Supporting Information

Development of a selective labeling probe for Bruton's tyrosine kinase quantification in live cells

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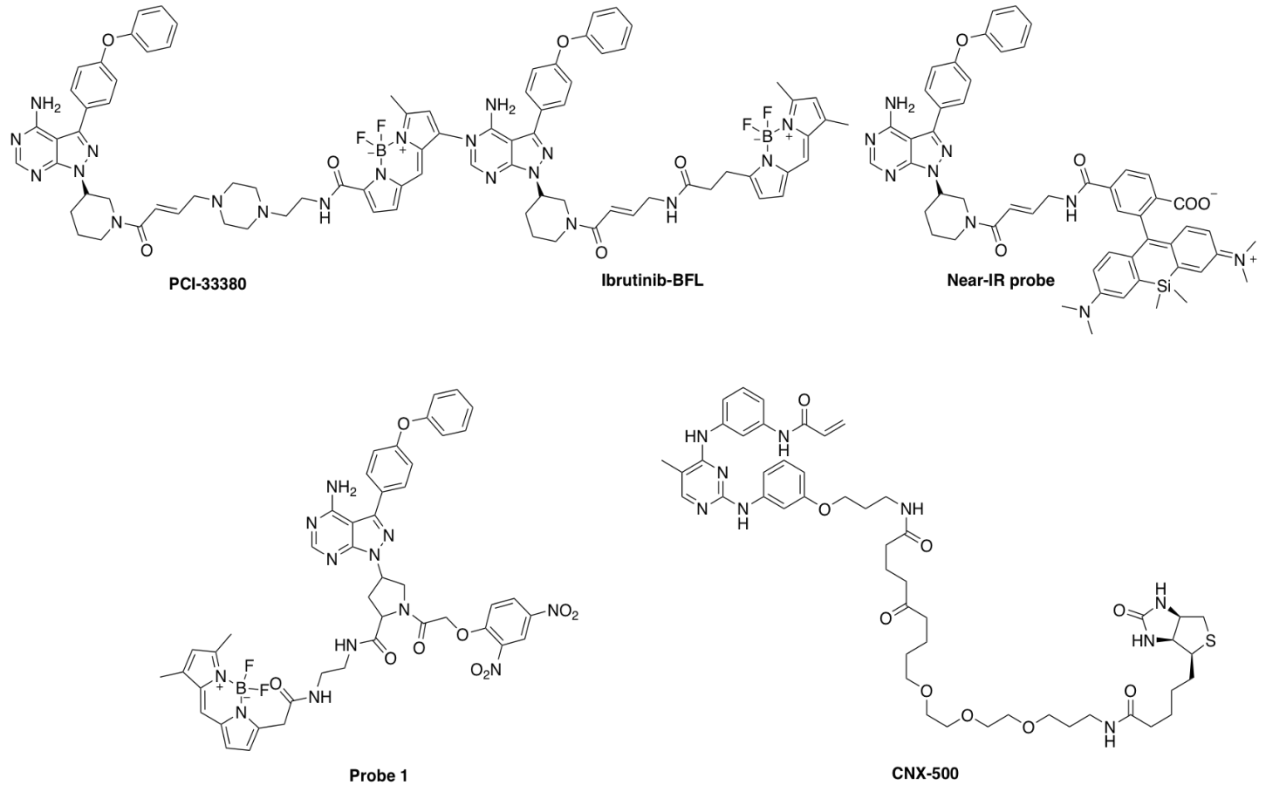
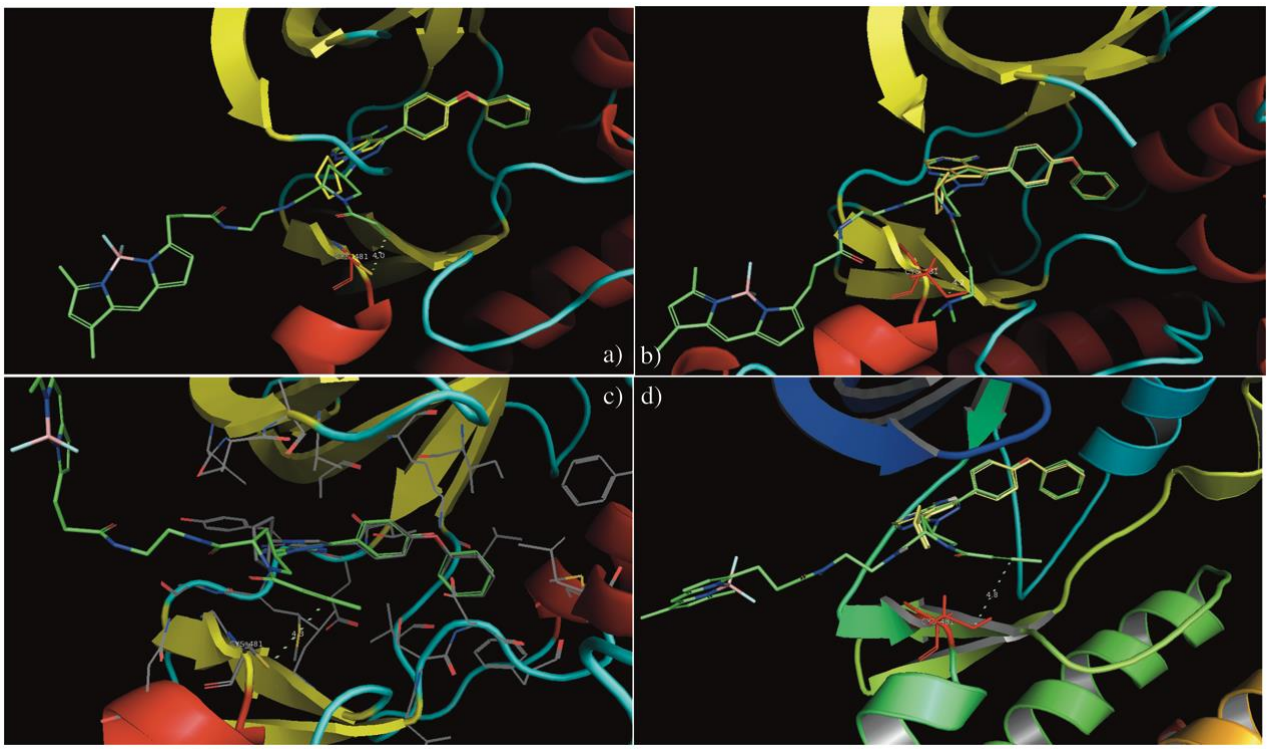
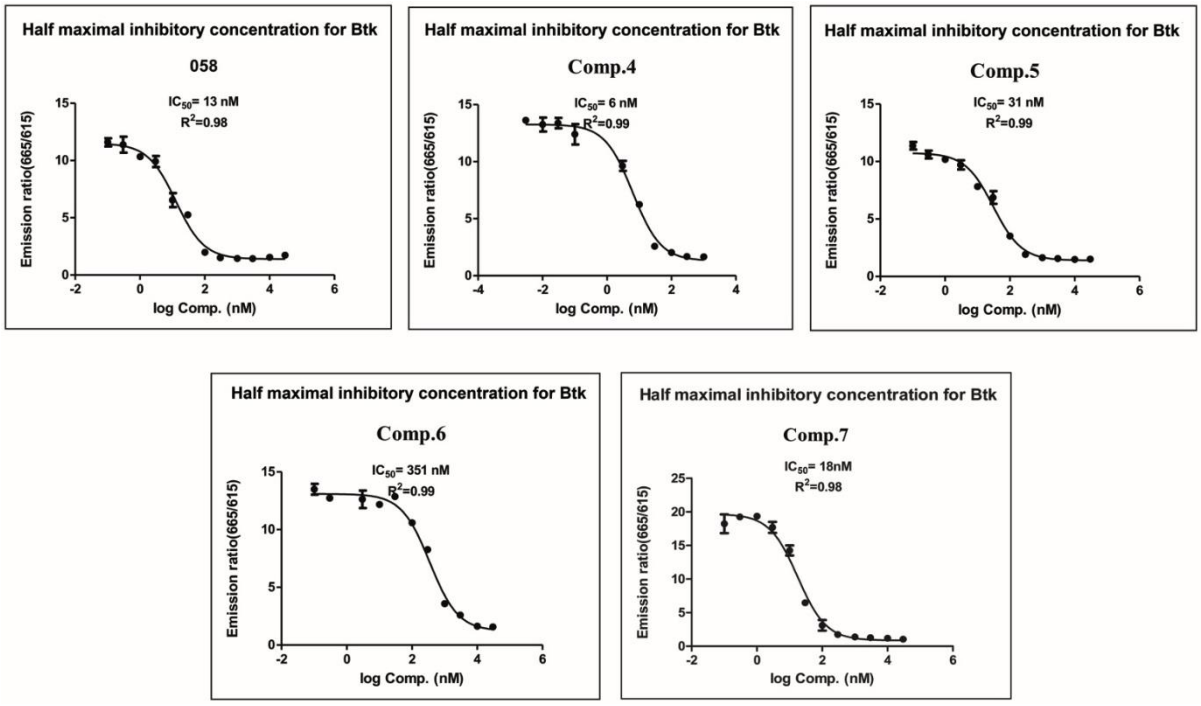
I. **Supporting Figures**



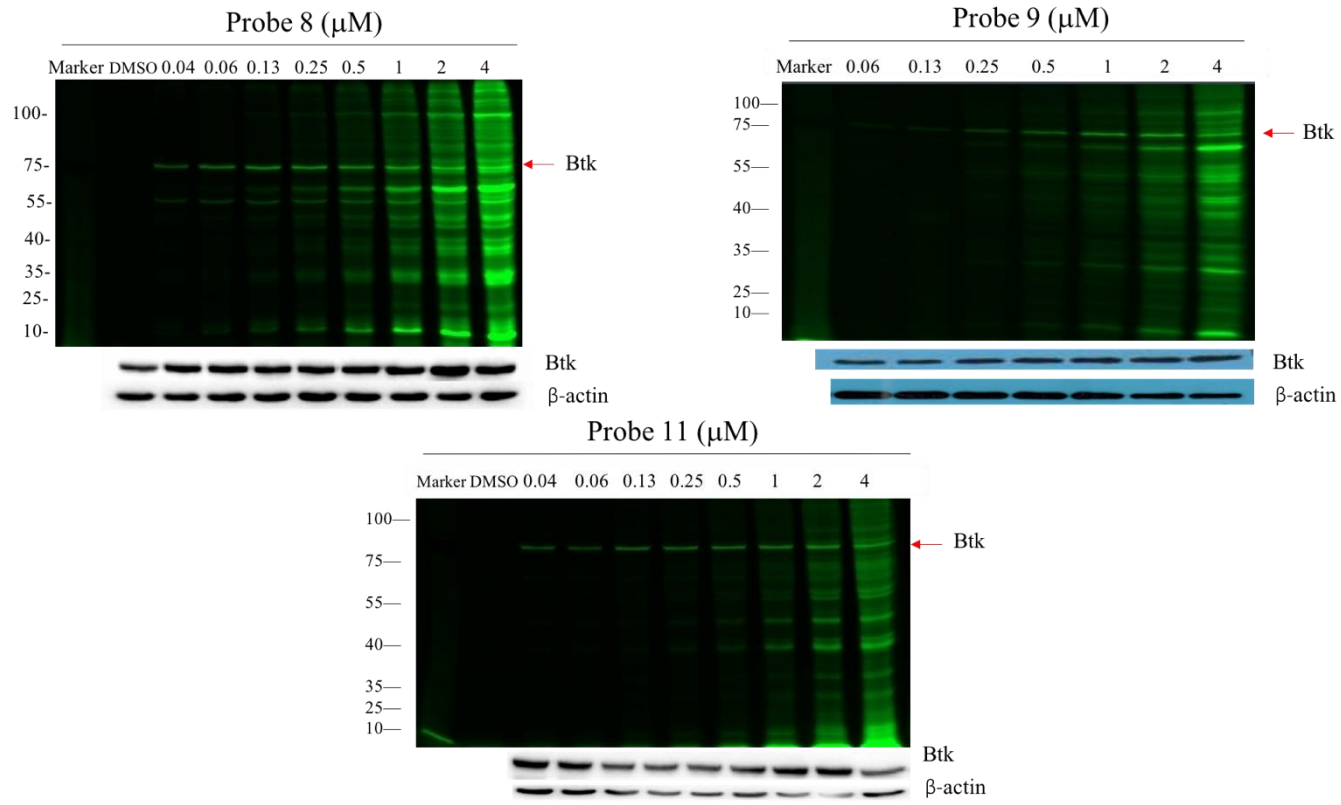
Supporting Figure 1. Structures of representative Btk probes.



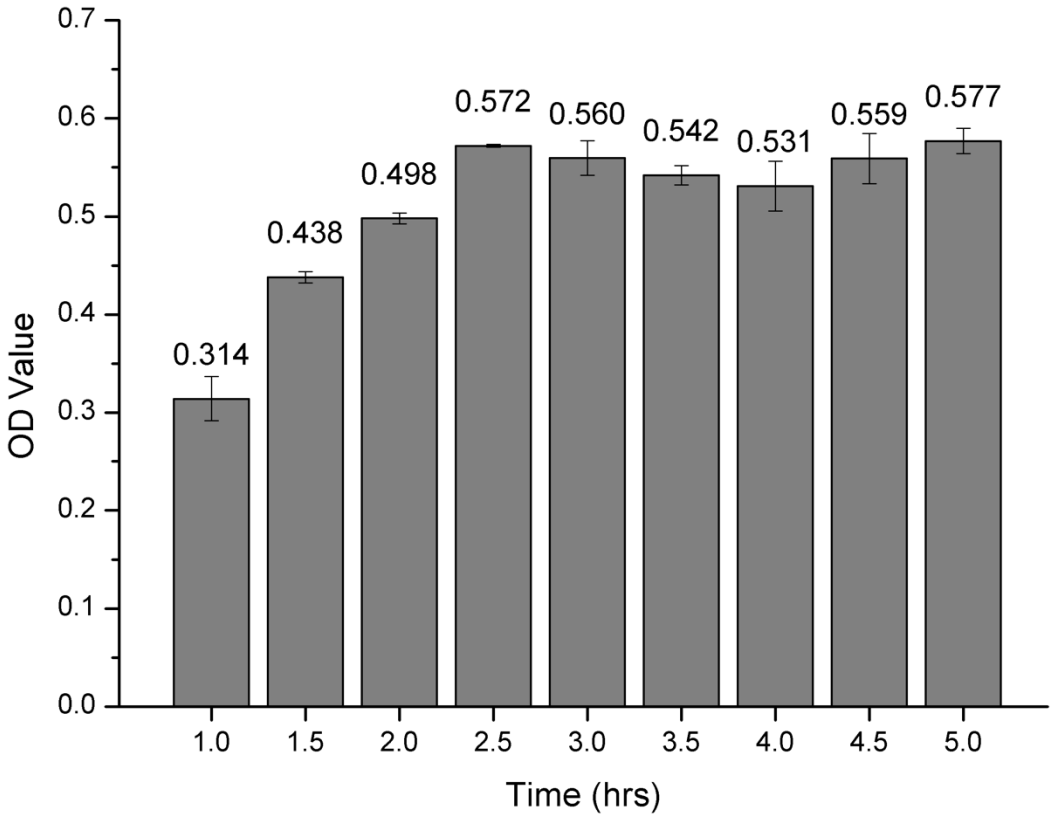
Supporting Figure 2. Docking models of probes in Btk's active site (carbon atoms in green, oxygen atom in red, nitrogen in blue). (Generated by MOE 2015)
a) probe **8** b) probe **9** c) probe **11** d) probe **10** Cys481 is at the C-terminus of the hinge region and the distances between the warhead and the residue were show in red. All probes were predicted to overlap with the original ligand and the reactive groups are about 4 Å away from the cysteine residue.



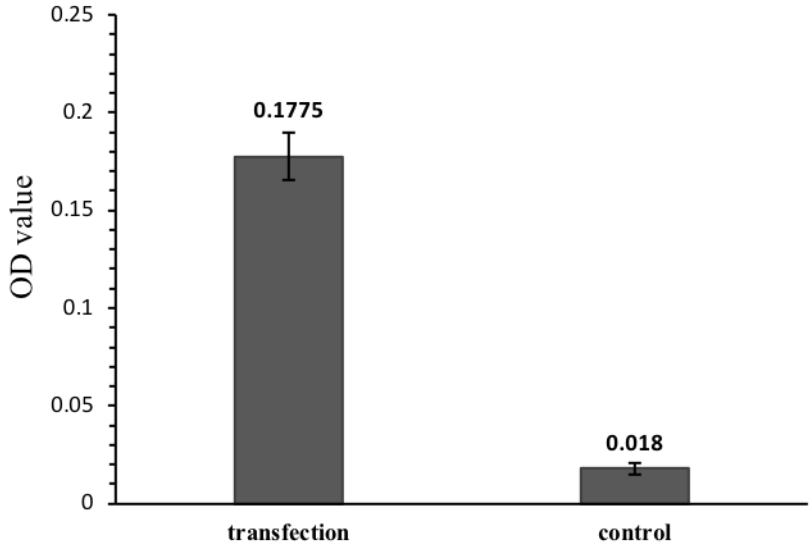
Supporting Figure 3. The IC_{50} values of the four probe precursors. Compound **058** is a known Btk inhibitor with a reported IC_{50} of 5 nM [compound **31** in *J Med Chem* **2014**, *57*, 5112].



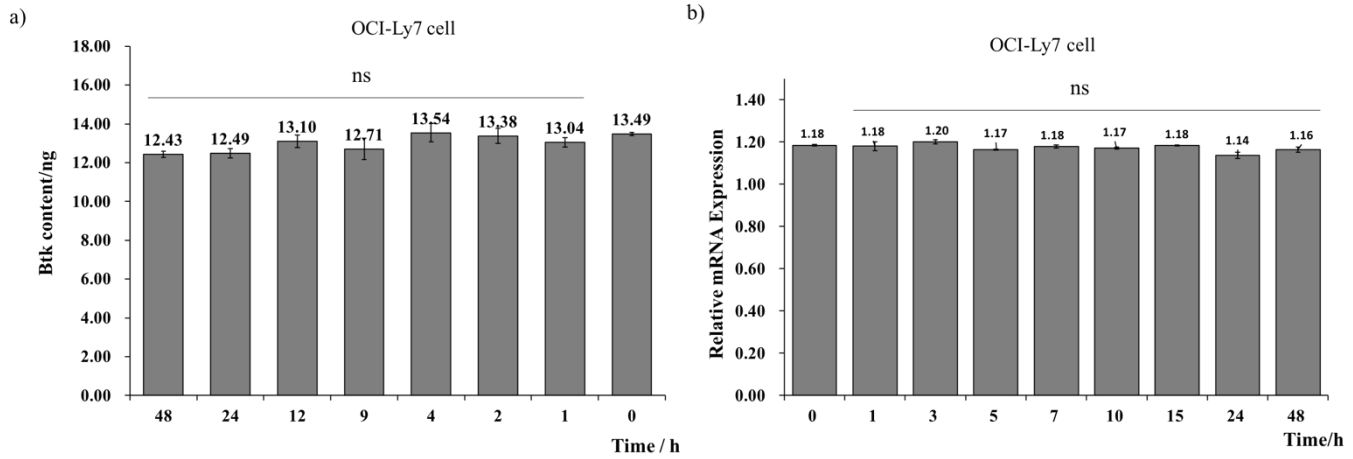
Supporting Figure 4. Btk in OCI-Ly7 cells were labelled by probes **8**, **9** and **11**.



Supporting Figure 5. The relationship between sample incubation time and fluorescence readout. The cell lysates in each well were incubated for different time from 1 to 5 hours. It requires 2.5 hours for the antibody to fully capture Btk protein under our experiment conditions.



Supporting Figure 6. The detection result of Btk transfection in Jurkat cells by ELISA.
The human Btk plasmid were transferred into 100 thousand Jurkat cells by Electroporation technology. After 48 hours in 37℃, 5%CO_2, the cells were incubated with probe **10** (2 μM). After 1 hour, the cells were harvested and detected by ELISA method. The result showed significant difference between the two samples.



Supporting Figure 7. a) The Btk content in OCI-Ly7 cell after BCR activation for 0 to 48 hours. b) The relative mRNA expression levels in OCI-Ly7 BCR activation. Differences were evaluated using Student's *t* test comparing the value of *T*= 0 hour with the later time points; ns, non-significant. All the data are mean ± s.e.m. of two independent experiments measured in duplicate (n=2).

Btk/ng	OD value	Std. Error Mean
300.00	2.7434	0.02
150.00	1.5548	0.03
75.00	0.7894	0.09
37.50	0.3658	0.08
18.75	0.2319	0.02
9.38	0.1775	0.01
4.69	0.1128	0.00
2.34	0.0985	0.02
1.17	0.0935	0.01
0.59	0.0898	0.01

Supporting Figure 8. The raw data of standard curve.

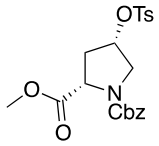
II. Chemistry

General Information

^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Advance 400 and 500 spectrometers at ambient temperature. Chemical shifts are reported in ppm relative to residual chloroform (δ_H 7.26 and δ_C 77.16) and dimethylsulfoxide (δ_H 2.50 and δ_C 39.52) as standards. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants and number of protons. Purity data were estimated by HPLC with UV detection at 254 nm. Mass spectrometric data were obtained using an AB Q-Star mass spectrometer.

