Electronic Supplementary Material (ESI) for Chemical Science.

Supplementary Information:

Caged circular siRNAs for photomodulation of gene expression in cells and mice

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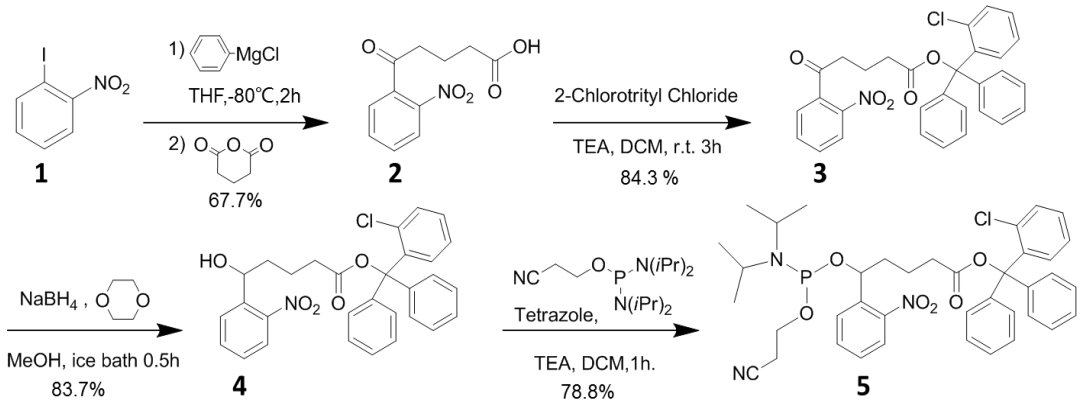
Synthesis of photocleavable phosphoramidite linker (PL)

General chemical experimental procedures. All chemical reactions for synthesis of photocleavable linker (**PL**) were performed under an inert atmosphere using dry reagents, solvents, and flame-dried glassware. Analytical thin-layer chromatography (TLC) was performed with Merck Kiselgel 60 F254 silica gel plates (0.24 mm thick). Column chromatography was performed with silica gel 60 (200-300 mesh). ^{1}H-NMR(400MHz) and ^{13}C-NMR(100MHz) spectra were taken on Bruker AVANCE III-400 spectrometers and standardized to the NMR solvent peak, chemical shifts were reported in parts per million (ppm). Mass spectra of small molecules were obtained on Waters Xevo TQD Mass Spectrometer using electrospray ionization (ESI). The reactions were conducted in a dark room when necessary.



Scheme S1. Synthetic route of new photocleavable phosphoroamidite linker （PL). dichloromethane (DCM), tetrahydrofuran (THF) and triethylamine (TEA).

5-(2-nitrophenyl)-5-oxopentanoic acid (2) Compound **1** (9.0 g, 36.1 mmol) was dissolved in dry THF (10 mL) and stirred at -80℃ and then 2 M phenylmagnesium chloride in THF (20.7 mL, 39.8 mmol) was added slowly. When the solution turned dark red, glutaric anhydride (5.04 g, 43.4 mmol) dissolved in dry THF (10 mL) was added dropwise. The mixture was stirred at -40℃ for another 2 h and then returned to 0 ºC overnight while stirring. After the addition of 40 mL saturated ammonium chloride to quench the reaction, the pH value of the

mixture solution was adjusted to 12 with sodium hydroxide. After extraction with ethyl acetate for three times, the water phase was adjusted to acid condition (pH=2) with HCl aqueous solution, and the product **2** was then extracted with ethyl acetate, following water washing. The organic phase was dried over Na_2SO_4, and was then evaporated under reduced pressure without further purification, which gave compound **2** (5.8 g, 67.7%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 (dd, 1H), 7.73 (td, 1H), 7.60 (td, 1H), 7.39 (dd, 1H), 2.88 (t, 2H), 2.54 (t, 2H), 2.16 – 2.06 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 201.63, 179.01, 145.50, 137.93, 134.36, 130.50, 127.26, 124.47, 41.63, 32.51, 18.58. m/z calcd for $[M+H]^+$:237.2. MS found: (M+Na) 260.3.

*(2-chlorophenyl)diphenylmethyl5-(2-nitrophenyl)-5-oxopentanoate(**3**)* Compound **2** (1.0 g, 4.2 mmol) was dissolved in methylene chloride (DCM, 20 mL), and then triethylamine (TEA, 3.05 mL, 21.0 mmol) and 2-chlorotrityl chloride (1.58 g, 5.04 mmol) were added slowly at room temperature. After stirring for another 3 h, the reaction solvent was removed under reduced pressure. Further purification by silica gel chromatography using 1:1 petroleum ether/ethyl acetate gave compound **3** (1.82 g, 3.54 mmol, 84.3 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 (dd, 1H), 7.68 (td, 1H), 7.62 – 7.54 (m, 1H), 7.34 – 7.16 (m, 15H), 2.86 (t, 2H), 2.73 (t, 2H), 2.13 (p, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 201.75, 171.26, 145.50, 142.52, 139.73, 137.95, 134.21, 133.65, 131.63, 131.43, 130.38, 129.14, 127.97, 127.86, 127.41, 127.28, 125.89, 124.39, 89.83, 41.69, 33.50, 18.89. m/z calcd for $[M+H]^+$:514.0. MS found: (M+Na) 536.5.

*(2-chlorophenyl)diphenylmethyl 5-hydroxy-5-(2-nitrophenyl)pentanoate (**4**)*. Compound **3** (1.82 g, 3.54 mmol) was dissolved in methanol/dioxane (3:2 by volume, 15 mL), and $NaBH_4$ (200 mg, 5.2 mmol) was then added slowly. After 30 min, acetone (5 mL) was added to consume the excess of $NaBH_4$. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in ethyl acetate. The organic solution was washed with brine and was then dried with anhydrous Na_2SO_4. After removal of organic solvents, the resulting brown powder was purified by silica gel chromatography using 3:1 petroleum ether/ethyl acetate to give the desired alcohol as a pale yellow powder **4** (1.52 g, 83.7%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 (dd, 1H), 7.79 (dd, 1H), 7.67 – 7.59 (m, 1H), 7.44 – 7.38 (m, 1H), 7.38 – 7.17 (m, 14H), 5.29 – 5.15 (m, 1H), 2.78 (d, 1H), 2.68 (dd, 2H), 1.99 – 1.71 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.89, 147.63, 142.55, 142.49, 140.14, 139.72, 133.68, 133.46, 131.62, 131.45,

129.12, 128.07, 128.00, 127.97, 127.83, 127.38, 125.85, 124.30, 89.82, 68.69, 37.56, 34.38, 21.09. MS: m/z calcd for $[M+H]^+$ 515.2, MS found: (M+Na) 538.5.

*5-(2-nitrophenyl)-5-oxopentanoic acid phosphoramidite (**5**)*

To a dry flask containing compound **4** (0.3 g, 0.58 mmol) and anhydrous tetrazole (81.5 mg, 1.16 mmol), 2-cyanoethyl-N, N, N′, N′-tetraisopropylphosphorodiamidite (700 mg, 2.3 mmol) dissolved in dichloromethane (5 mL) was added via syringe under nitrogen atmosphere. After another 0.5 h, the reaction mixture was subjected to purification with silica gel chromatography using 4:1 petroleum ether/ethyl acetate/2% triethylamine to give white foam **5** (0.328 g, 78.8%). The product contained two isomers, where the difference in isomeric phosphorus chemical shift is about 1.5 ppm on ^{31}P NMR spectra. Isomer 1: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 (d, 1H), 7.83 (d, 1H), 7.63 (t, 1H), 7.42 (dd, 1H), 7.38 – 7.18 (m, 14H), 5.47 (dd, 1H), 3.90 – 3.72 (m, 2H), 3.57 – 3.41 (m, 2H), 2.71 (dd, 1H), 2.56 – 2.50 (m, 2H), 2.04 – 1.83 (m, 4H), 1.15 (d, 6H), 0.89 (d, 6H). ^{13}C NMR (100 MHz, CDCl3) δ 171.40, 147.26, 142.69, 142.60, 139.80, 139.21, 133.67, 133.19, 131.66, 131.39, 129.10, 128.00, 127.94, 127.83, 127.36, 125.84, 124.06, 117.57, 89.62, 70.03, 69.85, 58.84, 58.64, 43.16, 43.03, 34.47, 24.59, 24.51, 24.05, 23.98, 21.04, 20.25, 20.19.^{31}P NMR (MHz, $CDCl_3$): 148.55 (s). Isomer 2: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92 (dd, 1H), 7.78 (d, 1H), 7.66 (t, 1H), 7.47 – 7.39 (m, 1H), 7.37 – 7.17 (m, 14H), 5.42 (d, 1H), 3.69 – 3.48 (m, 4H), 2.49 – 2.28 (m, 2H), 2.03 – 1.81 (m, 4H), 1.19 (dd, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.24, 147.35, 142.66, 142.57, 139.77, 139.29, 133.67, 133.12, 131.61, 131.38, 129.21, 129.03, 127.97, 127.93, 127.87, 127.78, 127.30, 125.79, 123.95, 117.23, 89.60, 71.12, 70.95, 58.10, 57.90, 43.26, 43.14, 34.64, 24.58, 24.55, 24.51, 24.47, 21.05, 20.03, 19.96. ^{31}P NMR (MHz, $CDCl_3$): 150.01 (s). MS: m/z calcd for $[M+H]^+$715.3, MS found: (M+Na) 738.3.

