Supplementary Material (ESI) for Chemical Communications

A New Dehydrogenase from *Clostridium acetobutylicum* for Asymmetric Synthesis: Dynamic Reductive Kinetic Resolution (DYRKR) Entry into the Taxotère Side Chain

Gregory A. Applegate, Ross W. Cheloha, David L. Nelson and David B. Berkowitz*
Department of Chemistry, University of Nebraska, Lincoln, NE 68588-0304 USA
dbb@unlserve.unl.edu

Supplementary Information

I. Protein Biochemistry

A: Recombinant *CaADH* Expression. An oligonucleotide coding for the sequence of the targeted gene (GI: 81775727 NCBI) was encoded in a pUC57 vector purchased from GenScript. The sequenced added a 5'-*Nde*I cut site (5' CATATG) and a 3'-*Xho*I cut site (5' CTCGAG). The gene sequence was ligated into a pET-28c(+) vector (EMD Biosciences) that features a hexa-histidine tag. The construct was ultimately transformed into BL21-CodonPlus(DE3)-RIL competent cells. Cultures were inoculated at 37°C and 250 rpm in 1L batches of LB-Lennox broth (10 g tryptone, 5 g NaCl, and 5 g yeast extract). Cells were grown to an OD_{600} of 0.6 to 0.8, and were subsequently induced with 0.5 mM IPTG. Temperature was reduced to 25°C for 16 h, in an effort to mitigate protein aggregation. Cells were harvested by centrifugation at 5000 rpm (4°C) and stored at -80°C.

B: Purification of *CaADH*. Cells were thawed on ice and resuspended in 100 mM H_2KPO_4/HK_2PO_4 buffer, pH 7.0, at a concentration of 1g wet cell weight per 2 mL of extraction buffer. Egg white lysozyme was added at a 1 mg/mL loading level. The suspension was sonicated (5 x 30 sec) and centrifuged at 10000 RPM (4°C for 10 min). The supernatant was transferred to a column of Co^{2+}-NTA agarose in the presence of 50 mM imidazole and 10% glycerol (v/v). Step elution with imidazole was completed in 50 mM increments to 200 mM. A final elution was completed at a concentration of 500 mM. Fractions eluting between 100 mM and 500 mM were pooled and concentrated to a final volume between 10 and 15 mL. The concentrate was dialyzed against H_2KPO_4/HK_2PO_4 buffer, pH 7.0.

C: Standard Assay - Determining Enzyme Activity. Units of enzyme activity were defined according to the following standard assay:
The enzymatic reduction of benzaldehyde (10 mM) was conducted with NADPH (0.2 mM) as cofactor, in H_2KPO_4/HK_2PO_4 buffer (100 mM, pH 7.0) containing DMSO (5% v/v) at 37°C. The reaction volume was 1 mL. One unit was defined as the amount of enzyme leading to the consumption of one µmole of NADPH/min ($\Delta\varepsilon_{340}$ = -6.22 min^{-1} in a quartz cell with 1 cm path length). Protein concentration was determined by the Lowry method. Activity and protein concentration are shown in the table below. Assays were carried out on a Shimadzu-UV2101PC UV-visible spectrophotometer, with Peltier (thermoelectric) temperature control, and multi-cell changer, unless otherwise noted.

Table S1: Purification table for *Clostridium acetobutylicum* dehydrogenase (*CaADH*).

Fraction	Units/mL	Total Units	mg/mL	Total mg	Units/mg	Yield	Pur Factor
Crude	38.5	463.1	19.7	236.4	1.9	-	1
Final	32.1	321.5	0.56	5.6	58.1	70%	30

D: Structure-Activity Profile. A structure-activity profile was constructed by measuring relative rates of reduction of various substrates by *CaADH*. Conditions for the screen are the same as listed above with one caveat; protein concentration was adjusted to provide activity slopes within the range of detection.

Each substrate rate observed was then corrected to account for the amount of enzyme used to give an appropriate measure of percentage activity, relative to benzaldehyde as standard substrate.

Substrate	**Rel. Rate***
Benzaldehyde	100
2-Pyridinecarboxaldehyde	81
3-Pyridinecarboxaldehyde	143
4-Pyridinecarboxaldehyde	445
Phenylacetaldehyde	14
Dihydrocinnamaldehyde	10
Furfural	8
3-Methyl-2-butenal	2
Propanal	3
Heptaldehyde	2
Cyclohexanone	6
2-Octanone	8
2-Acetylpyridine	2
Benzophenone	2
Benzyl acetone	8
Ethyl pyruvate	20
Ethyl benzoylacetate	16
Ethyl acetoacetate	9
Methyl 3-benzoylpropionate	33

Table S2: A structure-activity profile established for *Clostridium acetobutylicum* dehydrogenase, measuring the rates of reduction by NADPH for each substrate @ 10 mM concentration in 100 mM H_2KPO_4/HK_2PO_4 buffer, pH 7.0, with 5 vol% DMSO. *Rates are standardized to milligrams of protein used and rate is reported as a percentage of that of benzaldehyde.

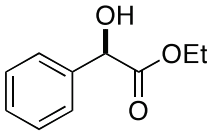
II. Organic Synthesis

A: General Experimental. Methanol was distilled over magnesium and iodine. Other reagents were obtained from commercial sources and used without further purification. Reaction progress was monitored by TLC. Flash chromatography was performed using Merck silica gel 60 (230-400 mesh). ^{1}H NMR spectra were acquired on Bruker-DRX-Avance-400 MHz instrument with chemical shifts reported relative to residual $CHCl_3$ (7.25 ppm). Coupling constants are reported in Hz. Proton-decoupled ^{13}C NMR spectra were recorded on Bruker-DRX-Avance-400 MHz instrument with chemical shifts reported relative to $CDCl_3$ (77.0 ppm). Optical rotation @ 589 nm was measured at room temperature in an Autopol polarimeter. Chiral HPLC analysis was performed on a Chiralcel OD column. Elution was carried out with hexane/isopropanol, in an isocratic fashion. Actual eluent compositions and flow rates are given with the corresponding HPLC traces (vide infra).

B: General Procedure for the Preparation of α-, β- and γ-Hydroxyester HPLC Standards. To a solution of ketoester (0.25 mmol) in methanol (2.5 mL, 0°C) was added $NaBH_4$ (4 mg, 0.1 mmol). Reaction

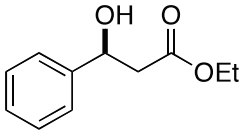
progress was monitored by TLC (30% EtOAc-hexanes). The reaction was quenched with saturated, aqueous ammonium chloride and extracted with ethyl acetate. Extracts were pooled and dried over sodium sulfate, filtered and evaporated in vacuo. Standards were purified by SiO_2 flash column chromatography (30% EtOAc-hexanes).

C: General Procedure for Asymmetric Reduction of α-, β,- and γ-Ketoesters, with Cofactor Recycling. A solution of $NADP^+$ (2 mg, 2.5 μmol), *CaADH* (8.0 benzaldehyde units), D-glucose (450 mg, 2.5 mmol) and glucose dehydrogenase from *Thermoplasma acidophilum* (10 SI units) in H_2KPO_4/HK_2PO_4 buffer (23.8 mL, 100 mM, pH 6.5) was stirred at room temperature. Keto ester (0.25 mmol, in 1.25 mL DMSO) was added and the resulting reaction mixture stirred magnetically. Reaction progress was monitored by TLC (typically 30% EtOAc-hexanes). Product was extracted thrice with ethyl acetate, and the combined organic layers dried over sodium sulfate. Following vacuum filtration, and concentration, the product was purified via SiO_2 flash column chromatography (EtOAc-hexanes), and characterized by NMR and HPLC analysis.



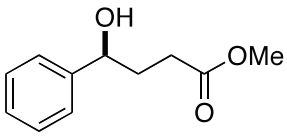
(R)-Ethyl 1-hydroxy-1-phenylacetate (2)
From ethyl benzoylformate (**1**, 45 mg, 0.25 mmol), following the procedure for biocatalytic reduction with nicotinamide cofactor regeneration, the title compound (**2**) was obtained (42 mg, 90% yield) as a clear, colorless oil in 90% ee, as determined by chiral HPLC (traces below).

^{1}H NMR (400 MHz, $CDCl_3$) δ 1.22 (t, J = 7.2 Hz, 3H), 3.48 (d, J = 3 Hz, OH), 4.16 (dq, J(d) = 10.8 Hz, J(q) = 7.2 Hz , 1H), 4.25 (dq, J(d) = 10.8 Hz, J(q) = 7.2 Hz, 1H), 5.15 (d, J = 3 Hz, 1H), 7.30-7.41 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.0, 62.2, 72.9, 126.5, 128.4, 128.5, 128.4, 173.7; $[\alpha]_D$(obs'd-90% ee) = -90.2° (c 1.0, $CHCl_3$), {lit[1]: $[\alpha]_D$ = -104.4° (c 1.0, $CHCl_3$)}. HRMS (EI) *m/z* calcd for $C_{10}H_{12}O_3$ $(M)^+$ 180.0786, obsd. 180.0792.



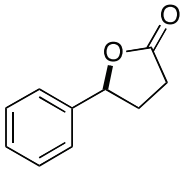
(S)-Ethyl 3-hydroxy-3-phenylpropionate (4)
From ethyl benzoylacetate (**3**, 48 mg, 0.25 mmol), following the procedure for biocatalytic reduction with nicotinamide cofactor regeneration, was obtained β-hydroxy ester **4** (45 mg, 93% yield), as a clear colorless oil in 99% ee, as determined by chiral HPLC (traces below).

^{1}H NMR (400 MHz, $CDCl_3$) δ 1.25 (t, J = 7.2 Hz, 3H), 2.72 (m, 2H), 3.25 (d, J = 3.2 Hz, OH), 4.17 (q, J = 7.2 Hz, 2H), 5.12 (m, 1H), 7.26-7.32 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1, 43.3, 60.9, 70.3, 125.7, 127.8, 128.5, 142.5, 172.4; $[\alpha]_D$(obs'd-99% ee) = -28.6° (c 1.0, $CHCl_3$), {lit[2]: $[\alpha]_D$ = -25.8° (c 1.3, $CHCl_3$)}. HRMS (EI) *m/z* calcd for $C_{11}H_{14}O_3$ $(M)^+$ 194.0943, obsd. 194.0951.



(S)-Methyl 4-hydroxy-4-phenylbutyrate (6)
From methyl 3-benzoylpropionate (**5**, 48 mg, 0.25 mmol) and following the procedure for biocatalytic reduction with nicotinamide cofactor regeneration, D-γ-hydroxy ester **6** was obtained (41 mg, 84% yield) as a mixture of alcohol and γ-lactone, in 99% ee, as determined by HPLC (traces below).

^{1}H NMR (400 MHz, $CDCl_3$) δ 2.06 (q, J = 7.2 Hz, 2H), 2.26 (br, 1H), 2.43 (t, J = 6.4 Hz, 2H), 3.65 (s, 3H), 4.74 (t, J = 6.4 Hz, 1H), 7.25-7.33 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 30.4, 33.8, 51.7, 73.5, 125.7, 127.7, 128.5, 144.0, 174.3. HRMS (EI) *m/z* calcd for $C_{11}H_{14}O_3$ $(M)^+$ 194.0943, obsd. 194.0945. Absolute stereochemistry was determined from the optical rotation of the corresponding lactone (vide infra).

