Electronic Supplementary Information

Acyl azide generation and amide bond formation in continuous-flow for the synthesis of peptides

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1. Materials and Methods

1.1 Solvents and Reagents

All solvents and reagents were obtained from standard commercial vendors and were used without any further purification.

1.2 NMR Spectra

^{1}H NMR spectra were recorded on a Bruker 300 MHz instrument. ^{13}C NMR spectra were recorded on the 300 MHz instrument at 75 MHz. Chemical shifts (δ) are expressed in ppm downfield from TMS as internal standard. The letters s, d, dd, t, q, and m are used to indicate singlet, doublet, doublet of doublets, triplet, quadruplet, and multiplet.

1.3 HPLC Analysis

HPLC analysis of the final products was performed on a Shimadzu HPLC system (DGU-20A5 degasser, SPD-M20A UV-VIS detector, LC-20AD pumps, CTO-20A column oven). The system was equipped with a C18 reversed-phase analytical column (150 × 4.6 mm, particle size 5 μm) at 37 °C. Mobile phases: A (water/MeCN 90:10 (v/v) + 0.1% TFA) and B (MeCN + 0.1% TFA) at a combined flow rate of 1.5 mL/min were used. A gradient was applied, which commenced from 30% of solvent B increasing to 100% over a 10 min runtime.

1.4 Chiral HPLC Analysis

Epimerization was determined by using a Shimadzu HPLC system (DGU-14A degasser, SCL-10A VP system controller, SPD-10 UV-VIS detector, LC-20AT pumps) and a Chiralpak® AD-H chiral column (particle size of 5 μm, dimensions of 4.6 mm x 250 mm). An isocratic mixture of hexane and iPrOH was used as eluent. The pump flow rates were 0.4 mL/min and 0.1 mL/min for hexane and iPrOH respectively. To enable epimerization studies, batch reactions were conducted with the D-enantiomer as starting material.

2. Experimental Details

Table S1. Preliminary batch experiments for investigation of acyl hydrazide formation.[a]

Entry	$N_2H_2.H_2O$ [equiv]	Solvent	T [°C]	t [min]	Yield [%][b]
1	2	PhMe	70	30	-
2	2	PhMe	80	30	-
3	2	MeOH	70	30	16
4	2	MeOH	80	30	23
5	4	MeOH	80	30	91
6	4	MeOH	80	40	94
7	4	MeOH	80	50	95
8	5	MeOH	80	40	98
9	5	MeOH	80	50	>99
10	3	MeOH	ambient	24	>99

[a]0.5 M *N*-Boc-protected methyl ester amino acid **1a** in 1 mL solvent. [b]Yield measured by GC-MS peak area integration. Entries 1 to 9 were performed in a microwave reactor.

2.1 General Procedure for the Preparation of Hydrazide Derivatives 2a-2d from *N*-Boc Protected Methyl Ester Amino Acids 1a-1d

CAUTION NOTE 1: Hydrazine is an explosive and toxic reagent. In the presence of water its explosive behavior can be reduced. Proper safety measures must be taken when removing hydrazine under reduced pressure. Water should be added to the crude reaction mixture prior to the evaporation. Also ensure that the cooling system of the rotary evaporator is on and that the rotary evaporator and its exhaust are all contained within a well-ventilated fumecupboard.

20 mL of a 0.5 M solution of *N*-Boc-protected methyl ester amino acid **1a-1d** (10.0 mmol) and hydrazine monohydrate (1.50 g, 30.0 mmol) in MeOH were stirred for 24 hours at room temperature. Subsequently, ultra-pure water (3 mL) was added to the reaction mixture. All volatiles were carefully removed under reduced pressure to obtain the desired hydrazide derivative **2a-2d**.

***N*-Boc-L-Ala-NH-NH_2 (2a).** (2.03 g, >99% yield). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.99 (s, 1H), 5.24 (d, *J* = 7.9 Hz, 1H), 4.17 (m, 4.22-4.13, 1H) 3.88 (s, 2H), 1.42 (s, 9H), 1.35 (d, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ 173.6, 155.6, 80.4, 48.9, 28.5, 18.5.

***N*-Boc-L-Ser-NH-NH_2 (2b).** (2.19 g, >99% yield). ^{1}H-NMR (300 MHz, DMSO) δ 9.02 (s, 1H), 6.58 (d, *J* = 8.4 Hz, 1H), 4.18 (s, 2H), 3.95-3.90 (m, 1H), 3.53 - 3.43 (m, 2H), 1.37 (s, 9H). ^{13}C-NMR (75 MHz, DMSO) δ 169.7, 155.1, 78.1, 61.9, 55.5, 28.2.

***N*-Boc-L-Phe-NH-NH_2 (2c).** (2.79 g, >99% yield). ^{1}H-NMR (300 MHz, DMSO) δ 9.12 (s, 1H), 7.27-7.17 (m, 5H), 6.93 (d, *J* = 8.7 Hz, 1H), 4.22 (s, 2H), 4.14-4.06 (m, 1H), 2.90-2.83 (m, 1H), 2.77-2.69 (m, 1H), 1.29-1.20 (m, 9H). ^{13}C-NMR (75 MHz, DMSO) δ 170.1, 155.1, 138.2, 129.2, 128.0, 126.2, 77.9, 54.5, 37.8, 28.2.

***N*-Boc-L-Pro-NH-NH_2 (2d).** (2.29 g, >99% yield). ^{1}H-NMR (300 MHz, DMSO) δ 9.03 (s, 1H), 4.14-3.93 (m, 3H), 3.42-3.18 (m, 1H), 2.08-1.97(m, 1H), 1.88-1.68 (m, 3H) 1.38-1.32 (m, 9H). ^{13}C-NMR (75 MHz, DMSO) δ 171.9, 171.6, 153.5, 153.2, 78.5, 78.4, 58.3, 58.2, 46.6, 46.4, 31.0, 30.1, 28.2, 28.0, 23.9, 23.3.

2.2 General Procedure for the Solvent Screen for the Dipeptide Synthesis in Batch

A flask, submerged within an ice bath at 0 ºC, was charged with *N*-Boc-L-Ala-$NHNH_2$ (**2a**) (100 mg, 0.492 mmol), X mL of solvent(s) (Table S1) and an aqueous solution of $NaNO_2$ (0.5 mL, 1.75 mmol, 3.5 M). An aqueous solution of HCl (0.590 mL, 1 M) was added. The reaction mixture was stirred for a specific time Y min (see Table S1) at 0 ºC. A solution of L-Ala-OBn·HCl (**4a**) (111 mg, 0.516 mmol) in H_2O (0.5 mL) was simultaneously added alongside neat Et_3N (103 µL, 0.738 mmol). The reaction mixture was stirred for a further 2 hours at 0 °C. In the case of monophasic environment, all volatiles were removed under reduced pressure. In the case of a two phase system then the phases were separated, organic phase dried over sodium sulfate, and then the volatiles removed under reduced pressure. Conversion and yields were determined by NMR integration against dioxane as an internal standard. N.D. = not detected.

Table S1. Screening of solvents in batch for acyl azide generation and peptide coupling.

Entry	Solvent	Time Y [min]	Conv. **2a** (%)	Yield **5a** (%)
1	1.5 mL H_2O	1.5	>99	90
2	1.5 mL MeOH	1.5	>99	25
3	1.5 mL DMA	1.5	>99	N.D.
4	1.5 mL DMA	15	>99	N.D.
5	1.5 mL Dioxane	15	70	50
6	1.2 mL H_2O + 0.3 mL Dioxane	15	>99	50
7	0.75 mL H_2O + 0.75 mL Dioxane	15	80	57
8	1.5 mL THF + 1 mL H_2O	15	>99	N.D.
9	1.5 mL MeTHF + 1 mL H_2O	15	>99	N.D.
10	1.5 mL Et_2O + 1 mL H_2O	7	>99	40
11	1.5 mL Toluene + 1 mL H_2O	7	>99	70
12	1.5 mL EtOAc + 1 mL H_2O	7	60	42
13	1.5 mL MTBE + 1 mL H_2O	7	75	41

2.3 General Procedure for the Investigation of Reaction Time for the Acyl Azide Generation and Dipeptide Coupling in Batch

A flask was charged with Boc-L-Ala-$NHNH_2$ (**2a**) (100 mg, 0.492 mmol), H_2O (1.5 mL), toluene (3.1 mL) and an aqueous solution of $NaNO_2$ (0.5 mL, 1.75 mmol, 3.5 M) at 0 ºC. An aqueous solution of HCl (0.59 mL, 1 M) was then added. The mixture was stirred for time A (Table S2) at 0 ºC. A 0.5 mL aqueous solution of L-Ala-OBn·HCl (**4a**) (114 mg, 0.516 mmol) was simultaneously added alongside neat Et_3N (103 µL, 0.738 mmol). The reaction mixture was stirred for time B at 0 °C. The phases were separated. The organic phase was dried over sodium sulfate and all volatiles removed under reduce pressure. Yield was calculated with NMR integration against dioxane as an internal standard.

Table S2. Screening of reaction time for acyl azide generation and peptide coupling.

Entry	Time A (min)	Time B (min)	Conv. (**2a**) (%)	Yield (**5a**) (%)
1	7	120	>99	69
2	7	60	>99	68
3	1.5	60	>99	72
4	1.5	30	>99	70
5	1.5	15	>99	72

2.4 General Procedure for the Investigation of Reaction Time for the Acyl Azide Generation and Dipeptide Coupling in Flow

Scheme S1. Continuous-flow system for acyl azide generation and amide coupling.

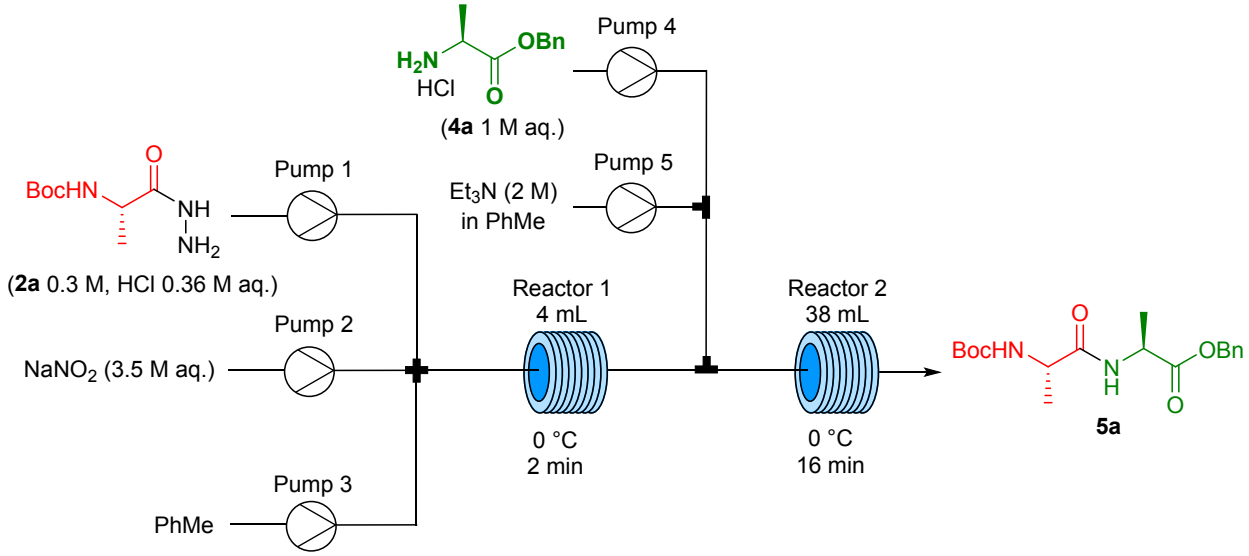


Table S3. Components of the continuous-flow setup.

Abbreviation	Name	Details	Function
P1, P2, P4, P5	Syringe pump	Syrris	Pumping of liquid feed
P3	HPLC pump	Knauer	Pumping of liquid feed
M1, M3	T-mixer	PEEK 0.5 mm i.d.	Mixing of liquid feeds
M2	T-mixer	PEEK 1 mm i.d.	Mixing of liquid feeds
R1	Reactor	PTFE 4 mL i.v., 0.5 mm i.d.	Reactor for acyl azide generation
R2	Reactor	PTFE 38 mL, 1 mm i.d.	Reactor for amidation reaction

The continuous-flow system (Scheme S1) comprised of 5 separate feeds. Feed 1 contained a 15 mL aqueous solution of the hydrazide precursor **2a-2d** (4.50 mmol, 0.3 M) and HCl (1.2 equiv). Feed 2 was a 3.5 M aqueous solution of $NaNO_2$. Feed 3 was neat toluene. Feed 4 was a 1 M aqueous solution of L-Ala-OBn·HCl (**4a**). Feed 5 was a 2 M solution of triethylamine in toluene. Feeds 1, 2 and 3 were mixed by using a PEEK cross-assembly (0.5 mm i.d.) prior to entering reactor 1 (4 mL internal volume, 0.5 mm i.d.). The effluent from reactor 1 was mixed with feeds

4 and 5 using a PEEK T-mixer (0.5 mm i.d.) resulting in a biphasic segmented flow regime before entering reactor 2 (38 mL internal volume, 1 mm i.d.). The whole system (excluding pumps) was submerged within an ice-bath at 0 °C. The crude reaction mixture was collected, and the organic phase was separated by using a separating funnel, dried over Na_2SO_4, filtered and evaporated under reduced pressure to give the desired Boc-protected dipeptide (**4a-4e**). The yield was calculated by NMR integration against an internal standard.

CAUTION NOTE 2: Excess sodium nitrite was used in flow so that the remaining azide could be quenched at the outlet by using hydrochloric acid. Hydrazoic acid (HN_3) is quenched by using the same method. The effluent was immediately quenched at the outlet.

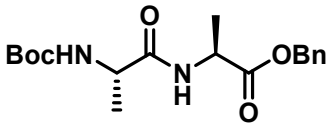
$NaNO_2 + HCl \rightarrow HNO_2 + NaCl$

$2NaN_3 + 2HNO_2 \rightarrow 3N_2\,(g) + 2NO\,(g) + 2NaOH$

$NO\,(g) + O_2\,(g) \rightarrow NO_2\,(g)$

Nitrogen dioxide (NO_2) is less toxic and highly preferable over remaining azide and hydrazoic acid. No gas bubbles were observed during experimentation. A nitrogen purge line was connected to the collection vessel as a safety precaution. Iodine-starch paper can be used to test for an excess of nitrite (goes blue) which indicates the quench is complete.

***N*-Boc-L-Ala-L-Ala-OBn (5a)**

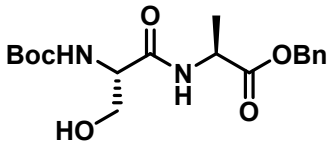


Flow rates: pump 1 = 0.769 mL/min, pump 2 = 0.231 mL/min, pump 3 = 1 mL/min, pump 4 = 0.242 mL/min, pump 5 = 0.173 mL/min. Reactor 1 residence time = 2 minutes. Reactor 2 residence time = 16 minutes. Total residence time = 18 minutes. Reaction mixture was collected during 10 minutes once steady-state conditions were reached. Application of the general work up described above provided a clear oil which crystallized as a white solid overnight (775 mg, 70% NMR assay yield using dioxane as an internal standard). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.36-7.34 (m, 5H) 6.76 (d, *J* = 7.4 Hz, 1H), 5.21-5.09 (m, 3H), 4.65-4.55 (m, 1H), 4.18 (s, 1H), 1.43 (s, 9H), 1.40 (d, *J* = 7.2 Hz, 3H), 1.33 (d, *J* = 7.1 Hz, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ 172.7, 172.4, 155.5, 135.4, 128.7, 128.6, 128.3, 80.2, 67.3, 50.1, 48.2, 28.4, 18.5, 18.4.

Long-run for *N*-Boc-D-Ala-L-Ala-OBn (9)

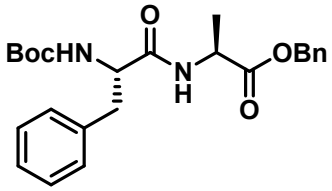
Feed 1 contained 82 mL aqueous solution of *N*-Boc- D-Ala-$NHNH_2$ (**8**) (5.00 g, 24.6 mmol, 0.3 M) and HCl (1.2 equiv.). The remaining feeds were unchanged. Flow rates: pump 1 = 0.769 mL/min, pump 2 = 0.231 mL/min, pump 3 = 1 mL/min, pump 4 = 0.242 mL/min, pump 5 = 0.173 mL/min. Reactor 1 residence time = 2 minutes. Reactor 2 residence time = 16 minutes. Total residence time = 18 minutes. Reaction mixture was collected during 93 minutes once steady-state conditions were reached. Application of the general work up described above provides a clear oil that recrystallizes as a white solid overnight (6.78 g, 70% NMR assay yield).

***N*-Boc- L-Ser-L-Ala-OBn (5b)**



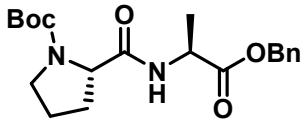
Flow rates: pump 1 = 0.385 mL/min, pump 2 = 0.115 mL/min, pump 3 = 0.5 mL/min, pump 4 = 0.121 mL/min, pump 5 = 0.087 mL/min. Reactor 1 residence time = 4 minutes. Reactor 2 residence time = 32 minutes. Total residence time = 36 minutes. Reaction mixture was collected during 20 minutes once steady state concentration was reached. Application of the general work-up described above provides a white solid (730 mg, 71% NMR assay yield). ^{1}H-NMR (300 MHz, DMSO) δ 8.27 (d, *J* = 7.1 Hz, 1H), 7.40-7.34 (m, 5H) 6.67 (d, *J* = 8.3 Hz, 1H), 5.11 (s, 2H), 4.77 (s, 1H), 4.34 (p, *J* = 7.2 Hz, 1H), 4.09-3.96 (m, 1H), 3.63-3.47 (m, 2H), 1.38 (s, 9H), 1.30 (d, *J* = 7.3 Hz, 3H). ^{13}C-NMR (75 MHz, DMSO) δ 172.3, 170.3, 155.2, 136.0, 128.9, 128.2, 128.0, 78.1, 66.9, 61.8, 56.7, 47.8, 28.2, 17.0.

***N*-Boc-L-Phe-L-Ala-OBn (5c)**



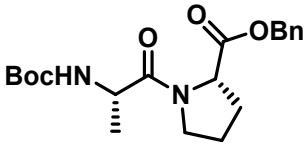
Due to the precipitation of the acyl azide intermediate **3e** inside of reactor 1 at the standard conditions, a variation to the general procedure for the synthesis of dipeptides was applied. Feed A contained a 24 mL aqueous solution of Boc-Phe-$NHNH_2$ (**2c**) (1.342 g, 4.80 mmol, 0.2 M) and HCl (1.2 equiv). Feed 3 contained a mixture toluene/EtOAc 6:1. Feed 2, 4 and 5 remain unchanged. Flow rates: pump 1 = 0.417 mL/min, pump 2 = 0.083 mL/min, pump 3 = 0.5 mL/min, pump 4 = 0.088 mL/min, pump 5 = 0.063 mL/min. Reactor 1 residence time = 4 minutes. Reactor 2 residence time = 32 minutes. Total residence time = 36 minutes. Reaction mixture was collected during 30 minutes once steady state concentration was reached. Application of the general work up described above provides a white solid (930 mg, NMR assay yield: 74%). ^{1}H-NMR (300 MHz, DMSO) δ 8.46 (d, *J* = 7.1 Hz, 1H), 7.37-7.33 (m, 5H), 7.27-7.19 (m, 5H), 6.91 (d, *J* = 8.8 Hz, 1H), 5.13 (s, 2H), 4.36 (p, *J* = 7.1 Hz, 1H), 4.23-4.12 (m, 1H), 2.96-2.63 (m, 2H), 1.35-1.21 (m, 12H). ^{13}C NMR (75 MHz, DMSO) δ 172.4, 172.9, 155.3, 138.3, 136.0, 129.2, 128.4, 128.2, 128.0, 127.8, 127.7, 78.0, 66.0, 55.4, 48.7, 37.4, 28.2, 16.9.

***N*-Boc-L-Pro-L-Ala-OBn (5d)**



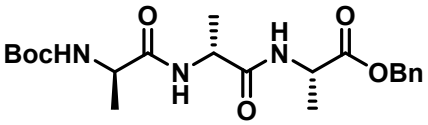
Flow rates: Pump 1 = 0.385 mL/min, pump 2 = 0.115 mL/min, pump 3 = 0.5 mL/min, pump 4 = 0.121 mL/min, pump 5 = 0.087 mL/min. Reactor 1 residence time = 4 minutes. Reactor 2 residence time = 32 minutes. Total residence time = 36 minutes. Reaction mixture was collected during 20 minutes once steady state concentration was reached. Application of the general work up described above gives a white solid (700 mg, NMR assay yield: 77%). ^{1}H-NMR (300 MHz, DMSO) δ 8.36-8.29 (m, 1H), 7.40 - 7.30 (m, 5H), 5.16-5.04 (m, 2H), 4.36-4.30 (m, 1H), 4.10-4.05 (m, 1H), 3.28-3.22 (m, 1H), 2.06-1.97 (m, 1H), 1.75-1.69 (m, 3H), 1.37 (s, 3H), 1.31-1.29 (m, 9H). ^{13}C NMR (75 MHz, DMSO) δ 172.4, 172.3, 172.2, 153.5, 153.2, 136.0, 128.4, 128.1, 127.8, 78.4, 65.9, 59.2, 58.8, 47.5, 46.6, 46.4, 30.7, 29.8, 28.2, 28.0, 23.7, 23.1, 16.9, 16.8.

