Electronic Supplementary Information

Effect of molecular structure on anticancer drug release rate from prodrug nanoparticles

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EXPERIMENTAL SECTION

General methods

All reactions were carried out in flame-dried glassware, under a nitrogen atmosphere, with dry solvents. Reactions were monitored by analytical thin layer chromatography (TLC) carried out on 0.25 mm silica gel plates. Visualization of the developed plate was performed using UV absorbance and aqueous cerium ammonium molybdate. Flash chromatography was performed on silica gel 60N (230–400 mesh) with the indicated solvent systems. IR spectra were recorded on a Varian FTS-7000 system equipped with an ATR unit. NMR spectra were recorded on a Bruker AVANCE-400 spectrometer and calibrated using residual undeuterated solvent as an internal reference ($CDCl_3$ at δ 7.26 ppm for ^1H, and δ 77.2 ppm for ^{13}C NMR). HRMS was performed using a micrOTOF-Q II-S1 using electrospray ionization (ESI) techniques. Zeta potential were measured using a Malvern Zetasizer nanoZS. SEM images were observed using JEOL JSM-6700F. Powder XRD patterns were measured by using Bruker AXS D8 Advance. Cell viability was evaluated using a microplate reader (Bio-Rad iMark microplate absorbance reader). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed using an HPLC (Agilent 1260 Infinity) connected to a mass spectrometer (Bruker HCT ultra-IMR).

Materials

Podophyllotoxin (PPT), dichloromethane (CH_2Cl_2), 4-dimethylaminopyridine (DMAP), ammonium chloride (NH_4Cl), chloroform ($CHCl_3$), deuterochloroform ($CDCl_3$), tetrahydrofuran (THF), acetonitrile, formic acid, phosphate-buffered saline (PBS), methanol (MeOH), and dithiothreitol (DTT) were purchased from Wako Pure Chemical Industry. Sebacic acid, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), and octadecanedioic acid were purchased from Tokyo Chemical Industry. Anhydrous magnesium sulfate ($MgSO_4$) was purchased from Kanto Chemical. 4,4'-dithiodibutyric acid and esterase from porcine liver were purchased from Sigma-Aldrich. Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies. Cell Counting Kit-8 was purchased from Dojindo. 5× Passive Lysis Buffer was purchased from Biotium. These reagents were used without further purification.

Silica gel plates (60F-254) were purchased from E. Merck. Silica gel 60N (230–400 mesh) was purchased from Kanto Chemical.

KPL-4 human breast cancer cells were provided by Prof. Noriaki Ohuchi, Prof. Kohsuke Gonda, Dr. Hiroshi Tada, and Dr. Liman Cong of the Graduate School of Medicine, Tohoku University.

Synthesis of PPT dimer C10

PPT (1.04 g, 2.51 mmol) was dissolved in CH_2Cl_2 (25 mL), and then sebacic acid (269 mg, 1.33 mmol), EDC (1.17 g, 6.10 mmol) and DMAP (35.6 mg, 0.294 mmol) were added sequentially at room temperature. After stirring for 1 h at room temperature, additional EDC (480 mg, 2.50 mmol)

and DMAP (22.5 mg, 0.186 mmol) were added, and stirring was continued for a further 4 h. The reaction mixture was quenched with a saturated aqueous solution of NH_4Cl and washed with water and brine. The mixture was then dried over $MgSO_4$, filtered, and the filtrate was concentrated under reduced pressure. The residue was purified using silica gel column chromatography with $CHCl_3$ to give PPT dimer C10 (1.03 g, 1.03 mmol, 82%) as a white solid. IR: ν_{max} 1779, 1730, 1588, 1504, 1484, 1461, 1419, 1331, 1239, 1171, 1126, 1037, 1000, 930, 864, 796, 763, 746 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.20–1.40 (8H, m), 1.65–1.69 (4H, m), 2.37–2.46 (4H, m), 2.78–2.94 (4H, m), 3.75 (12H, s), 3.80 (6H, s), 4.19 (2H, t, J = 9.6 Hz), 4.35 (2H, dd, ^3J = 9.2 Hz, ^4J = 7.2 Hz), 4.59 (2H, d, J = 4.4 Hz), 5.87 (2H, d, J = 9.2 Hz), 5.97 (4H, d, J = 5.2 Hz), 6.38 (4H, s), 6.53 (2H, s), 6.74 (2H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.1, 29.2, 34.5, 38.9, 43.9, 45.7, 56.3, 60.9, 71.5, 73.6, 101.7, 107.1, 108.2, 109.9, 128.5, 132.5, 135.0, 137.2, 147.7, 148.3, 152.8, 173.8, 174.3; HRMS (ESI) m/z calcd. for $C_{54}H_{58}O_{18}Na$ $[M + Na]^+$ 1017.3515, found 1017.3516.

Synthesis of PPT dimer C18

PPT (1.00 g, 2.41 mmol) was dissolved in CH_2Cl_2 (24 mL), and then octadecanedioic acid (417 mg, 1.33 mmol), EDC (933 mg, 4.87 mmol) and DMAP (30.1 mg, 0.248 mmol) were added sequentially at room temperature. After stir-ring for 1 h at room temperature, additional EDC (486 mg, 2.54 mmol) and DMAP (30.8 mg, 0.254 mmol) were added and stirring was continued for a further 5 h. The reaction mixture was quenched with a saturated aqueous solution of NH_4Cl and washed with water and brine. The mixture was then dried over $MgSO_4$, filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography with $CHCl_3$ to give PPT dimer C18 (1.17 g, 1.06 mmol, 88%) as a white solid. IR: ν_{max} 1779, 1732, 1588, 1506, 1484, 1464, 1419, 1330, 1239, 1171, 1126, 1037, 988, 930, 863, 797, 766 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.24–1.40 (24H, m), 1.65–1.69 (4H, m), 2.39–2.44 (4H, m), 2.89–2.94 (4H, m), 3.75 (12H, s), 3.80 (6H, s), 4.19 (2H, t, J = 9.6 Hz), 4.35 (2H, dd, ^3J = 9.2 Hz, ^4J = 6.8 Hz), 4.59 (2H, d, J = 4.4 Hz), 5.88 (2H, d, J = 9.2 Hz), 5.97 (4H, d, J = 5.2 Hz), 6.39 (4H, s), 6.53 (2H, s), 6.74 (2H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.1, 29.3, 29.25, 29.34, 29.5, 29.69, 29.72, 29.8, 34.5, 38.8, 43.8, 45.6, 56.2, 60.8, 71.5, 73.4, 101.7, 107.1, 108.1, 109.8, 128.5, 132.4, 134.9, 137.1, 147.7, 148.2, 152.7, 173.8, 174.4; HRMS (ESI) m/z calcd. for $C_{62}H_{74}O_{18}Na$ $[M + Na]^+$ 1129.4767, found 1129.4791.

