Supplementary Information

Peptidomimetic Vinyl-Heterocyclic Inhibitors of Cruzain Effect Anti-Trypanosomal Activity

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Part 1. Molecular Modeling

In addition to compound **9**, we also utilized Schrodinger to develop both covalent and non-covalent models of compounds **7**, **11-13** and **15** bound to cruzain, also based on the crystal structure of **K11777** covalently bound to cruzain as described in the Experimental Section. The covalent and non-covalent models are in general similar in terms of the orientation of the peptide sidechains. The modeled structures differ, however, by virtue of the nature of how the molecular modeling was performed, leading to the distal positioning of the vinyl group in the non-covalent structures from the active-site residues Cys_{25} and His_{162}. Compounds **12** and **15** which each contain an N-methyl-pyridine, indicate that the methyl group with its attending positive charge is directed to His_{162}, suggesting that the latter is neutral.

Table S1. Predicted affinity of covalent docking for select structures

Compound	Structure	Cdock Affinity (kcal/mol)	Predicted K_i (μM)
7	Cbz-Phe-Phe-vinyl-2Pyrmd	-7.11	6.11
9	NMePip-Phe-hPhe-vinyl-2Pyrmd	-7.01	7.23
11	Cbz-Phe-Phe-vinyl-2Pyr	-7.17	5.52
12	Cbz-Phe-Phe-vinyl-2PyrNMe	-8.00	1.36
13	Cbz-Phe-hPhe-vinyl-2Pyr	-7.63	2.54
15	Cbz-Phe-hPhe-vinyl-2PyrNMe	-8.32	0.79

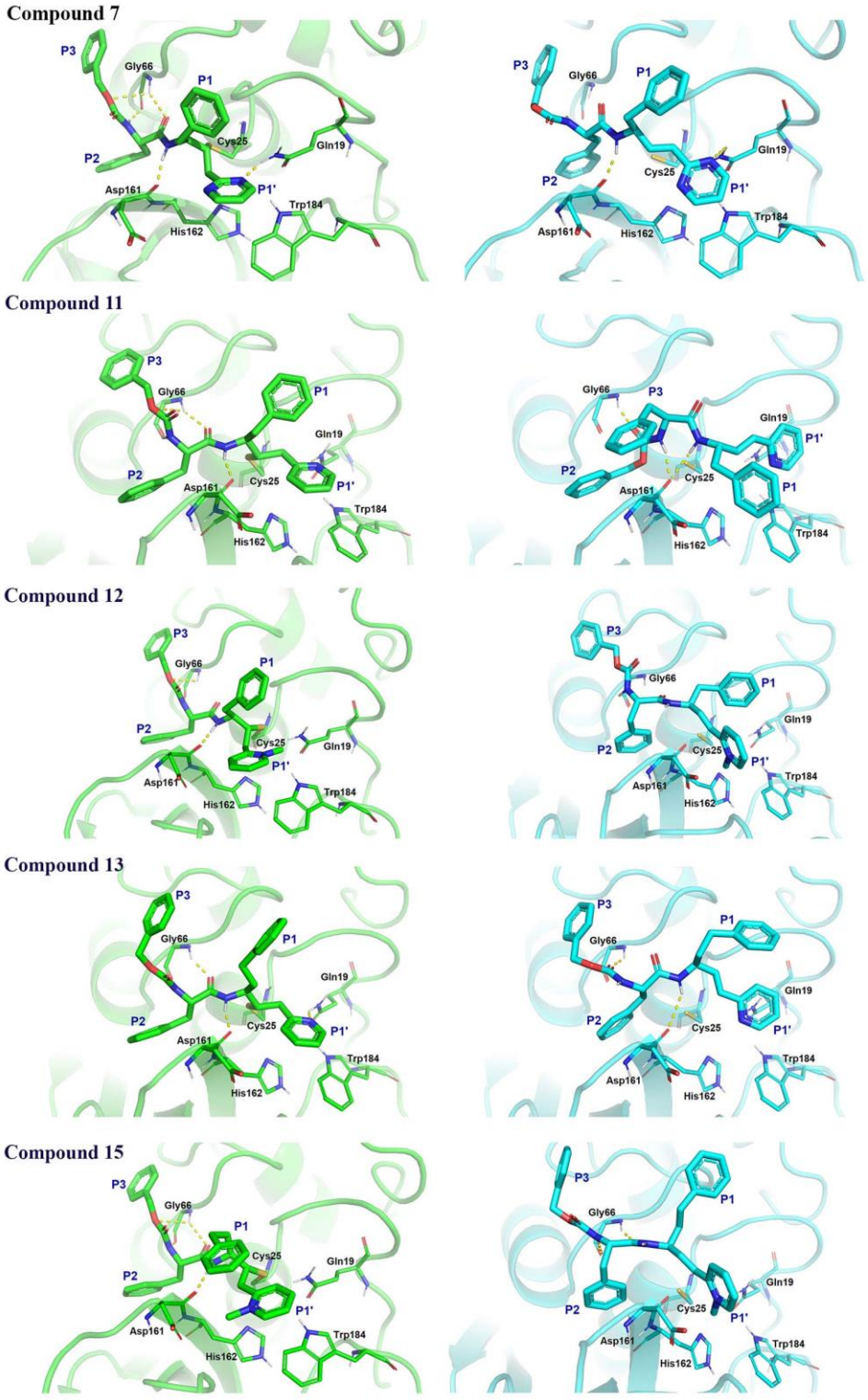
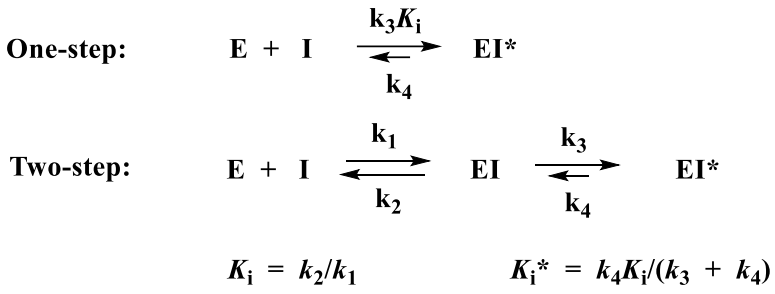


Figure S1. Molecular models of compound **7**, **11-13**. and **15** bound to cruzain created using Schrodinger. Left panels: binding poses in which a covalent bond is formed between the β-carbon of the vinyl group of the inhibitor. Right panels: binding poses in which no covalent bond is formed with Cys_{25}.

Part 2. Fitting of Time-Course Inhibition Data using Kintek Explorer®

In addition to data fitting as described in the Experimental Section, time courses for PVH inhibitors found in **Table 4** and elsewhere were fitted using Kintek Explorer, using both a single-step and two-step binding model (**Scheme S1**).



Scheme S1

Fitted parameters from the one-step model: Sigma: 91.608; k_3K_i = 0.0246 ± 0.000045 $\mu M^{-1} s^{-1}$, k_4 = 0.00175 ± 0.00005 s^{-1}, k_4/k_3K_i = 0.071 ± 0.001 μM. Fitted parameters from the two-step model: Sigma: 89.323; k_1 = 0.02 ± 0.02 $\mu M^{-1} s^{-1}$, k_2 = 0.07 ± 0.099 s^{-1}, k_3 = 0.0058 ± 0.001 $\mu M^{-1} s^{-1}$, k_4 = 0.0011 ± 0.0008 s^{-1}, K_i = 0.40 ± 0.02 μM, and K_i* = 0.06 μM. The lower value of Sigma for the two-step indicates a slightly better fit, albeit with more poorly determined individual rate constants. Note that when $k_4 << k_3$, $K_i^* \sim k_4K_i/k_3$, as is reflected in the values calculated from both models above: $k_4/k_3K_i \sim K_i^*$ = 0.071 ± 0.001 μM and K_i^* = 0.06 μM. These values of K_i* compared favorably with the value of K_i^* = 0.126 ± 0.004 μM determined by global fitting as reported in **Table 2.** Application of fitting by Kintek Explorer for potent cruzain inhibitors **11-13** provided respective values for the two models of: $k_4/k_3K_i \sim K_i^*$ = 0.23 ± 0.02 μM and K_i^* = 0.6 μM, $k_4/k_3K_i \sim K_i^*$ = 0.474 ± 0.002 μM and K_i^* = 0.4 μM, and $k_4/k_3K_i \sim K_i^*$ = 0.233 ± 0.008 μM and K_i^* = 0.2 μM, which again are comparable to their respective values of K_i* obtained by global data fitting as reported in **Table 2**.

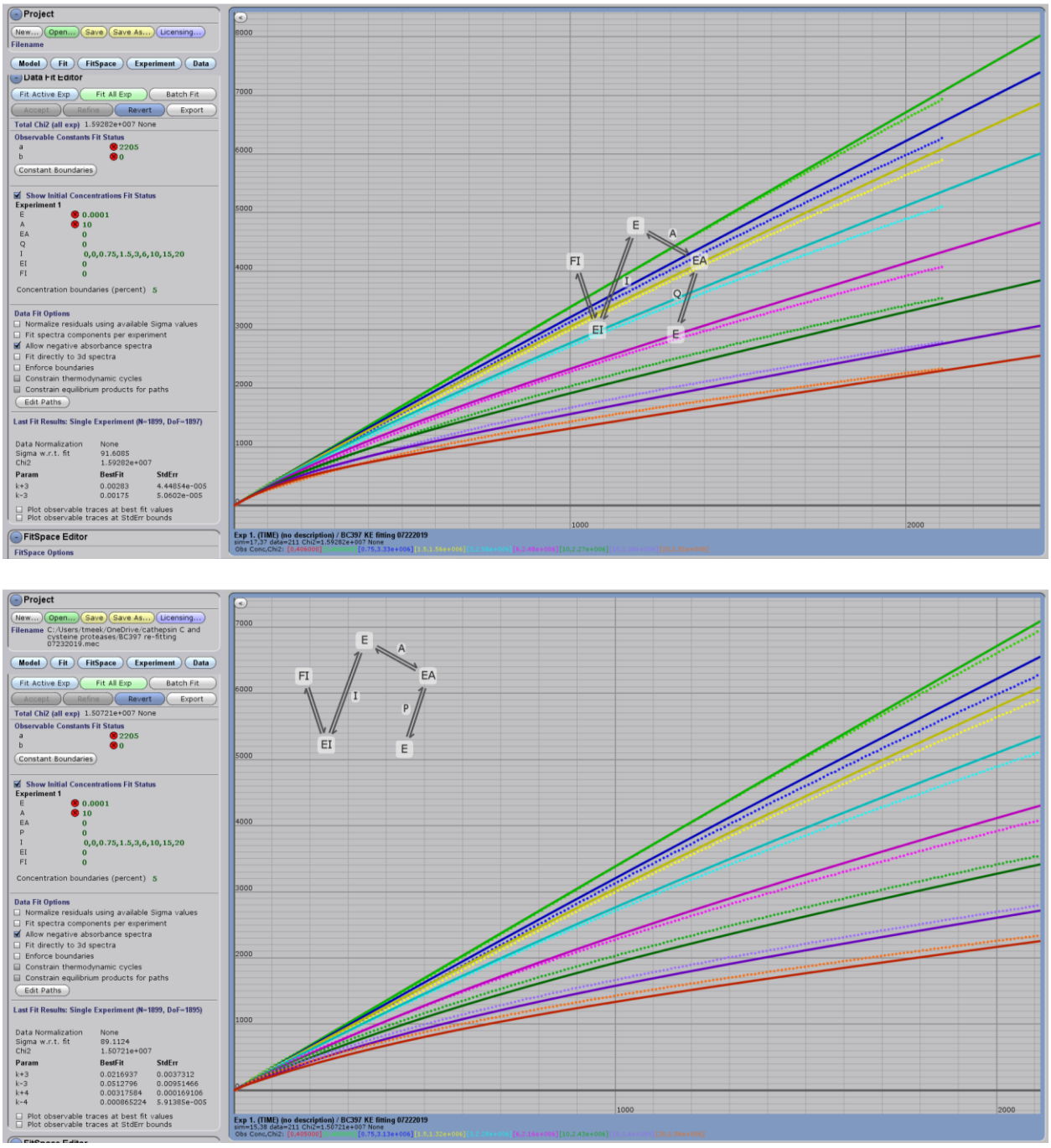


Figure S2. Time-dependent inhibition of cruzain by **15** depicting formation of product AMC vs. time for 0-2100 sec using the one-step model (top) and two-step model (bottom) at micromolar concentrations of **15** of 0 (green), 0.75 (blue), 1.5 (yellow), 3.0 (cyan), 6.0 (magenta), 10.0 (dark green), 15.0 (purple) and 20.0 (orange)**.** Fitting of time course using Kintek Explorer with resulting rate constants obtained from fitting of the 2-step model of **Scheme S1**.

Part 3. Thiolation of Cruzain Inhibitors by Glutathione

Table S2. Kinetic Constants of Thiolation of Cruzain Inhibitors[a]

Compound	Rate of Thiolation, k ($mM^{-1}min^{-1}$)	K_{eq} (M^{-1})
K11777	0.00028 ± 0.0004	NA
7	Negligible	NA
11	Negligible	NA
12	0.037 ± 0.002	7400
15	0.054 ± 0.004	2400
17[b]	Negligible	NA
25	Negligible	NA
26	0.015 ± 0.005	930

[a] 1 mM glutathione was mixed with 0.5 mM K11777 and compounds in Tris (pH 8.0), 10% DMSO (v/v) at room temperature. Aliquots were analyzed by LCMS as described; [b] 5mM glutathione was used for **17**; NA, not applicable.

Part 4. Synthesis and Characterization of AMC-Peptide Substrates

Boc-L-homophenylalanine-AMC: tert-butyl (S)-(1-((4-methyl-2-oxo-2H-chromen-7-yl)amino)-1-oxo-4-phenylbutan-2-yl)carbamate. A round bottom flask charged with Boc-L-homophenylalanine (1.5 g, 5.64 mmol) and 7-amino-4-methylcoumarin (627.2 mg, 3.58 mmol) was purged with N_2 gas yielding positive pressure. Anhydrous dichloromethane (50 ml) and tetrahydrafuran (15 mL) was added followed by the addition of N,N-diisopropylethylamine (915 μL, 5.25 mmol). Propylphosphonic anhydride in a 50% wt. solution of ethyl acetate (3.2 mL, 5.37 mmol) was added dropwise at 0°C resulting in a yellow color. The reaction was The reaction was allowed to react for 30 hours and was monitored by TLC (3:1 hexanes:ethyl acetate) or LC-MS analysis. Solvents were removed and the yellow oil was solubilized in DCM (~60 mL) and washed with water (25 mL, 4x) and brine (25 mL, 2x). The organic layer was dried over Na_2SO_4 and solvents removed under reduced pressure to yield an off white solid. The crude amide directly used for the next reaction without purification. LC-MS Rt: 5.59 min, *m/z* 437.3 ($[M+H]^+$, $C_{25}H_{28}N_2O_5^+$ Calcd 437.21

H_2N-L-homophenylalanine-AMC-TFA: (S)-1-((4-methyl-2-oxo-2H-chromen-7-yl)amino)-1-oxo-4-phenylbutan-2-aminium-TFA. A round bottom flask charged with Boc-L-homophenylalanine-AMC (60 mg, 0.137 mmol) was purged with N_2 gas yielding positive pressure. Anhydrous dichloromethane (6 mL) was added followed by the dropwise addition of trifluoroacetic acid (2 mL) at 0°C. The reaction was monitored via TLC (3:1 hexanes:ethyl acetate) and was complete following 1.5 hours. The solvents were removed under reduced pressure and co-evaporated with chloroform (15 mL, 4x) and ether (15 mL, 2x) to yield a tan solid. LC-MS analysis verified the formation of the desired amine. The product was directly used for the next reaction without purification.

(S2) Cbz-L-phenylalanine-L-homophenylalanine-AMC: Benzyl ((S)-1-(((S)-1-((4-methyl-2-oxo-2H-chromen-7-yl)amino)-1-oxo-4-phenylbutan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate. A round bottom flask charged with $^+H_3$N-L-homophenylalanine-TFA (60 mg, 0.138 mmol) and Cbz-phenylalanine-OH (49.5 mg, 0.166 mmol) was purged with N_2 gas yielding positive pressure. Anhydrous dichloromethane (4 mL) was added followed by the addition of N,N-diisopropylethylamine (120 μL, 0.69 mmol). Propylphosphonic anhydride in a 50% wt. solution of ethyl acetate (164 μL, 0.276 mmol) was added dropwise at 0°C resulting in a yellow color. The reaction was allowed to react for 45 minutes or until no starting material remained via TLC (3:1 hexanes:ethyl acetate v/v) and LC-MS analysis. Solvents were removed and the yellow oil was solubilized in DCM (~20 mL) and washed with water (10 mL, 4x) and brine (10 mL, 1x). The organic layer was dried over Na_2SO_4 and solvents removed under reduced pressure to yield a yellow solid. The crude was purified via preparative HPLC to yield the desired amide (50.1 mg, 59% yield) as a white powder. ^{1}H NMR (400 MHz, DMSO) δ 1.91 – 2.17 (m, 2H), 2.41 (d, *J* = 1.2 Hz, 3H), 2.56 – 2.89 (m, 3H), 3.07 (dd, *J* = 13.7, 4.1 Hz, 1H), 4.30 – 4.60 (m, 2H), 4.84 – 5.11 (m, 2H), 6.28

(d, *J* = 1.5 Hz, 1H), 7.10 – 7.39 (m, 14H), 7.45 – 7.60 (m, 2H), 7.68 – 7.85 (m, 2H), 8.40 (d, *J* = 7.7 Hz, 1H), 10.46 (s, 1H). LC-MS Rt: 5.88 min, *m/z* 618.4 ($[M+H]^+$, $C_{37}H_{35}N_3O_6^+$ Calcd 618.3.

(S3) Cbz-L-leucine-L-homophenylalanine-AMC: benzyl ((S)-4-methyl-1-(((S)-1-((4-methyl-2-oxo-2H-chromen-7-yl)amino)-1-oxo-4-phenylbutan-2-yl)amino)-1-oxopentan-2-yl)carbamate. A round bottom flask charged with ^+H_3N-L-homophenylalanine • TFA (64 mg, 0.142 mmol) and Cbz-Leucine-OH (49.1 mg, 0.185 mmol) was purged with N_2 gas yielding positive pressure. Anhydrous dichloromethane (4 mL) and tetrahydrafuran (4 mL) was added followed by the addition of N,N-diisopropylethylamine (124 µL, 0.71 mmol). Propylphosphonic anhydride in a 50% wt. solution of ethyl acetate (164 µL, 0.276 mmol) was added dropwise at 0°C resulting in a yellow color. The reaction was allowed to react for 45 minutes and no starting material remained via TLC (3:1 hexanes:ethyl acetate v/v) and LC-MS analysis. Solvents were removed and the yellow oil was solubilized in DCM (~20 mL) and washed with water (10 mL, 4x) and brine (10 mL, 1x). The organic layer was dried over Na_2SO_4 and solvents removed under reduced pressure to yield a yellow solid. The crude amide was purified via preparative HPLC to yield (48.9 mg, 59%) as a white powder. ^{1}H NMR (400 MHz, DMSO) δ 0.90 (t, *J* = 6.9 Hz, 6H), 1.50 (t, *J* = 7.3 Hz, 2H), 1.68 (t, *J* = 7.0 Hz, 1H), 2.02 (d, *J* = 32.8 Hz, 2H), 2.41 (s, 3H), 2.54 – 2.79 (m, 3H), 4.15 (q, *J* = 7.8 Hz, 1H), 4.47 (s, 1H), 5.06 (s, 2H), 6.27 (s, 1H), 7.09 – 7.41 (m, 9H), 7.49 (t, *J* = 8.9 Hz, 2H), 7.64 – 7.86 (m, 2H), 8.25 (d, *J* = 7.7 Hz, 1H), 10.42 (s, 1H). LC-MS Rt: 5.88 min, *m/z* 584.3 ($[M+H]^+$, $C_{34}H_{37}N_3O_6^+$ Calcd 584.3

Boc-L-4-pyridylalanine-AMC: tert-butyl (S)-(1-((4-methyl-2-oxo-2H-chromen-7-yl)amino)-1-oxo-3-(pyridin-4-yl)propan-2-yl)carbamate. A round bottom flask charged with Boc-L-4-pyridylalanine-OH (325 mg, 1.22 mmol) and 7-amino-4-methylcoumarin (164.5 mg, 0.939 mmol) was purged with N_2 gas yielding positive pressure. Anhydrous dichloromethane (6 mL) and tetrahydrafuran (14 ml) was added followed by the addition of N,N-diisopropylethylamine (600 µL, 3.44 mmol). Propylphosphonic anhydride in a 50% wt. solution of ethyl acetate (1.125 mL, 1.89 mmol) was added dropwise at 0°C resulting in a yellow color. The reaction was allowed to react for 15 hours and product formation was monitored via TLC (1% MeOH:DCM v/v) and LC-MS analysis. Solvents were removed under reduced pressure and the yellow oil was solubilized in DCM (~30 mL) and washed with water (15 mL, 4x) and brine (15 mL, 2x). The organic layer was dried over Na_2SO_4 and solvents were removed under reduced pressure. The resulting yellow solid was deemed pure enough (~94% via LC-MS, 425 mg) to proceed directly to the next step. LC-MS Rt: 2.84 min, *m/z* 424.26 ($[M+H]^+$, $C_{23}H_{25}N_3O_5^+$ Calcd 424.2

NH_2-L-4-pyridylalanine-AMC•TFA: (S)-1-((4-methyl-2-oxo-2H-chromen-7-yl)amino)-1-oxo-3-(pyridin-4-yl)propan-2-aminium•TFA. A round bottom flask charged with Boc-L-4-pyridylalanine-AMC (150 mg, 0.353 mmol) was purged with N_2 gas yielding positive pressure. Anhydrous dichloromethane (10 mL) was added followed by the dropwise addition of trifluoroacetic acid (3 mL) at 0°C. The reaction was monitored

via TLC (1% MeOH:DCM v/v) and was complete following 1.5 hours. The solvents were removed under reduced pressure and co-evaporated with chloroform and ether to yield a yellow solid. LCMS analysis verified the formation of the product. The product was directly used for the next reaction without purification.

(S5) Cbz-L-phenylalanine-L-4-pyridylalanine-AMC: Benzyl ((S)-1-(((S)-1-((4-methyl-2-oxo-2H-chromen-7-yl)amino)-1-oxo-3-(pyridin-4-yl)propan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate. A round bottom flask charged with $^{+}H_3N$-L-4-pyridylalanine ● TFA (154 mg, 0.352 mmol) and Cbz-phenylalanine-OH (137.3 mg, 0.459 mmol) was purged with N_2 gas yielding positive pressure. Anhydrous dichloromethane (10 mL) and tetrahydrafuran (2 ml) was added followed by the addition of N,N-diisopropylethylamine (367.9 μL, 2.11 mmol). Propylphosphonic anhydride in a 50% wt. solution of ethyl acetate (419.1 μL, 0.704 mmol) was added dropwise at 0°C resulting in a yellow color. The reaction was allowed to react for 45 minutes or until no starting material remained via TLC (1% MeOH:DCM v/v) and LC-MS analysis. Solvents were removed and the yellow oil was solubilized in DCM (~30 mL) and washed with water (15 mL, 4x) and brine (15 mL, 1x). The organic layer was dried over Na_2SO_4 and solvents removed under reduced pressure to yield a yellow solid. 100 mg of the crude was purified via preparative HPLC to yield (32.94 mg, 39%) as a white powder. ^{1}H NMR (400 MHz, DMSO) δ 2.33 – 2.45 (m, 3H), 2.72 (dd, *J* = 13.9, 10.5 Hz, 1H), 2.85 – 3.09 (m, 2H), 3.14 (dd, *J* = 13.9, 5.4 Hz, 1H), 4.14 – 4.37 (m, 1H), 4.68 – 4.89 (m, 1H), 4.96 (s, 2H), 6.29 (d, *J* = 1.5 Hz, 1H), 7.25 (ttd, *J* = 22.3, 14.7, 13.7, 6.4 Hz, 12H), 7.39 – 7.54 (m, 2H), 7.75 (d, *J* = 8.7 Hz, 2H), 8.25 – 8.56 (m, 3H), 10.52 (s, 1H). LC-MS Rt: 3.57 min, *m/z* 605.3 ($[M+H]^+$, $C_{35}H_{32}N_4O_6^+$ Calcd 605.23.

N-methyl-piperazine-L-phenylalanine-OH: (4-methylpiperazine-1-carbonyl)-L-phenylalanine. A round bottom flask charged with methyl (4-methylpiperazine-1-carbonyl)-L-phenylalanine (500 mg, 1.64 mmol) was purged with N_2 gas yielding positive pressure. Anhydrous methanol (8.5 mL) and tetrahydrofuran (8.5 mL) was added followed by the addition of 0.5 M LiOH in H_20 (17 mL, 8.5 mmol). The reaction was monitored by TLC (5% MeOH:DCM v/v) and after 3.5 hours the reaction was stopped and solvents were removed under reduced pressure. The reaction mixture was dissolved in acidified water (0.5 M HCl, 10 mL) to neutralize the unreacted LiOH and the solvent was removed under reduced pressure. The crude compound was then transferred to the next reaction step without purification.

(S7) N-methyl-piperazine-L-phenylalanine-L-homophenyl-alanine-AMC: 4-methyl-N-((S)-1-(((S)-1-((4-methyl-2-oxo-2H-chromen-7-yl)amino)-1-oxo-4-phenylbutan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)piperazine-1-carboxamide. A round bottom flask charged with $^{+}H_3N$-L-homophenylalanine • TFA (105 mg, 0.313 mmol) and N-methyl-piperazine-phenylalanine-OH (160 mg, 0.55 mmol) was purged with N_2 gas yielding positive pressure. Anhydrous dichloromethane (10 mL) was added followed by the addition of

N,N-diisopropylethylamine (274 μL, 1.57 mmol). Propylphosphonic anhydride in a 50% wt. solution of ethyl acetate (377 μL, 0.626 mmol) was added dropwise at 0°C resulting in a yellow color. The reaction was allowed to react for 45 minutes and no starting material remained via TLC (1:1 ethyl acetate: hexanes v/v) and LC-MS analysis. Solvents were removed and the yellow oil was solubilized in DCM (~20 mL) and washed with water (10 mL, 4x) and brine (10 mL, 1x). The organic layer was dried over Na_2SO_4 and solvents removed under reduced pressure to yield a yellow solid. The crude amide was purified via preparative HPLC to yield (21.1 mg, 11%) of a white powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.94 – 2.16 (m, 6H), 2.31 (s, 4H), 2.32 – 2.41 (m, 5H), 2.42 (d, *J* = 1.2 Hz, 4H), 2.73 (t, *J* = 7.4 Hz, 2H), 2.85 – 3.04 (m, 2H), 3.25 (ddd, *J* = 12.7, 10.6, 4.9 Hz, 3H), 3.41 (ddt, *J* = 12.5, 5.6, 2.9 Hz, 3H), 4.36 (dt, *J* = 8.7, 5.3 Hz, 1H), 4.60 (td, *J* = 8.5, 4.5 Hz, 1H), 4.78 (d, *J* = 4.4 Hz, 1H), 6.20 (d, *J* = 1.4 Hz, 1H), 6.60 (d, *J* = 8.2 Hz, 1H), 7.12 – 7.40 (m, 10H), 7.52 (d, *J* = 8.5 Hz, 1H), 7.68 – 7.82 (m, 2H), 9.16 (s, 1H). LC-MS Rt: 3.29 min, *m/z* 610.1 ($[M+H]^+$, $C_{35}H_{39}N_5O_5^+$ Calcd 610.3.

Boc-L-4-pyridylalanine-L-homophenylalanine-AMC: tert-butyl ((S)-1-(((S)-1-((4-methyl-2-oxo-2H-chromen-7-yl)amino)-1-oxo-4-phenylbutan-2-yl)amino)-1-oxo-3-(pyridin-4-yl)propan-2-yl)carbamate. A round bottom flask charged with $^+H_3$N-L-homophenylalanine • TFA (146 mg, 0.325 mmol) and Boc-L-4-pyridylalanine-OH (104 mg, 0.39 mmol) was purged with N_2 gas yielding positive pressure. Anhydrous dichloromethane (10 mL) was added followed by the addition of N,N-diisopropylethylamine (283 μL, 1.63 mmol). Propylphosphonic anhydride in a 50% wt. solution of ethyl acetate (387 μL, 0.65 mmol) was added dropwise at 0°C resulting in a yellow color. The reaction was allowed to react for 45 minutes and no starting material remained via TLC (1:1 hexanes:ethyl acetate v/v) and LC-MS analysis. Solvents were removed and the yellow oil was solubilized in DCM (~20 mL) and washed with water (10 mL, 4x) and brine (10 mL, 1x). The organic layer was dried over Na_2SO_4 and solvents removed under reduced pressure to yield a yellow solid. The crude amide was moved directly to the next step with no purification. LC-MS Rt: 3.57 min, *m/z* 581.7 ($[M+H]^-$, $C_{33}H_{36}N_4O_6^-$ Calcd 581.3.

NH_2-L-4-pyridylalanine-L-homophenylalanine-AMC•TFA: S)-1-(((S)-1-((4-methyl-2-oxo-2H-chromen-7-yl)amino)-1-oxo-4-phenylbutan-2-yl)amino)-1-oxo-3-(pyridin-4-yl)propan-2-aminium TFA. A round bottom flask charged with Boc-L-4-pyridylalanine-L-homophenylalanine-AMC (172 mg, 0.294 mmol) was purged with N_2 gas yielding positive pressure. Anhydrous dichloromethane (10 mL) was added followed by the dropwise addition of trifluoroacetic acid (5 mL) at 0°C. The reaction was monitored via TLC (1:1 hexanes:ethyl acetate) and was complete following 1.5 hours. The solvents were removed under reduced pressure and co-evaporated with chloroform (15 mL, 4x) and ether (15 mL, 2x) to yield a tan solid. LC-MS analysis verified the formation of the desired amine. The product was directly used for the next reaction without purification.

(S8) Cbz-L-4-pyridylalanine-L-homophenylalanine-AMC: Benzyl ((S)-1-(((S)-1-((4-methyl-2-oxo-2H-chromen-7-yl)amino)-1-oxo-4-phenylbutan-2-yl)amino)-1-oxo-3-(pyridin-4-yl)propan-2-yl)carbamate. A round bottom flask charged with NH_2- L-4-pyridylalanine-L-homophenylalanine-AMC-TFA (227 mg, 0.379 mmol; excess weight is due to residual TFA salts) was purged with N_2 gas yielding positive pressure. Anhydrous ethanol (4 mL) was added followed by the addition of a solution of sodium bicarbonate (127 mg, 1.52 mmol) in water (4 mL). Subsequently, a solution of benzyl chloroformate (107 uL, 0.76 mmol) in 1,4 dioxanes (4 mL) was added dropwise and the reaction was allowed to react overnight. Solvents were removed under reduced pressue and the reaction mixture was solubilized in dichloromethane (30 mL) and washed with H_2O (10 mL, 3x), brine (10 mL, 2x). The organic layer was dried over Na_2SO_4 and solvents removed under reduced pressure to yield an orange oil. The crude amide was purified via preparative HPLC to yield (45.9 mg, 20%) as a white powder. 1H NMR (400 MHz, MeOD) δ 1.88 – 2.27 (m, 2H), 2.44 (d, *J* = 1.3 Hz, 3H), 2.68 (ddddd, *J* = 20.0, 15.3, 12.7, 7.8, 3.3 Hz, 2H), 2.96 (dd, *J* = 14.1, 9.2 Hz, 1H), 3.22 (dd, *J* = 13.9, 5.3 Hz, 1H), 4.50 (dd, *J* = 8.8, 5.3 Hz, 1H), 4.57 (dd, *J* = 9.1, 5.3 Hz, 1H), 5.05 (d, *J* = 3.5 Hz, 2H), 6.21 (d, *J* = 1.5 Hz, 1H), 7.01 – 7.37 (m, 12H), 7.47 (dd, *J* = 8.7, 2.1 Hz, 1H), 7.55 – 7.72 (m, 1H), 7.78 (d, *J* = 2.0 Hz, 1H), 8.33 (d, *J* = 5.1 Hz, 2H).LC-MS Rt: 3.79 min, *m/z* 617.5 ($[M+H]^-$, $C_{36}H_{34}N_4O_6^-$ Calcd 617.3.

Boc-L-phenylalanine-AMC: tert-butyl (S)-(1-((4-methyl-2-oxo-2H-chromen-7-yl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate. A round bottom flask charged with Boc-L-phenylalanine (380 mg, 1.43 mmol) and 7-amino-4-methylcoumarin (193 mg, 1.1 mmol) was purged with N_2 gas yielding positive pressure. Anhydrous tetrahydrafuran (30 mL) was added followed by the addition of N,N-diisopropylethylamine (958 µL, 5.5 mmol). Propylphosphonic anhydride in a 50% wt. solution of ethyl acetate (1.31 mL, 2.2 mmol) was added dropwise at 0°C resulting in a yellow color. The reaction was allowed to react for 15 hours was monitored by TLC (3:1 hexanes:ethyl acetate) or LC-MS analysis. Solvents were removed and the yellow oil was solubilized in DCM (~80 mL) and washed with water (30 mL, 4x) and brine (30 mL, 2x). The organic layer was dried over Na_2SO_4 and solvents removed under reduced pressure to yield a yellow solid. The crude amide was purified via preparative HPLC (363 mg, 78%) LC-MS Rt: 5.31 min, *m/z* 423.3 ($[M+H]^+$, $C_{24}H_{26}N_2O_5^+$ Calcd 423.2.

H_2N-L-phenylalanine-AMC•TFA: (S)-1-((4-methyl-2-oxo-2H-chromen-7-yl)amino)-1-oxo-3-phenylpropan-2-aminium•TFA. A round bottom flask charged with Boc-L-phenylalanine-AMC (40 mg, 0.095 mmol) was purged with N_2 gas yielding positive pressure. Anhydrous dichloromethane (3 mL) was added followed by the dropwise addition of trifluoroacetic acid (1 mL) at 0°C. The reaction was monitored via TLC and was complete following 1.5 hours. The solvents were removed under reduced pressure and co-evaporated with chloroform (15 mL, 4x) and ether (15 mL, 2x) to yield a tan solid. LCMS analysis

verified the formation of the desired amine. The product was directly used for the next reaction without purification.

(S9) Cbz-L-phenylalanine-L-phenylalanine-AMC: benzyl ((S)-1-(((S)-1-((4-methyl-2-oxo-2H-chromen-7-yl)amino)-1-oxo-3-phenylpropan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate. A round bottom flask charged with $^{+}H_3N$-L-phenylalanine •TFA (40 mg, 0.092 mmol) and Cbz-phenylalanine-OH (33 mg, 0.11 mmol) was purged with N_2 gas yielding positive pressure. Anhydrous dichloromethane (5 mL) and was added followed by the addition of N,N-diisopropylethylamine (80 μL, 0.46 mmol). Propylphosphonic anhydride in a 50% wt. solution of ethyl acetate (110 μL, 0.18 mmol) was added dropwise at 0°C resulting in a yellow color. The reaction was allowed to react for 45 minutes and no starting material remained via TLC (3:1 hexanes:ethyl acetate v/v) and LC-MS analysis. Solvents were removed and the yellow oil was solubilized in dichloromethane (~20 mL) and washed with water (10 mL, 4x) and brine (10 mL, 1x). The organic layer was dried over Na_2SO_4 and solvents removed under reduced pressure to yield a yellow solid. The crude amide was purified via preparative HPLC to yield (34.2 mg, 62%) as a white powder. ^{1}H NMR (400 MHz, DMSO) δ 2.41 (s, 3H), 2.72 (dd, *J* = 13.9, 10.5 Hz, 1H), 2.87 – 3.05 (m, 2H), 3.11 (dd, *J* = 13.8, 5.8 Hz, 1H), 4.30 (td, *J* = 9.6, 9.0, 4.2 Hz, 1H), 4.73 (q, *J* = 7.5 Hz, 1H), 4.96 (s, 2H), 6.28 (s, 1H), 7.06 – 7.39 (m, 15H), 7.39 – 7.53 (m, 2H), 7.60 – 7.79 (m, 2H), 8.37 (d, *J* = 7.8 Hz, 1H), 10.47 (s, 1H). LC-MS Rt: 5.68 min, *m/z* 603.4 ($[M+H]^+$, $C_{36}H_{33}N_3O_6^+$ Calcd 604.2.