Synthesis of Compounds

Compound 1



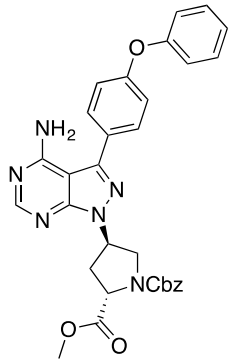
1

Thionyl chloride (9.5 ml, 133.5 mmol) was added to (4*S*)-4-Hydroxy-L-proline (5 g, 38.15 mmol) dissolved in 150 ml methanol for 1h. The reaction was stirred for 4h at room temperature. The mixture was concentrated and dissolved in 150 ml DCM. Cbz-OSU (11 g, 46 mmol) and Et_3N (9 ml, 76 mmol) were added to the mixture. The reaction was stirred for 4 hours at room temperature. The mixture was concentrated and extracted with ethyl acetate and water three times. The ethyl acetate layers were collected and washed with saturated aq. NaCl solution, dried over anhydrous Na_2SO_4, concentrated in vacuo and purified by flash column chromatography (EtOAc : Hexanes = 1:3) to give an intermediate as white solids (yield 98%).

P-TsCl (0.4 g, 2.2 mmol), DMAP (6 mg, 0.05 mmol) and Et_3N (0.3 ml, 2 mmol) were added to the intermediate (0.3 g, 1 mmol). The reaction was stirred for 4 hours at room temperature. The mixture was concentrated and extracted with ethyl acetate and water three times. The ethyl acetate layers were collected and washed with brine, dried over anhydrous Na_2SO_4, concentrated in vacuo and purified by flash column chromatography (EtOAc : Hexanes = 1:1) to give compound **1** as white oil (yield 74%).

^{1}HNMR (400 MHz, $CDCl_3$) δ 7.73 (d, *J* = 8.3 Hz, 2H), 7.37 – 7.20 (m, 7H), 5.20 – 4.94 (m, 3H), 4.47 (ddd, *J* = 24.8, 7.8, 3.9 Hz, 1H), 3.75 – 3.52 (m, 5H), 2.40 (qd, *J* = 8.5, 4.6 Hz, 5H).^{13}CNMR (101 MHz, $CDCl_3$) δ 171.56, 171.30, 154.39, 154.03, 145.27, 136.27, 133.56, 130.04, 128.53, 128.09, 127.75, 78.72, 77.83, 77.48, 77.16, 76.84, 67.38, 57.58, 57.33, 52.36, 52.00 – 51.72, 37.21, 36.12, 21.71. HRMS (m/z): $[M+H]^+$ calcd. for $C_{21}H_{24}NO_7S^+$, 434.1273; found: 434.1278.

Compound 2

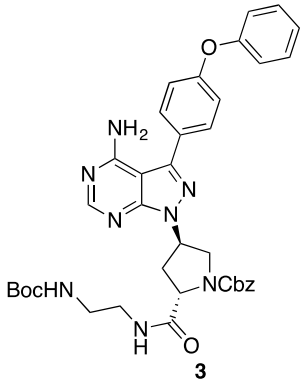


2

The 3-(4-Phenoxyphenyl)-1*H*-pyrazolo (3,4 d) pyridine-4-amine (1 g, 3.3 mmol) was dissolved in 25 ml DMF and stirred with potassium carbonate (0.9 g, 6.6 mmol) for 30 min. Compound **1** obtained in the previous step dissolved in 5 ml DMF was added to the reaction system. The reaction mixture was heated to 80°C and stirred overnight under argon atmosphere. The mixture was concentrated and extracted with ethyl acetate and water three times. The ethyl acetate layers were collected and washed with brine, dried over anhydrous Na_2SO_4, concentrated in vacuo and purified by flash column chromatography (EtOAc : Hexanes = 3:1) to give compound **2** as white solids (yield 90%).

^{1}HNMR (400 MHz, $CDCl_3$) δ 8.28 (s, 1H), 7.61 – 7.52 (m, 2H), 7.34 (dd, *J* = 11.5, 4.3 Hz, 2H), 7.30 – 7.18 (m, 5H), 7.16 – 6.98 (m, 5H), 6.04 (s, 2H), 5.68 – 5.51 (m, 1H), 5.15 (dd, *J* = 12.4, 7.0 Hz, 1H), 5.05 (dd, *J* = 12.4, 9.2 Hz, 1H), 4.81 – 4.70 (m, 1H), 4.13 – 3.93 (m, 2H), 3.75 (s, 1.5H), 3.60 (s, 1.5H), 3.05 – 2.90 (m, 1H), 2.52 – 2.40 (m, 1H).^{13}CNMR (101 MHz, $CDCl_3$) δ 172.75, 158.70, 158.15, 156.34, 155.90, 154.84, 154.61, 154.24, 144.46, 136.45, 130.01, 128.49, 128.18 – 127.71, 124.16, 119.67, 119.12, 98.75, 77.48, 77.16, 76.84, 67.31, 58.72, 54.45, 53.87, 53.52, 52.52, 51.66 (s), 50.86, 35.75, 34.81, 31.64, 22.71, 14.19. HRMS (m/z): $[M+H]^+$ calcd. for $C_{31}H_{29}N_6O_5^+$, 565.2194; found: 565.2194.

Compound 3.

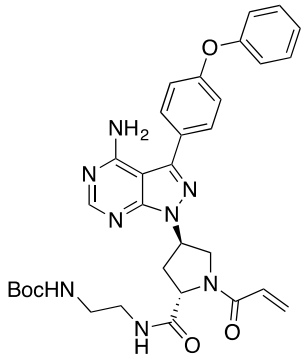


3

Compound **2** (1 g, 1.8 mmol) was dissolved in 15 ml tetrahydrofuran : methanol : water = 9 : 3 : 3 at 0°C. 1N LiOH (0.1 g, 4.55 mmol) was added slowly to the reaction mixture. After 4 hours, the mixture was concentrated and extracted with ethyl acetate and water. The aqueous layers were combined and acidified to pH = 1 with 1N HCl at 0°C. The reaction mixture was extracted with ethyl acetate and water three times. The organic layers were collected and dried over anhydrous Na_2SO_4 to give the carboxylic acid. EDCI (1.7 g, 9 mmol), HOBt(0.5 g, 3.6 mmol), DIPEA (15 ml, 9 mmol) and N-(2-aminoethyl) carbamic acid *tert*-butyl ester(0.1 g, 4.55 mmol) were added to the solution of the acid under argon atmosphere. The mixture was stirred for 4 hours and then extracted with ethyl acetate and water. The organic layer was washed with saturated NaCl solution, dried over anhydrous Na_2SO_4, concentrated and purified by flash column chromatography (EtOAc : Hexanes = 5:1) to give compound **3** as white solids (yield 90 %).

^{1}HNMR (500 MHz, $CDCl_3$) δ 8.31 (s, 1H), 7.63 – 7.55 (m, 2H), 7.41 – 7.27 (m, 7H), 7.18 – 7.14 (m, 1H), 7.12 (d, *J* = 8.6 Hz, 2H), 7.07 (dd, *J* = 8.6, 1.0 Hz, 2H), 5.99 (s, 2H), 5.54 – 5.37 (m, 1H), 5.22 (d, *J* = 12.3 Hz, 1H), 5.12 (d, *J* = 12.3 Hz, 1H), 4.50 – 4.40 (m, 1H), 4.11 (s, 2H), 3.04 (dd, *J* = 197.6, 78.4 Hz, 6H), 1.36 (s, 9H).^{13}CNMR (126 MHz, CDCl3) δ 158.95 – 158.66, 158.26 – 158.00, 156.40 – 156.38, 156.38 – 155.74, 154.85 – 154.41, 144.41 – 144.16, 136.22 – 135.93, 130.28 – 129.93, 128.80 – 128.51, 128.51 – 128.21, 128.21 – 128.02, 127.81 – 127.30, 124.43 – 124.15, 119.86 – 119.61, 119.17 – 118.97, 98.95 – 98.65, 77.41, 77.16, 76.91, 67.73, 60.40, 40.17, 28.46. HRMS (m/z): $[M+H]^+$ calcd. for $C_{37}H_{41}N_8O_6^+$, 693.3144; found: 693.3164.

Compound 4.



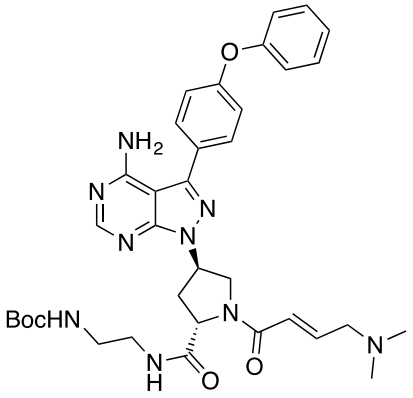
4

Compound **3** (220 mg, 0.32 mmol) was dissolved in 3 mL ethyl acetate and a few drops of methanol. A catalytic palladium hydroxide on carbon was added. The mixture was stirred under hydrogen at room temperature for 4 hours. The mixture was filtered with celatom and concentrated to give intermediate which was directly used in the next reaction without purification.

The reaction intermediate (60 mg, 0.1 mmol) was dissolved in a mixed solvent (1.5 ml tetrahydrofuran and two drops of water). Et_3N (28 mg, 40 μl, 0.27 mmol), acryloyl chloride (9 mg, 0.1 mmol) were added at 0°C. The mixture was stirred for 2 hours in room temperature. The mixture was concentrated and extracted with ethyl acetate and saturated NaCl solution three times. The organic layers was dried over anhydrous Na_2SO_4, filtered and concentrated by rotary evaporator in vacuo. The concentrate was purified by flash column chromatography (EtOAc : Hexanes = 3:1) to give the compound **4** as white solid (yield 70%).

^{1}HNMR (400 MHz, $CDCl_3$) δ 8.36 (s, 1H), 7.62 (d, *J* = 8.6 Hz, 2H), 7.39 (t, *J* = 7.9 Hz, 2H), 7.21–7.11 (m, 3H), 7.08 (d, *J* = 7.7 Hz, 3H), 6.54–6.41 (m, 2H), 5.79 (dd, *J* = 8.2, 3.9 Hz, 1H), 5.66 (s, 2H), 5.49 (dt, *J* = 15.3, 7.5 Hz, 1H), 5.16 (s, 1H), 4.68 (t, *J* = 7.9 Hz, 1H), 4.32 (dd, *J* = 10.5, 7.5 Hz, 1H), 4.27–4.18 (m, 1H), 3.39–3.20 (m, 2H), 3.13–2.88 (m, 2H), 2.78–2.61 (m, 1H), 1.39 (s, 9H).^{13}CNMR (101 MHz, $CDCl_3$) δ170.90 (s), 166.05 (s), 158.88 (s), 157.92 (s), 156.33 (s), 154.88 (s), 144.41 (s), 130.11 (d, *J* = 8.5 Hz), 127.95 (s), 127.48 (s), 119.77 (s), 119.14 (s), 99.00 (s), 79.30 (s), 77.48 (s), 77.16 (s), 76.84 (s), 59.78 (s), 54.68 (s), 51.05 (s), 40.40 (s), 40.11 (s), 33.25 (s), 28.53 (s). HRMS (m/z): $[M+H]^+$ calcd. for $C_{32}H_{37}N_8O_5^+$, 613.2881; found: 613.2897.

Compound 5.

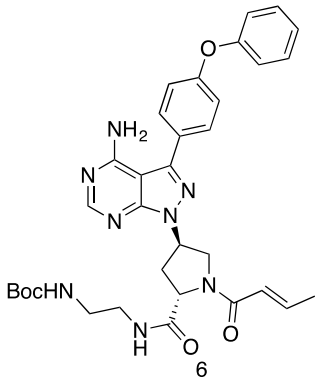


5

Compound **5** was synthesized with a similar procedure as compound **4**. (2*E*)-4-(Dimethylamino)-2-butenoic acid (31 mg, 0.36 mmol), HOBt (25 mg, 0.18 mmol), EDCI (86 mg, 0.45 mmol) and Et_3N (45 mg, 0.45 mmol) were added to the intermediate in 3 ml DCM and stirred for 6 hours. The mixture was purified with a similar procedure to give compound **5** as white solids (yield 55%).

^{1}HNMR (400 MHz, $CDCl_3$) δ 8.29 (s, 4H), 7.58 (t, *J* = 9.7 Hz, 8H), 7.35 (t, *J* = 7.9 Hz, 10H), 7.17–7.00 (m, 20H), 6.93 (dt, *J* = 15.1, 5.8 Hz, 4H), 6.31 (d, *J* = 15.2 Hz, 4H), 5.49–5.39 (m, 4H), 4.64 (t, *J* = 8.1 Hz, 4H), 4.24 (dt, *J* = 17.6, 10.1 Hz, 7H), 3.26 (ddd, *J* = 19.5, 13.7, 7.2 Hz, 8H), 3.16 (d, *J* = 5.1 Hz, 7H), 2.99 (ddd, *J* = 21.9, 11.8, 5.6 Hz, 15H), 2.75–2.58 (m, 3H), 2.20 (s, 24H), 1.34 (s, 36H).^{13}CNMR (101 MHz, $CDCl_3$) δ171.23, 165.81, 158.71, 158.16, 156.79–156.50, 156.10, 154.76, 144.44, 130.02, 127.47, 124.16, 122.48, 119.65, 119.06, 98.82, 79.15, 77.50, 77.18, 76.86, 60.61, 59.60, 54.49, 50.99, 45.59, 40.13, 31.62, 28.44, 22.69, 14.17. HRMS (m/z): $[M+H]^+$ calcd. for $C_{35}H_{44}N_9O_5^+$, 670.3460; found: 670.3460.

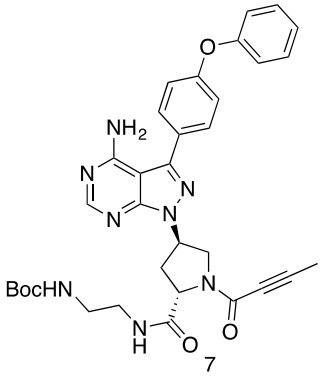
Compound 6.



Compound **6** was synthesized with a similar procedure as compound **4**. Crotonic acid (31 mg, 0.36 mmol), HOBt (25 mg, 0.18 mmol), EDCI (86 mg, 0.45 mmol) and Et_3N (45 mg, 0.45 mmol) were added to the intermediate in 3 ml DCM and stirred for 6 hours. The mixture was treated with similar procedure to give compound **6** as white solids (yield 76%).

^{1}HNMR (400 MHz, CDCl3) δ 8.33 (s, 1H), 7.60 (t, *J* = 8.2 Hz, 2H), 7.38 (t, *J* = 7.9 Hz, 2H), 7.19 – 7.05 (m, 5H), 6.99 (dd, *J* = 14.8, 7.0 Hz, 1H), 6.16 (d, *J* = 14.4 Hz, 1H), 5.48 (dd, *J* = 14.7, 7.4 Hz, 1H), 5.27 (d, *J* = 5.6 Hz, 1H), 4.66 (t, *J* = 7.9 Hz, 1H), 4.25 (dt, *J* = 17.8, 10.3 Hz, 2H), 3.34 – 3.19 (m, 2H), 3.10 – 2.92 (m, 2H), 2.89 – 2.54 (m, 3H), 1.88 (dd, *J* = 6.9, 1.4 Hz, 3H), 1.37 (s, 9H).^{13}CNMR (101 MHz, CDCl3) δ 171.22, 166.34, 158.89, 157.71, 156.36, 155.28, 154.60, 145.40 – 145.22, 144.15, 130.07, 127.29, 124.28, 122.35, 119.74, 119.12, 98.83, 79.09, 77.48, 77.16, 76.84, 59.60, 54.73, 50.96, 40.16, 33.31, 28.49, 18.35. HRMS (m/z): $[M+H]^+$ calcd. for $C_{33}H_{39}N_8O_5^+$, 627.3038; found: 627.3071.

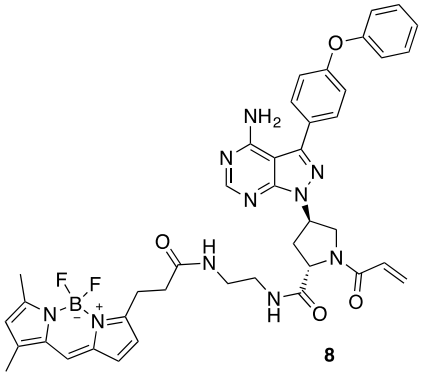
Compound 7.



Compound **7** was synthesized with a similar procedure as compound **4**. Butynoic acid (18.5 mg, 0.22 mmol) HOBt (25 mg, 0.18 mmol), EDCI (86 mg, 0.45 mmol) and Et_3N (45 mg, 0.45 mmol) were added to the intermediate in 3 ml DCM and stirred for 6 hours. The mixture was treated with similar procedure to give compound **7** as white solids (yield 85%).

^{1}HNMR (400 MHz, CDC_{l3}) δ 8.34 (s, 1H), 7.61 (dd, *J* = 12.2, 5.4 Hz, 2H), 7.43 – 7.36 (m, 2H), 7.21 – 7.11 (m, 3H), 7.11 – 7.04 (m, 2H), 6.21 (s, 2H), 5.53 – 5.42 (m, 1H), 5.05 (s, 1H), 4.61 (t, *J* = 7.9 Hz, 1H), 4.47 (dd, *J* = 11.1, 7.3 Hz, 1H), 4.27 (dd, *J* = 11.1, 8.1 Hz, 1H), 3.34 – 2.94 (m, 7H), 2.84 – 2.68 (m, 1H), 1.99 (s, 3H), 1.40 (s, 9H).^{13}CNMR (101 MHz, CDCl3) δ 170.38, 159.09, 157.41, 156.32, 154.99, 154.41, 144.81, 130.29 – 129.90, 127.04, 124.39, 119.82, 119.14, 98.74, 90.44, 79.52, 77.48, 77.16, 76.84, 73.72, 61.91, 58.87, 53.96, 52.30, 50.07, 40.47, 40.21, 36.29, 33.58, 29.71, 29.40, 28.48, 22.81, 4.09. HRMS (m/z): $[M+H]^+$ calcd. for $C_{33}H_{37}N_8O_5^+$, 625.2881; found: 625.2918.

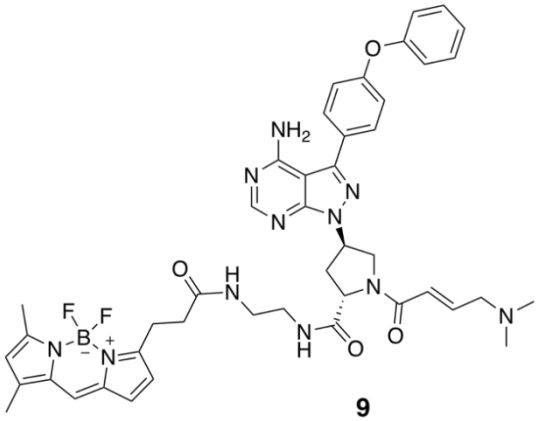
Compound 8.



Trifluoroacetic acid (35 mg, 0.31 mmol) was added to compound **4** (15 mg, 0.017 mmol) dissolved in 1 ml anhydrous DCM. The reaction was stirred at room temperature for 3 hours. The mixture was concentrated (theoretical: 8.8 mg, 0.017 mmol) and dissolved in 2 ml DMF which was directly used in the next reaction. HATU (19.4 mg, 0.051 mmol), BODIPY®FL (5 mg, 0.017 mmol) and DIPEA (11 mg, 15 µl, 0.085 mmol) were added to the intermediate at 0°C under argon atmosphere. The reaction was stirred for 6 hours at room temperature. The mixture was concentrated in vacuo and extracted with ethyl acetate and saturated NaCl solution three times. The organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated in vacuo. The mixture was purified by HPLC (gradient elution of acetonitrile from 50% to 95% in 15min) and lyophilized to give red solids (yield 42%, purity 93%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.34 (s, 1H), 7.61 (d, *J* = 8.6 Hz, 2H), 7.42 – 7.34 (m, 2H), 7.18 (t, *J* = 7.4 Hz, 2H), 7.13 (d, *J* = 8.6 Hz, 2H), 7.08 (dd, *J* = 9.9, 2.2 Hz, 3H), 6.89 (d, *J* = 4.0 Hz, 1H), 6.46 (d, *J* = 14.2 Hz, 1H), 6.42 – 6.37 (m, 2H), 6.30 (d, *J* = 4.0 Hz, 1H), 6.08 (s, 1H), 5.80 – 5.73 (m, 1H), 5.70 (dd, *J* = 8.8, 3.4 Hz, 1H), 5.56 (s, 2H), 4.86 (t, *J* = 6.2 Hz, 1H), 4.17 (ddd, *J* = 16.6, 10.3, 6.8 Hz, 2H), 3.40 (dd, *J* = 12.7, 9.8 Hz, 3H), 3.27 (t, *J* = 7.4 Hz, 3H), 2.79 (t, *J* = 6.8 Hz, 2H), 2.72 – 2.59 (m, 2H), 2.52 (s, 3H), 2.23 (d, *J* = 8.3 Hz, 3H).^{13}C NMR (101 MHz, $CDCl_3$) δ 172.56, 171.56, 171.21, 165.98, 165.98, 160.25, 158.61, 157.78, 157.63, 156.03, 155.98, 154.34, 144.35, 143.68, 141.06, 138.74, 135.05, 133.40, 130.14, 130.14, 130.04, 129.50, 129.43, 128.46, 128.46, 127.95, 127.77, 127.10, 124.29, 124.11, 123.94, 123.79, 120.45, 119.76, 119.76, 119.23, 119.15, 117.50, 98.53, 77.48, 77.48, 77.37, 77.16, 77.16, 76.84, 76.84, 59.83, 59.37, 55.13, 54.73, 51.52, 51.41, 40.22, 39.79, 39.24, 38.80, 35.80, 35.75, 33.10, 32.81, 24.94, 24.87, 14.88, 11.43, 11.27, 1.28, 1.16, -0.04. HRMS (m/z): $[M+H]^+$ calcd. for $C_{41}H_{42}BF_2N_{10}O_4^+$, 787.3446; found: 787.3443.

Compound 9.

