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SUPPORTING INFORMATION

Focusing on probe-modified peptides: a quick and effective way for target identification

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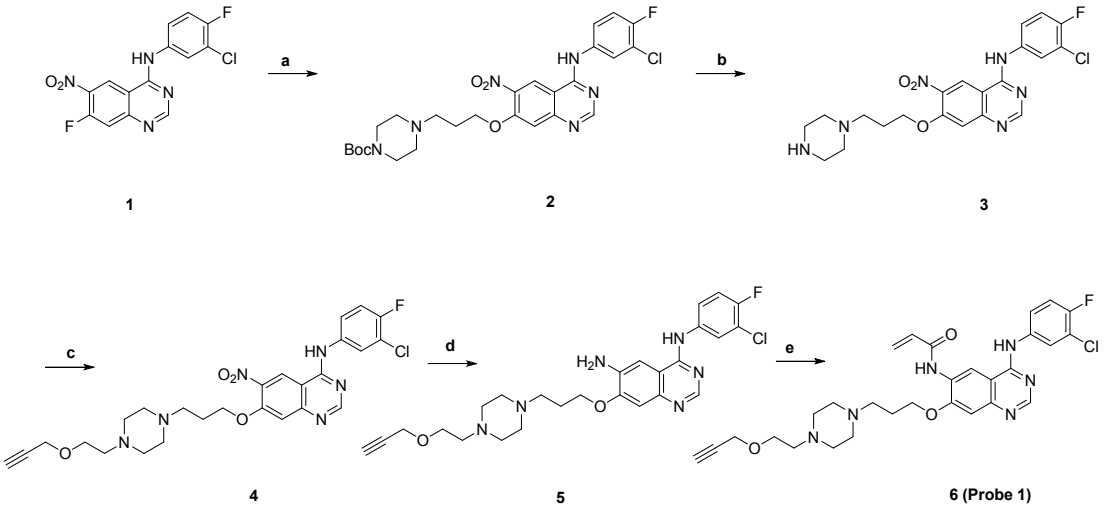
General materials and methods

All chemical reagents were used as supplied by Sigma-Aldrich (Pudong District, Shanghai, CN), J&K (Chaoyang District, Beijing, CN) and Alfa Aesar Chemicals (Dongcheng District, Beijing, CN). DCM, DMF, acetonitrile were distilled from calcium hydride; THF was distilled from sodium/benzophenone ketyl prior to use. Srepavidin-HRP(#3999), HER2 XP® Rabbit mAb(#4290s) were bought from Cell Signalling Technology(Pudong District, Shanghai, CN). Alexa Fluor® 555 conjugated Goat anti-Rabbit IgG (A-21428) was bought Life Technology. Anti β-Actin Mouse Monoclonal Anitibody (CW0096), HRP Conjugated Goat Anti-Mouse IgG (CW0102), HRP Conjugated Goat Anti-Rabbit IgG (CW0103) were bought from Cwbiotech (Changping District, Beijing, CN). CellTiter-Glo® Luminescent Cell Viability Assay (G7573) was bought from Promega Biotech Co (Dongcheng District, Beijing, CN). RPMI 1640 Medium(22400105), Fetal Bovine Serum (12483-020) and Penicillin-Streptomycin(15140-122) were bought from Life Technology (Dongcheng District, Beijing, CN). WESTERN LIGHTNING™ Plus-ECL (NEL103001EA) was bought from Perkin Elmer (American Fork, UT, US). High Capacity Strepavidin agrose (20361) was bought from Thermo Scientific (Dongcheng District, Beijing, CN). Cathepsin C (1071-CY-010) and Cathepsin L (952-CY-010) were bought from R&D (Akron, OH, US). Gly-Phe-AFC(SMAFC041) was bought from SM Biochemicals (Anaheim, CA, US). All the other biologic chemicals were purchased from Sigma-Aldrich (Pudong District, Shanghai, CN). The assay and cell culture plates were purchased from Corning (Pudong District, Shanghai, CN). HER2 kinase assay was detected by Reaction Biology Corporation (Malvern, PA, US).

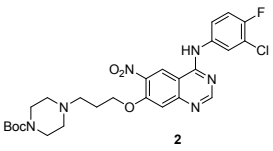
^{1}HNMR spectra were recorded on a Varian 400 MHz spectrometer (Varian, Inc., Palo Alto, CA, US) at ambient temperature with $CDCl_3$ as the solvent unless otherwise stated. ^{13}C NMR spectra were recorded on a Varian 100 MHz spectrometer (with complete proton decoupling) at ambient temperature. Chemical shifts are reported in parts per million relative to chloroform (1H, δ 7.26; 13C, δ77.00). Data for ^{1}H NMR are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), integration and coupling constants. All chemical reagents were used as supplied by Sigma-Aldrich (Pudong District, Shanghai, CN), J&K Chemical Ltd. (Pudong District, Shanghai, CN) and Alfa Aesar Chemicals (Fengxian District, Shanghai, CN). DCM, DMF, acetonitrile were distilled from calcium hydride; tetrahydrofuran was distilled from sodium/benzophenone ketyl prior to use. High-resolution mass spectra were obtained by Agilent Techmologies 6540 UHD Accurate-Mass Q-TOF LC/MS (Santa Clara, CA, US). The samples were analyzed by HPLC/MS on a Waters Auto Purification LC/MS system (3100 Mass Detector, 2545 Binary Gradient Module, 2767 Sample Manager, and 2998 Photodiode Array (PDA)Detector) which was purchased from Waters (Milford, MA, US). The system was equipped with a Waters C_{18} 5μm SunFire separation column(150*4.6 mm), equilibrated with HPLC grade water (solvent A) and HPLC grade acetonitrile (solvent B) with a flow rate of 0.3 mL/min. Quasielastic light scattering was detected in Institute of Biophysics of the Chinese Academy by DynaPro NanoStar (Serial No:271-DNP) of which was purchased from WYATT technology corporation (Santa Barbara, CA ,US).

Synthetic Procedures

Synthesis Scheme S1 for probe 1

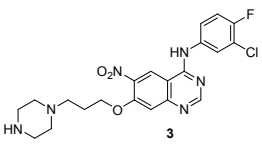


Scheme 1. Reagents and conditions: a. tert-butyl 4-(3-hydroxypropyl)piperazine-1-carboxylate, t-BuOK, THF, r.t, overnight, 64%; b. TFA, DCM, r.t, 2 h, 61%; c. Cs_2CO_3, 2-(prop-2-yn-1-yloxy)
ethyl 4-methylbenzenesulfonate, DMF/dioxane, 95 °C, overnight, 37%; d. Fe, NH_4Cl, EtOH, H_2O, 80 °C, 1.5 h, 96%; e. acryloyl chloride, Et_3N, THF, 0 °C, 1 h, 20%.



***tert*-butyl4-(3-((4-((3-chloro-4-fluorophenyl)amino)-6-nitroquinazolin-7-yl)oxy)propyl)piperazine-1-carboxylate(2):**

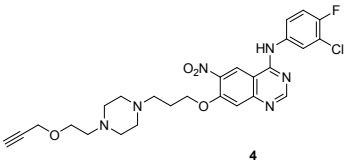
To a solution of compound **1** (2 g, 6 mmol), prepared from previously reported procedures[1], in anhydrous THF (30 mL), tert-butyl 4-(3-hydroxypropyl) piperazine-1-carboxylate (2.2 g, 9 mmol), *t*-BuOK (1.3 g, 12 mmol) were added. The reaction mixture was stirred at room temperature for overnight and then quenched with water (100 mL). The mixture was extracted with ethyl acetate (3×100 mL), the combined organic fractions were dried (Na_2SO_4) and concentrated in vacuo. The residue was purified by column chromatography on silica(DCM : MeOH =10:1) to give **2** (2.1 g, yield: 64 %) as a yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.39 (s, 9H), 1.91-1.96 (m, 2H), 2.27-2.34 (m, 6H), 3.42 (t, J = 4.8 Hz, 4H), 4.34 (t, J = 6.0 Hz, 2H), 7.45-7.50 (m, 2H), 7.78-7.82 (m, 1H), 8.16 (dd, J = 2.8 Hz, 6.8 Hz, 1H), 8.67 (s, 1H), 9.21 (s, 1H), 10.15 (s, 1H). HRMS *m/z* : calcd. for $C_{26}H_{30}ClFN_6O_5$ $[M+H]^+$:561.2023, found 561.2019.



***N*-(3-chloro-4-fluorophenyl)-6-nitro-7-(3-(piperazin-1-yl)propoxy)quinazolin-4-amine (3):**

To a solution of **2** (510 mg, 0.91 mmol) in DCM (3 mL), TFA (3 mL) was added, then the reaction mixture was stirred at room temperature for 2 h. The solvent was removed by evaporation, and the residue was diluted with water (20 mL) and slowly basified with 1 mol · L^{-1} sodium hydroxide adjust the reaction mixture pH >7, extracted with ethyl acetate (3×20 mL). The combined organic fractions were dried (Na_2SO_4) and concentrated in vacuo. The residue was

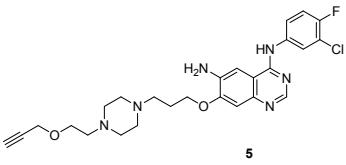
purified by column chromatography on silica(DCM : MeOH =10:1) to give compound **3** (311 mg, yield: 61%) as a a yellow solid.[1]H NMR (400 MHz,MeOD) δ: 2.06-2.12 (m, 2H), 2.69-2.75 (m, 6H), 3.24 (t, J = 4.8 Hz, 4H), 4.37 (t, J = 6.0 Hz, 2H), 7.28 (t, J = 8.8 Hz, 1H), 7.38 (s, 1H), 7.67-7.71 (m, 1H), 8.04-8.06 (m, 1H), 8.61 (s, 1H), 9.01 (s, 1H). ^{13}C NMR(400MHz, MeOD):δ26.8 (C), 44.7 (C), 50.8 (C), 55.2 (C), 68.7 (C), 108.9 (C), 109.9 (C), 117.0 (C), 117.2 (C), 120.9 (C), 121.1 (C), 122.4 (C), 123.1 (C), 124.9 (C), 136.6 (C), 140.4 (C), 153.4 (C), 154.4 (C), 155.7 (C), 158.0 (C), 159.1 (C). HRMS *m/z* : calcd. for $C_{21}H_{22}ClFN_6O_3$ $[M+H]^+$:461.1499, found 461.1509.



4

***N*-(3-chloro-4-fluorophenyl)-6-nitro-7-(3-(4-(2-(prop-2-yn-1-yloxy)ethyl)piperazin-1-yl)propoxy)quinazolin-4-amine(4):**

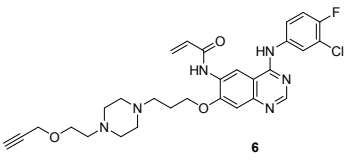
A suspension of 2-(prop-2-yn-1-yloxy)ethyl 4-methylbenzenesulfonate[2] (336 mg, 1.3 mmol), Cs_2CO_3 (1.2 g, 3.6 mmol) in DMF (1.5 mL) and dioxane (3 mL) was added compound **3** (550 mg, 1.2 mmol), then the reaction mixture was heated at 95 ^{o}C for overnight. Water (100 mL) was added to the crude product and the mixture was extracted with ethyl acetate (3×100 mL), the combined organic fractions were dried (Na_2SO_4) and concentrated in vacuo, the residue was purified by column chromatography on silica(DCM : MeOH =10:1) to give **4** (240 mg, yield:37%) as a yellow solid. ^{1}H NMR (400 MHz, DMSO- d_6) δ: 2.13-2.18 (m, 2H), 2.74-2.77 (m,12H), 3.56-3.57 (m, 1H), 3.77 (t, J =5.2 Hz, 2H), 4.24-4.25 (m, 2H), 4.31 (t, J =5.2 Hz, 2H), 7.18 (t, J =8.8 Hz, 1H), 7.35-7.36 (m,1H), 7.68-7.71 (m, 1H), 8.02 (dd, J = 1.6 Hz, 6.4 Hz, 1H), 8.74 (s, 1H), 8.98 (s, 1H), 9.17 (brs, 1H). HRMS *m/z* : calcd. for $C_{26}H_{28}ClFN_6O_4$ $[M+H]^+$:543.1917, found 543.1932.



5

N4-(3-chloro-4-fluorophenyl)-7-(3-(4-(2-(prop-2-yn-1-yloxy)ethyl)piperazin-1-yl)propoxy)quinazoline-4,6-diamine(5):

A suspension of **4** (240 mg,0.44 mmol) , iron powder (123 mg, 2.2 mmol) and NH_4Cl(28 mg, 0.53 mmol) in EtOH(6 mL)/H_2O(1 mL) was stirred at 80 ^{o}C for 1.5h. The precipitate (iron powder) was separated by filtration and the filtrate was concentrated under reduced pressure. Purification of the crude product by column chromatography (petroleum ether:EtOAc =1:1) to give **5** (219 mg, 96%) as a yellow solid. ^{1}H NMR (400 MHz, MeOD) δ: 2.23-2.25 (m, 2H), 2.93-2.94(m, 1H), 3.08-3.15 (m, 12H), 3.81(t, J =5.2 Hz, 2H), 4.22-4.23 (m, 2H), 4.26 (t, J =5.2 Hz, 2H), 7.05 (s, 1H), 7.23 (t, J =8.8 Hz, 1H), 7.48 (s, 1H), 7.68-7.70 (m, 1H), 7.93-7.94 (m, 1H), 8.47 (s, 1H). ^{13}C NMR(400MHz, MeOD):δ 25.9 (C), 51.9 (C), 52.3 (C), 54.9 (C), 57.3 (C), 59.1 (C), 68.0 (C), 76.6 (C), 80.2 (C), 100.7 (C), 102.4 (C), 110.1 (C), 117.2 (C), 117.4 (C), 121.1 (C), 121.3 (C), 125.1 (C), 126.7 (C), 135.2 (C), 135.7 (C), 142.0 (C), 147.4 (C), 155.0 (C), 155.4 (C), 157.8 (C), 158.0 (C). HRMS *m/z* : calcd. for $C_{26}H_{30}ClFN_6O_2$ $[M+H]^+$:513.2176, found 513.2174.



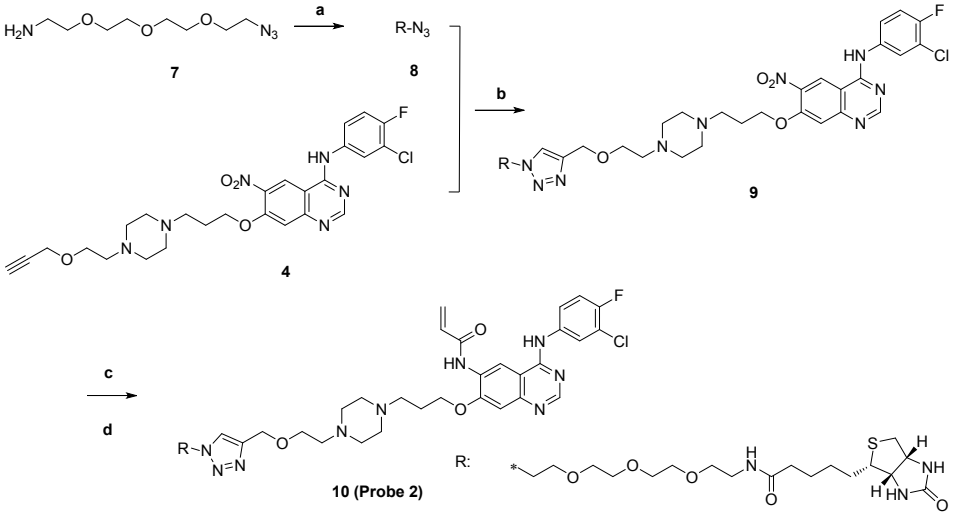
6

***N*-(4-((3-chloro-4-fluorophenyl)amino)-7-(3-(4-(2-(prop-2-yn-1-yloxy)ethyl)piperazin-1-yl)propoxy)quinazolin-6-yl)acrylamide(6):**

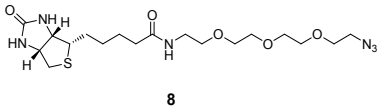
To a solution of **5** (40 mg, 0.042 mmol), Et_3N (12.7 mg, 0.13 mmol) in anhydrous THF (1 mL), acryloyl chloride (4.0 mg, 0.046 mmol) was added under ice-bath, the reaction mixture was stirred at 0 ^{o}C for 1h then quenched with saturated $NaHCO_3$ (20 mL). The mixture was extracted with ethyl acetate (3×20 mL), the combined organic fractions were dried (Na_2SO_4) and concentrated in vacuo. The crude product was purified using reversed-phase chromatography to give **6** as a yellow solid (8.3 mg, yield:20 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.13-2.20 (m, 2H), 2.43 (t, J =2.4 Hz, 1H), 2.66-2.73 (m, 12H), 3.68 (t, J =5.6 Hz, 2H), 4.17 (d, J =2.4 Hz, 2H), 4.22 (t, J =6.0 Hz, 2H), 5.83 (d, J =10.4 Hz, 1H), 6.46 (d, J =16.4 Hz, 1H), 6.56-6.63 (m, 1H), 7.09 (t, J =8.8 Hz, 1H), 7.20 (s, 1H), 7.26 (s, 1H), 7.45-7.49 (m, 1H), 7.78 (s, 1H), 7.84 (dd, J = 2.8 Hz, 6.8 Hz, 1H), 8.52 (s, 1H), 8.60 (s, 1H), 9.14 (s, 1H). ^{13}C NMR(400MHz, $CDCl_3$):δ 22.1 (C), 25.8 (C), 29.7 (C),

52.5 (C), 54.5 (C), 57.4 (C), 58.3 (C), 67.0 (C), 74.6 (C), 79.5 (C), 107.2 (C), 109.1 (C), 110.0 (C), 116.3 (C), 120.7 (C), 121.6 (C), 124.1 (C), 127.9 (C), 128.4 (C), 131.2 (C), 135.1 (C), 148.2 (C), 152.1 (C), 153.4 (C), 154.3 (C), 155.9 (C), 156.7 (C), 164.3 (C), 175.5 (C=O). HRMS *m/z* : calcd. for $C_{29}H_{32}ClFN_6O_3$ $[M+H]^+$:567.2281, found 567.2275.

