Discovery of Turn-On Fluorescent Probes for Detecting Bcl-2 Protein

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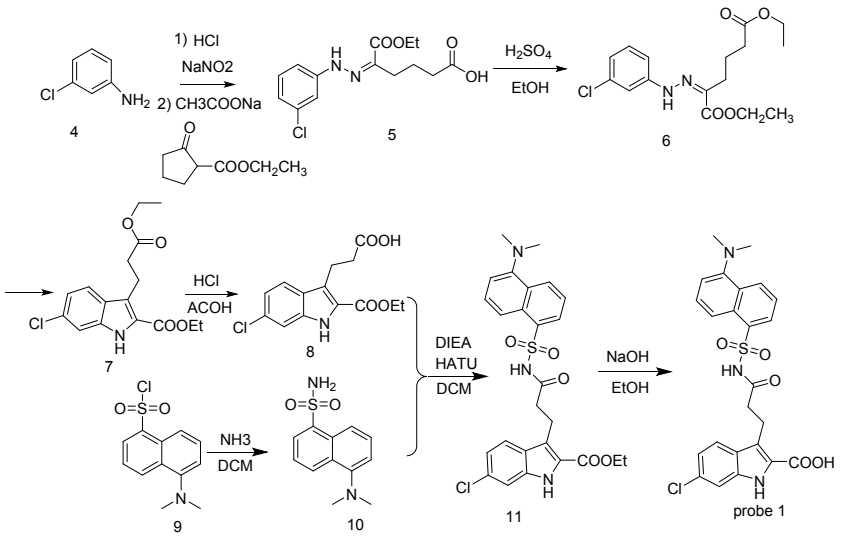
1. Synthesis



Scheme S1. Synthetic Route of Probe **1**

(Z)-5-(2-(3-chlorophenyl)hydrazono)-6-ethoxy-6-oxohexanoic acid(5)

1M HCl(7 mL) was added into a 50 mL round bottom flask with 9 mL water, then 3-chloroaniline(1.27 g, 10 mmol) was added in under ice bath, 5 min after NaNO3(1.38 g, 20 mmol) solution was droped in slowly. 2-Oxocyclopentate carboxylate(2.5 mL, 15 mmol) was dissolved in 4.2 mL EtOH and KOH(5.0 g, 90 mmol) with 5 mL water was droped in. 0.5 h after, the solution of 3-chloroaniline was droped into the solutionof 2-oxocyclopentate carboxylate slowly and the mixture was stirred under 40 °C for 1h. The reaction mixture was concentrated *in vacuo* to give the crude product **5** without further purification.

(Z)-diethyl 2-(2-(3-chlorophenyl)hydrazono)hexanedioate(6)

To a solution of compound **5**(3.3 g, 10 mmol) in EtOH(25 mL) was added con.H_2SO_4 3 mL, slowly. The mixture was refluxed for 2 h. The reaction mixture was cooled and EtOH was removed by distillation then extracted with EtOAc. The organic layer was washed with water, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The crude product was purified by column chromatography on silica gel (petroleum: ethyl acetate = 5:1) to give compound **6** as a pale yellow solid.

A pale yellow solid, Yield: 2.50 g, 40%. M.p.:81-83°C. ^{1}H-NMR (400 MHz, $CDCl_3$), δ 9.86(s, 1H), 7.40-7.39(m, 1H), 7.28(s, 1H), 7.23-7.22(m, 1H), 6.94-6.91(m, 1H), 4.36-4.27(m, 4H), 2.65-2.61(m, 2H), 2.50-2.47(m, 2H), 1.80-1.74(m, 2H), 1.43(t, *J* = 8.0 Hz, 3H), 1.37(t, *J* = 8.0 Hz, 3H).

ethyl 6-chloro-3-(3-ethoxy-3-oxopropyl)-*1H*-indole-2-carboxylate(7)

To a solution of compound **6**(0.68 g, 2 mmol) in toluene(10 mL) was added p-Toluenesulfonic acid (0.58 g, 3 mmol). The reaction mixture was refluxed for 12h. The cooled mixture was concemtrated *in vacuo* and extracted with EtOAc, dried and concentrated to get oil. The oil recrystallized from hexane to obtain intermediate **7** as an off-white solid.

A white solid, Yield: 0.53 g, 83%. M.p.: 93-95°C.^{1}H-NMR (400 MHz, $CDCl_3$), δ 9.91(d, *J* = 12.0 Hz, 1H), 7.68(d, *J* = 8.0 Hz, 1H), 7.39(s, 1H), 7.15-7.12(m, 1H), 4.46-4.42(m, 2H), 4.19-4.09(m, 2H),

3.76-3.72(m, 1H), 3.42(t, *J* = 8.0 Hz, 1H), 2.74-2.67(m, 2H), 1.47-1.44(m, 3H), 1.30(t, *J* = 8.0 Hz, 1H), 1.24(t, *J* = 8.0 Hz, 2H).

3-(6-chloro-2-(ethoxycarbonyl)-*1H*-indol-3-yl)propanoic acid(8)

Compound **7**(0.32 g, 1 mmol) was added to a 50 mL round bottom flask with 3.5 mL acetic acid. Then, 0.5 mL con.HCl was added and the mixture reacted under 80 °C. Three hours later, 10 mL water was added in and the solids were filtered to get a white compound **8**.

A white solid, Yield: 0.26 g, 89%. M.p.: 210-213°C. ^{1}H-NMR (400 MHz, $CDCl_3$), δ 12.09(s, 1H), 11.71(s, 1H), 7.74(d, *J* = 8.0 Hz, 1H), 7.42(t, *J* = 4.0 Hz, 1H), 7.11-7.07(m, 1H), 4.36-4.32(m, 2H), 4.27(t, *J* = 8.0 Hz, 2H), 2.53(t, *J* = 8.0 Hz, 2H), 1.37(t, *J* = 8.0 Hz, 3H).

5-(dimethylamino)naphthalene-1-sulfonamide(10)

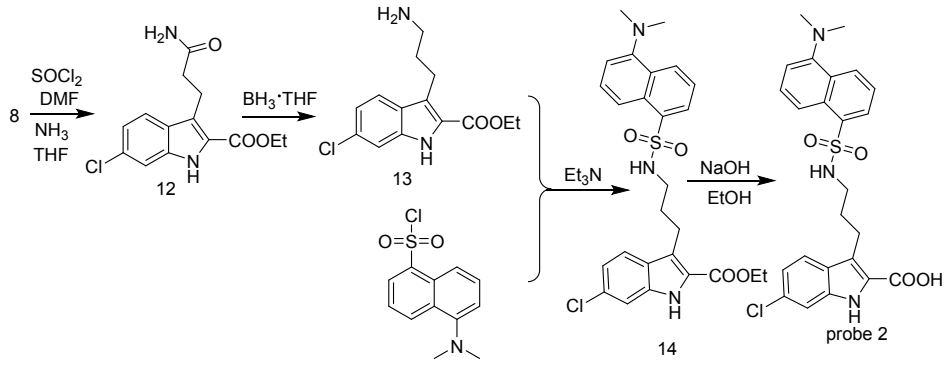
Compound **9**(0.5 g, 1.8 mmol) was dissolved in DCM(10 mL) under NH_3, 15 min later, some solvent was removed and the product (**10**) was filtered to obtain a white solid.