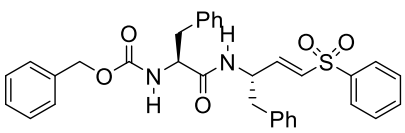
Cbz-L-arginine(Mtr)-L-homophenylalanine-AMC: Benzyl ((S)-5-(3-((4-methoxy-2,6-dimethylphenyl)sulfonyl) guanidino)-1-(((S)-1-((4-methyl-2-oxo-2H-chromen-7-yl)amino)-1-oxo-4-phenylbutan-2-yl)amino)-1-oxopentan-2-yl)carbamate. A round bottom flask charged with $^{+}H_3N$-L-homophenylalanine • TFA (120 mg, 0.266 mmol) and Cbz-Arginine (Mtr)-OH (180 mg, 0.346 mmol) was purged with N_2 gas yielding positive pressure. Anhydrous dichloromethane (10 mL) and was added followed by the addition of N,N-diisopropylethylamine (232 μL, 1.33 mmol). Propylphosphonic anhydride in a 50% wt. solution of ethyl acetate (317 μL, 0.532 mmol) was added dropwise at 0°C resulting in a yellow color. The reaction was allowed to react for 3 hours and no starting material remained via TLC (3:1 hexanes:ethyl acetate v/v) and LC-MS analysis. Solvents were removed and the yellow oil was solubilized in DCM (~40 mL) and washed with water (10 mL, 4x) and brine (10 mL, 1x). The organic layer was dried over Na_2SO_4 and solvents removed under reduced pressure to yield a yellow solid that was moved directly to the next step. LC-MS Rt: 5.73 min, *m/z* 837.9 ($[M+H]^-$, $C_{43}H_{48}N_6O_9S^-$ Calcd 837.3.

(S10) Cbz-L-arginine-L-homophenylalanine-AMC: benzyl ((S)-5-guanidino-1-(((S)-1-((4-methyl-2-oxo-2H-chromen-7-yl)amino)-1-oxo-4-phenylbutan-2-yl)amino)-1-oxopentan-2-yl)carbamate. A round bottom flask charged with Cbz-Arginine (Mtr)-L-homophenylalanine-AMC (130 mg, 0.158 mmol) and phenol (450 mg, 4.8 mmol, 5% wt/v) was purged with N_2 gas yielding positive pressure. Trifluoroacetic acid (6

mL) and was added followed by allowing the reaction to proceed for 12 hours. The solvent was removed under a flow of N_2 gas and the crude product was subsequently purified by preparative HPLC to yield (34 mg, 34% yield) as a white powder. ^{1}H NMR (400 MHz, MeOD) δ 1.58 – 2.00 (m, 4H), 2.00 – 2.33 (m, 2H), 2.34 – 2.53 (m, 3H), 2.52 – 2.84 (m, 2H), 3.20 (q, *J* = 6.3, 5.8 Hz, 2H), 4.23 (t, *J* = 6.5 Hz, 1H), 4.50 (dd, *J* = 9.2, 4.9 Hz, 1H), 5.13 (s, 2H), 6.23 (t, *J* = 5.5 Hz, 1H), 6.90 – 7.41 (m, 10H), 7.47 (d, *J* = 8.7 Hz, 1H), 7.66 (dt, *J* = 18.0, 8.3 Hz, 1H), 7.84 (d, *J* = 11.3 Hz, 1H), 8.53 (s, 1H). LC-MS Rt: 3.62 min, *m/z* 625.5 ($[M+H]^-$, $C_{34}H_{38}N_6O_6^-$ Calcd 625.3.

(S11) NMe-Pip-Phe-Phe-AMC: 4-methyl-N-((S)-1-(((S)-1-((4-methyl-2-oxo-2H-chromen-7-yl)amino)-1-oxo-3-phenylpropan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)piperazine-1-carboxamide. A round bottom flask charged with $^+H_3$N-L-phenylalanine •TFA (100 mg, 0.29 mmol) and N-methyl-piperazine-phenylalanine-OH (160 mg, 0.55 mmol) was purged with N_2 gas yielding positive pressure. Anhydrous dichloromethane (15 mL) and was added followed by the addition of N,N-diisopropylethylamine (253 µL, 1.45 mmol). Propylphosphonic anhydride in a 50% wt. solution of ethyl acetate (345 µL, 0.58 mmol) was added dropwise at 0°C resulting in a yellow color. The reaction was allowed to react for 45 minutes and no starting material remained via TLC (1:1 hexanes:ethyl acetate v/v) and LC-MS analysis. Solvents were removed and the yellow oil was solubilized in dichloromethane (~40 mL) and washed with water (10 mL, 4x) and brine (10 mL, 1x). The organic layer was dried over Na_2SO_4 and solvents removed under reduced pressure to yield a yellow solid. The crude amide was purified via preparative HPLC to yield (24.6 mg, 14%) ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.38 (d, *J* = 1.2 Hz, 3H), 2.54 (s, 3H), 2.55 – 2.78 (m, 4H), 2.81 – 3.04 (m, 1H), 3.02 – 3.28 (m, 3H), 3.32 – 3.60 (m, 4H), 4.47 (dt, *J* = 8.7, 5.5 Hz, 1H), 4.87 (q, *J* = 6.9 Hz, 1H), 5.65 (d, *J* = 5.7 Hz, 1H), 6.16 (d, *J* = 1.4 Hz, 1H), 6.83 – 7.40 (m, 15H), 7.46 (d, *J* = 8.7 Hz, 1H), 7.54 – 7.72 (m, 2H), 8.30 (s, 1H), 9.45 (s, 1H). LC-MS Rt: 3.15 min, *m/z* 596.2 ($[M+H]^+$, $C_{34}H_{37}N_5O_5^+$ Calcd 596.3.

Part 5. Structures of PVH Inhibitors 1-27



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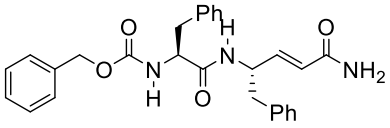

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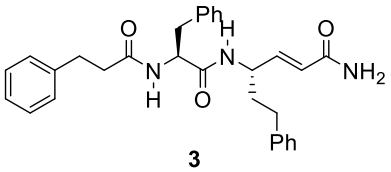

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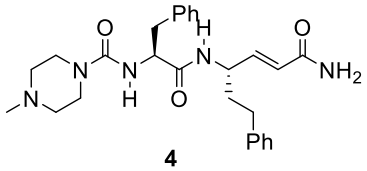

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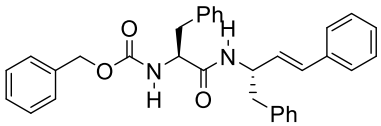

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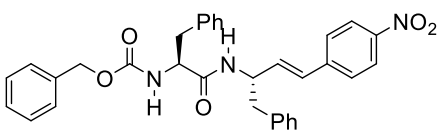

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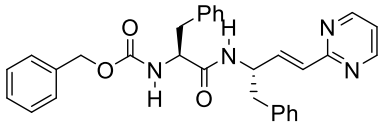

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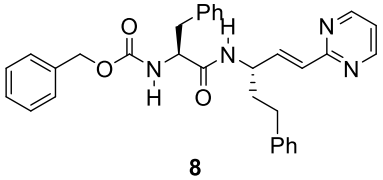

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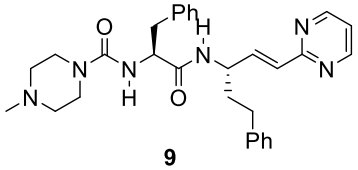

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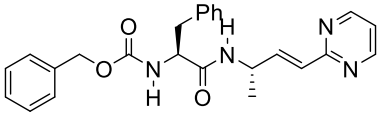

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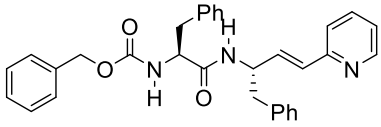

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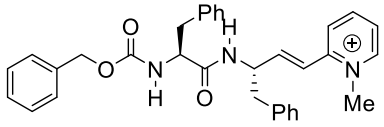

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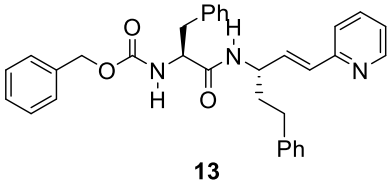

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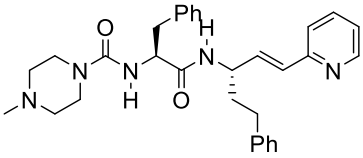

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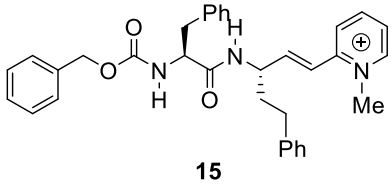

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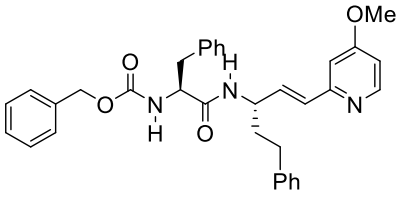

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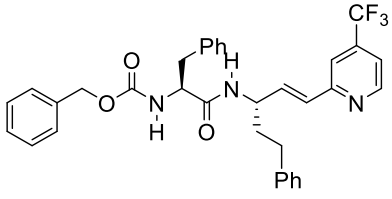

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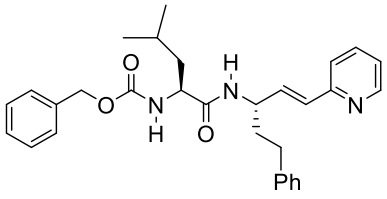

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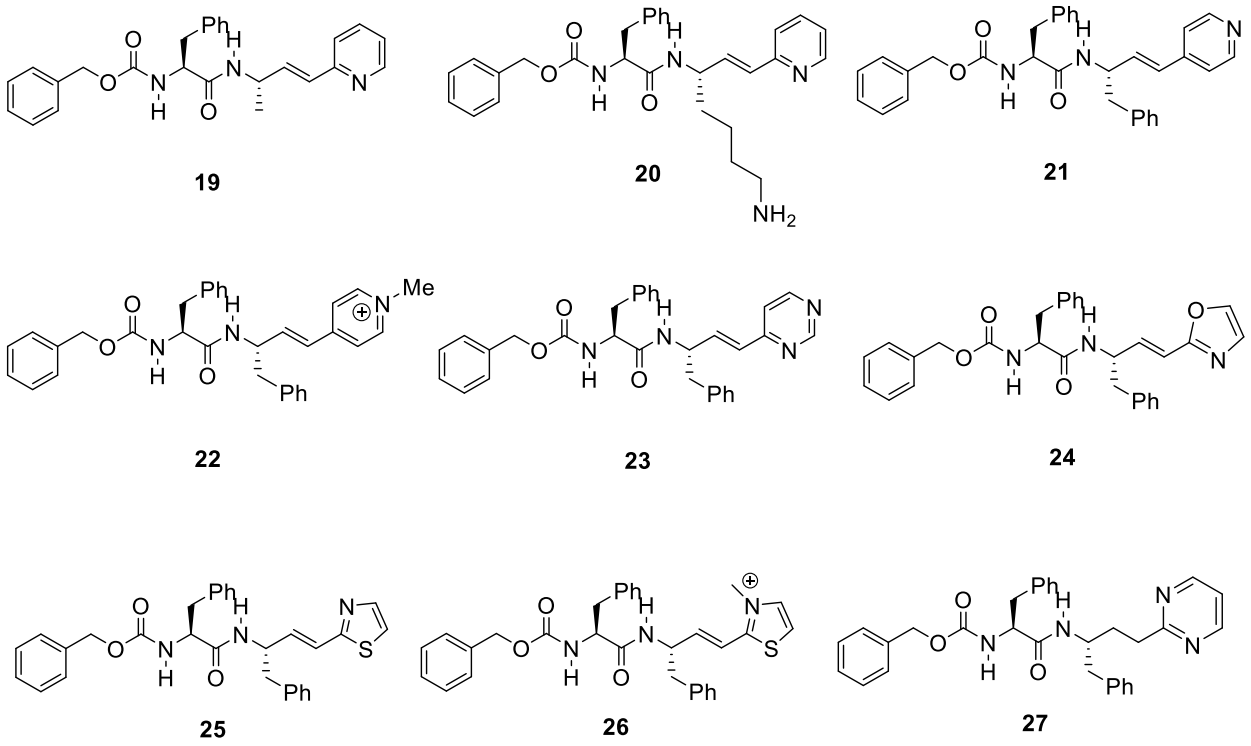
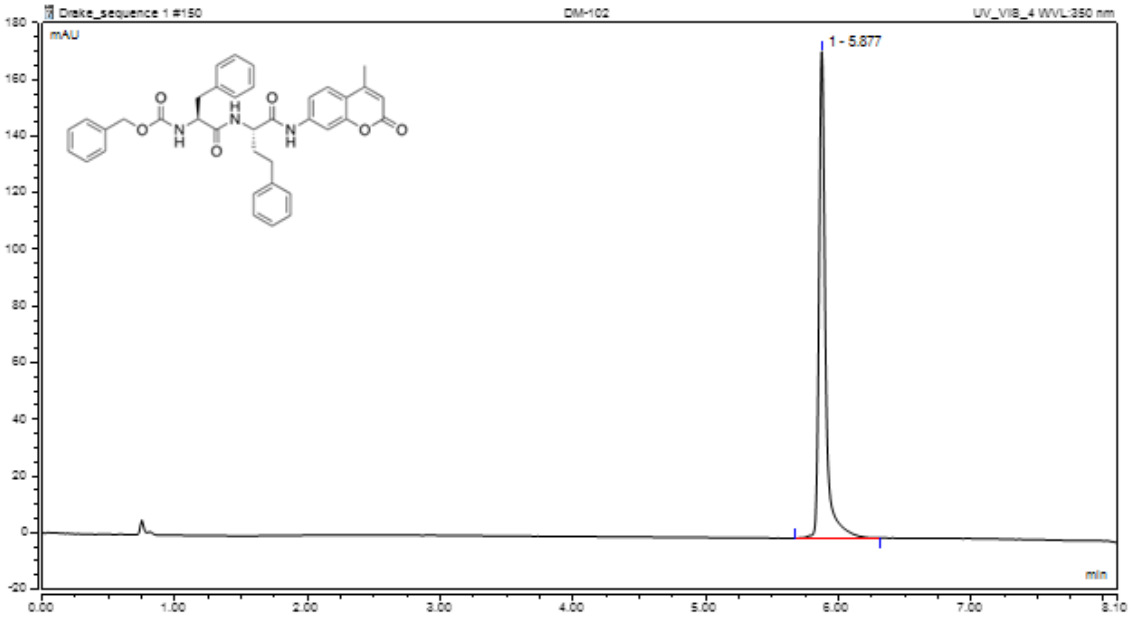


Figure S3. Structures of Inhibitors from Table 2 and Experimental Section.

Part 6. HPLC Traces of Synthesized Substrates and Inhibitors

All cruzain substrates and inhibitors have been analyzed by LCMS, and have determined as >95% analytical purity as demonstrated in the chromatograms below.

S2:



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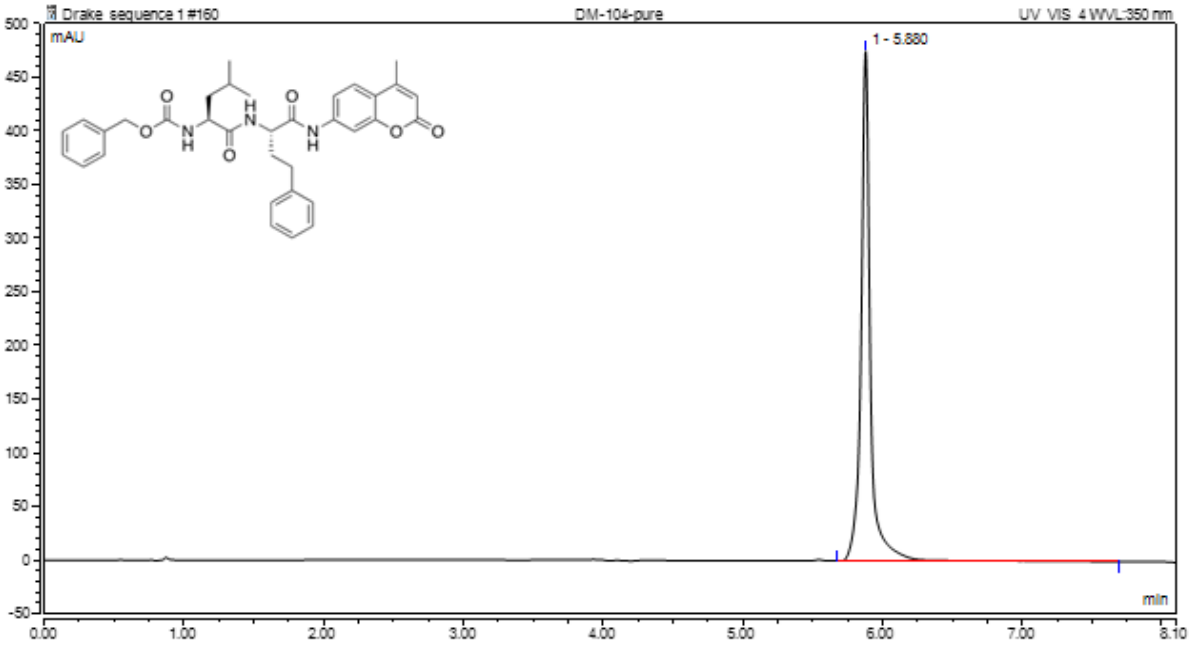

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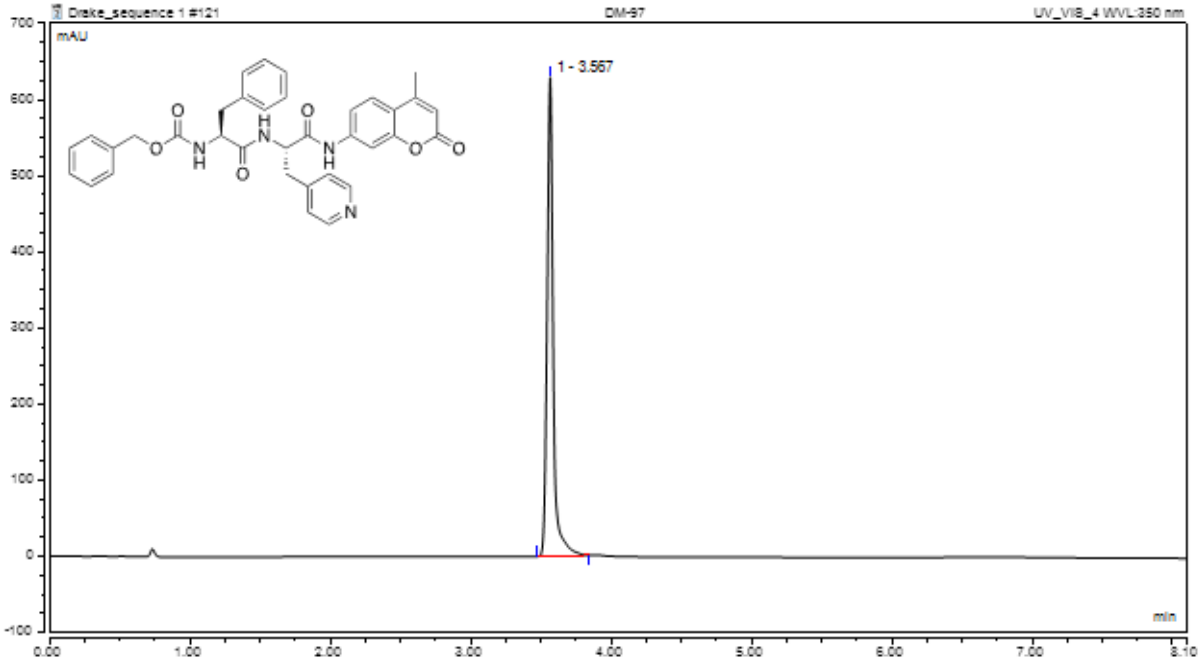
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min

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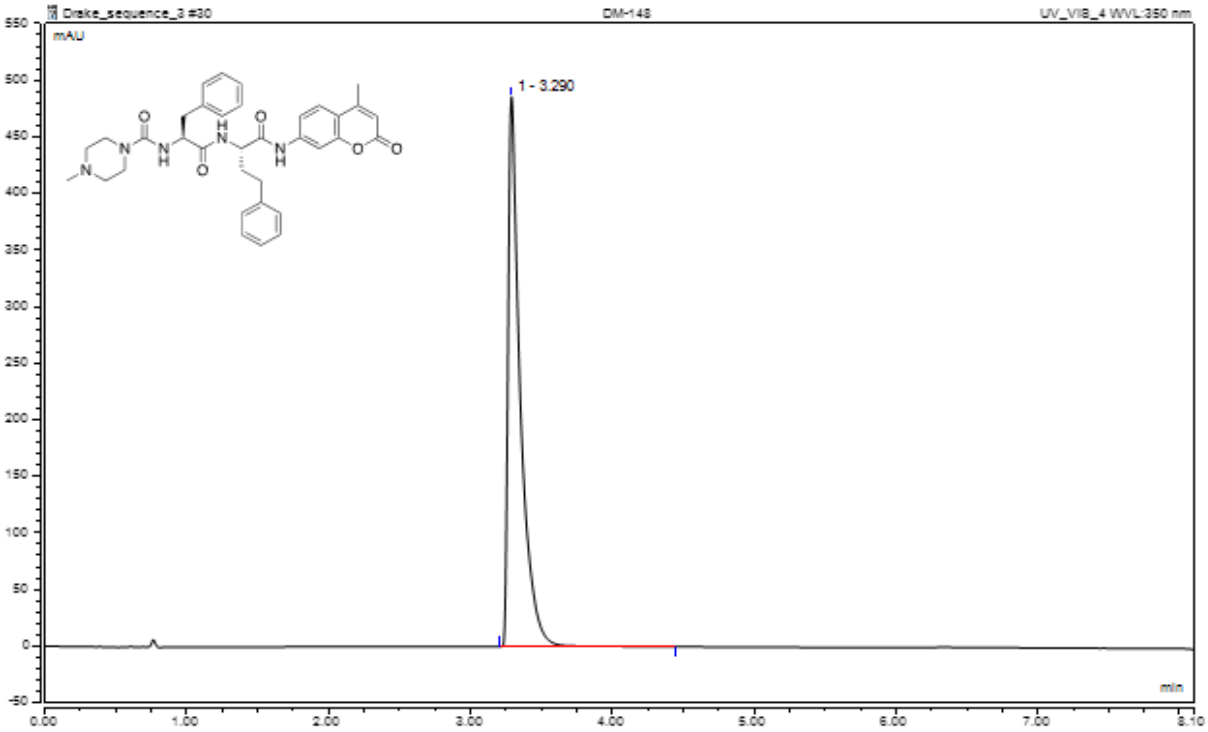
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UV_VIS_4 WVL:350 nm
mAU
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min

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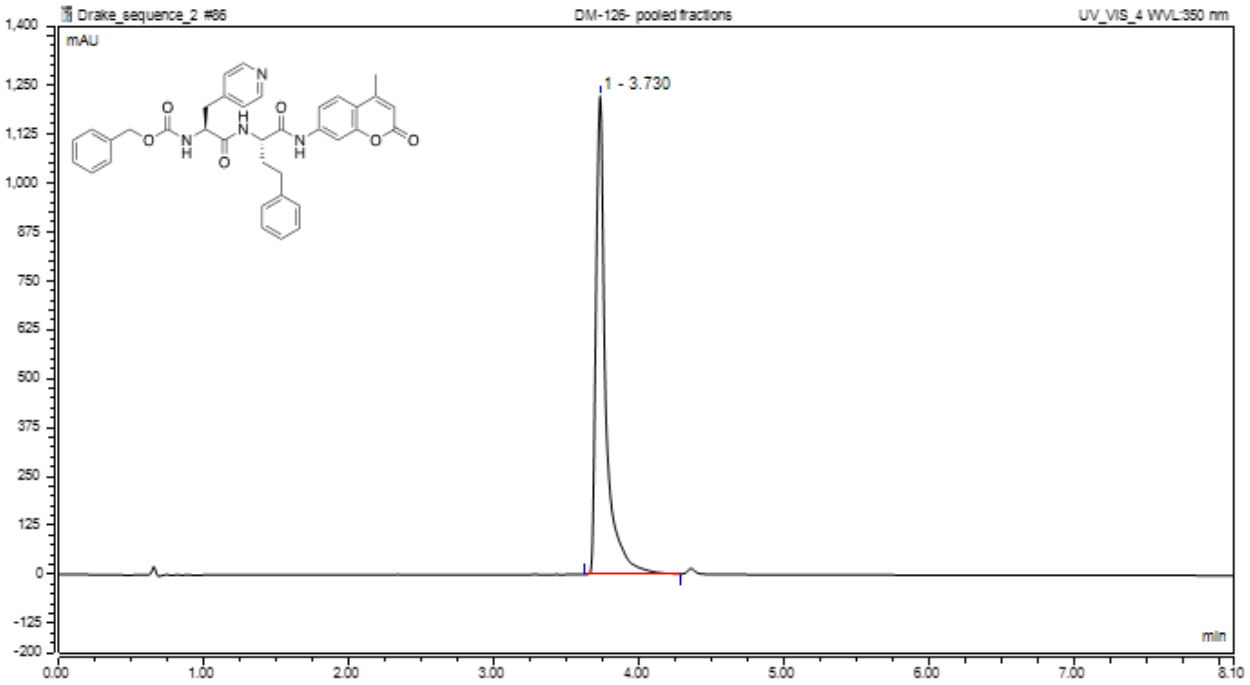
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min

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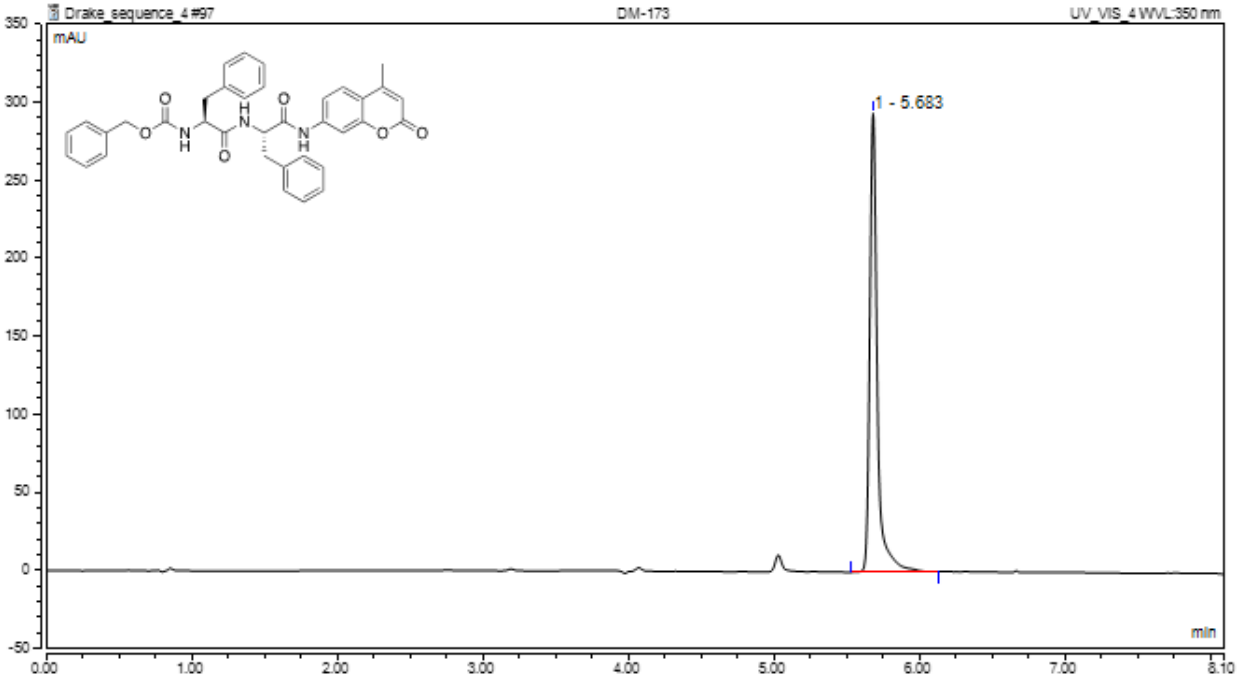
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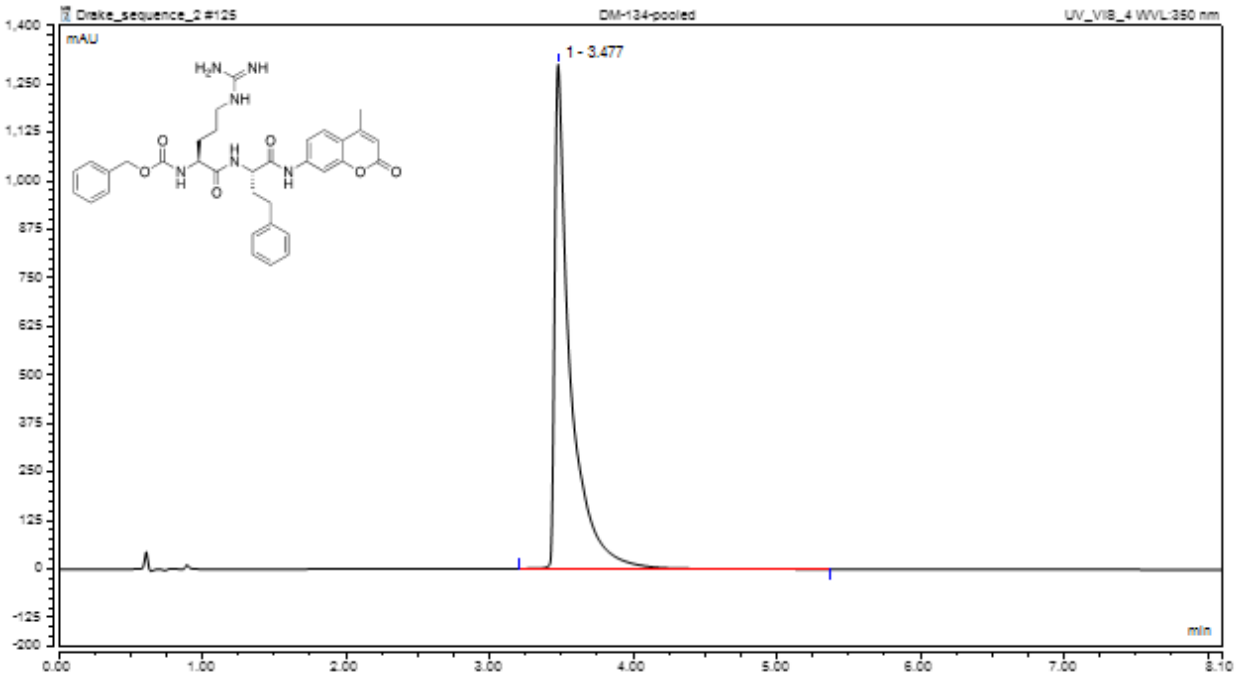
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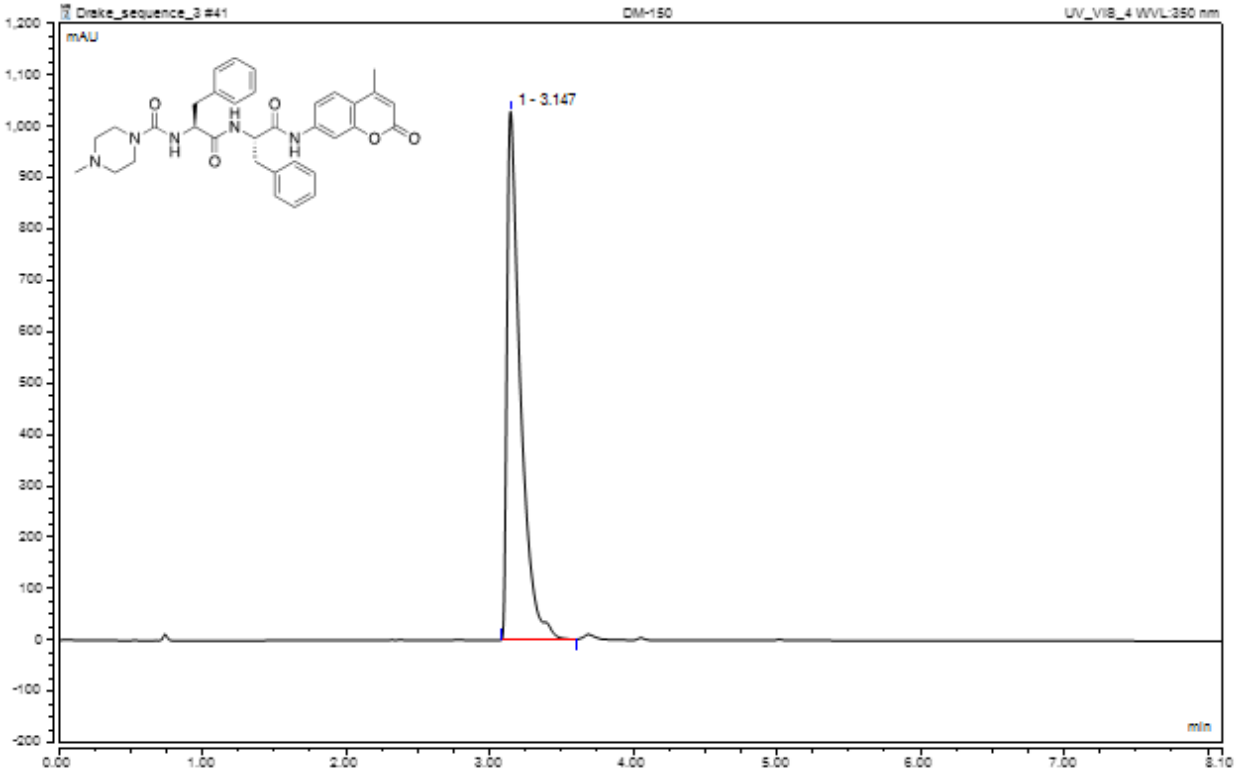
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min

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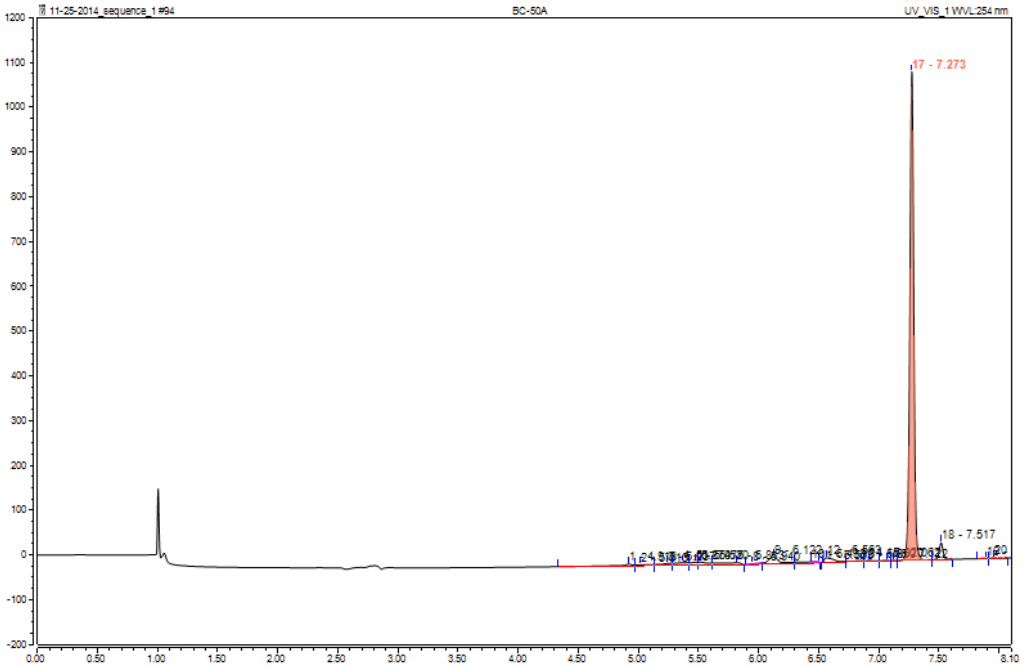