Synthesis Scheme S2 for probe 2



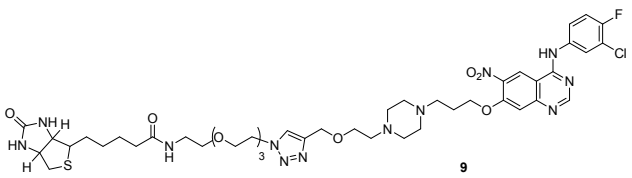
Scheme 2. Reagents and conditions: a. Biotin-NHS, Et_3N, DMF, 30 °C, overnight, 77% ; b. Et_3N, CuI, THF, 55 °C, under nitrogen, 2h, 71%; c. Fe, NH_4Cl, EtOH, H_2O, 80 °C, 1.5h; d. acryloyl chloride, Et_3N, THF, 0 °C, 1h, 20%.



8

_N_-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide(8):

A solution of 2,5-dioxopyrrolidin-1-yl-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno [3,4-d]imidazol-4-yl)pen-tanoate (2g, 5.9mmol), **7** (756mg, 8.8mmol), Et_3N (1.2g, 12mmol) in DMF (30ml) was stirred at 30 °C for overnight. The solvent was removed by evaporation and the crude product was dissolved in the minimum volume of methylene chloride/methanol (30ml,v/v=10:1). The mixture was heated at reflux for 30 min, after the solution was cooled to room temperature, the precipitate was collected by filtration, washed with methylene chloride (30ml) and dried under vacuum to give **8** (1.41g, 77%) as a white solid which was used in the next step without further purification. ^{1}H NMR(400MHz,DMSO-d_6): δ 1.23-1.65 (m, 6H), 2.06 (t, J =7.2 Hz, 2H), 2.57 (d, J =12.4 Hz, 1H), 2.82 (dd, J = 5.2 Hz, 12.4 Hz, 1H), 3.07-3.11 (m, 1H), 3.18 (dd, J = 5.6 Hz, 11.6 Hz, 2H), 3.37-3.41 (m, 4H), 3.48-3.57 (m, 8H), 3.60 (t, J =4.8 Hz, 2H), 4.11-4.14 (m, 1H), 4.28-4.32 (m, 1H), 6.37 (s, 1H), 6.44 (s, 1H), 7.81 (t, J =5.6 Hz, 1H). ^{13}C NMR(400MHz, DMSO-d_6): δ 25.7 (C), 28.4 (C), 28.6 (C), 35.5 (C), 38.8 (C), 40.3 (C), 50.4 (C), 55.8 (C), 59.6 (C), 61.5 (C), 69.6 (C), 69.7 (C), 69.9 (C), 70.1 (C), 70.2 (C), 70.3 (C), 163.2(C=O), 172.5 (C=O). HRMS *m/z* : calcd. for $C_{18}H_{32}N_6O_5S$ $[M+H]^+$: 445.2228, found: 445.2245.

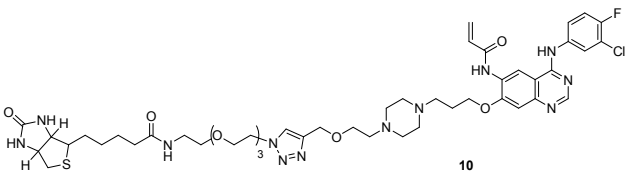


9

_N_-(2-(2-(2-(2-(4-((2-(4-(3-((4-((3-chloro-4-fluorophenyl)amino)-6-nitroquinazolin-7-yl)oxy)propyl)piperazin-1-yl)ethoxy)methyl)-1H-

1,2,3-triazol-1-yl)ethoxy)ethoxy)ethoxy)ethyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide(9):

To a solution of **4** (60 mg, 0.11 mmol) and **8** (59 mg, 0.13 mmol) in THF (2 ml), TEA (11 mg, 0.11 mmol) and CuI (2 mg, 0.011mmol) were added under nitrogen. The reaction mixture was stirred at 55 °C for 2h and then quenched with water (10 ml), extracted with DCM (3×10 ml). The combined organic fractions were dried (Na_2SO_4) and concentrated in vacuo. The residue was purified by column chromatography on silica (DCM : MeOH =20:1 to 10:1) to give **9** (77 mg, yield:71 %) as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.32-1.72 (m, 6H), 2.01-2.07 (m, 2H), 2.15-2.19 (m, 2H), 2.49-2.62 (m, 12H), 3.08-3.13 (m, 1H), 3.36-3.40 (m, 3H), 3.51 (t, J =5.2 Hz, 2H), 3.57-3.59 (m, 10H), 3.65 (t, J =5.2 Hz, 2H), 3.86 (t, J =5.2 Hz, 2H), 4.24 (t, J =5.2 Hz, 2H), 4.28-4.30 (m, 1H), 4.52 (t, J =4.8 Hz, 2H), 4.62 (s, 2H), 5.53 (s, 1H), 6.17 (s, 1H), 6.73 (t, J =5.2 Hz, 1H), 7.11 (t, J =8.8 Hz, 1H), 7.29 (s, 1H), 7.74 (s, 1H), 8.01 (dd, J = 2.4 Hz, 6.4 Hz, 1H), 8.65 (s, 1H), 9.19 (s, 1H). HRMS *m/z* : calcd. for $C_{44}H_{60}ClFN_{12}O_9S$ $[M+H]^+$:987.4072, found 987.4062.

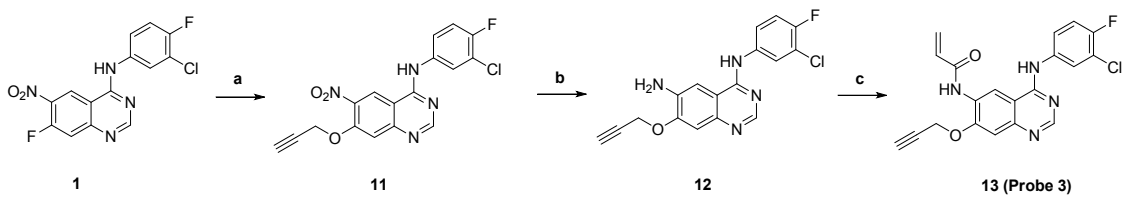


10

***N*-(2-(2-(2-(2-(4-((2-(4-(3-((6-acrylamido-4-((3-chloro-4-fluorophenyl)amino)quinazolin-7-yl)oxy)propyl)piperazin-1-yl)ethoxy)methyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethoxy)ethyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide(10):**

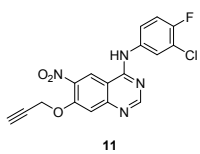
A suspension of compound **9** (68 mg, 0.069 mmol), iron powder (19 mg, 0.35 mmol) and NH_4Cl(18 mg, 0.35 mmol) in EtOH(4 mL)/H_2O(1 mL) was stirred at 80 °C for 1.5h. The precipitate (iron powder) was separated by filtration and the filtrate was concentrated under reduced pressure to give intermediate as a yellow solid.To a solution of intermediate (40 mg, 0.042 mmol) , Et_3N (12.7 mg, 0.13 mmol) in anhydrous THF (1 mL), acryloyl chloride (4.2 mg, 0.046 mmol) was added under ice-bath, the reaction mixture was stirred at 0 °C for 1h then quenched with saturated $NaHCO_3$ (2 mL). The mixture was extracted with ethyl acetate (3×20 mL), the combined organic fractions were dried (Na_2SO_4) and concentrated in vacuo. The crude product was purified using reversed-phase chromatography to give product **10** as a yellow solid (8.3 mg, yield:20%).^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.25-1.47 (m, 6H), 1.60-1.64 (m, 2H), 2.14-2.16 (m, 2H), 2.48-2.64 (m, 12H), 3.10-3.15 (m, 1H), 3.40-3.44 (m, 3H), 3.54 (t, J =5.2 Hz, 2H),3.58-3.61 (m, 10H) 3.67 (t, J =5.2 Hz, 2H), 3.88 (t, J =5.2 Hz, 2H), 4.29-4.32 (m, 2H) 4.47-4.50 (m, 1H), 4.54 (t, J =4.8 Hz, 2H), 4.65 (s, 2H), 5.05 (s,1H), 5.84-5.88 (m, 1H), 6.42-6.46 (m, 2H), 7.14 (t, J =8.8 Hz, 1H), 7.75 (s, 1H), 7.88 (s, 1H), 7.97 (dd, J = 2.4 Hz, 6.4 Hz, 1H), 8.63 (s, 1H), 9.11 (s, 1H). HRMS *m/z* : calcd. for $C_{47}H_{64}ClFN_{12}O_8S$ $[M+2H]^{2+}$:506.2255, found 506.2255.

Synthesis Scheme S3 for probe 3



1 11 12 13 (Probe 3)

Scheme 3. Reagents and conditions: a. Propargyl alcohol, *t*-BuOK, THF, r.t, 5h, 92%； b. Fe, NH_4Cl, EtOH, H_2O, 80 °C, 4h, 98%;c. Acrylyl chloride, Et_3N, DMF, 0 °C, 1h, 77%.

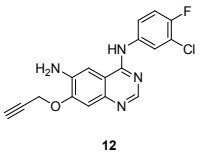


11

***N*-(3-chloro-4-fluorophenyl)-6-nitro-7-(prop-2-yn-1-yloxy)quinazolin-4-amine(11):**

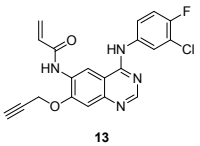
To a solution of **1** (700 mg,2.1 mmol) in THF (10 mL), propargyl alcohol (236 mg, 4.2 mmol) was added, the reaction mixture was stirred at r.t for 5 min, then potassium tert-

butanolate (236 mg, 2.1 mmol) was added under 0 °C. After stirring at room temperature for 5 h, water (100 mL) was added and the mixture was extracted with ethyl acetate (3×100 mL). The combined organic fractions were dried (Na_2SO_4) and concentrated in vacuo to give **11** (711mg, 92%) as a yellow solid which was used in the next step without further purification. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.77 (t, J =2.4 Hz, 1H), 5.18 (d, J =2.4 Hz, 2H), 7.43-7.48 (m, 1H), 7.54 (s, 1H), 7.74-7.78 (m, 1H), 8.13 (dd, J =2.8Hz, 6.8 Hz, 1H), 8.65 (s, 1H), 9.23 (s, 1H), 10.29 (bar, 1H). HRMS *m/z* : calcd. for $C_{17}H_{10}ClFN_4O_3$ $[M+H]^+$: 373.0498, found: 372. 0506.


12

***N*^{4}-(3-chloro-4-fluorophenyl)-7-(prop-2-yn-1-yloxy)quinazoline-4,6-diamine(12):**

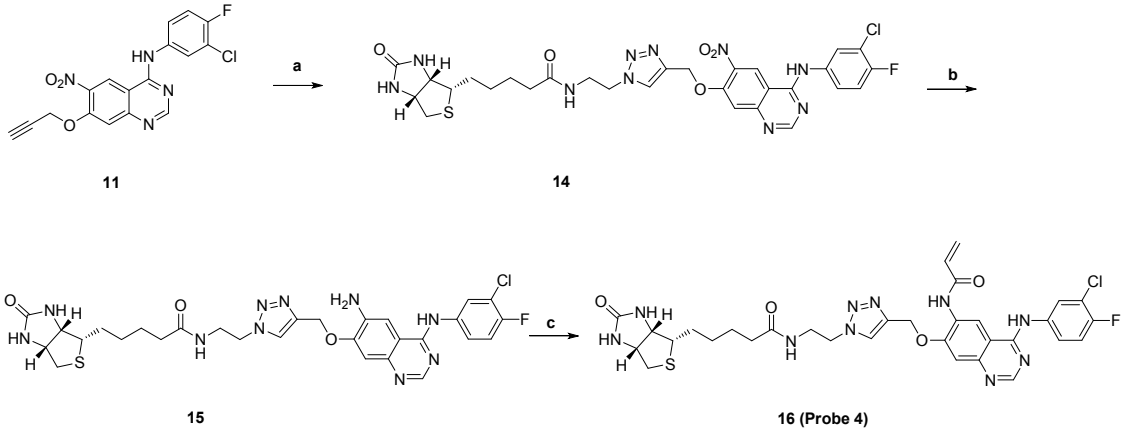
A suspension of **11** (500 mg, 1.3 mmol) , iron powder (376 mg, 6.5 mmol) and NH_4Cl(215 mg, 3.9 mmol) in EtOH(30 mL)/H_2O(1 mL) was stirred at 80 °C for 4 h. The precipitate (iron powder) was separated by filtration and the filtrate was concentrated under reduced pressure. Purification of the crude product by column chromatography (petroleum ether : EtOAc =1 : 1) to give **12** (450 mg, 98%) as a yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.69 (t, J =2.4 Hz, 1H), 5.03 (d, J =2.4 Hz, 2H), 5.38 (s, 2H), 7.21 (s, 1H), 7.37-7.41 (m, 2H), 7.78-7.82 (m, 1H), 8.18 (dd, J =2.4Hz, 6.8 Hz, 1H), 8.38 (s, 1H), 9.44 (s, 1H). HRMS *m/z* : calcd. for $C_{17}H_{12}ClFN_4O$ $[M+H]^+$: 343.0756, found: 343.0783.


13

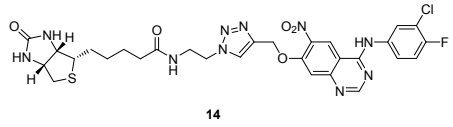
***N*-(4-((3-chloro-4-fluorophenyl)amino)-7-(prop-2-yn-1-yloxy)quinazolin-6-yl)acrylamide(13):**

To a solution of **12** (165 mg, 0.48 mmol) , Et_3N (97 mg, 0.96 mmol) in anhydrous DMF (2 mL), acrylyl chloride (43 mg, 0.48 mmol) was added under ice-bath, the reaction mixture was stirred at 0 °C for 1 h. water (10 mL) was added and the mixture was extracted with ethyl acetate (3×10 mL), the combined organic fractions were dried (Na_2SO_4) and concentrated in vacuo. Purification of the crude product by column chromatography (petroleum ether : EtOAc =1 : 1) to give **13** (136 mg, 77%) as a pale-yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.81 (t, J =2.4 Hz, 1H), 5.17 (d, J =2.4 Hz, 2H), 5.85 (dd, J =2.0Hz, 10.0 Hz, 1H), 6.34 (dd, J =2.0Hz, 16.8 Hz, 1H), 6.77 (dd, J =10.0Hz, 16.8 Hz, 1H), 7.45 (s, 1H), 7.51 (t, J =9.2 Hz, 1H), 7.69-7.73 (m, 1H), 8.03 (dd, J =2.4Hz, 6.8 Hz, 1H), 8.76 (s, 1H), 9.09 (s, 1H), 9.96 (s, 1H). ^{13}C NMR(MHz,DMSO-d_6): δ56.9 (C),78.8 (C), 79.9 (C), 108.7 (C), 109.7 (C), 117.0 (C), 117.4 (C), 119.1 (C), 119.3 (C), 122.9 (C), 124.1 (C), 127.5 (C), 132.1 (C), 137.2 (C), 149.1 (C), 152.4 (C), 153.7 (C), 154.5 (C), 157.3 (C), 164.1 (C=O). HRMS *m/z* : calcd. for $C_{20}H_{14}ClFN_4O_2$ $[M+H]^+$: 397.0862, found:397.0857.

Synthesis Scheme S4 for probe 4



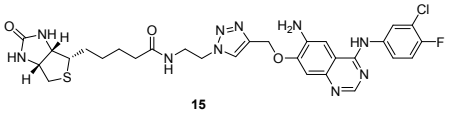
Scheme 4. Reagents and conditions: a. CuI, Et_3N, THF, DMF, 40 °C, overnight, under nitrogen, 98%; b. Fe, NH_4Cl, EtOH, H_2O, 80 °C, overnight, 98%; c. acetyl chloride, Et_3N, DMF, 0 °C to r.t, 2h, 75%.



