Supporting Information for

Copper-Free Sonogashira Cross-Coupling for Functionalization of Alkyne-Encoded Proteins in Aqueous Medium and in Bacterial Cells

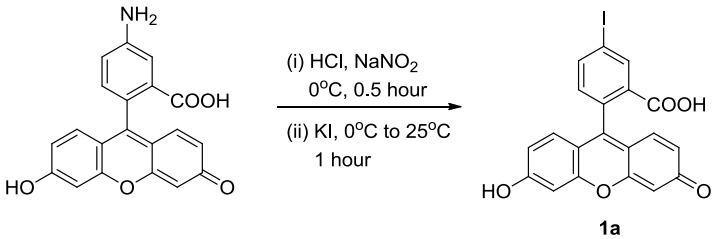
Nan Li, Reyna K. V. Lim, Selvakumar Edwardraja and Qing Lin*

Department of Chemistry, State University of New York at Buffalo, Buffalo, New York 14260-3000, United States, qinglin@buffalo.edu

General data

All Sonogashira cross-coupling reactions were carried out under ambient conditions using 0.5 mL plastic test tubes with magnetic stirring. Solvents and chemicals were purchased from commercial sources and used directly without further purification. Flash chromatography was performed with SiliCycle P60 silica gel (40-63 μm, 60 Å). ^{1}H NMR spectra were recorded with Inova-300 or -500 MHz spectrometers and chemical shifts were reported in ppm using either TMS or deuterated solvents as internal standards (TMS, 0.00; $CDCl_3$, 7.26; CD_3OD, 3.31; DMSO-d_6, 2.50). Multiplicity was reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, brs = broad. ^{13}C NMR spectra were recorded at 75 MHz and 125 MHz and chemical shifts were reported in ppm using the deuterated solvents as internal standards ($CDCl_3$, 77.0; CD_3OD, 49.0; DMSO-d_6, 39.5). Electrospray LC-MS analysis was performed using a Finnigan LCQ Advantage IonTrap mass spectrometry coupled with a Surveyor HPLC system. Protein liquid chromatography was performed using a Phenomenex Jupiter C4 column (5 μm, 300 Å, 2.00 × 50 mm) with a flow rate of 200 μL/min and a linear gradient of 5-95% ACN/H_2O containing 0.1% HCOOH.

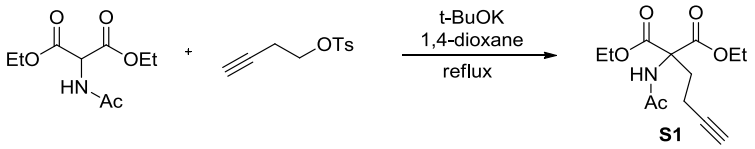
Synthesis of cross-coupling substrates



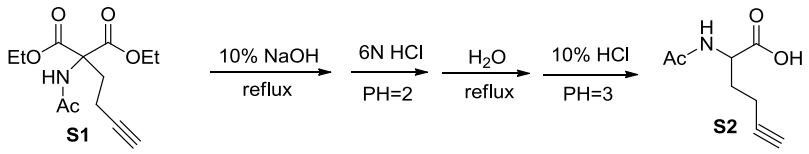
1a

2-(6-Hydroxy-3-oxo-3*H*-xanthen-9-yl)-5-iodobenzoic acid (1a): To a suspension of 5-aminofluorescein (200 mg, 0.576 mmol) in 2.0 mL of 12 N HCl was added 1.0 g of ice, and the

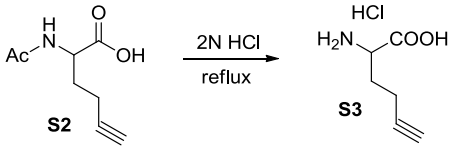
mixture was cooled with an ice-water bath. A solution of $NaNO_2$ (50 mg, 0.72 mmol) in 1.0 mL of water was added dropwise over 1 min, and the mixture was stirred at 0 °C for 30 minutes. A solution of KI (956 mg, 5.74 mmol) in 1.6 mL of water was added dropwise over 1 min with vigorous stirring at 0 °C. The ice-water bath was removed and the stirring was continued for additional one hour. The reaction mixture was extracted three times with *i*-PrOH/$CHCl_3$ (1:3). The combined organic extracts were washed with saturated $Na_2S_2O_3$ solution, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The resulting residue was purified by flash chromatography using 10% MeOH/CH_2Cl_2 as an eluent to afford the desired product as an orange solid (142 mg, 53% yield): ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.15 (br, 2H), 8.30 (d, J =1.1 Hz, 1H), 8.10 (dd, J = 8.0, 1.5 Hz, 1H), 7.09 (d, J = 8.0 Hz, 1H), 6.67 (d, J = 2.4 Hz, 2H), 6.61 (d, J = 8.6 Hz, 2H), 6.55 (dd, J = 8.7, 2.4 Hz, 2H); ^{13}C NMR (125 MHz, DMSO-*d*6) δ 167.15, 159.59, 151.82, 143.95, 133.00, 129.24, 128.42, 126.10, 112.66, 108.94, 102.23, 96.24, 83.41; MS (ESI) calcd for $C_{20}H_{11}IO_5$ 458.2 [M+H$^+$], found 458.1.



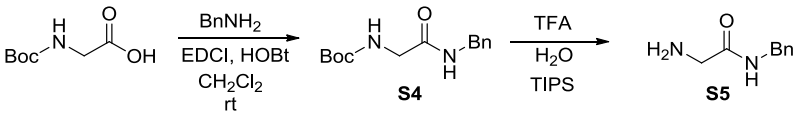
Diethyl 2-acetamido-2-(but-3-yn-1-yl)malonate (S1): To a solution of diethyl acetamidomalonate (2.10 g, 9.50 mmol) in dry dioxane (40 mL) was added *t*-BuOK (1.18 g, 10.44 mmol), and the mixture was vigorously stirred at 60 ºC for 2 hours before but-3-yn-1-yl 4-methylbenzenesulfonate (1.68 mL, 9.50 mmol) was added. After 72 hours, the suspension was filtered, and the filtrate was washed with Et_2O (3 × 20 mL) and concentrated under reduced pressure. The resulting residue was purified by flash chromatography using 10-30% EtOAc/hexanes as an eluent to afford the desired product as a pale yellow solid (1.347g, 35% yield): ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.81 (s, 1H), 4.25-4.19 (m, 4H), 2.59 (t, J = 7.4 Hz, 2H), 2.14-2.08 (m, 2H), 2.02 (s, 3H), 1.91-1.90 (m, 1H), 1.24 (t, J = 7.1 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.35, 167.83, 82.71, 69.10, 65.81, 62.77, 30.92, 23.12, 14.02, 13.45; MS (ESI) calcd for $C_{13}H_{19}NO_5$ 269.1 [M+H$^+$], found 269.1.



2-Acetamidohex-5-ynoic acid (S2): Diethyl 2-acetamido-2-(but-3-yn-1-yl)malonate (**S1**, 1.65 g, 6.1 mmol) was dissolved in 10% NaOH (10 mL) and the solution was refluxed for 5 hours. After cooling down to room temperature, the solution was acidified to pH 2 with 6 N HCl and concentrated. The resulting solid was dissolved in H_2O (20 mL), and the solution was refluxed for 5 hours. After filtering the mixture through a layer of active carbon, the filtrate was acidified to pH 3 with 10% HCl and extracted with EtOAc (5 × 30 mL). The combined organic layers were dried with anhydrous Na_2SO_4, filtered, and concentrated to afford the titled compound as a white solid (654 mg, 35% yield): ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.60 (brs, 1H), 8.12 (d, J = 7.7 Hz, 1H), 4.27-4.19 (m, 1H), 2.82 (t, J = 2.4 Hz, 1H), 2.23-2.18 (m, 2H), 1.92-1.86 (m, 1H), 1.84 (s, 3H), 1.78-1.66 (m, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 173.44, 169.45, 83.27, 71.85, 30.04, 22.38, 14.83; MS (ESI) calcd for $C_8H_{11}NO_3$ 169.1 [M+H$^+$], found 169.0.

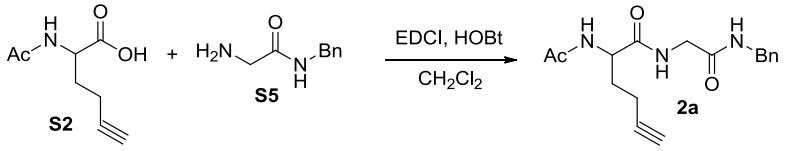


2-Aminohex-5-ynoic acid (S3): A solution of 2-acetamidohex-5-ynoic acid (**S2**, 653.9 mg, 3.87 mmol) in 15 mL 2 N HCl was refluxed for 2 hours before concentrated under reduced pressure. Addition of acetone to the resulting residue gave a white solid, which was filtered and washed with acetone to afford the titled compound (374 mg, 59% yield): ^{1}H NMR (500 MHz, D_2O) δ 4.16 (t, J = 6.5 Hz, 1H), 2.45-2.42 (m, 2H), 2.24-2.18 (m, 1H), 2.12-2.01 (m, 1H); ^{13}C NMR (125 MHz, D_2O) δ 171.84, 82.30, 71.12, 52.00, 28.45, 14.14; MS (ESI) calcd for $C_6H_9NO_2$ 127.1, [M+H$^+$] found 127.1.

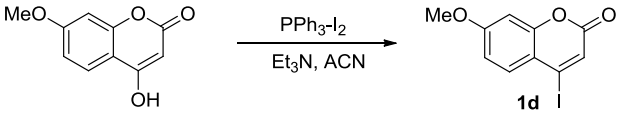


2-Amino-N-benzylacetamide (S5): A solution of 2-((*tert*-butoxycarbonyl)amino)acetic acid (700 mg, 4.0 mmol), $BnNH_2$ (440μL, 4.0 mmol), EDCI (764 mg, 4.0 mmol) and HOBt (540 mg, 4 mmol) in 15 mL CH_2Cl_2 was stirred at room temperature overnight. After reaction was complete, the organic layer was separated, washed successively with 1 N HCl (50 mL) and saturated $NaHCO_3$ (50 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The resulting

residue was purified by flash chromatography using 25% EtOAc/hexanes as an eluent to give the intermediate **S4** (1.02g, 97% yield): ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35-7.32 (m, 2H), 7.29-7.26 (m, 3H), 6.49 (brs, 1H), 5.19 (brs, 1H), 4.47 (d, *J* = 5.8 Hz, 2H), 3.83 (d, *J* = 5.5 Hz, 2H), 1.44 (s, 9H). To a solution of *tert*-Butyl (2-(benzylamino)-2-oxoethyl)carbamate (**S4**) dissolved in 1.5 mL TFA were added 50 μL TIPS and 50 μL H_2O, and the mixture was stirred at room temperature overnight. After reaction was complete, the solution was basified using saturated $NaHCO_3$ and the solvent was removed under a stream of dry air. The solid was extracted with MeOH/CH_2Cl_2 and the organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated to afford the titled compound, which was used directly without further purification: MS (ESI) calcd for $C_6H_{12}N_2O$ 164.1 [M+H$^+$], found 164.1.

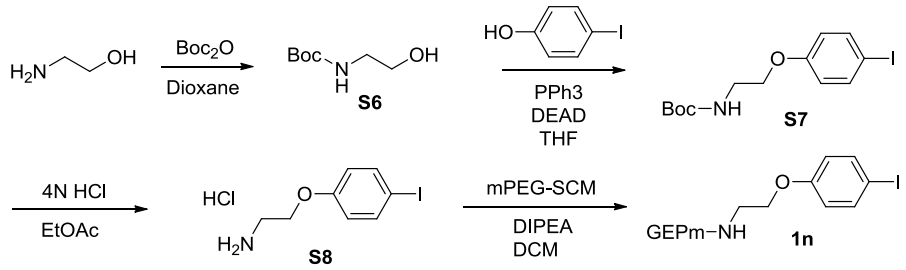


2-Acetamido-*N*-(2-(benzylamino)-2-oxoethyl)hex-5-ynamide (2a): A solution of 2-acetamido hex-5-ynoic acid (**S2**, 300 mg, 1.77 mmol), 2-amino-N-benzylacetamide (**S5**, 300 mg, 1.77 mmol), EDCI (350 mg, 1.83 mmol) and HOBt (247 mg, 1.83 mmol) in 10 mL CH_2Cl_2 was stirred at room temperature overnight. After reaction was complete, the organic layer was washed successively with 1 N HCl (30 mL) and saturated $NaHCO_3$ (30 mL), dried over anhydrous Na_2SO_4, filtered and concentrated. The resulting residue was re-dissolved in EtOAc and hexanes were added to precipitate the titled compound as a white solid (157 mg, 26% yield): ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.25-8.23 (q, 2H), 8.15 (d, *J* = 7.2 Hz, 1H), 7.30 (t, *J* = 7.3 Hz, 2H), 7.25-7.21 (q, 3H), 4.29-4.27 (q, 2H), 4.26-4.20 (m, 1H), 3.71 (d, *J* = 5.9 Hz, 2H), 2.80 (t, *J* = 2.6 Hz, 1H), 2.20-2.18 (q, 2H), 1.87-1.85 (m, 1H), 1.83 (s, 3H), 1.74-1.68 (m, 1H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 171.66,169.92, 168.70, 139.26, 128.23, 127.13, 126.74, 83.63,71.59, 52.26, 42.14, 41.97, 22.51, 14.67; MS (ESI) calcd for $C_{17}H_{21}N_3O_3$ 315.2 [M+Na$^+$], found 315.2.



4-Iodo-7-methoxy-2*H*-chromen-2-one (1d): A solution of I_2 (560 mg, 2.2 mmol) in dry ACN under argon was treated with PPh_3 (575 mg, 2.2 mmol), and the resulting yellow precipitate was

stirred at 25 ^{o}C for 15 minutes. Et_3N (0.3 mL, 2.2 mmol) and 4-hydroxy-7-methoxy-2*H*-chromen-2-one (384 mg, 2.0 mmol) were added and the mixture was refluxed for 15 minutes. The solvent was removed under reduced pressure and the residue was purified by flash chromatography using 10% EtOAc/ hexanes to give the titled compound as a pale yellow solid (38 mg, 6 % yield): ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.58 (d, *J* = 8.9 Hz, 1H), 7.02 (s, 1H), 6.89-6.86 (q, 1H), 6.76 (d, *J* = 2.5 Hz, 1H), 3.89 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.64, 158.66, 152.83, 133.63, 123.87, 119.89, 114.64, 113.25, 100.48, 55.88; MS (ESI) calcd for $C_{10}H_7IO_3$ 301.9 [M+H^{+}], found 302.1.

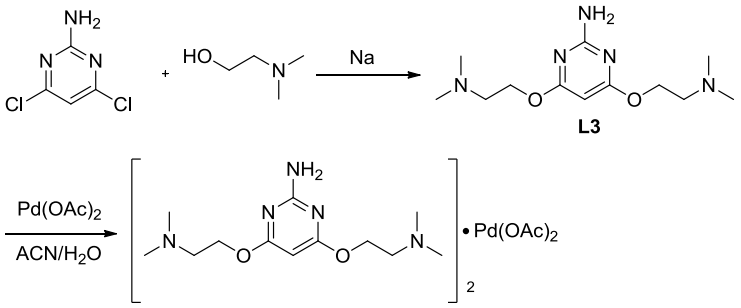


mPEG-phenyl iodide (1n): A solution of 2-aminoethanol (610 mg, 10 mmol) in dioxane (50 mL) was added $(Boc)_2O$ (2.4 g, 11 mmol), and the mixture was stirred at room temperature overnight. After reaction was complete, the solvent was removed reduced pressure and the residue was re-dissolved in 30 mL CH_2Cl_2. The solution was washed successively with 1% HCl (30 mL), brine (2 × 20 mL), H_2O (30 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated to yield **S6**, which used directly in subsequent steps without further purification: ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.99 (brs, 1H), 3.70-3.68 (q, 2H), 3.28 (d, *J* = 2.5 Hz, 1H), 6.76 (d, *J* = 5 Hz, 2H), 2.61 (brs, 1H),1.44 (s, 9H). To a solution of *tert*-butyl (2-hydroxyethyl)carbamate (**S6**, 837.2 mg, 5.2 mmol), 4-iodophenol (880 mg, 4.0 mmol) and PPh_3 (1.36 g, 5.2 mmol) in THF (6 mL) at 4 ^{o}C under argon was added 40% DEAD solution in toluene (2 mL), and the mixture was stirred at room temperature for 16 hours. The solvent was removed under reduced pressure and the residue was purified by flash chromatography using 25% EtOAc/ hexanes as an eluent to give intermediate **S7** as a pale yellow solid (1.088 g, 75% yield): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.58-7.53 (m, 2H), 6.69-6.65 (m, 2H), 4.96 (brs, 1H), 3.98 (t, *J* = 5.2 Hz, 2H), 3.53 (t, *J* = 5.3 Hz, 2H), 1.45(s, 9H); MS (ESI) calcd for $C_{13}H_{18}INO_3$ 363.0 [M+Na^{+}], found 363.1. To a solution of *tert*-butyl (2-(4-iodophenoxy)ethyl) carbamate (**S7**, 1.088g, 2.99 mmol) in 6 mL EtOAc was added 4 N HCl in EtOAc, and the mixture was stirred at room temperature for 2.5 hours. The solid formed was collected with a filter and dried in vacuum to give compound **S8** as

a white solid (816 mg, 91% yield): ^{1}H NMR (500 MHz, D_2O) δ 7.66 (d, *J* = 7.6 Hz, 2H), 6.82 (d, *J* = 7.6 Hz, 2H), 4.24 (t, *J* = 4.3 Hz, 2H), 3.41 (t, *J* = 4.4 Hz, 2H); ^{13}C NMR (125 MHz, D_2O) δ 157.47, 138.43, 116.94, 83.42, 63.86, 38.81; MS (ESI) calcd for $C_8H_{10}INO$ 263.0 [M+H$^+$], found 263.0. To a solution of **S8** (29.9 mg, 0.1 mmol) and DIPEA (70 μL, 0.4 mmol) in 2 mL CH_2Cl_2 was added dropwise a solution of mPEG-SCM (200 mg, ~5 kDa, 0.04 mmol) in 1 mL CH_2Cl_2, and the mixture was stirred at room temperature overnight. After adding pivaloyl chloride (50 μL, 0.04 mmol), the solution became clear after stirring for 30 minutes. To the above solution was added 10 mL anhydrous Et_2O and the mixture was cooled over dry ice. The white precipitate was collected by filtration, washed with anhydrous Et_2O, and dried in vacuum to afford the titled compound as a white powder (159 mg, 77% yield): ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.25 (brs, 21 H), 7.59-7.55 (m, 2H), 6.71-6.69 (m, 2H), 4.30 (s, 3H), 4.25-4.21 (m, 2H), 4.04 (t, J = 5.5, 2H), 4.02 (s, 1H), 3.81-3.77 (m, 5H), 3.73-3.62 (m, 381H), 3.58-3.55 (m, 4H), 3.54-3.50 (m, 4H), 3.39 (s, 3H), 3.13-3.08 (m, 10H), 1.92 (s, 6H), 1.59-1.55 (m, 33H), 1.49-1.46 (m, 22H), 1.27 (s, 3H).

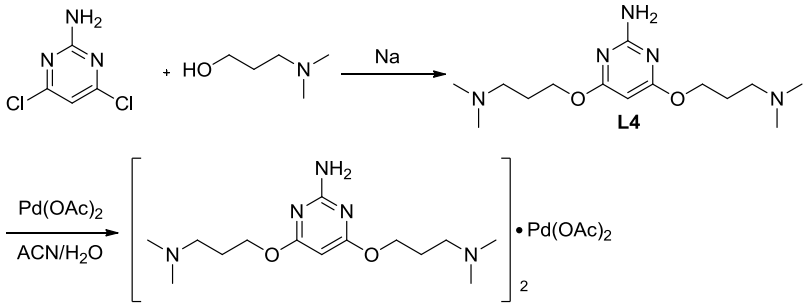
Preparation of palladium catalysts

The palladium catalysts with **L1**, **L2**, **L6** and **L7** ligands were prepared according to a literature procedure.[S1]

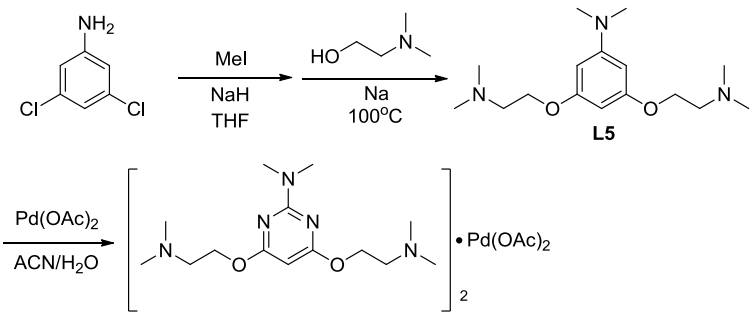


2,2'-((2-Aminopyrimidine-4,6-diyl)bis(oxy))bis(*N*,*N*-dimethylethanamine) (L3): A solution of sodium (2.3 g, 100 mmol) in 2-(dimethylamino)ethanol (50 mL, 500 mmol) was treated with 4,6-dichloropyrimidin-2-amine (8.15 g, 50 mmol). The mixture was stirred at 100 ^{o}C for 1 hour before cooling down to room temperature. After removing the solid with centrifugation, the solvent was evaporated and the residue was purified by flash chromatography using MeOH/CH_2Cl_2 containing 1% Et_3N as an eluent to afford **L3** as a pale yellow solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.51 (d, *J* = 0.7 Hz, 1H), 4.82 (brs, 2H), 4.28 (t, *J* = 5.8 Hz, 4H), 2.64 (t, *J* = 5.8 Hz, 4H), 2.29 (s,

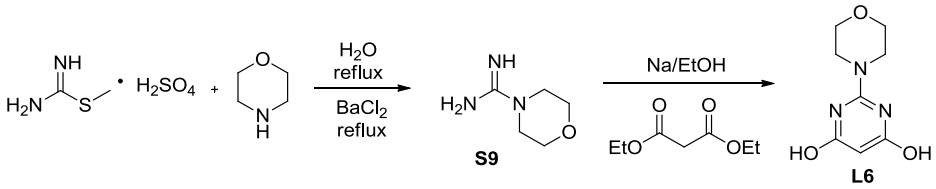
12H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.00, 162.18, 80.88, 64.20, 58.16, 45.96; MS (ESI) calcd for $C_{12}H_{23}N_5O_2$ 269.2 [M+H$^+$], found 269.2. **L3-palladium complex: L3** ligand (13.5 mg, 0.05 mmol) was dissolved in 0.5 mL ACN and the mixture was heated at 65 ^{o}C for 5 minutes. $Pd(OAc)_2$ (5.5 mg, 0.025 mmol) was then added and the heating continued for another 30 minutes before cooling to room temperature. The clear solution was diluted to 2.5 mL with ddH_2O to yield the desired palladium complex.



3,3'-((2-Aminopyrimidine-4,6-diyl)bis(oxy))bis(*N*,*N*-dimethylpropan-1-amine) (L4): A solution of sodium (0.23 g, 10 mmol) in 3-(dimethylamino)propan-1-ol (5.8 mL, 50 mmol) was treated with 4,6-dichloropyrimidin-2-amine (1.63 g, 10 mmol). The mixture was stirred at 100 ^{o}C for an hour before cooling down to room temperature. The solid was removed by centrifugation solid and the solution was concentrated. The resulting residue was purified by flash chromatography MeOH/CH_2Cl_2 containing 1% Et_3N as an eluent to afford **L4** as a pale yellow solid: ^{1}H NMR (300 MHz, DMSO-*d*6) δ 6.49 (brs, 2H), 5.29 (s, 1H), 4.16 (t, *J* = 6.7 Hz, 4H), 2.29 (t, *J* = 7.1 Hz, 4H), 2.13 (s, 12H), 1.81-1.72 (m, 4H); ^{13}C NMR (75 MHz, DMSO-*d*6) δ 171.32, 162.73, 78.21, 63.89, 55.60, 45.10, 26.58; MS (ESI) calcd for $C_{14}H_{27}N_5O_2$ 297.2 [M+H$^+$], found 297.2. **L4-palladium complex: L4** (29.8 mg, 0.10 mmol) was dissolved in 1.0 mL ACN, and the mixture was heated at 65 ^{o}C for 5 minutes. $Pd(OAc)_2$ (11.0 mg, 0.05 mmol) was then added and the heating was allowed to continue for another 30 minutes before cooling to room temperature. The clear solution was diluted to 5.0 mL with dd H_2O to yield the desired palladium complex.

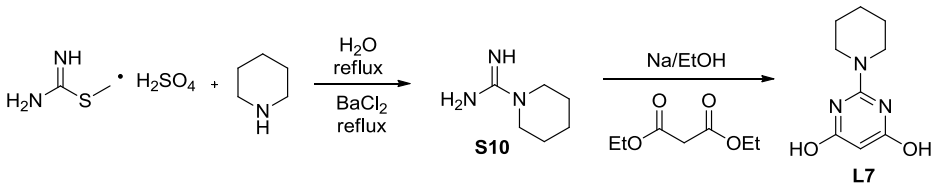


2,2'-((5-(Dimethylamino)-1,3-phenylene)bis(oxy))bis(*N*,*N*-dimethylethanamine) (L5): A solution of 4,6-dichloropyrimidin-2-amine (328 mg, 2 mmol) in THF (8 mL) at 0 °C was added NaH (320 mg, 8 mmol) followed by MeI (250 μL, 4 mmol; add slowly). After addition, the solution was warmed to room temperature and the reaction progress was monitored by TLC. After the reaction reached completion, the solvent was removed under reduced pressure at a temperature below 30 °C. The resulting solid was used directly without further purification. To a solution of sodium (184 mg, 8 mmol) in 2-(dimethylamino)ethanol (4 mL, 40 mmol) was added the crude product from the last step (382 mg). The mixture was stirred at 100 ^{o}C for an hour before cooling down to room temperature. The solid was removed by centrifugation solid and the solution was concentrated. The resulting residue was purified by flash chromatography MeOH/CH_2Cl_2 containing 1% Et_3N as an eluent to afford **L5** (61.1 mg, 10% yield over 2 steps): ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.37 (s, 1H), 4.35 (t, *J* = 6.1 Hz, 4H), 3.11 (s, 6H), 2.65 (t, *J* = 6.1 Hz, 4H), 2.29 (s, 12H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.37, 161.53, 78.29, 63.68, 8.25, 46.01, 36.91; MS (ESI) calcd for $C_{16}H_{29}N_3O_2$ 295.2 [M+H^+], found 295.3. **L5-palladium complex: L5** (14.85 mg, 0.05 mmol) was dissolved in 0.5 mL ACN, and the mixture was heated at 65 ^{o}C for 5 minutes. $Pd(OAc)_2$ (5.5 mg, 0.05 mmol) was added and the heating was allowed to continue for another 30 minutes before cooling down to room temperature. The clear solution was diluted to 2.5 mL with dd H_2O to yield the desired palladium complex.



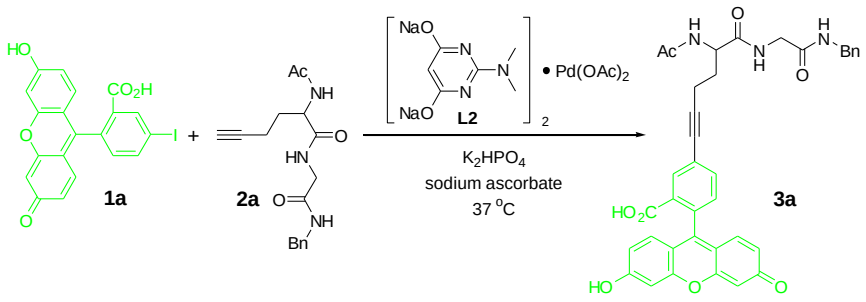
2-Morpholinopyrimidine-4,6-diol (L6): *S*-Methylisothiourea sulfate (13.9 g, 74 mmol), H_2O (36.7 mL) and morpholine (9 mL, 103 mmol) were added to a flask fitted with a reflux condenser. The mixture was slowly heated to boiling and the reflux was maintained for 5 minutes. A hot solution of $BaCl_2$ (12.2 g, 59 mmol) in 25 mL H_2O was added, and the mixture was then heated for additional 30 minutes. After filtration, the solvent was removed by applying a stream of dry air and the residue was re-dissolved in hot ethanol (10 mL) and acetone (75 mL). A while solid was collected with a filtration funnel to afford morpholine-4-carboximidamide **S9** as a hydrochloride salt (4.70 g, 49% yield): ^{1}H NMR (300 MHz, D_2O) δ 3.83 (t, *J* = 4.6 Hz, 4H), 3.51 (t, *J* = 4.1 Hz, 4H); ^{13}C NMR (75 MHz, D_2O) δ 156.57, 65.41, 45.17. Sodium (1.38 g, 60 mmol) was cut into small

pieces and added slowly into EtOH (40 mL). After all the sodium was completely dissolved, **S9** (3.3 g, 20 mmol) and diethylmalonate (3.05 mL, 20 mmol) were added. The mixture was refluxed for 3 hours before cooling down to room temperature. The solvent was removed under reduced pressure and 1 N HCl (64 mL) was added to the resulting white solid. The solid was dissolved initially and then gradually reappeared over a course of 1 hour. The solid was collected with a filter, washed with H_2O (3 × 30 mL), and dried to give the desired product as a white solid (1.57 g, 40% yield): ^{1}H NMR (300 MHz, D_2O) δ 10.69 (brs, 2H), 4.79 (s, 1H), 3.62-3.54 (m, 8H); ^{13}C NMR (75 MHz, D_2O) δ 168.82, 155.96, 79.57, 66.09, 44.78; MS (ESI) calcd for $C_8H_{11}N_3O_3$ 197.1 [M+H$^+$], found 197.2.