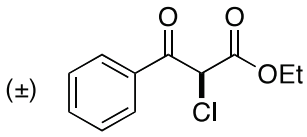


(S)-5-Phenyl-dihydro-2(3H)-furanone (15)

The title compound (**15**) was isolated from the reduction of methyl 3-benzoylpropionate (in mixture with γ-hydroxy ester **6**, as described above).

^{1}H NMR (400 MHz, $CDCl_3$) δ 2.09-2.28 (m, 1H), 2.59-2.70 (m, 3H), 5.49 (m, 1H), 7.31-7.45 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.0, 30.9, 81.2, 125.2, 128.4, 128.7, 139.3, 176.9; $[\alpha]_D$ (obsd-99% ee) = -34.1° (c 1.0, $CHCl_3$), {lit[3]: $[\alpha]_D$ = -35.5° (c 2.4, $CHCl_3$)}. HRMS (EI) *m/z* calcd for $C_{10}H_{10}O_2$ $(M)^+$ 160.0681, obsd. 162.0673.

D: Dynamic Reductive Kinetic Resolution (DYRKR)
Substrate Synthesis.

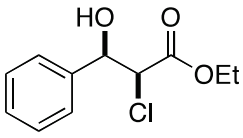


Ethyl 2-Chloro-3-oxo-3-phenylpropionate (13)

To a 2 M solution of SO_2Cl_2 in dichloromethane at 0°C was added ethyl benzoylacetate. The reaction was stirred overnight and warmed to room temperature. The reaction was quenched with H_2O and extracted with diethyl ether. The combined organic layers were dried over sodium sulfate, filtered, and evaporated, in vacuo. The α-chloro-β-keto ester product was purified to homogeneity with flash silica column chromatography (20% dicholoromethane-hexanes) (colorless oil; 80% yield):

^{1}H NMR (400 MHz, $CDCl_3$) δ 1.24 (t, J = 7.2 Hz, 3H), 4.28 (q, J = 7.2 Hz, 2H), 5.62 (s, 1H), 7.27-7.50 (m, 2H), 7.57 (t, J = 7.6 Hz, 1H), 8.01 (d, J = 7.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.8, 57.9, 63.1, 128.9, 129.2, 133.3, 134.3, 165.2, 188.2. HRMS (EI) *m/z* calcd for $C_{11}H_{11}ClO_3$ $(M)^+$ 226.0397, obsd 226.0395.

Procedure for DYRKR.



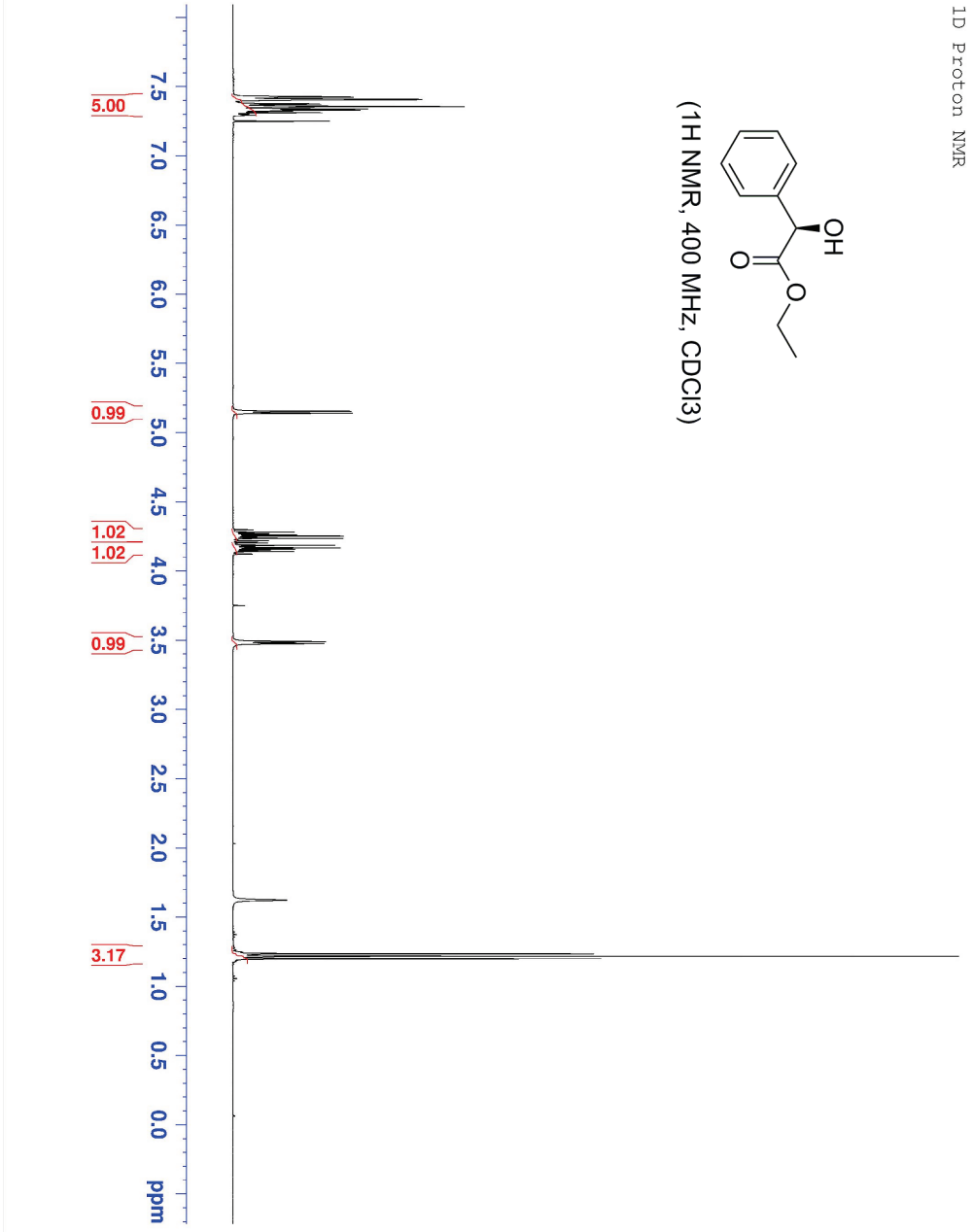
(2S,3R)-Ethyl 2-Chloro-3-hydroxy-3-phenylpropionate (14)

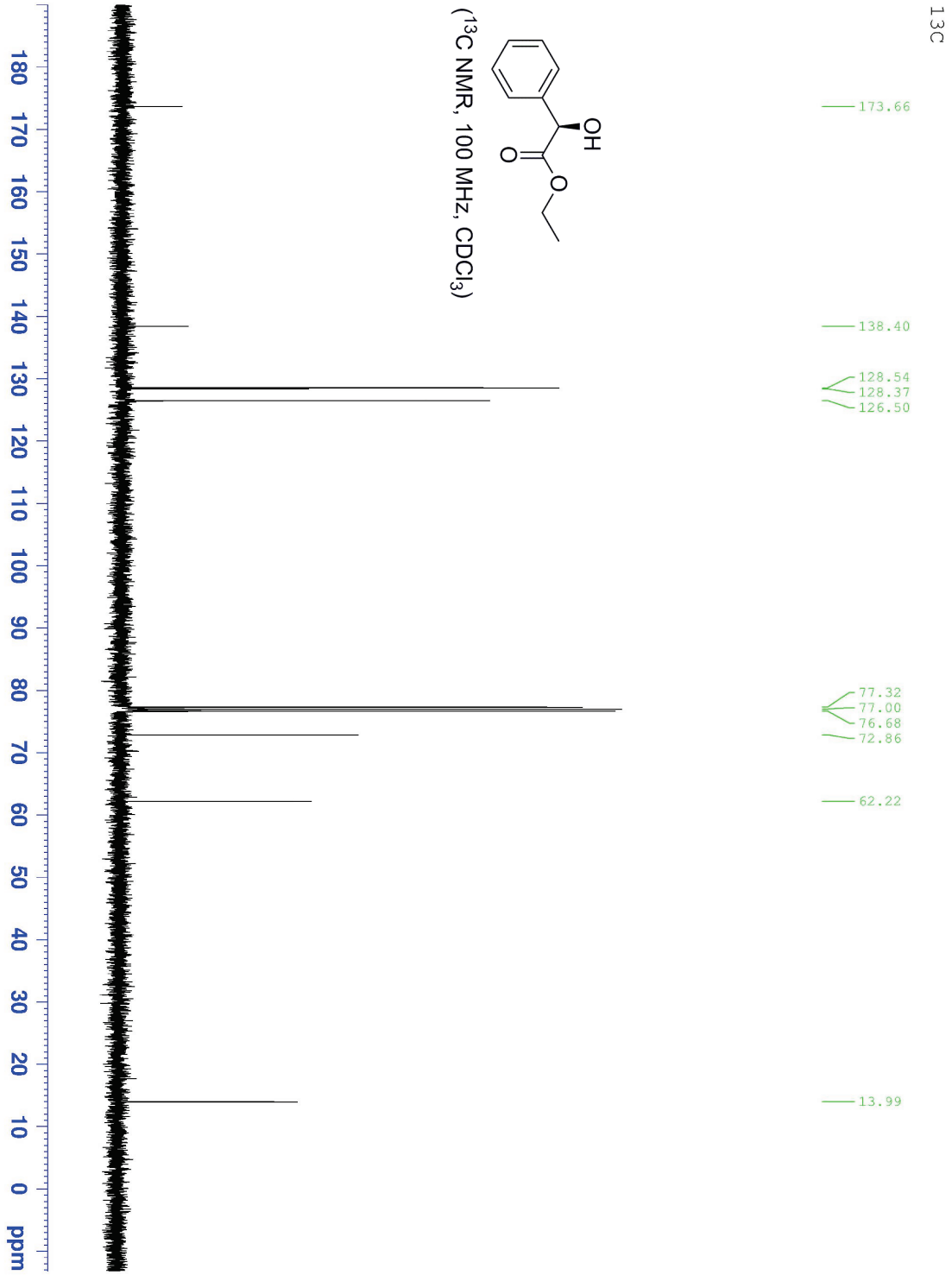
A solution of $NADP^+$ (7.9 mg, 10.1 μmol), *CaADH* (100 benzaldehyde units), D-glucose (7.3 g, 40.5 mmol), and glucose dehydrogenase from *Thermoplasma acidophilum* (38 SI units) in H_2KPO_4/HK_2PO_4 buffer (478 mL, 100 mM, pH 6.5) was stirred at room temperature. Ketone **13** (1.0 g (4.5 mmol) in 28 mL DMSO) was added, and the reaction mixture stirred magnetically. Reaction progress was monitored by TLC (30% EtOAc-hexanes). Upon completion, the reaction mixture was extracted with ethyl acetate, dried over sodium sulfate, filtered, and concentrated, in vacuo. The DYRKR product (**14**) was obtained as a colorless oil (952 mg, 95% yield), following SiO_2 flash chromatography (30% EtOAc-hexanes), and characterized by NMR and chiral HPLC (95% de, 99% ee). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.099 (t, J = 7.2 Hz, 3H), 3.27 (br, 1H), 4.07 (q, J = 7.2 Hz, 2H), 4.42 (d, J = 6.4, 1H), 5.08 (d, J = 6.4 Hz, 1H), 7.28-7.32 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.5, 62.1, 62.8, 74.5, 126.5, 128.3, 128.5, 138.2, 167.8; $[\alpha]_D$(obs'd- 95% de; 99% ee)= -3.1° (c 1.0, $CHCl_3$), {lit[4]: $[\alpha]_D$ = -3.0° (c 1.7, $CHCl_3$)}. HRMS (EI) *m/z* calcd for $C_{11}H_{13}ClO_3$ $(M)^+$ 228.0553, obsd 228.0555.

