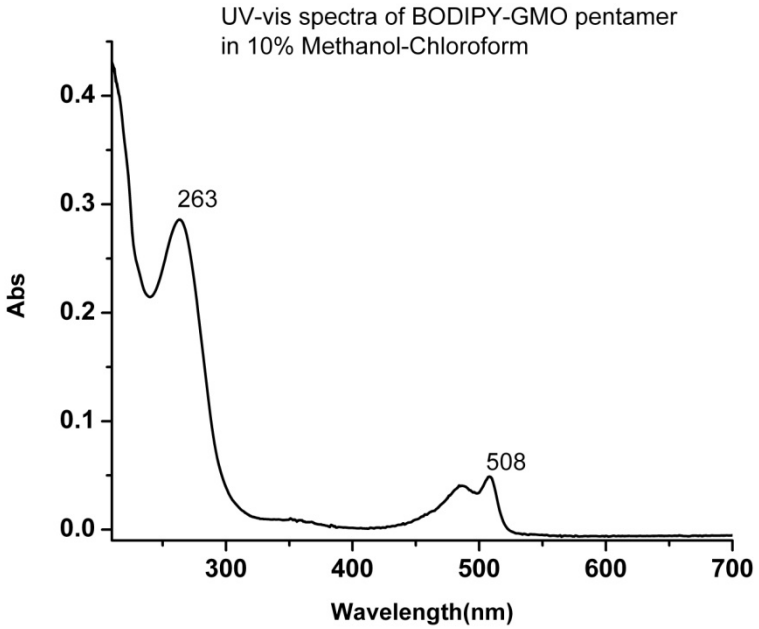
BODIPY-NHS ester **16** was prepared using a modified literature procedure as depicted in Scheme S3. A solution of **16** (2 µmol) in DCM was treated with propargyl amine (2.5 µmol) in presence of excess of triethyl amine (14 µL, 10 µmol). After reaction, the mixture was prolonged dried under vacuum, with small amount of DCM every time, until smell of propargyl amine fade away. The crude product **17** was directly used for the click reaction.

A mixture of the GMO pentamer **5** (2.5 mg, 1.25 nMol), acetylene BODIPY **17** (2 µmol), CuI (0.65 µmol), Na-ascorbate (1.26 µmol) and TBTA (1.26 µmol) in DMF-MeOH (200 µL, 1:1) was stirred for 24 h. This was purified through a short $SiO_2$ column and then with preparative thin layer chromatography to obtain the title product **18**. HRMS (ESI) *m/z* [M + Na]$^+$, calcd for $C_{118}H_{122}BF_2N_{29}O_{20}Na$ 2336.9380, found 2335.7480.

**7′-BODIPY-thymidyl -GMO pentamer (6).**

The BODIPY-conjugated and benzoyl protected GMO **18** was deprotected by 5% HOAC in TFE and the mass obtained was stirred with 100 µL of 25% $K_2CO_3$ solution in MeOH-$H_2O$ (1:1) for 24 h. The reaction mixture was neutralized with AcOH and the solvent was

reduced under vacuum. The residue was loaded to a small RP-silica gel (Merck) column and eluted with 0-20% of (8 % $NH_3$-MeOH) and acetonitrile. Uv-Vis spectra of the fractions were taken and fractions having absorption peak both at 262 nm and 506 nm were collected. The combined fractions were concentrated and the product **6** was again purified through preparative TLC and extracted with MeOH-DCM (1:1). The concentration of the stock was measured from the UV-Vis spectra. HRMS (ESI) *m/z* $[M + H]^{4+}$, calcd for $C_{71}H_{96}BF_2N_{29}O_{16}^{4+}$ 414.9407, found 413.1251.




UV-Visible spectrum of **6** (75 μM solution in 10% MeOH-$CHCl_3$)

**Synthesis of the cationic GMO-PMO conjugate by click chemistry (10)**

The azide moiety was intrinsically present in cationic GMO. To obtain the alkyne moiety, Gli1 PMO **7** (50 μL of a 1 mM soln, 50 nMole) was treated with large excess of hexynoic acid-NHS ester **8** (400 nMole X 3, total 24 equiv) in 250 μL of 0.1 M $NaHCO_3$ buffer for 26 hr in a microcentrifuge tube. Then eight volume excess of chilled acetone was added, vortexed and the oligo was precipitated. The mixture was kept in -20 $^oC$ for 4 hr and the palette of **9** was collected by centrifugation. It was washed thrice with 300 μL of chilled acetonitrile and centrifuged. The resulting palette was again dissolved in water (800 μL) and dialyzed for 24 h using a 3500 MW cut off dialysis bag to remove the remaining traces of low molecular weight reactants.

For the click reaction, Finn's protocol for click chemistry was followed.[3] The stock solutions prepared were $CuSO_4$ (20 mM in $H_2O$), Na-ascorbate (100 mM in $H_2O$), tris-

(benzyltriazolylmethyl)amine i.e TBTA (20 mM in DMSO) and diaminoguanidine (100 mM in $H_2O$). The azide GMO **1** stock was 5 mM in $H_2O$.

To a solution of **9** (50 nMole) in phosphate buffer (pH 7, 100 mM, 200 μL) were added the GMO **1** in 5 fold excess (250 nMole, 50 μL of the stock) and DMSO (100 μL) was added. The mixture was purged with argon to remove the dissolved oxygen. The above stock solution was added in such a way so that they reach the recommended final concentrations which are: premixed $CuSO_4$ (200 μM) and TBTA (1 mM), Na-ascorbate (5 mM); aminoguanidine hydrochloride (5 mM). The reaction mixture was vortexed for 30 hr and then precipitated with chilled acetone as discussed in case of **9** above. The resulting reddish palette containing product **10** was dissolved in 200 μL of water and incubated with 50 mM (50 μL) EDTA solution for 30 min to complex out the bound Cu. The mixture was dialysed for 24 h with a 3500 MW cut of dialysis bag using water. The concentration of the final product was measured by UV-Vis spectroscopy as a water solution. Recovered overall yield for **10** from **7** was 51 %. The product was concentrated in speed vac and redissolved in cell culture grade water to prepare stock solution for biological assay. In ESI-MS spectra (+ve ion mode, +4.5 KV ion spray voltage, temp 300 $^oC$) many multi-charged species corresponds to the desired molecular ion peak was accounted.

## Cell culture techniques

***CHO-K1 cells**:*

Chinese hamster ovary (CHO-K1) cells were obtained from cell repository NCCS, Pune. The cells were regularly maintained in DMEM medium (Gibco) supplemented with 10% FBS (Gibco), 100 μg/mL streptomycin and 100 units/mL penicillin. Cells were grown in 60 mm cell culture dishes and incubated at 37 °C in a humidified atmosphere of 5% $CO_2$ to approximately 70-80% confluence. A subculture was performed in every 4-5 days. After each 2 day, media was replaced with fresh media to eliminate the dead cells. For subculture, the adherent cells were detached from the surface of the culture dishes by trypsinization and splitted to new dishes.

***Shh-Light 2 cells**:* Shh-Light 2 (derived from mouse embryo NIH3T3 cell lines stably transfected with a Gli-dependent firefly luciferase and constitutive renilla luciferase reporters) cells were obtained as a gift from Professor JK Chen, Stanford University. The culture of Shh-Light II cells was performed in 10 % bovine calf serum (Gibco)-DMEM supplemented with 100 μg/mL streptomycin and 100 units/mL penicillin at 37 $^oC$ in a 5% $CO_2$ incubator in humidified atmosphere. For subculture, 0.25 % trypsin-EDTA was used and the cells were cryopreserved in 5 % DMSO in complete growth media at liquid $N_2$ temperature.

***HEK 293T cells:*** Human Embryonic Kidney 293T cells were kindly provided by Professor JK Chen, Stanford University. Regular culturing of 293T cells was done in a similar way as mentioned in case of CHO-K1 using the same 10% FBS –DMEM media.

***MCF-7 cells*:** Human breast adenocarcinoma MCF-7 cells were obtained from ATCC. MCF-7 cells were regularly cultured in DMEM supplemented with 10% FBS, 100 µg/mL streptomycin and 100 units/mL penicillin in a similar way as mentioned in case of CHO-K1 cells.

***PC-3 cells:*** Human prostate cancer cell line PC-3 was obtained from cell repository NCCS, Pune. PC-3 cells were cultured regularly in F12K nutrient mixture (Gibco) supplemented with 10 % FBS and 100 µg/mL streptomycin and 100 units/mL penicillin. For subculture, they were trypsinized with 0.25 % trypsin-EDTA.

## Cellular penetration study of BODIPY tagged-pentameric GMO

### *Live cell fluorescence imaging in CHO-K1 cells*

CHO-K1 cells were plated at 30000 cells/well in DMEM containing 10 % FBS and 100 µg/mL streptomycin and 100 units/mL penicillin in 24-well tissue culture plates (BD Falcon) and allowed to attach at 37 °C in a humid 5 % $CO_2$ atmosphere for 24 h. After removal of media, 500 µL of 0.5 % FBS in DMEM was added to each well. The BODIPY-conjugated pentameric GMO **6** in DMSO was added to each well so that 2.5 µM final concentration was achieved. Cells were incubated with BODIPY-GMO for 16 h, and the media was removed. The cells were washed twice with PBS and replaced with fresh media. The cells were directly visualised under an Olympus inverted fluorescent microscope with a 20X objective (**Figure S1**). The BODIPY-GMO intake was observed in almost all cells.

