Supporting Information

Self-assembled Nano-Photosensitizer for Targeted, Activatable, and Biosafe Cancer Phototheranostics

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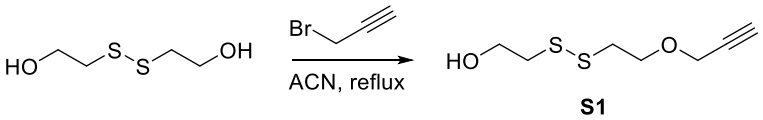
1. General Information

Reaction progress was monitored by TLC on pre-coated silica plates (Merck TLC Silica gel 60 F_{254}), and spots were visualized by UV, iodine, or other suitable stains. Flash column chromatography was carried out using silica gel. NMR (^{1}H-NMR, ^{13}C-NMR) spectra were recorded on the Bruker NMR spectrometers. High-resolution mass spectrometry (HRMS) was analyzed with the Bruker microTOF-Q Mass Spectrometer. HPLC analysis was carried out on the 1525 Waters HPLC system equipped with a 2489 UV/Visible detector. Water with 0.1% TFA and acetonitrile with 0.1% TFA were used as eluents, and the flow rate was set as 1 mL/min. Chemical shifts were reported in parts per million (ppm) referenced to appropriate internal standards or residual solvent peaks ($CDCl_3$ = 7.26 ppm, DMSO-d_6 = 2.50 ppm). The following abbreviations were used in reporting spectra, br s (broad singlet), s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublets). Cells were obtained from ATCC and cultured in the corresponding medium (Gibco) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco), 100 units/mL penicillin, 100 μg/mL streptomycin (Gibco) and maintained in a humidified 37°C incubator with 5% CO_2.

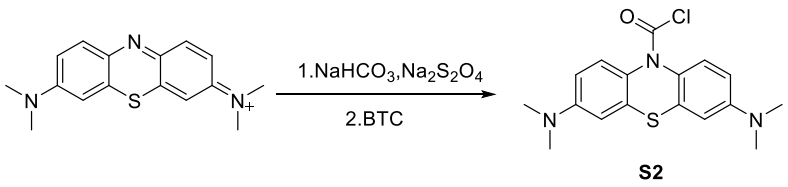
2. Chemical Synthesis



S1

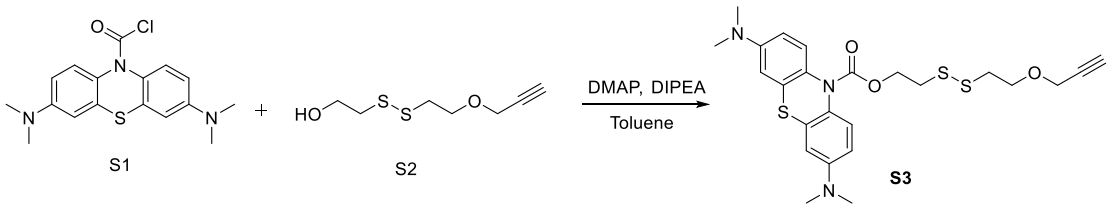
Scheme S1. Synthesis of **S1**.

2-((2-(prop-2-yn-1-yloxy)ethyl)disulfanyl)ethan-1-ol (S1). To a solution of 2,2'-disulfanediylbis(ethan-1-ol) (2.0 g, 12.99 mmol) in 20 mL of acetonitrile, 3-bromoprop-1-yne (1.85 g, 15.59 mmol) was added dropwise in 2 minutes. The mixture was then heated to reflux for 4 h, and TLC showed no starting material. Next, 50 mL of water was added to quench the reaction before the mixture was extracted with 3 × 20 mL of ethyl acetate. The organic phase was combined and dried over anhydrous sodium sulfate, concentrated in vacuum. The residue was then purified by column chromatography (PE : EA = 5 : 1) to offer **S1** as a light-yellow oil (1.62 g, 54.1%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.21 – 4.15 (m, 2H), 3.88 (t, J = 5.8 Hz, 2H), 3.79 (t, J = 6.4 Hz, 2H), 2.91 (t, J = 6.4 Hz, 2H), 2.86 (t, J = 5.8 Hz, 2H), 2.46 (t, J = 2.4 Hz, 1H), 2.23 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 79.32, 74.95, 68.13, 60.26, 58.25, 41.40, 38.34. HRMS: m/z $[M+H]^+$ calcd. for $C_7H_{13}O_2S_2^+$: 193.0357; found: 193.0457.



Scheme S2. Synthesis of **S2**.

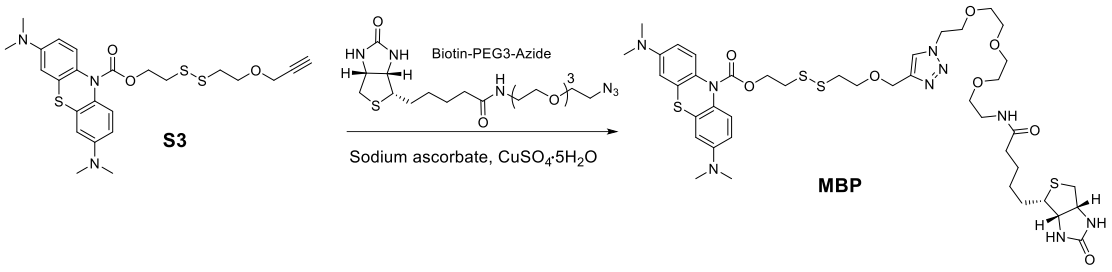
3,7-bis(dimethylamino)-10H-phenothiazine-10-carbonyl chloride (S2). Methylene blue (320.2 mg, 1.0 mmol) and $NaHCO_3$ (502.4 mg, 6.0 mmol) were dissolved in 10 mL of water and 10 mL of dichloromethane. Then sodium dithionite (697.2 mg, 4.0 mmol) dissolved in 5 mL water was added, and the mixture was stirred at 40 °C under a nitrogen atmosphere until the solution became yellow (typically within 15-30 min). The mixture was cooled with an ice-water bath, to which bis(trichloromethyl)carbonate (178.1 mg, 0.6 mmol) in 5 mL of dichloromethane was added dropwise. After addition, the mixture was stirred for another 1 h. The solution was poured into 25 mL of ice water while stirring, and the resulting mixture was extracted with 3 × 20 mL portions of dichloromethane. The combined extracts were washed with brine, dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure without heating. Column chromatography (PE : EA = 9 : 1) of the crude product over silica gel gave product **S2** as a white solid (215.2 mg, 62%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.39 (d, J = 8.7 Hz, 2H), 6.70 (d, J = 2.7 Hz, 2H), 6.62 (dd, J = 8.9, 2.7 Hz, 2H), 2.96 (s, 12H). HRMS: m/z $[M+H]^+$ calcd. for $C_{17}H_{19}ClN_3OS^+$: 348.0937; found: 348.1087.



Scheme S3. Synthesis of **S3**.

2-((2-(prop-2-yn-1-yloxy)ethyl)disulfanyl)ethyl-3,7-bis(dimethylamino)-10H-phenothiazine-10-carboxylate (S3). To a solution of **S1** (381.7 mg, 1.1 mmol) and **S2** (192 mg, 1 mmol) in 10 mL of dry toluene, N,N-diisopropylethylamine (522 μL, 3 mmol) and 4-dimethylaminopyridine (12.2 mg, 0.1 mmol) was added. The mixture was stirred at room temperature under nitrogen atmosphere overnight. The next day, the solvent was removed in vacuum, and the residue was purified by flash column (DCM : MeOH = 30 : 1) to offer **S3** as a colorless oil (298 mg, 59.2%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 (d, J = 8.8 Hz, 2H), 6.66 (d, J = 2.7 Hz, 2H), 6.62 (dd, J = 8.9, 2.8 Hz,

2H), 4.42 (s, 2H), 4.16 (d, *J* = 2.4 Hz, 2H), 3.75 (t, *J* = 6.3 Hz, 2H), 2.97 (t, *J* = 6.5 Hz, 2H), 2.92 (s, 12H), 2.87 (t, *J* = 6.3 Hz, 2H), 2.45 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.18, 148.79, 132.73, 128.11, 127.03, 111.11, 110.31, 79.49, 74.92, 68.00, 63.94, 58.23, 40.75, 38.59, 37.48. HRMS m/z $[M+H]^+$ calcd. for $C_{24}H_{30}N_3O_3S_3^+$: 504.1449; found: 504.1649.



Scheme S4. Synthesis of **MBP**.

2-((2-((1-(13-oxo-17-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-3,6,9-trioxa-12-azaheptadecyl)-1H-1,2,3-triazol-4-yl)methoxy)ethyl)disulfaneyl)-ethyl 3,7-bis (dimethylamino)-10H-phenothiazine-10-carboxylate (MBP). The synthesis of **MBP** was followed the previous literature[1],[2]. Biotin-PEG_3-Azide (44.5 mg, 0.1 mmol) and sodium ascorbate (4 mg, 0.02 mmol) were added to a solution of **S3** (50.4 mg, 0.1 mmol) in 5.0 mL of EtOH and MeOH (2 : 1). The reaction mixture was degassed for 15 min by purging argon gas. Then copper sulfate pentahydrate (2.5 mg, 0.01 mmol) in 3 mL of water was added. The mixture was stirred overnight at room temperature. Then the mixture was concentrated in vacuum and purified by column chromatography (DCM : MeOH= 20 : 1) to afford the desired final compound as light blue oil (63.5 mg, 67.9%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 – 7.71 (m, 1H), 7.33 (d, *J* = 8.6 Hz, 2H), 6.91 (s, 1H), 6.70 (s, 1H), 6.64 – 6.56 (m, 4H), 5.90 (s, 1H), 4.62 (s, 2H), 4.49 (t, *J* = 4.7 Hz, 2H), 4.45 – 4.33 (m, 3H), 4.26 – 4.22 (m, 1H), 3.82 (t, *J* = 4.7 Hz, 2H), 3.72 (t, *J* = 6.1 Hz, 2H), 3.62 (s, 2H), 3.57 – 3.51 (m, 6H), 3.41 – 3.35 (m, 3H), 3.08 (d, *J* = 4.6 Hz, 1H), 2.89 (s, 12H), 2.86 – 2.80 (m, 4H), 2.71 – 2.64 (m, 1H), 2.19 – 2.13 (m, 2H), 1.42 – 1.36 (m, 2H), 1.35 – 1.28 (m, 2H), 1.25 (s, 2H), 1.22 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.53, 154.13, 148.64, 144.67, 132.70, 128.17, 127.02, 123.93, 111.20, 110.40, 70.47, 70.38, 70.33, 70.03, 69.94, 69.40, 68.41, 64.34, 63.94, 61.79, 60.23, 55.72, 50.19, 40.81, 40.53, 39.13, 38.71, 37.27, 35.94, 28.31, 28.11, 25.68. HRMS: m/z $[M+H]^+$ calcd. for $C_{42}H_{62}N_9O_8S_4^+$: 948.3604; found: 948.3678.