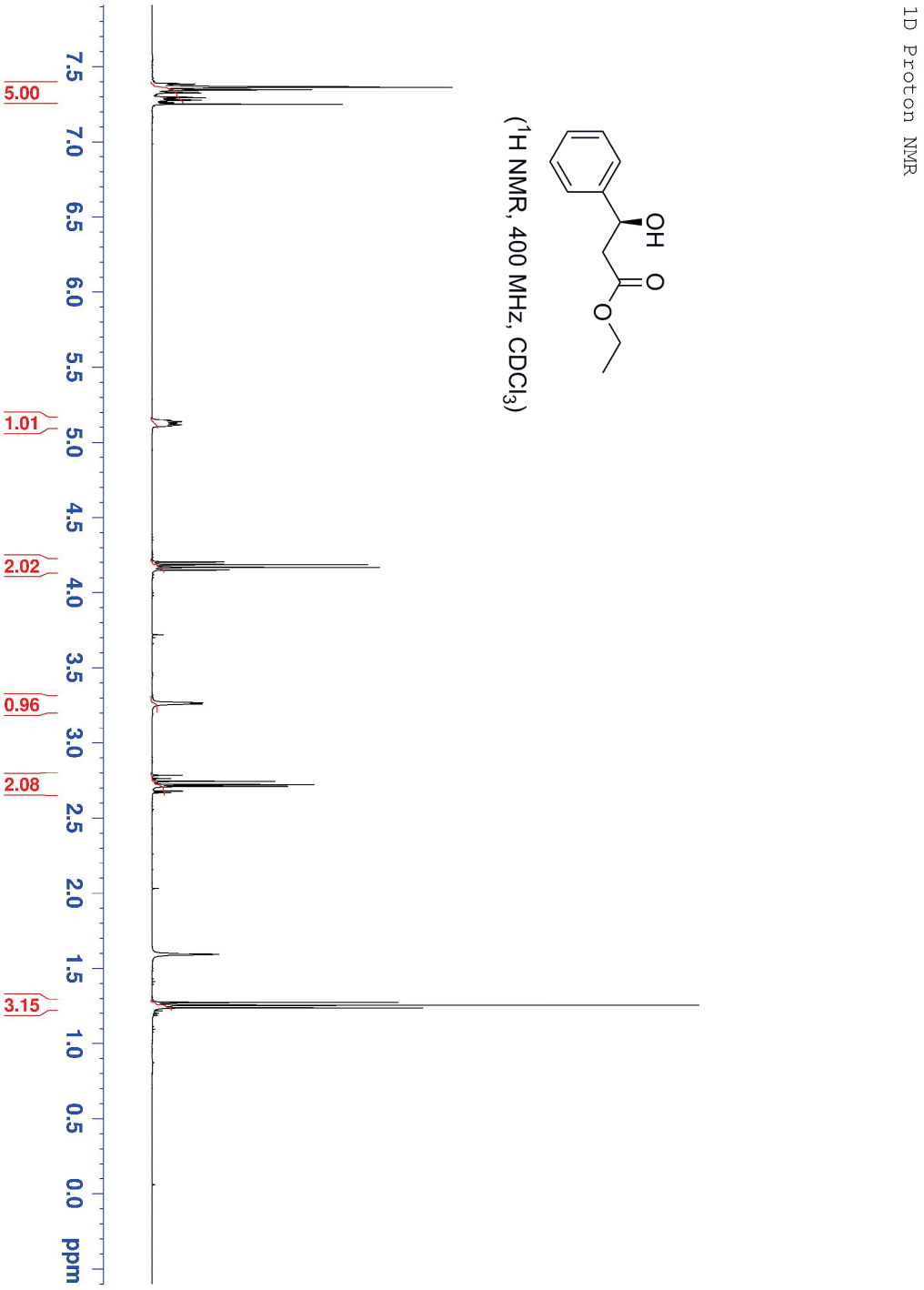
III. References

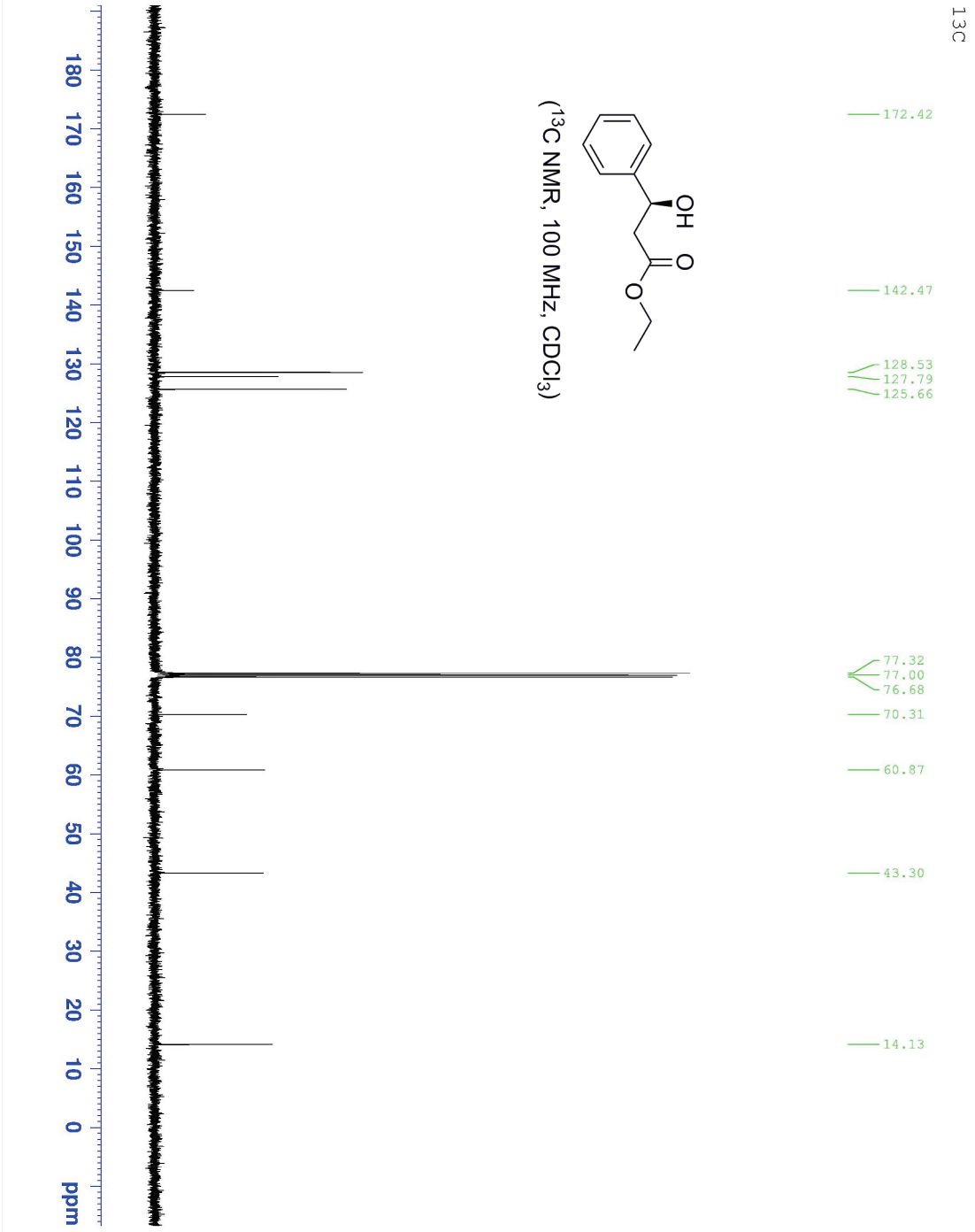
1) J. A. Dale, D. L. Duff and H.S. Mosher, *J. Org. Chem.,* 1968, ***33*****,** 3245.
2) B. S. Deol, D. D. Ridly and G. W. Simpson, *Aust. J. Chem*., 1976, **29,** 2459-2467.
3) C. Najera, M. Yus and D. Seebach, *Helv. Chim. Acta*, 1984, **67**, 289.
4) G. Jörg and M. Bertau, *ChemBioChem*, 2004, **4**, 87-92.

