



UNIVERSITAT DE
BARCELONA

Metal-dependent 2-oxoacid aldolases as versatile biocatalysts for the synthesis of chiral multifunctional compounds

Roser Marín Valls

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6.1 Molecular biology

6.1.1 Cloning of 3-methyl-2-oxobutanoate hydroxymethyltransferase gene (panB) from *E. coli* K-12 in pQE60 plasmid.

The gene *panB* from *E. coli* K-12 (NCBI data base accession number NC 000913.3) was amplified by PCR from genomic DNA and inserted into pQE60 using FastDigest NcoI and BglII (Thermo Scientific) as restriction enzymes with the primers: forward: 5'-ATCGATTAACCATGGACATG AAACCGACCACC-3'; reverse and complementary: 5'-GCAGGTACAAGATCTATGGAAAC TGTGTTC-3'.

A) Bases sequence of the *panB* gene insert in pQE60 and **B)** amino acid sequence of protein construct. Start methionine incorporated by open reading frame in pQE60 (black), 3-methyl-2-oxobutanoate hydroxymethyltransferase (KPHMT, blue) and 6x His tag (red).

A

```
ATGGACATGAAACCGACCACCATCTCCTTACTGCAGAAGTACAAACAGGAAAAAAA  
ACGTTTCGCGACCATCACCGCTTATGACTATAGCTTCGCCAAACTCTTTGCTGATG  
AAGGGCTTAACGTCATGCTGGTGGGCGATTTCGCTGGGCATGACGGTTCAGGGGCAC  
GACTCCACCCTGCCAGTTACCGTTGCCGATATCGCCTACCACACTGCCGCCGTACG  
TCGCGGCGCACCAAACTGCCTGCTGCTGGCTGACCTGCCGTTTATGGCGTATGCCA  
CGCCGGAACAAGCCTTCGAAAACGCCGCAACGGTTATGCGTGCCGGTGCTAACATG  
GTCAAATTTGAAGGCGGTGAGTGGCTGGTAGAAACCGTACAAATGCTGACCGAACG  
TGCCGTTTCTGTATGTGGTCACTTAGGTTTAAACACCACAGTCAGTGAATATTTTCG  
GTGGCTACAAAGTTCAGGGGCGCGGCGATGAAGCGGGCGATCAACTGCTCAGCGAT  
GCATTAGCCTTAGAAGCTGCTGGGGCACAGCTGCTGGTGCTGGAATGCGTGCCGGT  
TGAAGTGGCAAAACGTATTACCGAAGCACTGGCGATCCCGGTTATTGGCATTGGCG  
CAGGCAACGTCCTGACGGGCAGATCCTCGTGATGCACGACGCCTTTGGTATTACC  
GGCGGTCACATTCCTAAATTCGCTAAAAATTTCTTCGCCGAAACGGGCGACATCCG  
CGCGGCTGTGCGGCAGTATATGGCTGAAGTGGAGTCCGGCGTTTATCCGGGCGAAG  
AACACAGTTTCCATAGATCTCATCACCATCACCATCAC
```

B

```
MDMKPTTISLLQKYKQEKRFATITAYDYSFAKLFADEGLNVMLVGDSLGMTVQGH  
DSTLPVTVADIAYHTAAVRRGAPNCLLLADLPFMAYATPEQAFENAATVMRAGANM  
VKIEGGEWLVETVQMLTERAVPVCGLHLGLTPQSVNIFGGYKVQGRGDEAGDQLLSD  
ALALEAAGAQLLVLECVPELAKRITEALAIPIVIGIGAGNVTDGQILVMHDAFGIT  
GGHIPKFAKNFLAETGDIRAAVRQYMAEVESGVYPGEEHSFHRSHHHHHH
```


6.1.2 Megaprimer site-directed mutagenesis

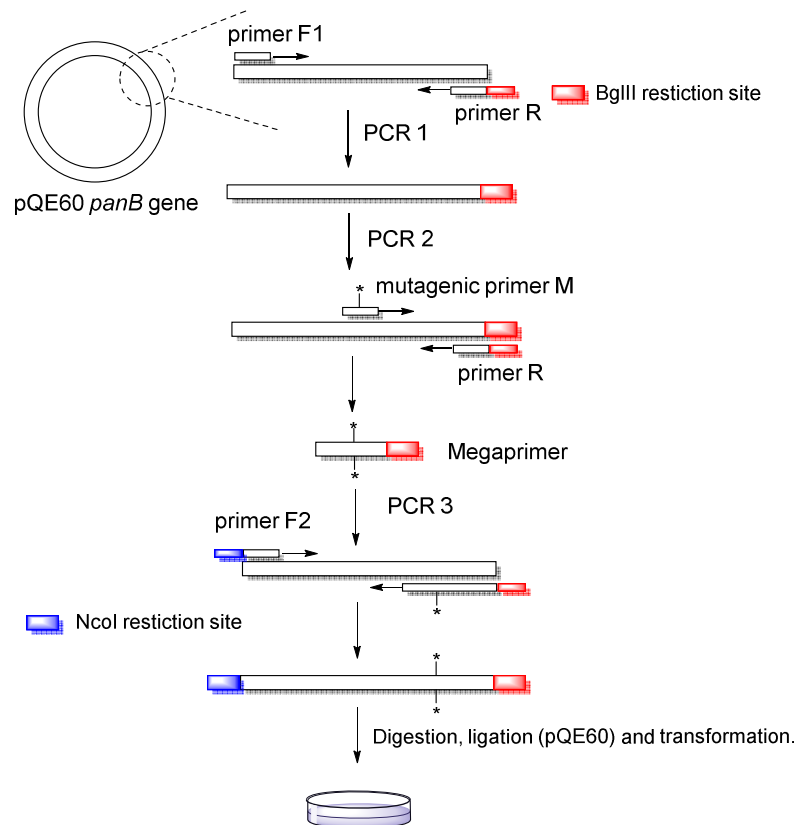


Figure S1 Schematic illustration of the megaprimer method for site-directed mutagenesis. The first-round PCR (PCR 1) was performed using external forward (F1) and reverse primer (R) with BglII restriction site to amplify the *panB* gene. The second-round PCR (PCR 2) was performed using the mutagenic primer (M) and external primer (R) to amplify the mutagenic “megaprimer” fragment from the DNA template. The third-round PCR (PCR 3) was performed using external primer (F2) with NcoI restriction site and "megaprimer" (purified product of second-round PCR).

6.1.3 Gibson assembly

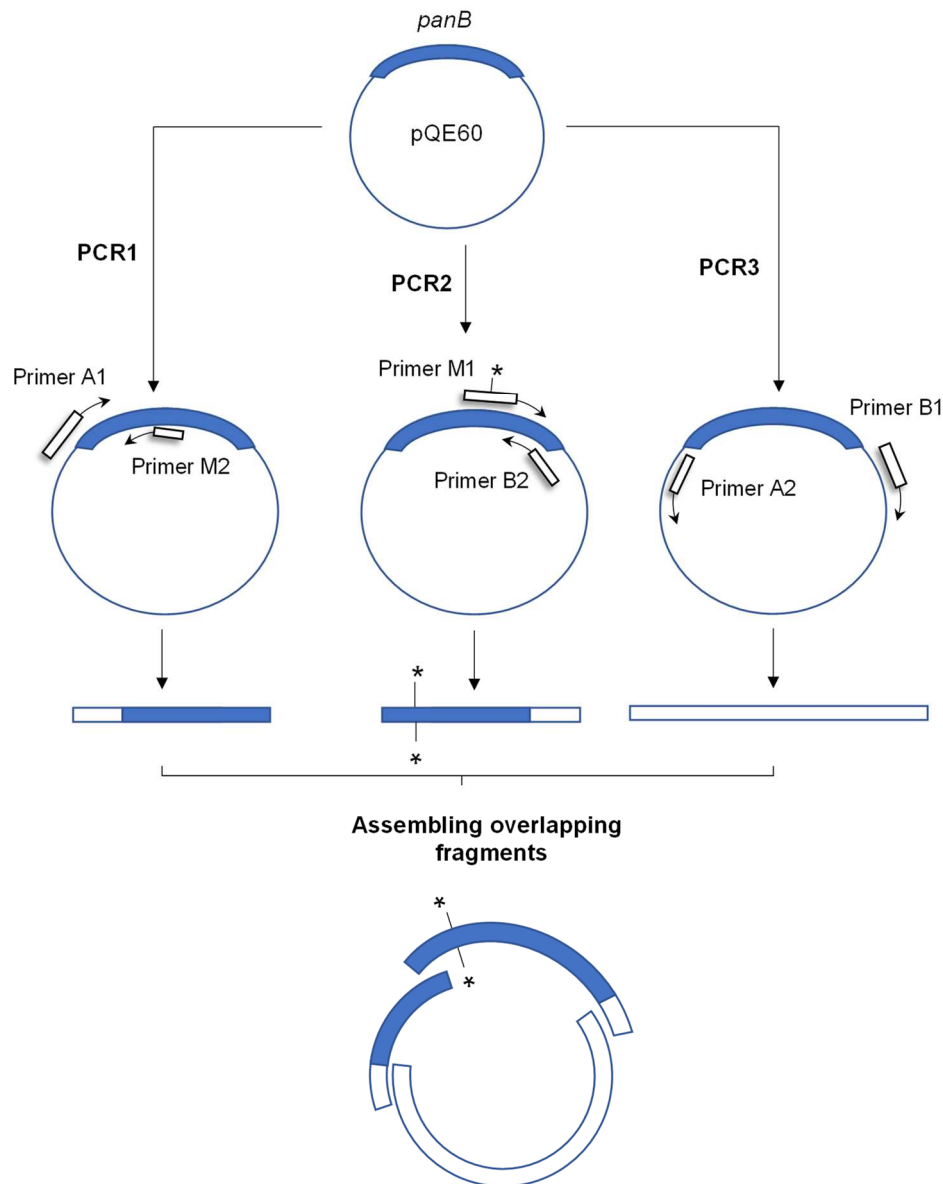


Figure S2 Schematic illustration of the Gibson assembly method for site-directed mutagenesis. The first PCR (PCR 1) was performed using external forward (A1) and reverse primer (M2) one half of the *panB* gene. The second PCR (PCR 2) was performed using the mutagenic primer (M1) and external primer (B2) to amplify the second half of the gene with the desired mutation. The third PCR (PCR 3) was performed using a forward primer (B1) with BglII restriction site and reverse primer (A2) with NcoI restriction site. The three PCR products have overlapping regions and were assembled with NEBuilder ® HiFi Assembly Master Mix (E2621S, New England BioLabs) kid.

6.1.4 KPHMT purification

Table S1 Yield of KPHMT variants after purification

KPHMT variant	Yield^a
Wild-type	172
L42A	102
I212A	148
L42A/I212A	156
I202A	170
V214G	88
I202A/V214G	205
I212A/V214G	200
I202A/I212A/V214G	92
I202A/I212A	94

^a mg protein L⁻¹ medium culture

Table S2 Molecular weight determination by ESI-MS of KPHMT variants.

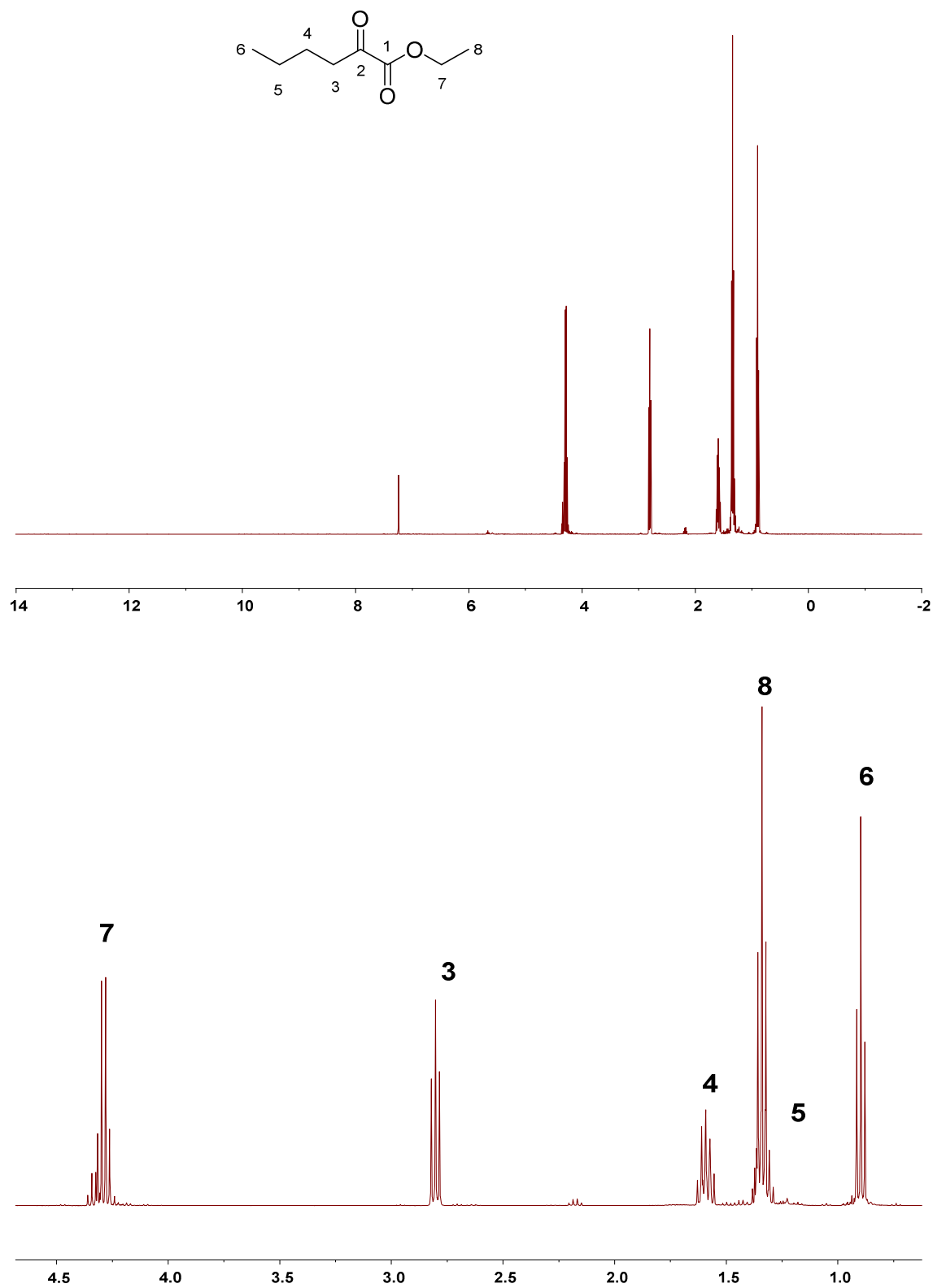
Enzyme variant	Molecular weight calculated/Da^a	Molecular weight measured/Da	Difference/Da
Wild-type	29549.83	29549,68	0.15
L42A	29507.75	29509,36	1.61
I212A	29507.75	29505,78	1.97
L42A/I212A	29465.67	29463,42	2.25
I202A	29507.75	29508,22	0.47

^a Molecular weight was calculated using ProtParam (<http://expasy.org/tools/protparam.html>).

6.2 Data from chapter 3.1

6.2.1 NMR spectra of ethyl 2-oxoesters (C2e-m) and 2-oxoacids (1e-m)

a)



b)

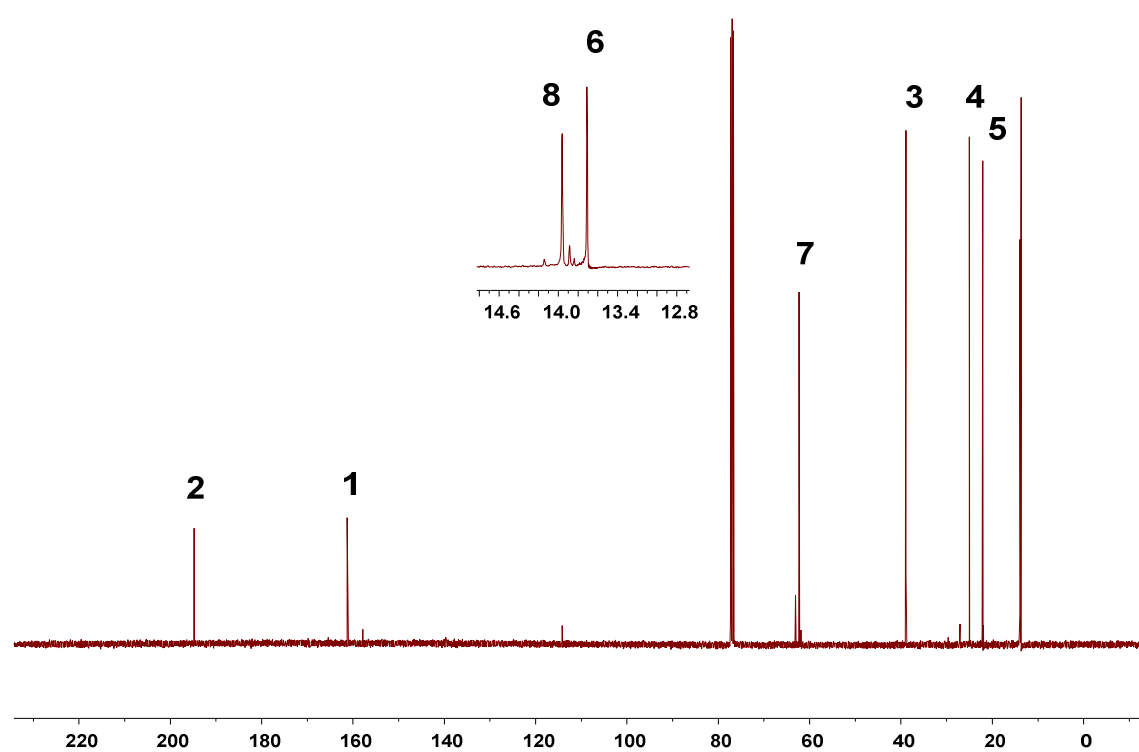
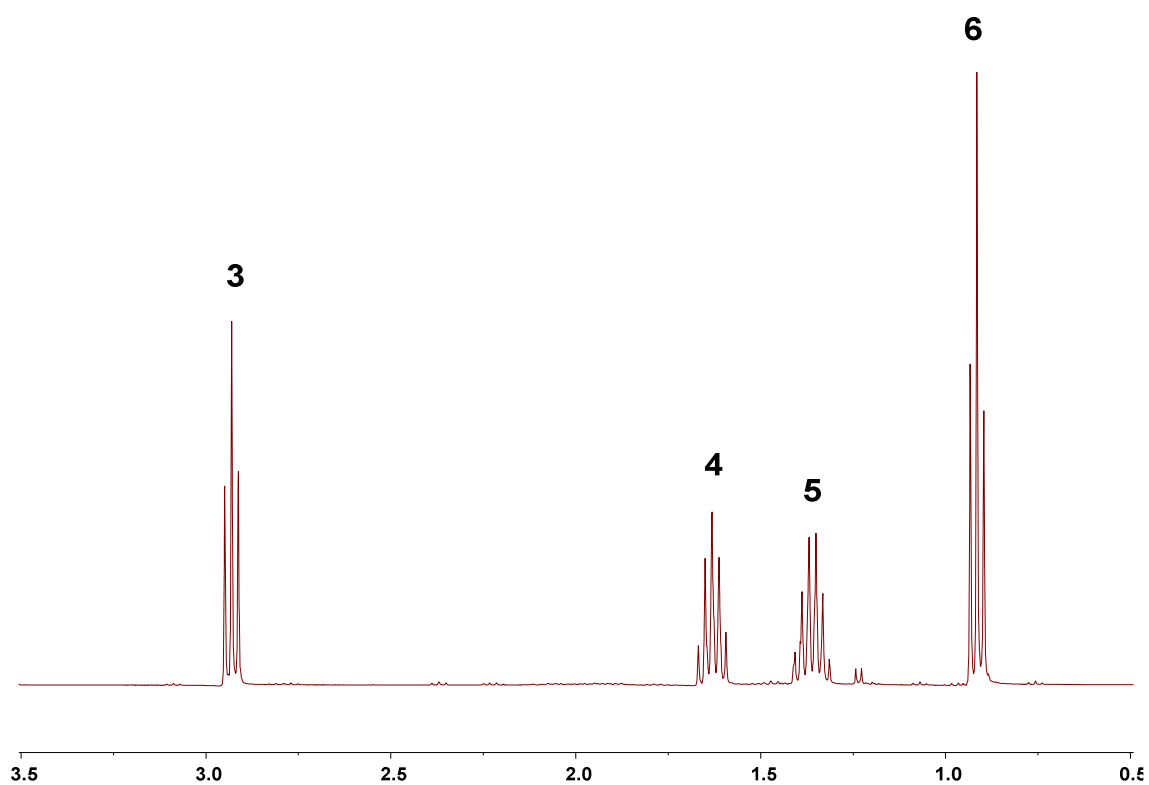
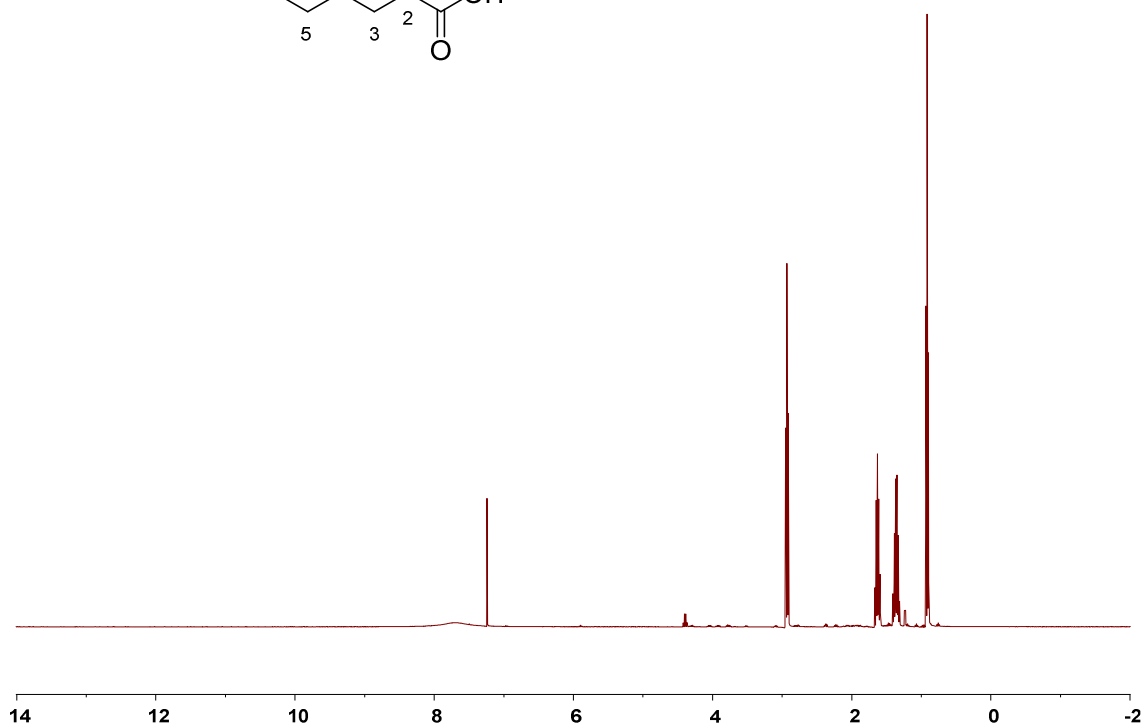
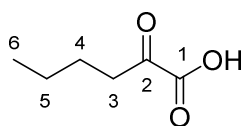


Figure S3 NMR spectra (CDCl_3) of **C2e**: a) ^1H and b) ^{13}C .

a)



b)

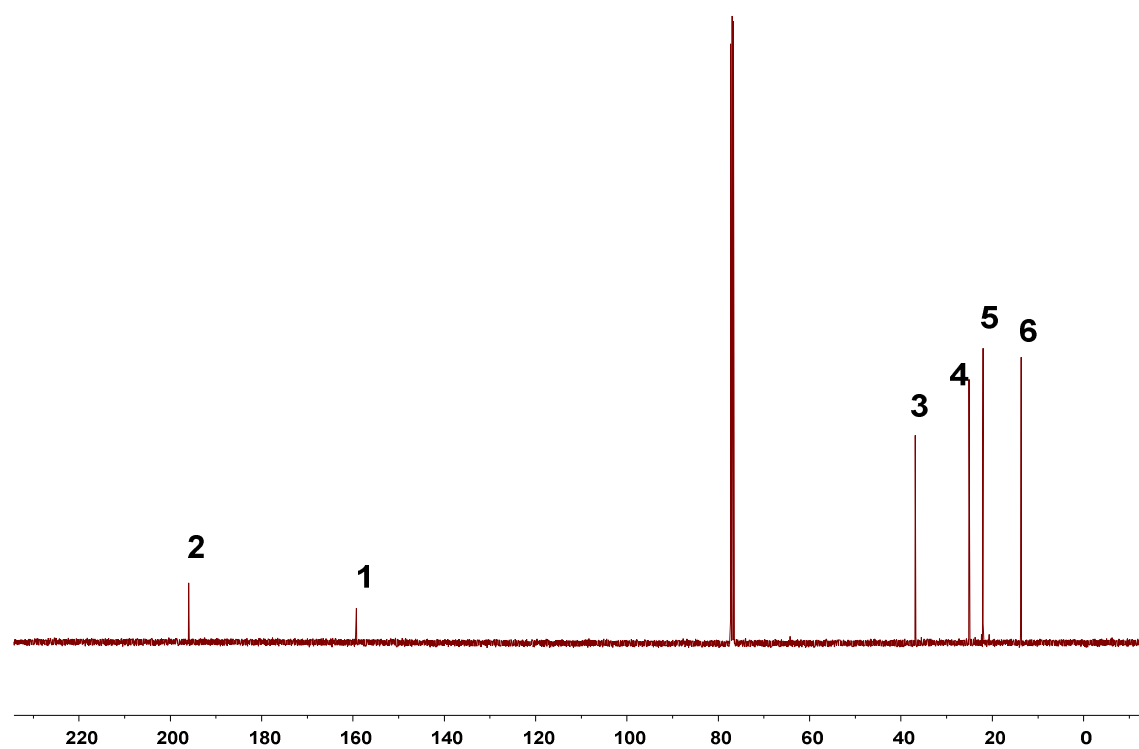
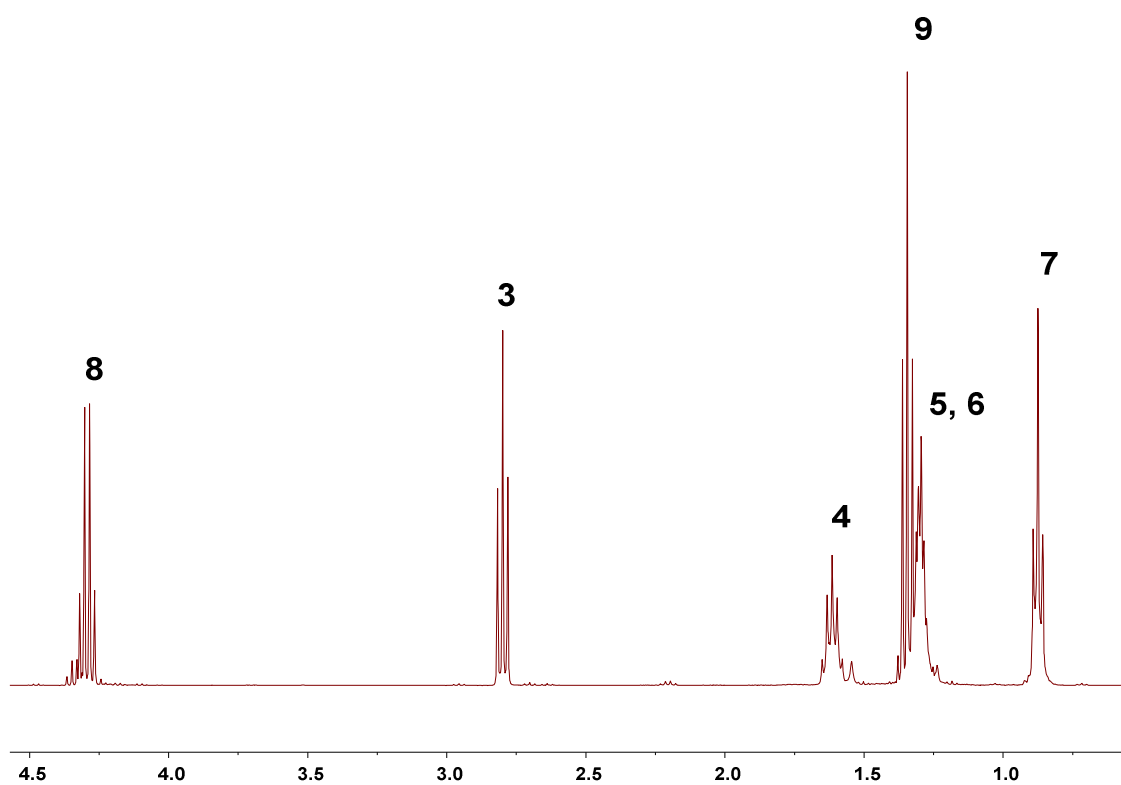
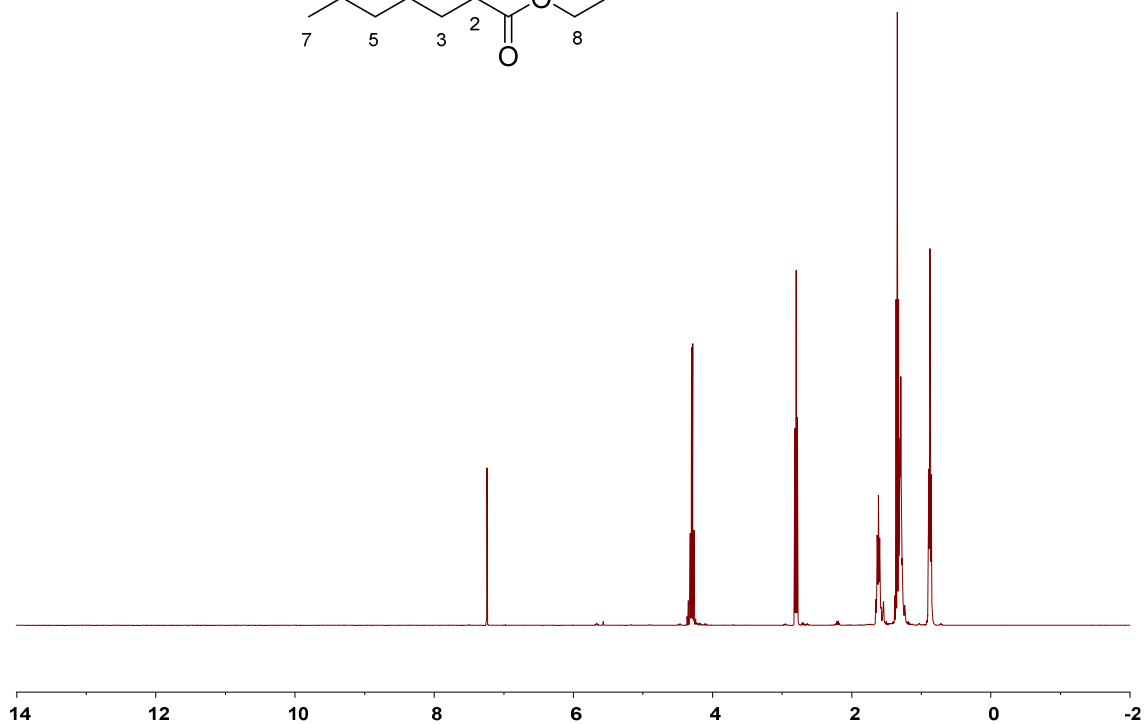
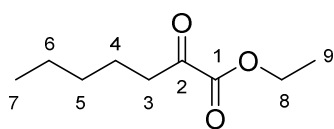


Figure S4 NMR spectra (CDCl_3) of **1e**: a) ^1H and b) ^{13}C .

a)



b)

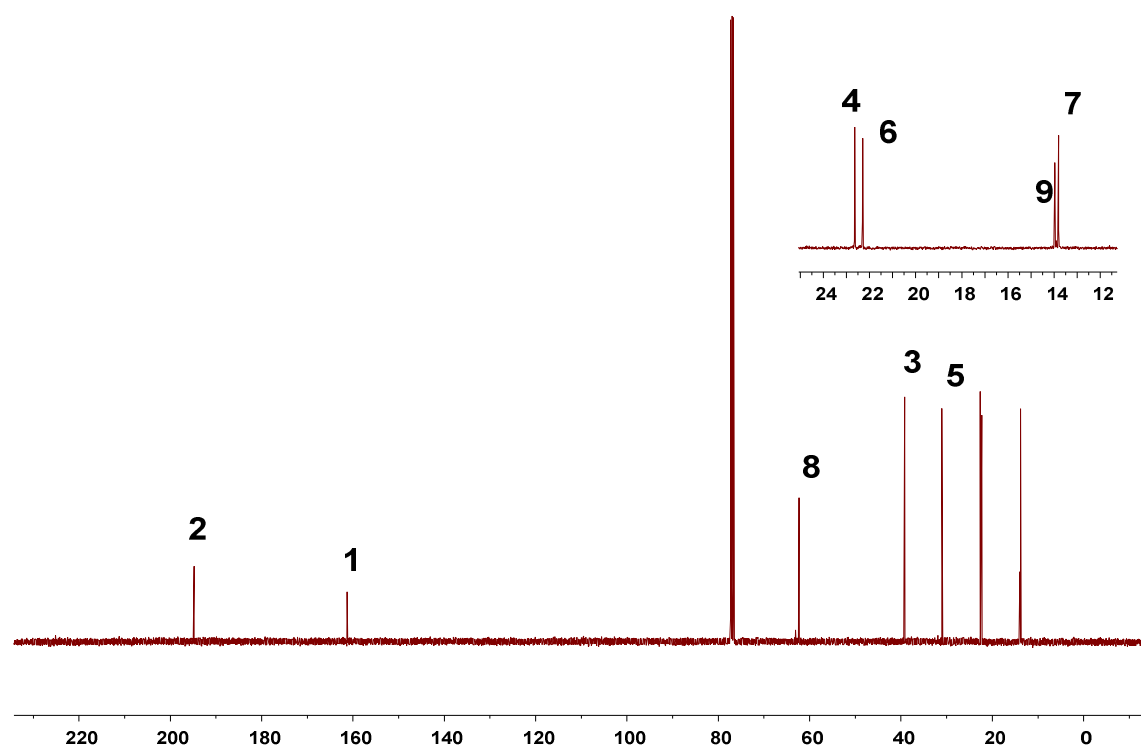
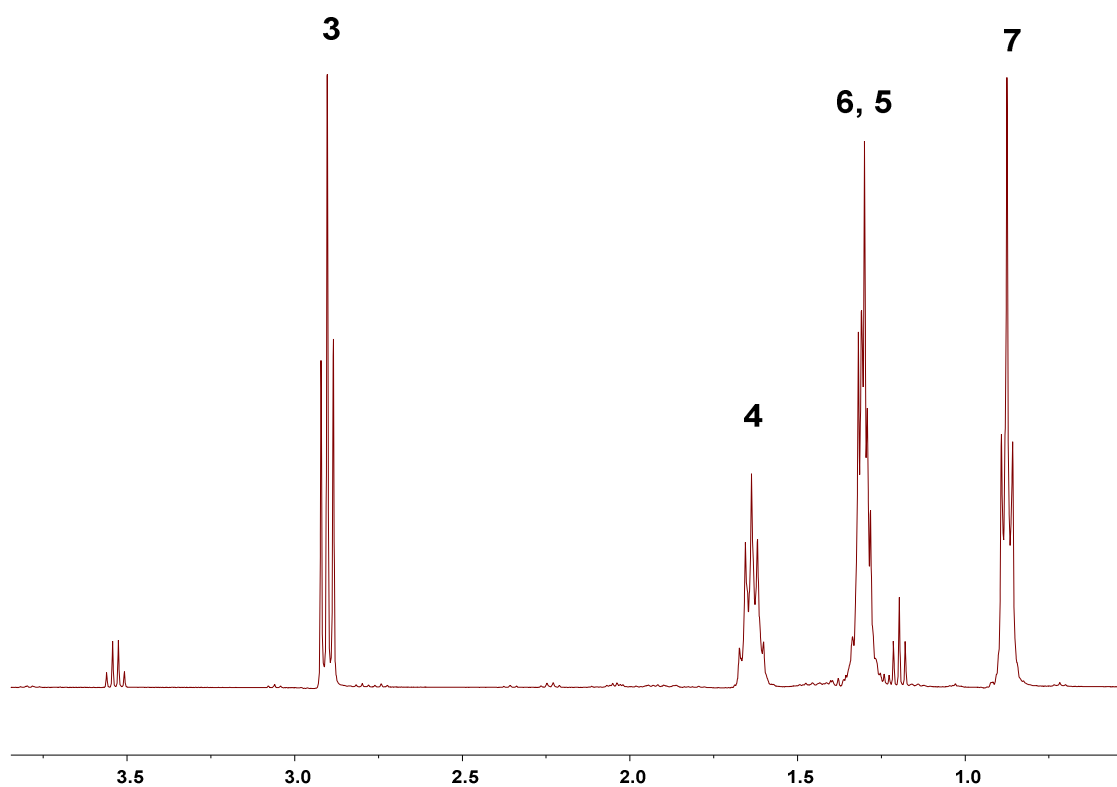
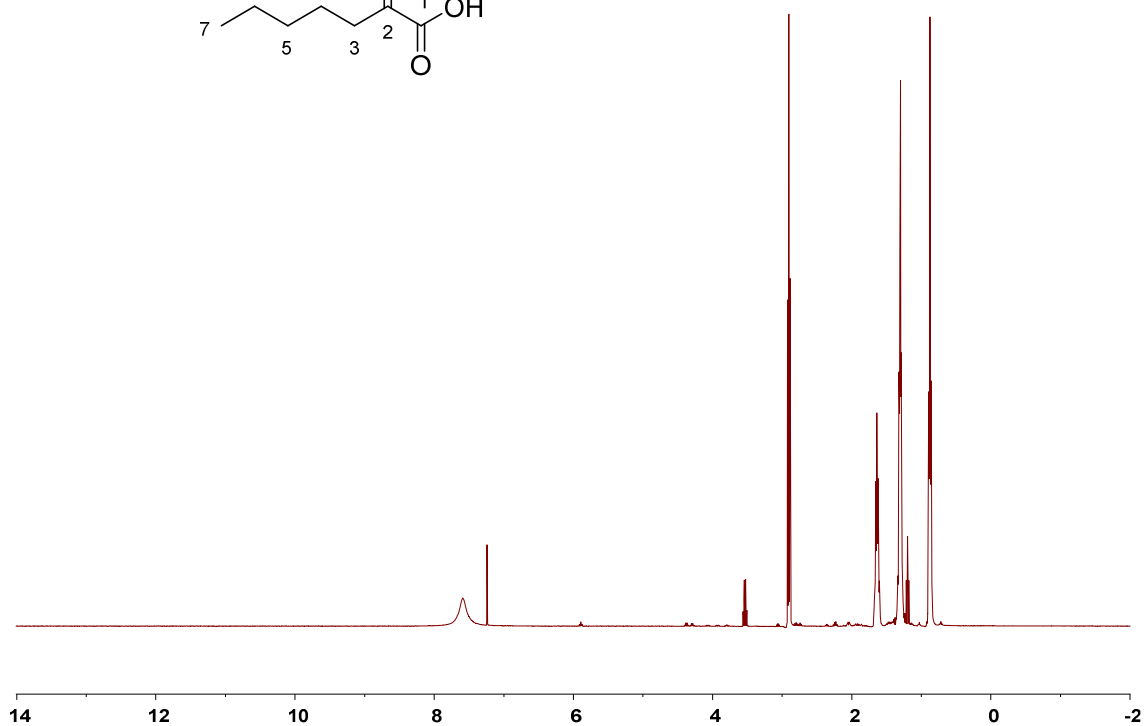
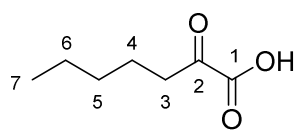


Figure S5 NMR spectra (CDCl_3) of **C2f**: a) ^1H and b) ^{13}C .

a)



b)

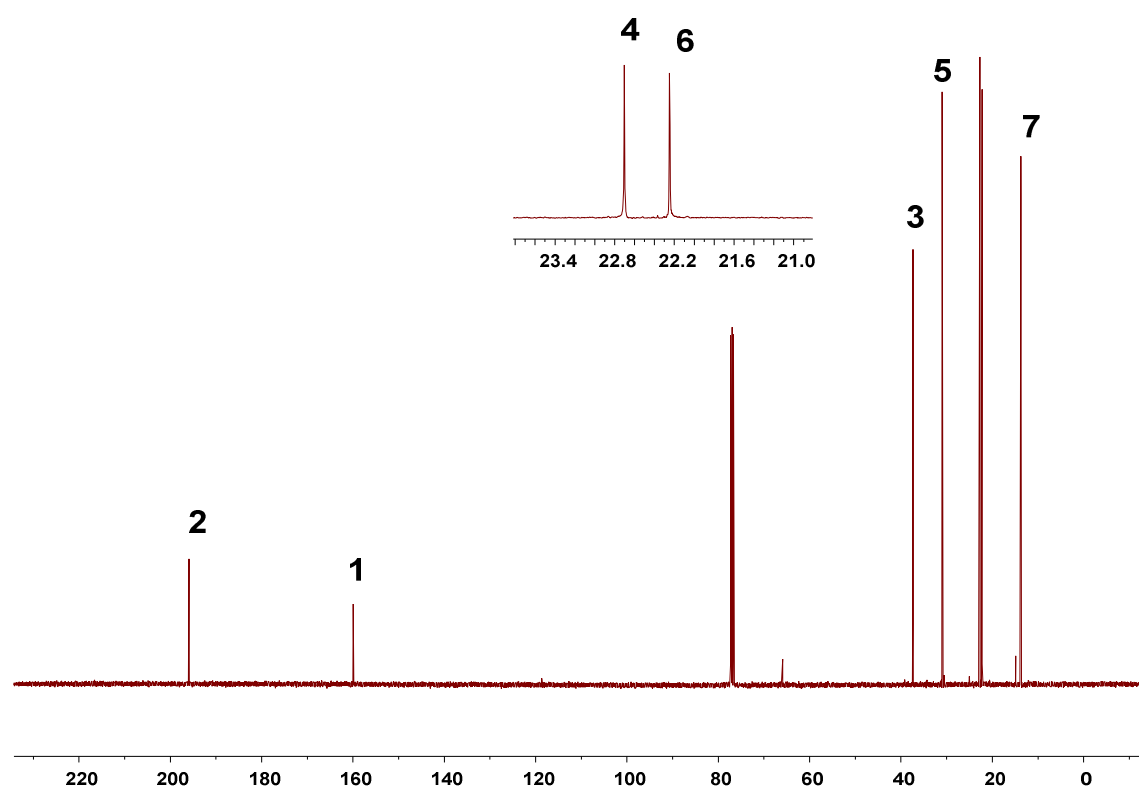
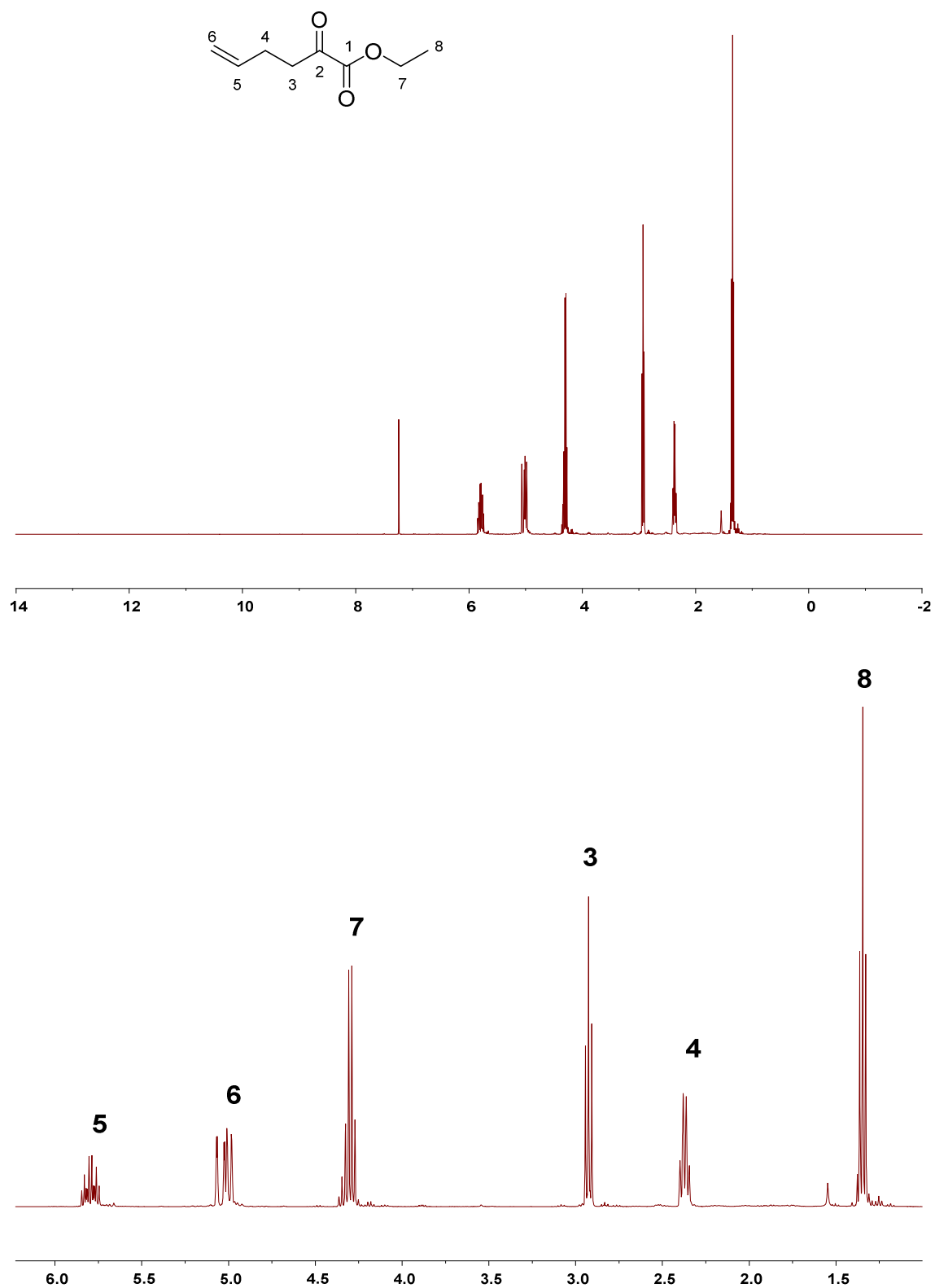


Figure S6 NMR spectra (CDCl_3) of **1f**: a) ^1H and b) ^{13}C .

a)



b)

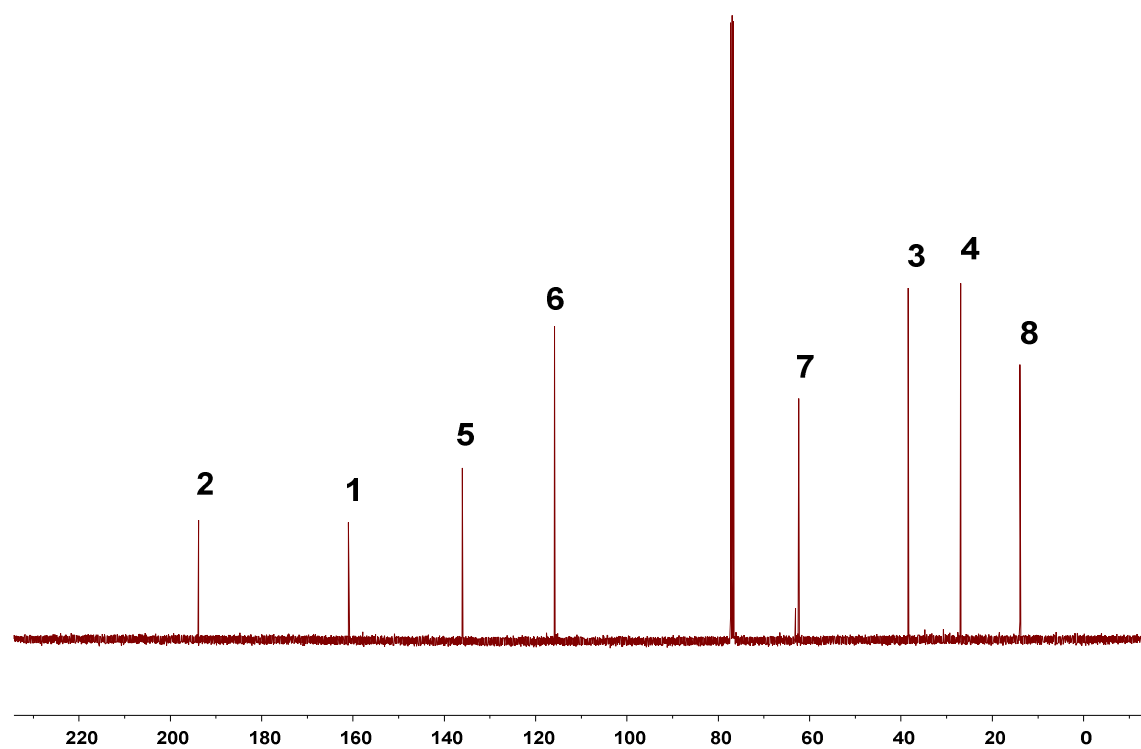
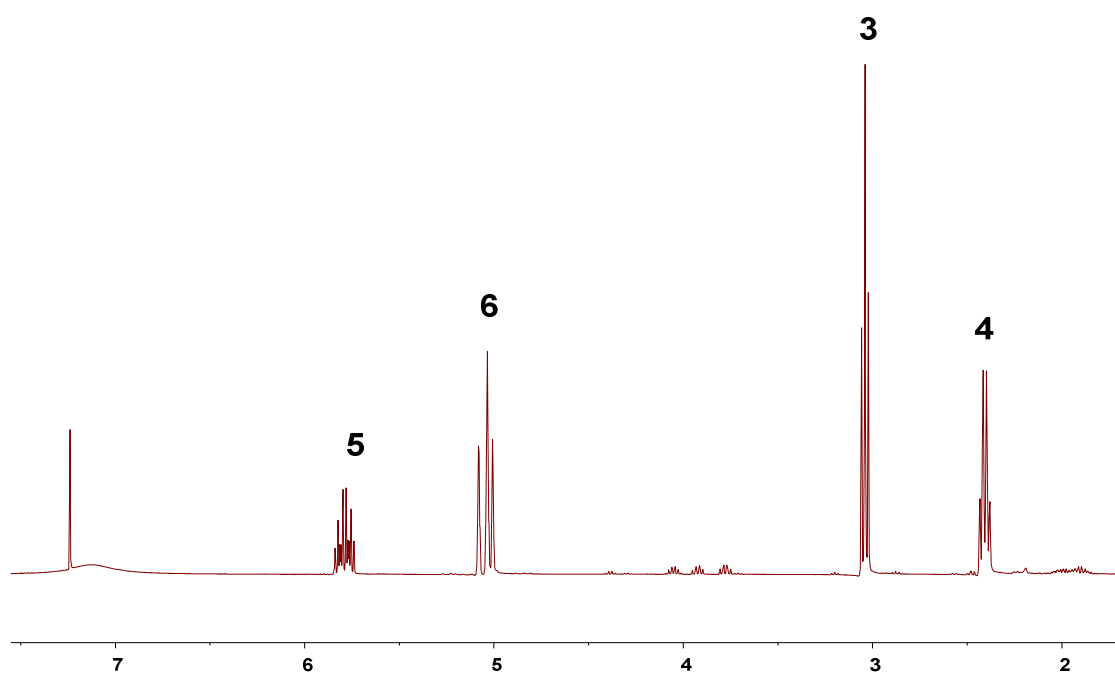
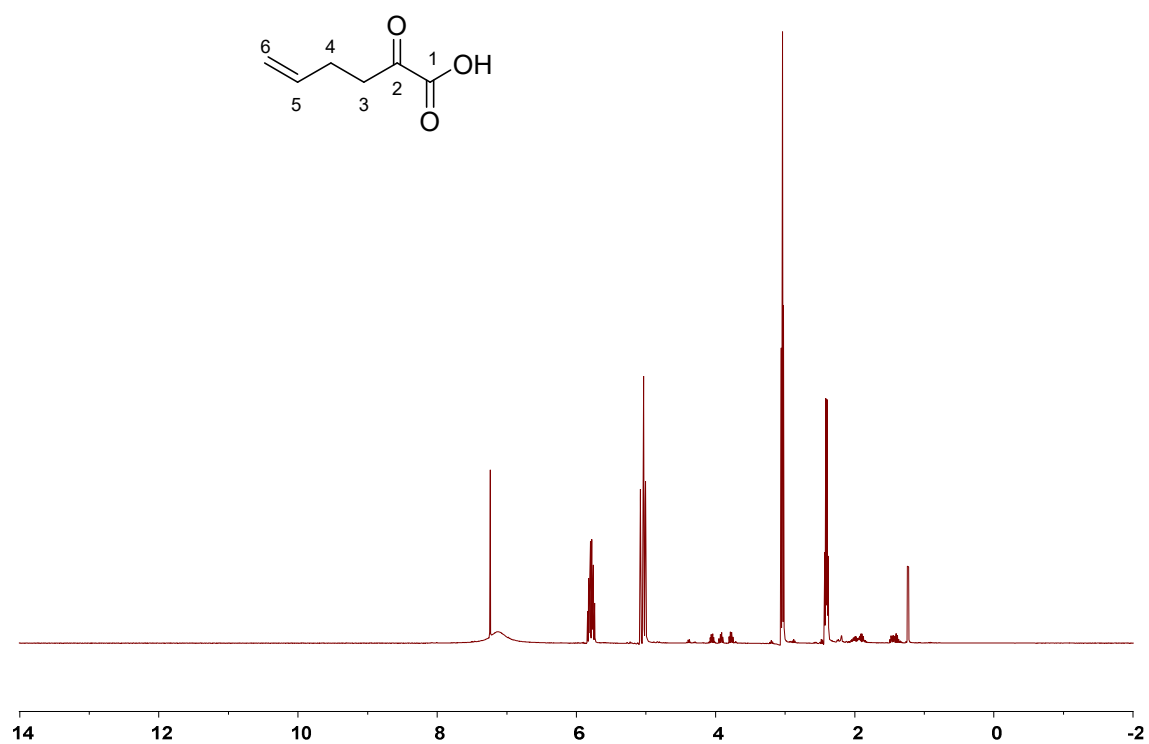


Figure S7 NMR spectra (CDCl_3) of **C2g**: a) ^1H and b) ^{13}C .

a)



b)

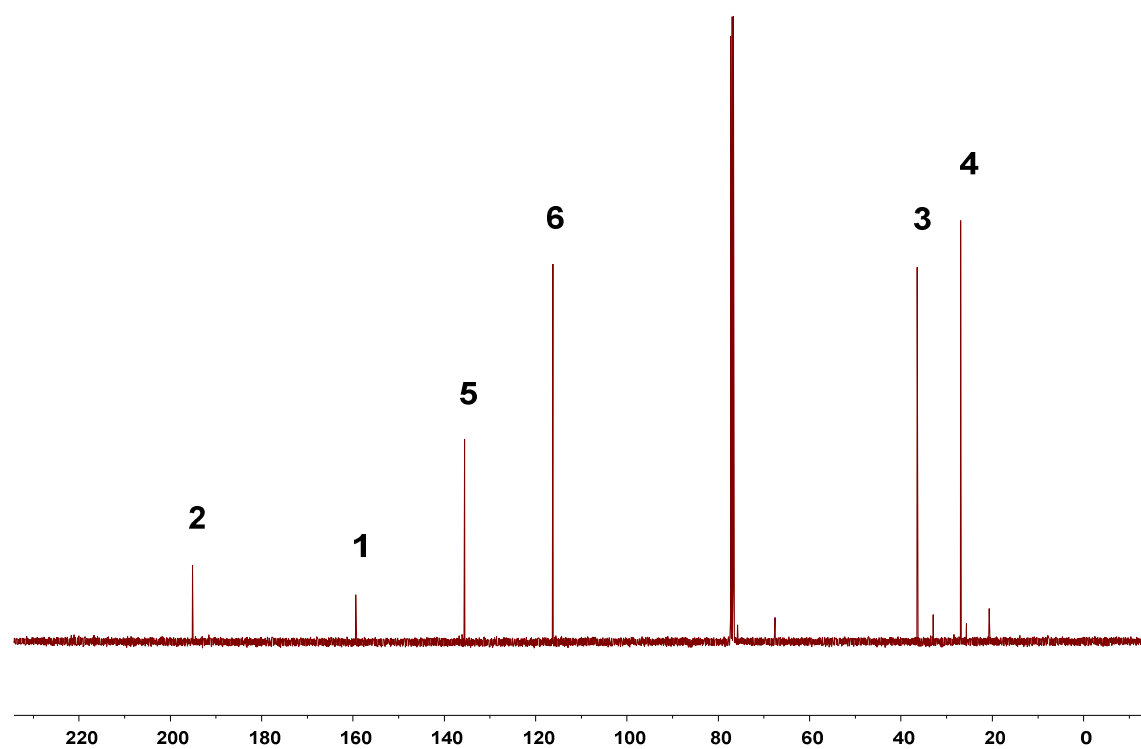
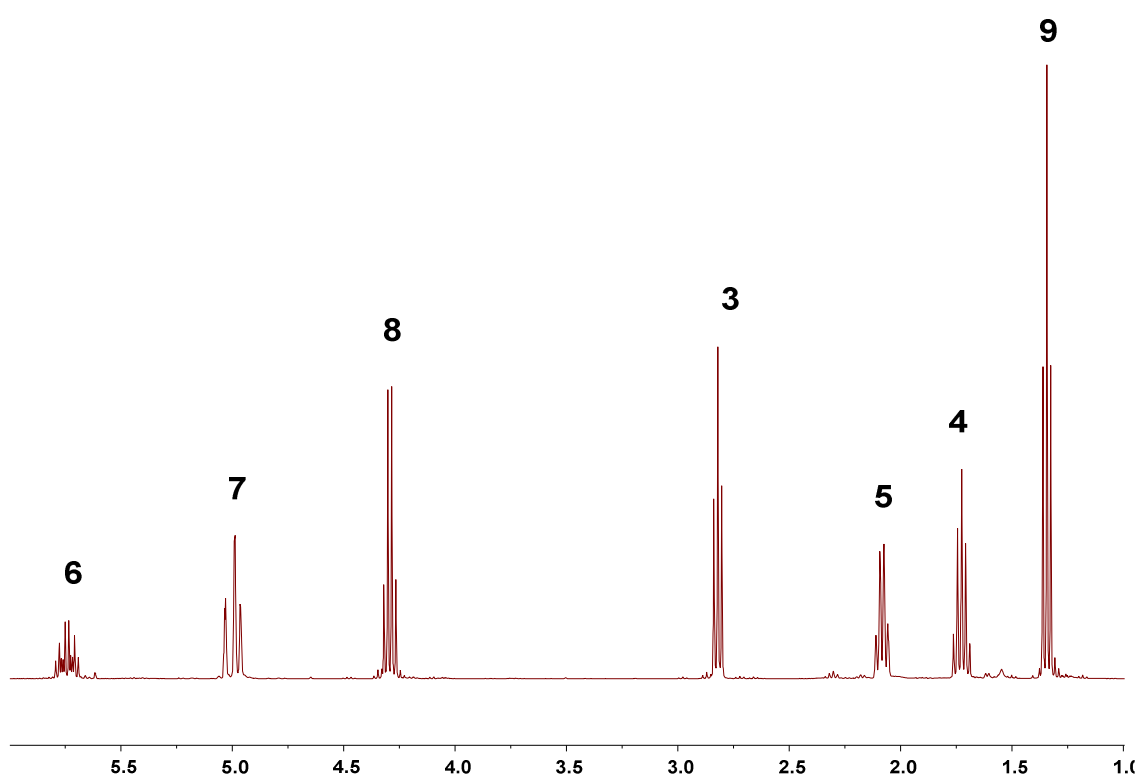
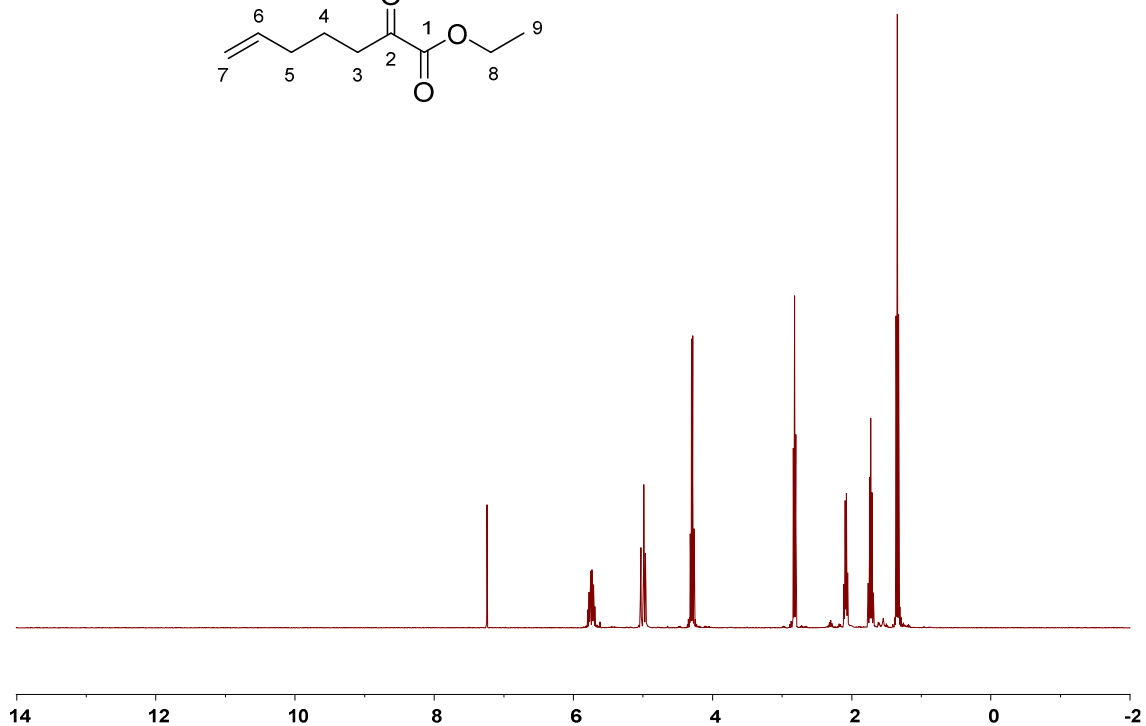
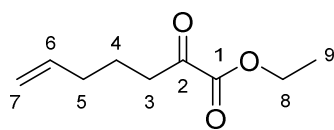


Figure S8 NMR spectra (CDCl_3) of **1g**: a) ^1H and b) ^{13}C .

a)



b)

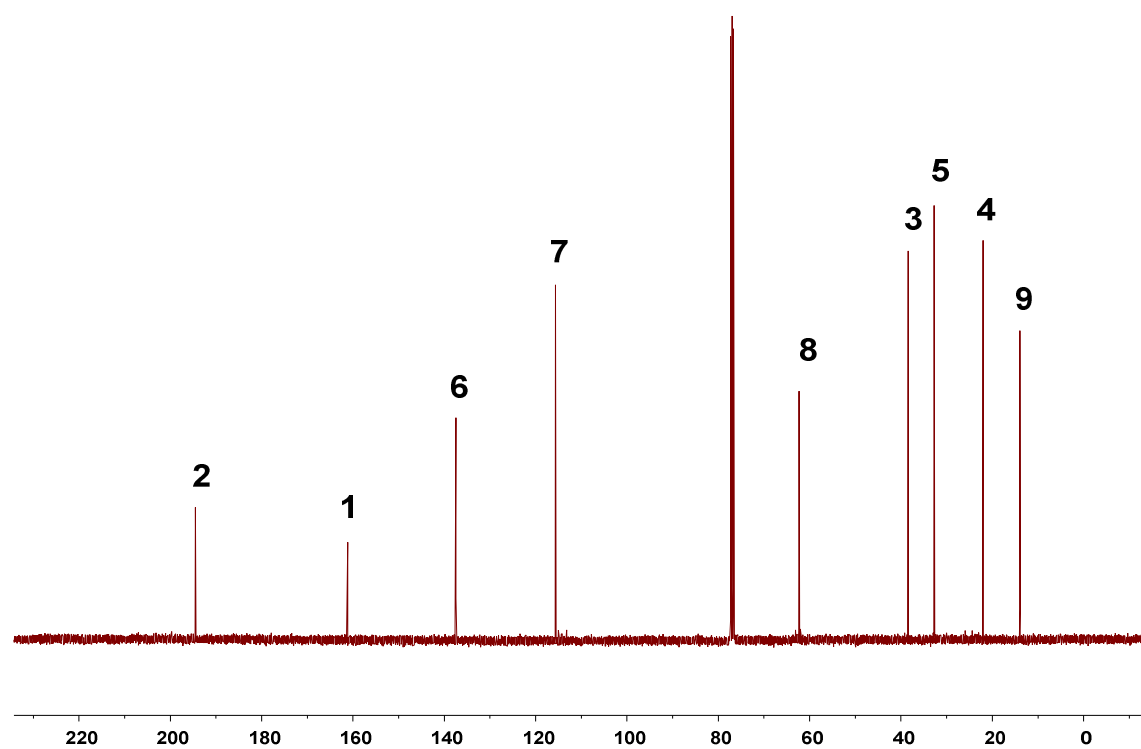
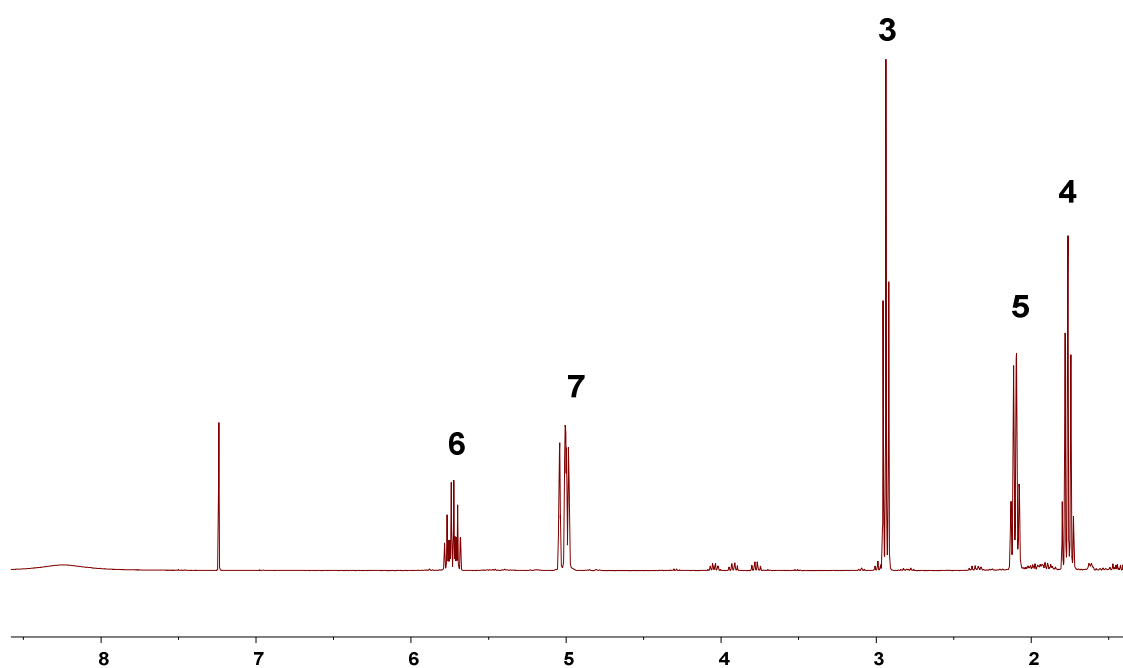
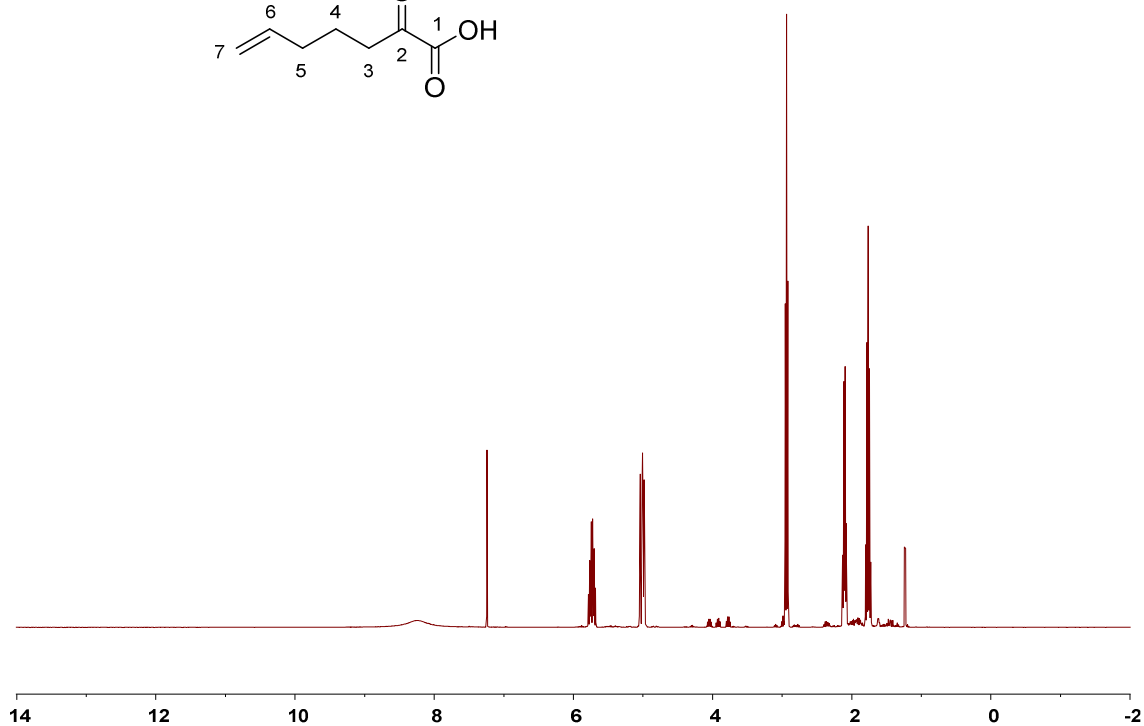
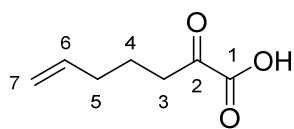


Figure S9 NMR spectra (CDCl_3) of **C2h**: a) ^1H and b) ^{13}C .

a)



b)

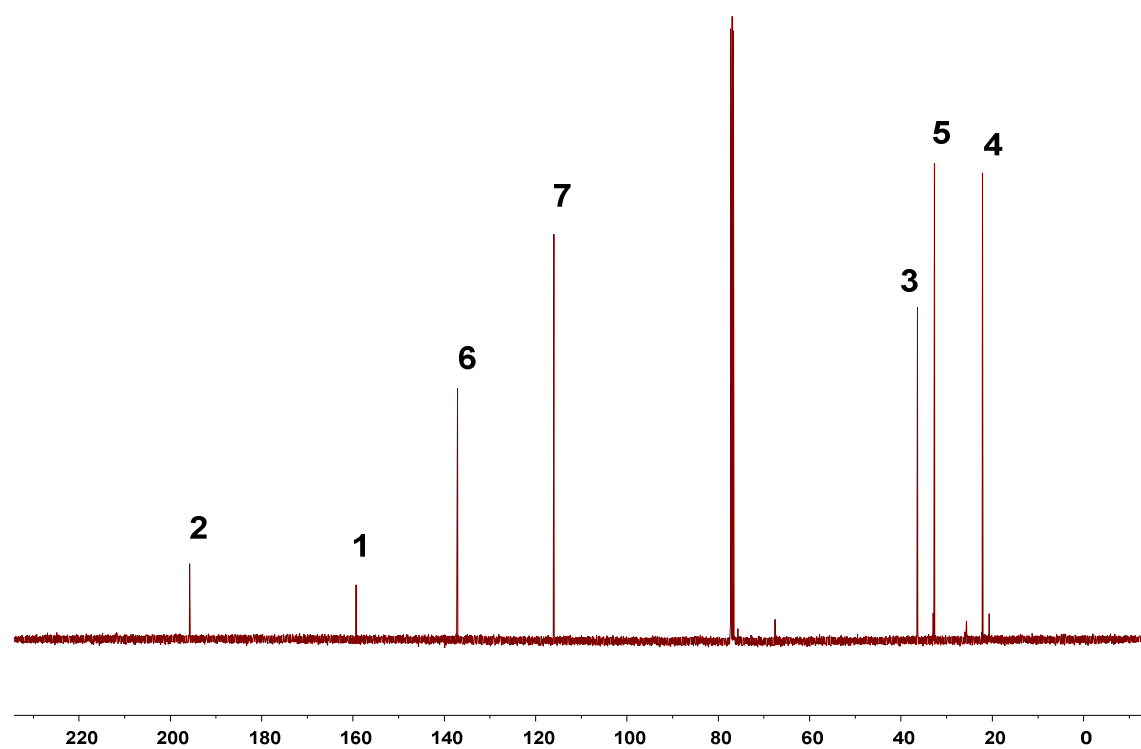
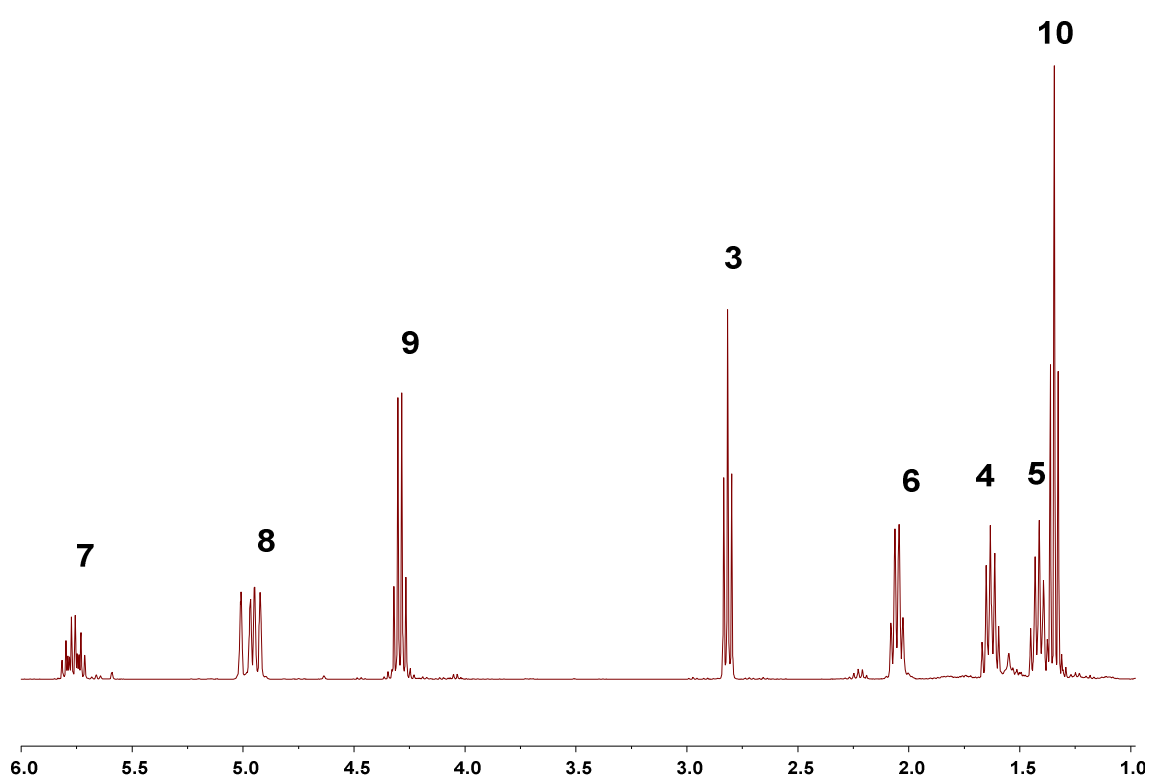
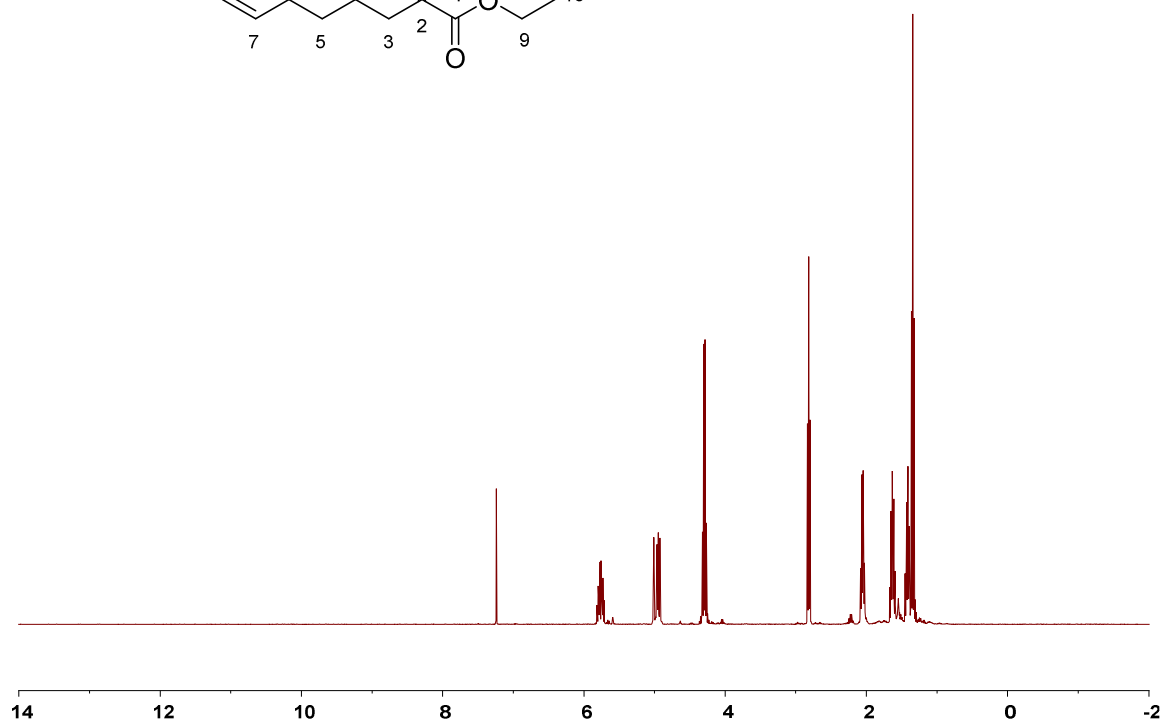
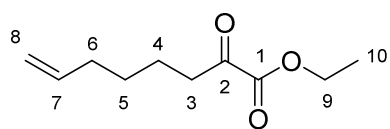


Figure S10 NMR spectra (CDCl_3) of **1h**: a) ^1H and b) ^{13}C .

a)



b)

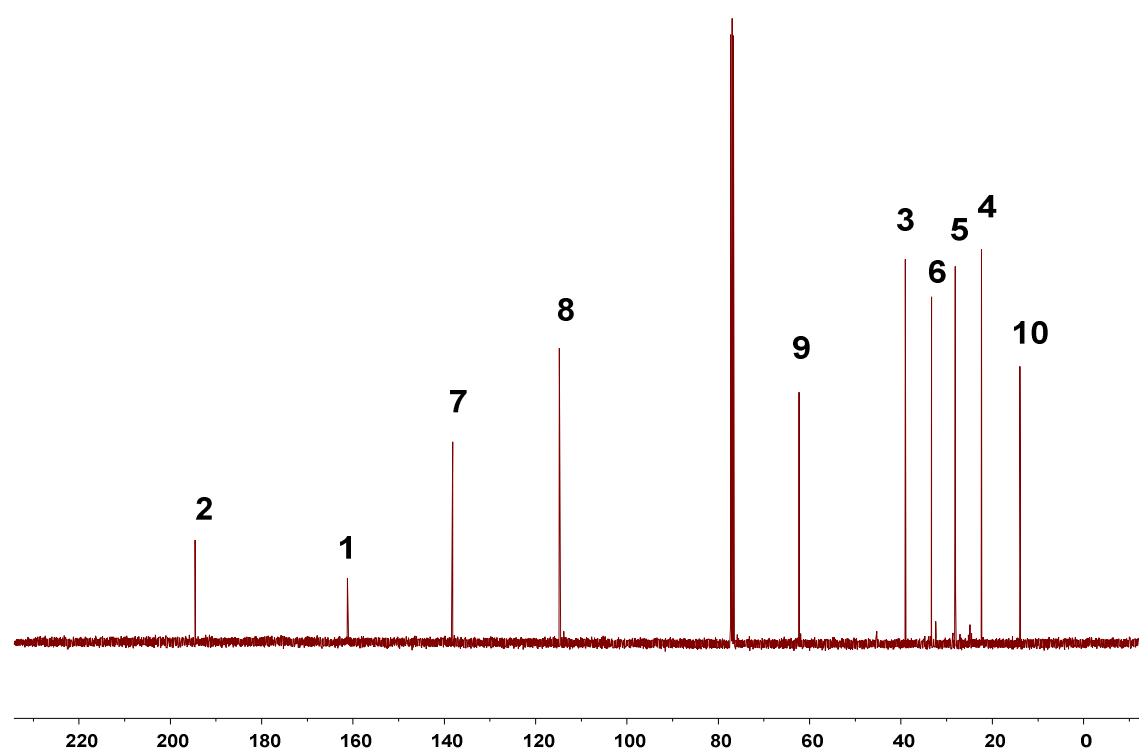
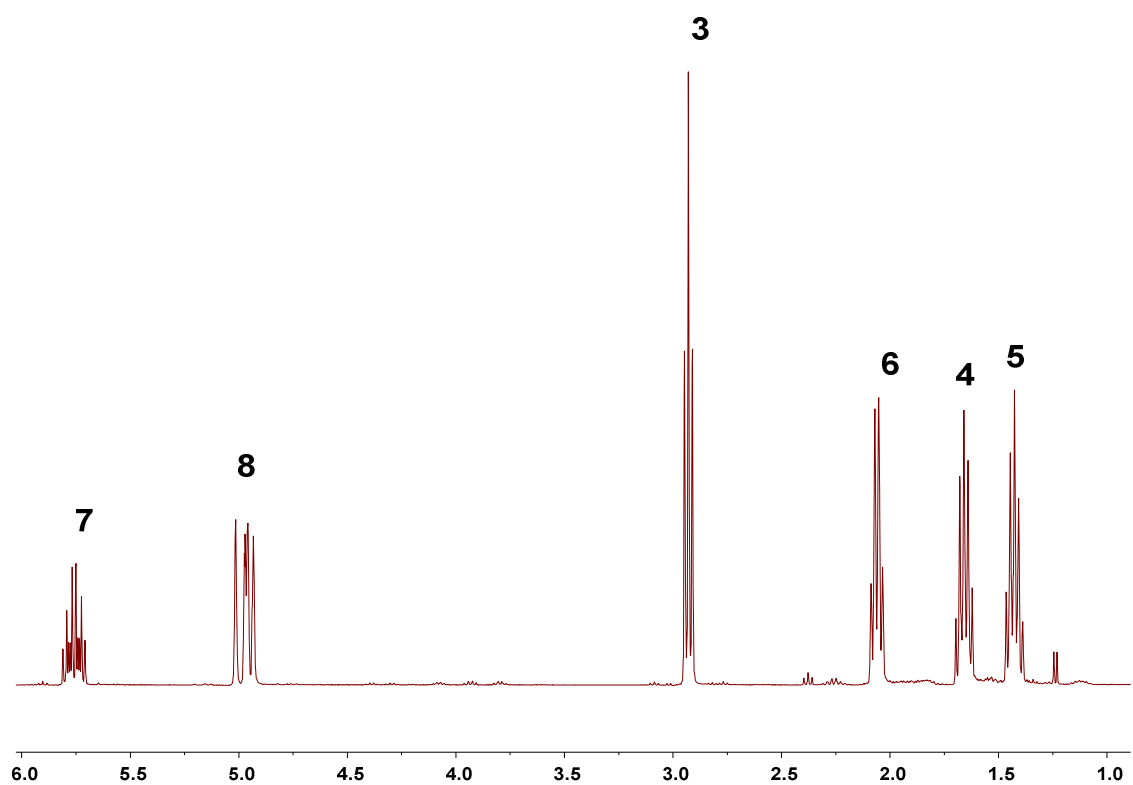
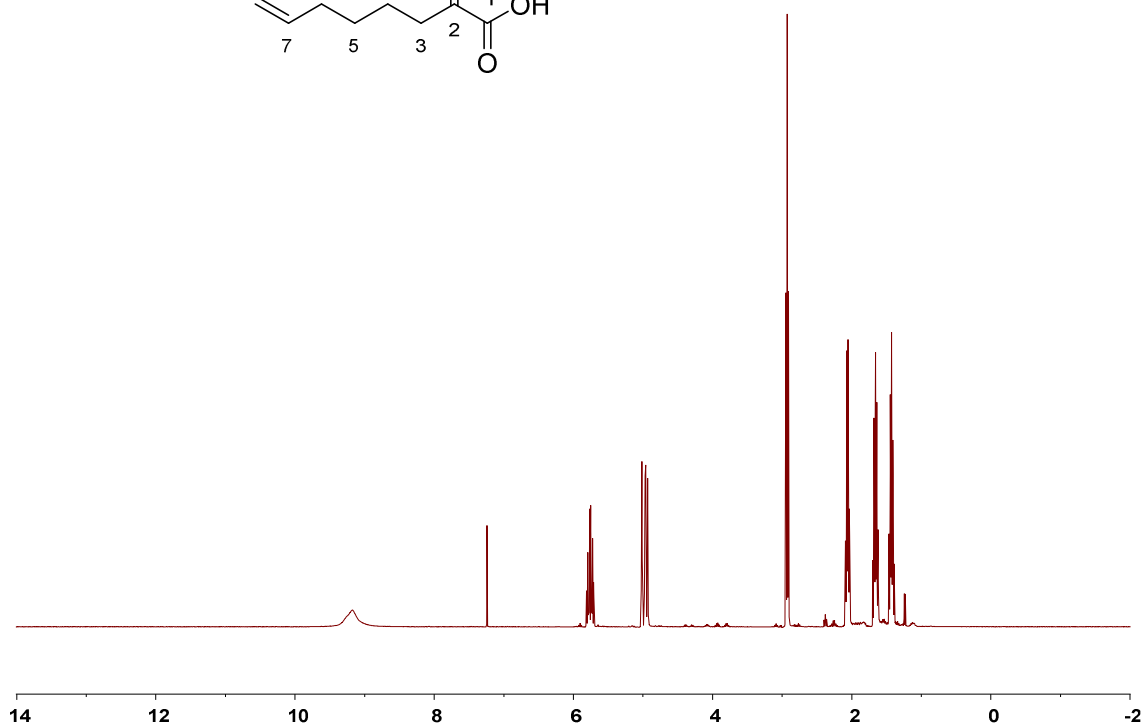
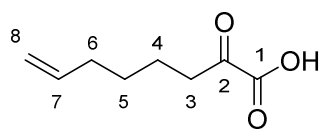


Figure S11 NMR spectra (CDCl_3) of **C2i**: a) ^1H and b) ^{13}C .

a)



b)

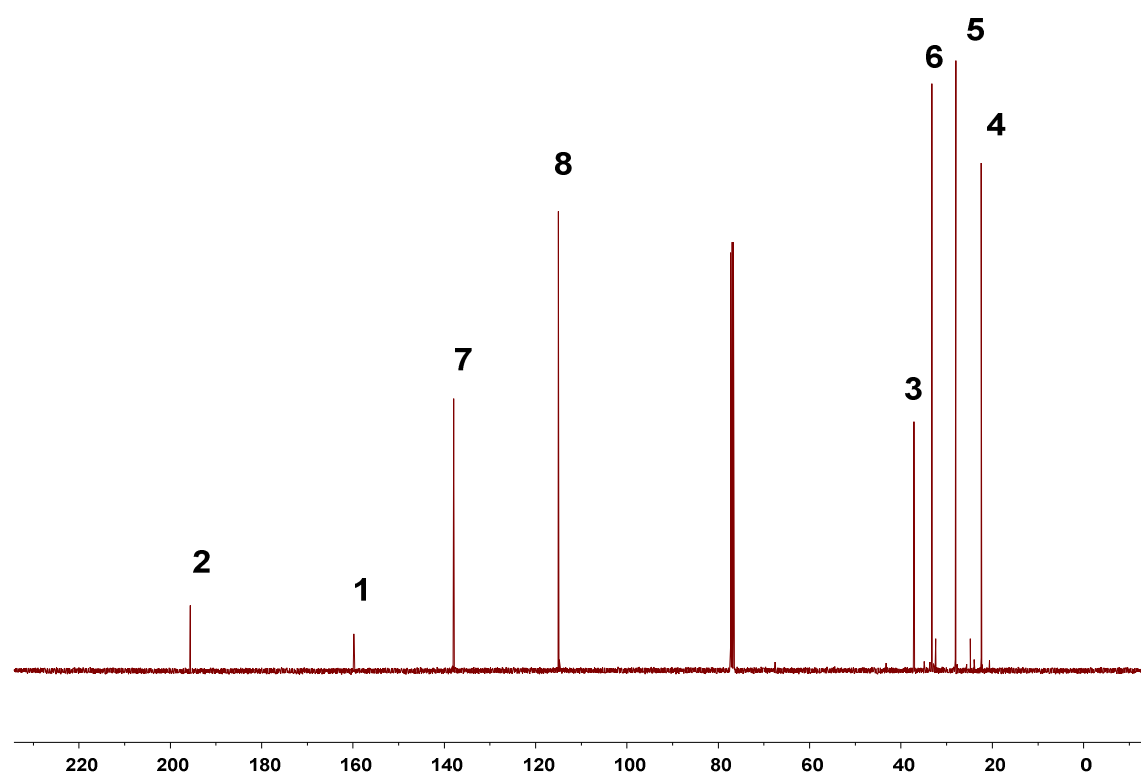
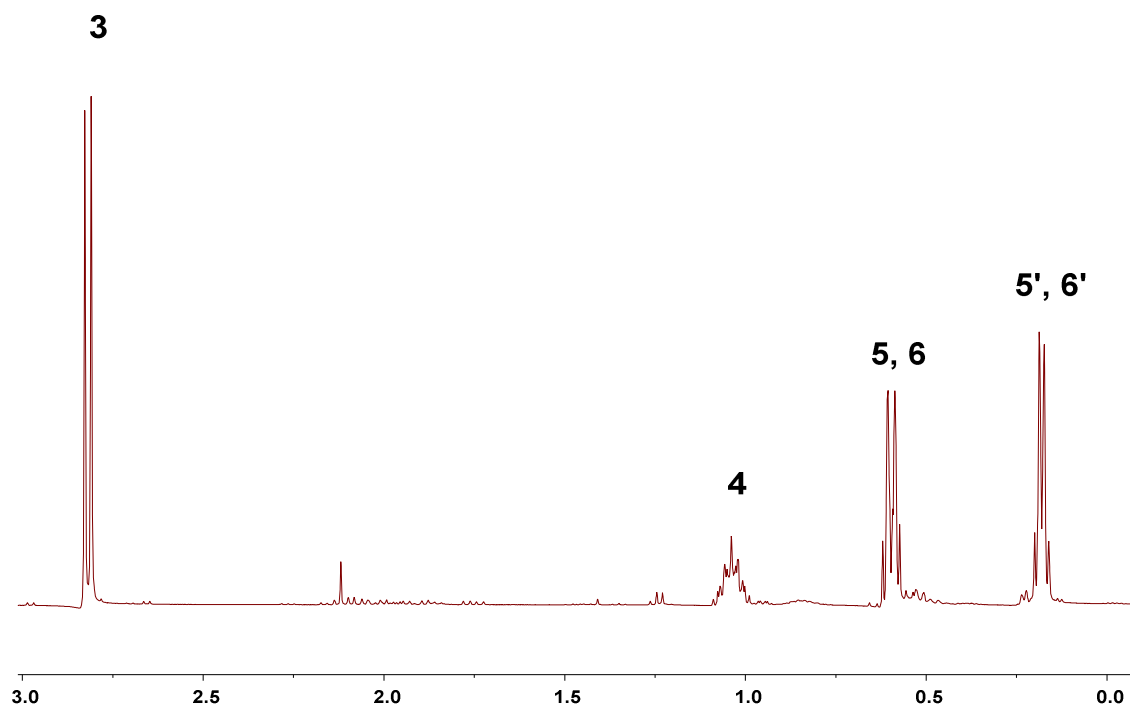
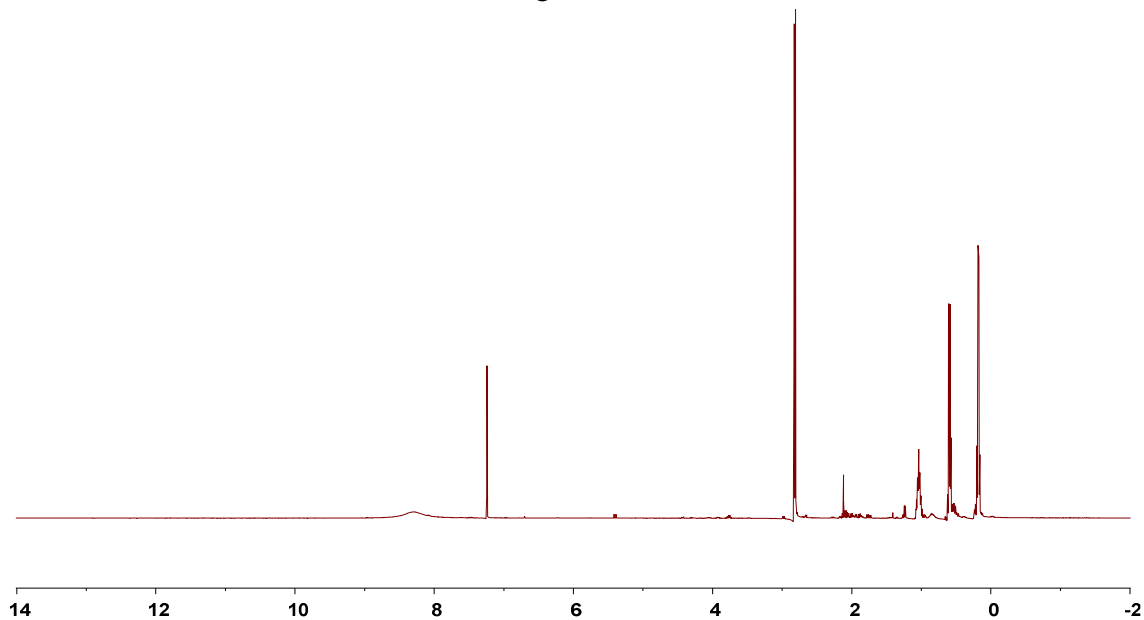
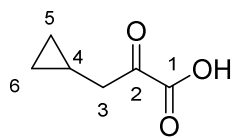


Figure S12 NMR spectra (CDCl_3) of **1i**: a) ^1H and b) ^{13}C .

a)



b)

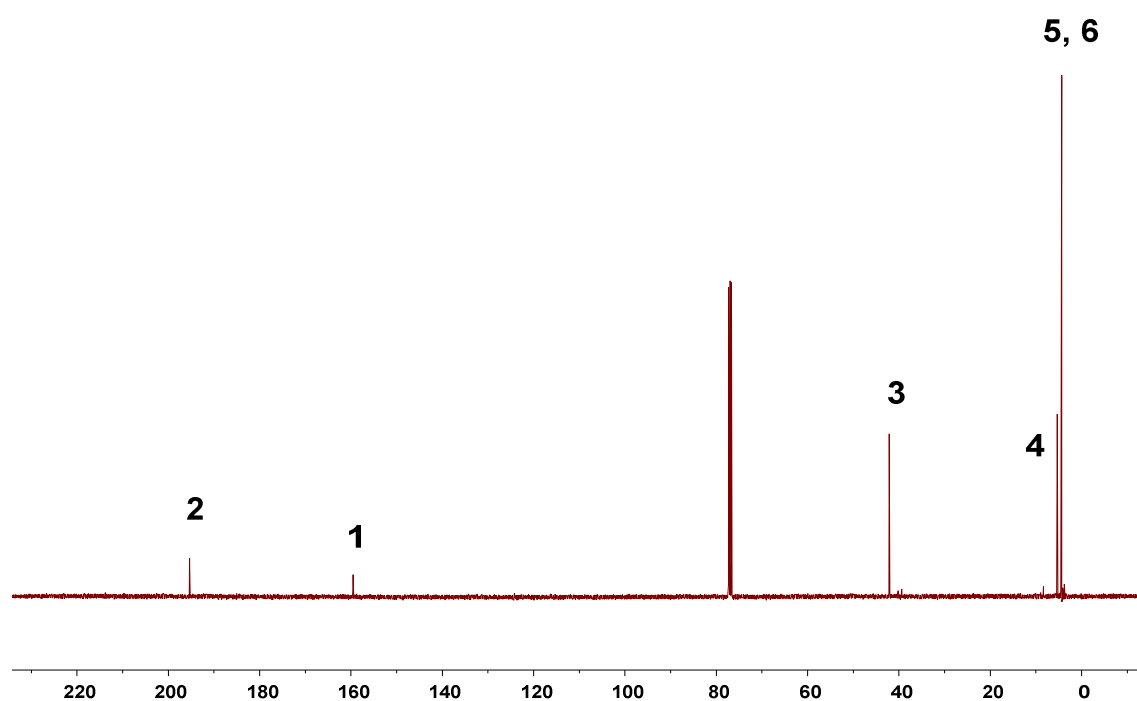
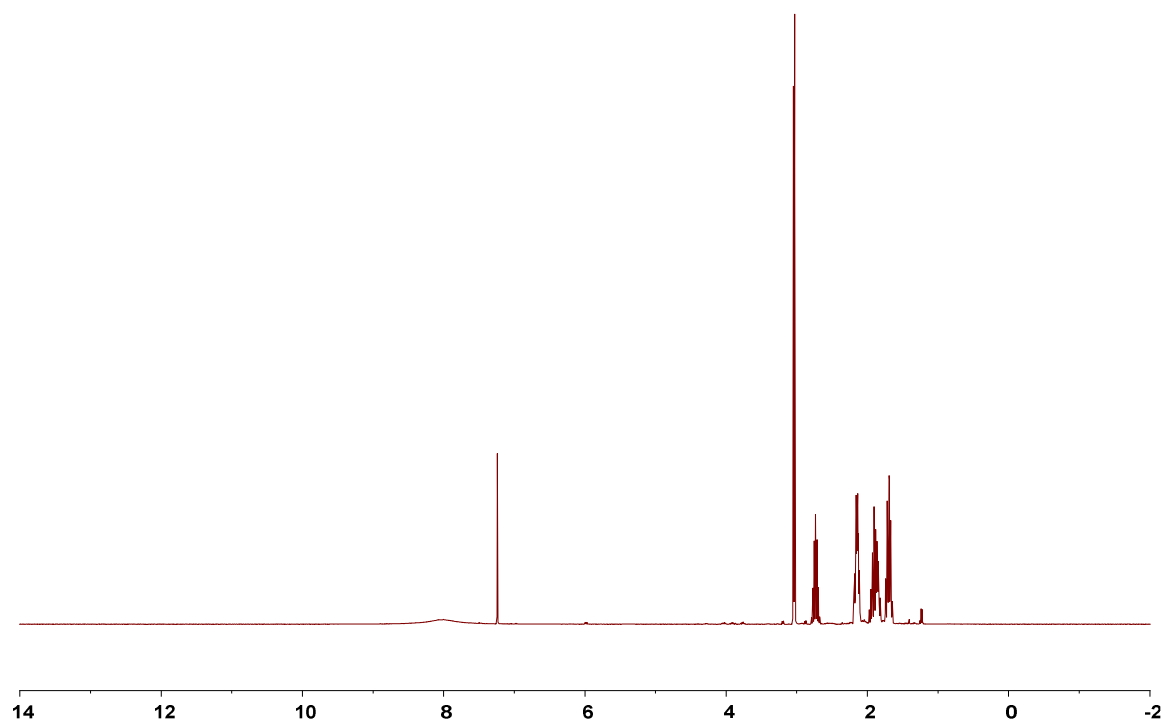
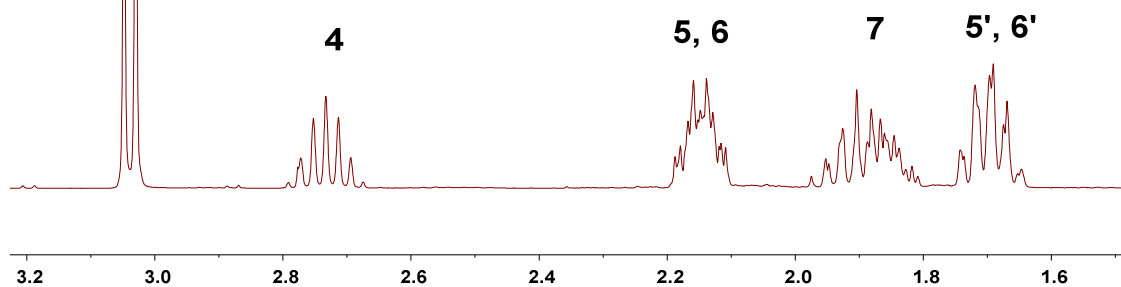
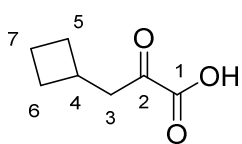


Figure S13 NMR spectra (CDCl_3) of **1k**: a) ^1H and b) ^{13}C .

a)



3



b)

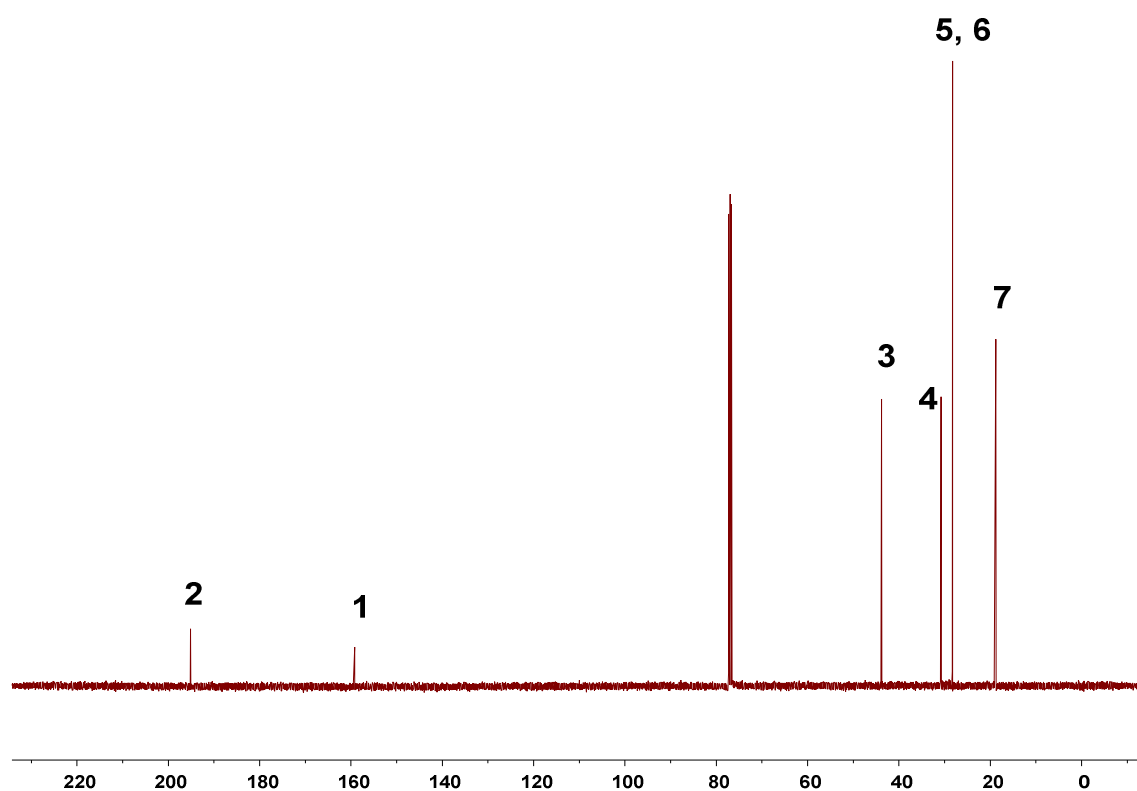
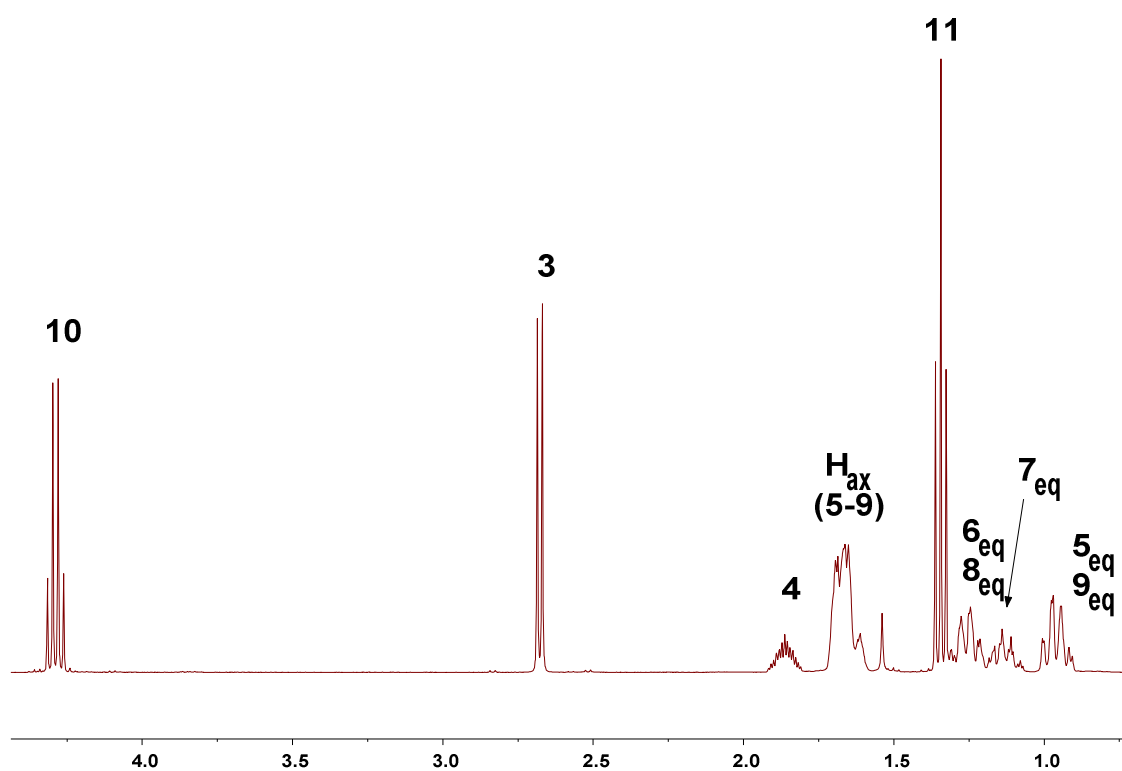
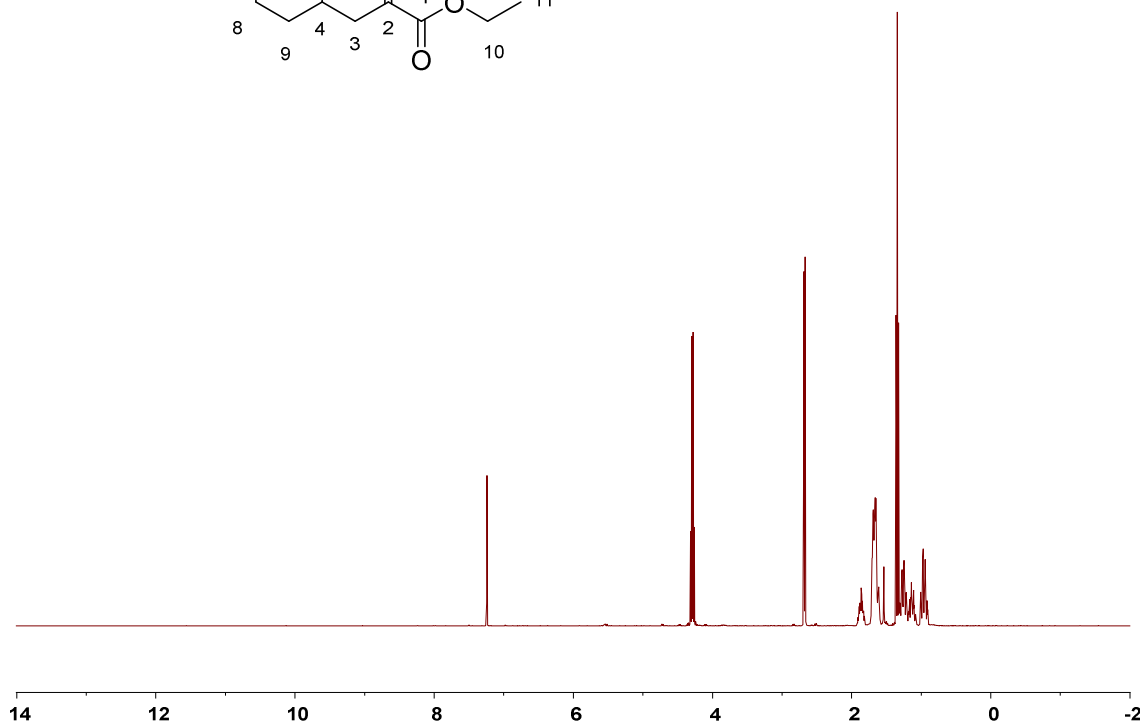
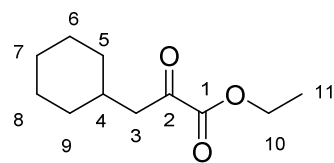


Figure S14 NMR spectra (CDCl_3) of **1l**: a) ^1H and b) ^{13}C .

a)



b)

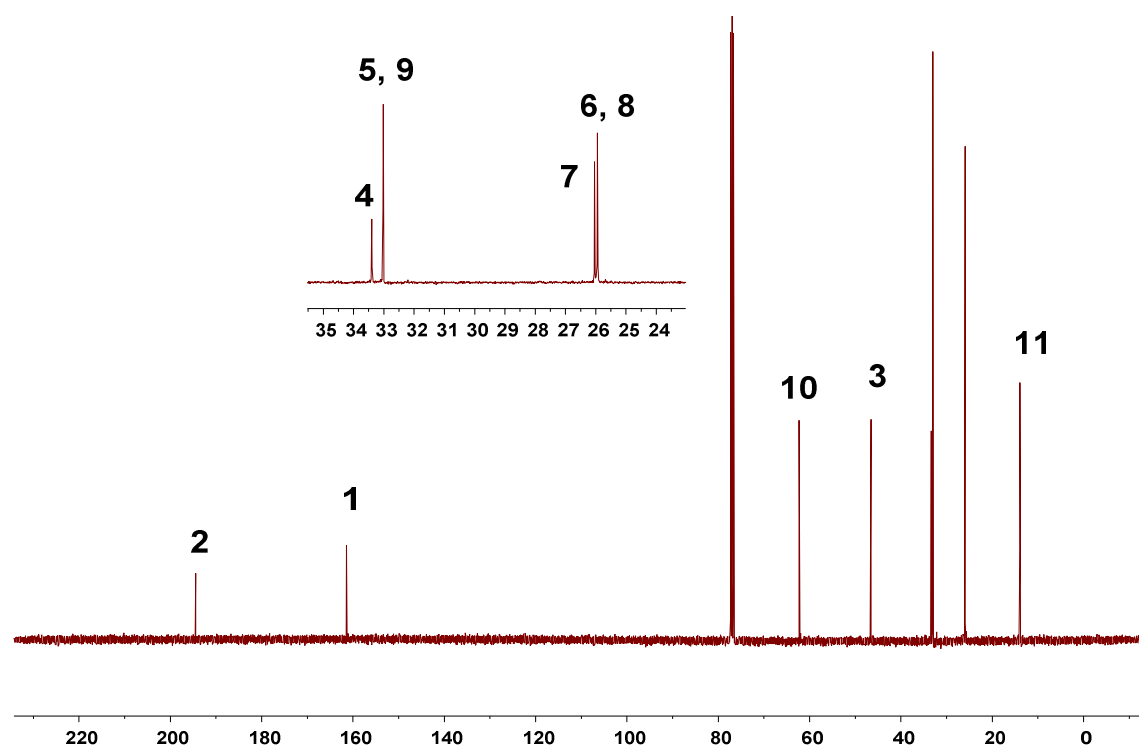
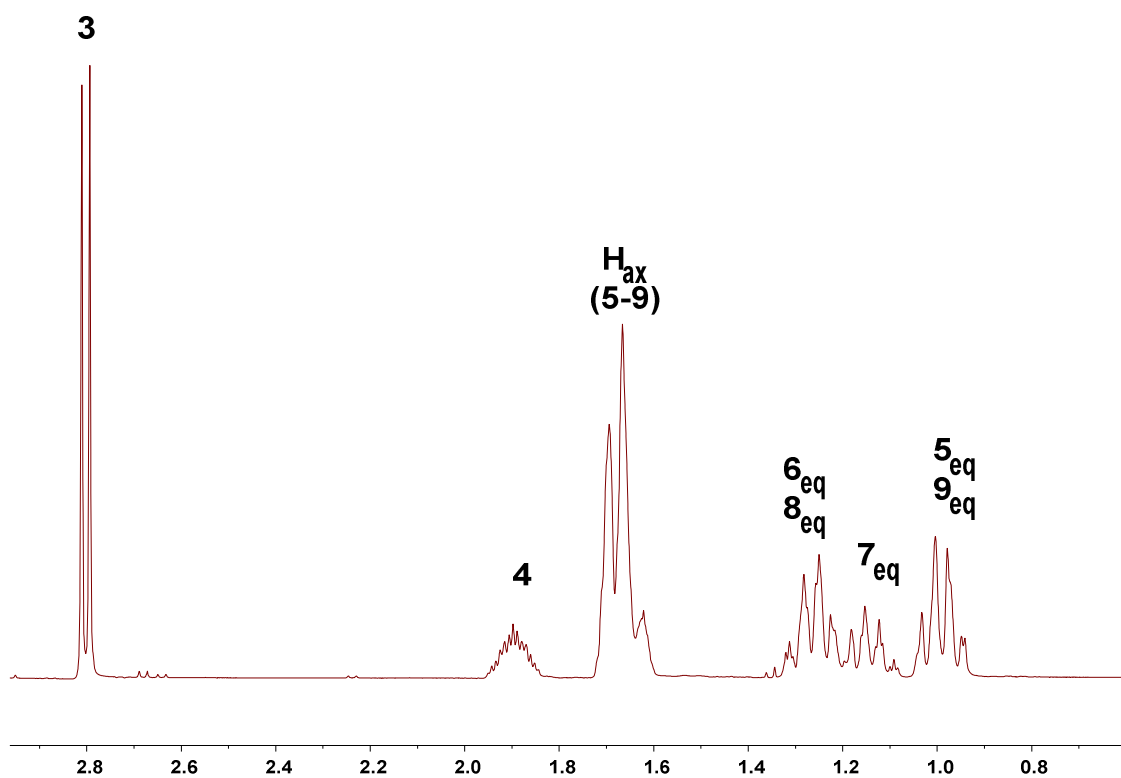
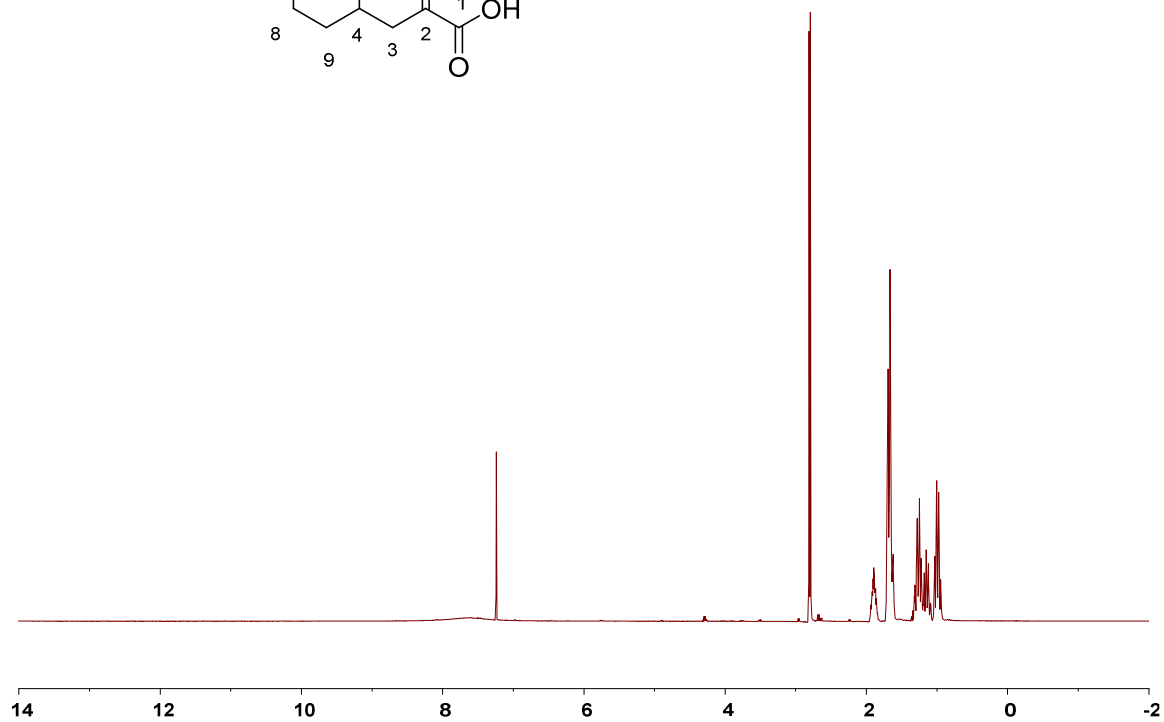
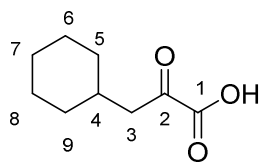


Figure S15 NMR spectra (CDCl_3) of **C2m**: a) ^1H and b) ^{13}C .

a)



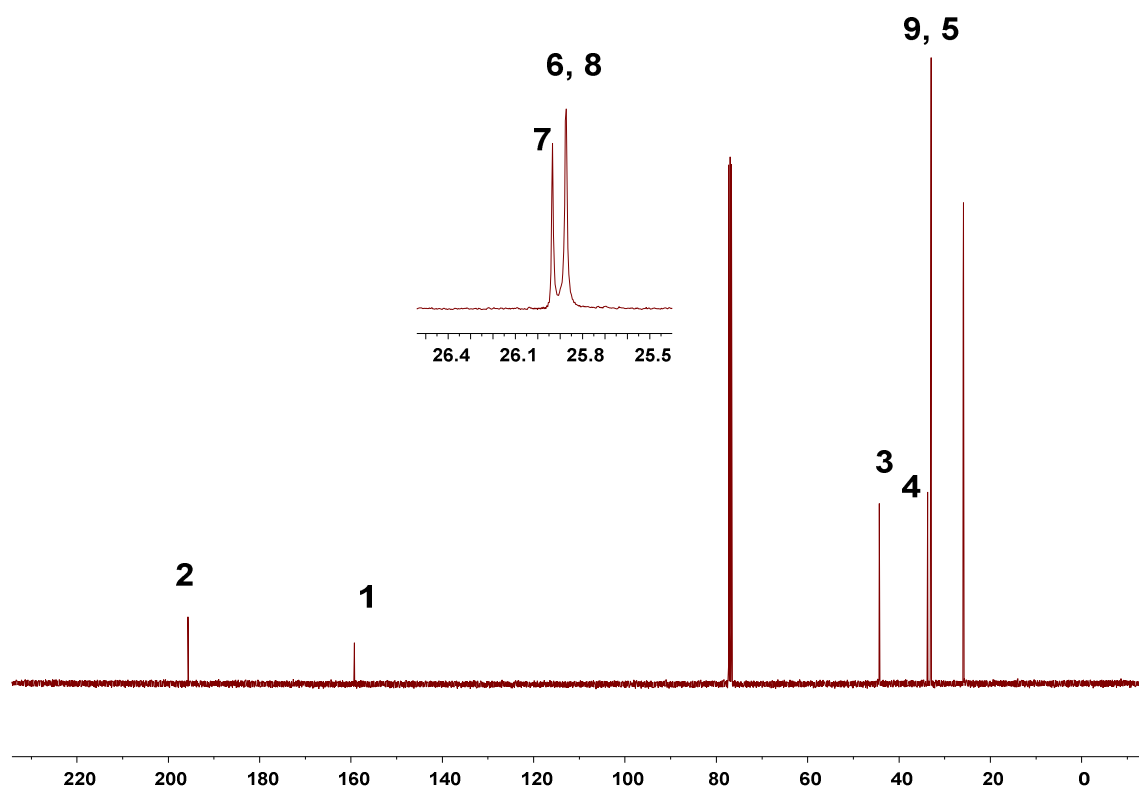


Figure S16 NMR spectra (CDCl_3) of **1m**: a) ^1H and b) ^{13}C .

6.2.2 Reaction monitoring for the synthesis of products S-4a-k

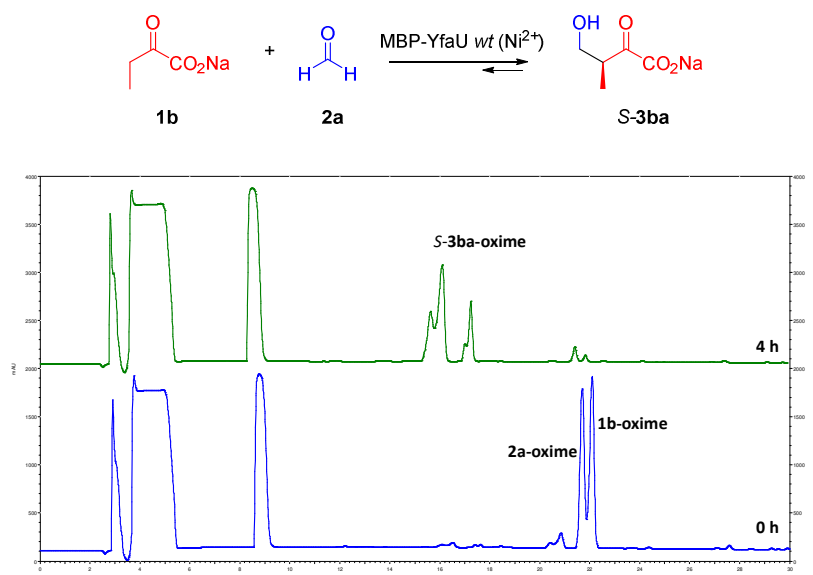
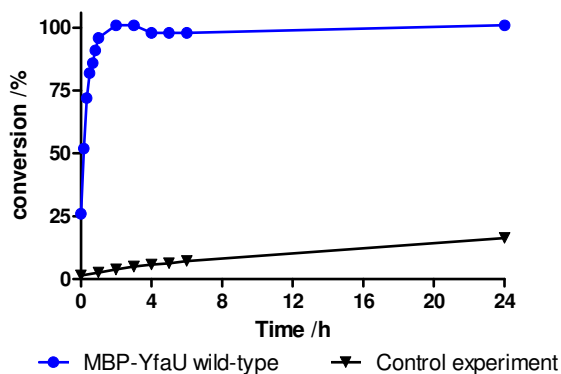


Figure S17 Aldol addition of sodium 2-oxobutanoate (**1b**) to formaldehyde (**2a**) catalyzed by MBP-YfaU wild-type (Ni^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 4 h (> 95% conversion).