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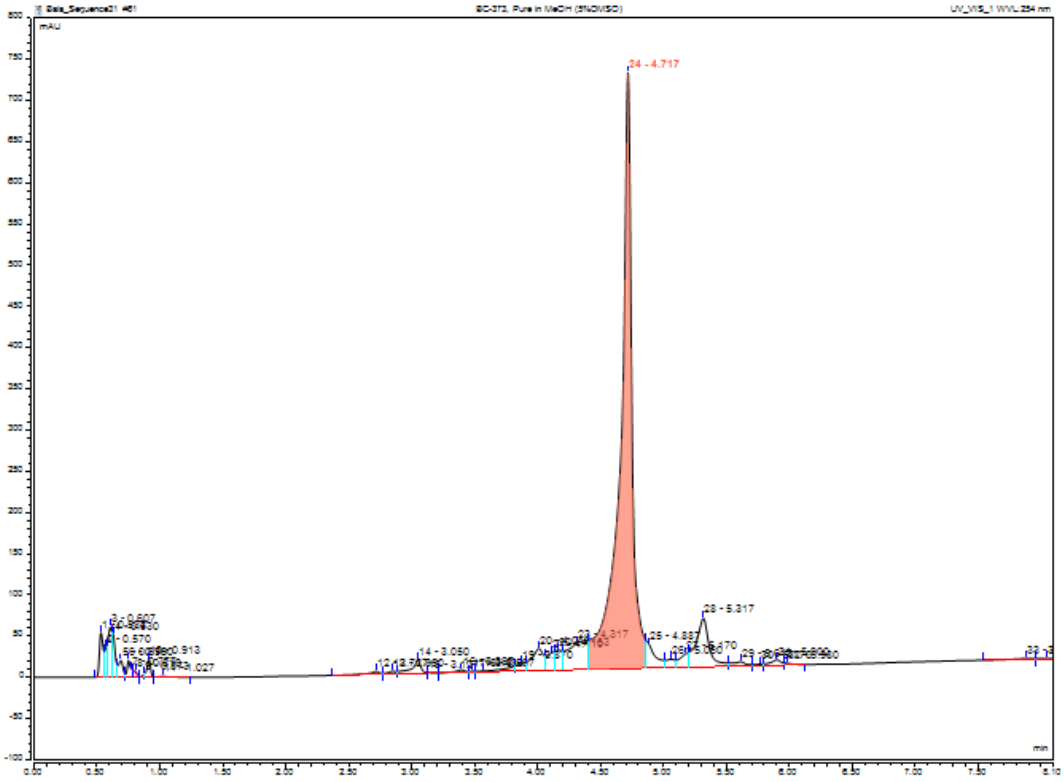

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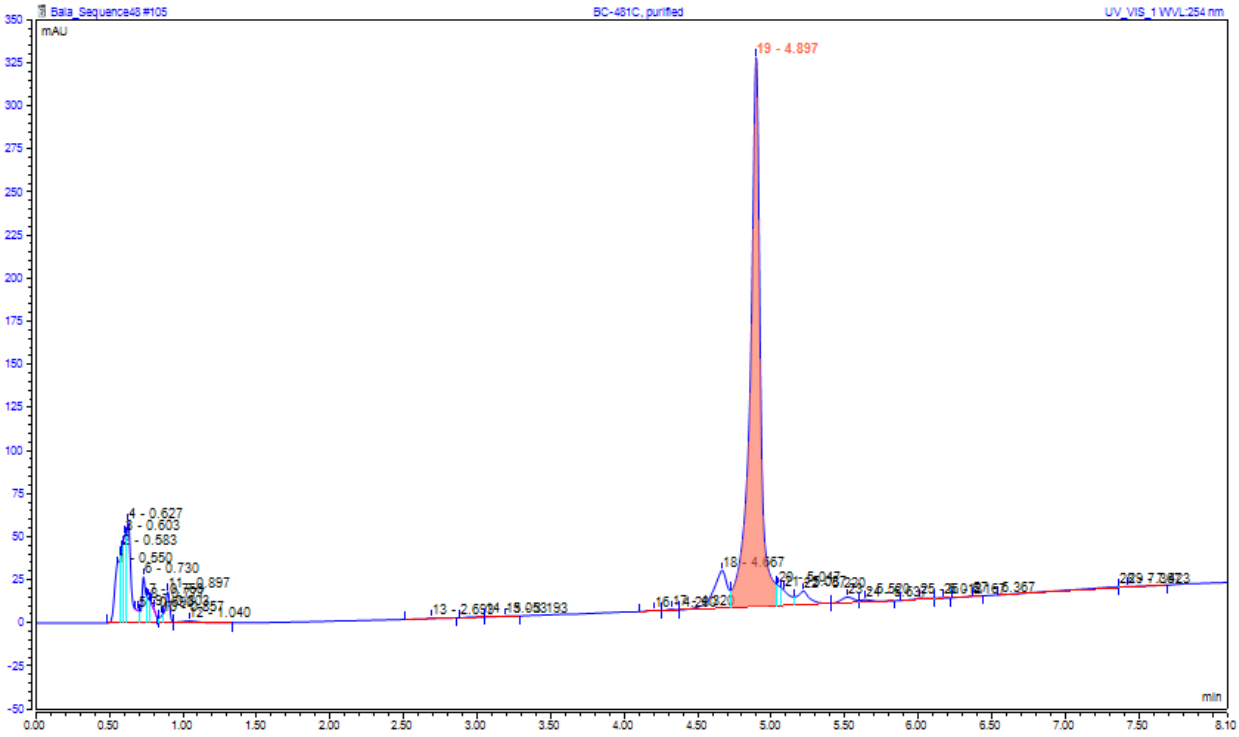

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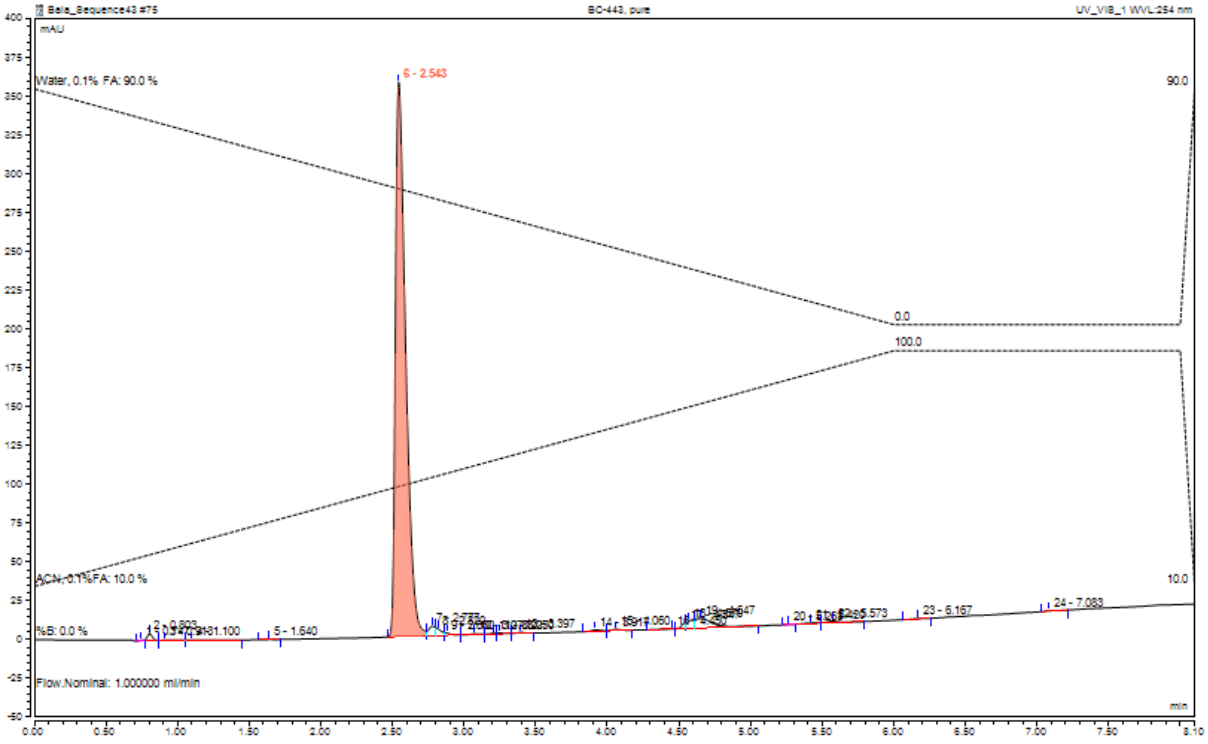

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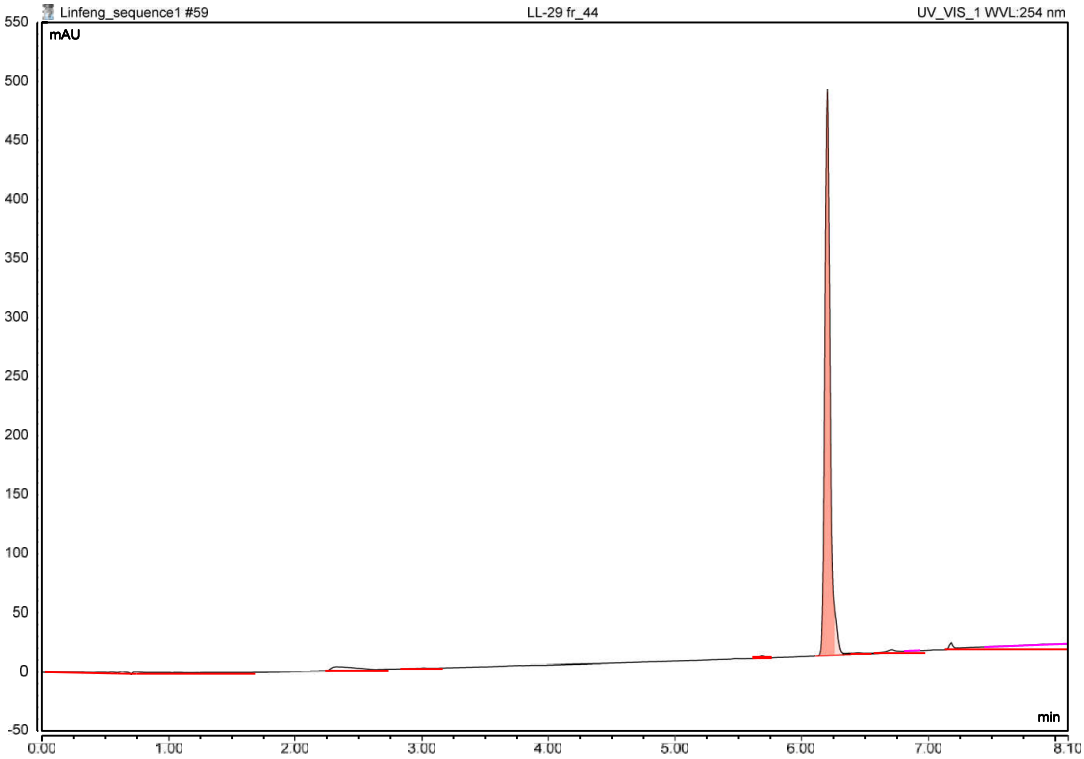

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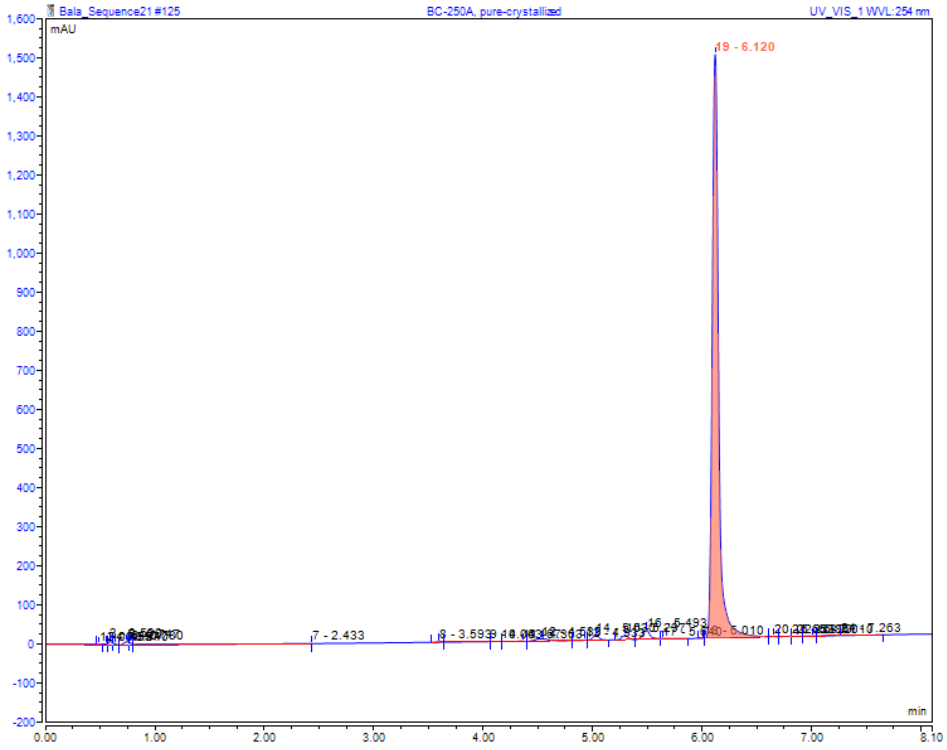

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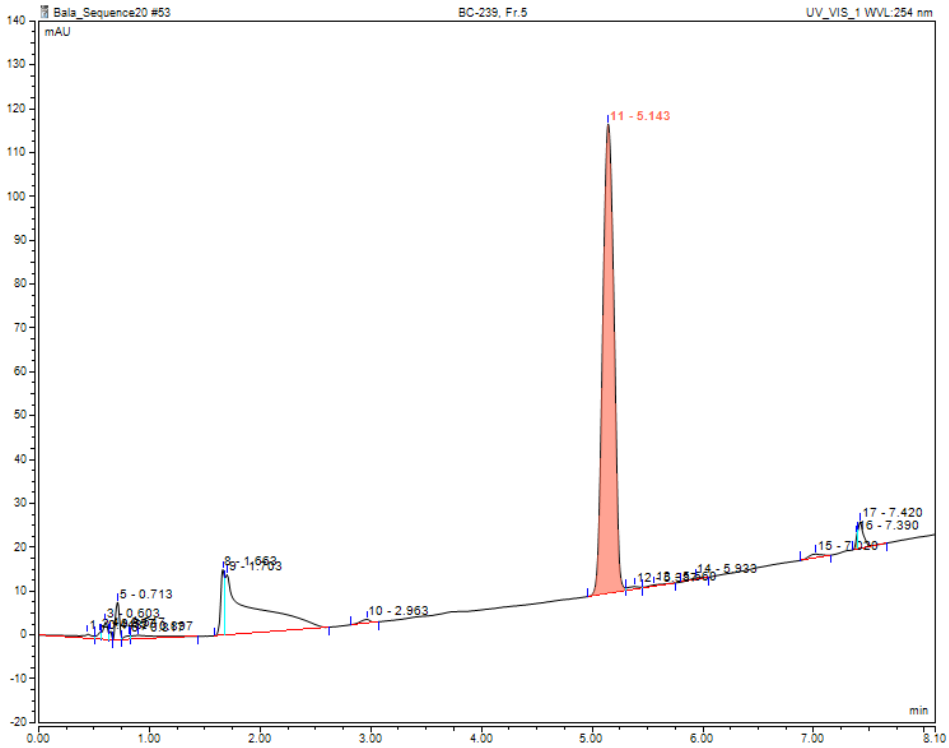

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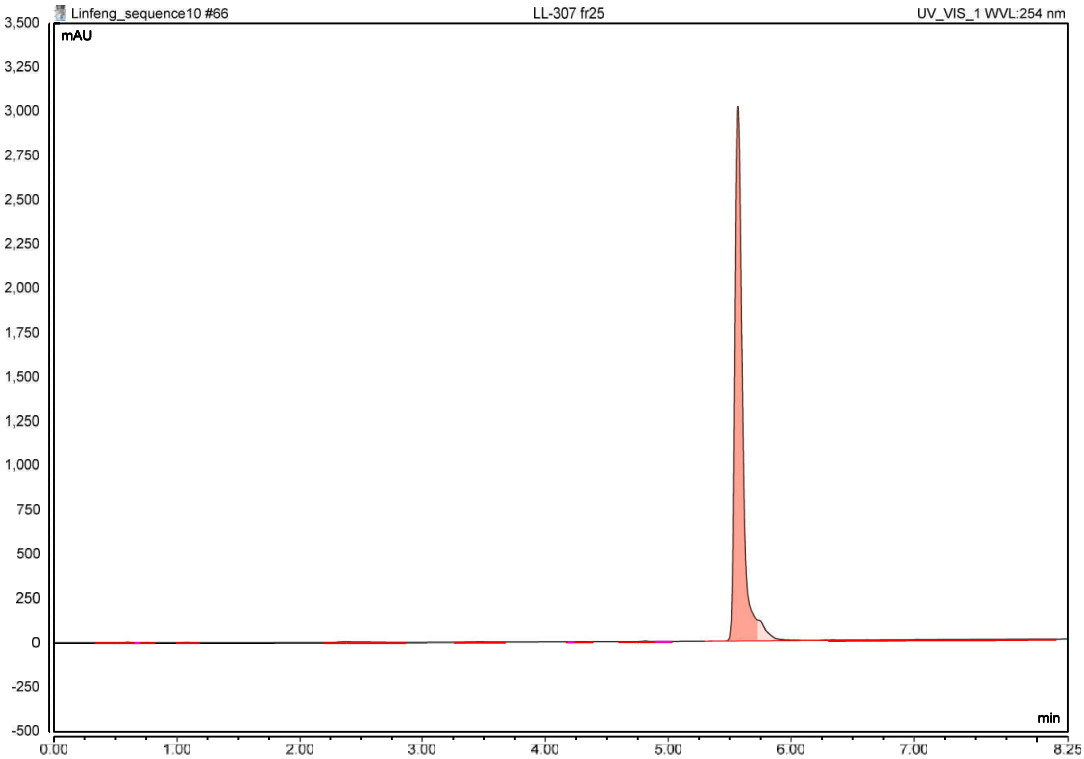

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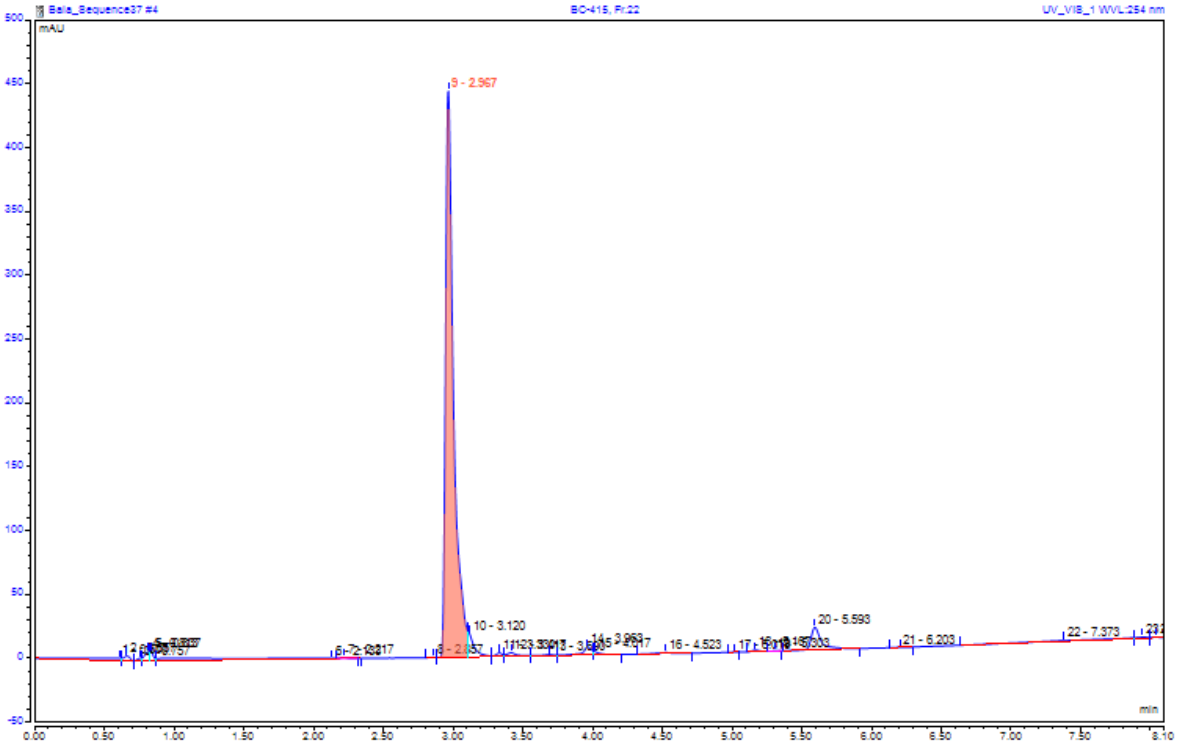

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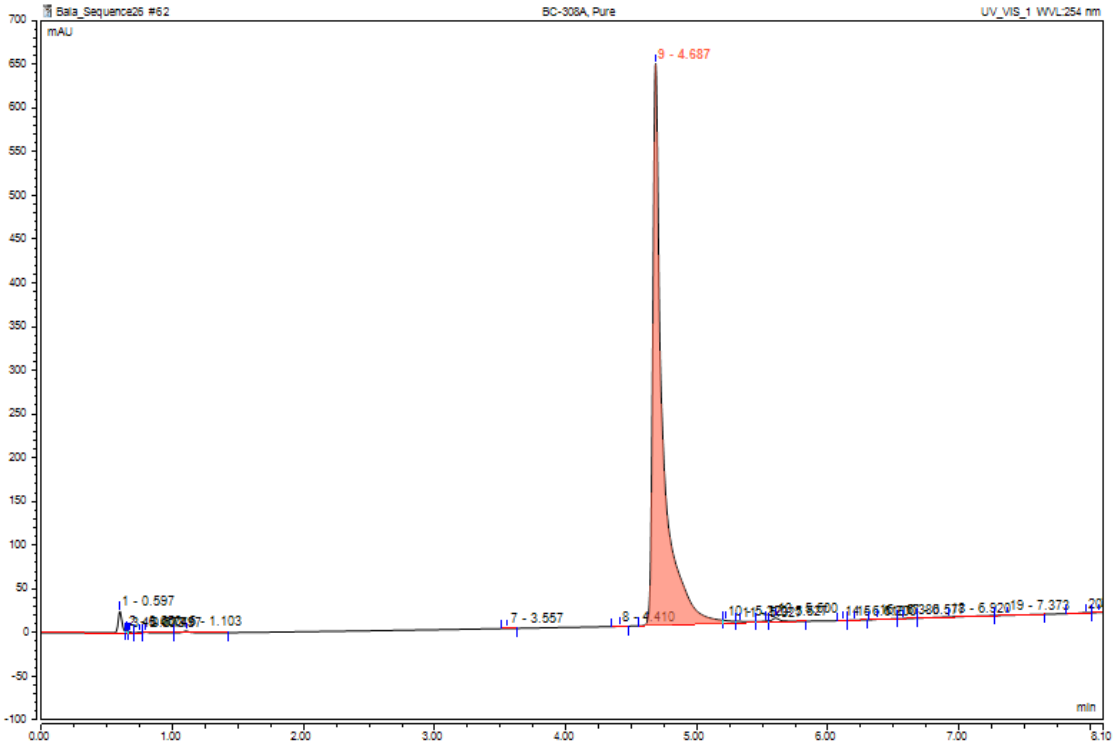

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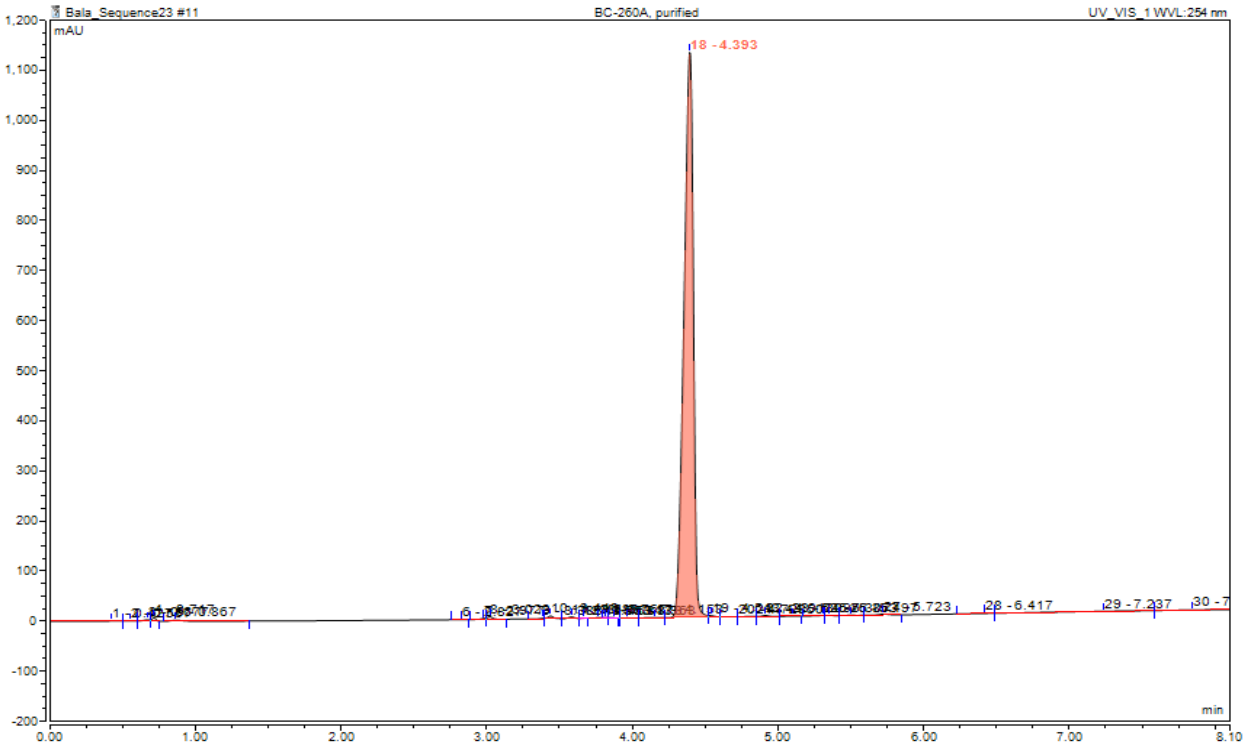

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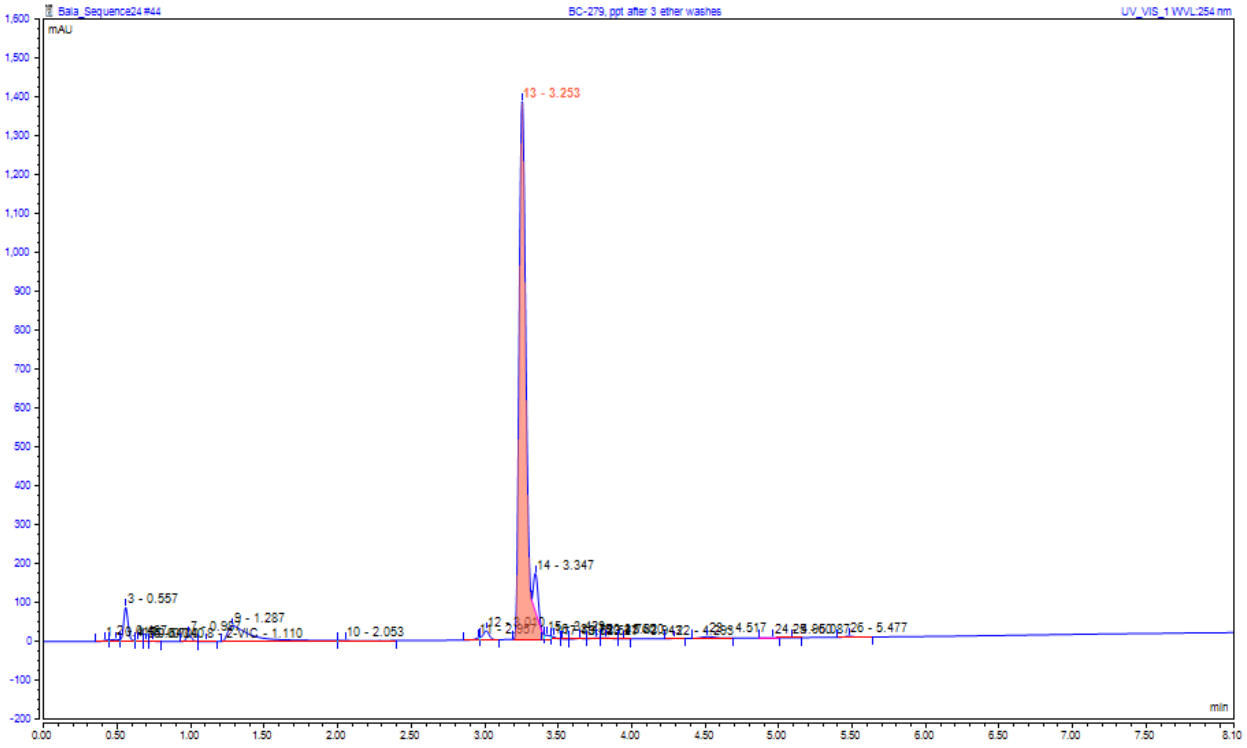

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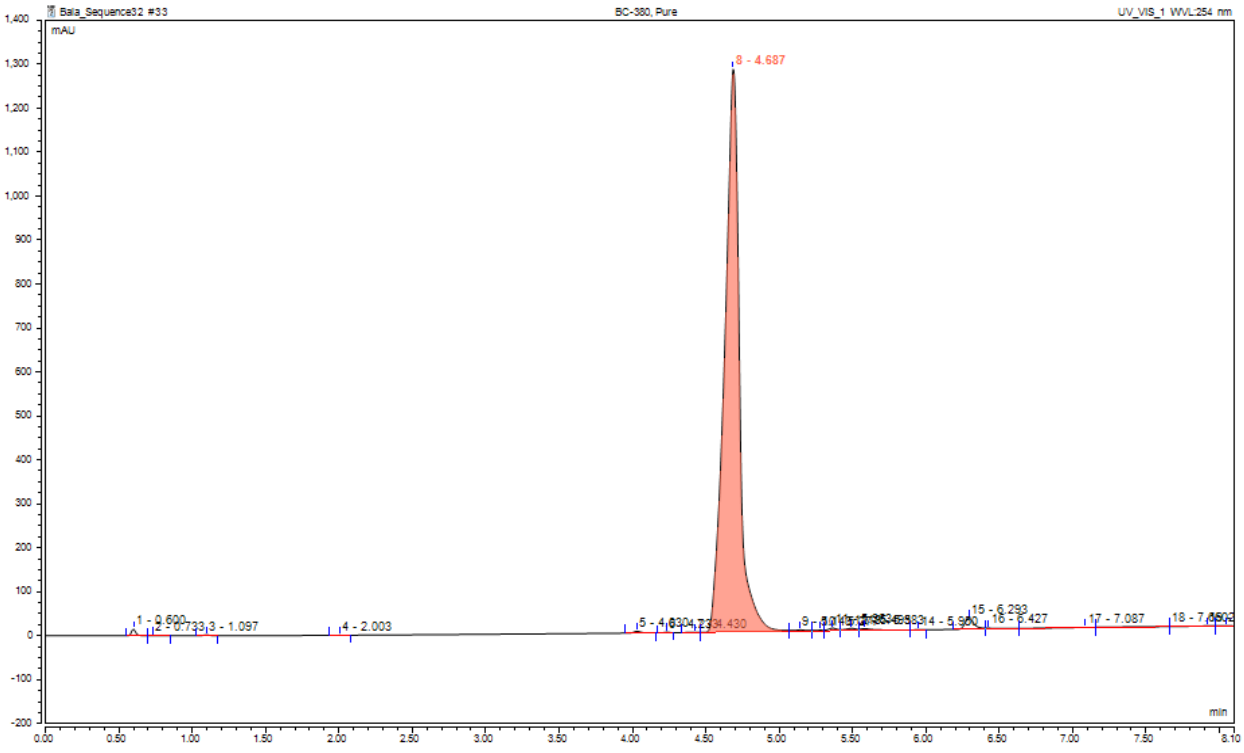
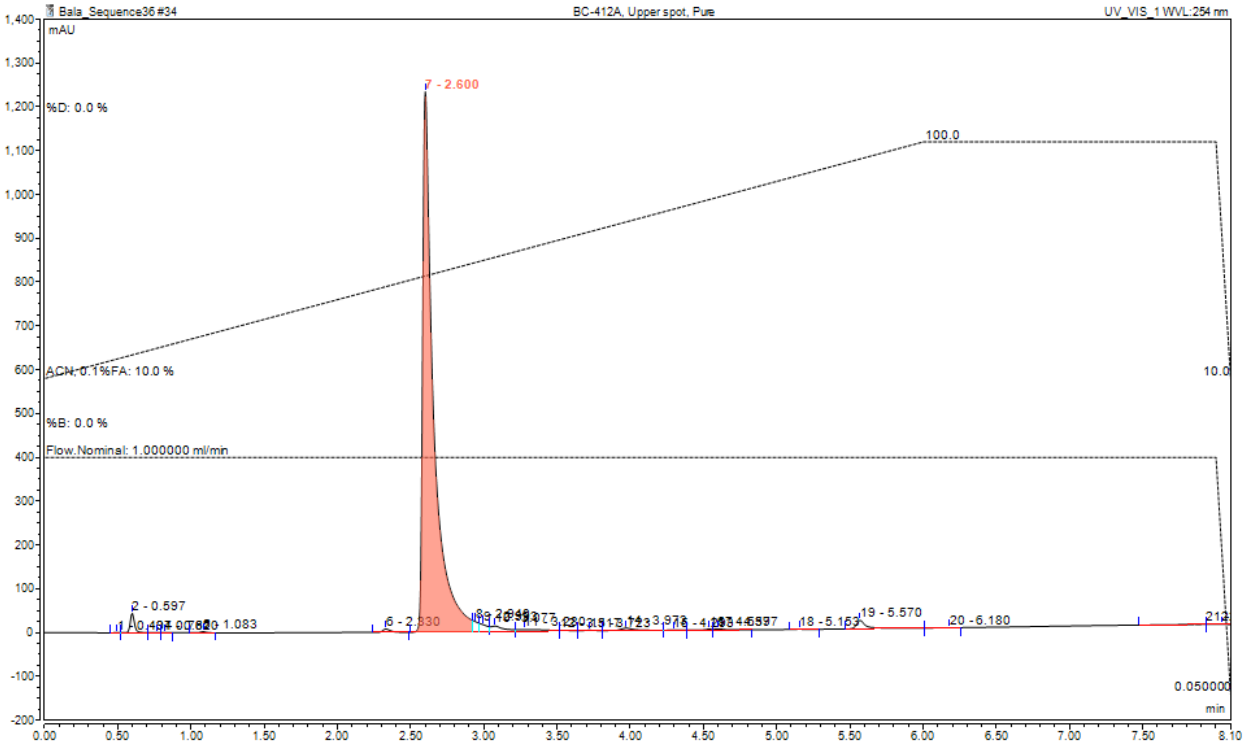
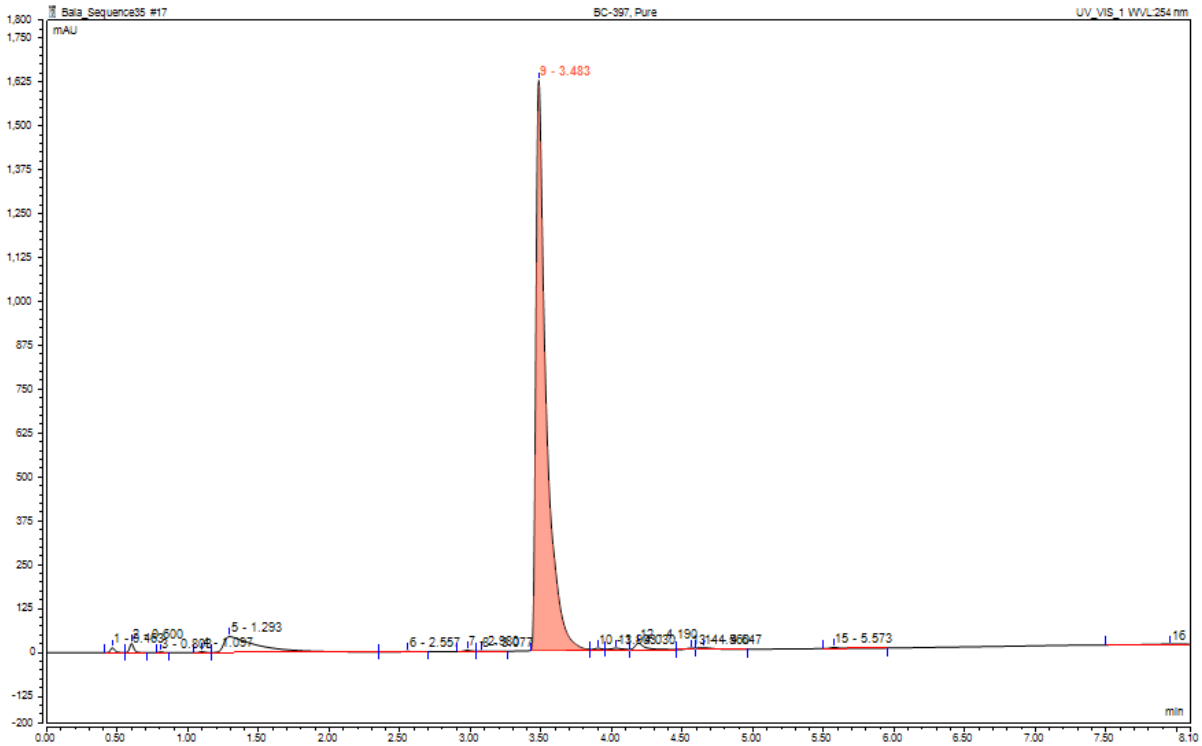