14

***N*-(2-(4-(((4-((3-chloro-4-fluorophenyl)amino)-6-nitroquinazolin-7-yl)oxy)methyl)-1H-1,2,3-triazol-1-yl)ethyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide(14):**

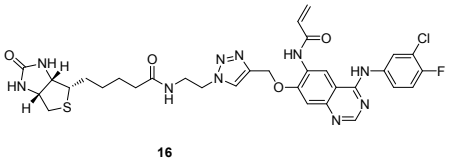
A stirred solution of **11** (500 mg, 1.3 mmol), N-(2-azidoethyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide[2] (625 mg, 2 mmol) and Et_3N (271 mg, 2.7 mmol) in THF (20 mL)/DMF (5 mL) was treated with CuI (26 mg, 0.13 mmol) under nitrogen at room temperature. The solution was stirred at 40 °C for overnight and then quenched with water (100 mL). The mixture was extracted with ethyl acetate (3×10 mL), the combined organic fractions were dried (Na_2SO_4) and concentrated in vacuo to give **14** (900 mg, 98%) as a yellow solid which was used in the next step without further purification. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.15-1.63 (m, 6H), 2.01 (t, J = 7.2 Hz, 2H), 2.55 (d, J = 12.4 Hz, 1H), 2.80 (dd, J=5.2Hz, 12.4Hz, 1H), 3.05-3.11 (m, 1H), 4.11 (dd, J=4.8Hz, 7.6Hz, 1H), 4.28 (dd, J=4.4Hz, 7.6Hz, 1H), 4.45 (t, J = 6.0 Hz, 2H), 5.5 (s, 2H), 6.34 (s, 1H), 6.40 (s, 1H), 7.48 (t, J = 9.2 Hz, 1H), 7.79 (s, 1H), 7.98 (t, J =5.6 Hz, 1H), 8.16 (dd, J = 2.8 Hz, 6.8 Hz, 1H), 8.24 (s, 1H), 8.72 (s, 1H), 9.23 (s, 1H), 10.25(s, 1H). ^{13}C NMR(400MHz,DMSO-d_6): δ25.5 (C), 28.4 (C), 28.5 (C), 35.5(C), 49.3 (C), 55.8 (C), 59.6 (C), 61.4 (C), 63.4 (C), 111.5 (C), 117.2 (C), 117.3 (C), 119.5 (C), 122.2 (C), 123.1 (C), 124.4 (C), 125.7 (C), 125.8 (C), 136.2 (C), 136.3 (C), 139.4 (C), 139.6 (C), 141.5 (C), 141.6 (C), 147.2 (C), 153.6 (C),158.3 (C), 163.2 (C=O), 173.0 (C=O). HRMS *m/z* : calcd. for $C_{29}H_{30}ClFN_{10}O_5S$ $[M+H]^+$: 685.1867, found: 685.1867.



15

***N*-(2-(4-(((6-amino-4-((3-chloro-4-fluorophenyl)amino)quinazolin-7-yl)oxy)methyl)-1H-1,2,3-triazol-1-yl)ethyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide(15):**

A suspension of **14** (900 mg, 1.3 mmol) , iron powder (368 mg, 6.5 mmol) and NH_4Cl(209 mg, 3.9 mmol) in EtOH (30 mL)/H_2O (1 mL) was stirred at 80 °C for overnight. The precipitate (iron powder) was separated by filtration and the filtrate was concentrated under reduced pressure. Purification of the crude product by column chromatography (petroleum ether : EtOAc =1 : 1) to give **15** (843 mg, 98%) as a yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.23-1.62 (m, 6H), 2.02 (t, J = 7.2 Hz, 2H), 2.55 (d, J = 12.4 Hz, 1H), 2.78 (dd, J=5.2Hz, 12.4Hz, 1H), 3.04-3.09 (m, 1H), 3.34-3.37 (m, 4H), 3.49-3.53 (m, 2H), 4.08-4.12 (m, 1H), 4.26 (dd, J = 4.8 Hz, 7.2 Hz, 1H), 4.45 (t, J = 5.6 Hz, 2H), 5.36 (s, 2H), 6.36 (s, 1H), 6.41 (s, 1H), 7.39 (t, J = 9.2 Hz, 1H), 7.43 (s, 1H), 7.79-7.83 (m, 1H), 7.98 (t, J =5.6 Hz, 1H), 8.20 (dd, J = 2.4 Hz, 6.8 Hz, 1H), 8.33 (s, 1H), 9.42 (s, 1H). ^{13}C NMR(400MHz,DMSO-d_6): δ25.6 (C), 28.5 (C), 28.6 (C), 35.5(C), 49.3 (C), 55.8 (C), 59.6 (C), 61.4 (C), 62.5 (C), 101.6 (C), 110.8 (C), 116.7 (C), 116.9 (C), 118.9 (C), 119.1 (C), 121.8 (C), 121.9 (C), 122.9 (C), 125.3(C), 137.9(C), 138.0 (C), 138.9 (C), 142.0 (C), 151.7 (C), 151.9 (C), 154.3 (C), 155.4 (C), 163.1 (C=O), 172.9 (C=O). HRMS *m/z* : calcd. for $C_{29}H_{32}ClFN_{10}O_3S$ $[M+H]^+$: 655.2125, found: 655.2117.

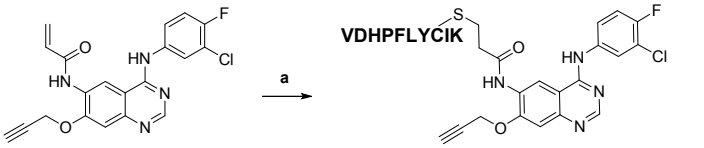


16

***N*-(2-(4-(((6-acrylamido-4-((3-chloro-4-fluorophenyl)amino)quinazolin-7-yl)oxy)methyl)-1H-1,2,3-triazol-1-yl)ethyl)-5-((3aS,4S,6aR)-3a,6a-dimethyl-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (16):**

To a solution of **15** (500 mg, 0.76 mmol) , Et_3N (154 mg, 1.5 mmol) in anhydrous DMF (5 mL), acetyl chloride (69 mg, 0.76 mmol) was added under ice-bath, the reaction mixture was stirred at 0 °C for 2h. Water (10 mL) was added and the mixture was extracted with ethyl acetate (3×10 mL), the combined organic fractions were dried (Na_2SO_4) and concentrated in vacuo. Purification of the crude product by column chromatography (petroleum ether: EtOAc =1:1) to give **16** (406 mg, 75%) as a pale-yellow solid. ^1H NMR(400MHz,DMSO-d_6): δ 1.16-1.57 (m, 6H), 2.00 (t, J = 7.2 Hz, 2H), 2.20 (t, J = 7.2 Hz, 1H), 2.77 (t, J = 4.8 Hz, 1H), 2.80 (d, J = 5.2 Hz, 1H), 3.03-3.12 (m, 2H), 4.09-4.14 (m, 2H), 4.27-4.32 (m, 2H), 4.45 (t, J = 6.0 Hz, 2H), 5.50 (s, 1H), 5.83 (dd, J=1.6Hz, 10.0Hz, 1H), 6.33 (dd, J = 2.0 Hz, 16.8Hz, 1H), 6.36-6.41 (m, 2H), 6.75 (dd, J = 10.0 Hz, 16.8 Hz, 1H), 7.50 (t, J = 8.8 Hz, 1H), 7.63-7.65 (m, 1H), 7.71-7.14 (m, 1H), 7.96 (t, J = 4.4 Hz, 1H), 8.03-8.04 (m, 1H), 8.31 (s, 1H), 8.77 (s, 1H), 9.09 (s, 1H), 9.82 (s, 1H). ^{13}C NMR(400MHz,DMSO-d_6): δ 25.0 (C), 25.5 (C), 25.7 (C), 28.4(C), 28.5 (C), 33.9 (C), 35.5 (C), 49.4 (C), 55.8 (C), 59.6 (C), 61.5 (C), 117.0 (C), 117.2 (C), 117.3 (C), 119.3 (C), 119.5 (C), 119.8 (C), 125.8(C), 128.2(C), 131.9(C), 141.8(C), 163.1(C), 163.2(C), 164.1, 173.0 (C=O), 173.2 (C=O), 174.9 (C=O). HRMS *m/z* : calcd. for $C_{34}H_{38}ClFN_{10}O_4S$ $[M+H]^+$: 709.2231, found: 709.2225.

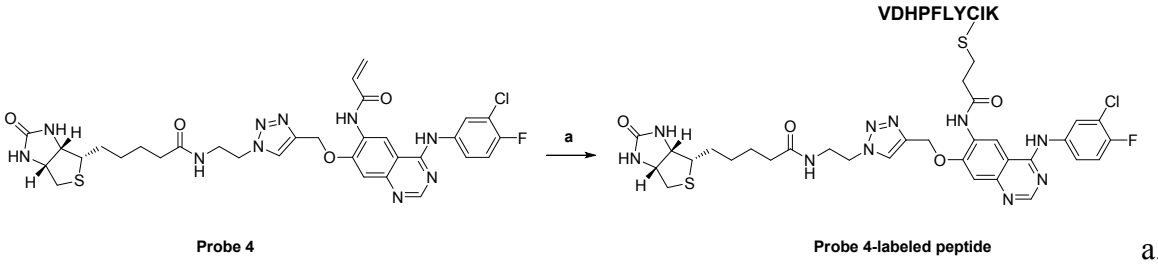
Synthesis of probe-labeled peptides



a. peptide, DMF, 40 °C, overnight, under nitrogen, 31.6 %.

Probe 3-labeled peptide:

A solution of peptide (10mg, 0.008mmol) and probe 3 (9.6mg, 0.024mmol) in DMF (1ml) under nitrogen was stirred at 40 °C for overnight. The solvent was removed by evaporation, and the crude product was purified using reversed-phase chromatography to give **probe 3-labeled peptide** as a pale-yellow solid (6.2 mg, yield: 31.6 %). HRMS *m/z* : calcd. for $C_{79}H_{101}ClFN_{17}O_{16}S$ $[M+2H]^{2+}$: 815.8576, found: 815.8565.



a. Et_3N, DMF, H_2O, 40 °C, overnight, 23.5%.

Probe 4-labeled peptide:

A solution of probe 4 (17mg, 0.024mmol) in Et_3N/DMF/H_2O (2.5ml, v/v/v=1:1:3) was added to the solution of peptide (10mg, 0.008mmol) in DMF (1ml) under nitrogen. The reaction mixture was stirred at 40 °C for overnight. The solvent was removed by evaporation, and the crude product was purified using reversed-phase chromatography to give **probe 4-labeled peptide** as a pale-yellow solid (3.7mg, yield: 23.5 %).

HRMS *m/z* : calcd. for $C_{91}H_{121}ClFN_{23}O_{18}S_2$ $[M+2H]^{2+}$: 971.9260, found 971.9252.

Experimental Protocols

Cell culture and western blotting

A human breast cancer cell line, Sk-Br3 cells were purchased from America type culture collection (ATCC, Chaoyang District, Beijing, CN). Sk-Br3 cells were grown at 37°C under a humidified 5% CO2 atmosphere, in a culture medium consisting of RPMI1640 supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were washed three times with phosphate-buffered saline (PBS) and harvested by trypsinization. The cell pellets were then lysed with RIPA lysis buffer (20 mM Tris pH7.5, 150 mM NaCl, 1 mM Na_2EDTA, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1% Triton X-100, 1 mM beta-glycerophosphate, 1 mM EGTA, 1 mM PMSF, and a phosphatase inhibitor cocktail) for 30 min. Cell lysates were centrifuged at 15,000 × g for 20 min at 4°C. The protein concentration was determined with a Bradford protein assay. Sample lysates were resolved in 4%-12% gradient SDS-polyacrylamide gels and transferred to nitrocellulose membranes for incubation with corresponding antibodies.

Cell Survival Assay

Sk-Br3 cells were seeded in density of 2000 cells/well, in 384-well cell culture plates for 24 h. Duplicate wells were exposed to 3-fold serial dilution concentration of probes for 72 hours as previously described[4]. Cell survival analysis was performed using a Cell Titer-Glo Luminescent Cell Viability Assay kit following manufacturer's instructions with minor modifications. In brief, 20 μL of Cell Titer-glo reagent was added to 30 μL of cell culture medium. Cell culture plates were place on a shaker for 10 min and then incubated at room temperature for an additional 10 min. Luminescence readings were taken with an EnSpire multimode plate reader (PerkinElmer, American Fork, UT, US). The data was analyzed using the Graphpad Prism program (GraphPad Software; www.graphpad.com). The curves were fitted using a non-linear regression model with a sigmoidal dose response.

Negative stain electron microscopy

Cell lysates were prepared in RIPA lysis buffer. For treatment, 200 μL lysates were precipitated by 1 mL MeOH and dissolved in 100 μL of 1% SDS with ultrasonic agitation. 2 μL of cell lysates samples, with or without treatment, were applied to glow discharged carbon coated 400-mesh copper grids(Beijing

Zhongjingkeyi Technology Co., Ltd., Haidian District, Beijing, CN), washed five times with Milli-Q water and negative stained with 2% uranyl acetate in water. Specimens were examined using an FEI Tecnai G2 Spirit TWIN electron microscope (FEI, Brno, Czech Republic) operating at 120 kV, and projection images were recorded on a Gatan US4000 UHS CCD camera (Gatan Corporation, Pleasanton, CA, US) at either 3,200x or 30,000x magnification.

Intact protein mass spectrometry analysis

The recombinant bacmid DNA containing DNA segment encoding residues 703-1027 of the human HER2 was kindly provided by Dr. Yun Caihong (Peking University). The HER2 KD (kinase domain) protein was expressed using a baculovirus/insect cell system, and purified using an Ni-NTA affinity column.For identification the covalent bond formation of Canertinib and HER2, 5uM HER2 KD was incubated with 100uM Canertinib in kinase assay buffer (10 mM $MgCl_2$, 1 mM EGTA, 2.5 mM DTT, and 0.5 mM Na_3VO_4 in Hepes buffer, pH 7.6) for 4 hours at room temperature. The protein-Canertinib mixture was then loaded onto a desalt trap column and desalted with 5% MeCN and 0.1% TFA in water, at a flow rate of 0.5 mL/min. Intact protein analyses were performed on a Xevo G2 QTOF system (Waters, Milford, MA, US) equipped with a standard electrospray source as previously described[5].

Concentration-dependence of probe 4 modification on HER2

2-fold serial dilution concentration of probe **4** (from 100 μM to 0.8 μM) was added to 5uM HER2 KD (10 mM $MgCl_2$, 1 mM EGTA, 2.5 mM DTT, and 0.5 mM Na_3VO_4 in Hepes buffer, pH 7.6). The above mixture was incubated for 4 hours at room temperature and precipitated by prichilled MeOH. The supernatant was removed and MeOH was added to wash one time. Precipitated proteins were solubilized in 1x SDS loading buffer for 10 min at 95 °C. The resulting proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Nitrocellulose membranes were blocked with PBS buffer containing 5% nonfat powdered milk and 1% triton for 1 h, incubated with anti-HER2 antibody(1:2000 dilution) for 1 hr, washed with 1x PBST buffer 5 min 3 times, then incubated with secondary antibody(anti-rabbit, 1:4000 dilution) for 30min, washed with 1x PBST buffer 5min 3 times and developed using WESTERN LIGHTNING™ *Plus*-ECL.

Mass spectrometry and data analysis of probe-labeled peptides

A peptide (sequence VDHPFLYCIK) was synthesized by GL Biochem Ltd (Minhang District, Shanghai, CN). 100 μM of probe **2** (or probe **4**) and 10 μM of peptides(sequence VDHPFLYCIK) were mixed in phosphate-buffered saline (PBS) at 37 °C, with vigorous shaking, for 2h. The probe-labeled peptides were separated by an analytical capillary column (50 μm × 15 cm) packed with 5 μm spherical C18 reversed phase material (YMC, Shimogyo-ku,Kyoto, JP). A Waters nanoAcquity UPLC system (Waters, Milford, MA, US) was used to generate the following HPLC gradient: 0-30% B in 40 min, 30-70% B in 15 min (A = 0.1% formic acid in water, B = 0.1% formic acid in acetonitrile). The eluted peptides were sprayed into an LTQ Orbitrap Velos mass spectrometer (ThermoFisher Scientific, Waltham, MA, US) equipped with a nano-ESI ion source. The mass spectrometer was operated in data-dependent mode with one MS scan followed by four CID (Collision Induced Dissociation) and four HCD (High-energy Collisional Dissociation) MS/MS scans for each cycle. Database searches were performed on an in-house Mascot server (Matrix Science Ltd., Westminster ,London, UK) against the IPI (International Protein Index) human protein database. The search parameters were: 7 ppm mass tolerance for precursor ions; 0.5 Da mass tolerance for product ions. Three missed cleavage sites were allowed for trypsin digestion, and the following variable

modifications were included: oxidation (+15.9949 m/z) on methionine, carbamidomethylation (+57.0215 m/z) on cysteine, probe **2** (+1010.4363 m/z) on cysteine, and probe **4** (+708.2158 m/z) on cysteine. The tandem mass spectra of the matched modified peptides were manually checked for their validity.

Characterization of the site of probe 4 modification on HER2

Probe **4** was used to label purified HER2 to evaluate the covalent labeling capacity of acrylamide. Reaction conditions for probe **4** ligation to HER2 KD were as follows: 5 μM HER2, 100 μM probe, 10 mM $MgCl_2$, 1 mM EGTA, 2.5 mM DTT, and 0.5 mM Na_3VO_4 in Hepes buffer (pH 7.6), reaction at room temperature for 4h. The resulting products were precipitated with pre-chilled MeOH. The supernatant was discarded, and the pellets were then dissolved in 8 M urea and reduced in 5 mM DTT at 60 °C for 30 min, followed by alkylation in 15 mM iodoacetamide in darkness for 1 h. The trypsin digestion was performed in 50 mM NH_4HCO_3 buffer, with an enzyme:substrate ratio of 1:50. The chymotrypsin digestion was performed in 100mM Tris-HCl (pH 8.0), 10mM $CaCl_2$, with an enzyme:substrate ratio of 1:10. For pepsin digestion, samples were digested in 10 mM HCl and a 1/5 pepsin (enzyme:substrate: 1:5) preparation was added. All samples were incubated at 37°C for 2 h, and the resulting peptides were analyzed via mass spectrometry.

