Supporting Information

# Cyclobutane-bearing restricted anchoring residues enabled geometry-specific hydrocarbon peptide stapling

Baobao Chen,# Chao Liu,# Wei Cong, Fei Gao, Yan Zou, Li Su, Lei Liu, Alexander Hillisch, Lutz Lehmann, Donald Bierer, Xiang Li,* Hong-Gang Hu*

**Abstract:** Stapled peptides are regarded as the promising next-generation therapeutics because of their improved secondary structure, membrane permeability and metabolic stability as compared with the prototype linear peptides. Usually, stapled peptides are obtained by hydrocarbon stapling technique, anchoring from paired olefin-terminated unnatural amino acids and the consequent ring-closing metathesis (RCM). To investigate the adaptability of rigid cyclobutane structure in RCM and expand the chemical diversity of hydrocarbon peptide stapling, we herein described the rational design and efficient synthesis of cyclobutane-based conformationally constrained amino acids, termed (*E*)-1-amino-3-(but-3-en-1-yl)cyclobutane-1-carboxylic acid (**E**$_7$) and (*Z*)-1-amino-3-(but-3-en-1-yl)cyclobutane-1-carboxylic acid (**Z**$_7$). All four combinations including **E**$_7$-**E**$_7$, **E**$_7$-**Z**$_7$, **Z**$_7$-**Z**$_7$ and **Z**$_7$-**E**$_7$, were proven to be applicable in RCM-mediated peptide stapling to afford the corresponding geometry-specific stapled peptides. With the aid of the combined quantum and molecular mechanics, **E**$_7$-**E**$_7$ combination was proven to be optimal in both RCM reaction and helical stabilization. With the spike protein of SARS-CoV-2 as the target, a series of cyclobutane-bearing stapled peptides were obtained. Among them, **E**$_7$**−E**$_7$ geometry-specific stapled peptides indeed exhibit higher α-helicity and cellular uptake, and thus stronger biological activity than canonical hydrocarbon stapled peptides. We believe this methodology possesses great potential to expand the scope of the existing peptide stapling strategy. These cyclobutane-bearing restricted anchoring residues served as effective supplements for the existing olefin-terminated unnatural amino acids and the resultant geometry-specific hydrocarbon peptide stapling provided more potentials for peptide therapeutics.

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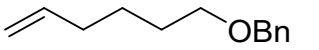
## Experimental Procedures

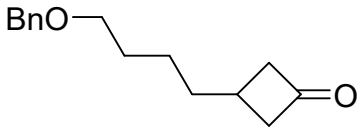
**Materials and instruments**. All the starting materials were purchased from commercial sources without further purification. The amino acids protected by Fmoc were purchased from GL Biochem (Shanghai) Co., Ltd. Rink Amide MBHA resin was purchased from Tianjin Nankai Hecheng Technology Co., Ltd. Other reagents and solvents were purchased from TCI (Shanghai), Energy Chemical or Sinopharm Chemical Reagent Co. Ltd. Dichloromethane (DCM) and THF were distilled over calcium hydride ($CaH_2$) or NaH under argon atmosphere. All reactions vessels were oven-dried before use. A Vydac C18 column (5 μm, 4.6 mm×250 mm) with a 1 mL/min flow rate was used for analytical RP-HPLC, and a Vydac C18 column (10 μm, 10 mm×250 mm or 22 mm×150 mm) with a 3-6 mL/min flow rate was used for semi-preparative RP-HPLC. The solvents systems were buffer A (0.1% TFA in water), buffer B (0.1% TFA in $CH_3CN$). Data were recorded and analyzed using the software system LC Solution. Reactions were monitored by thin-layer chromatography (TLC) and visualized by UV analyzer (254 nm). $^1$H- and $^{13}$C-NMR spectra was recorded on a Bruker 600 MHz instrument. Chemical shifts ($\delta$) were reported relative to TMS (0 ppm) for $^1$H-NMR and $^{13}$C-NMR spectra. The coupling constants (J) were displayed in Hertz (Hz) and the splitting patterns were defined as follows: singlet (s); broad singlet (s, br); doublet (d); doublet of doublet (dd); triplet (t); quartet (q); multiplet (m). ESI-MS was measured with a Bruker Esquire-LC mass spectrometer. High resolution mass spectra were measured on a Waters Xevo G2 QTOF mass spectrometer.

***Amino acids synthesis. ((hex-5-en-1-yloxy)methyl)benzene (4)***



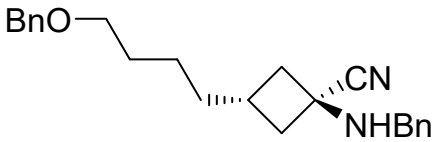

To a solution of hex-5-en-1-ol (3, 60 g, 0.6 mol) in THF was added NaH (21.6 g, 0.9 mol) at 0 ºC. The reaction was stirred for 60 min and BnBr (113 g, 0.66 mol) was added dropwise. The mixture was stirred at room temperature overnight. After quenched by anhydrous methanol, the mixture was evaporated to dryness and dissolved with EA (600 mL), washed with saturated $NaHCO_3$ (3×100 mL), brine (3×100 mL), dried over $Na_2SO_4$, filtered, concentrated and purified by silica gel column (100:1-10:1, PE/EtOAc) to afford 4 as a light-yellow oil (90 g, 79 %). $^1$H-NMR (600 MHz, $CDCl_3$): δ 7.34 (m, 4 H), 7.27 (m, 1 H), 5.84-5.77 (m, 1 H), 5.02-4.93 (m, 2 H), 4.50 (s, 2 H), 3.47 (t, J = 8.8 Hz, 2 H), 2.07 (q, J = 14.4 Hz, 2 H), 1.63 (m, 2 H), 1.48 (m, 2 H). $^{13}$C-NMR (150 MHz, $CDCl_3$): δ 133.74, 128.37, 127.64, 127.51, 114.54, 77.07, 72.90, 70.27, 33.59, 29.25, 25.54. ESI-MS m/z calcd for $C_{13}H_{18}O$ 190.13; found $[M+H]^+$ 191.17.

***3-(4-(benzyloxy)butyl)cyclobutan-1-one (5)***



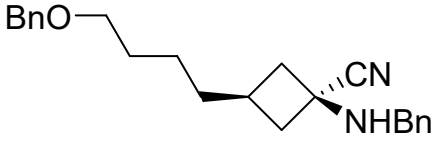

To a solution of 4 (60 g, 0.32 mmol) in THF (1 L) was added zinc-copper couple, (1-3% Cu, 86 g, 1.33 mol). This mixture was stirred at 0°C for 15 min and a mixed solution of trichloroacetyl chloride (130 g, 0.73 mol) and phosphorus oxychloride (110 g, 0.73 mol) was added dropwise. After stirring over night at 0°C, the reaction was diluted with EA (3 L) and the organic layer was successively washed with brine (3×2 L), saturated $NaHCO_3$ (3×2 L), brine (3×2 L), dried over $Na_2SO_4$, filtered and concentrated. The residue was redissolved with $AcOH/H_2O$ solution (500 mL, v/v=1:1) and zinc powder was added batchwise at 0°C. After refluxing for 4 hours, the reaction was diluted with EA (2 L) and the organic layer was successively washed with brine (3×2 L), saturated $NaHCO_3$ (3×2 L), brine (3×2 L), dried over $Na_2SO_4$, filtered, concentrated and purified by column chromatography (50:1-10:1, petro ether/EtOAc) to afford 5 as a light-yellow oil (40 g, 54%). $^1$H-NMR (600 MHz, $CDCl_3$): δ 7.34 (m, 4 H), 7.28 (m, 1 H), 4.50 (s, 2 H), 3.48 (t, J = 8.8 Hz, 2 H), 3.13 (m, 2 H), 2.66 (m, 2 H), 2.36 (m, 1 H), 1.67–1.63 (m, 2 H), 1.59 (q, J = 15.2 Hz, 2 H), 1.42 (m, 2 H). $^{13}$C-NMR (150 MHz, $CDCl_3$): δ 208.61, 138.55, 28.40, 127.67, 127.59, 72.98, 70.15, 52.54, 36.16, 29.57, 25.00, 23.86. ESI-MS m/z calcd for $C_{15}H_{20}O_2$ 232.15; found $[M+H]^+$ 233.15.

***(1s,3r)-1-(benzylamino)-3-(4-(benzyloxy)butyl)cyclobutane-1-carbonitrile (6a)***



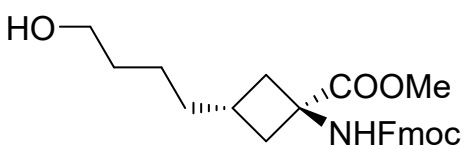

To a solution of 5 (10 g, 0.043 mol) was added $BnNH_2$ (5.1 g, 0.047 mol), the mixture was stirred at room temperature for 15 min. TMSCN (4.7g, 0.047 mol) was then added dropwise and the reaction was continually stirred for 30 min. The reaction was concentrated and purified by column chromatography (50:1-10:1, petro ether/EtOAc) to afford 6a (3.5 g, 23%) as a light-yellow oil. $^1$H-NMR (600 MHz, d-DMSO): δ 7.47 (d, J = 7.8 Hz, 2 H), 7.40 (t, J = 9.0 Hz, 2 H), 7.35 (m, 3 H), 7.31 (d, J = 6.6 Hz, 2 H), 7.28 (t, J = 13.8 Hz, 1 H), 4.44 (s, 2 H), 3.99 (s, 2 H), 3.41 (t, J = 12.6 Hz, 2 H), 2.55 (d, J = 10.2 Hz, 2 H), 2.49 (m, 1 H), 2.21 (m, 2 H), 1.52 (m, 2 H), 1.25 (m, 2 H). $^{13}$C-NMR (150 MHz, d-DMSO): δ 139.17, 129.73, 129.31, 129.10, 128.97, 128.68, 127.82, 72.28, 69.97, 52.43, 50.85, 48.22, 42.76, 38.46, 35.80, 35.55, 29.47, 28.30, 24.99, 23.71, 23.41. ESI-MS m/z calcd for $C_{15}H_{20}N_2O_2$ 348.22; found $[M+H]^+$ 349.50.

***(1r,3s)-1-(benzylamino)-3-(4-(benzyloxy)butyl)cyclobutane-1-carbonitrile (6b)***




From 5 (10 g, 0.043 mol), light yellow oil, 10 g, yield 67%. $^1$H-NMR (600 MHz, d-DMSO): δ 7.46 (d, J = 6.6 Hz, 2 H), 7.40 (t, J = 14.4 Hz, 2 H), 7.35 (m, 3 H), 7.30 (d, J = 7.2 Hz, 2 H), 7.28 (t, J = 14.4 Hz, 1 H), 4.44 (s, 2 H), 3.99 (s, 2 H), 3.40 (t, J = 12.6 Hz, 2 H), 2.61 (m, 2 H), 2.33 (m, 1 H), 2.11 (m, J = 9.0 Hz 2 H), 1.50 (m, 2 H), 1.42 (m, 2 H), 1.23 (m, 2 H). $^{13}$C-NMR (150 MHz, d-DMSO): δ 139.17, 129.79, 128.97, 128.68, 127.86, 127.78, 72.28, 69.94, 50.81, 48.20, 38.39, 35.52, 29.45, 28.29, 23.41. ESI-MS m/z calcd for $C_{15}H_{20}N_2O_2$ 348.22; found $[M+H]^+$ 349.55.

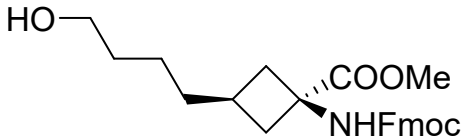
***methyl (1s,3r)-1-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-hydroxybutyl) cyclobutane-1-carboxylate (7a)***




6 M HCl (100 mL) was added to 6a (10 g, 0.024 mol), the mixture was refluxed for 12 h (monitored by HPCL). After cooling to rt, the aqueous phase was washed with DCM (3×100 mL) and concentrated. The residue was re-dissolved with MeOH (80 mL) and

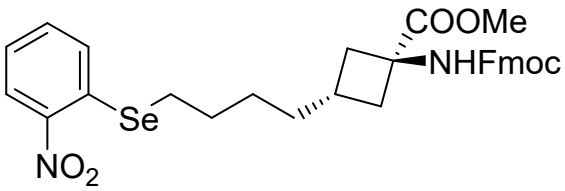
equipped with hydrogen balloons 10%. The hydrogenation was carried out for 12 hours catalyzed with 10% Pd/C (w/w). After removement of MeOH, the residue and $Na_2CO_3$（5.0 g, 0.048 mol）was re-dissolved with water (50 mL). FmocOSu（16.1 g, 0.048 mol）1,4-dioxane solution (50 mL) was added dropwise and this reaction was stirred at rt for 12 hours. After the reaction was completed, 1,4-dioxane was removed and the aqueous phase was washed with EA (3×200 mL), adjusted pH to 1-2, extracted with EA three times. The organic phase was washed with brine, dried over $Na_2SO_4$, filtered, concentrated and in MeOH/toluene (20 mL, 1:3, v/v) was added $TMSCH_2N_2$ (1.5 mL, 3.66 mmol) the reaction was stirred at rt for 1 h. After the reaction was completed, solvent was removed and the residue was purified by column chromatography (10:1-1:1, PE/EA) to afford 7a (4.36 g, 43%) as a white solid. $^1$H-NMR (600 MHz, d-DMSO): δ 8.10 (s, 1 H), 7.90 (d, J = 4.8 Hz, 2 H), 7.73 (d, J = 4.8 Hz, 2 H), 7.43 (t, J = 9.6 Hz, 2 H), 7.35 (t, J = 9.6 Hz, 2 H), 4.35 (m, 3 H), 4.24 (m, 1 H), 3.58 (s, 3 H), 3.39 (m, 2 H), 2.31 (m, 1 H), 2.25 (m, 2 H), 2.09 (t, J = 13.2 Hz, 2 H), 1.39 (m, 4 H), 1.21 (m, 2 H). $^{13}$C-NMR (150 MHz, d-DMSO): δ173.94, 155.25, 144.27, 141.34, 128.10, 127.52, 125.67, 120.59, 65.74, 61.14, 55.68, 52.50, 47.20, 36.32, 32.86, 29.12, 23.44. ESI-MS m/z calcd for $C_{25}H_{29}NO_5$ 423.20; found $[M+H]^+$ 424.40, $[M+Na]^+$ 424.45.

***methyl (1r,3s)-1-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-hydroxybutyl) cyclobutane-1-carboxylate (7b)***



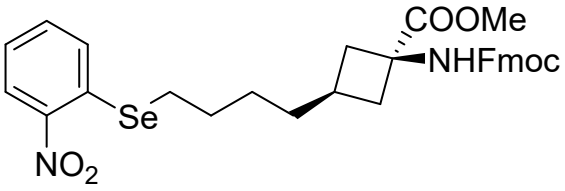

From 6b (10 g, 0.024 mol), white solid, 3.95 g, yield 39%. $^1$H-NMR (600 MHz, $CDCl_3$): δ8.10 (s, 1H), 7.89 (d, J = 5.2 Hz, 2H), 7.72 (d, J = 4.8 Hz, 2H), 7.42 (t, J = 10.0 Hz, 2H), 7.35 (t, J = 10.0 Hz, 2H), 4.32 (m, 2H), 4.24 (m, 1H), 3.62 (m, 3H), 3.41 (t, J = 8.8 Hz, 3H), 2.60 (t, J = 13.6Hz, 1H), 2.21 (m, 1H), 1.80 (t, J = 16.0 Hz, 2H), 1.41 (m, 4H), 1.20 (m, 2H). $^{13}$C-NMR (150 MHz, d-DMSO): δ174.65, 155.69, 144.35, 141.37, 128.08, 127.41, 125.29, 120.55, 65.72, 61.19, 54.61, 52.37, 47.21, 37.84, 37.08, 32.91, 28.57, 23.49. ESI-MS m/z calcd for $C_{25}H_{29}NO_5$ 423.20; found $[M+H]^+$ 424.40, $[M+Na]^+$ 446.45.

***methyl (1s,3r)-1-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-((2-nitrophenyl) selanyl) butyl)cyclobutane-1-carboxylate (8a)***



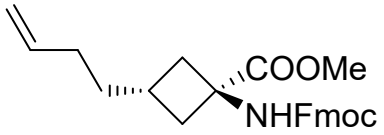

To a solution of 7a (846 mg, 2 mmol) in dry THF (5 mL) was added 2-nitrophenylselenocyanate (684 mg, 1.5 mmol) and tributyl phosphine (808 mg, 4 mmol), the reaction was stirred under Ar protection at rt for 12 h. After the reaction was completed, solvent was removed and the residue was purified by column chromatography (50:1-10:1, PE/EA) to afford 8a (920 mg, 76%) as a colorless oily liquid. $^1$H-NMR (600 MHz, d-DMSO): δ 8.26 (d, J = 5.2 Hz, 1 H), 8.09 (s, 1 H), 7.90 (d, J = 4.8 Hz, 2 H), 7.70 (m, 4 H), 7.43 (m, 3 H), 7.34 (d, J = 9.6 Hz, 2 H), 4.33 (d, J = 4.8 Hz, 2 H), 4.23 (t, J = 8.8 Hz, 2 H), 3.58 (m, 3 H), 3.00 (t, J = 9.6 Hz, 1 H), 2.31 (m, 1 H), 2.23 (m, 2 H), 2.09 (m, J = 13.2 Hz, 2 H), 1.66 (m, 2 H), 1.42 (m, 2 H), 1.34 (m, 2 H). $^{13}$C-NMR (150 MHz, d-DMSO): δ 173.89, 155.85, 147.04, 144.26, 141.24, 134.85, 132.69, 130.25, 128.10, 127.51, 126.75, 126.54, 120.59, 65.73, 55.66, 52.51, 47.20, 36.40, 35.61, 29.20, 28.42, 27.32, 26.10. ESI-MS m/z calcd for $C_{31}H_{32}N_2O_6Se$ 608.14; found $[M+H]^+$ 609.50, $[M+Na]^+$ 631.50 .

***(1r,3s)-1-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-((2-nitrophenyl)selanyl) butyl)cyclobutane-1-carboxylate (8b)***



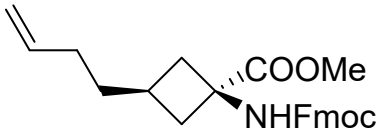

From 7b (800 mg, 1.89 mmol), colorless oily liquid, 815 mg, 71%. $^1$H-NMR (600 MHz, d-DMSO): δ 8.27 (d, J = 5.2 Hz, 1 H), 8.10 (s, 1 H), 7.90 (m, 2 H), 7.70 (m, 4 H), 7.43 (m, 3 H), 7.34 (d, J = 9.6 Hz, 2 H), 4.31 (m, 2 H), 4.22 (t, J = 13.2 Hz, 1 H), 3.59 (m, 3 H), 3.01 (t, J = 14.4 Hz, 1 H), 2.58 (m, 2 H), 2.09-2.25 (m, 2 H), 1.78 (m, 2 H), 1.66 (m, 2 H), 1.44 (m, 2 H), 1.32 (m, 2 H). $^{13}$C-NMR (150 MHz, d-DMSO): δ 174.60, 155.26, 146.98, 144.35, 141.35, 134.80, 130.17, 127.96, 127.41, 125.64, 120.50, 65.70, 60.22, 54.57, 52.56, 47.19, 37.77, 36.50, 28.42, 27.34, 26.08, 21.20, 14.53. ESI-MS m/z calcd for $C_{31}H_{32}N_2O_6Se$ 608.14; found $[M+H]^+$ 609.45, $[M+Na]^+$ 631.50 .

***methyl (1s,3r)-1-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(but-3-en-1-yl) cyclobutane-1- carboxylate (9a)***



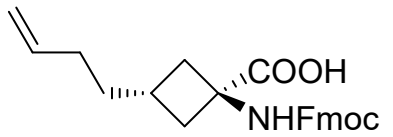

To a solution of 8a (1 g, 2.47 mmol) in THF (5 mL) was added $H_2O_2$ (5 mL), the reaction was stirred at rt for 12 h. After the stirring with 20 mL Sat. $Na_2S_2O_3$ for 10 min, the reaction was extracted with EA (3×20 mL). The organic layer was washed with brine (3×20 mL), concentrated and purified by column chromatography (50:1-10:1, petro ether/EtOAc) to afford 9a (834 mg, 83%) as a colorless oily liquid. $^1$H-NMR (600 MHz, d-DMSO): δ8.09 (s, 1 H), 7.90 (d, J = 5.2 Hz, 2 H), 7.72 (d, J = 5.2 Hz, 2 H), 7.41 (t, J = 10.0 Hz, 2 H), 7.34 (d, J = 10.0 Hz, 2 H), 5.78 (m, 1 H), 4.97 (m, 2 H), 4.30 (d, J = 4.4 Hz, 3 H), 4.23 (m, 1 H), 3.57 (s, 3 H), 2.09 (t, J = 10.4 Hz, 2 H), 1.94 (m, 2 H), 1.47 (q, J = 14.8 Hz, 2 H), 1.24 (m, 2 H). $^{13}$C-NMR (150 MHz, d-DMSO): δ173.91, 155.85, 144.27, 141.24, 138.94, 128.11, 127.52, 120.41, 115.28, 65.74, 55.63, 52.52, 47.18, 36.36, 35.46, 31.22, 28.84. ESI-MS m/z calcd for $C_{25}H_{27}NO_4$ 405.19; found $[M+H]^+$ 406.50, $[M+Na]^+$ 428.45.

