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Accounts and Rapid Communications in Chemical Synthesis

Supporting Information

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Hydrophilic α-Aryl-α-Diazoamides for Protein Esterification

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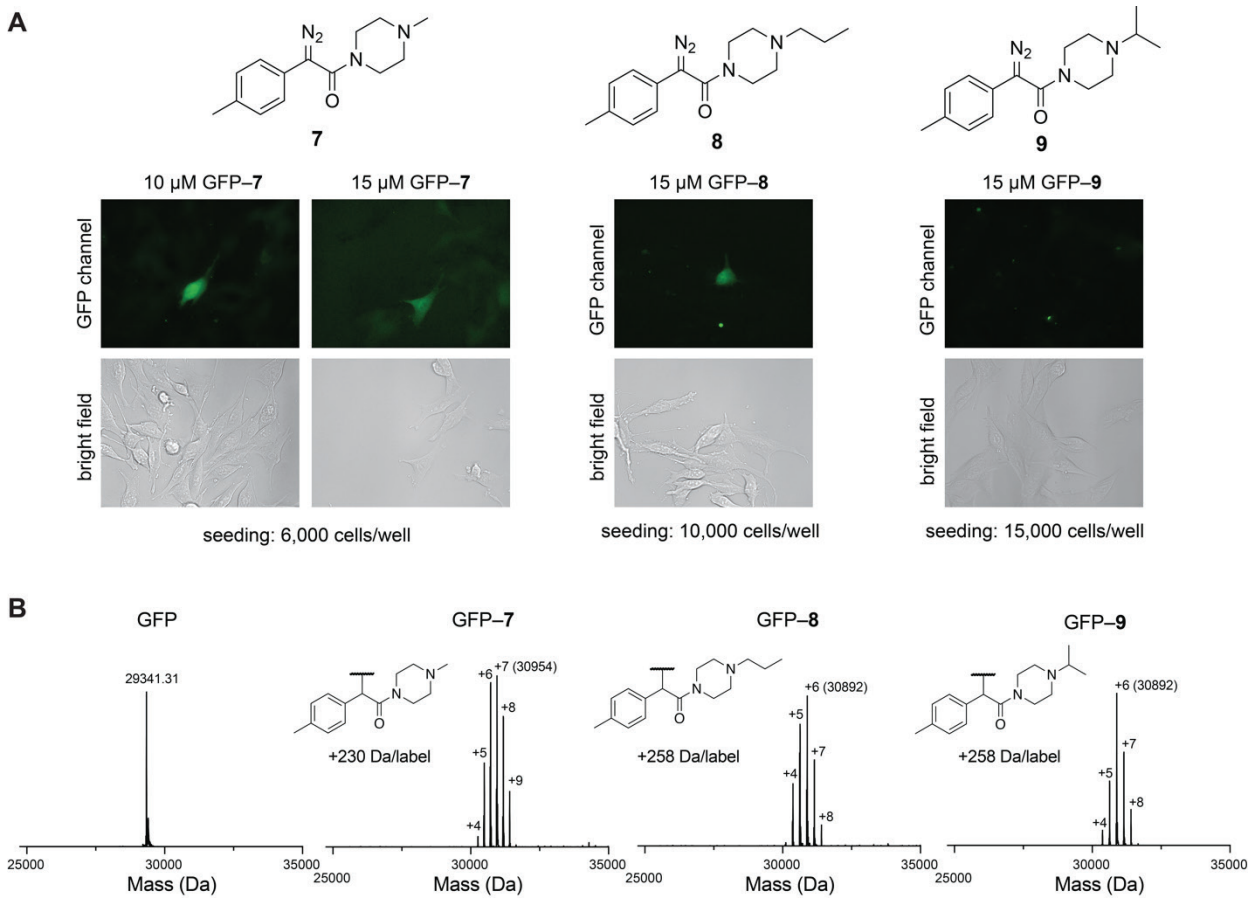


Figure S1. (A) Live-cell florescence images of M21 cells treated with GFP–**7**, GFP–**8**, or GFP–**9** in serum-free DMEM at 37 °C (*n* = 1 independent experiments per dosing condition). For incubation times and additional experimental details, see Section "VI. Live-Cell Microscopy." (B) Deconvoluted ESI Q-TOF mass spectra of the esterified proteins used in the experiment.

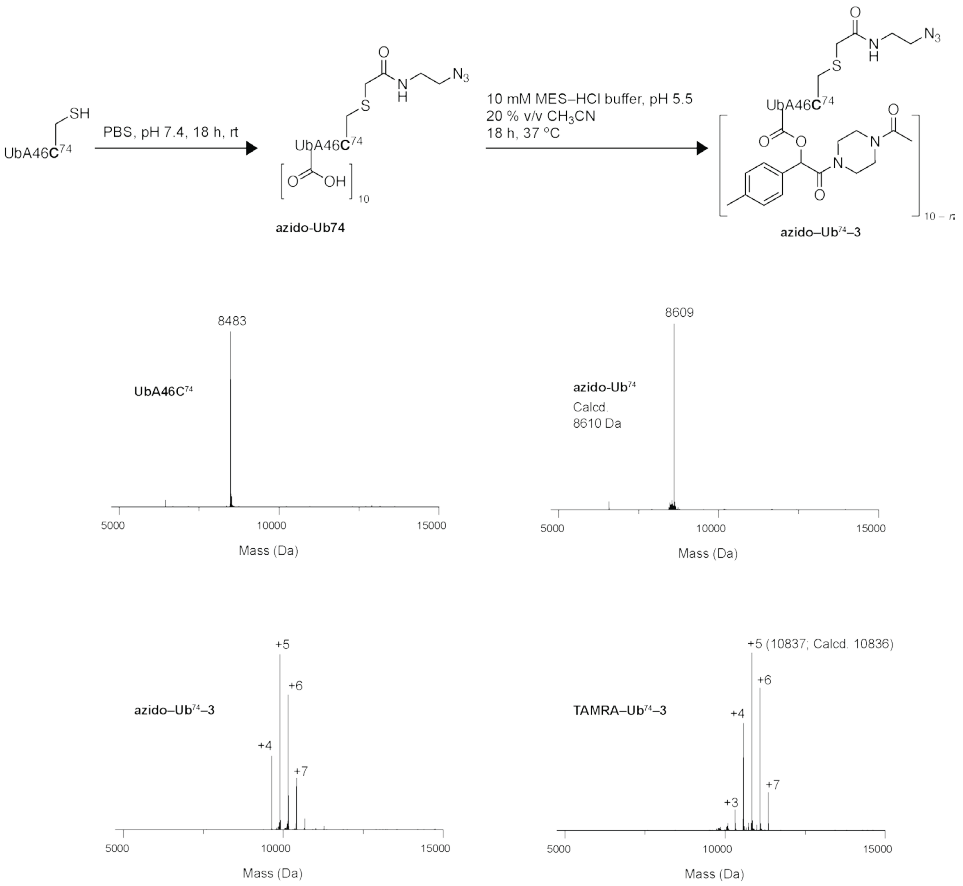


Figure S2 Preparation of TAMRA–Ub^{74}–**3**. Deconvoluted ESI Q–TOF mass spectra of UbA46C^{74}, azido–Ub^{74}, azido–Ub^{74}–**3**, and TAMRA–Ub^{74}–**3**.

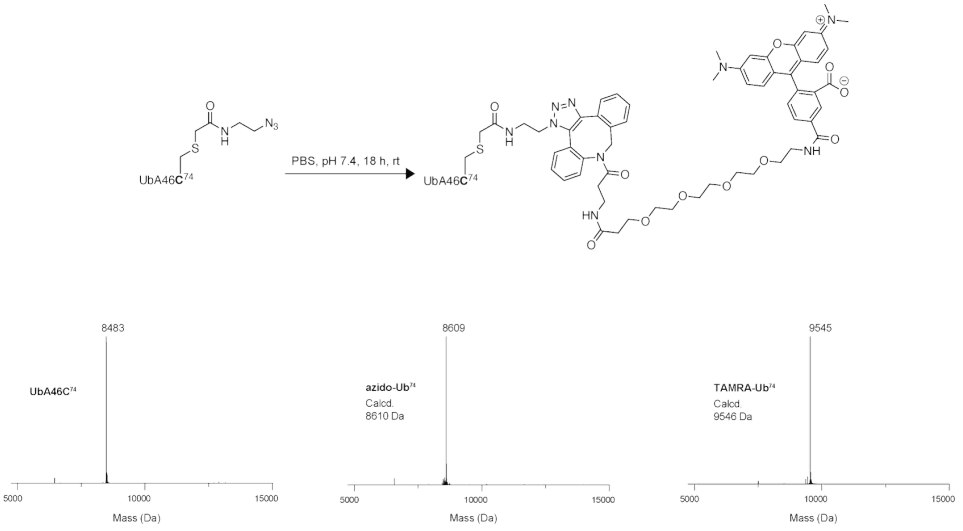


Figure S3. Preparation of TAMRA–Ub^{74}. Deconvoluted ESI Q-TOF mass spectra of UbA46C^{74}, azido–Ub^{74}, and TAMRA–Ub^{74}.

I. Abbreviations Used

CHO	Chinese hamster ovary
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCM	dichloromethane
DIPEA	*N*,*N*-diisopropylethylamine
DMEM	Dulbecco's modified Eagle's medium
ESI	electrospray ionization
FBS	fetal bovine serum
GFP	"super-folder" variant of the green fluorescent protein
HEPES	4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid
MES	2-(*N*-morpholino)ethanesulfonic acid
MWCO	molecular weight cut-off
PBS	phosphate-buffered saline
pI	isoelectric point
NMR	nuclear magnetic resonance
Q–TOF	quadruple–time-of-flight
RFP	red fluorescent protein
TAMRA	tetramethylrhodamine
TLC	thin-layer chromatography
$UbA46C^{74}$	A46C variant of human ubiquitin protein (1–74)
UV	ultraviolet
vis	visible
WGA	wheat-germ agglutinin

II. Chemical Synthesis

General Experimental procedures. All anhydrous air-free reactions were performed under a positive atmosphere of dry N_2(g) in oven-dried glassware equipped with a magnetic stir bar. All procedures are carried out at ambient temperature (~22 °C) and pressure (~1.0 atm) unless stated otherwise. Anhydrous solvents were obtained from an in-house Glass Contour Solvent System (SG Water, Nashua, NH). The term "concentrated under reduced pressure" refers to the removal of solvents and other volatile materials using a Buchi rotary evaporator (model R-210) at water aspirator pressure (<20 torr) while maintaining the water-bath temperature at 25–35 °C. Column chromatography was performed with Silicycle 40–60 Å silica (230–400 mesh); thin-layer chromatography (TLC) was performed with EMD Millipore Silica gel 60 F_{254} glass support plates. The visualization of TLC plates was performed using a hand-held UV lamp (254 nm) for UV-active compounds or by staining with an azide-staining solution to detect compounds bearing an azido group.[1]

Materials. All commercially sourced reagents were used as provided by the manufacturer without further purification. 4-iodotoluene, 4-methylbenzenesulfonhydrazide and 2-azidoethylamine hydrochloride were from Fisher Scientific (Hampton, NH). Glyoxylic acid monohydrate, *N*-hydroxysuccinimide, palladium(II) acetate, silver carbonate, 3-iodopyridine, 6-iodoquinoline, *N*,*N*-dicyclohexylcarbodiimide, *N*,*N*-dimethylamine (2.0 M in THF), morpholine, 1-acetylpiperazine, diisopropylethylamine (DIPEA), triethylamine, and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) were from Sigma–Aldrich (St. Louis, MO). 1-Methylpiperazine, 1-*n*-propylpiperazine dihydrobromide, 1-isopropylpiperazine, and iodoacetic anhydride were also from Sigma–Aldrich. 6-Iodoquinazoline was from AmBeed (Arlington Heights, IL). *N*-Succinimidyl 3-(diphenylphosphino)propionate and tri(2-furyl)phosphine were from TCI America (Portland, OR); anhydrous EtOAc was from ACROS Organics (Geel, Belgium). Phosphate-buffered saline (PBS) was at pH 7.4 and without Ca^{2+}/Mg^{2+}. PD MiniTrap G-25

columns were from Cytiva (Buckinghamshire, UK); Amicon® Ultra 0.5-mL centrifugal filters (3K MWCO) were from Sigma–Aldrich.

The "superfolder" variant of the green fluorescent protein (GFP)[2] and the A46C variant of human ubiquitin protein (1–74) (UbA46C)[3] were produced heterologously in *Escherichia coli* as described previously.

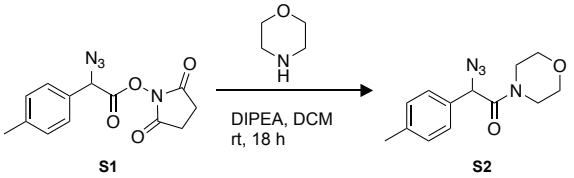
Instrumentation. ^{1}H and ^{13}C NMR spectroscopy were performed with a Bruker Avance Neo 400 MHz or Bruker Avance 500 MHz spectrometers. All chemical shifts are reported in parts per million (ppm, *δ),* and spectra were referenced using an internal trimethylsilane solvent peak or with residual undeuterated solvent peak, when appropriate. All ^{1}H NMR data are represented as chemical shift, multiplicity (s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, br = broad), coupling constant (*J,* Hz), and integration. Water obtained from a Milli-Q® IQ 7000 water purification system (MilliporeSigma, Burlington, MA) was used for the preparation of buffers.

High-resolution mass spectrometry (HRMS) of small molecules was performed with a JEOL AccuTOF 4G LC-plus mass spectrometer equipped with an ionSense Direct Analysis in Real Time (DART) source.

Intact protein ESI mass spectrometry was performed with a 6350 Accurate-Mass Q–TOF LC/MS (Agilent Technologies, Lexington, MA) equipped with a PLRP-S column (1000 Å pore size, 5-µm particle size, 50 mm length × 2.1 mm ID; Agilent Technologies). All Q–TOF LC/MS analyses were performed with a gradient of 5–95% v/v MeCN (containing 0.1% v/v formic acid) in water (containing 0.1% v/v formic acid) over 7 min. Protein concentrations were determined either by using a Pierce™ bicinchonic acid (BCA) protein assay kit (Thermo Scientific) on a Spark microplate reader from Tecan (Zürich, Switzerland) or with a DS-11 UV–vis spectrophotometer from DeNovix (Wilmington, DE).

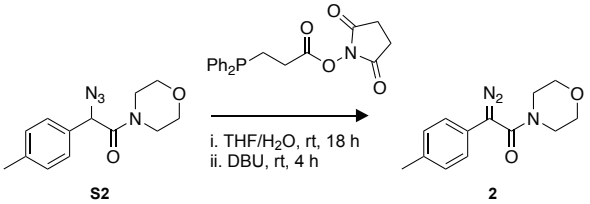
Safety

Caution Diazo compounds are potentially explosive when exposed to heat, light, pressure, or shock.[4] Although we did not encounter any issues, we recommend storing diazo compounds at a temperature of ≤0 °C in the dark.

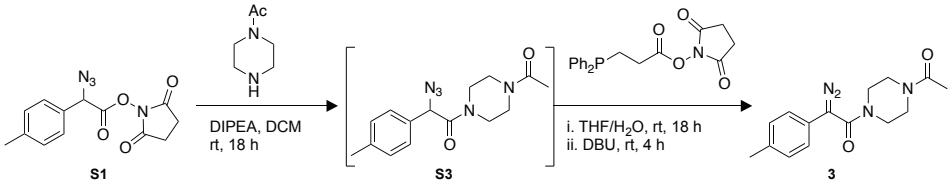


S1 **S2**

2-Azido-1-morpholino-2-(*p*-tolyl)ethan-1-one (S2). To an oven-dried round-bottom flask was added compound **S1**[5] (402 mg, 1.39 mmol) and DCM (15 mL). Morpholine (0.81 mL, 2.09 mmol) was added to the solution, followed by DIPEA (0.48 mL, 2.78 mmol), and the reaction mixture was stirred for 18 h. The reaction mixture was concentrated under reduced pressure, and the resulting residue was dissolved with ethyl acetate and washed sequentially with 1 N HCl (2×), $NaHCO_3$(sat'd aq) (2×), and brine (1×). The organic layer was dried over anhydrous Na_2SO_4(s), filtered, and concentrated under reduced pressure. The resulting residue was purified by silica gel chromatography (30% v/v EtOAc in hexanes) to give compound **S2** (340 mg, 94% yield). **^{1}H NMR** (400 MHz, $CDCl_3$, *δ*): 7.27–7.21 (m, 4H), 4.93 (s, 1H), 3.83–3.78 (m,1H), 3.72–3.67 (m, 1H), 3.61–3.53 (m, 2H), 3.50–3.45 (m, 1H), 3.32–3.25 (m, 1H), 3.20–3.13 (m, 2H), 2.38 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$, *δ*): 167.93, 139.87, 131.14, 130.61, 128.11, 67.12, 66.47, 64.29, 46.30, 43.06, 21.67. **HRMS** *m*/*z* calcd for $C_{13}H_{16}N_4O_2$ $[M + H]^+$, 261.1346; found, 261.1358.