A white solid, Yield: 0.46 g, 99%. M.p.: 210-213°C. ^{1}H-NMR (400 MHz, $CDCl_3$), δ 8.43(d, *J* = 8.0 Hz, 1H), 8.30(d, *J* = 8.0 Hz, 1H), 8.05(d, *J* = 8.0 Hz, 1H), 8.13(d, *J* = 8.0 Hz, 1H), 7.63-7.56(m, 4H), 7.26(d, *J* = 8.0 Hz, 1H), 2.83(s, 6H).

6-chloro-3-(3-(5-(dimethylamino)naphthalene-1-sulfonamido)-3-oxopropyl)-*1H*-indole-2-carboxylic acid(probe 1)

The solution of compound **8**(0.15 g, 0.5 mmol) in dry dichloromethane (10 mL) was treated with ethyldiisopropylamine (0.19 g, 1.5 mmol) under ice bath condition, 10 min later, 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate(0.23 g, 1.2 mmol) was added in and the cloudy reaction mixture was stirred for 30 min, then compound **10**(0.14 g, 0.55 mmol) was added. The solution was stirred over night and the solvent was removed and the concentrated solution was dissolved in DCM and washed with 2M citric acid, brine and dried, concentrated to yield yellow oil. Finally, the oil was simply purified by silica gel chromatography to generate crude compound **11** without further purification. The crude product **11** was dissolved in ethanol directly, 2 ml 50% NaOH aqueous solution was added in and the mixture was stirred over night at room temperature. Then, the solvent was removed and 10 mL water was added in. The mixture was acidified with 1 M HCl solution and the white compound was filtered to get the crude product. The product was recrystallized from ethanol and water to get a yellow powder.

A yellow solid, Yield: 0.06 g, 41%. M.p.: 158-161°C. ^{1}H-NMR (400 MHz, DMSO-d_6), δ 11.51(s, 1H), 8.44(d, *J* = 8.0 Hz, 1H), 8.32-8.27(m, 1H), 8.18 (d, *J* = 8.0 Hz, 1H), 7.62-7.52(m, 3H), 7.34(s, 1H), 7.22-7.15(m, 1H), 7.02(d, *J* = 8.0 Hz, 1H), 6.94(d, *J* = 12.0 Hz, 1H), 3.08(t, *J* = 8.0 Hz, 2H), 2.83(s, 6H), 2.41(s, 2H). ^{13}C-NMR (DMSO-d_6, 101 MHz): δ173.34, 163.52, 151.72, 137.84, 136.52, 129.95, 129.79, 129.68, 129.41, 129.28, 128.02, 126.19, 125.82, 123.91, 122.40, 121.56, 120.08, 119.57, 115.27, 112.03, 45.52, 38.29, 20.06 HRMS (ESI) m/z calcd for $C_{35}H_{38}BrN_8O_5S$ ([M + H] $^+$) 761.1869; found 761.1867.



Scheme S2. Synthetic Route of Probe **2**

Ethyl 3-(3-amino-3-oxopropyl)-6-chloro-*1H*-indole-2-carboxylate (12)

Compound **5**(0.30 g, 1 mmol) was dissolved in 15 mL THF, $SOCl_2$ (0.11 mL) and 2d DMF were added in subsequently. The reaction mixture was stirred for 2 h at room temperature. Then, ammonia was filled in for 20 min. After stirred for 1 h, the solvent was removed and a lot of water was added in. The solid was filtered to get the white product **12**.

A white powder, Yield: 0.27 g, 100%. M.p.: 228-230°C. ^{1}H-NMR (400 MHz, $CDCl_3$), δ 11.67(s, 1H), 7.75(d, *J* = 8.0 Hz, 1H), 7.41-7.39(m, 1H), 7.23(t, *J* = 8.0 Hz, 1H), 7.13-7.06(m, 1H), 6.74-6.71(m, 1H), 4.37-4.32(m, 2H), 3.24-3.16(m, 2H), 2.39-2.33(m, 2H), 1.38(t, *J* = 8.0 Hz, 3H).

Ethyl 3-(3-aminopropyl)-6-chloro-*1H*-indole-2-carboxylate (13)

To a solution of compound **12**(0.58 g, 2 mmol) in THF(5 mL) was added BH3 in THF (8 mL, 8 mmol) under nitrogen.The reaction mixture was stirred for 16h at 20°C and quenched by addition of EtOH and then concentrated *in vacuo*. 3 M HCl (4 mL) was added in and the mixture was refluxed for 1 h, then, the reaction solution was neutralized by NaOH solution and extracted with EtOAc, dried and concentrated to get the crude product. The product was washed by petroleum and ethyl acetate (10:1) to obtain compound **13** as a pale yellow solid.

To a solution of compound **13**(0.13 g, 0.5 mmol) in THF (20 mL) was added Et_3N (0.15 mL, 1.05 mmol). After stirred for 0.5 h, Dansyl chloride (0.15 g, 0.525 mmol) was added in and stirred overnight. Then, the solvent was evaporated and the residue was purified by column chromatography on silica gel (petroleum: ethyl acetate = 7:1) to give compound **14** as a yellow powder.

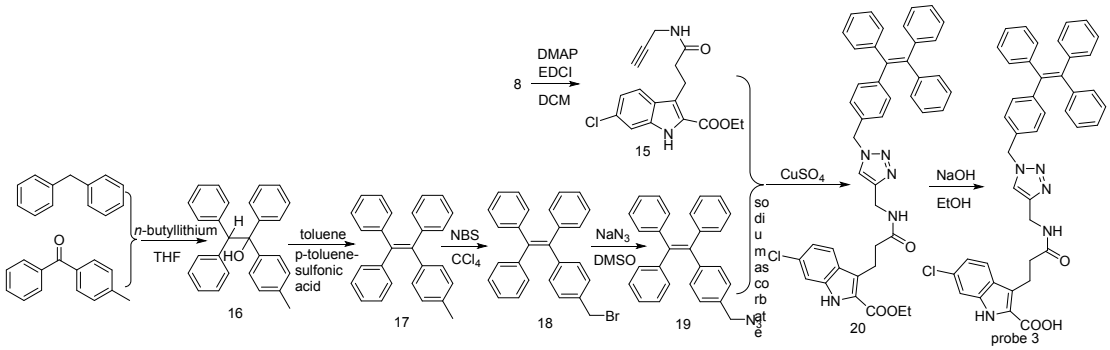
A yellow solid, Yield: 1.04 g, 84.0%. M.p.:118-120°C. ^{1}H-NMR (400 MHz, $CDCl_3$), δ 11.61(s, 1H), 8.45(d, *J* = 8.0 Hz, 1H), 8.32(d, *J* = 8.0 Hz, 1H), 8.05(d, *J* = 8.0 Hz, 1H), 7.94(s, 1H), 7.60(t, *J* = 8.0 Hz, 2H), 7.40-7.37(m, 2H), 7.26(d, *J* = 8.0 Hz, 1H), 6.99(d, *J* = 8.0 Hz, 1H), 4.28-4.23(m, 2H), 2.81(s, 10H), 1.56-1.53(m, 2H), 1.26-1.23(m, 3H).