***methyl (1r,3s)-1-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(but-3-en-1-yl) cyclobutane-1- carboxylate (9b)***



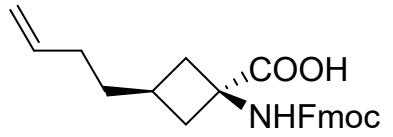

From 8b (800 mg, 80%), colorless oily liquid, 830mg, yield 83%. $^1$H-NMR (600 MHz, d-DMSO): δ8.09 (s, 1 H), 7.88 (d, J = 5.2 Hz, 2 H), 7.71 (d, J = 5.2 Hz, 2 H), 7.42 (t, J = 10.0 Hz, 2 H), 7.34 (d, J = 9.6 Hz, 2 H), 5.78 (m, 1 H), 4.97 (m, 2 H), 4.30 (d, J = 4.8 Hz, 3 H), 4.21 (m, 1 H), 3.60 (s, 3 H), 2.59 (t, J = 14.0 Hz, 2 H), 2.21 (m, 1 H), 1.92 (m, 2 H), 1.80 (m, 2 H), 1.4 (m, 2 H). $^{13}$C-NMR (150 MHz, d-DMSO): δ174.54, 155.25, 144.36, 141.36, 138.92, 128.09, 127.51, 125.30, 120.52, 115.25, 65.71, 54.57, 52.49, 47.18, 37.74, 36.35, 31.14, 28.07. ESI-MS m/z calcd for $C_{25}H_{27}NO_4$ 405.19; found $[M+H]^+$ 406.55, $[M+Na]^+$ 428.50.

(*1s,3r)-1-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(but-3-en-1-yl)cyclobutane-1-carboxylic acid ($E_7$)*




To a solution of 9b (101 mg, 0.25 mmol) in 1,2-dichloroethane (10 mL) was added trimethyltin hydroxide (135 mg, 0.75 mmol), the reaction was heated at 80 °C for 5 h. After completion of the reaction indicated by TLC, the mixture was concentrated in vacuo, and the residue was taken up in ethyl acetate (20 mL). The organic layer was washed with 1M HCl, brine, dried over $Na_2SO_4$, concentrated and purified by column chromatography (100:1-10:1, DCM/MeOH) to afford $E_7$ (80 mg, 82%) as a white solid. $^1$H-NMR (600 MHz, d-DMSO): δ12.38 (s, 1 H), 7.97 (s, 1 H), 7.90 (d, J = 4.8 Hz, 2 H), 7.73 (d, J = 4.8 Hz, 4 H), 7.42 (t, J = 9.6 Hz, 2 H), 7.34 (d, J = 9.6 Hz, 2 H), 5.78 (m, 1 H), 4.97 (m, 2 H), 4.25 (m, 3 H), 2.33 (m, 1 H), 2.24 (t, J = 13.6 Hz, 2 H), 2.08 (t, J = 10.0 Hz, 2 H), 1.94 (m, 2 H), 1.48 (m, 2 H). $^{13}$C-NMR (150 MHz, d-DMSO): δ 175.17, 155.87, 144.27, 141.19, 138.99, 128.12, 127.55, 125.73, 120.57, 115.24, 65.79, 55.50, 47.15, 36.18, 35.48, 31.23, 28.83. ESI-MS m/z calcd for $C_{24}H_{25}NO_4$ 391.18; found $[M+H]^+$ 392.50, $[M+Na]^+$ 414.50, $[M+K]^+$ 430.45.

*(1r,3s)-1-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(but-3-en-1-yl)cyclobutane-1-carboxylic acid ($Z_7$)*




From 9a (130 mg, 0.32 mmol), white solid, 95 mg, 76%. $^1$H-NMR (600 MHz, d-DMSO): δ12.39 (s, 1 H), 7.97 (s, 1 H), 7.90 (d, J = 4.8 Hz, 2 H), 7.72 (d, J = 4.8 Hz, 4 H), 7.42 (t, J = 10.0 Hz, 2 H), 7.34 (d, J = 10.0 Hz, 2 H), 5.78 (m, 1 H), 4.98 (m, 2 H), 4.23 (m, 3 H), 2.58 (m, 2 H), 2.22 (m, 2 H), 1.94 (m, 2 H), 1.78 (t, J = 14.0 Hz, 2 H), 1.48 (m, 2 H). $^{13}$C-NMR (150 MHz, d-DMSO): δ 175.80, 155.17, 144.30, 141.19, 139.37, 128.10, 127.54, 125.49, 120.53, 115.24, 65.75, 54.45, 47.15, 37.67, 36.48, 31.25, 28.18. ESI-MS m/z calcd for $C_{24}H_{25}NO_4$ 391.18; found $[M+H]^+$ 392.55, $[M+Na]^+$ 414.40, $[M+K]^+$ 430.40.

**Calculation of reaction Gibbs free energy.** Reaction Gibbs free energy (ΔrGm) is the difference between the chemical potential of the reactant and product in a standard state. Under isothermal isobaric conditions, the forward reaction will occur spontaneously when ΔrGm is less than zero, otherwise was the reverse reaction. According to van't Hoff isothermal formula (i.e., ΔrGm=-RTlnK), the equilibrium constant K, used to characters whether the reaction is thorough or not, is related to the index of reaction Gibbs free energy. Given that the investigated RCM reaction in this work occurs at room temperature and shows thermodynamic- rather than dynamic-driving characteristics, without requiring ultra-low temperature control, the occurrence difficulty can be predicted by calculating reaction Gibbs free energy variation between reactants and products. The reaction free energy is composed of thermodynamic and electrochemical ones. All the calculations are carried out with Gaussian 16, in which B3LYP functional was adopted to optimize the ground state structure. As a supplement, the 6-311 g (d, p) basis set is used to obtain a more accurate optimized structure. Based on the optimized ground state structure, frequency calculation is further carried out. The results show that no imaginary frequency appears, indicating that the optimized small molecule structures are all distributed at the local minimum of potential energy surface.

**Prediction of helicity.** Molecular dynamics (MD) simulation is an important method to explore protein folding, receptor-ligand interactions, and motion pattern of biological macromolecules. The basic principle is that, starting from a specific initial structure of biological macromolecules, following molecular force field and Newton's equation, the position, velocity and force at every moment are recorded, and then various thermodynamic and dynamic properties are calculated statistically. In this work, 300 ns comparative MD simulations were performed for the SARS-CoV-2 models using AMBER12 software package and AMBER ff14SB force field, whose parameters were fitted based on experimental values. For other calculation details, the SHAKE algorithm was used for constraining bonds involving hydrogen atoms, and TIP3P water model was adopted for solvent model; the particle mesh Ewald (PME) method was employed to evaluate long-range electrostatic interactions; temperature was controlled via Langevin thermostat method, and Berendsen barostat was used to regulate the pressure with a reference value of 1 atm; the integration step was set as 2 fs, with the obtained trajectories monitored by VMD software.

As one of the main forms of secondary structure, α-helical plays an important role in maintaining structural rigidity, cellular and even biological functions of the protein. In general, the higher the helicity is, the more stable the peptide is and may exhibit better transmembrane ability. In this work, helicity is defined as all the number of α-helices divided by the total number of secondary structures in MD trajectories. In order to better analyze the relationship between helicity and the structure of SARS-CoV-2 models, three self-defined parameters were introduced: (1) the distance D between anchoring residues (C3 and T7) Cα atoms; (2) the helix pitch T—the distance between two adjacent threads along the helix; (3) the abducting area S of the long aliphatic chain exposed to solvent. In the standard α-helical structure of five residues, the distance D ranges from 6.0 to 6.6 Å, and the pitch T retains around 6.0 Å.

**General procedures for the Fmoc solid phase peptide synthesis.** The starting amino acids were attached to the Rink Amide resin (loading capacity=0.43 mmol/g, 100 mg) with a single coupling procedure.

**(a) Standard pre-activation of resin protocol:** The resin was swollen in DCM/DMF mixture solvent for 10 min.

**(b)** Standard Fmoc-deprotection protocol: After treatment with 20% piperidine/DMF for 15 min, the resin was washed (5×DMF, 5×$CH_2Cl_2$, 5×DMF).

**(c) Standard coupling protocol:** HCTU (3 eq.), Fmoc-protected amino acid (3 eq.) and DIPEA (9 eq.) were dissolved in DMF, the solution was added to the resin. After 20 min, the resin was washed (5×DMF, 5×DCM, 5×DMF). The coupling reaction was monitored with the ninhydrin test.

**(d) Standard acetylation protocol:** 10 mL $Ac_2O$/Py (1:1, v/v) mixed solution was added to the resin. After mechanically stirring for 20 min, the resin was washed (5×DMF, 5×DCM, 5×DMF).

**(e) Standard on-resin cyclization protocol:** A solution of first-generation Grubbs' reagent (0.1 eq) in DCE was added to the resin. After overnight reaction, the resin was washed (5×DMF, 5×DCM, 5×DMF).

**(f) Standard cleavage protocol:** The cleavage cocktail (TFA/TIPS/EDT/$H_2O$= 95:2:2:1, v/v/v/v) was added to the resin at room temperature. After stirring for 2 hours, the cleavage cocktail was collected, and the resin was washed with the TFA cleavage cocktail (3×).

**(g) Standard purification and analysis protocol:** The collected TFA cocktails were bubbled with argon and then the chilled diethyl ether was added to the concentrated TFA solution to precipitate the crude peptides. The peptide suspensions were centrifuged for 3 min at 3000 rpm and then the clear solution was decanted. The step of precipitation, centrifugation and decantation operations was repeated three times. The resulting white residues were dissolved in $CH_3CN/H_2O$, analyzed by HPLC and HR-MS and purified by RP-HPLC.

**CD spectroscopy**. CD measurements were conducted using a JASCO J-820 spectropolarimeter (JASCO Corp., Ltd). Peptides were dissolved in a 50.0% TFE aqueous solution at a concentration of 0.1 mg/mL. UV spectra were obtained at 20°C within a quartz cell with a path length of 10.0 mm. The percent helicity was calculated using the following equation.

$$\alpha = \frac{[\theta]_{222}}{[\theta]_{max}} \times 100\%$$

$[\theta]_{222}$ is the molar ellipticity of 222 nm; $[\theta]_{max}$ =(-44000+250T) (1-k/n), k=4, n is the numbers of amino acids and T=20℃.

**Protease stability**. Solution A was prepared by dissolving purified peptides in DMSO to achieve a final concentration of 1 mM. For solution B, α-chymotrypsin was dissolved in a PBS buffer with a pH of 7.4, which contained 2 mM CaCl2, resulting in a final concentration of 0.5 ng/μL. The two solutions were then combined with 50 μL of solution A and 1950 μL of solution B. Subsequently, the percent residual peptide was assessed via HPLC after incubation at 37°C for 0, 1, 2, 4, 8, and 12 hours.

**Live SARS-Cov-2 infection**. The SARS-CoV-2 virus, identified with PubMed No: MT627325, was isolated, processed, and maintained within the ABSL-3 laboratory at the Second Military Medical University. Infections were carried out in 96-well plates, each containing 5×104 viruses per well, using the same batch of viruses for consistency. All experimental procedures involving live viruses were strictly conducted within the ABSL-3 laboratory, and samples were only removed from this facility once virus inactivation was complete. Uninfected samples were cultured for an equivalent duration as control experiments. Stapled peptides at the specified concentration were mixed with monolayer Vero-E6 cells for a 30-minute incubation period, followed by the addition of SARS-CoV-2 (MOI=0.1). After adsorption at 37 ℃, the supernatant was aspirated, and a 0.9% methyl cellulose overlay was applied to the cells. After 72 hours, the plates were fixed and stained, and plaques were enumerated by fixation with 4% paraformaldehyde and subsequent staining with 0.1% crystal violet.

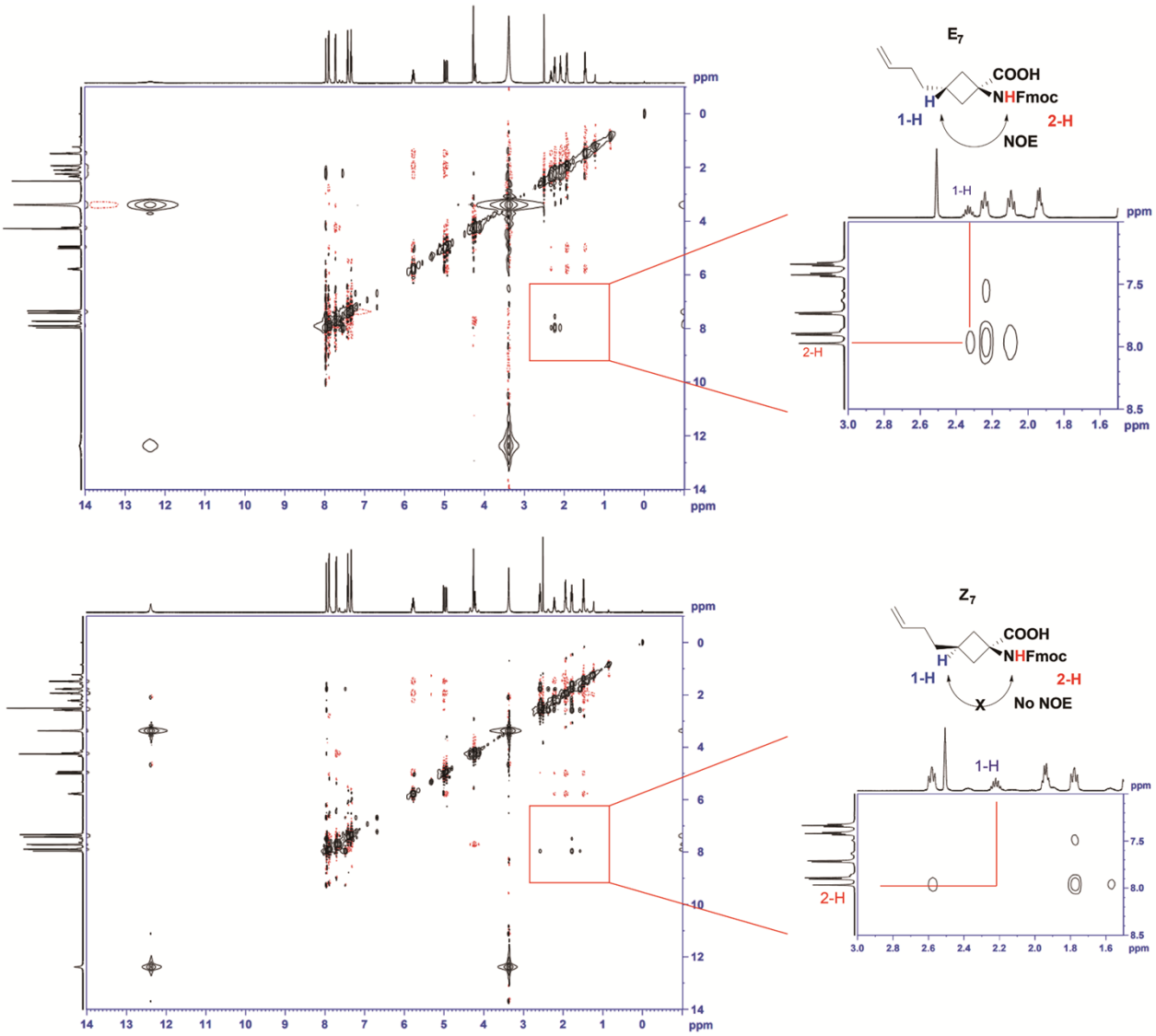



**Figure S1.** Results of the NOESY experiments for the configuration assignment of $\mathbf{E}_7$ and $\mathbf{Z}_7$.

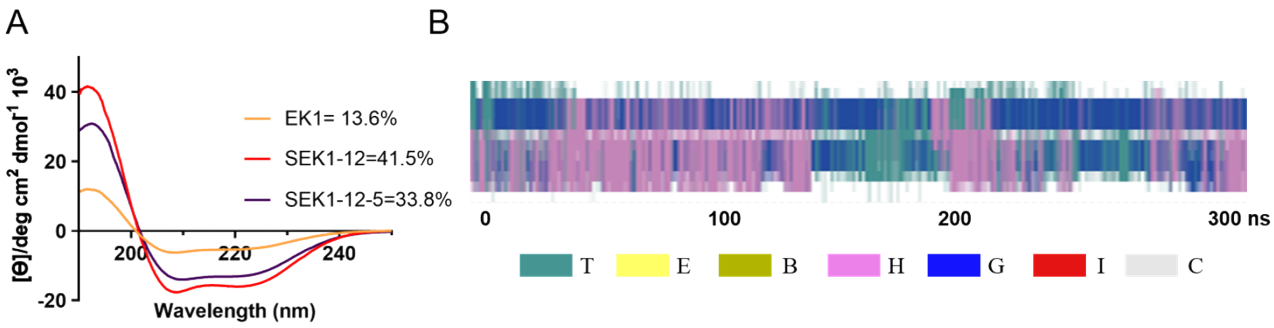


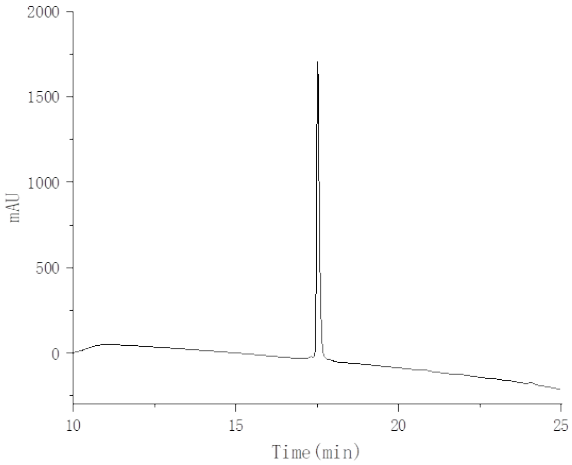

**Figure S2.** (A) The secondary structure of EK1, SEK1-12 and SEK1-12-5 were analyzed by CD. (B)The change of the secondary structure of SEK1-12-5 with simulation time.

## Peptide characterization and nuclear magnetic spectrum

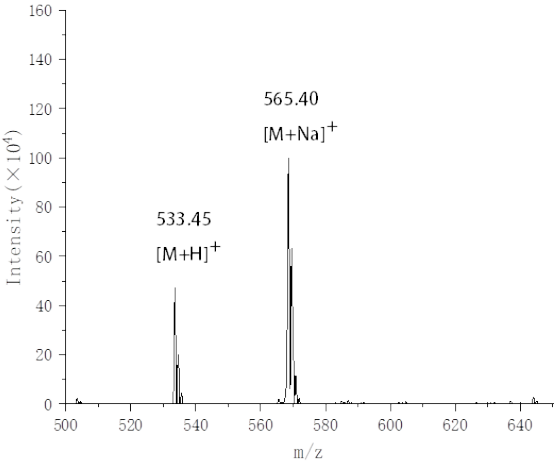
**A**

2a: [Cyc(1, 5)]Ac-$E_7$AGA$E_7$-$NH_2$

**B**




**C**

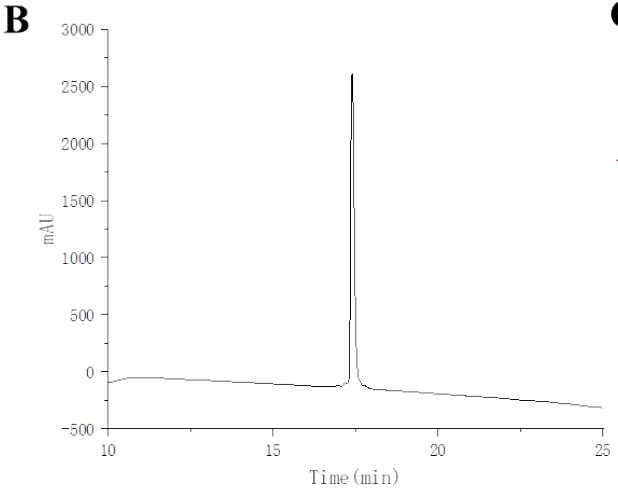



White lyophilized powder, 8.4 mg, 95% conversion, 38% yield. A) The structure of **2a**; B). The HPLC of purified **2a**. Gradient: 0-90% of buffer B in 30 min with C18 column (5 μm, 2.5 mm×250 mm); C) ESI-MS spectrum of **2a**, calcd. for $C_{26}H_{40}N_6O_6$ 532.3009; found $[M+H]^+$= 533.45; $[M+Na]^+$= 565.40.

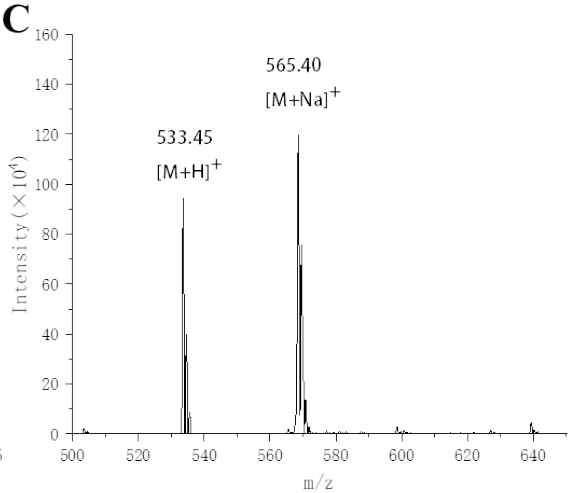
**A**

2b: [Cyc(1, 5)]Ac-$E_7$AGA$Z_7$-$NH_2$

**B**



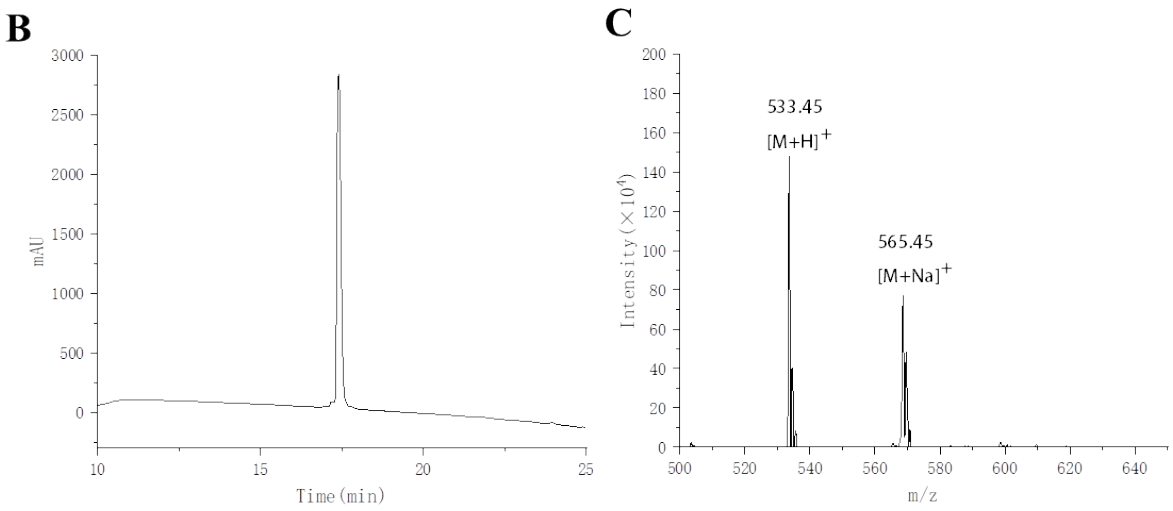

**C**




White lyophilized powder, 6 mg, 88% conversion, 27% yield. A) The structure of **2b**; B). The HPLC of purified **2b**. Gradient: 0-90% of buffer B in 30 min with C18 column (5 μm, 2.5 mm×250 mm); C) ESI-MS spectrum of **2b**, calcd. for $C_{26}H_{40}N_6O_6$ 532.3009; found $[M+H]^+$=533.45; $[M+Na]^+$=565.40.