Synthesis of PPT dimer SS

PPT (1.00 g, 2.41 mmol) was dissolved in CH_2Cl_2 (24 mL), and then 4,4'-dithiodibutyric acid (319 mg, 1.34 mmol), EDC (1.39 g, 7.25 mmol) and DMAP (64.5 mg, 0.532 mmol) were added sequentially at room temperature. After stirring for 2 h at room temperature, the reaction mixture was quenched with saturated aqueous solution of NH_4Cl and washed with water and brine. The mixture was then dried over $MgSO_4$, filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography with $CHCl_3$ to give PPT dimer SS (1.22 mg, 1.18 mmol, 98%) as a white solid. IR: ν_{max} 1778, 1730, 1588, 1504, 1484, 1420,

1330, 1239, 1173, 1125, 1038, 995, 932, 868, 799, 764, 749 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 2.04–2.12 (4H, m), 2.51–2.90 (12H, m) 3.72 (12H, s), 3.77 (6H, s), 4.18 (2H, t, J = 9.2 Hz), 4.35 (2H, dd, ^3J = 8.8 Hz, ^4J = 6.4 Hz), 4.58 (2H, d, J = 4.0 Hz), 5.87 (2H, d, J = 8.4 Hz), 5.97 (4H, d, J = 6.4 Hz), 6.37 (4H, s), 6.53 (2H, s), 6.76 (2H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 24.0, 32.5, 37.5, 38.7, 43.7, 45.5, 56.2, 60.7, 71.3, 73.8, 101.7, 107.0, 108.1, 109.8, 128.2, 132.4, 134.8, 137.1, 147.6, 148.2, 152.6, 173.4, 173.6; HRMS (ESI) m/z calcd. for $C_{52}H_{54}O_{18}S_2Na$ $[M + Na]^+$ 1053.2644, found 1053.2652.

Fabrication of nanoparticles

All PPT dimer nanoparticles were prepared using the reprecipitation method. At ambient temperature, 5 mM PPT dimer in THF (100 μL) was injected into stirring distilled water (10 mL). The size distribution of PPT dimer nanoparticles was derived from SEM images.

***In vitro* assay**

KPL-4 human breast cancer cells were maintained in DMEM supplemented with 5% FBS.

KPL-4 cells were seeded in 96-well plates at a density of 2×10^4 cells/well. After 1 day, PPT dimer nanoparticles in cell culture media (0.1–5 μM based on PPT monomer concentration) were added to the wells, and cells were cultured for 2 days. Cell viabilities were evaluated using Cell Counting Kit-8 and normalized to the $OD_{450}-OD_{620}$ value for the untreated cells. Assays were performed in triplicate.

LC-MS/MS measurements

The HPLC conditions for all samples were as follows: column, reverse-phase ODS column (Imtakt Cadenza CD-C_{18}, ø 2 × 100 mm); column temperature, 35 °C; mobile phase, gradient generated from acetonitrile with 0.1% formic acid/water with 0.1% formic acid (v/v) = 40/60 to 90/10 at 1 min; flow rate, 0.3 mL/min; injection volume, 1 μL; retention time, PPT 2.6 min, PPT dimer C4 5.2 min, PPT dimer C10 5.8 min, PPT dimer C18 9.6 min, and PPT dimer SS 5.3 min.

Precursor ions of PPT (m/z 397.30), PPT dimer C4 (m/z 928.30), PPT dimer C10 (m/z 1017.30), PPT dimer C18 (m/z 1124.50) and PPT dimer SS (m/z 1048.30) were used.

Cellular uptake

KPL-4 cells were seeded in 96-well plates at a density of 2×10^4 cells/well. After 1 day, 5 μM PPT dimer nanoparticles were added, and cancer cells were cultured for 3 h. Cancer cells were washed with PBS (-) three times and lysed using lysis buffer (100 μL). The split lysate (50 μL) was sonicated for 10 min, and then MeOH (450 μL) was added to the lysates. After centrifugation at 10000 rpm for 5 min, the supernatant was subjected to LC-MS/MS to evaluate the cellular uptake rate of PPT dimer nanoparticles.

Release rate of PPT

The rate of esterase-mediated release of PPT from PPT dimer nanoparticles was evaluated as follows. An aqueous dispersion of PPT dimer nanoparticles (50 μL) was added to 1000 units of esterase from porcine liver in a PBS (-) solution (450 μL). The mixture was heated at 37 °C in a water bath. After 1, 3, 6, 9, 12, 24, 30, and 36 hours, the reaction mixture (10 μL) were added to MeOH (90 μL), and centrifuged at 10000 rpm for 5 min. The supernatant was diluted by a factor of ten using a 9 : 1 mixture of MeOH and distilled water, and the hydrolysis rate of the samples was evaluated using LC-MS/MS.

The release rate of PPT from PPT dimer nanoparticles in PBS was evaluated as follows. An aqueous dispersion of PPT dimer nanoparticles (50 μL) was added to PBS (-) (450 μL). The mixture was heated at 37 °C in a water bath. After 6, 12, 24, and 36 hours, the reaction mixture (10 μL) was added to MeOH (90 μL), and centrifuged at 10000 rpm for 5 min. The supernatant was diluted by a factor of ten using a 9 : 1 mixture of MeOH and distilled water, and the hydrolysis rate of the samples was evaluated using LC-MS/MS.

The release rate of PPT from PPT dimer SS nanoparticles by DTT was evaluated as following. An aqueous dispersion of PPT SS dimer nanoparticles (50 μL) was added to 5 mM DTT PBS (-) solution (450 μL). The mixture was heated at 37 °C in a water bath. After 6, 12, 24, and 36 hours, the reaction mixture (10 μL) was added to MeOH (90 μL), and centrifuged at 10000 rpm for 5 min. The supernatant was diluted by a factor of ten using a 9 : 1 mixture of MeOH and distilled water, and the degradation rate of the samples was evaluated using LC-MS/MS.

