Electronic Supplementary Material (ESI) for Materials Chemistry Frontiers.

Supporting Information

A Glutathione-activated Carrier Free Nanodrug of Triptolide as Trackable Drug Delivery System for Monitoring and Improving Tumor Therapy

Ying Li†, [a, b] Lihua Zhou†, [b, c] Baode Zhu†, [d] Jingjing Xiang, [b] Jian Du, [e] Manwen He, [c] Xingxing Fan, [g] Pengfei Zhang*, [b] Ruosheng Zeng*, [a, f] and Ping Gong *[b]

a. School of Materials Science and Engineering, School of Life and Environmental Sciences, Guilin University of Electronic Technology, Guilin 541004, P. R. China.
b. Guangdong Key Laboratory of Nanomedicine, Shenzhen Engineering Laboratory of Nanomedicine and Nanoformulations, CAS Key Lab for Health Informatics, CAS-HK Joint Lab for Biomaterials, Institute of Biomedicine and Biotechnology, Shenzhen Institutes of Advanced Technology (SIAT), Chinese Academy of Sciences Shenzhen 518055, China. *E-mail: pf.zhang@siat.ac.cn and ping.gong@siat.ac.cn.
c. School of Applied Biology, Shenzhen Institute of Technology, No. 1 Jiangjunmao, Shenzhen 518116, P. R. China.
d. College of Chemistry Biology & and Environmental Engineering, Xiangnan University, Chenzhou, 423043, China.
e. The First Affiliated Hospital of Shandong First Medical University.
f. School of Physical Science and Technology, Guangxi University, Nanning 530004, China. * E-mail: zengrsh@guet.edu.cn.
g. State Key Laboratory of Quality Research in Chinese Medicine, Macau Institute for Applied Research in Medicine and Health, Macau University of Science and Technology, Macau, SAR, China.

† These authors contributed equally to this work.

*Corresponding authors:
Prof. Ping Gong (ping.gong@siat.ac.cn);
Prof. Ruosheng Zeng (zengrsh@guet.edu.cn);
Dr. Pengfei Zhang (pf.zhang@siat.ac.cn).

Experiment section

Chemicals and Materials

Heptamethine cyanine dye IR780 and Triptolide (TP) were purchased from sigma company (USA). Bis-(2-aminoethyl) disulfide dihydrochloride and Triethylamine (TEM) were obtained from J&K scientific Ltd. (China). N, N'-Dicyclohexyl carbodiimide (DCC), 1-hydroxy-5-pyrrolidinedione (NHS) and 4-(dimethylamino)-pyridin (DMAP) were purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd. Cell Counting Kit-8, Mitochondria Staining Kit and DAPI were purchased from sigma company (USA). All solvents and reagents were analytical grade and used without further purification. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker 400 MHz. UV-visible Spectra was recorded on Varian Cary 50 spectrometer. Fluorescence spectra were recorded on FP-600.

The Synthesis of TP-COONa

To a solution of Triptolide (TP) (360 mg, 1 mmol) in pyridine (2 mL) were added succinic anhydride (400 mg, 4 mmol) and DMAP (24 mg, 0.2 mmol). After stirring overnight, the mixture was diluted with ethyl acetate, then washed with saturated copper sulfate, water and brine, respectively. The organic layers were dried over Na_2SO_4 and filtered. The filtrate was concentrated using a rotary evaporator to give a residue. The residue was purified by silica gel column chromatography (CH_2Cl_2/CH_3OH, 15:1) to give TP-COONa (80%) as a white solid.^{1}H NMR (400 MHz, MeOD) δ 5.49 (s, 2H), 5.08 (s, 1H), 3.96 (d, J = 3.1 Hz, 1H), 3.63 (d, J = 3.1 Hz, 1H), 3.46 (d, J = 5.6 Hz, 1H), 2.67 (d, J = 5.8 Hz, 2H), 2.26 (dq, J = 18.3, 6.1 Hz, 2H), 1.97 – 1.84 (m, 2H), 1.51 (ddd, J = 12.6, 5.6, 1.6 Hz, 1H), 1.37 – 1.21 (m, 5H), 1.04 (s, 3H), 1.00 – 0.84 (m, 6H). ^{13}C NMR (101 MHz, MeOD) δ 176.09, 173.82, 173.63, 163.87, 125.55, 72.98, 71.98, 64.89, 64.33, 62.67, 61.01, 56.22, 54.78, 41.47, 36.81, 32.72, 30.83, 29.15, 24.18, 23.67, 17.90, 17.84, 14.40, 14.14.

The Synthesis of Cyss / Cycc

Bis-(2-aminoethyl) disulfide dihydrochloride (50.67 mg,0.225 mmol) was dissolved in anhydrous DMF (2 ml) with TEA (91 μL). Then, IR780 (30 mg,0.045 mmol) dissolved in anhydrous DMF (1 mL) was drop-wise added to the above solution under N_2 atmosphere at room temperature. The solvent was evaporated under reduced pressure to get crude product Cyss after 4 h later. The product was further purified by silica gel column chromatography with dichloromethane and methanol. Yield: 56% ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (d, *J* = 12.9 Hz, 2H), 7.34 – 7.28 (m, 7H), 7.08 (t, *J* = 7.5 Hz, 2H), 6.88 (d, *J* = 7.9 Hz, 2H), 5.66 (d, *J* = 12.9 Hz, 2H), 4.20 (t, *J* = 6.1 Hz, 2H), 3.81 (t, *J* = 7.4 Hz, 4H), 3.37 (t, *J* = 6.0 Hz, 2H), 3.17 (t, *J* = 6.4 Hz, 2H), 2.96 (t, *J* = 6.3 Hz, 2H), 2.49 (t, *J* = 6.4 Hz, 4H), 1.85 (p, *J* = 7.3 Hz, 6H), 1.74 (s, 12H), 1.05 (t, *J* = 7.4 Hz, 6H).^{13}C NMR (101 MHz, $CDCl_3$) δ 171.66, 143.46, 140.58, 140.57, 138.02, 138.02, 127.90, 122.68, 122.21, 122.06, 120.41, 108.18, 68.11, 47.65, 44.57, 39.04, 38.03, 29.83, 29.06, 25.74, 25.38, 21.72, 20.17, 20.05, 11.89, 11.86.

Cycc was obtained in the same way. Yield: 58% ^{1}H NMR (400 MHz, MeOD) δ 8.11 (d, *J* = 6.9 Hz, 2H), 7.96 (s, 1H), 7.77 (d, *J* = 13.0 Hz, 2H), 7.43 – 7.34 (m, 2H), 7.30 (t, *J* = 7.7 Hz, 2H), 7.17 – 7.04 (m, 4H), 6.96 (d, *J* = 6.9 Hz, 2H), 5.84 (d, *J* = 13.0 Hz, 2H), 3.92 (t, *J* = 7.3 Hz, 4H), 3.79 (t, *J* = 7.0 Hz, 2H), 3.23 (s, 6H), 2.95 (q, *J* = 7.8 Hz, 2H), 2.54 (t, *J* = 6.5 Hz, 4H), 1.82 (q, *J* = 7.3 Hz, 6H), 1.67 (d, *J* = 4.2 Hz, 12H), 1.03 (t, *J* = 7.4 Hz, 6H). ^{13}C NMR (101 MHz, MeOD) δ 169.21, 144.57, 141.21, 140.17,

129.36, 123.89, 123.09, 121.48, 110.16, 108.21, 95.71, 51.64, 40.69, 40.19, 32.41, 30.71, 29.24, 28.48, 27.49, 27.28, 26.03, 22.96, 21.12, 11.81.

The Synthesis of CyssTP / CyccTP

Under N_2 atmosphere, the TP-COOH (20 mg,0.043 mmol) activated by NHS (6 mg,0.052 mmol), DCC (10.74 mg,0.052 mmol) and TEA (54 μL), was added to Cyss (32 mg,0.052 mmol) dissolved in 2 mL DMF. The reaction was going overnight at room temperature. Finally, the CyssTP was purified by silica gel column chromatography with dichloromethane and methanol. Yield: 45%. The structures were analyzed by HRSM and NMR, respectively. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (dt, *J* = 15.1, 7.5 Hz, 2H), 7.34 – 7.17 (m, 6H), 7.03 (dt, *J* = 11.9, 7.4 Hz, 2H), 6.84 (dd, *J* = 14.8, 7.9 Hz, 2H), 5.62 (dd, *J* = 13.1, 9.4 Hz, 2H), 5.05 (d, *J* = 7.3 Hz, 1H), 4.66 (s, 2H), 4.12 (t, *J* = 6.4 Hz, 2H), 3.80 (dq, *J* = 10.6, 7.3, 6.0 Hz, 4H), 3.68 (d, *J* = 16.1 Hz, 2H), 3.59 – 3.49 (m, 2H), 3.42 (d, *J* = 10.0 Hz, 2H), 3.32 (t, *J* = 6.2 Hz, 2H), 2.89 (dd, *J* = 12.5, 6.3 Hz, 2H), 2.78 – 2.67 (m, 4H), 2.67 – 2.53 (m, 4H), 2.50 – 2.40 (m, 4H), 2.29 (dd, *J* = 15.4, 7.5 Hz, 2H), 1.80 (h, *J* = 7.6, 6.9 Hz, 7H), 1.67 (d, *J* = 15.1 Hz, 12H), 1.23 (s, 3H), 1.04 – 0.99 (m, 6H), 0.91 (t, *J* = 6.5 Hz, 3H), 0.80 (t, *J* = 6.7 Hz, 3H). ^{13}C NMR (101 MHz, MeOD) δ 179.70, 176.21, 174.43, 174.27, 173.58, 170.64, 170.05, 164.05, 163.90, 144.68, 141.63, 141.00, 131.00, 129.56, 126.27, 125.68, 124.33, 123.25, 121.86, 110.51, 101.54, 96.41, 73.06, 72.74, 72.12, 65.01, 64.94, 64.54, 64.37, 62.86, 62.75, 61.15, 56.88, 56.80, 56.38, 56.31, 54.95, 45.75, 41.62, 41.55, 39.80, 38.96, 38.69, 36.94, 36.68, 36.31, 33.20, 31.97, 31.05, 30.96, 30.87, 30.76, 30.60, 30.46, 29.47, 29.33, 29.27, 29.23, 28.25, 27.05, 26.02, 24.32, 23.87, 23.12, 18.07, 18.05, 17.32, 17.27, 14.59, 14.40, 14.33, 11.93.