Click chemistry of peptides in organic solvent

The probe **3** or probe **4** labeled peptides (sequence VDHPFLYCIK) were synthesized and purified via HPLC (see Supplementary Methods). 10 μM of probe **3**-labeled peptides were clicked to biotin tags under the following conditions: 1 mM Biotin-C_2-N_3, 0.4 mM TBTA, 4 mM CuI, 4 mM VcNa, and 2 mM DIPEA, with or without 3 mg/ml of cell lysate peptides, at 40 °C, for 12 h. The products were analyzed with a Finnigan TSQ Quantum DISCOVERY MAX mass spectrometer (ThermoFisher Scientific, Waltham, MA, US) following separation using a Kinetex-C18 110A column (3 mm * 30 mm ID., 2.6 um, Phenomenex (Torrance, CA US). The products measured by the TSQ instrument were calculated as absolute concentrations based on standard concentration curves prepared from analysis of a serial dilution of probe **-** labeled peptides. The solvent system used for the separation was composed of 0.1% FA in ACN and 0.1% FA in water. The amount of ACN in the gradient was increased in a stepwise fashion from 10% ACN to 100% ACN over 1.2 min, held for 1.4 min, and then re-equilibrated to 10% ACN over 1.4 min. Data acquisition used multiple reaction monitoring. Positively charged ions representing the [M+H]+/2 for the product and [M+H]+ for the internal standard were selected in MS1 and dissociated with argon (CID) to form specific product ions that were subsequently monitored in the MS2 analysis. The ion transition for the product-probe **4-**labeled peptides was 648.178→305.072, and the transition for the internal standard was 386.230 →122.107. The dynamic range of the assay ranged from 0.1 to 50 μM, based on a linear regression model.

Affinity capture of probe-labeled peptides via BTC-ABPP

Stock solutions of probe **3** were prepared to suitable concentrations by dilution with DMSO, and then diluted to final working concentrations in the culture medium. Cells were grown until 80-90% confluency and were then removed from the culture medium. The probe-containing medium was added, and cells were incubated at 37 °C for 4 h. Cells were then washed three times with PBS and collected following trypsinization. Cells were lysed in RIPA lysis buffer and the supernatant, after centrifugation, was

precipitated with prechilled MeOH. Precipitated proteins were then digested by pepsin or trypsin. The resulting peptides were lyophilized and were dissolved in 5 ml DMF to link them to biotin tags (1 mM Biotin-C_2-N_3, 0.4 mM TBTA, 4 mM CuI, 4 mM VcNa, and 2 mM DIPEA at 40 °C for 12 h). The DMF reaction buffer was diluted with ethanol/H2O (1:1) to 50 ml. Excessive biotin tags were removed by dialysis with 1000 Da interception dialysis bags in ethanol/H2O (1:1). When the concentration of biotin was below 10 nM according to UPLC analysis, the remaining solution in the dialysis bags was collected and dried by vacuum evaporation. The resulting powder was dissolved in 1 ml of DMSO and diluted to 10 ml with PBS. The samples were incubated with 200 μL of streptavidin T1 magnetic beads at room temperature for 2 h. The beads were washed four times with 10% DMSO PBS, washed three times with PBS, and were then boiled in 100 μL 10 mM EDTA pH 8.2 and 95% formamide at 90 °C for 10 min. The eluted peptides were then diluted with H2O to a final volume of 1 ml and dried to 50 μL by vacuum evaporation. These samples were then analyzed via mass spectrometry.

Pull-Down for target identification in proteome profiling

3 μL of the probe **3** (stock solution 10 mM in DMSO),Canertinib (stock solution 10 mM in DMSO) or DMSO was added to 10 ml medium of SK-Br3 cells; the final concentration of each compound was 3 μM. Canertinib, as competitor for probe **3** was firstly added to cells and incubated for 30 min then 3 μL of the probe **3** was added. The samples were then incubated for 4h at 37 °C. After cells collection and lysis, cell lysates were denatured in 1% SDS for 1 h. Then the samples were reacted with biotin tags (Biotin-C_2-N_3) in click buffer (1 mM Biotin-C_2-N_3, 1 mM TBTA, 10 mM $CuSO_4$, and 10 mM VcNa) at room temperature for 2 h. The samples were precipitated with MeOH and washed twice with MeOH. Following resolubilization and ultrasonic agitation in 200 μL of 2% SDS and dilution to 4 ml with PBS buffer, the samples were incubated overnight at 4°C with streptavidin agarose. The beads were then washed three times with 300 mM NaCl in cell lysis buffer, washed three times with 150 mM NaCl cell lysis buffer, and washed twice with PBS buffer. The beads were then boiled in 50 μL of 1x SDS loading buffer for 10 min. Samples were separated by SDS-PAGE and analyzed by western blotting or silver staining. The different lanes were cut and analyzed by LC-MS/MS.

CTSC enzymatic assay

The assay was carried out in a 96-well clear plate. 30 μL of 1.0 × 10^6/mL THP-1 cells in medium were added to all wells.[3] Then 10 μL of 5% (v/v) DMSO positive controls or 10 μL of 3-fold serial diluted compound were dispensed into the appropriate well, The plate was preincubated at 37°C for 4 h in a cell culture incubator, and then 10 μL of H-Gly-Phe-AFC (100 μM) was added to start the assay reaction. The plate was further incubated for 60 min at 37°C, and the product of the reaction was read in a fluorescence plate reader using Ex λ400 nm and Em λ505 nm for AFC. A standard irreversible and selective CTSC inhibitor **10** by AstraZeneca was used as an inhibitor control in the assay.[4]

Supporting Figures

No.	Enzyme(IC_{50} nM)	Cell (IC_{50} nM)	
	HER2	Ba/F3-HER2	SK-Br3
Canertinib	0.13	3	65
probe 1	0.38	1	33
probe 2	1.64	197	433
probe 3	1.50	21	349
probe 4	1.99	3010	2582

Figure S1. IC_{50} values measured in HER2 kinase assays and cell assays.

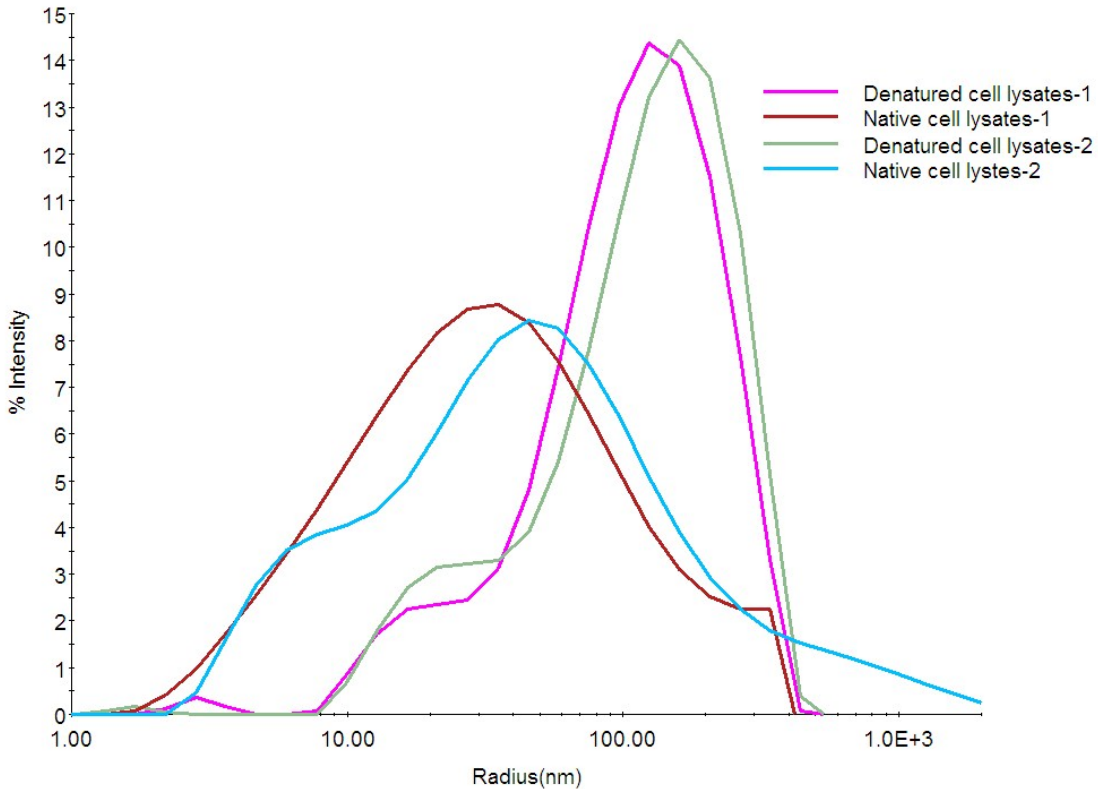


Figure S2. Analysis of proteins sizes by quasielastic light scattering. Samples of native cell lysates were dissolved in RIPA lysis buffer, and samples of denatured cell lysates were dissolved in 1% SDS PBS. Samples were detected by DynaPro NanoStar and analyzed by Dynamics software.

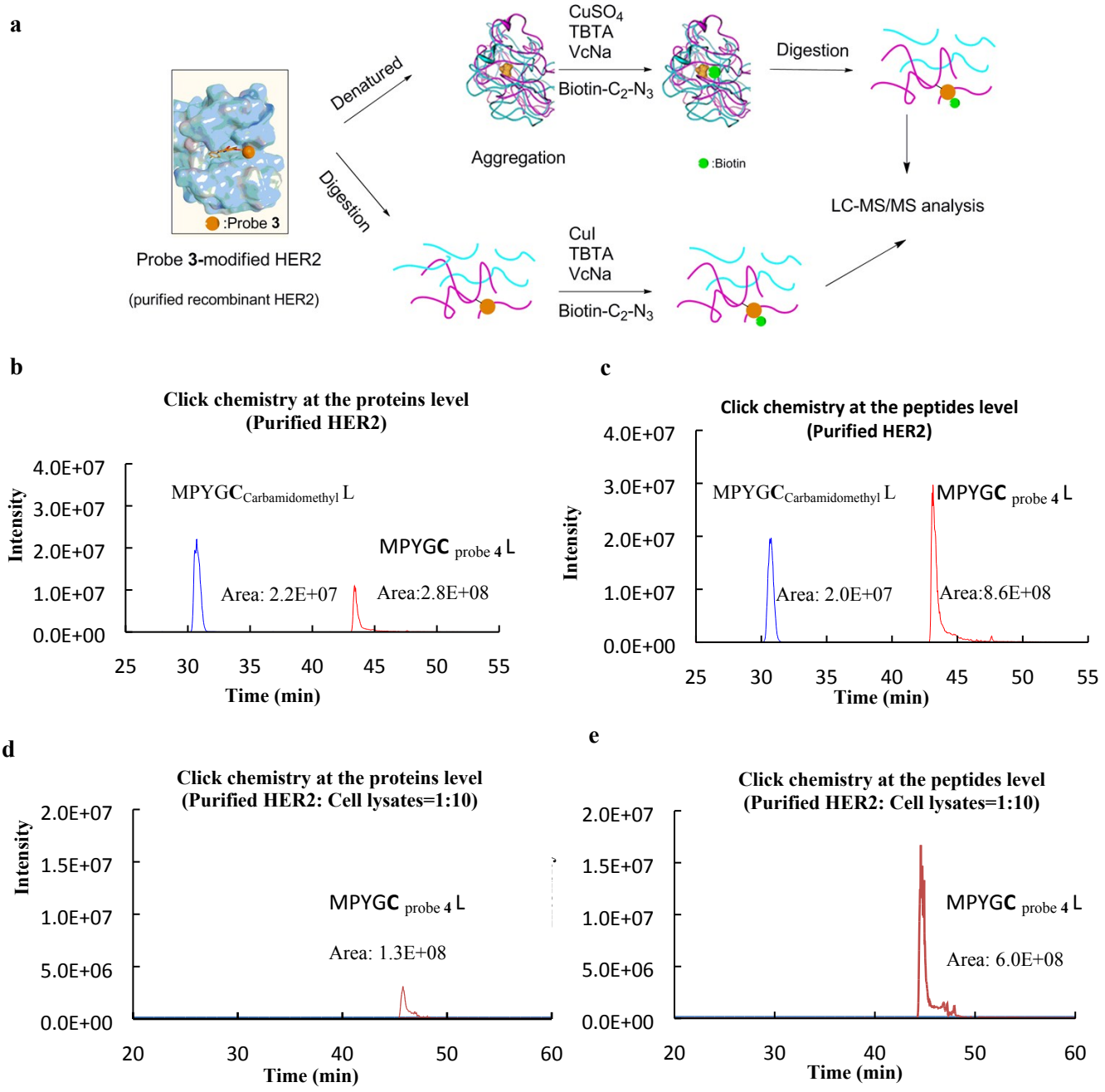
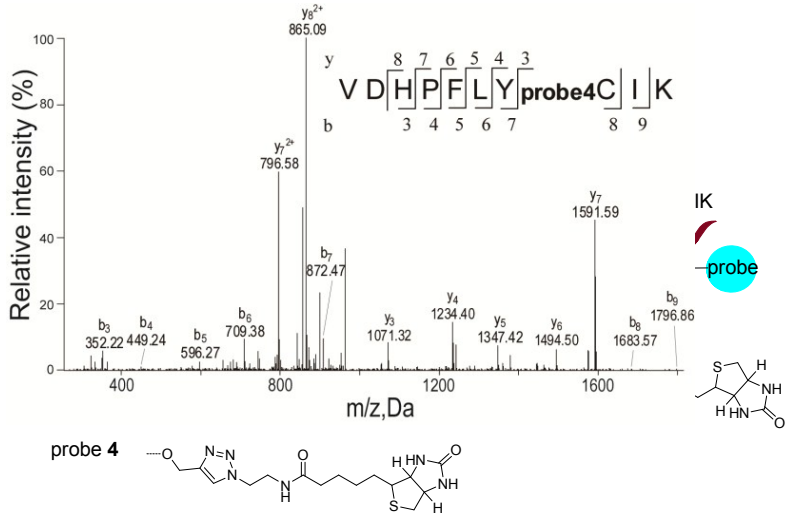


Figure S3. Comparison of click chemistry conjugation yields in different experimental systems. (a) Both workflows of click chemistry at the protein level and at the peptide level. 600 µL purified recombinant

HER2 (0.4 mg/ml) was incubated with 100 μM probe **3** at room temperature for 4 h. The HER2 proteins were precipitated by prechilled MeOH to remove excess probe **3**, and then divided into four aliquots. (b). One sample was dissolved in 300 μL 1% SDS followed by two steps, click conjugation with Biotin-C_2-N_3 tag (at the protein level) and pepsin digestion. (c).The second sample was digested by pepsin, and then conjugated with Biotin-C_2-N_3 tag in 300 μL DMF (at the peptide level). The rest two samples were mixed with 0.6 mg cell lysates respectively. (d). One sample was conjugated with Biotin-C_2-N_3 tag, followed by pepsin digestion. (e). The other was digested with pepsin followed by click conjugation. Four samples were diluted to 3 mL for dialysis, and then analyzed by LC-MS/MS. The last two samples (d,e) were enriched by streptavidin beads before MS analysis. (b,c) Extracted ion chromatograms of the probe **4**-modified peptide(MPYGC$_{probe\text{-}4}$L) and MPYGC$_{Carbamidomethyl}$L from first two samples, and MPYGC$_{Carbamidomethyl}$L was used as a internal standard. (d,e) Extracted ion chromatograms of the probe **4**-modified peptide(MPYGC$_{probe\text{-}4}$L) from last two samples mixed with cell lysates.

a



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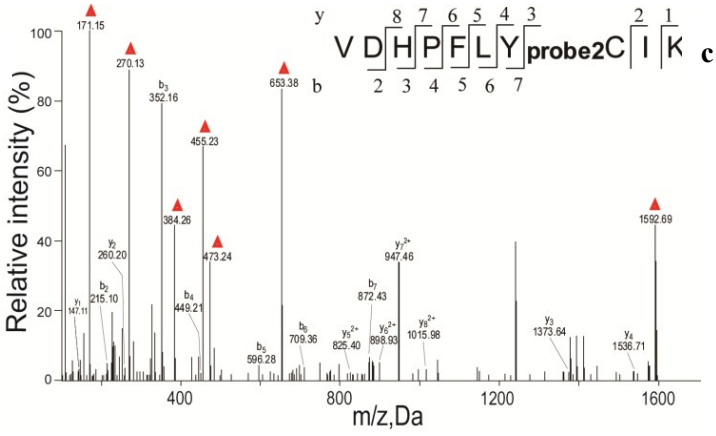


c

MW	Caculated fragment	MW(MS/MS)
171.15		171.15
270.13		270.13
473.19		473.24
653.38		653.38
1592.68	R: VDHPFLYCIK	1592.69

d

Figure S4. **Fragment of probe-labeled peptides in LC-MS/MS.** (a) The Michael addition reaction of probes (1 mM) to peptides (100 μM) in PBS buffer for 2 h at 37 °C. (b) Tandem MS spectrum of a probe **2**-modified peptide. ∆ mark peaks that were mostly derived from the biotin-linker-probe **2** and those peaks that could not be assigned (c) The table shows ∆ mark peaks in S4b. These fragments were mostly derived from the biotin-linker-probe **2** so they could not be assigned. (d) Tandem MS spectrum of a probe **4**-modified peptide.