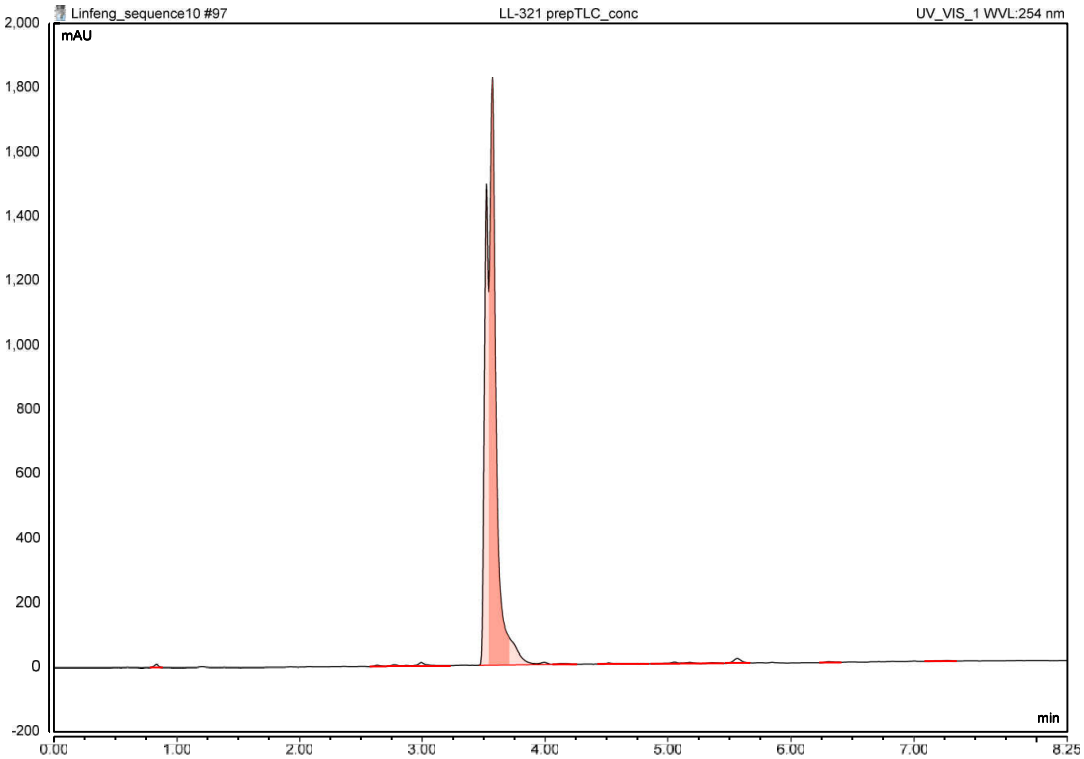
Compound **14**:



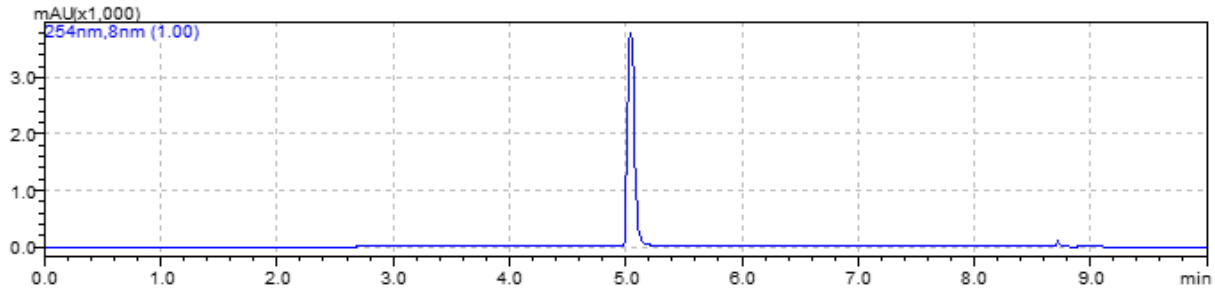
Compound **15**:



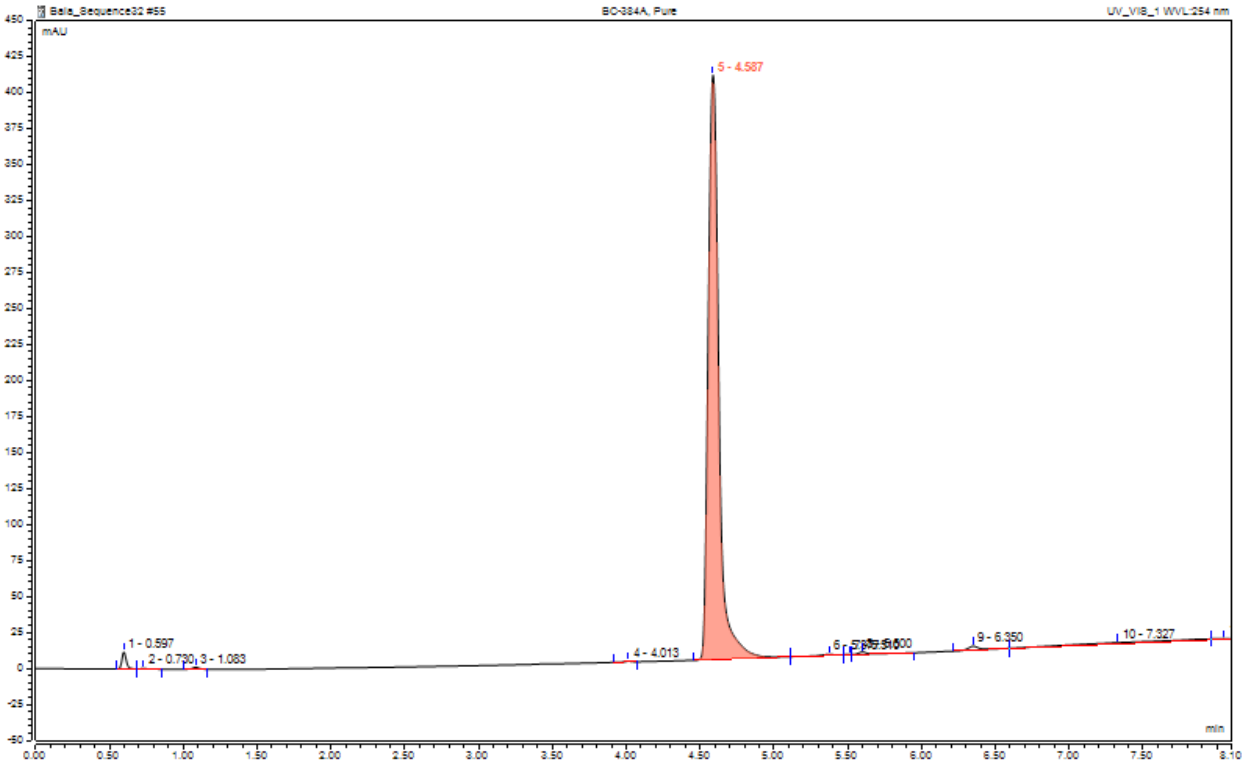
Compound **16**:



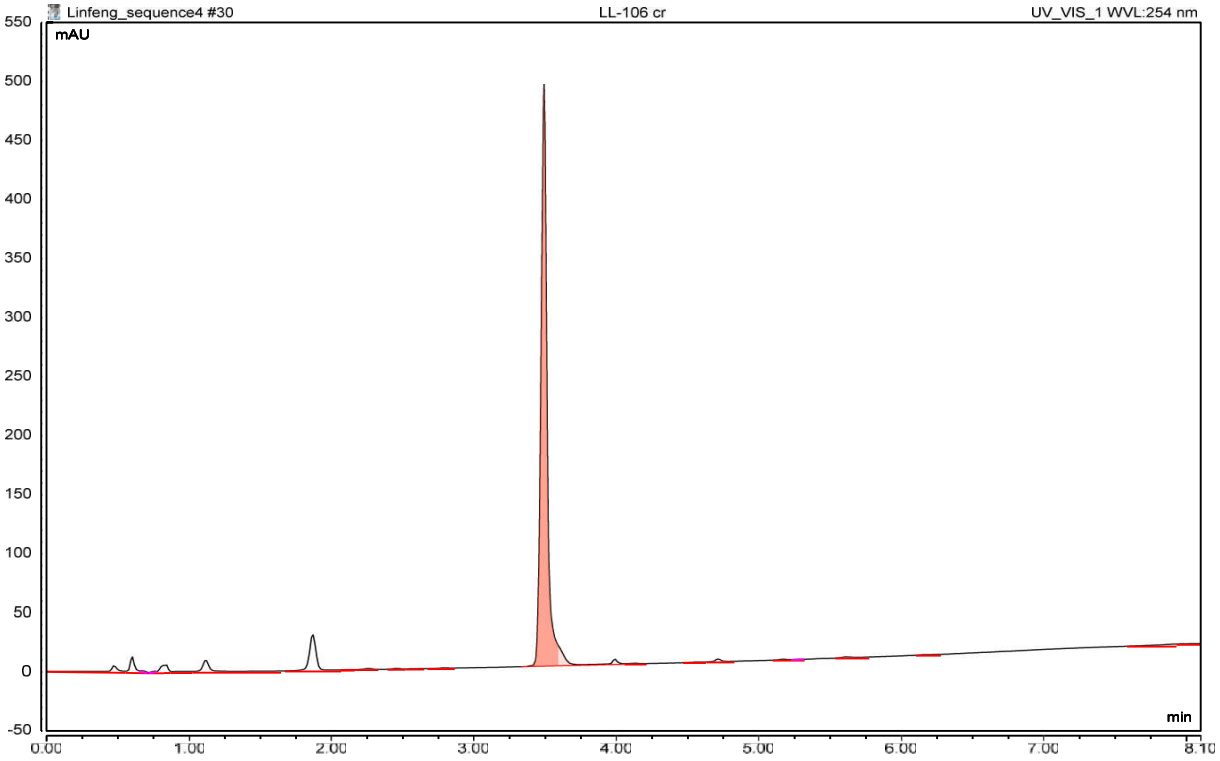
Compound **17**:



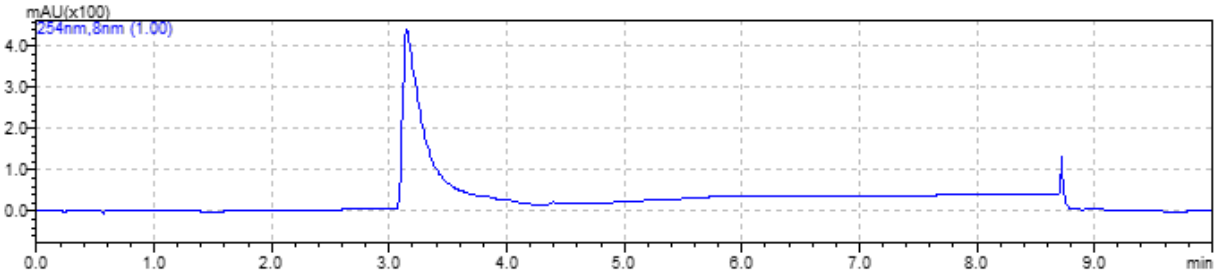
Compound **18**:



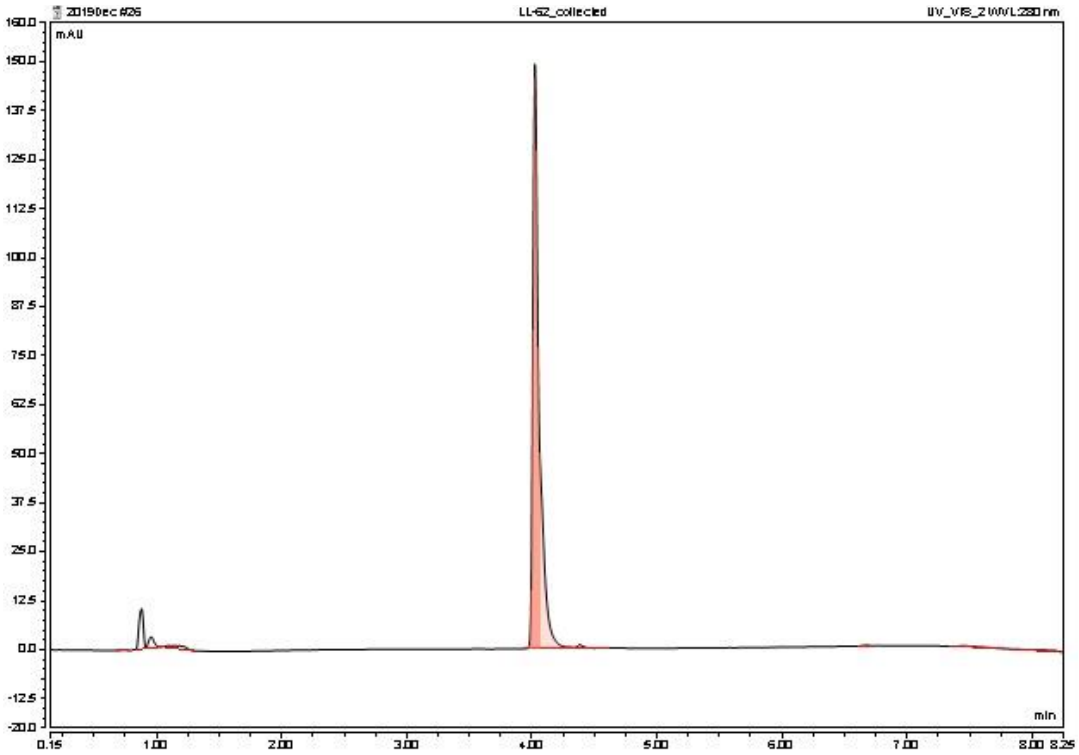
Compound **19**:



Compound **20**:



Compound **21**:



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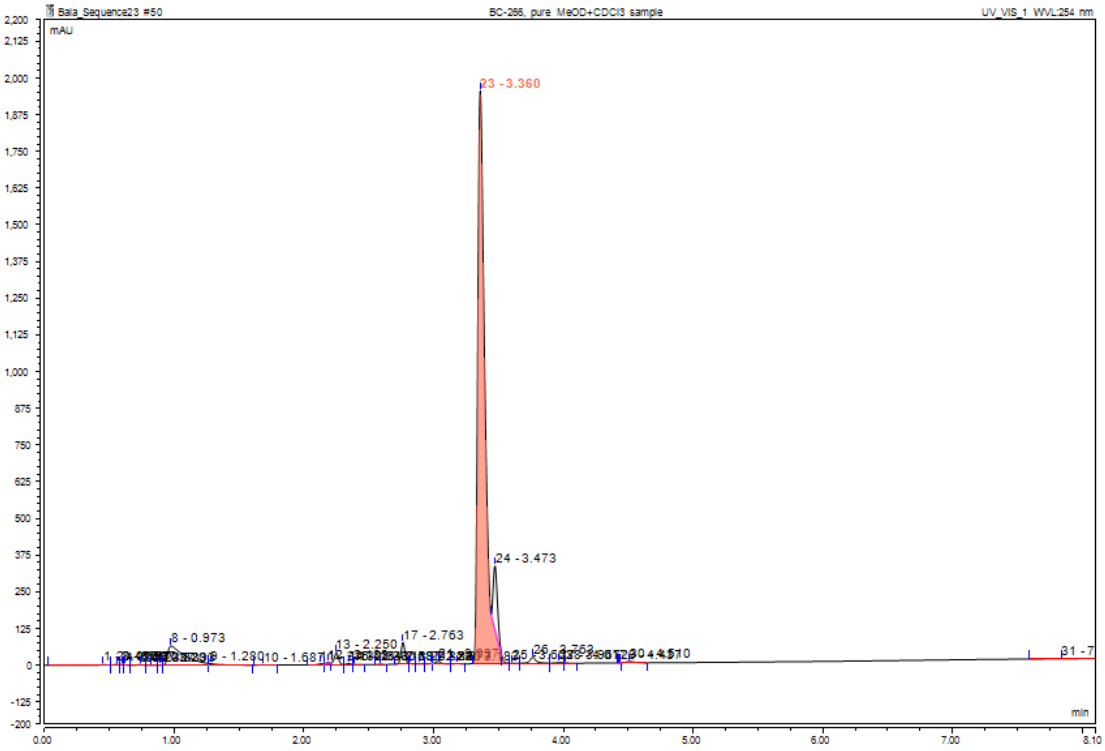
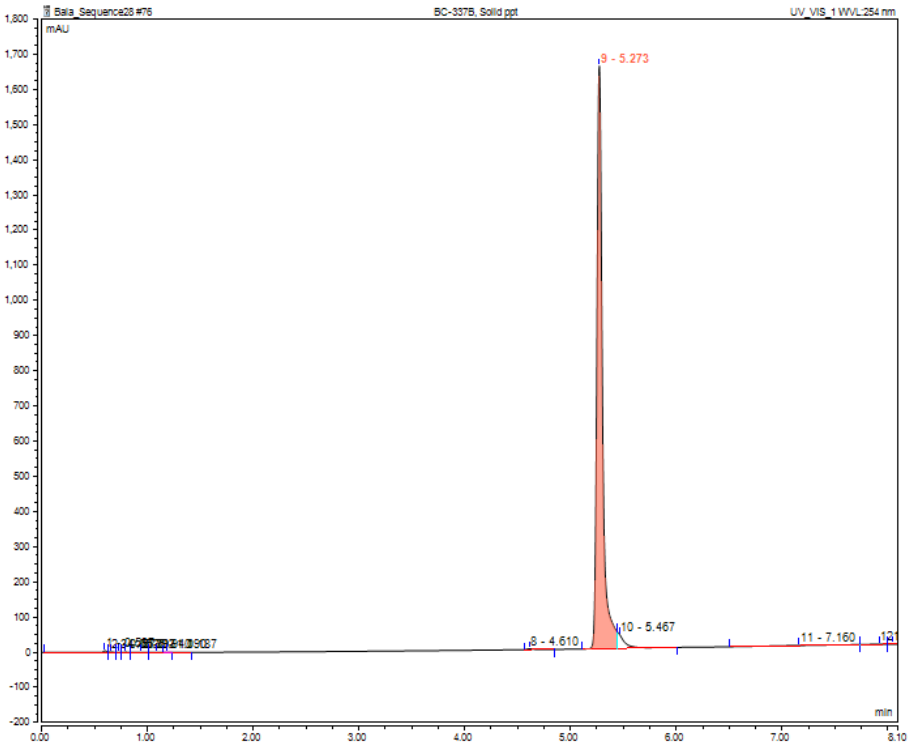


Compound **23**:



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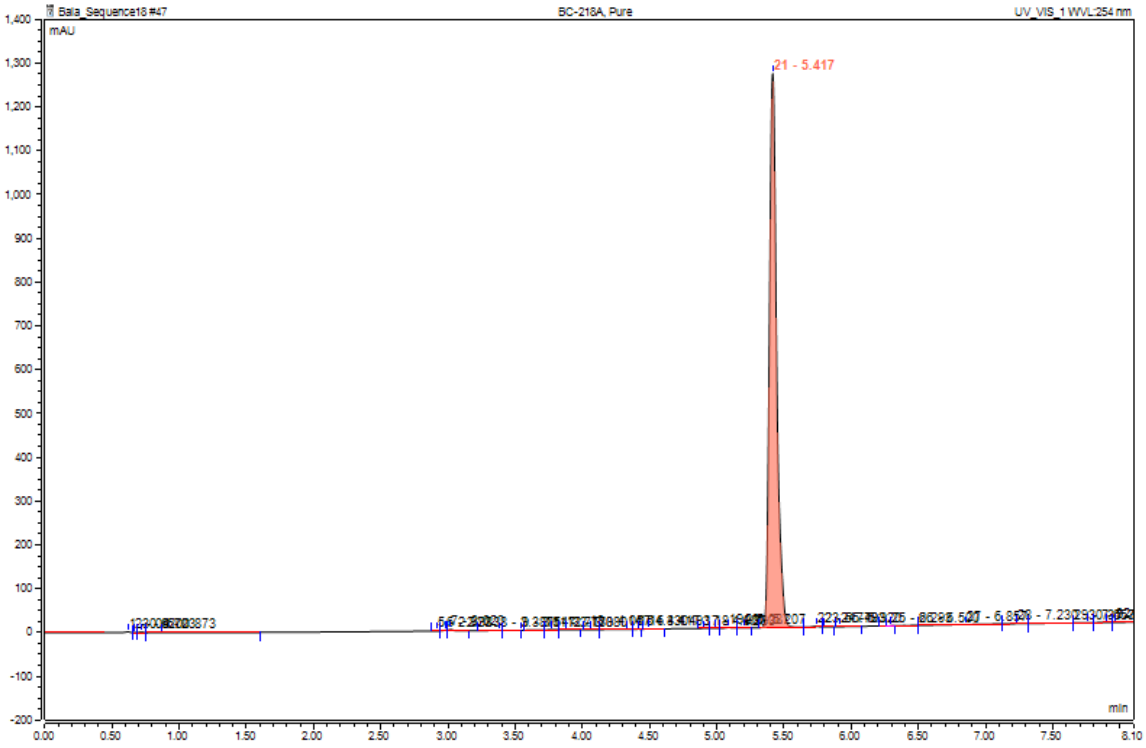

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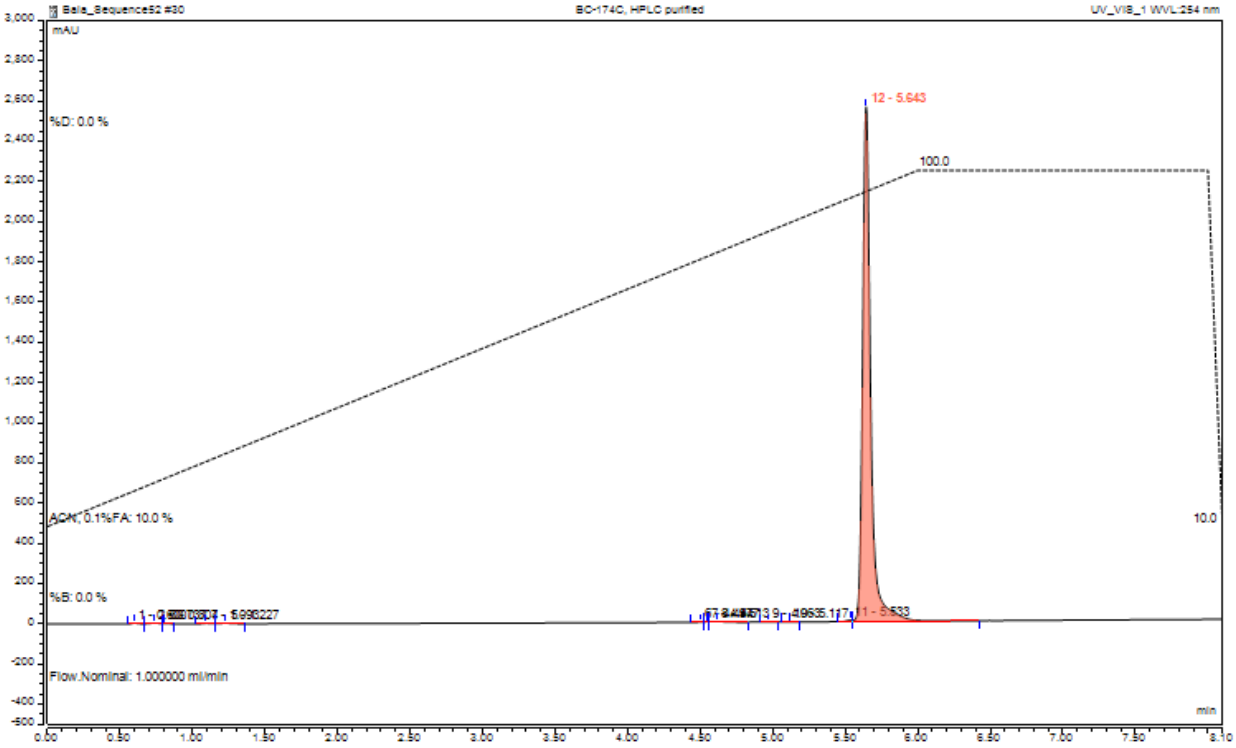
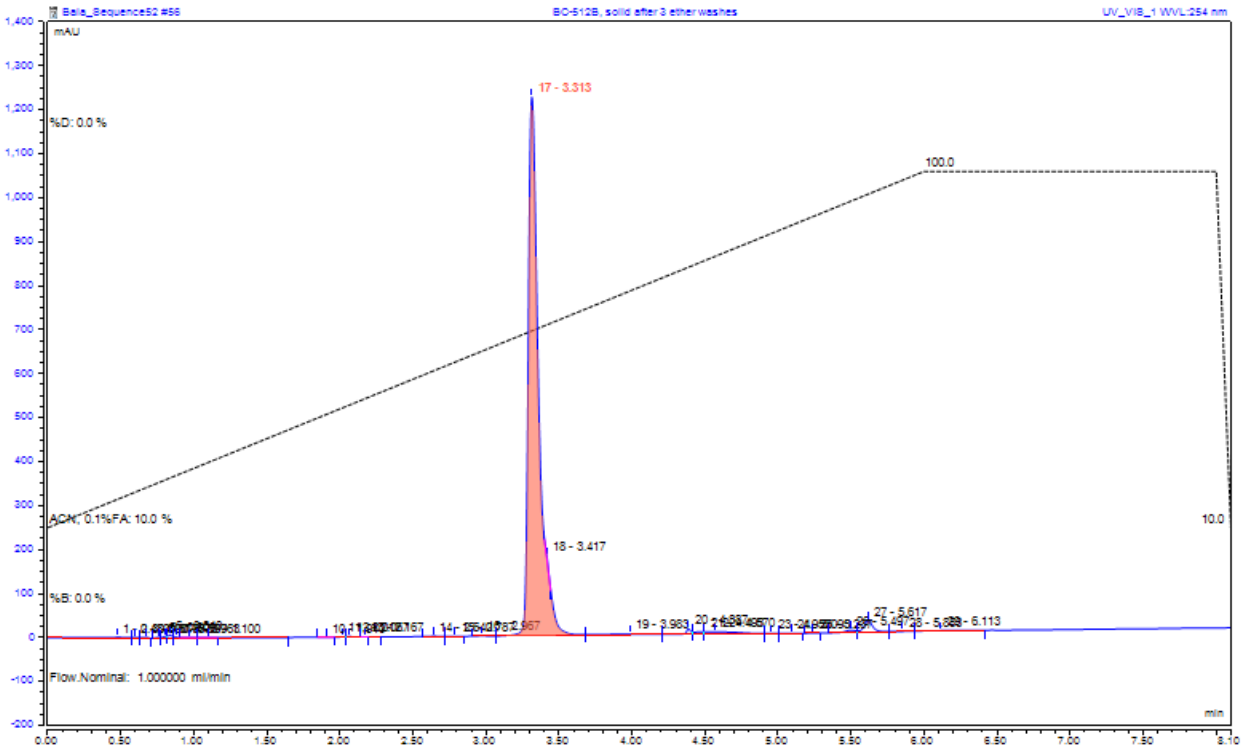
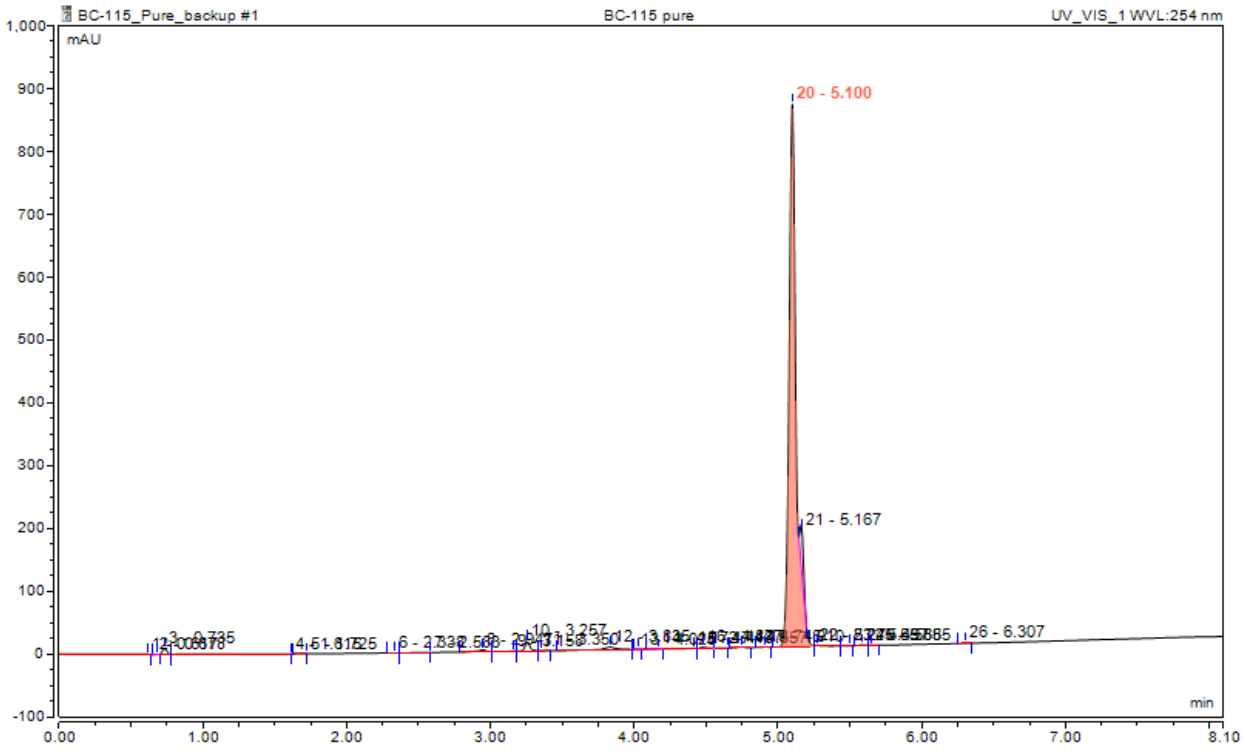


Compound **26**:



Compound **27**: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BC-115 pure
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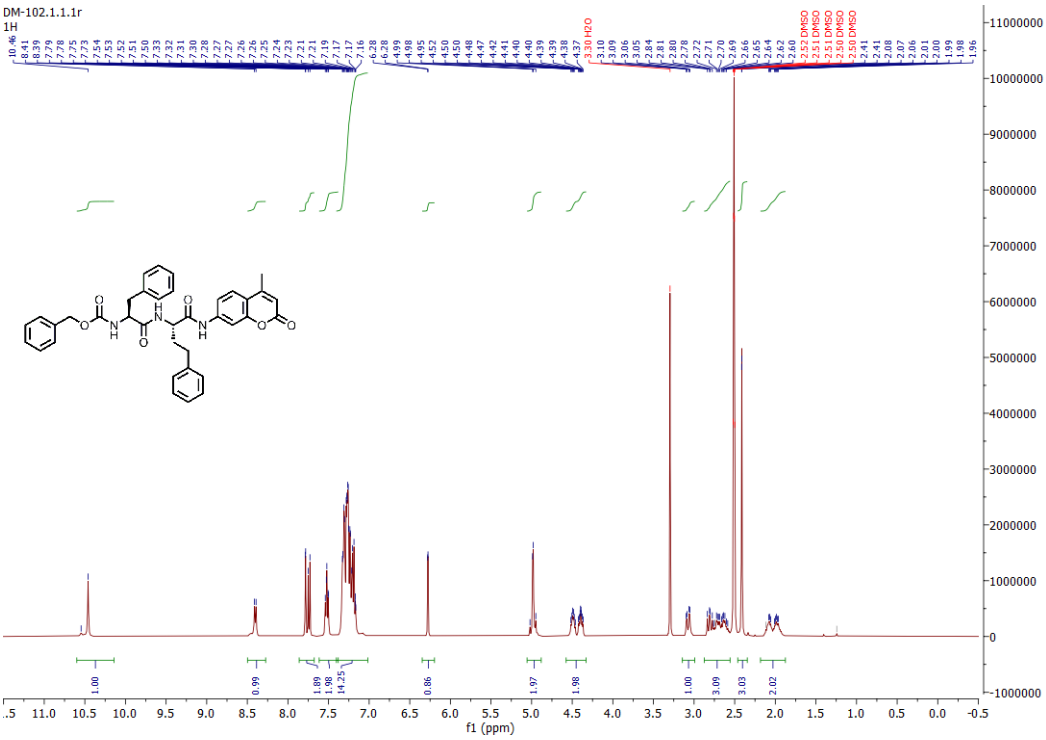
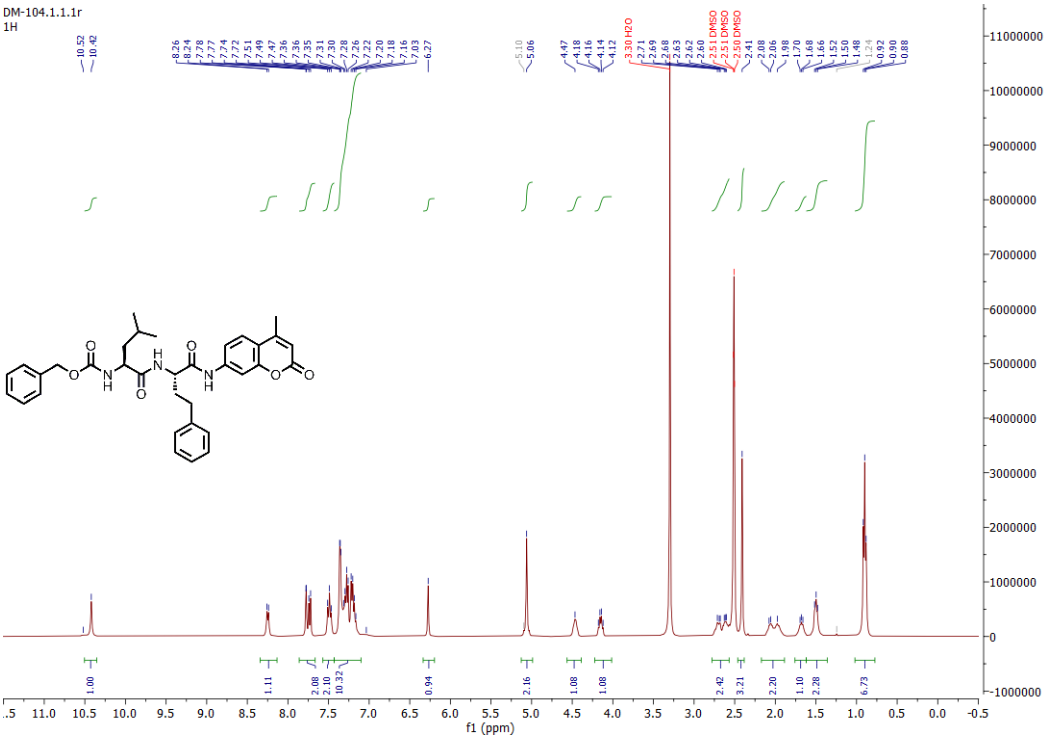
mAU
1,000
900
800
700
600
500
400
300
200
100
0
-100
20 - 5.100
21 - 5.167
26 - 6.307
min
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.10