***N*-Boc-L-Ala-L-Pro-OBn (5e)**



Feed 1 contained a 24.6 mL aqueous solution of *N*-Boc-L-Ala-$NHNH_2$ (**2a**) (1.5 g, 7.38 mmol, 0.3 M) and HCl (1.2 equiv). Feed 4 contained a 1 M stock solution of L-Pro-OBn·HCl (**4b**) in H_2O, the rest of the feeds were unchanged. Flow rates: Pump 1 = 0.769 mL/min, pump 2 = 0.231 mL/min, pump 3 = 1 mL/min, pump 4 = 0.242 mL/min, pump 5 = 0.173 mL/min. Reactor 1 residence time = 2 minutes. Reactor 2 residence time = 16 minutes. Total residence time = 18 minutes. Reaction mixture was collected during 22 minutes once steady state concentration was reached. Application of the general work up described above provides an orange oil (1.81 g, NMR assay yield: 83%). ^{1}H-NMR (300 MHz, DMSO) δ 7.40-7.32 (m, 5H), 7.00 (d, *J* = 7.5 Hz, 1H), 5.14-5.05 (m, 2H), 4.38 (dd, *J* = 8.7, 4.5 Hz, 1H), 4.28-4.19 (m, 1H), 3.68-3.60 (m, 1H), 3.55-3.48 (m, 1H), 2.23-2.14 (m, 1H), 1.97-1.79 (m, 3H), 1.36 (s, 9H), 1.11 (d, *J* = 7.0 Hz, 3H). ^{13}C NMR (75 MHz, DMSO) δ 171.7, 171.3, 155.0, 136.0, 128.4, 128.2, 127.7, 77.9, 65.8, 58.6, 47.4, 46.3, 28.5, 28.2, 24.7, 16.6.

***N*-Boc-D-Ala-D-Ala-L-Ala-OBn (11)**



Feed A contained a 24.6 mL of N-Boc-L-Ala-$NHNH_2$ (1.5 g, 7.38 mmol), HCl (1M aq. Solution, 8.86 mL) in H_2O, Feed D contained a 5 mL solution of D-Ala-L-Ala-OBn·HCl (**4a**, 1.44 g, 5 mmol) in H_2O, the rest of the feeds were unchanged. Flow rates: Pump 1 = 0.769 mL/min, pump 2 = 0.231 mL/min, pump 3 = 1 mL/min, pump 4 = 0.230 mL/min, pump 5 = 0.173 mL/min. Reactor 1 residence time = 2 minutes. Reactor 2 residence time = 16 minutes. Total residence time = 18 minutes. Reaction mixture was collected during 22 minutes once steady state concentration was reached. Application of the general work up described above provided an off-white solid (1 g, NMR assay yield: 77%). ^{1}H-NMR (300 MHz, DMSO) δ 8.42 - 8.30 (m, 1H), 7.81 (d, *J* = 7.6 Hz, 1H),7.37 - 7.35 (m, 5H) 7.02 (d, *J* = 7.4 Hz, 1H), 5.11 (s, 2H) 4.30 (p, *J* = 7.0 Hz, 2H), 4.00 - 3.84 (m, 1H), 1.37 (s, 9H), 1.29 (d, *J* = 7.3 Hz, 3H), 1.22 - 1.13 (m, 6H) .^{13}C NMR (75 MHz, DMSO)

δ 172.7, 172.7, 172.5, 155.6, 136.4, 128.9, 128.4, 128.1, 78.6, 66.3, 50.2, 48.3, 48.1, 28.6, 19.1, 18.4, 17.4.

2.5 General Procedure for the Boc-Cleavage in Batch

4 M HCl in 1,4-dioxane was added to the *N*-Boc-protected dipeptide (1 mmol) at 0 ºC under an argon atmosphere. The reaction mixture was stirred at 0 ºC for 4 minutes. The temperature was then increased to room temperature and left to stir until complete consumption of the starting material was observed by TLC. The reaction mixture was concentrated under reduced pressure at 30 ºC. The resultant oil was washed with Et_2O to afford the peptide.

L-Ala-L-Ala-OBn·HCl (7a). TLC (Eluent: DCM/MeOH 4:1) showed full conversion after 30 min. The application of the general procedure afforded a white solid (284 mg, 0.952 mmol, 95% yield). ^{1}H-NMR (300 MHz, D_2O) δ 7.42 (s, 1H), 5.24-5.15 (m, 2H), 4.47 (q, *J* = 7.3 Hz, 1H), 4.04 (q, *J* = 7.1 Hz, 1H), 1.43 (d, *J* = 3.3 Hz, 3H), 1.41 (d, *J* = 3.5 Hz, 3H). ^{13}C NMR (75 MHz, D_2O) δ 174.5, 171.2, 135.8, 129.4, 129.4, 129.0, 68.5, 49.5, 49.4, 17.0, 16.3.

L-Ser-L-Ala-OBn·HCl (7b). TLC (Eluent: DCM/MeOH 6:1) showed full conversion after 30 minutes. The application of the general procedure afforded a white solid (316 mg, 0.949 mmol, 95% yield). ^{1}H-NMR (300 MHz, D_2O) δ 7.42 (s, 5H), 5.23-5.14 (m, 2H), 4.51 (q, *J* = 7.3 Hz, 1H), 4.12-4.09 (m, 1H), 3.95-3.89 (m, 1H), 3.81-3.74 (m, 1H), 1.42 (d, J = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, D_2O) δ 174.4, 168.0, 135.8, 129.5, 129.4, 129.0, 68.5, 60.8, 55.1, 49.6, 16.5.

L-Phe-L-Ala-OBn·HCl (7c). TLC (Eluent: DCM/MeOH 6:1) showed full conversion after 60 minutes. The application of the general procedure afforded an off-white solid. (370 mg, 0.958 mmol, 96 % yield). ^{1}H-NMR (300 MHz, D_2O) δ 7.40-7.18 (m, 10H), 5.16 (s, 2H), 4.45 (q, *J* = 7.3 Hz, 1H), 4.19 (t, *J* = 7.1 Hz, 1H), 3.14 - 3.07 (m, 1H), 2.99-2.91 (m, 1H), 1.37 (d, *J* = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, D_2O) δ 174.1, 169.4, 135.8, 134.2, 130.1, 129.7, 129.5, 129.4, 129.1, 128.6, 68.4, 54.8, 49.5, 37.4, 16.6.

L-Pro-L-Ala-OBn·HCl (7d). TLC (Eluent: DCM/MeOH 7:1) showed full conversion after 30 minutes. The application of the general procedure provides a white solid. (319 mg, 0.958 mmol, 96% yield). ^{1}H-NMR (300 MHz, D_2O) δ 7.41 (s, 5H), 5.17 (AB, 2H), 4.46 (q, *J* = 7.3 Hz, 1H), 4.36-4.32 (m, 1H), 3.31 (t, *J* = 6.8 Hz, 2H), 2.34-2.26 (m, 1H), 1.96-1.83 (m, 3H), 1.42 (d, *J* = 7.3

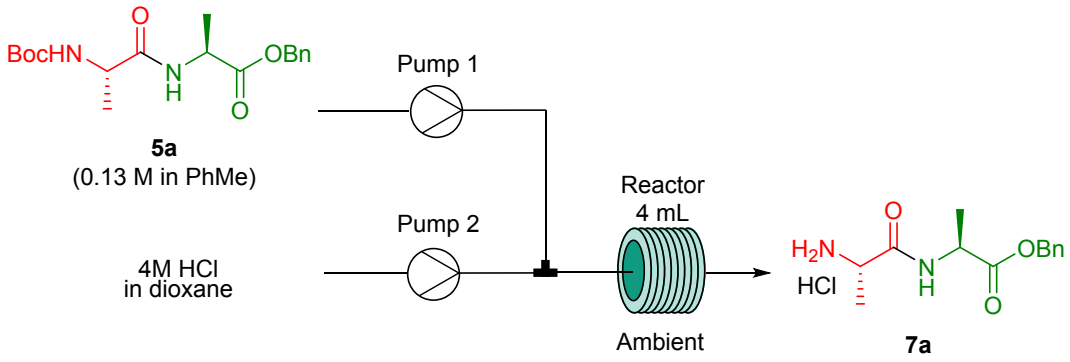
Hz, 3H). ^{13}C NMR (75 MHz, D_2O) δ 174.4, 170.1, 135.7, 129.5, 129.2, 68.5, 60.0, 49.7, 47.2, 30.3, 24.2, 16.2.

L-Ala-L-Pro-OBn·HCl (7e). TLC (Eluent: DCM/MeOH 7:1) showed full conversion after 30 minutes. The application of the general procedure afforded a white solid. (319 mg, 0.99 mmol, 99% yield). ^{1}H-NMR (300 MHz, D_2O) δ 7.42 (s, 5H), 5.24-5.18 (m, 2H), 4.52-4.48 (m, 1H), 4.35 (q, *J* = 7.1 Hz, 1H), 3.74-3.69 (m, 1H), 3.63-3.52 (m, 1H), 2.29-2.23 (m, 1H). 2.03-1.93 (m, 3H), 1.47 (d, J = 7.0 Hz, 3H). ^{13}C NMR (75 MHz, D_2O) δ 174.1, 169.7, 135.7, 129.4, 129.4, 128.9, 68.5, 60.4, 48.7, 47.9, 29.2, 24.8, 15.4.

D-Ala-L-Ala-L-Ala-OBn·HCl (12). 90.0 mg, 0.214 mmol scale. TLC (Eluent: DCM/MeOH 6:1) showed full conversion after 45 minutes. The application of the general procedure afforded an off-while solid. (78 mg, 0.203 mmol, 95% yield). ^{1}H-NMR (300 MHz, D_2O) δ 7.41 (s, 5H), 5.19 (s, 2H), 4.42-4.30 (m, 2H), 4.11-4.02 (m, 1H), 1.48 (d, *J* = 7.1 Hz, 3H), 1.39 (d, *J* = 7.3 Hz, 3H), 1.34 (d, *J* = 7.2 Hz, 3H). ^{13}C NMR (75 MHz, D_2O) δ 175.0, 174.7, 171.0, 135.9, 129.4, 129.3, 128.8, 68.3, 50.2, 49.5, 49.5, 17.2, 17.1, 16.5.

2.6 Boc-Cleavage in Flow Trial

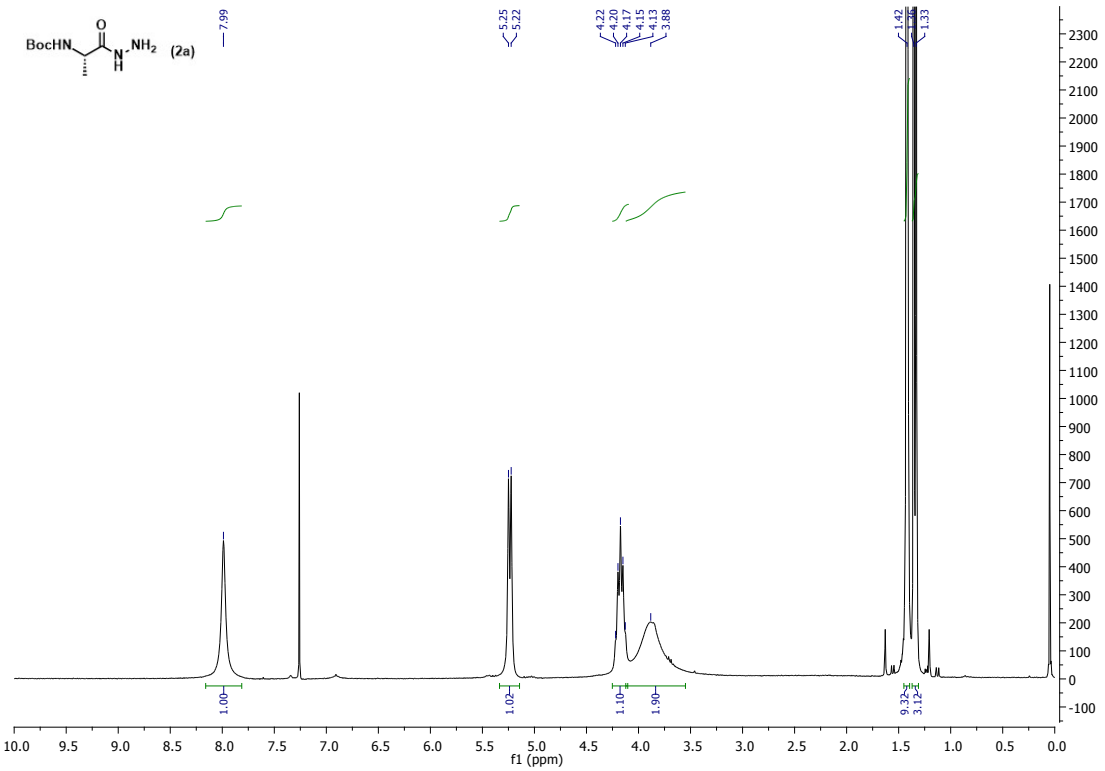
Scheme S2. Continuous-flow scheme for Boc-deprotection of compound **5a**.

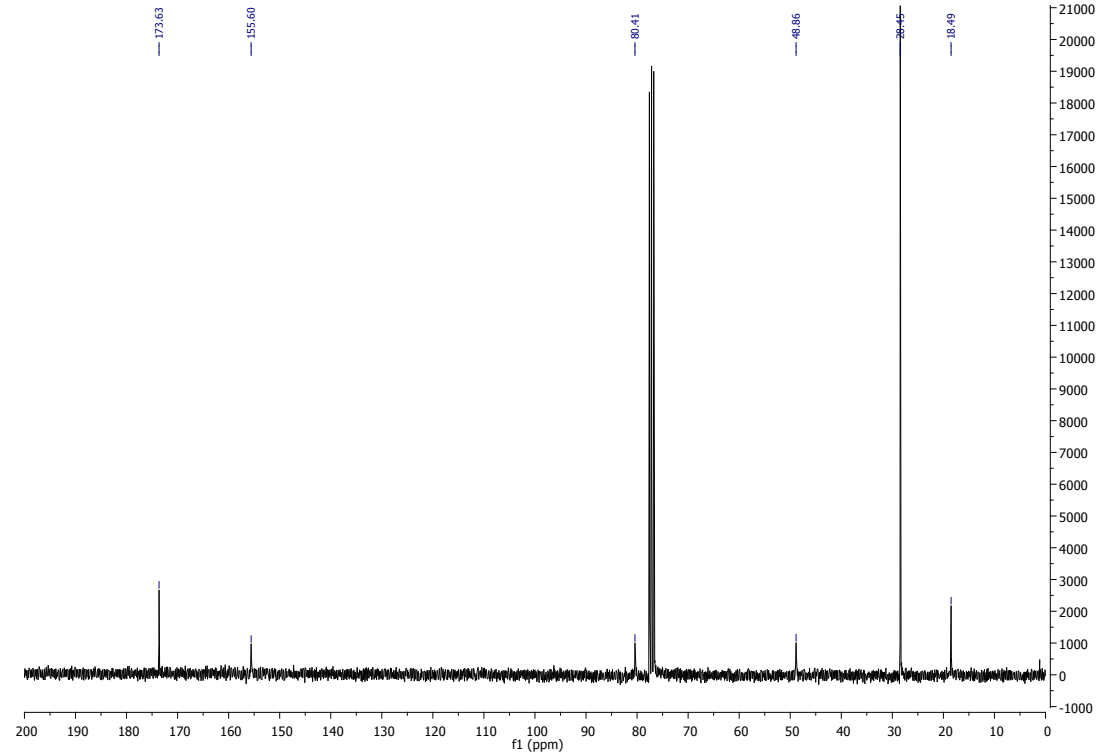


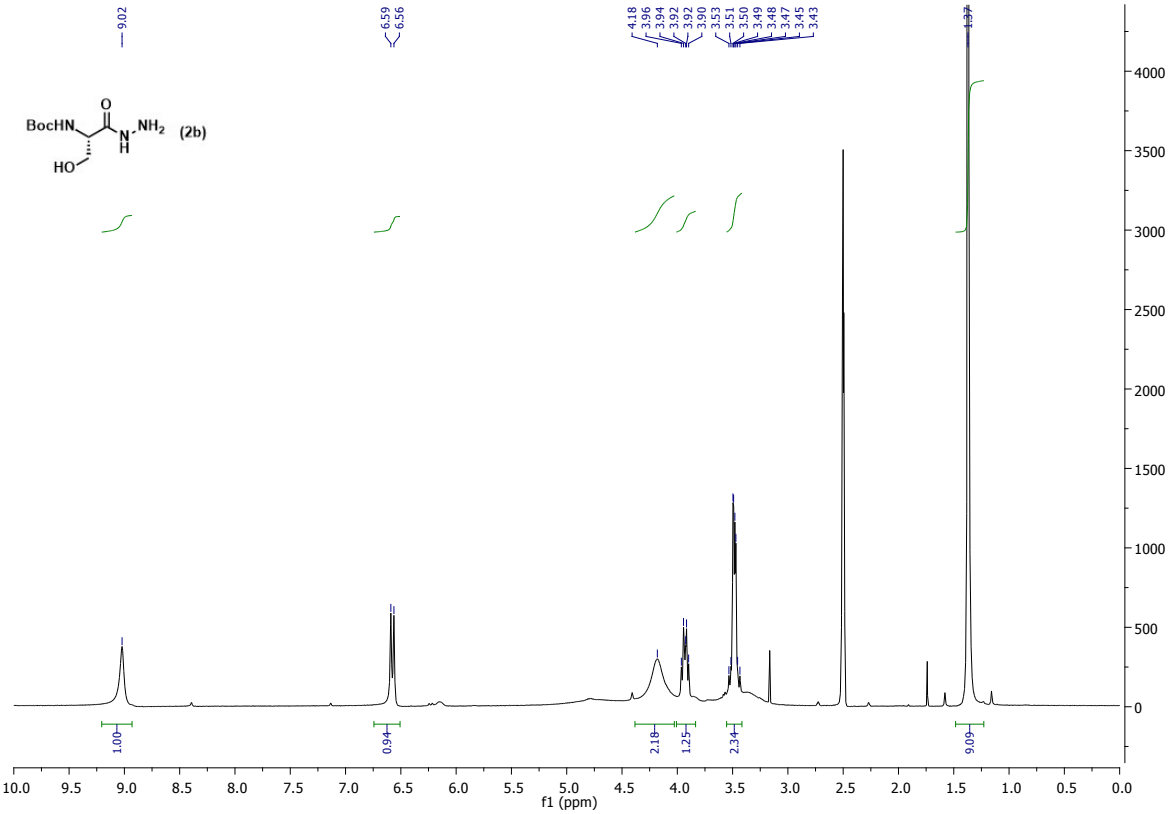
The continuous-flow system (Scheme S1) comprised of 2 separate feeds. Feed 1 contained 0.13 M **5a** in PhMe. Feed 2 contained 4 M HCl in dioxane. Feed 1 and feed 2 were pumped at 0.136 mL/min and 0.264 mL/min which corresponded to a residence time of 10 min. Feeds 1 and 2 were mixed prior to entering the reactor (4 mL internal volume, 0.5 mm i.d.) at an ambient temperature.

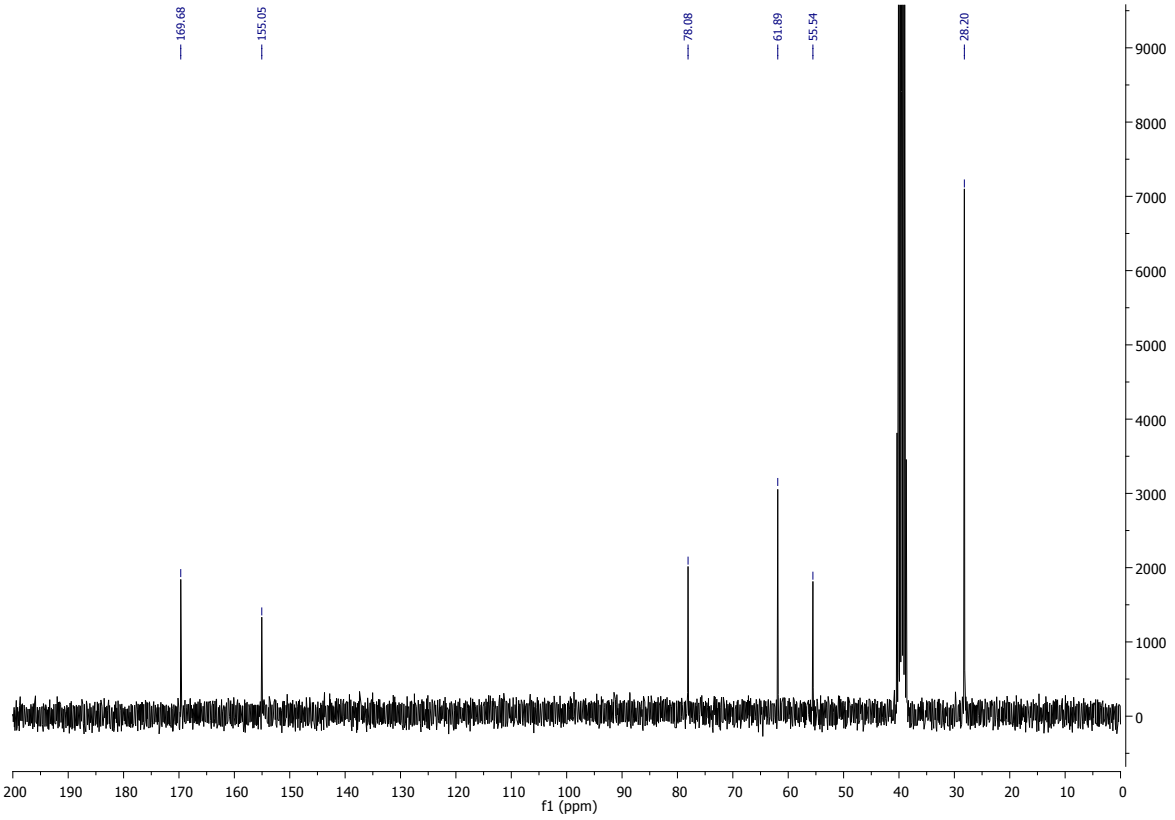
The reaction mixture was concentrated under reduced pressure at 30 ºC. The resultant oil was then treated with the procedure described above.