Dialysis of PPT dimer nanoparticles

A 100 μM aqueous dispersion of PPT dimer C4 or C18 nanoparticles (10 mL) was dialyzed in water (90 mL). After 1 day, the water outside the dialysis tube was subjected to LC-MS/MS to evaluate the concentration of free PPT dimers.

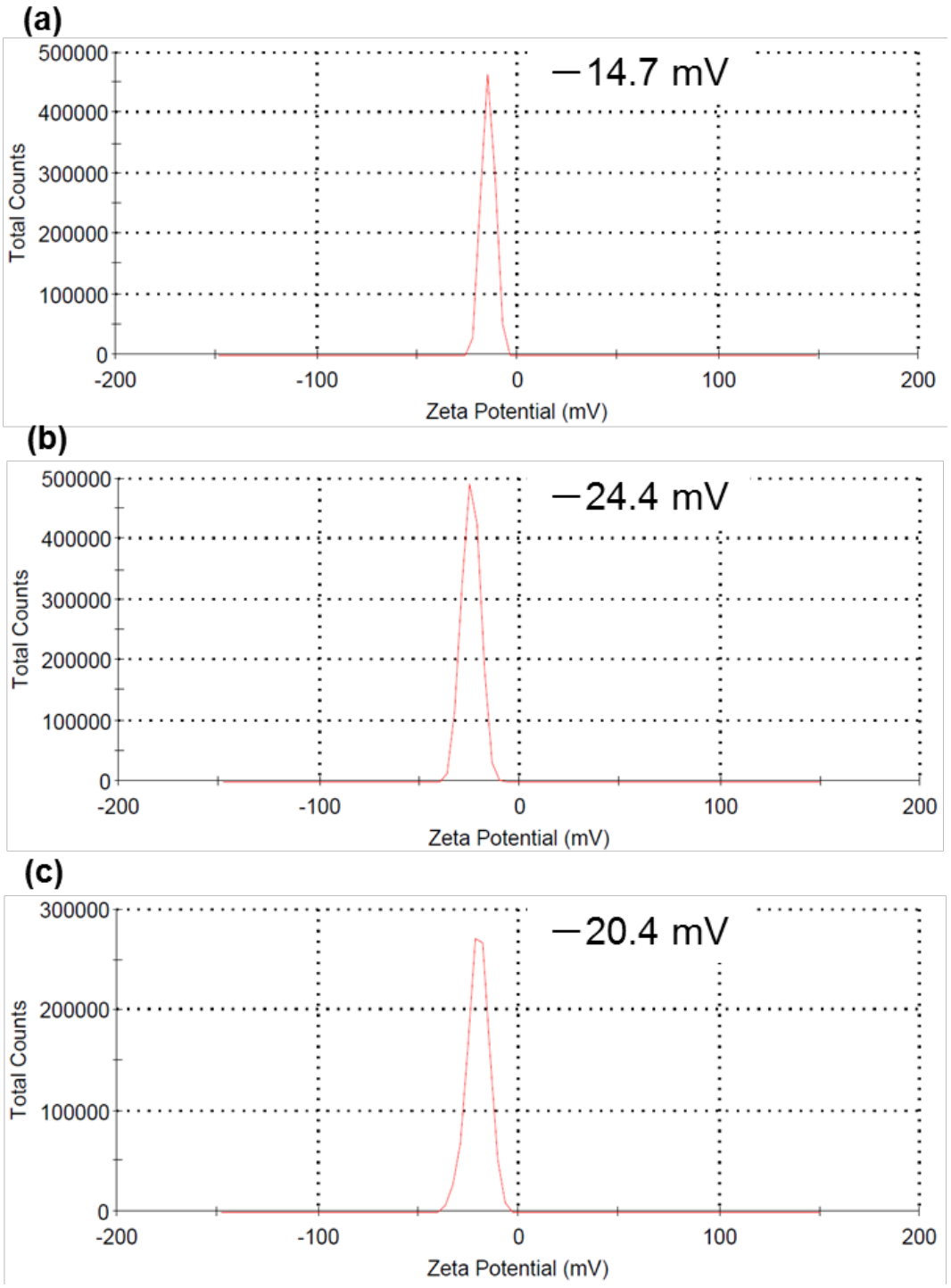


Figure S1. Zeta potencial of PPT dimer C4 (a), C10 (b) and C18 (c) nanoparticles.