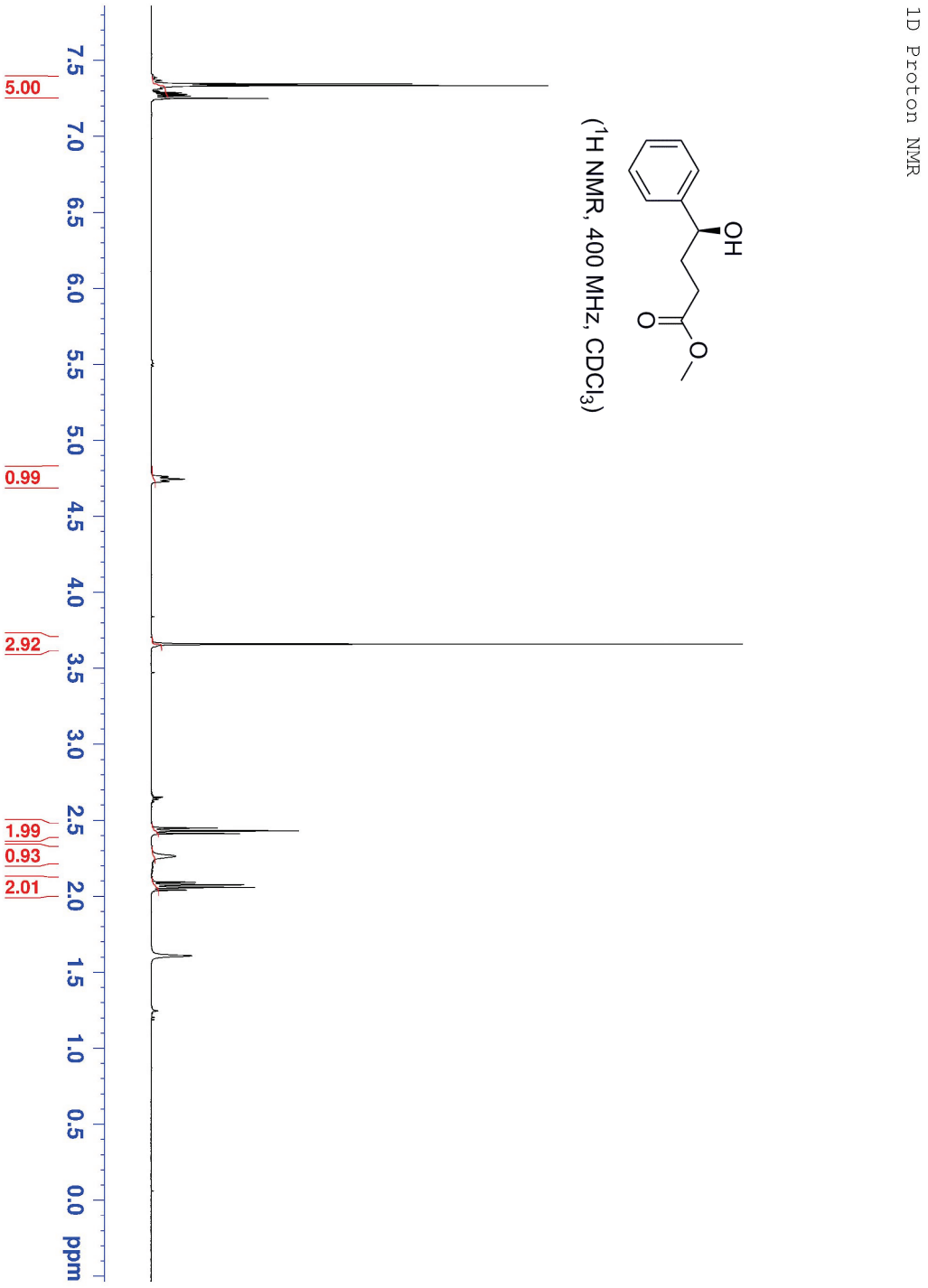
IV. NMR Spectra

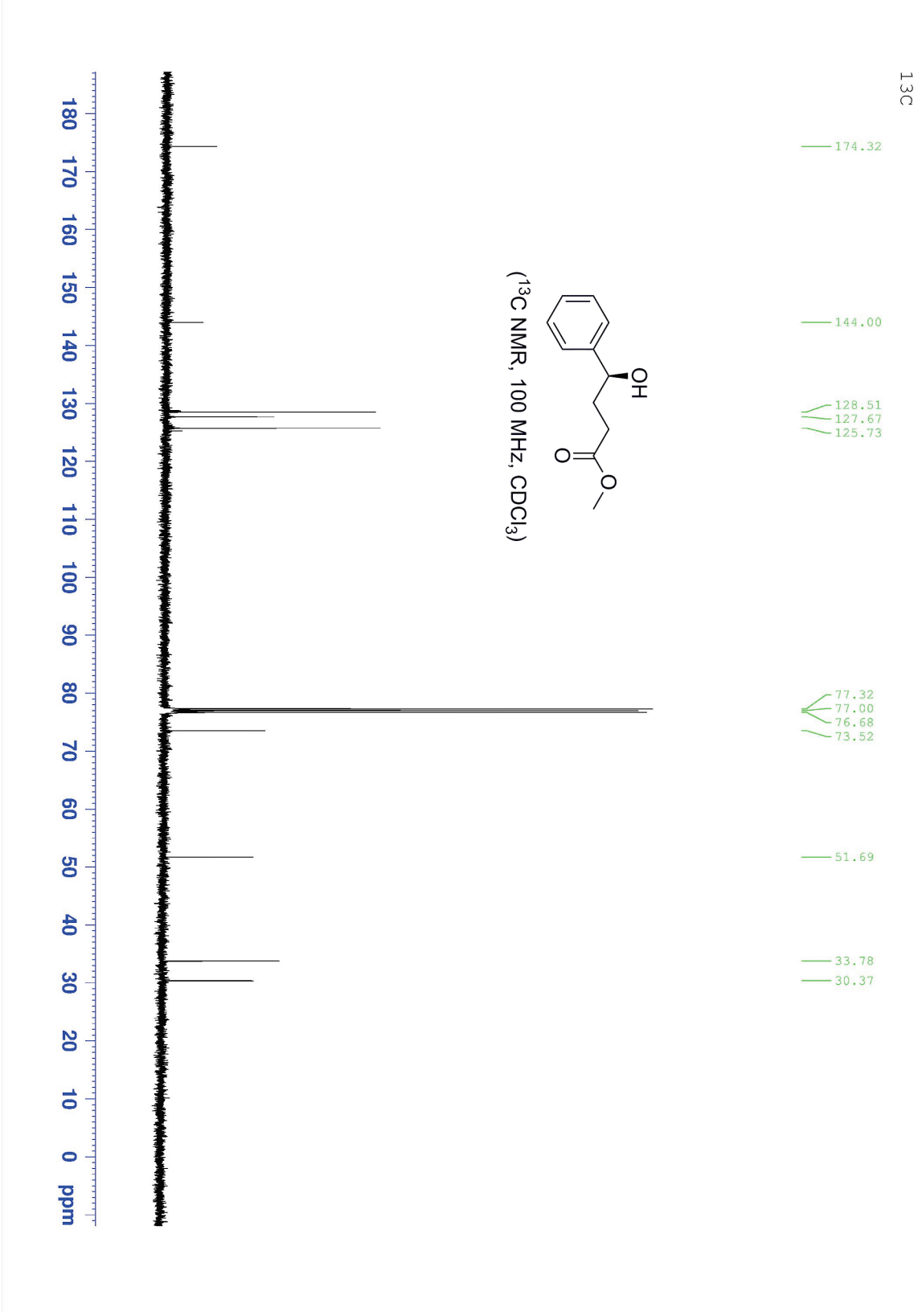


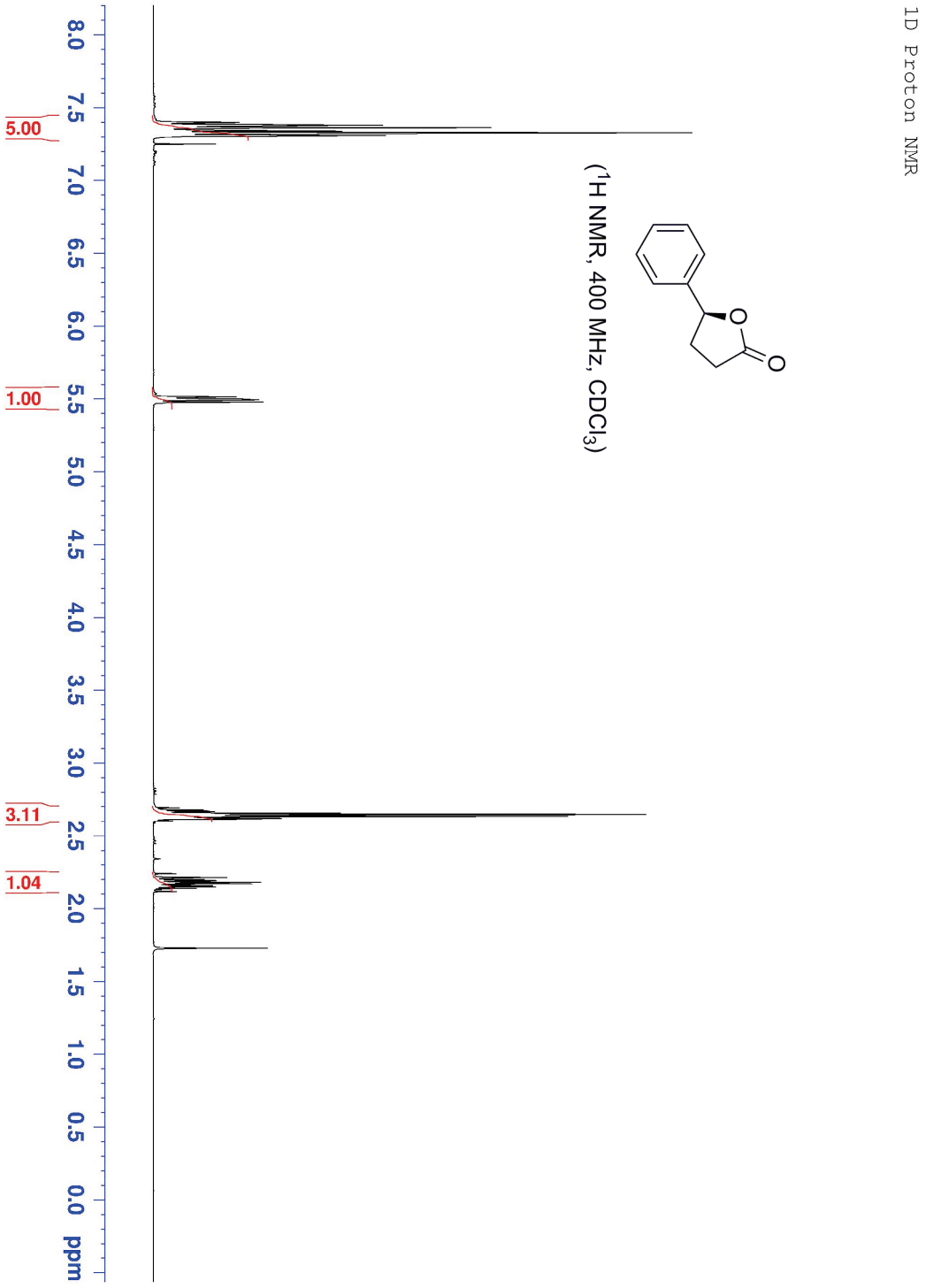

(^{13}C NMR, 100 MHz, $CDCl_3$)
OH
O
O
13C
173.66
138.40
128.54
128.37
126.50
77.32
77.00
76.68
72.86
62.22
13.99
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

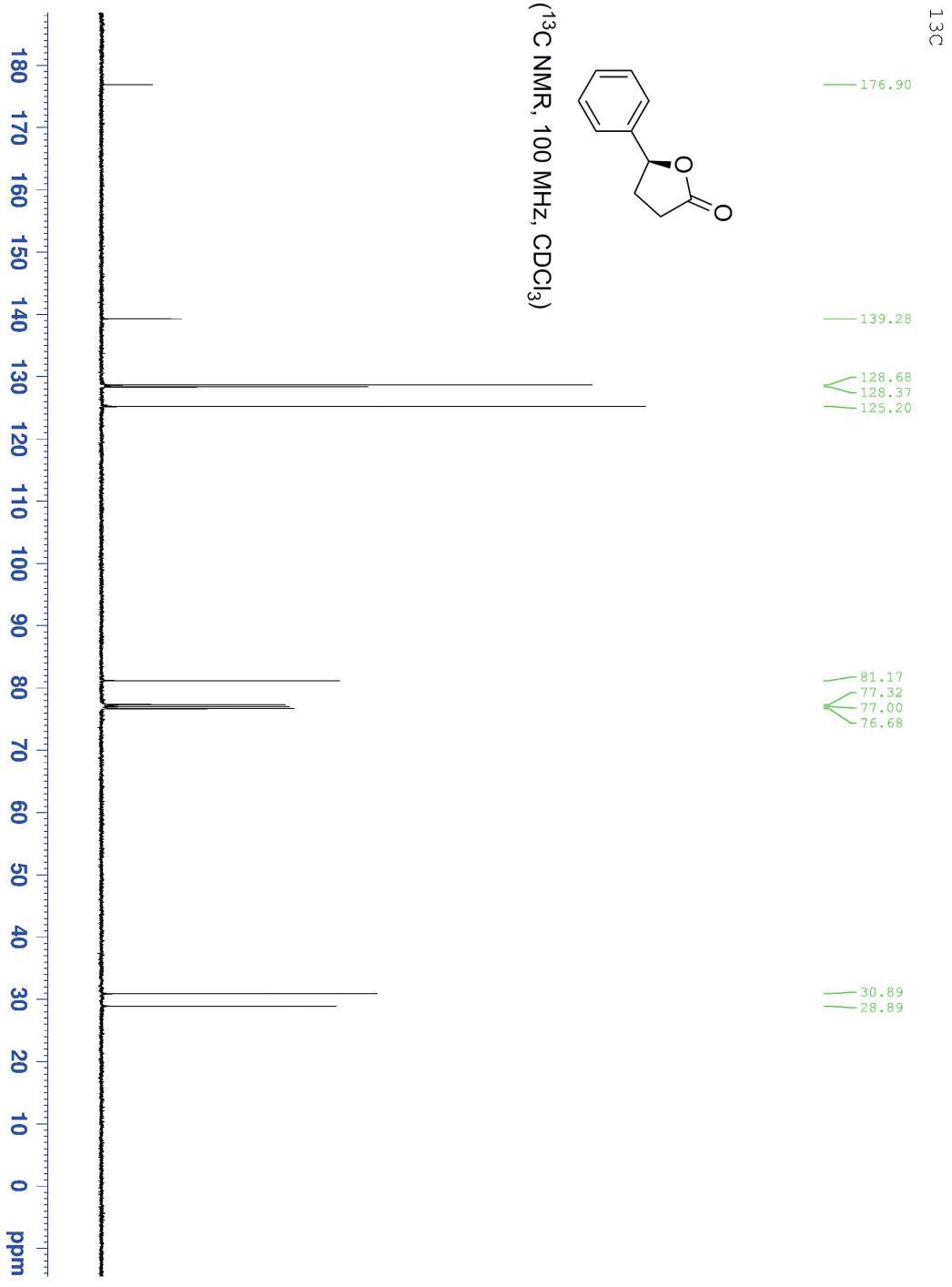

1D Proton NMR
(^{1}H NMR, 400 MHz, $CDCl_3$)
OH
O
O
5.00
1.01
2.02
0.96
2.08
3.15
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ppm

