Supplementary for:

Application of Biotechnology and Chiral Technology Methods in the Production of Ectoine Enantiomers

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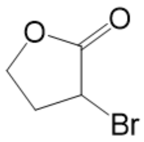
1. NMR spectra

Compound **1**



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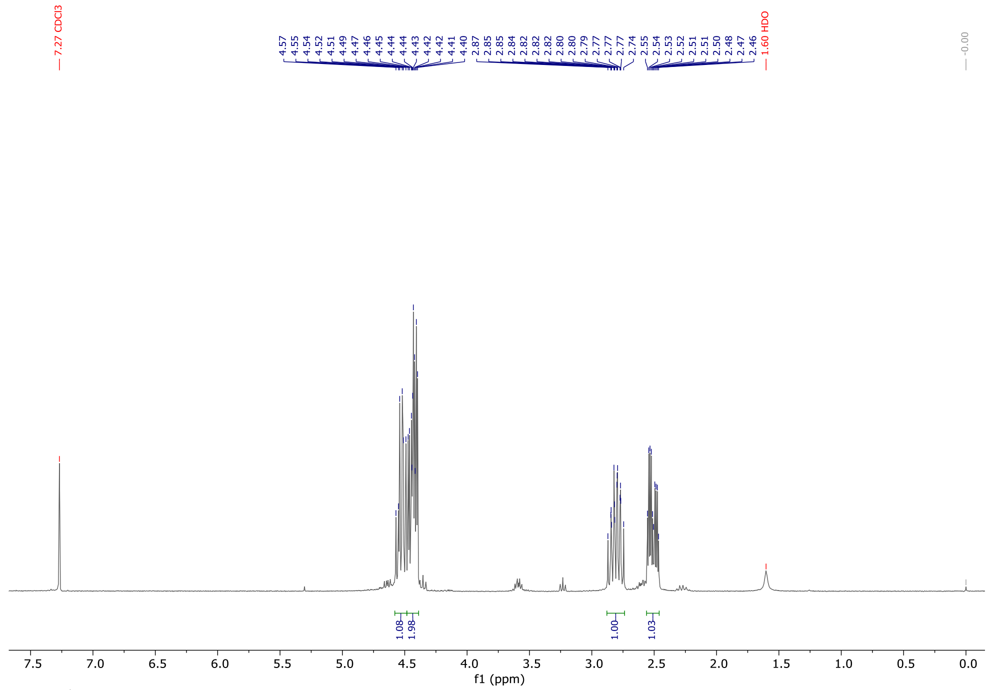


Figure S1.1. ^{1}H NMR ($CDCl_3$, 300 MHz) spectra of compound **1**.

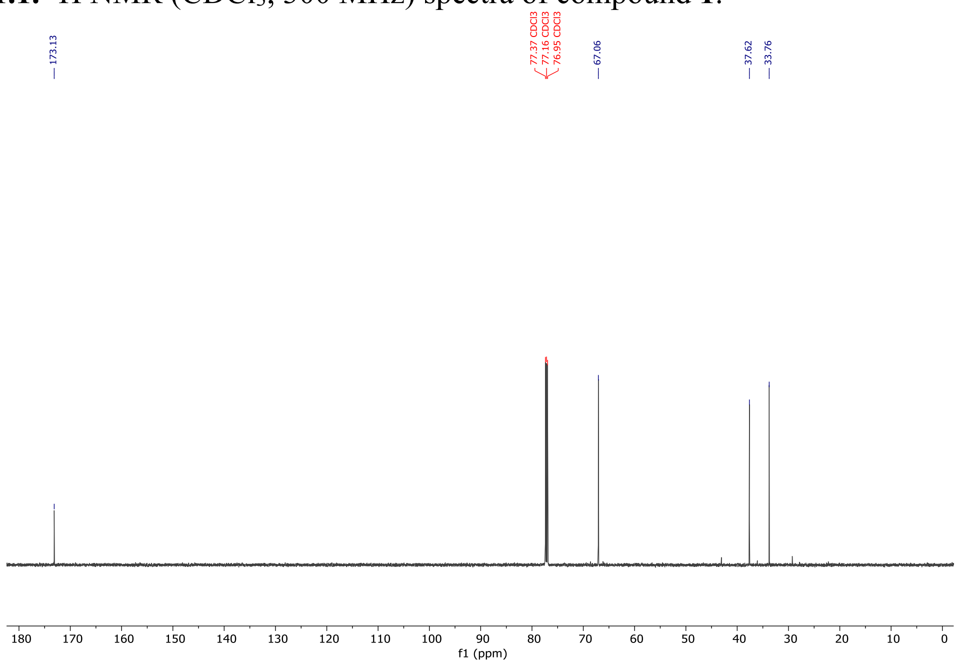


Figure S1.2. ^{13}C NMR ($CDCl_3$, 75 MHz) spectra of compound **1**.

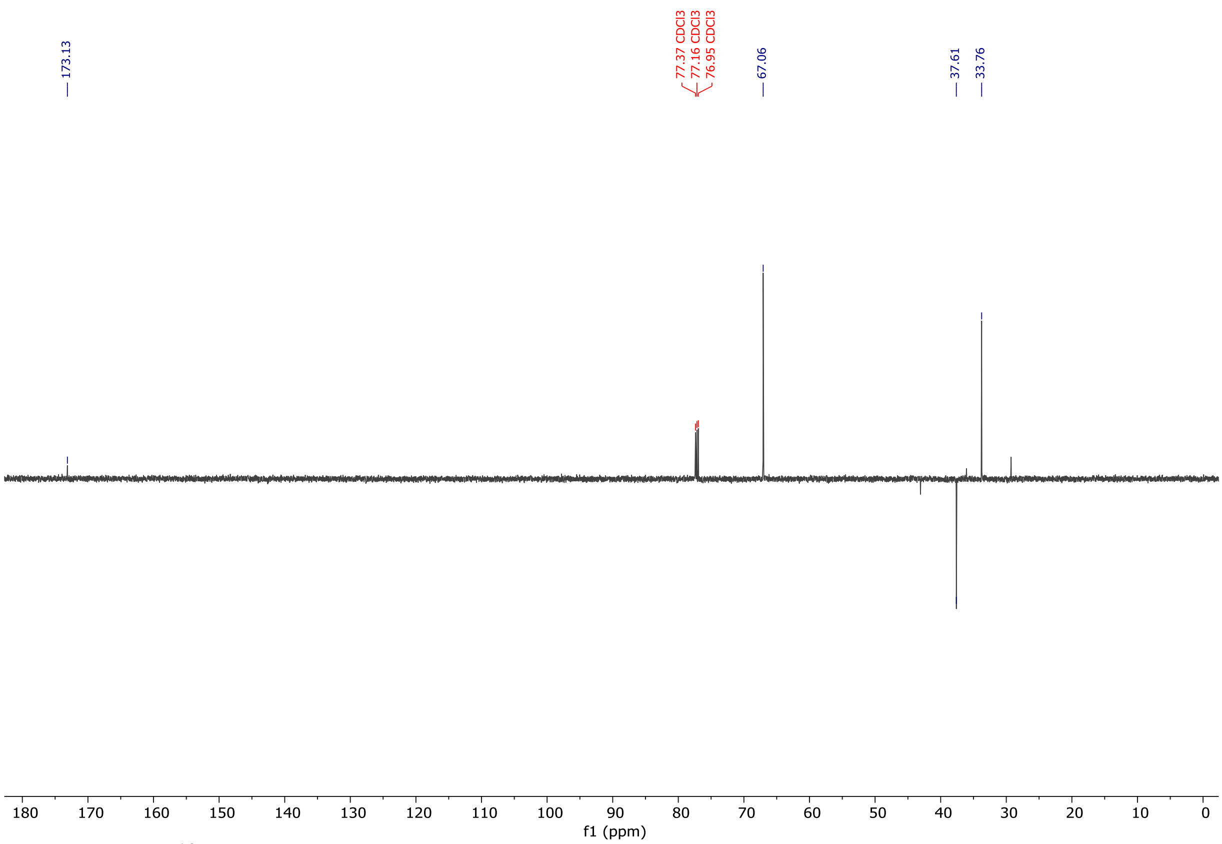
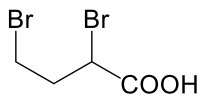


Figure S1.3. ^{13}C APT NMR ($CDCl_3$, 75 MHz) spectra of compound **1**.

2,4-Dibromobutyric acid



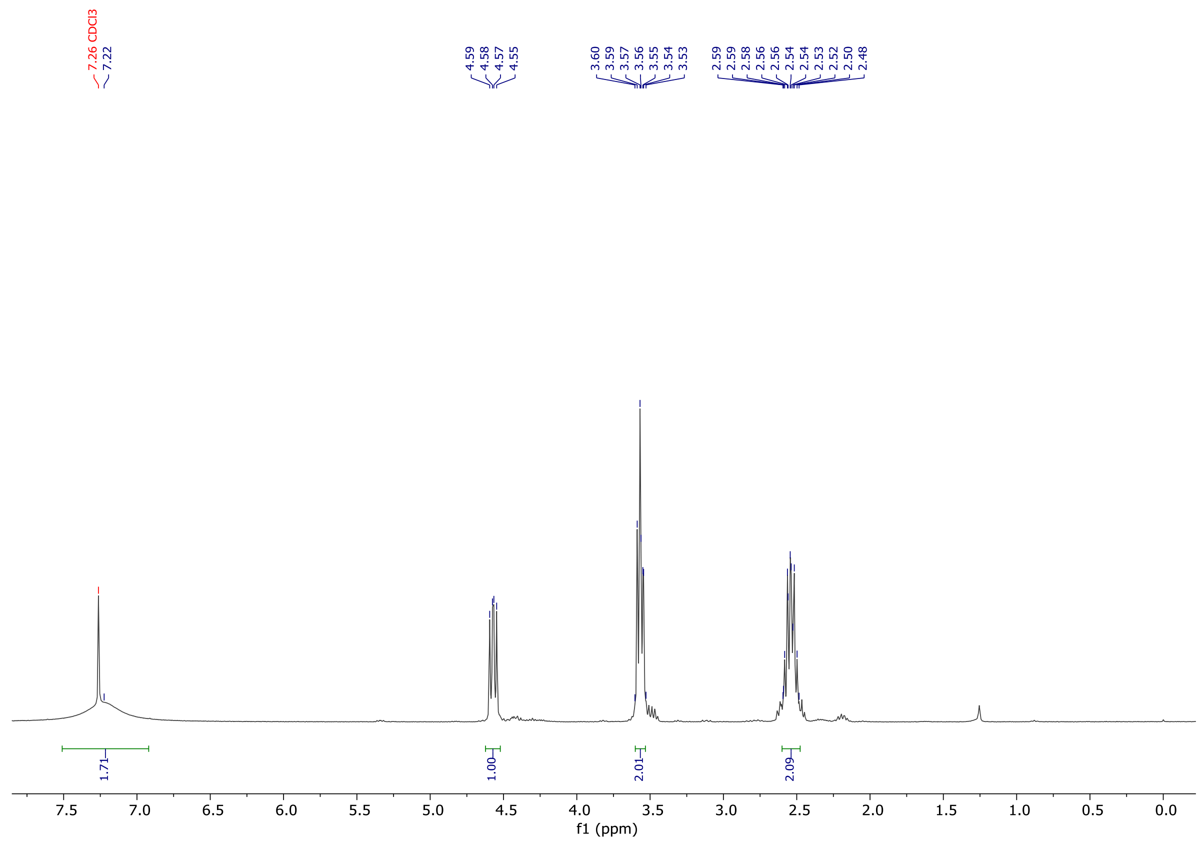


Figure S1.4. ^{1}H NMR ($CDCl_3$, 300 MHz) spectra of 2,4-dibromobutyric acid.

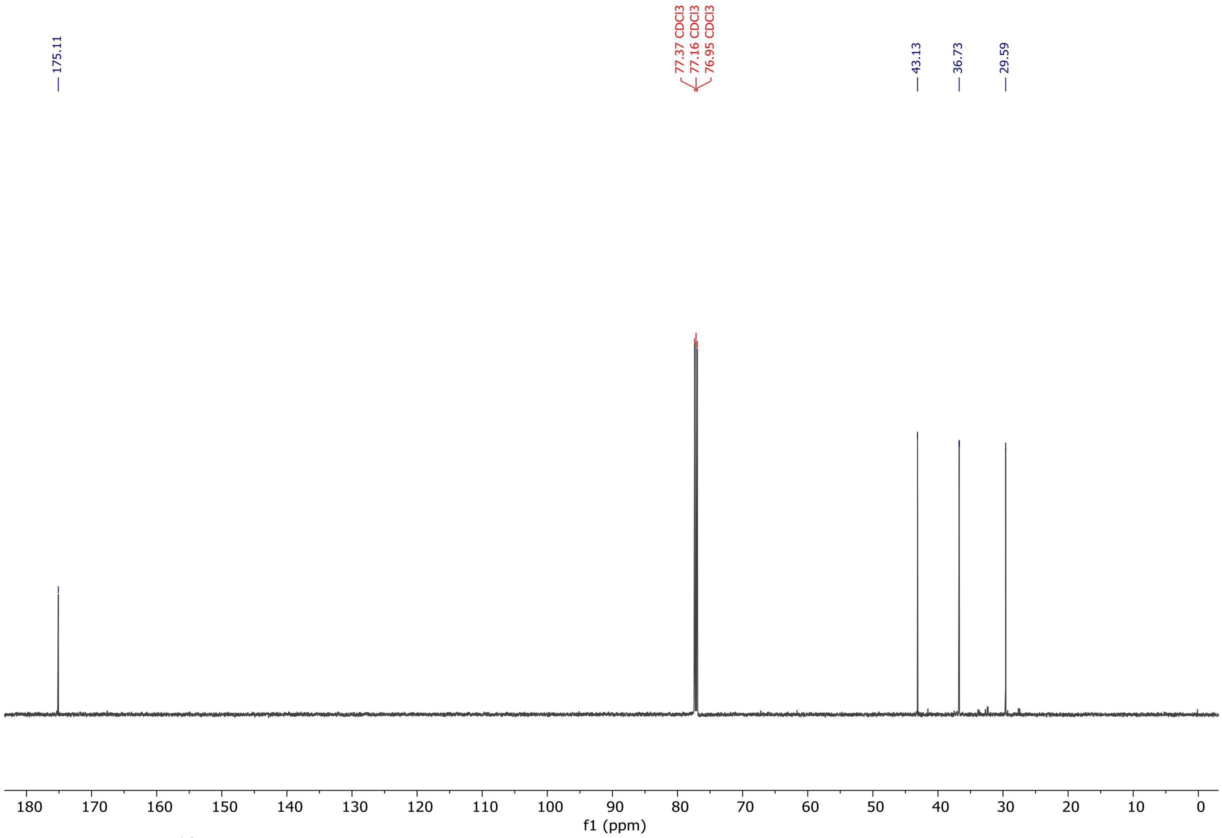


Figure S1.5. ^{13}C NMR ($CDCl_3$, 75 MHz) spectra of 2,4-dibromobutyric acid.

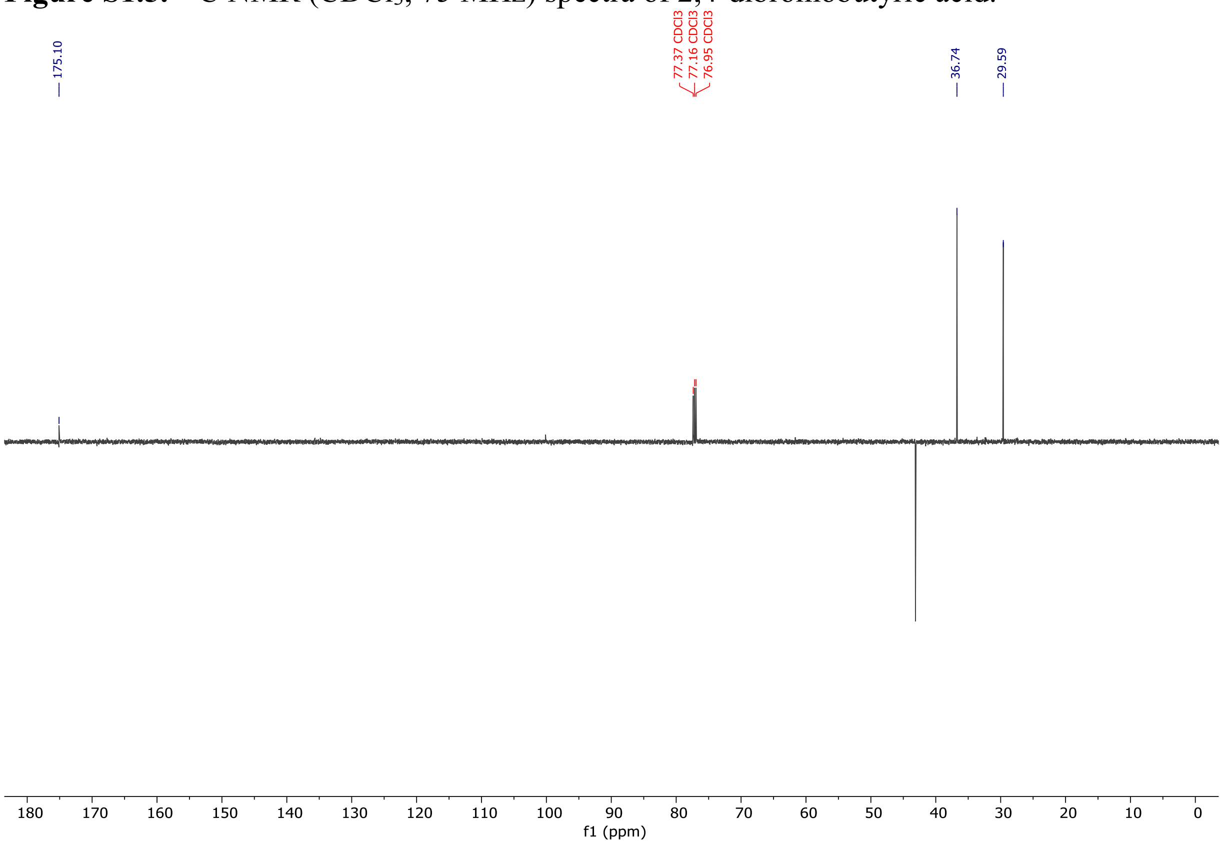
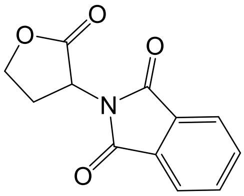


Figure S1.6. ^{13}C APT NMR ($CDCl_3$, 75 MHz) spectra of 2,4-dibromobutyric acid.

