Supporting information

Synthesis, biophysical characterization, and antitumor activity of antisense oligonucleotides with anisamide conjugation

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Synthesis of the Z-X-1 and Z-X-2 compounds

Compound 1: [(oxybis(ethane-2,1-diyl) bis(oxy)] bis(ethane-2,1-diyl) bis(4-methylbenzenesulfonate)

Tetraethylene glycol (1 g, 5.15 mmol) was dissolved in dichloromethane. 4-Toluenesulfonyl chloride (2.95 g, 15.45 mmol) dissolved in pyridine was dripped slowly into this solution and stirred overnight at room temperature. The progress of the raw material reaction was monitored by thin-layer chromatography (TLC). The reaction solution was then diluted with distilled water, and the water layer was extracted with dichloromethane. The organic layer was washed successively with hydrochloric acid and saturated NaCl solution, then dried with $MgSO_4$ and concentrated. The residue was purified with flash chromatography (petroleum ether:ethyl acetate = 1:1) to yield a colorless, transparent oily substance (2.32 g, 89.6%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.81–7.75 (m, 4H), 7.50–7.45 (m, 4H), 4.13–4.08 (m, 4H), 3.59–3.54 (m, 4H), 3.46–3.39 (m, 8H), 2.42 (s, 4H). MS m/z: $[M+H]^-$:503.14.

Compound 2: di-tert-butyl {[(oxybis(ethane-2,1-diyl)] bis(oxy)} bis(ethane-2,1-diyl) bis[(tert-butoxycarbonyl) carbamate]

Bis (tert-butoxycarbonyl) amine (0.87 g, 3.98 mmol) and cesium carbonate (1.3 g, 3.98 mmol) were dissolved in dimethyl sulfoxide. Compound 1 (1 g, 1.99 mmol) was then added. The mixture was stirred at 80°C for 2 h. The progress of the raw material reaction was monitored by TLC. The reaction solution was then diluted with distilled water, and the compound 2 was extracted with ethyl

acetate and washed with saturated NaCl solution. The organic layer was dried with anhydrous $MgSO_4$ and concentrated. The residue was purified with flash chromatography (petroleum ether: ethyl acetate = 5:1) to yield a colorless, transparent oily substance (0.32 g, 27.2%). ^{1}H NMR (600 MHz, DMSO-d6) δ 3.64 (t, J = 5.9 Hz, 4H), 3.50–3.46 (m, 12H), 1.43 (s, 36H). MS m/z $[M+Na]^-$: 615.36.

Compound 3: 2,2'-[(oxybis(ethane-2,1-diyl) bis(oxy)] bis(ethan-1-amine)

Compound 2 (0.1 g, 0.169 mmol) was added to hydrochloric acid/ethyl acetate (10 ml) and stirred overnight at room temperature. The solvent was then evaporated. The progress of the raw material reaction was monitored by TLC. The reaction solution was concentrated by rotatory evaporation to yield a yellow oily product (0.017 g, 52.4%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.37 (t, J = 5.6 Hz, 1H), 7.84–7.81 (m, 2H), 7.00–6.97 (m, 2H), 3.80 (s, 3H), 3.60–3.49 (m, 12H), 3.40 (q, J = 6.0 Hz, 3H), 2.96 (t, J = 5.3 Hz, 2H), 2.00 (s, 2H). MS m/z $[M+Na]^-$: 327.19.

Compound 4: N-(2-{2-[2-(2-aminoethoxy)ethoxy]ethoxy}ethyl)-4-methoxybenzamide

Compound 3 (0.5 g, 2.6 mmol) and pyridine were dissolved in dried dichloromethane. Under nitrogen protection in an ice bath, p-methoxybenzoyl chloride (0.15 g, 0.87 mmol) was dissolved in dried dichloromethane and then added to the solution drop by drop, followed by stirring in an ice bath for 1 h and at room temperature for 3 h. The progress of the raw material reaction was

monitored by TLC. The mixture was partitioned between saturated NaCl solution and CH_2Cl_2. The organic layer was dried with anhydrous $MgSO_4$ and concentrated. The residue was purified with flash chromatography (dichloromethane:methanol = 8:1) to yield a white crystalline substance (0.08 g, 29.5%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.37 (t, J = 5.6 Hz, 1H), 7.84–7.81 (m, 2H), 7.00–6.97 (m, 2H), 3.80 (s, 3H), 3.60–3.49 (m, 12H), 3.40 (q, J = 6.0 Hz, 3H), 2.96 (t, J = 5.3 Hz, 2H), 2.00 (s, 2H). MS m/z $[M+Na]^-$: 327.19.

Compound 5: N-(1-bromo-2-oxo-6,9,12-trioxa-3-azatetradecan-14-yl)-4-methoxybenzamide (Z-X-2)

Compound 4 (0.2 g, 0.61 mmol), triethylamine, and bromoacetyl bromide (0.12 g, 0.61 mmol) were dissolved in dry dichloromethane, followed by stirring overnight at room temperature. The progress of the raw material reaction was monitored by TLC. The reaction solution was diluted with dried dichloromethane, then washed successively with saturated Na_2CO_3 and NaCl solutions. The organic layer was dried with anhydrous $MgSO_4$ and concentrated. The residue was purified with flash chromatography (methylene chloride:methanol = 20:1) to yield a solid white substance (0.09 g, 33.0%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.35 (t, J = 5.6 Hz, 1H), 7.82 (dd, J = 8.8, 1.8 Hz, 2H), 6.98 (dd, J = 9.0, 2.9 Hz, 2H), 3.80 (d, J = 3.4 Hz, 3H), 3.54–3.46 (m, 7H), 3.39 (dq, J = 12.2, 5.8 Hz, 3H), 3.26–3.19 (m, 1H). MS m/z: $[M+H]^-$: 447.11.

Compound 6: 2-{2-[2-(2-hydroxyethoxy)ethoxy]ethoxy}ethyl-4-methoxybenzoate

Tetraethylene glycol (3.42 g, 17.59 mmol) and pyridine (0.5 ml) were dissolved in dried dichloromethane. Under nitrogen protection in an ice bath, p-methoxybenzoyl chloride (1 g, 5.86 mmol) was added drop by drop. The mixture was stirred at 0°C for 1 h and room temperature for 2 h. The progress of the raw material reaction was monitored by TLC. The mixture was extracted with ethyl acetate, then washed three times with distilled water and saturated NaCl solution. The organic layer was dried with anhydrous $MgSO_4$ and concentrated. The residue was purified with flash chromatography (dichloromethane:methanol = 20:1) to yield a colorless, transparent oily substance (2.32 g, 40.2%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.95–7.89 (m, 2H), 7.08–7.03 (m, 2H), 4.57 (t, J = 5.5 Hz, 1H), 4.37–4.32 (m, 2H), 3.84 (s, 3H), 3.76–3.71 (m, 2H), 3.59 (dd, J = 6.0, 3.6 Hz, 2H), 3.55–3.44 (m, 8H), 3.42–3.37 (m, 2H). MS m/z [M+H]- :329.16, $[M+Na]^-$:351.16.