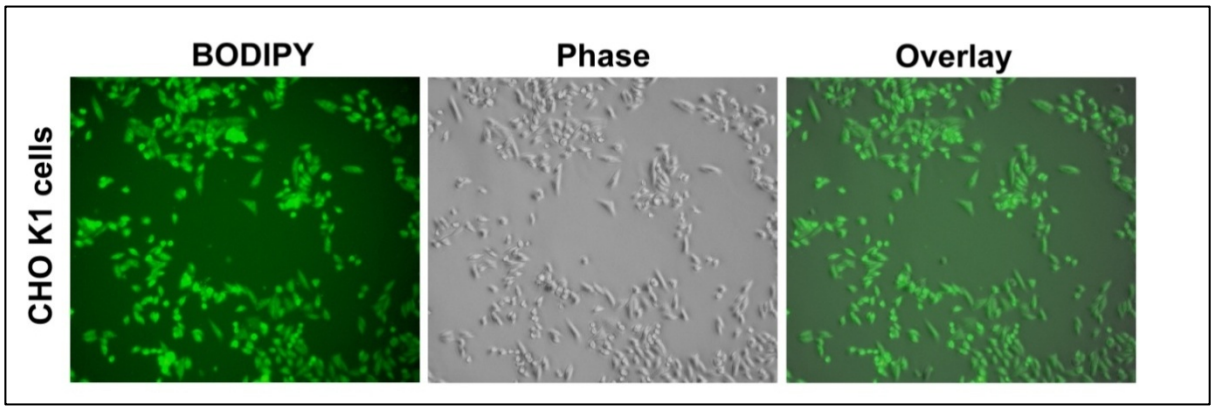



Figure S1. Live cell image (20X) of CHO K1 cells for cellular intake of BODIPY-GMO. Pentamer BODIPY-GMO **6** concentration: 2.5 µM, Incubation time 16 h.

***Fixed cell fluorescence imaging in 293T, MCF-7, PC-3 and Shh Light 2 cells***

HEK 293T, MCF-7, PC-3 and Shh LIGHT2 cells were seeded in 4 well chamber slides (BD Falcon) at a density of 10000 cells/well, in their respective growth medium and incubated in a humid 5% $CO_2$ incubator at 37 $^{o}$C. After 24 h, the media was replaced with 0.5 % FBS-DMEM containing 2.5 μM BODIPY-GMO **6**. The cells were incubated for 16 h at 37 $^{o}$C, and the culture medium containing BODIPY-GMO was removed. The cells were washed twice with PBS for 5 min and then gently fixed for 10 min using 3% paraformaldehyde in PBS. After fixation, the cells were washed with PBS containing 10 mM glycine and 0.2% sodium azide (PBS-GSA) and incubated with PBS-GSA for 5 min. The solution was removed and DAPI staining of nucleus was performed by incubating each well with 100 uL of 300 nM DAPI in PBS for 4 min. The cells were washed with PBS, the slide was then mounted with Invitrogen antifade reagent, covered with cover slip and visualised with a Leica DM3000 fluorescent microscope with 40X objective.

## Luciferase reporter assay

***Preparation of Sonic Hedgehog-conditioned medium***

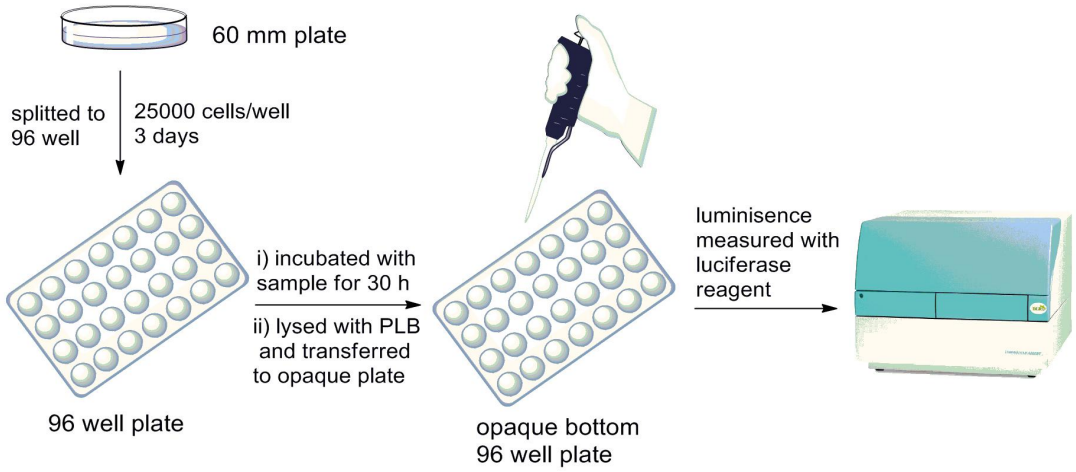
HEK 293T cells overexpressing Shh-N (N-terminal fragment of Shh without cholesterol modification) were cultured to 80 % confluence in DMEM supplemented with 10 % FBS, 100 μg/mL streptomycin and 100 units/mL penicillin. The media was replaced with 2 % FBS – DMEM containing 100 μg/mL streptomycin and 100 units/mL penicillin and cultured for another 24 h. The media was collected, centrifuged and filtered through 0.22 μ filter. The media was stored in -20 $^{o}$C and used to activate Shh pathway in Shh LIGHT2 cells.

***Cell based luciferase assay for hedgehog inhibition (Activation with Shh-conditioned media)***

Assays for Hh pathway activation in Shh-LIGHT2 cells, a clonal NIH3T3 cell line stably incorporating Gli-dependent firefly luciferase and constitutive *Renilla* luciferase reporters, were performed as described previously.[4] In a 96 well plate, 25000 Shh-LIGHT2 cells/well were cultured to confluence for 72 h in DMEM supplemented with 10 % bovine calf serum and 100 μg/mL streptomycin and 100 units/mL penicillin. According to the assay principle, the cells were co-exposed with Shh-conditioned media (1:10 dilution) in DMEM containing 0.5 % bovine calf serum, 100 μg/mL streptomycin and 100 units/mL penicillin to activate the Shh pathway. The stock solutions for the morpholinos **1**, **7** and **10** were prepared in cell culture grade water (Invitrogen) and introduced to the above incubation medium so that they reach the required concentration (Figure 4a). As a positive control Shh-condition media (1:10 dilution) in

incubation medium was used, negative controls were treated with cell culture water only. All the treatments are performed in duplicates.

After 30 h of incubation at standard condition, the media was carefully removed keeping the cell layer intact; the cells were washed once with PBS and lysed with 20 µL of 1X Passive lysis buffer (Promega) in each well. From there 10 µL/well of cell lysate was pipette into an opaque luminometer-compatible microplate. The Bright Glow Luciferase reagent (Promega; 40 µL in each well) was added using a robotic injecting system of the luminometer (Luminoscan, Thermo) and the light was measured for 10 sec after a lag time of 2 sec. The results are plotted (Figure 4a) to determine the inhibitions.




Luciferase assay with shh light II cells.

***Cell based luciferase assay for hedgehog inhibition (Activation with SAG)***

In another parallel assay like the above, a similar kind of experiment was repeated where the pathway activation was achieved with synthetic Hh agonist SAG instead of Shh-conditioned media. A 500 nM concentration of SAG was co-exposed with the oligos. Similar to the above experiment, inhibition of luciferase expression was observed in case of the conjugated morpholino (Figure S2).

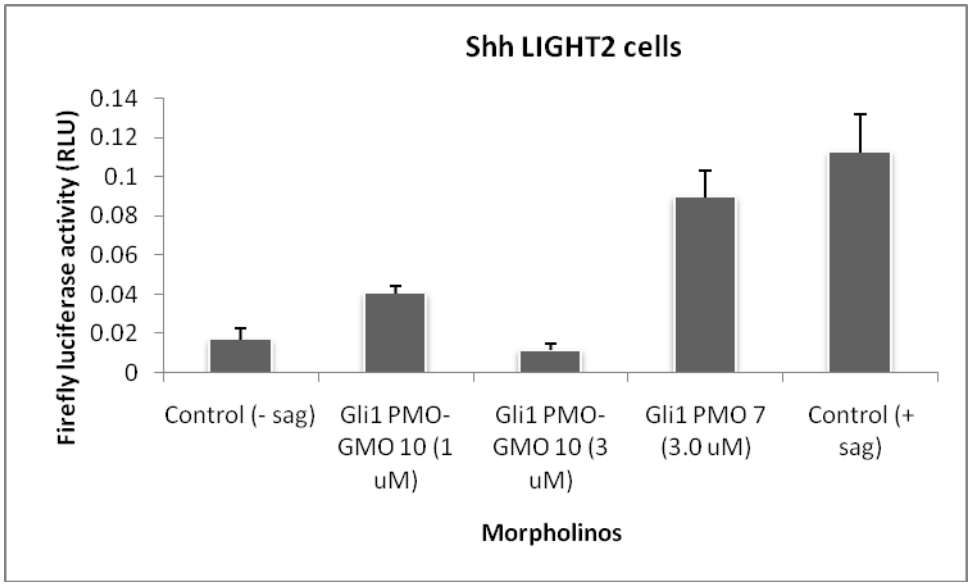



Figure S2. Inhibition of luciferase expression with morpholinos when the pathway has been activated with SAG.