MBP-YfaU	v_0^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	2.5	> 95
Control	$1.7 \cdot 10^{-2}$	16

^a $[\mathbf{1b}] = [\mathbf{2a}] = 100 \text{ mM}$, $[\text{enzyme}] = 4 \text{ mg mL}^{-1}$.

Figure S18 Reaction time course of aldol addition of sodium 2-oxobutanoate (**1a**) to formaldehyde (**2**) catalyzed by MBP-YfaU wild-type (Ni^{2+}) and control experiment performed under the same conditions without the enzyme.

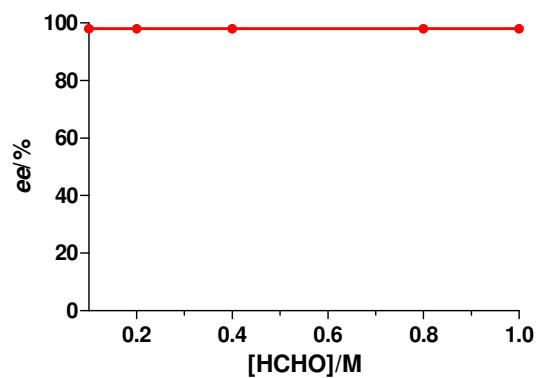


Figure S19 Effect of formaldehyde concentration on the enantiomeric excess of *S*-4a.

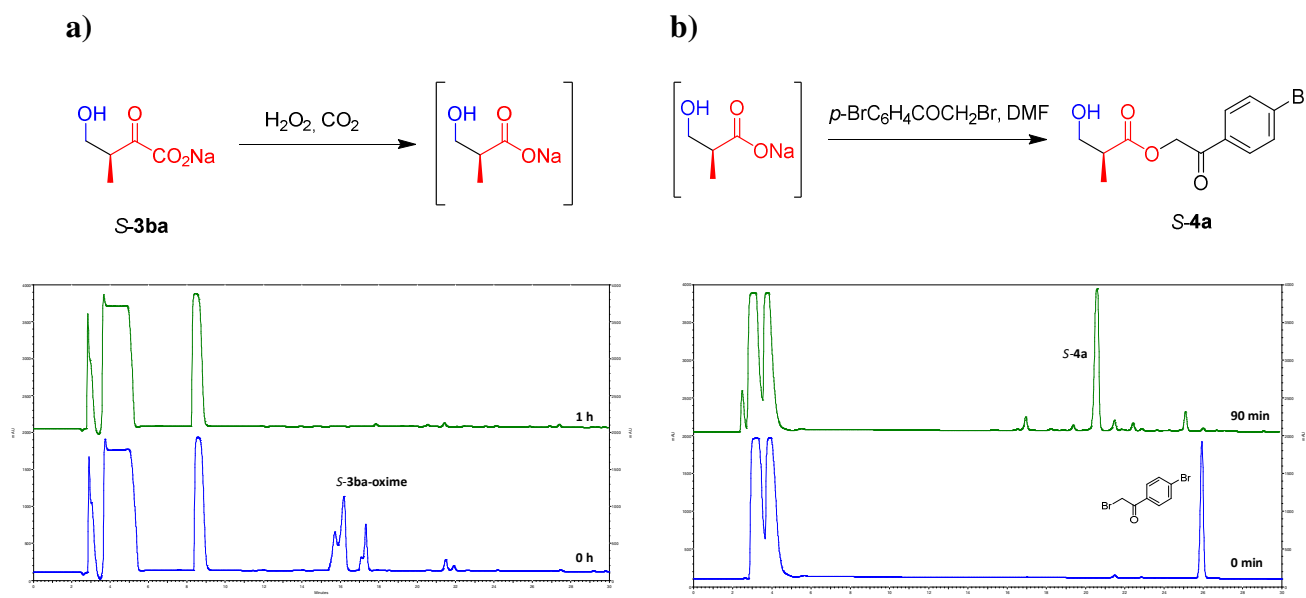


Figure S20 HPLC monitoring of oxidative decarboxylation of *S*-3ba (a) and synthesis of 4'-bromophenacyl ester *S*-4a (b).

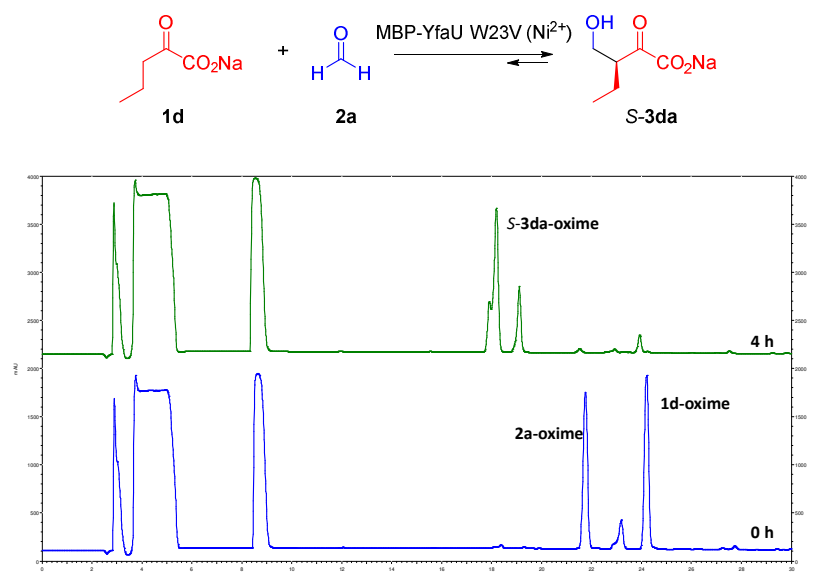
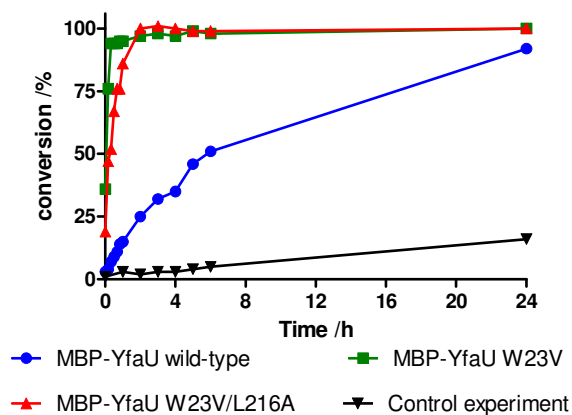


Figure S21 Aldol addition of sodium 2-oxopentanoate (**1d**) to formaldehyde (**2a**) catalyzed by MBP-YfaU W23V (Ni^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 4 h (> 95% conversion).



MBP-YfaU	v_0^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	0.1	92
W23V	6.9	>95
W23V/L216A	3.8	>95
Control	$1.2 \cdot 10^{-2}$	16

^a. $[\mathbf{1d}] = [\mathbf{2a}] = 100 \text{ mM}$, $[\text{enzyme}] = 4 \text{ mg mL}^{-1}$.

Figure S22 Reaction time course of aldol addition of sodium 2-oxopentanoate (**1d**) to formaldehyde (**2a**) catalyzed by MBP-YfaU (Ni^{2+}) wild-type, W23V and W23V/L216A variants. Control experiment was performed under the same conditions without the enzyme.

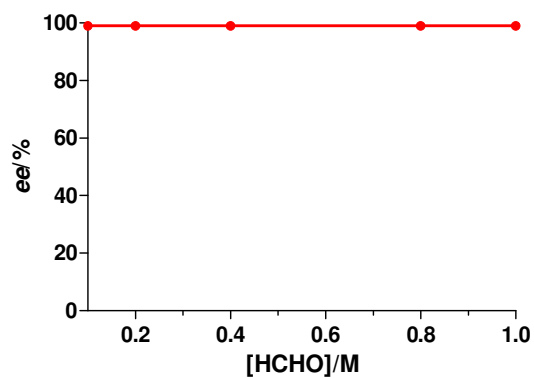


Figure S23 Effect of formaldehyde concentration on the enantiomeric excess of *S*-**4b**.

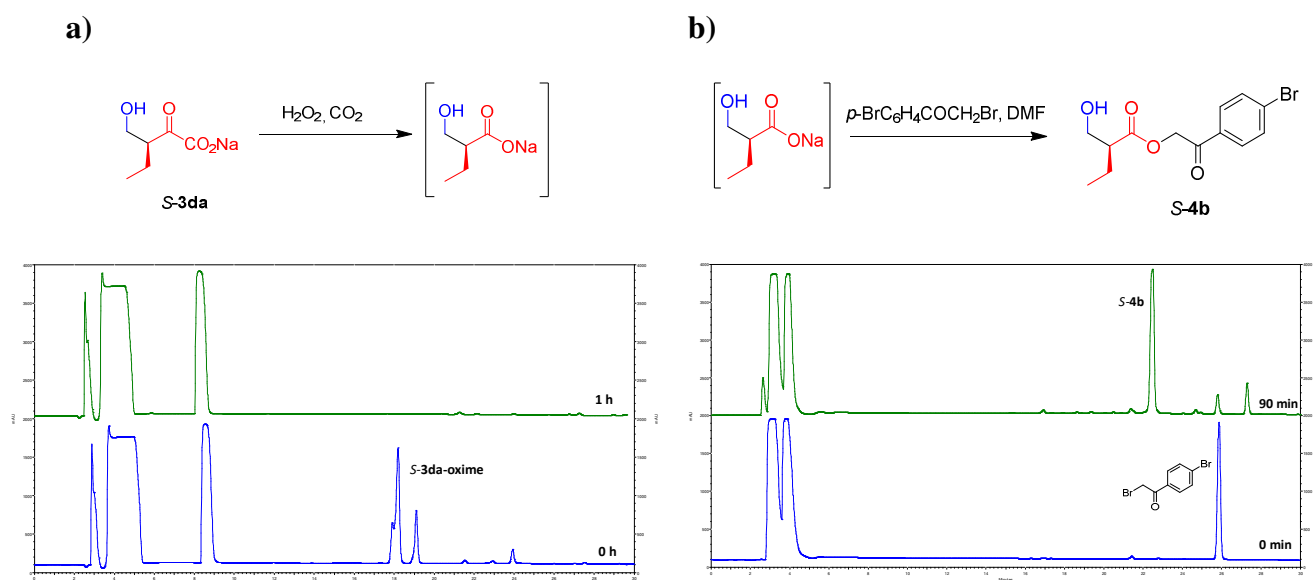


Figure S24 HPLC monitoring of oxidative decarboxylation of *S*-**3da** (a) and synthesis of 4'-bromophenacyl ester *S*-**4b** (b).

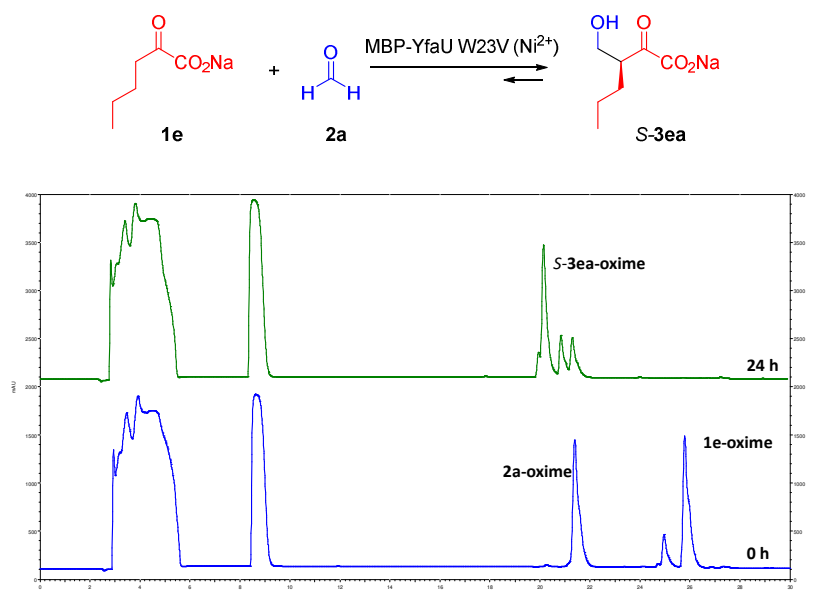
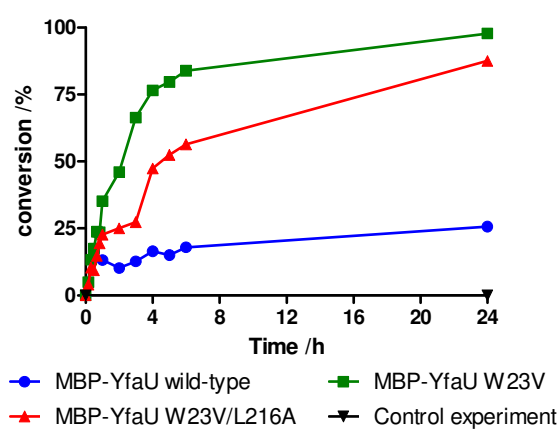


Figure S25 Aldol addition of sodium 2-oxohexanoate (**1e**) to formaldehyde (**2a**) catalyzed by MBP-YfaU W23V (Ni^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (> 95% conversion).



MBP-YfaU	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$1.3 \cdot 10^{-2}$	26
W23V	0.7	>95
W23V/L216A	0.5	88
Control	$_{-}^b$	$_{-}^b$

^a. [**1e**] = [**2a**] = 100 mM, [enzyme] = 4 mg mL^{-1} .

^b. Product not detected.

Figure S26 Reaction time course of aldol addition of sodium 2-oxohexanoate (**1e**) to formaldehyde (**2a**) catalyzed by MBP-YfaU (Ni^{2+}) wild-type, W23V and W23V/L216A variants. Control experiment was performed under the same conditions without the enzyme.

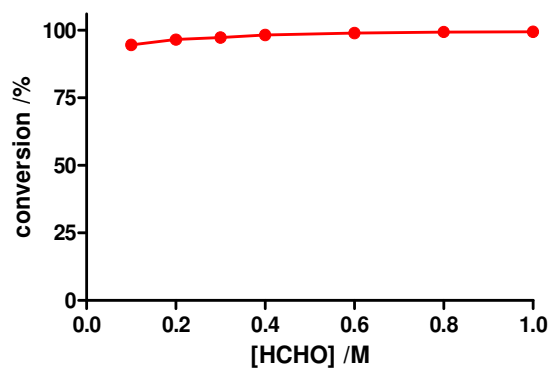


Figure S27 Effect of formaldehyde concentration on the yield of *S*-**3ea** in the aldol addition of **2a** to **1e** catalyzed by MBP-YfaU W23V (Ni²⁺).

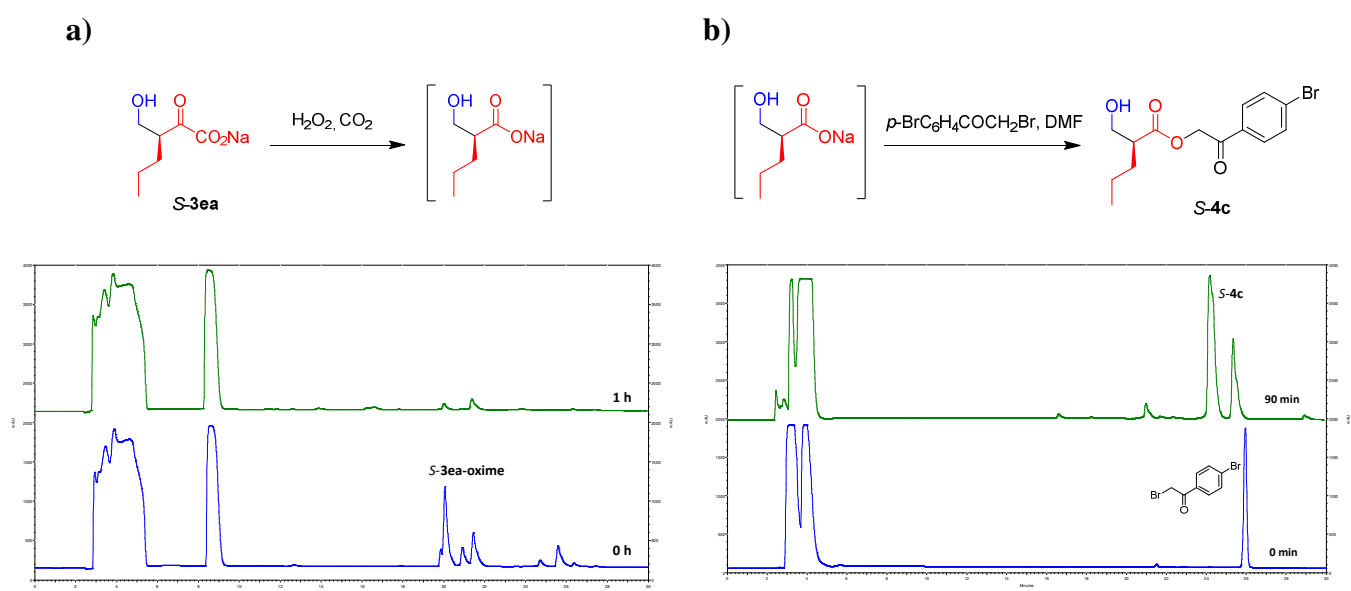


Figure S28 HPLC monitoring of oxidative decarboxylation of *S*-**3ea** (a) and synthesis of 4'-bromophenacyl ester *S*-**4c** (b).

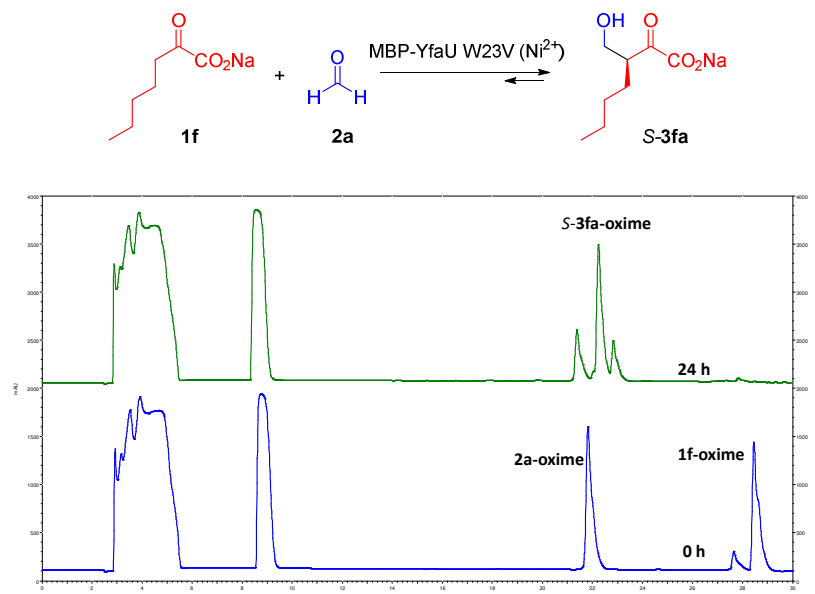
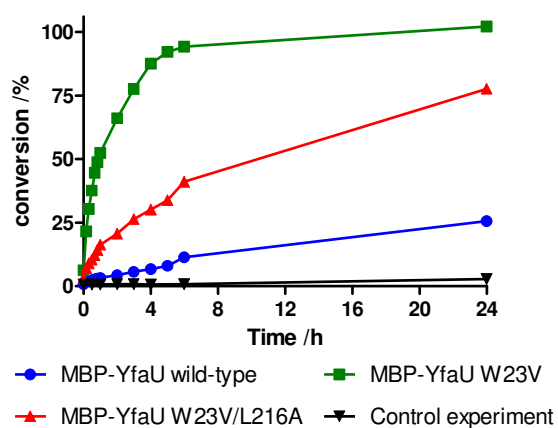


Figure S29 Aldol addition of sodium 2-oxoheptanoate (**1f**) to formaldehyde (**2a**) catalyzed by MBP-YfaU W23V (Ni^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (> 95% conversion).



MBP-YfaU	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$4,2 \cdot 10^{-2}$	26
W23V	1.1	>95
W23V/L216A	0.2	78
Control	$1,8 \cdot 10^{-3}$	<5

^a. $[\mathbf{1f}] = [\mathbf{2a}] = 100 \text{ mM}$, $[\text{enzyme}] = 4 \text{ mg mL}^{-1}$.

Figure S30 Reaction time course of aldol addition of sodium 2-oxoheptanoate (**1f**) to formaldehyde (**2a**) catalyzed by MBP-YfaU (Ni^{2+}) wild-type, W23V and W23V/L216A variants. Control experiment was performed under the same conditions without the enzyme.

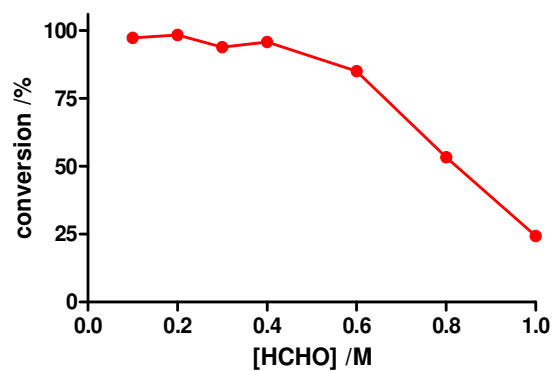


Figure S31 Effect of formaldehyde concentration on the yield of *S*-**3fa** in the aldol addition of **2a** to **1f** catalyzed by MBP-YfaU W23V (Ni²⁺).

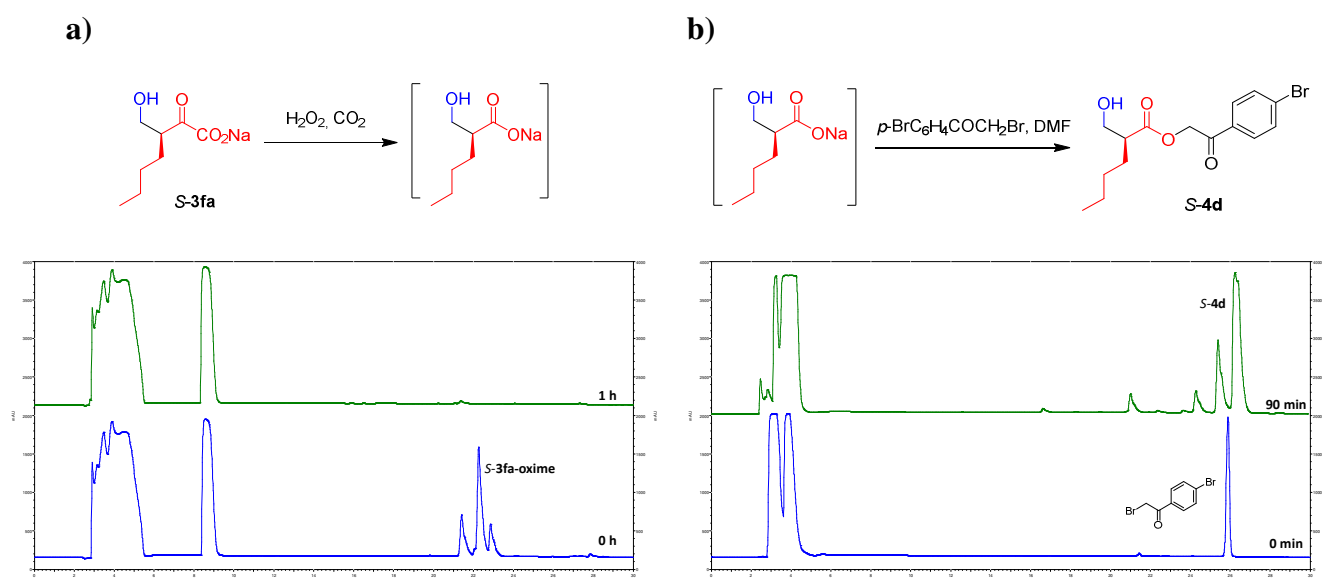


Figure S32 HPLC monitoring of oxidative decarboxylation of *S*-**3fa** (a) and synthesis of 4'-bromophenacyl ester *S*-**4d** (b).

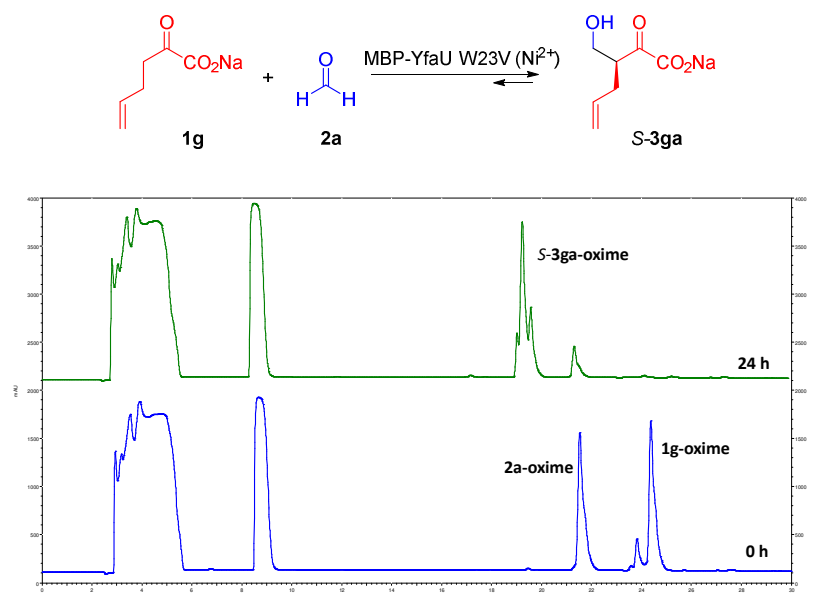
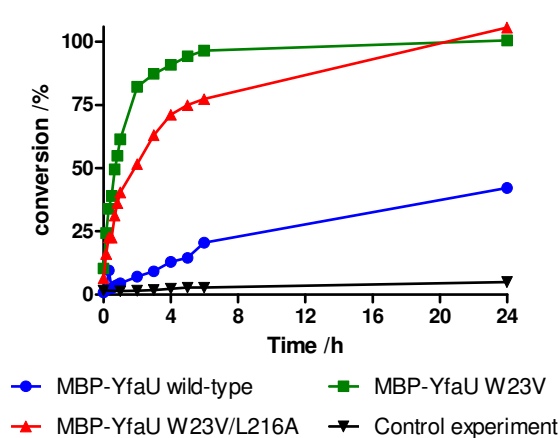


Figure S33 Aldol addition of sodium 2-oxohex-5-enoate (**1g**) to formaldehyde (**2a**) catalyzed by MBP-YfaU W23V (Ni^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (> 95% conversion).



MBP-YfaU	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$6.7 \cdot 10^{-2}$	42
W23V	1.1	>95
W23V/L216A	0.7	>95
Control	$3.4 \cdot 10^{-3}$	5

^a. [**1g**] = [**2a**] = 100 mM, [enzyme] = 4 mg mL^{-1} .

Figure S34 Reaction time course of aldol addition of sodium 2-oxohex-5-enoate (**1g**) to formaldehyde (**2a**) catalyzed by MBP-YfaU (Ni^{2+}) wild-type, W23V and W23V/L216A variants. Control experiment was performed under the same conditions without the enzyme.

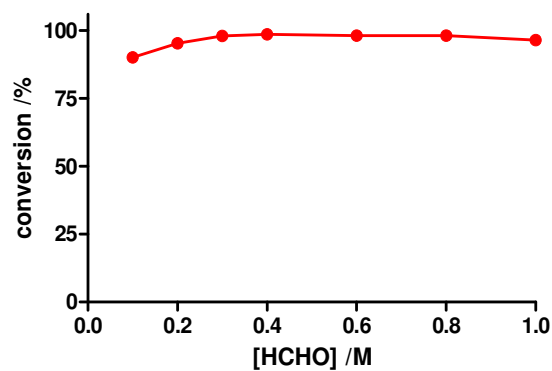


Figure S35 Effect of formaldehyde concentration on the yield of *S*-**3ga** in the aldol addition of **2a** to **1g** catalyzed by MBP-YfaU W23V (Ni²⁺).

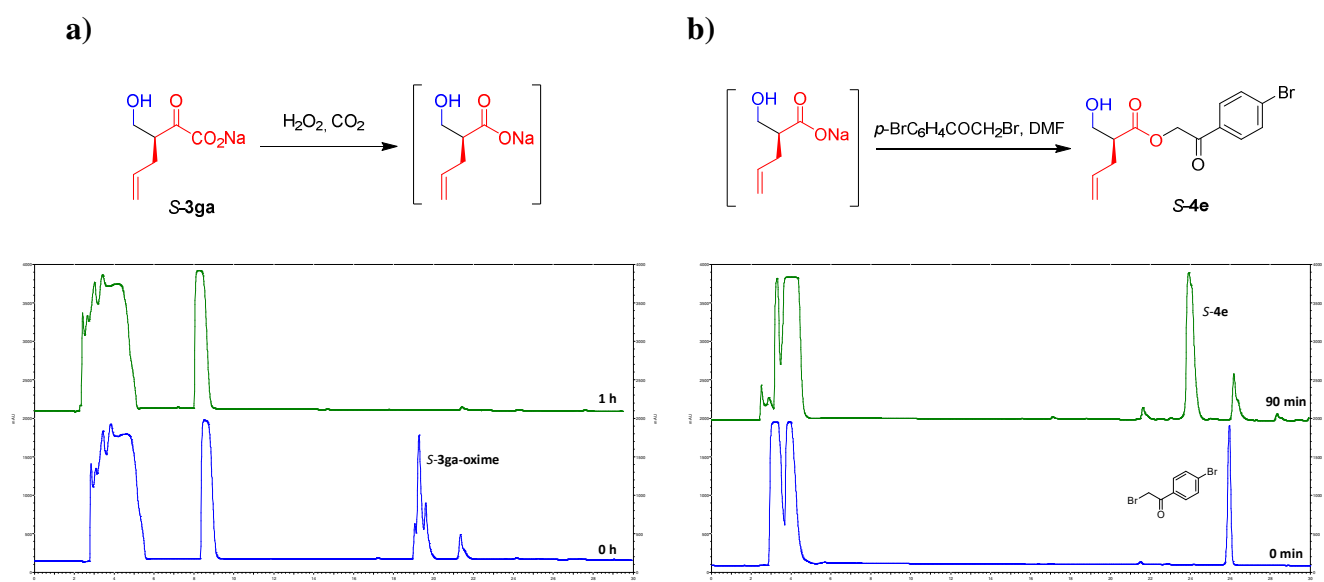


Figure S36 HPLC monitoring of oxidative decarboxylation of *S*-**3ga** (a) and synthesis of 4'-bromophenacyl ester *S*-**4e** (b).

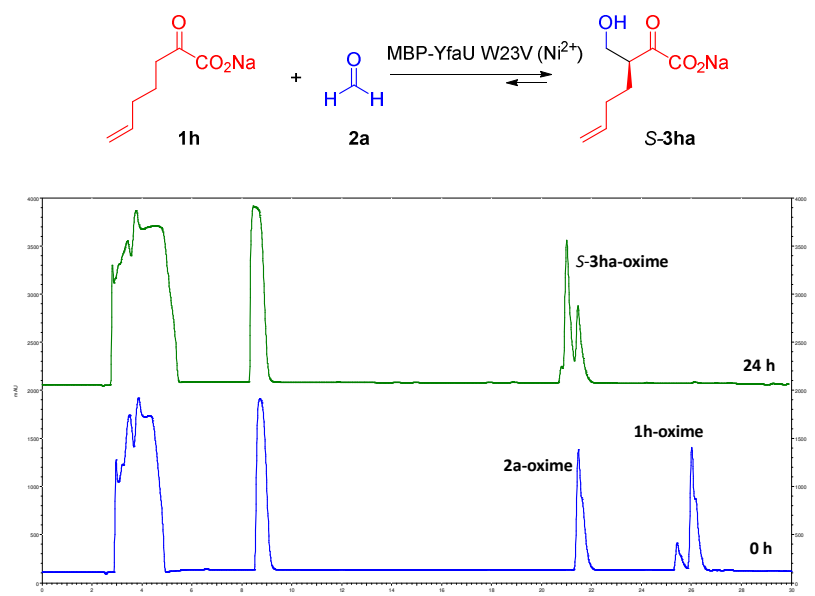
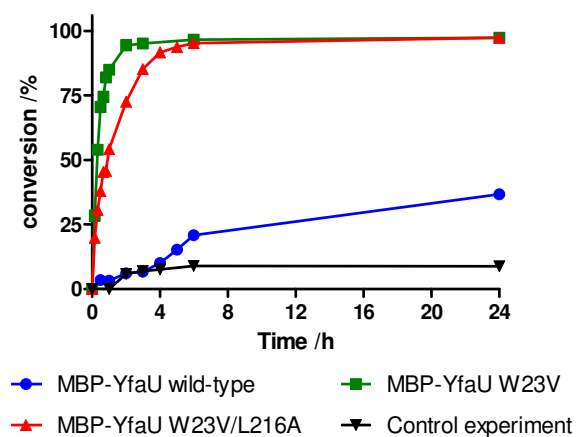


Figure S37 Aldol addition of sodium 2-oxohept-6-enoate (**1h**) to formaldehyde (**2a**) catalyzed by MBP-YfaU W23V (Ni^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (> 95% conversion).



MBP-YfaU	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$5.8 \cdot 10^{-2}$	37
W23V	2.3	>95
W23V/L216A	1.5	>95
Control	$4.9 \cdot 10^{-2}$	9

^a. $[\mathbf{1h}] = [\mathbf{2a}] = 100 \text{ mM}$, $[\text{enzyme}] = 4 \text{ mg mL}^{-1}$.

Figure S38 Reaction time course of aldol addition of sodium 2-oxohept-6-enoate (**1h**) to formaldehyde (**2a**) catalyzed by MBP-YfaU (Ni^{2+}) wild-type, W23V and W23V/L216A variants. Control experiment was performed under the same conditions without the enzyme.

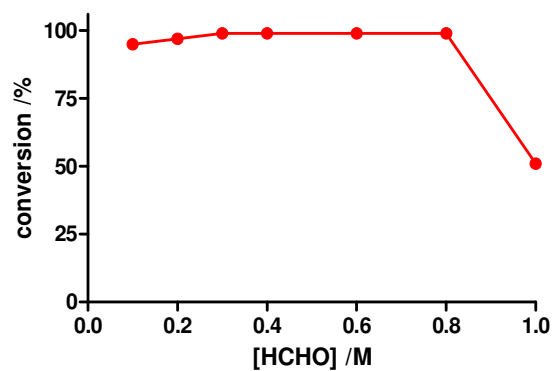


Figure S39 Effect of formaldehyde concentration on the yield of *S*-**3ha** in the aldol addition of **2a** to **1h** catalyzed by MBP-YfaU W23V (Ni²⁺).

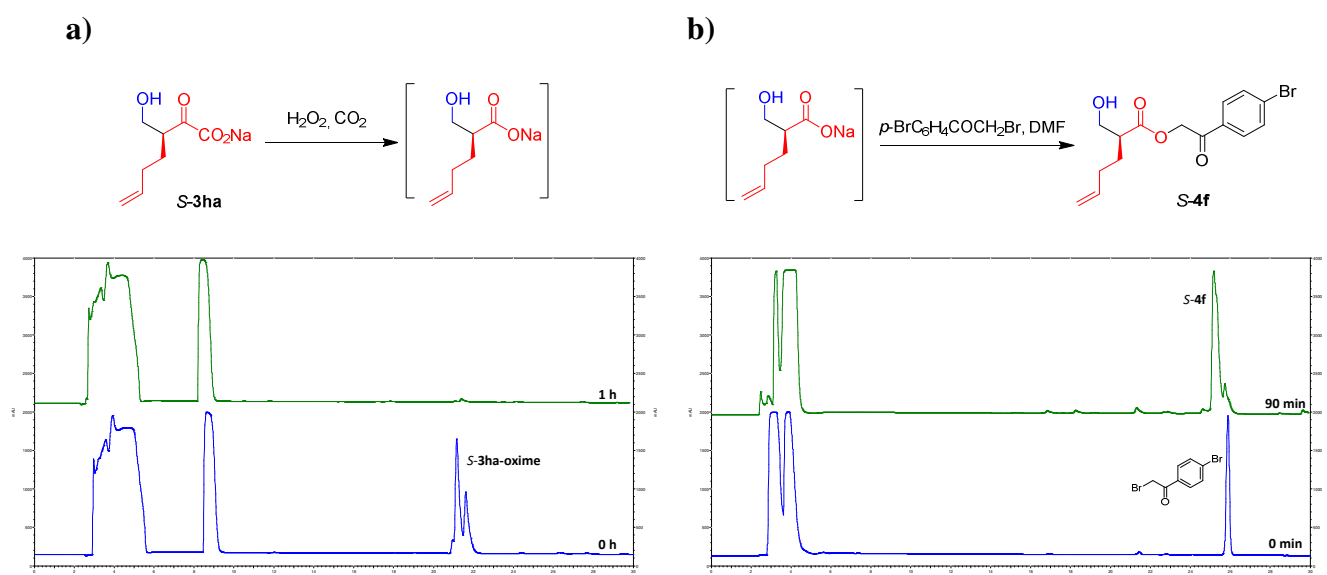


Figure S40 HPLC monitoring of oxidative decarboxylation of *S*-**3ha** (a) and synthesis of 4'-bromophenacyl ester *S*-**4f** (b).

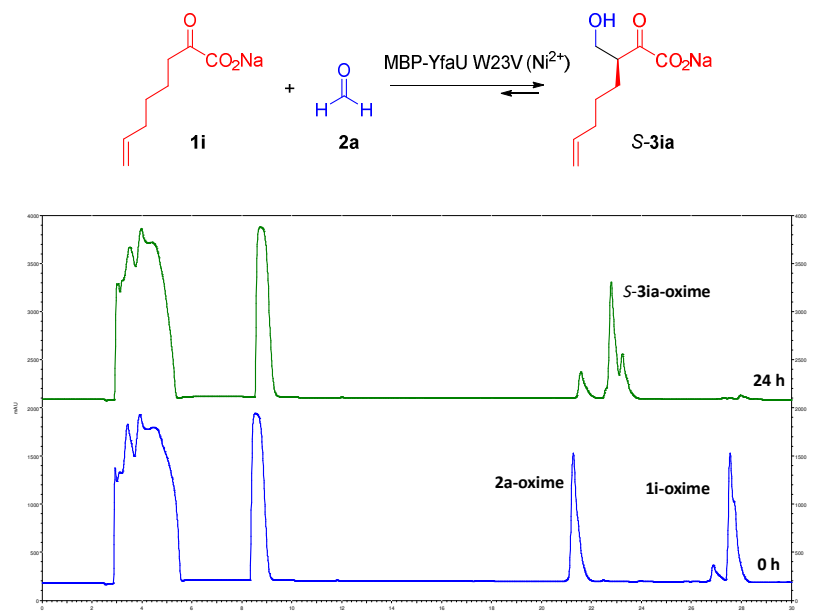
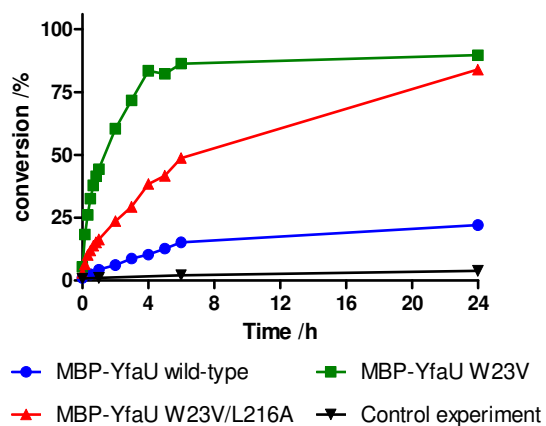


Figure S41 Aldol addition of sodium 2-oxooct-7-enoate (**1i**) to formaldehyde (**2a**) catalyzed by MBP-YfaU W23V (Ni^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (> 95% conversion).



MBP-YfaU	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$4.9 \cdot 10^{-2}$	22
W23V	1.1	90
W23V/L216A	0.4	84
Control	$5.6 \cdot 10^{-3}$	<5

^a. $[\mathbf{1i}] = [\mathbf{2a}] = 100 \text{ mM}$, $[\text{enzyme}] = 4 \text{ mg mL}^{-1}$.

Figure S42 Reaction time course of aldol addition of sodium 2-oxooct-7-enoate (**1i**) to formaldehyde (**2a**) catalyzed by MBP-YfaU (Ni^{2+}) wild-type, W23V and W23V/L216A variants. Control experiment was performed under the same conditions without the enzyme.

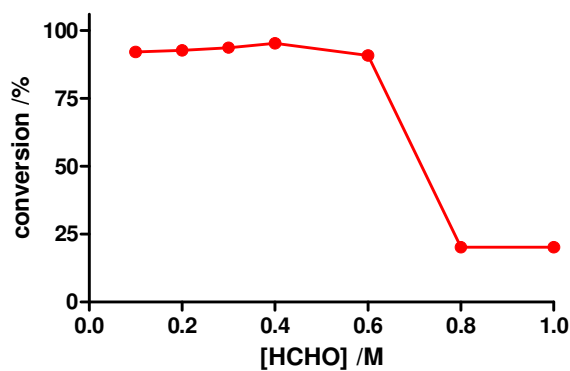


Figure S43 Effect of formaldehyde concentration on the yield of *S*-**3ia** in the aldol addition of **2a** to **1i** catalyzed by MBP-YfaU W23V (Ni²⁺).

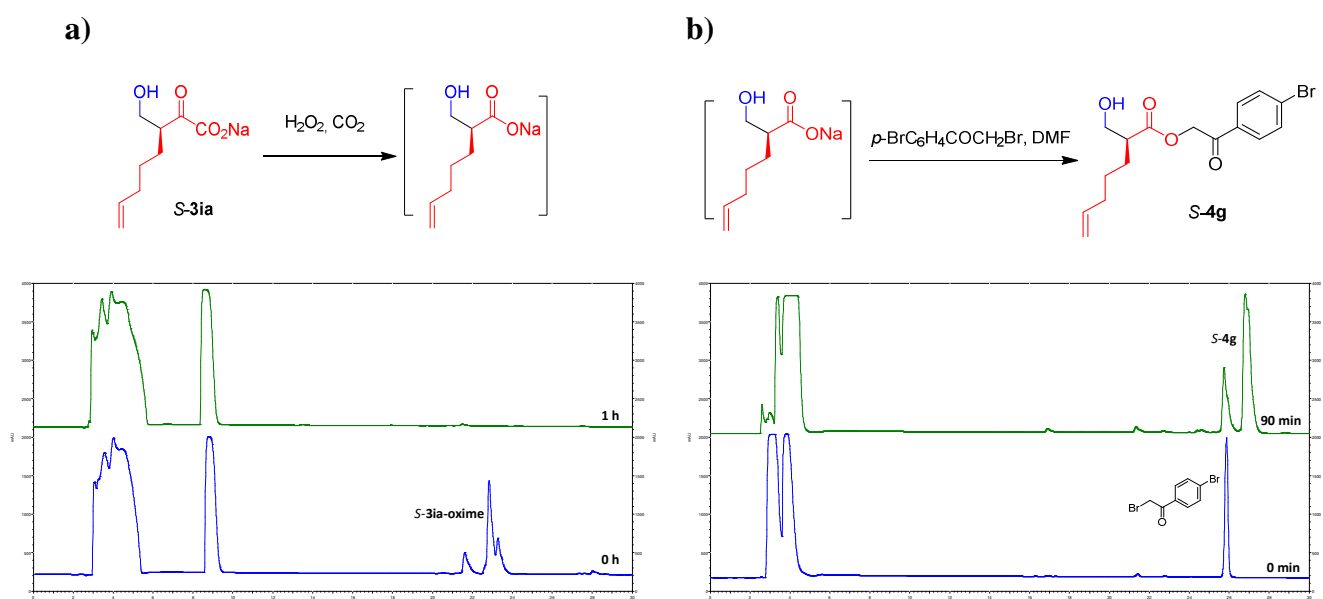


Figure S44 HPLC monitoring of oxidative decarboxylation of *S*-**3ia** (a) and synthesis of 4'-bromophenacyl ester *S*-**4g** (b).

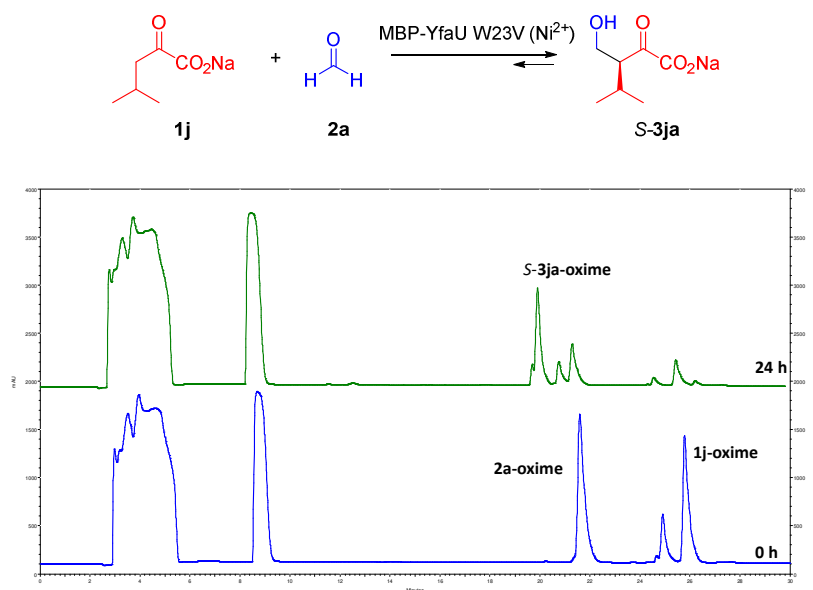
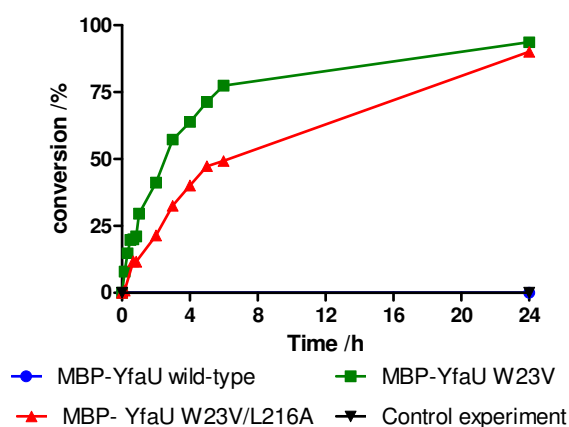


Figure S45 Aldol addition of sodium 4-methyl-2-oxopentanoate (**1j**) to formaldehyde (**2a**) catalyzed by MBP-YfaU W23V (Ni^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (> 95% conversion).



MBP-YfaU	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$_{-b}$	$_{-b}$
W23V	0.9	93
W23V/L216A	0.2	90
Control	$_{-b}$	$_{-b}$

^a. $[\mathbf{1j}] = [\mathbf{2a}] = 100 \text{ mM}$, $[\text{enzyme}] = 4 \text{ mg mL}^{-1}$.

^b. Product not detected.

Figure S46 Reaction time course of aldol addition of sodium 4-methyl-2-oxopentanoate (**1j**) to formaldehyde (**2a**) catalyzed by MBP-YfaU (Ni^{2+}) wild-type, W23V and W23V/L216A variants. Control experiment was performed under the same conditions without the enzyme.

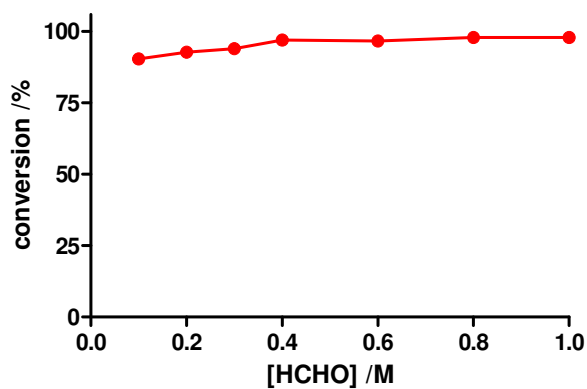


Figure S47 Effect of formaldehyde concentration on the yield of *S*-**3ja** in the aldol addition of **2a** to **1j** catalyzed by MBP-YfaU W23V (Ni²⁺).

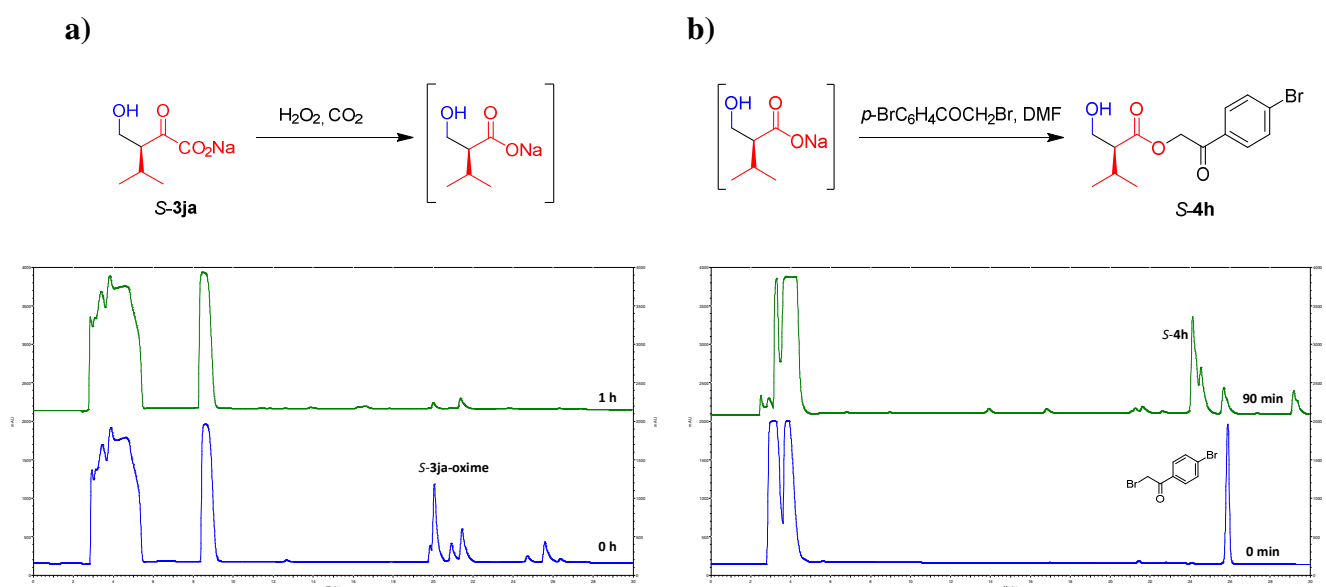


Figure S48 HPLC monitoring of oxidative decarboxylation of *S*-**3ja** (a) and synthesis of 4'-bromophenacyl ester *S*-**4h** (b).

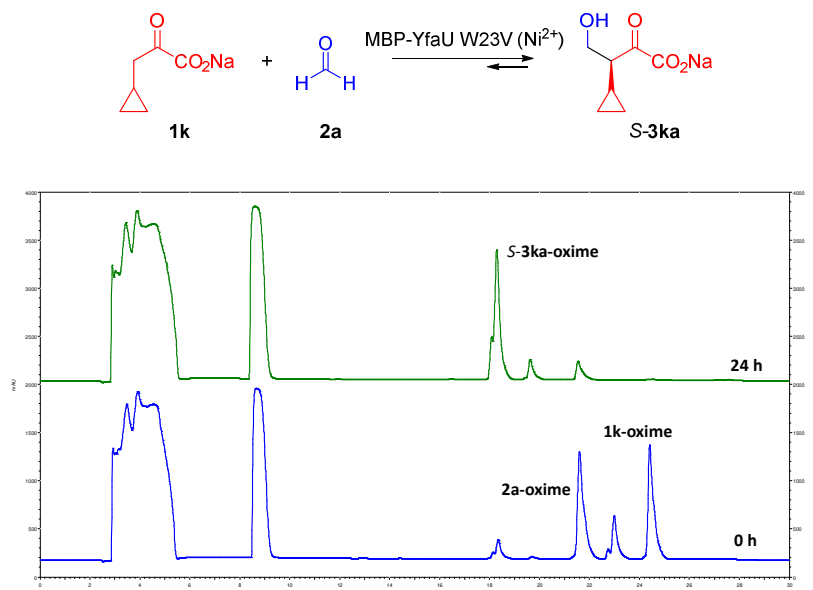
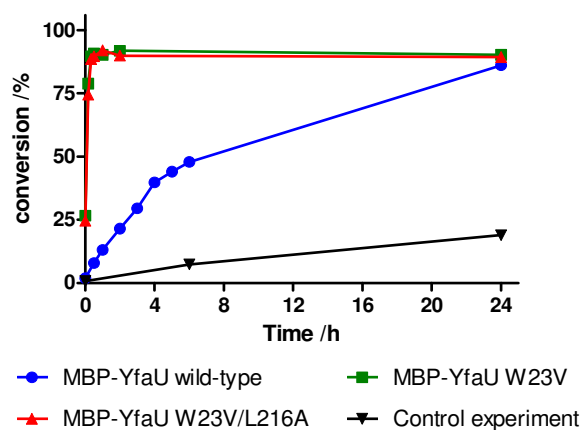


Figure S49 Aldol addition of sodium 3-cyclopropyl-2-oxopropanoate (**1k**) to formaldehyde (**2a**) catalyzed by MBP-YfaU W23V (Ni²⁺). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (> 95% conversion).



MBP-YfaU	v_o^a (mM·min ⁻¹)	Conv 24h (%)
wild-type	0.2	86
W23V	12.2	>95
W23V/L216A	9.1	>95
Control	$1.5 \cdot 10^{-2}$	19

^a. [1k] = [2a] = 100 mM, [enzyme] = 4 mg mL⁻¹.

Figure S50 Reaction time course of aldol addition of sodium 3-cyclopropyl-2-oxopropanoate (**1k**) to formaldehyde (**2a**) catalyzed by MBP-YfaU (Ni²⁺) wild-type, W23V and W23V/L216A variants. Control experiment was performed under the same conditions without the enzyme.

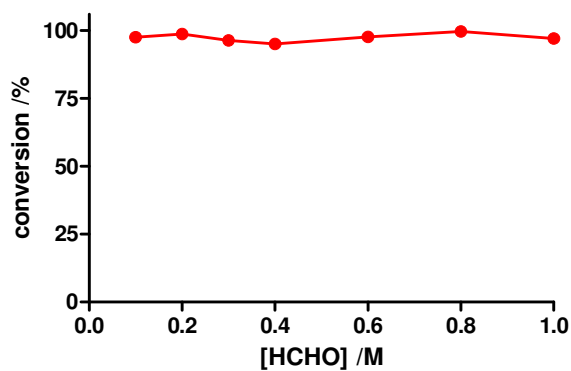


Figure S51 Effect of formaldehyde concentration on the yield of *S*-**3ka** in the aldol addition of **2a** to **1k** catalyzed by MBP-YfaU W23V (Ni²⁺).

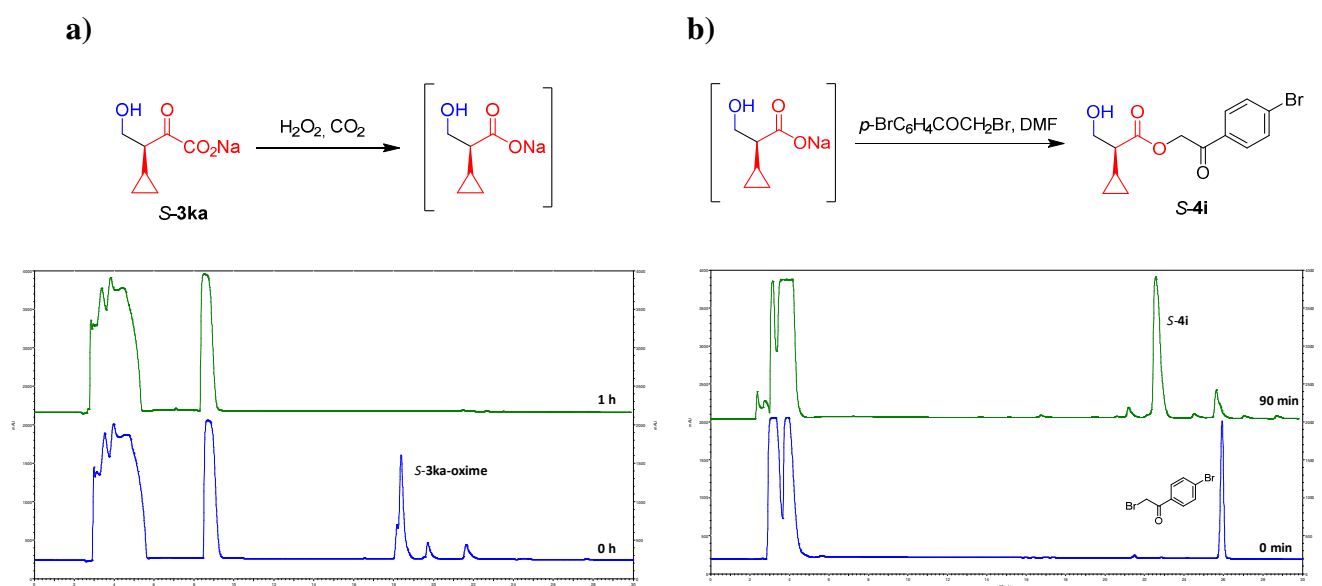


Figure S52 HPLC monitoring of oxidative decarboxylation of *S*-**3ka** (a) and synthesis of 4'-bromophenacyl ester *S*-**4i** (b).

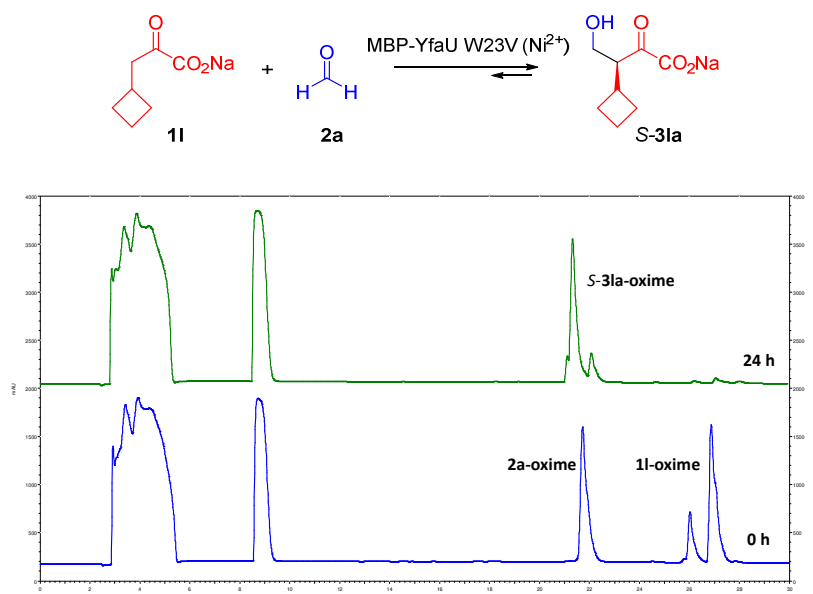
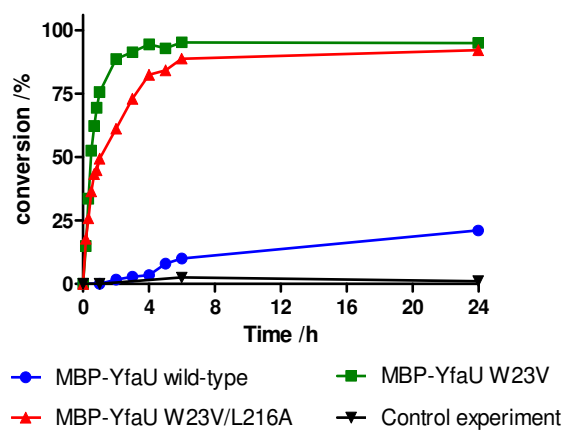


Figure S53 Aldol addition of sodium 3-cyclobutyl-2-oxopropanoate (**1I**) to formaldehyde (**2a**) catalyzed by MBP-YfaU W23V (Ni^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (> 95% conversion).



MBP-YfaU	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$2.0 \cdot 10^{-2}$	21
W23V	2.1	>95
W23V/L216A	1.5	92
Control	$_b$	$_b$

^a. $[\mathbf{1I}] = [\mathbf{2a}] = 100 \text{ mM}$, $[\text{enzyme}] = 4 \text{ mg mL}^{-1}$.

^b. Product not detected.

Figure S54 Reaction time course of aldol addition of sodium 3-cyclobutyl-2-oxopropanoate (**1I**) to formaldehyde (**2a**) catalyzed by MBP-YfaU (Ni^{2+}) wild-type, W23V and W23V/L216A variants. Control experiment was performed under the same conditions without the enzyme.

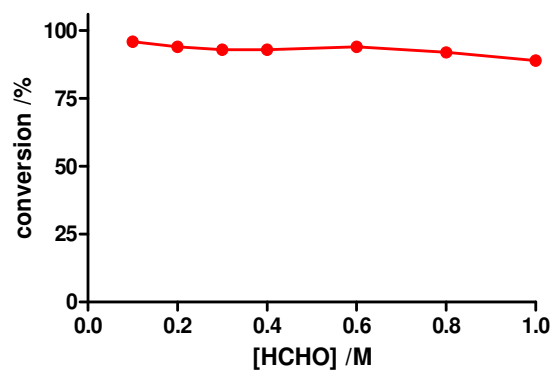


Figure S55 Effect of formaldehyde concentration on the yield of *S*-**3la** in the aldol addition of **2a** to **1l** catalyzed by MBP-YfaU W23V (Ni²⁺).

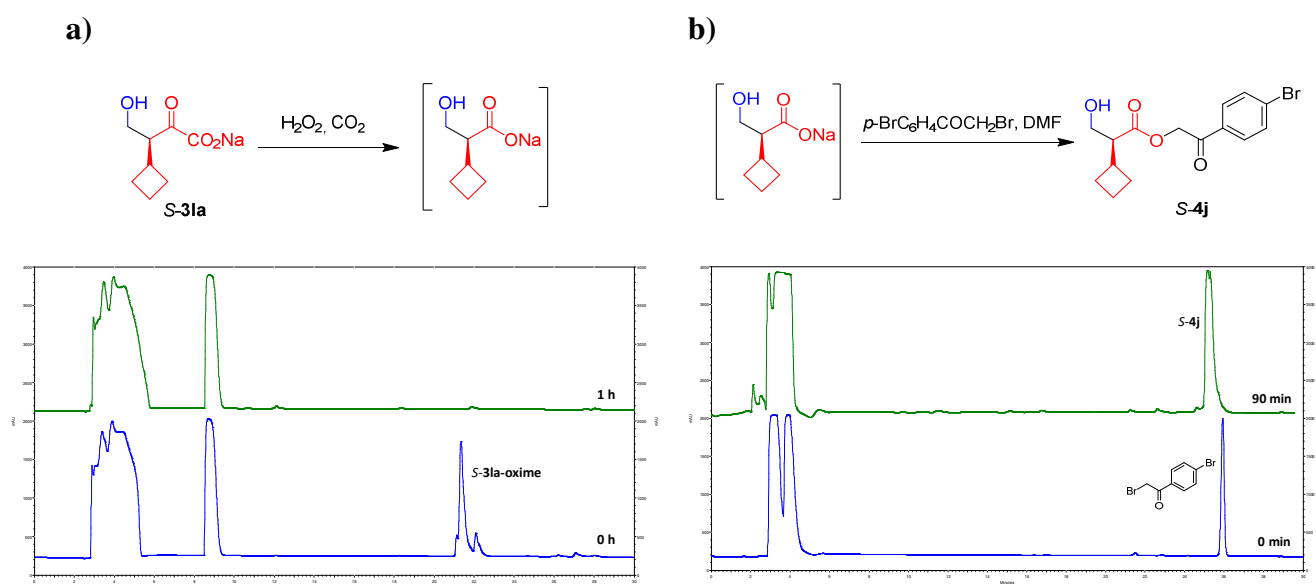


Figure S56 HPLC monitoring of oxidative decarboxylation of *S*-**3la** (a) and synthesis of 4'-bromophenacyl ester *S*-**4j** (b).

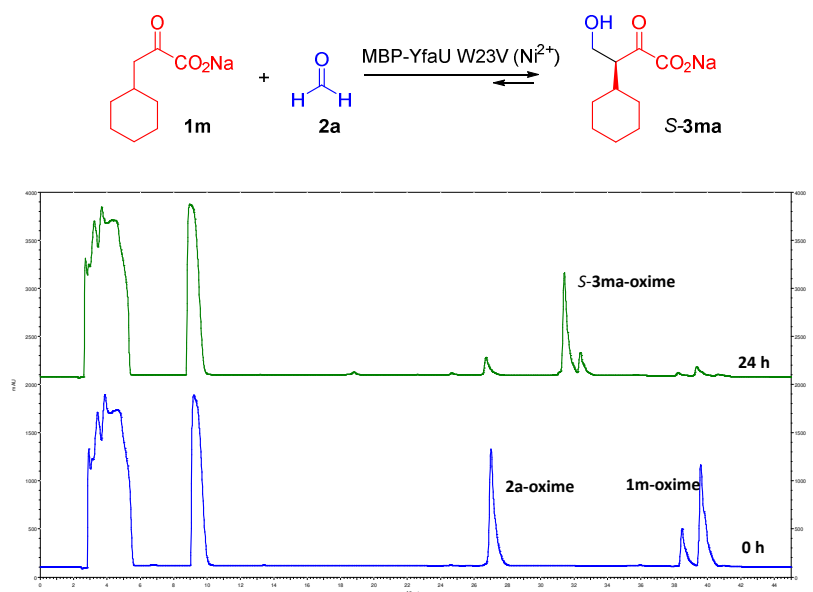
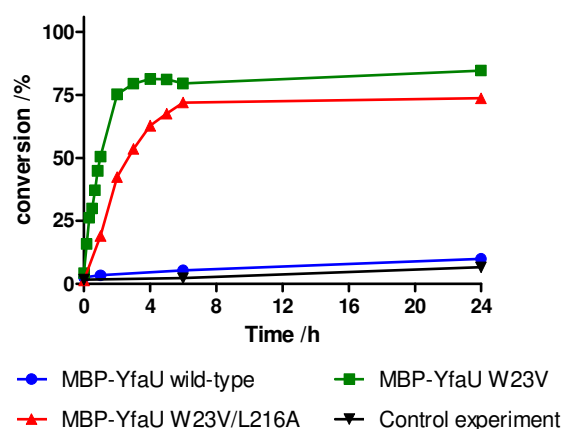


Figure S57 Aldol addition of sodium 3-cyclohexyl-2-oxopropanoate (**1m**) to formaldehyde (**2a**) catalyzed by MBP-YfaU W23V (Ni^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (> 95% conversion).



MBP-YfaU	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$1.1 \cdot 10^{-2}$	10
W23V	1.7	85
W23V/L216A	0.6	74
Control	$6.8 \cdot 10^{-3}$	7

^a. $[\mathbf{1m}] = [\mathbf{2a}] = 100 \text{ mM}$, $[\text{enzyme}] = 4 \text{ mg mL}^{-1}$.

Figure S58 Reaction time course of aldol addition of sodium 3-cyclohexyl-2-oxopropanoate (**1m**) to formaldehyde (**2a**) catalyzed by MBP-YfaU (Ni^{2+}) wild-type, W23V and W23V/L216A variants. Control experiment was performed under the same conditions without the enzyme.

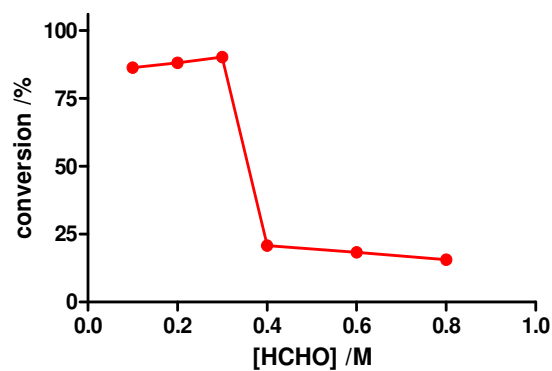


Figure S59 Effect of formaldehyde concentration on the yield of *S*-**3ma** in the aldol addition of **2a** to **1m** catalyzed by MBP-YfaU W23V (Ni²⁺).

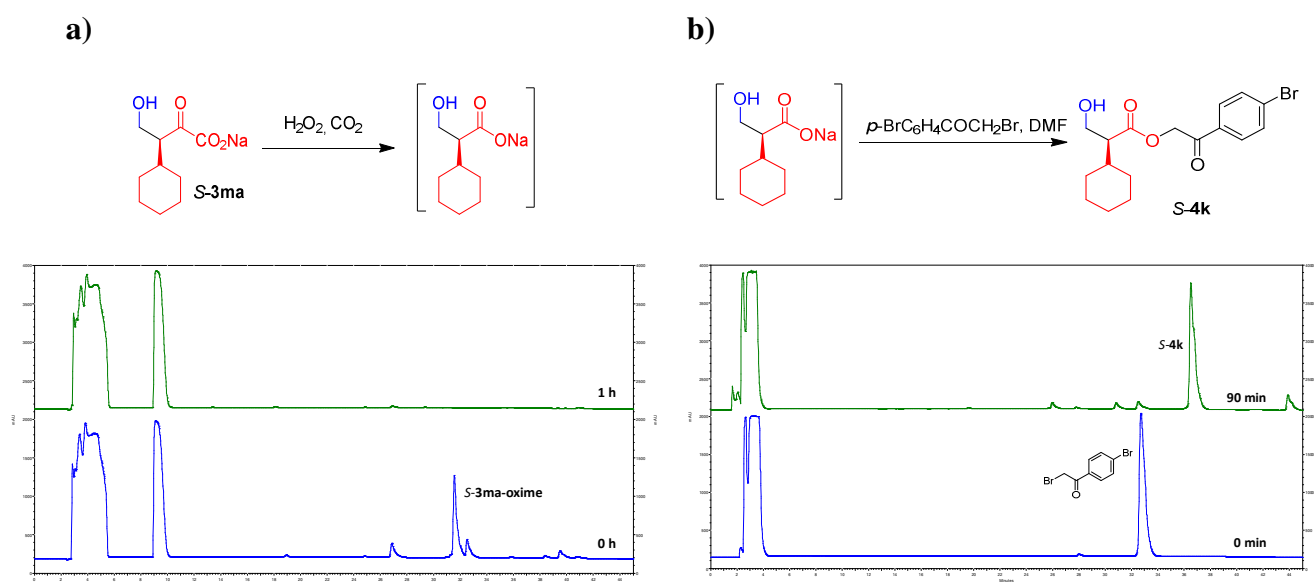


Figure S60 HPLC monitoring of oxidative decarboxylation of *S*-**3ma** (a) and synthesis of 4'-bromophenacyl ester *S*-**4k** (b).

Reaction monitoring for the synthesis of products *R*-4a-j

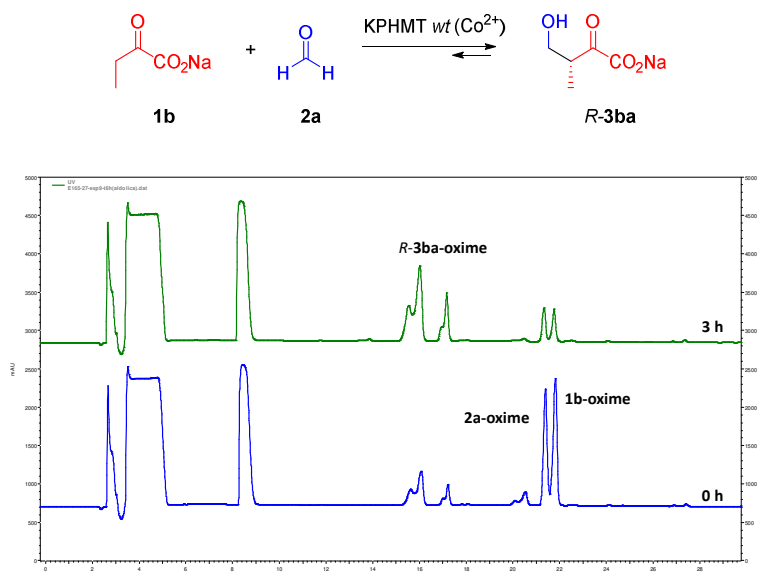
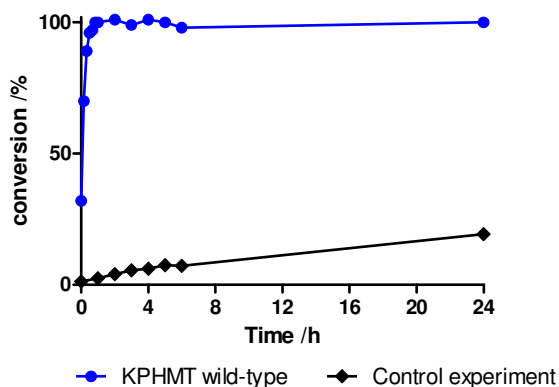


Figure S61 Aldol addition of sodium 2-oxobutanoate (**1b**) to formaldehyde (**2a**) catalyzed by KPHMT wild-type (Co²⁺). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 3 h (90% conversion).



KPHMT	v_o^a (mM·min ⁻¹)	Conv 24h (%)
wild-type	3.3	>95
Control	$1.3 \cdot 10^{-2}$	19

^a. [1b] = [2a] = 100 mM, [enzyme] = 4 mg mL⁻¹.

Figure S62 Reaction time course of aldol addition of sodium 2-oxobutanoate (**1b**) to formaldehyde (**2a**) catalyzed by KPHMT wild-type (Co²⁺) and control experiment performed under the same conditions without the enzyme.

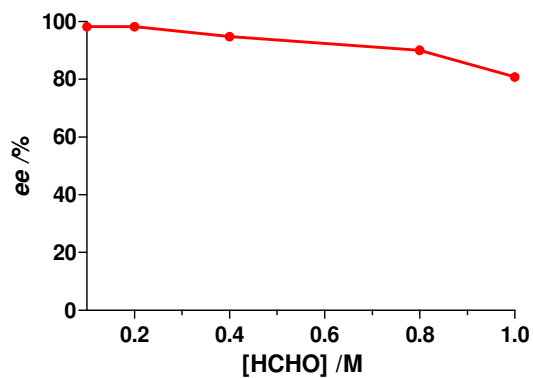


Figure S63 Effect of formaldehyde concentration on the enantiomeric excess of *R*-4a.

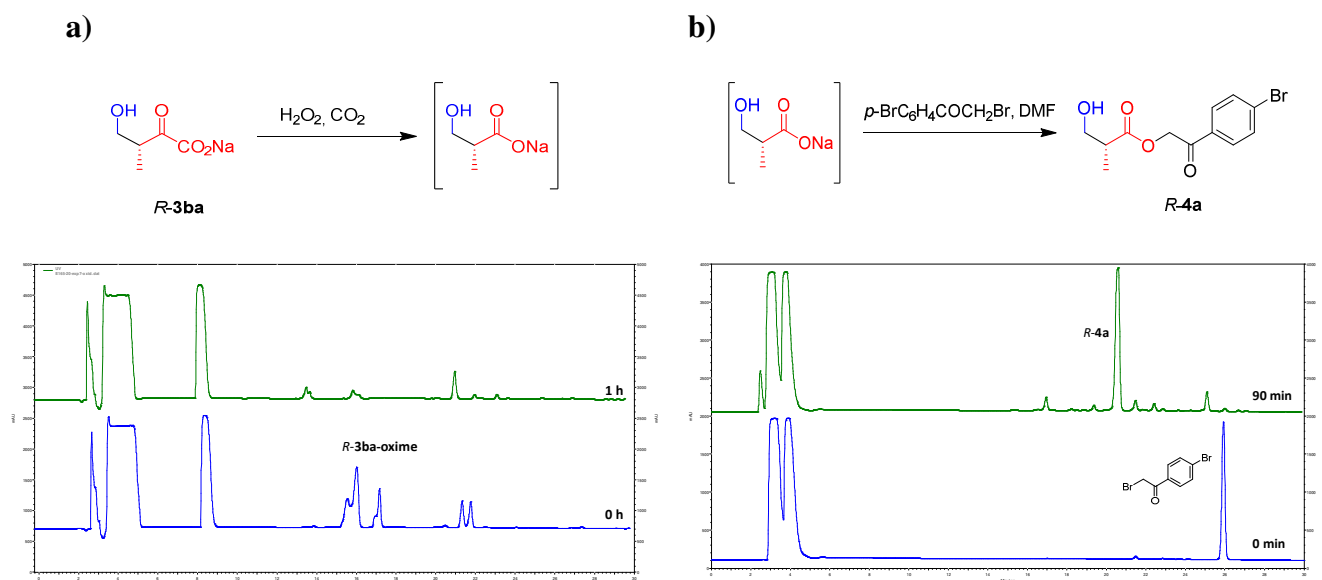


Figure S64 HPLC monitoring of oxidative decarboxylation of *R*-3ba (a) and synthesis of 4'-bromophenacyl ester *R*-4a (b).

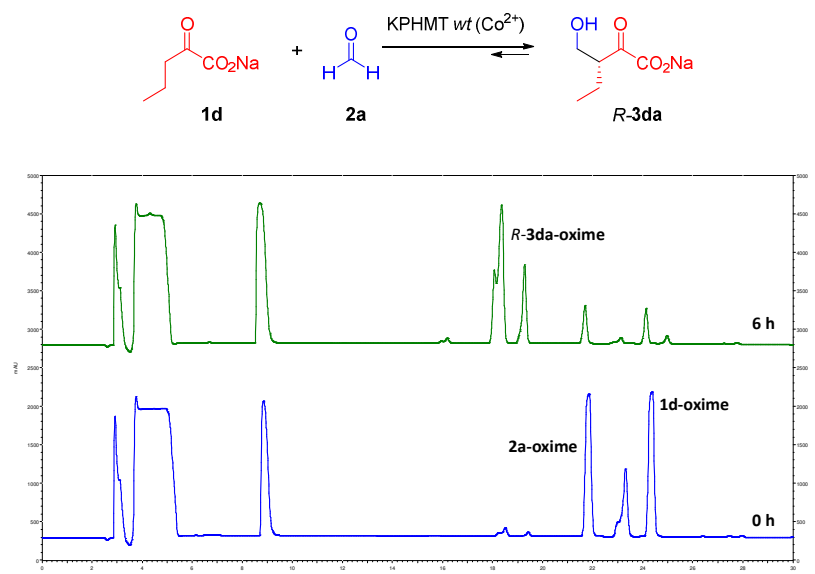
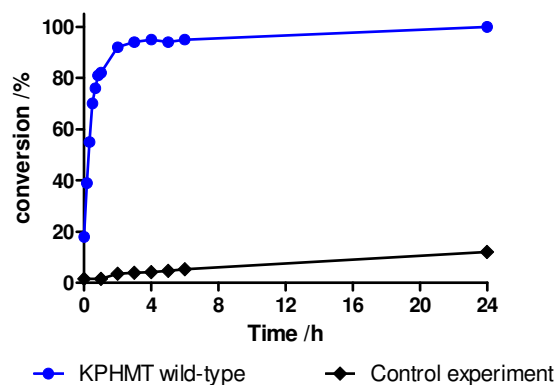


Figure S65 Aldol addition of sodium 2-oxopentanoate (**1d**) to formaldehyde (**2a**) catalyzed by KPHMT wild-type (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 6 h (95% conversion).



KPHMT	v_0^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	1.9	95
Control	$1.1 \cdot 10^{-2}$	12

^a. $[\mathbf{1d}] = [\mathbf{2a}] = 100 \text{ mM}$, $[\text{enzyme}] = 4 \text{ mg mL}^{-1}$.

Figure S66 Reaction time course of aldol addition of sodium 2-oxopentanoate (**1d**) to formaldehyde (**2a**) catalyzed by KPHMT wild-type (Co^{2+}) and control experiment performed under the same conditions without the enzyme.

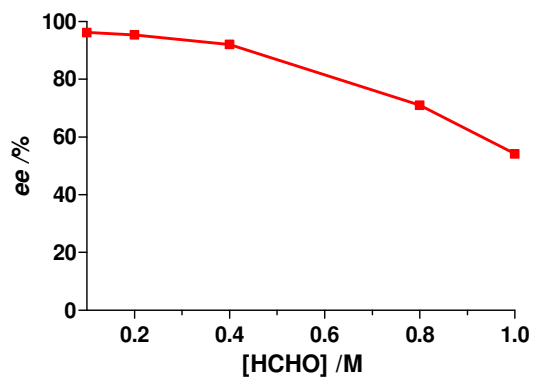


Figure S67 Effect of formaldehyde concentration on the enantiomeric excess of *R*-4b.

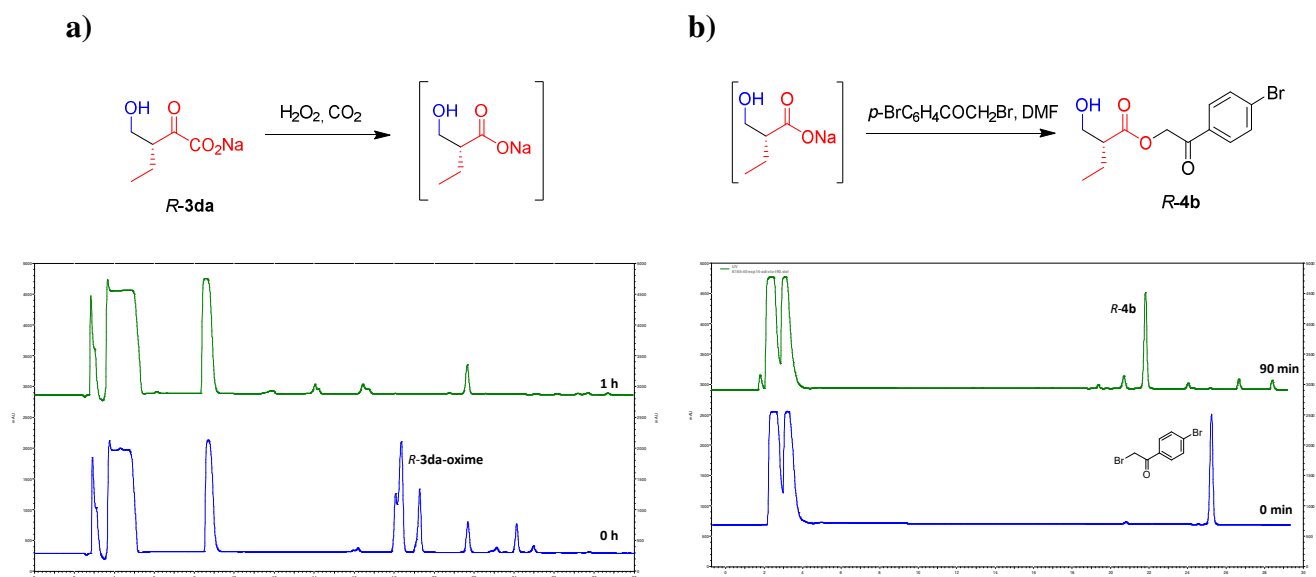


Figure S68 HPLC monitoring of oxidative decarboxylation of *R*-3da (a) and synthesis of 4'-bromophenacyl ester *R*-4b (b).

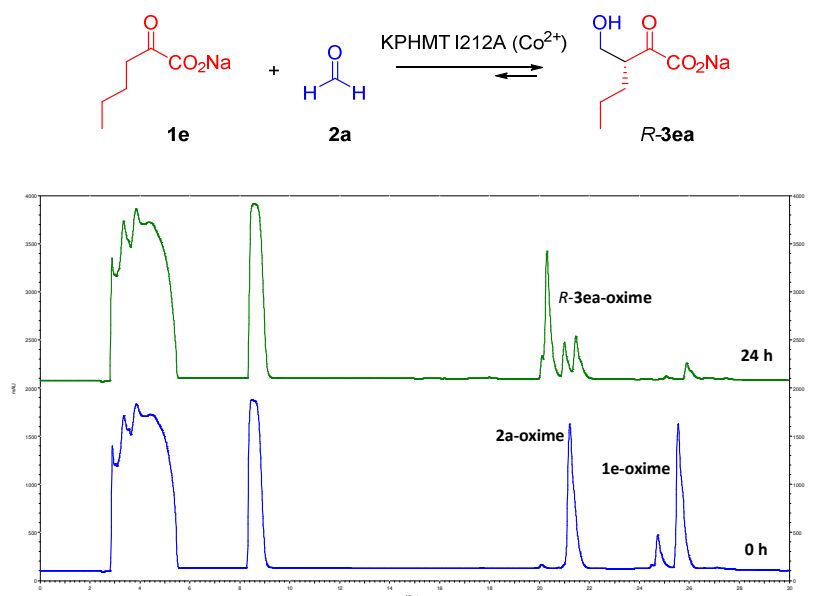
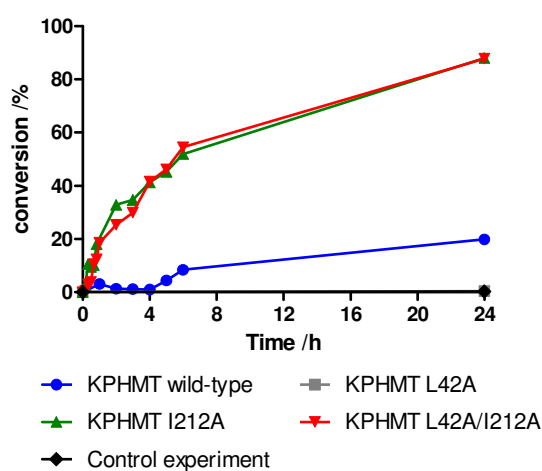


Figure S69 Aldol addition of sodium 2-oxohexanoate (**1e**) to formaldehyde (**2a**) catalyzed by KPHMT I212A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (85% conversion).



KPHMT	ν_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$1.6 \cdot 10^{-2}$	20
L42A	$_{-b}$	$_{-b}$
I212A	0.4	88
L42A/I212A	0.3	87
Control	$_{-b}$	$_{-b}$

^a. $[\mathbf{1e}] = [\mathbf{2a}] = 100 \text{ mM}$, $[\text{enzyme}] = 4 \text{ mg mL}^{-1}$.

^b. Product not detected.

Figure S70 Reaction time course of aldol addition of sodium 2-oxohexanoate (**1e**) to formaldehyde (**2a**) catalyzed by KPHMT (Co^{2+}) wild-type, L42A, I212A and L42A/I212A variants. Control experiment was performed under the same conditions without the enzyme.

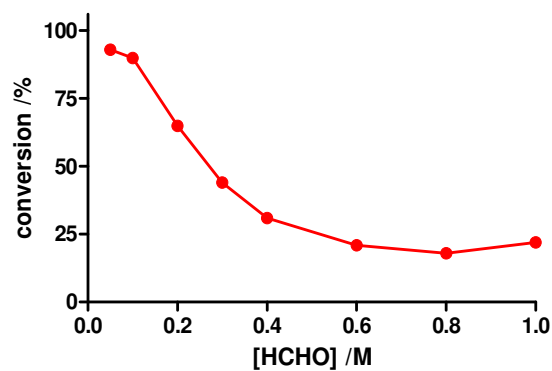


Figure S71 Effect of formaldehyde concentration on the yield of *R*-**3ea** in the aldol addition of **2a** to **1e** catalyzed by KPHMT I212A (Co²⁺).

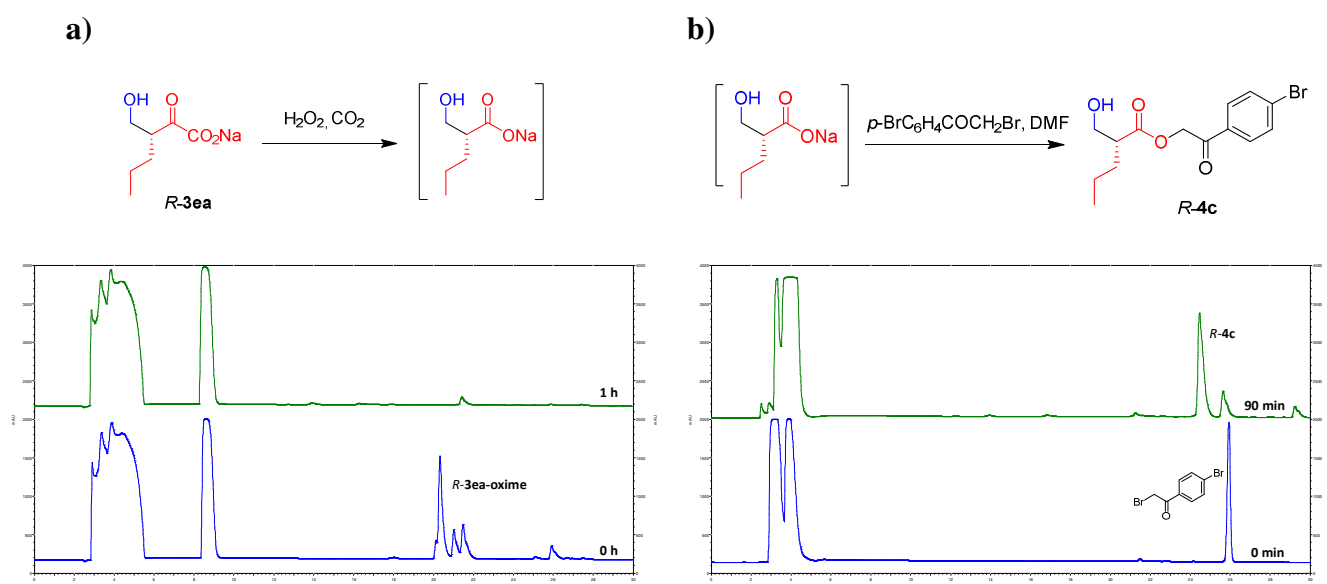


Figure S72 HPLC monitoring of oxidative decarboxylation of *R*-**3ea** (a) and synthesis of 4'-bromophenacyl ester *R*-**4c** (b).

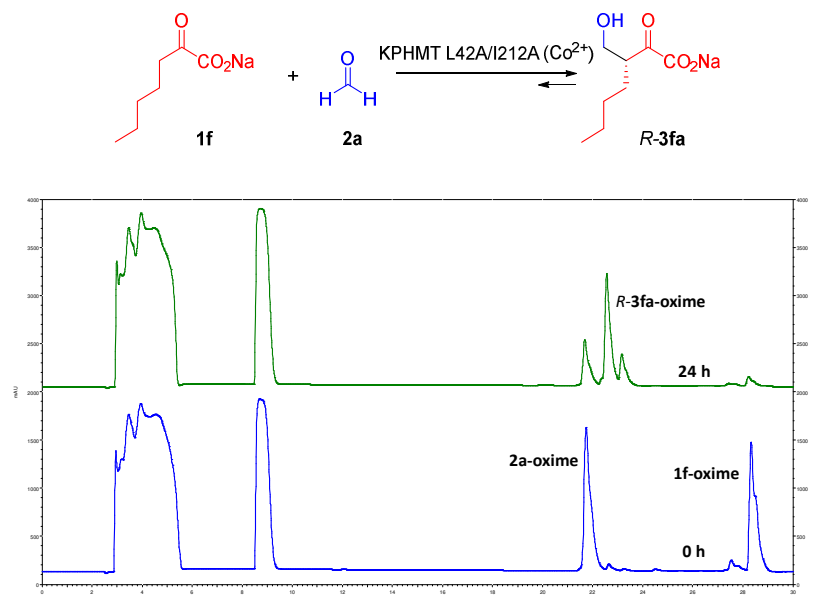
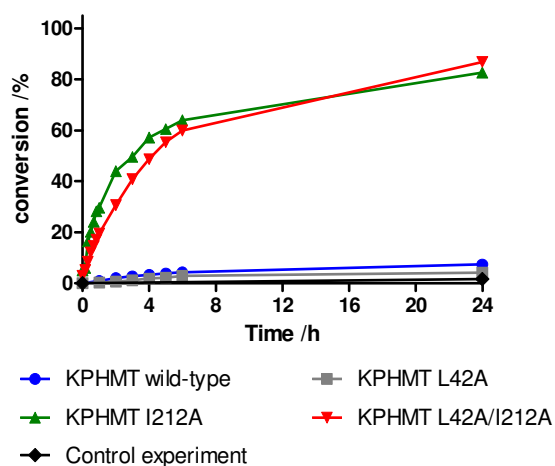


Figure S73 Aldol addition of sodium 2-oxoheptanoate (**1f**) to formaldehyde (**2a**) catalyzed by KPHMT L42A/I212A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (88% conversion).



KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$3.0 \cdot 10^{-3}$	7
L42A	$_{-}^b$	$_{-}^b$
I212A	0.5	83
L42A/I212A	0.3	87
Control	$_{-}^b$	$_{-}^b$

^a. $[\mathbf{1f}] = [\mathbf{2a}] = 100 \text{ mM}$, $[\text{enzyme}] = 4 \text{ mg mL}^{-1}$.

^b. Product not detected.

Figure S74 Reaction time course of aldol addition of sodium 2-oxoheptanoate (**1f**) to formaldehyde (**2a**) catalyzed by KPHMT (Co^{2+}) wild-type, L42A, I212A, L42A/I212A and I202A variants. Control experiment was performed under the same conditions without the enzyme.

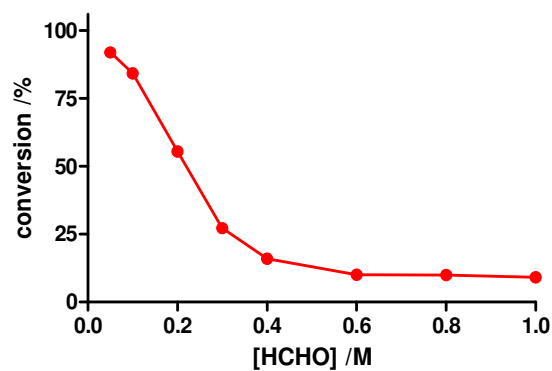


Figure S75 Effect of formaldehyde concentration on the yield of *R-3fa* in the aldol addition of **2a** to **1f** catalyzed by KPHMT L42A/I212A (Co²⁺).

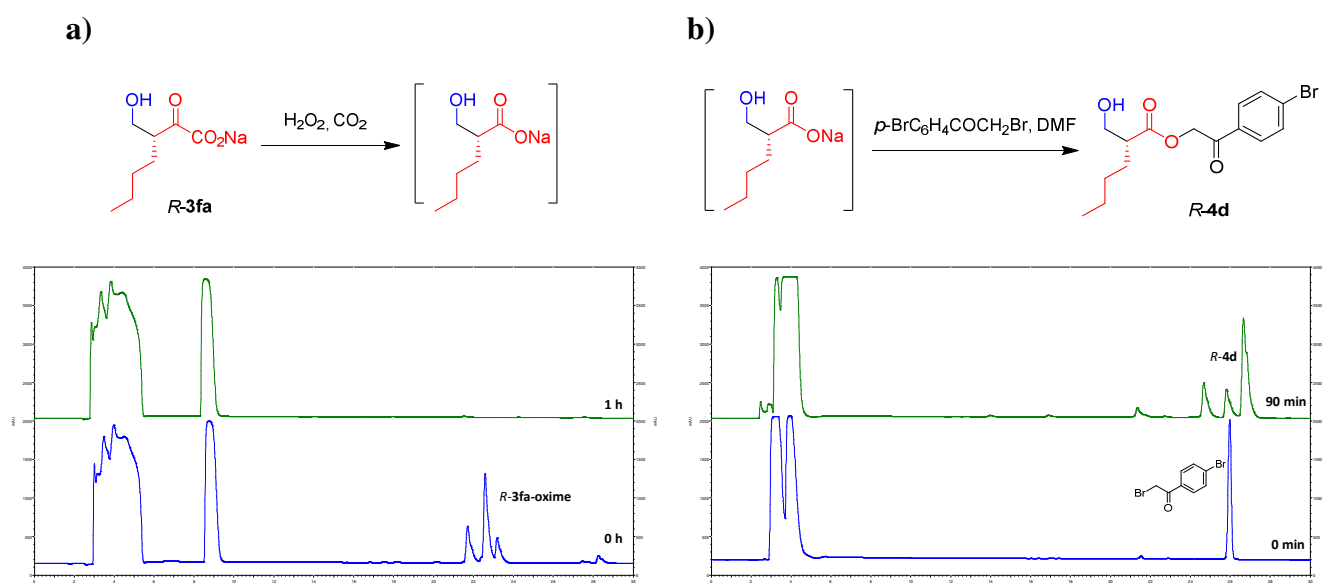


Figure S76 HPLC monitoring of oxidative decarboxylation of *R-3fa* (a) and synthesis of 4'-bromophenacyl ester *R-4d* (b).

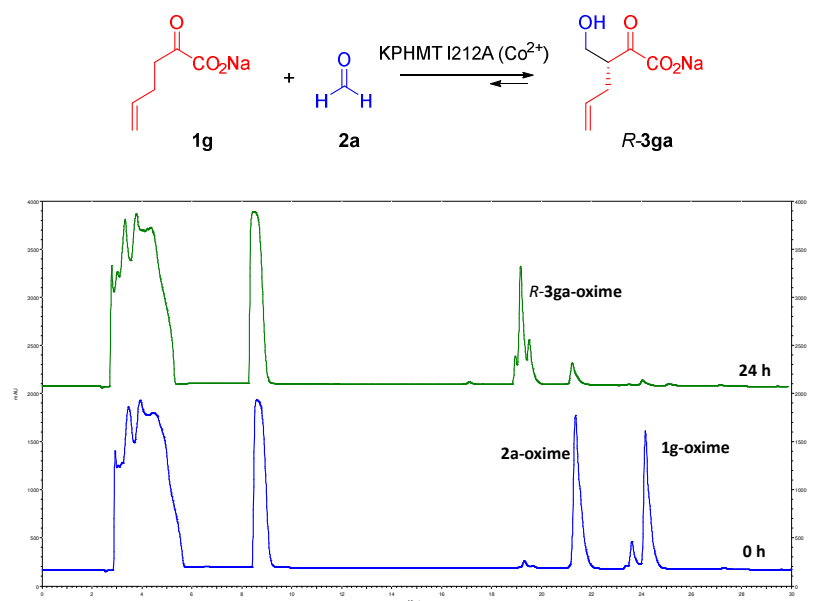
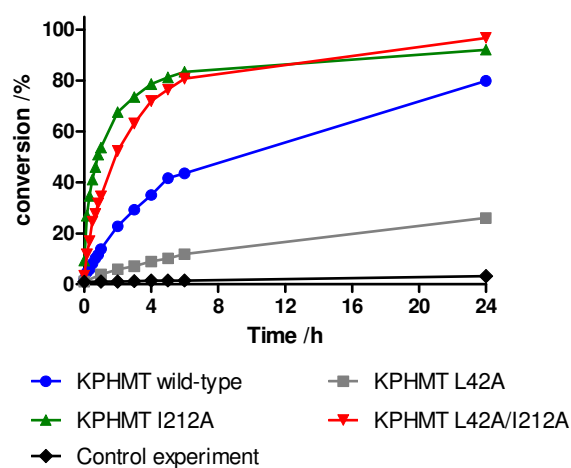


Figure S77 Aldol addition of sodium 2-oxohex-5-enoate (**1g**) to formaldehyde (**2a**) catalyzed by KPHMT I212A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (87% conversion).



KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	0.2	80
L42A	$3.7 \cdot 10^{-2}$	26
I212A	1.1	92
L42A/I212A	0.6	>95
Control	$_{-b}$	$_{-b}$

^a. $[\mathbf{1g}] = [\mathbf{2a}] = 100 \text{ mM}$, $[\text{enzyme}] = 4 \text{ mg mL}^{-1}$.

^b. Product not detected.

Figure S78 Reaction time course of aldol addition of sodium 2-oxohex-5-enoate (**1g**) to formaldehyde (**2a**) catalyzed by KPHMT (Co^{2+}) wild-type, L42A, I212A and L42A/I212A variants. Control experiment was performed under the same conditions without the enzyme.

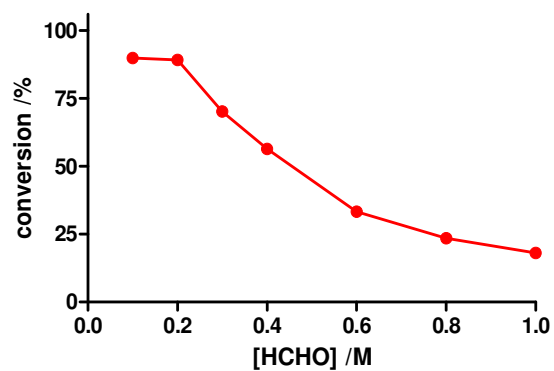


Figure S79 Effect of formaldehyde concentration on the yield of *R*-**3ga** in the aldol addition of **2a** to **1g** catalyzed by KPHMT I212A (Co²⁺).

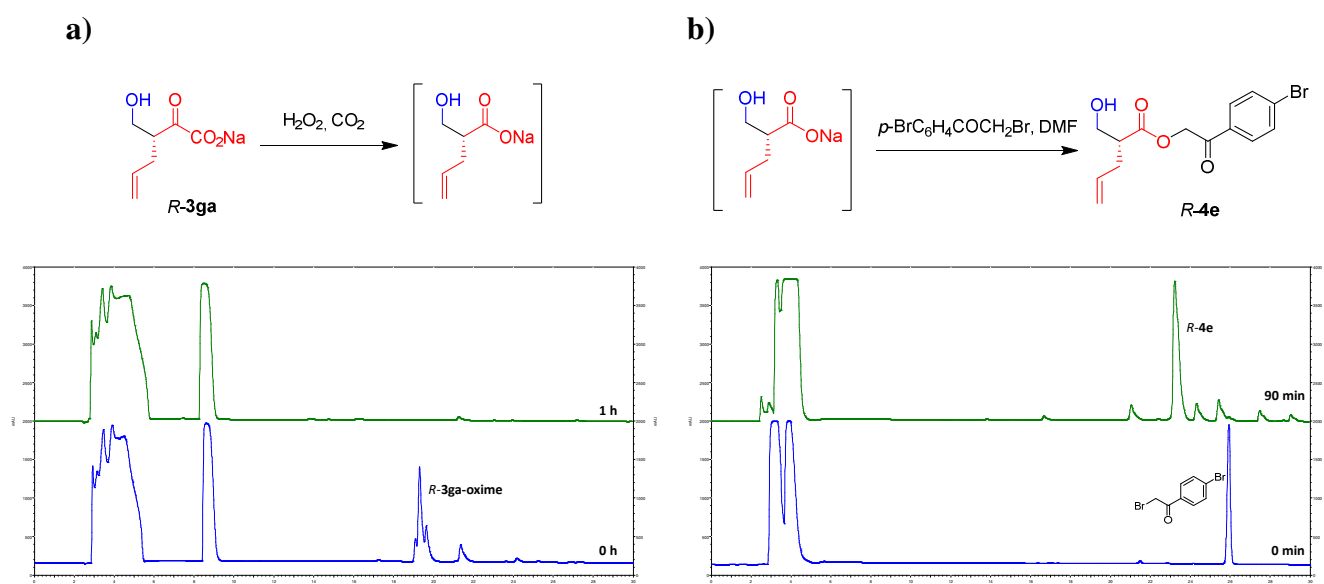


Figure S80 HPLC monitoring of oxidative decarboxylation of *R*-**3ga** (a) and synthesis of 4'-bromophenacyl ester *R*-**4e** (b).

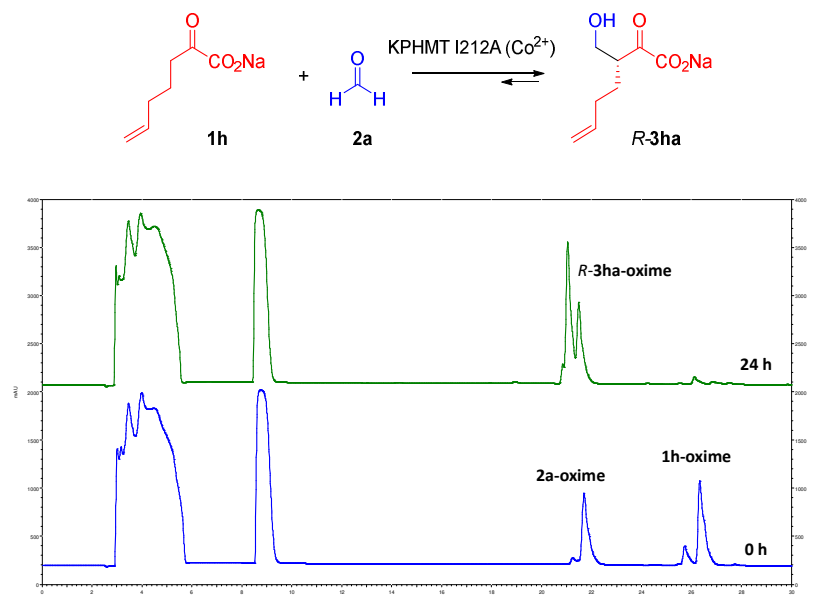
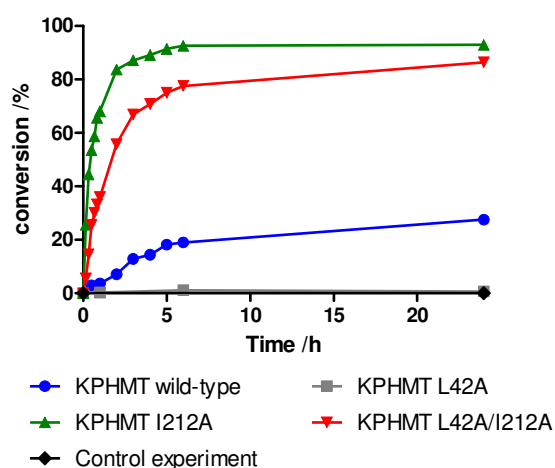


Figure S81 Aldol addition of sodium 2-oxohept-6-enoate (**1h**) to formaldehyde (**2a**) catalyzed by KPHMT I212A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (93% conversion).



KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$6.5 \cdot 10^{-2}$	28
L42A	$_{-}^b$	$_{-}^b$
I212A	2.2	93
L42A/I212A	1.0	86
Control	$_{-}^b$	$_{-}^b$

^a. $[\mathbf{1h}] = [\mathbf{2a}] = 100 \text{ mM}$, $[\text{enzyme}] = 4 \text{ mg mL}^{-1}$.

^b. Product not detected.

Figure S82 Reaction time course of aldol addition of sodium 2-oxohept-6-enoate (**1h**) to formaldehyde (**2a**) catalyzed by KPHMT (Co^{2+}) wild-type, L42A, I212A and L42A/I212A variants. Control experiment was performed under the same conditions without the enzyme.

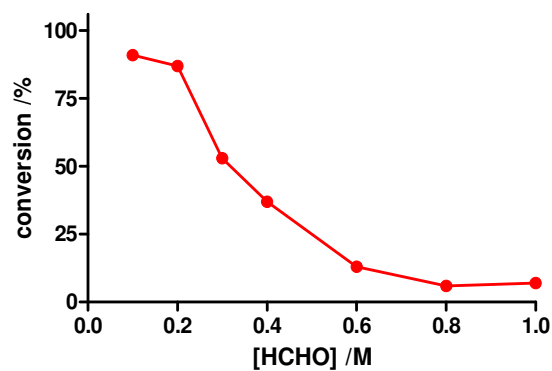


Figure S83 Effect of formaldehyde concentration on the yield of *R*-**3ha** in the aldol addition of **2a** to **1h** catalyzed by KPHMT I212A (Co²⁺).

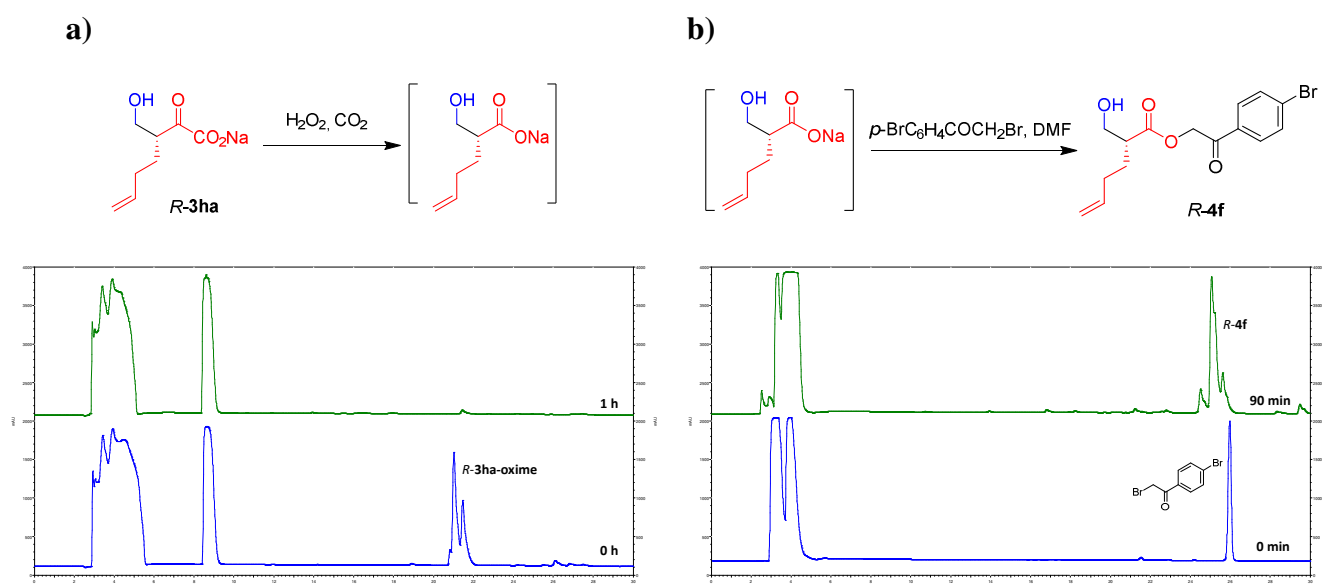


Figure S84 HPLC monitoring of oxidative decarboxylation reaction of *R*-**3ha** (a) and synthesis of 4'-bromophenacyl ester *R*-**4f** (b).

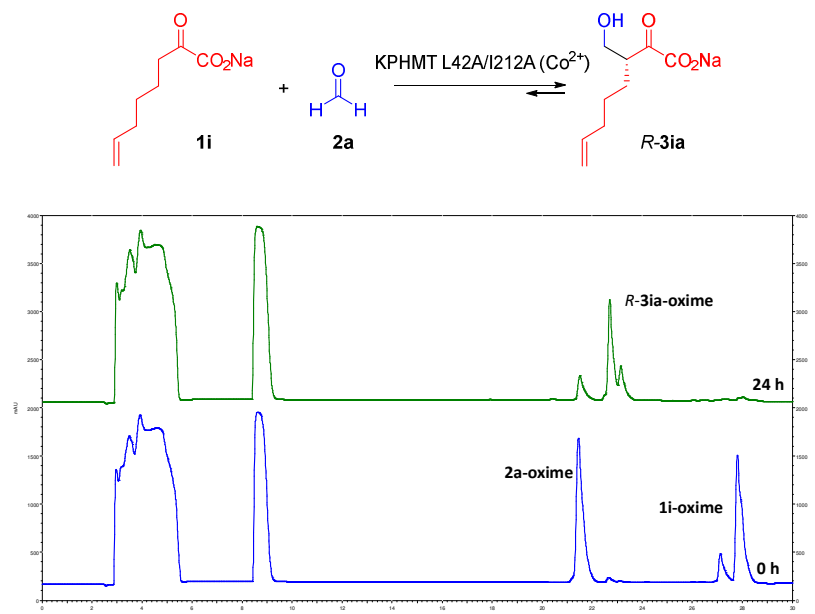
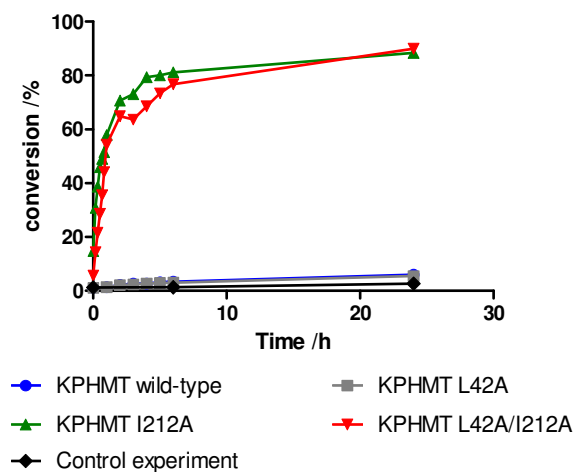


Figure S85 Aldol addition of sodium 2-oxooct-7-enoate (**1i**) to formaldehyde (**2a**) catalyzed by KPHMT L42A/I212A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (92% conversion).



KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$9.0 \cdot 10^{-3}$	6
L42A	$9.9 \cdot 10^{-3}$	5
I212A	1.5	88
L42A/I212A	1.0	90
Control	$-^b$	$-^b$

^a. $[\mathbf{1i}] = [\mathbf{2a}] = 100 \text{ mM}$, $[\text{enzyme}] = 4 \text{ mg mL}^{-1}$.

^b. Product not detected.

Figure S86 Reaction time course of aldol addition of sodium 2-oxooct-7-enoate (**1i**) to formaldehyde (**2a**) catalyzed by KPHMT (Co^{2+}) wild-type, L42A, I212A and L42A/I212A variants. Control experiment was performed under the same conditions without the enzyme.

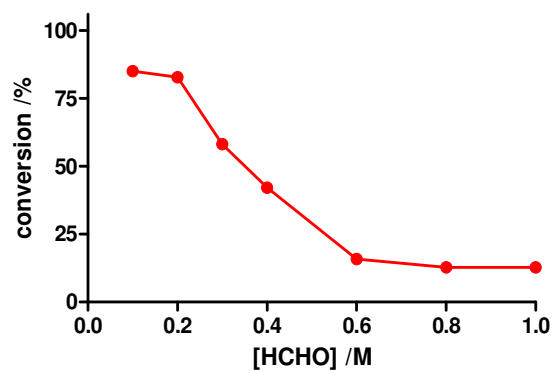


Figure S87 Effect of formaldehyde concentration on the yield of *R*-**3ia** in the aldol addition of **2a** to **1i** catalyzed by KPHMT L42A/I212A (Co²⁺).

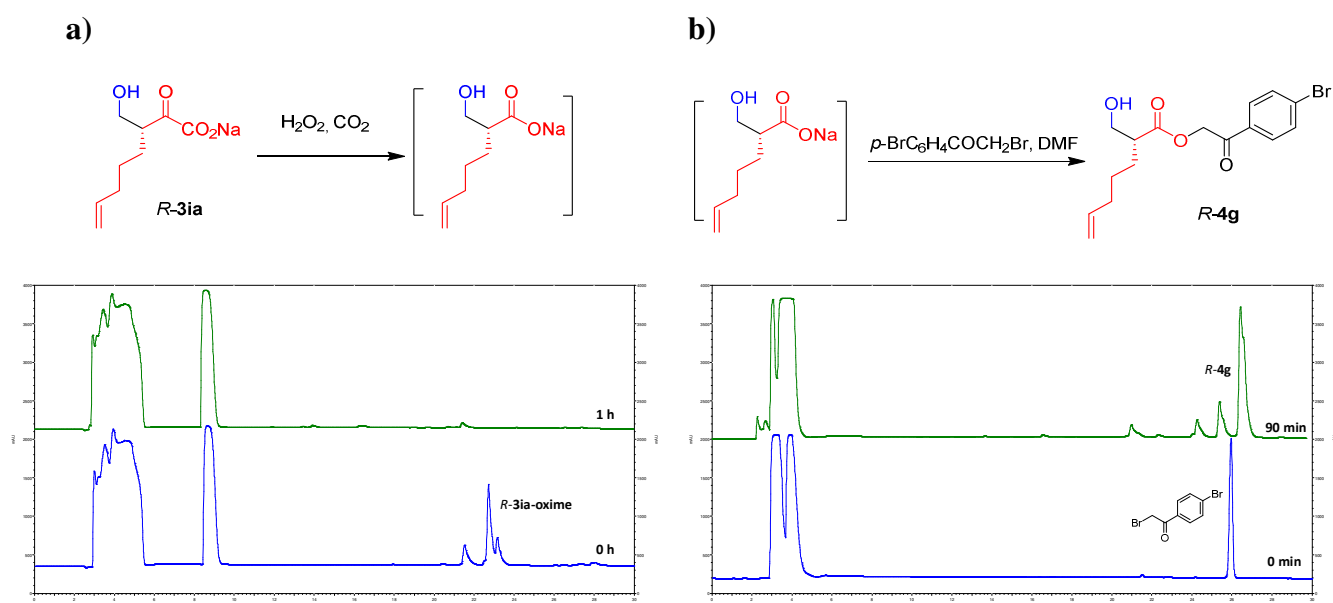


Figure S88 HPLC monitoring of oxidative decarboxylation of *R*-**3ia** (a) and synthesis of 4'-bromophenacyl ester *R*-**4g** (b).

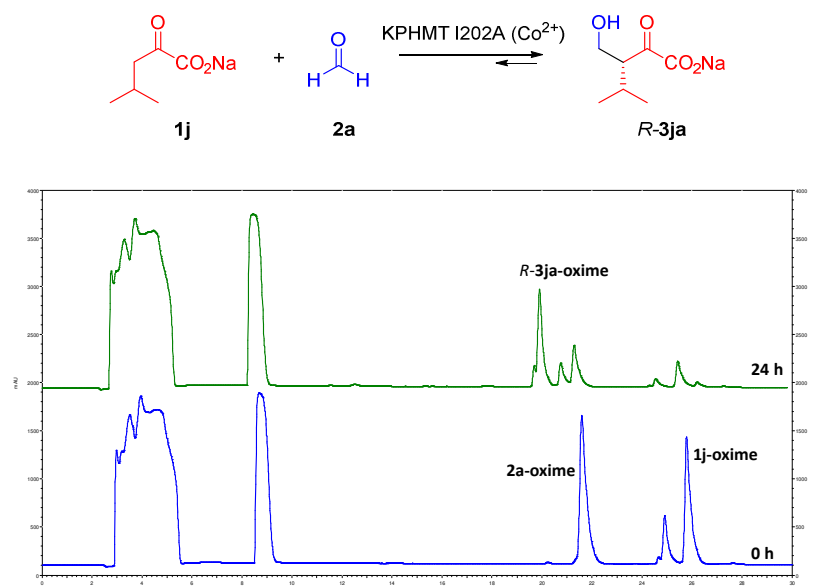
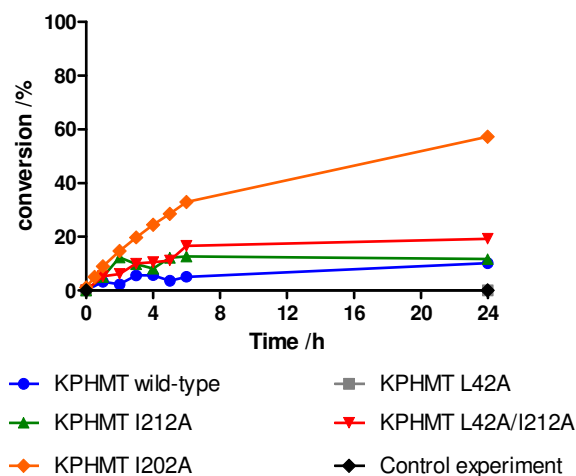


Figure S89 Aldol addition of sodium 4-methyl-2-oxopentanoate (**1j**) to formaldehyde (**2a**) catalyzed by KPHMT I202A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (70% conversion).



KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$7.4 \cdot 10^{-3}$	10
L42A	_b	_b
I212A	$7.6 \cdot 10^{-3}$	12
L42A/I212A	$4.7 \cdot 10^{-2}$	19
I202A	0.2	57
Control	_b	_b

^a $[\mathbf{1j}] = [\mathbf{2a}] = 100 \text{ mM}$, $[\text{enzyme}] = 4 \text{ mg mL}^{-1}$.

^b Product not detected.

Figure S90 Reaction time course of aldol addition of sodium 4-methyl-2-oxopentanoate (**1j**) to formaldehyde (**2a**) catalyzed by KPHMT (Co^{2+}) wild-type, L42A, I212A, L42A/I212A and L202A variants. Control experiment was performed under the same conditions without the enzyme.

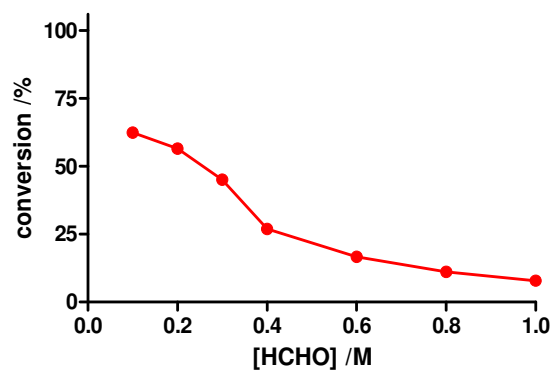


Figure S91 Effect of formaldehyde concentration on the yield of *R-3c* in the aldol addition of **2a** to **1j** catalyzed by KPHMT I202A (Co²⁺).

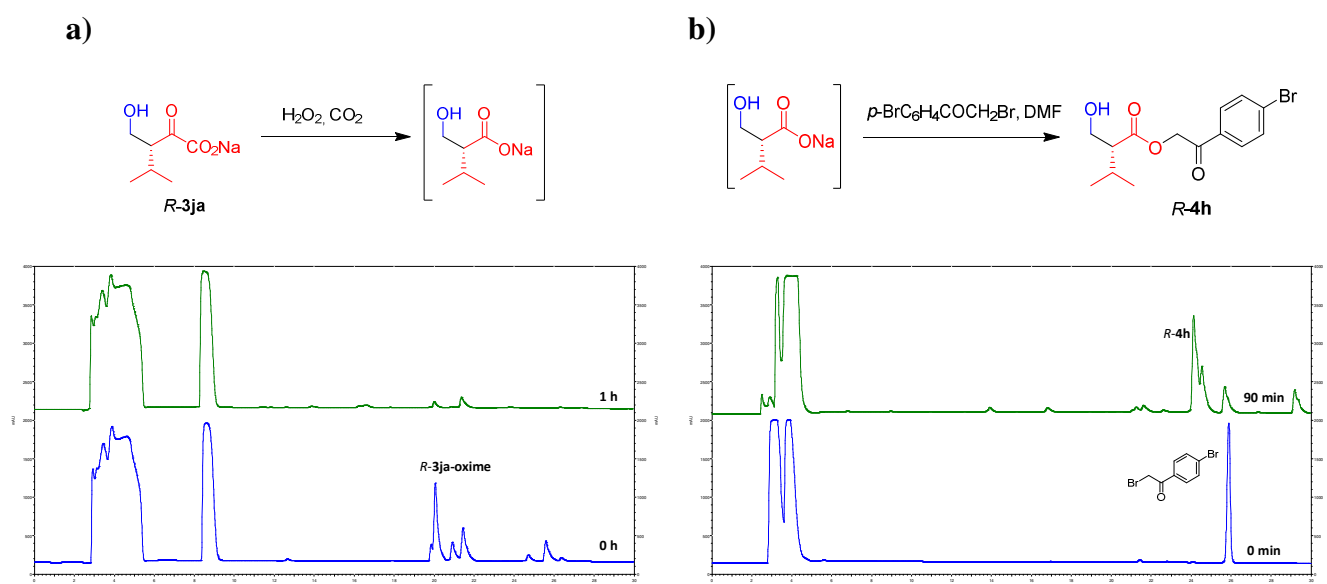


Figure S92 HPLC monitoring of oxidative decarboxylation of *R-3ja* (a) and synthesis of 4'-bromophenacyl ester *R-4h* (b).