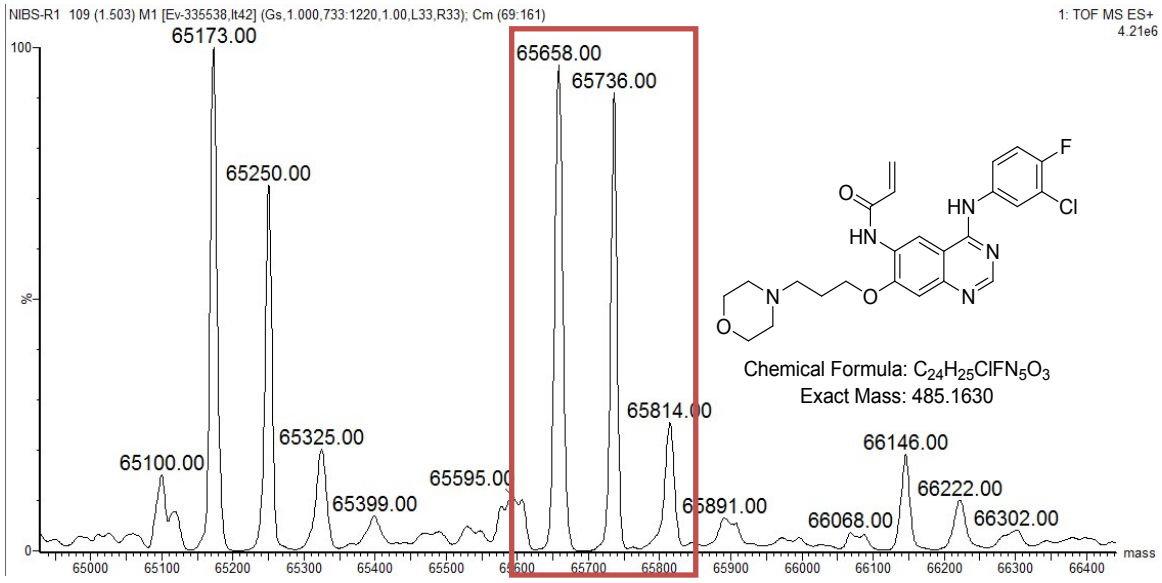


Figure S5. **Characterization of Canertinib-labeled recombinant HER2.** Reaction conditions for probe ligation to the HER2 were as follows: 5 μM HER2, 100 μM Canertinib, 10 mM $MgCl_2$, 1 mM EGTA, 2.5 mM DTT, and 0.5 mM Na_3VO_4 in Hepes buffer (pH 7.6) at room temperature for 4h. TOF-MS analysis showed the recombinant HER2 could be modified by Canertinib after 4 h incubation at room temperature.

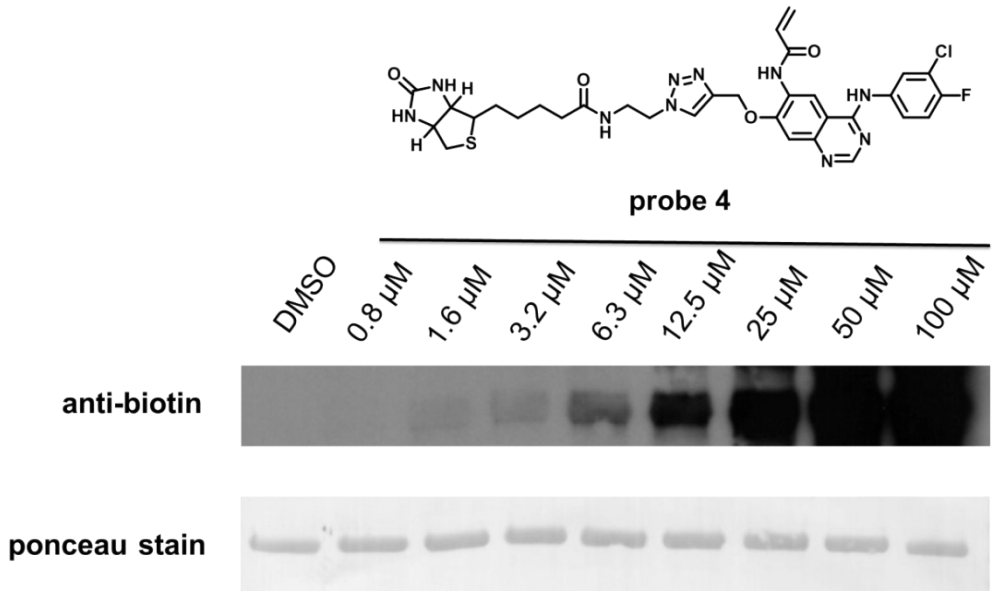
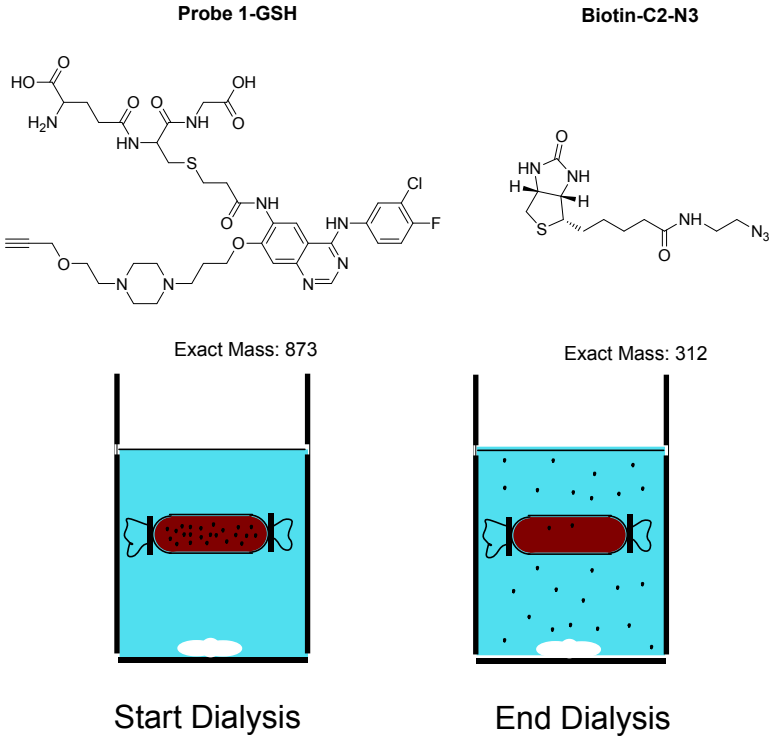


Figure S6. **Concentration-dependent labeling study of probe 4 to recombinant HER2.** Varying concentrations of probe **4**, from 0.8 µM to 100 µM, were incubated with 20 µl of 5 µM of the recombinant HER2 kinase domain for 4 h at room temperature.



Condition	Probe1-GSH	Biotin
Dialysis_control	100%	100%
Dialysis_30%_EtOH	78%	1%
Dialysis_40%_EtOH	99%	7%
Dialysis_50%_EtOH	99%	0%
Dialysis_60%_EtOH	90%	0%
Dialysis_70%_EtOH	105%	1%

Figure S7. Dialysis method for removing excess biotin tags. The starting material contained 100 μM of synthesized probe **1**-GSH and 1 mM of biotin tags. 1000 Da interception dialysis bags were soaked in different buffers (ranging from 30% to 70% ethanol) for 2 h. The starting solution was then added to each bag and dialyzed in the corresponding buffer for 12 h. The 20 μL residual solution in the dialysis bag was

then diluted to 200 μL with MeOH/H2O (1:1) and analyzed by LC-MS/MS. The starting solution was used as a control.

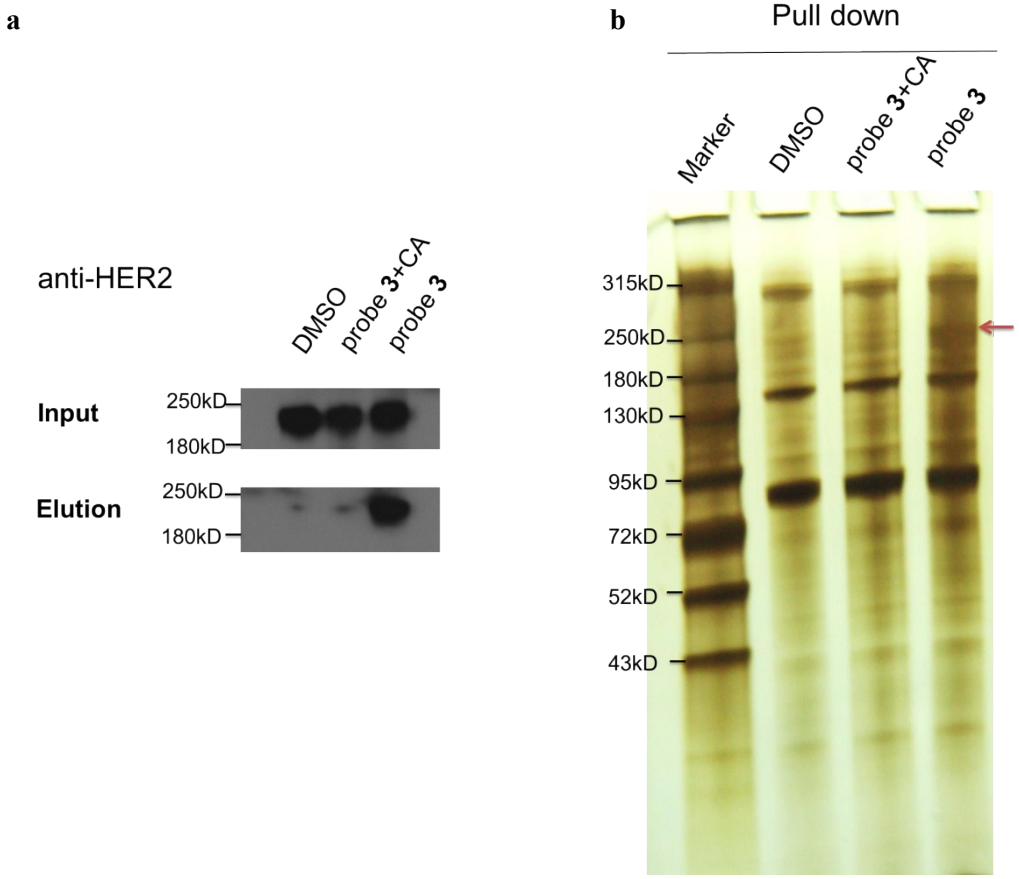


Figure S8. Pull-down of proteins by traditional CC-ABPP. SK-Br3 cells were treated with 3 μM probe **3**, 3 μM Canertinib(**CA**) and 3 μM probe **3** or an equal volume of DMSO for 4 h. The cells were then washed with PBS, lysed, and denatured in 1% SDS for 1 h. Following conjunction to biotin tags in click conditions for 2 h at room temperature, proteins were precipitated with prechilled MeOH, washed twice with MeOH, and redissolved in 2% SDS PBS buffer with ultrasonication. The concentration of SDS was diluted to 0.1% and the mixture solution was incubated with streptavidin beads for overnight. The beads were then collected and washed. Eluted proteins in 1× SDS loading buffer were separated by SDS-PAGE. DMSO as a negative control. (a) Western blotting of eluted proteins. (b) The silver staining of eluted proteins. The band in the location of the red arrow in silver staining was excised and identified via LC-MS/MS (see table S1).

TableS1. Proteins identified of the silver staining band in the red arrow.

	Accession number	**Protein description**	**Mascot score**	**Matched queries**	**Matched peptides**
1	IPI00220327	KRT1 Keratin, type II cytoskeletal 1	5337	144	46
2	IPI00019359	KRT9 Keratin, type I cytoskeletal 9	4487	115	36
3	IPI00217963	KRT16 Keratin, type I cytoskeletal 16	2389	78	33
4	IPI00021304	KRT2 Keratin, type II cytoskeletal 2 epidermal	2284	56	24
5	IPI00009865	KRT10 Keratin, type I cytoskeletal 10	2152	71	27
6	IPI00293665	KRT6B Keratin, type II cytoskeletal 6B	2134	82	34
7	IPI00384444	KRT14 Keratin, type I cytoskeletal 14	2130	77	34
8	IPI00300725	KRT6A Keratin, type II cytoskeletal 6A	1990	78	35
9	IPI00930073	KRT6C cDNA, FLJ93744, highly similar to Homo sapiens keratin 6E (KRT6E), mRNA	1846	71	32
10	IPI00009867	KRT5 Keratin, type II cytoskeletal 5	1633	63	27
11	IPI00013933	DSP Isoform DPI of Desmoplakin	1320	57	37
12	IPI00450768	KRT17 Keratin, type I	1117	47	20

		cytoskeletal 17			
13	IPI00789324	JUP Uncharacterized protein	1084	41	18
14	IPI00398625	HRNR Hornerin	993	19	9
15	IPI00300384	**ERBB2 Isoform 1 of Receptor tyrosine-protein kinase erbB-2**	809	34	18
16	IPI00554711	JUP Junction plakoglobin	583	20	13
17	IPI00011569	ACACA Isoform 1 of Acetyl-CoA carboxylase 1	409	13	9
18	IPI00103481	KRT72 Isoform 1 of Keratin, type II cytoskeletal 72	260	11	7
19	IPI00027547	DCD Dermcidin	207	6	2
20	IPI00025753	DSG1 Desmoglein-1	156	3	2
21	IPI00375843	KRT80 Isoform 3 of Keratin, type II cytoskeletal 80	155	3	2
22	IPI00022434	ALB Uncharacterized protein	100	2	1
23	IPI00000959	VIP Isoform 1 of VIP peptides	96	2	1
24	IPI00477227	KRT78 Isoform 1 of Keratin, type II cytoskeletal 78	83	3	2
25	IPI00514908	KPRP Keratinocyte proline-rich protein	80	3	2
26	IPI00334627	ANXA2P2 Putative annexin A2-like protein	57	2	2
27	IPI01025580	- Possible J 56 gene segment (Fragment)	51	2	1
28	IPI00397801	FLG2 Filaggrin-2	46	1	1
29	IPI00026256	FLG Filaggrin	44	1	1
30	IPI00376956	SPATA7 Isoform 2 of Spermatogenesis-associated protein 7	40	1	1
31	IPI00152881	SHROOM3 Isoform 1 of Protein Shroom3	40	1	1
32	IPI00955832	TFDP1 HCG23704, isoform CRA_a	37	1	1
33	IPI00103242	POF1B Isoform 1 of Protein POF1B	37	1	1
34	IPI00217511	HTR7P1 Seven transmembrane helix receptor	27	1	1
35	IPI00925214	CASP12 Uncharacterized protein	25	1	1
36	IPI00157860	AKAP4 Isoform 1 of A-kinase	21	1	1

		anchor protein 4			
37	IPI00375236	PDE4D Isoform 5 of cAMP-specific 3~,5~-cyclic phosphodiesterase 4D	21	1	1
38	IPI00152055	C8orf46 Isoform 1 of Uncharacterized protein C8orf46	21	1	1
39	IPI00021267	EPHA2 Ephrin type-A receptor 2	20	1	1
40	IPI00298306	ATM Serine-protein kinase ATM	15	1	1
41	IPI00641912	RTN4IP1 NOGO-interacting mitochondrial protein	15	1	1
42	IPI00103510	RXFP2 Relaxin receptor 2	14	1	1
43	IPI00059297	EMID2 Isoform 1 of Collagen alpha-1(XXVI) chain	14	1	1

Table S2. Proteins identified by proteomics analysis in the BTC-ABPP. SK-Br3 cells were treated with 3 μM probe **3** for 4 h, followed by digestion with pepsin, click chemistry, dialysis ,enrichment and analysis with LC-MS/MS. The sequence of matched peptides of proteins were separated by a semicolon. Probe **4**-modified peptides were marked in red.

	Accession number	**Protein description**	**Sequences**
1	IPI00003131	SEC22C Isoform 2 of Vesicle-trafficking protein SEC22c	ILNIMCAALNL+Carbamidomethyl (C); Oxidation (M)
2	IPI00844264	CSDE1 Putative uncharacterized protein DKFZp779B0247	SSSSTSSGT
3	IPI00300384	ERBB2 Isoform 1 of Receptor tyrosine-protein kinase erbB-2	**MPYGCL + Probe 4 (C)/ MPYGCL + Oxidation (M); Probe 4 (C)**
4	IPI00939931	IGSF1immunoglobulin superfamily member 1 isoform 3	LCCAISF + Carbamidomethyl (C)
5	IPI00747010	NPHS1 Isoform 1 of Nephrin	EPPSGPSGLPLL
6	IPI00910901	cDNA FLJ50001, moderately similar to Kelch-like protein 17	CPPGQS + Carbamidomethyl (C)
7	IPI00936821	TUBA1A cDNA FLJ53743, highly	MPAGSSTAW / VPYPRIHF

		similar to Tubulin alpha-3 chain	

a.