3. HPLC/MS Analysis of the MBNPs/GSH Reaction

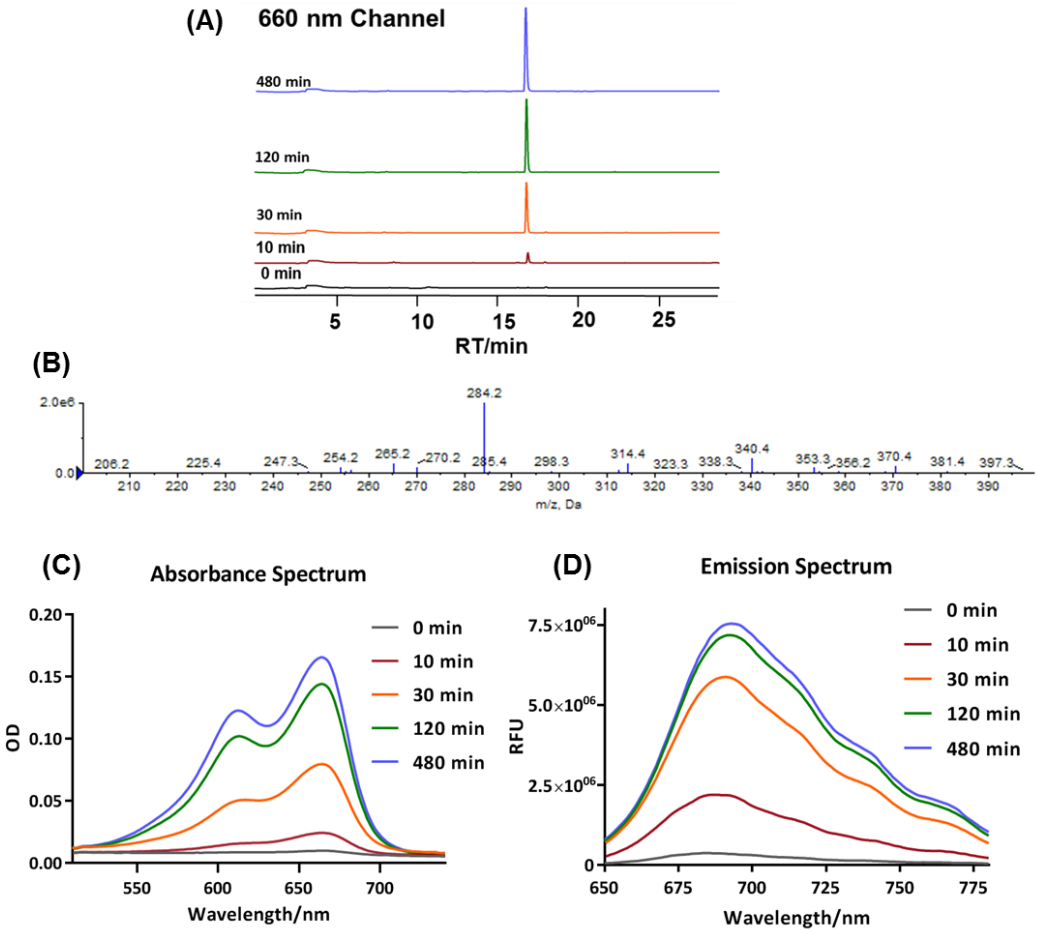


Figure S1. (A) The 660 nm channel of HPLC analysis. (B) MS analysis of rising peak in (A). (C) (D) Absorption and emission spectra of the reaction mixtures in HPLC analysis.

4. In Vitro Analysis of the MBNPs/GSH Reaction

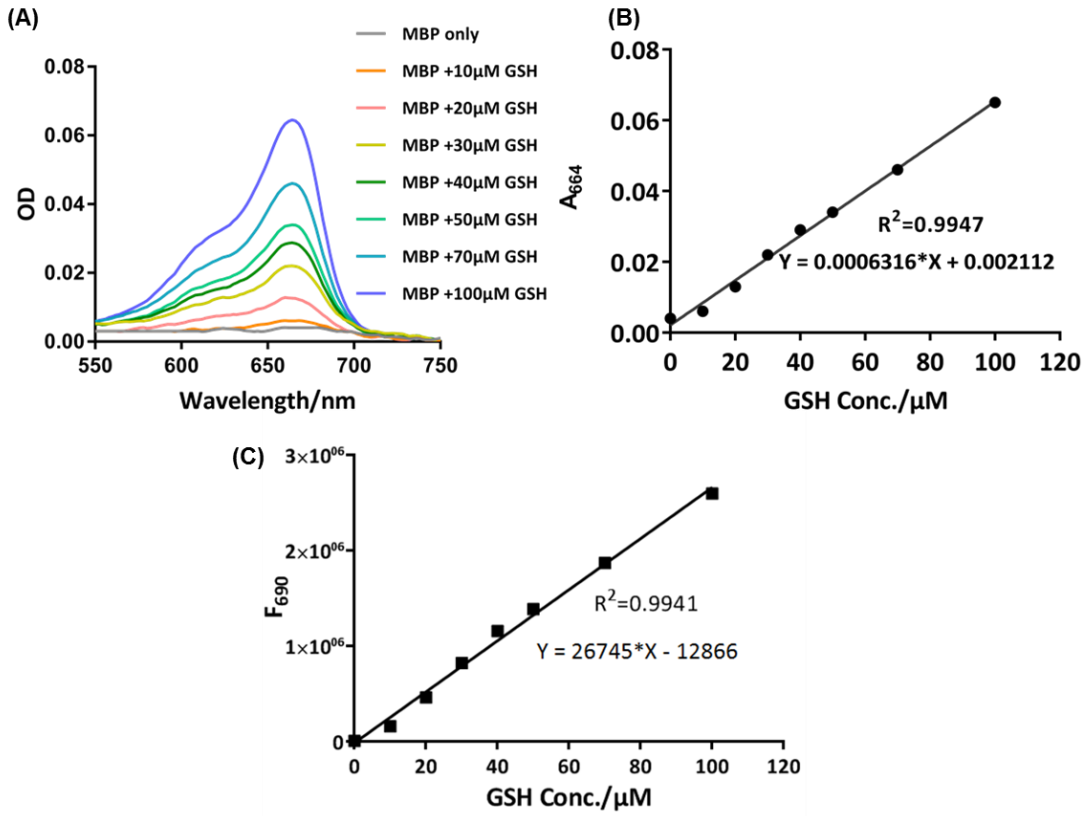


Figure S2. In vitro dose-dependent analyses of the **MBNPs**/GSH reaction. (A) Absorption spectra of the reaction mixtures. (B) (C) Analysis of the relations between GSH and absorbance (664 nm)/fluorescence (690 nm).

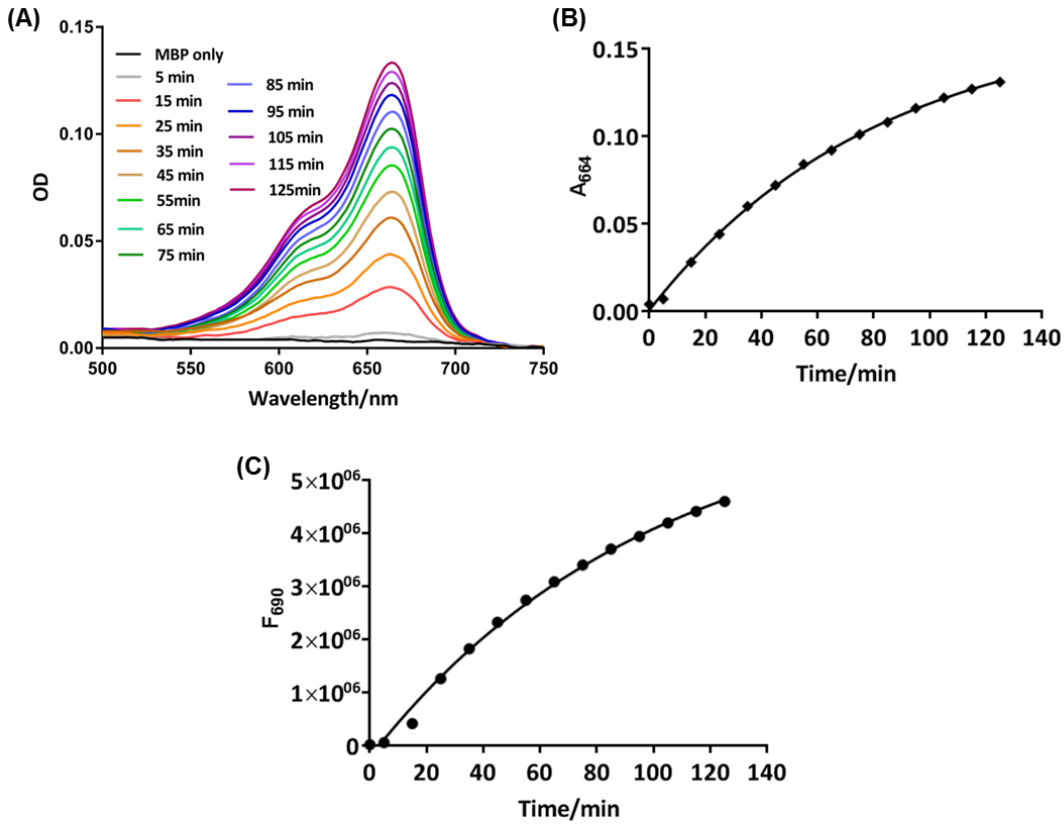


Figure S3. In vitro time-dependent analyses of the **MBNPs**/GSH reaction. (A) Absorption spectra of the reaction mixtures. (B) (C) Analysis of time-dependent absorbance (664 nm)/fluorescence intensity (690 nm).

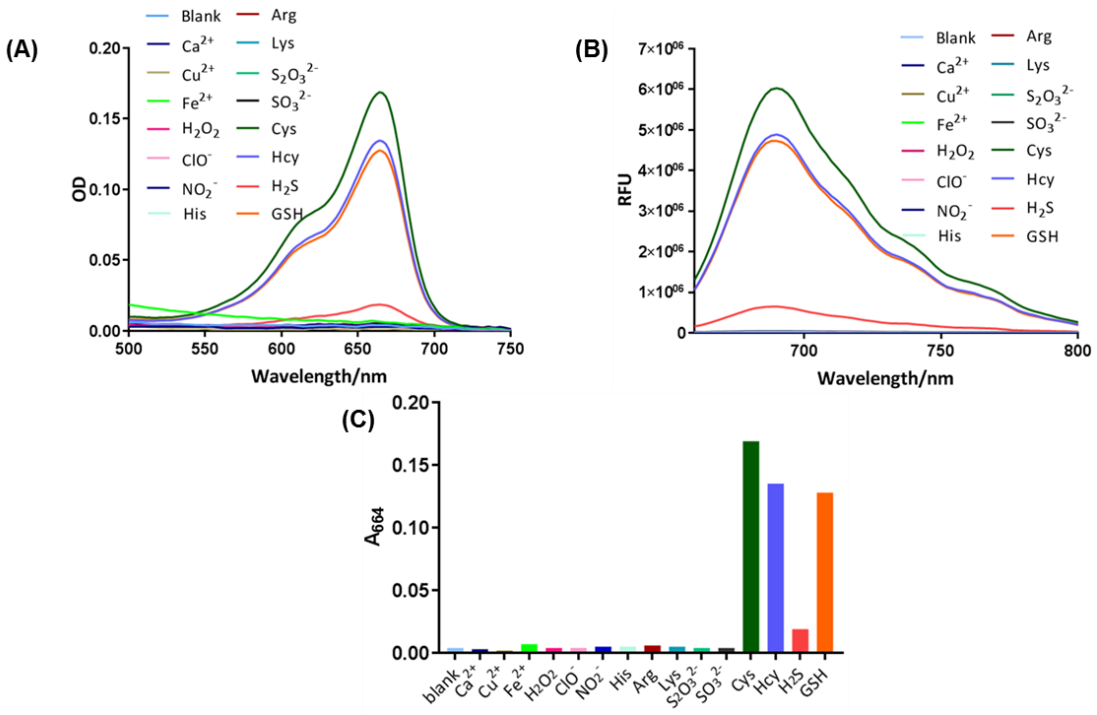


Figure S4. (A) (B) Absorption and emission spectra of **MBNPs** with different reactive species. (C) The 664 nm absorbance of (A).