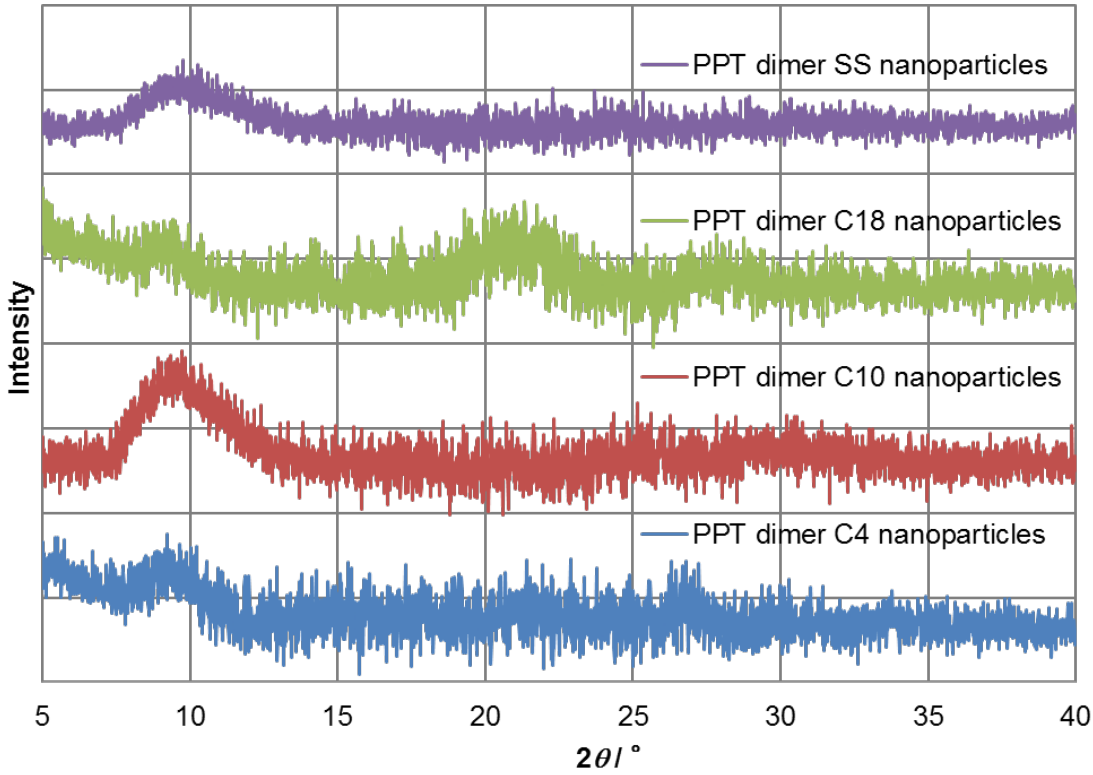


Figure S2. XRD measurement of PPT dimer nanoparticles.

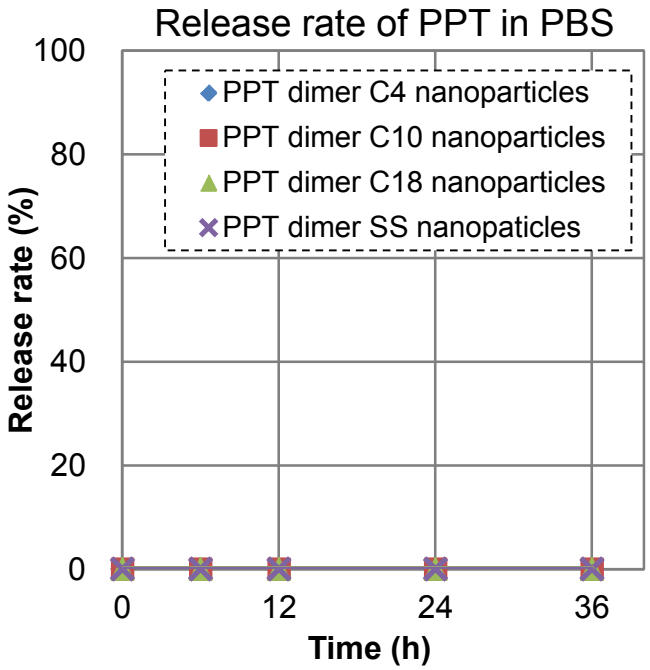


Figure S3. Hydrolysis rate of PPT dimer C4, C10, C18 and SS nanoparticles in PBS without esterase. All PPT dimer nanoparticles weren't hydrolyzed by heating at 37 °C.

Figure S4. ^{1}H NMR spectra of PPT dimer C10.

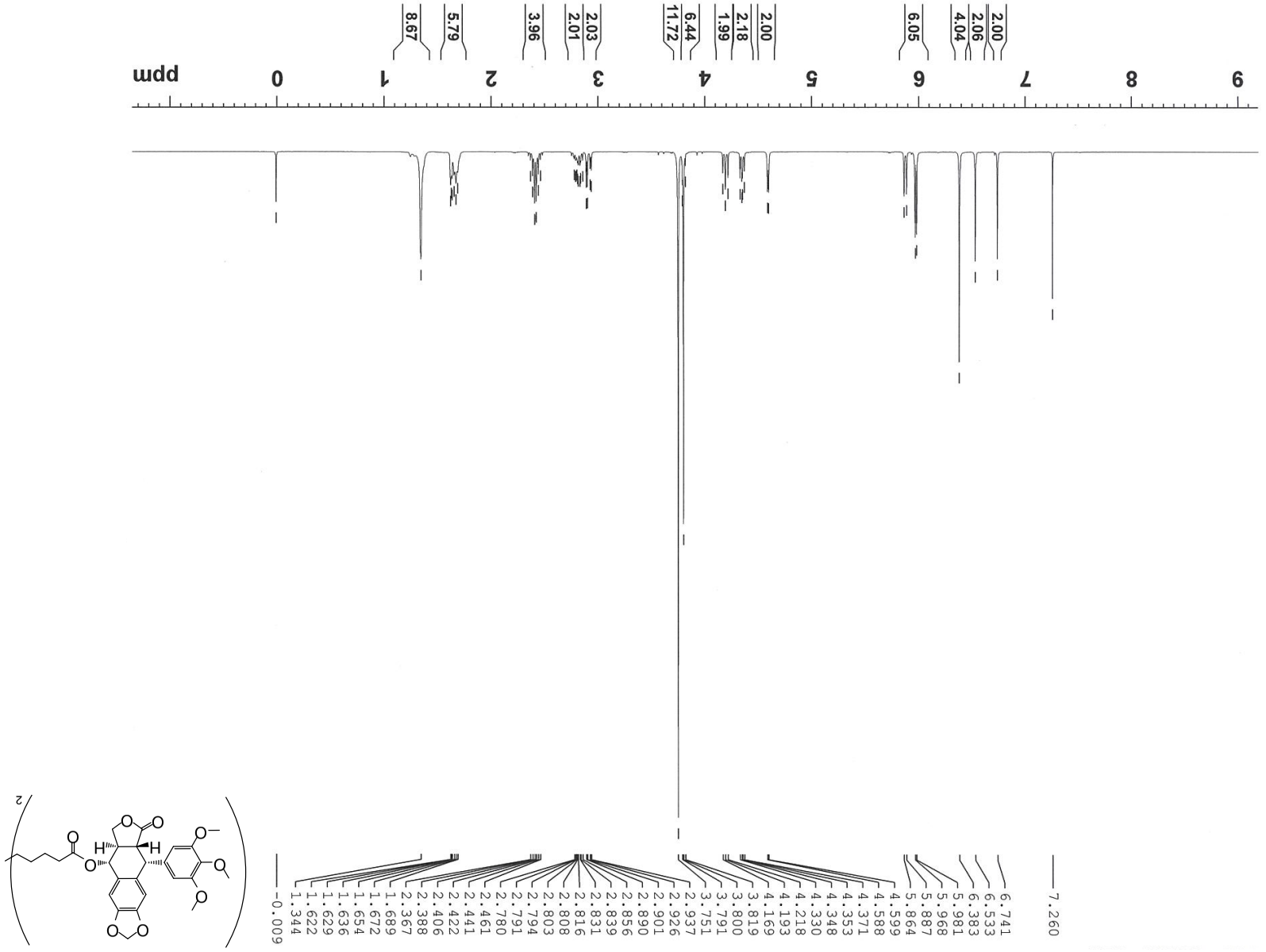


Figure S5. ^{1}H NMR spectra of PPT dimer C18.

