Supplementary Information

Reversible One-Step Acylation Facilitates Mitochondrial Delivery of Functional RNA

Yuli Zhuang[1,§], Xia Liu[2,§], Yuxuan Shen[1], Qiuyue Wang[1], Zhimin Lin[1], Qian Zeng[1], Xin Chen[1], Linye Jiang[1], Shao Q. Yao[2], Jingyan Ge[1,*]

[1]*State Key Laboratory of Green Chemical Synthesis and Conversion, Zhejiang Key Laboratory of Bioorganic Synthesis, College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou 310014, China, E-mail: gejy@zjut.edu.cn*

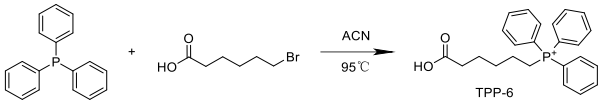
[2]*Department of Chemistry, National University of Singapore, Singapore 117543*

[§]*These authors were contributed equally to this work.*

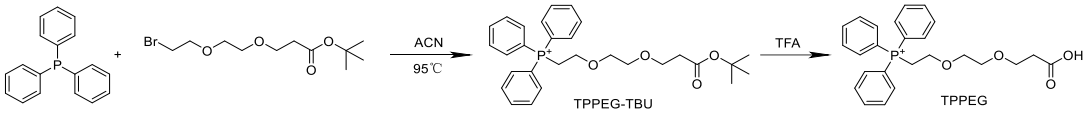
1. General materials

Dichloromethane (DCM), ethanol, methanol (MeOH), petroleum ether (PE), acetonitrile (ACN), and tetrahydrofuran (THF) were purchased from Hangzhou Fangping Chemical Co. Ether, dimethyl sulfoxide (DMSO), and citric acid were obtained from Sinopharm Chemical Reagent Co. Acetic acid, triphenylphosphine, 6-bromohexanoic acid, 1,1'-carbonyl diimidazole (CDI), tert-butyl(2-(2-bromoethoxy)ethyl)carbamate, and 3-(2-(2-(2-bromoethoxy)ethoxy)propyl)propyl tert-butyl ester, were purchased from Titan (Shanghai). 3-Carboxypropyl disulfide, *N*,*N*-dimethylglycine, 1-hydroxybenzotriazole (HOBt), and HBTU were obtained from Meryer (Shanghai). Sodium acetate (NaOAc), agarose, glutathione (GSH), and tris(hydroxymethyl)aminomethane (Tris) were purchased from Macklin (Shanghai). All siRNAs and TS-Gel Red nucleic acid stain were purchased from Tsingke Biotechnology. RNase A was obtained from Sangon Biotech (Shanghai). Cell Counting Kit-8 (CCK-8) was purchased from APExBIO Technology. Fetal bovine serum (FBS) was purchased from ExCell. 0.25% trypsin-EDTA solution was purchased from Solarbio (Beijing). DMEM and PBS (*p*H 7.4) were obtained from HyClone. MitoTracker Green, MitoTracker Deep Red, and LysoTracker Green were purchased from Thermo Fisher Scientific. Anti-MTCO1 and Anti-MTND1 antibodies were purchased from Abcam. Anti-Vinculin and goat anti-rabbit IgG antibodies were obtained from BioLegend. Mitochondrial membrane potential and reactive oxygen species (ROS) assay kits were purchased from Beyotime Biotechnology (Shanghai).

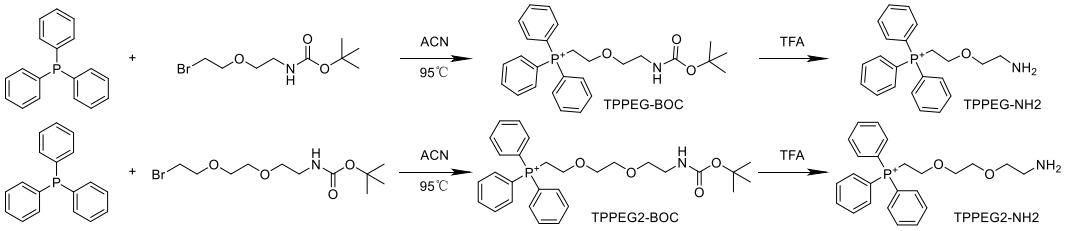
2. ***Synthesis of monomers***



6-Bromocaproic acid (1.0 g, 5.12 mmol) and triphenylphosphine (1.4 g, 5.43 mmol) were dissolved in acetonitrile (6 mL) and refluxed at 95°C for 24 h. After completion of the reaction, as monitored by TLC, the mixture was cooled to room temperature and cold ether (100 mL) was added slowly. The resulting precipitate was dissolved in dichloromethane, and the solvent was removed under reduced pressure to afford TPP-6 as a white powder (93.4% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.82 – 7.75 (m, 9H), 7.71 (td, *J* = 7.5, 3.5 Hz, 6H), 3.59 (td, *J* = 12.8, 6.1 Hz, 2H), 2.37 – 2.29 (m, 2H), 1.64 (q, *J* = 4.8, 4.3 Hz, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 175.95, 135.16, 133.63, 130.61, 118.11, 34.22, 29.54, 24.02, 22.54, 21.94. HRMS (ESI) calcd for $[M]^+$: 377.1665, found: 377.1666.



Compound TPPEG-TBU was prepared following the same procedure described for TPP-6, affording the product in 87% yield. TPPEG-TBU was then dissolved in dichloromethane (2 mL), and trifluoroacetic acid (0.2 mL, TFA) was added dropwise under cooling. The mixture was stirred at room temperature for 4 h, after which TFA was removed under reduced pressure. Cold ether (20 mL) was added to the resulting slurry, affording a white precipitate. The solid was collected, washed several times with ether, and dried under vacuum to yield TPPEG (80.5%).^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (dt, *J* = 15.6, 7.8 Hz, 9H), 7.65 (td, *J* = 7.9, 3.4 Hz, 6H), 3.96 (dt, *J* = 11.2, 5.5 Hz, 2H), 3.89 (dt, *J* = 22.5, 5.5 Hz, 2H), 3.52 (t, *J* = 6.0 Hz, 2H), 3.27 (q, *J* = 5.7 Hz, 4H), 2.52 (t, *J* = 6.0 Hz, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 173.58, 134.85, 134.02, 130.23, 118.94, 70.26, 70.16, 66.68, 64.03, 35.66, 25.40. HRMS (ESI) calcd for $[M]^+$: 423.1720, found: 423.1724.



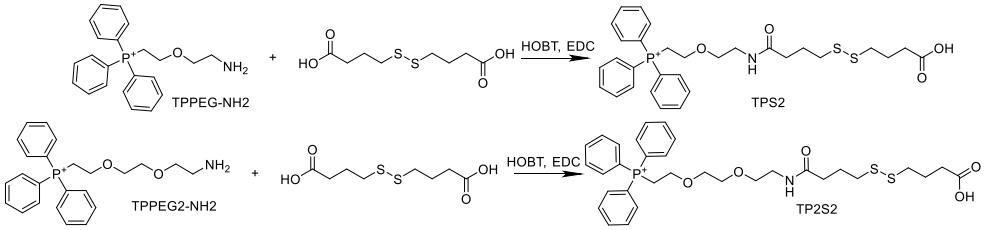
Compound TPPEG-Boc was prepared following the procedure described for TPP-6, affording the product as a yellow oil in 83% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.83 (ddd, *J* = 12.8, 8.4, 1.3 Hz, 6H), 7.80 – 7.74 (m, 3H), 7.67 (td, *J* = 7.7, 3.4 Hz, 6H), 4.37 (s, 1H), 4.26 (dt, *J* = 11.6, 5.6 Hz, 2H), 3.93 (d, *J* = 22.2 Hz, 2H), 3.23 (t, *J* = 5.3 Hz, 2H), 3.05 – 2.95 (m, 2H), 1.42 (s, 9H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 155.85, 134.67, 134.05, 130.05, 119.29, 118.71, 79.40, 70.31, 69.96, 69.79, 28.43, 25.38. HRMS (ESI) calcd for $[M]^+$: 450.2193, found: 450.2195.

TPPEG-NH2 was obtained using the procedure described for TPPEG, affording the product as a white

solid in 87% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.67 (s, 3H), 7.85 – 7.73 (m, 9H), 7.68 (td, *J* = 7.6, 3.4 Hz, 6H), 4.28 (d, *J* = 24.2 Hz, 2H), 4.22 (dd, *J* = 12.3, 6.0 Hz, 2H), 3.99 (dt, *J* = 21.0, 5.3 Hz, 2H), 3.68 – 3.63 (m, 2H), 3.02-2.94 (m, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 134.95, 133.91, 130.32, 118.65, 66.64, 64.08, 39.42, 24.35. HRMS (ESI) calcd for $[M+H]^{2+}$: 175.5871, found: 175.5875.

Compound TPPEG2-Boc was prepared following the procedure described for TPP-6, affording the product as a white solid in 83% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (ddd, *J* = 12.8, 7.1, 1.6 Hz, 6H), 7.79 – 7.71 (m, 3H), 7.65 (ddd, *J* = 9.0, 7.1, 3.5 Hz, 6H), 4.78 (s, 1H), 4.21 (dt, *J* = 11.6, 5.7 Hz, 2H), 3.98 (t, *J* = 5.7 Hz, 1H), 3.93 (t, *J* = 5.7 Hz, 1H), 3.36 – 3.31 (m, 2H), 3.25 (q, *J* = 4.8, 4.0 Hz, 4H), 3.18 (t, *J* = 5.0 Hz, 2H), 1.41 (s, 9H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 155.85, 134.67 (d, *J* = 3.1 Hz), 134.05 (d, *J* = 10.2 Hz), 130.05 (d, *J* = 12.9 Hz), 119.00 (d, *J* = 86.9 Hz), 79.40, 70.31, 69.96, 69.79, 64.17 (d, *J* = 7.5 Hz), 40.32, 28.43, 25.38 (d, *J* = 52.5 Hz). HRMS (ESI) calcd for $[M]^+$: 494.2455, found: 494.2460.

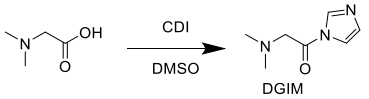
TPPEG2-NH2 was obtained using the procedure described for TPPEG, affording the product as a white solid in 87% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.52 (s, 3H), 7.80 (dd, *J* = 13.2, 7.7 Hz, 9H), 7.70 (td, *J* = 7.7, 3.3 Hz, 6H), 4.47 (s, 2H), 4.06 (ddt, *J* = 31.0, 20.6, 5.8 Hz, 4H), 3.72 (t, *J* = 5.2 Hz, 2H), 3.46 (q, *J* = 2.9 Hz, 2H), 3.41 (q, *J* = 2.9 Hz, 2H), 3.16 (q, *J* = 5.4 Hz, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 134.87 (d, *J* = 3.1 Hz), 133.89 (d, *J* = 10.2 Hz), 130.25 (d, *J* = 12.5 Hz), 118.81 (d, *J* = 86.8 Hz), 70.34, 70.22, 66.58, 64.07 (d, *J* = 5.9 Hz), 39.62, 24.80 (d, *J* = 52.4 Hz). HRMS (ESI) calcd for $[M+H]^{2+}$:197.6002, found:197.6005.



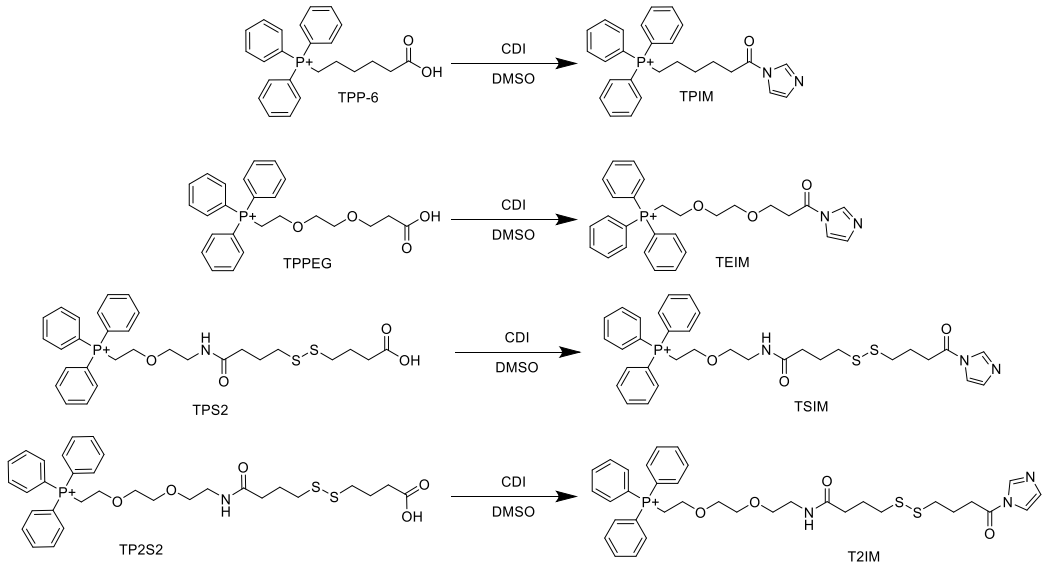
4,4′-Dithiodibutyric acid (92.2 mg, 0.387 mmol) was dissolved in tetrahydrofuran/dichloromethane (30 mL, v/v = 2:1). To this solution were added EDCI (74.2 mg, 0.387 mmol) and 1-hydroxybenzotriazole (HOBt, 104.6 mg, 0.774 mmol), and the mixture was stirred at room temperature for 30 min. TPPEG-NH2 (166 mg, 0.387 mmol), dissolved in tetrahydrofuran/dichloromethane (10 mL, v/v = 2:1), was added dropwise under a nitrogen atmosphere to the activated solution with stirring. The reaction mixture was then stirred at room temperature overnight. The reaction was washed successively with ice-cold saturated citric acid solution, ice water, and ice brine. The organic layer was collected, dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography. Elution with dichloromethane/methanol (40:1, v/v) gave fractions with Rf = 0.4-0.5. Evaporation of the solvent at 30°C afforded TPS2 as a white solid (62% yield). ^{1}H NMR (500 MHz, D_2O) δ 7.84 (td, *J* = 7.4, 1.8 Hz, 3H), 7.77 – 7.66 (m, 12H), 3.85 (dt, *J* = 20.3, 6.0 Hz, 2H), 3.64 (dt, *J* = 12.1, 5.9 Hz, 2H), 3.31 (t, *J* = 5.1 Hz, 2H), 3.13 (t, *J* = 5.1 Hz, 2H), 2.65 (q, *J* = 6.8 Hz, 4H), 2.40 (t, *J* = 7.3 Hz, 2H), 2.19 (t, *J* = 7.4 Hz, 2H), 1.86 (dp, *J* = 18.2, 7.2 Hz, 4H). ^{13}C NMR (151 MHz, D_2O) δ 177.75, 175.38, 134.94, 133.61, 129.97, 118.36, 68.60, 63.19, 39.05, 36.90, 36.89, 34.21, 32.30, 24.59, 23.70, 23.67. HRMS (ESI) calcd for $[M]^+$: 570.1901, found: 570.1896.