Compound 7: 14-bromo-13-oxo-3,6,9,12-tetraoxatetradecyl-4-methoxybenzoate (Z-X-2)

Compound 6 (0.6 g, 1.83 mmol), triethylamine (0.19 g, 1.83 mmol), and bromoacetyl bromide (0.37 g, 1.83 mmol) were dissolved in dry dichloromethane, followed by stirring overnight at room temperature. The progress of the raw material reaction was monitored by TLC. The reaction

solution was diluted with dried dichloromethane, then the compound 7 was washed successively with saturated Na_2CO_3 and NaCl solutions. The organic layer was dried with anhydrous $MgSO_4$ and concentrated. The residue was purified with flash chromatography (petroleum ether:ethyl acetate = 1:1) to yield a colorless, transparent oily substance (0.35 g, 42.7%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.94–7.89 (m, 2H), 7.08–7.03 (m, 2H), 4.37–4.32 (m, 2H), 4.23–4.17 (m, 2H), 4.16 (s, 2H), 3.84 (s, 3H), 3.75–3.71 (m, 2H), 3.59 (ddd, J = 9.7, 5.8, 3.8 Hz, 4H), 3.55–3.52 (m, 2H), 3.52 (s, 4H). MS m/z $[M+H+Na]^-$:473.06.

1. Supporting results and experimental raw data

2.1 ^{1}H NMR and HRMS spectra of the Z-X-1 and Z-X-2 compounds.