Chemical synthesis and purification of caged circular RNAs

Amino thymidine (C-6) 3′-lcaa controlled pore glass (CPG) 500 Å (Chem Genes Corp., cat. no. N-6054-05) was used for RNA oligonucleotide synthesis. All the caged single-stranded oligonucleotides were synthesized according to the standard RNA synthesis on ABI394 with 2-TBDMS RNA monomers. Following the procedures showed in Scheme S1, photocleavable phosphoramidite linker (PL) was coupled at 5′ terminal of RNA sequences according to the procedure of normal RNA synthesis. 2-Chlorotrityl protecting group was removed to release

terminal carboxylic acid on DMT-off mode. Synthesized oligonucleotides were then cleaved from CPG and deprotected using 0.3 mL concentrated ammonium hydroxide at room temperature for 24 hours. After removal of ammonia, the solution was centrifuged at 2500 rpm for 5 minutes to remove CPG. The supernatant was collected and concentrated using a Thermo Scientific Savant SPD2010 SpeedVac Concentrator. The obtain residue was dissolved in 100 μL DMSO, and then 100 μL TEA·3HF was added to remove TBDMS protecting group. After the mixture was shaken at 65ºC for 2.5 hours, the solution was cooled in ice bath and isopropoxytrimethylsilane (300 μL) was added to consume extra fluoride. Butyl alcohol (1.0 mL) was then added to above solution and the mixed solution were further stored at -80 ºC for 1 hour. After centrifugation and removal of supernatant, the precipitated white solid was washed with ether twice and collected for further purification.

The above white solid dissolved in buffer A was subject to HPLC purification (buffer A, 0.1M TEAB; buffer B, acetonitrile; 0–30% buffer B in 30 min; running temperature, 60 ºC). Due to the possible uncaging of the samples exposure to UV light during the HPLC separation, an analytical run was performed to obtain the elution time of the oligonucleotide. The subsequent preparative run was performed with the detector off. The main peak was collected and the solution was concentrated using a Thermo Scientific Savant SPD2010 SpeedVac Concentrators. The above separated oligonucleotides (about 3 nmol) were dissolved in 100 μL DMSO solutions containing 2 μL DIPEA and 50 mmol/L 4-(4, 6-dimethoxy [1.3.5] triazin-2-yl)-4-methyl morpholinium chloride (DMT-MM) at 25 ºC. The reaction solution was shaken at room temperature for 48 hours. DEPC-treated water (1.2 mL) was added to dilute the DMSO, the mixture was then desalted and concentrated using Millipore-amicon ultra-0.5 mL centrifugal filters (cutoff=3000). The crude products of white solid were collected after frozen-dry to remove water.

The crude products were dissolved in 1× PBS buffer and mixed with RNA loading buffer (0.25% bromophenol blue, 30% glycerol in DEPC-treated water). The solutions (6 μL /well) were loaded into 20% native polyacrylamide PAGE (8.6×6.8cm, and 1 mm thick) gels. The gels were then electrophoresed at 80V for 3 h, using 1×tris-borate-EDTA (TBE) buffer (pH 8.2). For each preparative gel, the two side sample lanes of the gel were cut and stained with 1×

SYBR Gold (Invitrogen) and then imaged. The images were printed out according to the same size of the gel which made it possible to mark the location of the gels without SYBR Gold stain. The gel zones at marked location were cut, crumbled into tiny particles and immersed into a 1 × TBE buffer at 37 °C overnight. After filtration of the solid particles, the solution of the RNA were desalted and concentrated using Millipore-amicon ultra-0.5 mL centrifugal filters (Cutoff =3000). The collected product was frozen-dried to remove water and gave the final caged circular single-stranded RNA. The yield of RNA cyclization step was usually 20-40%.

The caged circular single-stranded RNA was dissolved in 1×PBS buffer to make the 20 μmol/L stock solution and was then mixed with equal amount of the linear complementary RNA to form the caged circular siRNA duplex. This duplex was annealed by heating to 80 ℃ for 5 minutes and subsequent cooling to room temperature for at least 1 hour for further use.

Enzymetic synthesis and purification of caged circular RNAs

Linear RNA strands were synthesized according to the following sequence (5′ PO_4-UGCC GUU CTT-PL2-GUU CAC CUU GA, after cyclization, the circular oligonucleotide was irradiated to restore the original antisense RNA sequence: GUU CAC CUU GA UGCC GUU CTT) on a 1-μmol scale using an ABI394 DNA/RNA synthesizer on DMT-ON mode based on standard phosphoramidite chemistry. Photolinker2 (PL2) phosphoramidite was inserted in the middle of sequence and chemical phosphorylation reagent II (CPRII) was coupled at 5′ end. RNA oligonucleotides were then cleaved from CPG and deprotected using 0.5 mL 33% ammonium hydroxide solution at room temperature for 24 hours. Then the solution was centrifuged at 2500 rpm for 3 minutes to remove CPG. The supernatant was collected and concentrated using a Thermo Scientific Savant SPD2010 SpeedVac Concentrator. The obtained residue was re-dissolved in 100 μL DMSO, and then 100 μL TEA·3HF was added to remove TBDMS protecting group. After the mixture was shaken at 65°C for 2 hours to remove the TBDMS protecting groups at the 2′-position, the solution was cooled in ice bath and butyl alcohol (1.0 mL) and NaOAc (3M, 50 μL) was added to above solution. The mixed solution was then stored at -80 ℃ for 1 hour. After centrifugation at 13,000 rpm at 4 °C for 10 min and removal of supernatant, the precipitated white solid was washed with ether twice and collected for further purification.

The above white solid dissolved in DEPC water was then subject to HPLC purification using

reverse-phase HPLC column (C18) under the conditions: buffer A, 0.1M TEAB (0.05 M triethylammonium bicarbonate buffer, pH 8.5); buffer B, acetonitrile; 0–30% buffer B in 20 min, 30%-100% buffer B in 10 min; room temperature). The main peak was collected and the solution was concentrated. The obtained linear 5′-phosphate RNA was characterized.

The above RNA oligonucleotides was dissolved in water to make the final concentration of 143 μM. The cyclization of RNAs was carried out under following conditions: 7 μL RNA solution, 1 μL 10 mM ATP, 1 μL 10×reaction buffer and 1 μL T4 RNA ligase(10 units/μL). The mixed solution was placed in PCR at 37 ºC for 6 hours. The crude products were mixed with 6×RNA loading buffer (0.25% bromophenol blue, 30% glycerol in DEPC-treated water) and were then loaded to 20% native polyacrylamide PAGE (8.6×6.8 cm, and 1 mm thick) gels. The gels were then electrophoresed at 220 V for 50 minutes, using 1×tris-borate-EDTA (TBE) buffer (pH 8.2). For each preparative gel, the two side sample lanes of the gel were cut and stained with 1× SYBR Gold (Invitrogen), and then imaged using chemical luminescence gel imaging system. The images were printed according to the same size of the gel which made it possible to mark the location of the gels without SYBR Gold stain. The gel zones at marked location were cut, crumbled into tiny particles and immersed into a 1× TBE buffer. After shanking over a period of 24 h at room temperature on oscillator, the solid particles were filtered and the filtrate containing RNA was desalted and concentrated using Millipore-Amicon Ultra-0.5ml Centrifugal Filters (Cutoff =3000). The collected products were frozen-dried to remove water and gave the final circular single caged strand RNAs for further characterization.

Thermal denaturation studies

The oligonucleotides were mixed in 1×PBS to the final concentration of 2 μmol/ L (150 μL). Oligonucleotide solutions were hybridized by first heating at 85 ℃ for 5 min and then slowly cooled down to room temperature. The melting profiles started with a denaturing run from 20 ℃ to 85 ℃ at a rate of 1 ℃ /min. The absorbance at 260 nm was monitored at 1 ℃ intervals on a Beckman Series 800 UV spectrometer. The melting temperatures of the oligonucleotides were determined as the peak of the derivatives of the corresponding melting curves.

Figure S1. Chemical synthetic route and gel analysis of the caged circular single-stranded RNA

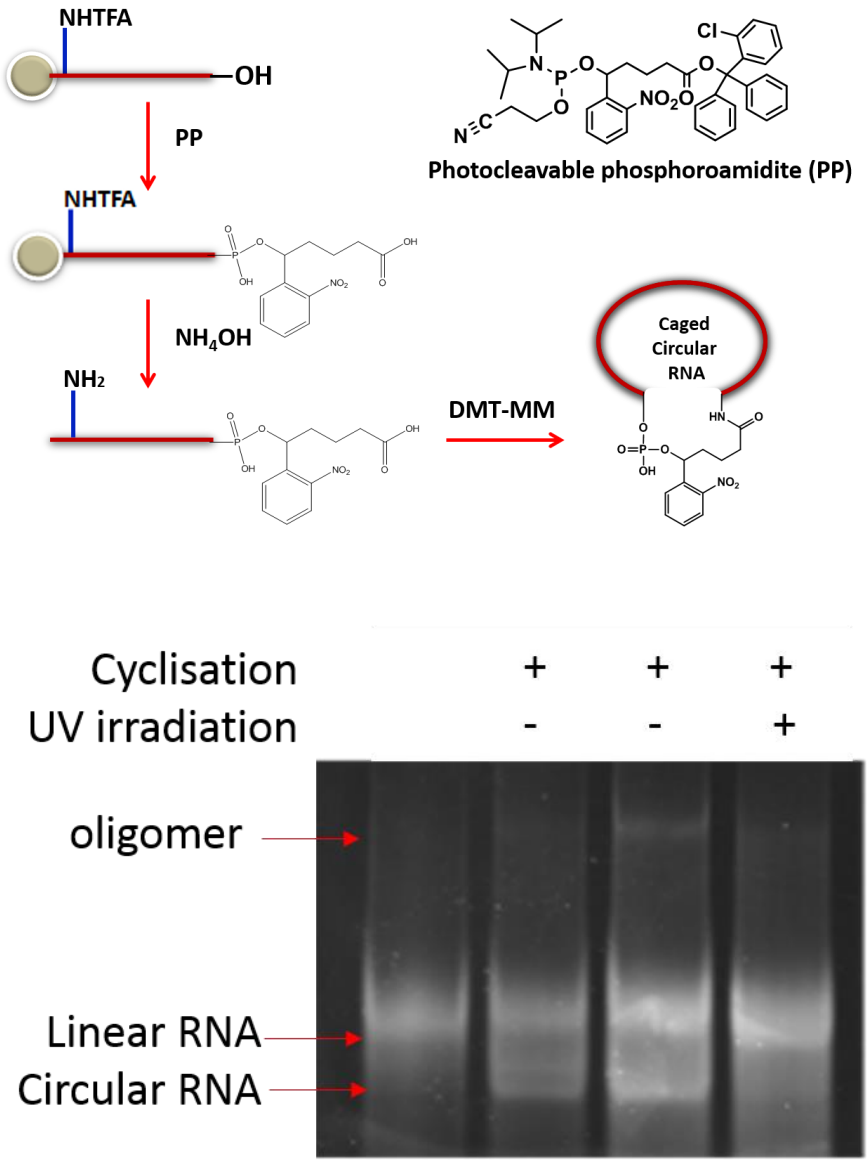
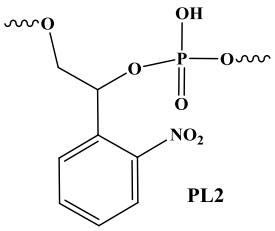


Table S1. The sequences of oligonucleotides used in the dual luciferase assay and their measured molecular weights (MW). Unmodified RNAs were purchased directly from GenePharma (Shanghai, China).