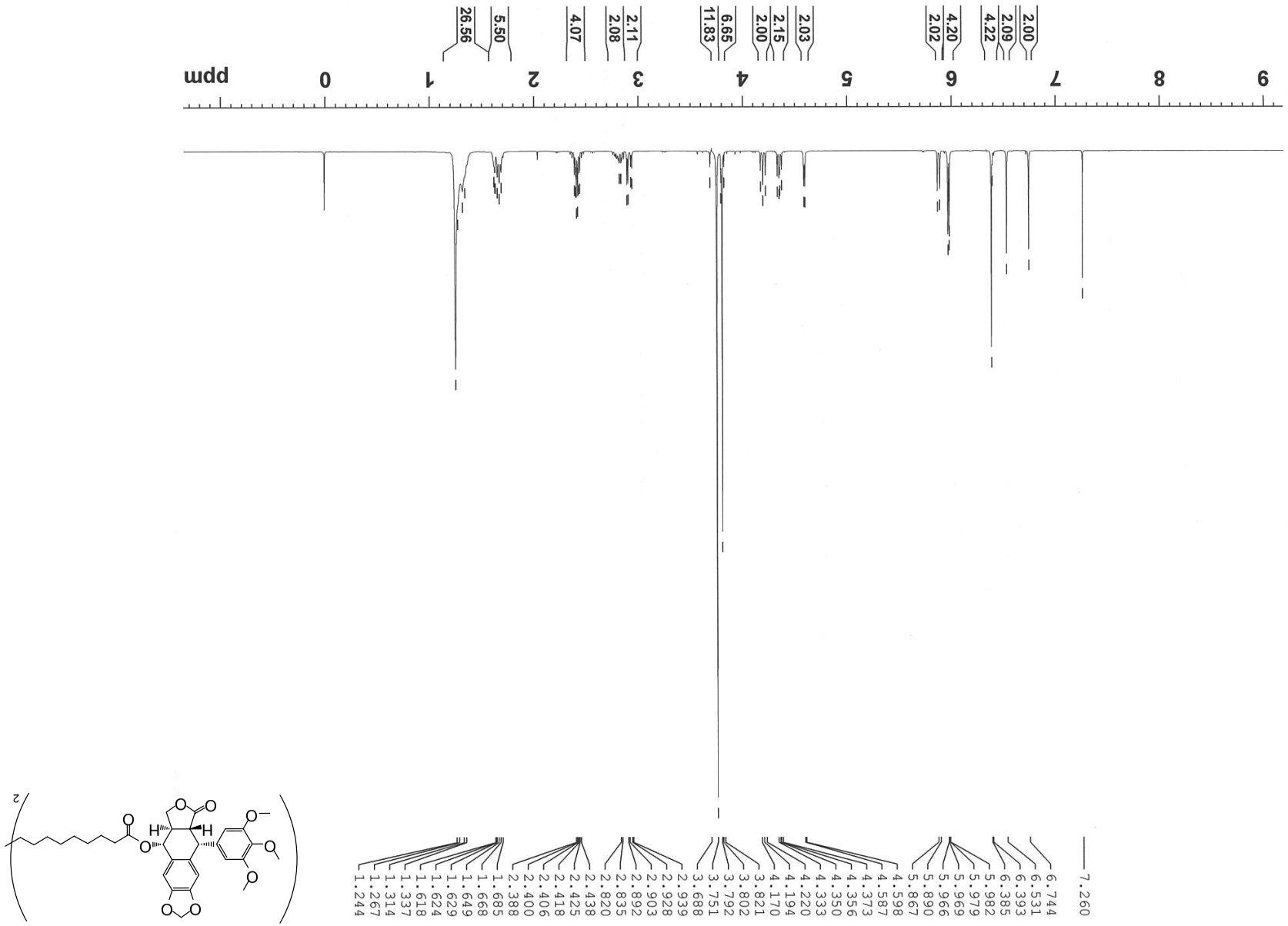
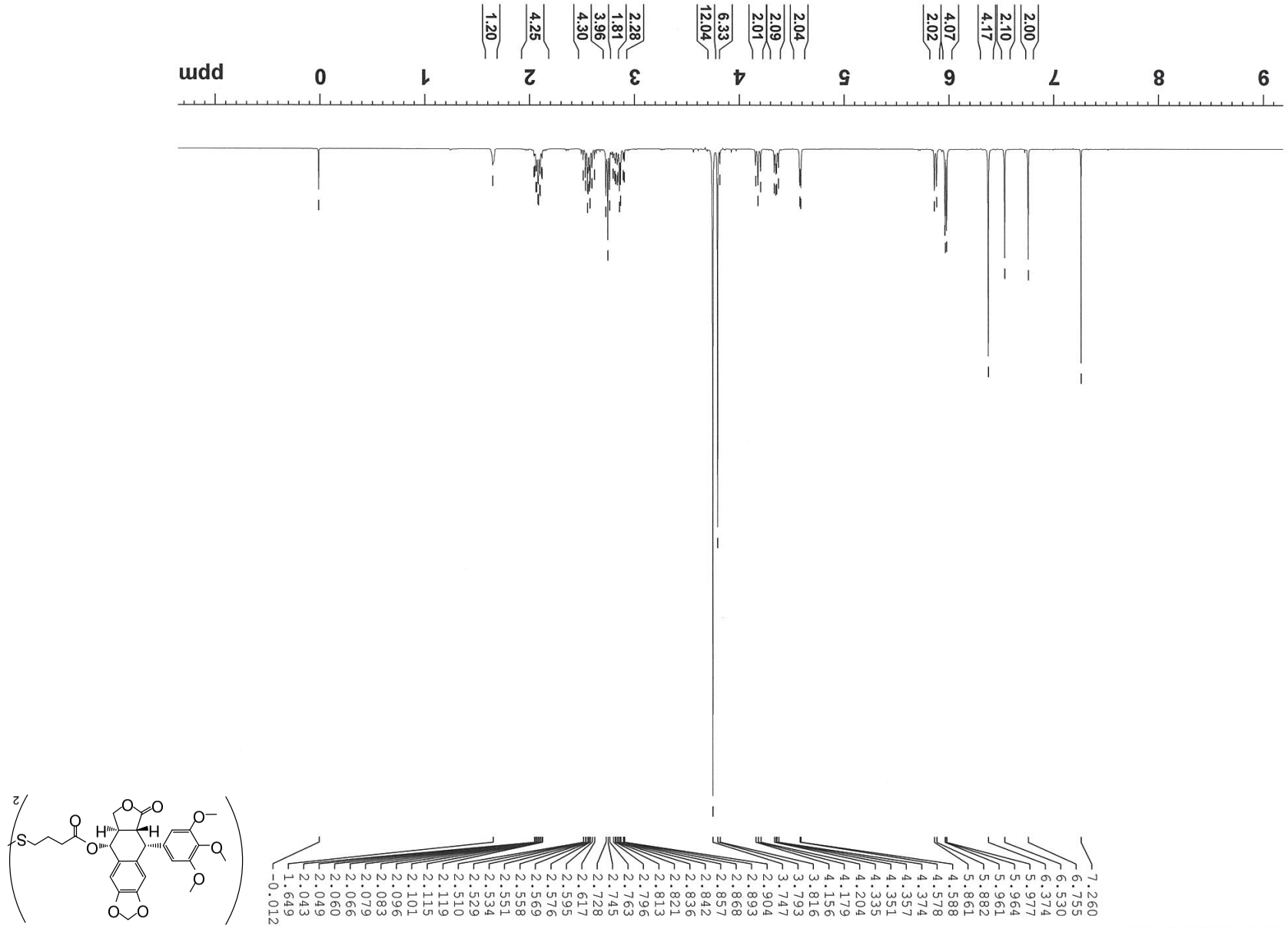