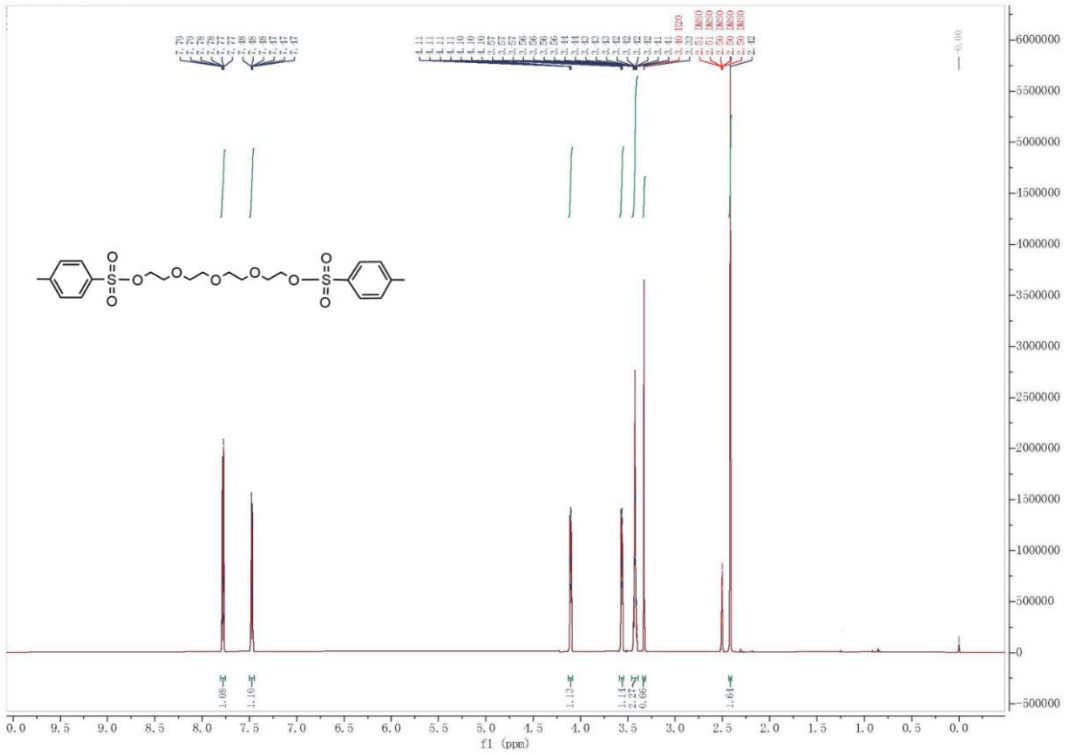

Figure S1. The ^{1}H NMR spectrum (600 MHz, DMSO) of compound 1

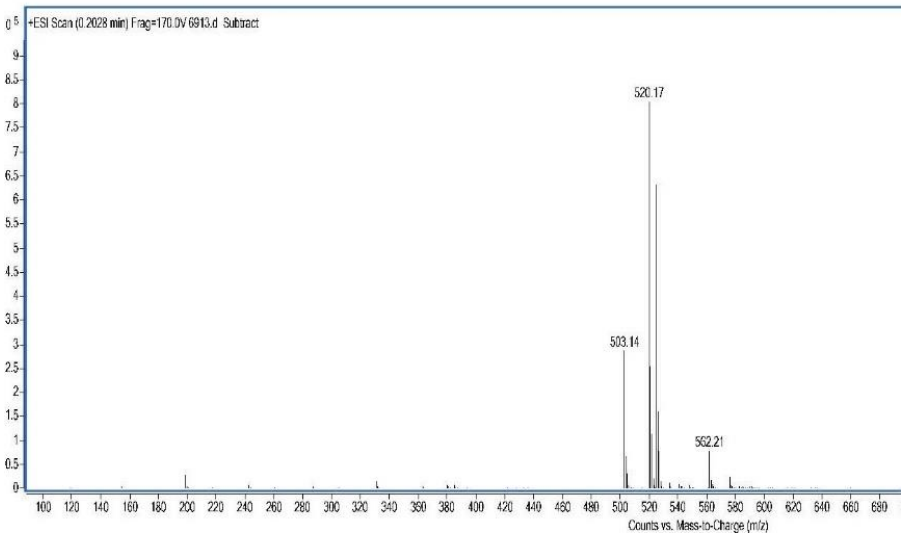

Figure S2. The HRMS spectra of compound 1

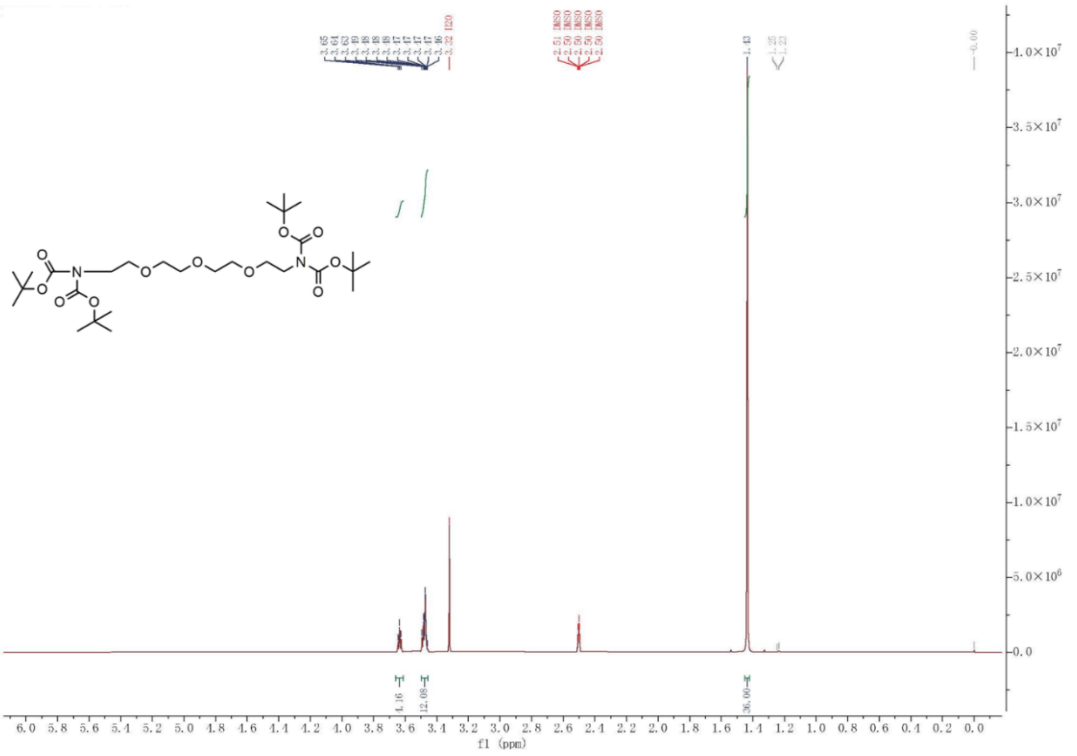

Figure S3. The ^{1}H NMR spectrum (600 MHz, DMSO) of compound 2

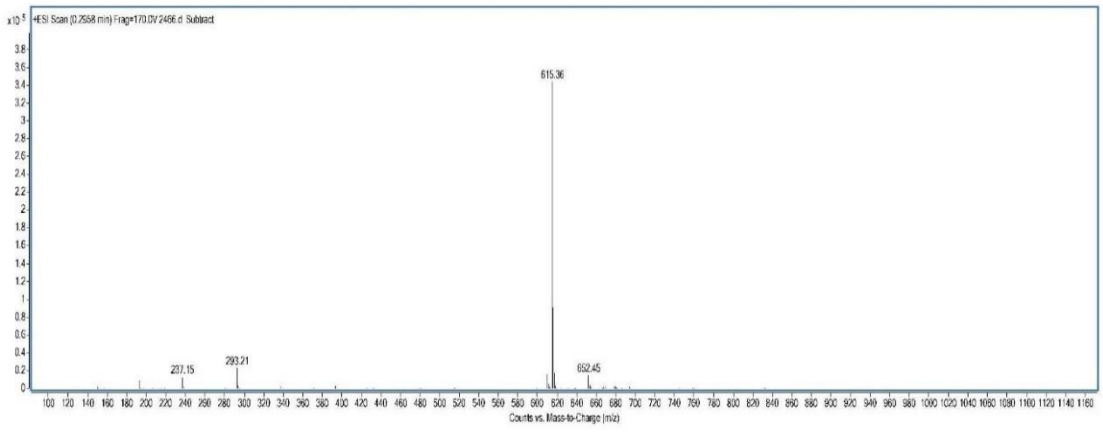

Figure S4. The HRMS spectra of compound 2

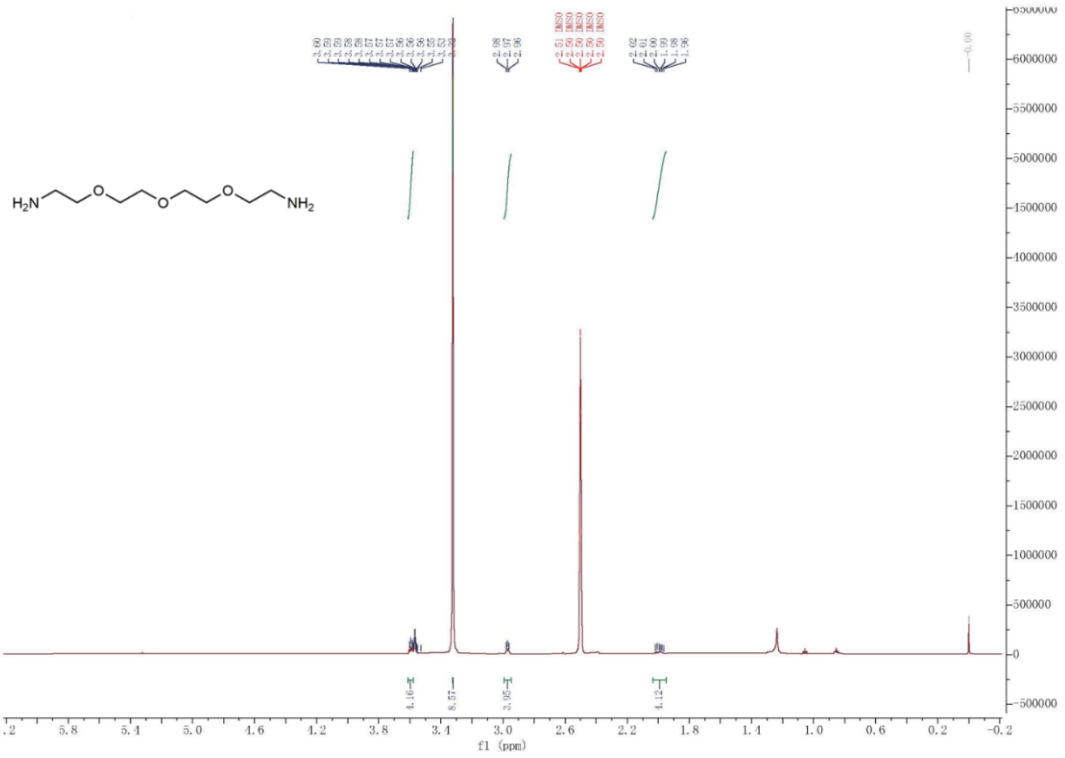