5. Reactive Oxygen Species (ROS) Evaluation

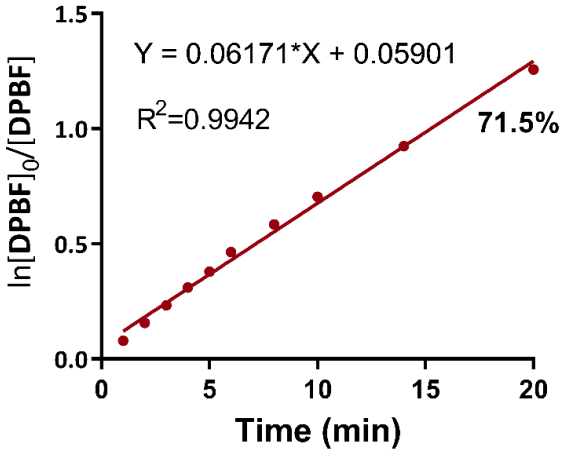


Figure S5. In vitro evaluation of ROS generation of **MBNPs**.

6. PA Analysis of MBNPs

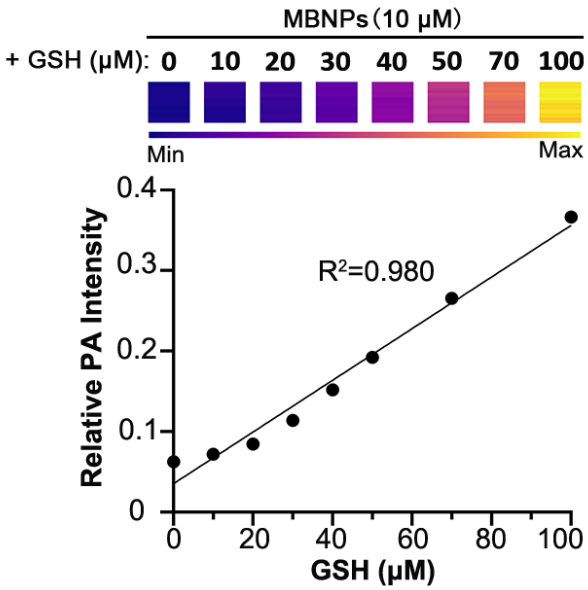


Figure S6. In vitro PA analysis of the **MBNPs** (10 μM) and GSH (0~100 μM).

7. Estimation of Intracellular GSH Levels

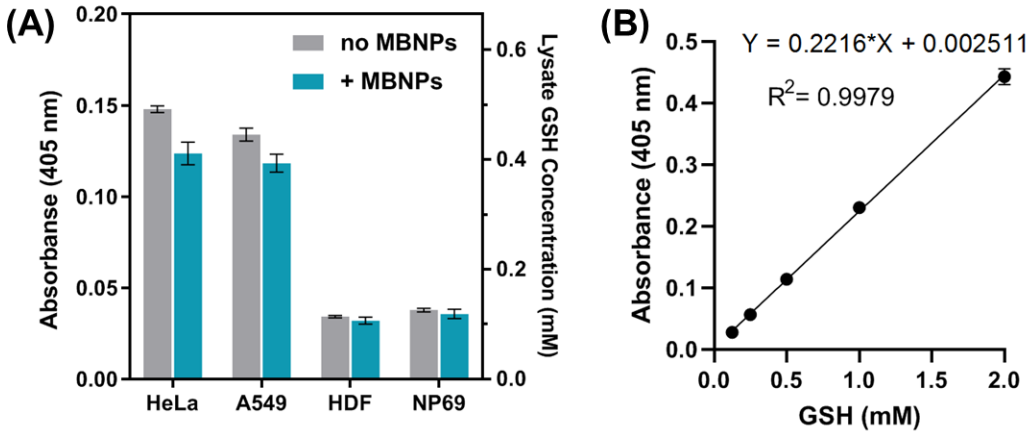


Figure S7. (A) Absorbance (405 nm)/GSH concentrations of cell lysates (10 μL) treated with 50 μL of DTNB (0.5 mM in PBS) with/without **MBNPs** (20 μM). GSH concentrations were converted based on the standard curve in (B). (B) Standard curve of GSH/DTNB in lysis buffer.

8. Cellular Imaging

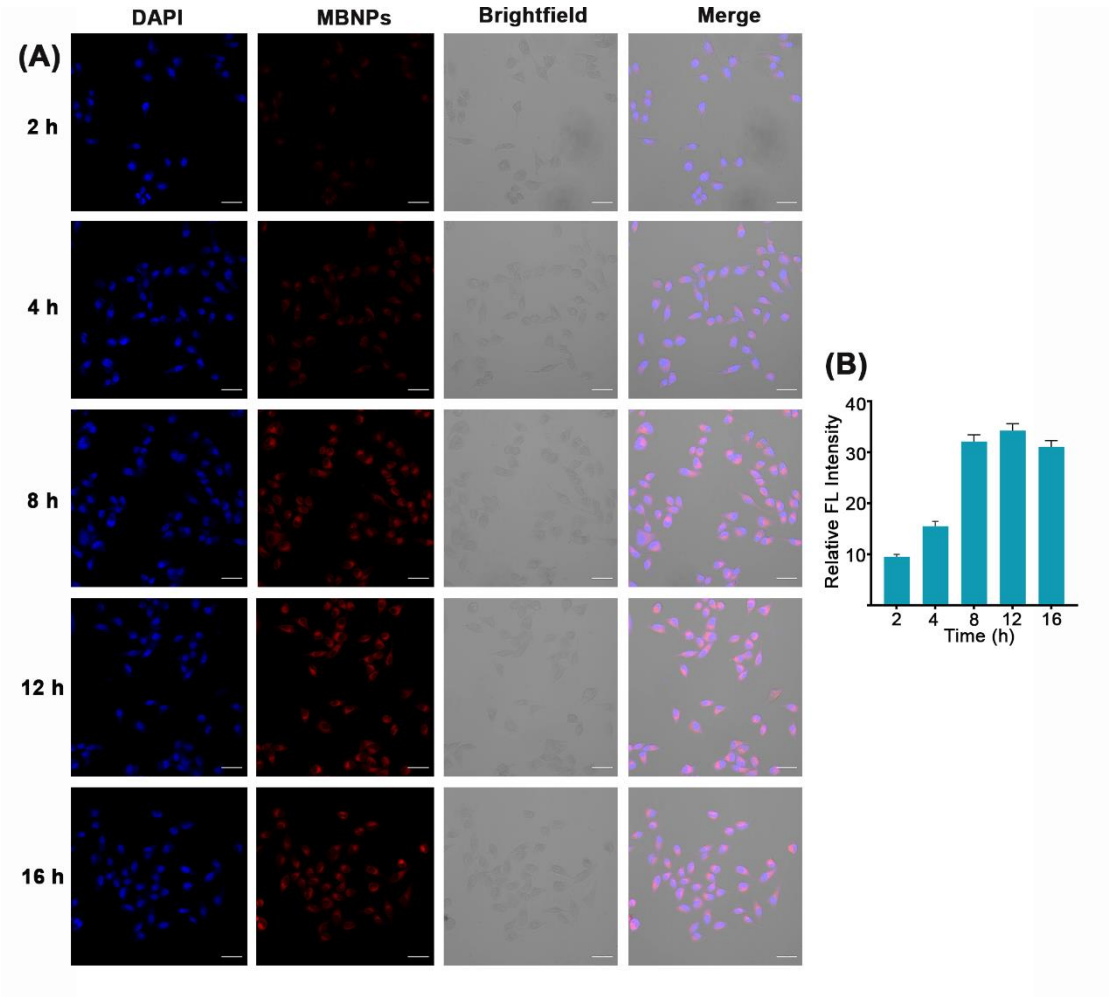


Figure S8. (A) Cellular imaging of **MBNPs** (2 μM) with HeLa cells at different stages. (scale bar = 50 μm) (B) Quantification of fluorescence in the red channel.

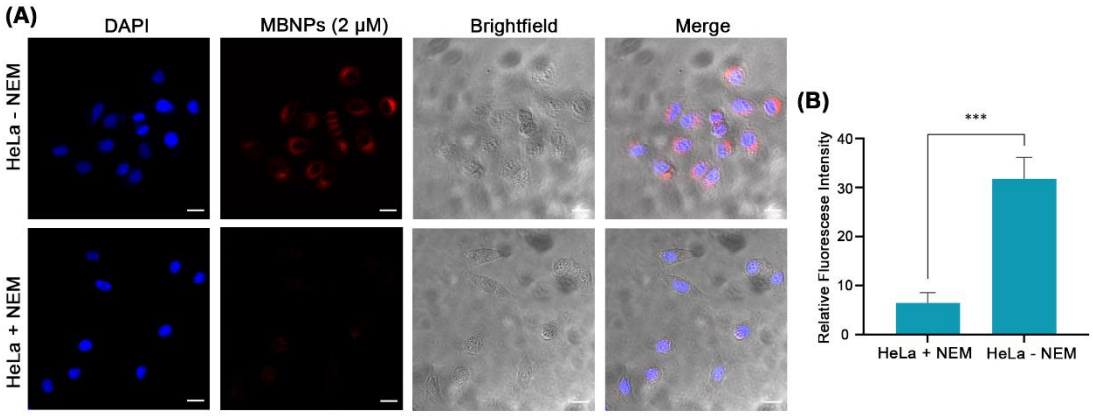


Figure S9. (A) Cellular imaging of **MBNPs** (2 μM) with Hela cells (12 h), with/without prior NEM (1 mM) treatment (30 min). (scale bar = 20 μm). (B) Quantification of fluorescence in the red channel of (A).