CyccTP was obtained in the same way. Yield: 48% ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 (s, 2H), 7.66 (d, *J* = 9.6 Hz, 2H), 7.29 (dt, *J* = 15.4, 7.5 Hz, 6H), 7.07 (t, *J* = 7.4 Hz, 2H), 6.87 (dd, *J* = 8.0, 4.1 Hz, 2H), 5.63 (d, *J* = 12.7 Hz, 2H), 5.08 (d, *J* = 5.9 Hz, 2H), 4.70 (s, 4H), 3.83 (d, *J* = 3.2 Hz, 4H), 3.75 – 3.72 (m, 2H), 3.72 – 3.69 (m, 2H), 3.67 (s, 1H), 3.49 (d, *J* = 5.6 Hz, 2H), 2.75 – 2.67 (m, 8H), 2.47 (d, *J* = 6.5 Hz, 4H), 1.92 (d, *J* = 13.9 Hz, 3H), 1.83 (p, *J* = 8.6, 7.3 Hz, 7H), 1.24 (d, *J* = 6.9 Hz, 6H), 1.04 (d, *J* = 8.0 Hz, 12H), 0.94 (d, *J* = 7.0 Hz, 6H), 0.82 (d, *J* = 6.9 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 176.40, 173.41, 172.17, 167.41, 162.79, 160.28, 143.13, 139.93, 137.87, 128.14, 125.46, 122.83, 122.19, 122.13, 120.06, 108.51, 71.27, 70.16, 63.56, 63.30, 61.23, 59.75, 58.35, 55.41, 54.94, 49.99, 47.74, 40.34, 39.37, 36.63, 35.65, 31.53, 30.99, 29.81, 29.69, 29.36, 29.11, 29.06, 27.96, 26.01, 25.43, 23.41, 21.43, 20.08, 18.38, 17.49, 17.05, 16.69, 13.73, 11.76.

Cell Culture

MCF-7 human breast adenocarcinoma cells, HeLa cells, L02 human hepatocytes cells, were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 ℃ under humidified environment with 5% CO_2.

Flow cytometry

Flow cytometric assay was employed to investigate cell viability. MCF-7 cells were seeded in 6-well plates at a density of 2-5 10^5×cells/well in 2 mL of media and grown in a humidified 5% CO_2 atmosphere at 37 ℃ for 48 h. Then, the medium was replaced with fresh medium containing different concentrations of CyssTPN (TP and CyccTPN as contrasts) for 15 min at 37℃. After 2 h incubation, cells were washed thrice with PBS, gently digested by trypsin and harvested by

centrifugation then resuspended in PBS. Infrared channel (APC-Cy7) was used with excitation at 633 nm and collection in the ranges of 750-810 nm.

***In Vitro* Cytotoxicity assay**

In vitro cytotoxicity of TP, CyccTPN, CyssTPN was evaluated by the Cell Counting Kit-8 assay. MCF-7 cells were dispensed into 96-well plates at a final concentration of (8×10^3cells/ well in a culture medium (200 μL) and incubated overnight before treatment. The culture medium was replaced with different concentrations (0-5000 nM) of TP, CyccTPN, CyssTPN. After 4h, rinsing three times with PBS, cells were incubated with new culture medium. CCk-8 assay was carried out to investigate the cell survival of different groups after 24 h.

LSCM fluorescence imaging

Laser Scanning Confocal Microscope (LSCM, Leica TCS SP5) imaging was applied to observe the distribution of CyssTPN in tumor cells under different conditions. MCF-7 cells were seeded in eight-well chambered cover glasses. The old medium at 24 h was changed by the medium with CyssTPN，the medium of contrast group (TP, CyccTPN) was changed by the medium containing equal concentration. After 2 h, cells were washed with PBS for 2 times, then with mito-tracker at 37℃ for 20 min and rinsed thrice with PBS. Finally, LSCM was used to observe cellular uptake and subcellular distribution. Both channels were excited at 633 nm and 747nm, respectively, and collected in the ranges of 670-800 nm and 750-800 nm.

Animals and Tumor Model

All experimental procedures involving animals were approved by the Ethics Committee of Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences (SIAT). Female BALB/c nude mice (5 weeks old and weighed 18–20 g) were purchased from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). To set up the tumor model, MCF-7 cells (0.5×10^6) in 0.2 mL of saline solution were administered by subcutaneous injection into the hind leg region.

***In Vivo* Monitoring the Activation of Prodrug**

Mice with tumor sizes about 300 mm^3 in volume were used and injected with 50 μl CyssTPN (1 mM). For fluorescence imaging, the above prodrug carriers were excited with two laser wavelengths at 740 nm. The semiquantitative analysis of fluorescence imaging were taken at 0.5/12/36/72 h. Histopathological analysis by hematoxylin and eosin (H&E) staining of heart, liver, spleen, lung, and kidney sections isolated from nude mice after treatment with control, TP, CyccTPN and CyssTPN.

Figures and tables

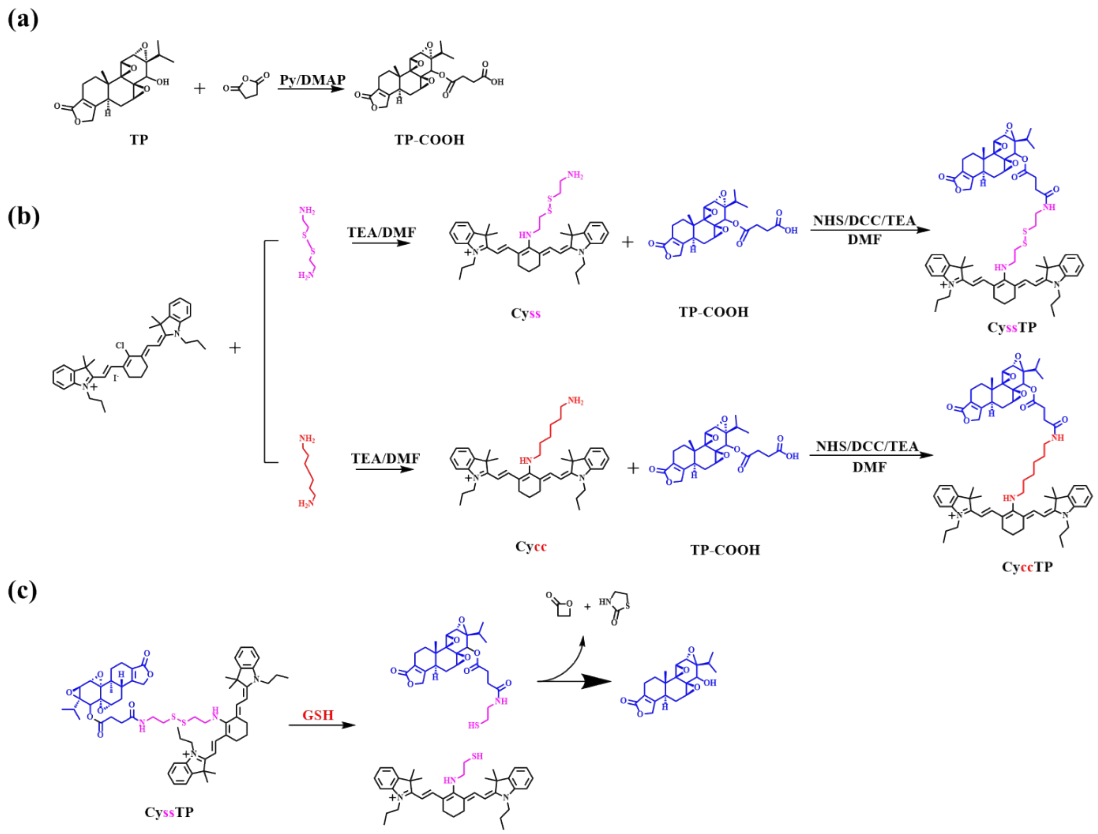
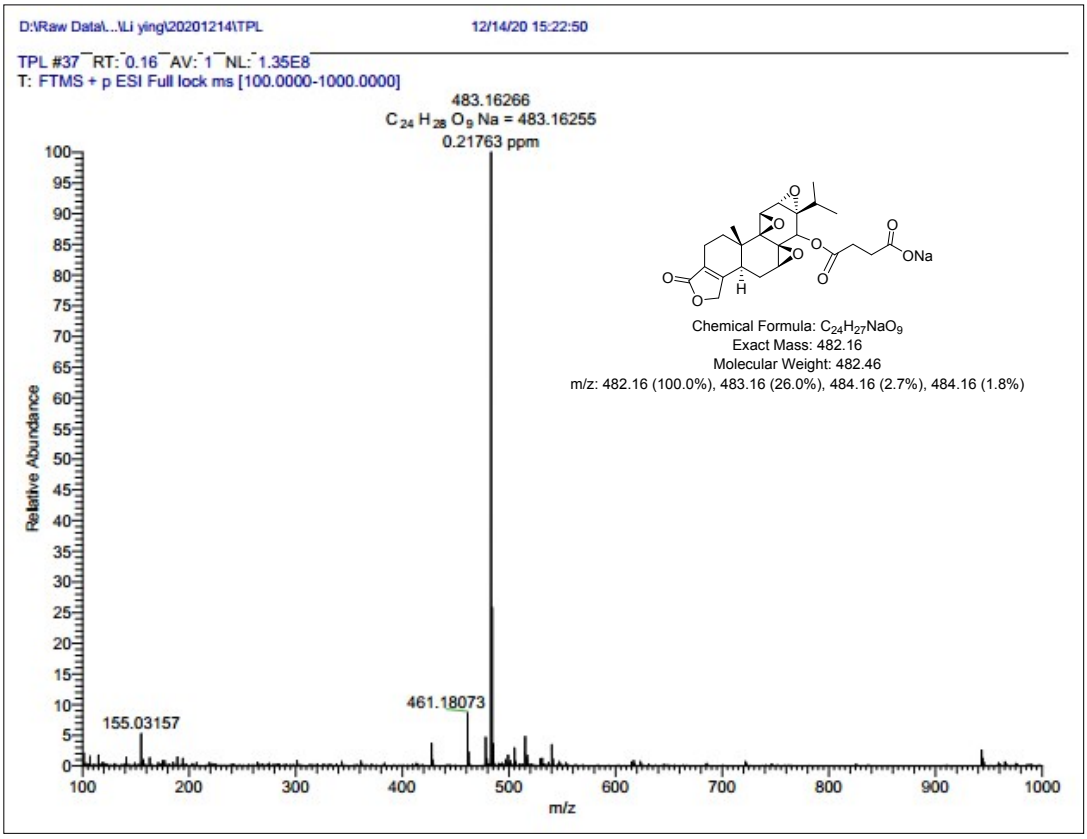


Fig. S1 Schematic diagram of TP transformation(a). Schematic of synthesis route of prodrug CyssTP and CyccTP (b). GSH response process diagram of CyssTP (c).