6-chloro-3-(3-(5-(dimethylamino) naphthalene-1-sulfonamido) propyl)-*1H*-indole-2-carboxylic acid (probe 2)

The product **14**(70 mg) was dissolved in ethanol, 1.5 ml 50% NaOH aqueous solution was added in and the mixture was stirred over night at room temperature. Then, the solvent was removed and 15 mL water was added in. The mixture was acidified with 1 M HCl solution and the white compound was filtered to get the crude product. The product was recrystallized from ethanol and water to get a yellow powder.

A yellow powder, Yield: 0.06 g, 86%. M.p.:70-73°C. ^{1}H-NMR (400 MHz, DMSO-d_6), δ 10.96 (s, 1H),

9.20(s,1H), 8.42(t, *J* = 8.0 Hz, 2H), 8.05(dd, J_1 = 8.0 Hz, J_2 = 4.0 Hz, 1H), 7.61-7.53(m, 2H), 7.31-7.28(m, 2H), 7.24(d, *J* = 8.0 Hz, 1H), 6.87(dd, J_1 = 8.0 Hz, J_2 = 4.0 Hz, 1H), 2.97-2.94(m, 2H), 2.81(s, 6H), 2.70-2.49(m, 2H), 1.54-1.47(m, 2H). ^{13}C-NMR (DMSO-d_6, 101 MHz): δ 163.51, 151.79, 136.63, 136.53, 129.81, 129.53, 129.49, 128.70, 128.27, 126.29, 125.36, 124.02, 122.35, 122.17, 120.12, 119.56, 115.54, 112.08, 45.49, 42.95, 31.12, 21.69. HRMS (ESI) m/z calcd. for $C_{35}H_{38}BrN_8O_5S$ ([M + H] $^+$) 761.1869; found 761.1867.



Scheme S3. Synthetic Route of Probe **3**

Ethyl6-chloro-3-(3-oxo-3-(prop-2-yn-1-ylamino)propyl)-*1H*-indole-2-carboxylate(15)

In a 250 mL round bottom flask, propargylamine (30 mg, 0.55 mmol), compound **8**(0.15 g, 0.5 mmol) and 4-dimethylaminopyridine (74 mg, 0.6 mmol) were dissolved in 100 mL of DCM. EDCI (0.29 g, 1.5 mmol) was added in gradually at 0 °C. After stirred overnight, the reaction mixture was extracted with DCM and washed by 1M citric acid solution. Then the organic layer was concentrated. The crude product **15** was purified by silica-gelchromatography using Petroleum ether/ ethyl acetate (v/v = 2:1) as eluent to give **15** as pink solid.

A pink solid, Yield: 0.07 g, 42.1%. M.p.: 180-183°C. ^{1}H-NMR (400 MHz, DMSO-d_6), *δ* 11.93 (s, 1H), 11.68(s, 1H), 7.73 (d, *J* = 8.0 Hz, 1H), 7.42-7.40 (m, 1H), 7.10-7.06 (m, 1H), 4.38-4.32 (m, 2H), 3.88-3.86 (m, 1H), 3.81-3.79 (m, 1H), 3.53-3.48 (m, 1H), 3.26 (t, *J* = 8.0 Hz, 1H), 3.10-3.07 (m, 1H), 2.43-2.36 (m, 2H), 1.38(t, *J* = 8.0 Hz, 3H).

1, 2, 2-triphenyl-1-(*p*-tolyl)ethanol(16)

10 mL of distilled THF was added into a 250 mL two necked round bottom flask and cooled to 0 °C under N_2, 5 mL (2.5 M in hexane) of *n*-butyllithium was added slowly by a syringe. Then, diphenylmethane(1.01 g, 10 mmol) was dropped in. After the mixture was stirred for 1 h at 0 °C, 1.63 g (8.3 mmol) of 4-methylbenzophenone was added into the reaction mixture. The reaction mixture was warmed to room temperature and stirred for one night. The mixture was quenched with a large amount of saturated NH_4Cl solution and extracted with DCM. Next, the organic layers were concentrated and petroleum ether (10 mL) was added in, then the product **16** was isolated as white solid.

A white solid, Yield: 2.6 g, 86.0%. M.p.: 138-141°C. ^{1}H-NMR (400 MHz, DMSO-d_6), *δ* 7.47 (t, *J* = 8.0 Hz, 5H), 7.40 (t, *J* = 8.0 Hz, 3H), 7.13-6.92 (m, 11H), 5.87 (s, 1H), 5.20 (s, 1H), 2.15 (s, 3H).

(2-(p-tolyl)ethene-1,1,2-triyl)tribenzene(17)

In a 50 mL round bottom flask, compound **16** (0.5 g, 10 mmol) and 0.01 g of *p*-toluenesulfonic acid were dissolved in 10 mL of toluene. The mixture was refluxed for 4 h. After cooled, the mixture was extracted with hexane. The solvent of hexane was collected and concentrated. Then, 5 mL

petroleum ether was added and the white solid was filtered to obtain intermediate **17**.

A white solid, Yield: 0.3 g, 78.0%. M.p.: 175-178°C. ^{1}H-NMR (400 MHz, DMSO-d_6), δ 7.15-7.09 (m, 9H), 6.98-6.93 (m, 8H), 6.85 (d, J = 8.0 Hz, 2H), 2.20 (s, 3H).

(2-(4-(bromomethyl)phenyl)ethene-1,1,2-triyl)tribenzene(18)

In a 100 mL round bottom flask, a solution of 0.7 g (2.0 mmol) of **18**, 0.40 g (2.2 mmol) of *N*-bromosuccinimide, 0.005 g of benzoyl peroxide in 12 mL of CCl_4 was refluxed for 12 h. After the reaction was finished, a lot of water was added and the mixture was extracted by DCM. The organic layers were combined and removed under reduced pressure. The product was purified by silica-gel chromatography using hexane as eluent to give **18** as white solid.

A white solid, Yield: 0.55 g, 64.0%. M.p.: 120-123°C. ^{1}H-NMR (400 MHz, DMSO-d_6), δ 7.13-7.09 (m, 11H), 7.03-6.98 (m, 8H), 4.42 (s, 2H).