Name	sequence	Calculated MW	Measured MW
Sense-normal (**SL**)	5′ - CCCUAUUCUCCUUCUUCGCtt	6416.8	-
Antisense-normal (**AL**)	5′ - GCGAAGAAGGAGAAUAGGGtt	6898.4	-
Sense strand caged circular RNA (**c-SL**)	5′ –(PL)- CCCUAUUCUCCUUCUUCGCtt*	6854.3	6853.0
Antisense strand caged circular RNA (**c-AL**)	5′ –(PL)- GCGAAGAAGGAGAAUAGGGtt*	7335.8	7335.0

Table S2. The sequences of oligonucleotides used in the GFP expression assay, their measured molecular weights (MW) using ESI-MS and the structure of the another linker.

Name	sequence	Calculated MW	Measured MW
Sense(**SG**)	5′- GAACGGCAUCAAGGUGAACtt	6759.2	6755.7
Antisense (**AG**)	5′- GUUCACCUUGAUGCCGUUCtt	6563.9	6561.3
Antisense circular (**c-AG**)	5′-(PL2)- GUUCACCUUGAUGCCGUUCtt	6872.9	6871.6



Scheme S2 Photocleavage of circular siRNA from chemical (left) and enzymatic (right) synthetic methods.

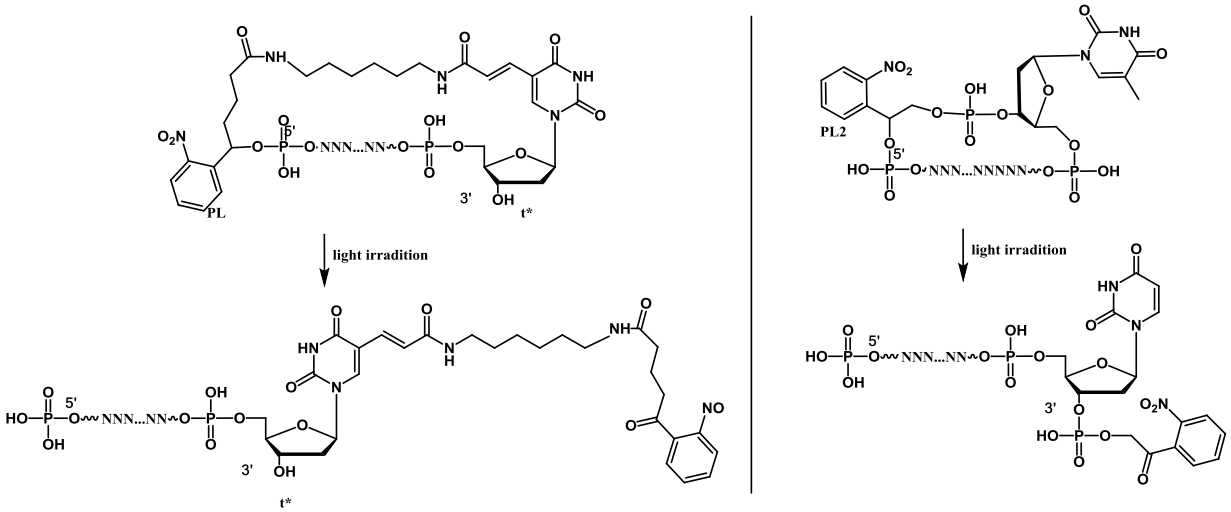


Figure S2. HPLC traces of single-stranded RNA . (A) The caged single-stranded RNA before cyclized, (B) The caged circular single-stranded RNA.

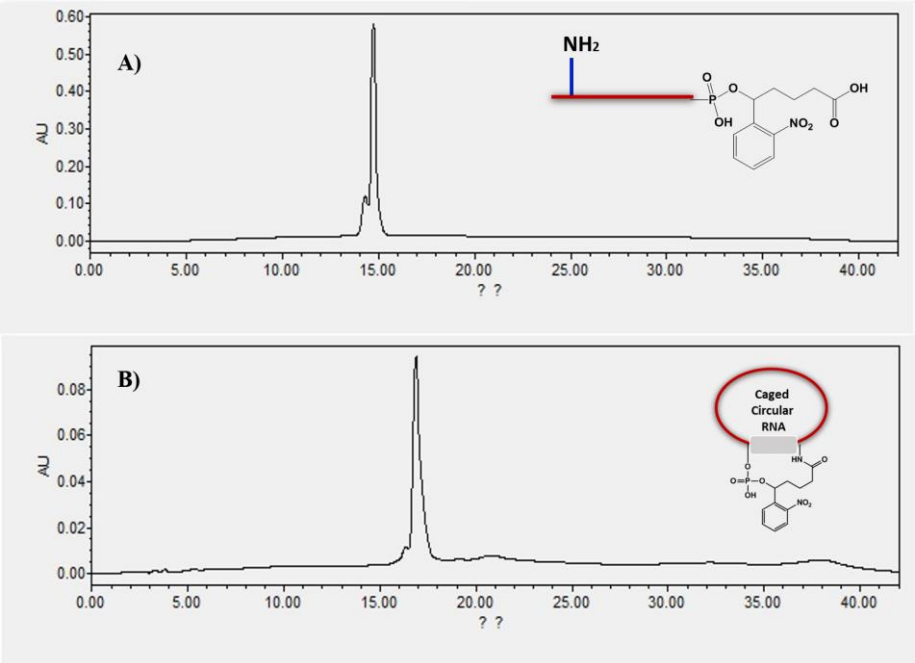


Figure S3. Native PAGE (20%) analysis of caged circular single-stranded RNA and photoactivated ones using a UV-LED for 3 minutes. Linear single-stranded RNA was restored upon light irradiation

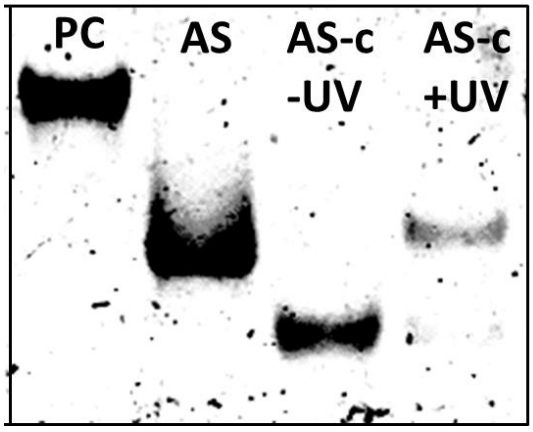


Figure S4. A) Evaluation of thermal stability of a caged circular siRNA duplex on native PAGE gel. Two fragment bands (B1 and B2) were observed on the PAGE gel. Further recollected fragments B1 and B2 were subject to PAGE gel analysis again, and the same two bands showed up on the gel. B) Two possible conformations of caged siRNA duplex.

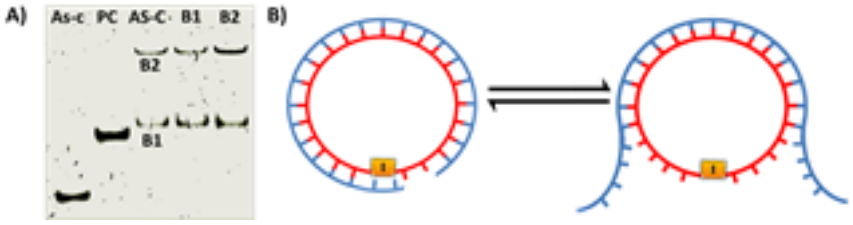


Figure S5. A) UV-melting profile at 260 nm of linear siRNA duplex (SG/AG), melting temperature is 70 ℃; B) UV-melting profile at 260 nm of caged circular siRNA duplex (SG/c-AG) , melting temperature is 62 ℃.

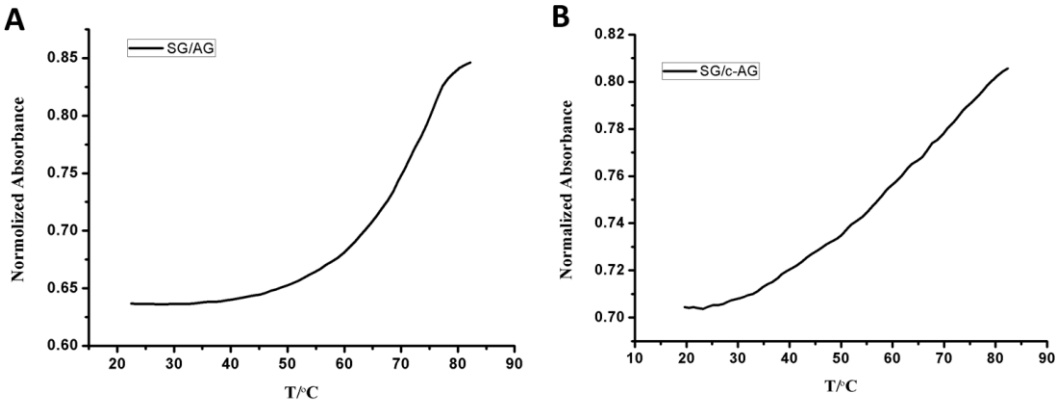


Figure S6. Cell viablity of HEK 293 using MTT assay at different UV irradiation time.