Compound **2**



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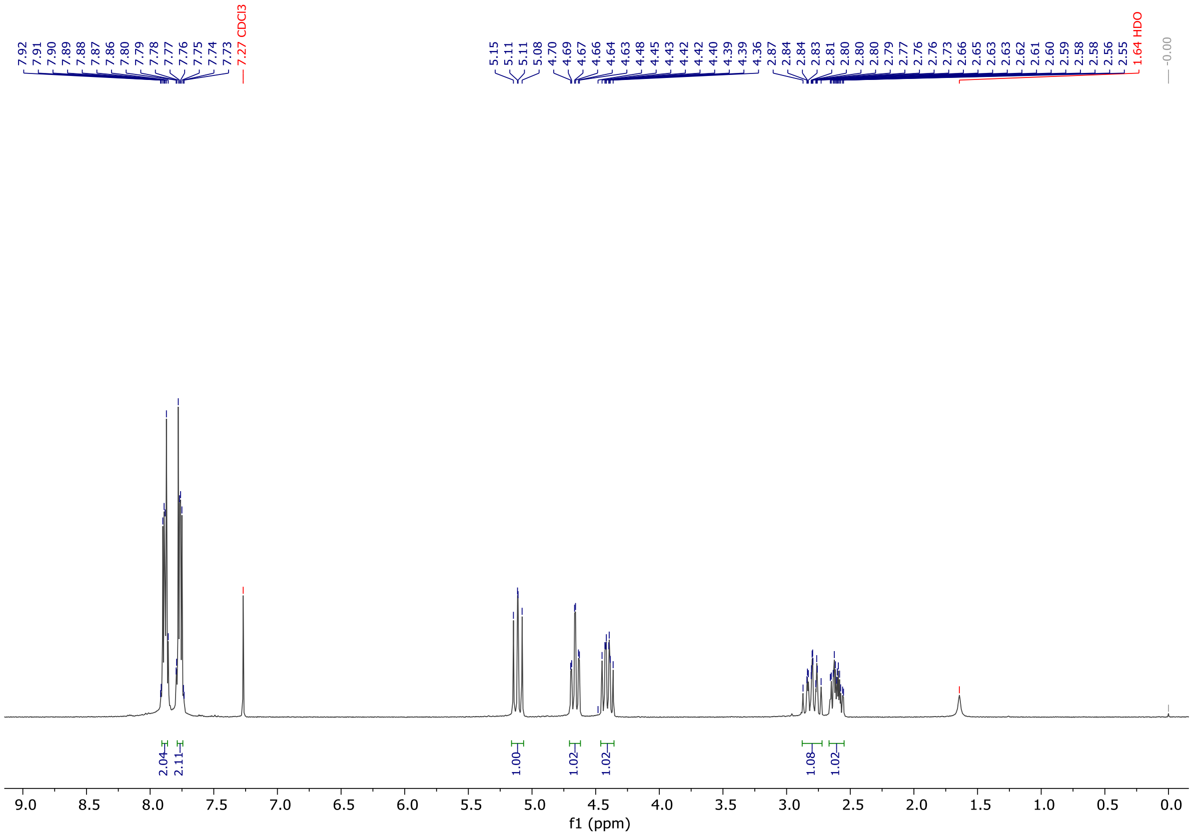


Figure S1.7. ^{1}H NMR ($CDCl_3$, 300 MHz) spectra of compound **2**.

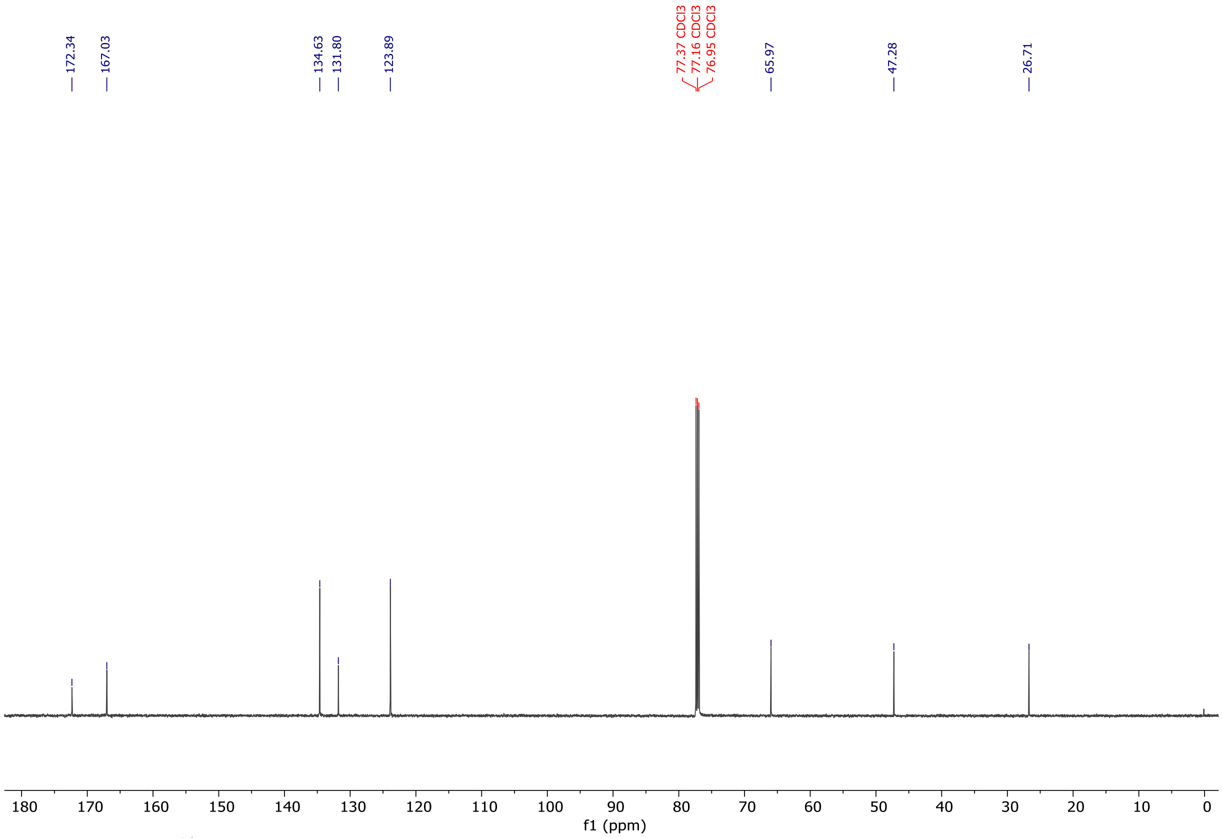


Figure S1.8. ^{13}C NMR ($CDCl_3$, 75 MHz) spectra of compound **2**.

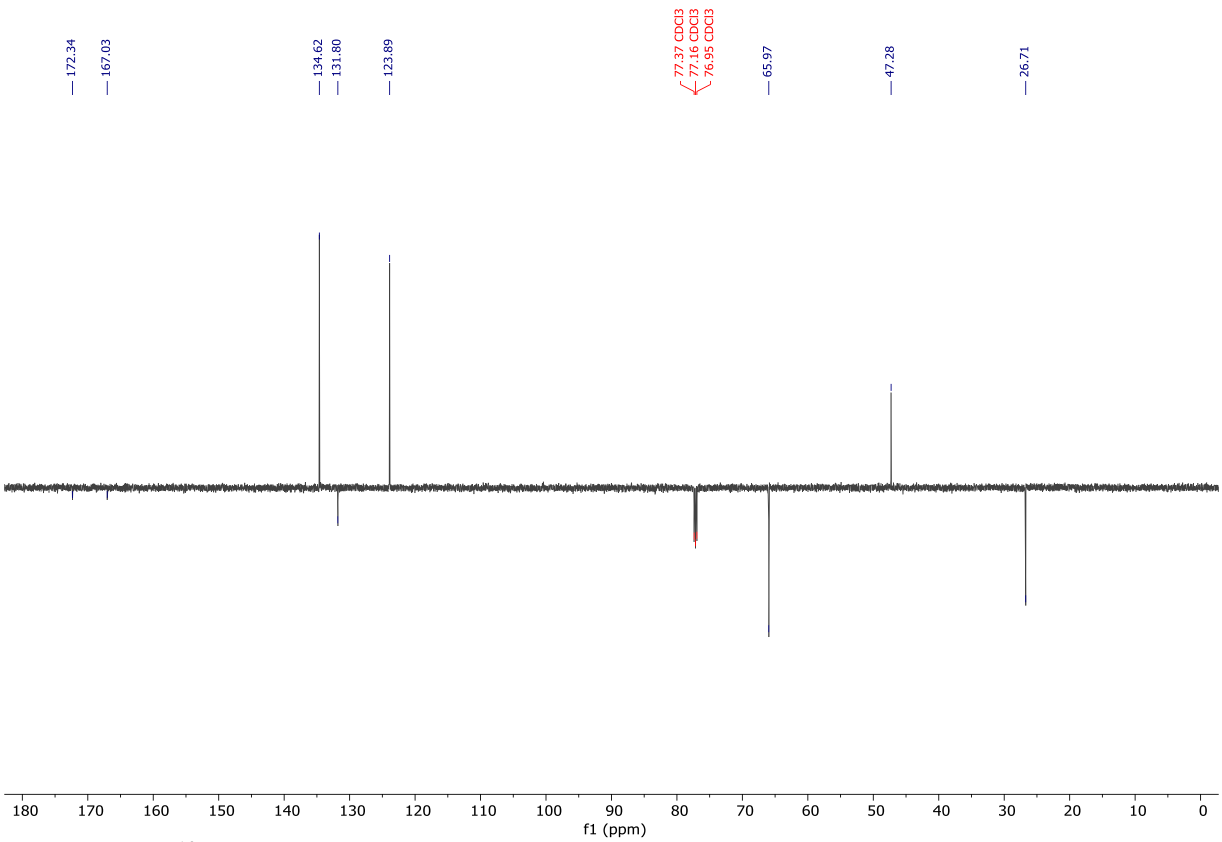
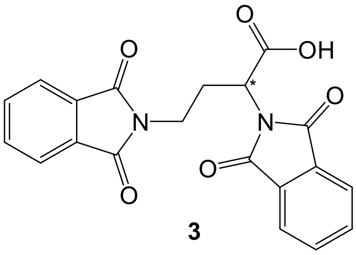


Figure S1.9. ^{13}C APT NMR ($CDCl_3$, 75 MHz) spectra of compound **2**.

Compound **3**



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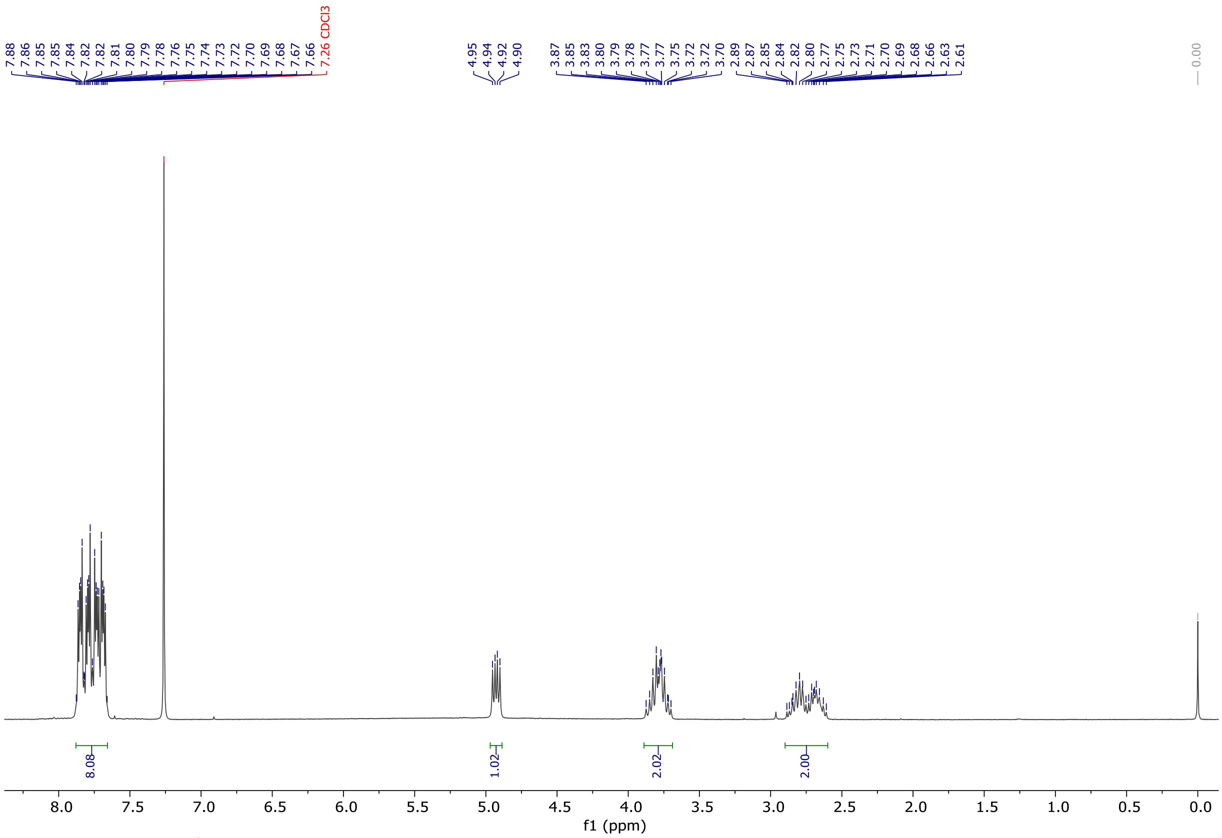


Figure S1.10. [1]H NMR ($CDCl_3$, 300 MHz) spectra of compound **3**.

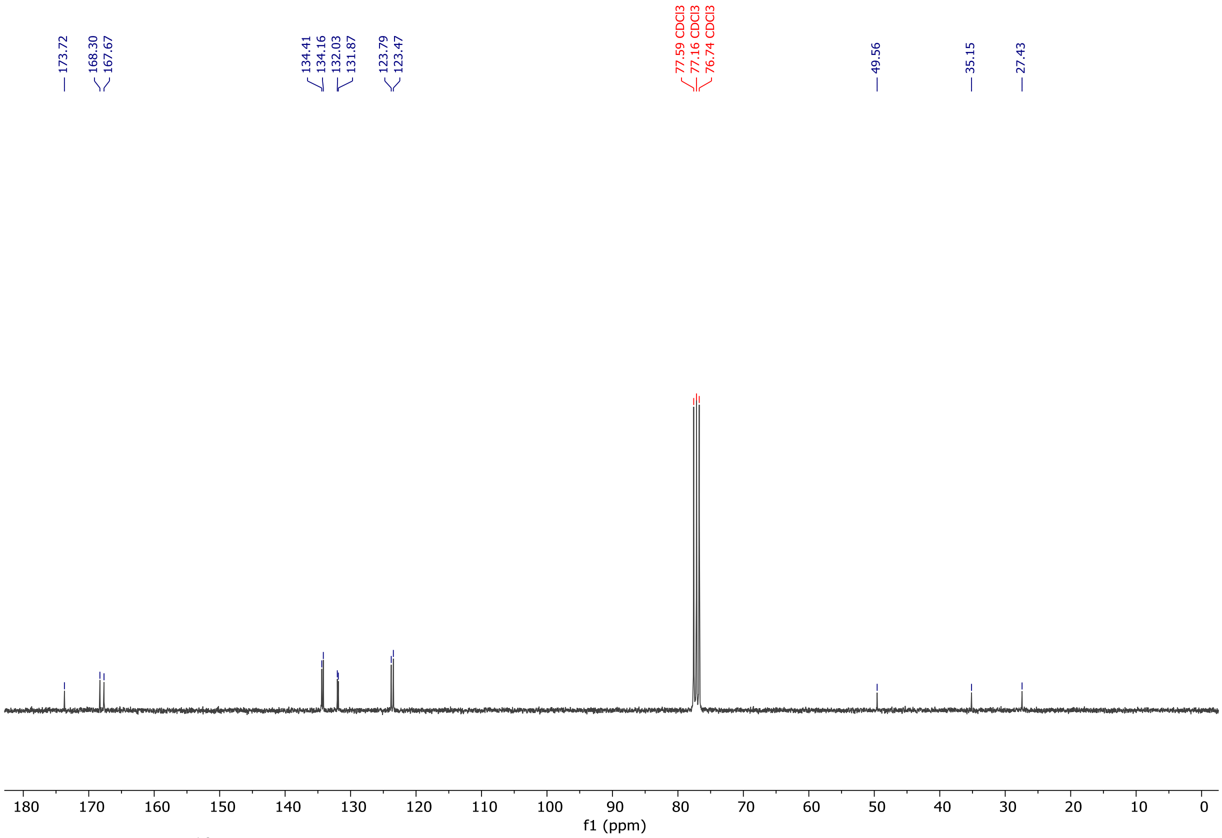


Figure S1.11. ^{13}C NMR ($CDCl_3$, 75 MHz) spectra of compound **3**.

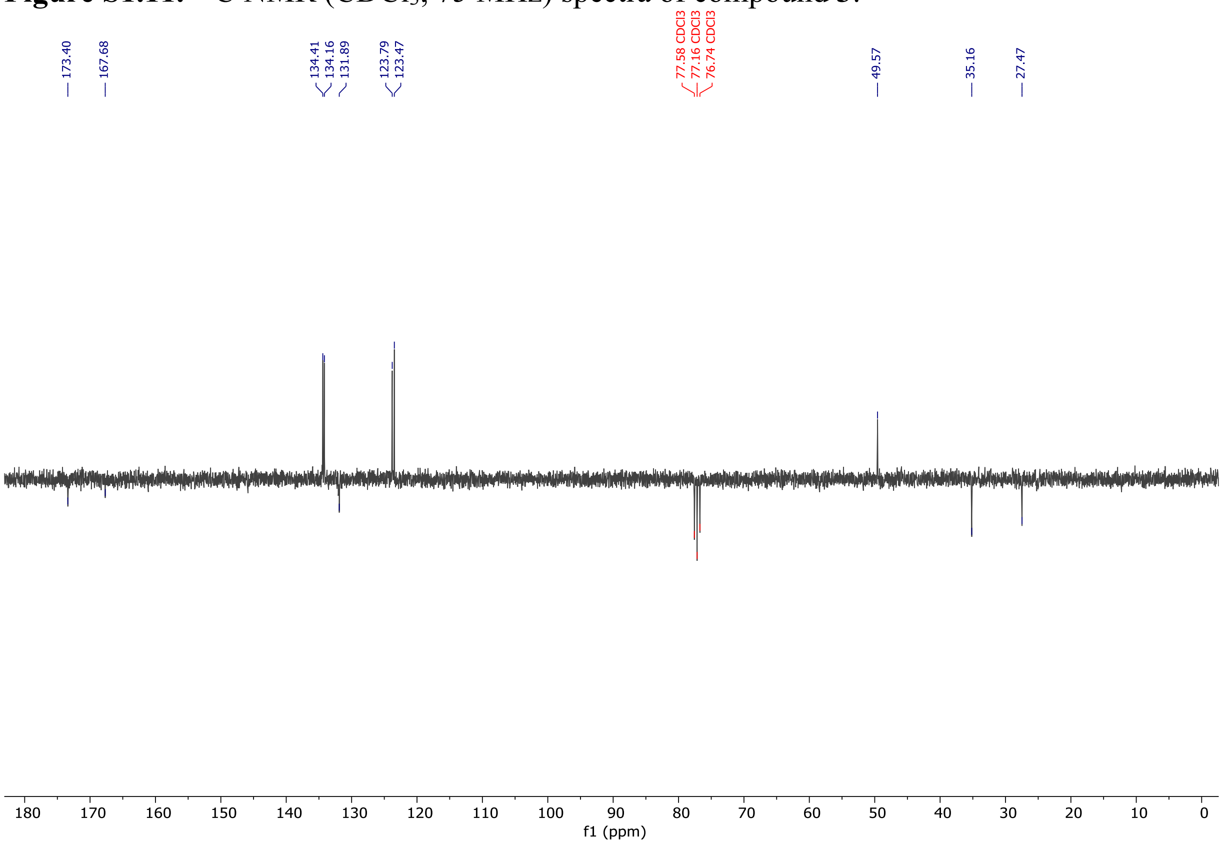
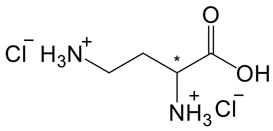


Figure S1.12. ^{13}C APT NMR ($CDCl_3$, 75 MHz) spectra of compound **3**.