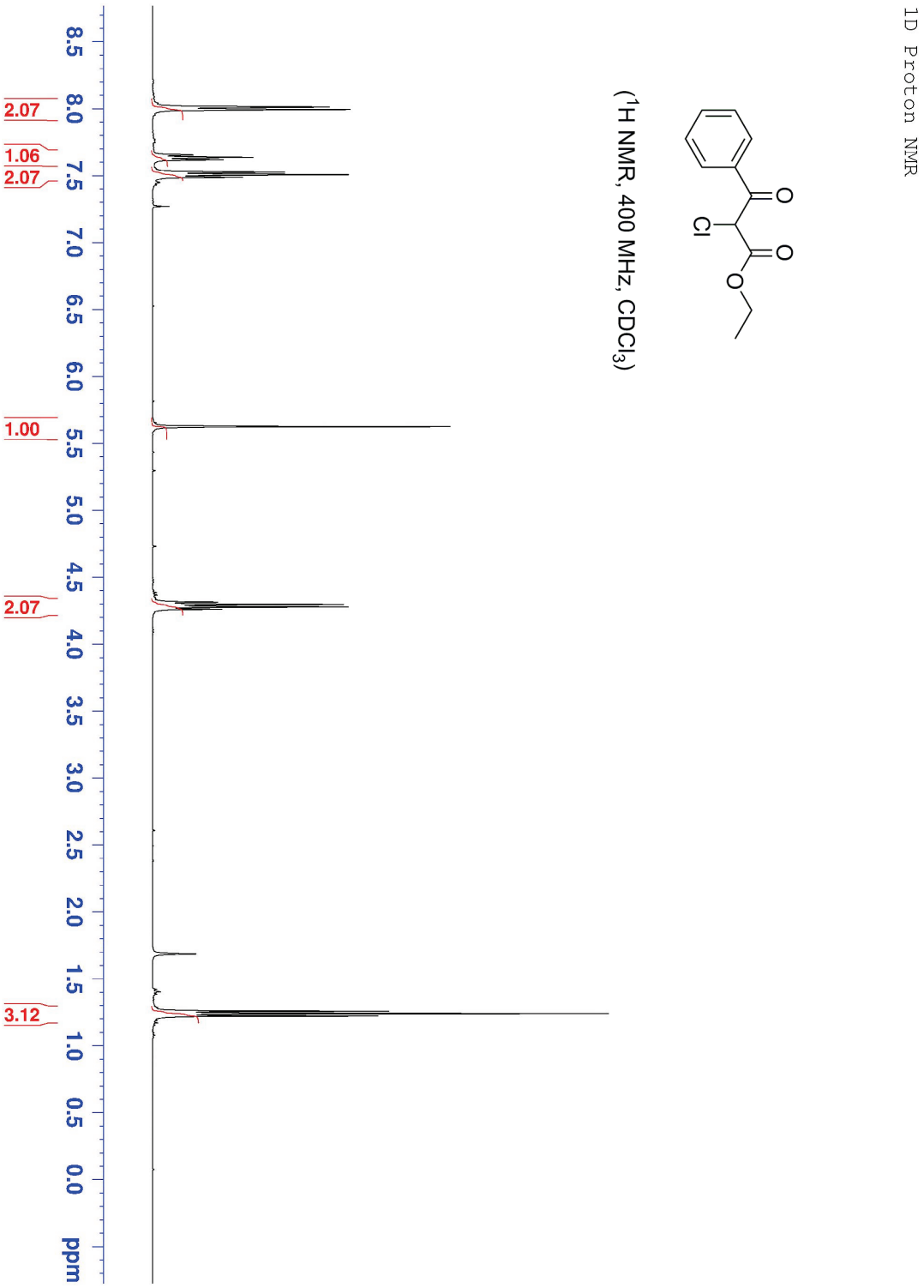

13C
172.42
142.47
128.53
127.79
125.66
77.32
77.00
76.68
70.31
60.87
43.30
14.13
OH
O
O
(^{13}C NMR, 100 MHz, $CDCl_3$)
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

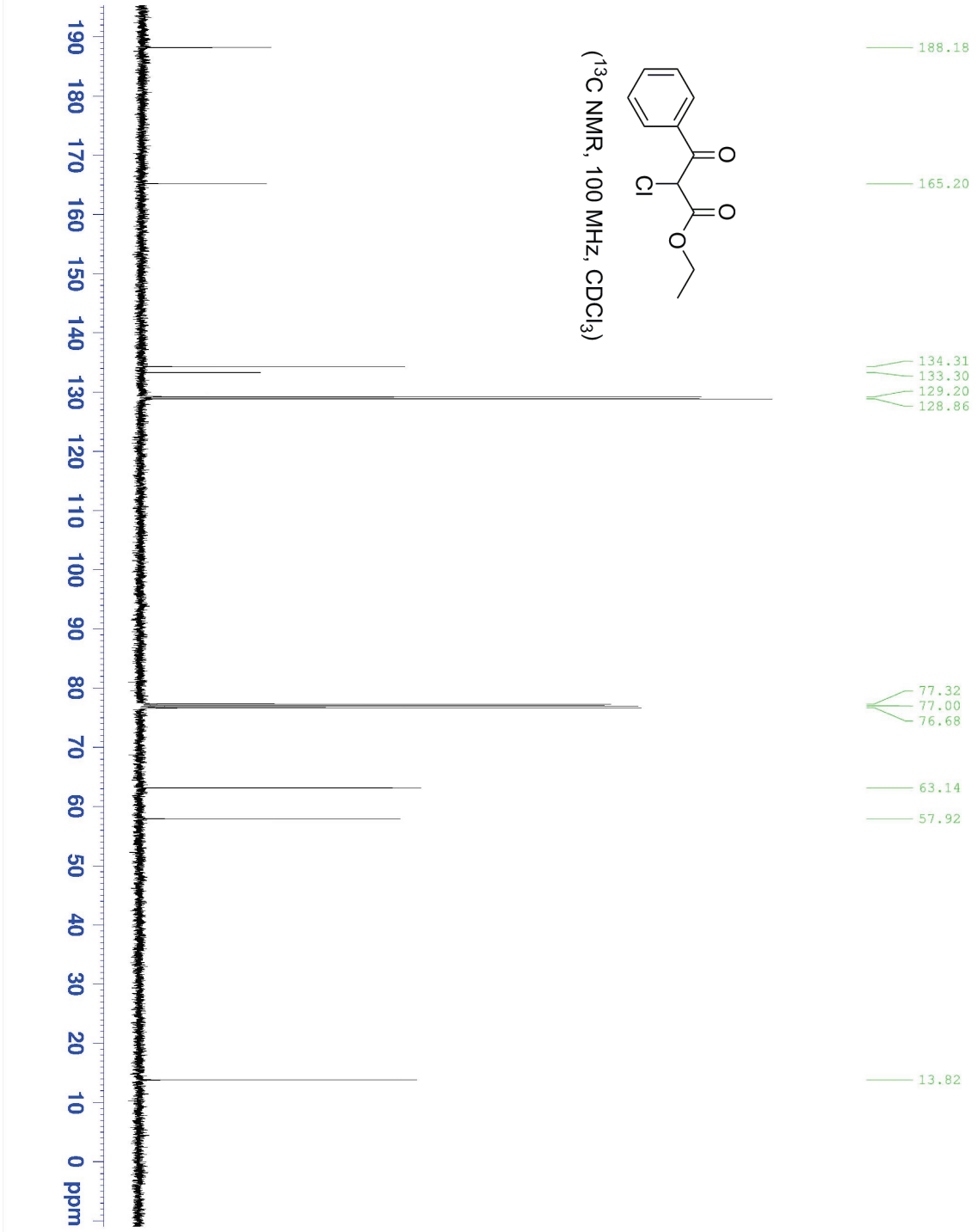

1D Proton NMR
(^{1}H NMR, 400 MHz, $CDCl_3$)
OH
O
O
5.00
0.99
2.92
1.99
0.93
2.01
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ppm