D:\Raw Data\...\Li ying\20201214\TPL
12/14/20 15:22:50
TPL #37 RT: 0.16 AV: 1 NL: 1.35E8
T: FTMS + p ESI Full lock ms [100.0000-1000.0000]
483.16266
C24 H28 O9 Na = 483.16255
0.21763 ppm
Relative Abundance
m/z
155.03157
461.18073
ONa
Chemical Formula: C24H27NaO9
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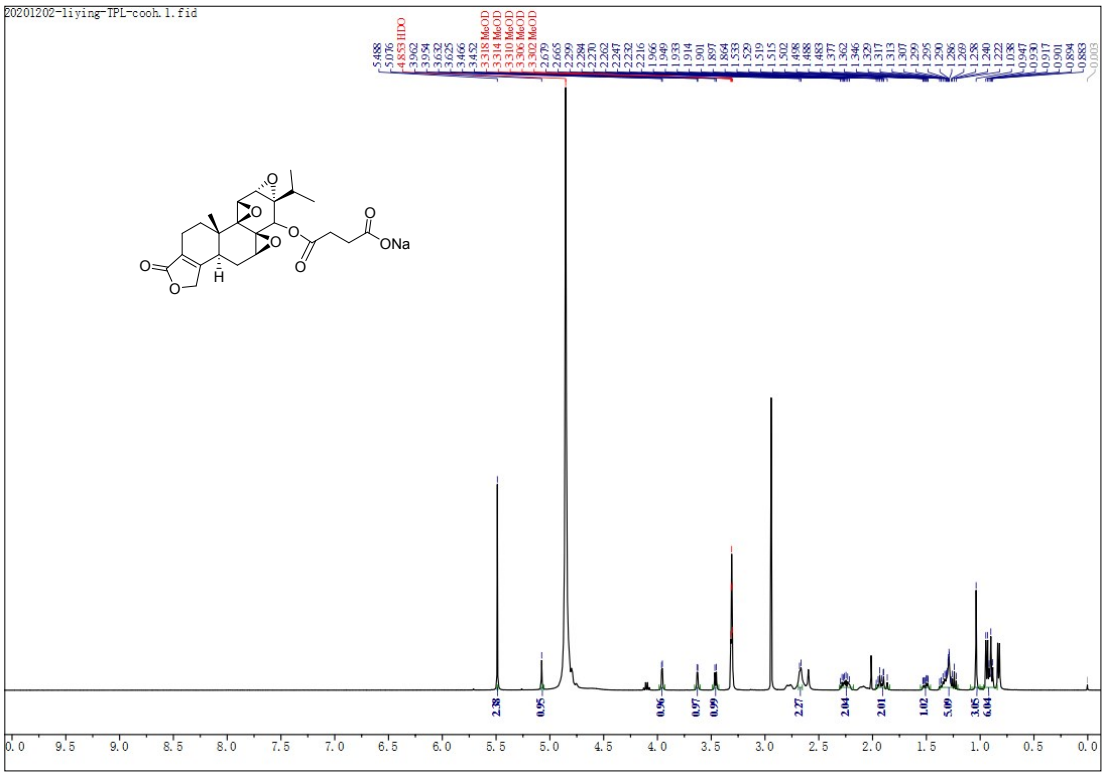
Molecular Weight: 482.46
m/z: 482.16 (100.0%), 483.16 (26.0%), 484.16 (2.7%), 484.16 (1.8%)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ONa

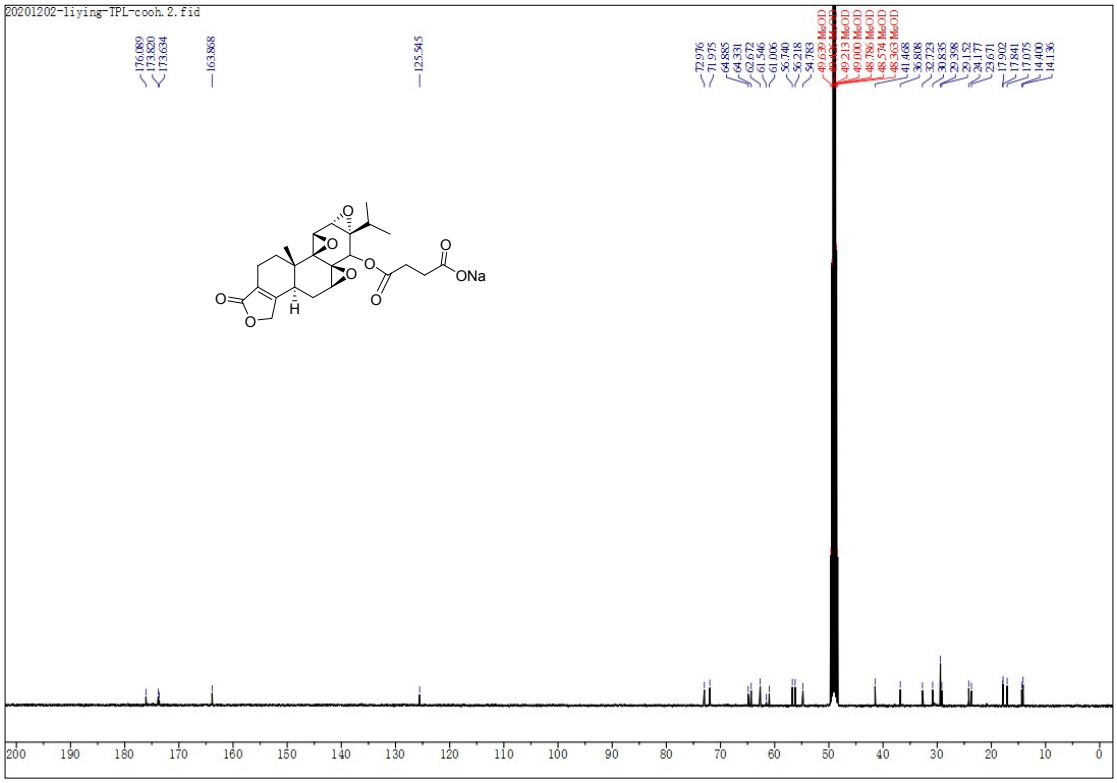
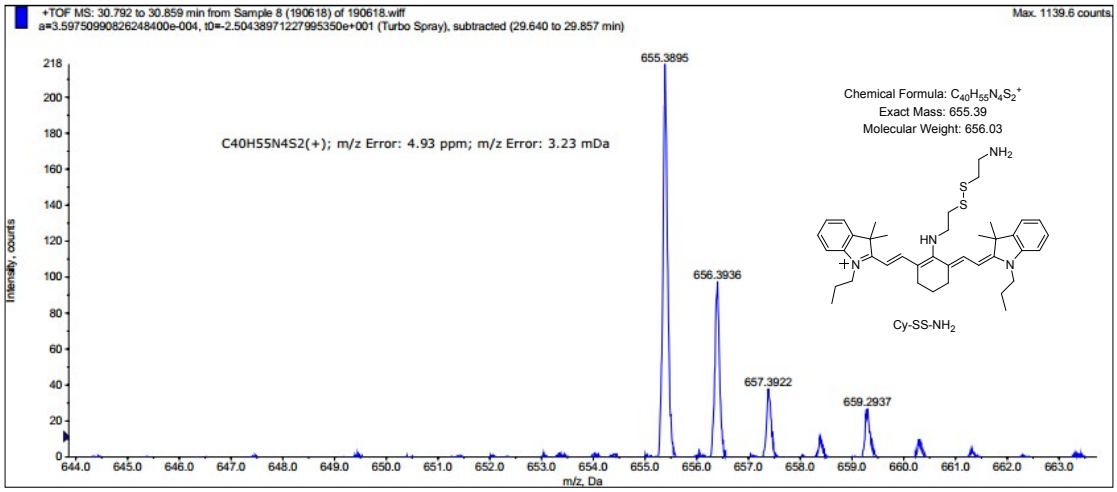