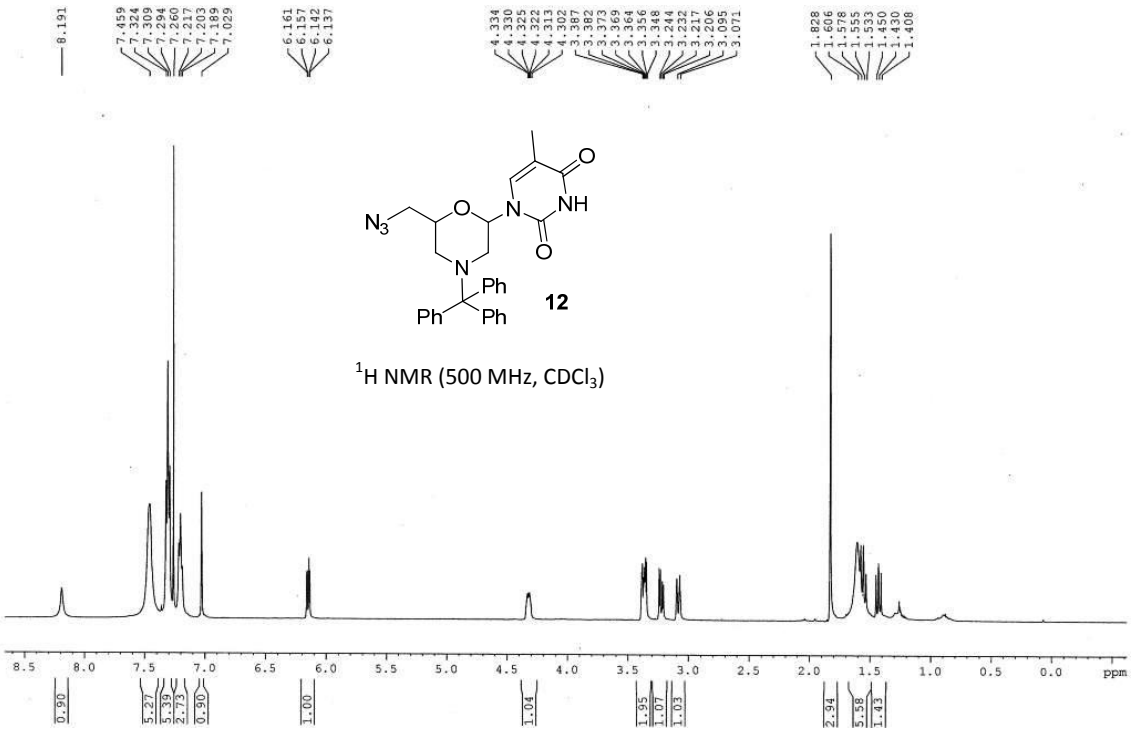
## Western Blot analysis

Shh LIGHT2 cells (200000 cells/well) in a 12 well plate were treated with morpholinos **1**, **7** and **10** in DMEM supplemented with 0.5 % Bovine Calf Serum, 100 µg/mL streptomycin and 100 units/mL penicillin in presence of Shh-conditioned media (1:5 dilution) for 40 h. The cells were briefly rinsed with PBS and lysed in 1X Laemmli sample buffer [50mM Tris (pH = 6.8), 10 % glycerol, 4 % SDS, 0.03 % bromophenol blue and 2.5 % β-mercaptoethanol]. The samples were boiled at 100 $^{o}$C for 5 min. Cell lysates were resolved by SDS-PAGE on 8 % polyacrylamide Tris-glycine gels and transferred onto polyvinyledene fluoride membrane (Millipore Corporation, Billerica, MA, USA) and blocked in 5% nonfat dry milk and 0.05 % tween-20 in phosphate buffered saline. The upper part of the membrane was probed with primary antibody against Gli1 (Cell Signaling) at 1:2000 dilution and the lower part of the membrane was probed with primary antibody against GAPDH (1:2000 dilution; Cell Signaling) at 4°C overnight, washed and probed with appropriate Anti-rabbit HRP-conjugated secondary antibody at 1:2000 dilution for two hours at room temperature. Bands were visualized using SuperSignal West Femto Trial Kit and the luminescence signal was captured on Biomax MR film (Eastman Kodak Co., Rochester, NY). The outcome has been shown in Figure 4c.

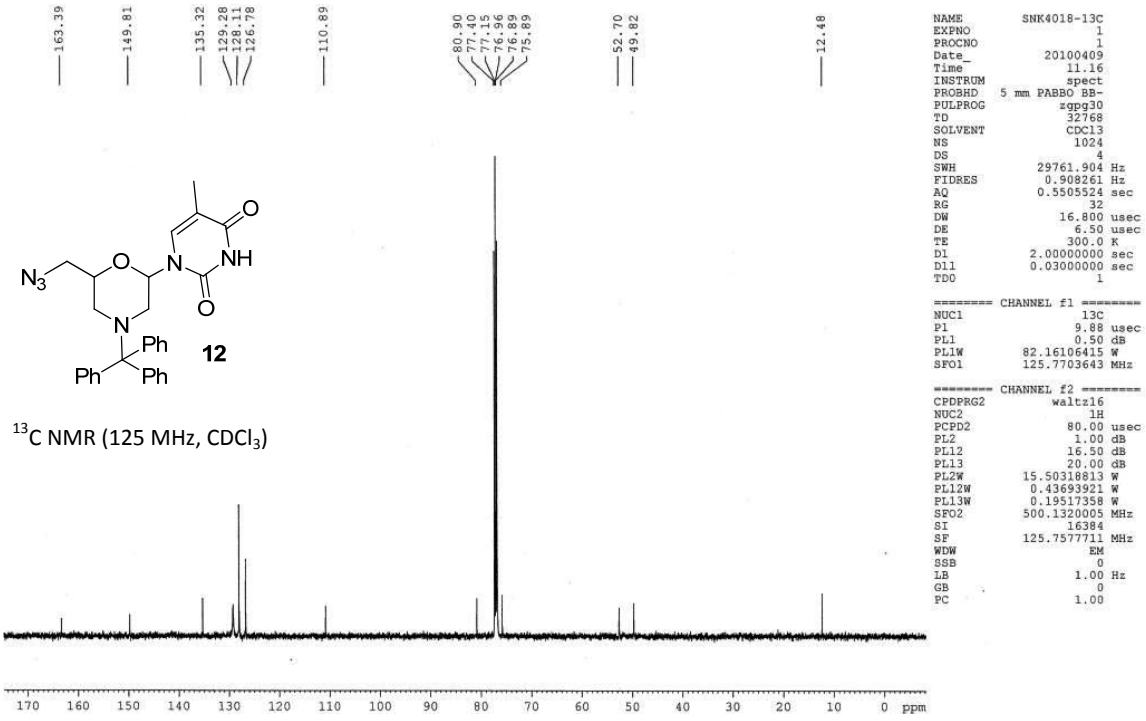
## Cell viability (MTT) assay

To determine the cellular toxicity of of the morpholinos in Shh LIGHT 2 cells, the oligomers were assayed with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability experiment. Shh-Light 2 cells were seeded into 96-well plates at a density of 12000

cells/well in normal growth medium containing 10 % calf serum (CS) in DMEM. After 24 hours, culture medium of each well was replaced with 100 µL fresh growth medium. Required amount of the reagent stocks of **1**, **7** and **10** were added to each well to reach the required concentration. The well plate was incubated under standard condition for 40 h and the media was then replaced with 100 µL of fresh media containing 1mg/mL MTT and incubated for four hour. The media containing the MTT was then aspirated and the purple formazan formed was dissolved in DMSO (100 µL/well). The absorption of each well was measured in a Thermo plate reader at 570 nm. The background absorption was deducted and the relative cell viability (%) relative to control wells was calculated by [A] test / [A] control × 100 and plotted in Figure 4b.