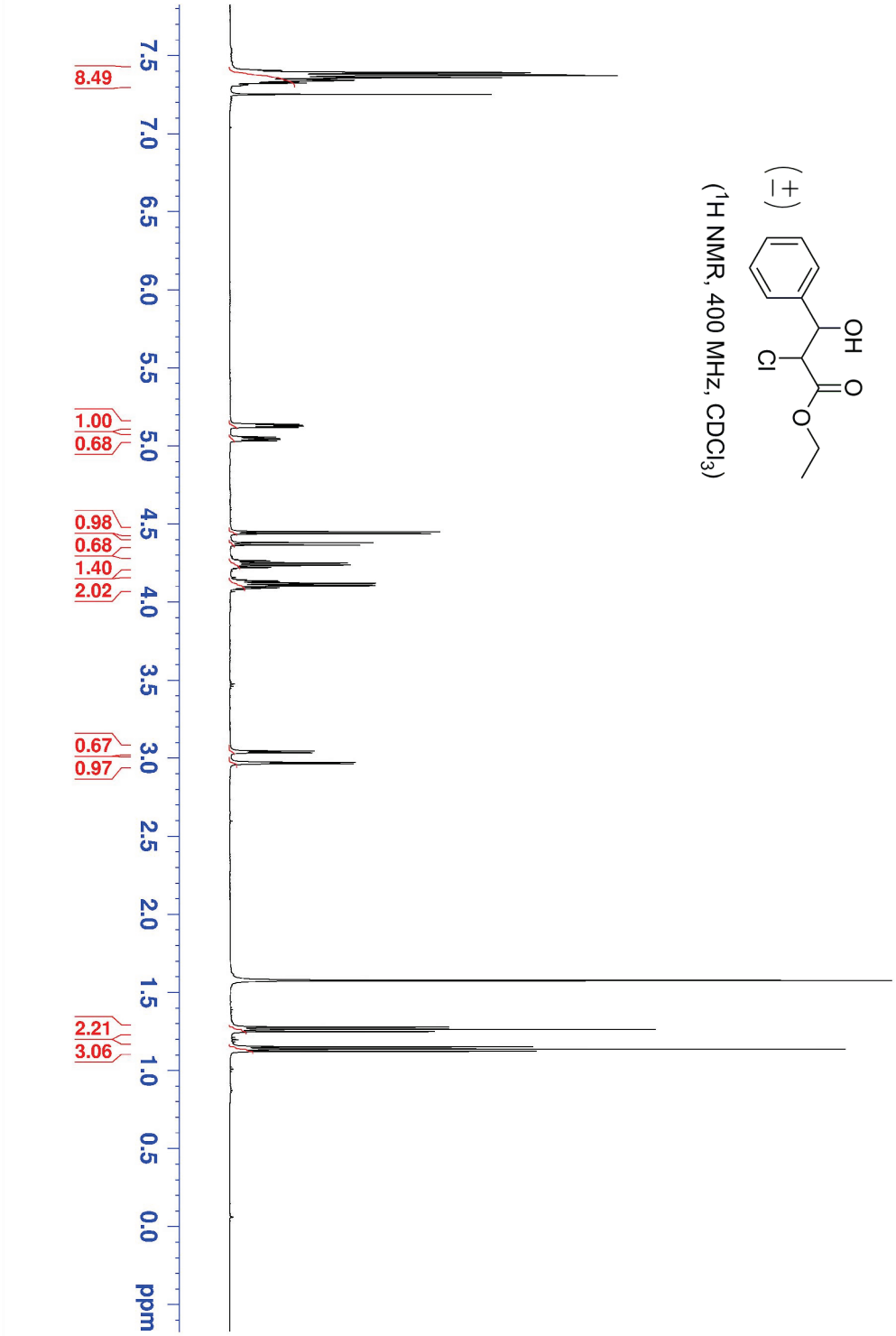

13C
174.32
144.00
128.51
127.67
125.73
77.32
77.00
76.68
73.52
51.69
33.78
30.37
OH
O
O
(13C NMR, 100 MHz, CDCl3)
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

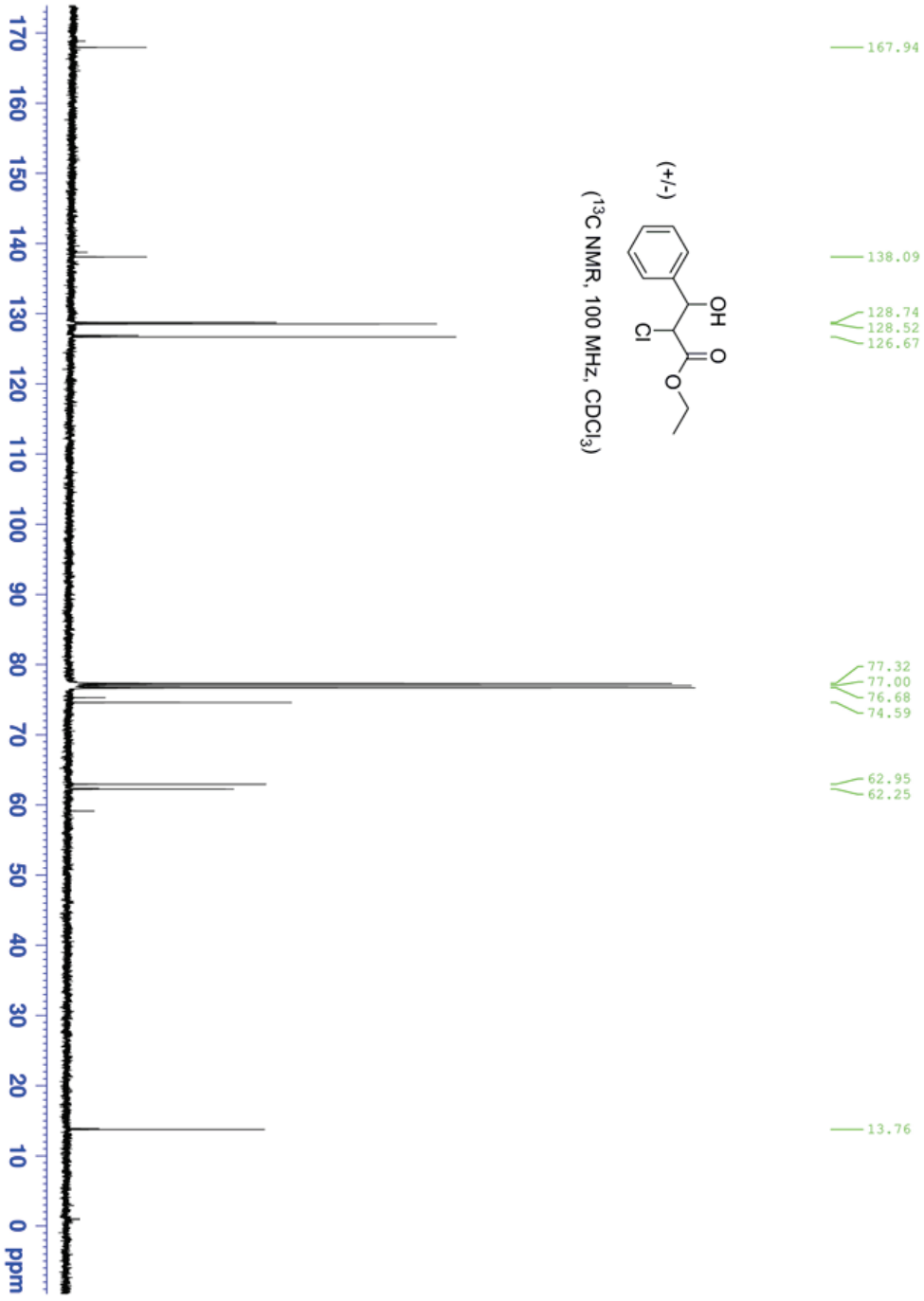

1D Proton NMR
(^{1}H NMR, 400 MHz, $CDCl_3$)
O
O
5.00
1.00
3.11
1.04
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ppm

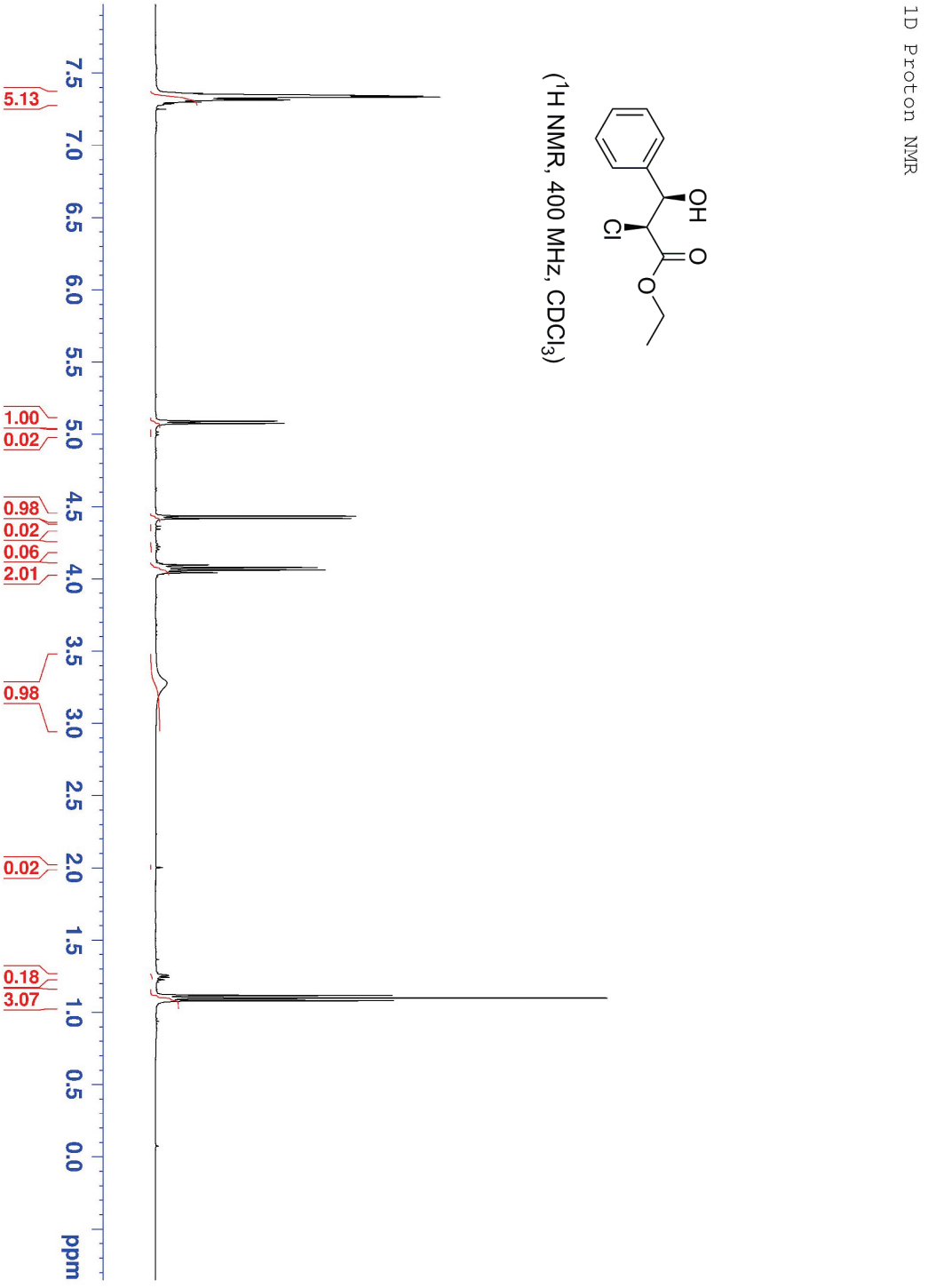

(^{13}C NMR, 100 MHz, CDCl$_3$)
13C
176.90
139.28
128.68
128.37
125.20
81.17
77.32
77.00
76.68
30.89
28.89
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