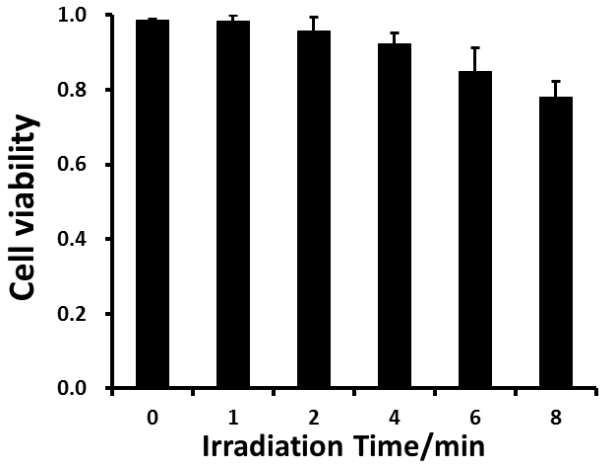


Table S3. The original data of Firefly or Renilla fluorescence signal upon exposure to and in the absence of light. The data showed that all siRNAs did not induce gene silencing activity of Rellina luciferase.

	Vector	PC	S-C	AS-C
Firefly	31478582	1429185	11742900	22031873
Firefly + UV	24685791	1177162	5471785	8207135
Renilla	6577713	7246392	7624195	6210396
Renilla + UV	5062478	5844188	6485614	5977201

Figure S7. Typical in vivo real-time fluorescent images at the indicated time point after intratumor injection of PBS on the left and linear siRNAs (L-siRNA) on the right respectively.

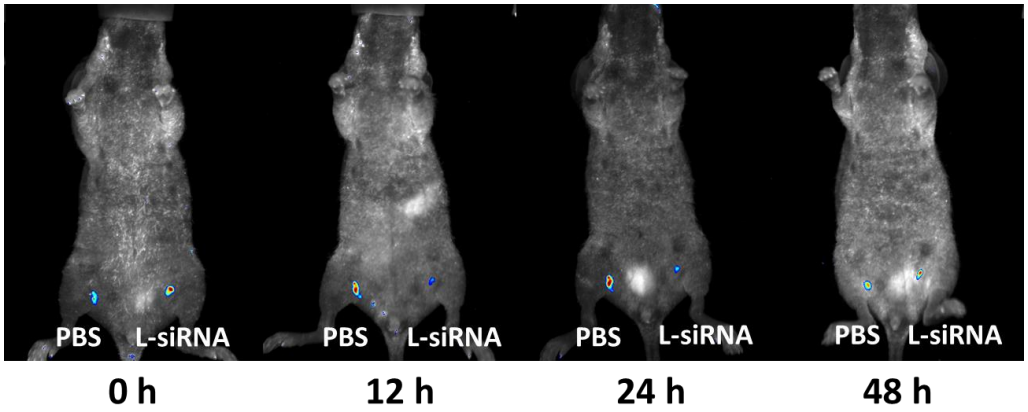


Figure S8. Representative images of xenograft tumors dissected from one of the mice which were injected with circular siRNAs and left tumor was subjected to light irradiation. They were quantified at 72h. (A) Bright field image; (B) Fluorescent image; (C) Overlapped image.

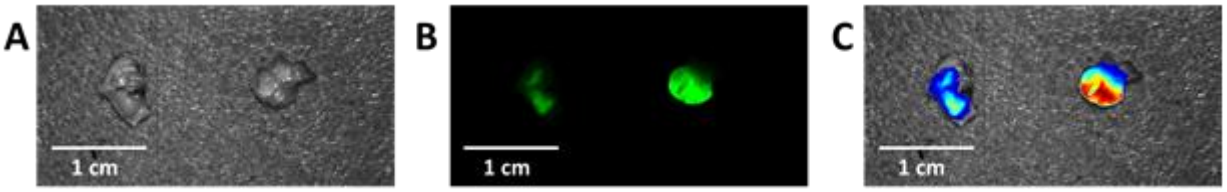
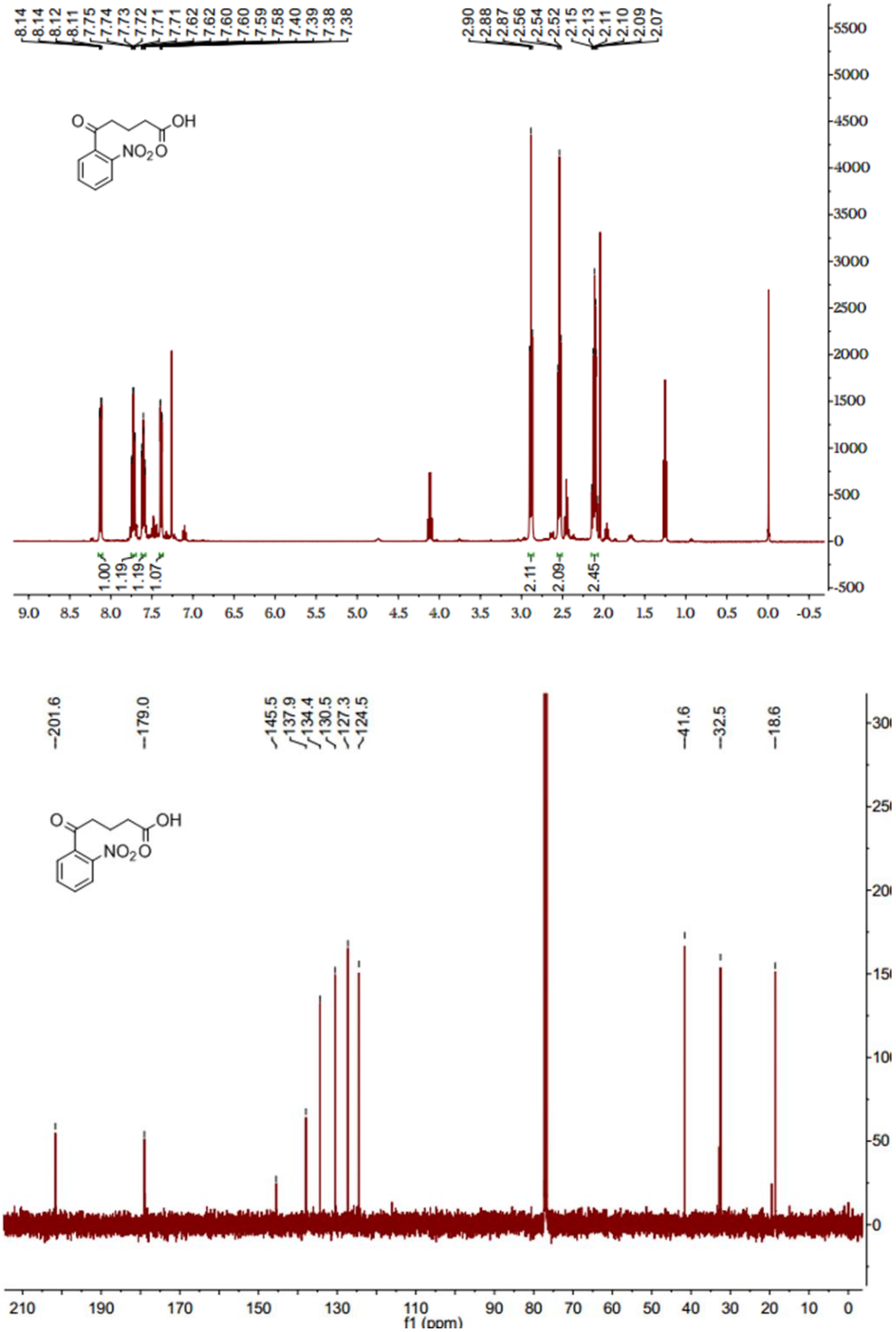
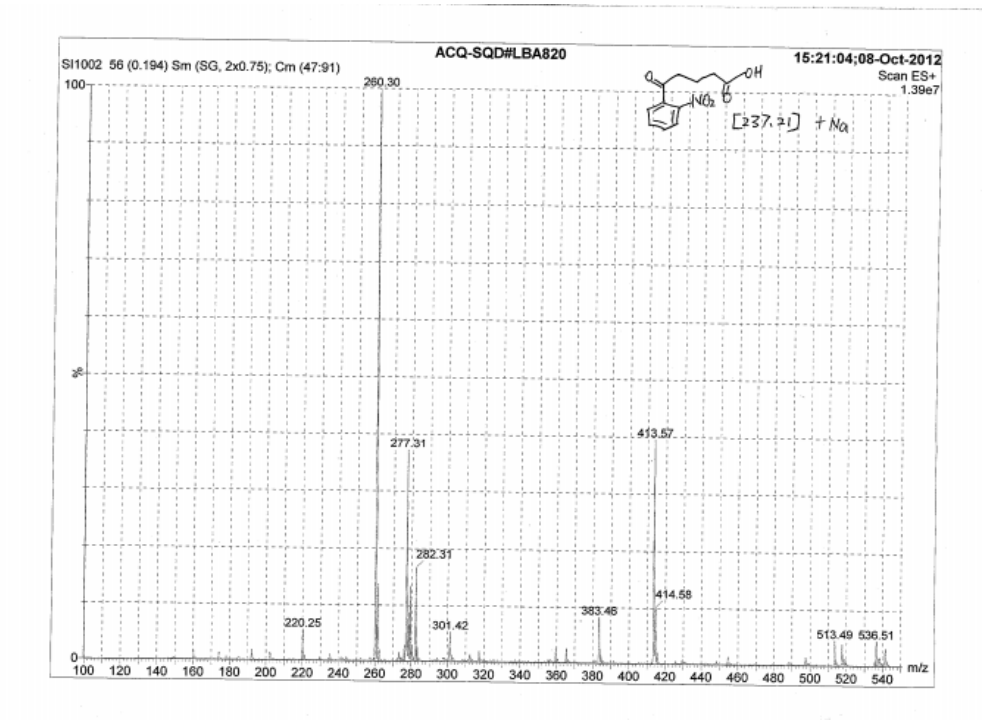