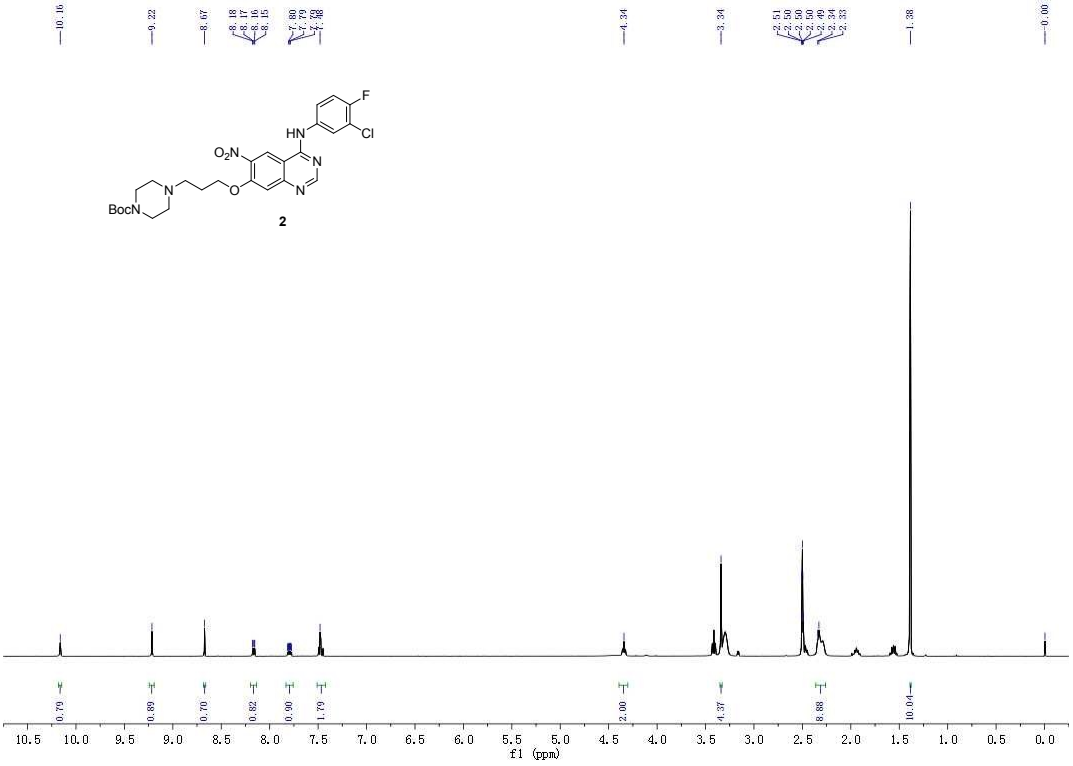
Candidates (Pepsin)	Identified peptides
HER2 (Receptor tyrosine-protein kinase erbB-2)	**MPYGC_{805}L**
FASN (Fatty acid synthase)	**RHAQPTC_{1558}PGAQL**
C16orf58 (Isoform 1 of UPF0420 protein C16orf58)	**VSGC_{256}PGF**
RPLP1 (60S acidic ribosomal protein P1)	**IC_{61}NVGAGGPAPAAGAAPAGGPAPST**
POLR3B (DNA-directed RNA polymerase III subunit RPC2)	**MISSDAFEVDVCGQC_{1083}GL**
CTSC (Isoform 1 of Dipeptidyl peptidase 1)	**VSPVRNQASCGSC_{234}YSF**
TRBV15 (T cell receptor beta variable 15)	**C_{50}ATSR**

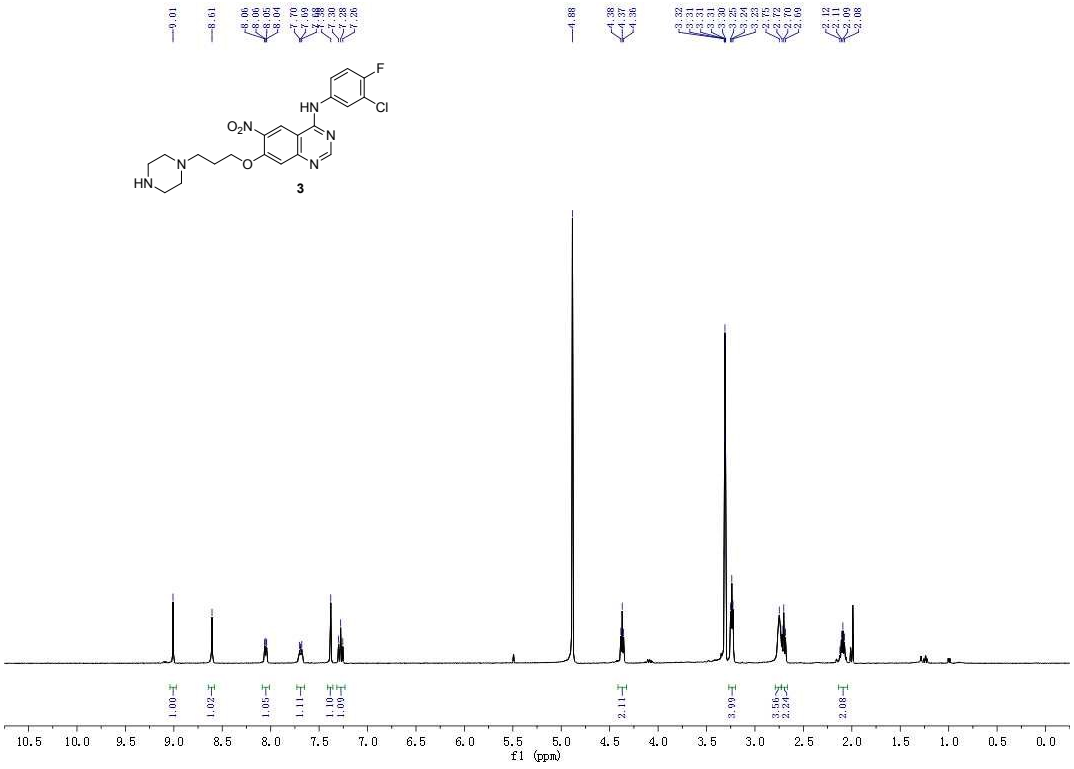
b.

Candidates (Trypsin)	Identified peptides
CTSC (Isoform 1 of Dipeptidyl peptidase 1)	**NQASCGSC_{234}Y**
VDAC2 (Voltage-dependent anion-selective channel protein 2)	**VC_{210}EDLDTS**
PCBP1 (Poly(rC)-binding protein 1)	**SSPVIC_{194}AGGQDR**
ERMP1 Uncharacterized protein	**VDGC_{43}SGGGR**
TTN Titin isoform CRA_a	**EG$C_{20935/26052}$EY**

Figure S9. Identification of modified peptides of target candidates. The tabel shows the identified probe **3**-labeled peptides resulting from digestion with pepsin (a) and trypsin (b). Red letters indicate the modification sites. The index numbers represent the site modified by probe **3** in the target proteins.

^{1}H and ^{13}C NMR Spectra

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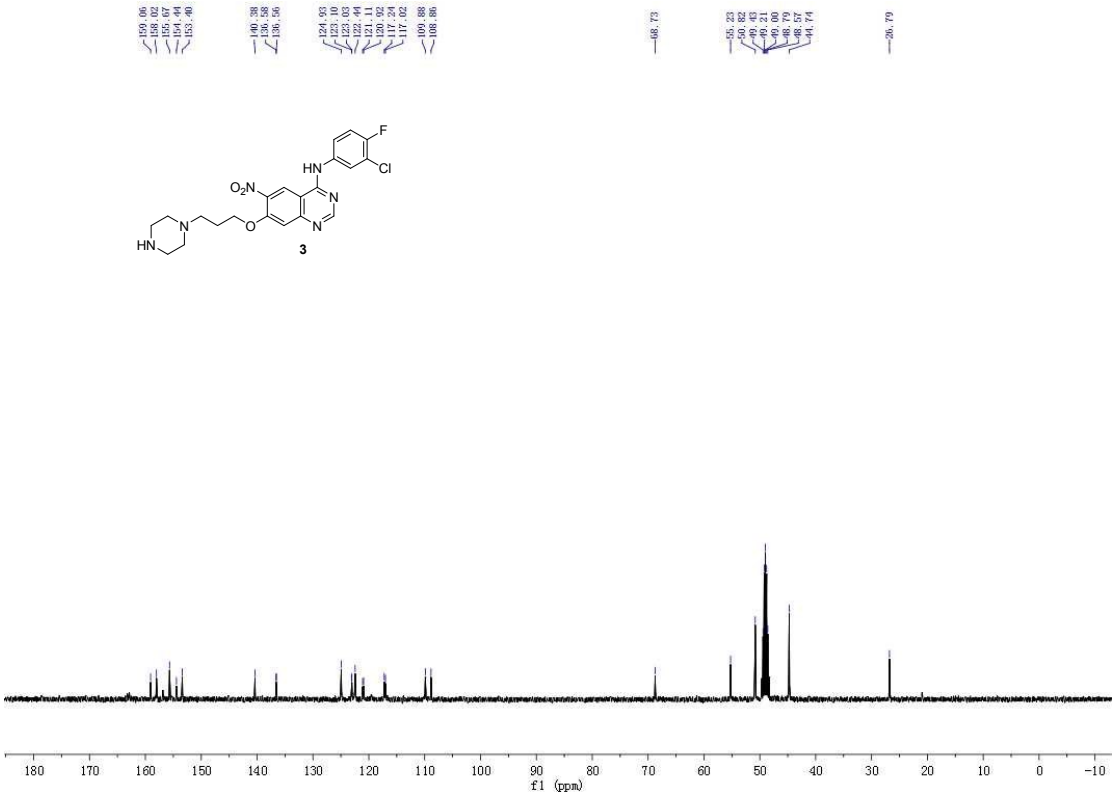
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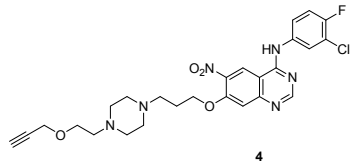
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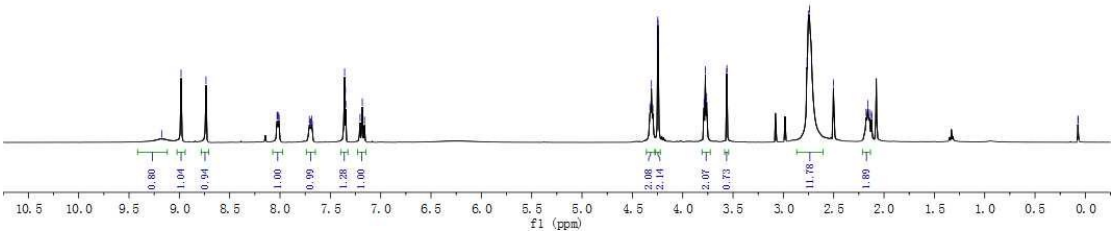
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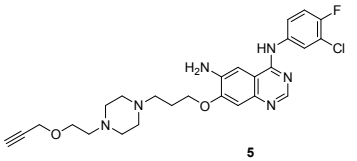
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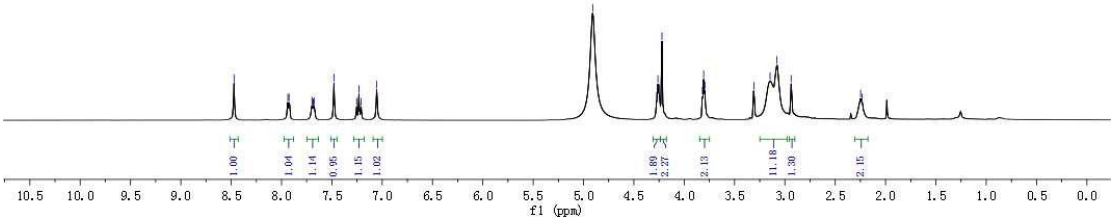
O

5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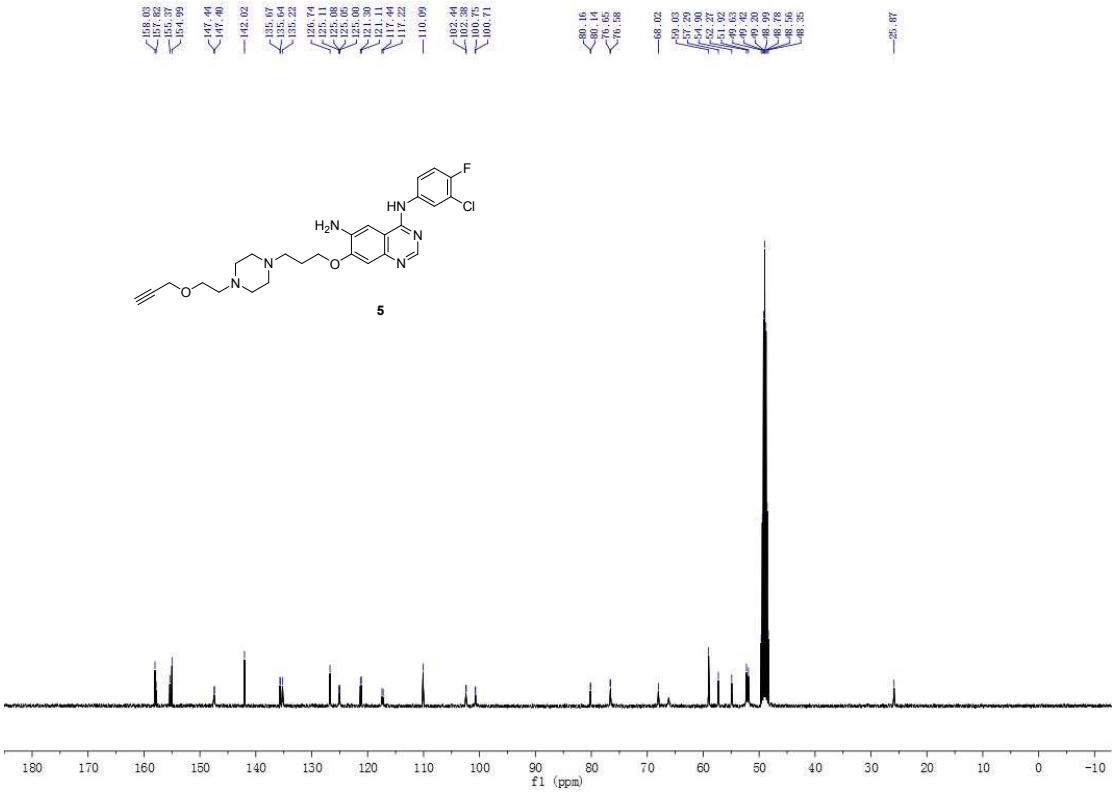
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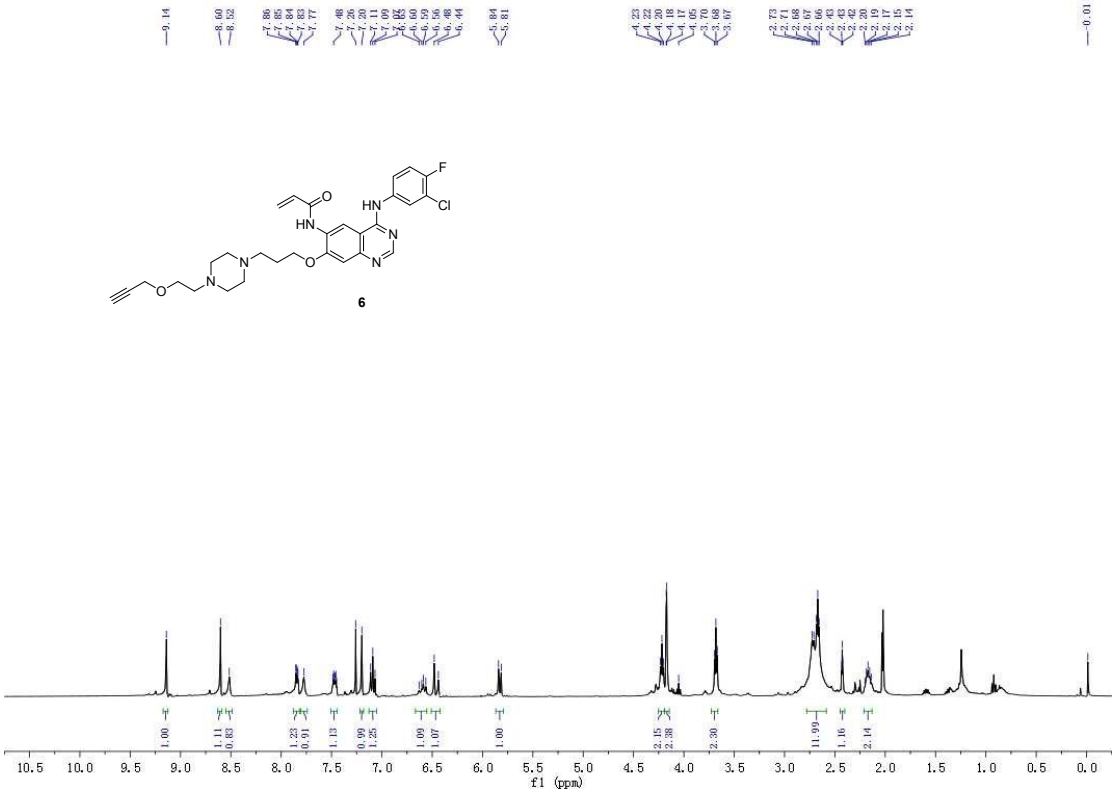
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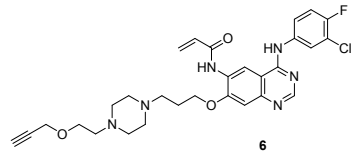

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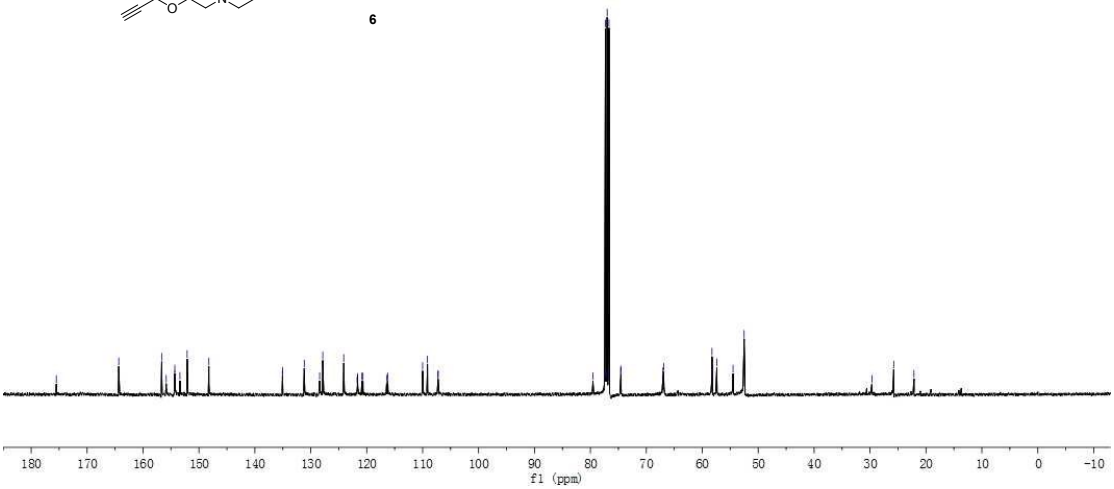