A

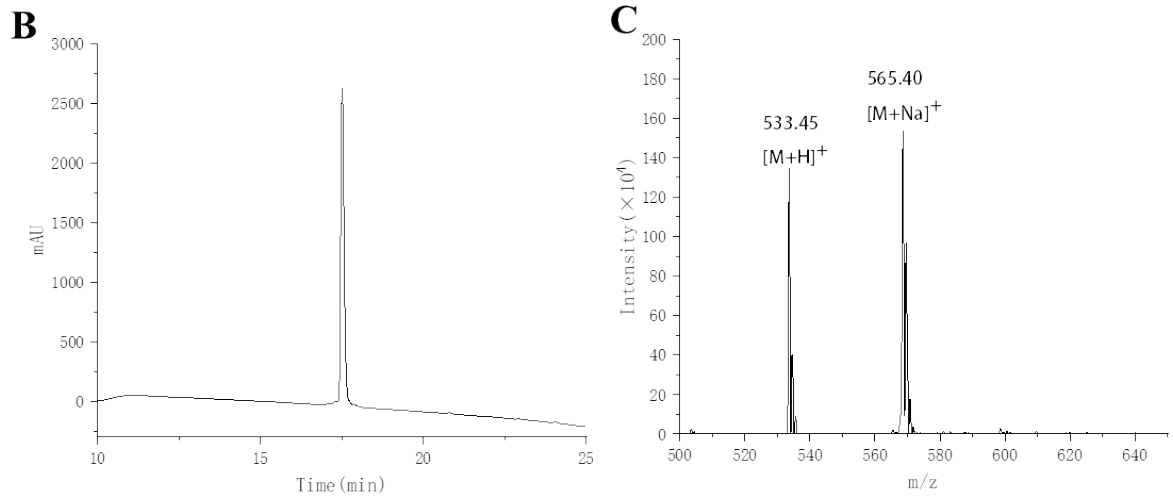
2c: [Cyc(1, 5)]Ac-$Z_7$AGA$Z_7$-$NH_2$




White lyophilized powder, 5.3 mg, 84% conversion, 24% yield. A) The structure of **2c**; B). The HPLC of purified **2c**. Gradient: 0-90% of buffer B in 30 min with C18 column (5 μm, 2.5 mm×250 mm); C) ESI-MS spectrum of **2c**, calcd. for $C_{26}H_{40}N_6O_6$ 532.3009; found $[M+H]^+$=533.45; $[M+Na]^+$=565.45.

A

2d: [Cyc(1, 5)]Ac-$Z_7$AGA$E_7$-$NH_2$

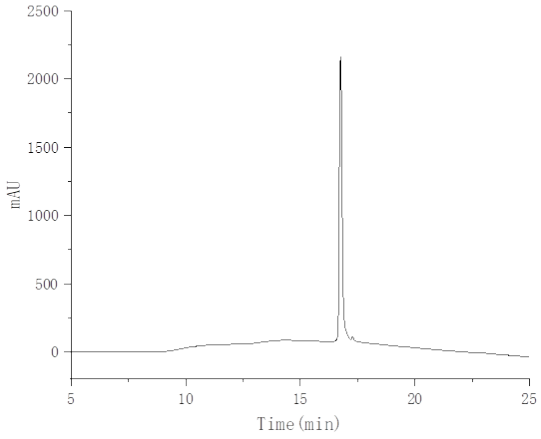



White lyophilized powder, 7.8 mg, 93% conversion, 35% yield. A) The structure of **2d**; B). The HPLC of purified **2d**. Gradient: 0-90% of buffer B in 30 min with C18 column (5 μm, 2.5 mm×250 mm); C) ESI-MS spectrum of **2d**, calcd. for $C_{26}H_{40}N_6O_6$ 532.3009; found $[M+H]^+$=533.45; $[M+Na]^+$=565.40.

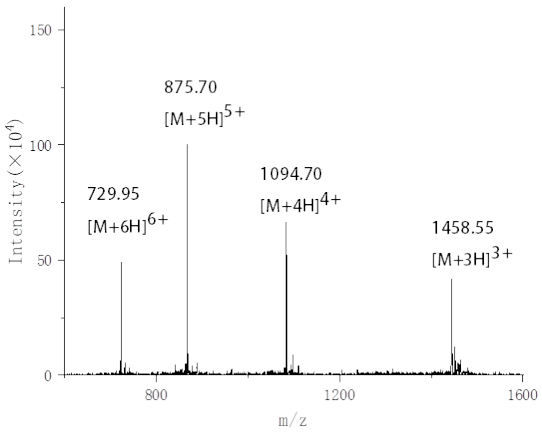
**A**

EK1: Ac-SLDQINVTFLDLEYEMKKLEEAIKKLEESYIDLKEL-$NH_2$

**B**




**C**

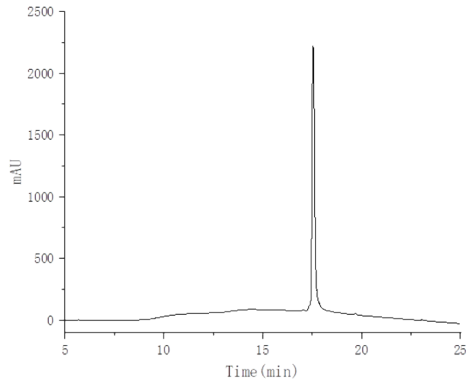



White lyophilized powder, 69 mg, 38% yield. A) The structure of **EK1**; B). The HPLC of purified **EK1.** Gradient: 0-90% of buffer B in 30 min with C18 column (5 μm, 2.5 mm×250 mm); C) ESI-MS spectrum of **EK1**, calcd. for $C_{203}H_{323}N_{43}O_{62}S$ 4370.2859; found $[M+3H]^{3+}$=1458.55; $[M+4H]^{4+}$=1094.70; $[M+5H]^{5+}$=875.70; $[M+6H]^{6+}$=729.95.

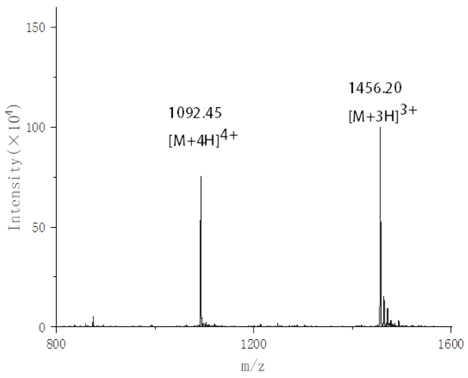
**A**

SEK1-12: [Cyc(21, 25)]Ac-SLDQINVTFLDLEYEMKKLE***S*$_5$AIK***S***$_5$LEESYIDLKEL-$NH_2$

**B**




**C**

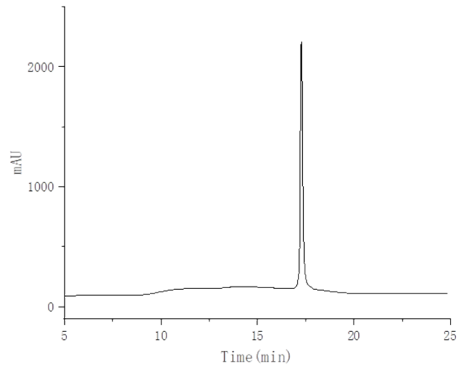



White lyophilized powder, 59 mg, 32% yield. A) The structure of **SEK1-12**; B). The HPLC of purified **SEK1-12**. Gradient: 0-90% of buffer B in 30 min with C18 column (5 μm, 2.5 mm×250 mm); C) ESI-MS spectrum of **SEK1-12**, calcd. for $C_{201}H_{323}N_{43}O_{62}S$ 4366.0940; found $[M+3H]^{3+}$=1456.20; $[M+4H]^{4+}$=1092.45.

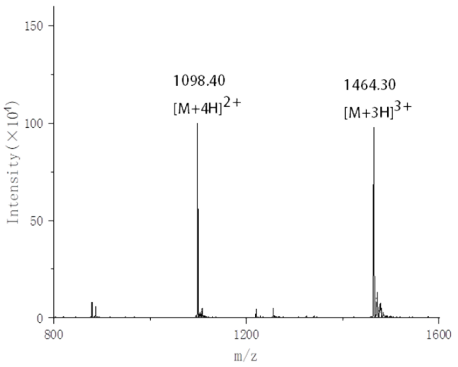
A

SEK1-12-1: [Cyc(21, 25)]Ac-SLDQINVTFLDLEYEMKKLE$E_7$AIK$E_7$LEESYIDLKEL-$NH_2$

B




C

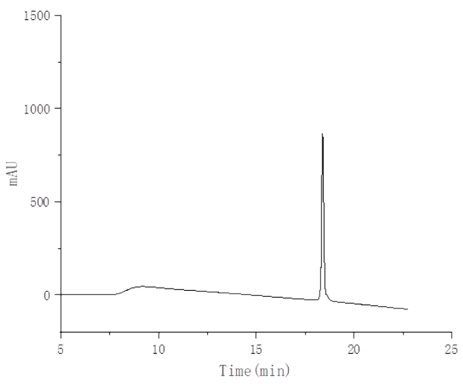



White lyophilized powder, 56 mg, 90% conversion, 31% yield. A) The structure of **SEK1-12-1**; B). The HPLC of purified **SEK1-12-1**. Gradient: 0-90% of buffer B in 30 min with C18 column (5 μm, 2.5 mm×250 mm); C) ESI-MS spectrum of **SEK1-12-1**, calcd. for $C_{203}H_{323}N_{43}O_{62}S$ 4390.1160; found $[M+3H]^{3+}$=1464.30; $[M+4H]^{4+}$=1098.40.

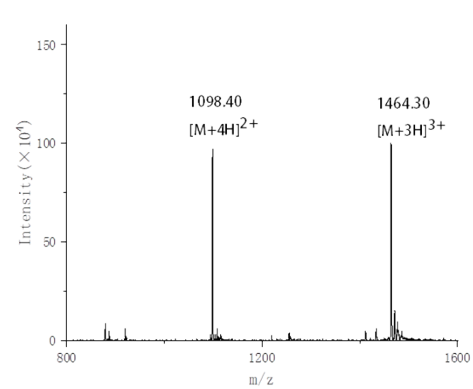
A

SEK1-12-2: [Cyc(21, 25)]Ac-SLDQINVTFLDLEYEMKKLE$E_7$AIK$Z_7$LEESYIDLKEL-$NH_2$

B




C

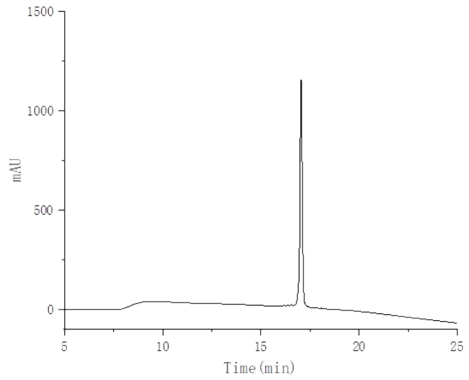



White lyophilized powder, 49 mg, 85% conversion, 28% yield. A) The structure of **SEK1-12-2**; B). The HPLC of purified **SEK1-12-2**. Gradient: 0-90% of buffer B in 30 min with C18 column (5 μm, 2.5 mm×250 mm); C) ESI-MS spectrum of **SEK1-12-2**, calcd. for $C_{203}H_{323}N_{43}O_{62}S$ 4390.1160; found $[M+3H]^{3+}$=1464.30; $[M+4H]^{4+}$=1098.40.

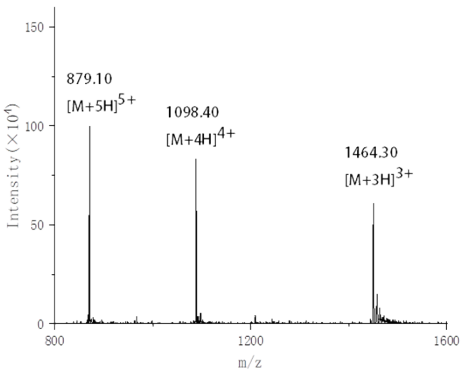
**A**

SEK1-12-3: [Cyc(21, 25)]Ac-SLDQINVTFLDLEYEMKKLE$Z_7$AIK$Z_7$LEESYIDLKEL-$NH_2$

**B**




**C**

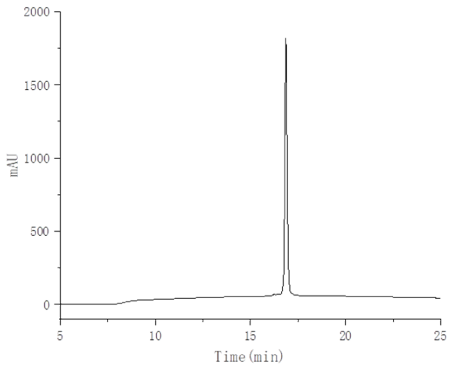



White lyophilized powder, 45 mg, 84% conversion, 26% yield. A) The structure of **SEK1-12-3**; B). The HPLC of purified **SEK1-12-3**. Gradient: 0-90% of buffer B in 30 min with C18 column (5 μm, 2.5 mm×250 mm); C) ESI-MS spectrum of **SEK1-12-3**, calcd. for $C_{203}H_{323}N_{43}O_{62}S$ 4390.1160; found $[M+3H]^{3+}$=1464.30; $[M+4H]^{4+}$=1098.40; $[M+5H]^{5+}$=879.10.

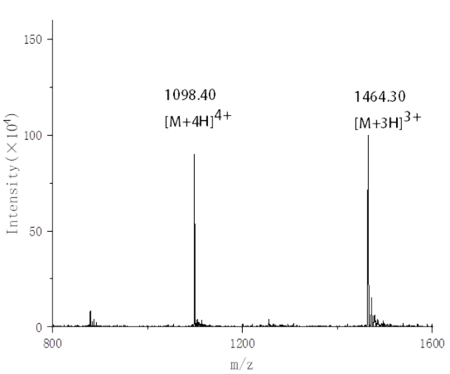
**A**

SEK1-12-4: [Cyc(21, 25)]Ac-SLDQINVTFLDLEYEMKKLE$Z_7$AIK$E_7$LEESYIDLKEL-$NH_2$

**B**




**C**

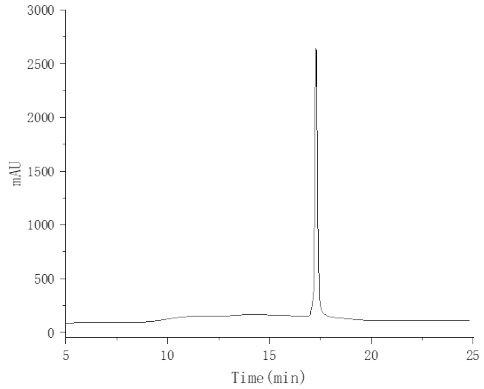



White lyophilized powder, 53 mg, 90% conversion, 29% yield. A) The structure of **SEK1-12-4**; B). The HPLC of purified **SEK1-12-4**. Gradient: 0-90% of buffer B in 30 min with C18 column (5 μm, 2.5 mm×250 mm); C) ESI-MS spectrum of **SEK1-12-4**, calcd. for $C_{203}H_{323}N_{43}O_{62}S$ 4390.1160; found $[M+3H]^{3+}$=1464.30; $[M+4H]^{4+}$=1098.40.