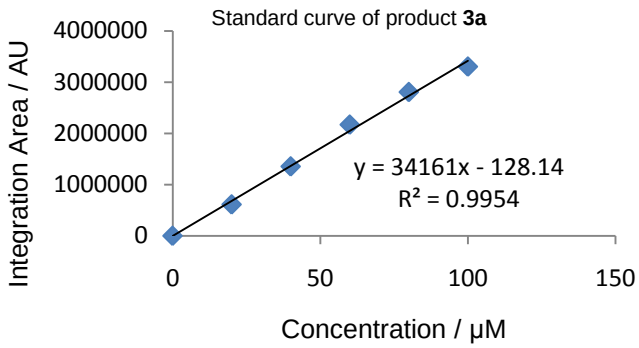
2-(Piperidin-1-yl)pyrimidine-4,6-diol (L7): *S*-Methylisothiourea sulfate (13.9 g, 74 mmol), H_2O (36.7 mL) and piperidine (10 mL, 103 mmol) were placed in a flask fitted with a reflux condenser. The mixture was slowly heated to boiling and the reflux was maintained for 5 minutes. A hot solution of $BaCl_2$ (12.2 g, 58.65) in 25 mL H_2O was added and the mixture was then heated for additional 30 minutes. After filtration, the solvent was removed by applying a stream of dry air and the residue was re-dissolved in hot ethanol (10 mL) and acetone (75 mL). A while solid was collected with a filtration funnel to afford 3.35 g piperidine-1-carboximidamide **S10** as a hydrochloride salt, which used directly for next step. Sodium (1.38 g, 60 mmol) was cut into small pieces and slowly added into EtOH (40 mL). After all the sodium was completely dissolved, **S10** (3.35 g, 21 mmol) and diethyl malonate (3.13 mL, 21 mmol) were added. The mixture was refluxed for 3 hours before cooling down to room temperature. The solvent was removed under reduced pressure and 1 N HCl (64 mL) was added to the resulting white solid. The solid was dissolved initially and then gradually reappeared over a course of 1 hour. The solid was collected with a filter, washed with H_2O (3 × 30 mL), and dried to give the desired product as a white solid (0.732 g, 5% yield over 2 steps): ^{1}H NMR (300 MHz, DMSO-*d*6) δ 10.55 (brs, 2H), 4.68 (s, 1H), 3.56 (t, *J* = 5.0 Hz, 4H), 1.58-1.47 (m, 6H); ^{13}C NMR (75 MHz, DMSO-*d*6) δ 168.16, 154.31, 78.46, 44.98, 25.15, 23.95; MS (ESI) calcd for $C_9H_{13}N_3O_2$ 195.1 [M+Na$^+$], found 195.2.

Screen of additional reaction conditions

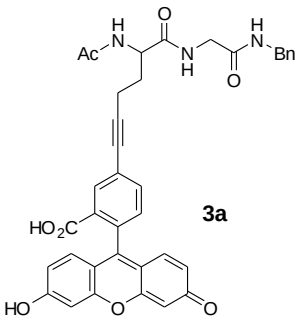


Entry	**1a** : **2a**	% palladium · ligand complex	Ligand	Time	Yield (%)[a]
8	1.05:2.40	30%	**L2**	40 min	91
9	1.10:1.00	20%	**L2**	30 min	58
10	1.20:1.00	20%	**L2**	30 min	55
11	1.30:1.00	20%	**L2**	30 min	46
12	1.00:1.05	20%	**L2**	30 min	58
13	1.00:1.10	20%	**L2**	30 min	86
14	1.00:1.15	20%	**L2**	30 min	78
15	1.00:1.20	20%	**L2**	30 min	84
16	1.00:1.25	20%	**L2**	30 min	80
17	1.00:1.30	20%	**L2**	30 min	73
18	1.00:1.40	20%	**L2**	30 min	76
19	1.00:1.50	20%	**L2**	30 min	74
20	1.00:1.10	5%	**L2**	30 min	3
21	1.00:1.10	10%	**L2**	30 min	27
22	1.00:1.10	15%	**L2**	30 min	64

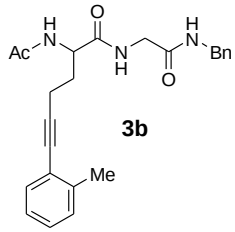
[a] Yields were derived by comparing the integration area of **3a** in their respective reverse-phase HPLC traces to that of a standard curve as follows:



Sonogashira cross-coupling from Tables 1 and 2

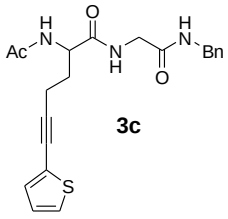


2-(6-Hydroxy-3-oxo-3*H*-xanthen-9-yl)-5-iodobenzoic acid (**1a**, 1.832 mg, 0.004 mmol), 2-acetamido-*N*-(2-(benzylamino)-2-oxoethyl)hex-5-ynamide (**2a**, 1.512 mg, 0.0048 mmol) in 20 μL DMSO, and K_2HPO_4 (21 mg, 0.12 mmol) were placed into an 1.5 mL glass vial followed by addition of deionized H_2O (41 μL). Afterwards, sodium ascorbate (0.55 mg, 0.0027 mmol) in 3.7 μL deionized H_2O and an aliquot of 60 μL of 10 mM $Pd(OAc)_2$· **L2** complex solution (0.6 μmol) were added by a micropipette. The vial was capped and the reaction was stirred at 37 ^oC for 20 minutes. A second batch of **2a** (1.512 mg, 0.0048 mmol) in 20 μL DMSO, sodium ascorbate (0.55 mg, 0.0027 mmol) in 3.7 μL deionized H_2O and a 60 μL of 10 mM $Pd(OAc)_2$· **L2** complex solution (0.6 μmol) were added and the mixture was stirred for additional 20 minutes. After a total of 40 minutes, the solution was cooled to room temperature, diluted with MeOH/H_2O (1:1), and analyzed by reverse-phage HPLC equipped with a C18 column and a UV-Vis detector. The yield was determined by comparing the integration area of absorption peak at 254 nm of product **3a** in the mixture to that of a standard curve (91%): ^{1}H NMR (300 MHz, CD_3OD) δ 8.31 (brs, 1H), 7.98 (brs, 1H), 7.75 (dd, *J* = 7.5 Hz, 1.4 Hz, 1H), 7.29-7.18 (m, 6H), 7.14 (d, *J* = 8.0 Hz, 1H), 6.67 (d, *J* = 2.2 Hz, 2H), 6.60-6.51 (m, 4H), 5.49 (s, 1H), 4.50-4.34 (q, 1H), 4.43-4.35 (m, 4H), 3.96-3.82 (m, 4H), 2.61 (t, *J* = 7.3 Hz, 2H), 2.20-2.14 (m, 1H), 1.97 (s, 1H), 1.95-1.92 (m, 1H); ^{13}C NMR (75 MHz, CD_3OD) δ 174.64, 174.09, 171.42, 170.71, 161.47, 154.07, 139.75, 139.42, 130.14, 129.48, 128.69, 128.47, 128.16, 127.11, 125.47, 113.67, 111.03, 103.56, 91.84, 80.94, 54.81, 54.59, 43.95, 43.69, 31.30, 22.47, 16.86; HRMS m/z (ESI) calcd for $C_{37}H_{31}N_3O_8$ 645.2184 [M+H$^+$], found 645.2189.



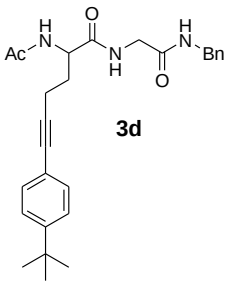
3b

The same procedure was used to obtain compound **3b** in 84% conversion:^{1}H NMR (500 MHz, CD_3OD) δ 7.33-7.07 (m, 9H), 4.46-4.34 (m, 3H), 3.89 (q, 2H),2.66-2.57 (m, 1H), 2.38 (s, 3H), 2.25 (d, *J* = 5.0 Hz, 1H), 2.19-2.12 (m, 1H), 2.00-1.90 (m, 4H); ^{13}C NMR (125 MHz, CD_3OD) δ 174.78, 174.01, 171.28, 167.27, 141.04, 139.61, 132.88, 130.31, 129.47, 128.88, 128.46, 128.14, 126.54, 124.68, 101.76, 93.17, 54.74, 43.86, 43.68, 31.71, 22.48, 16.95; HRMS m/z (ESI +) calcd for $C_{24}H_{27}N_3O_3$ 405.1945, [M + Na^+] found 405.1946.



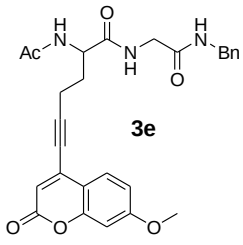
3c

The same procedure was used to obtain compound **3c** in 95% conversion: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.33-7.27 (m, 2H), 7.24-7.17 (m, 3H), 7.12-7.11 (q, 1H), 6.94-6.91 (q, 1H), 6.58 (d, *J* = 7.1 Hz, 1H), 4.57-4.34 (m, 3H), 4.08-3.85 (m, 2H), 2.61-2.40 (m, 2H), 2.15-2.04 (m, 1H), 1.99-1.92 (m, 1H), 1.90 (s, 3H), 1.75 (brs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.85, 171.14, 168.54, 138.00, 131.66, 128.79, 127.90, 127.63, 127.06, 126.63, 123.35, 92.41, 77.36, 75.29, 53.46, 43.64, 43.43, 30.55, 23.12, 16.46; HRMS (ESI) m/z calcd for $C_{24}H_{27}N_3O_3$ 397.1352 [M + Na^+], found 397.1357.



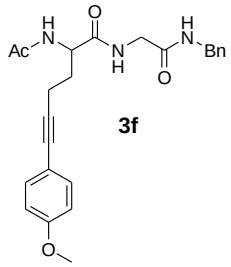
3d

The same procedure was used to obtain compound **3d** in 77% conversion: ^{1}H NMR (300 MHz, CD_3OD) δ 8.52 (brs, 3H), 7.35-7.24 (m, 9H), 4.46-4.31 (m, 3H), 3.89 (q, 2H), 2.53 (t, *J* = 7.0 Hz, 2H), 2.15-2.06 (m, 1H), 2.02-1.88 (m, 4H), 1.30 (s, 9H); HRMS (ESI) m/z calcd for $C_{27}H_{33}N_3O_3$ 447.2428 [M+Na^+], found 447.2422.



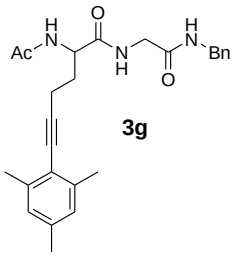
3e

The same procedure was used to obtain compound **3e** in 88% conversion: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.65 (d, *J* = 8.8 Hz, 1H), 7.52 (t, *J* = 5.6 Hz, 1H), 7.30-7.18 (m, 4H), 6.98 (t, *J* = 5.7 Hz, 1H),, 6.85-6.78 (m, 2H), 6.72 (d, *J* = 2.5 Hz, 1H), 6.29 (s, 1H), 4.70-4.63 (q, 1H), 4.40 (d, *J* = 5.9 Hz, 2H), 4.08-3.90 (m, 2H), 3.84 (s, 3H), 2.64-2.60 (m, 2H), 2.26-2.15 (m, 1H), 2.06-1.97 (m, 1H), 1.93 (s, 3H), 1.80 (brs, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.76, 171.09, 168.556, 163.28, 161.09, 155.31, 137.88, 137.81, 128.78, 127.81, 127.66, 115.21, 112.82, 112.32, 102.28, 100.82, 75.98, 55.96, 52.79, 43.67, 43.34, 30.96, 23.13, 16.62; HRMS (ESI) m/z calcd for $C_{27}H_{27}N_3O_6$ 489.1805 [M+Na^+], found 489.1799.



3f

A similar procedure was used to prepare compound **3f** in 70% conversion: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.34-7.29 (m, 4H), 7.28-7.27 (m, 2H), 7.25-7.24 (m, 1H), 6.87-6.77 (m, 4H),, 6.36 (d, *J* = 6.4 Hz, 1H), 4.54-4.36 (m, 3H), 4.11-3.85 (m, 2H), 3.80 (s, 3H), 2.54-2.45 (m, 2H), 2.16-2.07 (m, 1H), 2.02-1.95 (m, 1H), 1.90 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.81, 171.18, 168.43, 159.55, 142.30, 138.05, 133.07, 131.91, 128.79, 127.95, 127.93, 127.62, 117.31, 115.28, 114.11, 109.48, 86.61, 82.22, 55.43, 53.81, 43.64, 43.43, 30.37, 23.16, 16.25; HRMS (ESI) *m/z* calcd for $C_{24}H_{27}N_3O_4$ 421.1894 [M+Na^+], found 421.1886.



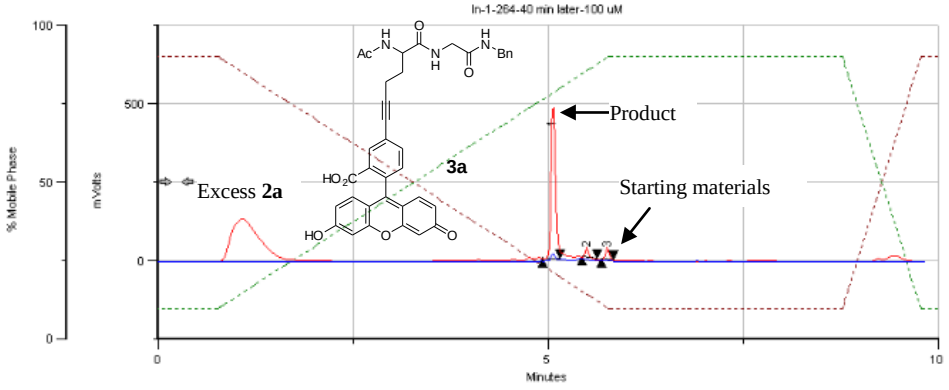
3g

A similar procedure was used to prepare compound **3g** in 72% conversion: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.52 (s, 1H), 7.29-7.26 (m, 5H), 6.83 (s, 1H), 4.47-4.29 (m, 3H), 3.97-3.80 (q, 2H), 3.07 (t, *J* = 1.7 Hz, 1H), 2.66-2.60 (m, 3H), 2.33 (s, 3H), 2.23-2.13 (m, 4H), 2.01-1.84 (m, 4H); HRMS (ESI) *m/z* calcd for $C_{26}H_{31}N_3O_3$ 433.2258 [M+Na^+], found 433.2262.

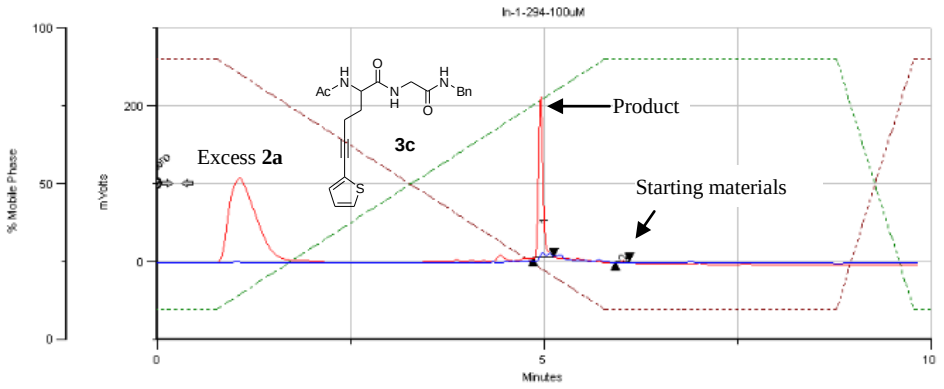
Representative HPLC traces for reactions in Table 2

The reaction mixture was resolved by reverse-phage HPLC equipped with a C18 column running a linear 10-90% acetonitrile/H_2O containing 0.1% formic acid gradient over 6 minutes. The conversions were calculated based on the disappearance of aryl iodides, whose amounts were quantified by their absorption at 254 nm (red line).

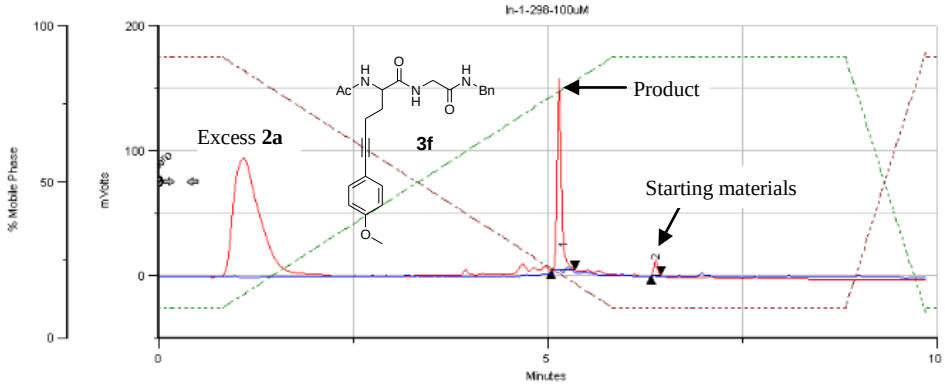
	Inj. Number	Peak Name	Sample Descrip.	R. Time	Area	Area %
1	2.00	*1	-40 min later	5.06	3102403.50	89.85
2	2.00	2	-40 min later	5.50	151335.39	4.38
3	2.00	3	-40 min later	5.76	199198.34	5.77
4						
5						



	Inj. Number	Peak Name	Sample Descrip.	R. Time	Area	Area %
1	3.00	*1	1-294-100ul	4.95	1404592.50	99.03
2	3.00	*2	1-294-100ul	6.02	13779.00	0.97
3						
4						
5						



	Inj. Number	Peak Name	Sample Descrip.	R. Time	Area	Area %
1	4.00	*1	1-298-100ul	5.14	993337.50	93.27
2	4.00	*2	1-298-100ul	6.39	71668.91	6.73
3						
4						
5						



Cloning of an elongated ubiquitin

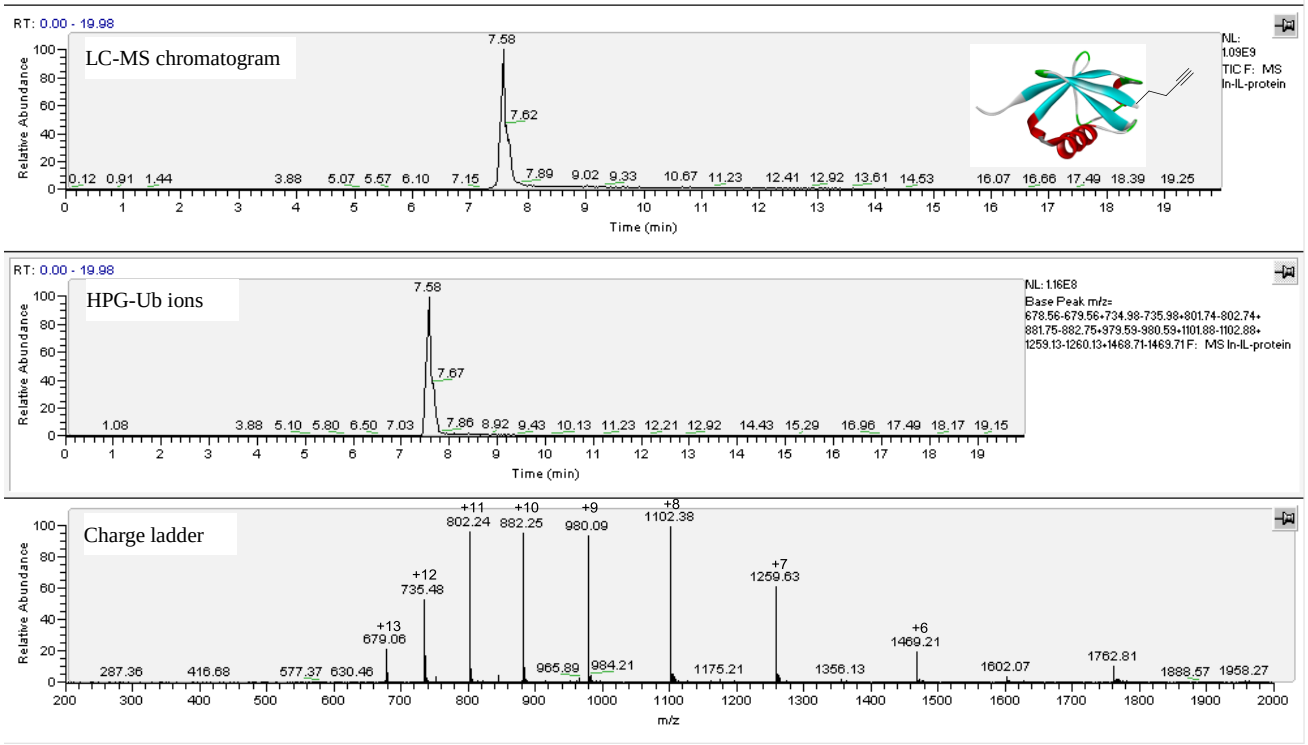
Using pET28a-Ub plasmid as a template, we added a human rhinovirus 3C protease recognition sequence after the His_6 tag and the Met-Gly-Gly sequence at the C-terminus of ubiquitin through PCR with a pair of primers: 5'-ATTATATGGATCCCTGGAAGTTCTGTTCCAGGGGCCCCAGATTTTTGTGAAAACCCTGAC-3' (forward primer with the BamHI site underlined) and 5'-ATATATTAAAGCTTTTATTAACCGCCCATACCGCCGCGCAGA-3' (reverse primer with the HindIII site underlined). The PCR fragment was digested with BamHI and HindIII restriction enzymes, and the digested fragment was ligated into the BamHI/HindIII sites of pQE80 to obtain ubiquitin expression plasmid pQE80L-PUbF. The clones containing the correct inserts were verified by DNA sequencing.

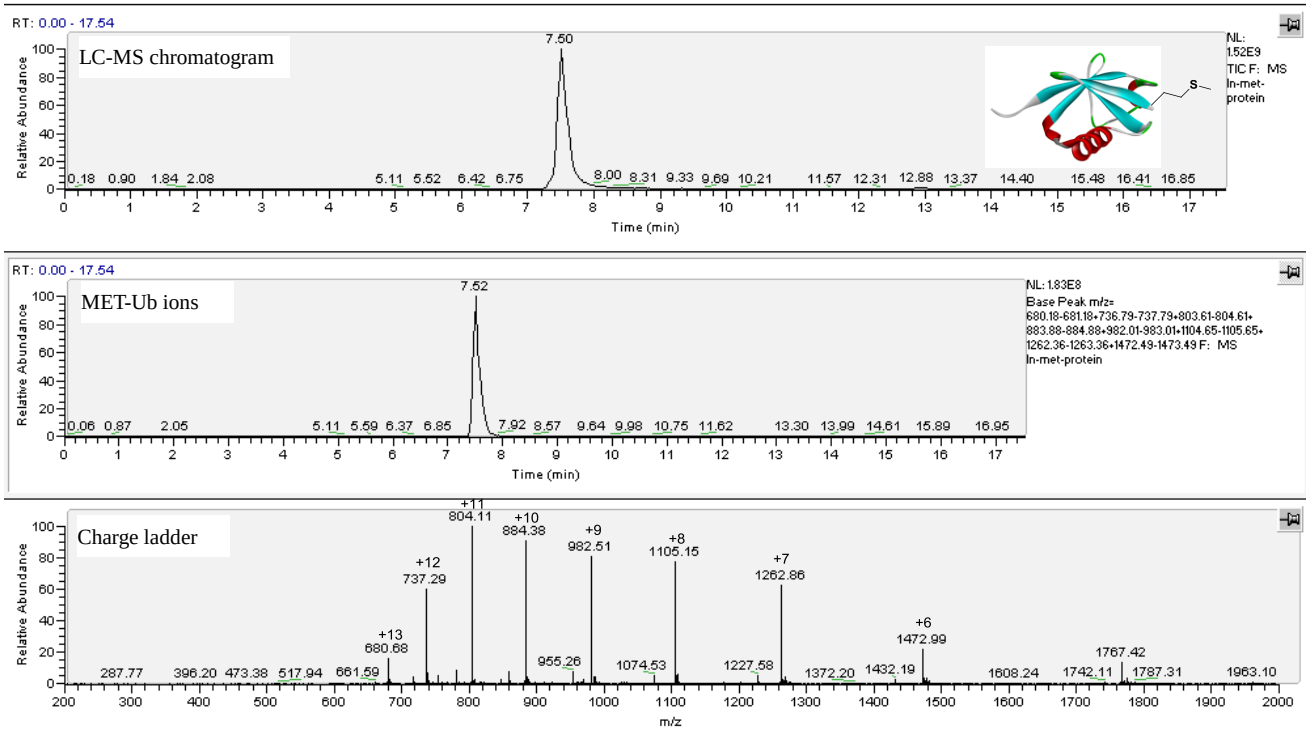
Expression and purification of HPG-encoded and wild-type ubiquitin

For expression of HPG-ubiquitin protein (HPG-Ub), a single colony of M15A—a methionine auxotroph—bearing pQE80L-PUbF plasmid was grown at 37 °C overnight in 8 mL of SelenoMet plus nutrient mix (a methionine-deficient medium) supplemented with 1 mM L-methionine and 100 μg/mL ampicillin. This starter culture was used to inoculate 800 mL of the same medium where cells were grown at 37^{o}C until the cell density (OD_{600}) reached 0.8-1.0. Afterwards, a medium shift was performed by first washing the cell pellet with 0.9% NaCl three times followed by re-suspending the cells in SelenoMet plus nutrient mix containing 100 μg/mL ampicillin. Cells were allowed to grow for additional 1 hour in order to deplete the intracellular methionine stock right before the induction of protein expression. Subsequently, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 1 mM homopropargylglycine (HPG) were added to the culture medium and the expression was allowed to continue at 37^{o}C for 4-5 hours. Cells were harvested by centrifugation at 6,000 rpm for 15 minutes and the cell pellets were stored at -20 °C. For expression of wild-type ubiquitin, the same procedure was followed except that LB broth containing ampicillin was used and that no medium shift was performed. Cell pellets were lysed by sonication and the supernatants were collected after removal of cell debris through centrifugation. The lysates were then subjected to Ni-NTA affinity chromatography to yield His_6-tagged ubiquitin proteins. To avoid possible interference from His-tag during the subsequent palladium-catalyzed cross-coupling reaction, an appropriate amount of PreScission protease was added to HAG-Ub or Ub in a Tris buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.0), and the mixture was incubated at 4 ^{o}C for 16 hours. The cleavage solutions were then adjusted to pH 8 before subjected to fast protein liquid chromatography (FPLC) using a Mono Q 5/50 GL column and a gradient of 20 mM Tris-HCl and 1.0 M NaCl, pH 8.0, as elution buffer. Fractions that correspond to the pure proteins were collected, desalted, and concentrated using Amicron Ultra centrifugal filters (MWCO ≈ 3K). Finally, the concentrations of both wild-type (Met-Ub) and mutant ubiquitin (HPG-Ub) were quantified via Bradford assay to be 0.86 mg/mL and 2.22 mg/mL, respectively.

Characterization of HPG-Ub and Met-Ub by LC-MS

Protein mass was determined by ESI-MS using a Jupiter C4 column (5 μ, 300 Å, 2.00 × 50 mm), a gradient of 5-95% ACN/H_2O containing 0.1% HCOOH over 25 min with the first 5 min going to the waste, and a flow rate of 200 μL. The molecular masses for both proteins were obtained by deconvoluting their respective multiply charged protein mass spectra (see the assigned charge ladders as follows). For HPG-Ub (sequence = GPQIFVKTLT GKTITLEVEP SDTIENVKAK IQDKEGIPPD QQRLIFAGKQ LEDGRTLSDY NIQKESTLHL VLRLRGGHpgGG), calcd mass 8811.1 Da, found 8812.1 ± 1.8 Da. The occupancy of HPG in ubiquitin was calculated to be 92% based on the following equation: occupancy % = $I_{\text{HPG-Ub}}/(I_{\text{HPG-Ub}} + I_{\text{Met-Ub}})$, where $I_{\text{HPG-Ub}}$ and $I_{\text{Met-Ub}}$ were ion counts of HAG-Ub and Met-Ub, respectively. For Met-Ub (sequence = GPQIFVKTLT GKTITLEVEP SDTIENVKAK IQDKEGIPPD QQRLIFAGKQ LEDGRTLSDY NIQKESTLHL VLRLRGGMGG), calcd mass 8833.1 Da, found 8833.8 ± 1.1 Da.





Sonogashira cross-couplings with HPG-Ub from Table 3

Three slightly different conditions were employed in conducting the cross-coupling reactions with HPG-Ub depending on the activity of specific aryl iodide structure.

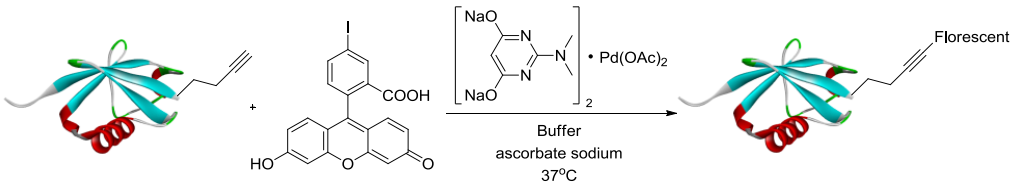
(1) For entries 1-4, 6, and 8-11, the following procedure was used: A stock solution of 10 mM aryl iodide was prepared by dissolving aryl iodide in DMSO, and a stock solution of 80 mM sodium ascorbate was prepared by dissolving 29.5 mg of sodium ascorbate in 1.875 mL ddH_2O. To an ice-chilled 0.75-mL eppendorf tube were added sequentially an aliquot of aryl iodide (24 μL, 0.25 μmol), an aliquot of 10 mM $Pd(OAc)_2$· **L2** complex solution (25 μL, 0.25 μmol), and an aliquot of sodium ascorbate (25 μL, 2.0 μmol), and the mixture was stirred at 37 °C for 60 minutes to obtain the activated reagent cocktail. Separately, to a 0.75-mL eppendorf tube containing 90.6 μL (for entry 8, 40.6 μL) solution of Na_2HPO_4 (15 nmol) in H_2O was added 2 μL of HPG-Ub protein stock (2.22 mg/mL in 50 mM Na_2HPO_4 buffer, pH 8.0; 0.5 nmol) and 3.7 μL of the activated reagent cocktail (25 equiv), and the mixture was stirred at 37 ^oC for 15 minutes before a second batch of the activated reagent cocktail (3.7 μL; 25 equiv) was added. The stirring was allowed to continue for another 15 minutes before the reaction was quenched by adding 20 μL of 0.5% 3-mercaptopropanoic acid solution in water (1.14 μmol). The reaction mixture was analyzed directly by LC-MS.

(2) For entries 7, 12-13, the following procedure was used: A stock solution of 10 mM aryl iodide was prepared by dissolving the corresponding aryl iodide in DMSO, and a stock solution of 80 mM sodium ascorbate was prepared by dissolving 29.5 mg of sodium ascorbate in 1.875 mL ddH_2O. To an ice-chilled 0.75-mL eppendorf tube were added sequentially an aliquot of aryl iodide (24 μL, 0.25 μmol), an aliquot of 10 mM $Pd(OAc)_2$· **L2** complex solution (25 μL, 0.25 μmol), and an aliquot of sodium ascorbate (25 μL, 2.0 μmol), and the mixture was stirred at 37 °C for 60 minutes to obtain the activated reagent cocktail. Separately, to a 0.75-mL eppendorf tube containing 40.6 μL solution of Na_2HPO_4 (15 nmol) in H_2O was added 2 μL of HPG-Ub protein stock (2.22 mg/mL in 50 mM Na_2HPO_4 buffer, pH 8.0; 0.5 nmol) and 3.7 μL of the activated reagent cocktail (25 equiv), and the mixture was stirred at 37 ^oC for 1.5 hours before a second batch of the activated reagent cocktail (3.7 μL, 25 equiv) was added. The stirring was allowed to continue for another 1.5 hours before the reaction was quenched by adding 20 μL of 0.5% 3-mercaptopropanoic acid solution in water (1.14 μmol). The reaction mixture was analyzed directly by LC-MS.