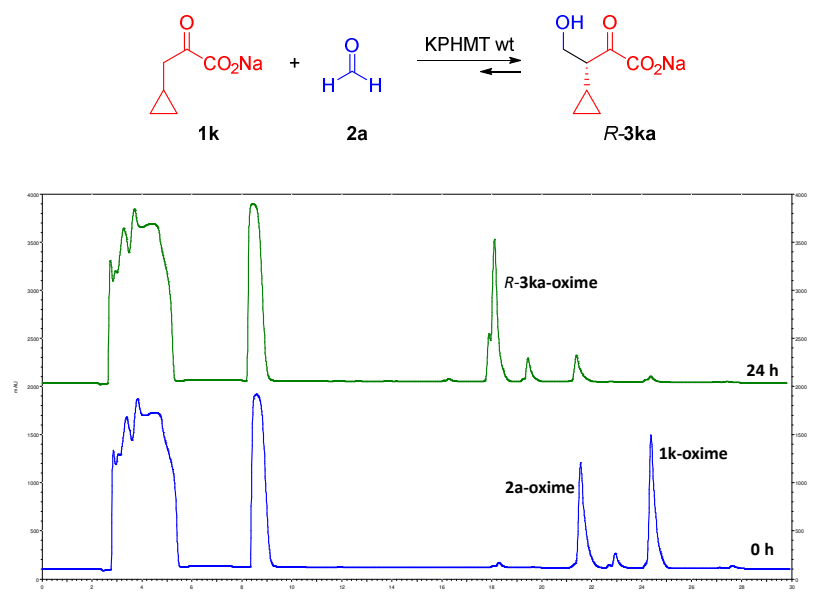
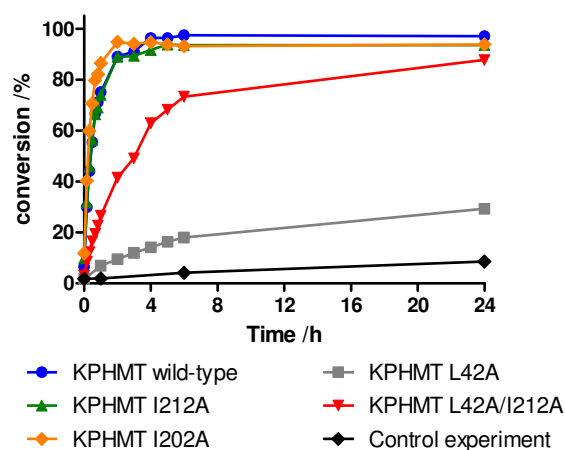


Figure S93 Aldol addition of sodium 3-cyclopropyl-2-oxopropanoate (**1k**) to formaldehyde (**2a**) catalyzed by KPHMT wild-type (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (>95% conversion).



KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	1.8	>95
L42A	$5.3 \cdot 10^{-2}$	29
I212A	1.9	94
L42A/I212A	0.5	88
I202A	3.0	94
Control	$6.5 \cdot 10^{-3}$	9

^a[**1k**] = [**2a**] = 100 mM, [enzyme] = 4 mg mL⁻¹.

Figure S94 Reaction time course of aldol addition of sodium 3-cyclopropyl-2-oxopropanoate (**1k**) to formaldehyde (**2a**) catalyzed by KPHMT (Co^{2+}) wild-type, L42A, I212A, L42A/I212A and I202A variants. Control experiment was performed under the same conditions without the enzyme.

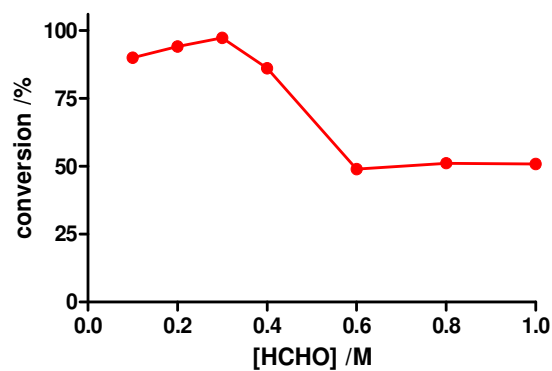


Figure S95 Effect of formaldehyde concentration on the yield of *R-3ka* in the aldol addition of **2a** to **1k** catalyzed by KPHMT wild-type (Co²⁺).

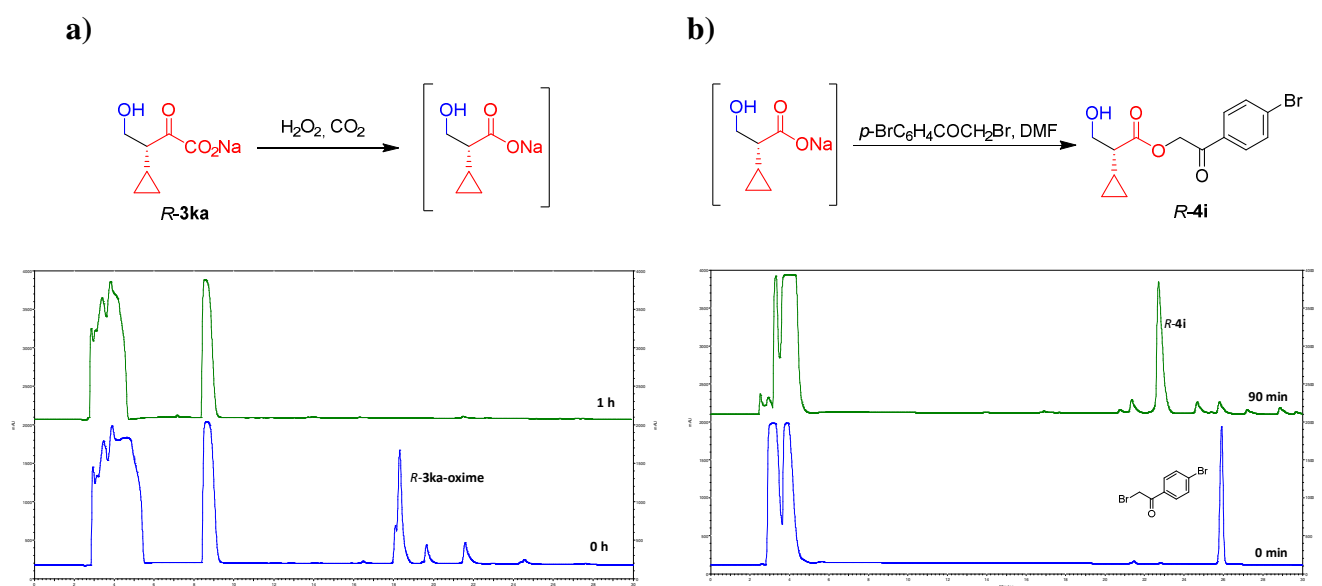


Figure S96 HPLC monitoring of oxidative decarboxylation of *R-3ka* (a) and synthesis of 4'-bromophenacyl ester *R-4i* (b).

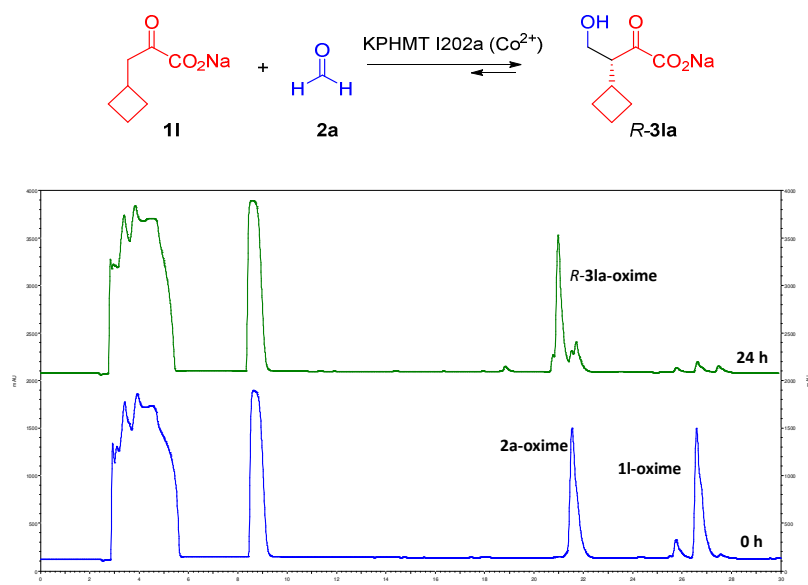
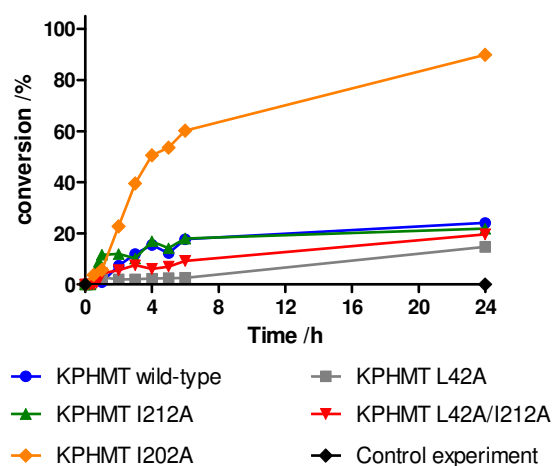


Figure S97 Aldol addition of sodium 3-cyclobutyl-2-oxopropanoate (**1I**) to formaldehyde (**2a**) catalyzed by KPHMT I202A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (90% conversion).



KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$8.3 \cdot 10^{-2}$	24
L42A	$1.3 \cdot 10^{-2}$	14
I212A	$8.9 \cdot 10^{-2}$	22
L42A/I212A	$6.2 \cdot 10^{-2}$	20
I202A	0.2	79
Control	$_{-}^b$	$_{-}^b$

^a. $[\mathbf{1I}] = [\mathbf{2a}] = 100 \text{ mM}$, $[\text{enzyme}] = 4 \text{ mg mL}^{-1}$.

^b. Product not detected.

Figure S98 Reaction time course of aldol addition of sodium 3-cyclobutyl-2-oxopropanoate (**1I**) to formaldehyde (**2a**) catalyzed by KPHMT (Co^{2+}) wild-type, L42A, I212A, L42A/I212A and I202A variants. Control experiment was performed under the same conditions without the enzyme.

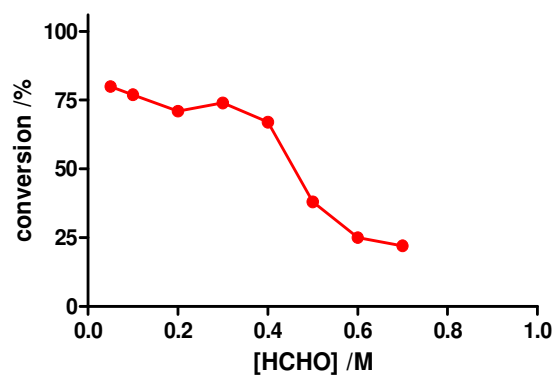


Figure S99 Effect of formaldehyde concentration on the yield of *R*-**3la** in the aldol addition of **2a** to **1l** catalyzed by KPHMT I202A (Co²⁺).

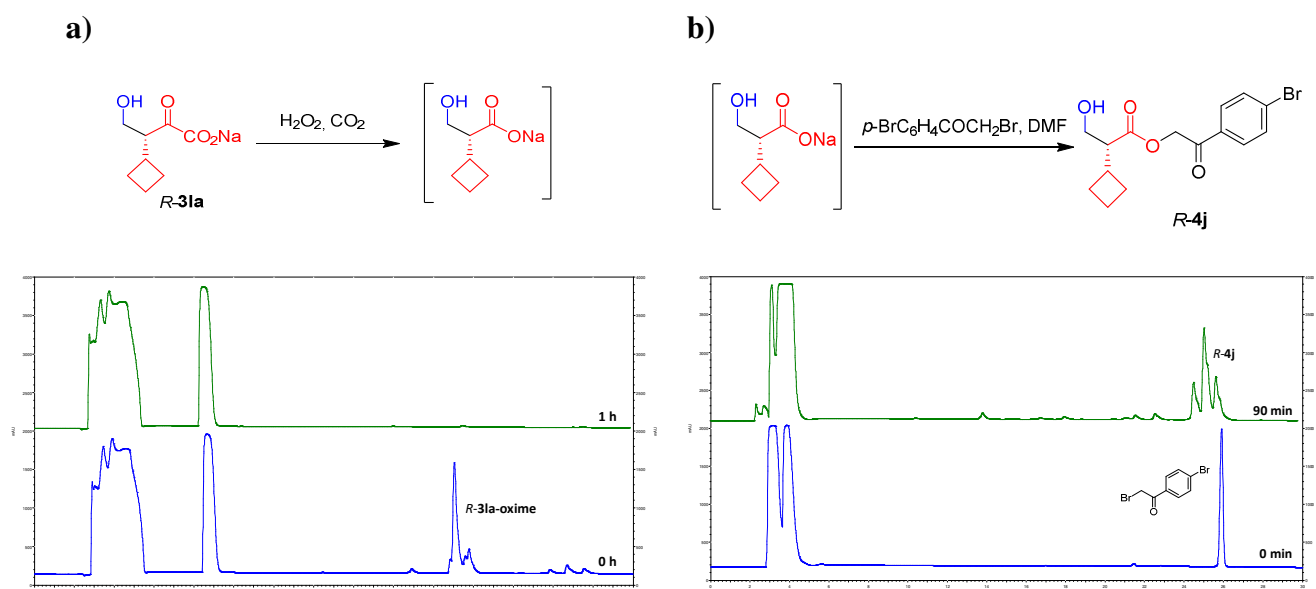
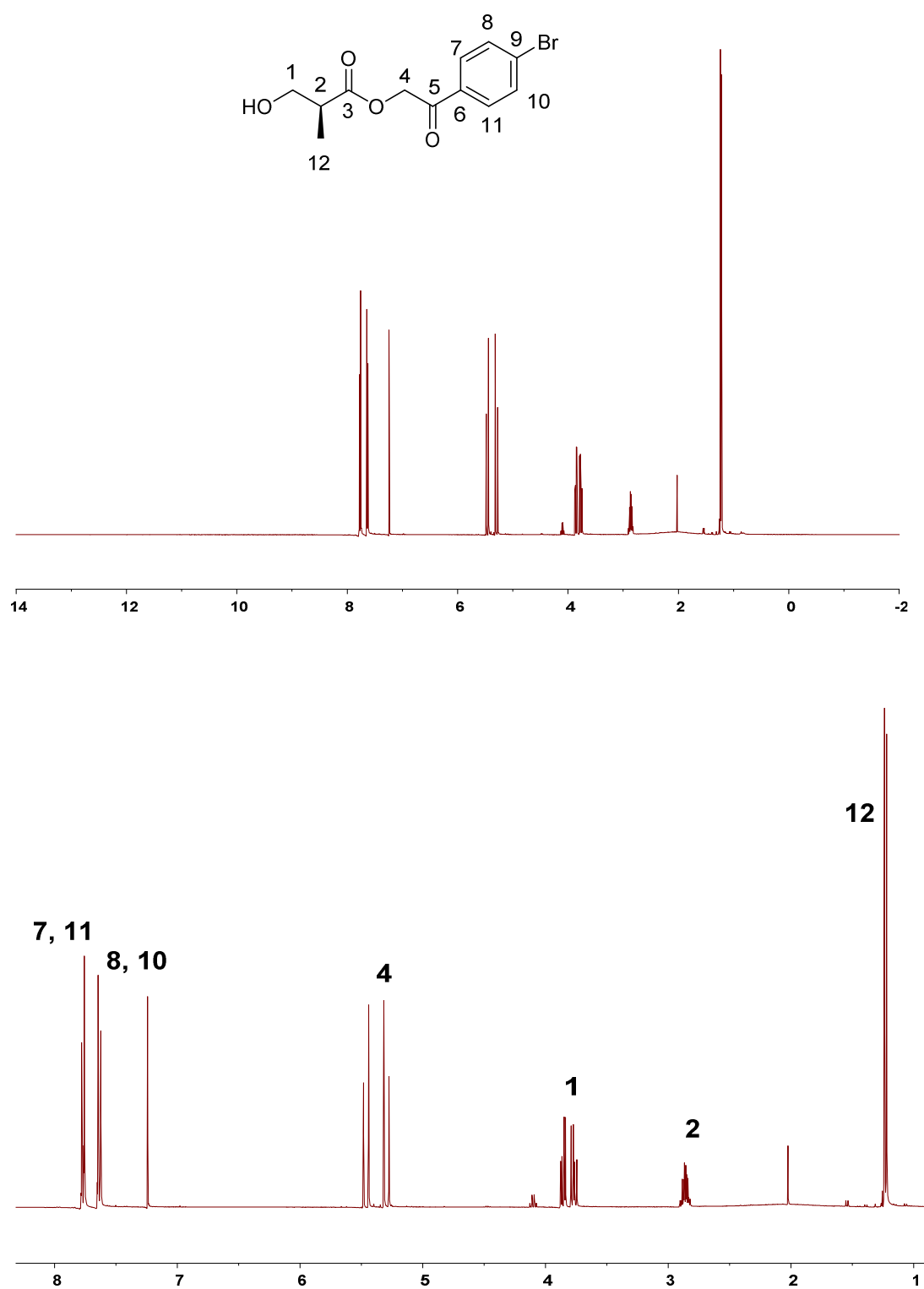


Figure S100 HPLC monitoring of oxidative decarboxylation of *R*-**3la** (a) and synthesis of 4'-bromophenacyl ester *R*-**4j** (b).

6.2.3 NMR spectra of 4'-bromophenacyl ester (4a-k)

The NMR spectra of the aldol adduct derivatives **4a-k** obtained with MBP-YfaU and KPHMT wild-type and variants were undistinguishable. For the sake of simplicity only the *S*-enantiomers are shown.

a)



b)

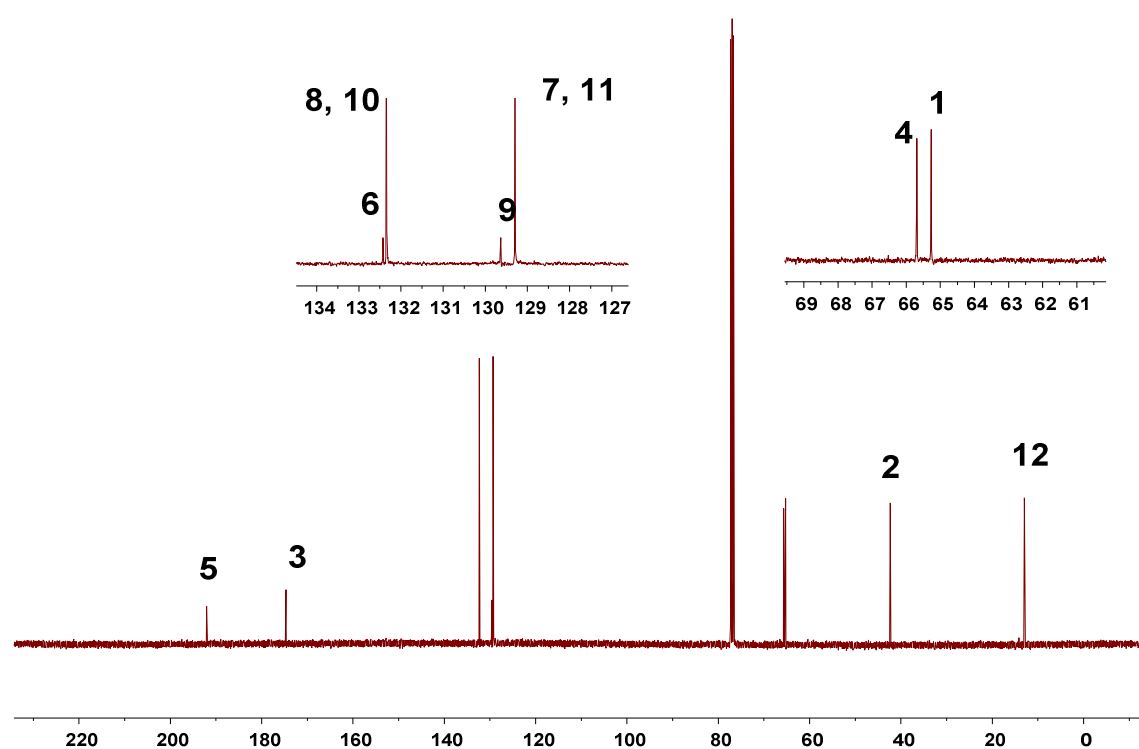
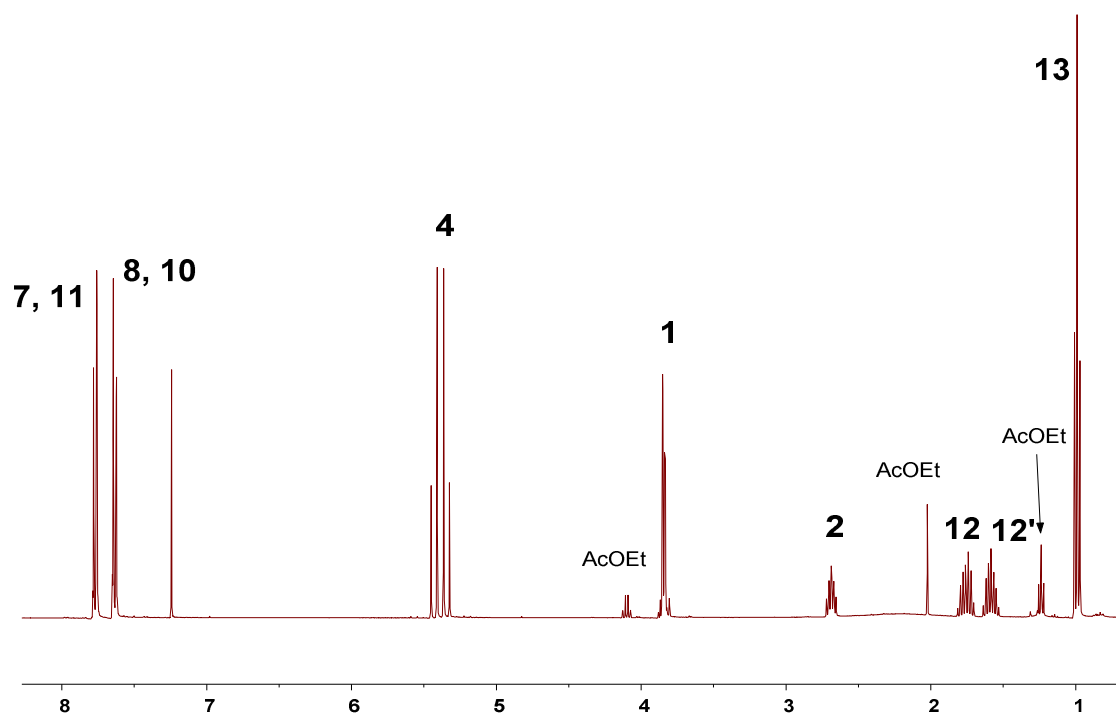
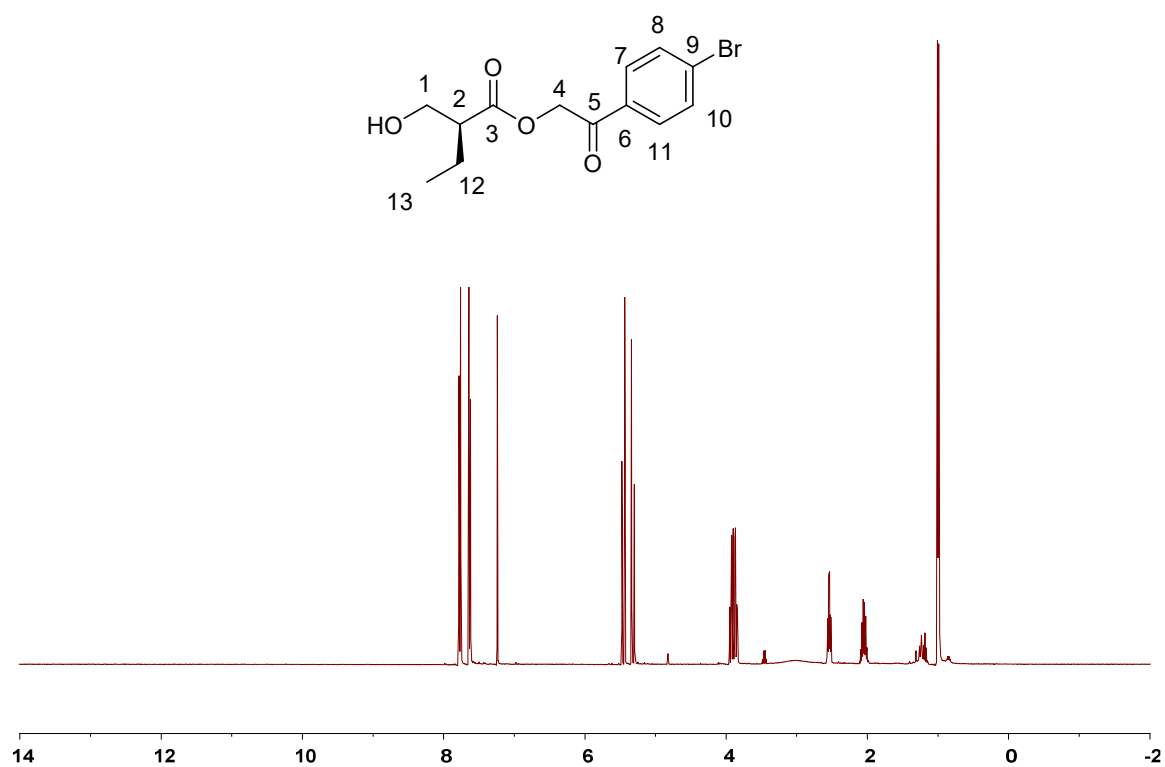


Figure S101 NMR spectra (CDCl_3) of *S*-4a: a) ^1H and b) ^{13}C .

a)



b)

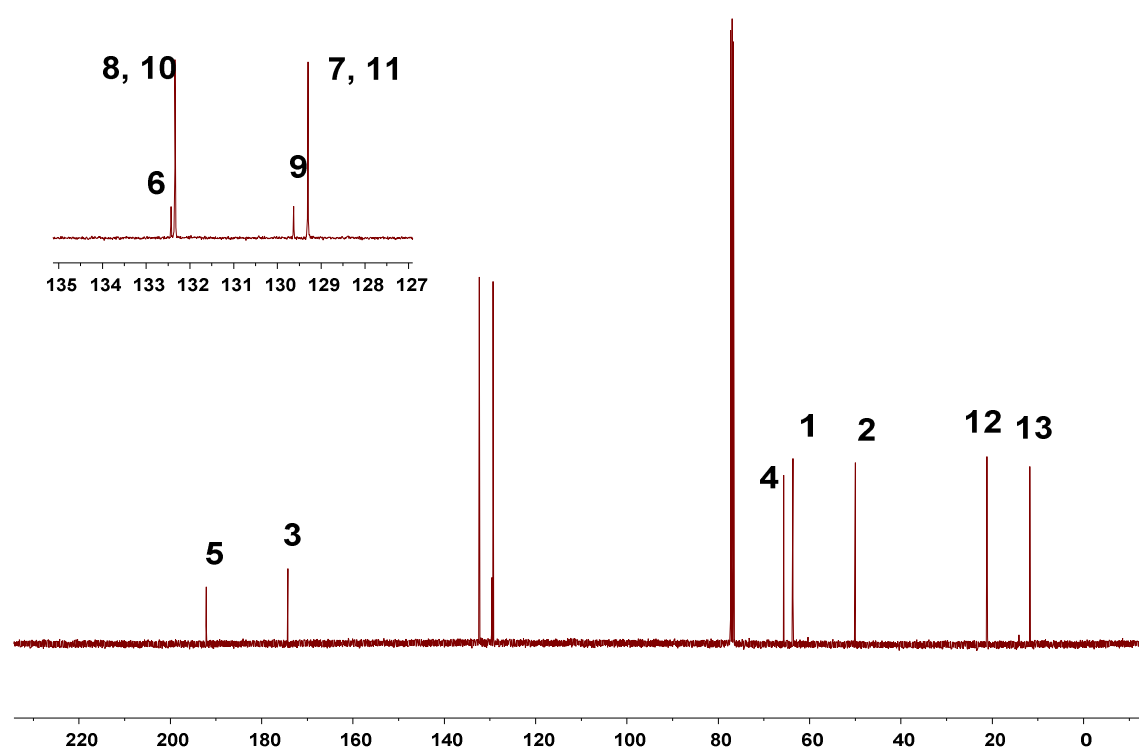
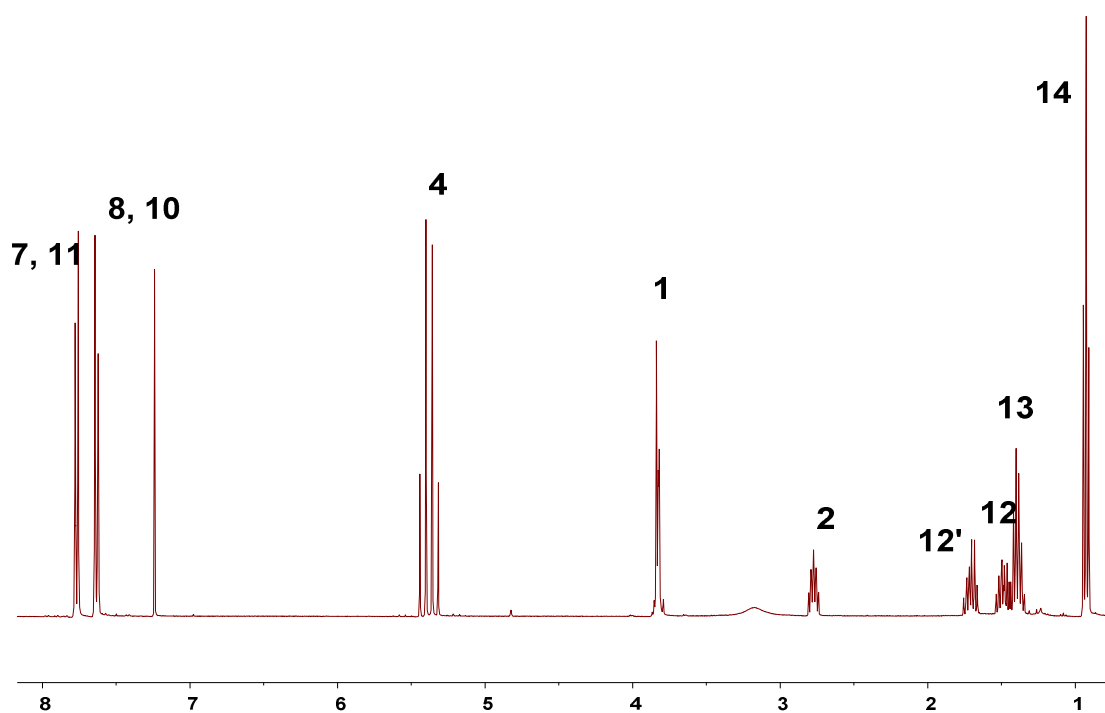
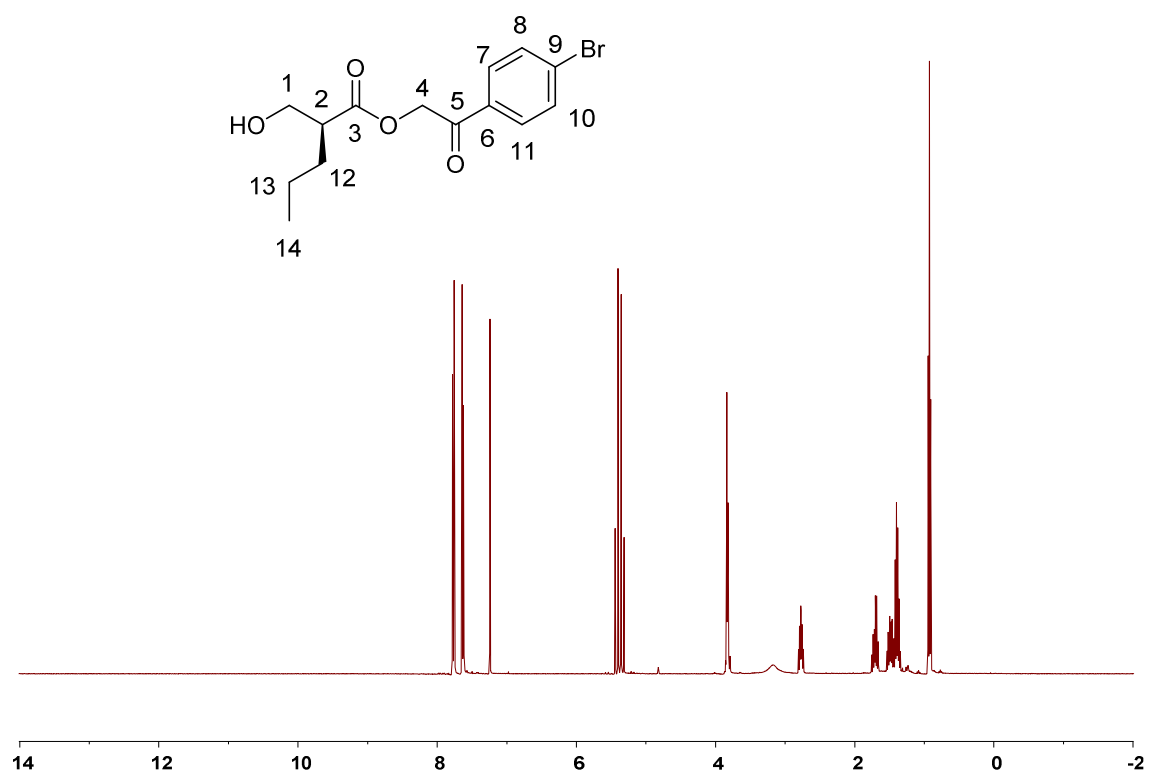


Figure S102 NMR spectra (CDCl_3) of *S*-4b: a) ^1H and b) ^{13}C .

a)



b)

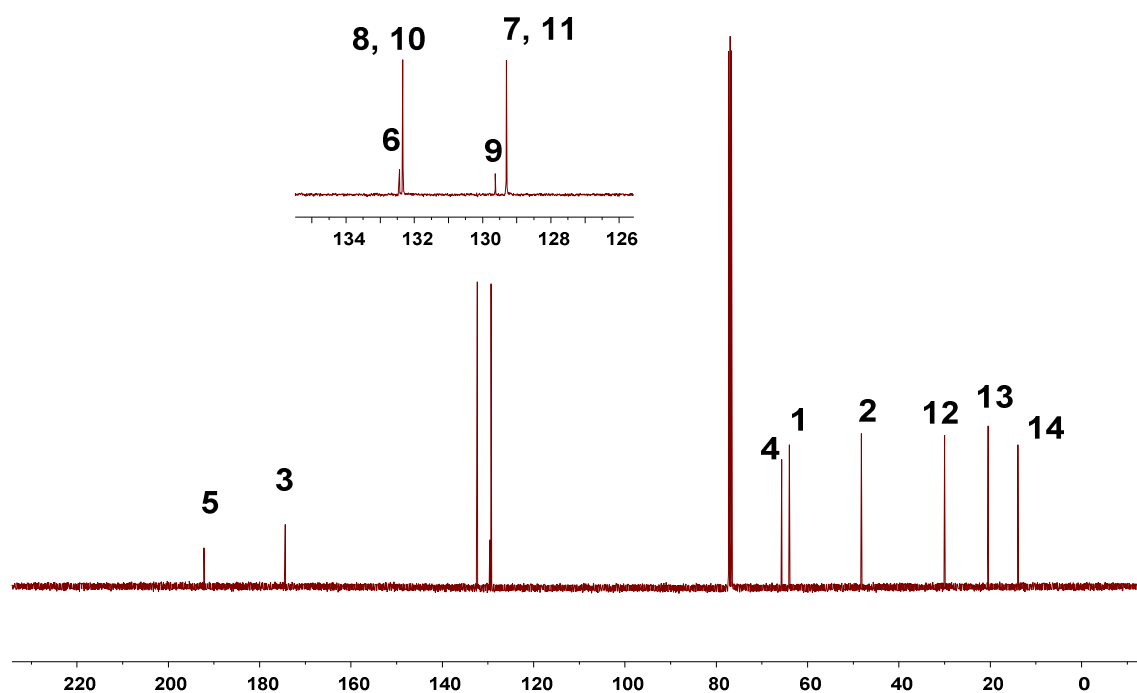
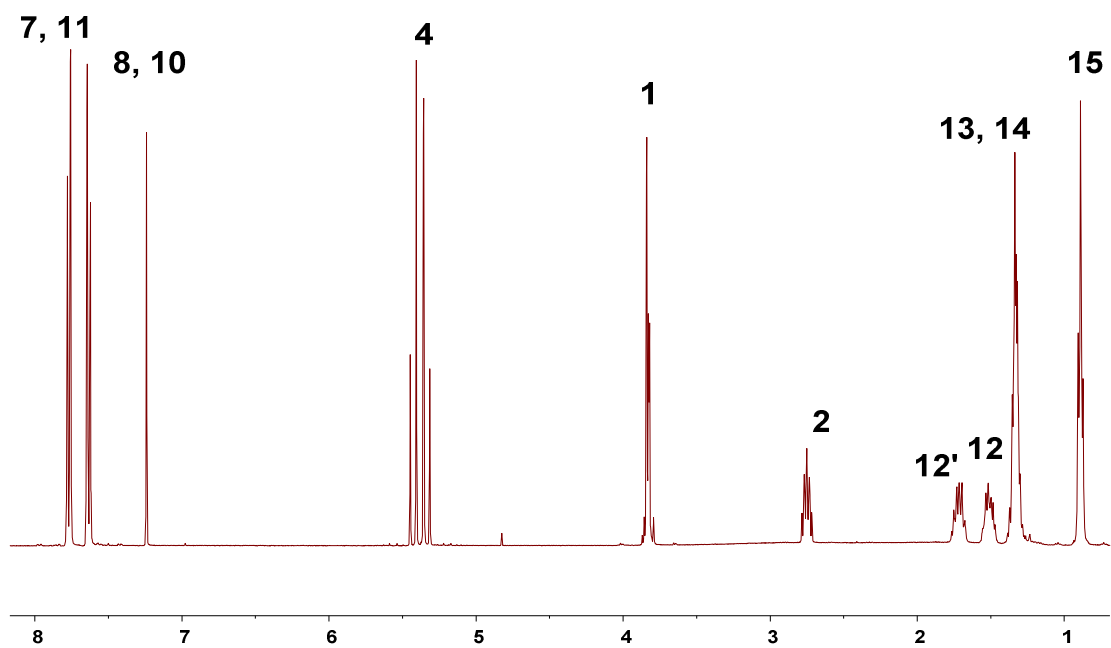
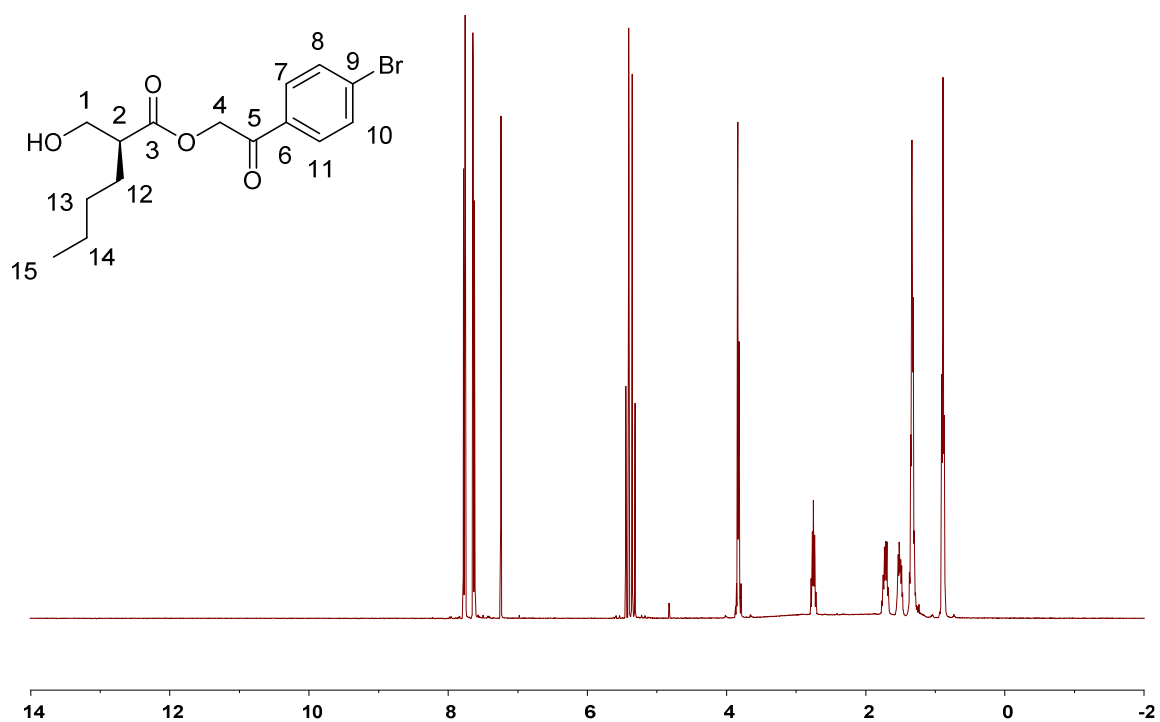


Figure S103 NMR spectra (CDCl_3) of *S*-4c: a) ^1H and b) ^{13}C .

a)



b)

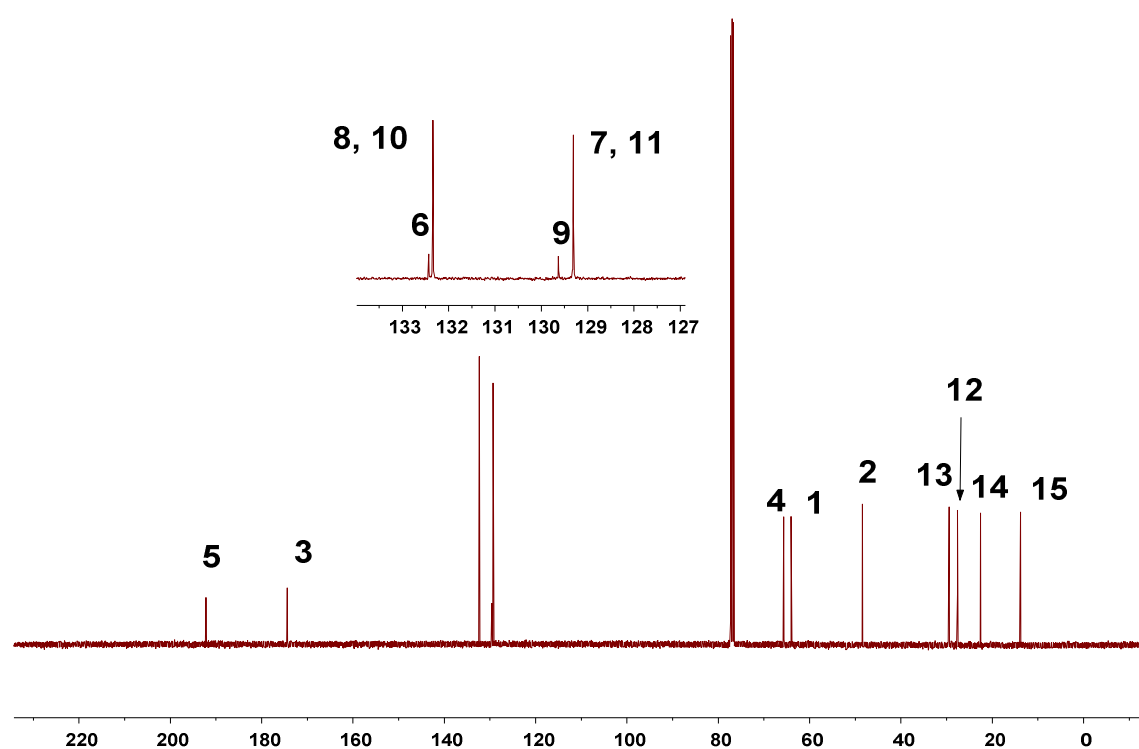
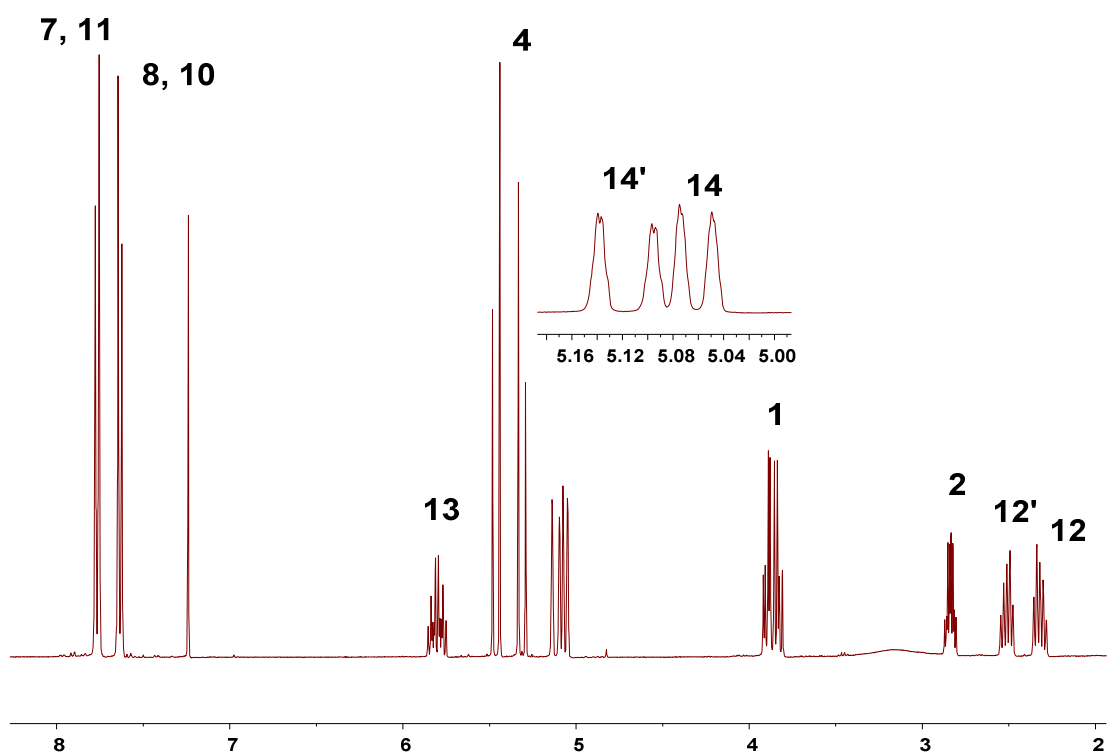
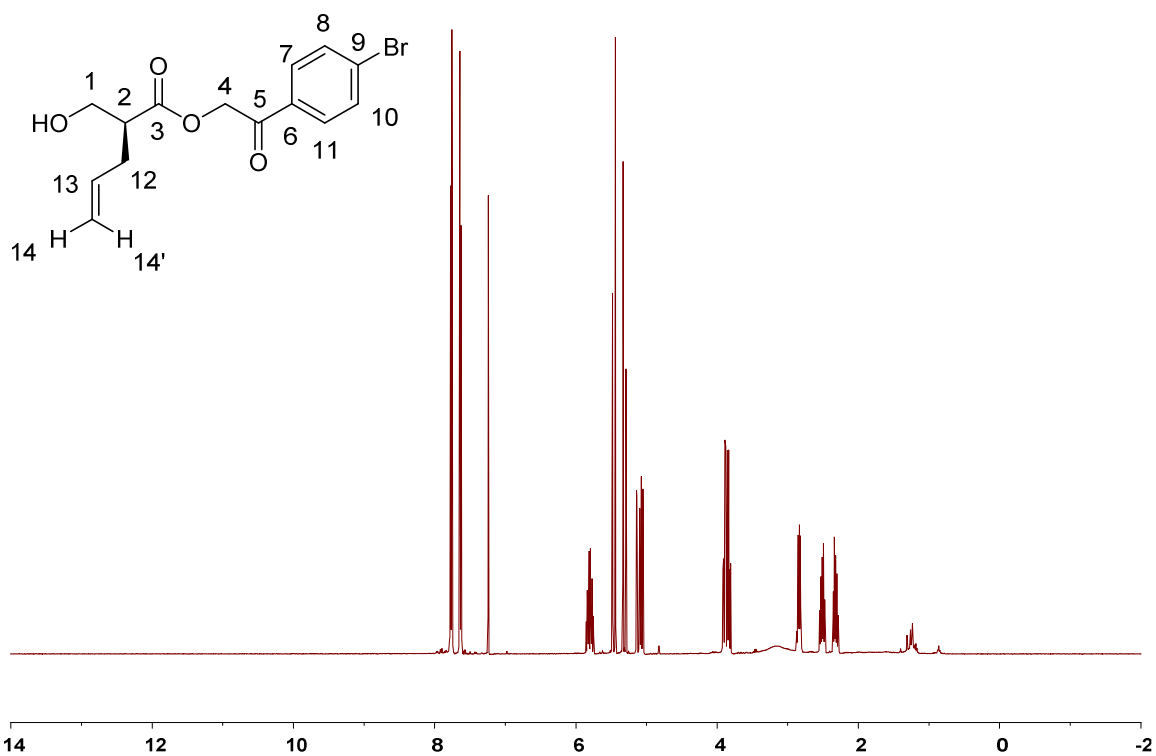


Figure S104 NMR spectra (CDCl_3) of *S*-4d: a) ^1H and b) ^{13}C .

a)



b)

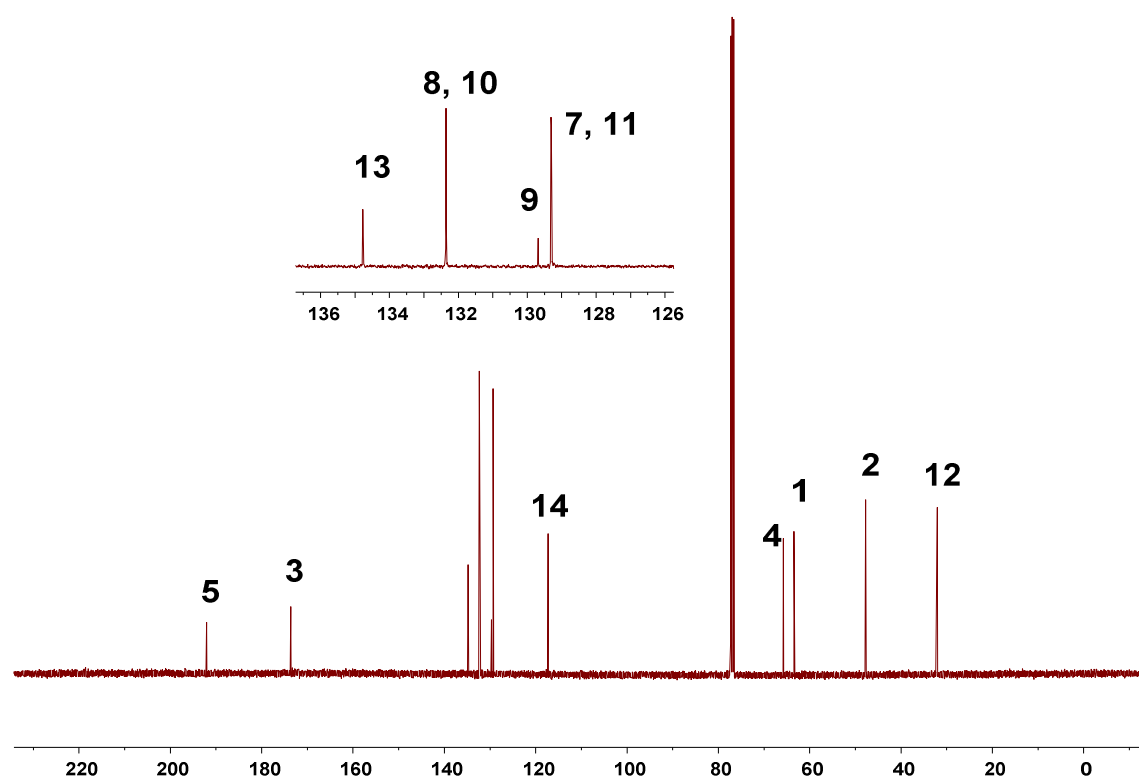
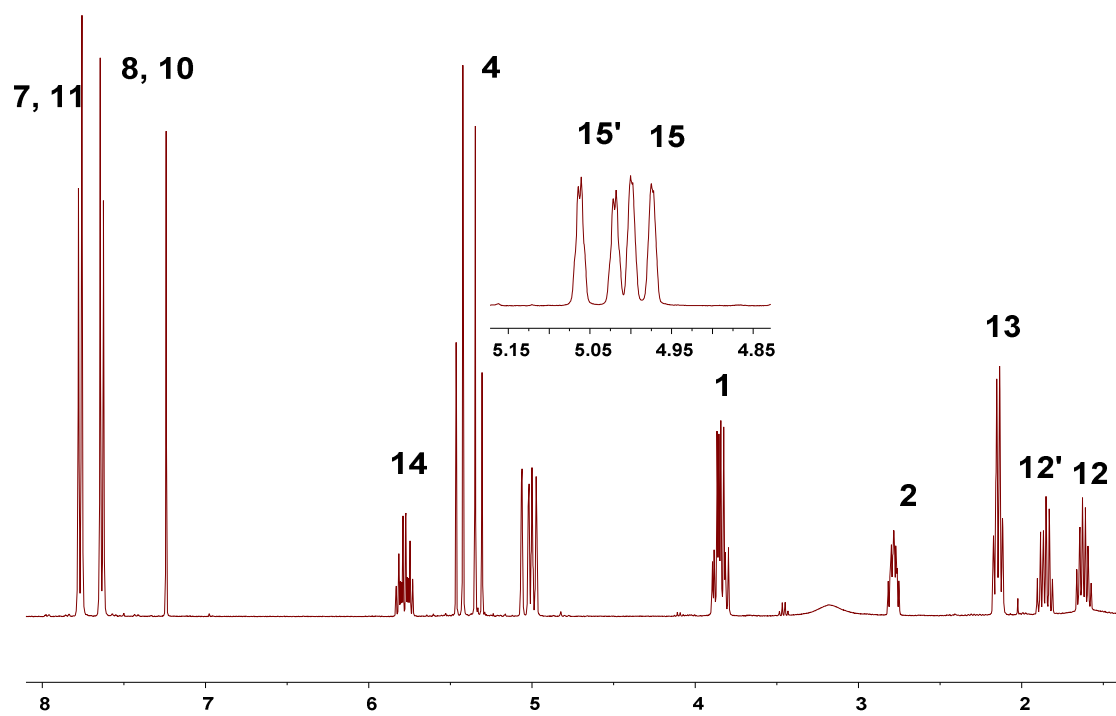
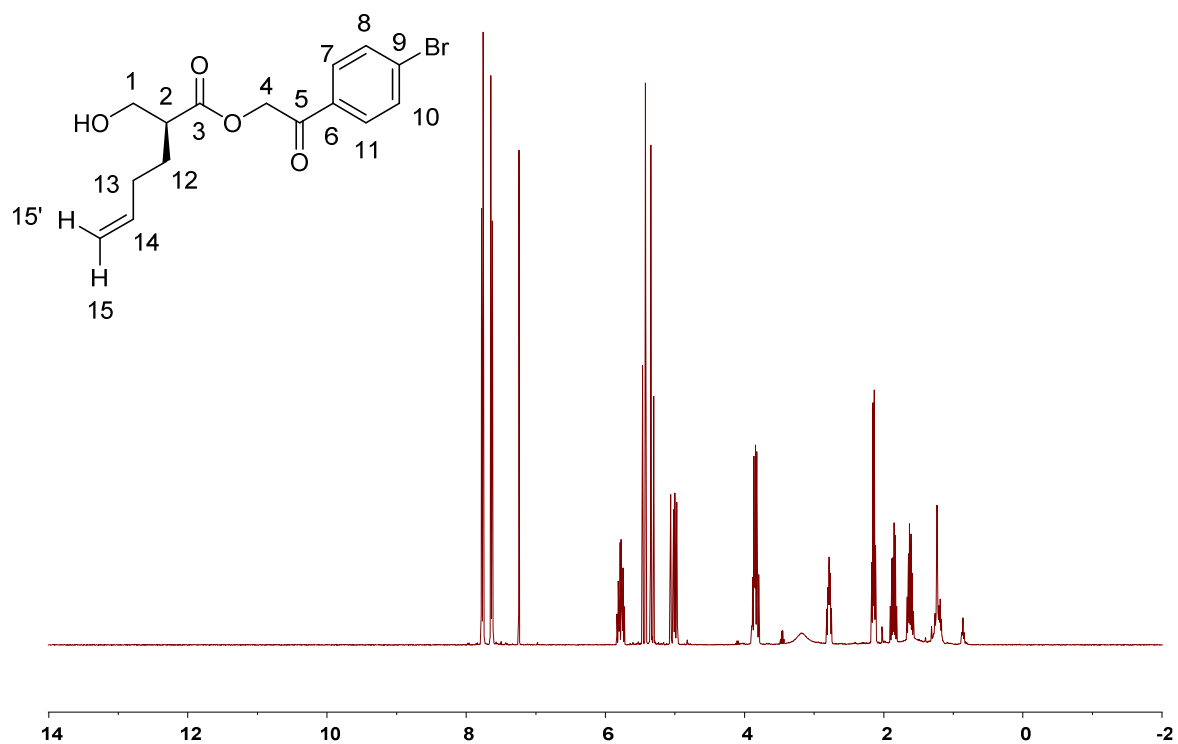


Figure S105 NMR spectra (CDCl_3) of *S*-4e: a) ^1H and b) ^{13}C .

a)



b)

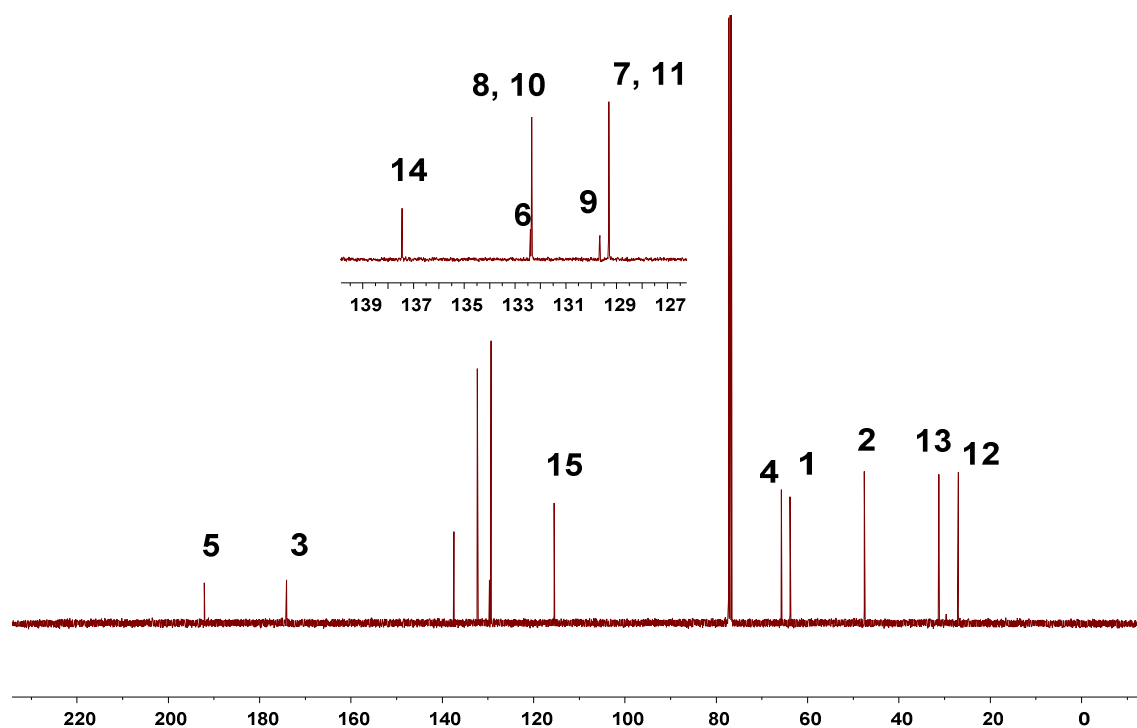
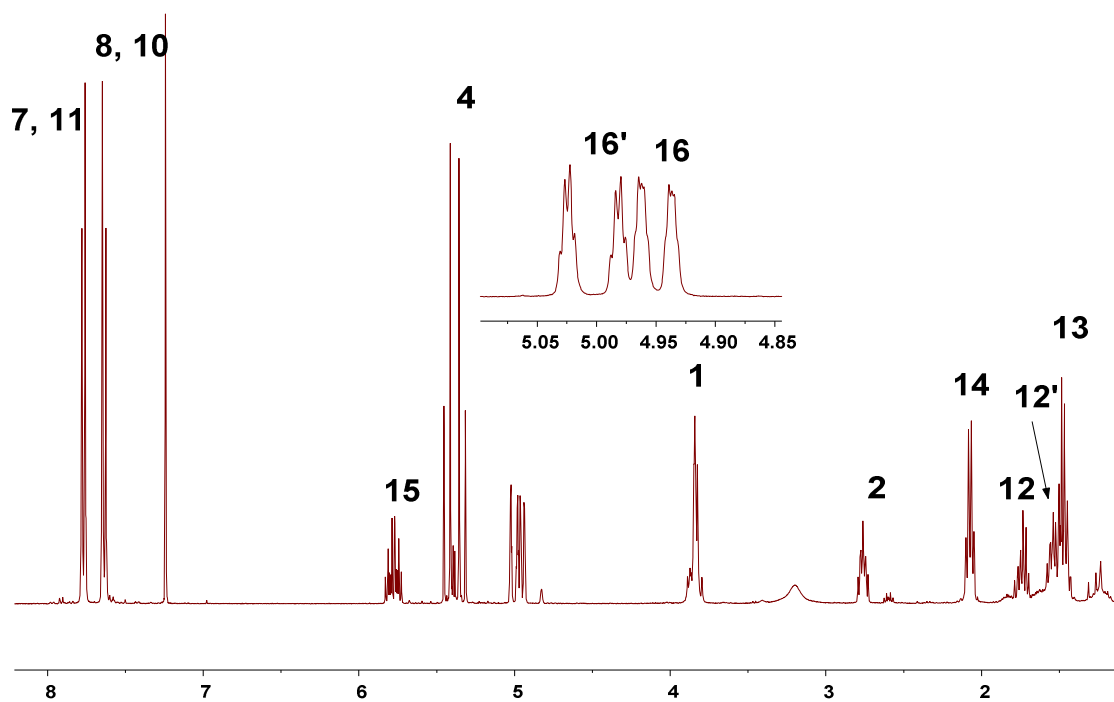
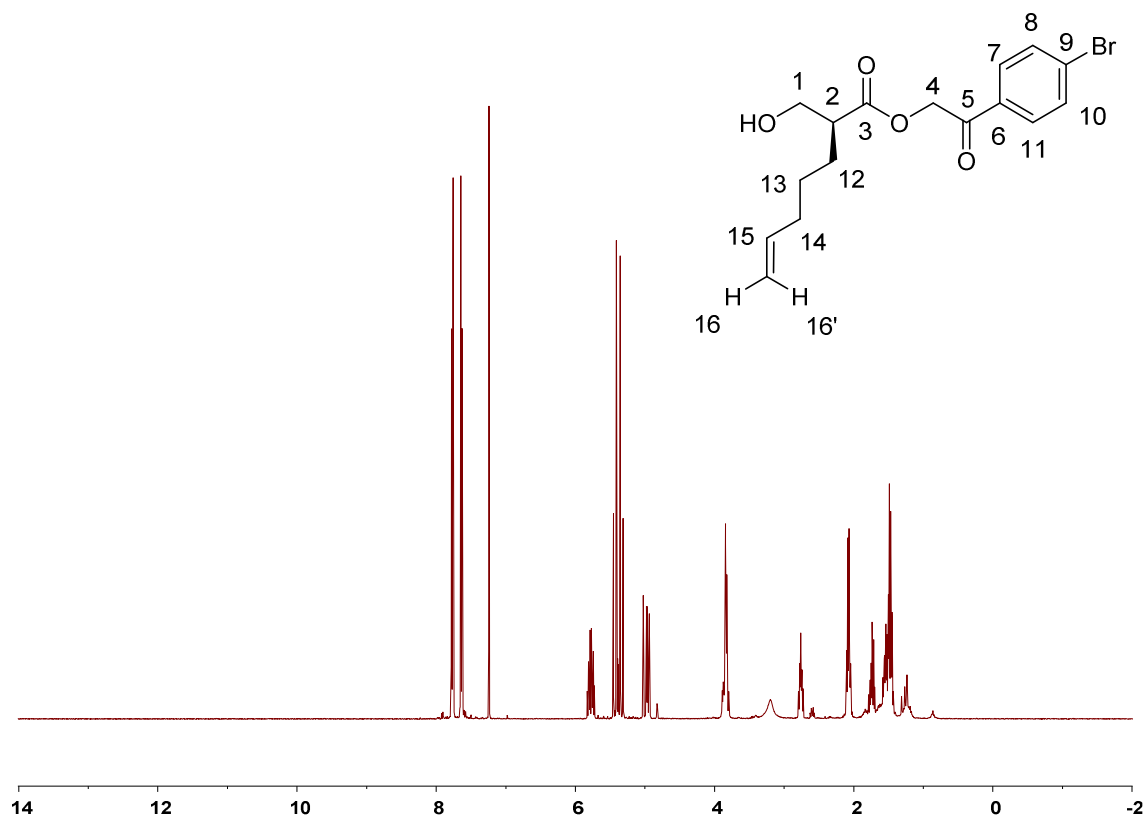


Figure S106 NMR spectra (CDCl₃) of *S*-4f: a) ¹H and b) ¹³C.

a)



b)

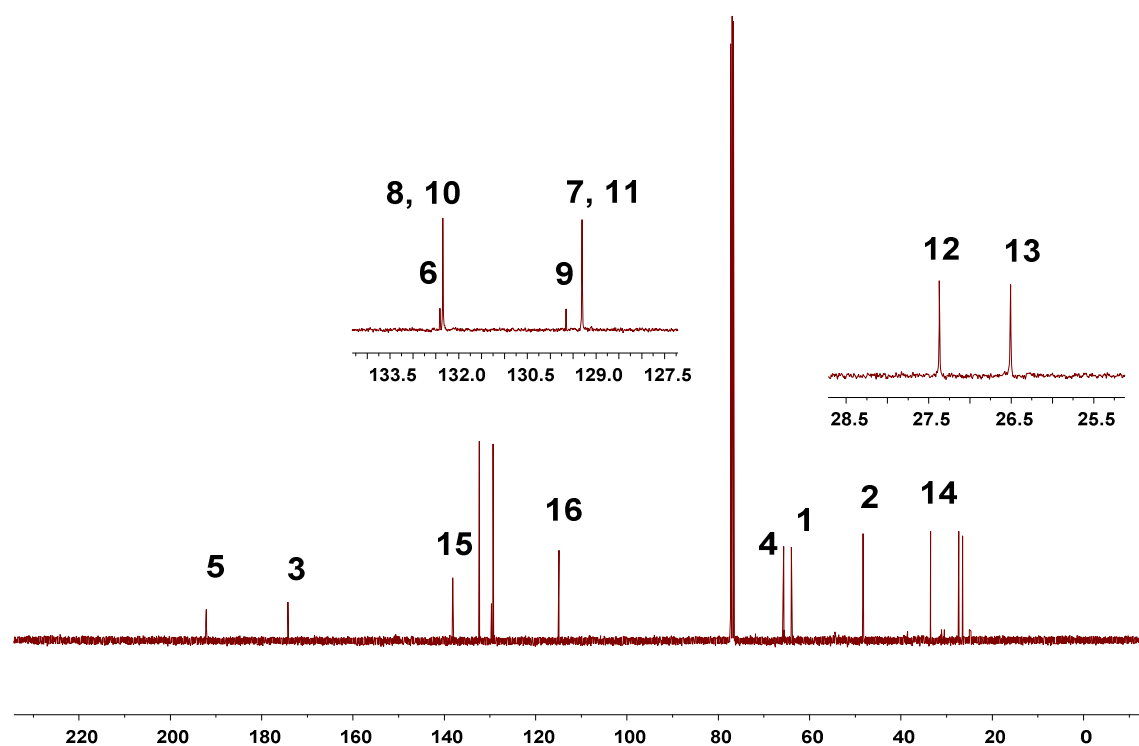
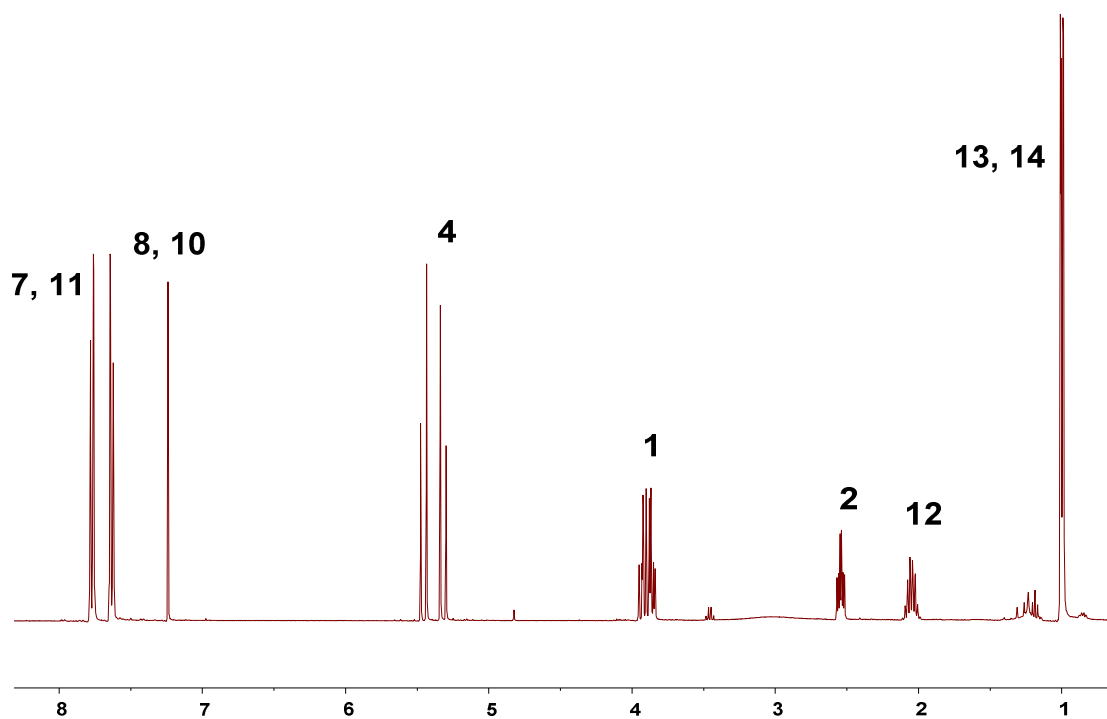
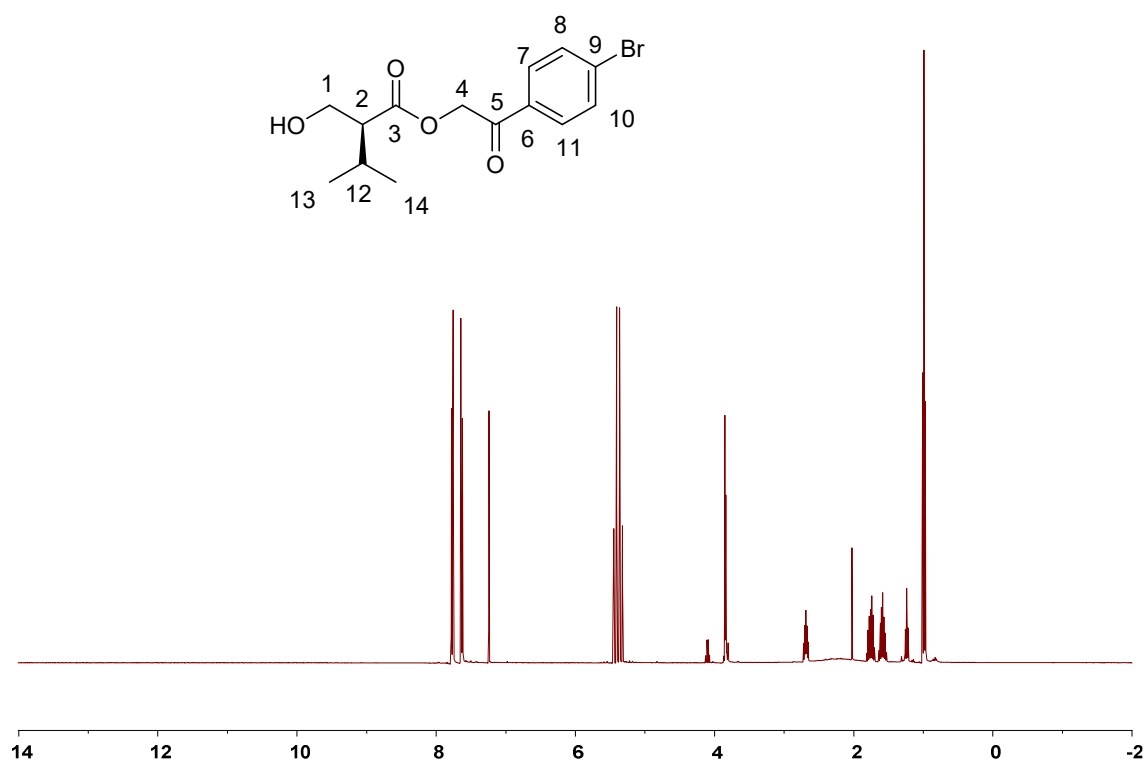


Figure S107 NMR spectra (CDCl_3) of *S*-4g: a) ^1H and b) ^{13}C .

a)



b)

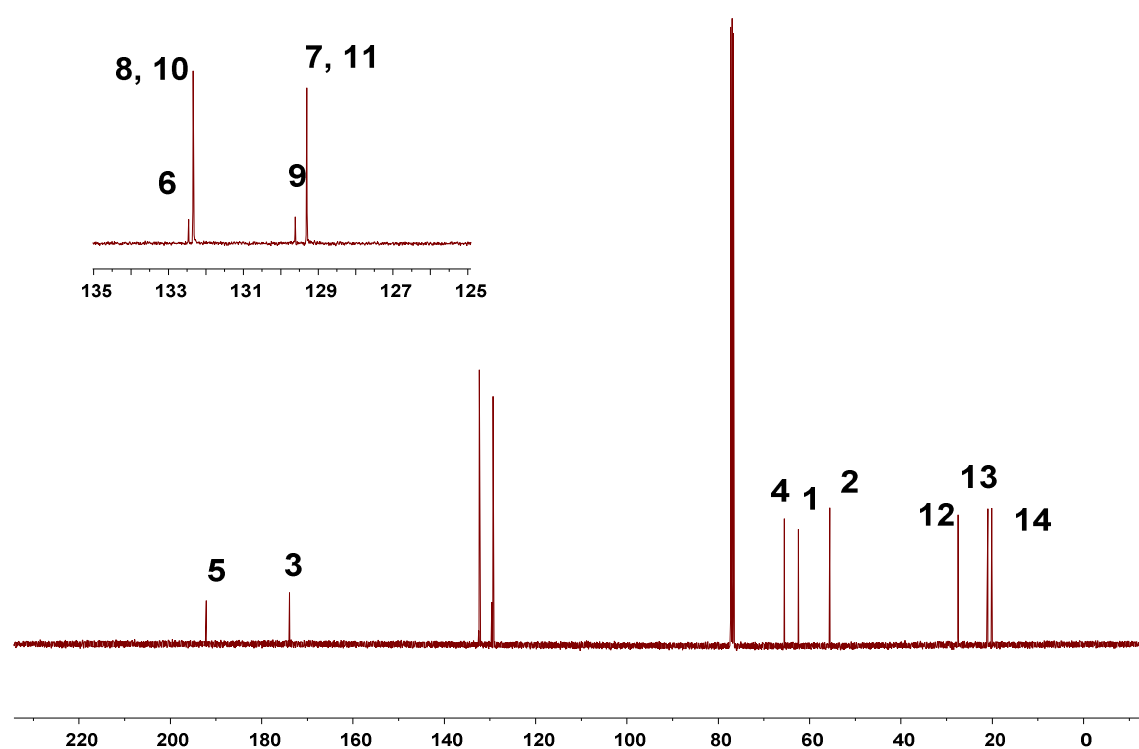
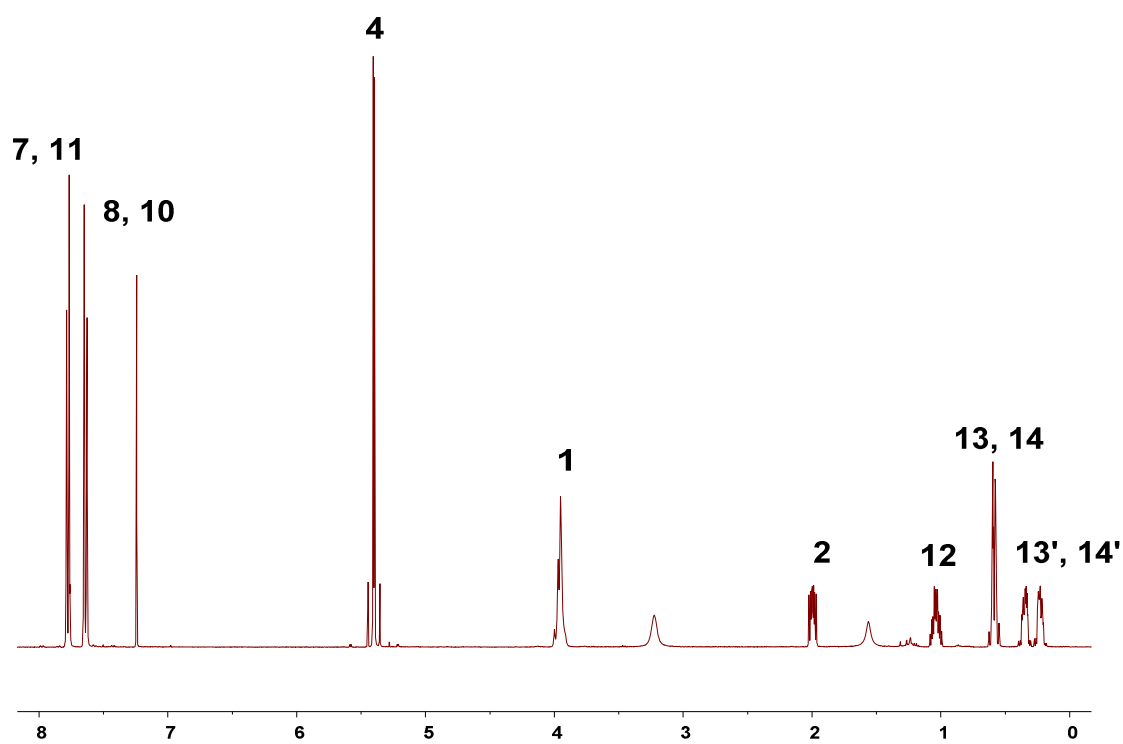
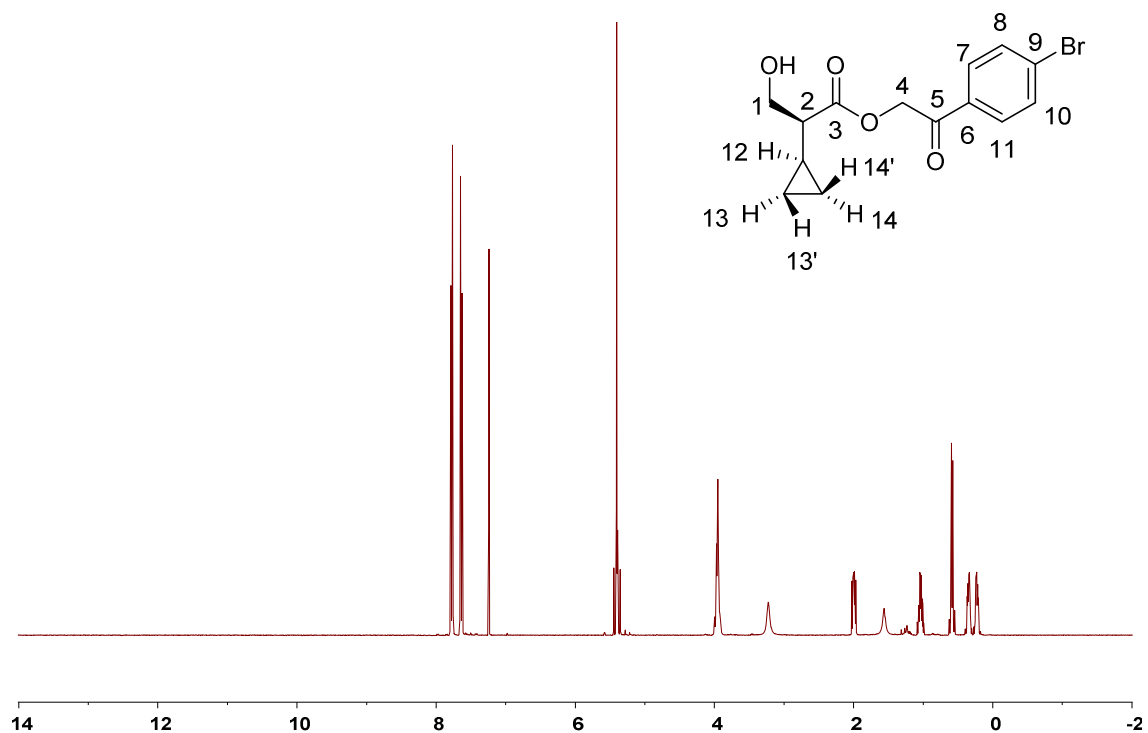


Figure S108 NMR spectra (CDCl_3) of *S*-4h: a) ^1H and b) ^{13}C .

a)



b)

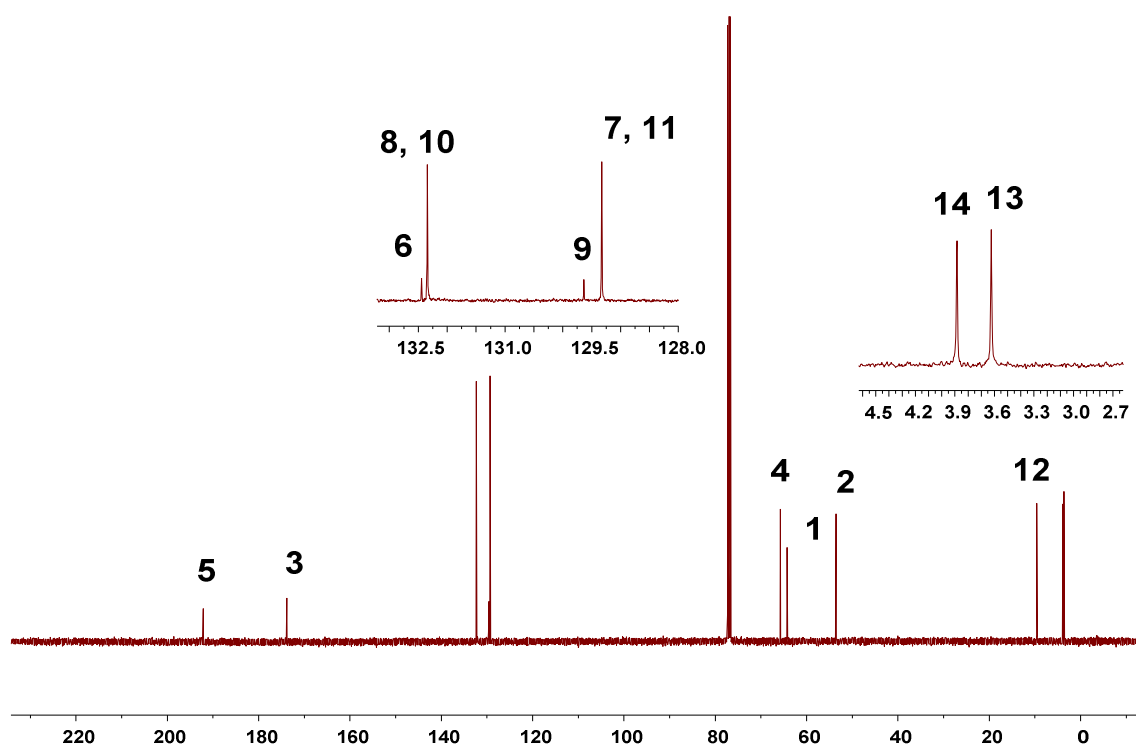
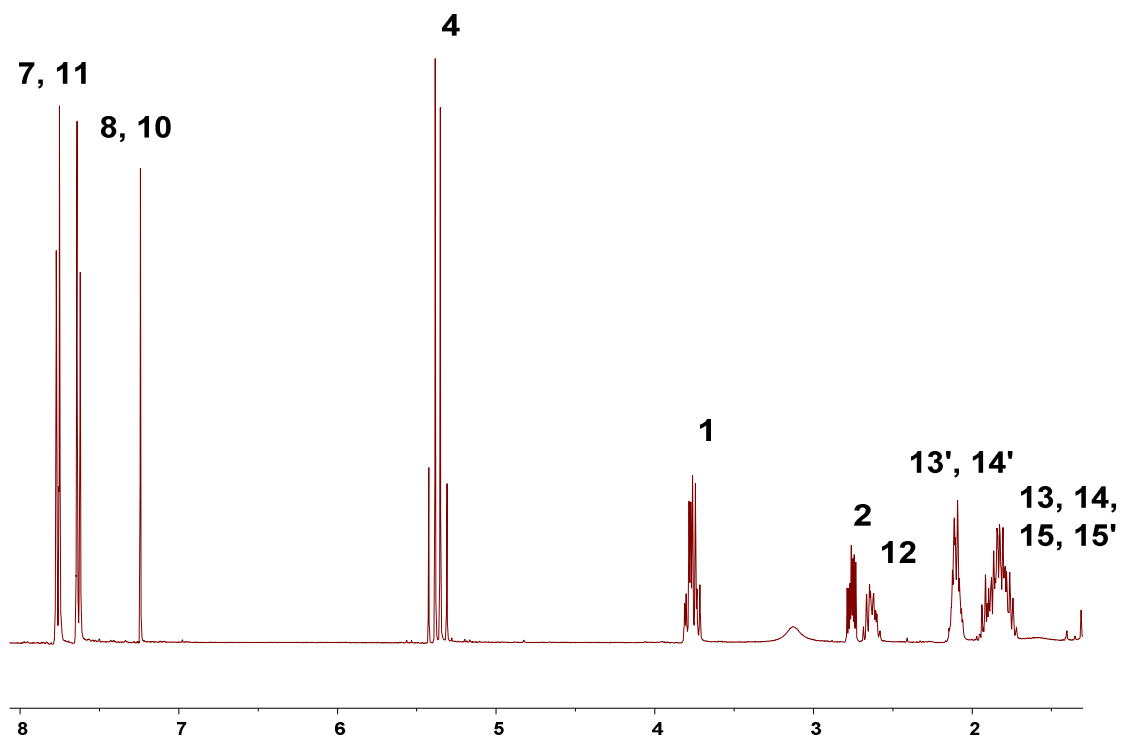
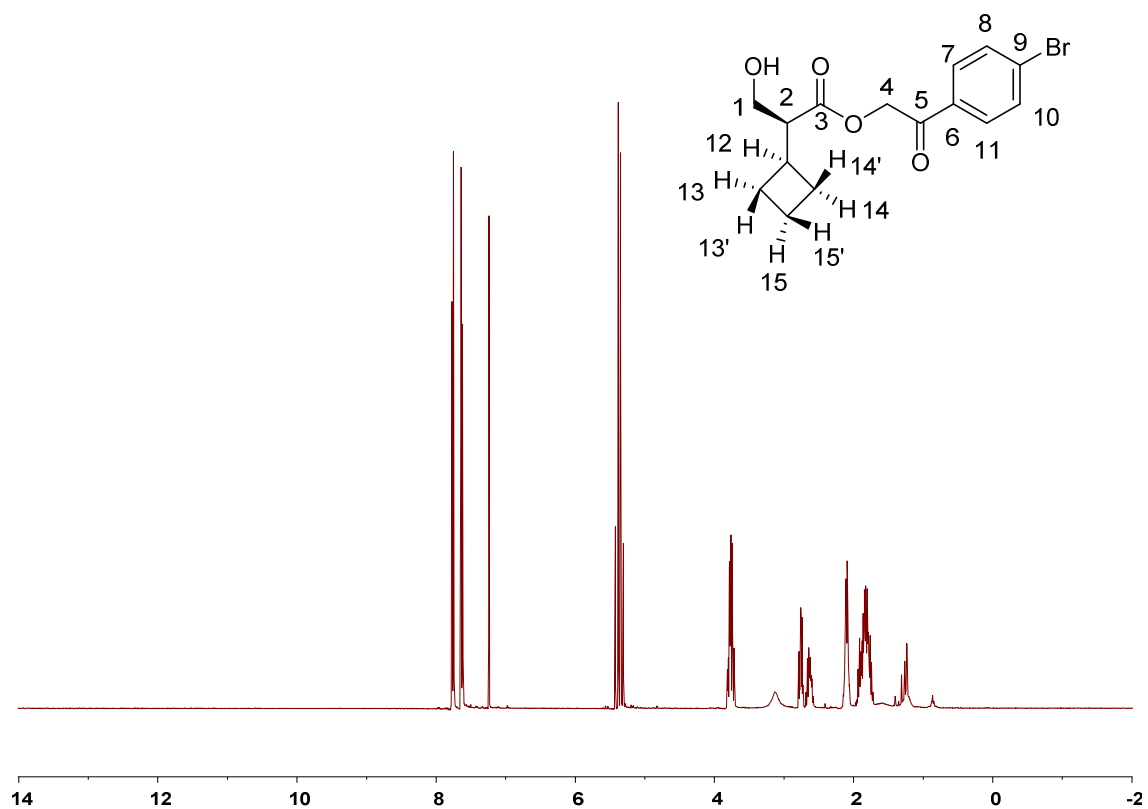


Figure S109 NMR spectra (CDCl_3) of *S*-4i: a) ^1H and b) ^{13}C .

a)



b)

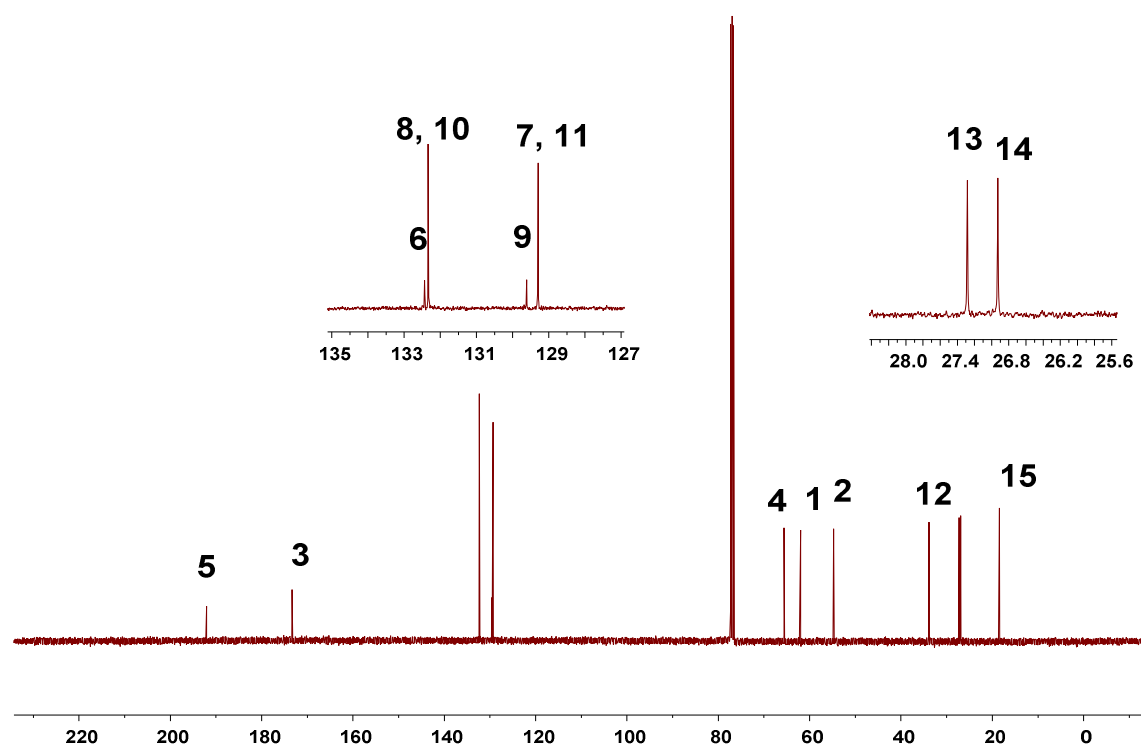
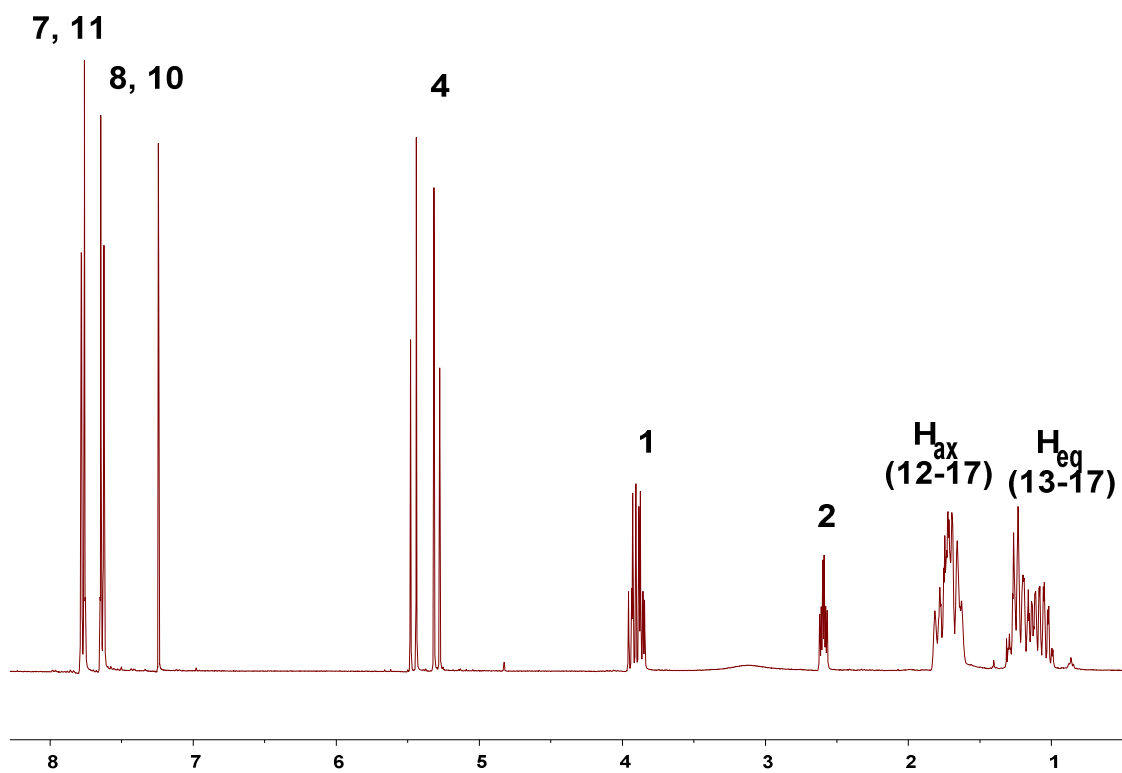
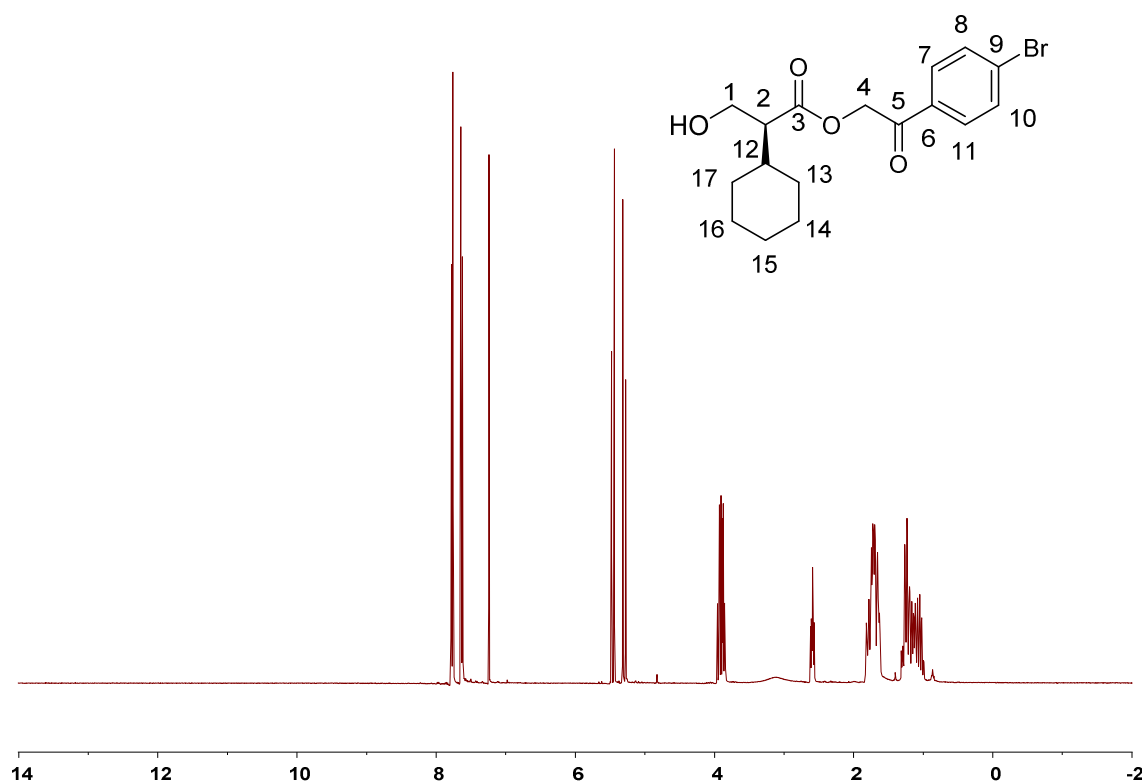


Figure S110 NMR spectra (CDCl_3) of *S*-4j: a) ^1H and b) ^{13}C .

a)



b)

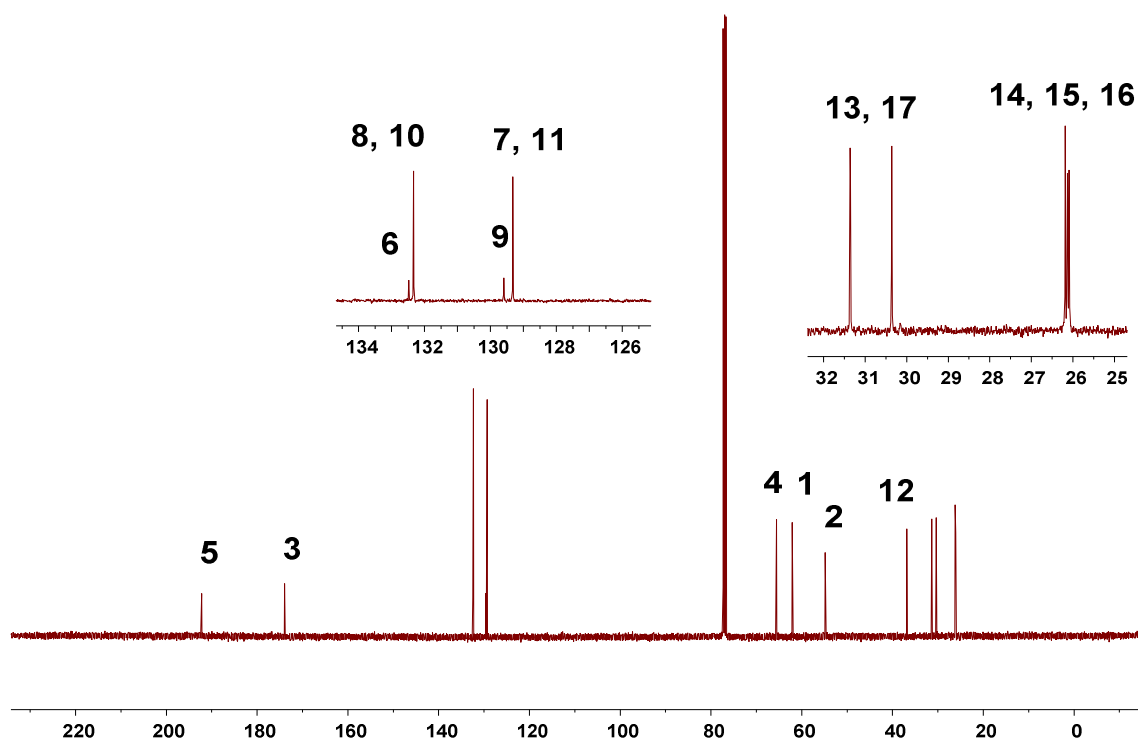
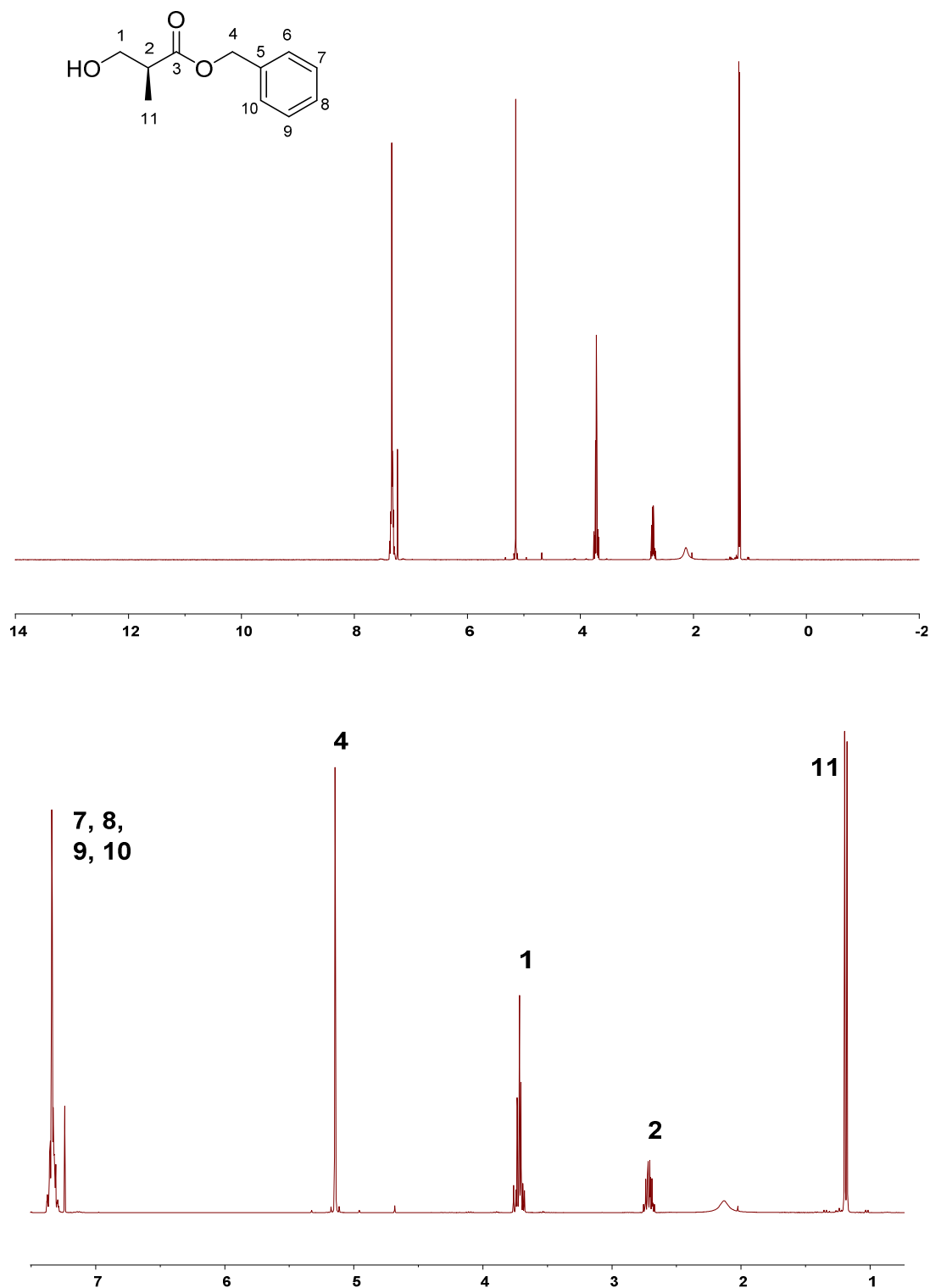


Figure S111 NMR spectra (CDCl_3) of *S*-4k: a) ^1H and b) ^{13}C .

6.2.4 NMR spectra of benzyl and 4-NO₂ benzyl esters

The NMR spectra of enantiomeric aldol adducts derivatives obtained with MBP-YfaU and KPHMT variants were undistinguishable. For the sake of simplicity only the *S*-enantiomers are shown.

a)



b)

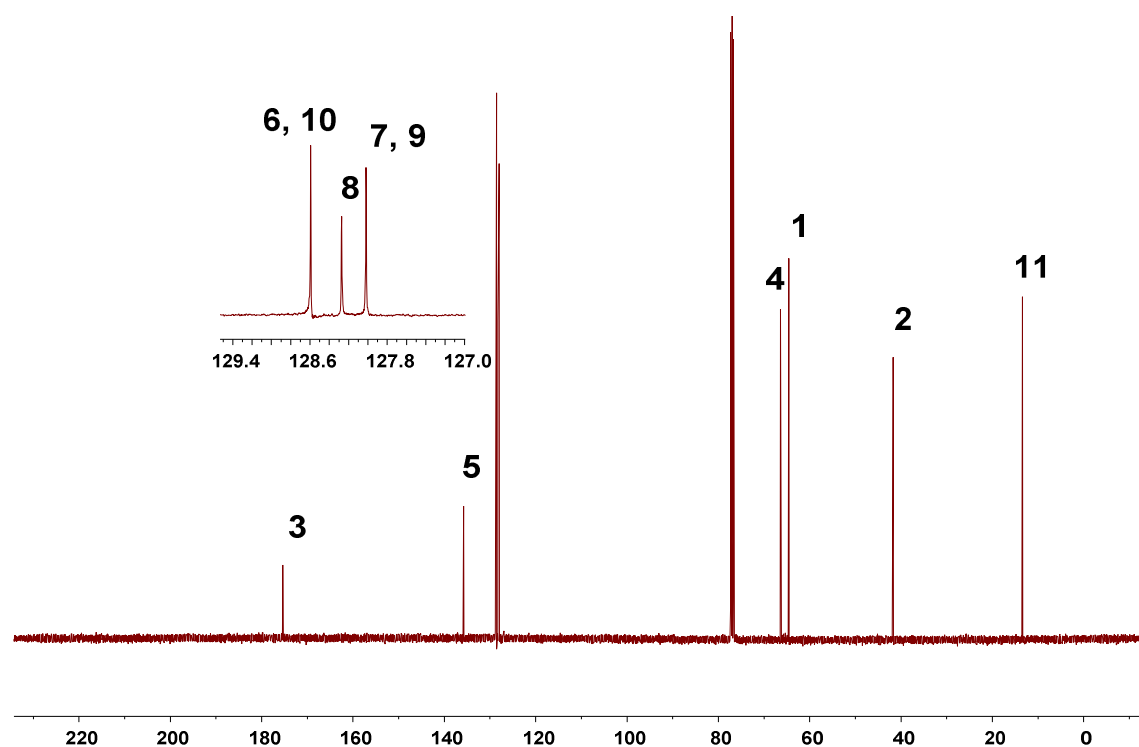
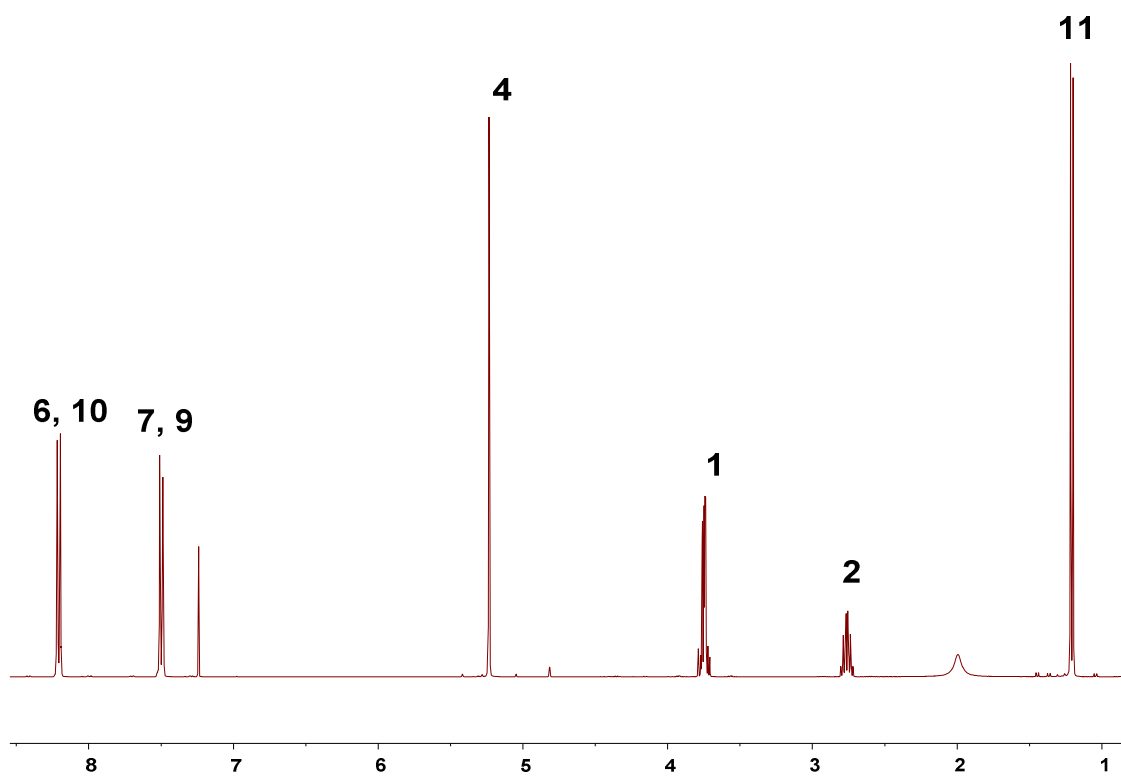
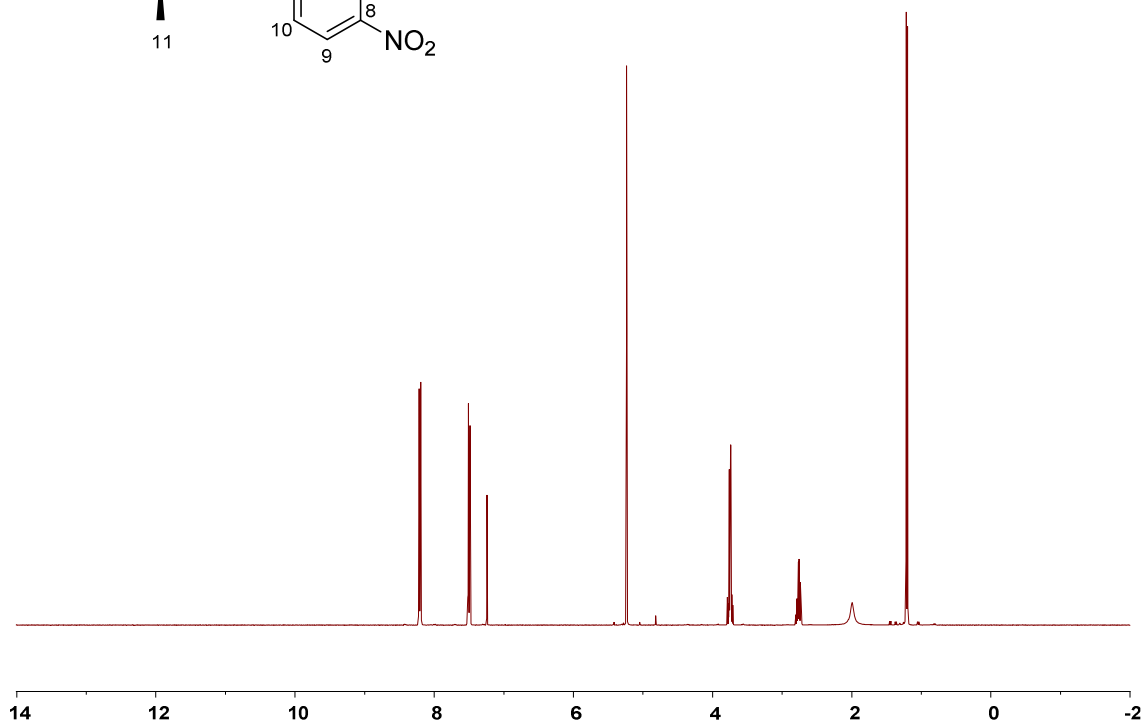
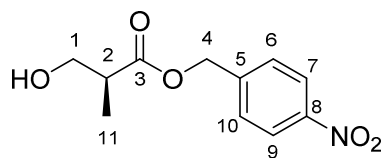


Figure S112 NMR spectra (CDCl_3) of *S*-5a: a) ^1H and b) ^{13}C .

a)



b)

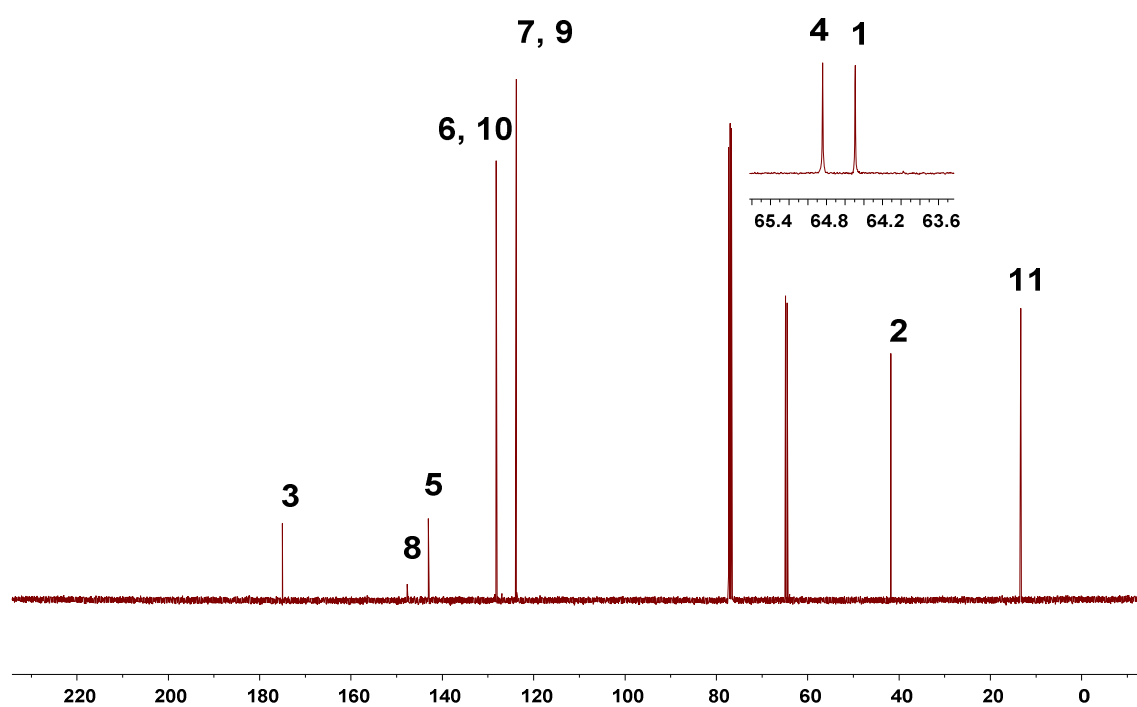
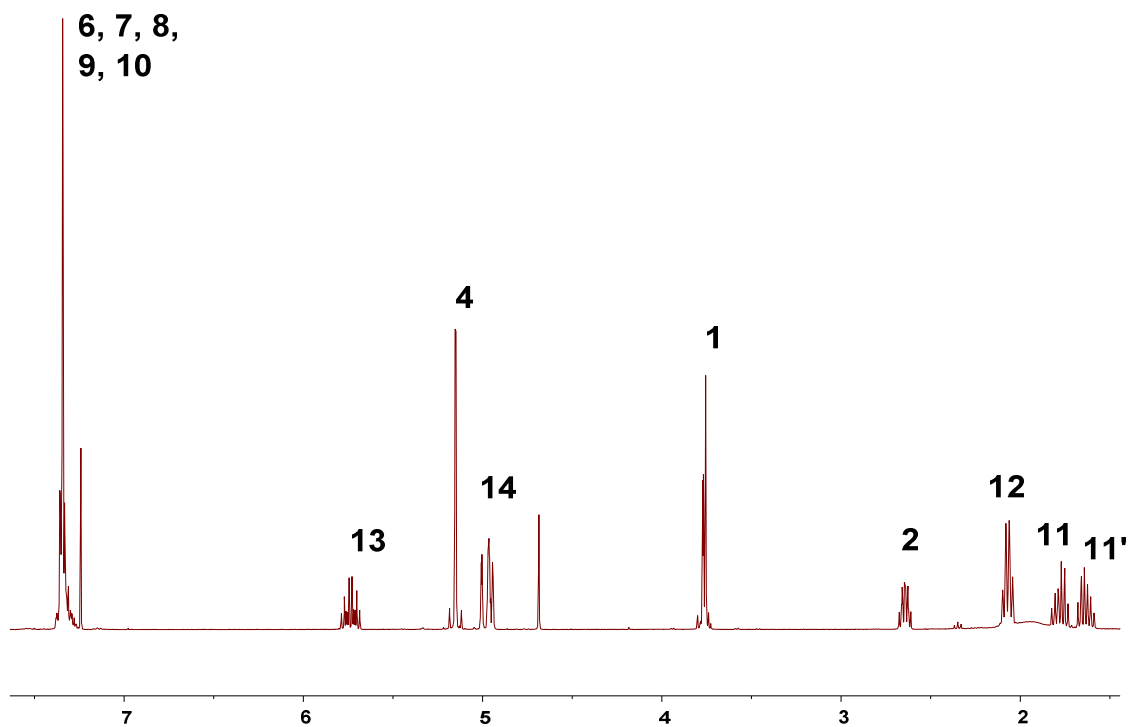
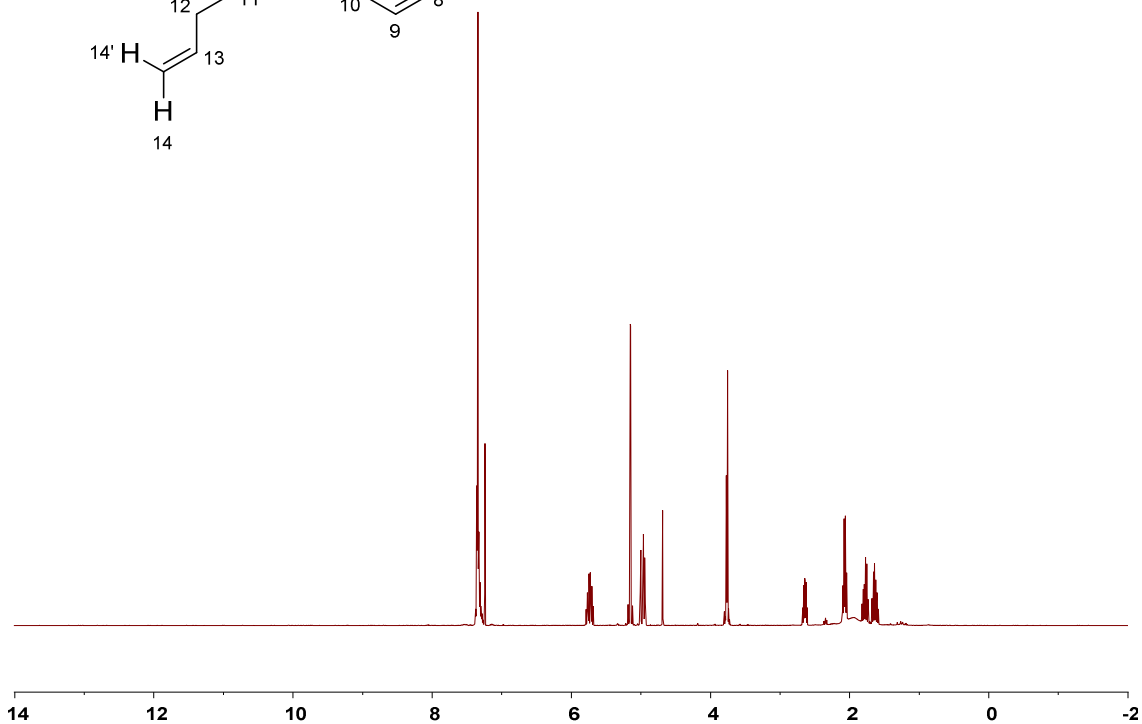
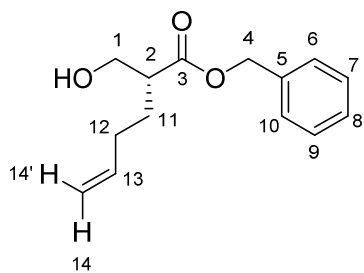


Figure S113 NMR spectra (CDCl₃) of *S*-**5b**: a) ¹H and b) ¹³C.

a)



b)

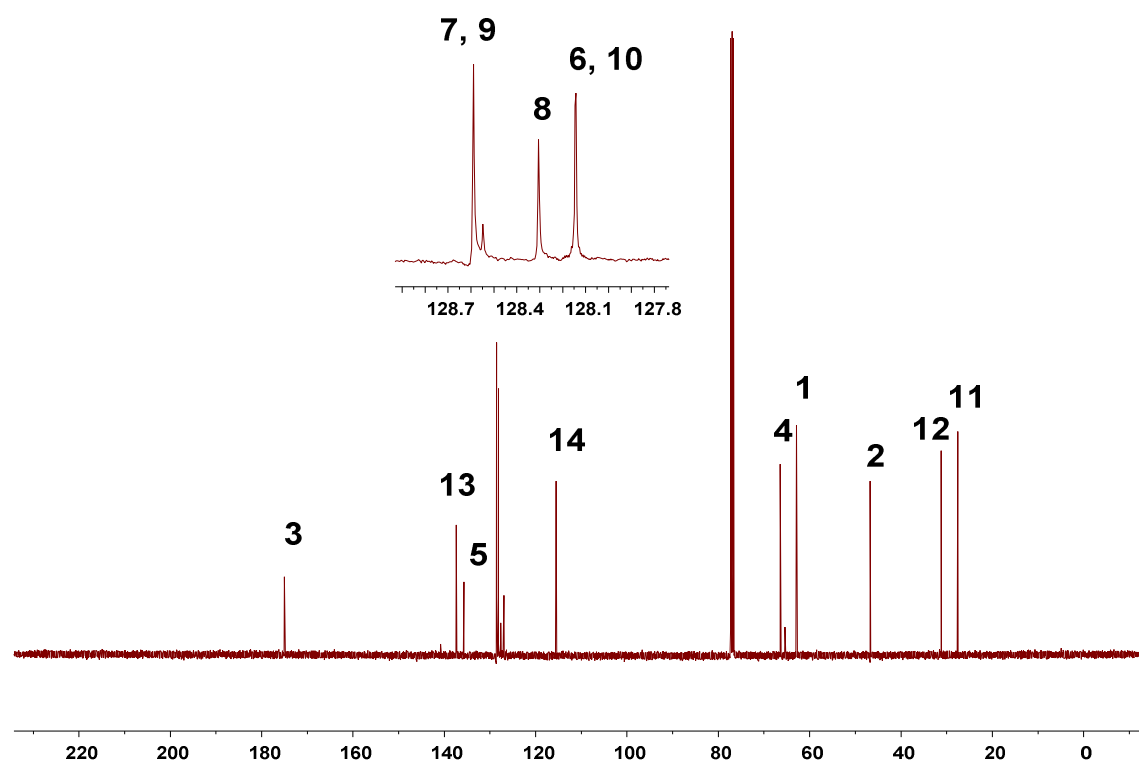
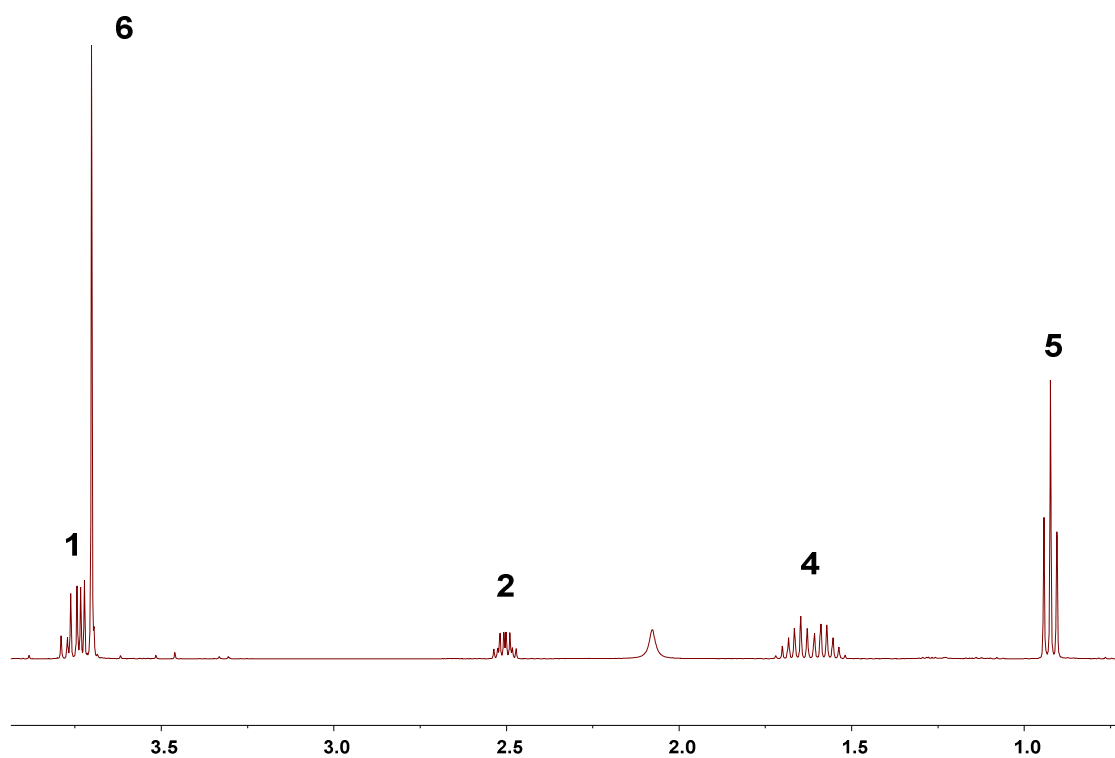
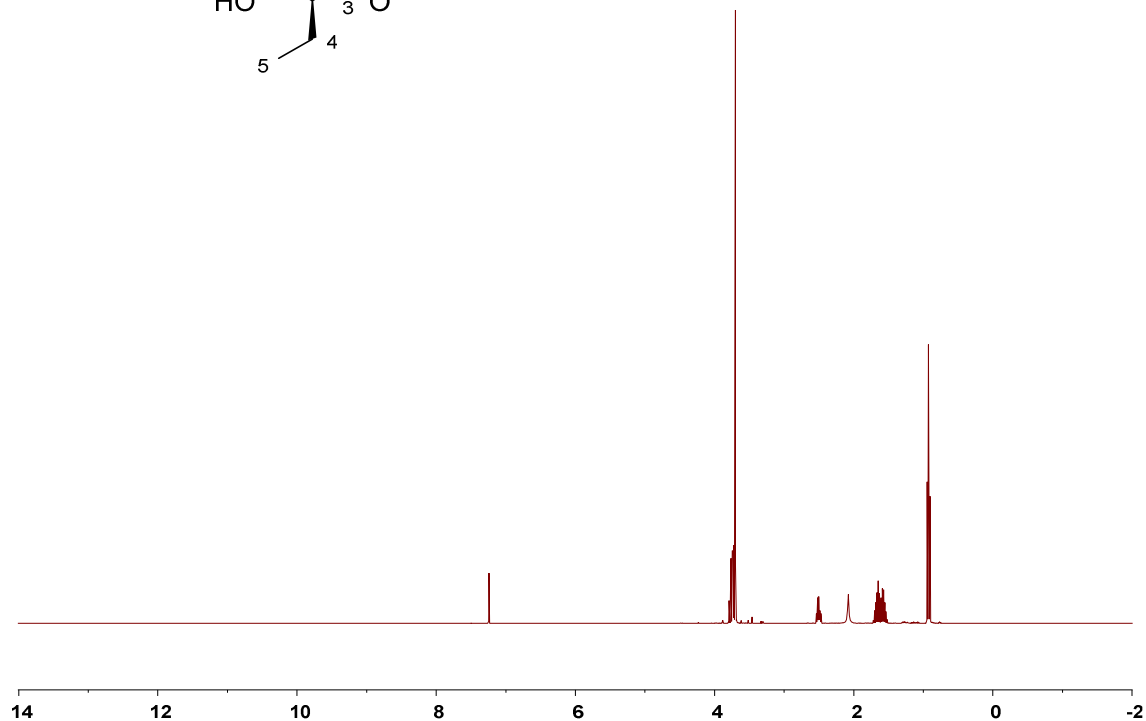
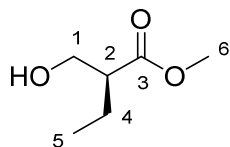


Figure S114 NMR spectra (CDCl_3) of *R*-5c: a) ^1H and b) ^{13}C .