Part 7. NMR Spectra of Synthesized Substrates and Inhibitors

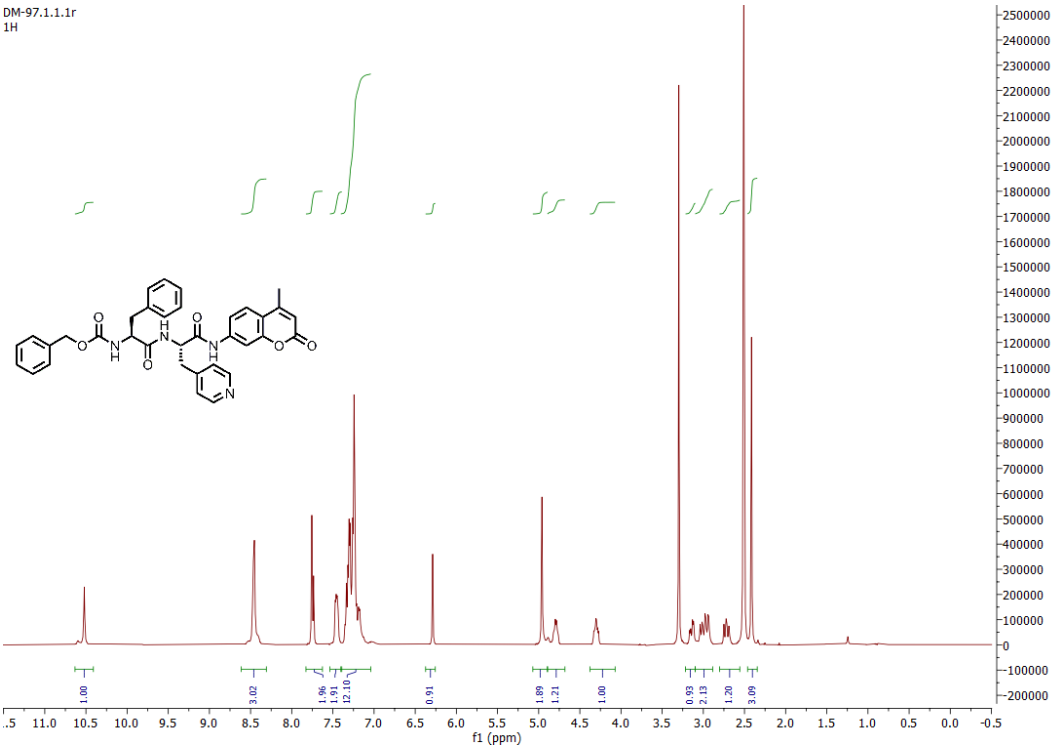
S2:



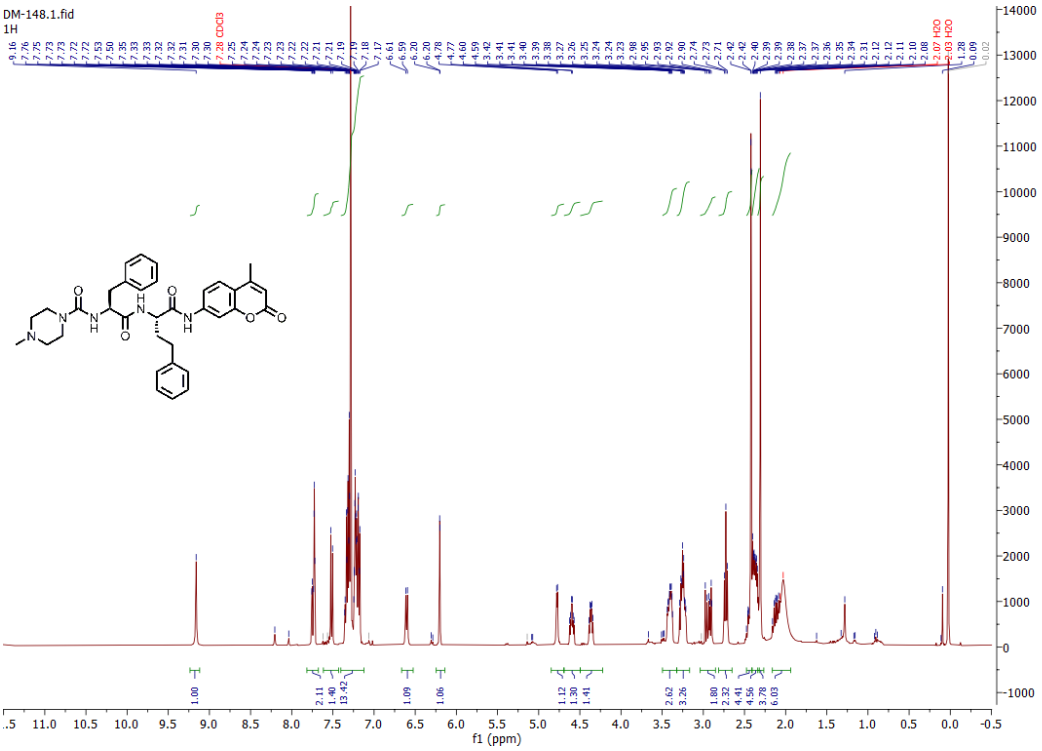
S3:



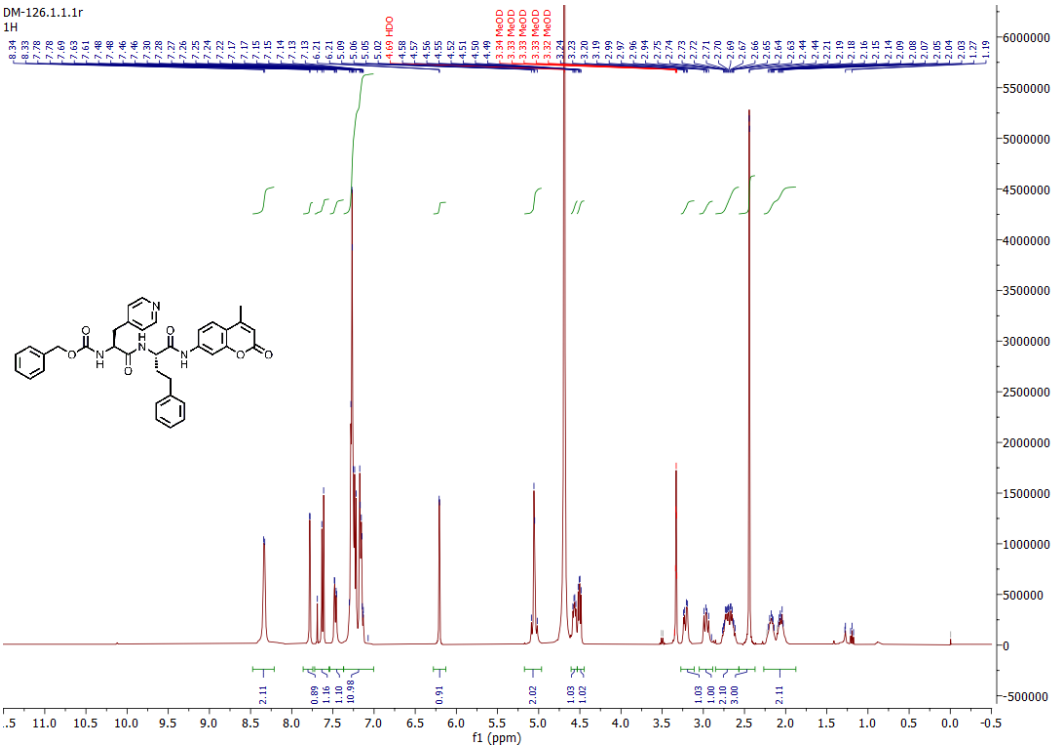
S5:



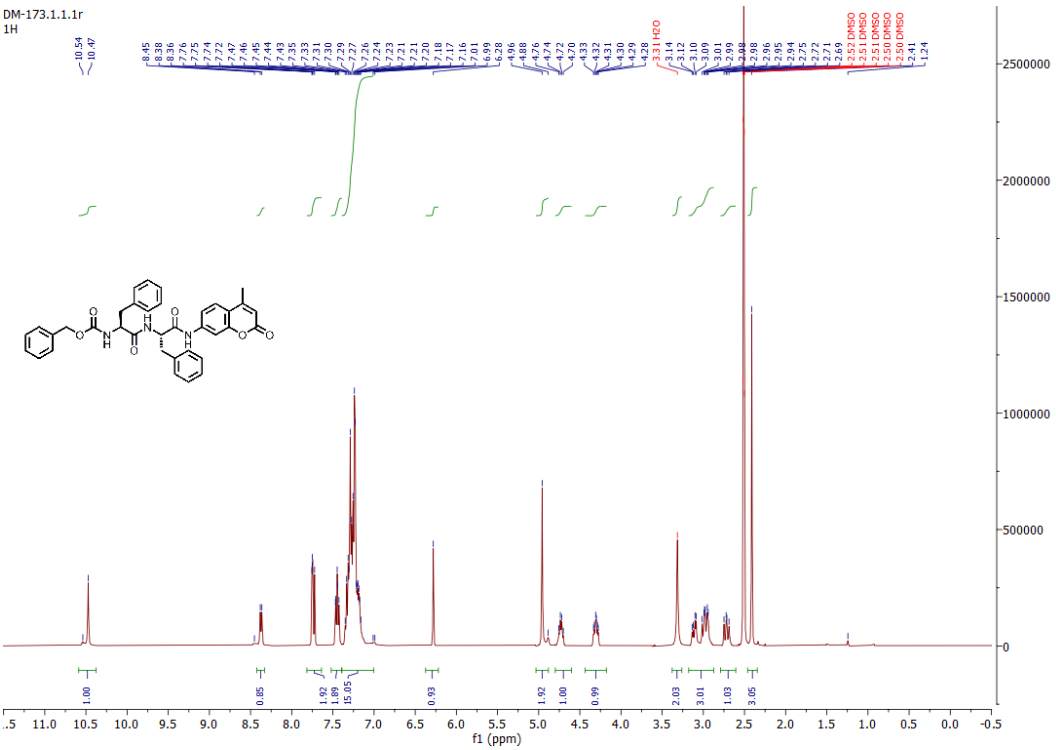
S7:



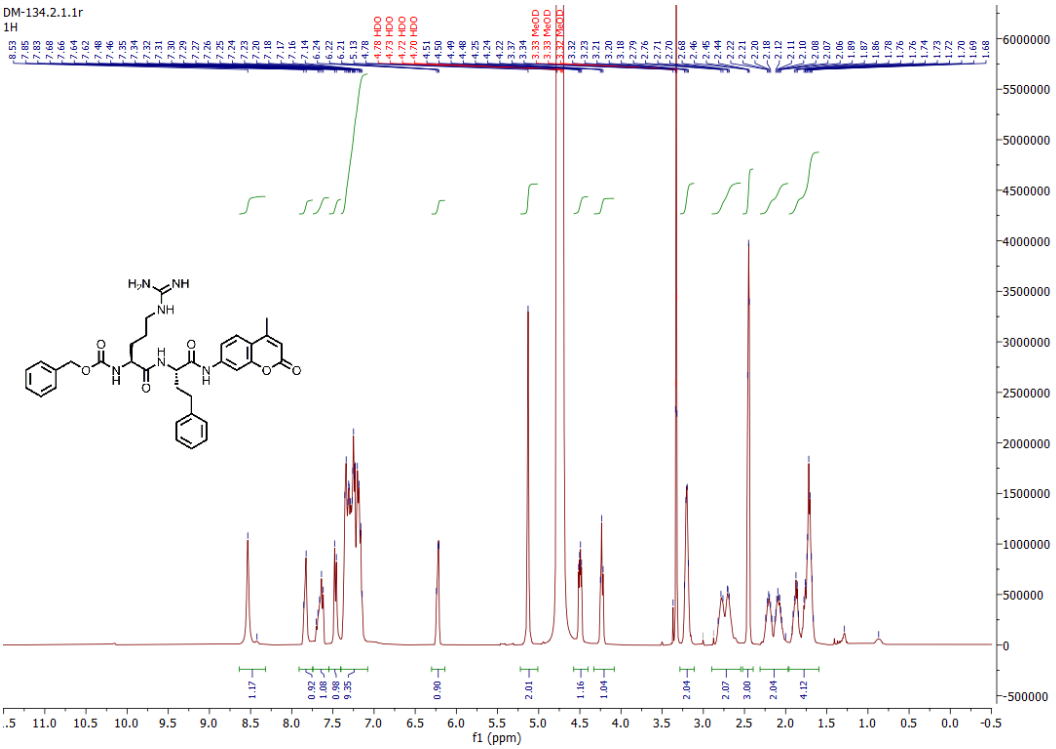
S8:



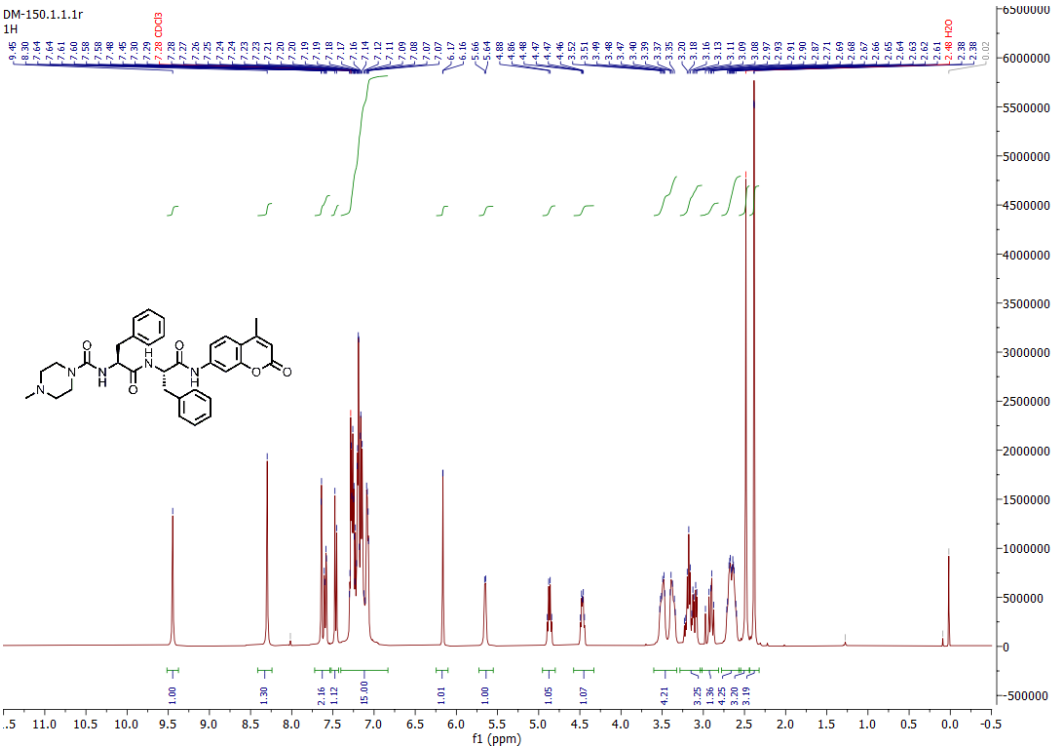
S9:



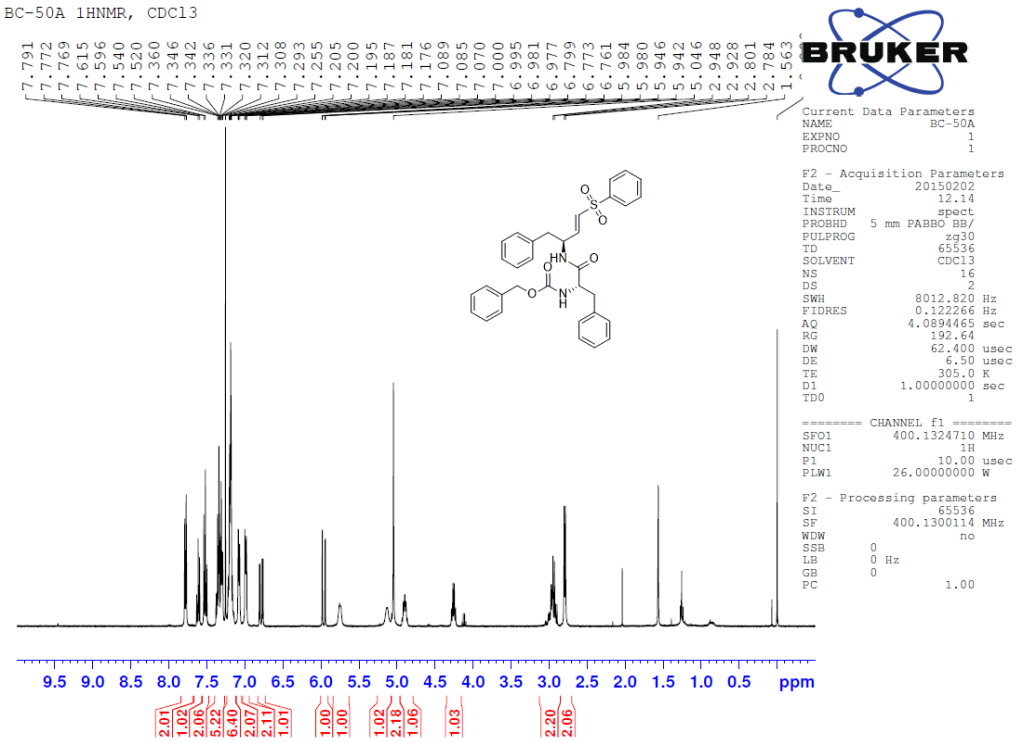
S10:



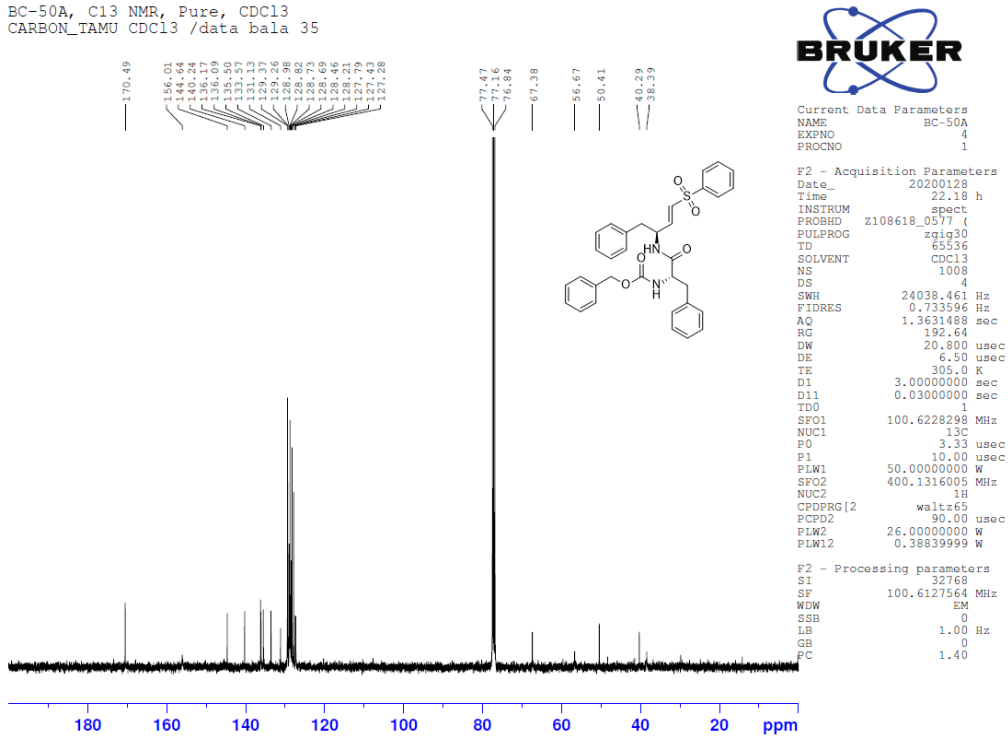
S11:



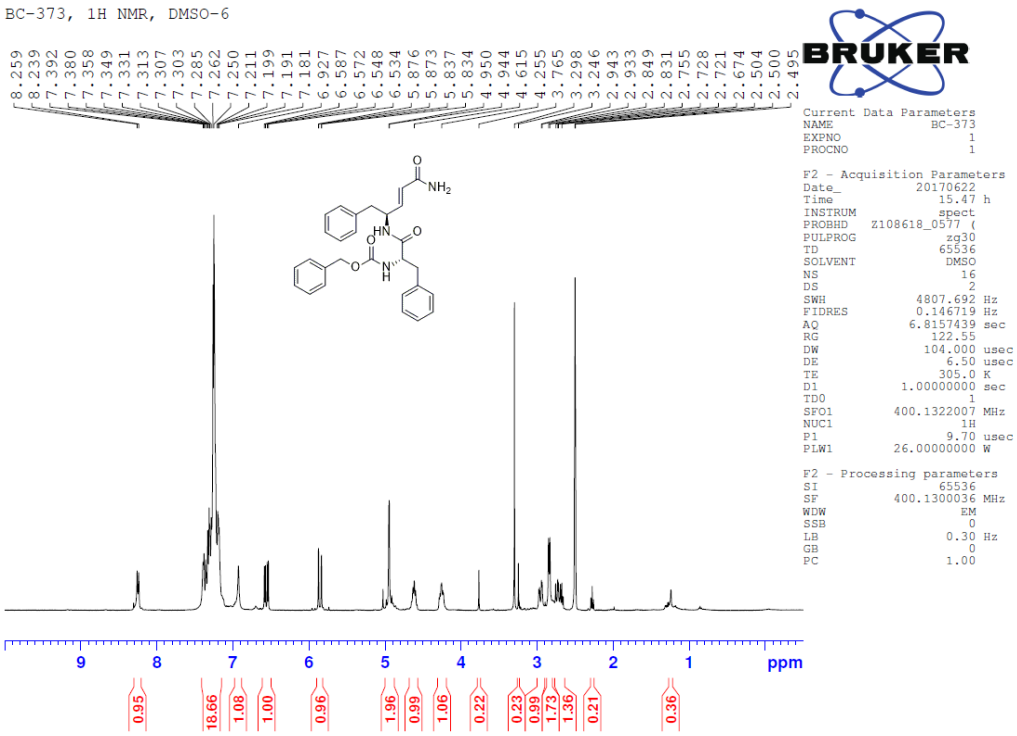
^{1}H NMR spectrum of compound **1**



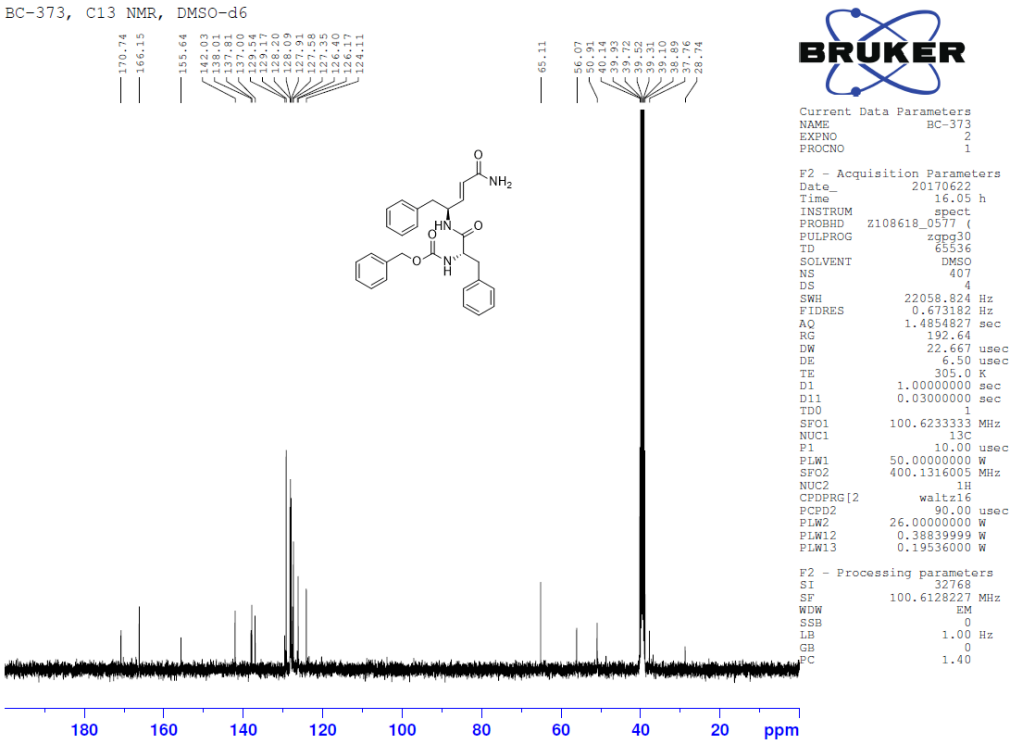
^{13}C NMR spectrum of compound **1**



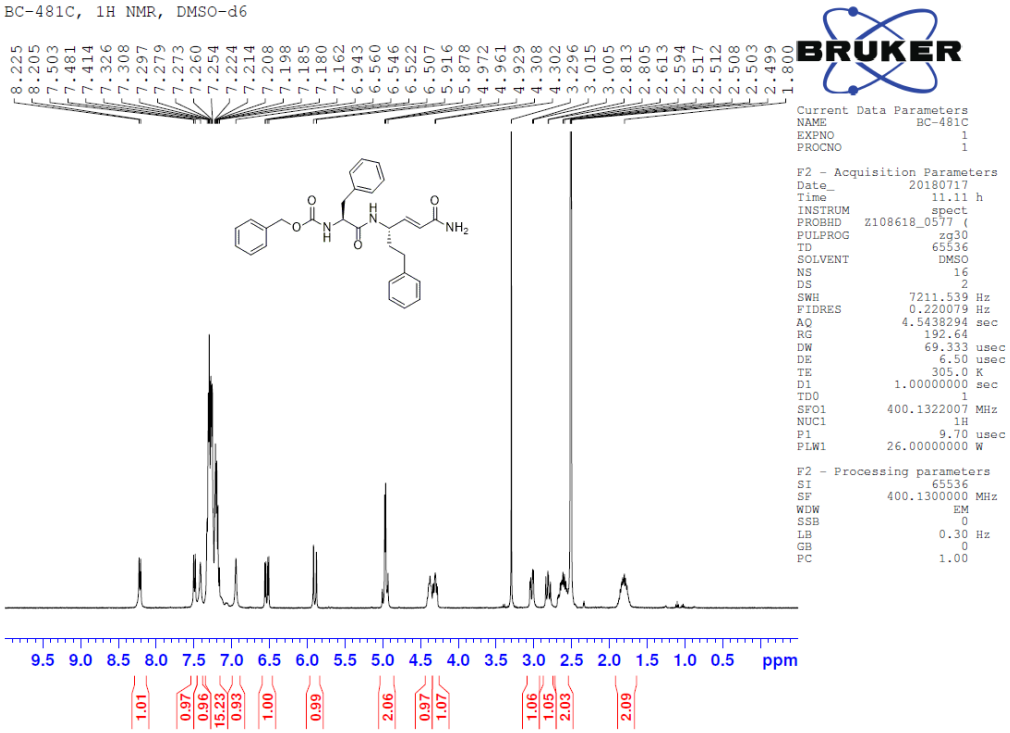
[1]H NMR spectrum of compound **2**



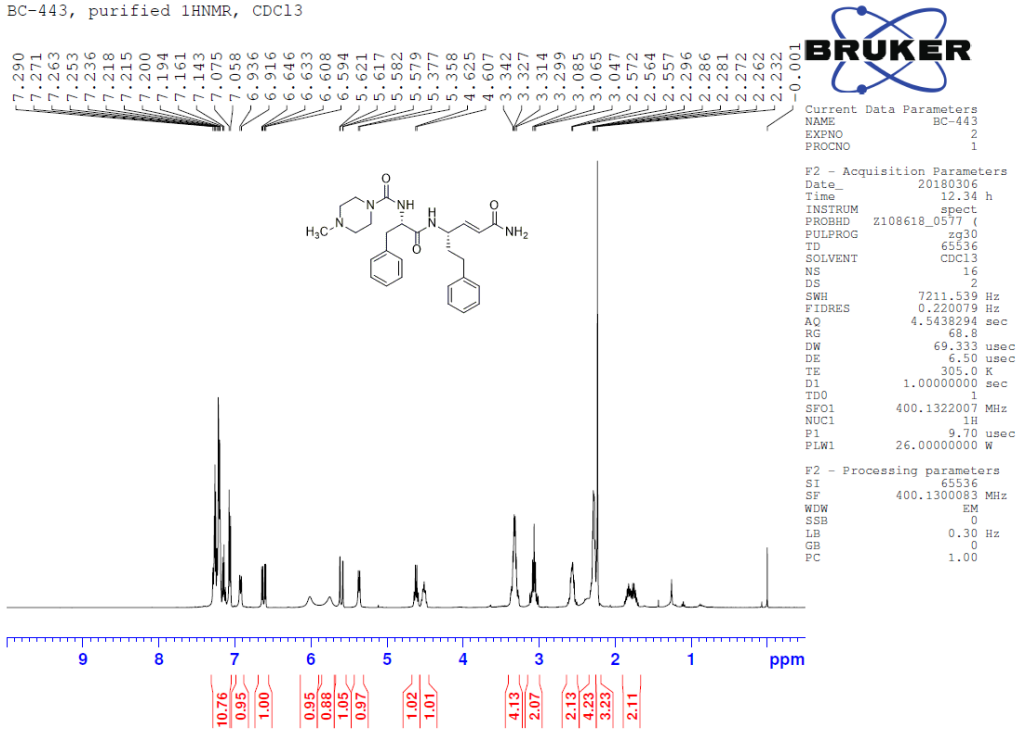
[13]C NMR spectrum of compound **2**



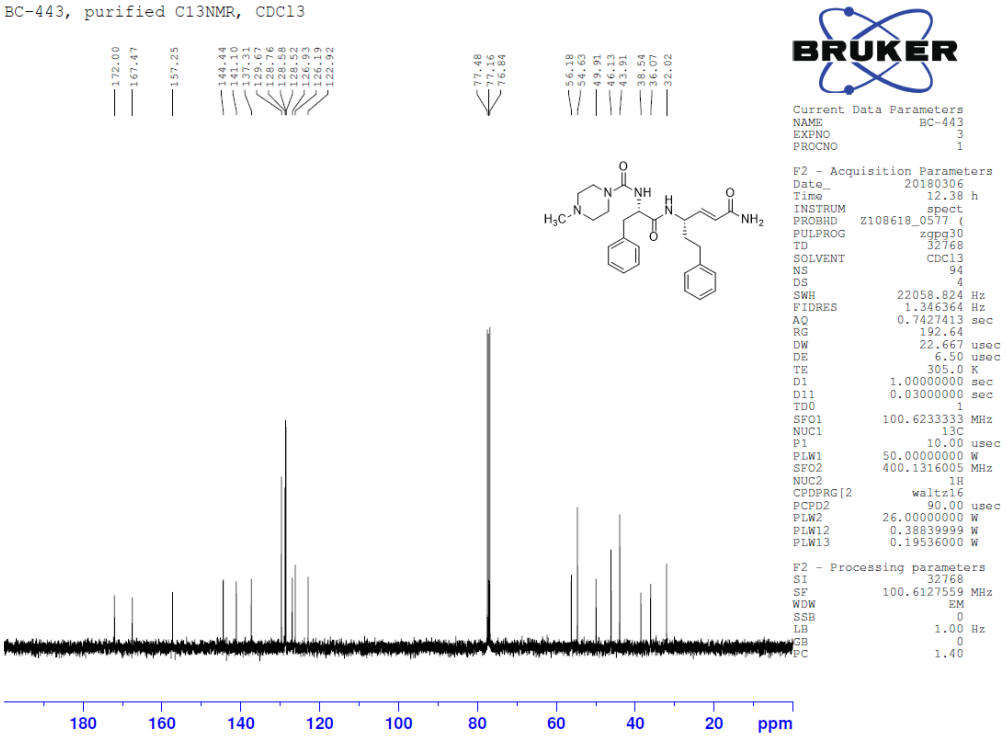
^{1}H NMR spectrum of compound **3**



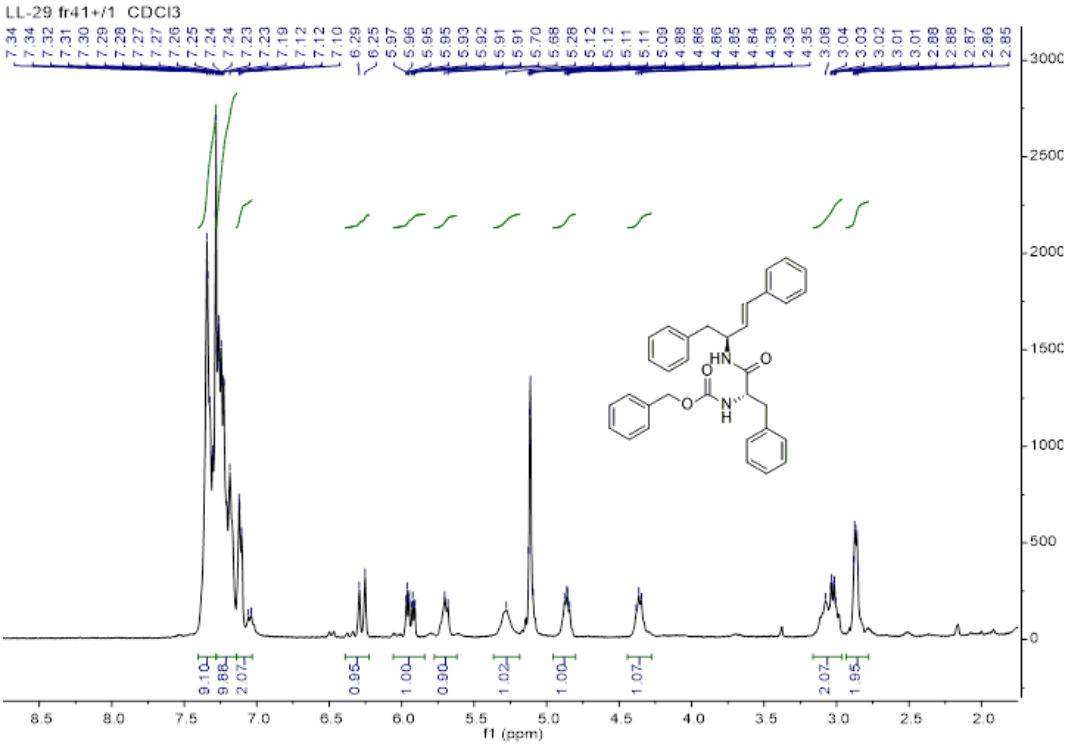
[1]H NMR spectrum of compound **4**



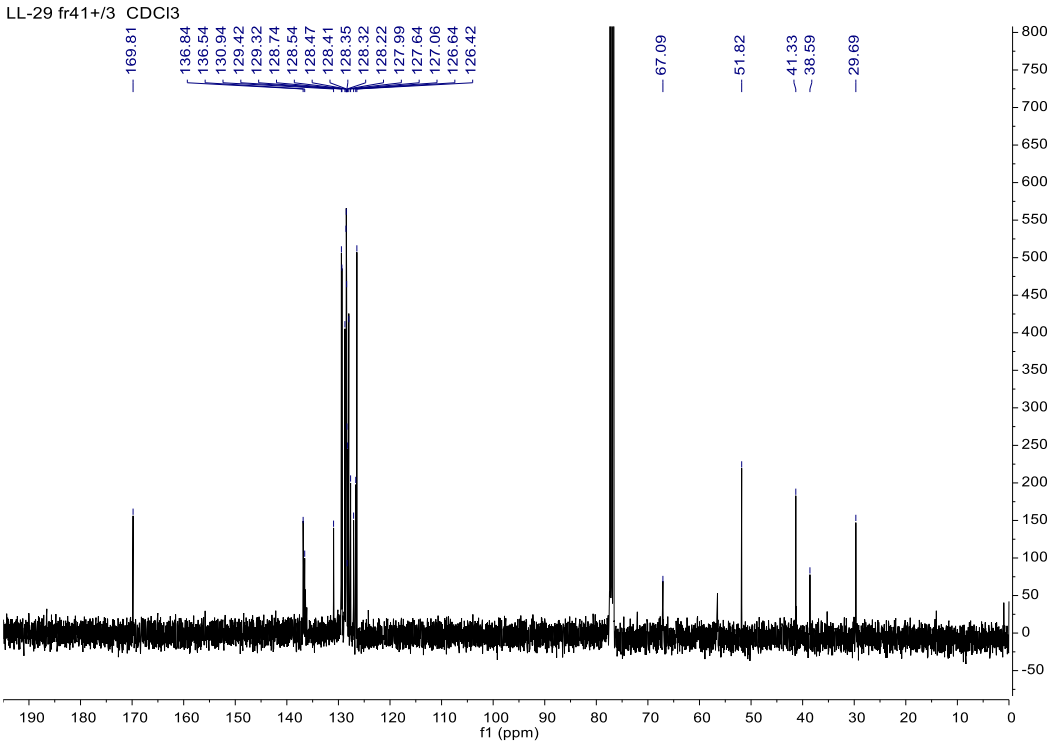
[13]C NMR spectrum of compound **4**



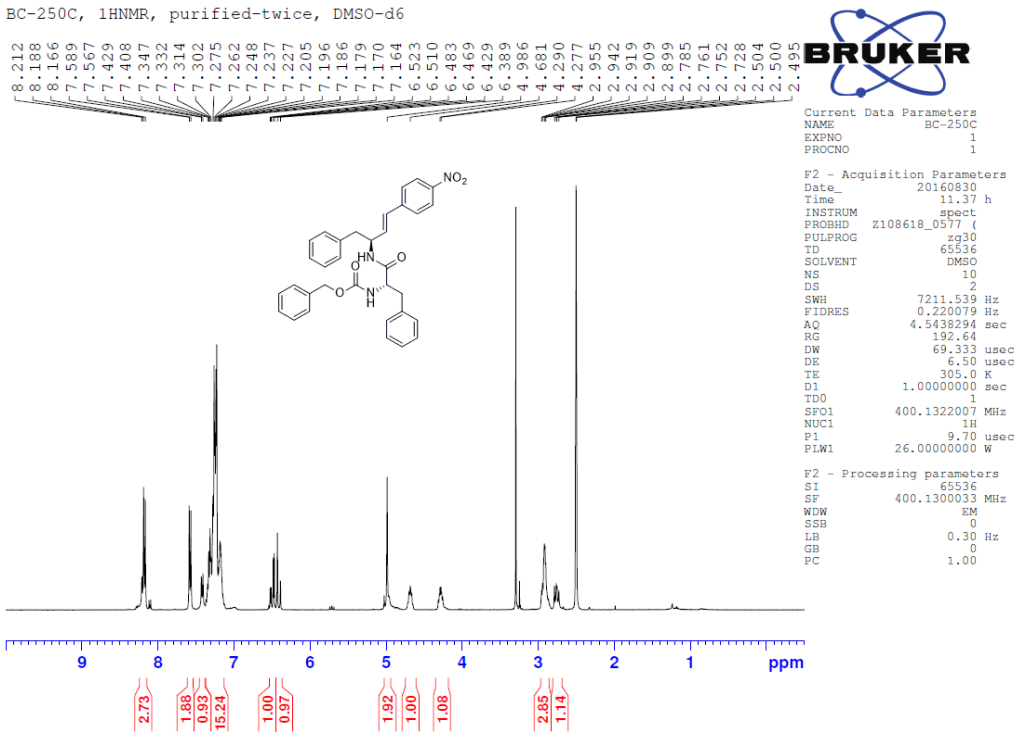
^{1}H NMR spectrum of compound **5**



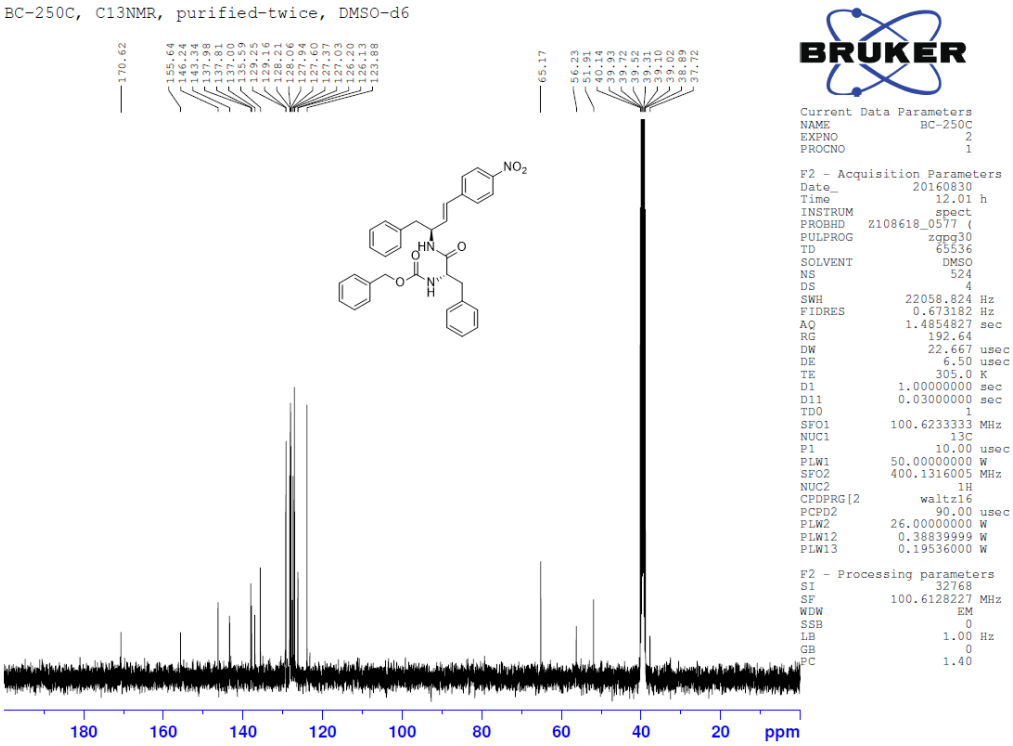
^{13}C NMR spectrum of compound **5**



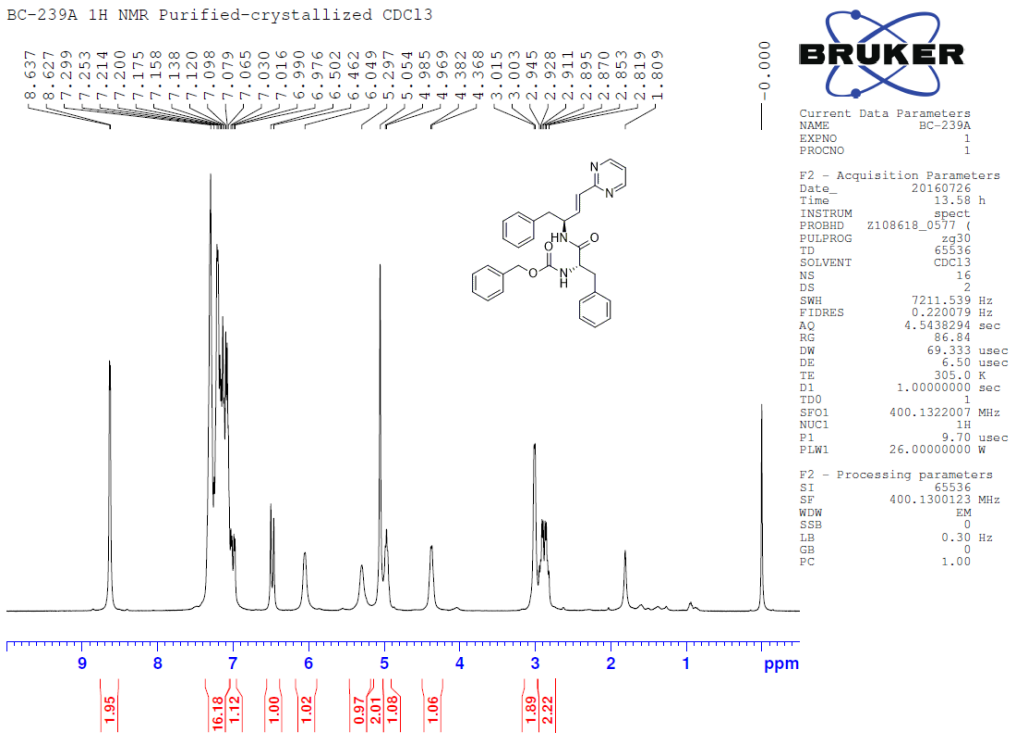
^{1}H NMR spectrum of compound **6**



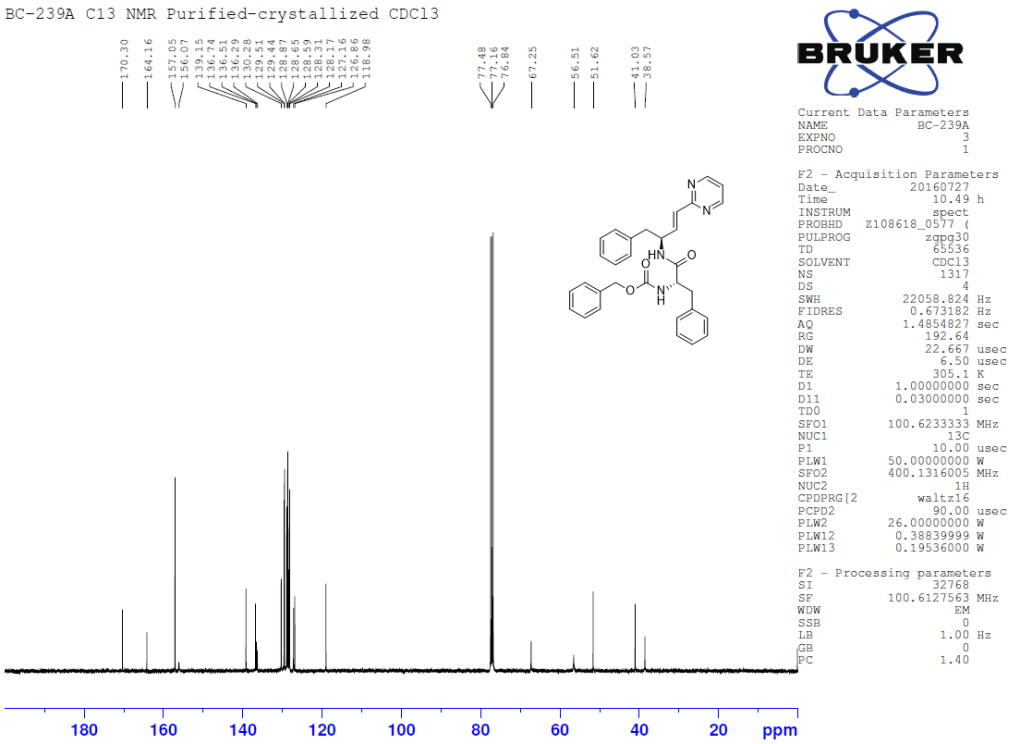
^{13}C NMR spectrum of compound **6**



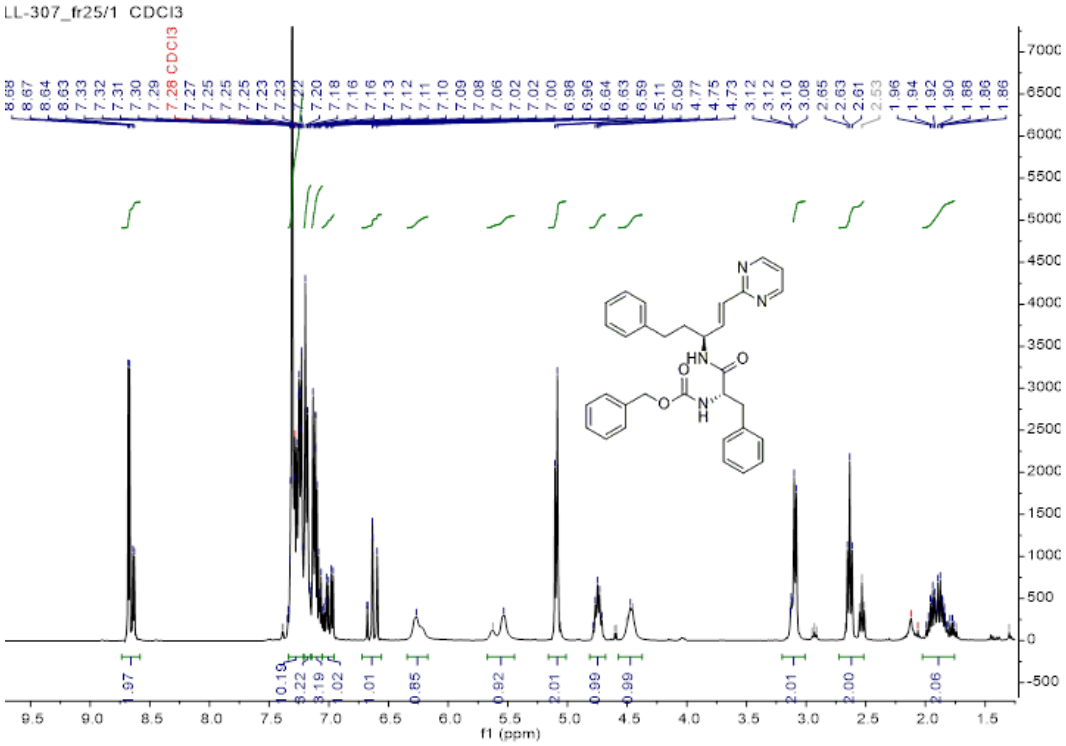
[1]H NMR spectrum of compound **7**



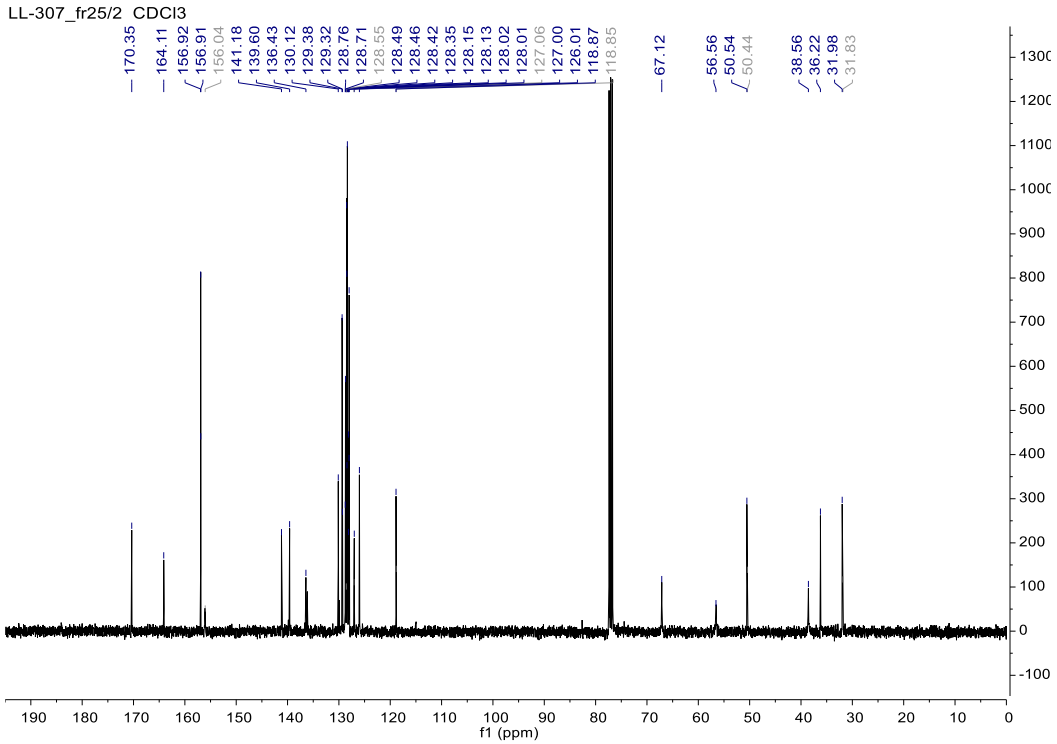
[13]C NMR spectrum of compound **7**



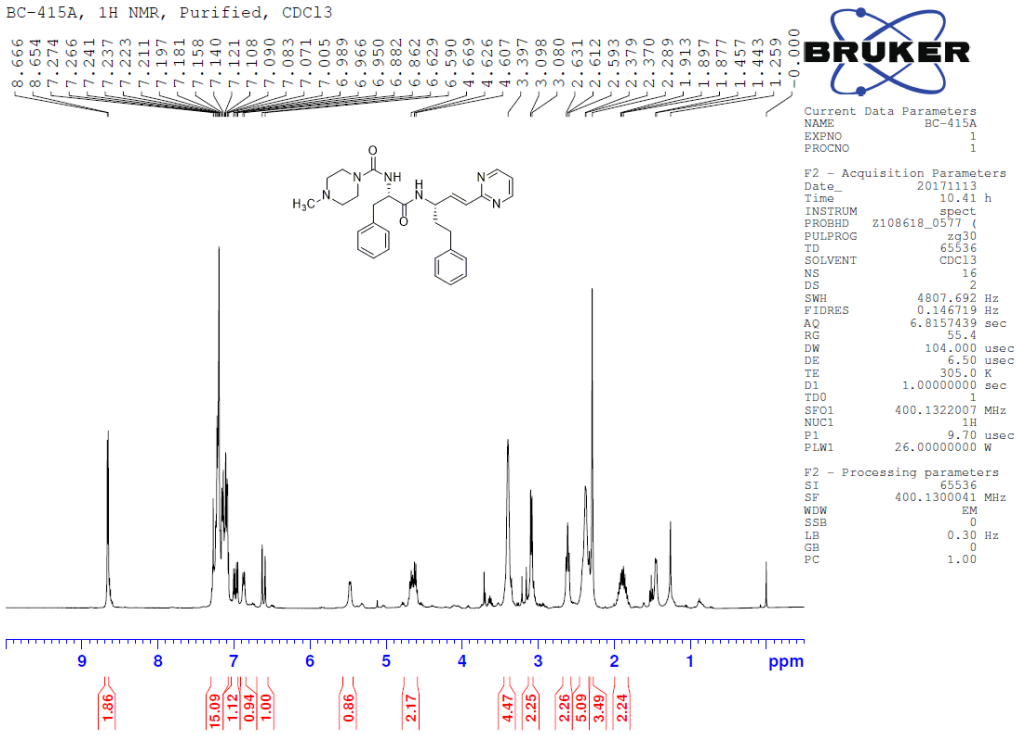
^{1}H NMR spectrum of compound **8**



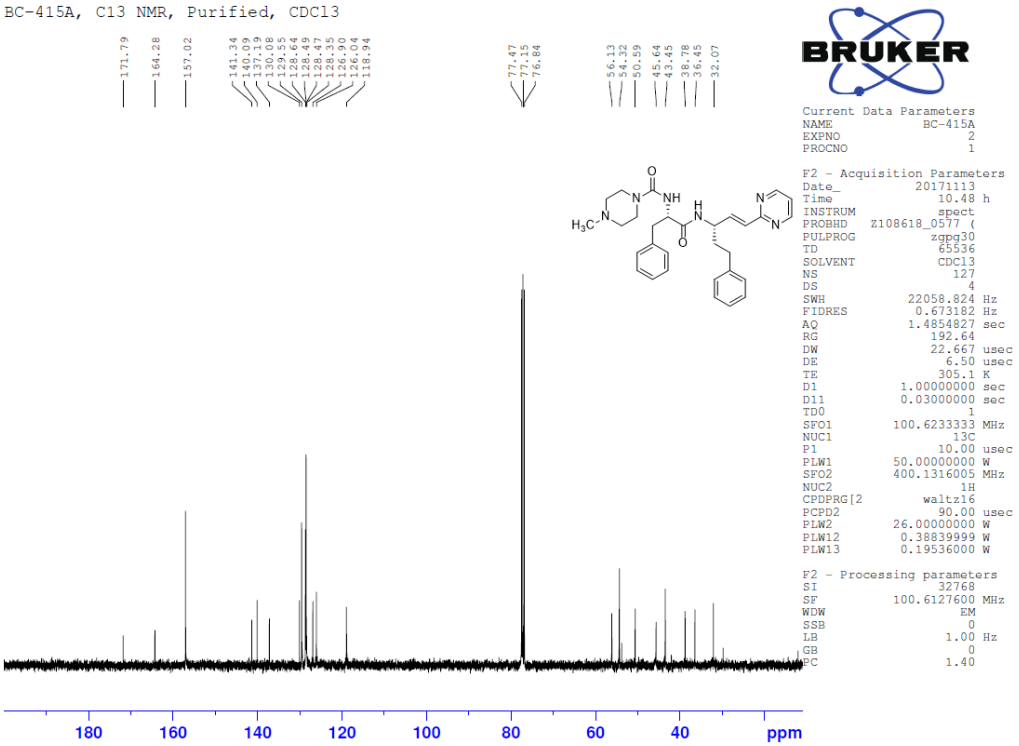
^{13}C NMR spectrum of compound **8**



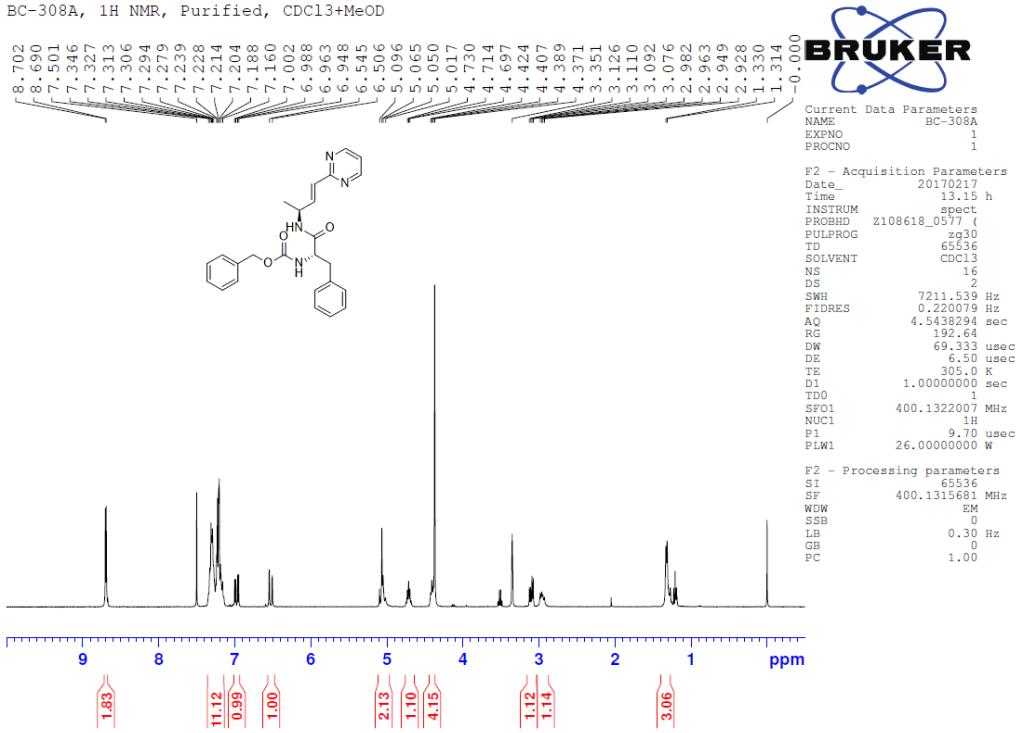
^{1}H NMR spectrum of compound **9**



^{13}C NMR spectrum of compound **9**



[1]H NMR spectrum of compound **10**



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PROCNO 1

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TD 65536
SOLVENT CDCl3
NS 16
DS 2
SWH 7211.539 Hz
FIDRES 0.220079 Hz
AQ 4.5438294 sec
RG 192.64
DW 69.333 usec
DE 6.50 usec
TE 305.0 K
D1 1.00000000 sec
TD0 1
SFO1 400.1322007 MHz
NUC1 1H
P1 9.70 usec
PLW1 26.00000000 W

F2 - Processing parameters
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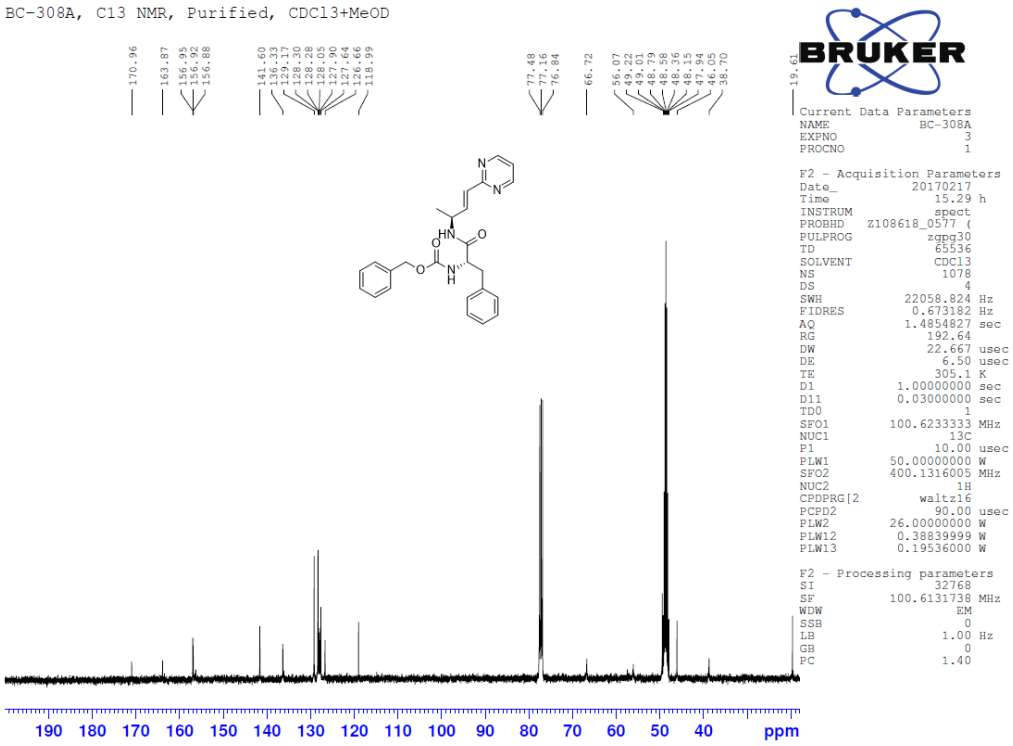
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

[13]C NMR spectrum of compound **10**



Current Data Parameters
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EXPNO 3
PROCNO 1

F2 - Acquisition Parameters
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TD 65536
SOLVENT CDCl3
NS 1078
DS 4
SWH 22058.824 Hz
FIDRES 0.673182 Hz
AQ 1.4854827 sec
RG 192.64
DW 22.667 usec
DE 6.50 usec
TE 305.1 K
D1 1.00000000 sec
D11 0.03000000 sec
TD0 1
SFO1 100.6233333 MHz
NUC1 13C
P1 10.00 usec
PLW1 50.00000000 W
SFO2 400.1316005 MHz
NUC2 1H
CPDPRG[2 waltz16
PCPD2 90.00 usec
PLW2 26.00000000 W
PLW12 0.38839999 W
PLW13 0.19536000 W

F2 - Processing parameters
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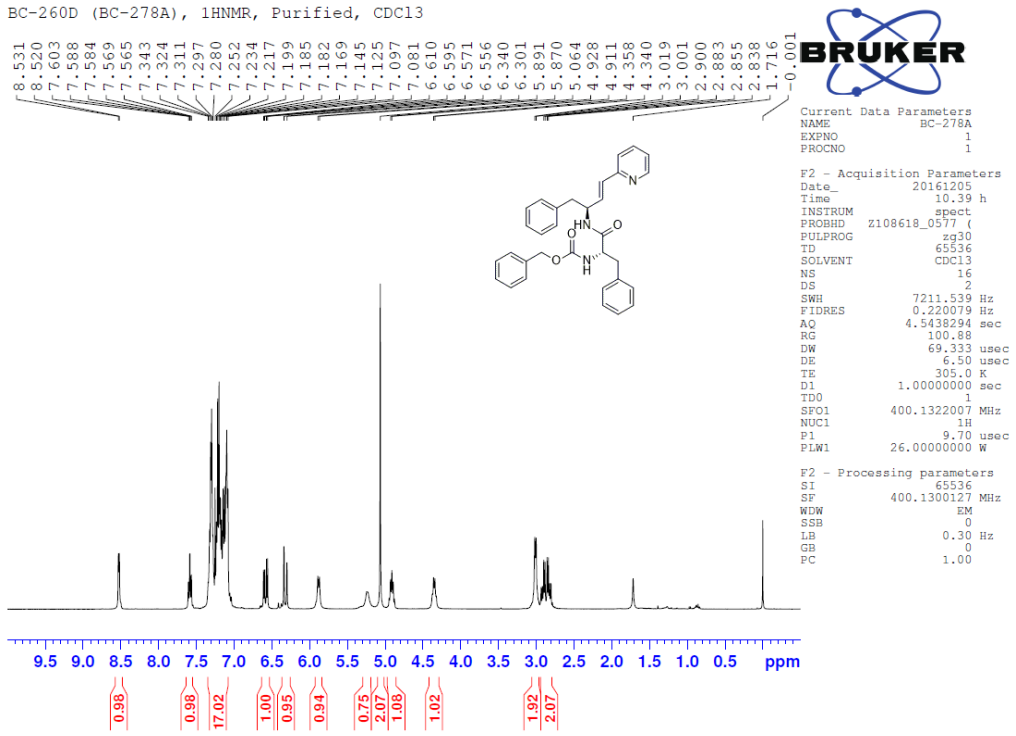
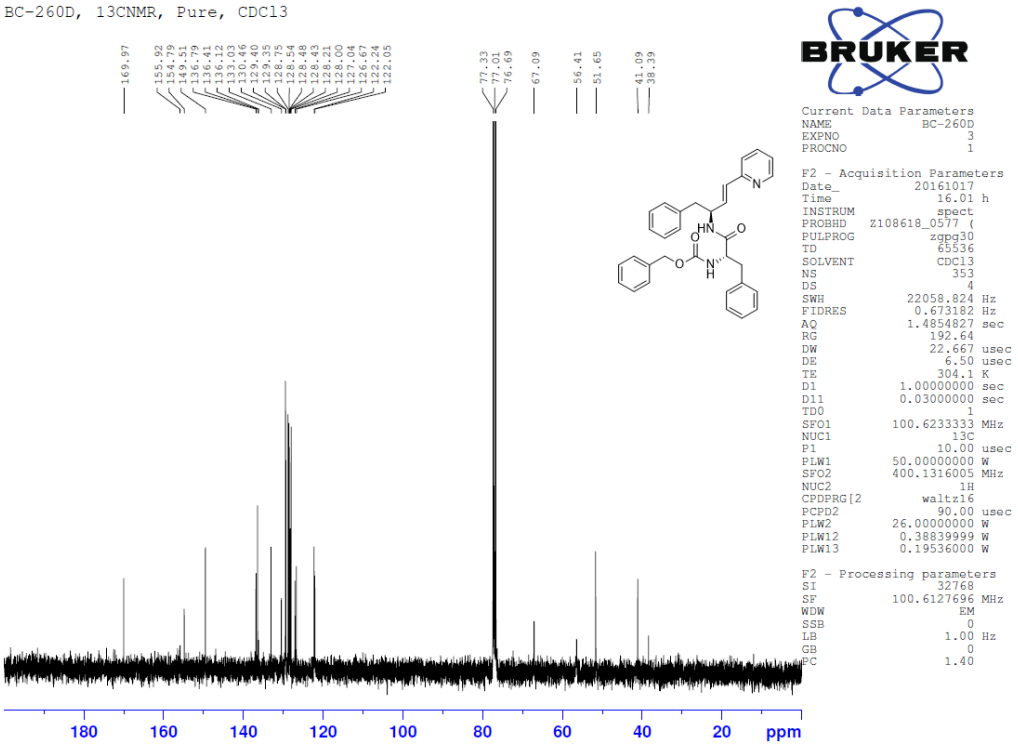
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40

