Supporting Information

The first ratiometric fluorescent probes for aminopeptidase N cell imaging

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1. Synthesis of 6c and 6d.

Methyl 6-bromo-2-ethyl-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (1a). The solution of 4-bromo-1,8-naphthalic anhydride (13.8 g, 50 mmol) and ethanamine (3.91 g, 60 mmol) in 500 mL 1,4-dioxane was refluxed at 120°C for 4 h. Then the mixture was cooled and poured to 500 mL water. The yieled yellowish sediment were collected by filtration and dried under 110°C air atmosphere to yeild a yellowish solid, 14 g, yield = 91.0%, mp: 159.2-160.1 °C.

6-amino-2-ethyl-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (2a). The mixture of **1a** (1.5 g, 5 mmol), conc. ammonin (10 mL), copper power (0.032 g, 0.5 mmol) and a stirring bar were sealed in a 30 mL screwed tube and stirred in a oil bath at 100°C for 10 h. After cooling to r.t., the suspension was extraced with 50 mL AcOEt, to the residue was added 50 mL water and extraced by another 50 mL AcOEt. The combination was washed with brine (100 mL*3) and dried over anhydrous $MgSO_4$ for 24 h. The solution was concentrated under reduced pressure and then purified by combiflash to yield a yellow power, 0.32 g, yield = 34.0%, mp: 280.0-282.3 °C.

2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)propanoic acid (3c). To the solution of Ala (3.5 g, 40 mmol) in 120 mL 10% Na_2CO_3 was added the solution of FmocOSu (13.5 g, 40 mmol) in acetone (100 mL) at 0°C. The mixture was stirred at r.t. over night. Acetone was evaporated under reduced pressure and the residue was wased by ether (100 mL*3). The water phase was adjusted pH to 2 with Conc. HCl and extracted with AcOEt (100 mL*3). The organic phase was dried over anhydrous $MgSO_4$ for 24 h. The solution was concentrated under reduced pressure and then recrystallized with AcOEt-*n*-hexane to yield a white solid, 10 g, yield = 80.4%, mp: 138.2-139.7 °C.

2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)pentanoic acid (3d). The synthesis is analogous to **3c** with Nva as the starting material. The product was obtained as a white solid. Yield = 79.4%, mp:134.1-135.4 °C.

(9*H*-fluoren-9-yl)methyl (1-chloro-1-oxopropan-2-yl)carbamate (4c). To the solution of **3c** (0.5 g, 1.6 mmol) in CH_2Cl_2 (8 mL) was added $SOCl_2$ (4 mL), the mixture was stirred at r.t. for 2 h. The solution was concentrated under reduced pressure and recrystallized with CH_2Cl_2-*n*-hexane to yield a white solid, 0.35 g, yield = 66.5%, mp: 94.5-96 °C.

(9*H*-fluoren-9-yl)methyl (1-chloro-1-oxopentan-2-yl)carbamate (4d). The synthesis is analogous to **4c** with **3d** as the starting material. The product was obtained as a white solid. Yield = 71.4%, mp: 95.3-97.1 °C.

(9*H*-fluoren-9-yl)methyl (1-((2-ethyl-1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-6-yl)amino)-1-oxopropa

n-2-yl)carbamate (5c). The suspension of **4c** (0.33 g, 1.0 mmol) and **2a** (0.12 g, 0.5 mmol) in AcOH (10 mL) was refluxed for 2 h. The mixture was cooled and poured into 50 mL water, the yield precipitation was filtered to yield a yellow power. The yellow power was purified by silica gel column to yield a pale-yellow power, 0.11 g, Yield = 41.3%, mp:112.7-113.4 °C.

(9*H*-fluoren-9-yl)methyl (1-((2-ethyl-1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-6-yl)amino)-1-oxopentan-2-yl)carbamate (5d). The synthesis is analogous to **5a** with **4d** and **2a** as the starting material. The product was obtained as a white solid. Yield = 73.5%, mp:95.3-97.1 °C.

2-amino-N-(2-ethyl-1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-6-yl)propanamide hydrochloride (6c). The mixture of **5c** (0.06 g, 0.11 mmol) and 5 mL 20% piperidine-DMF was stirred at r.t. for 30 min then concentrated under reduced pressure at 90°C to remove excess piperidine and DMF. The residue was purified first by silica gel column and then recrystallization with AcOEt-*n*-hexane to yield a yellow solid. 5 mL EtOAc solution saturated with HCl gas was added and stirred at r.t. for 1 h. The yield precipitation was filtered and washed with AcOEt to yield a light yellow power, 20 mg, yield = 51.1%, mp: 255.3-257.8°C, $[\alpha]_D^{20}$ = 31.51 (c = 0.073, MeOH). ^{1}H-NMR (D_2O, 300 MHz): δ 8.07 (d, 1 H, J = 7.2 Hz), 7.99 (t, 2 H, J = 8.1 Hz), 7.71 (d, 1 H, J = 8.1 Hz), 7.51 (t, 1 H, J = 7.5 Hz), 4.38 (q, 1 H, J = 6.9 Hz), 3.81 (q, 2 H, J = 7.2 Hz), 1.73 (d, 3H, J = 6.9 Hz), 1.12 (t, 3 H, J = 7.2 Hz); ^{13}C-NMR (DMSO, 150 MHz): δ 170.3, 163.7, 163.1, 139.5, 131.8, 131.5, 129.9, 128.7, 127.2, 125.2, 122.9, 121.1, 119.2, 49.5, 39.2, 17.8, 13.6; HRMS (ESI) m/z calcd. for $C_{17}H_{18}N_3O_3$ ($[M + H]^+$) 312.1343; found 312.1338.