NMR spectra of methyl ester and carboxylic acid derivatives (6a–e)

a)



b)

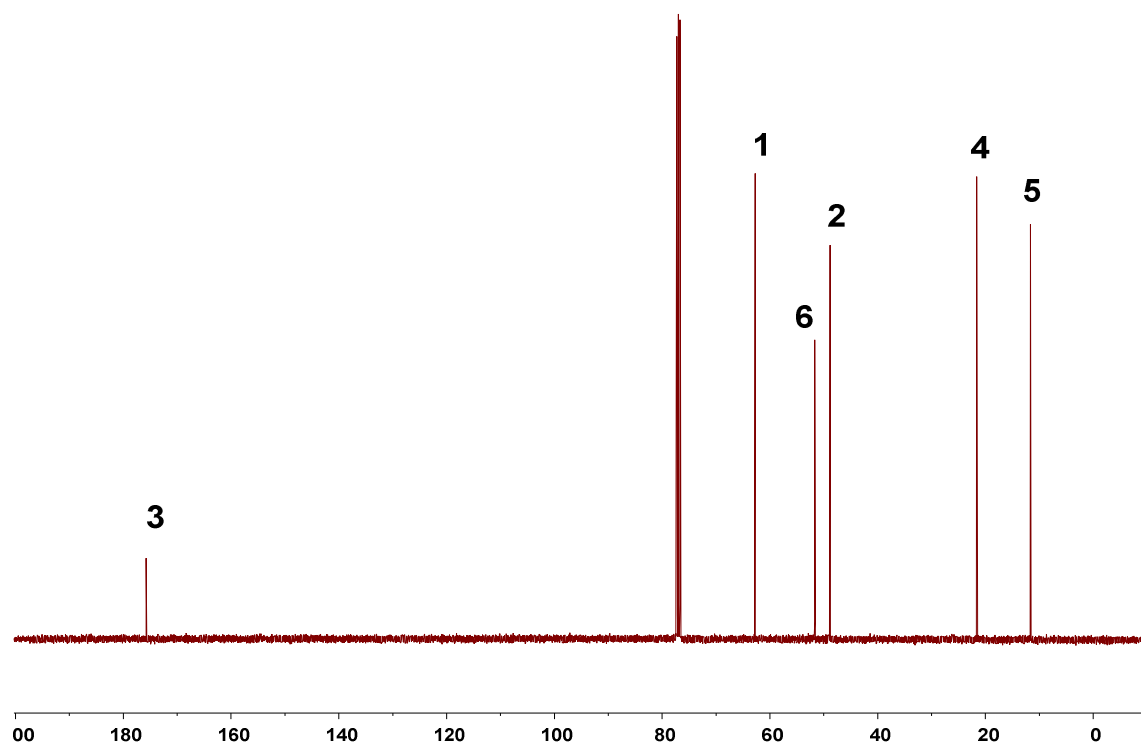
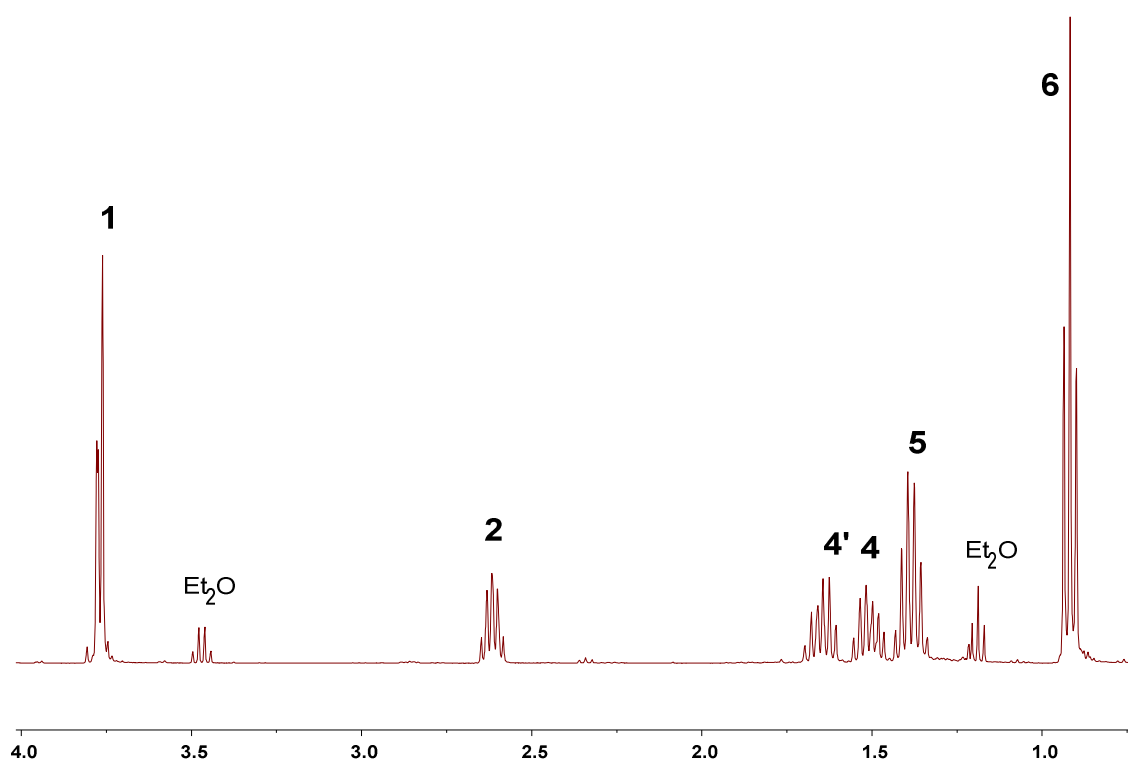
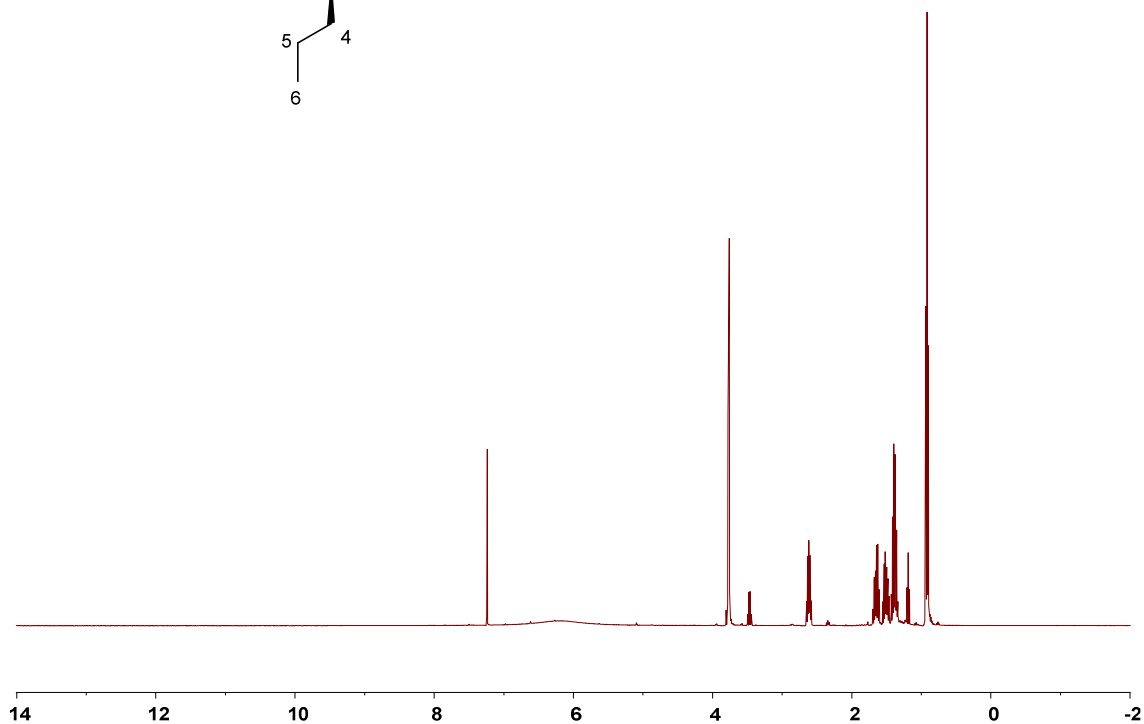
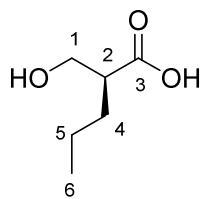


Figure S115 NMR spectra (CDCl_3) of *S*-6a: a) ^1H and b) ^{13}C .

a)



b)

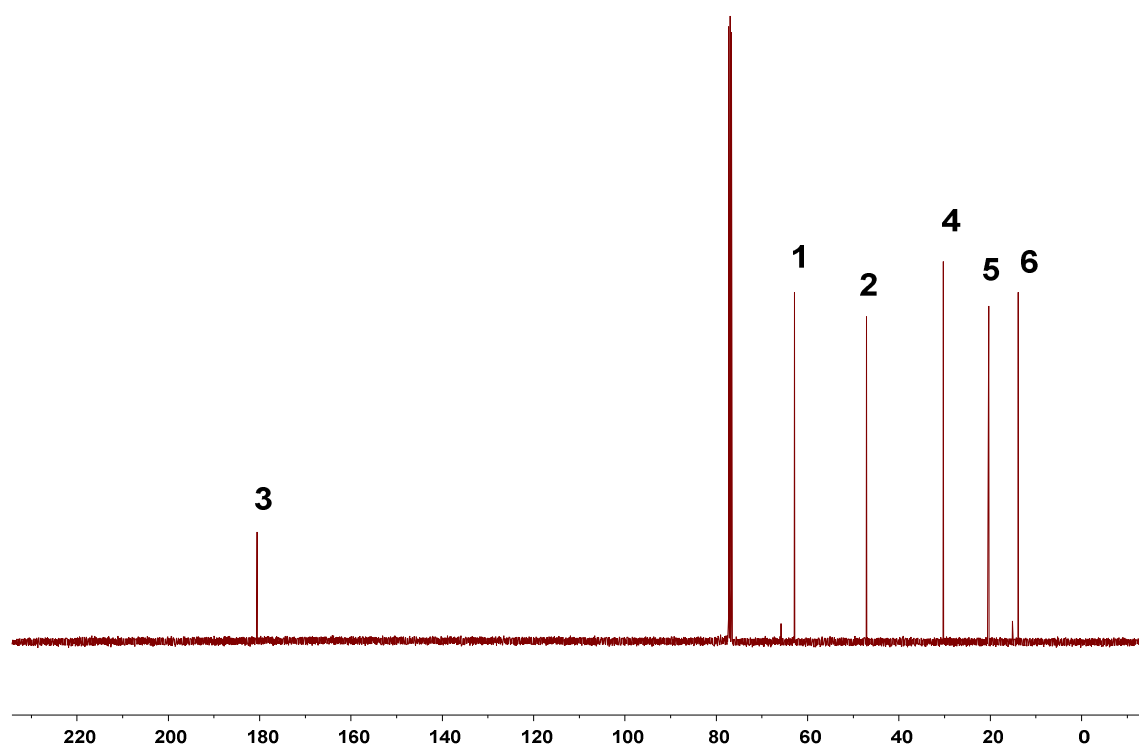
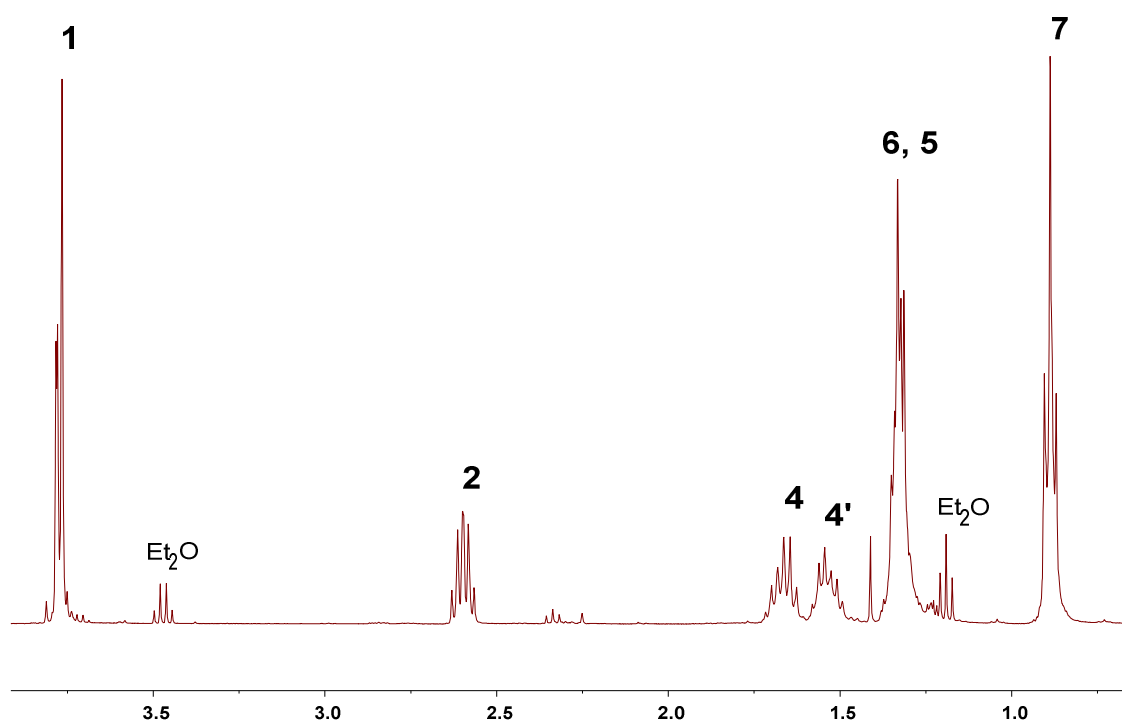
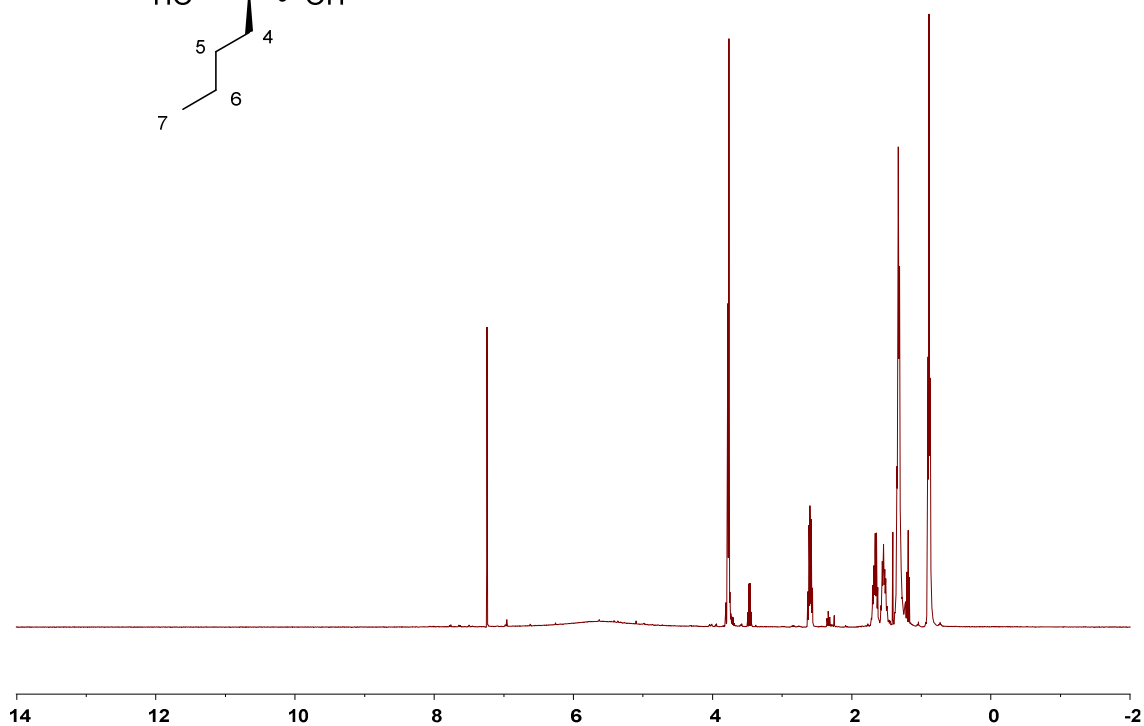
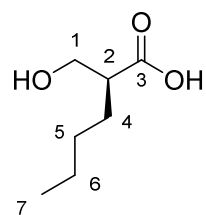


Figure S116 NMR spectra (CDCl_3) of *S*-6b: a) ^1H and b) ^{13}C .

a)



b)

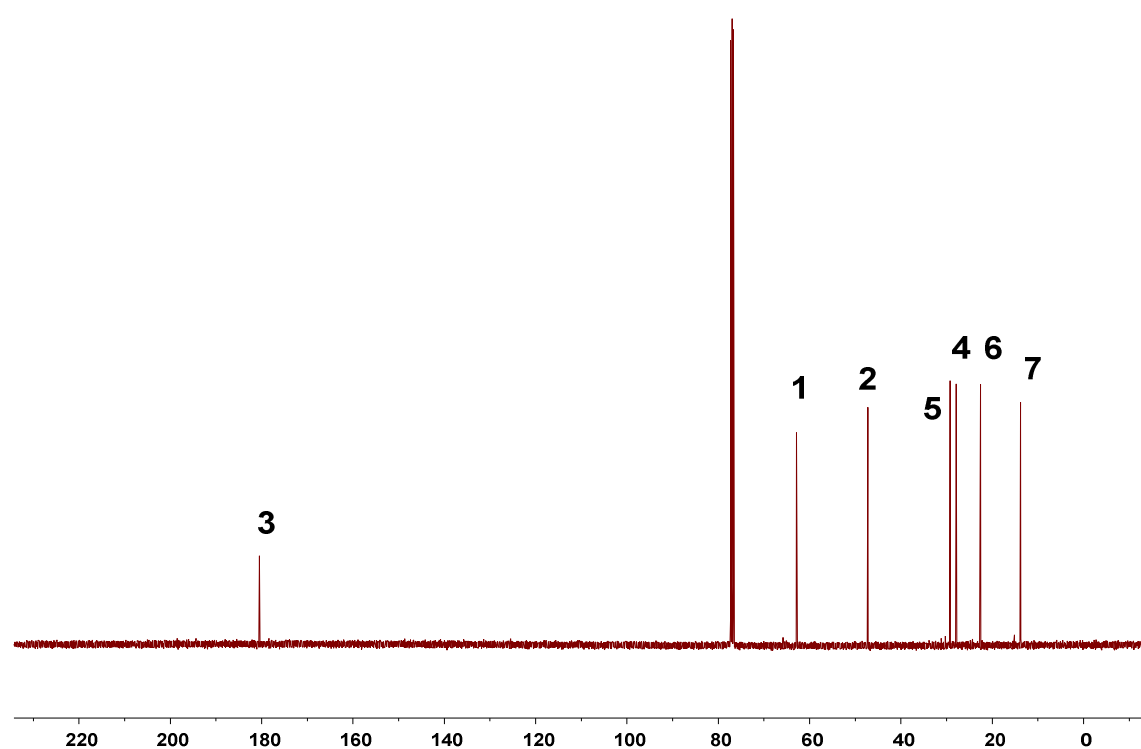
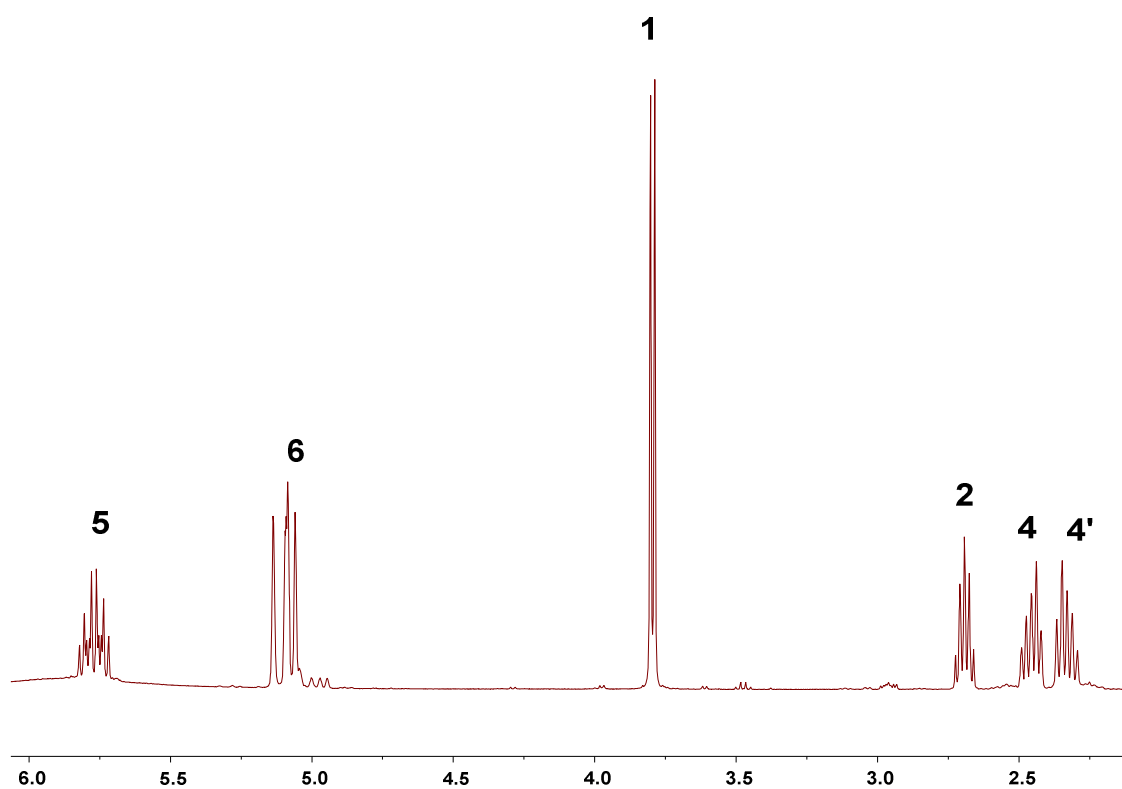
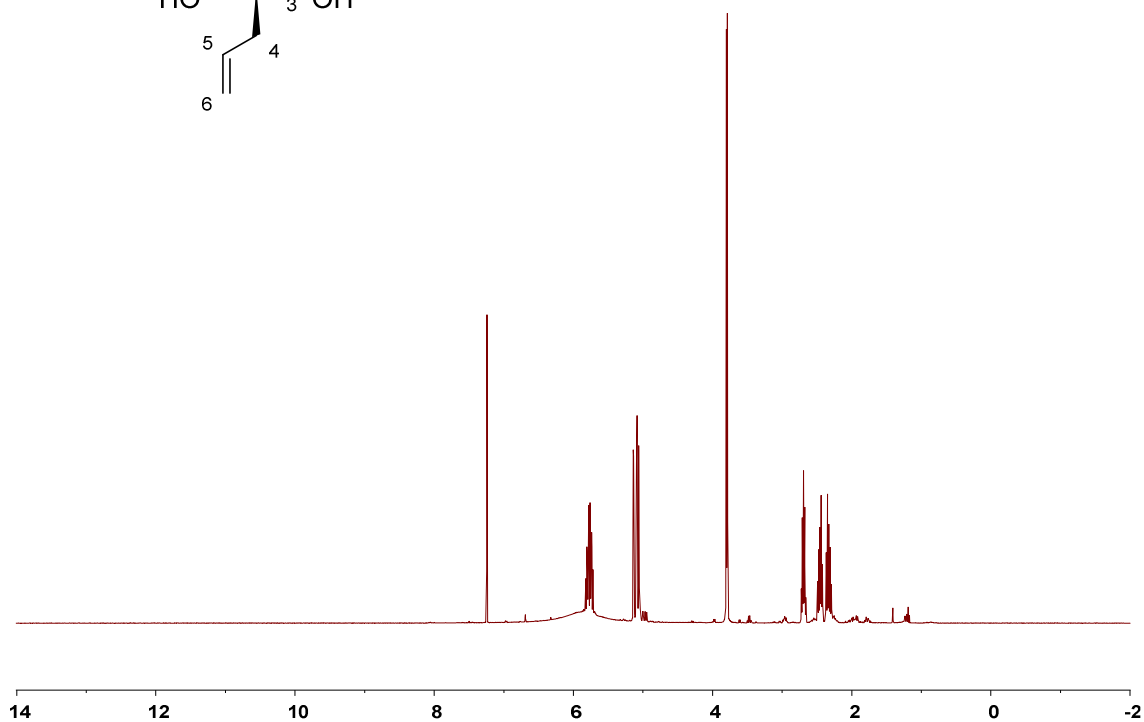
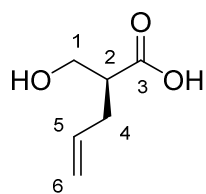


Figure S117 NMR spectra (CDCl_3) of *S*-6c: a) ^1H and b) ^{13}C .

a)



b)

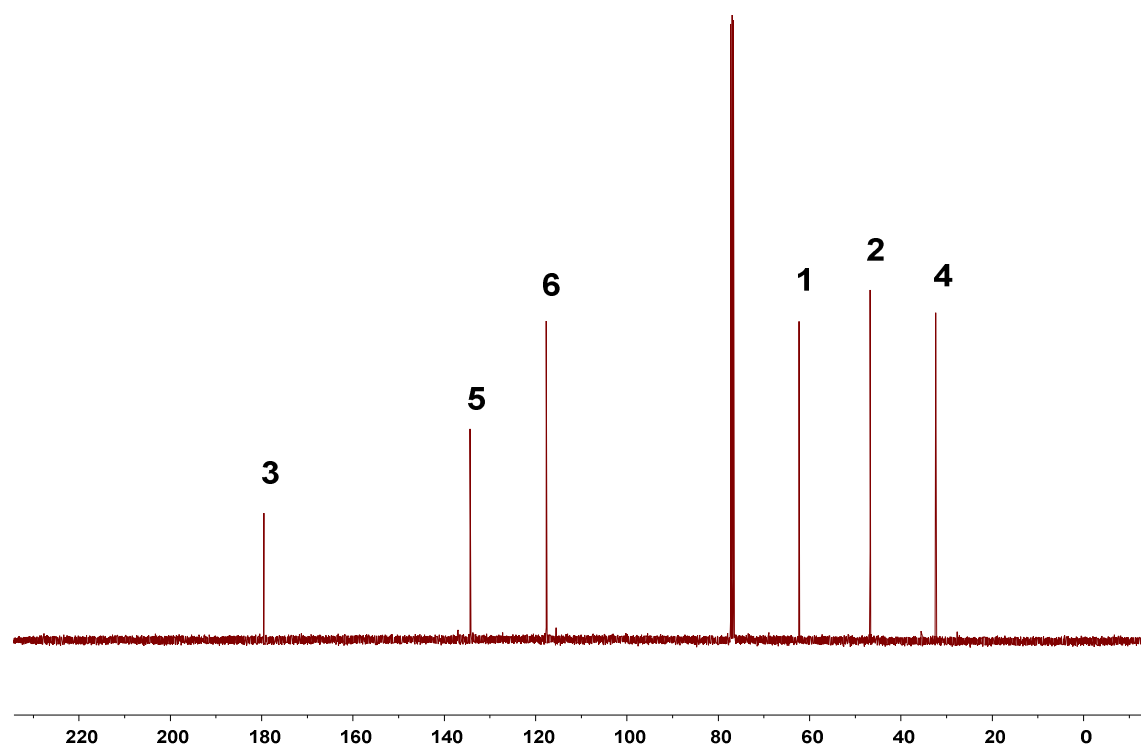
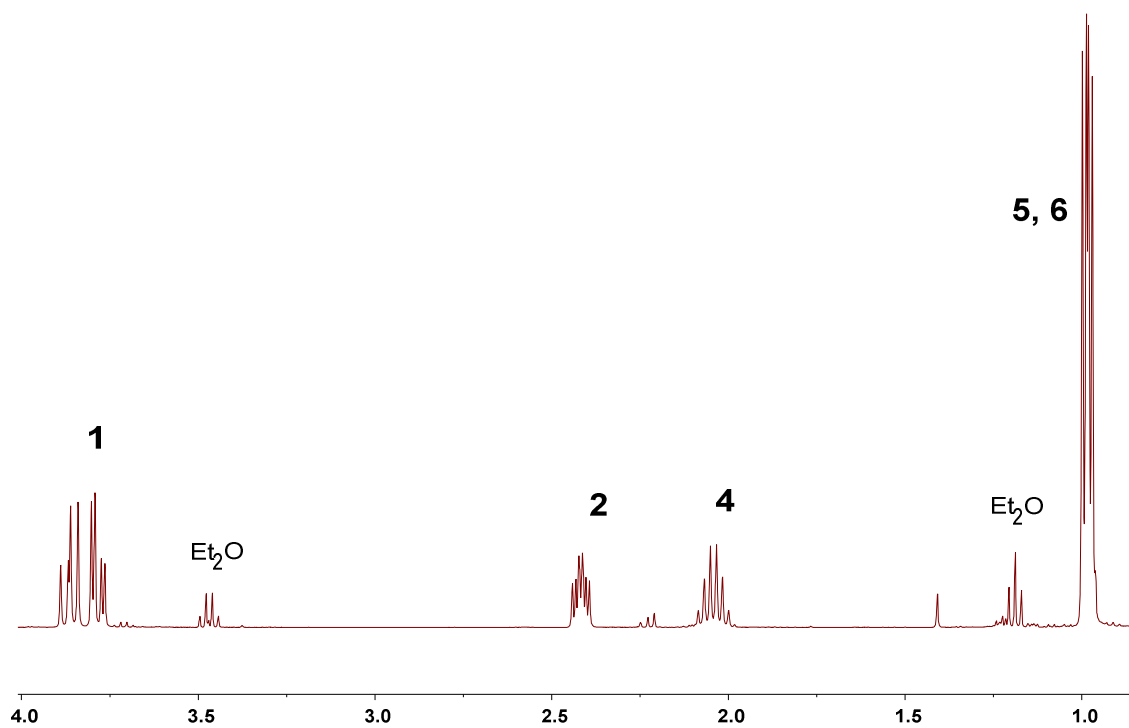
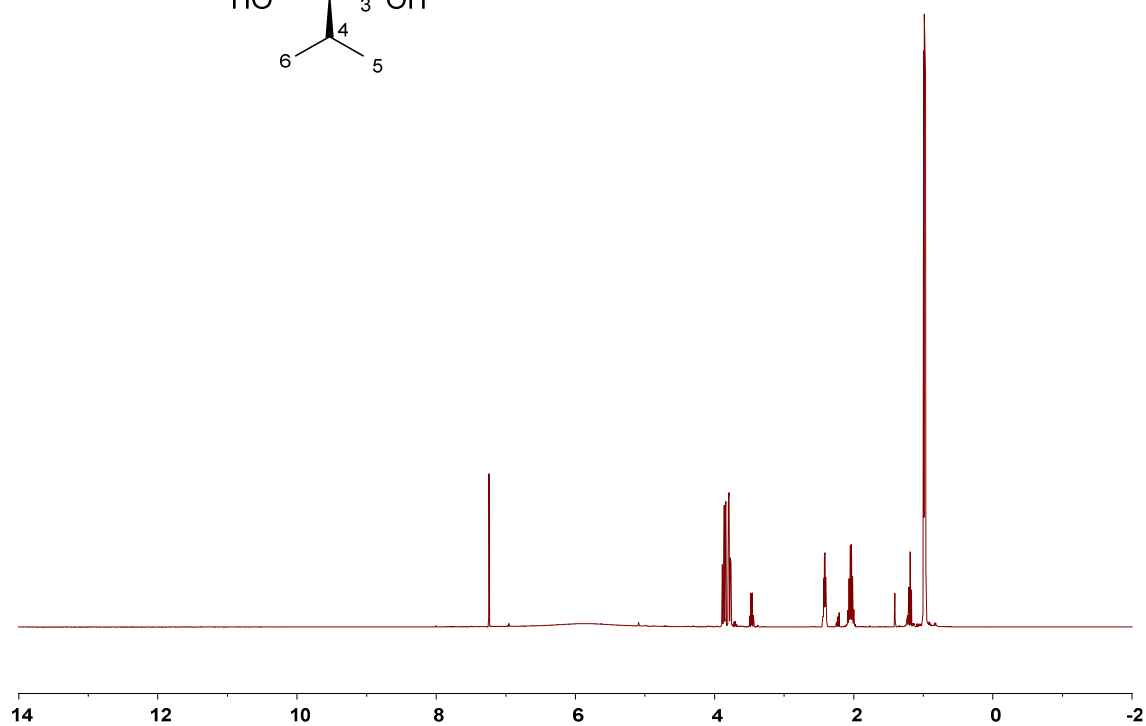
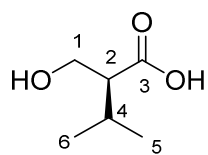


Figure S118 NMR spectra (CDCl_3) of *S*-6d: a) ^1H and b) ^{13}C .

a)



b)

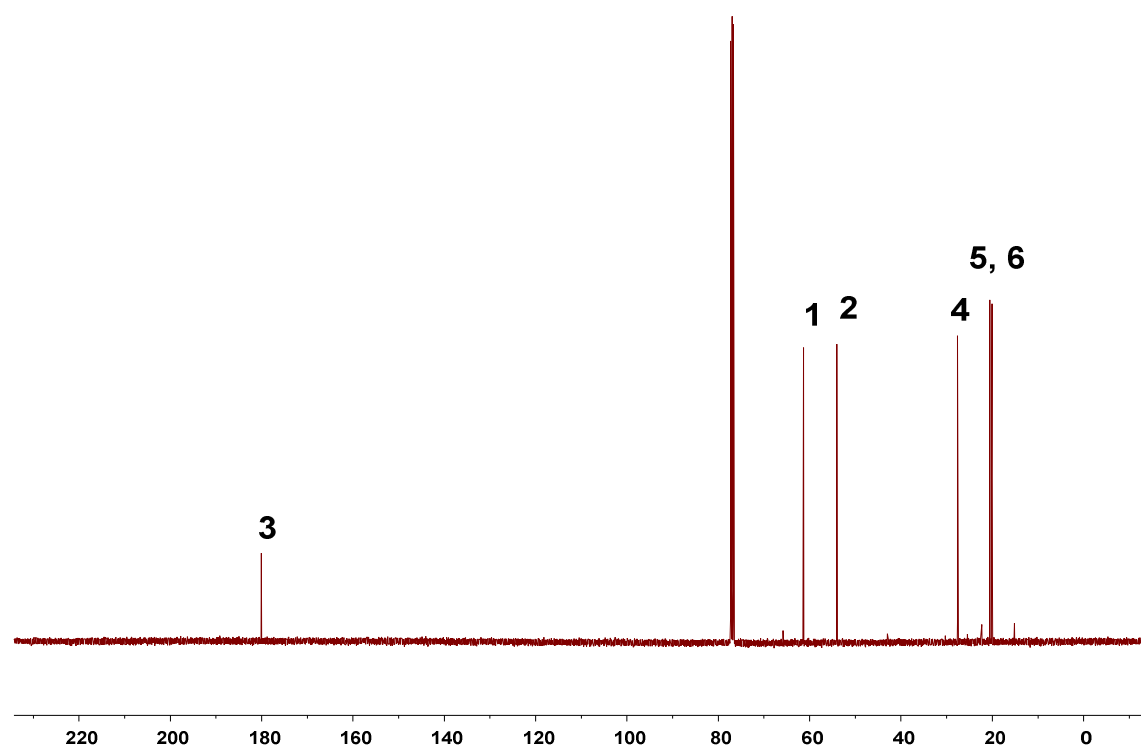
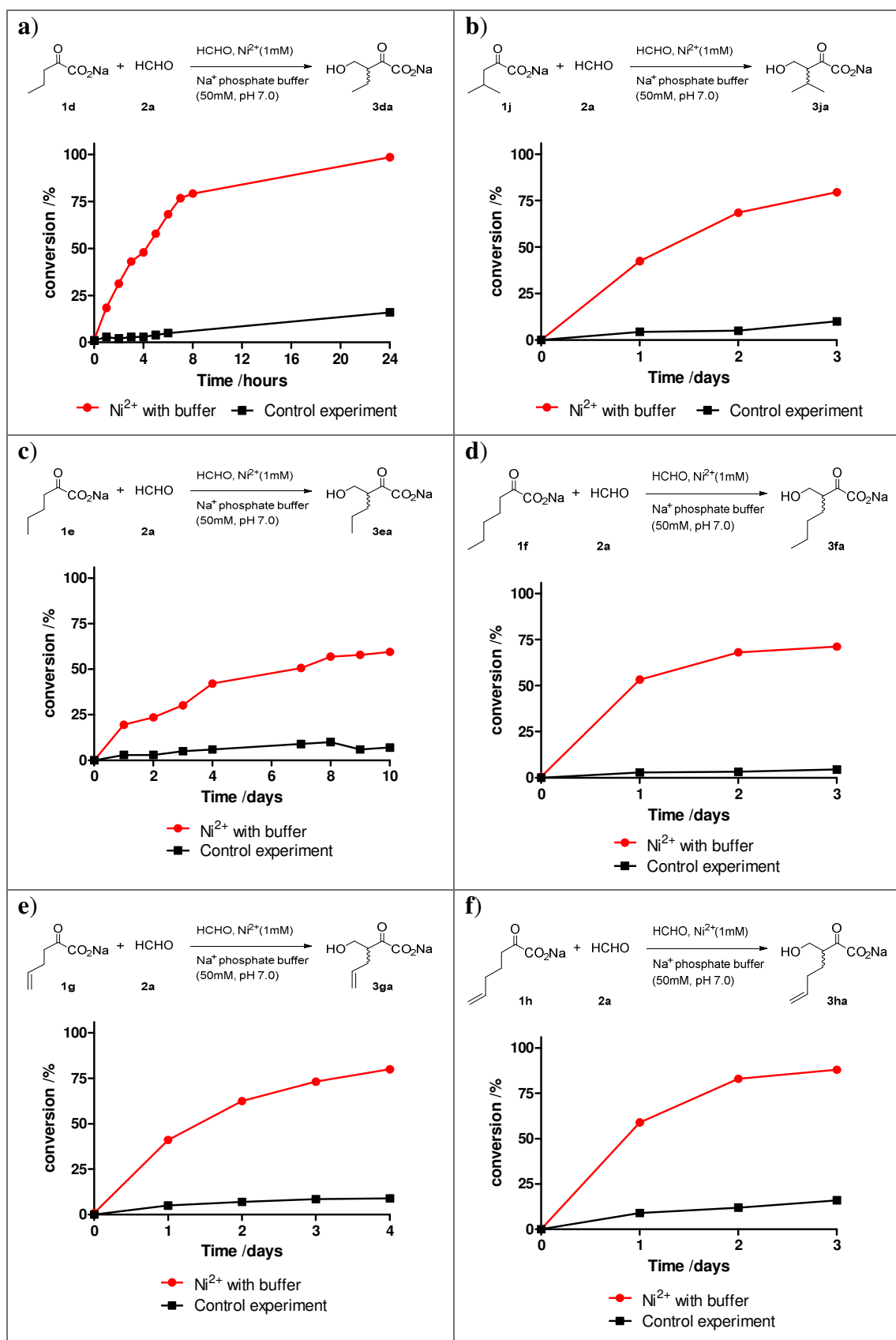


Figure S119 NMR spectra (CDCl_3) of *S*-6e: a) ^1H and b) ^{13}C .

6.2.5 Synthesis of racemic mixtures of products 4b-k



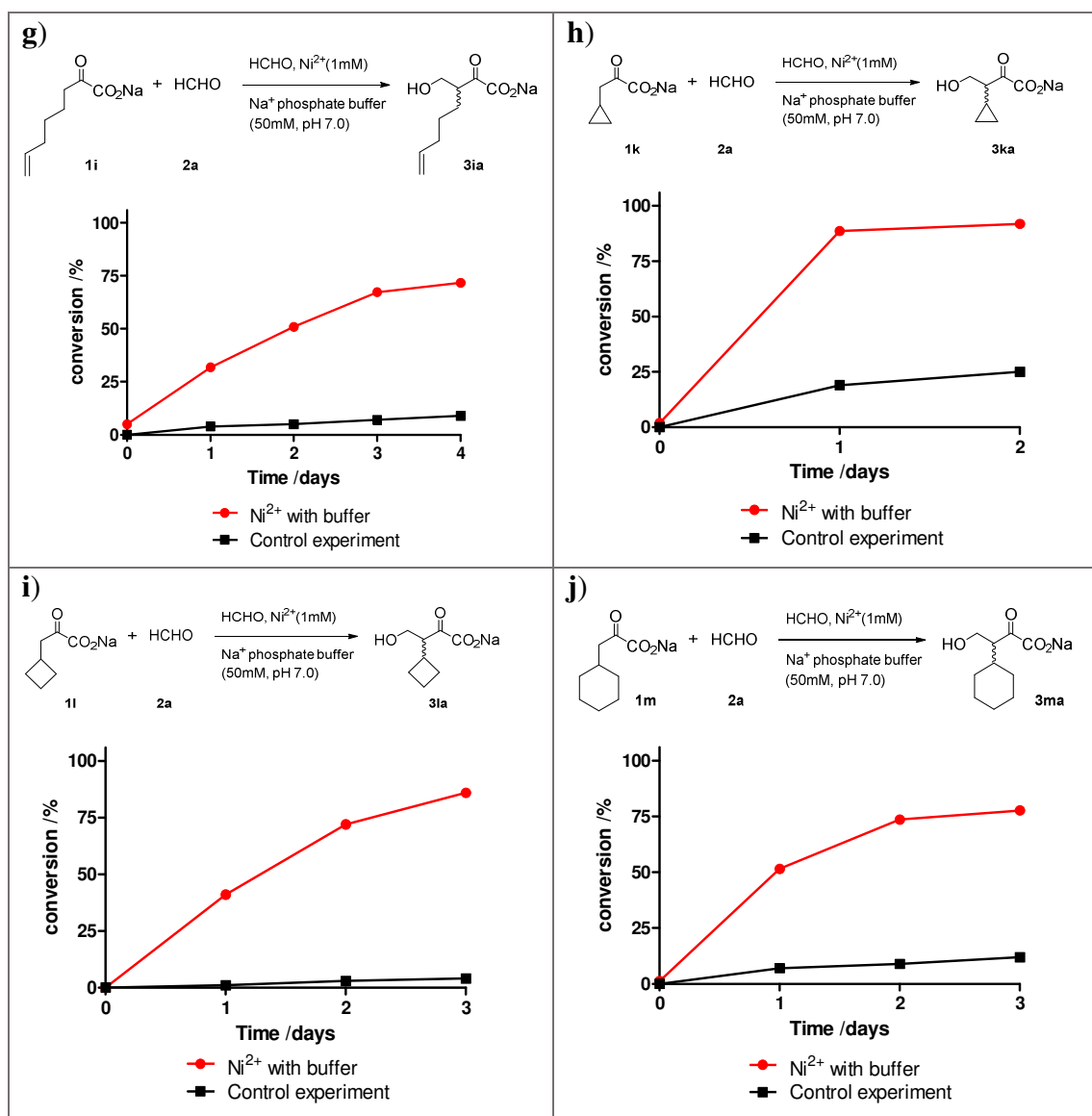
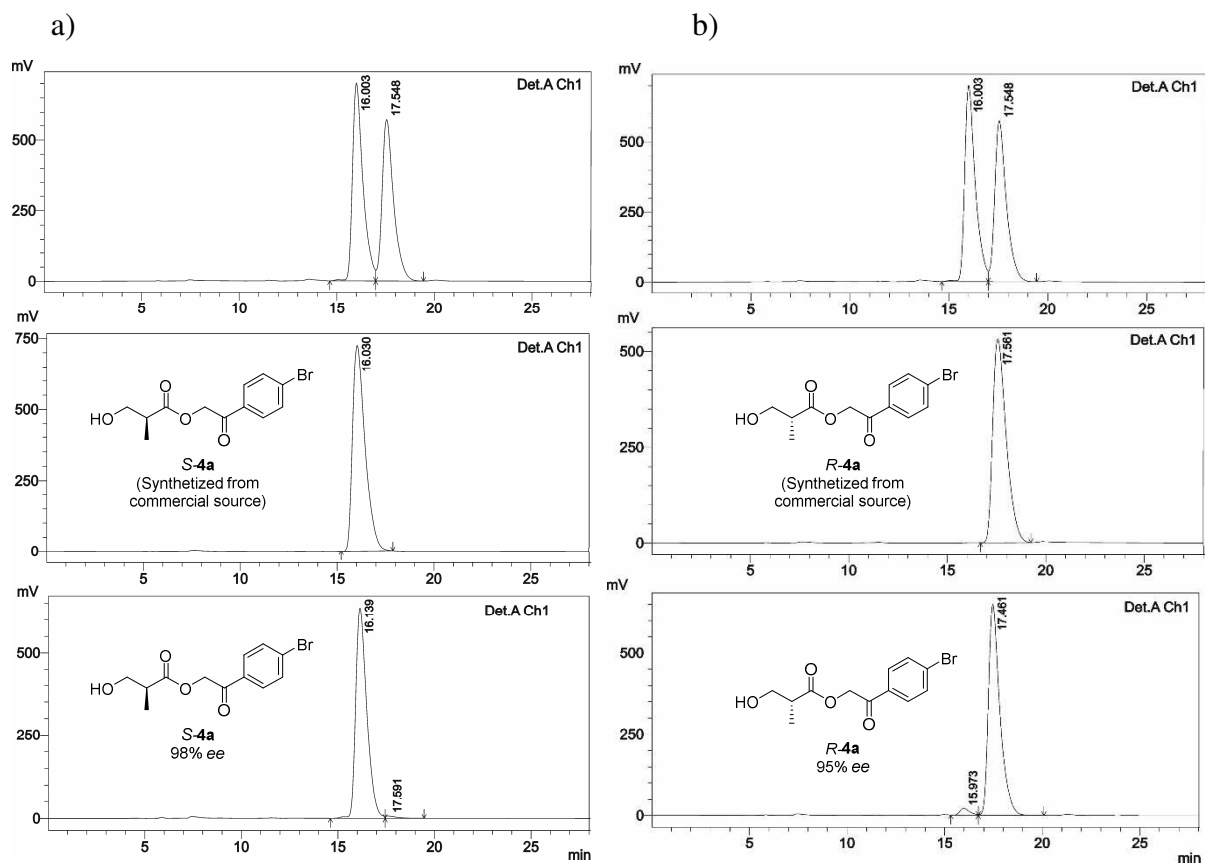
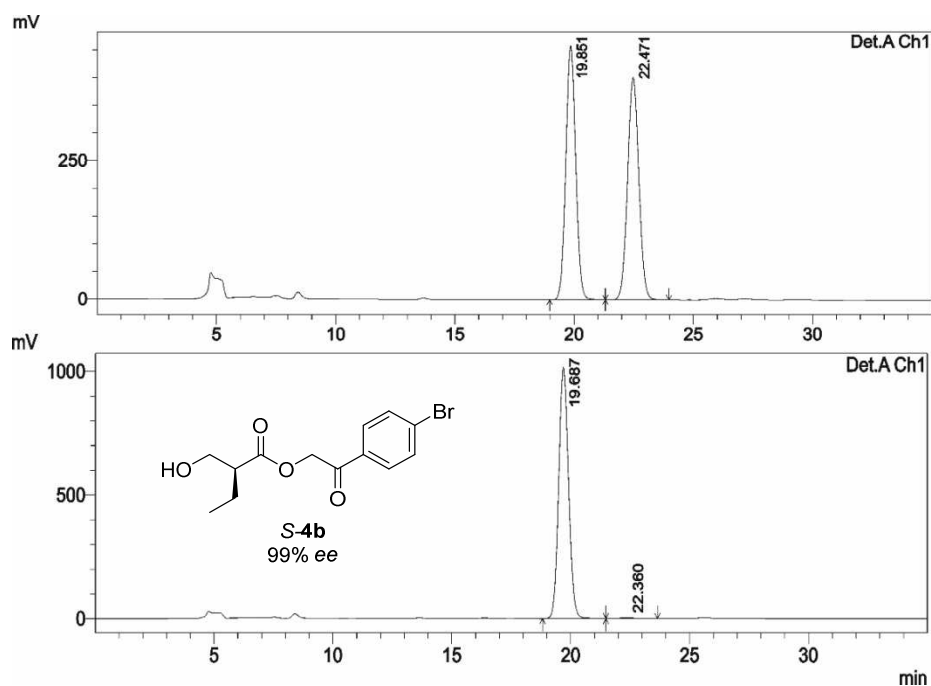


Figure S120 Reaction time course of the aldol addition of a) **1d**, b) **1j** c) **1e**, d) **1f**, e) **1g**, f) **1h**, g) **1i**, h) **1k**, i) **1l** and j) **1m** to formaldehyde (**2a**) catalyzed by Ni²⁺ (1mM) in sodium phosphate buffer (50mM, pH 7.0) and control experiment

6.2.6 Chiral HPLC analysis



a)



b)

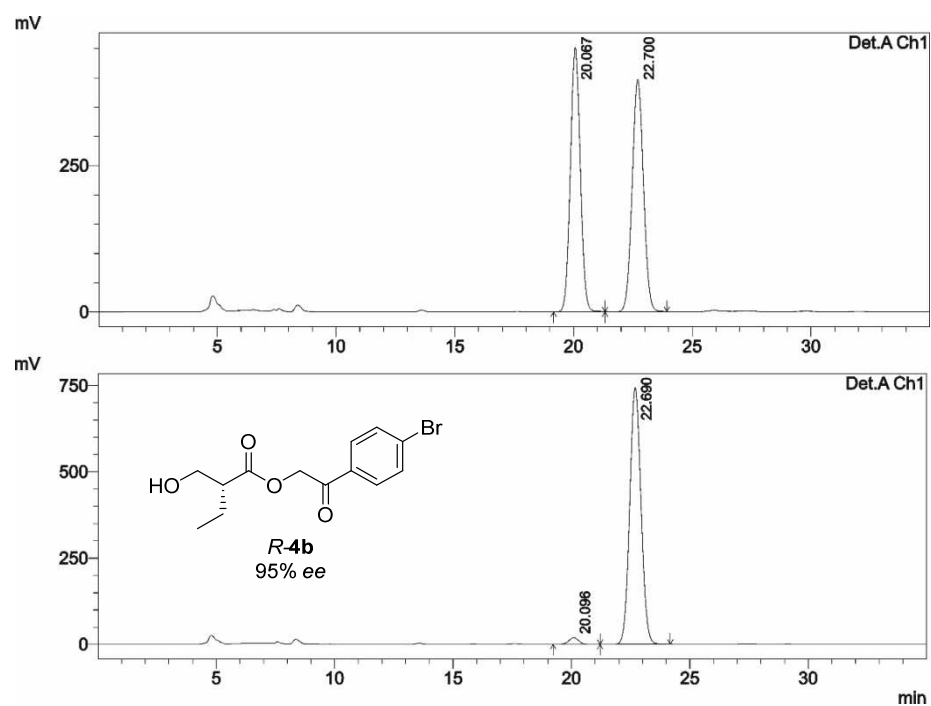


Figure S122 Chiral HPLC analysis chromatogram of **S-4b** synthesized with MBP-YFAU(Ni²⁺) wild-type (a) and **R-4b** synthesized with KPHMT wild-type (b). Conditions: CHIRALPACK® IC 46 x 250 mm column, 5 μ m, flow rate 0.7 mL min⁻¹ at 20 °C and UV detection (254 nm). Isocratic elution hexane:iPrOH 80:20.

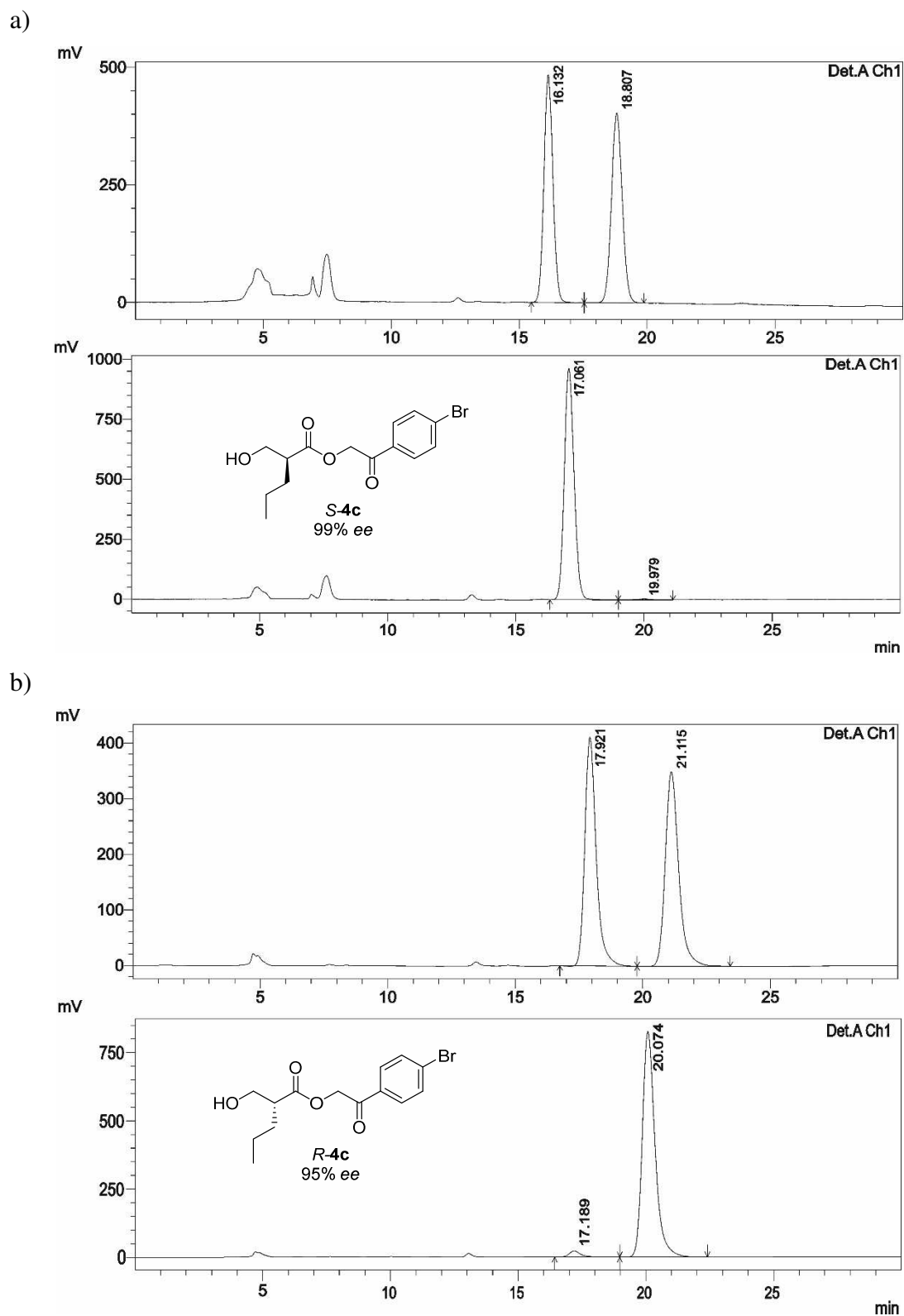


Figure S123 Chiral HPLC analysis chromatogram of *S*-**4c** synthesized with MBP-YfaU W23V.(a) and *R*-**4c** synthesized with KPHMT I212A (b). Conditions: CHIRALPACK® IC 46 x 250 mm column, 5 μ m, flow rate 0.7 mL min⁻¹ at 20 °C and UV detection (254 nm). Isocratic elution hexane:iPrOH 75:25.

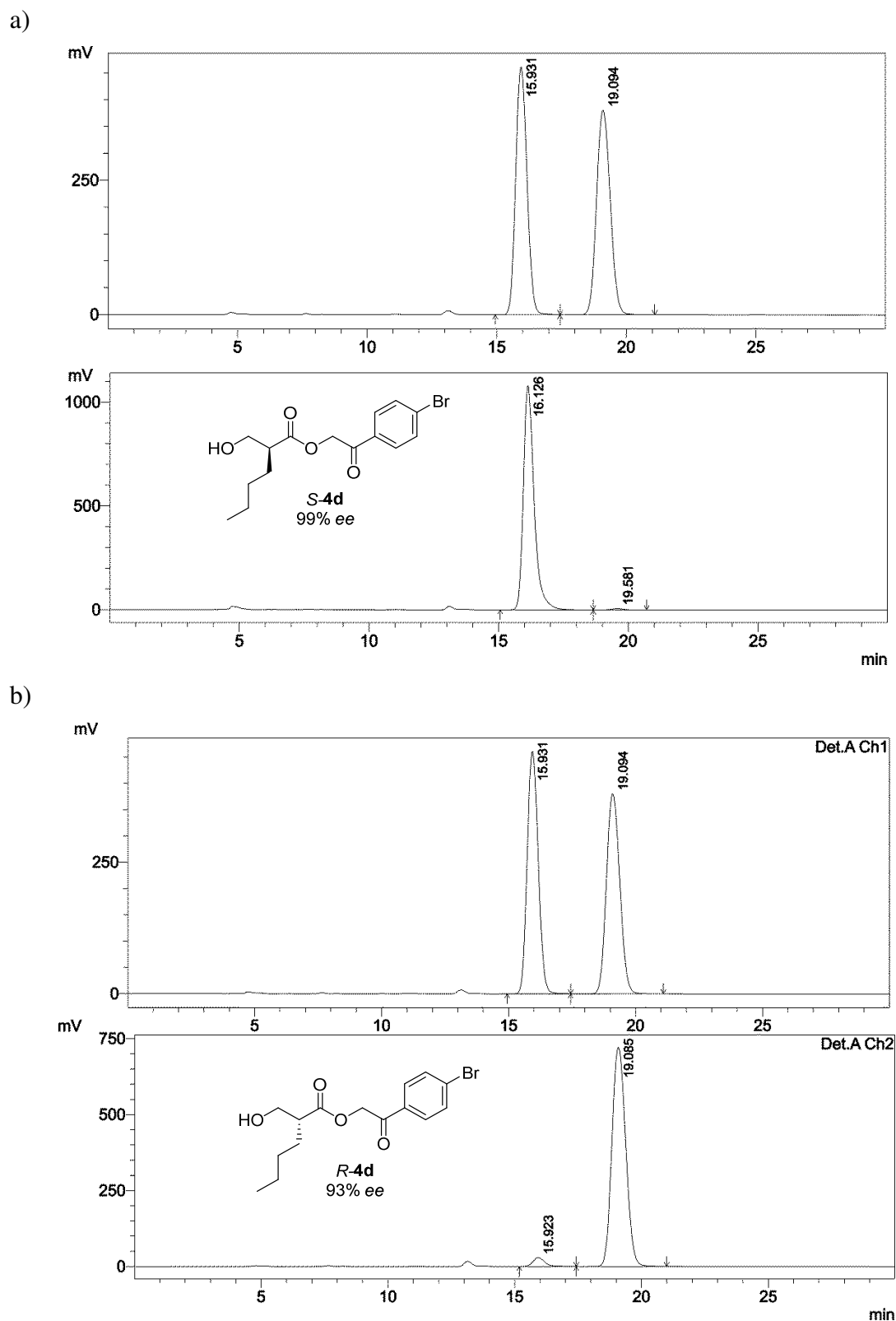
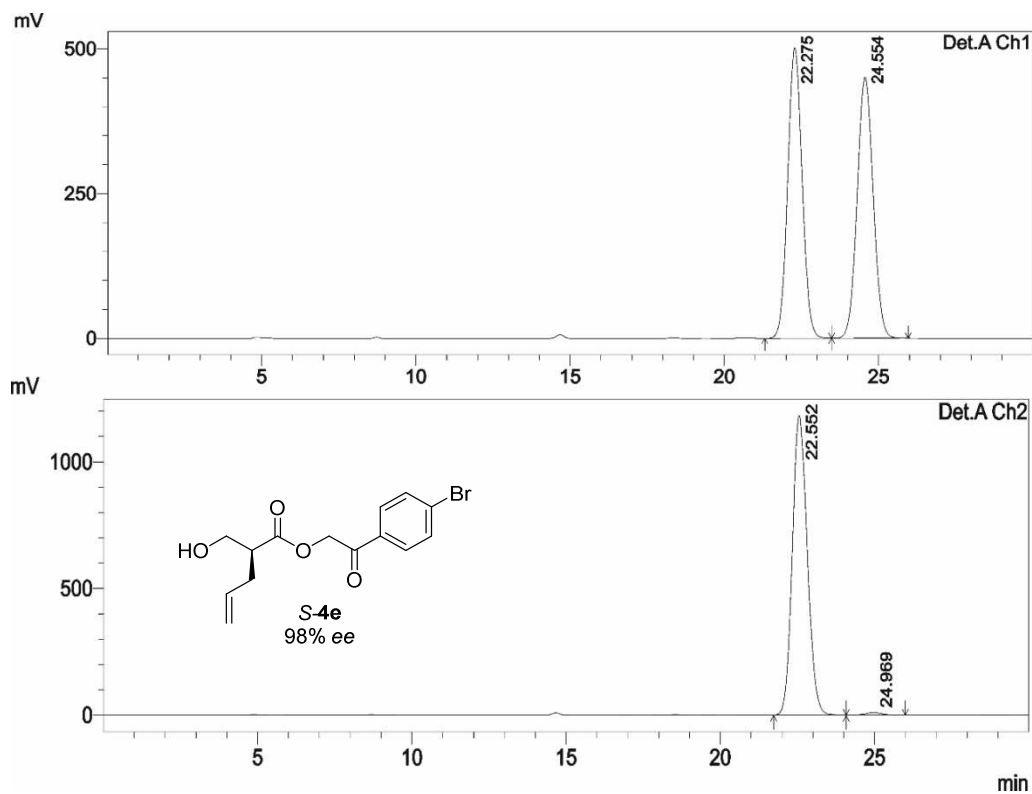
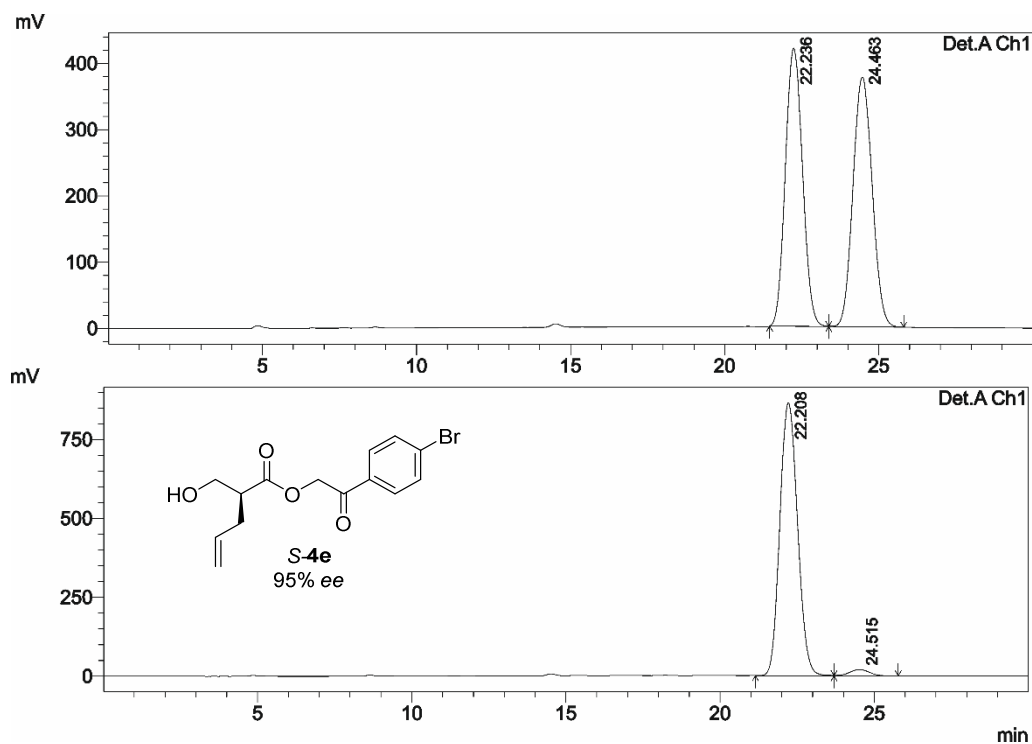


Figure S124 Chiral HPLC analysis chromatogram of *S*-**4d** synthesized with MBP-YfaU W23V (a) and *R*-**4d** synthesized with KPHMT L42A/I212A (b). Conditions: CHIRALPACK® IC 46 x 250 mm column, 5 μ m, flow rate 0.7 mL min⁻¹ at 20 °C and UV detection (254 nm). Isocratic elution hexane:iPrOH 75:25.

a)



b)



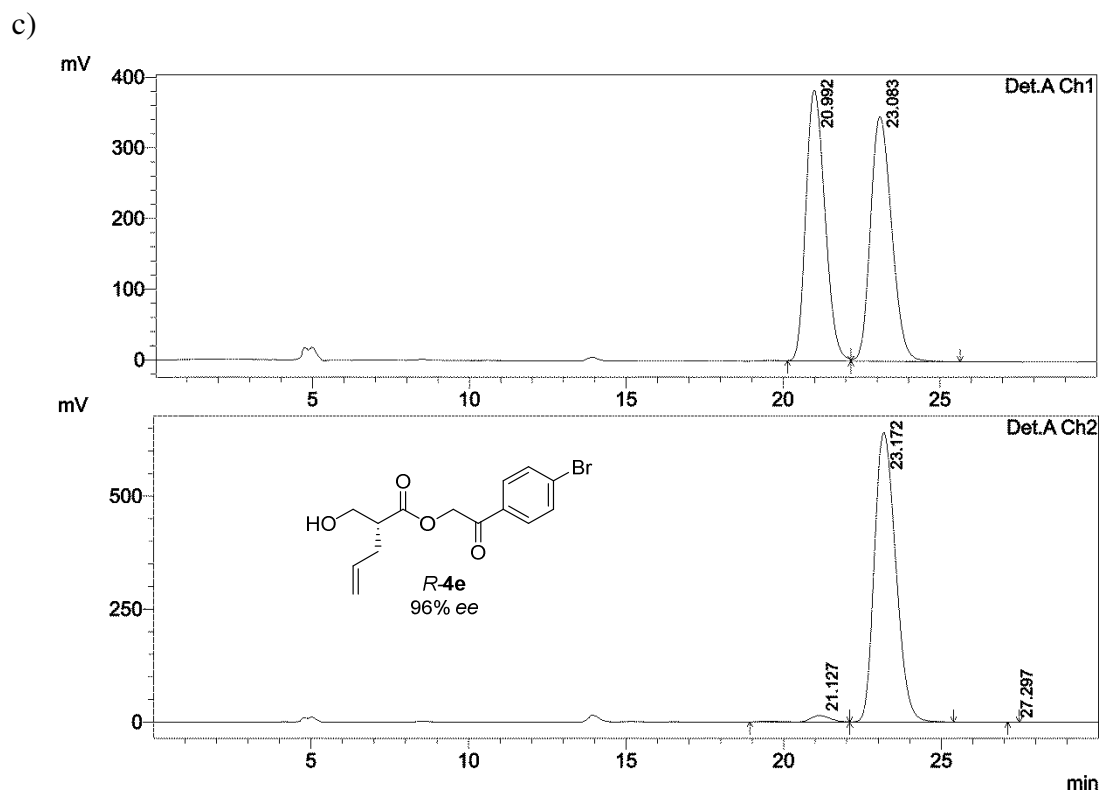


Figure S125 Chiral HPLC analysis chromatogram of *S*-**4e** synthesized with MBP-YfaU W23V (a), *S*-**4e** synthesized with MBP-YfaU W23V (b) and *R*-**4e** synthesized with KPHMT I212A (c). Conditions: CHIRALPACK® IC 46 x 250 mm column, 5 μ m, flow rate 0.7 mL min⁻¹ at 20 °C and UV detection (254 nm). Isocratic elution hexane:iPrOH 80:20.

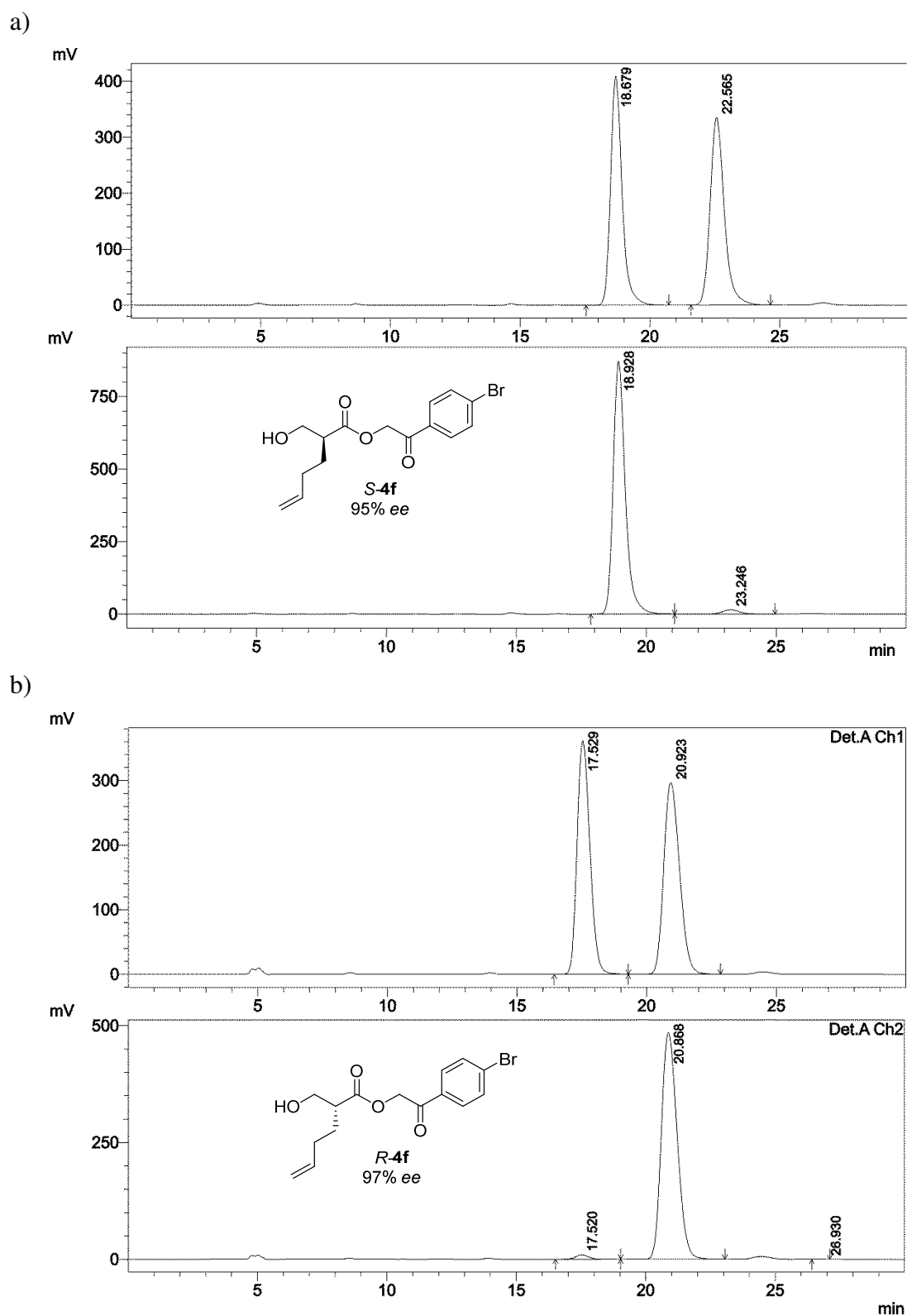


Figure S126 Chiral HPLC analysis chromatogram of *S*-**4f** synthesized with MBP-YfaU W23V (a) and *R*-**4f** synthesized with KPHMT I212A (b). Conditions: CHIRALPACK® IC 46 x 250 mm column, 5 μ m, flow rate 0.7 mL min⁻¹ at 20 °C and UV detection (254 nm). Isocratic elution hexane:iPrOH 80:20.

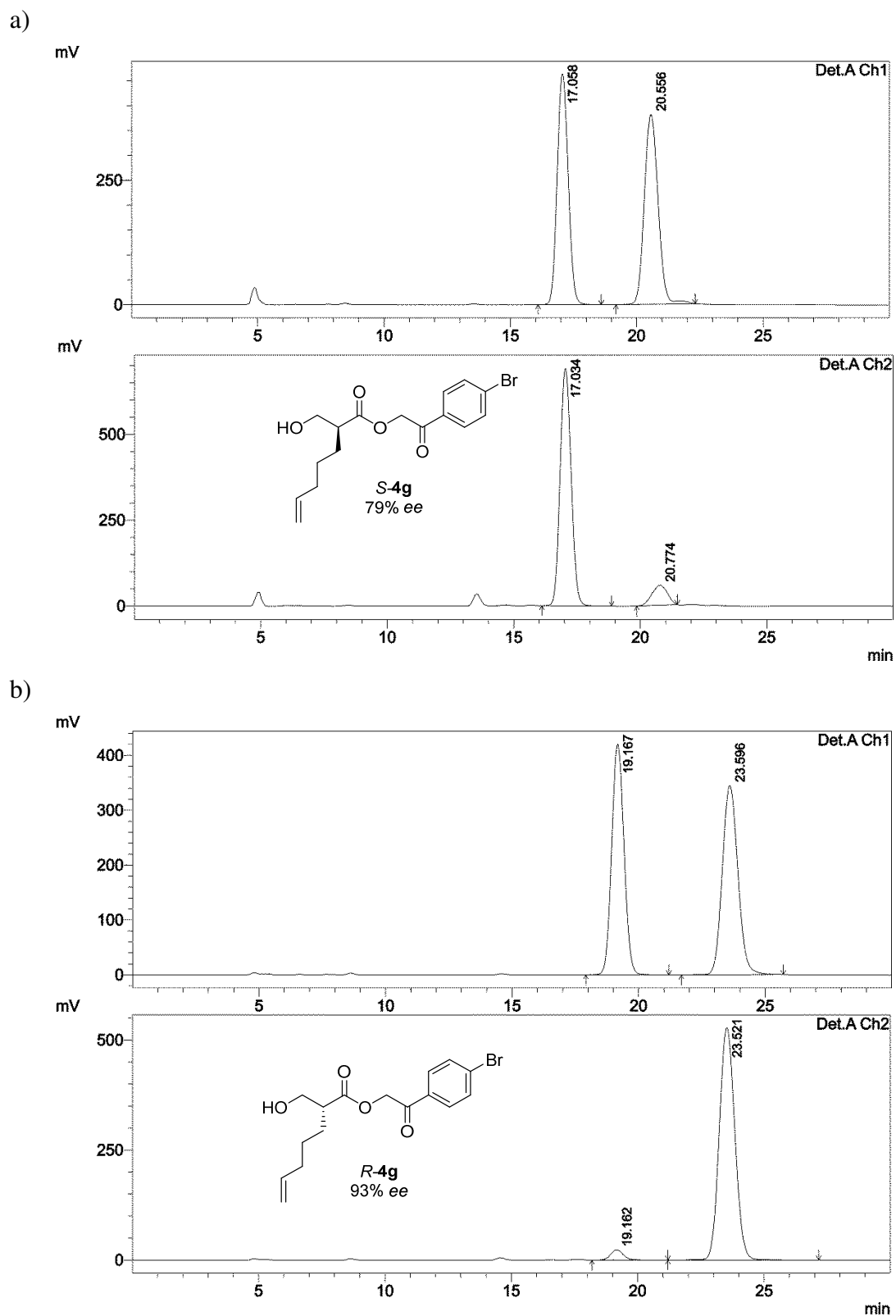


Figure S127 Chiral HPLC analysis chromatogram of **S-4g** synthesized with MBP-YfaU W23V (a) and **R-4g** synthesized with KPHMT L42A/I212A (b). Conditions: CHIRALPACK® IC 46 x 250 mm column, 5 μ m, flow rate 0.7 mL min⁻¹ at 20 °C and UV detection (254 nm). Isocratic elution hexane:iPrOH 80:20.

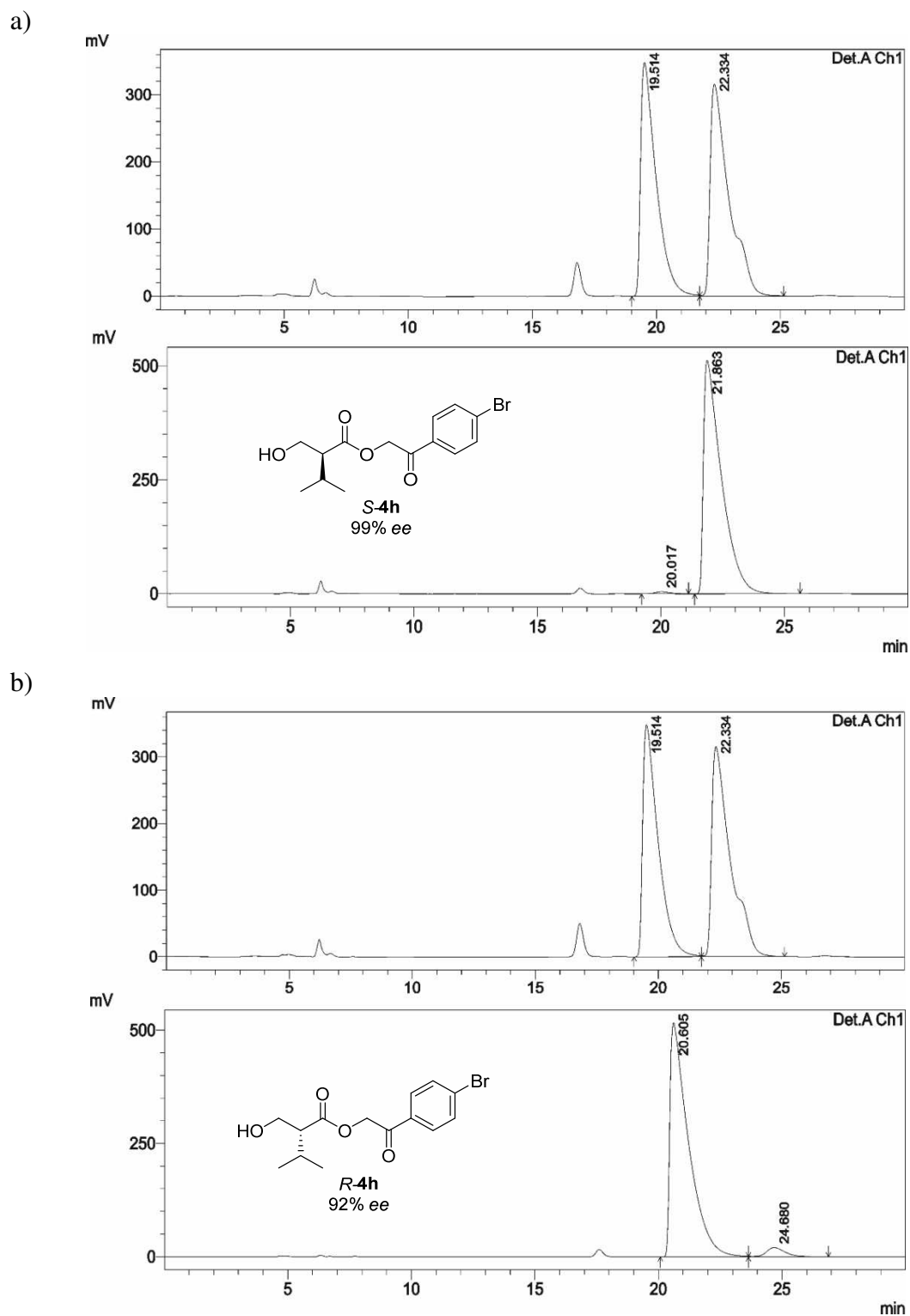
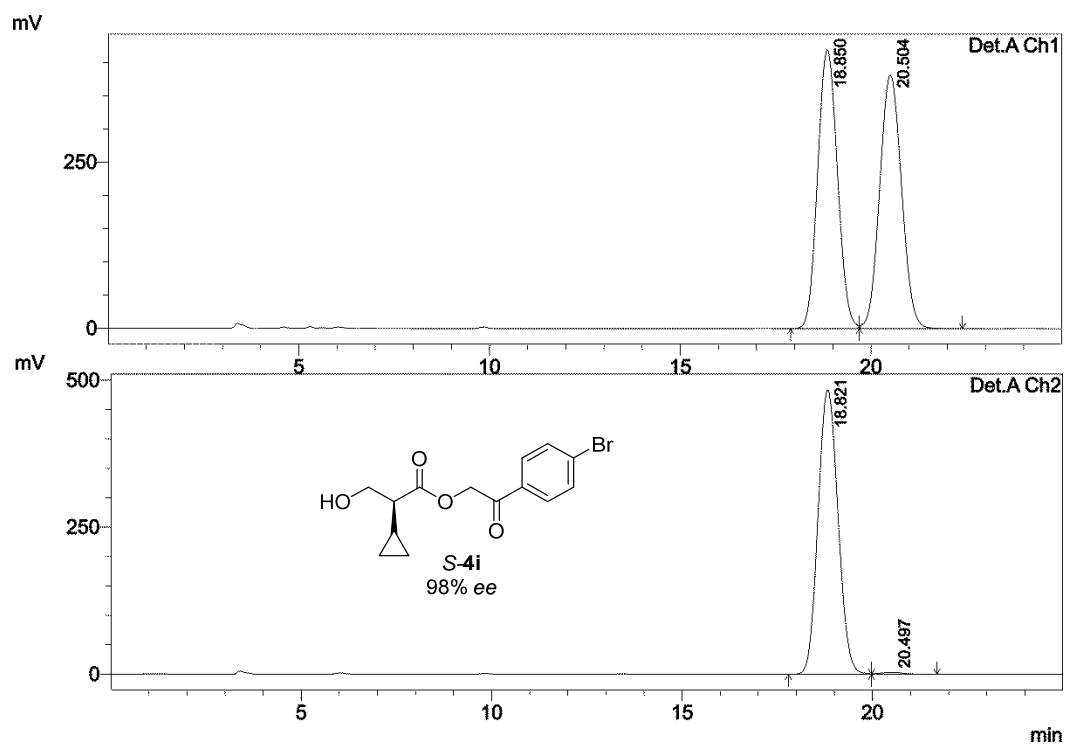
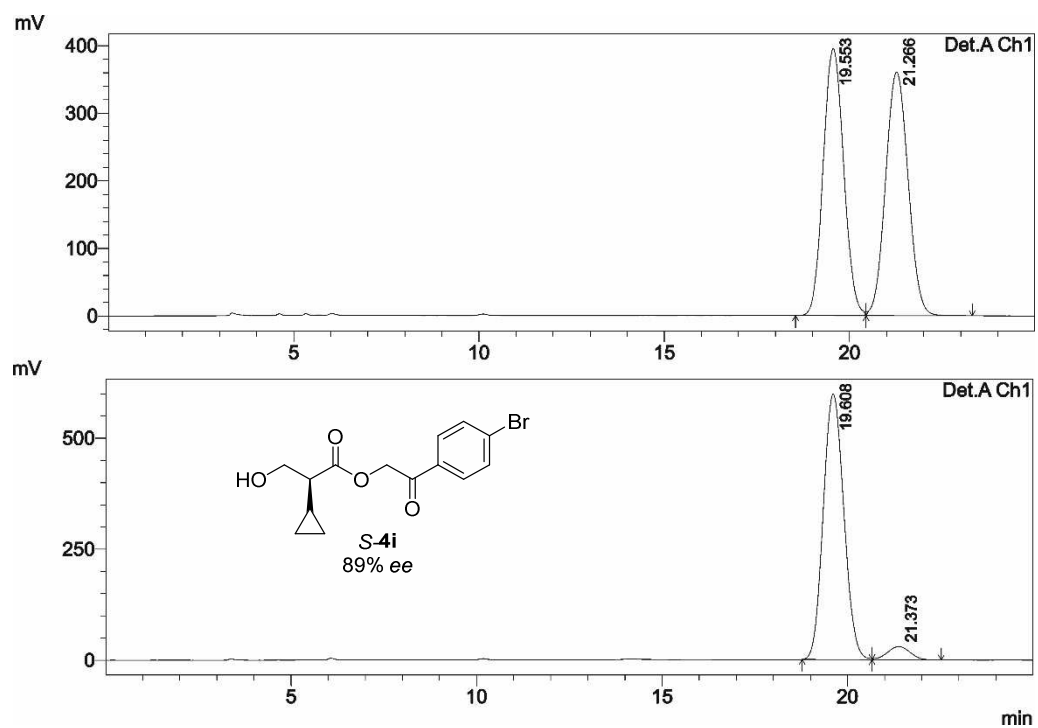


Figure S128 Chiral HPLC analysis chromatogram of *S*-**4h** synthesized with MBP-YfaU W23V (a) and *R*-**4h** synthesized with KPHMT I202A (b). Conditions: CHIRALPACK® ID 46 x 250 mm column, 5 μ m, flow rate 0.7 mL min⁻¹ at 20 °C and UV detection (254 nm). Isocratic elution hexane:iPrOH 75:25.

a)



b)



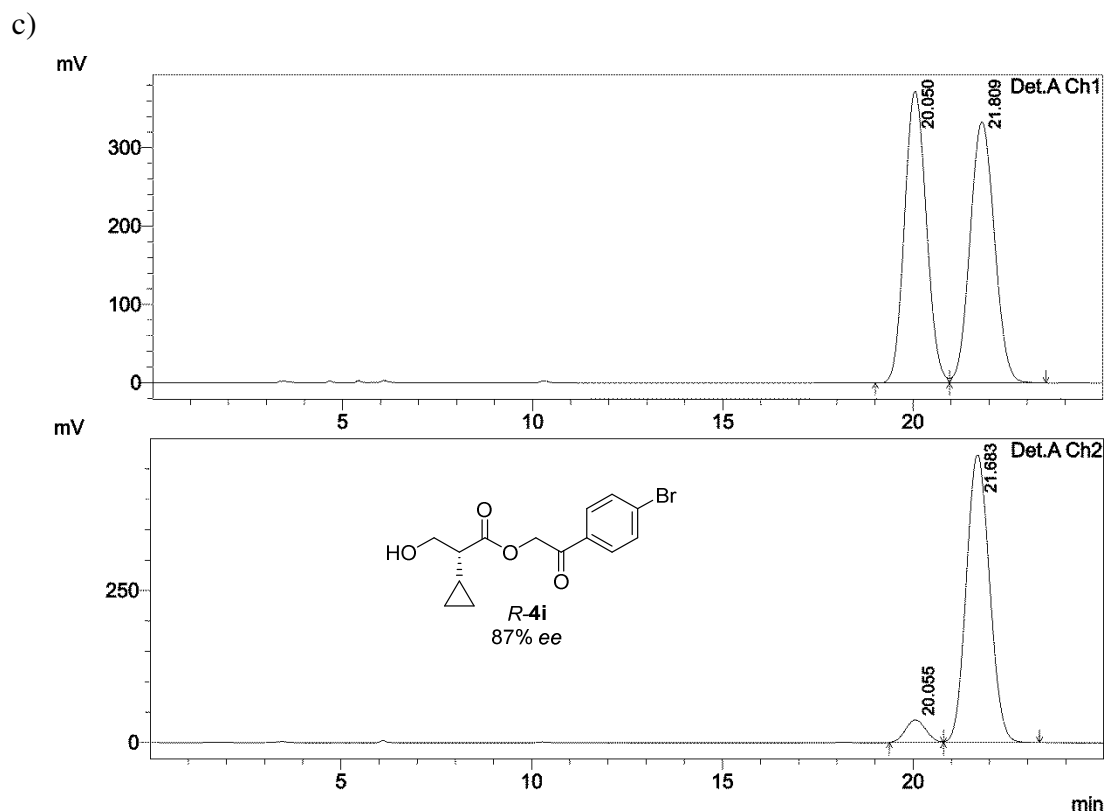


Figure S129 Chiral HPLC analysis chromatogram of *S*-4i synthesized with MBP-YfaU W23V (a), *S*-4i synthesized with MBP-YfaU wild-type (b) and *R*-4i synthesized with KPHMT wild-type (c). Conditions: CHIRALPACK® IC 46 x 250 mm column, 5 μ m, flow rate 1 mL min⁻¹ at 20 °C and UV detection (254 nm). Isocratic elution hexane:iPrOH 80:20.

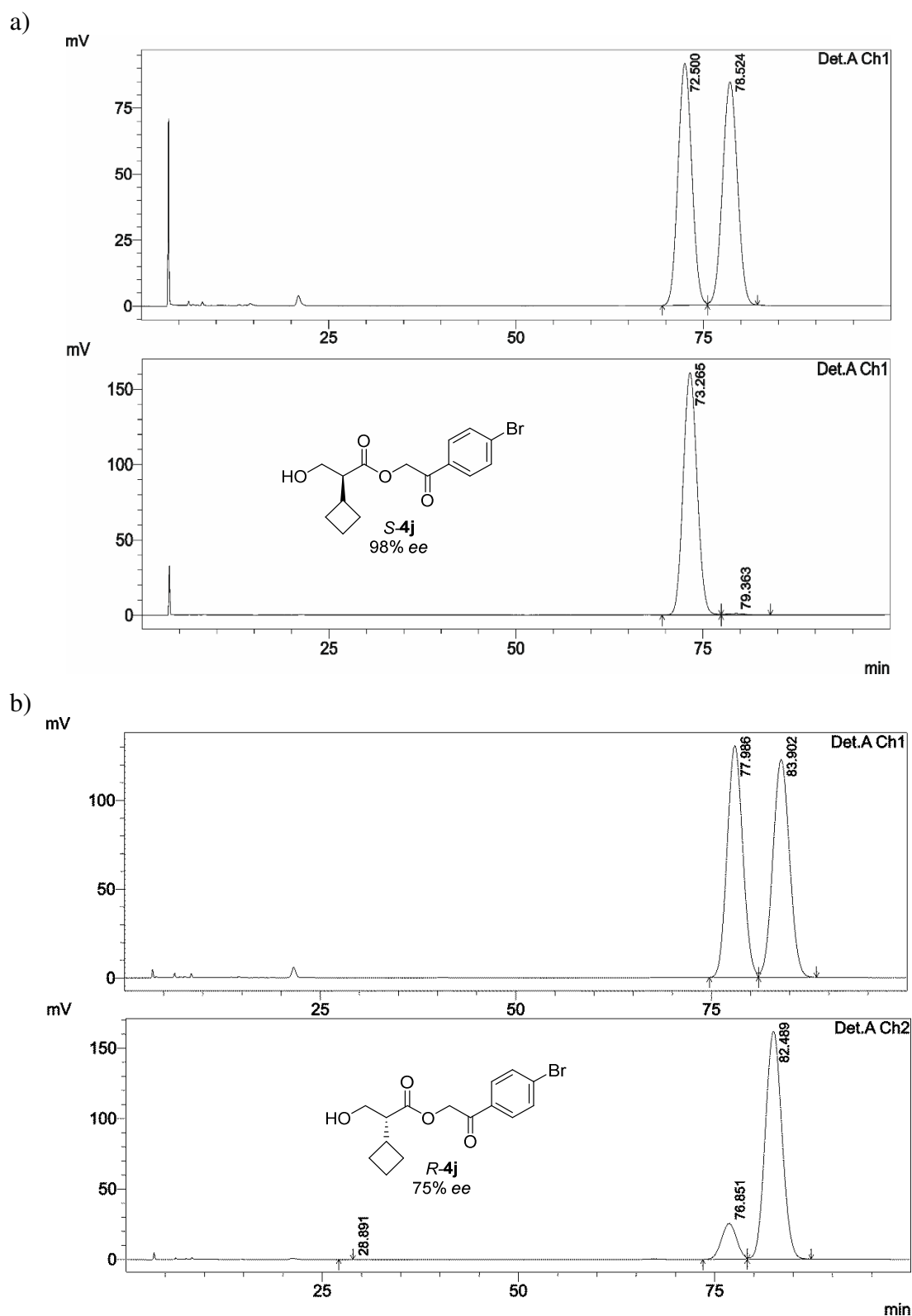


Figure S130 Chiral HPLC analysis chromatogram of *S*-**4j** synthesized with MBP-YfaU W23V (a) and *R*-**4j** synthesized with KPHMT I202A (b). Conditions: CHIRALPACK® IC 46 x 250 mm column, 5 μ m, flow rate 1 mL min⁻¹ at 20 °C and UV detection (254 nm). Isocratic elution hexane:iPrOH 95:5.

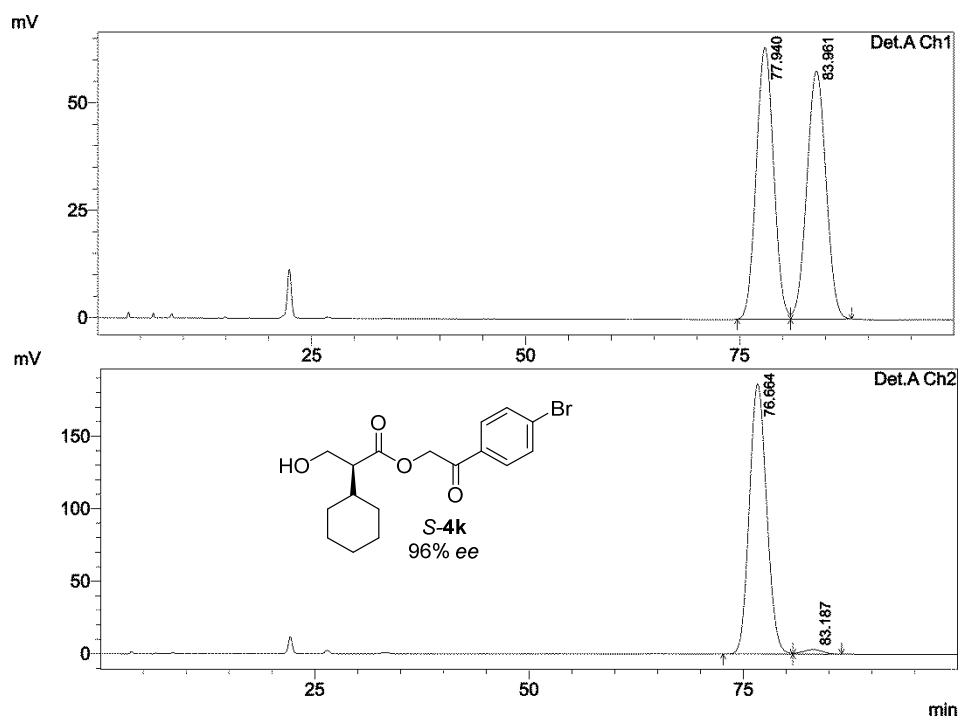
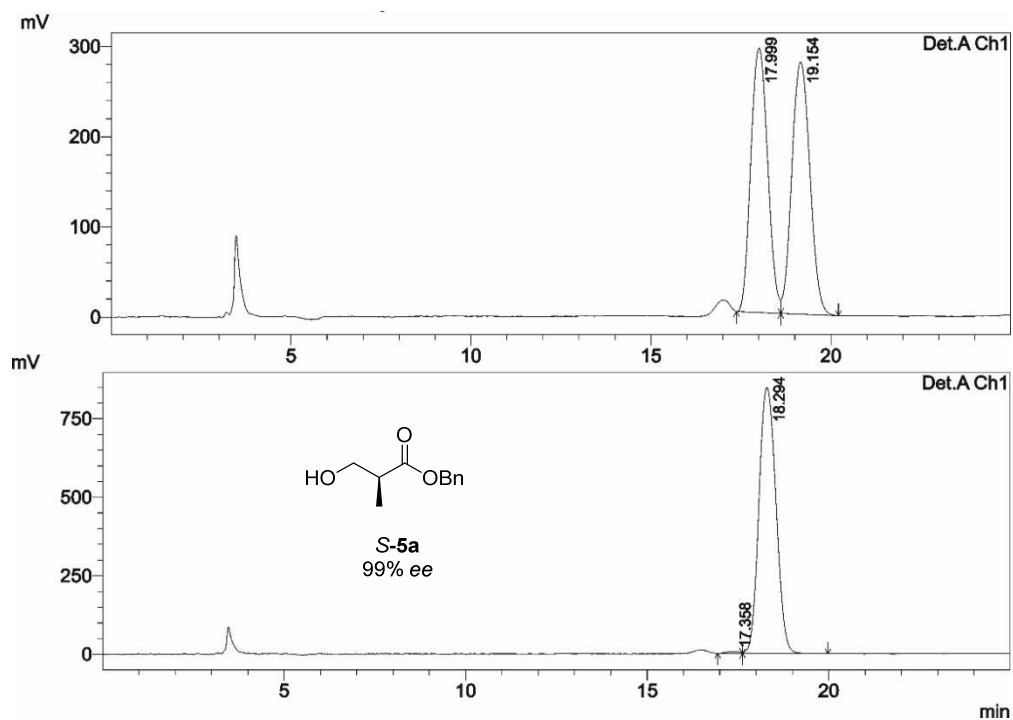


Figure S131 Chiral HPLC analysis chromatogram of *S*-4k synthesized with MBP-YfaU W23V. Conditions: CHIRALPACK® IC 46 x 250 mm column, 5 μ m, flow rate 1 mL min⁻¹ at 20 °C and UV detection (254 nm). Isocratic elution hexane:iPrOH 95:5.

a)



b)

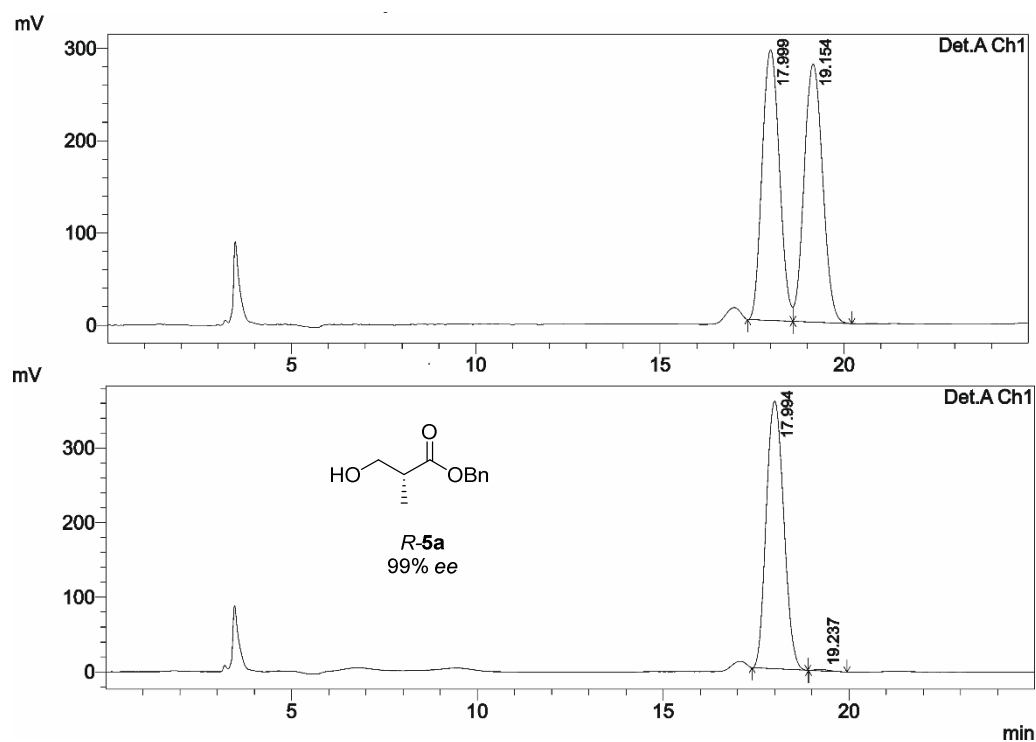


Figure S132 Chiral HPLC analysis chromatogram of *S*-5a synthesized with MBP-YfaU wild-type (a) and *R*-5a synthesized with KPHMT wild-type (b). Conditions: CHIRALPACK® IB 46 x 250 mm column, 5 μ m, flow rate 1 mL min⁻¹ at 20 °C and UV detection (209 nm). Isocratic elution hexane:iPrOH 98:2.

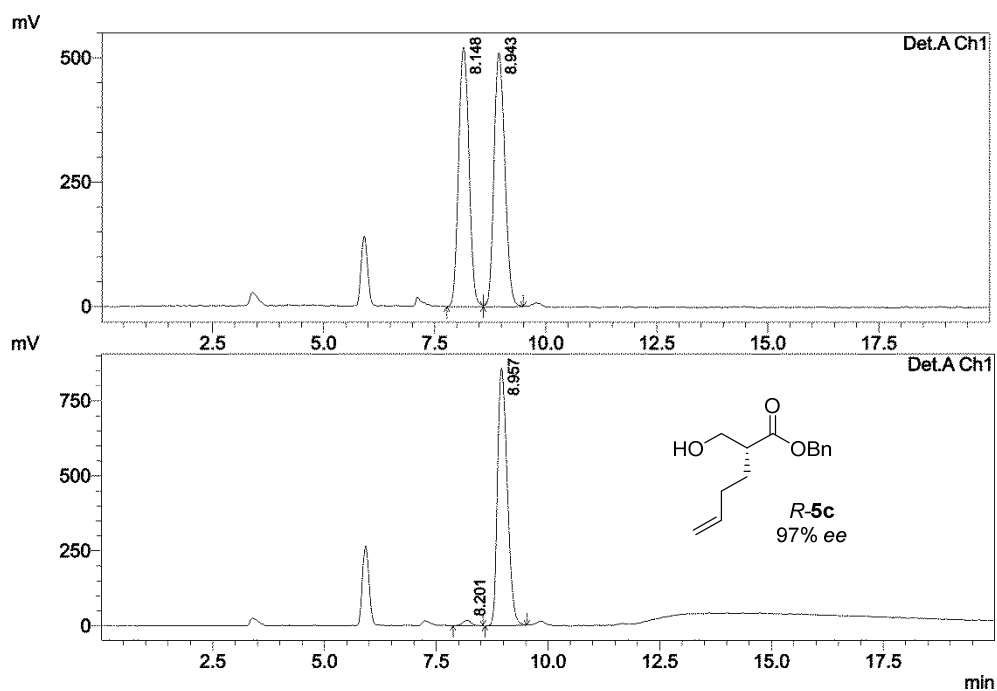
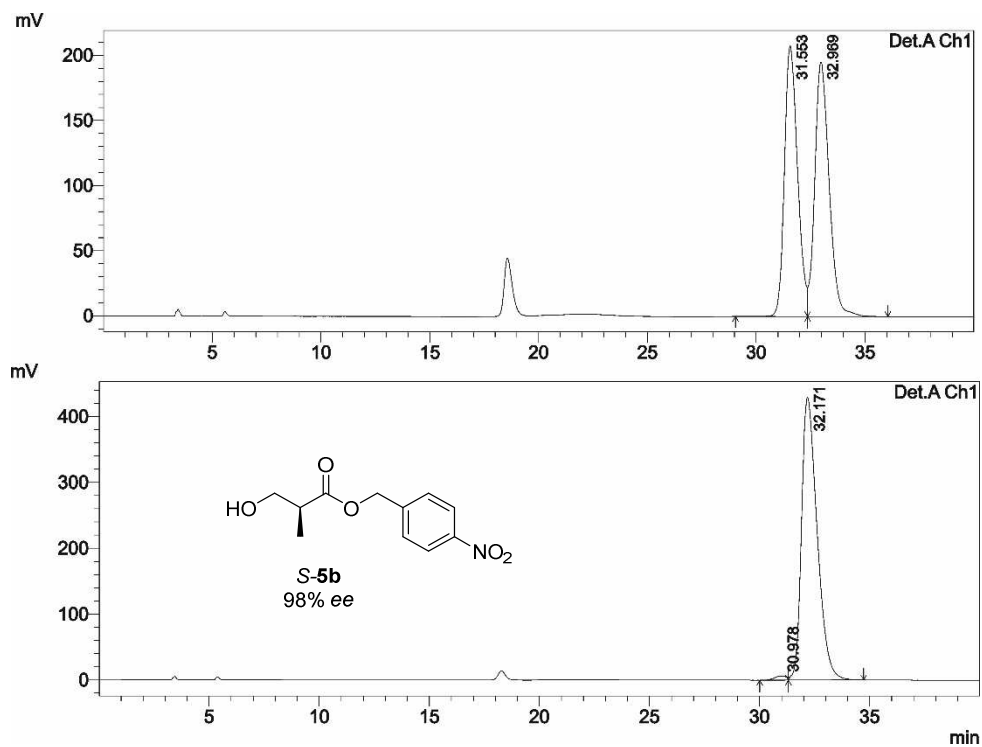


Figure S133 Chiral HPLC analysis chromatogram of *R*-5c synthesized with KPHMT I212A. Conditions: CHIRALPACK® ID 46 x 250 mm column, 5 μ m, flow rate 1 mL min⁻¹ at 20 °C and UV detection (209 nm). Isocratic elution hexane:iPrOH 90:10.

a)



b)

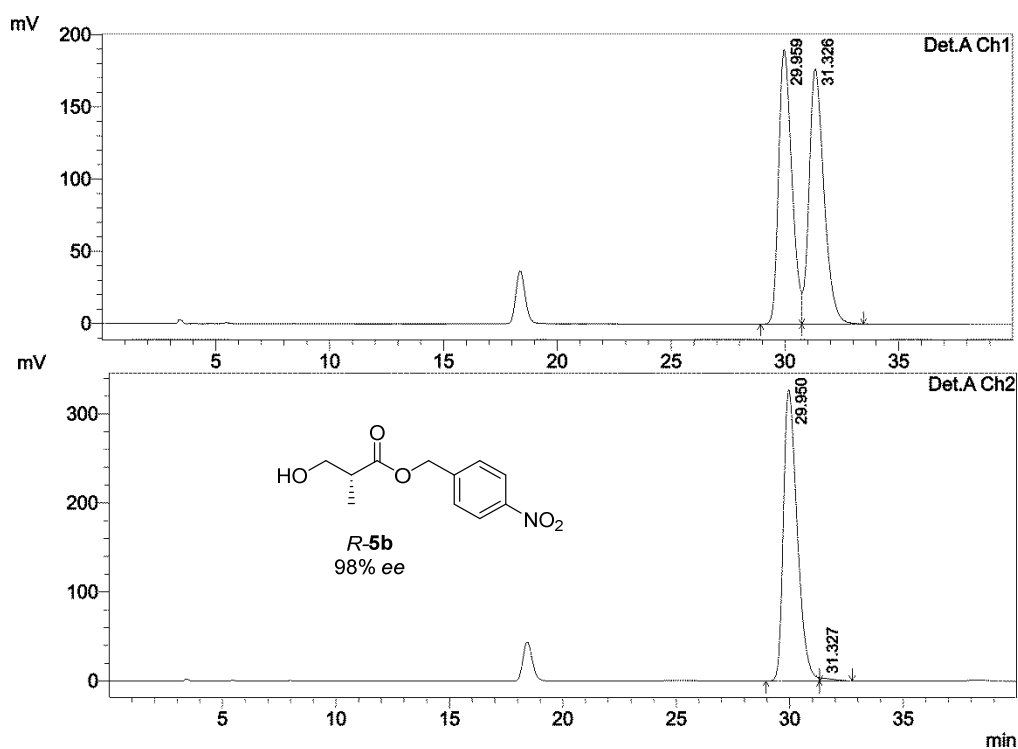
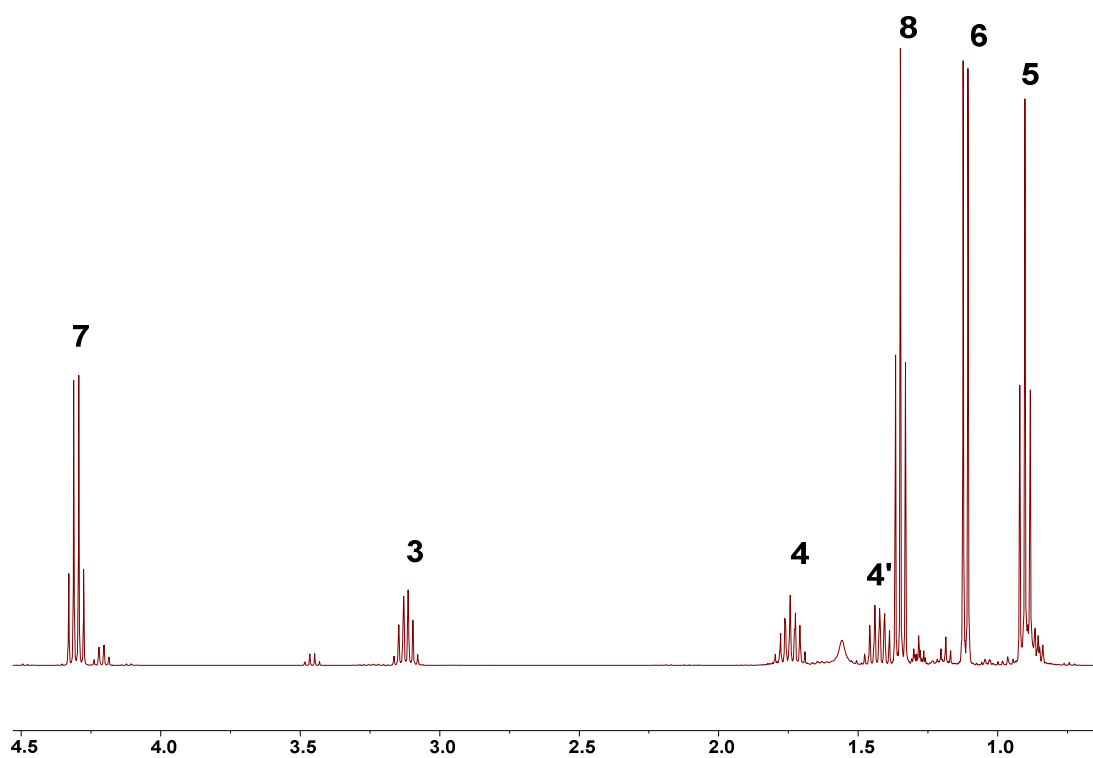
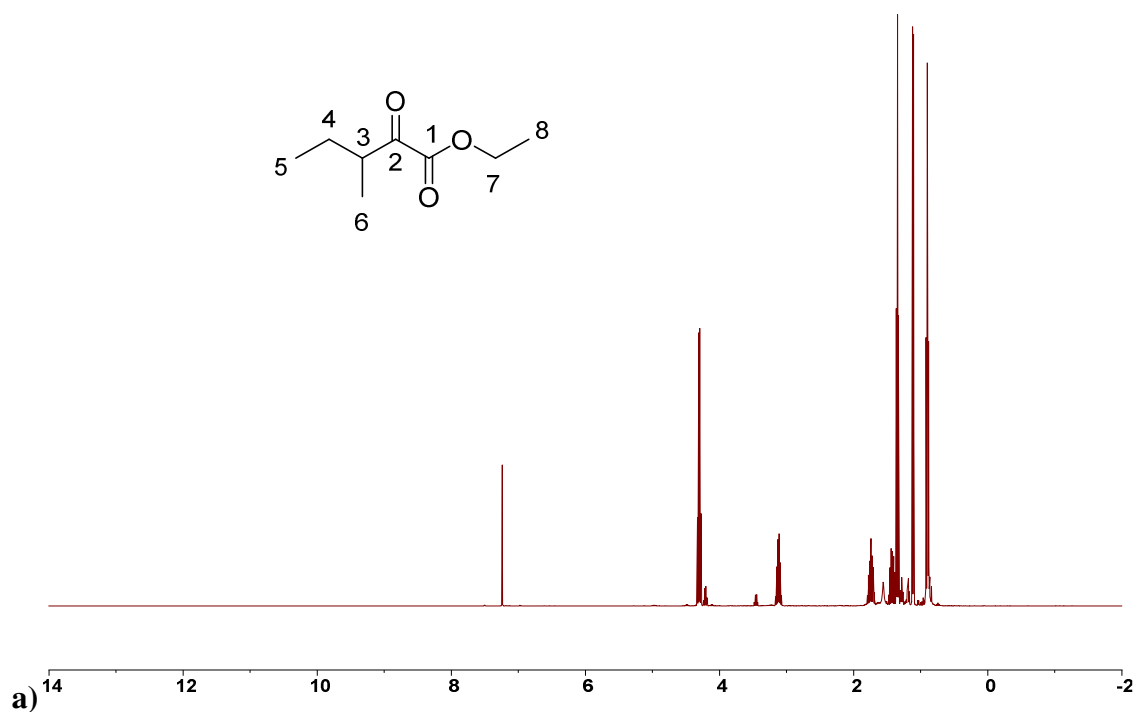


Figure S134 Chiral HPLC analysis chromatogram of a) **S-5b** synthesized with MBP-YfaU wild-type (a) and **R-5b** synthesized with KPHMT wild-type (b). Conditions: CHIRALPACK® IB 46 x 250 mm column, 5 μm , flow rate 1 mL min⁻¹ at 20 °C and UV detection (209 nm). Isocratic elution hexane:iPrOH 95:5.