2-Diazo-1-morpholino-2-(*p*-tolyl)ethan-1-one (2). To an oven-dried round-bottom flask was added compound **S2** (334 mg, 1.28 mmol), THF/H_2O (20:3, 13 mL; degassed H_2O), and *N*-succinimidyl 3-(diphenylphosphino)propionate (502 mg, 1.41 mmol), and the reaction mixture was stirred overnight under N_2(g). DBU (0.38 mL, 2.56 mmol) was added to the reaction mixture, which was stirred for 1 h. The reaction mixture was diluted with brine and extracted with DCM (2×). The organic layer was dried over anhydrous Na_2SO_4(s), filtered, and concentrated under reduced pressure. The resulting residue was purified by silica gel chromatography (30% v/v EtOAc in hexanes) to give diazoamide **2** (47.6 mg, 15% yield). **^{1}H NMR** (400 MHz, $CDCl_3$, *δ*): 7.20 (d, *J* = 8.1 Hz, 2H), 7.10 (d, *J* = 8.3 Hz, 2H), 3.64 (t, *J* = 4.0 Hz, 4H), 3.44 (t, *J* = 4.0 Hz, 4H), 2.35 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$, *δ*): 166.00, 136.37, 130.20, 125.04, 124.39, 66.82, 46.10, 21.19. The diazo carbon was not observed by ^{13}C NMR spectroscopy.[6] **HRMS** *m*/*z* calcd for $C_{13}H_{16}NO$ [M − N_2 + H]$^+$, 218.1084; found, 218.1087.

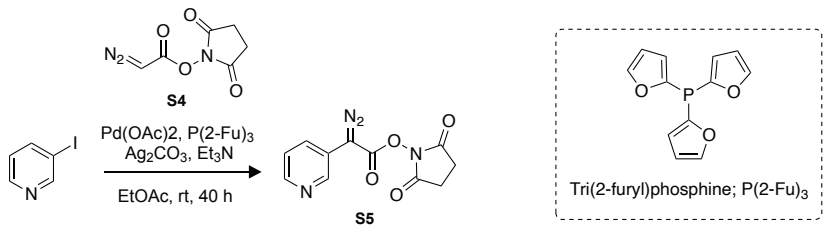


1-(4-Acetylpiperazin-1-yl)-2-diazo-2-(*p*-tolyl)ethan-1-one (3). To an oven-dried round-bottom flask was added compound **S1** (150 mg, 0.520 mmol) and DCM. 1-Acetylpiperazine (100 mg, 0.781 mmol) was added to the solution, followed by DIPEA (0.18 mL, 1.04 mmol), and the reaction mixture was stirred for 18 h. The reaction mixture was poured into a separatory funnel and washed with $NaHCO_3$(sat'd aq) (1×). The organic layer was dried over anhydrous Na_2SO_4(s), filtered, and concentrated under reduced pressure. The resulting crude residue (115 mg) was dissolved in THF/H_2O (20:3, 4.0 mL; degassed H_2O), *N*-succinimidyl 3-(diphenylphosphino)propionate (150 mg, 0.420 mmol) was added, and the reaction mixture was stirred overnight. DBU (0.11 mL, 0.764 mmol) was added to the reaction mixture, which was stirred for 1 h. The reaction mixture was diluted with brine and extracted with DCM (3×). The organic layer was dried over anhydrous Na_2SO_4(s), filtered, and concentrated under reduced pressure. The resulting residue was purified by silica gel chromatography (30% v/v EtOAc in pentane) to give diazoamide **3** (35.7 mg, 33% yield). **^{1}H NMR** (400 MHz, $CDCl_3$, *δ*): 7.20 (d, *J* = 8.2 Hz, 2H), 7.10 (d, *J* = 8.3 Hz, 2H), 3.57 (t, *J* = 5.3 Hz, 2H), 3.50–3.43 (m, 4H), 3.37 (dd, *J* = 6.6, 4.0 Hz, 2H), 2.35 (s, 3H), 2.09 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$, *δ*): 169.28, 166.41, 136.66, 130.29, 125.24, 124.27, 46.13, 45.76, 45.35, 41.33, 21.50, 21.20. The diazo carbon was not observed by ^{13}C NMR spectroscopy. **HRMS** *m*/*z* calcd for $C_{15}H_{18}N_2O_2$ [M − N_2 + H]$^+$, 259.1441; found, 259.1461.

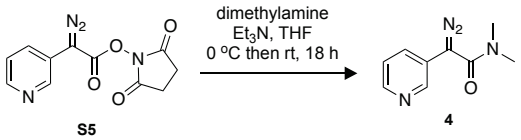
General Procedure A: Synthesis of *N*-succinimidyl 2-aryl-2-diazoacetates

Compound **S4**, tri(2-furyl)phosphine, Ag_2CO_3, EtOAc were added to an oven-dried 20-mL scintillation vial capped with a TFE septum. The reaction vessel was purged and backfilled with dry Ar(g), and the reaction mixture was stirred. Aryl iodide and $Pd(OAc)_2$ were added to two separate oven-dried scintillation vials under an atmosphere of dry Ar(g) and dissolved in EtOAc (0.1–0.4 mL). The dissolved aryl iodide, $Pd(OAc)_2$, and Et_3N were then added simultaneously to

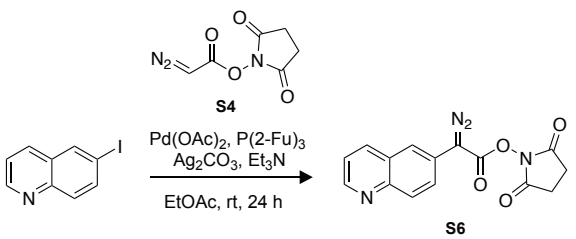
the reaction mixture, and the reaction vessel was again purged and backfilled with Ar(g). The reaction mixture was stirred, and progress of the reaction was monitored by TLC. At the completion of the reaction, the reaction mixture was filtered through a short (2 cm) bed of silica gel and the filtrate was concentrated under reduced pressure. The resulting residue was purified by silica gel chromatography (EtOAc in pentane).



2,5-Dioxopyrrolidin-1-yl 2-diazo-2-(pyridin-3-yl)acetate (S5). The title compound was synthesized using General Procedure A with compound **S4** (50 mg, 0.273 mmol), 3-iodopyridine (84 mg, 0.410 mmol), tri(2-furyl)phosphine (13 mg, 0.055 mmol, 20 mol%), $Pd(OAc)_2$ (6 mg, 0.03 mmol, 10 mol%), Ag_2CO_3 (38 mg, 0.137 mmol), Et_3N (57 µL, 0.410 mmol), and EtOAc (5.5 mL). The reaction mixture was stirred for 40 h. Purification by silica gel chromatography (pentane/EtOAc; 50–70% v/v) gave compound **S5** (40.5 mg, 57% yield).**^{1}H NMR** (500 MHz, $CDCl_3$, *δ*): 8.69 (s,1H), 8.54–8.47 (m, 1H), 7.82 (dt, *J* = 8.2, 2.0 Hz, 1H), 7.36 (dd, *J* = 8.2, 4.8 Hz, 1H), 2.90 (s, 4H). **^{13}C NMR** (126 MHz, $CDCl_3$, *δ*): 169.23, 159.94, 148.17, 145.28, 131.86, 123.87, 120.67, 25.68. The diazo carbon was not observed by ^{13}C NMR spectroscopy. **HRMS** *m*/*z* calcd for $C_{11}H_9N_4O_4$ $[M + H]^+$, 261.0618; found, 261.0653.

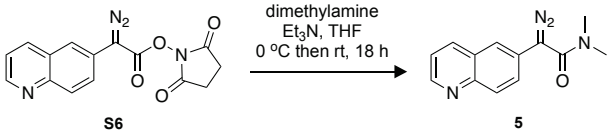


2-Diazo-*N*,*N*-dimethyl-2-(pyridin-3-yl)acetamide (4). To an oven-dried round-bottom flask was added compound **S5** (15 mg, 0.0580 mmol) and THF (1.6 mL) and cooled in an ice bath. Dimethylamine (0.0696 mmol; from 2.0 M in THF) was added to the solution, followed by triethylamine (16 µL, 0.116 mmol), and the reaction mixture was stirred for 18 h. The reaction mixture was concentrated under reduced pressure, and the resulting residue was purified by silica gel chromatography (EtOAc in pentane, 50–80% v/v) to give diazoamide **4** (4.2 mg, 38% yield). **^{1}H NMR** (500 MHz, $CDCl_3$, *δ*): 8.51 (s, 1H), 8.42–8.38 (m, 1H), 7.61 (ddd, *J* = 8.1, 2.5, 1.5 Hz, 1H), 7.31–7.27 (m, 1H), 3.02 (s, 6H). **^{13}C NMR** (126 MHz, $CDCl_3$, *δ*): 164.90, 146.80, 145.51, 131.54, 124.70, 123.76, 37.86. The diazo carbon was not observed by ^{13}C NMR spectroscopy. **HRMS** *m*/*z* calcd for $C_9H_{11}N_4O$ $[M + H]^+$, 191.0941; found, 191.0959.



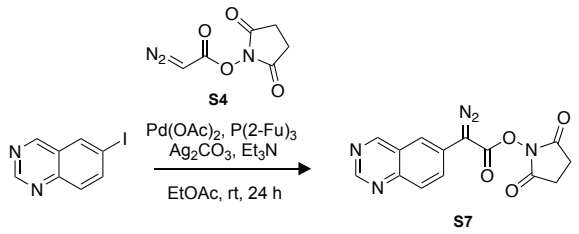
2,5-Dioxopyrrolidin-1-yl 2-diazo-2-(quinolin-6-yl)acetate (S6). The title compound was synthesized using General Procedure A with compound **S4** (50 mg, 0.273 mmol), 6-iodoquinoline (105 mg, 0.410 mmol), tri(2-furyl)phosphine (13 mg, 0.055 mmol, 20 mol%), $Pd(OAc)_2$ (6 mg, 0.03

mmol, 10 mol%), Ag_2CO_3 (38 mg, 0.137 mmol), Et_3N (57 µL, 0.410 mmol), and EtOAc (5.5 mL). The reaction mixture was stirred for 24 h. Purification by silica gel chromatography (EtOAc in penane; 50% then 80% v/v) gave compound **S6** (28.6 mg, 34% yield). **^{1}H NMR** (400 MHz, $CDCl_3$, *δ*): 8.95–8.87 (m, 1H), 8.19–8.10 (m, 2H), 7.99 (d, *J* = 2.2 Hz, 1H), 7.70 (dd, *J* = 8.9, 2.3 Hz, 1H), 7.43 (dd, *J* = 8.3, 4.2 Hz, 1H), 2.92 (s, 4H). **^{13}C NMR** (101 MHz, $CDCl_3$, *δ*): 169.35, 160.37, 150.95, 147.02, 135.91, 130.82, 128.64, 125.13, 123.24, 122.19, 121.66, 25.72. The diazo carbon was not observed by ^{13}C NMR spectroscopy. **HRMS** *m*/*z* calcd for $C_{15}H_{11}N_4O_4$ $[M + H]^+$, 311.0736; found, 311.0754.



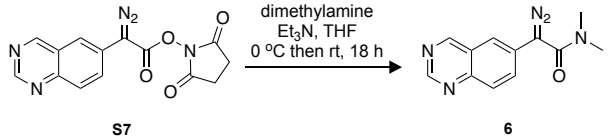
S6 5

2-Diazo-*N*,*N*-dimethyl-2-(quinolin-6-yl)acetamide (5). To an oven-dried round-bottom flask was added compound **S6** (21 mg, 0.0677 mmol) and THF (1.9 mL) and cooled in an ice bath. Dimethylamine (0.135 mmol; from 2.0 M in THF) was added to the solution, followed by triethylamine (19 µL, 0.135 mmol), and the reaction mixture was stirred for 18 h. The reaction mixture was concentrated under reduced pressure, and the resulting residue was purified by silica gel chromatography (EtOAc in pentane, 50–80% v/v) to give diazoamide **5** (6.2 mg, 38% yield). **^{1}H NMR** (500 MHz, $CDCl_3$, *δ*): 8.86 (dd, *J* = 4.3, 1.7 Hz, 1H), 8.12–8.06 (m, 2H), 7.72 (d, *J* = 2.1 Hz, 1H), 7.55 (dd, *J* = 8.9, 2.2 Hz, 1H), 7.39 (dd, *J* = 8.3, 4.2 Hz, 1H), 3.05 (s, 6H). **^{13}C NMR** (126 MHz, $CDCl_3$, *δ*): 165.41, 150.20, 146.66, 135.54, 130.48, 128.87, 126.11, 126.03, 122.13, 121.94, 37.88. The diazo carbon was not observed by ^{13}C NMR spectroscopy. **HRMS** *m*/*z* calcd for $C_{13}H_{13}N_4O$ $[M + H]^+$, 241.1084; found, 241.1105.



S4 S7

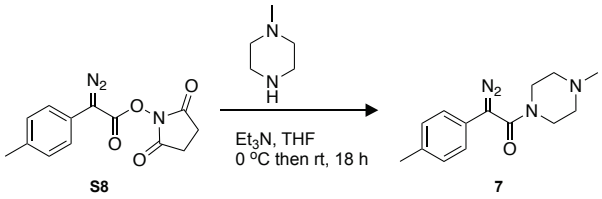
2,5-Dioxopyrrolidin-1-yl 2-diazo-2-(quinazolin-6-yl)acetate (S7). The title compound was synthesized using General Procedure A with compound **S4** (30 mg, 0.164 mmol), 6-iodoquinazoline (63 mg, 0.246 mmol), tri(2-furyl)phosphine (8 mg, 0.033 mmol, 20 mol%), $Pd(OAc)_2$ (4 mg, 0.03 mmol, 10 mol%), Ag_2CO_3 (23 mg, 0.082 mmol), Et_3N (34 µL, 0.246 mmol), and EtOAc (3.4 mL). The reaction mixture was stirred for 24 h. Purification by silica gel chromatography (EtOAc in pentane; 50–80% v/v) gave compound **S7** (27 mg, 53% yield). **^{1}H NMR** (400 MHz, $CDCl_3$, *δ*): 9.39 (s, 1H), 9.33 (s, 1H), 8.12 (dd, *J* = 5.6, 3.3 Hz, 2H), 7.91 (dd, *J* = 9.1, 2.2 Hz, 1H), 2.93 (s, 4H). **^{13}C NMR** (101 MHz, $CDCl_3$, *δ*): 169.23, 160.11, 160.06, 155.64, 148.73, 129.84, 129.44, 125.43, 123.71, 122.04, 25.72. The diazo carbon was not observed by ^{13}C NMR spectroscopy. **HRMS** *m*/*z* calcd for $C_{14}H_{10}N_5O_4$ $[M + H]^+$, 312.0727; found, 312.0747.



S7 6

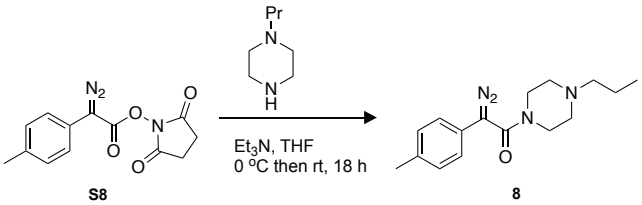
2-Diazo-*N*,*N*-dimethyl-2-(quinazolin-6-yl)acetamide (6). To an oven-dried round-bottom flask was added compound **S7** (22 mg, 0.0707 mmol) and THF (2.0 mL), and cooled in an ice bath.

Dimethylamine (0.0848 mmol; from 2.0 M in THF) was added to the solution, followed by triethylamine (20 µL, 0.141 mmol), and the reaction mixture was stirred for 18 h. The reaction mixture was concentrated under reduced pressure, and the resulting residue was purified by silica gel chromatography (pentane/EtOAc, 50% then 100% v/v EtOAc) to give diazoamide **6** (11.4 mg, 67% yield). Note: The purity of the title compound was compromised due to on-column degradation (see ^{1}H NMR spectrum on page S33). Hence, the compound was used immediately without further purification. **^{1}H NMR** (500 MHz, $CDCl_3$, δ): 9.35 (s, 1H), 9.27 (s, 1H), 8.06 (d, J = 8.9 Hz, 1H), 7.87 (d, J = 2.2 Hz, 1H), 7.78 (dd, J = 9.0, 2.2 Hz, 1H), 3.08 (s, 6H). **HRMS** *m*/*z* calcd for $C_{12}H_{12}N_3O$ $[M - N_2 + H]^+$, 214.0975; found, 214.0989.



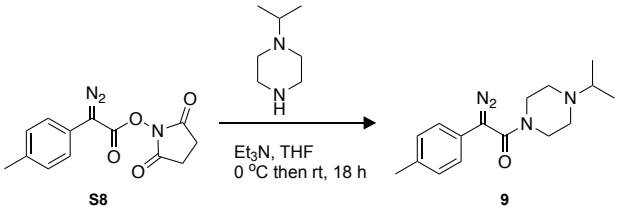
S8 7

2-Diazo-1-(4-methylpiperazin-1-yl)-2-(*p*-tolyl)ethan-1-one (7). To an oven-dried round-bottom flask was added compound **S8**[6] (50 mg, 0.183 mmol) and THF (5.0 mL) and cooled in an ice bath. 1-Methylpiperazine (27 mg, 0.274 mmol) was added to the solution, followed by triethylamine (76 µL, 0.548 mmol), and the reaction mixture was stirred for 18 h. The reaction mixture was concentrated under reduced pressure, and the resulting residue was purified by silica gel chromatography (DCM/MeOH, 2–3% v/v) to give diazoamide **7** (22 mg, 47% yield). **^{1}H NMR** (400 MHz, $CDCl_3$, δ): 7.19 (d, J = 8.3 Hz, 2H), 7.09 (d, J = 8.3 Hz, 2H), 3.46 (dd, J = 6.6, 3.5 Hz, 4H), 2.38–2.33 (m, 7H), 2.29 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$, δ): 165.69, 136.05, 130.08, 124.84, 124.57, 54.90, 46.15, 45.56, 21.14. The diazo carbon was not observed by ^{13}C NMR spectroscopy. **HRMS** *m*/*z* calcd for $C_{14}H_{19}N_2O$ $[M - N_2 + H]^+$, 231.1492; found, 231.1496.