$^{1}$H NMR (500 MHz, $CDCl_3$)

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TD                   32768
SOLVENT              CDCl3
NS                    1024
DS                       4
SWH              29761.904 Hz
FIDRES           0.908261 Hz
AQ               0.5505524 sec
RG                      32
DW                  16.800 usec
DE                   6.50 usec
TE                  300.0 K
D1             2.00000000 sec
D11            0.03000000 sec
TD0                      1

======== CHANNEL f1 ========
NUC1                   13C
P1                    9.88 usec
PL1                   0.50 dB
PL1W         82.16106415 W
SFO1        125.7703643 MHz

======== CHANNEL f2 ========
CPDPRG2          waltz16
NUC2                   1H
PCPD2              80.00 usec
PL2                 1.00 dB
PL12               16.50 dB
PL13               20.00 dB
PL2W         15.50318813 W
PL12W         0.43693921 W
PL13W         0.19517358 W
SFO2        500.1320005 MHz
SI                  16384
SF          125.7577711 MHz
WDW                    EM
SSB                     0
LB                   1.00 Hz
GB                      0
PC                   1.00
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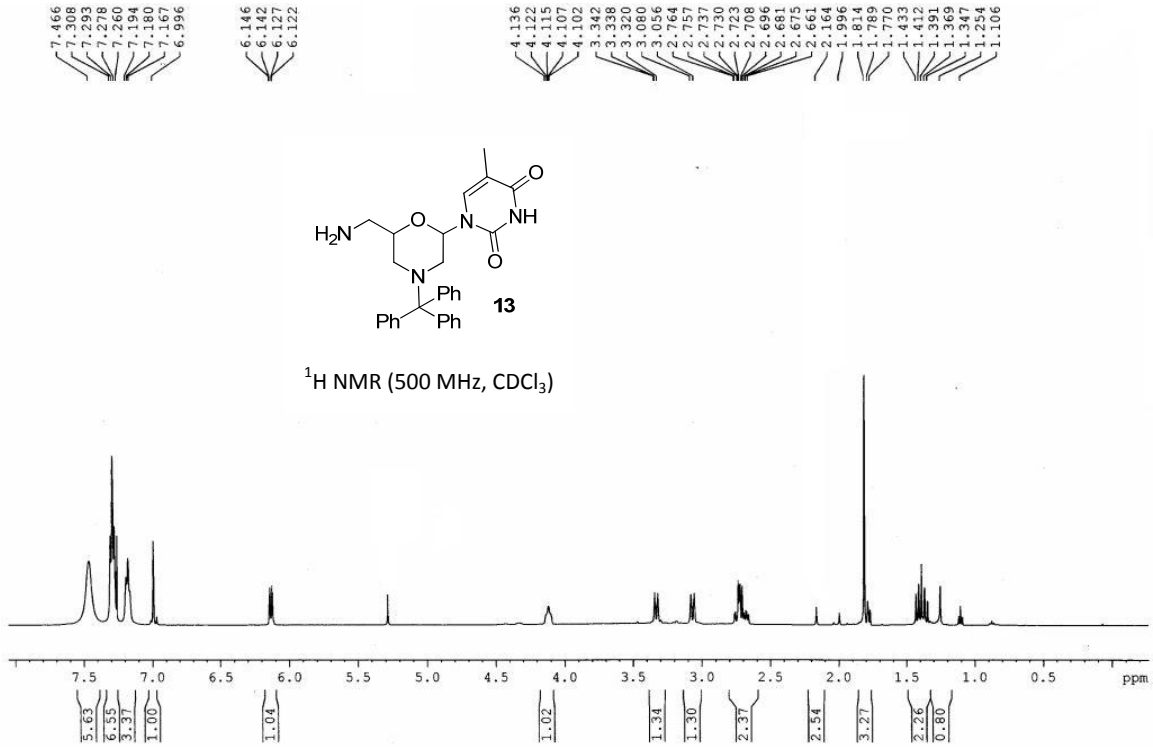
$^{13}$C NMR (125 MHz, $CDCl_3$)




$^{1}$H NMR (500 MHz, $CDCl_3$)

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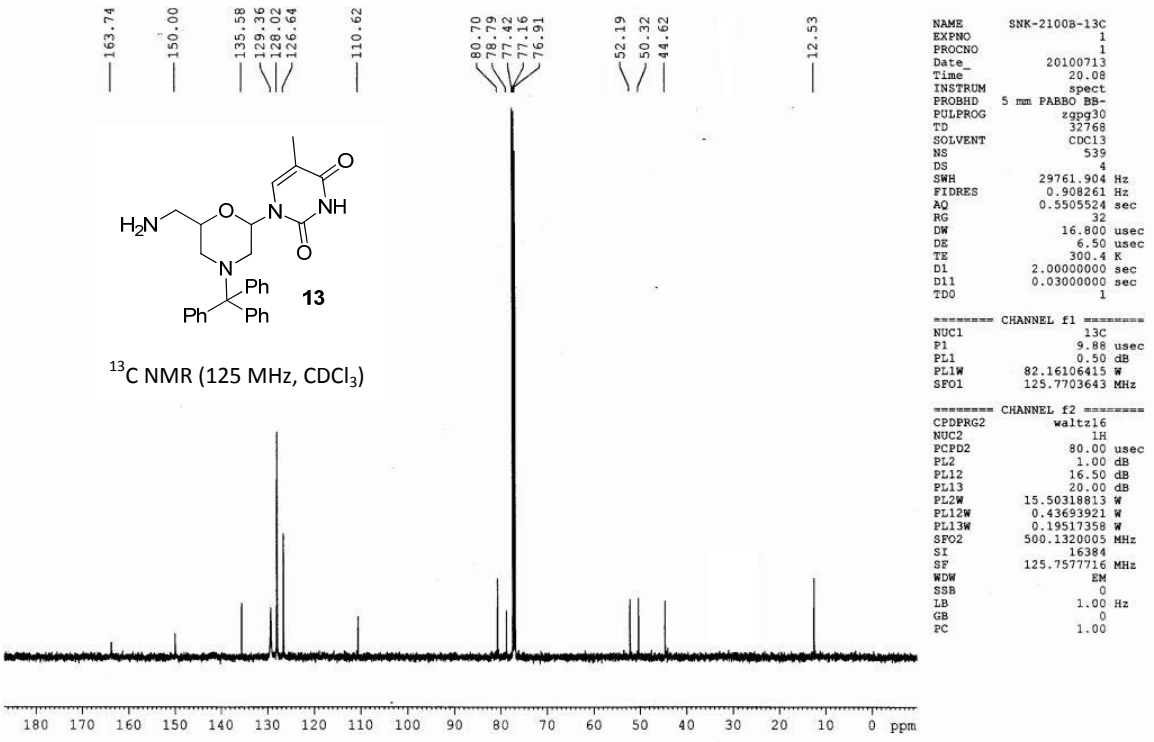


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TD                   32768
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NS                     539
DS                       4
SWH              29761.904 Hz
FIDRES           0.908261 Hz
AQ               0.5505524 sec
RG                      32
DW                 16.800 usec
DE                  6.50 usec
TE                 300.4 K
D1            2.00000000 sec
D11           0.03000000 sec
TD0                      1

======== CHANNEL f1 ========
NUC1                   13C
P1                  9.88 usec
PL1                 0.50 dB
PL1W          82.16106415 W
SFO1         125.7703643 MHz

======== CHANNEL f2 ========
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NUC2                   1H
PCPD2              80.00 usec
PL2                 1.00 dB
PL12               16.50 dB
PL13               20.00 dB
PL2W          15.50318813 W
PL12W          0.43693921 W
PL13W          0.19517358 W
SFO2         500.1320005 MHz
SI                  16384
SF           125.7577716 MHz
WDW                    EM
SSB                     0
LB                 1.00 Hz
GB                      0
PC                 1.00
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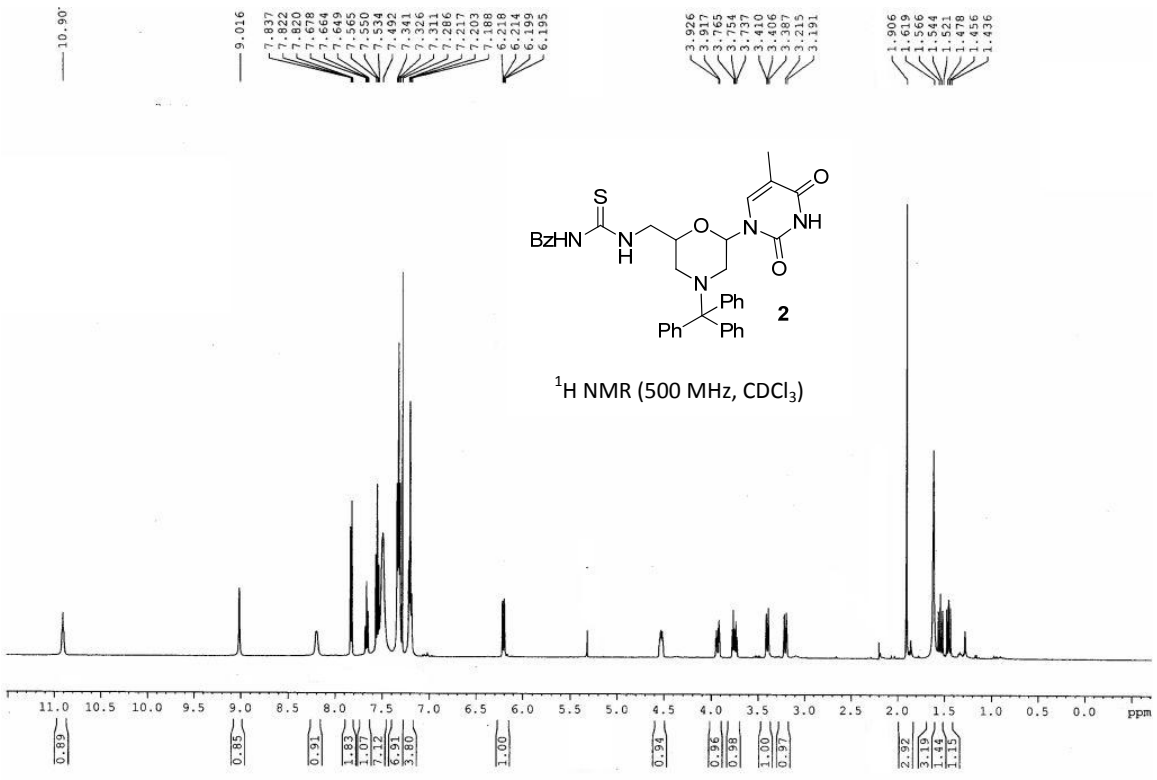
$^{13}$C NMR (125 MHz, $CDCl_3$)