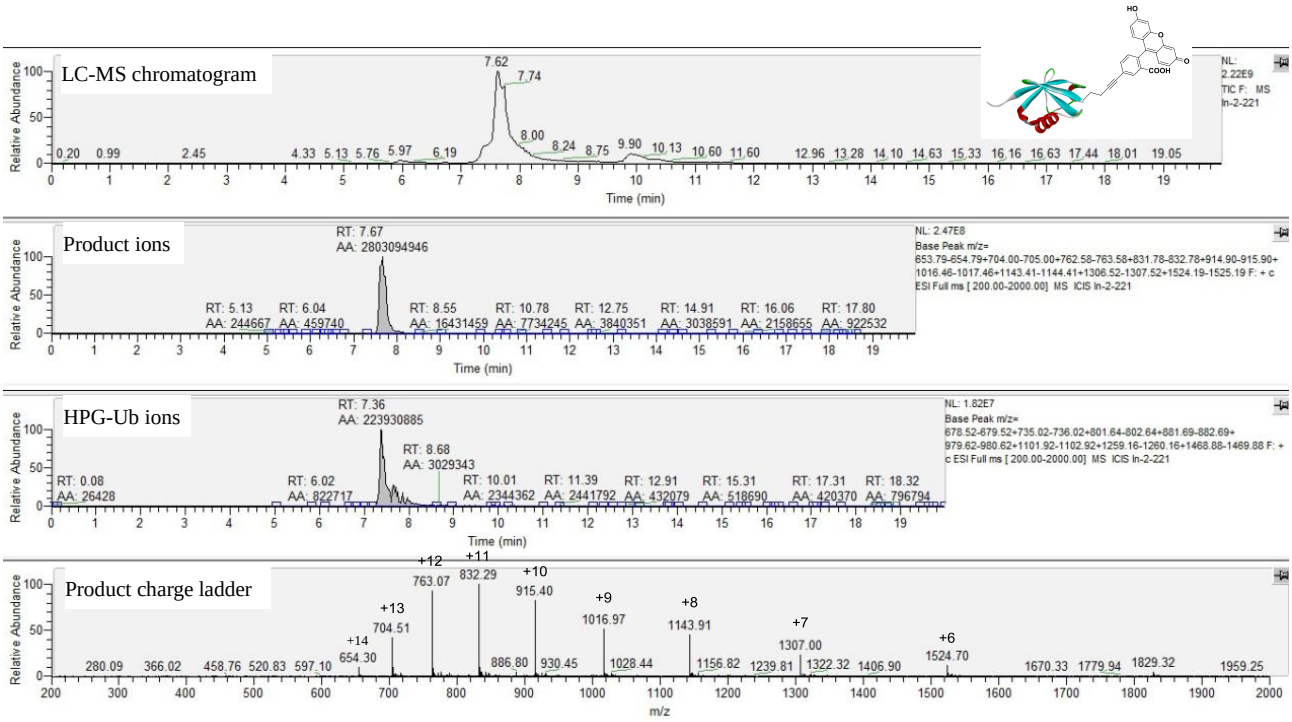
(3) For entry 5, the following procedure was used: A stock solution of 10 mM 4-iodo-7-methoxy-2*H*-chromen-2-one was prepared by dissolving 0.79 mg of 4-iodo-7-methoxy-2*H*-chromen-2-one in 0.523 mL acetonitrile. To an ice-chilled 0.75-mL eppendorf tube were added successively an aliquot of 4-iodo-7-methoxy-2*H*-chromen-2-one (50 μL, 0.25 μmol) and an aliquot of 10 mM $Pd(OAc)_2$· **L2** complex solution (25 μL, 0.25 μmol), and the mixture was stirred at 37 °C for 60 minutes to derive the activated reagent cocktail. Separately, to a 0.75-mL eppendorf tube containing 40 μL solution of Na_2HPO_4 (250 nmol) in H_2O was added 2 μL of HPG-Ub protein stock (2.22 mg/mL in 50 mM Na_2HPO_4 buffer, pH 8.0; 0.5 nmol) and 3.8 μL of the activated reagent cocktail (25 equiv), and the mixture was stirred at 37 ^oC for 2 hours before a second batch of the activated reagent cocktail (3.8 μL, 25 equiv) was added. The stirring was allowed to continue for additional 2 hours before the reaction was quenched by adding 20 μL of 0.5% 3-mercaptopropanoic acid solution in water (1.14 μmol). The reaction mixture was then analyzed directly by LC-MS.

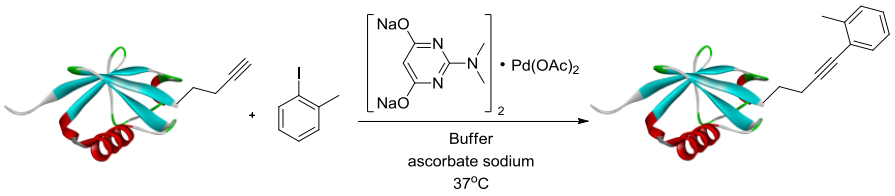
For LC-MS analysis, we used a Finnigan LCQ Advantage IonTrap mass spectrometry coupled with a Surveyor HPLC system with ion counts as readout (no UV-Vis detector was attached). Protein liquid chromatography was performed using a Phenomenex Jupiter C4 column (5 μm, 300 Å, 2.00 × 50 mm) with a flow rate of 200 μL/min and a linear gradient of 5-95% ACN/H_2O containing 0.1%

HCOOH. After obtaining the LC-MS raw data, we identified the masses of individual protein species (Met-Ub, HPG-Ub, and cross-coupling product) by performing ion extraction using the respective m/z values of the base peaks followed by area integration to obtain the amounts of the individual protein ions. When an appreciable amount of side product was detected which can be attributed to HPG-Ub, the area integration of the side product was also included in the yield calculation. The following equations were used in determining the reaction yield: yield % = $I_{product}/(I_{HPG\text{-}Ub} + I_{product})$ when there were no detectable side products, or yield % = $I_{product}/(I_{HPG\text{-}Ub} + I_{product} + I_{side\ product})$ when side products were detected; where $I_{HPG\text{-}Ub}$, $I_{product}$, and $I_{side\ product}$ represent the ion counts of the remaining HPG-Ub, product, and side product, respectively.

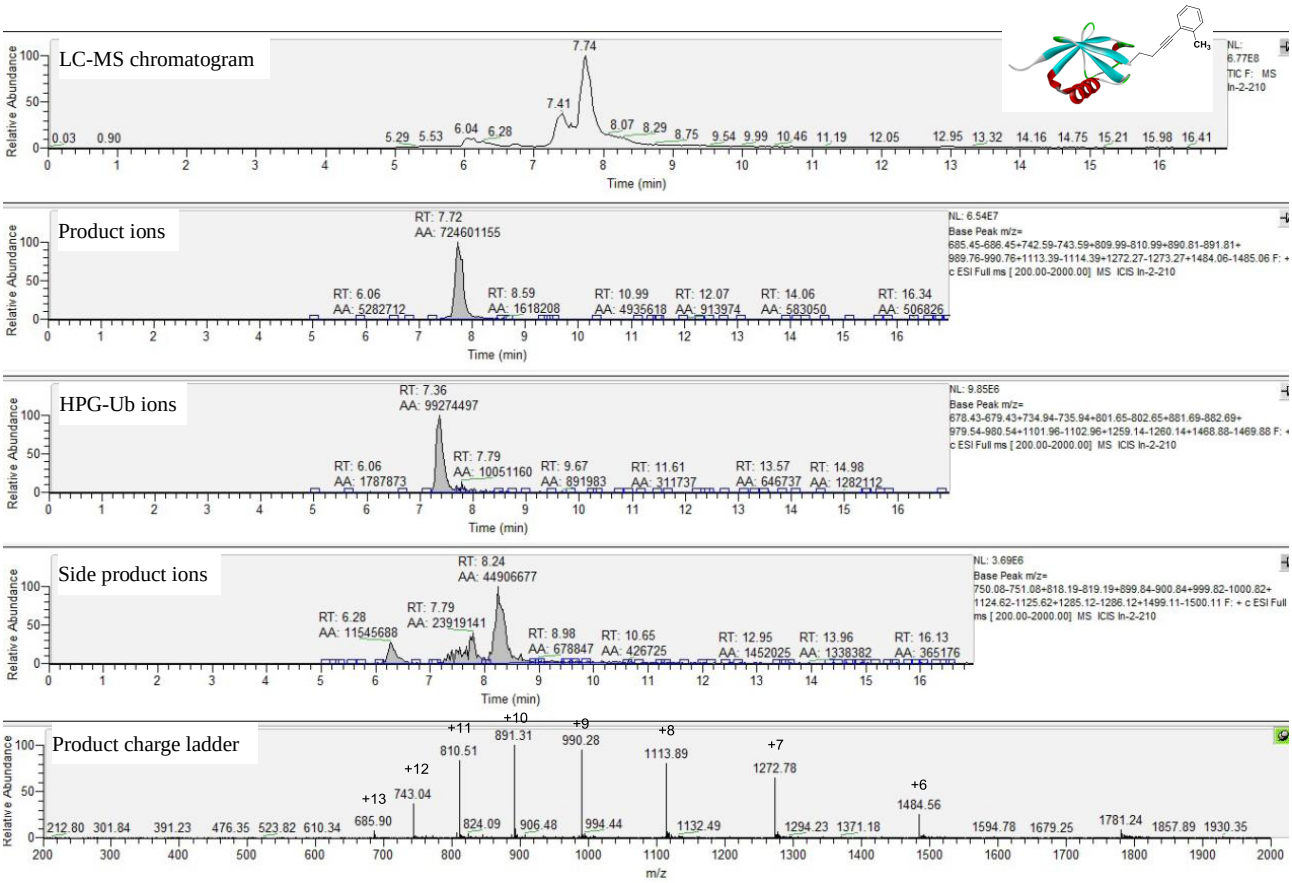


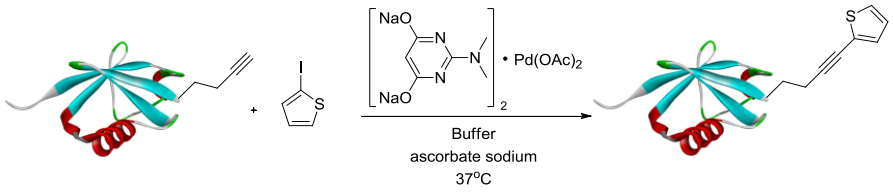
The charge ladder for the desired product is shown as follows: calcd mass 9142.1 Da, found 9143.7 ± 1.3 Da. The reaction yield was determined to be 93% by LC-MS based on the following equation: yield % = $I_{product}/(I_{HPG\text{-}Ub} + I_{product})$.



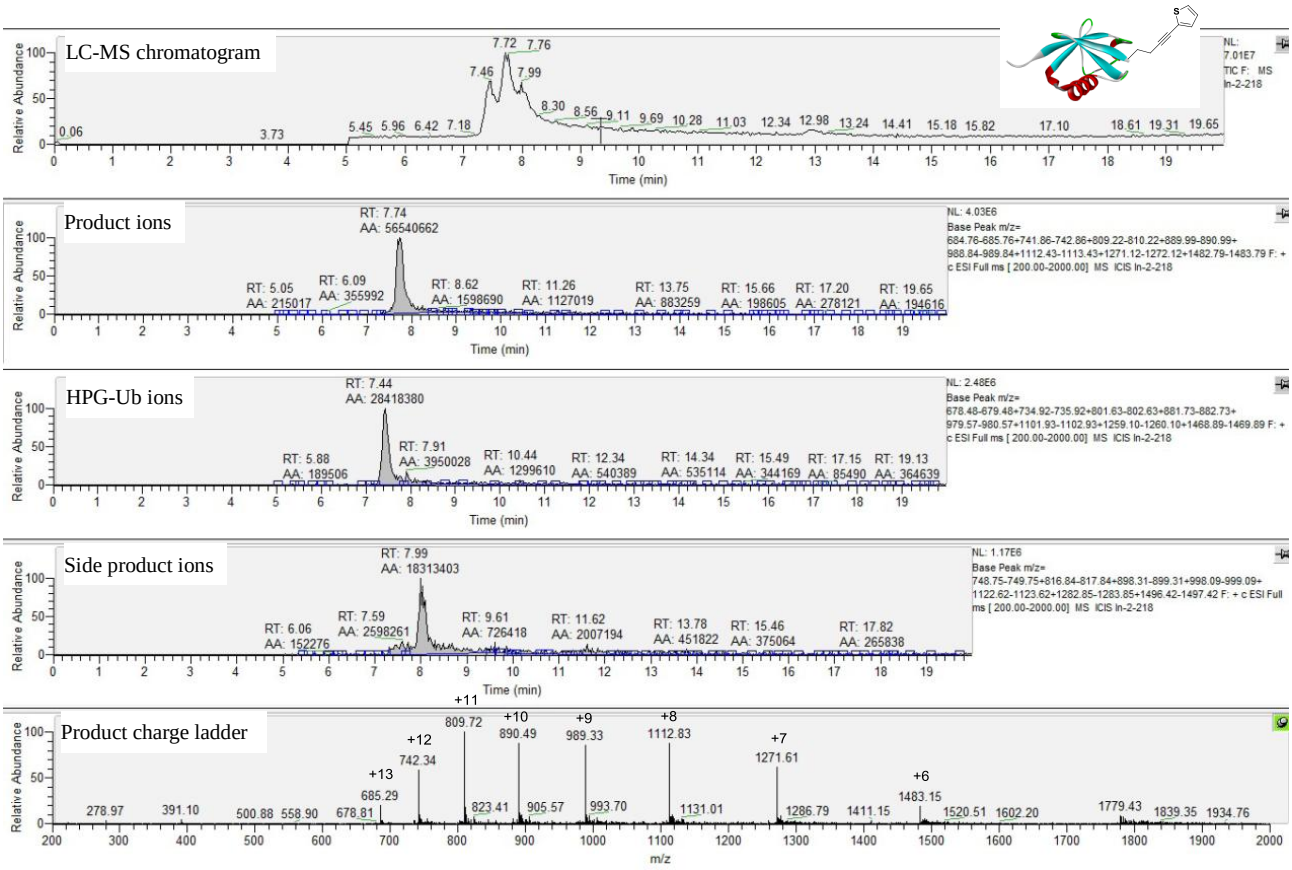


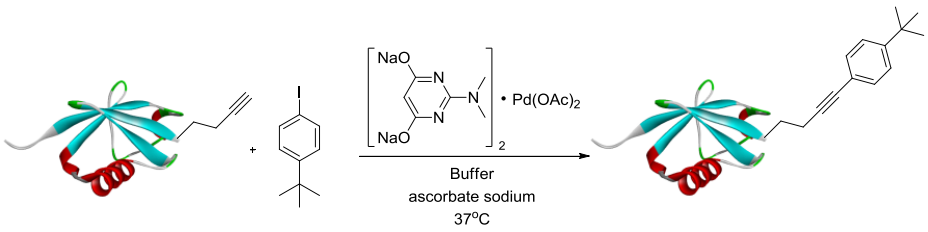
The charge ladder for the desired product is shown as follows: calcd mass 8902.1 Da, found 8903.4 ± 1.2 Da. A side product with a mass of 8993.4 ± 1.2 Da was detected and included in the yield calculation; the yield was determined to be 83% based on the following equation: yield % = $I_{\text{product}}/(I_{\text{HPG-Ub}} + I_{\text{product}} + I_{\text{side product}})$.



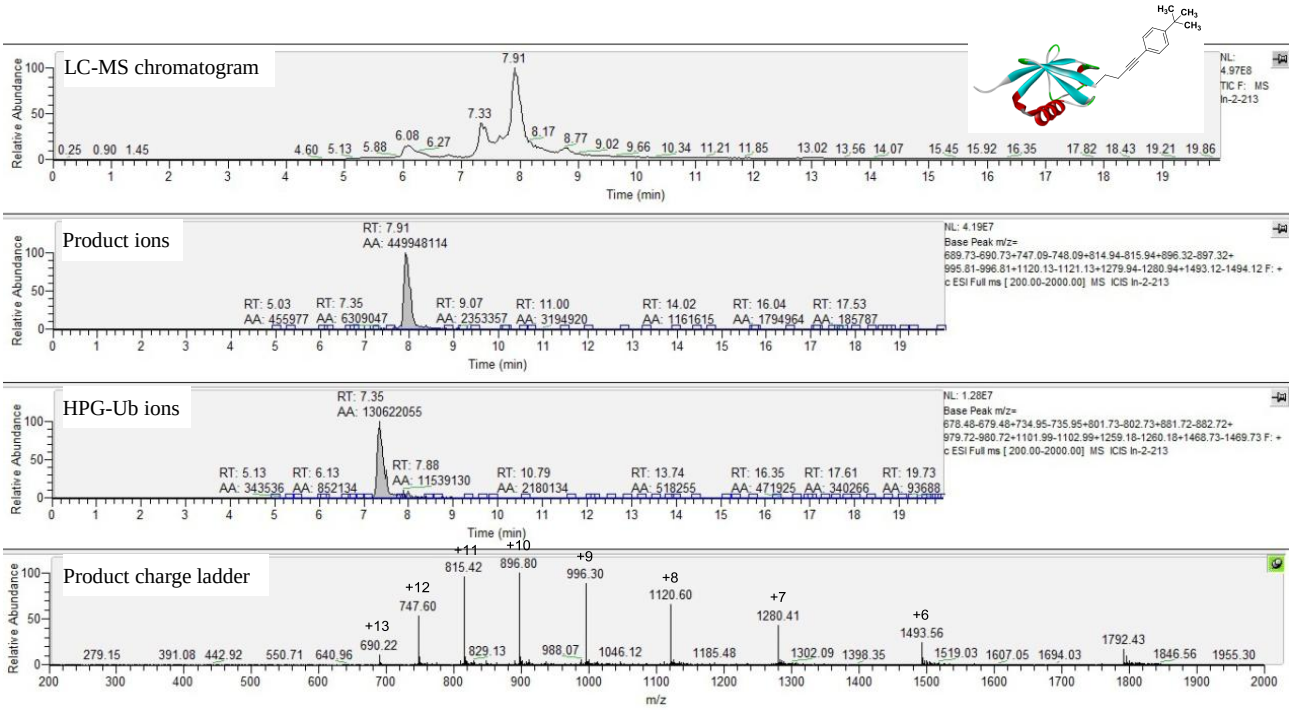


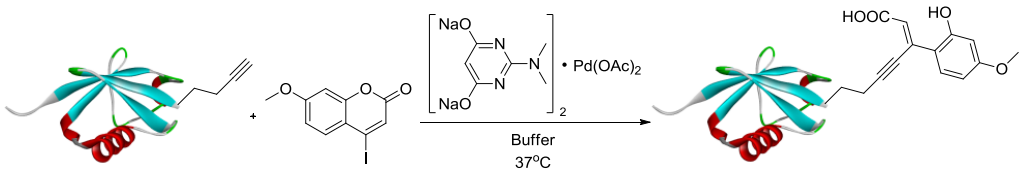
The charge ladder for the desired product is shown as follows: calcd mass 8894.1 Da, found 8894.9 ± 0.9 Da. A side product with a mass of 8977.7 ± 1.5 Da was detected and included in the yield calculation; The yield was determined to be 55% based on the following equation: yield % = $I_{product}/(I_{HPG\text{-}Ub} + I_{product} + I_{side\ product})$.



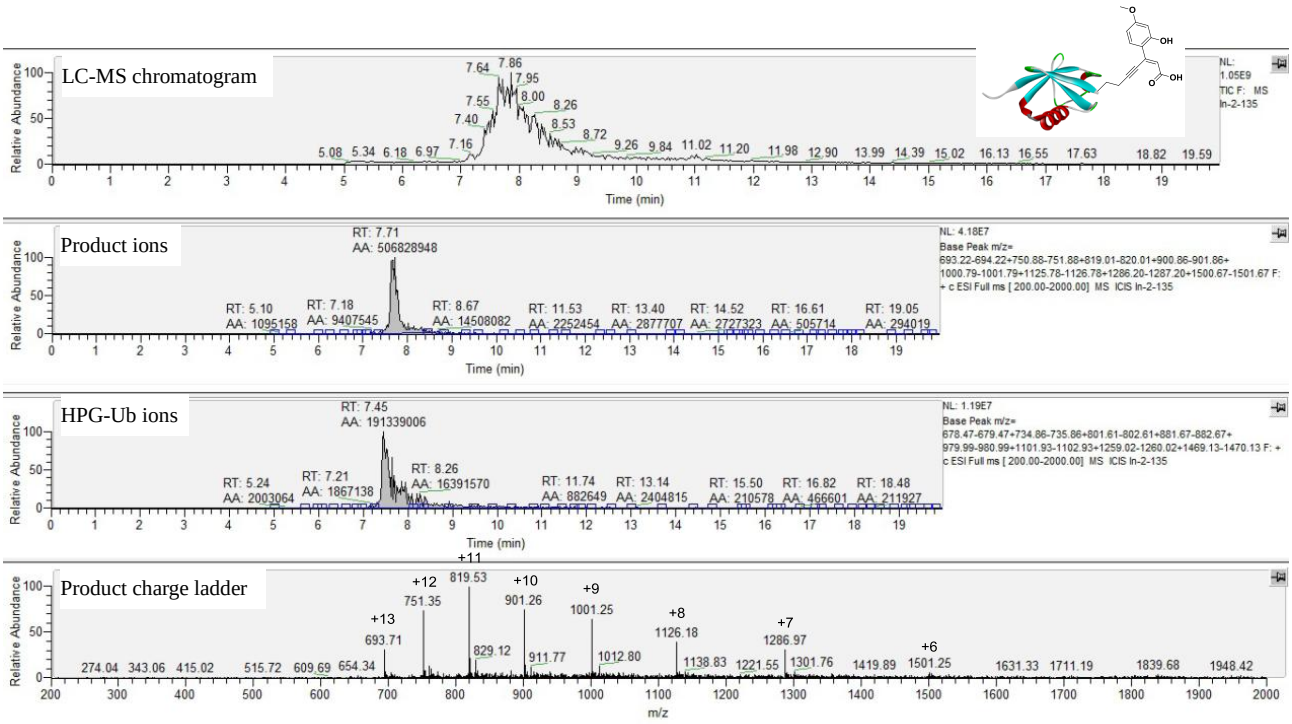


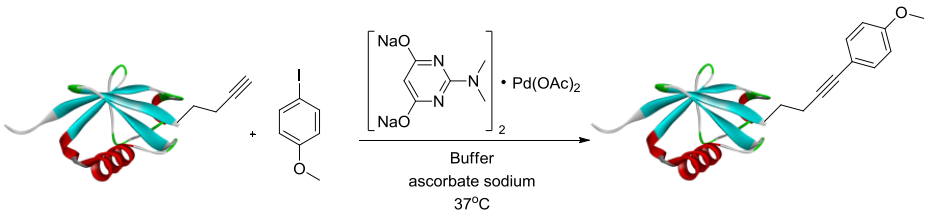
The charge ladder for the desired product is shown as follows: calcd mass 8944.1 Da, found 8945.5 ± 1.2 Da. The reaction yield was determined to be 86% based on the following equation: yield % = $I_{product}/(I_{HPG\text{-}Ub} + I_{product})$.



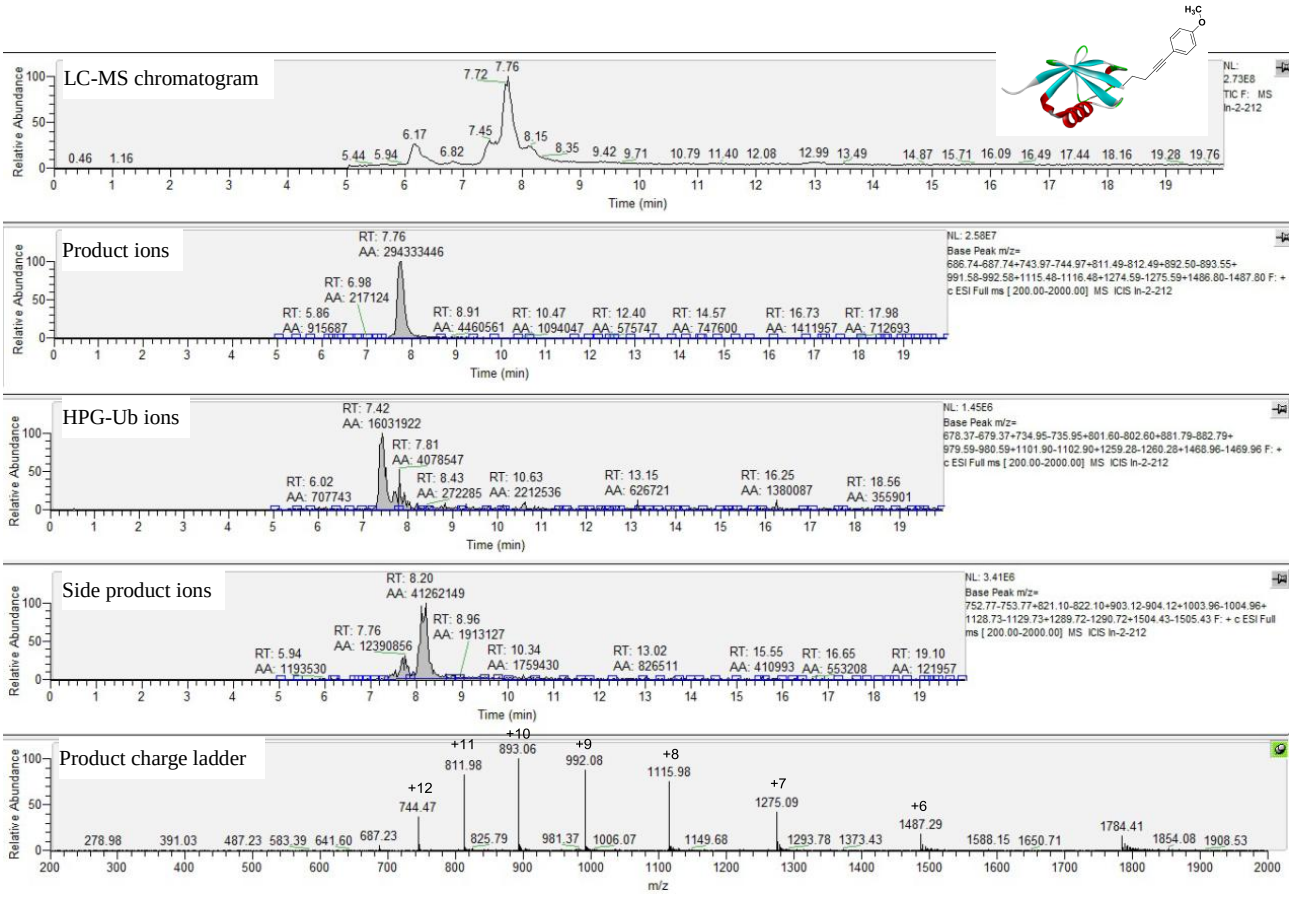


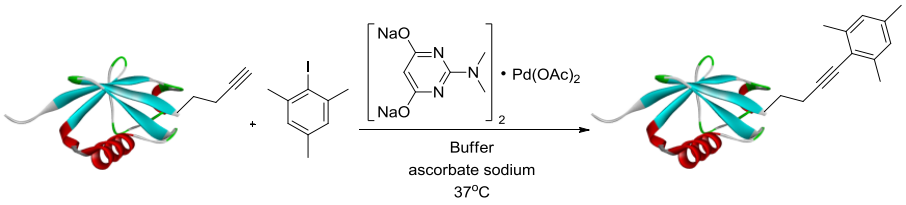
The charge ladder for the cross-coupled product is shown as follows; calcd mass 8986.1 Da, found 8986.1 + 18 Da (M + H_2O). The reaction yield was calculated to be 73% based on the following equation: yield % = $I_{product}/(I_{HPG\text{-}Ub} + I_{product})$.



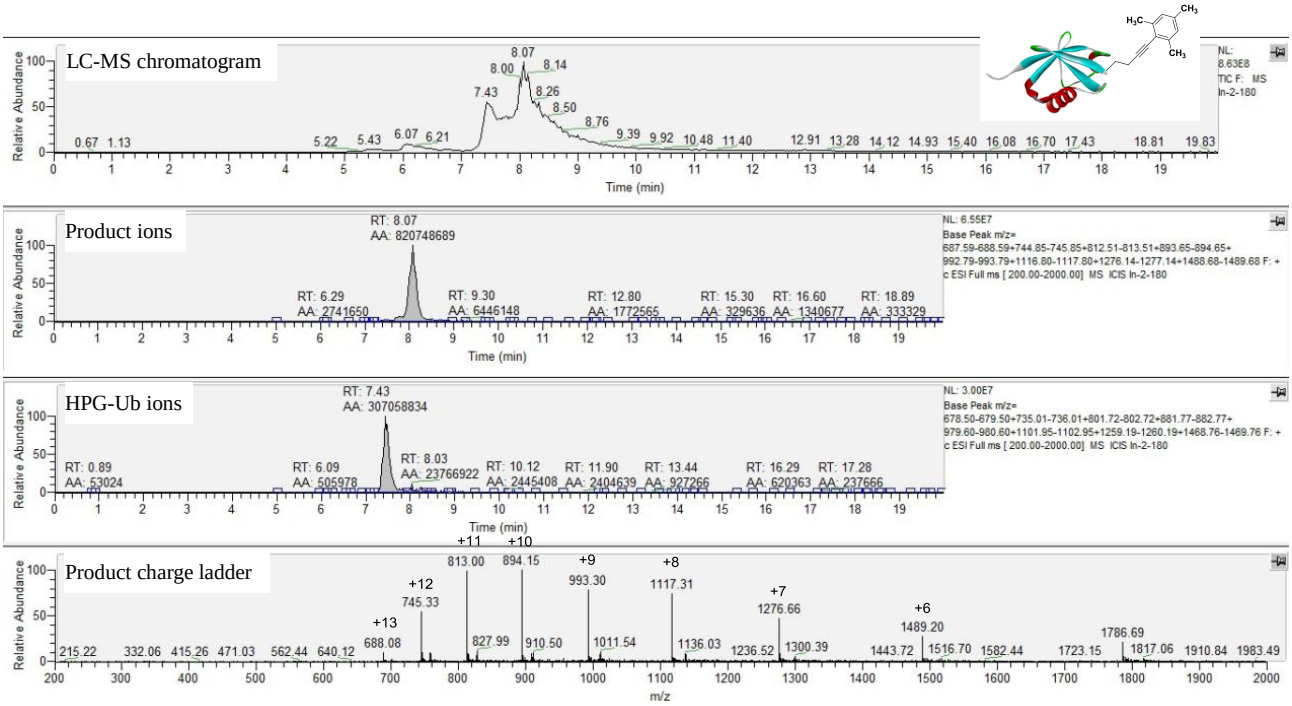


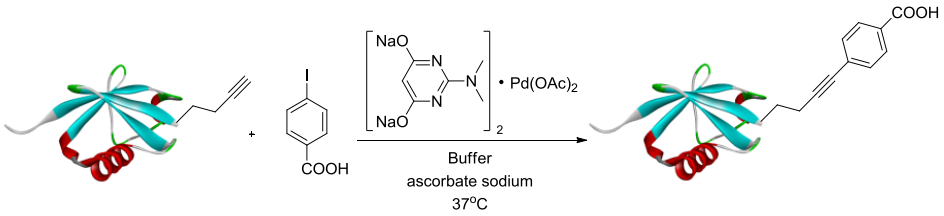
The charge ladder for the desired product is shown as follows: calcd mass 8918.1 Da, found 8919.9 ± 1.3. A side product with a mass of 9025.80 ± 1.8 Da was detected and included in the yield calculation; the yield was determined to be 83% based on the following equation: yield % = $I_{product}$/($I_{HPG\text{-}Ub}$ + $I_{product}$ + $I_{side\ product}$).