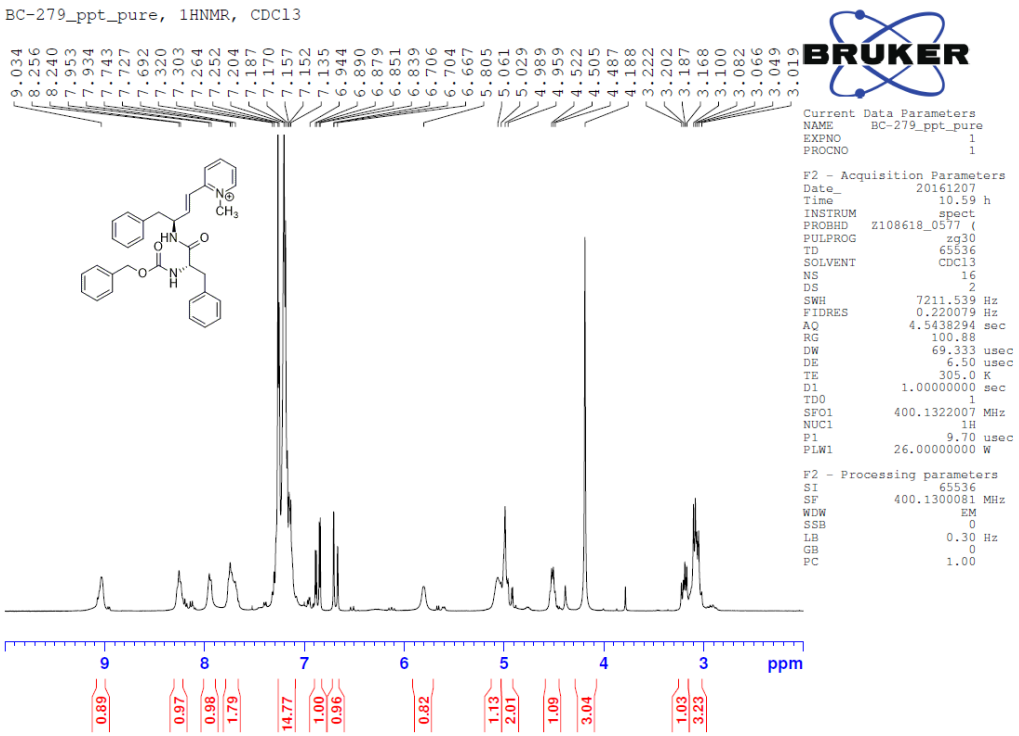
^{1}H NMR spectrum of compound **11**



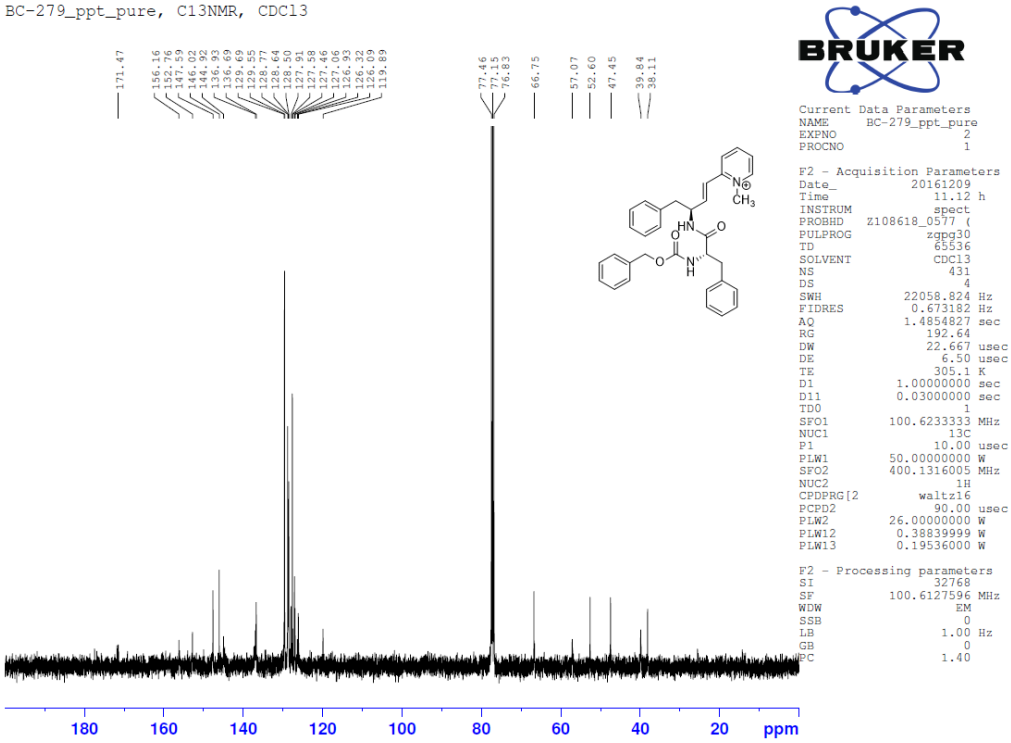
^{13}C NMR spectrum of compound **11**



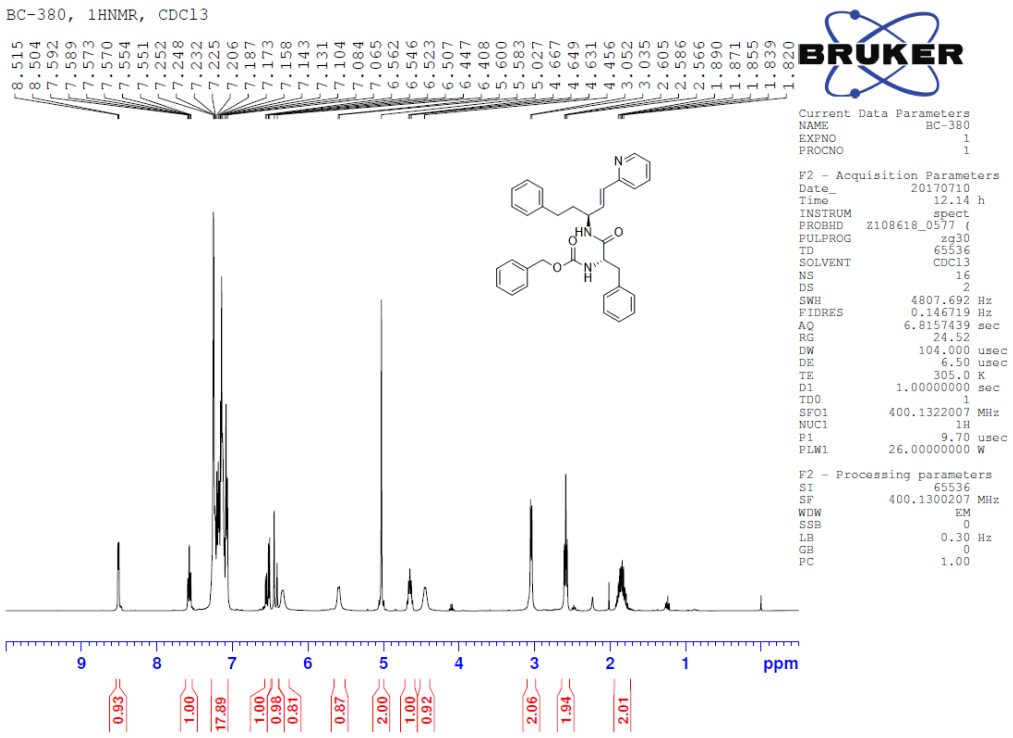
[1]H NMR spectrum of compound **12**



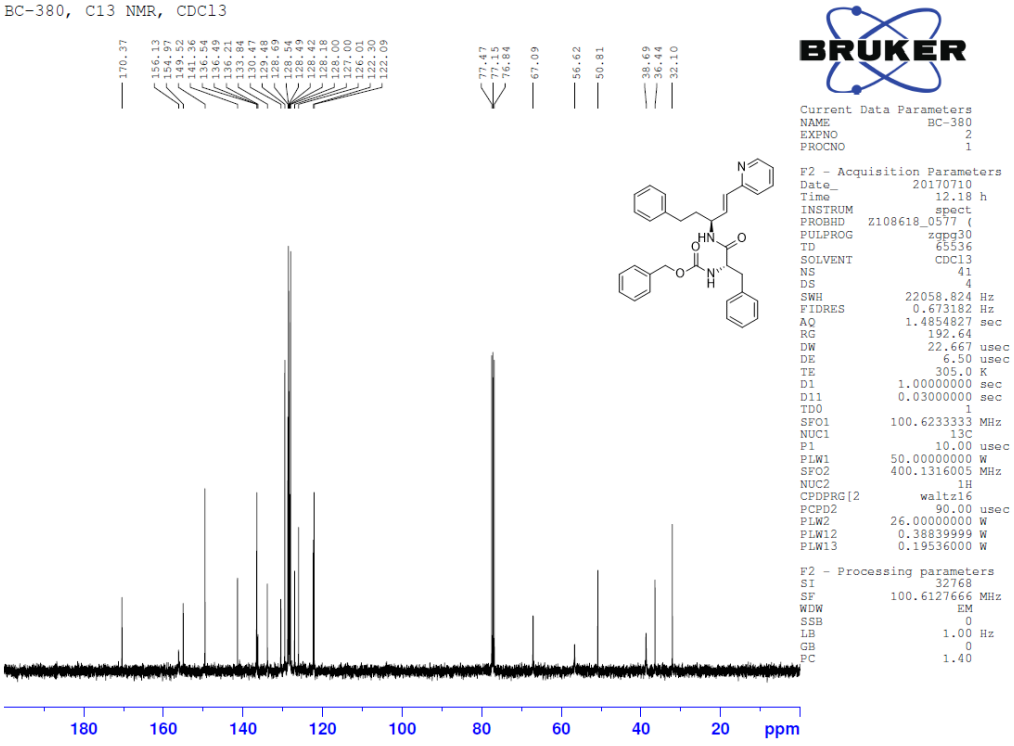
[13]C NMR spectrum of compound **12**



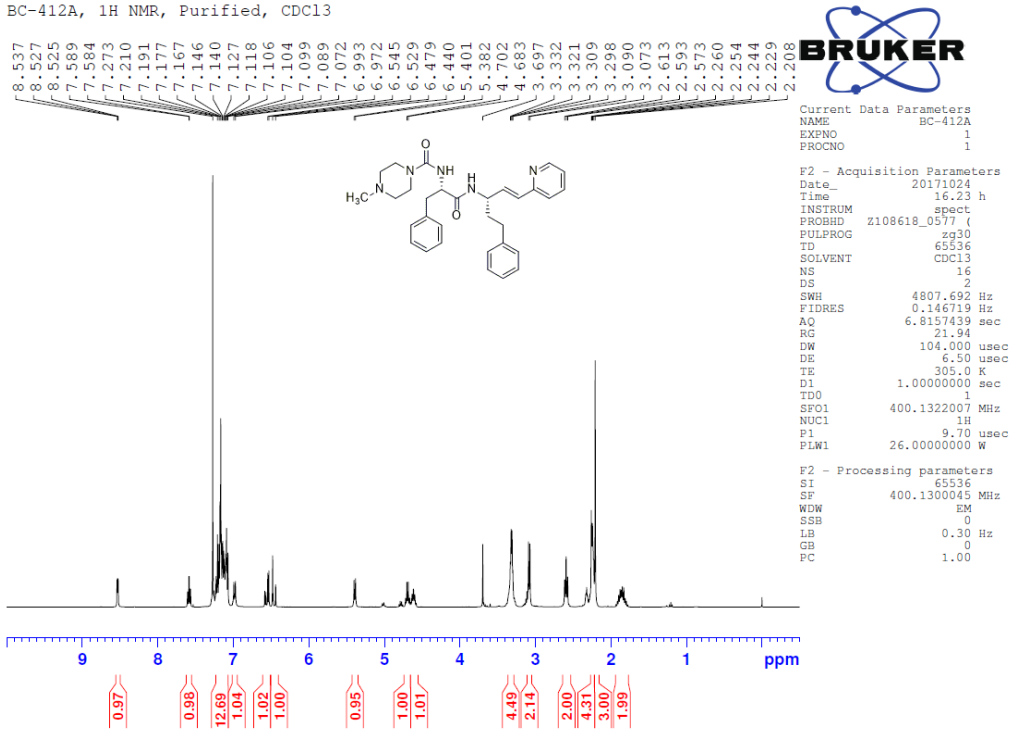
[1]H NMR spectrum of compound **13**



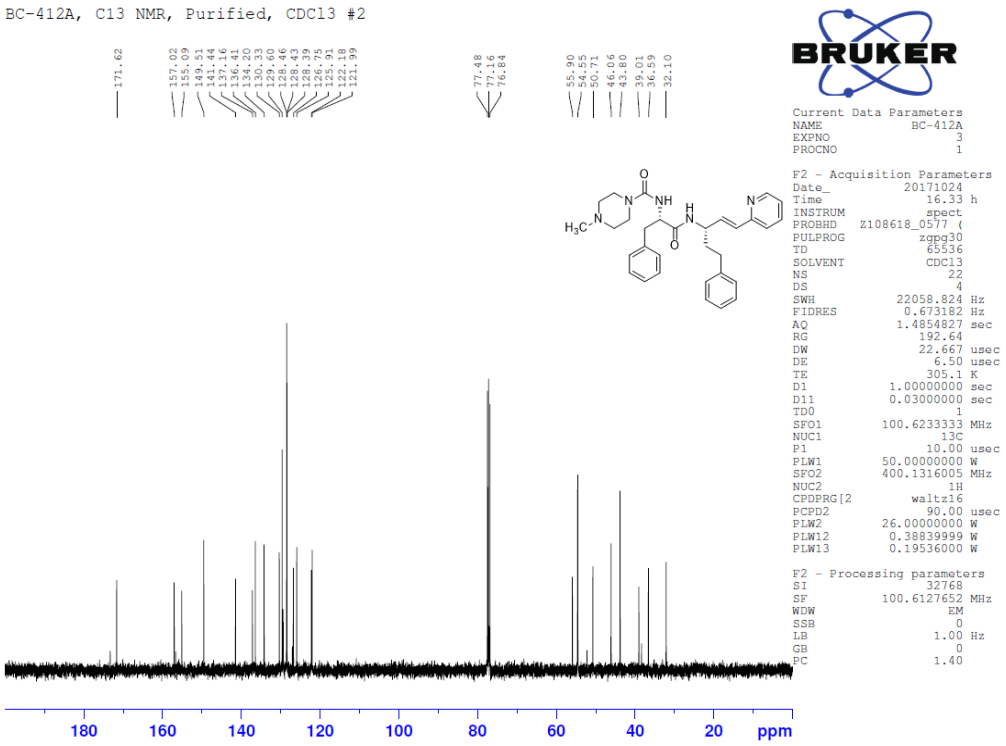
[13]C NMR spectrum of compound **13**



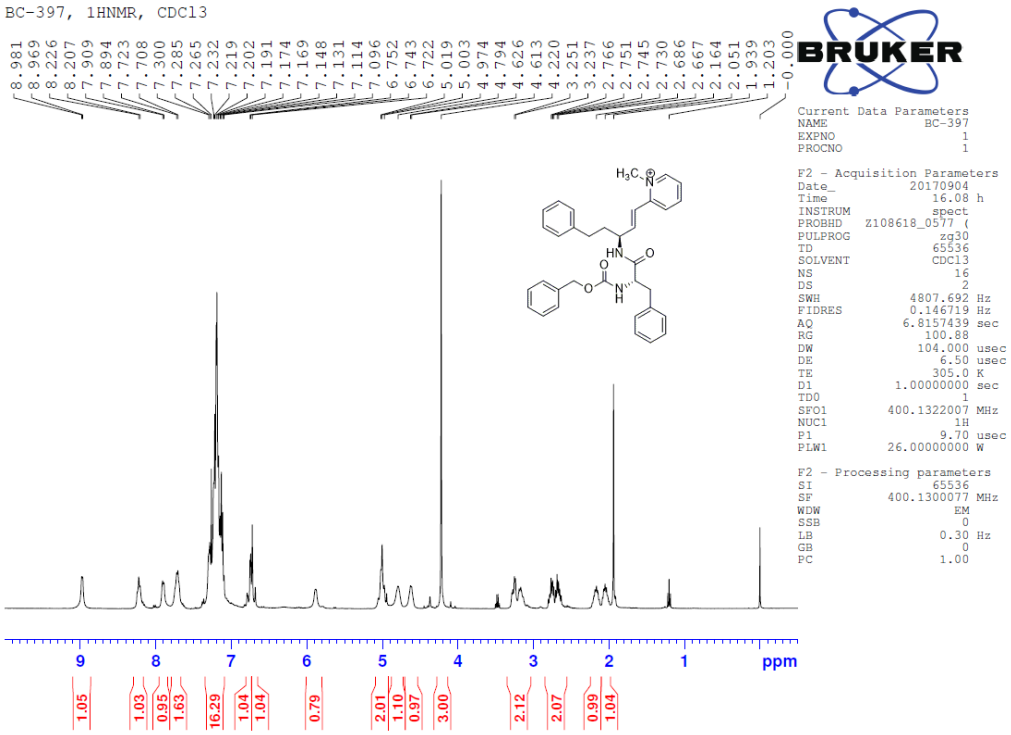
^{1}H NMR spectrum of compound **14**



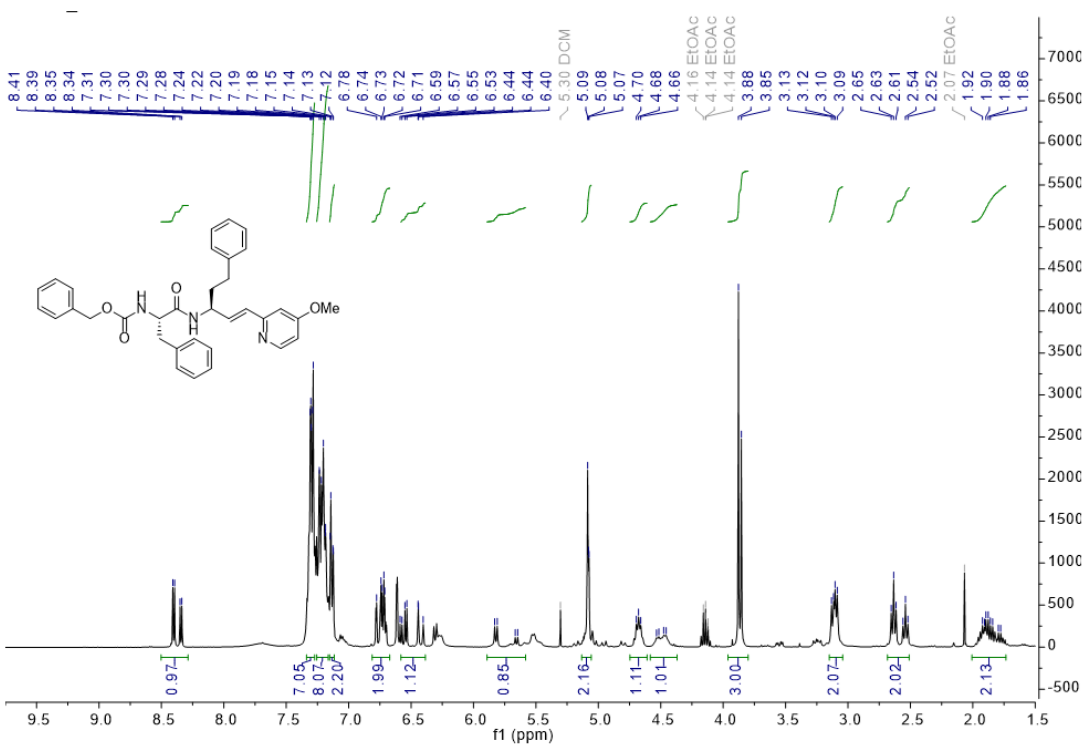
^{13}C NMR spectrum of compound **14**

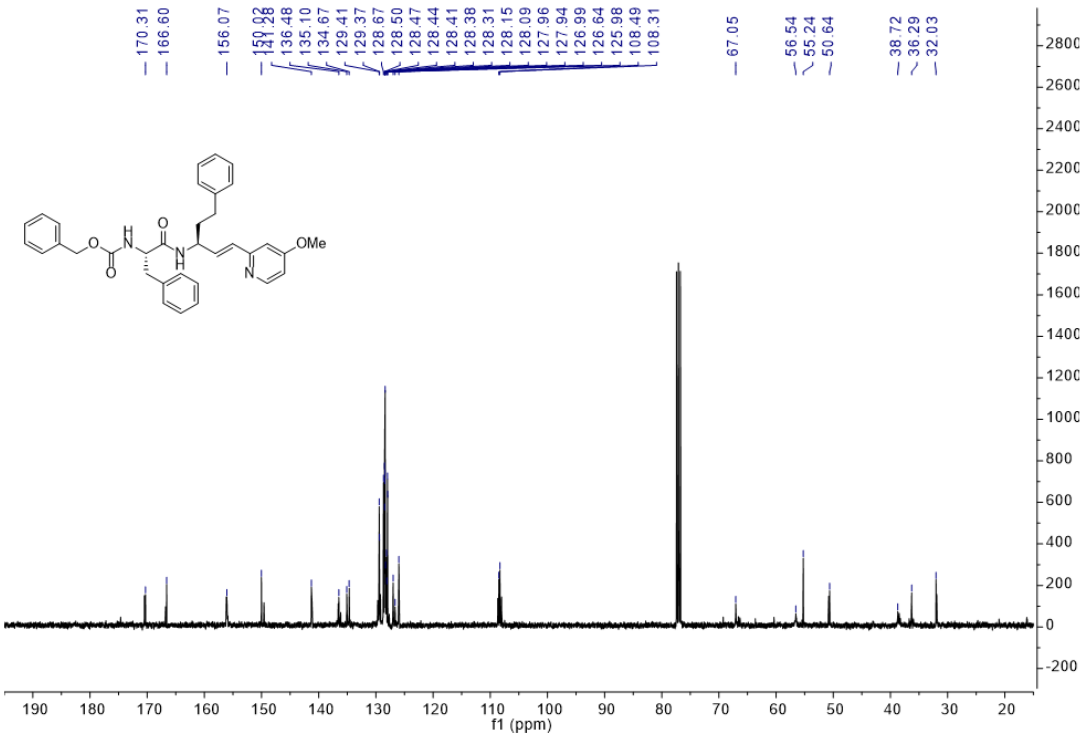


[1]H NMR spectrum of compound **15**

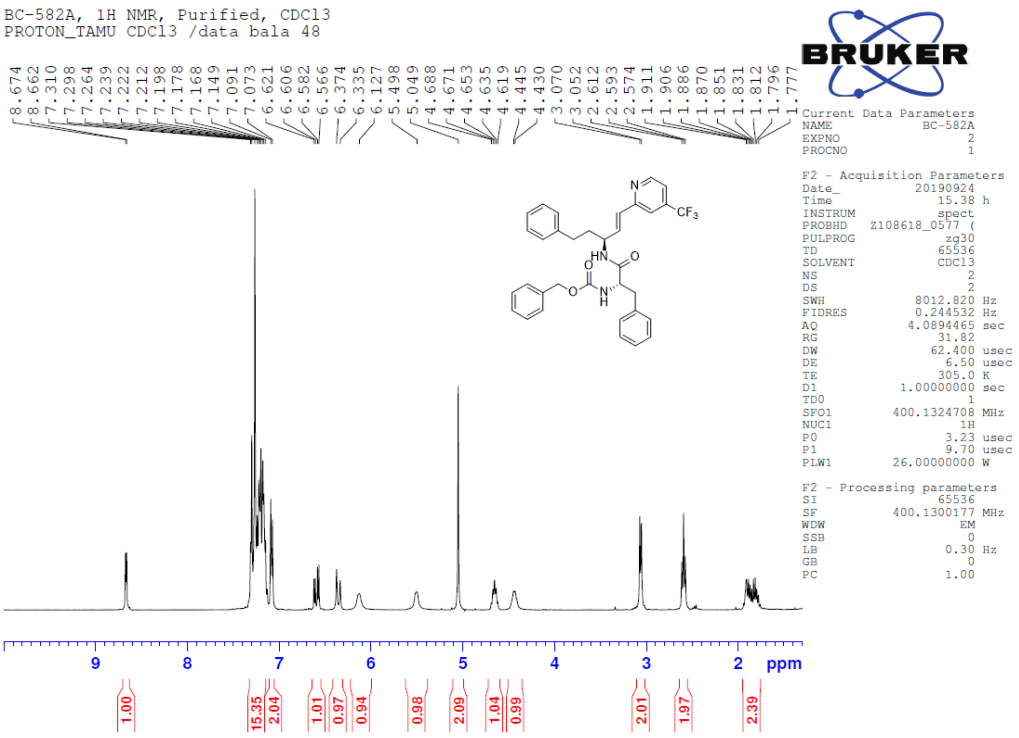


^{1}H NMR spectrum of compound **16**

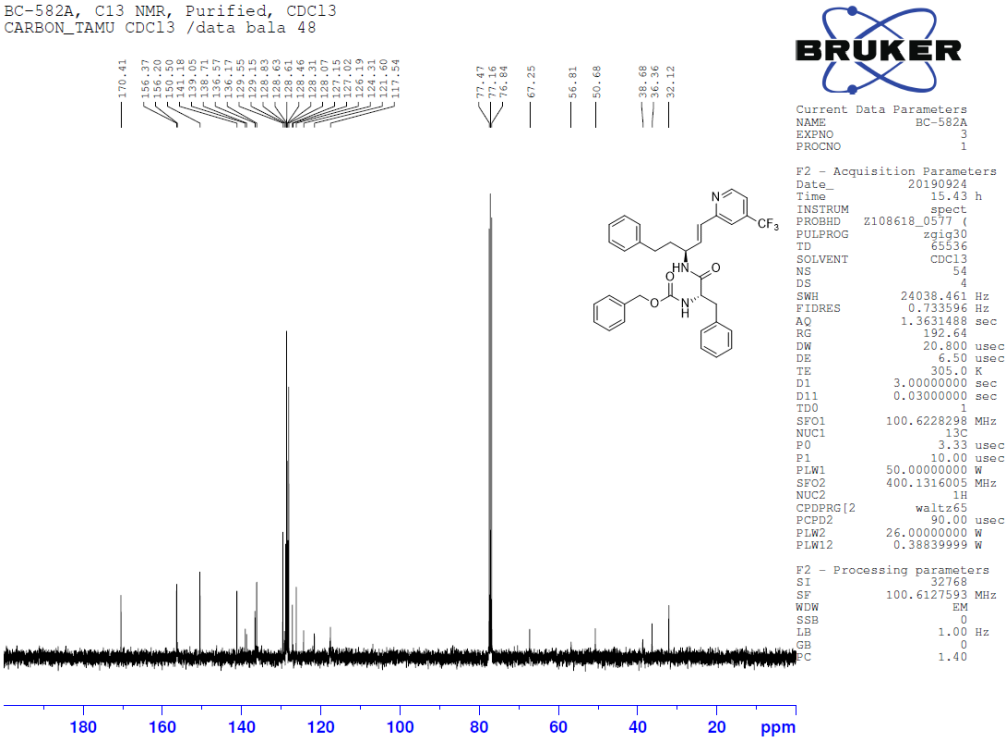




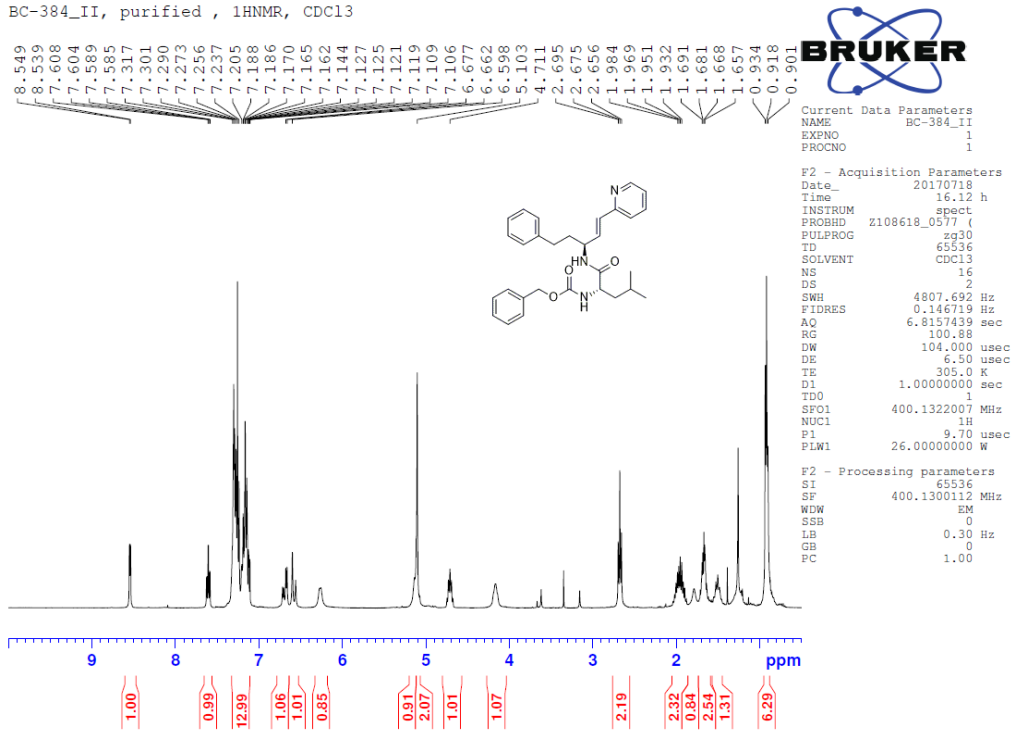
^{1}H NMR spectrum of compound **17**



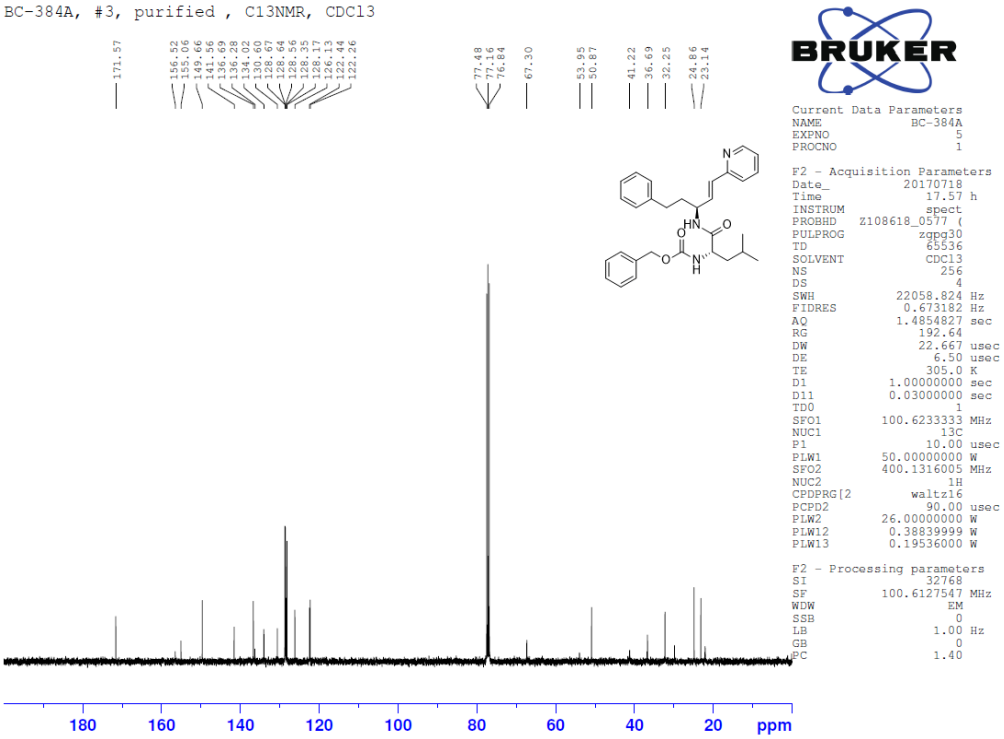
^{13}C NMR spectrum of compound **17**



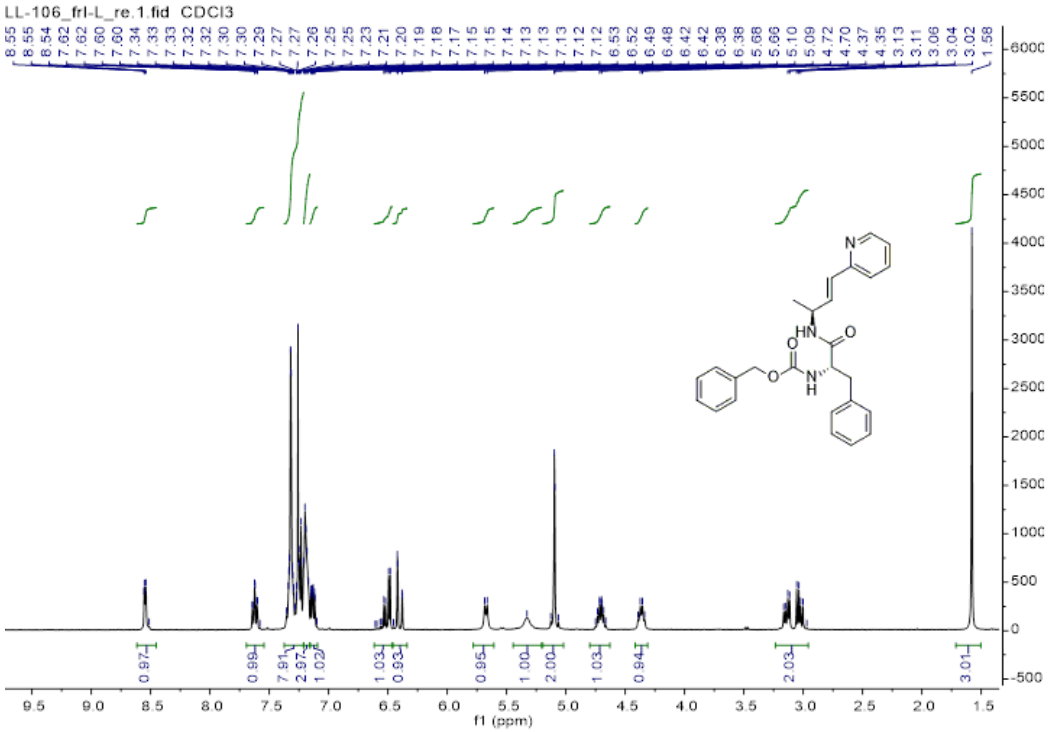
[1]H NMR spectrum of compound **18**



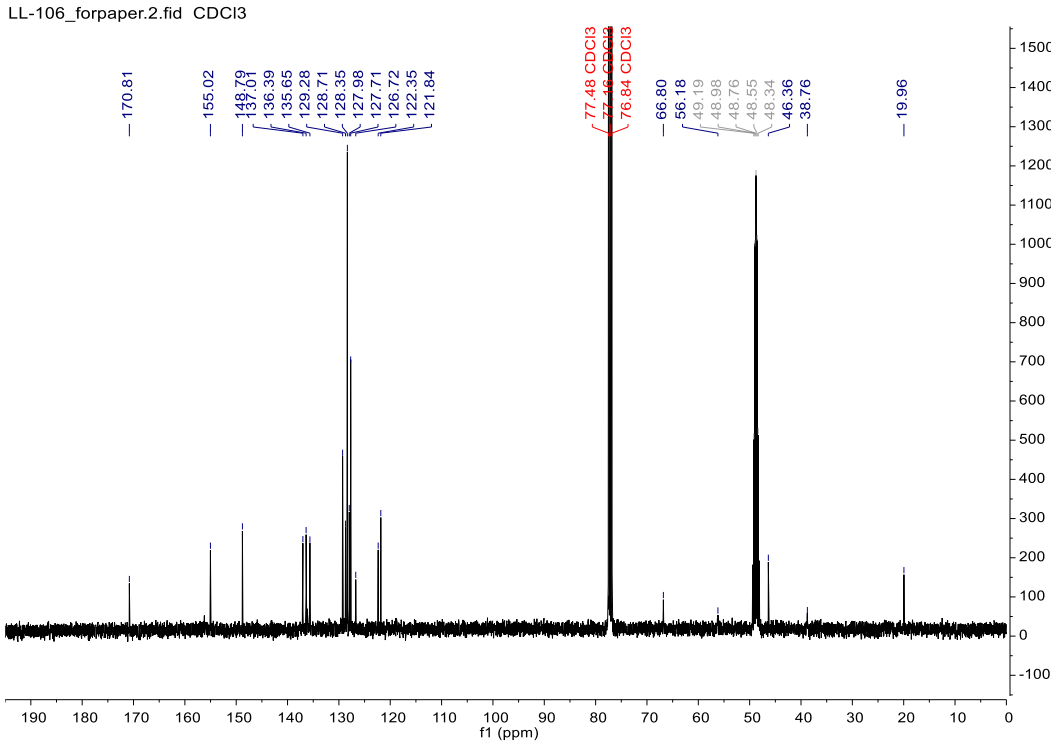
[13]C NMR spectrum of compound **18**



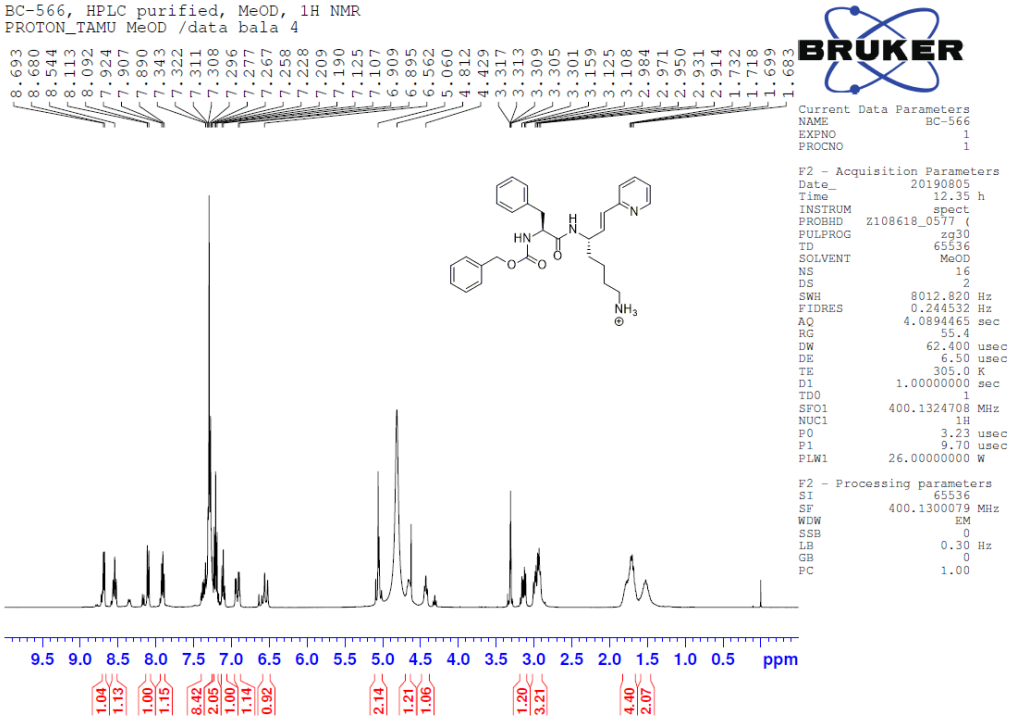
^{1}H NMR spectrum of compound **19**



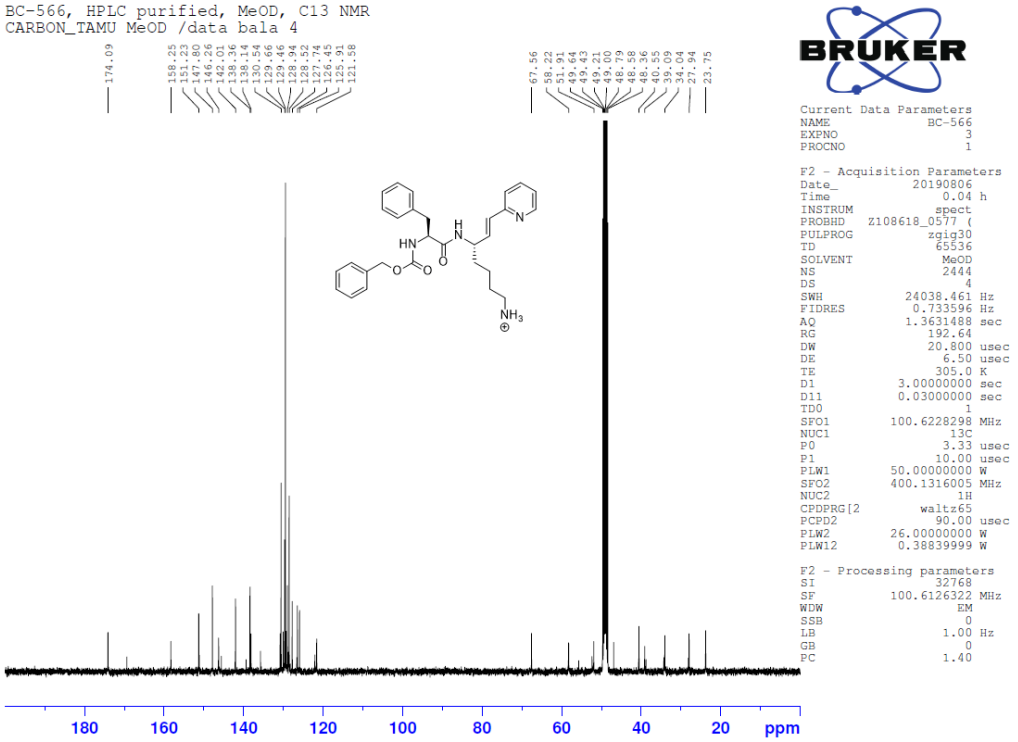
^{13}C NMR spectrum of compound **19**



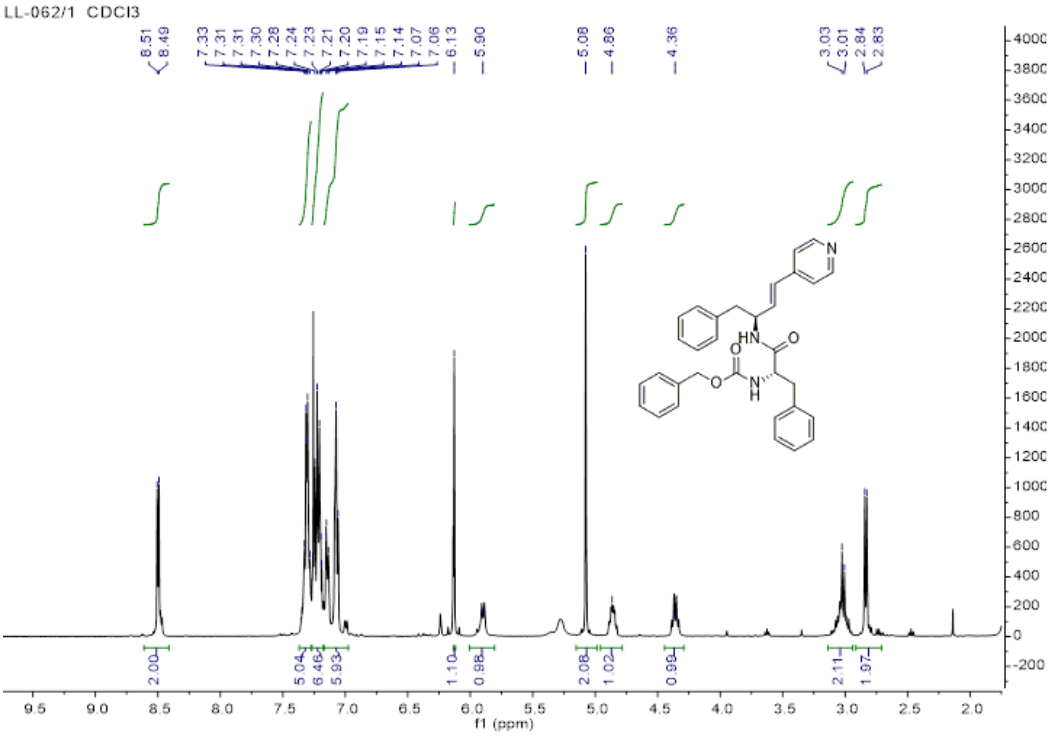
[1]H NMR spectrum of compound **20**