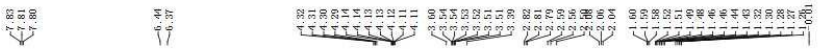

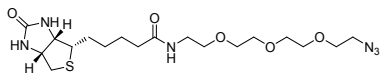

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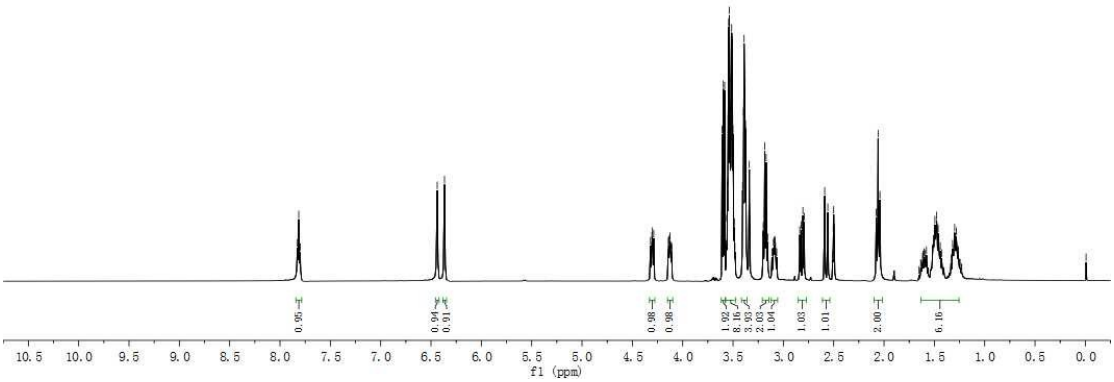

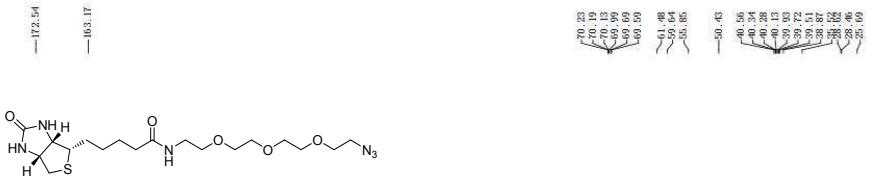

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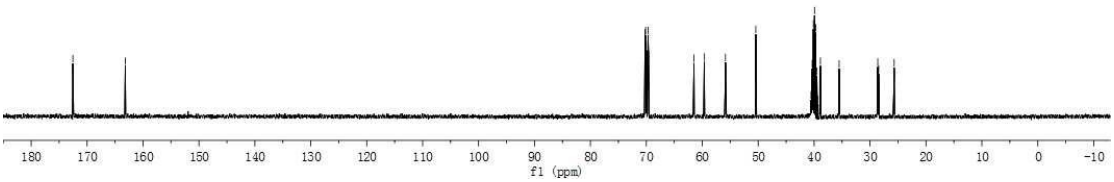

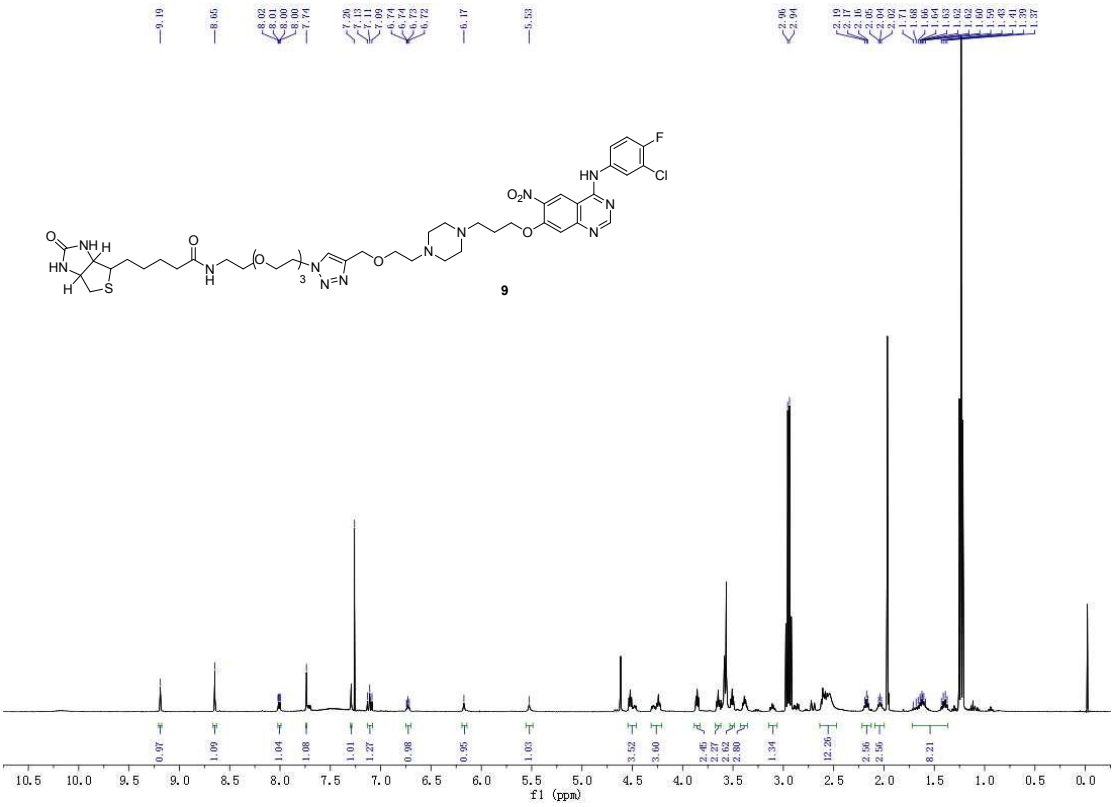

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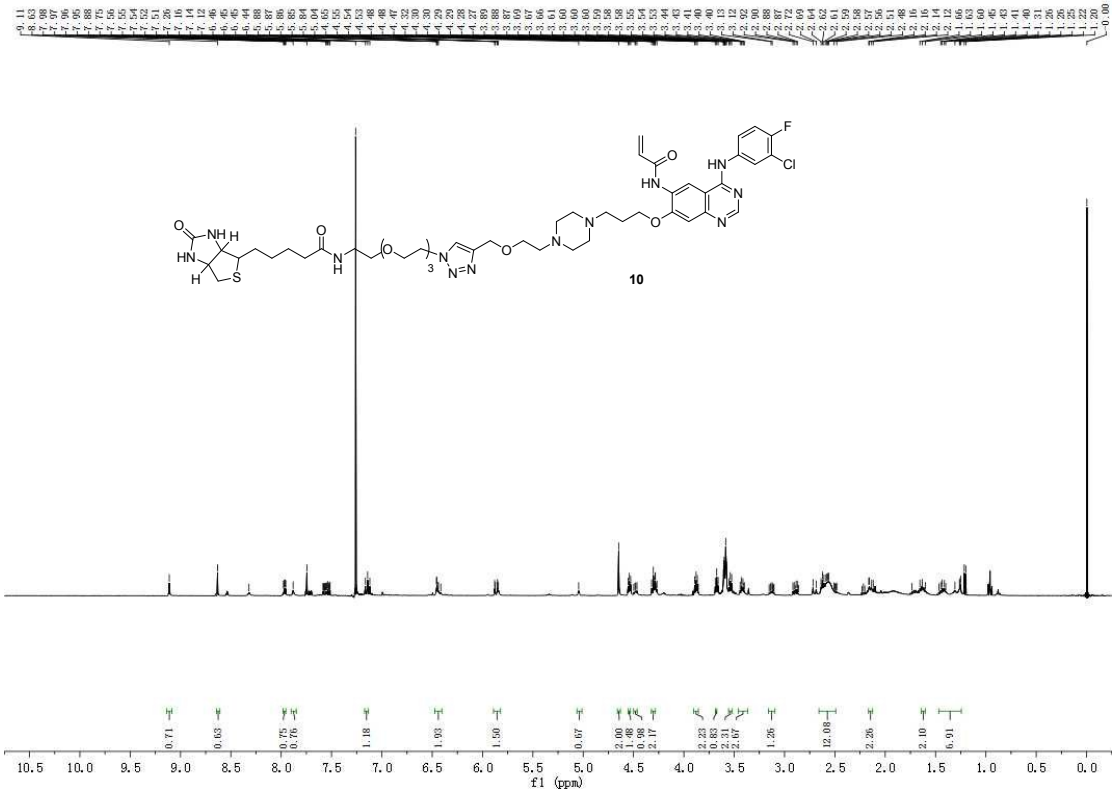

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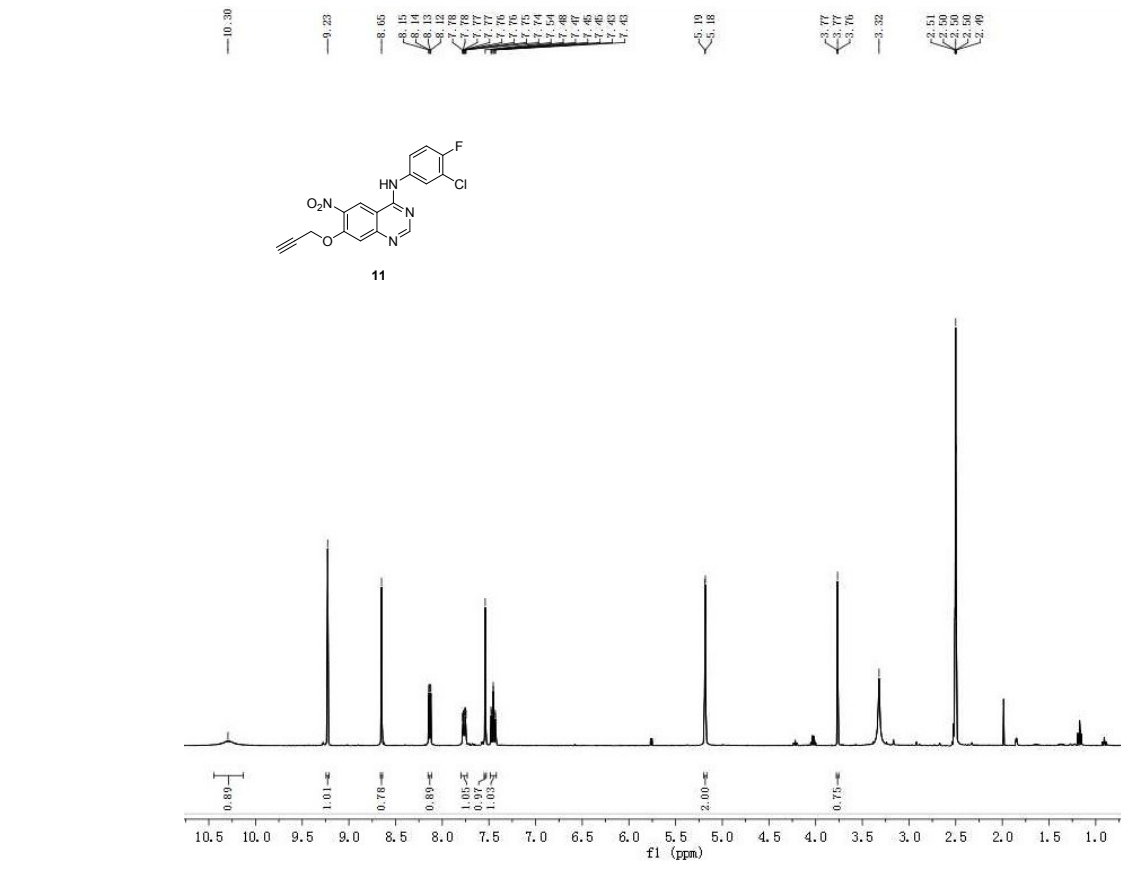

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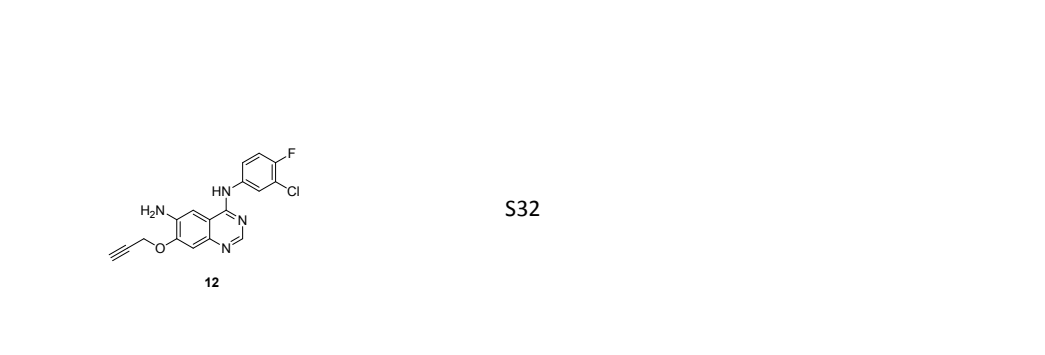

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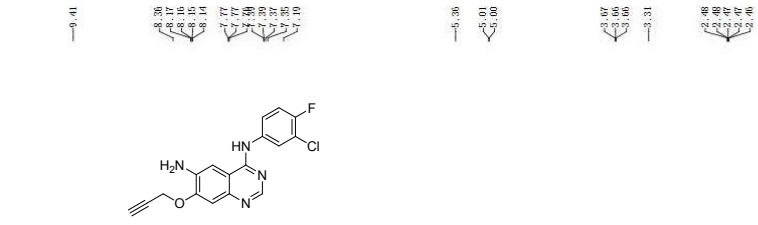

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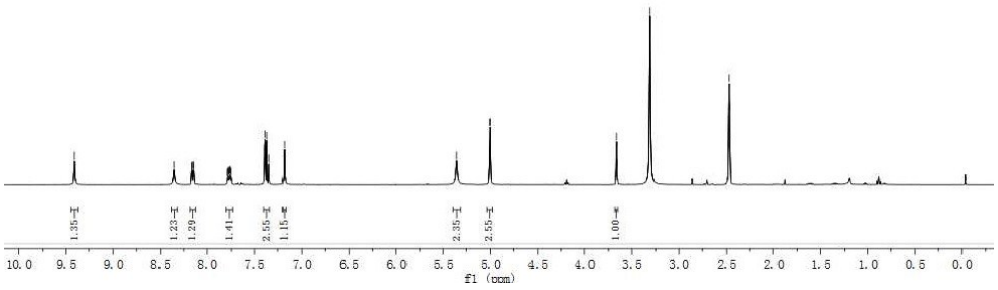

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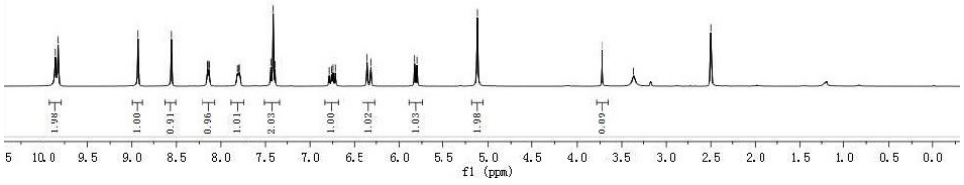