Compound **4**



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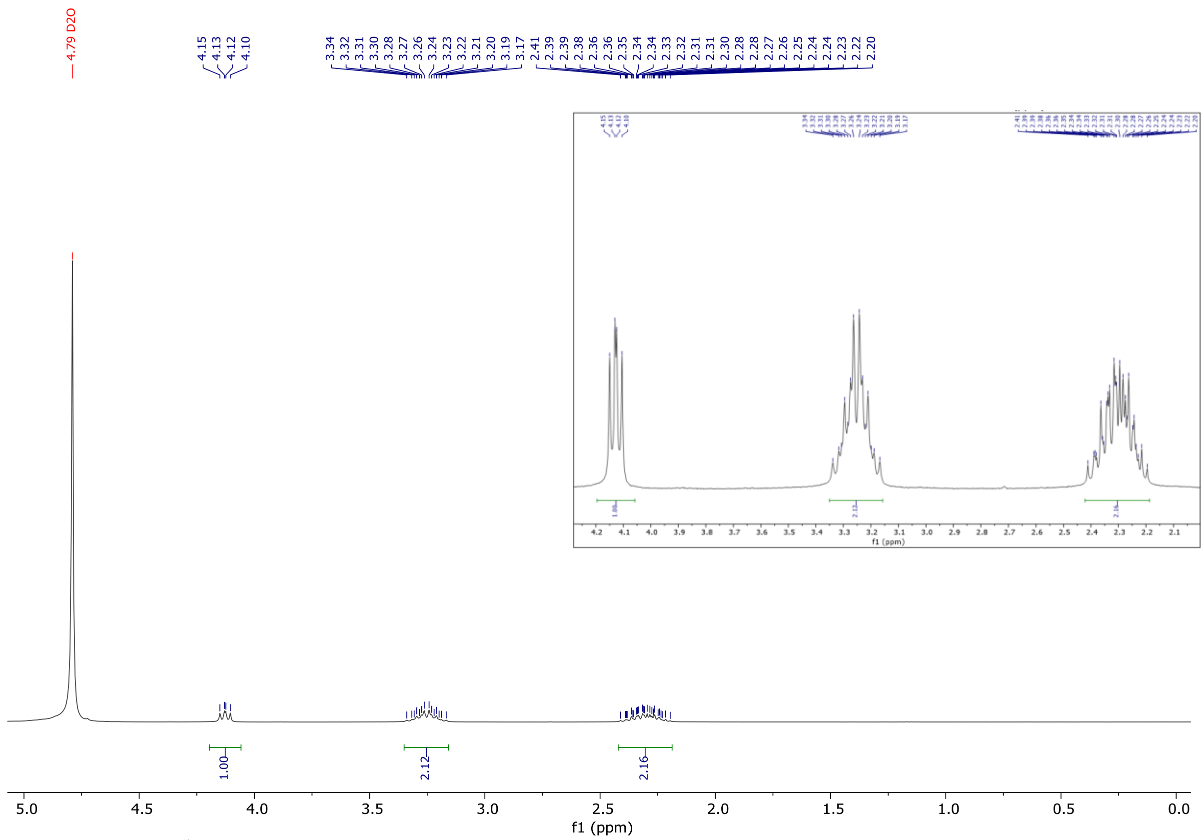


Figure S1.13. ^{1}H NMR ($CDCl_3$, 300 MHz) spectra of compound **4**.

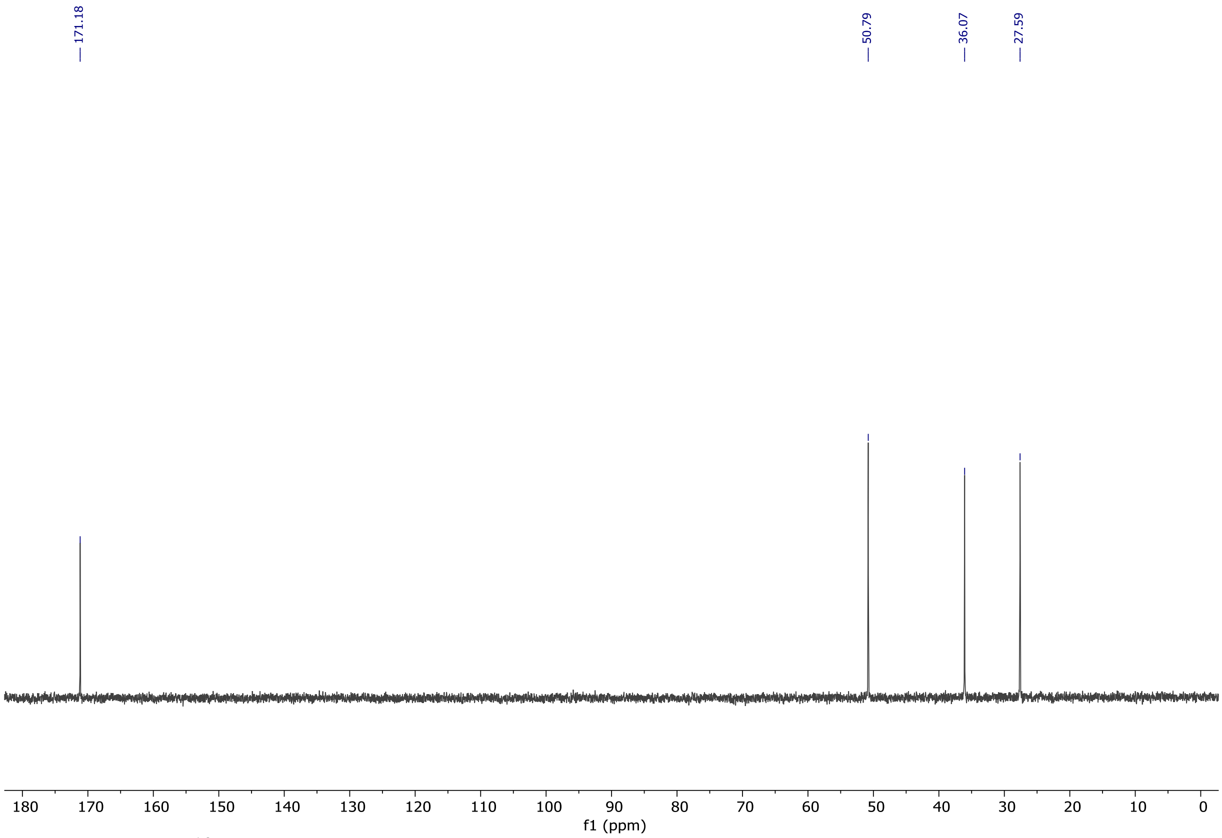


Figure S1.14. ^{13}C NMR ($CDCl_3$, 75 MHz) spectra of compound **4**.

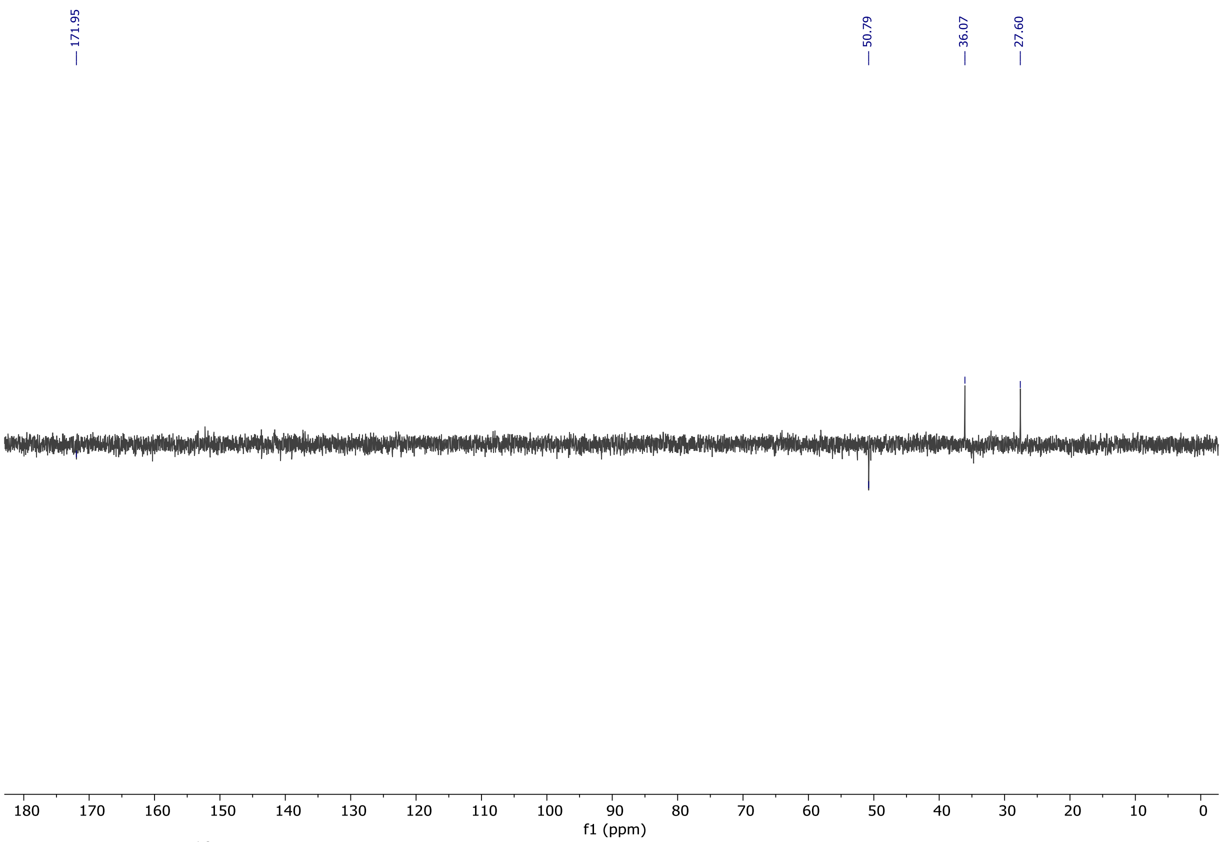
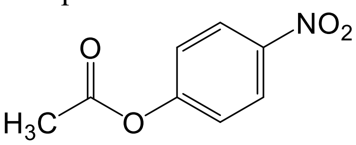


Figure S1.15. ^{13}C APT NMR ($CDCl_3$, 75 MHz) spectra of compound **4**.

Compound **5**



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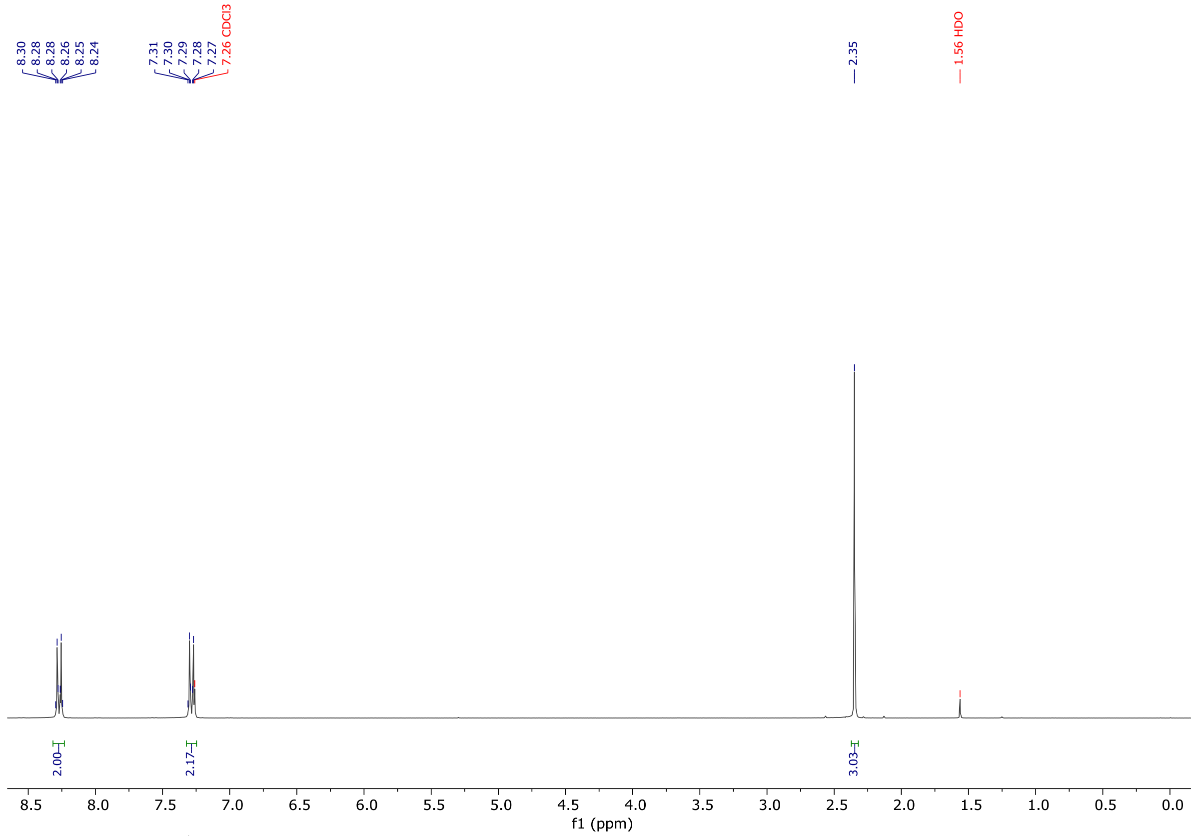


Figure S1.16. [1]H NMR ($CDCl_3$, 300 MHz) spectra of compound **5**.

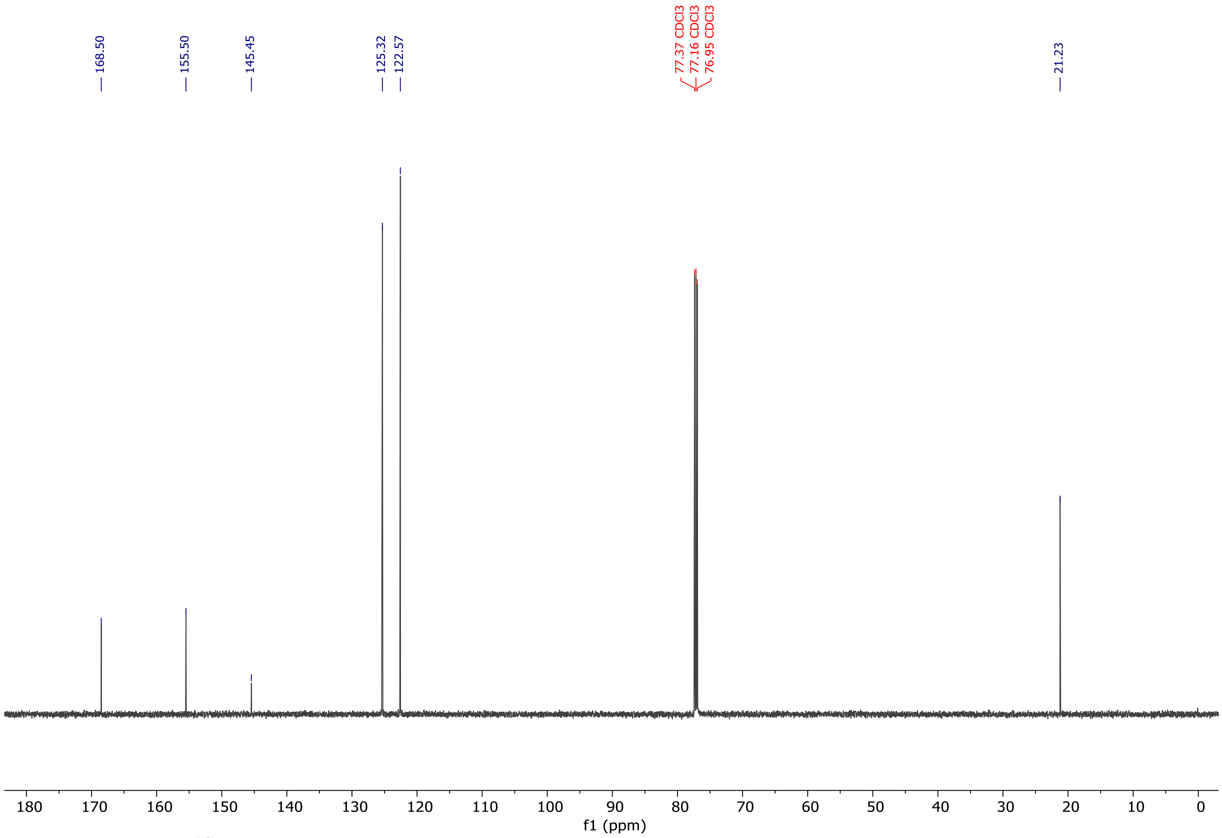


Figure S1.17. ^{13}C NMR ($CDCl_3$, 75 MHz) spectra of compound **5**.

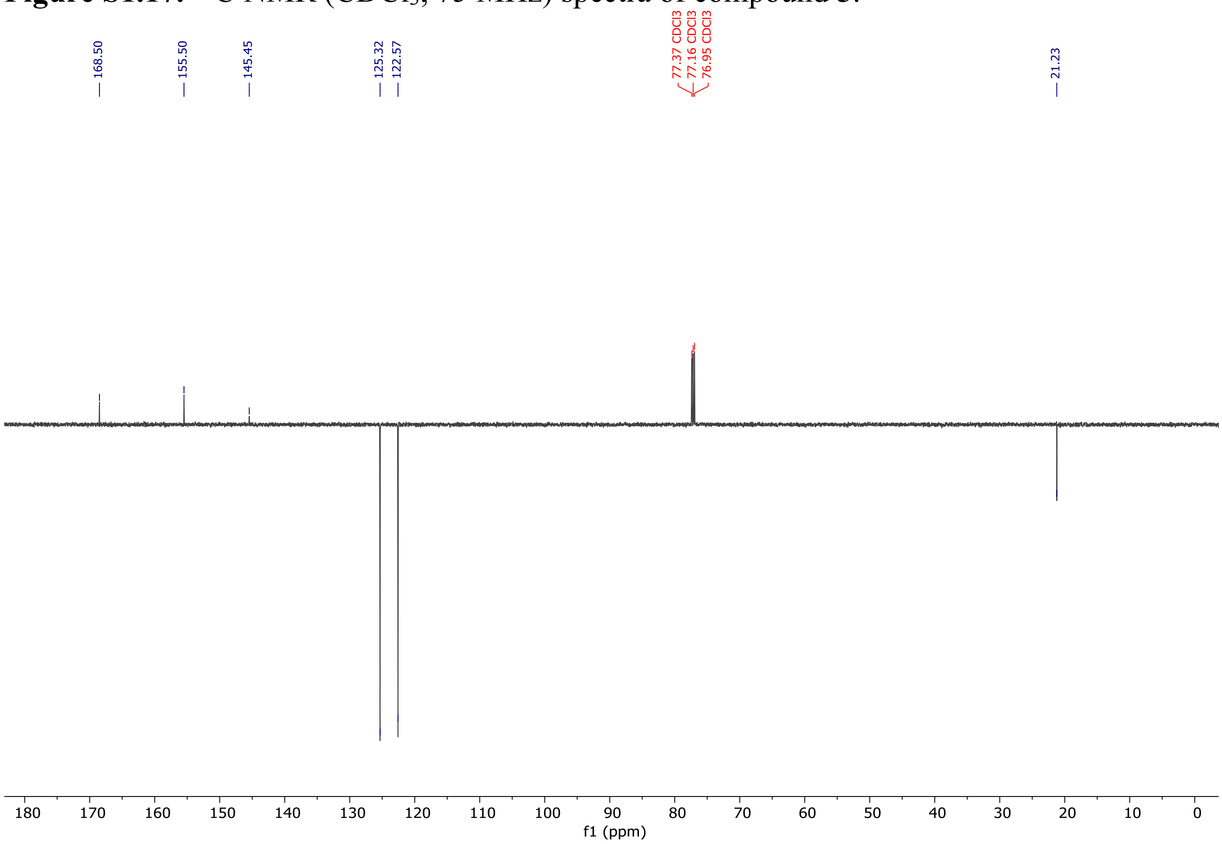
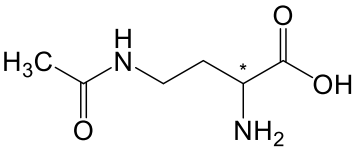


Figure S1.18. ^{13}C APT NMR ($CDCl_3$, 75 MHz) spectra of compound **5**.