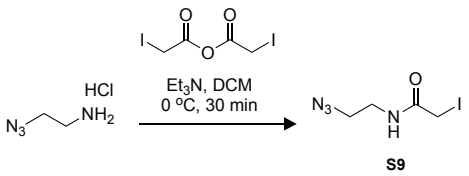
S8 8

2-Diazo-1-(4-propylpiperazin-1-yl)-2-(*p*-tolyl)ethan-1-one (8). To an oven-dried round-bottom flask was added compound **S8** (50 mg, 0.183 mmol) and THF (5.0 mL) and cooled in an ice bath. 1-*n*-Propylpiperazine dihydrobromide, (75 mg, 0.274 mmol) was added to the solution, followed by triethylamine (0.15 mL, 1.10 mmol), and the reaction mixture was stirred for 18 h. The reaction mixture was concentrated under reduced pressure, and the resulting residue was purified by silica gel chromatography (80% v/v EtOAc in pentane) to give diazoamide **8** (30 mg, 58% yield). **^{1}H NMR** (500 MHz, $CDCl_3$, δ): 7.18 (d, J = 8.0 Hz, 2H), 7.09 (d, J = 8.0 Hz, 2H), 3.46 (t, J = 5.0 Hz, 4H), 2.39 (t, J = 5.1 Hz, 4H), 2.34 (s, 3H), 2.32–2.27 (m, 2H), 1.48 (dt, J = 15.0, 7.5 Hz, 2H), 0.89 (t, J = 7.4 Hz, 3H). **^{13}C NMR** (126 MHz, $CDCl_3$, δ): 165.53, 135.99, 130.06, 124.83, 124.64, 60.58, 53.10, 45.64, 21.14, 20.05, 11.97. The diazo carbon was not observed by ^{13}C NMR spectroscopy. **HRMS** *m*/*z* calcd for $C_{16}H_{23}N_2O$ $[M - N_2 + H]^+$, 259.1805; found, 259.1819.



S8 9

2-Diazo-1-(4-isopropylpiperazin-1-yl)-2-(*p*-tolyl)ethan-1-one (9). To an oven-dried round-bottom flask was added compound **S8** (50 mg, 0.183 mmol) and THF (5.0 mL), and the flask was cooled in an ice bath. 1-Isopropylpiperazine (35 mg, 0.274 mmol) was added to the solution, followed by triethylamine (76 µL, 0.548 mmol), and the reaction mixture was stirred for 18 h. The reaction mixture was concentrated under reduced pressure, and the resulting residue was purified by silica gel chromatography (80% v/v EtOAc in pentane) to give diazoamide **9** (20 mg, 38% yield). **^{1}H NMR** (400 MHz, $CDCl_3$, *δ*): 7.18 (d, *J* = 8.0 Hz, 2H), 7.09 (d, *J* = 8.2 Hz, 2H), 3.45 (t, *J* = 5.0 Hz, 4H), 2.69 (p, *J* = 6.6 Hz, 1H), 2.47 (t, *J* = 5.0 Hz, 4H), 2.34 (s, 3H), 1.03 (d, *J* = 6.5 Hz, 6H). **^{13}C NMR** (101 MHz, $CDCl_3$, *δ*): 165.46, 135.97, 130.06, 124.84, 124.68, 54.65, 48.68, 46.00, 21.16, 18.54. The diazo carbon was not observed by ^{13}C NMR spectroscopy. **HRMS** *m*/*z* calcd for $C_{16}H_{23}N_2O$ $[M - N_2 + H]^+$, 259.1805; found, 259.1822.



S9

***N*-(2-Azidoethyl)-2-iodoacetamide S9.** To an oven-dried round-bottom flask was added 2-azidoethylamine hydrochloride, DCM, and cooled to 0 °C. Iodoacetic anhydride was added dropwise, and the reaction mixture was stirred at 0 °C for 30 min. The reaction mixture was diluted with $NaHCO_3$(sat'd aq), poured into a separatory funnel, and extracted with EtOAc (3×). The organic layer was concentrated under reduced pressure to yield compound **S9**, which was used without further purification. **^{1}H NMR** (400 MHz, $CDCl_3$, *δ*): 6.41 (s, 1H), 3.72 (s, 2H), 3.51–3.42 (m, 4H).

Diazoamide **1** was synthesized as reported previously.[5]

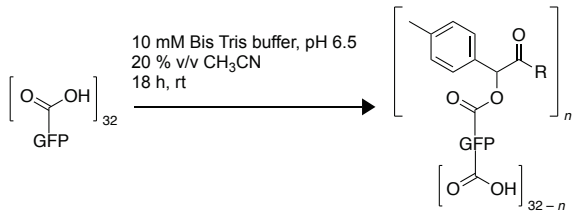
Compound **S4** was synthesized as reported previously.[7]

Compound **S8** was synthesized as reported previously.[6]

III. Protein Esterification Protocols

Note: We recommend preparing fresh working stocks of diazoamide compounds (in acetonitrile) prior to use. Unused stock solutions should be stored at −20 °C and monitored for a color change. A change in color from orange to clear indicates degradation of diazo compounds.

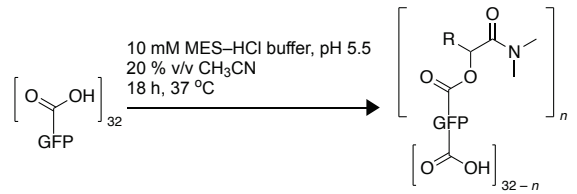
General protocol for esterification of GFP with diazoamides 1–3
GFP was exchanged into 10 mM Bis–Tris buffer, pH 6.5, using an Amicon Ultra centrifugal filter (10K MWCO), before esterification. Buffers containing inorganic phosphate or phosphoryl groups should not be used for protein esterification reactions because they react with diazoamides and quench their reactivity toward carboxyl groups on proteins.



To GFP (10.0 nmol) in an Eppendorf tube was added the appropriate amount of diazo compound, dissolved in acetonitrile so that the final concentration of acetonitrile was 20% v/v (see Table 1 for molar equivalence of diazo compounds relative to GFP). The final concentration of GFP in the reaction mixture was 200 μM. The reaction mixture was mixed by pipetting and shaken for 18 h. The reaction was quenched by adding 100 μL of PBS and filtering with a 0.22-μm Corning® Costar® Spin-X® centrifuge tube filter to remove any precipitated protein. The reaction mixture was then exchanged into PBS using a PD MiniTrap G-25 column. Eluted protein was concentrated using an Amicon Ultra filter (10K MWCO), and the esterification efficiency was characterized by ESI Q–TOF mass spectrometry.

General protocol for esterification of GFP with diazoamides 4–6

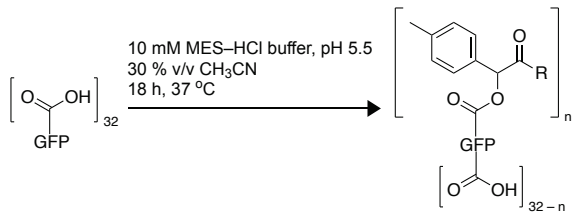
GFP was exchanged into 10 mM MES–HCl buffer, pH 5.5, before esterification, using an Amicon Ultra centrifugal filter (10K MWCO).



To GFP (10.0 nmol) in an Eppendorf tube was added the appropriate amount of diazo compound, dissolved in acetonitrile so that the final concentration of acetonitrile was 20% v/v (see Table 1 for molar equivalence of diazo compounds relative to GFP). The final concentration of GFP in the reaction mixture was 200 μM. The reaction mixture was mixed by pipetting and shaken for 18 h at 37 °C. The reaction was quenched by adding 100 μL of PBS and filtering with a 0.22-μm Corning® Costar® Spin-X® centrifuge tube filter to remove any precipitated protein. The reaction mixture was then exchanged into PBS using a PD MiniTrap G-25 column. Eluted protein was concentrated using an Amicon Ultra filter (10K MWCO), and the esterification efficiency was characterized by ESI Q–TOF mass spectrometry.

General protocol for esterification of GFP with compound 7–9

GFP was exchanged into 10 mM MES–HCl, pH 5.5, before esterification, using an Amicon Ultra centrifugal filter (10K MWCO).



To GFP (10.0 nmol) in an Eppendorf tube was added the appropriate amount of a diazo compound, dissolved in acetonitrile so that the final concentration of acetonitrile was 30% v/v (see Table 2 for molar equivalence of diazo compounds relative to GFP). The final concentration of GFP in the reaction mixture was at 200 μM. The reaction mixture was mixed by pipetting and shaken for 18 h at 37 °C. The reaction was quenched by adding 100 μL of PBS and filtering with

a 0.22-μm Corning® Costar® Spin-X® centrifuge tube filter to remove any precipitated protein. The reaction mixture was then exchanged into PBS using a PD MiniTrap G-25 column. Eluted protein was finally concentrated using an Amicon Ultra filter (10K MWCO), and the esterification efficiency was characterized by ESI Q–TOF mass spectrometry.

Tandem esterification with diazoamides 4 and 1
Before esterification, GFP was exchanged into 10 mM MES–HCl buffer, pH 5.5, with an Amicon Ultra centrifugal filter (10K MWCO).

Step 1: Esterification of GFP with diazoamide **4**
To GFP (5.0 nmol), in an Eppendorf tube was added the appropriate amount of diazo compound **4** (at 100, 200, 300, or 400 equiv relative to GFP), dissolved in acetonitrile so that the final concentration of acetonitrile was 20% v/v. The final concentration of GFP in the reaction mixtures was at 100 μM (50 μL reaction volume). The reaction mixtures were mixed by pipetting and shaken for 4 h at 37 °C. The reaction was quenched by adding 100 μL of PBS and exchanged into 10 mM Bis–Tris buffer, pH 6.5, using Amicon Ultra centrifugal filters (10K MWCO). A small aliquot of esterified GFP (GFP–**4**) from each reaction was removed for characterization by ESI Q–TOF.

Step 2: Esterification of GFP–**4** with diazoamide **1**
To the ensuing GFP–**4** was added diazoamide **1** (100 equiv), dissolved in acetonitrile, so that the final concentration of acetonitrile was 20% v/v. The reaction mixture was mixed by pipetting and shaken for 18 h. The reaction was quenched by adding 100 μL of PBS and filtering with a 0.22-μm Corning® Costar® Spin-X® centrifuge tube filter to remove any precipitated protein. The reaction mixtures were then exchanged into PBS using a Zeba spin desalting columns, and the eluted soluble GFP–**4**-**1** was adjusted to the same volume for all reactions. The concentrations of the soluble protein product were determined by measuring the absorbance at 488 nm on a DeNovix DS-11 UV–vis spectrophotometer.

IV. Protein Conjugation Protocols

Preparation of azido–Ub^{74}
UbA46C^{74} (2.6 mg, 306 nmol) in an Eppendorf tube was dissolved in degassed PBS, and to the resulting solution was added compound **S9** (20.0 equiv, dissolved in DMSO). The final concentration of DMSO was ~1%. The reaction vessel was purged with N_2(g), and the reaction mixture was shaken for 18 h. The reaction mixture was exchanged into PBS (pH 7.4) and characterized by ESI Q–TOF mass spectrometry.

Preparation of azido–Ub^{74}–3
To azido–Ub^{74} (15.0 nmol in MES–HCl buffer, pH 5.5) in an Eppendorf tube was added diazoamide **3** (4.5 μmol, 300 equiv) dissolved in acetonitrile so that the final concentration of acetonitrile was 20% v/v. The final reaction volume was 100 μL. The reaction mixture was mixed by pipetting and shaken for 18 h at 37 °C. The reaction was quenched by adding 200 μL of PBS and filtering with a 0.22-μm Corning® Costar® Spin-X® centrifuge tube filter to remove any precipitated protein. The reaction mixture was then exchanged into PBS using a PD MiniTrap G-25 column. Eluted azido–Ub^{74}–**3** was concentrated using an Amicon Ultra centrifugal filter (3K MWCO) and then characterized by ESI Q–TOF mass spectrometry.

Preparation of TAMRA–Ub^{74}–3
To azido–Ub^{74}–**3** (from the previous step) in an Eppendorf tube was added TAMRA–DBCO (5.0 equiv) dissolved in DMSO. The reaction volume was 500 μL, and the final concentration of DMSO was <1% v/v. The reaction mixture was mixed by pipetting and shaken for 18 h. Unreacted TAMRA dye was removed by exchange into PBS using a PD MiniTrap G-25 column. The eluted

protein was concentrated in an Amicon Ultra centrifugal filter (3K MWCO) and then characterized by ESI Q-TOF mass spectrometry.

Preparation of TAMRA–Ub^{74}
To azido–Ub^{74} (20.0 nmol in PBS) in an Eppendorf tube was added TAMRA–DBCO (5.0 equiv, dissolved in DMSO). The reaction volume was 500 µL, and the final concentration of DMSO was <1% (v/v). The reaction mixture was mixed by pipetting and shaken for 18 h. Unreacted TAMRA dye was removed by exchange into PBS using a PD MiniTrap G-25 column. The eluted protein was concentrated in an Amicon Ultra centrifugal filter (3K MWCO) and then characterized by ESI Q–TOF mass spectrometry.

V. Cell Lines and Cell Culture

Cell Culture. Human melanoma M21 cells[8] was a gift from Dr. Oscar Ortiz (Merck KGaA, Darmstadt, Germany). HeLa Cells were from the Koch Institute's High-Throughput Sciences Facility's cell line repository and Chinese Hamster Ovary cells (CHO-K1) was from American Tissue Culture Collection. All cell lines were cultured in sterile culture flasks in a cell culture incubator at 37 °C under CO_2(g) (5% v/v). All cell lines tested negative for mycoplasma using the Lonza MycoAlert Plus kit. The number of cell passages was limited to fewer than twenty to minimize genetic drift. HeLa cells were sustained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with FBS (10% v/v), penicillin (100 units/mL), and streptomycin (100 µg/mL). M21 cells were sustained in high-glucose DMEM (Catalog Number: 12100046) from Thermo Fisher Scientific supplemented with sodium bicarbonate (1.5 g/L), FBS (5–10% v/v), penicillin (100 units/mL), and streptomycin (100 µg/mL). CHO-K1 cells were sustained in F12-K medium supplemented with FBS (10% v/v), penicillin (100 units/mL), streptomycin (100 µg/mL), and glutamine (2 mM). Seeding density was determined after cell counting using a Countess II FL Automated Cell Counter from Thermo Fisher Scientific.

VI. Live-Cell Microscopy

CHO-K1 cells. Cells were seeded at 5,000 cells/well 24 h before the day of the experiment. The culture medium was removed before the start of the experiment, and cells were washed twice with warm PBS, followed by an additional wash with warm complete F12-K medium. The esterified protein was diluted to the desired concentration in serum-free medium, filtered with a 0.22-µm Corning® Costar® Spin-X® centrifuge tube filter to remove any protein aggregates, before incubating with cells for 2 h at 37 °C or 4 °C. Next, the medium was removed, and the cells were washed with PBS (3×). Cells were then kept in 200 µL of Invitrogen Live Cell Imaging Solution (140 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 20 mM HEPES, pH 7.4). Cells were next treated with Hoechst 33342 at 2 µg/mL for 5 min, and Invitrogen wheat-germ agglutinin–AlexaFluor 647™ at 5 µg/mL for 15 min on ice, then washed twice with 200 µL of Invitrogen Live Cell Imaging Solution before a final 200 µL of Invitrogen Live Cell Imaging Solution was added to each well. Images were recorded on an Andor Spinning Disk Confocal Microscope using a 1.4 NA, 60× Plan Apo objective. Sample excitation was with a 50-mW 405 nm, 50-mW 488 nm, 50-mW 561 nm, or 100-mW 640 nm wavelength laser and recorded with either an Andor Zyla sCMOS camera or Andor iXion+ EMCCD camera. Images were acquired with the MetaMorph acquisition software. Background subtraction and image analysis were performed with ImageJ software.[9]

M21 cells. **Imaging experiment with GFP–7.** 6,000 cells were seeded in serum-supplemented (10% v/v FBS) DMEM (catalog number: 12100046), 24 h in advance of the experiment. Immediately before the experiment, cells were washed with warm serum-free DMEM (2×). Esterified protein in PBS was diluted to the desired treatment concentration in serum-free DMEM (<18% PBS), filtered with a 0.22 µm Corning Costar Spin-X centrifuge tube filter to remove any

protein aggregates, and incubated with the cells at 37 °C for 3 h. After the indicated time, the medium was aspirated, and the cells were rinsed with PBS (2×) and with serum-free DMEM (2×). The cells were then placed in serum-free medium and incubated for 5 h at 37 °C. Cells were washed with warm PBS (2×) and with warm FluoroBrite DMEM (2×). Finally, cells were placed in FluoroBrite DMEM without serum from Thermo Fischer Scientific (A1896701) and imaged with an EVOS M7000 Imaging System (product #AMF7000) from Thermo Fisher Scientific. Background subtraction and analysis of acquired images were performed using ImageJ software.[9]