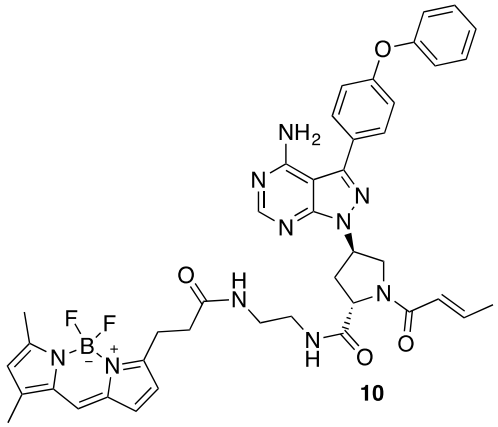


9

Compound **9** was synthesized with a similar procedure as compound **8**. Red solids (yield 30%, purity 98.5%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 11.69 (s, 2H), 8.20 (s, 2H), 7.53 (t, *J* = 7.8 Hz, 4H), 7.41 (t, *J* = 7.8 Hz, 4H), 7.21 (d, *J* = 7.3 Hz, 2H), 7.09 (dd, *J* = 23.7, 7.6 Hz, 9H), 6.86 (s, 4H), 6.71 (d, *J* = 13.5 Hz, 1H), 6.58 (s, 1H), 6.24 (d, *J* = 21.2 Hz, 3H), 6.11 (s, 2H), 5.49 (s, 1H), 4.66 (s, 3H), 4.30 (s, 5H), 3.98 (s, 2H), 3.81 (s, 3H), 3.71 (s, 2H), 3.43 (s, 2H), 3.30 (s, 6H), 3.14 (s, 5H), 2.91 (d, *J* = 29.7 Hz, 3H), 2.85 (s, 4H), 2.78 (s, 9H), 2.60 – 2.38 (m, 10H), 2.24 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 160.53, 159.99, 157.33, 157.14, 155.90, 153.91, 152.39, 147.18, 146.51, 146.42, 144.57, 135.39, 133.38, 132.09, 131.73, 130.58, 130.30, 129.96, 128.49, 125.19, 124.74, 124.05, 120.81, 120.02, 119.32, 116.91, 97.78, 97.73, 77.41, 77.16, 76.91, 59.81, 59.29, 57.37, 55.52, 54.17, 51.04, 42.71, 42.66, 42.55, 40.34, 38.98, 36.12, 35.13, 34.95, 33.62, 24.65, 15.05, 11.40. HRMS (m/z): $[M+H]^+$ calcd. for $C_{44}H_{49}BF_2N_{11}O_4^+$, 844.4025; found: 844.4027.

Compound 10.

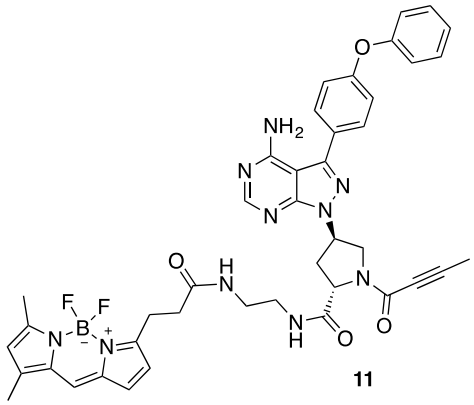


10

Compound **10** was synthesized with a similar procedure as compound **8**. Red solids (yield 25%, purity 98%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 11.66 (s, 1H), 8.10 (d, *J* = 13.8 Hz, 1H), 7.52 (t, *J* = 9.1 Hz, 2H), 7.42 (t, *J* = 7.9 Hz, 3H), 7.22 (t, *J* = 7.4 Hz, 1H), 7.15 (d, *J* = 8.5 Hz, 2H), 7.12 – 7.06 (m, 3H), 7.06 – 6.93 (m, 1H), 6.90 (d, *J* = 3.8 Hz, 1H), 6.70 (s, 1H), 6.27 (d, *J* = 3.9 Hz, 2H), 6.15 – 6.06 (m, 2H), 5.86 – 5.76 (m, 1H), 4.87 (s, 2H), 4.17 (ddd, *J* = 27.7, 18.6, 11.7 Hz, 3H), 3.42 (t, *J* = 19.5 Hz, 3H), 3.24 (d, *J* = 25.3 Hz, 3H), 2.82 – 2.60 (m, 4H), 2.48 (s, 3H), 2.24 (s, 3H), 1.85 (d, *J* = 6.3 Hz, 3H), 1.28 (dt, *J* = 14.3, 5.5 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.76, 155.43, 153.32, 151.92, 147.19, 145.95, 144.57, 144.16, 135.06, 133.04, 129.99, 129.46, 128.11, 124.63, 124.49, 123.82, 120.35, 119.78, 119.02, 116.74, 97.03, 77.16, 77.05, 76.85, 76.53, 59.13, 55.24, 50.72, 39.73, 39.35, 35.19, 32.91, 24.46, 18.12, 14.72, 11.11, -0.18. HRMS (m/z): $[M+H]^+$ calcd. for $C_{42}H_{44}BF_2N_{10}O_4^+$, 801.3603; found: 801.3657.

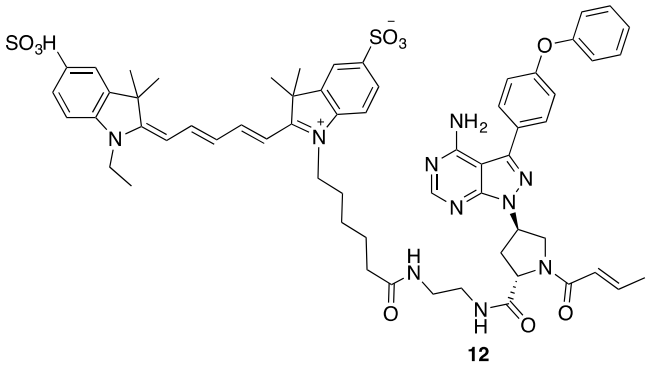
Compound 11.



Compound **11** was synthesized with a similar procedure as compound **8**. Red solids (yield 25%, purity 94%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 (s, 14H), 7.59 – 7.50 (m, 42H), 7.42 (t, *J* = 8.0 Hz, 42H), 7.22 (s, 28H), 7.18 – 7.12 (m, 44H), 7.09 (d, *J* = 7.3 Hz, 55H), 6.90 (d, *J* = 4.0 Hz, 19H), 6.36 (s, 17H), 6.30 (d, *J* = 3.8 Hz, 19H), 6.26 – 6.16 (m, 25H), 6.09 (s, 13H), 5.82 – 5.69 (m, 19H), 4.81 (d, *J* = 7.8 Hz, 17H), 4.23 (d, *J* = 5.8 Hz, 40H), 3.40 (s, 62H), 3.31 – 3.12 (m, 93H), 2.87 (d, *J* = 15.8 Hz, 55H), 2.76 – 2.57 (m, 111H), 2.51 (d, *J* = 12.1 Hz, 75H), 2.24 (s, 68H), 1.97 (d, *J* = 13.6 Hz, 58H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.06, 155.78, 153.86, 152.15, 147.46, 146.39, 144.04, 135.42, 135.05, 133.40, 122.14, 117.13, 97.38, 90.77, 73.58, 64.39, 58.93, 55.04, 52.88, 40.26, 39.43, 35.64, 33.76, 24.85, 21.17, 15.04, 11.44, 5.26, 4.16, 0.14. HRMS (m/z): $[M+H]^+$ calcd. for $C_{42}H_{42}BF_2N_{10}O_4^+$, 799.3446; found:799.3449.

Compound 12.



Trifluoroacetic acid (35 mg, 0.31 mmol) was added to compound **6** (13 mg, 0.02 mmol) dissolved in 1 ml anhydrous DCM. The reaction was stirred at room temperature for 3 hours. The mixture was concentrated (theoretical: 9 mg, 0.02 mmol) and dissolved in 2 ml DMF which was directly used in the next reaction. TSTU (3.6 mg, 0.012 mmol), compound Cy5 (5 mg, 0.0075 mmol) and Et_3N (7 μl, 0.051 mmol) were added to the intermediate at 0°C under argon atmosphere. The reaction was stirred overnight at room temperature. The reaction was monitored by HPLC. Petroleum ether (5 ml) was added to the reaction. The precipitate was dissolved in DMSO and purified by HPLC (gradient elution of acetonitrile from 30% to 95% in 30min) and lyophilized to give blue solids (yield 38%, purity 93%).

^{1}H NMR (500 MHz, DMSO) δ 8.35 (t, *J* = 12.9 Hz, 17H), 8.26 (t, *J* = 5.2 Hz, 11H), 7.81 (s, 22H), 7.64 (dd, *J* = 6.5, 2.1 Hz, 36H), 7.46 – 7.39 (m, 25H), 7.33 – 7.23 (m, 33H), 7.22 – 7.04 (m, 75H), 6.74 – 6.62 (m, 12H), 6.57 (t, *J* = 12.3 Hz, 9H), 6.30 (dd, *J* = 13.6, 9.6 Hz, 22H), 5.58 (dd, *J* = 21.4, 14.8 Hz, 8H), 4.62 (dd, *J* = 8.3, 4.8 Hz, 7H), 4.22 – 3.97 (m, 48H), 3.97 – 3.83 (m, 10H), 3.16 – 3.03 (m, 54H), 2.05 (dd, *J* = 12.7, 5.4 Hz, 19H), 1.84 – 1.73 (m, 32H), 1.68 (s, 107H), 1.52 (dd, *J* = 13.9, 6.5 Hz, 22H), 1.26 – 1.20 (m, 62H). ^{13}C NMR (126 MHz, DMSO) δ 173.20, 172.38, 172.05, 171.23, 163.98, 157.88, 157.17, 156.17, 155.64, 154.21, 145.37, 143.54, 141.85, 141.38, 140.53, 140.38, 130.02, 129.94, 128.38, 127.68, 127.40, 126.03, 125.55, 123.72, 123.27, 122.46, 119.86, 118.90, 118.87, 110.02, 103.43, 103.06, 97.45, 59.11, 58.85, 54.31, 50.89, 48.82, 48.78, 45.66, 40.02, 39.85, 39.69, 39.52, 39.35, 39.19, 39.02, 38.58, 38.50, 38.38, 38.03, 36.16, 35.67, 35.10, 34.07, 31.03, 30.69, 28.97, 28.88, 28.84, 28.69, 28.54, 28.45, 27.85, 27.02, 26.91, 26.55, 26.45, 25.63, 24.99, 24.67, 21.94, 17.49, 13.79, 13.70, 11.96, 8.47, 0.68. HRMS (m/z): $[M+H]^+$ calcd. for $C_{61}H_{69}N_{10}O_{10}S_2^+$, 1165.4640; found: 1165.4637.

III. Biology

General Information

All the fluorescence intensity analysis was recorded by Perkin Elmer Envision 2104 Multilabel Reader (Em =450 nm) using striwell™ microplate (costar 42592).

All the fluorescence gel scanning was conducted with PharasFX™ Plus Molecular Imager (Bio-Rad, Ex = 480nm and Em = 530nm, Ex = 635nm and Em =695nm) using Quantity One™ software.

Experimental Procedures

The detection efficiency of probe in cell samples.

Sample A and B of Figure 3b: 3×10^6/ml OCI-Ly7 cells were grown in six-well plates with probe **10** (1 μM) or probe **12** (1 μM) for 1 hour. Then DMSO (5 μl) was added into each well for 1 hour. All the cells were harvested.

Sample C and D of Figure 3b: 3×10^6/ml OCI-Ly7 cells were grown in six-well plates with probe **10** (1 μM) or DMSO for 1 hour. Then the lysate labelled by probe 12 (16 μM) for 1 hour either.

All the samples were heated at 100°C for 10 min and loaded on SDS-page gels (10%), and the gels were treated with the same fluorescence scanning and western blot steps as the probe labelling experiment. (Figure 3b)

Detection of the residual Btk in cell sample which have undergone ELISA detection.

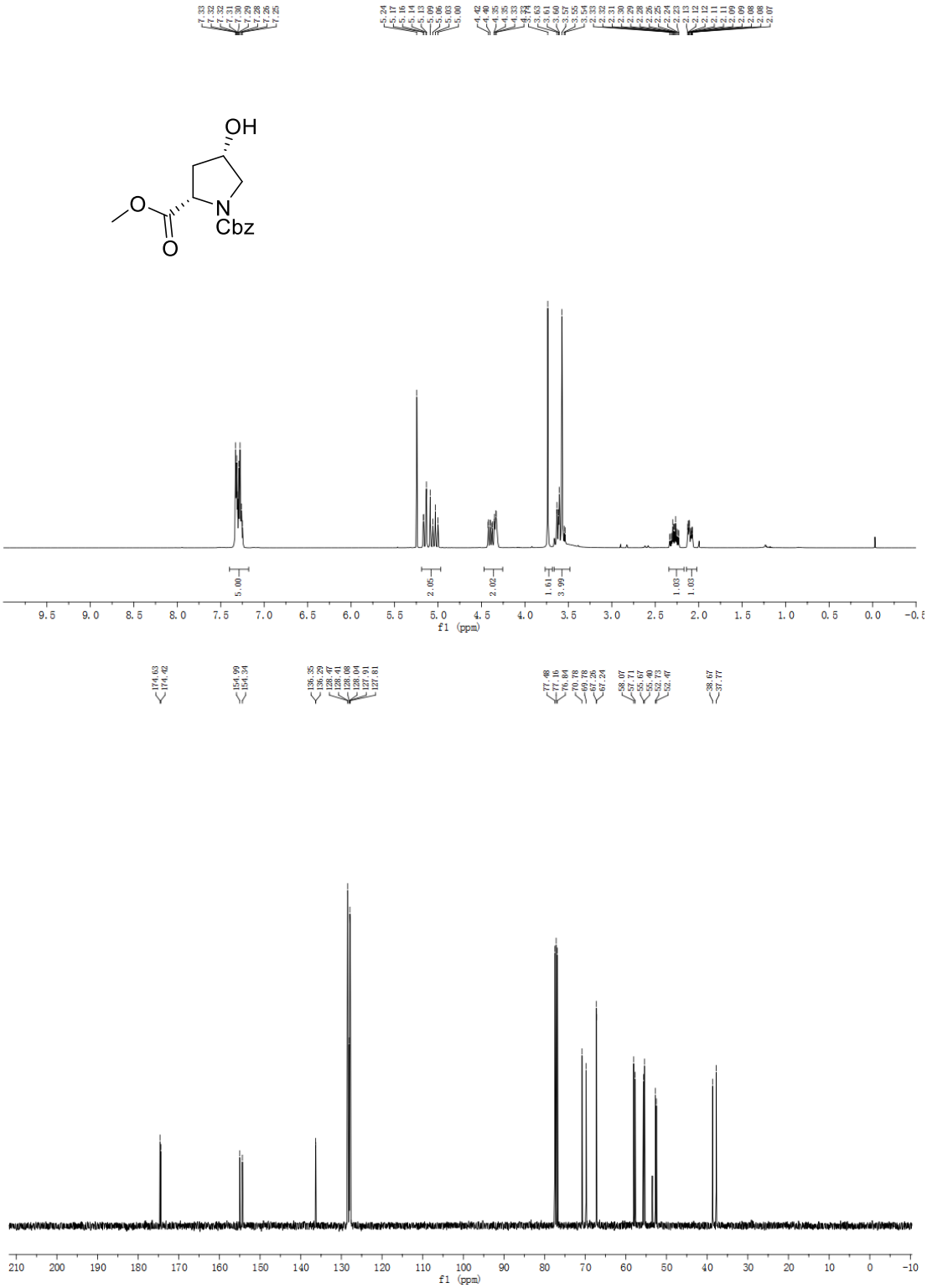
The cell samples were harvested after the ELISA detection. After heating at 100°C for 10 minutes, the denatured samples were loaded on SDS-page gels (10%), and was scanned for fluorescence signals, and also by western blotting. (Figure 3c)

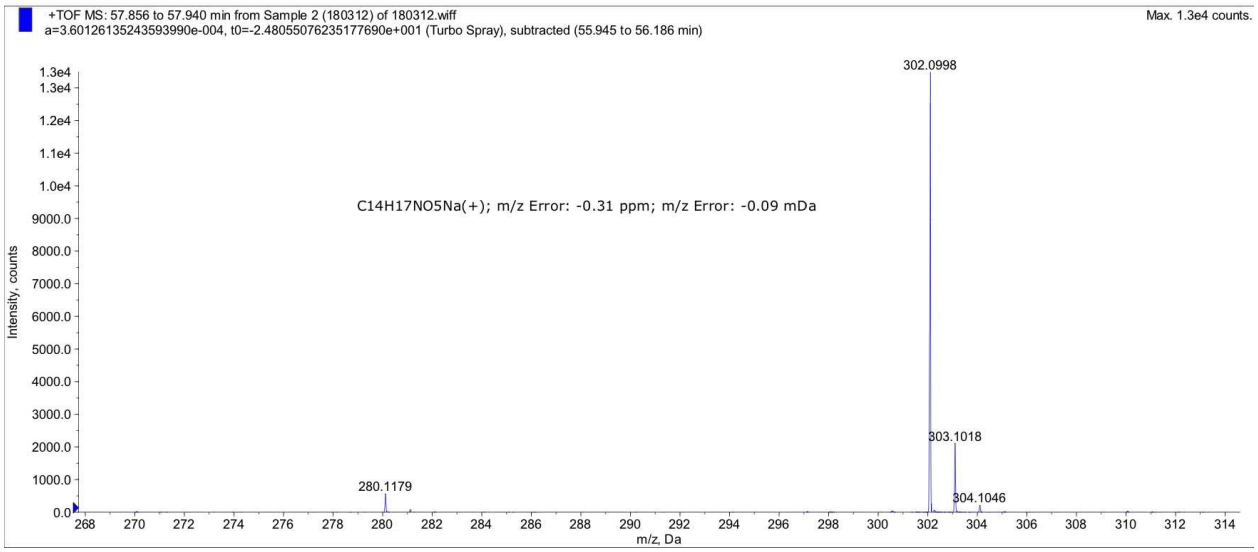
RT-qPCR experiment

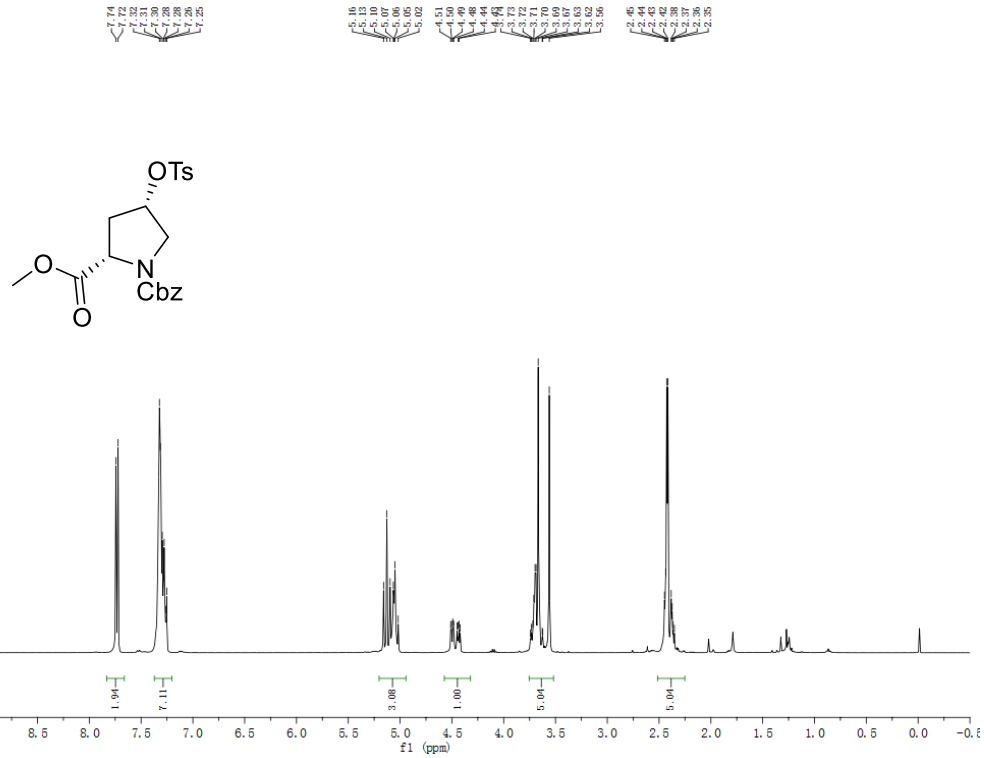
20×10^3 Ramos and OCI-Ly7 cells were incubated in 1ml of RPMI 1640 medium containing 9% fetal bovine serum and activated with 200μg/ml IgM (R&D G-105-C) for 0h, 1h, 2h, 4h, 9h, 12h and 48h. Total RNA was extracted from each group using TRIzol reagent according to the manufacturer's instructions (ambion 15596026). The RNA quality was assessed by determining the ratio of spectrophotometric absorbance of the sample at 260nm to that of 280nm using Nanodrop 2000. All samples had an A260/A280 absorption ratio > 1.6. cDNA was reverse transcribed from RNA using Revert Aid First Strand cDNA Synthesis kit (Thermo scientific #K1621). Primers for RT-qPCR assays of Btk were from sanggo biotech (9406063704 forward and 9406063705 reverse). Primers of β-actin used were from BBI life sciences (BN61102-0001 forward and BN61102-0002 reverse). All RT-qPCR reactions containing 2ul cDNA were performed using the FastStar Universal SYBR Green Master (Rox) (Roche 04913914001) on BIO-RAD CFX Connect™ real time system which was programmed as 50°C for 2min, 95°C for 10min, followed by 40 cycles at 90°C