Compound **6**



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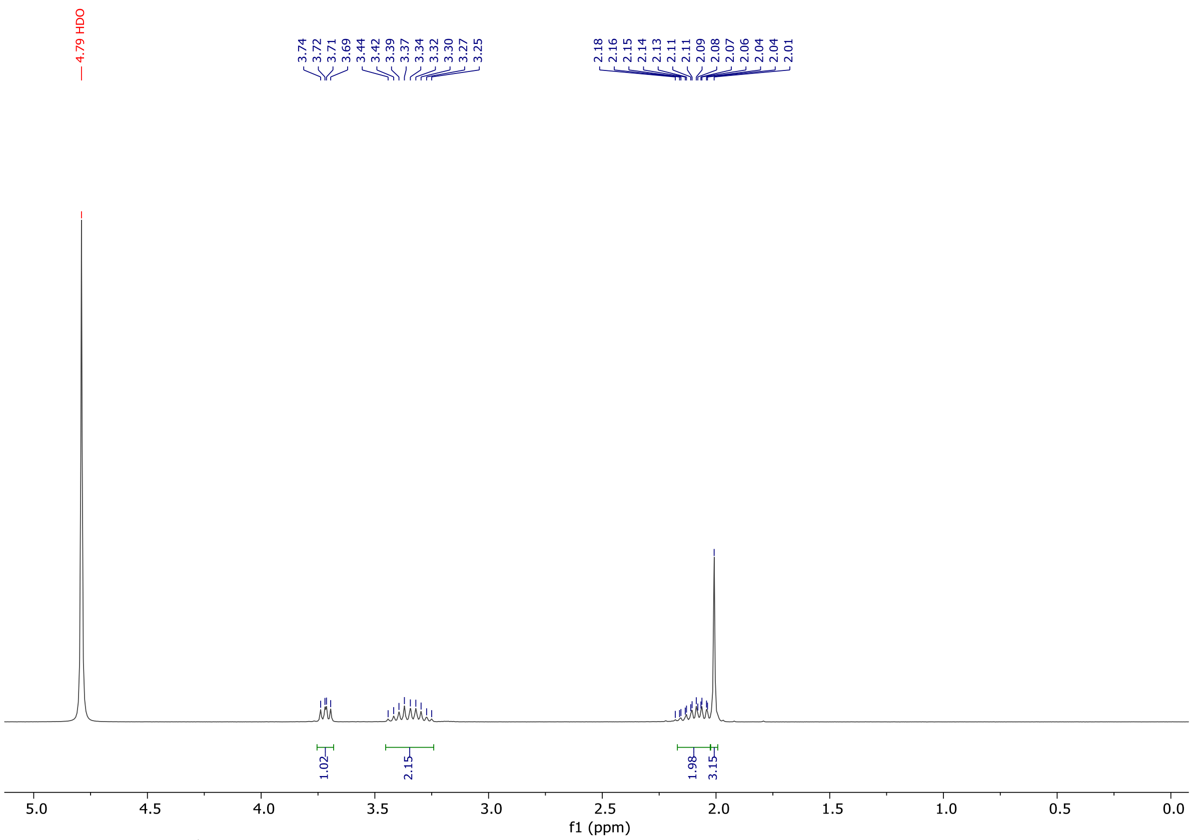


Figure S1.19. ^{1}H NMR (D_2O_3, 300 MHz) spectra of compound **6**.

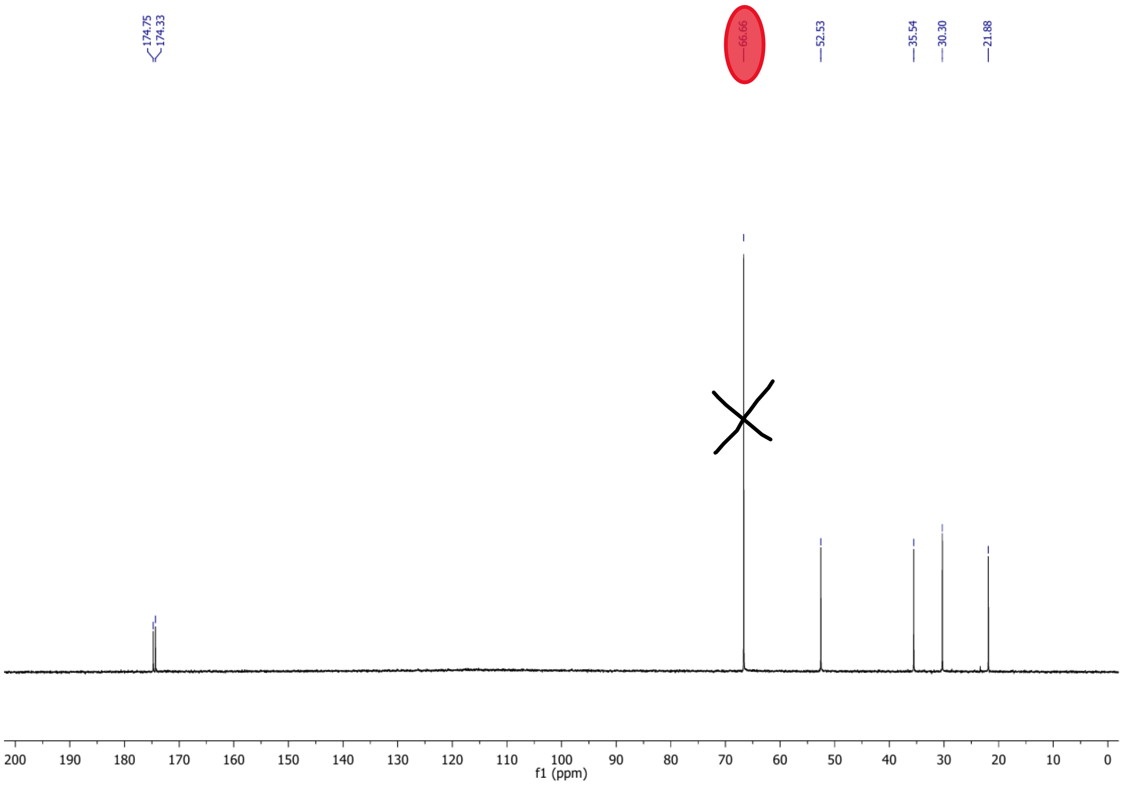
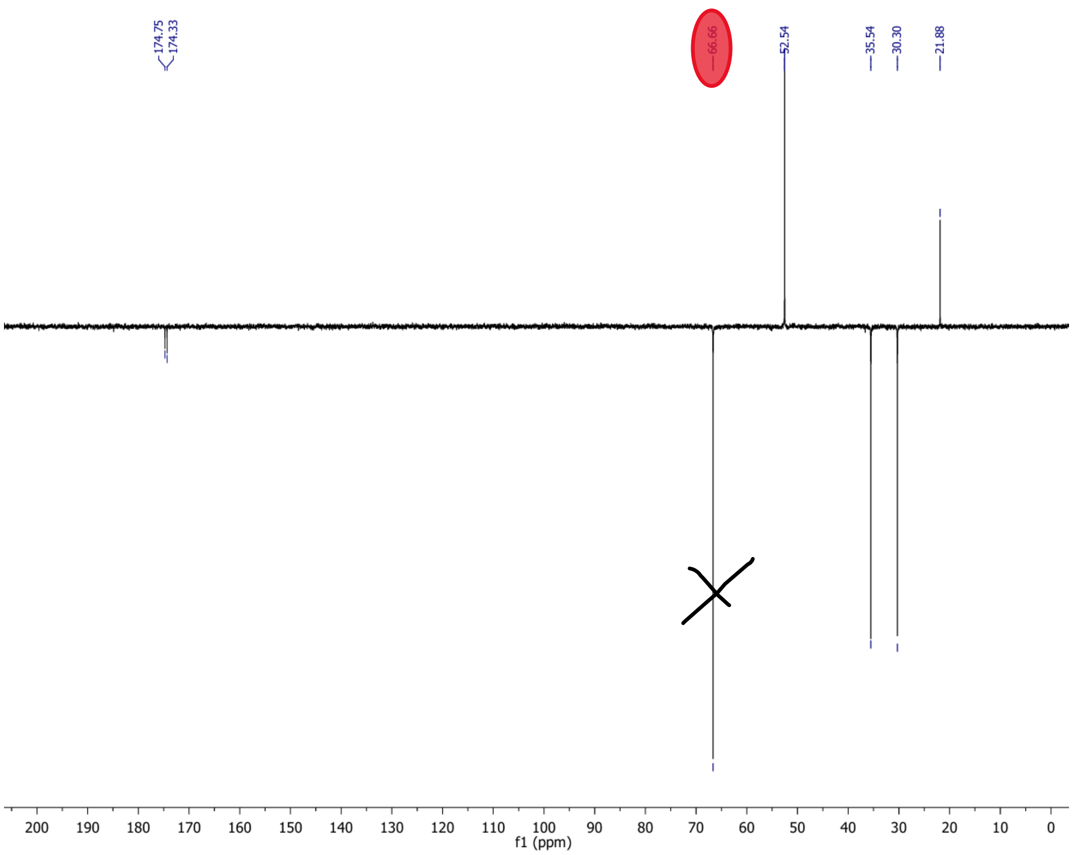
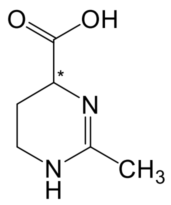


Figure S1.20. ^{13}C NMR (D_2O+dioxane-d_8, 75 MHz) spectra of compound **6**.



Slika D1.21. ^{13}C APT NMR (D_2O+dioxane-d_8, 75 MHz) spectra of compound **6**.

Compound **7**



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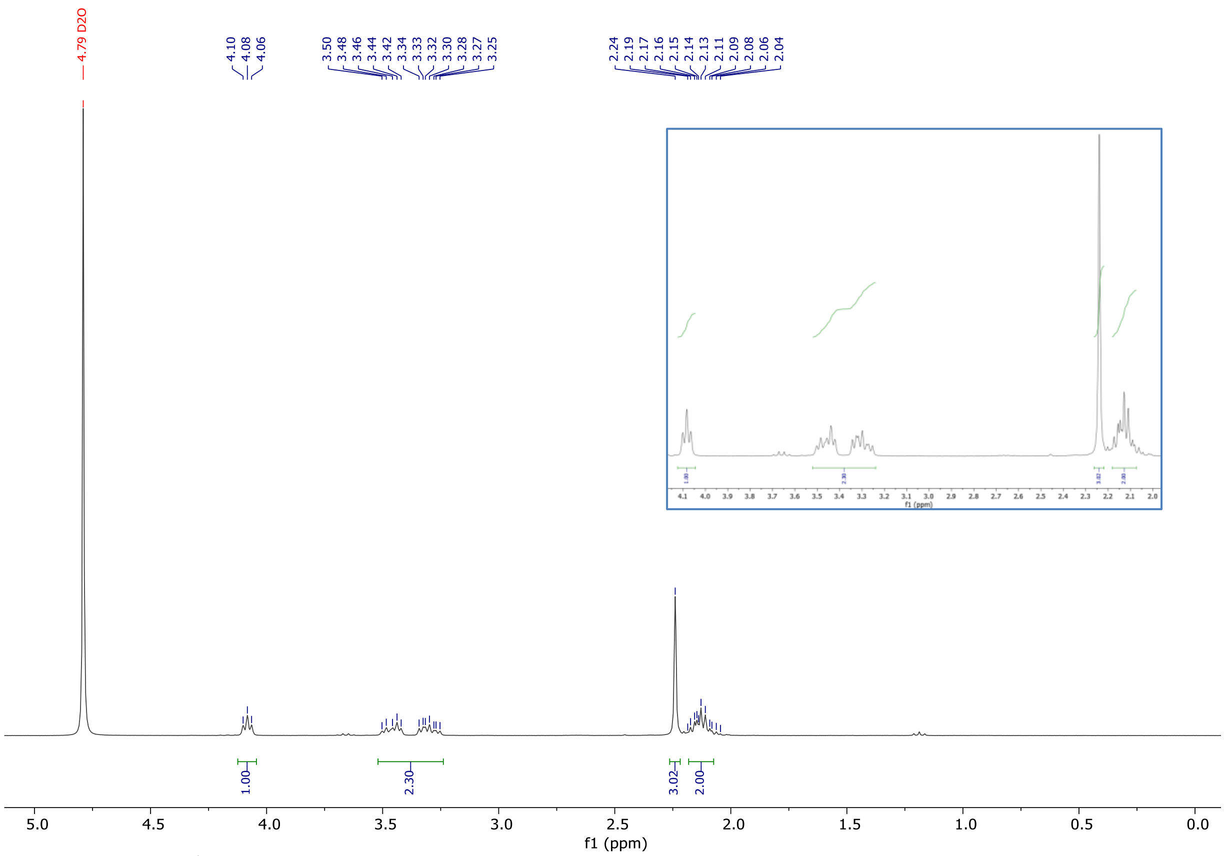

Figure S1.22. ^{1}H NMR (D_2O, 300 MHz) spectra of compound **7.**

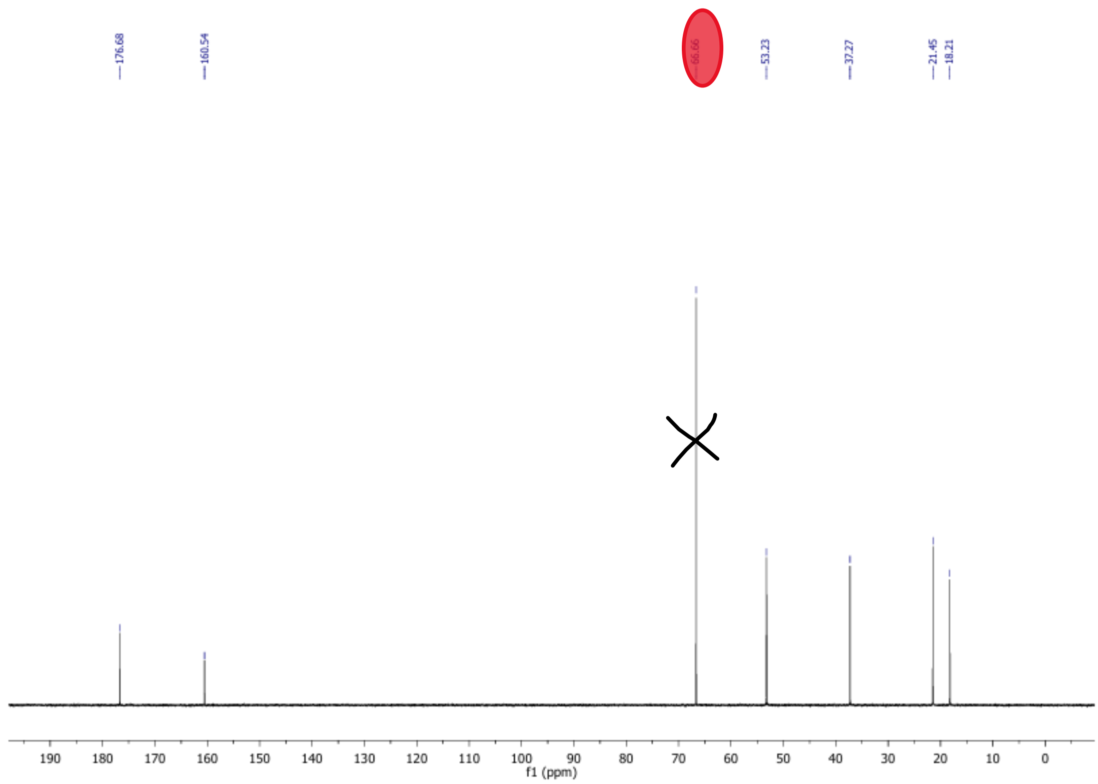

Figure S1.23. ^{13}C NMR (D$_2$O+dioxane-d_8, 75 MHz) spectra of compound **7.**

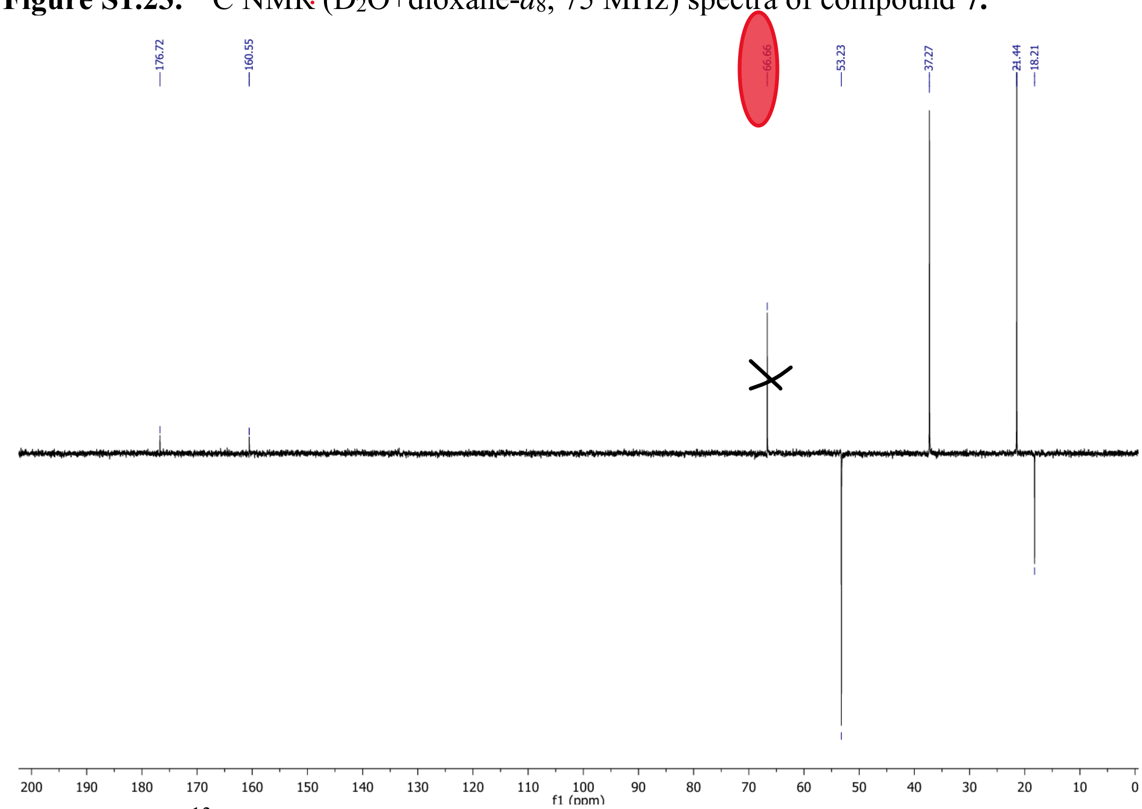

Figure S1.24. ^{13}C APT NMR (D$_2$O+dioxane-d_8, 75 MHz) spectra of compound **7.**

2. IR spectra

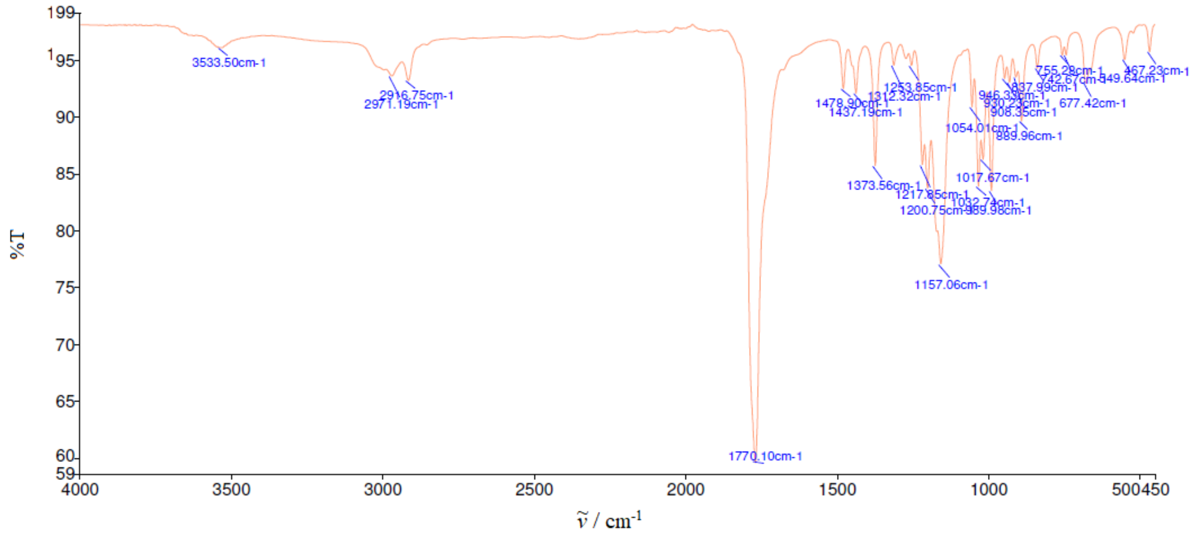

Figure S2.1. IR spectra of compound **1**.