TP2S2 was synthesized according to the same procedure, affording the product as a yellow oil in 54% yield. ^{1}H NMR (400 MHz, D_2O) δ 7.74 (td, *J* = 7.3, 1.7 Hz, 3H), 7.70 – 7.62 (m, 6H), 7.58 (td, *J* = 7.7, 3.6 Hz, 6H), 3.76 (dt, *J* = 19.9, 6.0 Hz, 2H), 3.53 (dt, *J* = 12.1, 5.9 Hz, 2H), 3.32 – 3.23 (m, 6H), 3.17 (t, *J* = 5.4 Hz, 2H), 2.54 (td, *J* = 7.1, 5.3 Hz, 4H), 2.29 (t, *J* = 7.3 Hz, 2H), 2.20 (t, *J* = 7.3 Hz, 2H), 1.79 (dt, *J* = 20.4, 7.1 Hz, 4H). ^{13}C NMR (151 MHz, D_2O) δ 177.76, 175.65, 134.94, 133.69, 129.94, 118.28, 69.84, 69.37, 68.80, 63.30, 38.95, 36.91, 36.87, 34.22, 32.26, 24.65, 23.67, 23.64. HRMS (ESI) calcd for $[M]^+$: 614.2158, found: 614.2159.

3. Preparation of acylation precursor



Preparation of DGIM stock solution. According to the literature,[1,2] Dimethylglycine (103.2 mg, 1.0 mmol) was dissolved in DMSO to afford a 4 M solution. Separately, CDI (162.2 mg, 1.0 mmol) was dissolved in DMSO to a final concentration of 4 M. The dimethylglycine solution (4 M) was slowly added to the CDI solution (4 M) with stirring, and the mixture was stirred vigorously at room temperature for 1 h. The resulting reaction mixture was used directly as a 2 M DGIM stock solution without further purification.



TPIM, TEIM, TSIM, and T2IM were prepared according to the same procedure described for DGIM. Related ^{1}H and ^{13}C NMR spectrums in *d6*-DMSO were included in the final.

Table S1 The main siRNA sequences

siRNA	Sequence
siCOX1 or siCOX1^{TMR}	5'-(TAMRA)CGGUCACCCUGAAGUUUAUA(dT)(dT)-3' 5'-AAUAUAAACUUCAGGGUGACCG(dT)dT)-3'
siND1 or siND1^{TMR}	5'-(TAMRA)CUUCUAACCUCCCUGUUCUUA(dT)9dT)-3' 5-UAAGAACAGGGAGGUUAGAAG(dT)(dT)-3'
siNC or siND1^{FAM}	5'-(FAM)ACGUGACACGUUCGGAGAA(dT)(dT)-3' 5'-UUCUCCGAACGUGUCACGU(dT)(dT)-3'
siMIF	5'-GGGUCUACAUCAACUAUUA(dT)(dT)-3' 5'-UAAUAGUUGAUGUAGACCC(dT)(dT)-3'

4. RNA@IM preparation and purification

DGIM, TPIM, TEIM, TSIM, and T2IM were each prepared at an initial concentration of 2 M in DMSO. The RNA stock solution, including single strand RNA and pre-annealed double-stranded RNA, was prepared in water at an initial concentration of 100 μM. For the double-stranded siRNA used, each strand was present at 100 μM. Each IM compound was then added to 8 μL RNA solution to achieve the desired final concentrations (DGIM at 800 mM, and the others at 100 mM). The mixture was pipetted thoroughly to mix and incubated at 37°C with shaking for 4 h. The reaction was cooled on ice, followed by the addition of 0.1 volume of 3 M sodium acetate solution. After mixing thoroughly, 40 volumes of pre-chilled ethanol were added, and the mixture was vortexed briefly before incubation at -80°C overnight. The precipitate was collected by centrifugation at 21,100 rpm for 1 h at 4 °C. The pellet was washed twice with 200 μL of 75% cold ethanol on ice, then air-dried at room temperature with the tube lid open for ~30 min. After drying, the RNA@IM pellet was dissolved in RNase-free water and stored at -80°C until further use. The concentration of purified RNA was estimated based on nucleic acid band intensity in agarose gel electrophoresis.

5. MALDI-TOF MS analysis

1 μL of a saturated 3-HPA matrix in acetonitrile/water (1:1 v/v) with 10 mg/mL ammonium citrate dibasic was deposited and dried on the target plate. Next, 1 μL of purified RNA@IM was added and dried. Finally, a second 1 μL of matrix was overlaid and crystallized to form a sandwich structure. Spectra were acquired in negative ion mode (BRUKER autoflex® maX) and processed with FlexAnalysis 3.4 for baseline correction and peak identification.

6. Deacetylation experiment

For each 500 ng of IM@RNA, dissolve the sample in 8 μL of water. To the IM@RNA solution, add the 1 M Tris (pH 8.8) or 100 mM GSH stock solutions to obtain final concentrations of 200 mM Tris or 20 mM GSH, respectively. Mix thoroughly by pipetting, then incubate the mixture on a shaker at 37°C for 24 h. After incubation, analyze the reaction products by agarose gel electrophoresis.

7. Stability to RNase

For each 200 ng of IM@RNA^{FAM}, dissolve the sample in 3 μL of water. Heat the solution at 95 °C for 2 min, then immediately place it at 4 °C. Subsequently, add 1 μL of RNase A (100 μg/mL) to the solution. After incubation for 30 min, analyze the reaction products by agarose gel electrophoresis without GelRed and capture images.

8. Detection of RNA@IM cell localization

To evaluate the cell localization of IM@RNA, confocal fluorescence microscopy was performed. TAMRA-labeled RNA was used for imaging, and IM@RNA^{TMR}was prepared as described in Section 4. Cells were incubated with IM@RNA^{TMR} at the indicated concentrations for the designated time periods. Subsequently, organelle-specific trackers were added and incubated for 30 min. After incubation, cells were washed several times with DMEM to remove unbound probes prior to imaging. MitoTracker Green (λ_{ex} = 490 nm, λ_{em} = 516 nm) or MitoTracker Deep Red (λ_{ex} = 644 nm, λ_{em} = 665 nm) were used for mitochondrial staining, while LysoTracker Green (λ_{ex} = 504 nm, λ_{em} = 511 nm) was used for lysosomal staining. The TAMRA signal was detected in the red

channel (λ_{ex} = 552 nm, λ_{em} = 578 nm). Fluorescence images were acquired using a Leica DM8 confocal laser scanning microscopy, and colocalization was assessed by overlaying the signals from the TAMRA channel with the corresponding organelle tracker channels.

9. Flow cytometry

The cell was seeded in a 12-well plate with a density of 3×10^5. The RNA used in this experiment was modified with FAM fluorescent groups and named RNA^{FAM}. After cell attachment, 100 nM $IM@RNA^{FAM}$ was added into the cell medium. After 24 h-incubation, cells were washed with PBS three time. Further using trypsin to detach cells, cells were collected and resuspended in PBS to analyze on a BD FACS Canto™ III flow cytometer.

10. Immunoblotting (WB)

Following SDS-PAGE, the target protein region was excised using a molecular weight marker reference and transferred to a PVDF membrane. The membrane was blocked with 5% skim milk in TBST for 1.5 h, washed thoroughly, and incubated with specific primary antibodies (anti-MTCO1 1:2000, anti-MTND1 1:5000, anti-Vinculin 1:10000, diluted in 3% BSA/TBST) for 1.5 h at room temperature. After TBST washes, HRP-conjugated goat anti-rabbit IgG secondary antibody (1:7500) was applied for 1 h, followed by additional washes. Protein bands were visualized using FDbio-Dura ECL kit, detected with an Invitrogen iBright 1500 imaging system, and analyzed using ImageJ software.

11. Reactive oxygen species (ROS) detection

Cell reactive oxygen species were tested according to the kit procedure (Reactive Oxygen Species Assay Kit; Beyotime, Cat No: S0033S6) In short, cells after treatment with the corresponding IM@RNA, DCFH-DA diluted with PBS (1:1000) was added into cells. After incubation at 37℃ for 20 min in CO_2 incubator, cells were washed with PBS and take fluorescent image at λ_{ex} = 488 nm and λ_{em} = 525 nm. Cells were treated with Rosup (50 mg/mL) diluted with serum-free DMEM (1:1000) for 20 min as positive control.

12. Mitochondrial membrane potential detection

Mitochondrial membrane potential detection was carried out according to the kit procedure (Mitochondrial membrane potential assay kit with JC-1; Beyotime; Cat No: C2006). Commercial membrane potential dyes (JC-1) solution was incubated with cells at 37°C for 20 min in CO_2 incubator after cells treated with IM@RNA before washing with PBS twice and finally analyzed by CLSM. Cells treated with 10 μM CCCP (carbonyl cyanide m-chlorophenyl hydrazone) for 20 min were served as a positive control.

13. Cell viability

HeLa cells (5000 cells) were seeded into 100 μL medium on a 96-well plate at 37 °C for 24 h. Then, cells were incubated with the different concentrations of IM@RNAs in 100 μL medium 37 ° C for 72 h. After that, cells were treated with CCK-8 reagent (ApexBio Technology) following the instruction. The absorbances were then measured at 450 nm using BioTek Cytation 5. Results are processed by GraphPad Prism 8.0 software. Data are presented as mean ± SD (n=3).

14. Wound-healing experiment

The 6-well plate was seeded at a density of 500,000 cells per well. After cells adhering to form a monolayer. A yellow pipette tip was used to scratch from one side of the well to the other, ensuring the scratch is perpendicular to the straight lines drawn on the back of the plate. Fresh medium with inhibitors was added and cells were further incubated at 37°C with 5% CO_2 in a saturated humidity incubator. Images of the scratches were captured at 0 h and as indicated incubation time to document cell migration.

13. Animal experiment

Three-week-old BALB/c nude mice were purchased from Hangzhou Qizhen Laboratory Animal Technology Co., Ltd. (Zhejiang, China). After a one-week acclimation period, HepG2 cells (4×10^6 cells in 100 μL PBS) were subcutaneously injected into the right shoulder. When tumor volumes reached approximately 150 mm^3, the mice were randomly divided into three groups (n = 6 per group) and received intratumoral injections every day with one of the following treatments: (1) PBS, (2) DGIM@siND1, or (3) T2IM@siND1. Body weight and tumor volume were measured prior to each injection. Tumor volume (V) was calculated using the formula: $V = L \times W^2 \times 0.52$, where L is tumor length and W is tumor width, measured by digital caliper. When tumors became smaller than approximately 100 mm^3, mice were sacrificed, and tumors were excised and weighed. For tissue protein extraction, 0.1 g of tumor tissue from each group was washed with PBS and homogenized in RIPA buffer supplemented with 1 mM protease inhibitor (Beyotime). The homogenates were incubated on ice for 1 h, followed by centrifugation at 12,000 rpm for 10 min at 4°C to remove debris. Equal amounts of supernatant were subjected to Western blotting analysis. For histological analysis, portions of tumor tissues were fixed in formalin, embedded in paraffin, sectioned, and subjected to immunofluorescence staining and TUNEL assay (Wuhan Servicebio). All animal experimental procedures were approved by the Ethics Committee of Zhejiang University of Technology (Approval number: MGS20250514055) and conducted in accordance with the Guide for the Care and Use of Laboratory Animals in ZJUT and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1996).

Reference

[1] 1. Fang, L.; Xiao, L.; Jun, Y. W.; Onishi, Y.; Kool, E. T. Reversible 2'-OH acylation enhances RNA stability. *Nat. Chem.* 2023, **15**, 1296-1305.

[2] Guo, J.; Chen, S.; Onishi, Y.; Shi, Q.; Song, Y.; Mei, H.; Chen, L.; Kool, E. T.; Zhu, R. Y. RNA control via redox-responsive acylation. *Angew. Chem. Int. Ed.* 2024, **63**, e202402178.