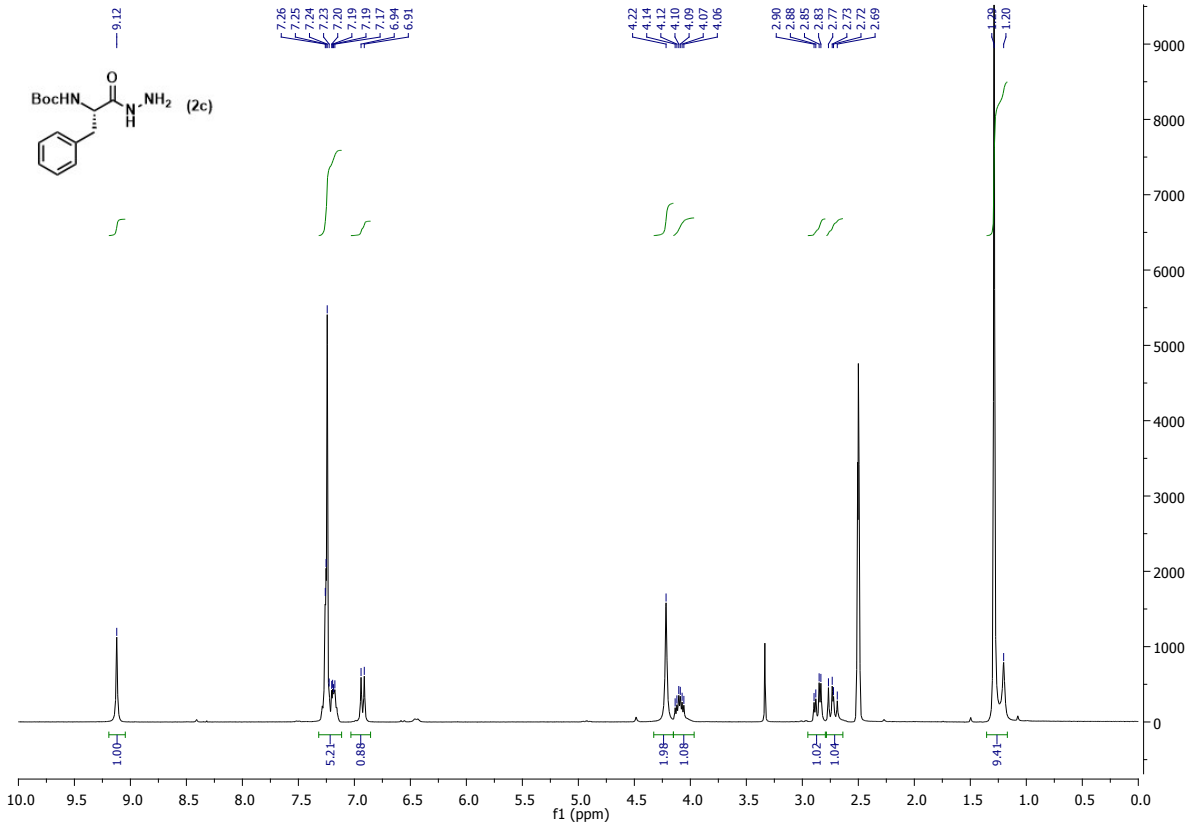
2.7 NMR Spectra

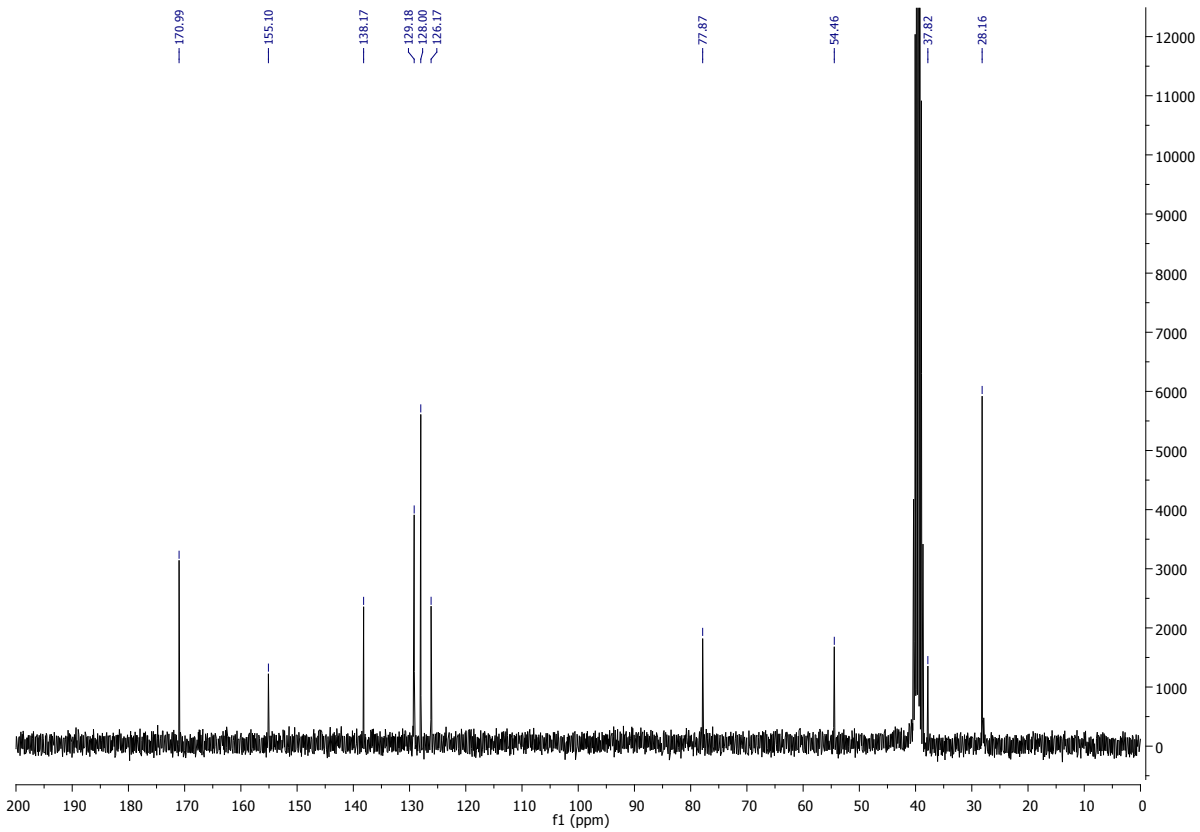





9.02
6.59
6.56
4.18
3.96
3.94
3.92
3.92
3.90
3.53
3.51
3.50
3.49
3.48
3.47
3.45
3.43
1.37
BocHN
O
NH2
N
H
HO
(2b)
1.00
0.94
2.18
1.25
2.34
9.09
f1 (ppm)


169.68
155.05
78.08
61.89
55.54
28.20
f1 (ppm)


9.12
7.26
7.25
7.24
7.23
7.20
7.19
7.19
7.17
6.94
6.91
4.22
4.14
4.12
4.10
4.09
4.07
4.06
2.90
2.88
2.85
2.83
2.77
2.73
2.72
2.69
1.29
1.20
BocHN
O
NH2
(2c)
1.00
5.21
0.88
1.98
1.08
1.02
1.04
9.41
f1 (ppm)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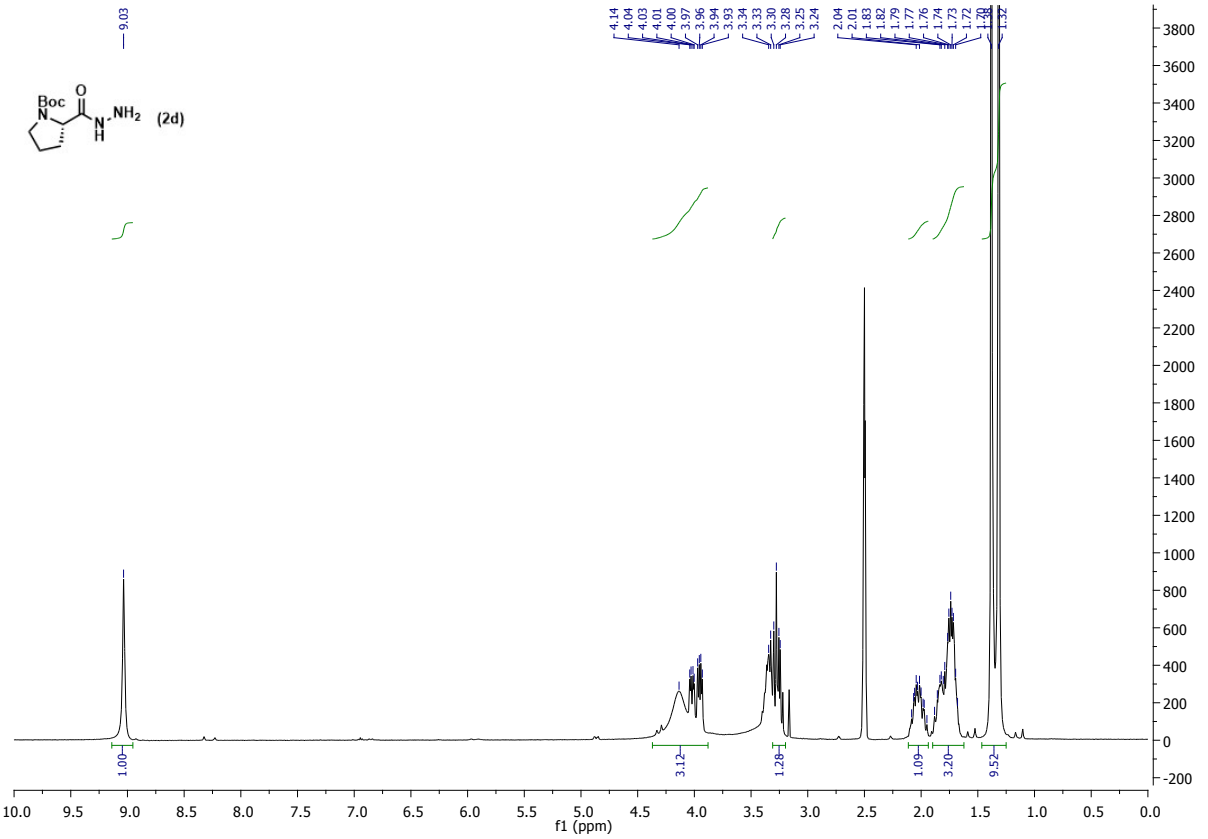
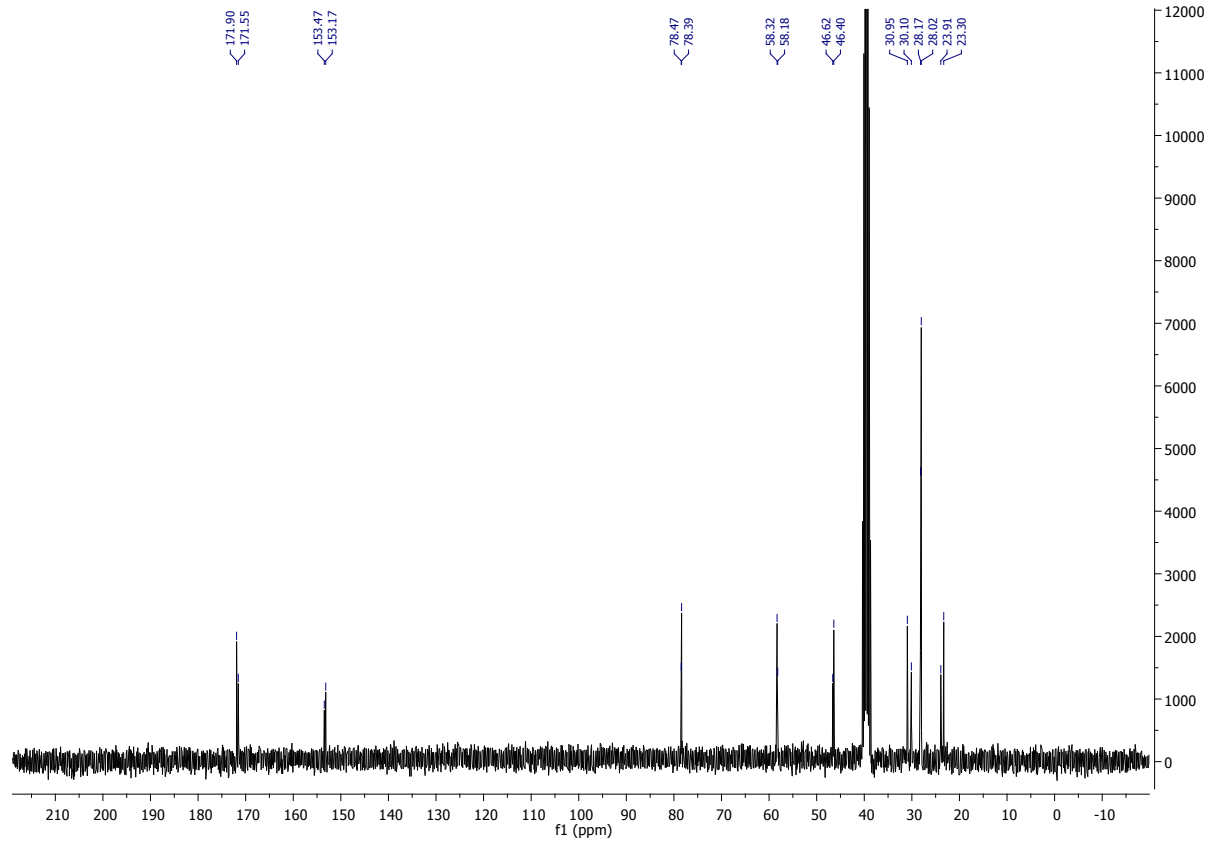
155.10
138.17
129.18
128.00
126.17
77.87
54.46
37.82
28.16
f1 (ppm)


9.03
4.14
4.04
4.03
4.01
4.00
3.97
3.96
3.94
3.93
3.34
3.33
3.30
3.28
3.25
3.24
2.04
2.01
1.83
1.82
1.79
1.77
1.76
1.74
1.73
1.72
1.70
1.32
Boc
N
O
N
H
NH2
(2d)
1.00
3.12
1.28
1.09
3.20
9.52
f1 (ppm)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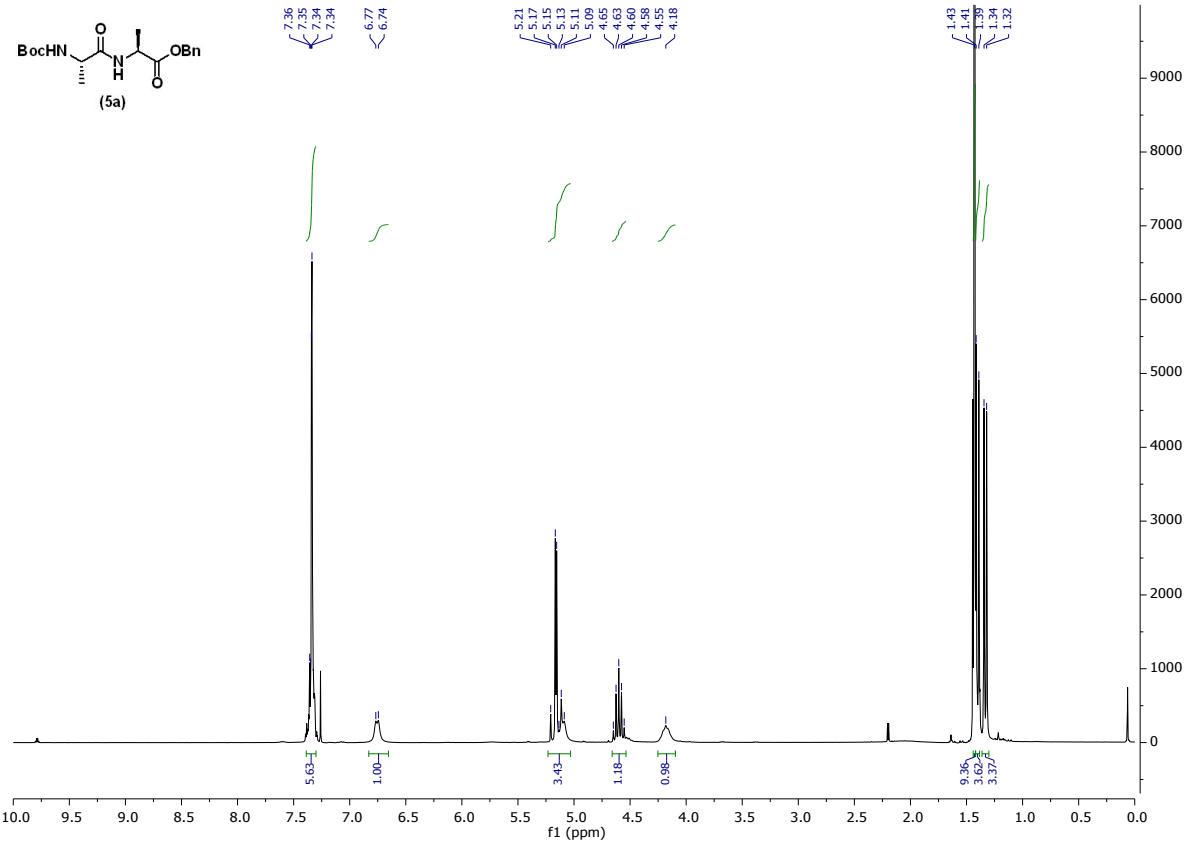
171.55
153.47
153.17
78.47
78.39
58.32
58.18
46.62
46.40
30.95
30.10
28.17
28.02
23.91
23.30
f1 (ppm)


BocHN
OBn
(5a)
7.36
7.35
7.34
7.34
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6.74
5.21
5.17
5.15
5.13
5.11
5.09
4.65
4.63
4.60
4.58
4.55
4.18
1.43
1.41
1.39
1.34
1.32
5.63
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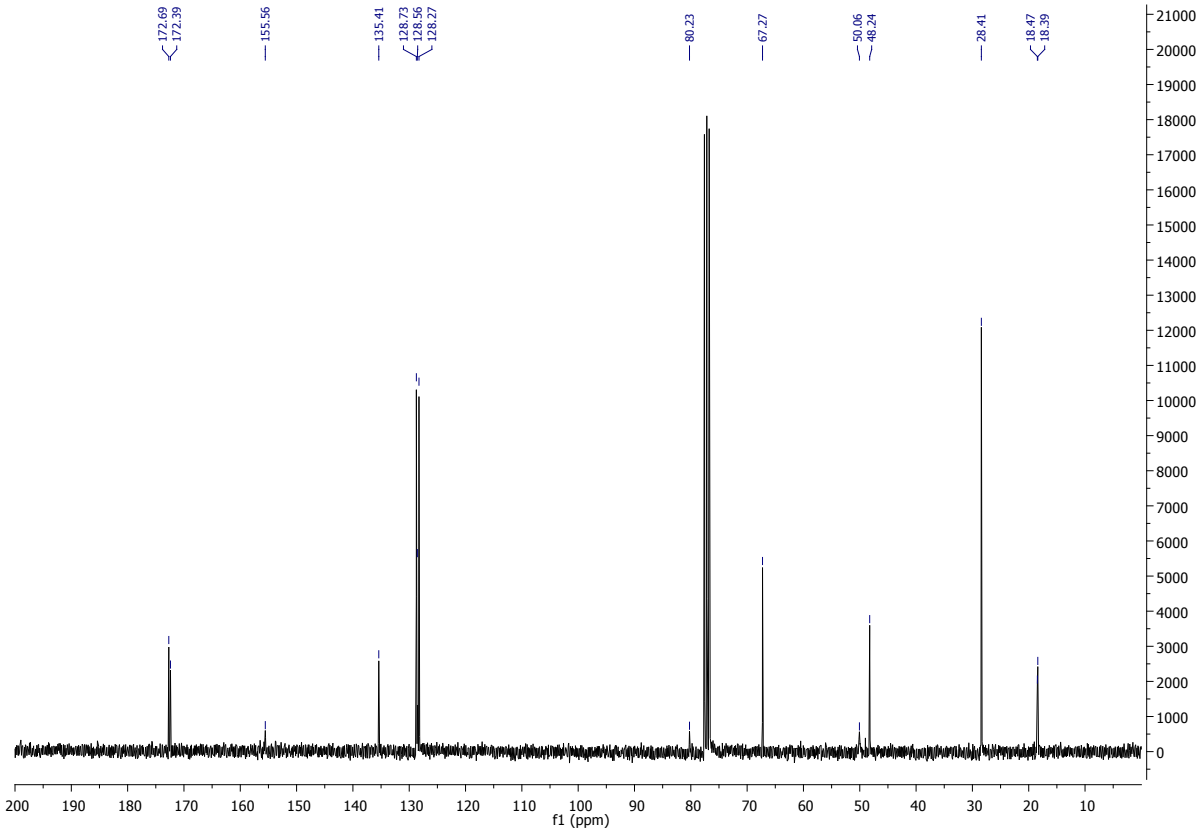
3.43
1.18
0.98
9.36
3.62
3.37
f1 (ppm)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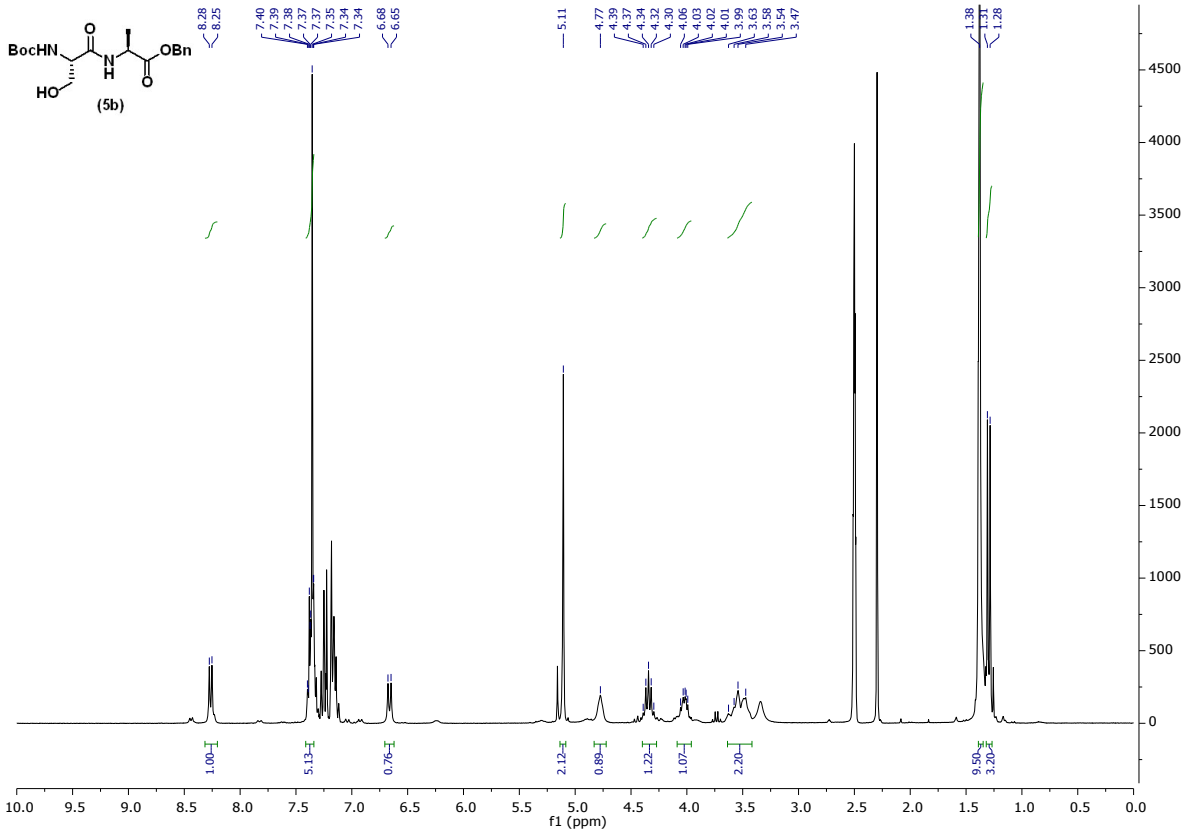
128.56
128.27
80.23
67.27
50.06
48.24
28.41
18.47
18.39
f1 (ppm)