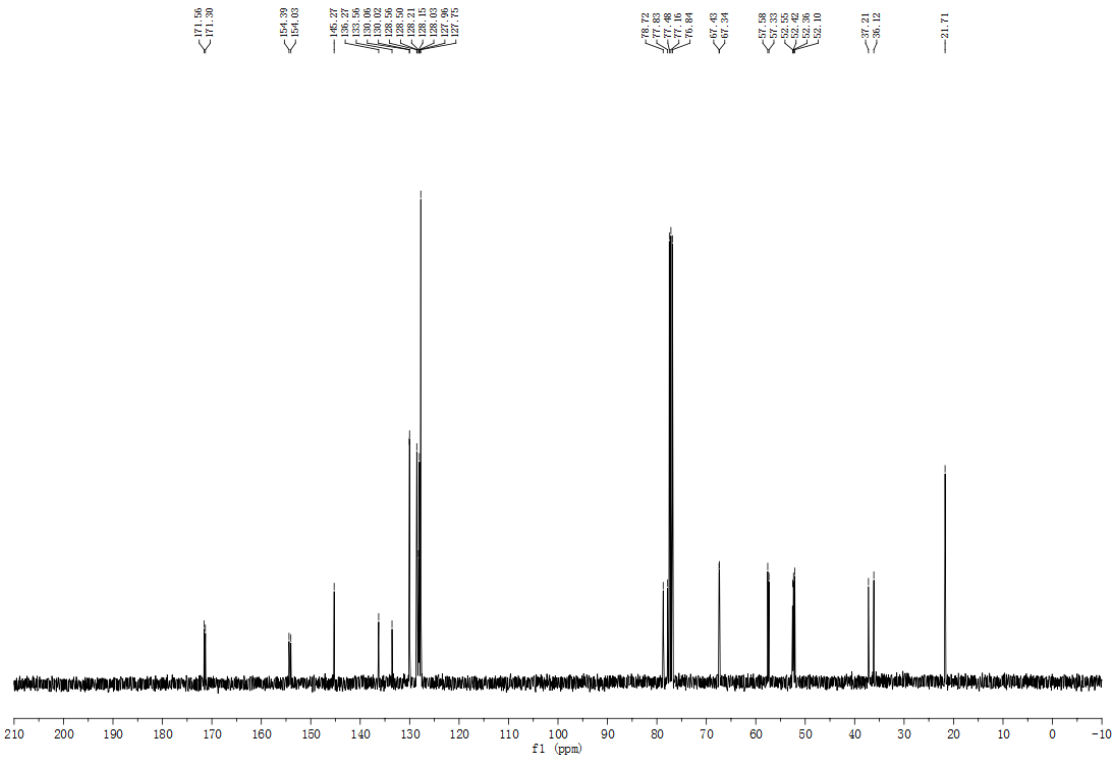
for 10s, 60°C for 30s, finally subjecting to the melting temperature to check the amplification curve. (Supporting Figure 7)

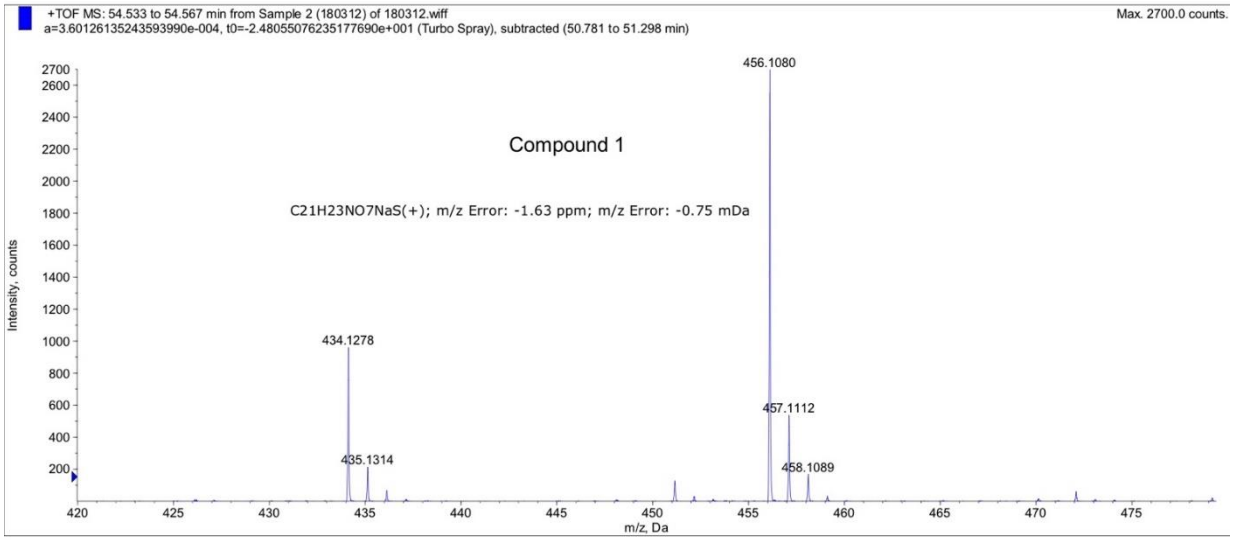
IV. ^{1}H NMR, ^{13}C NMR and HRMS Spectra of Compounds

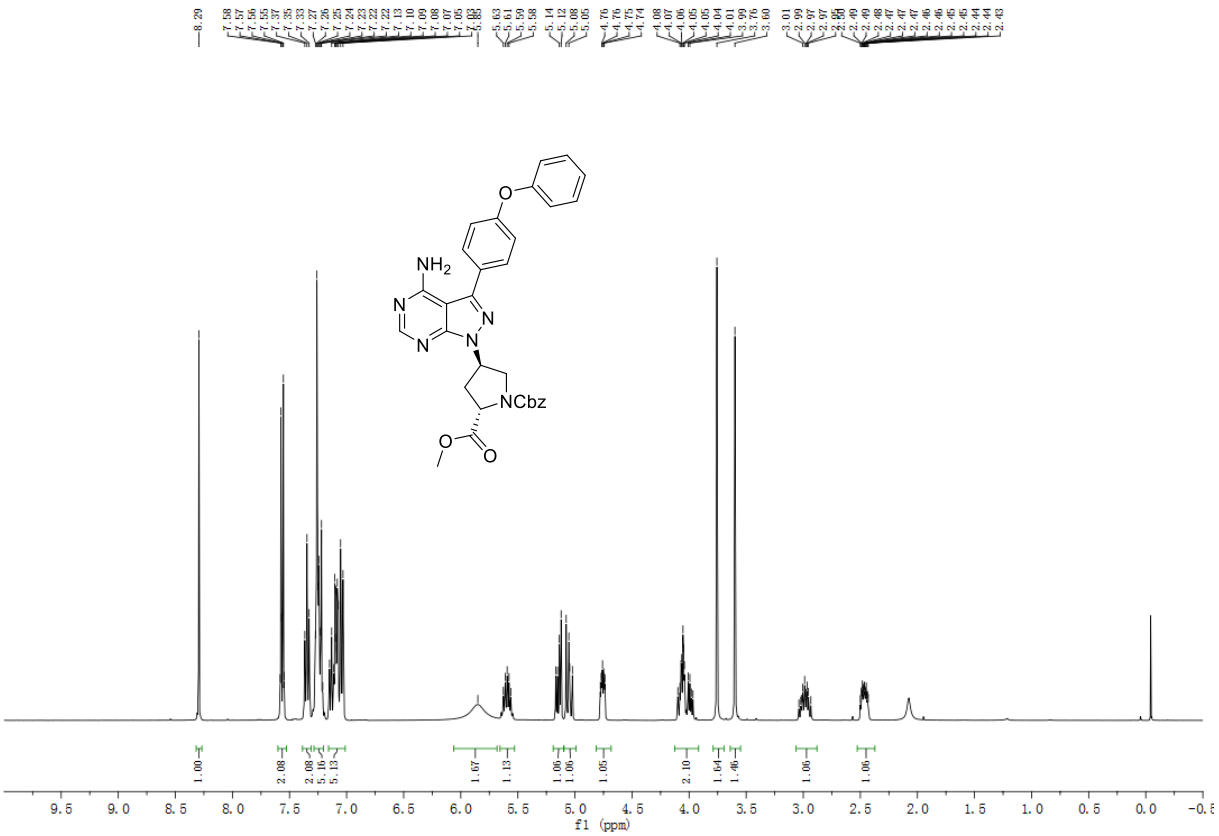


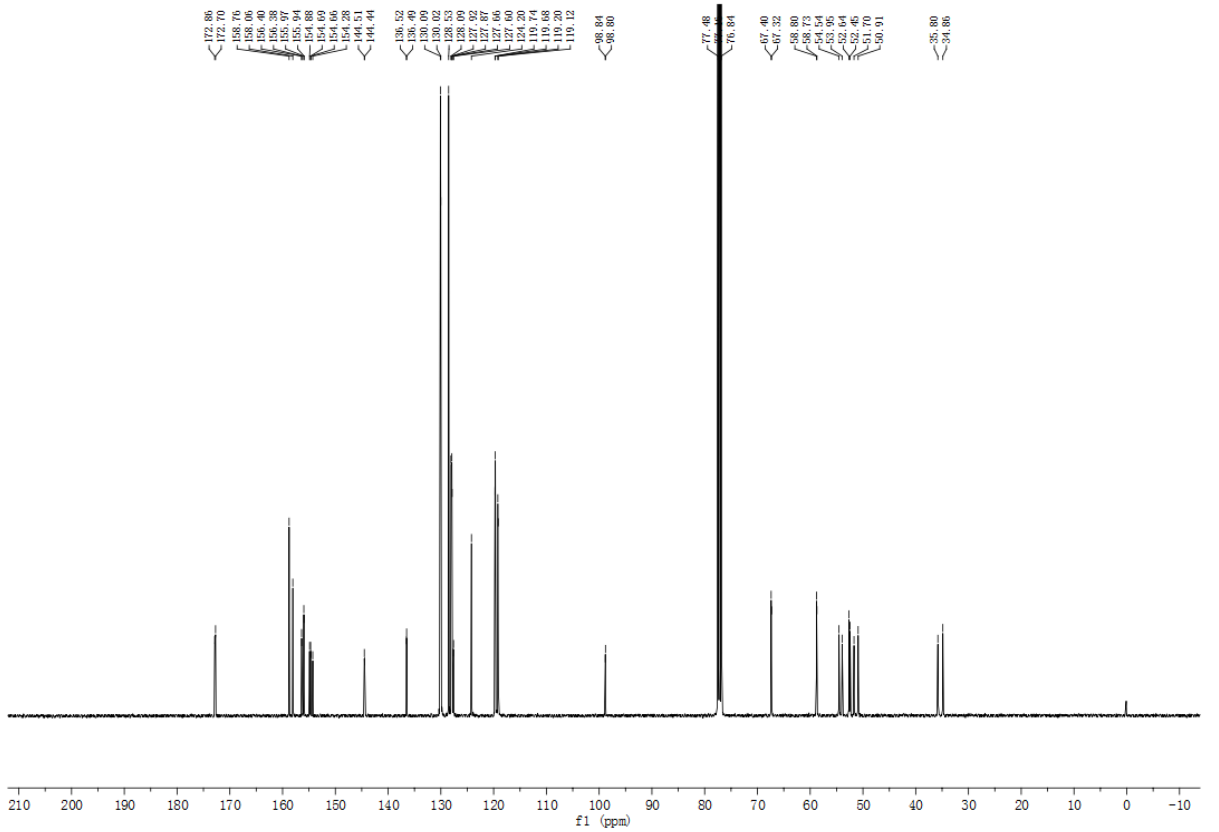

+TOF MS: 57.856 to 57.940 min from Sample 2 (180312) of 180312.wiff
a=3.60126135243593990e-004, t0=-2.48055076235177690e+001 (Turbo Spray), subtracted (55.945 to 56.186 min)
Max. 1.3e4 counts.
C14H17NO5Na(+); m/z Error: -0.31 ppm; m/z Error: -0.09 mDa
302.0998
303.1018
304.1046
280.1179
Intensity, counts
m/z, Da

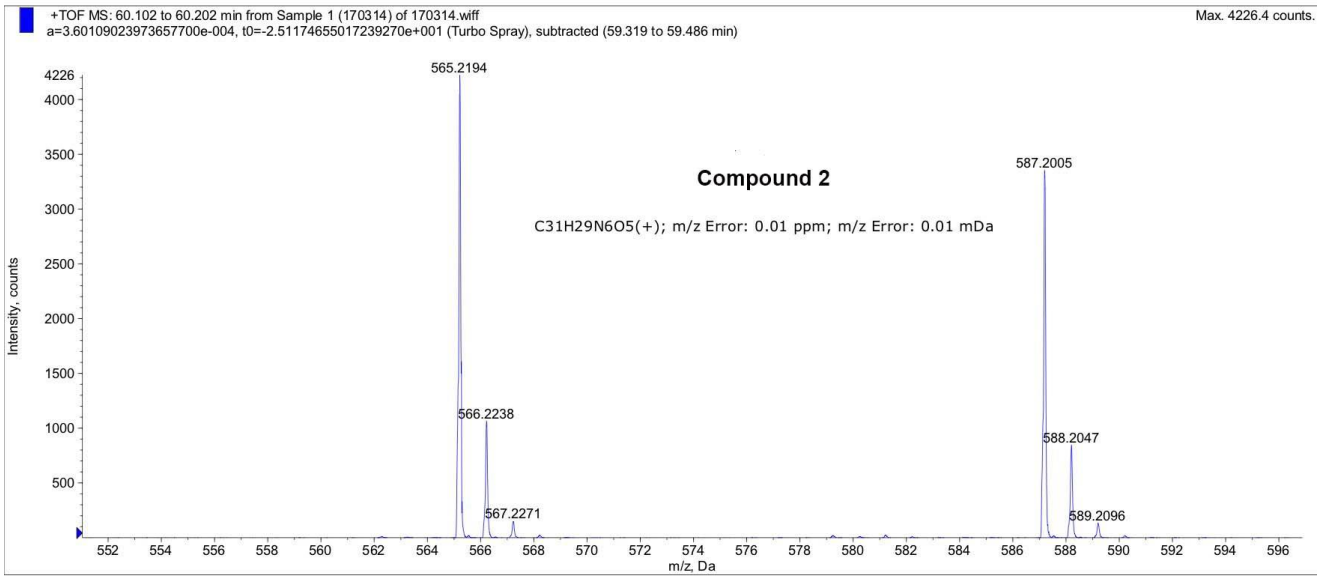

OTs
O
N
Cbz
O
f1 (ppm)

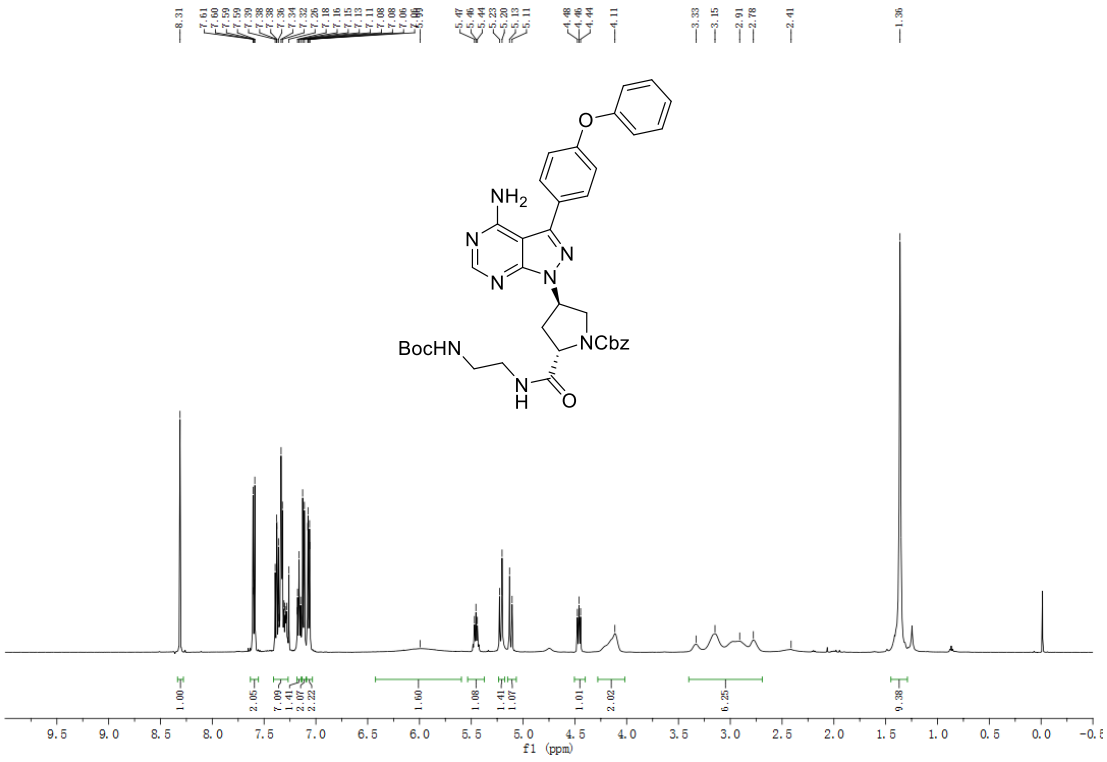

f1 (ppm)

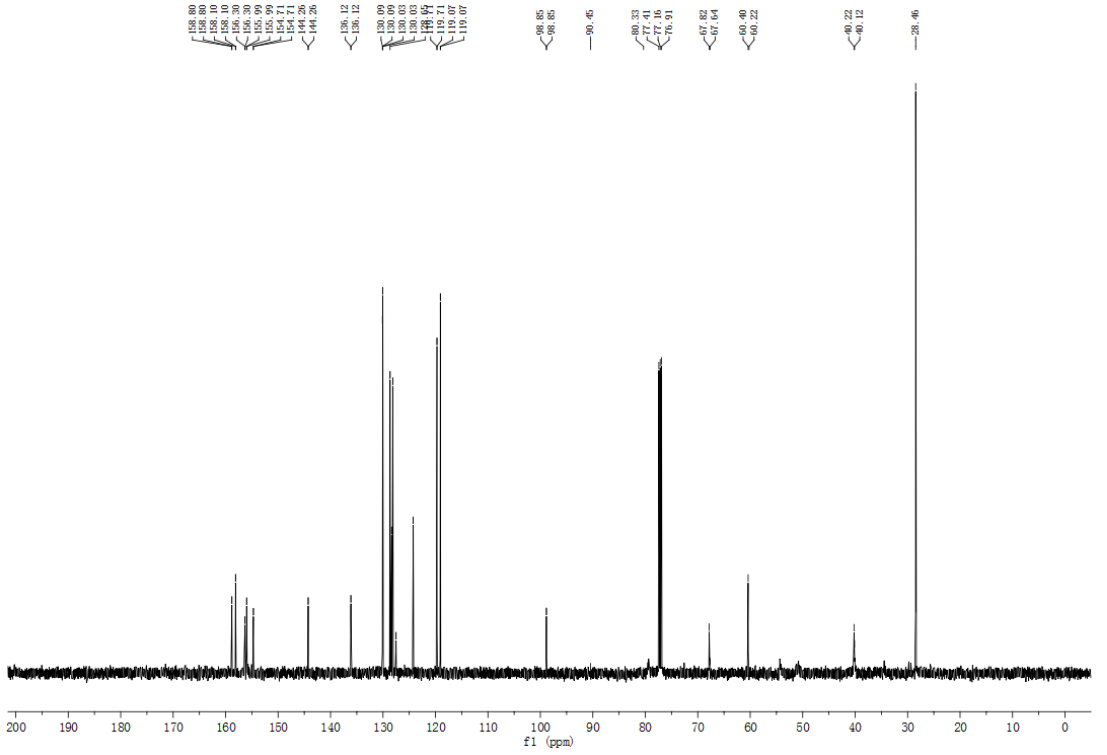

+TOF MS: 54.533 to 54.567 min from Sample 2 (180312) of 180312.wiff
a=3.6012613524359390e-004, t0=-2.4805507623517769 0e+001 (Turbo Spray), subtracted (50.781 to 51.298 min)
Max. 2700.0 counts.
Compound 1
C21H23NO7NaS(+); m/z Error: -1.63 ppm; m/z Error: -0.75 mDa
456.1080
434.1278
457.1112
435.1314
458.1089
Intensity, counts
m/z, Da


NH2
N
N
N
N
O
O
O
NCbz
f1 (ppm)


f1 (ppm)


+TOF MS: 60.102 to 60.202 min from Sample 1 (170314) of 170314.wiff
a=3.60109023973657700e-004, t0=-2.51174655017239270e+001 (Turbo Spray), subtracted (59.319 to 59.486 min)
Max. 4226.4 counts.
Compound 2
C31H29N6O5(+); m/z Error: 0.01 ppm; m/z Error: 0.01 mDa
565.2194
566.2238
567.2271
587.2005
588.2047
589.2096
Intensity, counts
m/z, Da


8.31
7.61
7.60
7.59
7.59
7.39
7.38
7.38
7.36
7.34
7.32
7.26
7.18
7.16
7.15
7.13
7.11
7.08
7.08
7.06
5.47
5.46
5.44
5.23
5.20
5.13
5.11
4.48
4.46
4.44
4.11
3.33
3.15
2.91
2.78
2.41
1.36
NH2
N
N
N
N
O
NCbz
BocHN
N
H
O
1.00
2.05
7.09
1.41
1.07
2.22
1.60
1.08
1.41
1.07
1.01
2.02
6.25
9.38
f1 (ppm)