14. Supporting figures

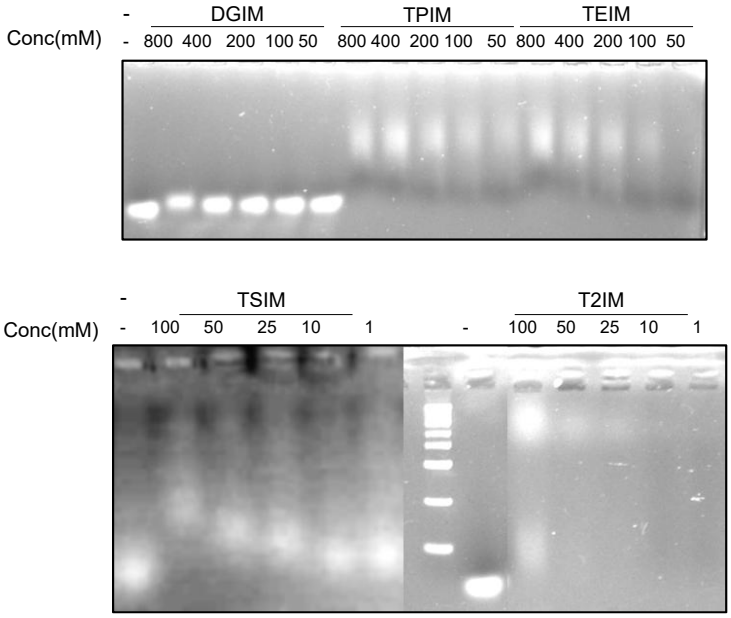


Figure S1 Electrophoretic analysis of one-step siRNA cloaking *via* acylation. 500 ng siNC was incubated with a concentration gradient of different acylation reagents (DGIM, TPIM, TEIM, TSIM, and T2IM) at 37 °C for 4 h, followed by agarose gel electrophoresis.

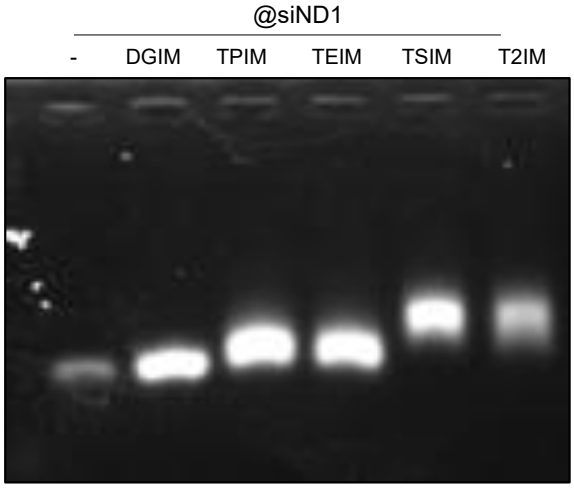


Figure S2 Electrophoretic analysis of siND1 cloaking via acylation using different reagents.

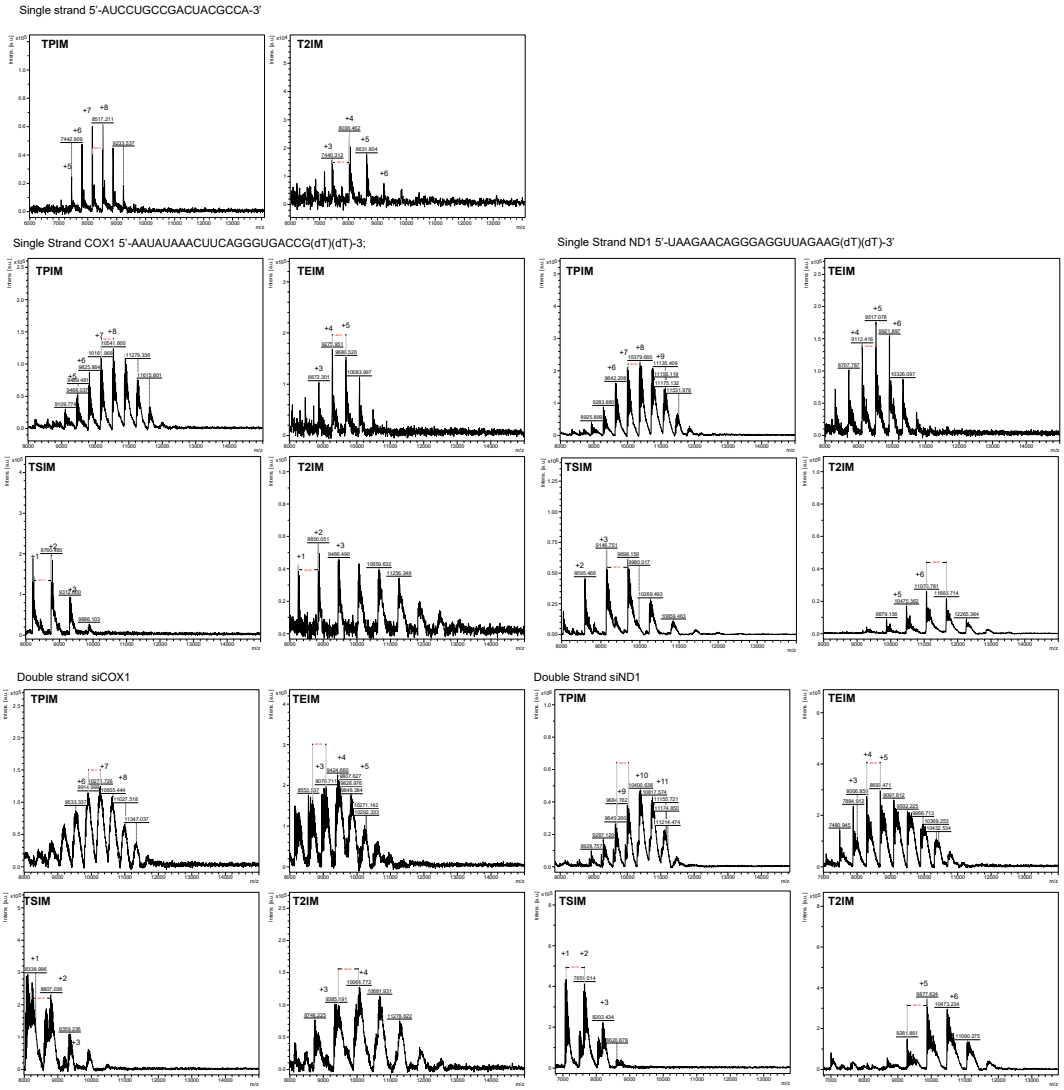


Figure S3 MALDI-TOF mass spectra of cloaked single/double strand RNA.

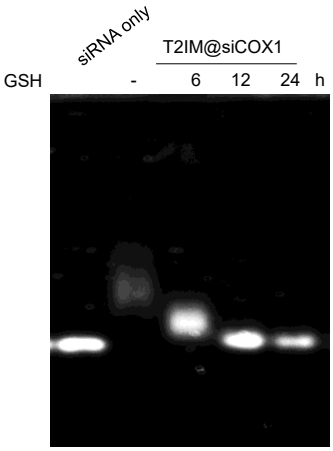


Figure S4 Electrophoretic analysis of acylated siCOX1 after GSH (50 mM) treatment.

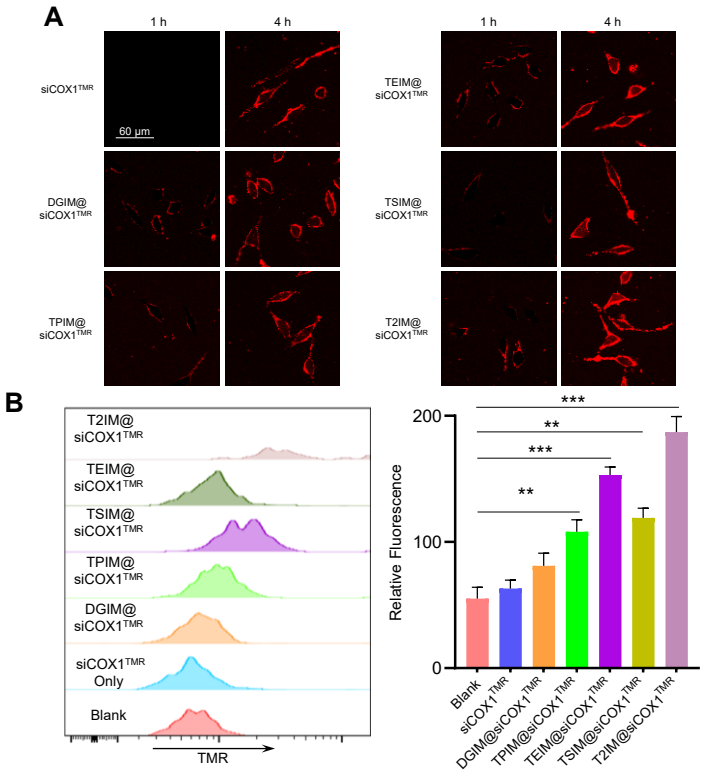


Figure S5 A) Fluorescence confocal microscopy imaging of acylated siCOX1^{TMR} uptake in HeLa cells. Cells were incubated with acylated siCOX1^{TMR} (final concentration: 100 nM) for 1 and 4 h, respectively. Imaging was performed using E_x/E_m wavelengths of 552/(570-600) nm. B) Flow cytometry analysis of acylated siCOX1^{TMR} uptake in HeLa cells. C) Quantification of uptake after 4 h incubation is shown on the right as a column plot of relative mean fluorescence intensity. Statistical significance was determined using an unpaired *t*-test (n = 3). **P ≤ 0.01; ***P ≤ 0.001.

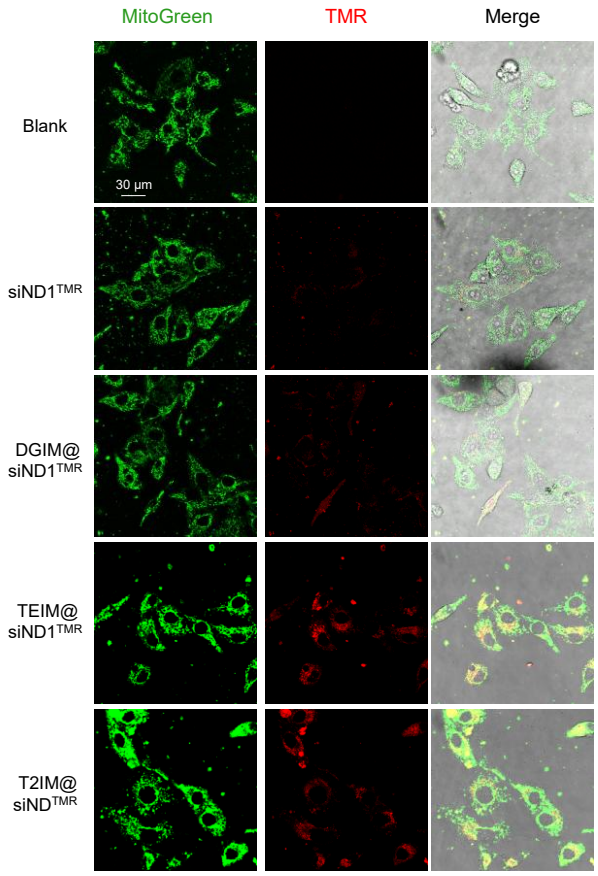


Figure S6 Fluorescence confocal microscopy imaging of siND1^{TMR} uptake in HepG2 cells. Cells were incubated with siND1^{TMR} (final concentration: 100 nM) for 48 h, followed by co-staining with Mito Greem Tracker prior to imaging.

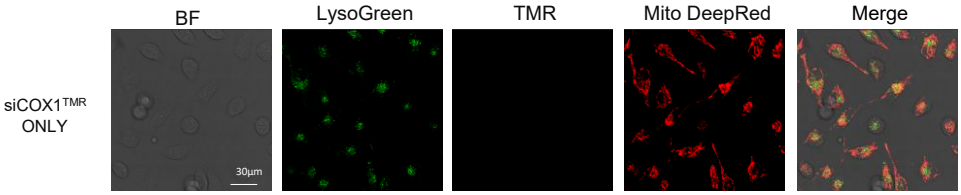


Figure S7 Fluorescence confocal microscopy imaging of siCOX1^{TMR} uptake in HeLa cells. Cells were incubated with siCOX1^{TMR} (final concentration: 100 nM) for 48 h, followed by co-staining with Lysosome Green and Mito DeepRed prior to imaging. This is a Supplementary Figure related to Figure 3 of the maintext.

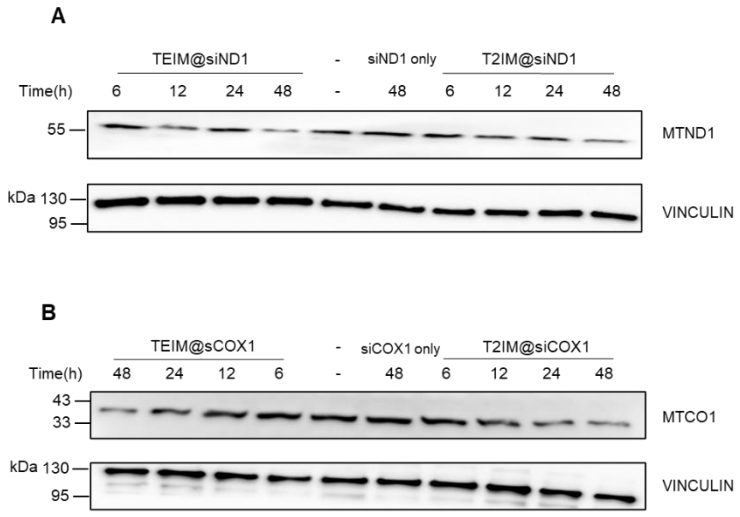


Figure S8 Time-dependent Western blot analysis of mitochondrial protein expression. Protein levels of (A) MTND1 and (B) MTCO1 were assessed in HeLa cells following treatment with TEIM@siND1, T2IM@siND1, TEIM@siCOX1, or T2IM@siCOX1. Vinculin was used as a loading control.

