Supporting Information

Identification of c[D-Trp-Phe-β-Ala-β-Ala], the First κ-Opioid Receptor-Specific Negative Allosteric Modulator

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Table S1. RP HPLC and ESI MS analyses of the linear precursors and of the CPs **5-13**, and respective cyclization yields.

Linear peptide	Purity (%) [a]	ESI MS [M+1] found/calcd. [b]	CP	CP sequence	Cyclization Yield (%) [c]	CP ES-MS [M+1] found/calcd. [b]	CP Purity (%) [c,d]
H-Gly-D-Trp-Phe-GlyOH	80	466.2/466.2090	**5**	c[D-Trp-Phe-Gly-Gly]	68	448.2/448.1985	94
H-Ala-D-Trp-Phe-GlyOH	85	480.2/480.2247	**6**	c[D-Trp-Phe-Gly-Ala]	69	462.2/462.2141	96
H-D-Ala-D-Trp-Phe-GlyOH	78	480.2/480.2247	**7**	c[D-Trp-Phe-Gly-D-Ala]	63	462.4/462.2141	96
H-Gly-D-Trp-Phe-β-AlaOH	81	480.3/480.2247	**8**	c[D-Trp-Phe-β-Ala-Gly]	80	462.2/462.2141	95
H-D-Trp-Phe-AhaOH [e]	83	465.3/465.2502	**9**	c[D-Trp-Phe-Aha] [e]	74	447.4/447.2396	95
See Scheme 1	77	472.2/473.2553	**10**	c[D-Trp-Phe-Ah-3-ea] [f]	75	445.4/445,5430	96
H-β-Ala-D-Trp-Phe-β-AlaOH	83	494.2/494.2403	**11**	c[D-Trp-Phe-β-Ala-β-Ala]	78	476.2/476.2298	96
H Gly-D-Trp-Phe-GABAOH	81	494.3/494.2403	**12**	c[D-Trp-Phe-GABA-Gly]	81	476.4/476.2298	98
H-GABA-D-Trp-Phe-GlyOH	79	494.2/494.2403	**13**	c[D-Trp-Phe-Gly-GABA]	77	476.4/476.2298	96

[a] Determined by analytical RP-HPLC on a C18 column Phenomenex Gemini 3μ C18 110 Å 100 3 3.0 mm, mobile phase from 9:1 H_2O/CH_3CN/0.1% HCOOH to 2:8 H_2O/CH_3CN/0.1% HCOOH in 20 min at a flow rate of 1.0 mL min^{-1}. [b] MS single quadrupole HP 1100MSD detector. [c] Determined after semi-preparative RP HPLC on a C18 RP column ZORBAX Eclipse XDBC18 PrepHT cartridge 21.2 3x150 mm 7μ, mobile phase from 8:2 H_2O/CH_3CN to 100% CH_3CN, in 10 min, flow rate 12 mL min^{-1}. [d] Determined by analytical RP-HPLC, same stationary phase as for [a], mobile phase from 9:1 H_2O/CH_3CN to 2:8 H_2O/CH_3CN in 20 min, flow rate 1.0 mL min^{-1}. [e] Aha, 6-aminohexanoic acid. [f] Ah-4-ea, 6-aminohex-4-enoic acid.

c[D-Trp-Phe-Ah-3-ea] (***10***). Boc-Phe-OH (0.29 g, 1.1 mmol) was dissolved in DCM/DMF (3 mL/1 mL), then HOBt (0.15 g, 1.1 mmol) was added and the mixture was stirred for 10 min, followed by EDC hydrochloride (0.21 g, 1.1 mmol), allylamine (63 mg, 1.1 mmol), triethylamine (0.32 mL, 2.2 mmol). The mixture was stirred overnight at RT. Afterwards, the reaction was concentrated at reduced pressure and the residue was suspended in EtOAc (30 mL). The mixture was washed with 1M HCl (6 mL), and with saturated Na_2CO_3 (6 mL). The organic layer was collected and dried over anhydrous Na_2SO_4, and the solvent was removed at reduced pressure to obtain crude Boc-Phe-allylamine (0.24 g, 0.79 mmol, 72%) as an oily residue.

To remove Boc group, the crude Boc-Phe-allylamine (0.24 g, 0.79 mmol) was dissolved in TFA/DCM (1 mL/3 mL), and the mixture was stirred for 2 hours at RT. After removing the volatiles at reduced pressure, the residue was triturated with ice-cold Et_2O to give H-Phe-allylamine·TFA salt (0.23 g, 0.72 mmol, 91 %) as a white powder, which was used directly for next coupling reaction without further purifications.

Boc-D-Trp-OH (0.26g, 0.86 mmol) and HOBt (0.11 g, 0.86 mmol) were dissolved in DCM/DMF (3 mL/1 mL), and the mixture was stirred for 10 min, followed by EDC hydrochloride (0.16 g, 0.86 mmol), H-Phe-allylamine·TFA (0.23 g, 0.72 mmol), triethylamine (0.25 mL, 1.7 mmol) in sequence. The mixture was stirred overnight at RT, and work-up and Boc deprotection procedures were performed as described above, giving crude H-Trp-Phe-allylamine·TFA (0.34 g, 0.68 mmol, 94 %) as a white powder.

Subsequently, 4-pentenoic acid (0.10 g, 1.0 mmol) and HOBt (0.14 g, 1.0 mmol) were dissolved in DCM/DMF (3 mL/1 mL), and the mixture was stirred for 10 min, followed by EDC hydrochloride (0.19 g, 1.0 mmol), H-D-Trp-Phe-allylamine (0.34 g, 0.68 mmol), triethylamine (0.29 mL, 2.1 mmol). The mixture was stirred overnight at RT, and work-up procedure was repeated as described above, giving crude 4-pentenoic-D-Trp-Phe-allylamine (0.28 g, 0.60 mmol, 88 %) as a waxy solid.

RCM reaction. Grubb's 1 catalyst (9 mg, 8.5% mol/mol) was dissolved in anhydrous DCM (20 mL) and the mixture was stirred for 10 min under nitrogen. Then, a solution of 4-pentenoic-D-Trp-Phe-allylamine (0.060 g, 0.13 mmol) in DCM (3 mL) was added, and the mixture was stirred for 30 min at rt, then it was refluxed for 36h. The completion of the reaction was monitored by TLC. After filtration through celite, the filtrate was evaporated at reduced pressure, and the residue was purified by semipreparative RP HPLC (General methods), giving **10** (0.044 g, 0.1 mmol, 77%, 96% pure as determined by analytical RP HPLC, General methods).