Figure S6. ^{1}H NMR spectra of PPT dimer SS.



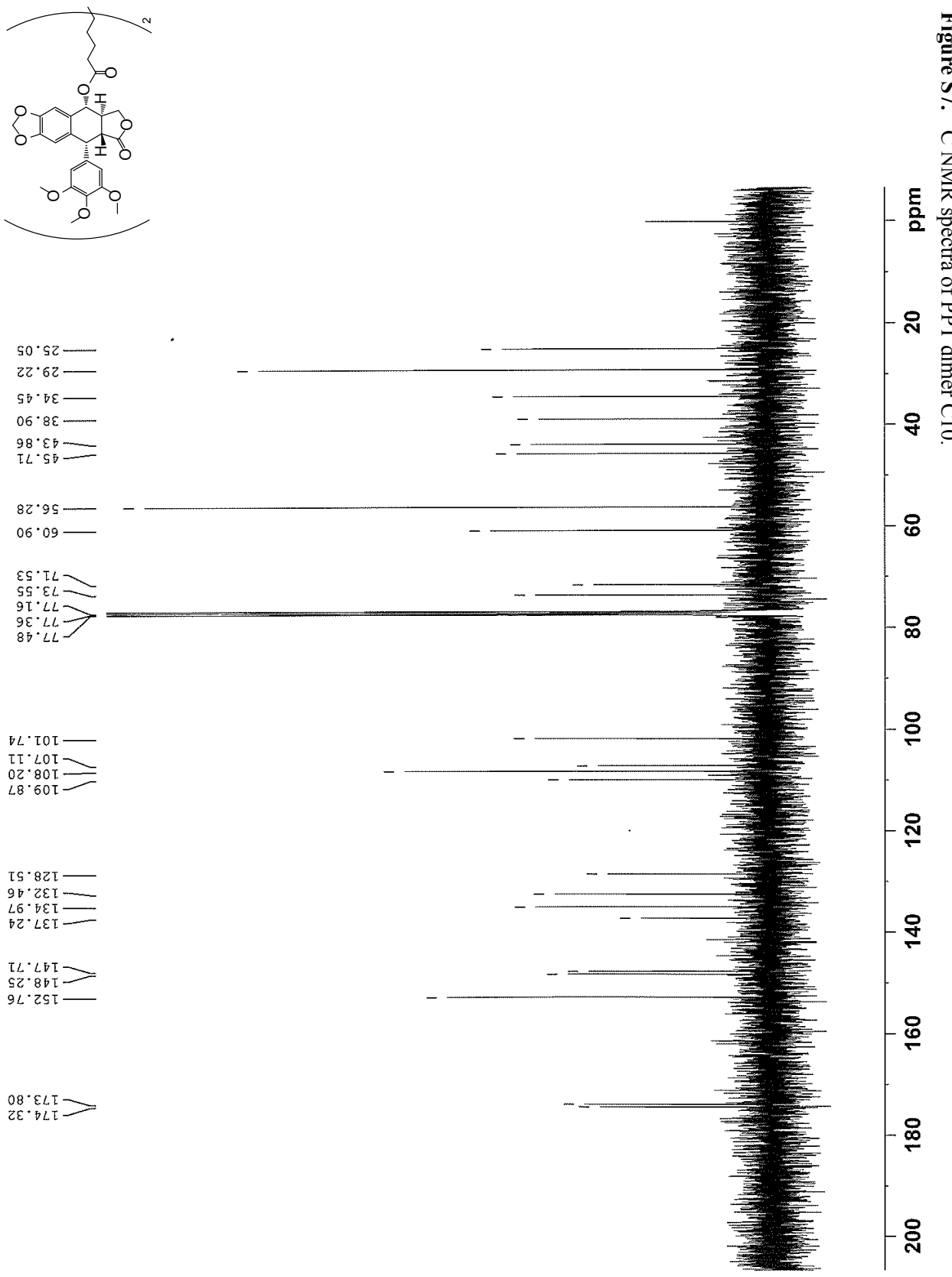


Figure S7. ^{13}C NMR spectra of PPT dimer C10.

