Supporting Information

Chemical Trigger-enabled Bioconjugate Reaction

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1. Materials and Methods

Compounds **1**[1] and **9**[2] were synthesized according to the previously reported methods. Compounds **4**, **10** and 2-mercaptoethanol were obtained from Sigma-Aldrich. Tz-BODIPY FL **(18)** and Tetrazine-Cy5 **(19)** were purchased from Lumiprobe Corporation (Hunt Valley, Maryland 21030, USA). All other reagents were purchased from commercial sources and used as received without further purification. PBS buffer solutions were adjusted to pH = 6.0, 6.3, 6.7, 7.0, 7.4, 7.8 through standard protocol, separately.

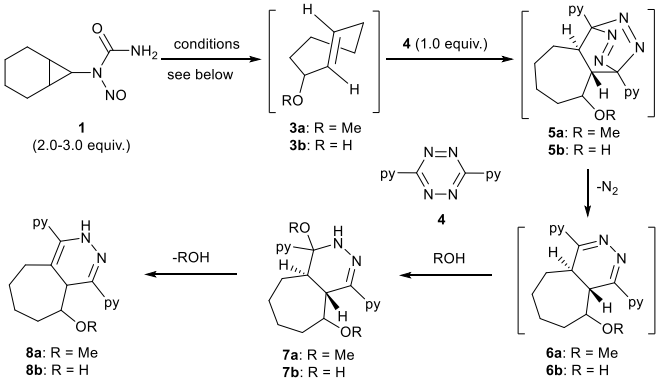
If no further details are given, the reaction was performed under ambient atmosphere and temperature. Analytical thin layer chromatography (TLC) was performed on silica gel-coated plates (Merck, 60 F_{254}) with the indicated solvent mixture, visualization was done using ultraviolet (UV) irradiation (λ = 254 nm) and/or staining with aqueous $KMnO_4$. Purification by column chromatography was carried out using silica gel 60 (Merck, 0.040-0.063 mm), reversed phase column chromatography was carried out using C_{18} Functional, Irregulair Silica (Screening Devices, 0.040-0.063 mm. 60Å, 12% functionalization).

^{1}H NMR spectra were recorded on a Bruker Avance III 400 (400 MHz) spectrometer. TMS (δH 0.00) or the NMR solvent residual peak of $(CD_3)_2CO$ ((C_3HD_5O) δH 2.05), $CDCl_3$ (($CHCl_3$) δH 7.26), $(CD_3)_2SO$ ((C_2HD_5SO) δH 2.50), CD_3OD ((CHD_3O) δH 3.31) or D_2O ((HDO) δH 4.79) were used as the internal reference. ^{13}C NMR spectra were recorded on a Bruker Advance III 400 (100MHz) spectrometer in $(CD_3)_2CO$ (δC 29.8), $CDCl_3$ (δC 77.16), CD_3OD (δC 49.0) or $(CD_3)_2SO$ (δC 39.5) using their central resonance as the internal reference. All ^{13}C NMR spectra were proton decoupled. Absorbance measurements were performed at the Infinite M200 Pro plate reader (Tecan), if not stated otherwise. Low-resolution mass spectra (LRMS) were recorded on Thermo LCQ Advantage Max (Electrospray Ionization (ESI)). A Thermo Finnigan LCQ Fleet ESI ion-trap mass spectrometer, which is equipped with a Shimadzu HPLC (C18-column,150 x 3 mm, particle size 3 µm, acetonitrile/water gradient 5 - 100%, 1 - 35 minutes and a flow of 0.2 mL/min) and a PDA detector, was used to separated organic compounds and record a low-resolution mass spectra. GC-MS spectra were recorded on a Thermo Finnigan Trace GC PolarisQ with a HP-5MS capillary column (30 m x 0.25 mm x 0.25 µm) using method 100°C (3 min) 15°C/min) 300°C (4 min). High-resolution mass spectra (HRMS) of small molecules were recorded on a JEOL AccuTOF JMS-T100CS (ESI). Q-TOF mass spectrometry of proteins were recorded on a SYNAPTTM G2-Si HDMS (Waters). Fluorescent signal in SDS-PAGE gels was measured on a FluorChem® FC3 imager (Alpha Innotech).

2. Synthetic Procedures and Spectroscopic Data

2.1 Model Reaction between Bicyclic N-Nitrosourea (BNU) Derivative (1) and 3,6-Di(pyridin-2-yl)-1,2,4,5-tetrazine (4)

Scheme S1. IEDDA reaction between **1** and **4**.



Entry	Conditions	Time[c]	Conversion of **4**[d]
1[a]	$NaHCO_3$ (4.0 equiv.), MeOH, 25 °C	20 min	>95%
2[a]	$NaHCO_3$ (4.0 equiv.), H_2O/MeCN(1/1), 25 °C	12 min	>95%
3[a]	Na_2CO_3 (4.0 equiv.), H_2O/MeCN(1/1), 25 °C	15 sec	>95%
4[a]	Na_2HPO_4 (4.0 equiv), H_2O/MeCN(1/1), 25 °C	10 min	>95%
5[b]	PBS (pH = 7.8)/MeCN(9/1), 37 °C	1.5 min	>95%
6[b]	PBS (pH = 7.3)/MeCN(9/1), 37 °C	5 min	>95%
7[b]	PBS (pH = 7.0)/MeCN(9/1), 37 °C	8 min	>95%
8[b]	PBS (pH = 6.5)/MeCN(9/1), 37 °C	15 min	>95%
9[b]	PBS (pH = 6.0)/MeCN(9/1), 37 °C	35 min	>95%
10[b]	H_2O/MeCN(9/1), 37 °C (pH = 7.3)	8 h	>95%
11[b]	NaH_2PO_4 solution (0.2M)/MeCN (9/1) (pH = 4.7), 37 °C	12 h	>95%

[a] 2.0 equiv. BNU (C = 0.04M); [b] 3.0 equiv. BNU (C = 0.003M); [c] The reaction time was judged by the complete dispearence of **4**; [d] The conversion was evaluated based on the consumption of **4**.

General procedure a

$NaHCO_3$ (32.6 mg, 0.388 mmol, 4 equiv), Na_2CO_3 (41.1 mg, 0.388 mmol, 4 equiv), or $Na_2HPO_4 \cdot 12H_2O$ (139.0 mg, 0.388 mmol, , 4 equiv) was added to a solution of BNU (**1**) (35.5 mg, 0.194 mmol, 2 equiv) and 3,6-di-(2-pyridyl)-1,2,4,5-tetrazine (**4**) (23.4 mg, 0.097 mmol, 1 equiv) in CH_3OH (5 mL) or MeCN/H_2O solution (1:1, 5 mL) at 25°C. Upon mixing, nitrogen evolved immediately and the color changed from fuchsia to pale yellow indicating the reaction was completed. The reaction mixture was concentrated using a rotary evaporator and purified by silica column to obtain compound **7a** or **8b** as major product.

General procedure b

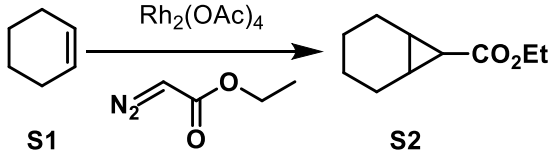
BNU (**1**) (12.0 mg, 0.065 mmol, 3 equiv) was added to a solution 3,6-di-(2-pyridyl)-1,2,4,5-tetrazine (**4**) (5.3 mg, 0.022 mmol, 1 equiv) in MeCN/PBS (pH = 7.8, 7.3, 7.0, 6.5, 6.0) buffer soulution, MeCN/H_2O solution, or MeCN/NaH_2PO_4 solution (1:9, 20 mL) at 37°C. Upon mixing, nitrogen evolved immediately and the color changed from fuchsia to pale yellow, indicating the reaction was completed. The reaction mixture was concentrated using a rotary evaporator and purified by silica column to obtain compound **8b** as major product.

Note: In entry 1, a mixture of compound **7a** and **8a** were obtained when the reaction products were purified using acetone- petroleum ether solvent system and only compound **7a** were obtained when the reaction products were purified using 5% methanol in methylene chloride as eluents. In entry 2-11, a mixture of **7b** and **8b** were obtained when the reaction products were purified using MeOH-DCM solvent system and only compound **8b** were obtained when the reaction products were purified using ether:acetone (2:1) as eluents

1,5-Dimethoxy-1,4-di(pyridin-2-yl)-2,4a,5,6,7,8,9,9a-octahydro-1H-cyclohepta[d]pyridazine (7a) ^{1}H NMR (400 MHz, MeOD) δ 8.58 (ddd, *J* = 4.9, 1.6, 0.8 Hz, 1H), 8.52 – 8.46 (m, 1H), 7.92 – 7.84 (m, 2H), 7.78 (td, *J* = 7.7, 1.8 Hz, 1H), 7.70 – 7.64 (m, 1H), 7.37 (ddd, *J* = 7.5, 4.9, 1.1 Hz, 1H), 7.30 (ddd, *J* = 7.4, 5.0, 1.2 Hz, 1H), 3.78 – 3.73 (m, 1H), 3.35 (s, 1H), 3.12 (s, 3H), 2.57 (s, 3H), 2.47 (dd, *J* = 10.1, 7.7 Hz, 1H), 2.10 – 2.02 (m, 1H), 1.95 (d, *J* = 13.2 Hz, 1H), 1.79 – 1.67 (m, 1H), 1.61 – 1.42 (m, 4H), 1.34 – 1.17 (m, 2H). ^{13}C NMR (100 MHz, MeOD) δ 160.1, 157.7, 153.5, 149.5, 149.1, 138.2, 138.0, 124.2, 124.2, 123.8, 122.5, 88.7, 80.3, 58.2, 55.2, 50.8, 37.2, 35.6, 30.3, 28.1, 22.2. HRMS (ESI) *m/z* calcd for $C_{21}H_{27}N_4O_2$ $[M+H]^+$ 367.2134, found 367.2133.

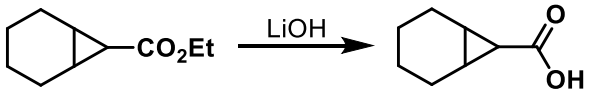
1,4-Di(pyridin-2-yl)-4a,5,6,7,8,9-hexahydro-2H-cyclohepta[d]pyridazin-5-ol (8b) ^{1}H NMR (400 MHz, MeOD) δ 8.64 (ddd, *J* = 4.9, 1.7, 0.9 Hz, 1H), 8.56 (ddd, *J* = 5.0, 1.7, 0.9 Hz, 1H), 8.09 – 8.06 (m, 1H), 7.92 (td, *J* = 7.8, 1.8 Hz, 1H), 7.88 – 7.83 (m, 1H), 7.60 (d, *J* = 7.9 Hz, 1H), 7.42 (ddd, *J* = 7.6, 4.9, 1.1 Hz, 1H), 7.38 (ddd, *J* = 7.4, 5.0, 1.1 Hz, 1H), 4.64 (s, 1H), 3.89 – 3.81 (m, 2H), 2.79 – 2.72 (m, 1H), 1.98 (m, 1H), 1.93 – 1.85 (m, 3H), 1.69 (m, 2H). ^{13}C NMR (100 MHz, MeOD) δ 157.0, 153.6, 150.4, 148.6, 140.6, 138.9, 138.5, 138.4, 135.0, 125.5, 124.8, 124.2, 122.8, 73.7, 45.7, 36.7, 32.9, 31.5, 22.2. HRMS (ESI) *m/z* calcd for $C_{19}H_{20}N_4O$ $[M+H]^+$ 321.1715, found 321.1718.

2.2 Synthesis of BNU (1)



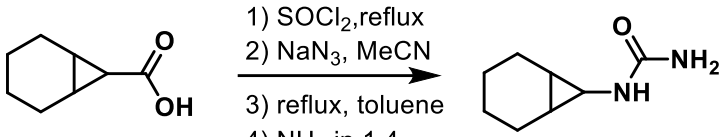
Ethyl bicyclo[4.1.0]heptane-7-carboxylate (S2): To a solution of cyclohexene **S1** (2.6 mL, 25.4 mmol) and $Rh_2(OAc)_4$ (28 mg, 0.06 mmol) in anhydrous DCM (50 mL) was added ethyl 2-diazoacetate (1.4 mL, 12.7 mmol) through a syringe pump over a period of 16 h. After the addition, the mixture was kept stirring under inert atmosphere until the diazo compound consumed completely. The solvent and excess olefins were removed in vacuo, and the residue was filtered on a column of silica gel to remove the metal catalyst. The crude product was purified by column chromatography (petroleum ether-EA 30:1) to get **S2** (1.049 g, 49%) as a

colorless oil. The NMR spectrums agree with the reported data[3].



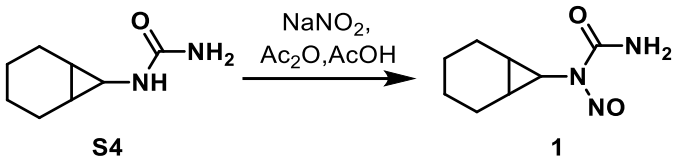
S2 S3

Bicyclo[4.1.0]heptane-7-carboxylic acid (S3): To a solution of **S2** (6.369 g, 37.87 mmol) in methanol and water (4:1, 60 mL) at room temperature was added $LiOH \cdot H_2O$ powder in small portions (7.940 g, 189.33 mmol), and the mixture was stirred at room temperature for 15 h. The reaction mixture was then acidified with 1N HCl to pH = 1, then extracted with ethyl acetate (2×100 mL) and then DCM (100 mL), dried over sodium sulfate, and evaporated to dryness. The crude product was separated on silica gel (DCM-MeOH-TFA 400:20:0.2) to yield **S3** (4.921 g, 93%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.96 – 1.85 (m, 2H), 1.76 – 1.69 (m, 2H), 1.67 – 1.64 (m, 2H), 1.39 (t, J = 4.2 Hz, 1H), 1.35 – 1.24 (m, 2H), 1.23 – 1.13 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 181.8, 25.6, 23.6, 22.7, 20.9. HRMS (ESI) *m/z* calcd for $C_8H_{11}O_2$ $[M-H]^+$ 139.0759, found 139.0753.



S3 S4

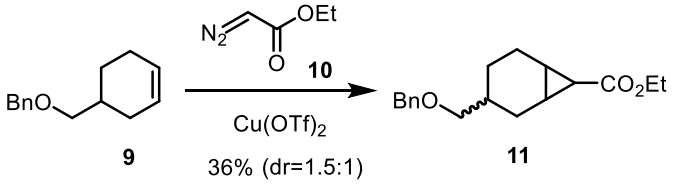
1-(Bicyclo[4.1.0]heptan-7-yl)urea (S4): A solution of the acid **S3** (950 mg, 6.78 mmol) in thionyl chloride (10 mL) was heated under reflux for 2 h. After cooling, the reaction mixture was concentrated under reduced pressure to give a yellow oil. A powder of NaN_3 (595 mg, 9.15 mmol) was added at 0°C to a stirred solution of the resulting acid chloride in CH_3CN (30 mL). The reaction mixture was stirred for a period of 8 h at this temperature, poured into ice-cold water (30 mL). The resulting mixture was extracted with diethyl ether (3 × 20 mL), and the combined organic phases were dried at 0°C. After concentration under reduced pressure at 0°C, the residue was taken up with anhydrous toluene (30 mL), and the solution was heated at 90°C for 2 h. After cooling, NH_3 in 1,4-dioxane (0.4 M, 34 mL) was added at 0°C. The obtained precipitate was recrystallized in EA to give **S4** (605 mg, 58% yield) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.29 (brs, 1H, NH), 5.08 (brs, 2H, NH_2), 2.12 (s, 1H), 1.96 – 1.84 (m, 2H), 1.70 – 1.60 (m, 2H), 1.27 – 1.19 (m, 2H), 1.13 – 1.02 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 160.6, 34.6, 22.3, 21.2, 19.7. HRMS (ESI) *m/z* calcd for $C_8H_{15}N_2O$ $[M+H]^+$ 155.1184, found 155.1181.



S4 1

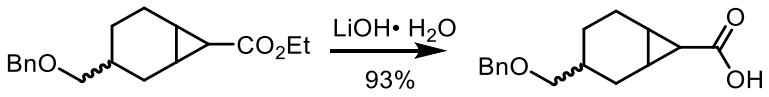
1-(Bicyclo[4.1.0]heptan-7-yl)-1-nitrosourea (1): A 100 mL round-bottomed flask was placed in an ice/water bath and equipped with a magnetic stirrer. To the flask were added **S4** (645 mg, 4.18 mmol) and 9 mL of a 2:1 mixture of acetic acid and acetic anhydride. $NaNO_2$ (288.6 mg, 4.18 mmol) was dissolved in 8 mL of water and added to the solution via syringe. The mixture was stirred for 50 min. To the solution was added 20 mL of ice water, and the solution was stirred for an additional 30 min in the ice bath. A yellow precipitate was formed during this period. The solution was extracted with DCM and then purified by chromatography with DCM to yield **1** (542 mg, 71%) as a yellow powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.75 (brs, 1H, NH_2), 5.38 (brs, 1H, NH_2), 2.01 (t, J = 3.8 Hz, 1H), 1.91 – 1.87 (m, 2H), 1.32 – 1.23 (m, 4H), 1.20 – 1.08 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.8, 33.8, 21.9, 21.2, 20.2. Note: The HRMS of compound **1** failed to give its molecular weight.

2.3 Synthesis of the Functionalized BNU Derivatives



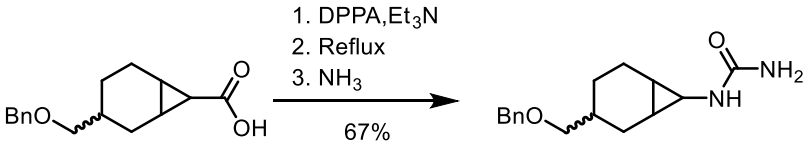
9 11

Ethyl 3-((benzyloxy)methyl)bicyclo[4.1.0]heptane-7-carboxylate (11): Ethyl diazoacetate (2.72 mL, 25.9 mmol) was added to a solution of compound **9** (10.487 g, 51.8 mmol) and copper(II) trifluoromethanesulfonate (936.7 mg, 2.59 mmol) in anhydrous DCM through a syringe pump over a period of 12 h. After the addition, the mixture was kept stirring until the diazo compound consumed completely. The solvent was removed in vacuo and the residue was purified on silica gel (petroleum ether-DCM 3:1, then DCM) to yield **11 (**4.725 g, 32%, dr = 1.5:1) as a colorless oil, together with substantial amounts of recovered compound **9** (5.517g, 53% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 – 7.27 (m, 5H), 4.48 – 4.45 (m, 2H), 4.10 (q, J = 7.2 Hz, 2H), 3.30 – 3.15 (m, 2H), 2.23 – 2.04 (m, 1H), 2.00 (d, J = 9.4 Hz, 1H), 1.80 (ddd, J = 18.2, 9.2, 4.7 Hz, 1H), 1.66 – 1.41 (m, 5H), 1.36 – 1.22 (m, 5H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.7, 138.6, 133.7, 128.4, 127.6, 75.4, 73.1, 60.3, 34.3, 26.9, 25.7, 23.4, 22.9, 22.2, 21.5, 14.4 (major isomer); 174.7, 138.6, 133.7, 128.4, 127.6, 75.3, 73.1, 60.3, 32.1, 26.2, 25.5, 23.1, 22.6, 22.2, 21.0, 14.4 (minor isomer). HRMS (ESI) *m/z* calcd for $C_{18}H_{25}O_3$ $[M+H]^+$ 289.1804, found 289.1812.



11 **12**

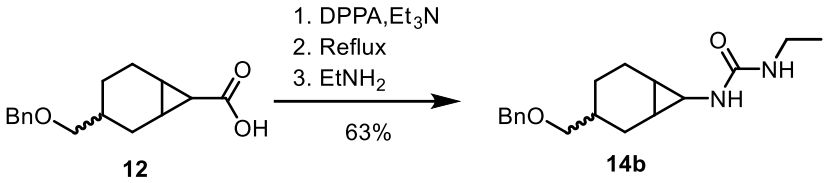
3-((Benzyloxy)methyl)bicyclo[4.1.0]heptane-7-carboxylic acid (12): To a solution of **11** (4.022 g, 13.95 mmol) in MeOH/H_2O (4:1, 40 mL) at room temperature was added LiOH·H_2O in small portions (2.926 g, 69.73 mmol), and the mixture was stirred for 14 h. The reaction mixture was then acidified with 1N HCl to pH=1, extracted with ethyl acetate (2 × 80 mL) and then DCM (80 mL), dried over sodium sulfate, and evaporated to dryness. The crude product was separated on silica gel (DCM-MeOH-TFA 400:20:0.2) to yield **12** (3.377 g, 93%, dr = 1.5:1) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.26 (m, 5H), 4.49 – 4.45 (m, 2H), 3.30 – 3.15 (m, 2H), 2.27 – 2.05 (m, 1H), 2.04 – 1.98 (m, 1H), 1.87 – 1.75 (m, 1H), 1.72 – 1.63 (m, 2H), 1.60 – 1.42 (m, 3H), 1.39 – 1.33 (m, 1H), 1.03 – 0.91 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 181.2, 138.4, 128.4, 127.6, 75.3, 73.1, 34.2, 26.7, 26.1, 25.3, 24.1, 22.7, 22.2 (major isomer); 181.2, 138.4, 128.4, 127.6, 75.1, 73.1, 31.9, 26.1, 25.5, 24.4, 23.2, 22.6, 22.1 (minor isomer). HRMS (ESI) *m/z* calcd for $C_{16}H_{19}O_3$ $[M-H]^+$ 259.1334, found 259.1323.



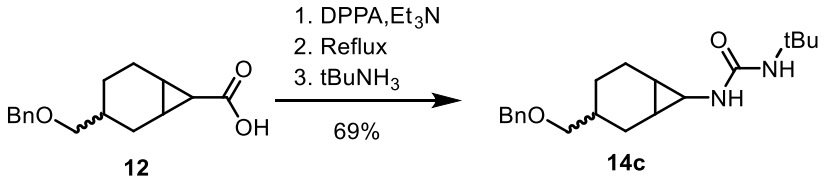
12 **14a**

1-(3-((Benzyloxy)methyl)bicyclo[4.1.0]heptan-7-yl)urea (14a): To a solution of **12** (2.965 g, 11.39 mmol) in toluene (25 mL) was sequentially added diphenylphosphene azide (3.19 mL, 14.81 mmol) and Et_3N (2.40 mL, 17.09 mmol). The mixture was stirred at room temperature for 0.5 h and then refluxed at 90°C for 4 h. After cooling to 0°C, NH_3 in 1,4-dioxane (0.4 M, 40 ml) was added and stirred for another 1 h. The solvent was removed, and the residue was purified by chromatography on silica gel with a gradient of DCM/MeOH (20:1) to give **14a** (2.094 g, 67% yield, dr = 1.5:1) as a yellow oil. Major diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 – 7.24 (m, 5H), 5.51 (br s, 1H, NH), 5.28 (br s, 1H, NH_2), 5.18 (br s, 1H, NH_2), 4.47 (s, 2H), 3.24 (d, *J* = 6.4 Hz, 2H), 2.46 – 2.19 (m, 1H), 2.16 – 2.05 (m, 1H), 2.03 – 1.97 (m, 1H), 1.89 – 1.69 (m, 1H), 1.61 – 1.36 (m, 3H), 1.35 – 1.22 (m, 1H), 1.17 – 1.03 (m, 2H). ^{13}C NMR (100 MHz, CDCl3) δ 160.8, 138.5, 128.4, 127.5, 127.5, 75.3, 73.1, 34.8, 34.0, 26.6, 25.6, 22.0, 20.4, 19.2. Minor diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 – 7.24 (m, 5H), 5.51 (brs, 1H, NH), 5.28 (brs, 1H, NH_2), 5.18 (brs, 1H, NH_2), 4.45 (s, 2H), 3.17 (dd, *J* = 6.3, 1.9 Hz, 2H), 2.46 – 2.19 (m, 1H), 2.16 – 2.05 (m, 1H), 2.03 – 1.97 (m, 1H), 1.89 – 1.69 (m, 1H), 1.61 – 1.36 (m, 3H), 1.35 – 1.22 (m, 1H), 1.17 – 1.03 (m, 2H). ^{13}C NMR (100 MHz,

CDCl3) δ 160.8, 138.4, 128.4, 127.5, 127.5, 75.3, 73.0, 34.4, 32.8, 25.4, 24.2, 21.9, 20.4, 18.8. HRMS (ESI) *m/z* calcd for $C_{16}H_{23}N_2O_2$ $[M+H]^+$ 275.1760, found 275.1753.

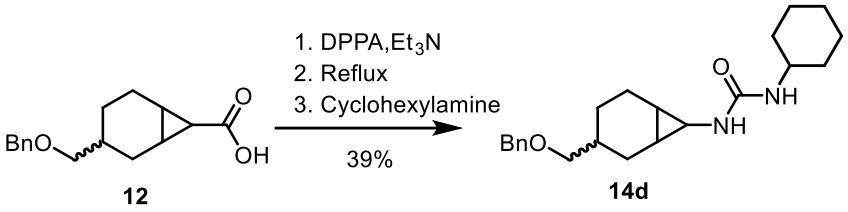


1-(3-((benzyloxy)methyl)bicyclo[4.1.0]heptan-7-yl)-3-ethylurea (14b): To a solution of **12** (300 mg, 1.15 mmol) in toluene (3 mL) was sequentially added diphenylphosphene azide (0.34 mL, 1.49 mmol) and Et_3N (0.25 mL, 1.72 mmol). The mixture was stirred at room temperature for 0.5 h and then refluxed at 90°C for 4 h. After cooling to 0 ℃, Ethylamine in THF (2.0 M, 1.2 mL) and toluene (6 mL) was added and stirred at r.t. overnight. The solvent was removed, and the residue was purified by chromatography on silica gel with a gradient of DCM/MeOH (40:1) to give **14b** (228 mg, 63% yield, dr = 1.5:1) as a brown oil. Major diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 – 7.24 (m, 5H), 5.01 (br s, 1H, NH), 4.86 (br s, 1H, NH), 4.48 (s, 2H), 3.25 (d, *J* = 6.4 Hz, 2H), 3.20 (d, *J* = 6.3 Hz, 2H), 2.30 – 2.12 (m, 1H), 2.10 – 1.98 (m, 2H), 1.71 – 1.83 (m, 1H), 1.60 – 1.55 (m, 1H), 1.40 – 1.52 (m, 2H), 1.39 – 1.24 (m, 1H), 1.15 (t, *J* = 7.1 Hz, 3H), 1.11 – 1.05 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.3, 138.5, 128.4, 127.6, 127.5, 75.4, 73.1, 35.0, 34.0, 32.9, 26.7, 25.7, 22.0, 20.5, 19.4, 15.7. Minor diastereoisomer:^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 – 7.24 (m, 5H), 5.01 (br s, 1H, NH), 4.86 (br s, 1H, NH), 4.47 (s, 2H), 3.25 (d, *J* = 6.4 Hz, 2H), 3.20 (d, *J* = 6.3 Hz, 2H), 2.30 – 2.12 (m, 1H), 2.10 – 1.98 (m, 2H), 1.71 – 1.83 (m, 1H), 1.60 – 1.55 (m, 1H), 1.40 – 1.52 (m, 2H), 1.39 – 1.24 (m, 1H), 1.15 (t, *J* = 7.1 Hz, 3H), 1.11 – 1.05 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.3, 138.5, 128.3, 127.6, 127.5, 75.3, 73.0, 35.0, 34.0, 32.9, 25.5, 24.3, 21.9, 20.5, 19.0, 15.7. HRMS (ESI) *m/z* calcd for $C_{18}H_{27}N_2O_2$ $[M+H]^+$ 303.2067, found 303.2066.

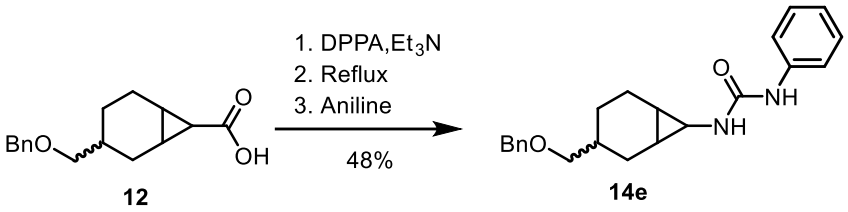


1-(3-((benzyloxy)methyl)bicyclo[4.1.0]heptan-7-yl)-3-(tert-butyl)urea (14c): To a solution of 12 (300 mg, 1.15 mmol) in toluene (3 mL) was sequentially added diphenylphosphene azide (0.34 mL, 1.49 mmol) and Et_3N (0.25 mL, 1.72 mmol). The mixture was stirred at room temperature for 0.5 h and then refluxed at 90°C for 4 h. After cooling to 0 ℃, tert-Butylamine (0.25 mL, 2.30 mmol) and toluene (6 mL) was added and stirred at r.t. overnight. The solvent was removed, and the residue was purified by chromatography on silica gel with a gradient of

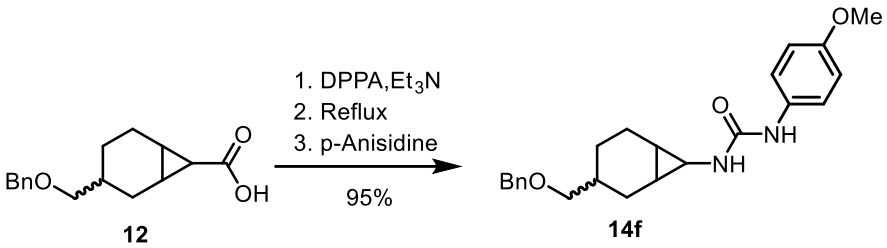
DCM/MeOH (40:1) to give **14c** (261 mg, 69% yield, dr = 1.5:1) as a yellow oil. Major diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.24 (m, 5H), 4.74 (br s, 1H, NH), 4.59 (br s, 1H, NH), 4.50 (s, 2H), 4.48 (s, 2H), 3.33 – 3.17 (m, 2H), 2.32 – 2.13 (m, 1H), 2.08 – 1.93 (m, 2H), 1.85 – 1.69 (m, 1H), 1.65 – 1.56 (m, 1H), 1.53 – 1.43 (m, 2H), 1.37 (s, 9H), 1.17 – 1.03 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.4, 138.5, 128.3, 127.5, 127.5, 75.3, 73.1, 50.2, 34.7, 34.3, 29.5, 26.6, 25.7, 22.1, 20.8, 19.7. Minor diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.24 (m, 5H), 4.74 (br s, 1H, NH), 4.59 (br s, 1H, NH), 4.48 (s, 2H), 3.33 – 3.17 (m, 2H), 2.32 – 2.13 (m, 1H), 2.08 – 1.93 (m, 2H), 1.85 – 1.69 (m, 1H), 1.65 – 1.56 (m, 1H), 1.53 – 1.43 (m, 2H), 1.37 (s, 9H), 1.17 – 1.03 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.4, 138.5, 128.3, 127.5, 127.5, 75.2, 73.0, 50.2, 34.0, 32.9, 29.5, 25.4, 24.3, 21.8, 20.7, 19.3. HRMS (ESI) *m/z* calcd for $C_{20}H_{31}N_2O_2$ $[M+H]^+$ 331.2380, found 331.2375.



1-(3-((benzyloxy)methyl)bicyclo[4.1.0]heptan-7-yl)-3-cyclohexylurea (14d): To a solution of 12 (300 mg, 1.15 mmol) in toluene (3 mL) was sequentially added diphenylphosphene azide (0.34 mL, 1.49 mmol) and Et_3N (0.25 mL, 1.72 mmol). The mixture was stirred at room temperature for 0.5 h and then refluxed at 90°C for 4 h. After cooling to 0 ℃, Cyclohexylamine (0.30 ml, 2.30 mmol) and toluene (6 ml) was added and stirred at r.t. overnight. The solvent was removed, and the residue was purified by chromatography on silica gel with a gradient of DCM/MeOH (40:1) to give **14d** (165 mg, 39% yield, dr = 1.5:1) as a pink oil. Major diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.24 (m, 5H), 4.96 (br s, 1H, NH), 4.81 (br s, 1H, NH), 4.48 (s, 2H), 3.68 – 3.54 (m, 1H), 3.30 – 3.15 (m, 2H), 2.32 – 2.08 (m, 1H), 2.07 – 1.86 (m, 4H), 1.72 – 1.55 (m, 4H), 1.49 – 1.29 (m, 5H), 1.25 – 1.00 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.6, 138.5, 128.3, 127.5, 127.5, 75.3, 73.0, 48.4, 34.5, 34.1, 33.8, 26.7, 25.7, 24.8, 24.3, 22.1, 20.6, 19.5. Minor diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.24 (m, 5H), 4.96 (br s, 1H, NH), 4.81 (br s, 1H, NH), 4.47 (s, 2H), 3.68 – 3.54 (m, 1H), 3.30 – 3.15 (m, 2H), 2.32 – 2.08 (m, 1H), 2.07 – 1.86 (m, 4H), 1.72 – 1.55 (m, 4H), 1.49 – 1.29 (m, 5H), 1.25 – 1.00 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.6, 138.5, 128.3, 127.5, 127.5, 75.3, 73.0, 48.4, 34.0, 33.8, 32.9, 25.7, 25.4, 24.7, 24.3, 21.9, 20.6, 19.1. HRMS (ESI) *m/z* calcd for $C_{22}H_{33}N_2O_2$ $[M+H]^+$ 357.2537, found 357.2536.

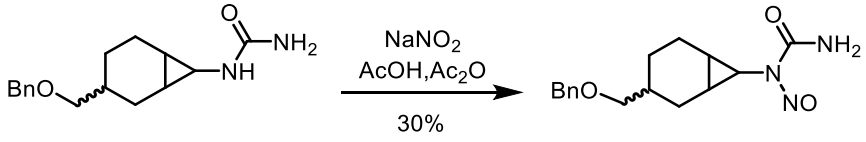


1-(3-((benzyloxy)methyl)bicyclo[4.1.0]heptan-7-yl)-3-phenylurea (14e): To a solution of 12 (200 mg, 0.77 mmol) in toluene (1.8 mL) was sequentially added diphenylphosphene azide (0.22 mL, 1.00 mmol) and Et_3N (0.16 mL, 1.15 mmol). The mixture was stirred at room temperature for 0.5 h and then refluxed at 90°C for 4 h. After cooling to 0 ℃, Aniline (0.14 mL, 1.54 mmol) and toluene (4 mL) was added and stirred at r.t. overnight. The solvent was removed, and the residue was purified by chromatography on silica gel with a gradient of DCM/MeOH (60:1) to give **14e** (120 mg, 48% yield, dr = 1.5:1) as a brown oil. Major diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 – 7.25 (m, 9H), 7.04 (t, *J* = 7.3 Hz, 1H), 5.53 (br s, 1H, NH), 4.57 – 4.43 (m, 2H), 3.33 – 3.13 (m, 2H), 2.34 – 2.01 (m, 3H), 1.89 – 1.69 (m, 1H), 1.65 – 1.26 (m, 4H), 1.22 – 1.02 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.9, 138.9, 138.6, 129.0, 128.4, 127.6, 127.6, 123.0, 119.6, 75.4, 73.1, 34.5, 34.1, 26.7, 25.6, 22.1, 20.4, 19.3. Minor diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 – 7.25 (m, 9H), 7.04 (t, *J* = 7.3 Hz, 1H), 5.53 (br s, 1H, NH), 4.57 – 4.43 (m, 2H), 3.33 – 3.13 (m, 2H), 2.34 – 2.01 (m, 3H), 1.89 – 1.69 (m, 1H), 1.65 – 1.26 (m, 4H), 1.22 – 1.02 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.9, 138.9, 138.6, 129.0, 128.4, 127.6, 127.6, 123.0, 119.6, 75.3, 73.1, 34.0, 32.9, 25.5, 24.3, 21.9, 20.4, 19.0. HRMS (ESI) *m/z* calcd for $C_{22}H_{27}N_2O_2$ $[M+H]^+$ 351.2067, found 351.2066.



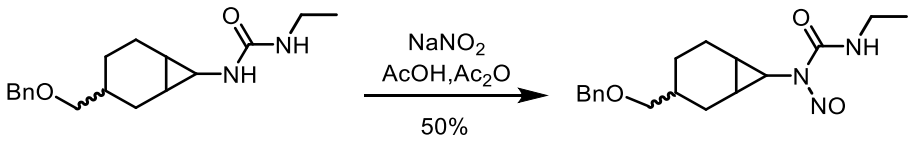
1-(3-((benzyloxy)methyl)bicyclo[4.1.0]heptan-7-yl)-3-(4-methoxyphenyl)urea (14f): To a solution of 12 (200 mg, 0.77 mmol) in toluene (1.8 mL) was sequentially added diphenylphosphene azide (0.22 mL, 1.00 mmol) and Et_3N (0.16 mL, 1.15 mmol). The mixture was stirred at room temperature for 0.5 h and then refluxed at 90 ℃ for 4 h. After cooling to 0 ℃, p-Anisidine (190 mg, 1.54 mmol) was added and stirred at r.t. overnight. The solvent was removed, and the residue was purified by chromatography on silica gel with a gradient of DCM/MeOH (60:1) to give **14f** (278 mg, 95% yield, dr = 1.5:1) as a brown oil. Major diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.22 (m, 7H), 6.97 (br s, 1H, NH), 6.83 (d, *J* = 8.9 Hz, 2H), 5.25 (br s, 1H, NH), 4.50 (s, 2H), 3.78 (s, 3H), 3.32 – 3.15 (m, 2H), 2.33 –

2.12 (m, 2H), 2.10 – 2.02 (m, 1H), 1.86 – 1.71 (m, 1H), 1.63 – 1.32 (m, 3H), 1.20 – 1.05 (m, 2H), 0.99 – 0.85 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.2, 156.0, 138.6, 131.7, 128.4, 127.5, 127.5, 122.3, 114.2, 75.4, 73.1, 55.5, 34.5, 32.9, 26.7, 25.7, 22.1, 20.5, 19.4. Minor diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.22 (m, 7H), 6.97 (br s, 1H, NH), 6.83 (d, *J* = 8.9 Hz, 2H), 5.25 (br s, 1H, NH), 4.49 (s, 2H), 3.78 (s, 3H), 3.32 – 3.15 (m, 2H), 2.33 – 2.12 (m, 2H), 2.10 – 2.02 (m, 1H), 1.86 – 1.71 (m, 1H), 1.63 – 1.32 (m, 3H), 1.20 – 1.05 (m, 2H), 0.99 – 0.85 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ157.1, 156.0, 138.6, 131.7, 128.4, 127.5, 127.5, 122.3, 114.2, 75.3, 73.1, 55.5, 34.1, 32.9, 25.5, 24.3, 21.9, 20.5, 19.0. HRMS (ESI) *m/z* calcd for $C_{23}H_{29}N_2O_2$ $[M+H]^+$ 381.2173, found 381.2173.