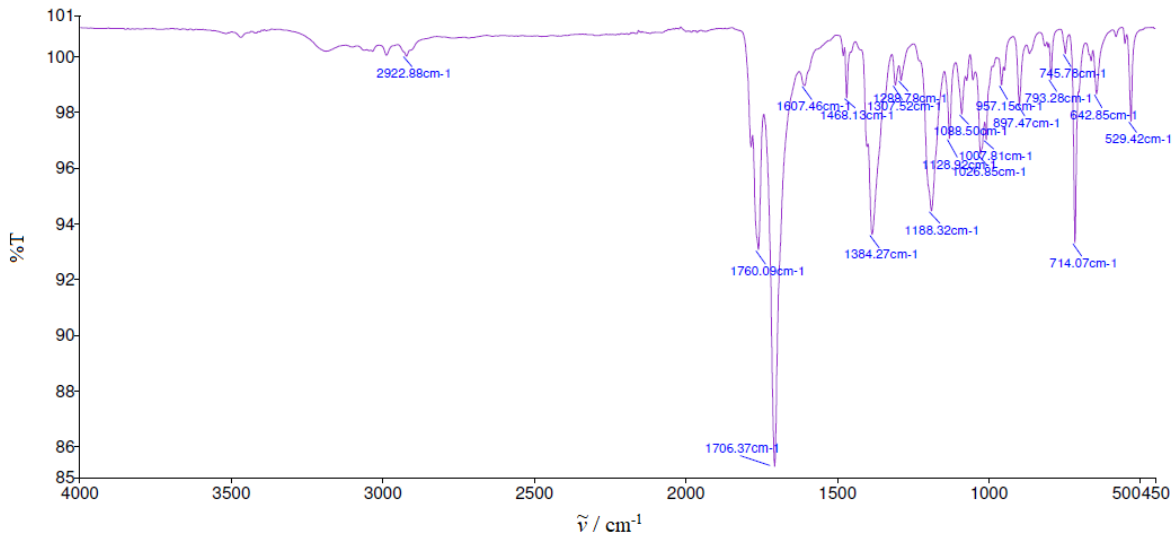

Figure S2.2. IR spectra of compound **2**.

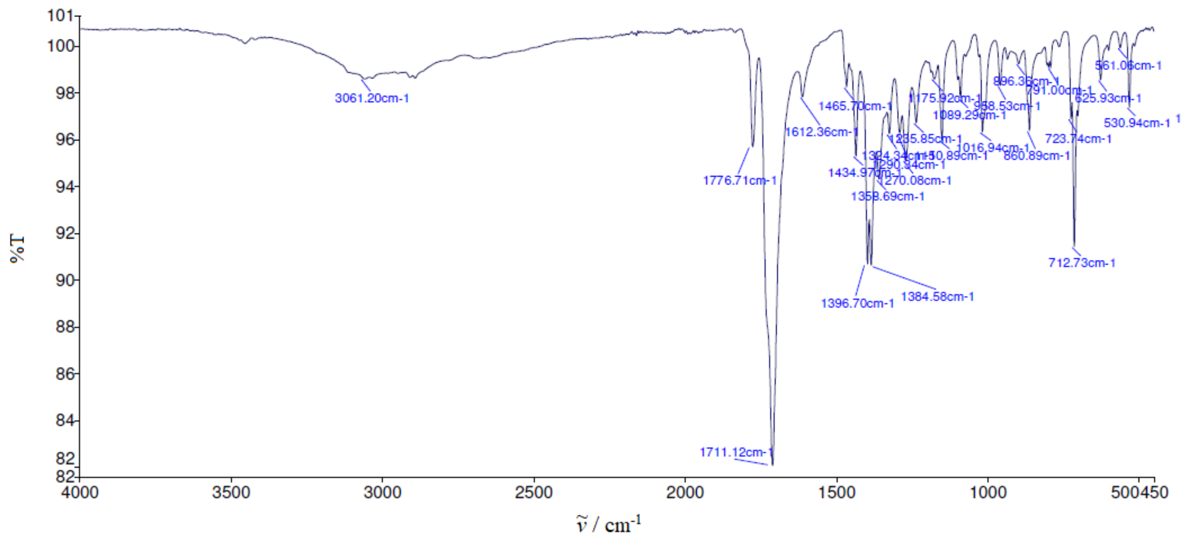

Figure S2.3. IR spectra of compound **3**.

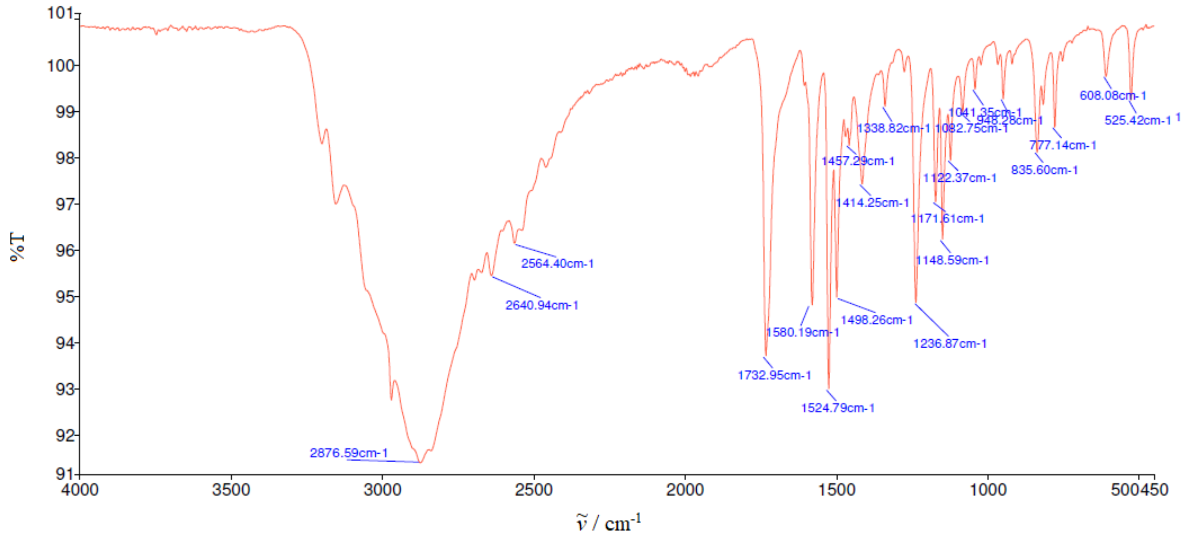

Figure S2.4. IR spectra of compound **4**.

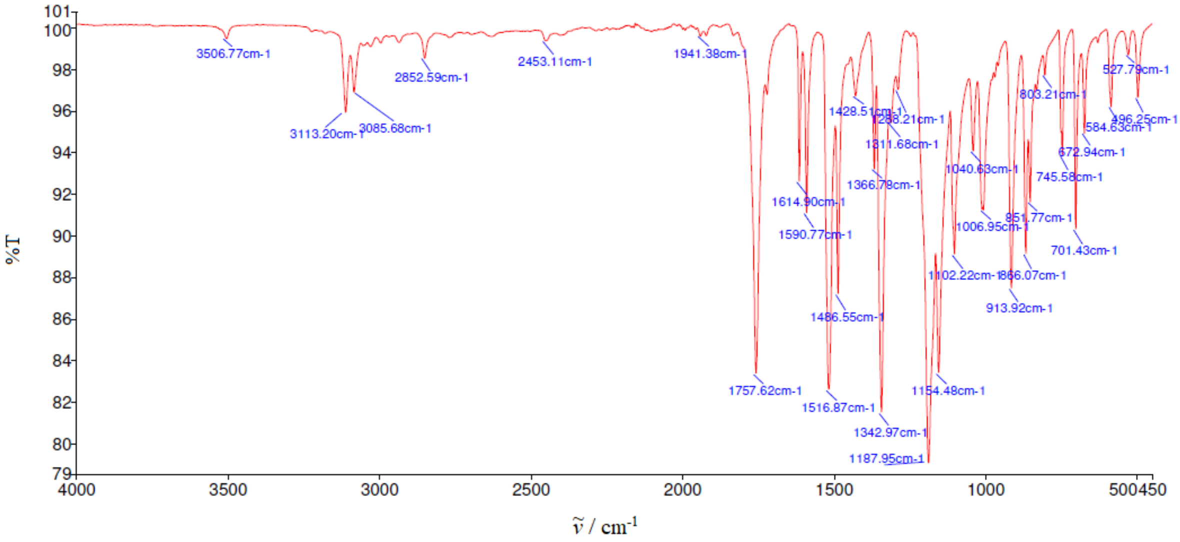

Figure S2.5. IR spectra compound **5**.

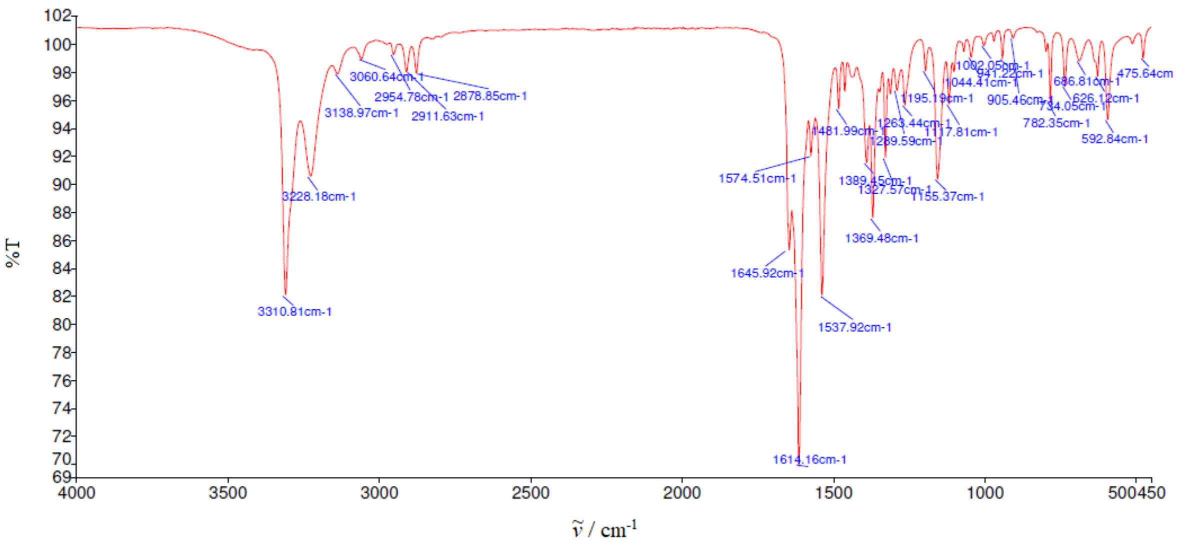

Figure S2.6. IR spectra of complex Cu(II) and compound **6**.

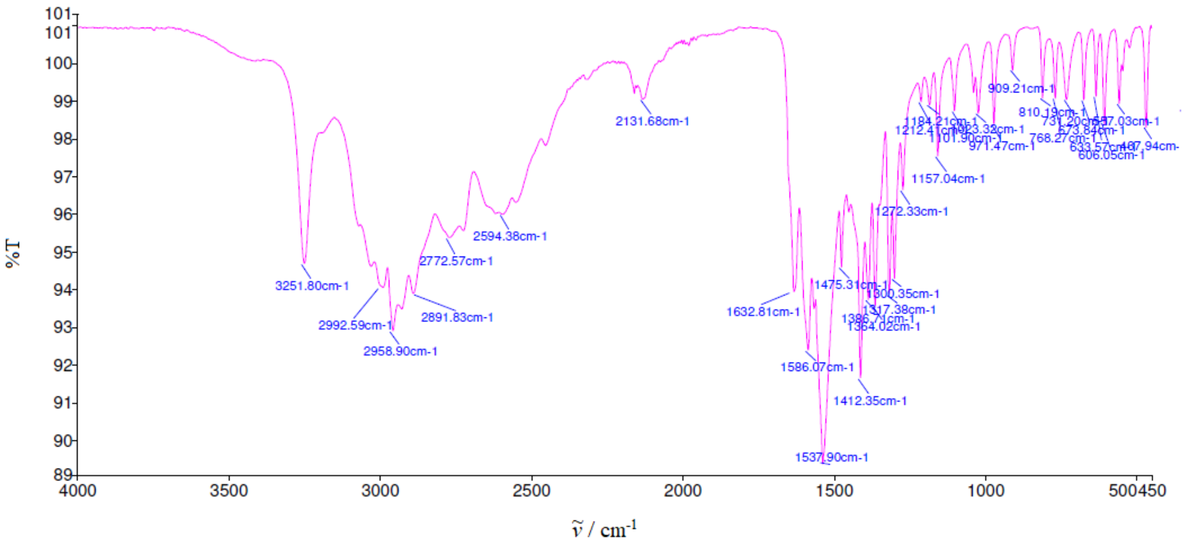

Figure S2.7. IR spectra of compound **6**.

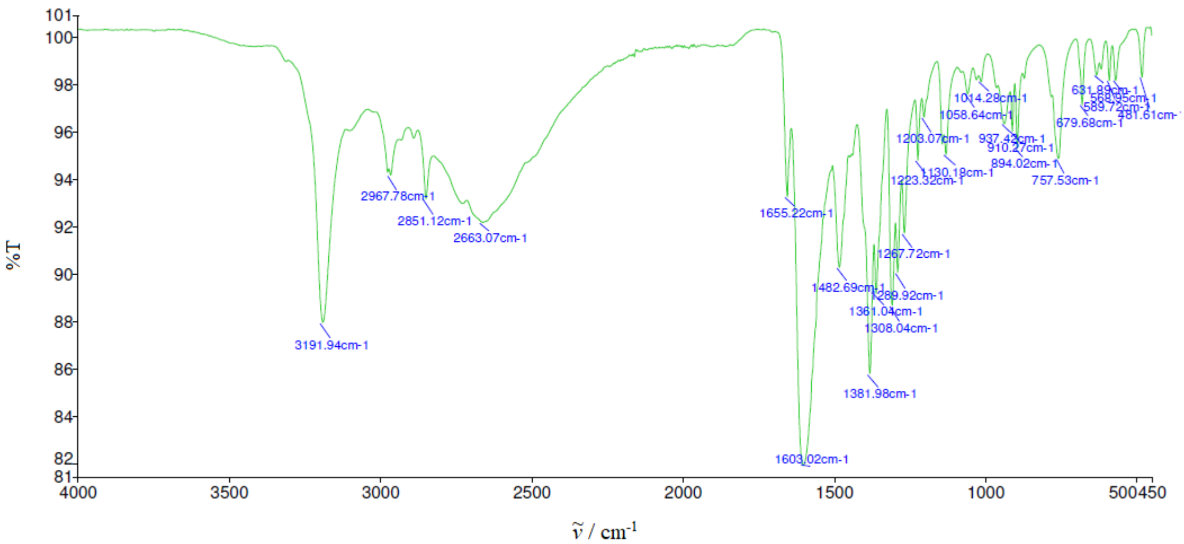

Figure S2.8. IR spectra of compound **7**.

3. Separation of (±)-ectoine enantiomeres

The solvents used for analyses were HPLC purity: MeOH, ACN, Milli-Q H_2O. All parameters used are listed in Table S3.1. UV detection was performed at 210 nm. The concentrations of (±)-ectoine used were 0.25 mg/mL. The injection volumes were 10 µL. Commercially available (+)-ectoine was used as a standard.

Table S1. Methods and conditions for (±)-ectoine enantioseparation.

Method	Column	Mobile phase*	Flow rate [mL/min]	T [°C]
1	(*R*,*R*)-WhelkO1	MeOH/ 0.1 % AcOH	1	25
2	(*R*,*R*)-WhelkO1	MeOH:H_2O = 1:1 / 0.1 % AcOH	1	25
3	(*R*,*R*)-WhelkO1	MeOH:H_2O = 7:3 / 0.1 % AcOH	1	25
4	(*R*,*R*)-WhelkO1	ACN:H_2O = 7:3	1	25
5	(*R*,*R*)-WhelkO1	ACN:H_2O = 15:85	1	25
6	Chiralcel OJ-RH	ACN:H_2O = 5:95	1	25
7	Chiralcel OJ-RH	MeOH:H_2O = 1:1/0.1 % AcOH	1	25
8	Chiralcel OJ-RH	ACN:H_2O = 15:85	1	25
9	Chiralcel OJ-RH	ACN:H_2O = 15:85/0.1 % AcOH	1	25
10	Chiralcel OJ-RH	ACN:H_2O = 1:9	1	25
11	Chiralcel OJ-RH	ACN:H_2O = 3:7	1	25
12	Chiralcel OJ-RH	ACN:H_2O = 7:3	1	25
13	Chiralcel OD-RH	ACN:H_2O = 15:85	1	25
14	Chiralcel OD-RH	MeOH/0.1 % AcOH	1	25
15	Chirallica PST-4	MeOH/0.1 % AcOH	1	25
16	Chiral-T	MeOH	1	30
17	Chiral-T	MeOH/0.1 % FA	1	25
18	Chiral-T	MeOH/0.01 % FA	1	25
19	Chiral-T	MeOH 0.01 % TEA	1	25
20	Chiral-T	MeOH:H_2O = 9:1	1	30
21	Chiral-T	MeOH:H_2O = 9:1	1	35
22	Chiral-T	MeOH:H_2O = 9:1	1	40
23	Chiral-T	MeOH:H_2O = 7.5:2.5	0.5	30
24	Chiral-T	MeOH:H_2O = 7.5:2.5	0.5	40
25	Chiral-T	MeOH:H_2O = 7.5:2.5	1	40
26	Chiral-T	MeOH:H_2O = 7.5:2.5	1.2	40
27	Chiral-T	MeOH:H_2O = 1:1	0.5	40
28	Chiral-T	MeOH:H_2O = 1:1	0.75	40
29	Chiral-T	MeOH:H_2O = 1:1	1	30
30	Chiral-T	MeOH:H_2O = 1:1	1	35
31	Chiral-T	MeOH:H_2O = 1:1	1	40
32	Chiral-T	MeOH:H_2O = 1:1	1	45
33	Chiral-T	MeOH:NH_4COOH (25 mM, pH = 3.0) = 7:3	1	40
34	Chiral-T	MeOH:NH4COOH (25 mM, pH = 3.0) = 1:1	1	40
35	Chiral-T	ACN:H2O = 8:2	1	30

*volume ratio v/v

4. Detection of (+)-ectoine in fermentation broths of salt-tolerant marine *Streptomyces*

4.1. *Streptomyces* strains

Table S2. *Streptomyces* strains isolated from marine invertebrates and sediments used in this study.