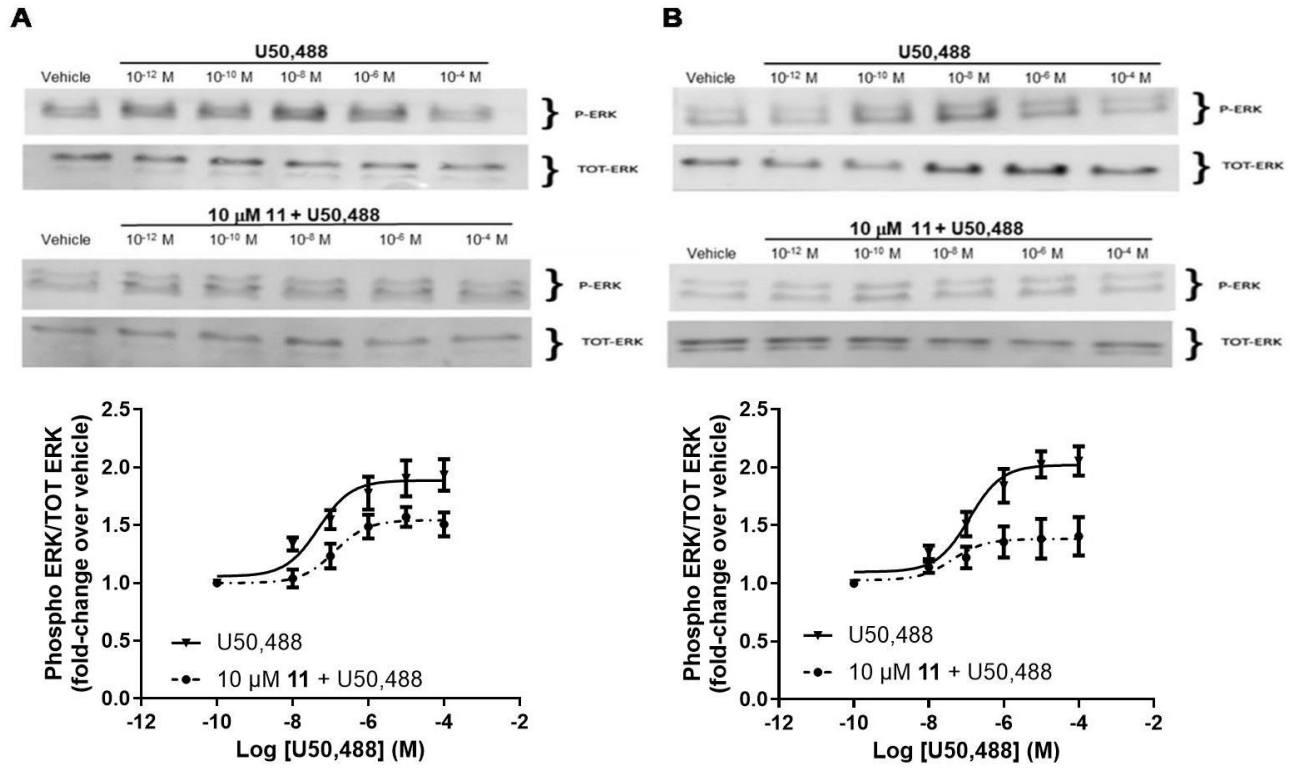


Figure S1. KOR-dependent activation of ERK1/2 phosphorylation determined by U50,488 (1 pM to 100 μM), alone or co-administered with 10 μM **11**, in (**A**) HEK-293/hKOR cells, and in (**B**) U87-MG cells. Data is displayed as mean ± SD of 6 independent experiments.

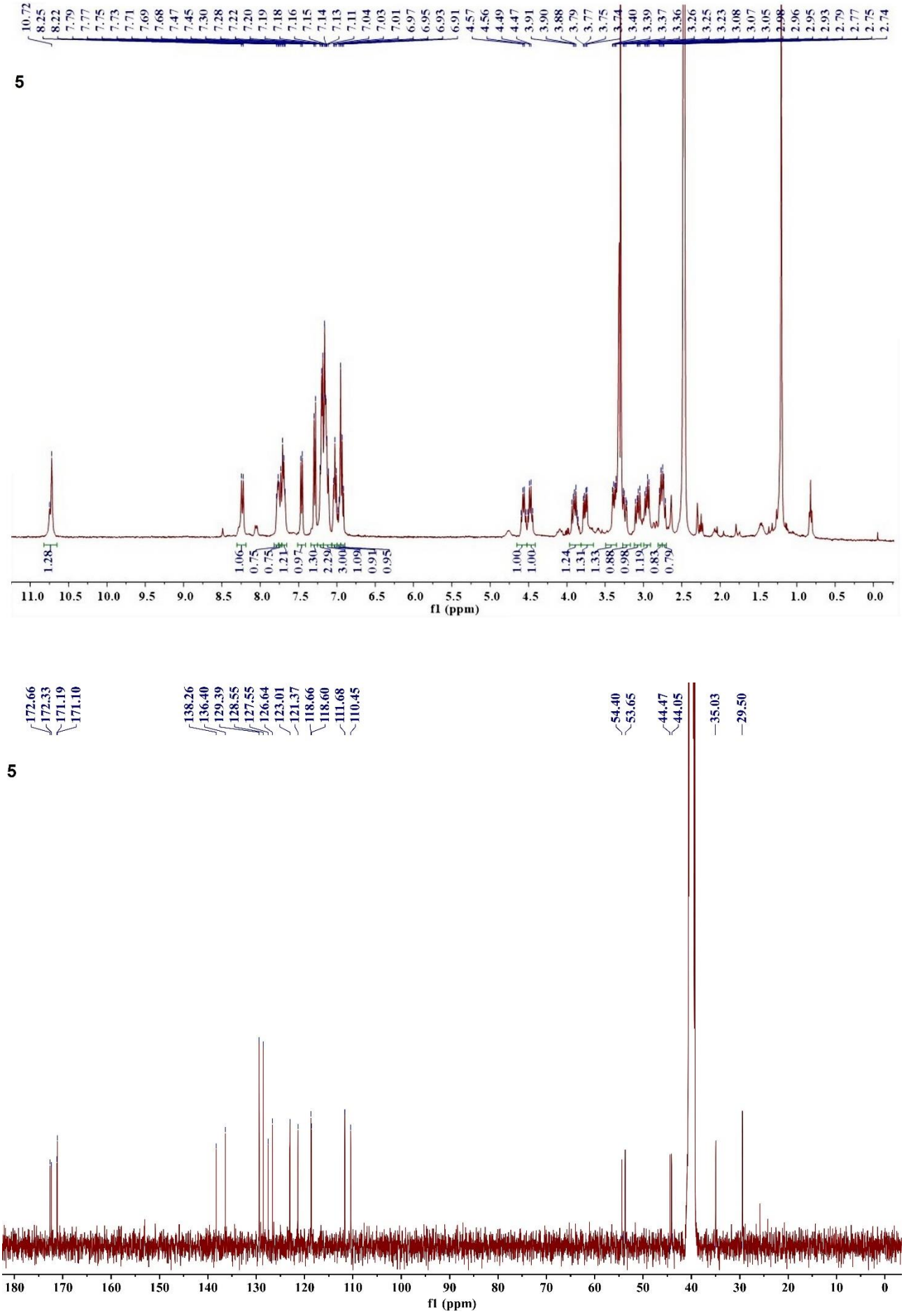


Figure S2. ^{1}H, ^{13}C NMR of c[D-Trp-Phe-Gly-Gly] (**5**).