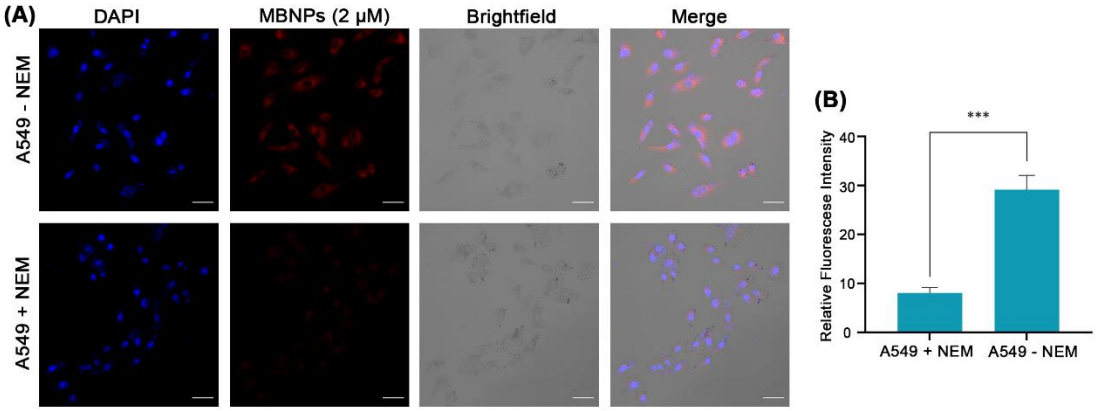


Figure S10. (A) Cellular imaging of **MBNPs** (2 μM) with A549 cells (12 h), with/without prior NEM (1 mM) treatment (30 min). (scale bar = 50 μm). (B) Quantification of fluorescence in the red channel of (A).

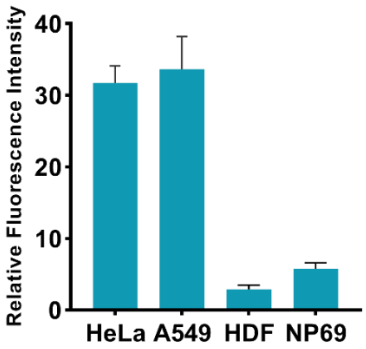


Figure S11. Fluorescence quantification for cellular imaging of **MBNPs** treated with different cells (red channel of **Figure 4**).

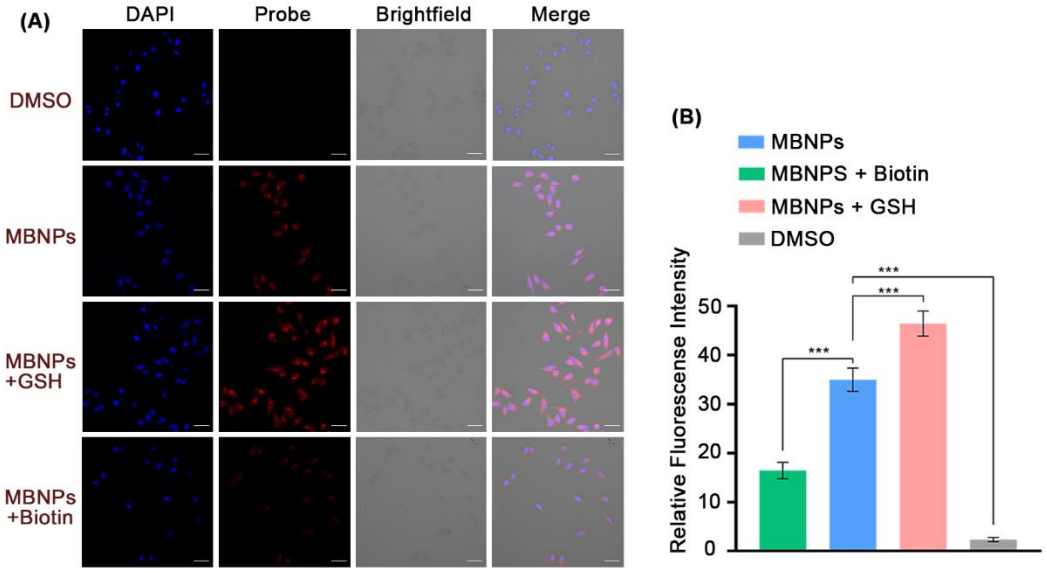


Figure S12. (A) Cellular imaging of **MBNPs** (2 μM) with HeLa cells (12 h) in the presence of exogenous GSH (1 mM) or biotin (1 mM). (scale bar = 50 μm). (B) Quantification of fluorescence in the red channel of (A).

9. Cytotoxicity

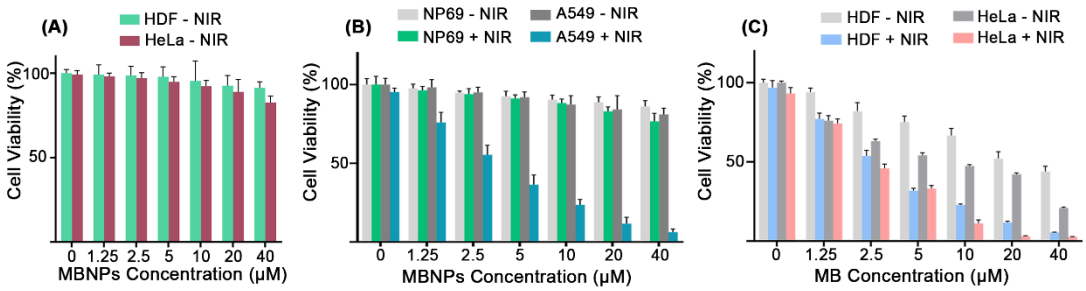


Figure S13. (A) Dark cytotoxicity of **MBNPs** towards HDF and HeLa cells (24 h). (B) Dark and photo cytotoxicity of **MBNPs** towards NP69 and A549 cells (24 h). (C) Dark and photo cytotoxicity of **MB** towards HDF and HeLa cells (24 h). Data represent mean ± SEM (n = 5).

10. Cellular ROS

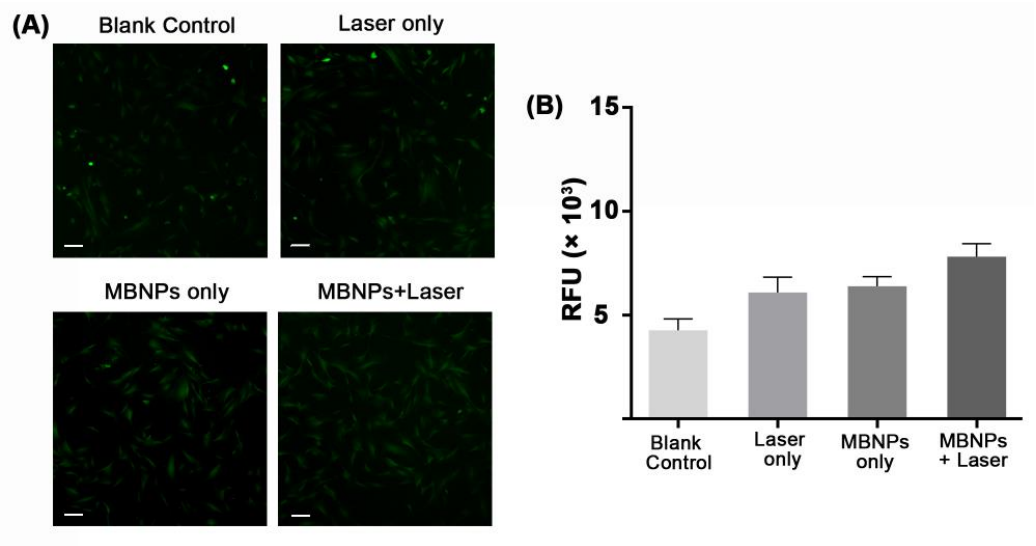


Figure S14. (A) Cellular ROS evaluation of **MBNPs** (20 μM) with H_2DCFDA (10 μM) by fluorescence imaging in HDF cells. Scale bar = 100 μm. (B) Fluorescence quantification of (A).

11. Histopathological and Immunohistochemical Analysis

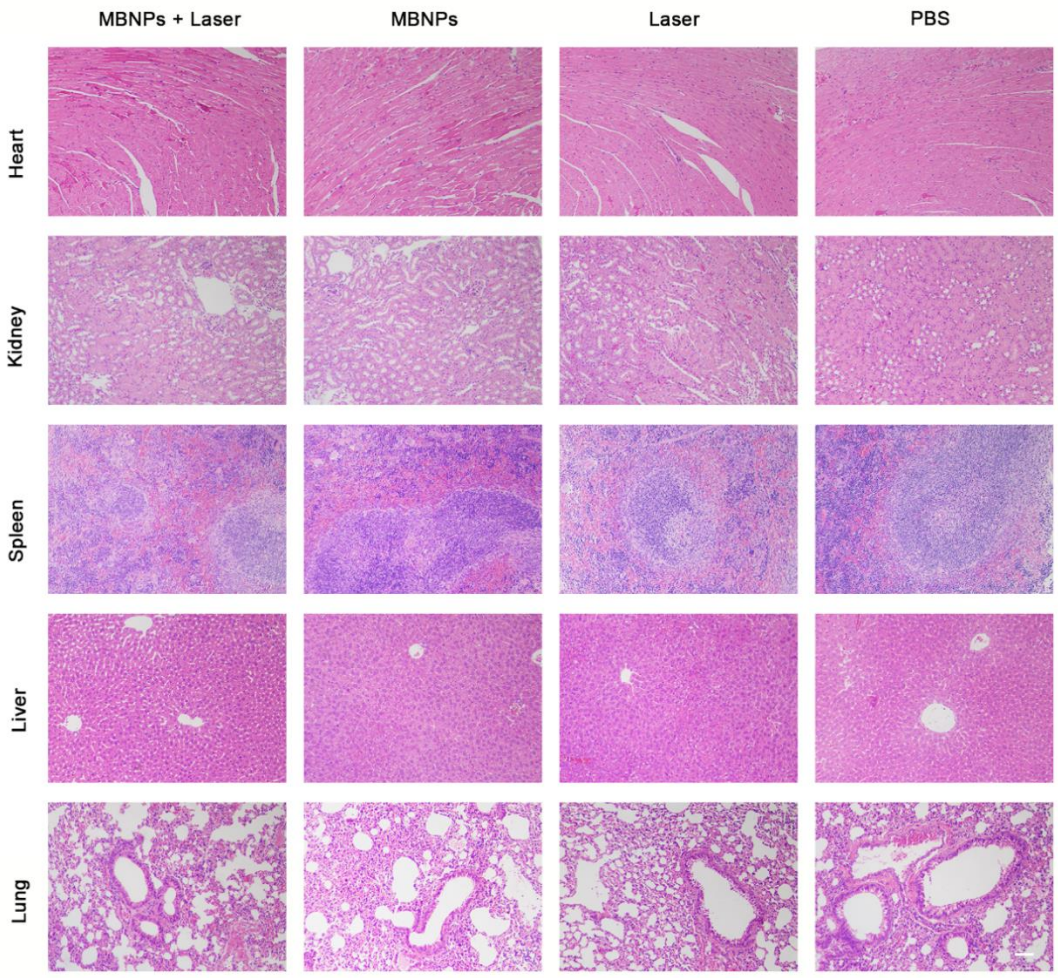


Figure S15. H&E stained histological images of tissue sections from major organs in different treatments after 14 d. (scale bar = 100 μm).