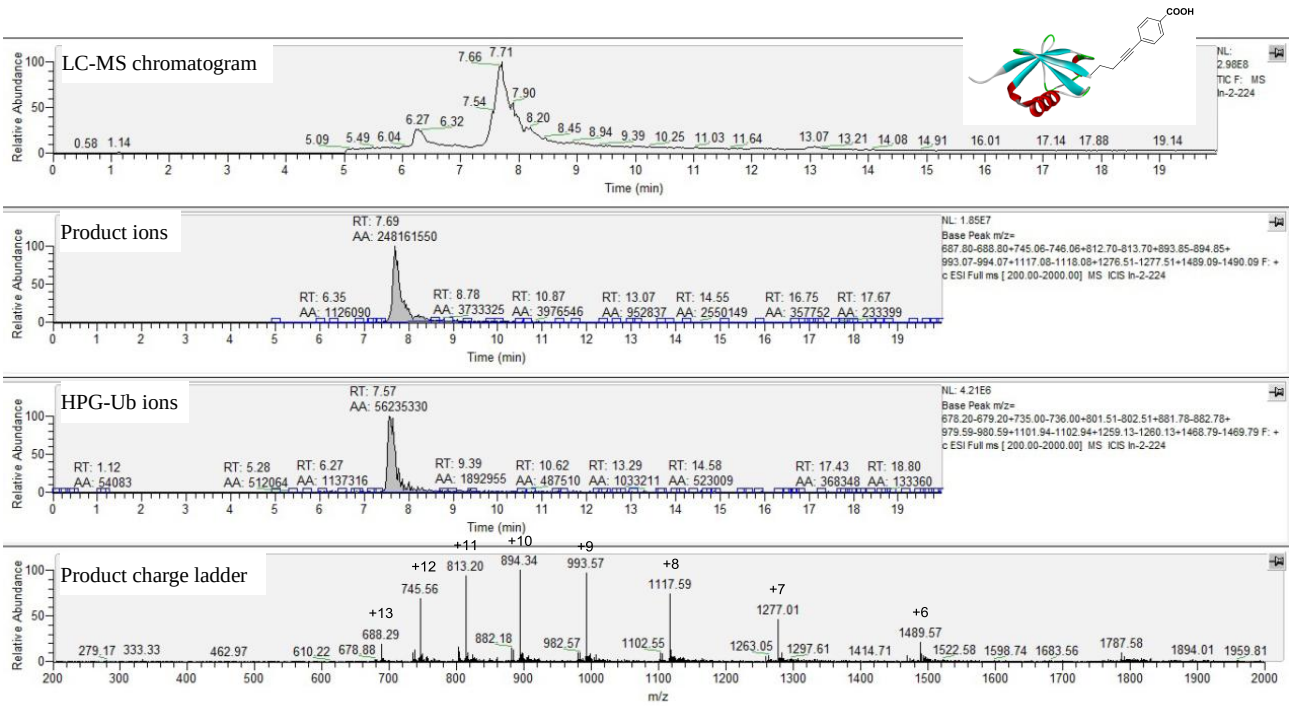


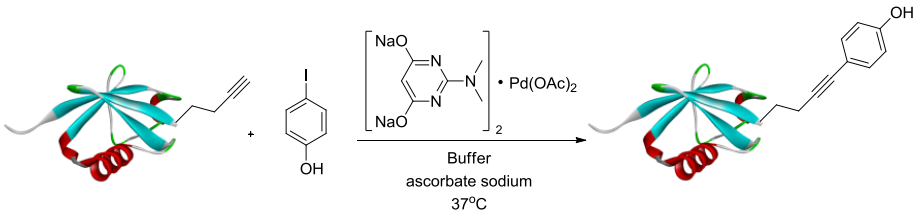
The charge ladder for the desired product is shown as follows: calcd mass 8930.1 Da, found 8930.9 ± 1.2. The reaction yield was calculated to be 73% based on the following equation: yield % = $I_{product}/(I_{HPG\text{-}Ub} + I_{product})$.



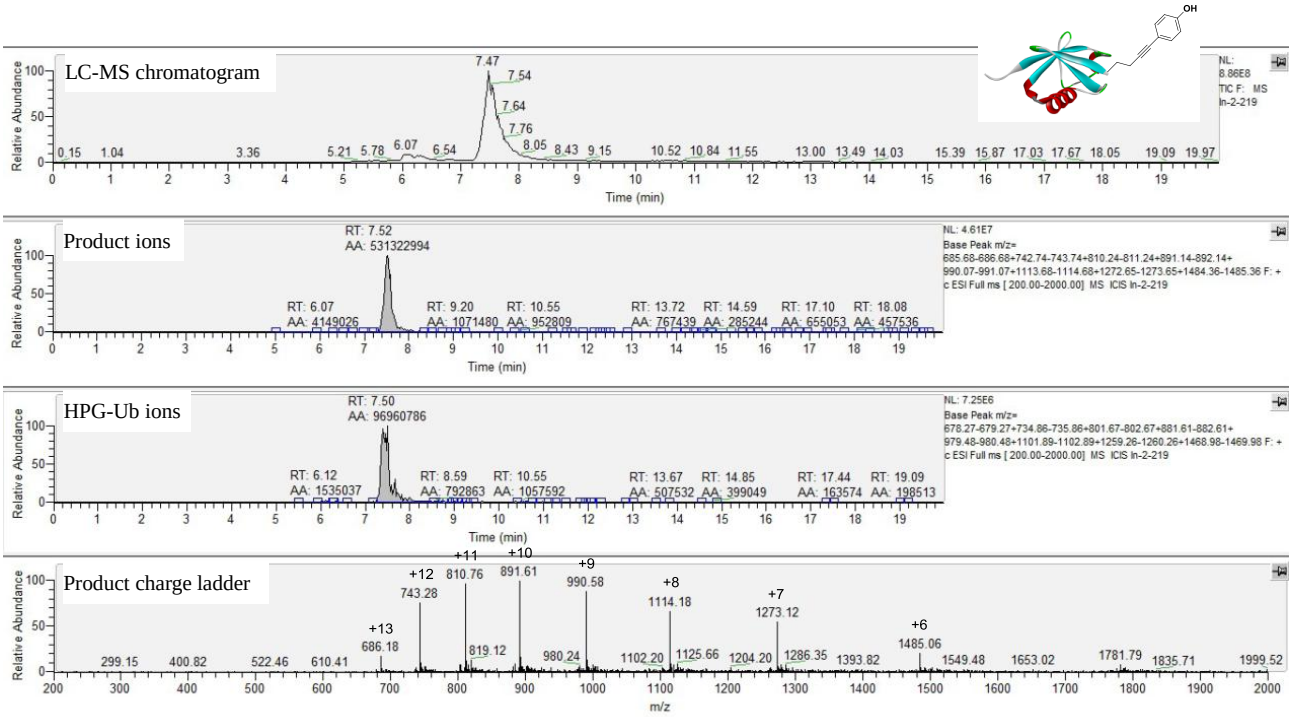


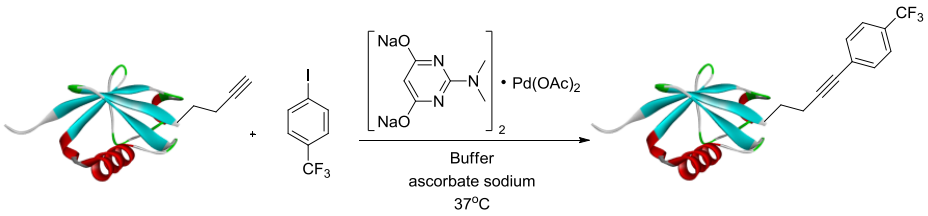
The charge ladder for the cross-coupled product is shown as follows: calcd mass 8932.1 Da, found 8933.2 ± 1.1. The reaction yield was determined to be 82% based on the following equation: yield % = $I_{product}/(I_{HPG\text{-}Ub} + I_{product})$.



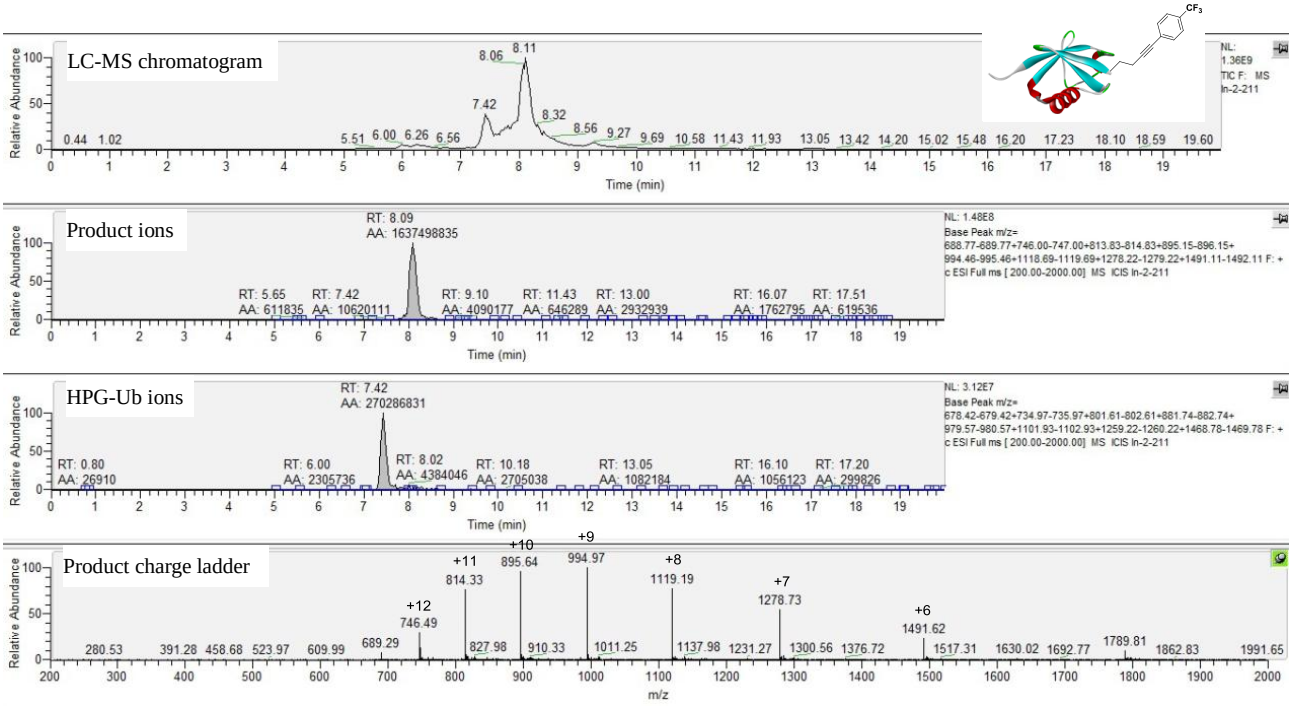


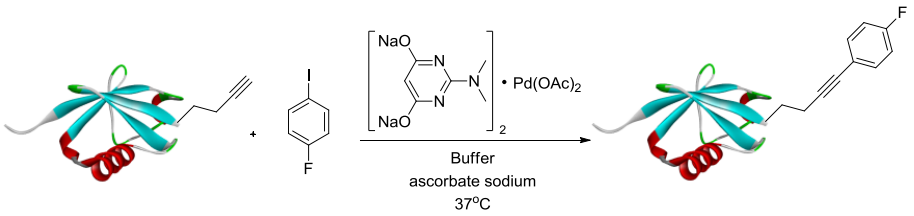
The charge ladder for the cross-coupled product is shown as follows; calcd mass 8904.1 Da, found 8906.1 ± 1.2. The reaction yield was calculated to be 85% based on the following equation: yield % = $I_{product}/(I_{HPG\text{-}Ub} + I_{product})$.



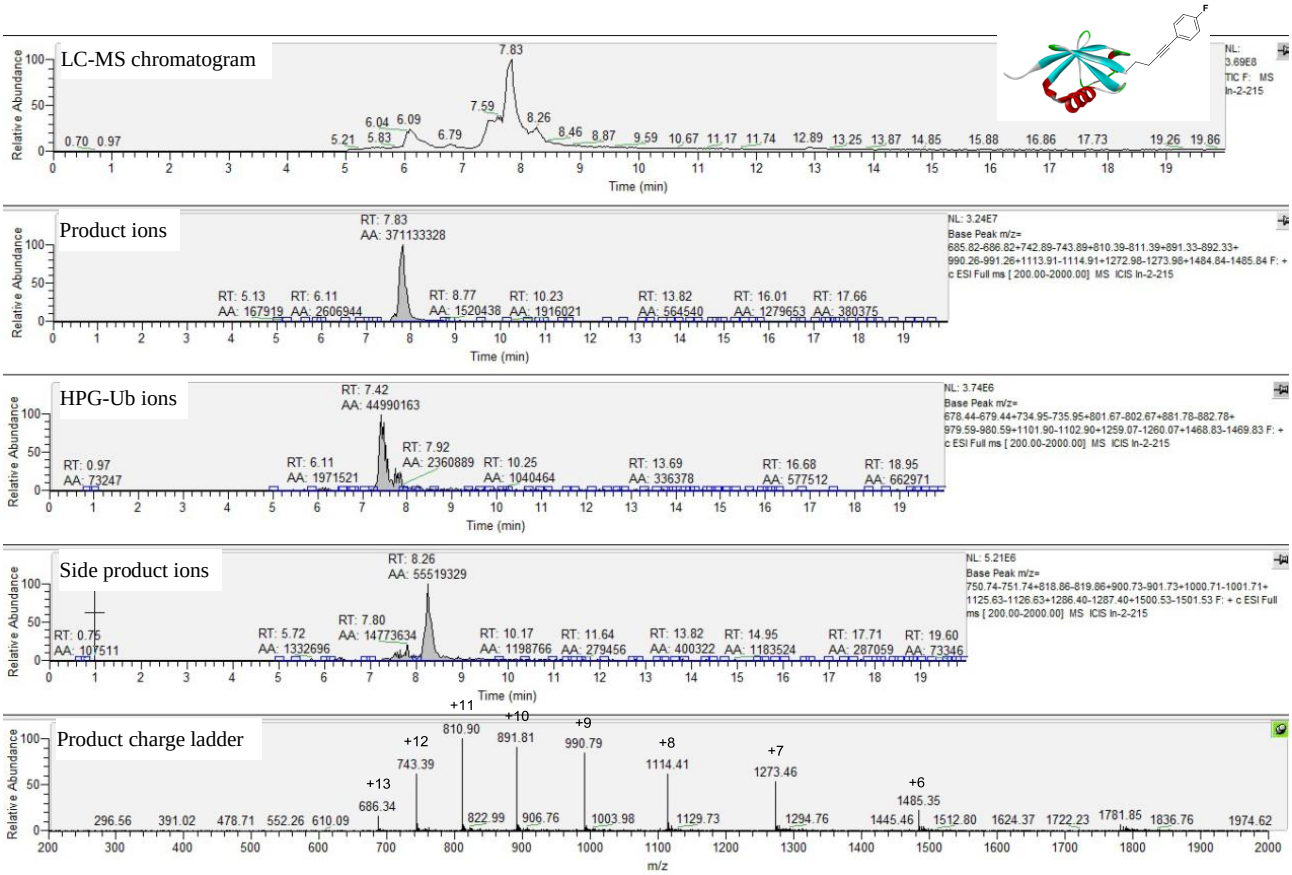


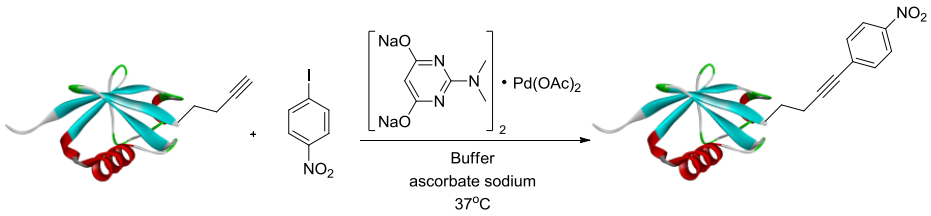
The charge ladder for the desired product is shown as follows: calcd mass 8956.1 Da, found 8957.7 ± 1.5. The reaction yield was determined to be 78% based on the following equation: yield % = $I_{product}$/($I_{HPG\text{-}Ub}$ + $I_{product}$).



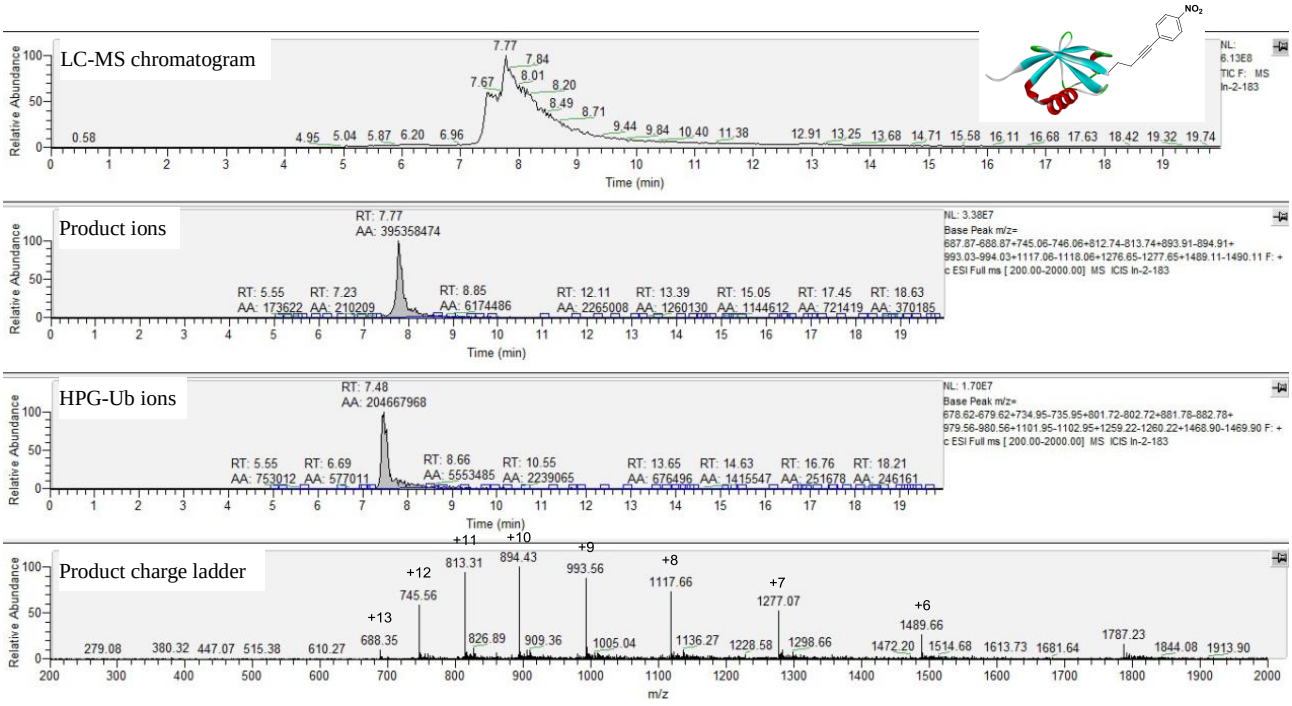


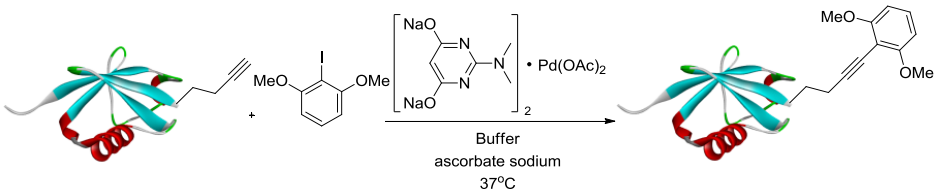
The charge ladder for the desired product is shown as follows: calcd mass 8906.1 Da, found 8907.9 ± 1.0. A side product with a mass of 9001.65 ± 0.9 Da was detected and included in the yield calculation. The yield was determined to be 79% based on the following equation: yield % = $I_{\text{product}}/(I_{\text{HPG-Ub}} + I_{\text{product}} + I_{\text{side product}})$.



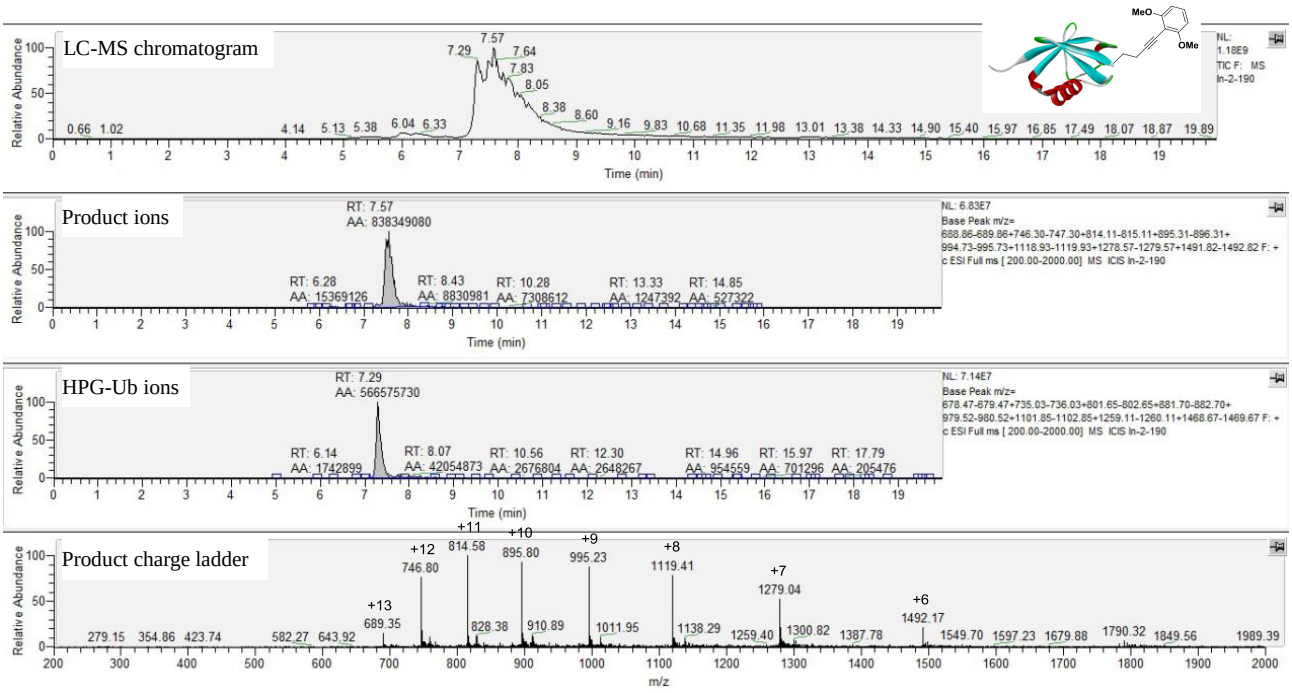


The charge ladder for the desired product is shown as follows: calcd mass 8933.1 Da, found 8933.9 ± 1.3. The reaction yield was calculated to be 66% based on the following equation: yield % = $I_{product}/(I_{HPG\text{-}Ub} + I_{product})$.

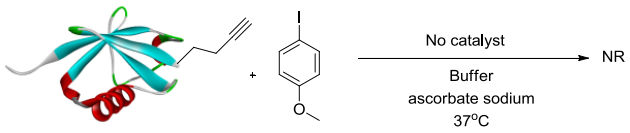




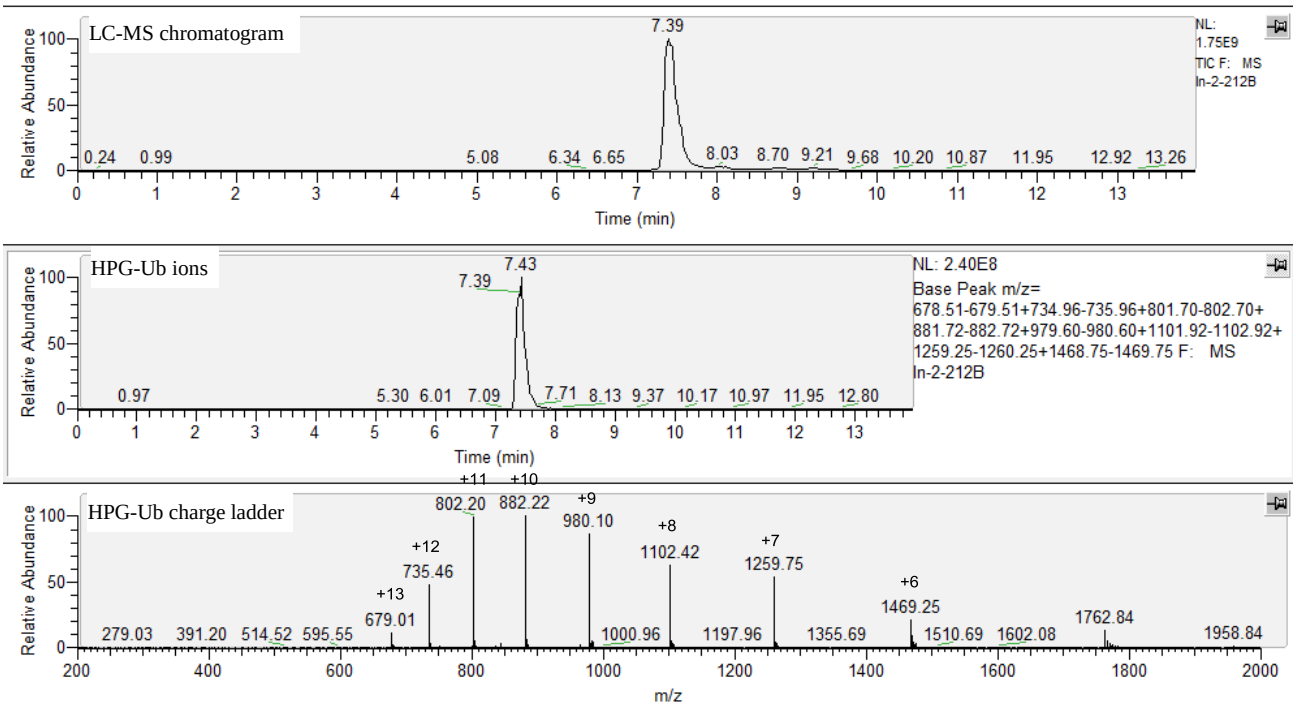
The charge ladder for the desired product is shown as follows; calcd mass 8948.1 Da, found 8948.0 ± 1.0. The reaction yield was calculated to be 60% based on the following equation: yield % = $I_{product}/(I_{HPG\text{-}Ub} + I_{product})$.

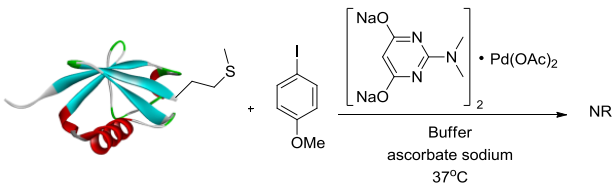


Control experiments for Sonogashira cross-coupling with protein substrates

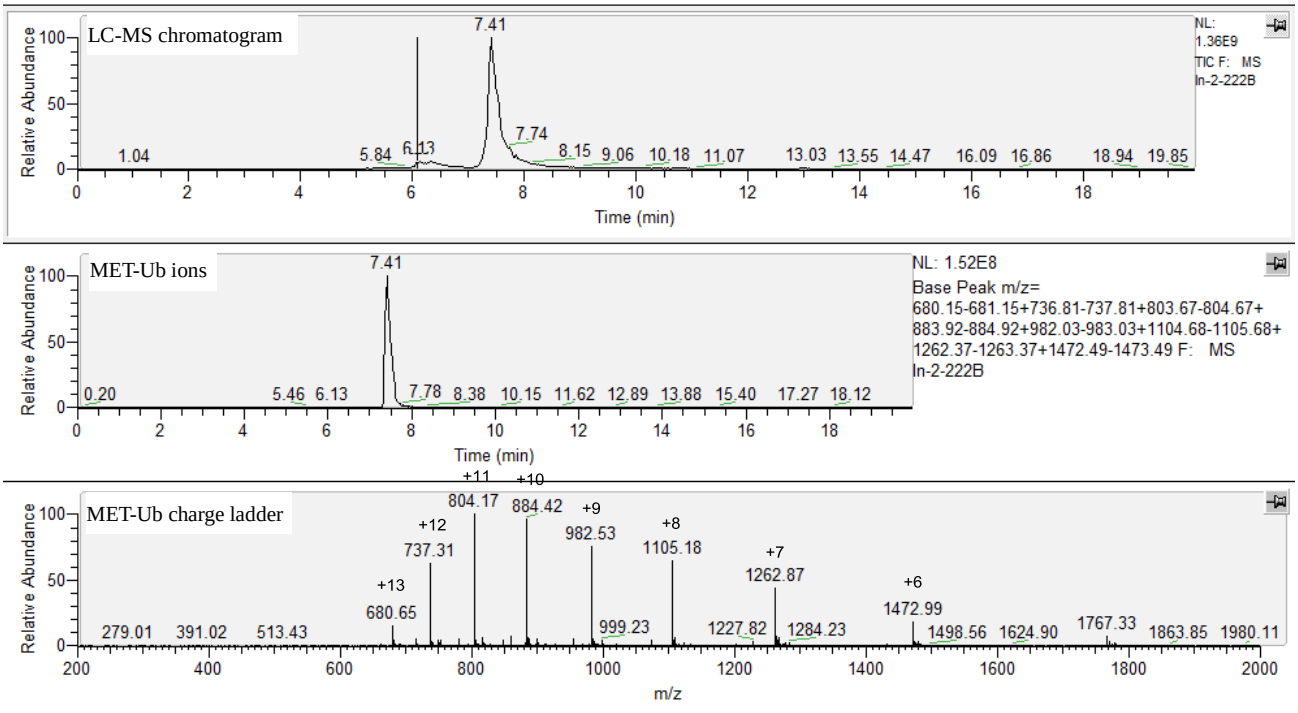


To an ice-chilled 0.75-mL eppendorf tube were added sequentially an aliquot of **1f** (24 μL, 0.25 μmol), an aliquot of sodium ascorbate (25 μL, 2.0 μmol), an aliquot of 25 μL of H_2O, and the mixture was stirred at 37 °C for 60 minutes to derive the activated reagent cocktail. Separately, to a 0.75-mL eppendorf tube containing 90.6 μL solution of Na_2HPO_4 (15 nmol) in H_2O was added 2 μL of HPG-Ub protein stock (2.22 mg/mL in 50 mM Na_2HPO_4 buffer, pH 8.0, 0.5 nmol) and 3.7 μL of the activated reagent cocktail, and the mixture was stirred at 37 °C for 15 minutes before a second batch of the activated reagent cocktail (3.7 μL) was added. The stirring was allowed to continue for another 15 minutes before the reaction was quenched by adding 20 μL of 0.5% 3-mercaptopropanoic acid solution in water (1.14 μmol). The reaction mixture was then analyzed directly by LC-MS equipped with a Jupiter C4 column; only the unreacted HPG-Ub was detected in the mass spectrum.