6.3 Data from chapter 3.2

6.3.1 NMR Spectra of ethyl 2-oxoesters and 2-oxoacids



b)

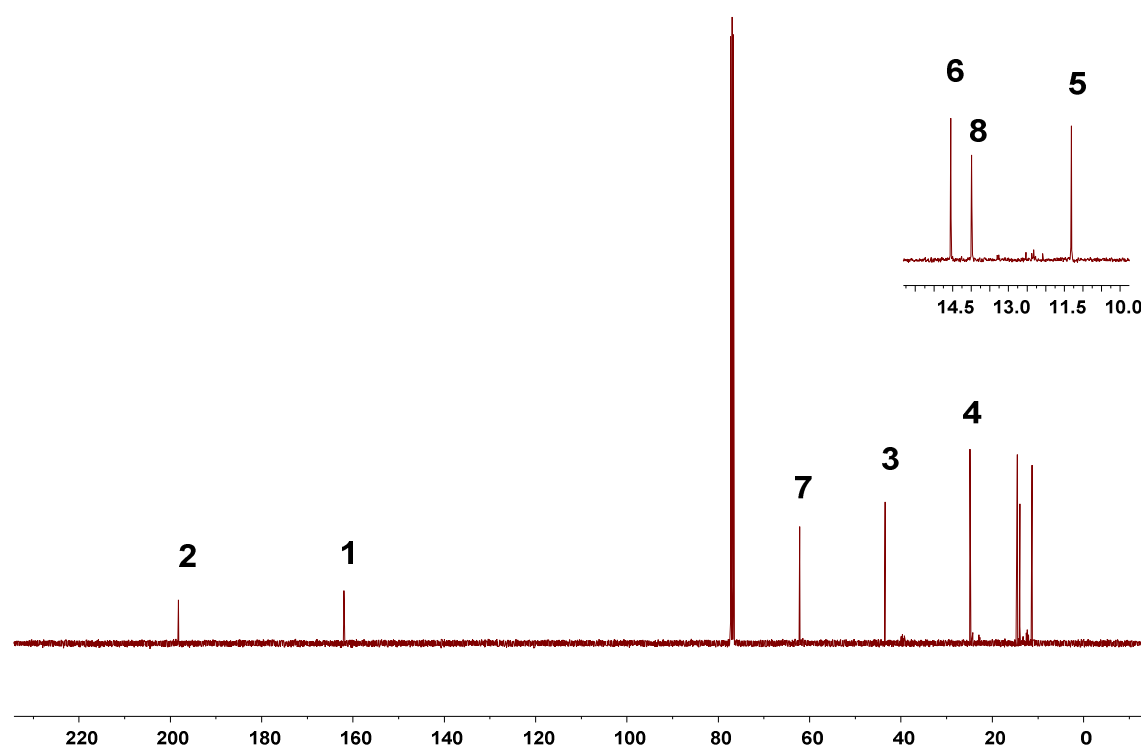
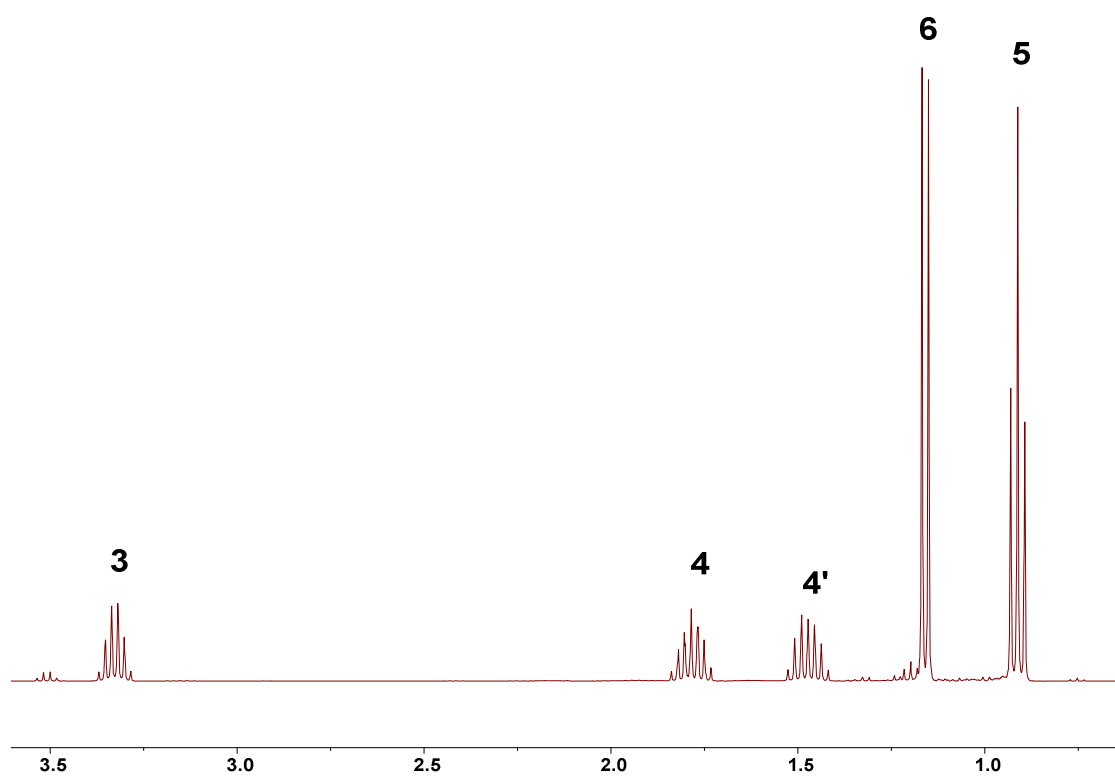
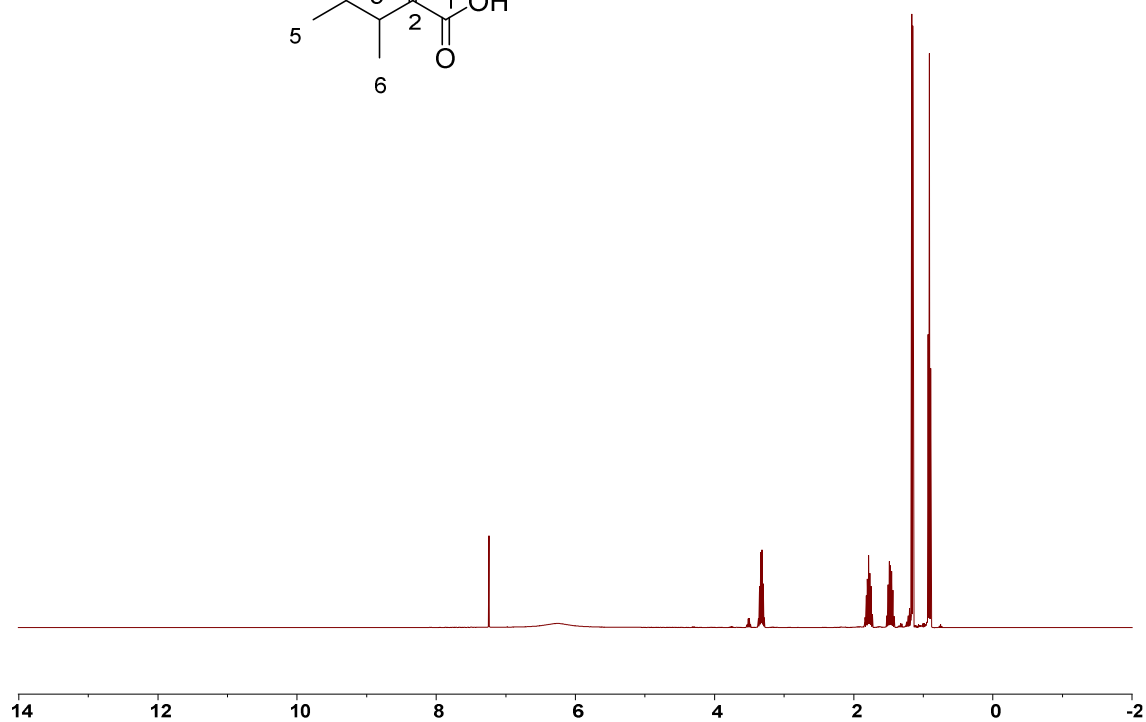
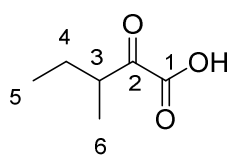


Figure S135 NMR spectra (CDCl_3) of the ester precursor of **C2n**: a) ^1H and b) ^{13}C .

a)



b)

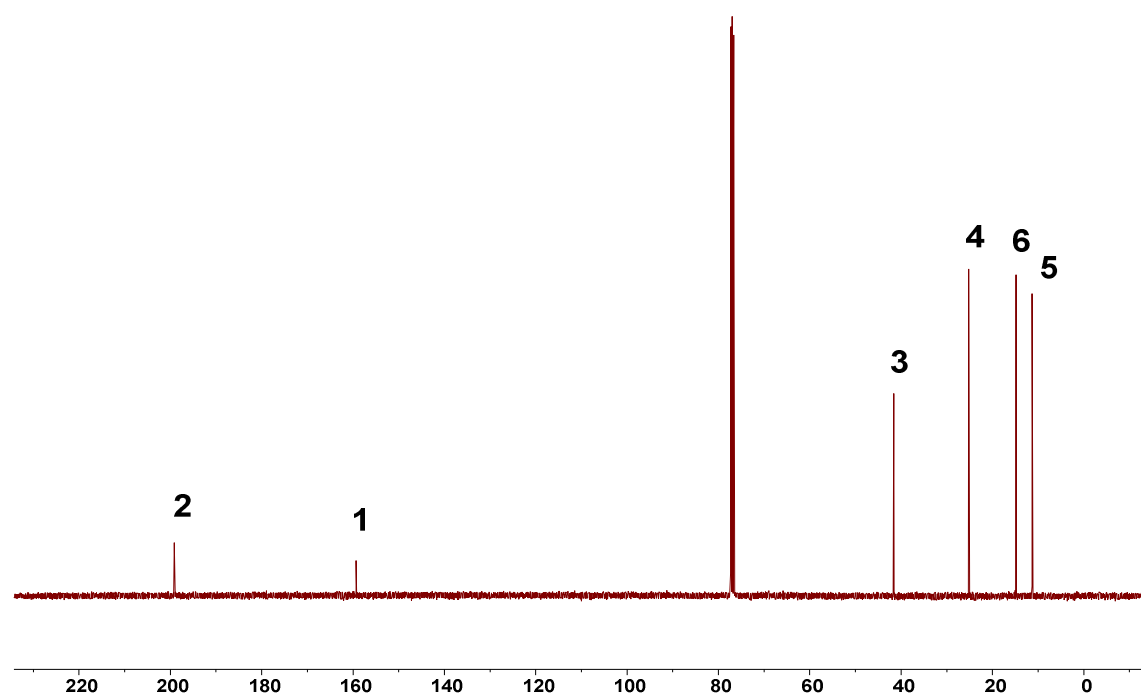
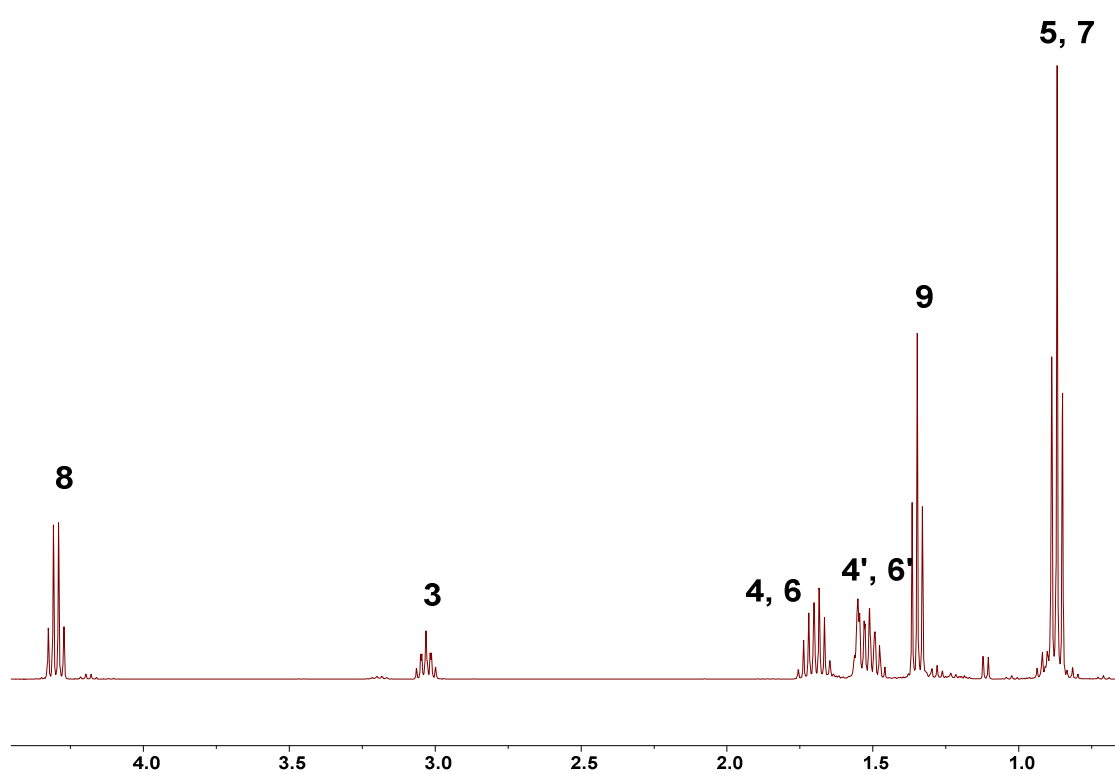
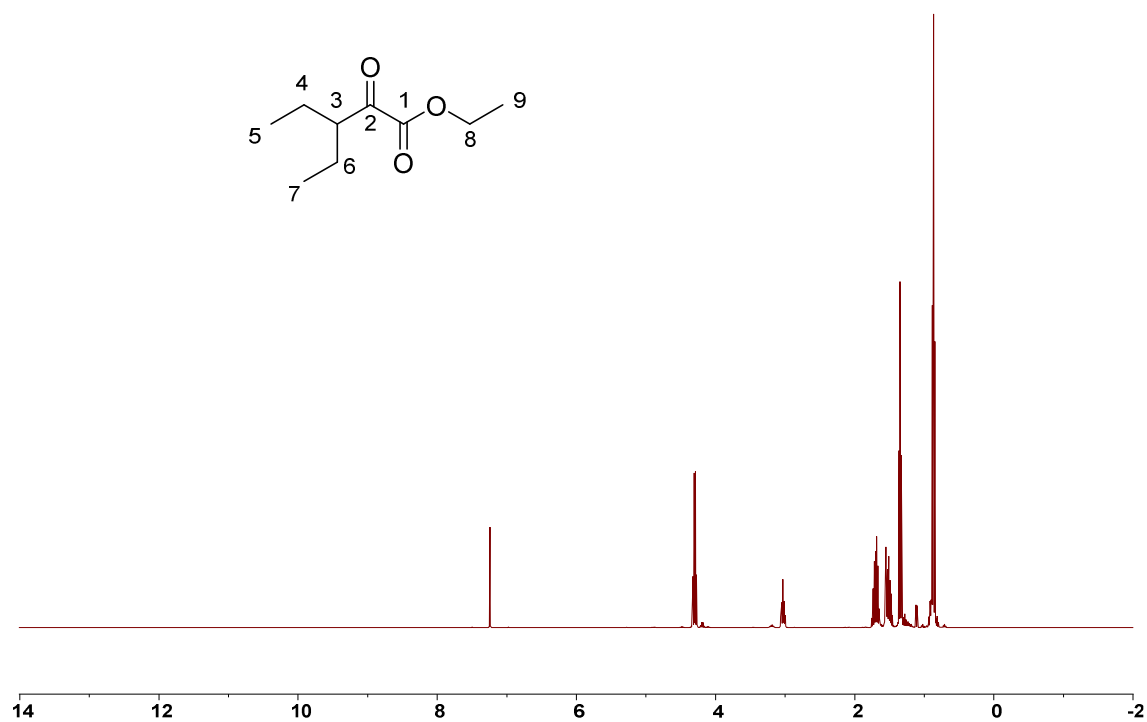


Figure S136 NMR spectra (CDCl_3) of *rac*-**1n**: a) ^1H and b) ^{13}C .

a)



b)

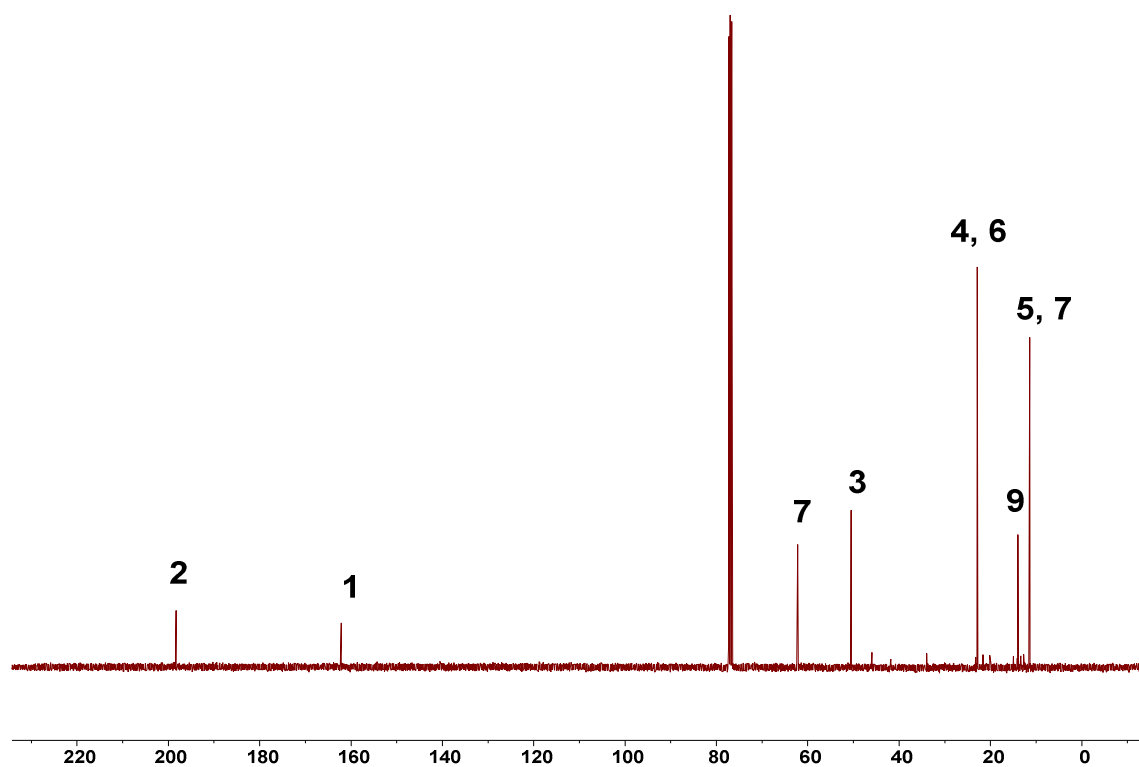
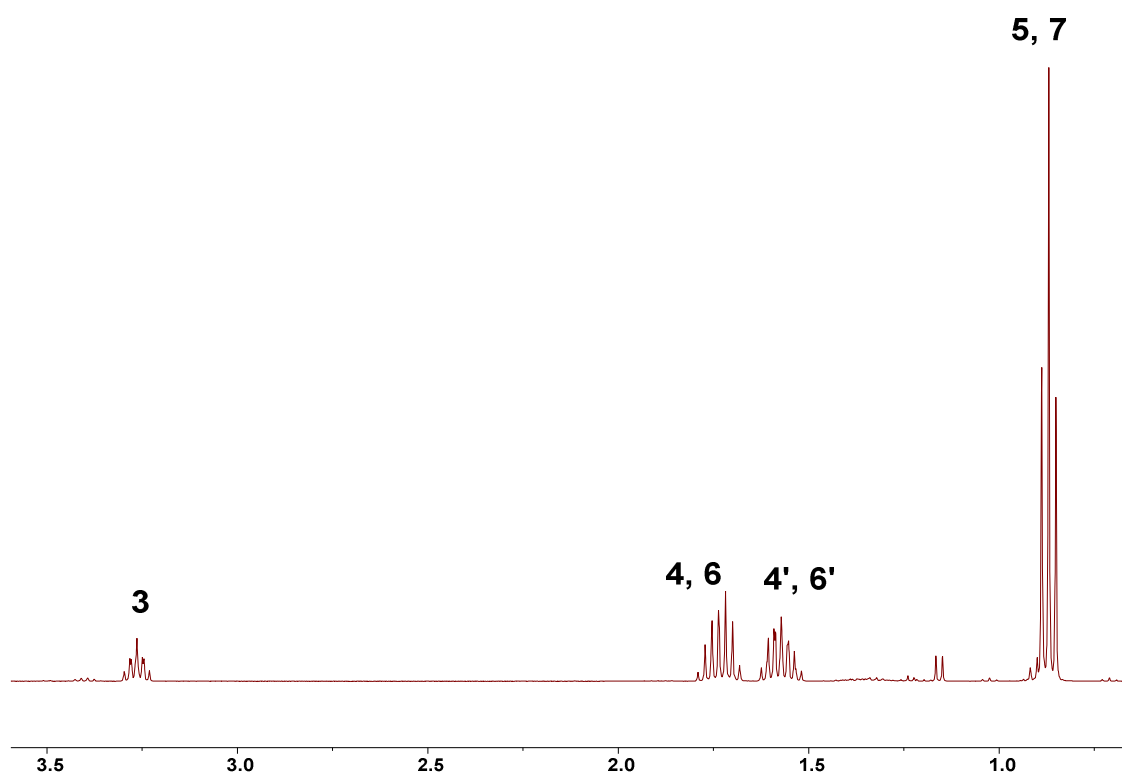
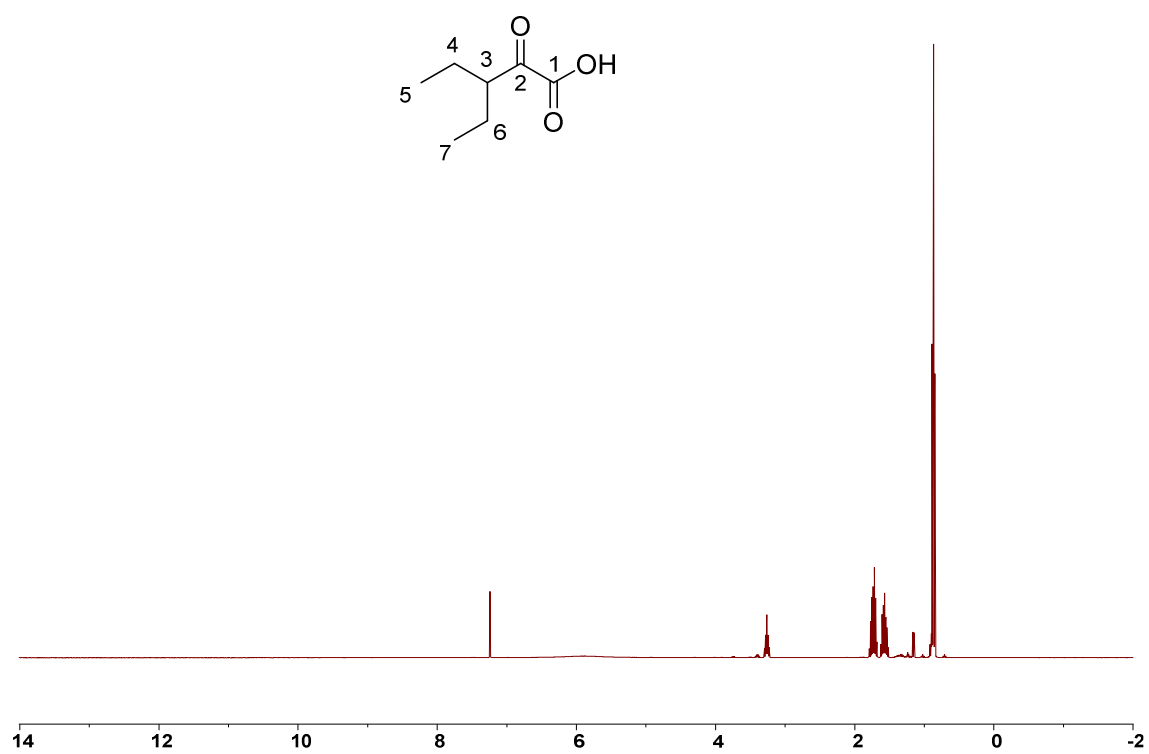


Figure S137 NMR spectra (CDCl_3) of the ester precursor of **C2o**: a) ^1H and b) ^{13}C .

a)



b)

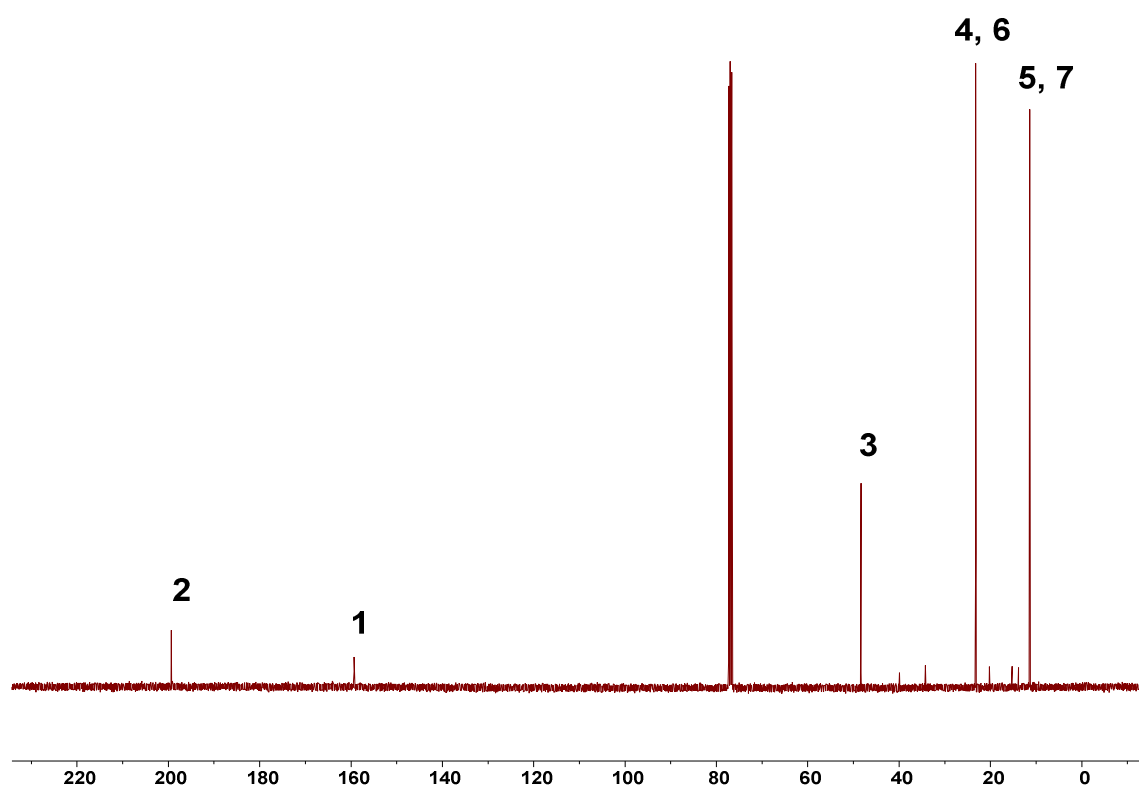
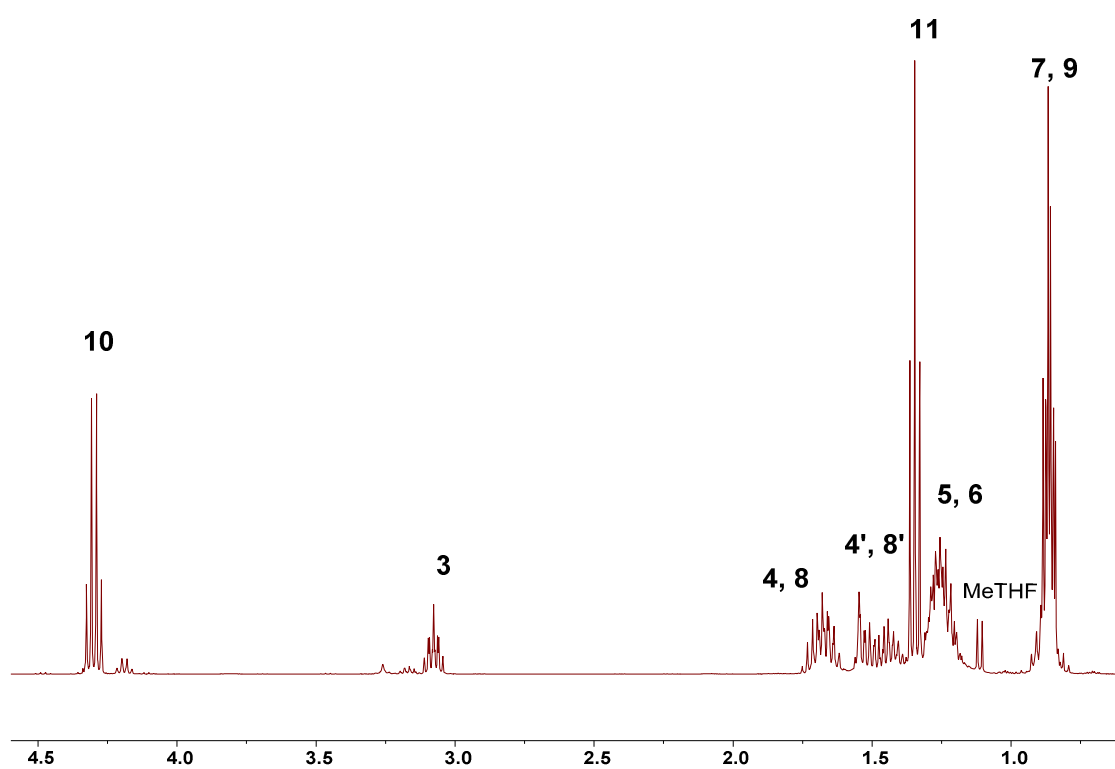
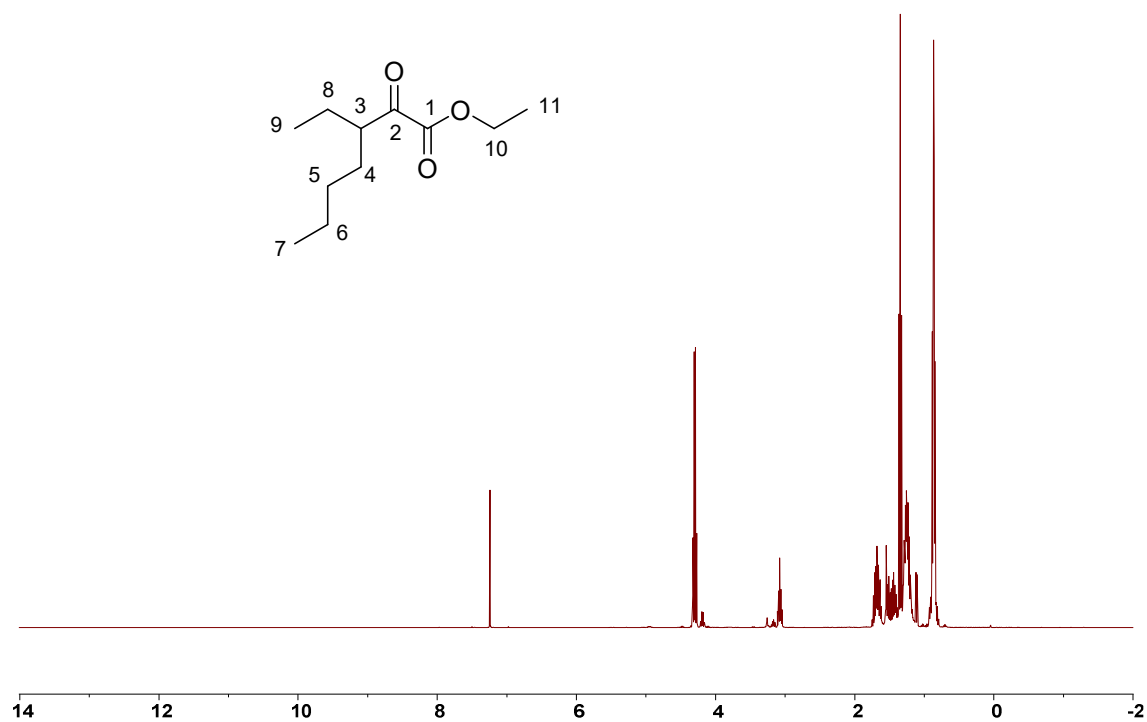


Figure S138 NMR spectra (CDCl_3) of **1o**: a) ^1H and b) ^{13}C .

a)



b)

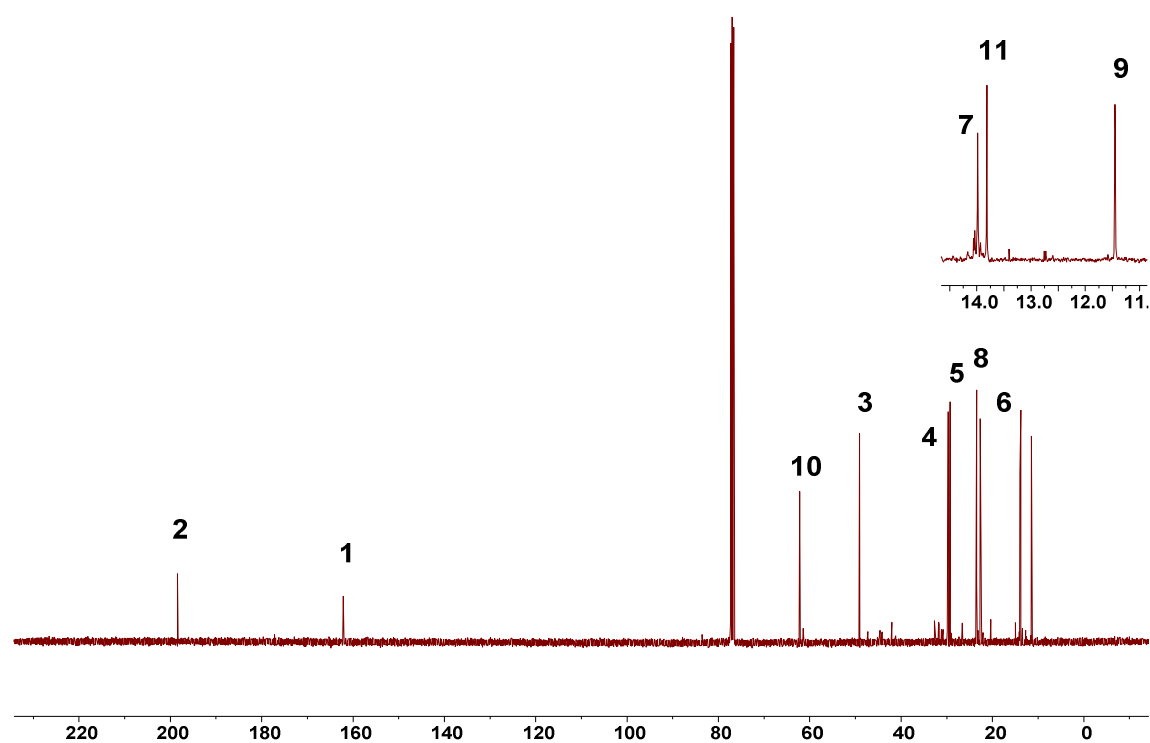
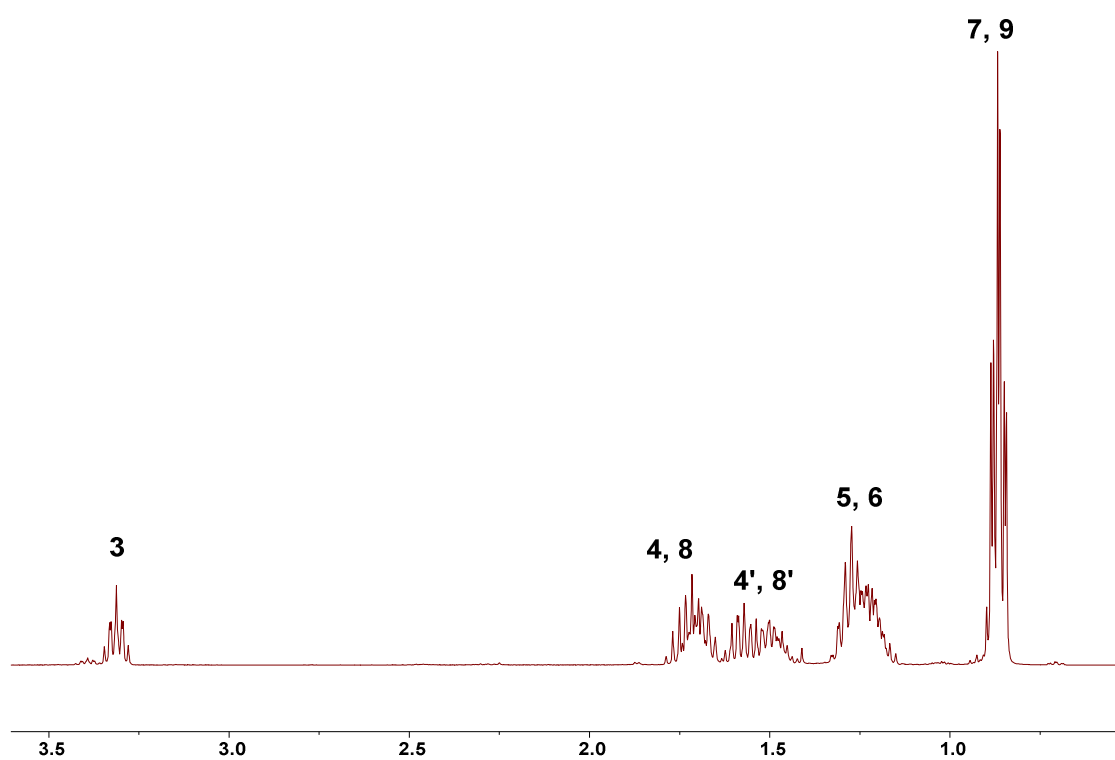
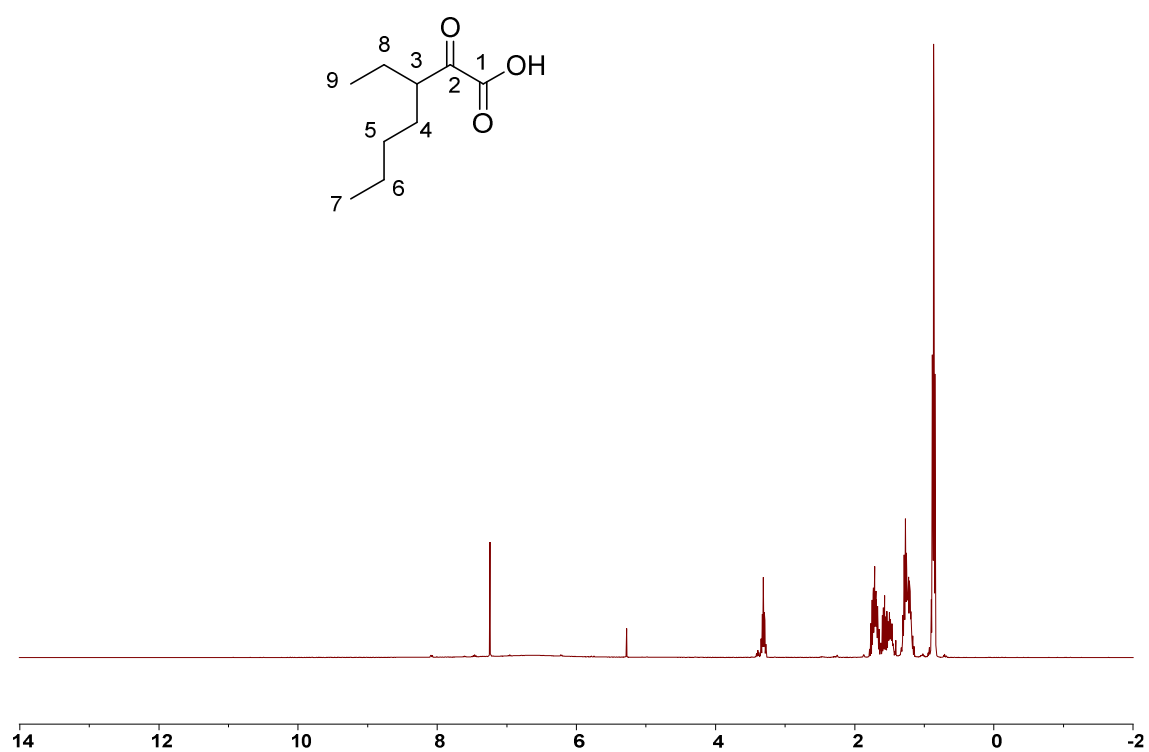


Figure S139 NMR spectra (CDCl_3) of the ester precursor of **C2p**: a) ^1H and b) ^{13}C .

a)



b)

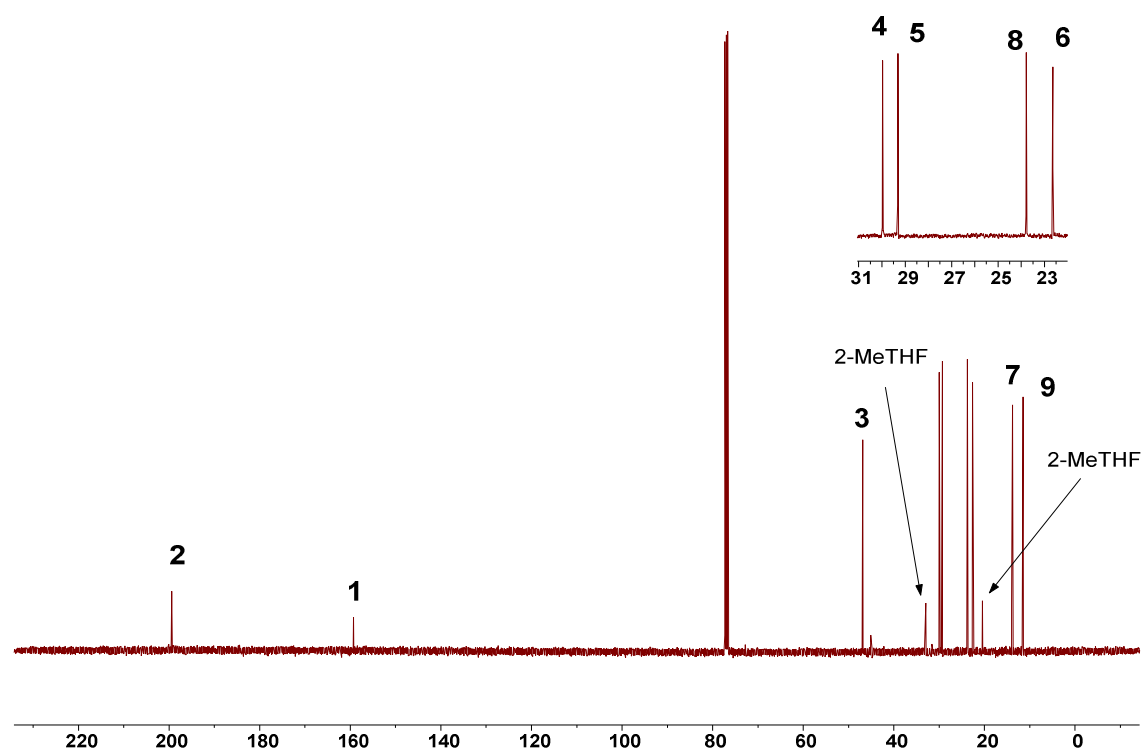
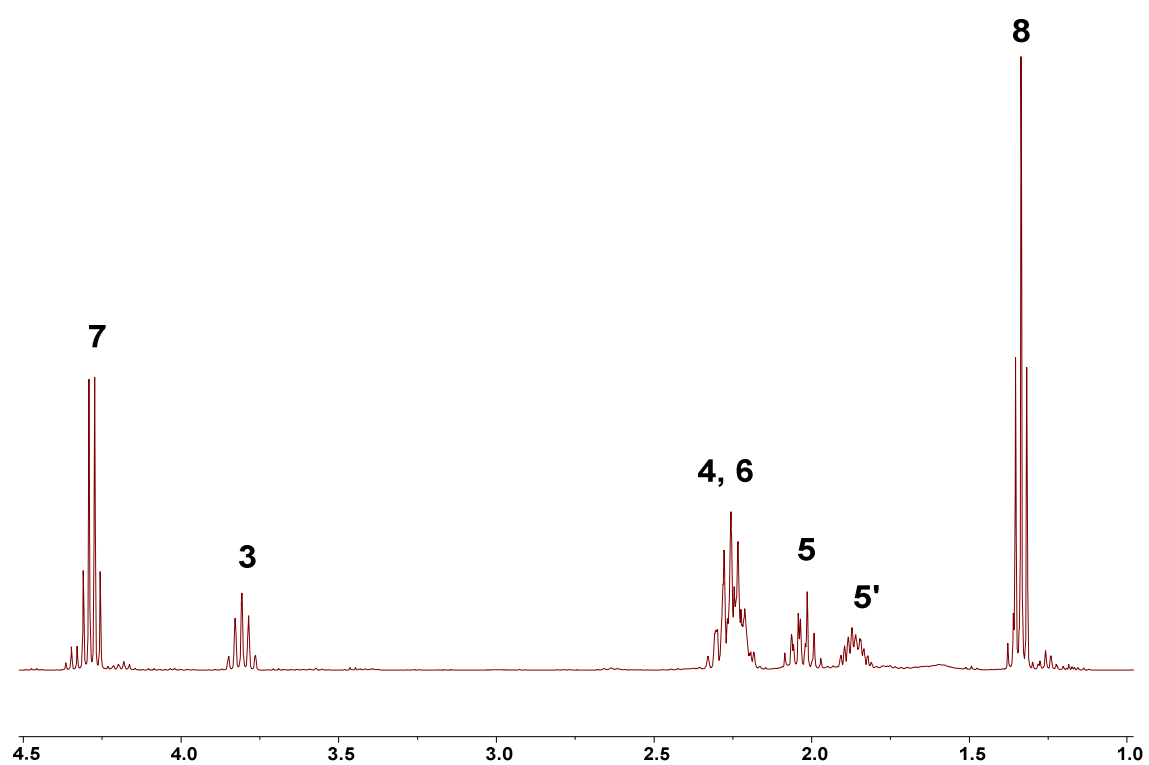
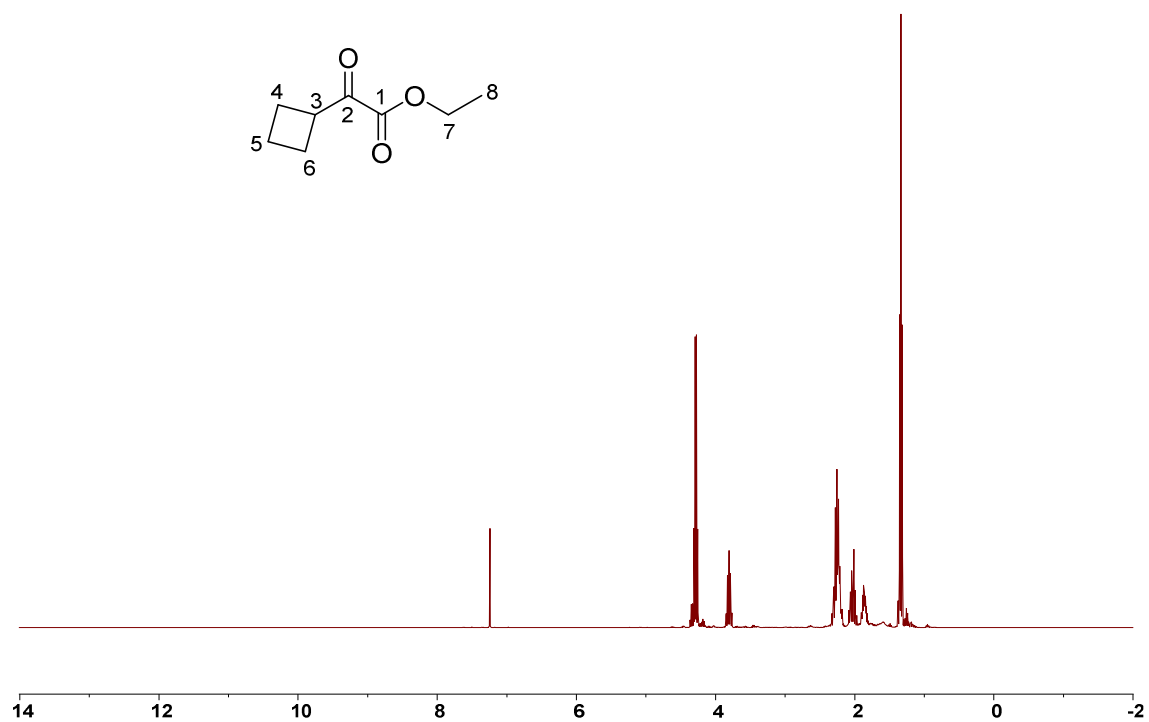


Figure S140 NMR spectra (CDCl_3) of *rac*-**1p**: a) ^1H and b) ^{13}C .

a)



b)

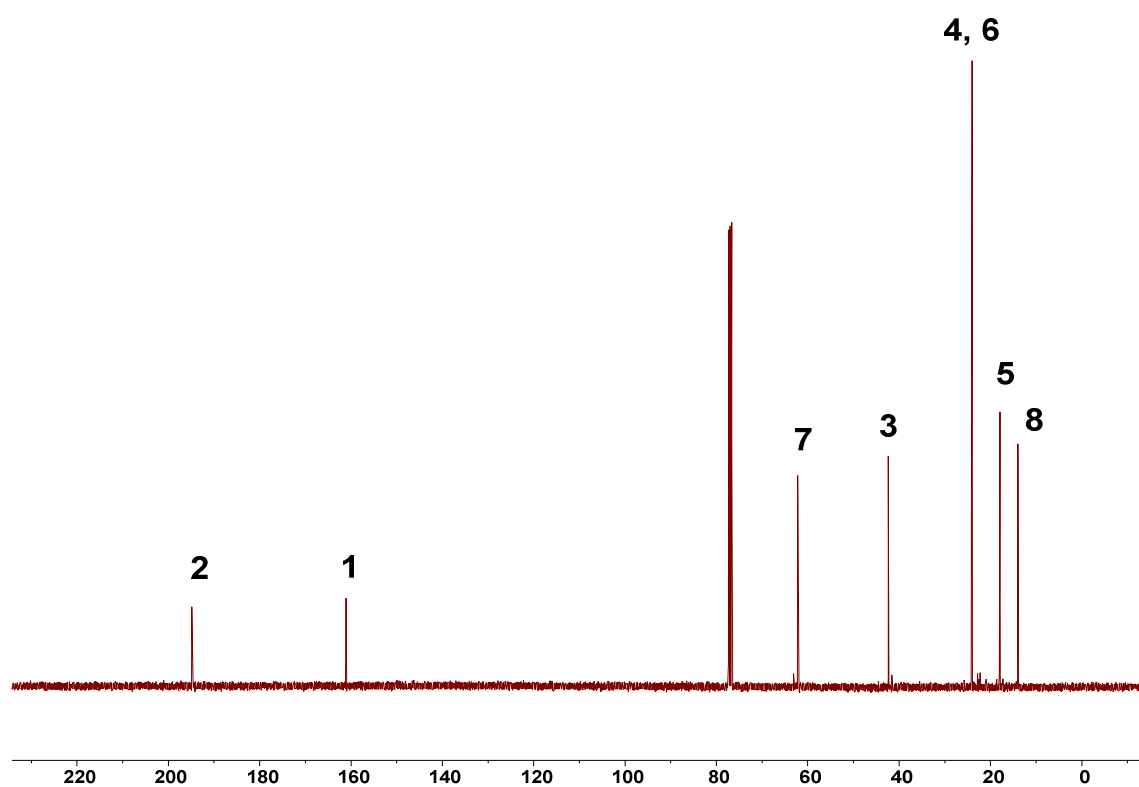
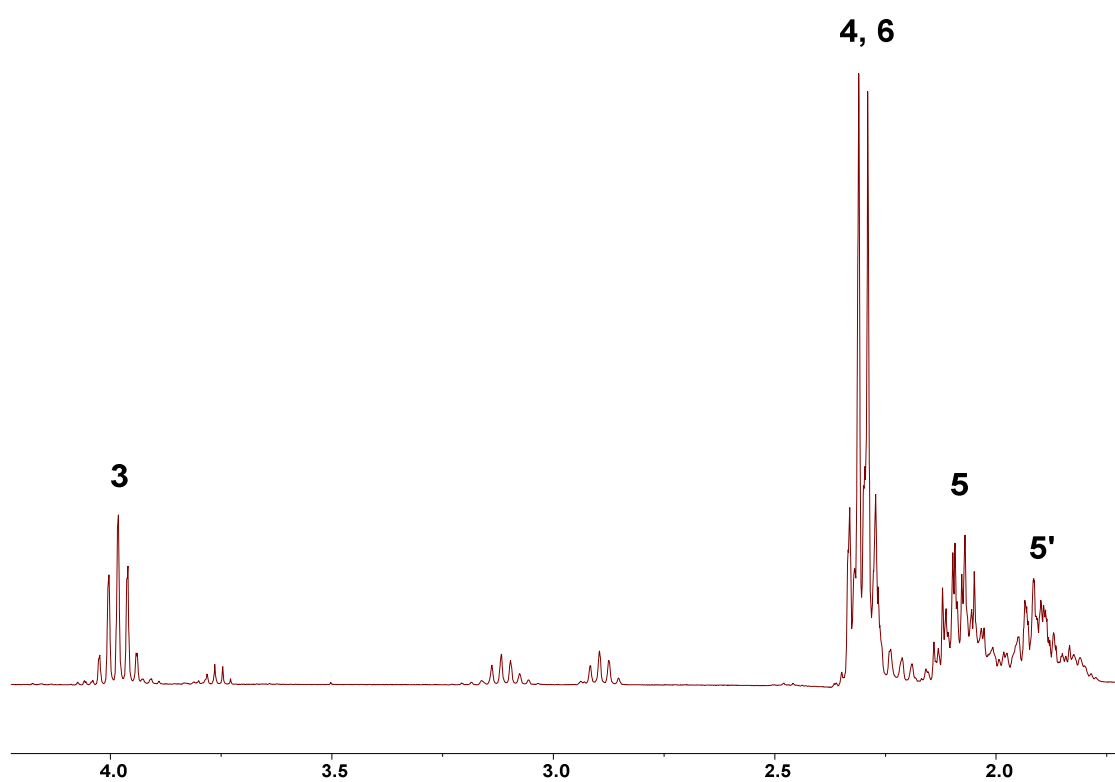
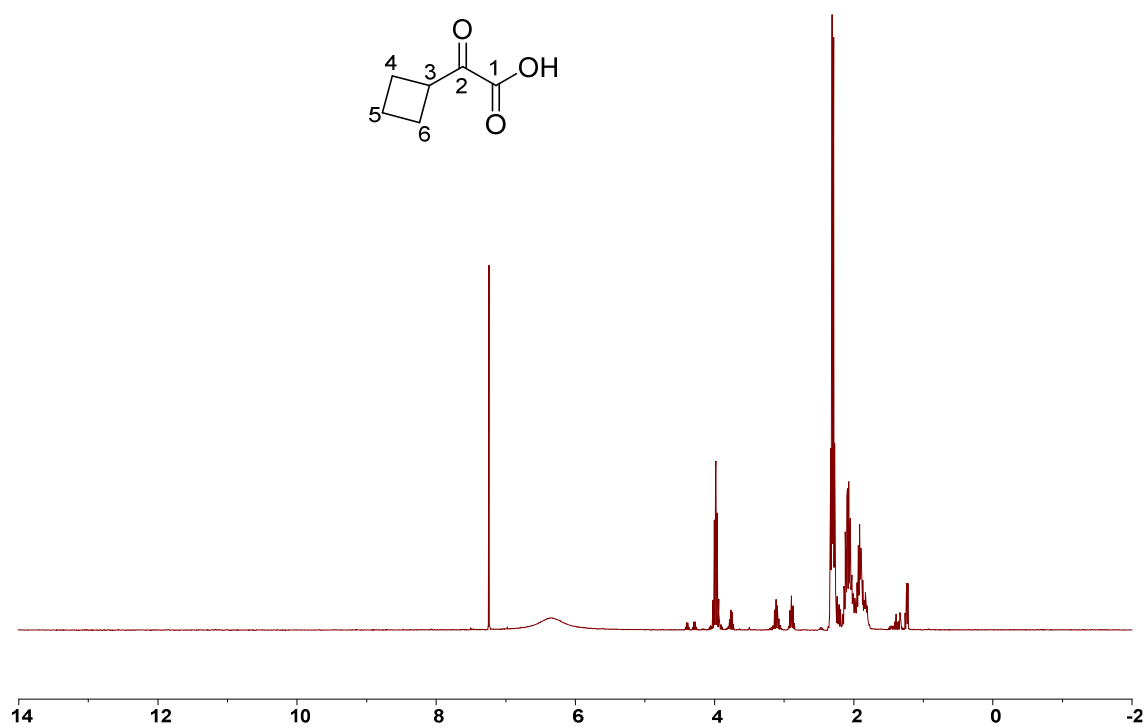
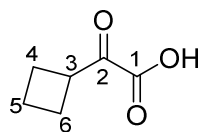


Figure S141 NMR spectra (CDCl₃) of the ester precursor of **C2q**: a) ¹H and b) ¹³C.

a)



b)

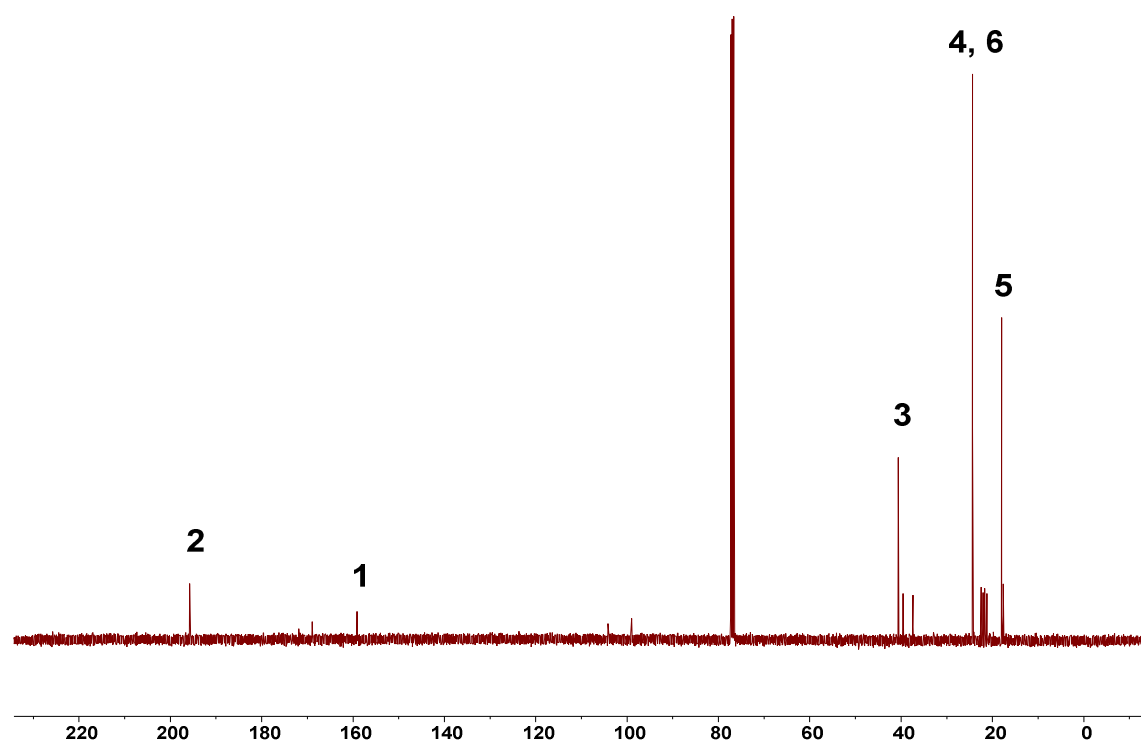
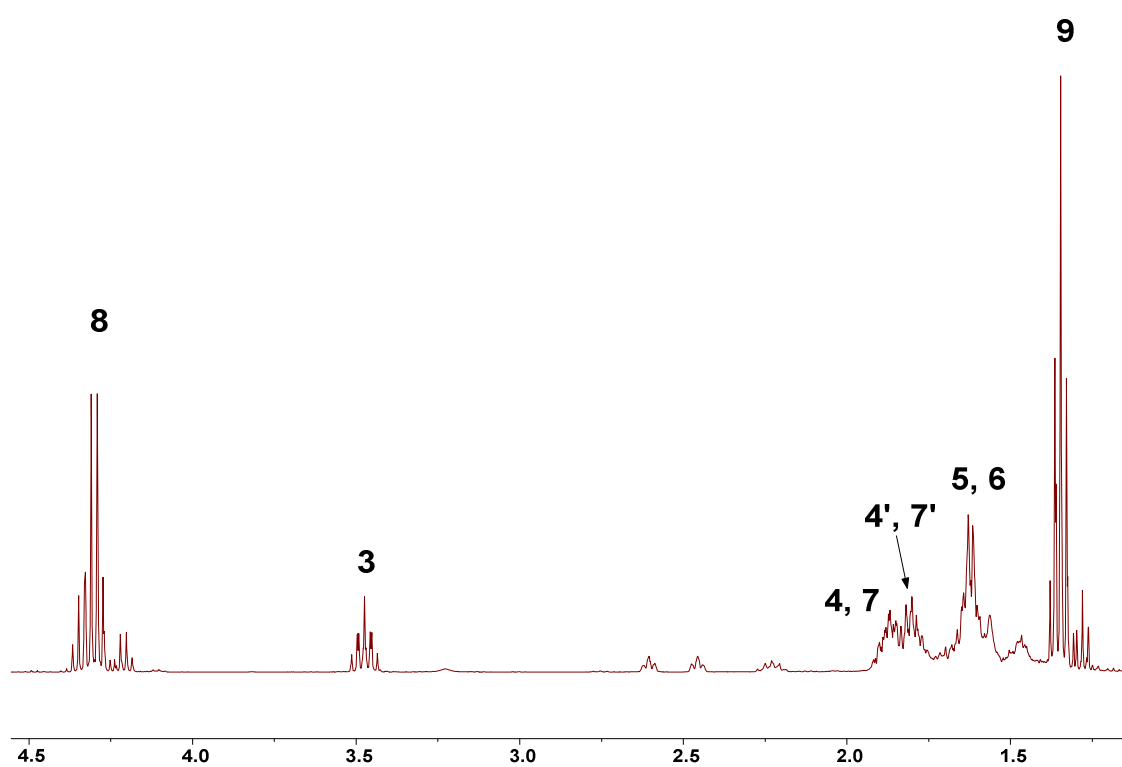
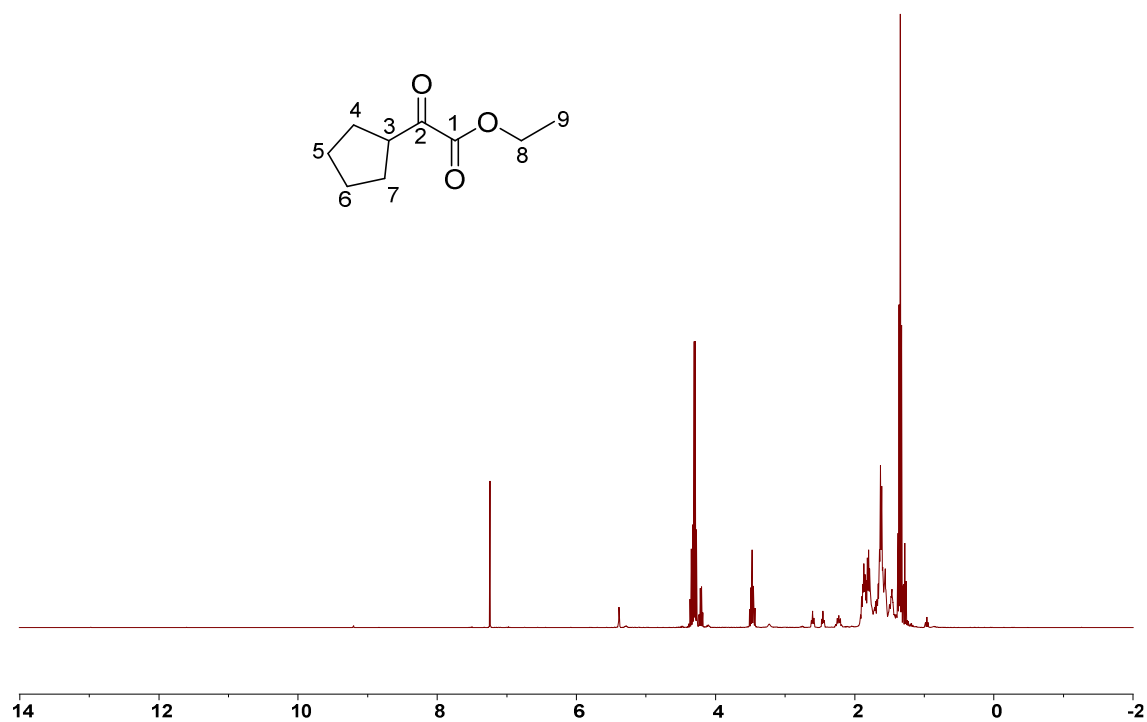


Figure S142 NMR spectra (CDCl_3) of **1q**: a) ^1H and b) ^{13}C .

a)



b)

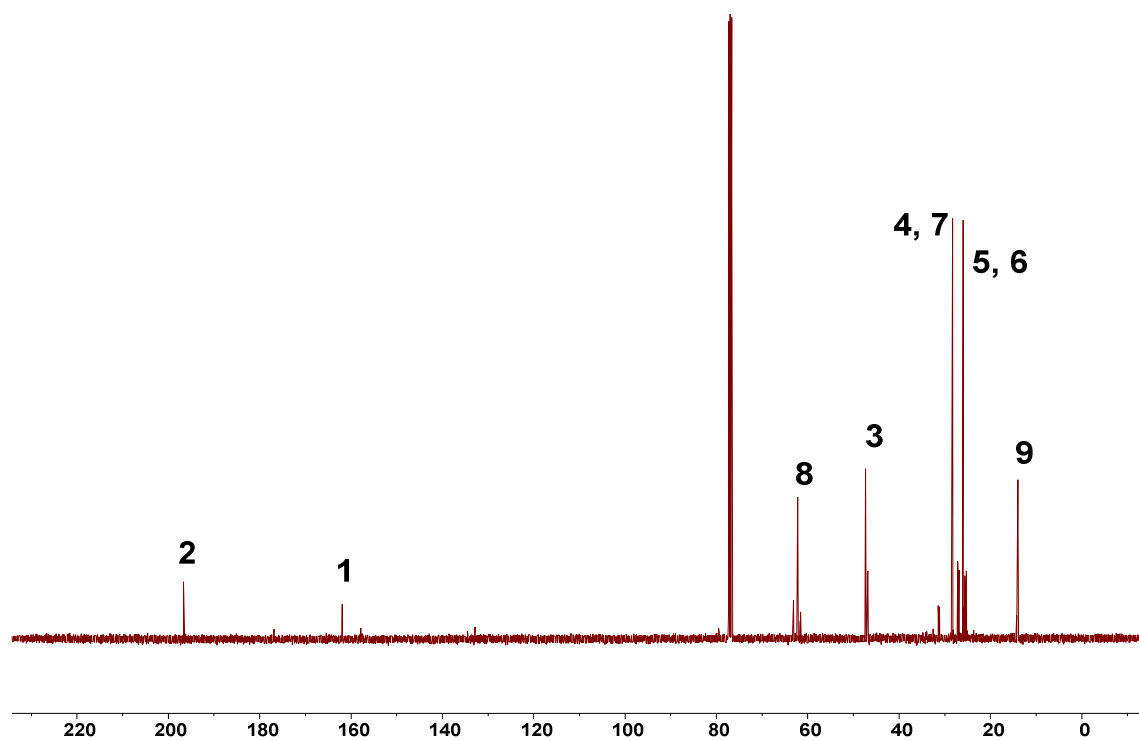
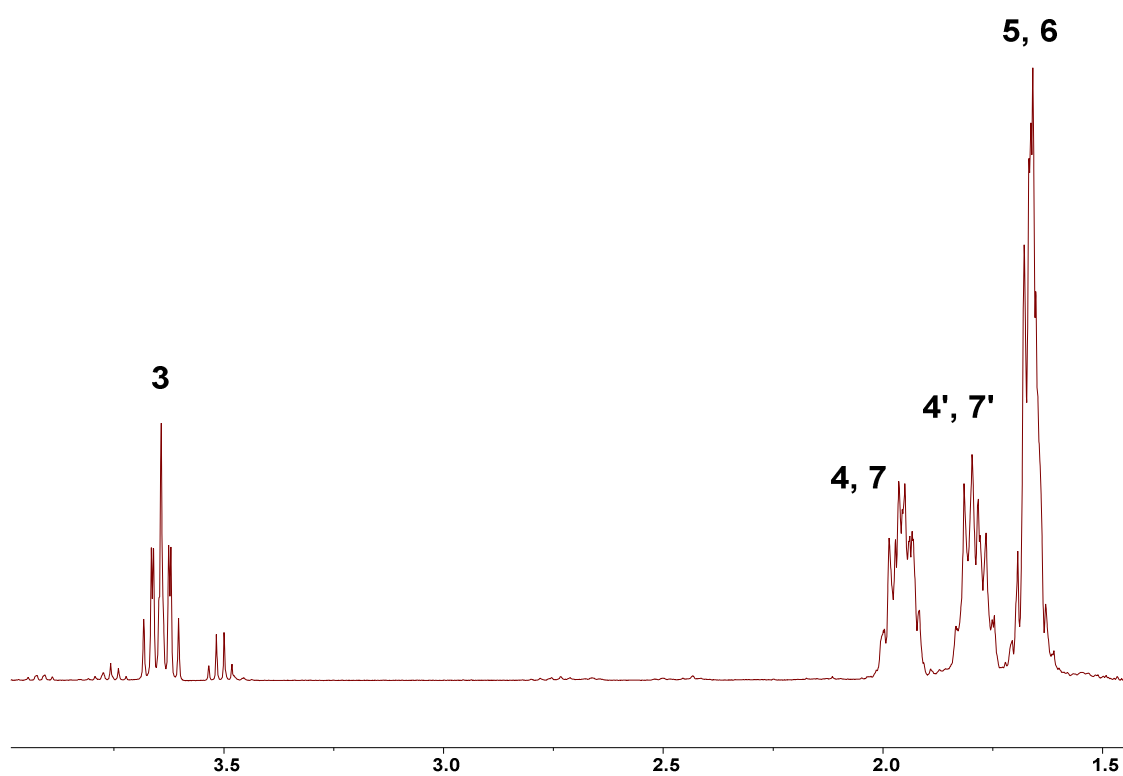
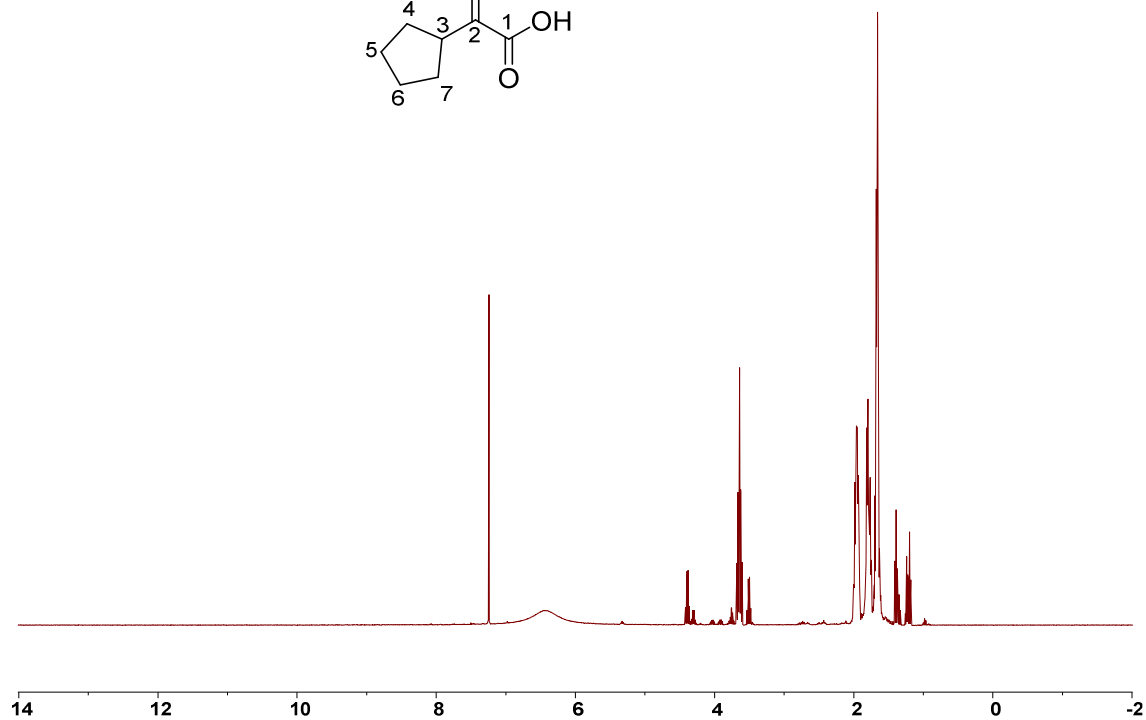
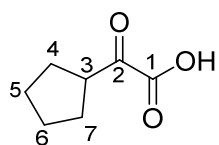


Figure S143 NMR spectra (CDCl_3) of the ester precursor of **C2r**: a) ^1H and b) ^{13}C .

a)



b)

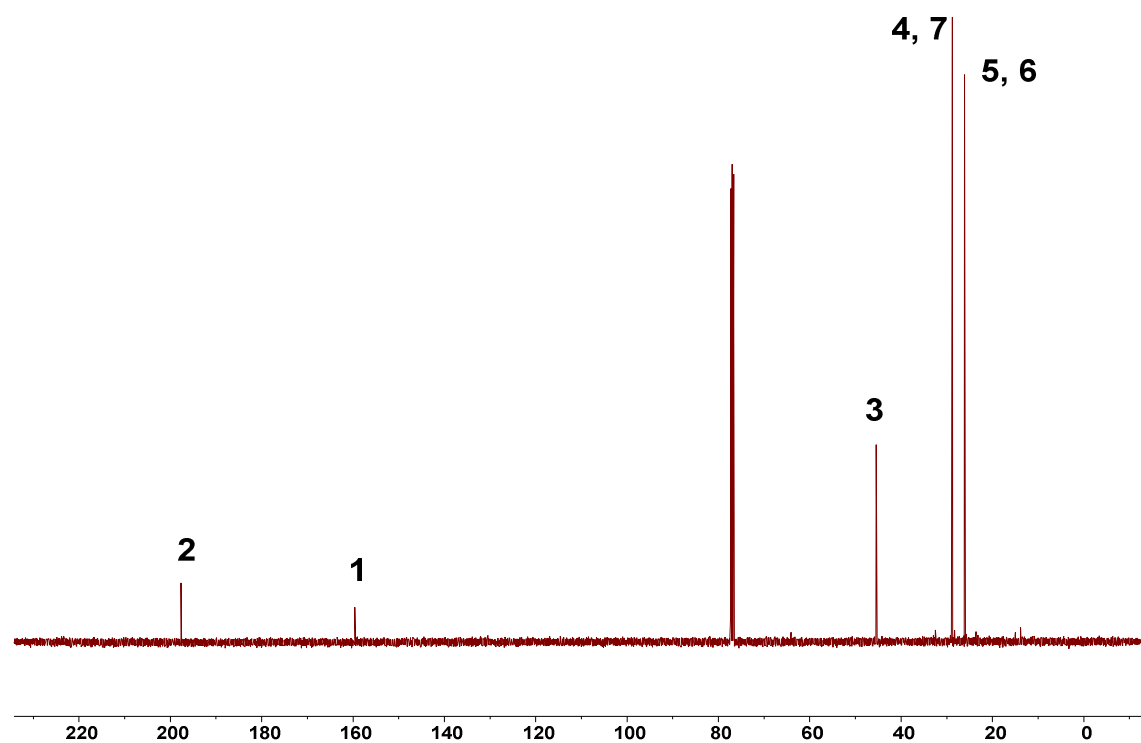
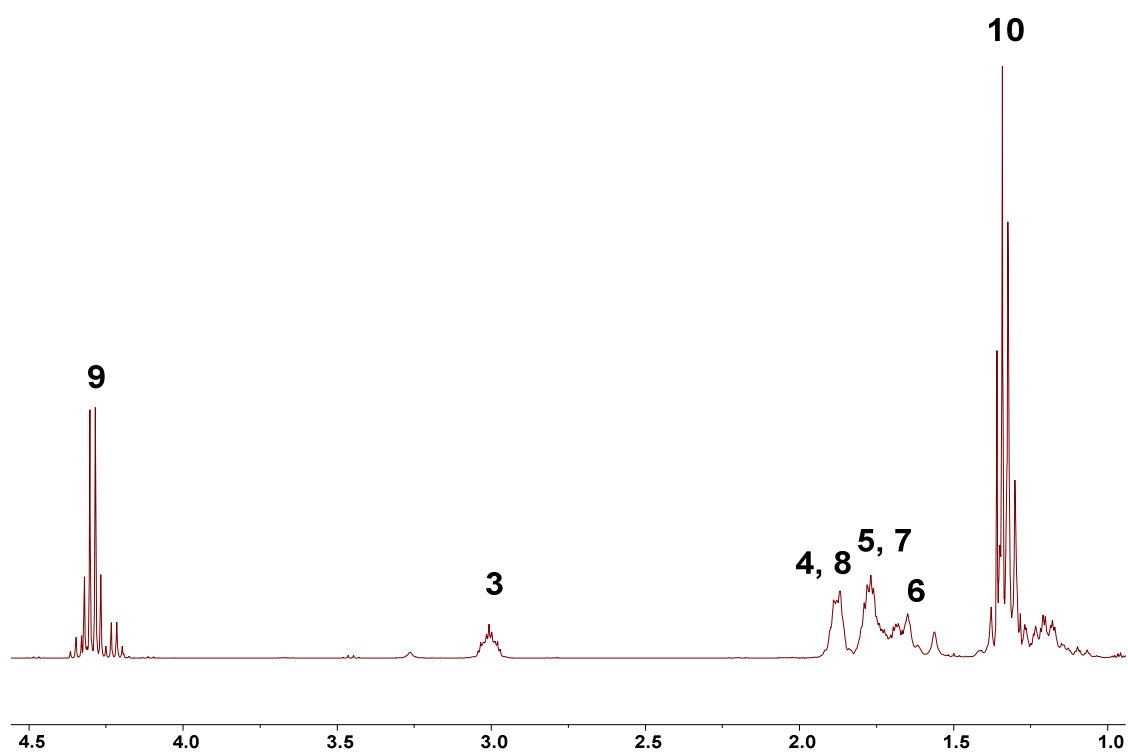
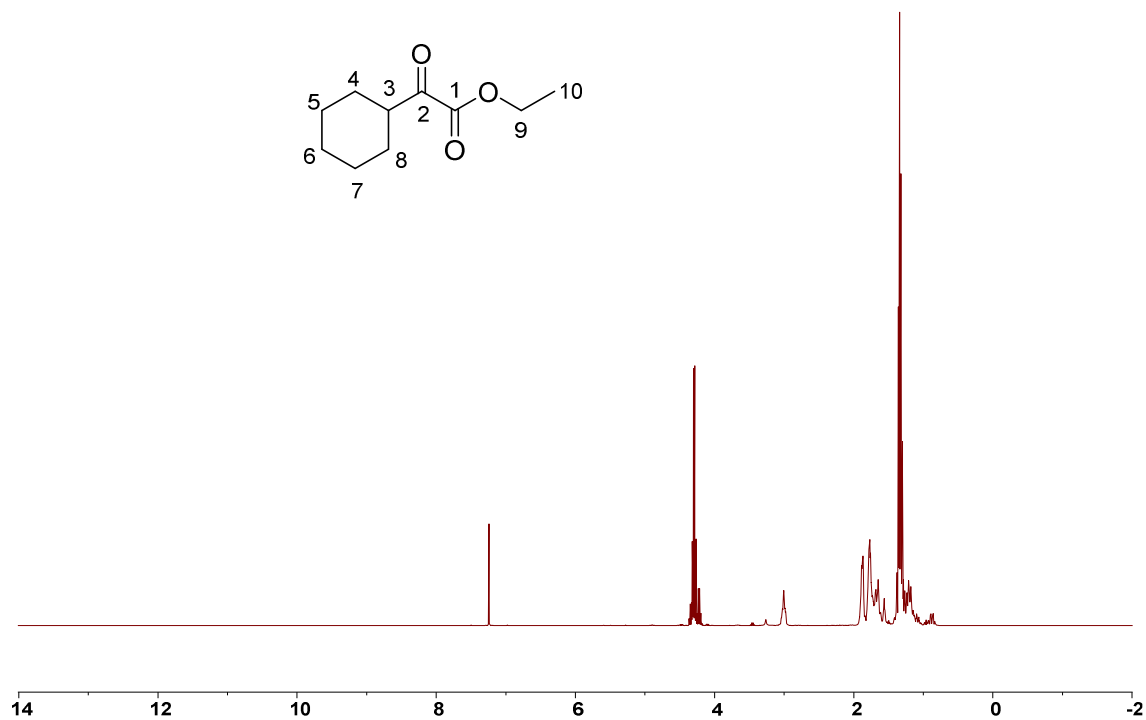
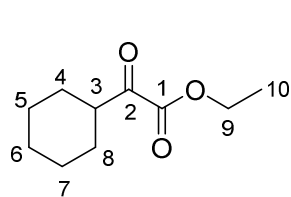


Figure S144 NMR spectra (CDCl_3) of **1r**: a) ^1H and b) ^{13}C .

a)



b)

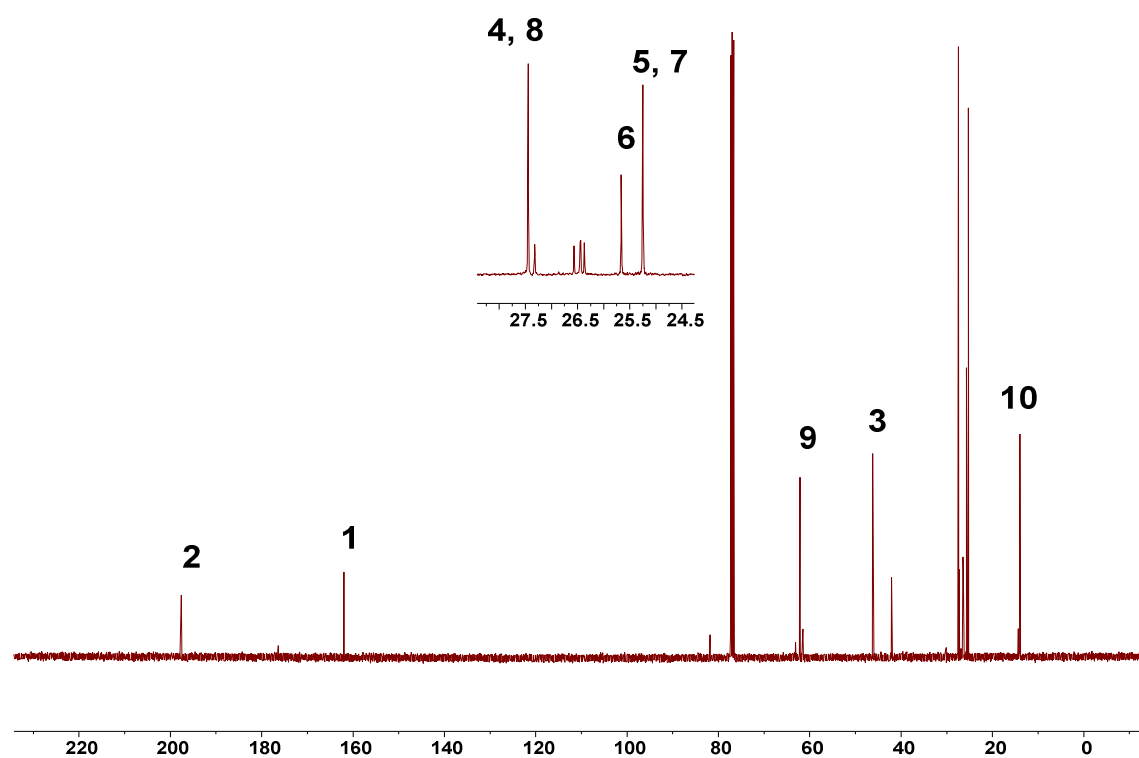
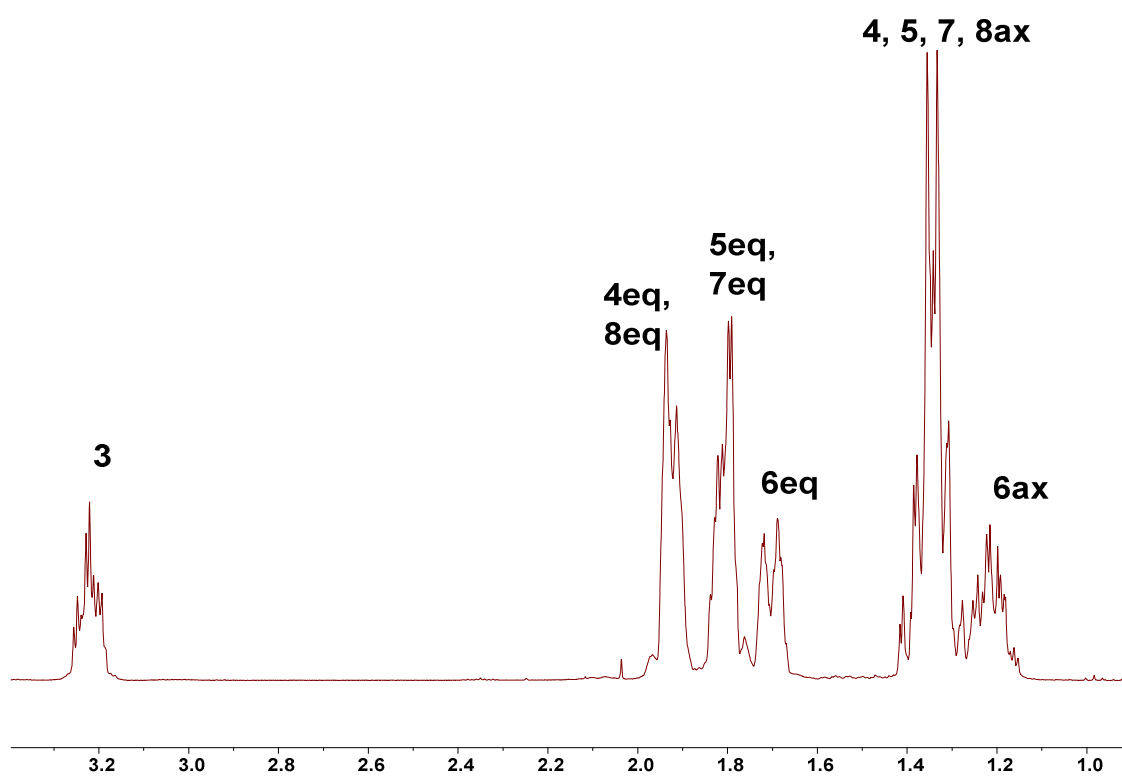
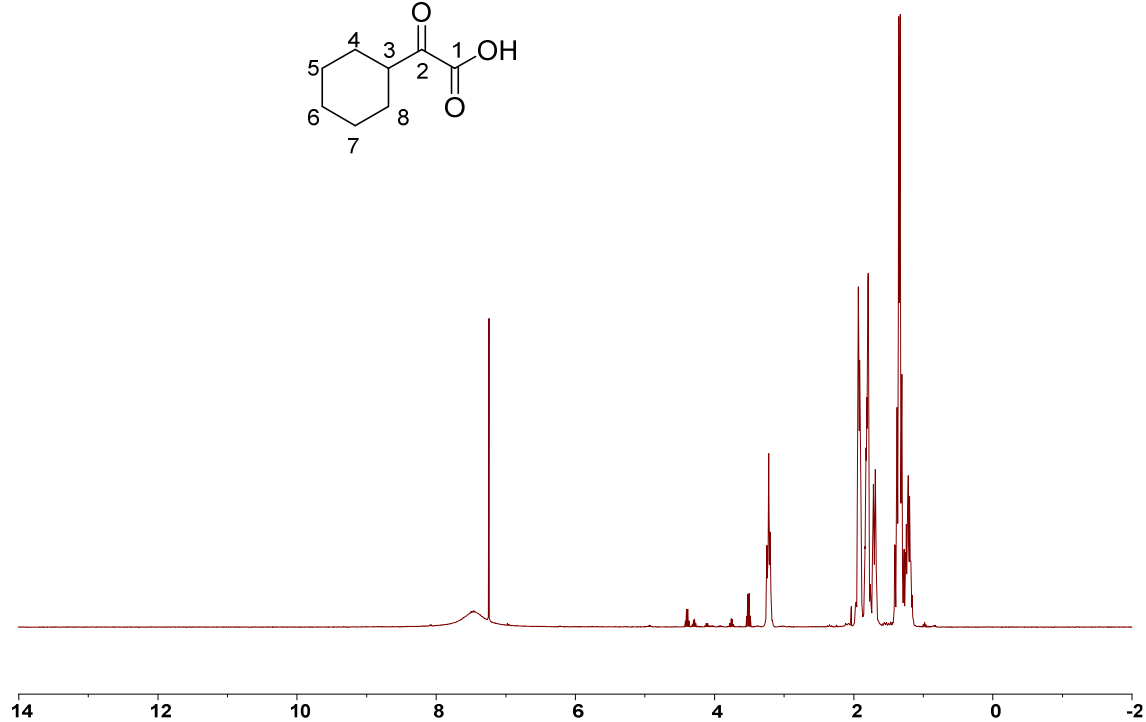
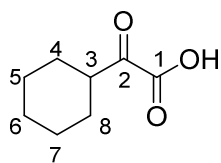


Figure S145 NMR spectra (CDCl_3) of the ester precursor of **C2s**: a) ^1H and b) ^{13}C .

a)



b)

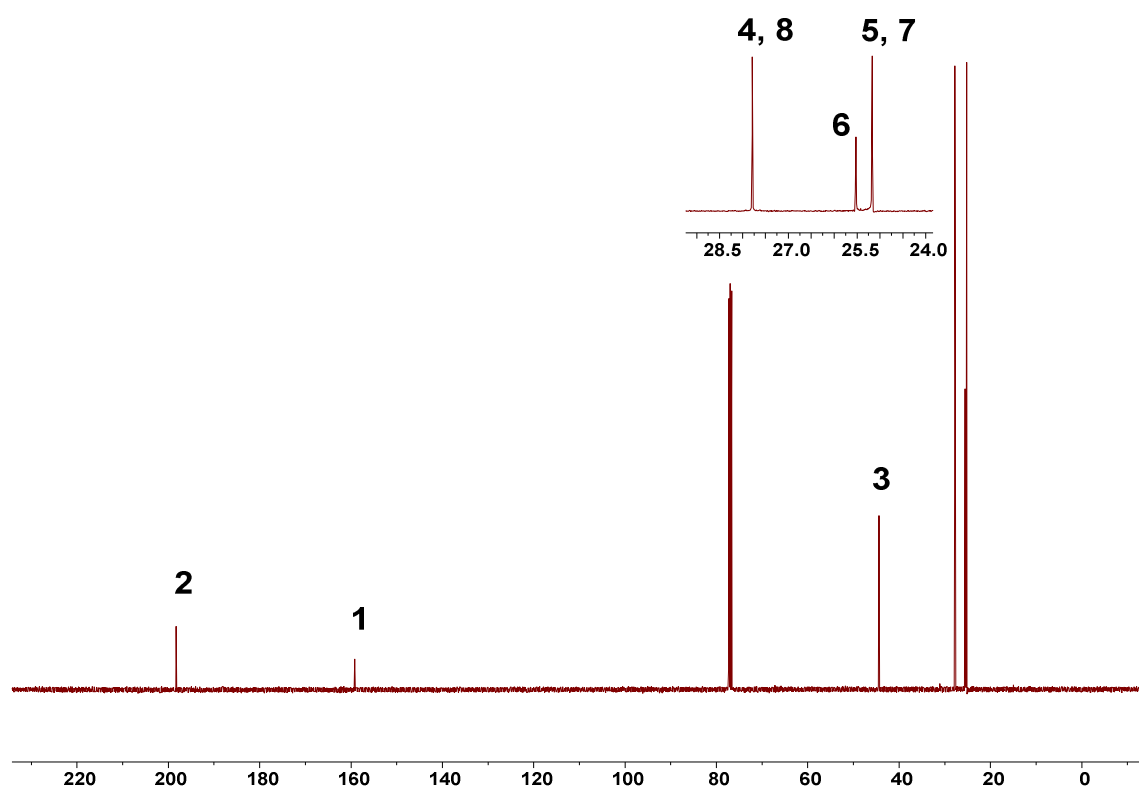
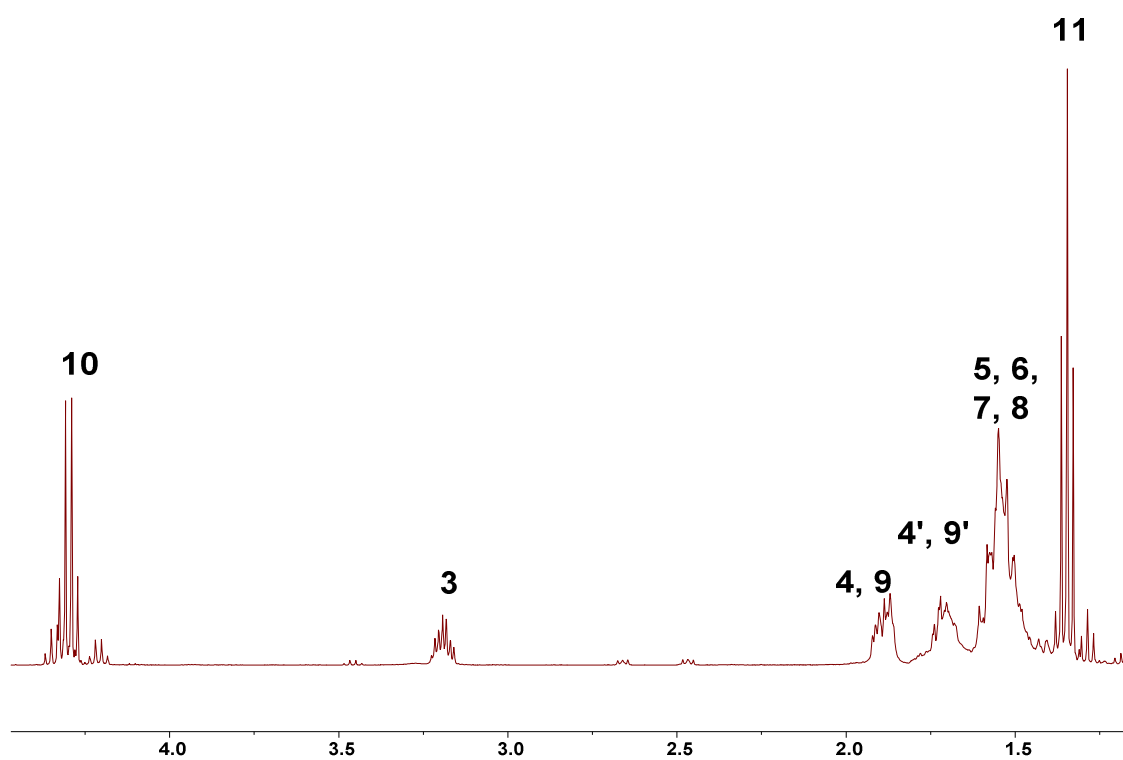
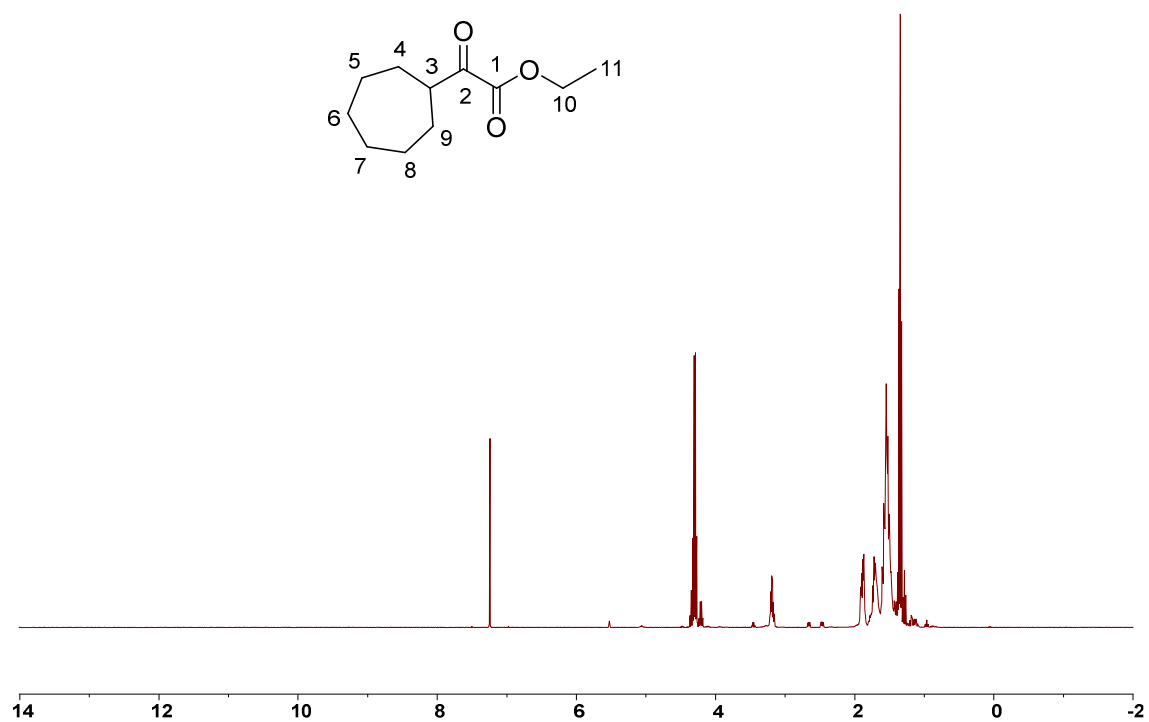


Figure S146 NMR spectra (CDCl_3) of **1s**: a) ^1H and b) ^{13}C .

a)



b)

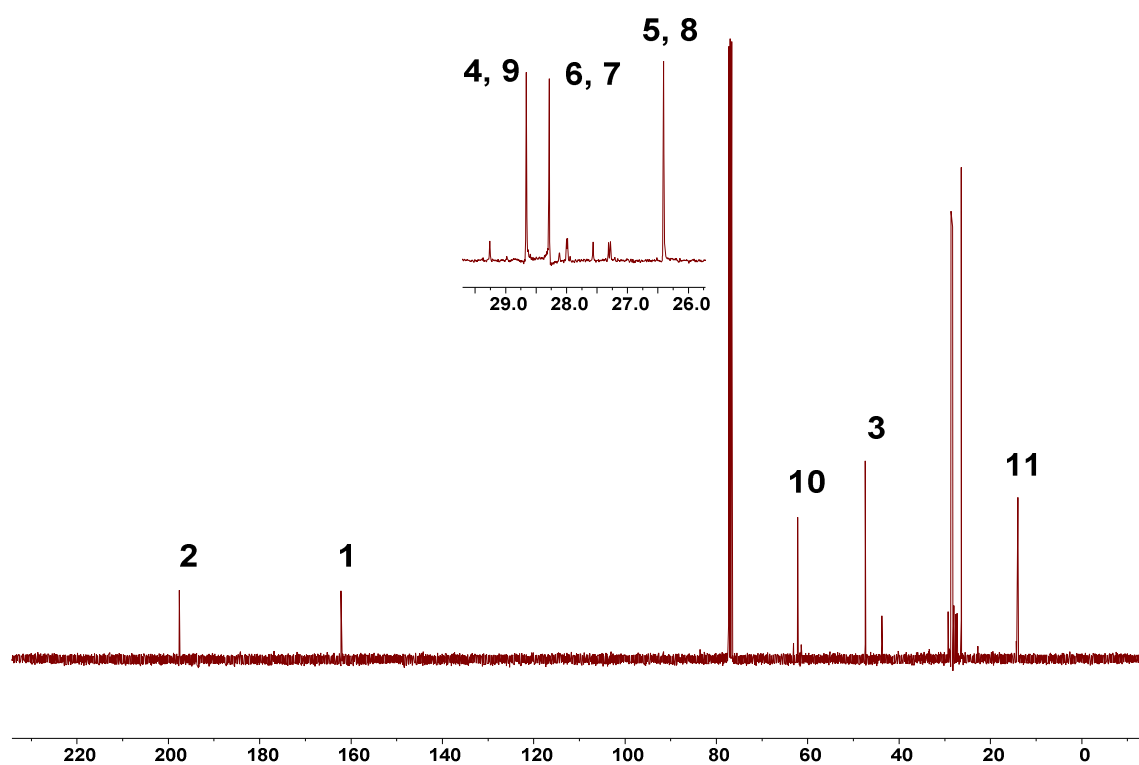
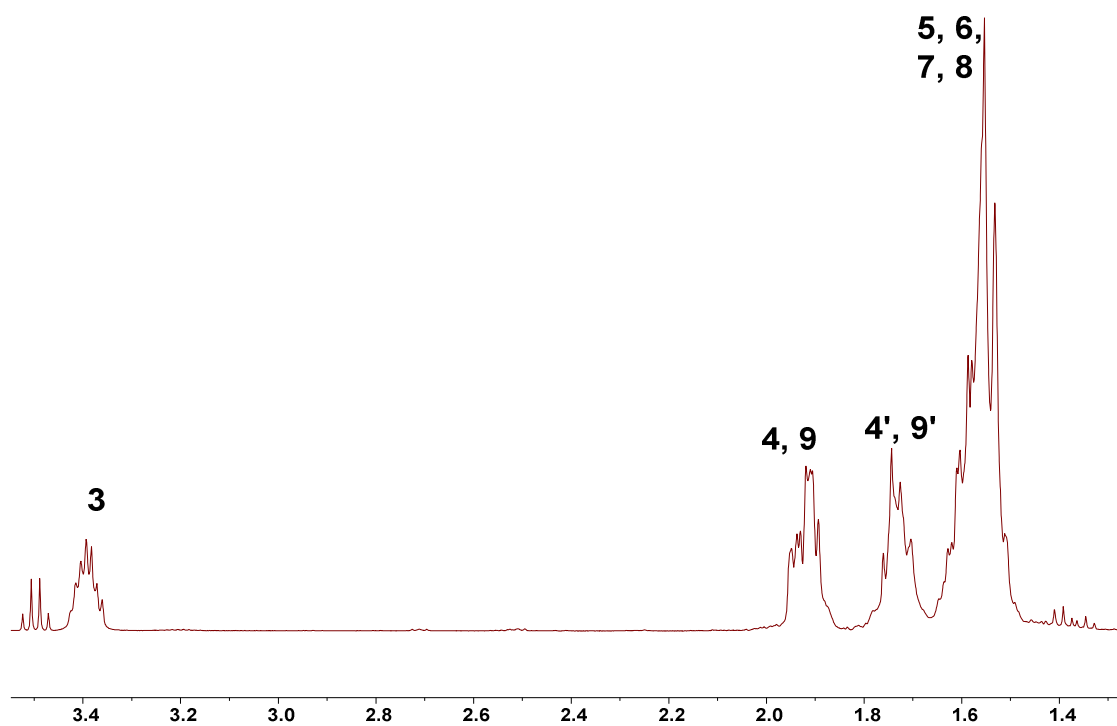
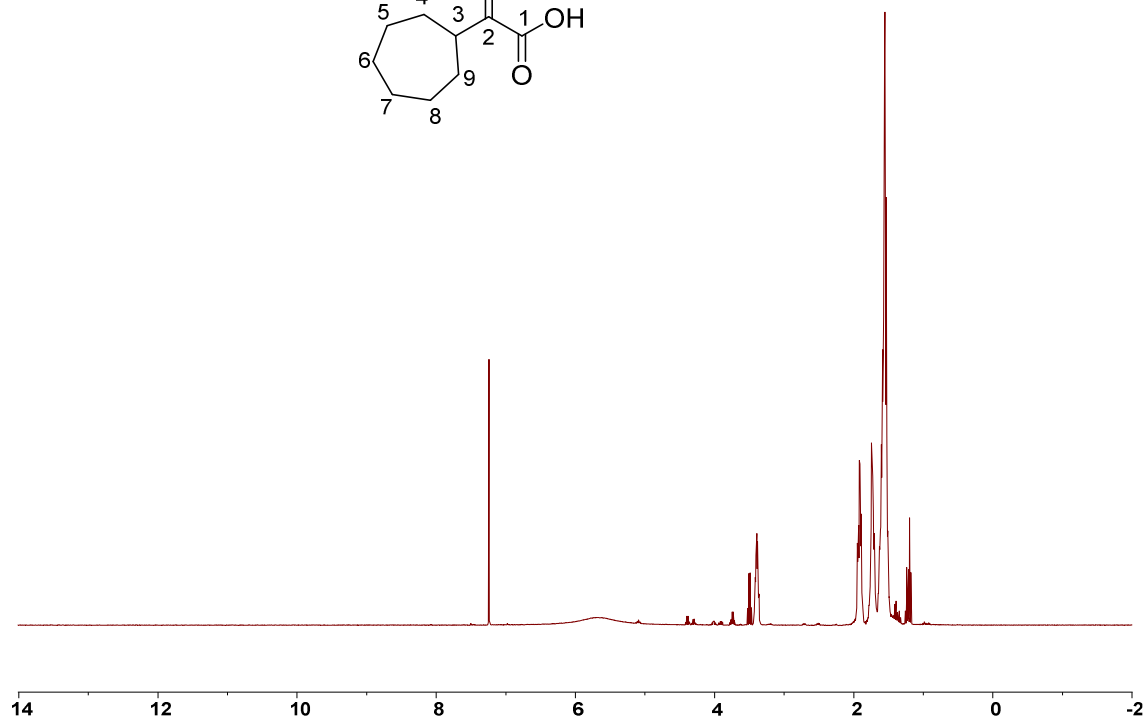
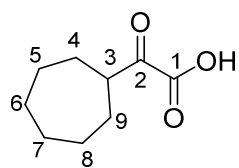


Figure S147 NMR spectra (CDCl_3) of the ester precursor of **C2t**: a) ^1H and b) ^{13}C .

a)



b)

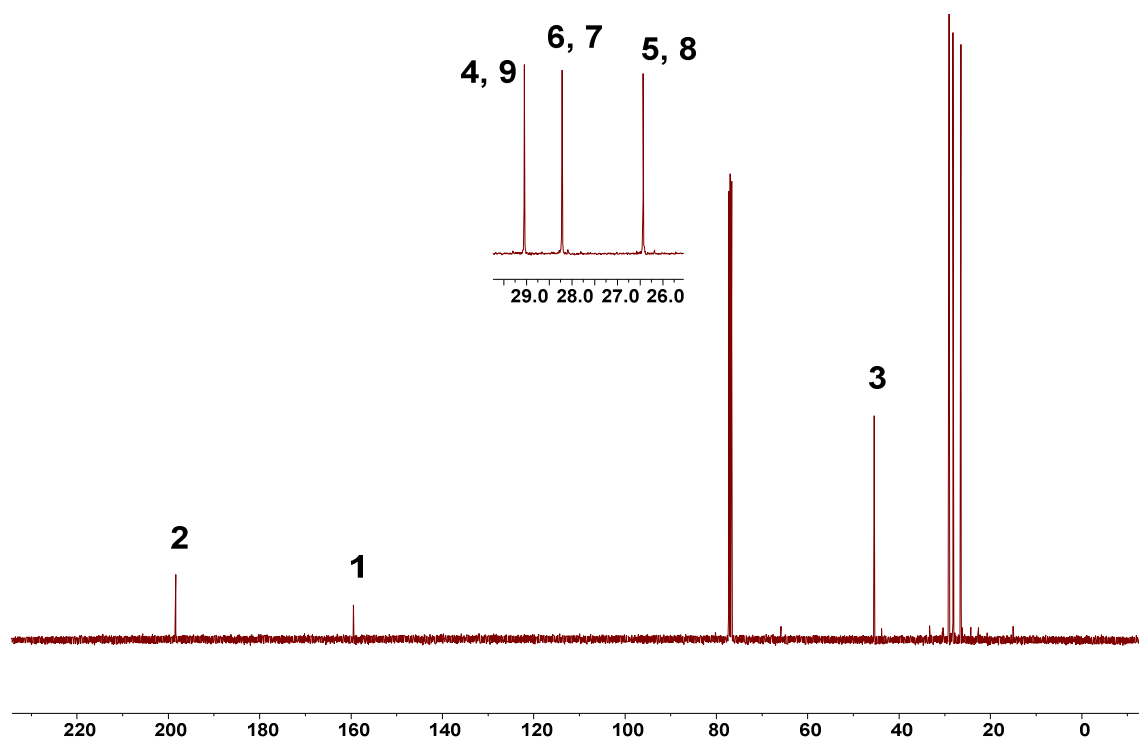


Figure S148 NMR spectra (CDCl_3) of **1t**: a) ^1H and b) ^{13}C .

6.3.2 Reaction monitoring for the synthesis of aldol adducts intermediates 3

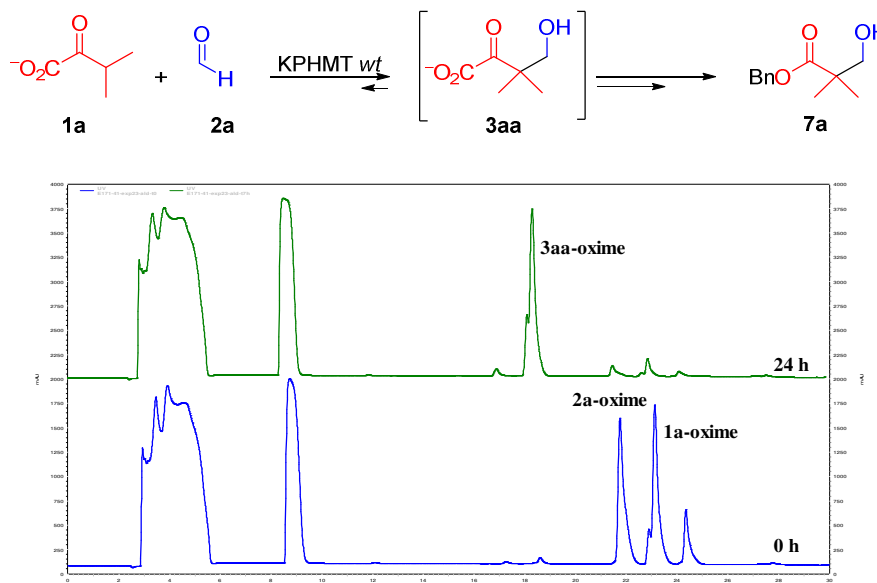
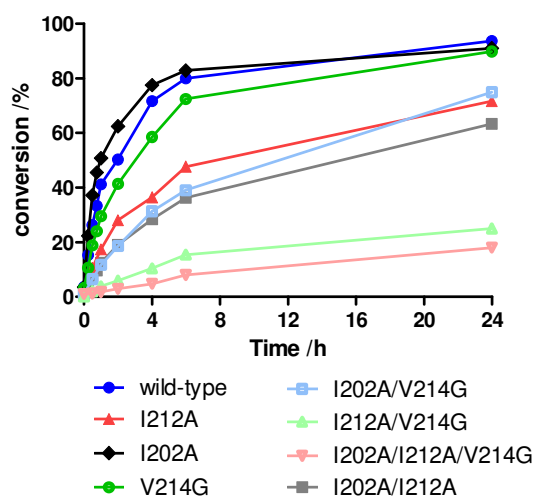


Figure S149 Aldol addition of 3-methyl-2-oxobutanoate (**1a**) to formaldehyde (**2a**) catalyzed by KPHMT wild-type (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (94% conversion).



KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	1.0 ± 0.16	95
I212A	$0.3 \pm 1.8 \cdot 10^{-2}$	71
I202A	1.26 ± 0.13	91
V214G	$0.6 \pm 3.9 \cdot 10^{-2}$	90
I202A/V214G	$0.15 \pm 1.0 \cdot 10^{-2}$	75
I212A/V214G	$7.8 \cdot 10^{-2} \pm 2 \cdot 10^{-3}$	25
I202A/I212A/ V214G	$2.1 \cdot 10^{-2} \pm 1 \cdot 10^{-3}$	18
I202A/I212A	$0.23 \pm 7 \cdot 10^{-3}$	63
Control	$_b$	$_b$

^a $[\mathbf{1a}] = [\mathbf{2a}] = 100 \text{ mM}$, $[\text{enzyme}] = 2 \text{ mg mL}^{-1}$.

^b not detected.

Figure S150 Reaction time course of aldol addition of 3-methyl-2-oxobutanoate (**1a**) to formaldehyde (**2a**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme.

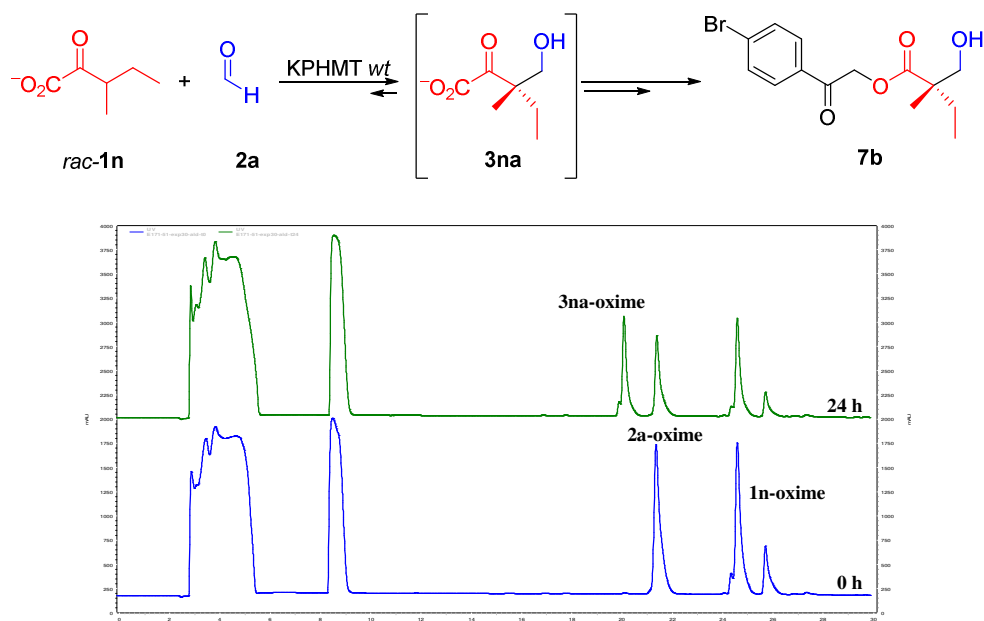
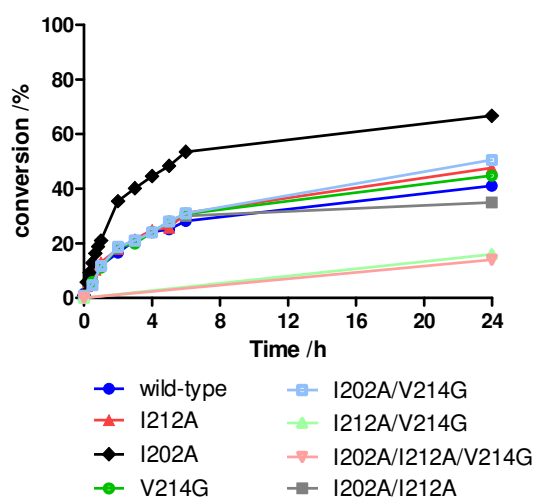


Figure S151 Aldol addition of 3-methyl-2-oxopentanoate (*rac*-**1n**) to formaldehyde (**2a**) catalyzed by KPHMT wild-type (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (43% conversion).



KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$0.28 \pm 6.0 \cdot 10^{-2}$	41
I212A	$0.38 \pm 2.1 \cdot 10^{-2}$	47
I202A	$0.51 \pm 2.8 \cdot 10^{-2}$	67
V214G	$0.19 \pm 7 \cdot 10^{-3}$	41
I202A/V214G	$0.20 \pm 2.0 \cdot 10^{-2}$	46
I212A/V214G	$1.1 \cdot 10^{-2} \pm 8 \cdot 10^{-3}$	16
I202A/I212A/ V214G	$9 \cdot 10^{-3} \pm 2 \cdot 10^{-3}$	14
I202A/I212A	$4.4 \cdot 10^{-2} \pm 7 \cdot 10^{-3}$	35
Control	$_b$	$_b$

^a $[\mathbf{1n}] = [\mathbf{2a}] = 100 \text{ mM}$, $[\text{enzyme}] = 2 \text{ mg mL}^{-1}$.

^b not detected.

Figure S152 Reaction time course of aldol addition of 3-methyl-2-oxopentanoate (*rac*-**1n**) to formaldehyde (**2a**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme.

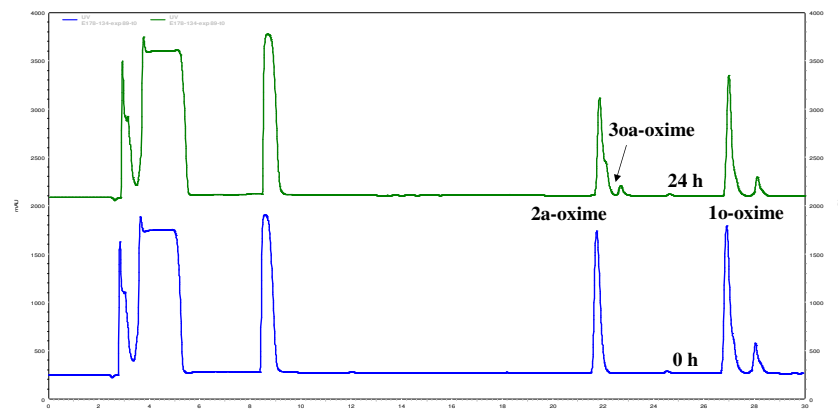
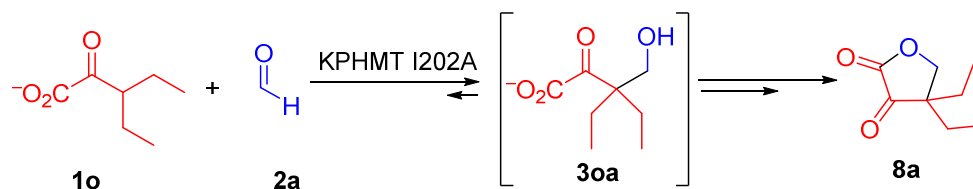
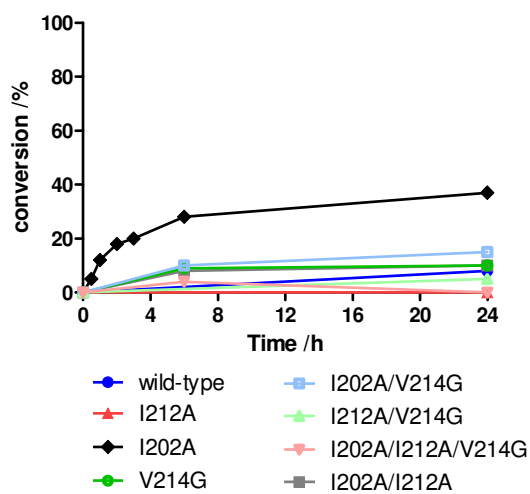


Figure S153 Aldol addition of 3-ethyl-2-oxopentanoate (**1o**) to formaldehyde (**2a**) catalyzed by KPHMT I202A (Co²⁺). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (37% conversion).



KPHMT	v_o^a (mM·min ⁻¹)	Conv 24h (%)
wild-type	$7 \cdot 10^{-3} \pm 2 \cdot 10^{-3}$	8
I212A	_b	_b
I202A	$0.25 \pm 3.2 \cdot 10^{-2}$	37
V214G	$1.2 \cdot 10^{-2} \pm 3 \cdot 10^{-3}$	10
I202A/V214G	$9 \cdot 10^{-3} \pm 2 \cdot 10^{-3}$	15
I212A/V214G	$7 \cdot 10^{-3} \pm 3 \cdot 10^{-3}$	5
I202A/I212A/ V214G	_b	_b
I202A/I212A	$1.5 \cdot 10^{-2} \pm 2 \cdot 10^{-3}$	10
Control	_b	_b

^a [**1o**] = [**2a**] = 100 mM, [enzyme] = 2 mg mL⁻¹.

^b not detected.

Figure S154 Reaction time course of aldol addition of 3-ethyl-2-oxopentanoate (**1o**) to formaldehyde (**2a**) catalyzed by KPHMT (Co²⁺) variants and control experiment performed under the same conditions without the enzyme.

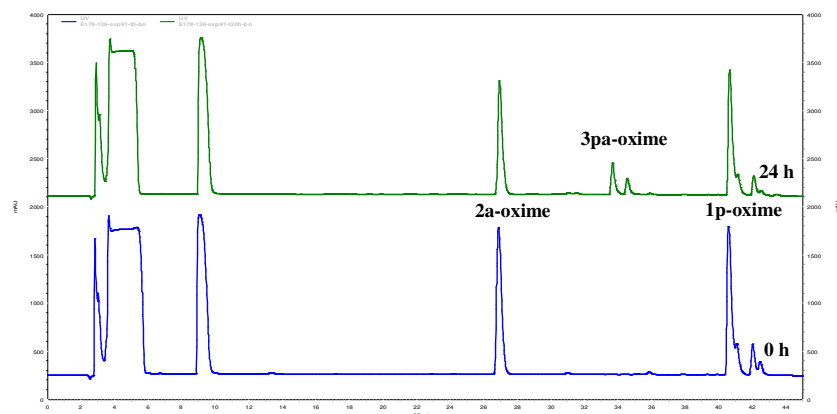
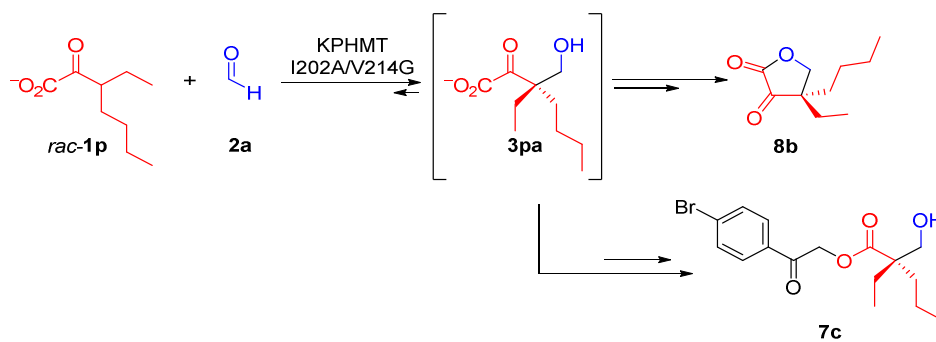
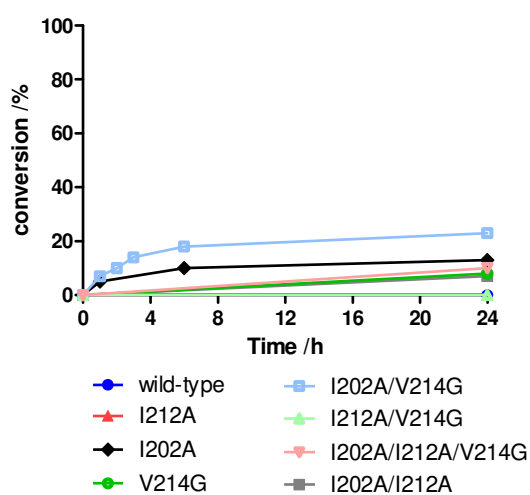


Figure S155 Aldol addition of 3-ethyl-2-oxoheptanoate (*rac*-**1p**) to formaldehyde (**2a**) catalyzed by KPHMT I202A/V214G (Co²⁺). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (23% conversion).



KPHMT	v_o^a (mM·min ⁻¹)	Conv 24h (%)
wild-type	_b	_b
I212A	_b	_b
I202A	$1.5 \cdot 10^{-2} \pm 3 \cdot 10^{-3}$	13
V214G	$1.1 \cdot 10^{-2} \pm 4 \cdot 10^{-3}$	8
I202A/V214G	$7.5 \cdot 10^{-2} \pm 5 \cdot 10^{-3}$	23
I212A/V214G	_b	_b
I202A/I212A/ V214G	$1.2 \cdot 10^{-2} \pm 3 \cdot 10^{-3}$	10
I202A/I212A	$9 \cdot 10^{-3} \pm 2 \cdot 10^{-3}$	7
Control	_b	_b

^a [**1p**] = [**2a**] = 100 mM, [enzyme] = 2 mg mL⁻¹.

^b not detected.

Figure S156 Reaction time course of aldol addition of 3-ethyl-2-oxoheptanoate (*rac*-**1p**) to formaldehyde (**2a**) catalyzed by KPHMT (Co²⁺) variants and control experiment performed under the same conditions without the enzyme.

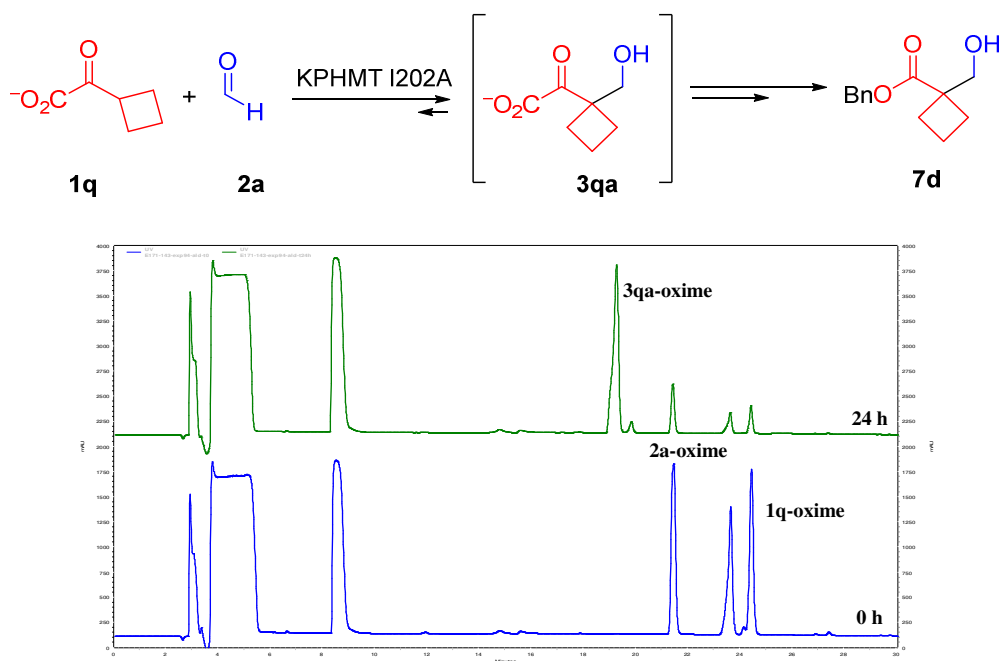
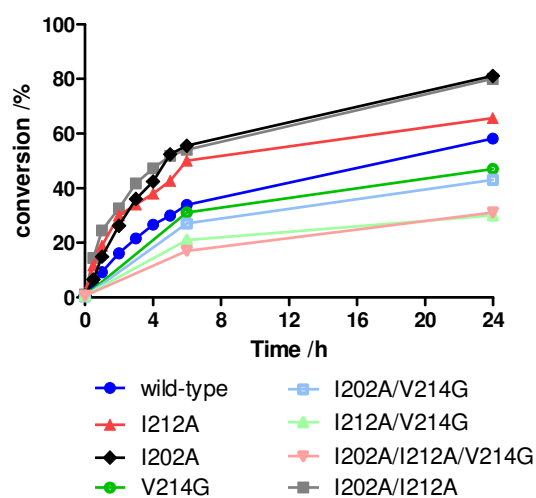


Figure S157 Aldol addition of 2-cyclobutyl-2-oxoetanoate (**1q**) to formaldehyde (**2a**) catalyzed by KPHMT wild-type (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (82% conversion).



KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$0.19 \pm 4 \cdot 10^{-3}$	53
I212A	$0.33 \pm 3.0 \cdot 10^{-2}$	63
I202A	$0.32 \pm 1.2 \cdot 10^{-2}$	82
V214G	$0.11 \pm 2 \cdot 10^{-3}$	47
I202A/V214G	$8.3 \cdot 10^{-2} \pm 1 \cdot 10^{-3}$	43
I212A/V214G	$6.1 \cdot 10^{-2} \pm 9 \cdot 10^{-3}$	30
I202A/I212A/ V214G	$4.7 \cdot 10^{-2} \pm 6 \cdot 10^{-3}$	31
I202A/I212A	$0.43 \pm 5.6 \cdot 10^{-2}$	72
Control	$_b$	$_b$

^a $[\mathbf{1q}] = [\mathbf{2a}] = 100 \text{ mM}$, $[\text{enzyme}] = 2 \text{ mg mL}^{-1}$.

^b not detected.

Figure S158 Reaction time course of aldol addition of 2-cyclobutyl-2-oxoetanoate (**1q**) to formaldehyde (**2a**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme.