2-amino-N-(2-ethyl-1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-6-yl)pentanamide hydrochloride (6d). The synthesis is analogous to **6c** with **5d** as the starting material. The product was obtained as a light yellow power. Yield =49.5%, mp:277.4-280.0°C. $[\alpha]_D^{20}$ = 40.00 (c = 0.080, MeOH). ^{1}H-NMR (D_2O, 300 MHz): δ 8.04 (d, 1 H, J = 7.5 Hz), 7.97 (t, 2 H, J = 8.1 Hz), 7.69 (d, 1 H, J = 8.1 Hz), 7.49 (t, 1 H, J = 8.1 Hz), 4.32 (t, 1 H, J = 6.6 Hz), 3.79 (q, 2 H, J = 6.9 Hz), 2.00-2.09 (m, 2 H), 1.49-1.59 (m, 2 H), 1.02-1.14 (m, 6 H); ^{13}C-NMR (DMSO, 150 MHz): δ 169.5, 163.6, 163.1, 139.2, 131.8, 131.5, 129.7, 128.7, 127.3, 125.2, 122.9, 121.3, 119.4, 54.4, 34.5, 32.3, 18.2, 14.1, 12.8; HRMS (ESI) m/z calcd. for $C_{19}H_{22}N_3O_3$ ($[M + H]^+$) 340.1656; found 340.1658.

2. Time course scan of 6c, 6d to APN

To the solution of **6c** or **6d** (80 μL, 250 μM) in 0.1 M tris-HCl buffer at pH 7.5 was added the solution of APN (20 μL, 10 IU/mL). The mixture was scanned for 30 min with excitation at 400 nm every two minutes by Varioskan microplate reader.

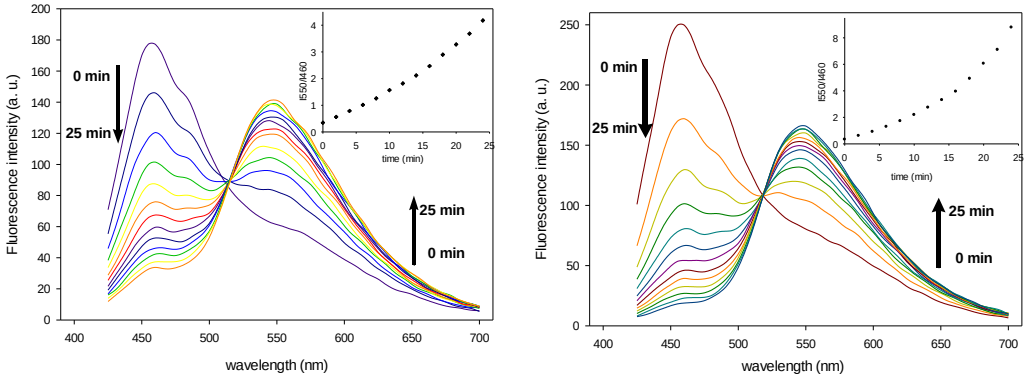
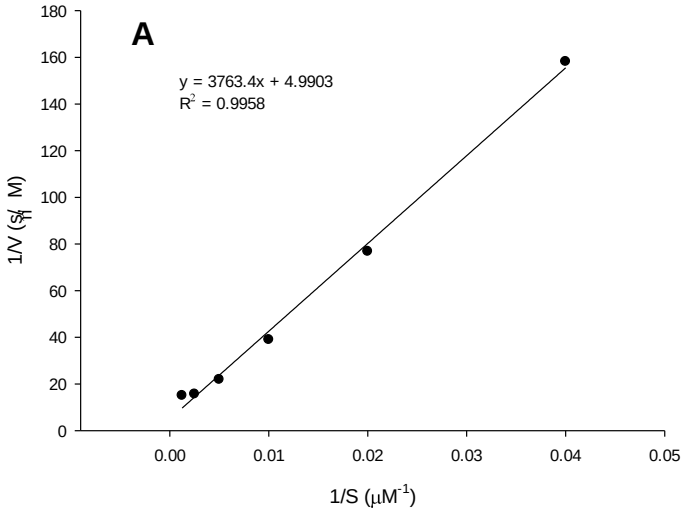


Figure S1 Emission spectra of **6c** (left) and **6d** (right) (200 μM) in the presence of APN (0.2 IU) in a tris-HCl buffer (100 mM, pH 7.5). Insets: ratios of fluorescent intensities at 550 and 460 nm as a function of time after adding APN (λ_{ex} = 400 nm).