12. Blood Routine Examination

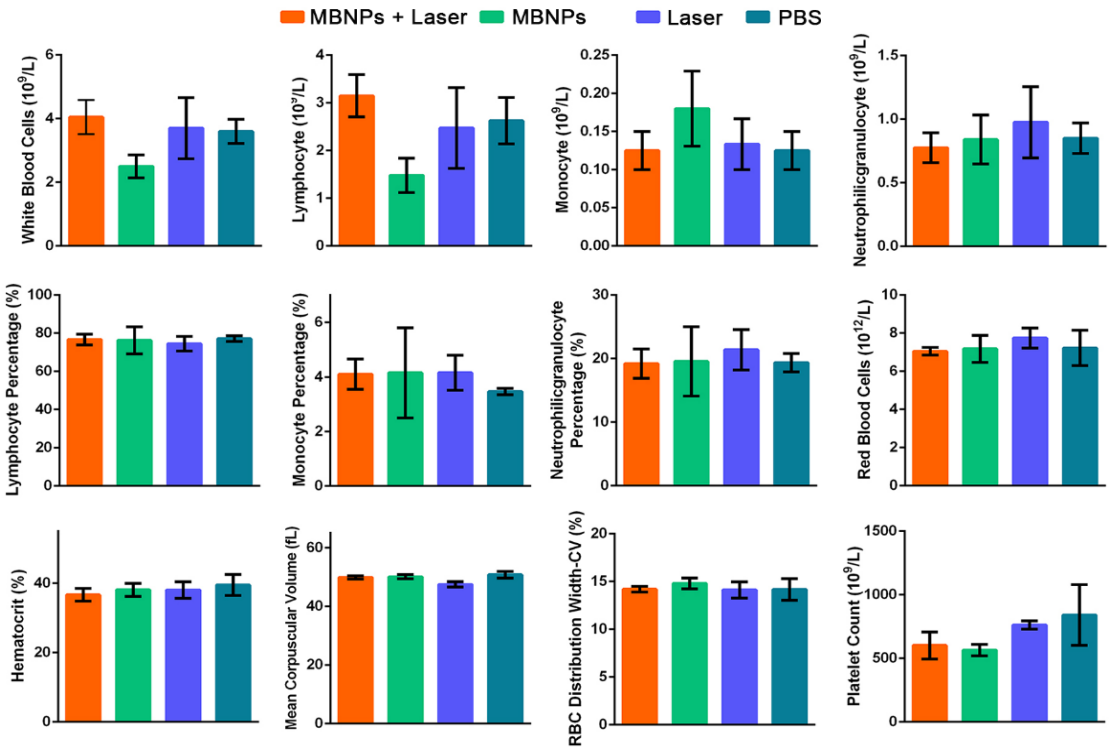


Figure S16. Hematological analysis of mice underwent the **MBNPs**-based PDT.

13. Statistical Analysis

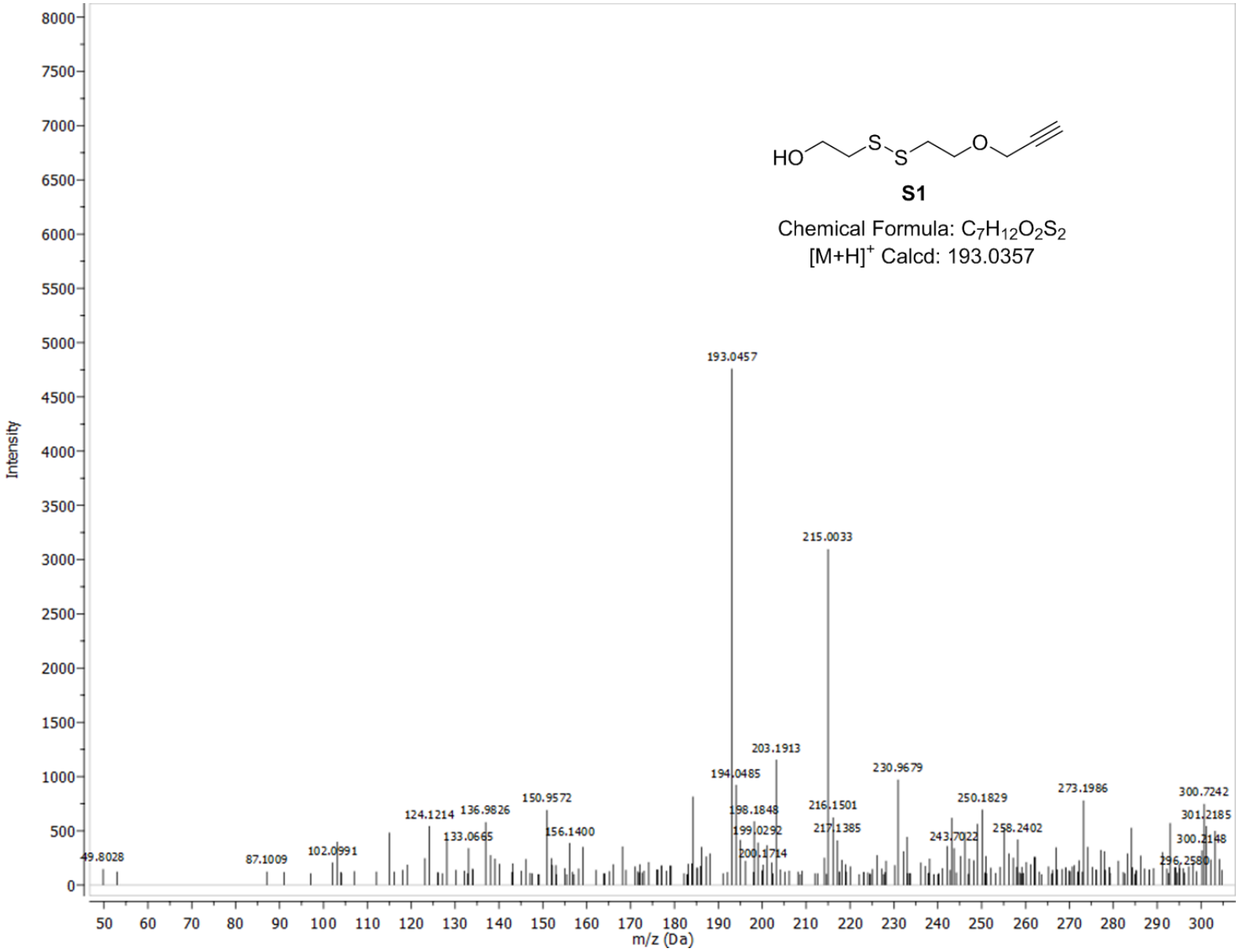
The results were presented as means ± SEM. The statistical analysis was performed with the t-test. p (p-value) < 0.05 was considered as statistically significant. ***p < 0.001; **p < 0.01; *p < 0.05.

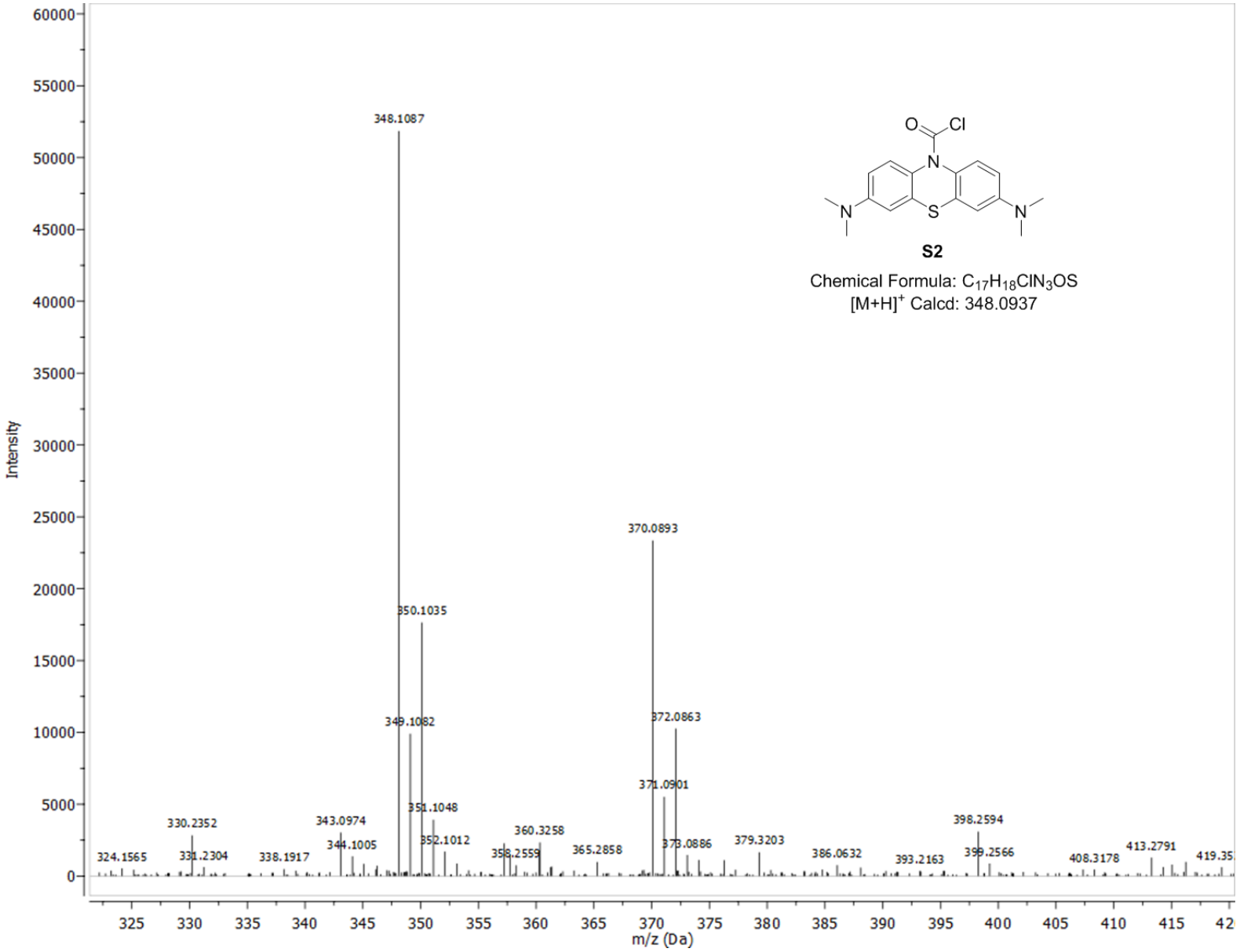
14. Reference

[1] R. Guo, F. Huang, B. Zhang, Y. Yan, J. Che, Y. Jin, Y. Zhuang, R. Dong, Y. Li, B. Tan, R. Song, Y. Hu, X. Dong, X. Li, N. Lin, *Theranostics* **2019**, 9, 3515.

[2] X. Liu, K. Jia, Y. Wang, W. Shao, C. Yao, L. Peng, D. Zhang, X. Y. Hu, L. Wang, *ACS Appl. Mater. Interfaces* **2017**, 9, 4843.