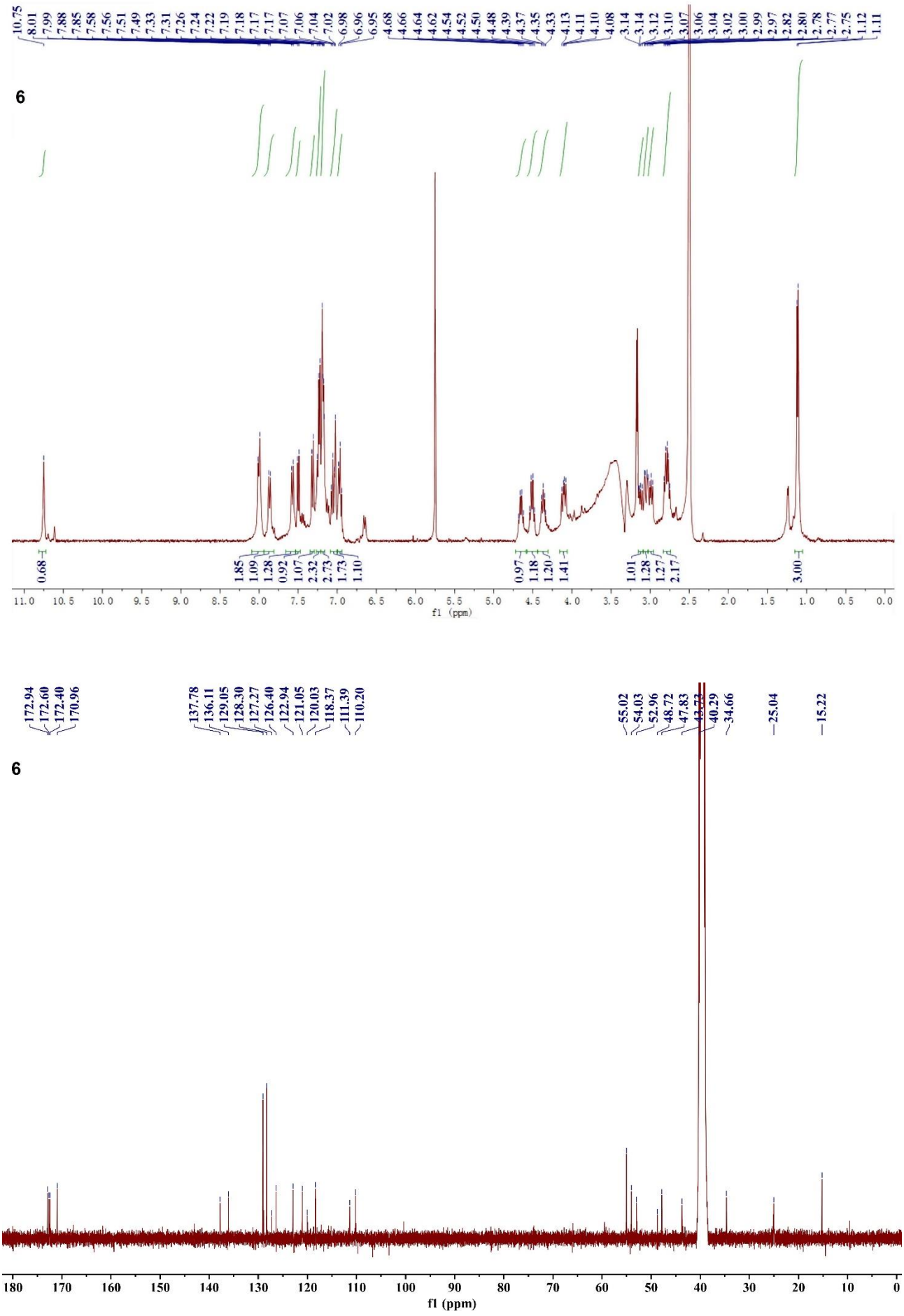


Figure S3. ^{1}H, ^{13}C NMR of c[D-Trp-Phe-Gly-Ala] (**6**).

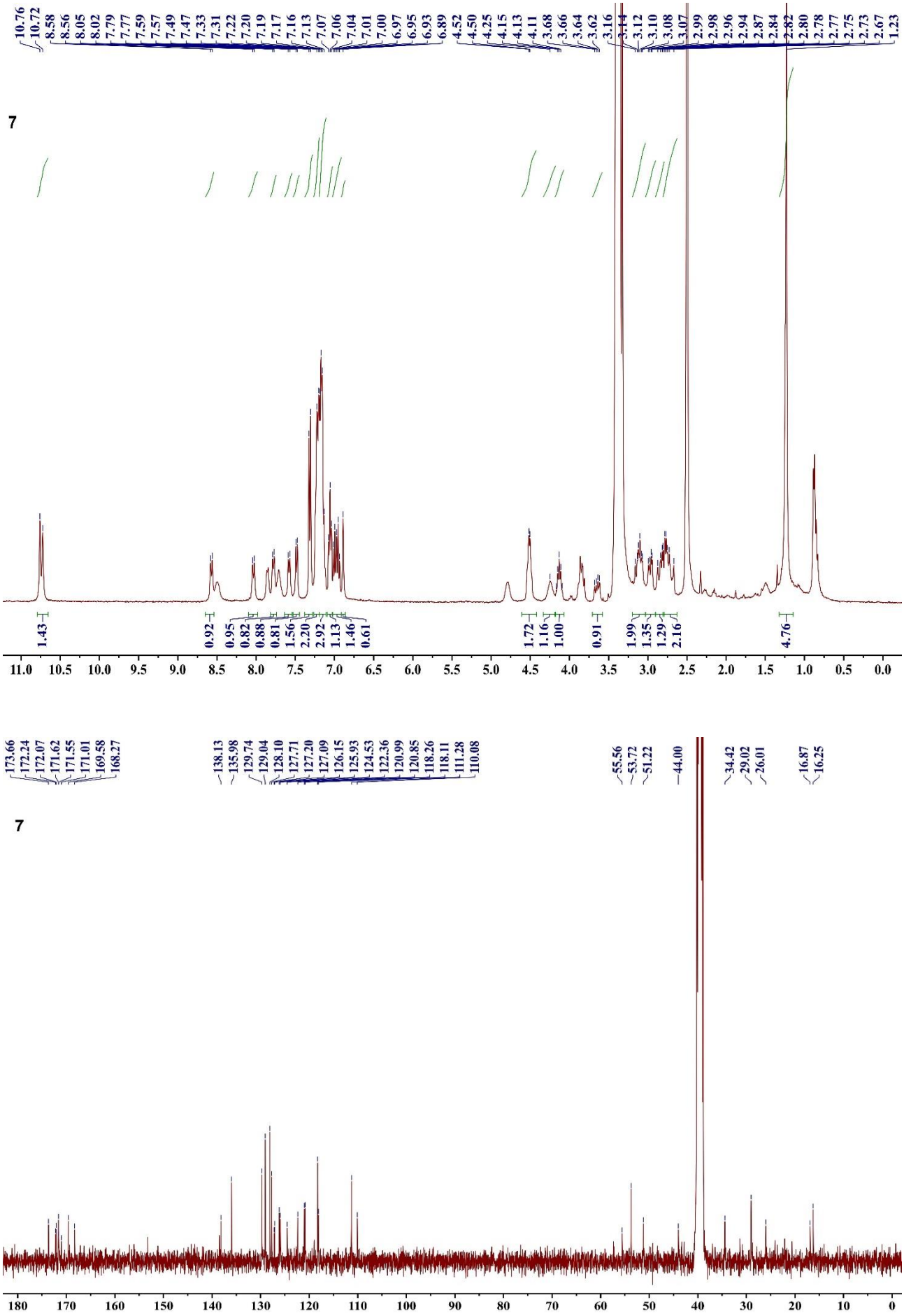


Figure S4. ^{1}H, ^{13}C NMR of c[D-Trp-Phe-Gly-D-Ala] (**7**).