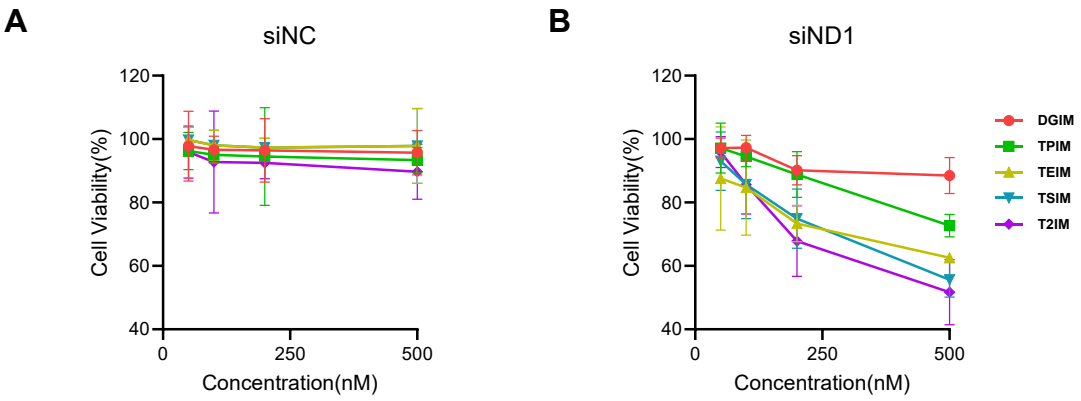


Figure S9 Cell viability of HeLa cells treated with various concentrations of acylated siNC (A) and acylated siND1 (B).

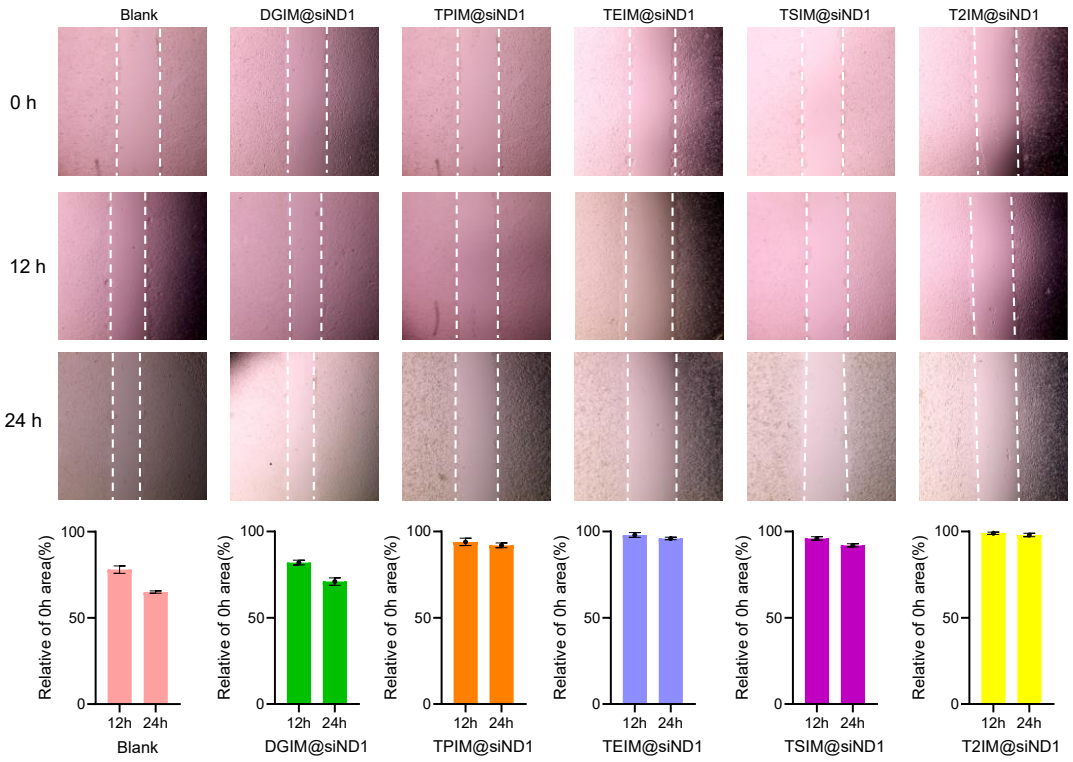


Figure S10 Scratch/wound-healing assay of cell migration in HeLa cells treated with different acylation reagents. Cell imaging above was taken at 12 h and 24 h post-treatment. The graph below shows the relative wound area over time, normalized to the initial wound area (0 h).

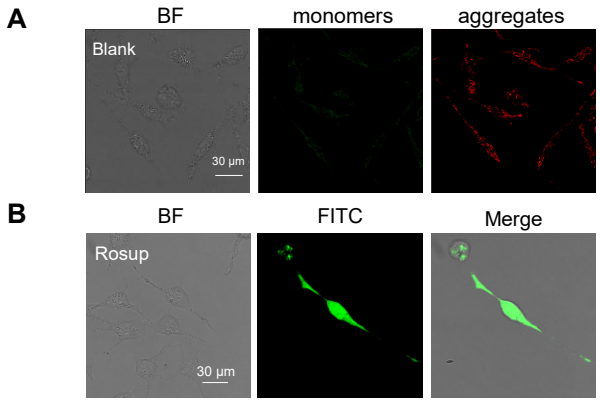


Figure S11 This is a Supplementary Figure related to Figure 5B and 5C of the maintext. Generally, cells were treated nothing or Rosup before staining with JC-1 (A) and DCFH-DA probe (B), respectively.

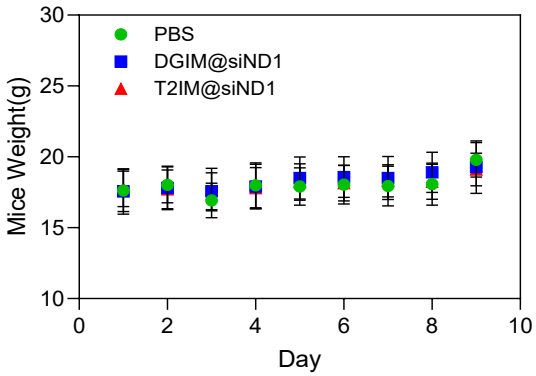


Figure S12 Body weight monitoring of mice during treatment with acylating reagents (n = 6).

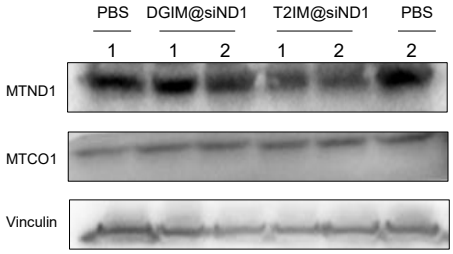


Figure S13 Western blot analysis of MTND1, MTCO1, and Vinculin expression in tumor samples. Representative blots from three independent samples (n=3) are shown. Results from one sample are presented in the maintext (Figure 6F).

UNCROPPED WB IMAGE

Figure 5A

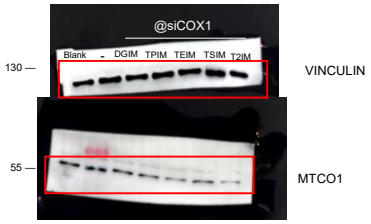


Figure 5B

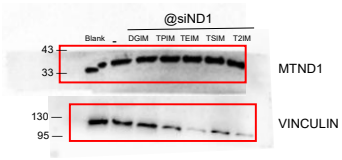


Figure 5C/S7B

Figure 5D/S7A

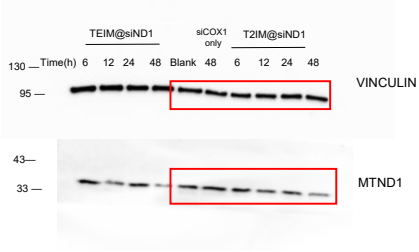


Figure 5E

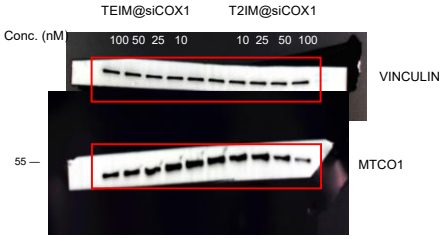


Figure 5F

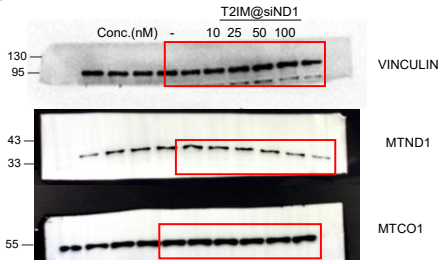


Figure 4G

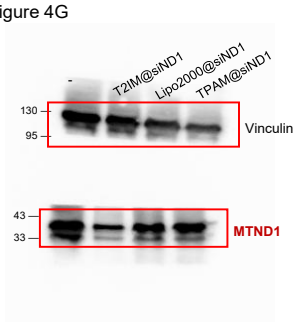


Figure 4H

Figure 7F

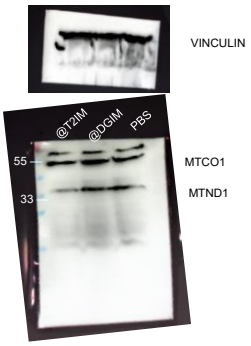
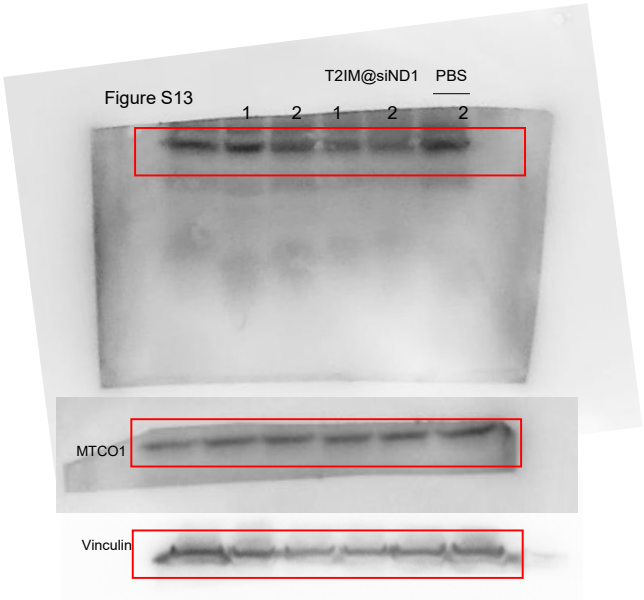
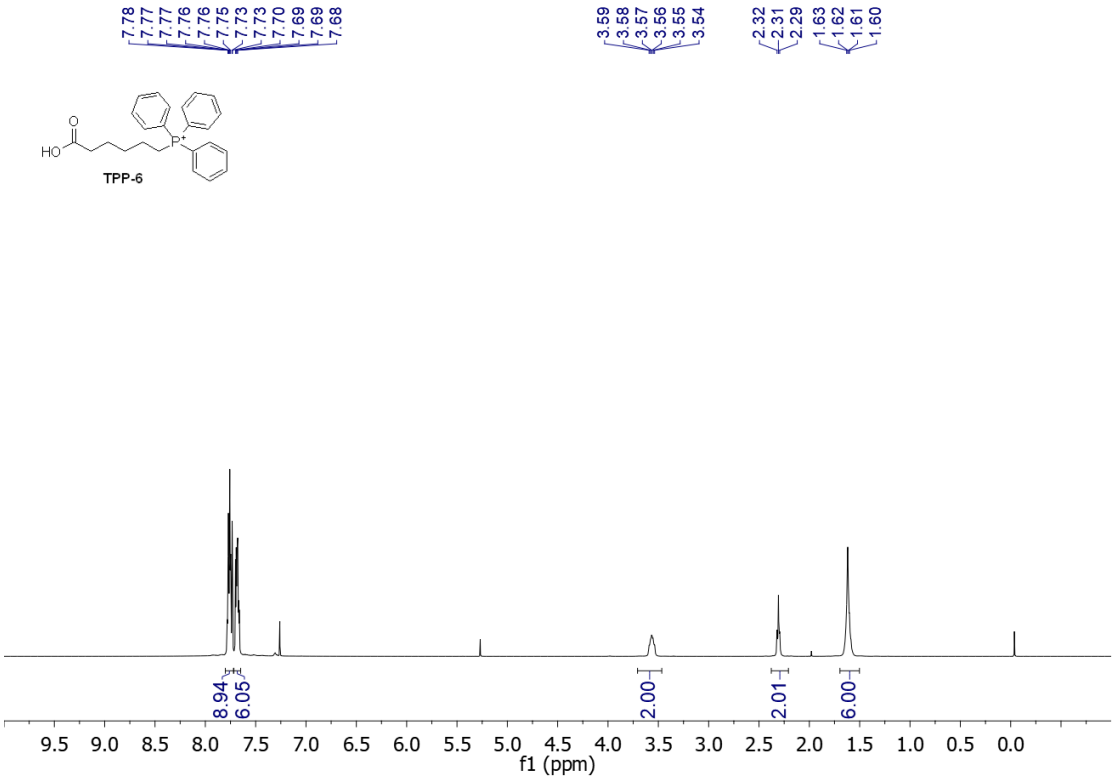
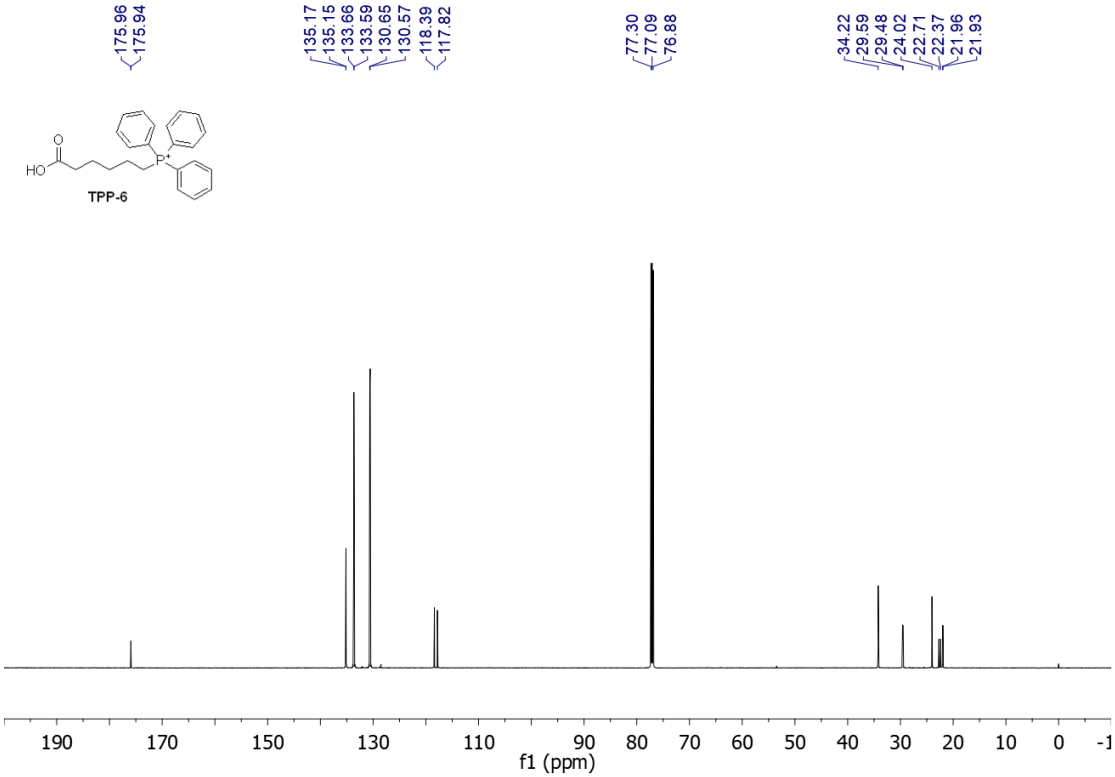