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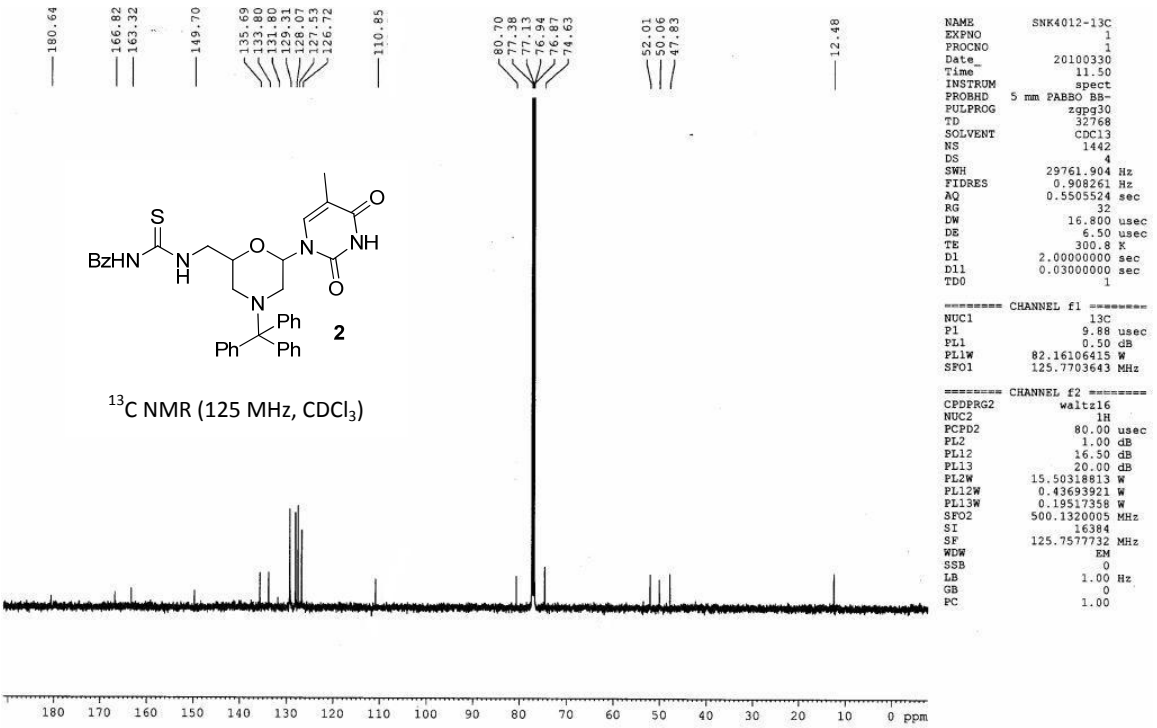