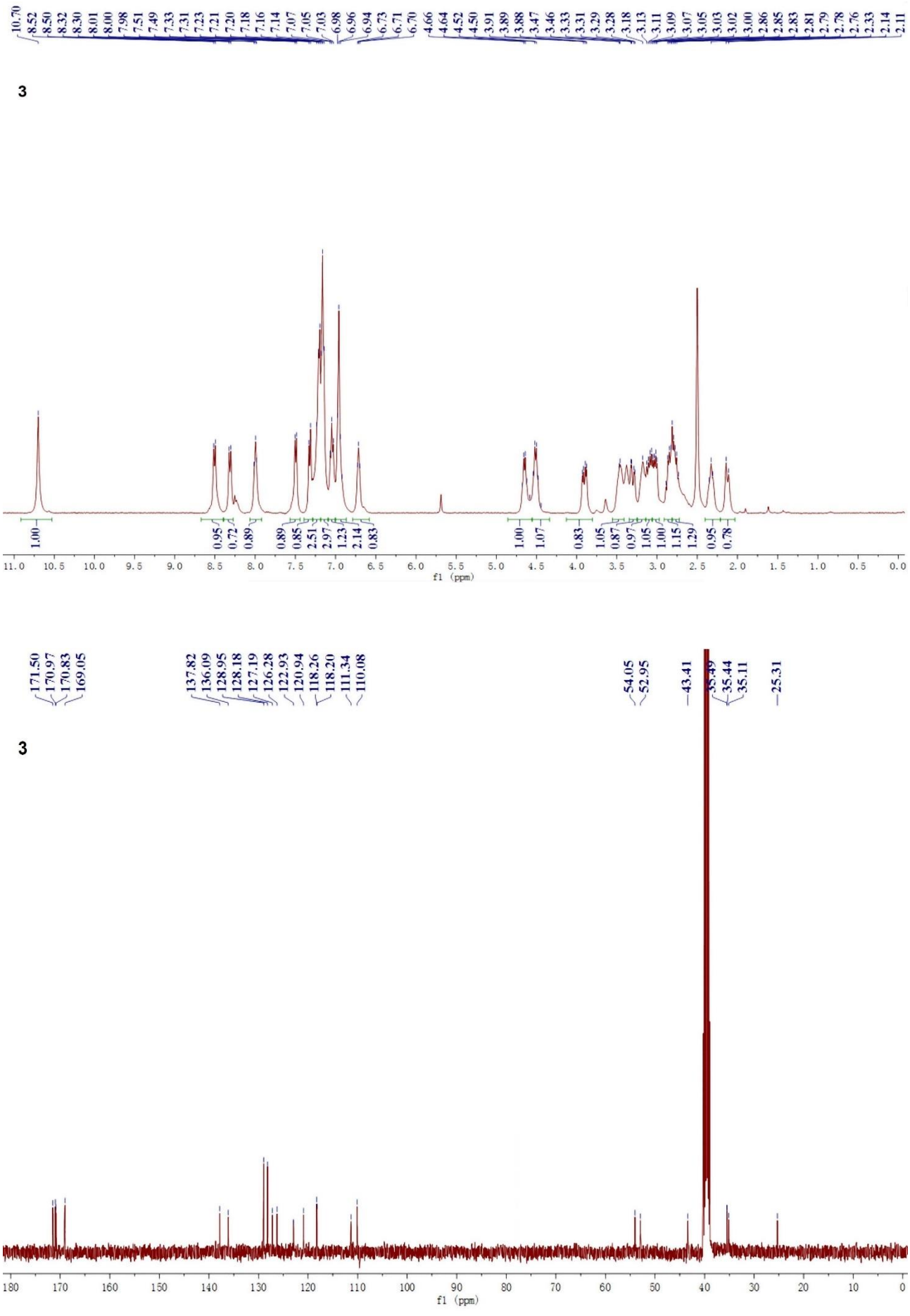
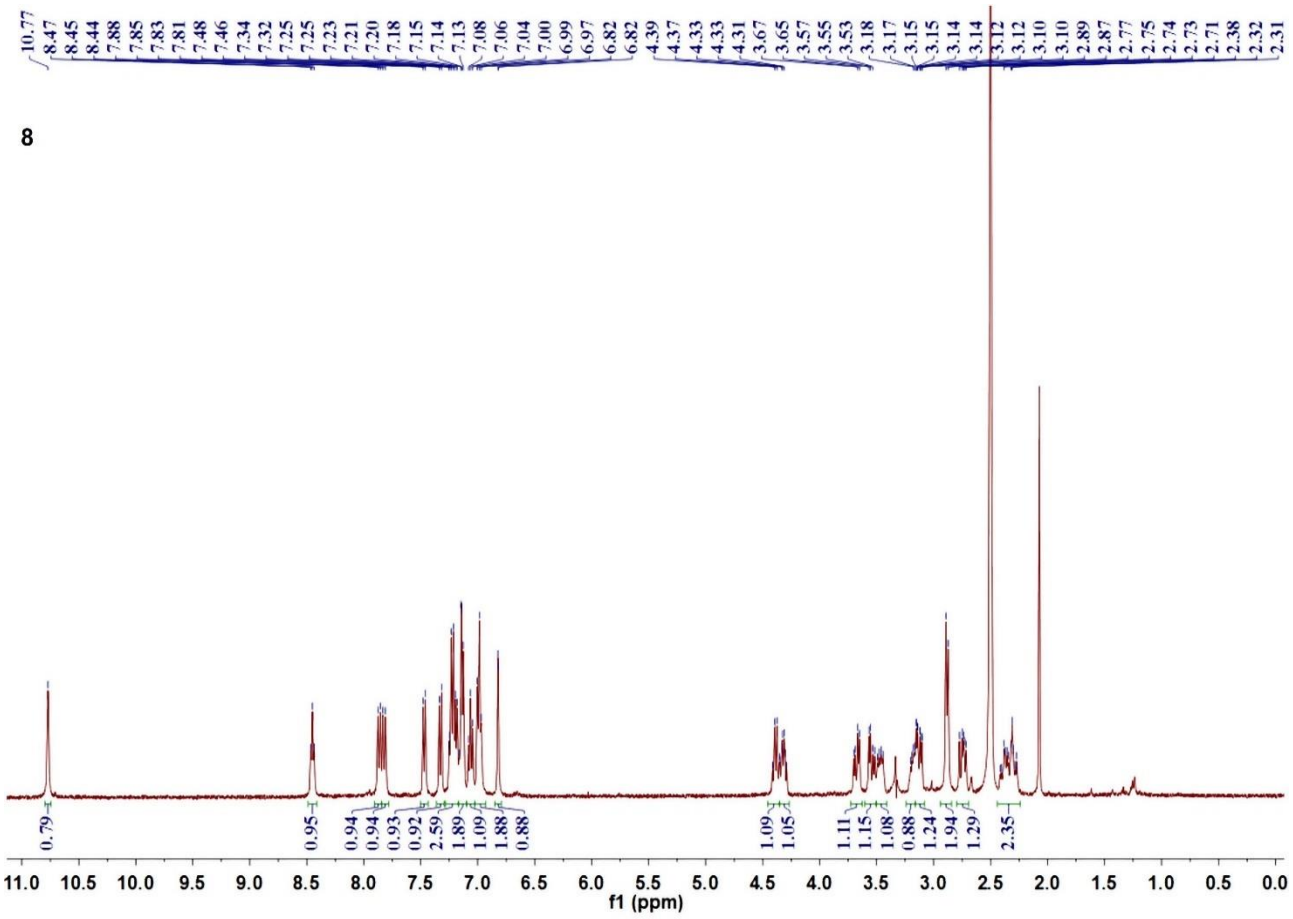


Figure S5. ^{1}H, ^{13}C NMR of c[D-Trp-Phe-Gly-βAla] (**3**).