BocHN
OBn
HO
(5b)
8.28
8.25
7.40
7.39
7.38
7.37
7.37
7.35
7.34
7.34
6.68
6.65
5.11
4.77
4.39
4.37
4.34
4.32
4.30
4.06
4.03
4.02
4.01
3.99
3.63
3.58
3.54
3.47
1.38
1.31
1.28
1.00
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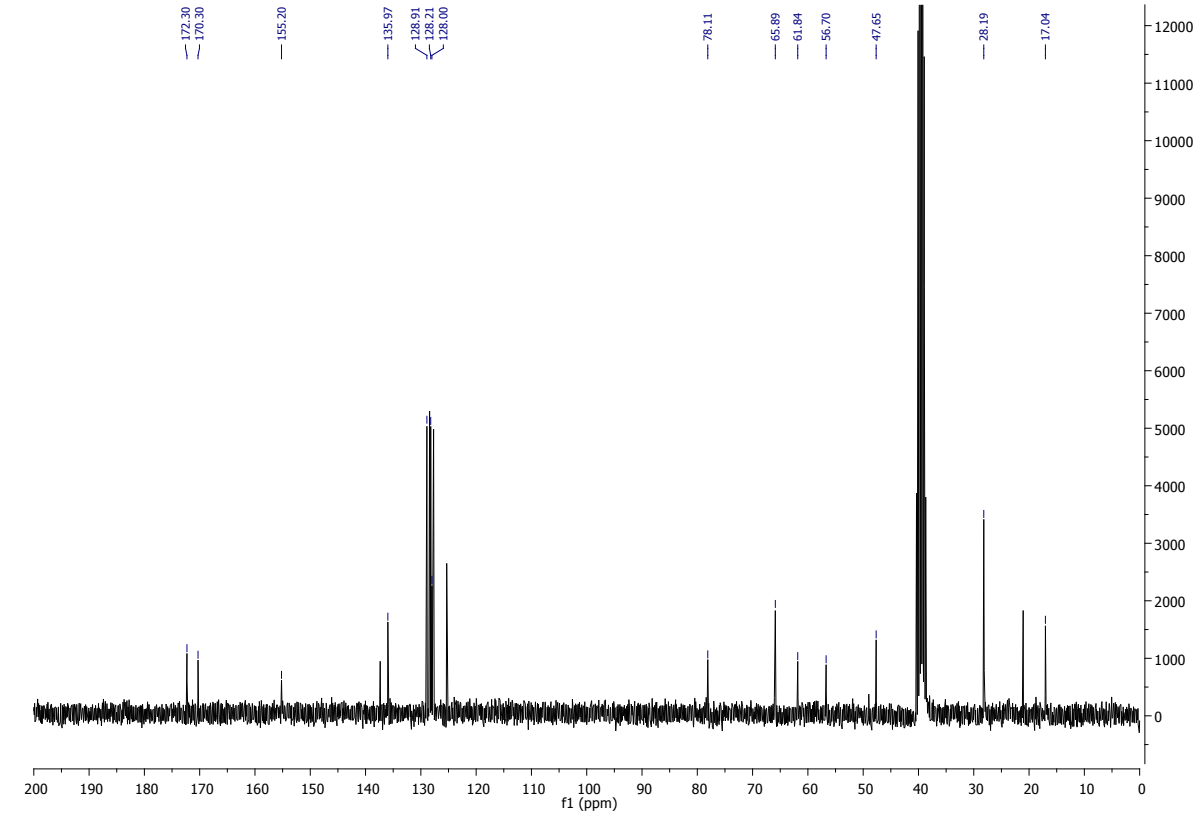
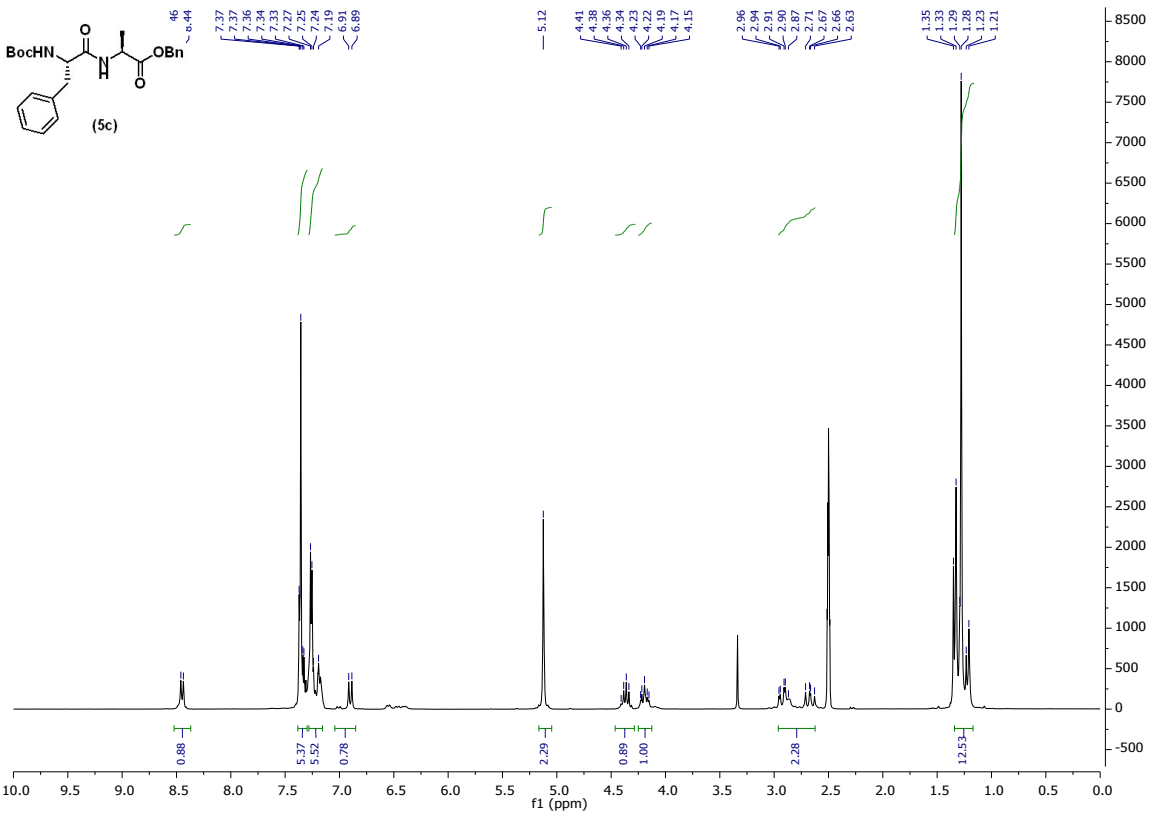
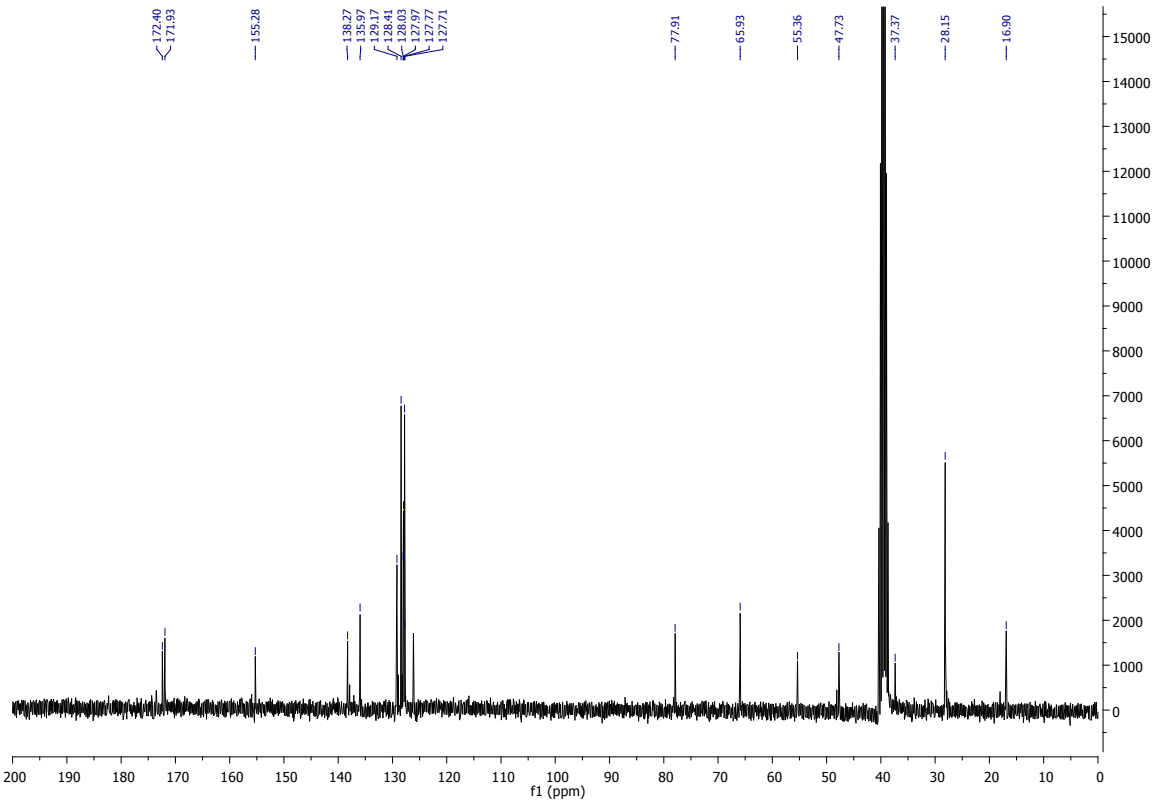
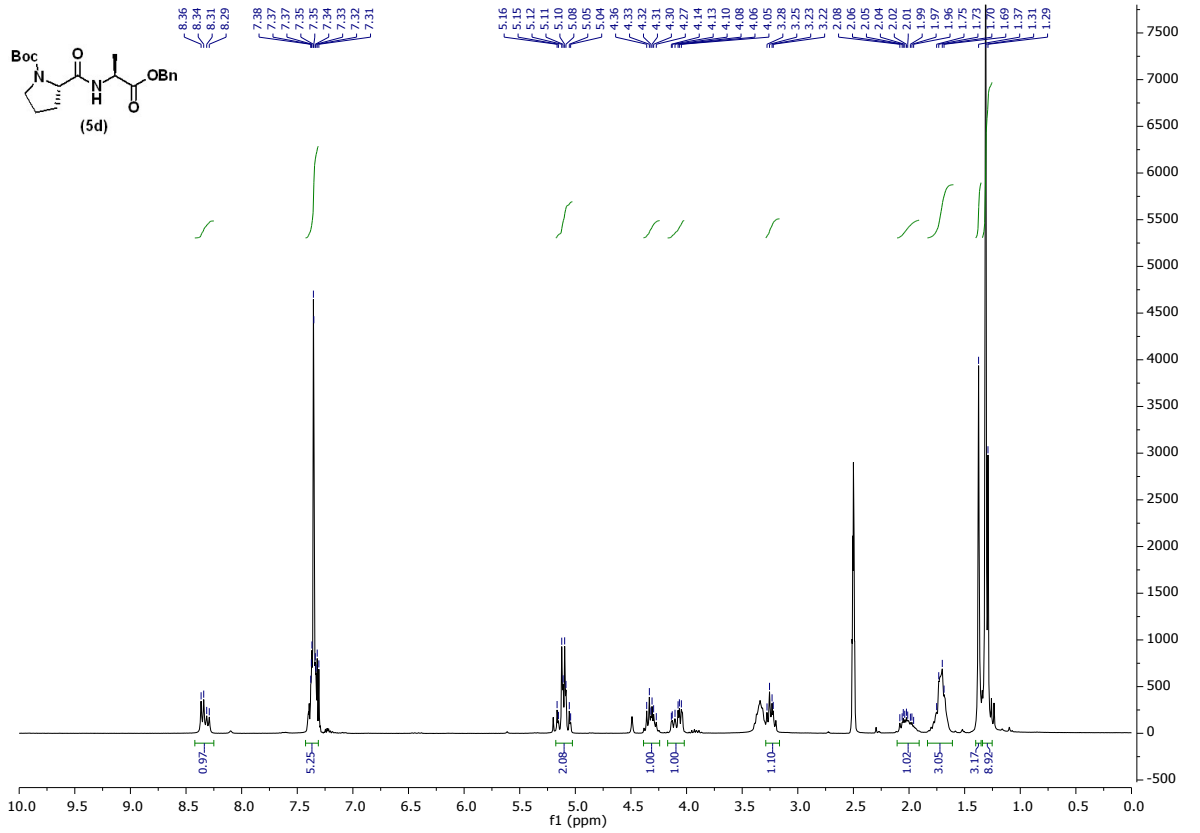
0.76
2.12
0.89
1.22
1.07
2.20
9.50
3.20
f1 (ppm)


172.30
170.30
155.20
135.97
128.91
128.21
128.00
78.11
65.89
61.84
56.70
47.65
28.19
17.04
f1 (ppm)


BocHN
OBn
(5c)
f1 (ppm)


f1 (ppm)


Boc
N
O
N
H
O
OBn
(5d)
8.36
8.34
8.31
8.29
7.38
7.37
7.37
7.35
7.35
7.34
7.33
7.32
7.31
5.16
5.15
5.12
5.11
5.10
5.08
5.05
5.04
4.36
4.33
4.32
4.31
4.30
4.27
4.14
4.13
4.10
4.08
4.06
4.05
3.28
3.25
3.23
3.22
2.08
2.06
2.05
2.04
2.02
2.01
1.99
1.97
1.96
1.75
1.73
1.70
1.69
1.37
1.31
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1.00
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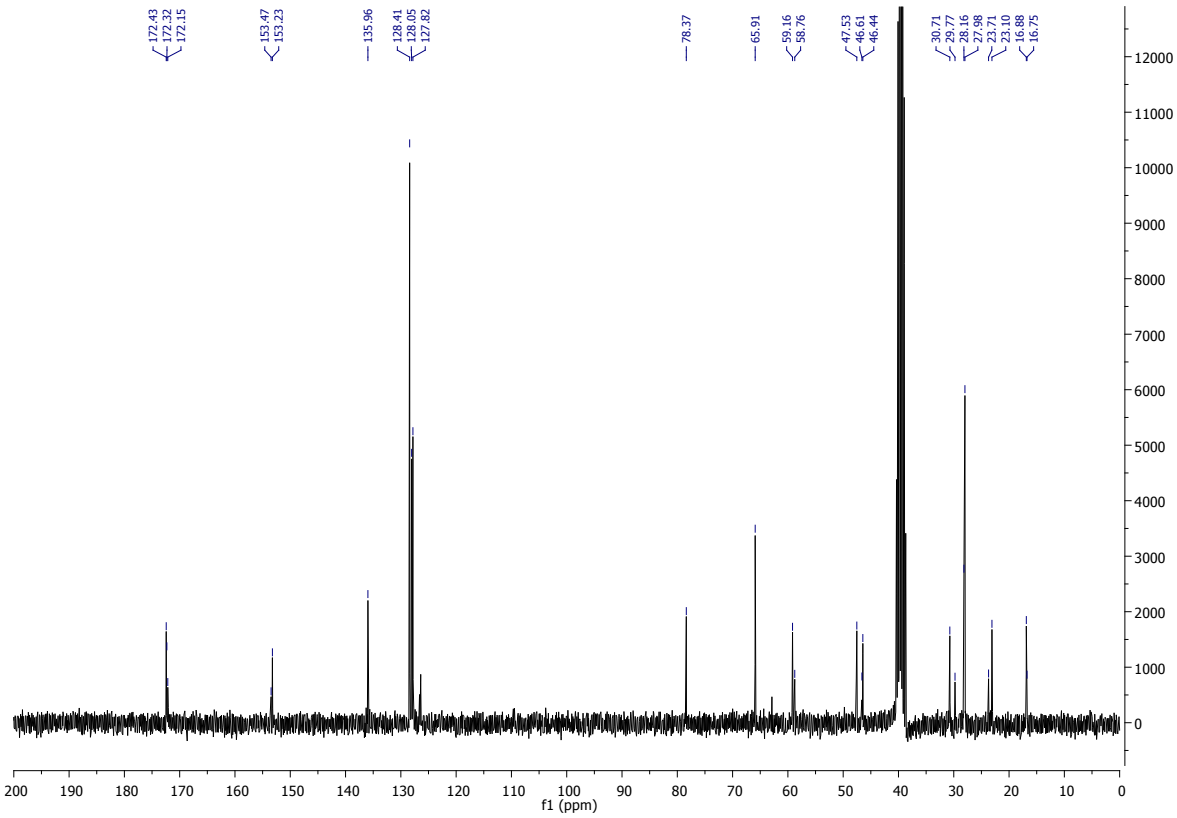
f1 (ppm)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153.23
135.96
128.41
128.05
127.82
78.37
65.91
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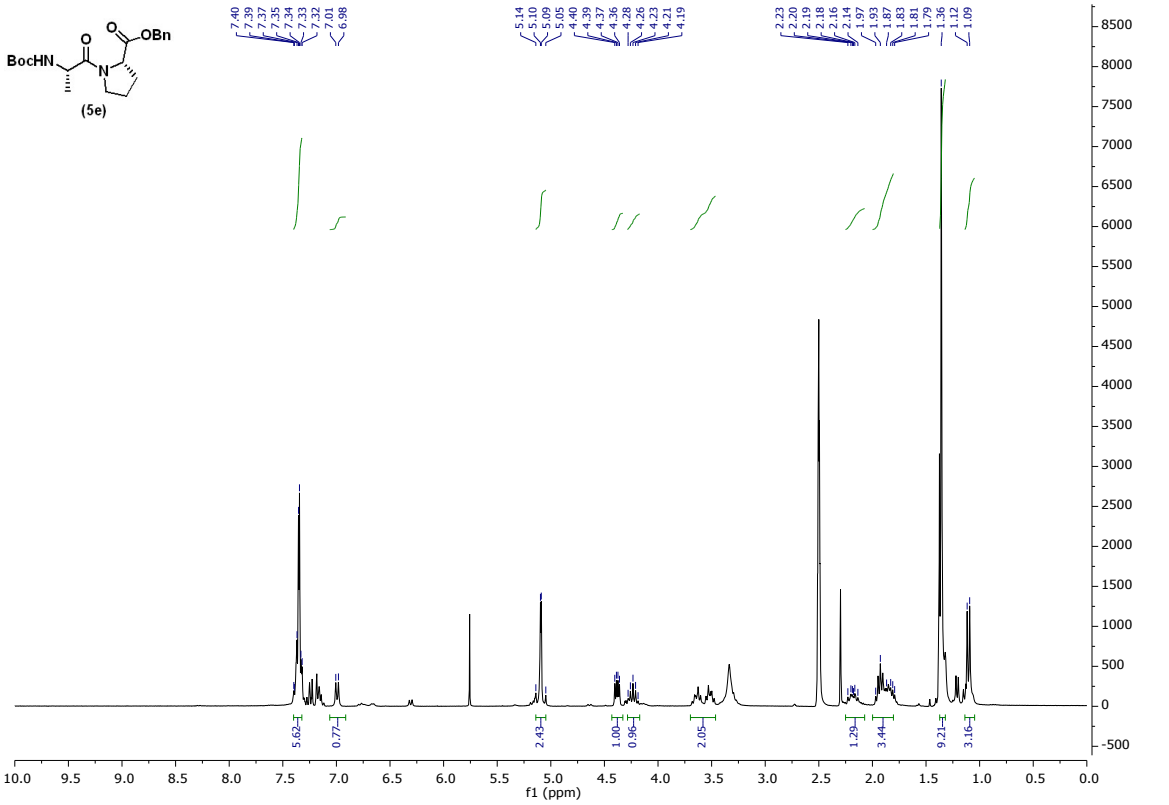
58.76
47.53
46.61
46.44
30.71
29.77
28.16
27.98
23.71
23.10
16.88
16.75
f1 (ppm)