158.80
158.60
158.10
158.10
156.30
156.30
155.99
155.99
154.71
154.71
154.26
144.26
136.12
136.12
130.09
130.09
130.03
130.03
119.71
119.71
119.07
119.07
98.85
98.85
90.45
80.33
77.41
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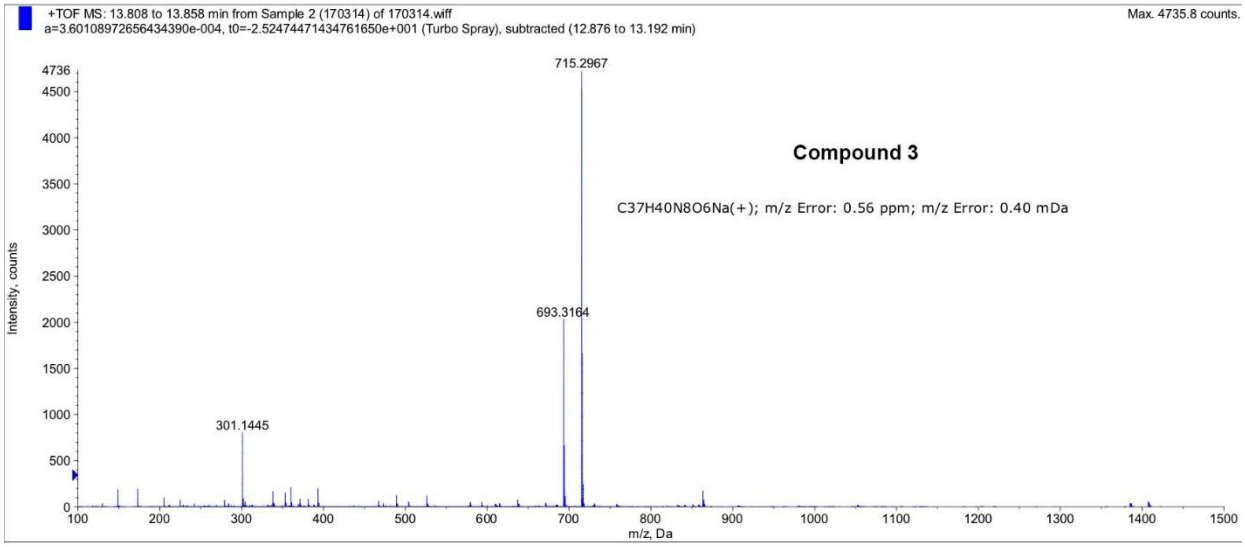
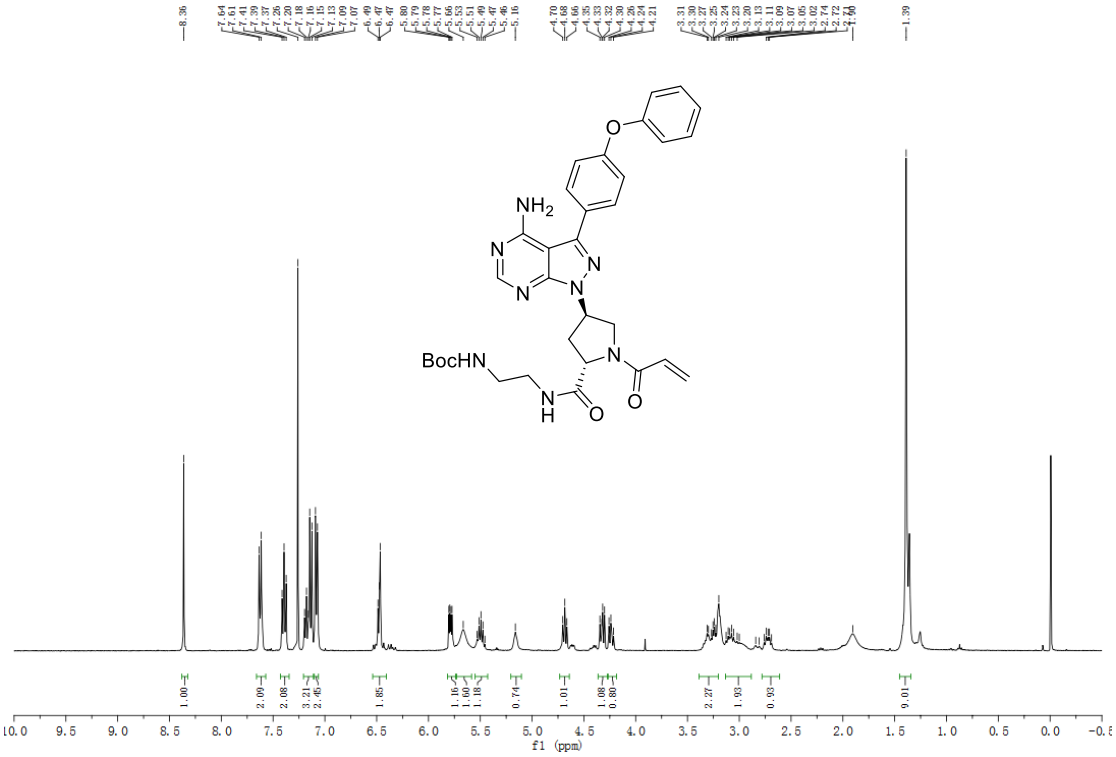
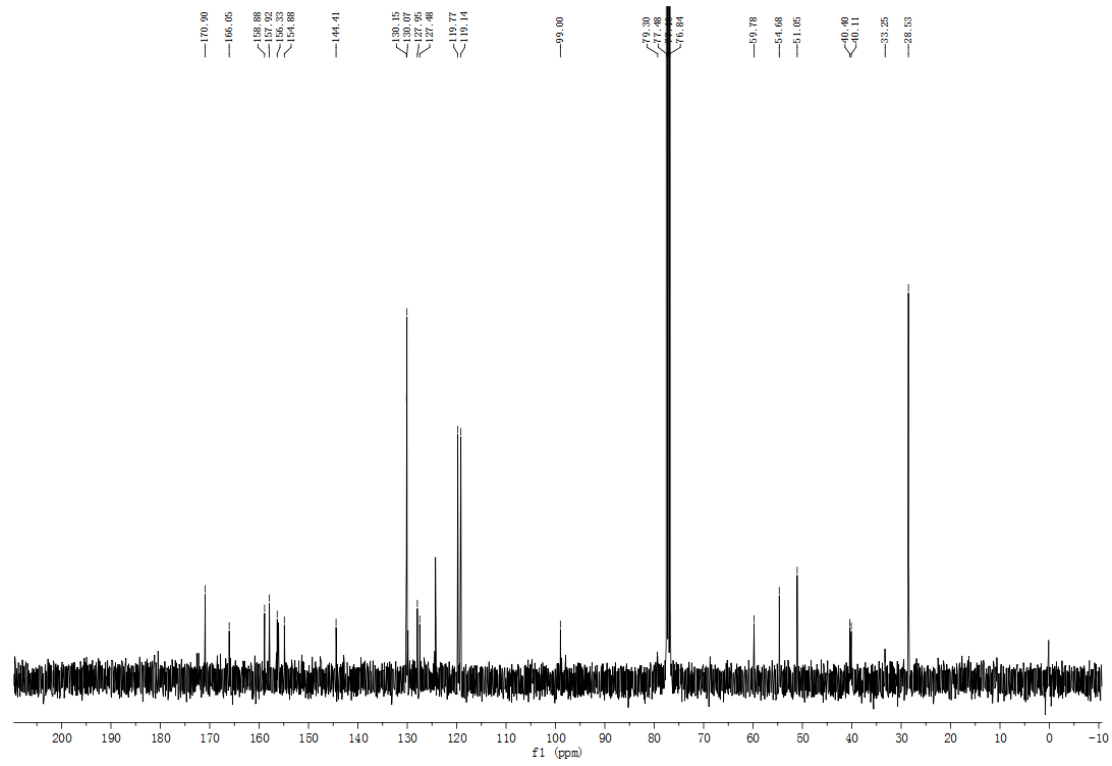
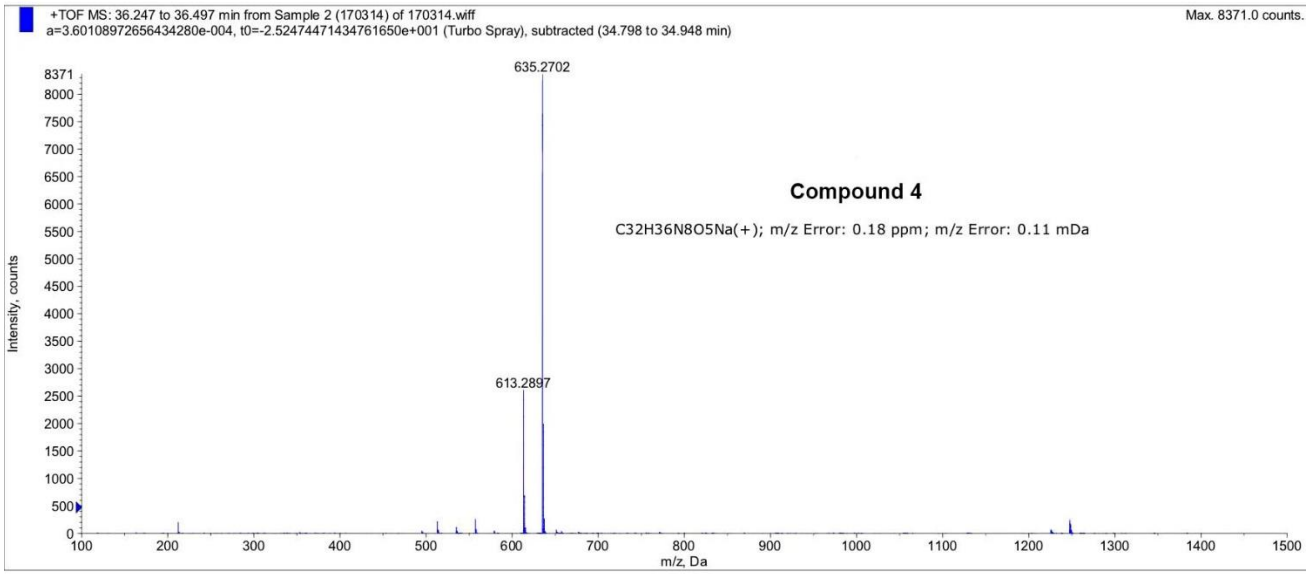
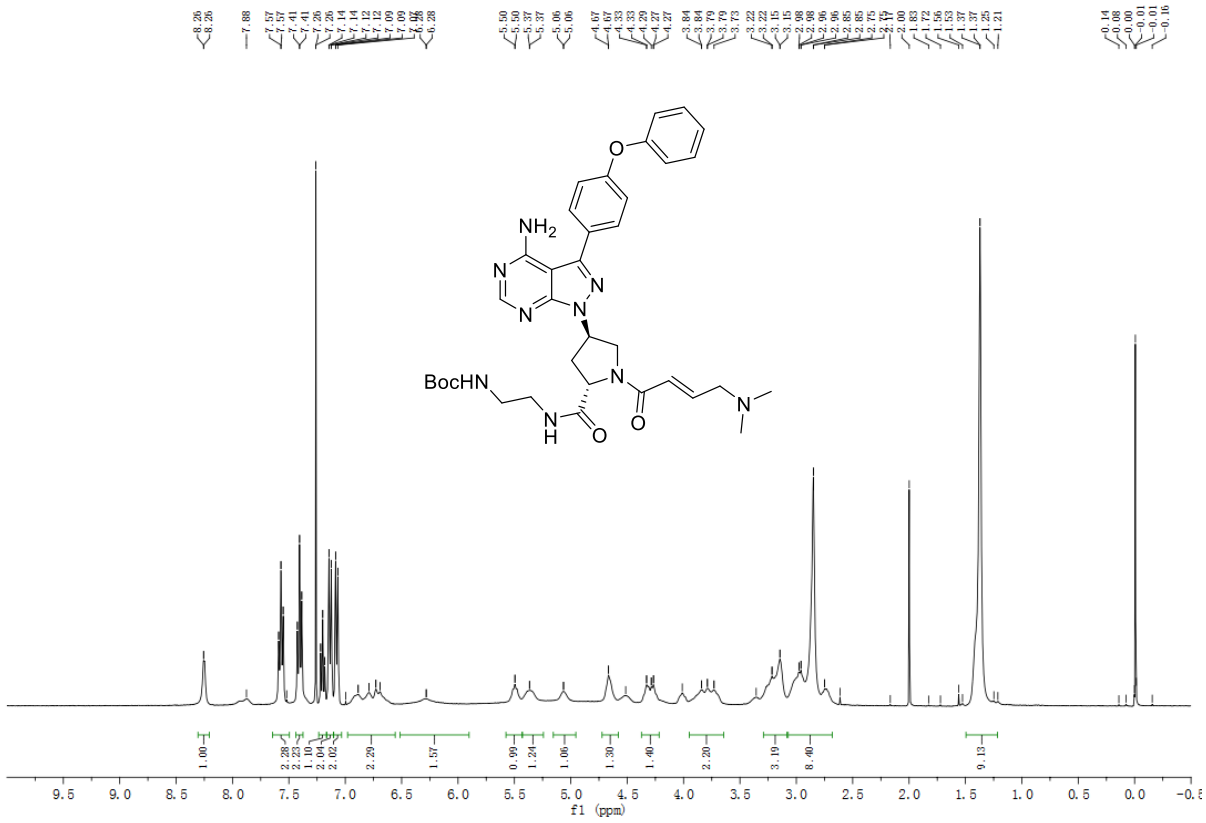
67.64
60.40
60.22
40.22
40.12
28.46
f1 (ppm)

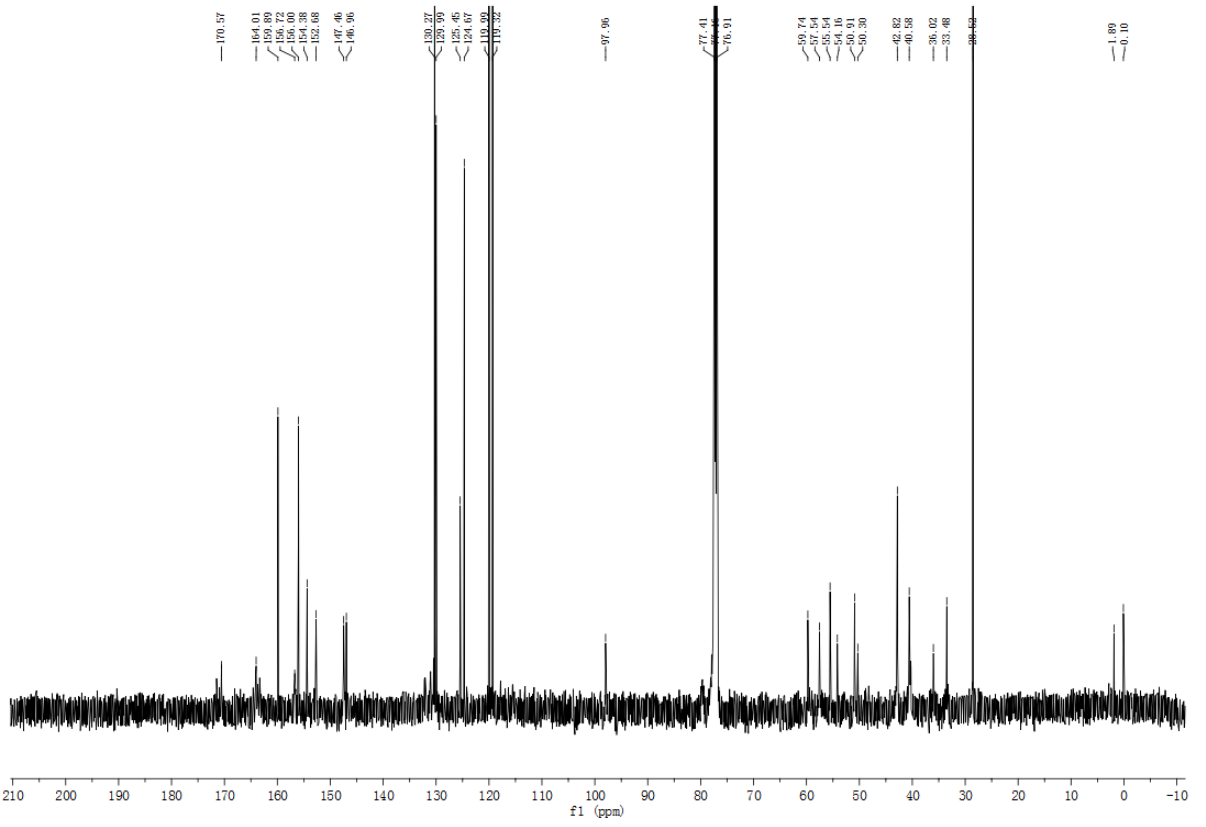

+TOF MS: 13.808 to 13.858 min from Sample 2 (170314) of 170314.wiff
a=3.60108972656434390e-004, t0=-2.52474471434761650e+001 (Turbo Spray), subtracted (12.876 to 13.192 min)
Max. 4735.8 counts.
715.2967
693.3164
301.1445
Compound 3
C37H40N8O6Na(+); m/z Error: 0.56 ppm; m/z Error: 0.40 mDa
Intensity, counts
m/z, Da

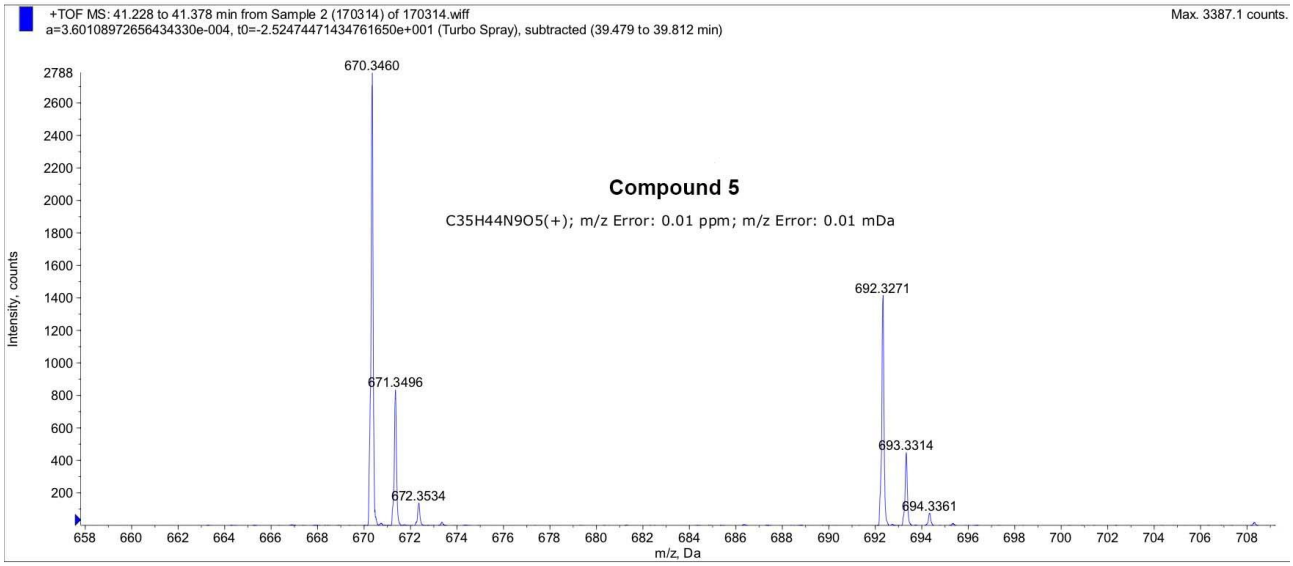

NH2
N
N
N
N
O
BocHN
N
H
O
O
f1 (ppm)

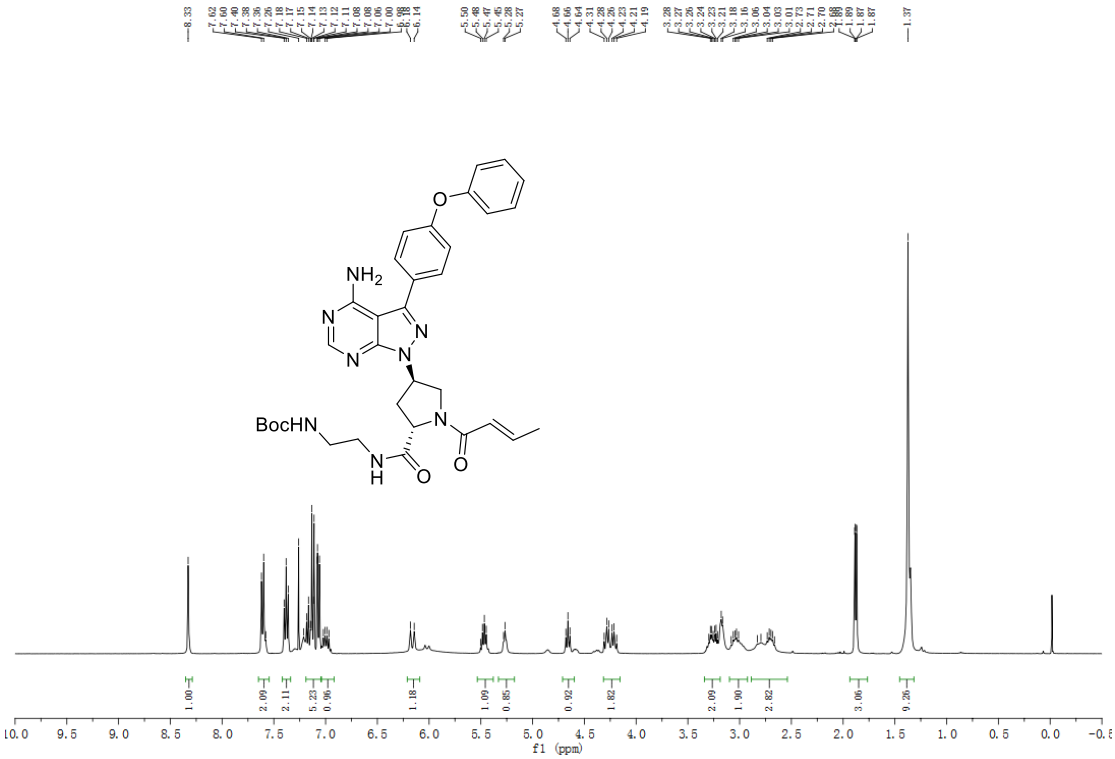

f1 (ppm)