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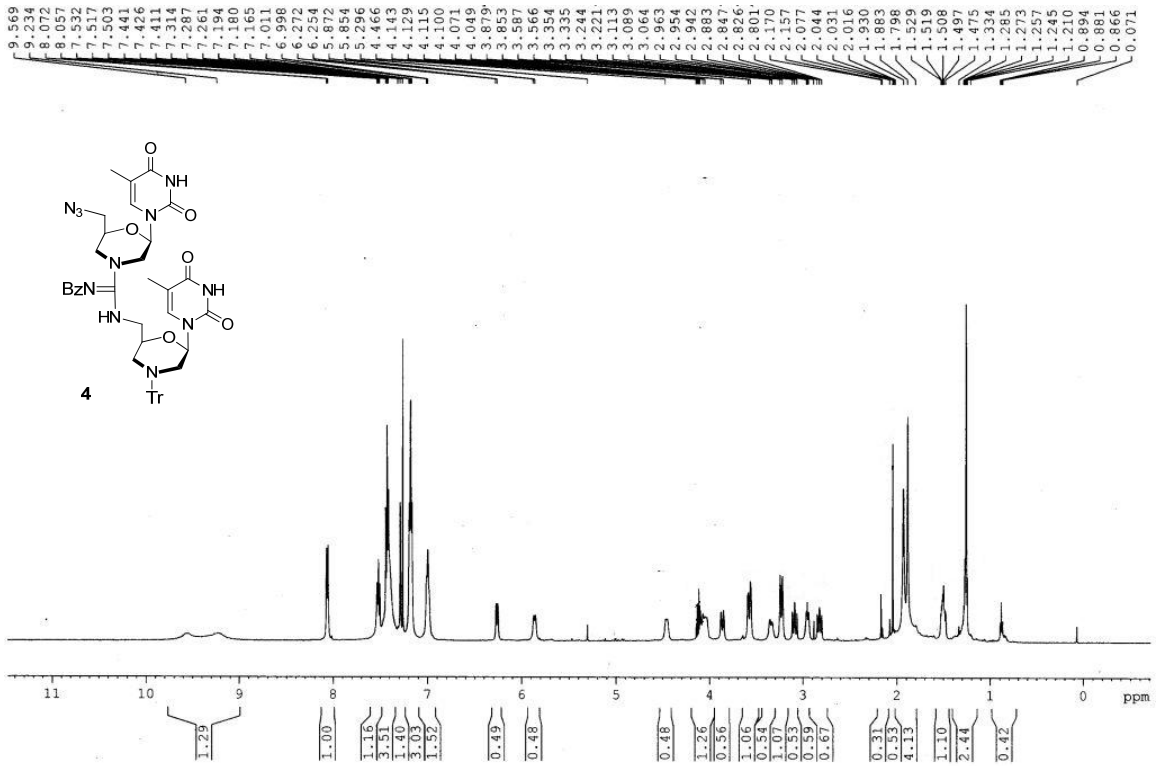
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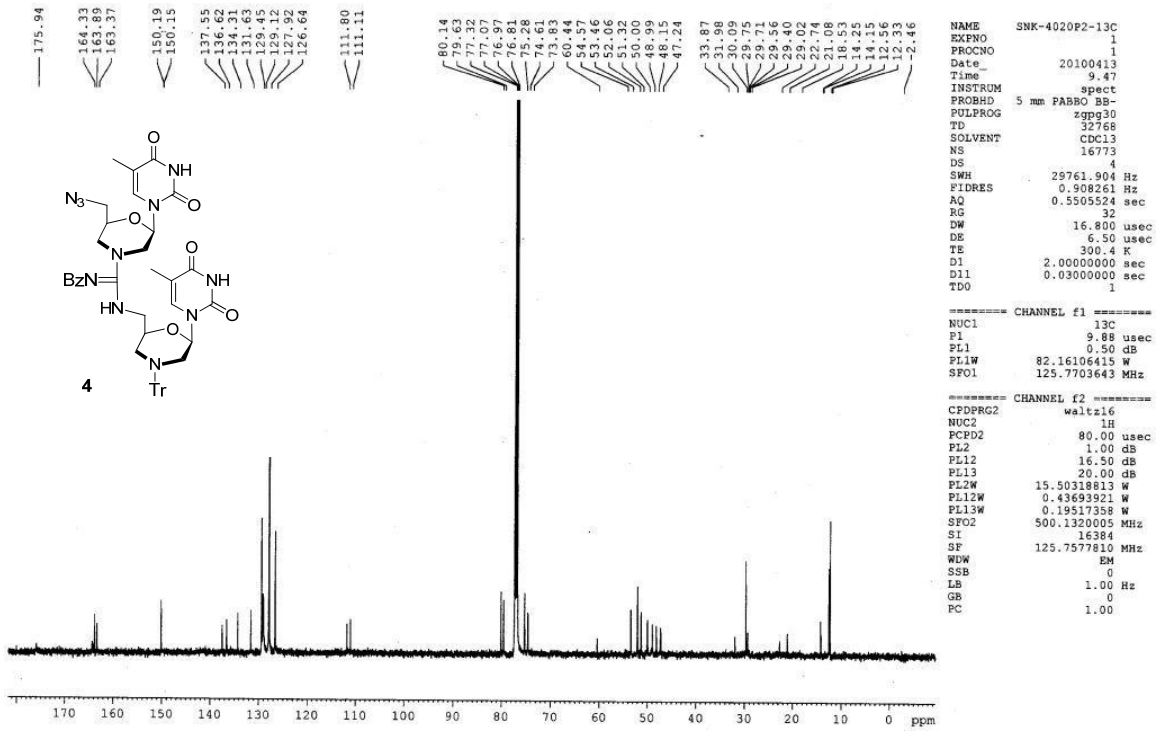
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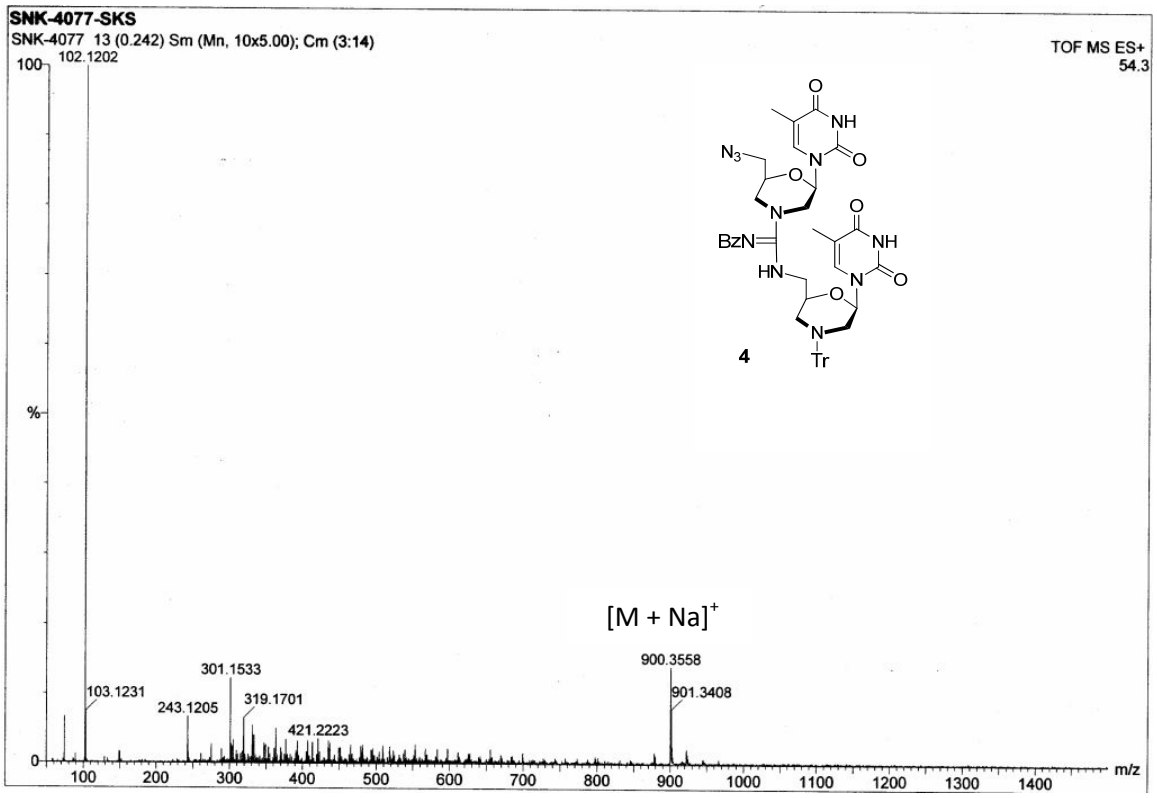
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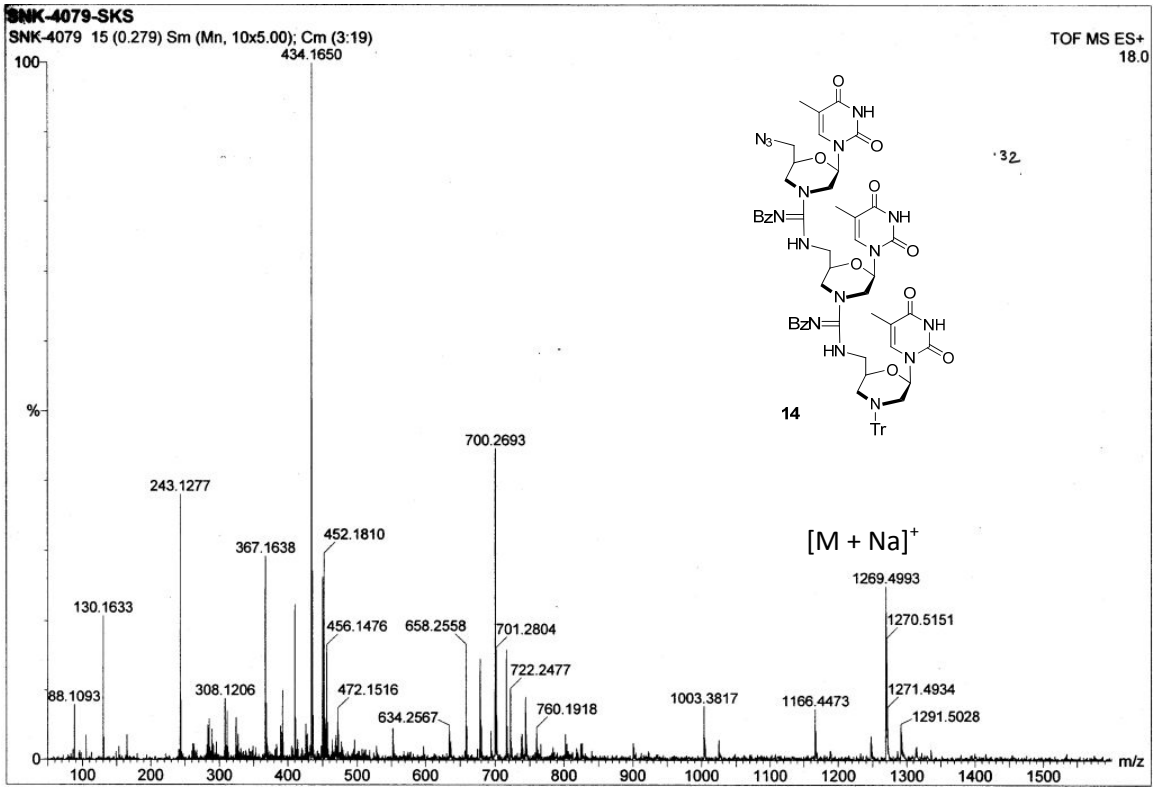
m/z
4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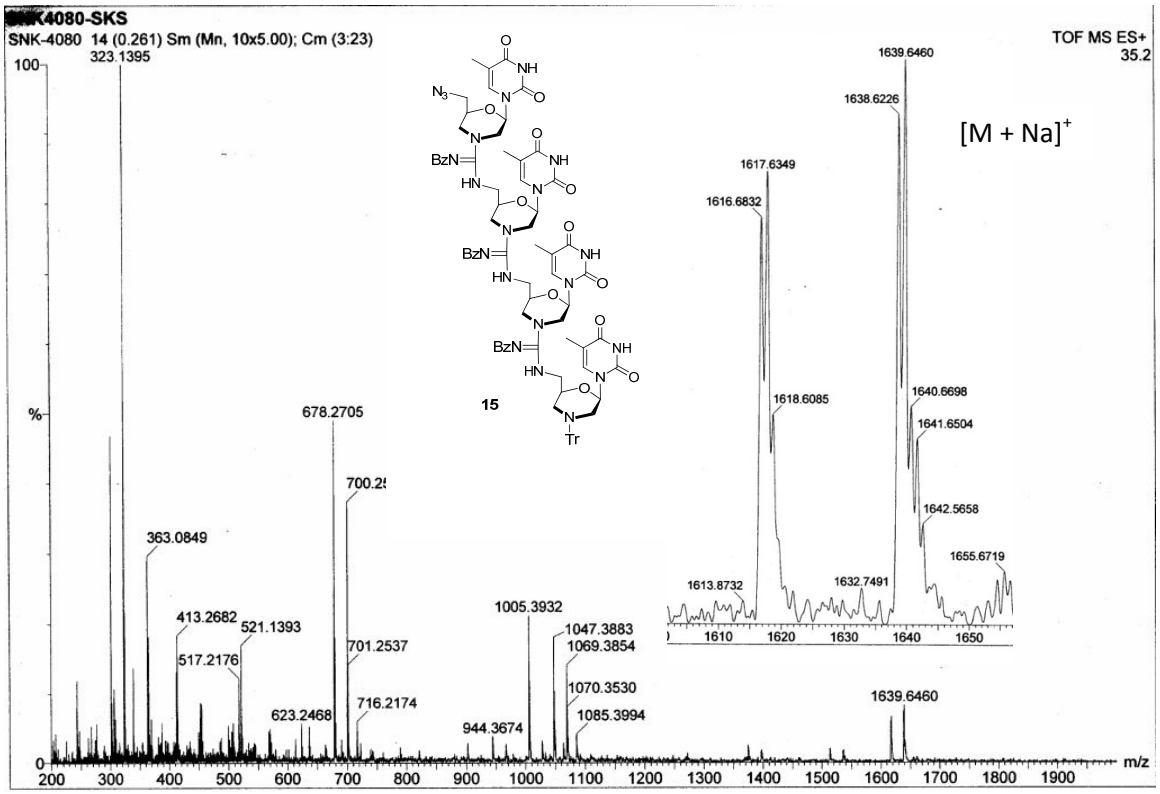
14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[M + Na]+