To an ice-chilled 0.75-mL eppendorf tube were added sequentially an aliquot of **1f** (24 μL, 0.25 μmol), an aliquot of 10 mM $Pd(OAc)_2$· **L2** complex solution (25 μL, 0.25 μmol), and an aliquot of sodium ascorbate (25 μL, 2.0 μmol), and the mixture was stirred at 37 °C for 60 minutes to derive the activated reagent cocktail. Separately, to a 0.75-mL eppendorf tube containing 90.6 μL solution of Na_2HPO_4 (15 nmol) in H_2O was added 5 μL of Met-Ub protein stock (0.86 mg/mL in 50 mM Na_2HPO_4 buffer, pH 8.0, 0.5 nmol) and 3.7 μL of the activated reagent cocktail, and the mixture was stirred at 37 °C for 15 minutes before a second batch of the activated reagent cocktail (3.7 μL) was added. The stirring was allowed to continue for another 15 minutes before the reaction was quenched by adding 20 μL of 0.5% 3-mercaptopropanoic acid solution in water (1.14 μmol). The reaction mixture was then analyzed directly by LC-MS equipped with a Jupiter C4 column; only the unreacted Met-Ub was detected in the mass spectrum.



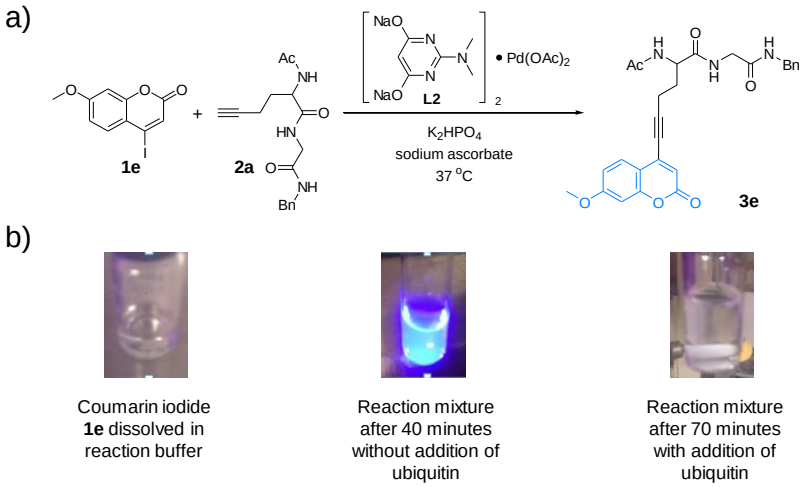


Figure S1. Effect of non-specific sequestration of palladium-ligand complexes by ubiquitin on the cross-coupling reaction: (a) The scheme of a reaction involving 0.02 μmol of **1e**, 0.02 μmol of **2a**, and 0.04 μmol of palladium-**L2** complex (2 equiv). (b) Images of the reaction mixtures upon excitation with a handheld UV lamp, λ_{ex} = 365 nm. For sequestration of palladium complex by proteins, wild-type ubiquitin (0.17 mg, 0.02 μmol, 1 equiv) was added to the reaction mixture.

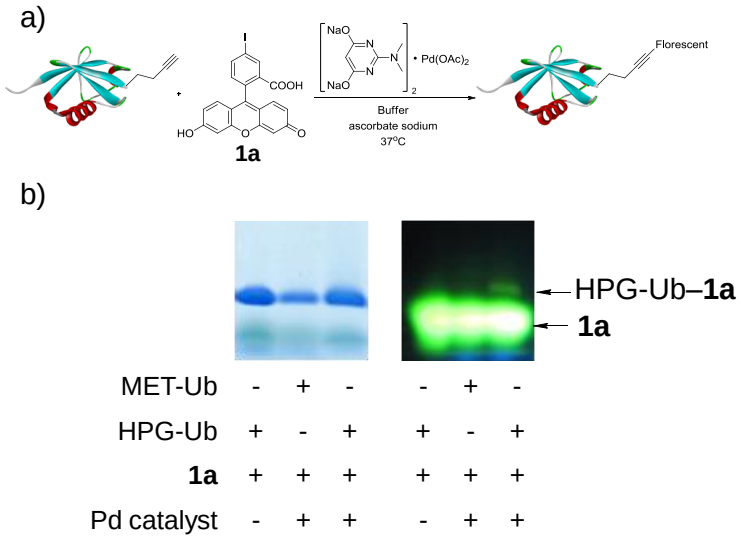
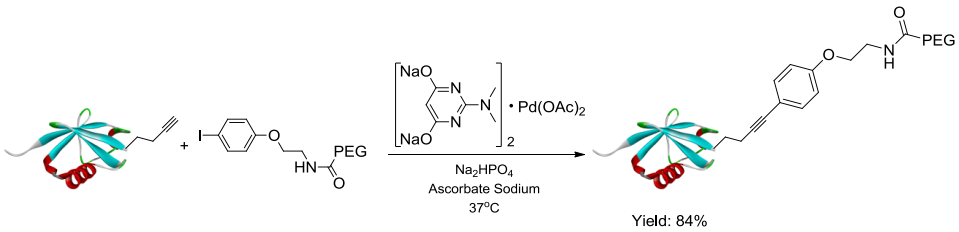


Figure S2. Selective florescent labeling of the HPG-encoded protein by fluorescein iodide **1a** *via* Sonogashira cross-coupling: (a) reaction scheme; (b) SDS-PAGE analysis of the reaction mixtures. Left, Coomassie blue stain; right, in-gel fluorescence of the same gel, λ_{ex} = 365 nm. The labeled product, HPG-Ub–**1a**, was indicated with an arrow; strongly fluorescent bands were excess fluorescent iodide **1a**.

Selective PEGylation of HPG-Ub via Sonogashira cross-coupling



A stock solution of mPEG-phenyl iodide (**1n**) was prepared by dissolving 64.3 mg of **1n** in 120 μL DMSO, and a stock solution of 80 mM sodium ascorbate was prepared by dissolving 29.5 mg of sodium ascorbate in 1.875 mL ddH_2O. To an ice-chilled 0.75-mL eppendorf tube were added sequentially an aliquot of **1n** (34 μL, 0.25 μmol), an aliquot of 10 mM $Pd(OAc)_2$· **L2** complex solution (25 μL, 0.25 μmol), and an aliquot of sodium ascorbate (25 μL, 2.0 μmol), and the mixture was stirred at 37 °C for 60 minutes to derive the activated reagent cocktail. Separately, to a 0.75-mL eppendorf tube containing Na_2HPO_4 (15 nmol) in 39.6 μL H_2O was added 2 μL of HPG-Ub protein stock (2.22 mg/mL in 50 mM Na_2HPO_4 buffer, pH 8.0, 0.5 nmol) and 4.2 μL of the activated reagent cocktail, and the mixture was stirred at 37 °C for 15 minutes before a second batch of the activated reagent cocktail (4.2 μL) was added. The stirring was allowed to continue for another 15 minutes before the reaction was quenched by adding 20 μL of 0.5% 3-mercaptopropanoic acid solution in water (1.14 μmol). The reaction mixture was then analyzed directly by SDS-PAGE. For control experiments, either palladium catalyst was dropped out or HPG-Ub was replaced by Met-Ub while the rest of conditions stayed the same.

Sonogashira cross-coupling reaction in bacterial cells

A starter culture of M15A (5 mL) was used to inoculate 100 mL of SelenoMet plus nutrient mix (a methionine-deficient medium) supplemented with L-methionine (1 mM) and ampicillin (100 μg/mL), and the cells were allowed to grow at 37 °C until OD_{600} reached 0.8-1.0. Afterwards, a medium shift was performed by first washing the cell pellet with 0.9% NaCl three times followed by re-suspension in SelenoMet plus nutrient mix containing 100 μg/mL of ampicillin. Cells were incubated at 37 °C with shaking for additional 1 hour to deplete the intracellular methionine stock. Then, 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) and 1 mM of HPG were added to the culture medium and the incubation was allowed to continue for additional 4 hours. M15A cells

overexpressing HPG-Ub were subjected to three separate treatments.

For preparation of the sample for lane 1 in Figure 2 (without palladium catalyst), 20 mL M15A cells were treated with **1a** (2.20 mg in 230 μL DMSO; final concentration = 100 μM), ddH_2O (2.4 mL), sodium ascorbate (23.76 mg in 600 μL ddH_2O; final concentration = 5 mM), and Na_2HPO_4 (12.86 mg in 770 μL ddH_2O; final concentration = 2 mM), and the mixture was incubated at 37 ^oC for 4 hours before centrifugation to afford cell pellet 1.

For preparation of the sample for lane 2 in Figure 2 (without pre-activation), 20 mL M15A cells were treated with **1a** (2.20 mg in 230 μL DMSO; final concentration = 100 μM), palladium complex (2.4 mL of 10 mM $Pd(OAc)_2$· **L2** complex solution; final concentration = 1 mM), sodium ascorbate (23.76 g in 600 μL ddH_2O; final concentration = 5 mM), and Na_2HPO_4 (12.86 g in 770 μL ddH_2O; final concentration = 2 mM), and the mixture was incubated at 37 ^oC for 4 hours before centrifugation to afford cell pellet 2.

For preparation of the sample for lane 3 in Figure 2 (with pre-activation), 20 mL M15A cells were treated with an activated reagent cocktail in which **1a** (2.20 mg in 230 μL DMSO; final concentration = 100 μM), palladium complex (2.4 mL of 10 mM $Pd(OAc)_2$· **L2** complex solution; final concentration = 1 mM) and sodium ascorbate (23.76 mg in 600 μL ddH_2O; final concentration = 5 mM) in a 5-mL tube were stirred at 37 ^oC for 1 hour, and Na_2HPO_4 (12.86 g in 770 μL ddH_2O; final concentration = 2 mM), and the mixture was incubated at 37 ^oC for 4 hours before centrifugation to afford cell pellet 3.

All cell pellets were washed with 0.9% NaCl solution (50 mL × 3) before subjected to fluorescent imaging and subsequent SDS-PAGE analysis. The results of fluorescent labeling of HPG–Ub by fluorescein iodide **1a** in *E. coli* cells *via* copper-free Sonogashira cross-coupling were shown in Figure 3 as well as Figure S3 in the following.

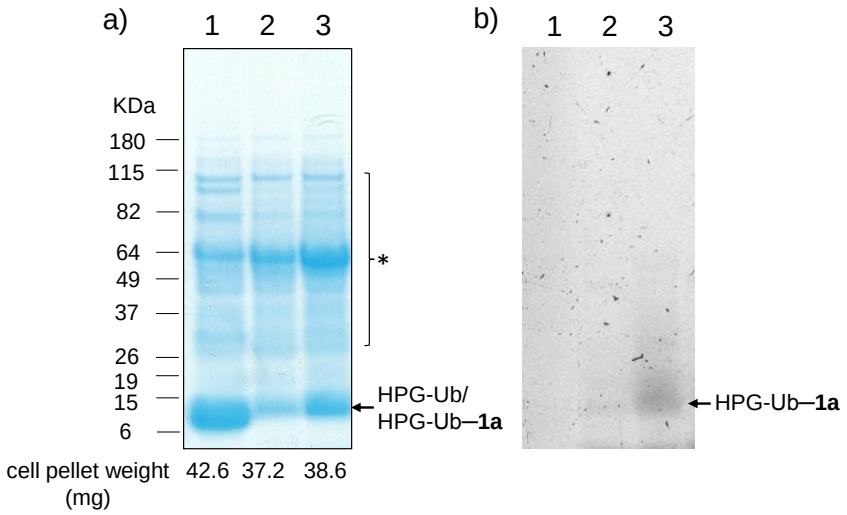


Figure S3. A repeat of fluorescent labeling of HPG–Ub by fluorescein iodide **1a** in *E. coli* cells via copper-free Sonogashira cross-coupling. (a) Coomassie blue stain of the proteins captured by Ni-NTA agarose beads. Lane 1: *E. coli* cells collected after treatment with 100 μM fluorescein iodide **1a** but not palladium complex; Pellet 2, cells collected after treatment with 100 μM **1a**, 1 mM palladium complex, 5 mM sodium ascorbate in Na_2HPO_4 solution at 37 °C for 4 h; Pellet 3, cells collected after treatment with the pre-activated mixture of palladium complex (1 mM) and **1a** (100 μM). Star (*) indicates proteins that were captured through non-specific binding to Ni-NTA-agarose beads. (b) In-gel fluorescence analysis of SDS-PAGE of the proteins captured by Ni-NTA-agarose beads from the lysates of the three cell pellets.

Cytotoxicity studies of palladium-ligand complexes to *E. coli*

A single colony of XL1-Blue was allowed to grow in 3 mL tetracycline-containing LB medium overnight. This starter culture was used to inoculate 50 mL of the same medium and the cells were allowed to grow at 37 ^{o}C until OD_{600} reaches 0.99. Afterward, five equal portions of 4.5 mL bacterial culture were treated with the following reagents respectively: (1) blank: 50 μL DMSO and 550 μL ddH_2O; (2) palladium complex: 500 μL of 10 mM $Pd(OAc)_2$·**L2** complex, 50 μL DMSO, and 50 μL ddH_2O; (3) a mixture of palladium complex and **1a** without pre-activation containing 500 μL of 10 mM $Pd(OAc)_2$·**L2** complex, **1a** (2.29 mg in 50 μL DMSO), and sodium ascorbate (7.92 mg in 50 μL ddH_2O); (4) an pre-activated reagent cocktail where a mixture of **1a** (2.29 mg in 50 μL DMSO), 500 μL of 10 mM $Pd(OAc)_2$·**L2** complex, and sodium ascorbate (7.92 mg in 50 μL ddH_2O) were stirred in a 2-mL tube at 37 ^{o}C for 1 hour before addition; (5) fluorescein iodide **1a** (2.29 mg dissolved in 50

μL DMSO and 110 μL ddH_2O. The cultures were allowed to continue for 4 hours before the aliquots of 100 μL were withdrawn and diluted 10^6 times. The aliquots of the diluted cultures (100 μL) were plated onto LB-agar plate. After overnight incubation at 37 °C, the colony numbers in each plate were counted and the colony forming units were calculated and plotted in Figure S4a. Separately, changes in cell density (OD_{600}) were measured in 1-hour intervals during the course of the treatments, and the results were plotted in Figure S4b.

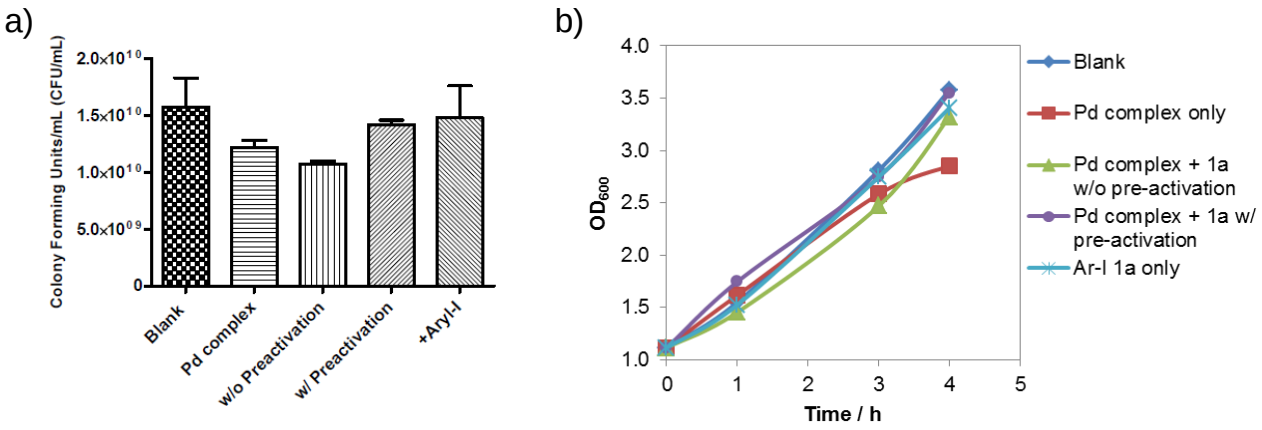
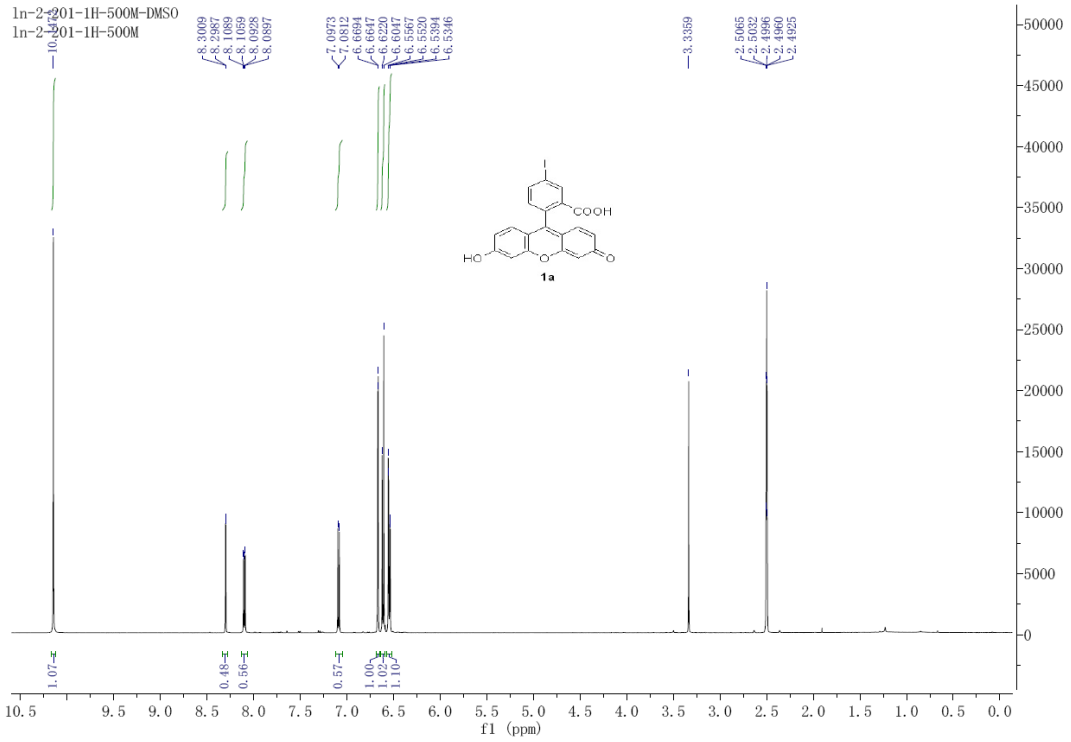


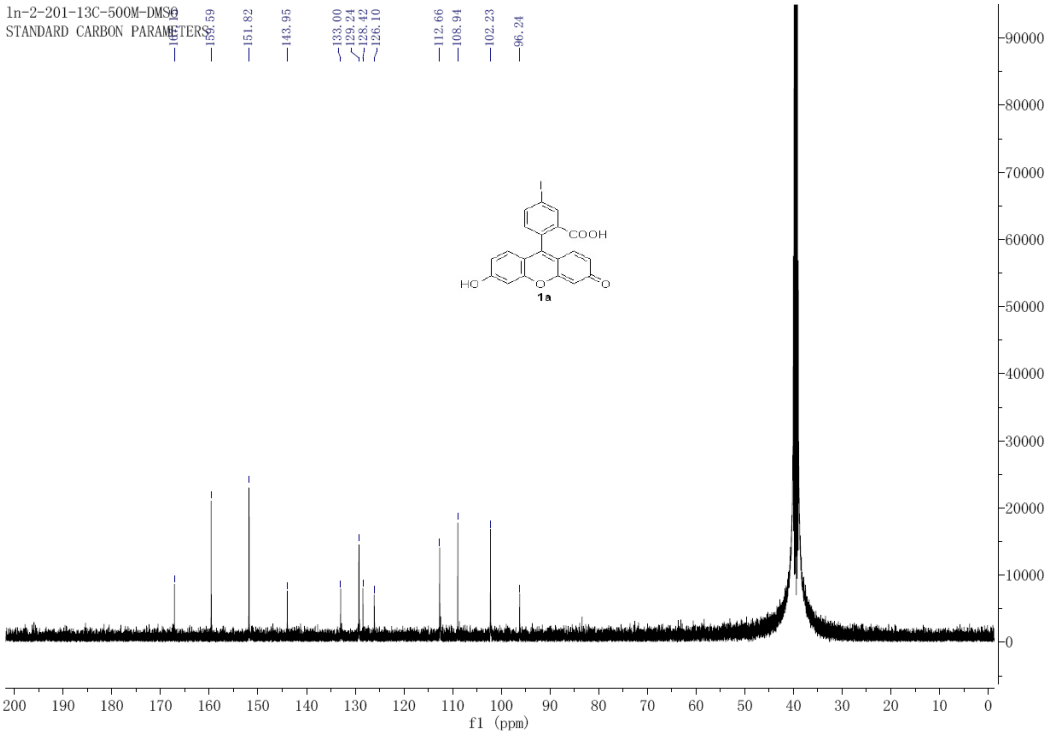
Figure S4. Assessment of cytotoxicity of the palladium-ligand complexes to *E. coli* through colony formation assay (a) and growth curve measurement (b) of XL-Blue cells after the treatments. The conditions for the five treatments were listed in the experimental procedures.

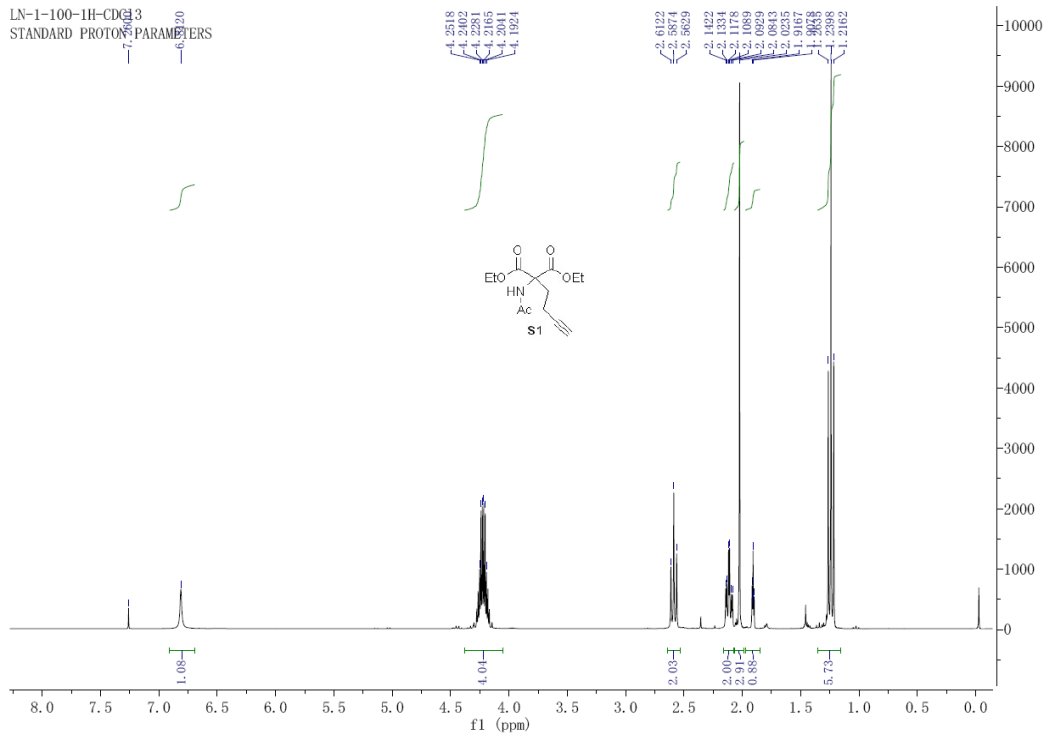
Reference

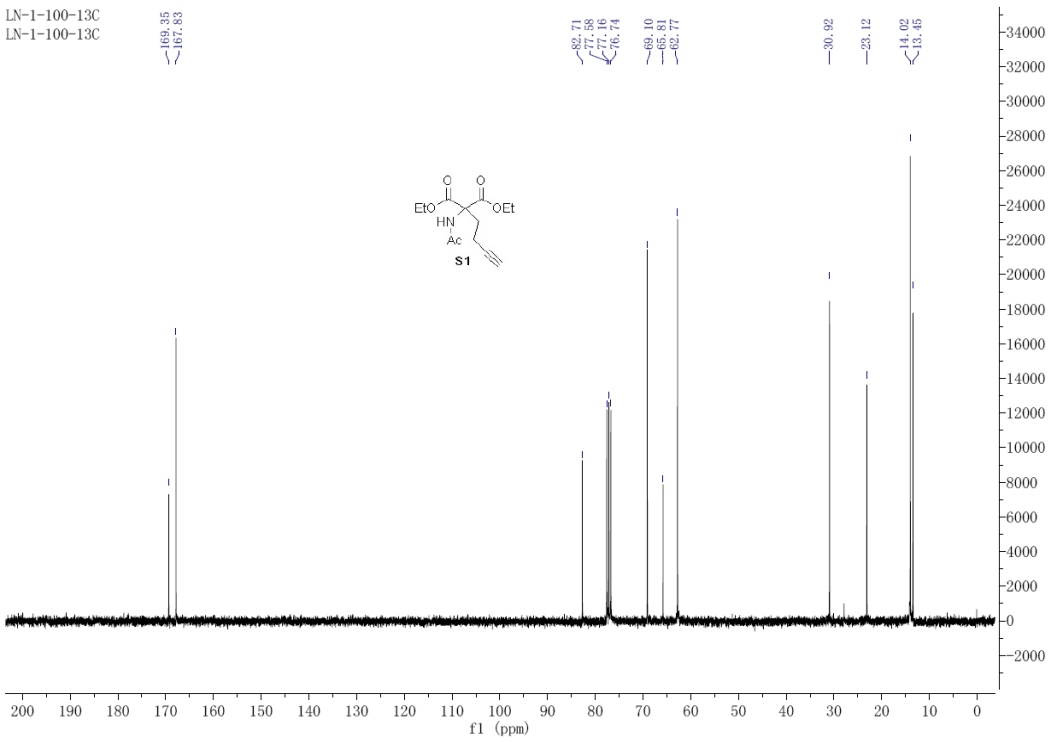
[S1] Chalker, J. M.; Wood, C. S. C.; Davis, B. G. *J. Am. Chem. Soc.* **2009**, *131*, 16346.