HR-MS Spectra

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349.1082
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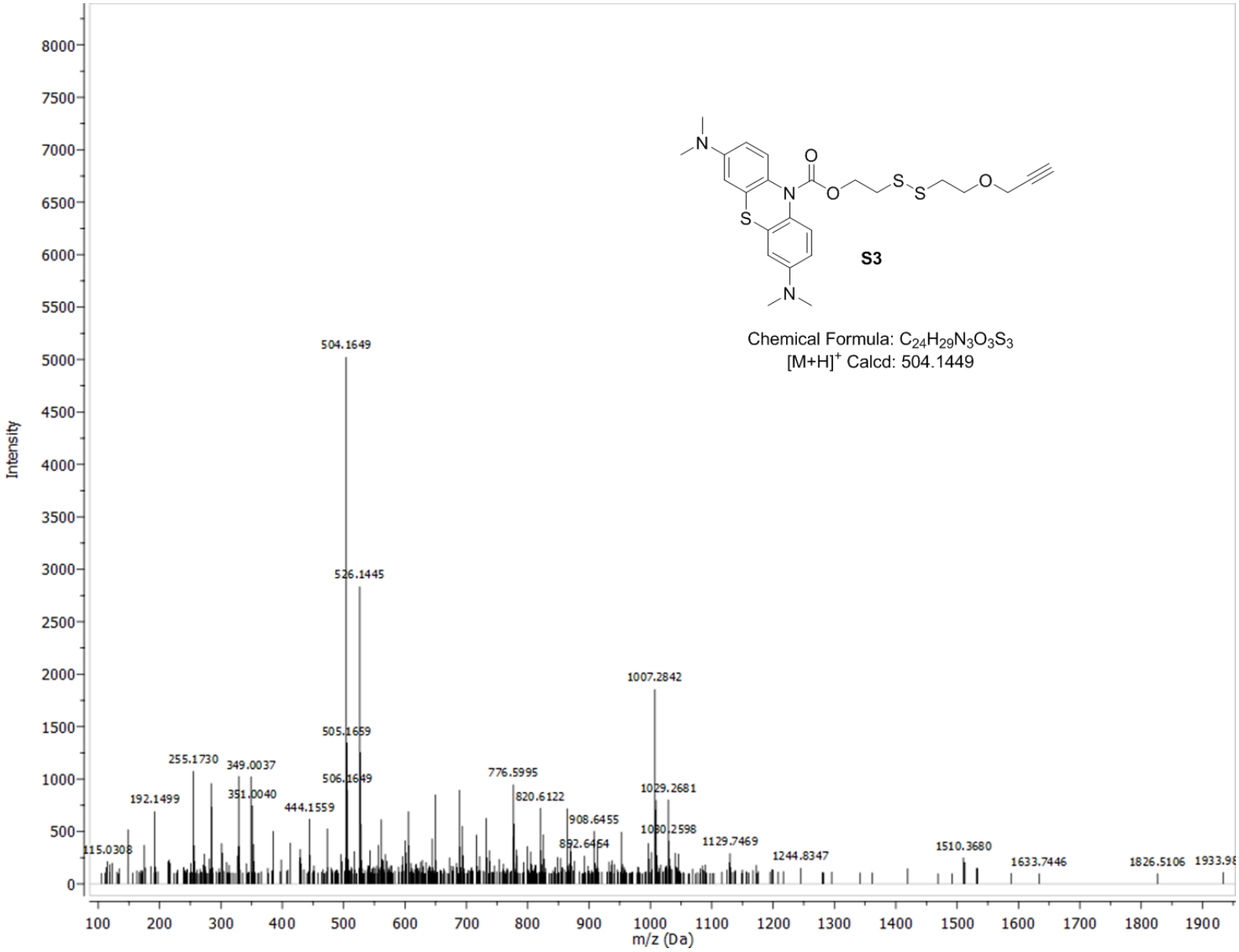
360.3258
358.2559
365.2858
373.0886
379.3203
386.0632
393.2163
398.2594
399.2566
408.3178
413.2791
324.1565
331.2304
338.1917
Intensity
m/z (Da)
S2
Chemical Formula: $C_{17}H_{18}ClN_3OS$
$[M+H]^+$ Calcd: 348.0937


S3
Chemical Formula: $C_{24}H_{29}N_3O_3S_3$
$[M+H]^+$ Calcd: 504.1449
Intensity
m/z (Da)
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351.0040
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776.5995
820.6122
908.6455
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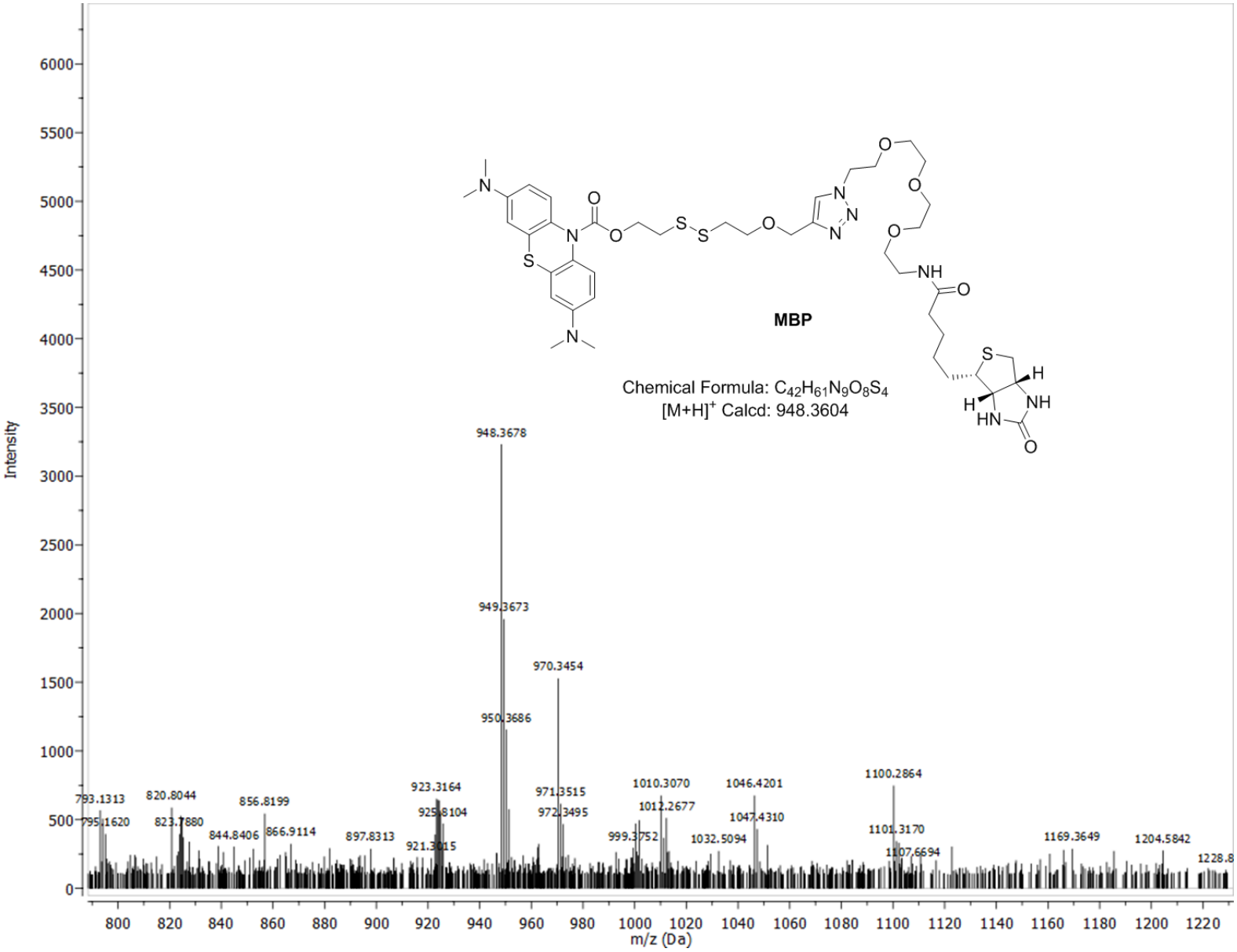
115.0308


MBP
Chemical Formula: $C_{42}H_{61}N_9O_8S_4$
$[M+H]^+$ Calcd: 948.3604
Intensity
m/z (Da)
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793.1313
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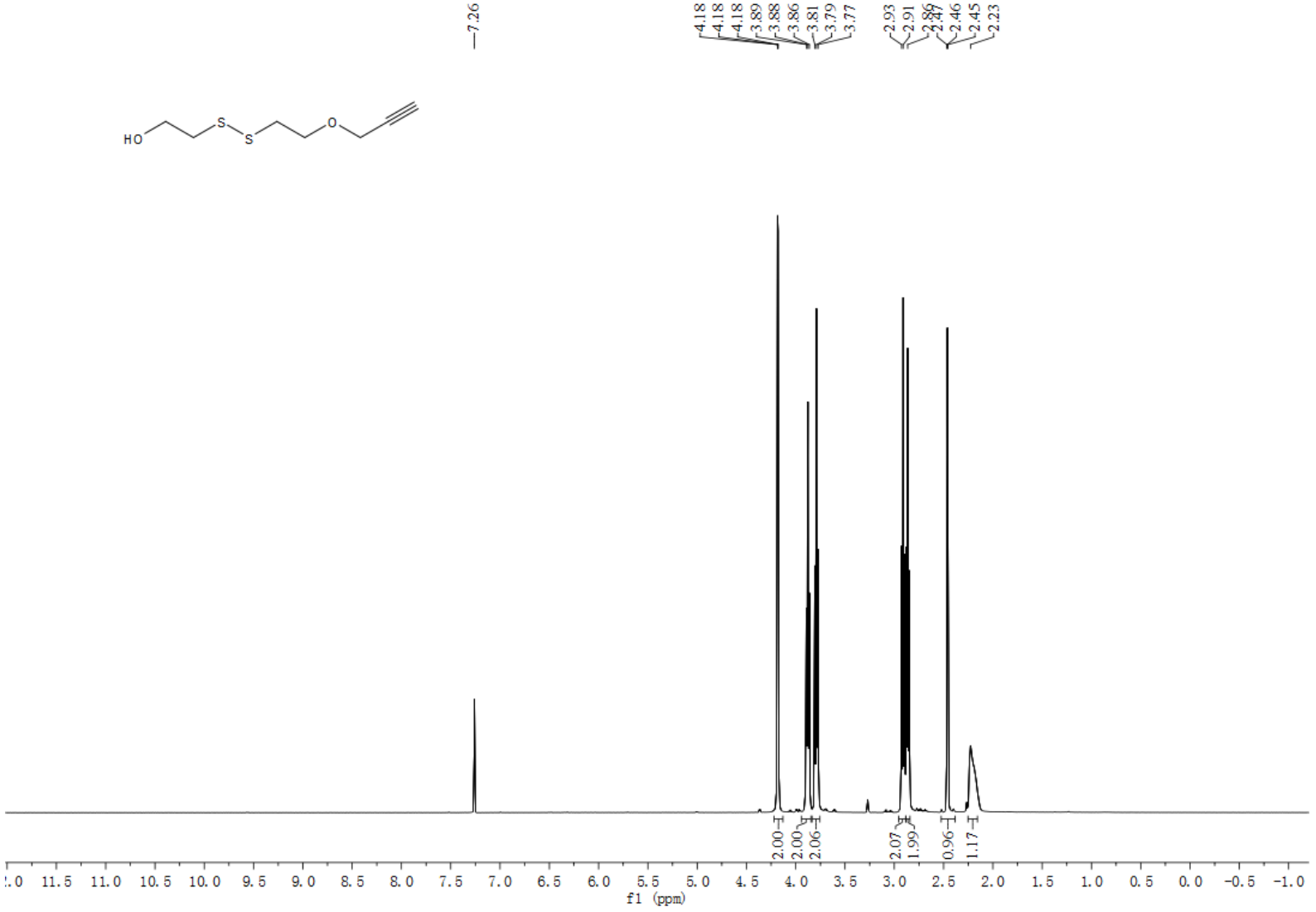
921.3015