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678.2705
700.2
363.0849
413.2682
521.1393
517.2176
701.2537
623.2468
716.2174
1005.3932
1047.3883
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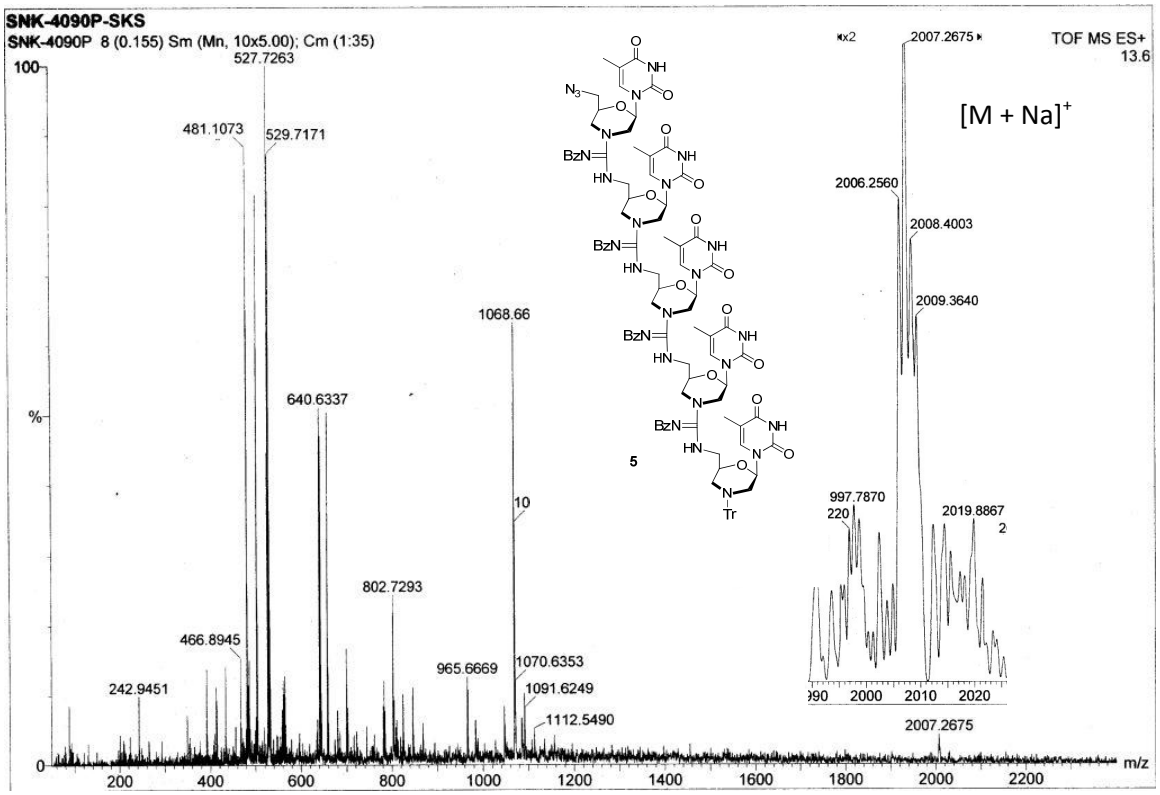
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[M + Na]+
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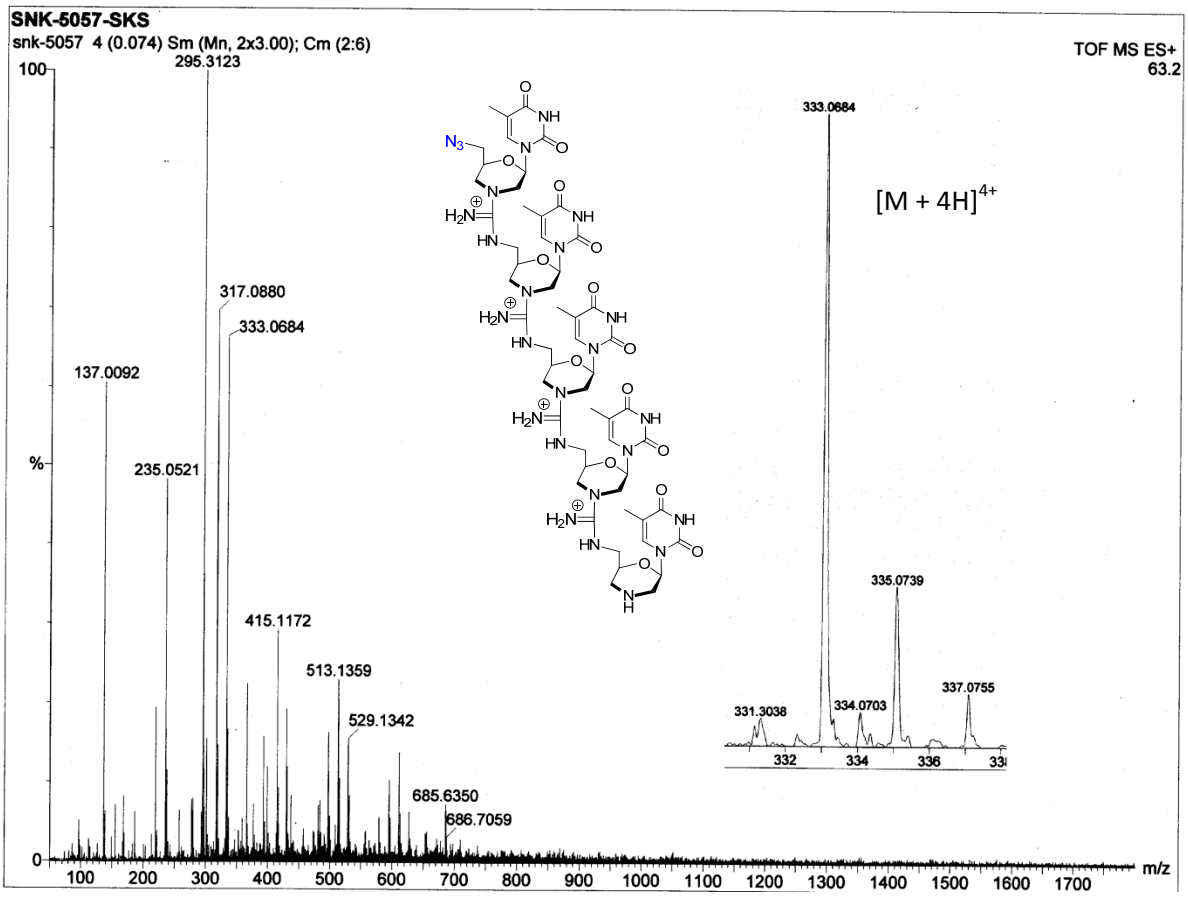
m/z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333.0684
137.0092
235.0521
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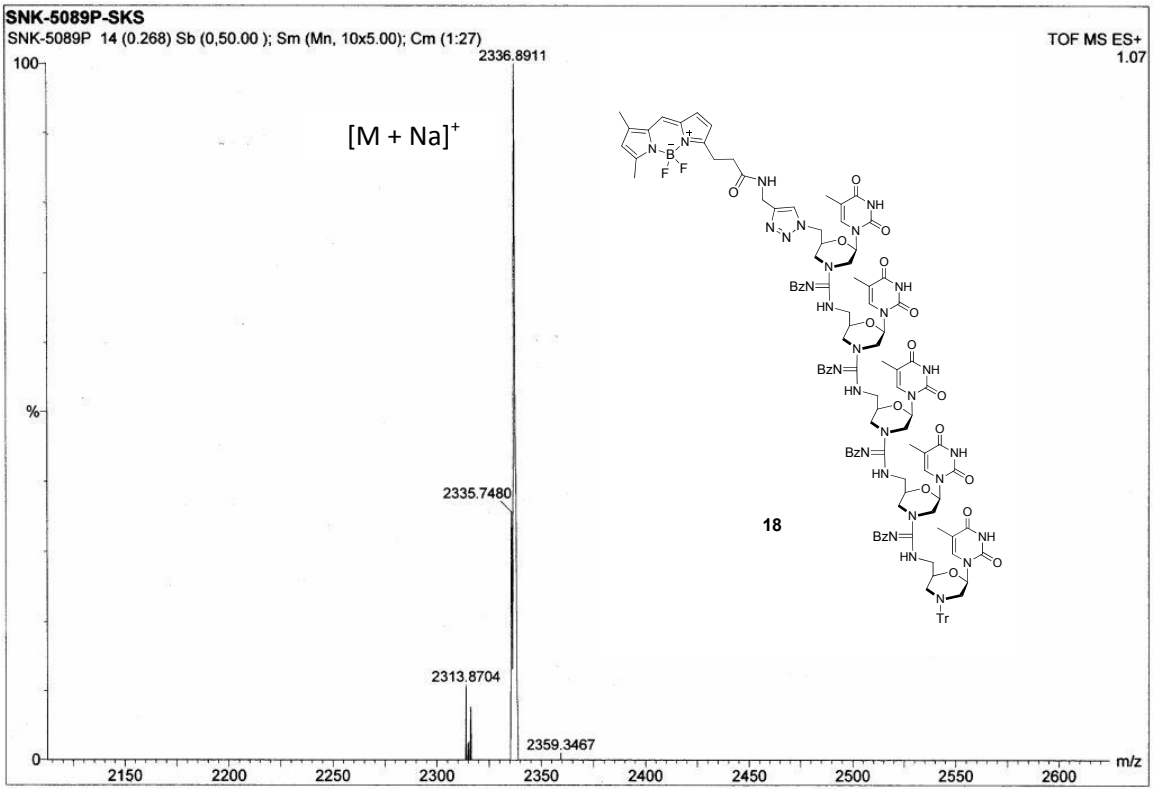
m/z