(2-(4-(azidomethyl)phenyl)ethene-1,1,2-triyl)tribenzene(19)

In a 100 mL two-neck round-bottom flask, 0.21 g (0.5 mmol) of **18** and 0.048 g (7.5 mmol) of sodium azide were dissolved in 10 mL DMSO under N_2. The reaction mixture was stirred overnight. A large amount (200 mL) of water was added, and the solution was extracted with DCM. The organic layers were then combined, dried over $MgSO_4$, and concentrated. The product **19** was purified by silica gel chromatography using petroleum ether/ethyl acetate (v/v = 150:1) as eluent.

A white solid, Yield: 0.25 g, 50.0%. M.p.: 105-108°C. ^{1}H-NMR (400 MHz, DMSO-d_6), δ 7.04-7.02 (m, 9H), 6.97-6.94 (m, 10H), 4.18 (s, 2H).

6-chloro-3-(3-oxo-3-(((1-(4-(1,2,2-triphenylvinyl)benzyl)-1H-1,2,3-triazol-4-yl)methyl)amino)propyl)-1H-indole-2-carboxylic acid(probe 3)

A solution of **12**(0.07 g, 0.2 mmol) and compound **19**(0.086 g, 0.22 mmol) in 10.0 mL *t*-BuOH/H_2O (2:1). 0.1 M sodium ascorbate (10 mL) and 0.1 M $CuSO_4$ (1.6 mL) were added. The cloudy mixture was heated to 50 °C for 1 h in the dark, and then cooled to room temperature. The solvent was removed and DCM was added. The organic layer was dried and evaporated. The residue was purified simply to get the crude product **20**.

To a solution of compound **20**(0.10 g) in EtOH (5 mL) was added 2 ml 50% NaOH aqueous solution. After stirred for 12 h, the solvent was removed and 20 mL water was added in. The reaction mixture was acidified by 1 M HCl solution and the white compound was filtered. The crude product was then recrystallized from ethanol and water to get a pale powder.

A pale solid, Yield: 0.07 g, 73.0%. M.p.: 208-210°C. ^{1}H-NMR (400 MHz, DMSO-d_6), δ 11.34 (d, J = 8.0 Hz, 1H), 9.16 (s 1H), 8.87 (s 1H), 7.78 (d, J = 12.0 Hz, 1H), 7.61 (d, J = 8.0 Hz, 1H), 7.35-7.30(m, 1H), 7.15-7.02 (m, 11H), 6.98-6.93 (m, 9H), 5.45 (d, J = 8.0 Hz, 2H), 5.43 (dd, J_1 = 16.0 Hz, J_2 = 8.0 Hz, 2H), 3.56 (t, J = 8.0 Hz, 2H), 2.45 (t, J = 8.0 Hz, 1H), 1.91(s, 1H).

2. Fluorescence Spectroscopy Test

Probes **1-3** were dissolved in DMSO to give 10 mM stock solution. Then, the stock solution of each probe was diluted by PBS (PH = 7.4) and acetonitrile-PBS to acquired 5 μM solution and different proportion of acetonitrile solutions respectively. The fluorescent properties of probes **1-3** were conducted on the Thermo-Fisher Varioskan microplate reader. Additionally, the quantum yield of these probes were calculated under PBS solution through the comparison with fluorescein in 0.1 M NaOH (Φ_{ST} = 0.92) which was considered as the reference using the following equation:

$$\Phi_X = \Phi_{ST} (A_{ST}/A_X) (F_X/F_{ST}) (\eta_X/\eta_{ST})^2$$

Where the subscripts ST and X denote standard and test respectively, Φ is the quantum yield, F is the integrated area under the fluorescence spectra, A is the absorbance, η is the refractive index of the solvent.

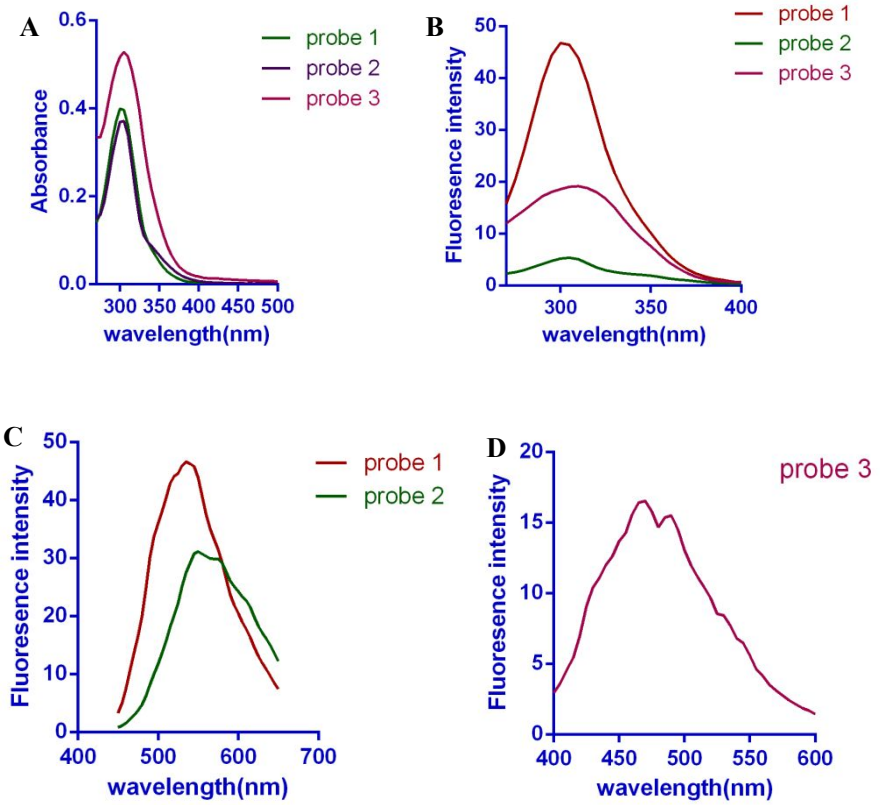


Figure S1. (**A**) Absorption spectra of probes; (**B**) Fluorescence excitation spectrum with each probe in PBS buffer; (**C**) Fluorescence emission spectrum of probes **1-2**; (**D**) Fluorescence emission spectrum of probe **3**.