Figure S5. The ^{1}H NMR spectrum (600 MHz, DMSO) of compound 3

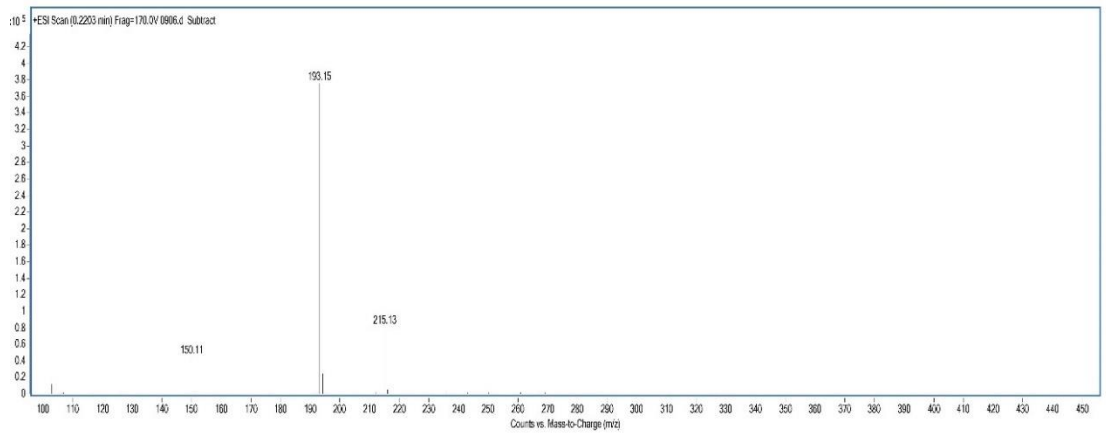

Figure S6. The HRMS spectra of compound 3

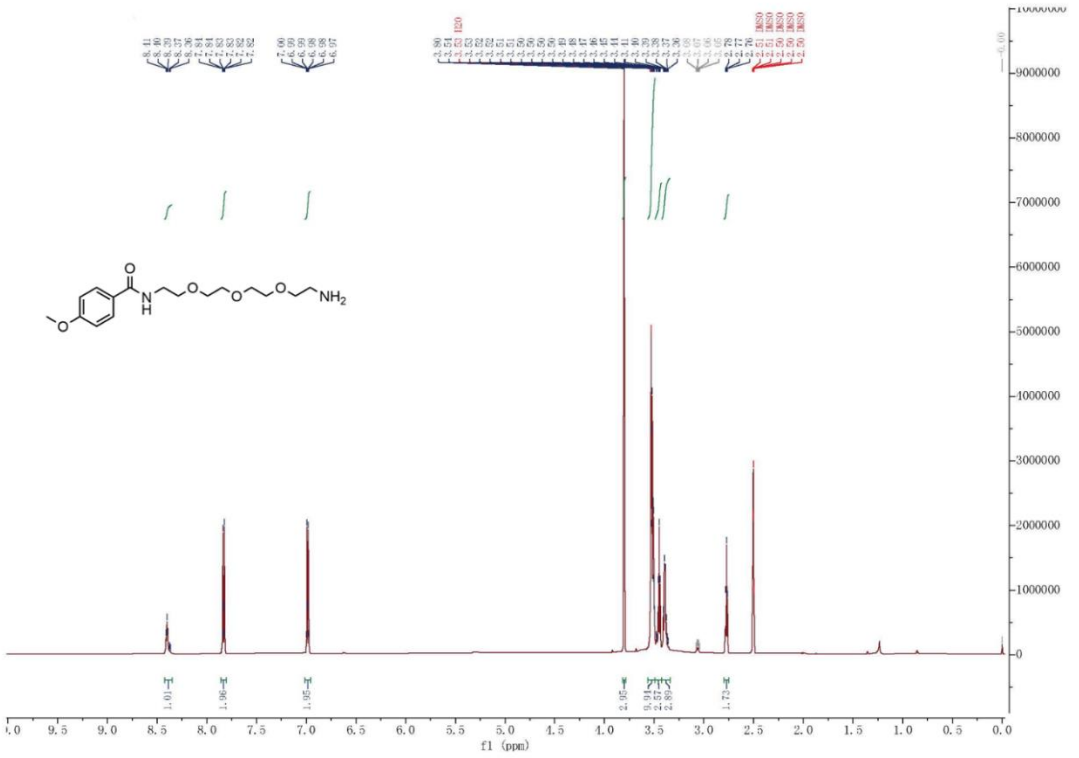

Figure S7. The ^{1}H NMR spectrum (600 MHz, DMSO) of compound 4

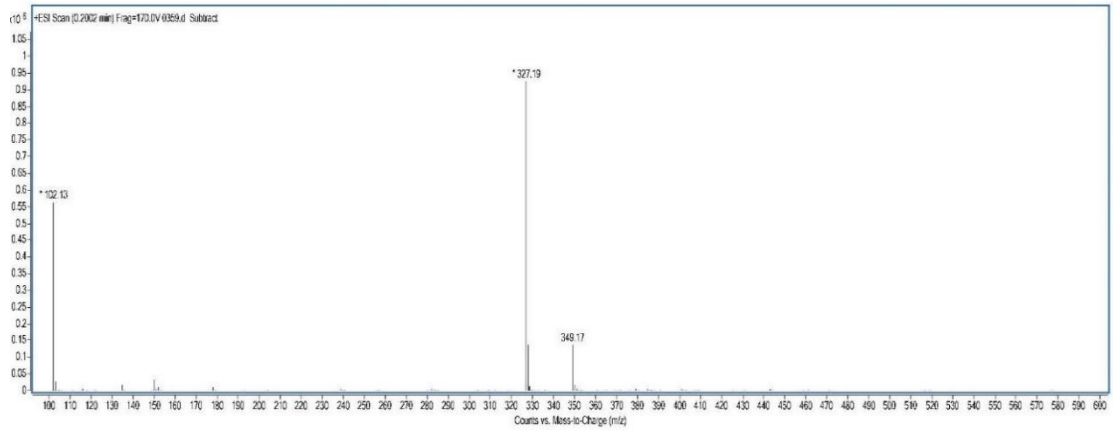

Figure S8. The HRMS spectra of compound 4

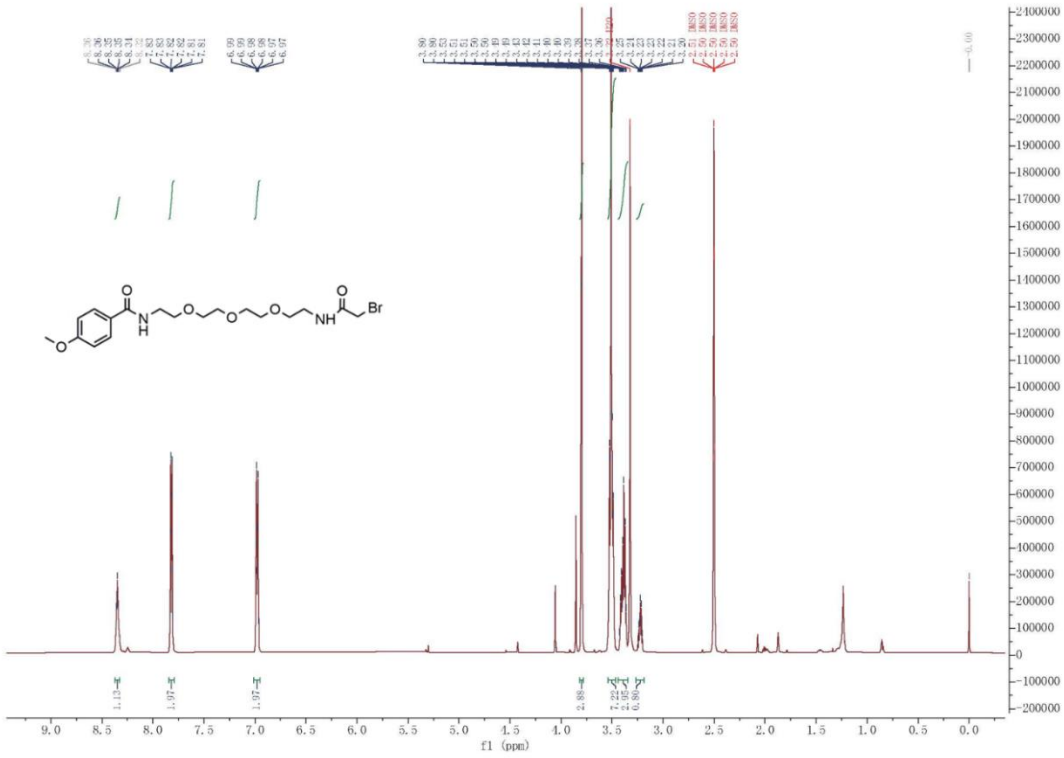

Figure S9. The ^{1}H NMR spectrum (600 MHz, DMSO) of compound 5