3. Lineweaver-Burk plot for V_{max} and K_m measurement



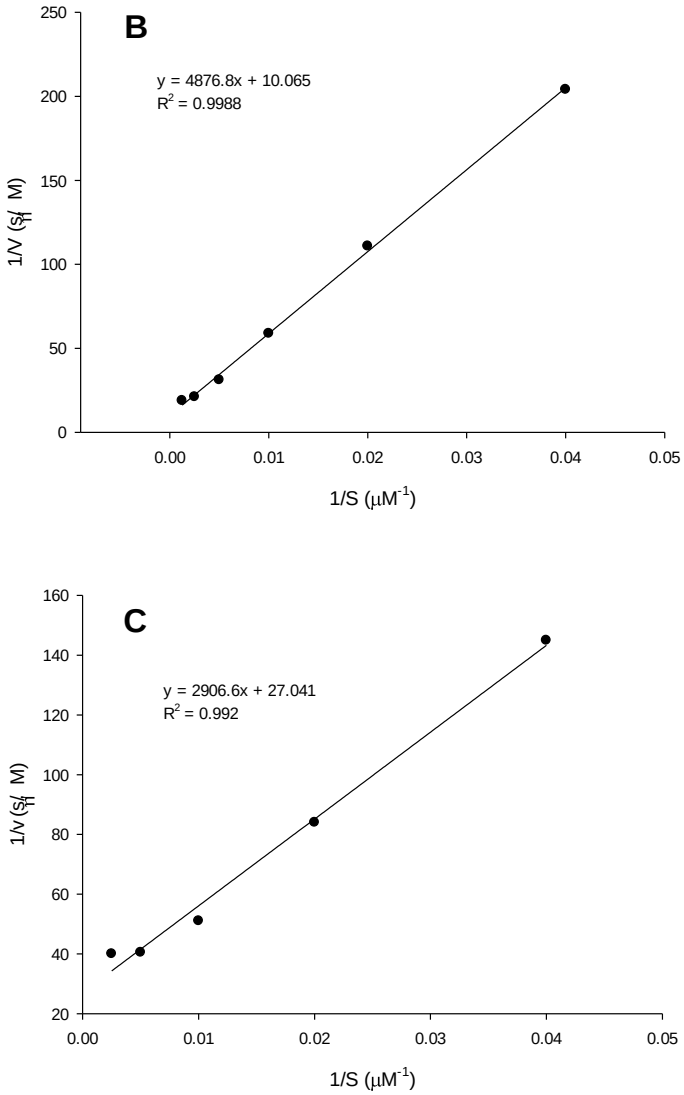


Figure S2 Lineweaver-Burk plot of **6c** (**A**), **6d** (**B**) and **6e** (**C**).

4. Stability of 6c, 6d and 6e

Stability of **6c**, **6d** and **6e** in tris-HCl buffer, artificial gastric juice and artificial intestinal juice was tested by measurement of the time course for the changing of ratios of fluorescent intensities at 550 and 460 nm (excitation at 400 nm) in a solution of 100 μM.

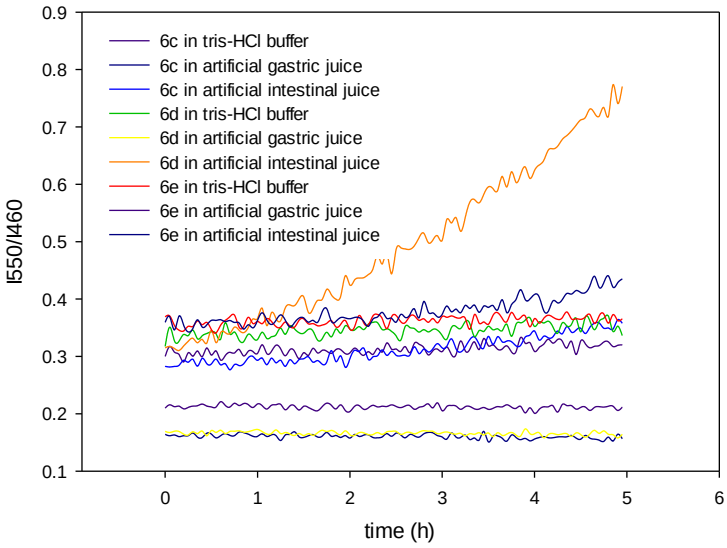


Figure S3 Time course for the generation of ratios of fluorescent intensities at 550 and 460 nm (excitation at 400 nm) of **6c**, **6d** and **6e** in the solution of tris-HCl buffer (100 mM, pH=7.5), artificial gastric juice and artificial intestinal juice (100μM).