3. Inhibition assay

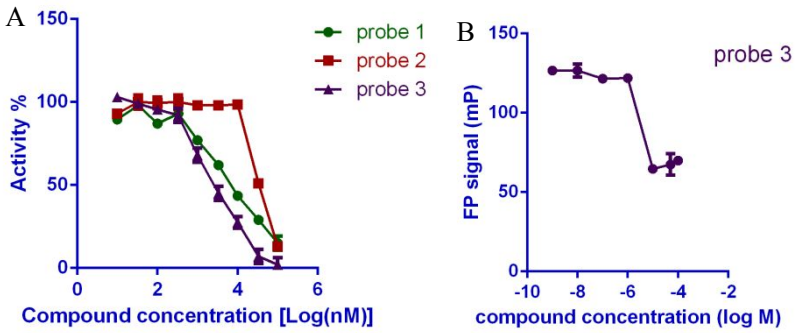


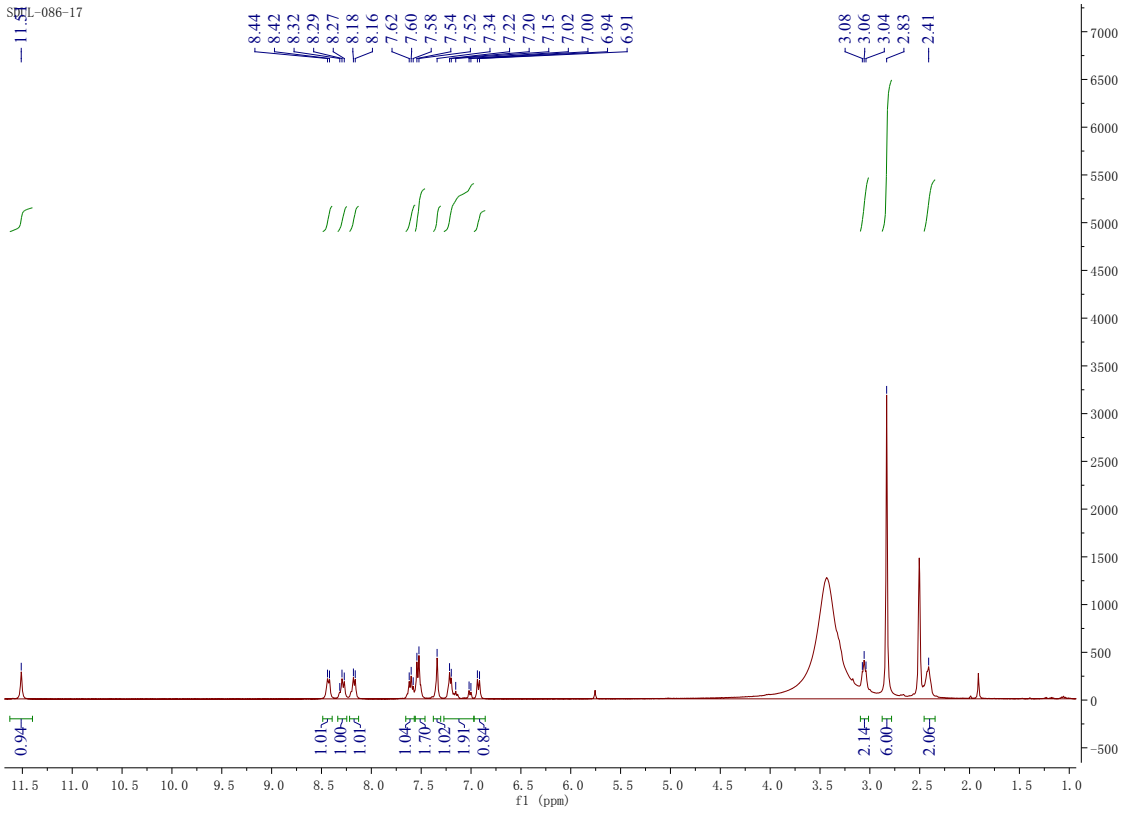
Figure S2. (**A**, **B**)Biological evaluation of probes for Bcl-2 proteins.

The binding assay for Bcl-2 protein of probe **1-3** was performed by TR-FRET technology using a peptide-ligand substrate and a recombinant BCL-2. The TR-FRET signal from the binding assay is correlated with the number of Ligand binding to BCL-2 protein. All of the binding assays were conducted at room temperature. Bcl-2 protein, ligand, the indicated amount of the inhibitor, and the reaction dyes were mixed in 20 μl buffer. Then, the reaction mixture was used for reading the

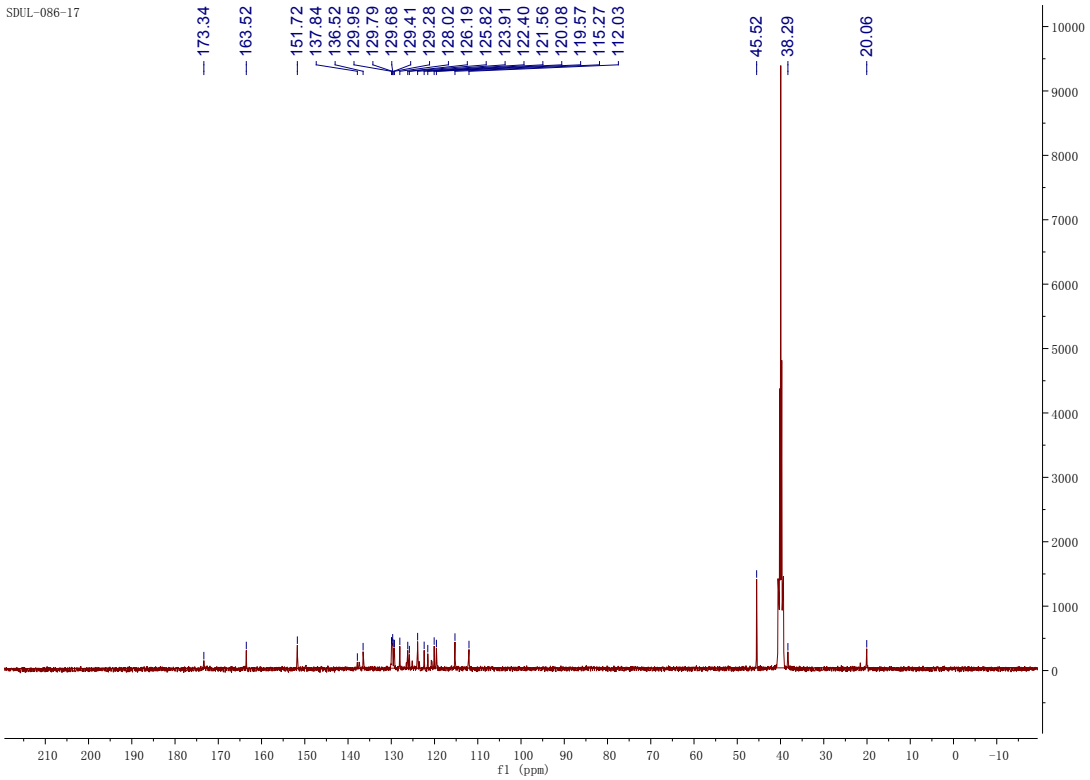
TR-FRET signal after incubated for 120 min. For the background, ligand was replaced with the assay buffer. Fluorescence signals for both the acceptor and donor dyes were measured using a Tecan Infinite M1000 plate reader. TR-FRET was recorded as the ratio of the fluorescence of the acceptor and the donor dyes (acceptor/donor).