Figure S8. The NMR and MS of the **PL** and its intermediates

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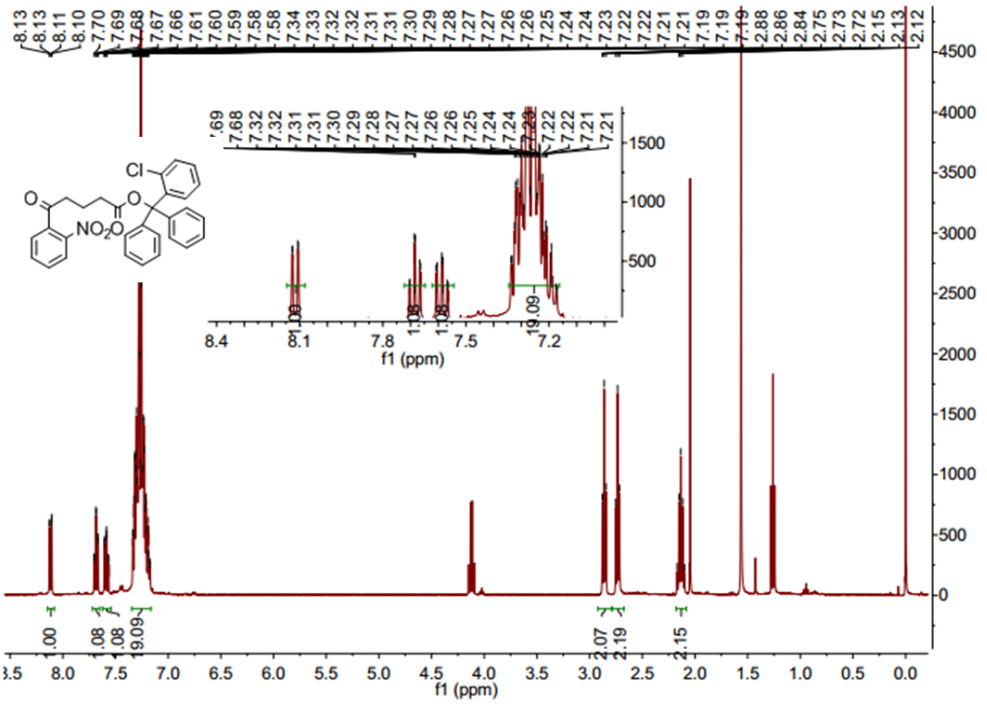
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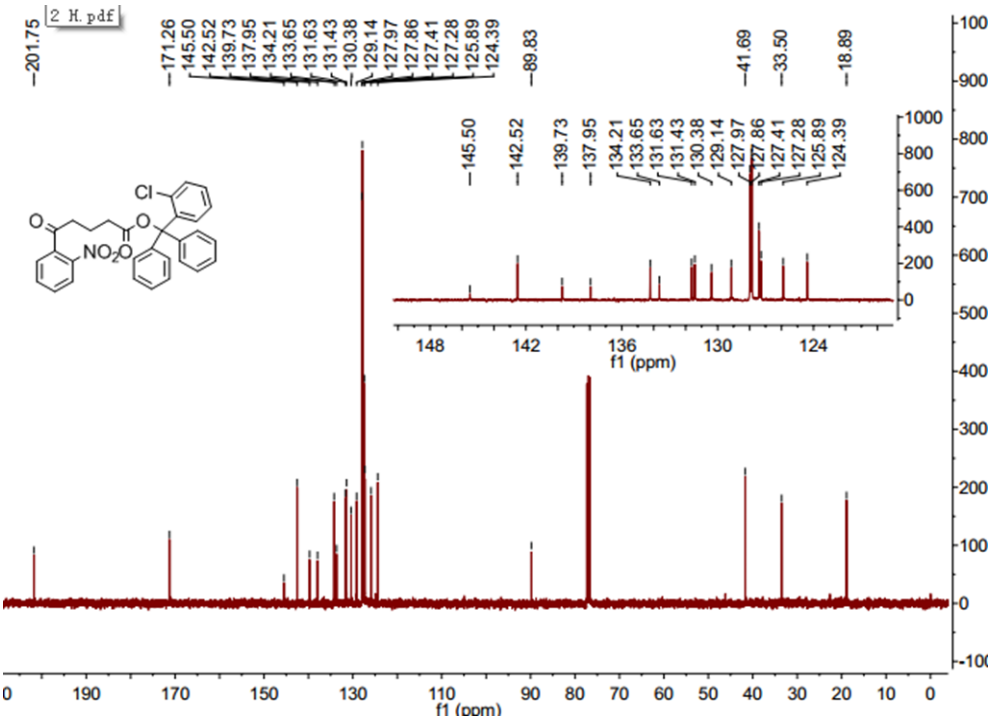
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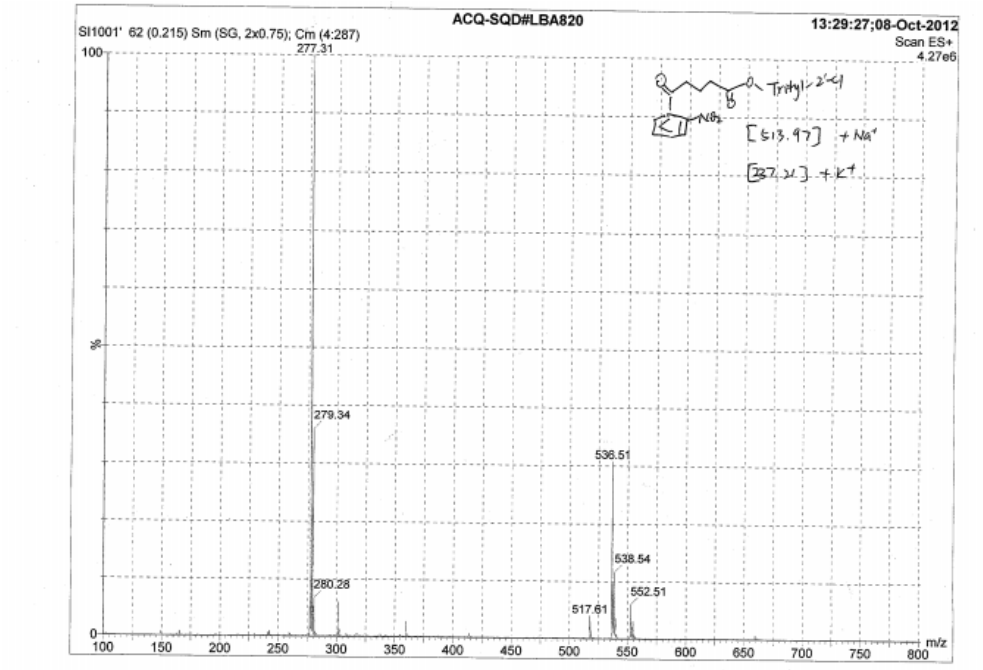
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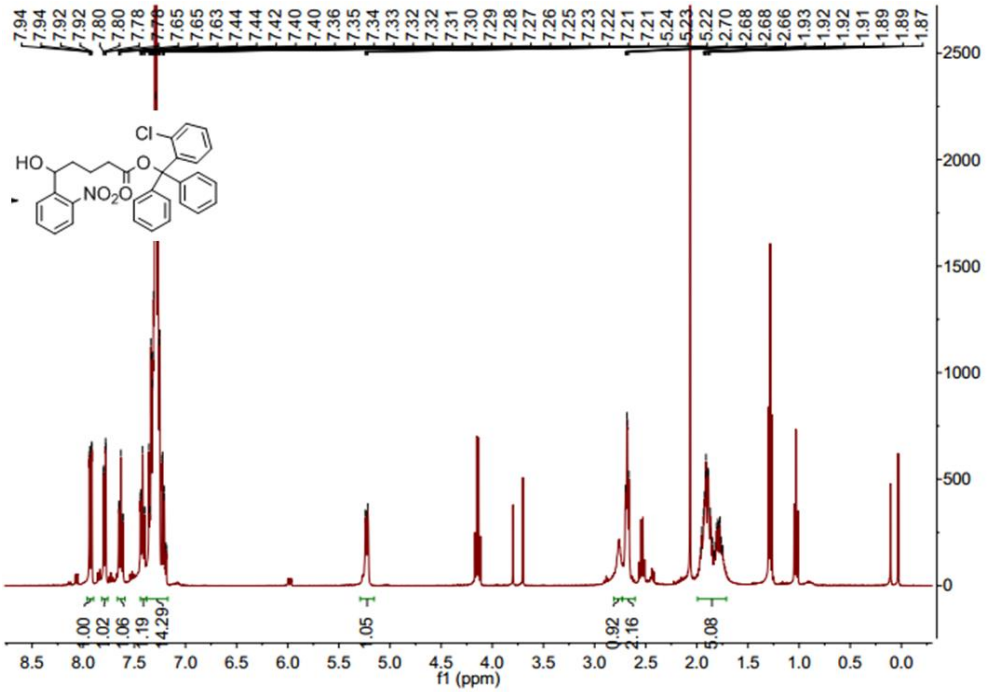