BocHN
OBn
(5e)
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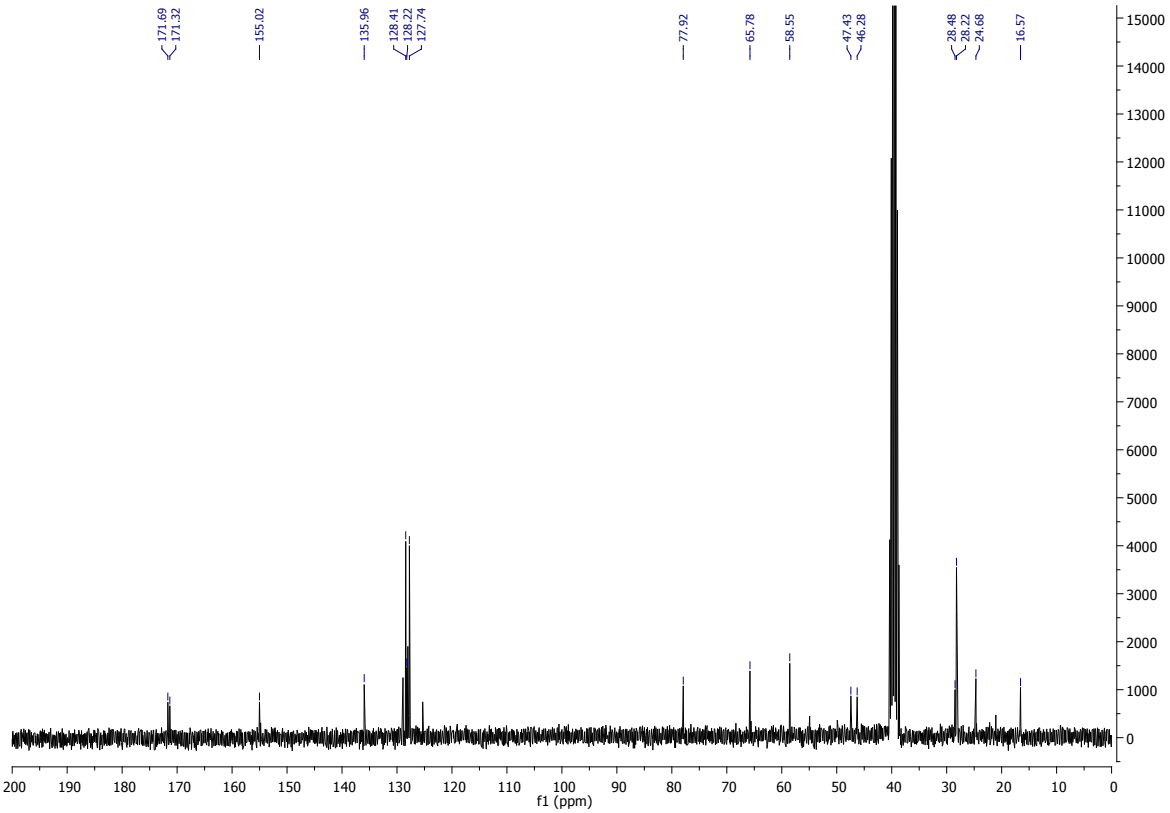
7.39
7.37
7.35
7.34
7.33
7.32
7.01
6.98
5.14
5.10
5.09
5.05
4.40
4.39
4.37
4.36
4.28
4.26
4.23
4.21
4.19
2.23
2.20
2.19
2.18
2.16
2.14
1.97
1.93
1.87
1.83
1.81
1.79
1.36
1.12
1.09
5.62
0.77
2.43
1.00
0.96
2.05
1.29
3.44
9.21
3.16
f1 (ppm)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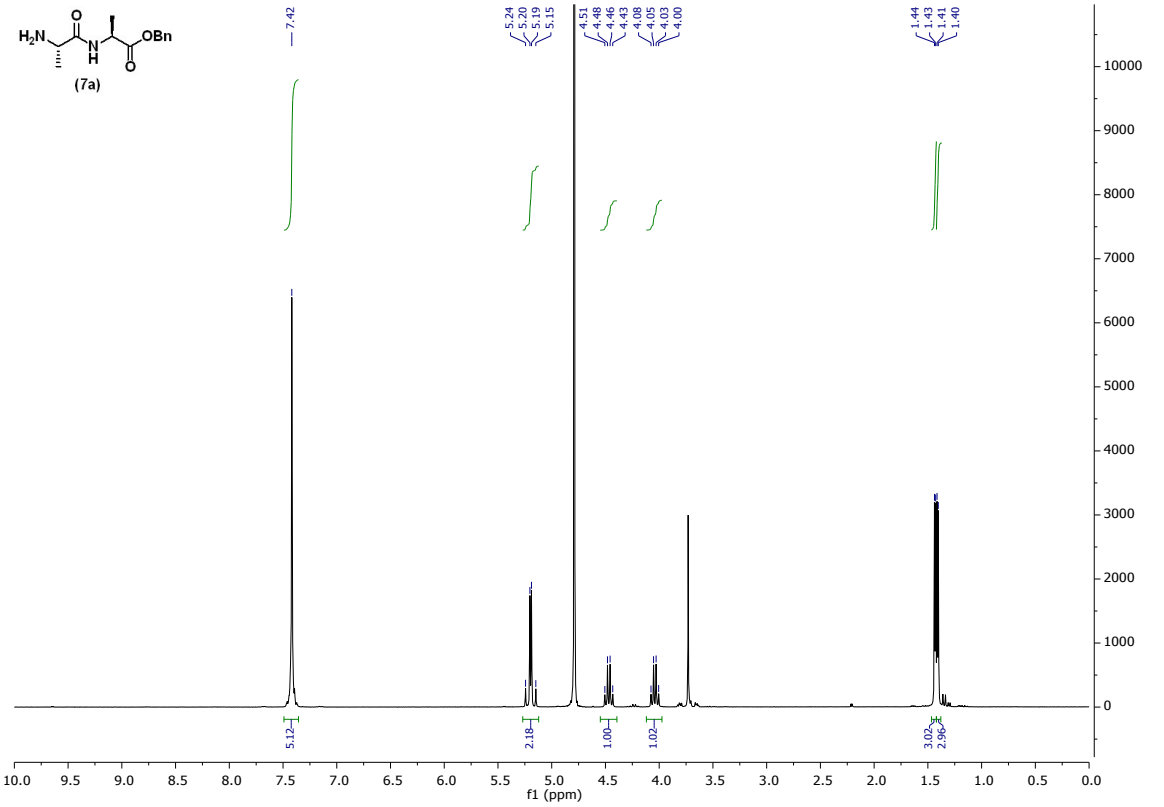
28.22
24.68
16.57
f1 (ppm)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5.19
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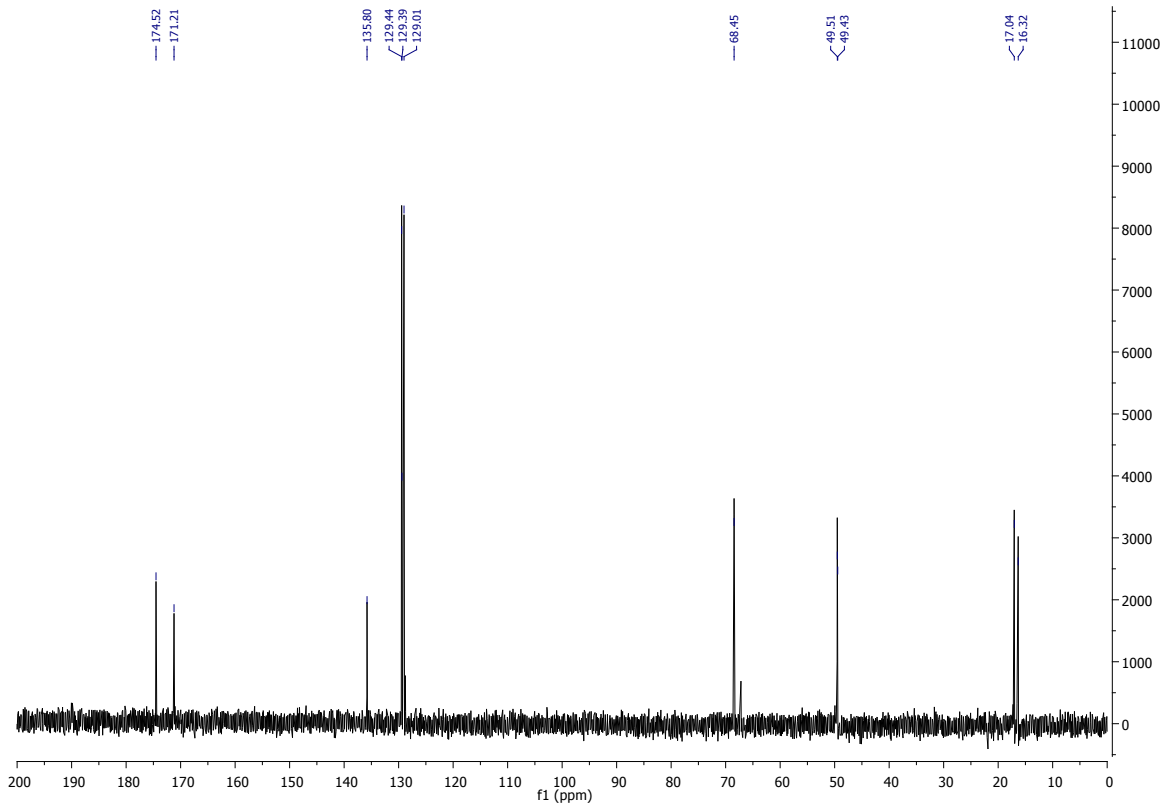
4.48
4.46
4.43
4.08
4.05
4.03
4.00
1.44
1.43
1.41
1.40
5.12
2.18
1.00
1.02
3.02
2.96
f1 (ppm)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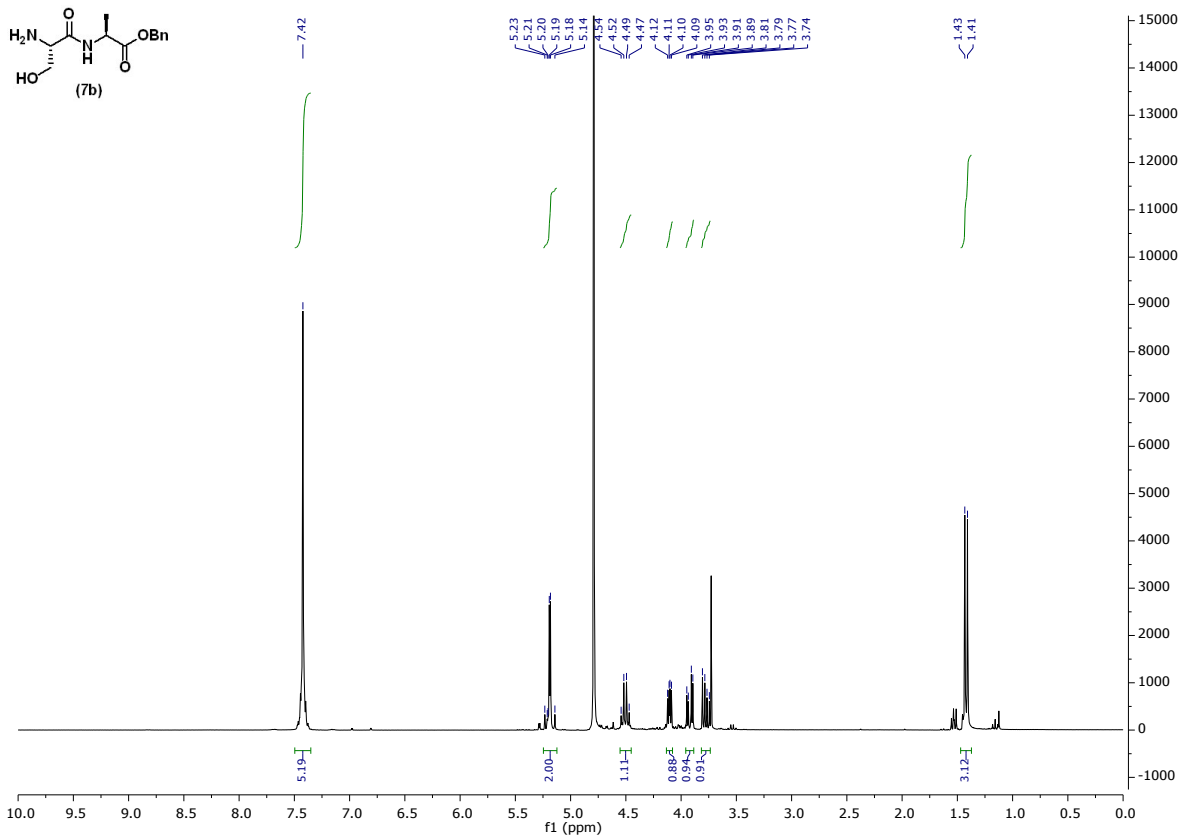
129.01
68.45
49.51
49.43
17.04
16.32
f1 (ppm)


(7b)
7.42
5.23
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5.14
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4.52
4.49
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4.12
4.11
4.10
4.09
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3.93
3.91
3.89
3.81
3.79
3.77
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1.41
5.19
2.00
1.11
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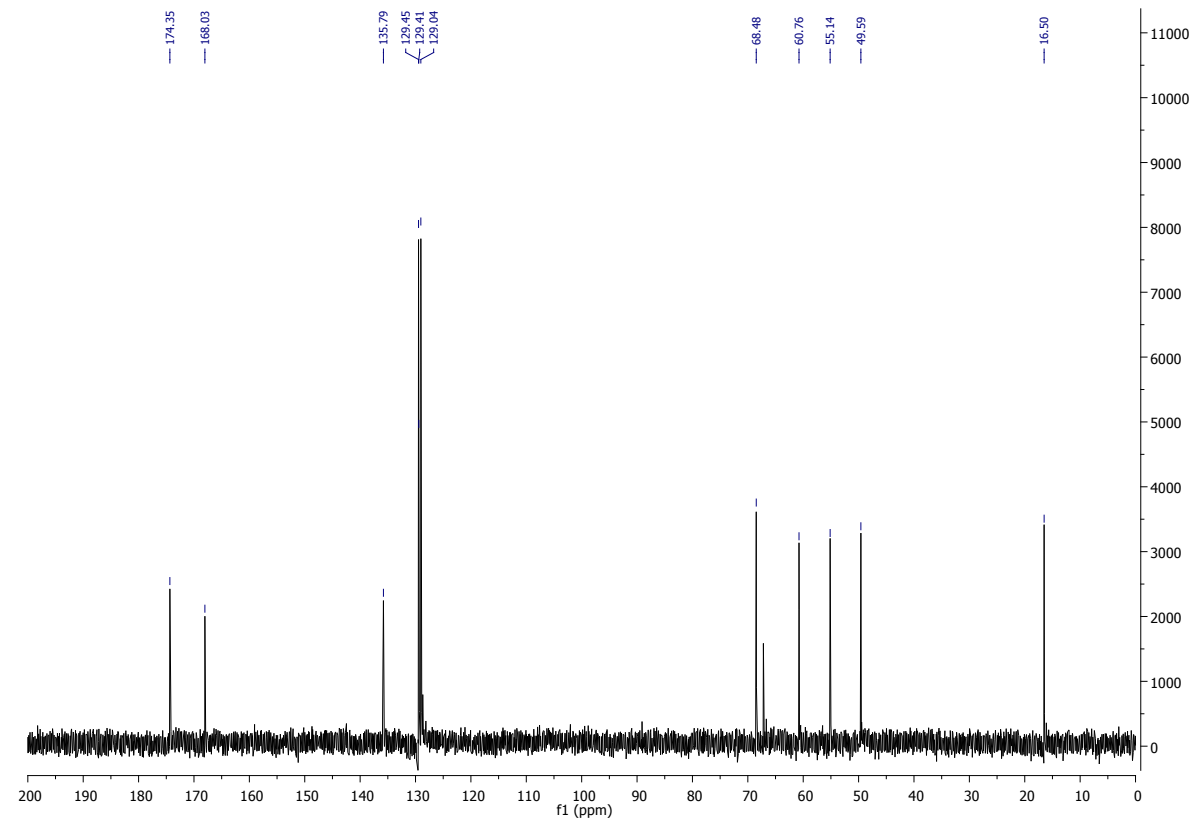
0.94
0.91
3.12
f1 (ppm)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129.04
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55.14
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16.50
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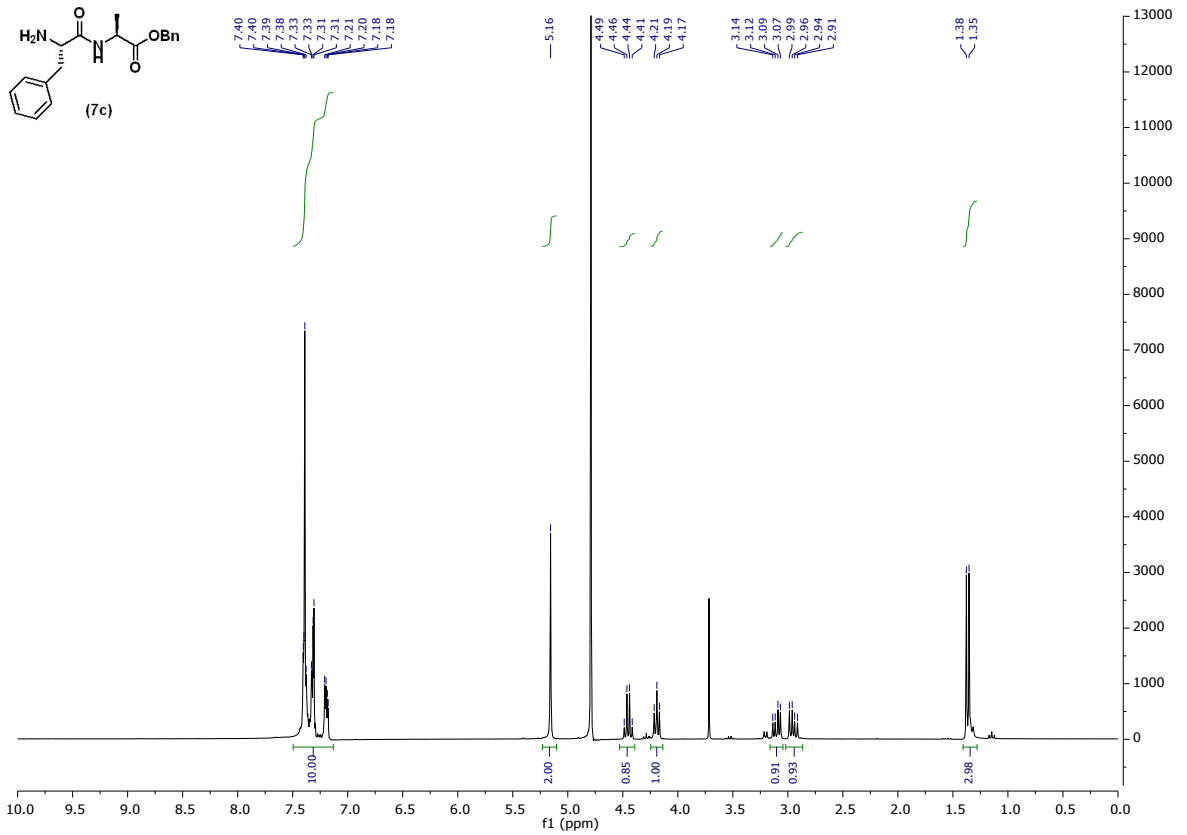
(7c)
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7.40
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7.33
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7.21
7.20
7.18
7.18
5.16
4.49
4.46
4.44
4.41
4.21
4.19
4.17
3.14
3.12
3.09
3.07
2.99
2.96
2.94
2.91
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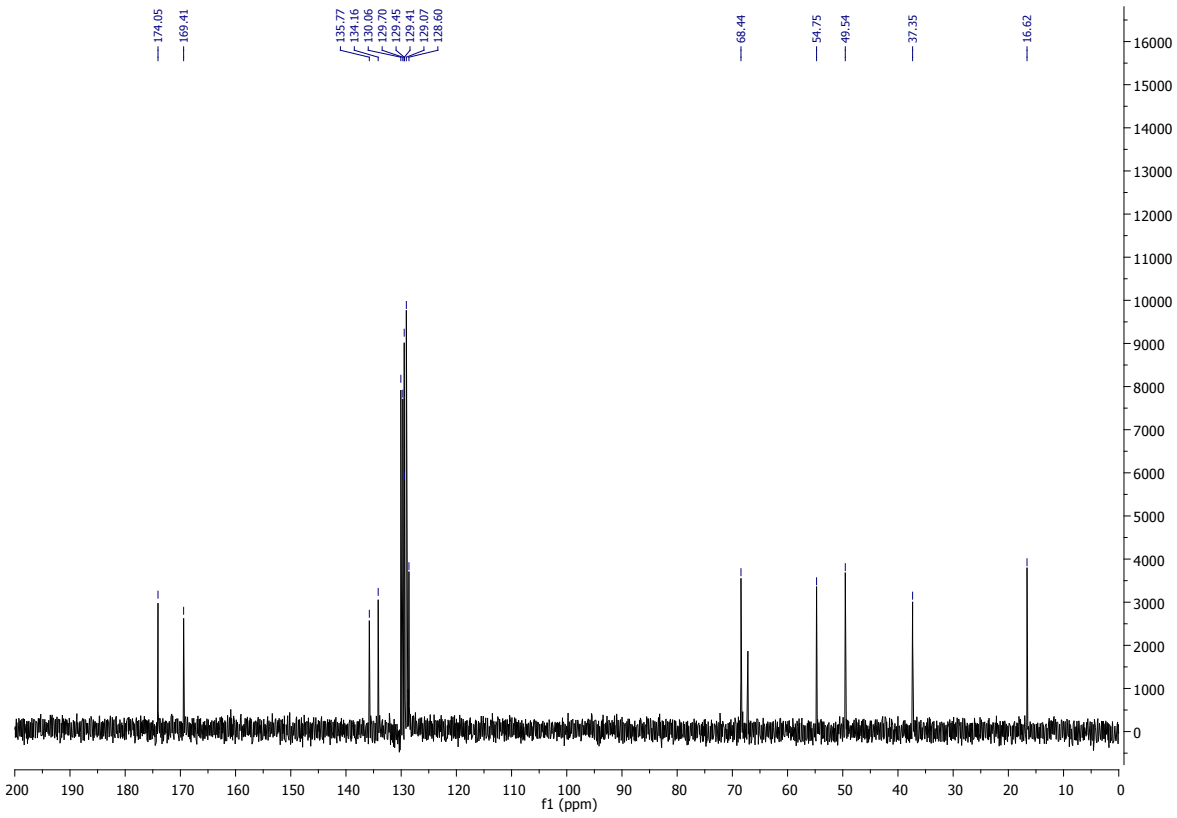
1.35
10.00
2.00
0.85
1.00
0.91
0.93
2.98
f1 (ppm)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129.07
128.60
68.44
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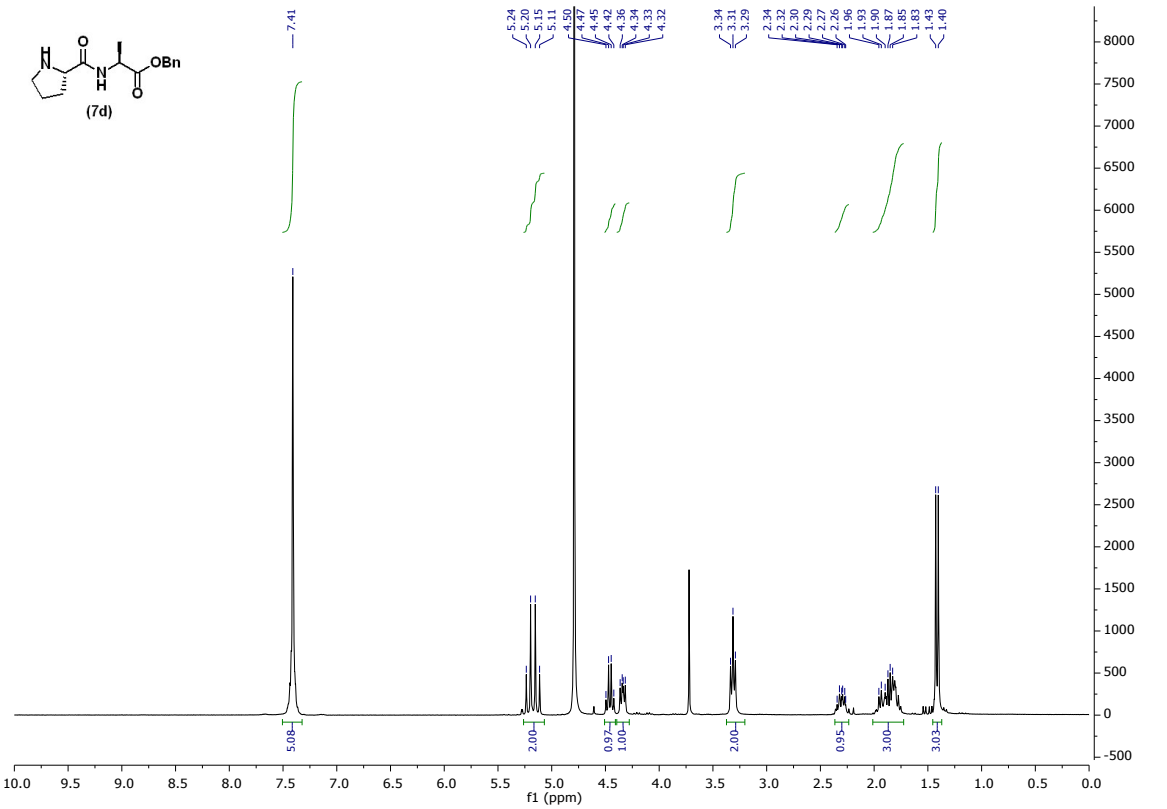
49.54
37.35
16.62
f1 (ppm)