Fluorescence polarization assay (FPA) was chosen to be used in the binding assay for Mcl-1 protein and Bcl-xL protein. In this assay, the Bid BH3 peptide with a fluorophore was considered as the tracer. The binding affinities of tested compounds were characterized quantitatively according to the changes of FP signals by the addition of the compounds at different concentrations. For further details, a 26-reside BH3 peptide (QEDIIRNIARHLAQVGDSMDRSIPPG) from Bid protein was labeled by 5-carboxyfluorescein succinimidyl ester (FAM) at the N-terminus[1]. Mcl-1 protein and these tested probes were preincubated for 30 min in the 10 mM PBS buffer in dark and room temperature. Next, 20 μL 5-FAM-Bid-BH3 peptide solutions in 10 mM PBS buffer were added into the above solutions to obtain a total volume of 200 μL. The flat-bottom, corning 384-well, black plates was selected for the assay. After incubated for 20 min, 60 μL mixtures were added into each well and every three wells with the same concentration of each sample. Afterward, the polarization values can be read from the Tecan GENios-Pro Injector Reader. Finally, the competitive inhibition constant (K_i) of tested probes was obtained using the method developed by Wang *et al*[2].

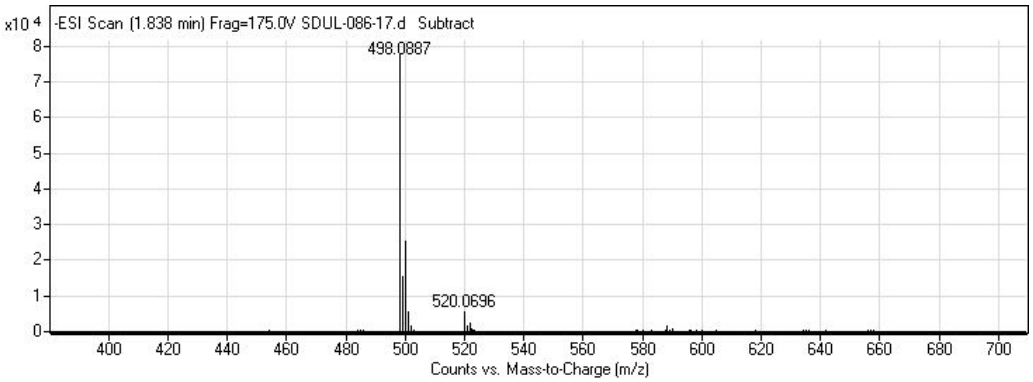
4. Charaterization of probes



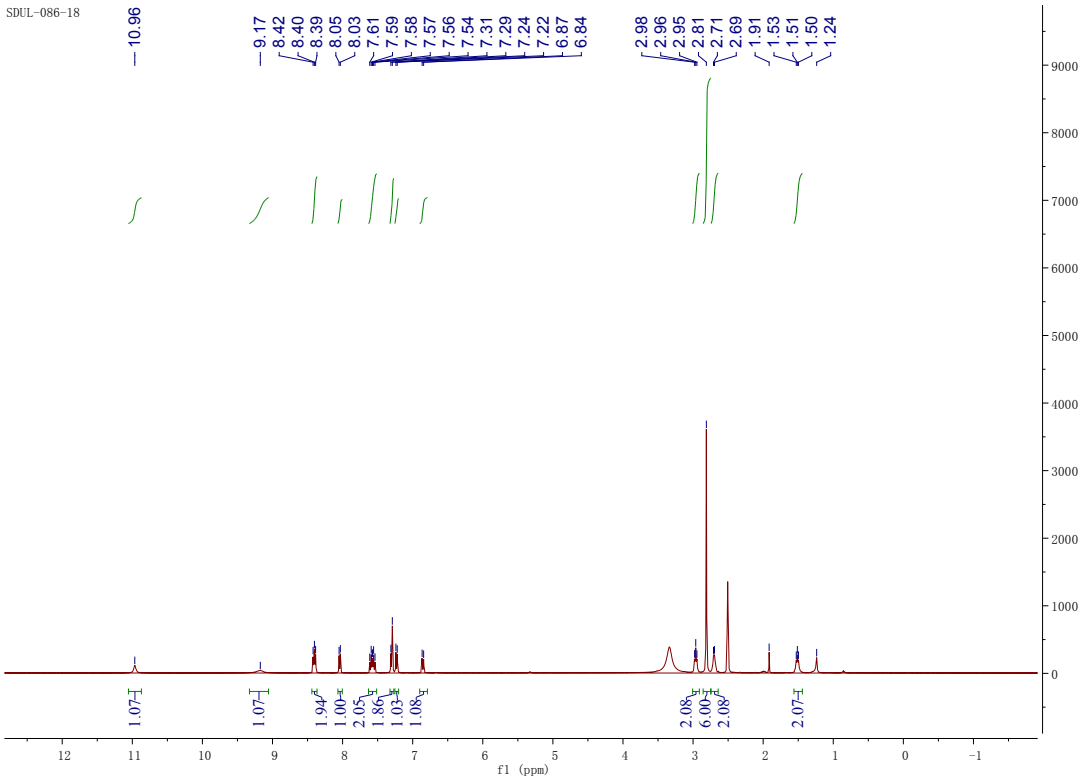
^{1}H-NMR spectra of probe **1**



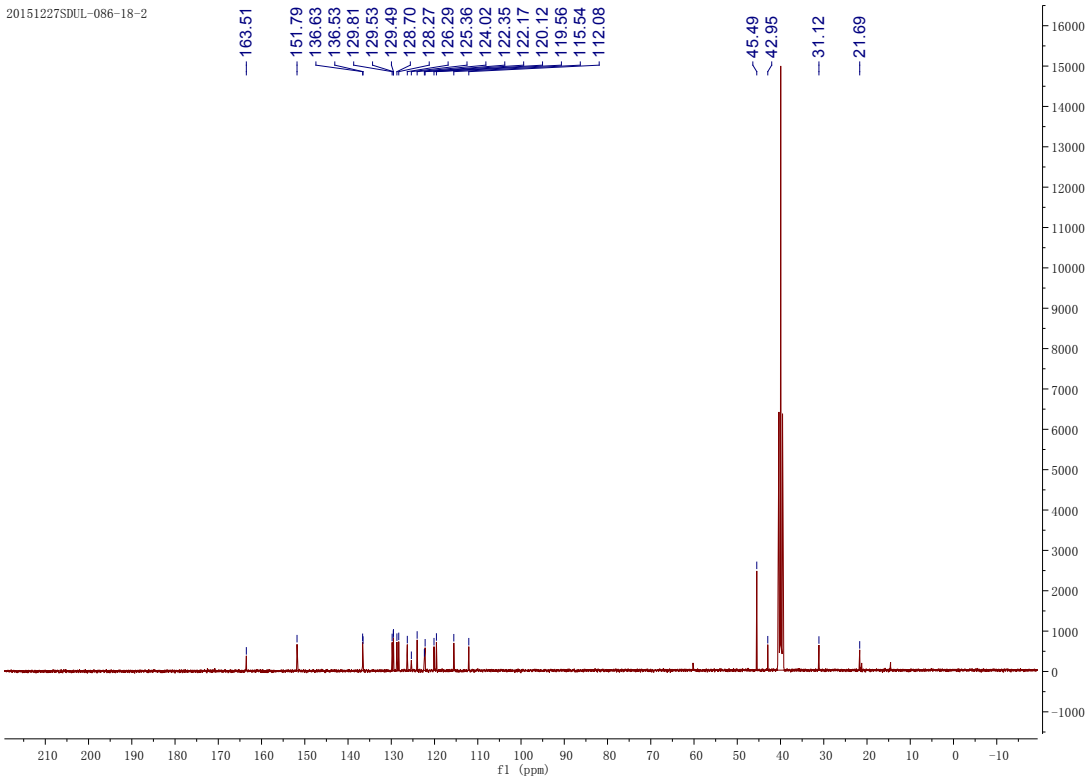
^{13}C-NMR spectra of probe **1**



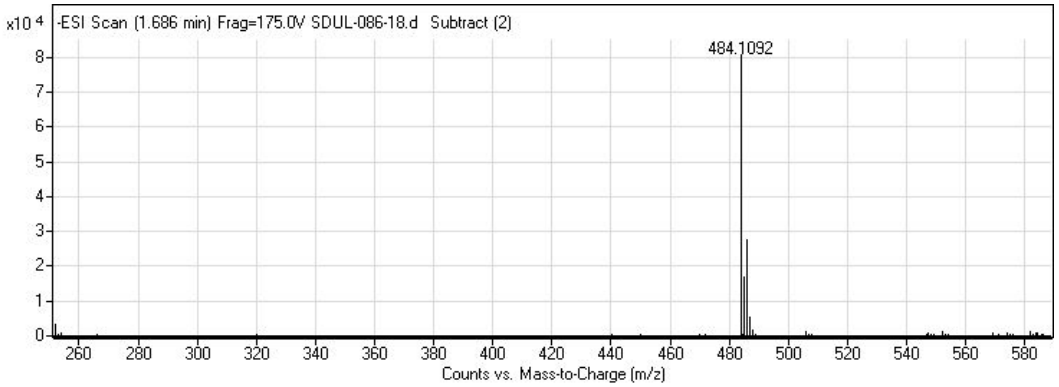
ESI-HRMS spectra of probe **1**



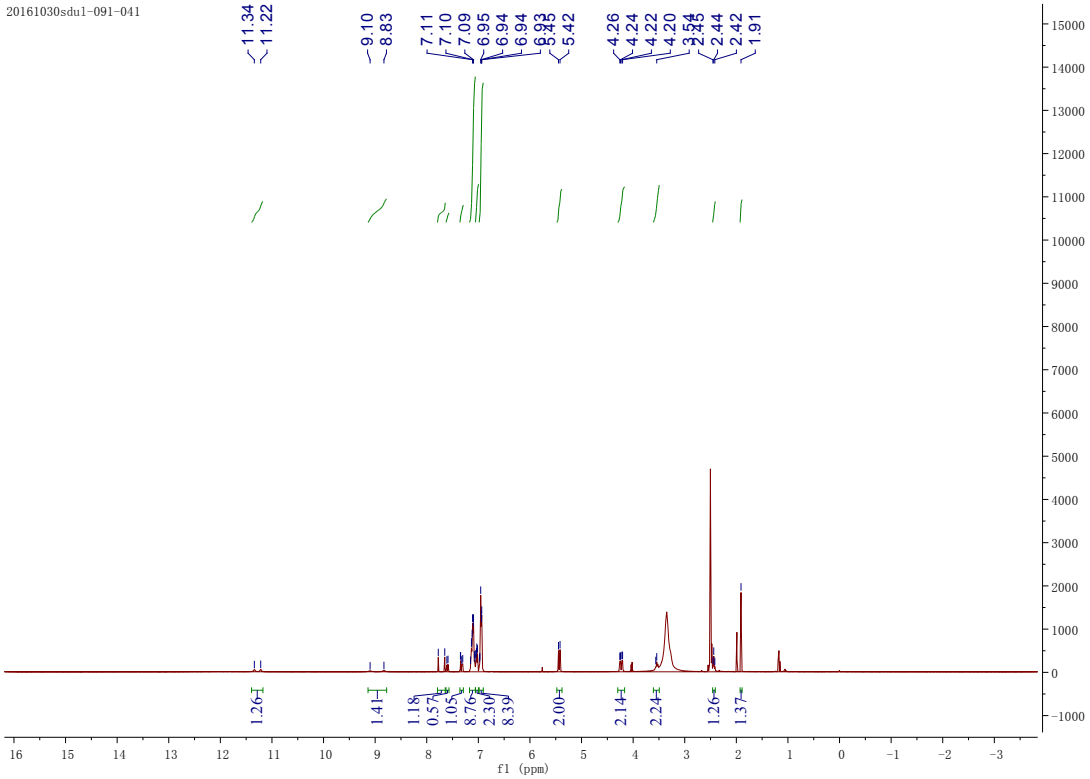
^{1}H-NMR spectra of probe **2**