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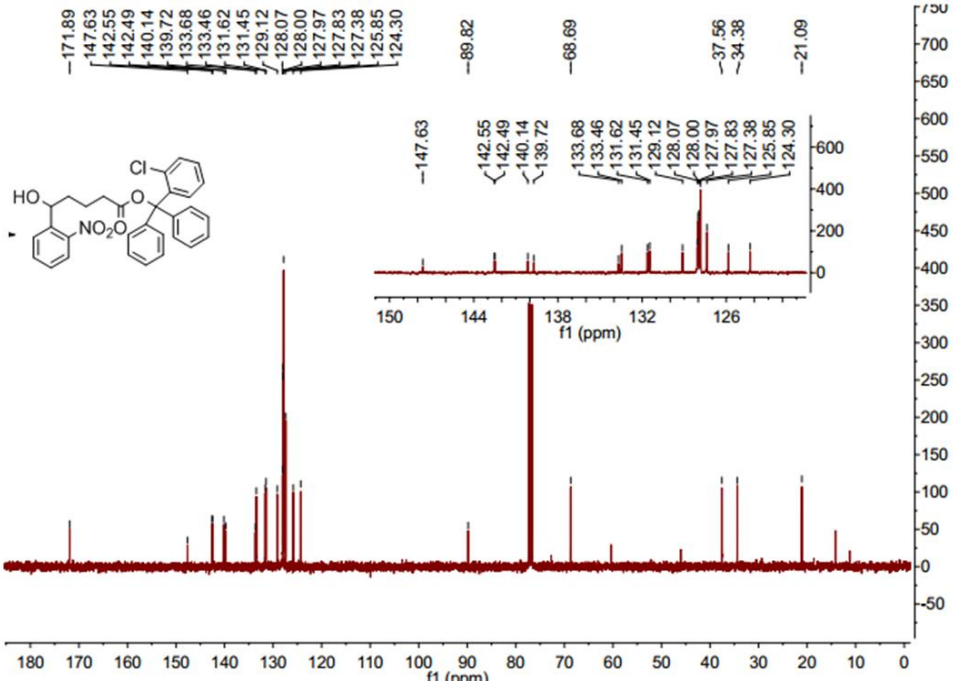
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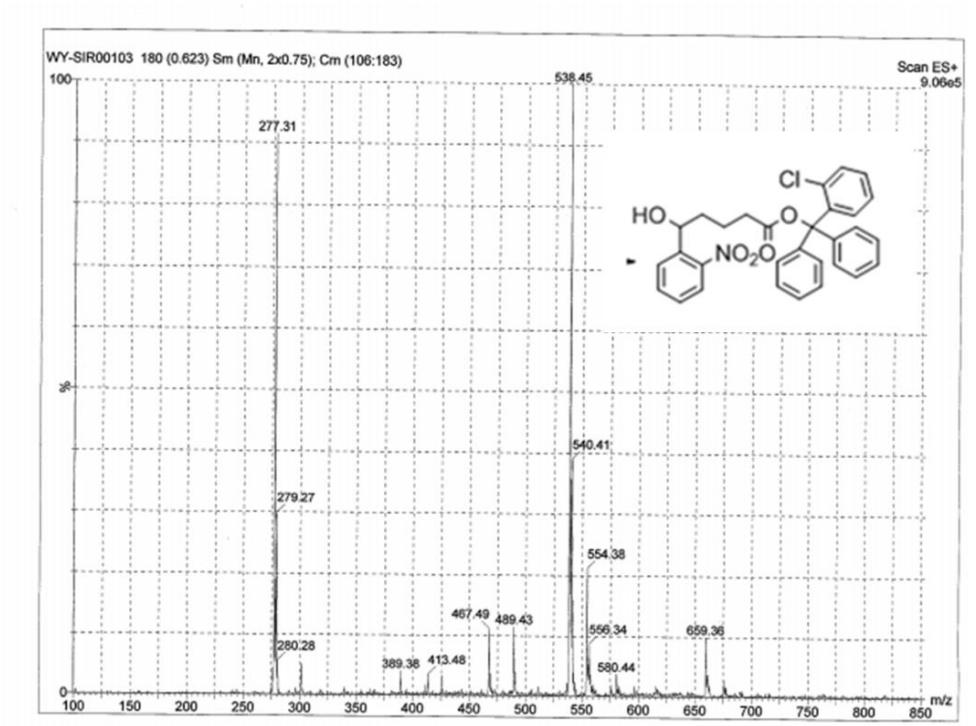
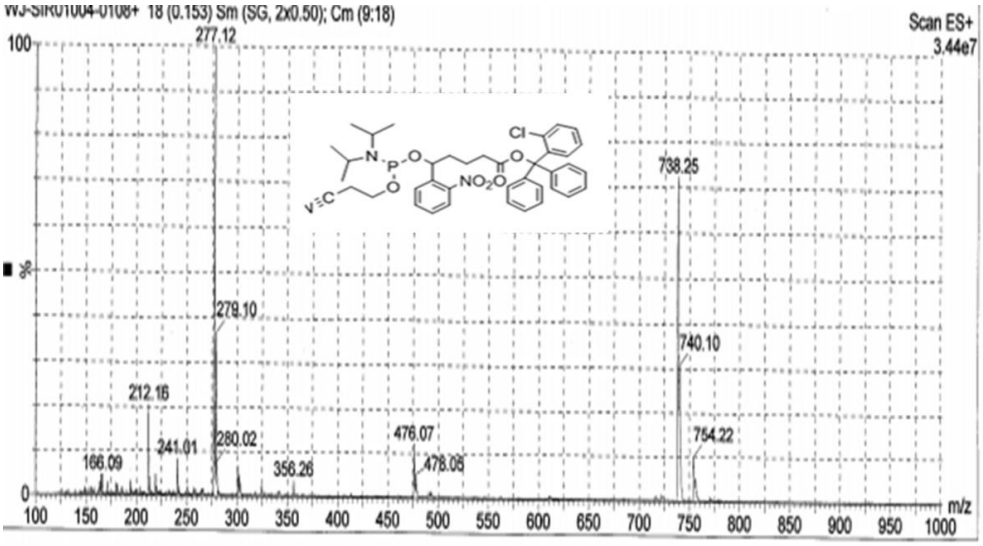
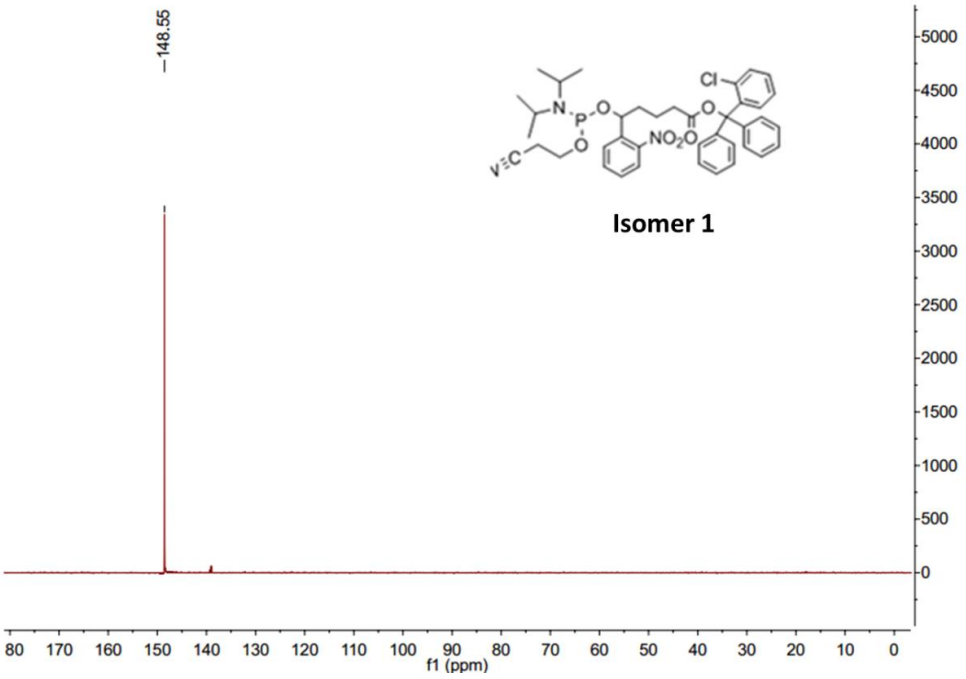
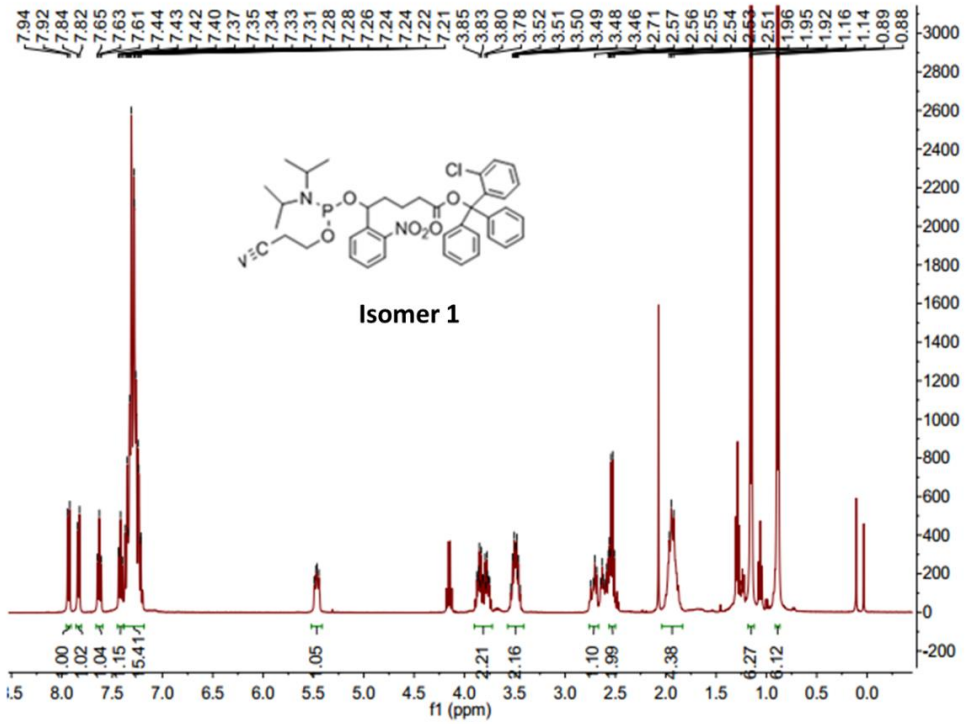
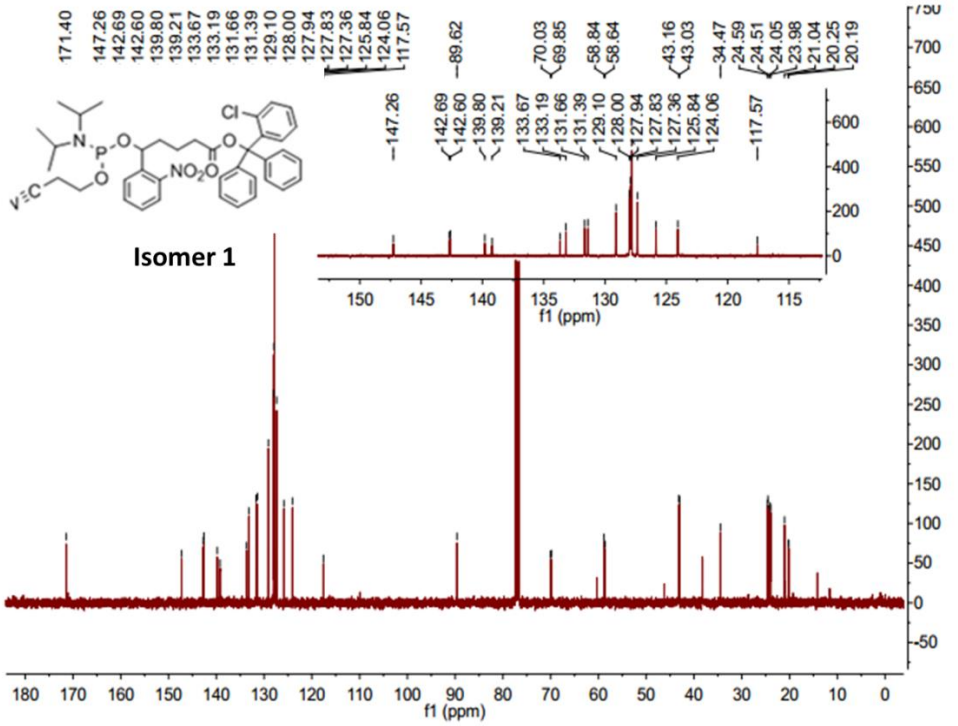
NO2
f1 (ppm)