5. ^{1}H NMR, ^{13}C NMR and HR-MS spectra

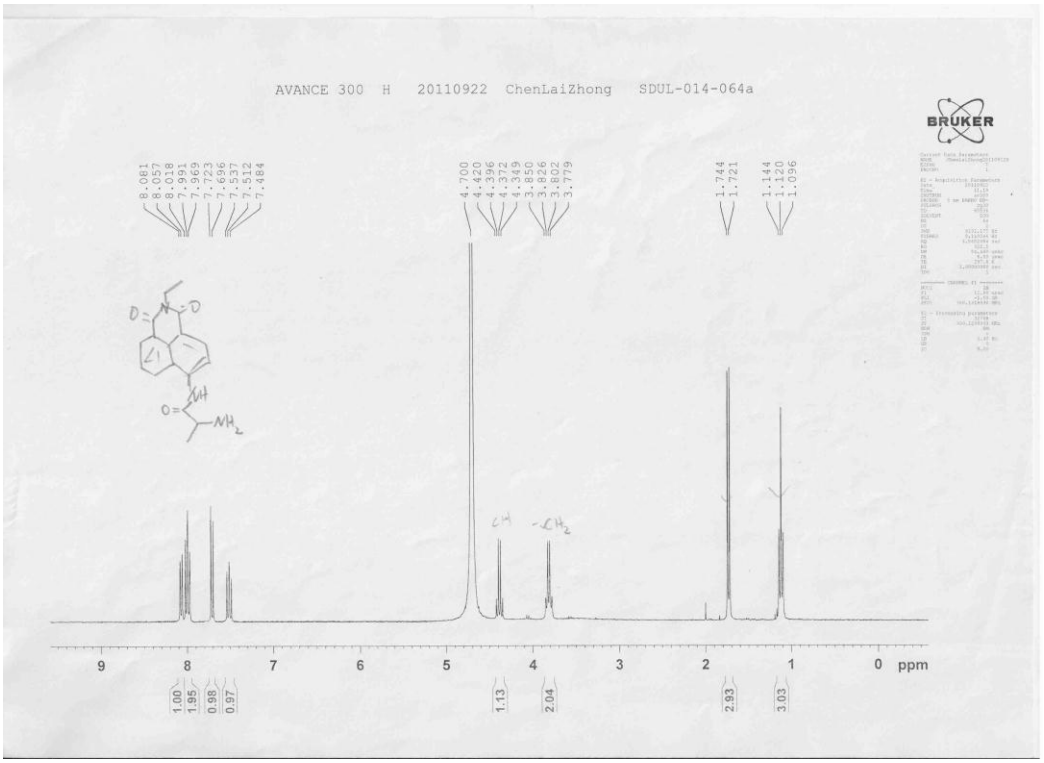


Figure S4 ^{1}H NMR (300 MHz) spectrum of Probe **6c**.