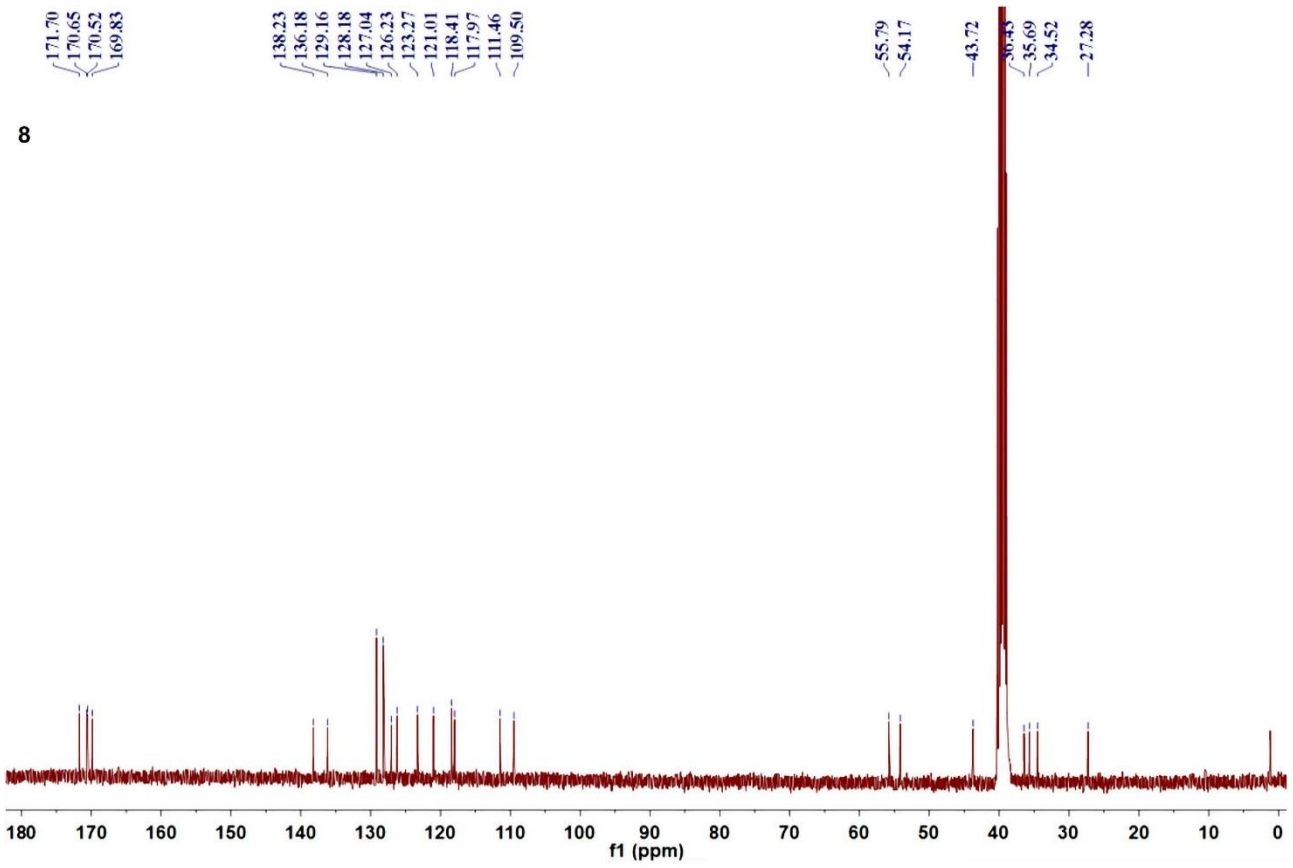


Figure S6. ^{1}H, ^{13}C NMR of c[D-Trp-Phe-β-Ala-Gly] (**8**).

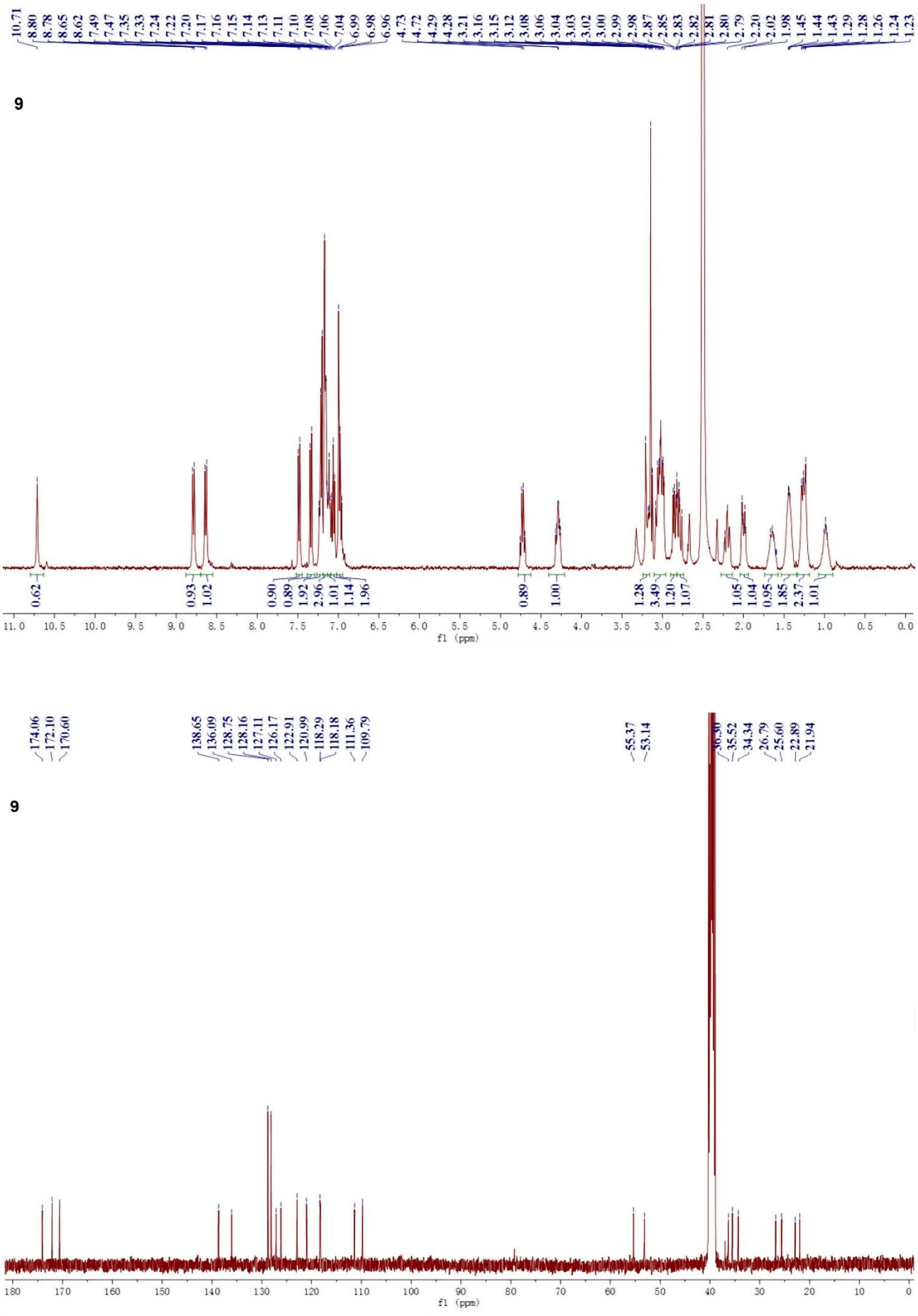


Figure S7. ^{1}H, ^{13}C NMR of c[D-Trp-Phe-Aha] (**9**).