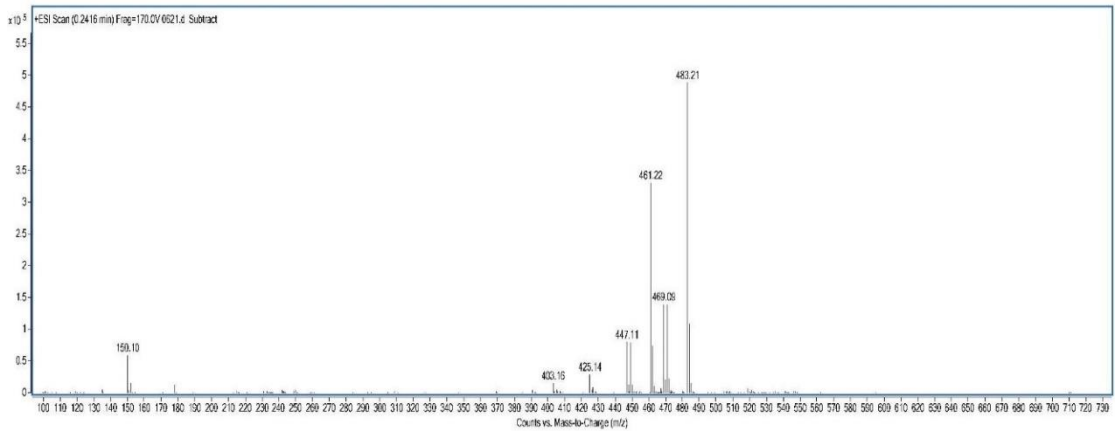

Figure S10. The HRMS spectra of compound 5

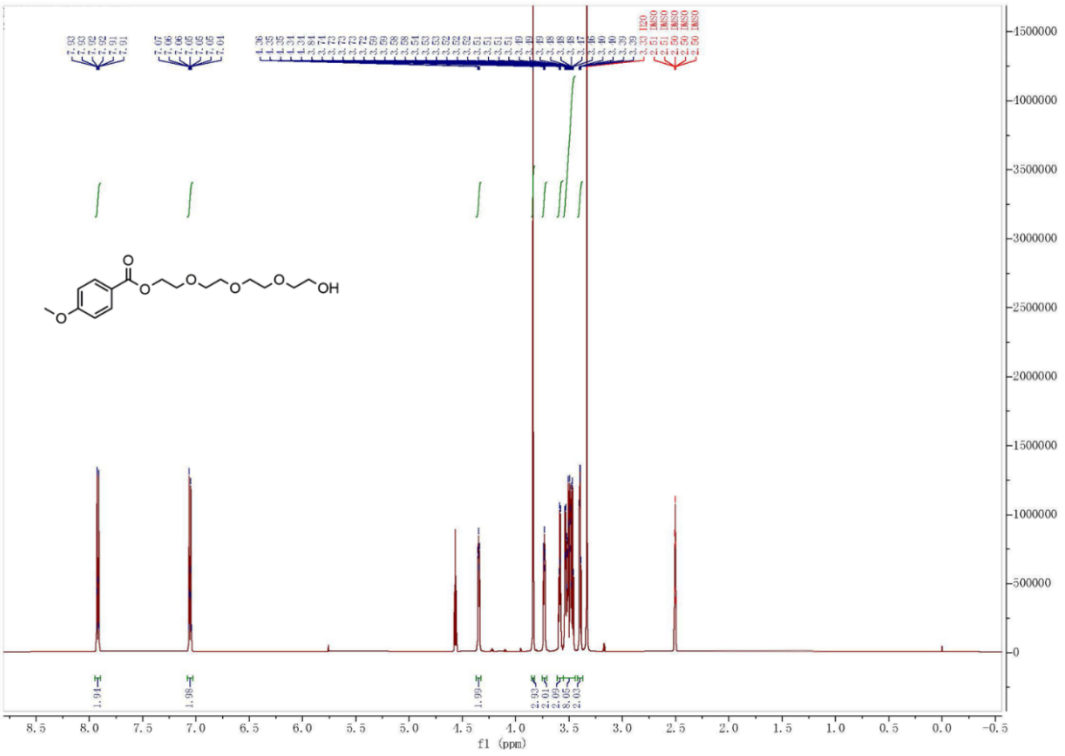

Figure S11. The ^{1}H NMR spectrum (600 MHz, DMSO) of compound 6

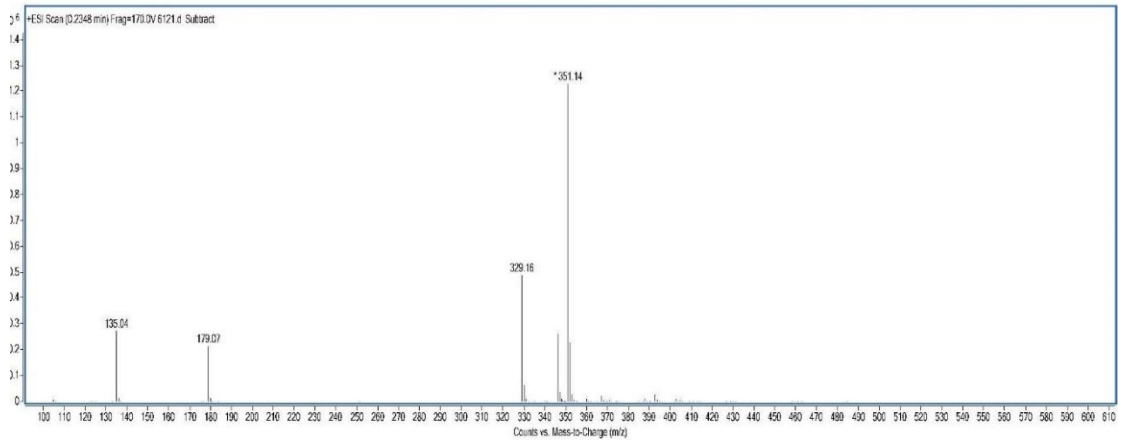

Figure S12. The HRMS spectra of compound 6

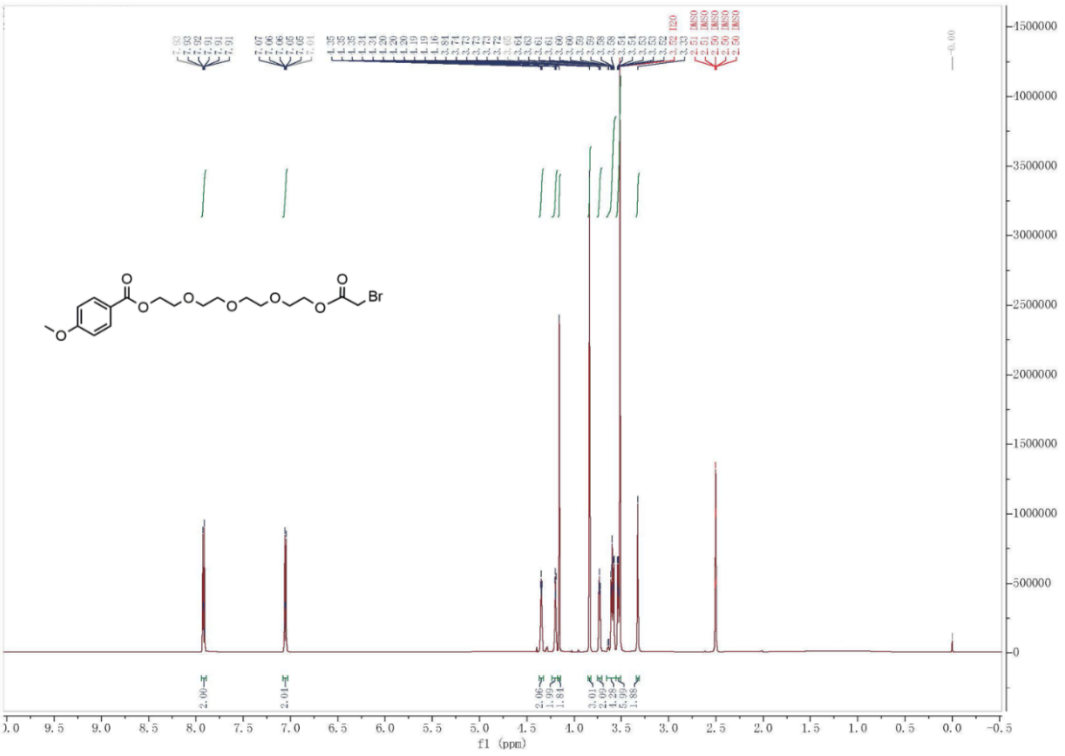

Figure S13. The ^{1}H NMR spectrum (600 MHz, DMSO) of compound 7