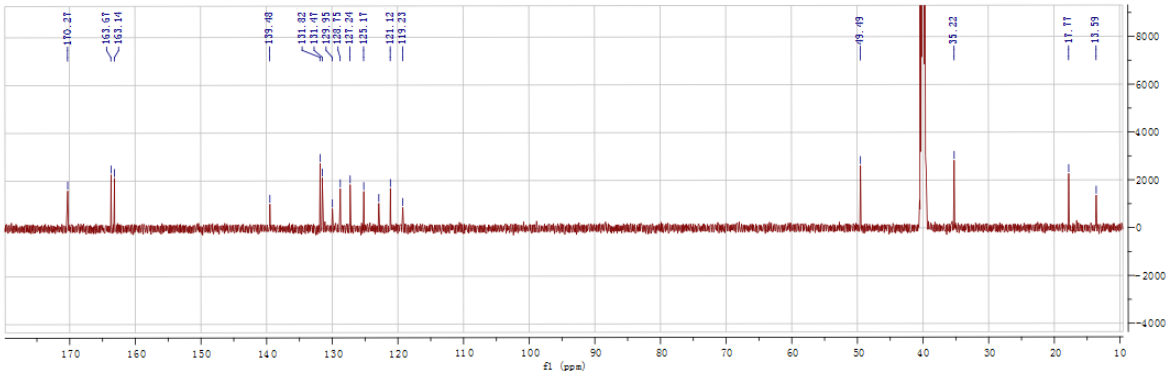
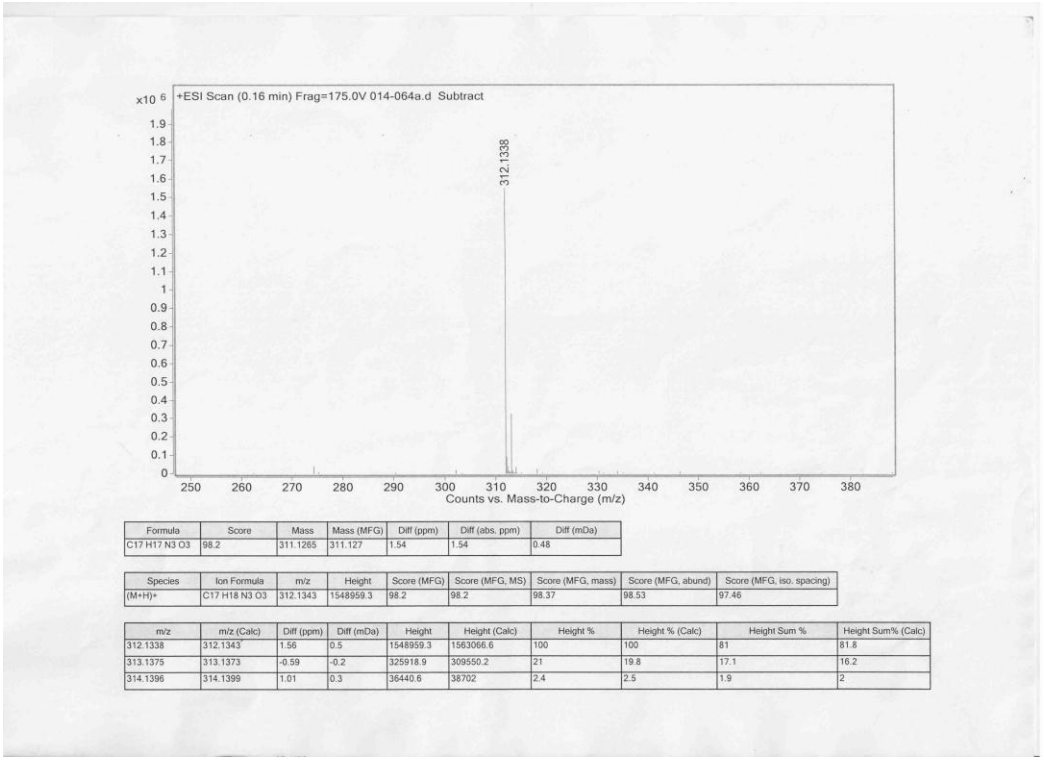


Figure S5 ^{13}C NMR (150 MHz) spectrum of Probe **6c**.



Formula	Score	Mass	Mass (MFG)	Diff (ppm)	Diff (abs. ppm)	Diff (mDa)
C17 H17 N3 O3	98.2	311.1265	311.127	1.54	1.54	0.48

Species	Ion Formula	m/z	Height	Score (MFG)	Score (MFG, MS)	Score (MFG, mass)	Score (MFG, abund)	Score (MFG, iso. spacing)
(M+H)+	C17 H18 N3 O3	312.1343	1548959.3	98.2	98.2	98.37	98.53	97.46

m/z	m/z (Calc)	Diff (ppm)	Diff (mDa)	Height	Height (Calc)	Height %	Height % (Calc)	Height Sum %	Height Sum% (Calc)
312.1338	312.1343	1.56	0.5	1548959.3	1563066.6	100	100	81	81.8
313.1375	313.1373	-0.59	-0.2	325918.9	309550.2	21	19.8	17.1	16.2
314.1396	314.1399	1.01	0.3	36440.6	38702	2.4	2.5	1.9	2

Figure S6 High resolution mass spectrum of Probe **6c**.