Imaging experiment with GFP–8. 10,000 cells were seeded in serum-supplemented (10% v/v FBS) DMEM, 24 h in advance of the experiment. Immediately before the experiment, cells were washed with warm serum-free DMEM. Esterified protein in PBS was diluted to the desired treatment concentration in serum-free DMEM (<16% PBS), filtered with a 0.22 μm Corning Costar Spin-X centrifuge tube filter to remove any protein aggregates, and incubated with the cells at 37 °C for 2.5 h. After the indicated time, the medium was aspirated, and the cells were washed with warm PBS (2×) and with warm FluoroBrite DMEM (2×). The cells were placed in FluoroBrite DMEM without serum and incubated for another 2.5 h. Finally, the cells were imaged with an EVOS M7000 Imaging System. Background subtraction and analysis of acquired images were performed using ImageJ software.[9]

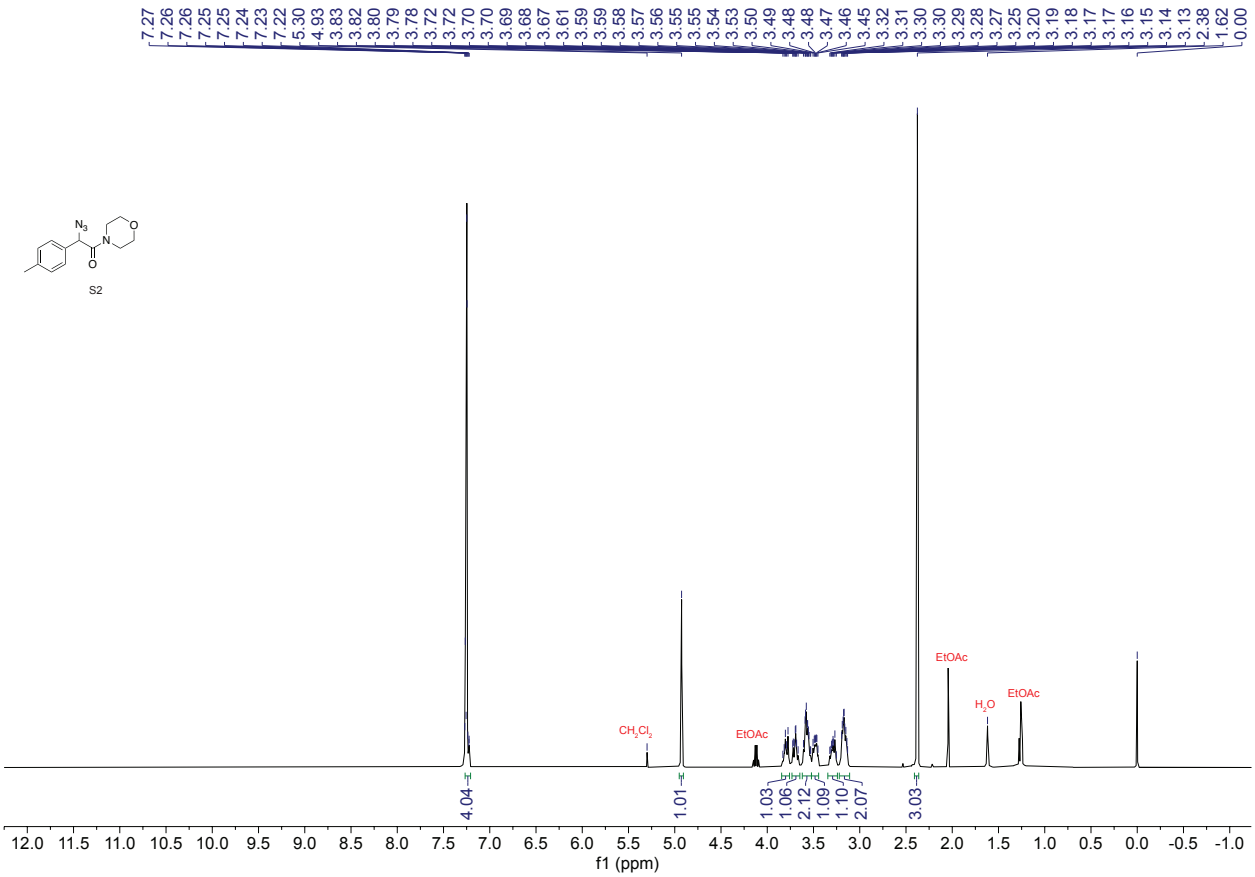
Imaging experiment with GFP–9. 15,000 cells were seeded in serum-supplemented (10% v/v FBS) DMEM, 24 h in advance of the experiment. Immediately before the experiment, cells were washed with warm serum-free FluoroBrite DMEM. Esterified protein in PBS was diluted to the desired treatment concentration in serum-free FluoroBrite DMEM (<19% v/v PBS), filtered with a 0.22 μm Corning Costar Spin-X centrifuge tube filter to remove any protein aggregates, and incubated with the cells at 37 °C for 3 h. After the indicated time, the medium was aspirated, and the cells were washed with warm PBS (2×) and with warm DMEM supplemented with 10% v/v FBS (2×). The cells were placed in DMEM with 10% v/v FBS and incubated for 1 h. Finally, the cells were washed with PBS (2×), FluoroBrite DMEM (2×), and placed in FluoroBrite DMEM. An EVOS M7000 Imaging System was used for imaging. Background subtraction and analysis of acquired images were performed using ImageJ software.[9]

HeLa cells. Cells (5,000 cells/well) were grown in complete DMEM, 24 h before the experiment. Immediately before the experiment, cells were washed twice with warm PBS and once with warm serum-free DMEM. Esterified protein was diluted to the desired treatment concentration in serum-free medium, filtered with a 0.22-μm Corning® Costar® Spin-X® centrifuge tube filter to remove any protein aggregates, and incubated with the cells at 37 °C for 2 h. Cells were aspirated and placed in complete medium and incubated for 2 h at 37 °C. Cells were washed two times with warm PBS and twice with warm FluoroBrite DMEM. Finally, cells were placed in FluoroBrite DMEM and imaged with an EVOS M7000 Imaging System using the RFP light cube for excitation (λ_{ex} = 531/40 nm, λ_{em} = 593/40 nm). Background subtraction and image analysis were performed with ImageJ software.[9]

Note: The concentration of vehicle (PBS) was <20% v/v for all imaging experiments.

VII. NMR Spectra

^{1}H NMR (400 MHz, $CDCl_3$)



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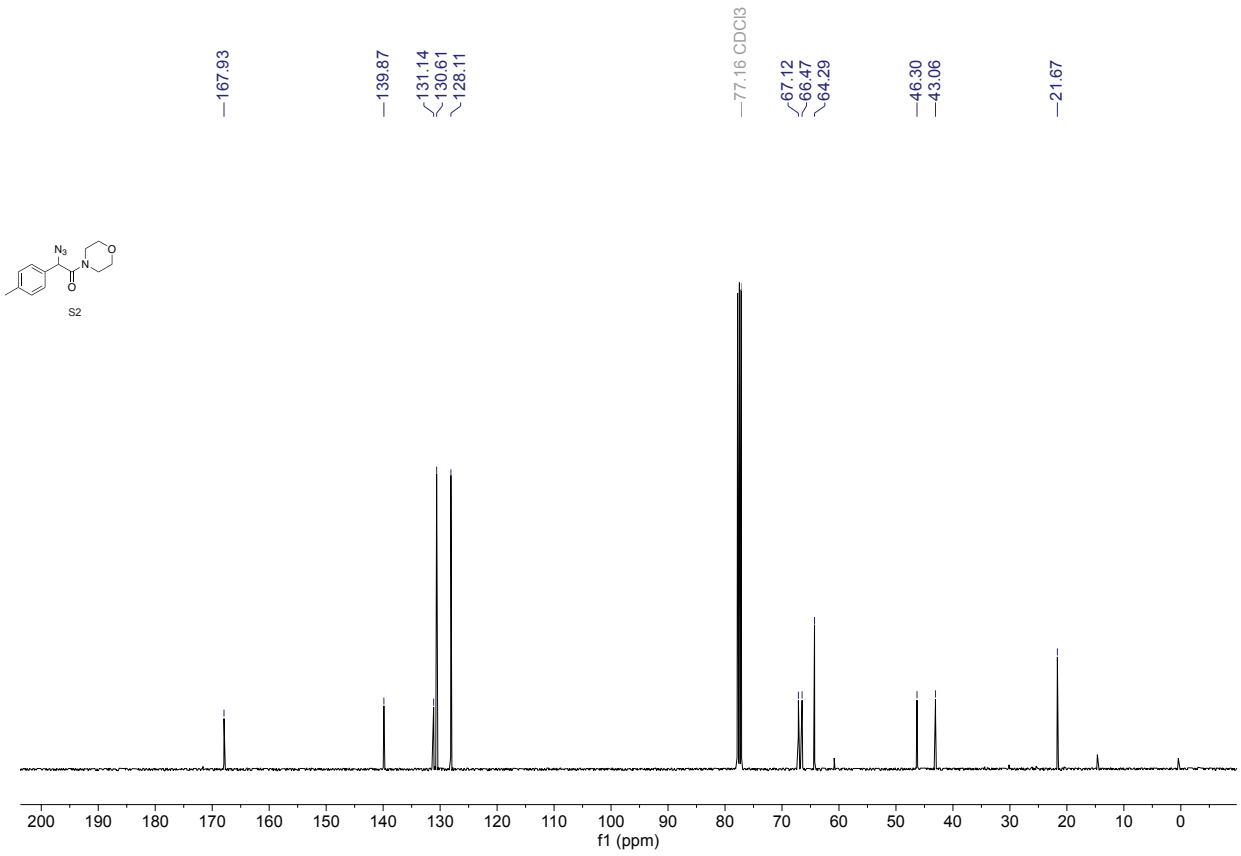

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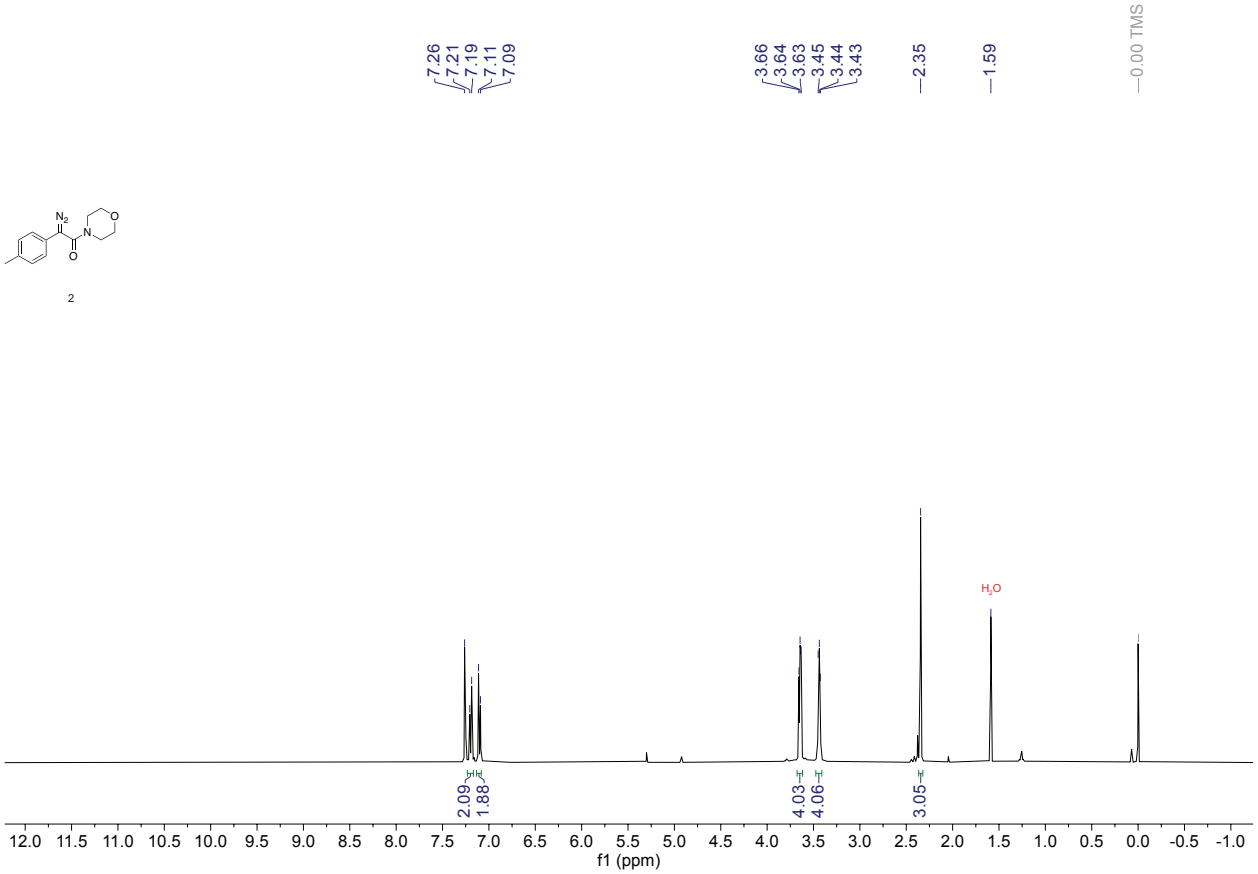

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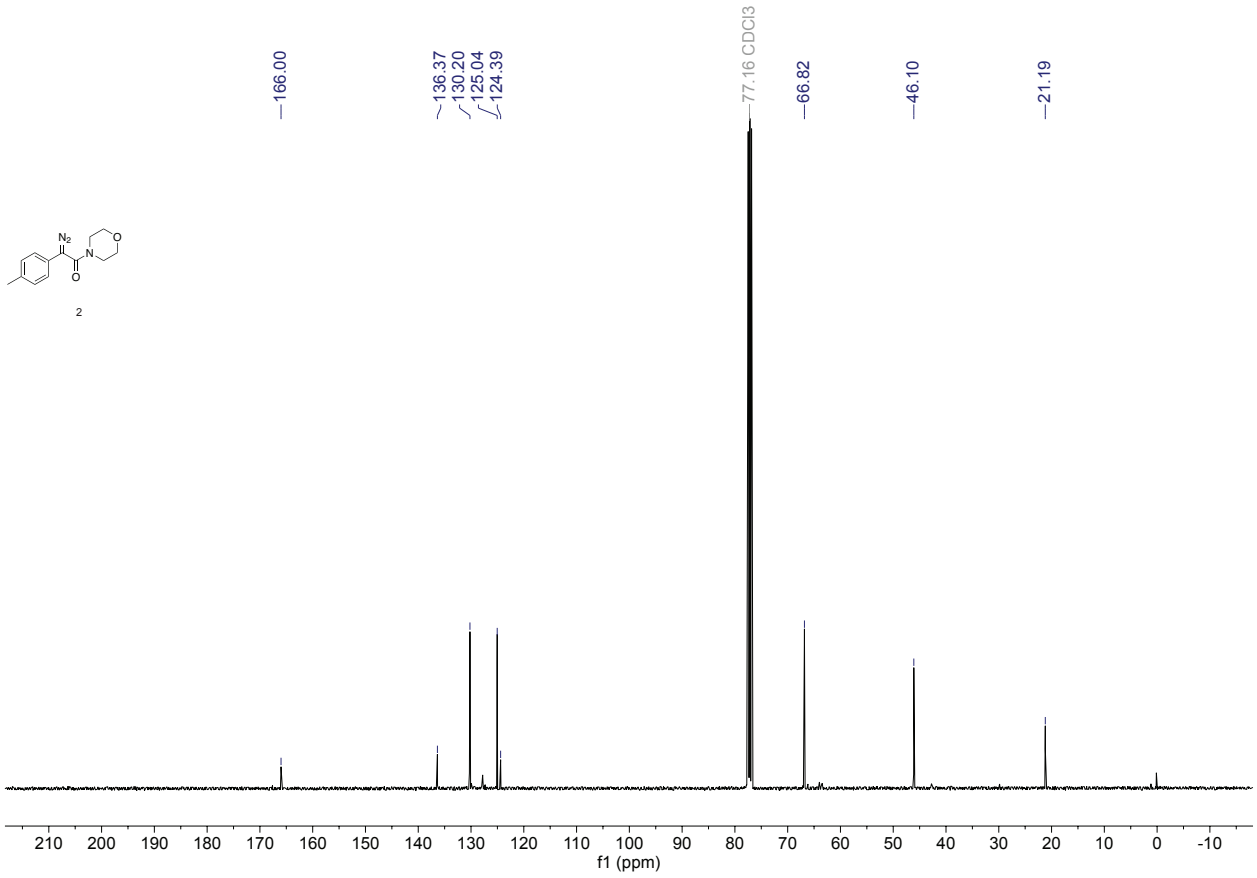