NMR Spectra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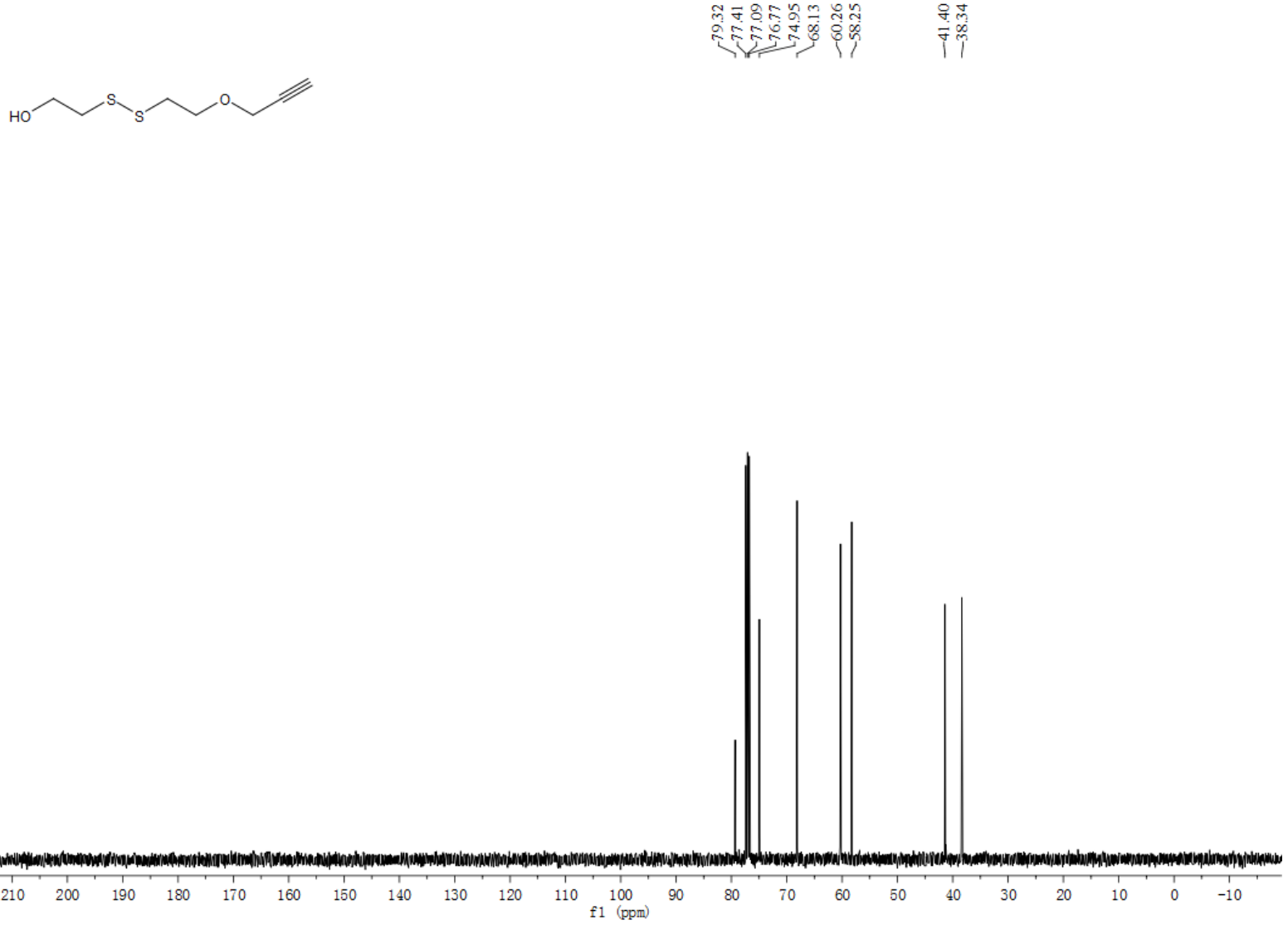
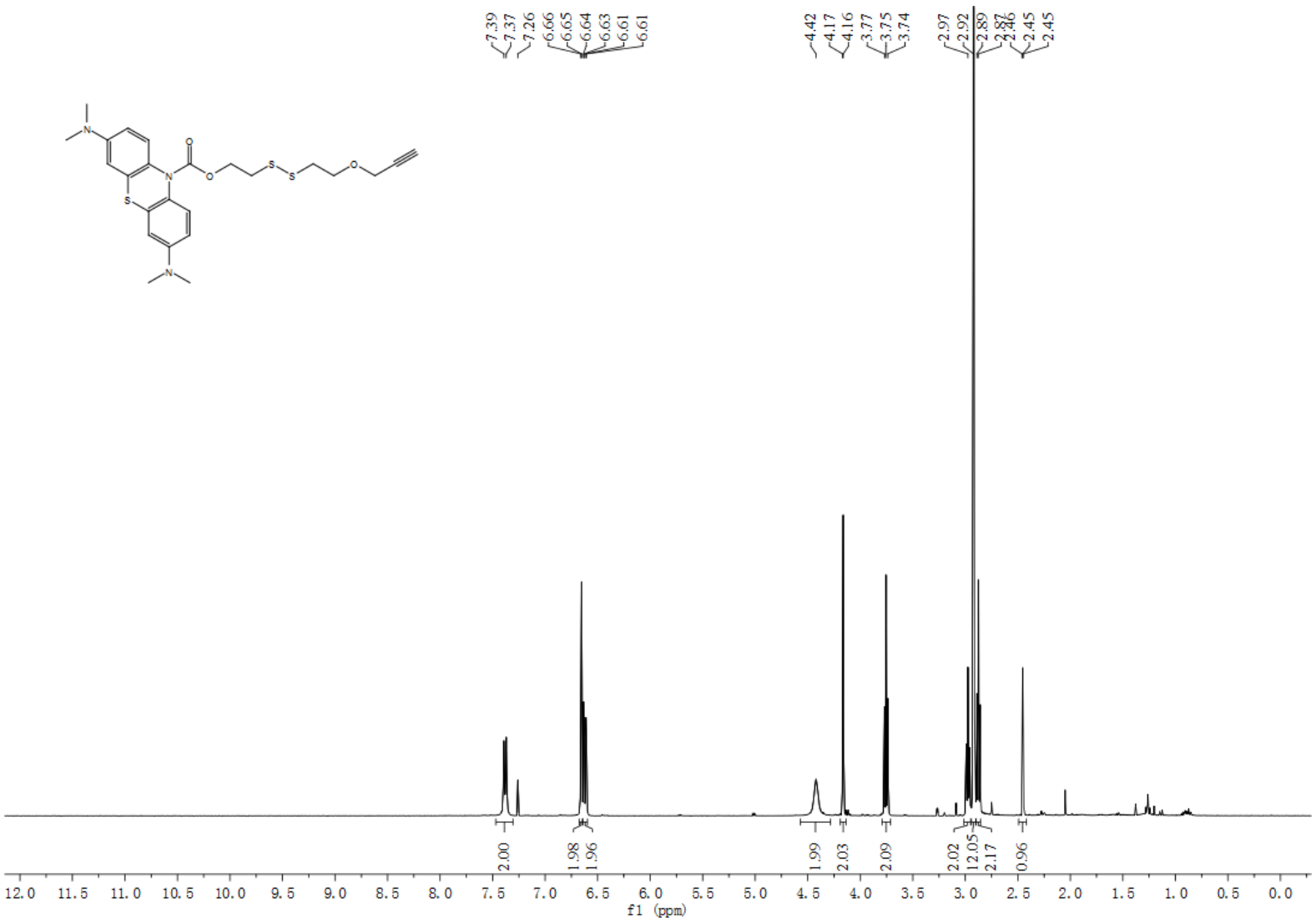
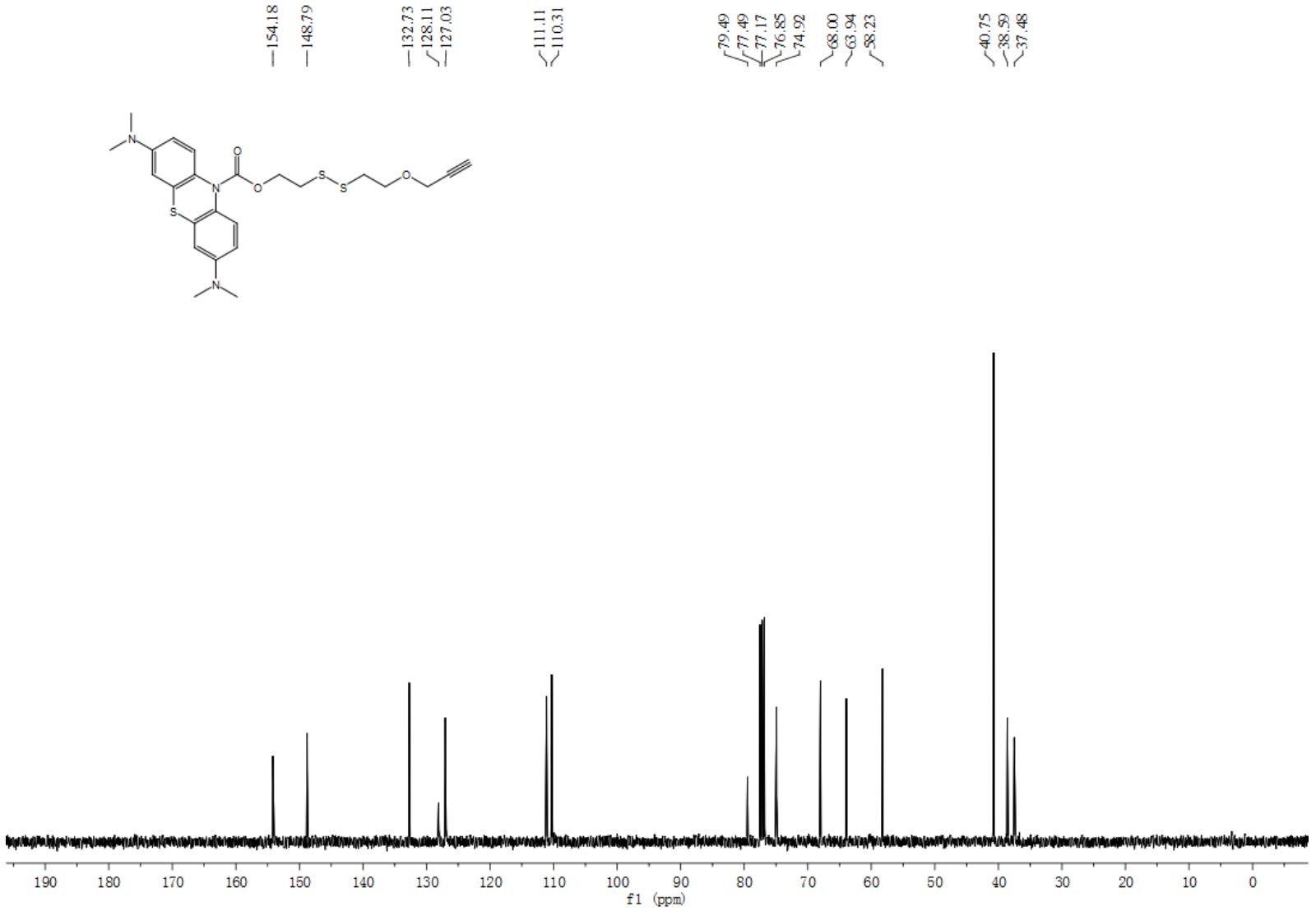
4.18
4.18
4.18
3.89
3.88
3.86
3.81
3.79
3.77
2.93
2.91
2.86
2.47
2.46
2.45
2.23
HO
S
S
O
2.00
2.00
2.06
2.07
1.99
0.96
1.17
f1 (ppm)