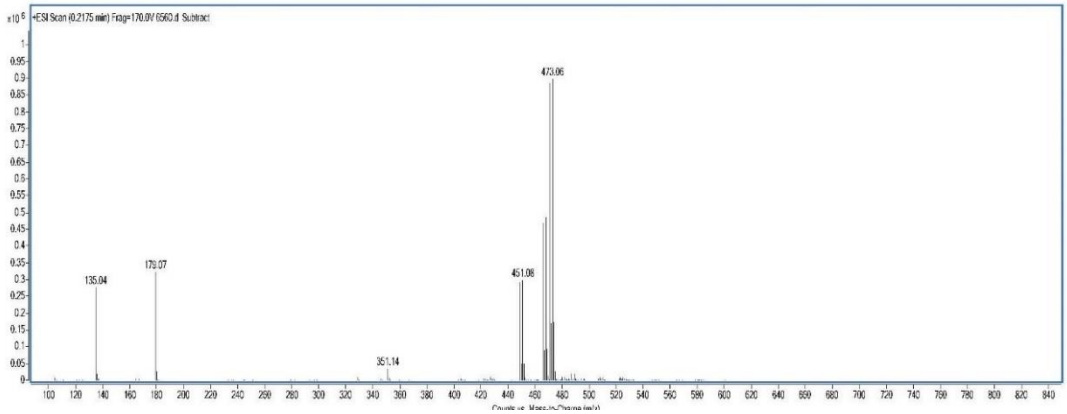

Figure S14. The HRMS spectra of compound 7

2.2 The sequences and molecular weights of Z1-Z4

Table S1. the sequences and molecular weights of Z1-Z4.

No.	Sequence (5′-3′)	Molecular weight (estimated)	Molecular weight (actual)
Z1	GGCTAAATCGCTCCACCAA*G	6088.0	6087.6
Z2	G*GCTAAATCGCTCCACCAAG	6088.0	6087.6
Z3	GGCTAAATCG*CTCCACCAAG	6088.0	6087.6
Z4	G*GCTAAATCGCTCCACCAA*G	6195.8	6196.8