14a **15a**

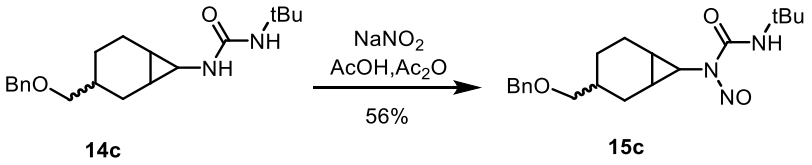
1-(3-((benzyloxy)methyl)bicyclo[4.1.0]heptan-7-yl)-1-nitrosourea (15a): To a flask charged with **14a** (127 mg, 0.463 mmol) and AcOH-Ac_2O (2:1 ratio, 3.3 mL) was added a solution of $NaNO_2$ (48 mg, 0.695 mmol) in 1.5 mL water through a syringe pump. After stirring at 0 ℃ for 40 min, 7.3 mL of ice water was added, and the resulting mixture was stirred for an additional 30 min. A yellow precipitate formed during this period. The solution was extracted with DCM, and then purified by chromatography (DCM-MeOH 80:1) to yield **15a** (42.1 mg, 30%, dr = 1.5:1) as a yellow oil. Major diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.21 (m, 5H), 6.71 (br s, 1H, NH), 5.55 (br s, 1H, NH), 4.47 (s, 2H), 3.32 – 3.12 (m, 2H), 2.34 – 2.09 (m, 2H), 2.00 – 1.93 (m, 1H), 1.85 – 1.58 (m, 2H), 1.55 – 1.05 (m, 5H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.7, 138.6, 128.4, 127.5, 127.5, 75.4, 73.1, 34.1, 34.0, 26.0, 25.4, 21.7, 20.9, 19.7. Minor diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.21 (m, 5H), 6.71 (br s, 1H, NH), 5.55 (br s, 1H, NH), 4.48 (s, 2H), 3.32 – 3.12 (m, 2H), 2.34 – 2.09 (m, 2H), 2.00 – 1.93 (m, 1H), 1.85 – 1.58 (m, 2H), 1.55 – 1.05 (m, 5H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.7, 138.5, 128.4, 127.5, 127.5, 75.3, 73.1, 33.6, 32.5, 25.0, 26.1, 21.3, 20.8, 19.3. HRMS (ESI) *m/z* calcd for $C_{16}H_{20}N_3O_3$ $[M-H]^-$ 302.1510, found 302.1509.



14b **15b**

1-(3-((benzyloxy)methyl)bicyclo[4.1.0]heptan-7-yl)-3-ethyl-1-nitrosourea (15b): To a flask

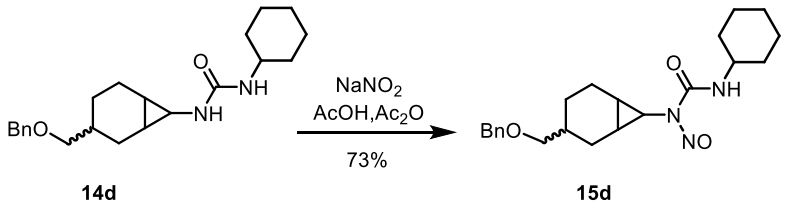
charged with **14b** (228 mg, 0.754 mmol) and AcOH-Ac_2O (2:1 ratio, 5.4 mL) was added a solution of $NaNO_2$ (78 mg, 1.13 mmol) in 2.4 mL water through a syringe pump. After stirring at 0 ℃ for 40 min, 12 mL of ice water was added, and the resulting mixture was stirred for an additional 30 min. A yellow precipitate formed during this period. The solution was extracted with DCM, and then purified by chromatography (DCM-MeOH 80:1) to yield **15b** (159.9 mg, 64%, dr = 1.5:1) as a yellow oil. Major diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 – 7.25 (m, 5H), 6.76 (br s, 1H, NH), 4.47 (s, 2H), 3.53 – 3.43 (m, 2H), 3.33 – 3.15 (m, 2H), 2.32 – 2.09 (m, 2H), 2.01 – 1.96 (m, 1H), 1.80 – 1.39 (m, 5H), 1.26 (t, *J* = 7.2 Hz, 3H), 1.21 – 1.16 (m, 1H), 1.14 – 1.06 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 153.5, 138.6, 128.3, 127.5, 127.5, 75.5, 73.1, 35.6, 34.4, 33.9, 26.0, 25.1, 21.7, 20.9, 19.7, 14.9. Minor diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 – 7.25 (m, 5H), 6.76 (br s, 1H, NH), 4.47 (s, 2H), 3.53 – 3.43 (m, 2H), 3.33 – 3.15 (m, 2H), 2.32 – 2.09 (m, 2H), 2.01 – 1.96 (m, 1H), 1.80 – 1.39 (m, 5H), 1.26 (t, *J* = 7.2 Hz, 3H), 1.21 – 1.16 (m, 1H), 1.14 – 1.06 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 153.5, 138.6, 128.3, 127.5, 127.5, 75.3, 73.1, 34.2, 32.5, 29.7, 25.4, 23.8, 21.3, 20.8, 19.3, 14.9. HRMS (ESI) *m/z* calcd for $C_{18}H_{26}N_3O_3$ $[M+H]^+$ 332.1969, found 332.1968.



14c 15c

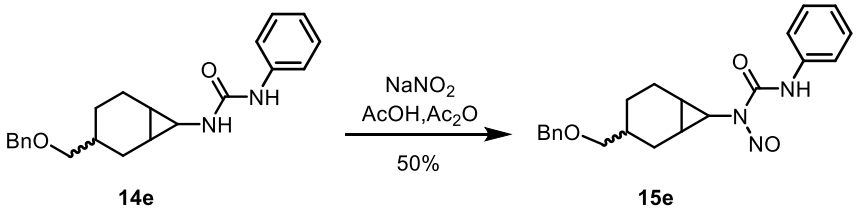
1-(3-((benzyloxy)methyl)bicyclo[4.1.0]heptan-7-yl)-3-(tert-butyl)-1-nitrosourea (15c): To a flask charged with **14c** (126 mg, 0.381 mmol) and AcOH-Ac_2O (2:1 ratio, 2.7 mL) was added a solution of $NaNO_2$ (39 mg, 0.565 mmol) in 1.2 mL water through a syringe pump. After stirring at 0 ℃ for 40 min, 6 mL of ice water was added, and the resulting mixture was stirred for an additional 30 min. A yellow precipitate formed during this period. The solution was extracted with DCM, and then purified by chromatography (DCM-MeOH 80:1) to yield **15c** (76.7 mg, 56%, dr = 1.5:1) as a yellow oil. Major diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 – 7.28 (m, 5H), 6.76 (br s, 1H, NH), 4.47 (s, 2H), 3.35 – 3.14 (m, 2H), 2.28 – 2.11 (m, 2H), 2.09 – 2.01 (m, 2H), 1.97 – 1.89 (m, 1H), 1.80 – 1.57 (m, 2H), 1.56 – 1.49 (m, 1H), 1.45 (s, 9H), 1.20 – 1.09 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 151.8, 138.6, 128.3, 127.5, 127.5, 75.5, 73.1, 51.9, 34.2, 33.6, 28.9, 26.0, 25.5, 21.7, 20.8, 19.6. Minor diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 – 7.28 (m, 5H), 6.76 (br s, 1H, NH), 4.47 (s, 2H), 3.35 – 3.14 (m, 2H), 2.28 – 2.11 (m, 2H), 2.09 – 2.01 (m, 2H), 1.97 – 1.89 (m, 1H), 1.80 – 1.57 (m, 2H), 1.56 – 1.49 (m, 1H), 1.45 (s, 9H), 1.20 – 1.09 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 151.8, 138.6, 128.3, 127.5, 127.5, 75.3, 73.0, 51.9, 34.1, 32.5, 28.9, 25.2, 23.8, 21.3, 20.8, 19.2. HRMS (ESI)

m/z calcd for $C_{20}H_{30}N_3O_3$ $[M+H]^+$ 360.2282, found 360.2282.



14d **15d**

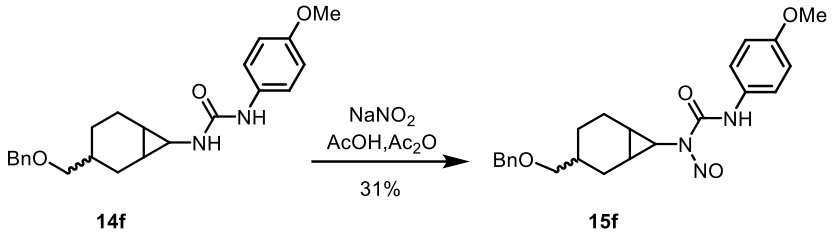
1-(3-((benzyloxy)methyl)bicyclo[4.1.0]heptan-7-yl)-3-cyclohexyl-1-nitrosourea (15d): To a flask charged with **14d** (165 mg, 0.545 mmol) and AcOH-Ac_2O (2:1 ratio, 3.0 mL) was added a solution of $NaNO_2$ (48 mg, 0.695 mmol) in 1.5 mL water through a syringe pump. After stirring at 0 ℃ for 40 min, 7.3 mL of ice water was added, and the resulting mixture was stirred for an additional 30 min. A yellow precipitate formed during this period. The solution was extracted with DCM, and then purified by chromatography (DCM-MeOH 80:1) to yield **15d** (131.8 mg, 73%, dr = 1.5:1) as a yellow oil. Major diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.22 (m, 5H), 6.68 (br s, 1H, NH), 4.47 (s, 2H), 3.91 – 3.78 (m, 1H), 3.36 – 3.14 (m, 2H), 2.33 – 2.10 (m, 2H), 2.08 – 1.86 (m, 3H), 1.77 – 1.58 (m, 4H), 1.56 – 1.15 (m, 8H), 1.15 – 1.06 (m, 1H), 1.04 – 0.80 (m, 1H), 0.72 – 0.57 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 152. 7, 138.6, 128.3, 127.5, 127.5, 75.5, 73.1, 49.9, 34.4, 33.9, 33.1, 26.0, 25.4, 24.8, 23.8, 21.7, 20.9, 19.7. Minor diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.22 (m, 5H), 6.68 (br s, 1H, NH), 4.47 (s, 2H), 3.91 – 3.78 (m, 1H), 3.36 – 3.14 (m, 2H), 2.33 – 2.10 (m, 2H), 2.08 – 1.86 (m, 3H), 1.77 – 1.58 (m, 4H), 1.56 – 1.15 (m, 8H), 1.15 – 1.06 (m, 1H), 1.04 – 0.80 (m, 1H), 0.72 – 0.57 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 152.7, 138.6, 128.3, 127.5, 127.5, 75.3, 73.0, 49.9, 34.2, 33.1, 32.5, 25.4, 25.1, 24.8, 23.8, 21.3, 20.8, 19.3. HRMS (ESI) *m/z* calcd for $C_{22}H_{32}N_3O_3$ $[M+H]^+$ 386.2438, found 386.2436.



14e **15e**

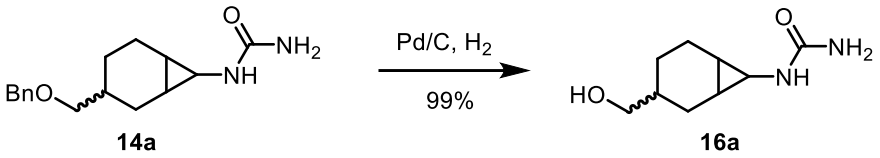
1-(3-((benzyloxy)methyl)bicyclo[4.1.0]heptan-7-yl)-1-nitroso-3-phenylurea (15e): To a flask charged with **14e** (120 mg, 0.342 mmol) and AcOH-Ac_2O (2:1 ratio, 2.4 mL) was added a solution of $NaNO_2$ (35 mg, 0.507 mmol) in 1.1 mL water through a syringe pump. After stirring at 0 ℃ for 40 min, 5.4 mL of ice water was added, and the resulting mixture was stirred for an additional 30 min. A yellow precipitate formed during this period. The solution was

extracted with DCM, and then purified by chromatography (DCM-MeOH 80:1) to yield **15e** (64.9 mg, 50%, dr = 1.5:1) as a yellow oil. Major diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.76 (br s, 1H, NH), 7.66 – 7.54 (m, 1H), 7.44 – 7.26 (m, 8H), 7.23 – 7.07 (m, 1H), 4.52 (s, 2H), 3.45 – 3.06 (m, 2H), 2.37 – 2.15 (m, 2H), 2.12 – 2.03 (m, 1H), 1.86 – 1.64 (m, 2H), 1.61 – 1.38 (m, 2H), 1.34 – 1.15 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 150.9, 138.6, 136.8, 129.2, 128.4, 127.6, 124.8, 120.0, 75.4, 73.1, 34.2, 33.8, 26.0, 25.2, 21.7, 21.3, 19.9. Minor diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.76 (br s, 1H, NH), 7.66 – 7.54 (m, 1H), 7.44 – 7.26 (m, 8H), 7.23 – 7.07 (m, 1H), 4.52 (s, 2H), 3.45 – 3.06 (m, 2H), 2.37 – 2.15 (m, 2H), 2.12 – 2.03 (m, 1H), 1.86 – 1.64 (m, 2H), 1.61 – 1.38 (m, 2H), 1.34 – 1.15 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 150.9, 138.6, 136.8, 129.2, 128.4, 127.6, 124.8, 120.0, 75.3, 73.1, 34.2, 32.5, 25.4, 23.8, 21.7, 21.1, 19.5. HRMS (ESI) *m/z* calcd for $C_{22}H_{24}N_3O_3$ $[M-H]^-$ 378.1823, found 378.1822.

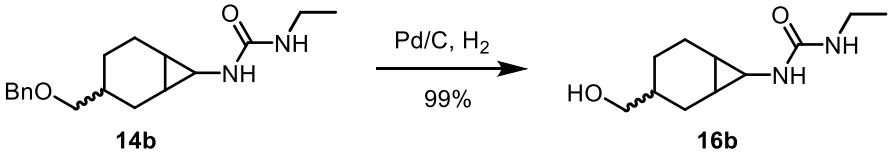


14f 15f

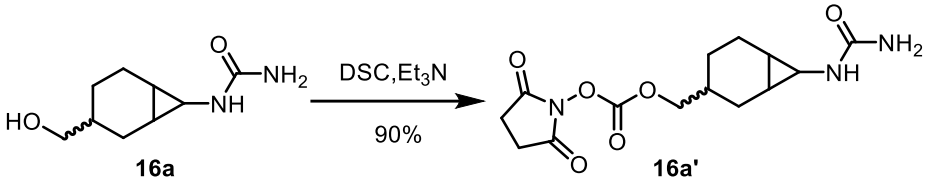
1-(3-((benzyloxy)methyl)bicyclo[4.1.0]heptan-7-yl)-3-(4-methoxyphenyl)-1-nitrosourea (15f): To a flask charged with **14f** (799 mg, 2.10 mmol) and AcOH-Ac_2O (2:1 ratio, 15 mL) was added a solution of $NaNO_2$ (217 mg, 3.145 mmol) in 6.7 mL water through a syringe pump. After stirring at 0 ℃ for 40 min, 33.2 mL of ice water was added, and the resulting mixture was stirred for an additional 30 min. A yellow precipitate formed during this period. The solution was extracted with DCM, and then purified by chromatography (DCM-MeOH 80:1) to yield **15f** (266.9 mg, 31%, dr = 1.5:1) as a yellow oil. Major diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.67 (br s, 1H, NH), 7.64 – 6.75 (m, 9H), 4.51 (s, 2H), 3.83 (s, 3H), 3.48 – 3.16 (m, 2H), 2.35 – 2.11 (m, 2H), 2.07 – 1.64 (m, 4H), 1.61 – 1.34 (m, 2H), 1.34 – 1.14 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ156.8, 151.2, 138.6, 129.8, 128.4, 127.6, 125.6, 122.1, 114.4, 75.4, 73.1, 55.5, 34.2, 32.5, 26.0, 25.3, 24.6, 21.3, 19.8. Minor diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.67 (br s, 1H, NH), 7.64 – 6.75 (m, 9H), 4.51 (s, 2H), 3.83 (s, 3H), 3.48 – 3.16 (m, 2H), 2.35 – 2.11 (m, 2H), 2.07 – 1.64 (m, 4H), 1.61 – 1.34 (m, 2H), 1.34 – 1.14 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ156.8, 151.2, 138.6, 129.8, 128.4, 127.6, 125.6, 122.1, 114.4, 75.3, 73.1, 55.5, 33.8, 32.5, 25.4, 23.8, 21.7, 21.0, 19.4. HRMS (ESI) *m/z* calcd for $C_{23}H_{26}N_3O_3$ $[M-H]^-$ 408.1929, found 408.1925.



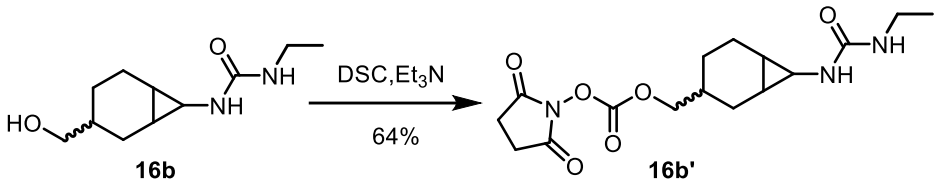
1-(3-(Hydroxymethyl)bicyclo[4.1.0]heptan-7-yl)urea (16a): To a solution of **14a** (2.094 g, 7.63 mmol) in MeOH (30 mL) was added palladium on carbon (209.7 mg), and the mixture was stirred under H_2 atmosphere overnight. The mixture was filtered and evaporated to dryness. The crude product was separated on silica gel (DCM-MeOH 20:1) to yield **16a** (1.405 g, 99%, dr= 1.5:1) as a white solid. ^{1}H NMR (400 MHz, MeOD) δ 5.49 (brs, 1H, NH), 4.61 (br s, 2H, NH2), 3.39 – 3.34 (m, 1H), 3.28 – 3.24 (m, 1H), 2.24 – 2.16 (m, 1H), 2.12 – 1.97 (m, 2H), 1.80 – 1.69 (m, 1H), 1.59 – 1.51 (m, 2H), 1.47 – 1.33 (m, 2H), 1.19 – 1.11 (m, 2H), 1.09 – 1.05 (m, 1H). ^{13}C NMR (100 MHz, MeOD) δ 163.3, 68.2, 37.7, 36.4, 35.6, 27.4, 26.4, 25.0, 23.2 (major isomer); 163.3, 68.1, 37.7, 36.4, 35.1, 27.4, 26.4, 25.0, 23.1 (minor isomer). HRMS (ESI) *m/z* calcd for $C_9H_{17}N_2O_2$ [M+H]+ 185.1290, found 185.1286.



1-ethyl-3-(3-(hydroxymethyl)bicyclo[4.1.0]heptan-7-yl)urea (16b): To a solution of **14b** (493 mg, 1.63 mmol) in MeOH (4.5 mL) was added palladium on carbon (45 mg), and the mixture was stirred under H_2 atmosphere overnight. The mixture was filtered and evaporated to dryness. The crude product was separated on silica gel (DCM-MeOH 20:1) to yield **16b** (342.6 mg, 99%, dr= 1.5:1) as a colorless-oil. Major diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.79 (br s, 1H, NH), 4.76 (br s, 1H, NH), 3.48 – 3.33 (m, 2H), 3.32 – 3.23 (m, 2H), 2.28 – 2.10 (m, 2H), 2.09 – 1.97 (m, 2H), 1.86 – 1.72 (m, 1H), 1.63 – 1.54 (m, 1H), 1.55 – 1.28 (m, 2H), 1.16 (t, *J* = 7.2 Hz, 3H), 1.14 – 1.07 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.2, 67.7, 36.2, 35.2, 34.4, 26.1, 25.3, 22.0, 20.7, 19.5, 15.6. Minor diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.79 (br s, 1H, NH), 4.76 (br s, 1H, NH), 3.48 – 3.33 (m, 2H), 3.32 – 3.23 (m, 2H), 2.28 – 2.10 (m, 2H), 2.09 – 1.97 (m, 2H), 1.86 – 1.72 (m, 1H), 1.63 – 1.54 (m, 1H), 1.55 – 1.28 (m, 2H), 1.16 (t, *J* = 7.2 Hz, 3H), 1.14 – 1.07 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.2, 67.7, 36.2, 35.1, 34.0, 25.0, 23.8, 22.0, 20.7, 19.1, 15.6. HRMS (ESI) *m/z* calcd for $C_{11}H_{21}N_2O_2$ $[M+H]^+$ 213.1598, found 213.1599.

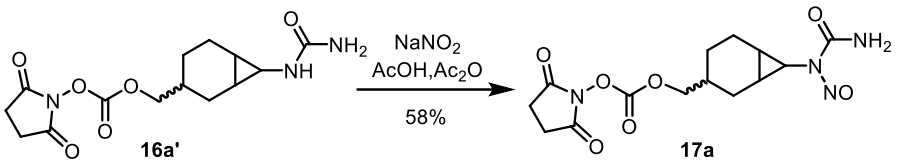


2,5-Dioxopyrrolidin-1-yl ((7-ureidobicyclo[4.1.0]heptan-3-yl)methyl) carbonate (16a'): To a solution of **16a** (144.7 mg, 0.79 mmol) and Et_3N (0.33 mL, 2.36 mmol) in 10 mL anhydrous acetonitrile at room temperature was added N,N'-disuccinimidyl carbonate (DSC) (221.5 mg, 0.864 mmol) in small portions, and the mixture was stirred under nitrogen for 15 h. The reaction mixture was concentrated under reduced pressure, and the residue was purified by chromatography (DCM-MeOH 10:1) to give **16a'** (231.0 mg, 90% yield, dr= 1.5:1) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.60 (brs, 1H, NH), 5.23 (brs, 2H, NH2), 4.11 – 3.94 (m, 2H), 2.75 (s, 4H), 2.31 – 1.90 (m, 3H), 1.85 – 1.61 (m, 1H), 1.60 – 1.31 (m, 3H), 1.22 – 0.98 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.2, 160.7, 151.6, 75.4, 34.7, 33.1, 31.9, 25.8, 25.5, 24.7, 23.4, 21.6 (major isomer); 169.0, 160.7, 151.6, 75.3, 34.3, 33.1, 31.9, 25.8, 25.5, 24.5, 23.4, 21.5 (minor isomer). HRMS (ESI) *m/z* calcd for $C_{14}H_{20}N_3O_6$ $[M+H]^+$ 326.1352, found 326.1343.

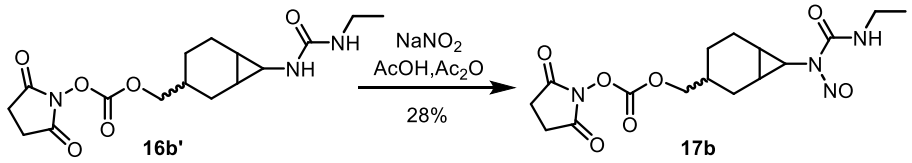


2,5-dioxopyrrolidin-1-yl ((7-(3-ethylureido)bicyclo[4.1.0]heptan-3-yl)methyl) carbonate (16b'): To a solution of **16b** (300 mg, 1.41 mmol) and Et_3N (0.60 mL, 4.28 mmol) in 18 mL anhydrous acetonitrile at room temperature was added N,N'-disuccinimidyl carbonate (DSC) (395 mg, 1.55 mmol) in small portions, and the mixture was stirred under nitrogen overnight. The reaction mixture was concentrated under reduced pressure, and the residue was purified by chromatography (DCM-MeOH 30:1) to give **16b'** (318.9 mg, 64% yield, dr= 1.5:1) as a white-solid. Major diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.75 (br s, 1H, NH), 4.74 (br s, 1H, NH), 4.20 – 4.03 (m, 2H), 3.30 – 3.19 (m, 2H), 2.85 (s, 4H), 2.34 – 2.16 (m, 1H), 2.13 – 1.98 (m, 2H), 1.88 – 1.75 (m, 1H), 1.69 – 1.43 (m, 3H), 1.17 (t, *J* = 7.2 Hz, 3H), 1.15 – 1.09 (m, 2H), 1.05 – 0.95 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.7, 158.9, 151.6, 75.3, 35.1, 34.4, 33.1, 25.9, 25.5, 24.8, 21.7, 20.3, 19.1, 15.7. Minor diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.75 (br s, 1H, NH), 4.74 (br s, 1H, NH), 4.20 – 4.03 (m, 2H), 3.30 – 3.19 (m, 2H), 2.85 (s, 4H), 2.34 – 2.16 (m, 1H), 2.13 – 1.98 (m, 2H), 1.88 – 1.75 (m, 1H), 1.69 – 1.43 (m,

3H), 1.17 (t, J = 7.2 Hz, 3H), 1.15 – 1.09 (m, 2H), 1.05 – 0.95 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.7, 158.9, 151.6, 75.2, 35.1, 34.0, 32.0, 25.5, 24.5, 23.5, 21.5, 20.0, 18.6, 15.7. HRMS (ESI) *m/z* calcd for $C_{16}H_{24}N_3O_6$ $[M+H]^+$ 354.1660, found 354.1660.



2,5-Dioxopyrrolidin-1-yl((7-(1-nitrosoureido)bicyclo[4.1.0]heptan-3-yl)methyl) carbonate (17a): To a flask charged with **16a'** (205.2 mg, 0.631 mmol) and AcOH-Ac_2O (2:1 ratio, 4.5 mL) was added a solution of $NaNO_2$ (43.5 mg, 0.631 mmol) in 2 mL water through a syringe pump. After stirring at 0 ℃ for 40 min, 10 mL of ice water was added, and the resulting mixture was stirred for an additional 30 min. A yellow precipitate formed during this period. The solution was extracted with DCM, and then purified by chromatography (DCM-MeOH 60:1) to yield **17a** (129.4 mg, 58%, dr = 1.5:1) as a yellow powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.76 (brs, 1H, NH2), 5.39 (brs, 1H, NH2), 4.21 – 4.01 (m, 2H), 2.84 (s, 4H), 2.31 – 2.19 (m, 2H), 2.01 – 1.95 (m, 1H), 1.84 – 1.68 (m, 2H), 1.56 – 1.48 (m, 2H), 1.44 – 1.35 (m, 1H), 1.20 – 1.09 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.7, 154.5, 151.7, 75.4, 33.9, 33.2, 25.5, 25.2, 24.4, 21.4, 20.5, 19.3 (major isomer); 168.7, 154.5, 151.7, 75.3, 33.4, 31.7, 25.5, 24.6, 23.1, 21.0, 20.3, 18.8 (minor isomer). HRMS (ESI) *m/z* calcd for $C_{14}H_{19}N_4O_7$ $[M+H]^+$ 355.1254, found 355.1244.



2,5-dioxopyrrolidin-1-yl ((7-(3-ethyl-1-nitrosoureido)bicyclo[4.1.0]heptan-3-yl)methyl) carbonate (17b) : To a flask charged with **16b'** (106 mg, 0.300 mmol) and AcOH-Ac_2O (2:1 ratio, 2.1 mL) was added a solution of $NaNO_2$ (31 mg, 0.449 mmol) in 0.95 mL water through a syringe pump. After stirring at 0 ℃ for 40 min, 5 mL of ice water was added, and the resulting mixture was stirred for an additional 30 min. A yellow precipitate formed during this period. The solution was extracted with DCM, and then purified by chromatography (PE-acetone 10:1 to 3:1) to yield **17b** (32.1 mg, 28%, dr = 1.5:1) as a yellow oil. Major diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.75 (br s, 1H, NH), 4.13 – 3.91 (m, 2H), 3.47 – 3.34 (m, 2H), 2.77 (s,

4H), 2.28 – 2.10 (m, 2H), 1.98 – 1.89 (m, 1H), 1.80 – 1.41 (m, 6H), 1.19 (t, J = 7.0 Hz, 3H), 1.11 – 1.04 (m, 1H). Minor diastereoisomer: ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.7, 153.4, 151.6, 75.4, 35.6, 34.2, 33.1, 25.5, 25.1, 24.3, 21.3, 20.4, 19.2, 14.9. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.75 (br s, 1H, NH), 4.13 – 3.91 (m, 2H), 3.47 – 3.34 (m, 2H), 2.77 (s, 4H), 2.28 – 2.10 (m, 2H), 1.98 – 1.89 (m, 1H), 1.80 – 1.41 (m, 6H), 1.19 (t, J = 7.0 Hz, 3H), 1.11 – 1.04 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.7, 153.4, 151.6, 75.2, 35.6, 33.7, 31.6, 25.5, 24.5, 23.1, 20.8, 20.2, 18.7, 14.9. HRMS (ESI) *m/z* calcd for $C_{16}H_{23}N_4O_7$ $[M+H]^+$ 383.1561, found 383.1562.

3. Determination the Kinetics and Stability of BNU

3.1 Determination of the Stability of BNU 1 in the Presence of 2-Mercaptoethanol

To determine the shelf stability to biological nucleophiles, a mixture of **1** (5.0 mg, 1 eq) and 2-mercaptoethanol (17 mg, 8 eq) was dissolved in $CDCl_3$ (0.6 mL), and then the sample was monitored over time (at RT) by ^{1}H-NMR.[4] It was turned out that BNU **1** was rather stable in the presence of strong nucleophile such as 2-mercaptoethanol, with most of the starting material kept intact after days.

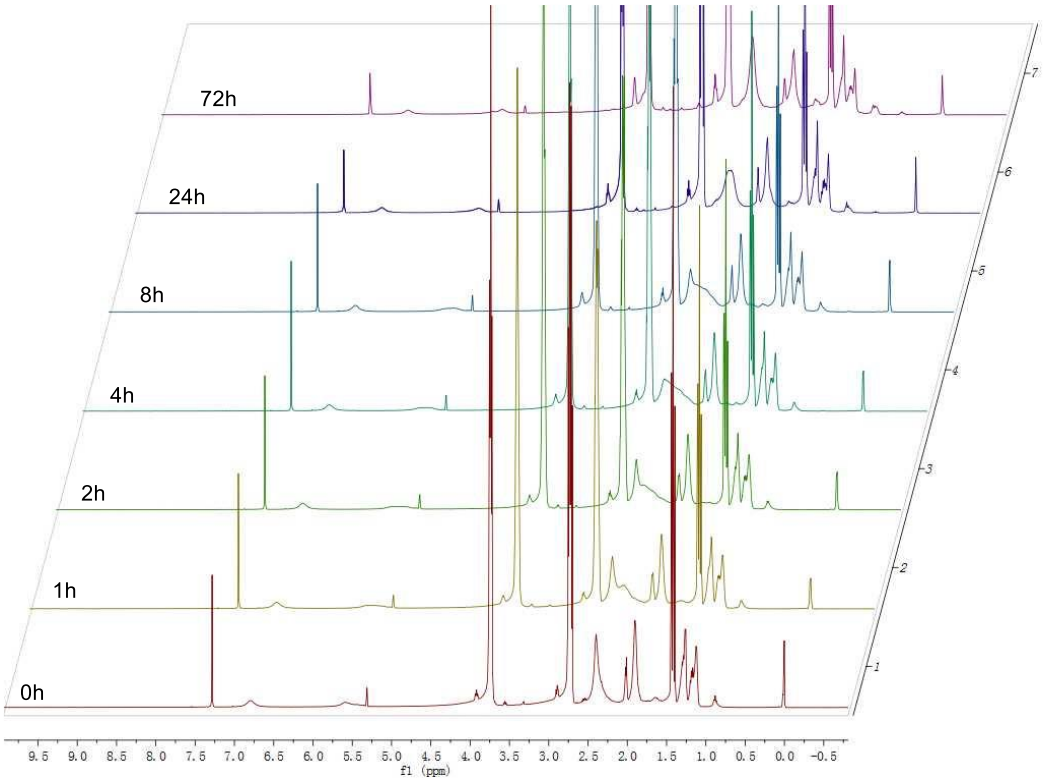


Figure S1. Stability of BNU **1** towards 2-mercaptoethanol.

3.2 Determination the Kinetics of Hydrolysis of 15a-f by UV-Vis Method (pH = 7.0)

The hydrolysis of BNU derivatives **15a-f** in pH = 7.0 were followed on a plate reader at 36.8°C, by measuring the absorbance decay of **15a-f** at indicated wavelength.[3] Sample stock solution of **15a-f** were prepared in very carefully dried methanol. The concentration of **15a-f** in the kinetic experiments was 0.2 mM. After mixing stock solution and buffer solution (0.2 M PBS buffer solution, pH = 7.0) together, the measurement was started directly. Absorbance measurements were recorded every 2 s over 10 min to 12 h time separately using a BioTek Epoch plate reader equipped with Gen5 software. All reactions were performed in triplo. The first-order rate constants were determined using Originlab 9.0 (Originlab Software, Inc).

Pseudo-First-Order Rate Constant

The pseudo first-order rate constants k_{obs} for BNU derivatives **15a-f** in pH = 7.0 was determined.

The decay of the absorbance of the **15a-f** was plotted against time (s) for pH = 7.0 of the buffer solutions. The k_{obs} was determined by fitting an exponential 'one phase decay' (nonlinear regression) using Originlab 9.0 whereby Y= (Y_0- plateau)*exp(-k_{obs}*time(s)) + plateau.

R / 36.8℃	$10^4 K_{obs}/s^{-1}$ (pH=7.0)	$t_{1/2}$ (pH=7.0)
15a (257nm)	63.1	1.8 min
15b (255nm)	6.90107	16.7 min
15c (275nm)	0.411197	4.6 h
15d (265nm)	0.591102	3.3 h
15e (348nm)	13.3	8.7 min
15f (348nm)	10.8	10.7 min

Figure S2. a) 0.2M of phosphate buffer; b) 0.2mM of nitrosourea derivatives; c) 1-5%volume ratio of nitrosourea derivatives solution to buffer solution d) all reactions were performed in triplo, $R^2 > 0.99$.

3.3 Determination the Kinetics of Hydrolysis of 15b with different pH (pH = 7.0, 7.4 and 8.0 under 36.8 ℃) and in the Presence of Biological Nucleophiles by UV-Vis Method

The hydrolysis of BNU derivative **15b** in pH = 7.0, 7.4, 8.0 were followed on a plate reader at 36.8°C, by measuring the absorbance decay of **15b** at 255nm in a similar protocol as **3.4**. To determine the shelf stability to biological nucleophiles, the hydrolysis of BNU derivative **15b** were followed on a plate reader at 36.8°C, by measuring the absorbance decay of **15b** at indicated wavelength in the Presence of Glycine, 2-Mercaptoethanol and glutathione separately in a similar protocol as **3.4**.

T / 36.8 °C	pH=7.0		pH=7.4		pH=8.0	
Substrate	**10^4 K_{obs}/s^{-1}**	**$T_{1/2}$/min**	**10^4 K_{obs}/s^{-1}**	**$T_{1/2}$/min**	**10^4 K_{obs}/s^{-1}**	**$T_{1/2}$/min**
15b	6.90107	16.7	17.7	6.5	64.3	1.8

Glycine	1 mM	7.30708	15.8	19.2	6.0	71.3	1.6
2-Mercaptoethanol	1 mM	7.6743	15.1	20.9	5.5	81.2	1.4
	10 mM	7.79479	14.8	21.5	5.4	81.7	1.4
GSH	1 mM	7.8137	14.8	22.2	5.2	83.6	1.4
	10 mM	7.89985	14.6	22.4	5.2	83.5	1.4

Figure S3. a) 0.2 M of phosphate buffer; b) 0.2 mM of **15b**; c) The volume of **15b** dissolved in methanol solution accounts for 1-5% of total volume of phosphate buffer; The volume of glycine, 2-Mercaptoethanol or GSH dissolved in water solution accounts for 0.4% of total volume of phosphate buffer; d) Measured by UV-vis at 255 nm.

4. Biological Assays

General materials for biological experiments: all biological reagents were purchased from Sigma-Aldrich, Tokyo Chemical Industry (TCI), Wako Pure Chemical Industries, Sasaki Chemical, Bio-Rad, Thermo Fisher Scientific, Nacalai Tesque or Watanabe Chemical Industries, and used without further purification, unless otherwise noted. FreeStyle™ 293-F cells were obtained from American Type Culture Collection (ATCC; Manassas, VA). FreeStyle™ 293 Expression Medium was obtained from Invitrogen. For cell culture volumes from 15–1200 mL, disposable polycarbonate erlenmeyer flasks from Corning (Tewksbury, MA) were used. MC-38 cells (mouse colon cancer cells) were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and cultured in Dulbecco's Modified Eagle's medium (DMEM) (Gbico) supplemented with 10% heat inactivated FBS (BioInd), 1% penicillin/streptomycin (Beyotime) at 37°C and 5% CO_2.

4.1 *In Vitro* Labeling of BNU-Modified BSA

Protein Modification

Bovine Serum Albumin (BSA) conjugates were prepared by treating the proteins with BNU

ester **17b**. In brief, BSA (500 µL of a 5 mg/mL solution in 0.2 M PBS buffer, pH = 7.4) was treated with **17b** (37 µL of a 100 mM solution in DMSO, 100 eq, added in 2 portions). Sample of BSA solution was incubated at 4°C (with shaking) for 2-3 h, modified protein sample was purified by centrifuge filtration (3×) using PBS (pH = 7.4) buffer solution, concentrated to 50 mg/mL. Then the BNU-modified BSA was subsequently reacted with **18** separately at 37°C.

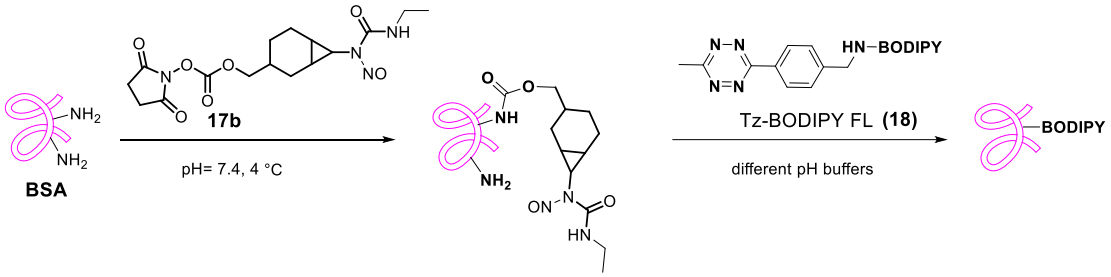


Figure S4. (A) Modification of free lysine residues on BSA (5 mg/mL) with **17b** (2.5 mg/mL) at pH = 7.4 and subsequent labeling with Tz-BODIPY FL (**18**).