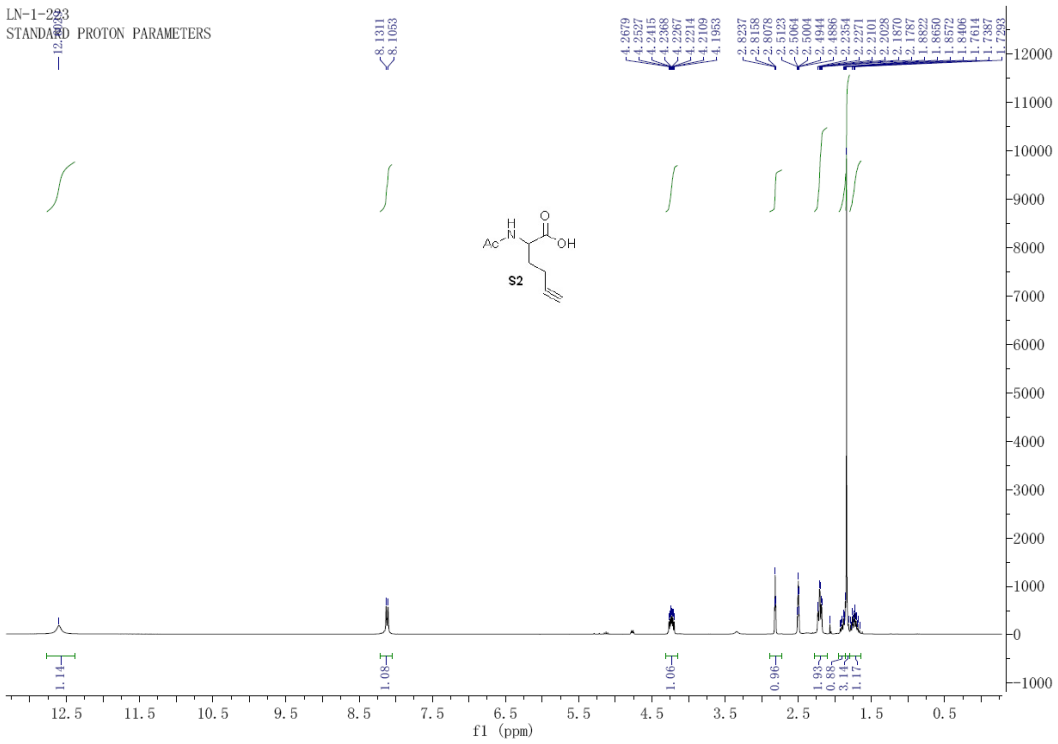
^{1}H NMR and ^{13}C NMR Spectra

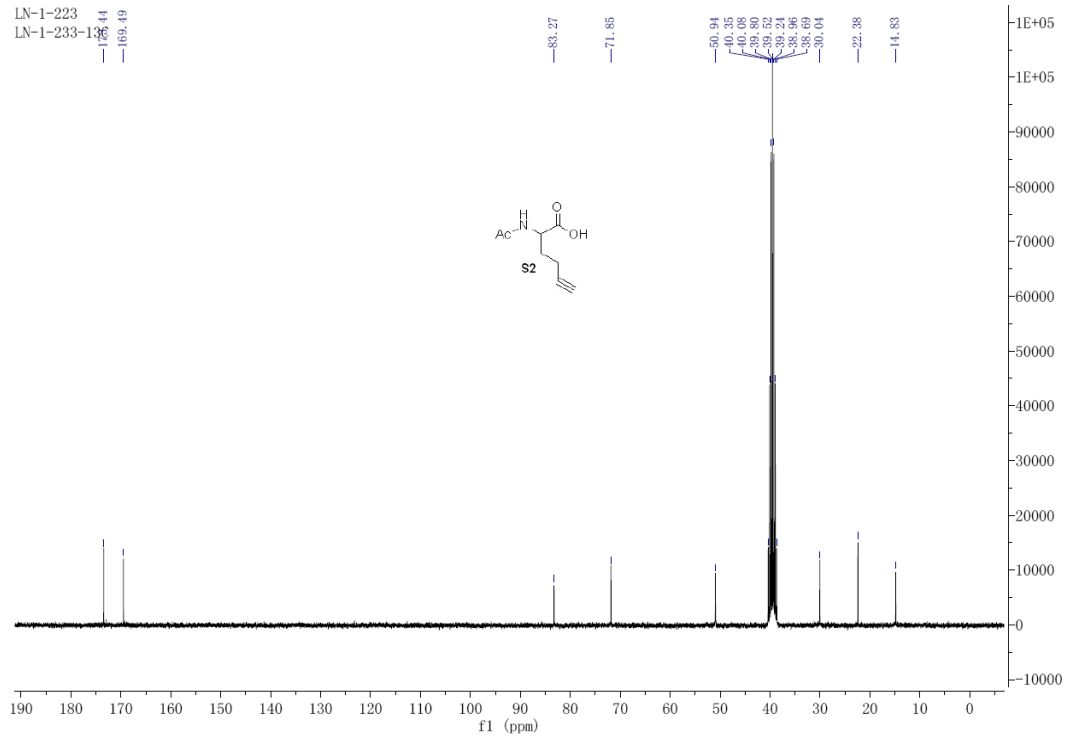


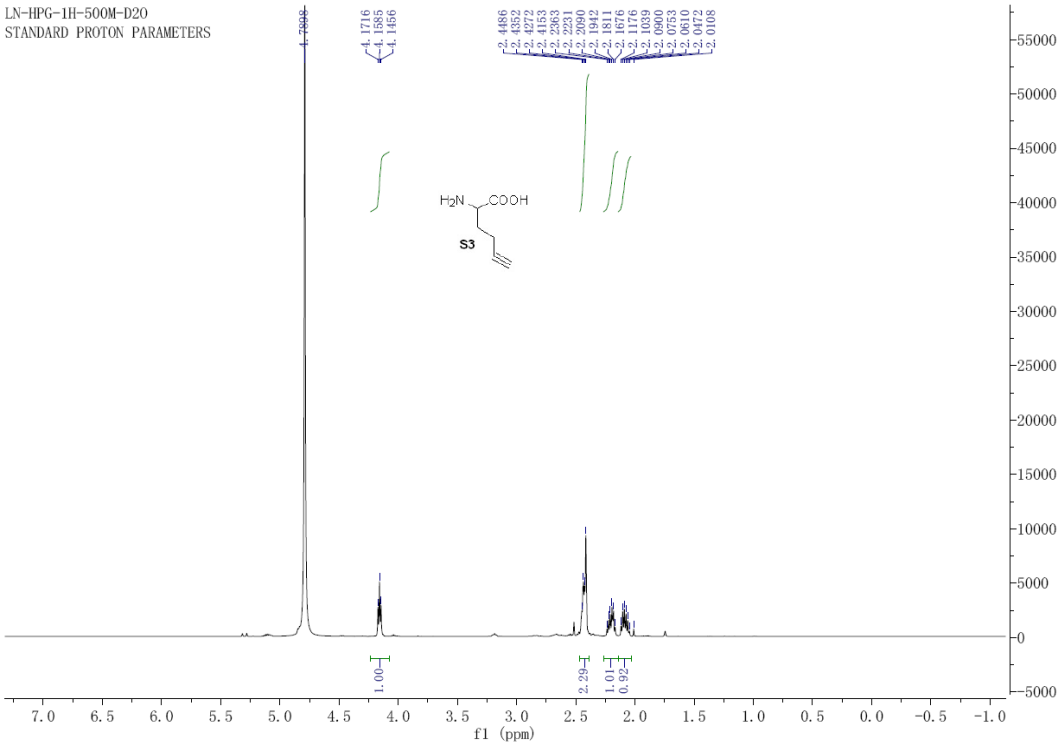


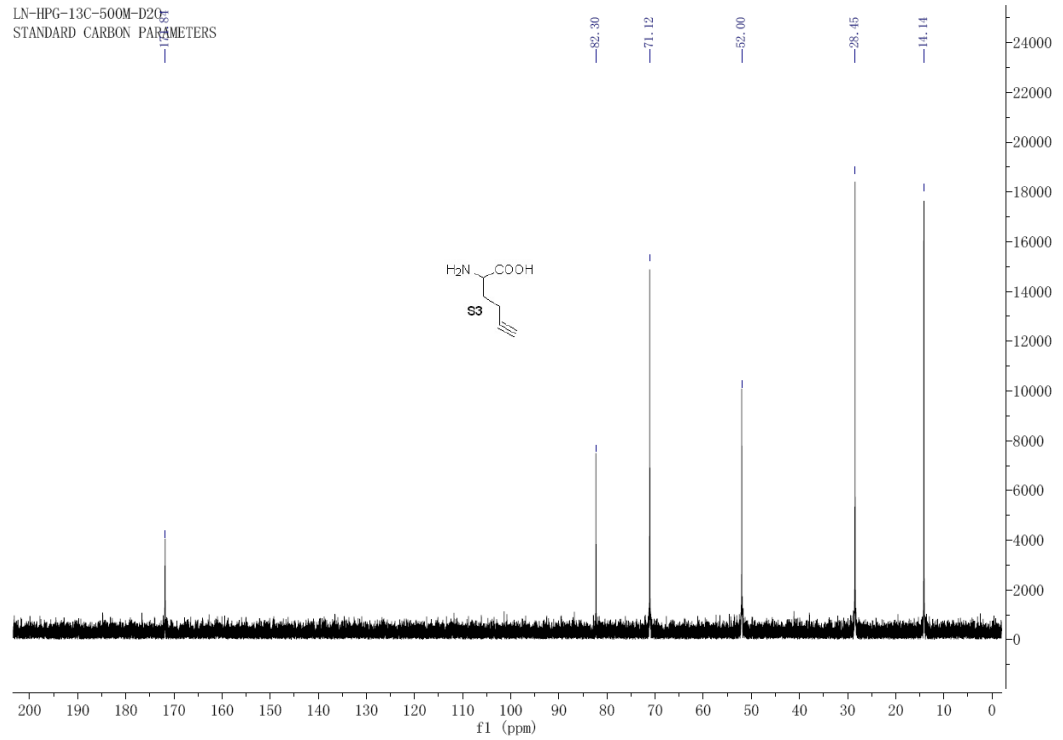

LN-1-100-1H-CDCl3
STANDARD PROTON PARAMETERS
EtO
OEt
HN
Ac
S1
f1 (ppm)

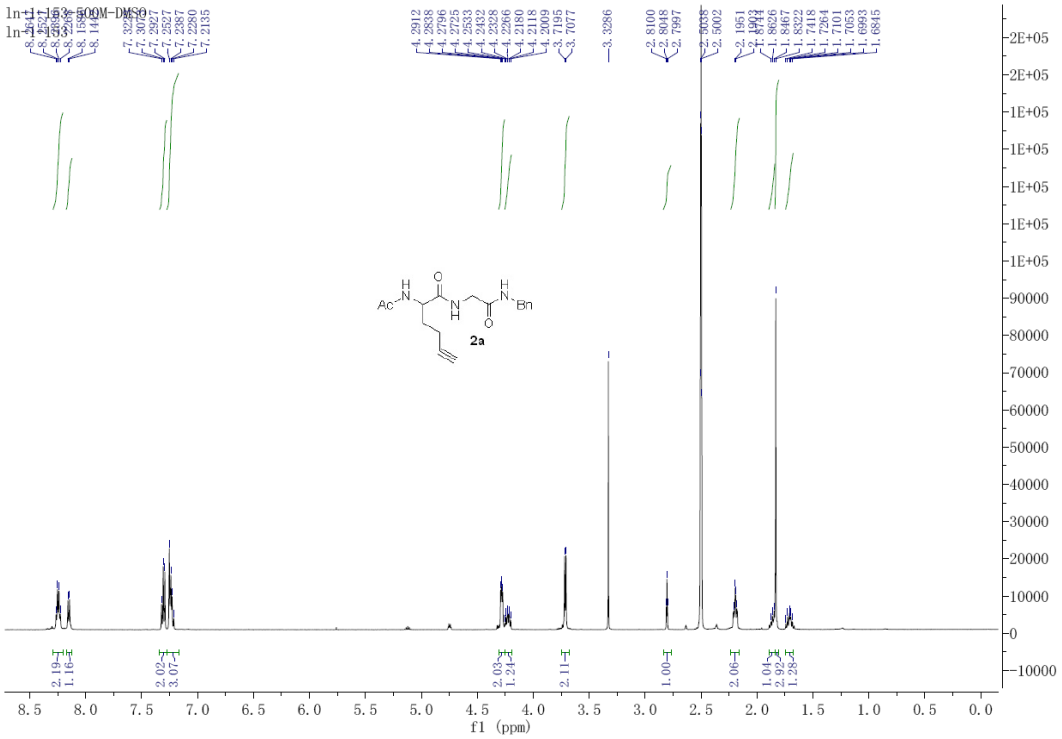

LN-1-100-13C
LN-1-100-13C
EtO
OEt
HN
Ac
S1
f1 (ppm)

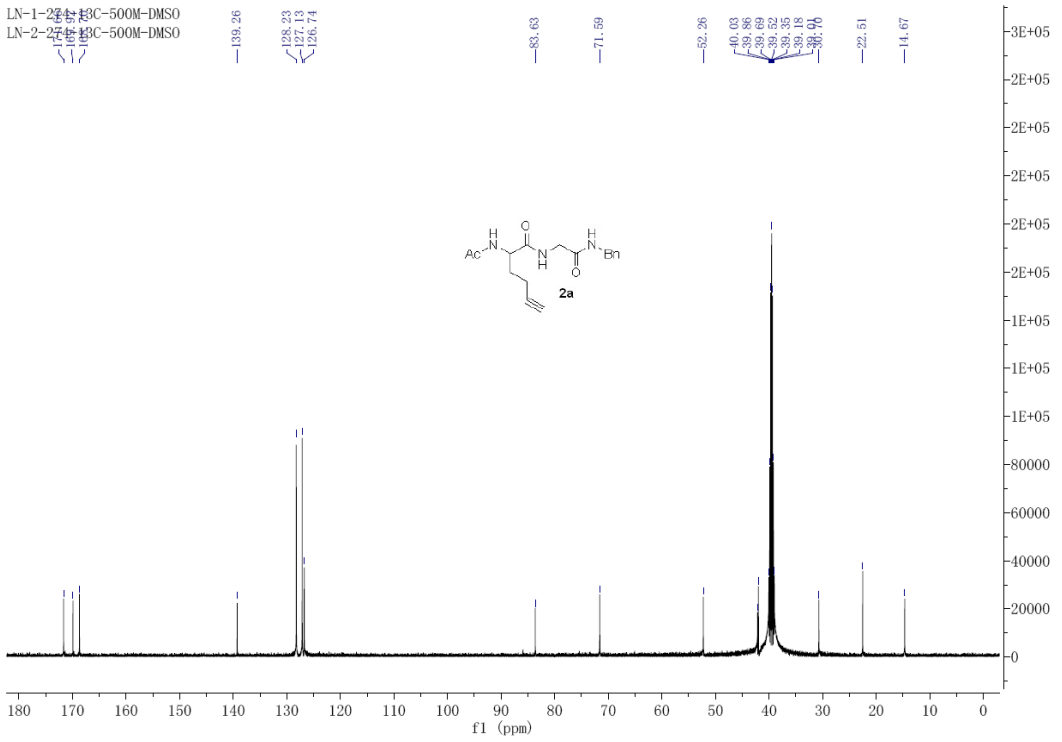

LN-1-223
STANDARD PROTON PARAMETERS
12.488
8.1311
8.1053
4.2679
4.2527
4.2415
4.2368
4.2267
4.2214
4.2109
4.1963
2.8237
2.8158
2.8078
2.5233
2.5064
2.5004
2.4944
2.4886
2.2354
2.2271
2.2101
2.2028
2.1870
2.1787
1.8822
1.8650
1.8572
1.8406
1.7614
1.7387
1.7293
Ac
N
H
O
OH
S2
1.14
1.08
1.06
0.96
1.93
0.88
3.14
1.17
f1 (ppm)

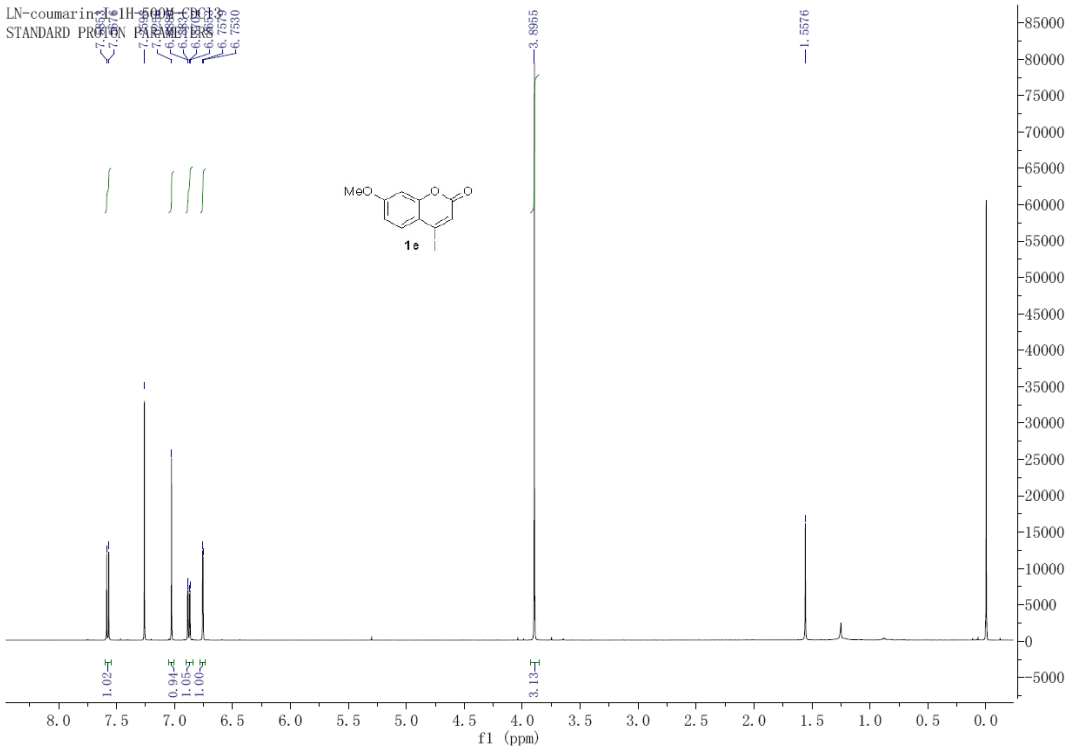

LN-1-223
LN-1-233-13C
176.44
169.49
83.27
71.85
50.94
40.35
40.08
39.80
39.52
39.24
38.96
38.69
30.04
22.38
14.83
Ac
N
H
O
OH
S2
f1 (ppm)

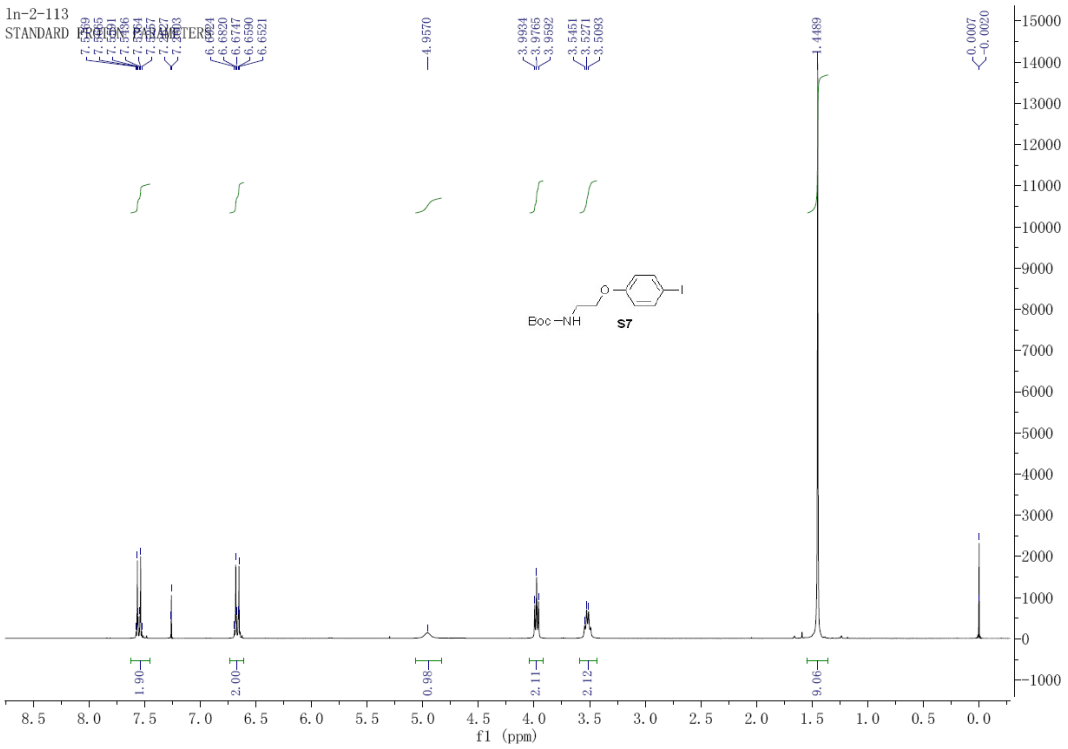

LN-HPG-1H-500M-D2O
STANDARD PROTON PARAMETERS
4.7898
4.1716
4.1585
4.1456
2.4486
2.4352
2.4272
2.4163
2.2963
2.2831
2.2090
2.1942
2.1811
2.1676
2.1176
2.1039
2.0900
2.0753
2.0610
2.0472
2.0108
H2N
COOH
S3
1.00
2.29
1.01
0.92
f1 (ppm)

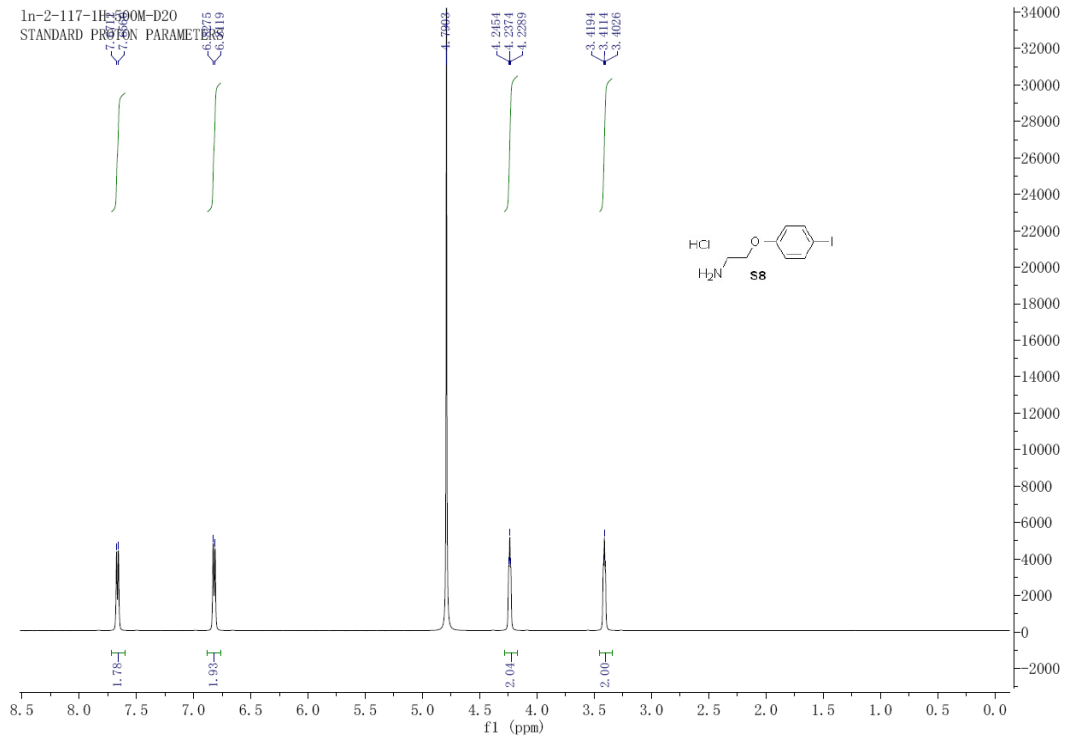

LN-HPG-13C-500M-D2O
STANDARD CARBON PARAMETERS
171.81
82.30
71.12
52.00
28.45
14.14
H2N
COOH
S3
f1 (ppm)

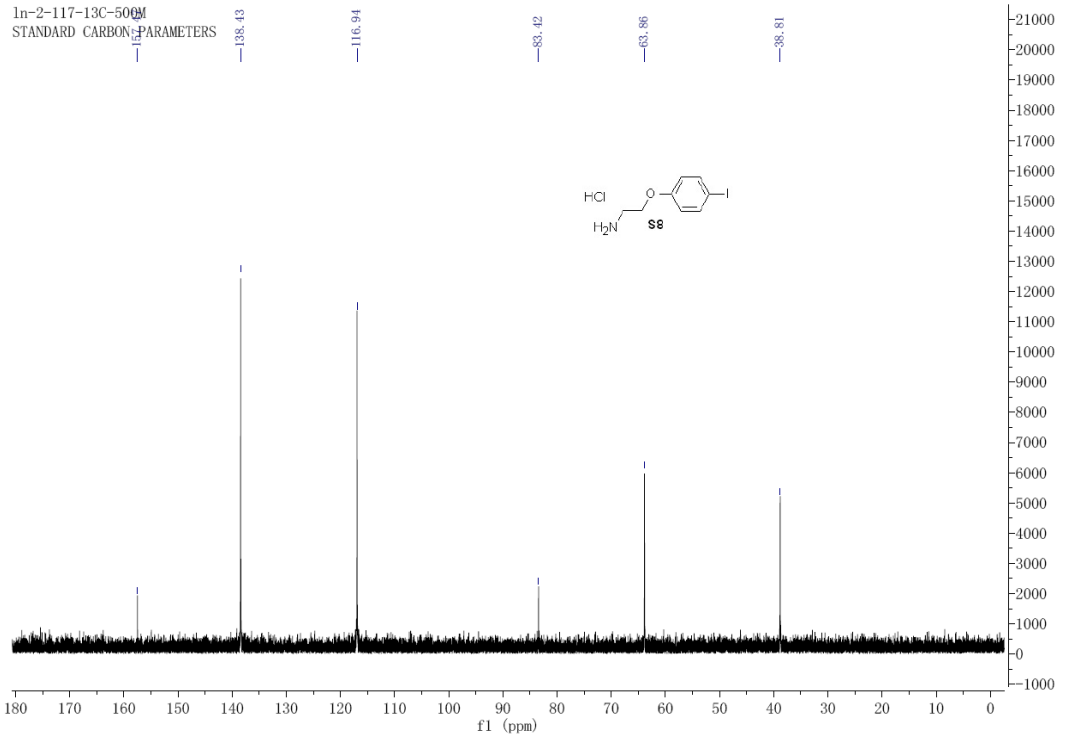

2a
f1 (ppm)

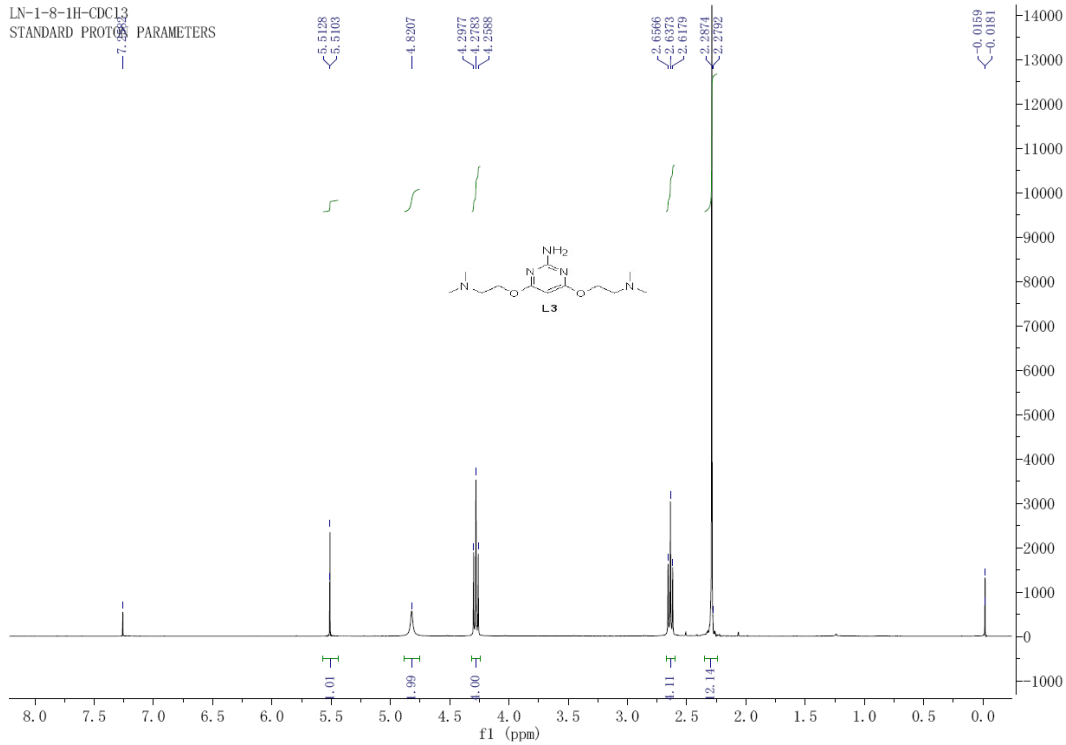

2a
f1 (ppm)

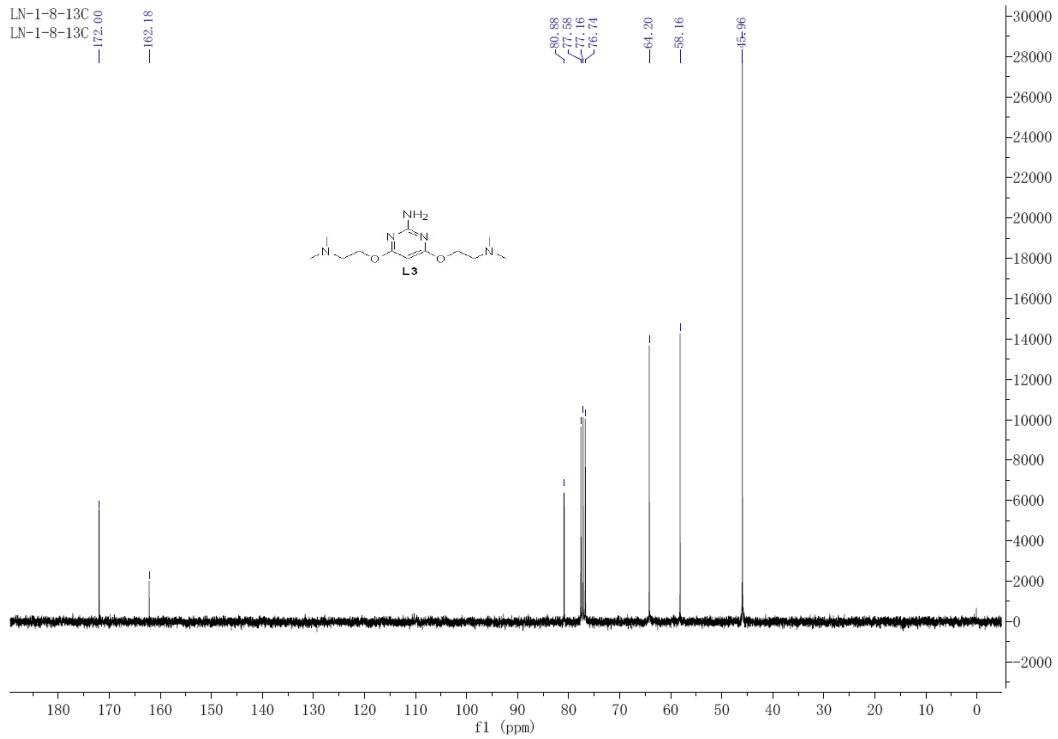

LN-coumarin-I-1H-500M-CDCl3
STANDARD PROTON PARAMETERS
7.5517
7.5317
7.5117
7.4917
7.4717
7.4517
7.4317
7.4117
7.3917
7.3717
7.3517
7.3317
7.3117
7.2917
7.2717
7.2517
7.2317
7.2117
7.1917
7.1717
7.1517
7.1317
7.1117
7.0917
7.0717
7.0517
7.0317
7.0117
6.9917
6.9717
6.9517
6.9317
6.9117
6.8917
6.8717
6.8517
6.8317
6.8117
6.7917
6.7717
6.7517
6.7317
6.7117
6.6917
6.6717
6.6517
6.6317
6.6117
6.5917
6.5717
6.5517
6.5317
6.5117
6.4917
6.4717
6.4517
6.4317
6.4117
6.3917
6.3717
6.3517
6.3317
6.3117
6.2917
6.2717
6.2517
6.2317
6.2117
6.1917
6.1717
6.1517
6.1317
6.1117
6.0917
6.0717
6.0517
6.0317
6.0117
5.9917
5.9717
5.9517
5.9317
5.9117
5.8917
5.8717
5.8517
5.8317
5.8117
5.7917
5.7717
5.7517
5.7317
5.7117
5.6917
5.6717
5.6517
5.6317
5.6117
5.5917
5.5717
5.5517
5.5317
5.5117
5.4917
5.4717
5.4517
5.4317
5.4117
5.3917
5.3717
5.3517
5.3317
5.3117
5.2917
5.2717
5.2517
5.2317
5.2117
5.1917
5.1717
5.1517
5.1317
5.1117
5.0917
5.0717
5.0517
5.0317
5.0117
4.9917
4.9717
4.9517
4.9317
4.9117
4.8917
4.8717
4.8517
4.8317
4.8117
4.7917
4.7717
4.7517
4.7317
4.7117
4.6917
4.6717
4.6517
4.6317
4.6117
4.5917
4.5717
4.5517
4.5317
4.5117
4.4917
4.4717
4.4517
4.4317
4.4117
4.3917
4.3717
4.3517
4.3317
4.3117
4.2917
4.2717
4.2517
4.2317
4.2117
4.1917
4.1717
4.1517
4.1317
4.1117
4.0917
4.0717
4.0517
4.0317
4.0117
3.9917
3.9717
3.9517
3.9317
3.9117
3.8917
3.8717
3.8517
3.8317
3.8117
3.7917
3.7717
3.7517
3.7317
3.7117
3.6917
3.6717
3.6517
3.6317
3.6117
3.5917
3.5717
3.5517
3.5317
3.5117
3.4917
3.4717
3.4517
3.4317
3.4117
3.3917
3.3717
3.3517
3.3317
3.3117
3.2917
3.2717
3.2517
3.2317
3.2117
3.1917
3.1717
3.1517
3.1317
3.1117
3.0917
3.0717
3.0517
3.0317
3.0117
2.9917
2.9717
2.9517
2.9317
2.9117
2.8917
2.8717
2.8517
2.8317
2.8117
2.7917
2.7717
2.7517
2.7317
2.7117
2.6917
2.6717
2.6517
2.6317
2.6117
2.5917
2.5717
2.5517
2.5317
2.5117
2.4917
2.4717
2.4517
2.4317
2.4117
2.3917
2.3717
2.3517
2.3317
2.3117
2.2917
2.2717
2.2517
2.2317
2.2117
2.1917
2.1717
2.1517
2.1317
2.1117
2.0917
2.0717
2.0517
2.0317
2.0117
1.9917
1.9717
1.9517
1.9317
1.9117
1.8917
1.8717
1.8517
1.8317
1.8117
1.7917
1.7717
1.7517
1.7317
1.7117
1.6917
1.6717
1.6517
1.6317
1.6117
1.5917
1.5717
1.5517
1.5317
1.5117
1.4917
1.4717
1.4517
1.4317
1.4117
1.3917
1.3717
1.3517
1.3317
1.3117
1.2917
1.2717
1.2517
1.2317
1.2117
1.1917
1.1717
1.1517
1.1317
1.1117
1.0917
1.0717
1.0517
1.0317
1.0117
0.9917
0.9717
0.9517
0.9317
0.9117
0.8917
0.8717
0.8517
0.8317
0.8117
0.7917
0.7717
0.7517
0.7317
0.7117
0.6917
0.6717
0.6517
0.6317
0.6117
0.5917
0.5717
0.5517
0.5317
0.5117
0.4917
0.4717
0.4517
0.4317
0.4117
0.3917
0.3717
0.3517
0.3317
0.3117
0.2917
0.2717
0.2517
0.2317
0.2117
0.1917
0.1717
0.1517
0.1317
0.1117
0.0917
0.0717
0.0517
0.0317
0.0117
-0.0083
-0.0283
-0.0483
-0.0683
-0.0883
-0.1083
-0.1283
-0.1483
-0.1683
-0.1883
-0.2083
-0.2283
-0.2483
-0.2683
-0.2883
-0.3083
-0.3283
-0.3483
-0.3683
-0.3883
-0.4083
-0.4283
-0.4483
-0.4683
-0.4883
-0.5083
-0.5283
-0.5483
-0.5683
-0.5883
-0.6083
-0.6283
-0.6483
-0.6683
-0.6883
-0.7083
-0.7283
-0.7483
-0.7683
-0.7883