SNK-5089P-SKS
SNK-5089P 14 (0.268) Sb (0,50.00 ); Sm (Mn, 10x5.00); Cm (1:27)
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[M + Na]+
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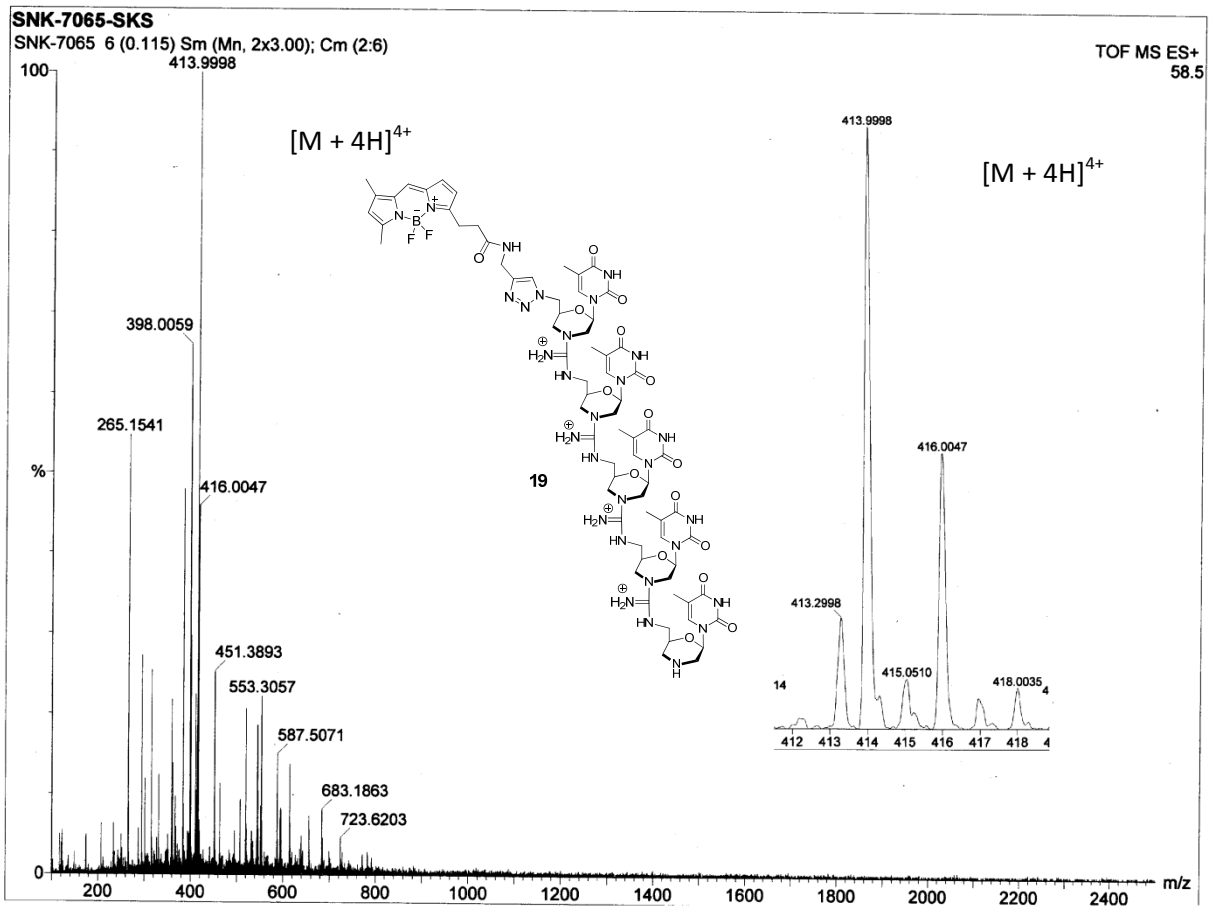
18
m/z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SNK-7065 6 (0.115) Sm (Mn, 2x3.00); Cm (2:6)
TOF MS ES+
58.5
100
%
0
413.9998
398.0059
265.1541
416.0047
451.3893
553.3057
587.5071
683.1863
723.6203
[M + 4H]4+
19
[M + 4H]4+
413.9998
416.0047
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600
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1400
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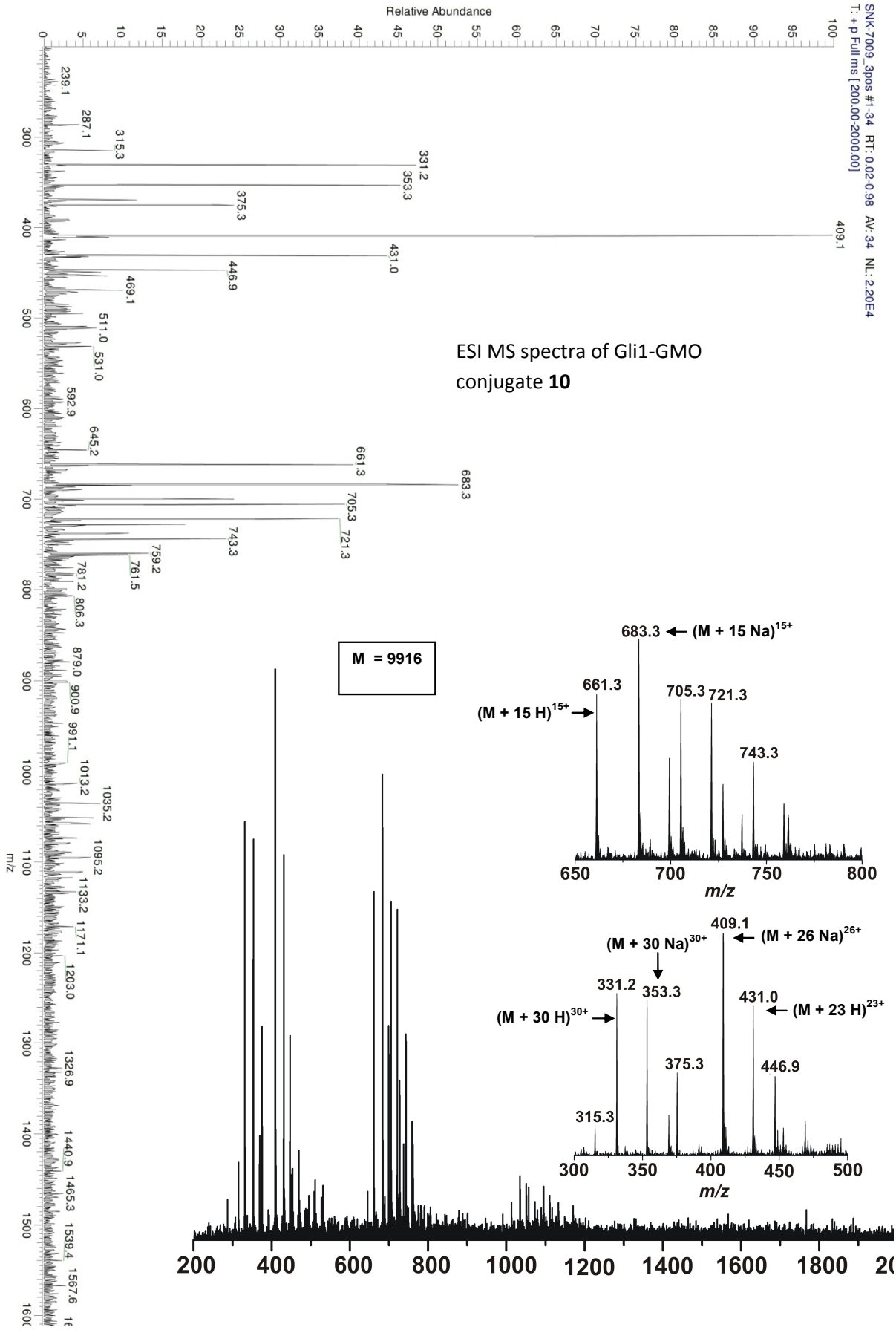


ESI MS spectra of Gli1-GMO conjugate **10**

## REFERENCES

[1] W. L. Armarego, C. Chai, *Purification of laboratory chemicals*, Butterworth-Heinemann, **2012**.
[2] K. Gießler, H. Griesser, D. Göhringer, T. Sabirov, C. Richert, *Eur. J. Org. Chem.* **2010**, *2010*, 3611-3620.
[3] V. Hong, S. I. Presolski, C. Ma, M. Finn, *Angew. Chem. Int. Ed.* **2009**, *48*, 9879-9883.
[4] J. Taipale, J. K. Chen, M. K. Cooper, B. Wang, R. K. Mann, L. Milenkovic, M. P. Scott, P. A. Beachy, *Nature* **2000**, *406*, 1005-1009.