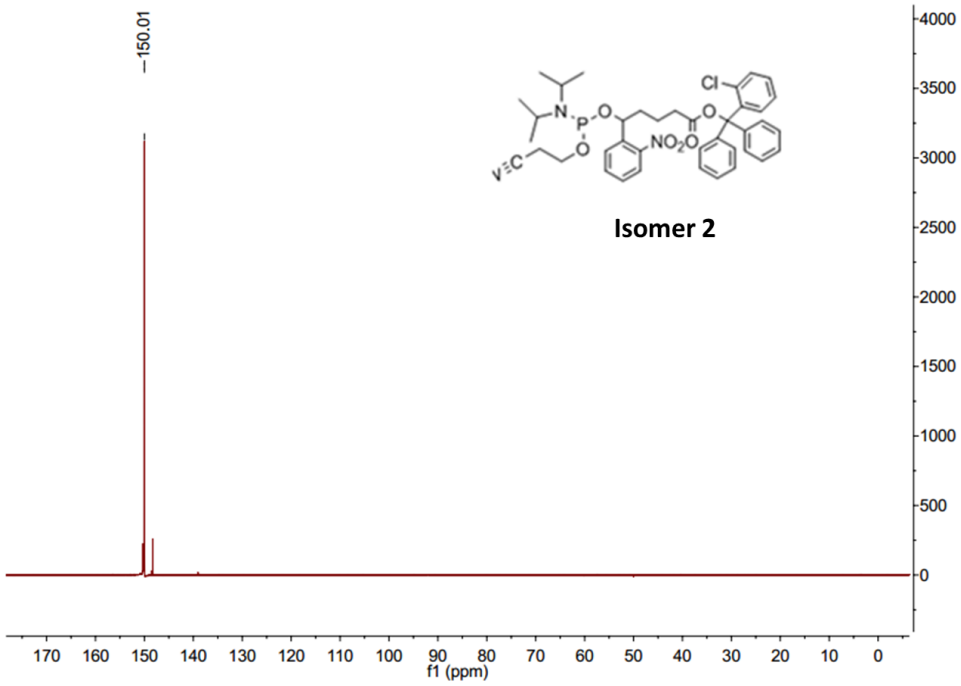

WY-SIR00103 180 (0.623) Sm (Mn, 2x0.75); Cm (106:183)
Scan ES+
9.06e5
538.45
277.31
540.41
279.27
554.38
467.49
489.43
556.34
659.36
280.28
389.38
413.48
580.44
Cl
HO
O
NO2
O
%
m/z

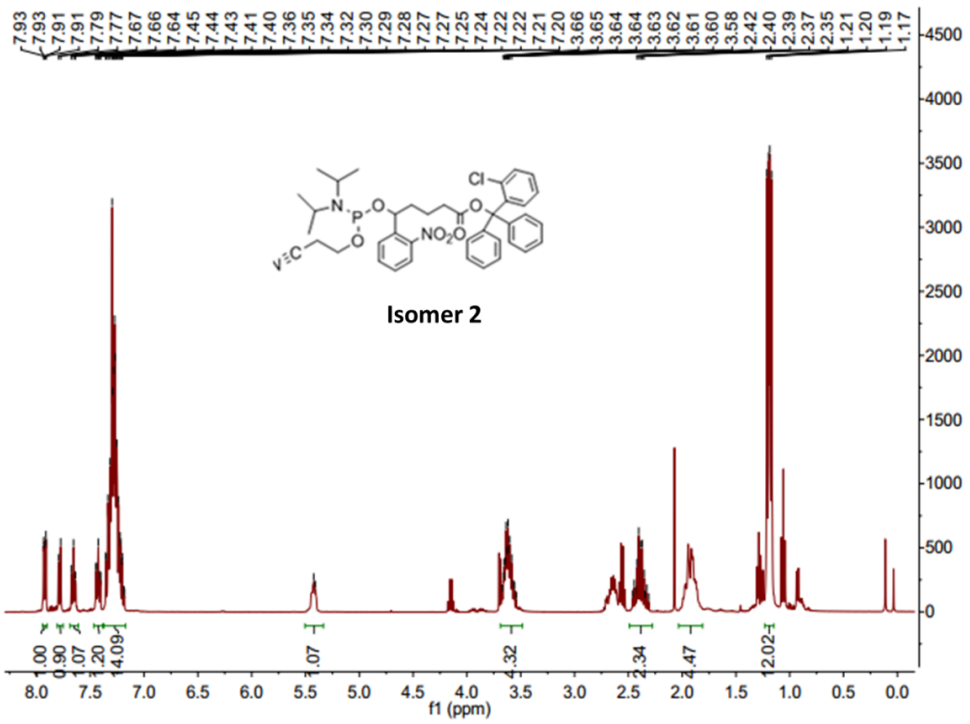

Scan ES+
3.44e7
277.12
738.25
279.10
740.10
212.16
280.02
476.07
754.22
241.01
166.09
356.26
478.05
N
P
O
Cl
NO2
%
m/z


148.55
Cl
N
P
O
NO2
Isomer 1
f1 (ppm)


Cl
N
P
O
NO2
Isomer 1
f1 (ppm)


171.40
147.26
142.69
142.60
139.80
139.21
133.67
133.19
131.66
131.39
129.10
128.00
127.94
127.83
127.36
125.84
124.06
117.57
89.62
70.03
69.85
58.84
58.64
43.16
43.03
34.47
24.59
24.51
24.05
23.98
21.04
20.25
20.19
Cl
NO2
Isomer 1
f1 (ppm)


150.01
Cl
NO2
Isomer 2
f1 (ppm)


Isomer 2
f1 (ppm)