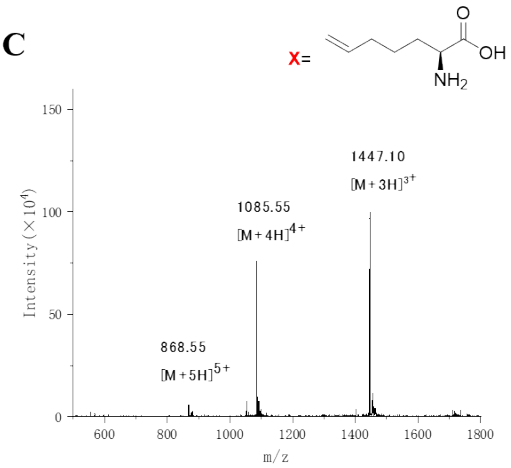
**A**

SEK1-12-5: [Cyc(21, 25)]Ac-SLDQINVTFLDLEYEMKKLE***X***AIK***X***LEESYIDLKEL-$NH_2$

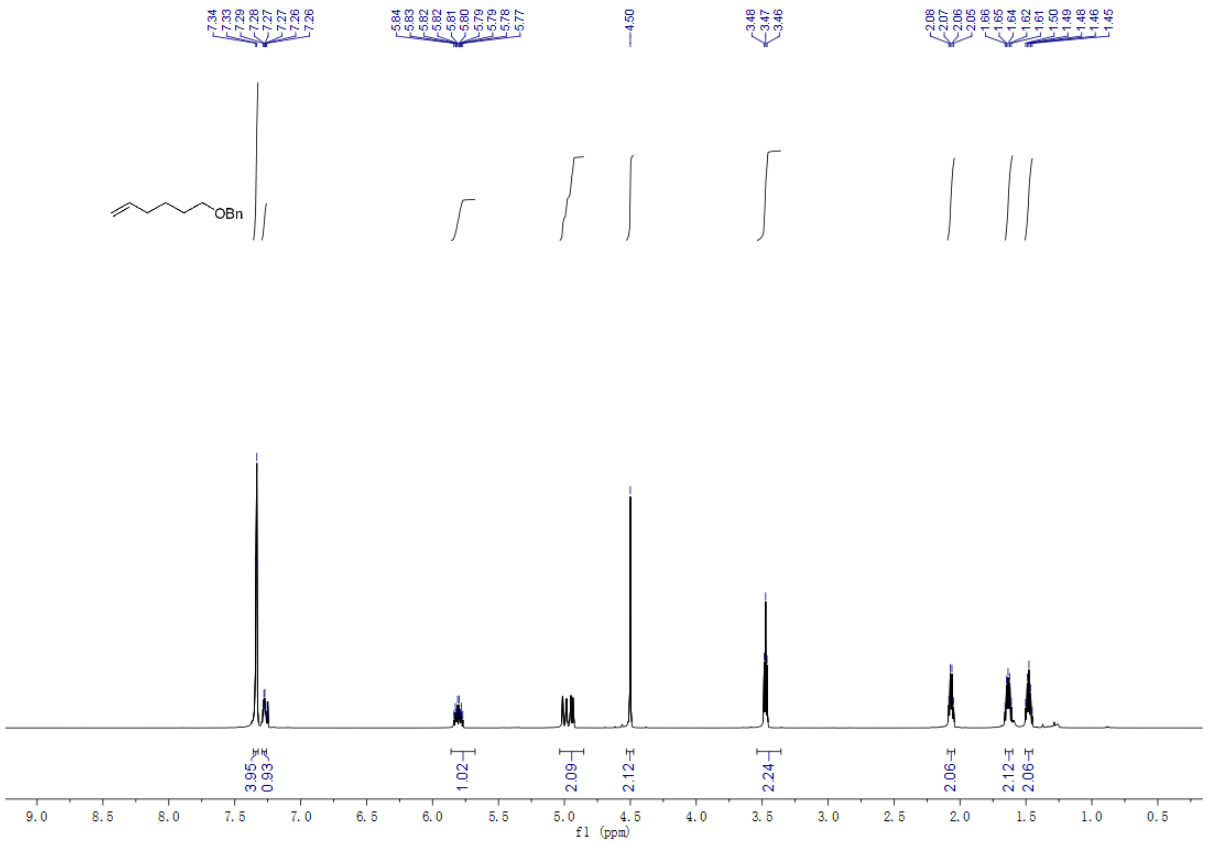
**B**



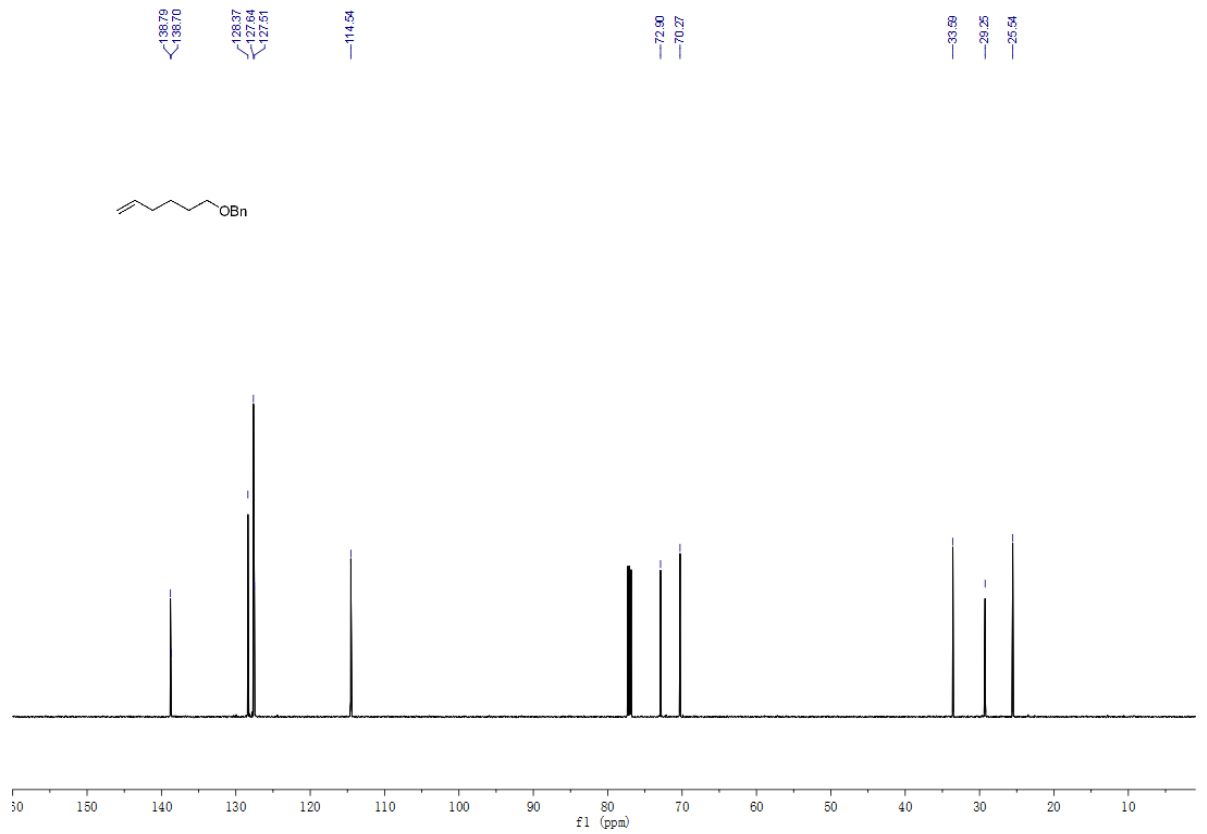

**C**



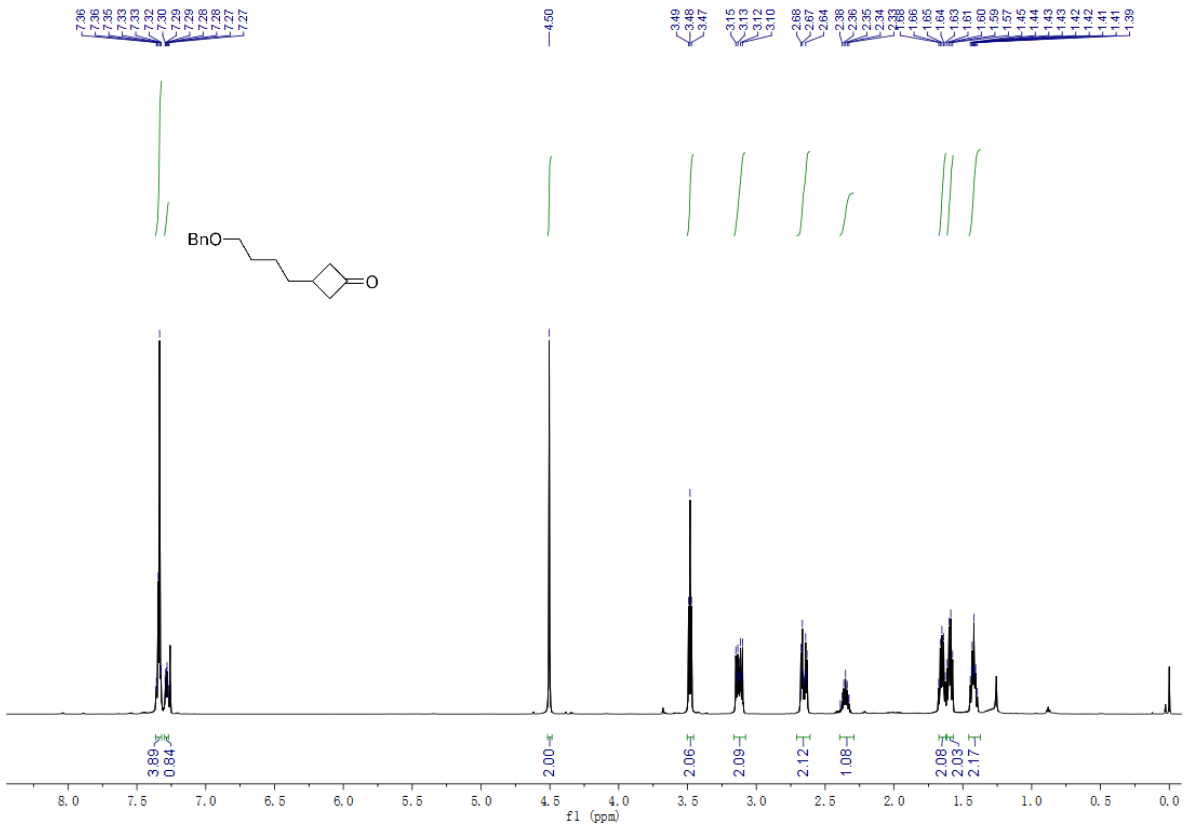

White lyophilized powder, 48 mg, 29% yield. A) The structure of **SEK1-12-5**; B). The HPLC of purified **SEK1-12-5**. Gradient: 0-90% of buffer B in 30 min with C18 column (5 μm, 2.5 mm×250 mm); C) ESI-MS spectrum of **SEK1-12-5**, calcd. for $C_{199}H_{319}N_{43}O_{62}S$ 4335.2851; found $[M+3H]^{3+}$=1447.10; $[M+4H]^{4+}$=1085.55; $[M+5H]^{5+}$=868.55.