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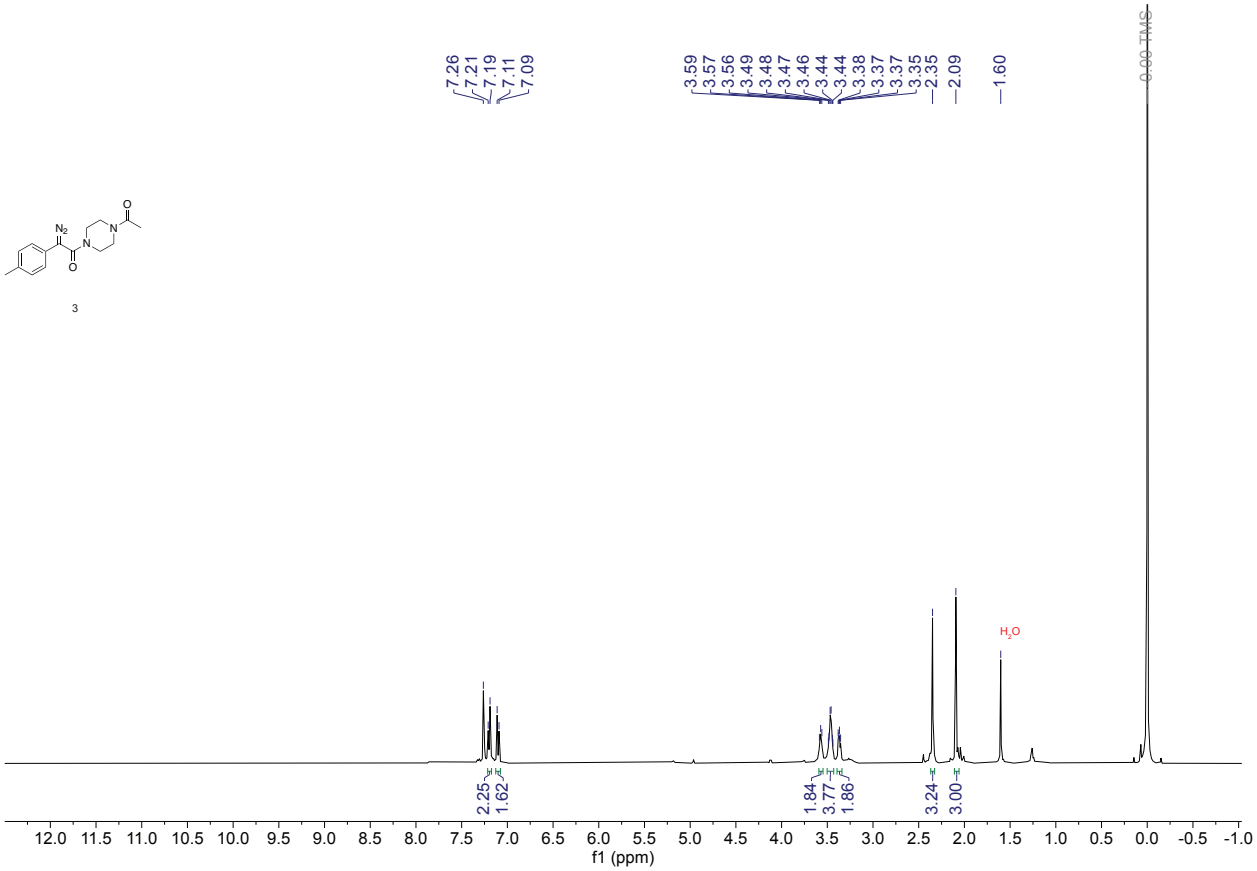

3

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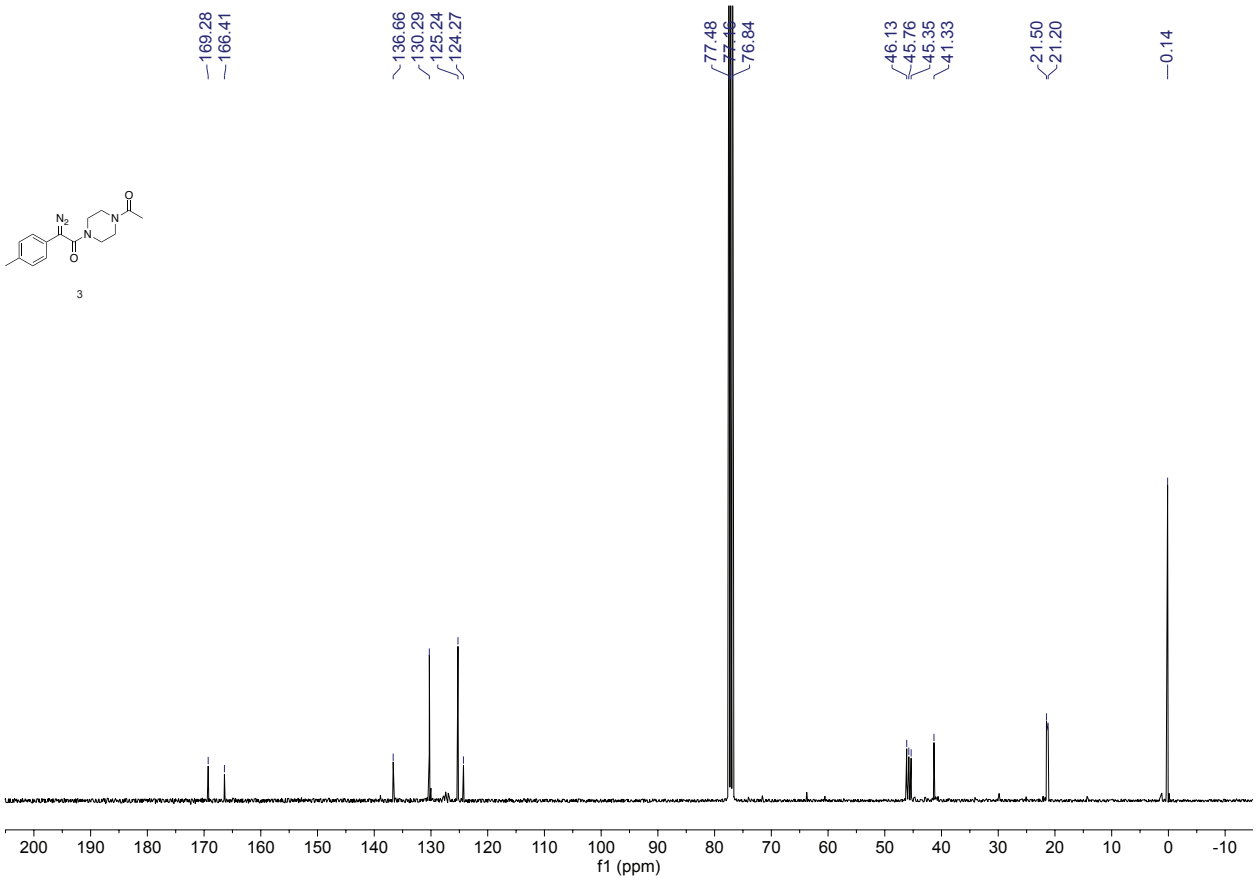

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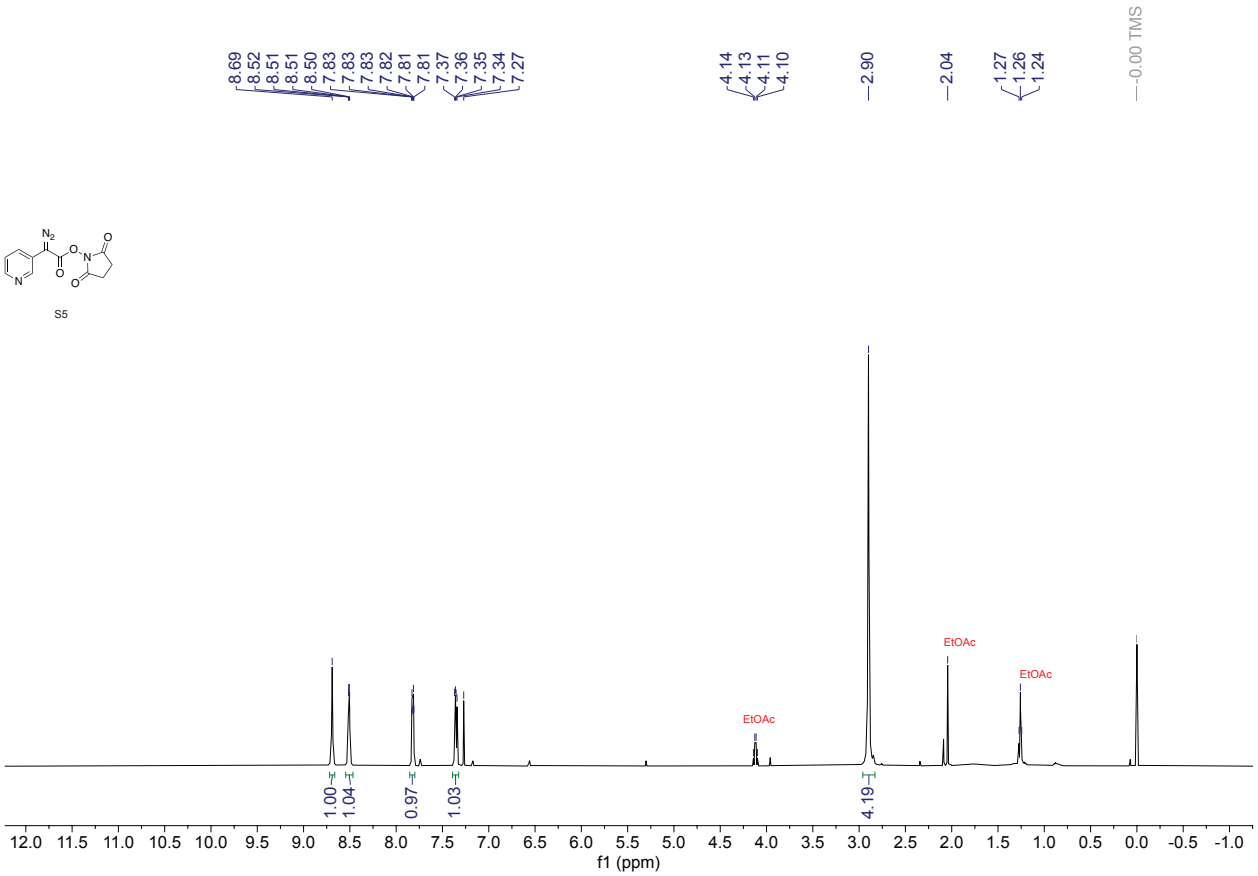
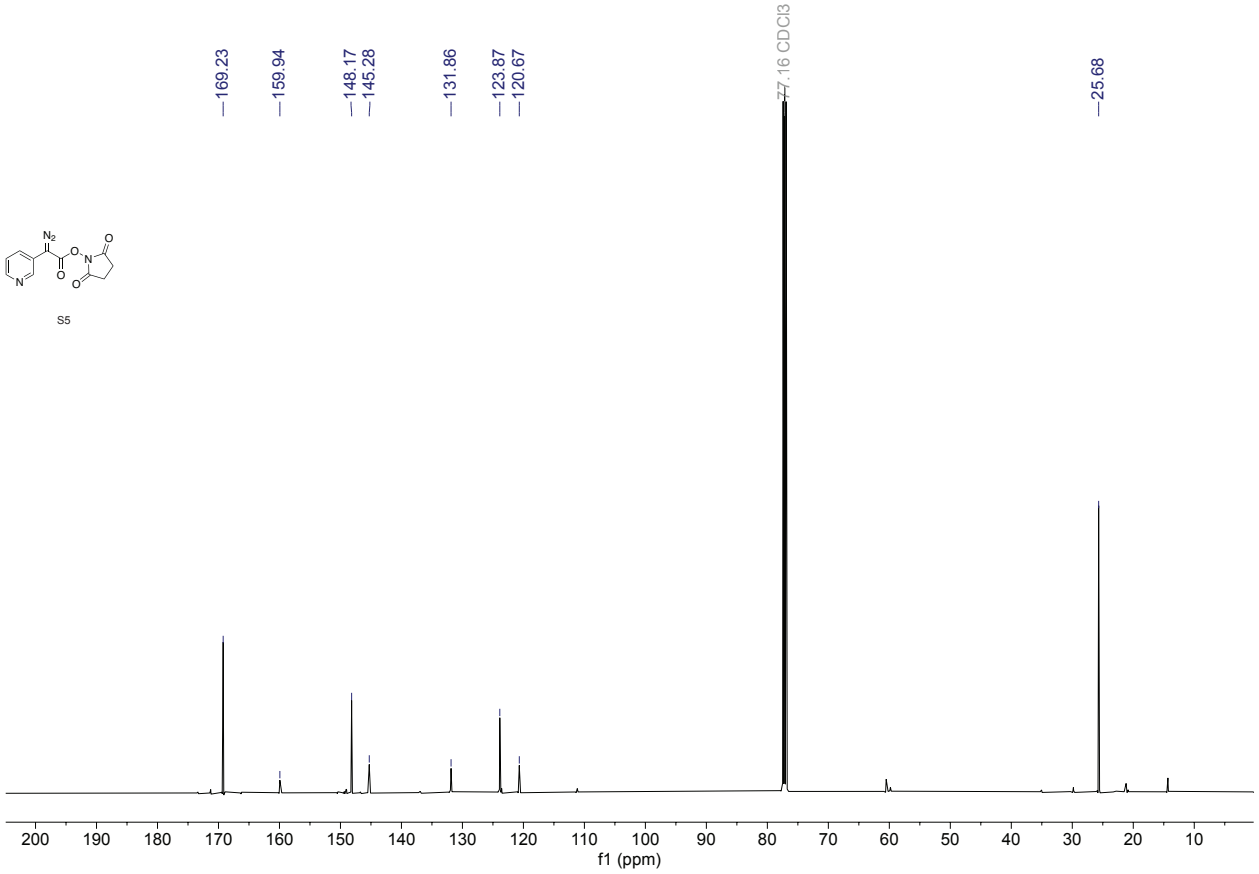


^{13}C NMR (126 MHz, $CDCl_3$)



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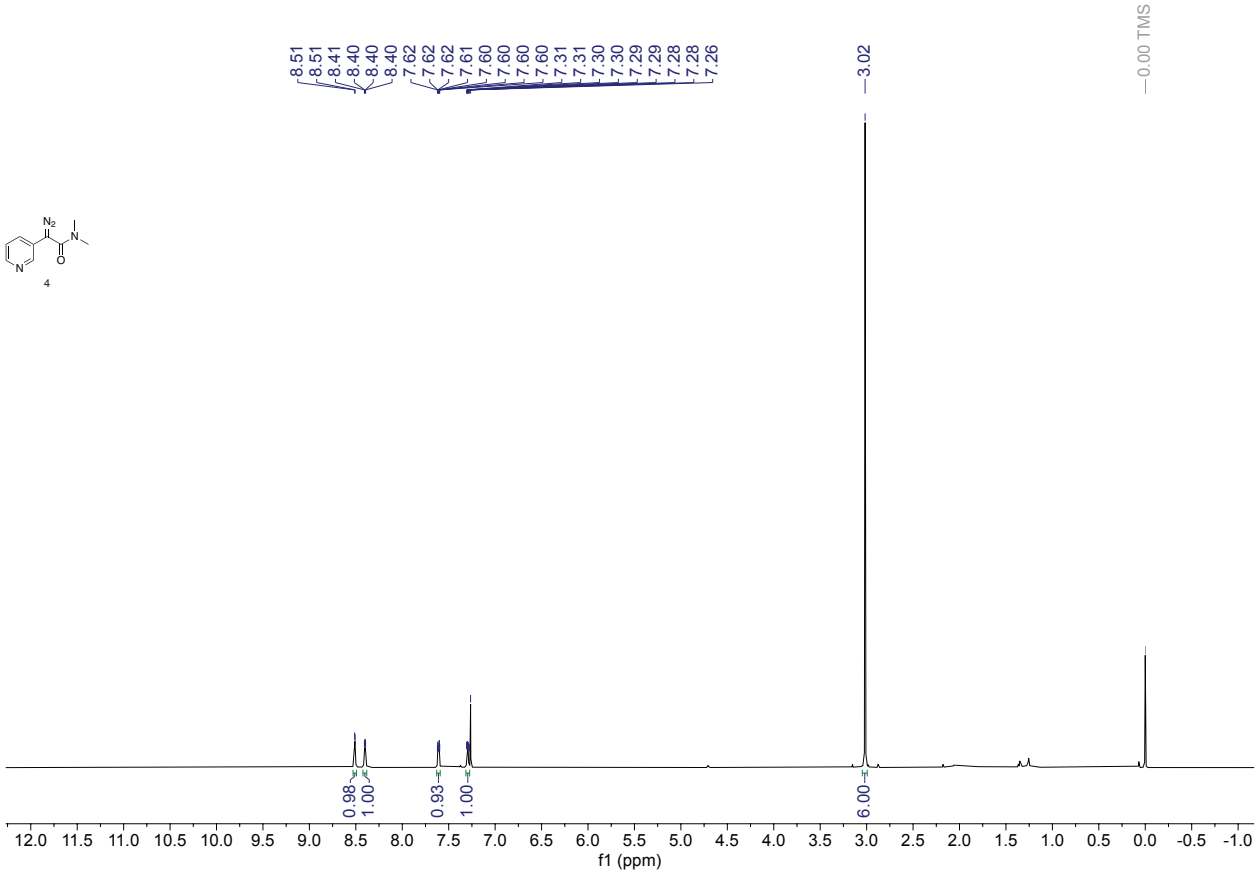