***** represented PS modification.

2.3 The MALDI-TOF-MS spectra of Z1-Z4.

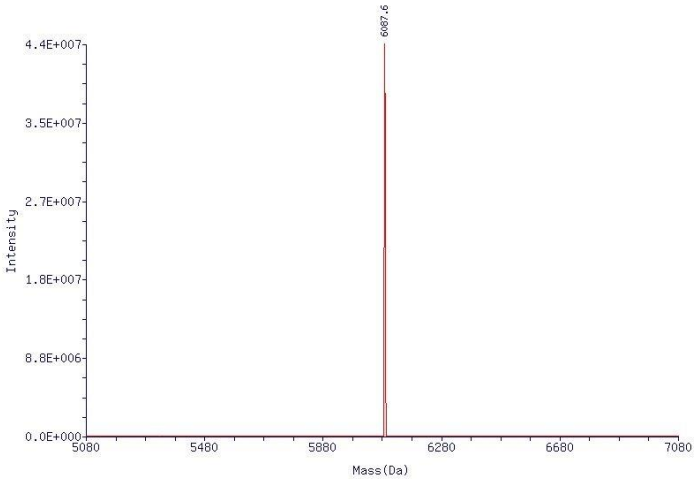

Figure S15. The MALDI-TOF-MS of Z1

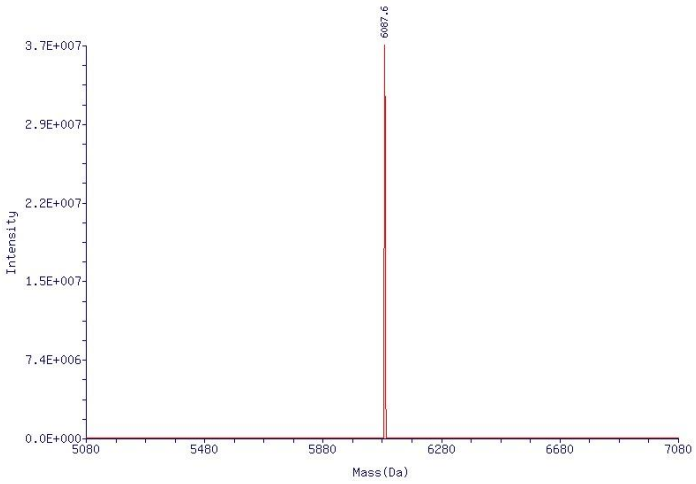

Figure S16. The MALDI-TOF-MS of Z2

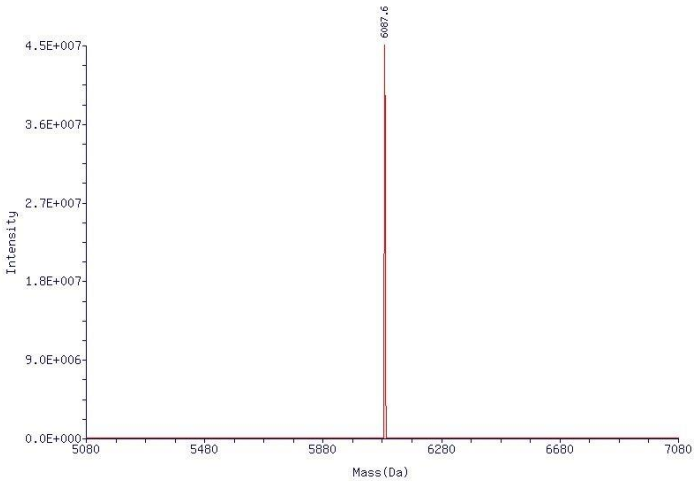

Figure S17. The MALDI-TOF-MS of Z3

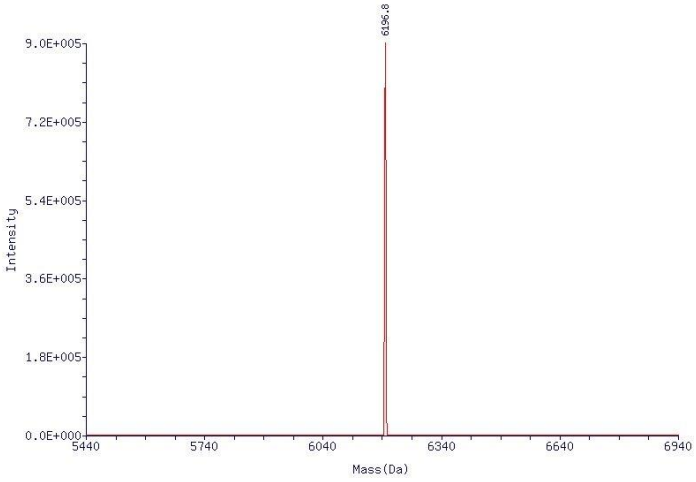

Figure S18. The MALDI-TOF-MS of Z4

2.4 The MALDI-TOF-MS spectra of T1-T6.

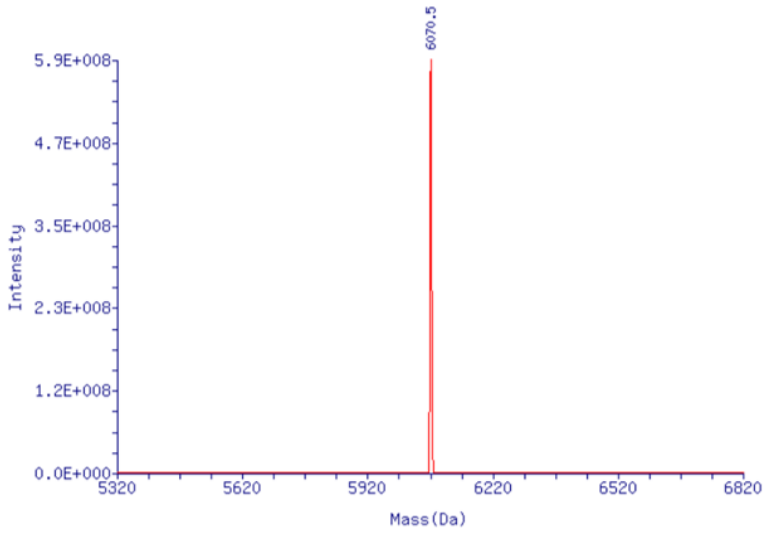

Figure S19. The MALDI-TOF-MS of T1

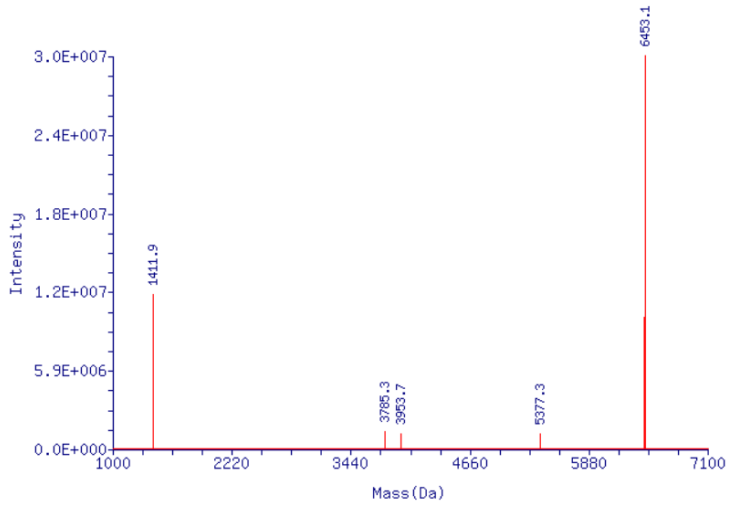

Figure S20. The MALDI-TOF-MS of T2

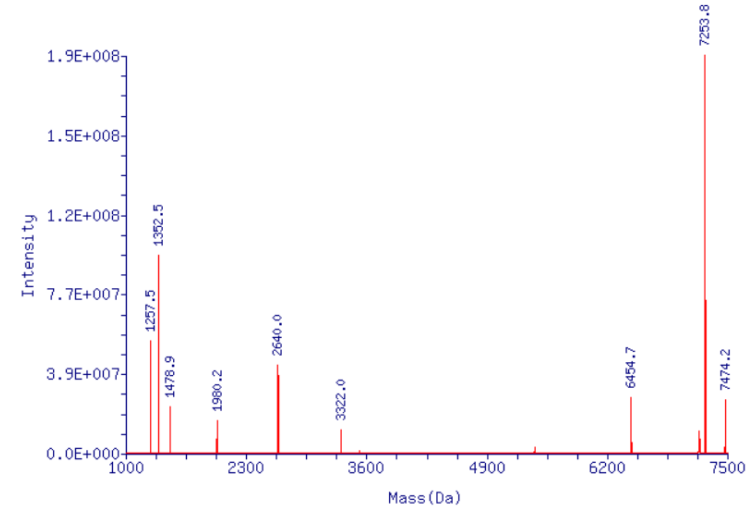

Figure S21. The MALDI-TOF-MS of T3

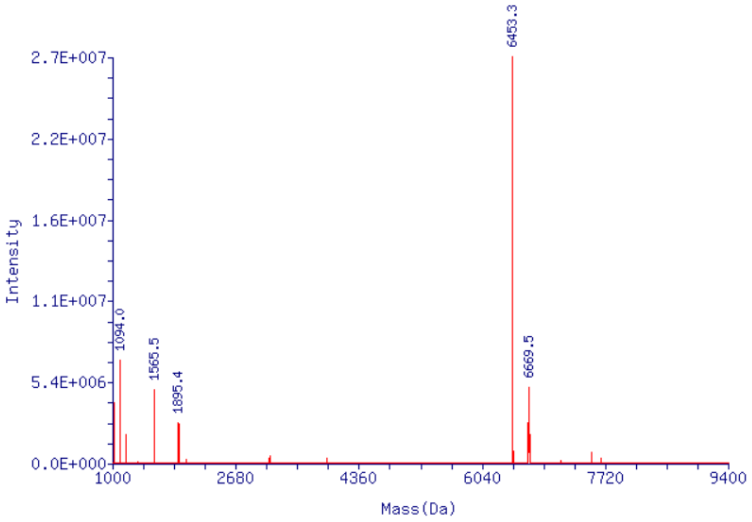