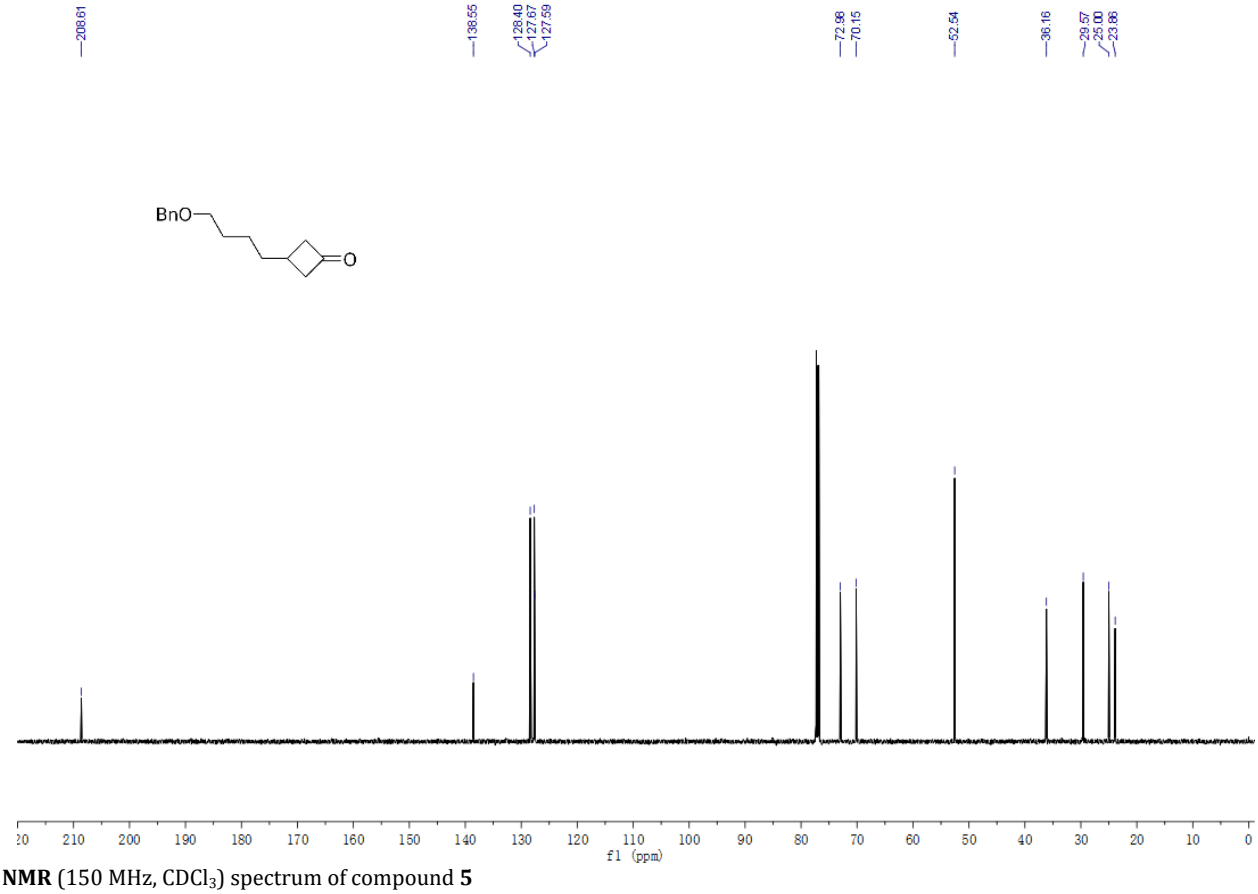

**$^{1}$H NMR** (600 MHz, $CDCl_3$) spectrum of compound **4**



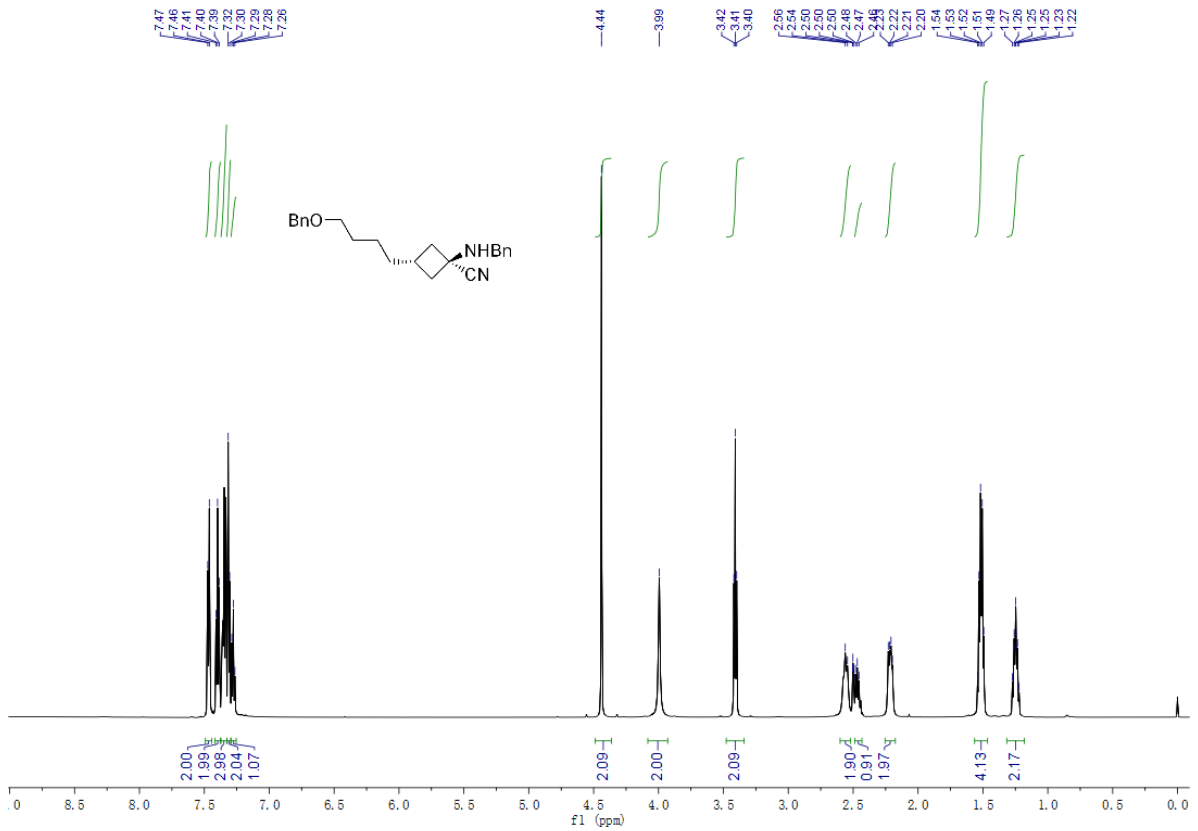

**$^{13}$C NMR** (150 MHz, $CDCl_3$) spectrum of compound **4**



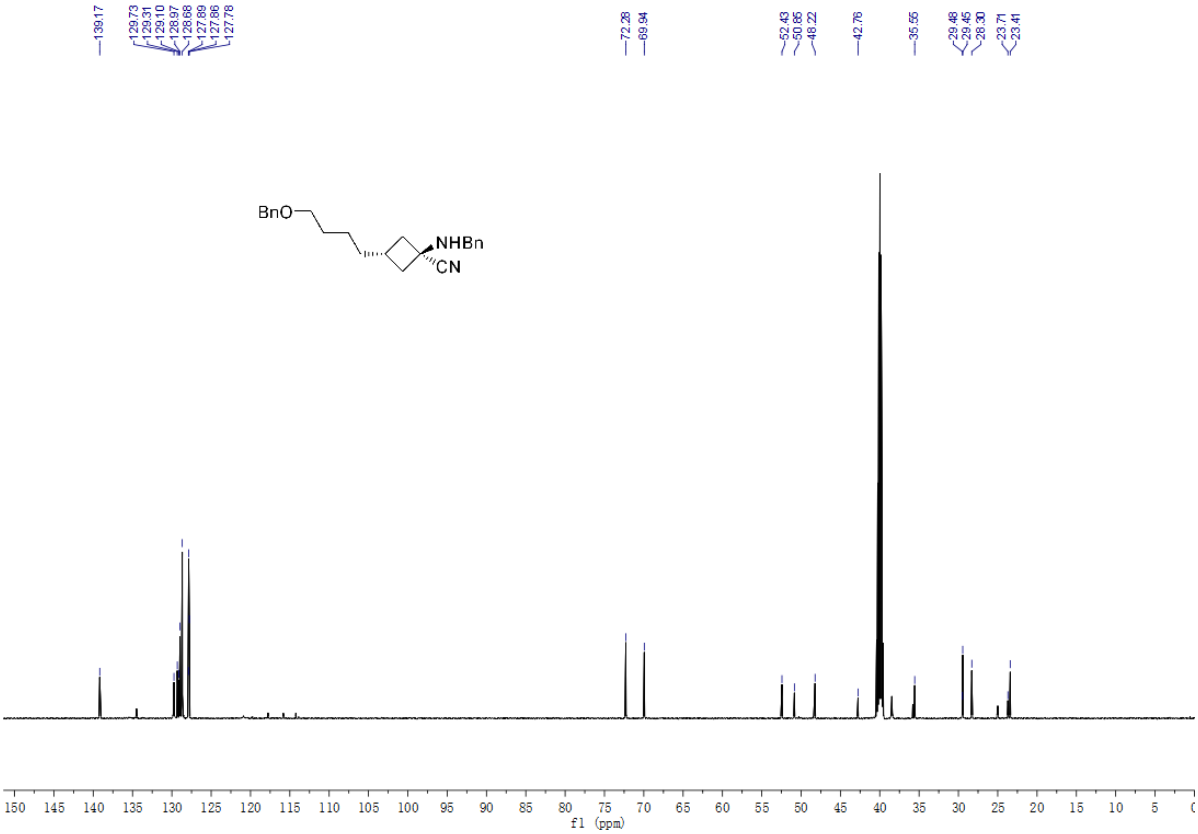

**$^{1}$H NMR** (600 MHz, $CDCl_3$) spectrum of compound **5**



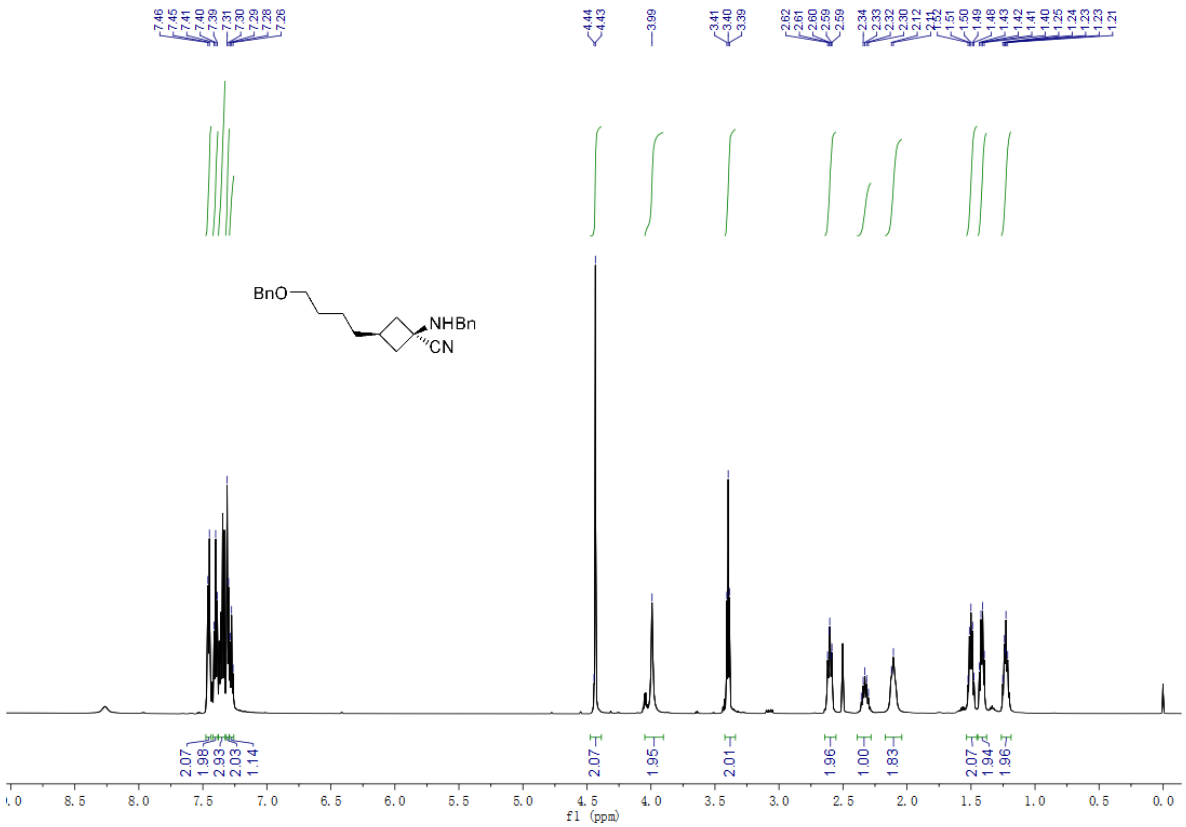

**$^{13}$C NMR** (150 MHz, $CDCl_3$) spectrum of compound **5**



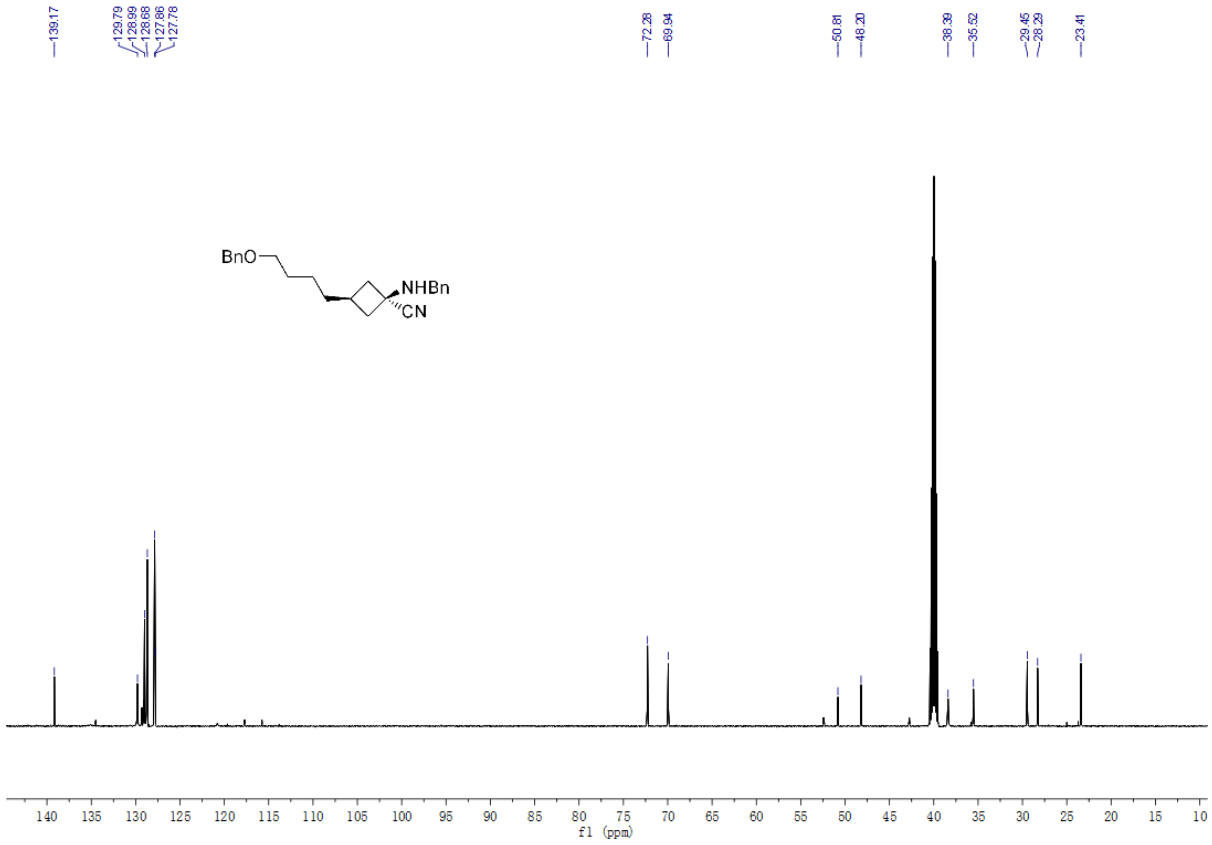

**$^{1}$H NMR** (600 MHz, *d*-DMSO) spectrum of compound **6a**



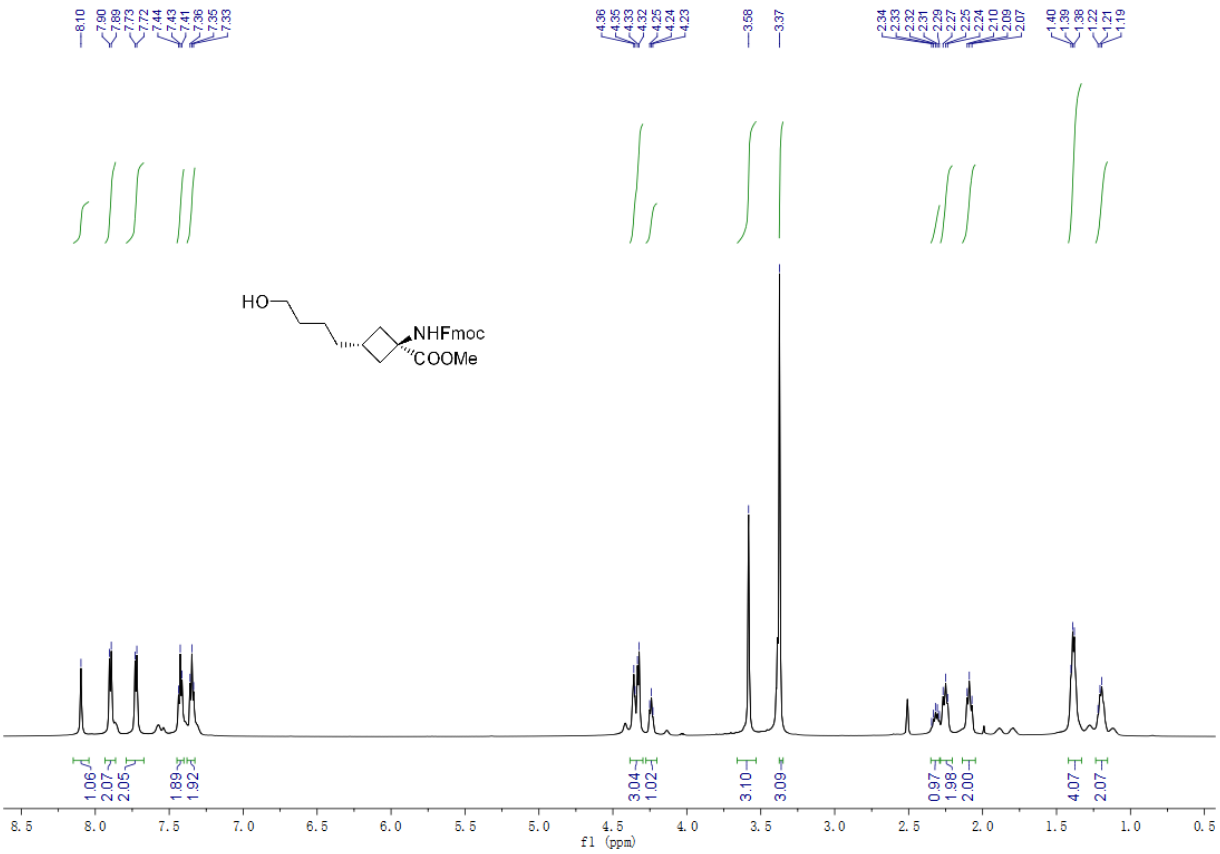

**$^{13}$C NMR** (150 MHz, *d*-DMSO) spectrum of compound **6a**



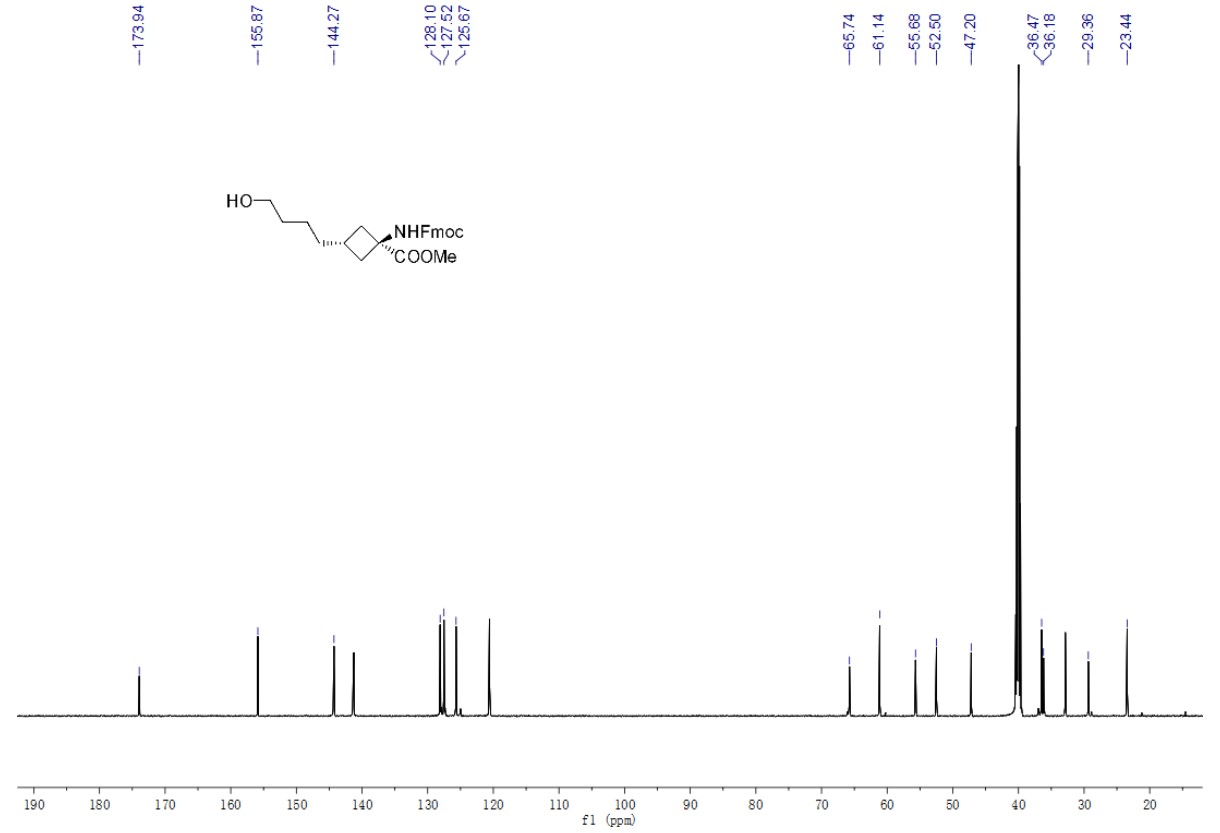

**$^{1}$H NMR** (600 MHz, *d*-DMSO) spectrum of compound **6b**



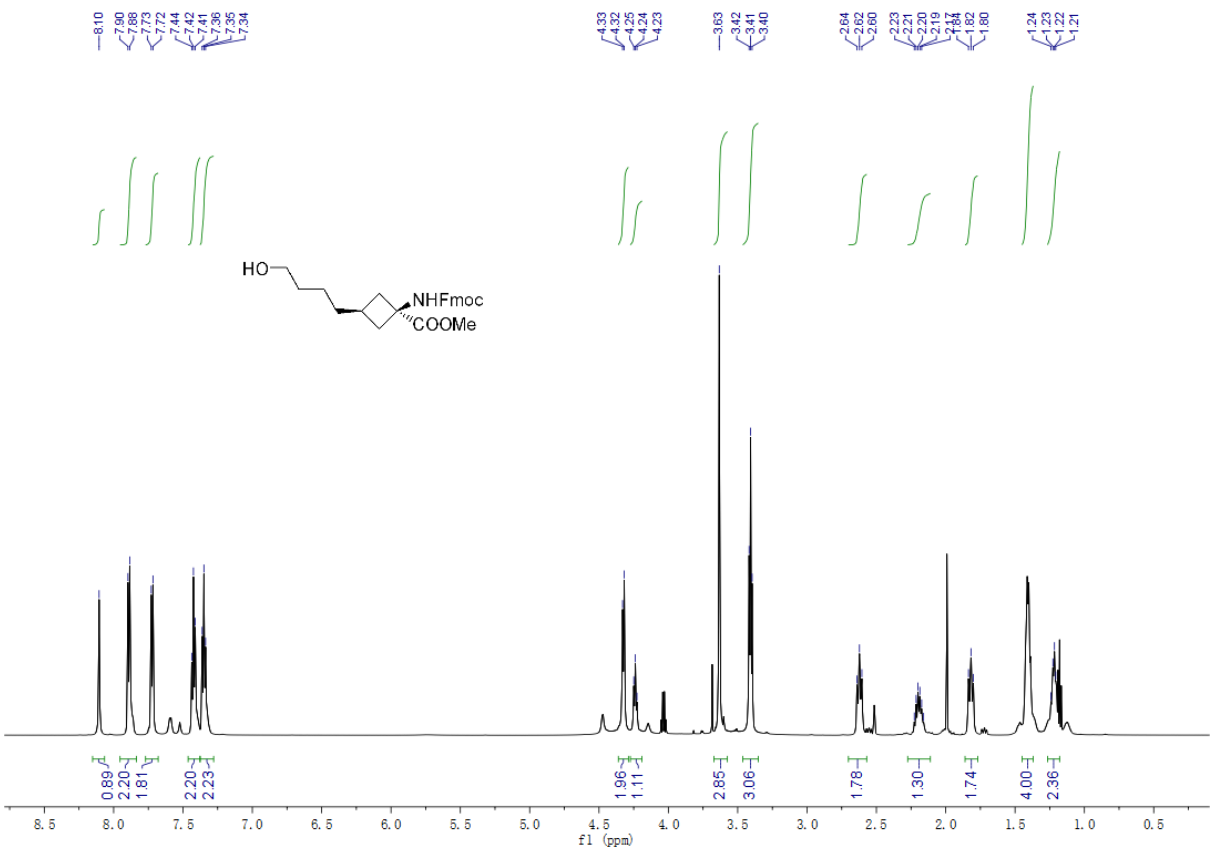

**$^{13}$C NMR** (150 MHz, *d*-DMSO) spectrum of compound **6b**



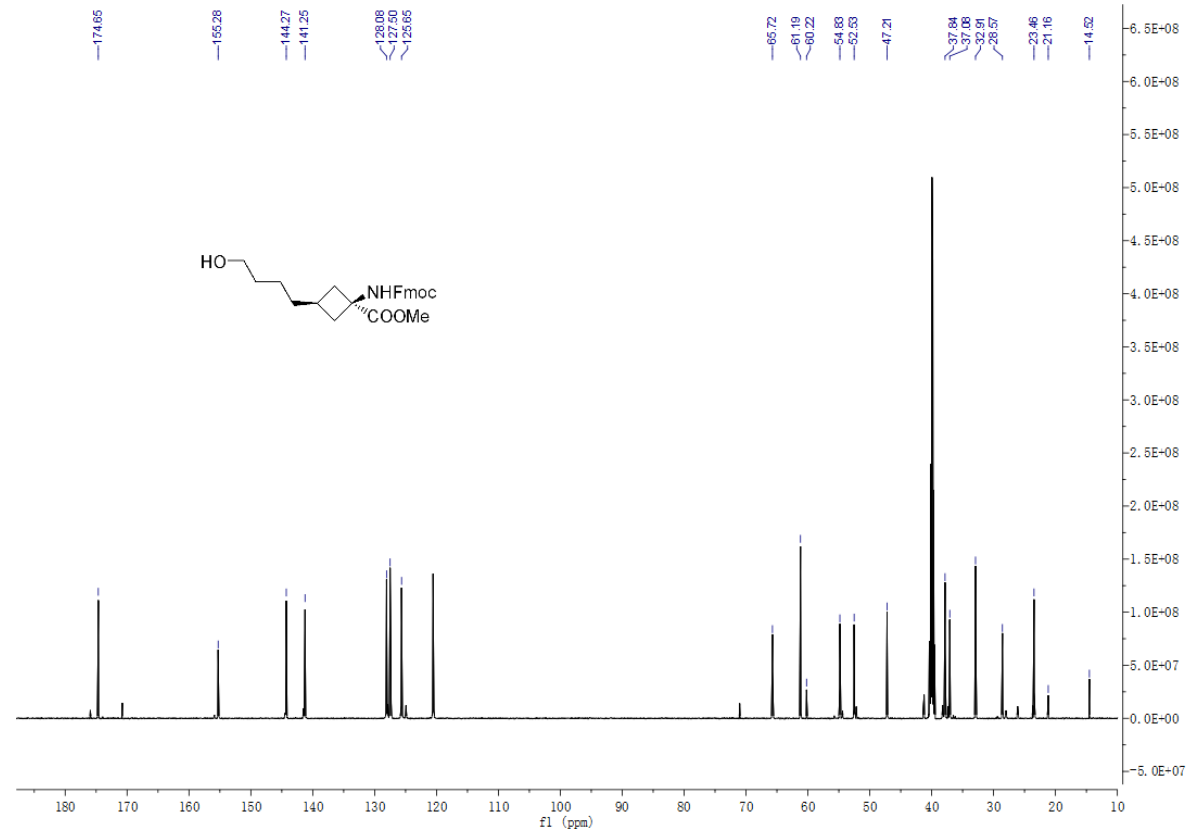

**$^{1}$H NMR** (600 MHz, *d*-DMSO) spectrum of compound **7a**



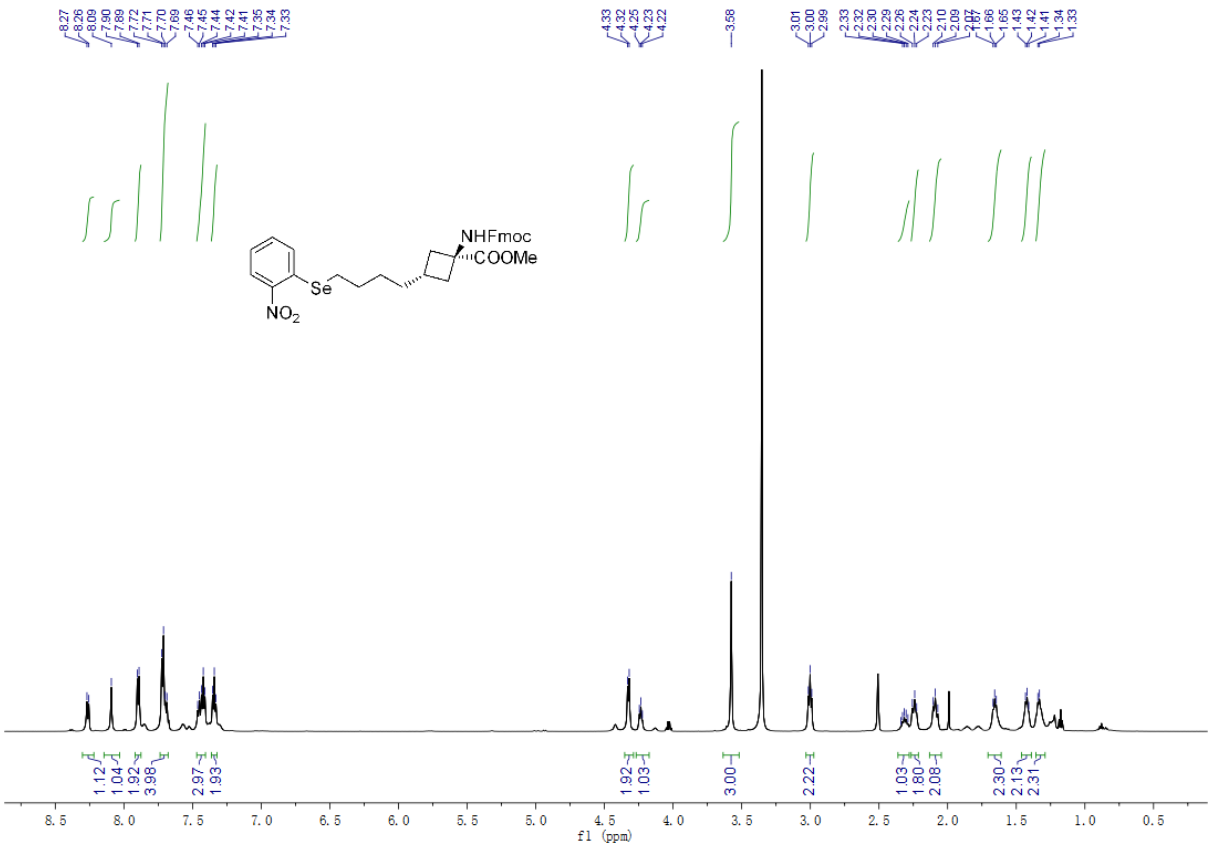

**$^{13}$C NMR** (150 MHz, *d*-DMSO) spectrum of compound **7a**



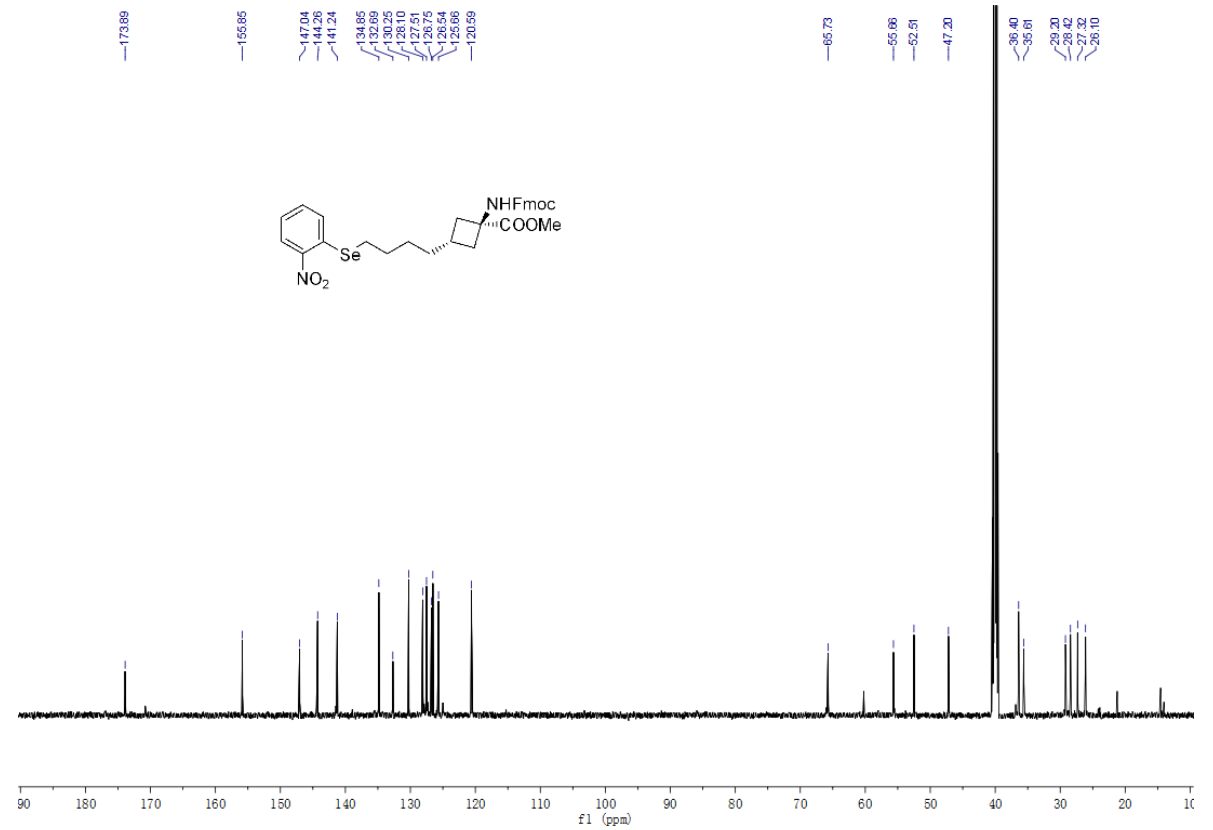