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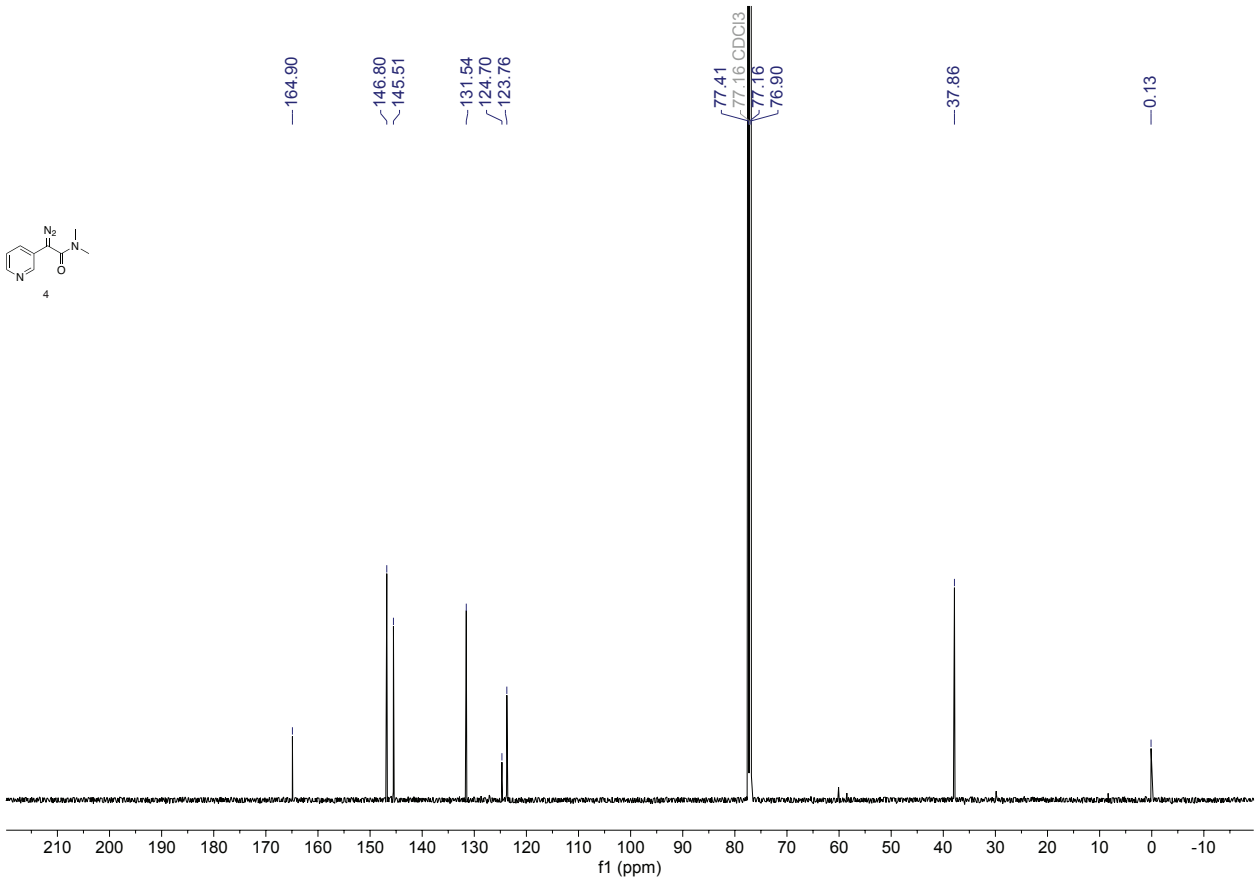

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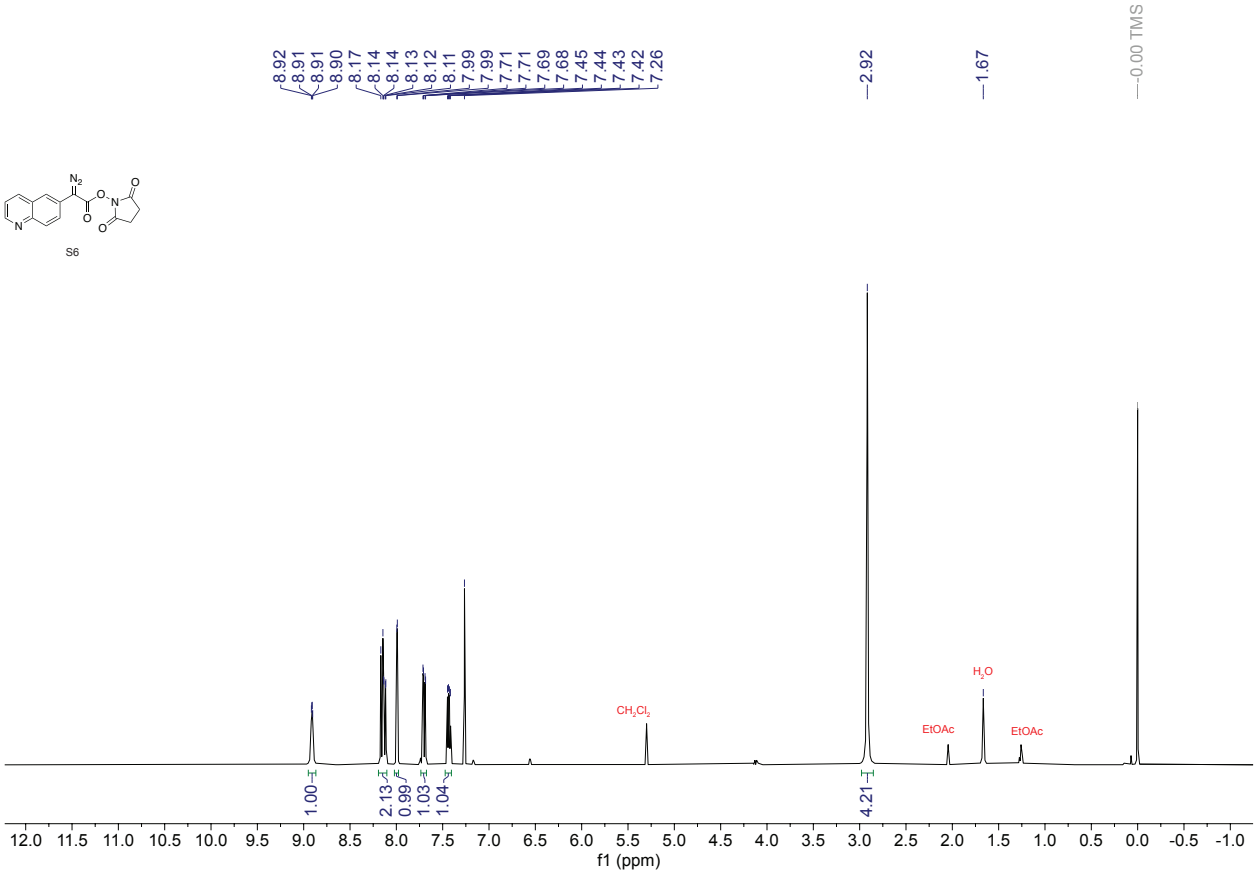

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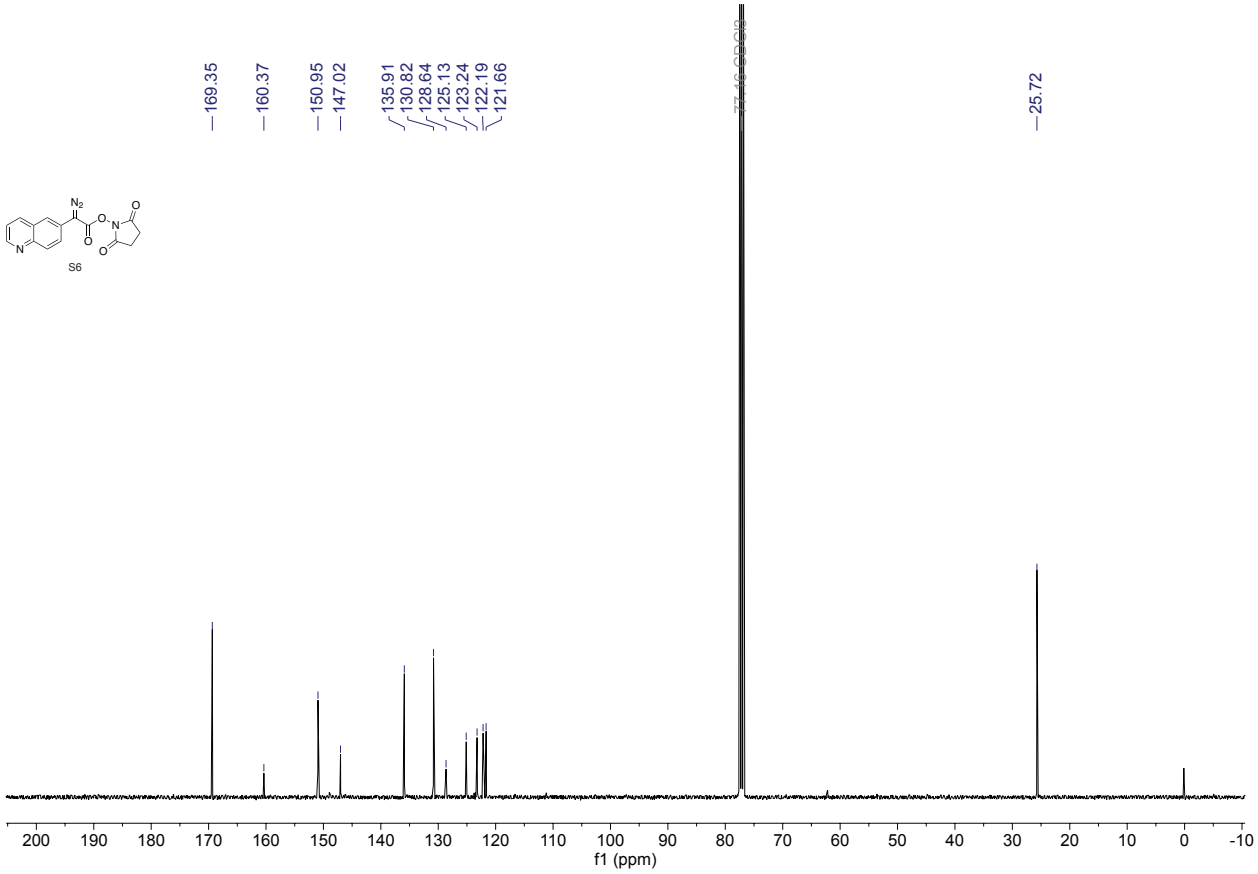

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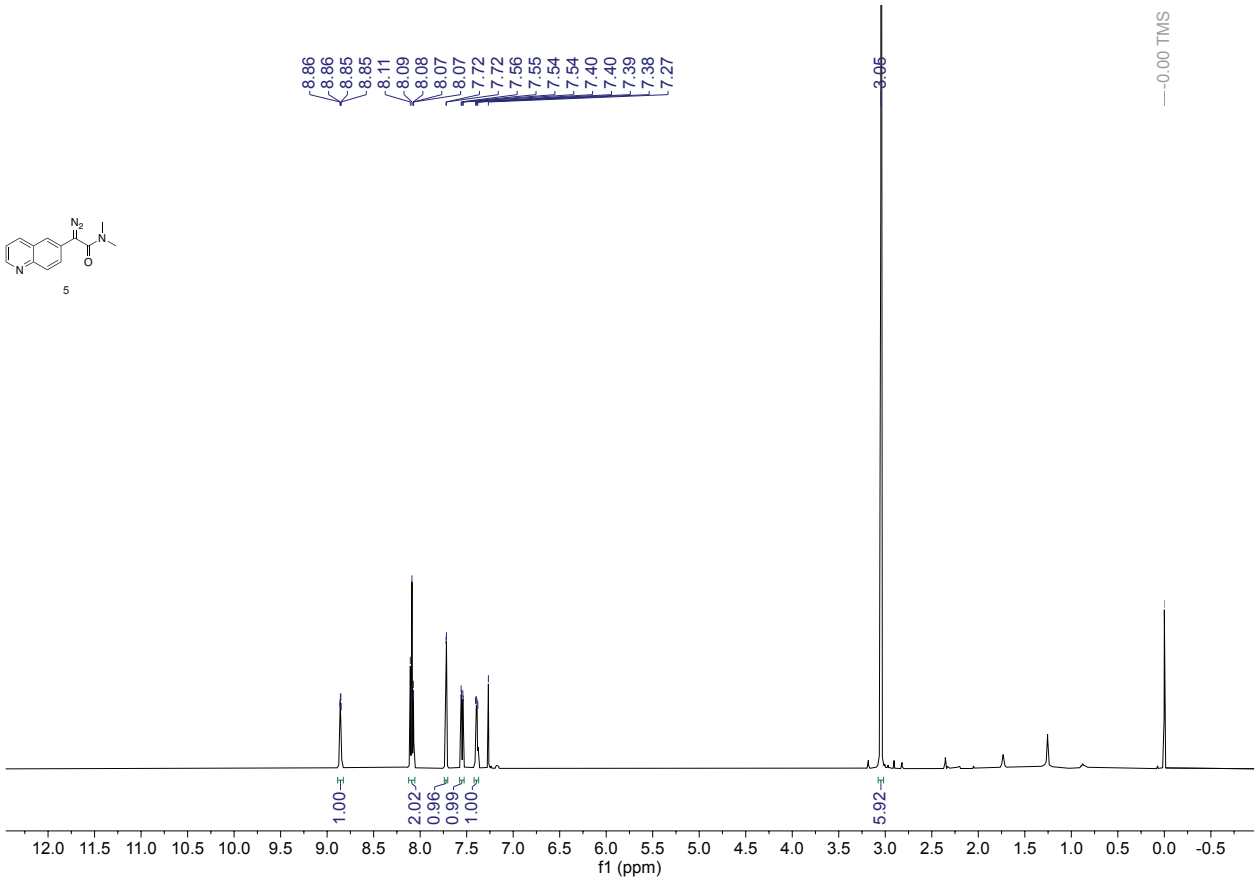
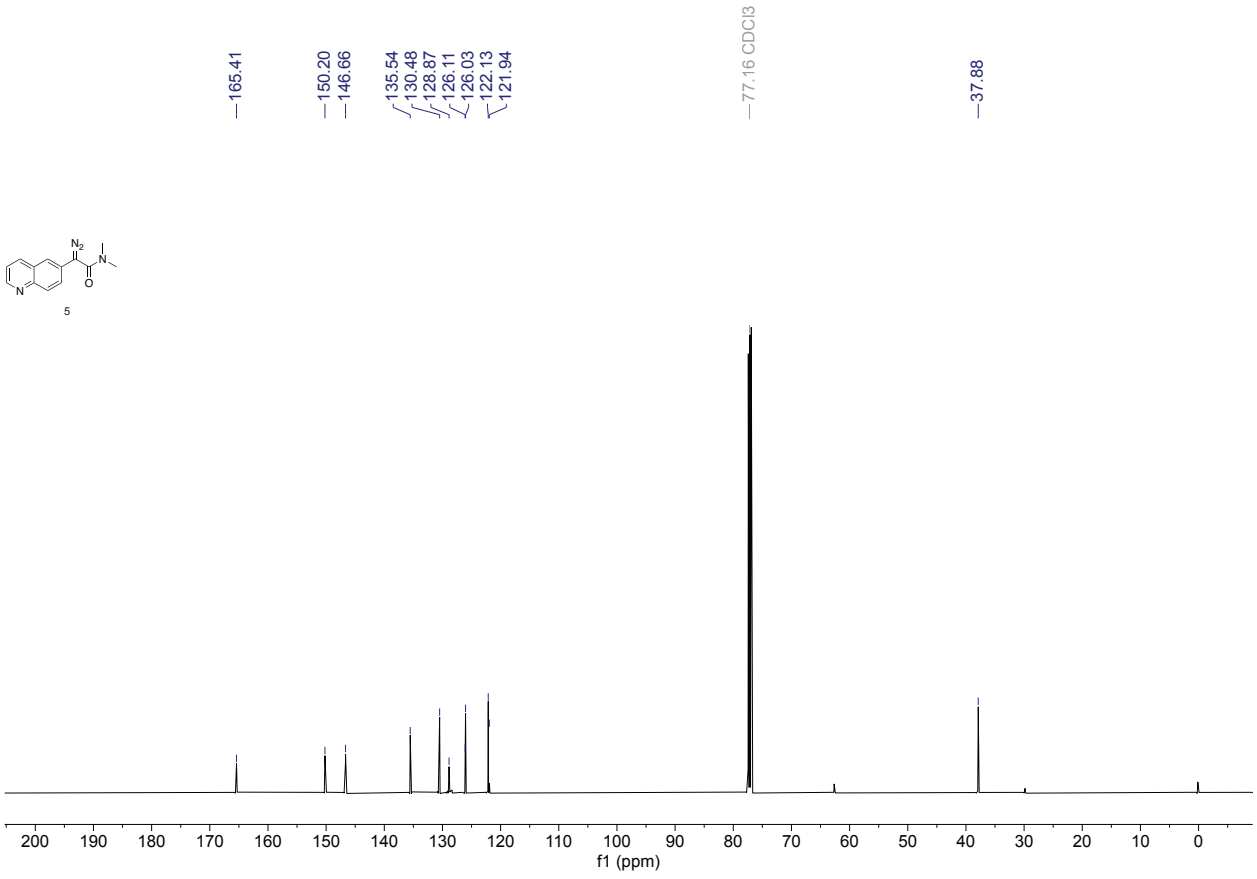
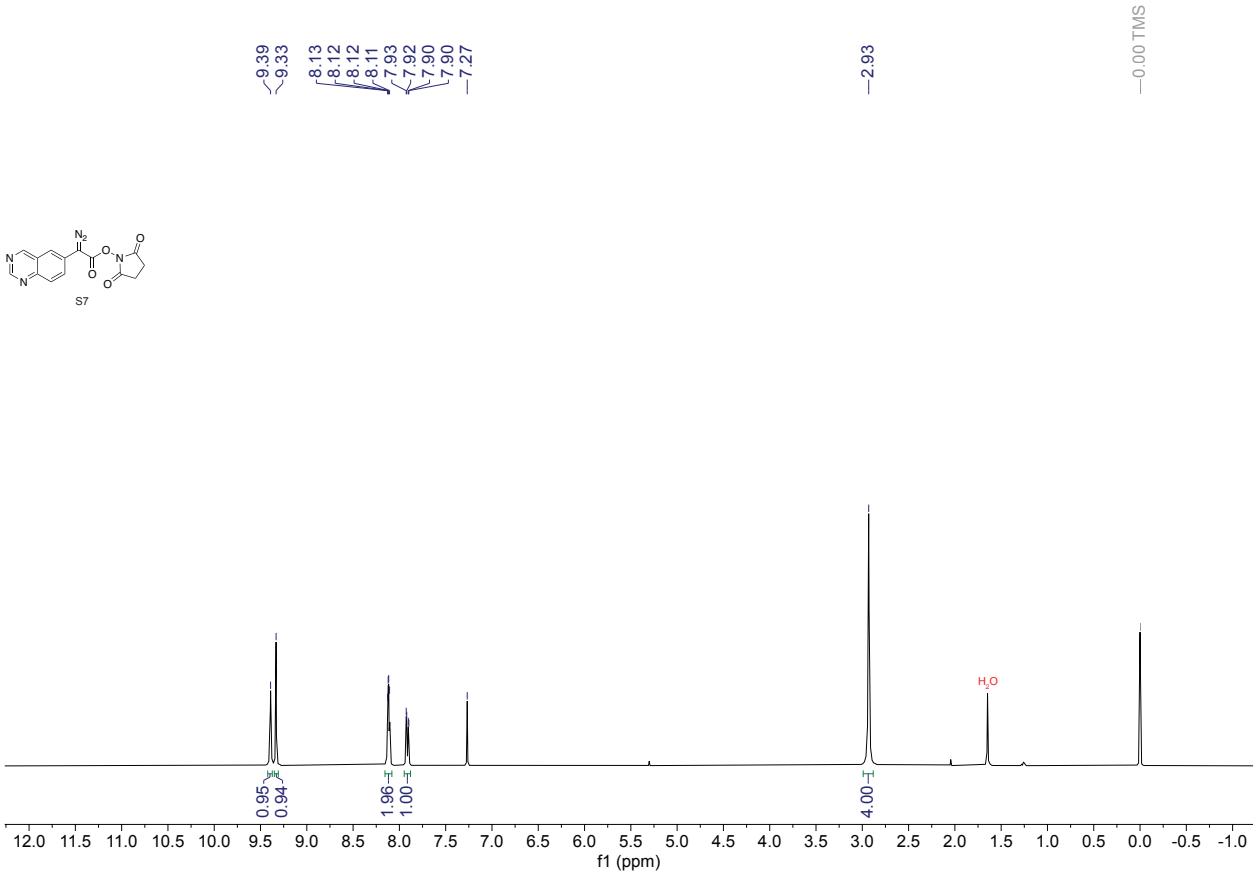