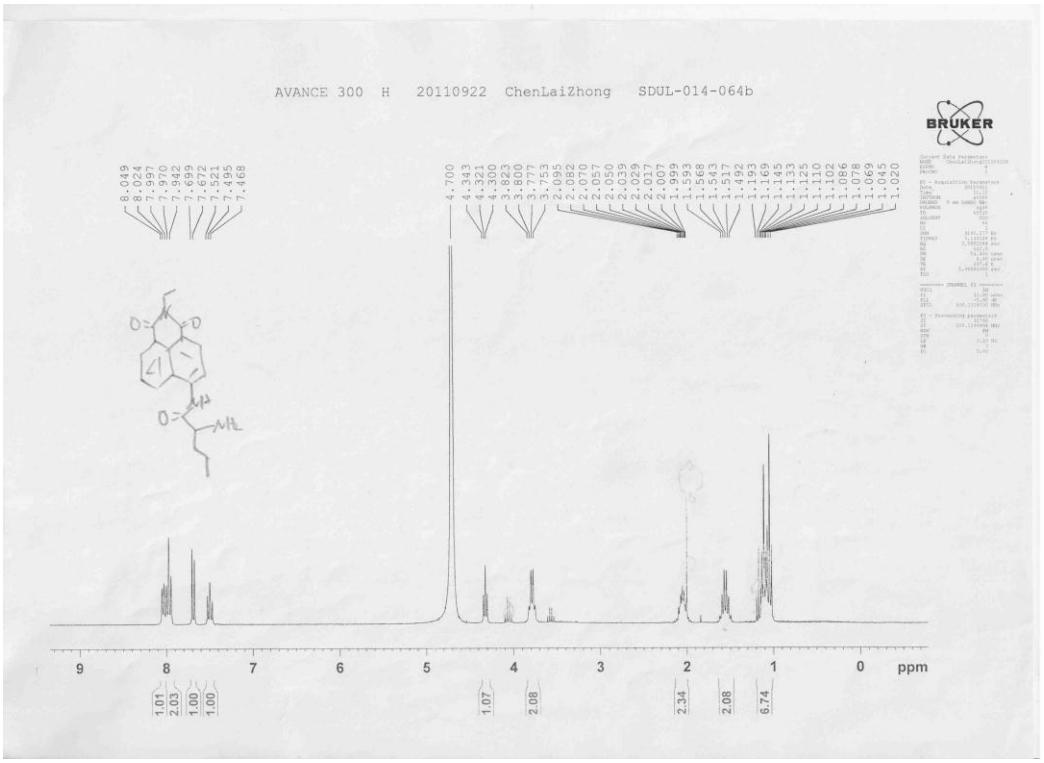


Figure S7 ^{1}H NMR (300 MHz) spectrum of Probe **6d**.

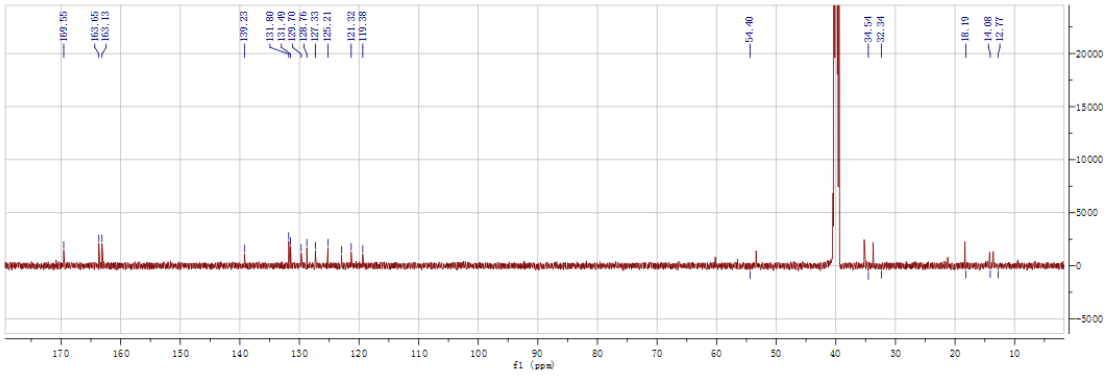
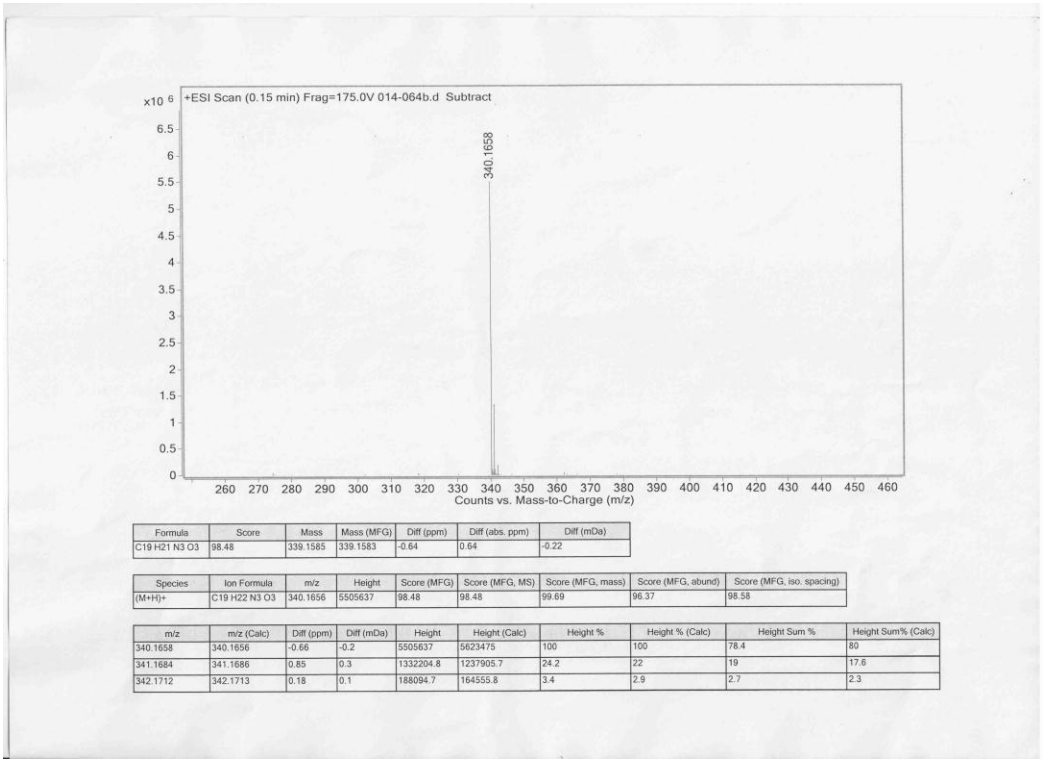


Figure S8 ^{13}C NMR (150 MHz) spectrum of Probe **6d**.