Figure S13

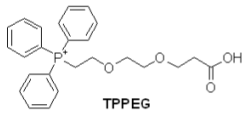


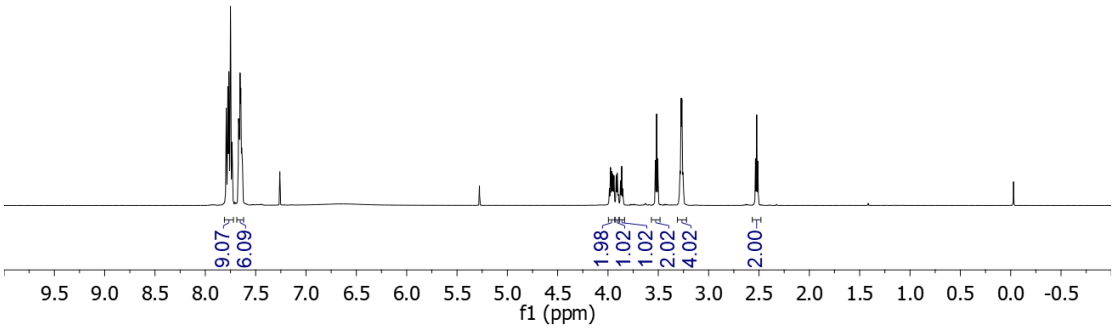
NMR of compounds



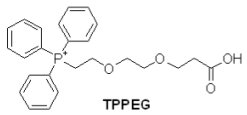


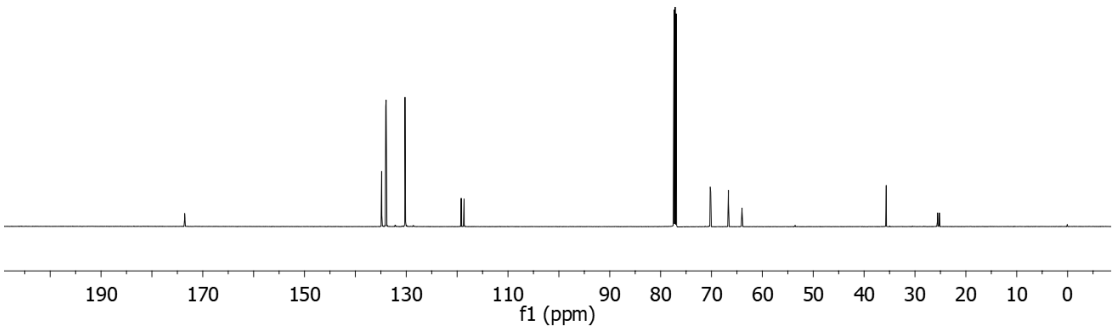

7.79
7.78
7.77
7.75
7.73
7.67
7.66
7.65
7.65
7.64
7.63
3.99
3.97
3.96
3.95
3.94
3.92
3.91
3.90
3.89
3.88
3.86
3.85
3.53
3.52
3.51
3.29
3.29
3.27
3.27
2.54
2.52
2.51

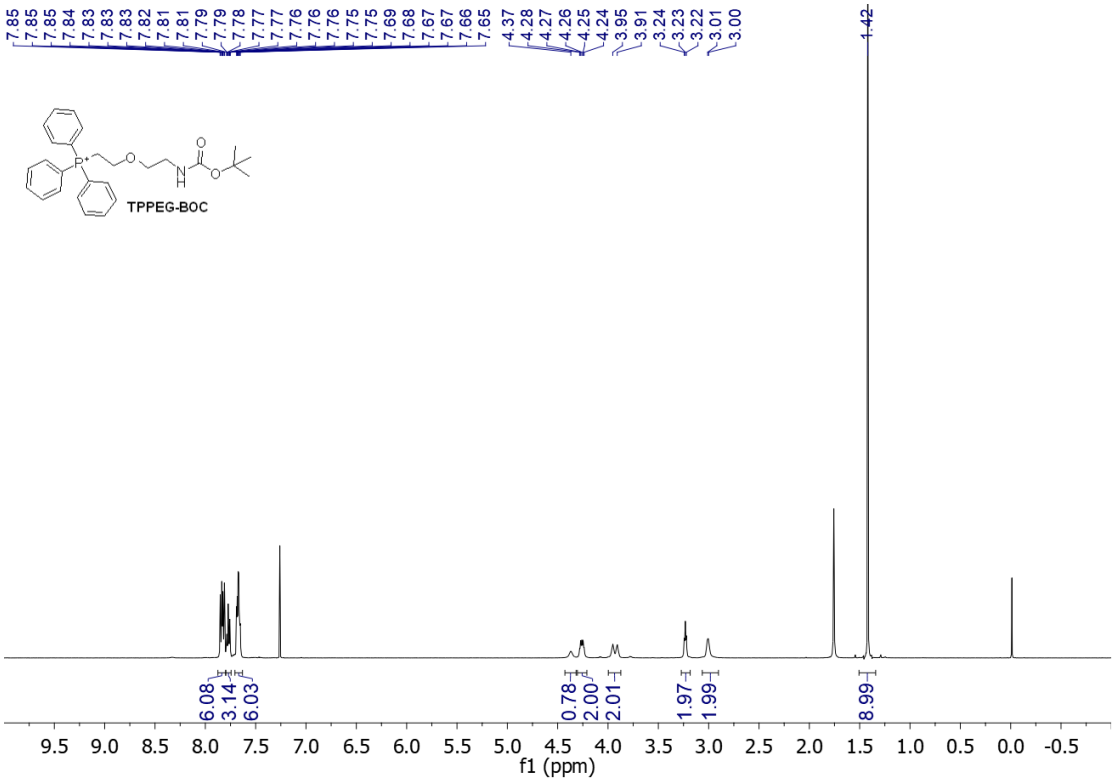

OH
O
TPPEG

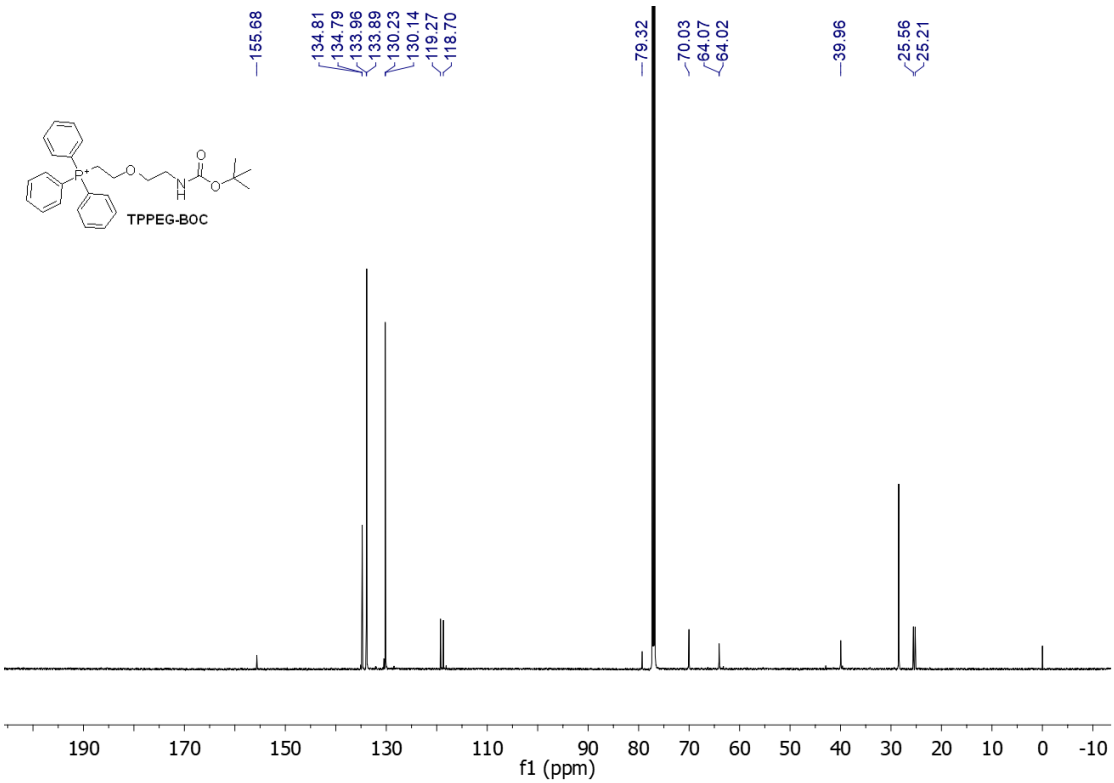

9.07
6.09
1.98
1.02
1.02
2.02
4.02
2.00
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)

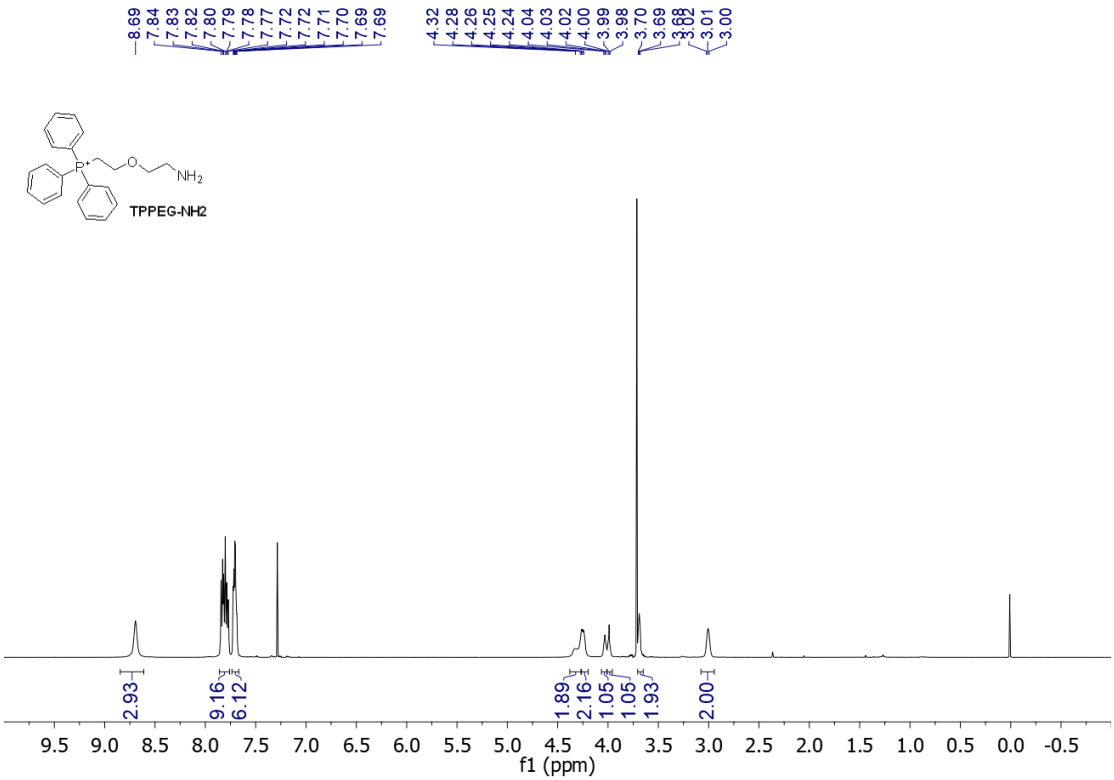

173.58
173.57
134.86
134.84
134.05
133.98
133.97
130.27
130.19
119.22
118.65
70.26
70.16
66.68
64.05
64.01
35.66
25.57
25.22