79.32
77.41
77.09
76.77
74.95
68.13
60.26
58.25
41.40
38.34
HO
S
S
O
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)


7.40
7.37
7.26
6.71
6.70
6.64
6.63
6.61
6.60
5.30
2.96
O
Cl
N
S
N
N
2.00
2.13
2.14
12.49
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
f1 (ppm)


7.39
7.37
7.26
6.66
6.65
6.64
6.63
6.61
6.61
4.42
4.17
4.16
3.77
3.75
3.74
2.97
2.92
2.89
2.87
2.46
2.45
2.45
2.00
1.98
1.96
1.99
2.03
2.09
2.02
12.05
2.17
0.96
12.0 11.5 11.0 10.5 10.0 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
f1 (ppm)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148.79
132.73
128.11
127.03
111.11
110.31
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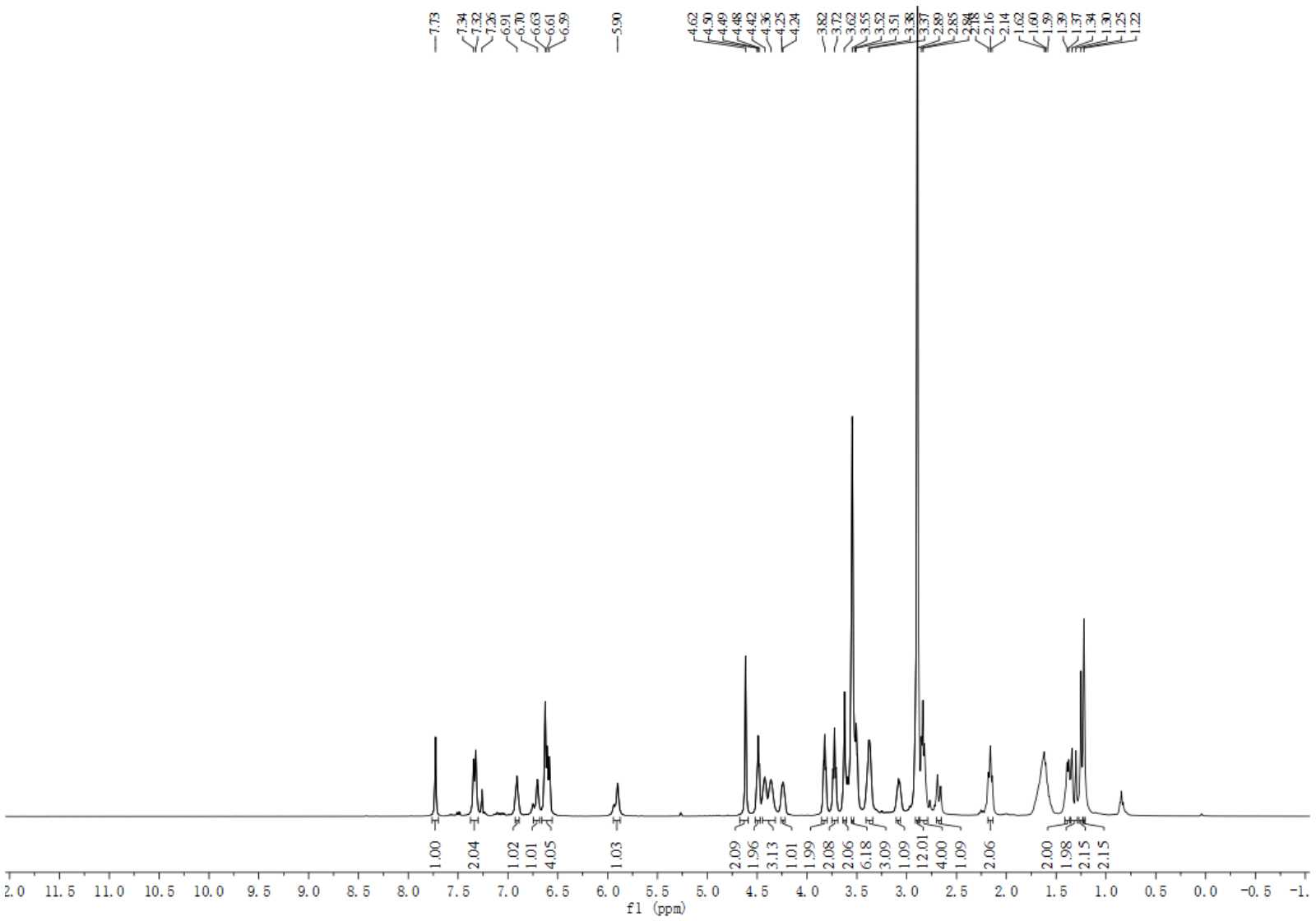
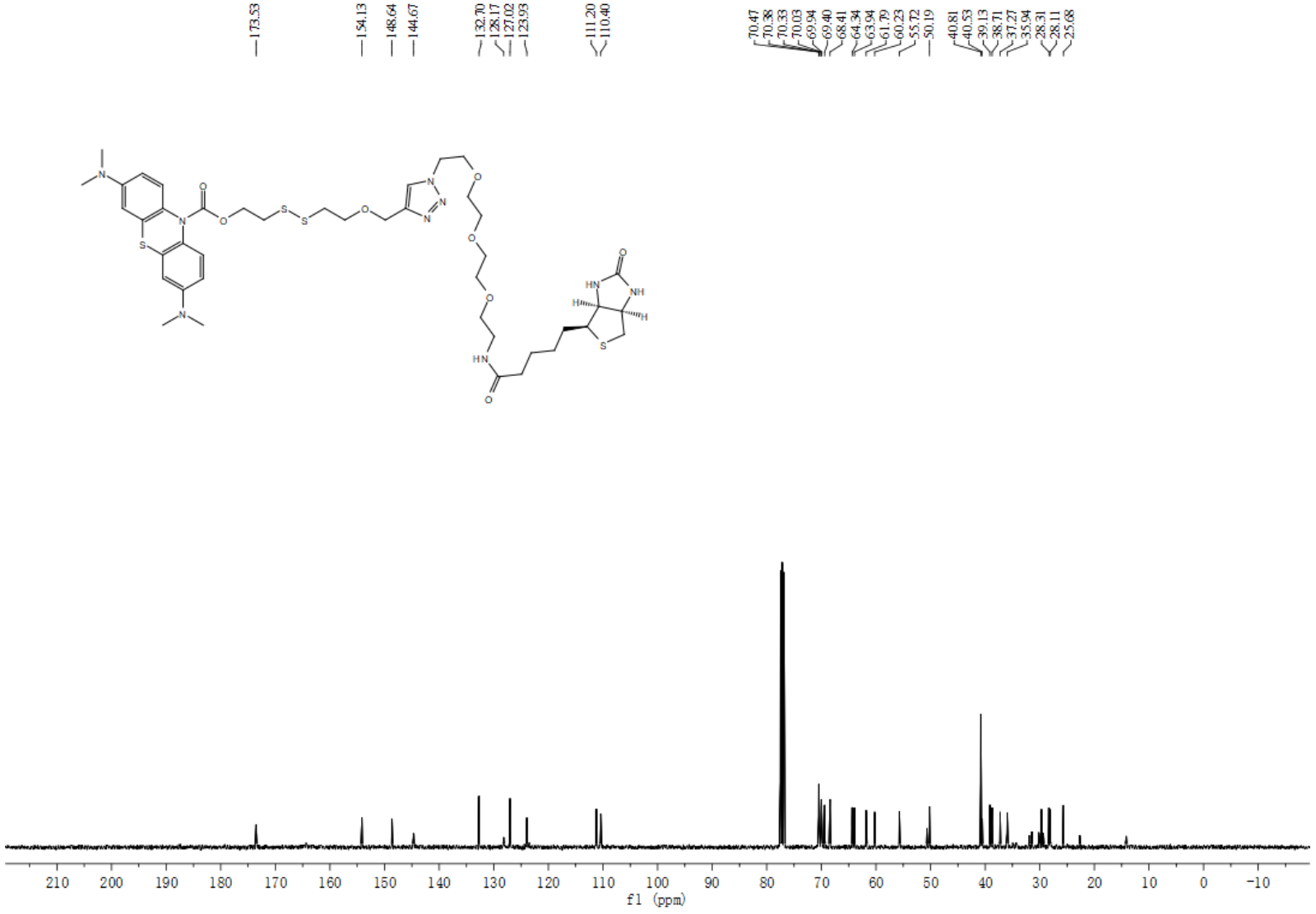
68.00
63.94
58.23
40.75
38.59
37.48
f1 (ppm)


7.73
7.34
7.32
7.26
6.91
6.70
6.63
6.61
6.59
5.90
4.62
4.50
4.49
4.48
4.42
4.36
4.25
4.24
3.82
3.72
3.62
3.55
3.52
3.51
3.38
3.37
2.89
2.85
2.16
2.14
1.62
1.60
1.59
1.39
1.37
1.34
1.30
1.25
1.22
1.00
2.04
1.02
1.01
4.05
1.03
2.09
1.96
3.13
1.01
1.99
2.08
2.06
6.18
3.09
1.09
12.01
4.00
1.09
2.06
2.00
1.98
2.15
2.15
f1 (ppm)


173.53
154.13
148.64
144.67
132.70
128.17
127.02
123.93
111.20
110.40
70.47
70.38
70.33
70.03
69.94
69.40
68.41
64.34
63.94
61.79
60.23
55.72
50.19
40.81
40.53
39.13
38.71
37.27
35.94
28.31
28.11
25.68
f1 (ppm)