**$^{1}$H NMR** (600 MHz, *d*-DMSO) spectrum of compound **7b**



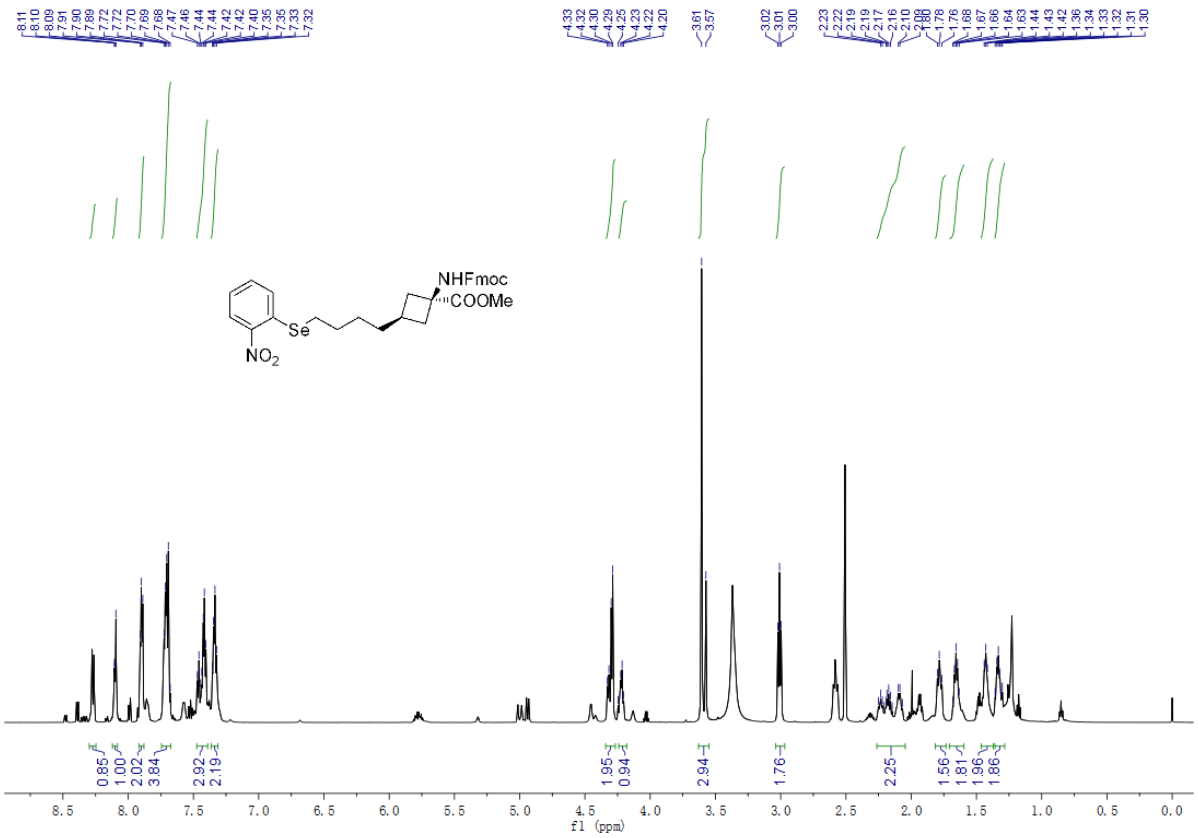

**$^{13}$C NMR** (150 MHz, *d*-DMSO) spectrum of compound **7b**



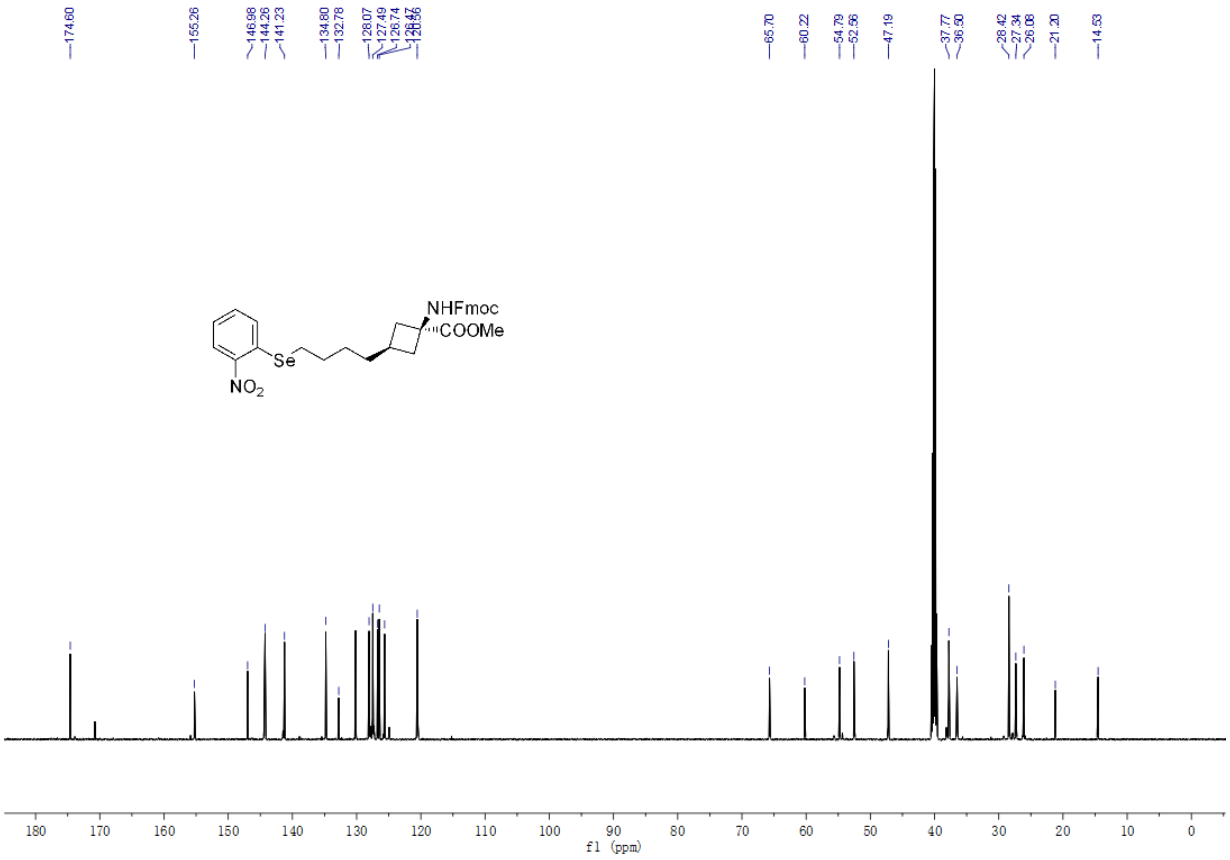

**$^{1}$H NMR** (600 MHz, *d*-DMSO) spectrum of compound **8a**



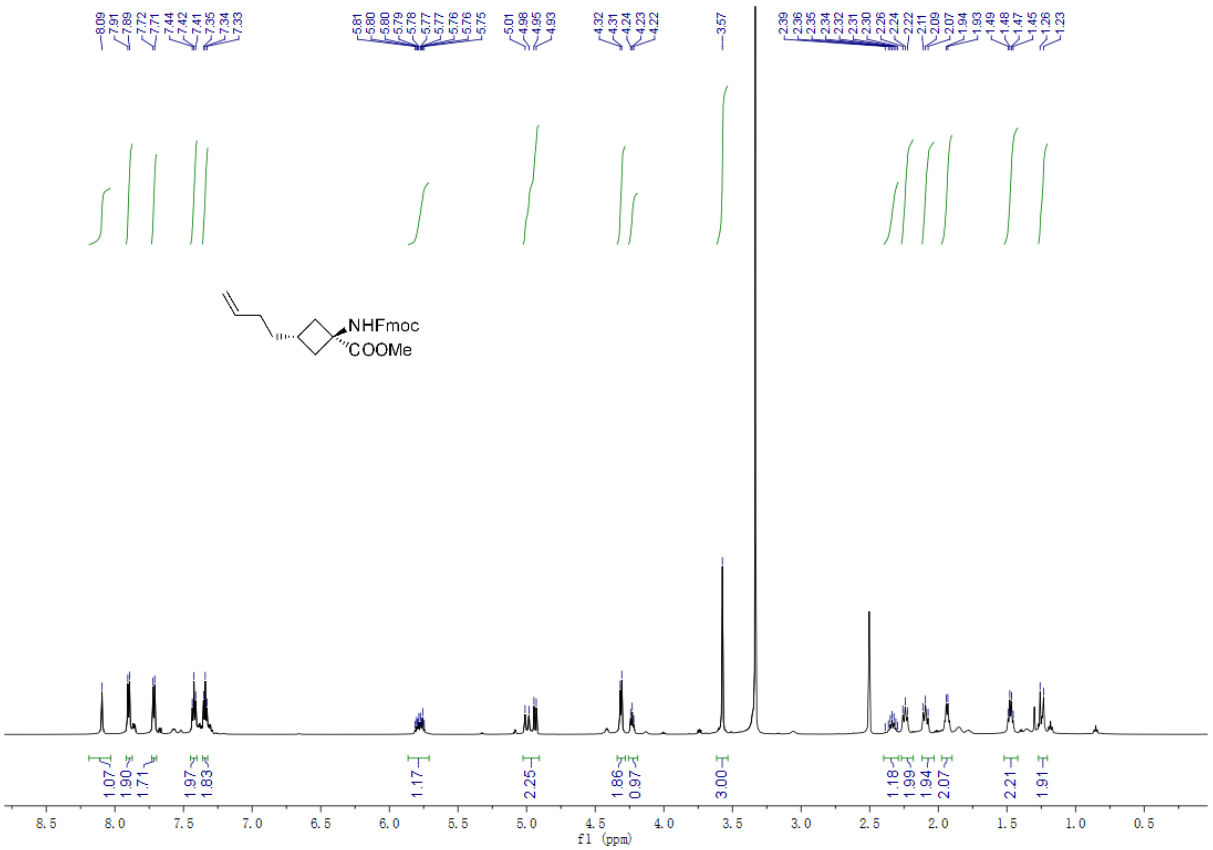

**$^{13}$C NMR** (150 MHz, *d*-DMSO) spectrum of compound **8a**



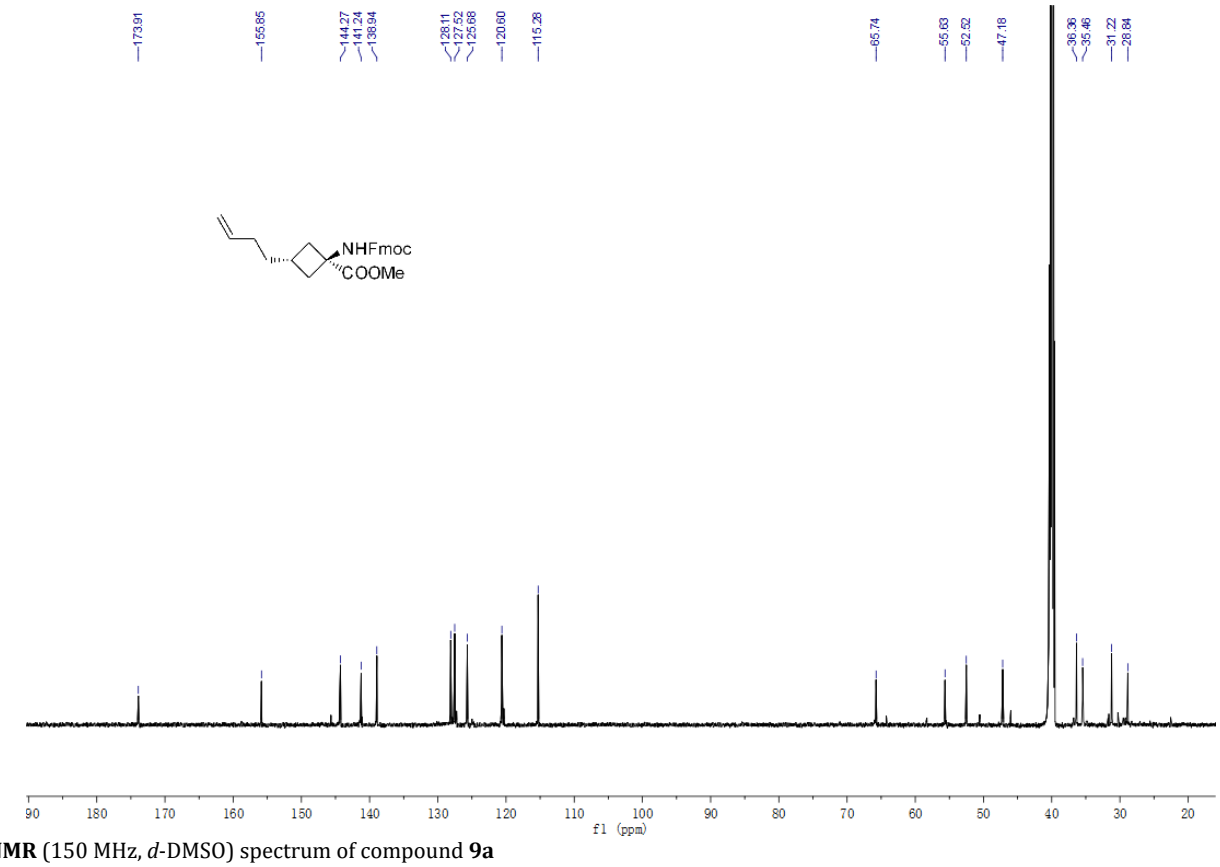

**$^{1}$H NMR** (600 MHz, *d*-DMSO) spectrum of compound **8b**



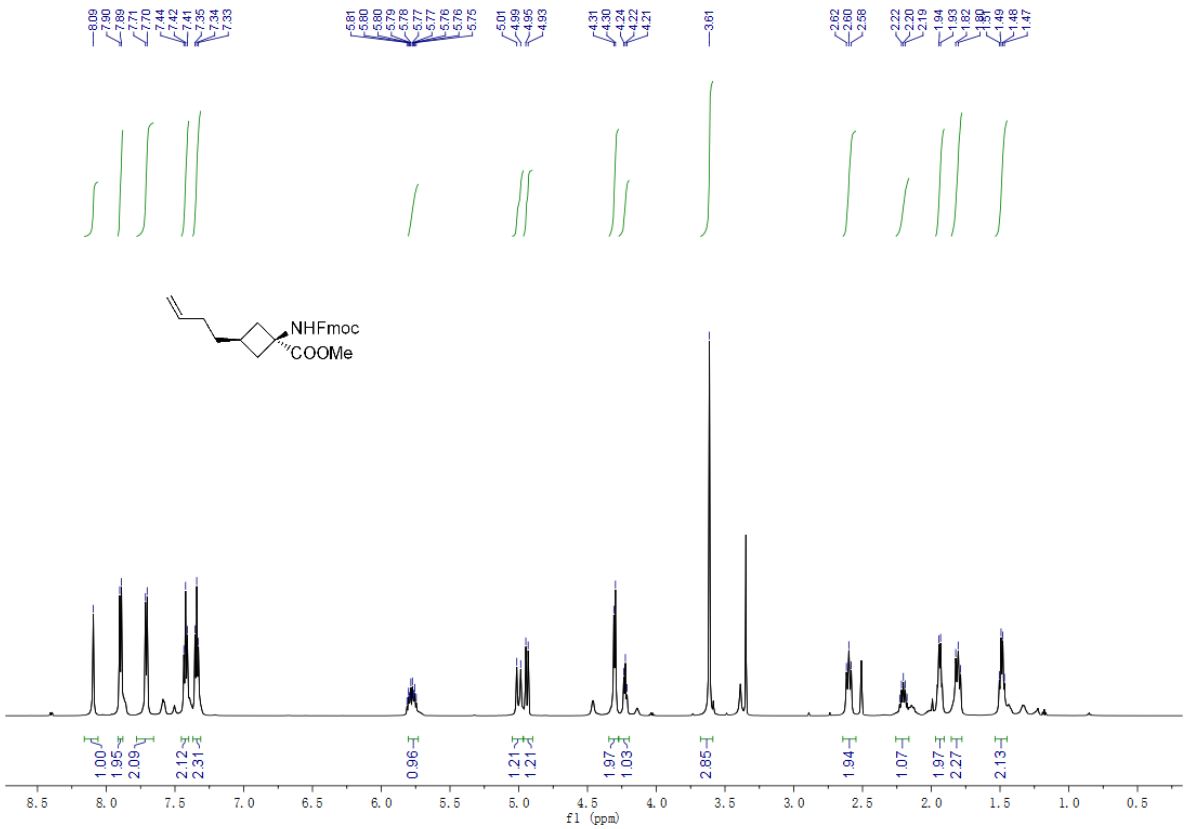

**$^{13}$C NMR** (150 MHz, *d*-DMSO) spectrum of compound **8b**



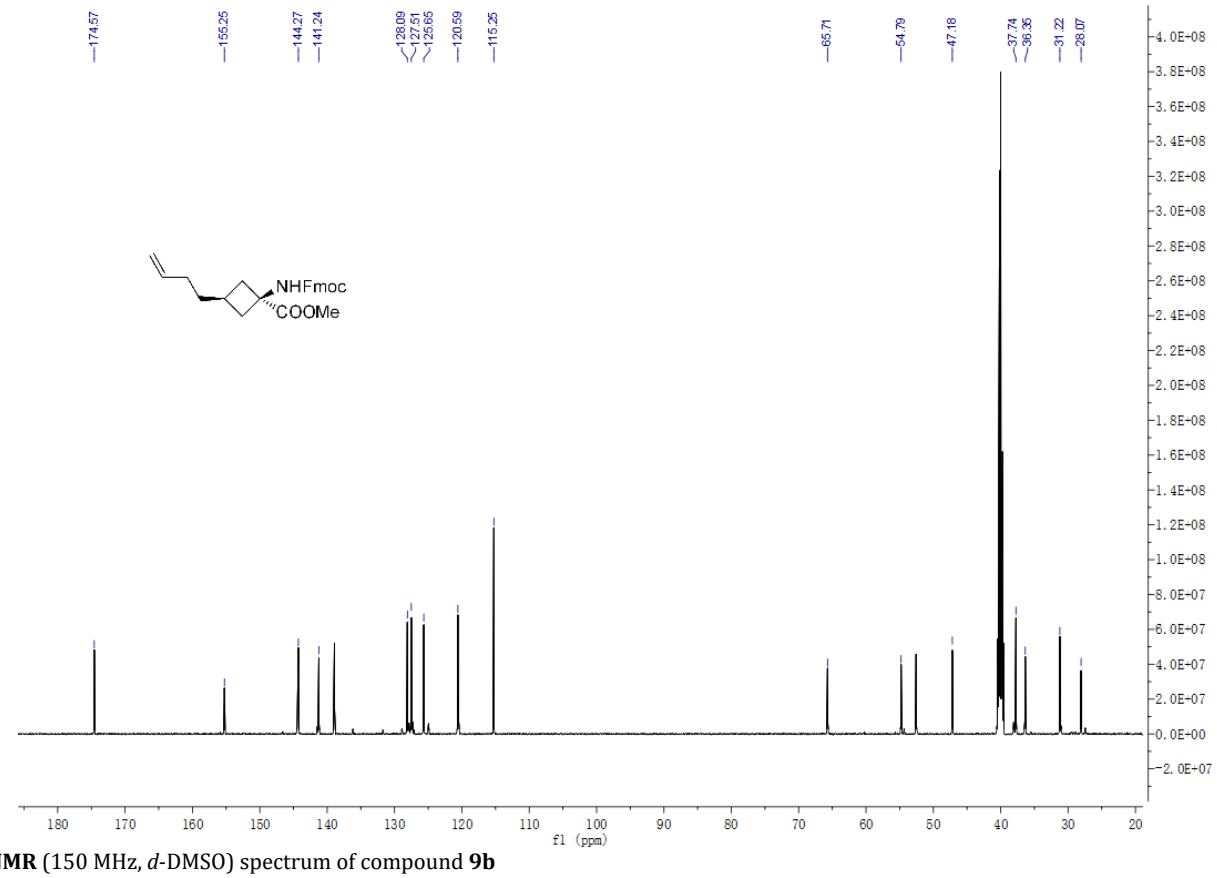

**$^{1}$H NMR** (600 MHz, *d*-DMSO) spectrum of compound **9a**



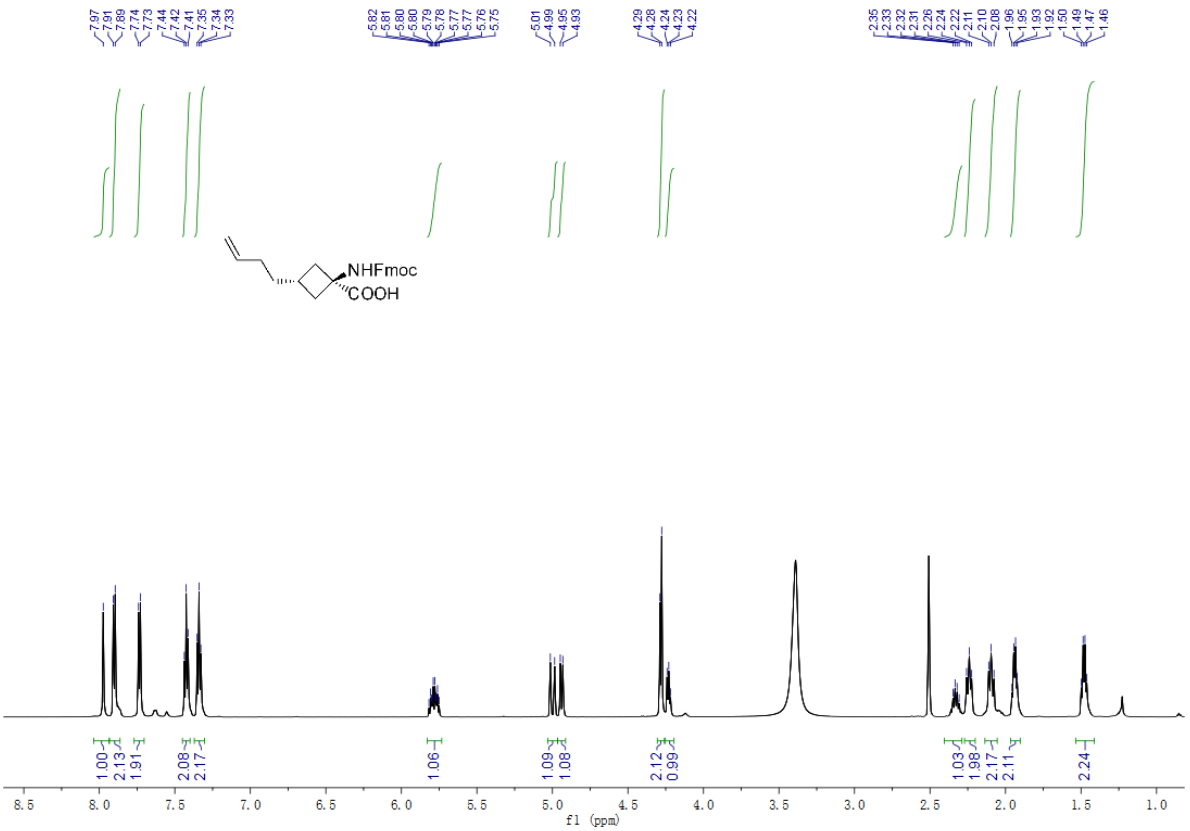

**$^{13}$C NMR** (150 MHz, *d*-DMSO) spectrum of compound **9a**



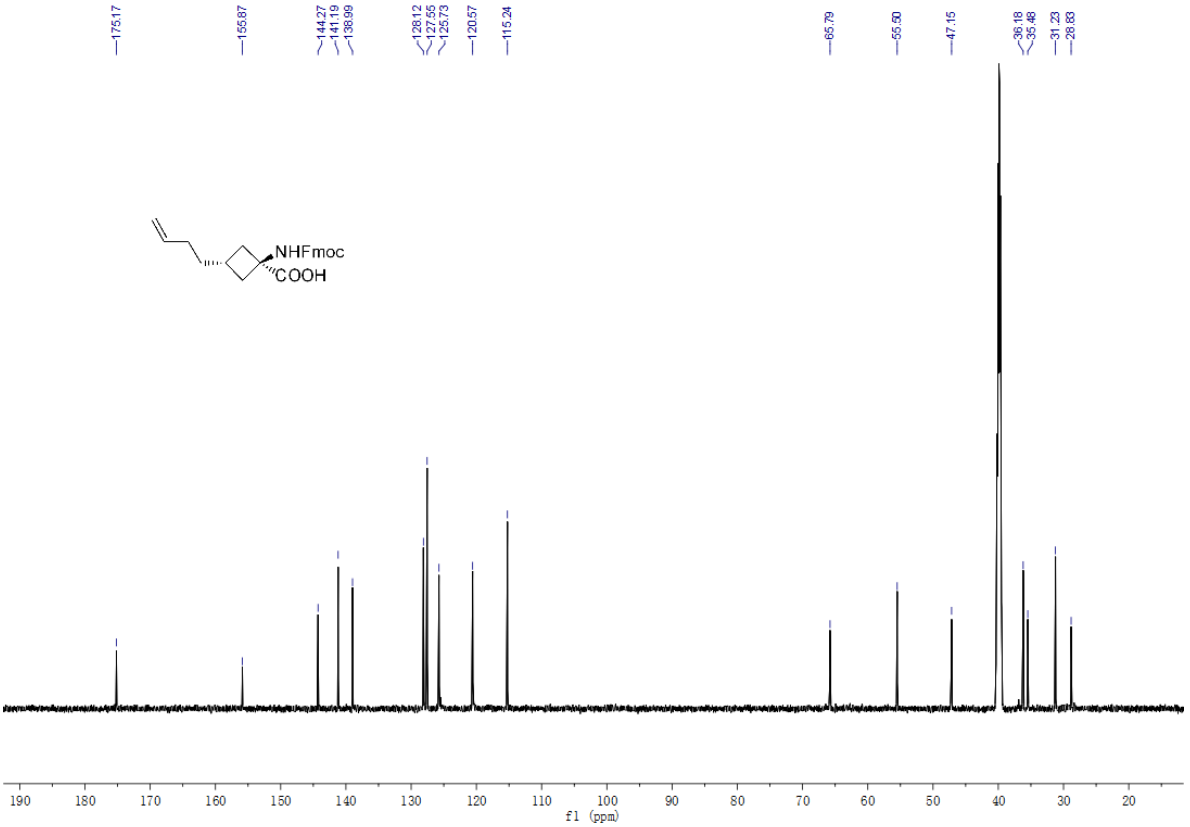

**$^{1}$H NMR** (600 MHz, *d*-DMSO) spectrum of compound **9b**



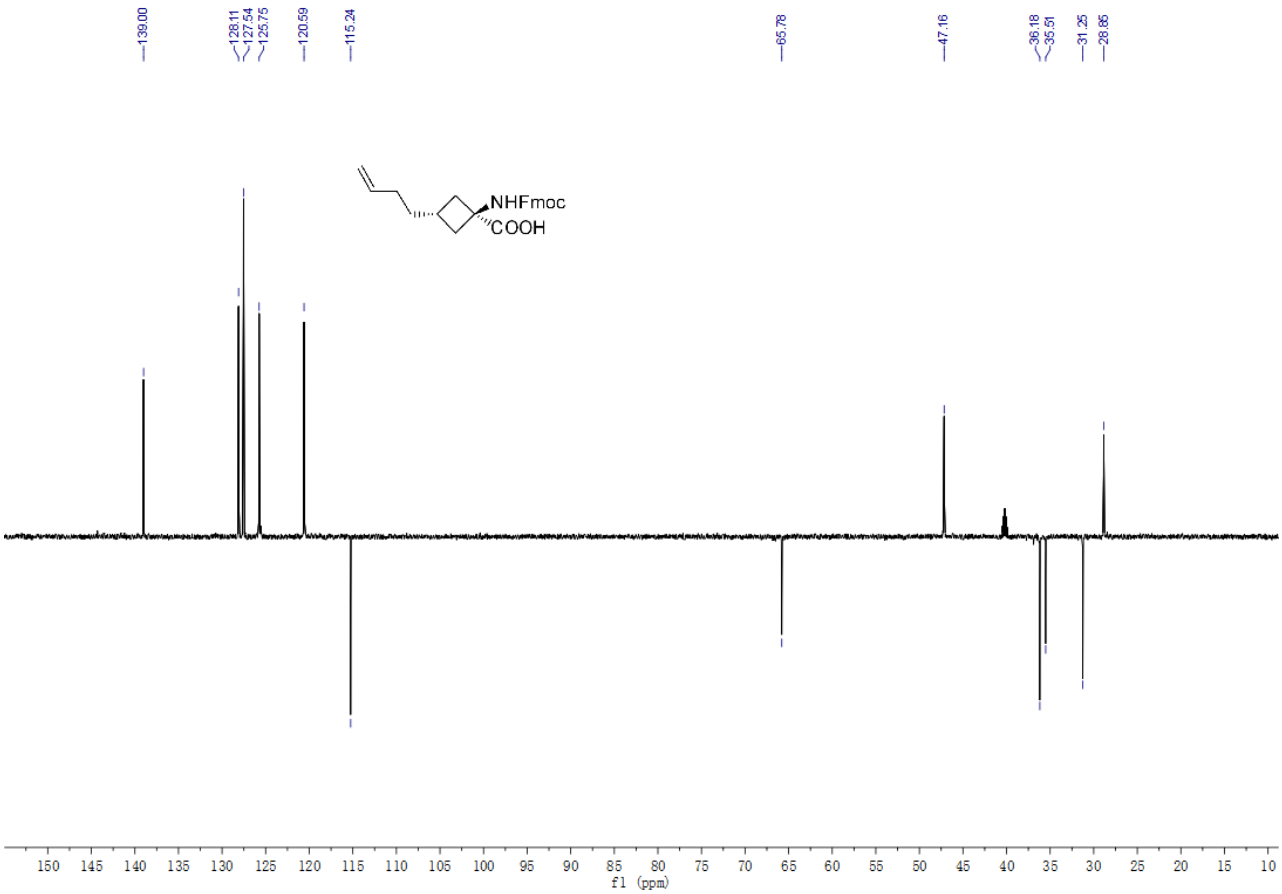

**$^{13}$C NMR** (150 MHz, *d*-DMSO) spectrum of compound **9b**



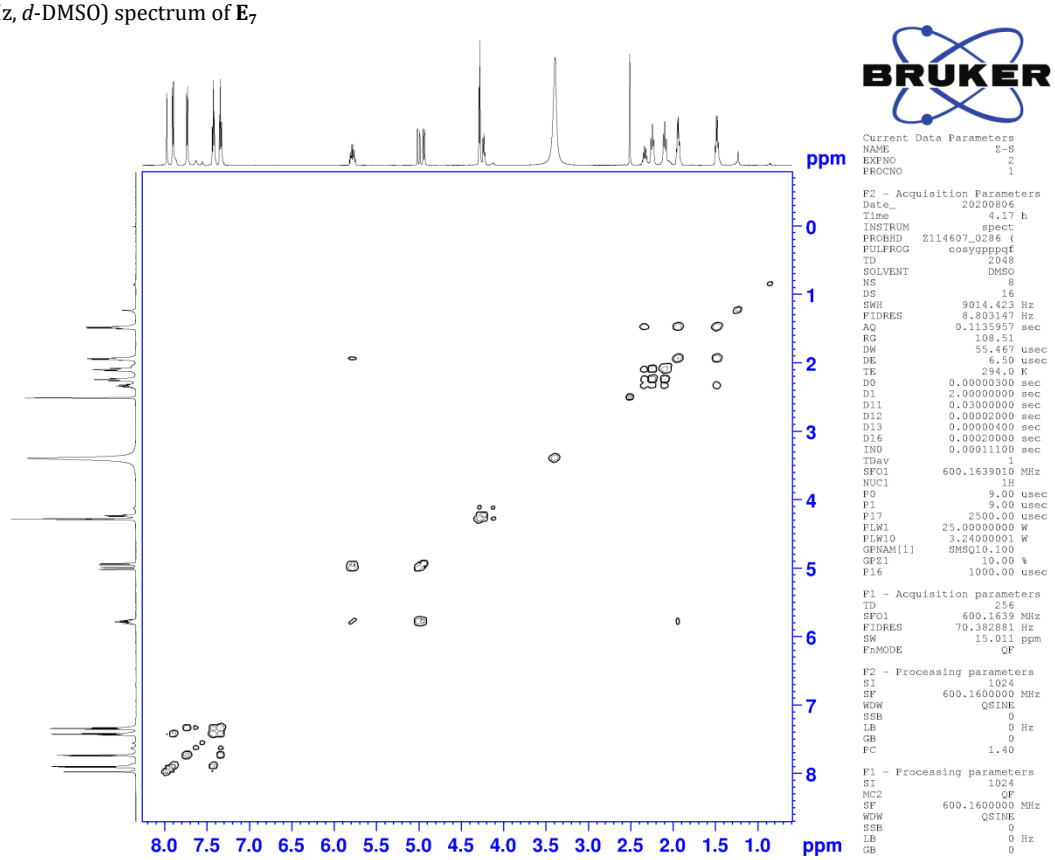