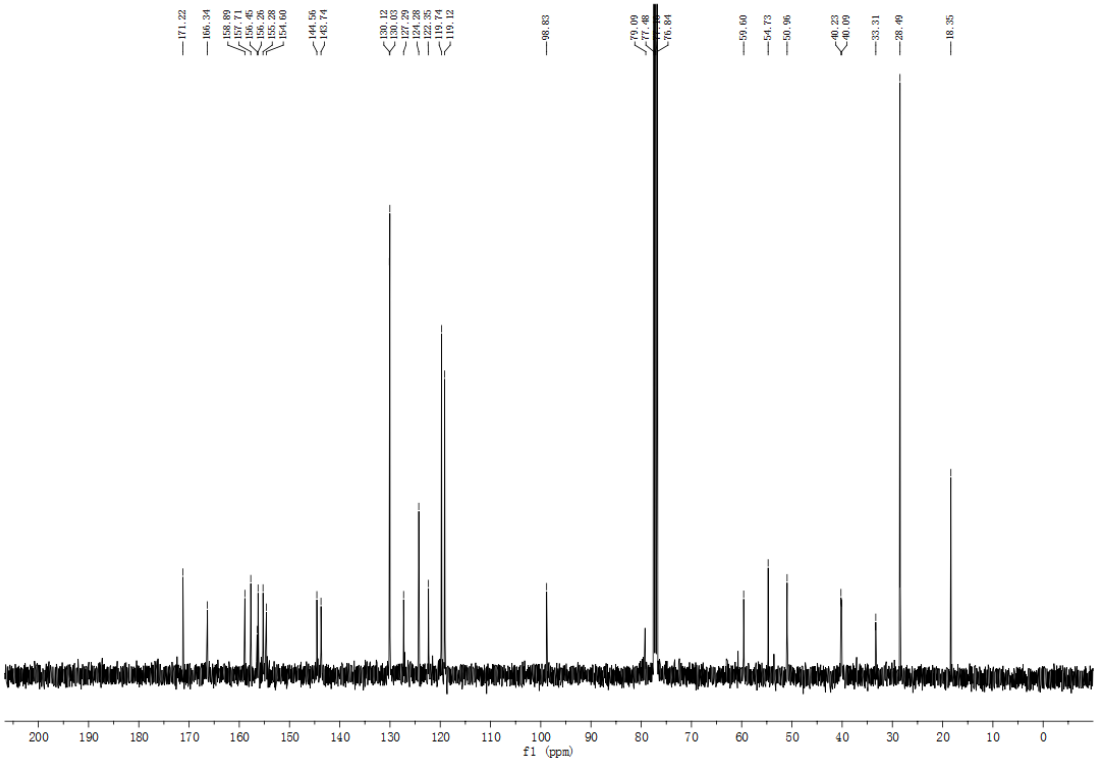

+TOF MS: 36.247 to 36.497 min from Sample 2 (170314) of 170314.wiff
a=3.60108972656434280e-004, t0=-2.52474471434761650e+001 (Turbo Spray), subtracted (34.798 to 34.948 min)
Max. 8371.0 counts.
635.2702
613.2897
Compound 4
C32H36N8O5Na(+); m/z Error: 0.18 ppm; m/z Error: 0.11 mDa
Intensity, counts
m/z, Da

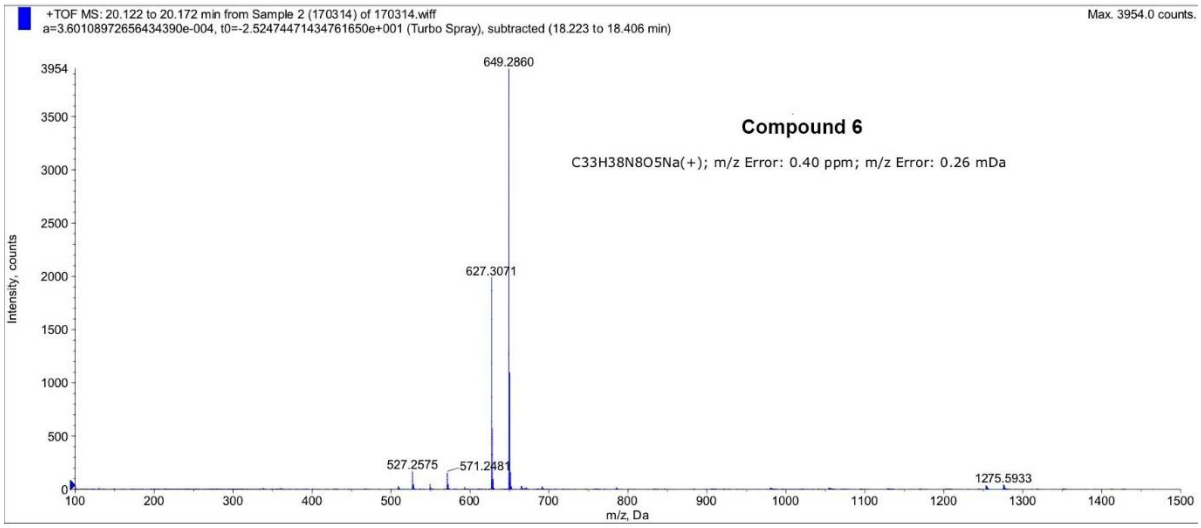

NH2
BocHN
f1 (ppm)

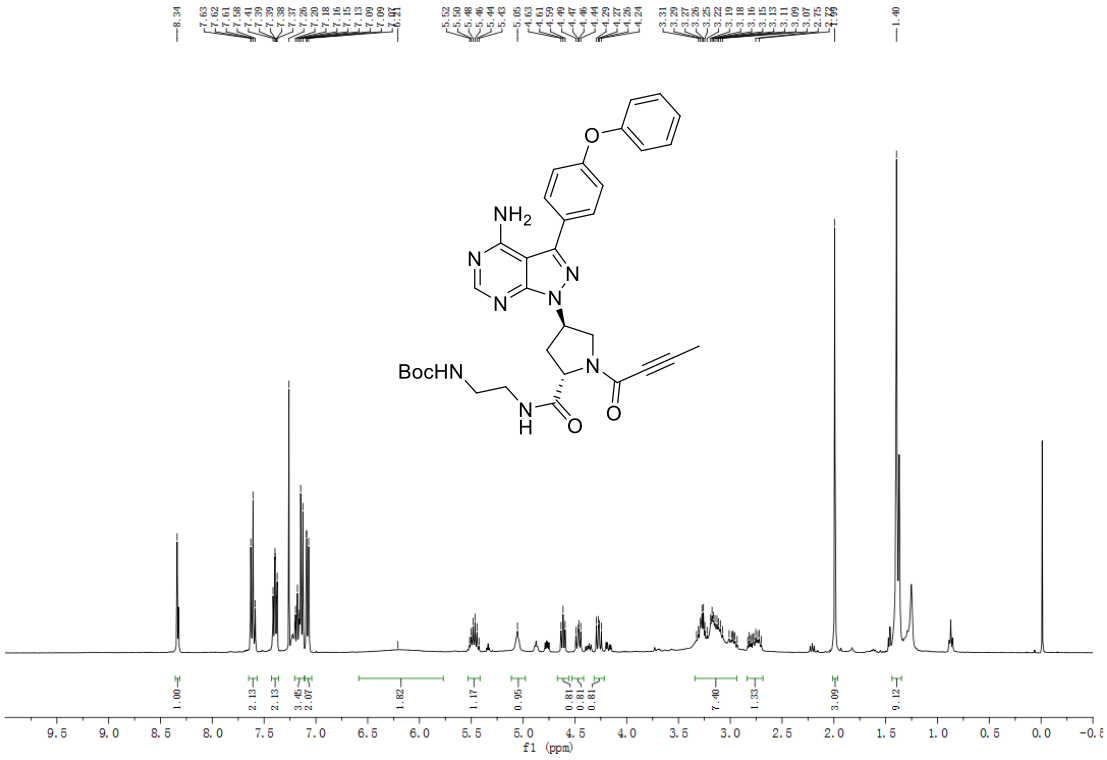

f1 (ppm)

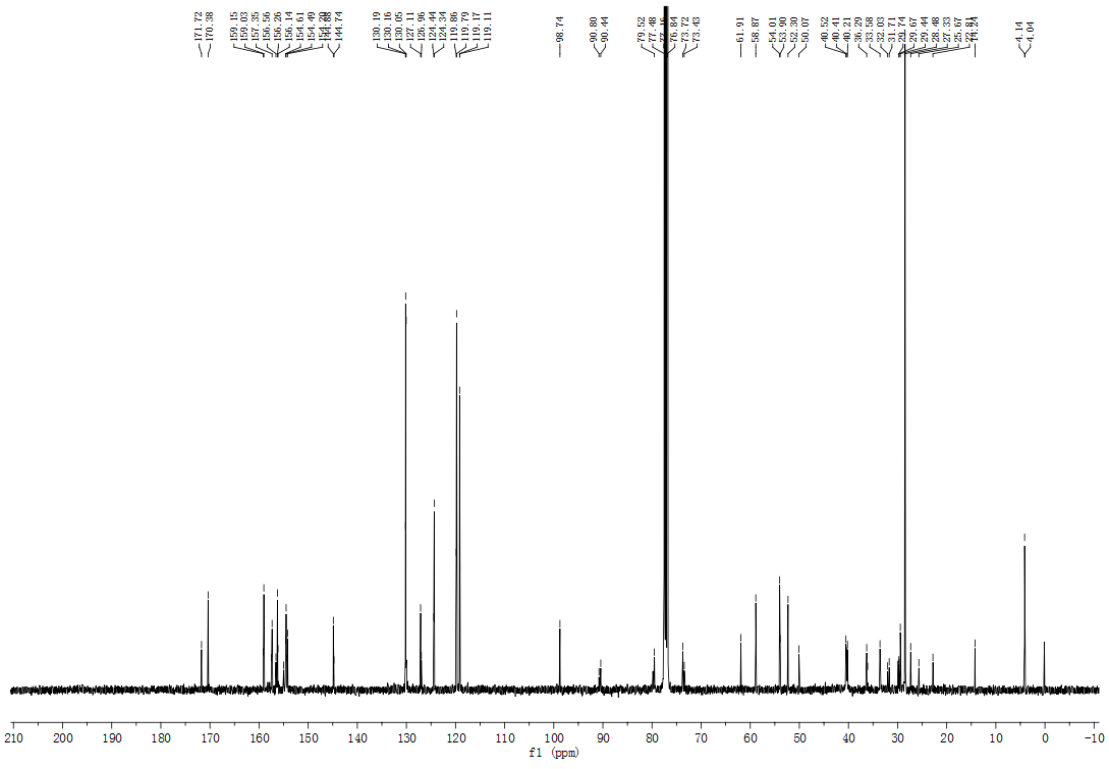

+TOF MS: 41.228 to 41.378 min from Sample 2 (170314) of 170314.wiff
a=3.60108972656434330e-004, t0=-2.52474471434761650e+001 (Turbo Spray), subtracted (39.479 to 39.812 min)
Max. 3387.1 counts.
Compound 5
C35H44N9O5(+); m/z Error: 0.01 ppm; m/z Error: 0.01 mDa
670.3460
671.3496
672.3534
692.3271
693.3314
694.3361
Intensity, counts
m/z, Da

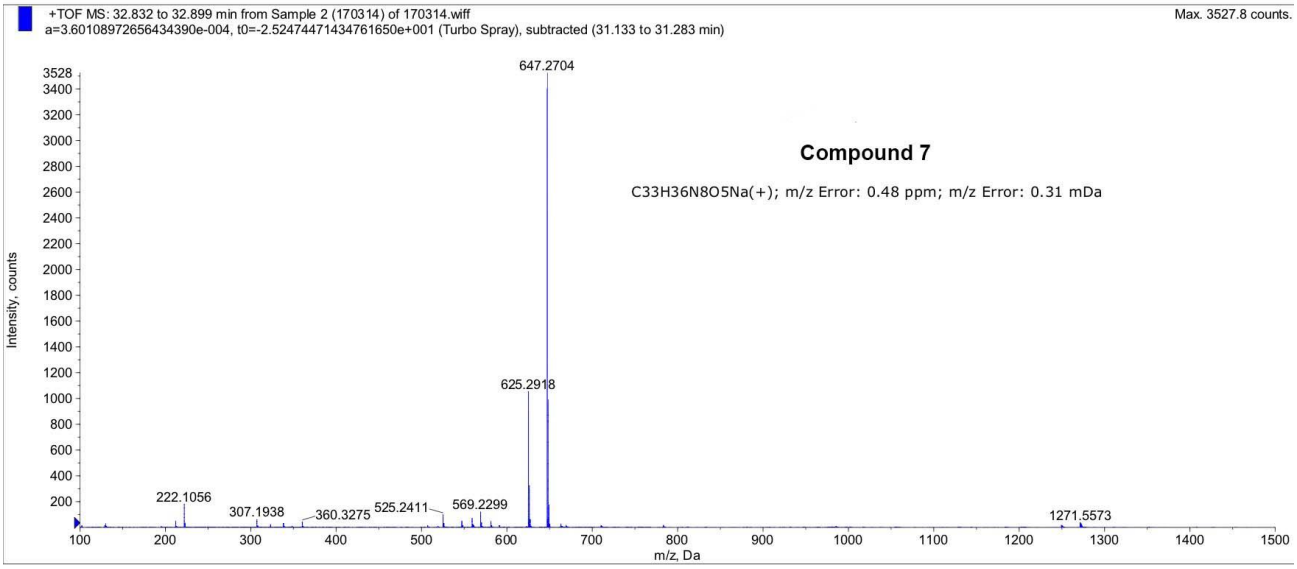

NH2
N
N
N
N
O
BocHN
N
H
O
O
f1 (ppm)

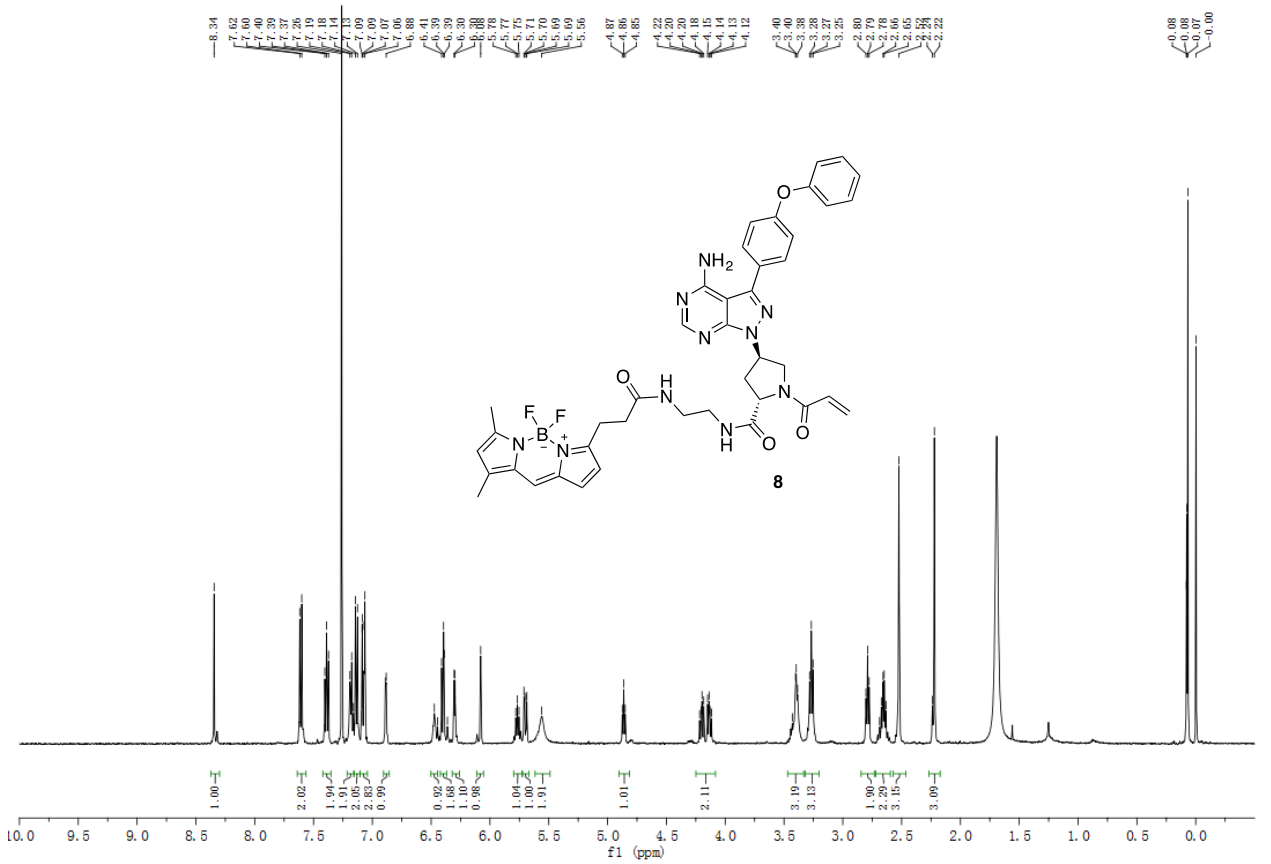

f1 (ppm)

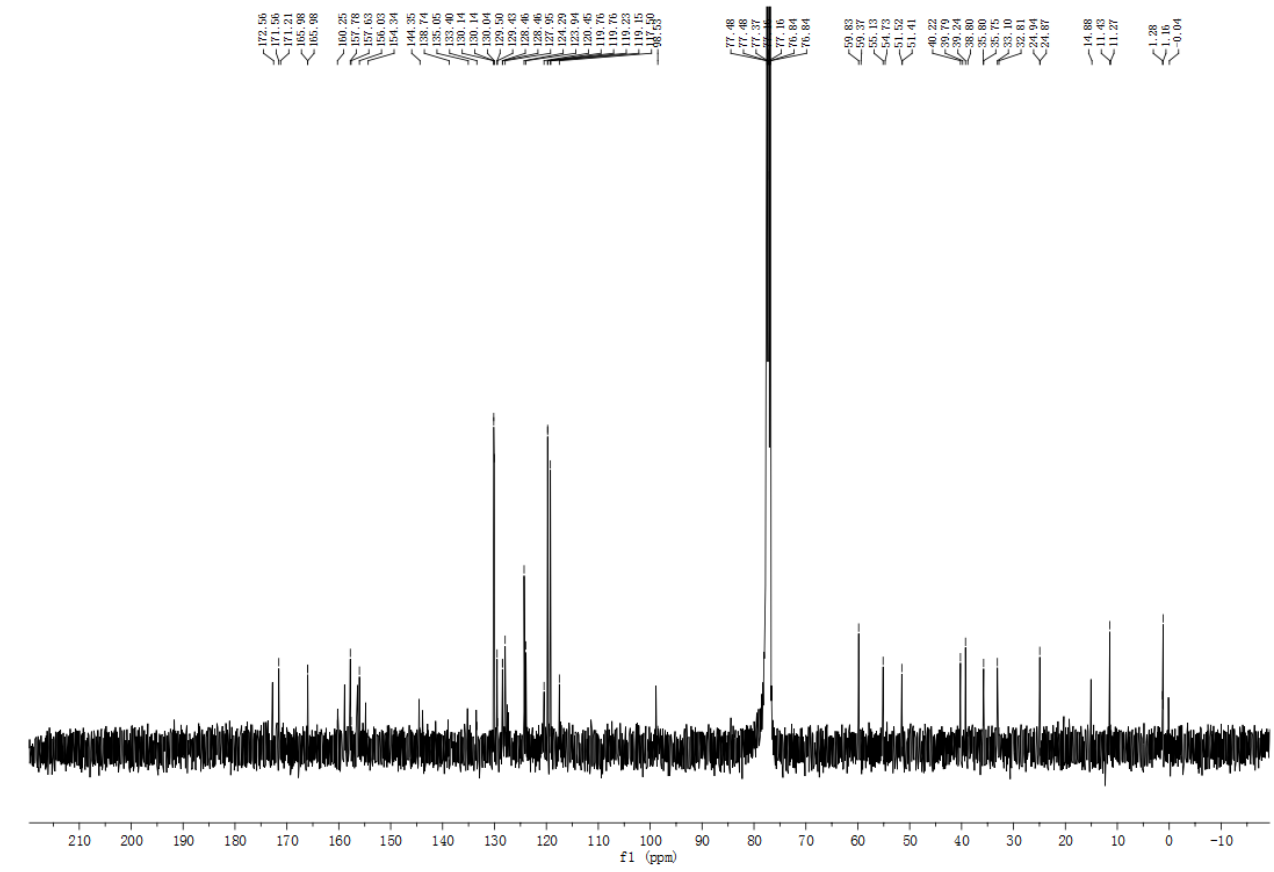

+TOF MS: 20.122 to 20.172 min from Sample 2 (170314) of 170314.wiff
a=3.60108972656434390e-004, t0=-2.52474471434761650e+001 (Turbo Spray), subtracted (18.223 to 18.406 min)
Max. 3954.0 counts.
Compound 6
C33H38N8O5Na(+); m/z Error: 0.40 ppm; m/z Error: 0.26 mDa
649.2860
627.3071
527.2575
571.2481
1275.5933
Intensity, counts
m/z, Da