(7d)
7.41
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5.11
4.50
4.47
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4.42
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3.29
2.34
2.32
2.30
2.29
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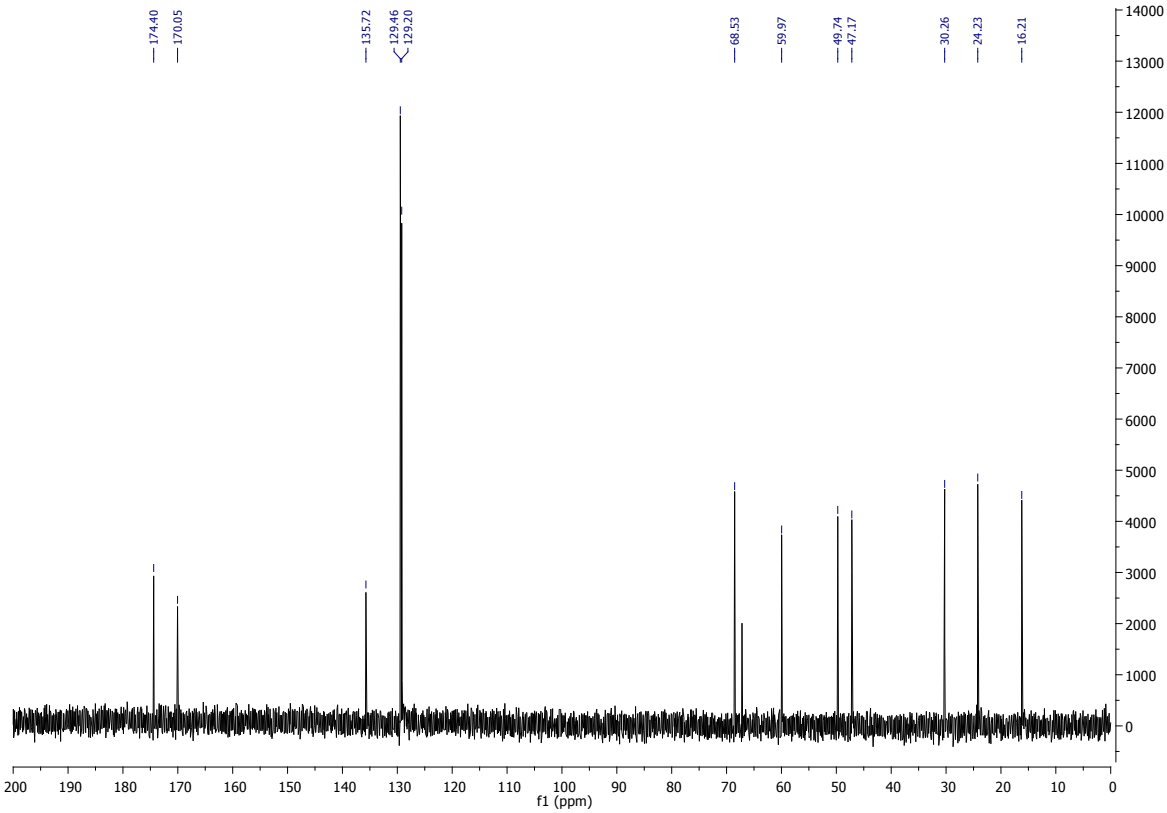
2.26
1.96
1.93
1.90
1.87
1.85
1.83
1.43
1.40
5.08
2.00
0.97
1.00
2.00
0.95
3.00
3.03
f1 (ppm)


174.40
170.05
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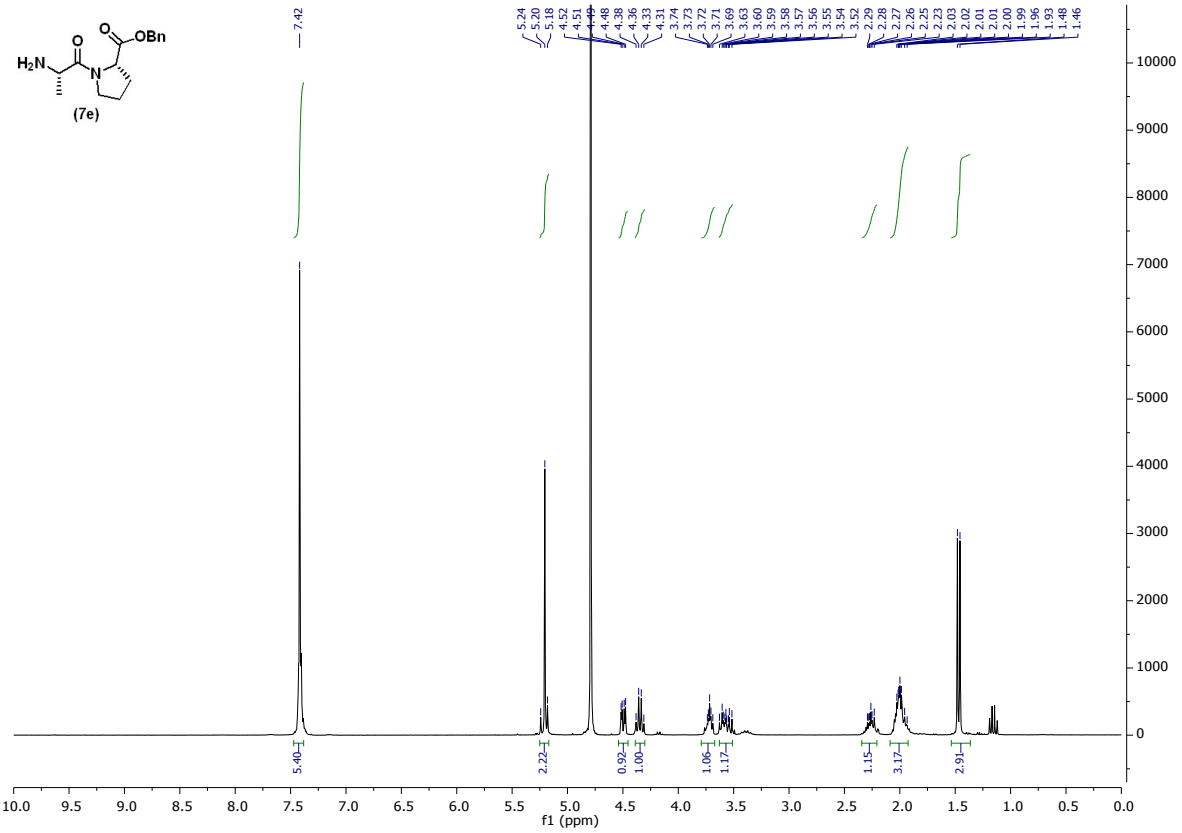
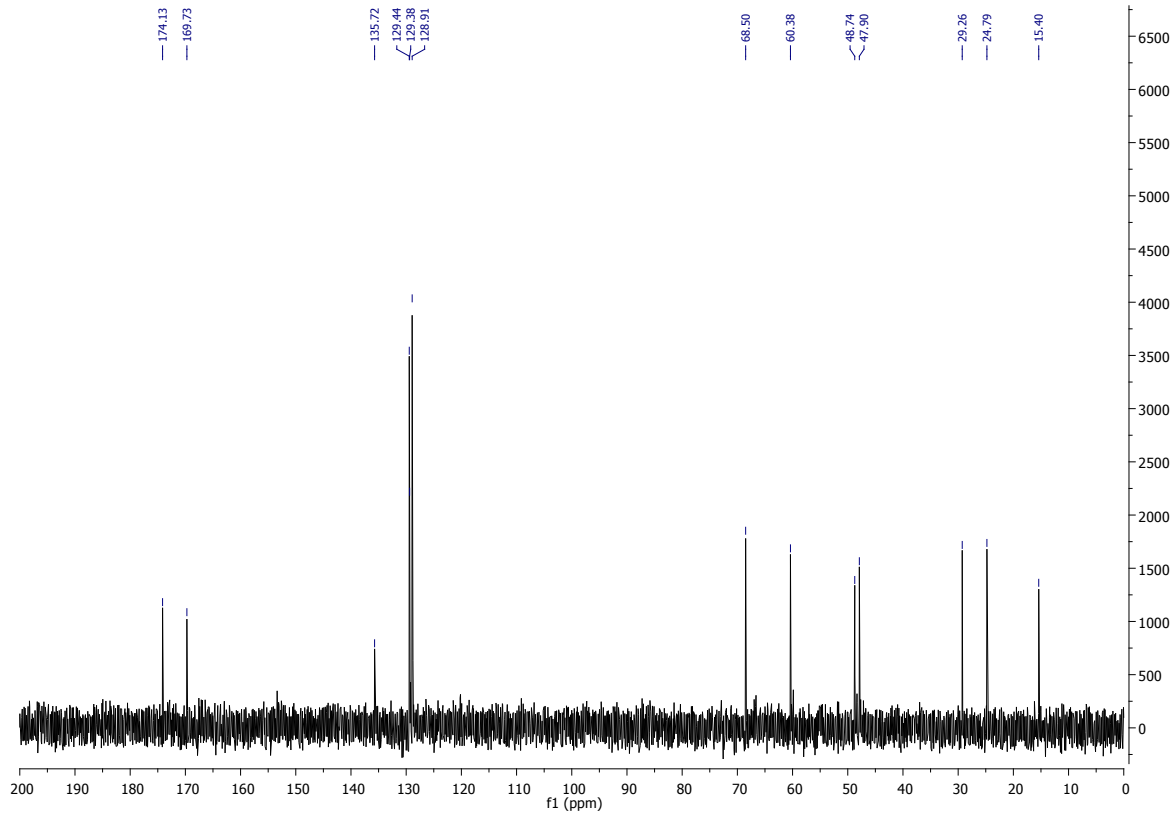
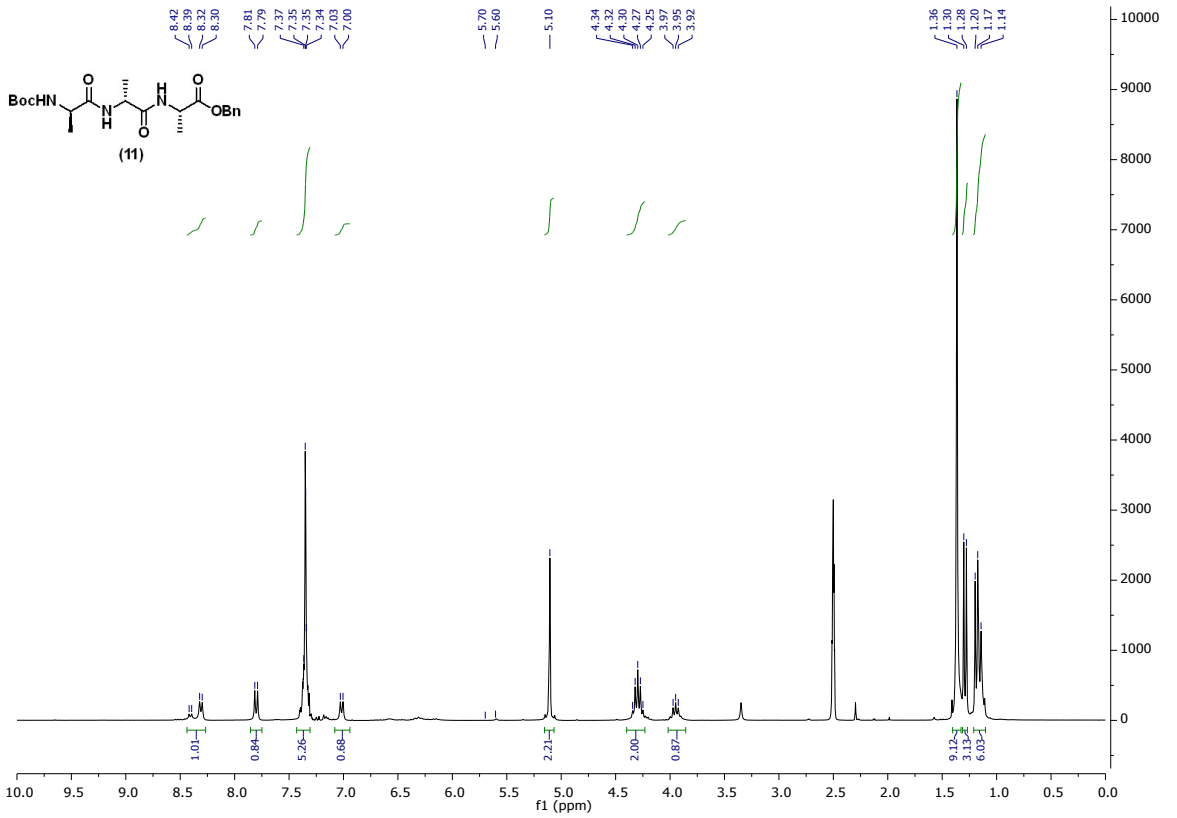
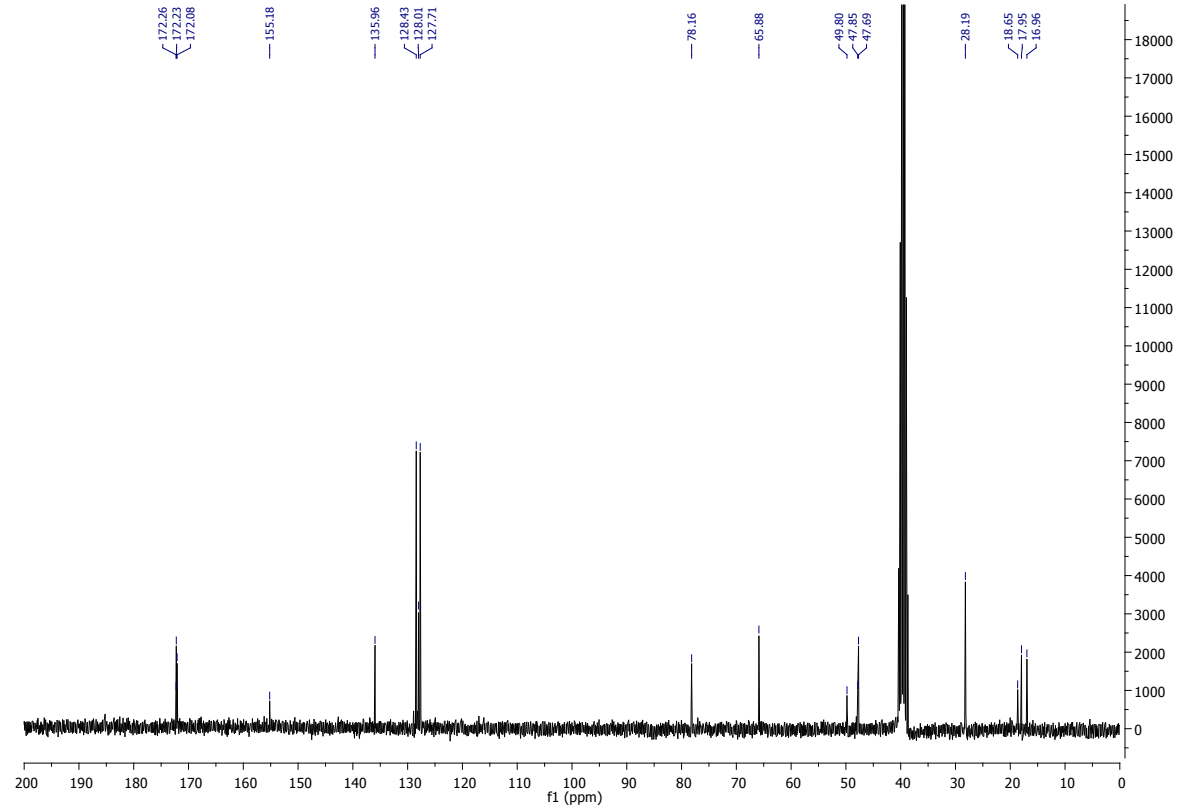
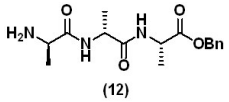
129.20
68.53
59.97
49.74
47.17
30.26
24.23
16.21
f1 (ppm)


(7e)
7.42
5.24
5.20
5.18
4.52
4.51
4.48
4.38
4.36
4.33
4.31
3.74
3.73
3.72
3.71
3.69
3.63
3.60
3.59
3.58
3.57
3.56
3.55
3.54
3.52
2.29
2.28
2.27
2.26
2.25
2.23
2.03
2.02
2.01
2.01
2.00
1.99
1.96
1.93
1.48
1.46
5.40
2.22
0.92
1.00
1.06
1.17
1.15
3.17
2.91
f1 (ppm)


174.13
169.73
135.72
129.44
129.38
128.91
68.50
60.38
48.74
47.90
29.26
24.79
15.40
f1 (ppm)