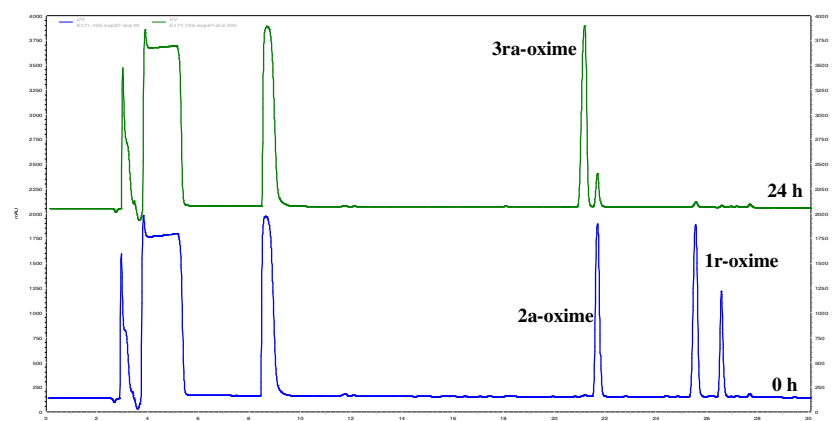
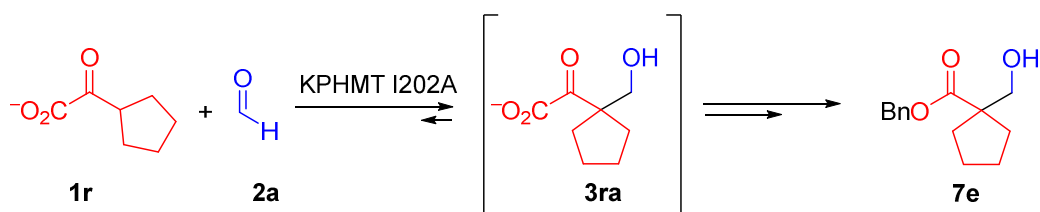
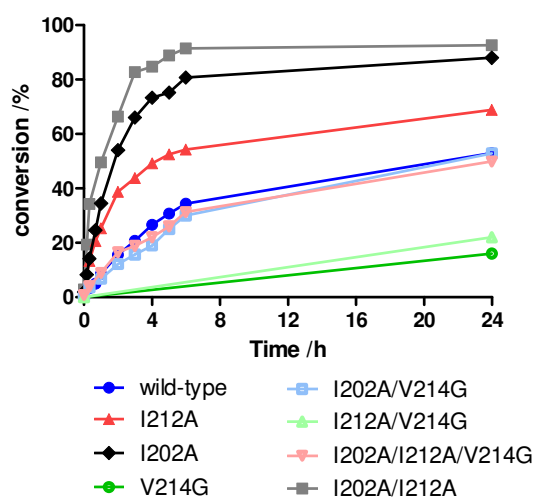


Figure S159 Aldol addition of 2-cyclopentyl-2-oxoetanoate (**1r**) to formaldehyde (**2a**) catalyzed by KPHMT I202A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (95% conversion).



KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$0.16 \pm 8 \cdot 10^{-3}$	49
I212A	$0.57 \pm 6.6 \cdot 10^{-2}$	67
I202A	$0.71 \pm 1.0 \cdot 10^{-2}$	87
V214G	$3.0 \cdot 10^{-2} \pm 4 \cdot 10^{-3}$	16
I202A/V214G	$0.11 \pm 6 \cdot 10^{-3}$	49
I212A/V214G	$5.5 \cdot 10^{-2} \pm 6 \cdot 10^{-3}$	22
I202A/I212A/ V214G	$0.17 \pm 7 \cdot 10^{-3}$	54
I202A/I212A	$0.78 \pm 8.0 \cdot 10^{-2}$	90
Control	$_b$	$_b$

^a $[\mathbf{1r}] = [\mathbf{2a}] = 100 \text{ mM}$, $[\text{enzyme}] = 2 \text{ mg mL}^{-1}$.

^b not detected.

Figure S160 Reaction time course of aldol addition of 2-cyclopentyl-2-oxoetanoate (**1r**) to formaldehyde (**2a**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme.

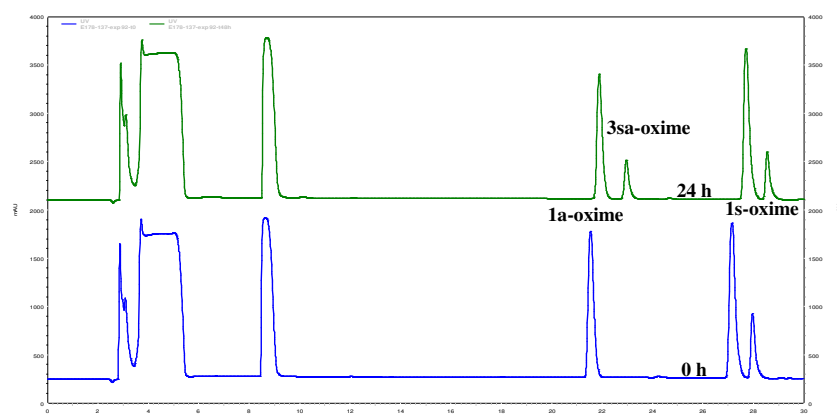
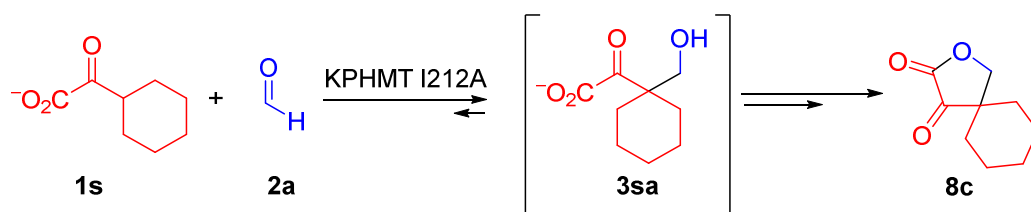
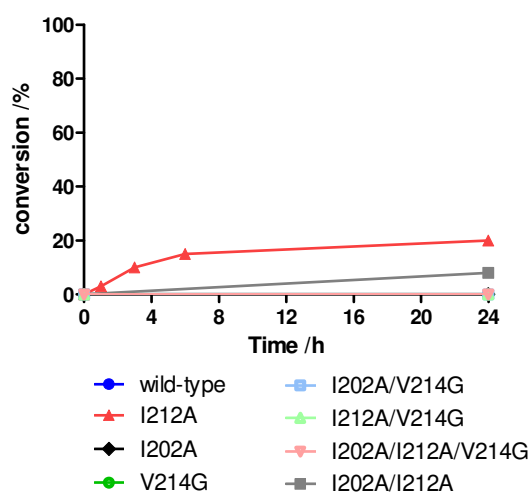


Figure S161 Aldol addition of 2-cyclohexyl-2-oxoetanoate (**1s**) to formaldehyde (**2a**) catalyzed by KPHMT I212A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (20% conversion).



KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	--_b	--_b
I212A	$5.5 \cdot 10^{-2} \pm 6 \cdot 10^{-3}$	20
I202A	--_b	--_b
V214G	--_b	--_b
I202A/V214G	--_b	--_b
I212A/V214G	--_b	--_b
I202A/I212A/ V214G	--_b	--_b
I202A/I212A	$1.1 \cdot 10^{-2} \pm 4 \cdot 10^{-3}$	8
Control	--_b	--_b

^a $[\mathbf{1s}] = [\mathbf{2a}] = 100 \text{ mM}$, $[\text{enzyme}] = 2 \text{ mg mL}^{-1}$.

^b not detected.

Figure S162 Reaction time course of aldol addition of 2-cyclohexyl-2-oxoetanoate (**1s**) to formaldehyde (**2a**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme.

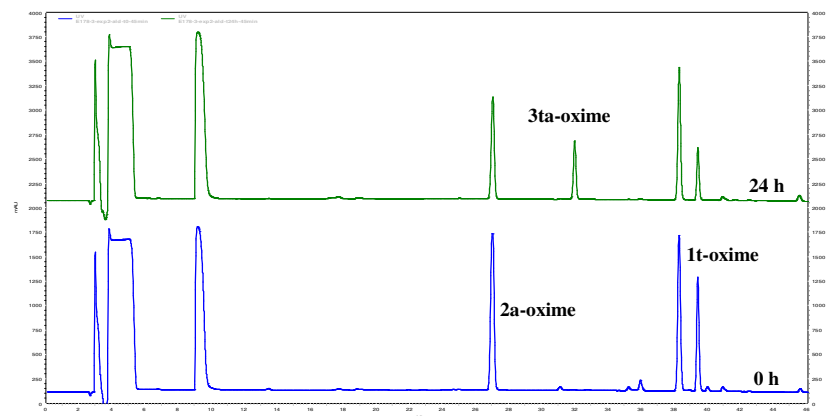
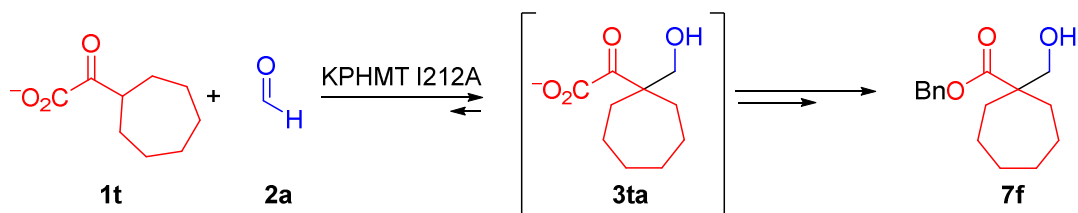
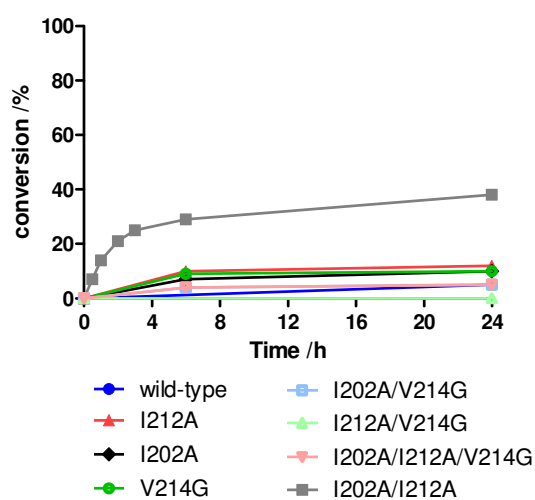


Figure S163 Aldol addition of 2-cycloheptyl-2-oxoetanoate (**1t**) to formaldehyde (**2a**) catalyzed by KPHMT I202A/I212A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (30% conversion).



KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$7 \cdot 10^{-3} \pm 3 \cdot 10^{-3}$	5
I212A	$1.8 \cdot 10^{-2} \pm 8 \cdot 10^{-3}$	12
I202A	$1.1 \cdot 10^{-2} \pm 2 \cdot 10^{-3}$	10
V214G	$1.2 \cdot 10^{-2} \pm 3 \cdot 10^{-3}$	10
I202A/V214G	$6 \cdot 10^{-3} \pm 3 \cdot 10^{-3}$	5
I212A/V214G	$_{-b}$	<5
I202A/I212A/ V214G	$6 \cdot 10^{-3} \pm 1 \cdot 10^{-3}$	5
I202A/I212A	$7.4 \cdot 10^{-2} \pm 4 \cdot 10^{-3}$	40
Control	$_{-b}$	$_{-b}$

^a $[\mathbf{1t}] = [\mathbf{2a}] = 100 \text{ mM}$, $[\text{enzyme}] = 2 \text{ mg mL}^{-1}$.

^b not detected.

Figure S164 Reaction time course of aldol addition of 2-cycloheptyl-2-oxoetanoate (**1t**) to formaldehyde (**2a**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme

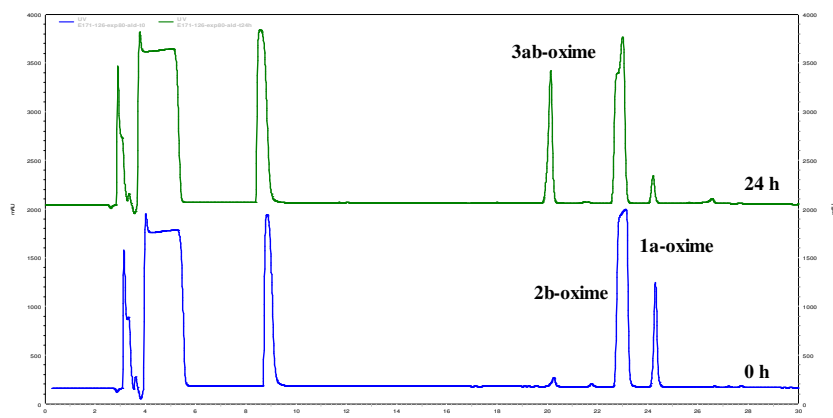
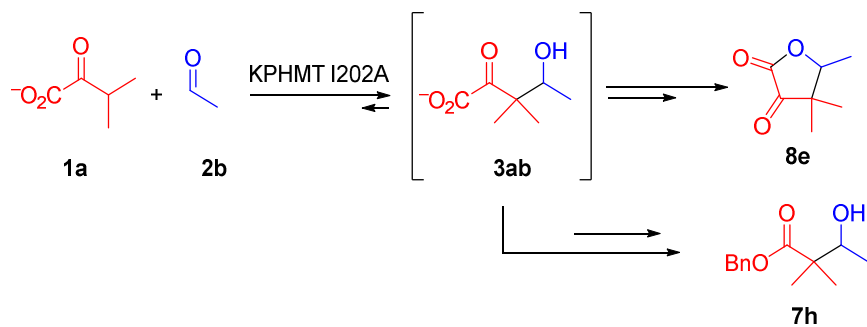
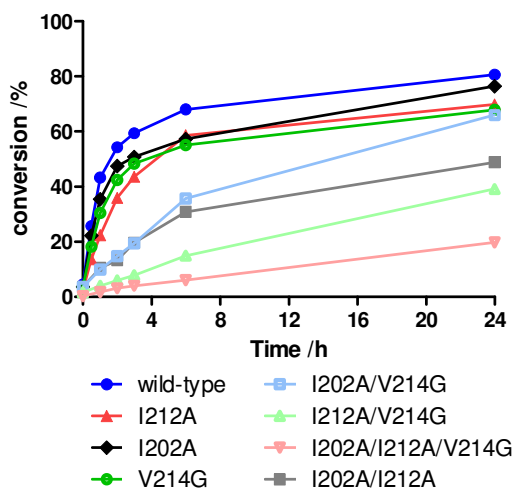


Figure S165 Aldol addition of 3-methyl-2-oxobutanoate (**1a**) to acetaldehyde (**2b**) catalyzed by KPHMT I202A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (86% conversion).



KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$0.64 \pm 2.3 \cdot 10^{-2}$	81
I212A	$0.27 \pm 2.4 \cdot 10^{-2}$	70
I202A	$0.35 \pm 7.0 \cdot 10^{-2}$	76
V214G	$0.32 \pm 5.1 \cdot 10^{-2}$	68
I202A/V214G	$8.7 \cdot 10^{-2} \pm 4 \cdot 10^{-3}$	66
I212A/V214G	$3.4 \cdot 10^{-2} \pm 1 \cdot 10^{-3}$	39
I202A/I212A/ V214G	$2.1 \cdot 10^{-2} \pm 1 \cdot 10^{-3}$	20
I202A/I212A	$8.4 \cdot 10^{-2} \pm 9 \cdot 10^{-3}$	49
Control	$_b$	$_b$

^a $[\mathbf{1a}] = 100 \text{ mM}$, $[\mathbf{2b}] = 200 \text{ mM}$ [enzyme] = 2 mg mL^{-1} . ^b not detected.

Figure S166 Reaction time course of aldol addition of 3-methyl-2-oxobutanoate (**1a**) to acetaldehyde (**2b**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme

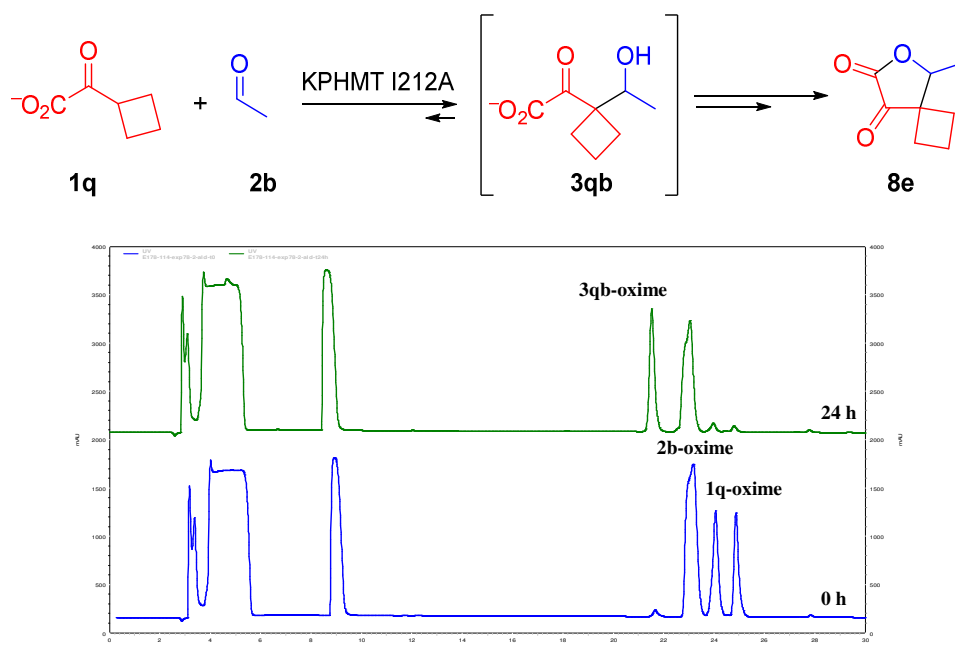
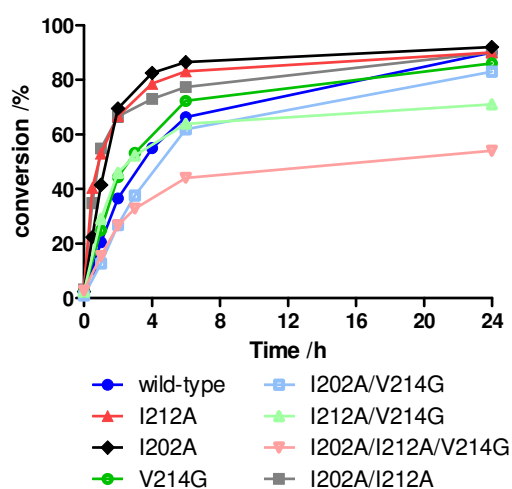


Figure S167 Aldol addition of 2-cyclobutyl-2-oxoetanoate (**1q**) to acetaldehyde (**2b**) catalyzed by KPHMT I212A (Co²⁺). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (94% conversion).



KPHMT	v_o^a (mM·min ⁻¹)	Conv 24h (%)
wild-type	$0.43 \pm 1.2 \cdot 10^{-2}$	90
I212A	$1.22 \pm 4.1 \cdot 10^{-2}$	90
I202A	$0.77 \pm 5.7 \cdot 10^{-2}$	92
V214G	$0.47 \pm 1.3 \cdot 10^{-2}$	86
I202A/V214G	$0.29 \pm 1.3 \cdot 10^{-2}$	83
I212A/V214G	$0.51 \pm 6.3 \cdot 10^{-2}$	71
I202A/I212A/ V214G	$0.25 \pm 5 \cdot 10^{-3}$	54
I202A/I212A	1.1 ± 0.1	90
Control	– ^b	– ^b

^a [1q] = 100 mM, [2b] = 200 mM, [enzyme] = 2 mg mL⁻¹. ^b not detected.

Figure S168 Reaction time course of aldol addition of 2-cyclobutyl-2-oxoetanoate (**1q**) to acetaldehyde (**2b**) catalyzed by KPHMT (Co²⁺) variants and control experiment performed under the same conditions without the enzyme

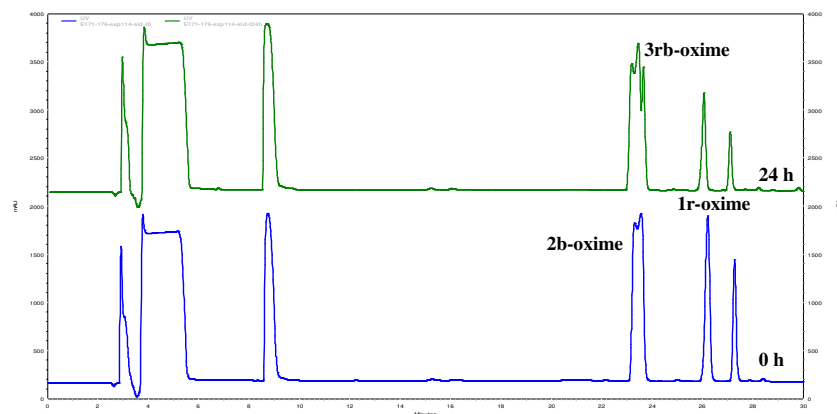
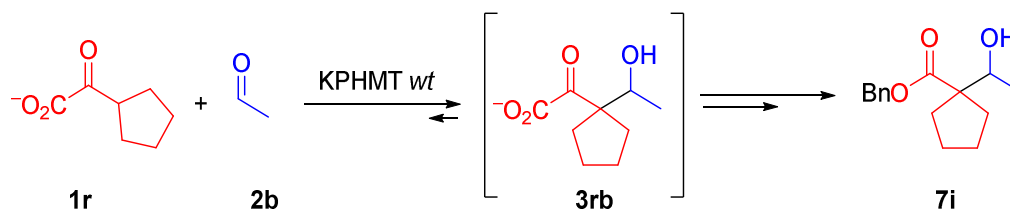
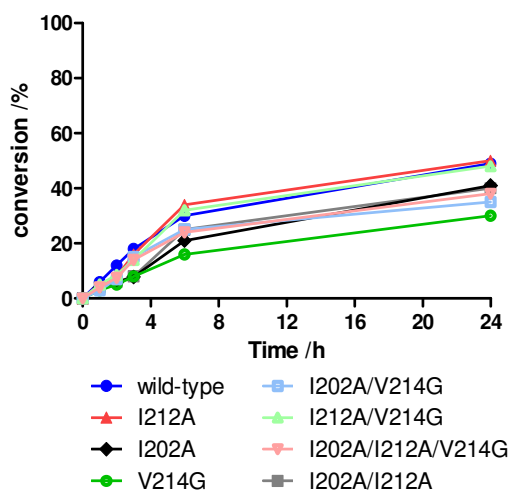


Figure S169 Aldol addition of 2-cyclopentyl-2-oxoetanoate (**1q**) to acetaldehyde (**2b**) catalyzed by KPHMT wild type (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (50% conversion).



KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$0.2 \pm 1.3 \cdot 10^{-2}$	49
I212A	$0.24 \pm 1.9 \cdot 10^{-2}$	50
I202A	$0.11 \pm 8 \cdot 10^{-3}$	41
V214G	$6.8 \cdot 10^{-2} \pm 4 \cdot 10^{-3}$	30
I202A/V214G	$7.3 \cdot 10^{-2} \pm 6 \cdot 10^{-3}$	35
I212A/V214G	$0.19 \pm 9 \cdot 10^{-3}$	48
I202A/I212A/ V214G	$9.6 \cdot 10^{-2} \pm 1.1 \cdot 10^{-2}$	38
I202A/I212A	$0.12 \pm 8 \cdot 10^{-3}$	40
Control	$-^b$	$-^b$

^a [**1q**] = 100 mM, [**2b**] = 200 mM, [enzyme] = 2 mg mL^{-1} . ^b not detected.

Figure S170 Reaction time course of aldol addition of 2-cyclopentyl-2-oxoetanoate (**1q**) to acetaldehyde (**2b**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme

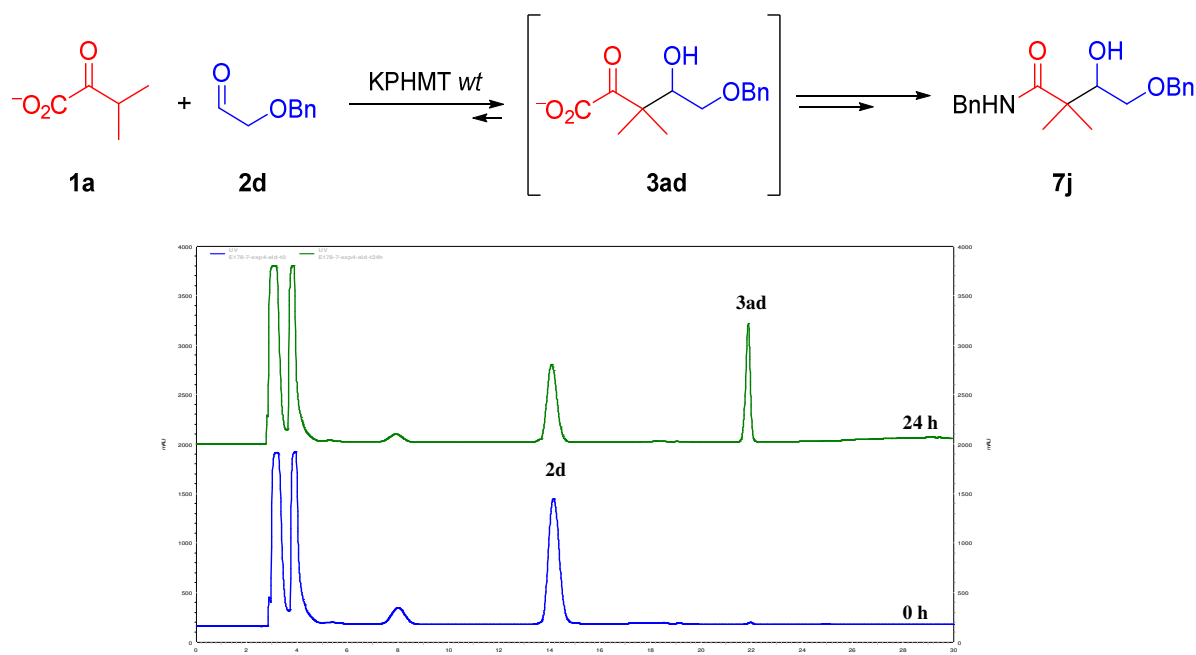


Figure S171 Aldol addition of 3-methyl-2-oxobutanoate (**1a**) to benzyloxyacetaldehyde (**2d**) catalyzed by KPHMT wild type (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (40% conversion).

KPHMT	Conv 24h ^a (%)
wild-type	44
I212A	37
I202A	43
V214G	42
I202A/V214G	35
I212A/V214G	<5
I202A/I212A/V214G	- ^b
I202A/I212A	12
Control	- ^b

^a $[\mathbf{1a}] = [\mathbf{2d}] = 100 \text{ mM}$, $[\text{enzyme}] = 2 \text{ mg mL}^{-1}$. ^b not detected.

Table S4. Conversion after 24 h of aldol addition reaction of 3-methyl-2-oxobutanoate (**1a**) to benzyloxyacetaldehyde (**2d**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme. The initial reaction rate was not determined.

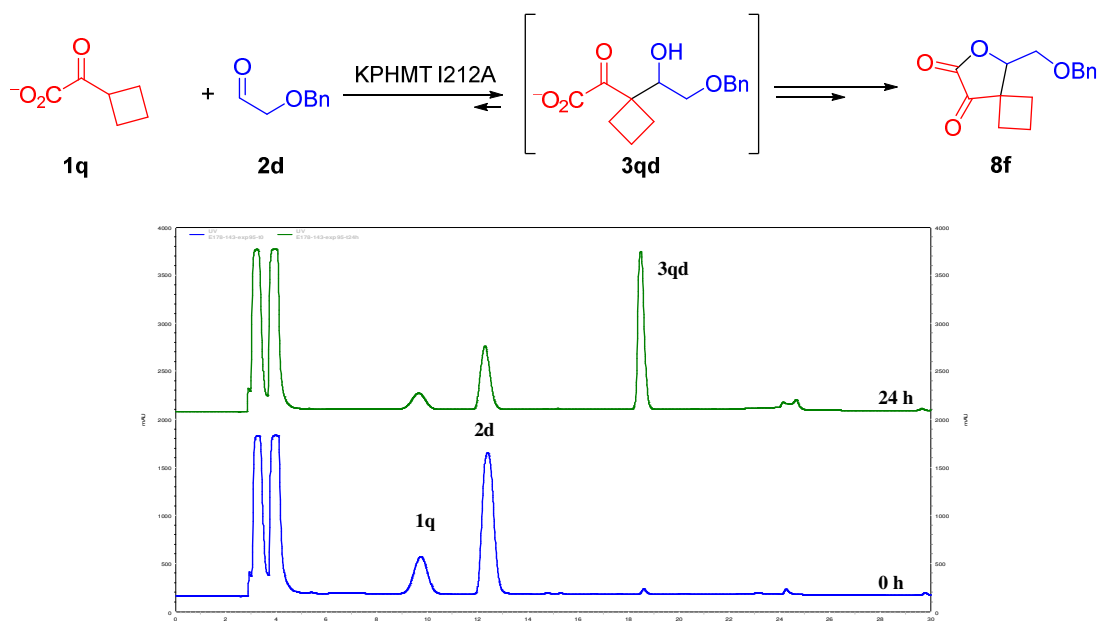


Figure S172 Aldol addition of 2-cyclobutyl-2-oxoetanoate (**1q**) to benzyloxyacetaldehyde (**2d**) catalyzed by KPHMT I212A (Co²⁺). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (57% conversion).

KPHMT	Conv 24h ^a (%)
wild-type	42
I212A	60
I202A	46
V214G	29
I202A/V214G	30
I212A/V214G	41
I202A/I212A/V214G	21
I202A/I212A	54
Control	– ^b

^a [**1q**] = [**2d**] = 100 mM, [enzyme] = 2 mg mL⁻¹. ^b not detected.

Table S5. Conversion after 24 h of aldol addition reaction of 2-cyclobutyl-2-oxoetanoate (**1q**) to benzyloxyacetaldehyde (**2d**) catalyzed by KPHMT (Co²⁺) variants and control experiment performed under the same conditions without the enzyme. The initial reaction rate was not determined.

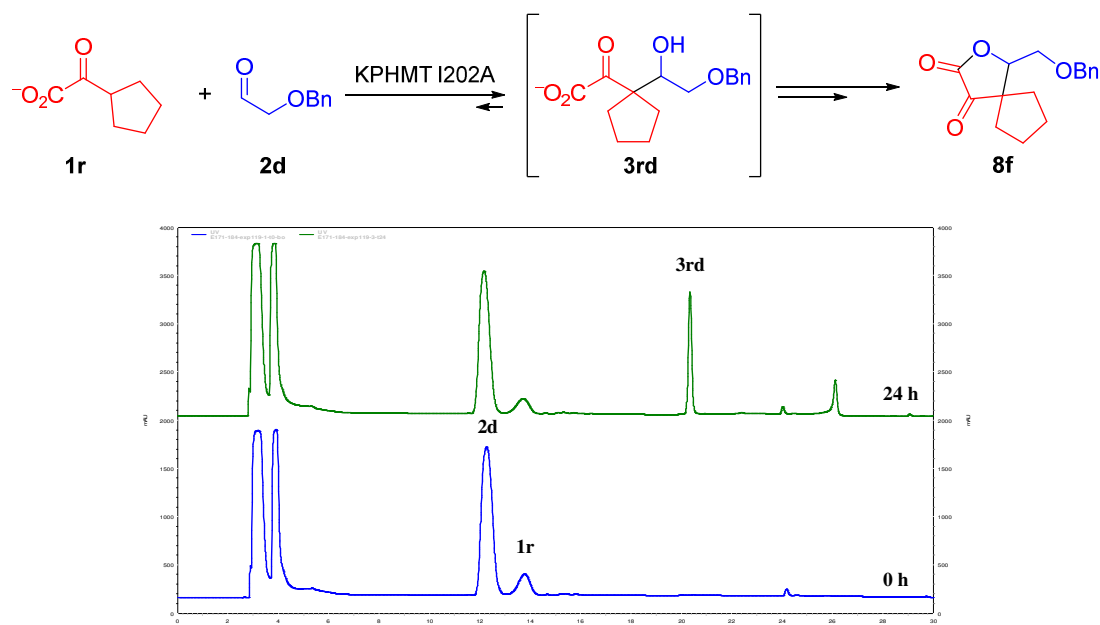


Figure S173 Aldol addition of 2-cyclopentyl-2-oxoetanoate (**1r**) to benzyloxyacetaldehyde (**2d**) catalyzed by KPHMT I202A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (26% conversion).

KPHMT	Conv 24h ^a (%)
wild-type	10
I212A	20
I202A	25
V214G	<5
I202A/V214G	– ^b
I212A/V214G	– ^b
I202A/I212A/V214G	– ^b
I202A/I212A	<5
Control	– ^b

^a $[\mathbf{1r}] = [\mathbf{2d}] = 100 \text{ mM}$, $[\text{enzyme}] = 2 \text{ mg mL}^{-1}$. ^b not detected.

Table S6. Conversion after 24 h of aldol addition reaction of 2-cyclopentyl-2-oxoetanoate (**1r**) to benzyloxyacetaldehyde (**2d**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme. The initial reaction rate was not determined.

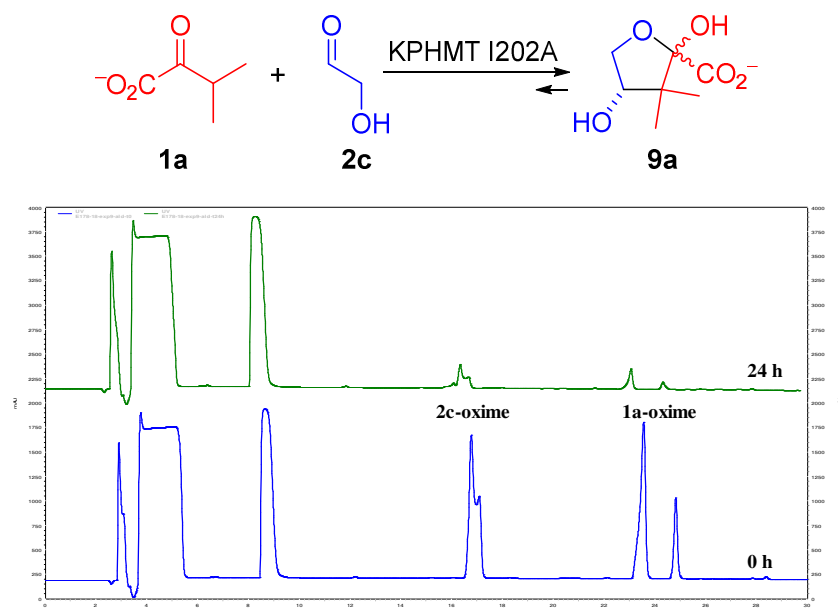
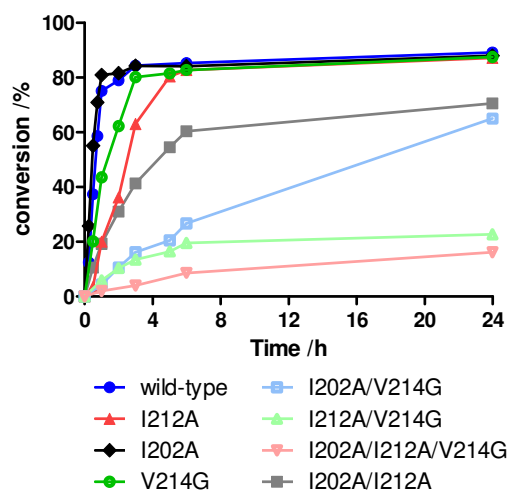


Figure S174 Aldol addition of 3-methyl-2-oxobutanoate (**1a**) to glycolaldehyde (**2c**) catalyzed by KPHMT I202A (Co²⁺). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (87% conversion). Product **9a** was not detected by HPLC because it was not derivatized by *O*-benzylhydroxylamine under the conditions employed. Only the substrate consumption could be monitored.



KPHMT	v_o^a (mM·min ⁻¹)	Conv 24h (%)
wild-type	$0.74 \pm 4 \cdot 10^{-3}$	89
I212A	$0.19 \pm 2.6 \cdot 10^{-2}$	87
I202A	$0.92 \pm 7.4 \cdot 10^{-2}$	88
V214G	$0.43 \pm 1.8 \cdot 10^{-2}$	86
I202A/V214G	$6 \cdot 10^{-2} \pm 2 \cdot 10^{-3}$	65
I212A/V214G	$5 \cdot 10^{-2} \pm 4 \cdot 10^{-3}$	23
I202A/I212A/ V214G	$1.6 \cdot 10^{-2} \pm 1 \cdot 10^{-3}$	16
I202A/I212A	$0.17 \pm 1.8 \cdot 10^{-2}$	71
Control	_{-b}	_{-b}

^a [1a] = [2c] = 100 mM, [enzyme] = 2 mg mL⁻¹.

^b not detected.

Figure S175 Reaction time course of aldol addition of 3-methyl-2-oxobutanoate (**1a**) to glycolaldehyde (**2c**) catalyzed by KPHMT (Co²⁺) variants and control experiment performed under the same conditions without the enzyme.

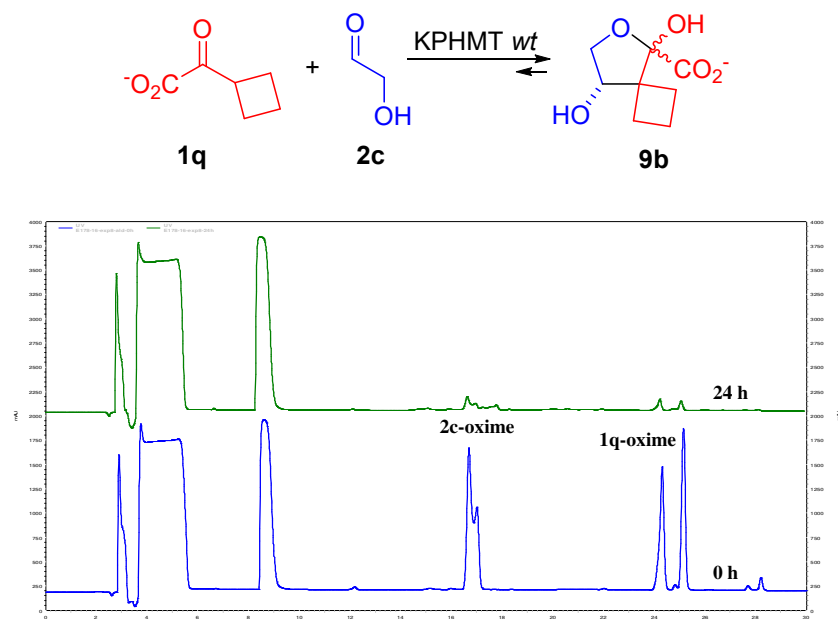
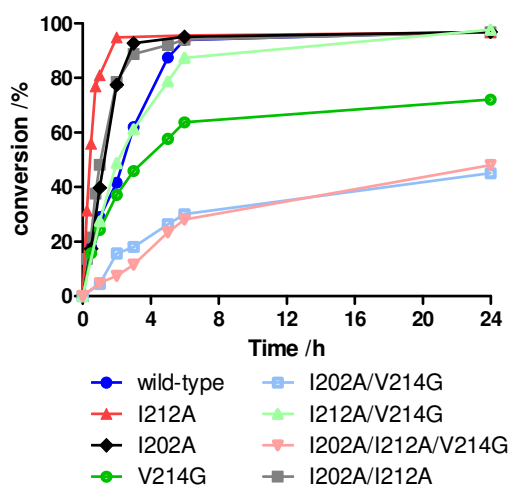


Figure S176 Aldol addition of 2-cyclobutyl-2-oxoetanoate (**1q**) to glycolaldehyde (**2c**) catalyzed by KPHMT wild-type (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (92% conversion). Product **9b** was not detected by HPLC because it was not derivatized by *O*-benzylhydroxylamine under the conditions employed. Only the substrate consumption could be monitored



KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$0.47 \pm 7.1 \cdot 10^{-2}$	95
I212A	$1.42 \pm 9.2 \cdot 10^{-2}$	91
I202A	$0.53 \pm 1.7 \cdot 10^{-2}$	96
V214G	$0.29 \pm 2.6 \cdot 10^{-2}$	60
I202A/V214G	$8.2 \cdot 10^{-2} \pm 1.9 \cdot 10^{-2}$	43
I212A/V214G	$0.31 \pm 4.1 \cdot 10^{-2}$	90
I202A/I212A/ V214G	$6.5 \cdot 10^{-2} \pm 4 \cdot 10^{-3}$	40
I202A/I212A	$0.69 \pm 6.2 \cdot 10^{-2}$	93
Control	$_{-b}$	$_{-b}$

^a $[\mathbf{1q}] = [\mathbf{2c}] = 100 \text{ mM}$, $[\text{enzyme}] = 2 \text{ mg mL}^{-1}$.

^b not detected.

Figure S177 Reaction time course of aldol addition of 2-cyclobutyl-2-oxoetanoate (**1q**) to glycolaldehyde (**2c**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme.

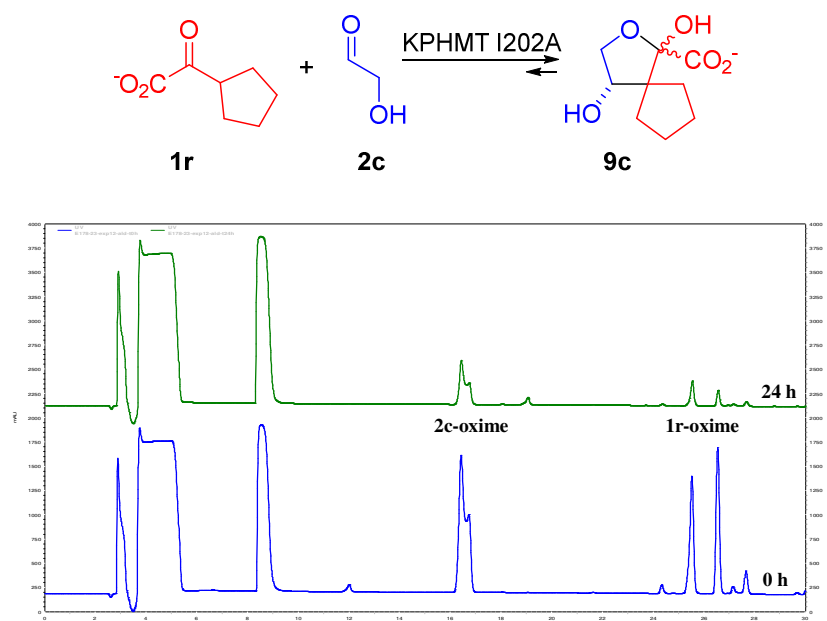
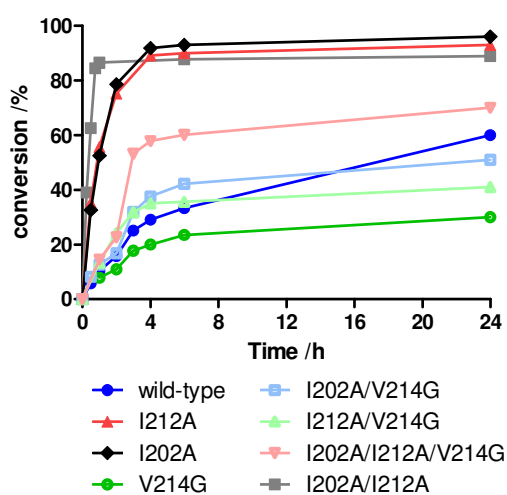


Figure S178 Aldol addition of 2-cyclopentyl-2-oxoetanoate (**1r**) to glycolaldehyde (**2c**) catalyzed by KPHMT I202A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (80% conversion). Product **9c** was not detected by HPLC because it was not derivatized by *O*-benzylhydroxylamine under the conditions employed. Only the substrate consumption could be monitored.



^a $[\mathbf{1r}] = [\mathbf{2c}] = 100 \text{ mM}$, $[\text{enzyme}] = 2 \text{ mg mL}^{-1}$.

^b not detected.

KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$0.13 \pm 1.8 \cdot 10^{-2}$	60
I212A	0.87 ± 0.13	93
I202A	0.85 ± 0.11	93
V214G	$6.5 \cdot 10^{-2} \pm 1.0 \cdot 10^{-2}$	20
I202A/V214G	$0.11 \pm 1.1 \cdot 10^{-2}$	40
I212A/V214G	$0.12 \pm 2.6 \cdot 10^{-2}$	38
I202A/I212A/ V214G	$0.19 \pm 1.3 \cdot 10^{-2}$	70
I202A/I212A	1.66 ± 0.17	85
Control	_b	_b

Figure S179 Reaction time course of aldol addition of 2-cyclopentyl-2-oxoetanoate (**1r**) to glycolaldehyde (**2c**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme.

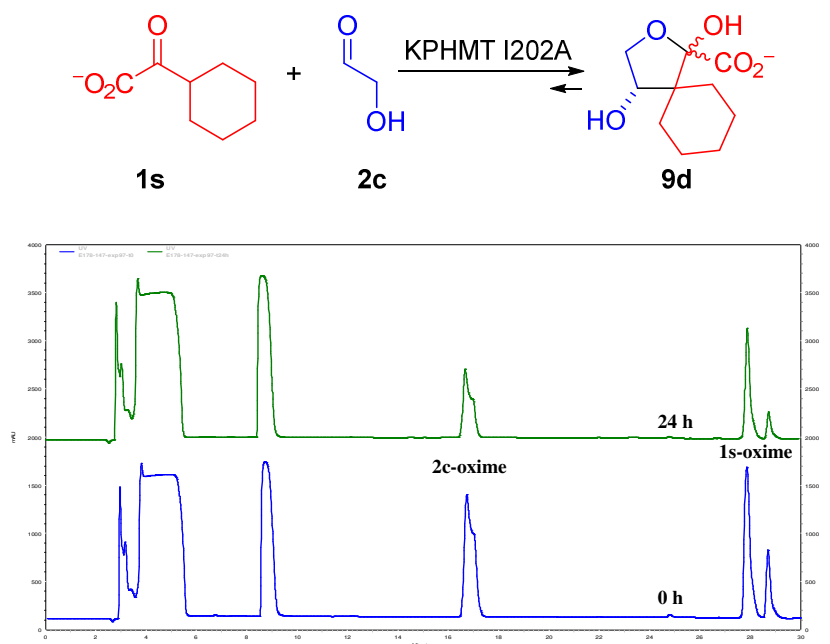


Figure S180 Aldol addition of 2-cyclohexyl-2-oxoetanoate (**1s**) to glycolaldehyde (**2c**) catalyzed by KPHMT I212A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (47% conversion). Product **9d** was not detected by HPLC because it was not derivatized by *O*-benzylhydroxylamine under the conditions employed. Only the substrate consumption could be monitored.

KPHMT	Conv 24h ^a (%)
wild-type	<5
I212A	40
I202A	– ^b
V214G	– ^b
I202A/V214G	– ^b
I212A/V214G	5
I202A/I212A/V214G	– ^b
I202A/I212A	7
Control	– ^b

^a [1s] = [2c] = 100 mM, [enzyme] = 2 mg mL⁻¹. ^b not detected.

Table S7. Conversion after 24 h of aldol addition reaction of 2-cyclohexyl-2-oxoetanoate (**1s**) to glycolaldehyde (**2c**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme. Initial reaction rate not determined.

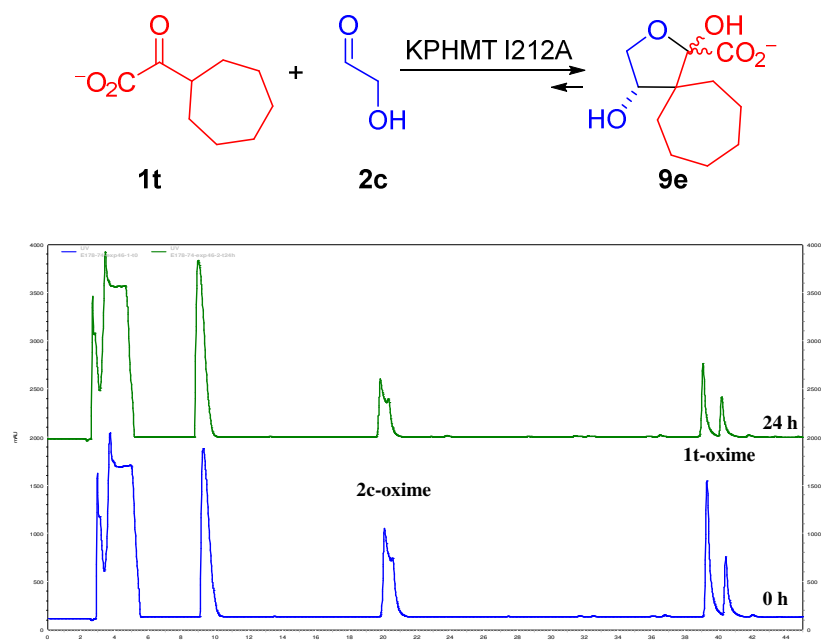


Figure S181 Aldol addition of 2-cycloheptyl-2-oxoetanoate (**1t**) to glycolaldehyde (**2c**) catalyzed by KPHMT I212A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (33% conversion). Product **9e** was not detected by HPLC because it was not derivatized by *O*-benzylhydroxylamine under the conditions employed. Only the substrate consumption could be monitored.

KPHMT	Conv 24h ^a (%)
wild-type	<5
I212A	42
I202A	– ^b
V214G	5
I202A/V214G	14
I212A/V214G	15
I202A/I212A/V214G	21
I202A/I212A	7
Control	– ^b

^a $[\mathbf{1t}] = [\mathbf{2c}] = 100 \text{ mM}$, $[\text{enzyme}] = 2 \text{ mg mL}^{-1}$. ^b not detected.

Table S8. Conversion after 24 h of aldol addition reaction of 2-cycloheptyl-2-oxoetanoate (**1t**) to glycolaldehyde (**2c**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme. Initial reaction rate not determined.

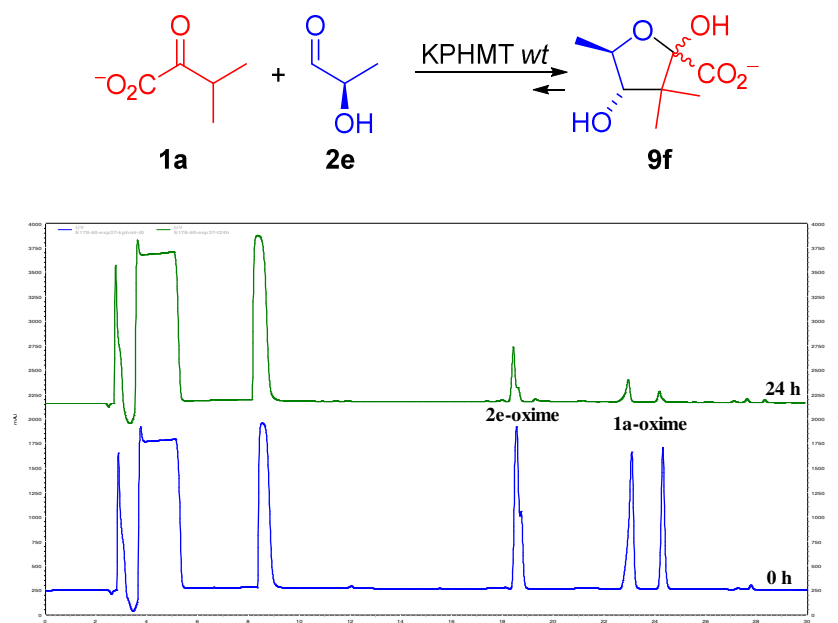
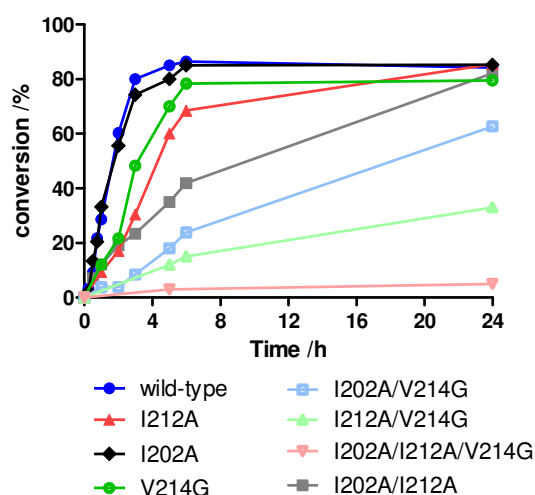


Figure S182 Aldol addition of 3-methyl-2-oxobutanoate (**1a**) to D-lactaldehyde (**2e**) catalyzed by KPHMT wild-type (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (85% conversion). Product **9f** was not detected by HPLC because it was not derivatized by *O*-benzylhydroxylamine under the conditions employed. Only the substrate consumption could be monitored.



KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$0.77 \pm 4.0 \cdot 10^{-2}$	84
I212A	$0.24 \pm 1.6 \cdot 10^{-2}$	85
I202A	$0.84 \pm 9.7 \cdot 10^{-2}$	85
V214G	$0.38 \pm 5.5 \cdot 10^{-2}$	80
I202A/V214G	$4.1 \cdot 10^{-2} \pm 8 \cdot 10^{-3}$	62
I212A/V214G	$2.6 \cdot 10^{-2} \pm 3 \cdot 10^{-3}$	33
I202A/I212A/ V214G	$1.5 \cdot 10^{-2} \pm 2 \cdot 10^{-3}$	5
I202A/I212A	$0.34 \pm 7.5 \cdot 10^{-2}$	82
Control	$_b$	$_b$

^a $[\mathbf{1a}] = [\mathbf{2e}] = 100 \text{ mM}$, $[\text{enzyme}] = 2 \text{ mg mL}^{-1}$.

^b not detected.

Figure S183 Reaction time course of aldol addition of 3-methyl-2-oxobutanoate (**1a**) to D-lactaldehyde (**2e**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme.

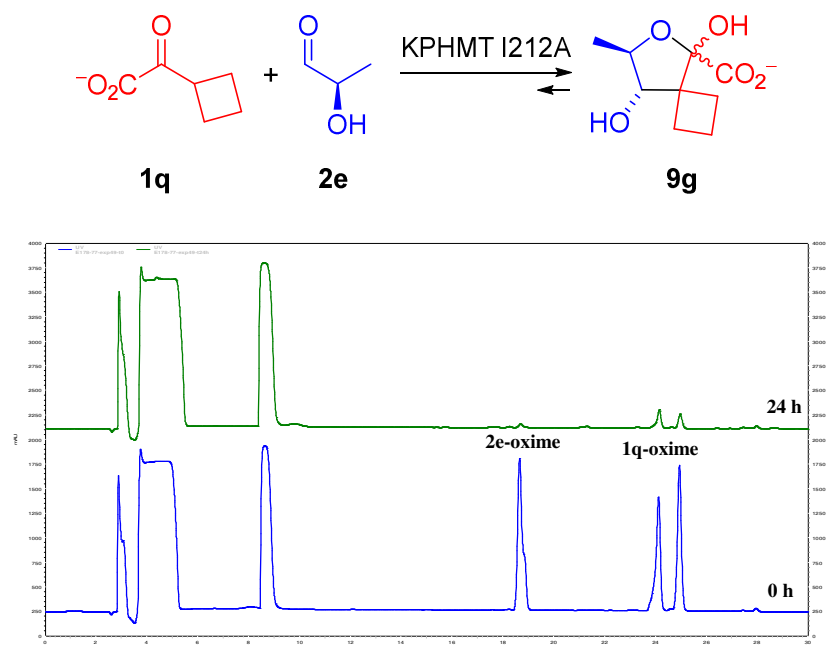
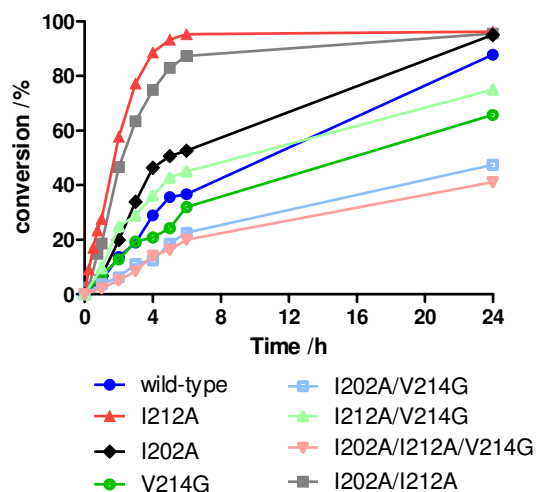


Figure S184 Aldol addition of 2-cyclobutyl-2-oxoetanoate (**1q**) to D-lactaldehyde (**2e**) catalyzed by KPHMT I212A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (95% conversion). Product **9g** was not detected by HPLC because it was not derivatized by *O*-benzylhydroxylamine under the conditions employed. Only the substrate consumption could be monitored.



^a $[\mathbf{1q}] = [\mathbf{2e}] = 100 \text{ mM}$, $[\text{enzyme}] = 2 \text{ mg mL}^{-1}$. ^b not detected.

KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$0.10 \pm 6 \cdot 10^{-3}$	86
I212A	$0.47 \pm 2.6 \cdot 10^{-2}$	97
I202A	$0.18 \pm 1.9 \cdot 10^{-2}$	95
V214G	$0.10 \pm 2 \cdot 10^{-3}$	61
I202A/V214G	$5.6 \cdot 10^{-2} \pm 5 \cdot 10^{-3}$	44
I212A/V214G	$0.17 \pm 2.4 \cdot 10^{-2}$	72
I202A/I212A/ V214G	$4.3 \cdot 10^{-2} \pm 2 \cdot 10^{-3}$	38
I202A/I212A	$0.32 \pm 1.8 \cdot 10^{-2}$	95
Control	_b	_b

Figure S185 Reaction time course of aldol addition of 2-cyclobutyl-2-oxoetanoate (**1q**) to D-lactaldehyde (**2e**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme.

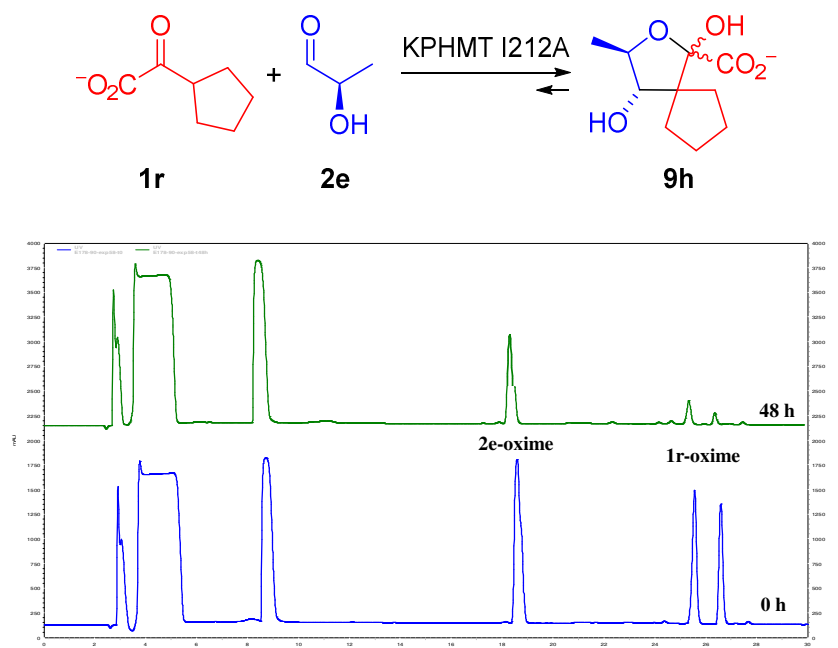
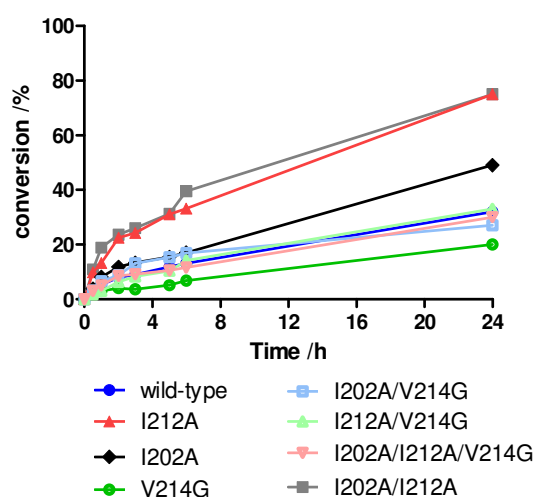


Figure S186 Aldol addition of 2-cyclopentyl-2-oxoetanoate (**1r**) to D-lactaldehyde (**2e**) catalyzed by KPHMT I212A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 48 h (88% conversion). Product **9h** was not detected by HPLC because it was not derivatized by *O*-benzylhydroxylamine under the conditions employed. Only the substrate consumption could be monitored.



^a $[\mathbf{1r}] = [\mathbf{2e}] = 100 \text{ mM}$, $[\text{enzyme}] = 2 \text{ mg mL}^{-1}$.

^b not detected. ^c 48h reaction.

KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$6.8 \cdot 10^{-2} \pm 9 \cdot 10^{-3}$	32
I212A	$0.17 \pm 2.7 \cdot 10^{-2}$	75/88 ^c
I202A	$9.8 \cdot 10^{-2} \pm 1.4 \cdot 10^{-2}$	49
V214G	$3.1 \cdot 10^{-2} \pm 7 \cdot 10^{-3}$	20
I202A/V214G	$7.2 \cdot 10^{-2} \pm 1.4 \cdot 10^{-2}$	27
I212A/V214G	$5.3 \cdot 10^{-2} \pm 2 \cdot 10^{-3}$	33
I202A/I212A/ V214G	$6.9 \cdot 10^{-2} \pm 6 \cdot 10^{-3}$	30
I202A/I212A	$0.19 \pm 4.9 \cdot 10^{-2}$	75/83 ^c
Control	_b	_b

Figure S187 Reaction time course of aldol addition of 2-cyclopentyl-2-oxoetanoate (**1f**) to D-lactaldehyde (**2e**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme.

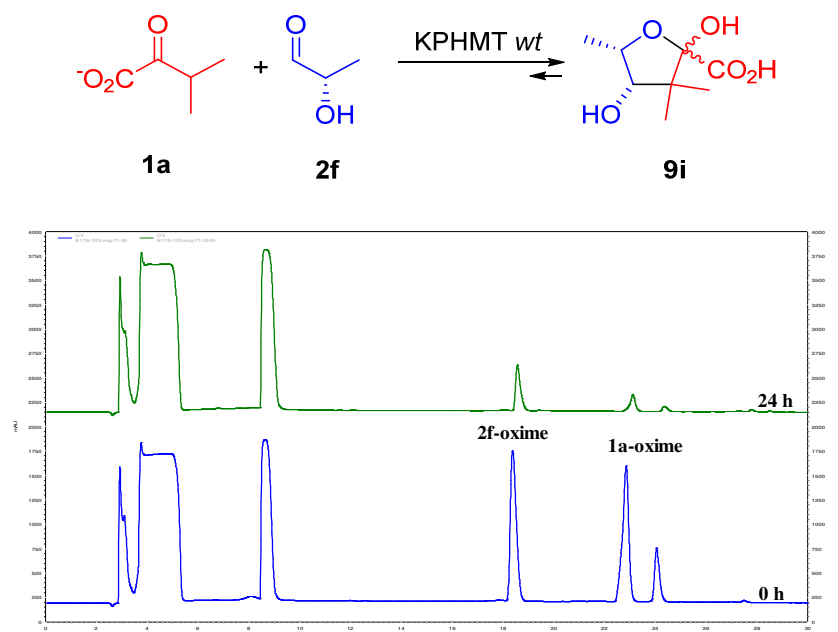
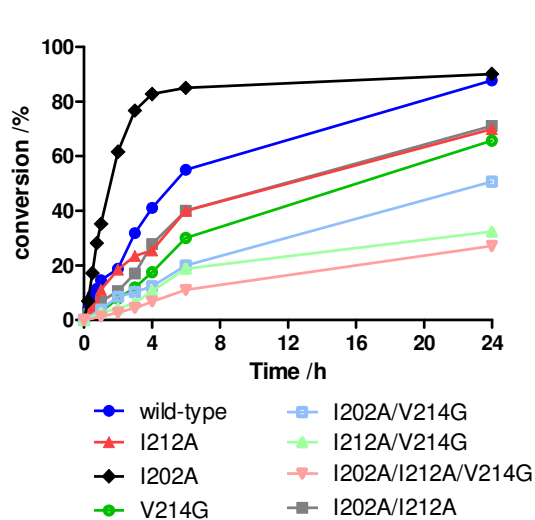


Figure S188 Aldol addition of 3-methyl-2-oxobutanoate (**1a**) to L-lactaldehyde (**2f**) catalyzed by KPHMT wild-type (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (88% conversion). Product **9i** was not detected by HPLC because it was not derivatized by *O*-benzylhydroxylamine under the conditions employed. Only the substrate consumption could be monitored.



KPHMT	ν_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$0.20 \pm 1.3 \cdot 10^{-2}$	87
I212A	$0.11 \pm 9 \cdot 10^{-3}$	73
I202A	$0.48 \pm 2.2 \cdot 10^{-2}$	90
V214G	$6.2 \cdot 10^{-2} \pm 9 \cdot 10^{-3}$	67
I202A/V214G	$5.0 \cdot 10^{-2} \pm 6 \cdot 10^{-3}$	43
I212A/V214G	$2.9 \cdot 10^{-2} \pm 2 \cdot 10^{-3}$	32
I202A/I212A/ V214G	$2.1 \cdot 10^{-2} \pm 1 \cdot 10^{-3}$	27
I202A/I212A	$9.8 \cdot 10^{-2} \pm 4 \cdot 10^{-3}$	67
Control	_b	_b

^a $[\mathbf{1a}] = [\mathbf{2f}] = 100 \text{ mM}$, $[\text{enzyme}] = 2 \text{ mg mL}^{-1}$.

^b not detected.

Figure S189 Reaction time course of aldol addition of 3-methyl-2-oxobutanoate (**1a**) to L-lactaldehyde (**2f**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme.

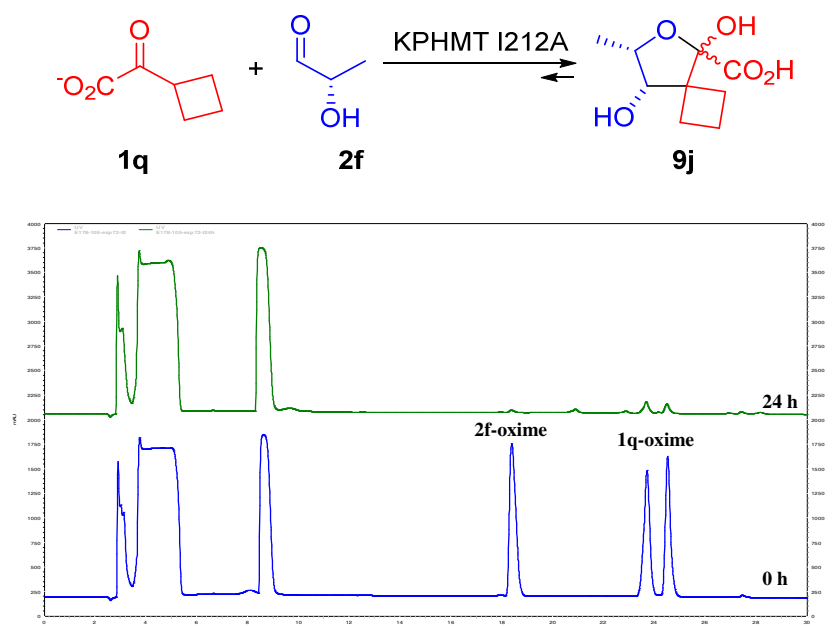
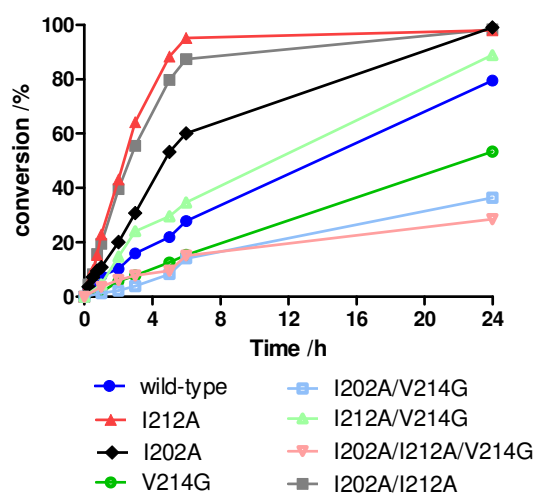


Figure S190 Aldol addition of 2-cyclobutyl-2-oxoetanoate (**1q**) to L-lactaldehyde (**2f**) catalyzed by KPHMT I212A (Co²⁺). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (95% conversion). Product **9j** was not detected by HPLC because it was not derivatized by *O*-benzylhydroxylamine under the conditions employed. Only the substrate consumption could be monitored.



^a [1q] = [2f] = 100 mM, [enzyme] = 2 mg mL⁻¹.

^b not detected.

KPHMT	v_o^a (mM·min ⁻¹)	Conv 24h (%)
wild-type	0.14±1.0·10 ⁻²	80
I212A	0.34±1.8·10 ⁻²	95
I202A	0.21±1.5·10 ⁻²	95
V214G	4.4·10 ⁻² ±5·10 ⁻³	63
I202A/V214G	1.9·10 ⁻² ±1·10 ⁻³	40
I212A/V214G	0.12±1.3·10 ⁻²	83
I202A/I212A/ V214G	4.3·10 ⁻² ±5·10 ⁻³	39
I202A/I212A	0.32±3.4·10 ⁻²	96
Control	^b	^b

Figure S191 Reaction time course of aldol addition of 2-cyclobutyl-2-oxoetanoate (**1q**) to L-lactaldehyde (**2f**) catalyzed by KPHMT (Co²⁺) variants and control experiment performed under the same conditions without the enzyme.

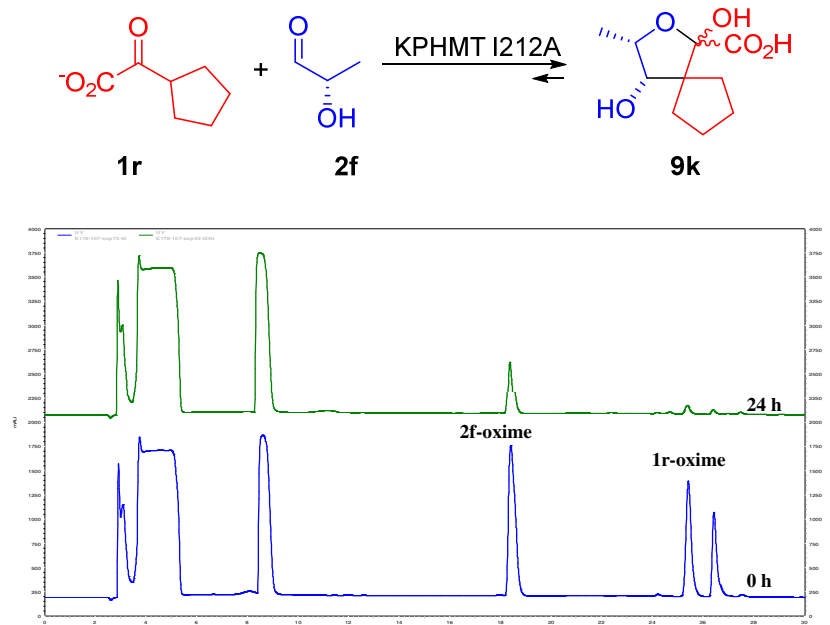
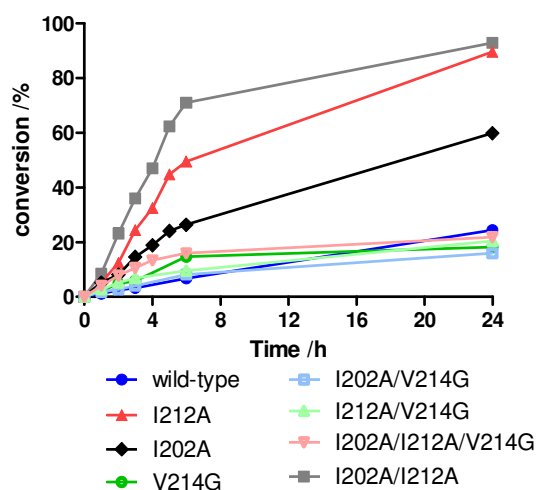


Figure S192 Aldol addition of 2-cyclopentyl-2-oxoetanoate (**1r**) to L-lactaldehyde (**2f**) catalyzed by KPHMT I212A (Co²⁺). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (90% conversion). Product **9k** was not detected by HPLC because it was not derivatized by *O*-benzylhydroxylamine under the conditions employed. Only the substrate consumption could be monitored.



KPHMT	v_o^a (mM·min ⁻¹)	Conv 24h (%)
wild-type	$2.0 \cdot 10^{-2} \pm 2 \cdot 10^{-3}$	26
I212A	$0.14 \pm 1.8 \cdot 10^{-2}$	82
I202A	$8.2 \cdot 10^{-2} \pm 8 \cdot 10^{-3}$	65
V214G	$3.6 \cdot 10^{-2} \pm 4 \cdot 10^{-3}$	12
I202A/V214G	$2.1 \cdot 10^{-2} \pm 1 \cdot 10^{-3}$	20
I212A/V214G	$3.84 \cdot 10^{-2} \pm 3 \cdot 10^{-3}$	20
I202A/I212A/ V214G	$6.4 \cdot 10^{-2} \pm 4 \cdot 10^{-3}$	27
I202A/I212A	$0.20 \pm 1.5 \cdot 10^{-2}$	80
Control	$_b$	$_b$

^a [1r] = [2f] = 100 mM, [enzyme] = 2 mg mL⁻¹.

^b not detected.

Figure S193 Reaction time course of aldol addition of 2-cyclopentyl-2-oxoetanoate (**1r**) to L-lactaldehyde (**2f**) catalyzed by KPHMT (Co²⁺) variants and control experiment performed under the same conditions without the enzyme.

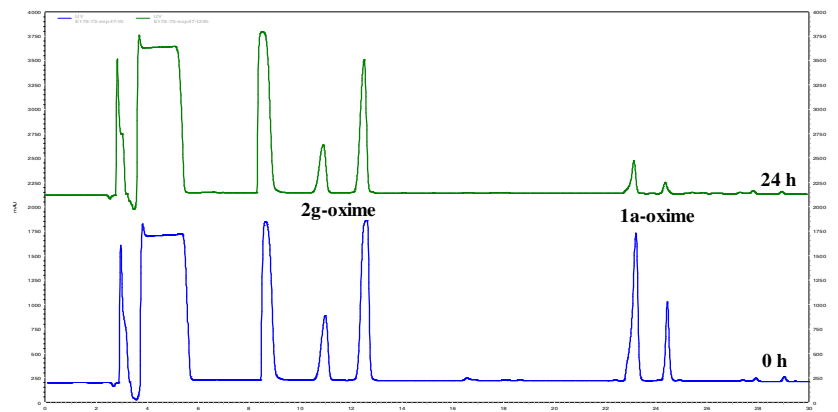
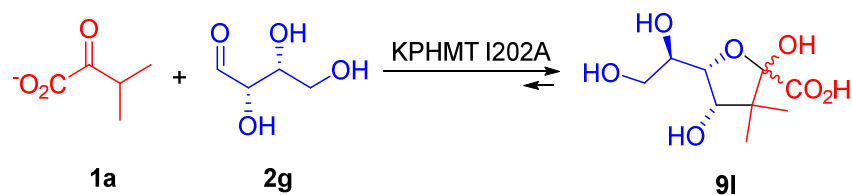
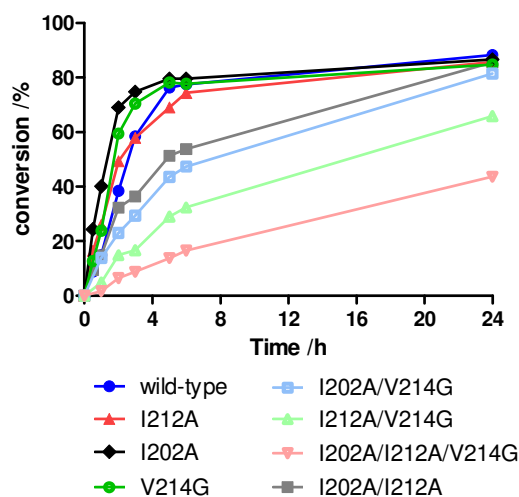


Figure S194 Aldol addition of 3-methyl-2-oxobutanoate (**1a**) to D-threose (**2g**) catalyzed by KPHMT I202A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (88% conversion). Product **9l** was not detected by HPLC because it was not derivatized by *O*-benzylhydroxylamine under the conditions employed. Only the substrate consumption could be monitored.



KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$0.33 \pm 1.5 \cdot 10^{-2}$	82
I212A	$0.40 \pm 2.5 \cdot 10^{-2}$	79
I202A	$0.58 \pm 5.0 \cdot 10^{-2}$	80
V214G	$0.44 \pm 3.3 \cdot 10^{-2}$	81
I202A/V214G	$0.15 \pm 1.9 \cdot 10^{-2}$	74
I212A/V214G	$0.10 \pm 1.7 \cdot 10^{-2}$	47
I202A/I212A/ V214G	$5.0 \cdot 10^{-2} \pm 2 \cdot 10^{-3}$	30
I202A/I212A	$0.19 \pm 1.0 \cdot 10^{-2}$	79
Control	$_b$	$_b$

^a $[\mathbf{1a}] = 100 \text{ mM}$, $[\mathbf{2g}] = 200 \text{ mM}$, $[\text{enzyme}] = 2 \text{ mg mL}^{-1}$. ^b not detected.

Figure S195 Reaction time course of aldol addition of 3-methyl-2-oxobutanoate (**1a**) to D-threose (**2g**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme.

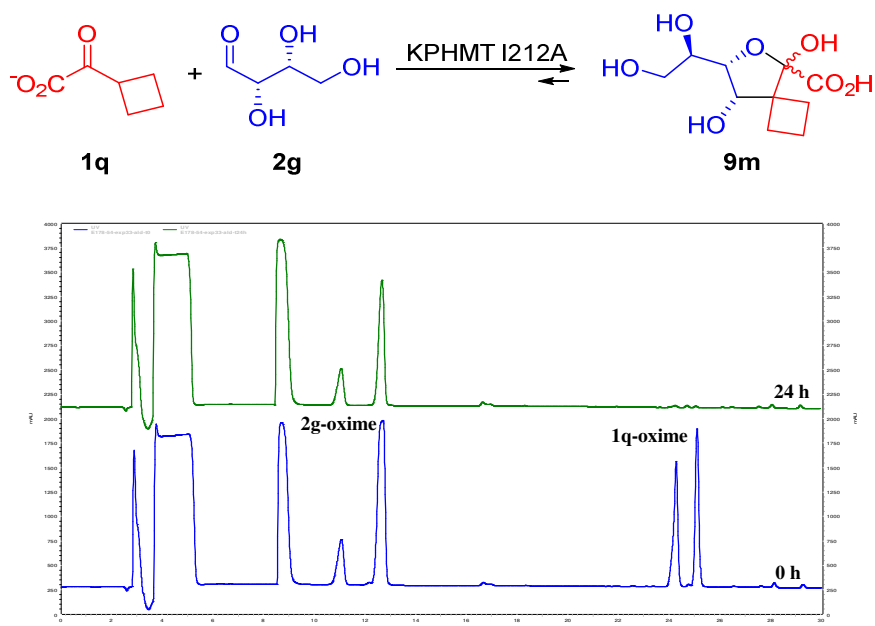
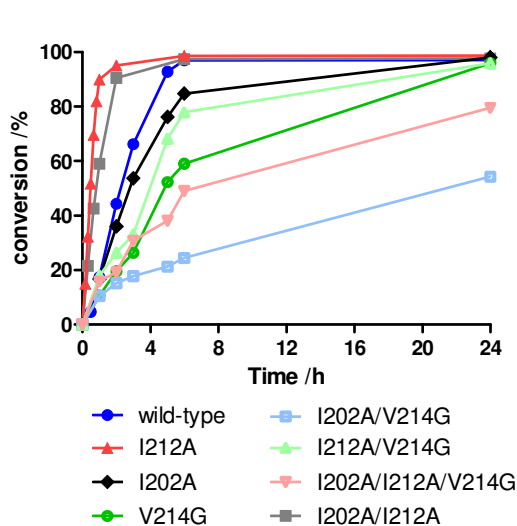


Figure S196 Aldol addition of 2-cyclobutyl-2-oxoetanoate (**1q**) to D-threose (**2g**) catalyzed by KPHMT I212A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (>95% conversion). Product **9m** was not detected by HPLC because it was not derivatized by *O*-benzylhydroxylamine under the conditions employed. Only the substrate consumption could be monitored.



KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$0.43 \pm 2.4 \cdot 10^{-2}$	98
I212A	$1.66 \pm 4.6 \cdot 10^{-2}$	98
I202A	$0.34 \pm 6 \cdot 10^{-3}$	98
V214G	$0.15 \pm 4 \cdot 10^{-3}$	97
I202A/V214G	$0.10 \pm 2.3 \cdot 10^{-2}$	53
I212A/V214G	$0.19 \pm 3.3 \cdot 10^{-2}$	93
I202A/I212A/ V214G	$0.13 \pm 2.8 \cdot 10^{-2}$	91
I202A/I212A	$1.0 \pm 4.2 \cdot 10^{-2}$	98
Control	$_{-b}$	$_{-b}$

^a $[\mathbf{1q}] = 100 \text{ mM}$, $[\mathbf{2g}] = 200 \text{ mM}$, $[\text{enzyme}] = 2 \text{ mg mL}^{-1}$. ^b not detected.

Figure S197 Reaction time course of aldol addition of 2-cyclobutyl-2-oxoetanoate (**1e**) to D-threose (**2g**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme.

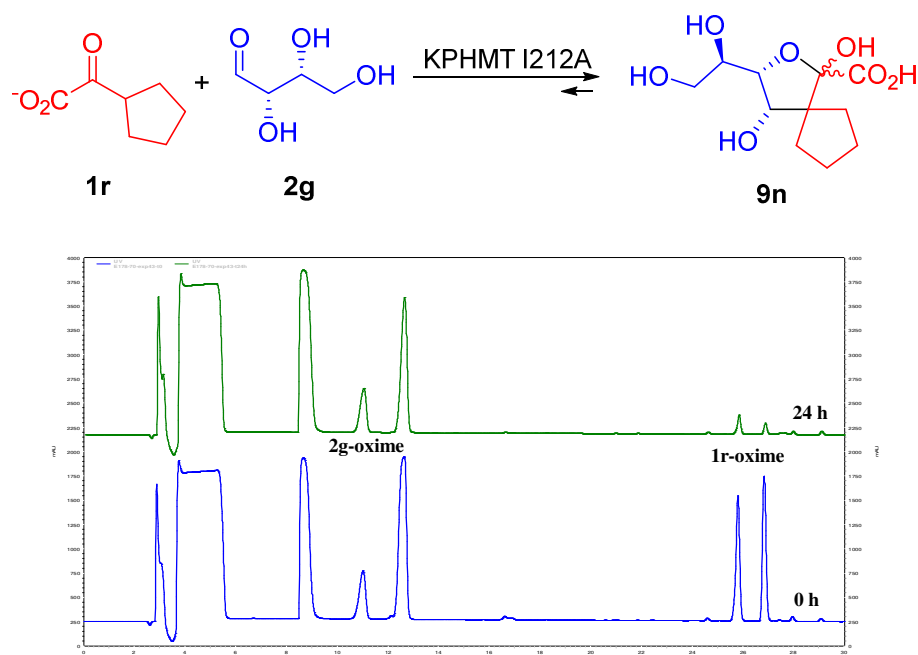
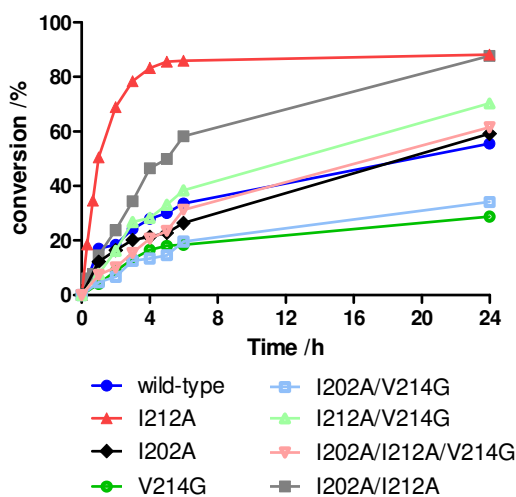


Figure S198 Aldol addition of 2-cyclopentyl-2-oxoethanoate (**1r**) to D-threose (**2g**) catalyzed by KPHMT I212A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (90% conversion). Product **9n** was not detected by HPLC because it was not derivatized by *O*-benzylhydroxylamine under the conditions employed. Only the substrate consumption could be monitored.



KPHMT	v_0^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$8.1 \cdot 10^{-2} \pm 1.3 \cdot 10^{-2}$	63
I212A	$0.73 \pm 1.9 \cdot 10^{-2}$	91
I202A	$0.10 \pm 2.2 \cdot 10^{-2}$	67
V214G	$6.6 \cdot 10^{-2} \pm 1 \cdot 10^{-3}$	31
I202A/V214G	$5.8 \cdot 10^{-2} \pm 7 \cdot 10^{-3}$	40
I212A/V214G	$0.13 \pm 6 \cdot 10^{-3}$	80
I202A/I212A/ V214G	$6.7 \cdot 10^{-2} \pm 4 \cdot 10^{-3}$	67
I202A/I212A	$0.16 \pm 1.4 \cdot 10^{-2}$	90
Control	$_b$	$_b$

^a $[\mathbf{1r}] = 100 \text{ mM}$, $[\mathbf{2g}] = 200 \text{ mM}$, $[\text{enzyme}] = 2 \text{ mg mL}^{-1}$. ^b not detected.

Figure S199 Reaction time course of aldol addition of 2-cyclopentyl-2-oxoethanoate (**1r**) to D-threose (**2g**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme.

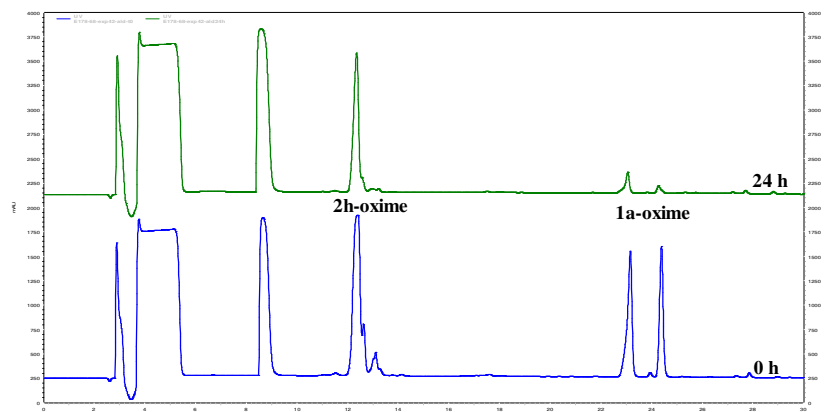
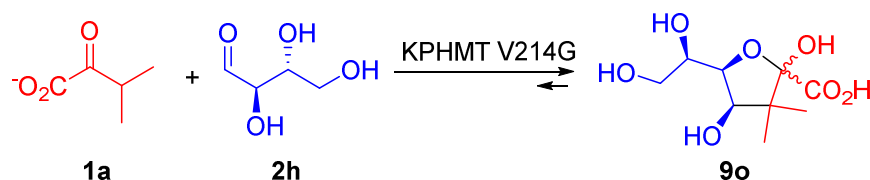
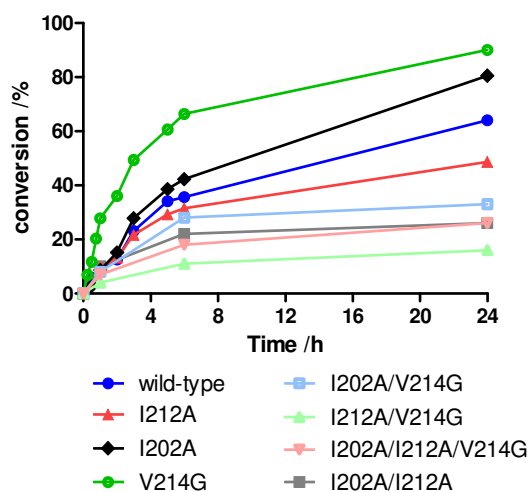


Figure S200 Aldol addition of 3-methyl-2-oxobutanoate (**1a**) to D-erythrose (**2h**) catalyzed by KPHMT V214G (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (88% conversion). Product **9o** was not detected by HPLC because it was not derivatized by *O*-benzylhydroxylamine under the conditions employed. Only the substrate consumption could be monitored.



^a **[1a]** = 100 mM, **[2h]** = 200 mM, [enzyme] = 2 mg mL⁻¹. ^b not detected.

KPHMT	v_o^a (mM·min ⁻¹)	Conv 24h (%)
wild-type	$0.11 \pm 1.2 \cdot 10^{-2}$	60
I212A	$0.87 \cdot 10^{-2} \pm 8 \cdot 10^{-3}$	42
I202A	$0.12 \pm 2.9 \cdot 10^{-2}$	73
V214G	$0.4 \pm 1.9 \cdot 10^{-2}$	90
I202A/V214G	$2.3 \cdot 10^{-2} \pm 8 \cdot 10^{-3}$	33
I212A/V214G	$1.1 \cdot 10^{-2} \pm 3 \cdot 10^{-3}$	15
I202A/I212A/ V214G	$1.8 \cdot 10^{-2} \pm 4 \cdot 10^{-3}$	26
I202A/I212A	$2.0 \cdot 10^{-2} \pm 3 \cdot 10^{-3}$	26
Control	_b	_b

Figure S201 Reaction time course of aldol addition of 3-methyl-2-oxobutanoate (**1a**) to D-erythrose (**2h**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme.

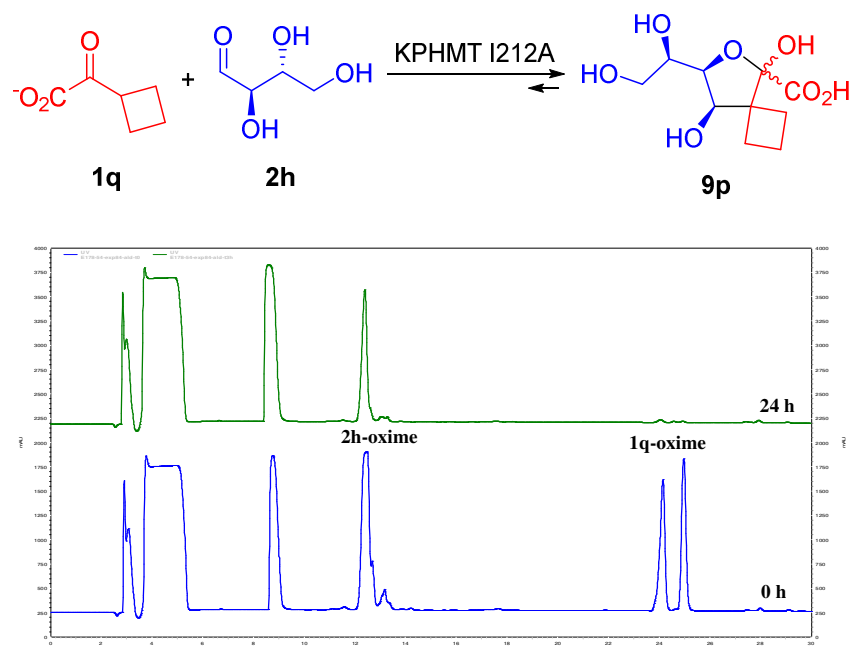
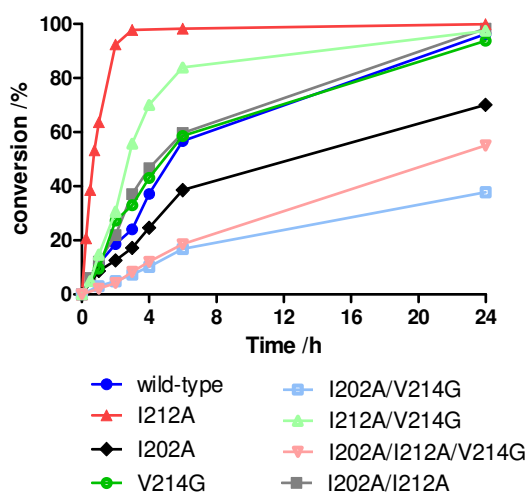


Figure S202 Aldol addition of 2-cyclobutyl-2-oxoetanoate (**1q**) to D-erythrose (**2h**) catalyzed by KPHMT I212A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h (>95% conversion). Product **9p** was not detected by HPLC because it was not derivatized by *O*-benzylhydroxylamine under the conditions employed. Only the substrate consumption could be monitored.



KPHMT	v_o^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$0.11 \pm 8 \cdot 10^{-3}$	97
I212A	$1.15 \pm 6.1 \cdot 10^{-2}$	98
I202A	$8.0 \cdot 10^{-2} \pm 8 \cdot 10^{-3}$	61
V214G	$0.18 \pm 2.4 \cdot 10^{-2}$	91
I202A/V214G	$3.0 \cdot 10^{-2} \pm 2 \cdot 10^{-3}$	27
I212A/V214G	$0.29 \pm 1.8 \cdot 10^{-2}$	98
I202A/I212A/ V214G	$4.0 \cdot 10^{-2} \pm 5 \cdot 10^{-3}$	53
I202A/I212A	$0.18 \pm 9 \cdot 10^{-3}$	98
Control	$_b$	$_b$

^a $[\mathbf{1q}] = 100 \text{ mM}$, $[\mathbf{2h}] = 200 \text{ mM}$, $[\text{enzyme}] = 2 \text{ mg mL}^{-1}$. ^b not detected.

Figure S203 Reaction time course of aldol addition of 2-cyclobutyl-2-oxoetanoate (**1q**) to D-erythrose (**2h**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme.

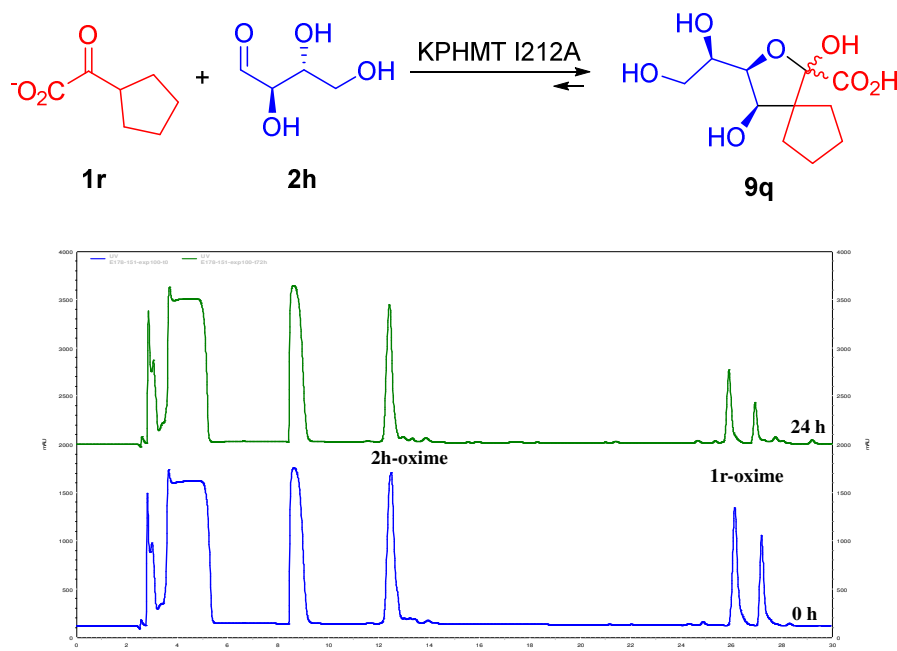
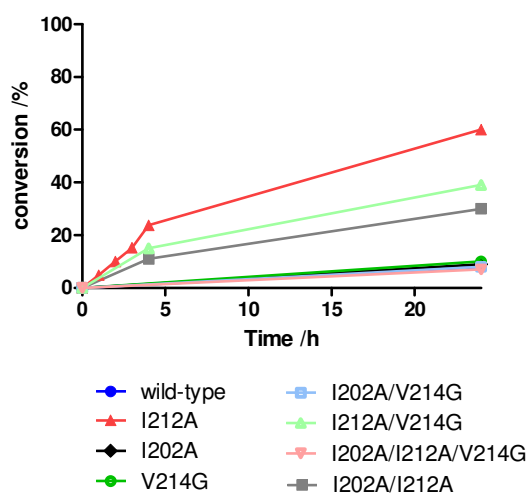


Figure S204 Aldol addition of 2-cyclopentyl-2-oxoetanoate (**1r**) to D-erythrose (**2h**) catalyzed by KPHMT I212A (Co^{2+}). Reaction was monitored by HPLC immediately after the enzyme addition (0 h) and after 24 h. Product **9q** was not detected by HPLC because it was not derivatized by *O*-benzylhydroxylamine under the conditions employed. Only the substrate consumption could be monitored.



KPHMT	v_0^a ($\text{mM} \cdot \text{min}^{-1}$)	Conv 24h (%)
wild-type	$5 \cdot 10^{-3} \pm 1 \cdot 10^{-3}$	8
I212A	$0.11 \pm 2.1 \cdot 10^{-2}$	64
I202A	$6 \cdot 10^{-3} \pm 2 \cdot 10^{-3}$	9
V214G	$7 \cdot 10^{-3} \pm 3 \cdot 10^{-3}$	10
I202A/V214G	$5 \cdot 10^{-3} \pm 2 \cdot 10^{-3}$	8
I212A/V214G	$2.7 \cdot 10^{-2} \pm 6 \cdot 10^{-3}$	39
I202A/I212A/ V214G	$4 \cdot 10^{-3} \pm 1 \cdot 10^{-3}$	7
I202A/I212A	$2.1 \cdot 10^{-2} \pm 9 \cdot 10^{-3}$	30
Control	$_b$	$_b$

^a [**1r**] = 100 mM, [**2h**] = 200 mM, [enzyme] = 2 mg mL⁻¹. ^b not detected.

Figure S205 Reaction time course of aldol addition of 2-cyclopentyl-2-oxoetanoate (**1r**) to D-erythrose (**2h**) catalyzed by KPHMT (Co^{2+}) variants and control experiment performed under the same conditions without the enzyme.

6.3.3 Initial reaction rates

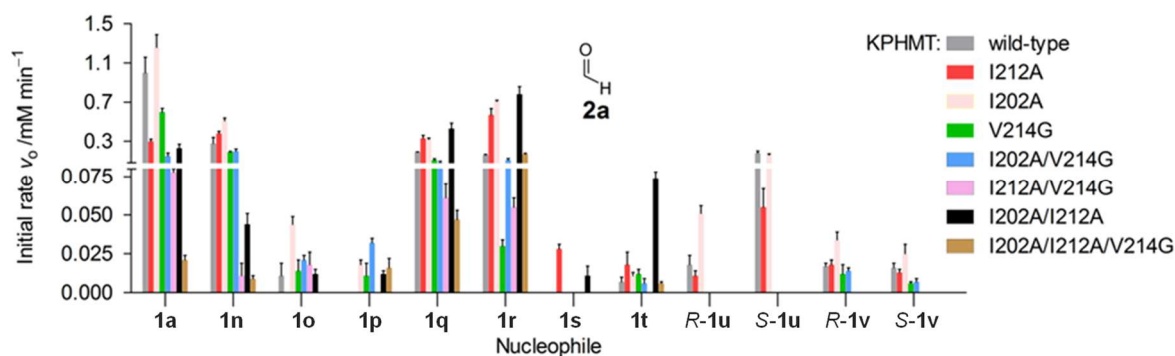


Figure S206 Initial reaction rates (v_0) of wild-type KPHMT and variants-catalyzed aldol addition of selected nucleophiles (**1a,n–v**) to methanal (**2a**). Nucleophiles *R* and *S-1x* were not converted by any KPHMT catalysts and are not shown in the Figure.

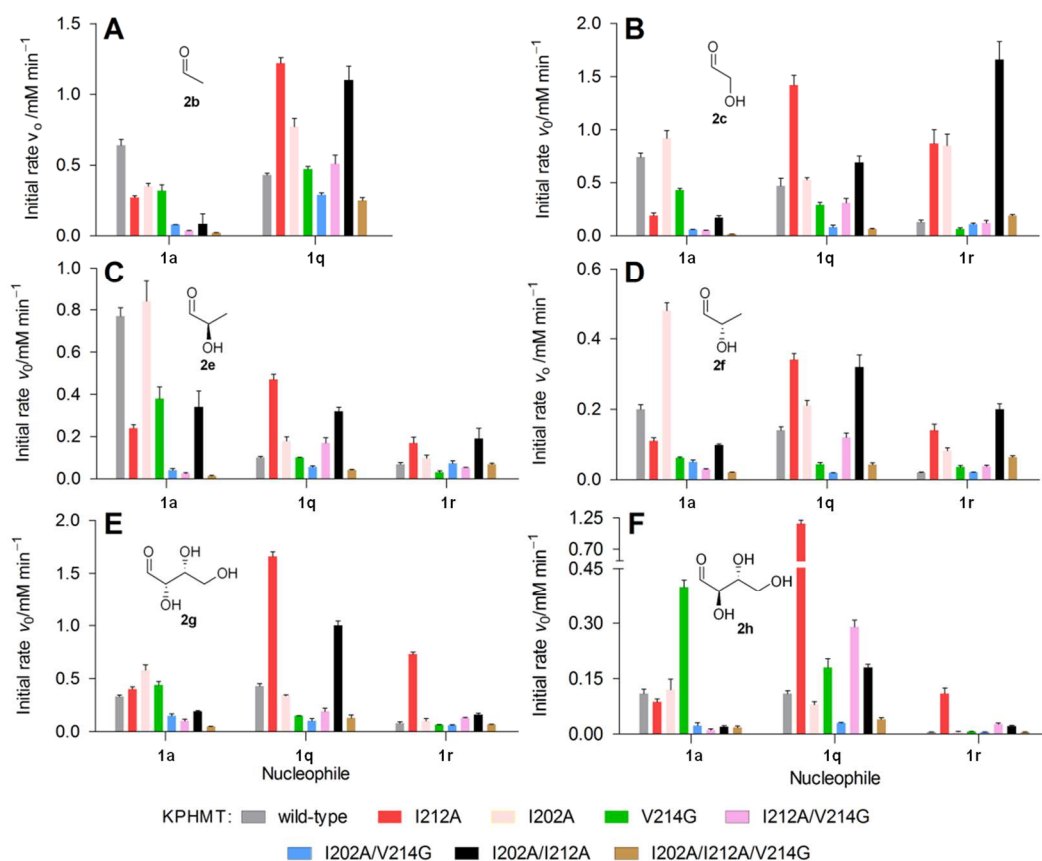
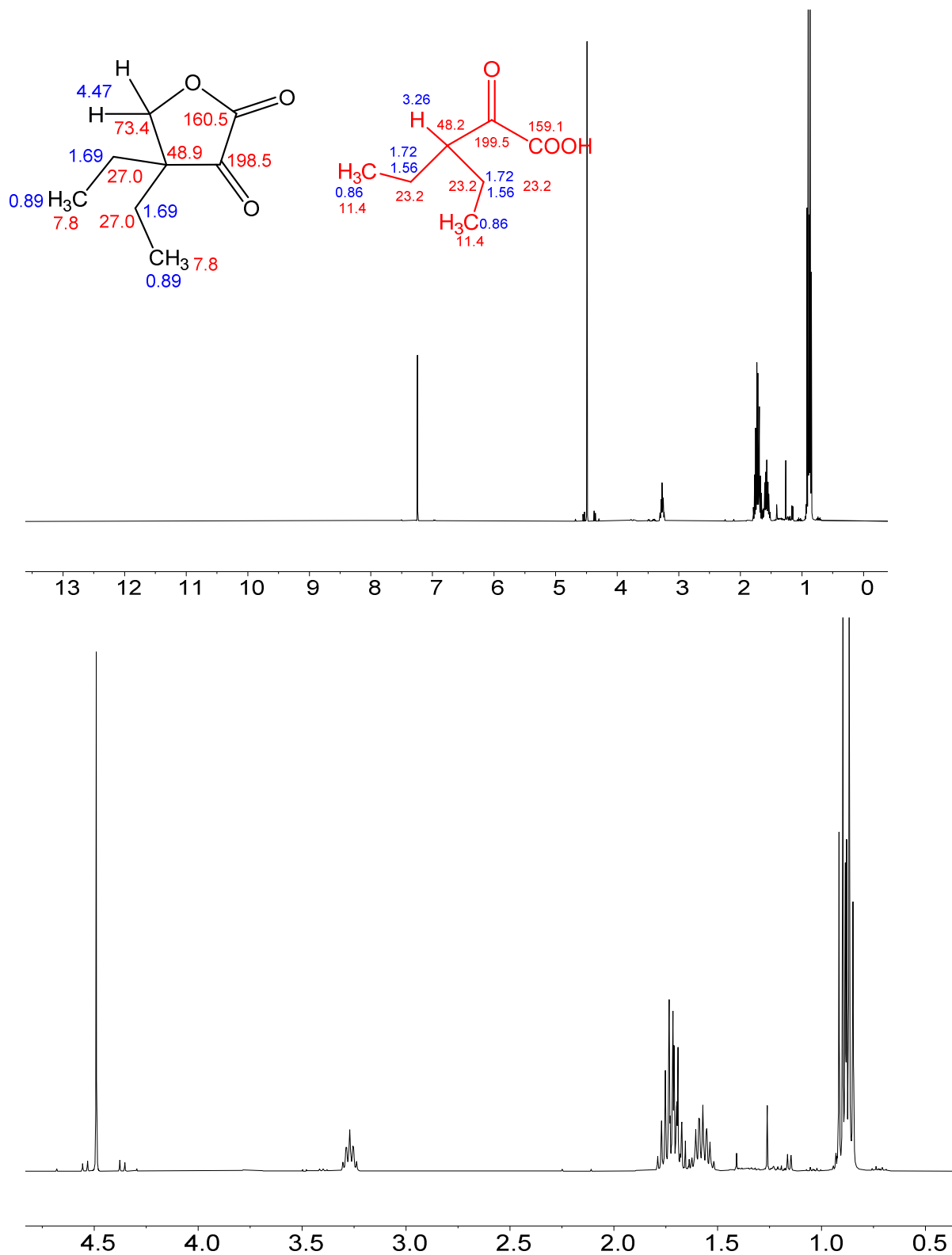


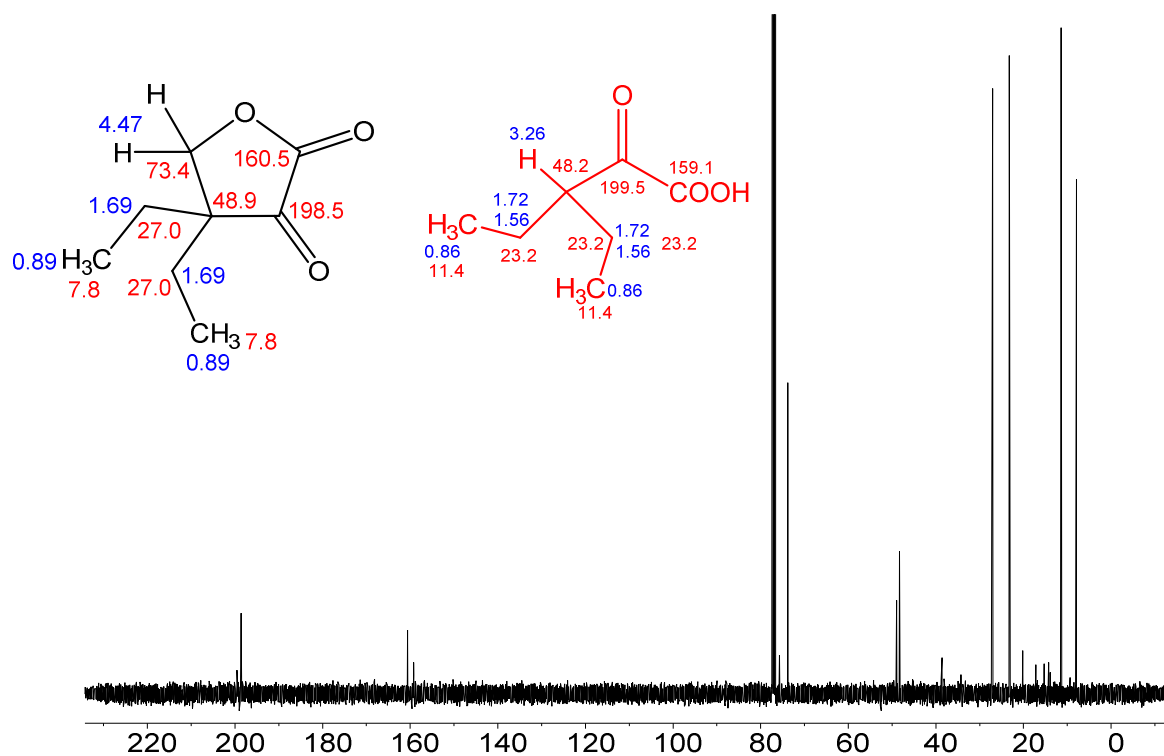
Figure S207 Initial reaction rates (v_0) of wild-type KPHMT and variants-catalyzed aldol addition of selected nucleophiles, **1a**, **1q** and **1r**, to (A) ethanal (**2b**), (B) hydroxyethanal (**2c**), (C) D-lactaldehyde (**2e**), (D) L-lactaldehyde (**2f**), (E) D-threose (**2g**) and (F) D-erythrose (**2h**).

6.3.4 NMR Spectra of products from the enzymatic synthesis 7, 8, 9 and 10

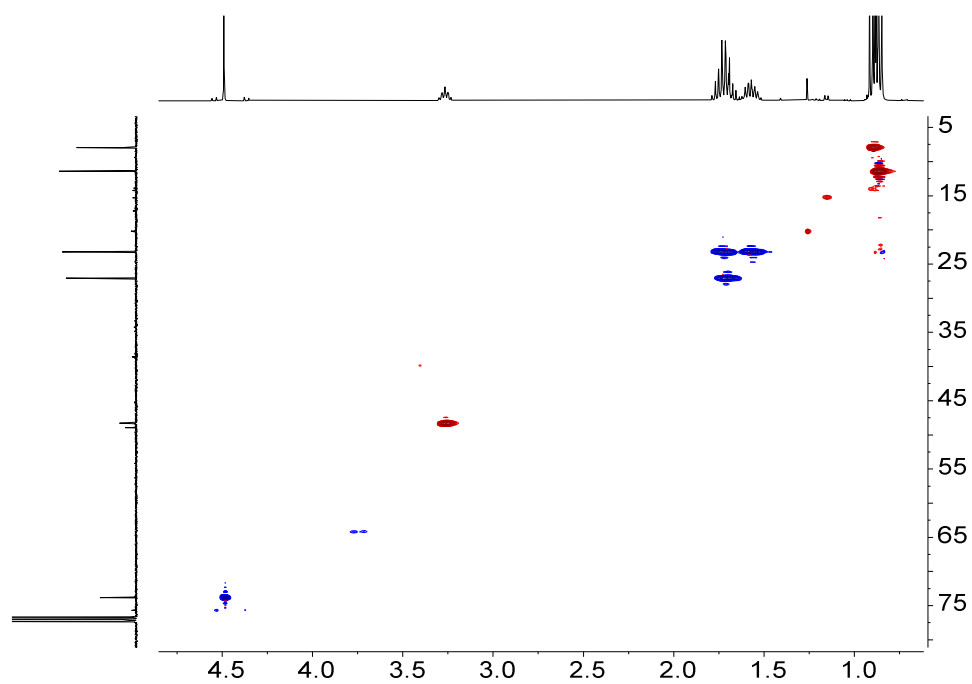
a)



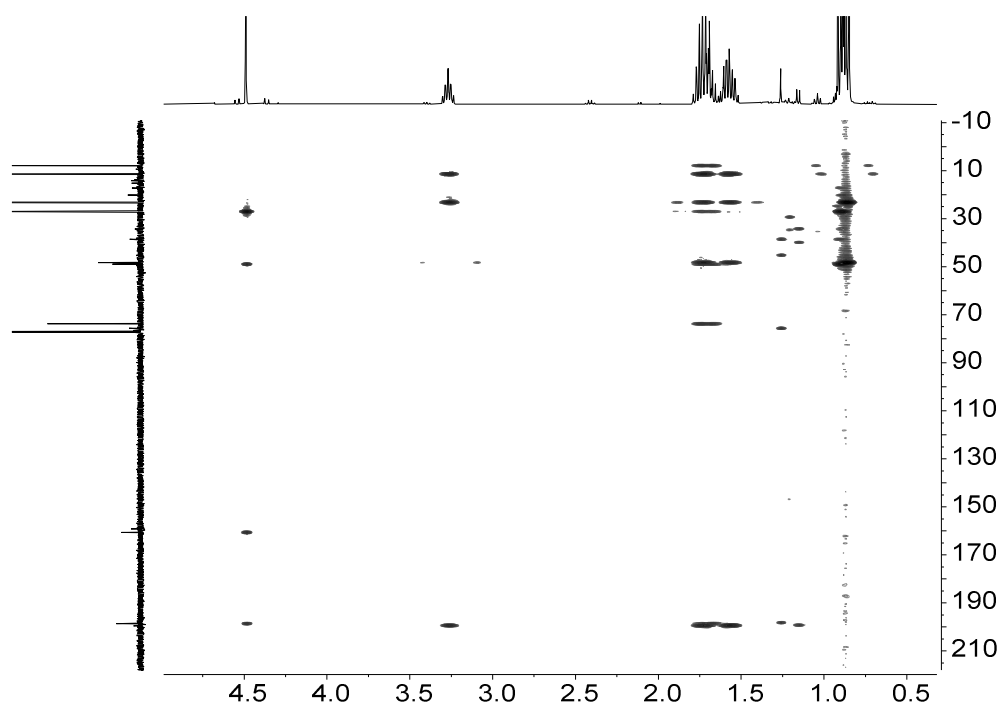
b)



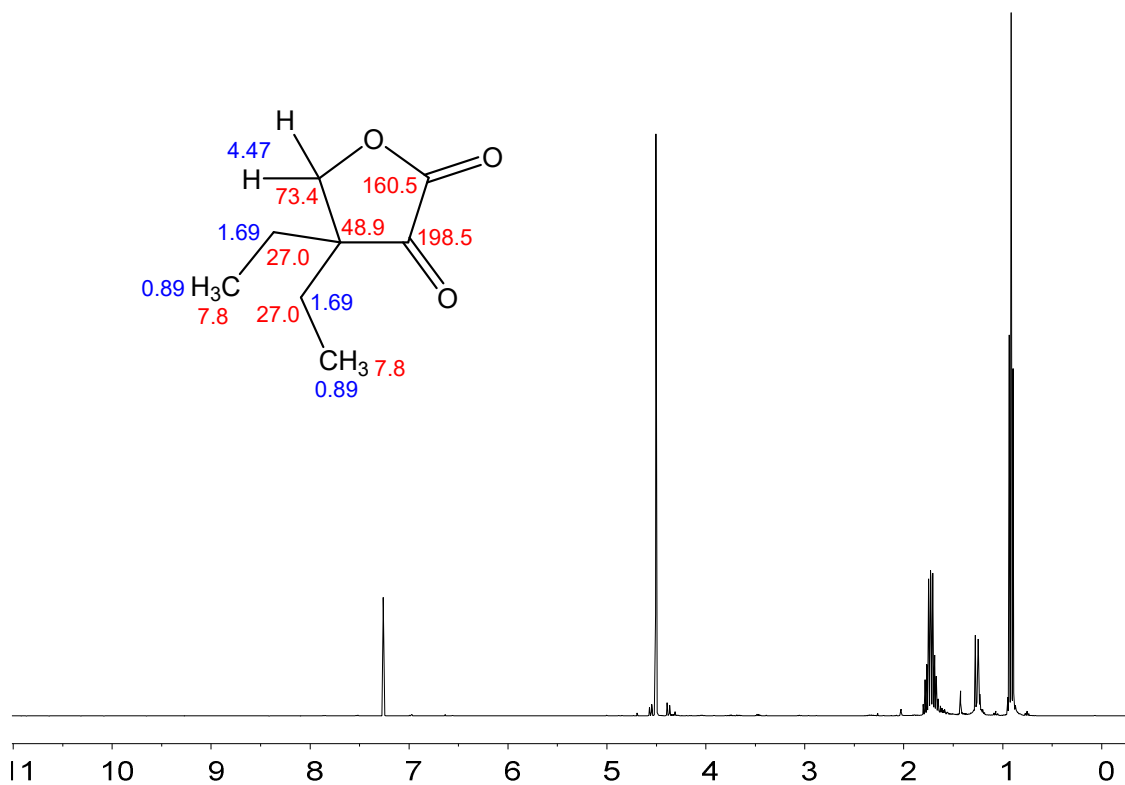
c)



d)



e)



f)

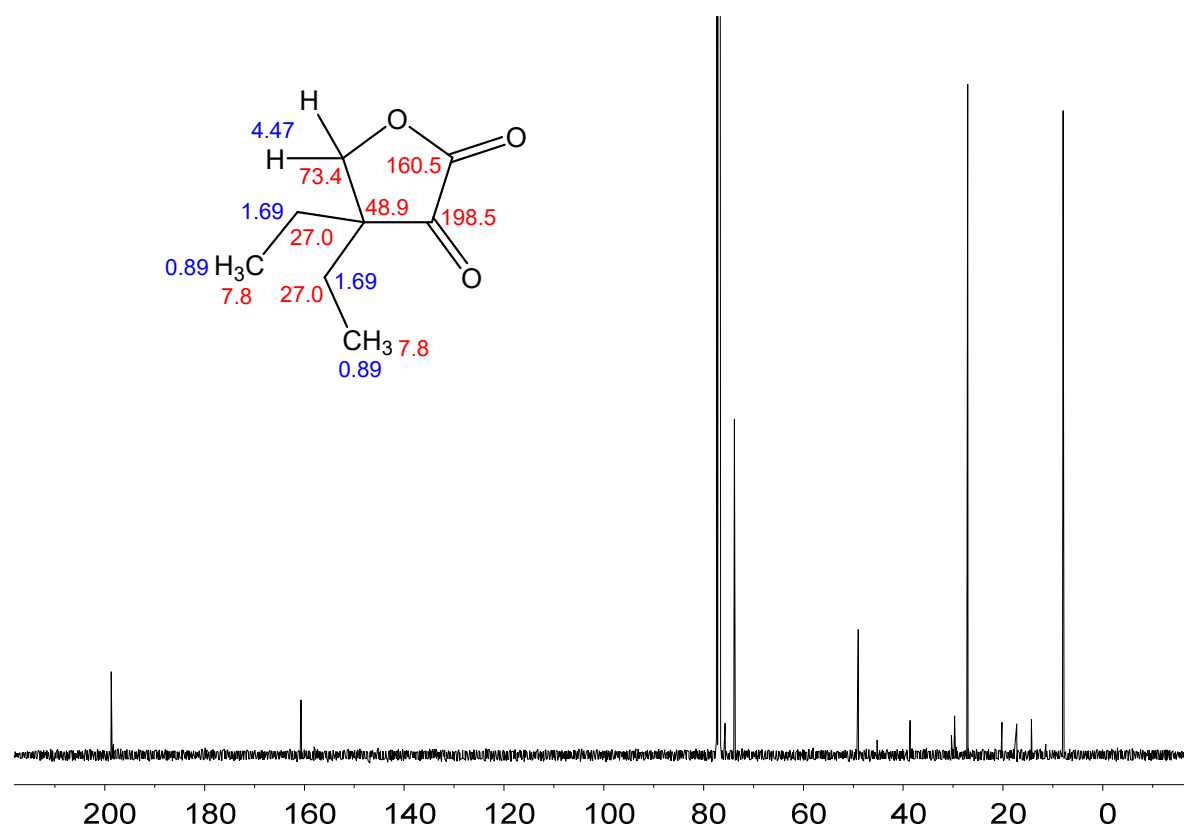


Figure S208 NMR spectra (CDCl_3) of unpurified **8a**: a) ^1H , b) ^{13}C , c) gHSQC, d) HMBC and pure **8a** e) ^1H , f) ^{13}C . E178-134-exp89.

The figure displays two ^{13}C NMR spectra of 2-methyl-2-oxocyclohexanecarboxylic acid in CDCl_3 . The left spectrum is the experimental data, and the right spectrum is the calculated spectrum. The chemical structure is shown with carbon atoms numbered 1 through 13.

Chemical Structure and Carbon Labeling:

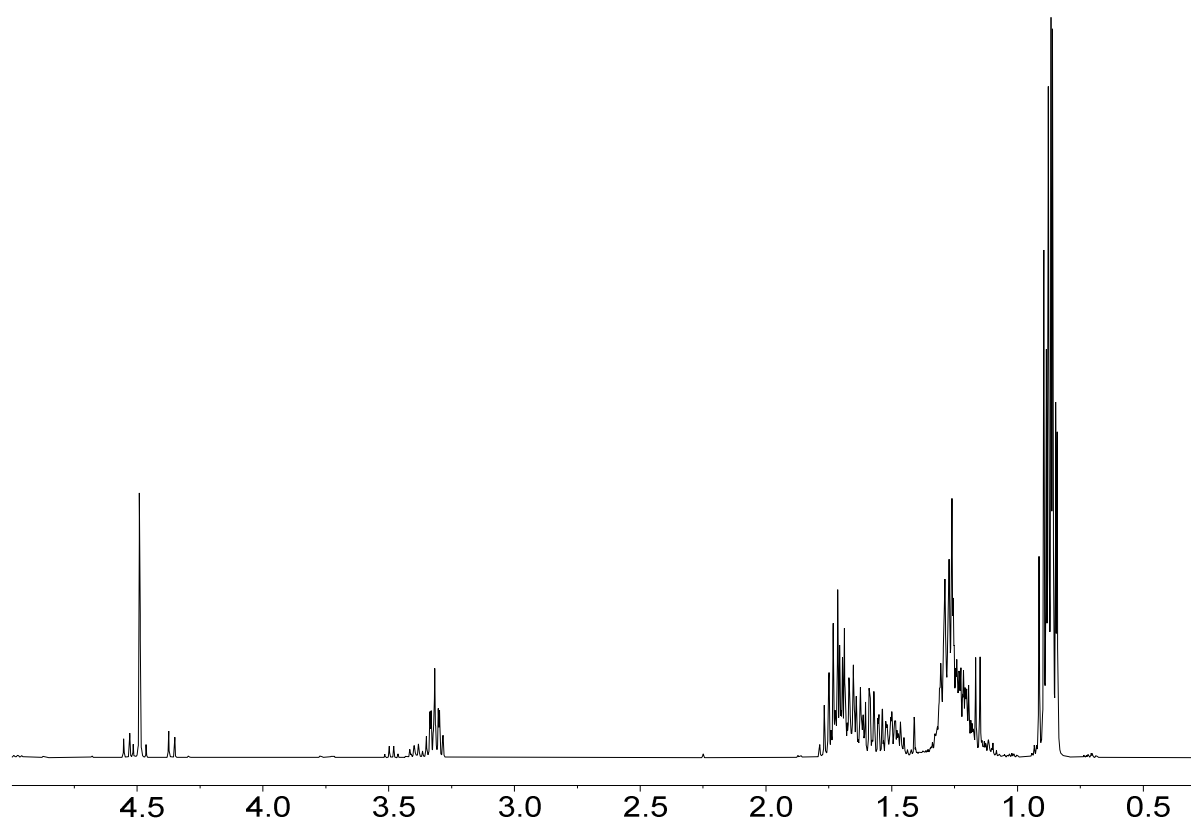
- 1: Carbonyl carbon of the cyclohexanone ring.
- 2: Quaternary carbon of the cyclohexanone ring, bonded to a methyl group (13) and a carboxylic acid group (159.0).
- 3: Methylene carbon of the cyclohexanone ring.
- 4: Methylene carbon of the cyclohexanone ring.
- 5: Methylene carbon of the cyclohexanone ring.
- 6: Methylene carbon of the cyclohexanone ring.
- 7: Methylene carbon of the cyclohexanone ring.
- 8: Methyl carbon of the cyclohexanone ring.
- 9: Carbonyl carbon of the carboxylic acid group.
- 10: Methyl carbon of the carboxylic acid group.
- 11: Methyl carbon of the carboxylic acid group.
- 12: Methyl carbon of the carboxylic acid group.
- 13: Methyl carbon of the carboxylic acid group.