Fig. S2 High-Resolution Mass Spectrometer, ^{1}H NMR and ^{13}C NMR of TP-COONa.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Max. 1139.6 counts.
655.3895
656.3936
657.3922
659.2937
C40H55N4S2(+); m/z Error: 4.93 ppm; m/z Error: 3.23 mDa
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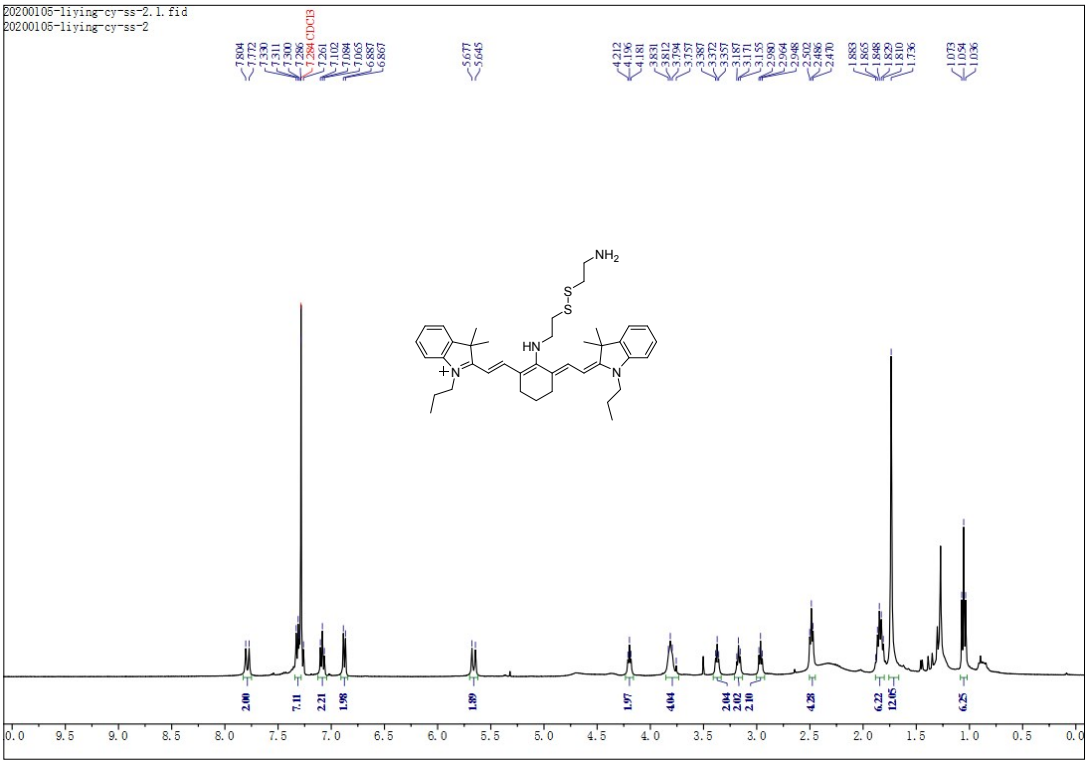
Exact Mass: 655.39
Molecular Weight: 656.03
Cy-SS-NH2
Intensity, counts
m/z, Da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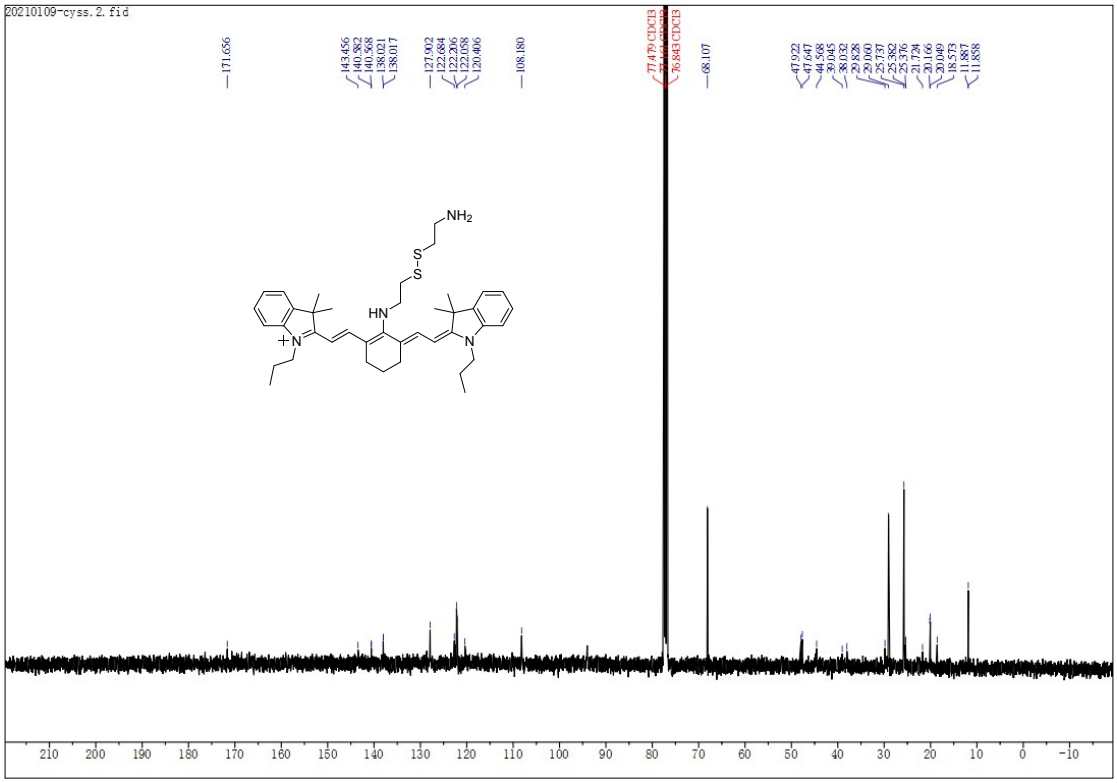
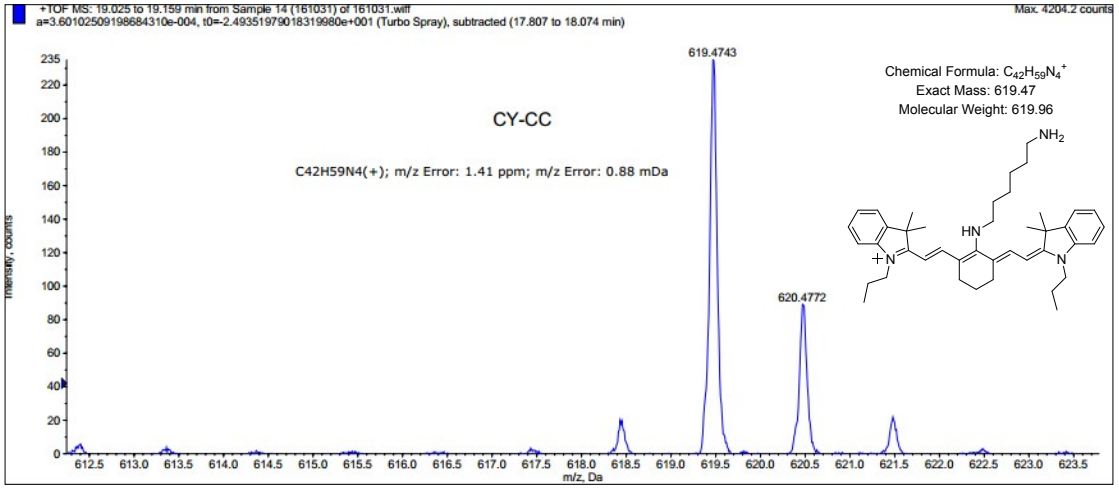


Fig. S3 High-Resolution Mass Spectrometer, ^{1}H NMR and ^{13}C NMR of Cyss.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Max. 4204.2 counts
CY-CC
C42H59N4(+); m/z Error: 1.41 ppm; m/z Error: 0.88 mDa
619.4743
620.4772
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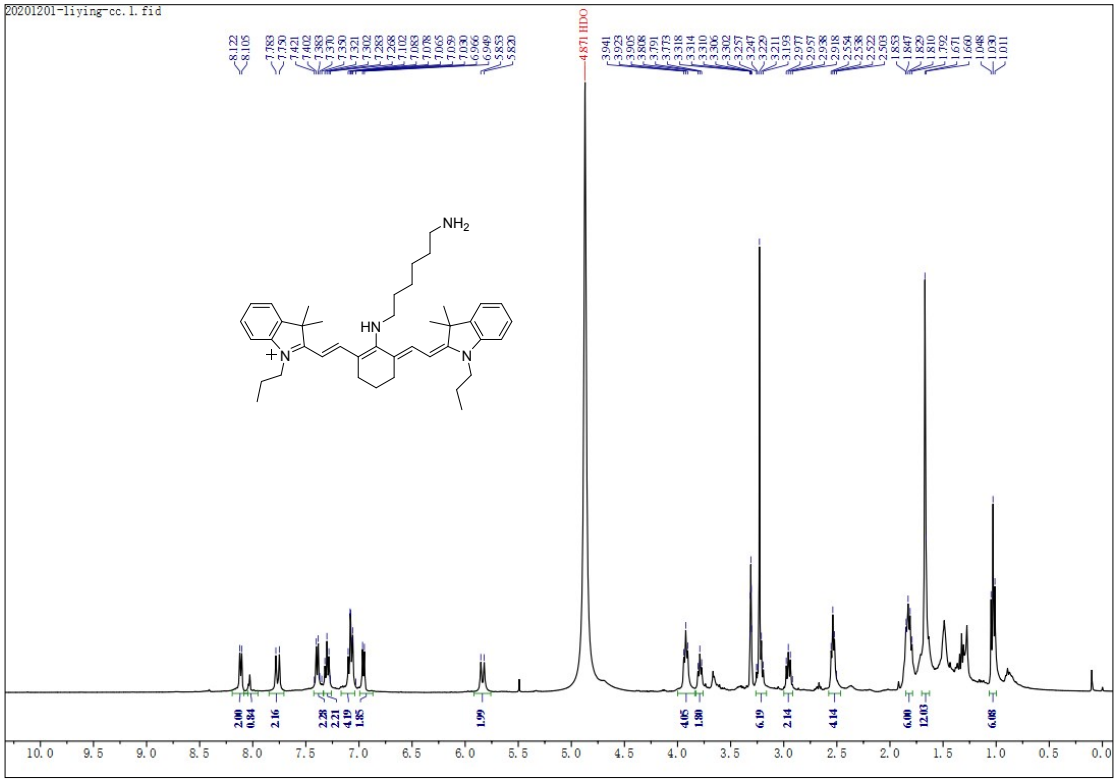
Molecular Weight: 619.96
NH2
HN
N
Intensity, counts
m/z, Da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HN
N
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1.80
6.19
2.14
4.14
6.00
12.03
6.08

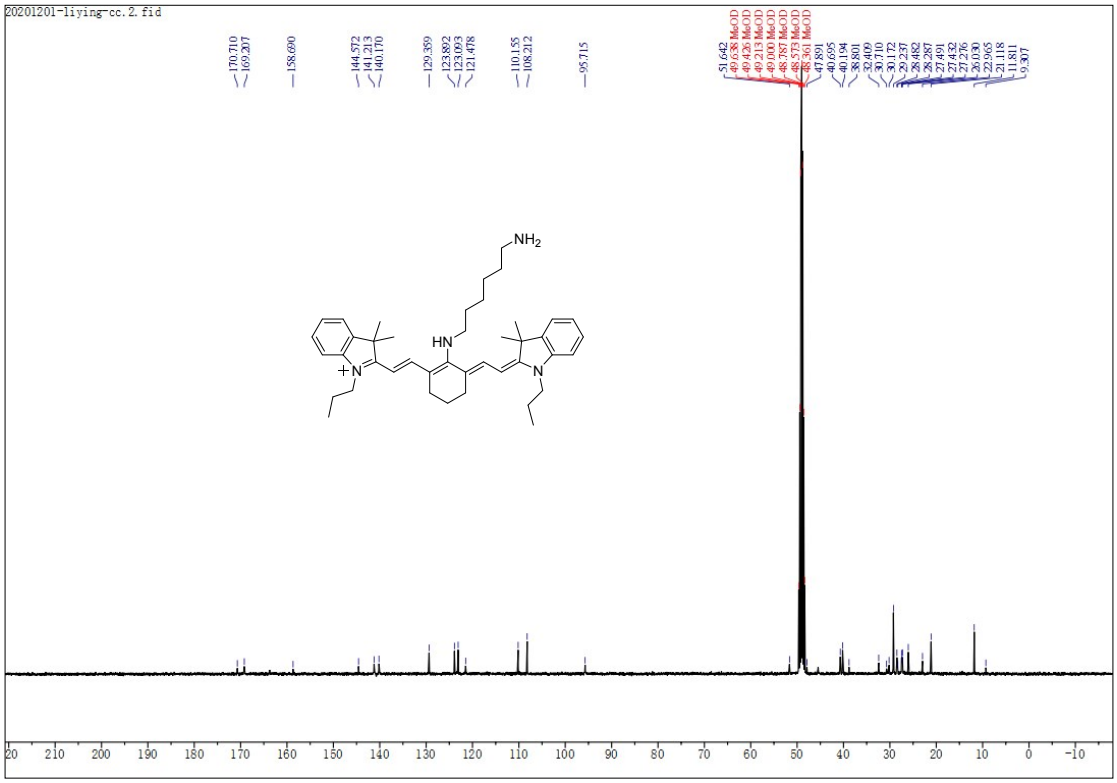
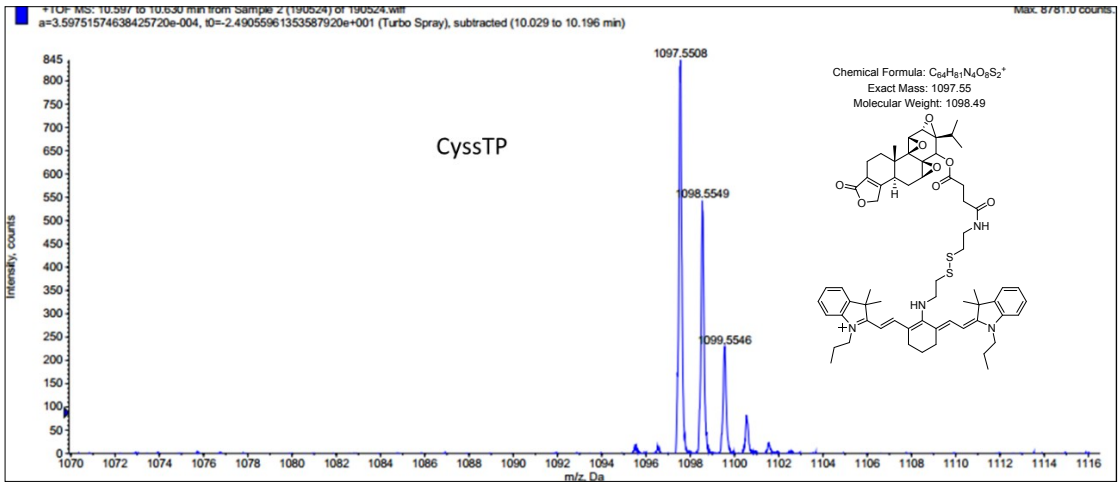