BSA Labeling at Different pH Conditions

1 µL BNU-modified BSA samples (made in 0.2 M PBS buffer solution, pH = 7.4) were added to 49 µL 0.2 M PBS buffer solutions (pH = 6.0, 6.3, 6.7, 7.0, 7.4, 7.8) separately for a final concentration of the functionalized BSA (1 mg/mL, final pH = 6.0, 6.3, 6.7, 7.0, 7.4, 7.8 separately, monitored by pH measuring apparatus) samples. In parallel, 1 µL BSA samples (50 mg/mL, made in 0.2 M PBS buffer solution, pH = 7.4) were added 49 µL 0.2 M PBS buffer solution (pH = 7.4) for a final concentration of the blank control BSA (1 mg/mL, final pH=7.4, monitored by pH measuring apparatus) sample. Tetrazine **18** (1 µL, 10 mM in DMSO) was then added and the reaction sample was shaken for 5 min, then 3,6-dipyridyl-s-tetrazine **4** (1.8 µL, 84 mM in DMSO, 200 equiv) was added to stop the reaction. The mixture was shaken at 37°C for 1 h and centrifuged for 2 min at 13 000 rpm. The modified BSA samples were measured using a BCA protein assay kit (Pierce). Protein isolates were analyzed by gel electrophoresis using 12% polyacrylamide gels. Gels were analyzed by in-gel fluorescence measurements on a FluorChem® FC3 imager (Alpha Innotech). Fluorescence was measured with a blue light excitation wavelength (475 nm) and a green filter emission (537 nm). Total protein loading was confirmed by subsequent staining with Coomassie Brilliant Blue.

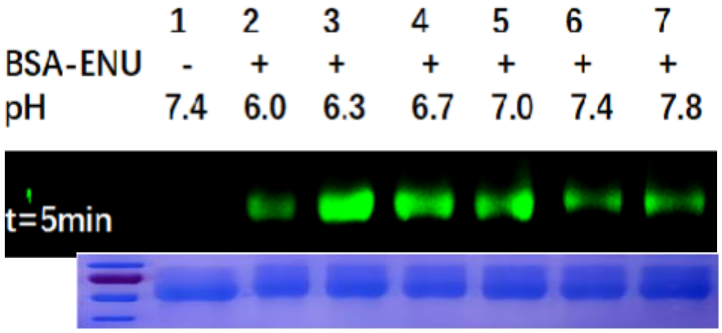


Figure S5. Gel analysis of BNU-modified BSA labeled at different pH buffers (pH = 6.0, 6.3, 6.7, 7.0, 7.4, 7.8) with Tz-BODIPY FL (**18**) (200 μM) at 37 ℃ for 5 min. Protein loading was assessed by Coomassie staining (lower panel).

BSA Labeling with Different Reaction Time

1 μL BNU-modified BSA samples (made in 0.2 M PBS buffer solution, pH=7.4) were added to 49 μL 0.2 M PBS buffer solutions (pH = 6.0, 6.3, 6.7, 7.0, 7.4, 7.8) separately for a final concentration of the functionalized BSA (1 mg/mL, final pH = 6.0, 6.3, 6.7, 7.0, 7.4, 7.8 separately, monitored by pH measuring apparatus) samples. In parallel, 1 μL BSA samples (50 mg/mL, made in 0.2 M PBS buffer solution, pH = 7.4) were added 49 μL 0.2 M PBS buffer solution (pH = 7.4) for a final concentration of the blank control BSA (1 mg/mL, final pH = 7.4, monitored by pH measuring apparatus) sample. Tetrazine **18** (1 μL, 10 mM in DMSO) was then added and the reaction sample was shaken for 10, 15, 35 min, then 3,6-dipyridyl-s-tetrazine **4** (1.8 μL, 84 mM in DMSO, 200 equiv) was added to stop the reaction. The mixture was shaken at 37°C for 1 h and centrifuged for 2 min at 13 000 rpm. The modified BSA samples were measured using a BCA protein assay kit (Pierce). Protein isolates were analyzed by gel electrophoresis using 12% polyacrylamide gels. Gels were analyzed by in-gel fluorescence measurements on a FluorChem® FC3 imager (Alpha Innotech). Fluorescence was measured with a blue light excitation wavelength (475 nm) and a green filter emission (537 nm). Total protein loading was confirmed by subsequent staining with Coomassie Brilliant Blue.

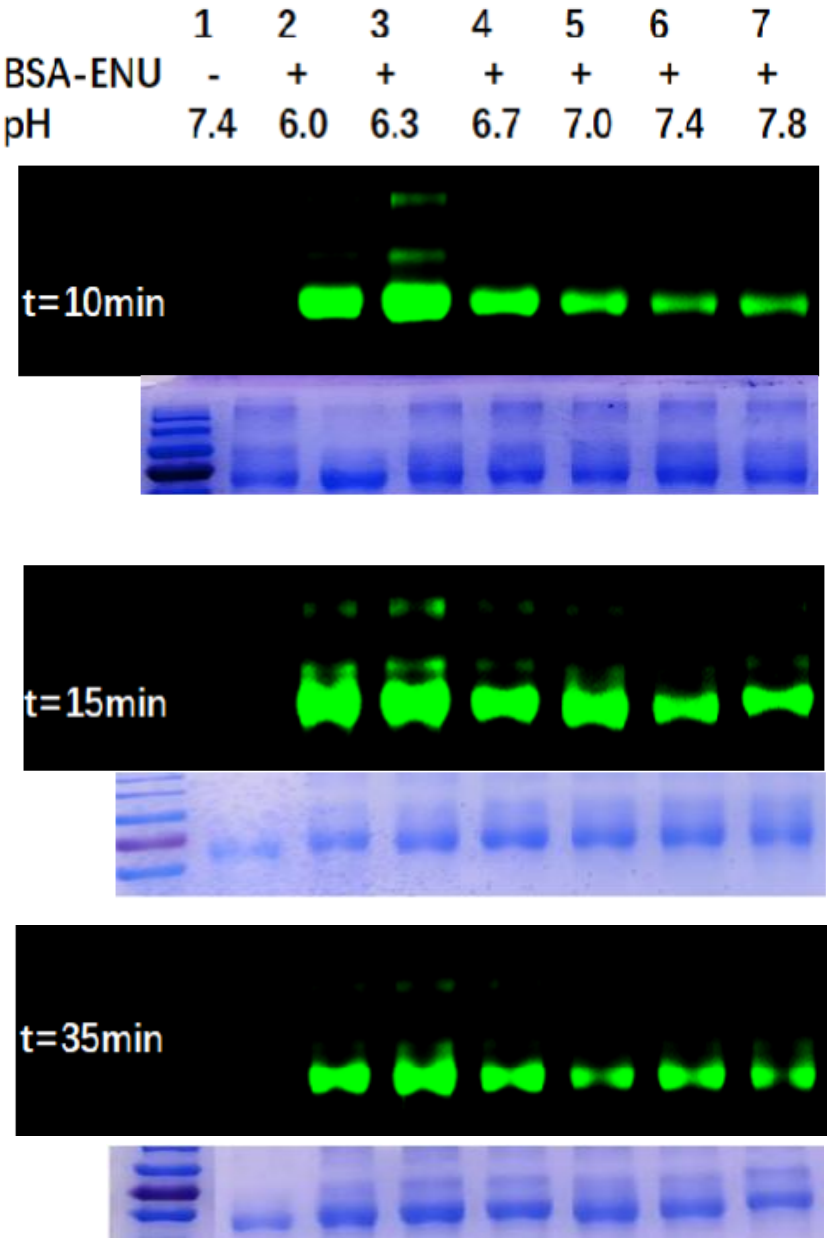


Figure S6. Gel analysis of BNU-modified BSA incubated with Tz-BODIPY FL (**18)** (200 μM) at 37°C for 10, 15, 35 min. Protein loading was assessed by Coomassie staining (lower panel).

Quantitative analysis of the protein fluorescence with the Image J software

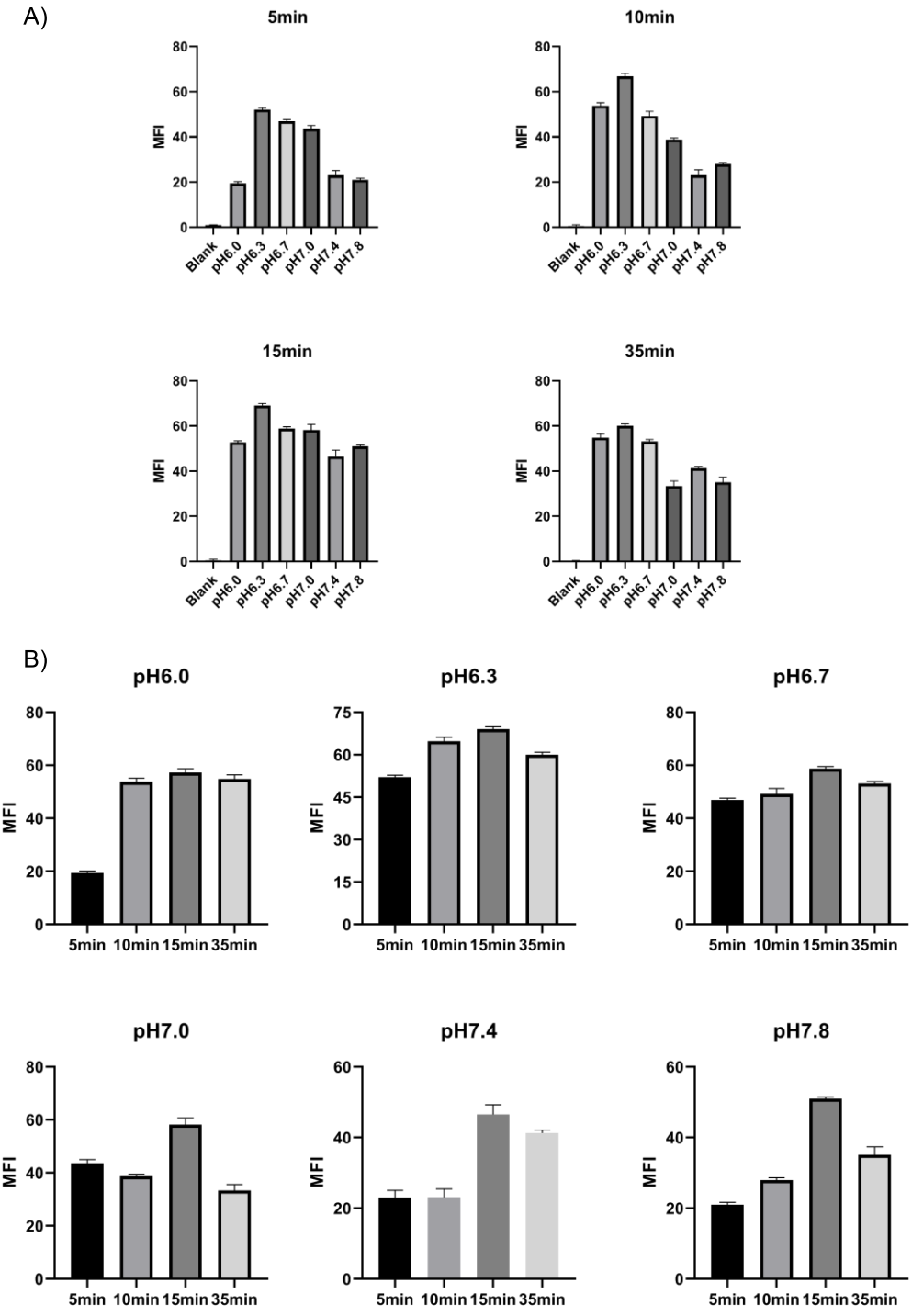


Figure S7. Quantitative analysis of protein labeling through TCH-mediated IEDDA reaction with the indicated pH and reaction time

4.2 Cell Labeling through TCH-mediated IEDDA Reaction

Expression, Purification and Characterization of Anti-PD-L1 Antibody (Avelumab)

The codon-optimized DNA for the heavy chain or light chain of anti-PD-L1 antibody (Avelumab) was synthesized by Genescript and cloned into the pFUSE-Fc vector6. HEK293F

cells were cultured in FreeStyle TM 293 Expression Medium (Gibco) under 5% CO_2 in an orbital shaker at 150 rpm at 37 ℃. When the cell density reached 1 × 10^6 cells/mL, the plasmid was transfected according to a protocol modified from Cold Spring Harbor Protocols. Briefly, 66 μL sterile 1 mg/mL 25-kDa linear polyethylenimines (PEIs) (Polysciences) was added to 3.3 mL PBS solution containing, 33 μg plasmids (22 μg heavy chain and 11 μg light chain). After votexing, the DNA/PEI mixture was incubated at room temperature for 30 min and then added to 27 mL cell culture in a 125 mL shake flask. Transfected cells were cultured for 4–6 days before harvesting. Antibody in the supernatant was purified with protein G (GE Healthcare Life Sciences) chromatography. The purity of antibody was examined by SDS-PAGE under reduced and non-reduced conditions.

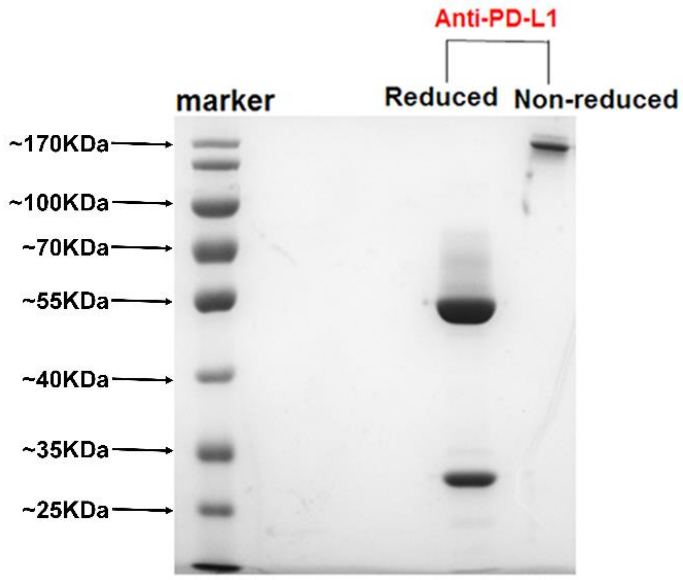


Figure S8. Characterization of Avelumab. The purity of antibody was examined by SDS-PAGE under reduced and non-reduced conditions.

Antibody Modification

Avelumab (1 mg/mL, 1.3 mL) in PBS (pH = 7.4) buffer solution was incubated with BNU derivative **17b** (100 mM, 8.7 μL) at 4 ℃ for 3 h. The conjugated antibody (Avel-BNU) was purified by centrifuge filtration (3×) using PBS buffer (pH = 7.4) solution, concentrated to 1 mg/mL. Control Avelumab was treated in an identical manner without **17b**.

Cell Labeling and Pretargted Cell Imaging

MC-38 cells were seeded into six-well plates at a density of 1×10^5 cells/well and cultured at 37°C for 24 h. Prior to the experiment, cells were washed twice with PBS buffer (pH = 7.4) to remove the remnant growth medium, and re-suspended in 500 μL 4% paraformaldehyde solution at 4°C for 20 minutes. Then, cells were washed twice with PBS buffer (pH = 7.4) to remove the paraformaldehyde solution. 10^5 MC-38 cells were incubated at 4°C with 1 mg/mL

Avelumab (control group) or 1 mg/mL BNU-modified Avelumab (Avel-BNU) in PBS buffer (pH = 7.4) for 60 minutes and then washed with cold PBS buffer (pH = 7.4). Tetrazine labeling was then performed by incubating the treated or untreated cells for 10 or 15 minutes at 37 °C in PBS buffer (pH = 6.3, 6.7) containing 100 μM of tetrazine-Cy5 (**19**), separately. Finally, all cells were washed twice with PBS buffer (pH = 7.4), counterstained with Hoechst 33342 (1 mM) for 15 minutes, washed twice with PBS buffer (pH = 7.4) again, and then visualized with a confocal microscope (Zeiss LSM780, 63×oil-immersion objective).

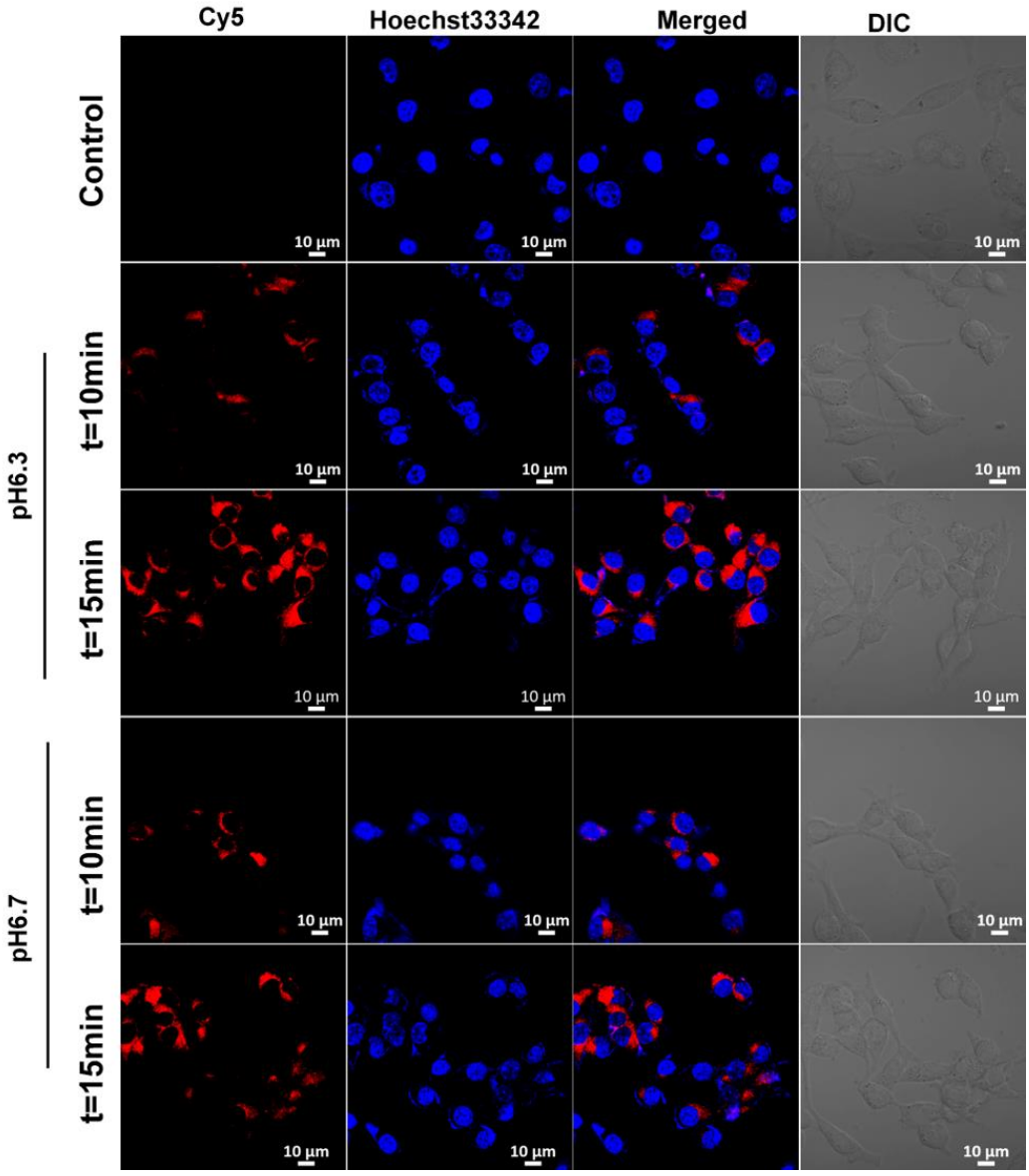


Figure S9. Confocal microscope images of MC-38 cells with different treatments (from top to down): cells treated with Avelumab and exposed to **19** at pH = 6.3 for 15 min; cells treated with Avel-BNU and exposed to **19** at pH = 6.3 for 10 min; cells treated with Avel-BNU and exposed to **19** at pH = 6.3 for 15 min; cells treated with Avel-BNU and exposed to **19** at pH = 6.7 for 10

min; cells treated with Avel-BNU and exposed to **19** at pH = 6.7 for 15 min. Scale bar: 10 μm.

Control Experiments of Cell Labeling

PD-L1 Localization Experiment: Avelumab (1 mg/mL, 200 μL) in 0.1 M $NaHCO_3$ solution (pH = 8.3) was incubated with Alexa Fluor 647 (66.67 mM, 1.76 μL) at 25 ℃ for 2 h. The conjugated antibody (Avel-AF647) was purified by centrifuge filtration (6×) using PBS (pH = 6.7) buffer solution, concentrated to 1 mg/mL. MC-38 cells were seeded into culture dishes at a density of 1×10^5 cells/well and cultured at 37°C for 24 h. Prior to the experiment, cells were washed twice with PBS buffer (pH = 7.4) to remove the remnant growth medium, and fixed by 500 μL 4% paraformaldehyde solution at 4°C for 20 minutes. Then, cells were washed twice with PBS buffer (pH = 7.4) to remove the paraformaldehyde solution. MC-38 cells were incubated with 500 nM Avel-AF647 in PBS buffer (pH = 6.7) at 4℃ overnight. Finally, all cells were washed twice with PBS buffer (pH = 7.4), counterstained with Hoechst 33342 (1 mM) for 15 minutes, washed twice with PBS buffer (pH = 7.4) again, and then visualized with a confocal microscope (Zeiss LSM780, 63×oil-immersion objective).

Note: In order to further confirm the localization of PD-L1 receptor in MC-38 cells, we conducted the above reaction to fluorescently label Avelumab through a conventional method. Due to its instant availability, we chose Alexa Fluor 647 as the fluorescence probe, which has similar ex/em wavelength as Cy5. We followed the same protocol to incubate the labeled Avelumab (Avel-AF647) with MC-38 cells. The fluorescence image (**Figure S10**) shows similar cytosolic distribution as that observed in the previous experiments.

Control Experiment with 15b: MC-38 cells were seeded into culture dishes at a density of 1×10^5 cells/well and cultured at 37°C for 24 h. Prior to the experiment, cells were washed twice with PBS buffer (pH = 7.4) to remove the remnant growth medium, and fixed by 500 μL 4% paraformaldehyde solution at 4°C for 20 minutes. Then, cells were washed twice with PBS buffer (pH = 7.4) to remove the paraformaldehyde solution. MC-38 cells were incubated with 0.6 μM and 6 μM (10×higher than the estimated conc.) **15b** in PBS buffer (pH = 6.7) at 4℃ for 1 h, and then with 10 μM tetrazine-Cy5 (**19**) (with washing steps after each treatment) for 15 minutes. Finally, all cells were washed twice with PBS buffer (pH = 7.4), counterstained with Hoechst 33342 (1 mM) for 15 minutes, washed twice with PBS again buffer (pH = 7.4), and then visualized with a confocal microscope (Zeiss LSM780, 63×oil-immersion objective).

Note: We conducted the above reaction, with the aim to exclude the possibility that the observed fluorescence signal was arouse from the reaction between the remaining **17b (**and its hydrolysis product**)** and tetrazine-Cy5 (**19**). The BNU derivative **15b**, a more stable homolog of **17b**, was used as a model compound. We followed the same protocol to treat the MC-38 cells with 0.6 μM and 6 μM (10×higher than the estimated conc.) of **15b** and subsequently with tetrazine-

Cy5 (**19**) (with washing steps after each treatment). The fluorescence images (**Figure S10**) shows no observable fluorescence. Taking together, we could conclude that the detected fluorescence in the cells did not come from the remaining **17b (**and its hydrolysis product**)**.

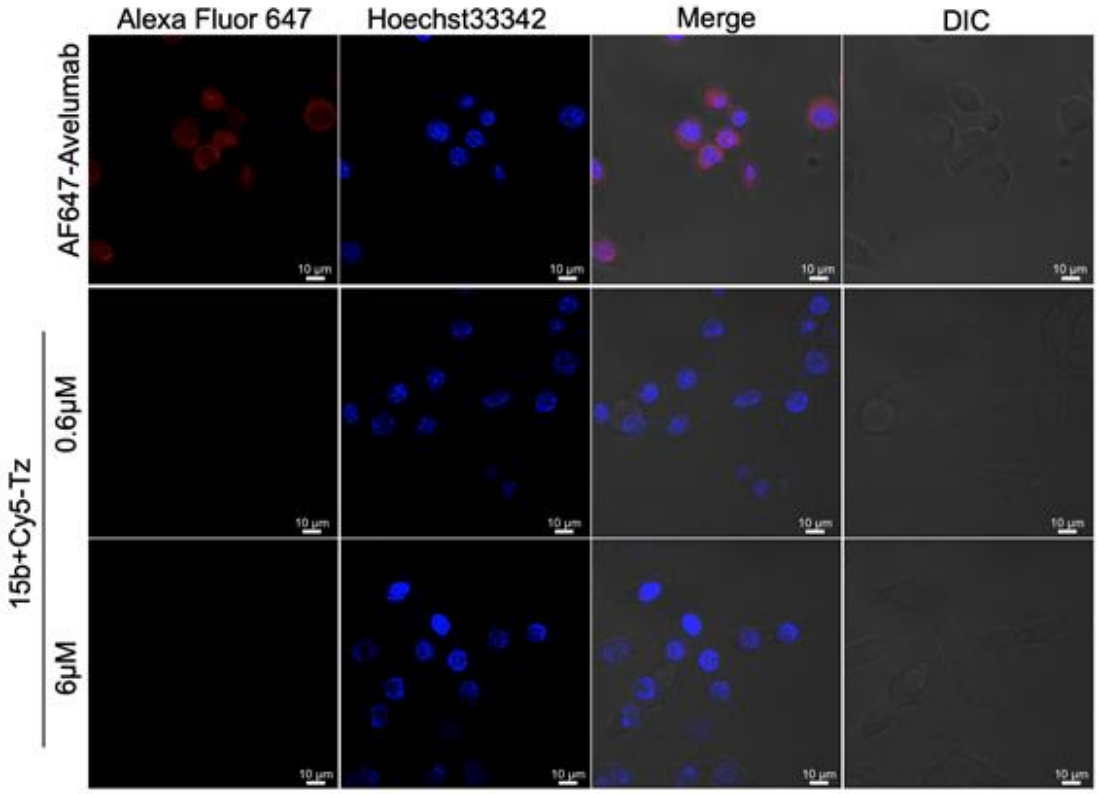


Figure S10. Confocal microscope images of MC-38 cells with different treatments. Up (PD-L1 Localization Experiment): cells treated with Avel-AF647 at pH = 6.7 overnight. Down (Control Experiment with **15b**): cells treated with **15b** and exposed to **19** at pH = 6.7 for 15 min with the indicated concentrations. Scale bar: 10 μm.

Flow Cytometry Analysis of Cell Labeling

200 μL 1x10^5 cells/mL MC-38 cells in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. MC-38 cells were seeded into six-well plates at a density of 1×10^5 cells/well and cultured at 37°C for 24 h. Prior to the experiment, cells were washed with PBS buffer (pH = 7.4) to remove the remnant growth medium, and re-suspended in 500 μL 4% paraformaldehyde solution at 4 ℃ for 20 minutes. Then, cells were washed twice with PBS buffer (pH = 7.4) to remove the paraformaldehyde solution. MC-38 cells were incubated at 4°C with 1 mg/mL Avelumab (control group) or 1 mg/mL BNU-modified Avelumab (Avel-BNU) in PBS buffer (pH = 7.4) for 60 minutes and then washed with cold PBS buffer (pH = 7.4). Tetrazine labeling was then performed by incubating the treated or untreated cells for 5, 10, 15 or 35 minutes at 37°C in PBS buffer (pH = 6.0, 6.3, 6.7, 7.0, 7.4 and 7.8) containing 100 μM of tetrazine-Cy5 (**19**), separately. Finally, all cells were washed

twice with PBS buffer (pH = 7.4), and then assayed by fluorescence-activated cell sorting (BD FACSCanto II) and analyzed using a Flowjo software (BD). Blank means cells treated with Avelumab and exposed to **19** at indicated pH and time.

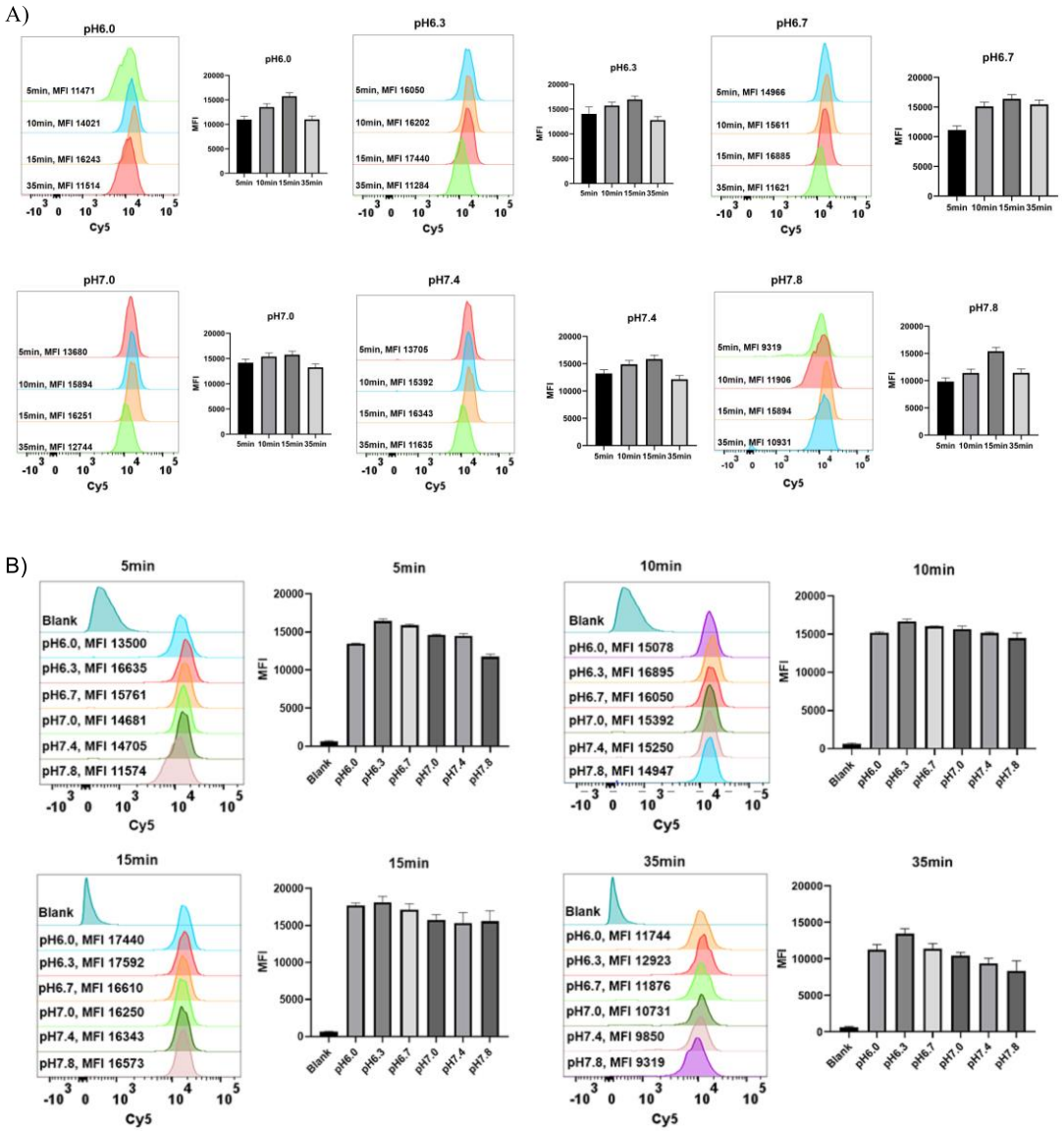


Figure S11. Flow cytometry analysis of MC-38 cells treated with Avel-BNU and exposed to **19** at pH = 6.0, 6.3, 6.7, 7.0, 7.4, 7.8 for 5, 10, 15 and 35 min; blank means cells treated with Avelumab and exposed to **19** at indicated pH and time. Error bars denote the standard deviation from three replicated experiments. The data show mean ± s.d. from a representative experiment (n=3).

Cytotoxicity of representative BNU derivatives 15d and 17b

293T cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Gibco) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) (BioInd), 1% penicillin/streptomycin (Beyotime) at 37°C and 5% CO_2. Raji and Ramos cells were cultured

in RPMI 1640 medium (Gibco) supplemented with 10% heat inactivated FBS (BioInd) and 1% penicillin/streptomycin (Beyotime) at 37°C and 5% CO_2. 293T, Raji and Ramos were seeded at 5000 cells into 96-well plates, pre-incubated for 24 h, then incubated with **15d** and **17b** for 48 h at concentrations from 0.04 mM to 1 mM at 37°C with 98% humidity and 5% CO_2. Afterwars 10 μL 0.1% Alamar Blue solution was added into each well and incubated for 2 h at 37°C. The results were read at excitation: 550 nm, emission: 585 nm using a cytation3 imaging reader (Biotek instrument, Inc.). Untreat cells were used as control. All experiments were carried out with four replicates.

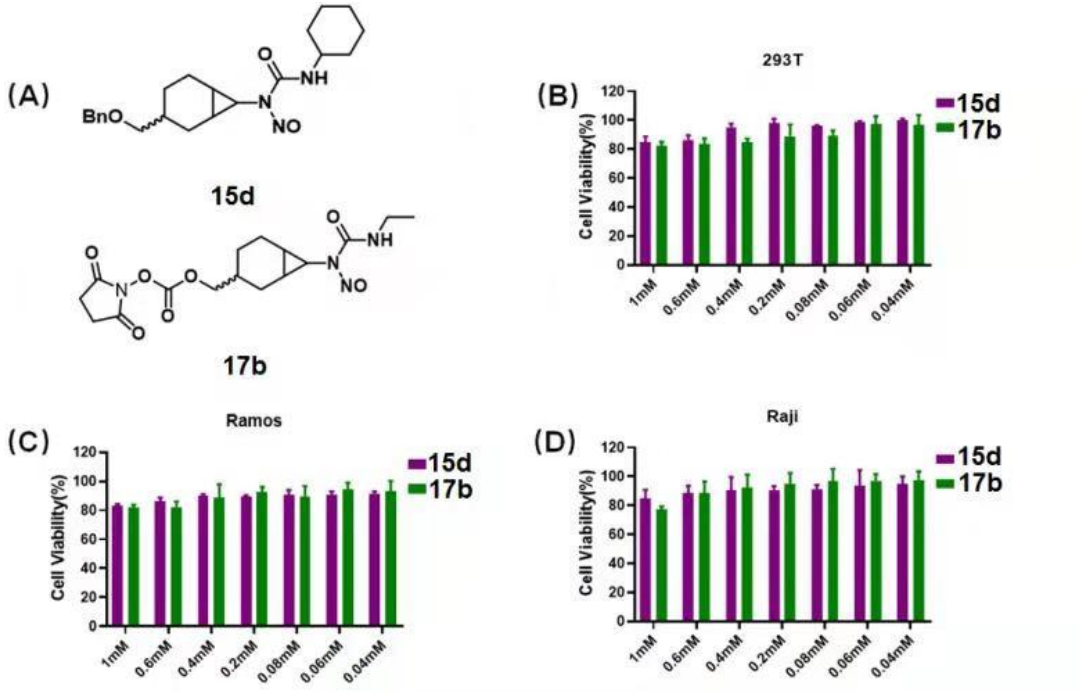


Figure S12. Cytotoxicity of representative BNU derivatives **15d** and **17b**.