Experimental Spectrum (Left):

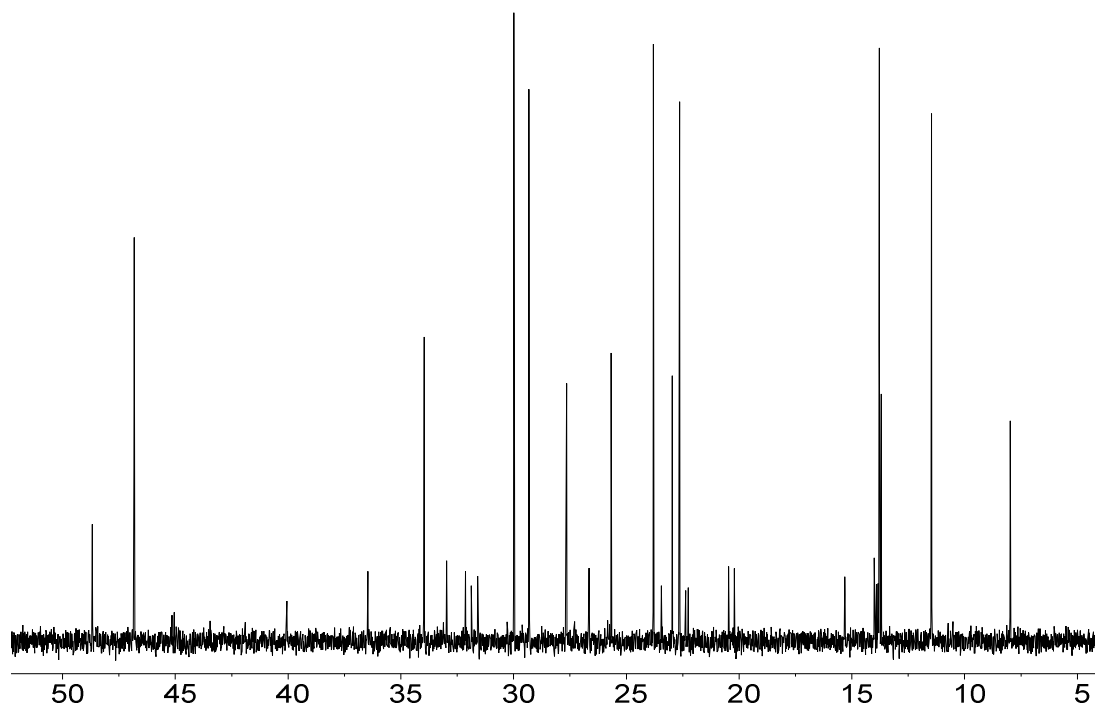
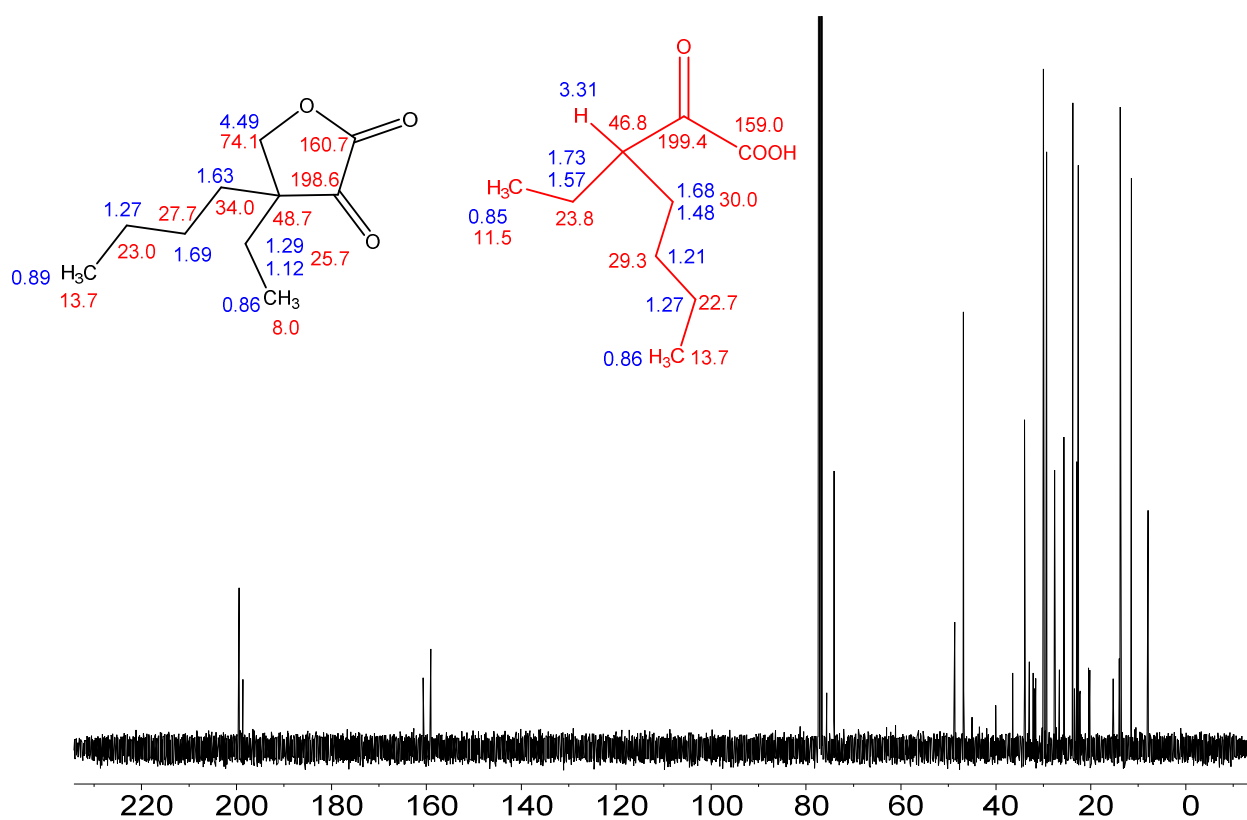
- Peak at 159.0 ppm: Carboxylic acid carbonyl carbon.
- Peak at 13.7 ppm: Methyl carbon of the carboxylic acid group.
- Peak at 11.5 ppm: Methyl carbon of the carboxylic acid group.
- Peak at 8.0 ppm: Methyl carbon of the cyclohexanone ring.
- Peak at 3.31 ppm: Methylene carbon of the cyclohexanone ring.
- Peak at 2.27 ppm: Methylene carbon of the cyclohexanone ring.
- Peak at 1.68 ppm: Methylene carbon of the cyclohexanone ring.
- Peak at 1.48 ppm: Methylene carbon of the cyclohexanone ring.
- Peak at 1.21 ppm: Methylene carbon of the cyclohexanone ring.
- Peak at 1.29 ppm: Methylene carbon of the cyclohexanone ring.
- Peak at 1.12 ppm: Methylene carbon of the cyclohexanone ring.
- Peak at 1.086 ppm: Methylene carbon of the cyclohexanone ring.
- Peak at 0.85 ppm: Methyl carbon of the cyclohexanone ring.
- Peak at 0.89 ppm: Methyl carbon of the cyclohexanone ring.

Calculated Spectrum (Right):

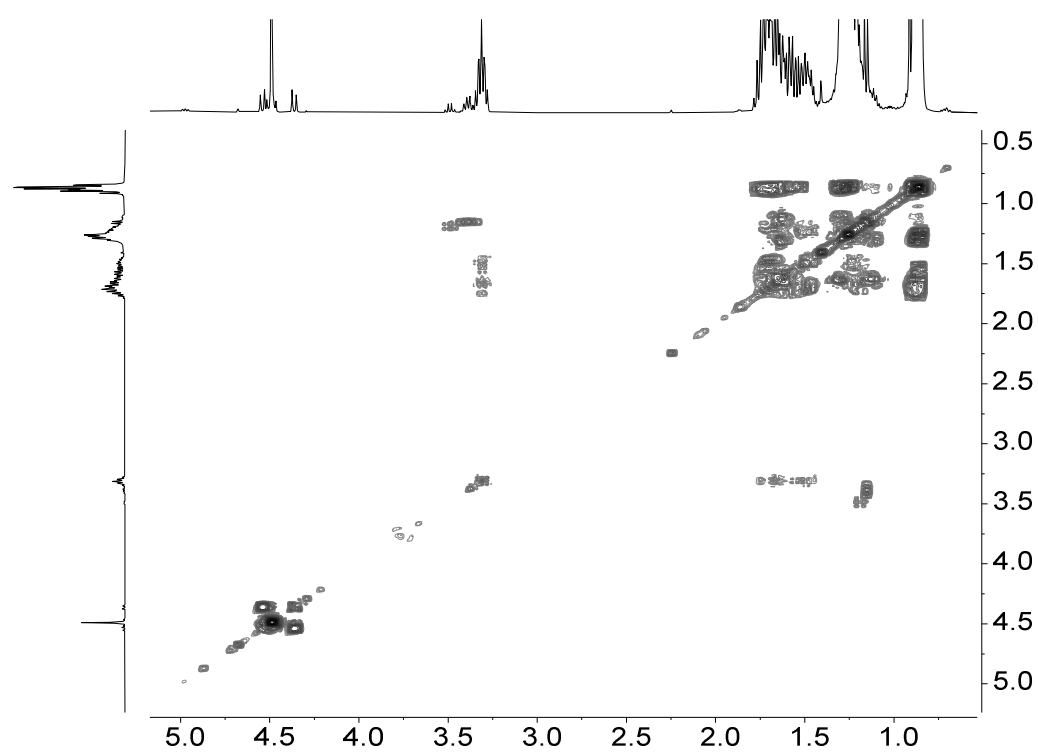
- Peak at 159.0 ppm: Carboxylic acid carbonyl carbon.
- Peak at 13.7 ppm: Methyl carbon of the carboxylic acid group.
- Peak at 11.5 ppm: Methyl carbon of the carboxylic acid group.
- Peak at 8.0 ppm: Methyl carbon of the cyclohexanone ring.
- Peak at 3.31 ppm: Methylene carbon of the cyclohexanone ring.
- Peak at 2.27 ppm: Methylene carbon of the cyclohexanone ring.
- Peak at 1.68 ppm: Methylene carbon of the cyclohexanone ring.
- Peak at 1.48 ppm: Methylene carbon of the cyclohexanone ring.
- Peak at 1.21 ppm: Methylene carbon of the cyclohexanone ring.
- Peak at 1.29 ppm: Methylene carbon of the cyclohexanone ring.
- Peak at 1.12 ppm: Methylene carbon of the cyclohexanone ring.
- Peak at 1.086 ppm: Methylene carbon of the cyclohexanone ring.
- Peak at 0.85 ppm: Methyl carbon of the cyclohexanone ring.
- Peak at 0.89 ppm: Methyl carbon of the cyclohexanone ring.



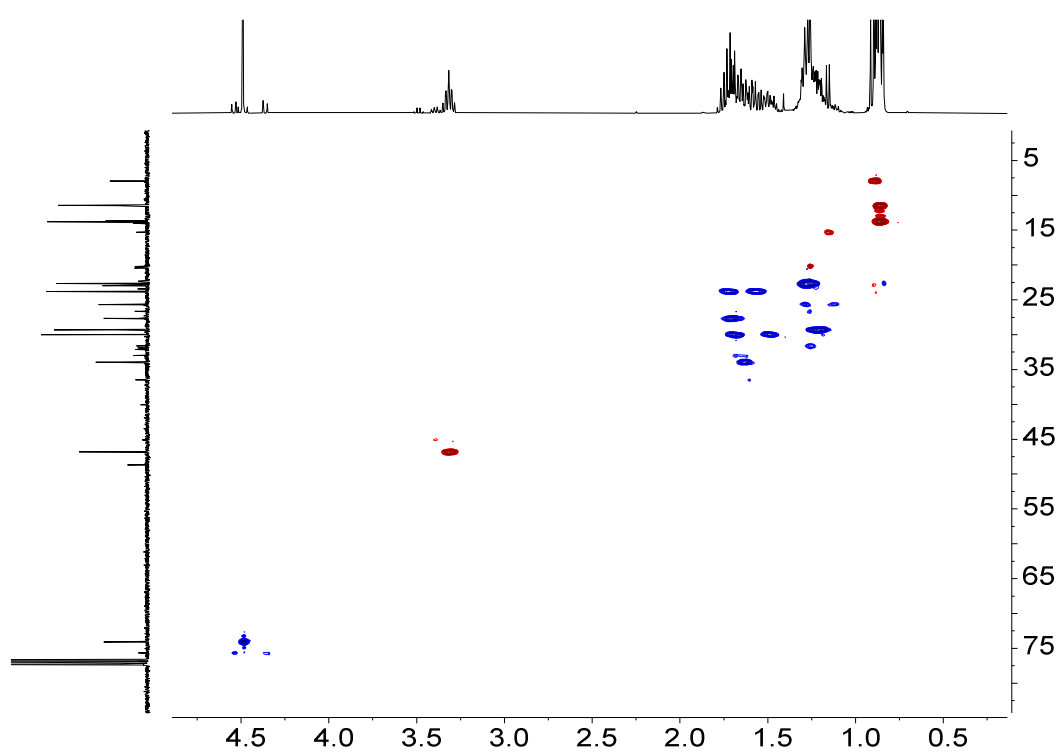
b)



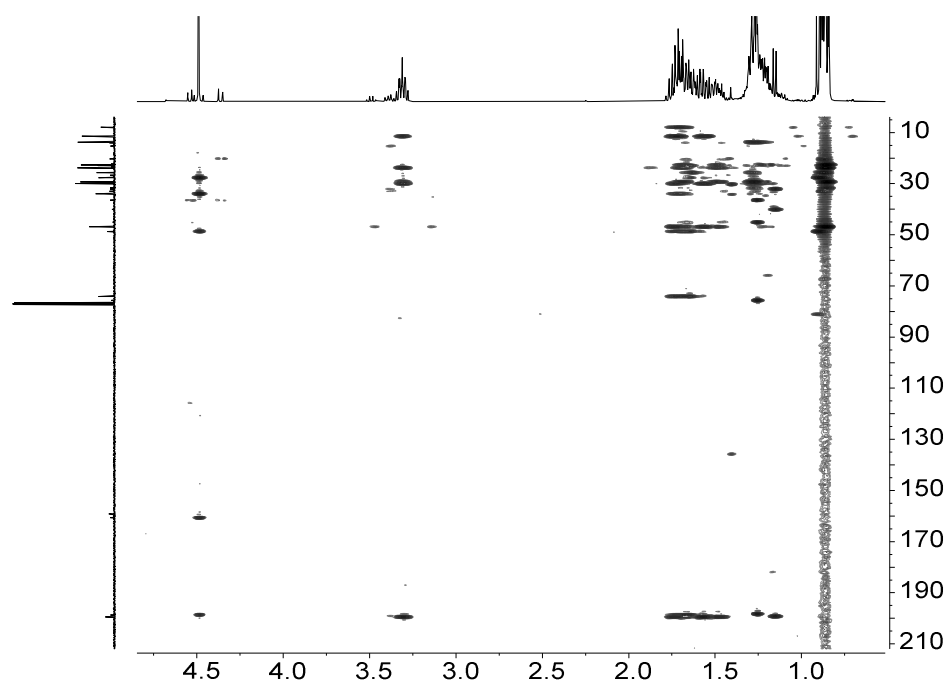
c)



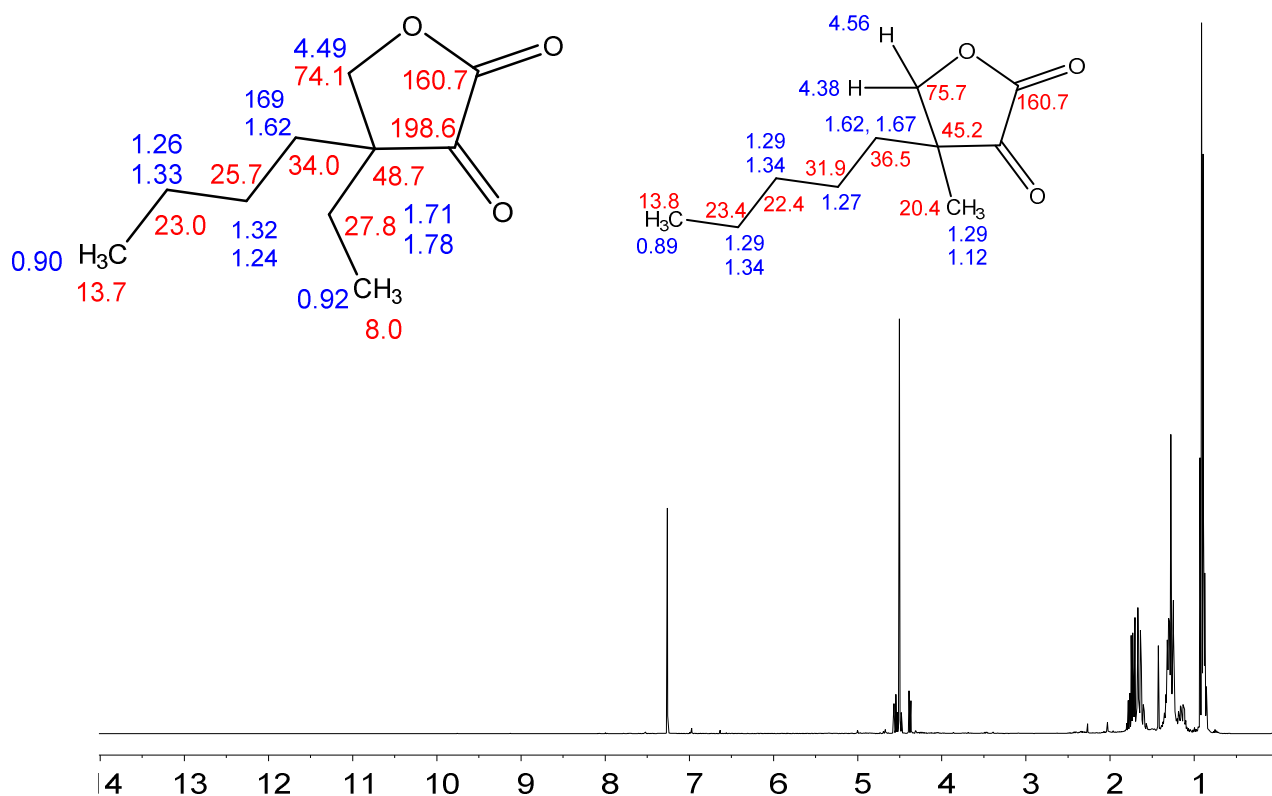
d)



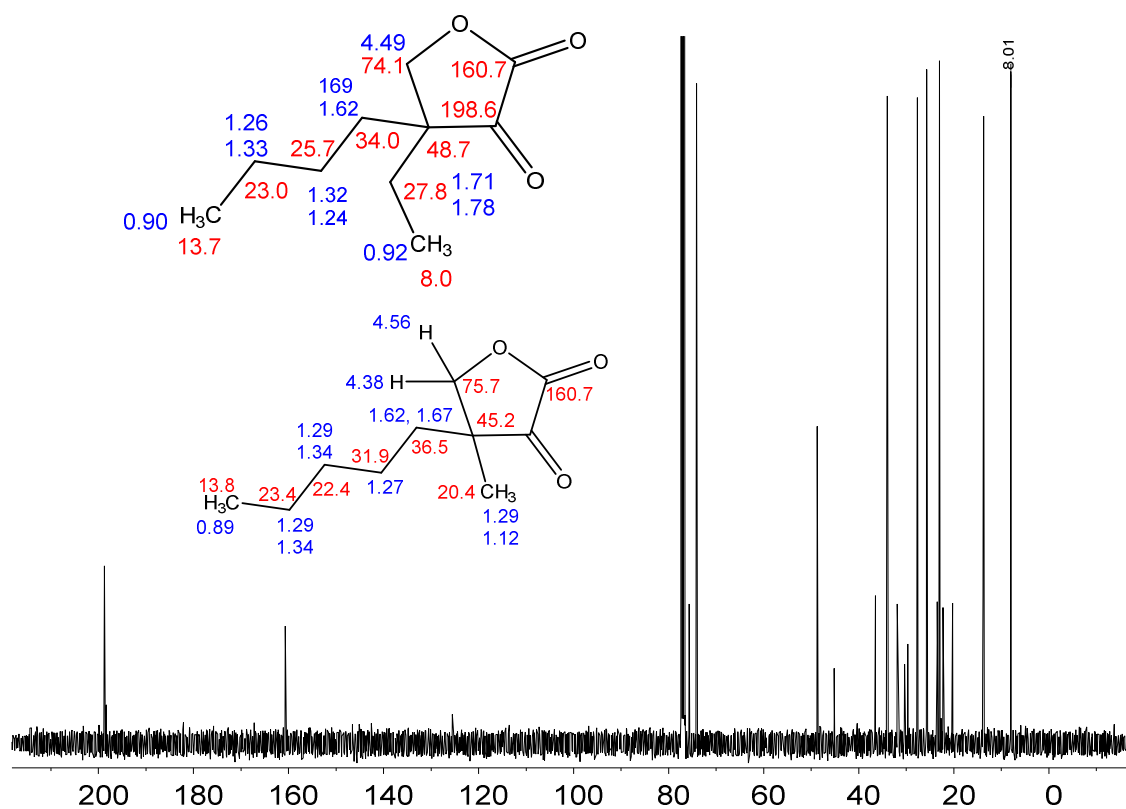
e)



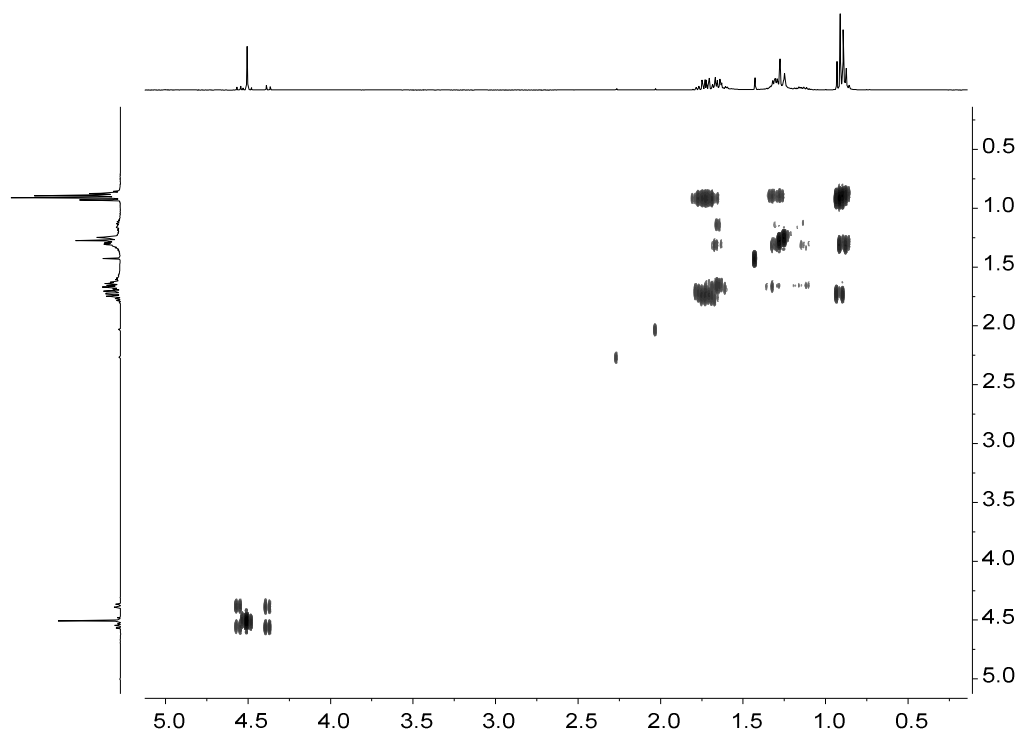
f)



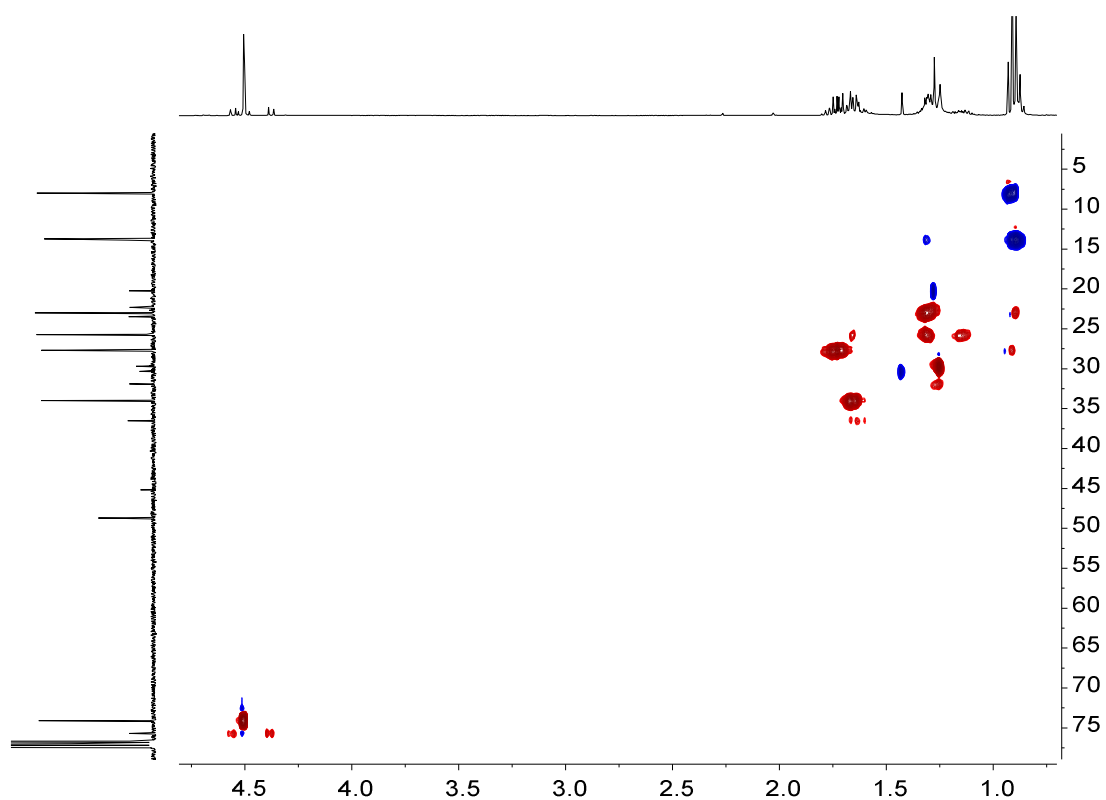
g)



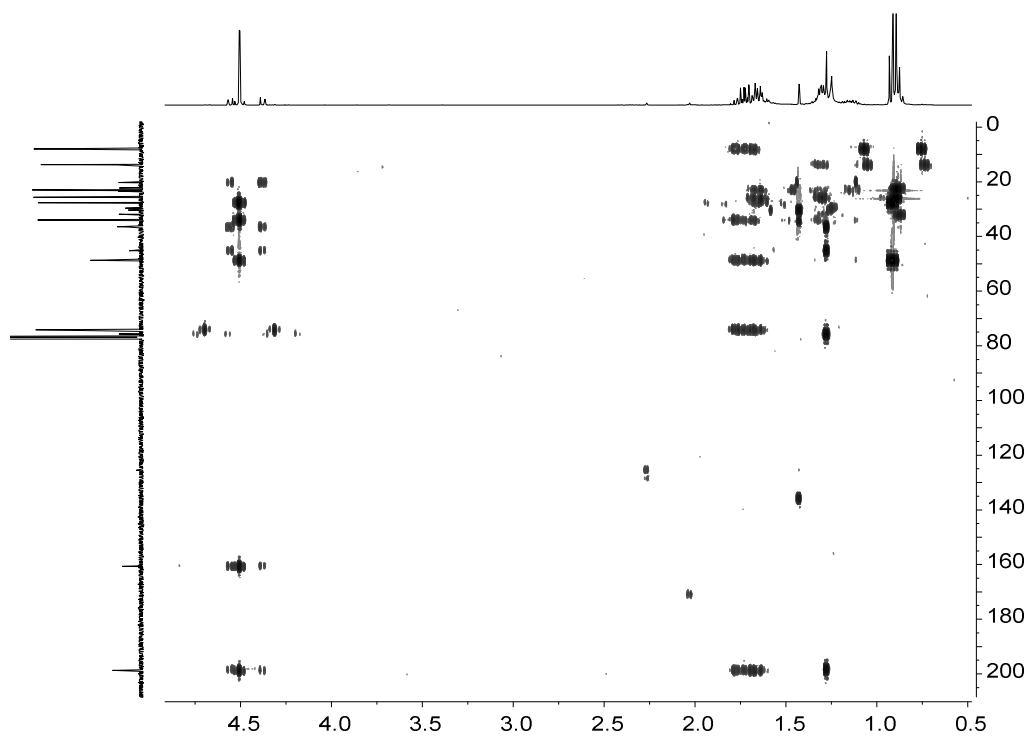
h)



i)



j)



k)

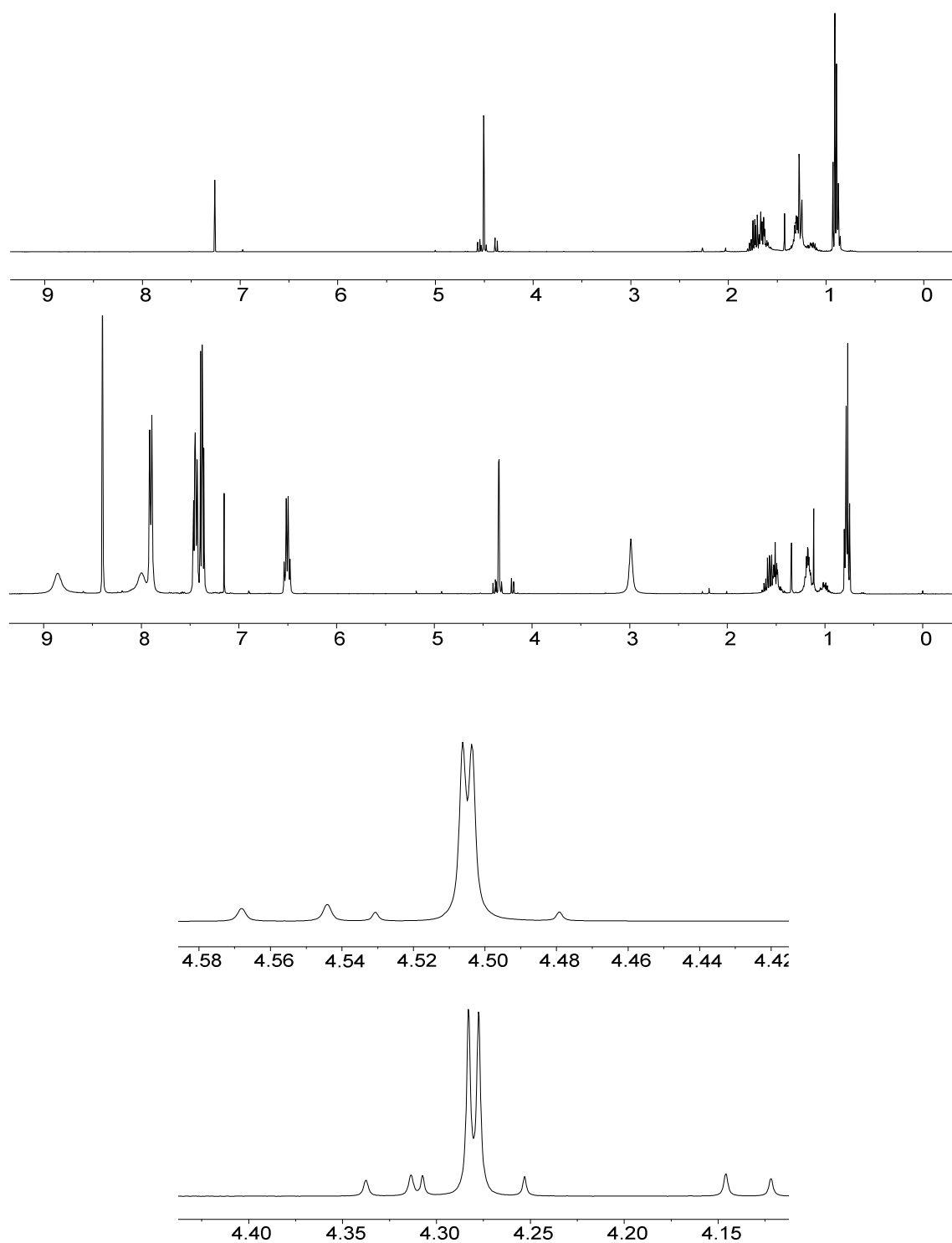


Figure S209 NMR spectra (CDCl_3) of unpurified **8b**: a) ^1H , b) ^{13}C , c) gCOSY d) gHSQC, e) HMBC and pure **8b** f) ^1H , g) ^{13}C , h) gCOSY, i) gHSQC, j) HMBC and k) original spectra and after adding 6 eq of *R* configured Pirkle's alcohol. E178-136-exp91.

The figure displays the ^{13}C NMR spectrum of 2,2,2,3,3,3-hexadeuterio-3-oxocyclohexanecarboxylic acid. The chemical structure is shown on the left, with carbon atoms labeled with their corresponding chemical shifts in ppm. The spectrum on the right shows the experimental peaks, with assignments for each carbon type.

Chemical Structure and Assignments:

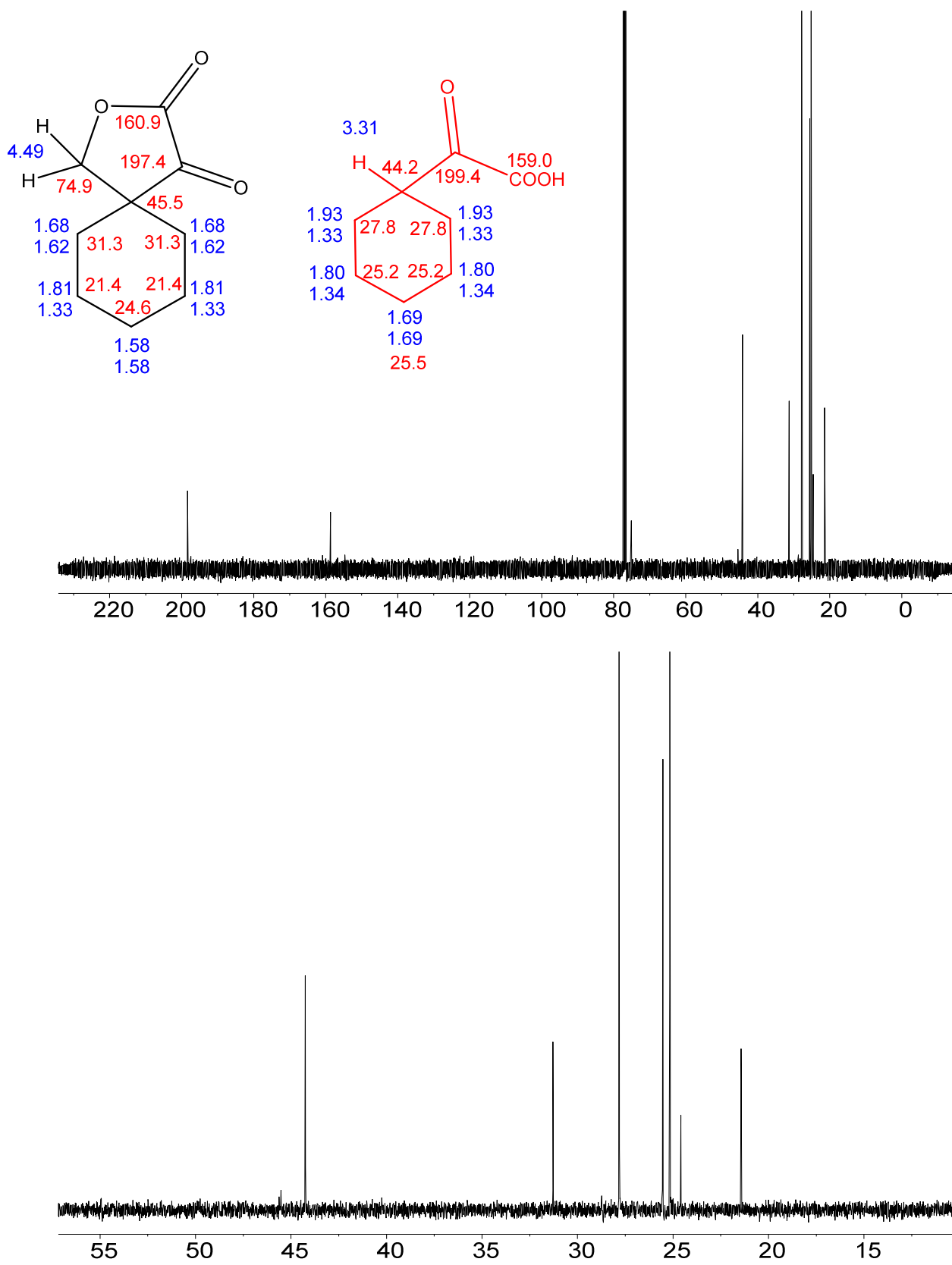
- Carboxylic Acid Group:**
 - COOH: 159.0 ppm
 - Carbonyl (C=O): 199.4 ppm
- Cyclohexane Ring:**
 - C1 (CH): 44.2 ppm
 - C2 (CD₂): 27.8 ppm
 - C3 (CD₂): 27.8 ppm
 - C4 (CD₂): 25.2 ppm
 - C5 (CD₂): 25.2 ppm
 - C6 (CD₂): 25.5 ppm

^{13}C NMR Spectrum (ppm):

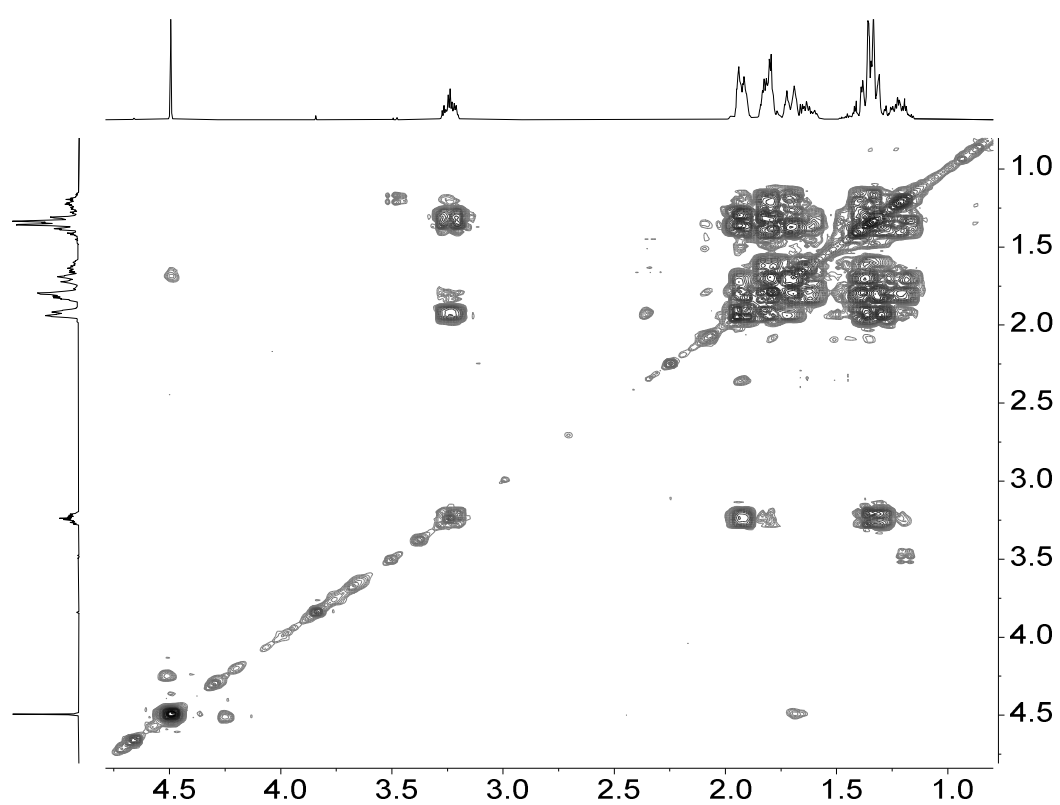
- 159.0 (COOH)
- 199.4 (C=O)
- 44.2 (CH)
- 27.8 (CD₂)
- 27.8 (CD₂)
- 25.2 (CD₂)
- 25.2 (CD₂)
- 25.5 (CD₂)



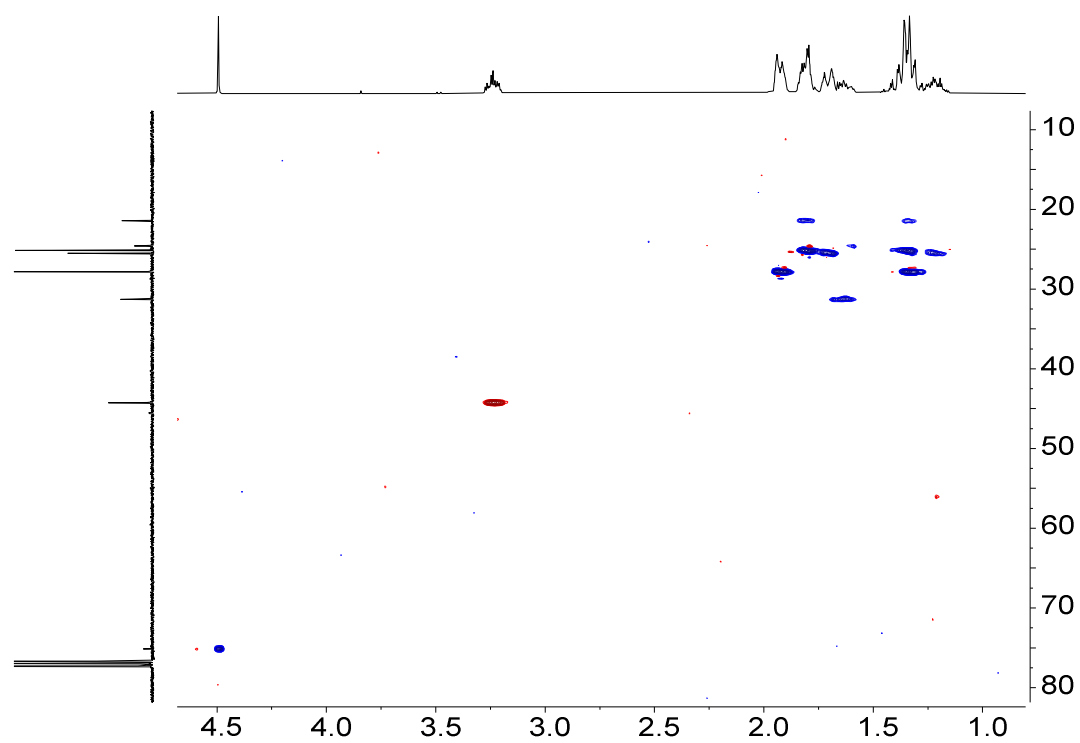
b)



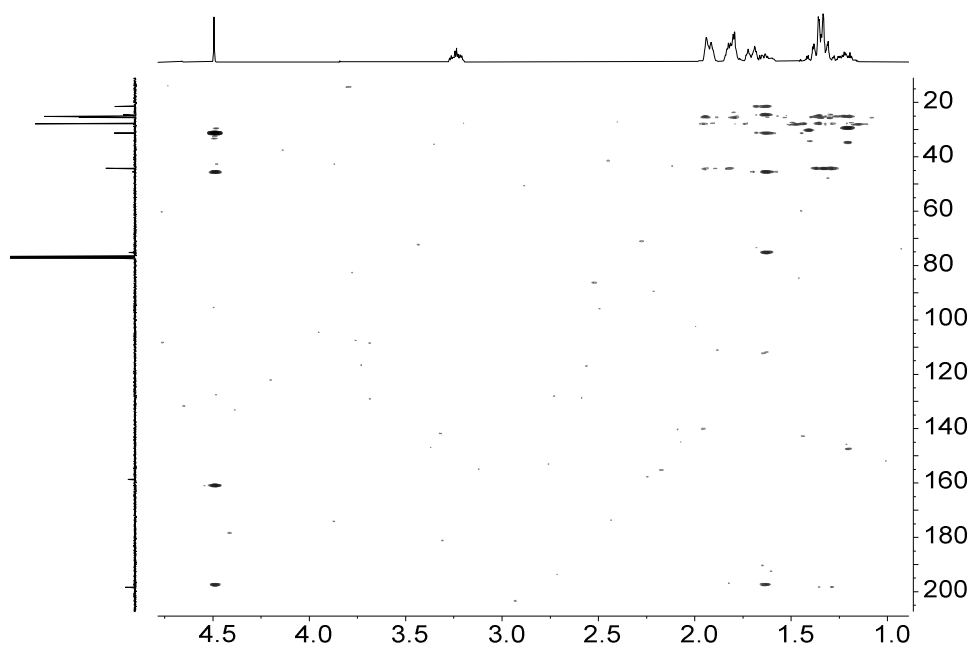
c)



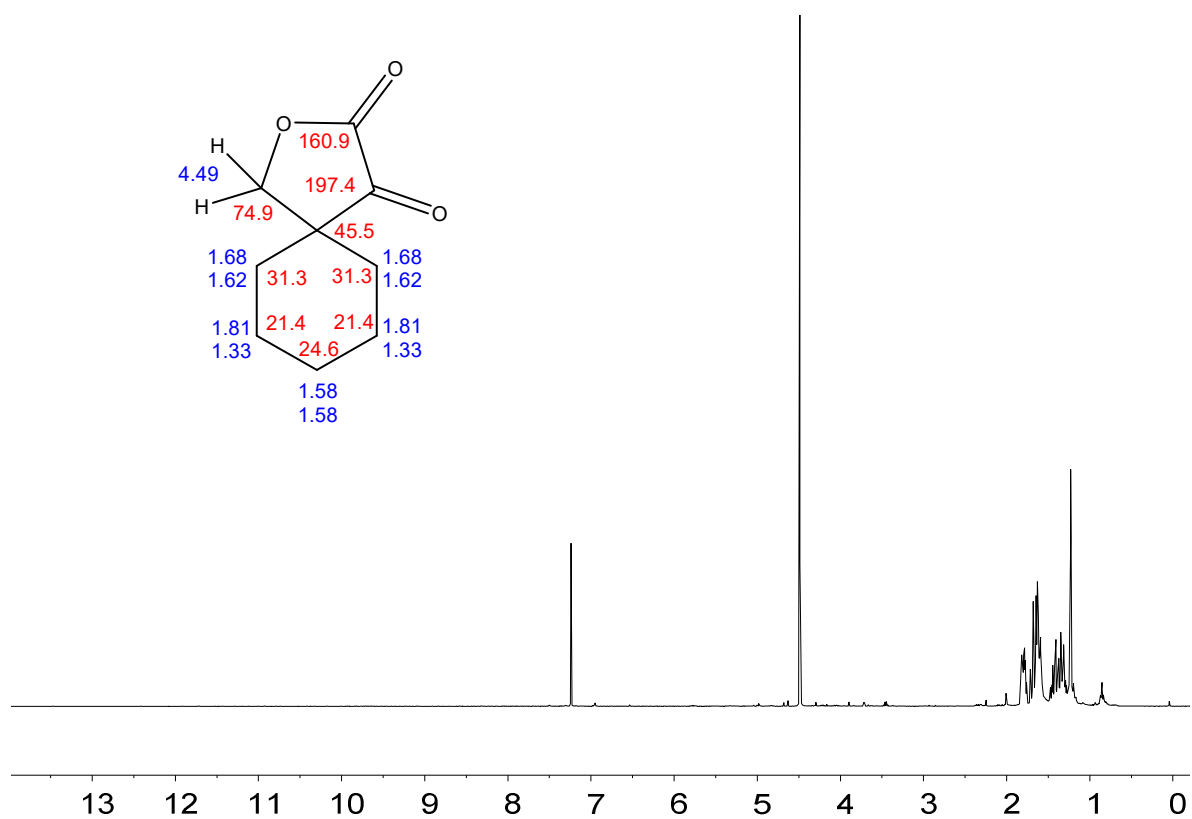
d)



e)



f)



g)

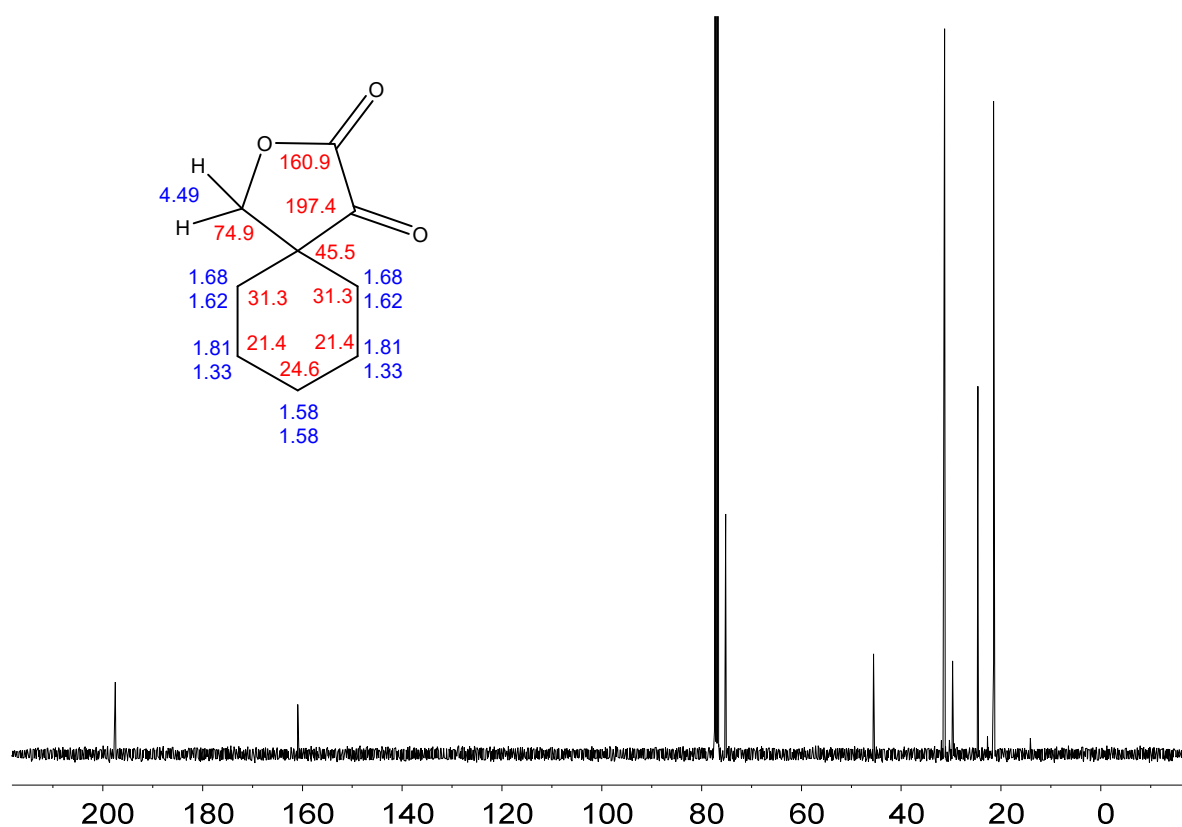
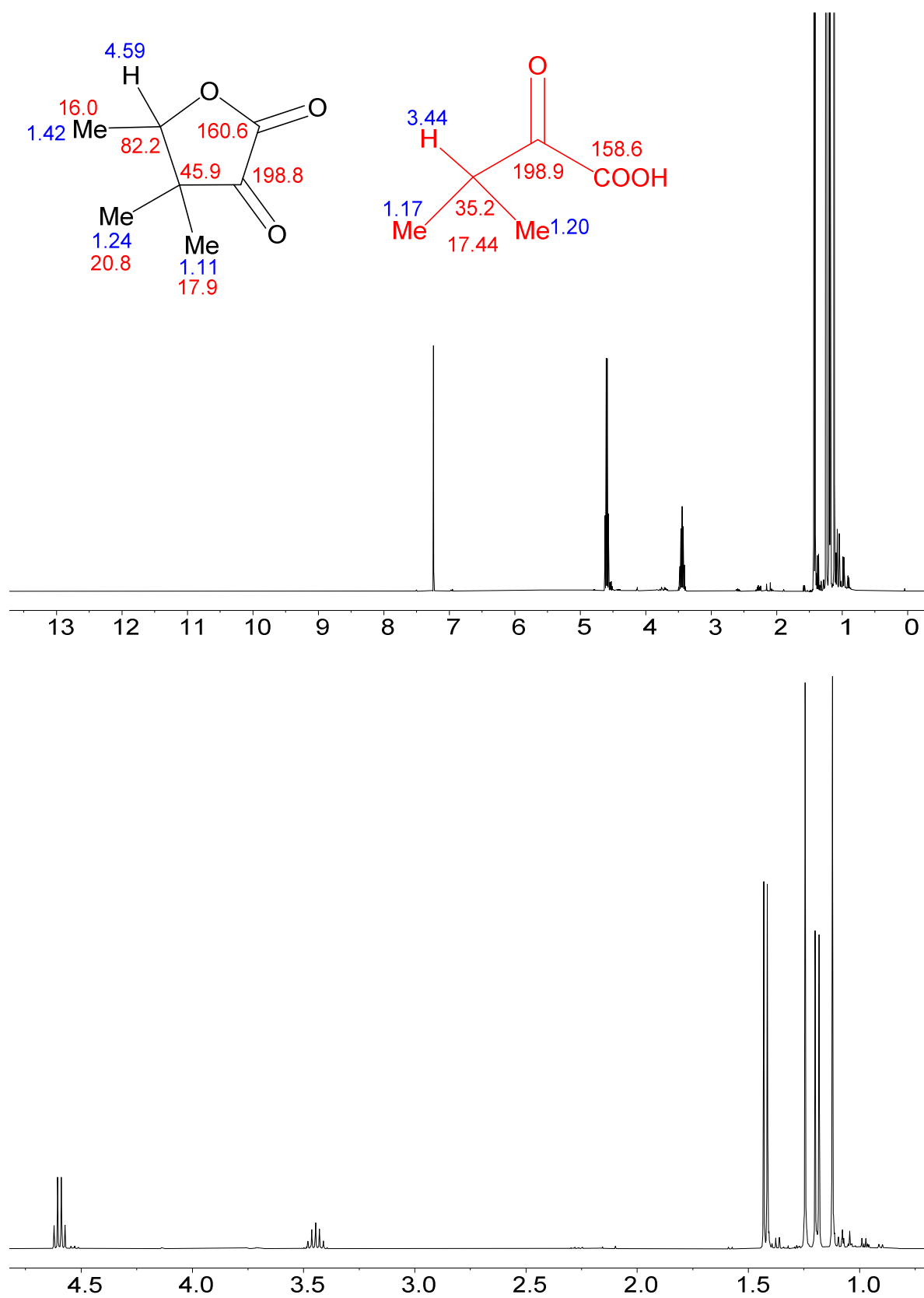
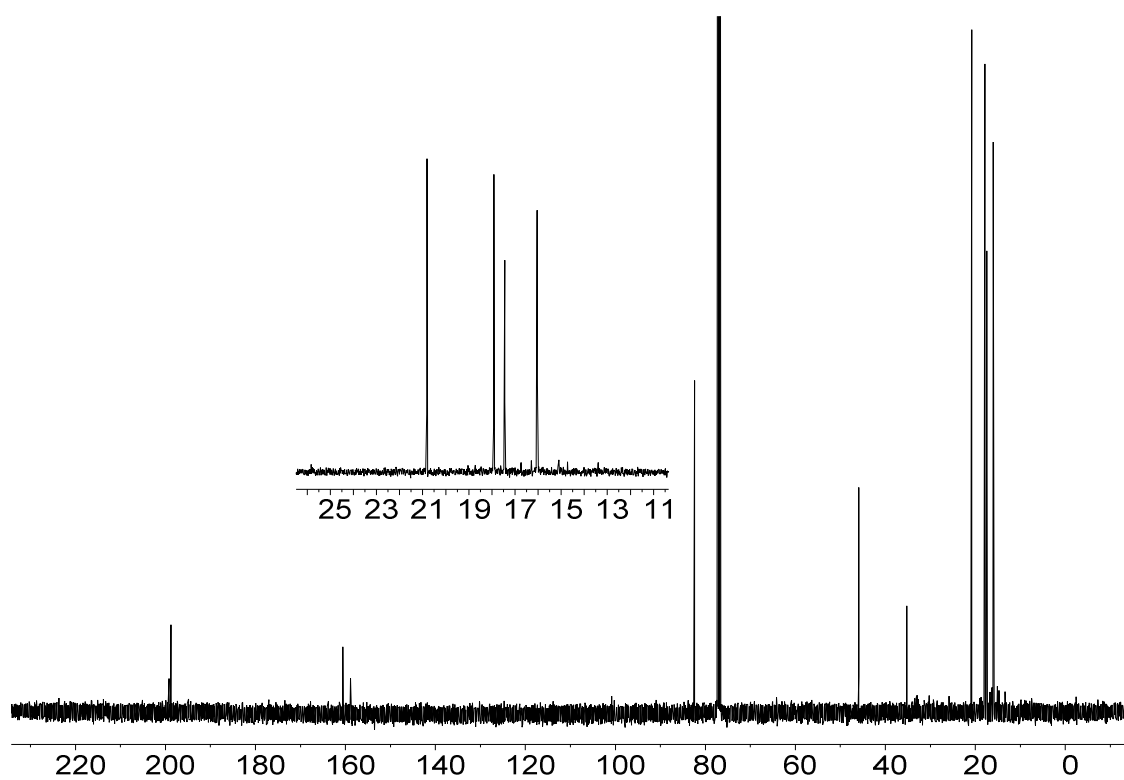


Figure S210 NMR spectra (CDCl_3) of unpurified **8c**: a) ^1H , b) ^{13}C , c) gCOSY d) gHSQC, e) HMBC and pure **8c** f) ^1H , g) ^{13}C . E178-137-exp92.

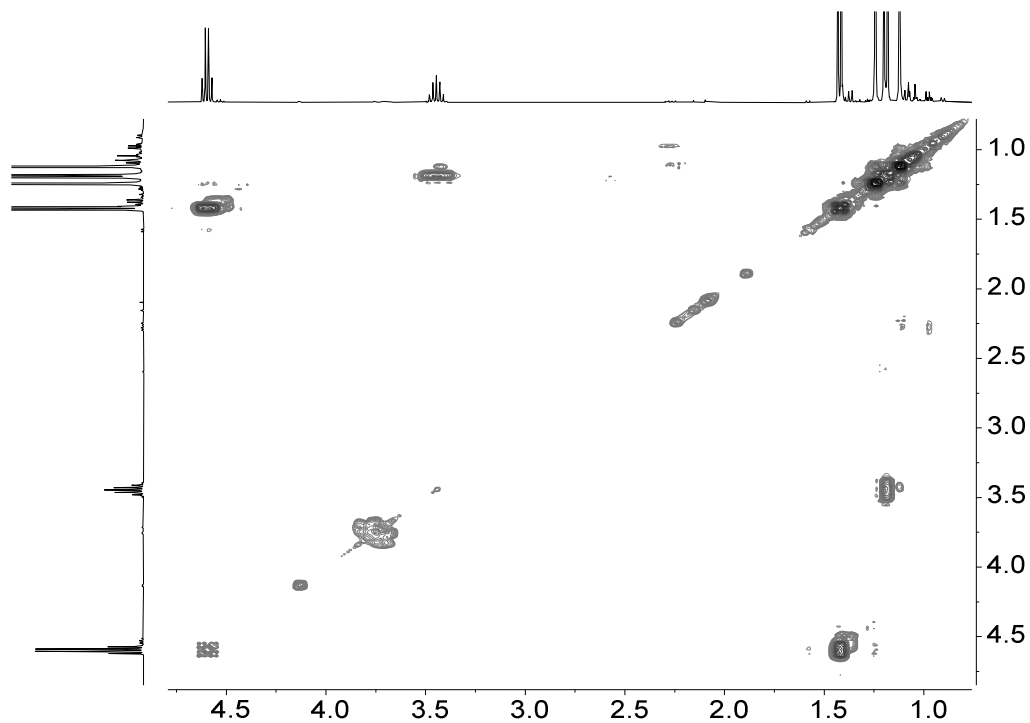
a)



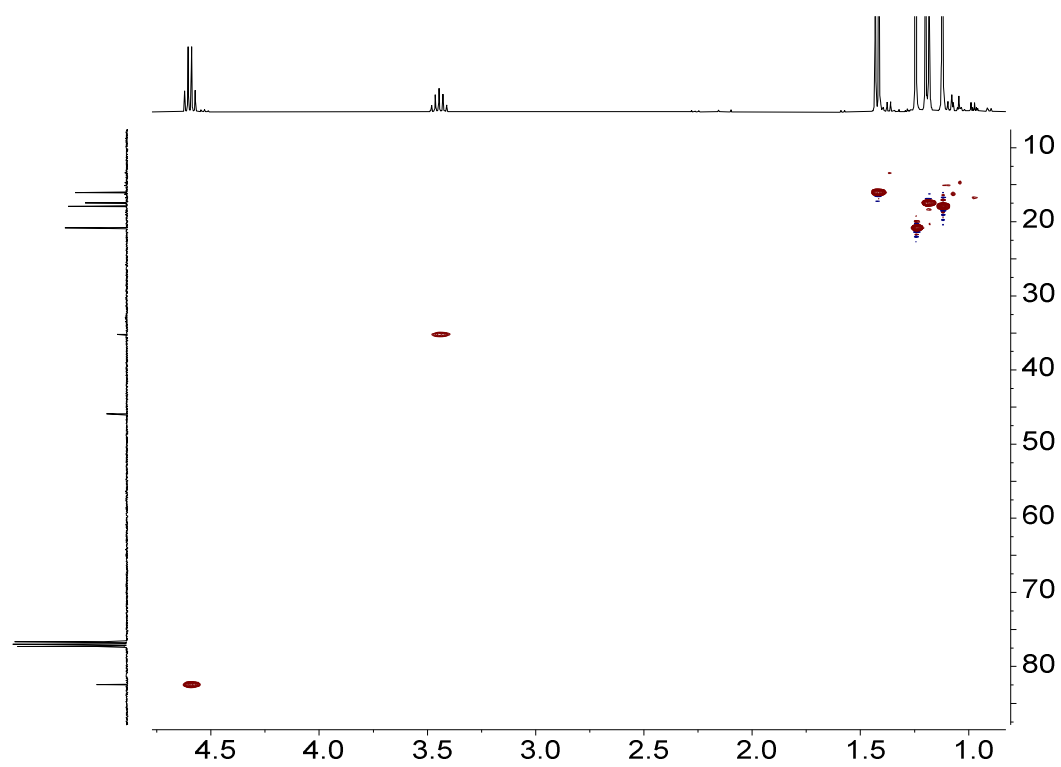
b)



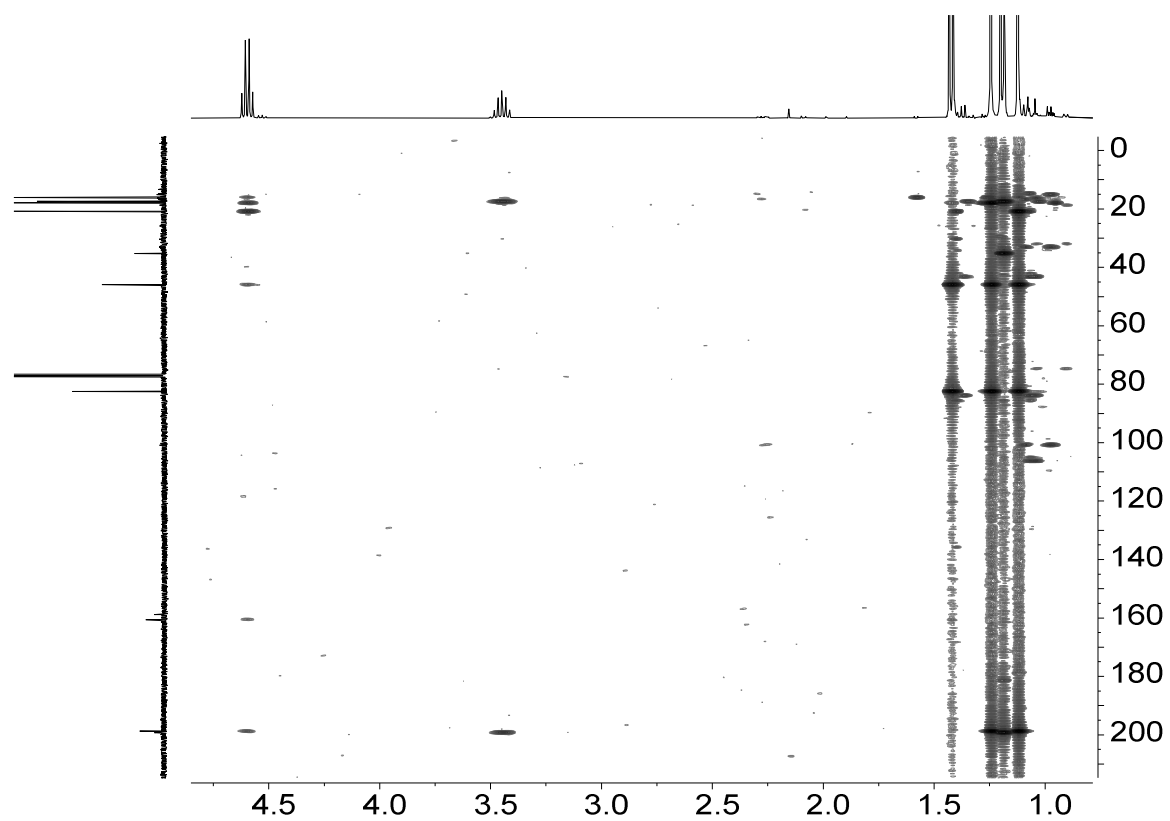
c)



d)



e)



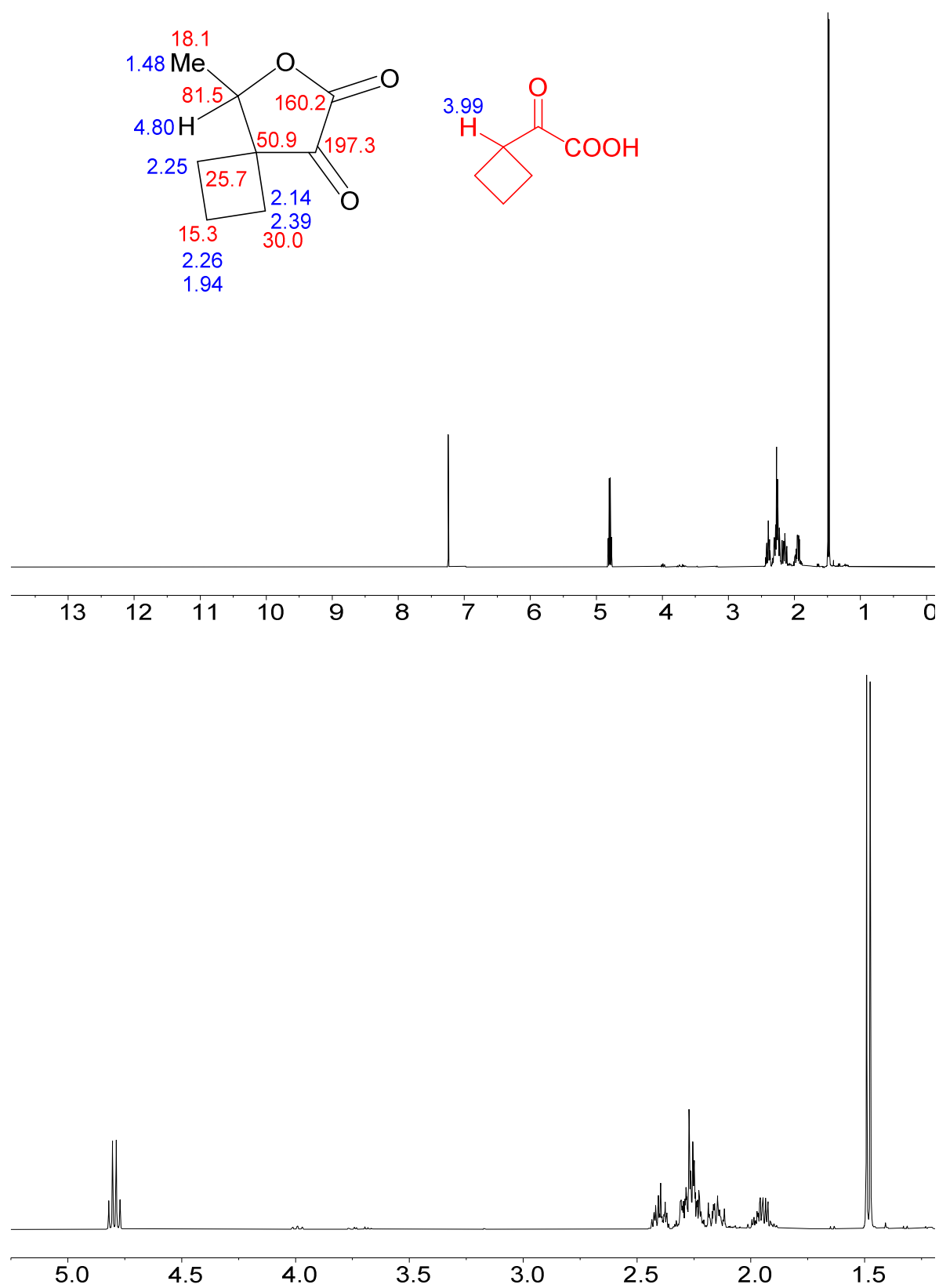
The figure displays the chemical structure of 2,2,4-trimethyl-1,3-dioxolane-5-carboxylic acid and its corresponding ¹³C NMR spectrum. The chemical structure is a five-membered ring containing an oxygen atom, with three methyl groups attached to the ring and a carboxylic acid group. The ¹³C NMR spectrum shows peaks at 16.0, 16.6, 198.8, 45.9, 82.2, 17.9, 20.8, 1.42, 1.24, 1.11, and 4.59 ppm. The peaks are assigned to the following carbons in the structure: 16.0 ppm (CH₃), 16.6 ppm (CH₃), 198.8 ppm (C=O), 45.9 ppm (CH), 82.2 ppm (CH₂), 17.9 ppm (CH₃), 20.8 ppm (CH₃), 1.42 ppm (CH₃), 1.24 ppm (CH₃), 1.11 ppm (CH₃), and 4.59 ppm (CH₂).

Mass spectrum of compound 10. The x-axis represents the mass-to-charge ratio (m/z) from 0 to 220, and the y-axis represents relative intensity from 0 to 100. The base peak is at m/z 81. Other significant peaks are observed at m/z 201, 161, 77, 49, 23, and 19.

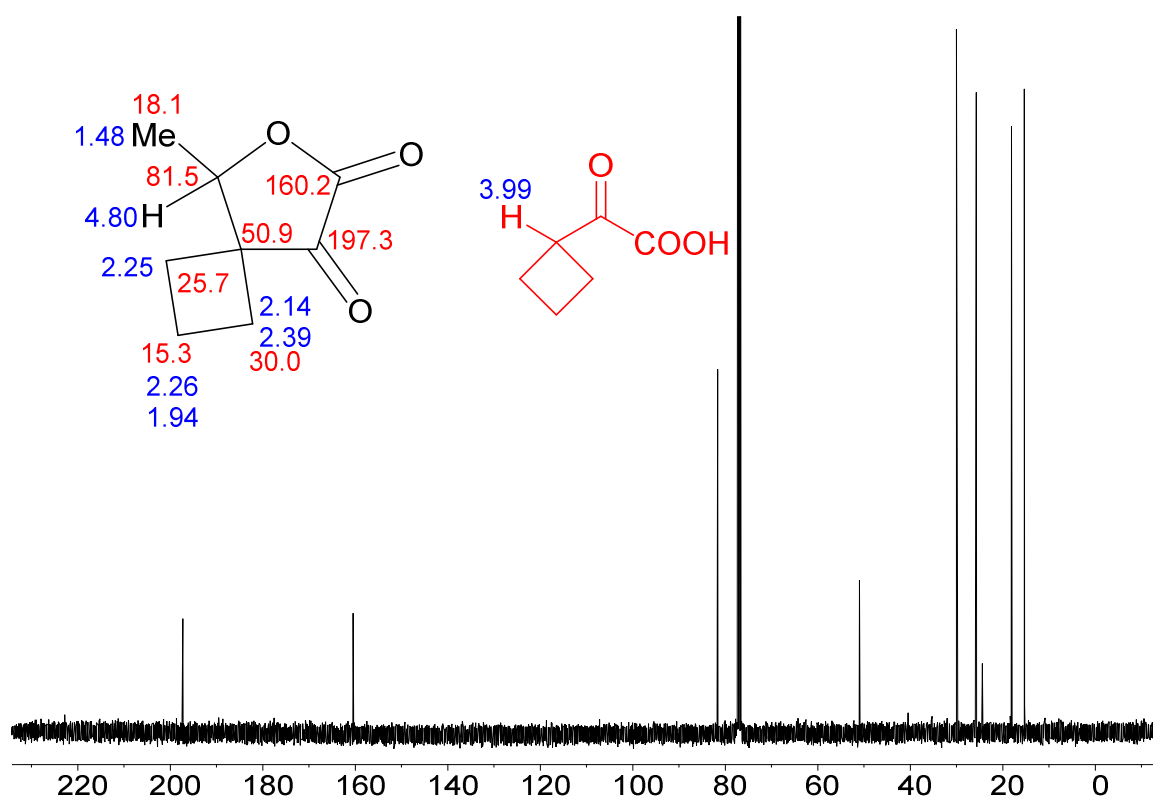
m/z	Relative Intensity (%)
201	~15
161	~10
81	100
77	~10
49	~35
23	~95
19	~85

210

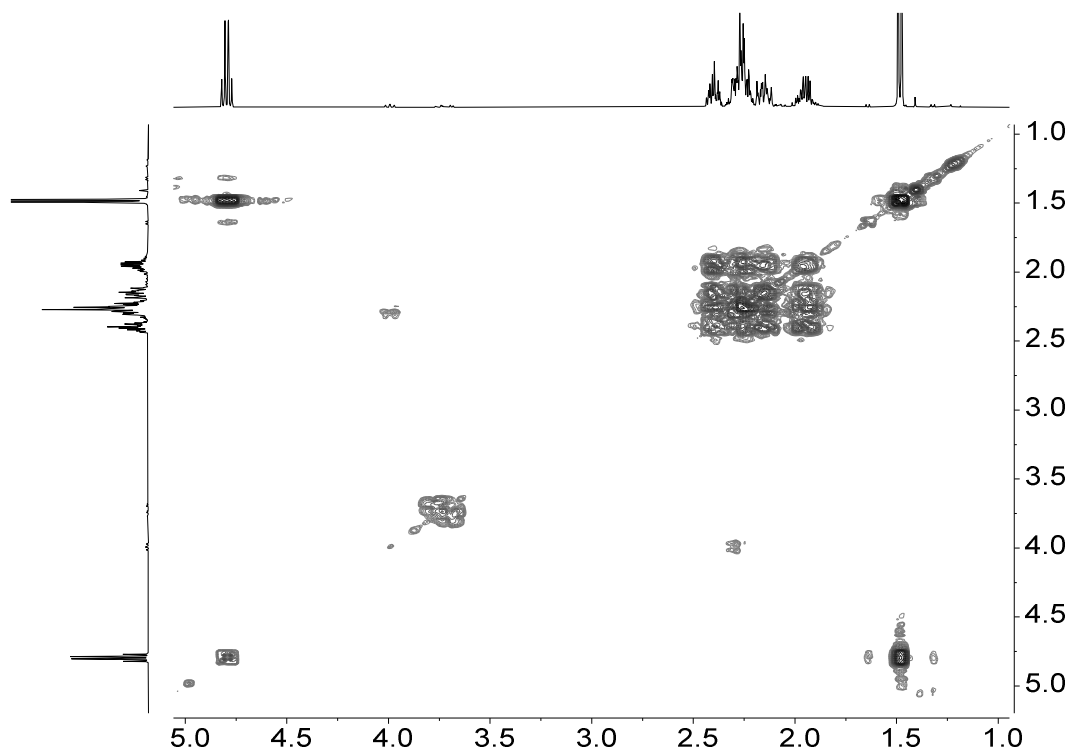
a)



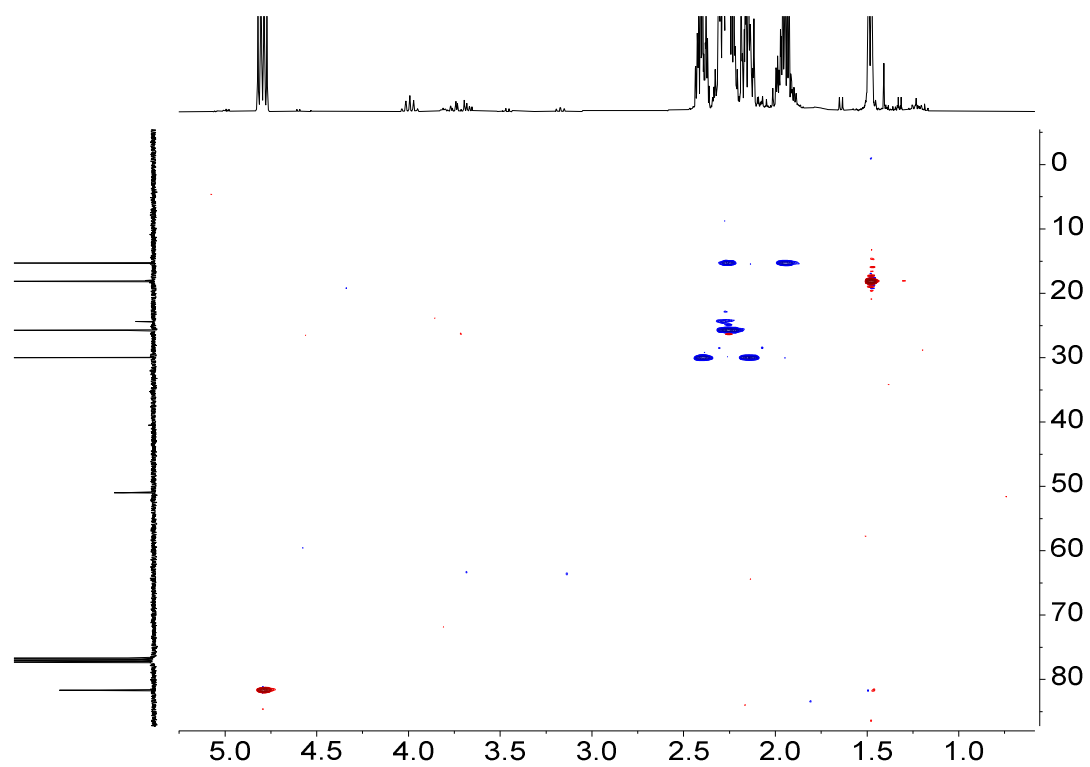
b)



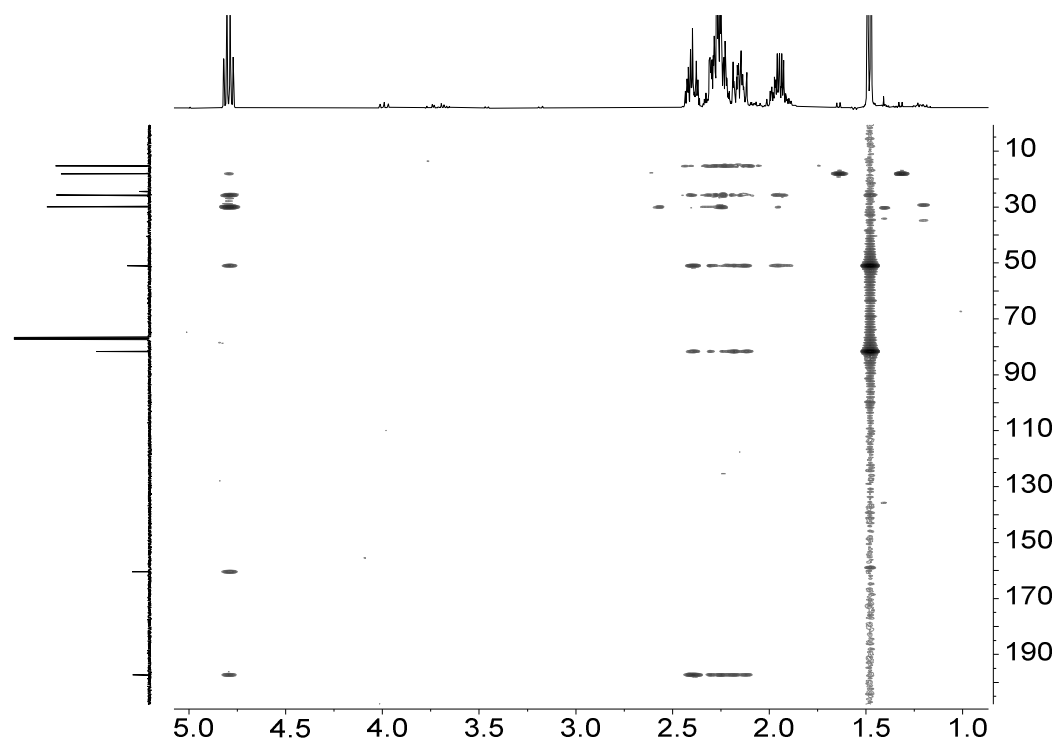
c)



d)



e)



f)

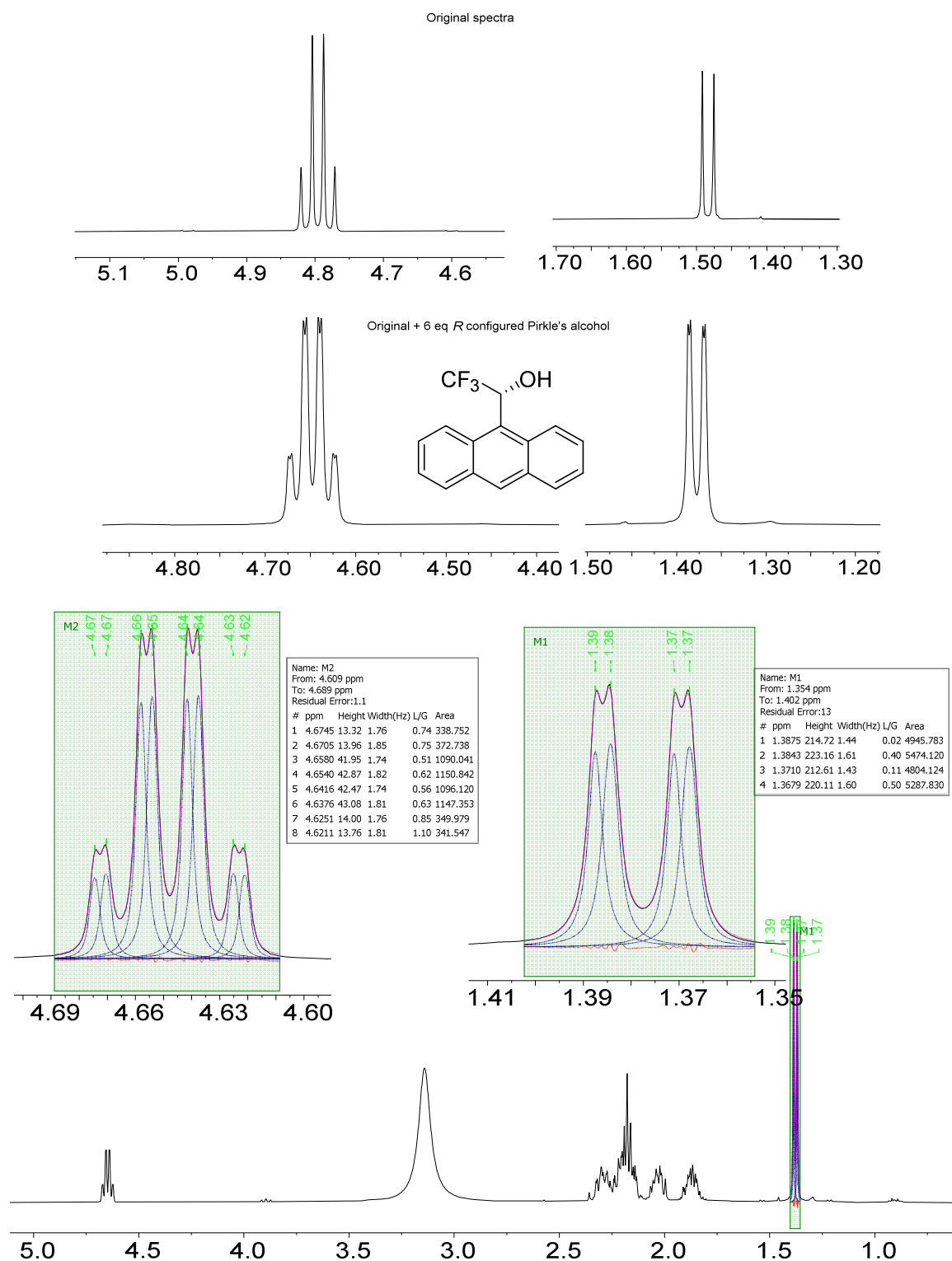
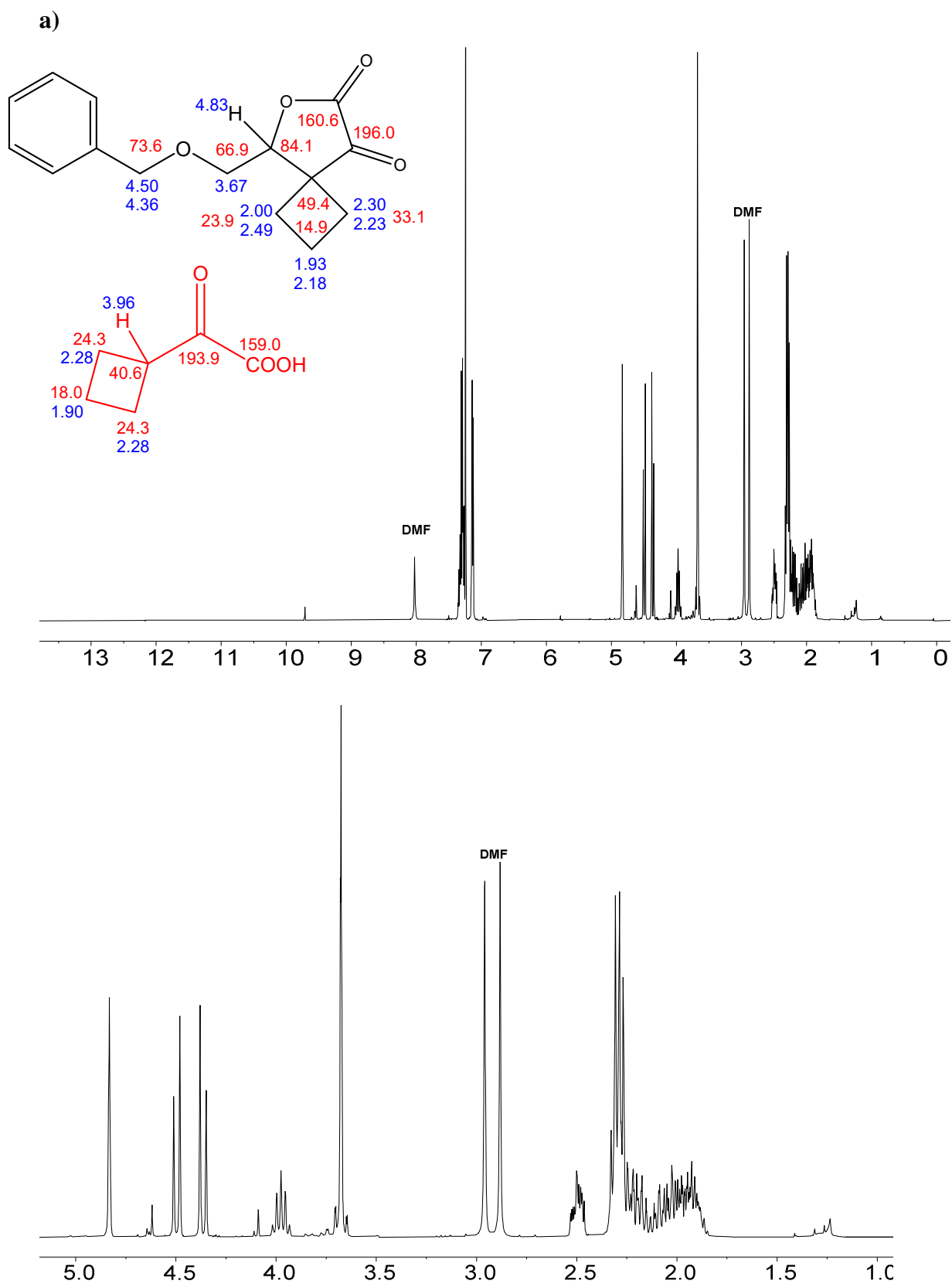
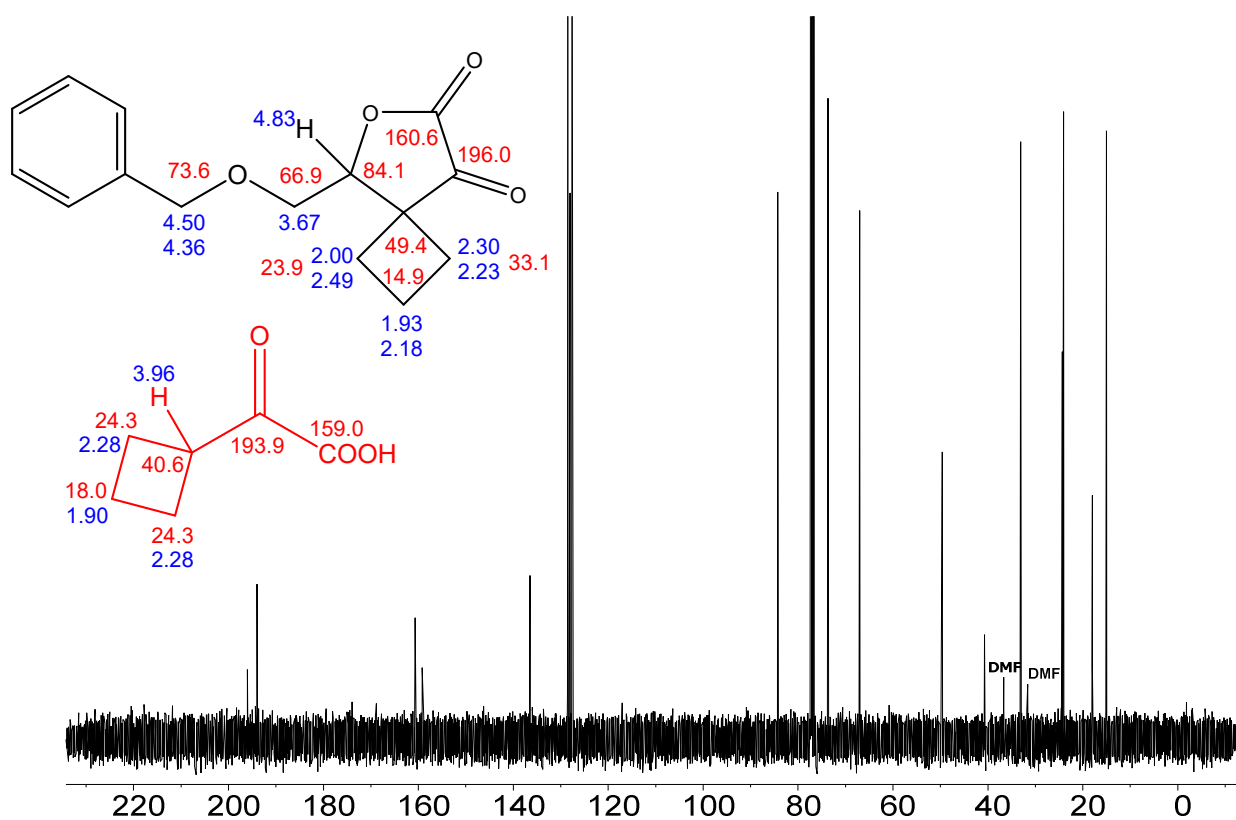


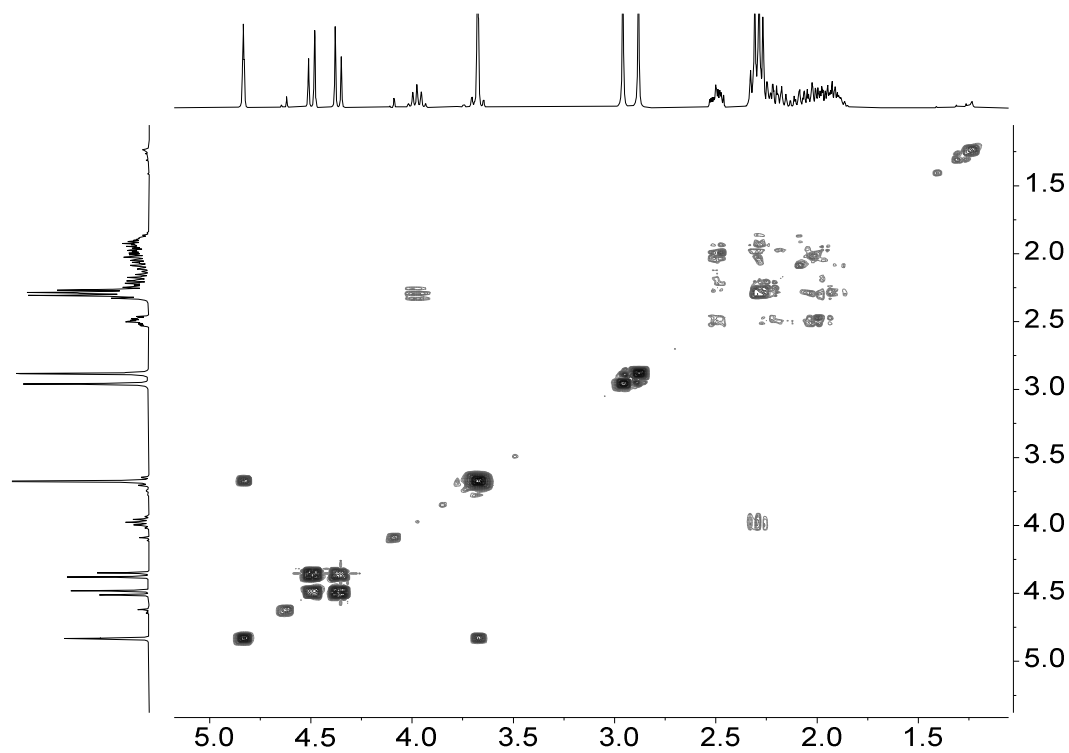
Figure S212 NMR spectra (CDCl₃) of pure **8e**: a) ¹H, b) ¹³C, c) gCOSY d) gHSQC, e) HMBC and f) Original spectra and after adding 6 eq of *R* configured Pirkle's alcohol E178-115-exp79.



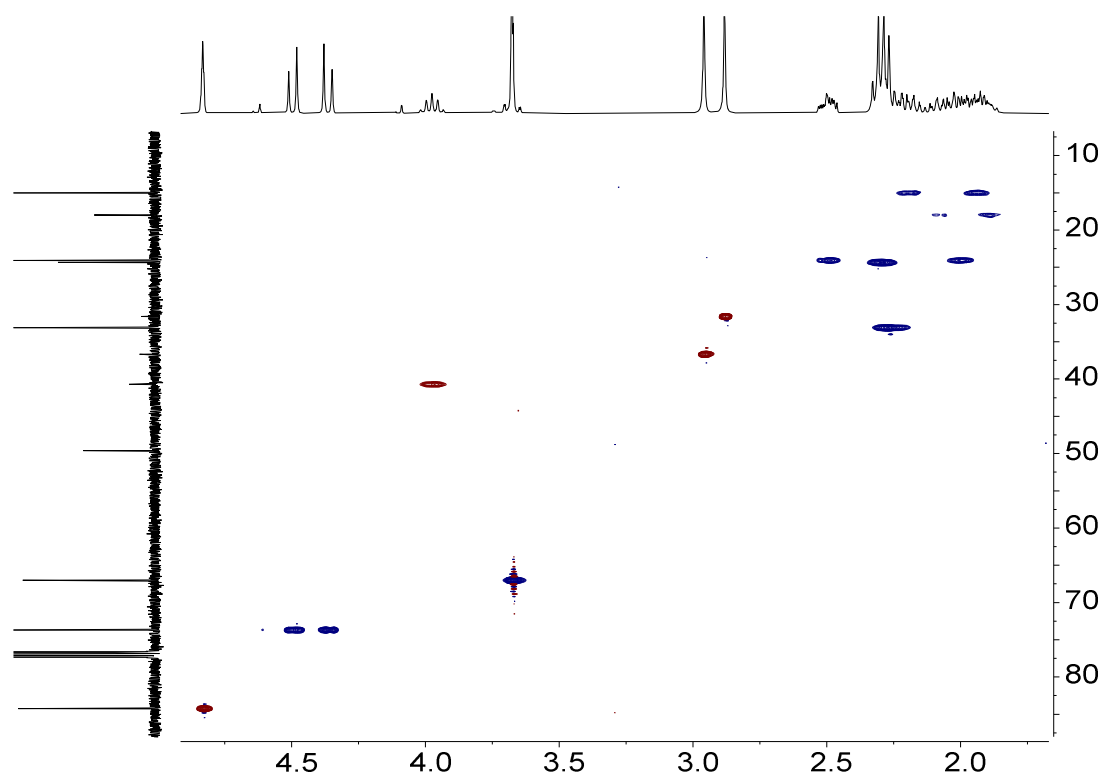
b)



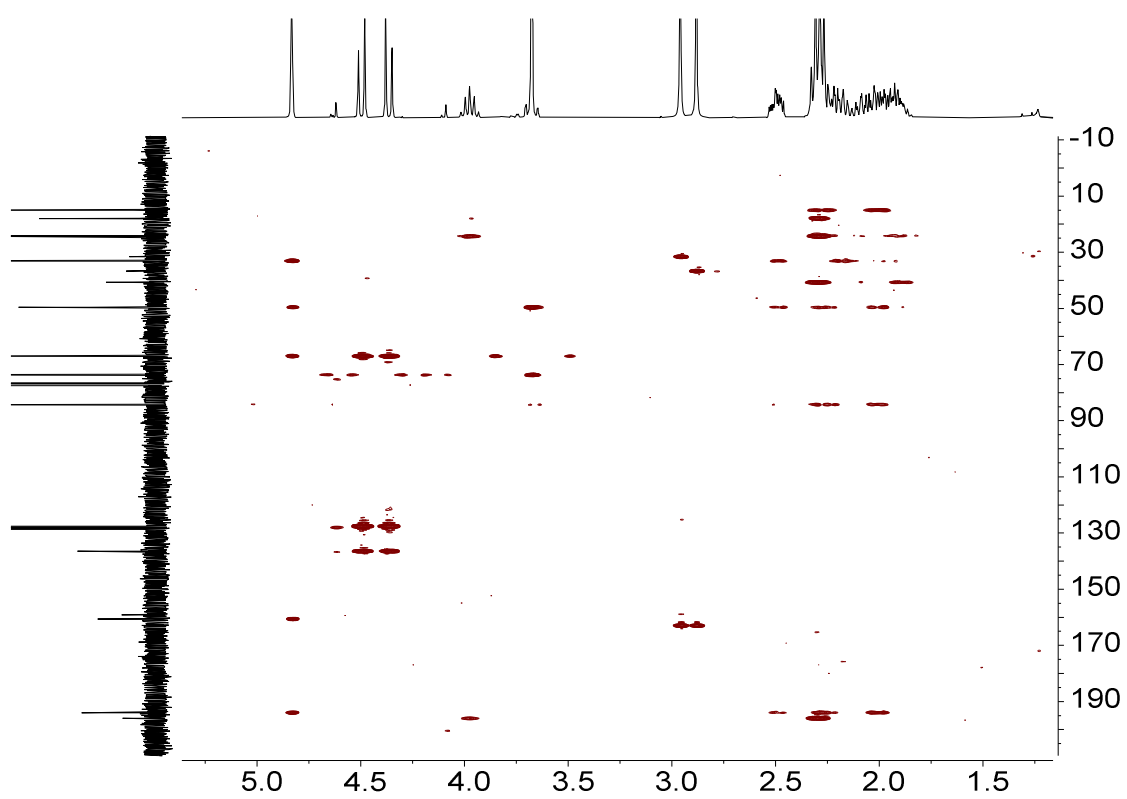
c)



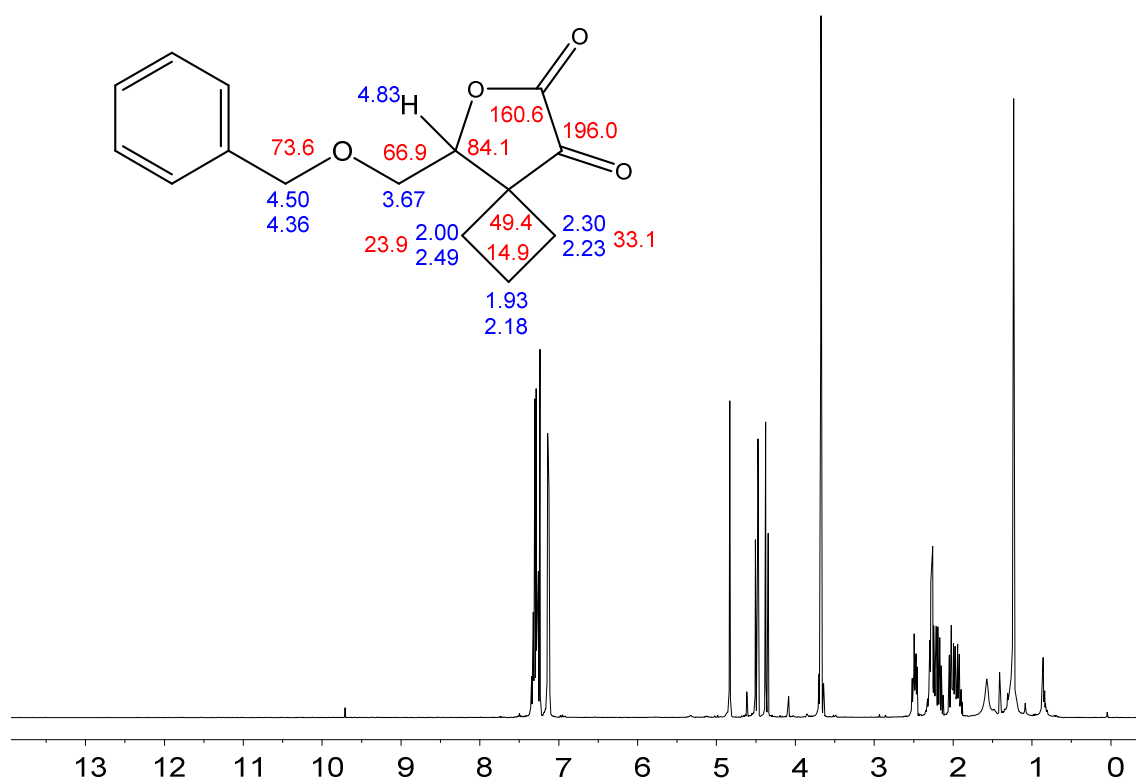
d)



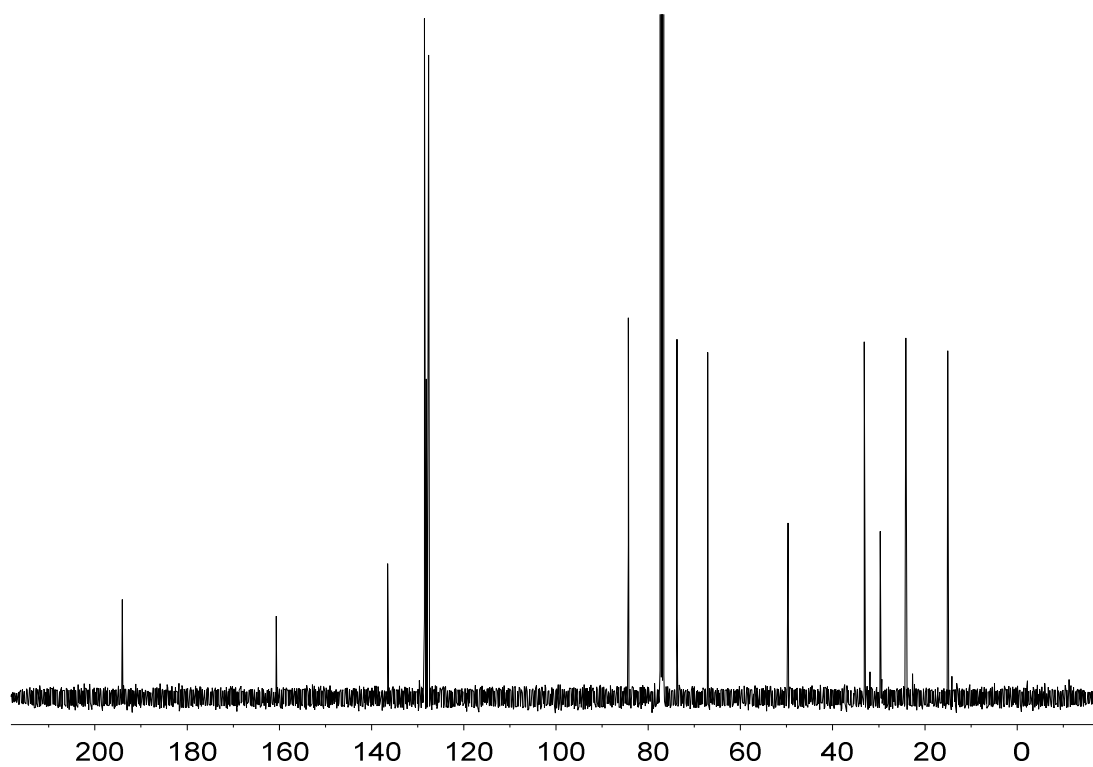
e)



f)



g)



h)

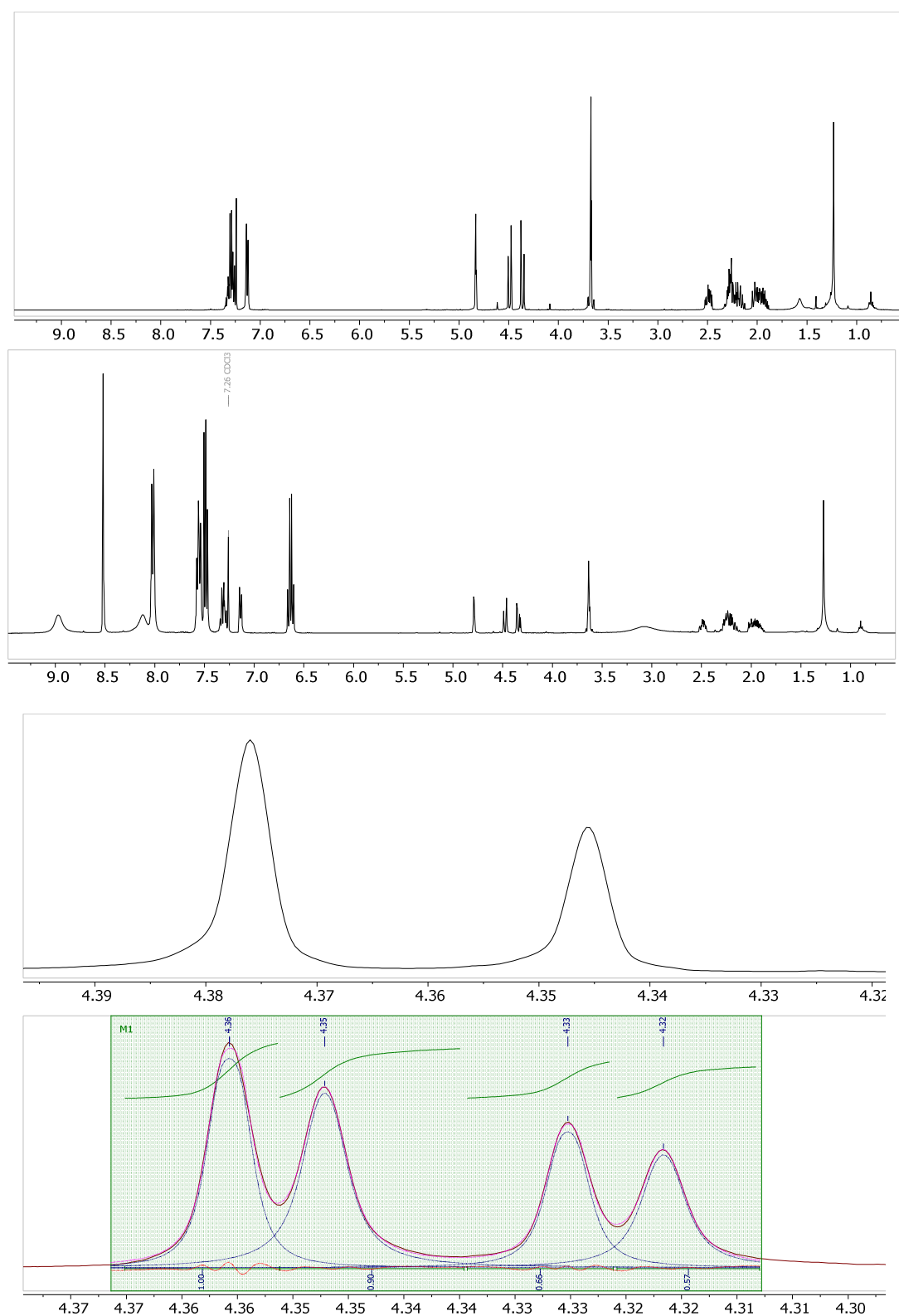
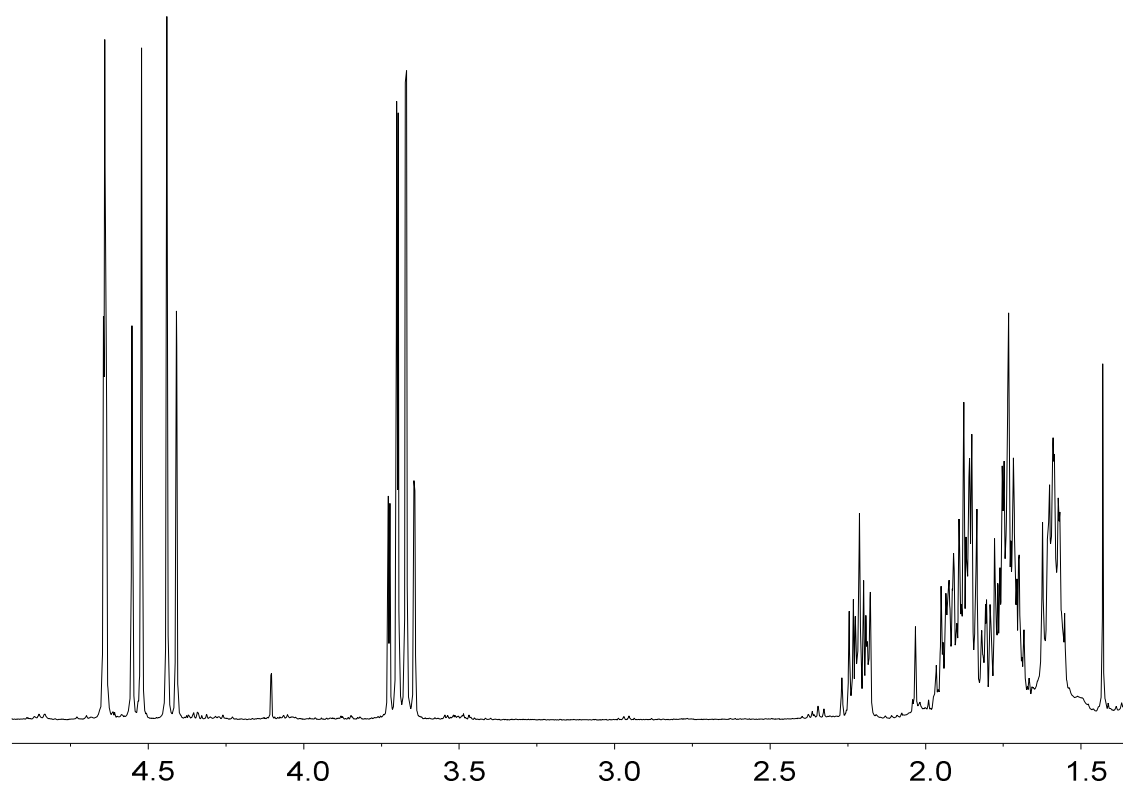
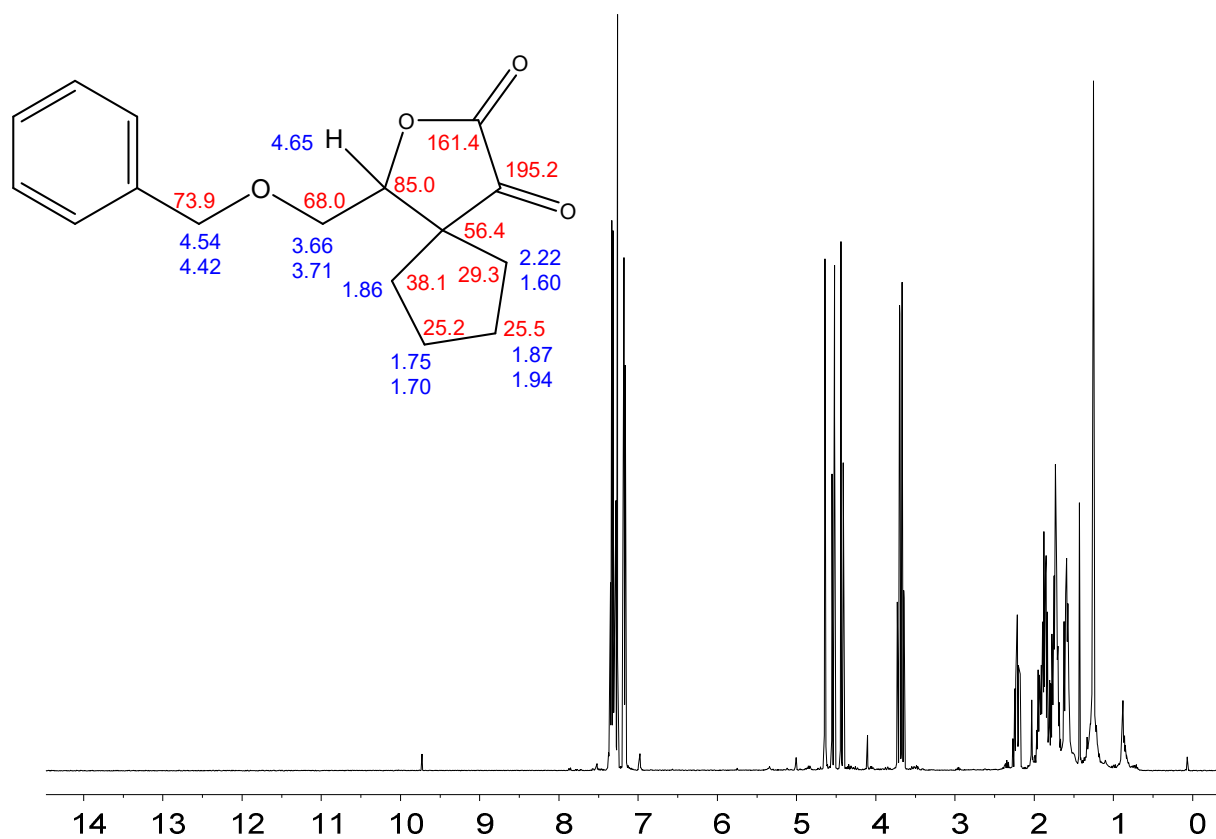
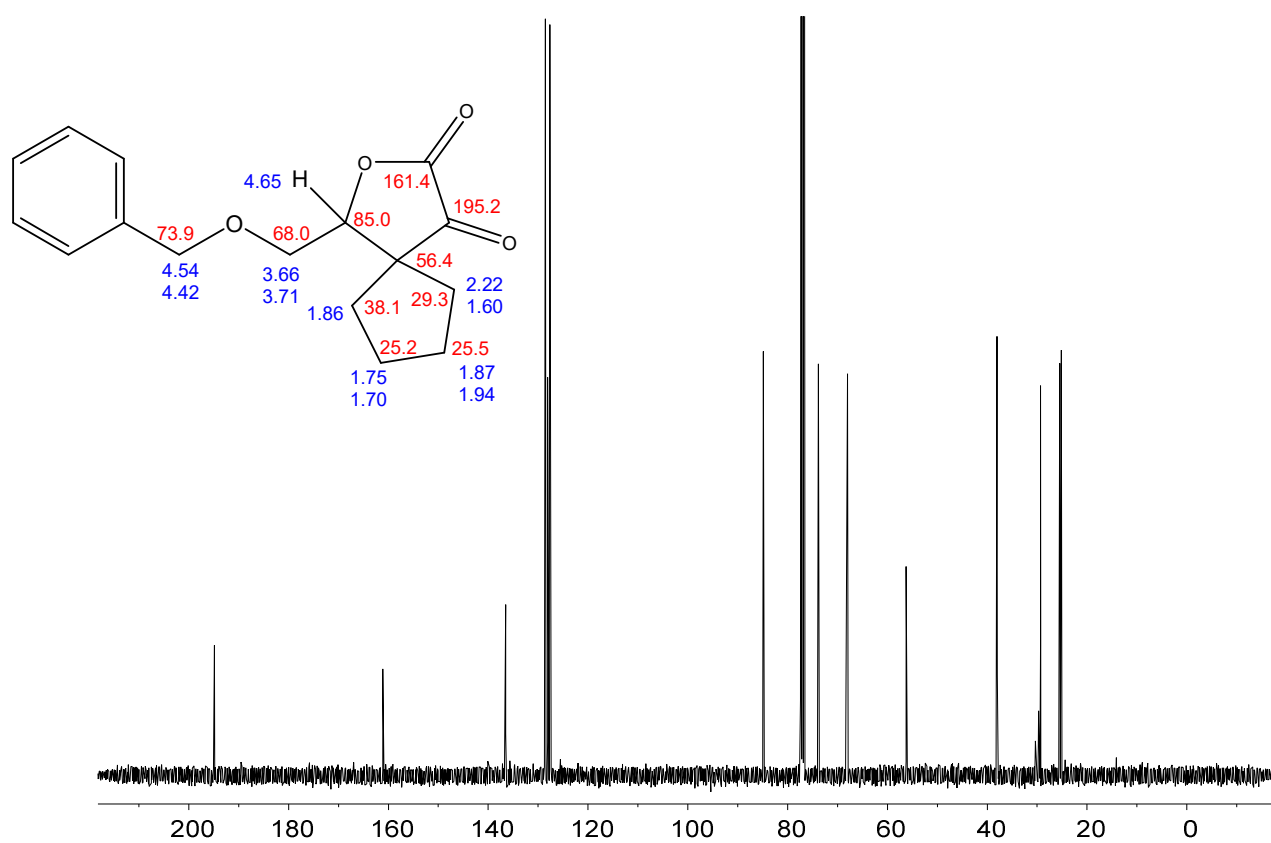


Figure S213 NMR spectra (CDCl_3) of unpurified **8f**: a) ^1H , b) ^{13}C , c) gCOSY d) gHSQC, e) HMBC and pure **8f** f) ^1H , g) ^{13}C and h) Original spectra and after adding 4 eq of *R* configured Pirkle's alcohol. E178-143-exp95.