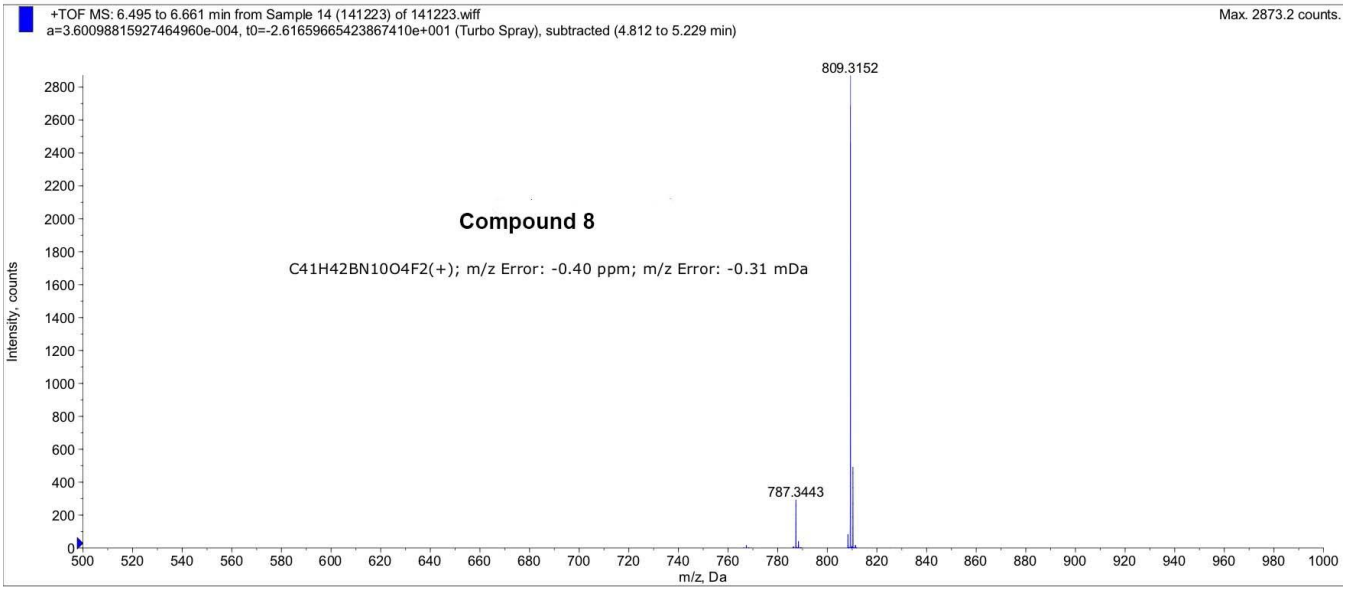

NH2
N
N
N
N
O
BocHN
N
H
O
O
f1 (ppm)

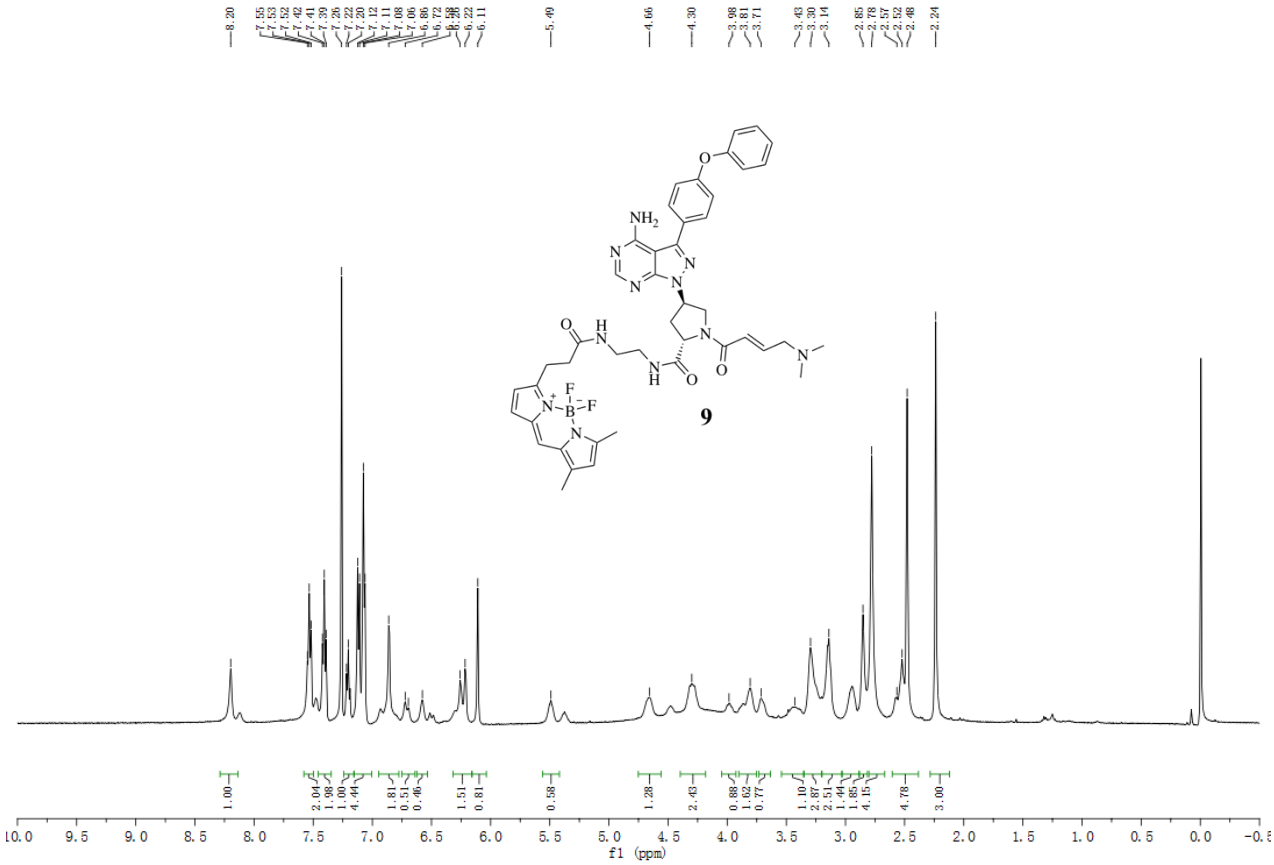

f1 (ppm)

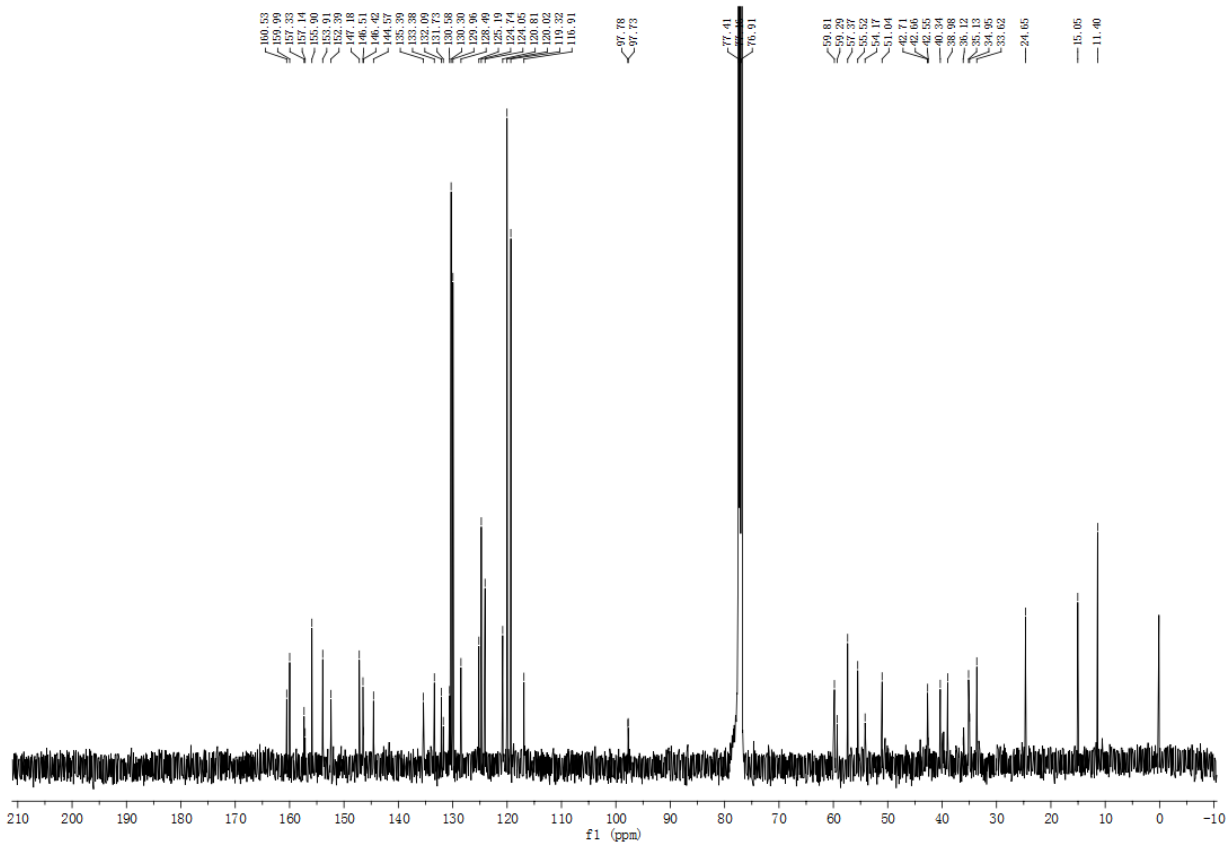

+TOF MS: 32.832 to 32.899 min from Sample 2 (170314) of 170314.wiff
a=3.60108972656434390e-004, t0=-2.52474471434761650e+001 (Turbo Spray), subtracted (31.133 to 31.283 min)
Max. 3527.8 counts.
Compound 7
C33H36N8O5Na(+); m/z Error: 0.48 ppm; m/z Error: 0.31 mDa
Intensity, counts
m/z, Da
647.2704
625.2918
222.1056
307.1938
360.3275
525.2411
569.2299
1271.5573

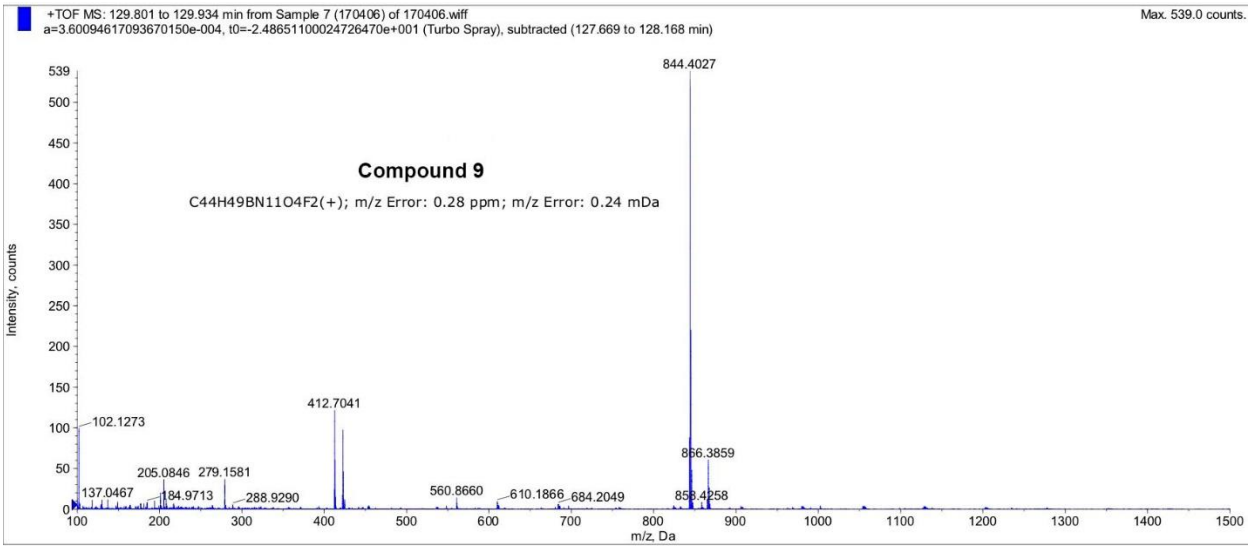

8.34
7.62
7.60
7.40
7.39
7.37
7.36
7.19
7.18
7.14
7.13
7.09
7.09
7.07
7.06
6.41
6.39
6.39
6.30
6.28
5.78
5.77
5.75
5.71
5.70
5.69
5.69
5.56
4.87
4.86
4.85
4.22
4.20
4.20
4.18
4.15
4.14
4.13
4.12
3.40
3.40
3.38
3.28
3.27
3.25
2.80
2.79
2.78
2.66
2.65
2.33
2.32
2.22
0.08
0.08
0.07
-0.00
NH2
O
N
N
N
N
H
N
O
F
F
B
N
N+
O
H
N
O
8
1.00
2.02
1.94
1.91
2.05
2.02
0.99
0.92
1.68
1.10
0.98
1.04
1.00
1.91
1.01
2.11
3.19
3.13
1.90
2.29
3.15
3.09
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)

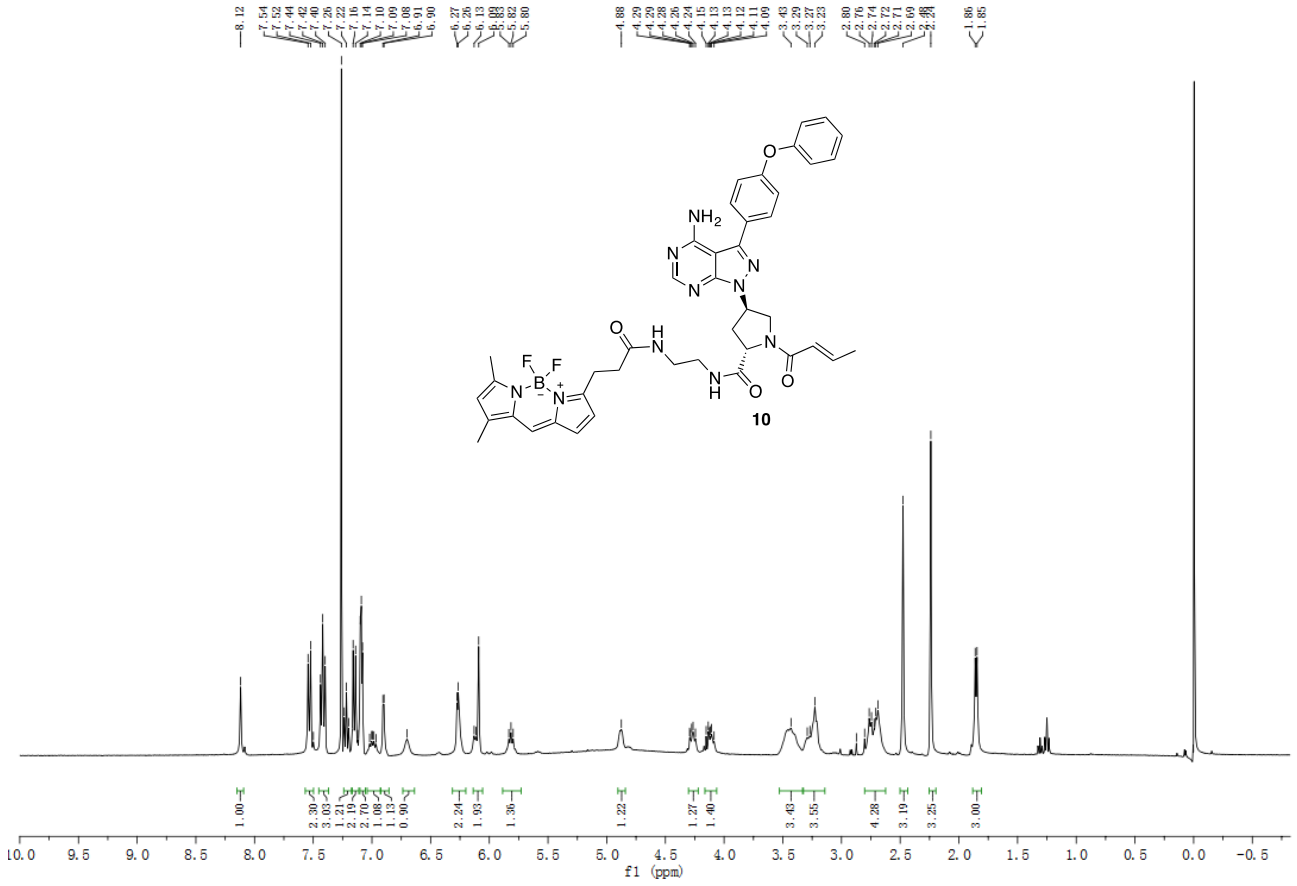

172.56
171.56
171.21
165.98
165.98
160.25
157.78
157.63
156.03
154.34
144.35
138.74
135.05
133.40
130.14
130.04
129.50
129.43
128.46
128.46
127.95
124.29
123.94
120.45
119.76
119.76
119.23
119.15
98.70
98.53
77.48
77.48
77.37
77.16
76.84
76.84
59.83
59.37
55.13
54.73
51.52
51.41
40.22
39.79
39.24
38.80
35.80
35.75
33.10
32.81
24.94
24.87
14.88
11.43
11.27
1.28
1.16
-0.04
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)

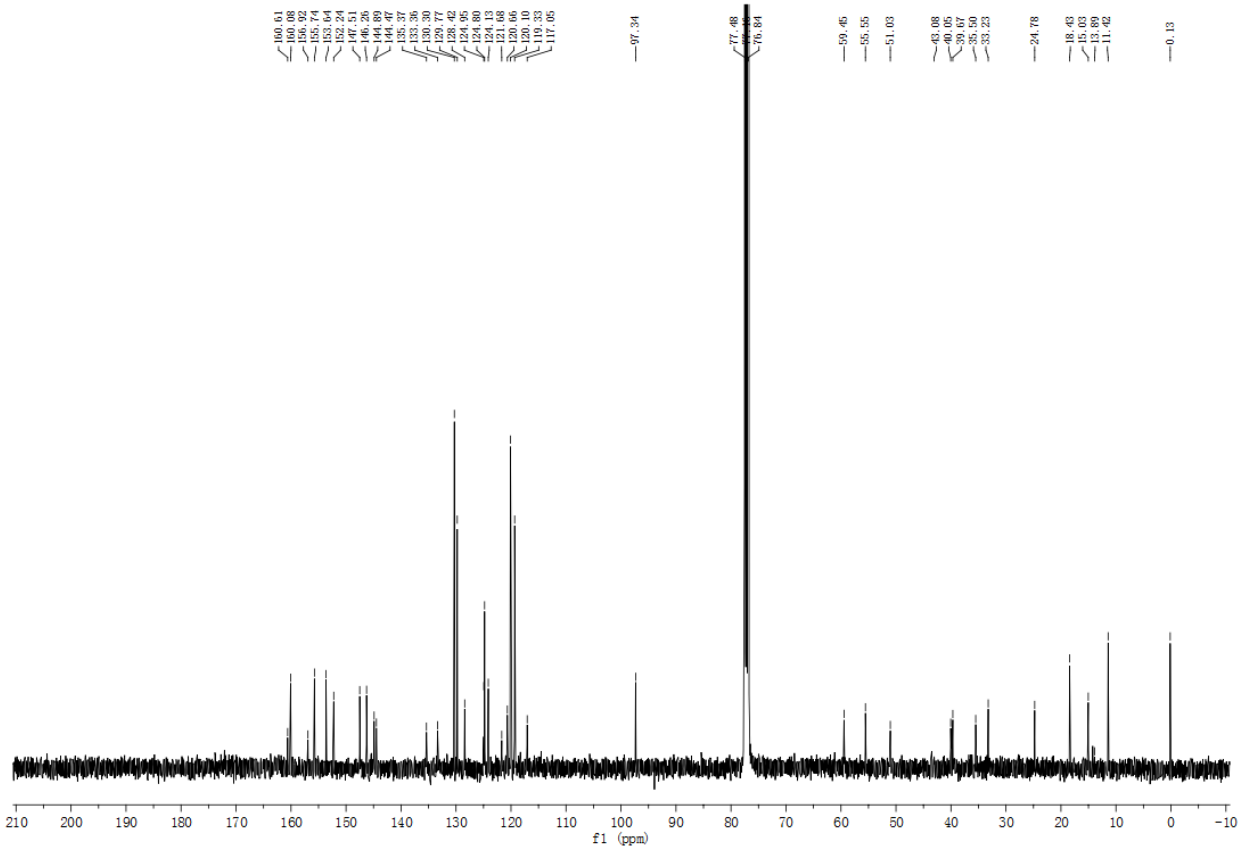

+TOF MS: 6.495 to 6.661 min from Sample 14 (141223) of 141223.wiff
a=3.60098815927464960e-004, t0=-2.61659665423867410e+001 (Turbo Spray), subtracted (4.812 to 5.229 min)
Max. 2873.2 counts.
809.3152
787.3443
Compound 8
C41H42BN10O4F2(+); m/z Error: -0.40 ppm; m/z Error: -0.31 mDa
Intensity, counts
m/z, Da

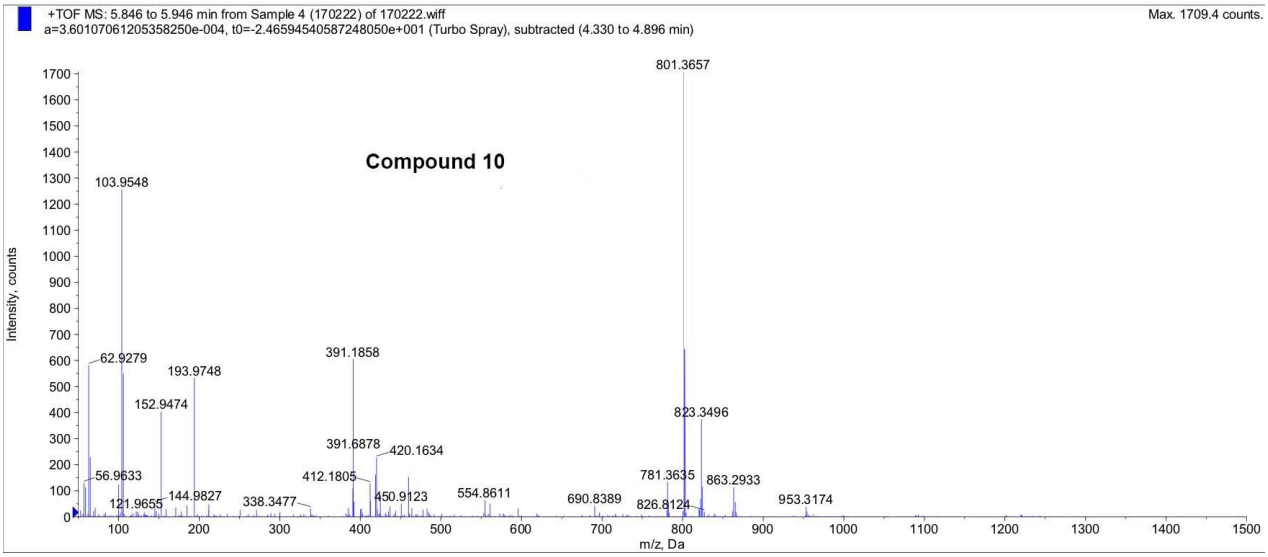

8.20
7.55
7.53
7.52
7.42
7.41
7.39
7.26
7.22
7.20
7.12
7.11
7.08
7.06
6.86
6.72
6.22
6.11
5.49
4.66
4.30
3.98
3.81
3.71
3.43
3.30
3.14
2.85
2.78
2.57
2.52
2.48
2.24
9
1.00
2.04
1.98
1.00
4.44
1.81
0.51
0.46
1.51
0.81
0.58
1.28
2.43
0.88
1.62
0.77
1.10
2.87
2.51
1.44
1.85
4.15
4.78
3.00
f1 (ppm)