Strain	Closest match[a]	Identity (%)	(Gene Bank Acc. No.)[b]	Type of sample	Sampling site	Sampling date
BC81	*Streptomyces hydrogenans* strain Kris3	100	PP784949	Sponge – *Geodia cydonium*	Adriatic Sea, Croatia, Dugi otok, Dragove 44°07'10.8"N 14°55'43.5"E	14.07.2019
BC121	*Streptomyces hydrogenans* strain Kris3	100	PP784953	Tunicate – *Phallusia mammillata*	Adriatic Sea, Croatia, Zadar, Tankerkomerc Marina 44°07'08.8"N 15°13'41.9"E	20.02.2020
BC123	*Streptomyces sp.* strain BSP1	100	PP784954	Tunicate – *Phallusia mammillata*		
BC126	*Streptomyces diastaticus* subsp. diastaticus strain YNF17-1	100	PP784955	Tunicate – *Phallusia mammillata*	Adriatic Sea, Croatia, Zaton, Šepurine 44°12'42.3"N 15°09'31.3"E	
BC131	*Streptomyces anulatus* strain NBC 01759	100	PP784956	Mollusca – *Mytilus galloprovincialis*	Adriatic Sea, Croatia, Zadar, Uvala Bregdetti Marina 44°05'58.4"N 15°14'56.3"E	18.02.2020
BC104	*Streptomyces diastaticus* subsp. diastaticus strain YNF17-1	100	PP784950	Sediment	Adriatic Sea, Croatia, Zaton, Šepurine 44°12'42.3"N 15°09'31.3"E	23.05.2019
BC114	*Streptomyces albidoflavus* strain R-53649	100	PP784951	Sediment	Adriatic Sea, Croatia, Zadar, Kolovare 44°06'15.5"N 15°14'06.6"E	23.05.2019
BC115	*Streptomyces hydrogenans* strain Kris3	100	PP784952	Sediment		
BC156	*Streptomyces sp.* strain HBUM206428	100	PP784957	Sediment	Adriatic Sea, Croatia, Zaton, Šepurine 44°12'42.3"N 15°09'31.3"E	21.02.2020
BC160	*Streptomyces sp.* strain BSP1	100	PP784958	Sediment		
BC167	*Streptomyces sp.* JC498	100	PP784959	Sediment		

[a] Based on partial 16S rRNA sequence alignment (> 900 bp) with sequences from NCBI BLAST.
[b] The sequences were submitted to GeneBank (Accesssion number will be added in proof)

4.2. HPLC chromatograms of (+)-ectoine in fermentation broths of salt-tolerant marine *Streptomyces*

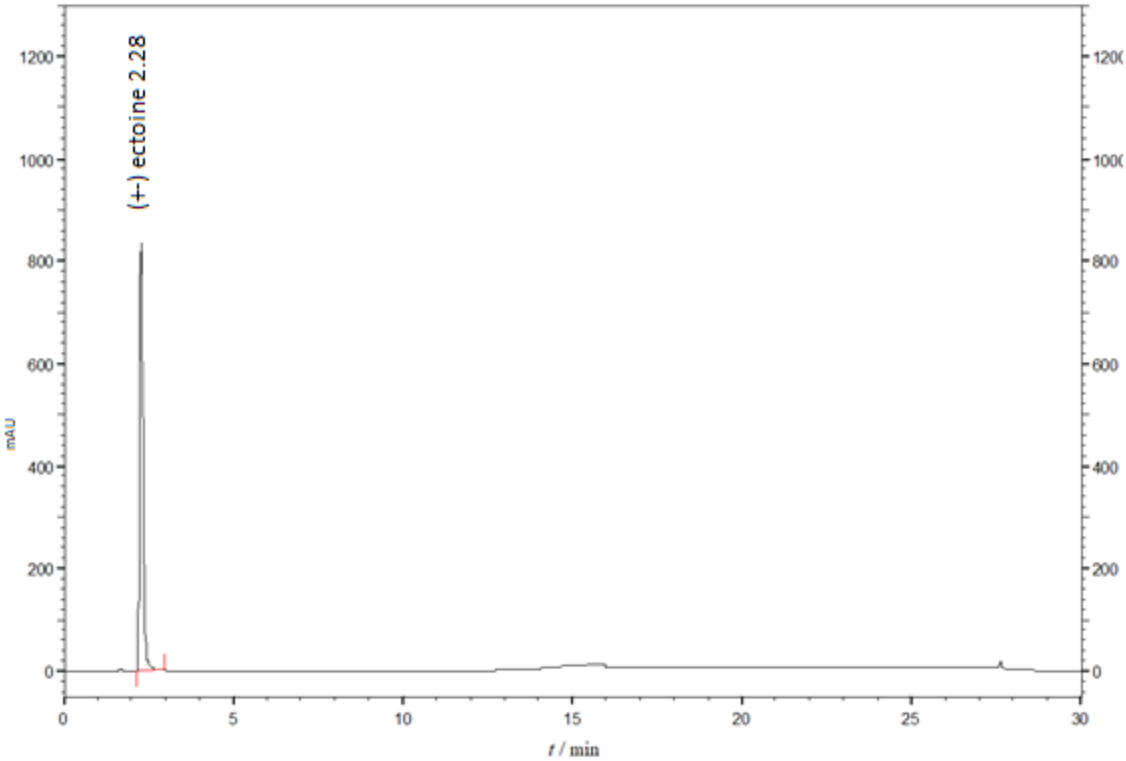

Figure S4.2.1. RP-HPLC chromatogram of (±)-ectoine at 220 nm.

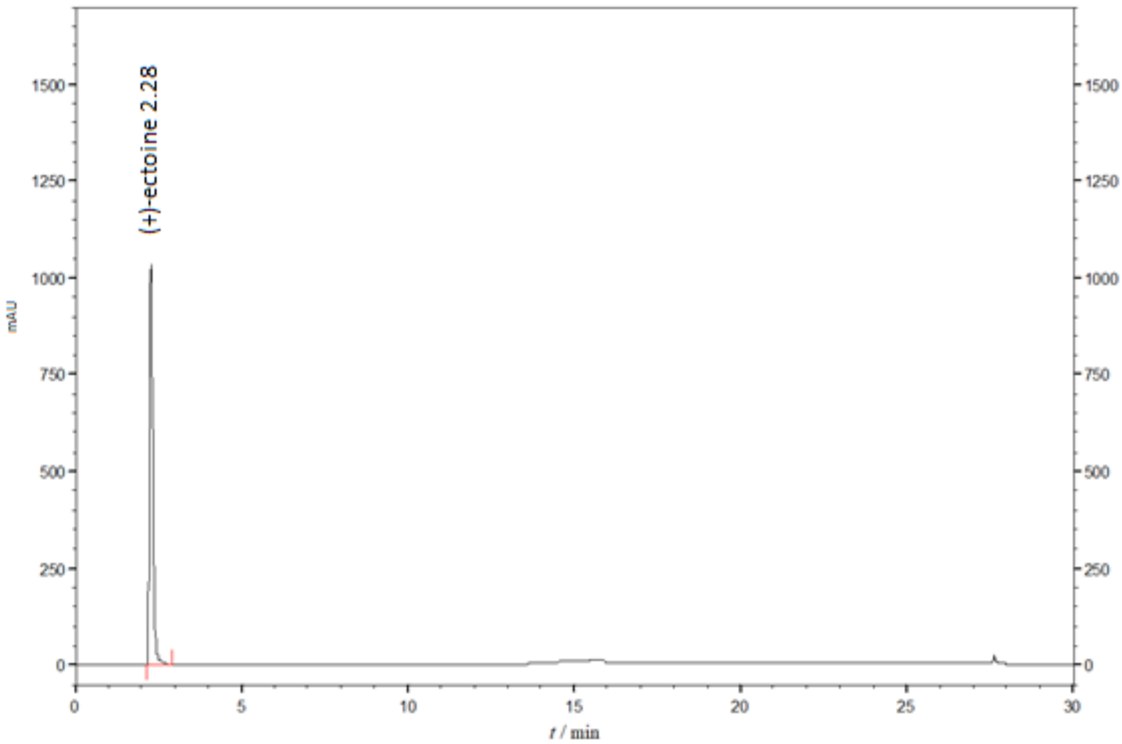

Figure S4.2.2. RP-HPLC chromatogram of (+)-ectoine at 220 nm.

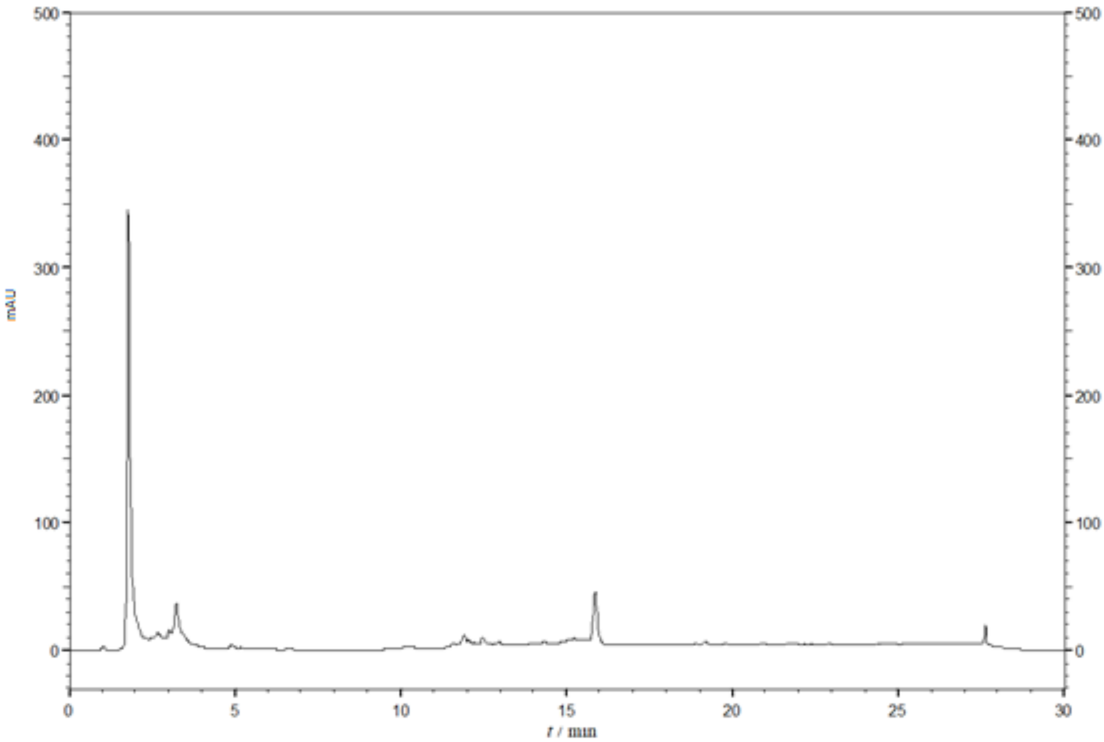

Figure S4.2.3. RP-HPLC chromatogram of sample **1** at 220 nm.

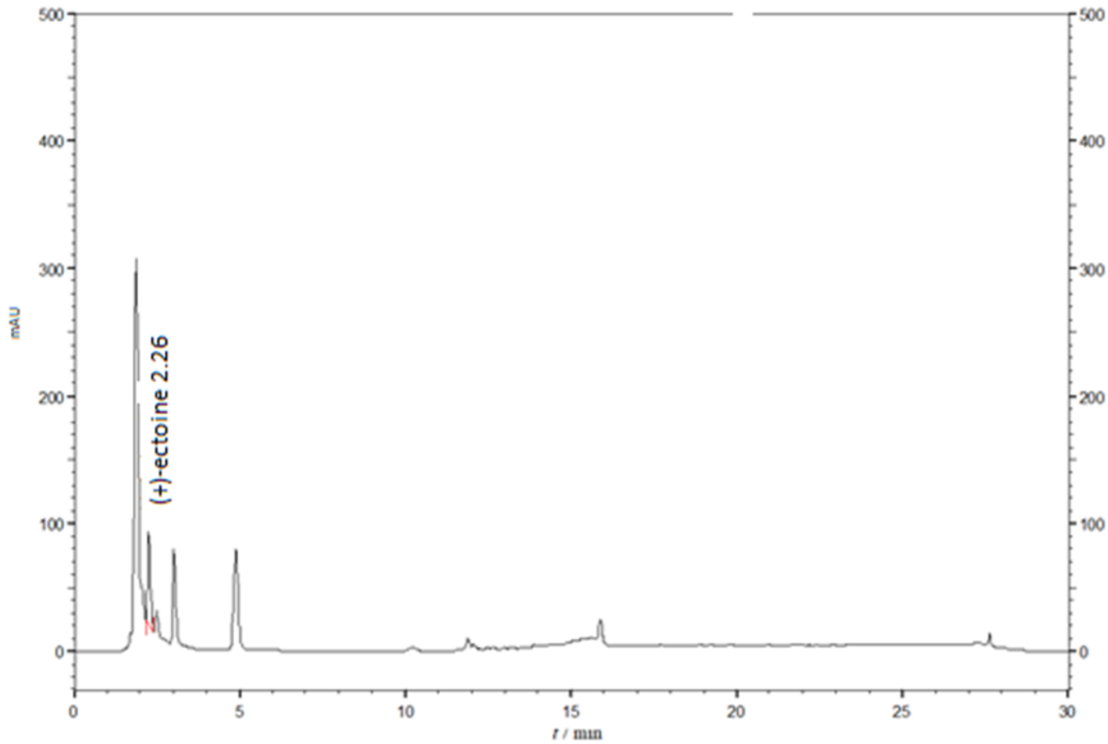

Figure S4.2.4. RP-HPLC chromatogram of sample **2** at 220 nm.

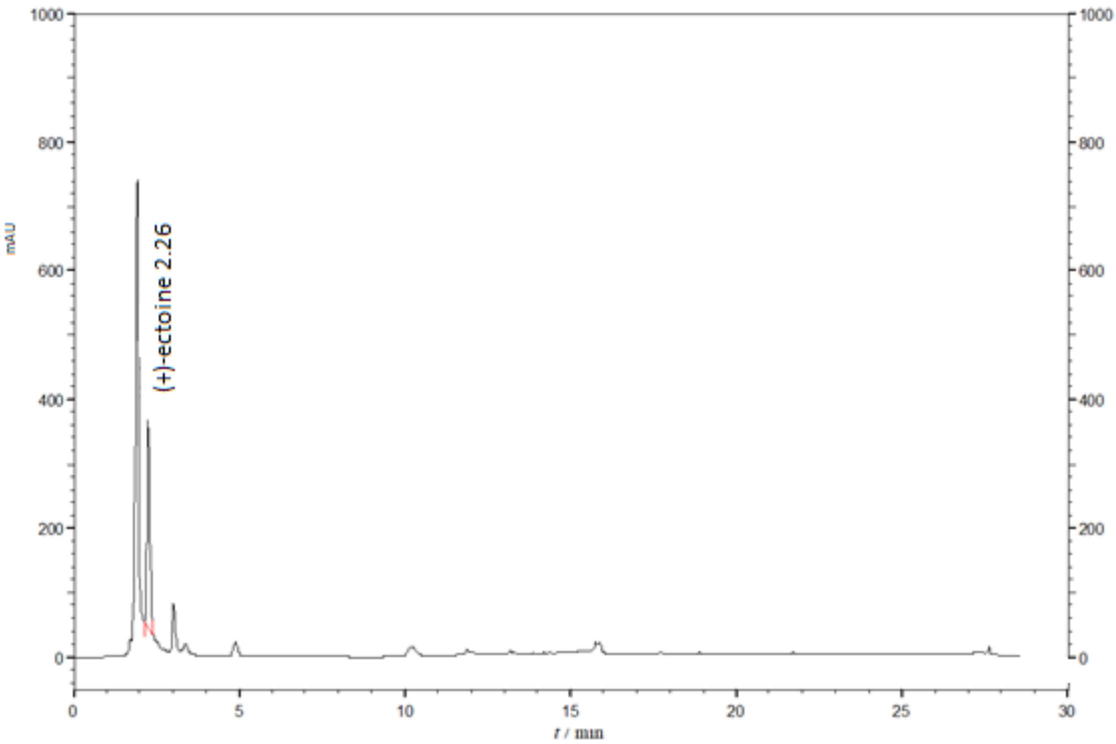

Figure S4.2.5. RP-HPLC chromatogram of sample **3** at 220 nm.

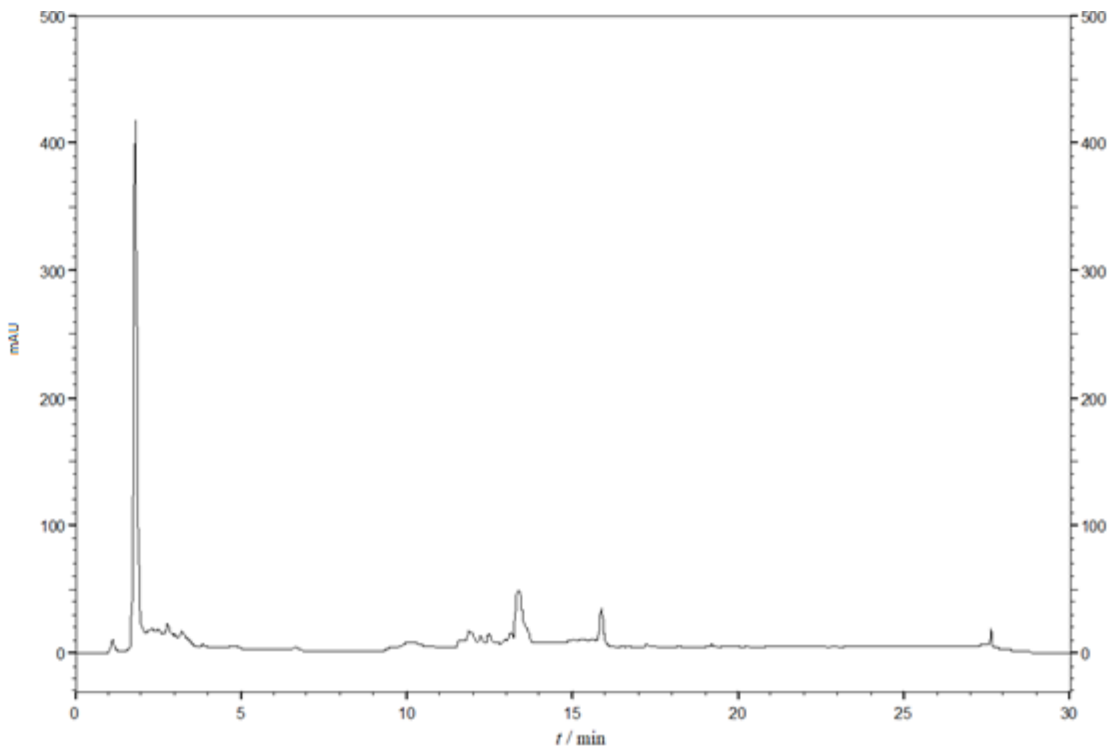

Figure S4.2.6. RP-HPLC chromatogram of sample **4** at 220 nm.