Fig. S4 High-Resolution Mass Spectrometer, ^{1}H NMR and ^{13}C NMR of Cycc.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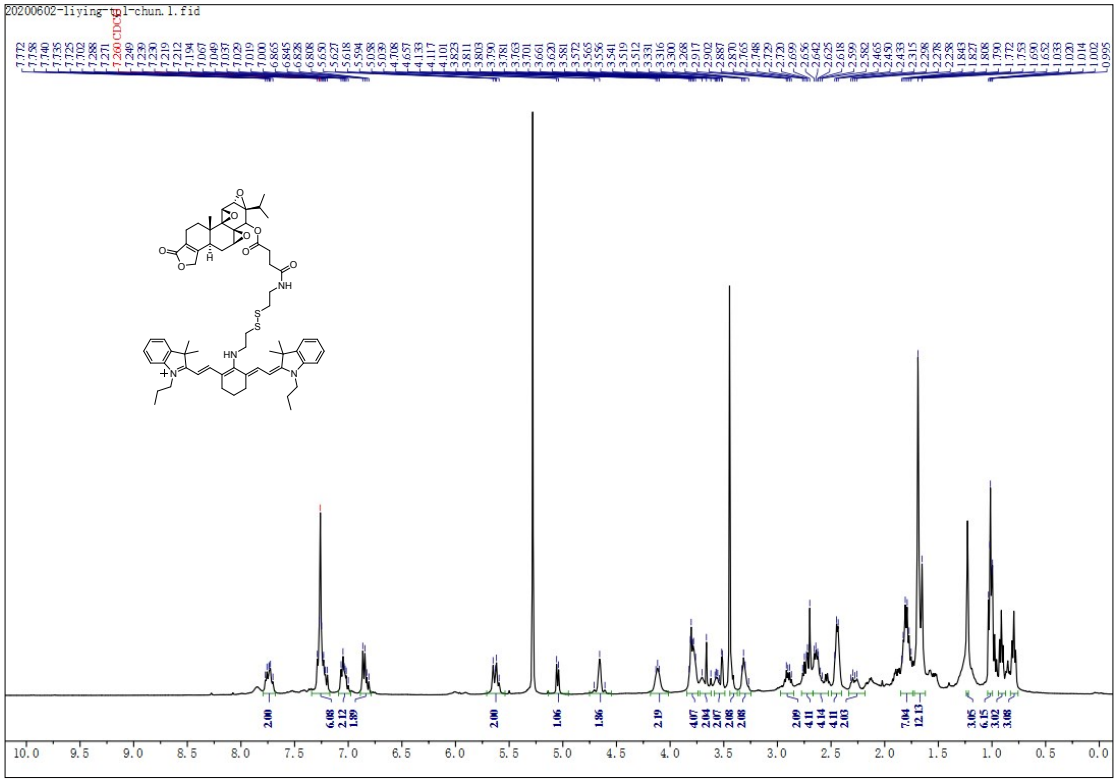
Max. 8781.0 counts.
CyssTP
1097.5508
1098.5549
1099.5546
Chemical Formula: C64H81N4O8S2+
Exact Mass: 1097.55
Molecular Weight: 1098.49
Intensity, counts
m/z, Da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9.0
8.5
8.0
7.5
7.0
6.5
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2.0
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0.5
0.0

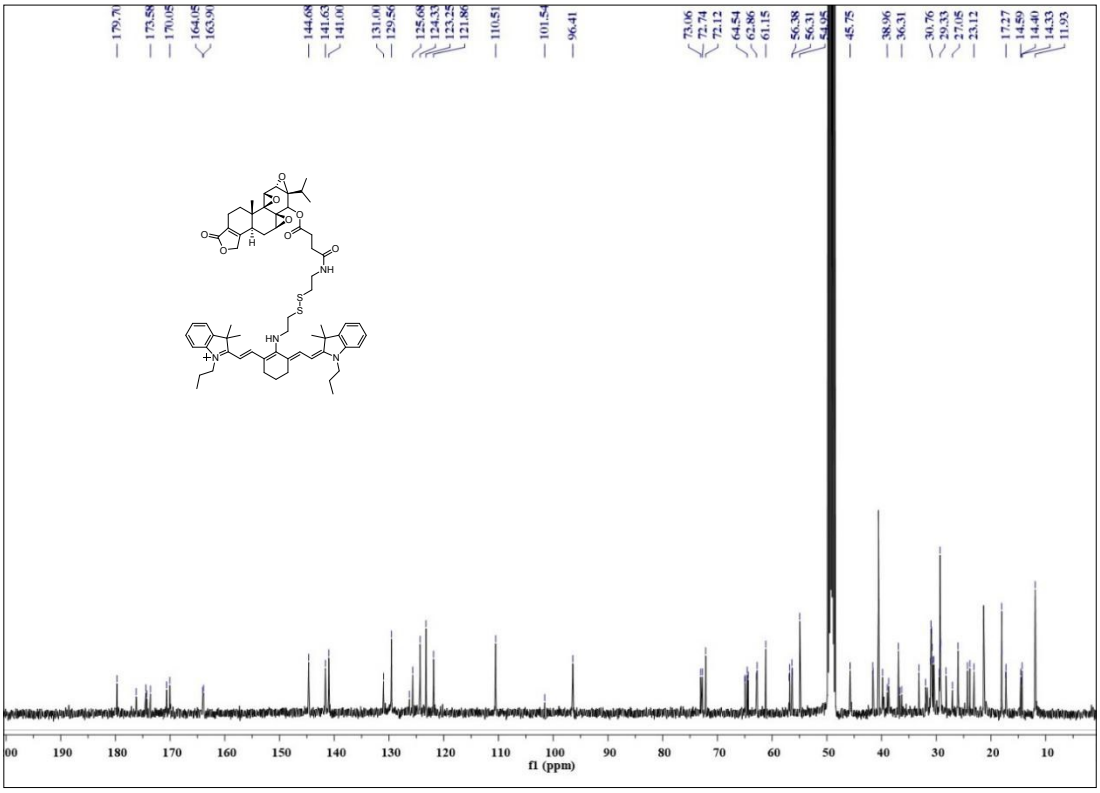
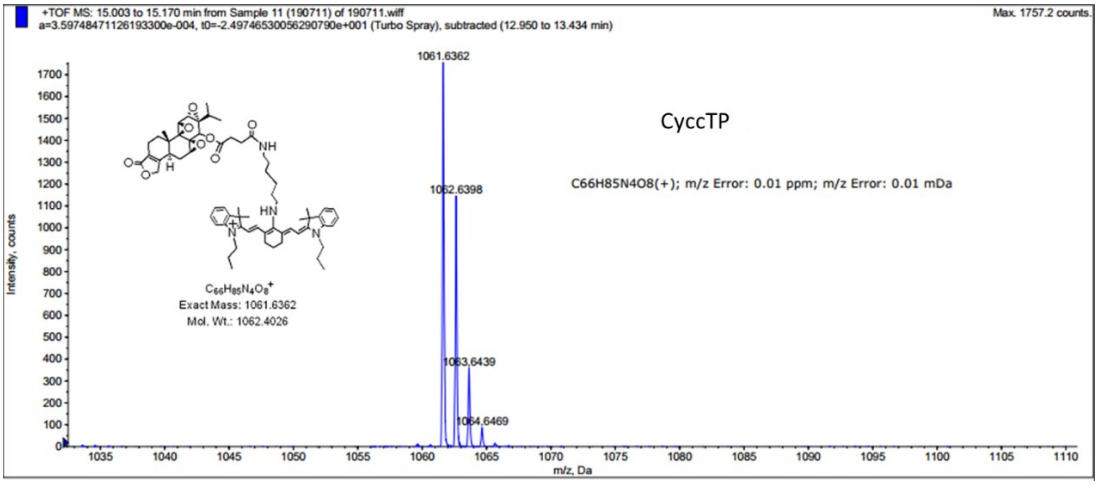