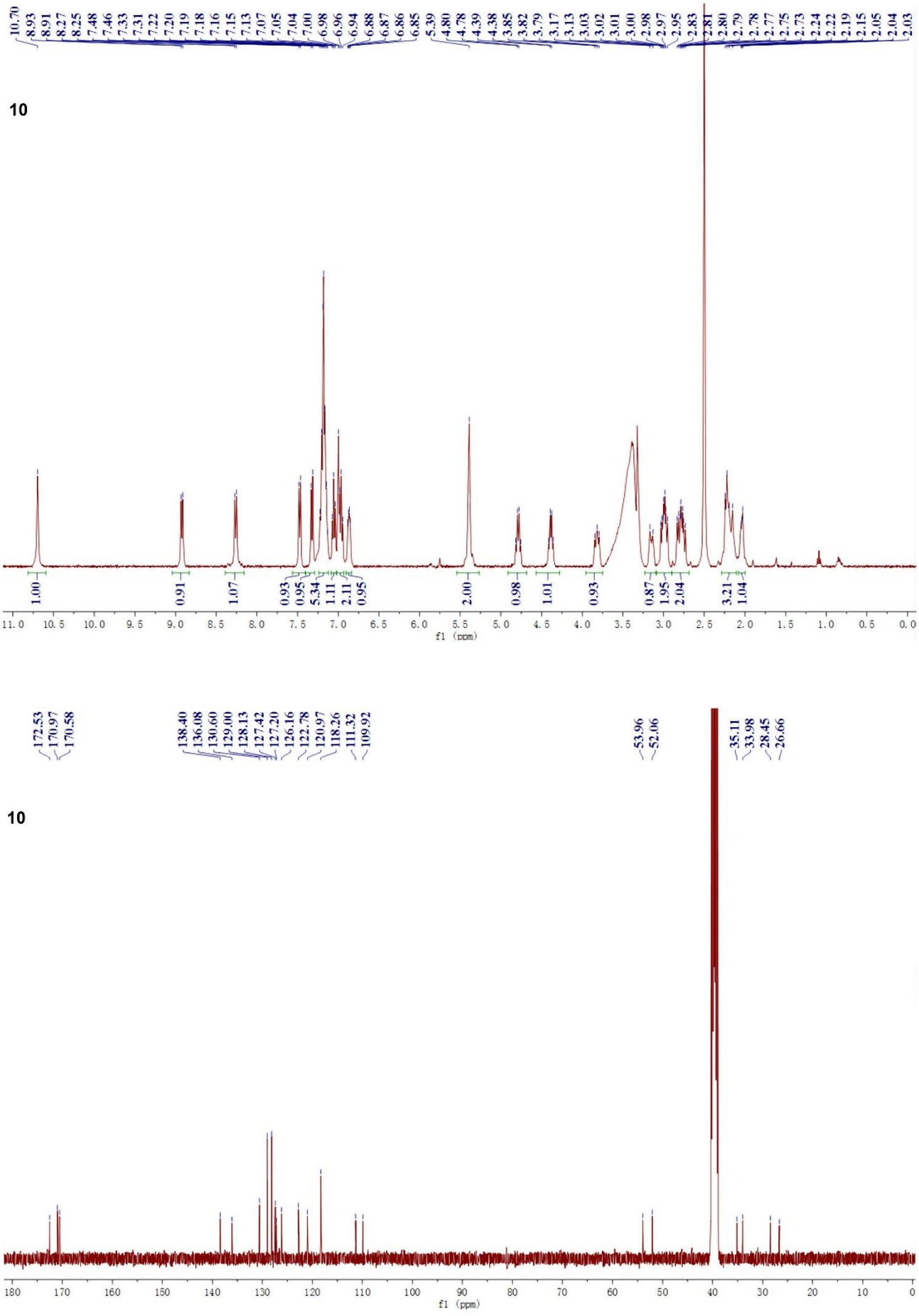


Figure S8. ^{1}H, ^{13}C NMR of c[D-Trp-Phe-Ah-4-ea] (**10**).

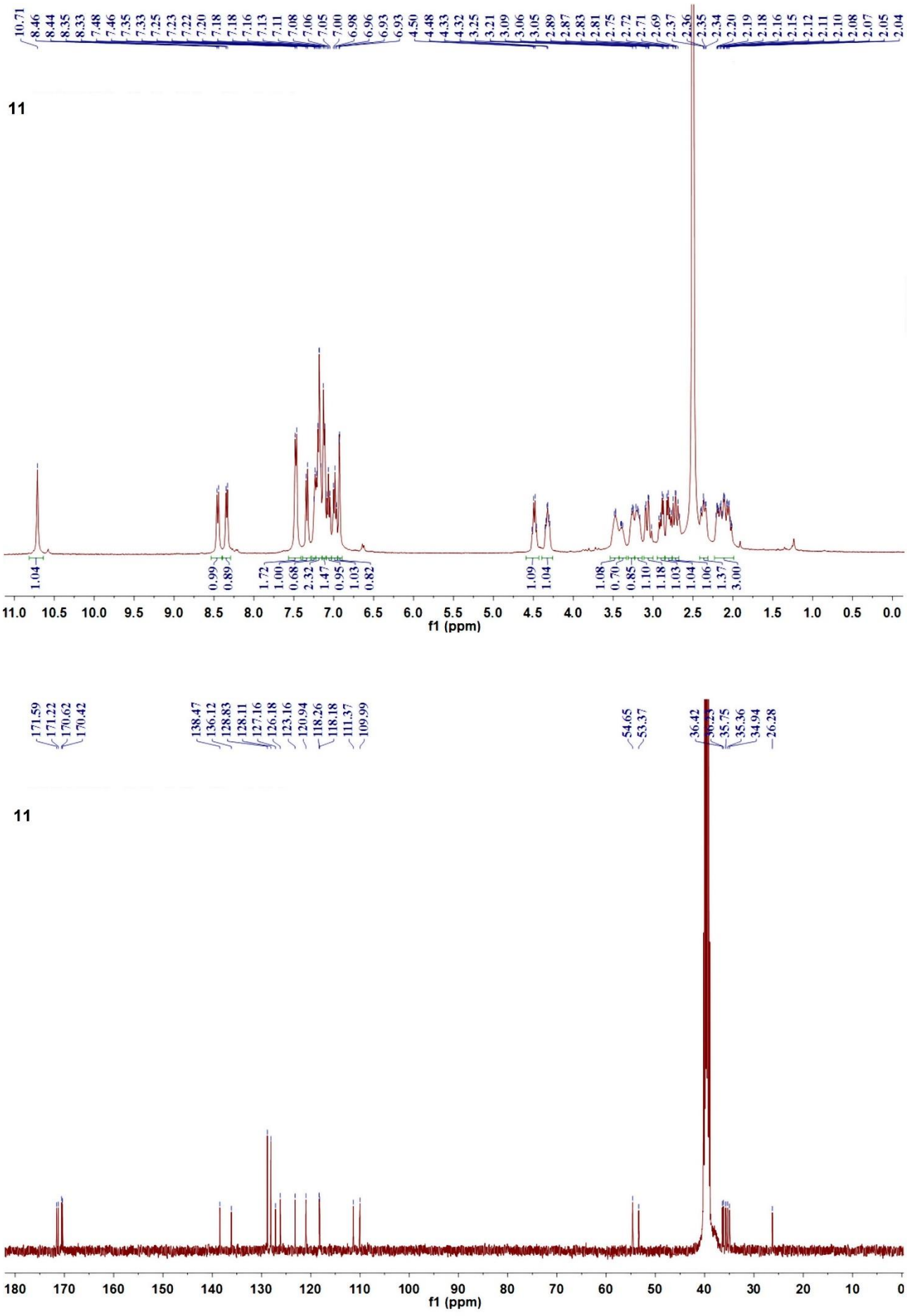


Figure S9. ^{1}H, ^{13}C NMR of c[D-Trp-Phe-β-Ala-β-Ala] (**11**).

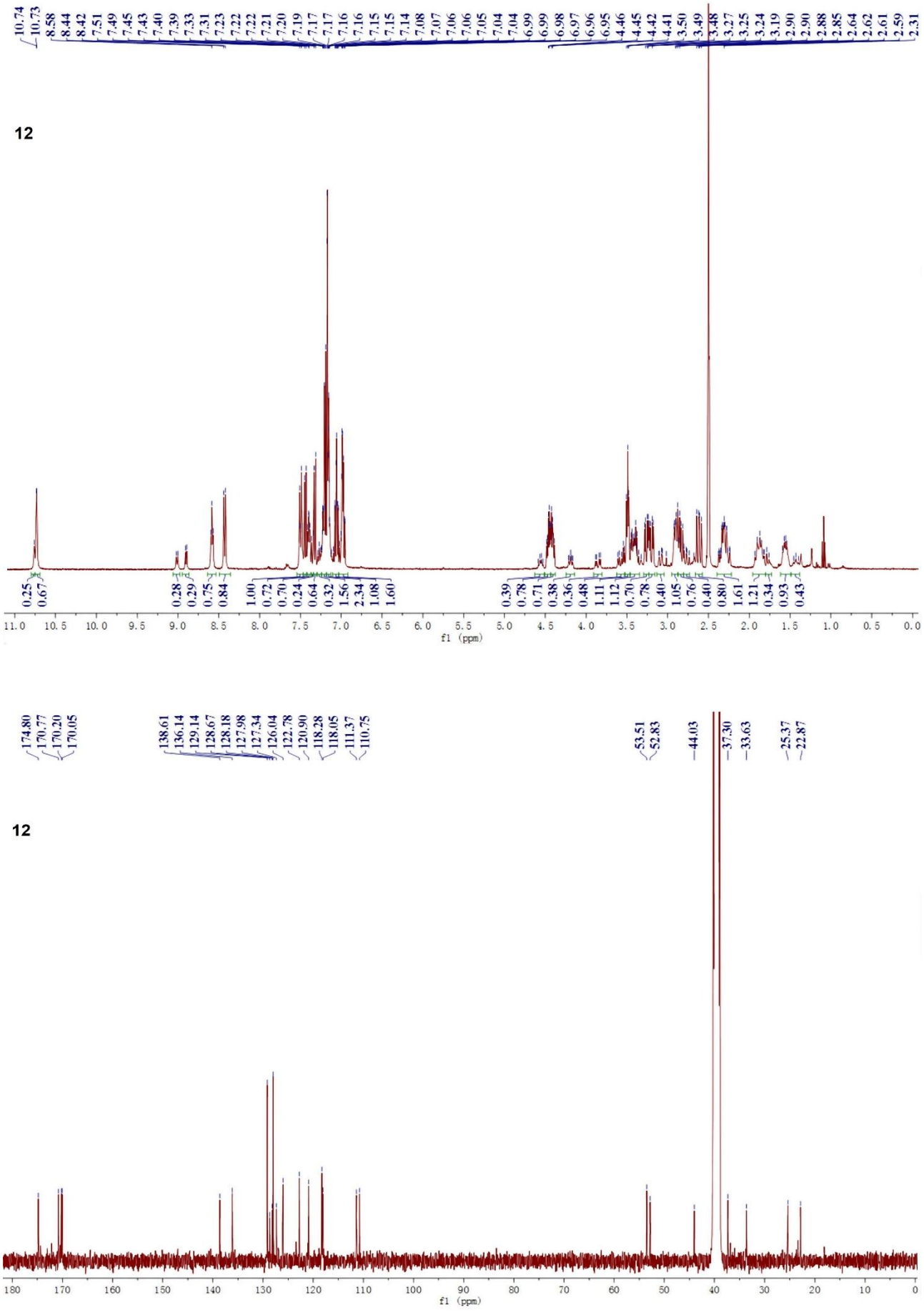


Figure S10. ^{1}H, ^{13}C NMR of c[D-Trp-Phe-GABA-Gly] (**12**).