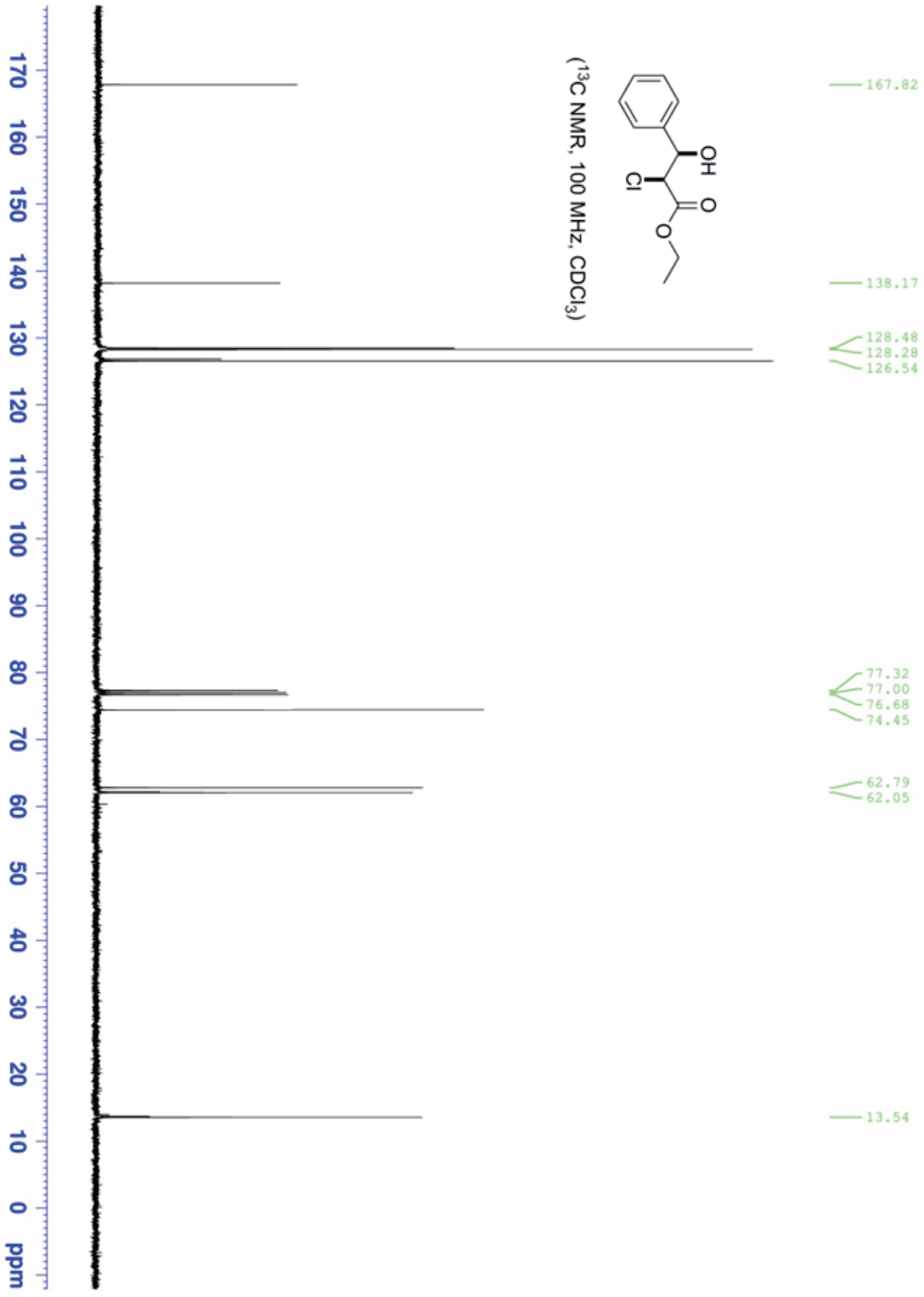

1D Proton NMR
(^{1}H NMR, 400 MHz, $CDCl_3$)
Cl
O
O
O
2.07
1.06
2.07
1.00
2.07
3.12
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ppm


(^{13}C NMR, 100 MHz, CDCl$_3$)
Cl
O
O
O
188.18
165.20
134.31
133.30
129.20
128.86
77.32
77.00
76.68
63.14
57.92
13.82
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm


(±)
OH
Cl
O
O
(¹H NMR, 400 MHz, CDCl3)
8.49
1.00
0.68
0.98
0.68
1.40
2.02
0.67
0.97
2.21
3.06
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ppm


(+/-)
(^{13}C NMR, 100 MHz, $CDCl_3$)
OH
O
Cl
O
167.94
138.09
128.74
128.52
126.67
77.32
77.00
76.68
74.59
62.95
62.25
13.76
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0 ppm

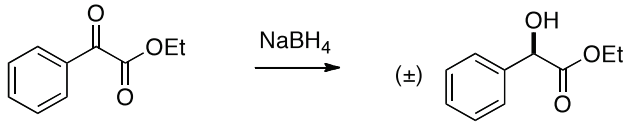

1D Proton NMR
(^{1}H NMR, 400 MHz, $CDCl_3$)
OH
Cl
O
O
5.13
1.00
0.02
0.98
0.02
0.06
2.01
0.98
0.02
0.18
3.07
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ppm

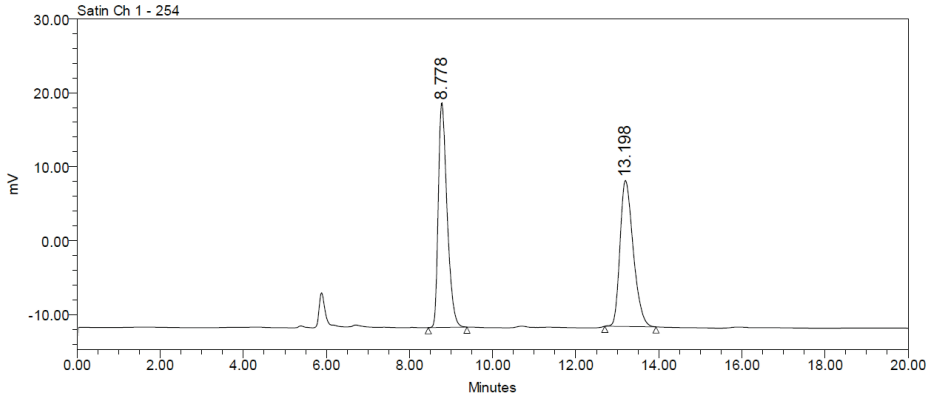

(^{13}C NMR, 100 MHz, $CDCl_3$)
OH
Cl
O
O
167.82
138.17
128.48
128.28
126.54
77.32
77.00
76.68
74.45
62.79
62.05
13.54
170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

V. Chiral HPLC Traces

Conditions: *Column*-Chiracel OD; *Eluent*-hexane/isopropanol 95:5; *Flow rate*: 1 mL/min; λ = 254 nm.

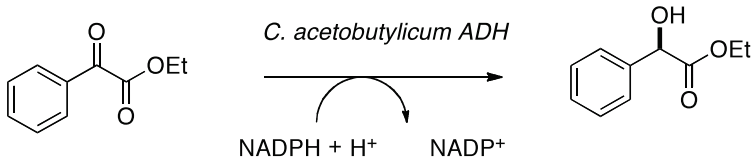
Racemic standard (from $NaBH_4$ reduction):

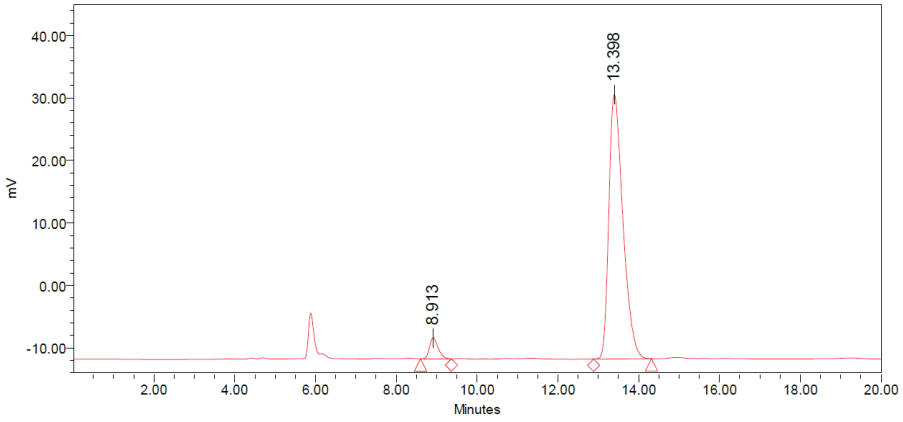




	RT	Area	% Area	Height
1	8.778	447474	50.64	30410
2	13.198	436196	49.36	19771

Product of CaADH-mediated reduction:

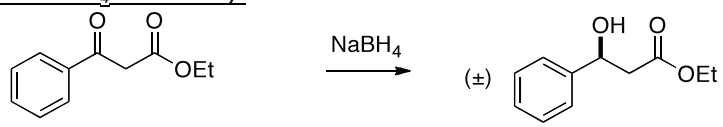


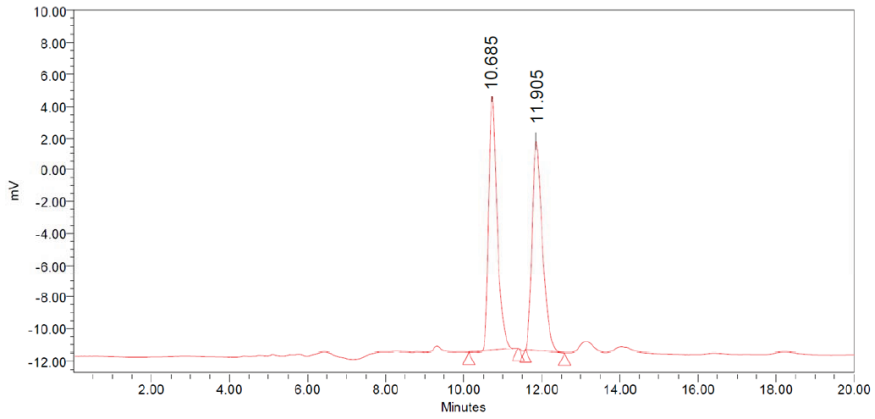


	RT	Area	% Area	Height
1	8.913	49977	4.65	3374
2	13.398	1023899	95.35	42364

Conditions: *Column*-Chiracel OD; *Eluent*-hexane/isopropanol 95:5; *Flow rate*: 1 mL/min; λ = 254 nm.

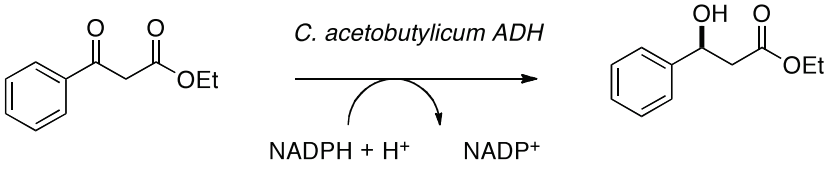
Racemic standard (from $NaBH_4$ reduction):

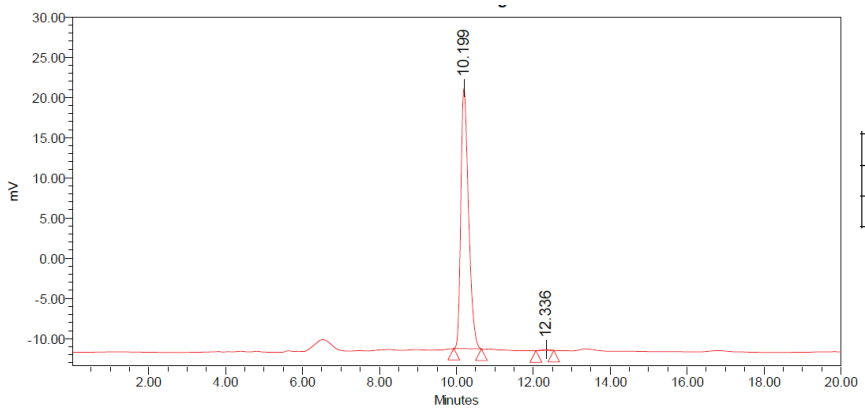




	RT	Area	% Area	Height
1	10.685	259365	51.76	16012
2	11.905	241705	48.24	14945

Product of CaADH-mediated reduction:

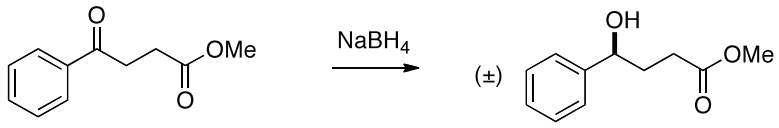


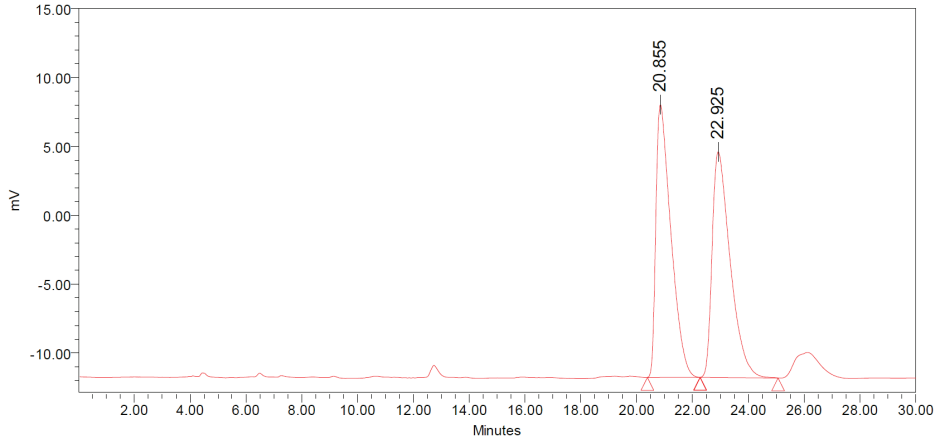


	RT	Area	% Area	Height
1	10.199	450304	99.62	32433
2	12.336	1643	0.36	117

Conditions: *Column*-Chiracel OD; *Eluent*-hexane/isopropanol 97.5:2.5; *Flow rate*: 1 mL/min; λ = 254 nm.

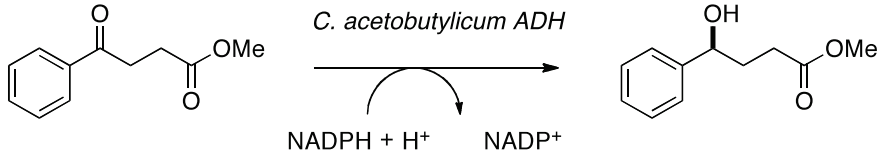
Racemic standard (from $NaBH_4$ reduction):

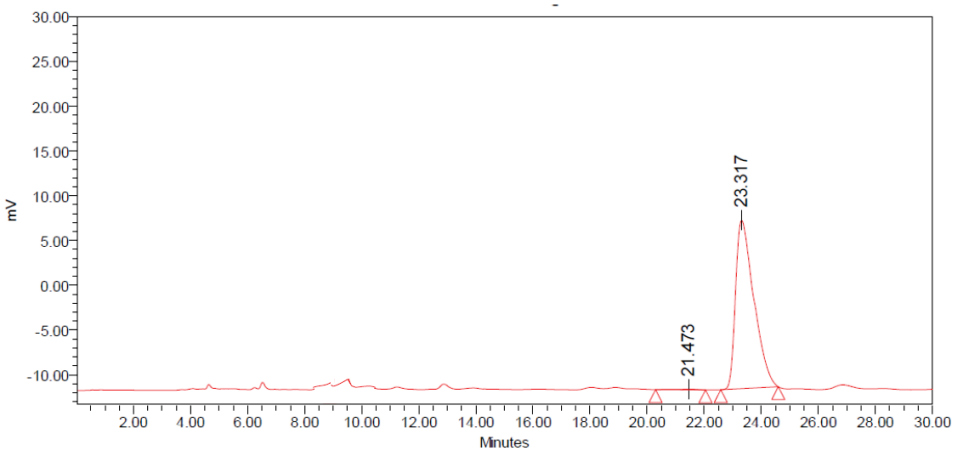




	RT	Area	% Area	Height
1	20.855	740877	50.10	19805
2	22.925	737873	49.90	16387

Product of CaADH-mediated reduction:

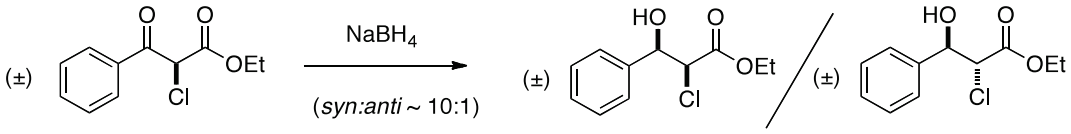


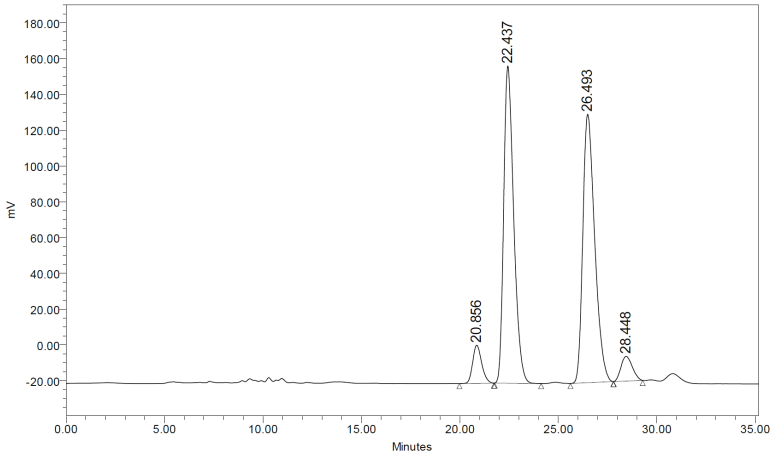


	RT	Area	% Area	Height
1	21.473	3808	0.46	76
2	23.317	833746	99.54	18787

Conditions: *Column*-Chiracel OD; *Eluent*-hexane/isopropanol 97:3; *Flow rate*: 1 mL/min; λ = 254 nm.

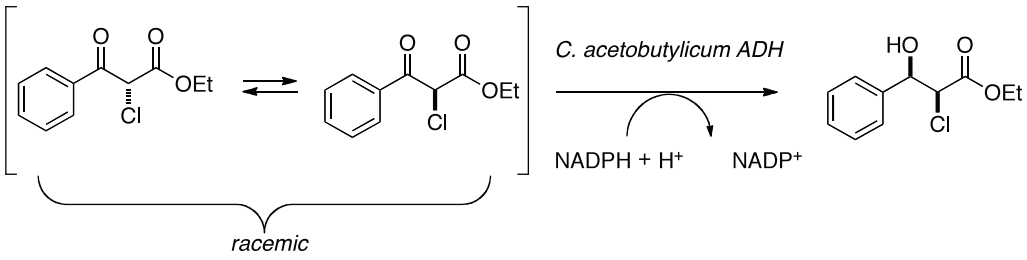
With $NaBH_4$:

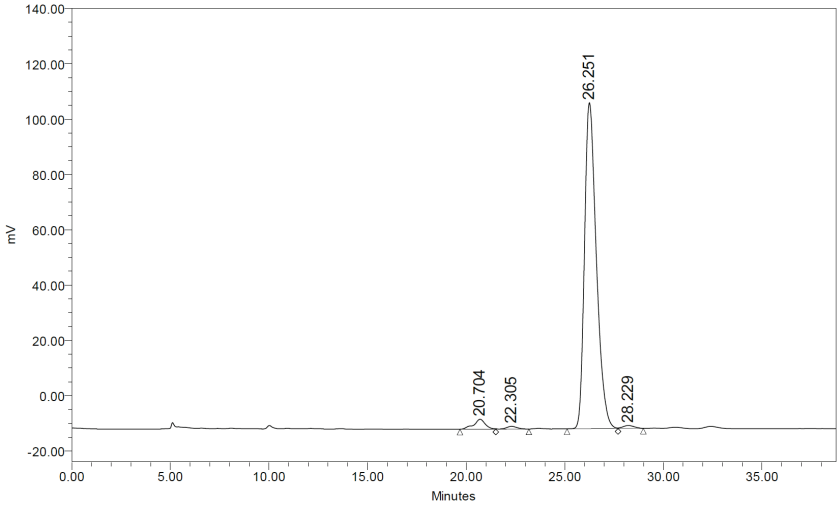




	RT	Area	% Area	Height
1	20.856	678279	4.91	21346
2	22.437	6274847	45.44	177382
3	26.493	6302826	45.64	150062
4	28.448	553519	4.01	13847

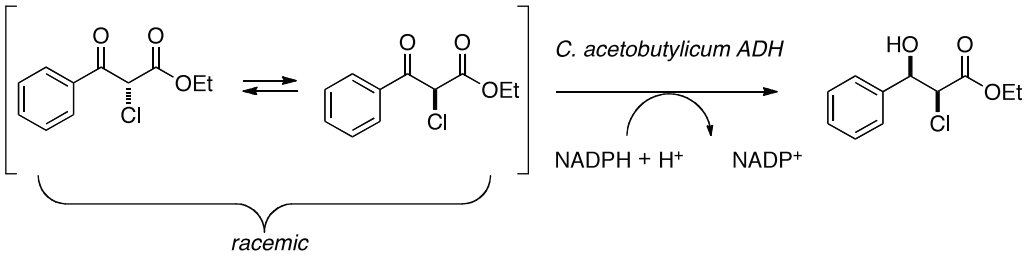
With Purified Enzyme:

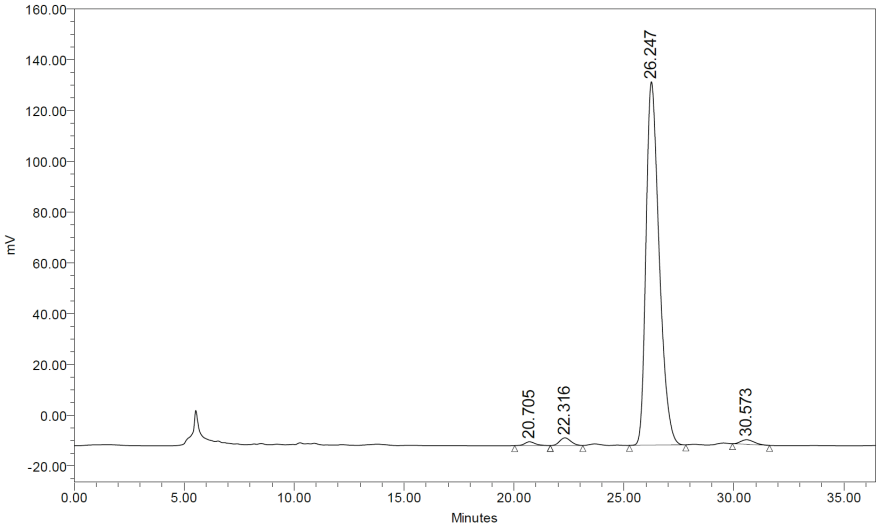




	RT	Area	% Area	Height
1	20.704	144781	2.81	3584
2	22.305	35970	0.70	984
3	26.251	4921886	95.65	117900
4	28.229	43321	0.84	1079

With Crude Cell Lysate:





	Peak Name	RT	Area	% Area	Height
1	Peak1	20.705	48442	0.78	1486
2	Peak2	22.316	105672	1.70	3030
3	Peak3	26.247	5971960	96.27	143213
4	Peak4	30.573	77409	1.25	1820