**$^{1}$H NMR** (600 MHz, *d*-DMSO) spectrum of **$E_7$**




**$^{13}$C NMR** (150 MHz, *d*-DMSO) spectrum of **$E_7$**




**Dept** (600 MHz, *d*-DMSO) spectrum of $\mathbf{E_7}$





Current Data Parameters
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EXPNO 2
PROCNO 1

F2 - Acquisition Parameters
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PROBHD Z114607_0286 (
PULPROG cosygpppqf
TD 2048
SOLVENT DMSO
NS 8
DS 16
SWH 9014.423 Hz
FIDRES 8.803147 Hz
AQ 0.1135957 sec
RG 108.51
DW 55.467 usec
DE 6.50 usec
TE 294.0 K
D0 0.00000300 sec
D1 2.00000000 sec
D11 0.03000000 sec
D12 0.00002000 sec
D13 0.00000400 sec
D16 0.00020000 sec
IN0 0.00011100 sec
TDav 1
SFO1 600.1639010 MHz
NUC1 1H
P0 9.00 usec
P1 9.00 usec
P17 2500.00 usec
PLW1 25.00000000 W
PLW10 3.24000001 W
GPNAM[1] SMSQ10.100
GPZ1 10.00 %
P16 1000.00 usec

F1 - Acquisition parameters
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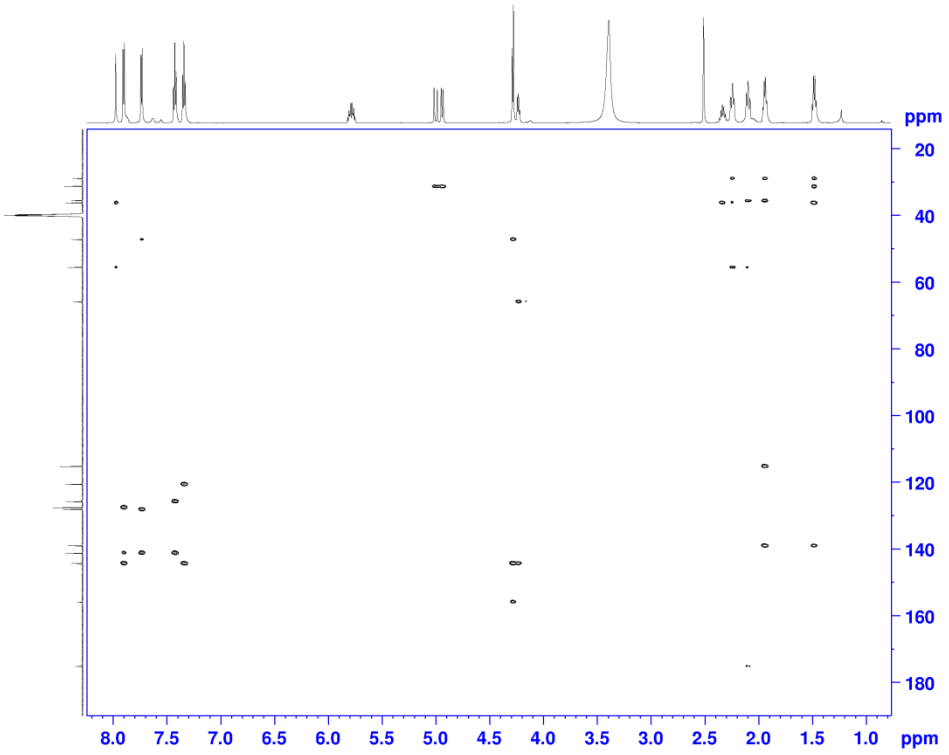
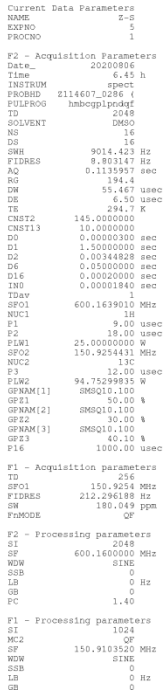
FIDRES 70.382881 Hz
SW 15.011 ppm
FnMODE QF

F2 - Processing parameters
SI 1024
SF 600.1600000 MHz
WDW QSINE
SSB 0
LB 0 Hz
GB 0
PC 1.40

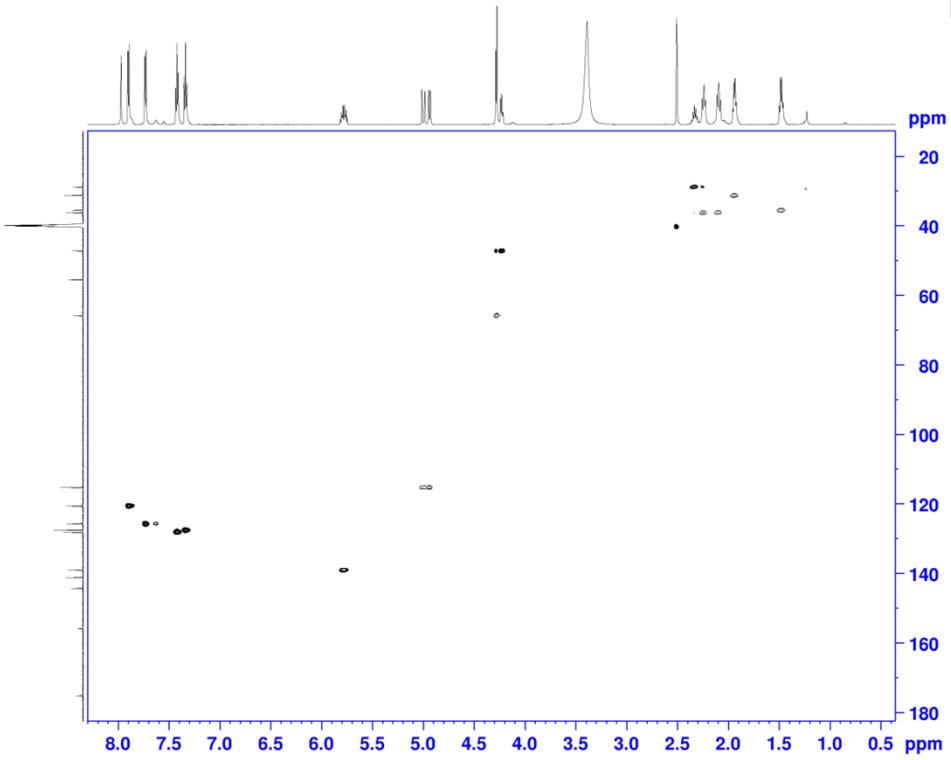
F1 - Processing parameters
SI 1024
MC2 QF
SF 600.1600000 MHz
WDW QSINE
SSB 0
LB 0 Hz
GB 0