5

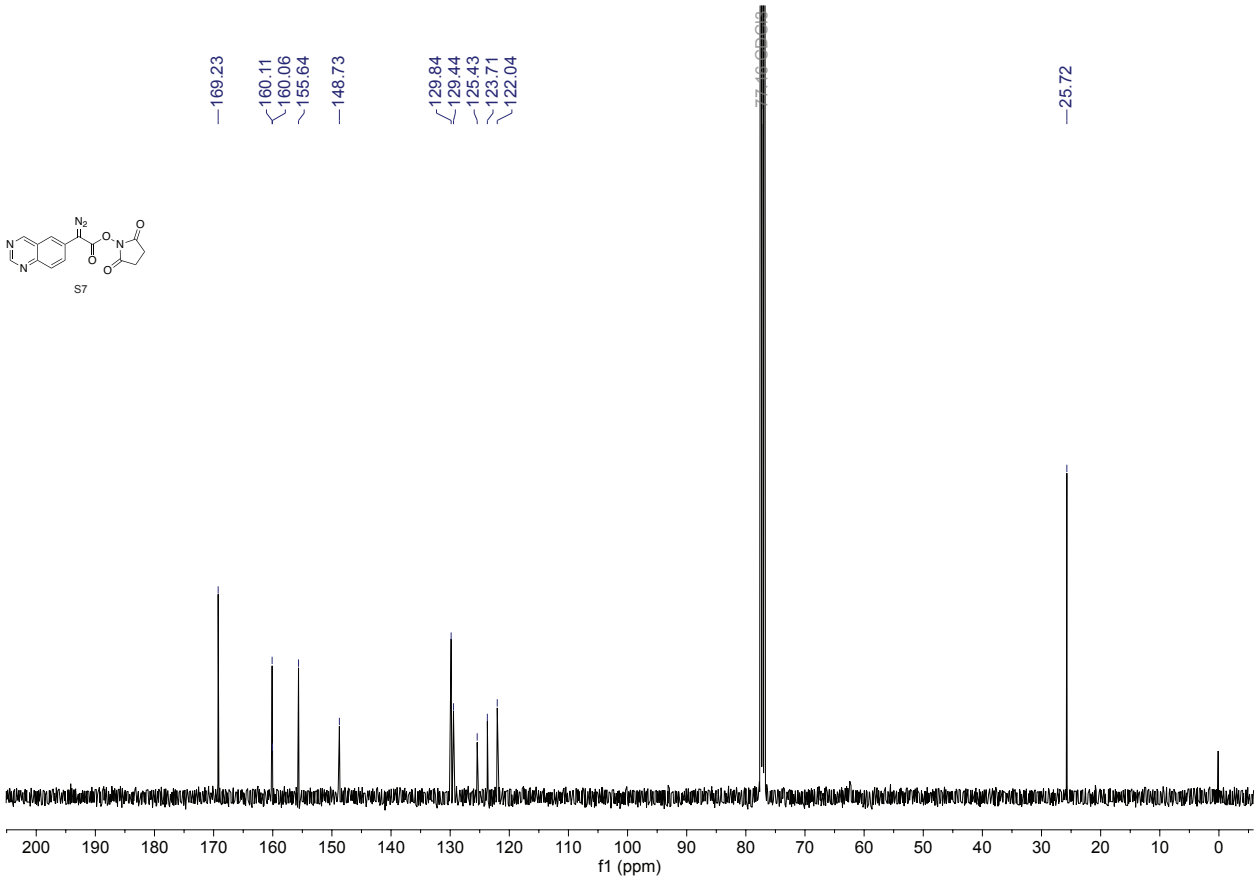
^{13}C NMR (126 MHz, $CDCl_3$)



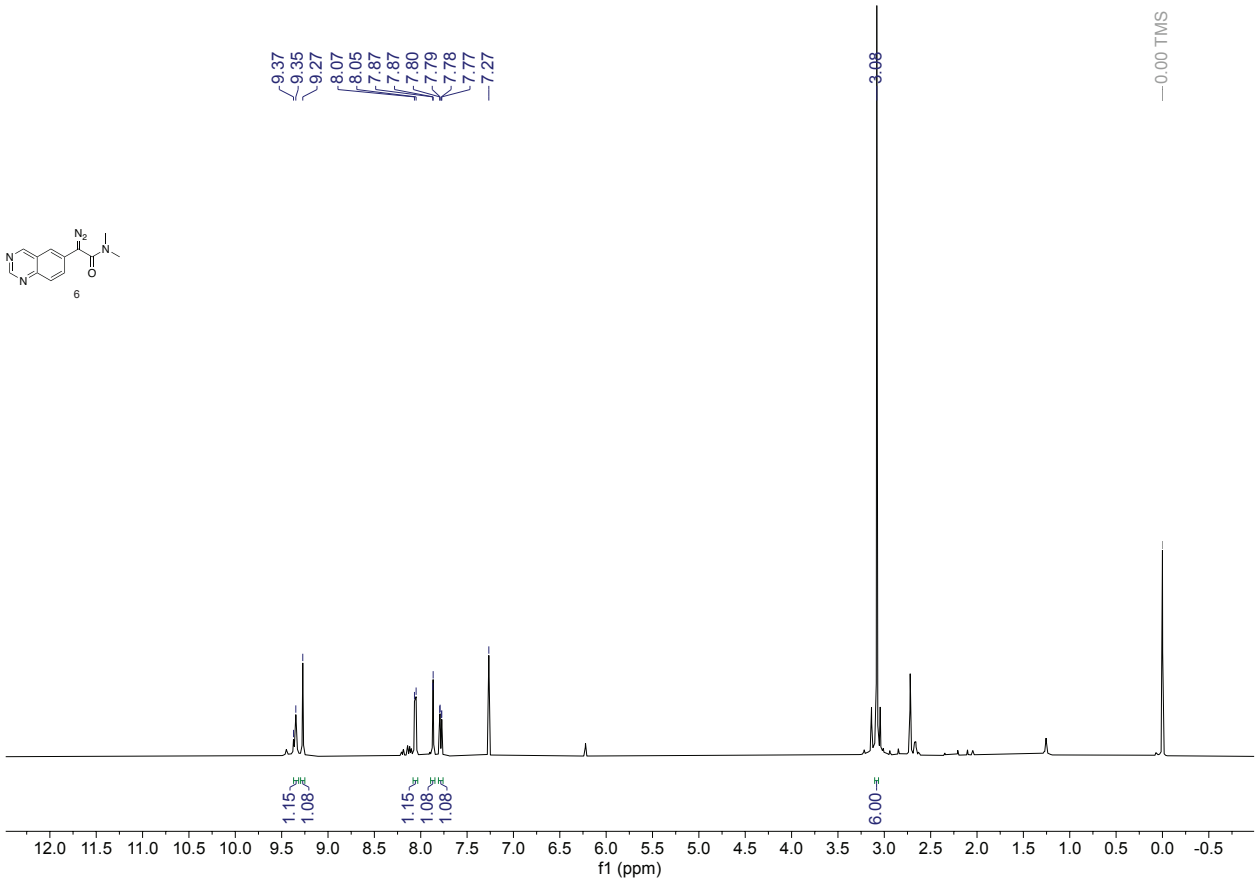
^{1}H NMR (400 MHz, $CDCl_3$)



^{13}C NMR (101 MHz, $CDCl_3$)



^{1}H NMR (500 MHz, $CDCl_3$)



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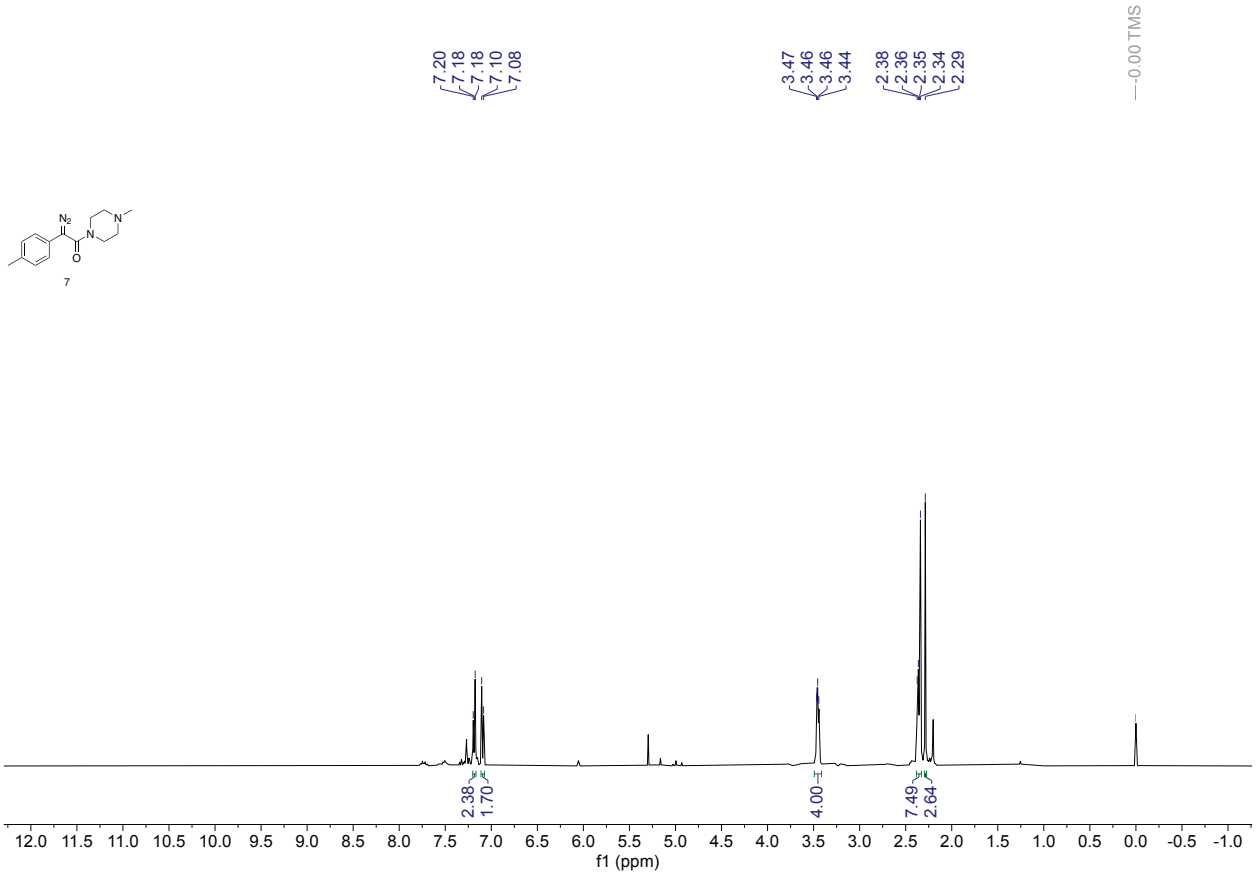
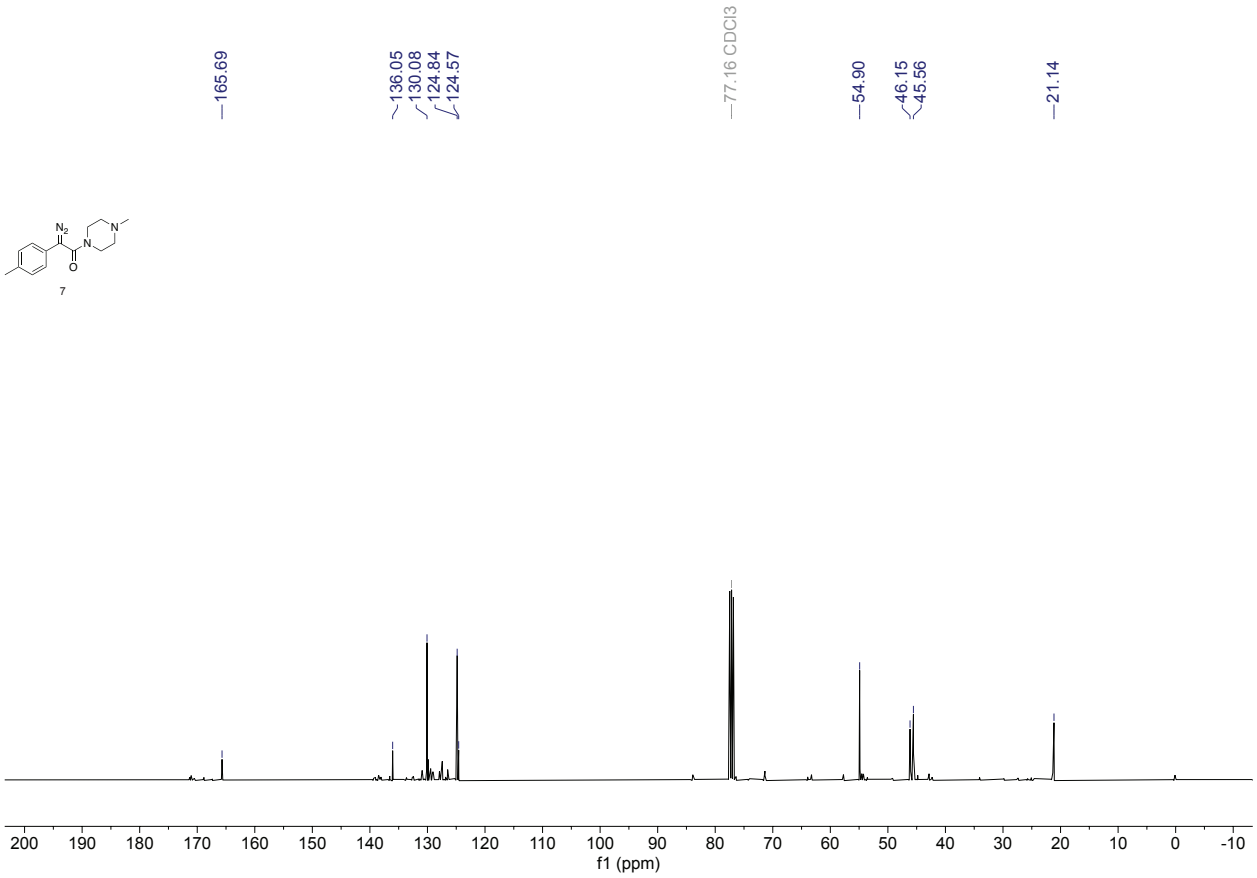
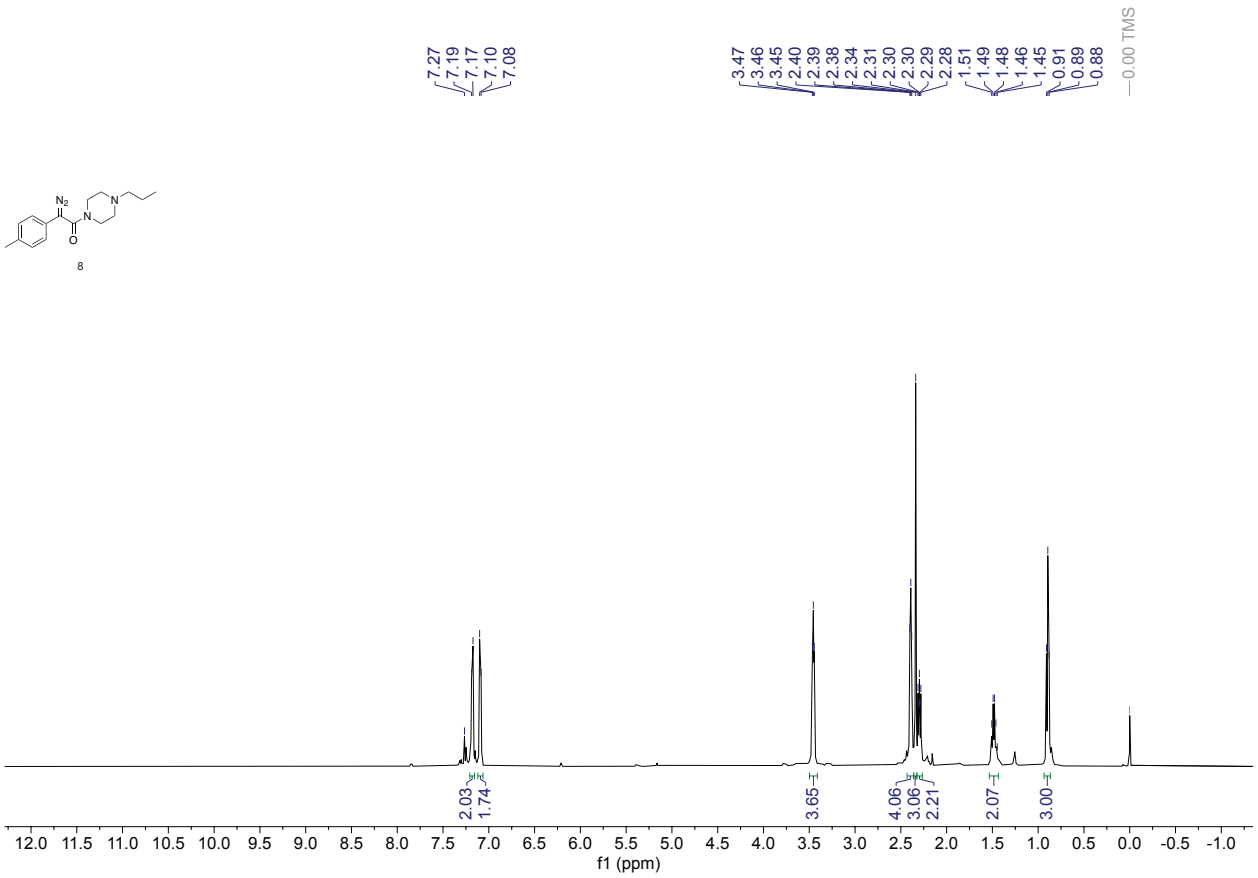
^{1}H NMR (400 MHz, $CDCl_3$)