Formula	Score	Mass	Mass (MFG)	Diff (ppm)	Diff (abs. ppm)	Diff (mDa)
C19 H21 N3 O3	98.48	339.1585	339.1583	-0.64	0.64	-0.22

Species	Ion Formula	m/z	Height	Score (MFG)	Score (MFG, MS)	Score (MFG, mass)	Score (MFG, abund)	Score (MFG, iso. spacing)
(M+H)+	C19 H22 N3 O3	340.1656	5505637	98.48	98.48	99.69	96.37	98.58

m/z	m/z (Calc)	Diff (ppm)	Diff (mDa)	Height	Height (Calc)	Height %	Height % (Calc)	Height Sum %	Height Sum% (Calc)
340.1658	340.1656	-0.66	-0.2	5505637	5623475	100	100	78.4	80
341.1684	341.1686	0.85	0.3	1332204.8	1237905.7	24.2	22	19	17.6
342.1712	342.1713	0.18	0.1	188094.7	164555.8	3.4	2.9	2.7	2.3

Figure S9 High resolution mass spectrum of Probe **6d**.

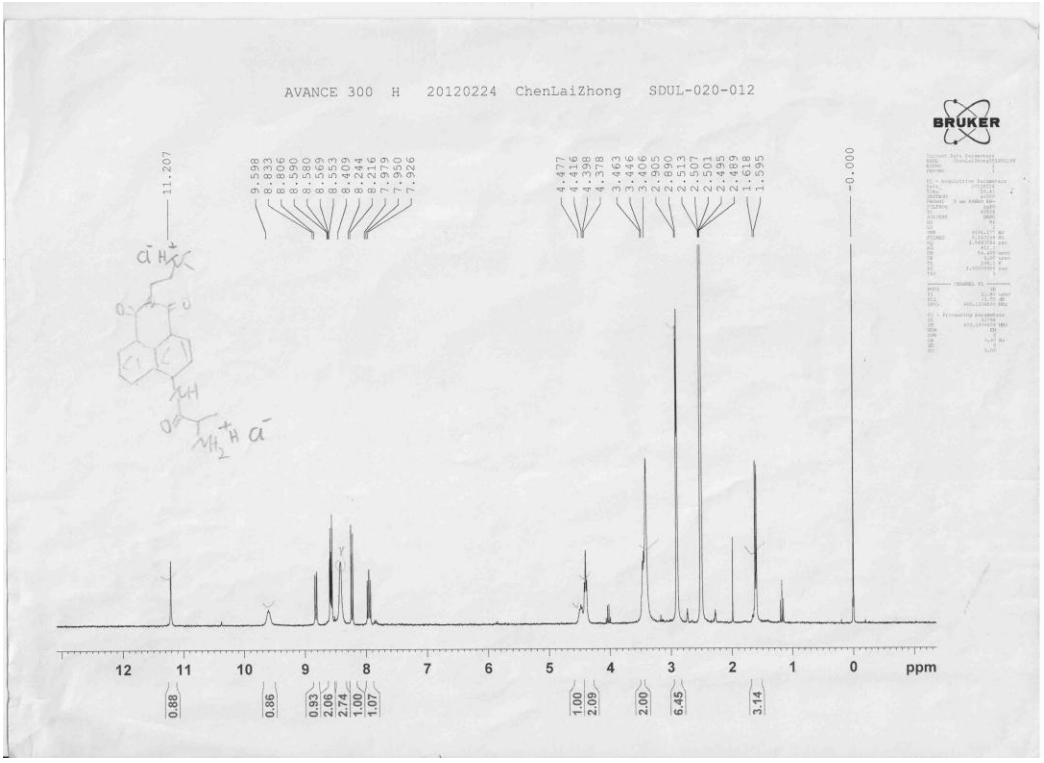


Figure S10 ^{1}H NMR (300 MHz) spectrum of Probe **6e**.