Figure S22. The MALDI-TOF-MS of T4

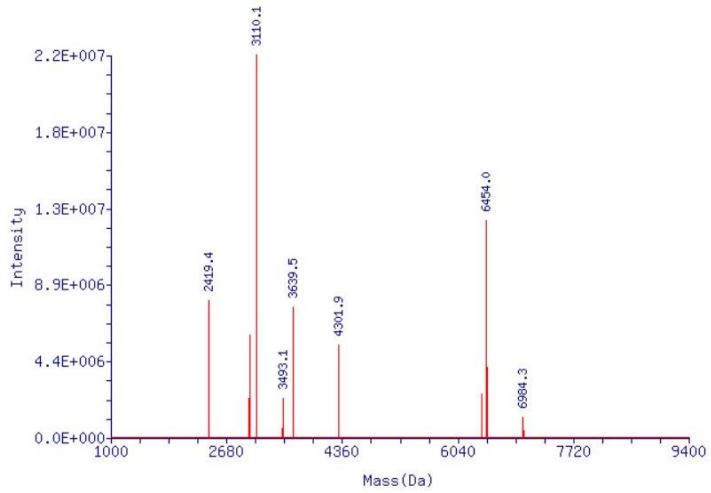

Figure S23. The MALDI-TOF-MS of T5

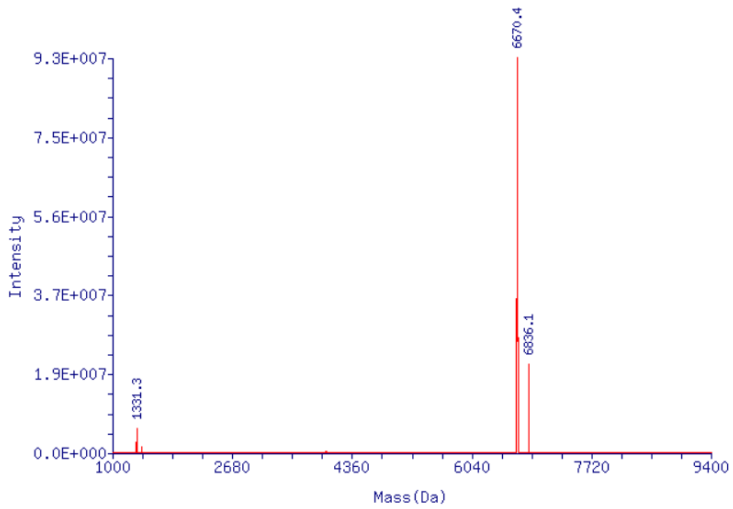

Figure S24. The MALDI-TOF-MS of T6

2.5 The cellular uptake ability of T1–T3.

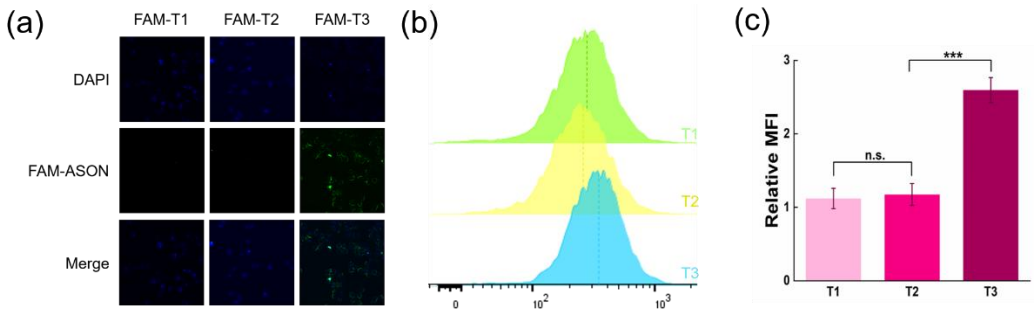

Figure S25. The cellular uptake ability of T1, T2 and T3. (a) Confocal images and (b) flow cytometry analysis of MCF-7 cells treated with PBS and T1–T3 [nuclei were labeled with DAPI (blue) and oligonucleotides was labeled with FAM (green); scale bars = 40 μm]. (c) Relative MFIs of treated MCF-7 cells. n.s., $p > 0.05$; ***$p < 0.001$. The data are presented as means ± standard deviations ($n = 3$).