5. Reference

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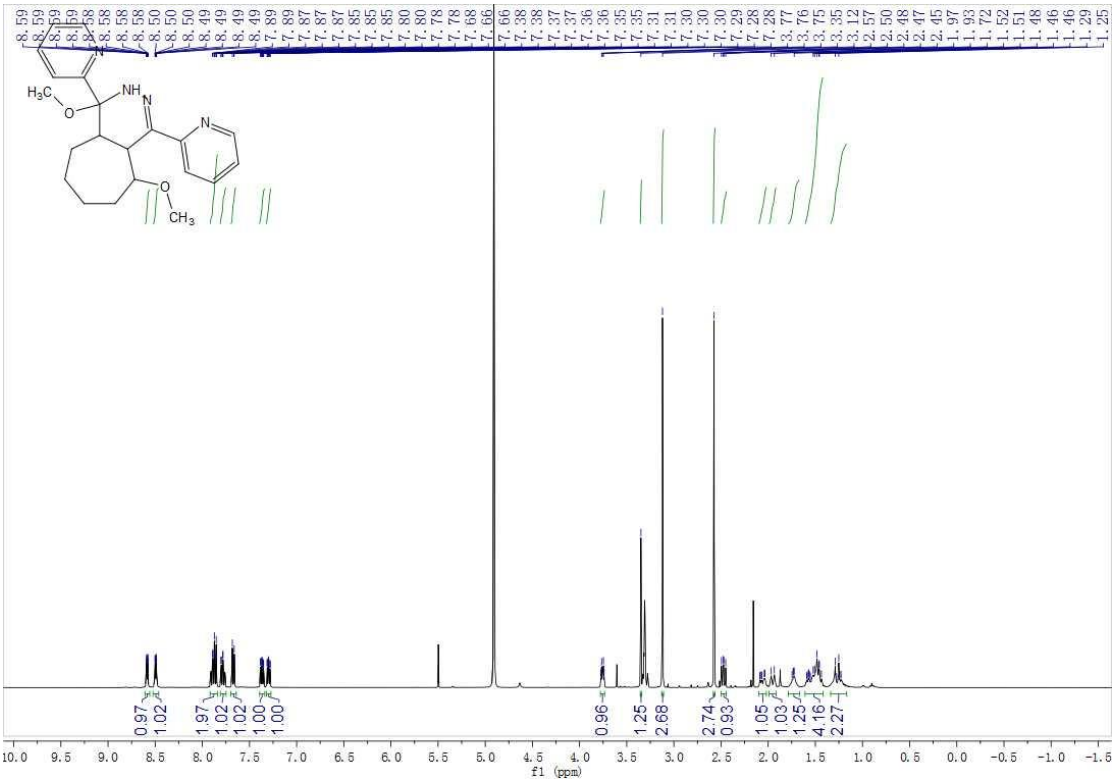
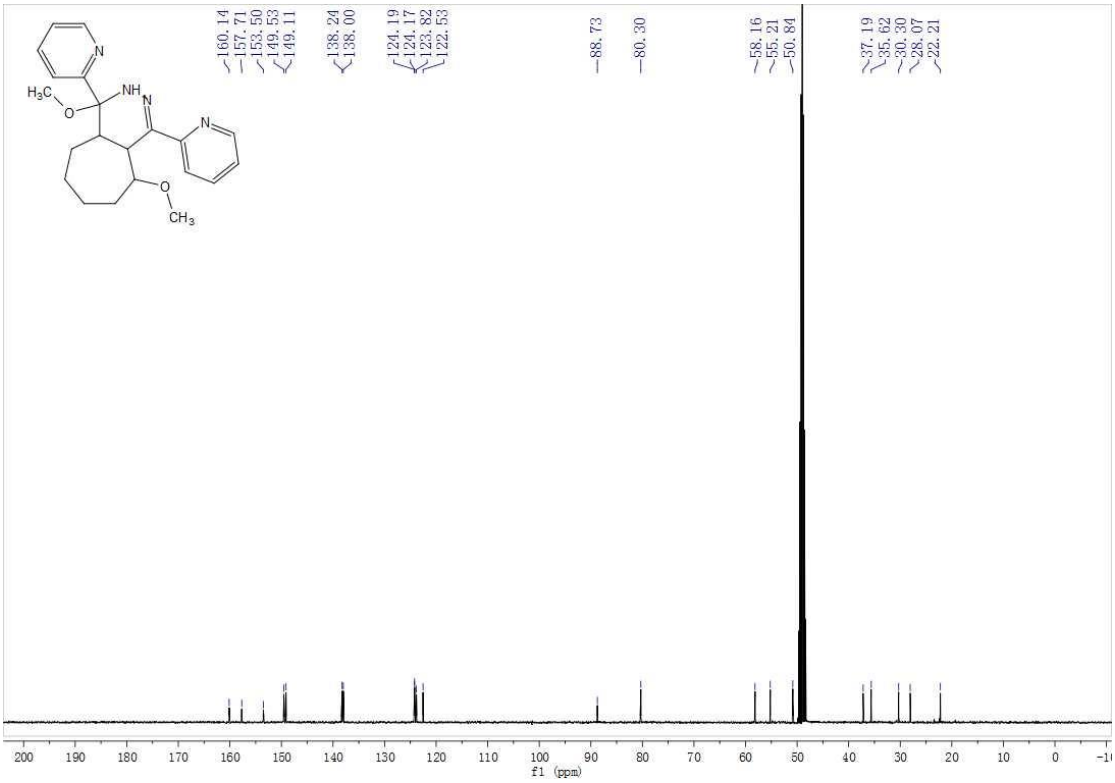
6. ^{1}H and ^{13}C NMR Spectra

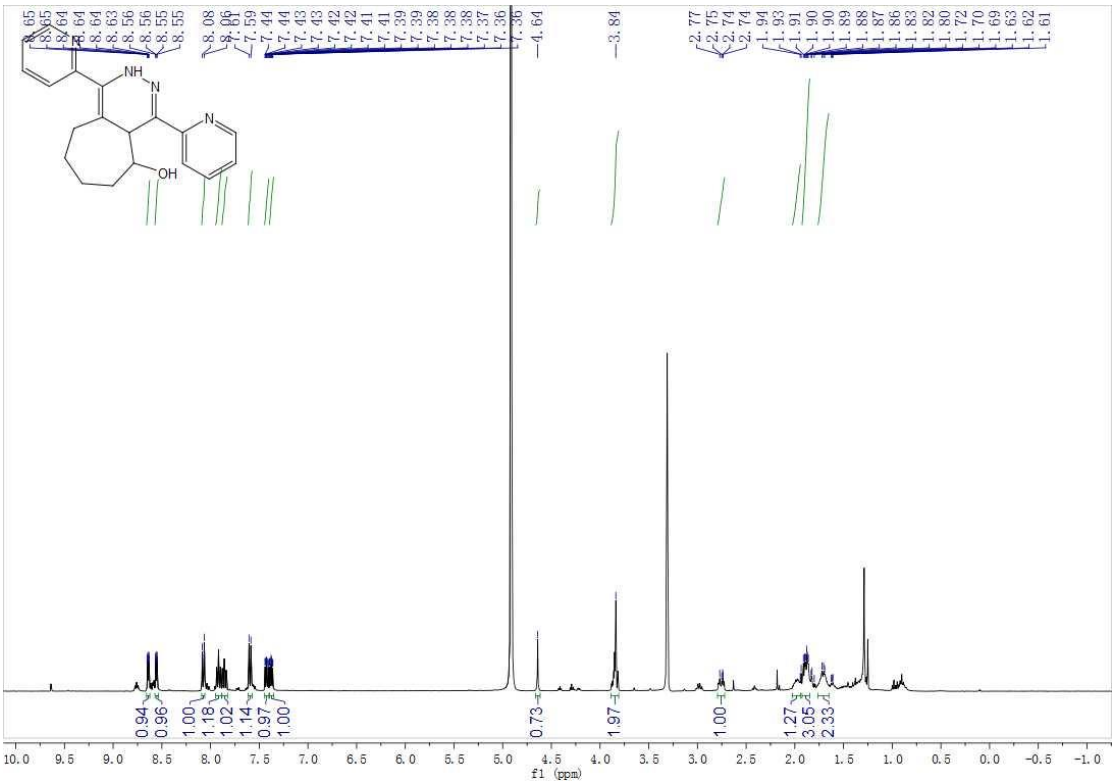
^{1}H NMR spectrum for **7a** (MeOD, 400 MHz)



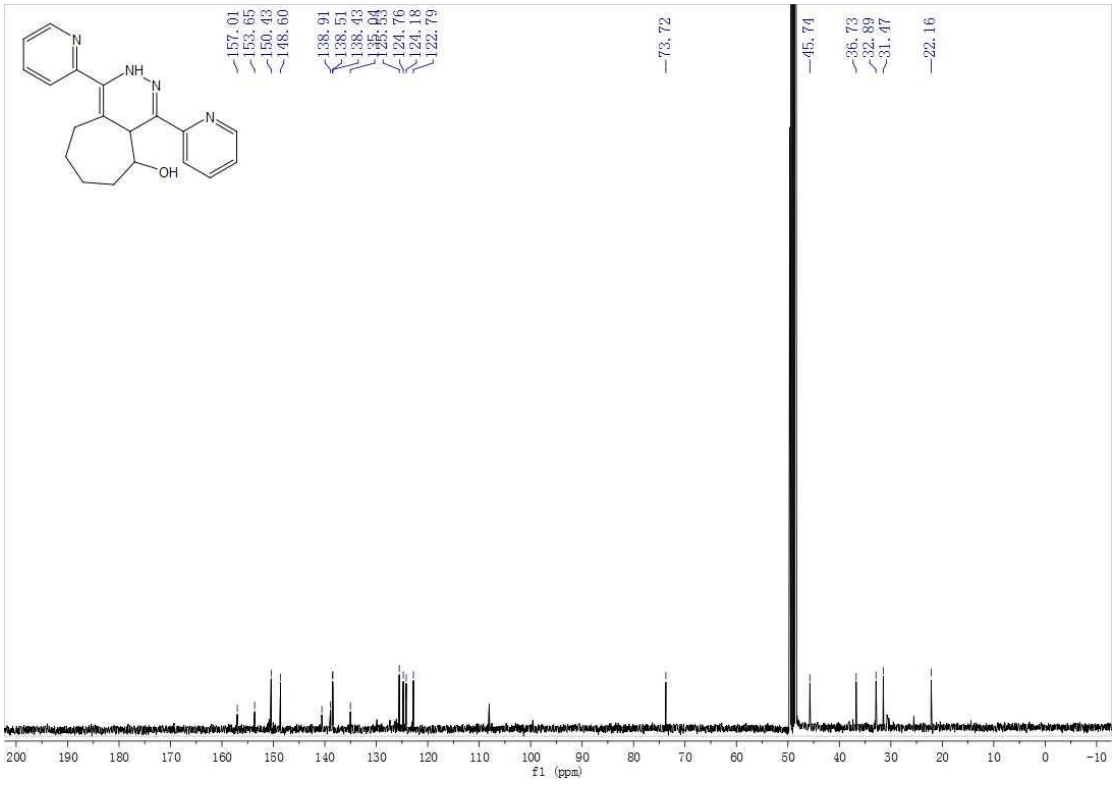
^{13}C NMR spectrum for **7a** (MeOD, 100 MHz)



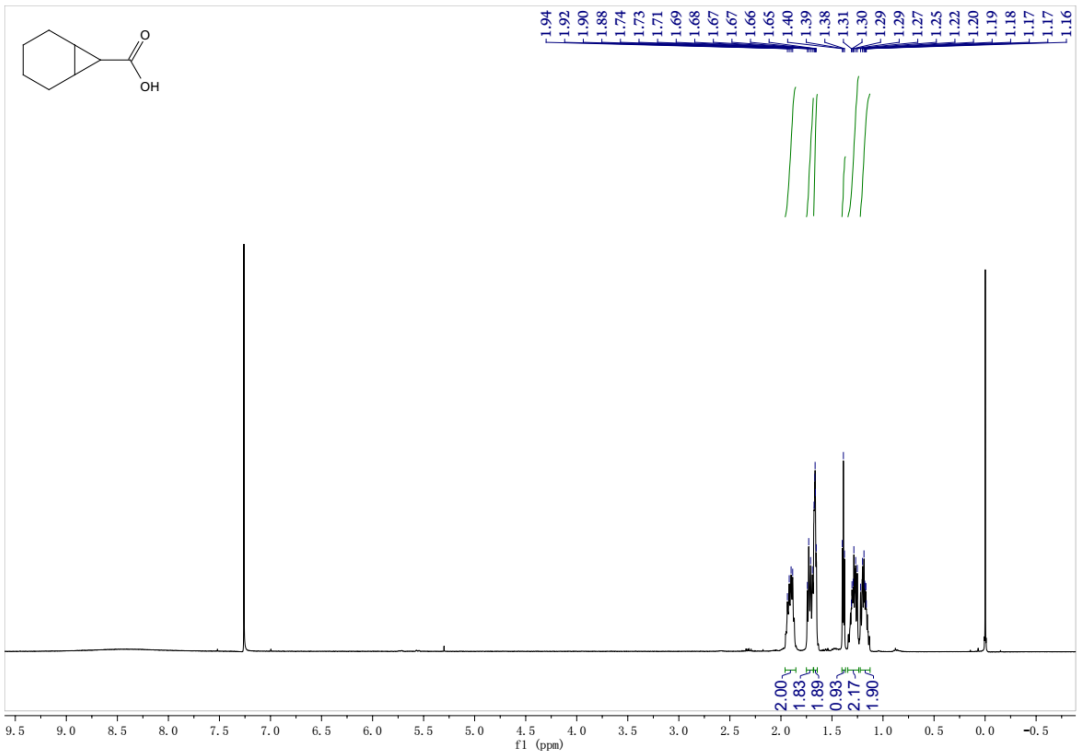
^{1}H NMR spectrum for **8b** (MeOD, 400 MHz)



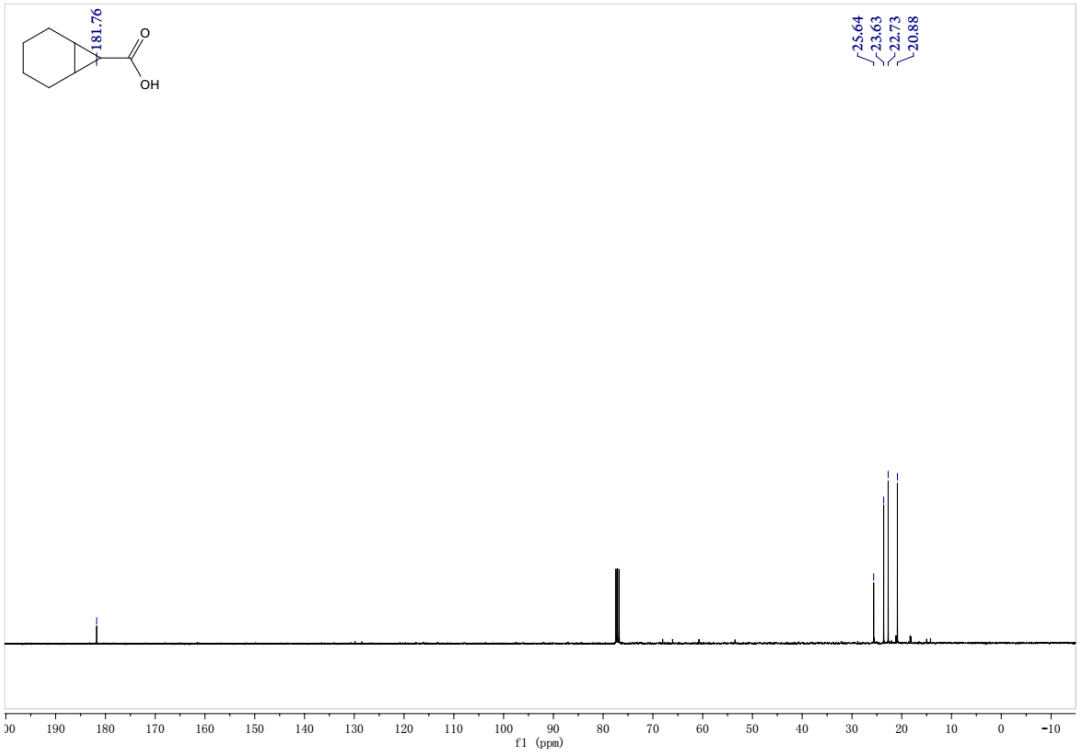
^{13}C NMR spectrum for **8b** (MeOD, 100 MHz)



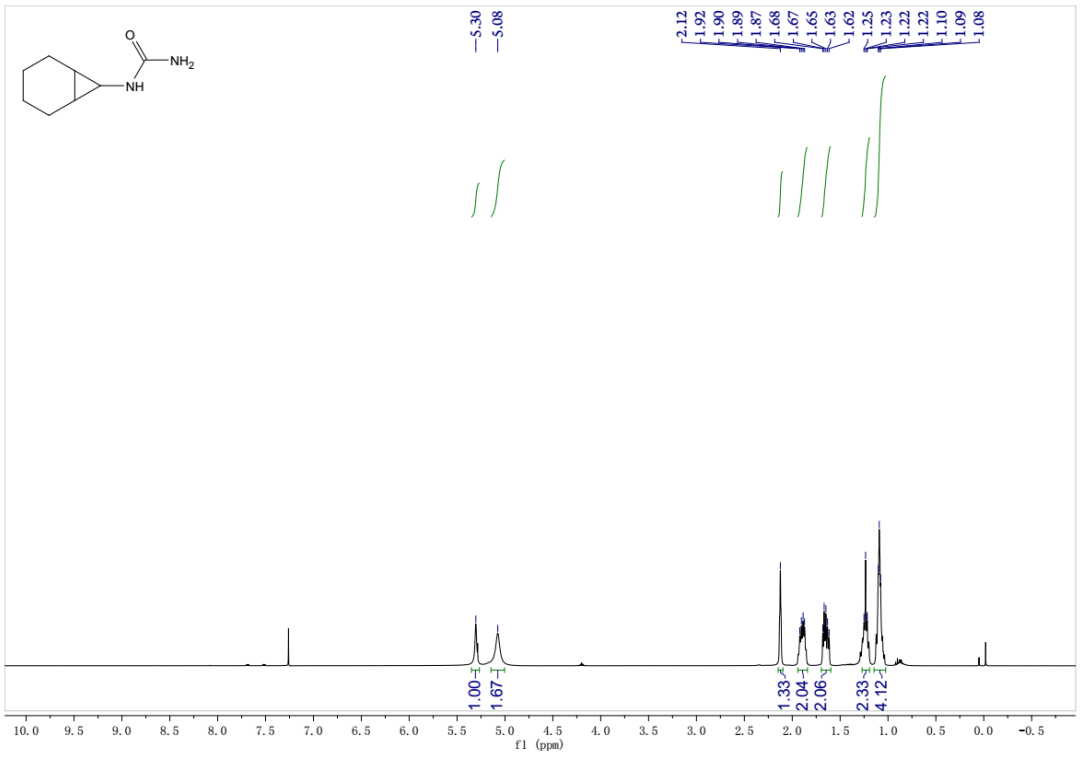
^{1}H NMR spectrum for **S3** ($CDCl_3$, 400 MHz)



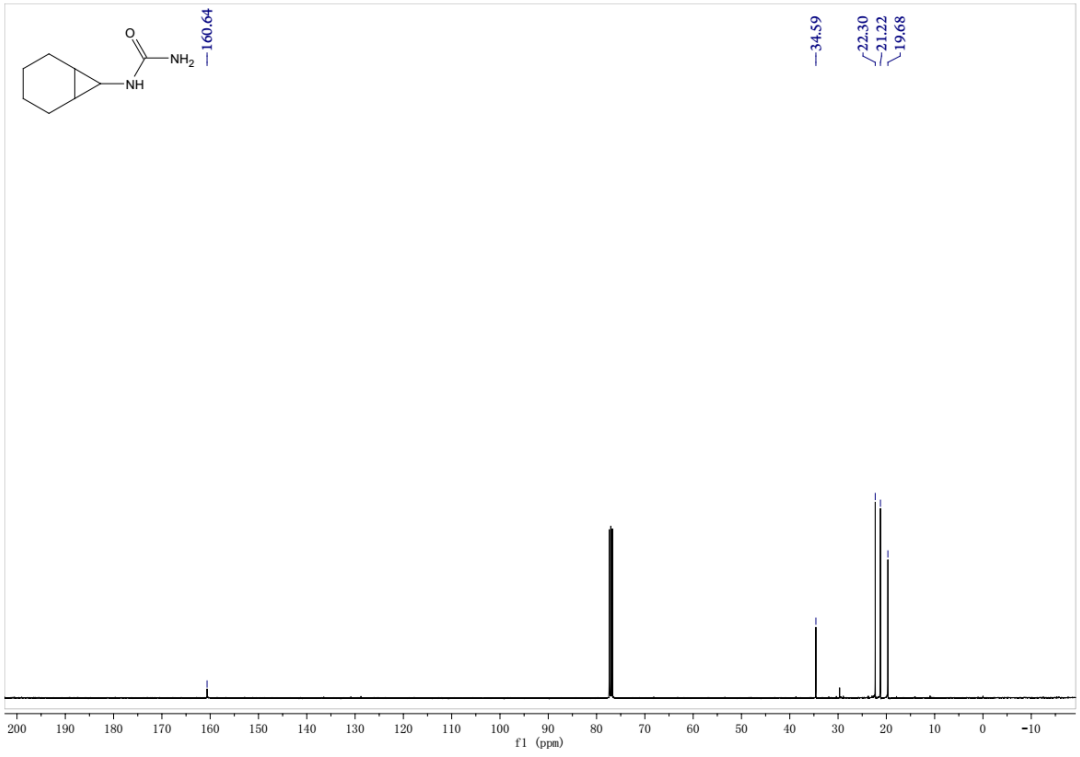
^{13}C NMR spectrum for **S3** ($CDCl_3$, 100 MHz)



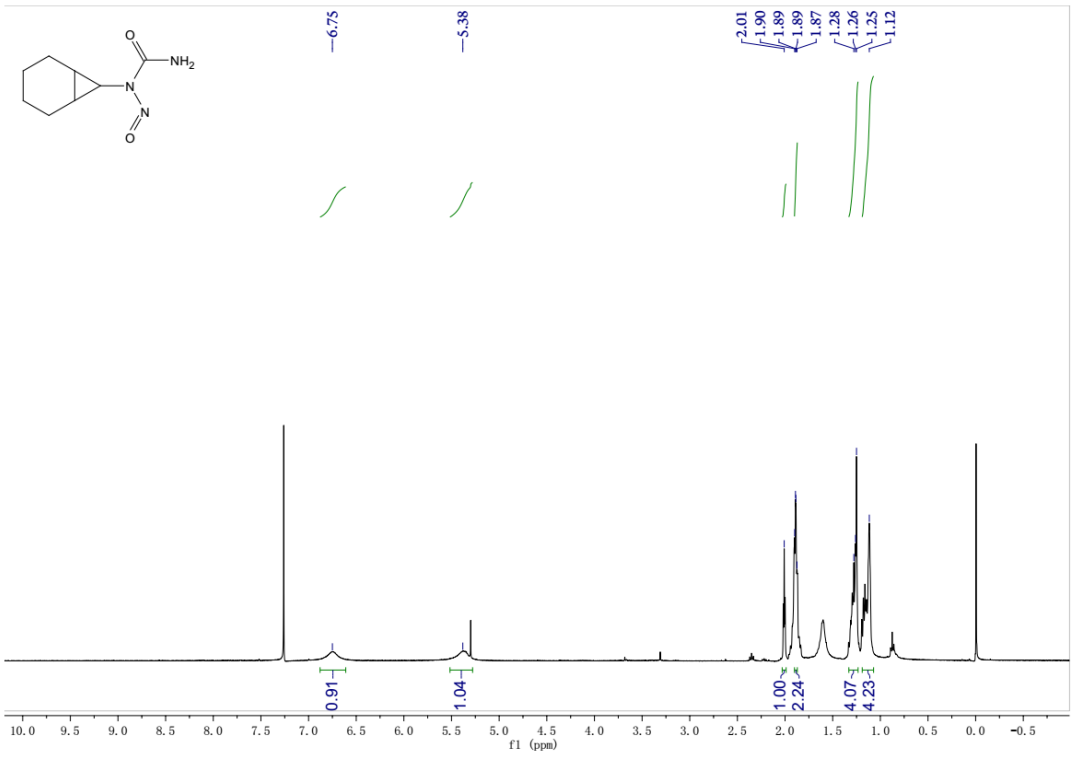
^{1}H NMR spectrum for **S4** ($CDCl_3$, 400 MHz)



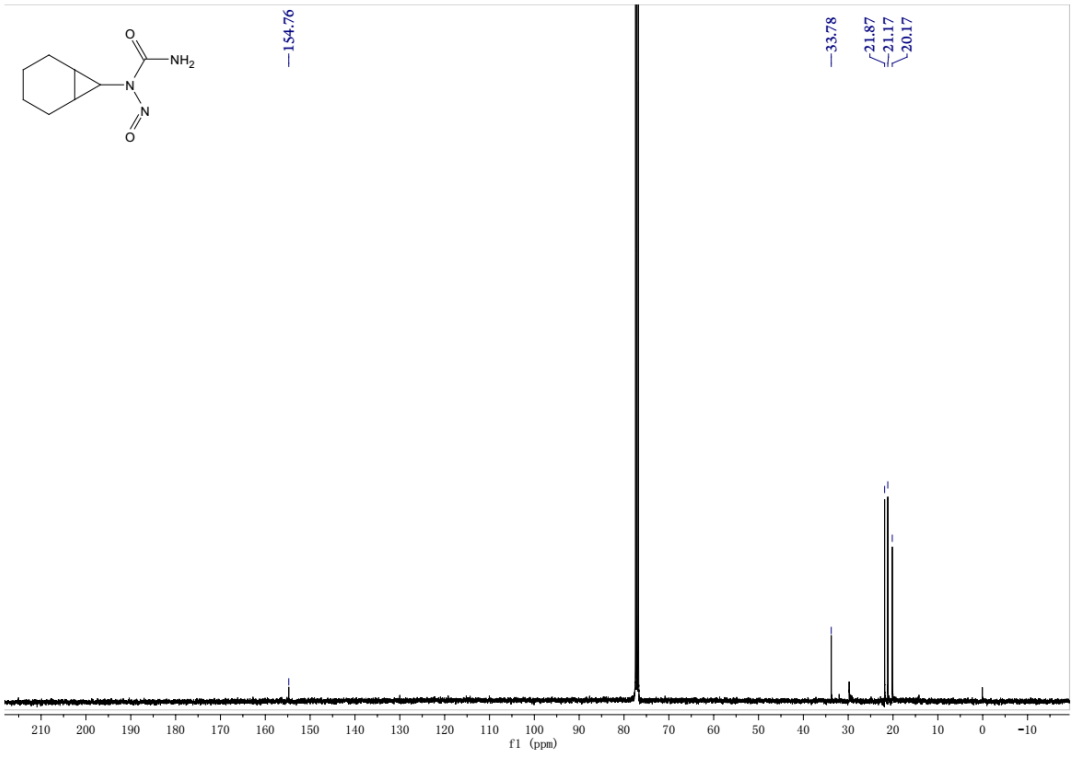
^{13}C NMR spectrum for **S4** ($CDCl_3$, 100 MHz)



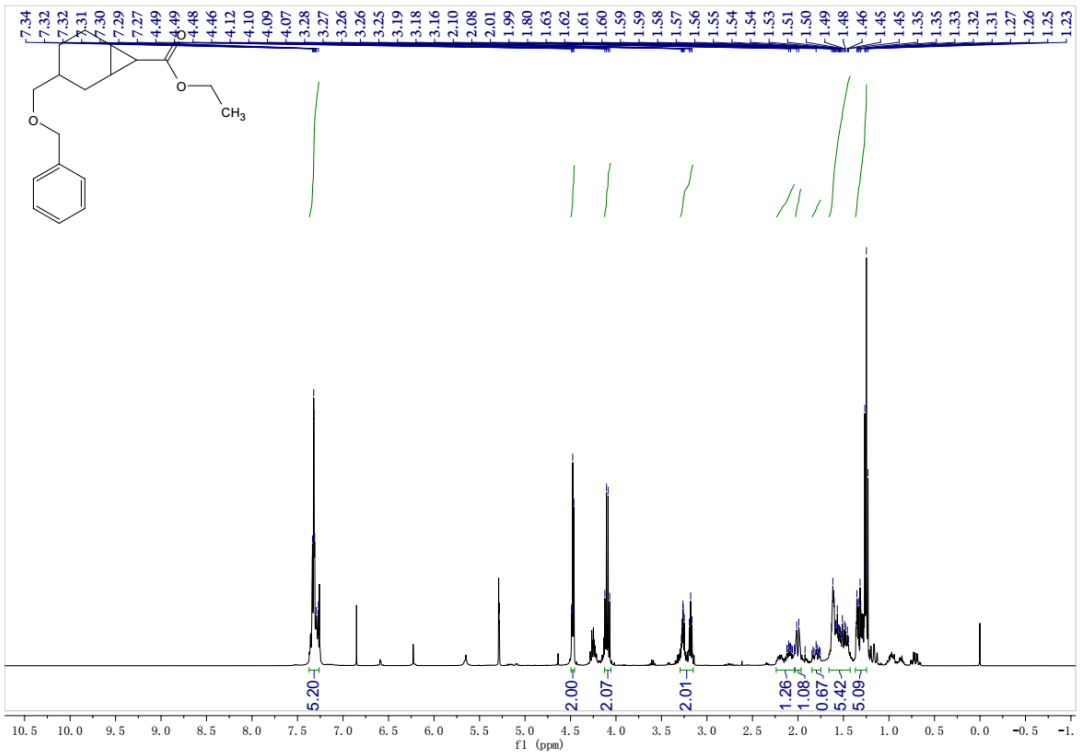
^{1}H NMR spectrum for **1** ($CDCl_3$, 400 MHz)



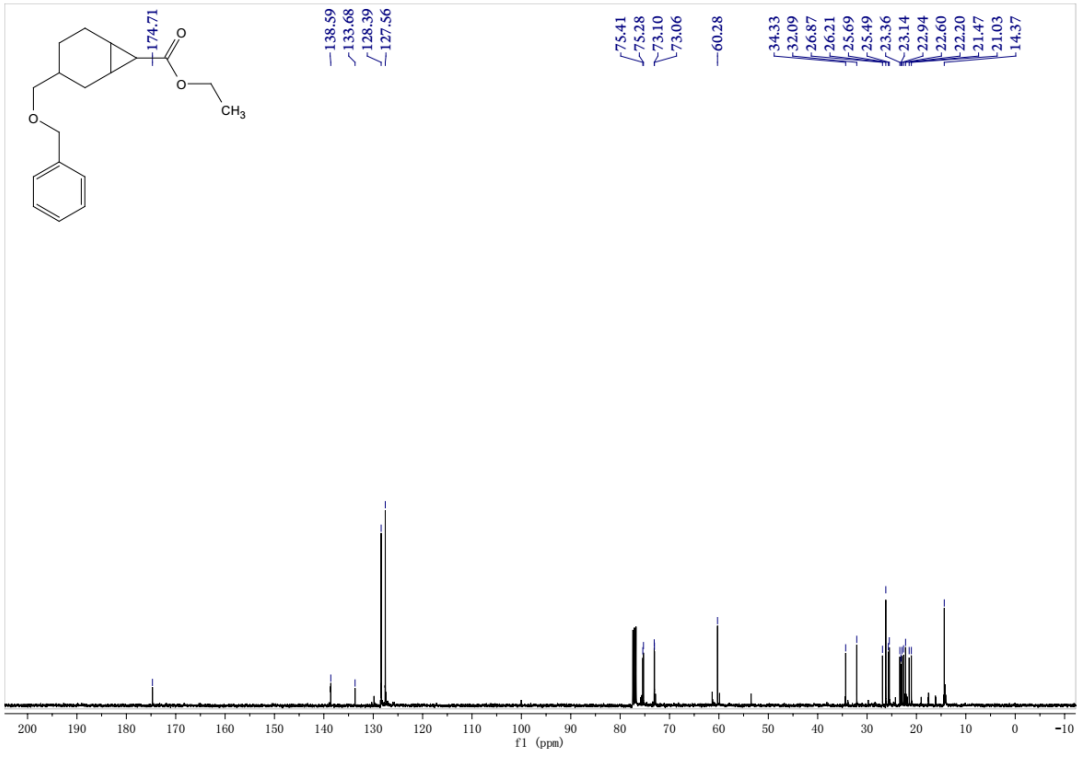
^{13}C NMR spectrum for **1** ($CDCl_3$, 100 MHz)



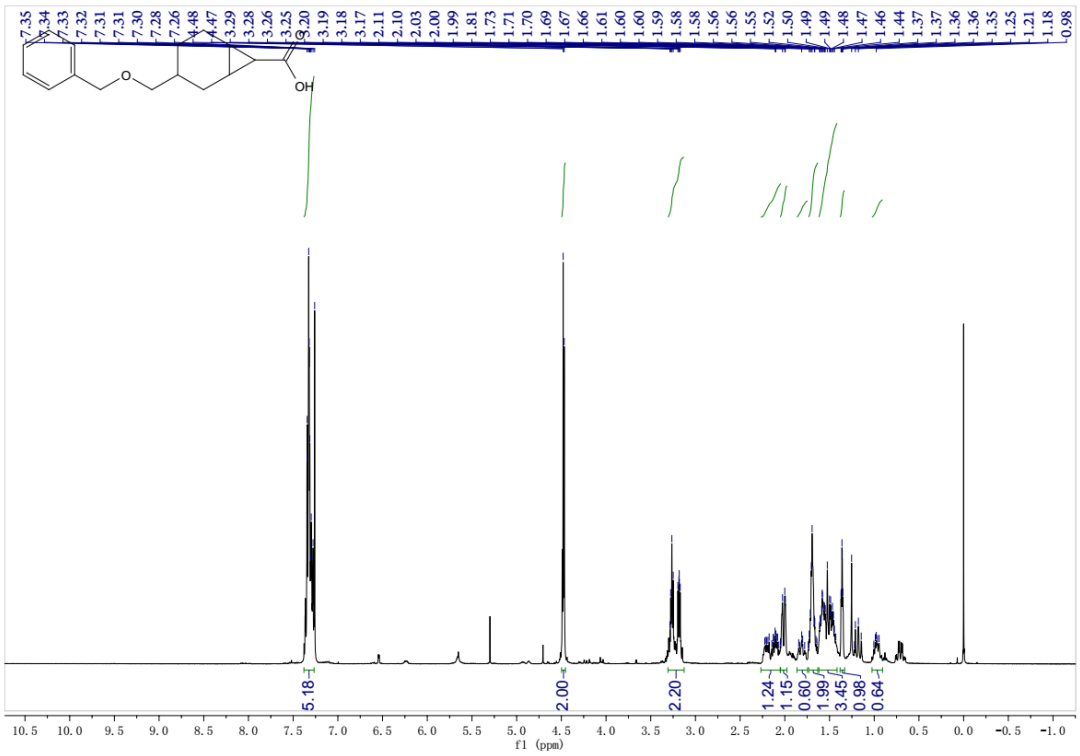
^{1}H NMR spectrum for **11** ($CDCl_3$, 400 MHz)



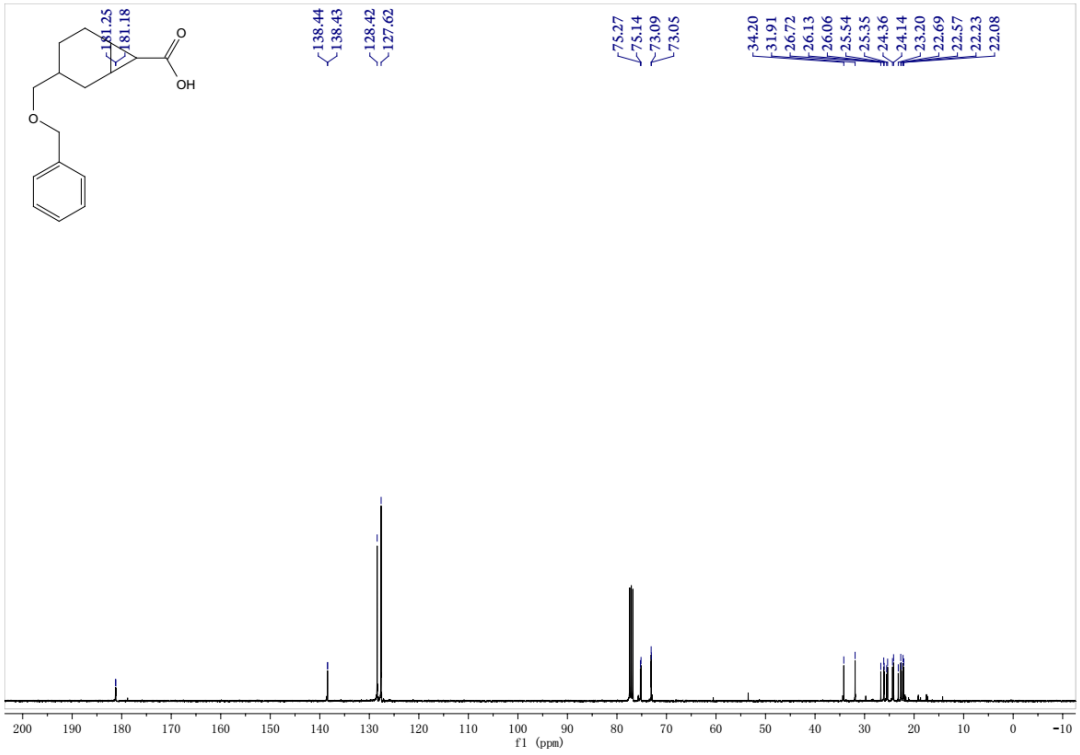
^{13}C NMR spectrum for **11** ($CDCl_3$, 100 MHz)



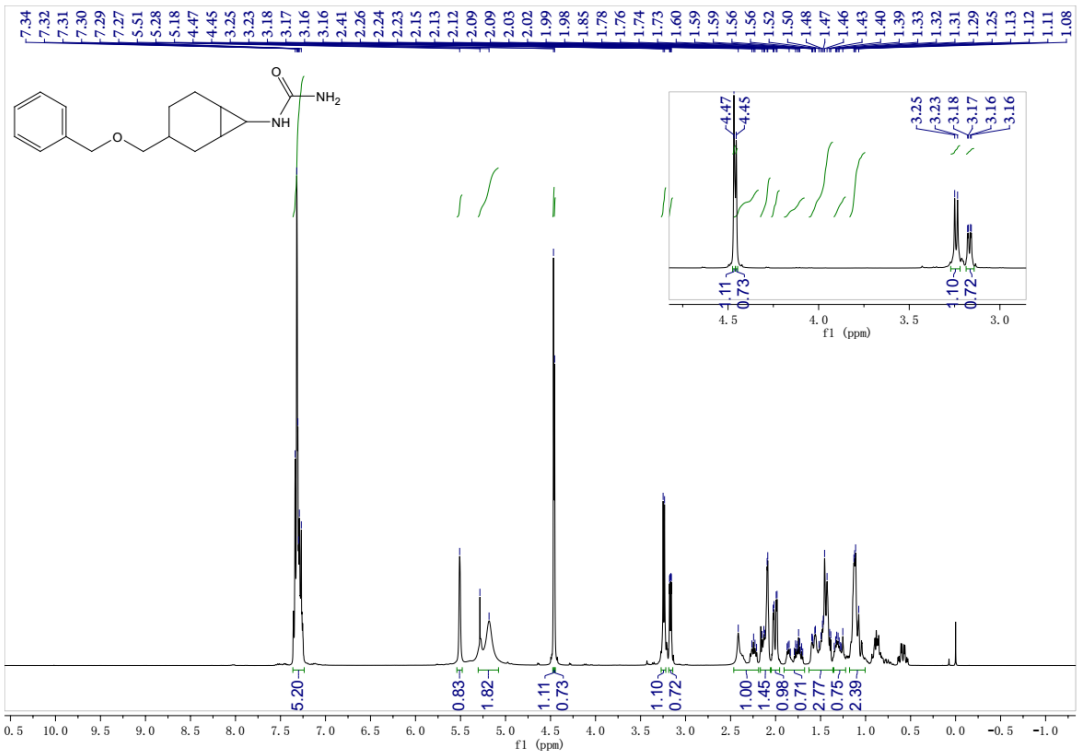
^{1}H NMR spectrum for **12** ($CDCl_3$, 400 MHz)