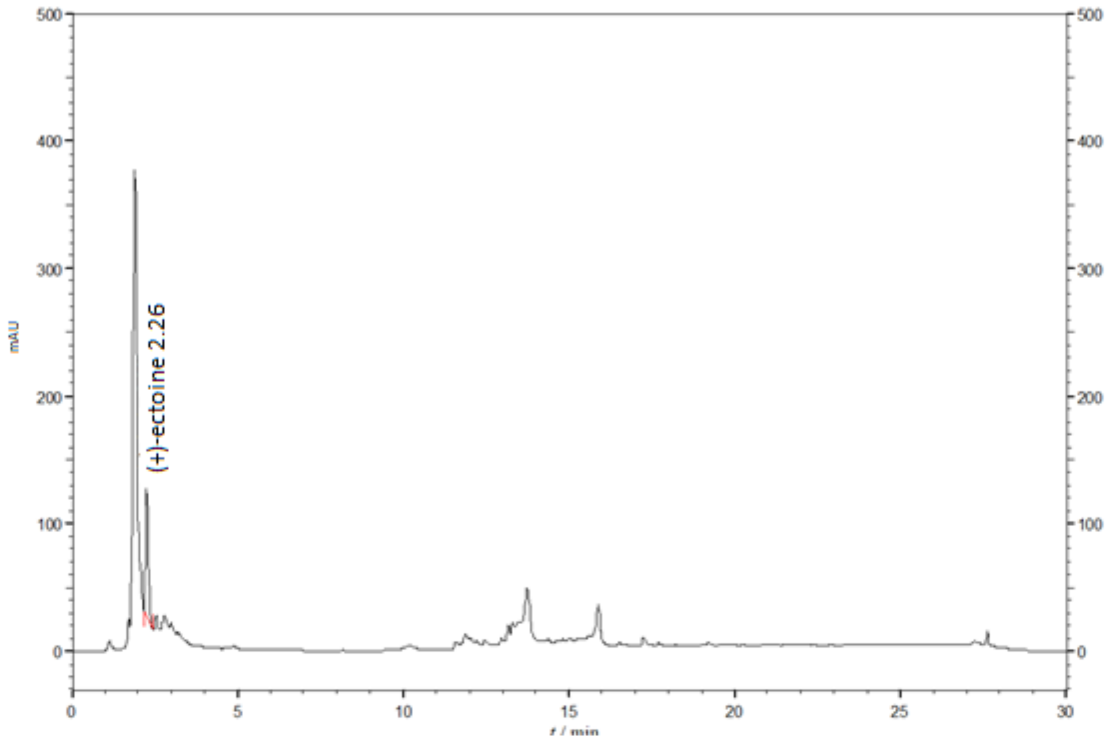

Figure S4.2.7. RP-HPLC chromatogram of sample **5** at 220 nm.

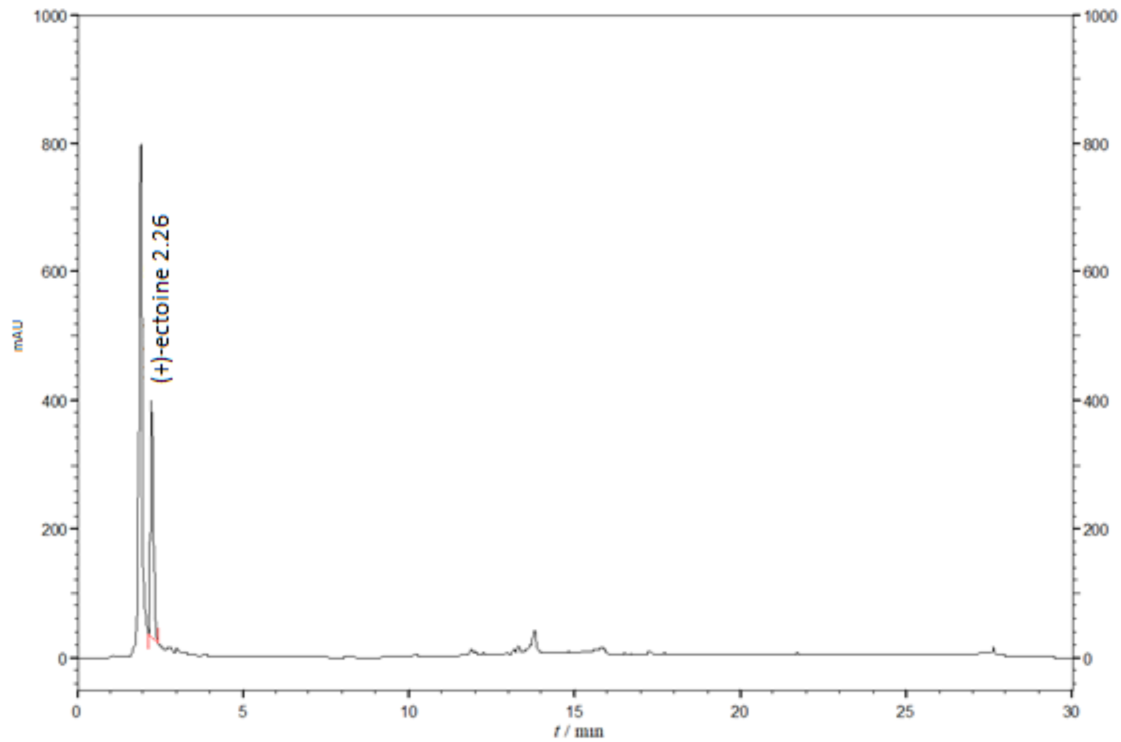

Figure S4.2.8. RP-HPLC chromatogram of sample **6** at 220 nm.

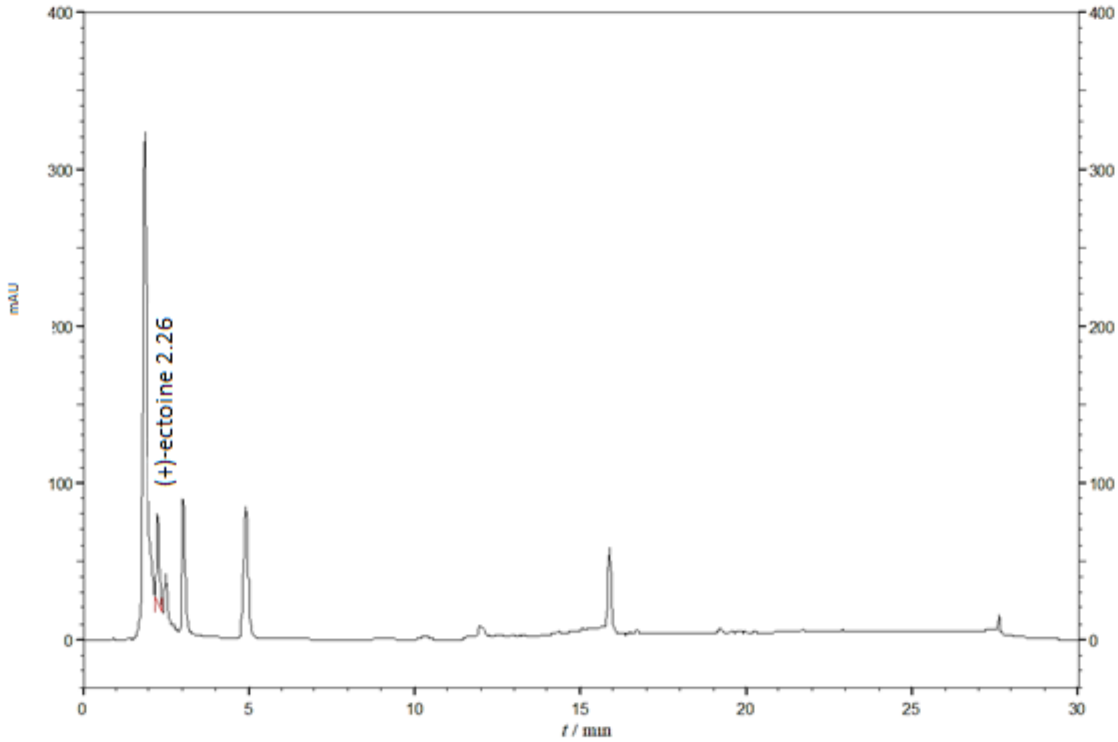

Figure S4.2.9. RP-HPLC chromatogram of sample **7** at 220 nm.

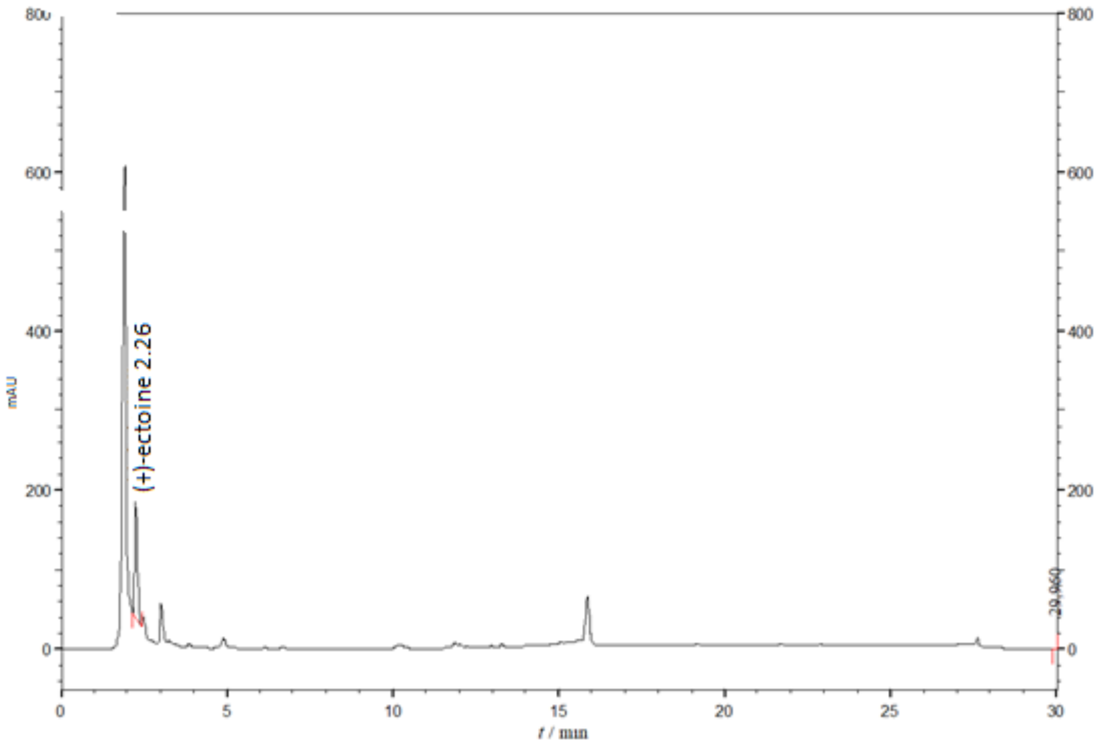

Figure S4.2.10. RP-HPLC chromatogram of sample **8** at 220 nm.

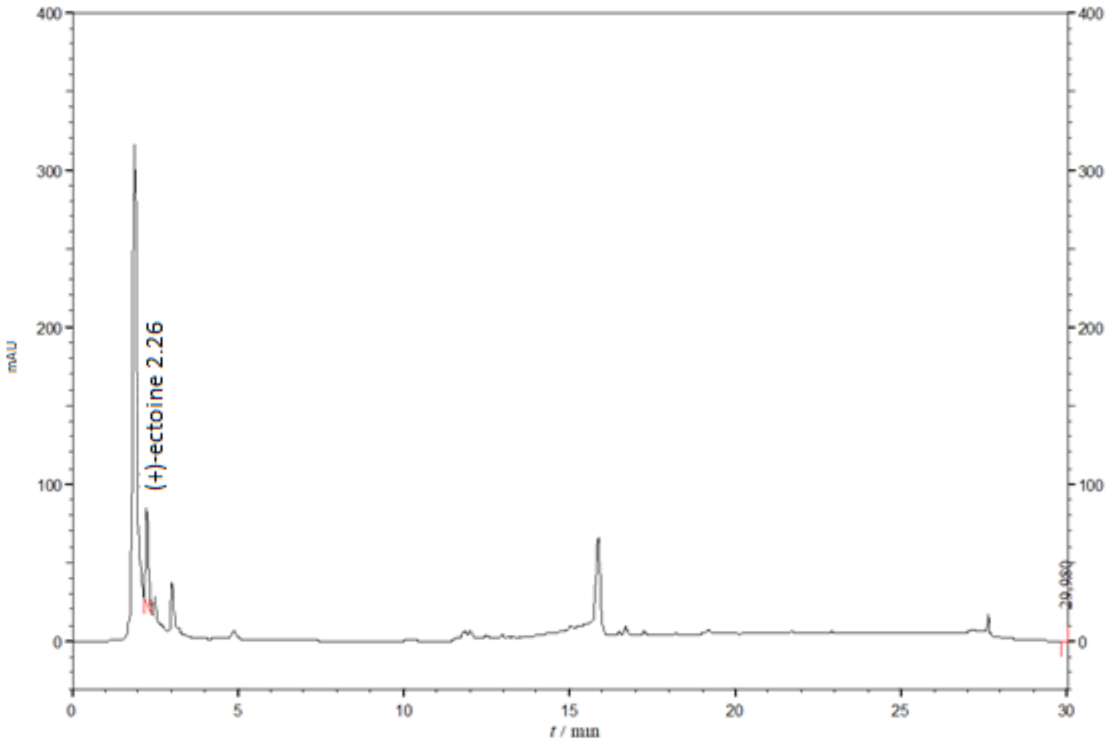

Figure S4.2.11. RP-HPLC chromatogram of sample **9** at 220 nm.

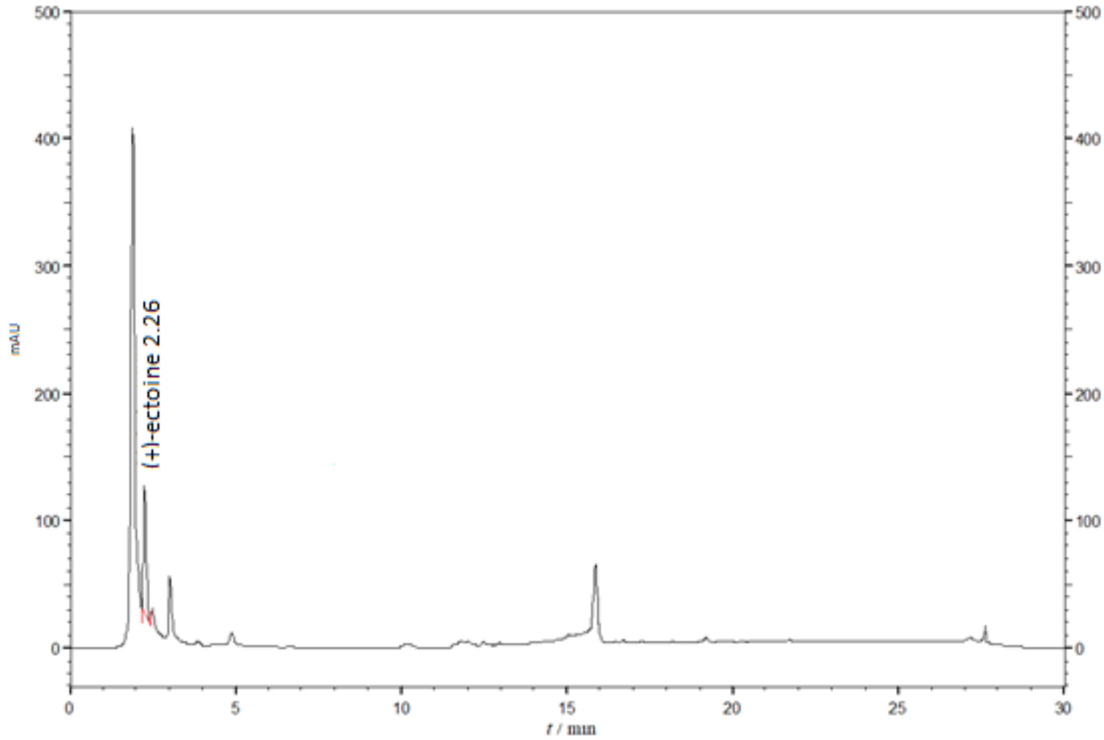

Figure S4.2.12. RP-HPLC chromatogram of sample **10** at 220 nm.

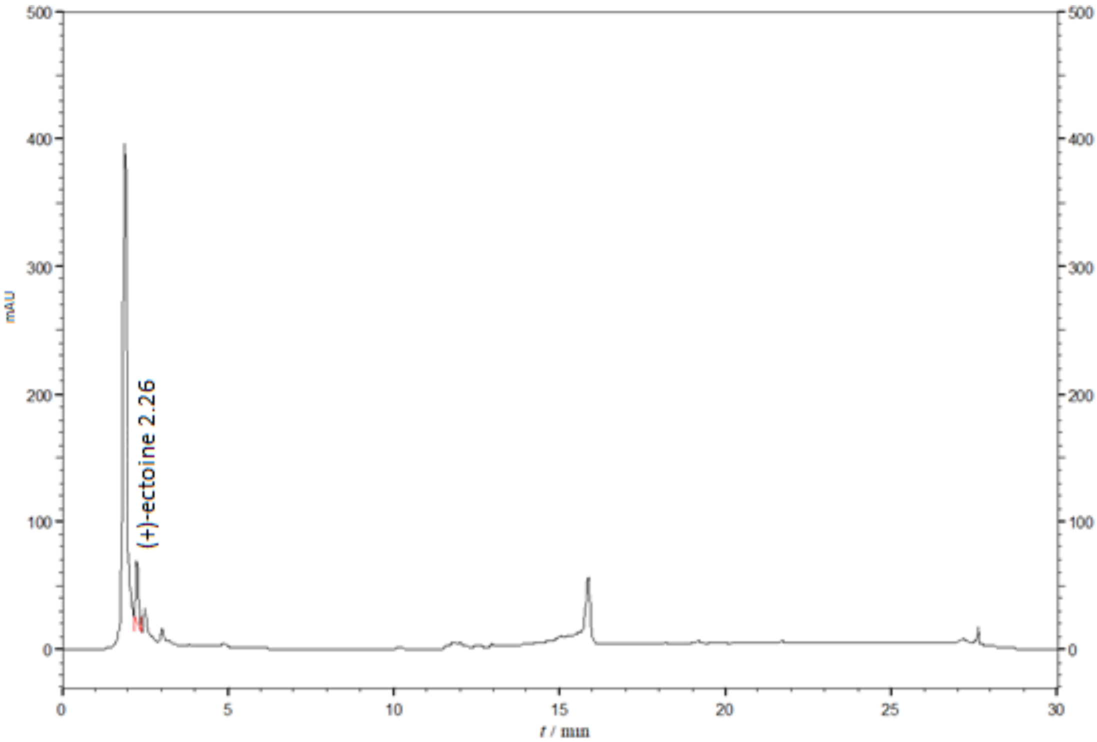

Figure S4.2.13. RP-HPLC chromatogram of sample **11** at 220 nm.

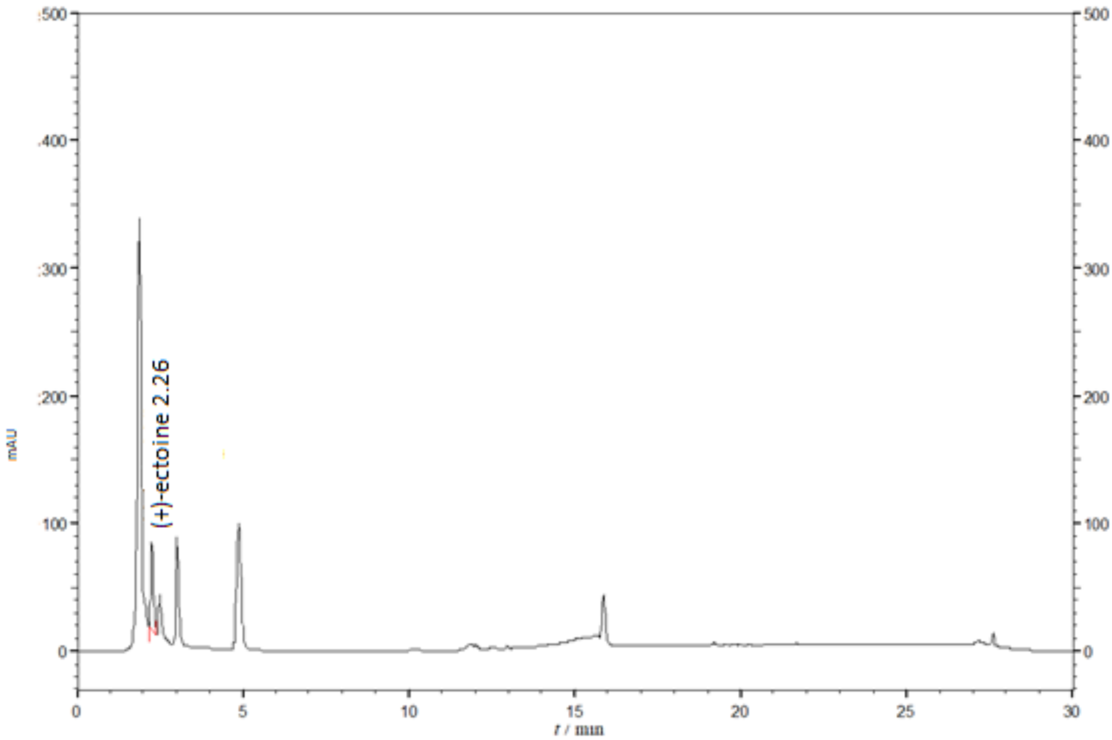

Figure S4.2.14. RP-HPLC chromatogram of sample **12** at 220 nm.