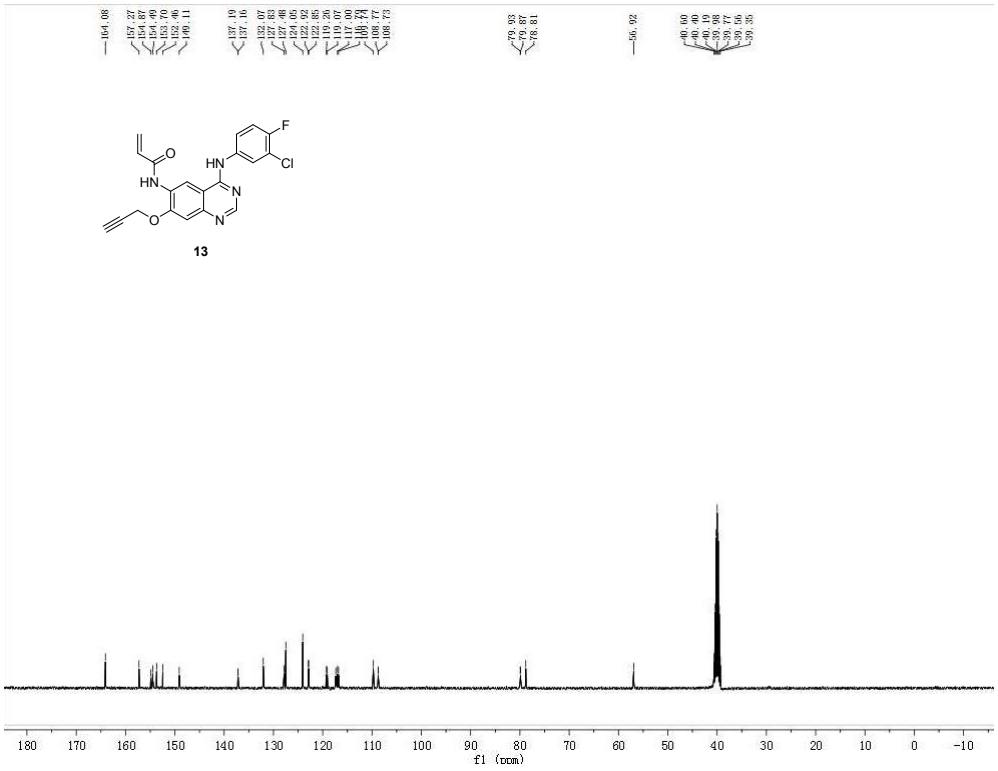
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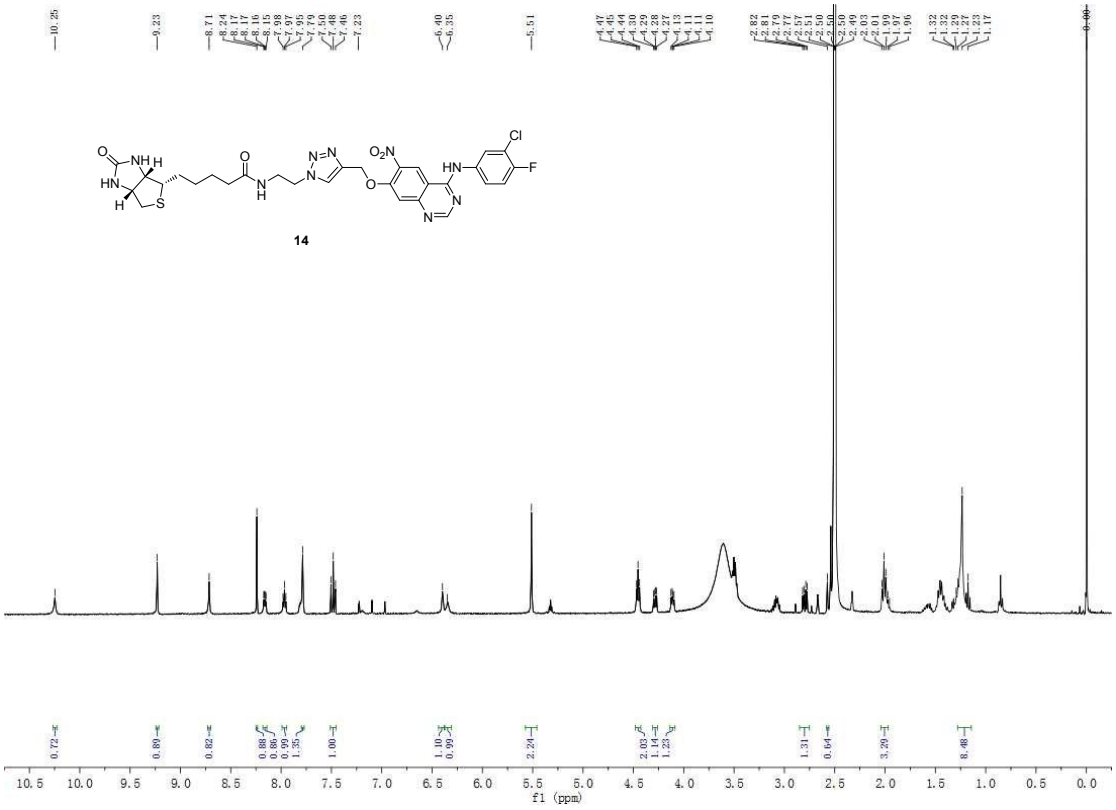
154.87
154.40
153.70
152.46
149.11
137.19
137.16
132.07
127.83
127.48
124.65
122.92
122.85
119.26
119.07
117.00
116.74
116.47
108.77
108.73
79.93
79.87
78.81
56.92
40.60
40.40
40.19
39.98
39.77
39.56
39.35
F
Cl
HN
O
HN
N
N
O
13
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)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8.17
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8.15
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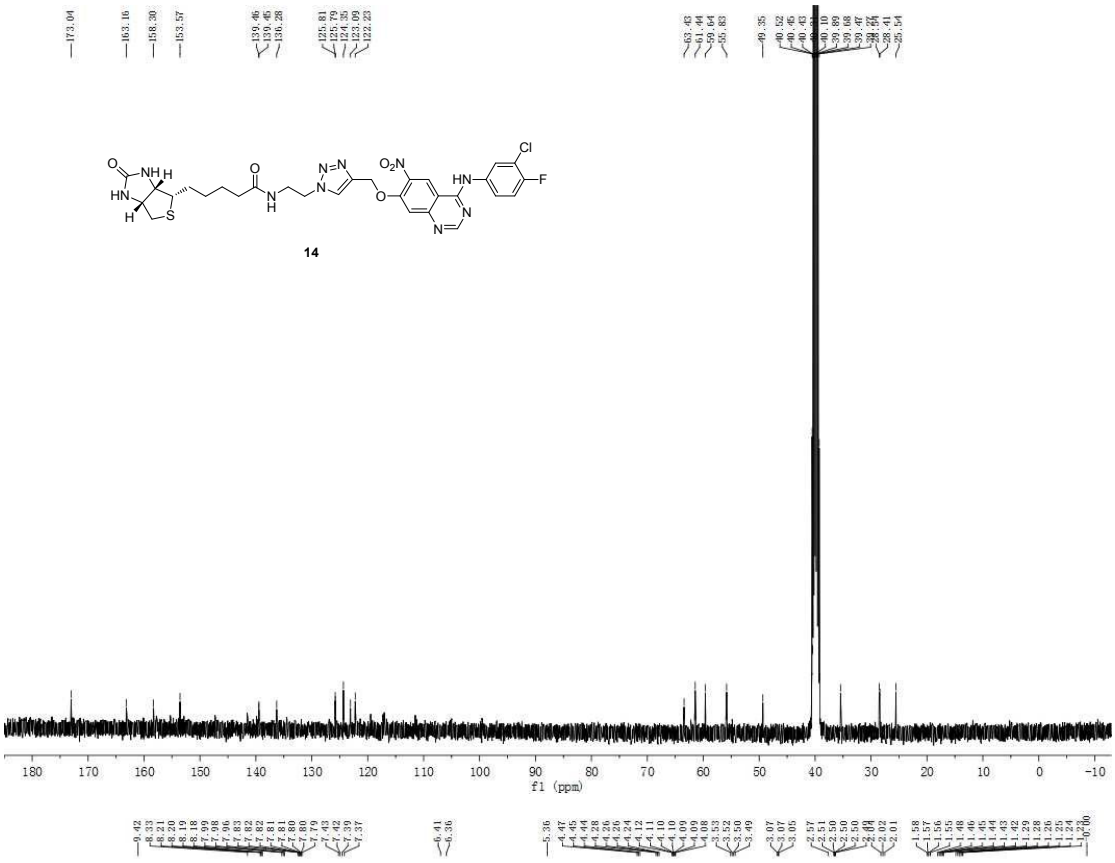
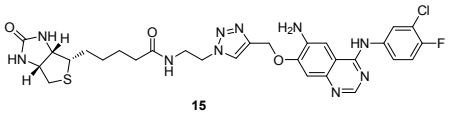
7.97
7.96
7.79
7.50
7.48
7.46
7.23
6.40
6.35
5.51
4.47
4.46
4.44
4.30
4.29
4.28
4.27
4.15
4.11
4.11
4.10
2.82
2.81
2.79
2.77
2.57
2.51
2.50
2.50
2.49
2.03
2.01
1.99
1.97
1.96
1.32
1.32
1.29
1.29
1.27
1.23
1.17
0.00
O
NH
H
HN
H
S
O
N
H
N=N
N
O2N
O
HN
Cl
F
N
N
14
0.72
0.89
0.82
0.88
0.86
0.90
1.35
1.00
1.10
0.99
2.24
2.03
1.14
1.25
1.31
0.64
3.29
8.48
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)



14



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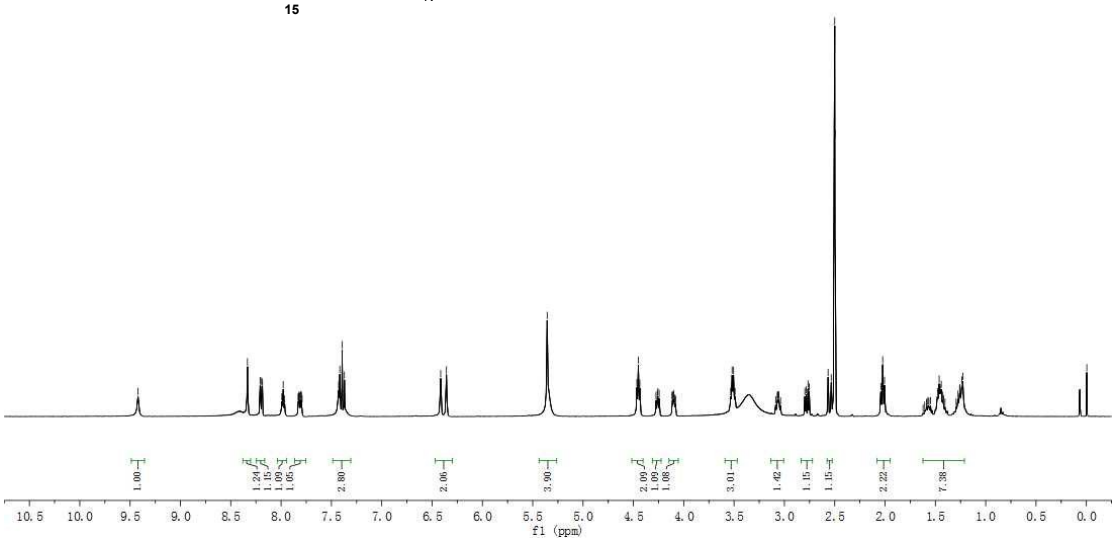

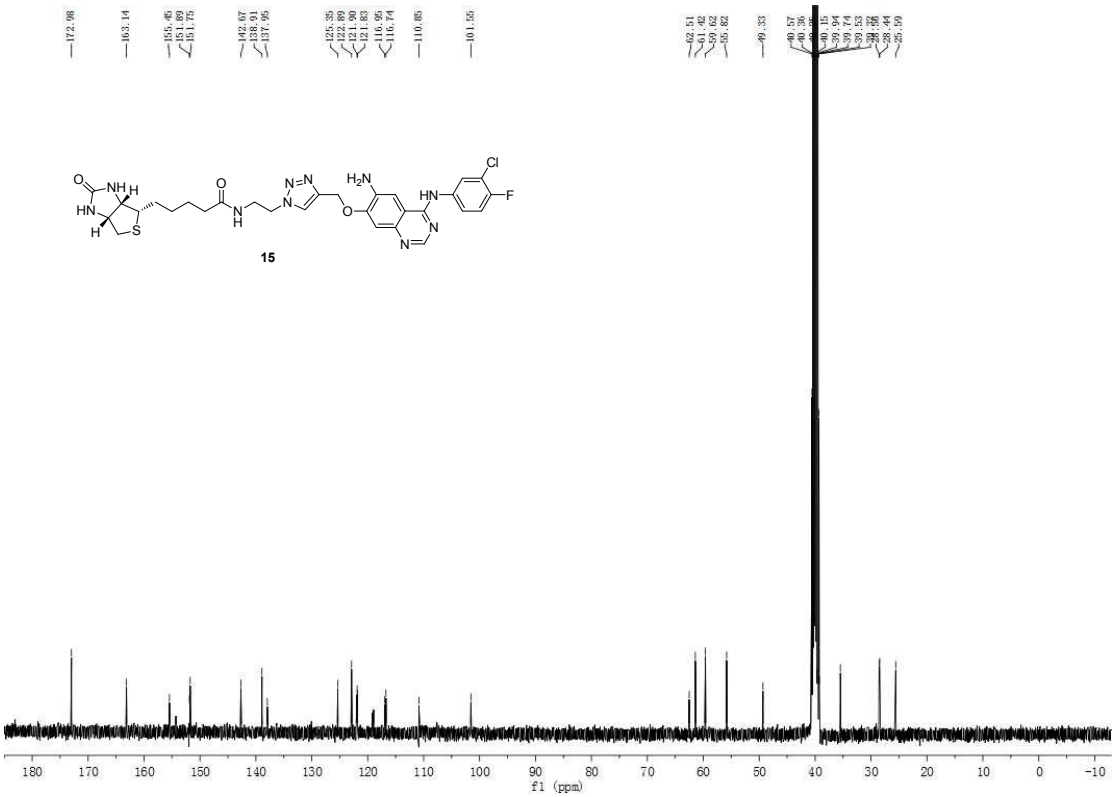
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137.95
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123.89
121.90
121.83
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110.85
101.55
62.51
61.42
59.62
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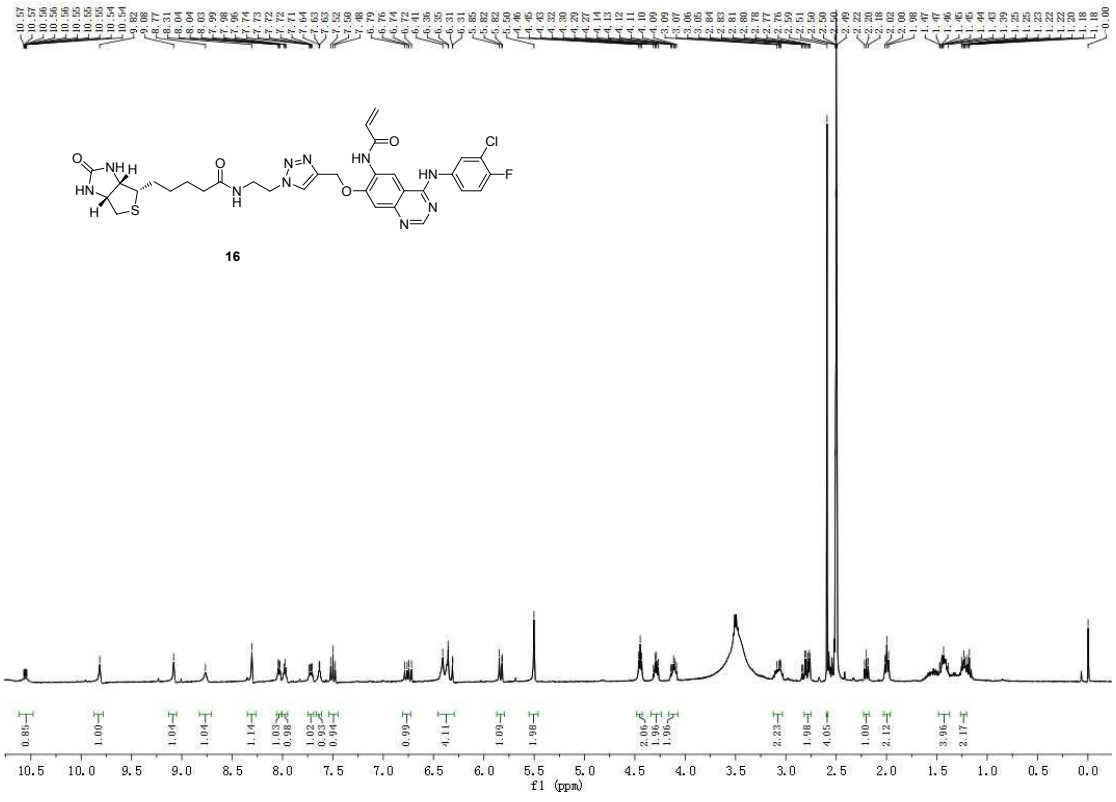
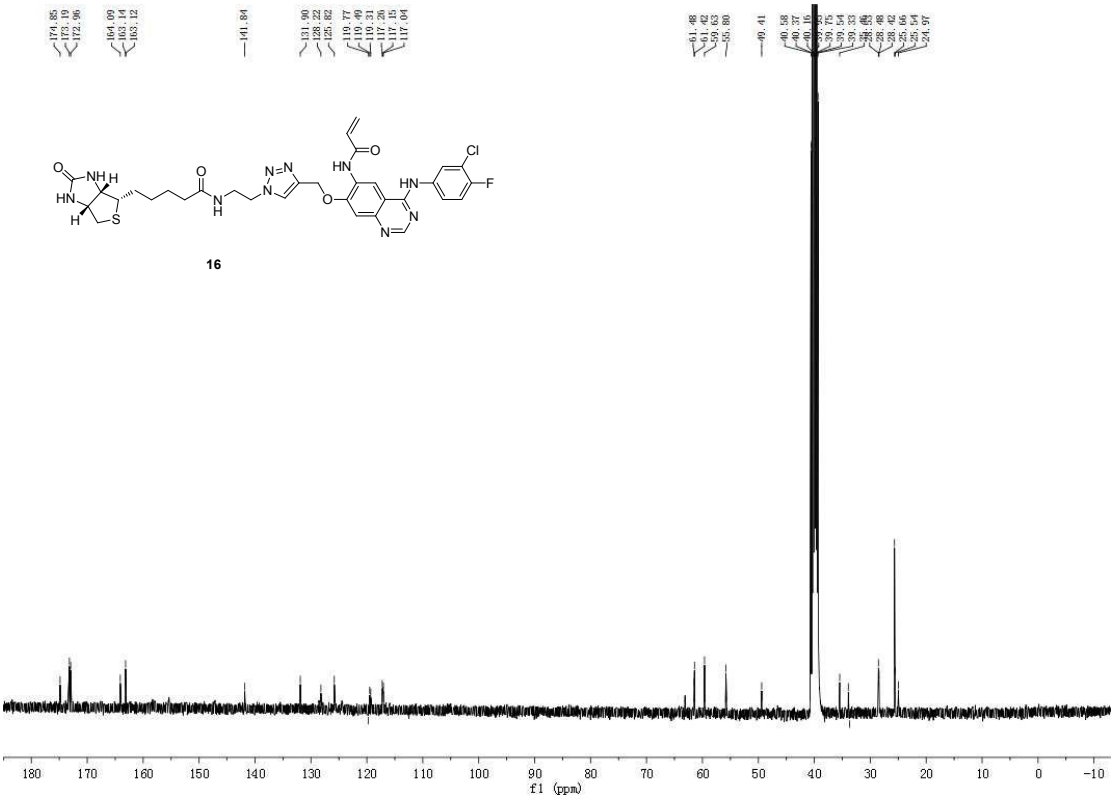
39.53
38.46
38.24
25.59
15
f1 (ppm)


16
f1 (ppm)



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2. T. Mayer and M. E. Maier, *European Journal of Organic Chemistry*, 2007, **2007**, 4711-4720.
3. B. Thong, J. Pilling, E. Ainscow, R. Beri and J. Unitt, *Journal of biomolecular screening*, 2011, **16**, 36-43.
4. M. Furber, A.-K. Tiden, P. Gardiner, A. Mete, R. Ford, I. Millichip, L. Stein, A. Mather, E. Kinchin and C. Luckhurst, *J. Med. Chem.*, 2014, **57**, 2357-2367.