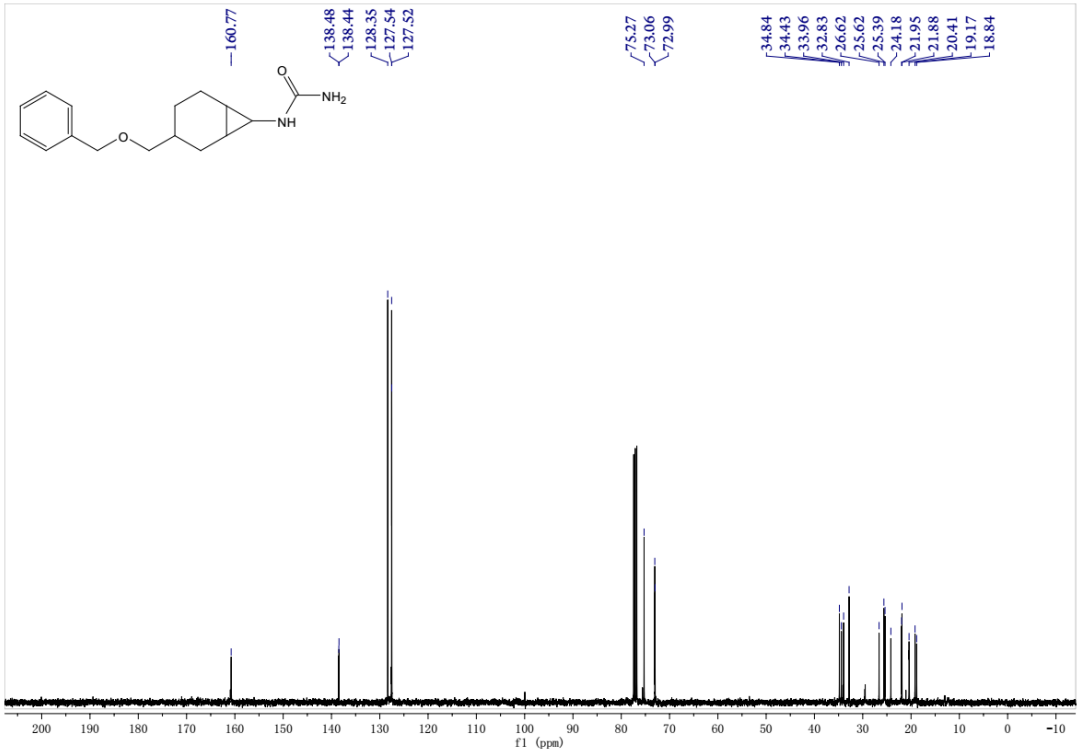
^{13}C NMR spectrum for **12** ($CDCl_3$, 100 MHz)



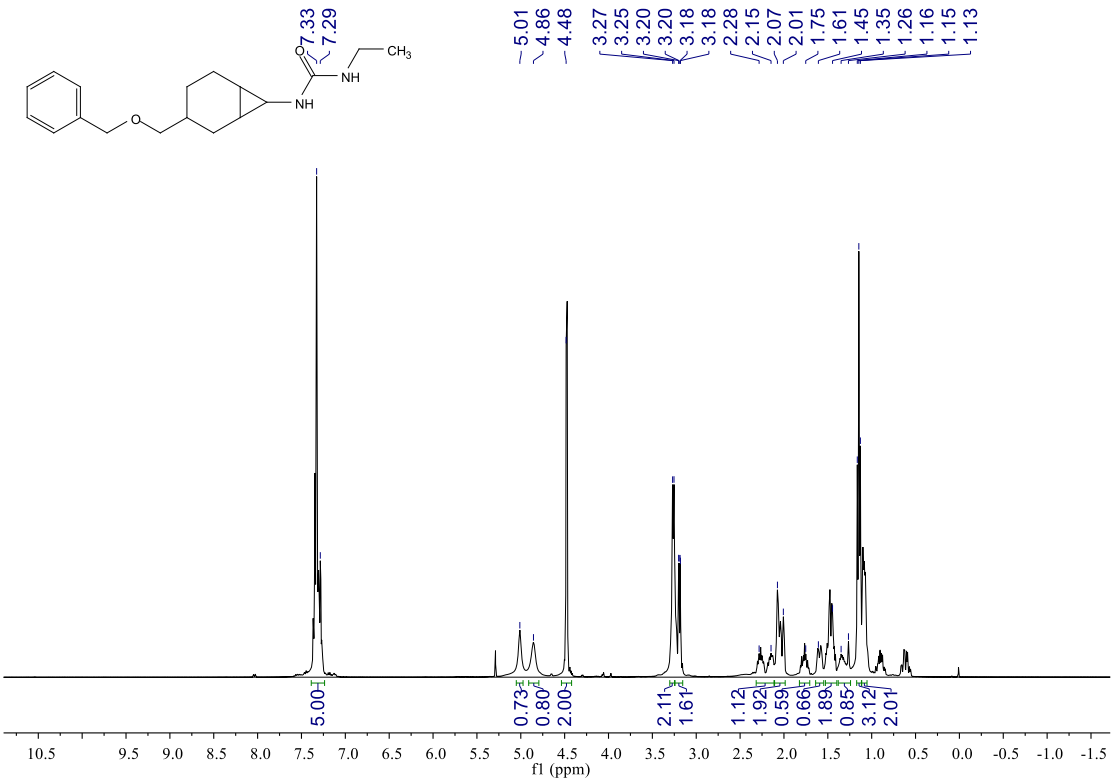
^{1}H NMR spectrum for **14a** ($CDCl_3$, 400 MHz)



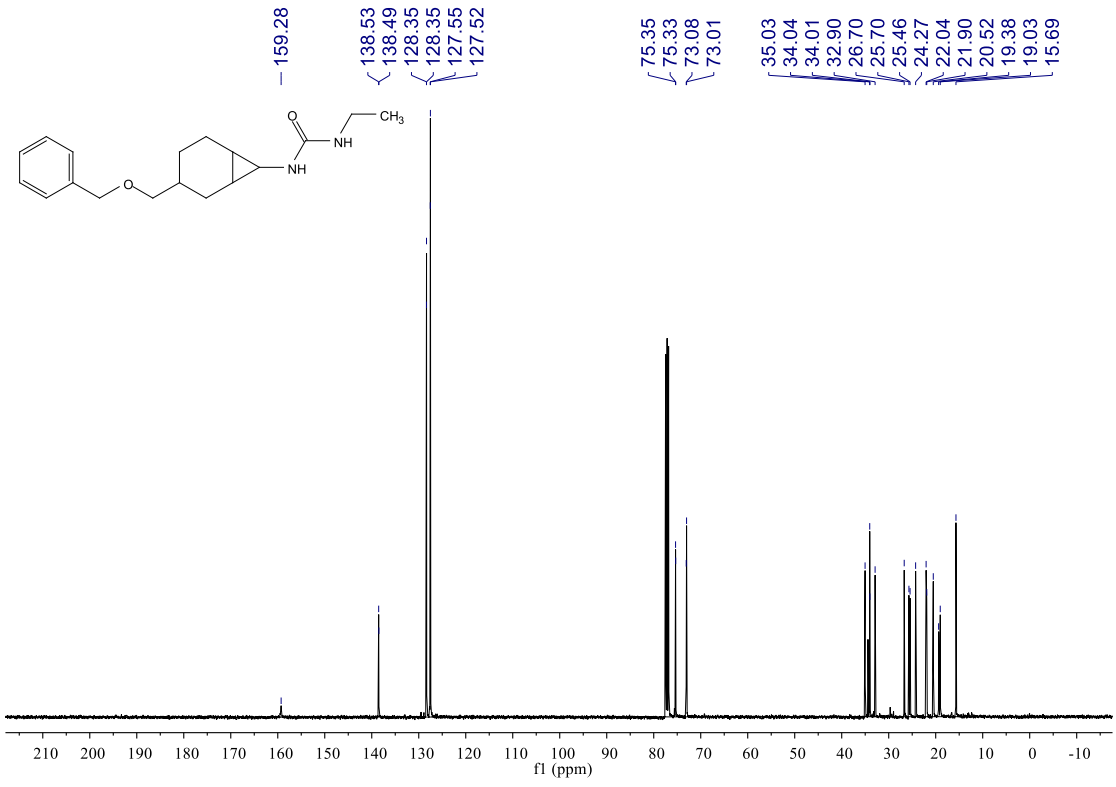
^{13}C NMR spectrum for **14a** ($CDCl_3$, 100 MHz)



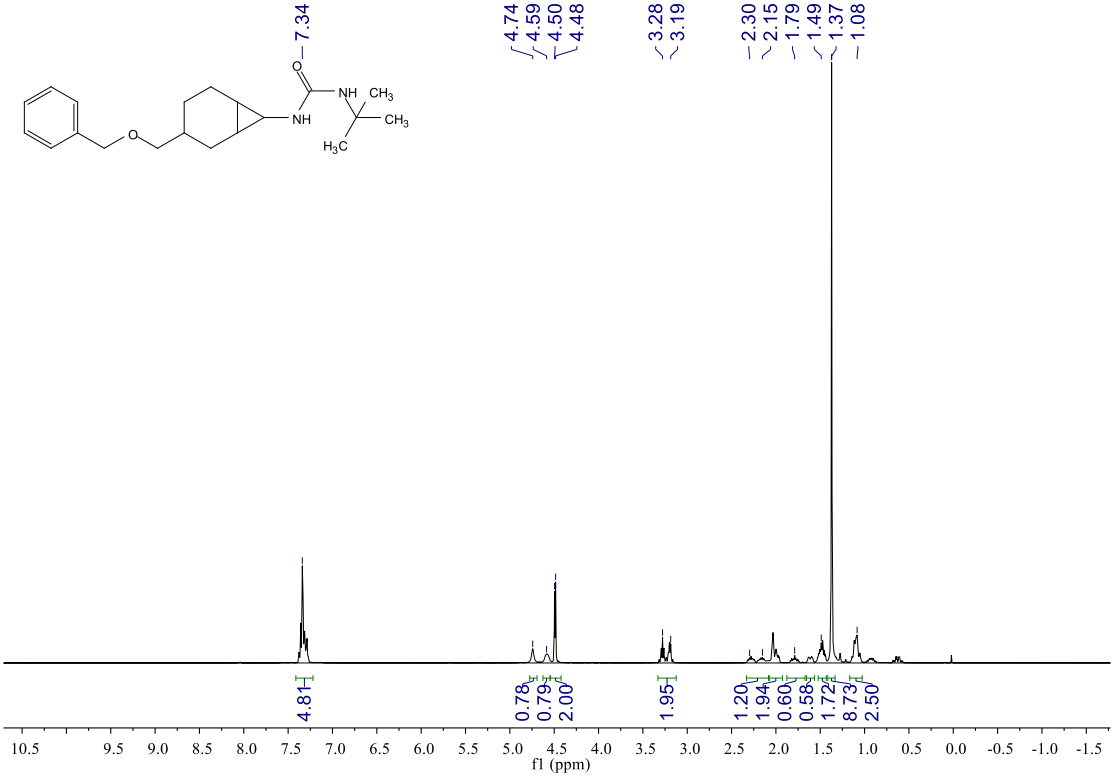
^{1}H NMR spectrum for **14b** ($CDCl_3$, 400 MHz)



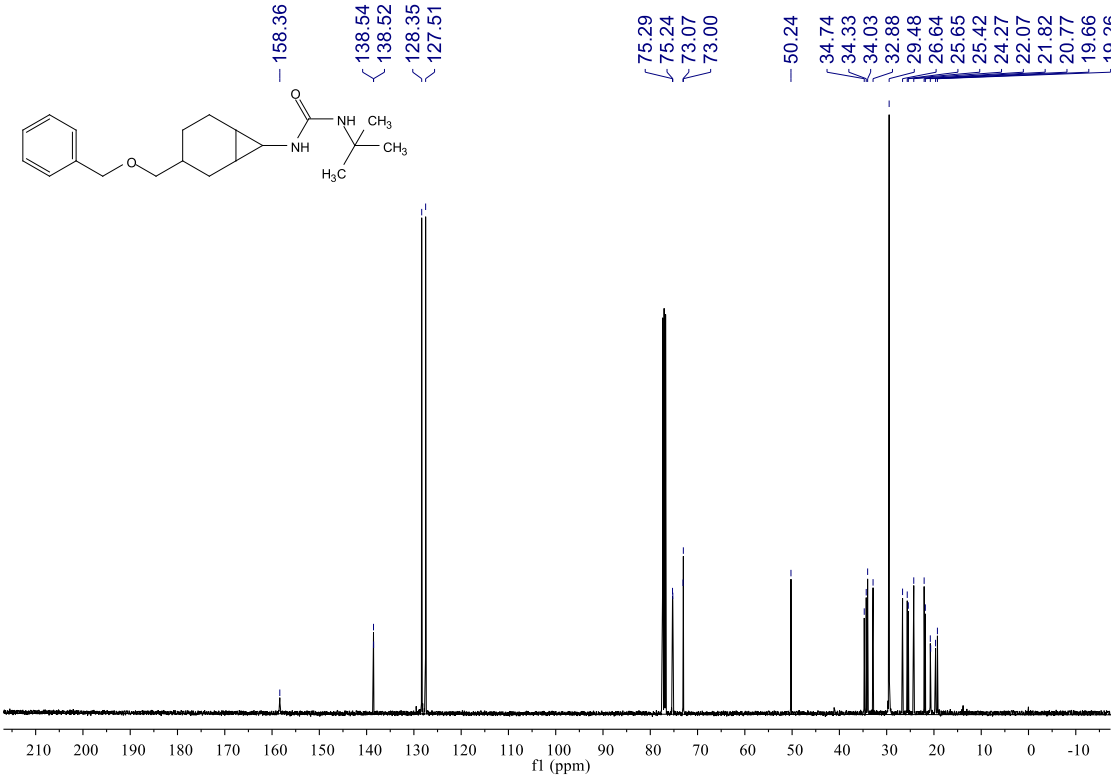
^{13}C NMR spectrum for **14b** ($CDCl_3$, 100 MHz)



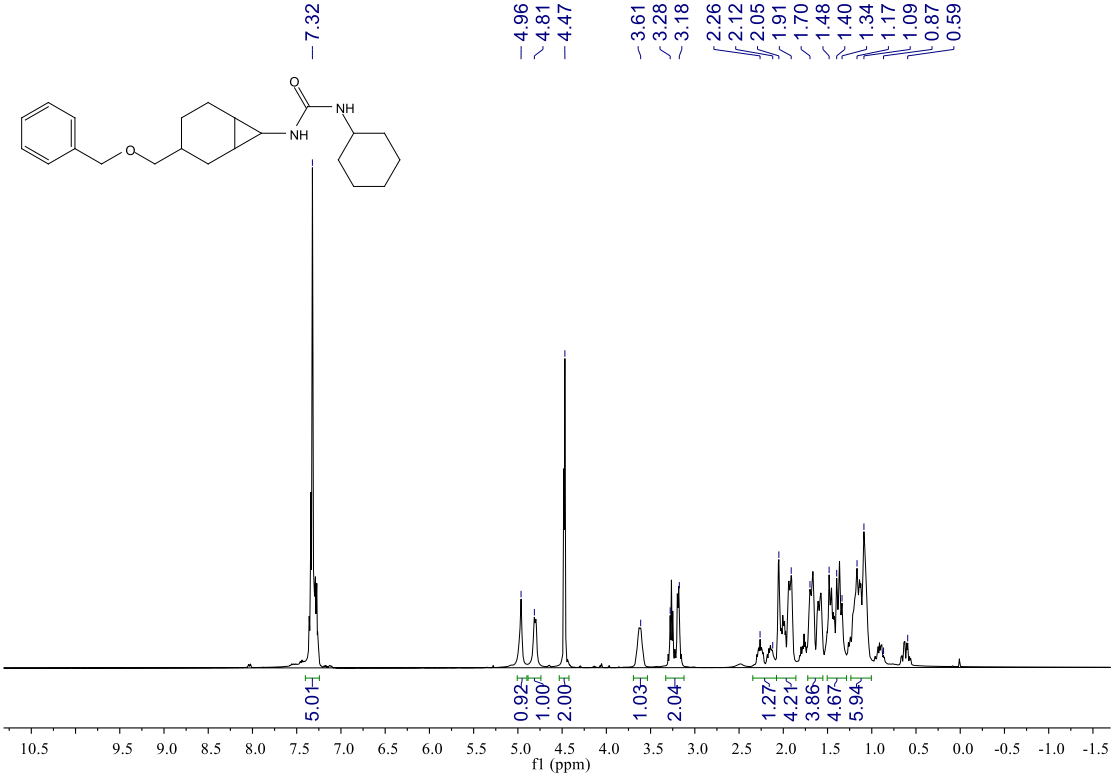
^{1}H NMR spectrum for **14c** ($CDCl_3$, 400 MHz)



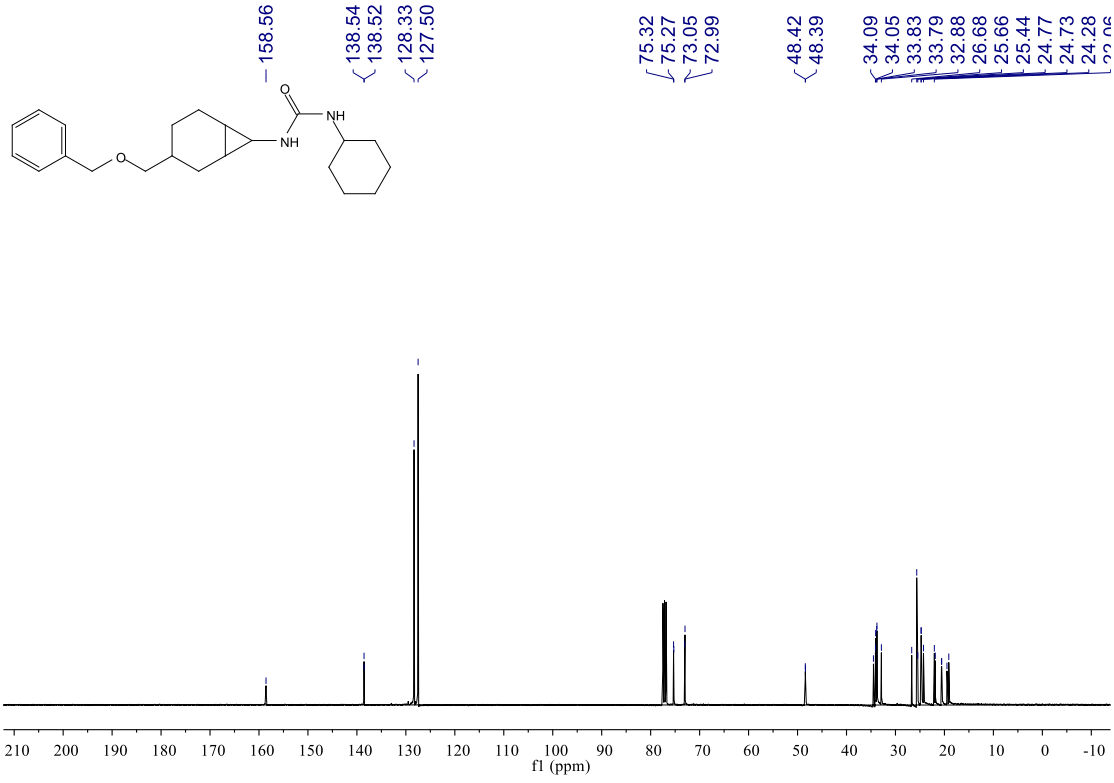
^{13}C NMR spectrum for **14c** ($CDCl_3$, 100 MHz)



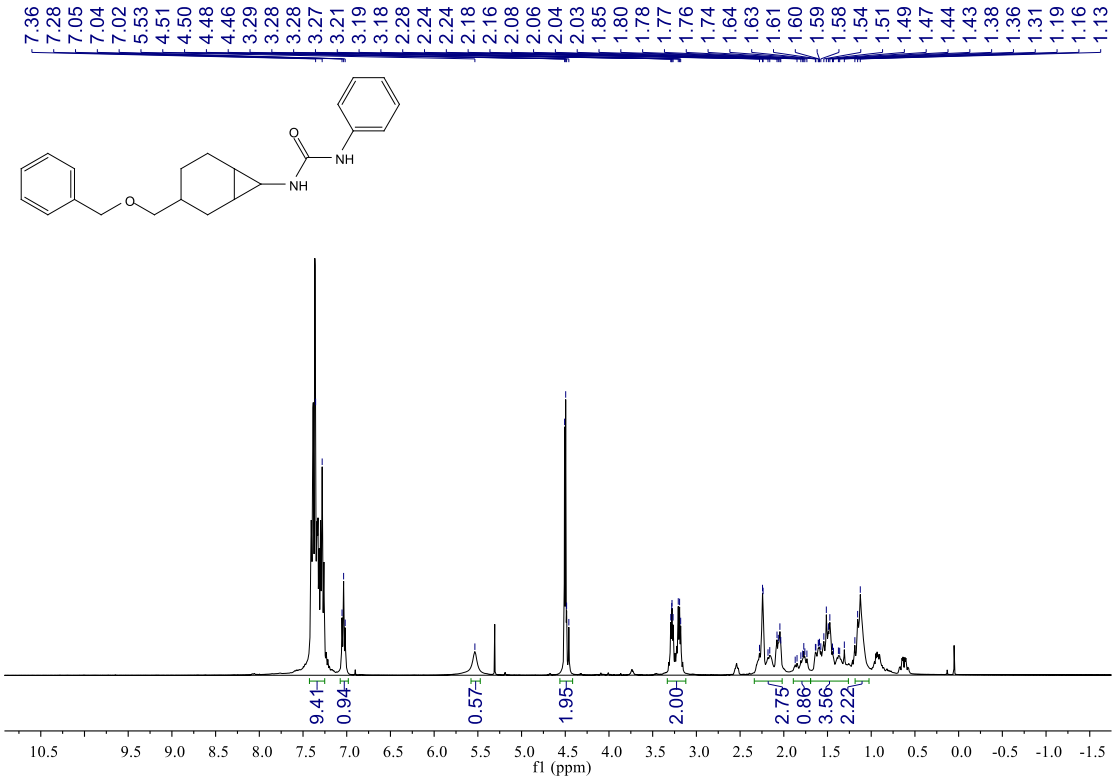
^{1}H NMR spectrum for **14d** ($CDCl_3$, 400 MHz)



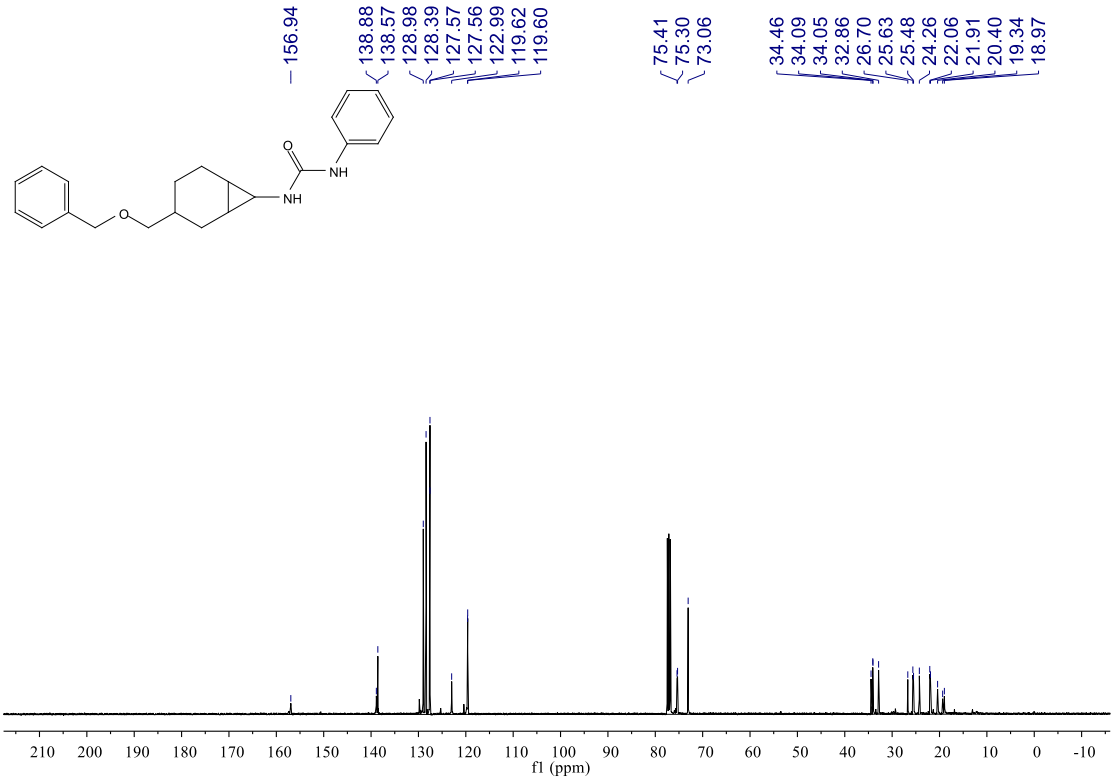
^{13}C NMR spectrum for **14d** ($CDCl_3$, 100 MHz)



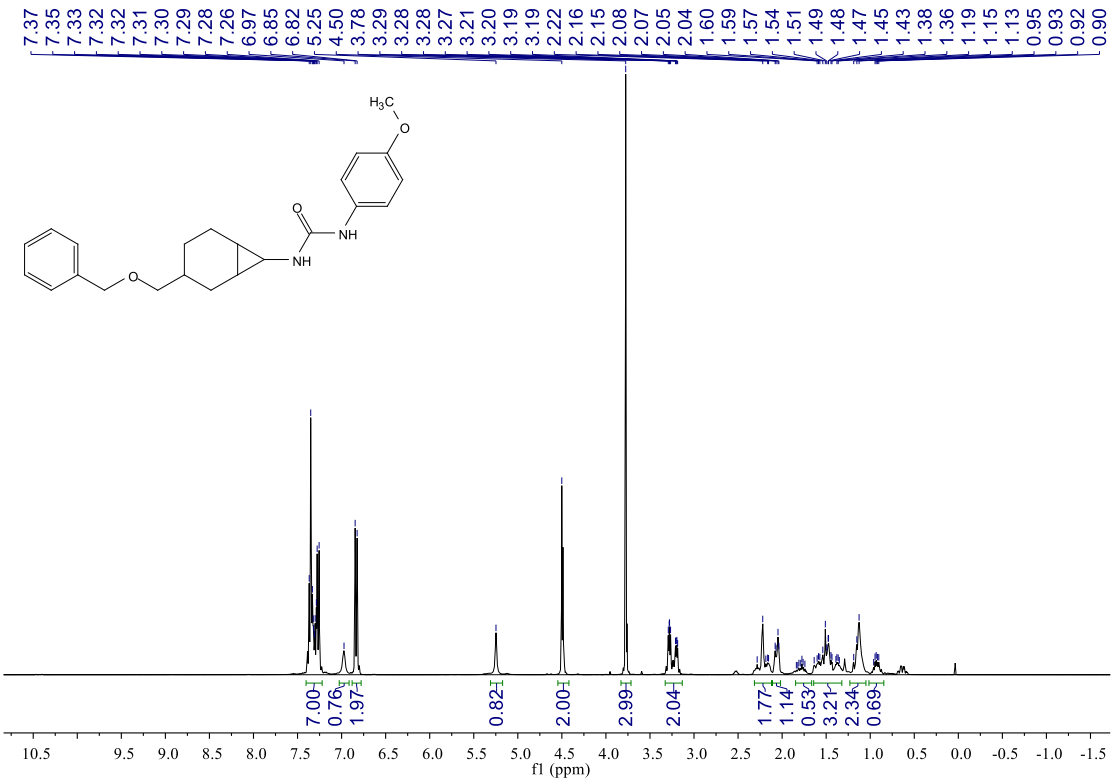
^{1}H NMR spectrum for **14e** ($CDCl_3$, 400 MHz)



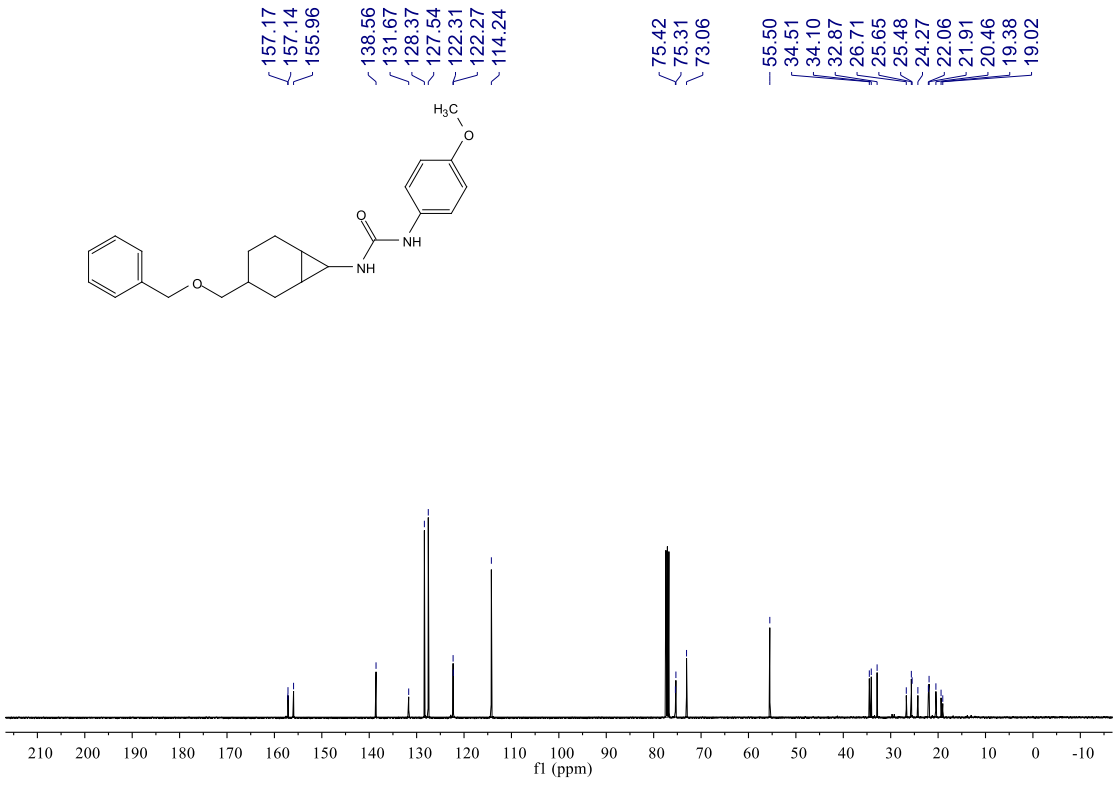
^{13}C NMR spectrum for **14e** ($CDCl_3$, 100 MHz)



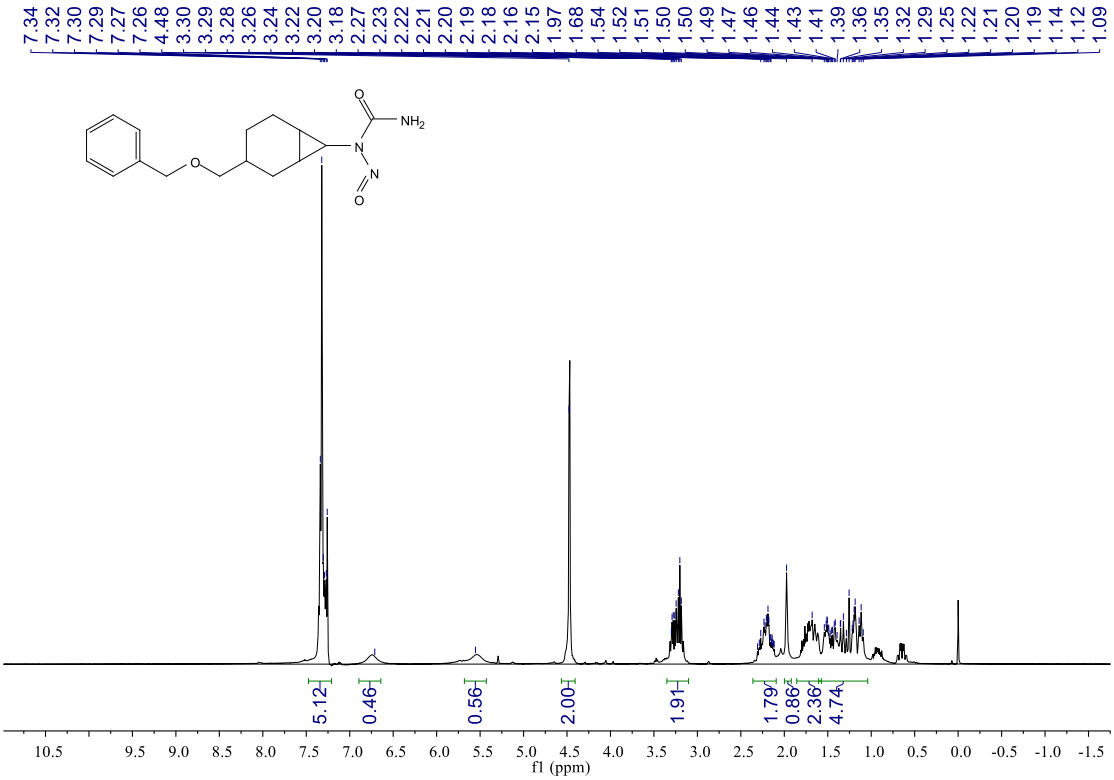
^{1}H NMR spectrum for **14f** ($CDCl_3$, 400 MHz)



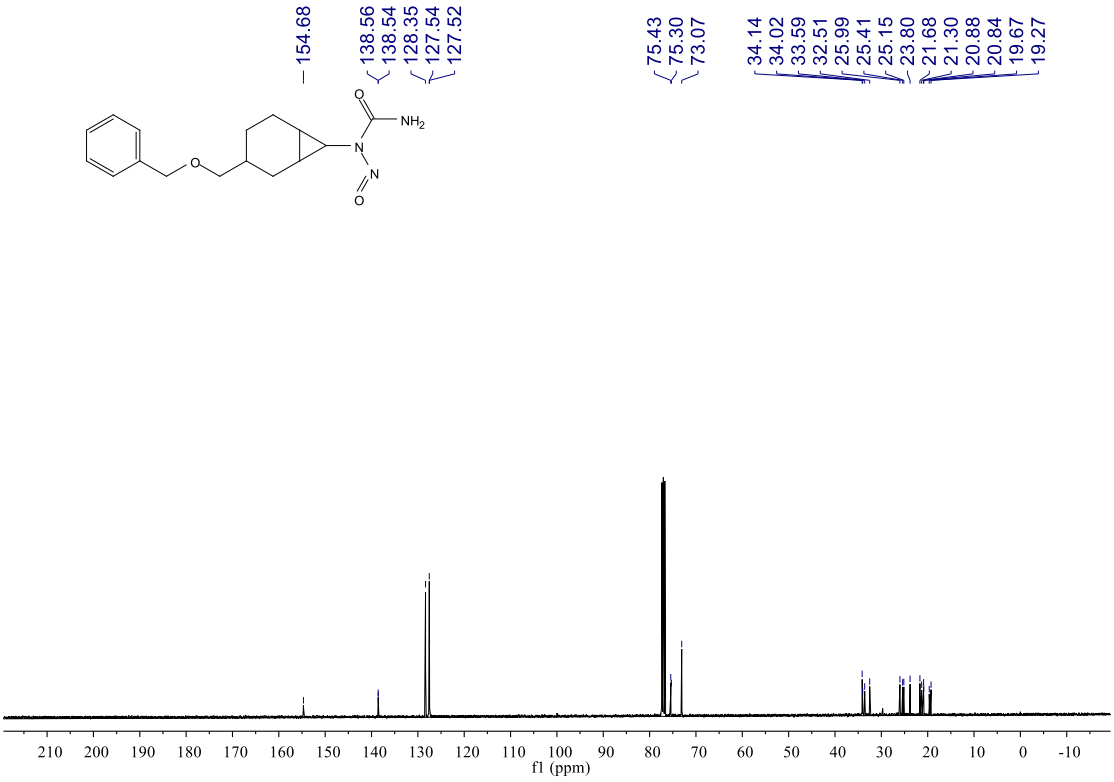
^{13}C NMR spectrum for **14f** ($CDCl_3$, 100 MHz)



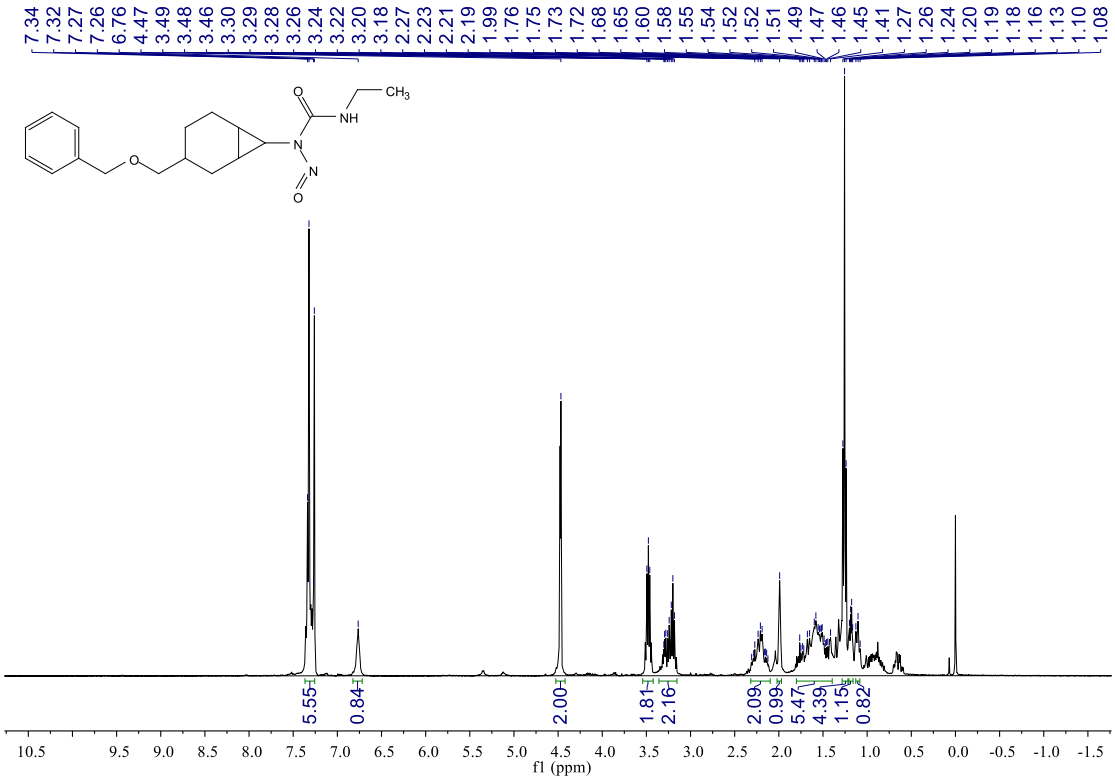
^{1}H NMR spectrum for **15a** ($CDCl_3$, 400 MHz)