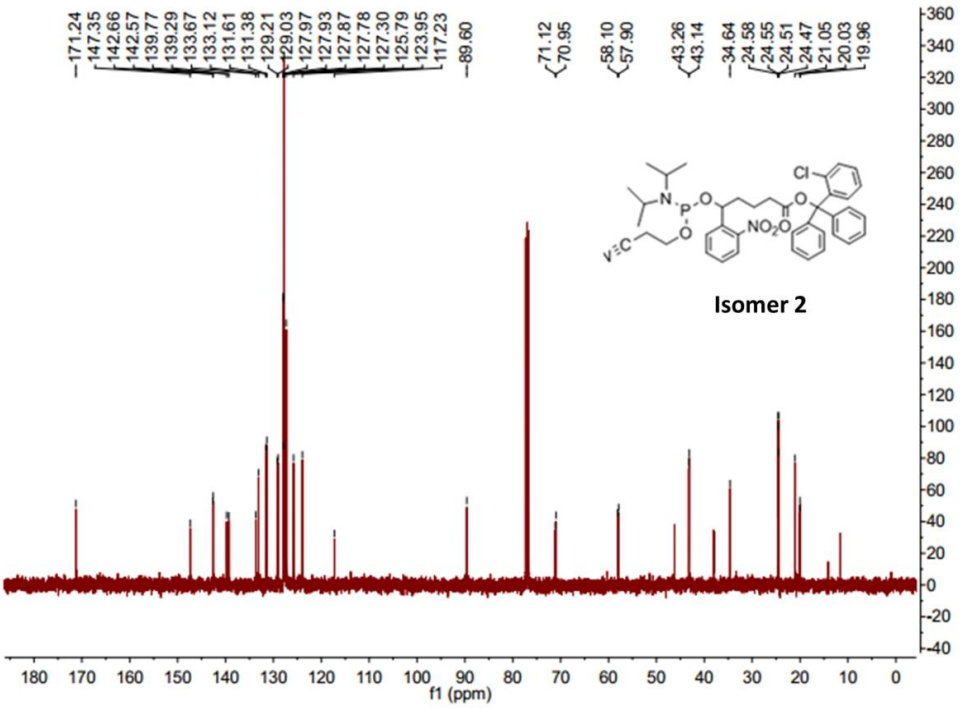
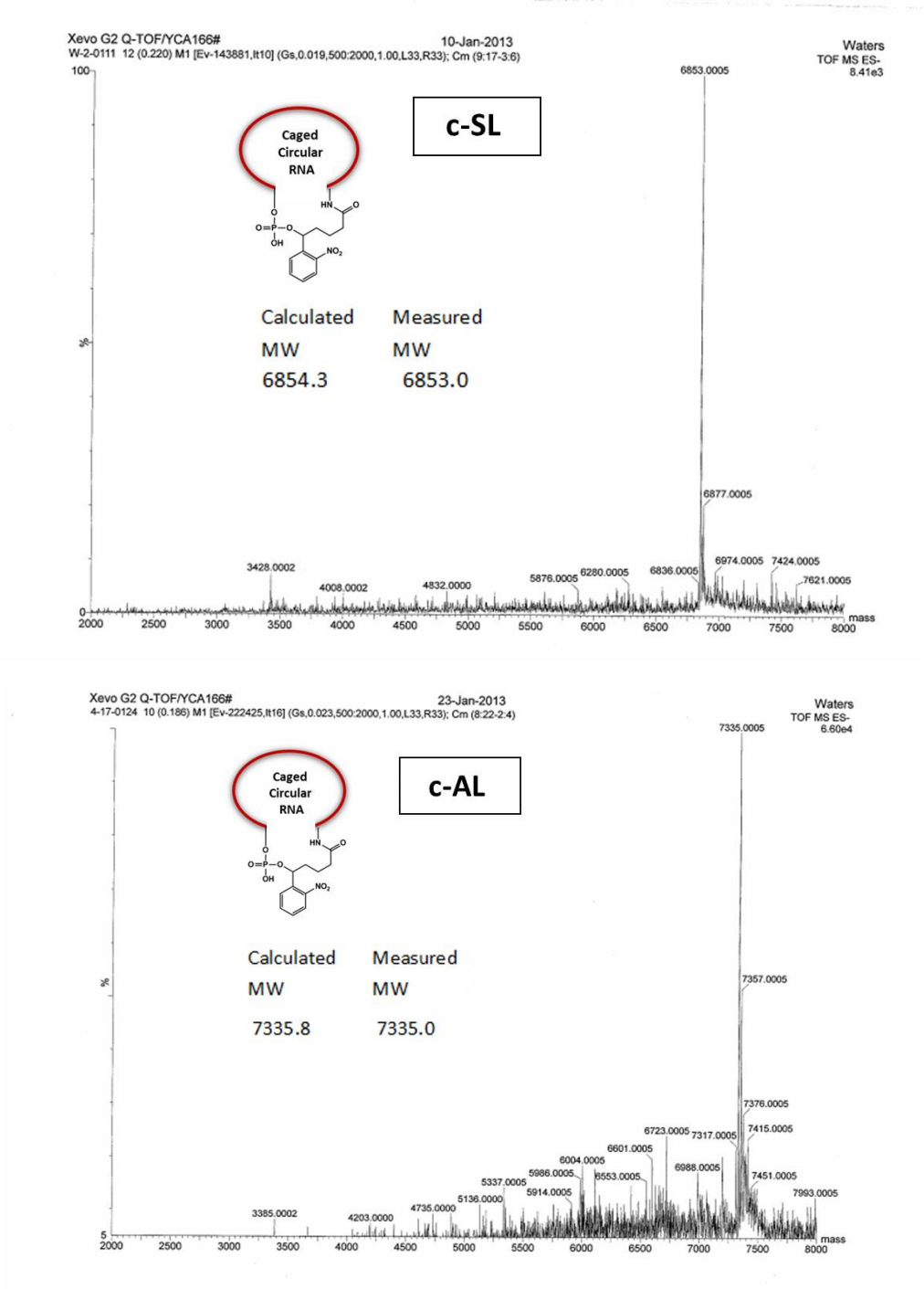
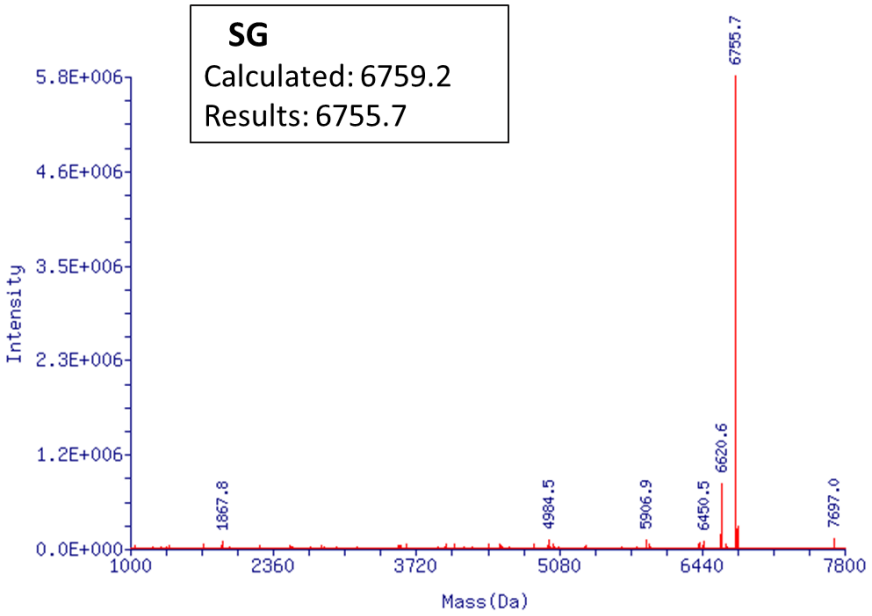
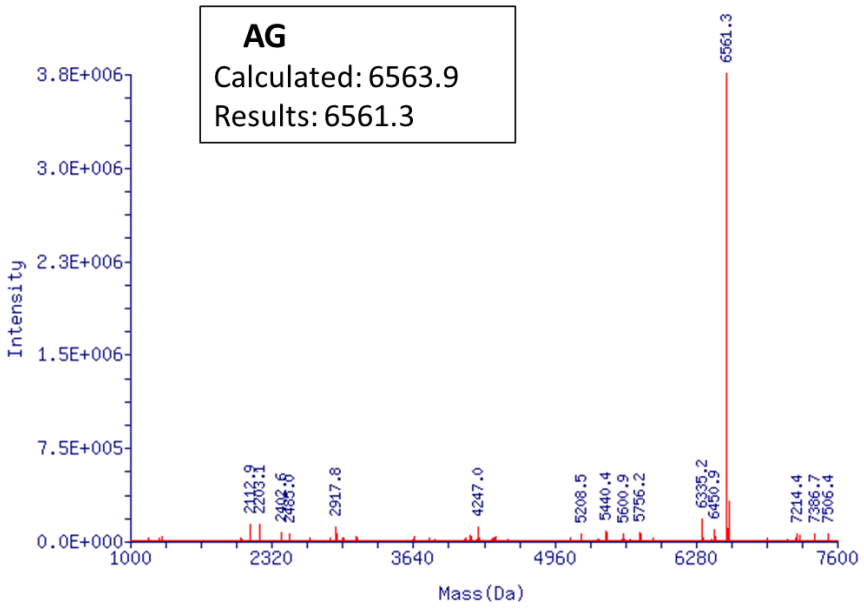
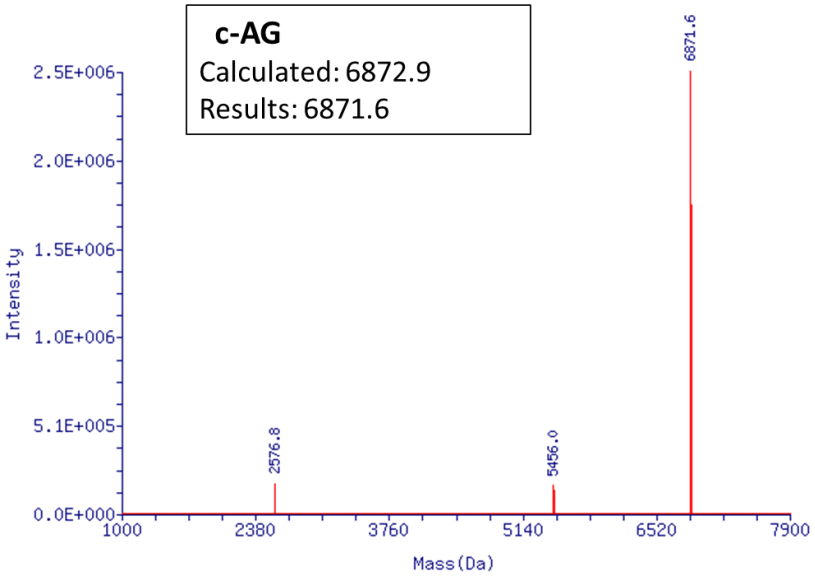

Isomer 2
f1 (ppm)

Figure S9. The MS of single-stranded RNA with ESI-MS.




SG
Calculated: 6759.2
Results: 6755.7
Intensity
Mass(Da)
5.8E+006
4.6E+006
3.5E+006
2.3E+006
1.2E+006
0.0E+000
1000
2360
3720
5080
6440
7800
1867.8
4984.5
5906.9
6450.5
6620.6
6755.7
7697.0


AG
Calculated: 6563.9
Results: 6561.3
Intensity
Mass(Da)
3.8E+006
3.0E+006
2.3E+006
1.5E+006
7.5E+005
0.0E+000
1000
2320
3640
4960
6280
7600
2917.8
4247.0
5208.5
5440.4
5600.9
5756.2
6335.2
6450.9
6561.3
7214.4
7386.7
7506.4


c-AG
Calculated: 6872.9
Results: 6871.6
2.5E+006
2.0E+006
1.5E+006
1.0E+006
5.1E+005
0.0E+000
Intensity
2576.8
5456.0
6871.6
1000
2380
3760
5140
6520
7900
Mass(Da)