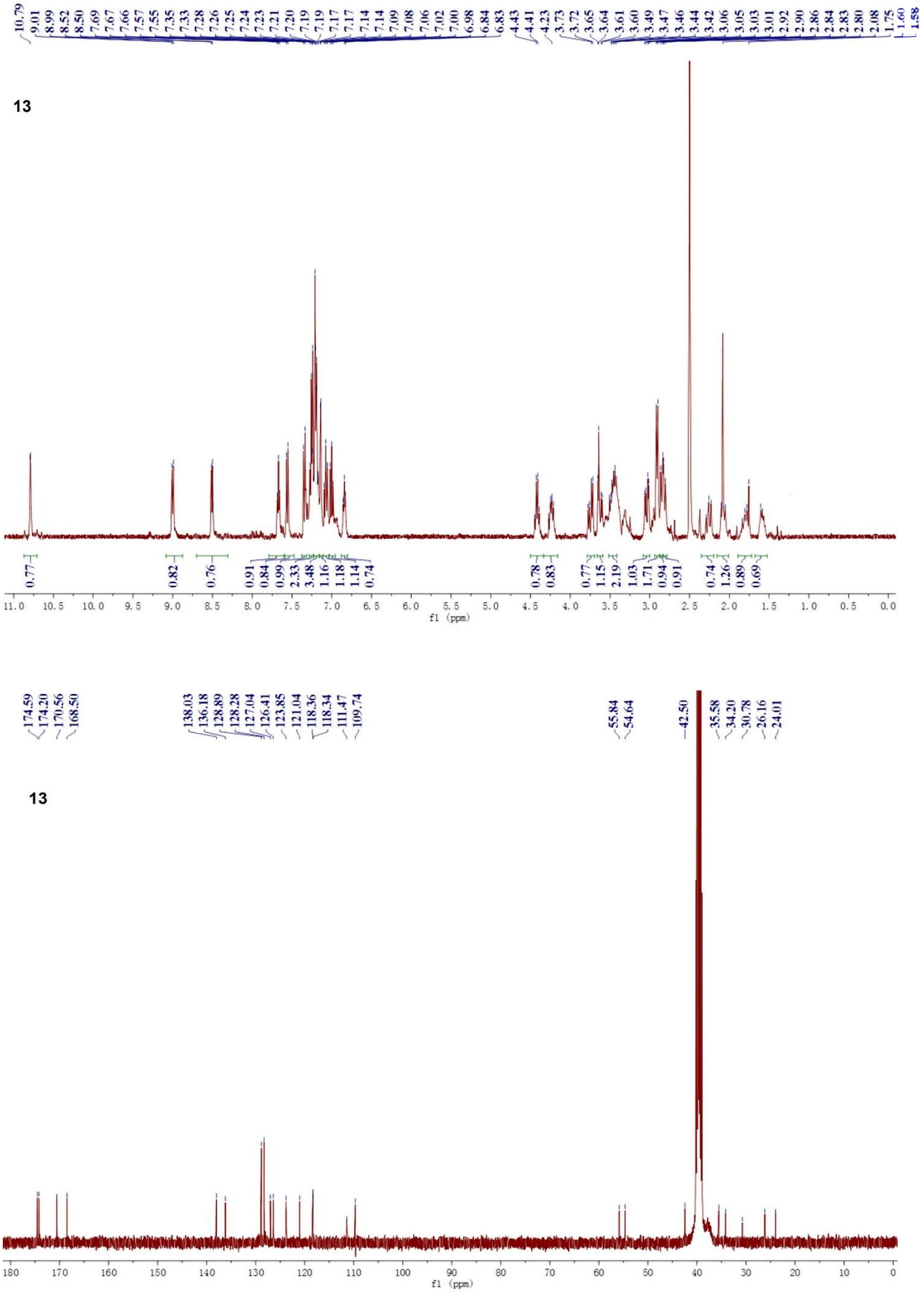
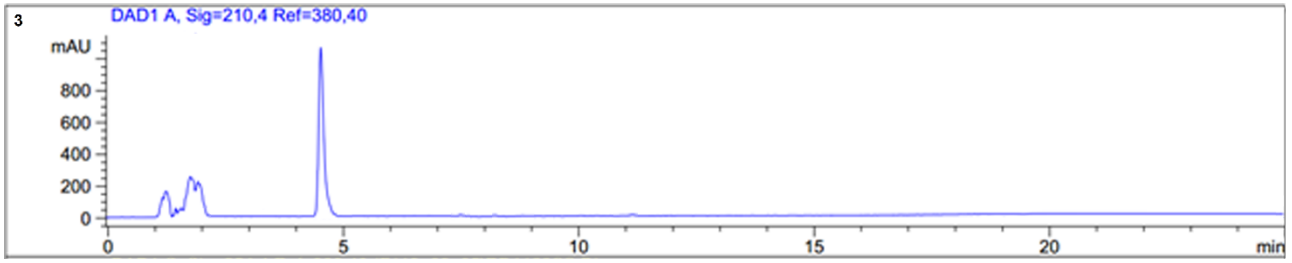