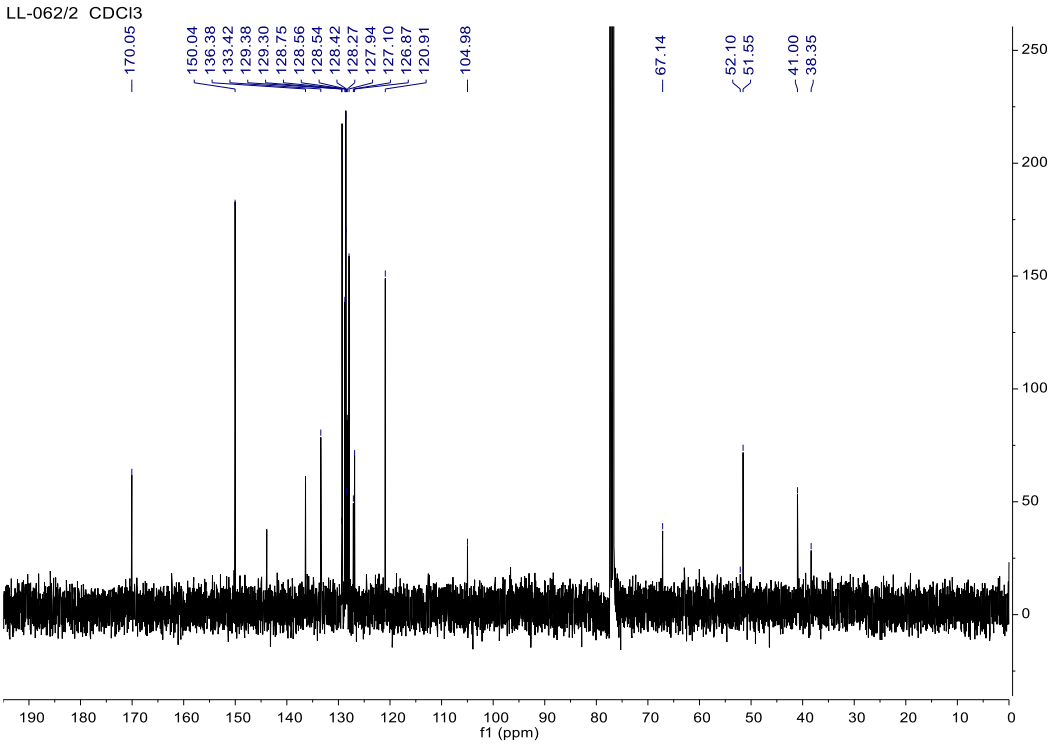
[13]C NMR spectrum of compound **20**



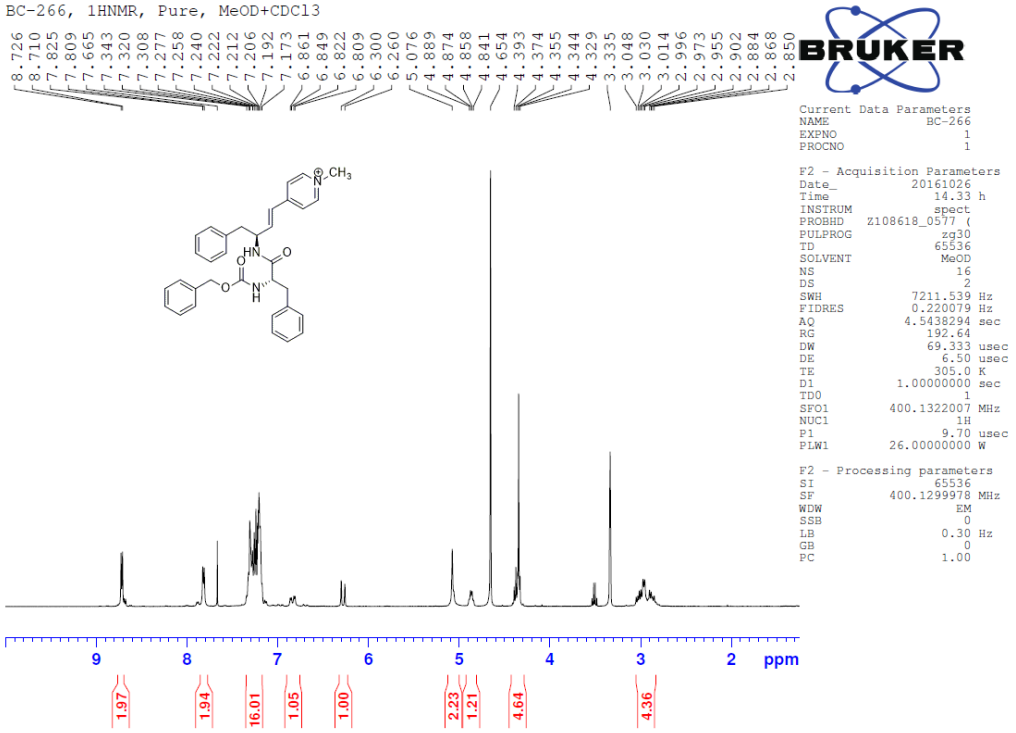
^{1}H NMR spectrum of compound **21**



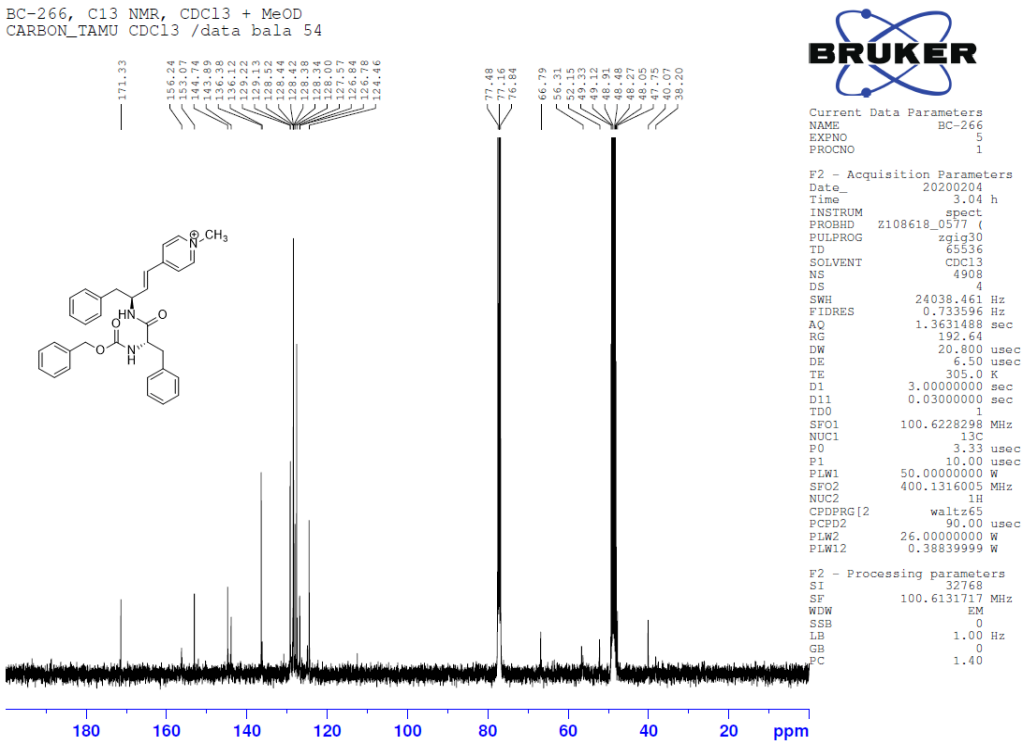
^{13}C NMR spectrum of compound **21**



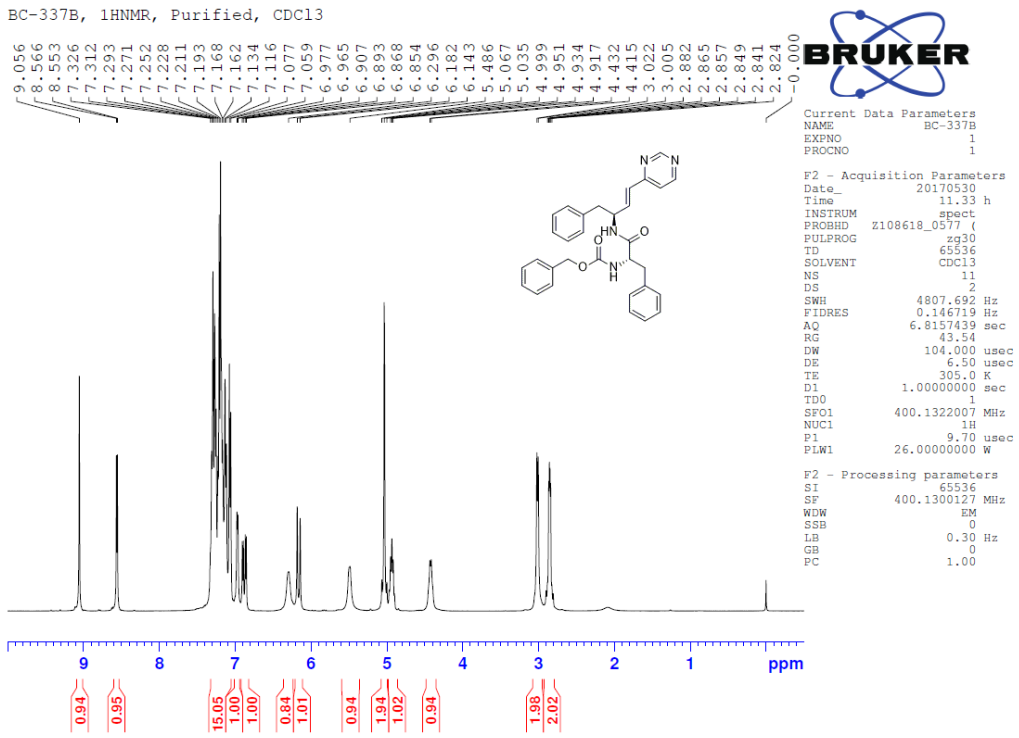
^{1}H NMR spectrum of compound **22**



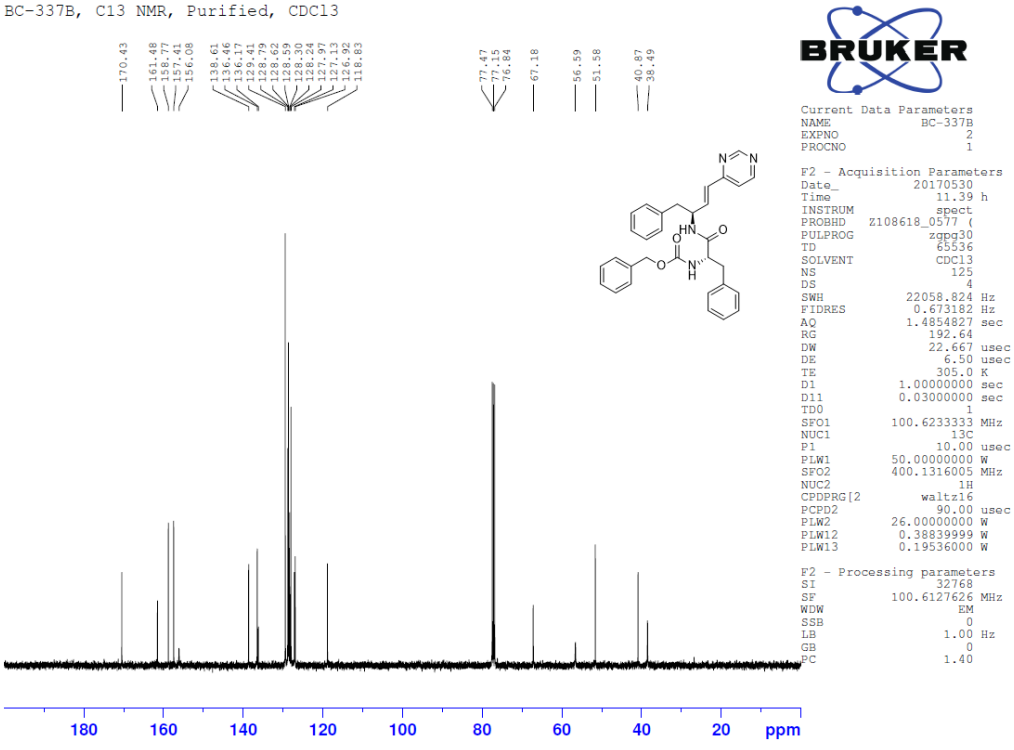
^{13}C NMR spectrum of compound **22**



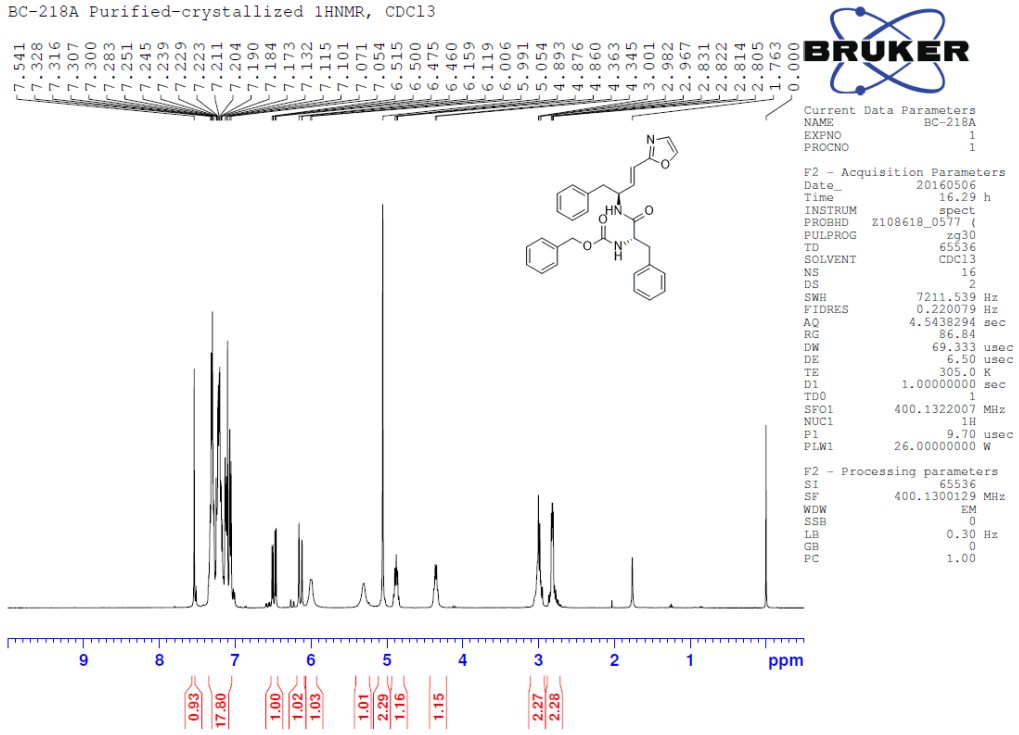
[1]H NMR spectrum of compound **23**



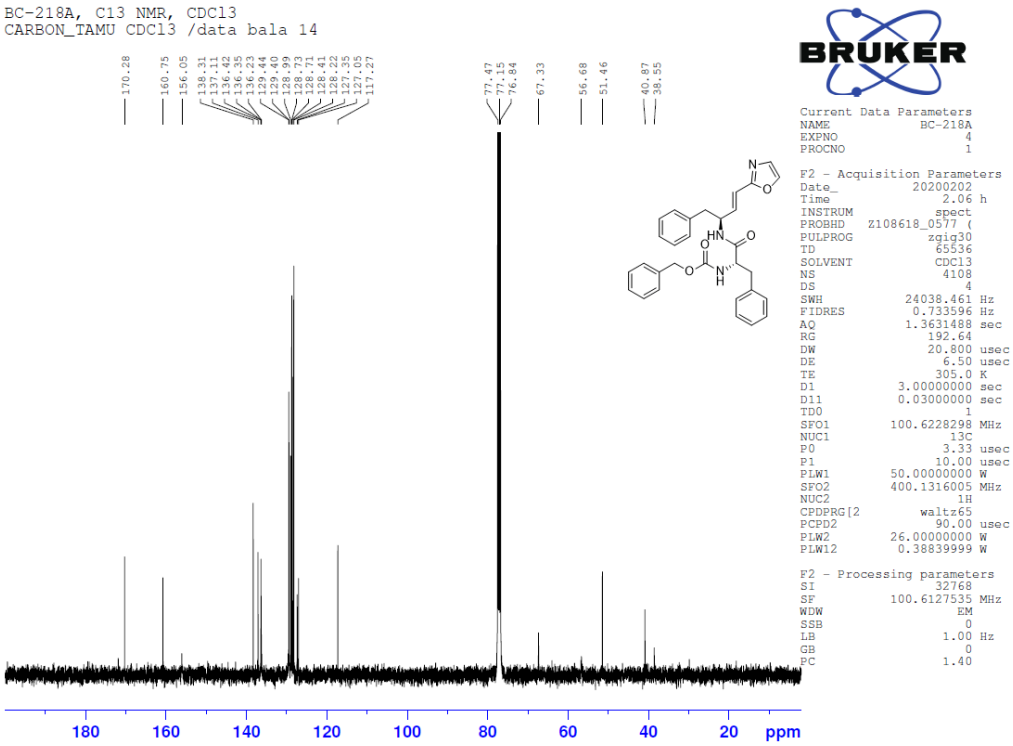
[13]C NMR spectrum of compound **23**



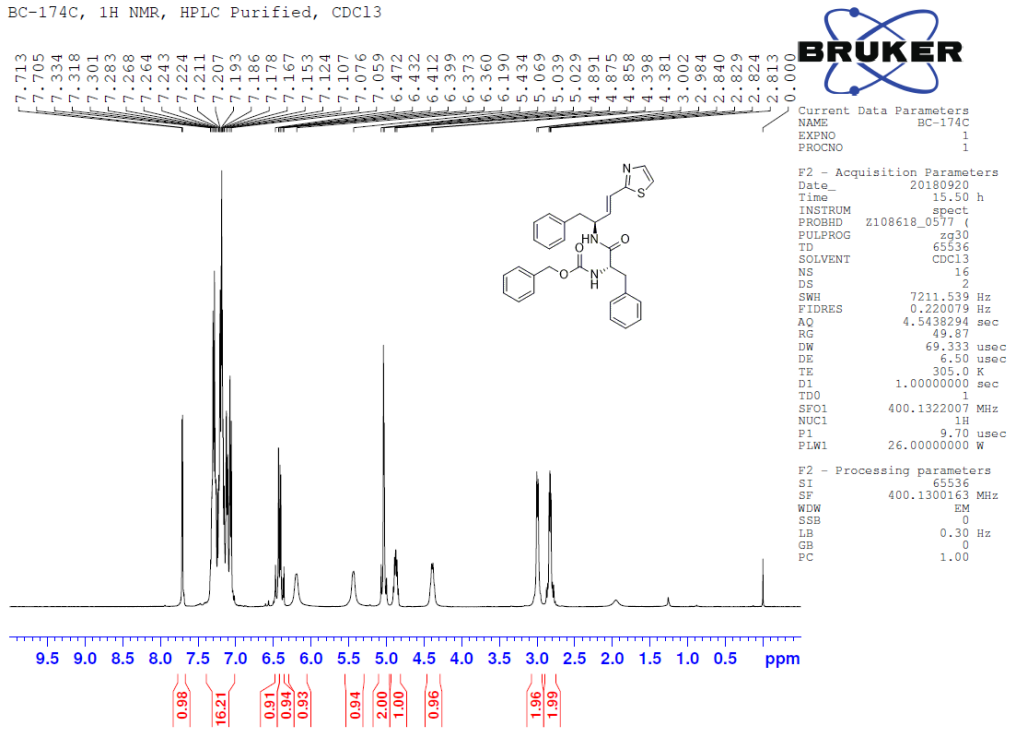
[1]H NMR spectrum of compound **24**



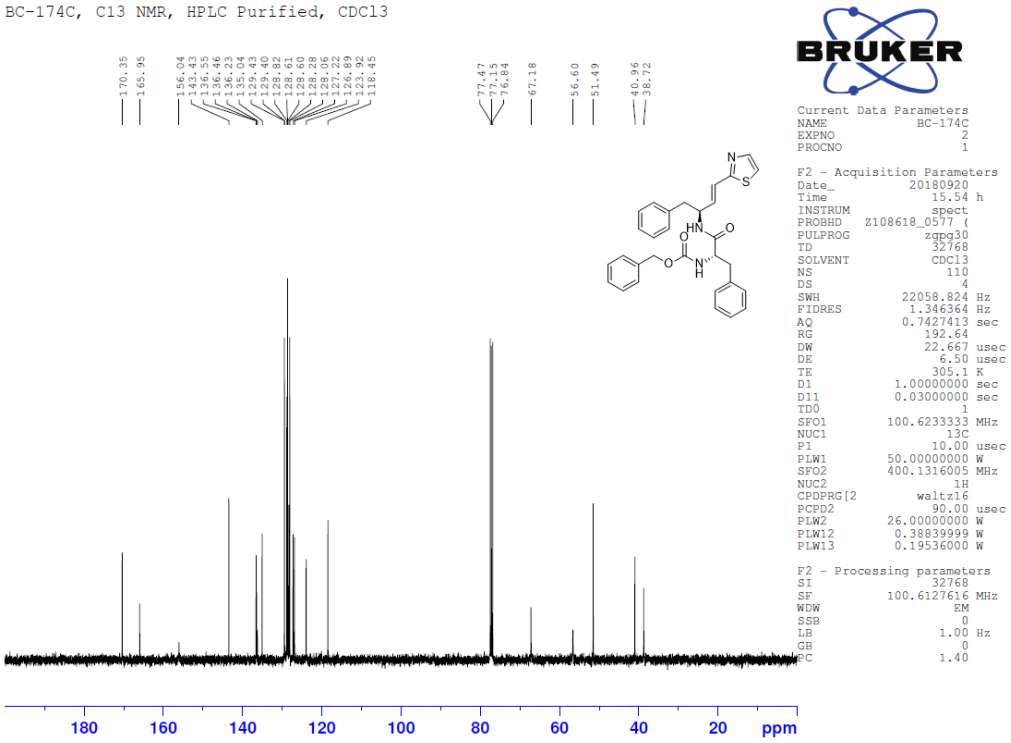
[13]C NMR spectrum of compound **24**



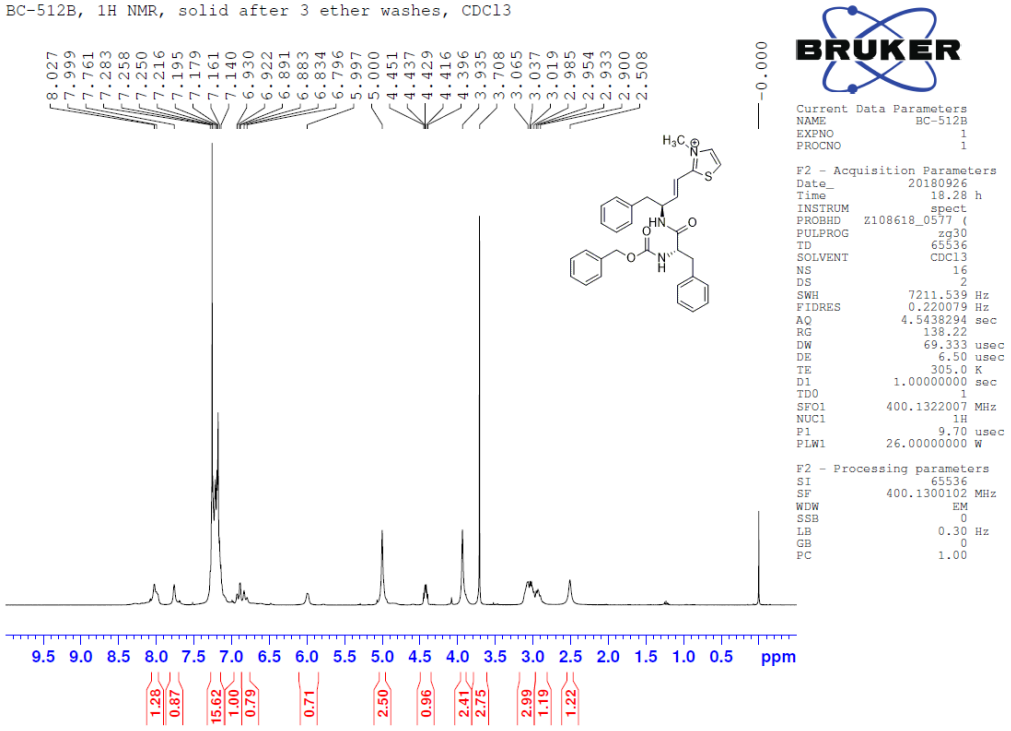
[1]H NMR spectrum of compound **25**



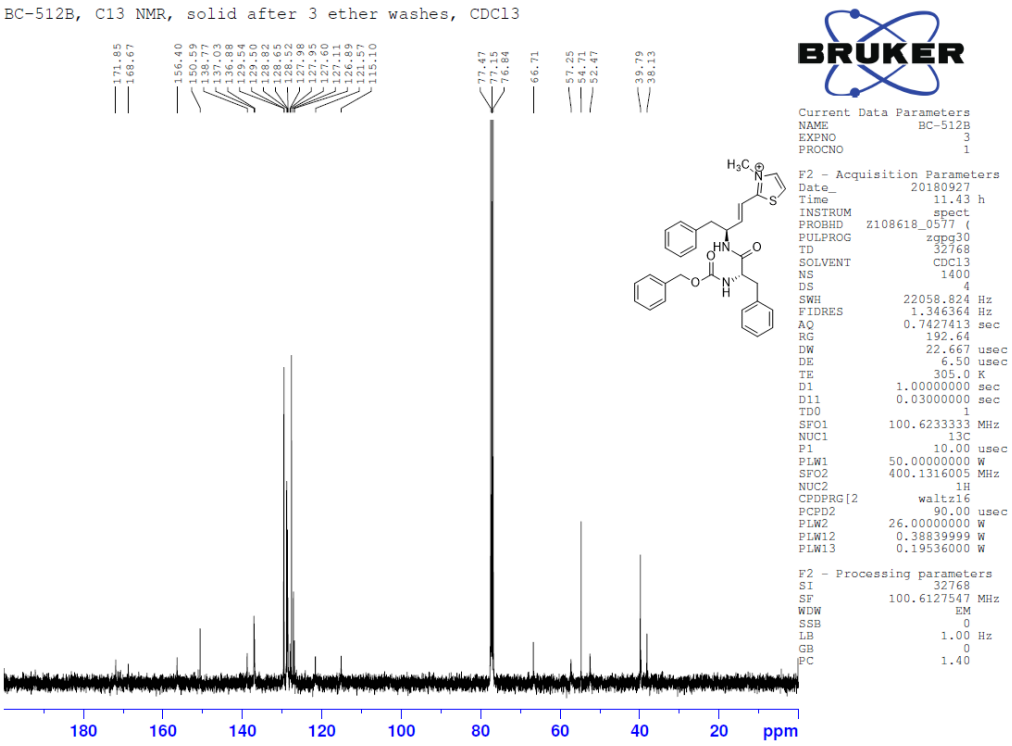
[13]C NMR spectrum of compound **25**



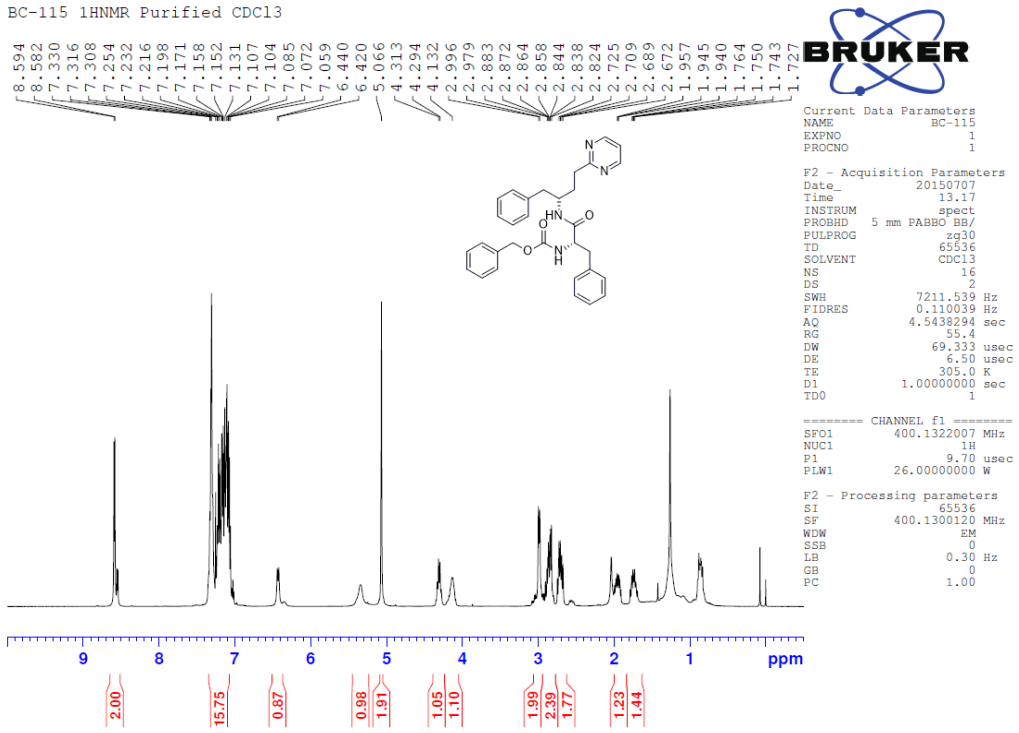
[1]H NMR spectrum of compound **26**



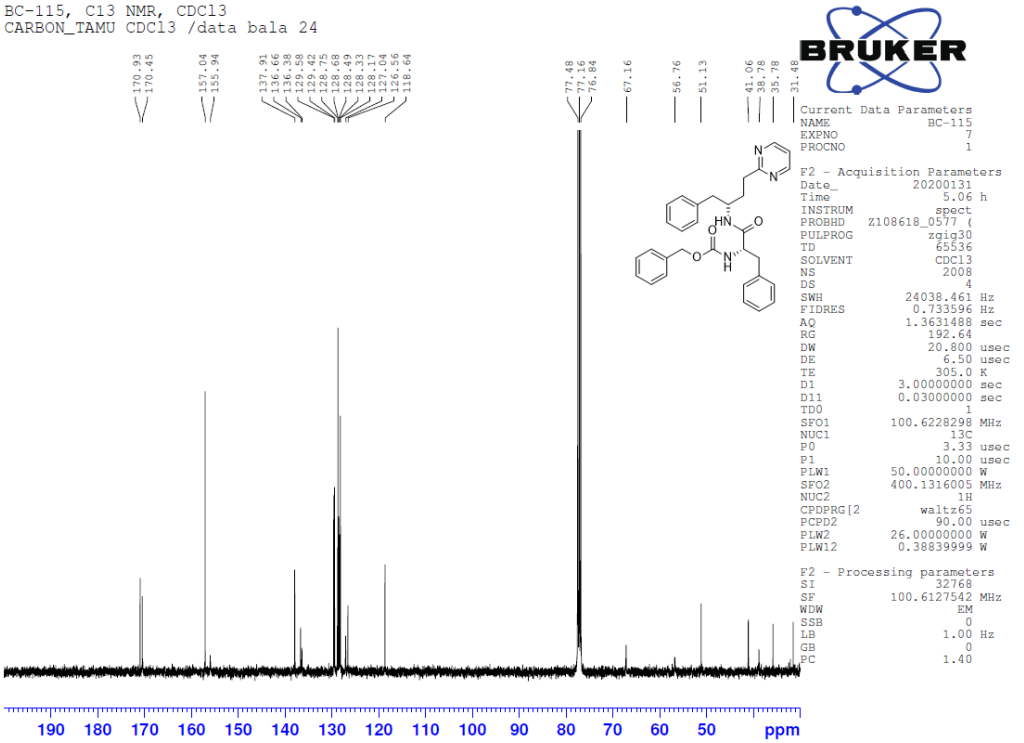
[13]C NMR spectrum of compound **26**



^{1}H NMR spectrum of compound **27**



^{13}C NMR spectrum of compound **27**



Part 8. Unpublished Data

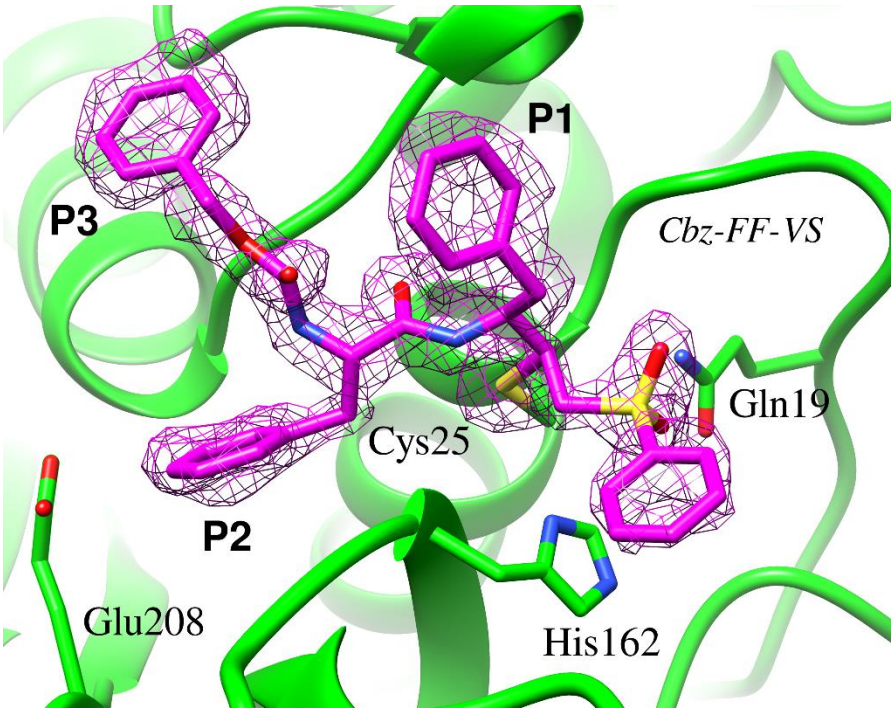


Figure S4. Crystal structure of cruzain bound to compound **1**. A covalent bond is evidently formed between the sulfur of Cys_{25} and the β-carbon of the former olefin bond (Tang, *et al.*).