Fig. S5 High-Resolution Mass Spectrometer, ^{1}H NMR and ^{13}C NMR of CyssTP.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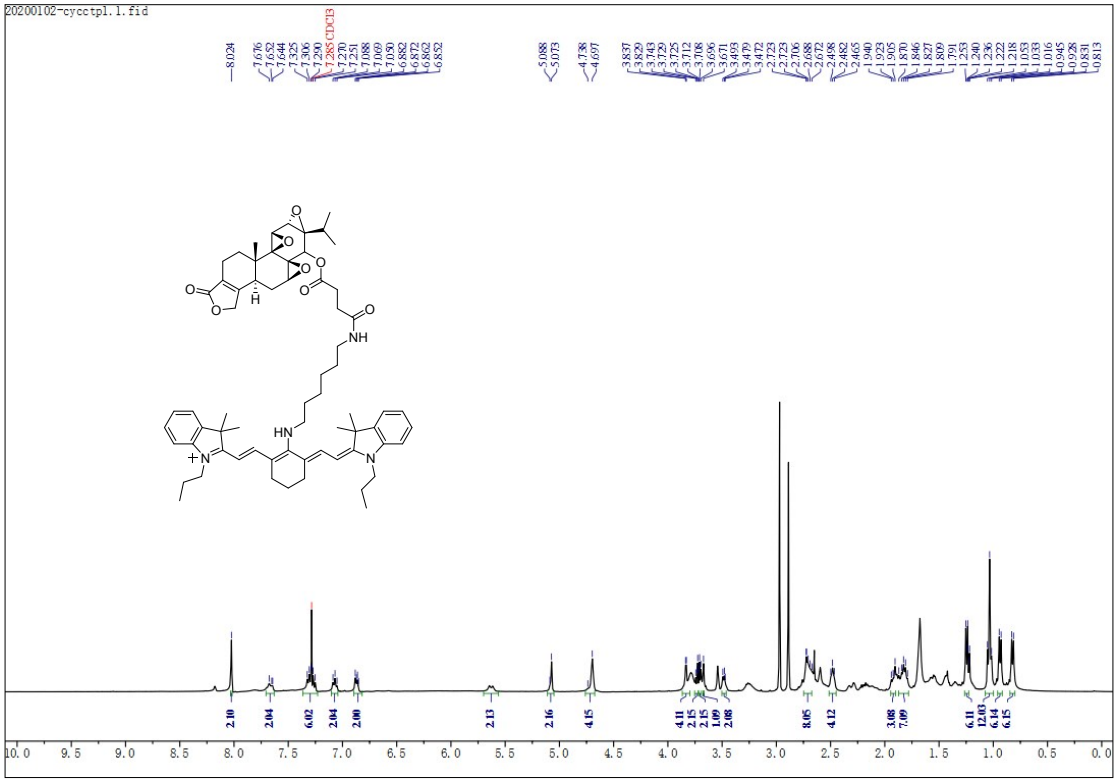
Max. 1757.2 counts.
1061.6362
1062.6398
1063.6439
1064.6469
CyccTP
C66H85N4O8(+); m/z Error: 0.01 ppm; m/z Error: 0.01 mDa
C66H85N4O8+
Exact Mass: 1061.6362
Mol. Wt.: 1062.4026
Intensity, counts
m/z, Da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7.676
7.652
7.644
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7.296
7.290
7.285 CDCl3
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7.099
7.090
6.882
6.872
6.862
6.852
5.088
5.073
4.738
4.697
3.837
3.829
3.743
3.729
3.725
3.712
3.706
3.696
3.671
3.493
3.479
3.472
2.723
2.713
2.706
2.688
2.672
2.498
2.482
2.465
1.940
1.923
1.905
1.870
1.846
1.827
1.809
1.791
1.253
1.240
1.236
1.222
1.218
1.033
1.018
1.016
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0.928
0.831
0.813
2.10
2.04
6.02
2.04
2.00
2.13
2.16
4.15
4.11
2.15
2.15
1.09
2.08
8.05
4.12
3.08
7.09
6.11
12.00
6.14
6.15

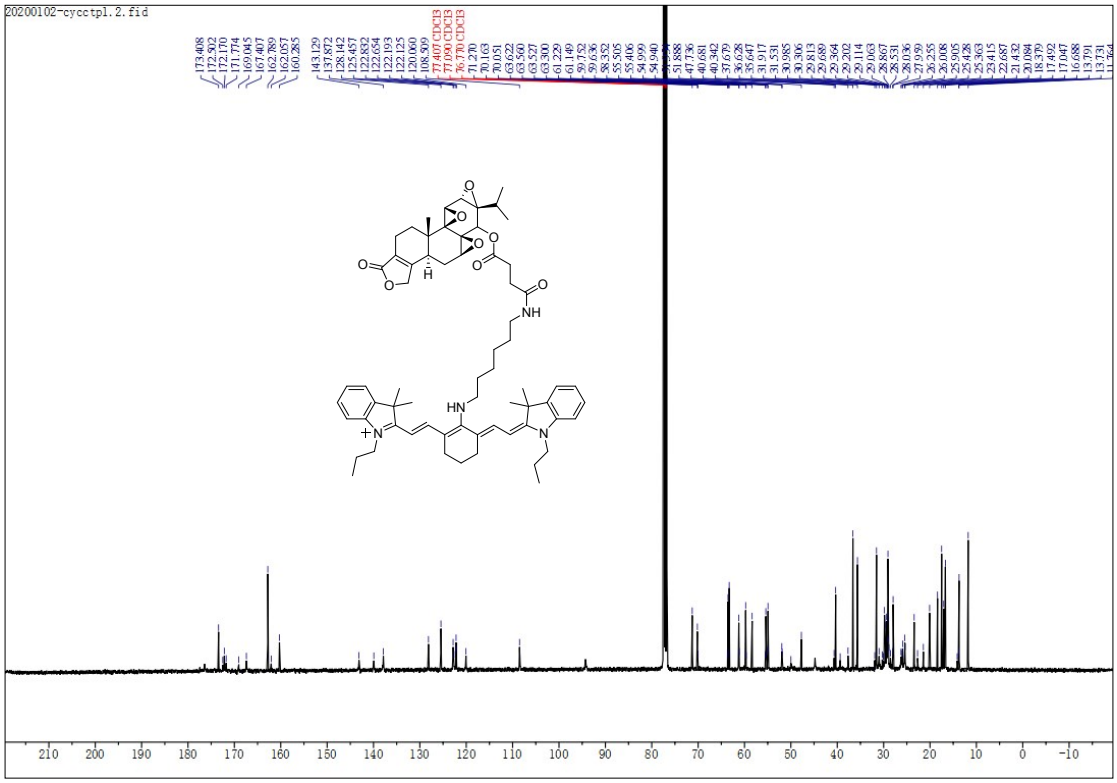

Fig. S6 High-Resolution Mass Spectrometer, ^{1}H NMR and ^{13}C NMR of CyccTP.

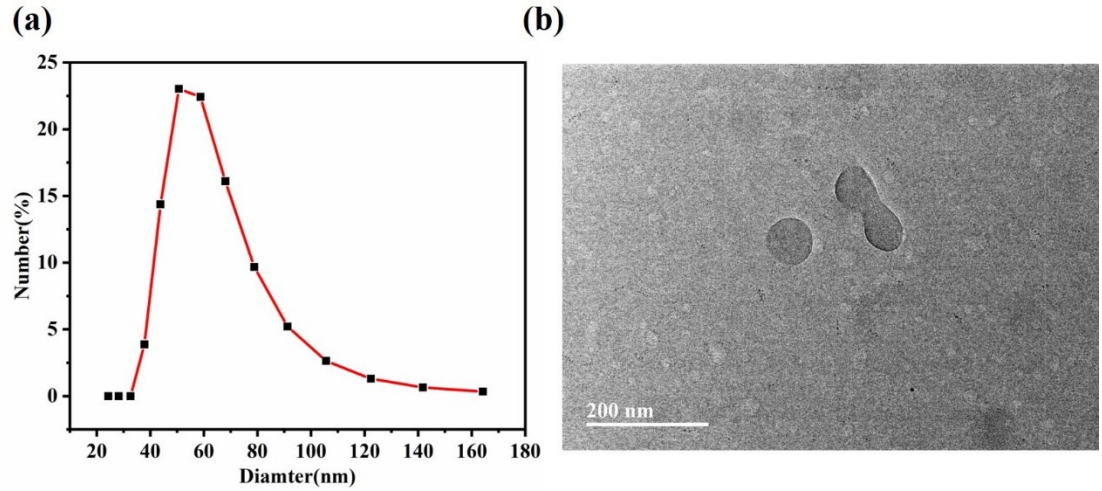

Fig. S7 The particle size(a) and TEM image(b) of CyccTPN.

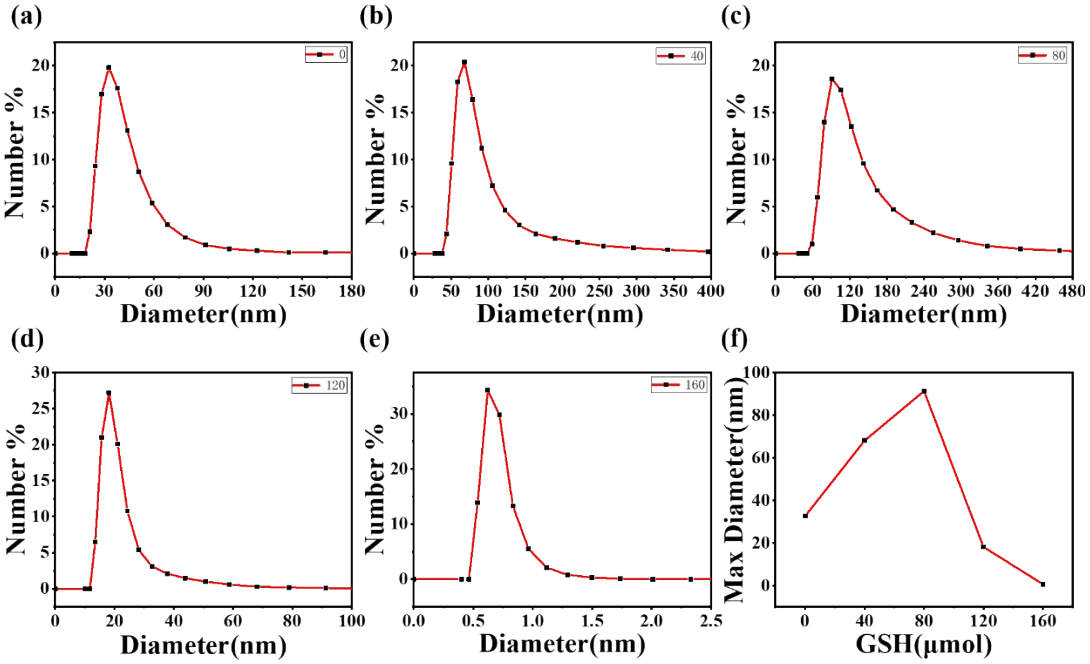

Fig. S8 The dynamic laser scattering (DLS) size of CyssTPN is 0-160 μM (a-e) due to the influence of GSH concentration. The maximum particle size (f) corresponding to the GSH concentration.

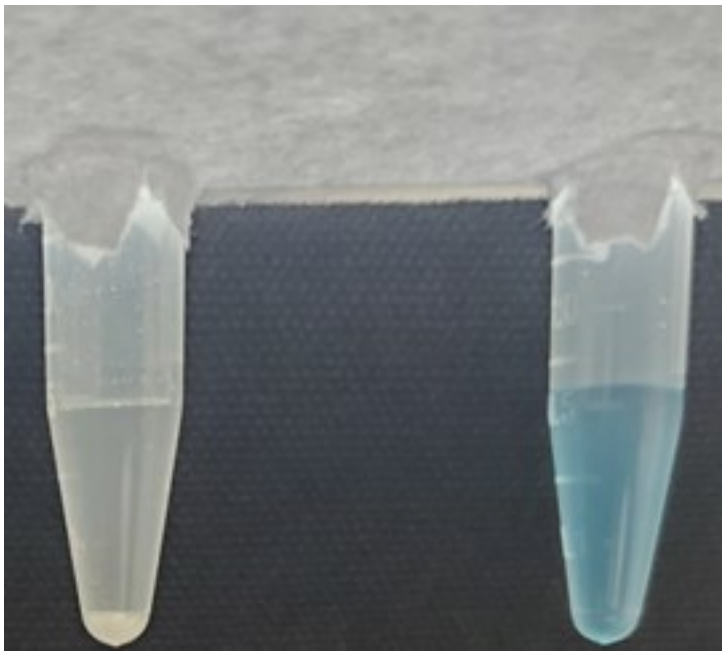

Fig.S9 Solubility of TP and CyssTPN in PBS buffer. (a)TP; (b)CyssTPN.