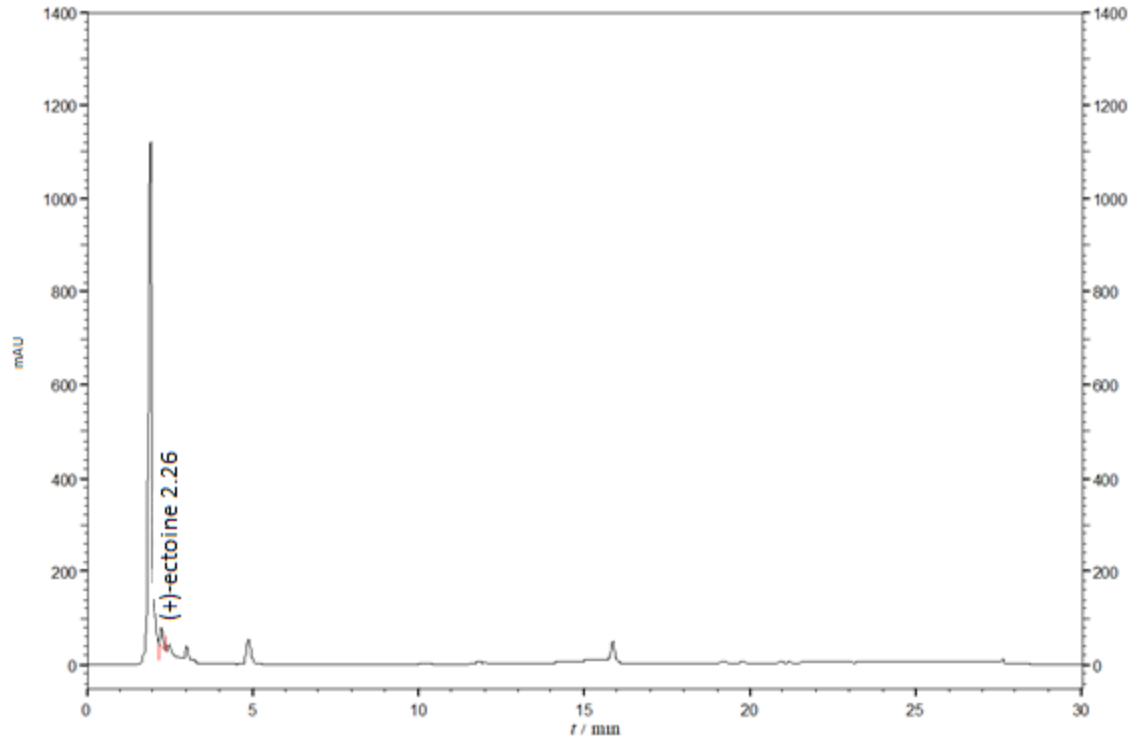

Figure S4.2.15. RP-HPLC chromatogram of sample **13** at 220 nm.

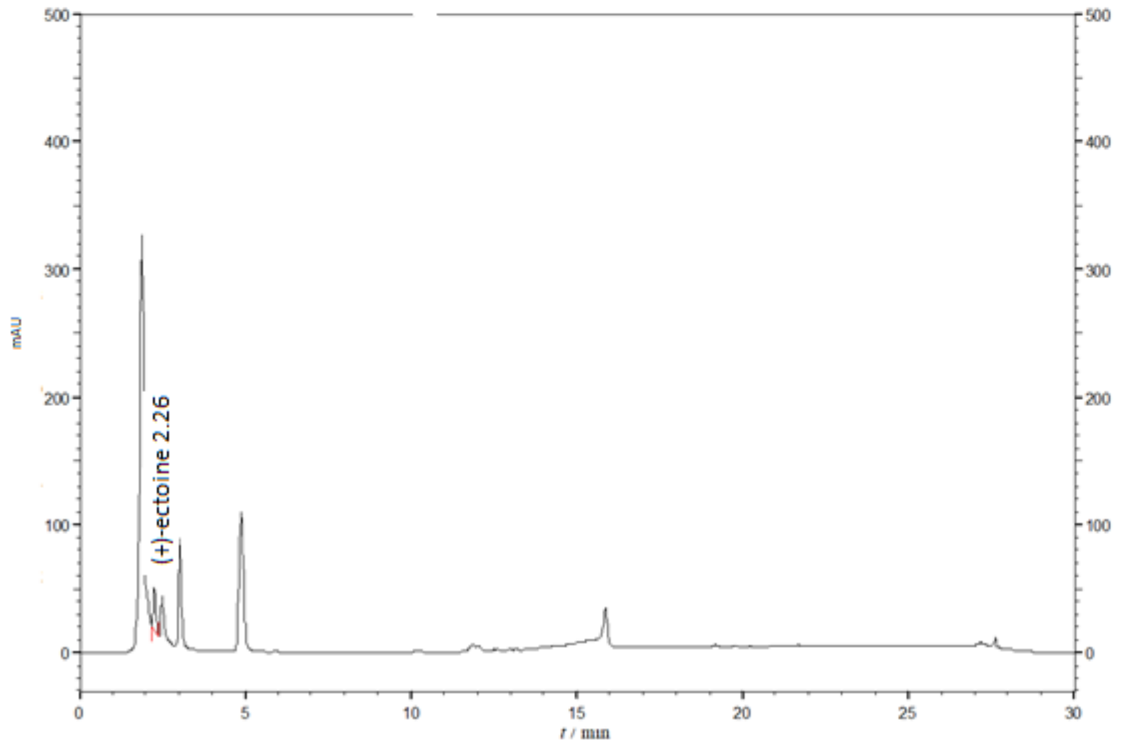

Figure S4.2.16. RP-HPLC chromatogram of sample **14** at 220 nm.

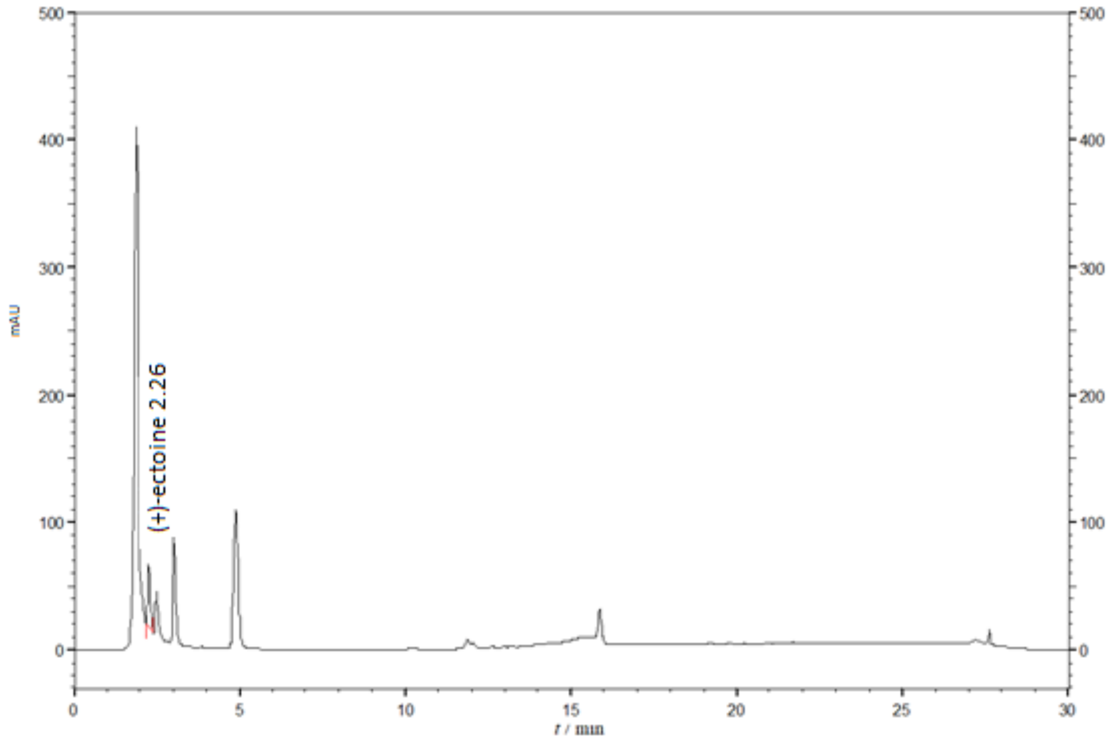

Figure S4.2.17. RP-HPLC chromatogram of sample **15** at 220 nm.

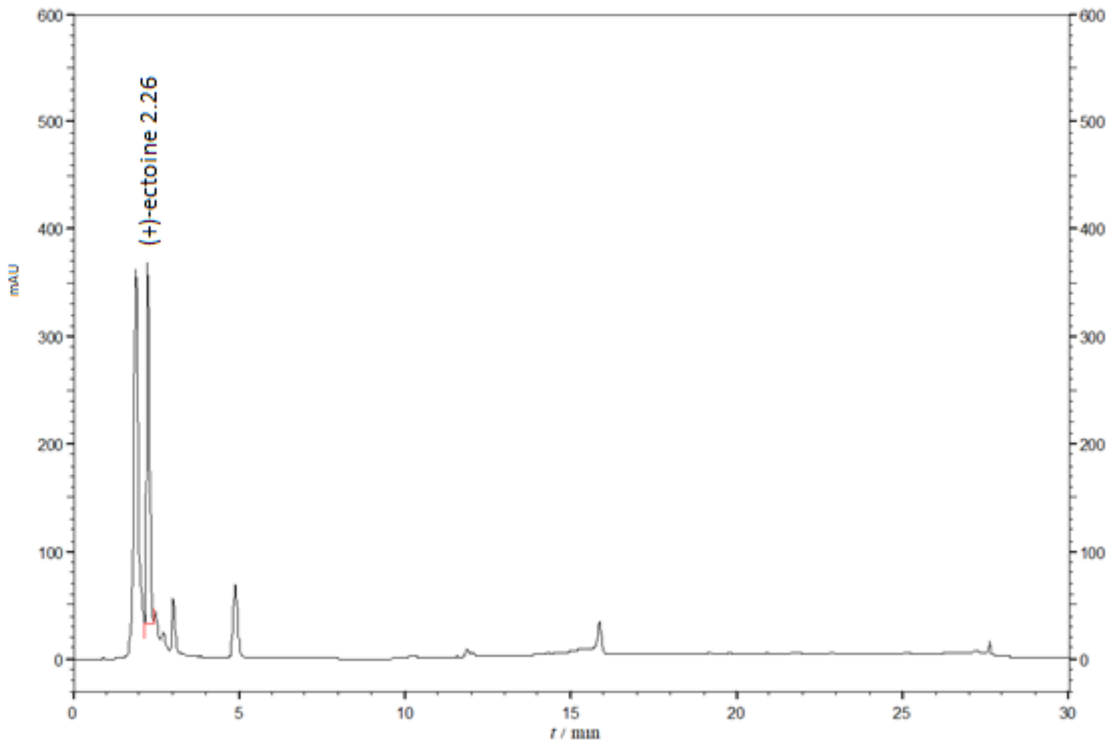

Figure S4.2.18. RP-HPLC chromatogram of sample **16** at 220 nm.

5. MS spectra of compound 7 and standard (+)-ectoine

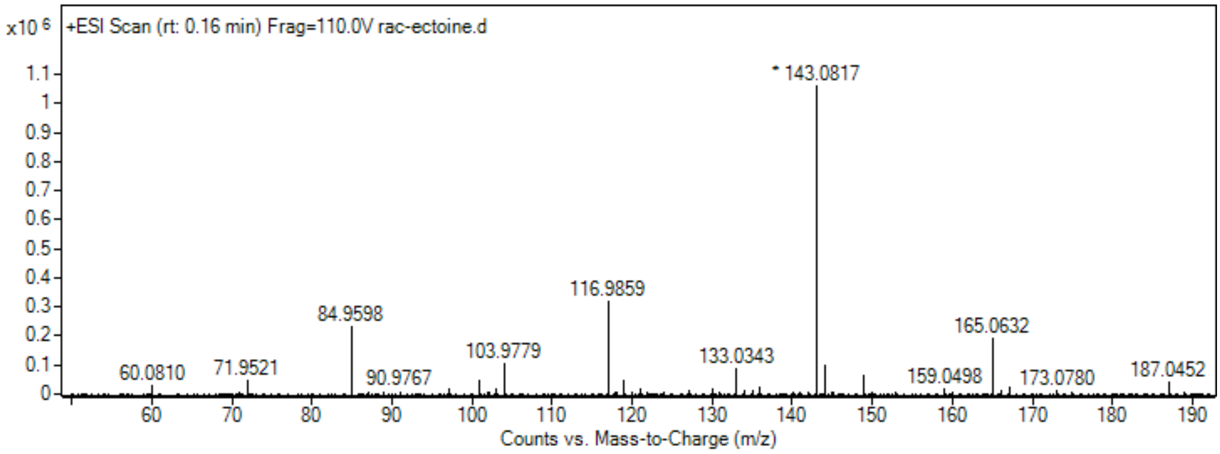

Figure S5.1. HRMS spectrum of compound **7** [(±)-ectoine].

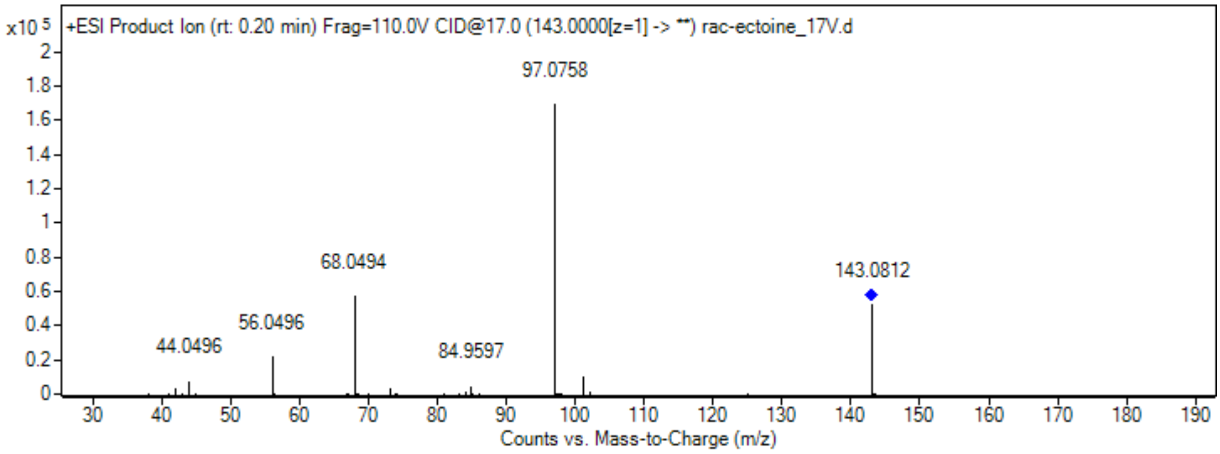

Figure S5.2. MS/MS Fragmentation of product ion *m/z* 143 (compound **7**).

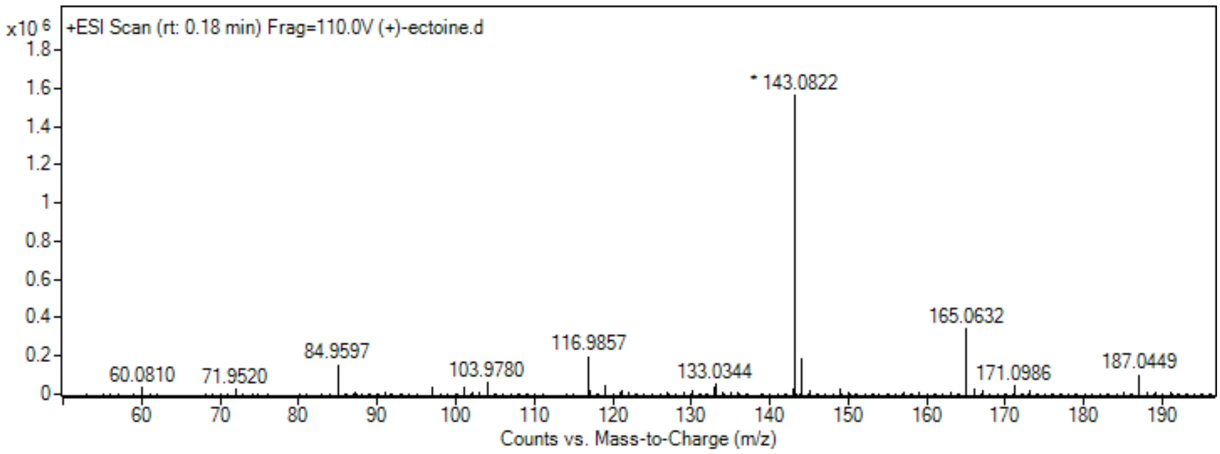

Figure S5.3. HRMS spectrum of standard (+)-ectoine.

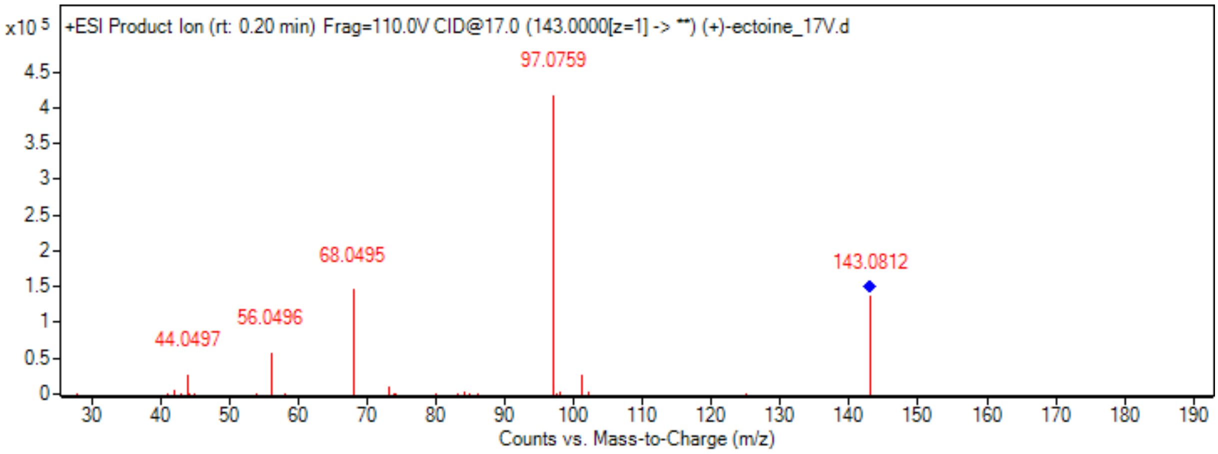

Figure S5.4. MS/MS Fragmentation of product ion *m/z* 143 (standard, (+)-ectoine).