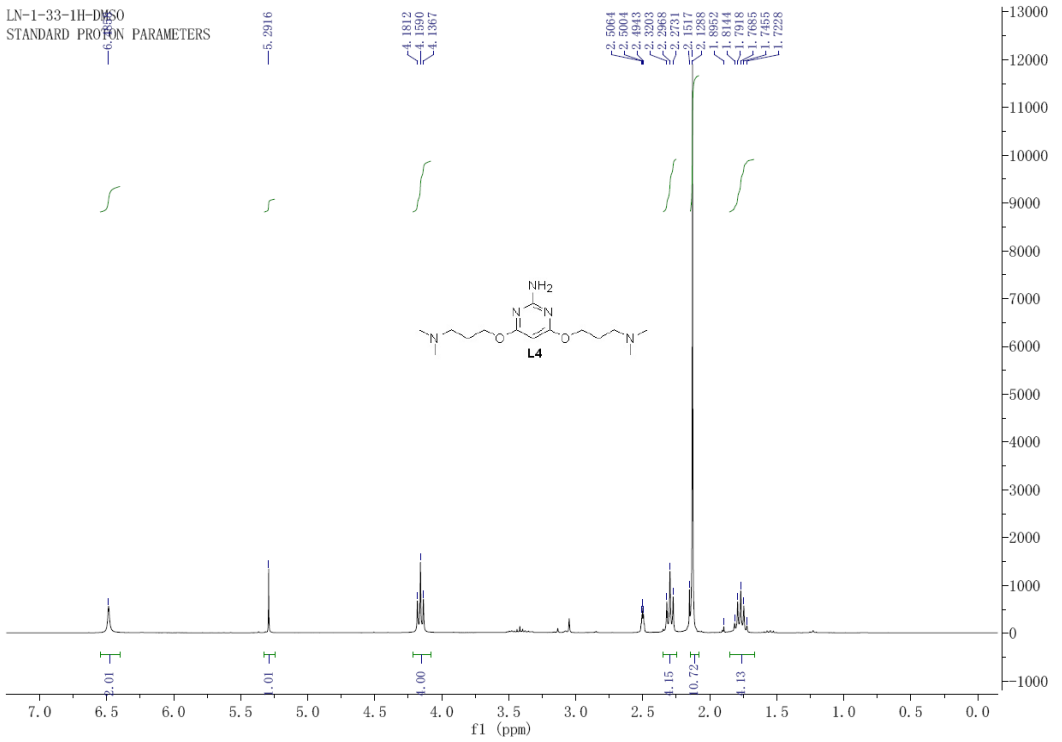

ln-2-113
STANDARD PROTON PARAMETERS
7.5489
7.5465
7.5441
7.5418
7.5394
7.5370
7.5347
7.5323
7.5299
6.6894
6.6820
6.6747
6.6590
6.6521
4.9570
3.9934
3.9765
3.9592
3.5451
3.5271
3.5093
1.4489
0.0007
-0.0020
Boc—NH
O
I
S7
1.90
2.00
0.98
2.11
2.12
9.06
f1 (ppm)

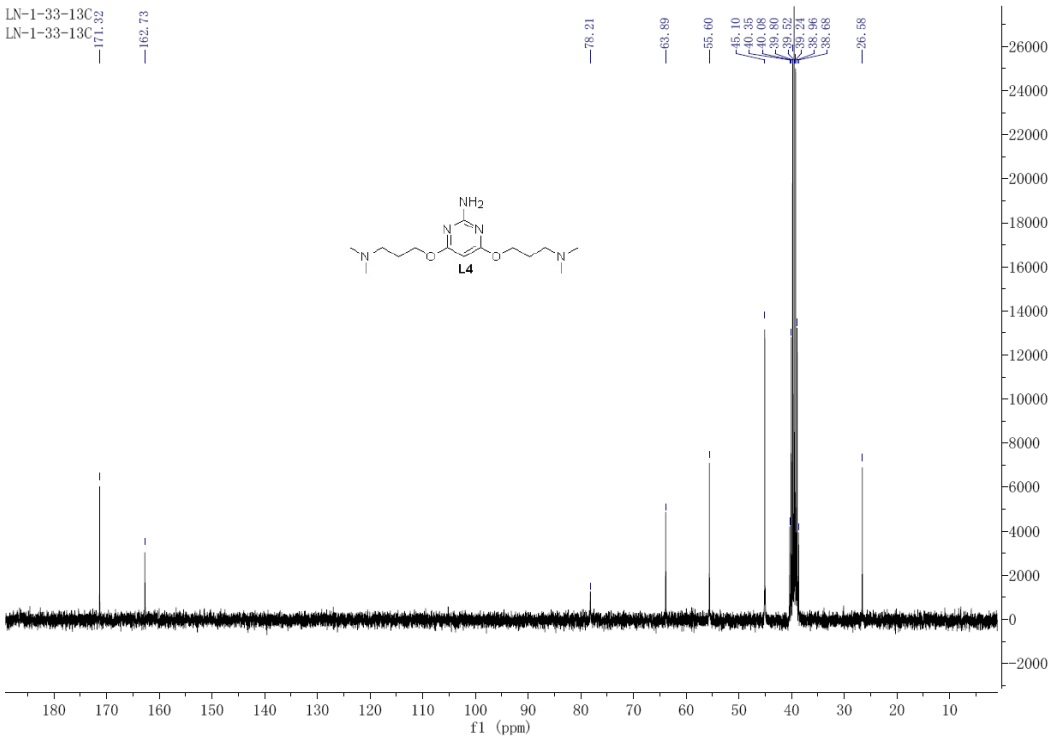

ln-2-117-1H-500M-D2O
STANDARD PROTON PARAMETERS
7.7903
6.8275
6.8119
4.2454
4.2374
4.2289
3.4194
3.4114
3.4026
HCl
H_2N
O
I
S8
1.78
1.93
2.04
2.00
f1 (ppm)

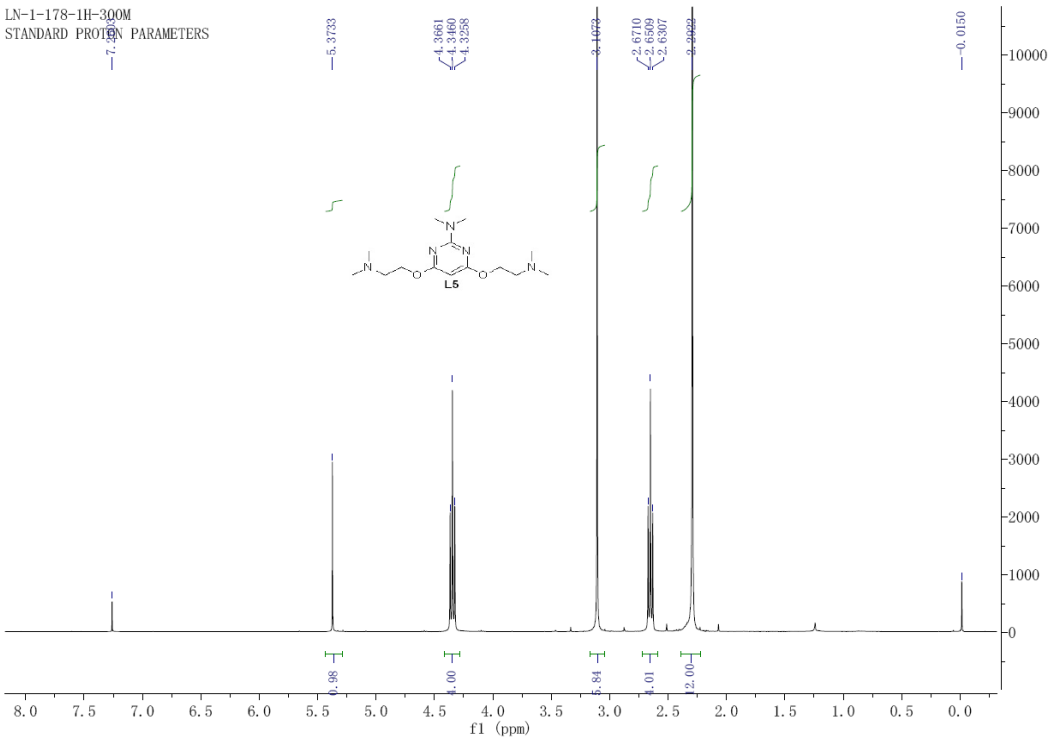

ln-2-117-13C-500M
STANDARD CARBON PARAMETERS
158.52
138.43
116.94
83.42
63.86
38.81
HCl
O
I
H2N
S8
f1 (ppm)

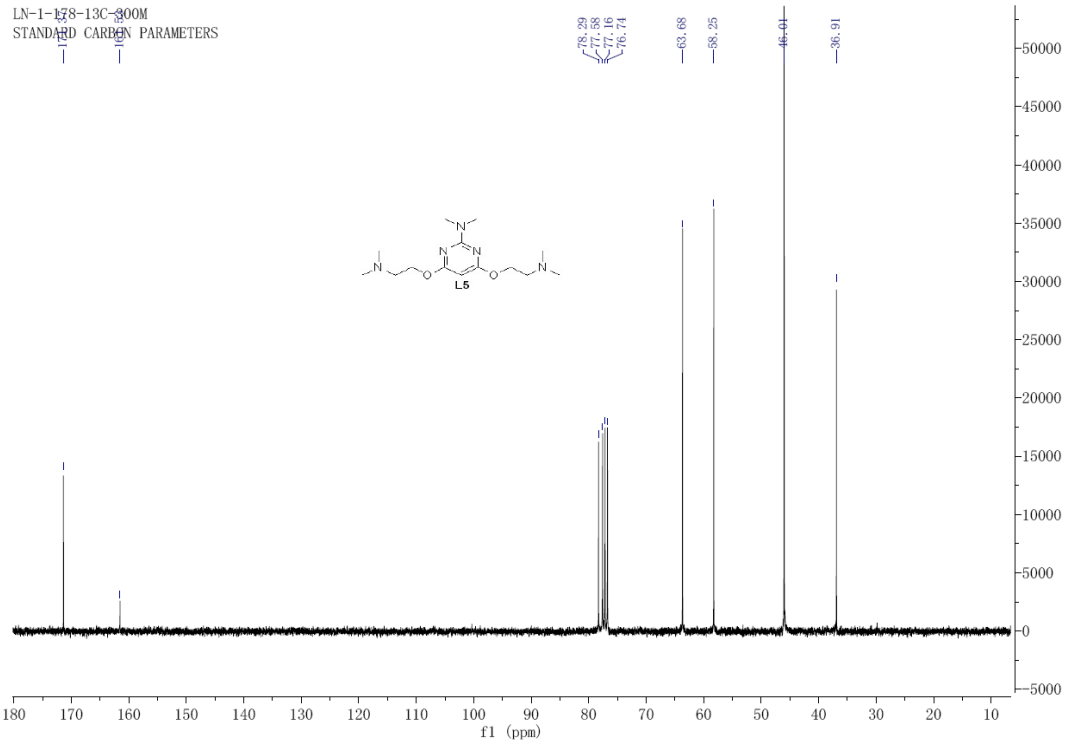

LN-1-8-1H-CDCl3
STANDARD PROTON PARAMETERS
7.2582
5.5128
5.5103
4.8207
4.2977
4.2783
4.2588
2.6566
2.6373
2.6179
2.2874
2.2792
0.0159
0.0181
NH2
N
O
L3
1.01
1.99
3.00
4.11
12.14
f1 (ppm)

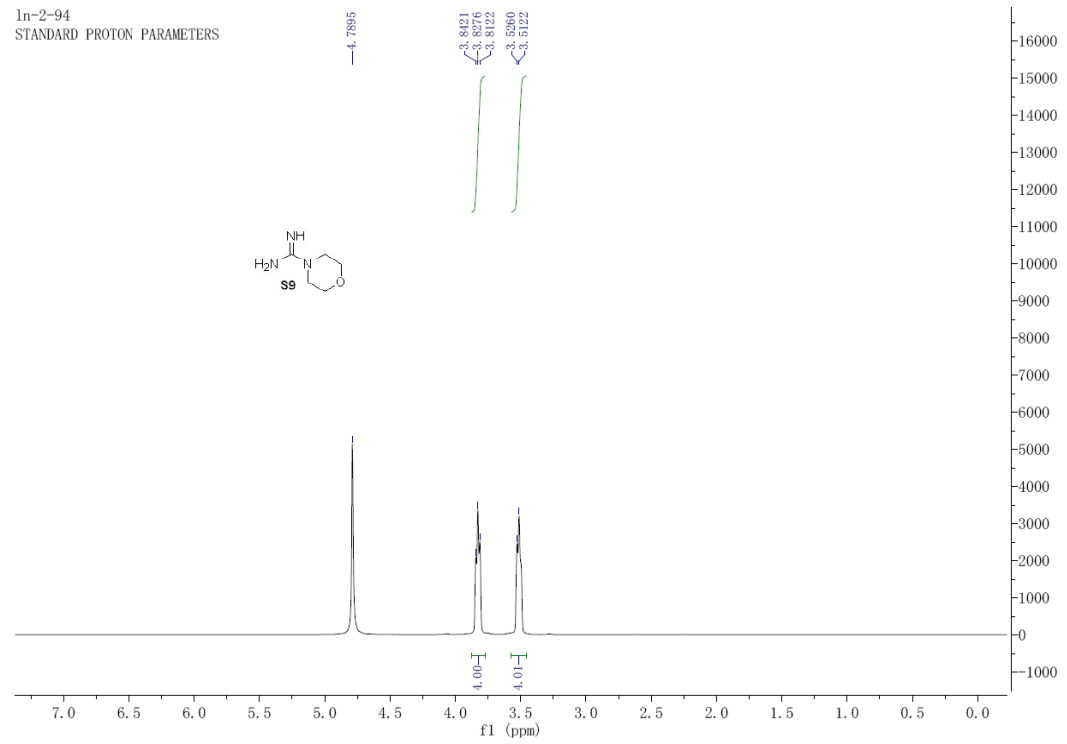

LN-1-8-13C
LN-1-8-13C
172.00
162.18
80.88
77.58
77.16
76.74
64.20
58.16
45.96
L3
f1 (ppm)

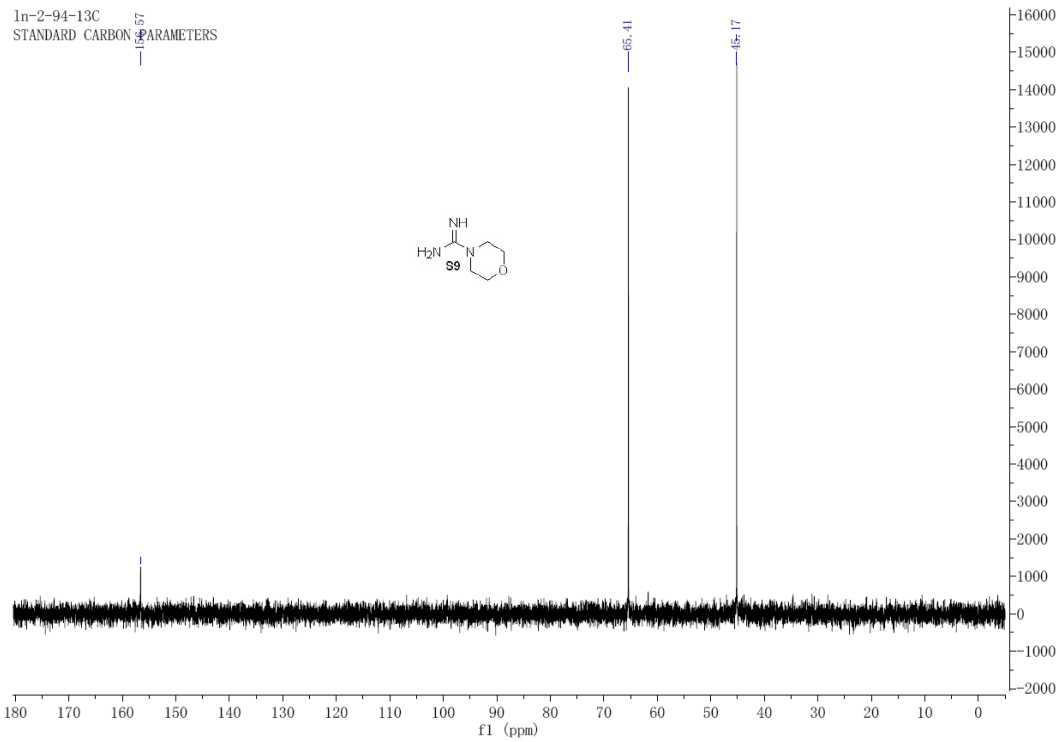

LN-1-33-1H-DMSO
STANDARD PROTON PARAMETERS
6.6852
5.2916
4.1812
4.1590
4.1367
2.5064
2.5044
2.4943
2.3203
2.2968
2.2731
2.1517
2.1288
1.8952
1.8144
1.7918
1.7685
1.7455
1.7228
L4
2.01
1.01
4.00
4.15
10.72
4.13
f1 (ppm)

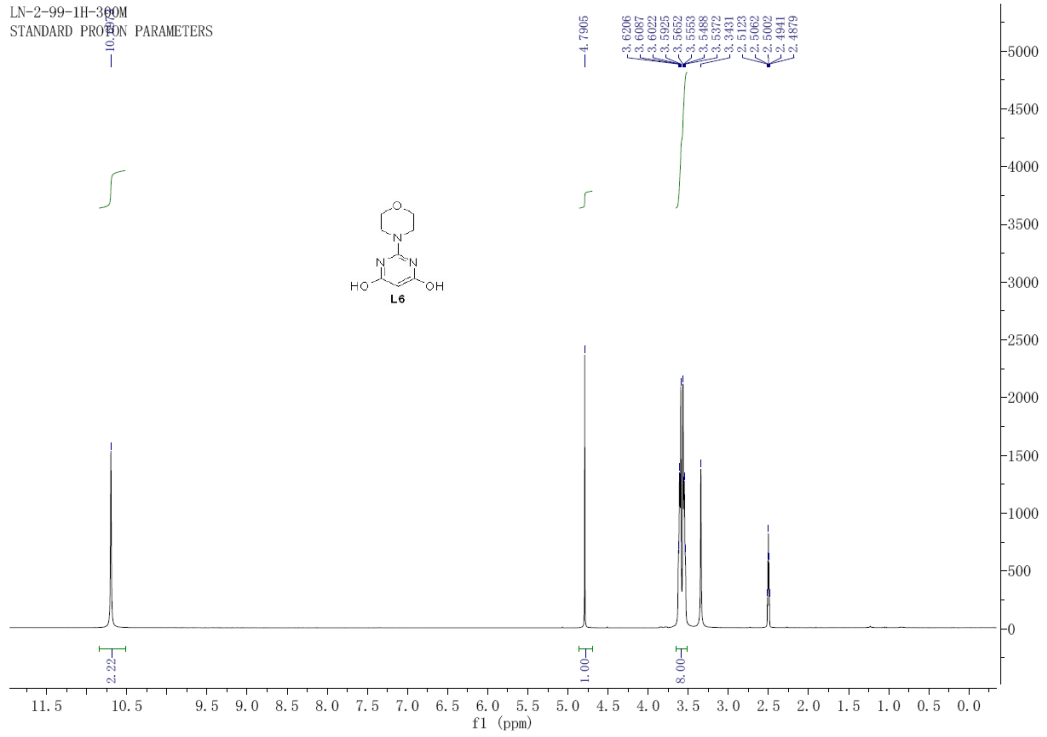

LN-1-33-13C
LN-1-33-13C
171.82
162.73
78.21
63.89
55.60
46.10
40.35
40.08
39.80
39.52
39.24
38.96
38.68
26.58
NH2
L4
f1 (ppm)

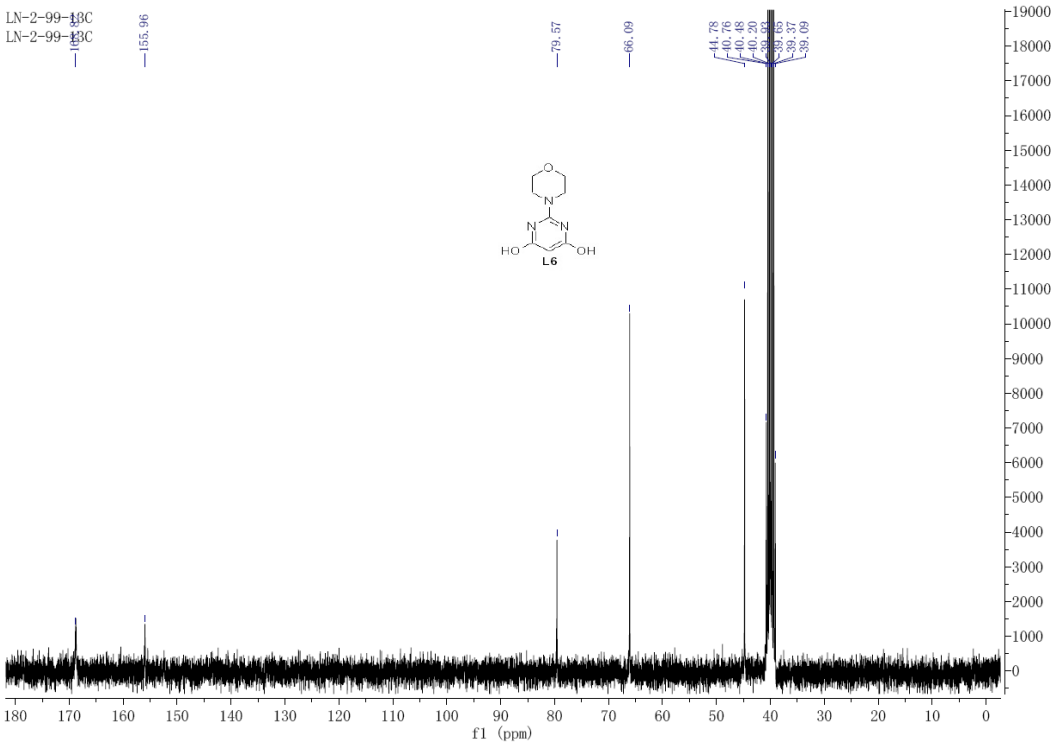

LN-1-178-1H-300M
STANDARD PROTON PARAMETERS
7.2605
5.3733
4.3661
4.3460
4.3258
3.1073
2.6710
2.6509
2.6307
2.3022
0.0150
L5
0.98
4.00
5.84
4.01
12.00
f1 (ppm)

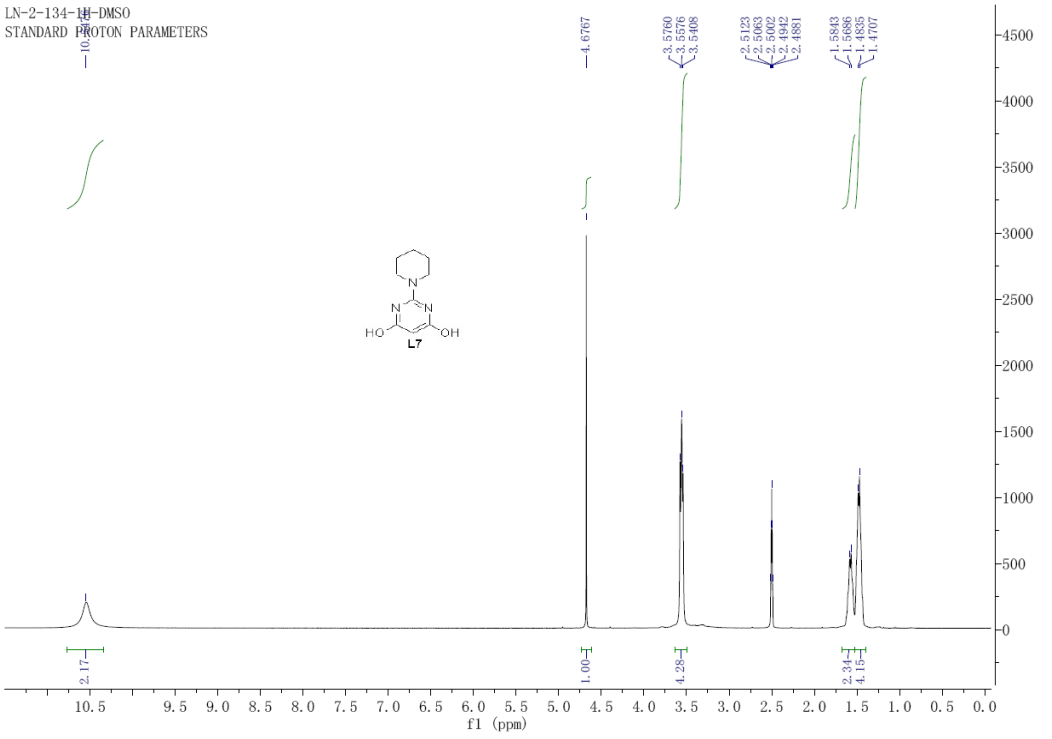

LN-1-178-13C-300M
STANDARD CARBON PARAMETERS
78.29
77.58
77.16
76.74
63.68
58.25
46.01
36.91
L5
f1 (ppm)

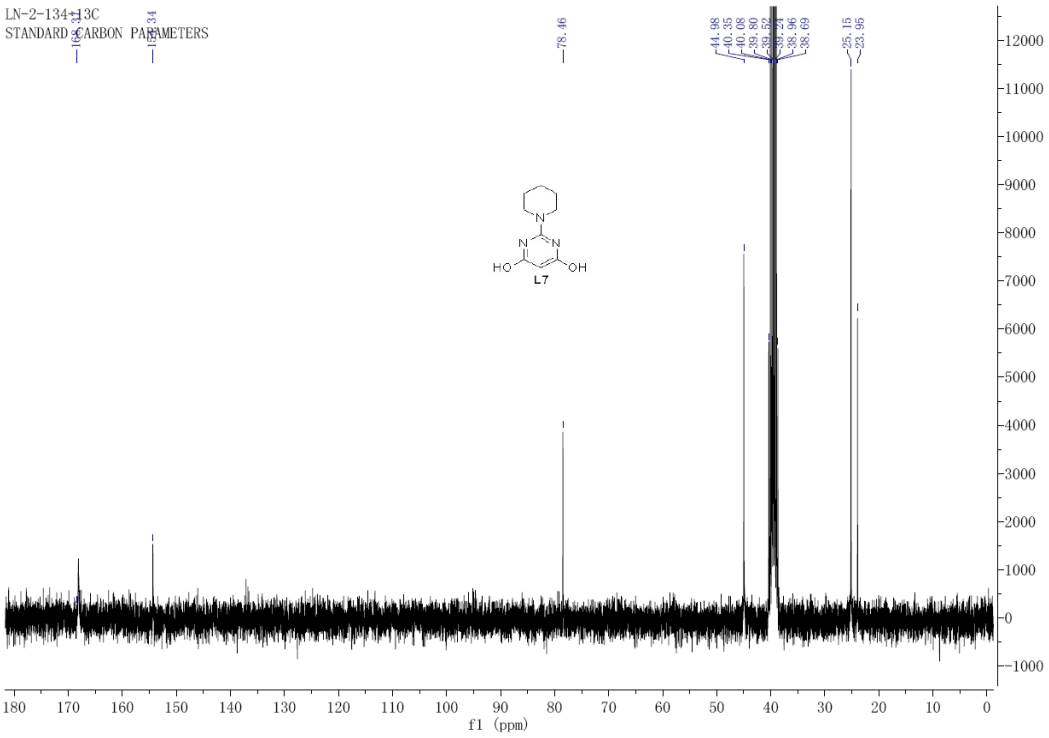

ln-2-94
STANDARD PROTON PARAMETERS
4.7895
3.8421
3.8276
3.8122
3.5260
3.5122
S9
4.00
4.01
f1 (ppm)