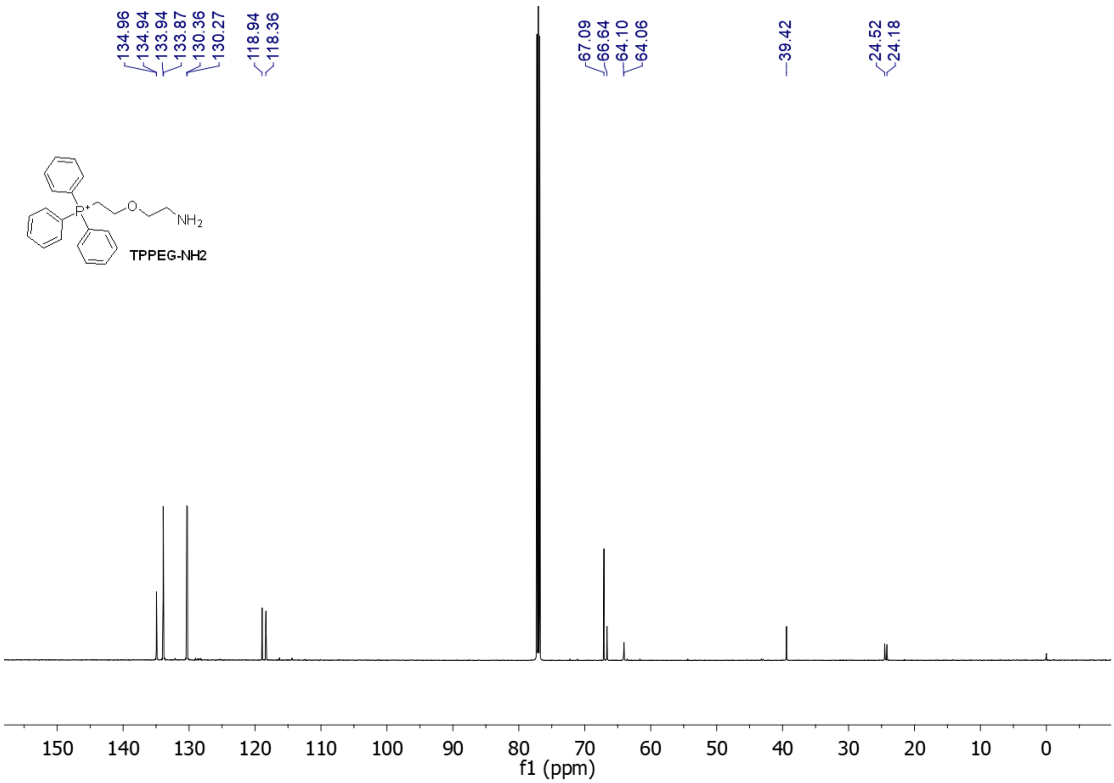

OH
O
TPPEG

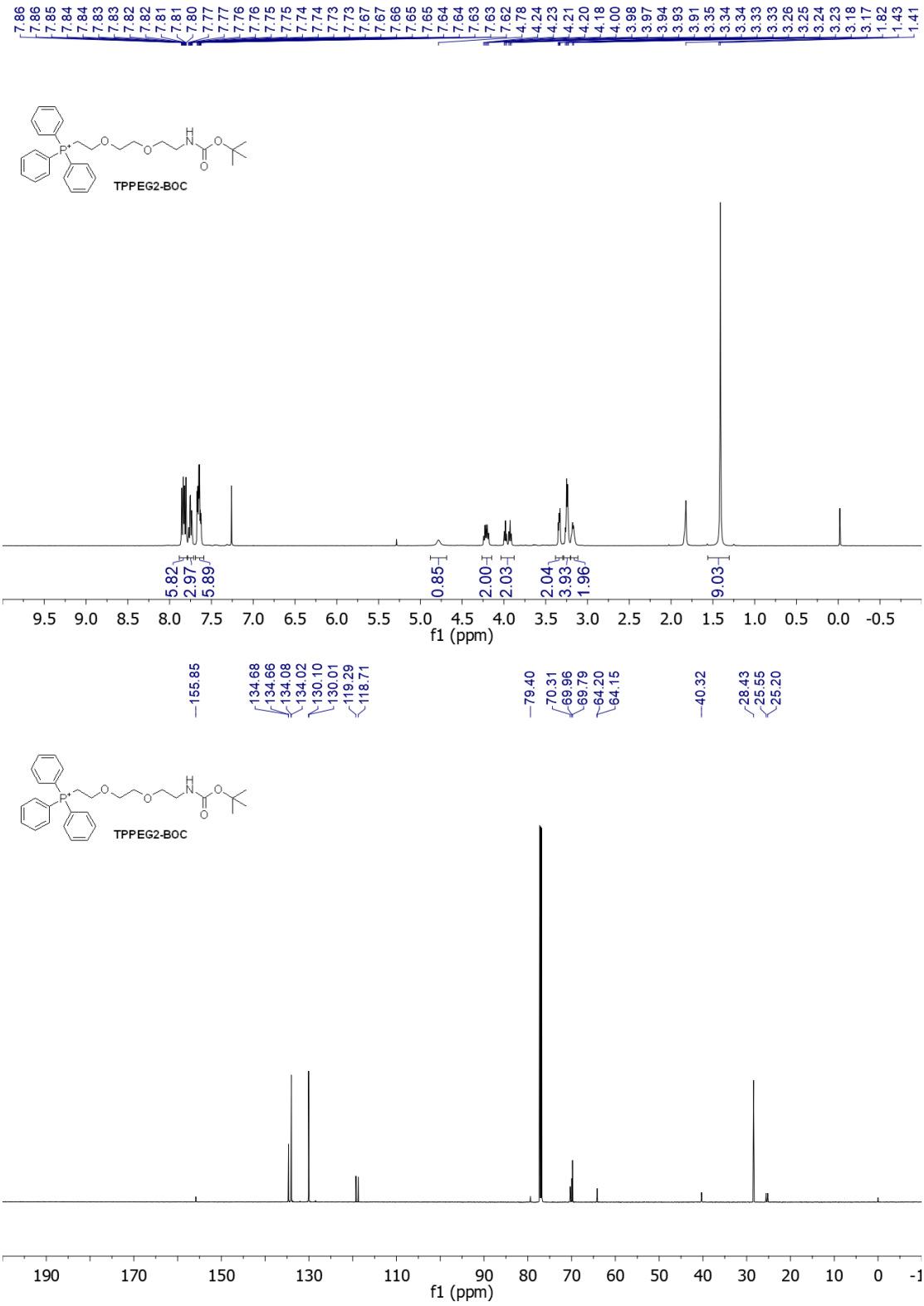

190 170 150 130 110 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

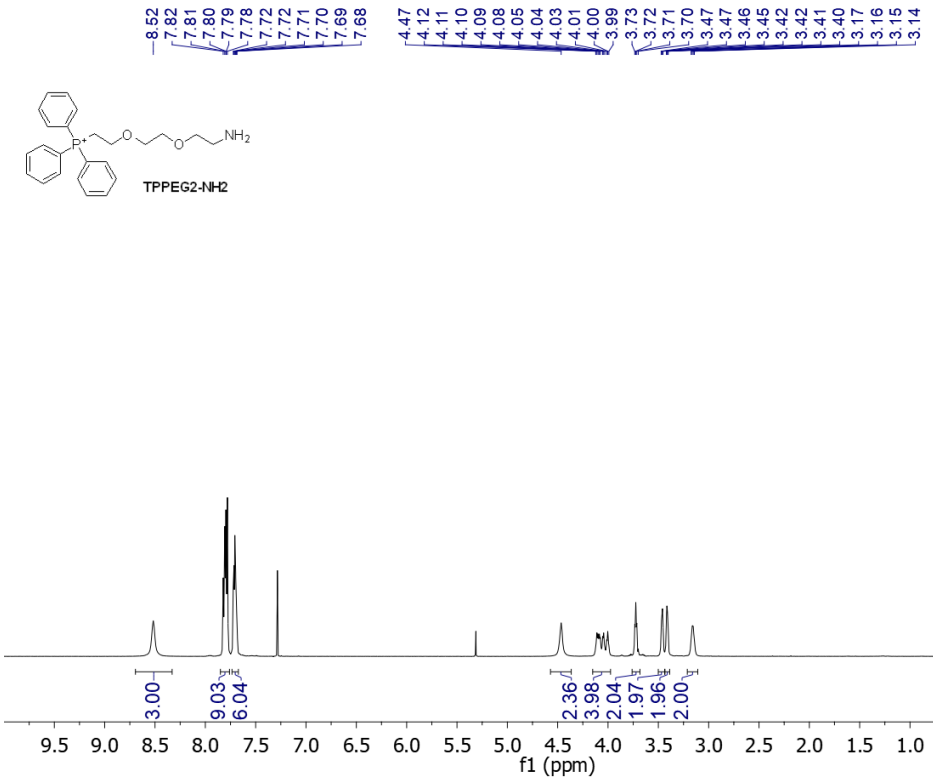

7.85
7.85
7.85
7.84
7.83
7.83
7.83
7.82
7.81
7.81
7.79
7.79
7.78
7.77
7.77
7.76
7.76
7.76
7.75
7.75
7.69
7.68
7.67
7.67
7.66
7.65
4.37
4.28
4.27
4.26
4.25
4.24
3.95
3.91
3.24
3.23
3.22
3.01
3.00
1.42
TPPEG-BOC
6.08
3.14
6.03
0.78
2.00
2.01
1.97
1.99
8.99
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)

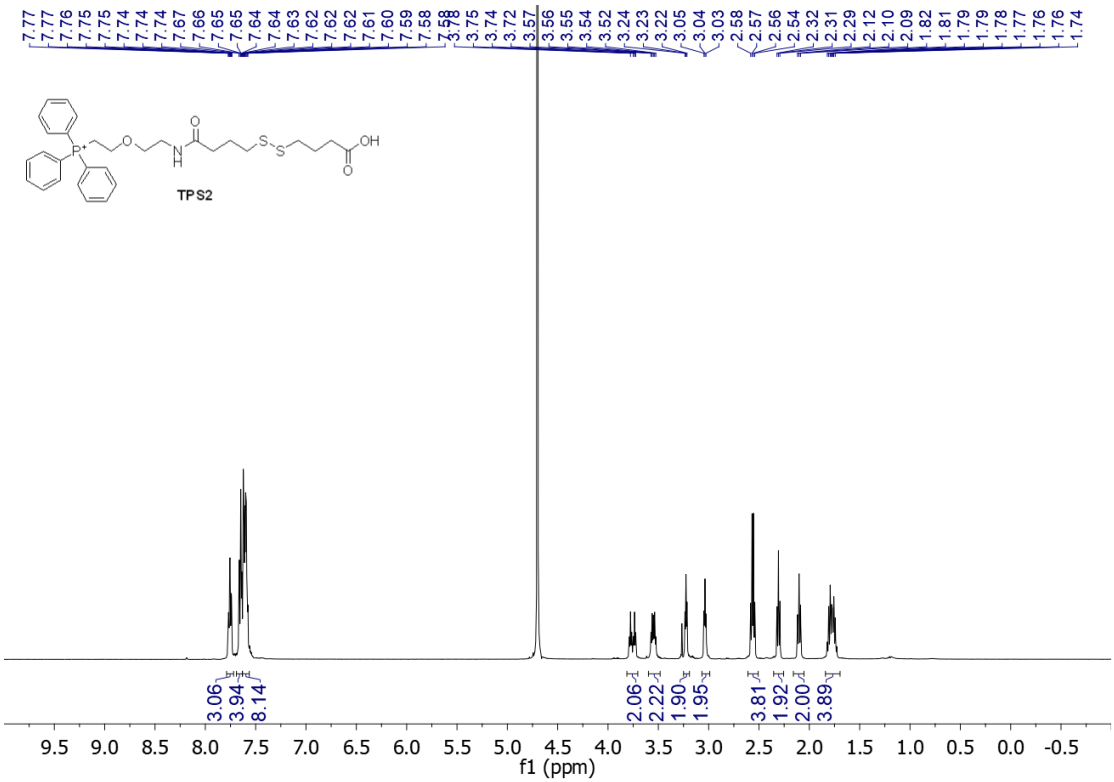

155.68
134.81
134.79
133.96
133.89
130.23
130.14
119.27
118.70
79.32
70.03
64.07
64.02
39.96
25.56
25.21
TPPEG-BOC
190 170 150 130 110 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