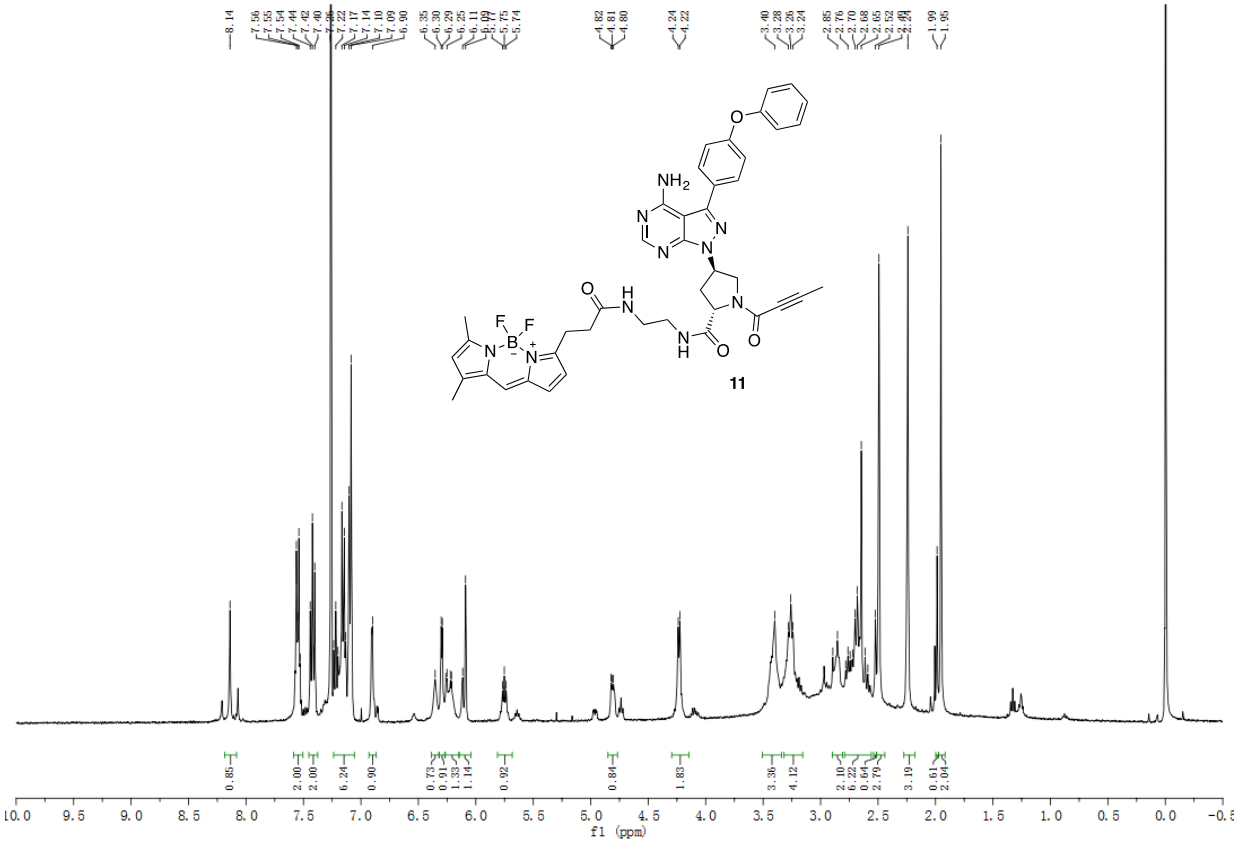

160.53
159.99
157.33
157.14
155.96
153.91
152.39
147.18
146.51
146.42
144.57
135.39
133.38
132.09
131.73
130.58
130.30
129.96
128.49
125.19
124.74
124.05
120.81
120.02
119.32
116.91
97.78
97.73
77.41
76.91
59.81
59.29
57.37
55.52
54.17
51.04
42.71
42.66
42.55
40.34
38.98
36.12
35.13
34.95
33.62
24.65
15.05
11.40
f1 (ppm)

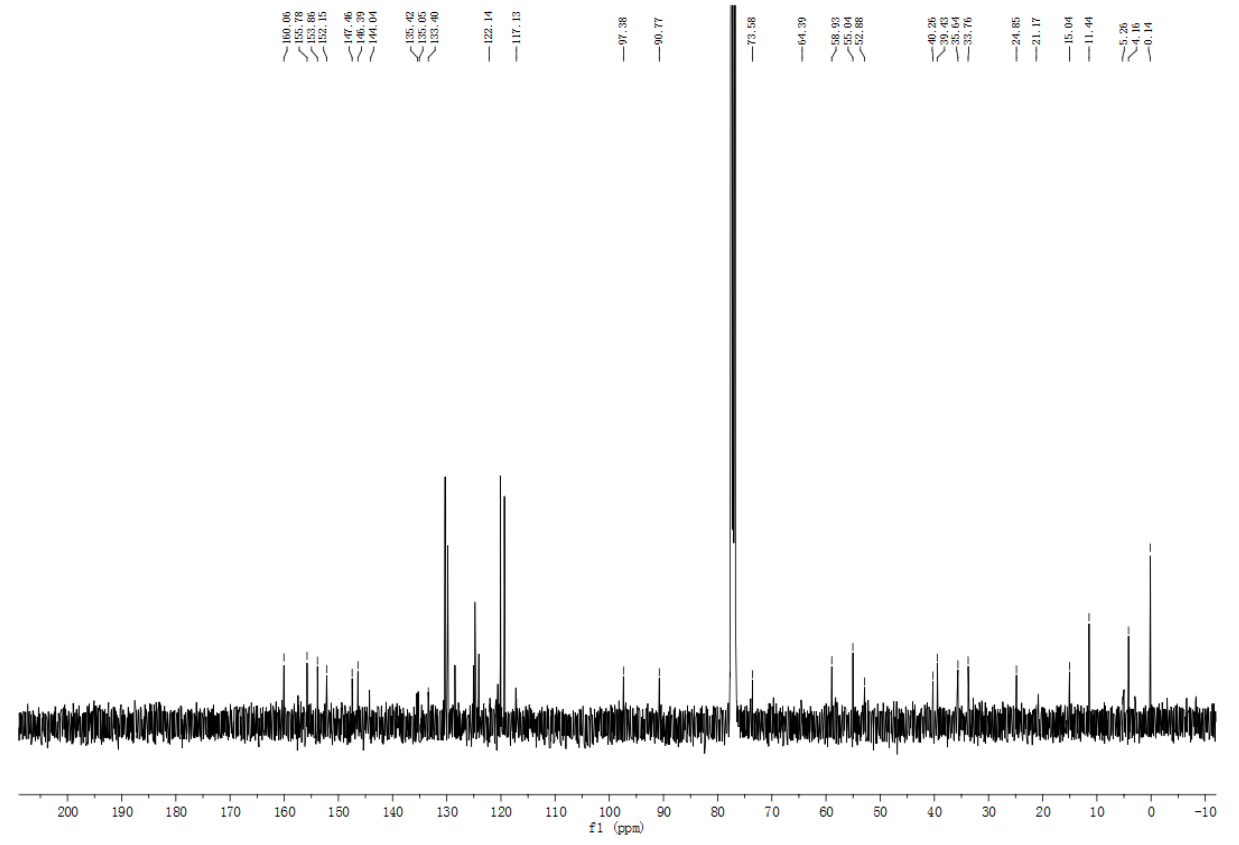

+TOF MS: 129.801 to 129.934 min from Sample 7 (170406) of 170406.wiff
a=3.60094617093670150e-004, t0=-2.48651100024726470e+001 (Turbo Spray), subtracted (127.669 to 128.168 min)
Max. 539.0 counts.
Compound 9
C44H49BN11O4F2(+); m/z Error: 0.28 ppm; m/z Error: 0.24 mDa
Intensity, counts
m/z, Da
844.4027
412.7041
102.1273
866.3859
205.0846
279.1581
137.0467
184.9713
288.9290
560.8660
610.1866
684.2049
858.4258

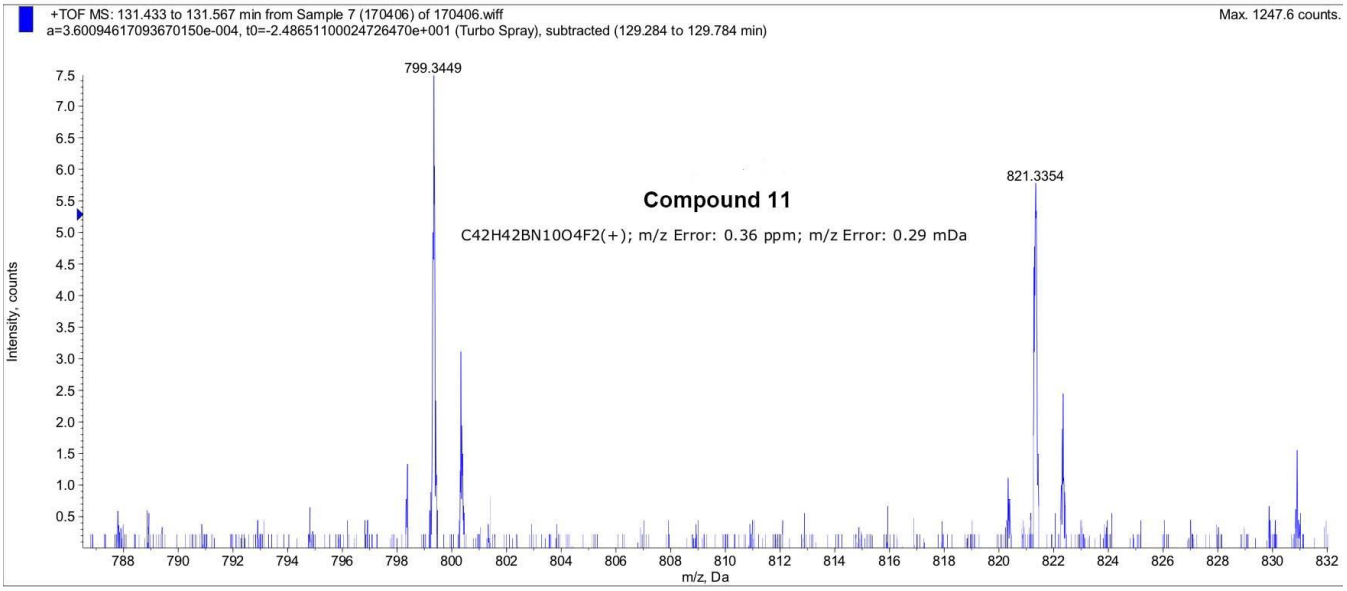

10
f1 (ppm)

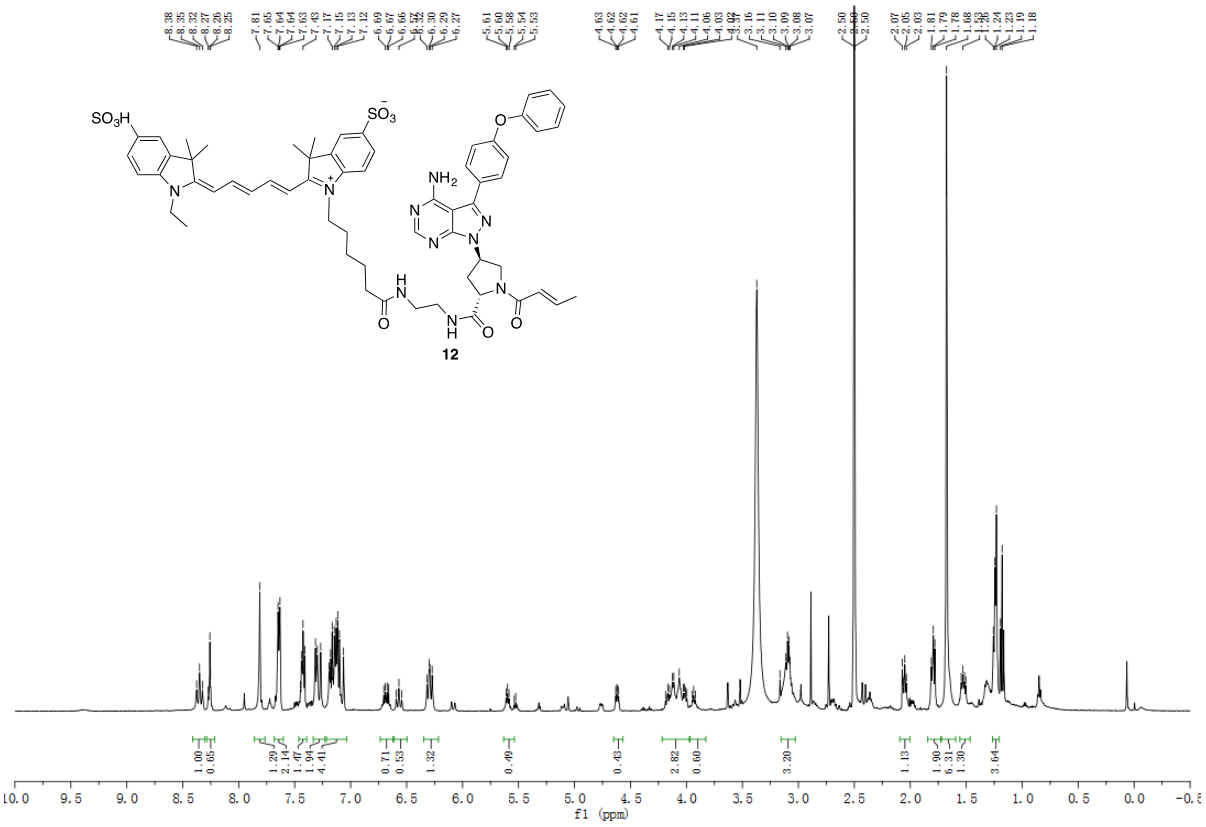

f1 (ppm)

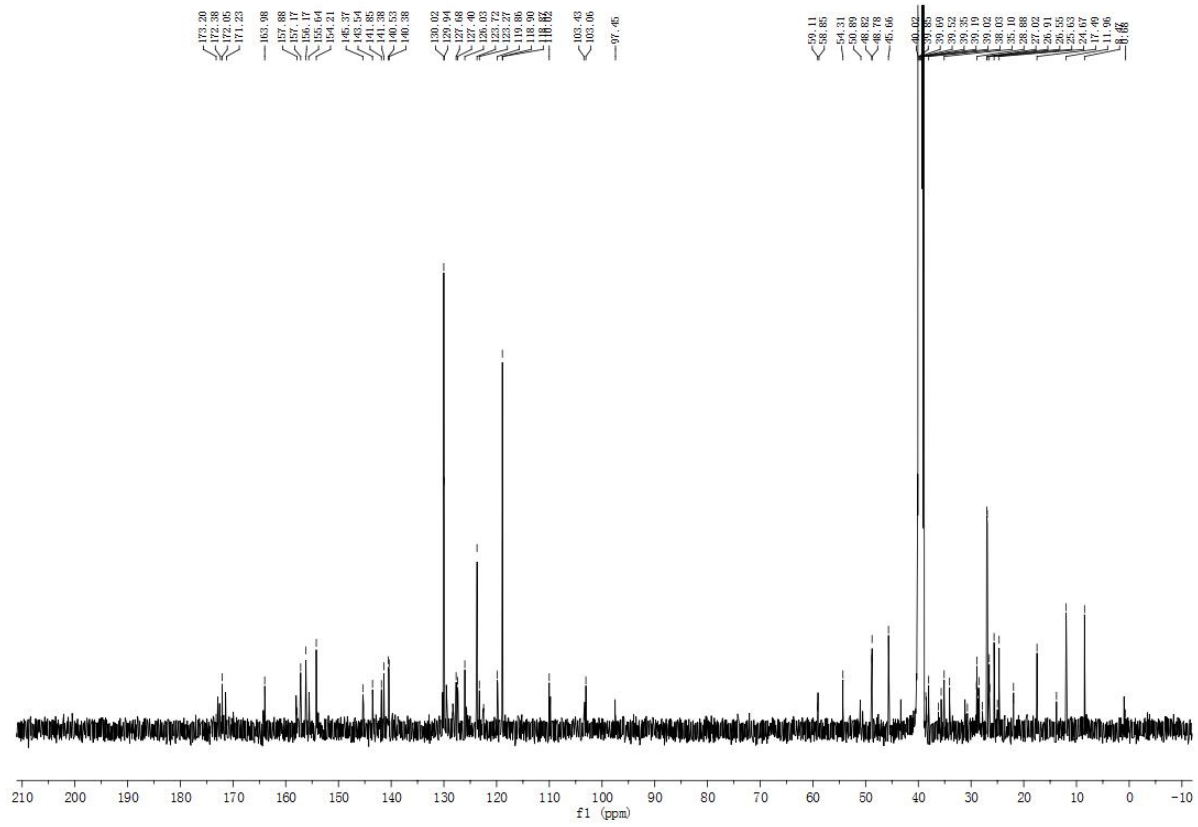

+TOF MS: 5.846 to 5.946 min from Sample 4 (170222) of 170222.wiff
a=3.6010706120535825 0e-004, t0=-2.4659454058724805 0e+001 (Turbo Spray), subtracted (4.330 to 4.896 min)
Max. 1709.4 counts.
Compound 10
Intensity, counts
m/z, Da
801.3657
103.9548
62.9279
193.9748
152.9474
391.1858
391.6878
420.1634
56.9633
412.1805
781.3635
863.2933
823.3496
121.9655
144.9827
338.3477
450.9123
554.8611
690.8389
826.8124
953.3174

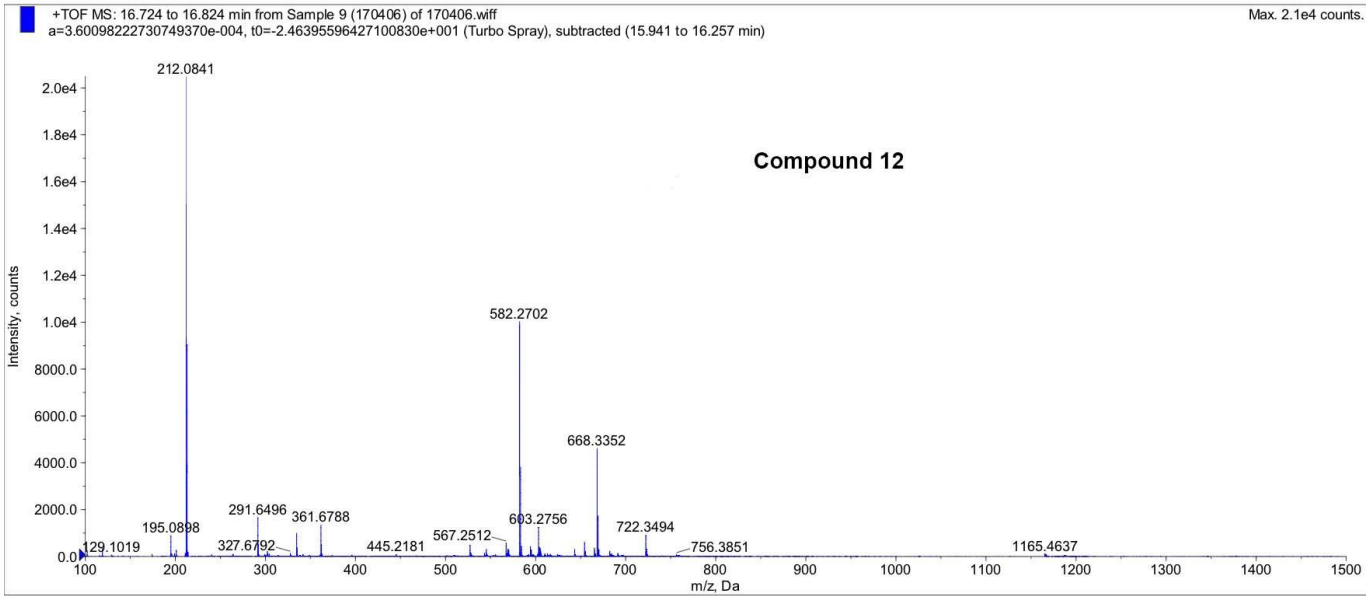

11
f1 (ppm)


f1 (ppm)


+TOF MS: 131.433 to 131.567 min from Sample 7 (170406) of 170406.wiff
a=3.60094617093670150e-004, t0=-2.48651100024726470e+001 (Turbo Spray), subtracted (129.284 to 129.784 min)
Max. 1247.6 counts.
799.3449
821.3354
Compound 11
C42H42BN10O4F2(+); m/z Error: 0.36 ppm; m/z Error: 0.29 mDa
Intensity, counts
m/z, Da


12
f1 (ppm)


f1 (ppm)


+TOF MS: 16.724 to 16.824 min from Sample 9 (170406) of 170406.wiff
a=3.6009822273074937 0e-004, t0=-2.4639559642710083 0e+001 (Turbo Spray), subtracted (15.941 to 16.257 min)
Max. 2.1e4 counts.
Compound 12
Intensity, counts
m/z, Da
212.0841
582.2702
668.3352
291.6496
361.6788
195.0898
603.2756
722.3494
567.2512
129.1019
327.6792
445.2181
756.3851
1165.4637