Figure S11. ^{1}H, ^{13}C NMR of c[D-Trp-Phe-Gly-GABA] (**13**).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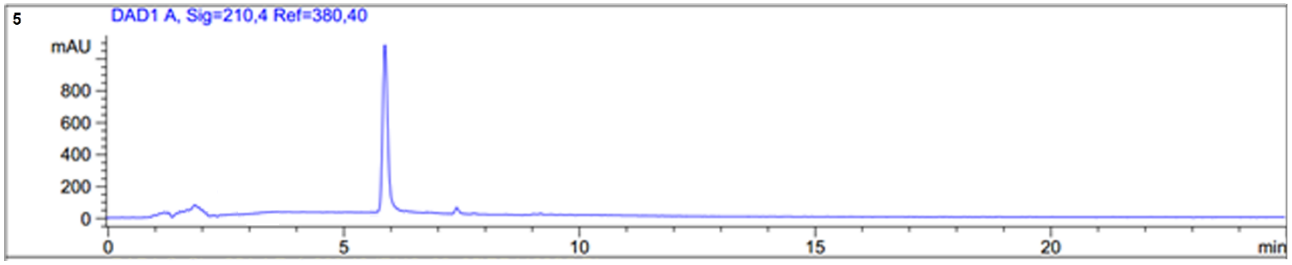
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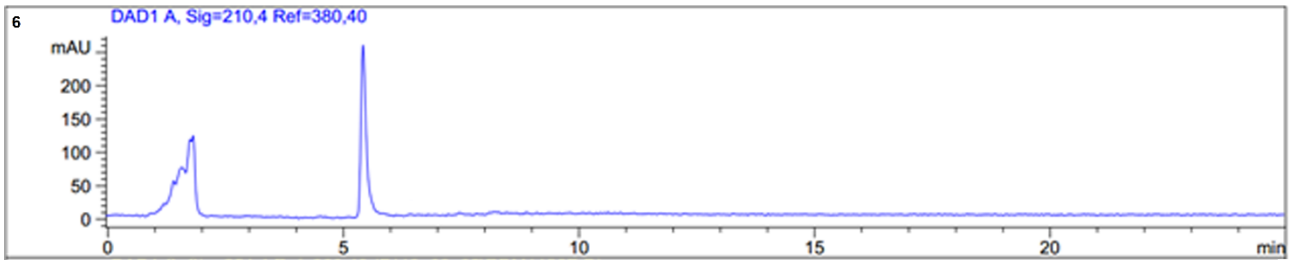
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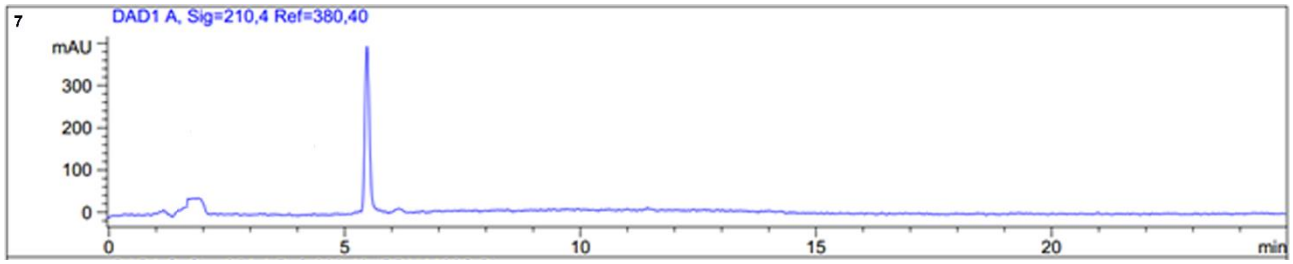
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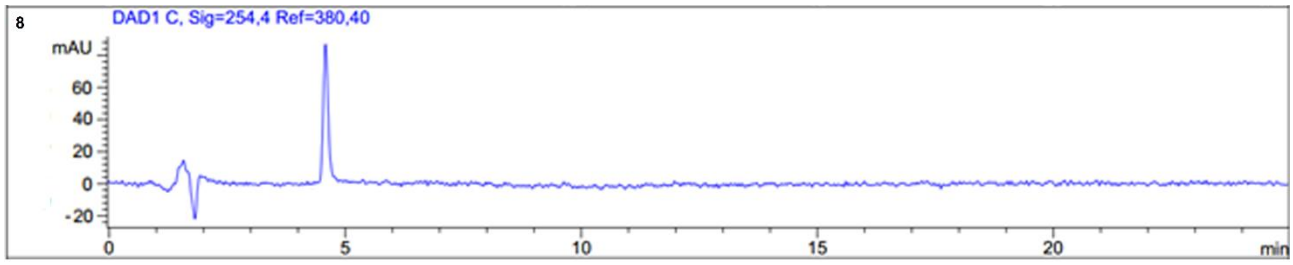
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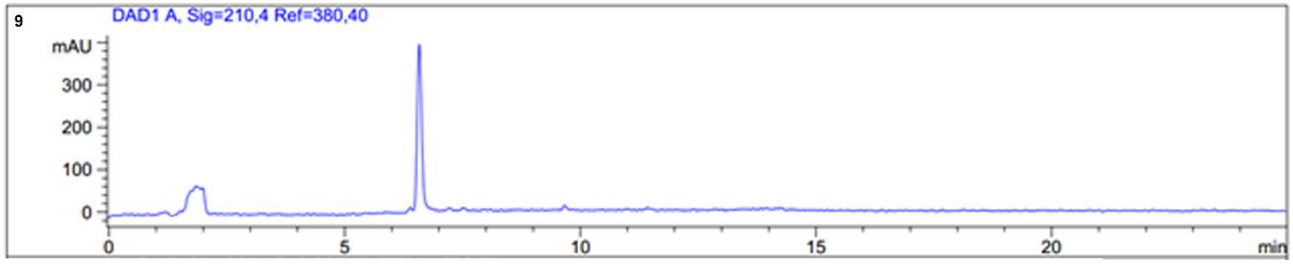
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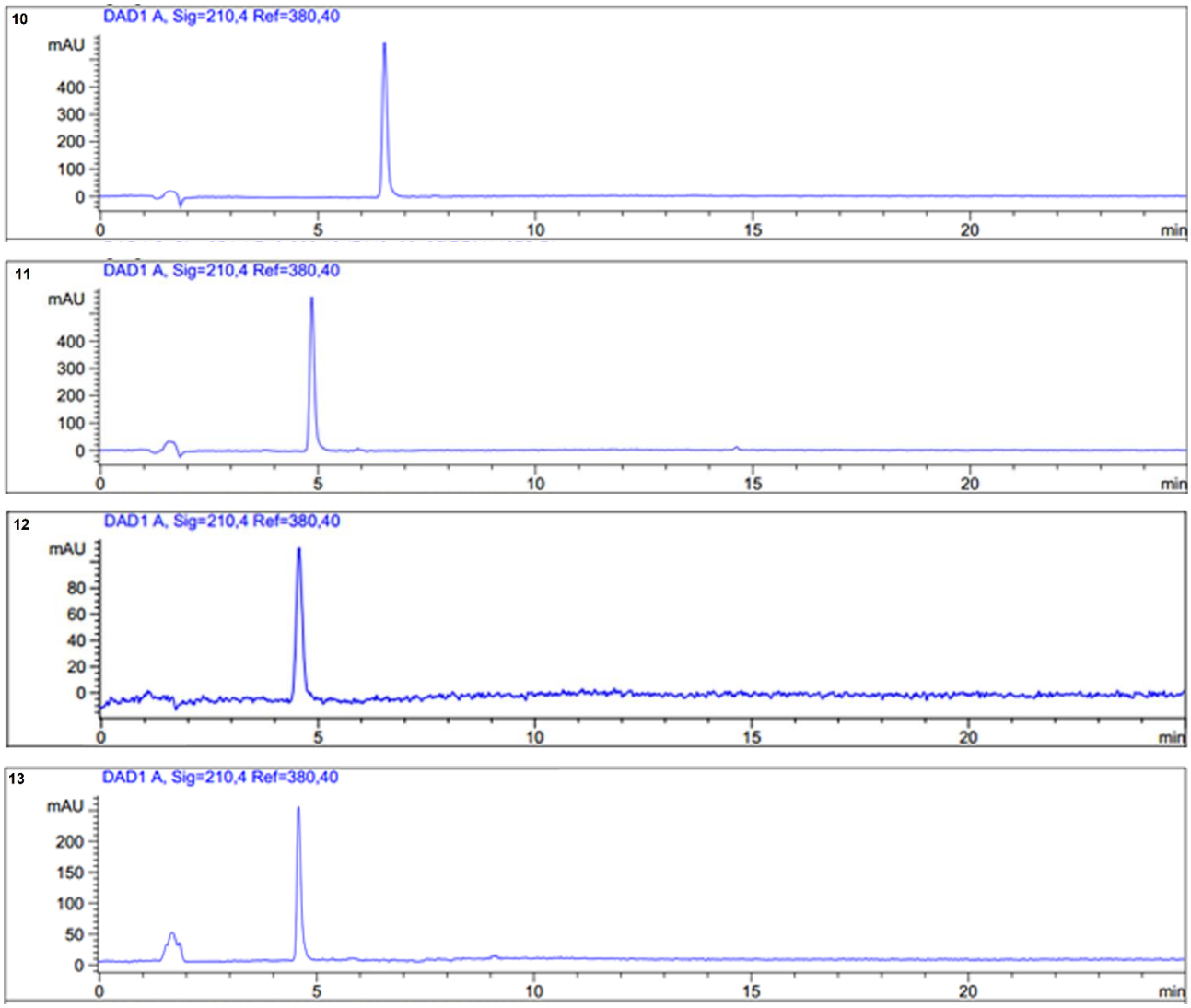

Figure S12. RP HPLC analyses