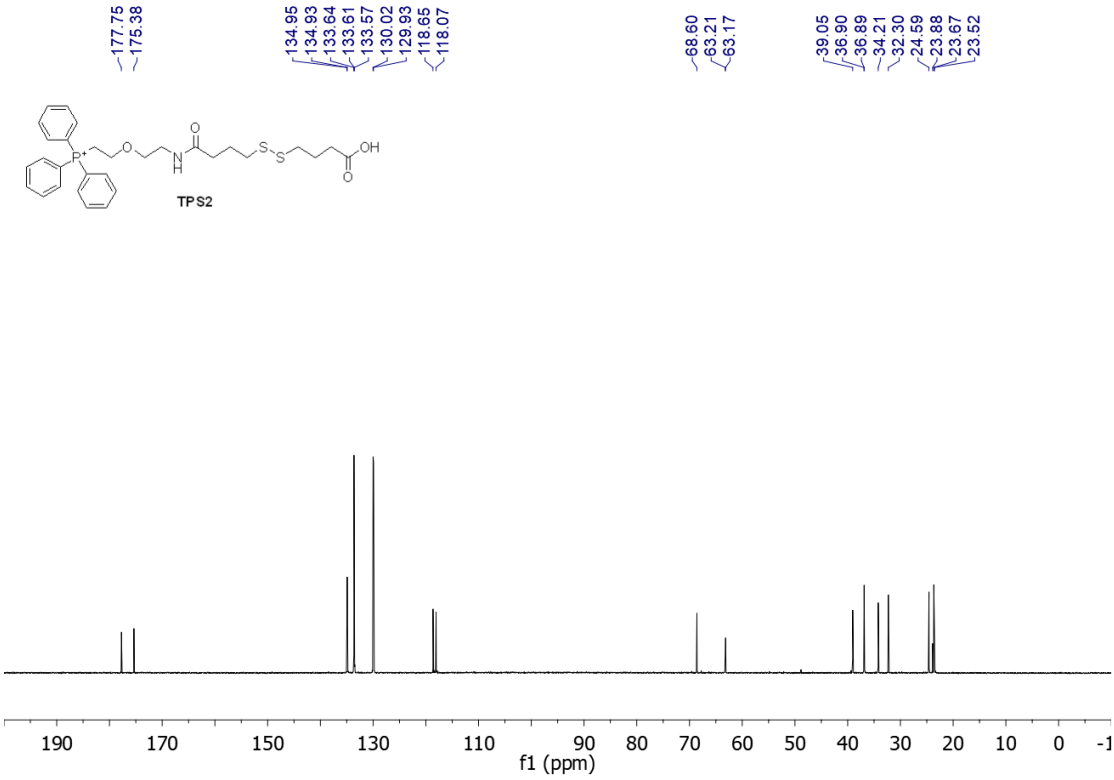

8.69
7.84
7.83
7.82
7.80
7.79
7.78
7.77
7.72
7.72
7.71
7.70
7.69
7.69
4.32
4.28
4.26
4.25
4.24
4.04
4.03
4.02
4.00
3.99
3.98
3.70
3.69
3.68
3.02
3.01
3.00
TPPEG-NH2
2.93
9.16
6.12
1.89
2.16
1.05
1.05
1.93
2.00
f1 (ppm)

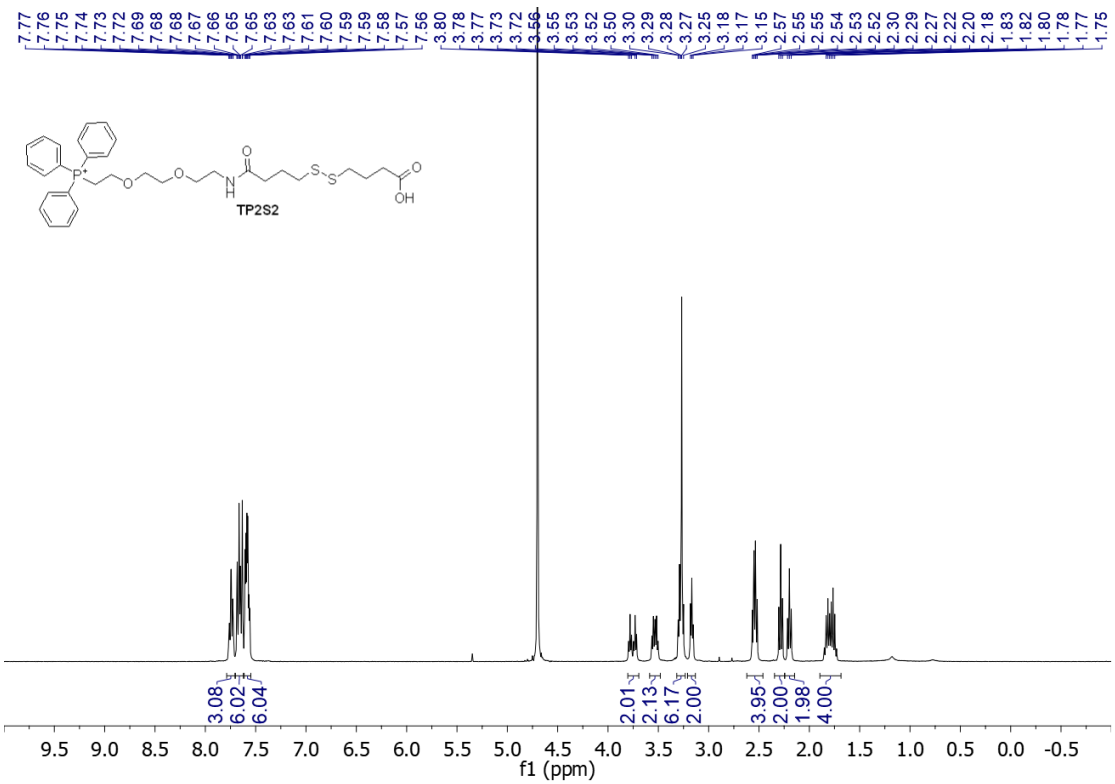

134.96
134.94
133.94
133.87
130.36
130.27
118.94
118.36
67.09
66.64
64.10
64.06
39.42
24.52
24.18
TPPEG-NH2
f1 (ppm)

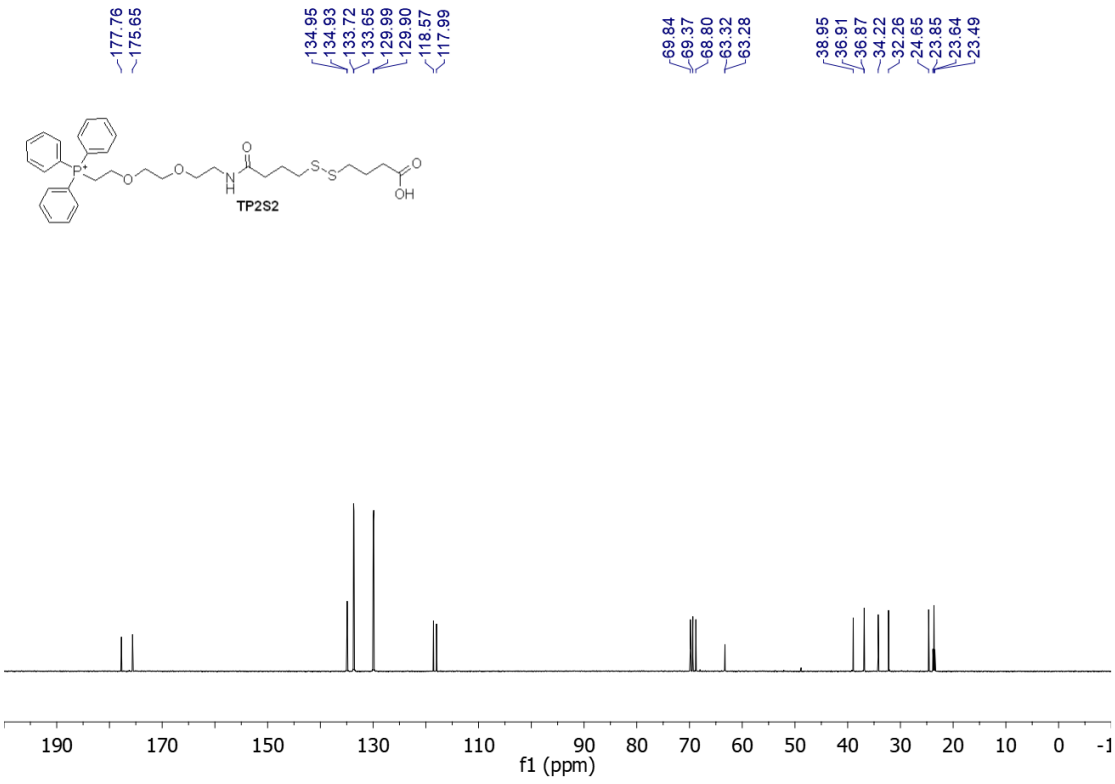

TPPEG2-BOC
5.82
2.97
5.89
0.85
2.00
2.03
2.04
3.93
1.96
9.03
f1 (ppm)
TPPEG2-BOC
f1 (ppm)

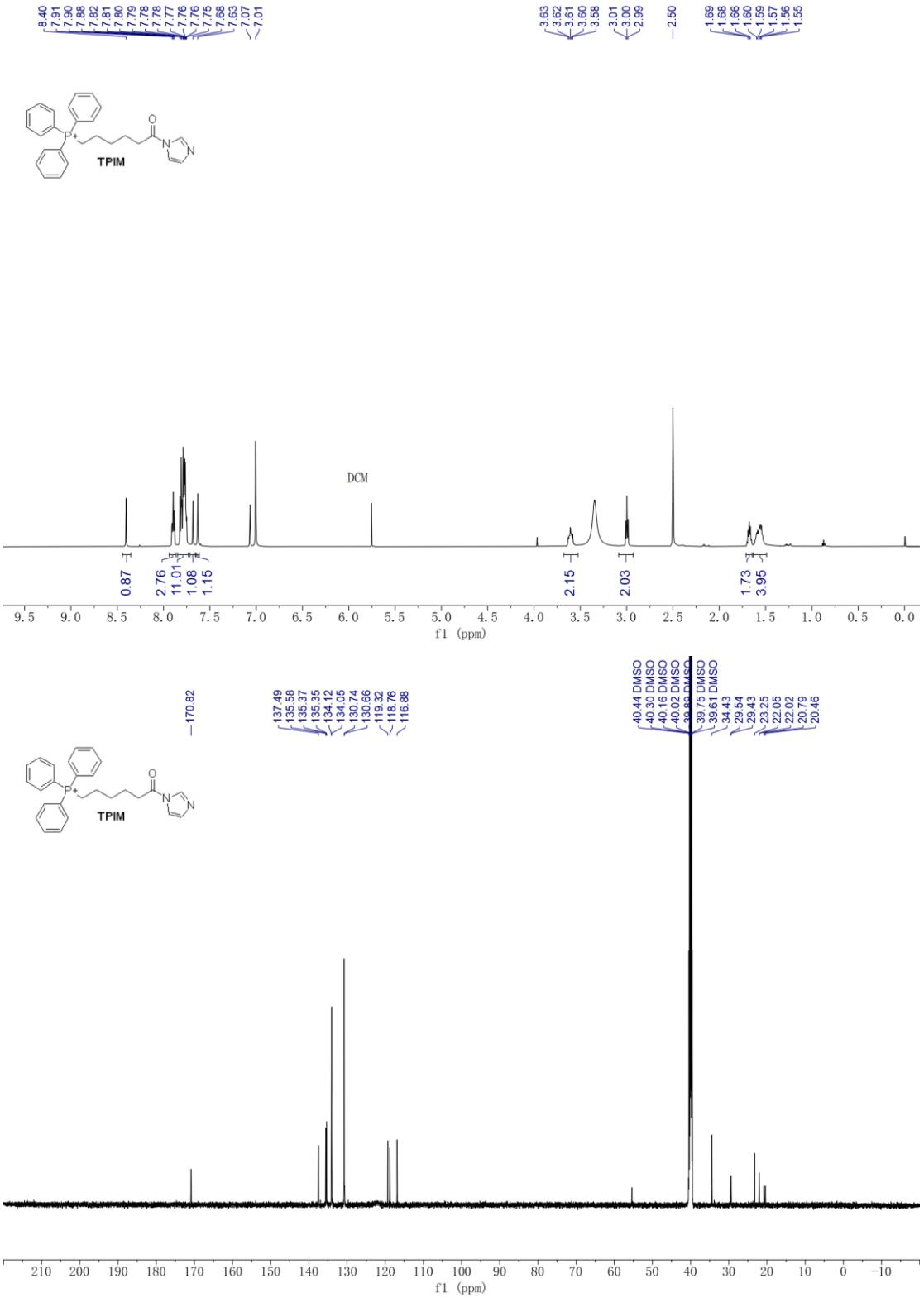

8.52
7.82
7.81
7.80
7.79
7.78
7.72
7.72
7.71
7.70
7.69
7.68
4.47
4.12
4.11
4.10
4.09
4.08
4.05
4.04
4.03
4.01
4.00
3.99
3.73
3.72
3.71
3.70
3.47
3.47
3.46
3.45
3.42
3.42
3.41
3.40
3.17
3.16
3.15
3.14
NH2
TPPEG2-NH2
3.00
9.03
6.04
2.36
3.98
2.04
1.97
1.96
2.00
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)
134.88
134.86
133.93
133.86
130.29
130.21
119.10
118.52
77.25
77.04
76.83
70.34
70.22
66.58
64.09
64.05
39.62
24.98
24.63
NH2
TPPEG2-NH2
150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