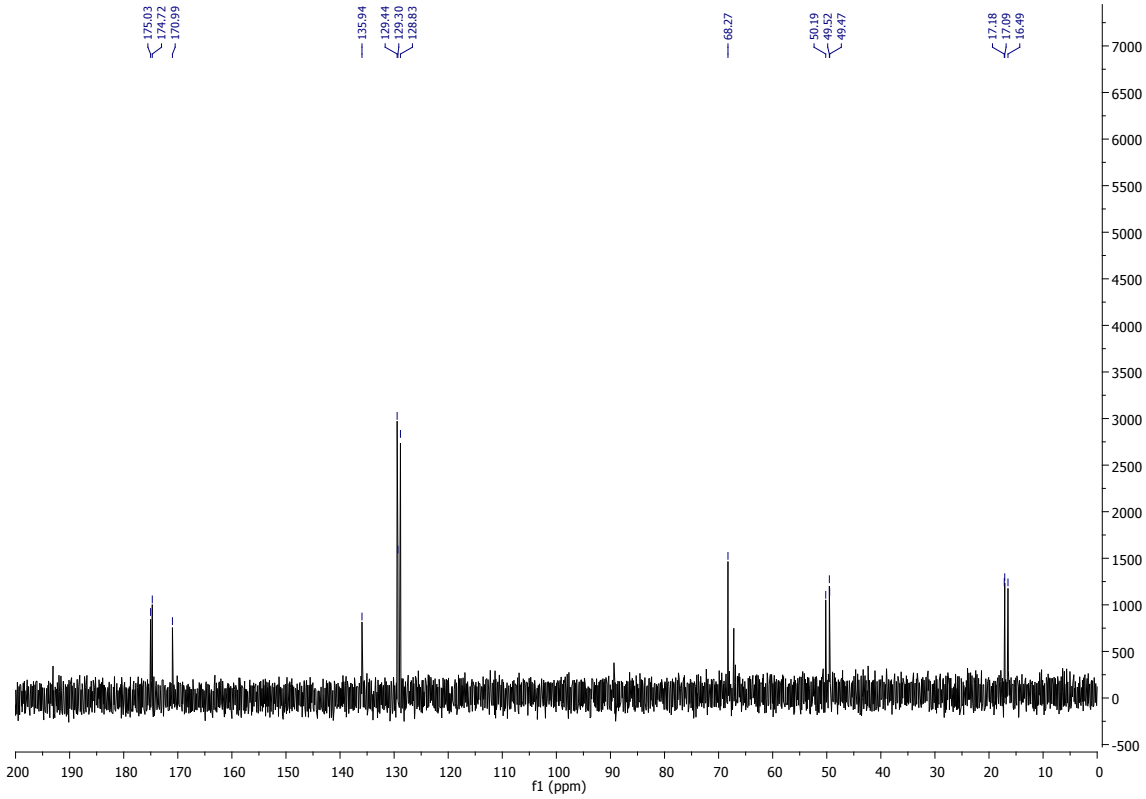

8.42
8.39
8.32
8.30
7.81
7.79
7.37
7.35
7.35
7.34
7.03
7.00
5.70
5.60
5.10
4.34
4.32
4.30
4.27
4.25
3.97
3.95
3.92
1.36
1.30
1.28
1.20
1.17
1.14
BocHN
OBn
(11)
1.01
0.84
5.26
0.68
2.21
2.00
0.87
9.12
3.13
6.03
f1 (ppm)


172.26
172.23
172.08
155.18
135.96
128.43
128.01
127.71
78.16
65.88
49.80
47.85
47.69
28.19
18.65
17.95
16.96
f1 (ppm)


H2N
O
N
H
O
N
H
O
OBn

(12)

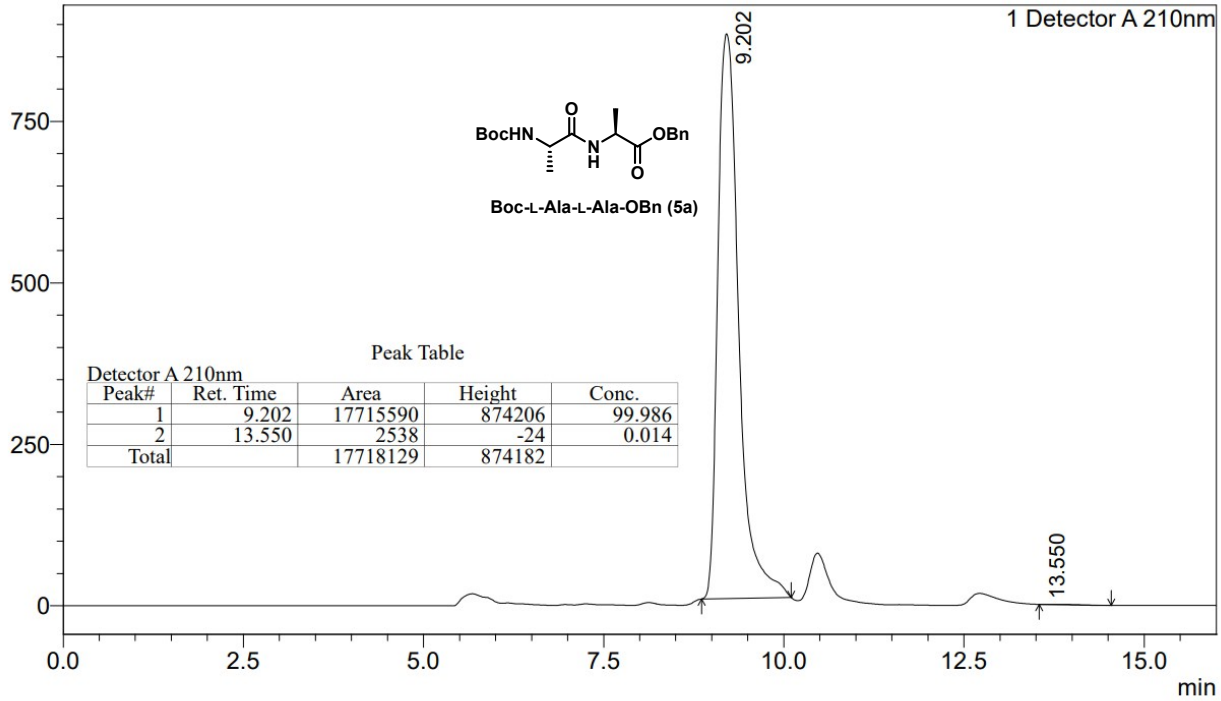
7.41
5.19
4.42
4.40
4.37
4.35
4.32
4.30
4.11
4.09
4.06
4.04
4.02
1.50
1.47
1.40
1.38
1.36
1.33
5.34
2.35
2.00
1.00
2.81
3.20
2.90
f1 (ppm)


175.03
174.72
170.99
135.94
129.44
129.30
128.83
68.27
50.19
49.52
49.47
17.18
17.09
16.49
f1 (ppm)

2.8 Chiral HPLC Chromatograms

Chiral HPLC analysis was used to determine the level of epimerization during the reaction. The chiral HPLCs were conducted using the reaction product after phase separation and evaporation. The sample was dissolved in iPrOH/n-hexane (1:1). Even though there were other peaks observed in the chiral HPLCs these compounds could be removed during the Boc cleavage protocol. A mixed sample of both possible epimers was prepared for each dipeptide to check that the undesired epimer was not formed during the flow process.

Chiral HPLC chromatograph from flow protocol to synthesize *N*-Boc-L-Ala-L-Ala-OBn (5a)

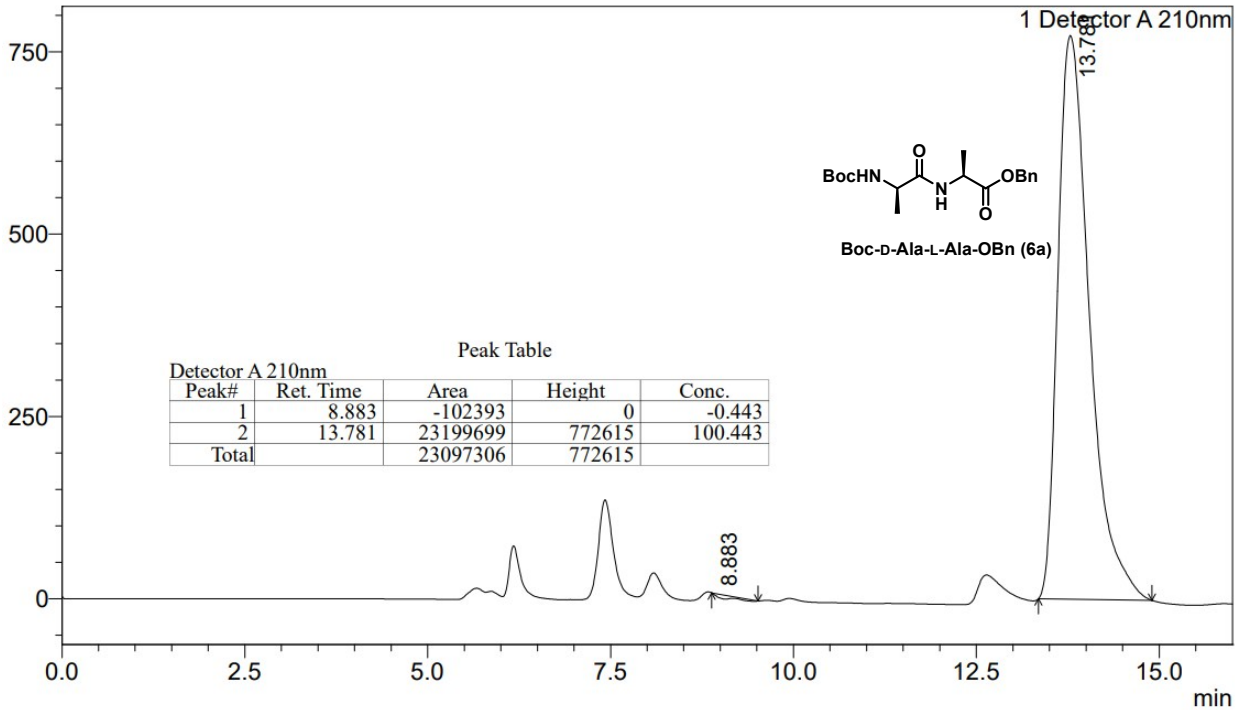


Peak Table

Detector A 210nm

Peak#	Ret. Time	Area	Height	Conc.
1	9.202	17715590	874206	99.986
2	13.550	2538	-24	0.014
Total		17718129	874182	

Chiral HPLC chromatograph from flow protocol to synthesize *N*-Boc-D-Ala-L-Ala-OBn (6a)

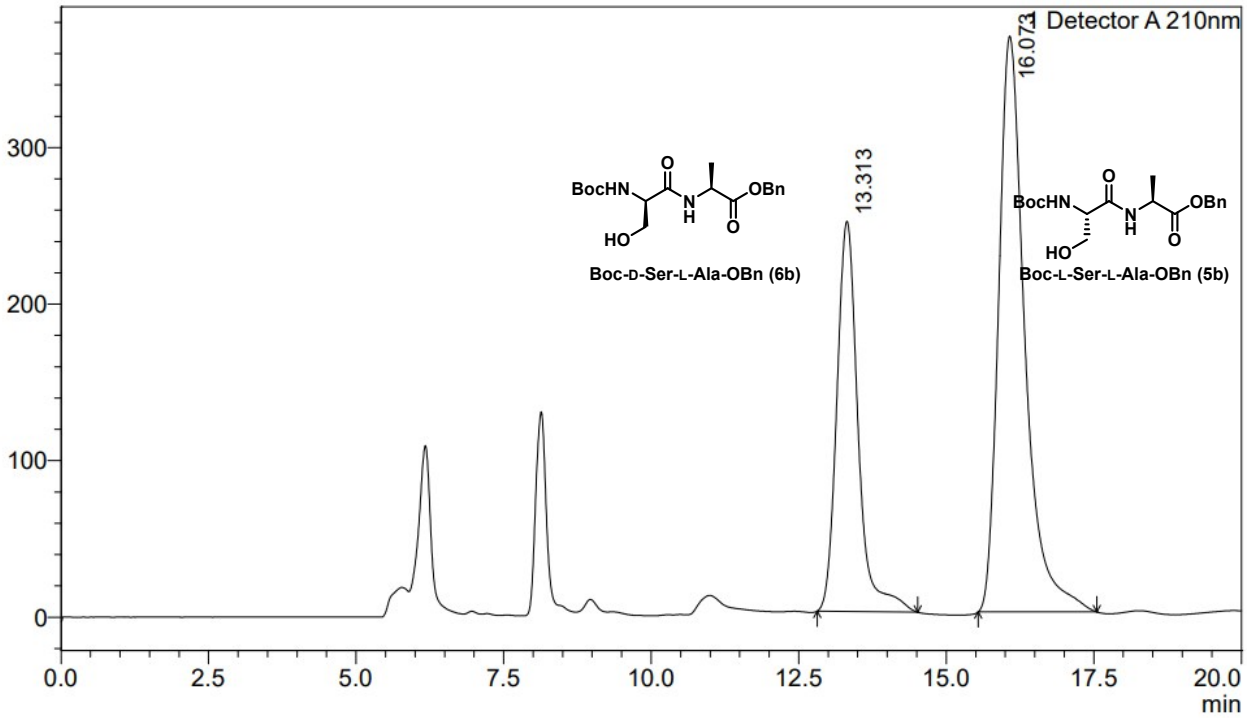


Peak Table

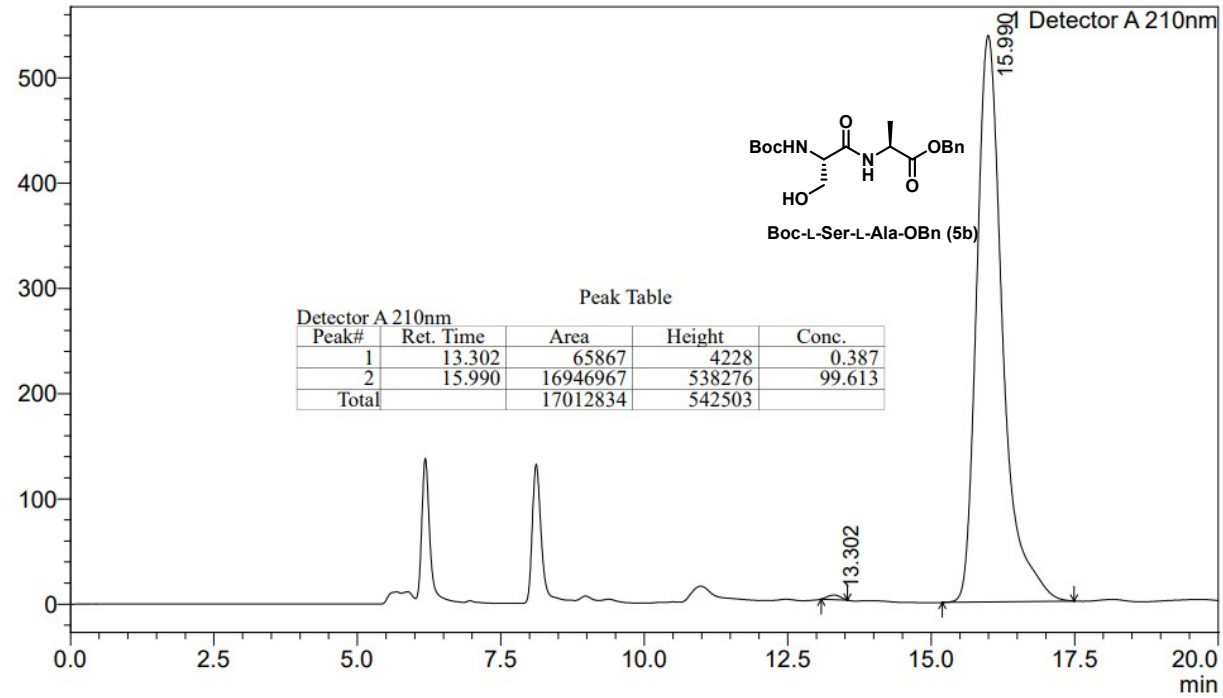
Detector A 210nm

Peak#	Ret. Time	Area	Height	Conc.
1	8.883	-102393	0	-0.443
2	13.781	23199699	772615	100.443
Total		23097306	772615	

Chiral HPLC chromatograph of *N*-Boc-L-Ser-L-Ala-OBn (5b) and *N*-Boc-D-Ser-L-Ala-OBn (6b) mixture



Chiral HPLC chromatograph from flow protocol to synthesize *N*-Boc-L-Ser-L-Ala-OBn (5b)

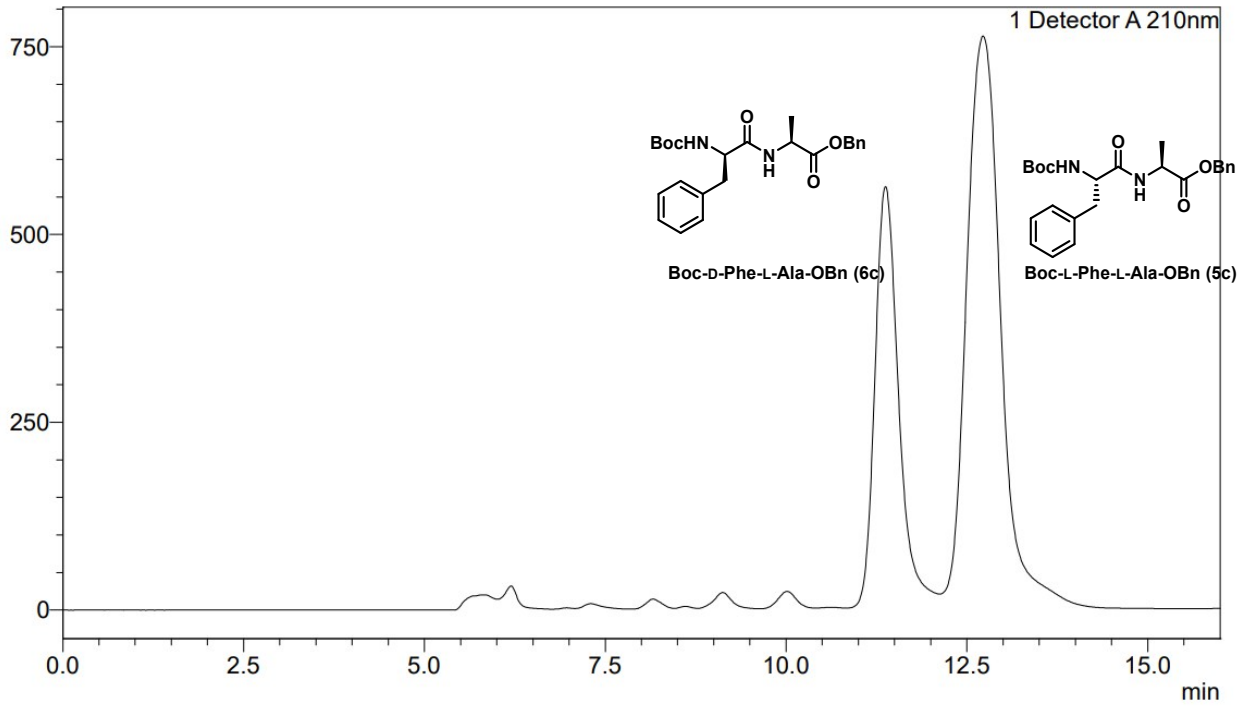


Peak Table

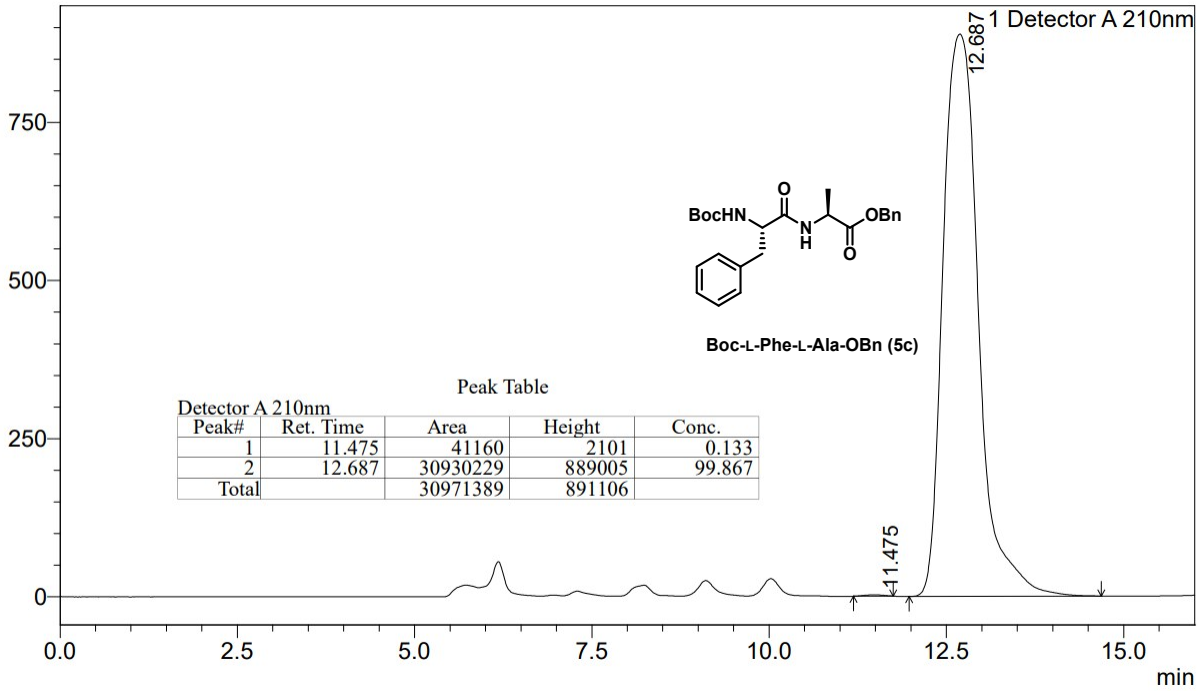
Detector A 210nm

Peak#	Ret. Time	Area	Height	Conc.
1	13.302	65867	4228	0.387
2	15.990	16946967	538276	99.613
Total		17012834	542503	

Chiral HPLC chromatograph of *N*-Boc-L-Phe-L-Ala-OBn (5c) and *N*-Boc-D-Phe-L-Ala-OBn (6c) mixture.