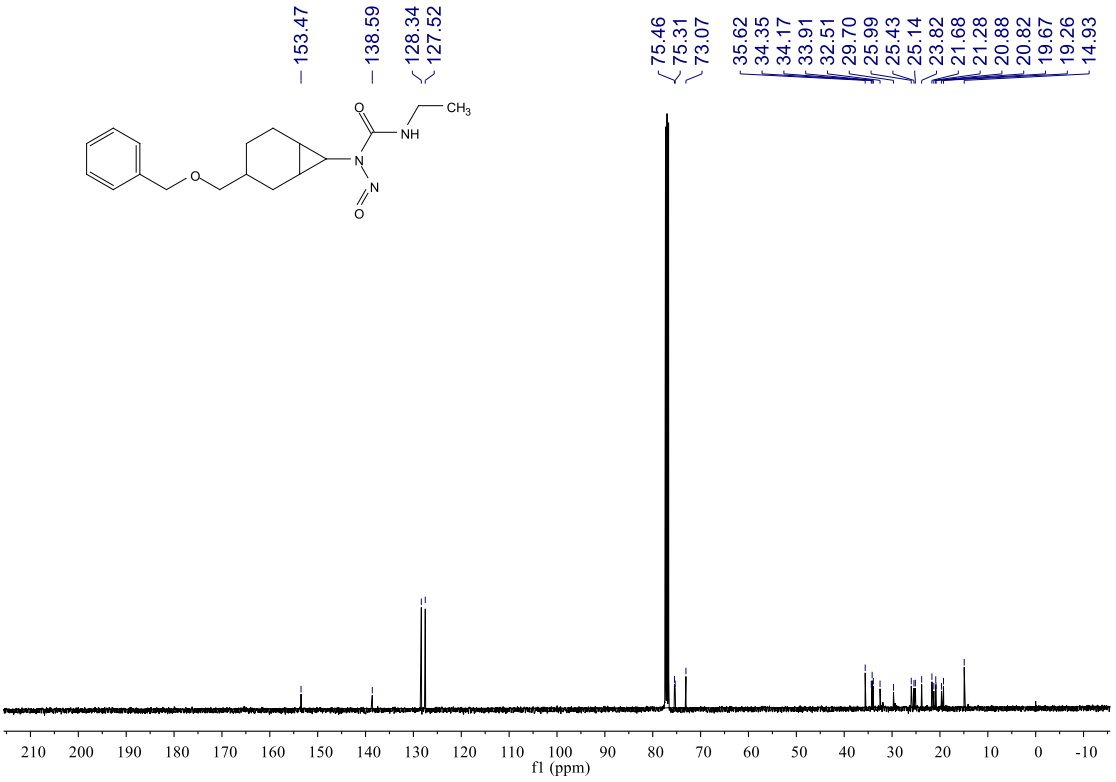
^{13}C NMR spectrum for **15a** ($CDCl_3$, 100 MHz)



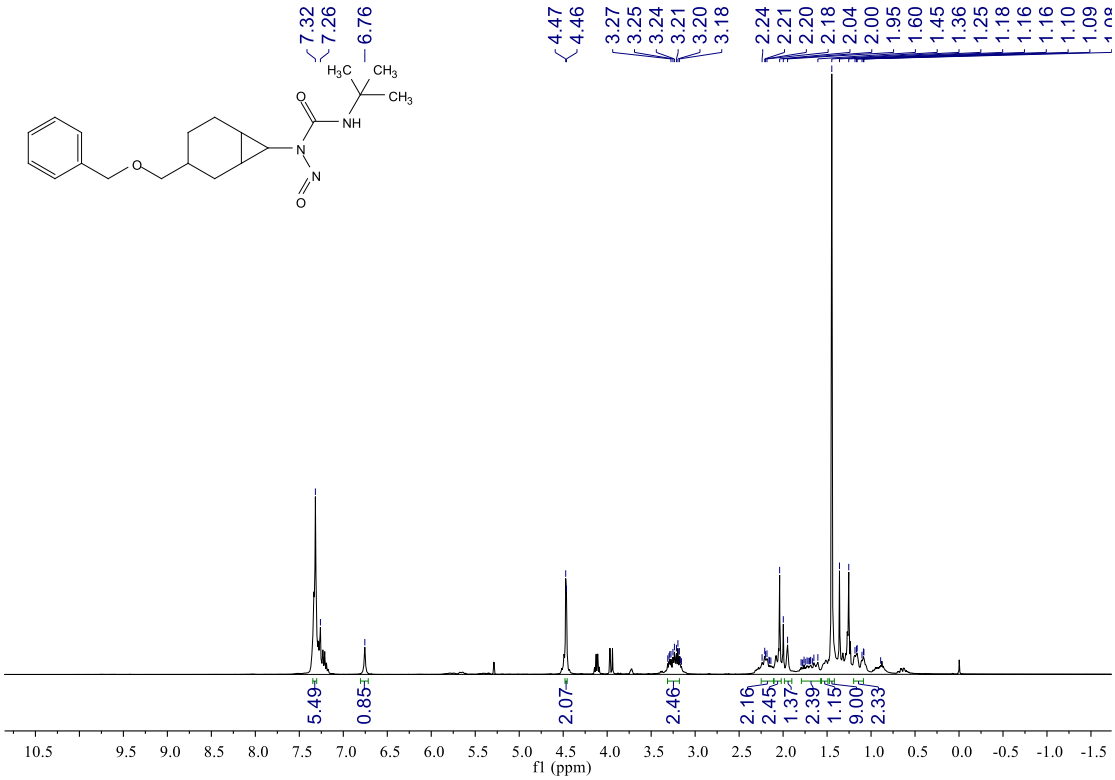
^{1}H NMR spectrum for **15b** ($CDCl_3$, 400 MHz)



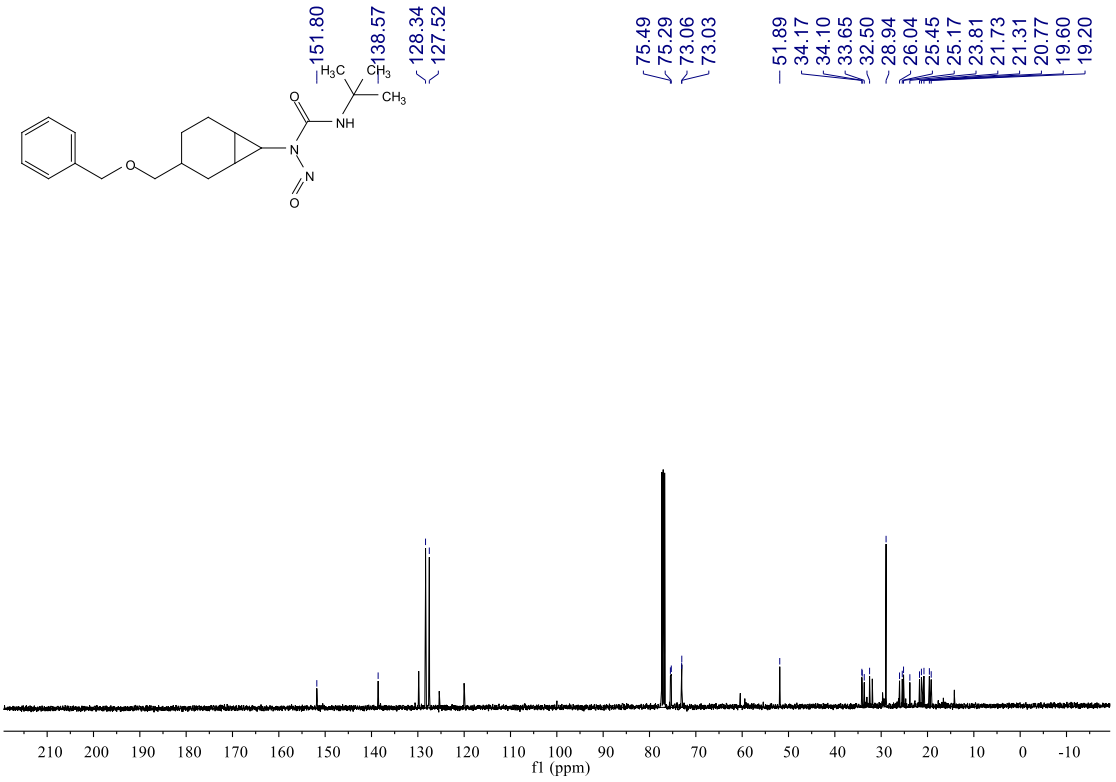
^{13}C NMR spectrum for **15b** ($CDCl_3$, 100 MHz)



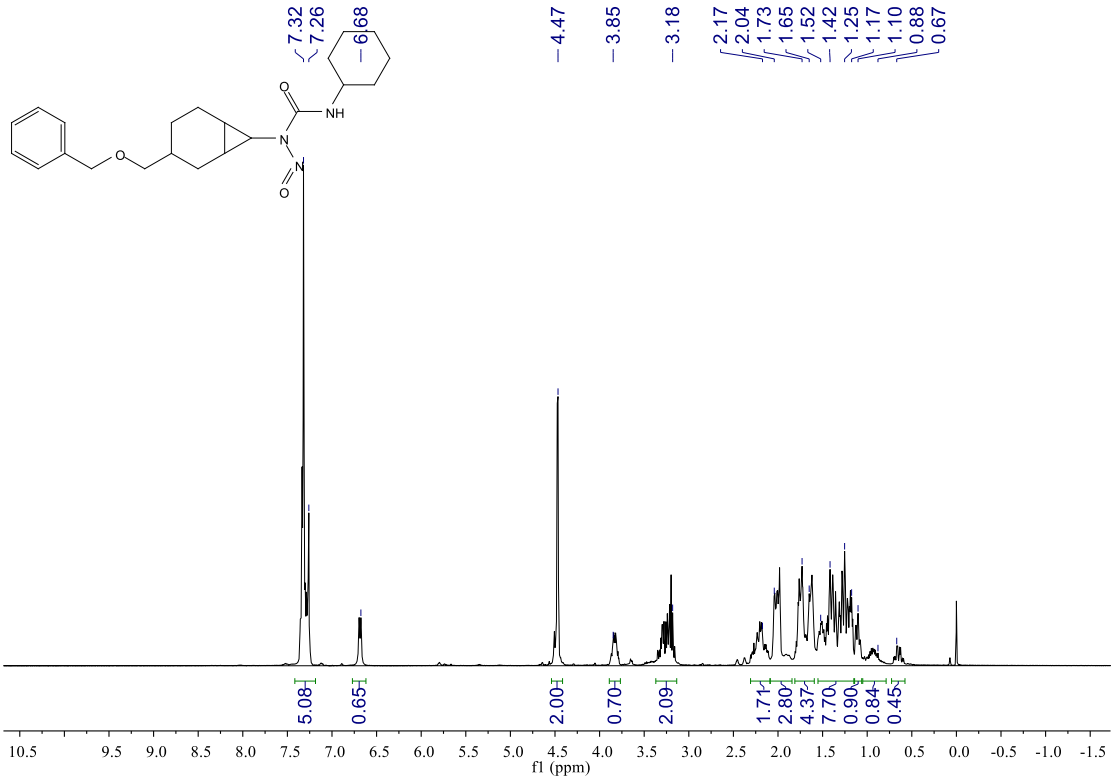
^{1}H NMR spectrum for **15c** ($CDCl_3$, 400 MHz)



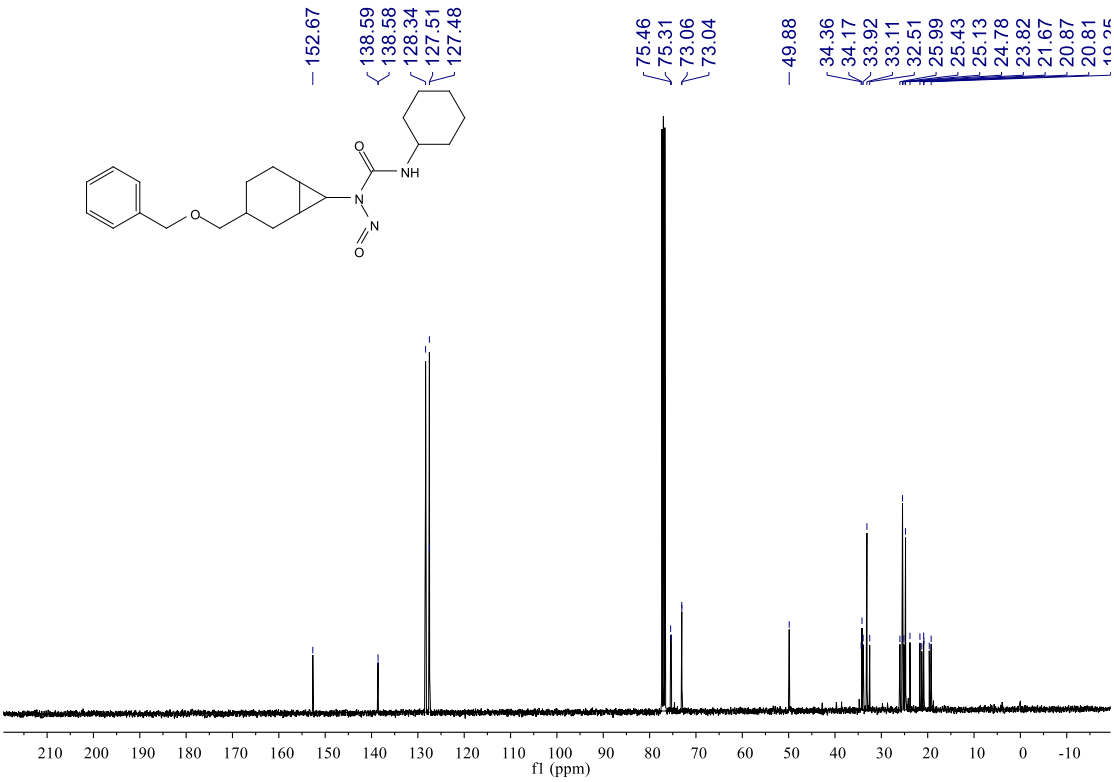
^{13}C NMR spectrum for **15c** ($CDCl_3$, 100 MHz)



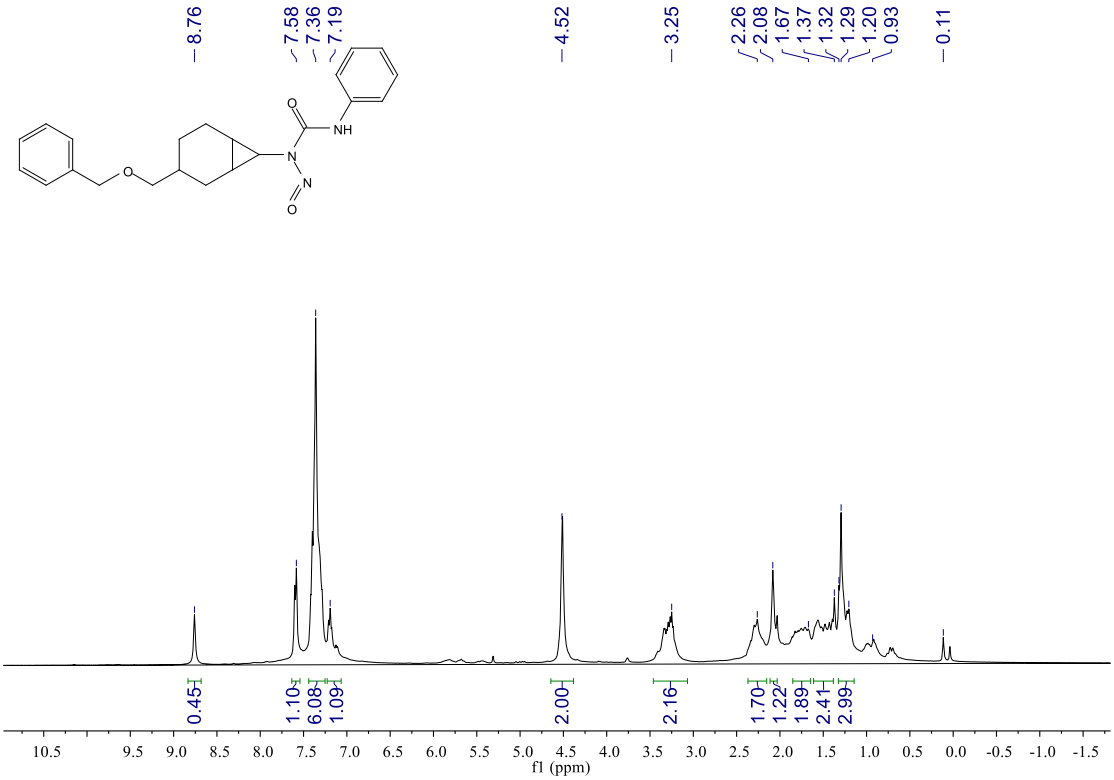
^{1}H NMR spectrum for **15d** ($CDCl_3$, 400 MHz)



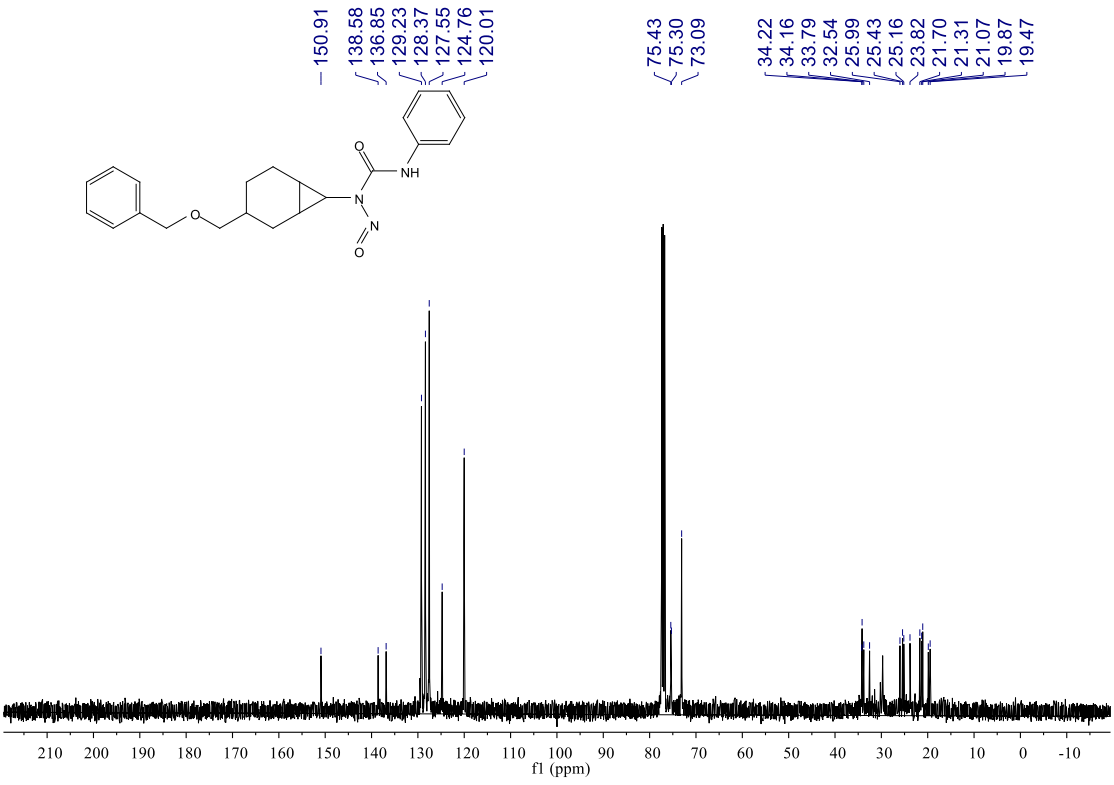
^{13}C NMR spectrum for **15d** ($CDCl_3$, 100 MHz)



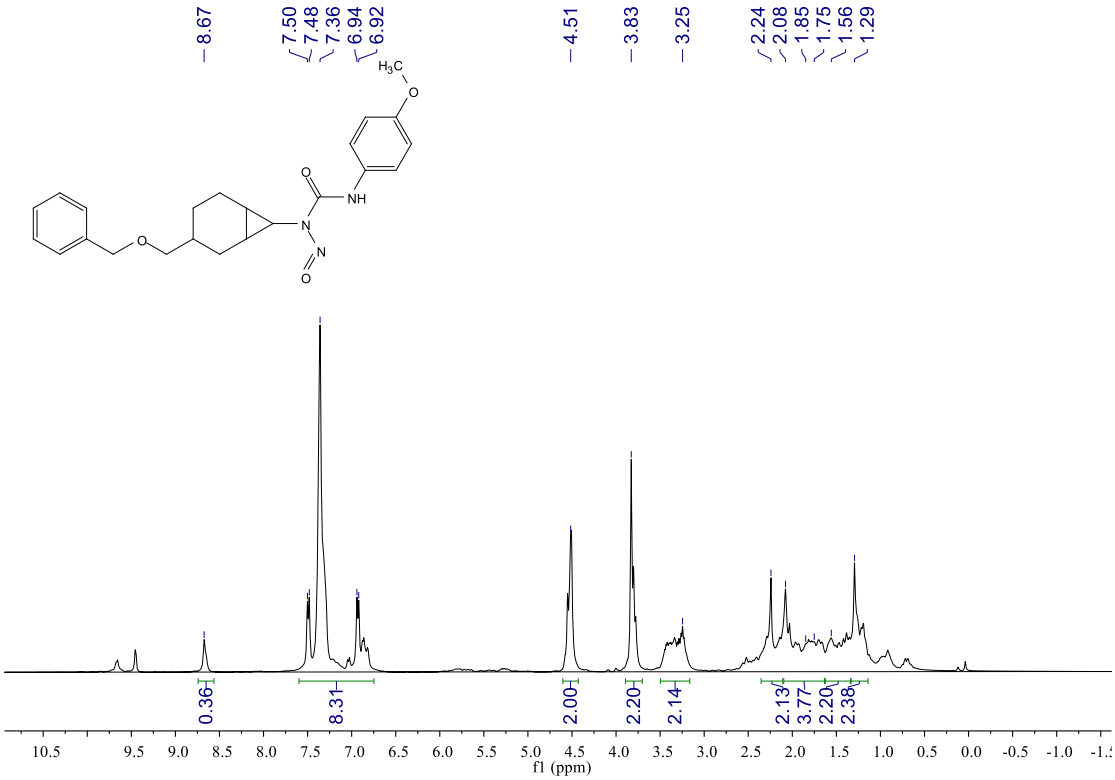
^{1}H NMR spectrum for **15e** ($CDCl_3$, 400 MHz)



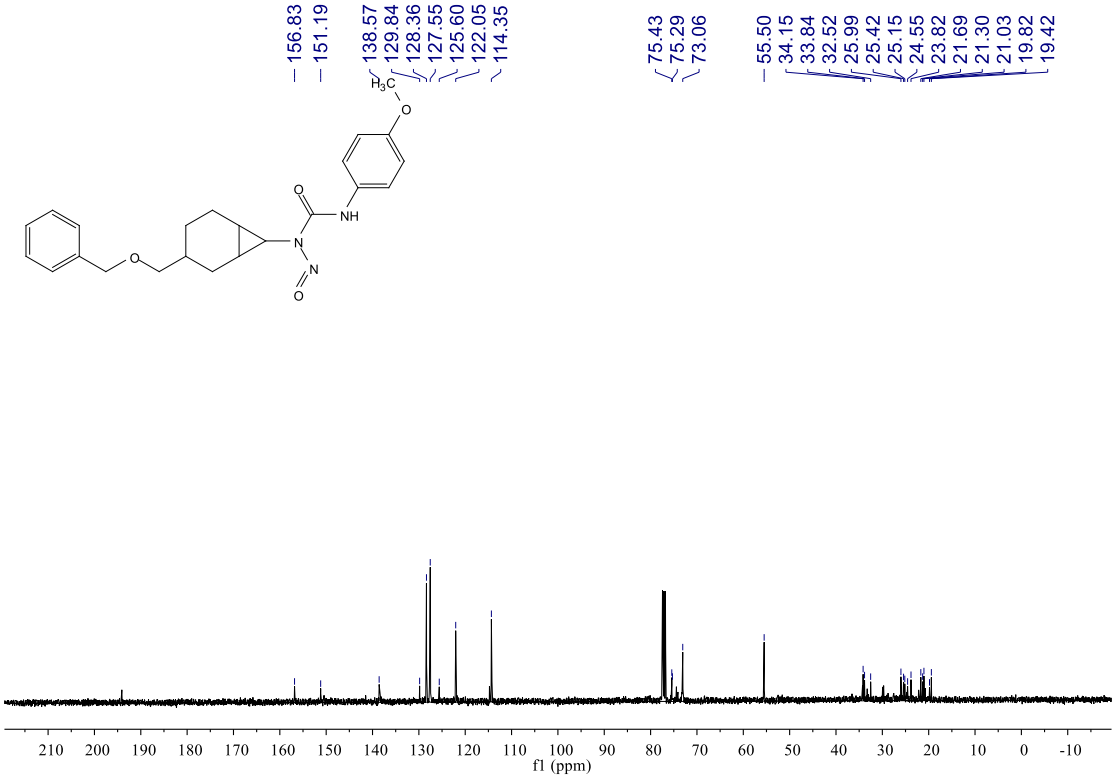
^{13}C NMR spectrum for **15e** ($CDCl_3$, 100 MHz)



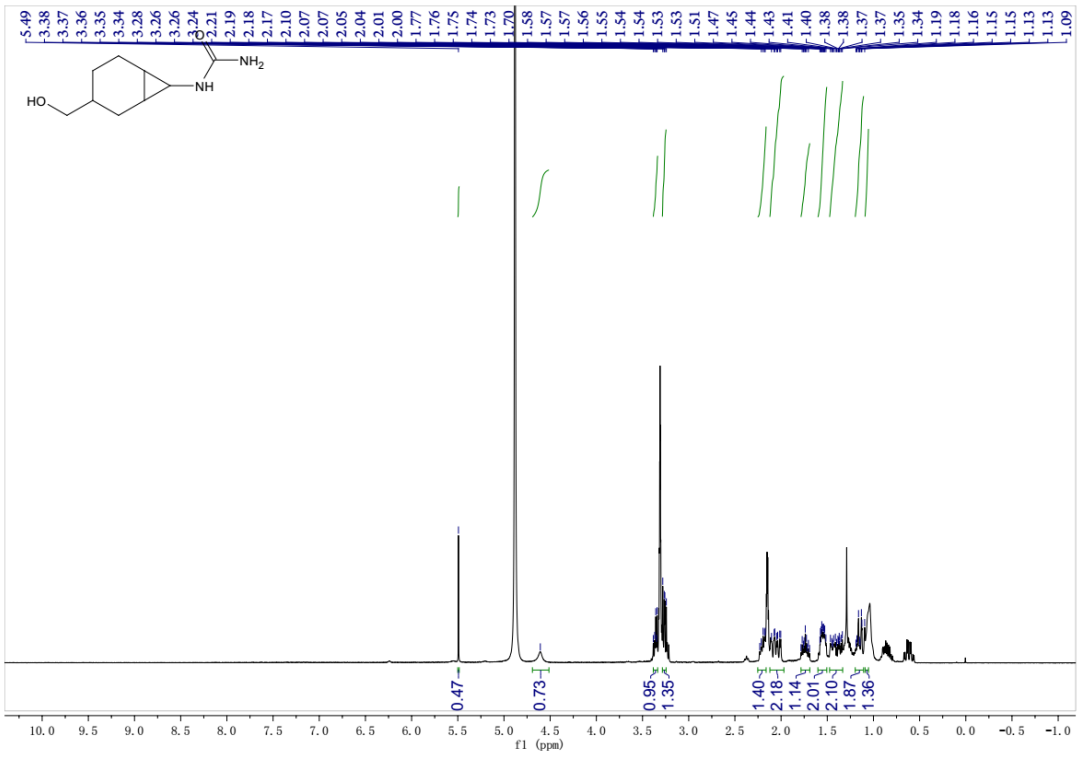
^{1}H NMR spectrum for **15f** ($CDCl_3$, 400 MHz)



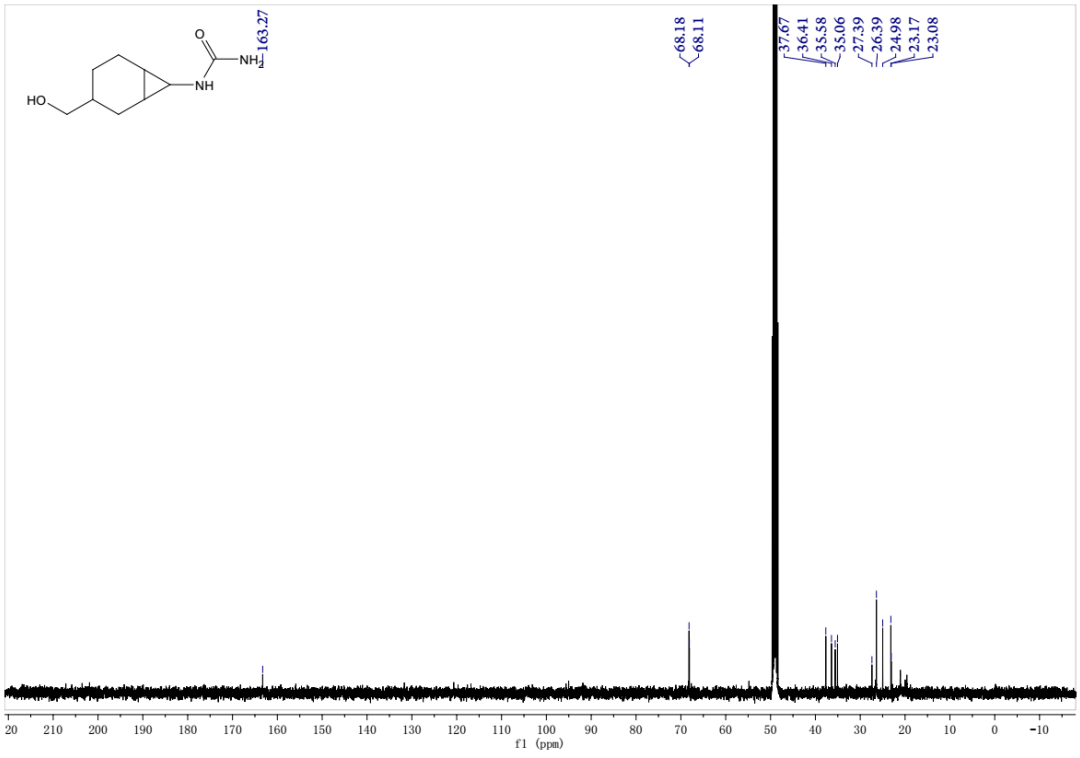
^{13}C NMR spectrum for **15f** ($CDCl_3$, 100 MHz)



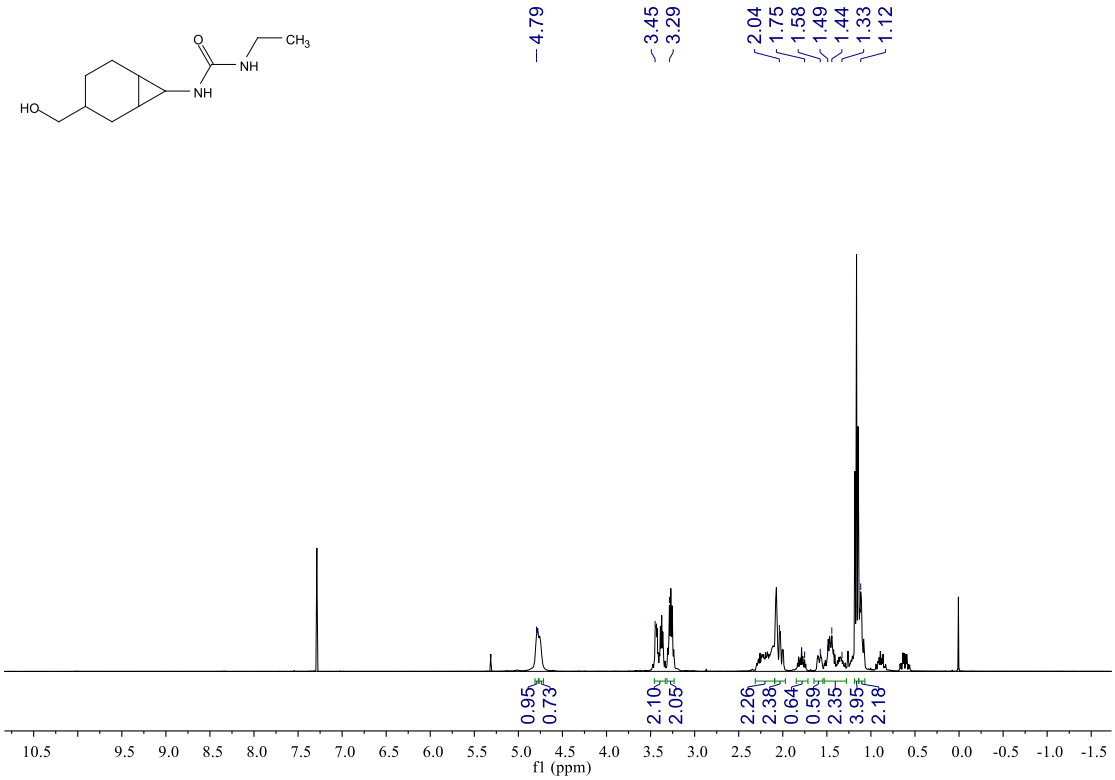
^{1}H NMR spectrum for **16a** (MeOD, 400 MHz)



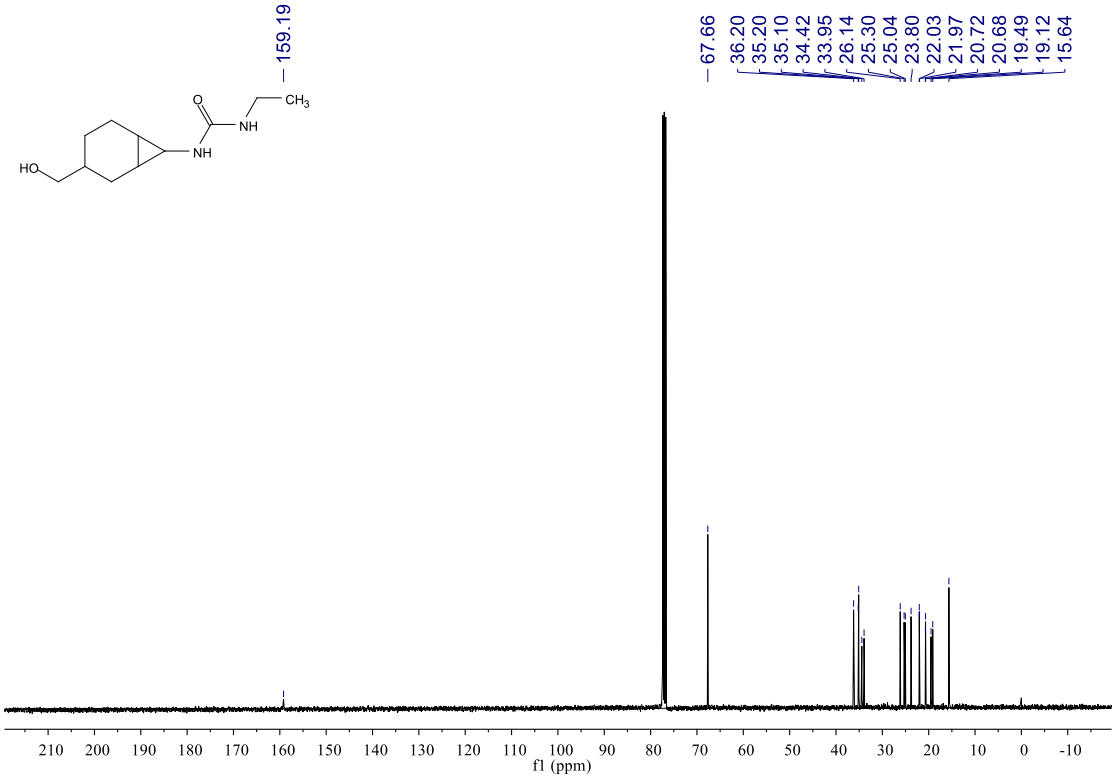
^{13}C NMR spectrum for **16a** (MeOD, 100 MHz)



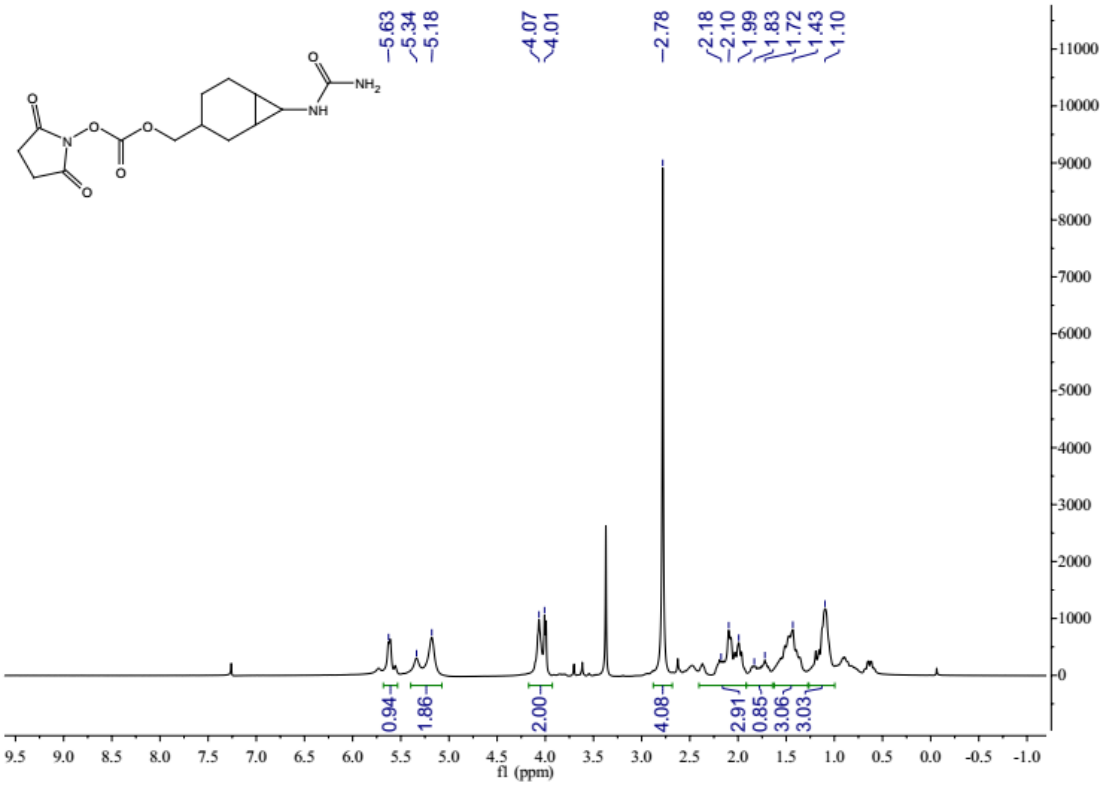
^{1}H NMR spectrum for **16b** ($CDCl_3$, 400 MHz)