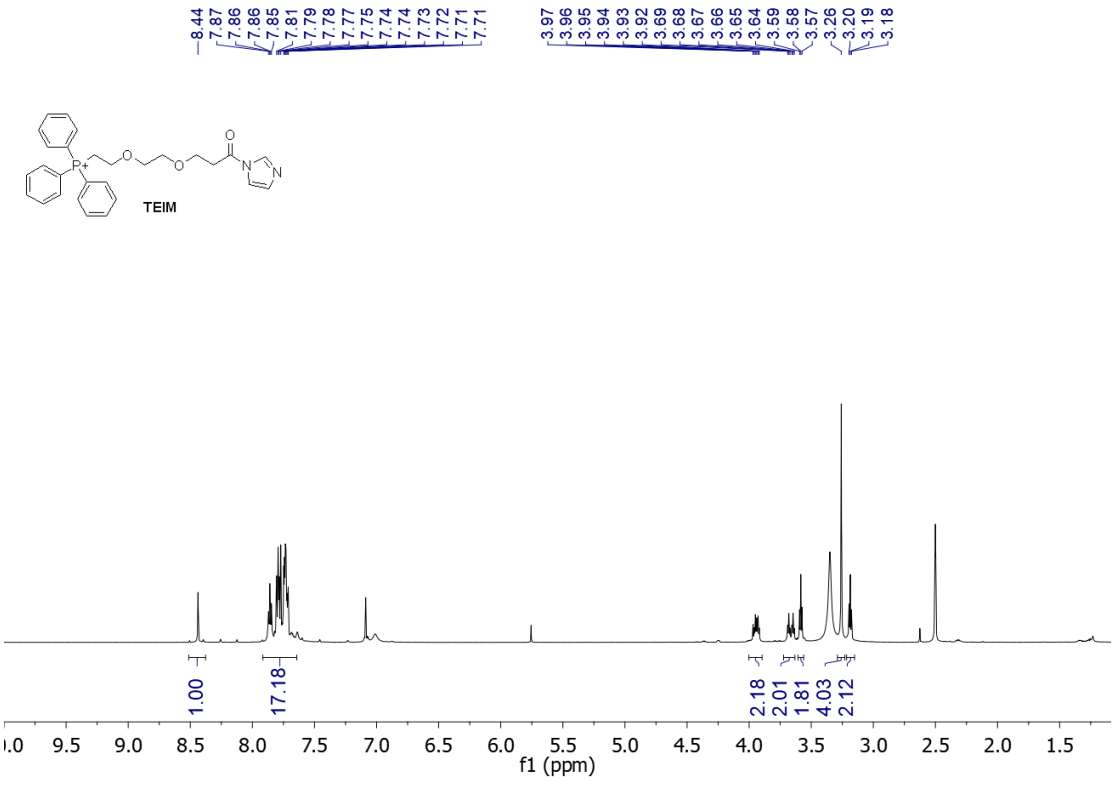

7.77 7.77 7.76 7.75 7.75 7.74 7.74 7.74 7.67 7.66 7.65 7.65 7.64 7.64 7.63 7.62 7.62 7.62 7.61 7.60 7.59 7.58 7.58 3.75 3.74 3.72 3.57 3.56 3.55 3.54 3.52 3.24 3.23 3.22 3.05 3.04 3.03 2.58 2.57 2.56 2.54 2.32 2.31 2.29 2.12 2.10 2.09 1.82 1.81 1.79 1.79 1.78 1.77 1.76 1.76 1.74
TPS2
3.06 3.94 8.14 2.06 2.22 1.90 1.95 3.81 1.92 2.00 3.89
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)

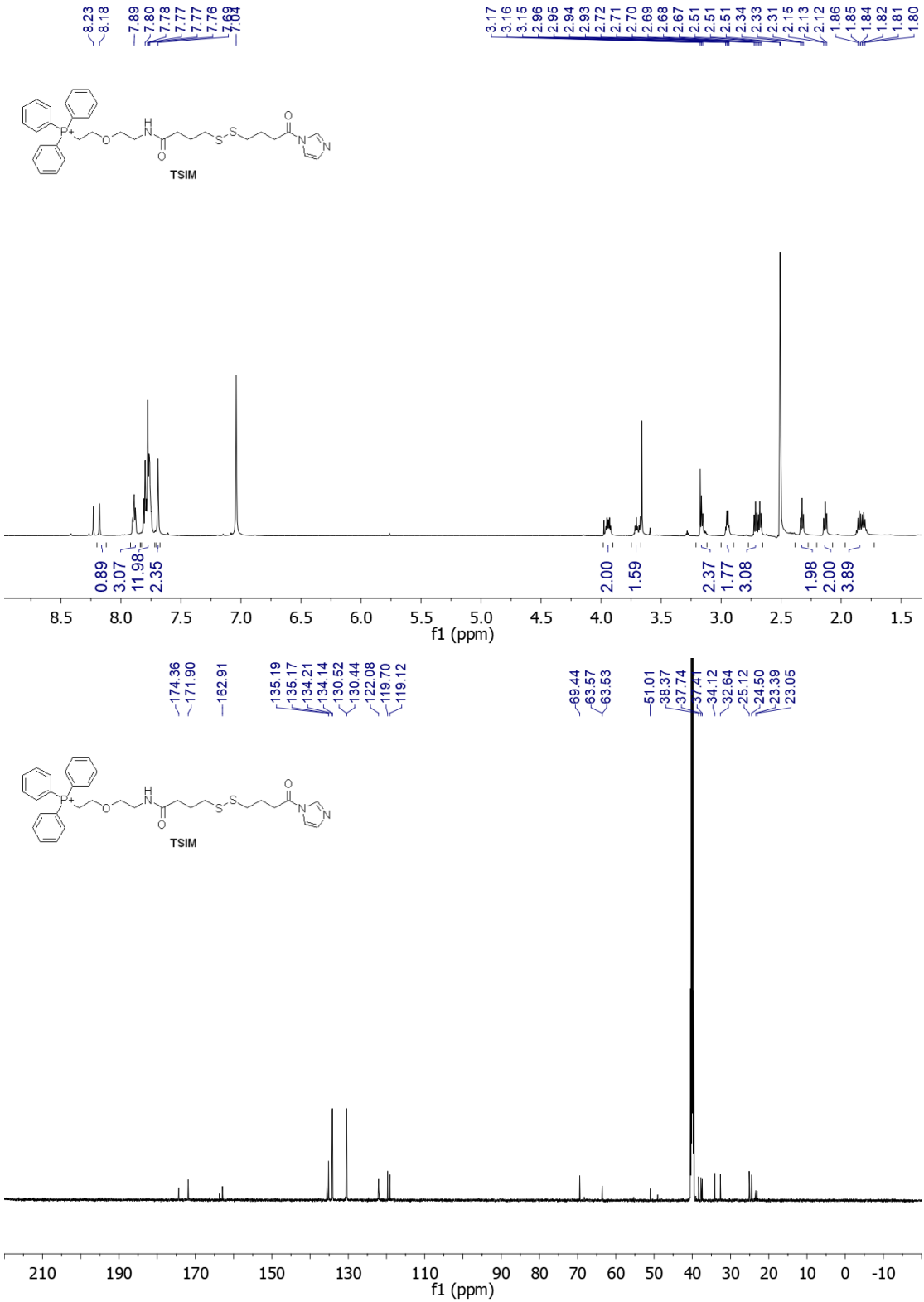

177.75 175.38 134.95 134.93 133.64 133.61 133.57 130.02 129.93 118.65 118.07 68.60 63.21 63.17 39.05 36.90 36.89 34.21 32.30 24.59 23.88 23.67 23.52
TPS2
190 170 150 130 110 90 80 70 60 50 40 30 20 10 0 -1
f1 (ppm)


7.77 7.76 7.75 7.74 7.73 7.72 7.69 7.68 7.68 7.67 7.66 7.65 7.65 7.63 7.63 7.61 7.60 7.59 7.59 7.58 7.57 7.56
3.80 3.78 3.77 3.73 3.72 3.56 3.55 3.53 3.52 3.50 3.30 3.29 3.28 3.27 3.25 3.18 3.17 3.15 2.57 2.55 2.55 2.54 2.53 2.52 2.30 2.29 2.27 2.22 2.20 2.18 1.83 1.82 1.80 1.78 1.77 1.75
TP2S2
3.08 6.02 6.04 2.01 2.13 6.17 2.00 3.95 2.00 1.98 4.00
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)

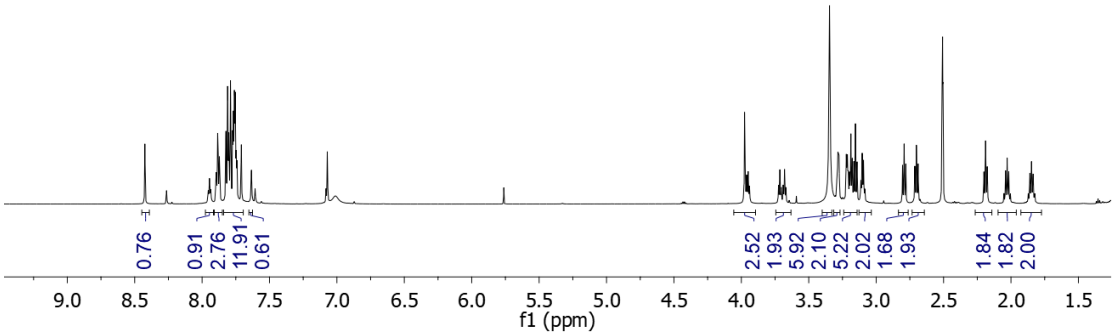

177.76 175.65
134.95 134.93 133.72 133.65 129.99 129.90 118.57 117.99
69.84 69.37 68.80 63.32 63.28
38.95 36.91 36.87 34.22 32.26 24.65 23.85 23.64 23.49
TP2S2
190 170 150 130 110 90 80 70 60 50 40 30 20 10 0 -1
f1 (ppm)

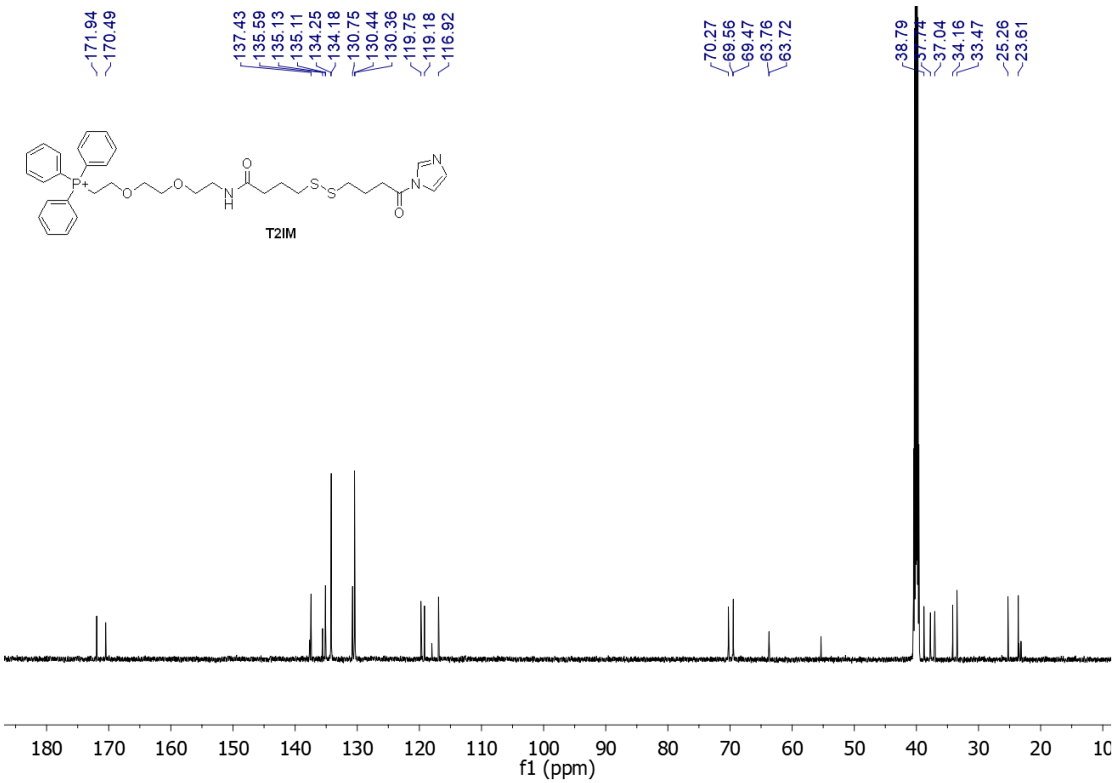

8.40
7.91
7.90
7.88
7.82
7.81
7.80
7.79
7.78
7.78
7.77
7.76
7.76
7.75
7.68
7.63
7.07
7.01
3.63
3.62
3.61
3.60
3.58
3.01
3.00
2.99
2.50
1.69
1.68
1.66
1.60
1.59
1.57
1.56
1.55
TPIM
DCM
0.87
2.76
11.01
1.08
1.15
2.15
2.03
1.73
3.95
f1 (ppm)
170.82
137.49
135.58
135.37
135.35
134.12
134.05
130.74
130.66
119.32
118.76
116.88
40.44 DMSO
40.30 DMSO
40.16 DMSO
40.02 DMSO
39.88 DMSO
39.75 DMSO
39.61 DMSO
34.43
29.54
29.43
23.25
22.05
22.02
20.79
20.46
TPIM
f1 (ppm)


8.44
7.87
7.86
7.86
7.85
7.81
7.79
7.78
7.77
7.75
7.74
7.74
7.73
7.72
7.71
7.71
3.97
3.96
3.95
3.94
3.93
3.92
3.69
3.68
3.67
3.66
3.65
3.64
3.59
3.58
3.57
3.26
3.20
3.19
3.18
TEIM
1.00
17.18
2.18
2.01
1.81
4.03
2.12
f1 (ppm)
169.14
137.64
135.11
135.09
134.24
134.17
130.87
130.42
130.34
119.75
119.18
116.93
70.20
69.80
65.65
63.75
63.71
40.44
40.30
40.17
39.89
39.75
23.47
23.13
TEIM
f1 (ppm)


8.23
8.18
7.89
7.80
7.78
7.77
7.77
7.76
7.69
7.64
3.17
3.16
3.15
2.96
2.95
2.94
2.93
2.72
2.71
2.70
2.69
2.68
2.67
2.51
2.51
2.51
2.34
2.33
2.31
2.15
2.13
2.12
1.86
1.85
1.84
1.82
1.81
1.80
TSIM
0.89
3.07
11.98
2.35
2.00
1.59
2.37
1.77
3.08
1.98
2.00
3.89
f1 (ppm)
174.36
171.90
162.91
135.19
135.17
134.21
134.14
130.52
130.44
122.08
119.70
119.12
69.44
63.57
63.53
51.01
38.37
37.74
37.41
34.12
32.64
25.12
24.50
23.39
23.05
TSIM
f1 (ppm)


T2IM


0.76
0.91
2.76
11.91
0.61
2.52
1.93
5.92
2.10
5.22
2.02
1.68
1.93
1.84
1.82
2.00
f1 (ppm)


T2IM
f1 (ppm)