Chiral HPLC chromatograph from flow protocol to synthesize *N*-Boc-L-Phe-L-Ala-OBn (5c)

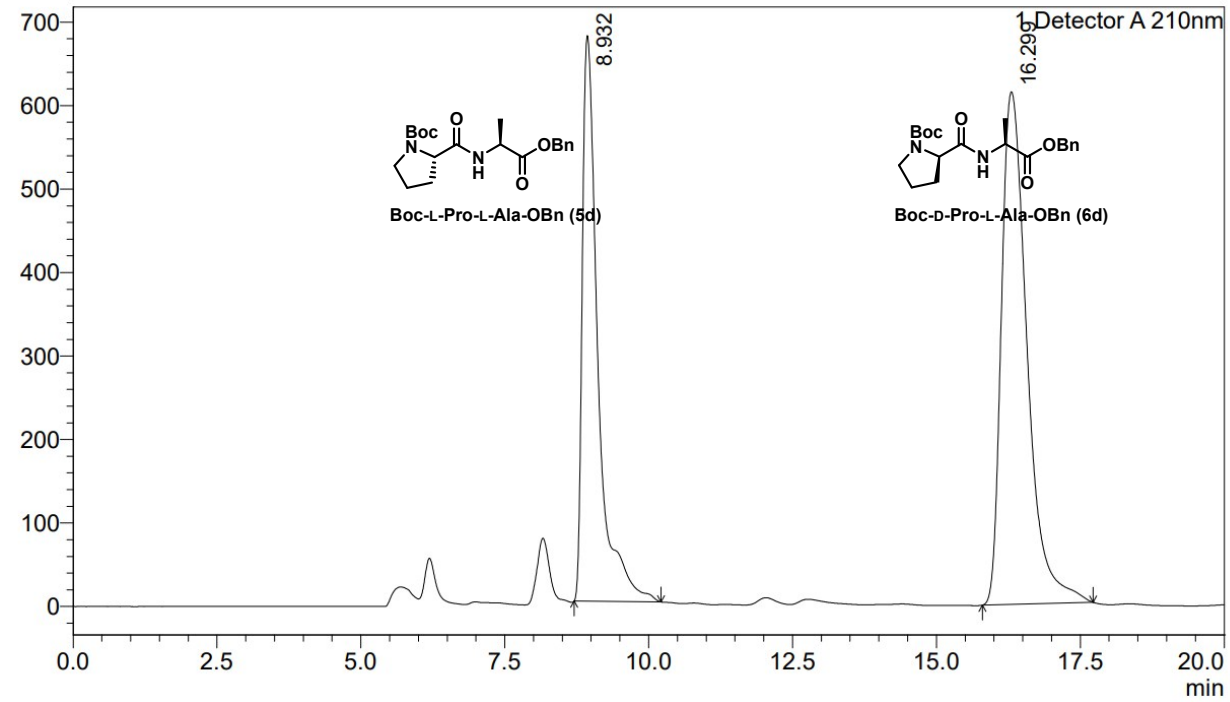


Peak Table

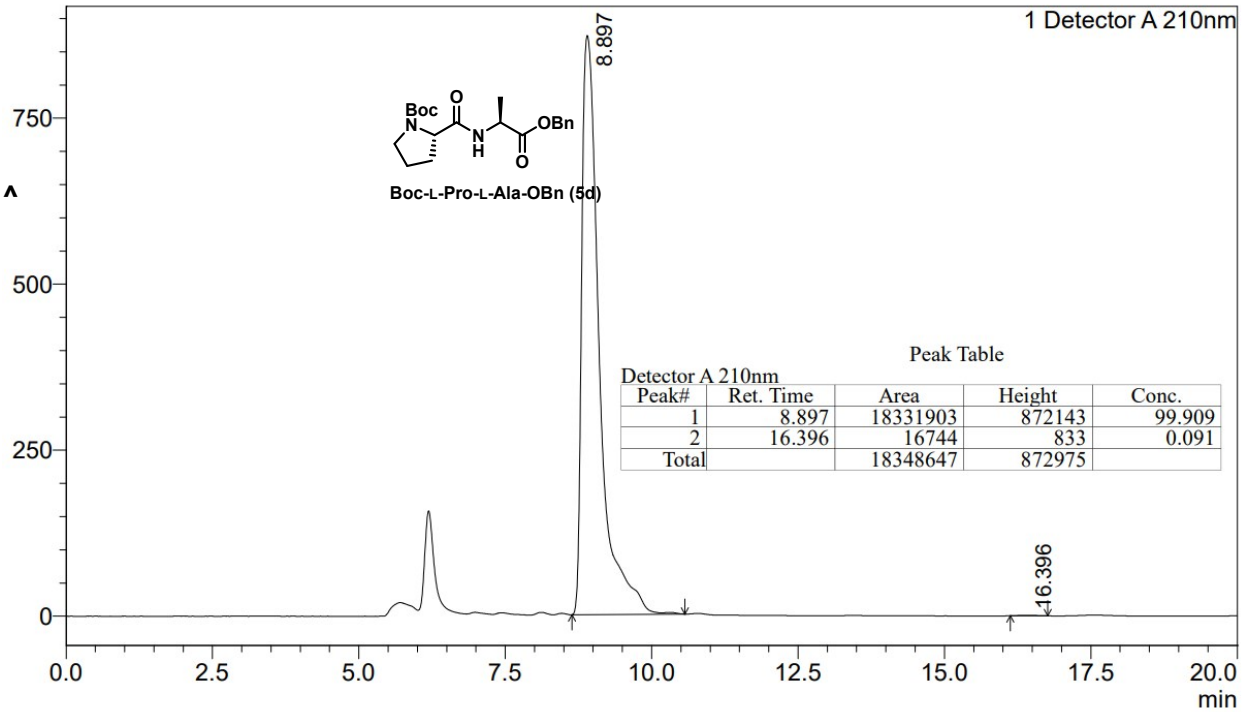
Detector A 210nm

Peak#	Ret. Time	Area	Height	Conc.
1	11.475	41160	2101	0.133
2	12.687	30930229	889005	99.867
Total		30971389	891106	

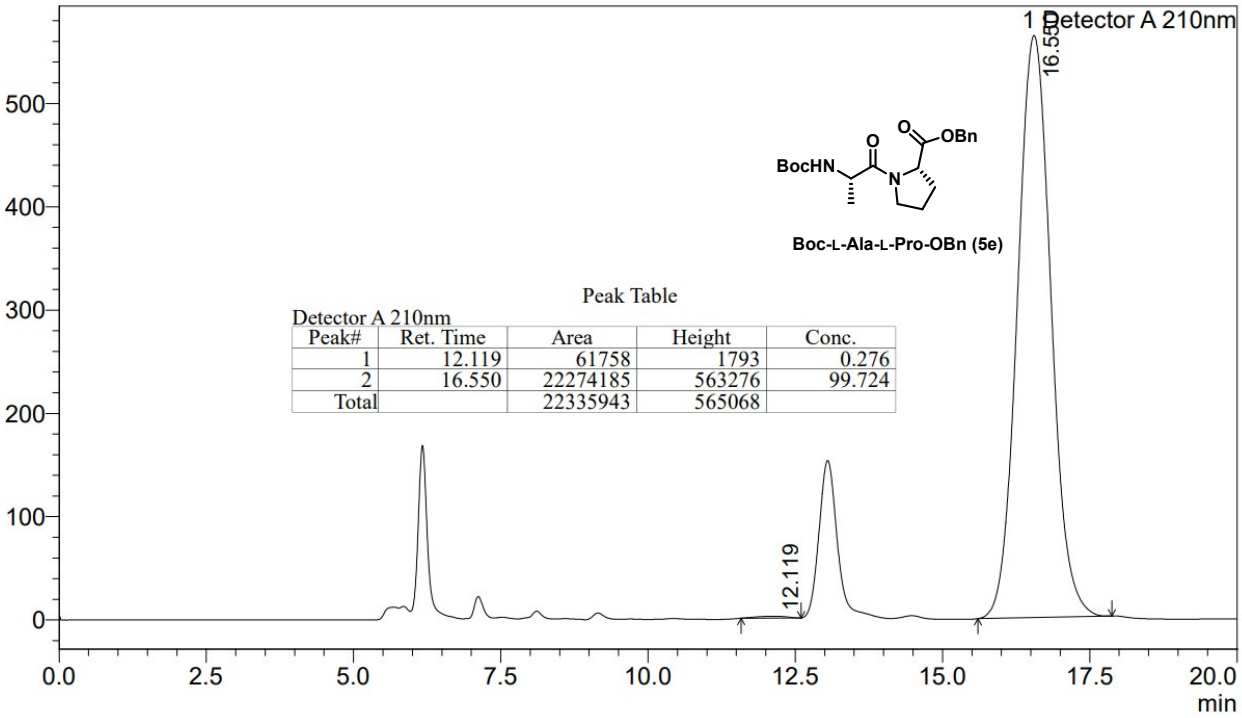
Chiral HPLC chromatograph of *N*-Boc-L-Pro-L-Ala-OBn (5d) and *N*-Boc-D-Pro-L-Ala-OBn (6d) mixture.



Chiral HPLC chromatograph from flow protocol to synthesize *N*-Boc-L-Pro-L-Ala-OBn (5d)



Chiral HPLC chromatograph from flow protocol to synthesize *N*-Boc-L-Ala-L-Pro-OBn (5e)

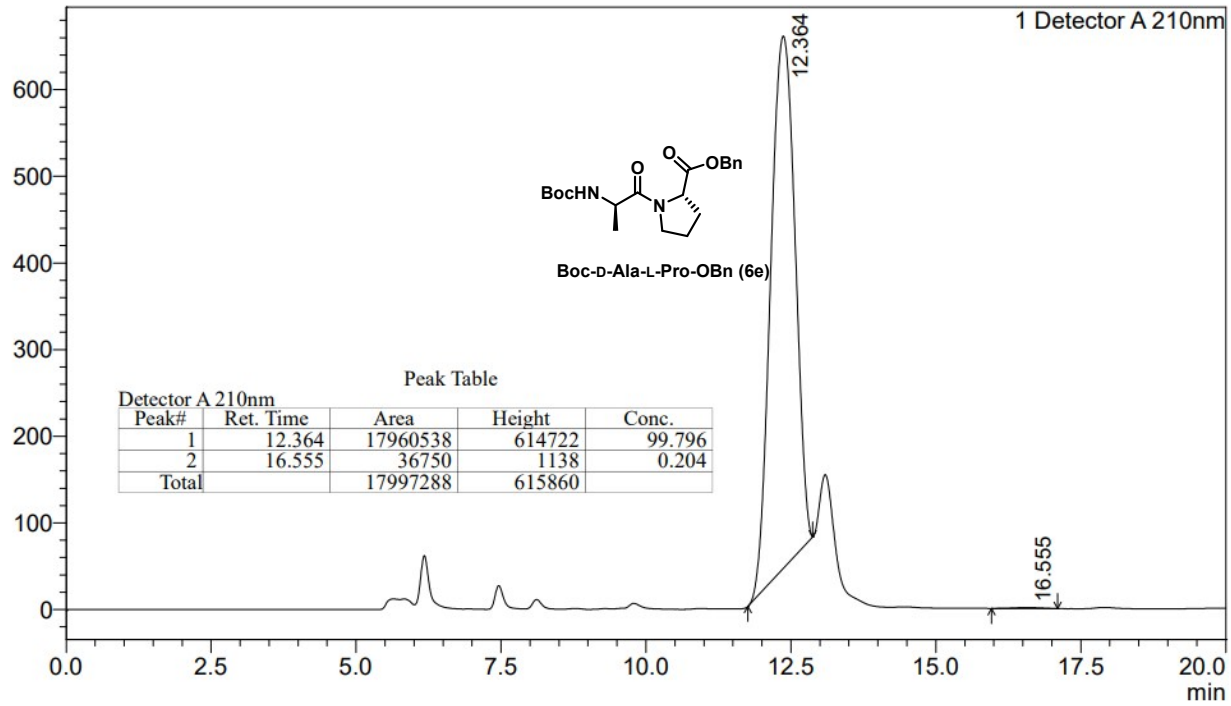


Peak Table

Detector A 210nm

Peak#	Ret. Time	Area	Height	Conc.
1	12.119	61758	1793	0.276
2	16.550	22274185	563276	99.724
Total		22335943	565068	

Chiral HPLC chromatograph from flow protocol to synthesize *N*-Boc-D-Ala-L-Pro-OBn (6e)



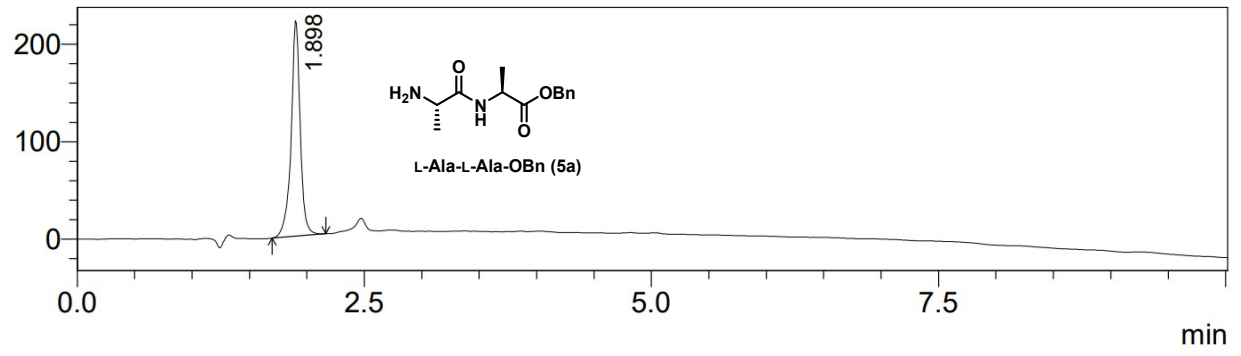
Peak Table

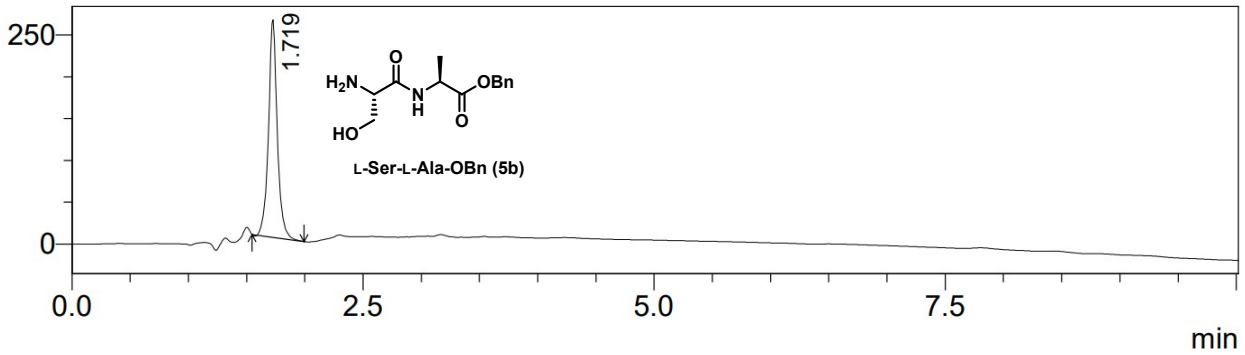
Detector A 210nm

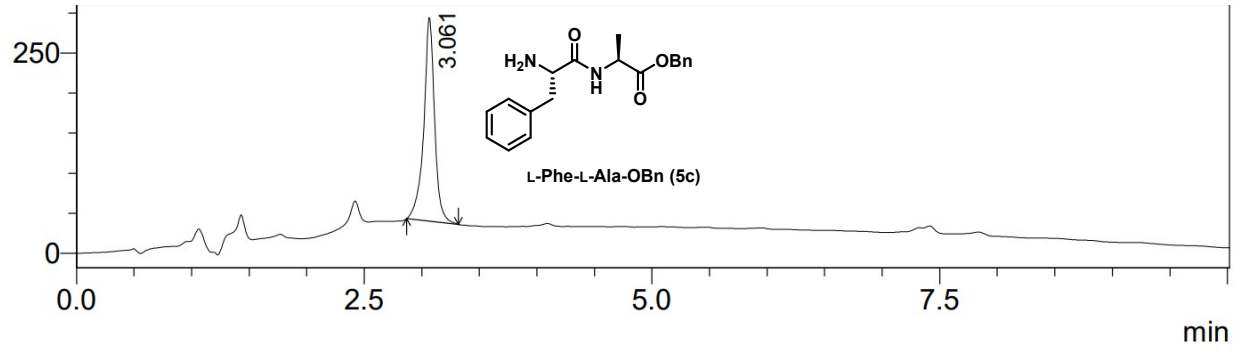
Peak#	Ret. Time	Area	Height	Conc.
1	12.364	17960538	614722	99.796
2	16.555	36750	1138	0.204
Total		17997288	615860	

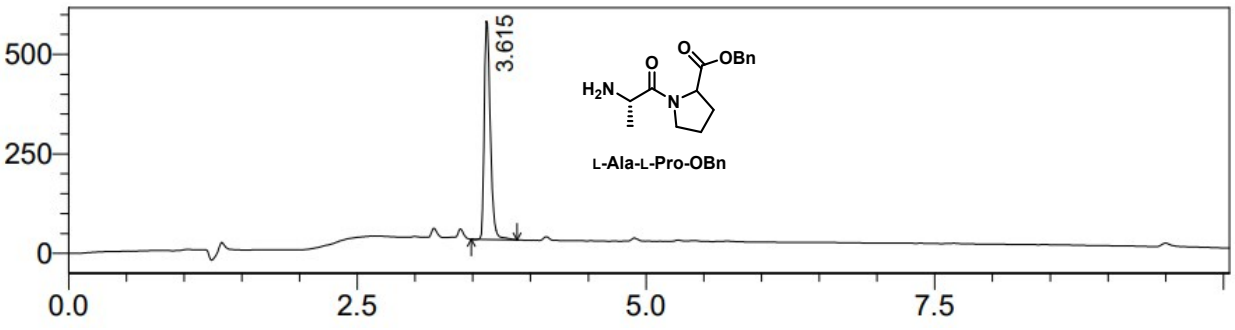
2.9 HPLC Chromatograms

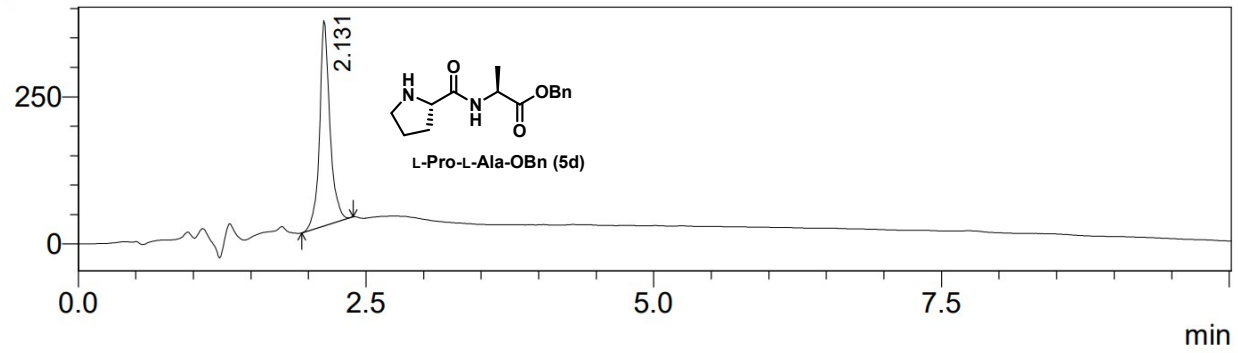
The Boc cleavage acted as a method to purify the peptides from the acyl azide generation and amide coupling stage. HPLCs of the final benzyl ester peptides after Boc cleavage are shown below.

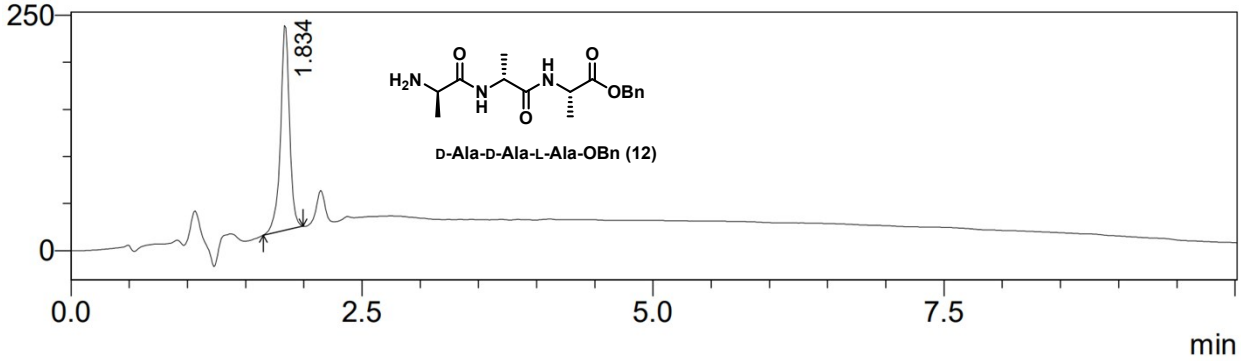









2.131
250
0
0.0
2.5
5.0
7.5
min
L-Pro-L-Ala-OBn (5d)


1.834
250
0
0.0
2.5
5.0
7.5
min
D-Ala-D-Ala-L-Ala-OBn (12)