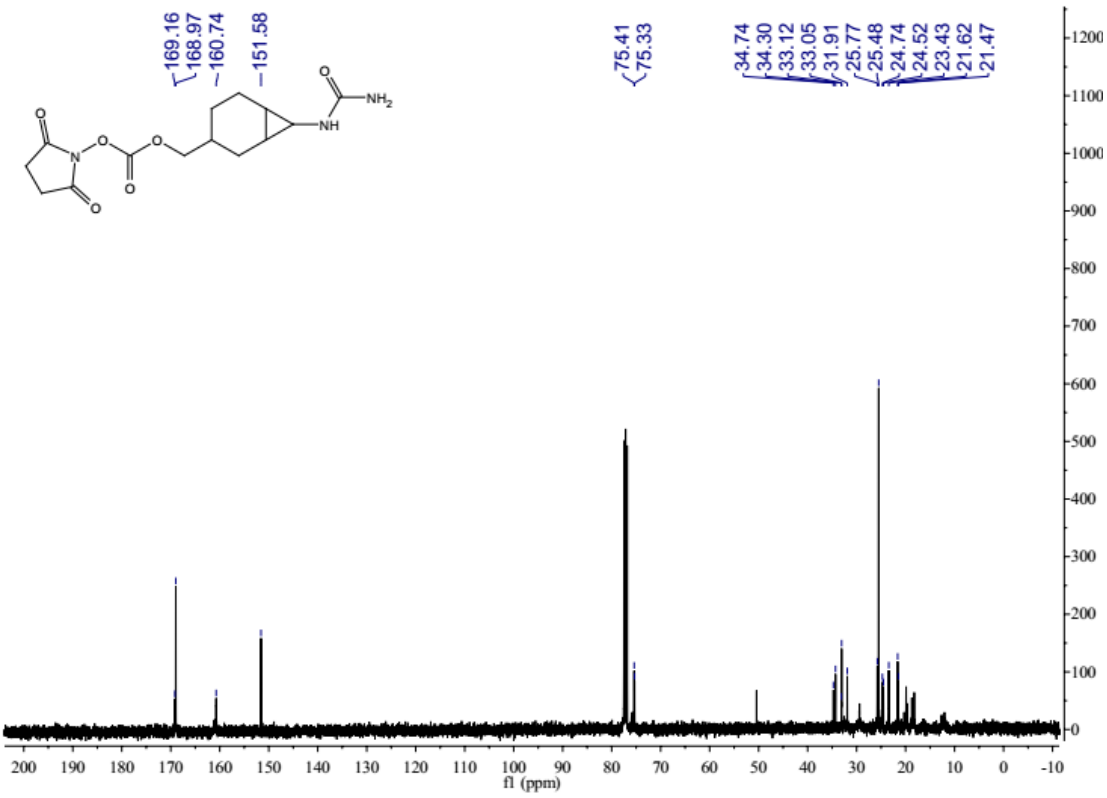
^{13}C NMR spectrum for **16b** ($CDCl_3$, 100 MHz)



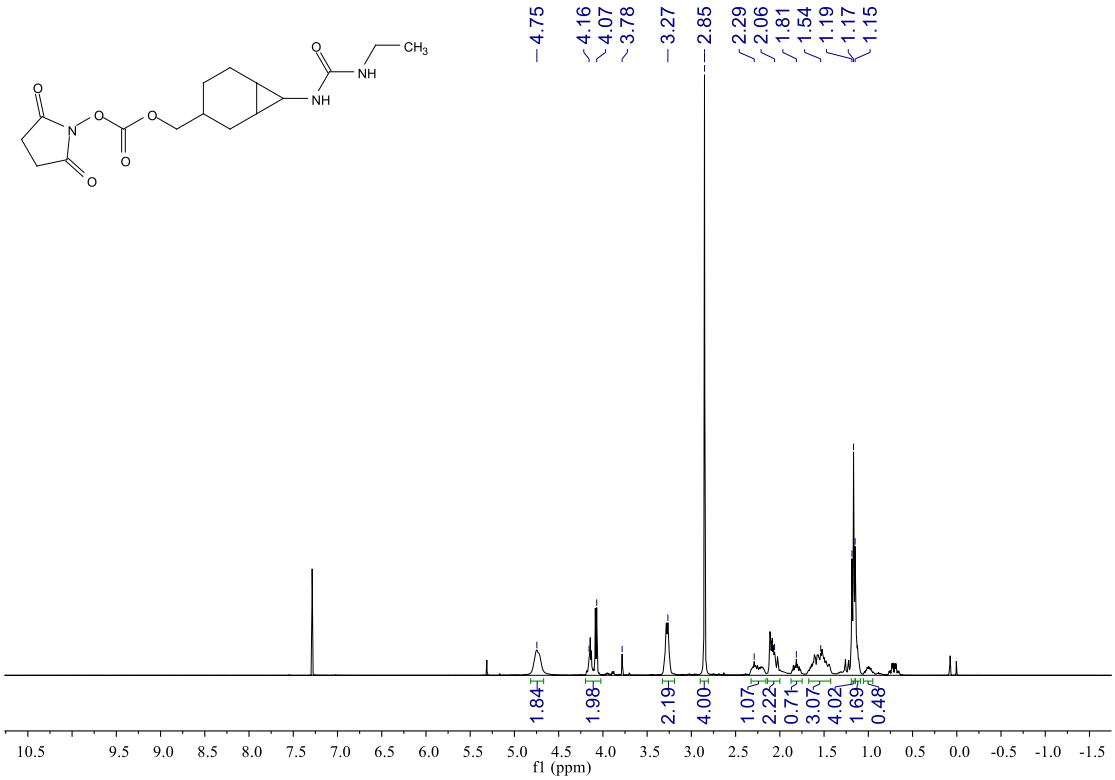
^{1}H NMR spectrum for **16a'** ($CDCl_3$, 400 MHz)



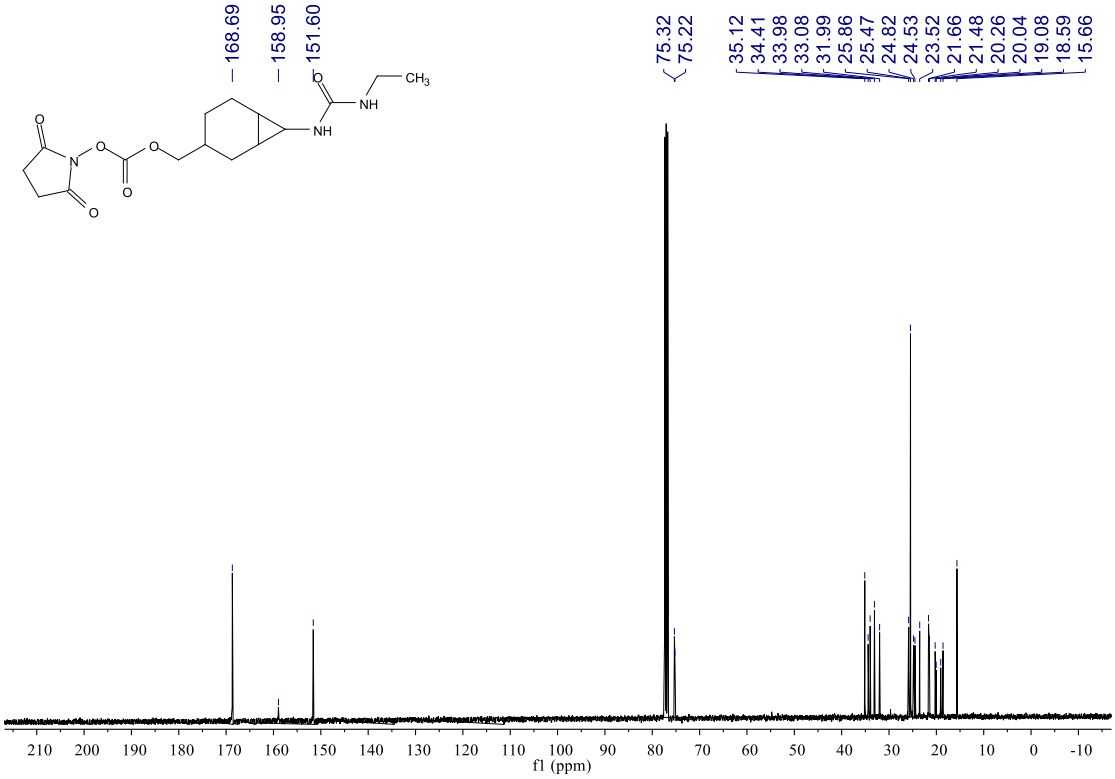
^{13}C NMR spectrum for **16a'** ($CDCl_3$, 100 MHz)



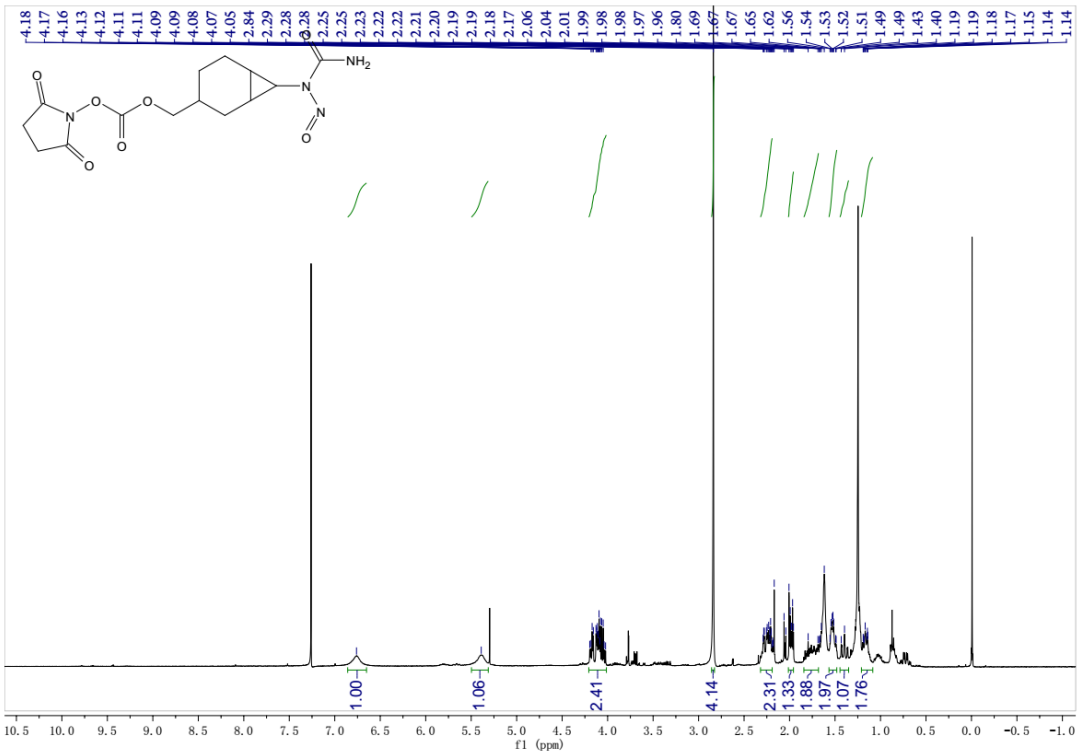
^{1}H NMR spectrum for **16b'** ($CDCl_3$, 400 MHz)



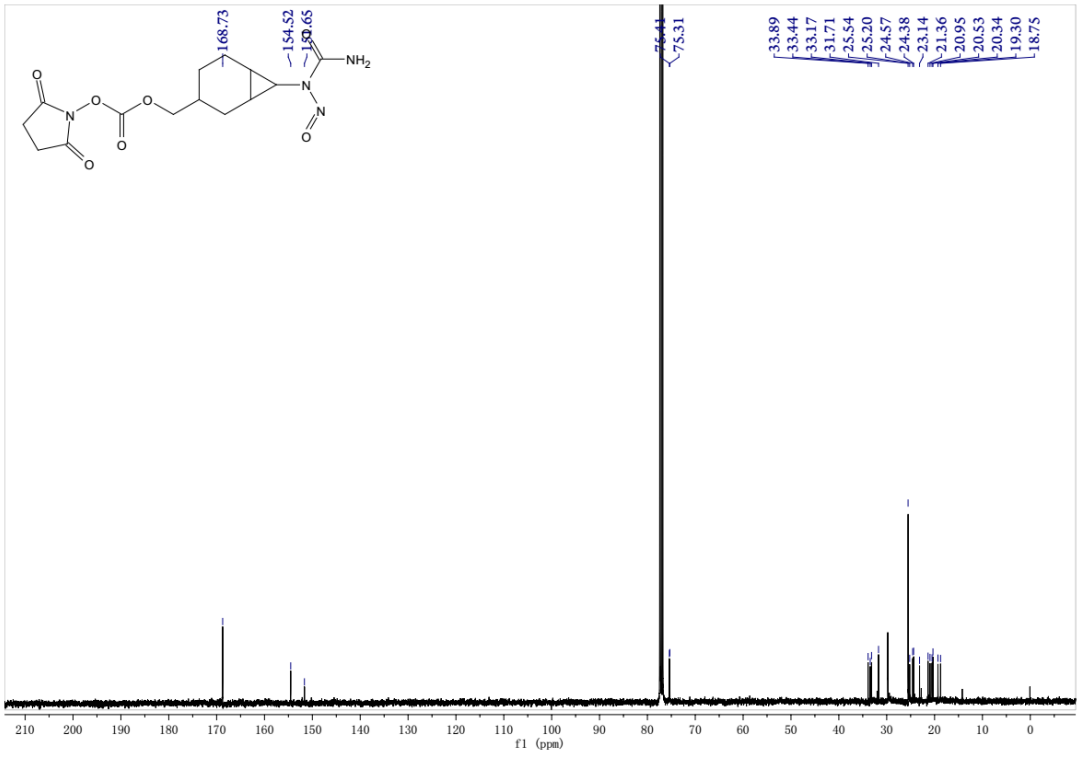
^{13}C NMR spectrum for **16b'** ($CDCl_3$, 100 MHz)



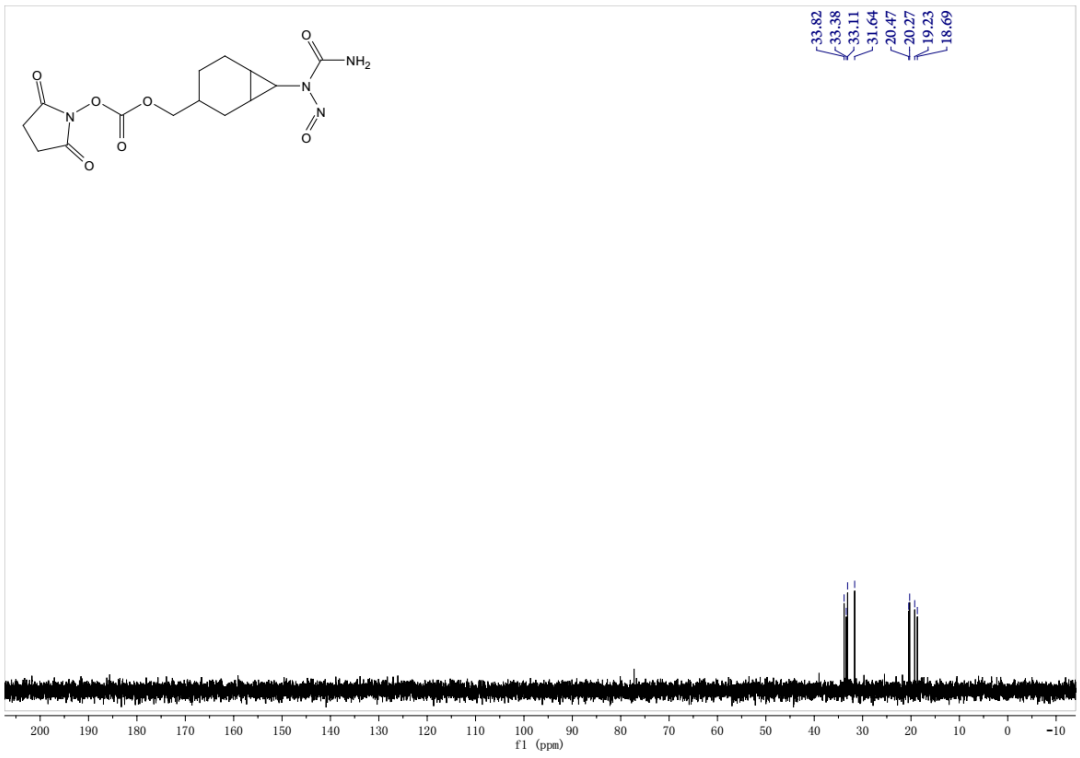
^{1}H NMR spectrum for **17a** ($CDCl_3$, 400 MHz)



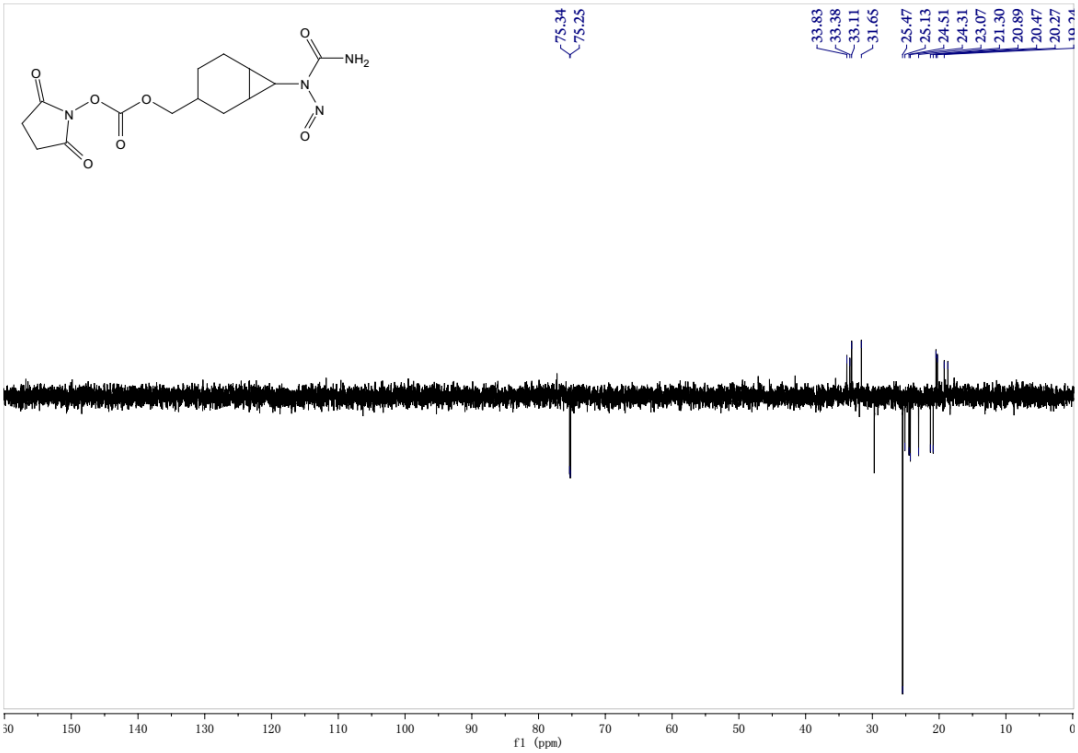
^{13}C NMR spectrum for **17a** ($CDCl_3$, 100 MHz)



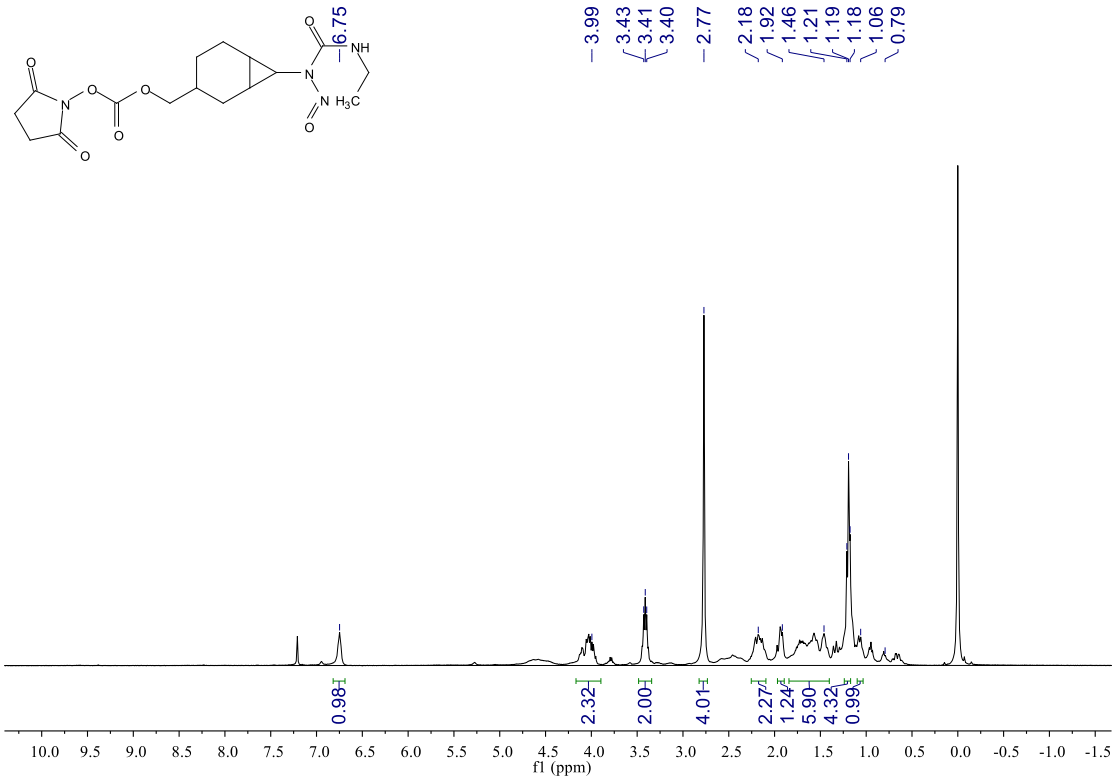
^{13}C DEPT-90 NMR spectrum for **17a** ($CDCl_3$, 100 MHz)



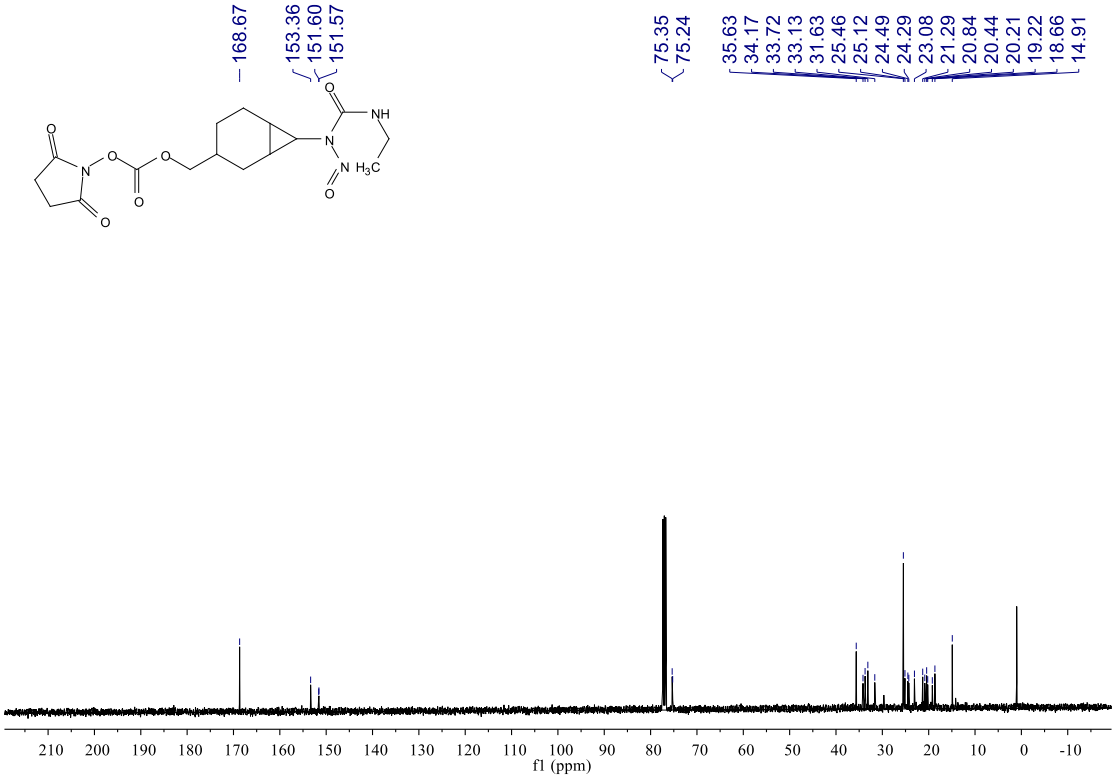
^{13}C DEPT-135 NMR spectrum for **17a** ($CDCl_3$, 100 MHz)



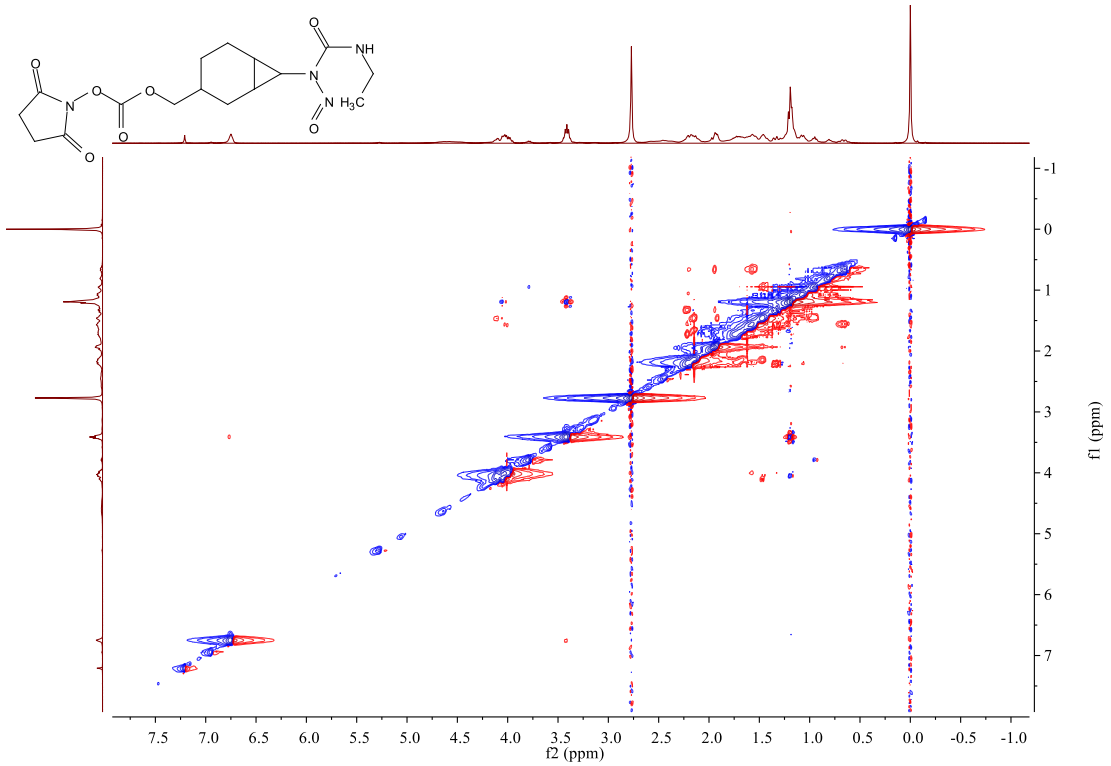
^{1}H NMR spectrum for **17b** ($CDCl_3$, 400 MHz)



^{13}C NMR spectrum for **17b** ($CDCl_3$, 100 MHz)

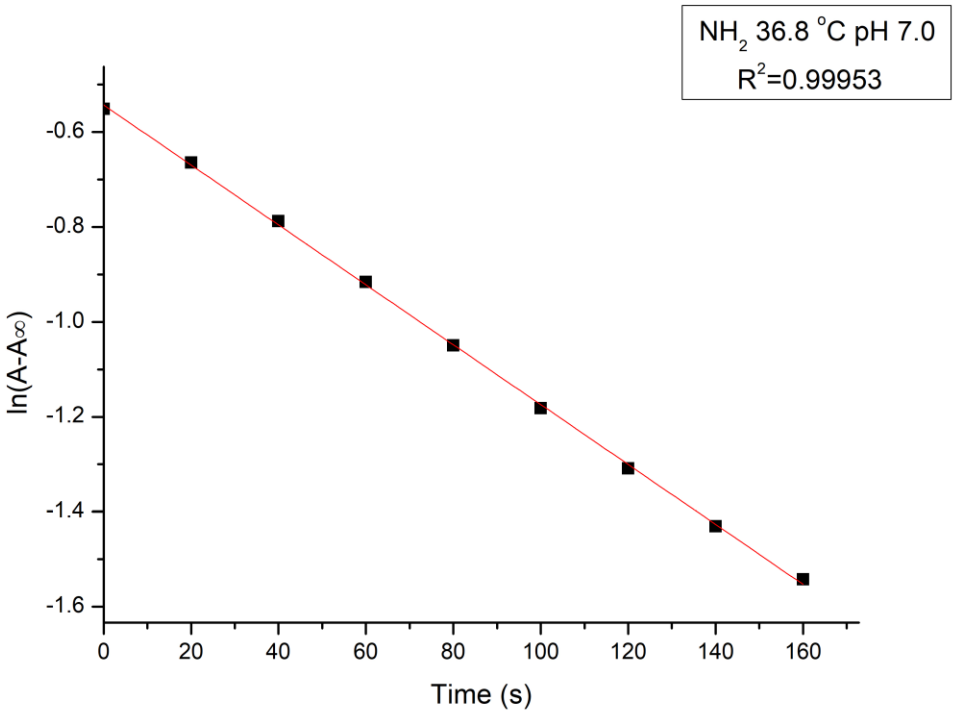


NOESYPHSW spectrum for **17b** ($CDCl_3$, 400 MHz)

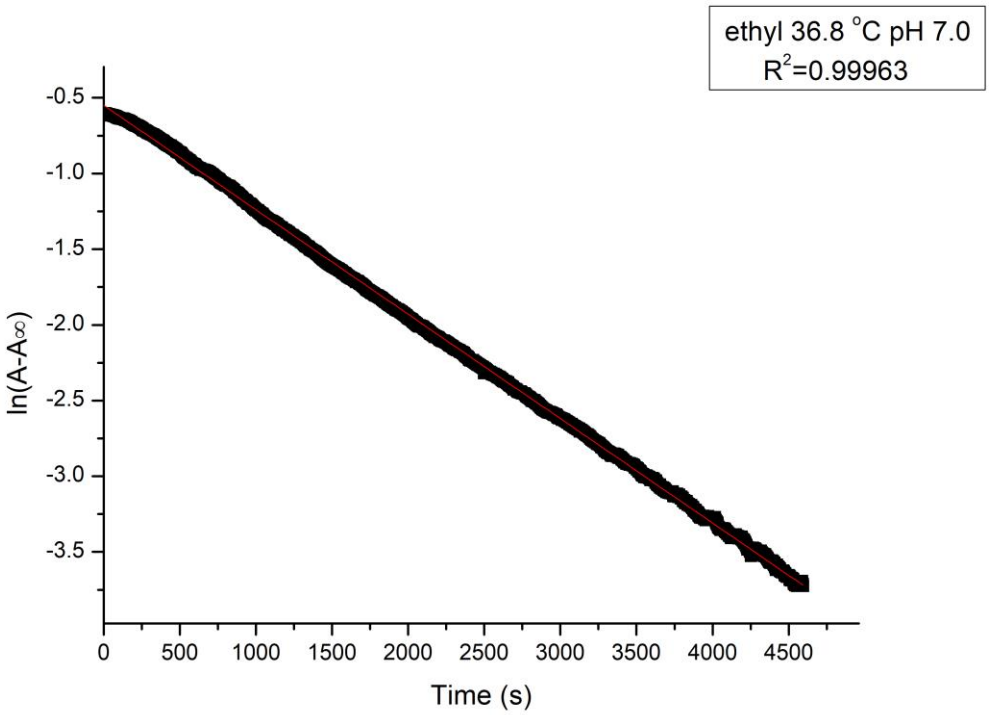


7. Appendix

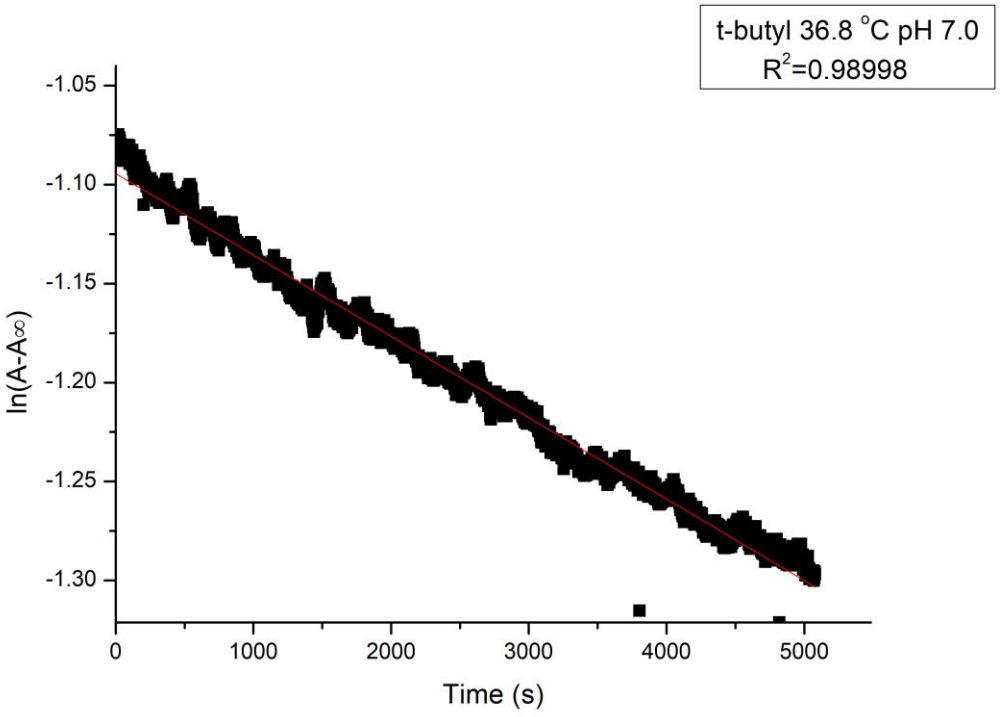
Kinetic data for 3.2 Determination the Kinetics of Hydrolysis of 15a-f by UV-Vis Method (pH = 7.0)



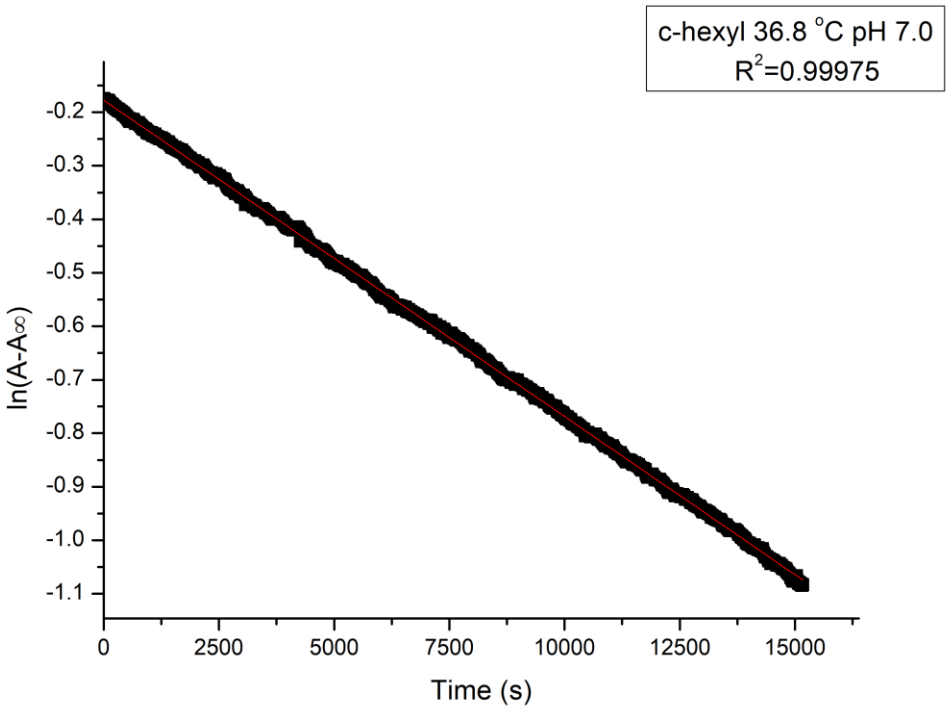
Relationship between ln (A - A∞) and time for the decomposition of **15a** in pH 7.0 at 36.8 ℃



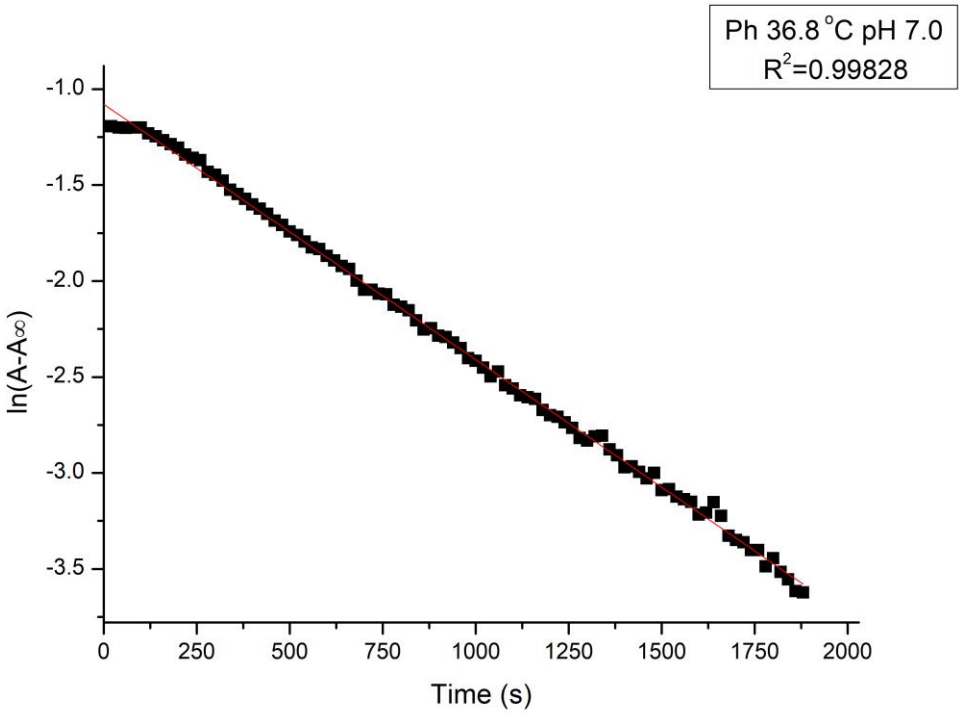
Relationship between ln (A - A∞) and time for the decomposition of **15b** in pH 7.0 at 36.8 ℃