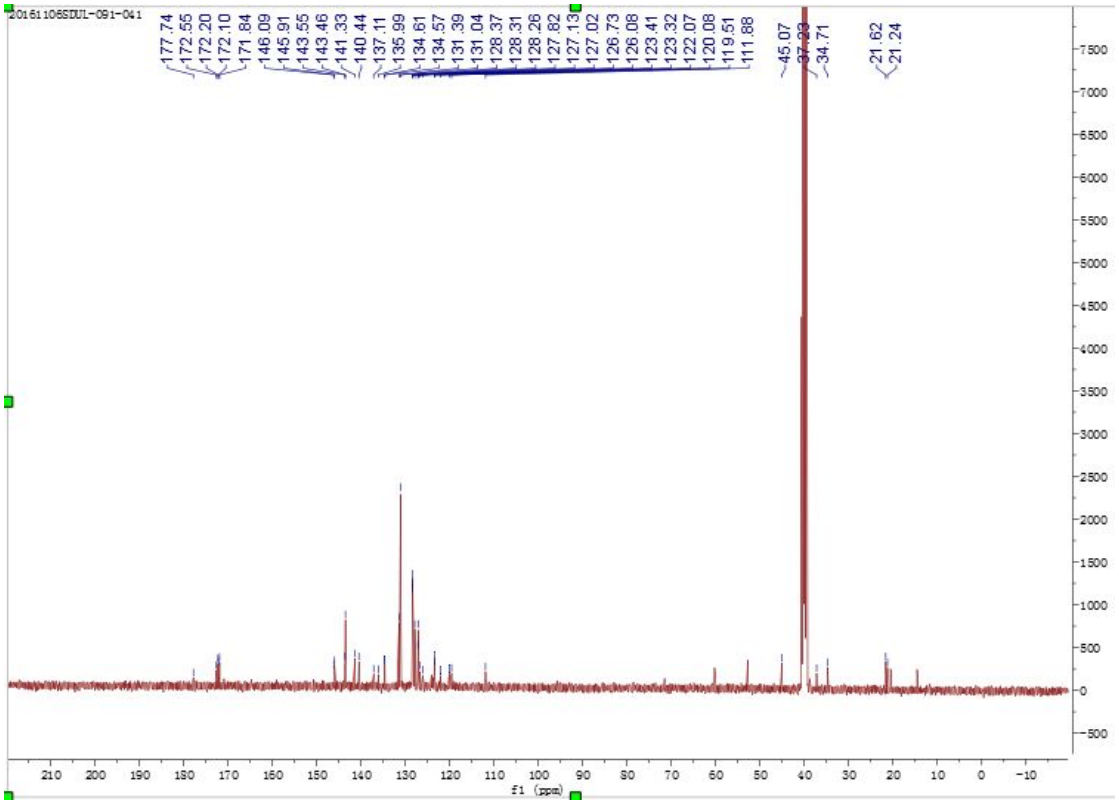
^{13}C-NMR spectra of probe **2**



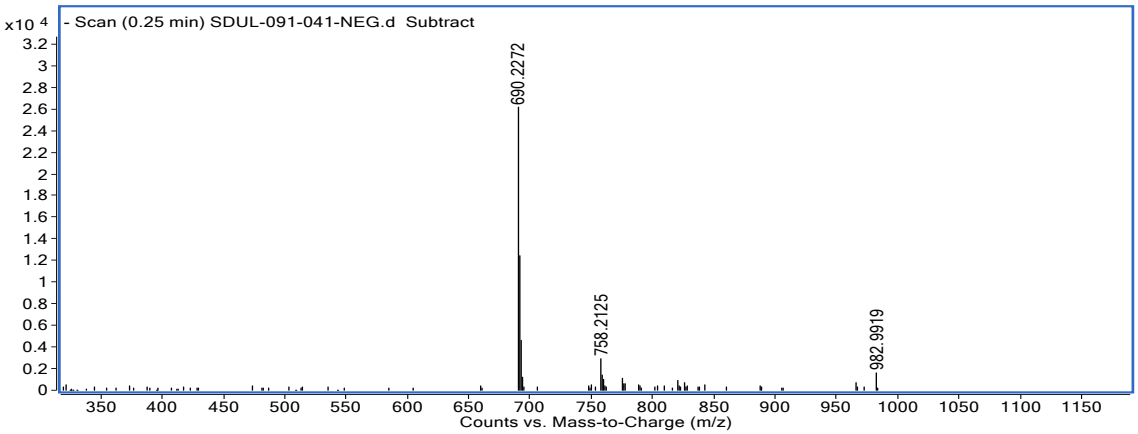
ESI-HRMS spectra of probe **2**



^{1}H-NMR spectra of probe **3**



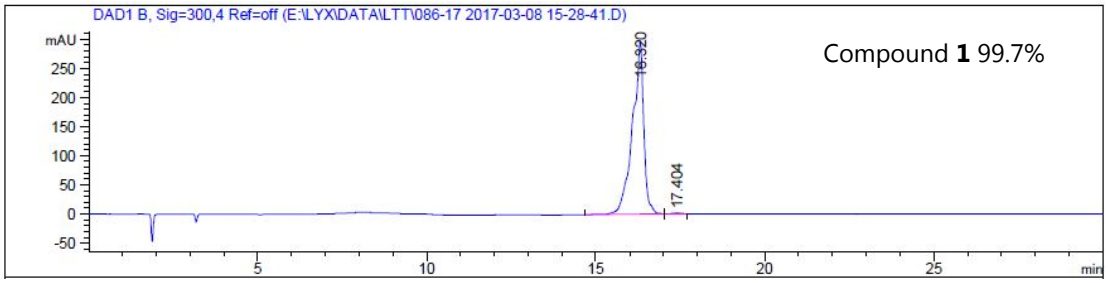
^{13}C-NMR spectra of probe **3**

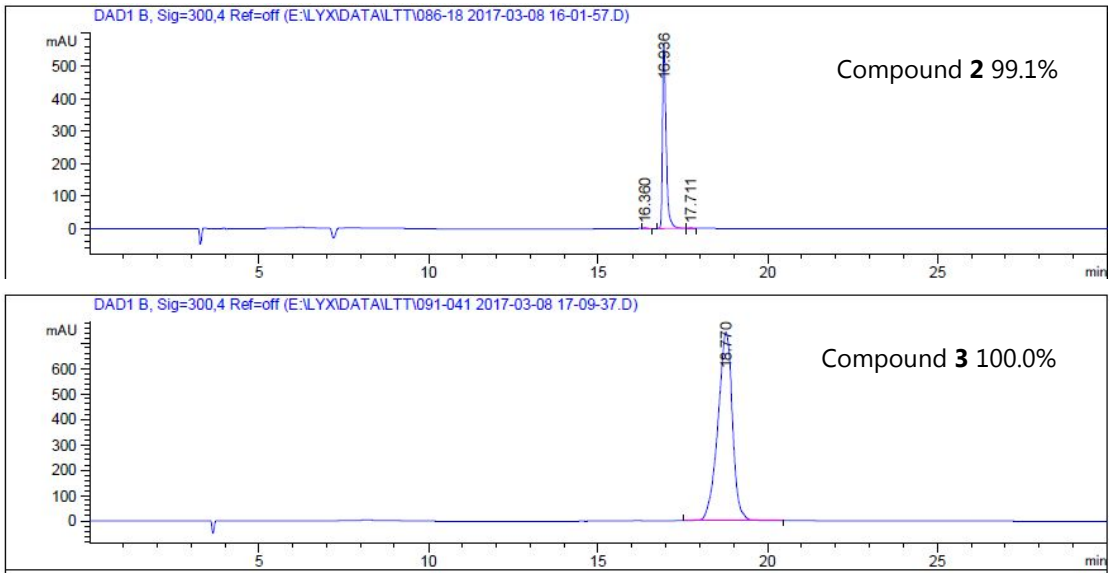


ESI-HRMS spectra of probe **3**

HPLC assessment of purity.

Probes with a purity of >99% was used for subsequent biological assays. We provided the spectra of HPLC assay as below.





5. References

(1)Zhou, B.; Li, X.; Li, Y.; Xu, Y.; Zhang, Z.; Zhou, M.; Zhang, X.; Liu, Z.; Zhou, J.; Cao, C. *Chemmedchem* **2011**, *6*, 904-921.

(2)Nikolovska-Coleska, Z.; Wang, R.; Fang, X.; Pan, H.; Tomita, Y.; Li, P.; Roller, P. P.; Krajewski, K.; Saito, N. G.; Stuckey, J. A. *Anal. Biochem.* **2004**, *332*, 261-273.