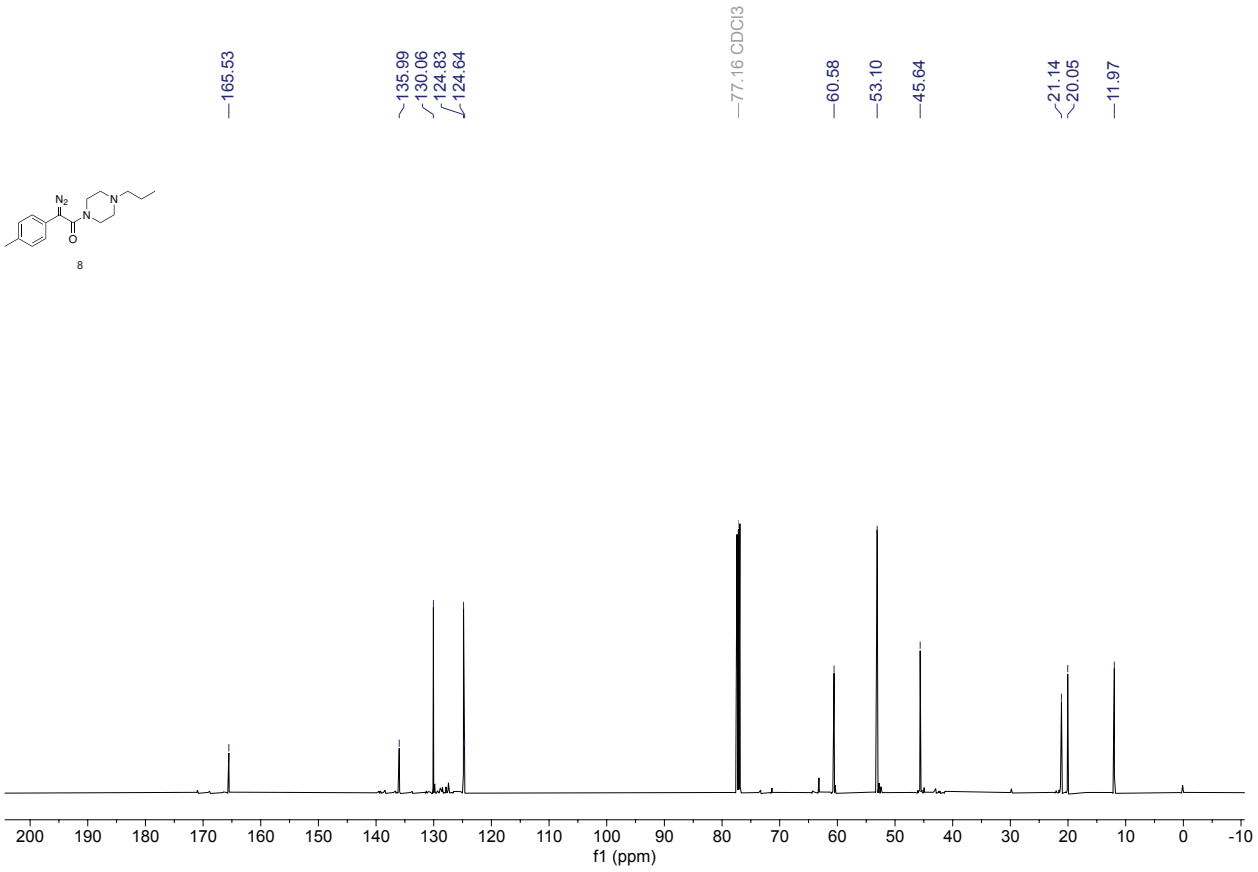
^{13}C NMR (101 MHz, $CDCl_3$)



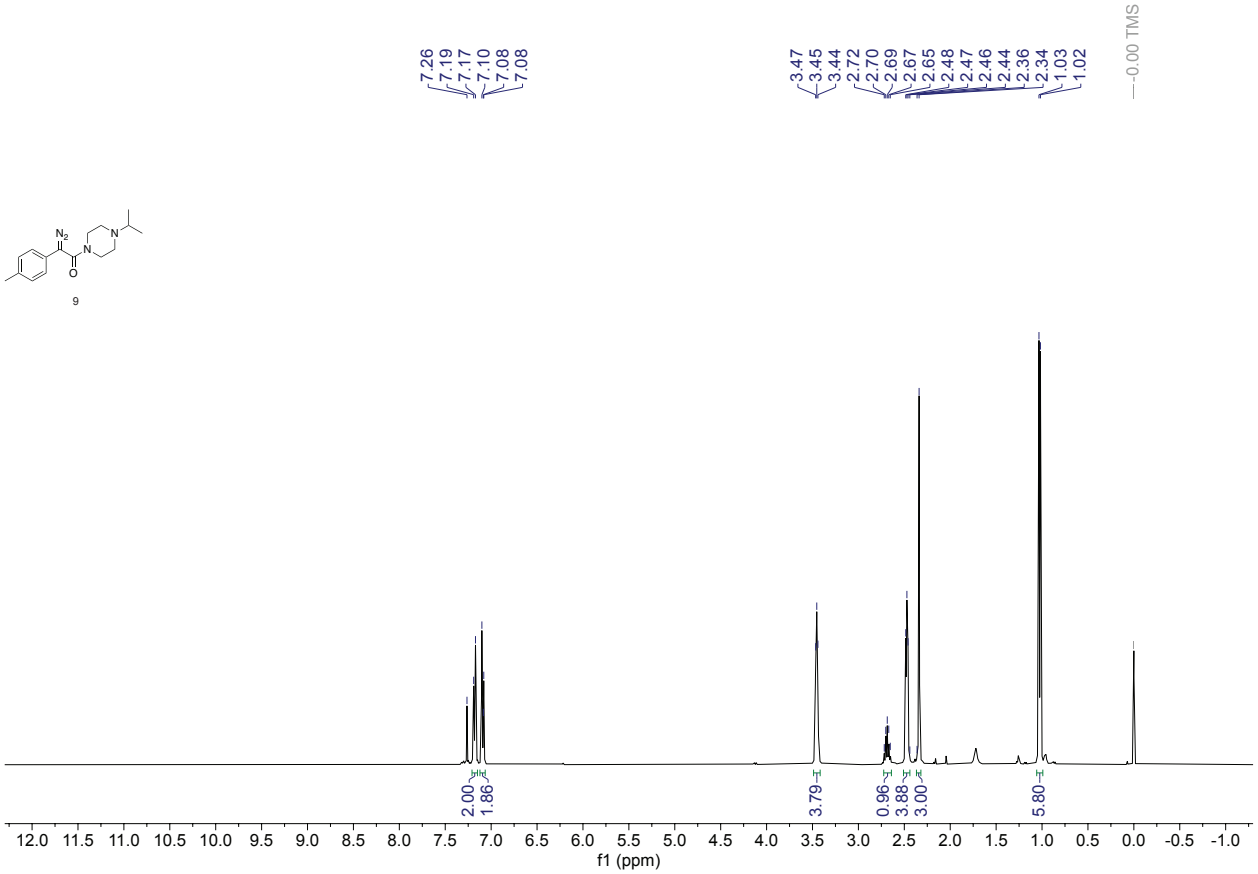
^{1}H NMR (500 MHz, $CDCl_3$)



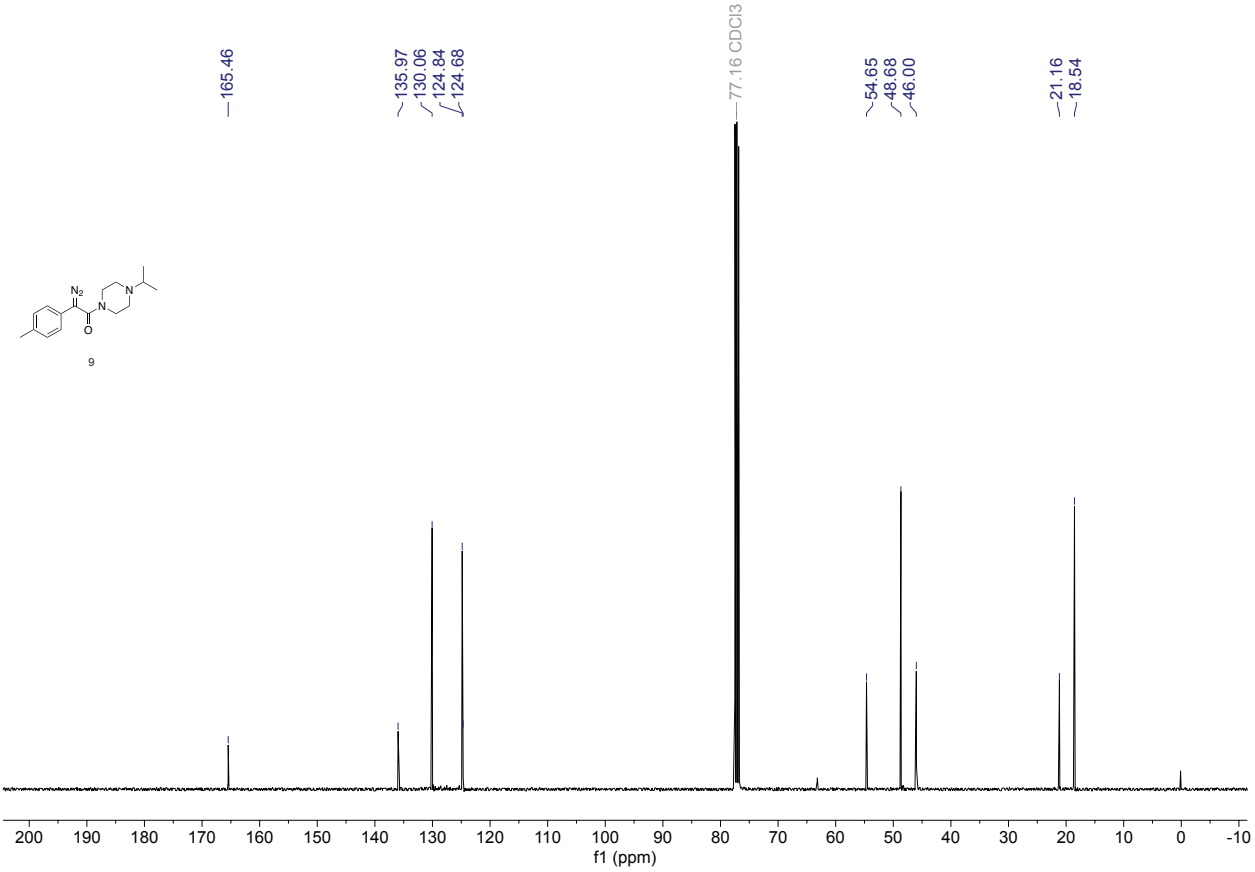
^{13}C NMR (126 MHz, $CDCl_3$)



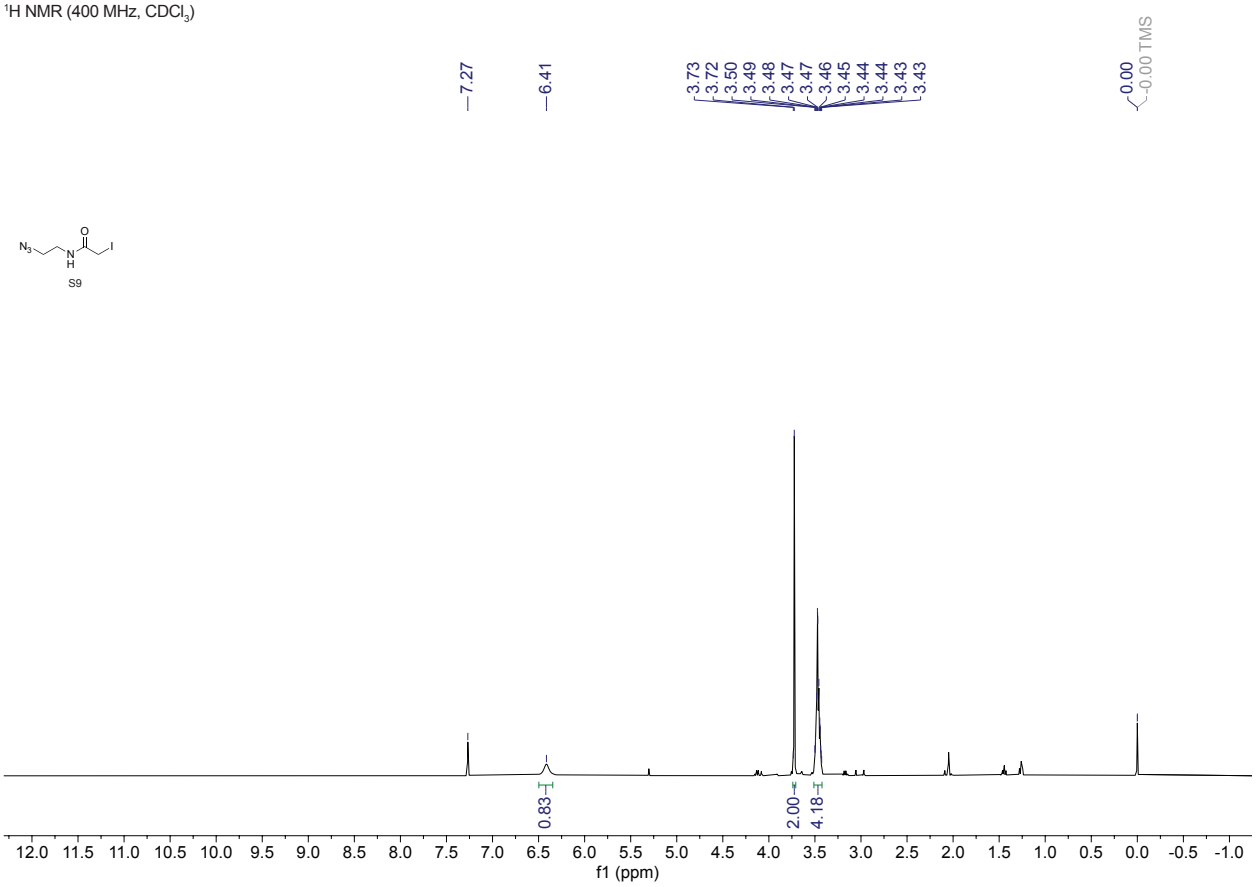
^{1}H NMR (400 MHz, $CDCl_3$)



^{13}C NMR (101 MHz, $CDCl_3$)



^{1}H NMR (400 MHz, $CDCl_3$)



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VIII. References

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