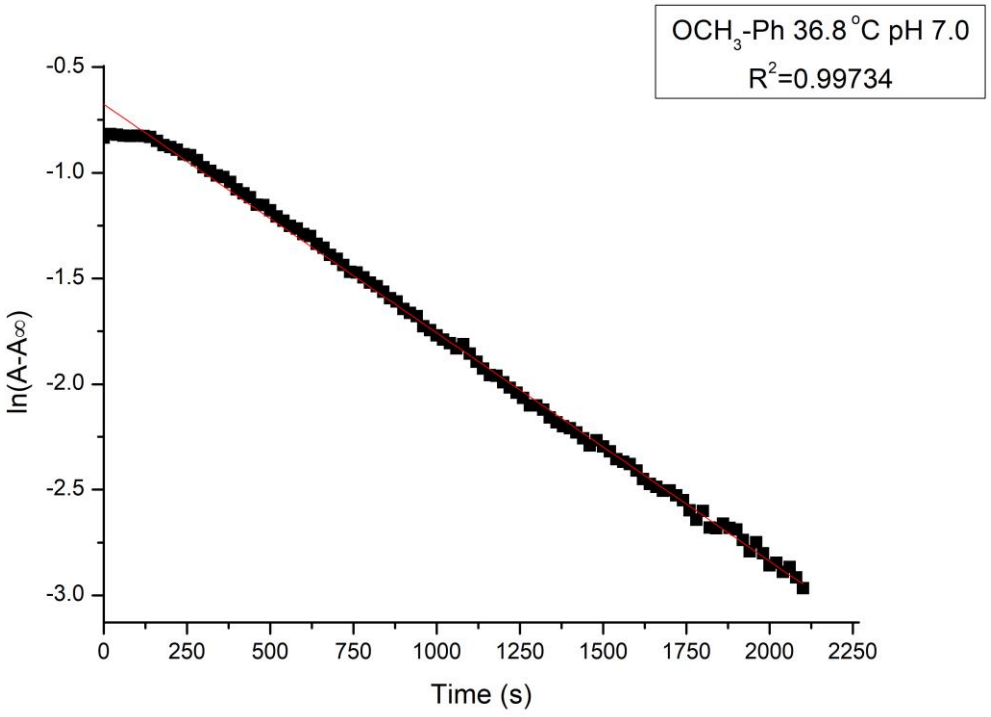
Relationship between ln (A - A_∞) and time for the decomposition of **15c** in pH 7.0 at 36.8 ℃



Relationship between ln (A - A_∞) and time for the decomposition of **15d** in pH 7.0 at 36.8 ℃

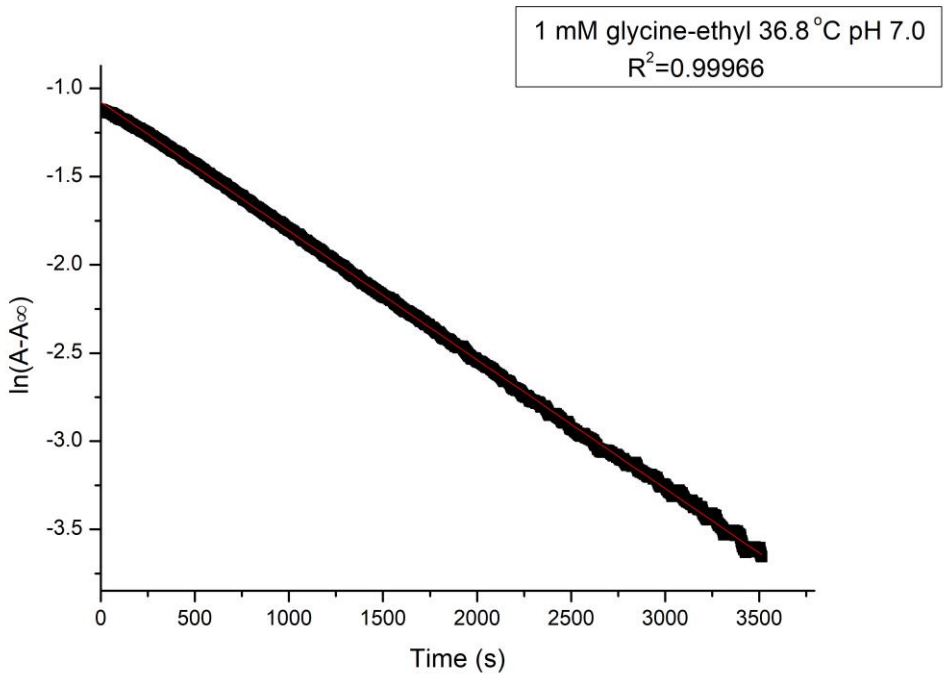


Relationship between ln (A - A_∞) and time for the decomposition of **15e** in pH 7.0 at 36.8 ℃

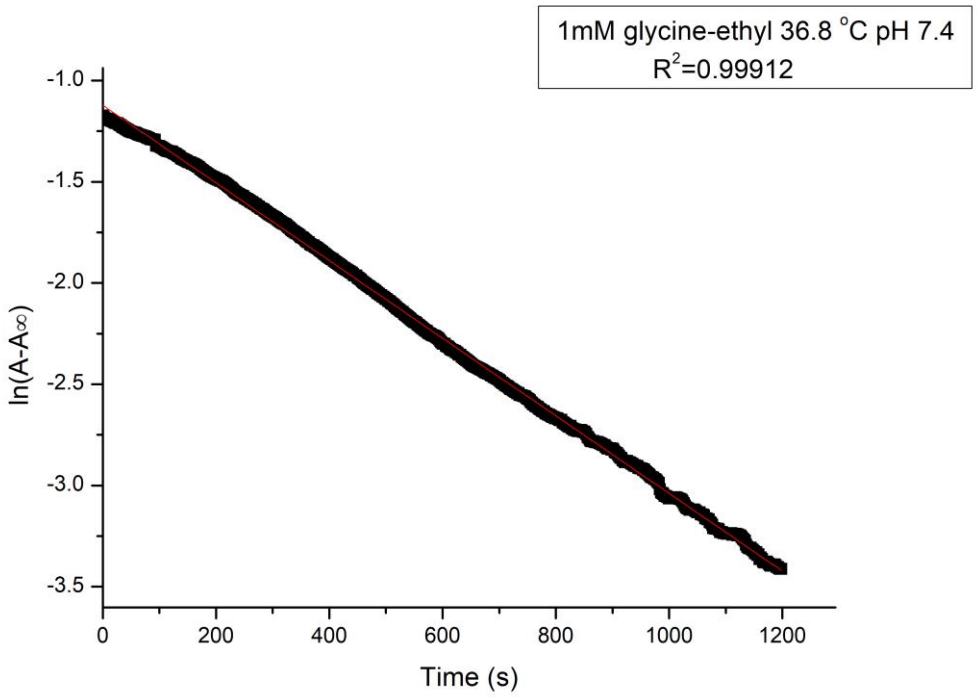


Relationship between ln (A - A_∞) and time for the decomposition of **15f** in pH 7.0 at 36.8 ℃

Kinetic data for 3.3 Determination the Kinetics of Hydrolysis of 15b with different pH (pH=7.0, 7.4 and 8.0 under 36.8 ℃) and Biological Nucleophiles by UV-Vis Method

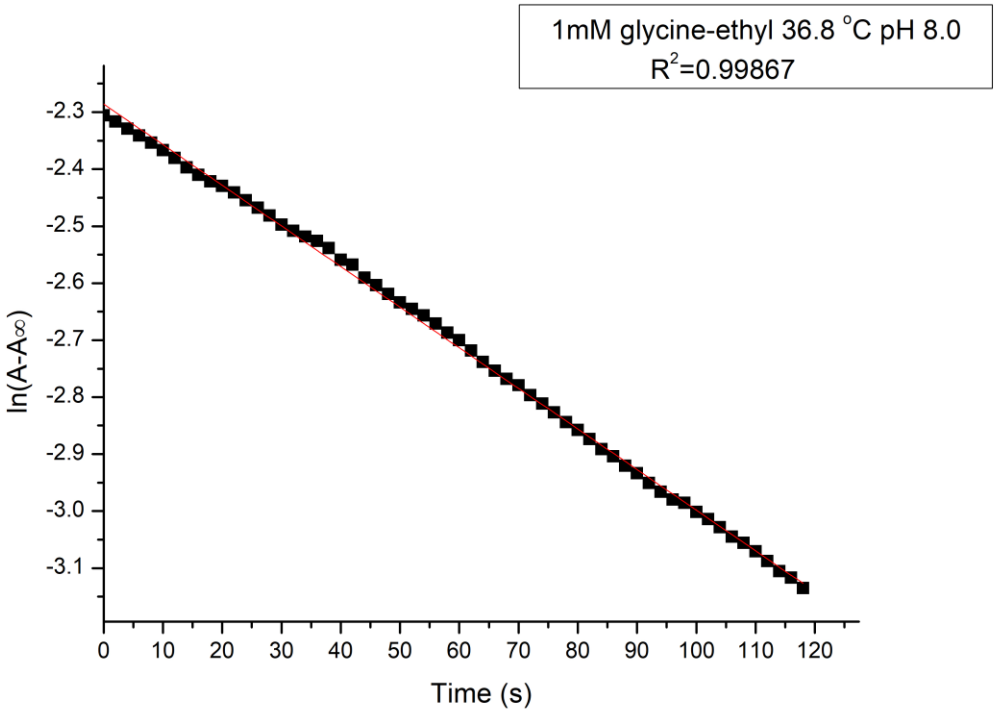


Relationship between ln (A - A_∞) and time for the decomposition of **15b** in pH 7.0 at 36.8 °C with 1 mM glycine

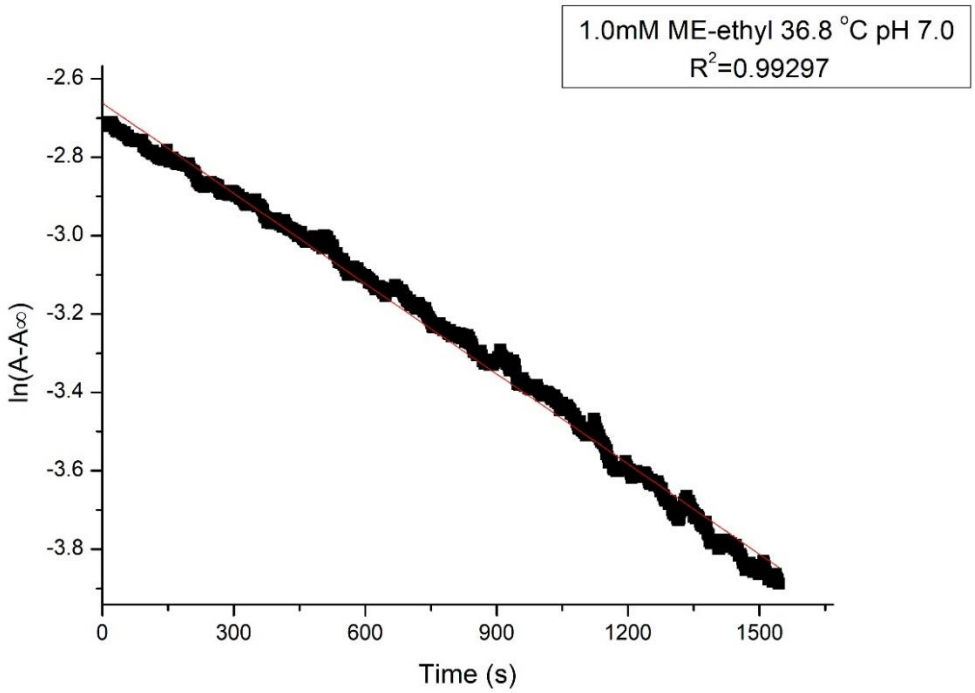


Relationship between ln (A - A_∞) and time for the decomposition of **15b** in pH 7.4 at 36.8 °C

with 1 mM glycine

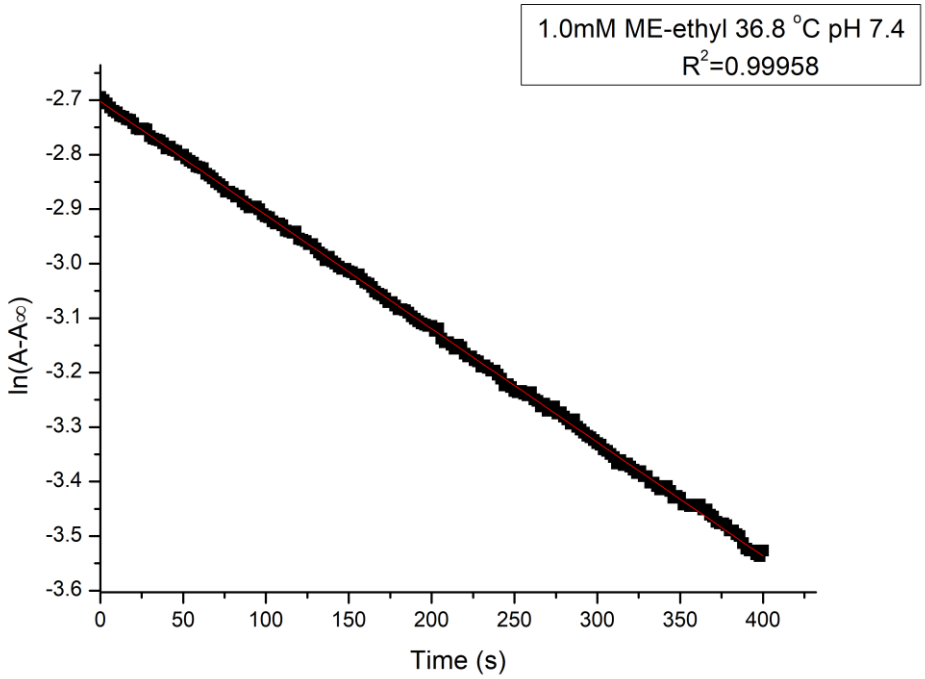


Relationship between ln (A - A_∞) and time for the decomposition of **15b** in pH 8.0 at 36.8 °C with 1 mM glycine

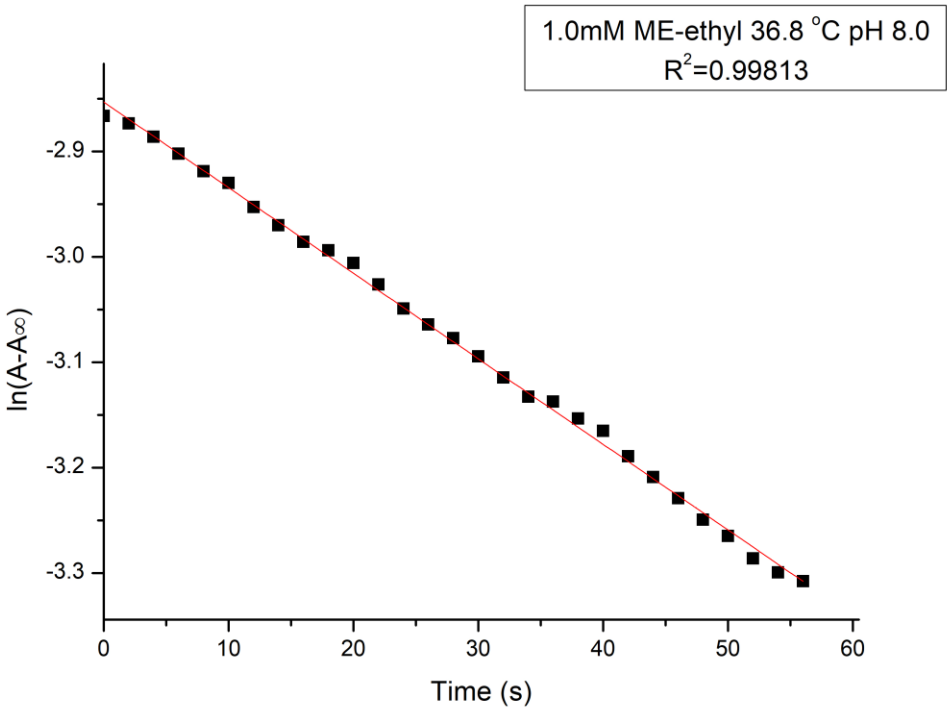


Relationship between ln (A - A_∞) and time for the decomposition of **15b** in pH 7.0 at 36.8 °C

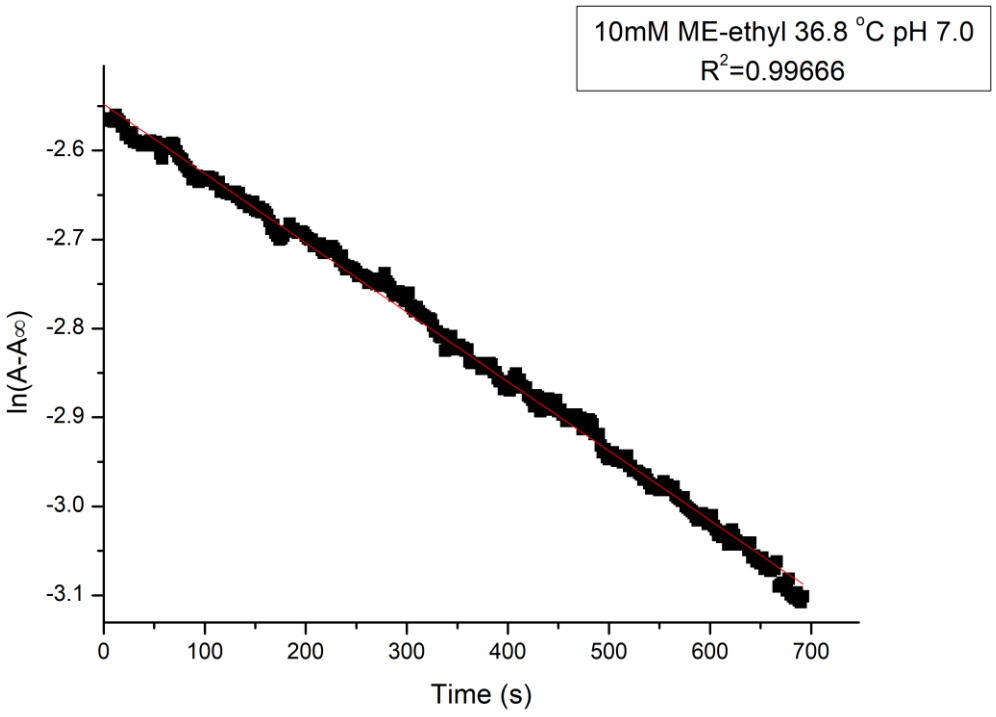
with 1 mM 2-Mercaptoethanol



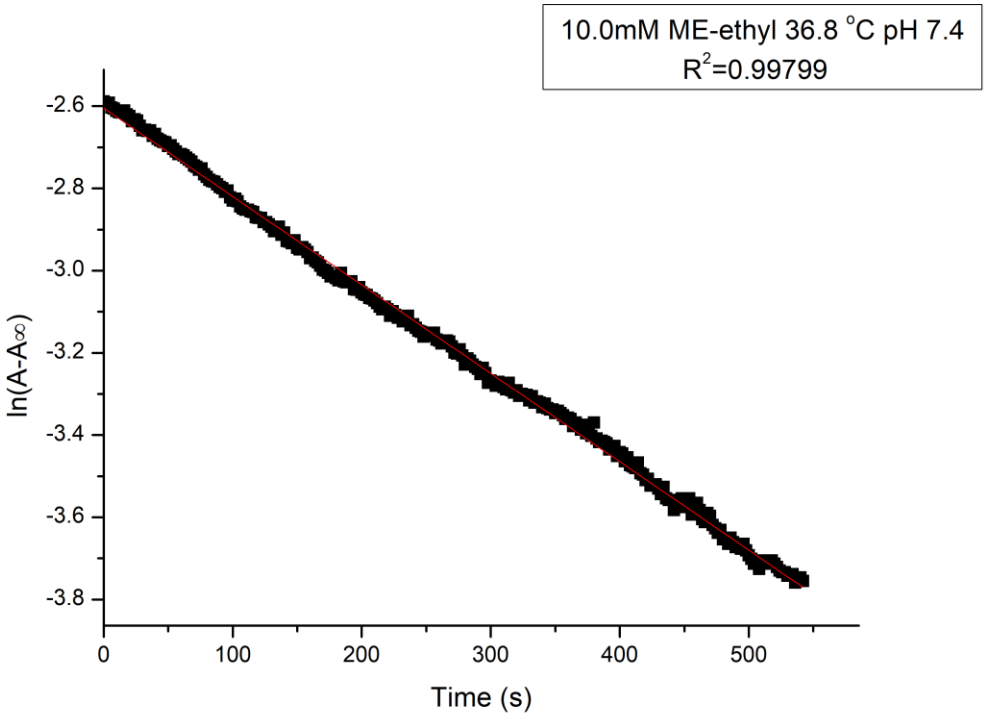
Relationship between ln (A - A∞) and time for the decomposition of **15b** in pH 7.4 at 36.8 °C with 1 mM 2-Mercaptoethanol



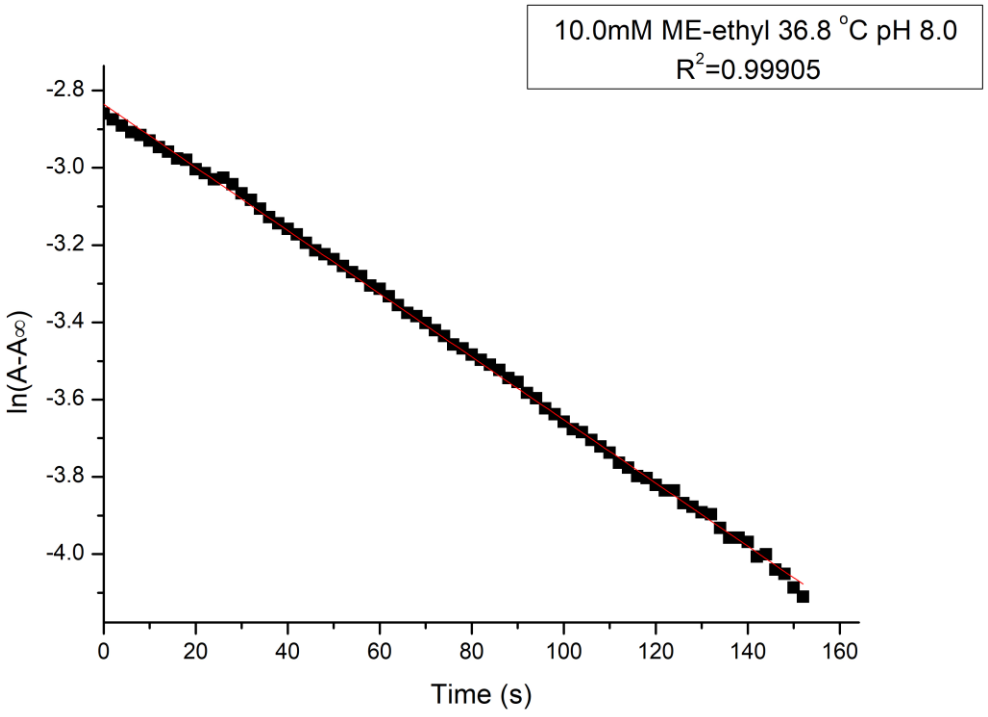
Relationship between ln (A - A∞) and time for the decomposition of **15b** in pH 8.0 at 36.8 °C with 1 mM 2-Mercaptoethanol



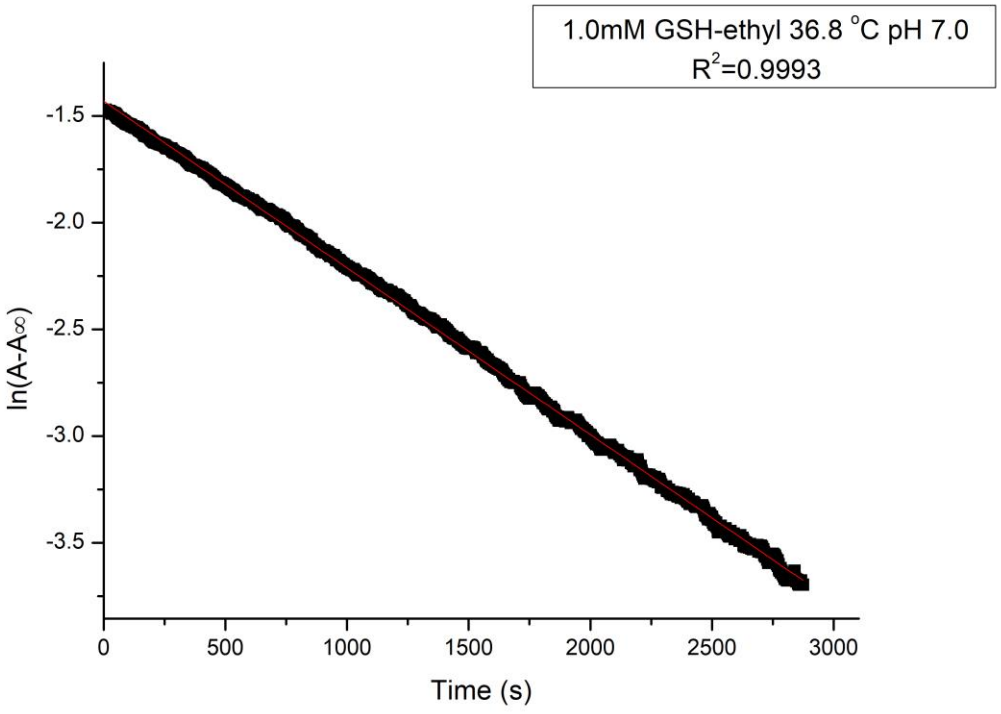
Relationship between ln (A - A_∞) and time for the decomposition of **15b** in pH 7.0 at 36.8 °C with 10 mM 2-Mercaptoethanol



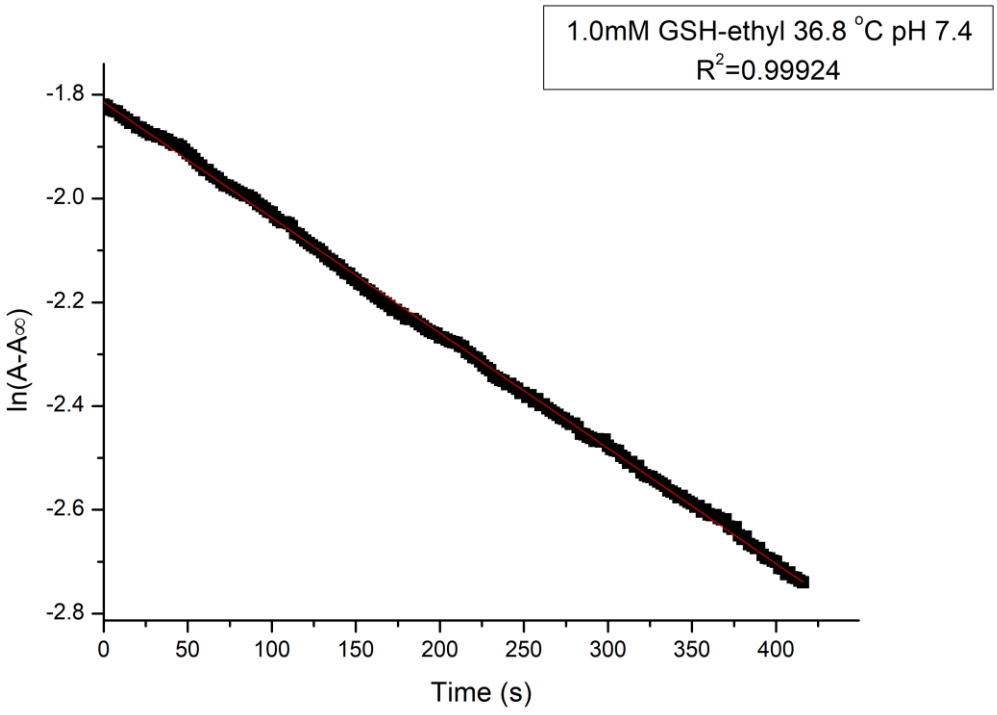
Relationship between ln (A - A_∞) and time for the decomposition of **15b** in pH 7.4 at 36.8 °C with 10 mM 2-Mercaptoethanol



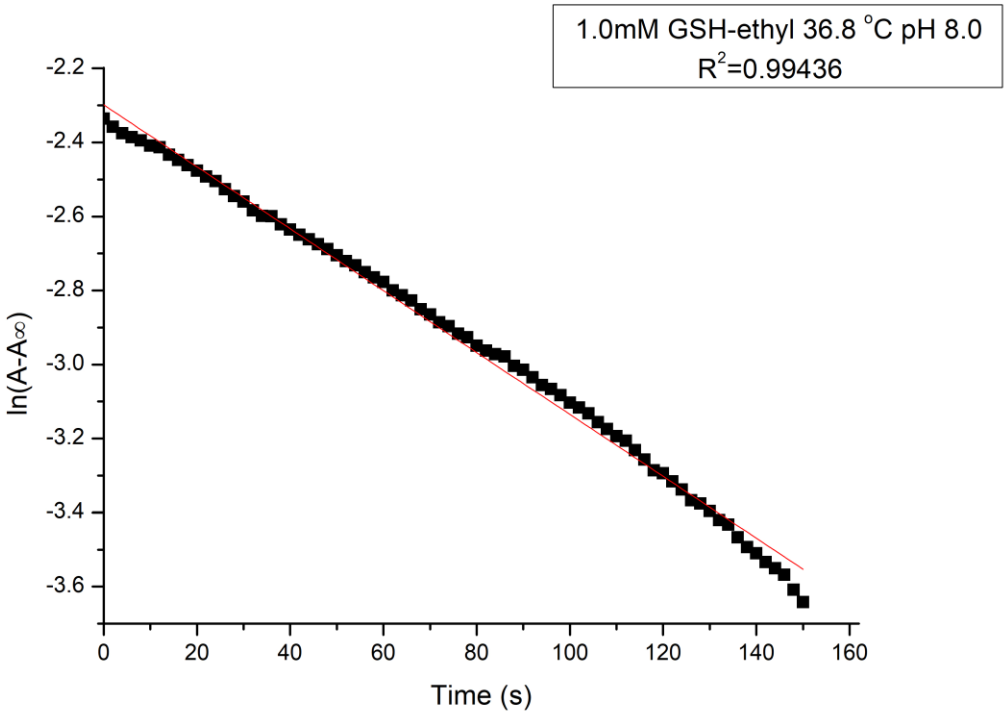
Relationship between ln (A - A_∞) and time for the decomposition of **15b** in pH 8.0 at 36.8 °C with 10 mM 2-Mercaptoethanol



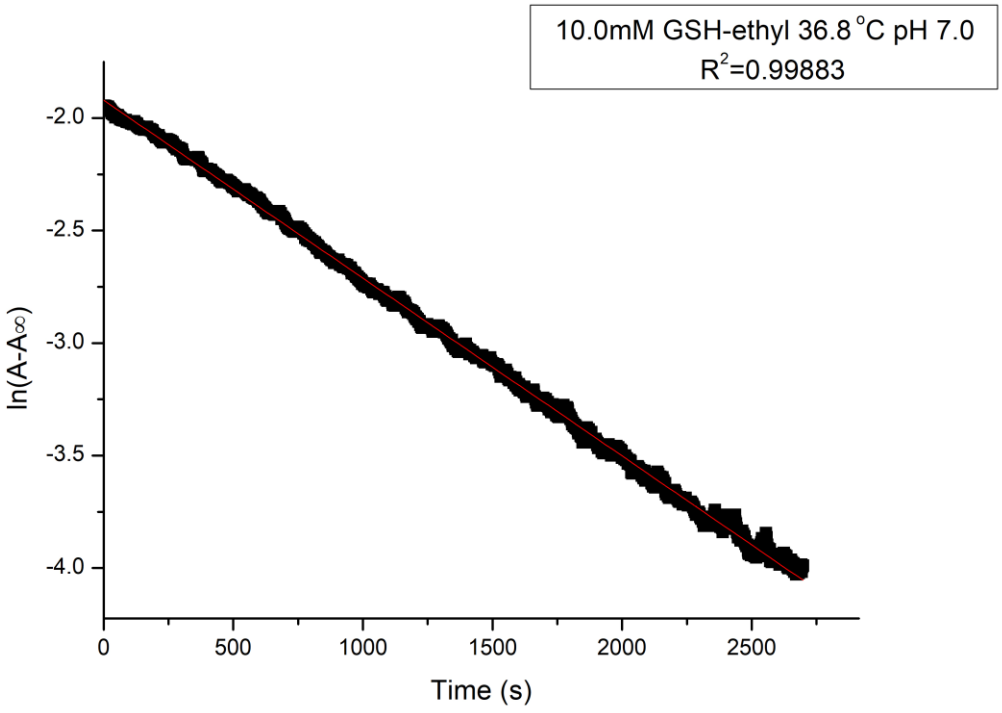
Relationship between ln (A - A_∞) and time for the decomposition of **15b** in pH 7.0 at 36.8 °C with 1 mM Glutathione



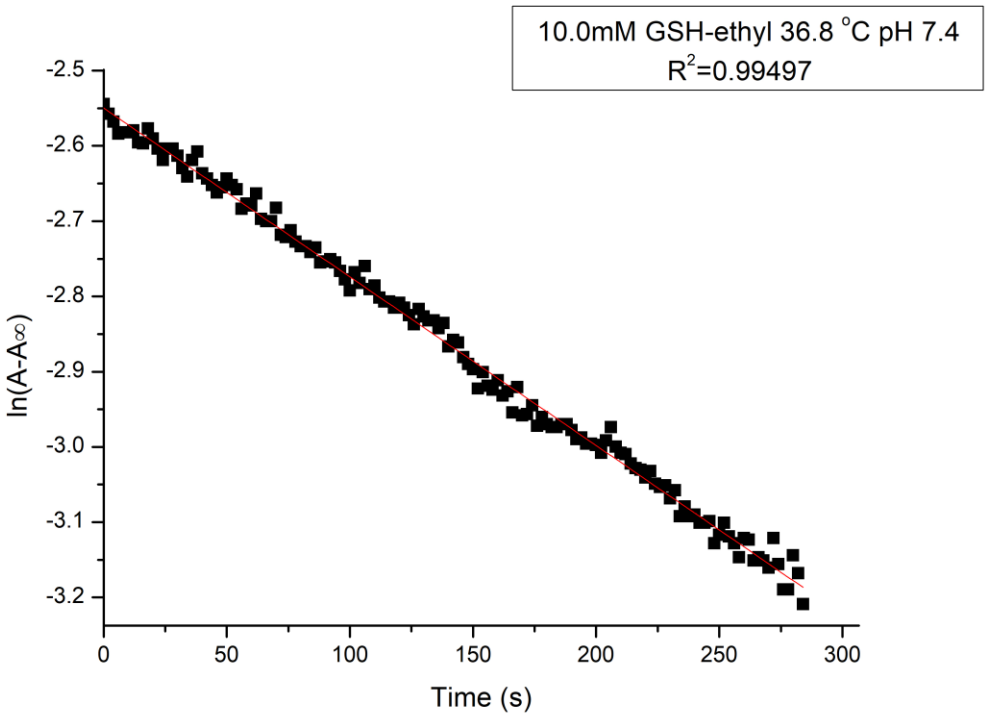
Relationship between ln (A - A_∞) and time for the decomposition of **15b** in pH 7.4 at 36.8 °C with 1 mM Glutathione



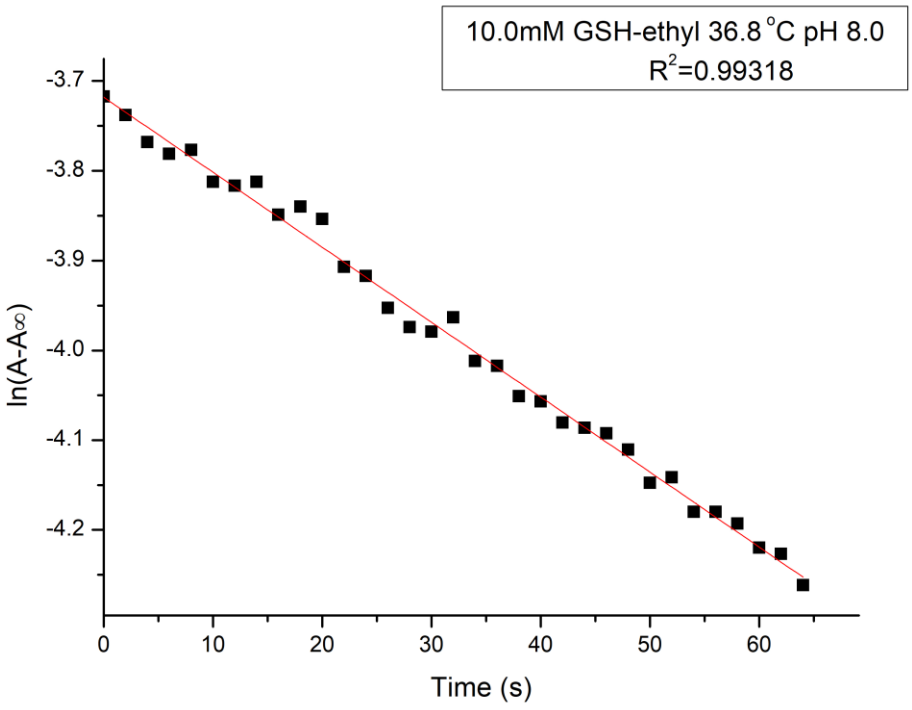
Relationship between ln (A - A_∞) and time for the decomposition of **15b** in pH 8.0 at 36.8 °C with 1 mM Glutathione



Relationship between ln (A - A_∞) and time for the decomposition of **15b** in pH 7.0 at 36.8 °C with 10 mM Glutathione



Relationship between ln (A - A_∞) and time for the decomposition of **15b** in pH 7.4 at 36.8 °C with 10 mM Glutathione



Relationship between ln (A - A_∞) and time for the decomposition of **15b** in pH 8.0 at 36.8 °C with 10 mM Glutathione