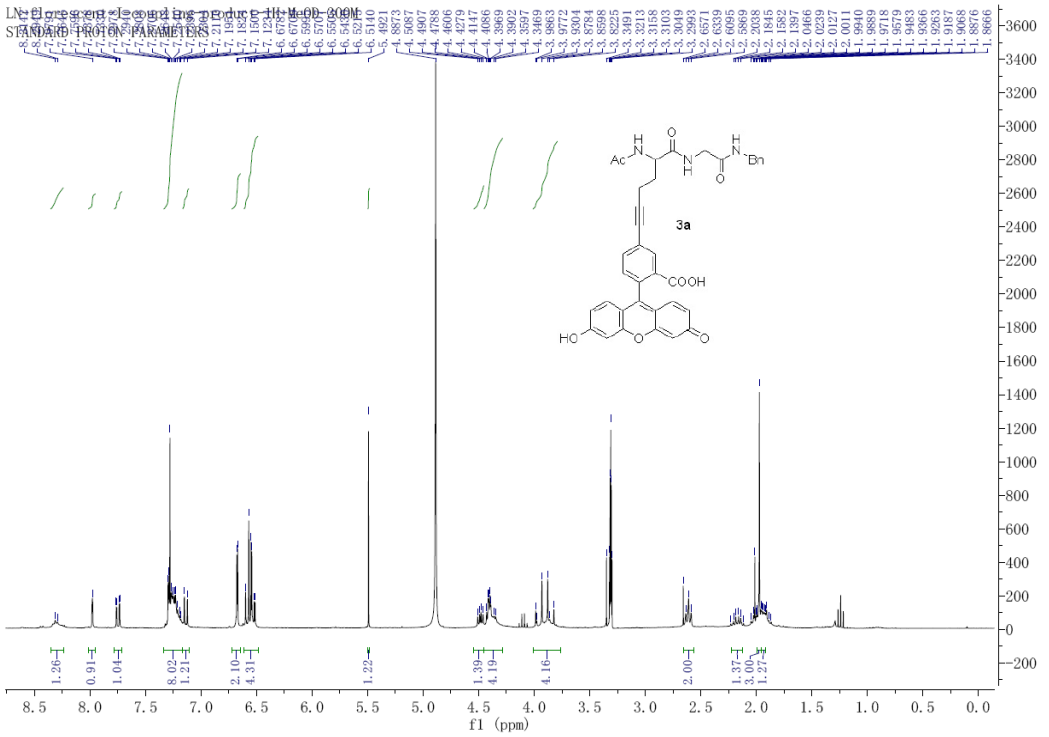

ln-2-94-13C
STANDARD CARBON PARAMETERS
157.57
65.41
45.17
NH
H2N
N
O
S9
f1 (ppm)

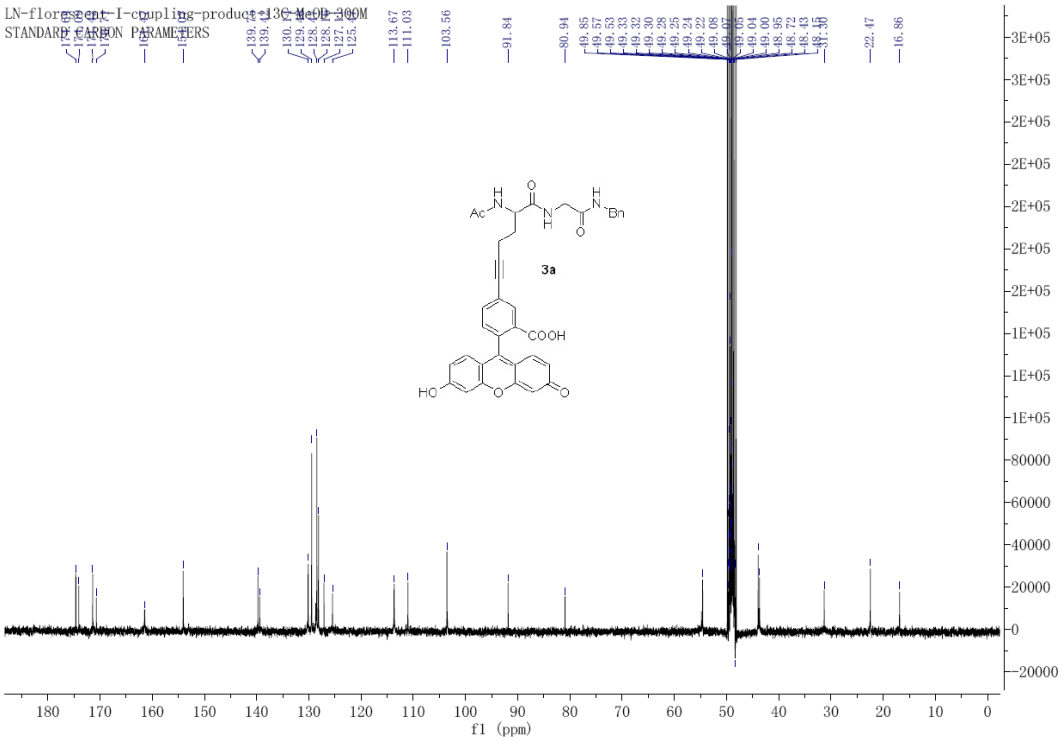

LN-2-99-1H-300M
STANDARD PROTON PARAMETERS
10.8707
4.7905
3.6306
3.6087
3.6022
3.5925
3.5652
3.5553
3.5488
3.5372
3.3431
2.5123
2.5062
2.5002
2.4941
2.4879
N
O
N
HO
OH
L6
2.22
1.00
8.00
f1 (ppm)

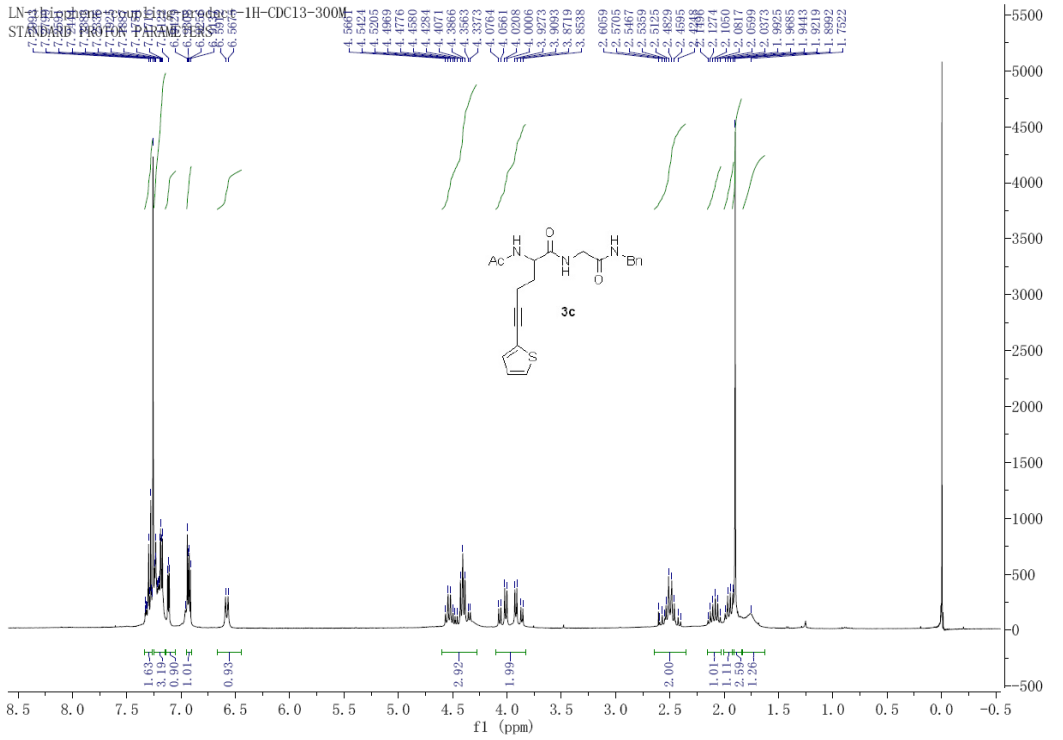

LN-2-99-13C
LN-2-99-13C
155.96
79.57
66.09
44.78
40.76
40.48
40.20
39.93
39.65
39.37
39.09
L6
f1 (ppm)

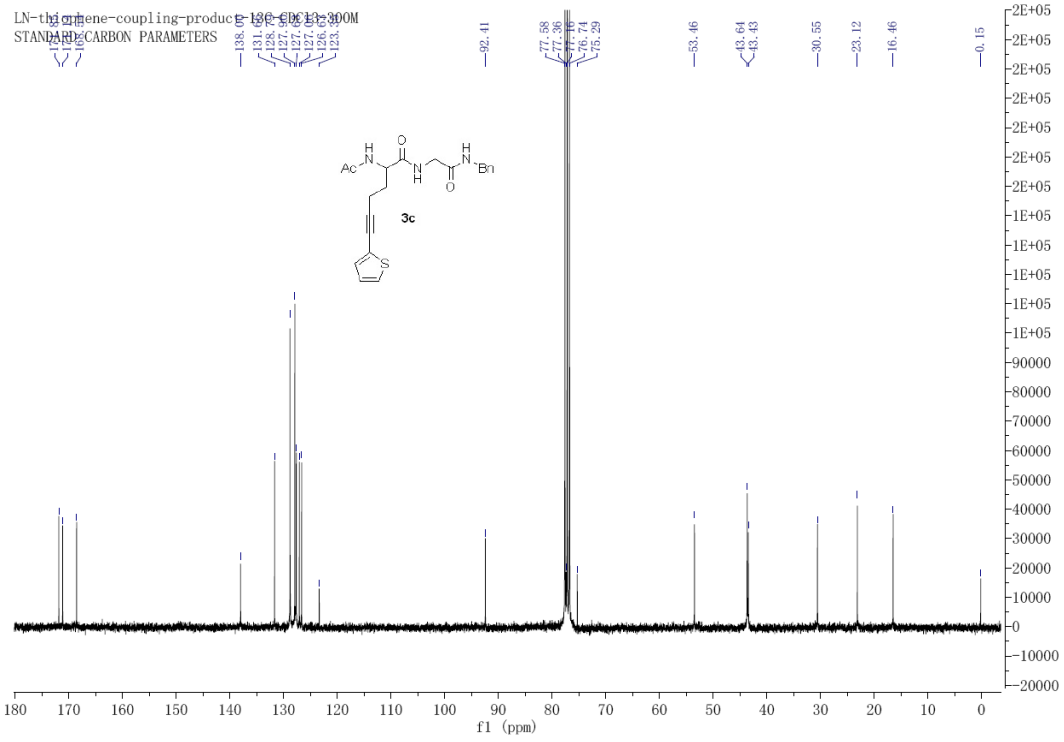

LN-2-134-1H-DMSO
STANDARD PROTON PARAMETERS
4.6767
3.5760
3.5576
3.5408
2.5123
2.5063
2.5002
2.4942
2.4881
1.5843
1.5686
1.4835
1.4707
L7
2.17
1.00
4.28
2.34
4.15
f1 (ppm)

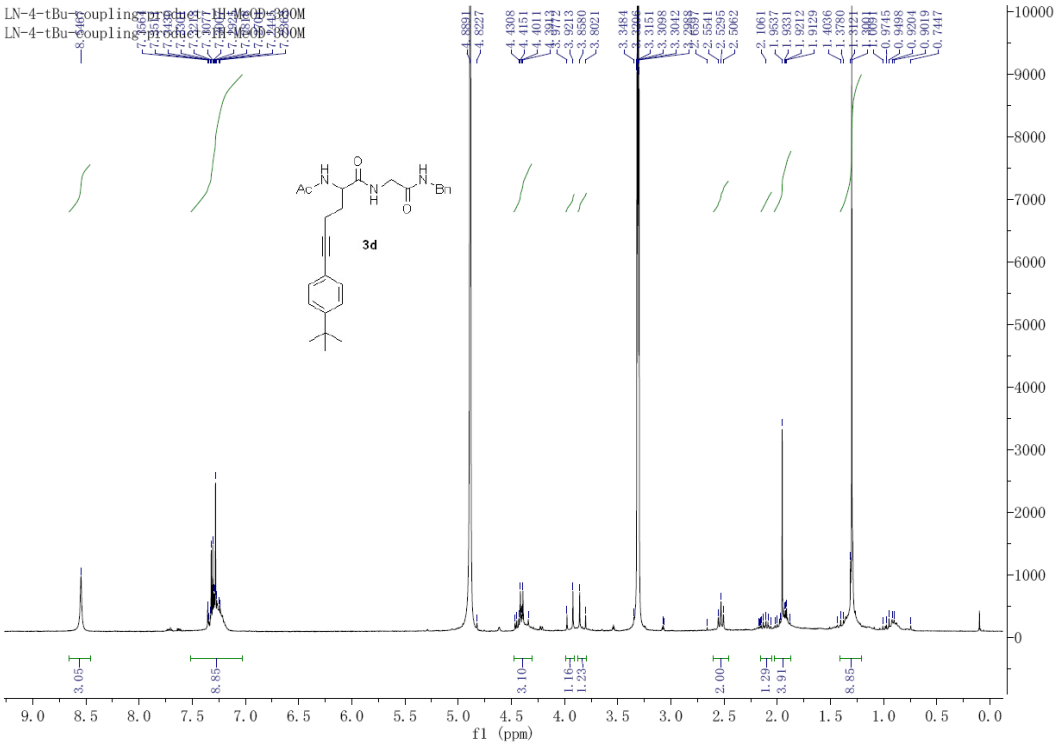

LN-2-134-13C
STANDARD CARBON PARAMETERS
78.46
41.98
40.35
40.08
39.80
39.52
38.96
38.69
25.15
23.96
L7
f1 (ppm)

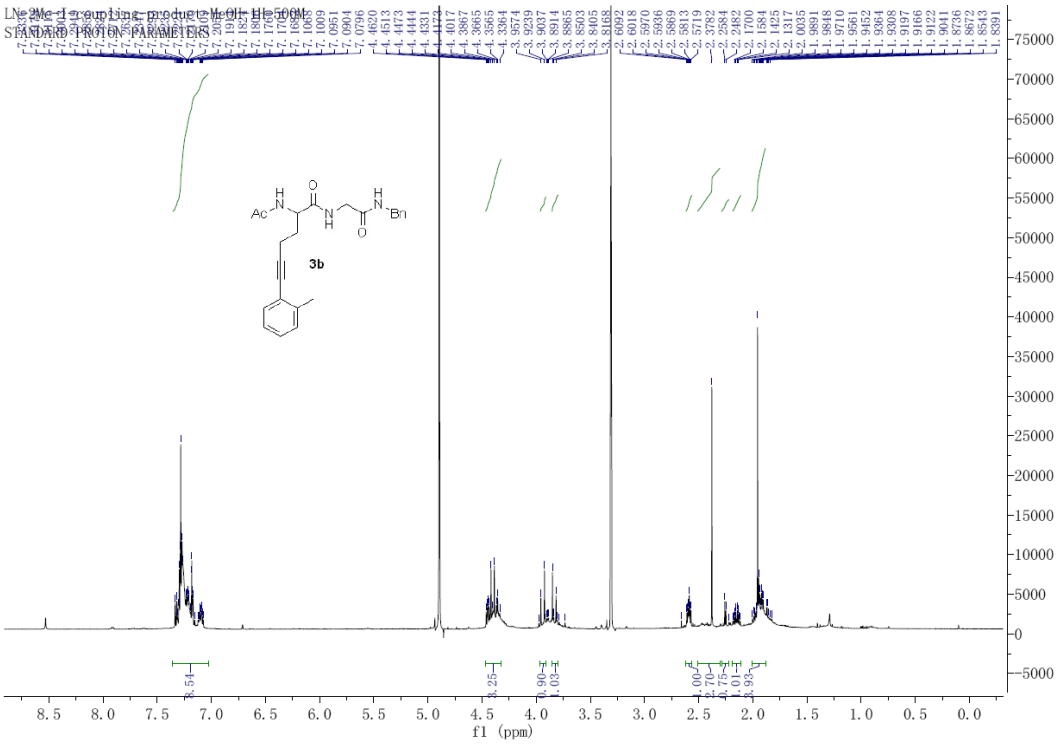

STANDARD PROTON PARAMETERS
3a
f1 (ppm)

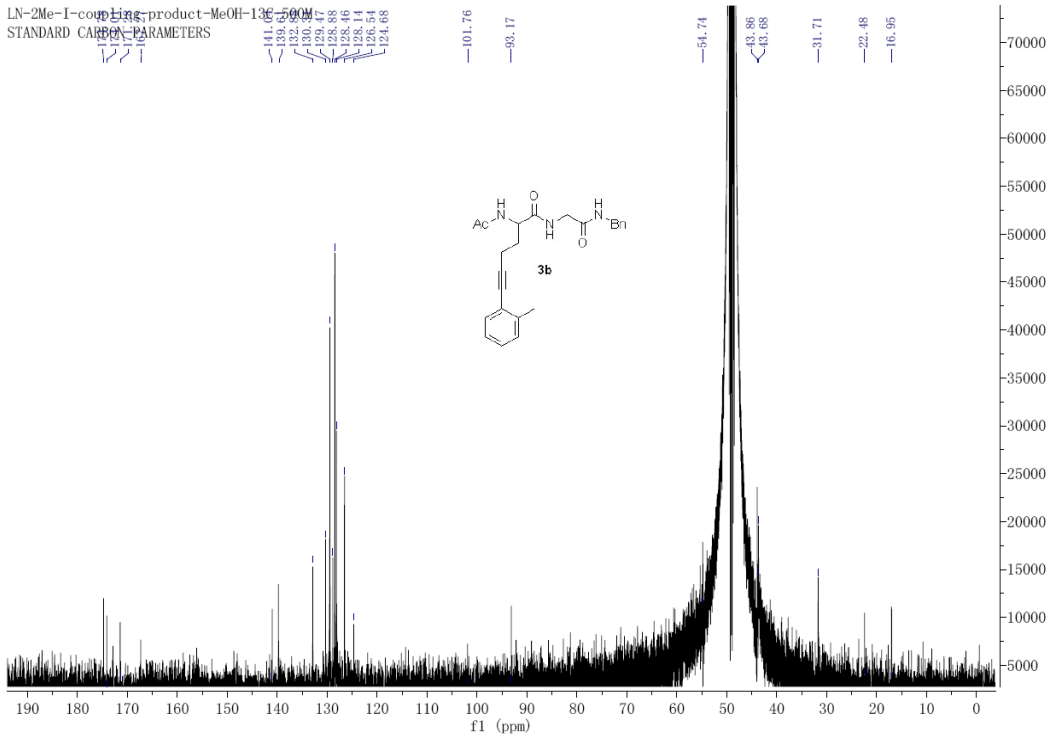

LN-florescent-I-coupling-product-13C-MeOD-300M
STANDARD CARBON PARAMETERS
3a
f1 (ppm)

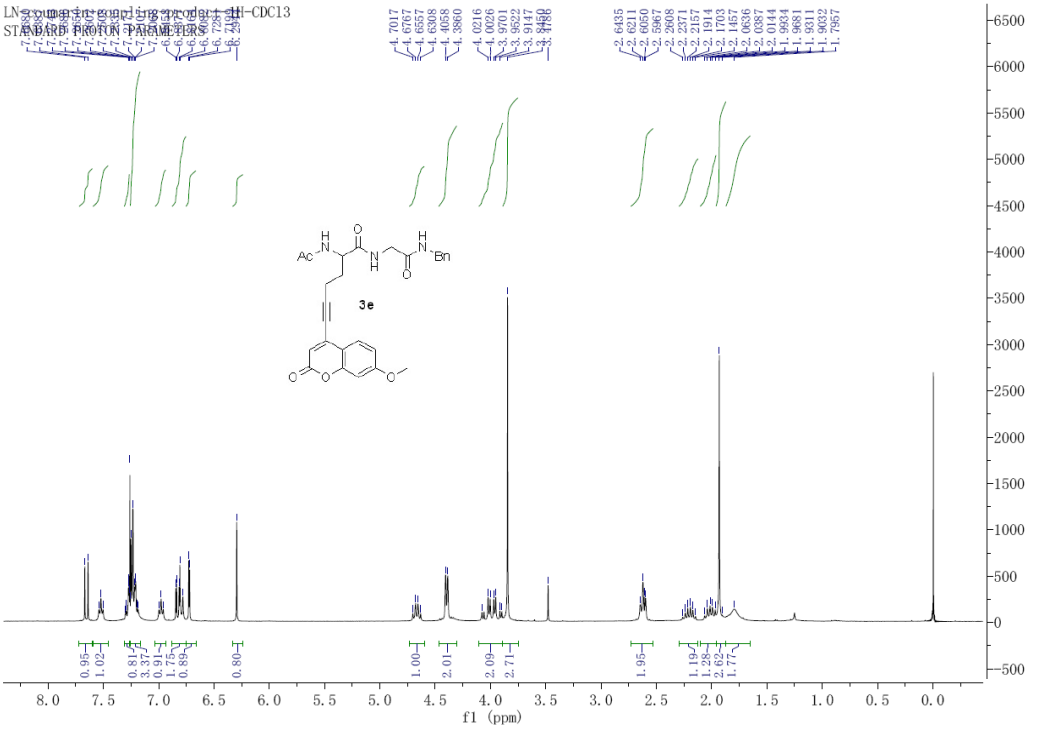

LN-thiophene-coupling-product-1H-CDCl3-300M
STANDARD PROTON PARAMETERS
3c
f1 (ppm)

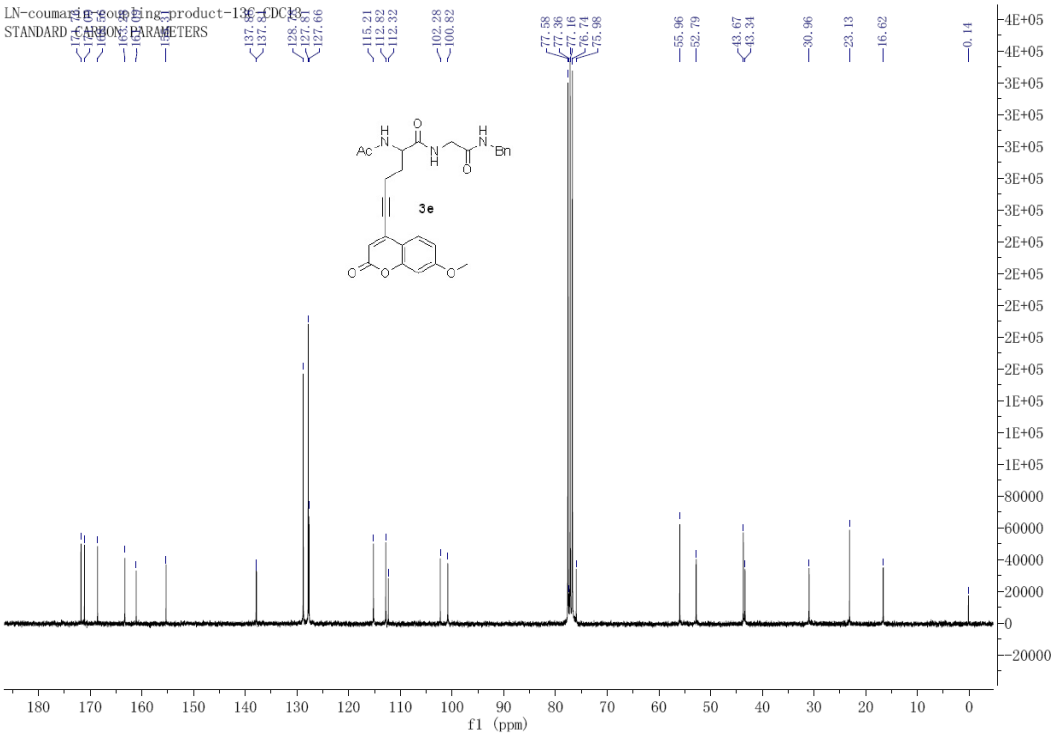

LN-thiophene-coupling-product-13C-CDCl3-300M
STANDARD 13C CARBON PARAMETERS
3c
f1 (ppm)

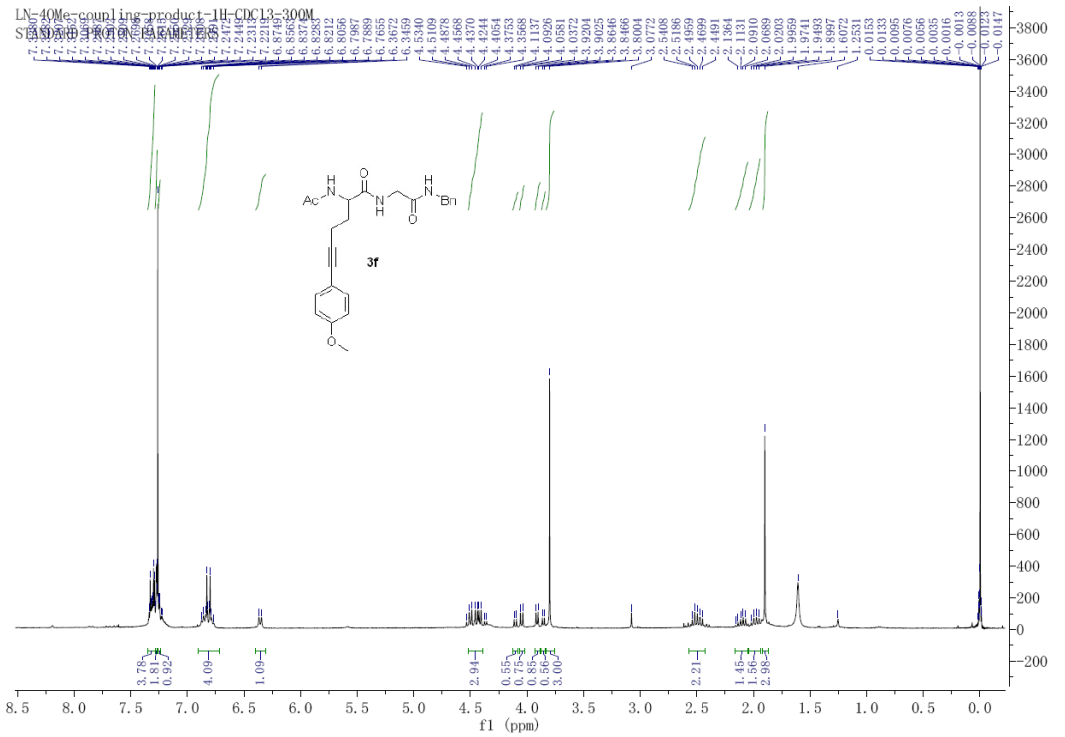

LN-4-tBu-coupling-product-1H-MeOD-300M
LN-4-tBu-coupling-product-1H-MeOD-300M
3d
f1 (ppm)

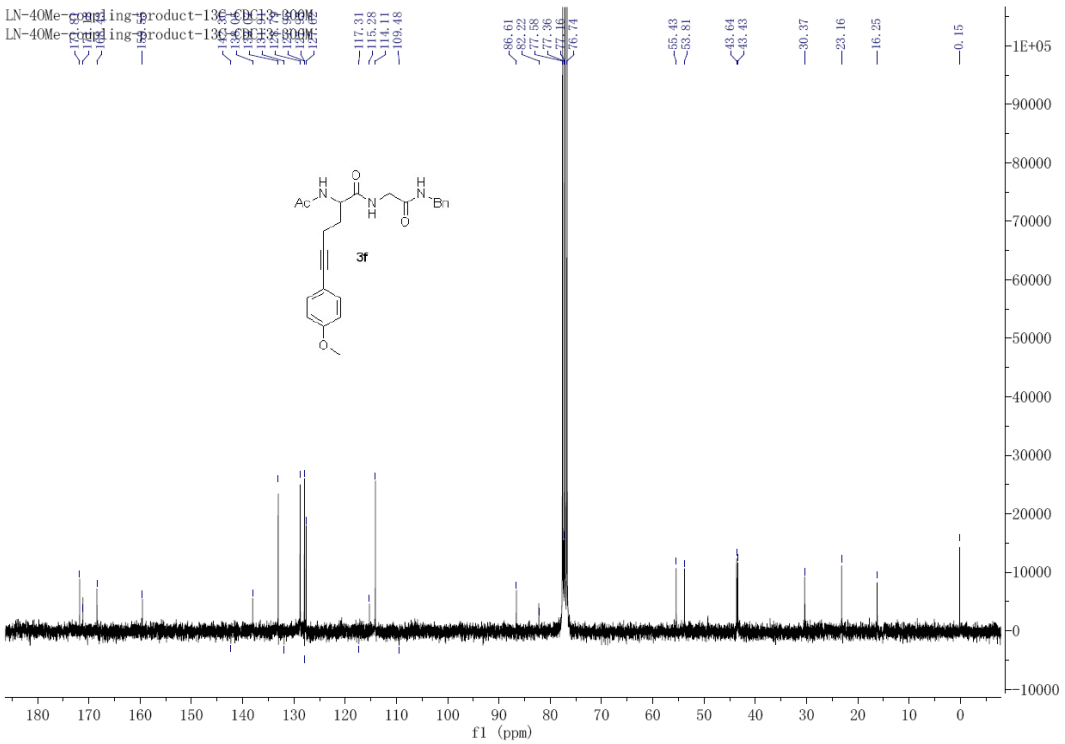

3b
f1 (ppm)

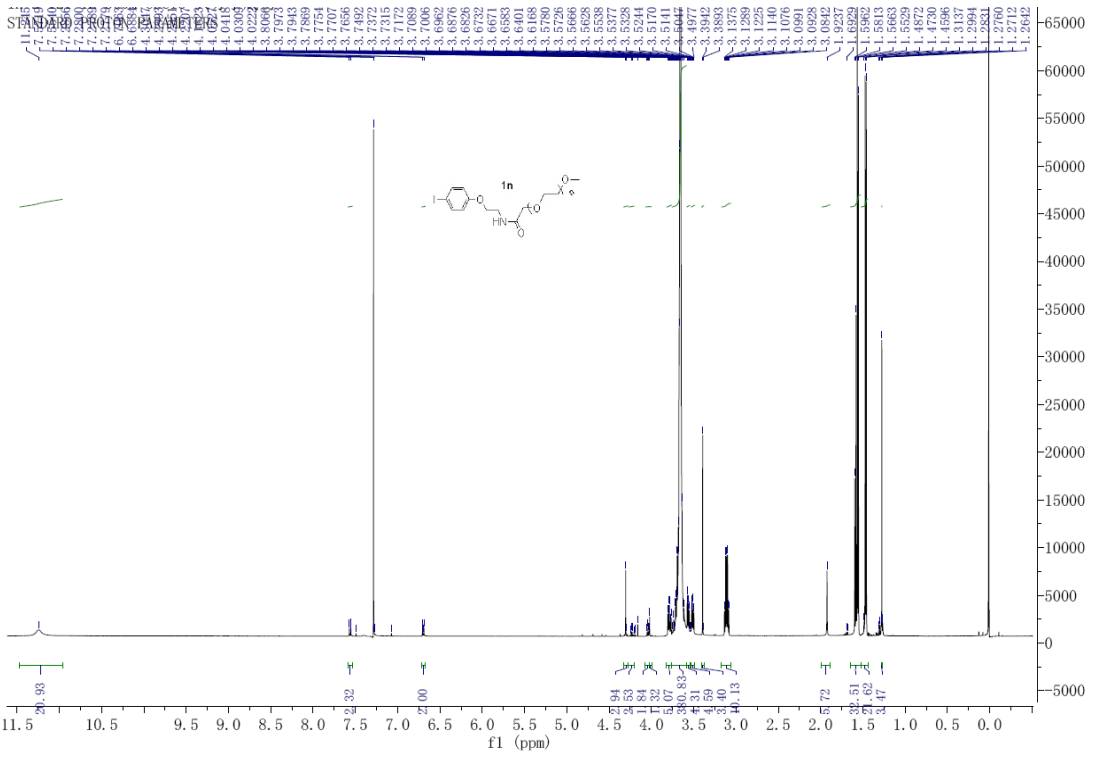

3b
f1 (ppm)


STANDARD PROTON PARAMETERS
3e
f1 (ppm)


STANDARD CARBON PARAMETERS
3e
f1 (ppm)


LN-40Me-coupling-product-1H-CDCl3-300M
f1 (ppm)


LN-40Me-coupling-product-13C-CDCl3-300M
f1 (ppm)


1n
f1 (ppm)