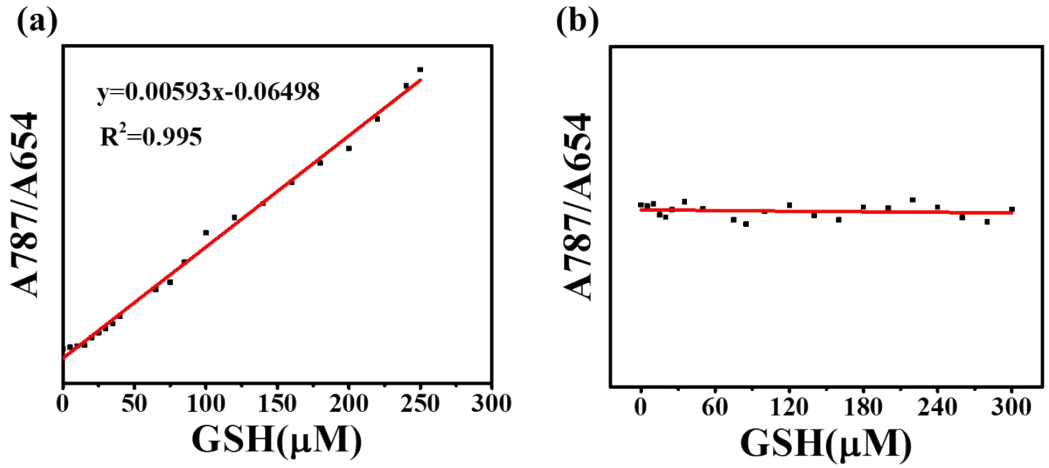

Fig. S10 The rate value of absorption of CyssTPN (787 nm/654 nm) (a)/ CyccTPN (787 nm/654 nm) (b) as function of GSH.

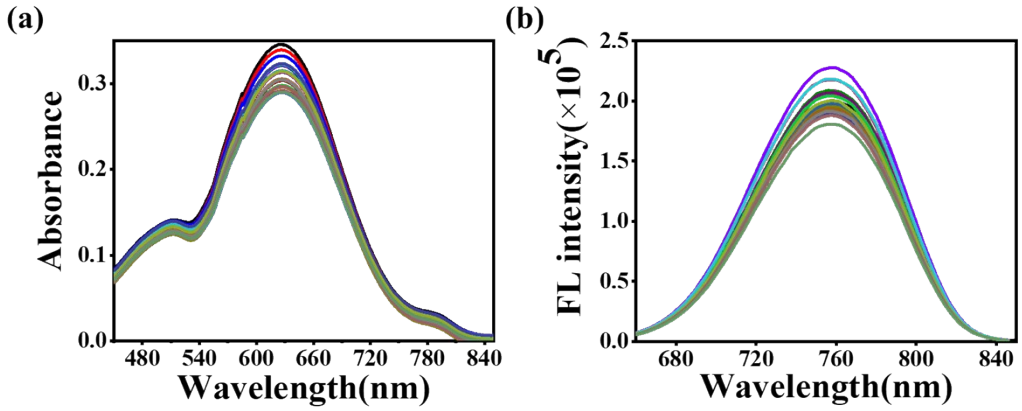

Fig. S11 (a) UV-Vis absorption of CyccTPN (100 μM) in the presence of GSH in the DMSO/PBS (1/1, v/v, PH = 7.4). (b) Fluorescence spectra of CyccTPN (100 μM) to GSH (λex = 646 nm/λem = 757 nm. GSH:0-250 μM.

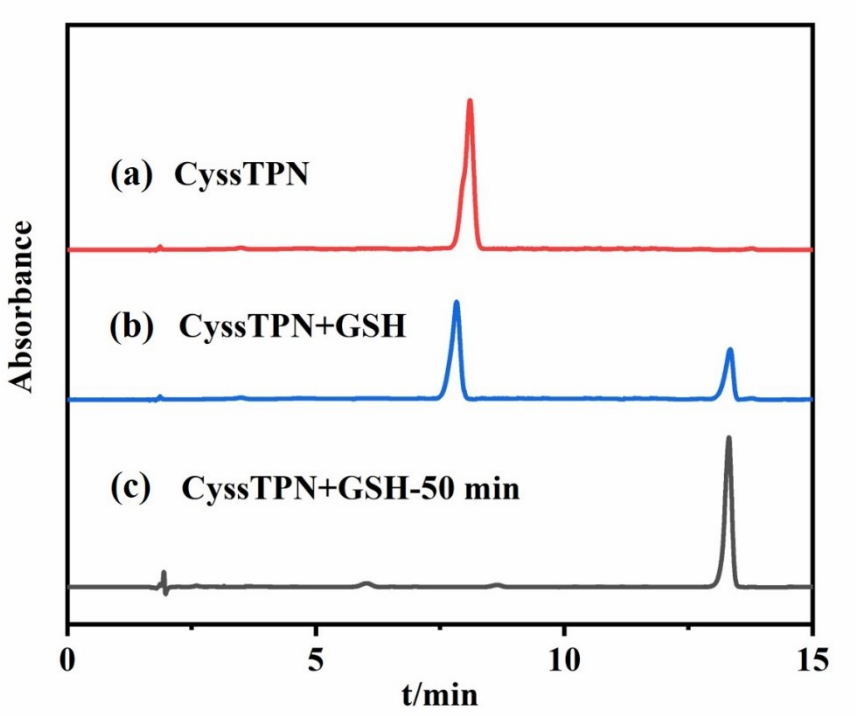

Fig. S12 Chromatograms of different reaction systems. (a) CyssTPN (100 μM); (b) CyssTPN (100 μM) treated with GSH; (c) CyssTPN (100 μM) treated with GSH for 50 min. The peaks eluted from the column were monitored at 254 nm with acetonitrile and water as eluents.

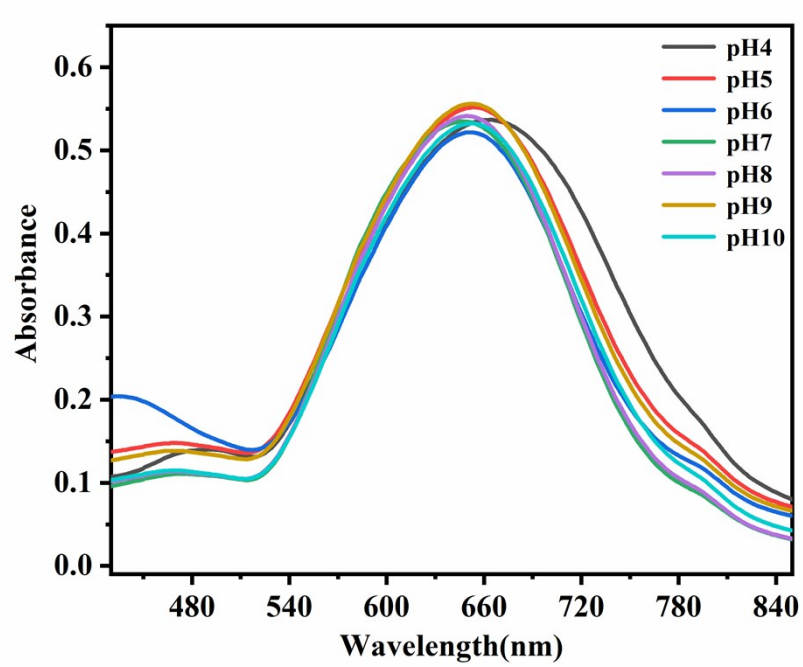

Fig. S13 the absorbance of CyssTPN (DMSO: H_2O = 1:1, 100 μM) in different pH buffer.

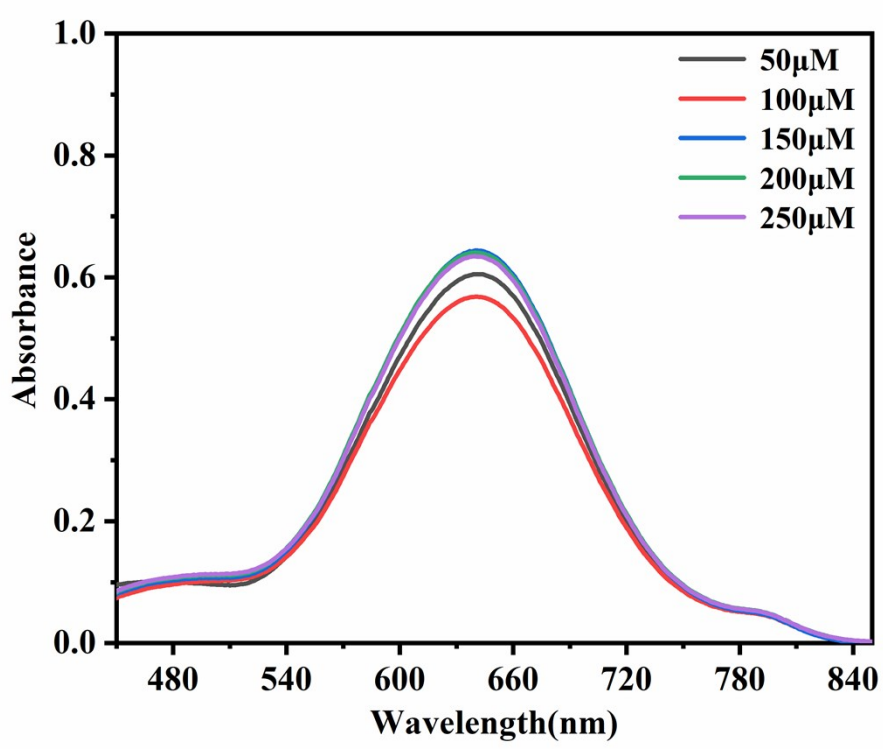

Fig. S14 the absorbance of CyssTPN (DMSO: H_2O = 1:1, 100 μM) in different concentration of Na^+.