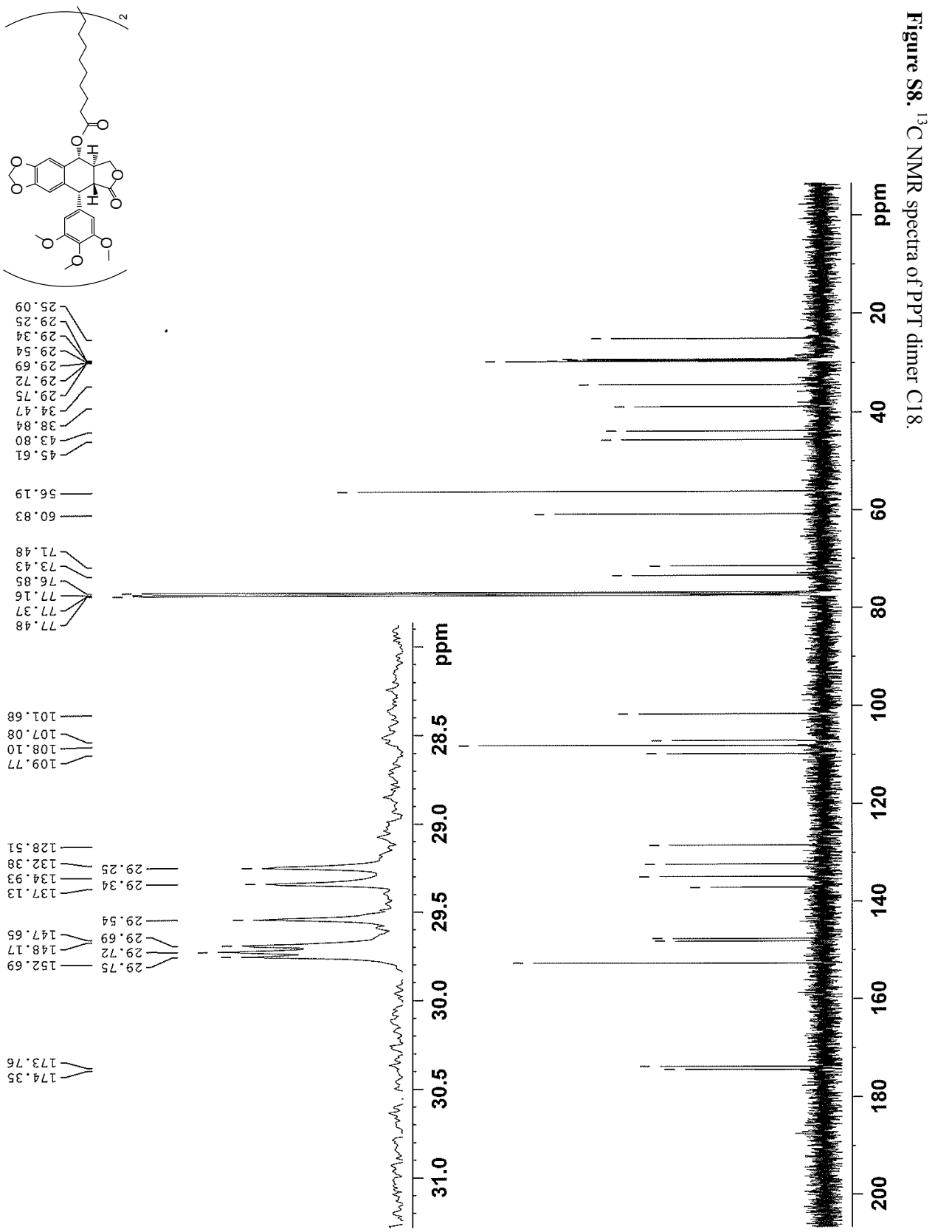
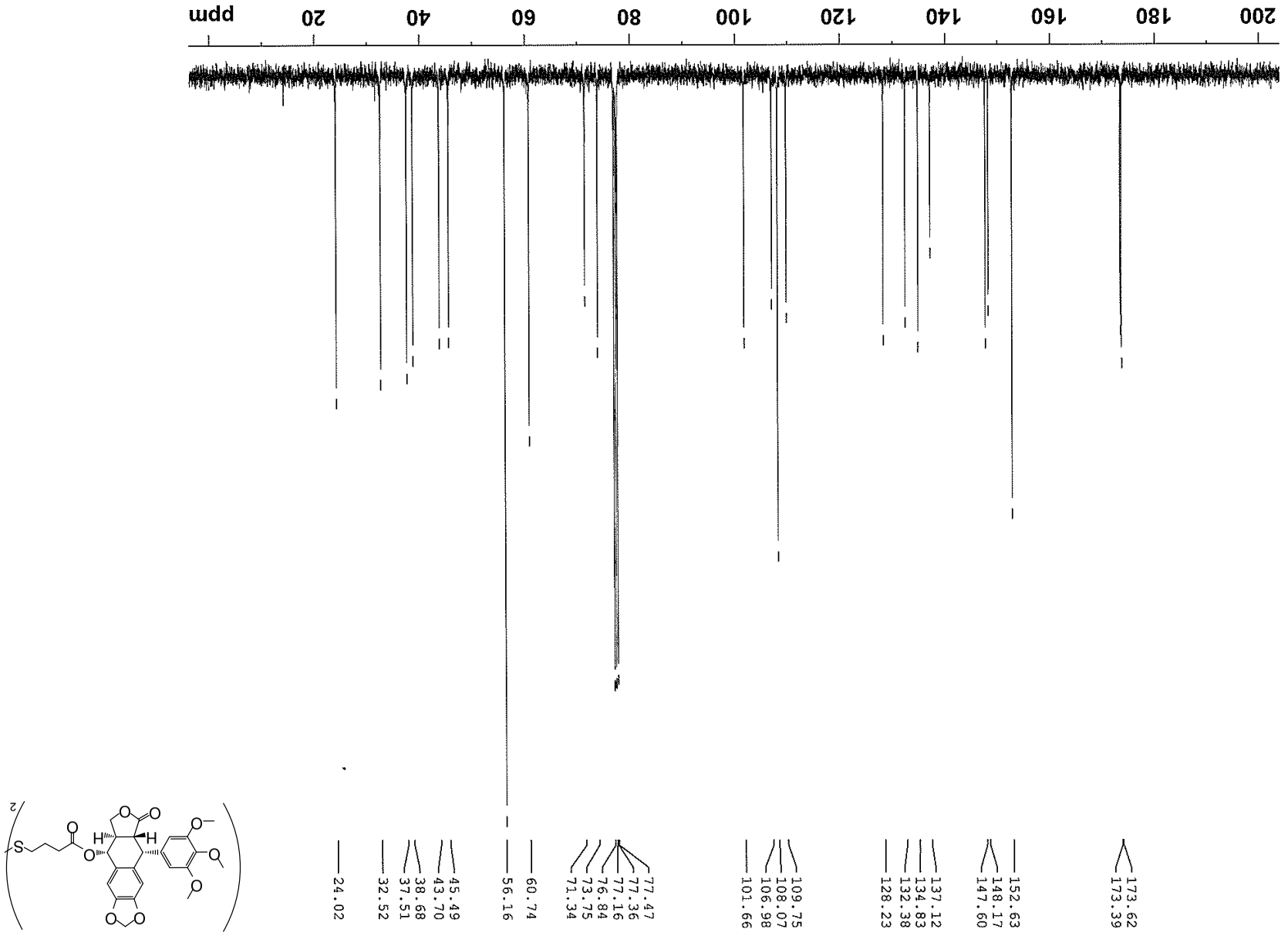