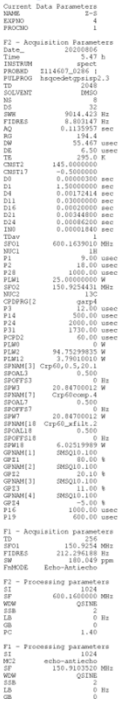

**Cosy** (600 MHz, *d*-DMSO) spectrum of $\mathbf{E_7}$



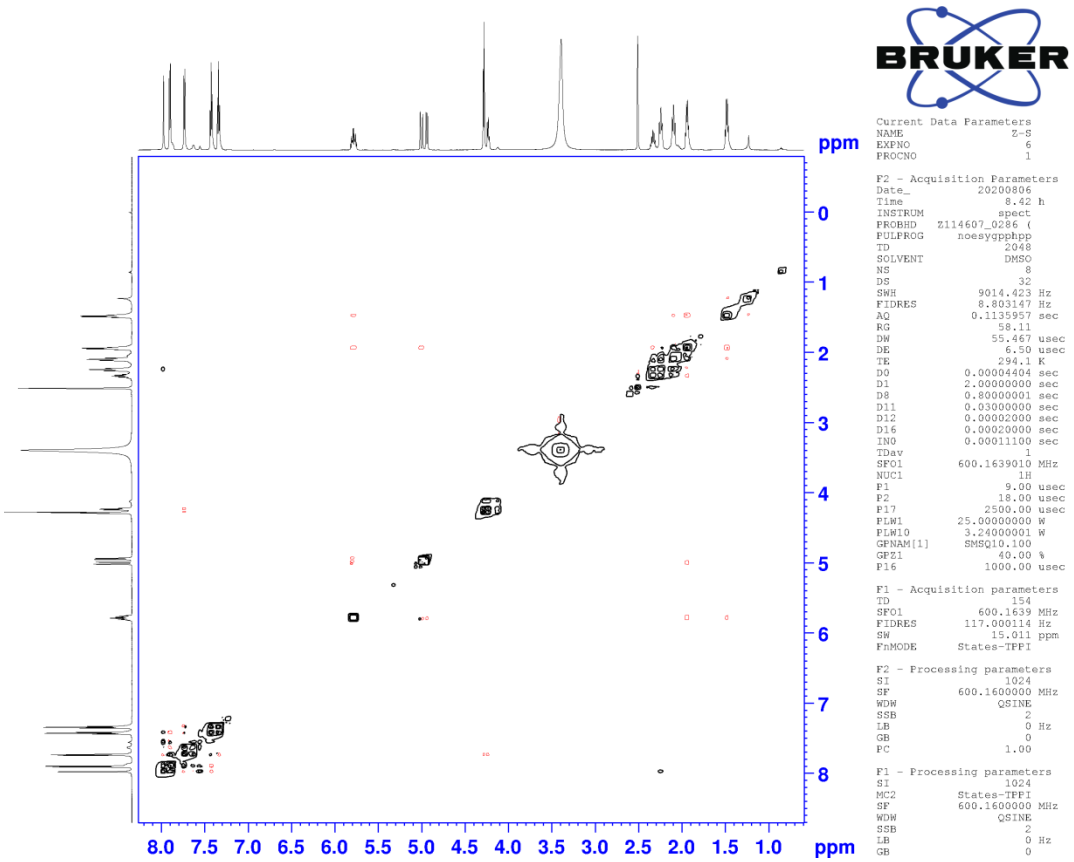




**Hmbc** (600 MHz, *d*-DMSO) spectrum of **E$_7$**

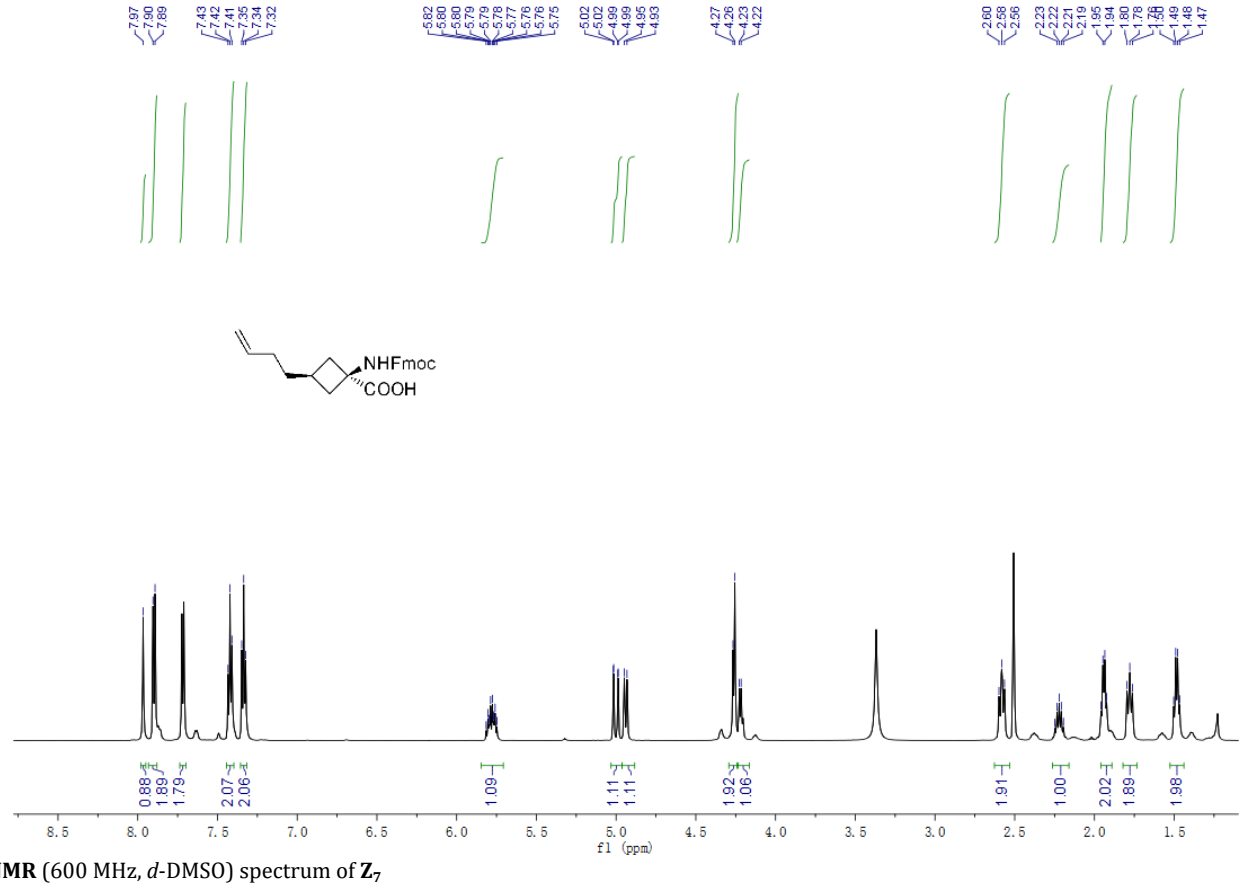


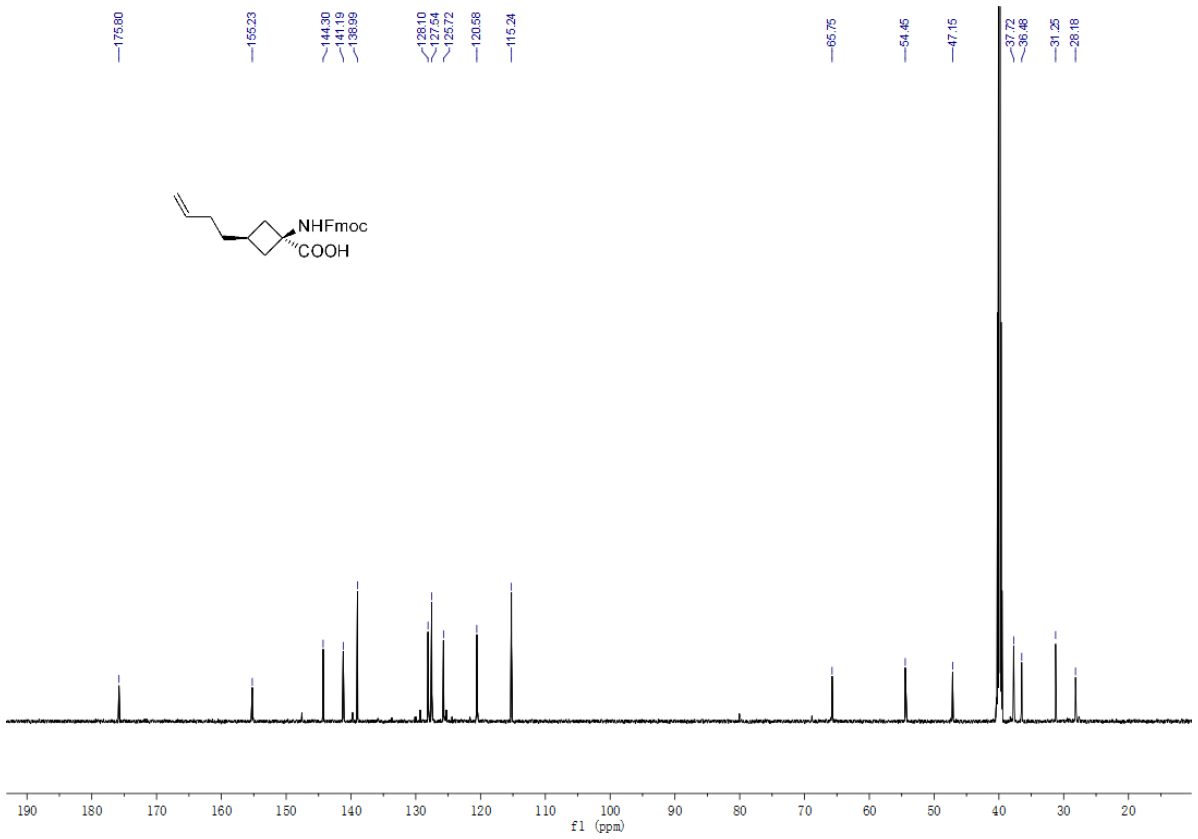

**Hsqc** (600 MHz, *d*-DMSO) spectrum of **E$_7$**



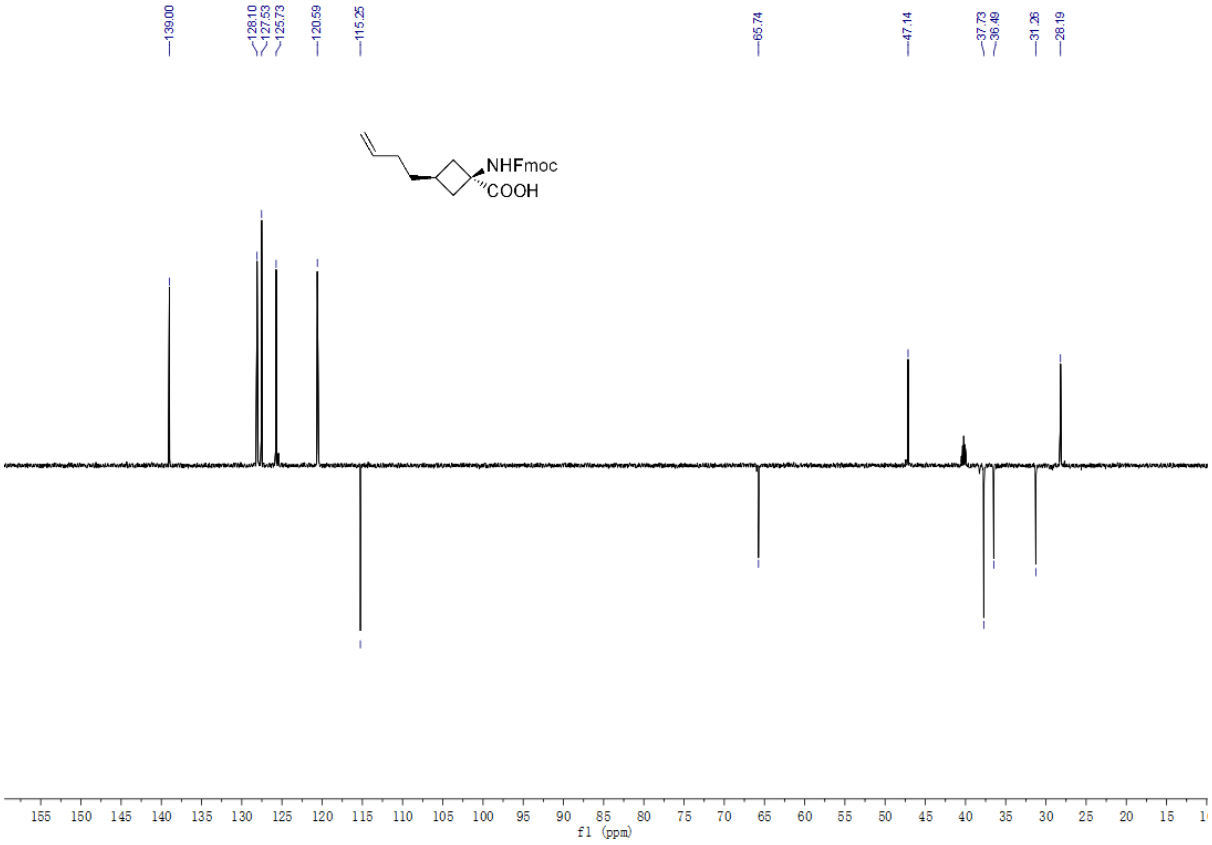

**Noesy** (600 MHz, *d*-DMSO) spectrum of $\mathbf{E_7}$



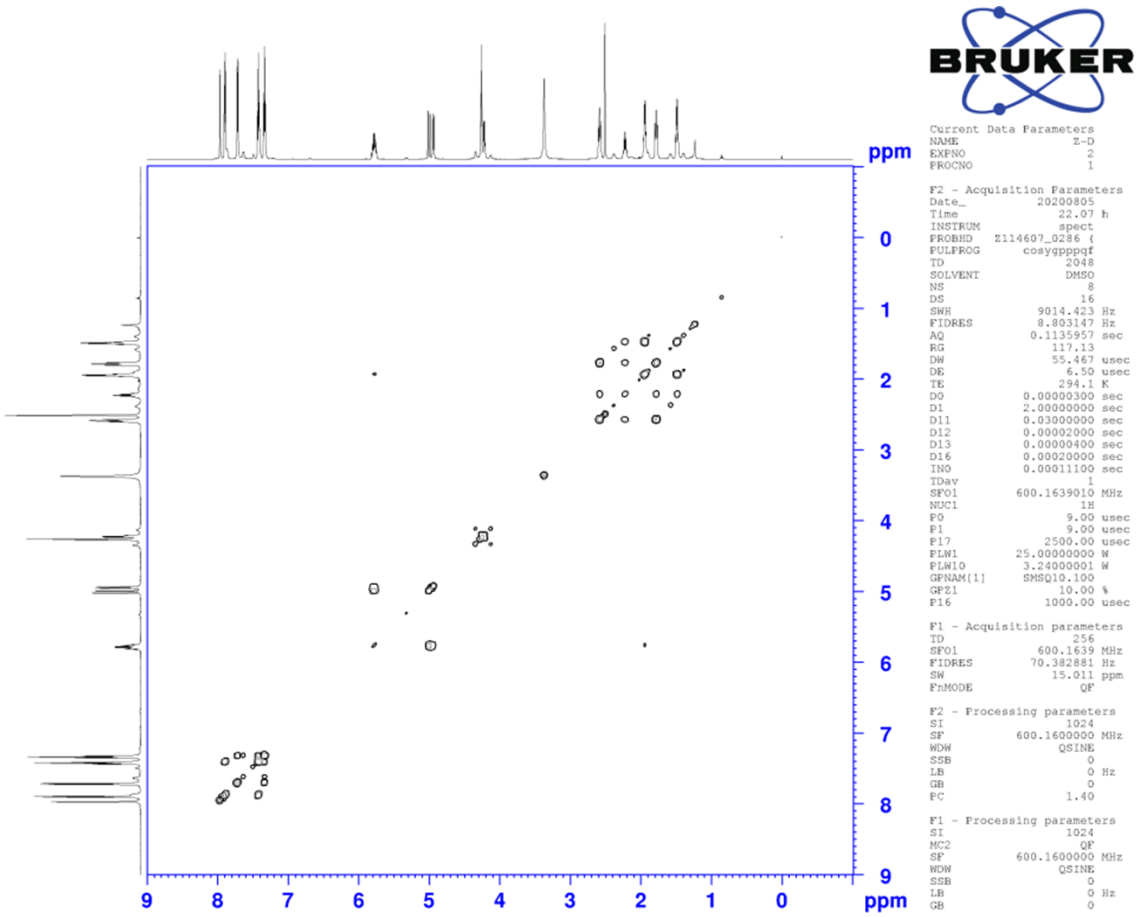

**$^1$H NMR** (600 MHz, *d*-DMSO) spectrum of $\mathbf{Z_7}$



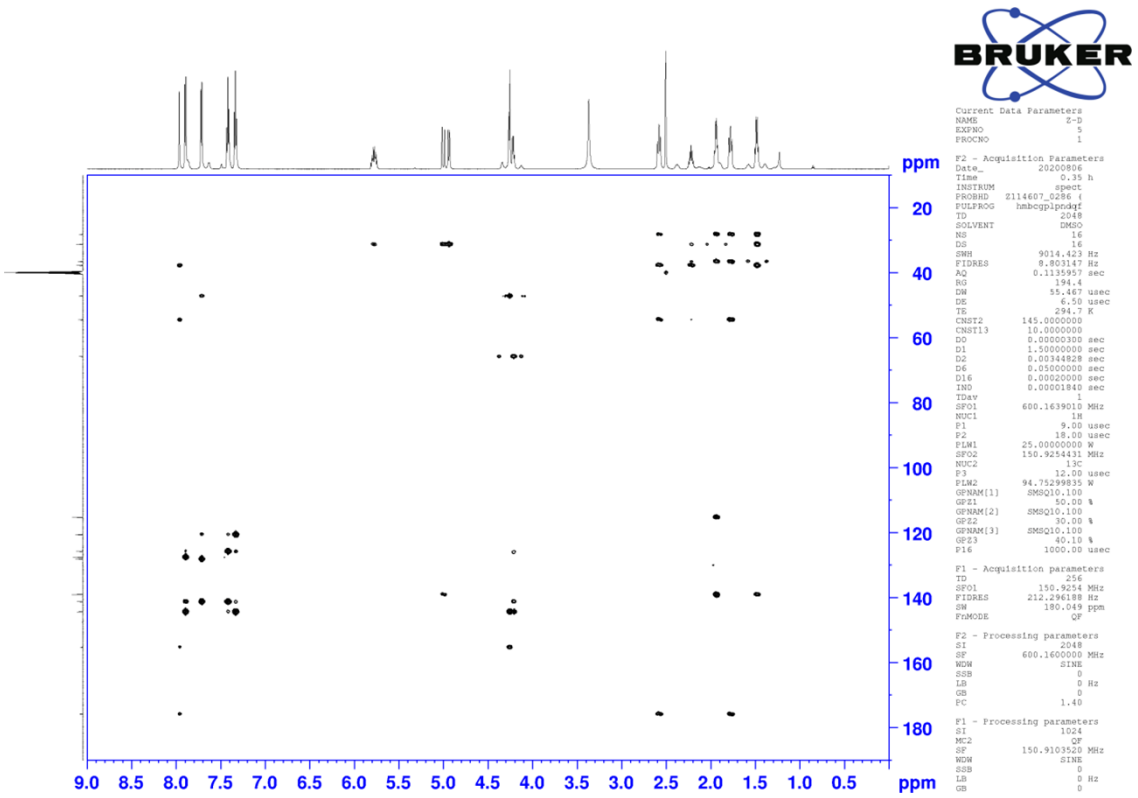

$^{13}$C NMR (150 MHz, *d*-DMSO) spectrum of **Z$_7$**



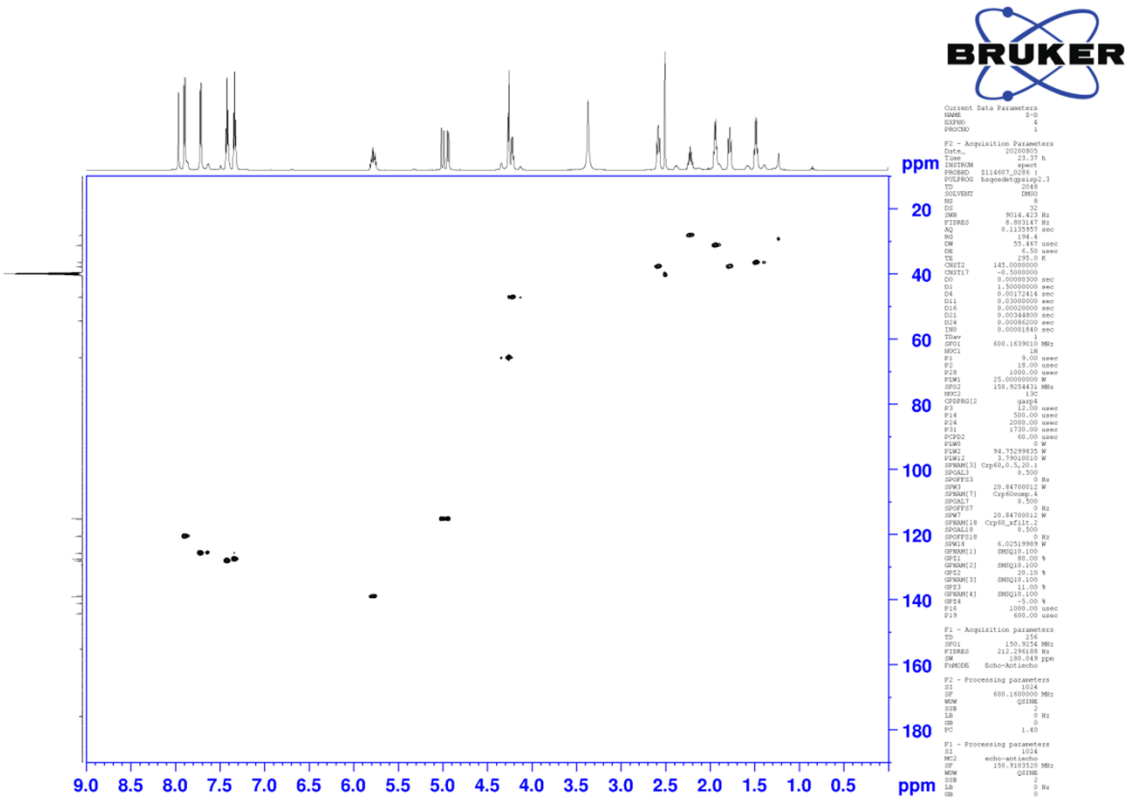

**Dept** (600 MHz, *d*-DMSO) spectrum of **Z$_7$**



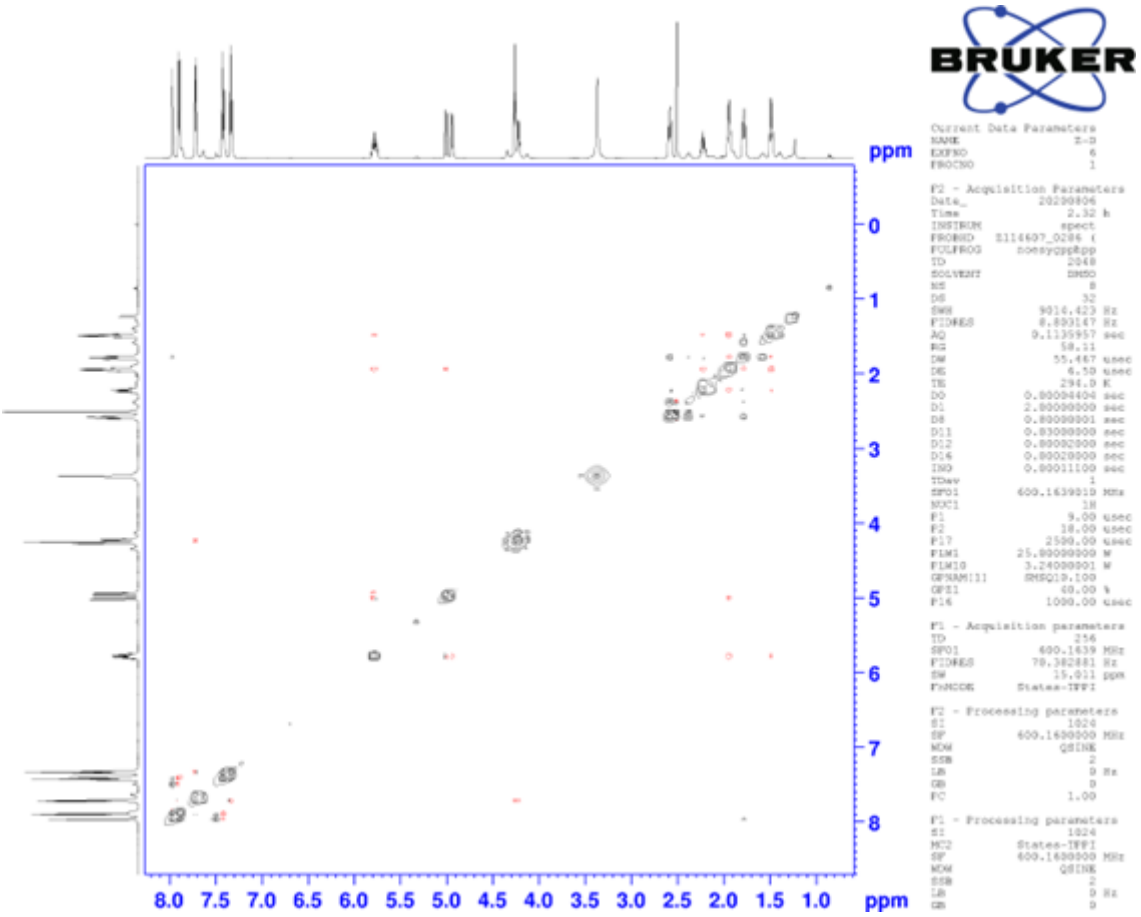

**Cosy** (600 MHz, *d*-DMSO) spectrum of **$Z_7$**




**Hmbc** (600 MHz, *d*-DMSO) spectrum of **$Z_7$**




**Hsqc** (600 MHz, *d*-DMSO) spectrum of $\mathbf{Z_7}$




**Noesy** (600 MHz, *d*-DMSO) spectrum of $\mathbf{Z_7}$