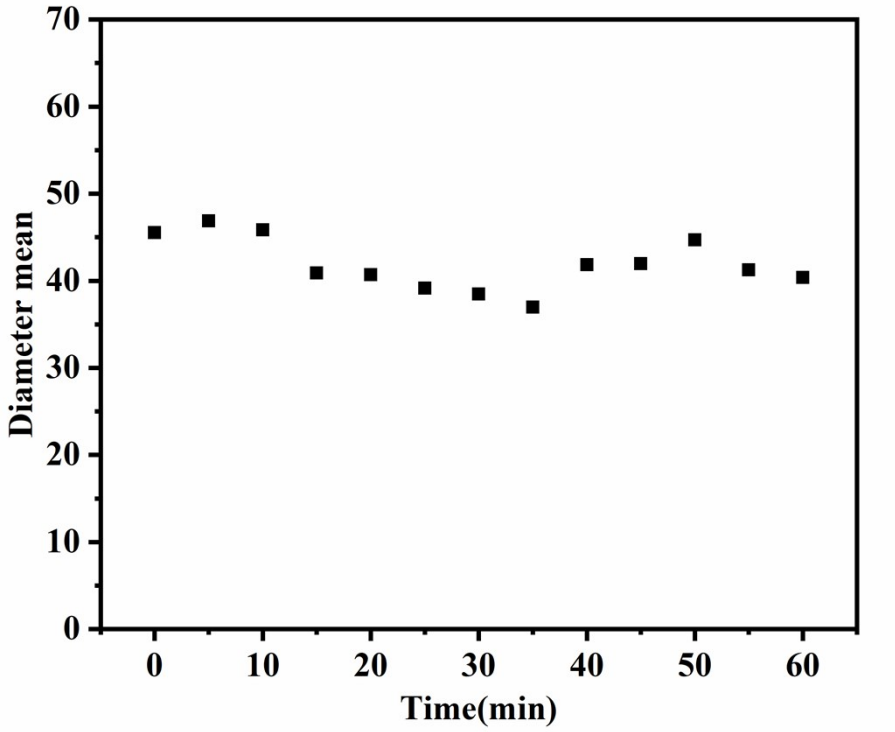

Fig. S15 Diameter mean of CyssTPN at different times (0-60 min) under illumination.

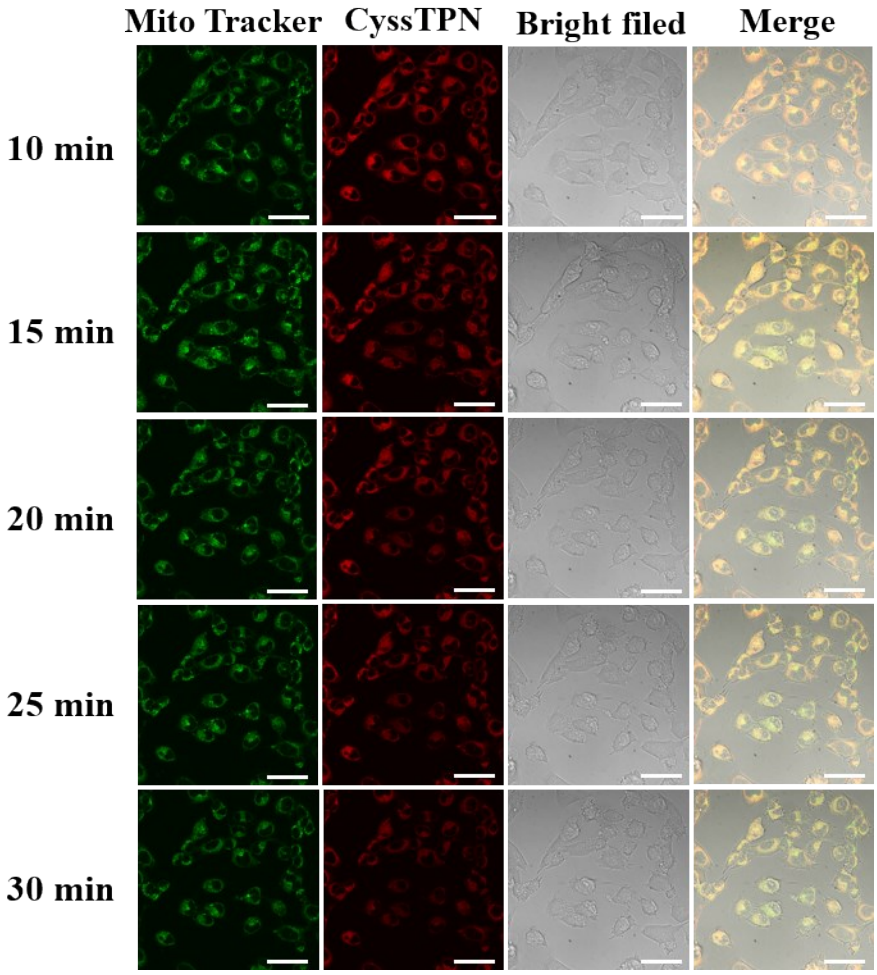

Fig. S16 Time-dependent Fluorescence confocal microscope images for tracking the activation of prodrug CyssTPN and CyccTPN. (b)Images were obtained at 10/15/20/25/30 min after cultivating CyssTPN with cells for 30 min. Scale bar: 20 μm.

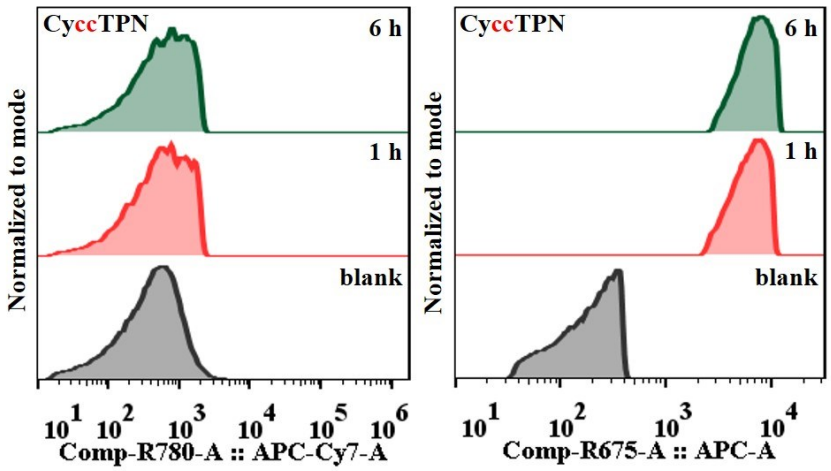

Fig. S17 Time-dependent Flow cytometry analysis of cellular uptake of CyccTPN real-timely tracking TP activation and the shift of fluorescence intensity after activation. Cancer cells: MCF-7.

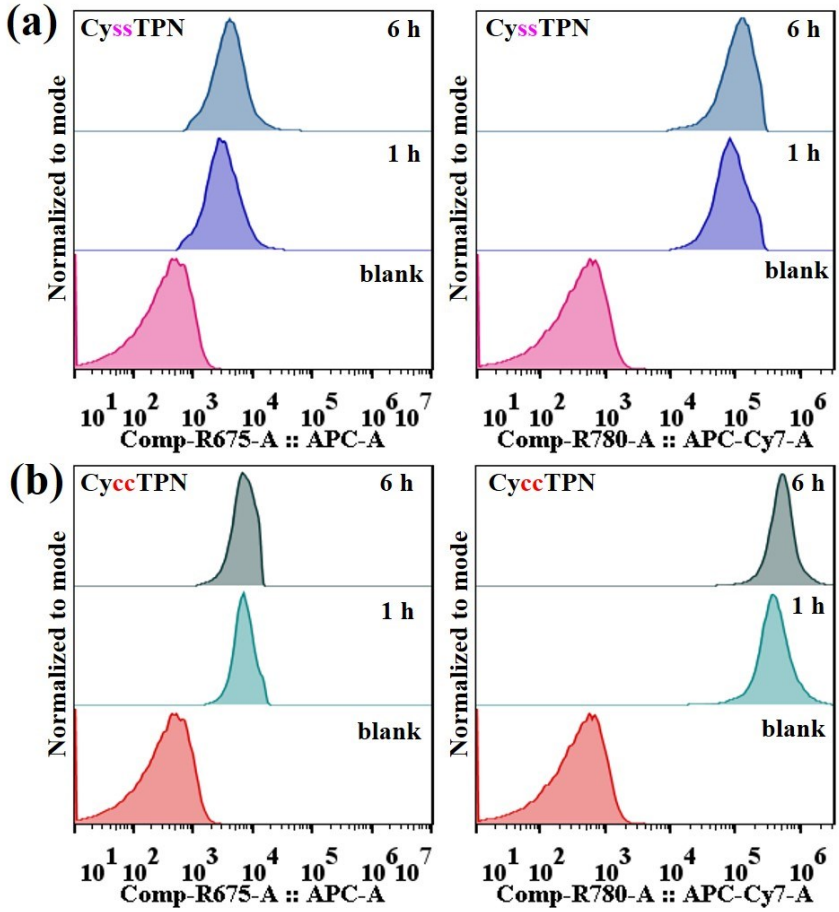

Fig. S18 Time-dependent Flow cytometry analysis of cellular uptake of CyssTPN (a)CyccTPN (b)real-timely tracking TP activation and the shift of fluorescence intensity after activation. Normal cells: L02.

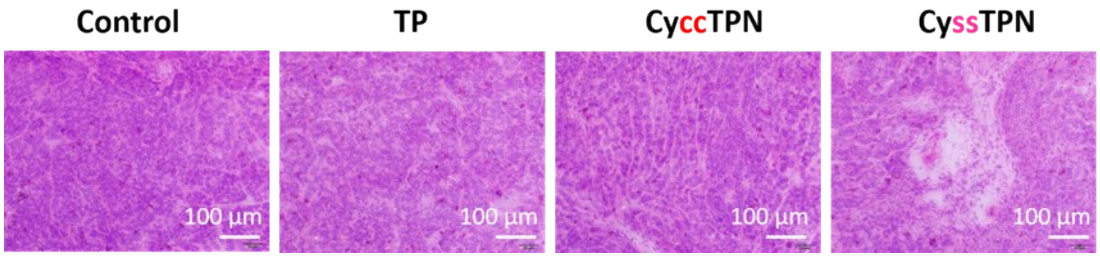

Fig. S19 *In vivo* inhibition using CyssTPN on MCF-7 tumor xenografts. H&E staining histological sections of tumor at 3 d after treatments with saline, TP, CyccTPN, CyssTPN. Scale bar:100 μm

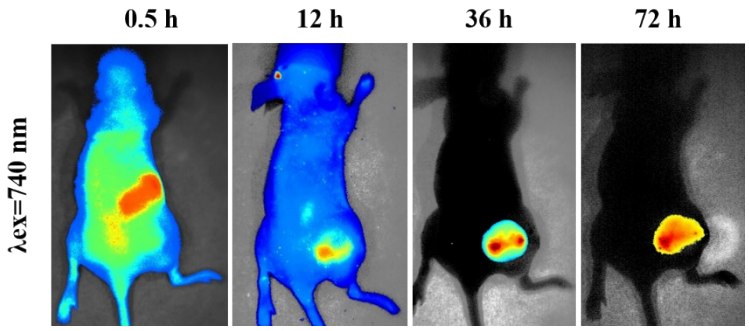

Fig. S20 *In vivo* biodistribution and NIRF imaging of CyssTPN in tumor-bearing mice at 0.5/12/48/72 h. The groups injected by CyssTPN excited by 740 nm.