Figure S8. ^{13}C NMR spectra of PPT dimer C18.

Figure S9. ^{13}C NMR spectra of PPT dimer SS.



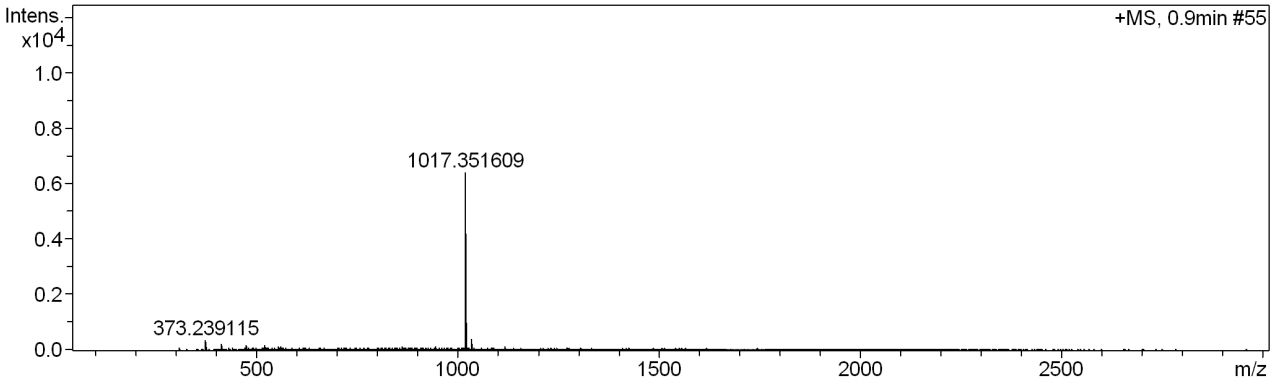


Figure S10. HRMS spectra of PPT dimer C10.

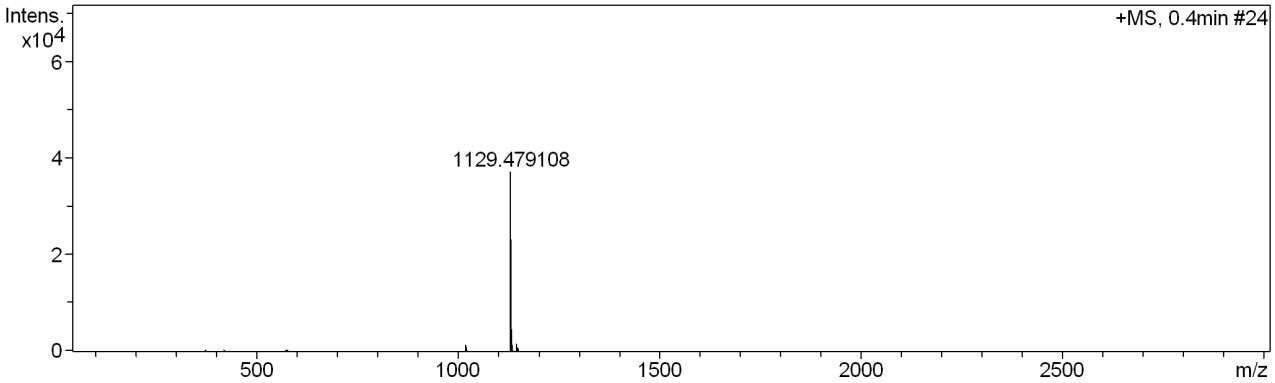


Figure S11. HRMS spectra of PPT dimer C18.

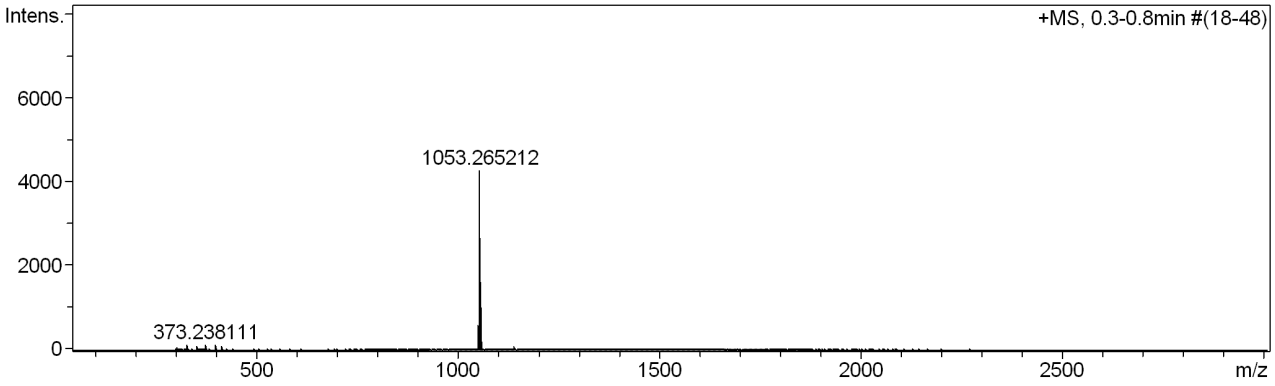


Figure S12. HRMS spectra of PPT dimer SS.