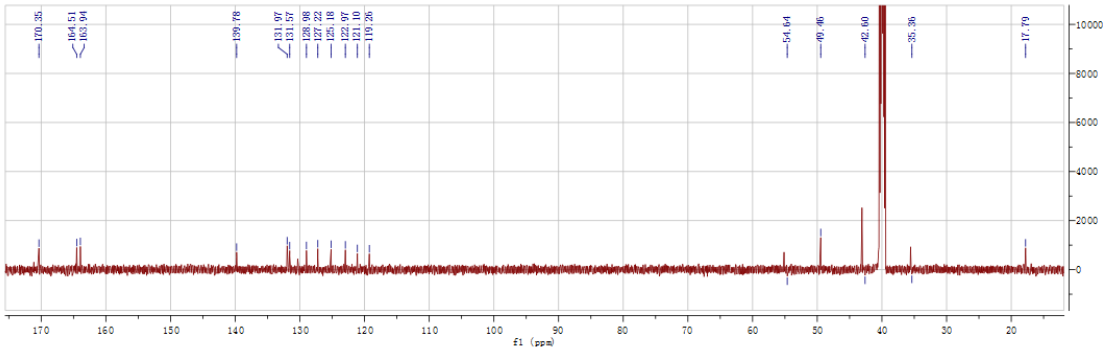
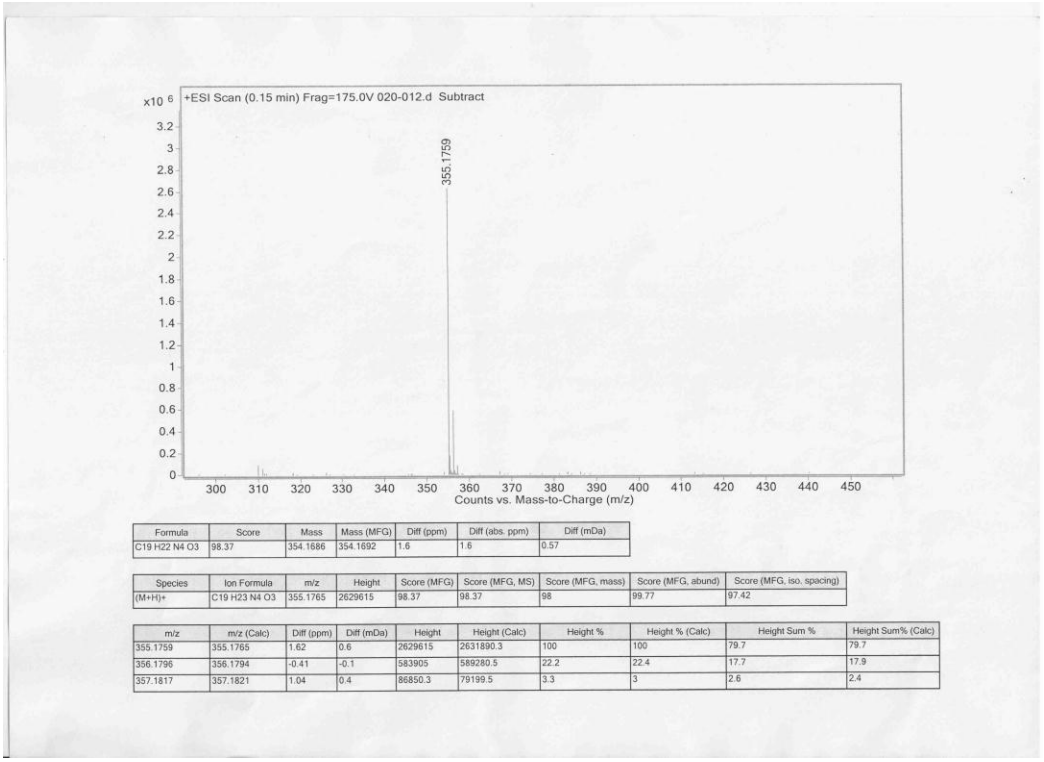


Figure S11 ^{13}C NMR (150 MHz) spectrum of Probe **6e**.



Formula	Score	Mass	Mass (MFG)	Diff (ppm)	Diff (abs. ppm)	Diff (mDa)
C19 H22 N4 O3	98.37	354.1686	354.1692	1.6	1.6	0.57

Species	Ion Formula	m/z	Height	Score (MFG)	Score (MFG, MS)	Score (MFG, mass)	Score (MFG, abund)	Score (MFG, iso. spacing)
(M+H)+	C19 H23 N4 O3	355.1765	2629615	98.37	98.37	98	99.77	97.42

m/z	m/z (Calc)	Diff (ppm)	Diff (mDa)	Height	Height (Calc)	Height %	Height % (Calc)	Height Sum %	Height Sum% (Calc)
355.1759	355.1765	1.62	0.6	2629615	2631890.3	100	100	79.7	79.7
356.1796	356.1794	-0.41	-0.1	583905	589280.5	22.2	22.4	17.7	17.9
357.1817	357.1821	1.04	0.4	86850.3	79199.5	3.3	3	2.6	2.4

Figure S12 High resolution mass spectrum of Probe **6e**.