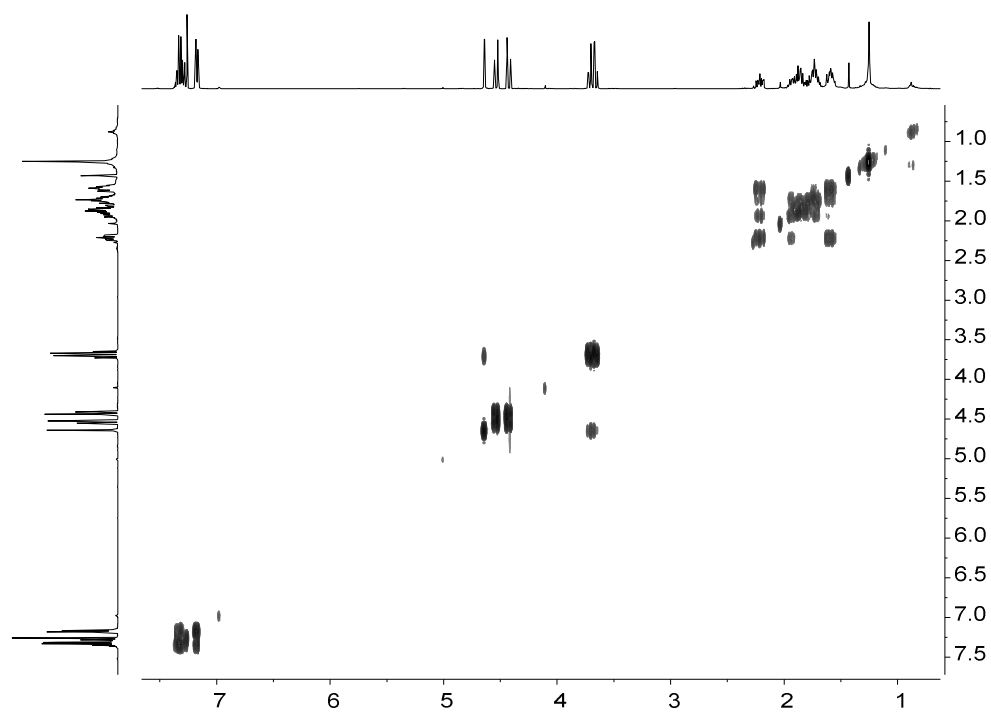
a)



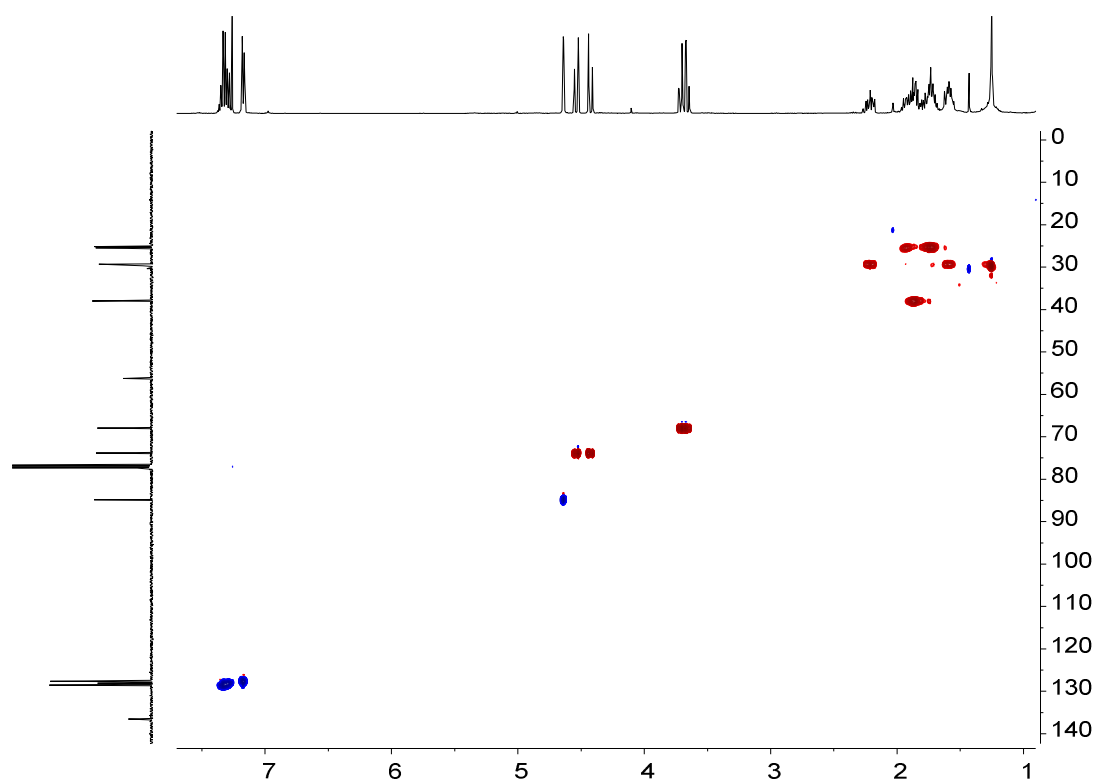
b)



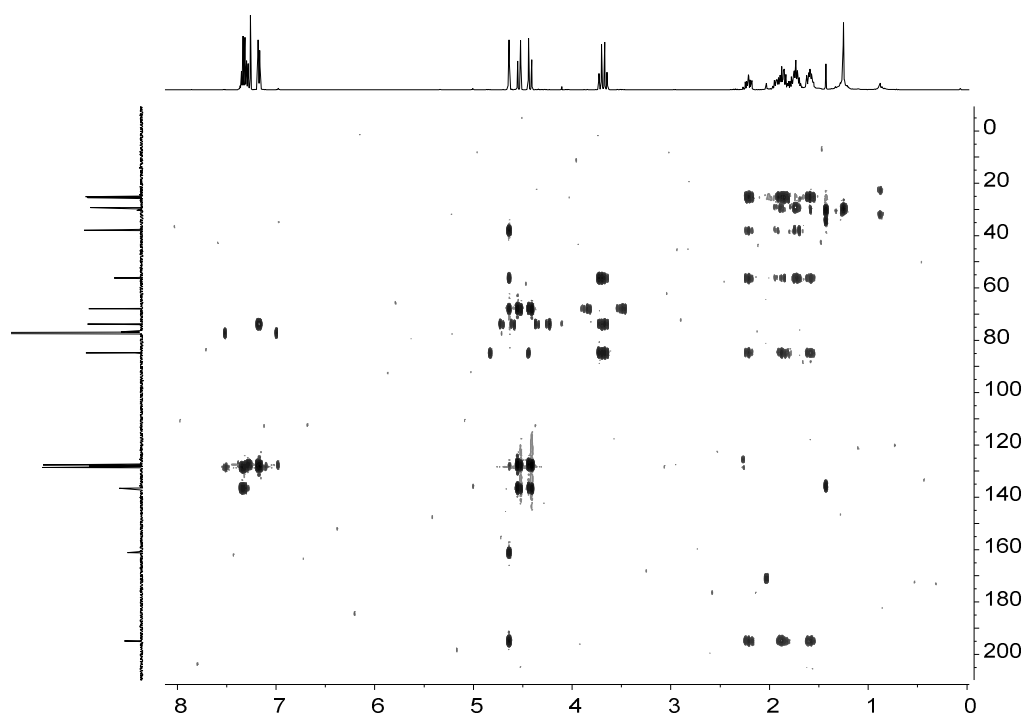
c)



d)



e)



f)

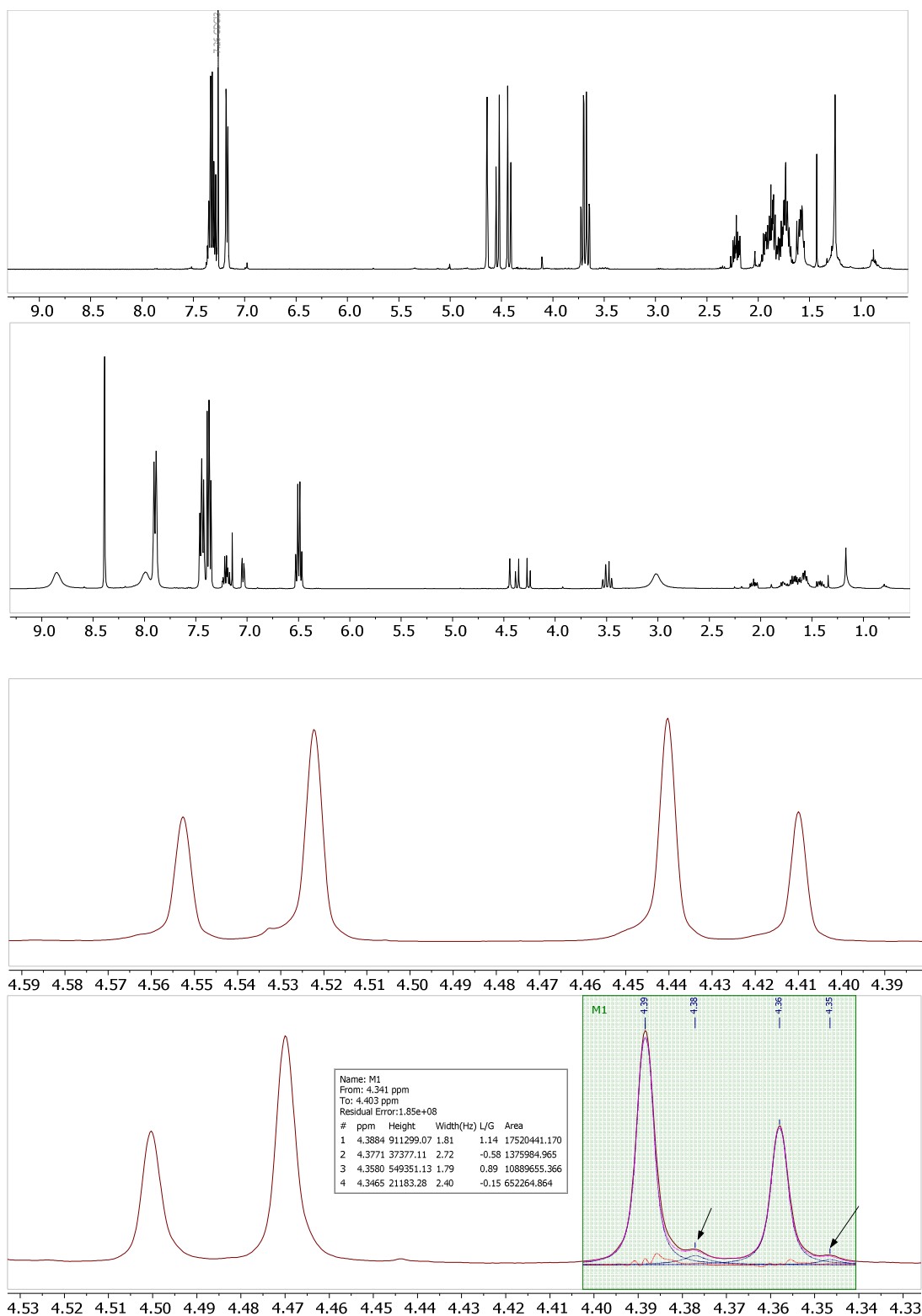
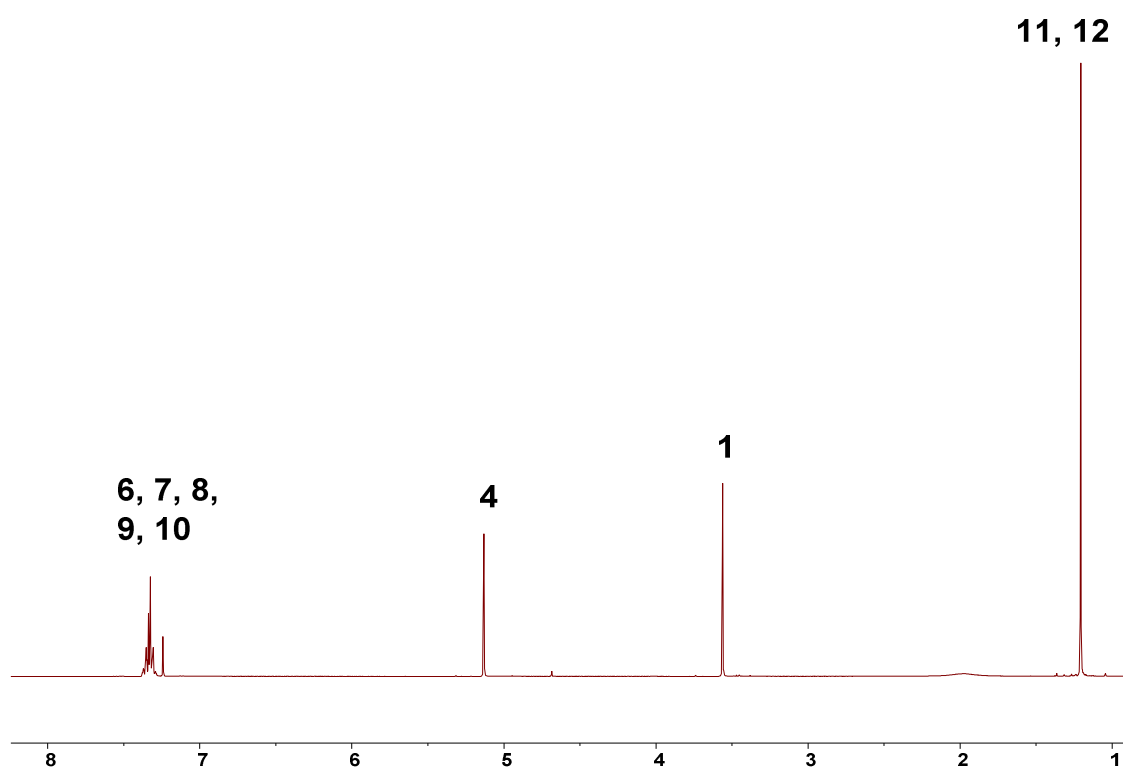
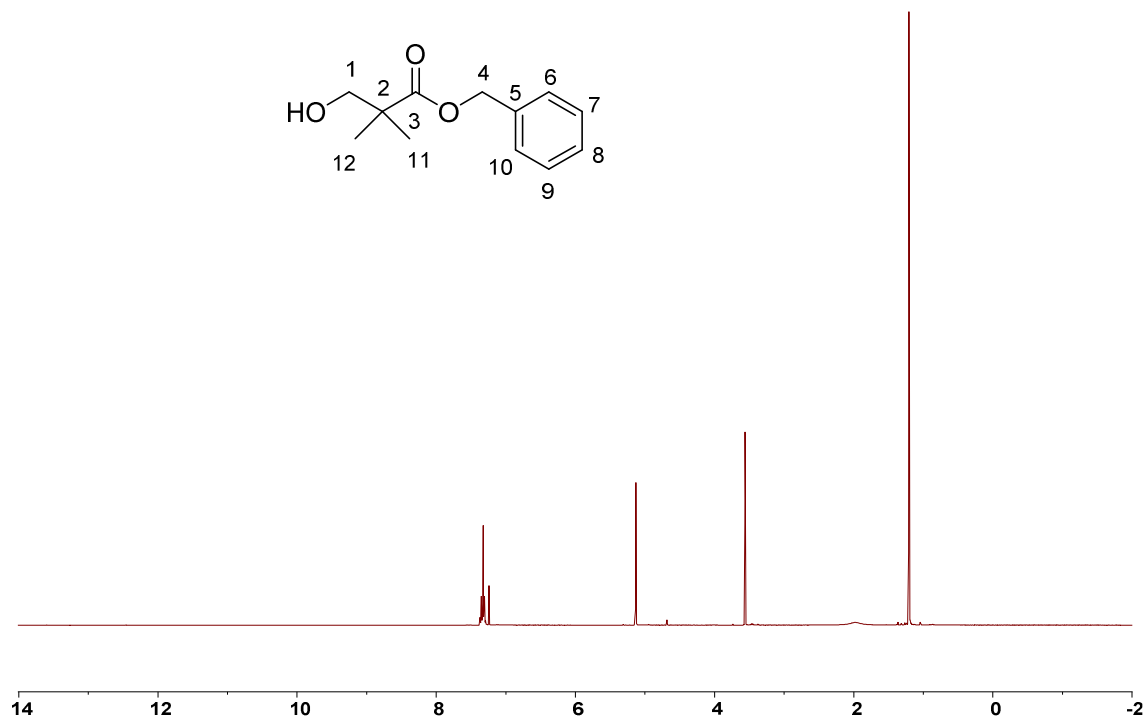
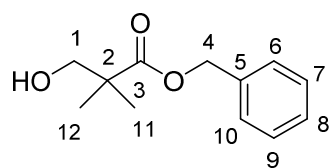


Figure S214 NMR spectra (CDCl₃) of **8g**: a) ¹H, b) ¹³C, c) gCOSY d) gHSQC, e) HMBC and f) Original spectra and after adding 6 eq of *R* configured Pirkle's alcohol. E178-153-exp101.

a)



b)

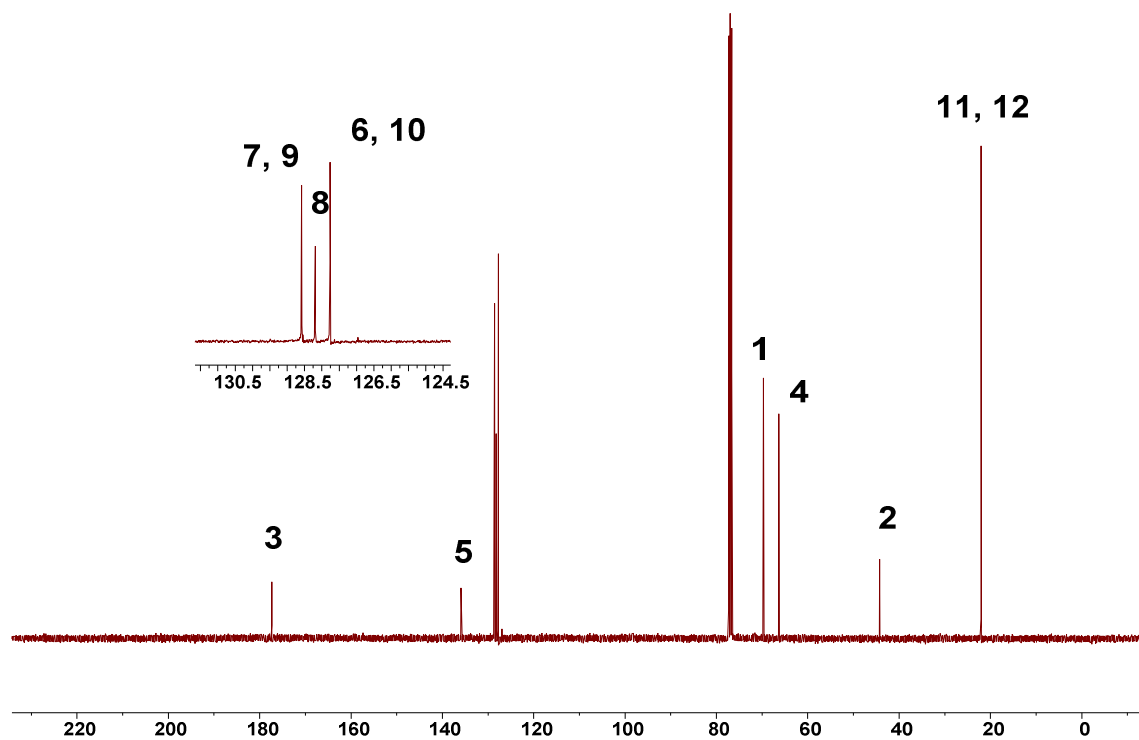
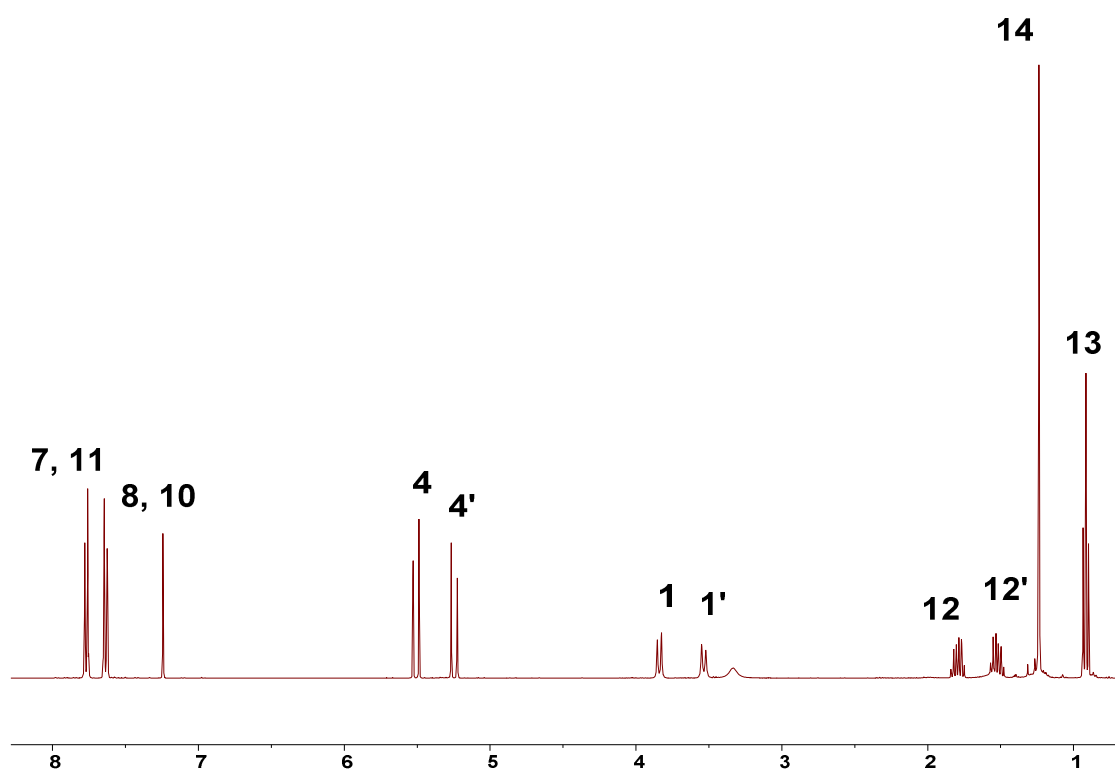
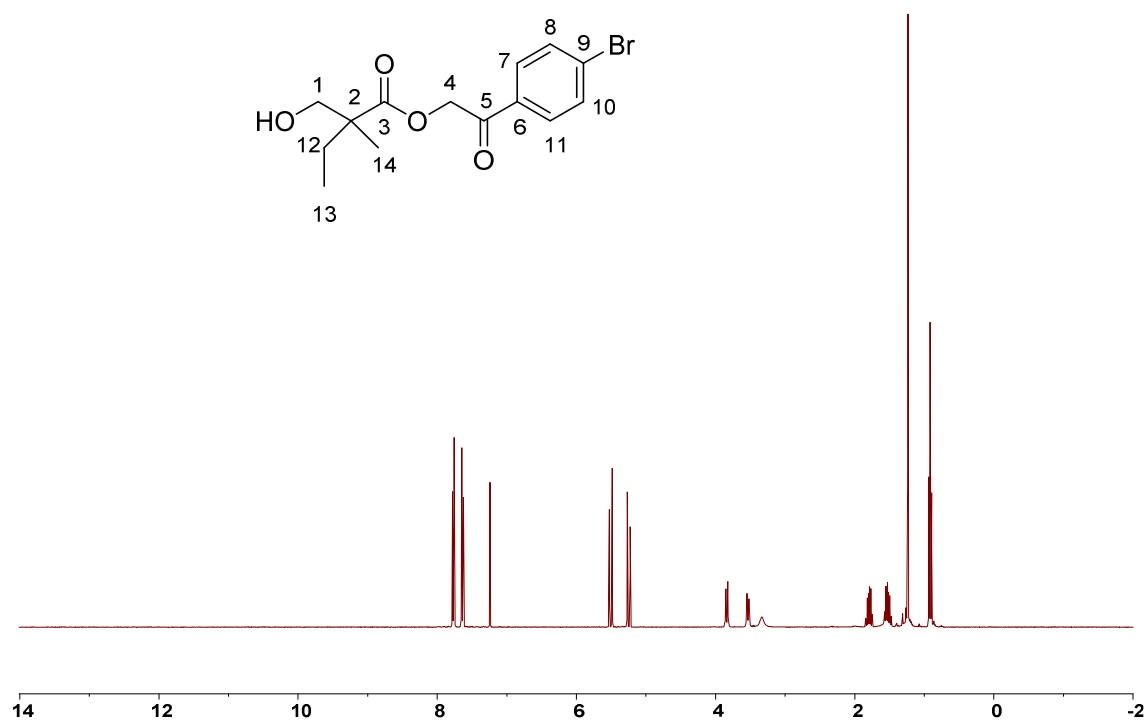


Figure S215 NMR spectra (CDCl_3) of **7a**: a) ^1H and b) ^{13}C .

a)



b)

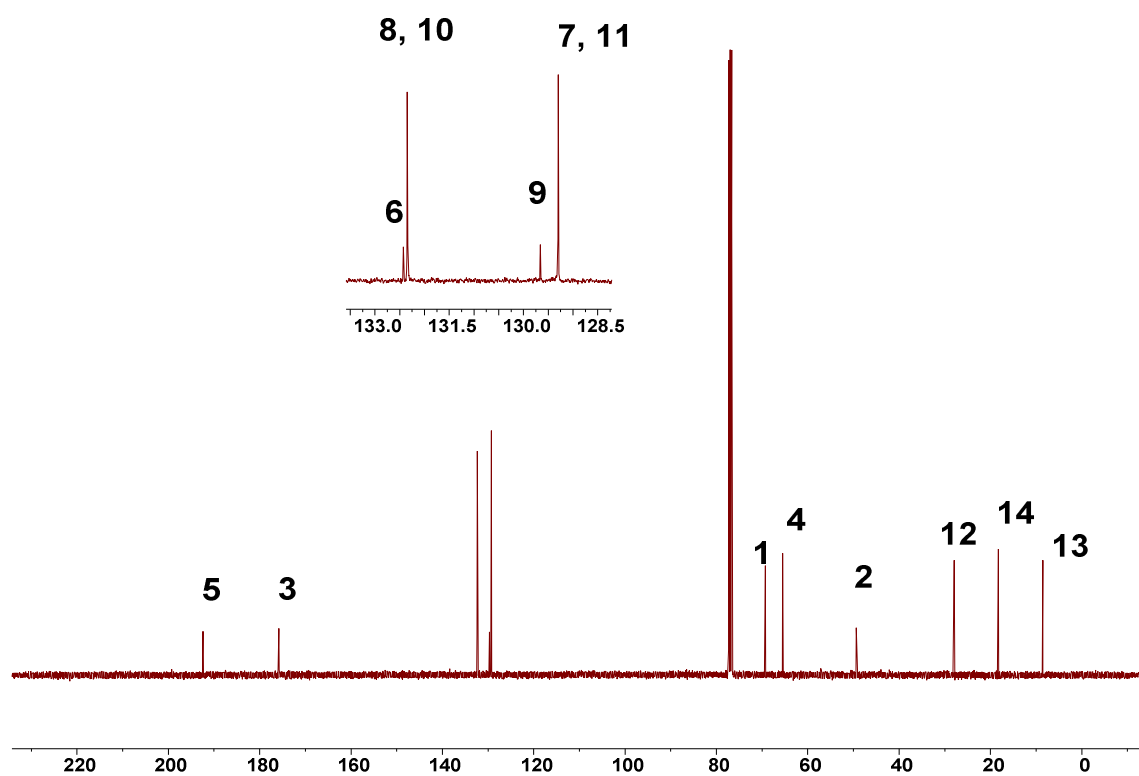
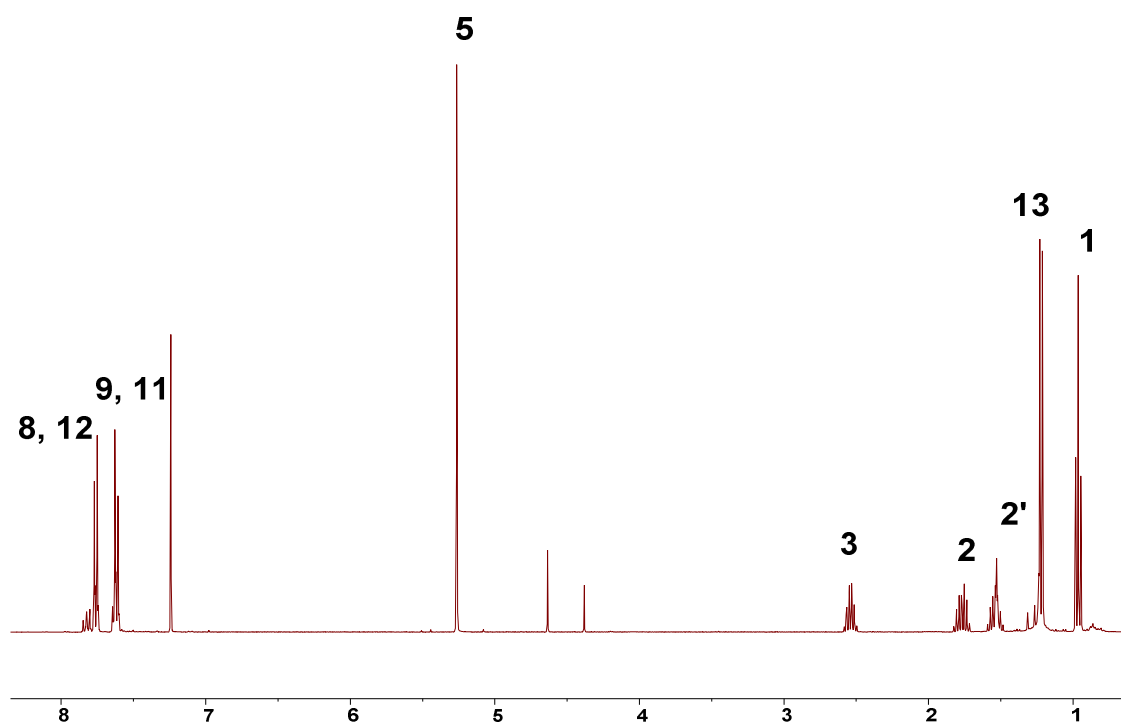
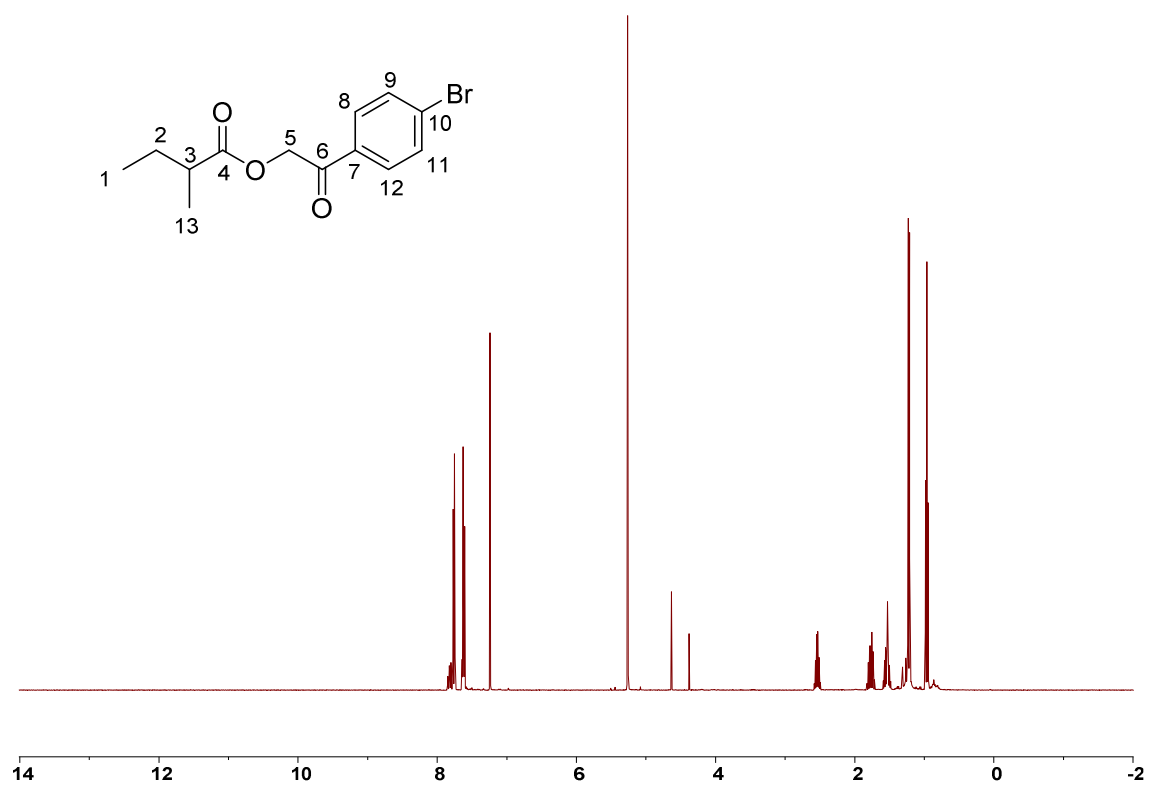


Figure S216 NMR spectra (CDCl_3) of **7b**: a) ^1H and b) ^{13}C .

a)



b)

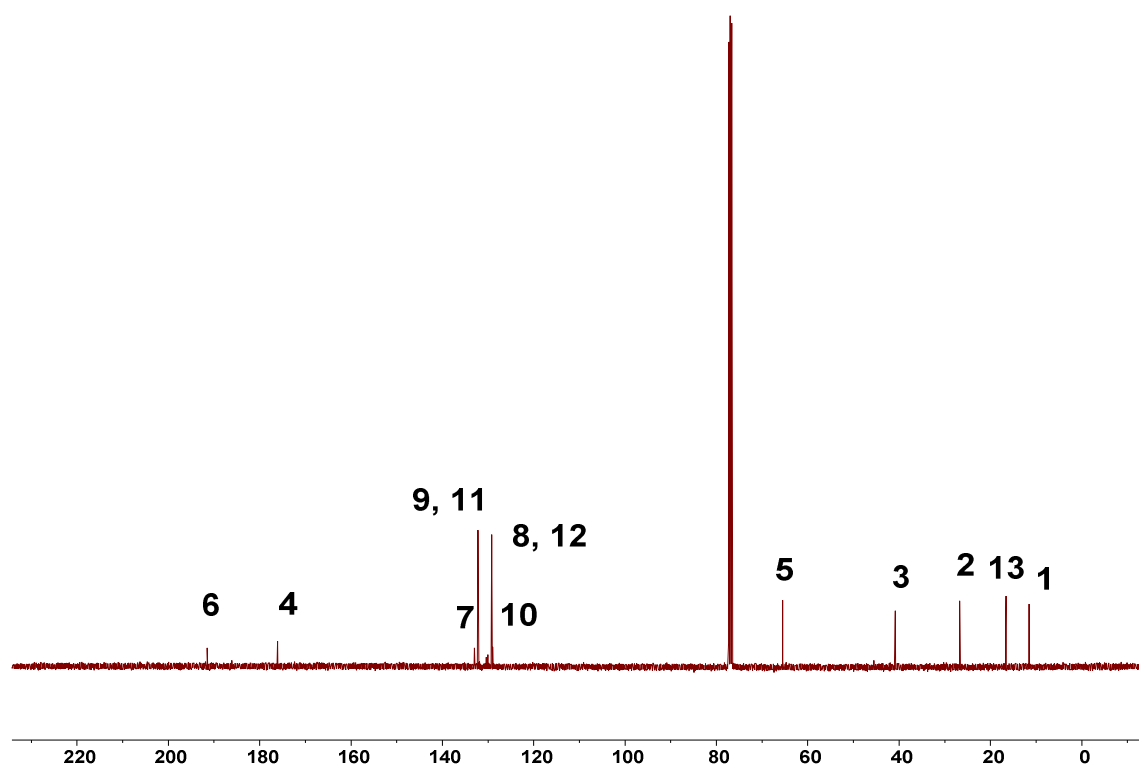
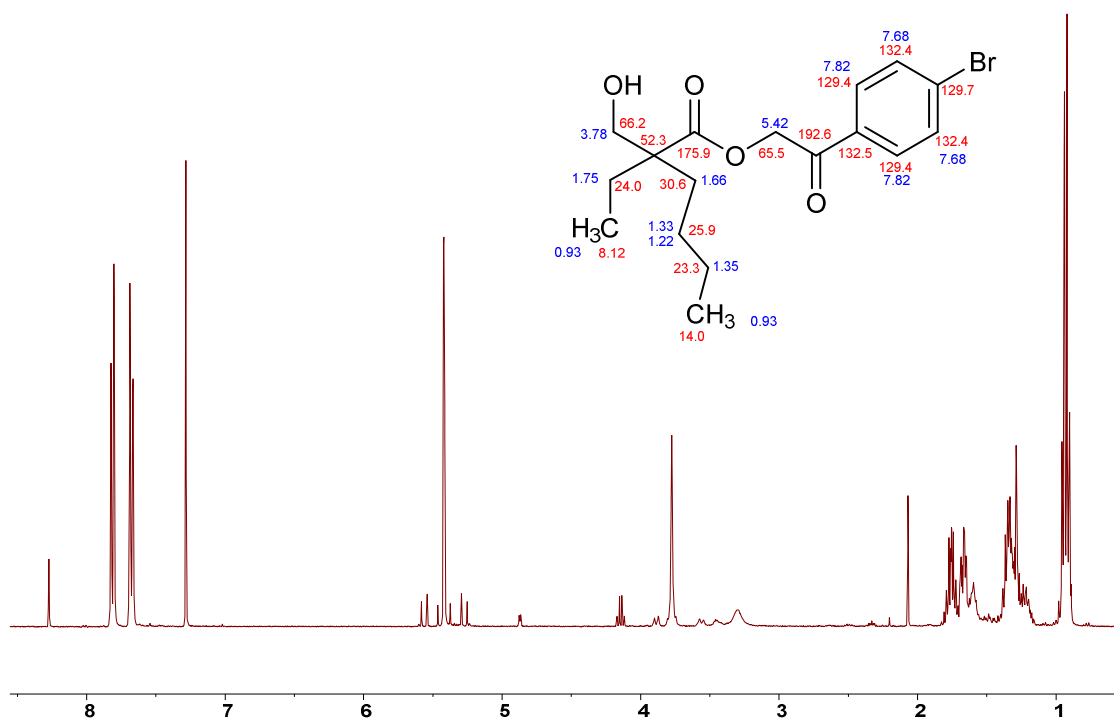
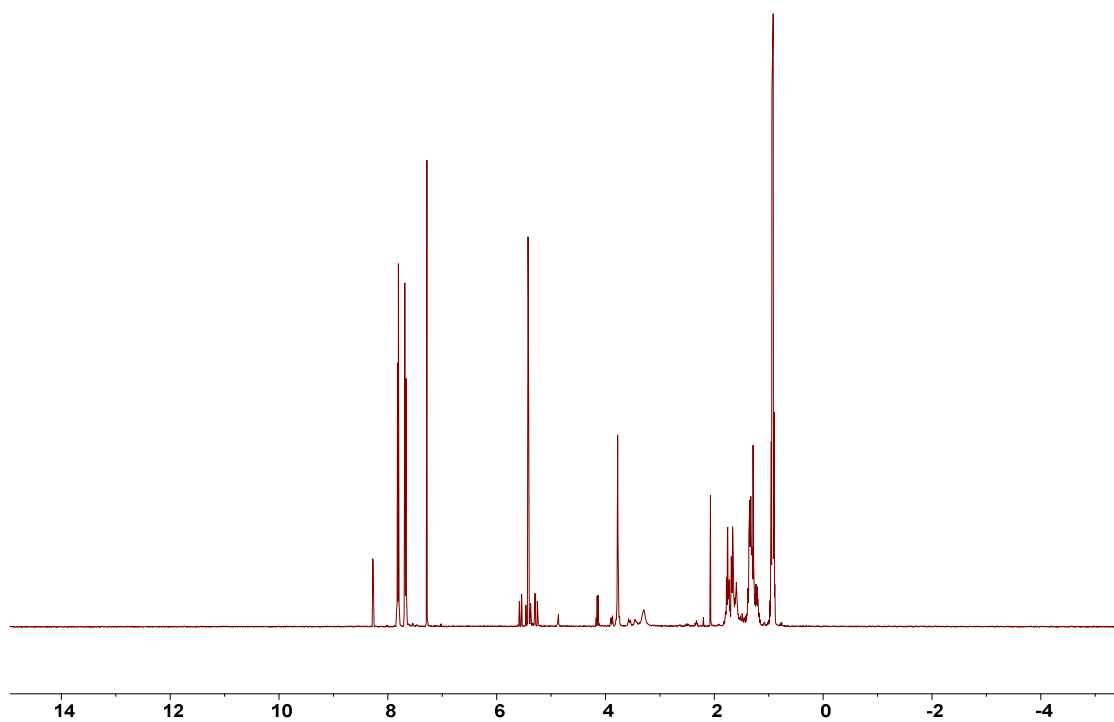
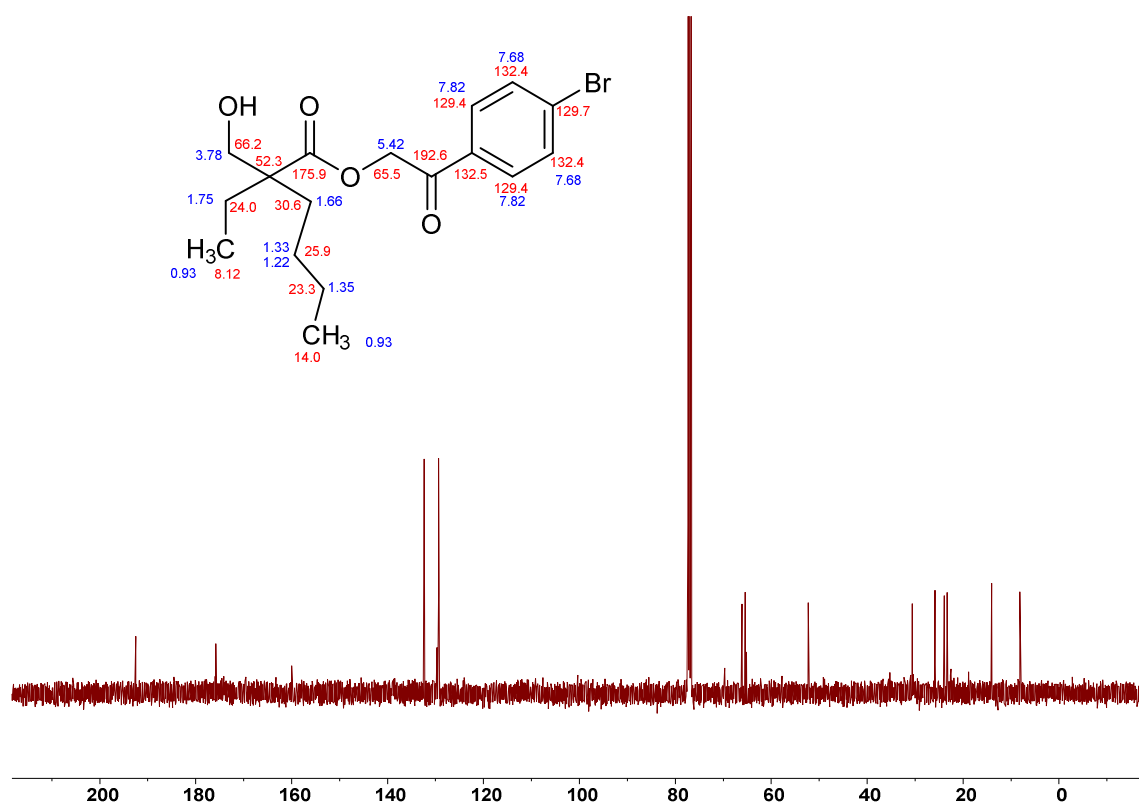


Figure S217 NMR spectra (CDCl₃) of **C5**: a) ¹H and b) ¹³C.

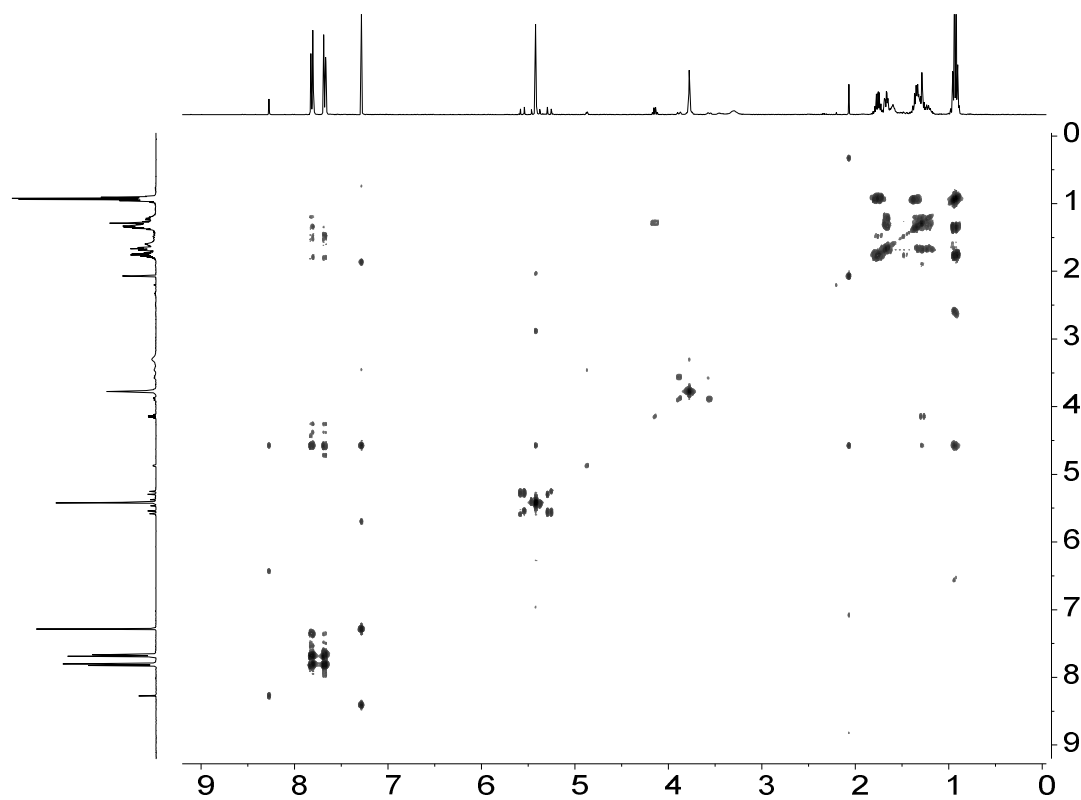
a)



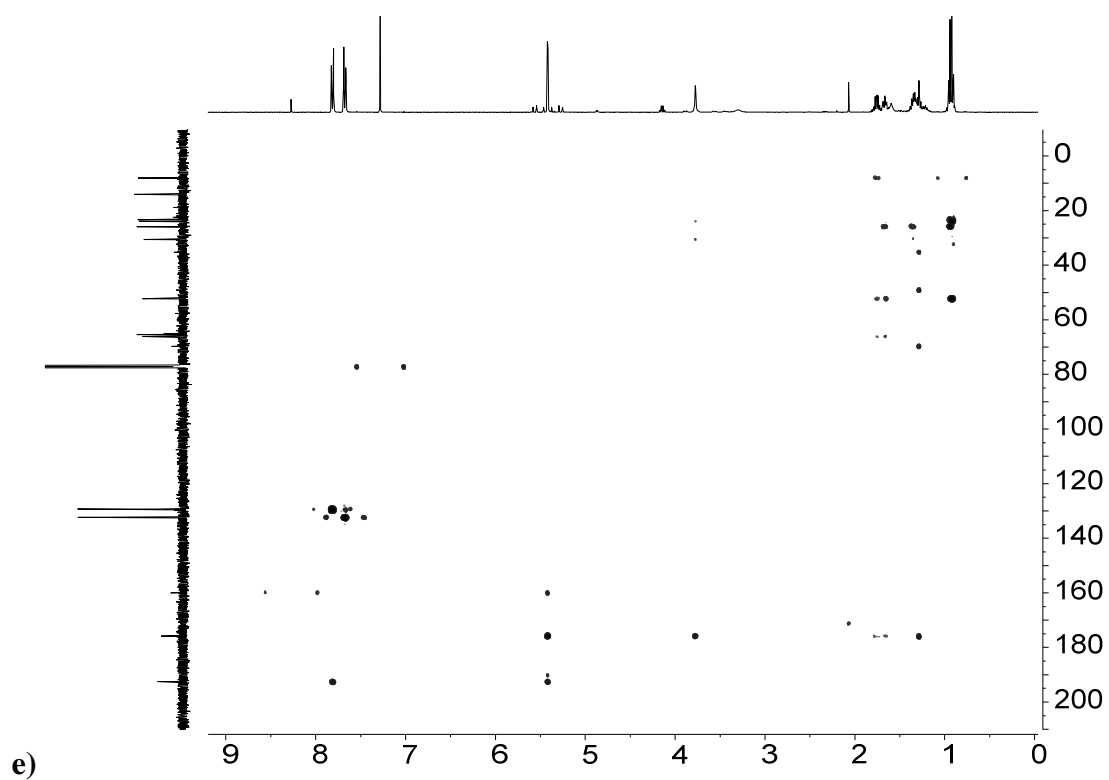
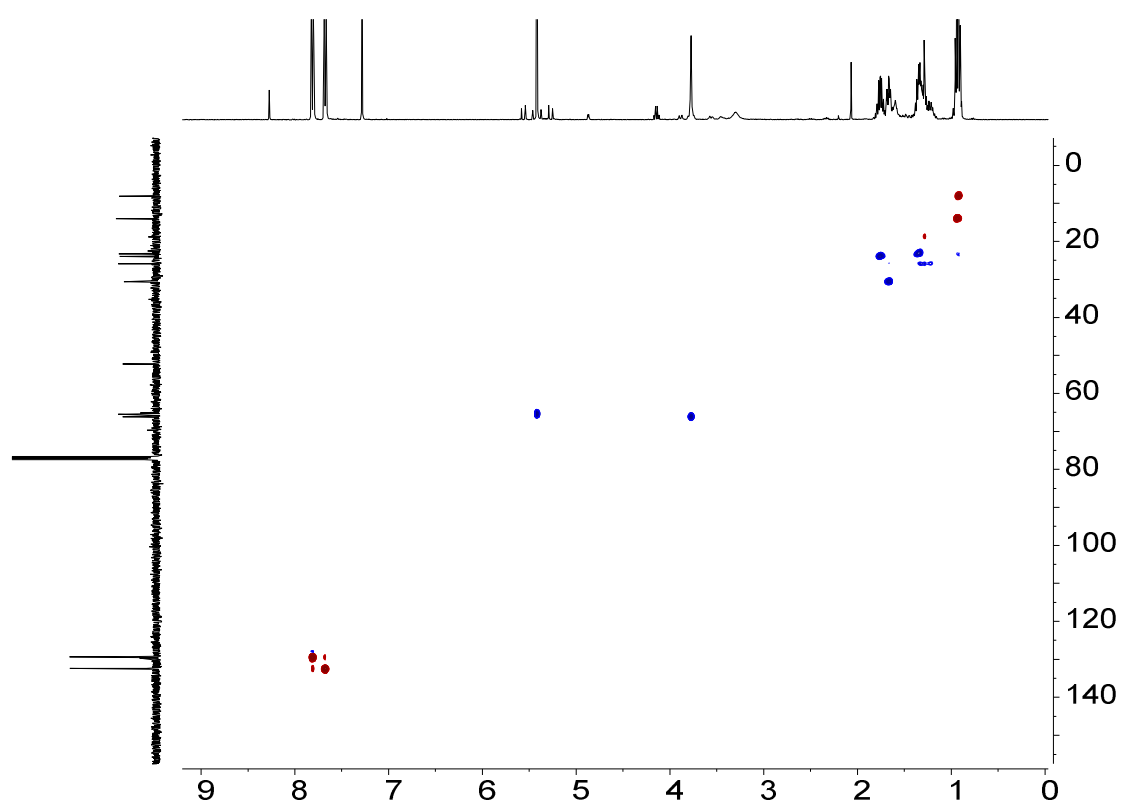
b)



c)



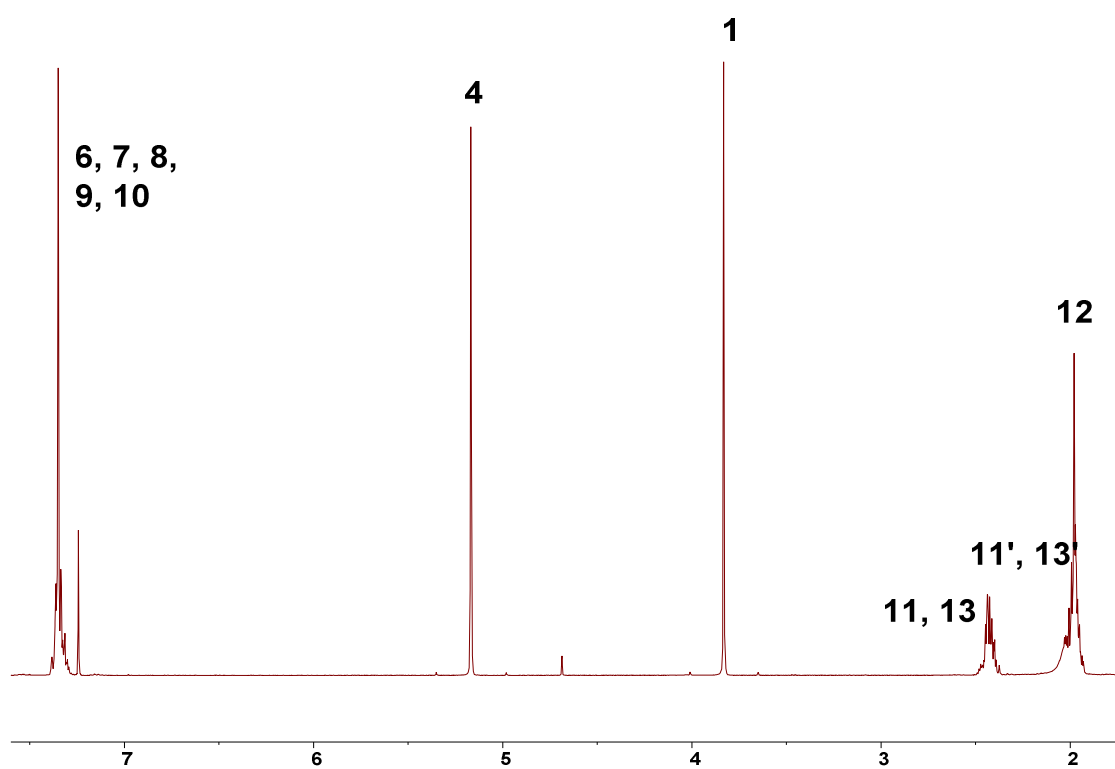
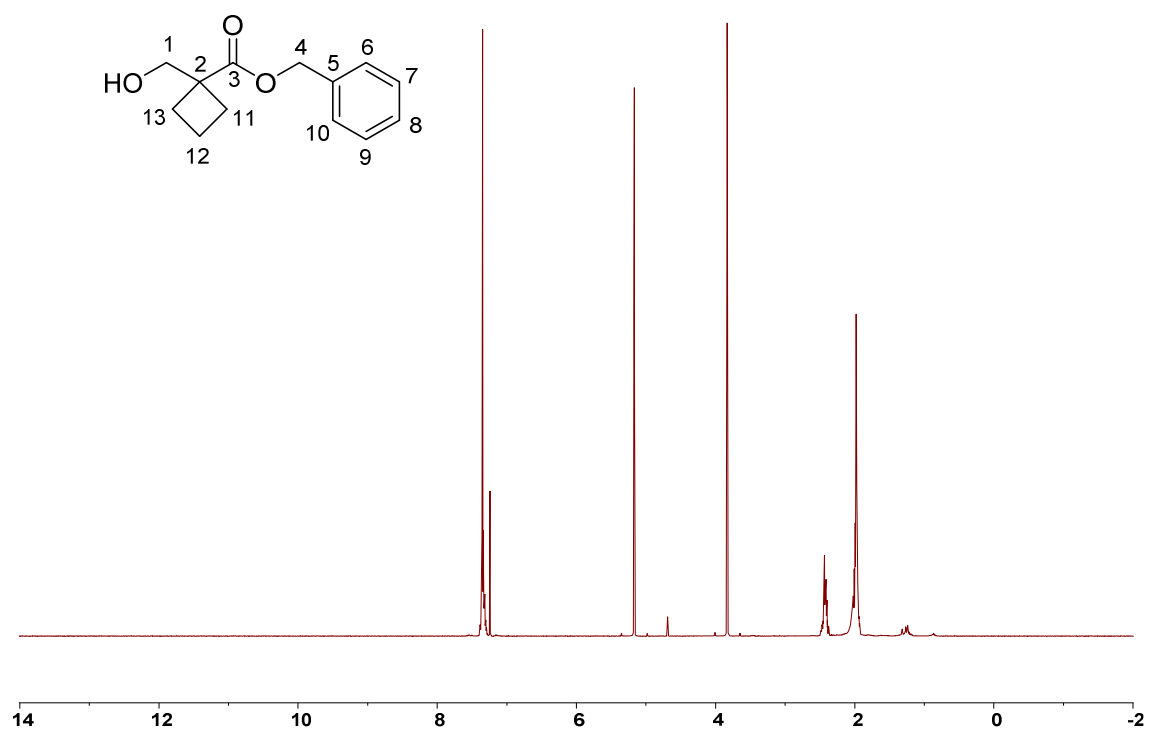
d)



e)

Figure S218 NMR spectra (CDCl₃) of **7c**: a) ¹H, b) ¹³C, c) gCOSY, d) HSQC and e) HMBC.

a)



b)

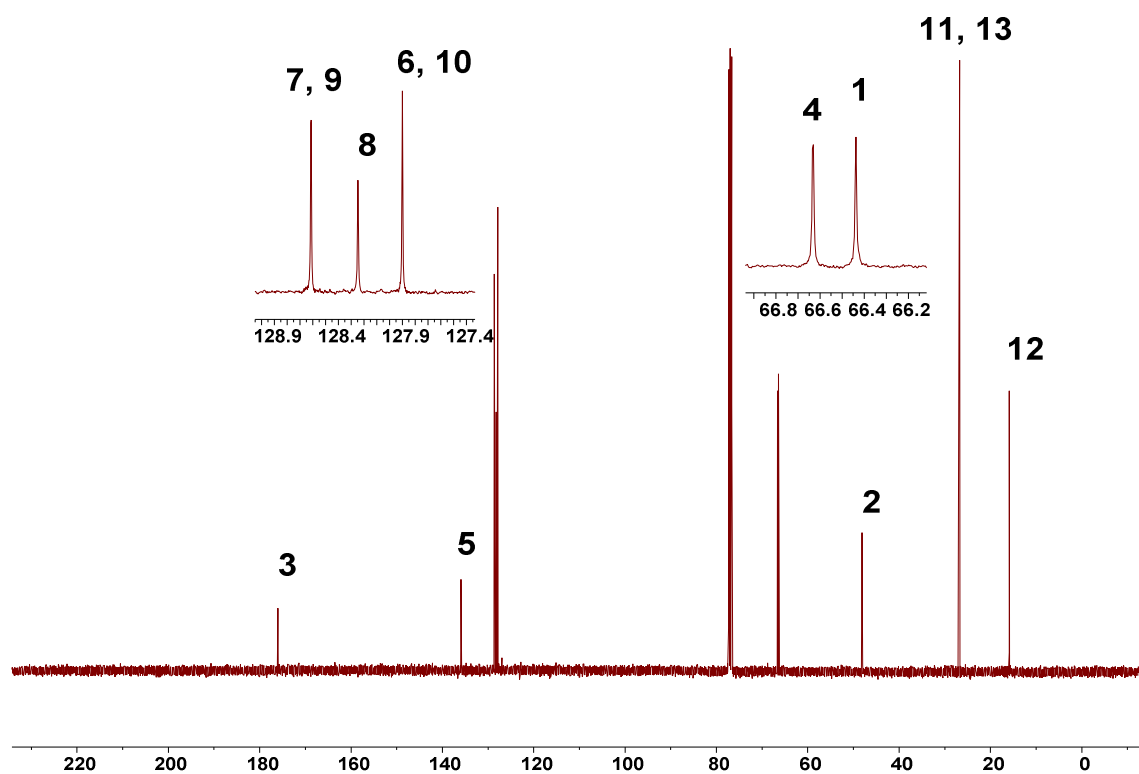
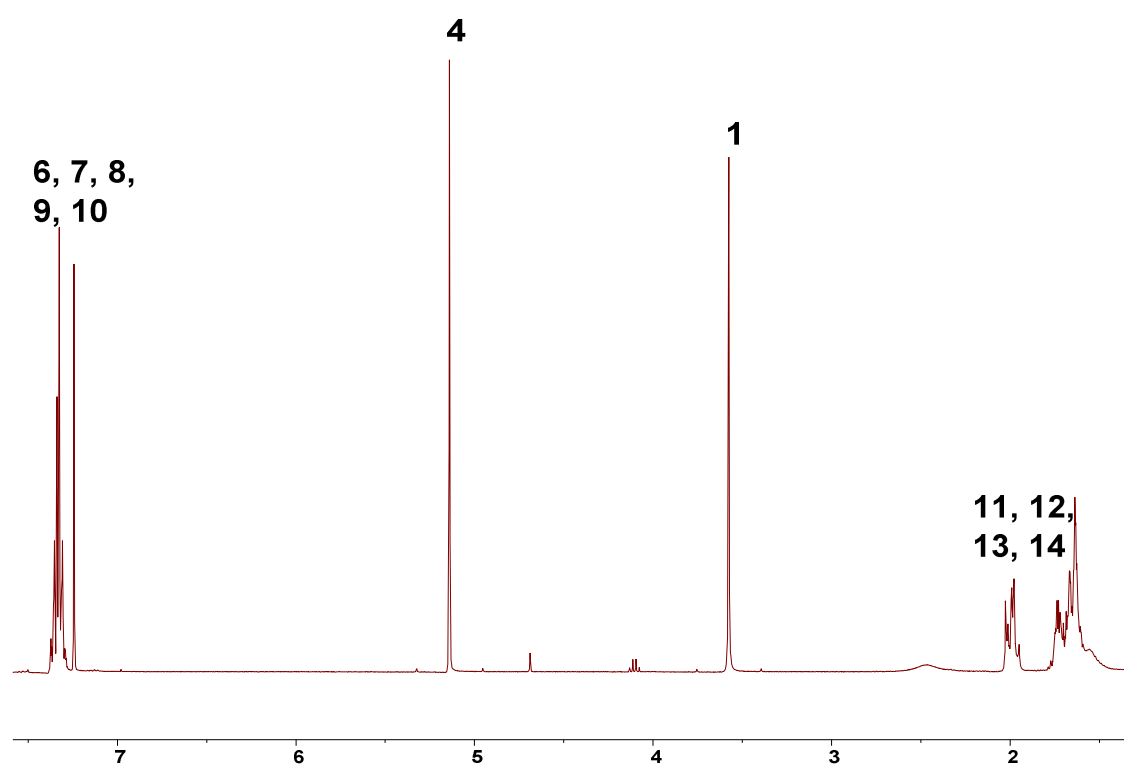
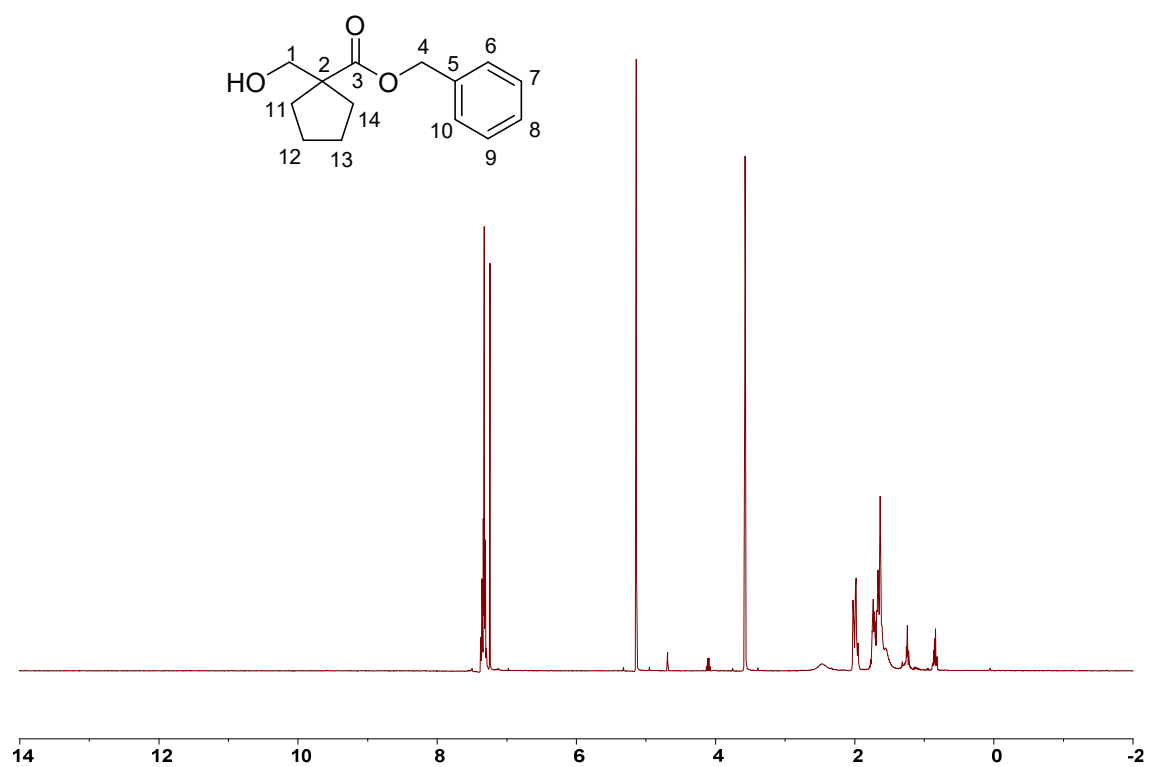


Figure S219 NMR spectra (CDCl_3) of **7d**: a) ^1H and b) ^{13}C .

a)



b)

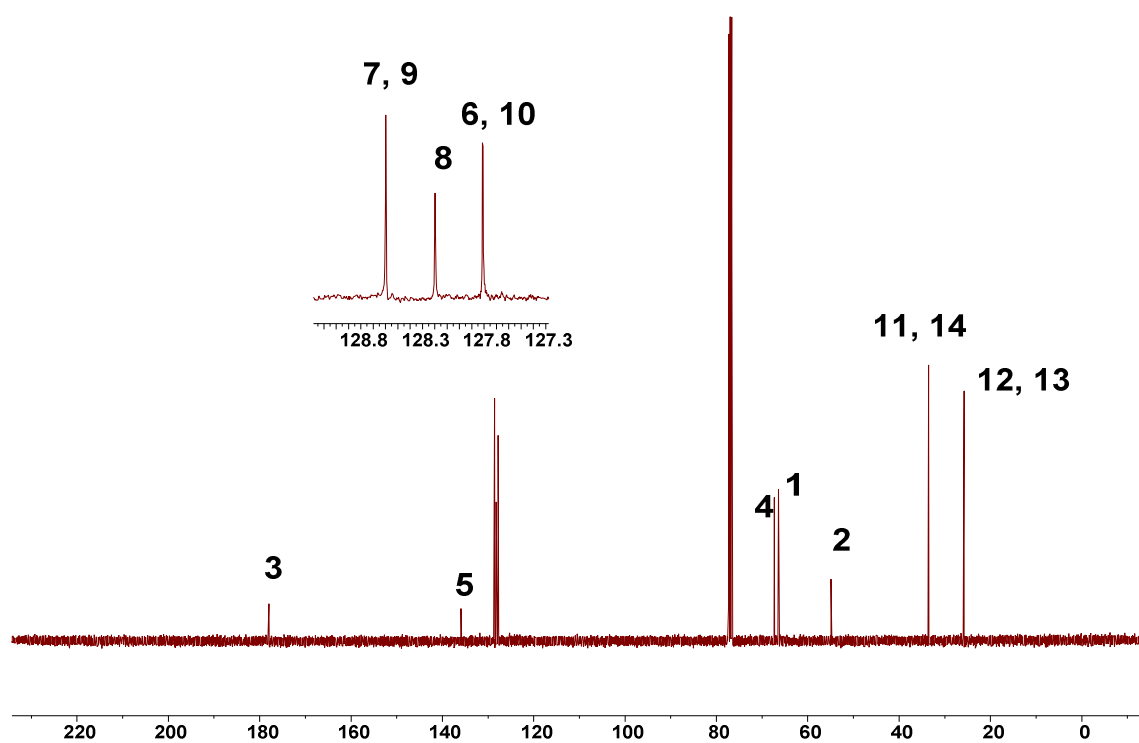
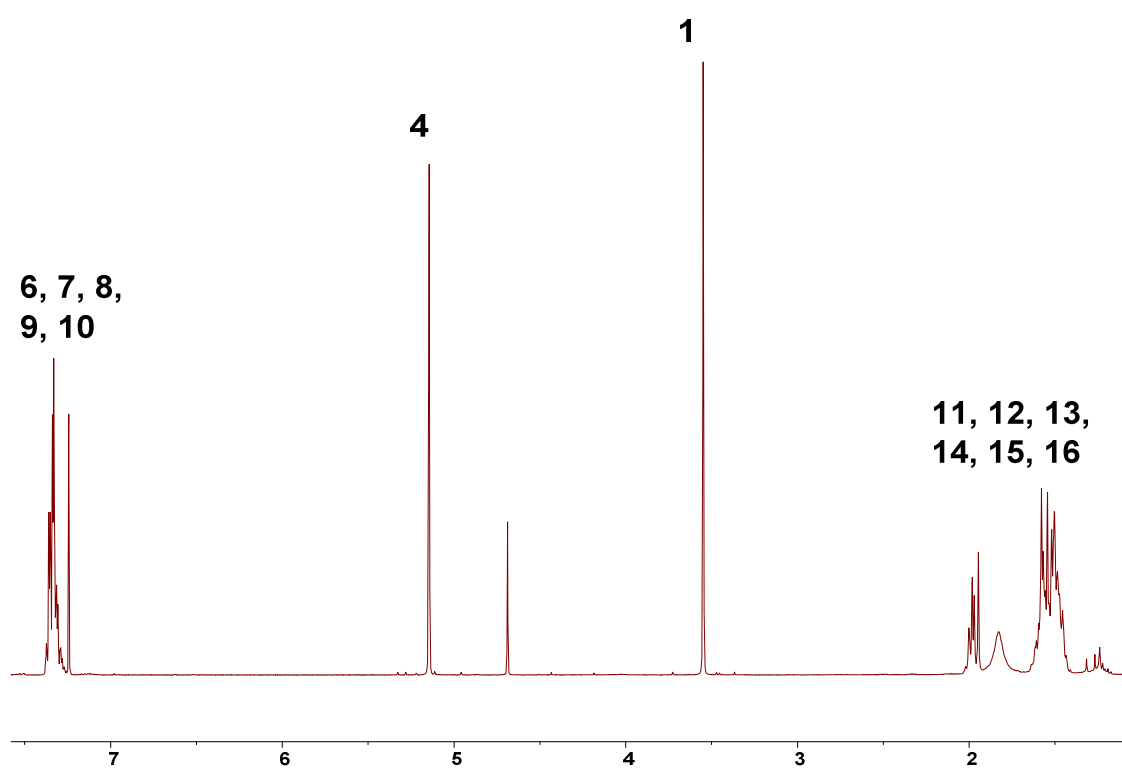
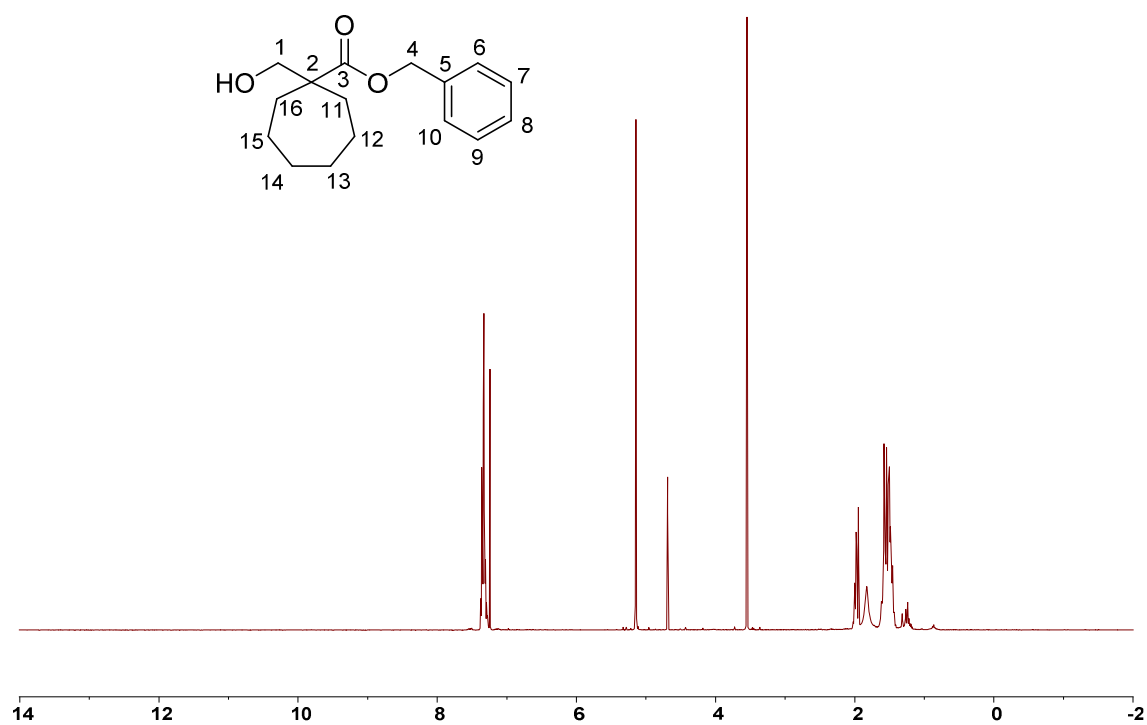


Figure S220 NMR spectra (CDCl_3) of **7e**: a) ^1H and b) ^{13}C .

a)



b)

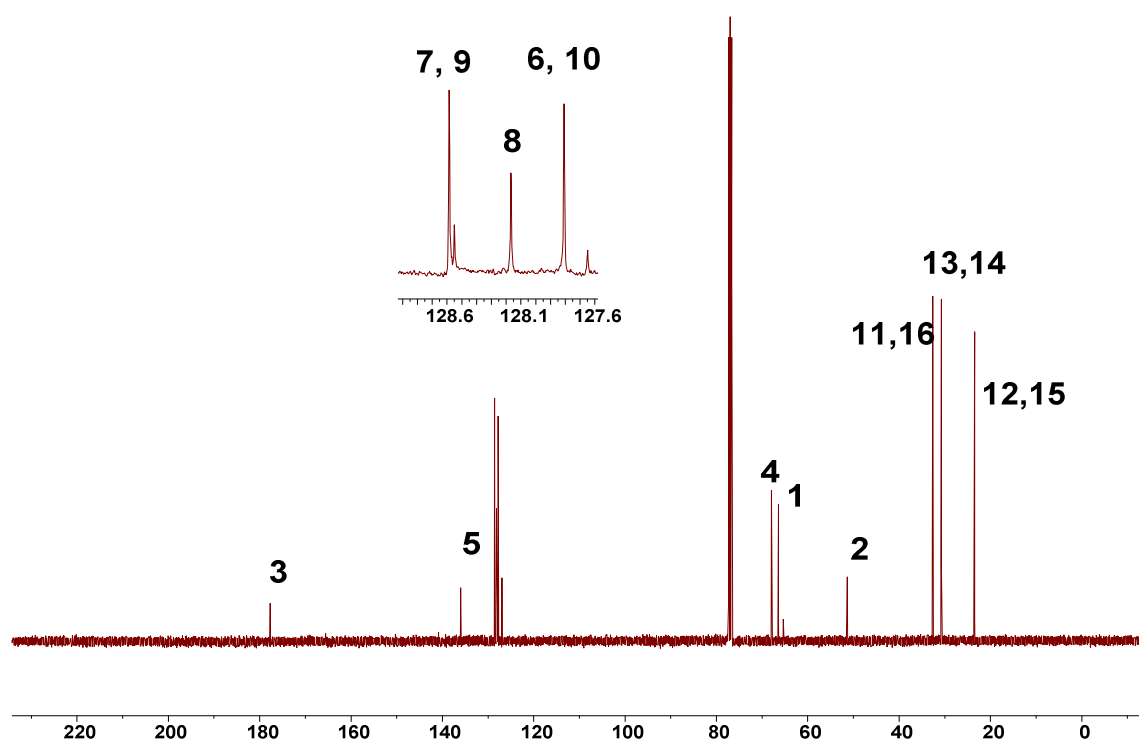
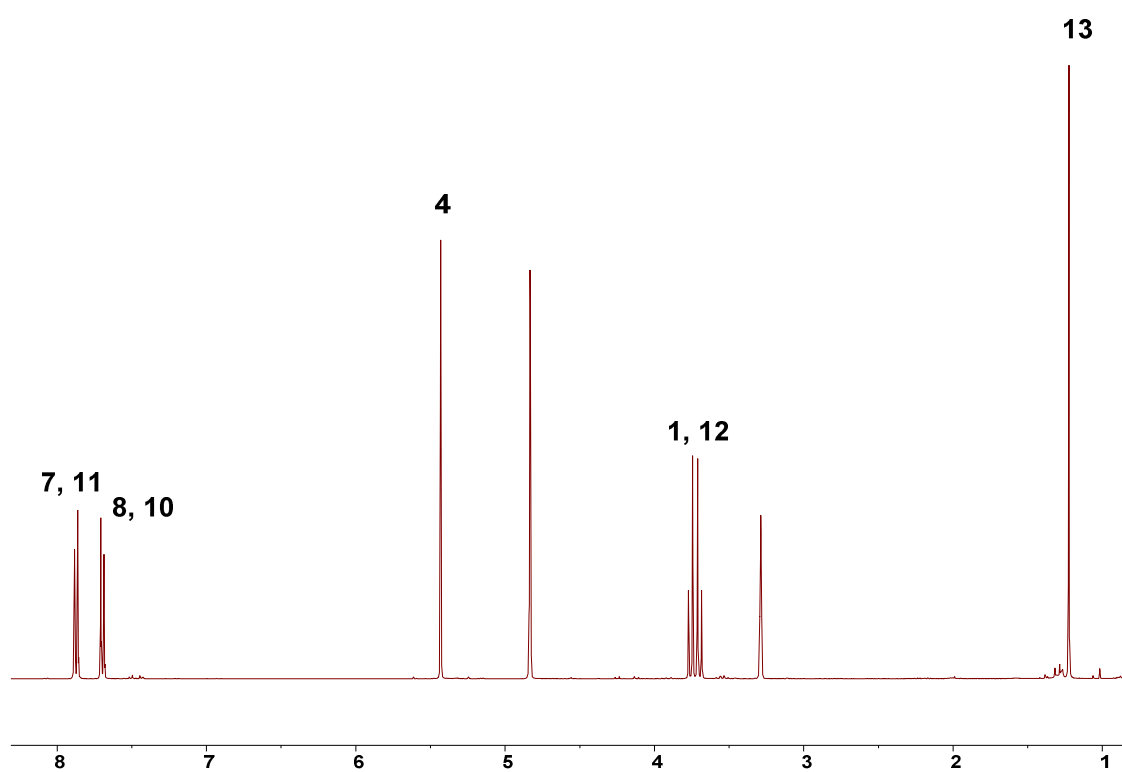
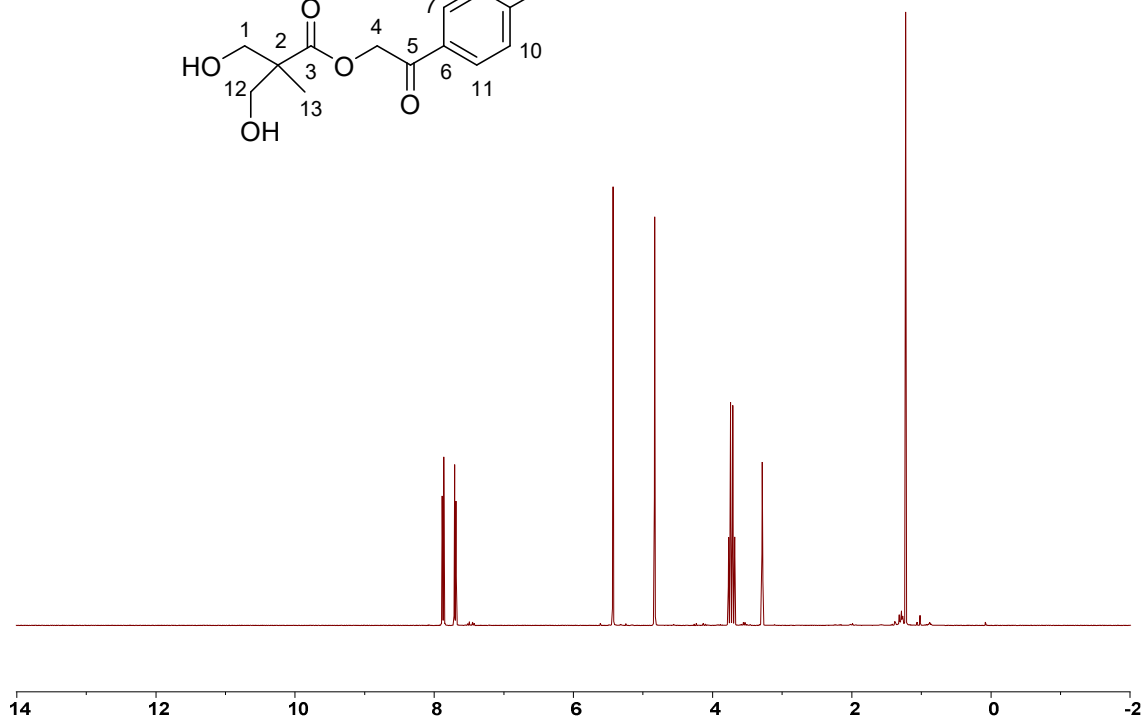
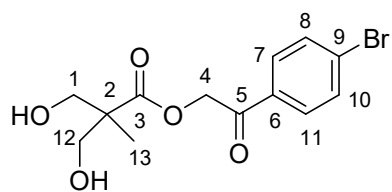


Figure S221 NMR spectra (CDCl_3) of **7f**: a) ^1H and b) ^{13}C .

a)



b)

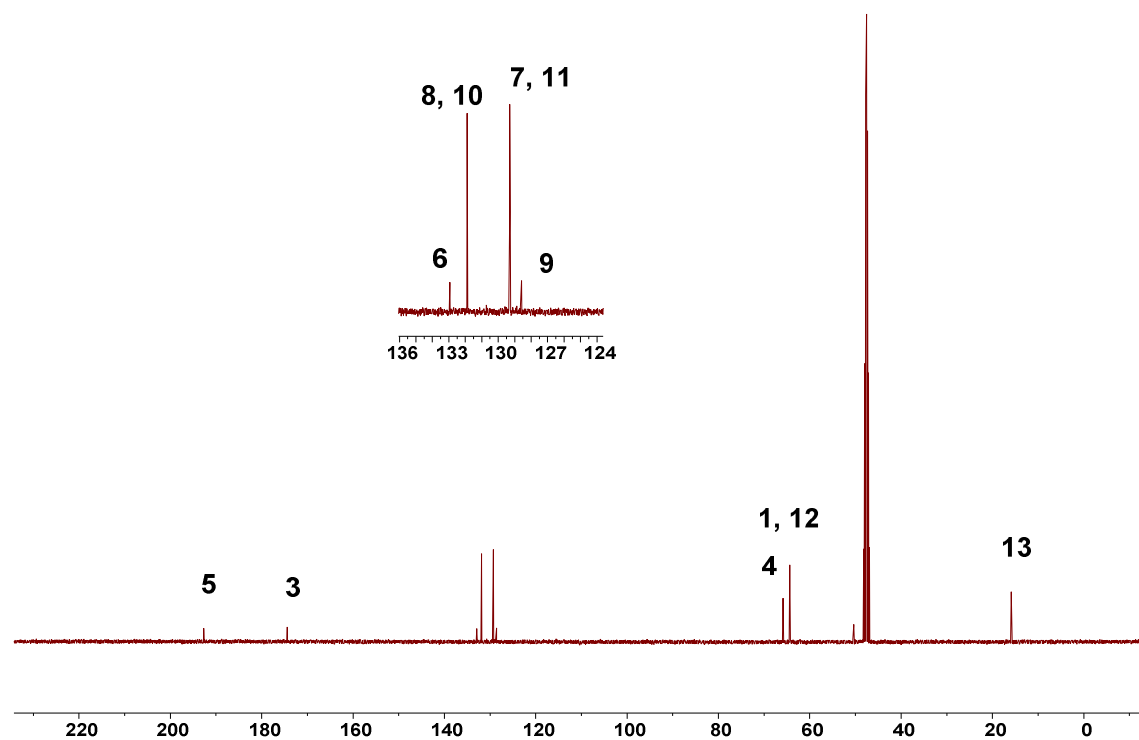
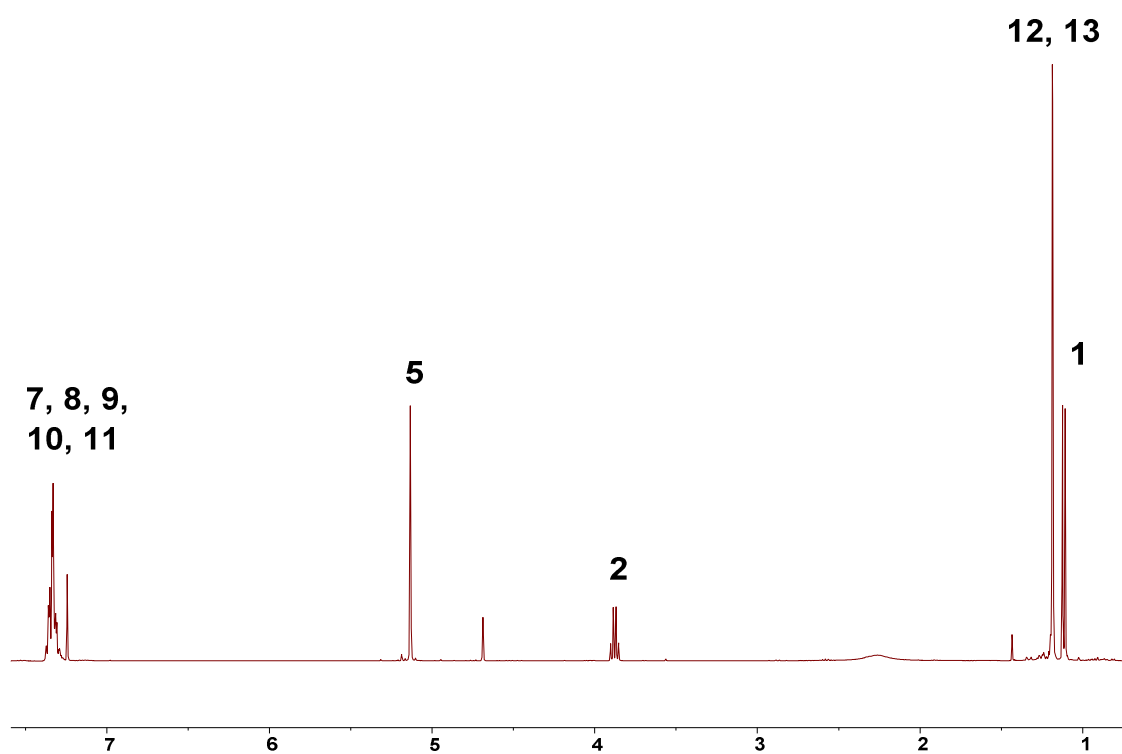
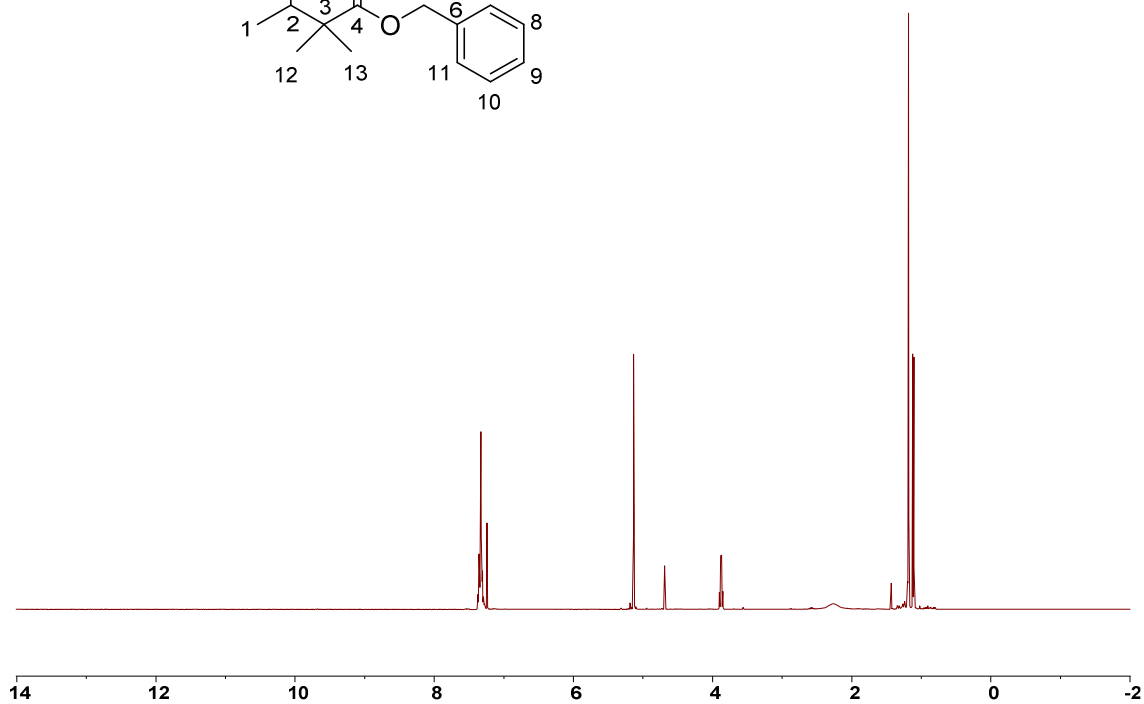
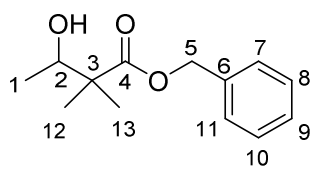
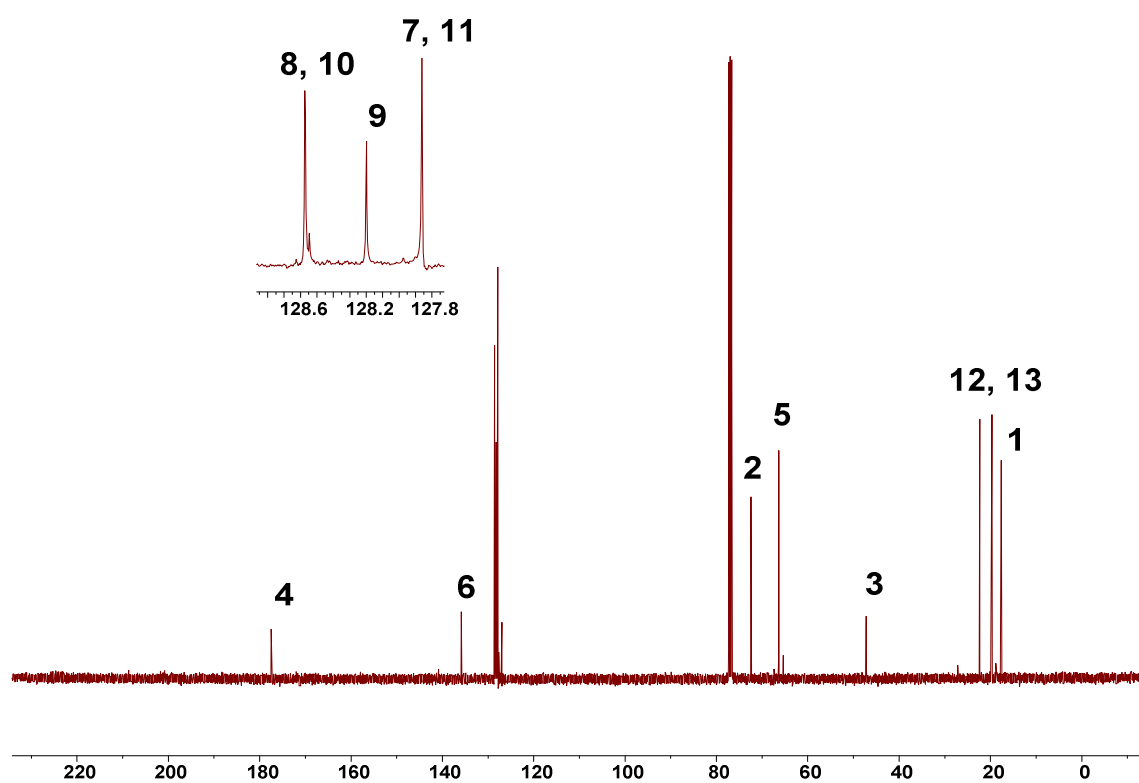


Figure S222 NMR spectra (CDCl_3) of **7g**: a) ^1H and b) ^{13}C .

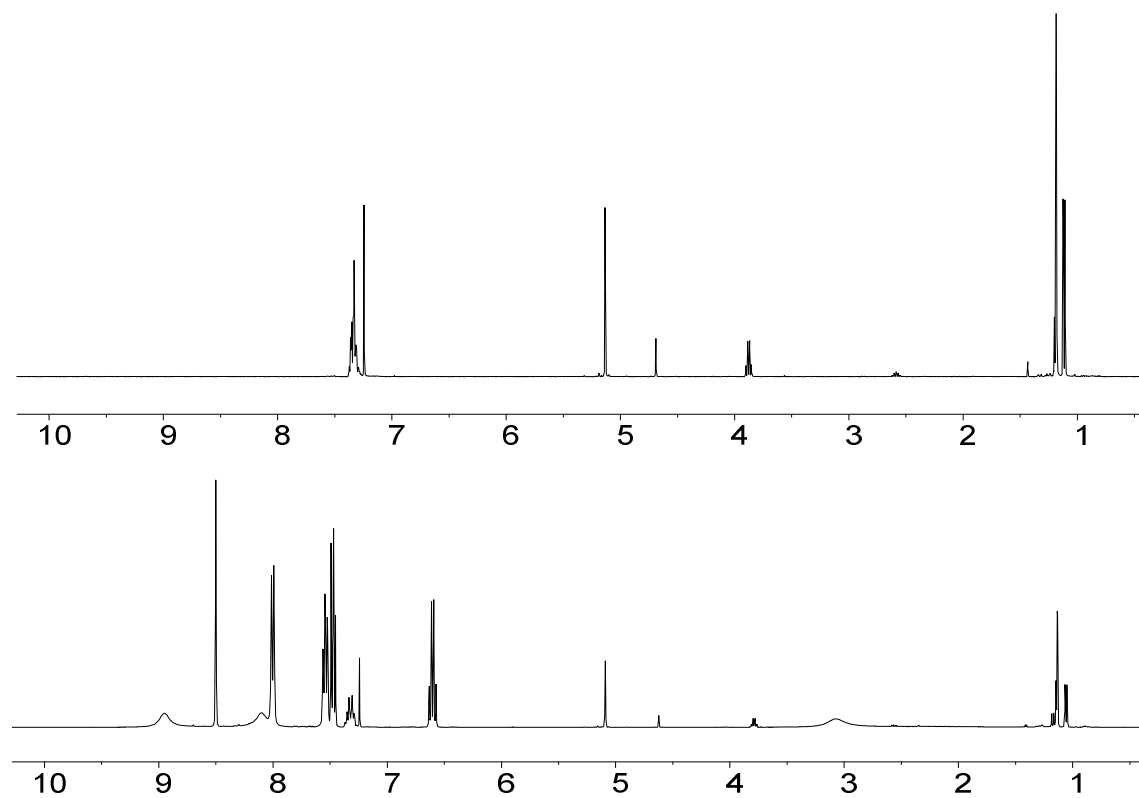
a)



b)



c)



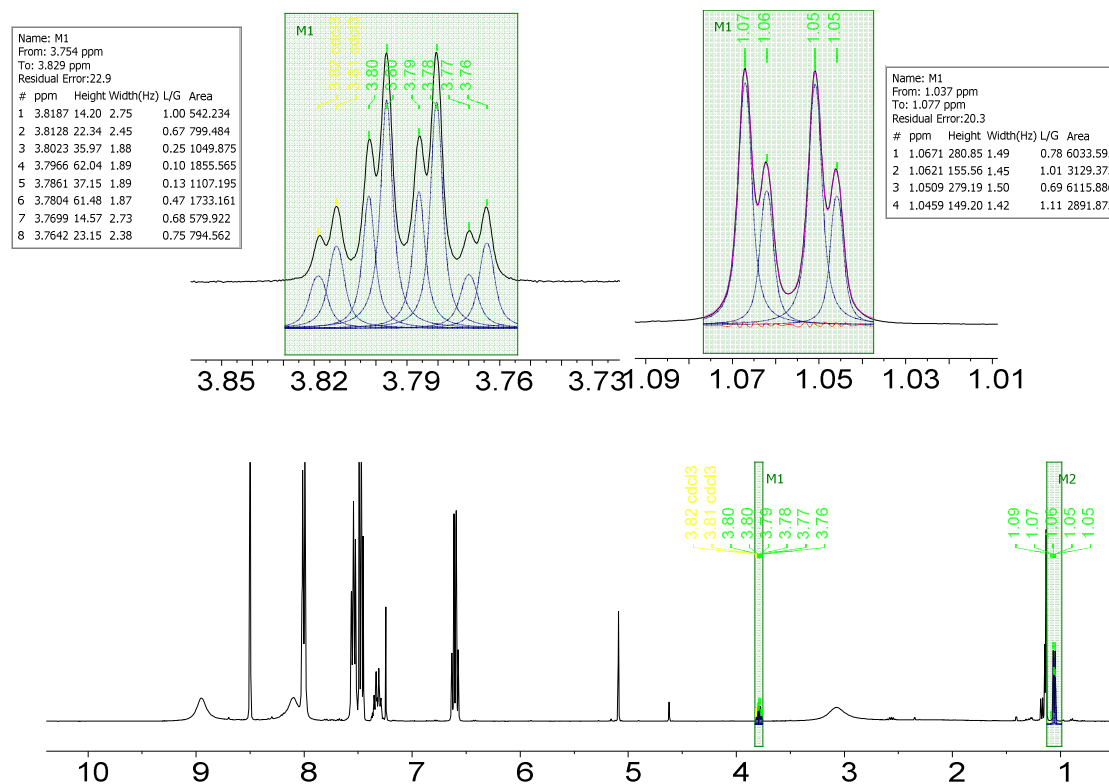
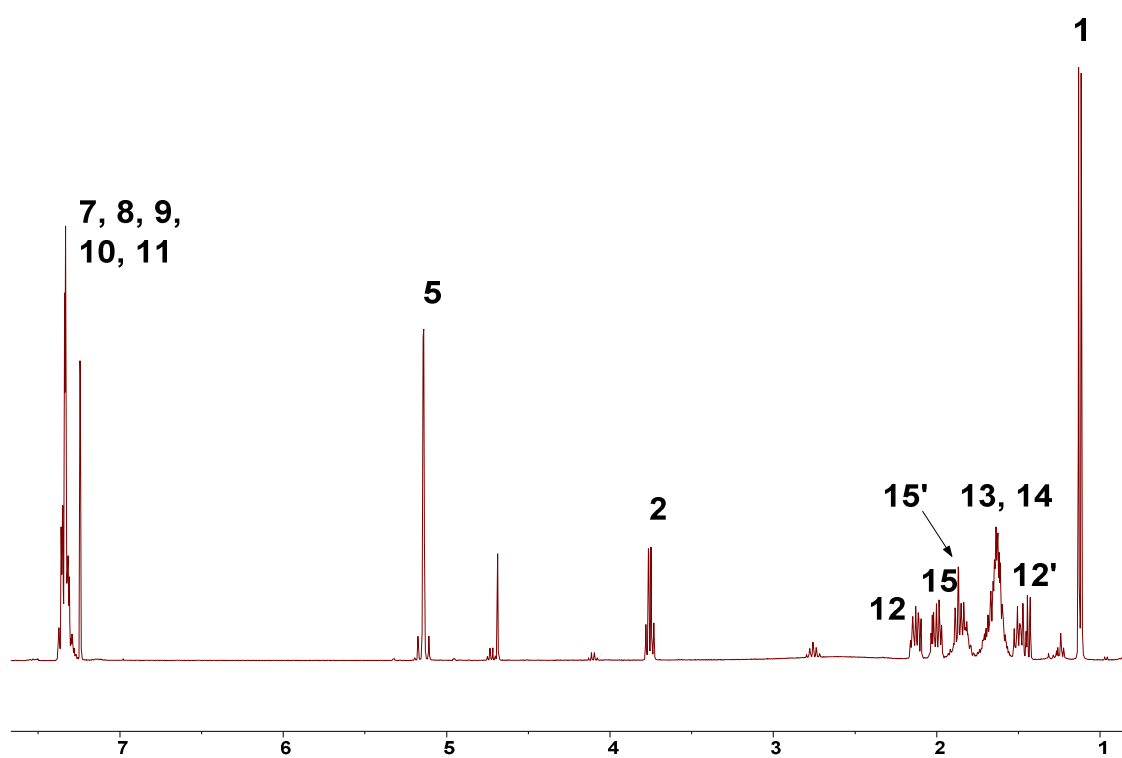
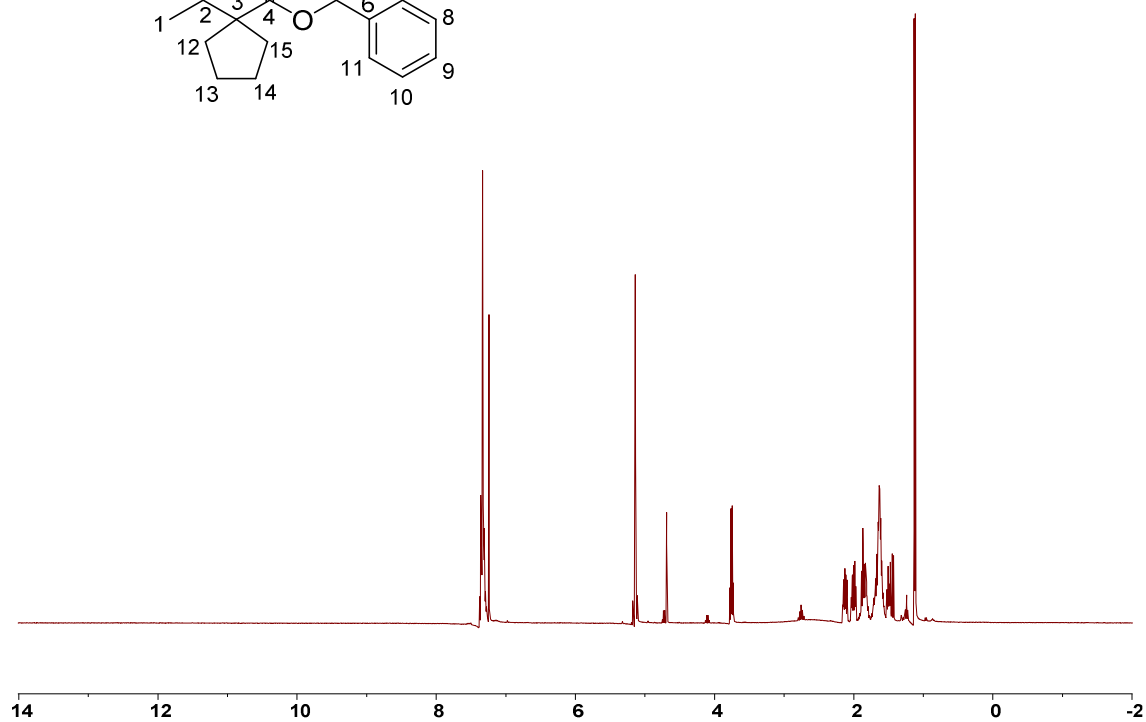
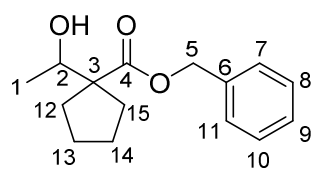


Figure S223 NMR spectra (CDCl₃) of **7h**: a) ¹H, b) ¹³C and c) Original spectra and after adding 5 eq of *R* configured Pirkle's alcohol.

a)



b)

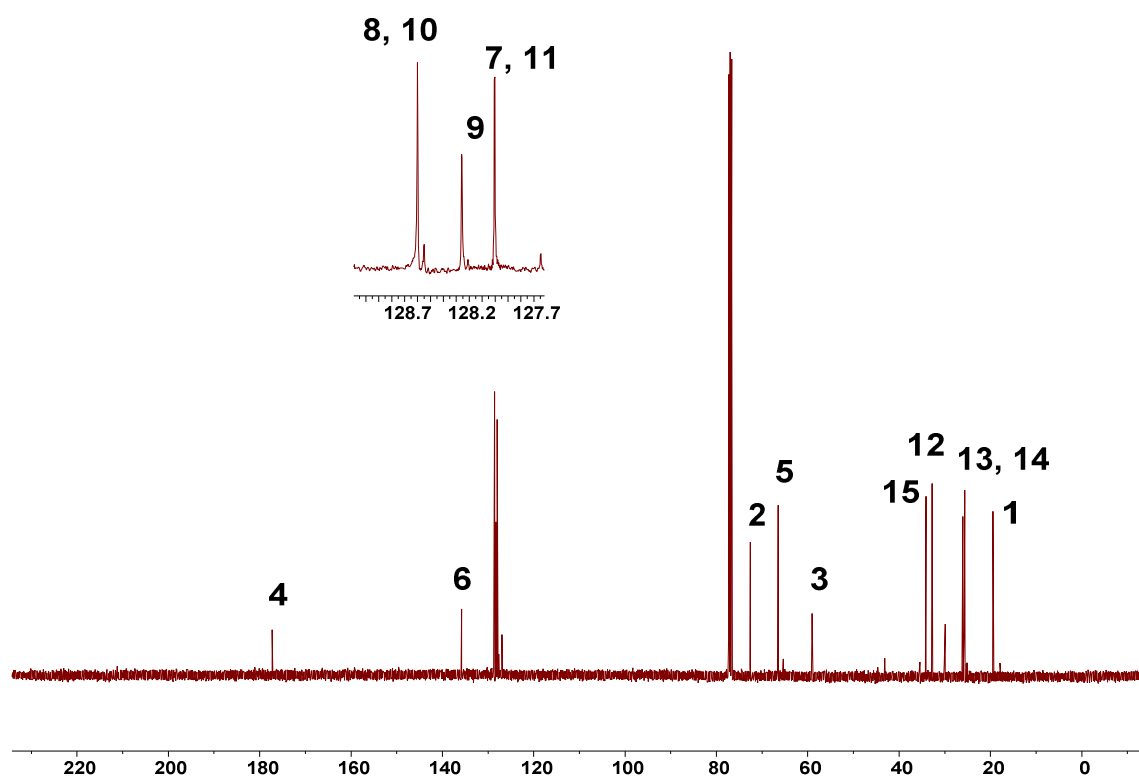
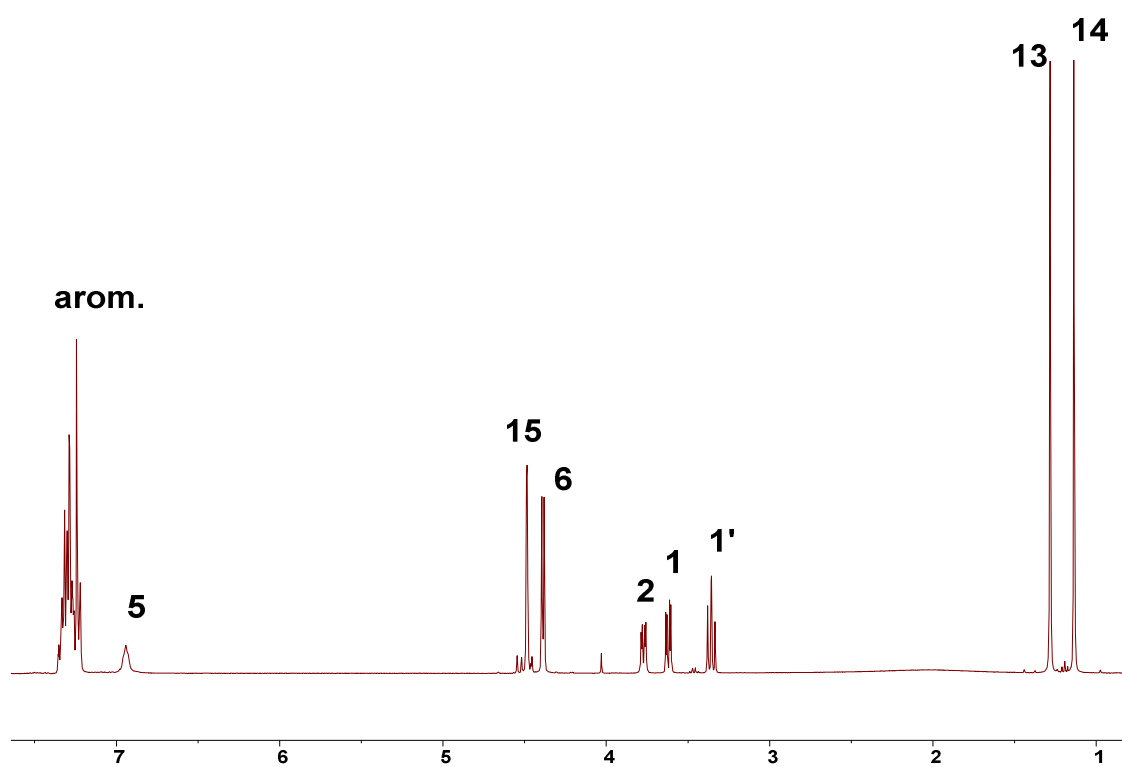
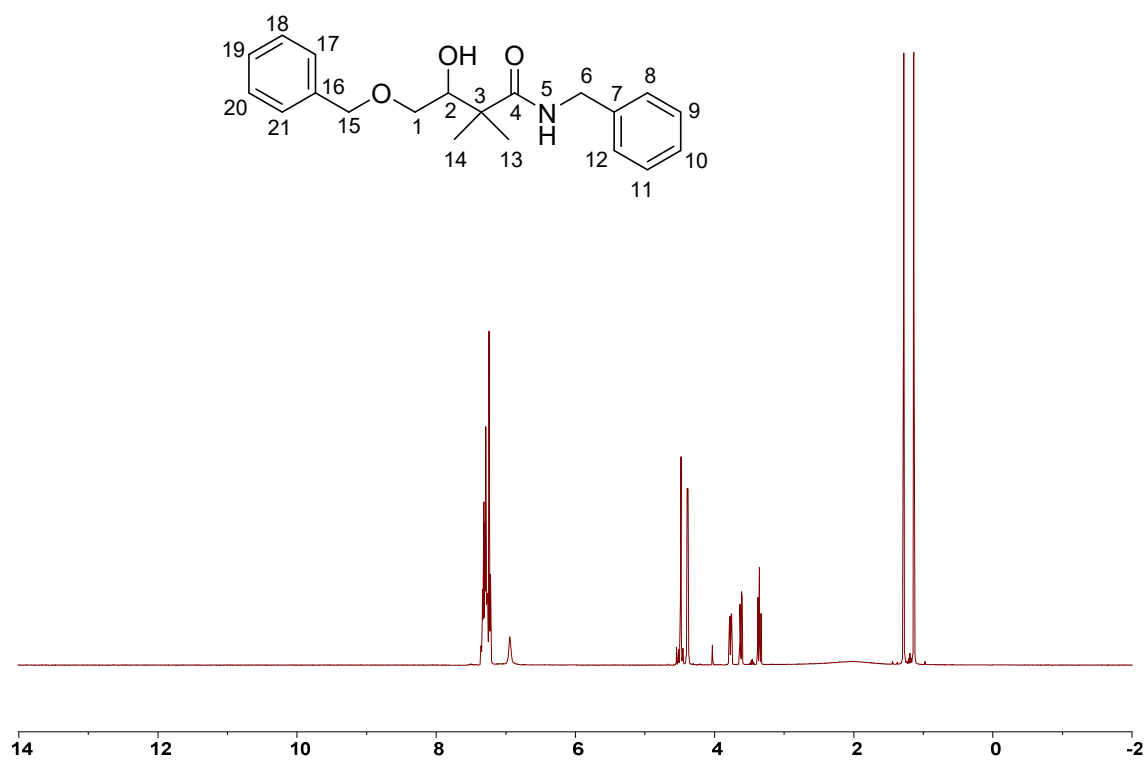
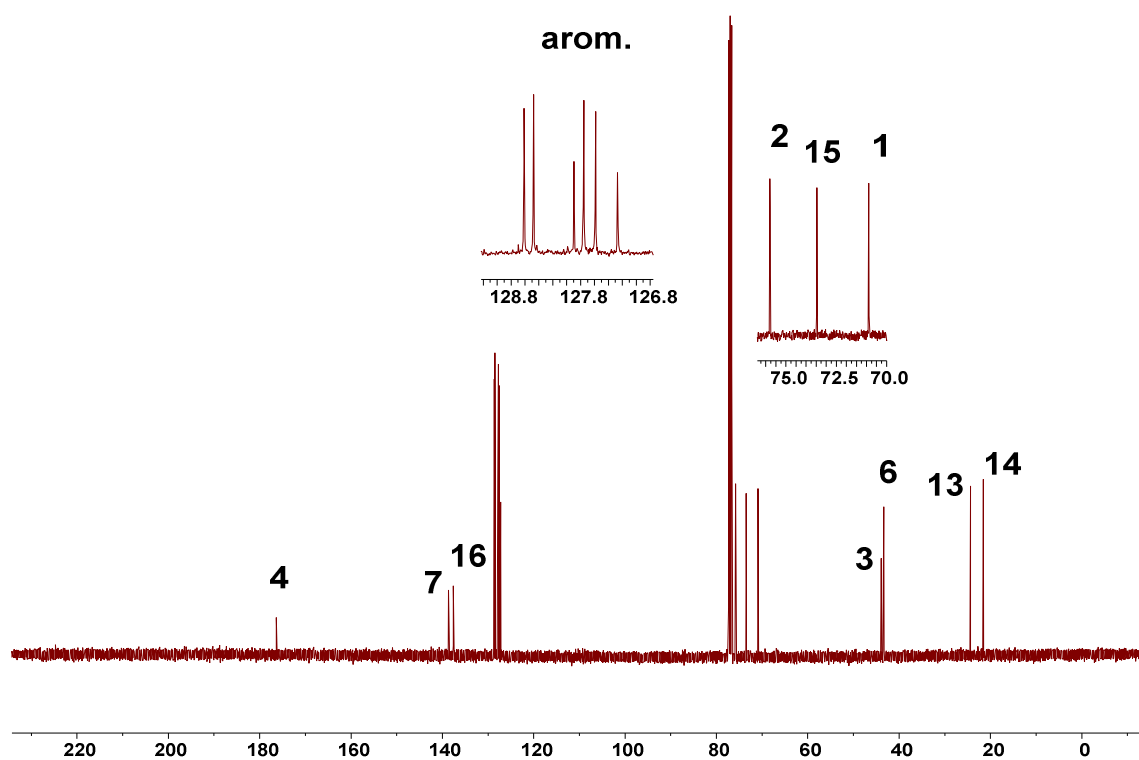


Figure S224 NMR spectra (CDCl_3) of **7i**: a) ^1H and b) ^{13}C .

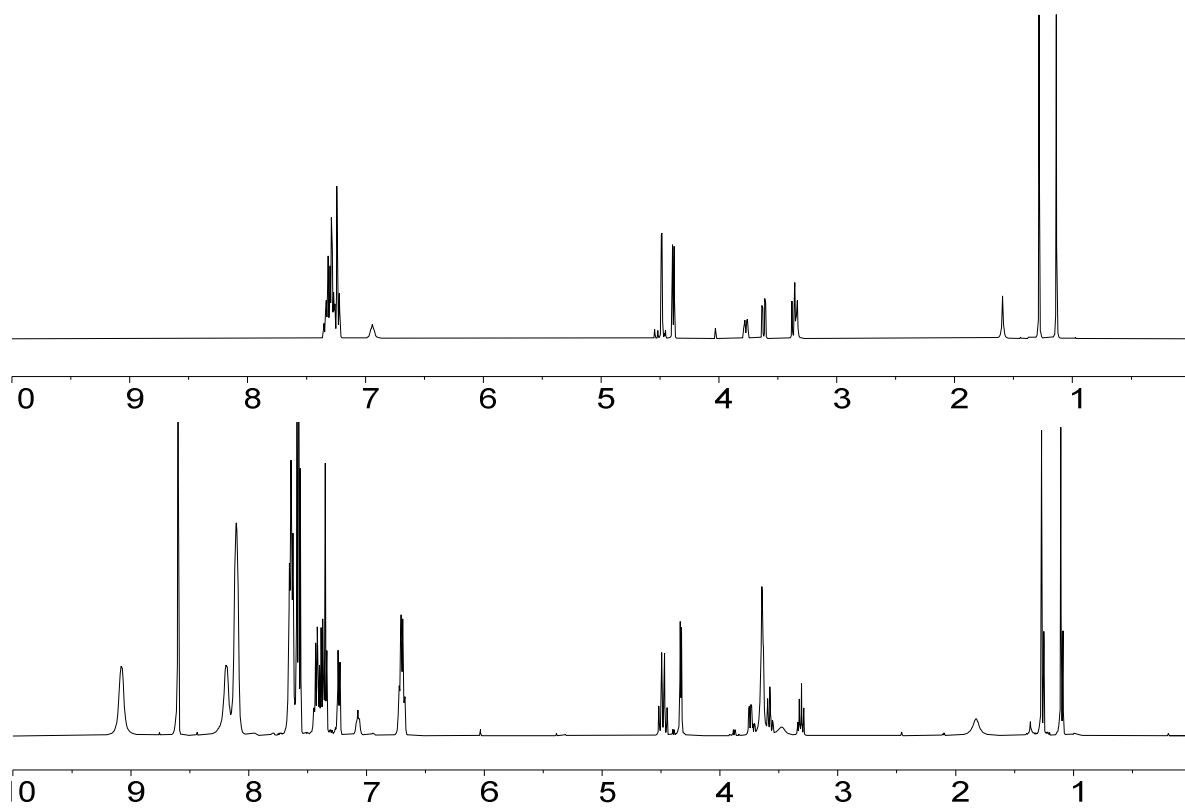
a)



b)



c)



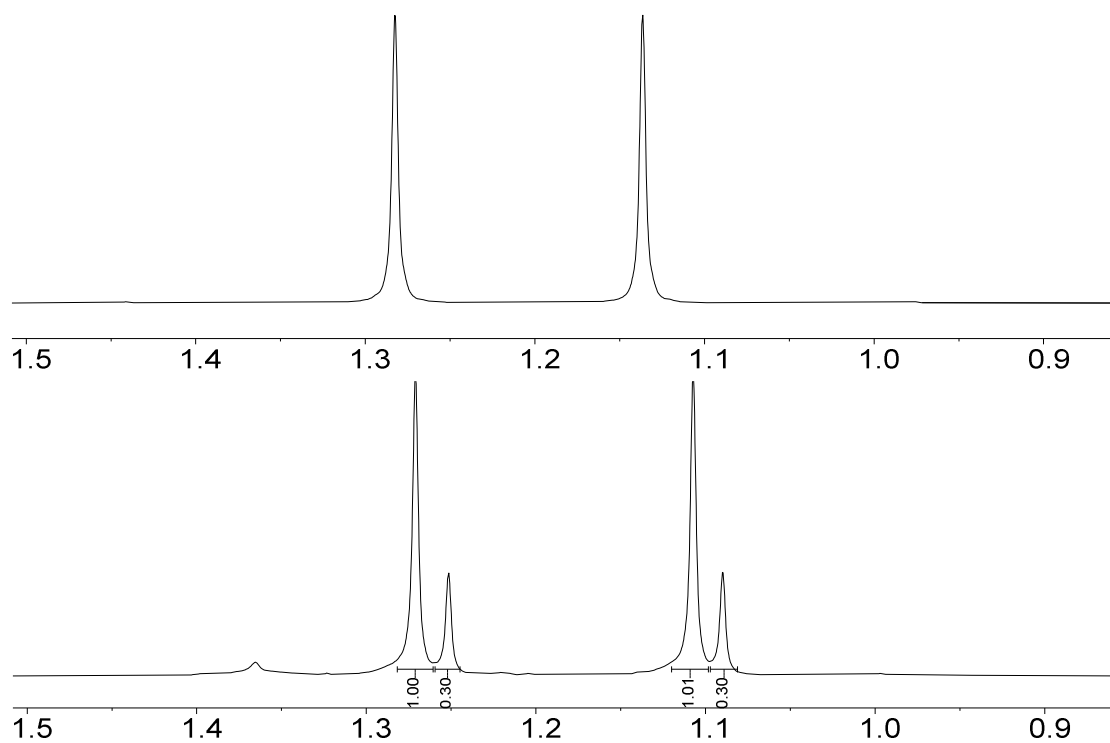
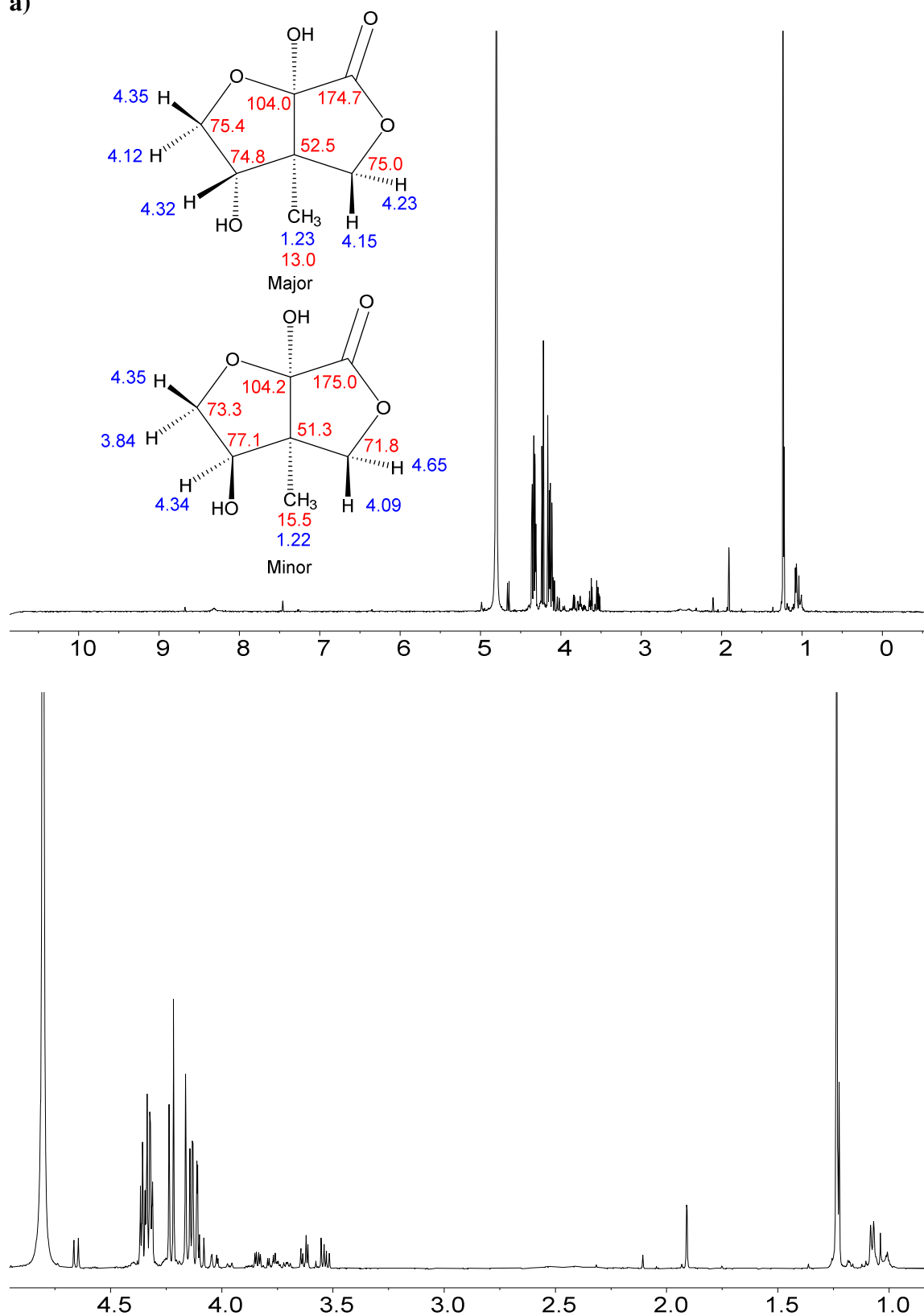
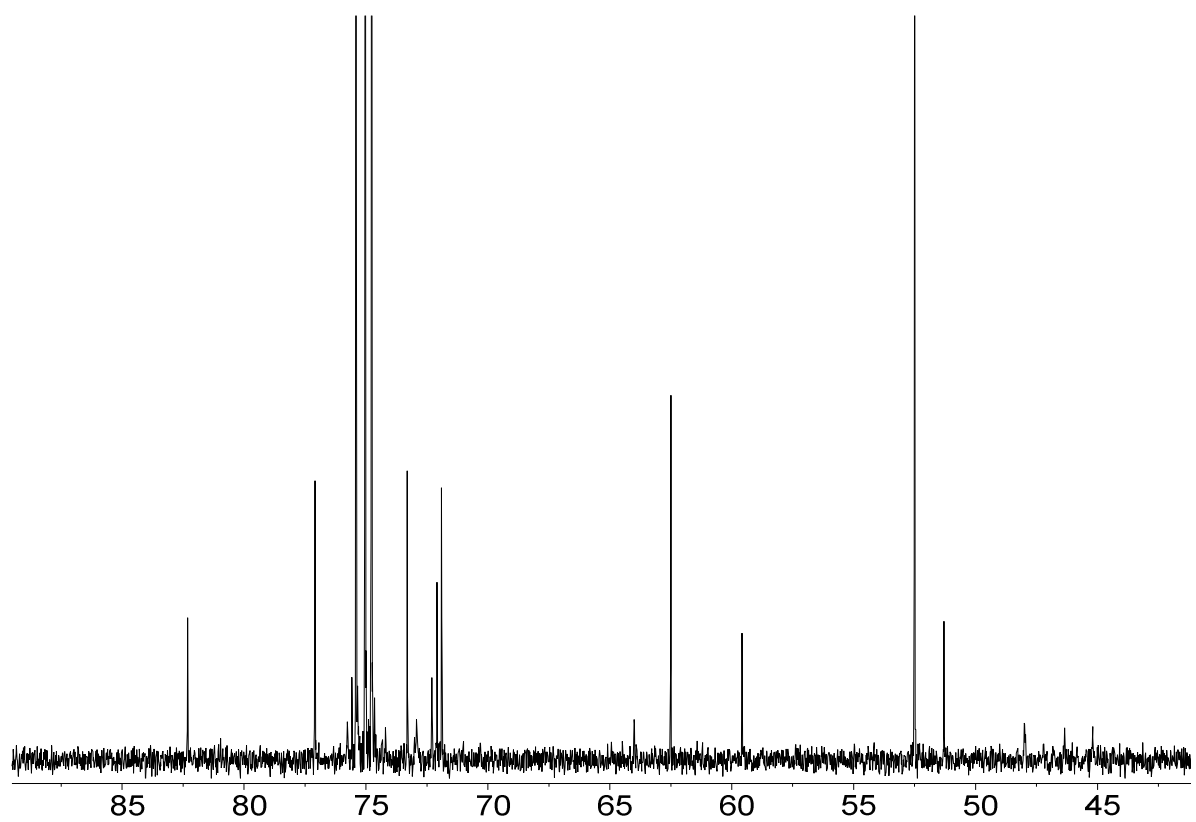
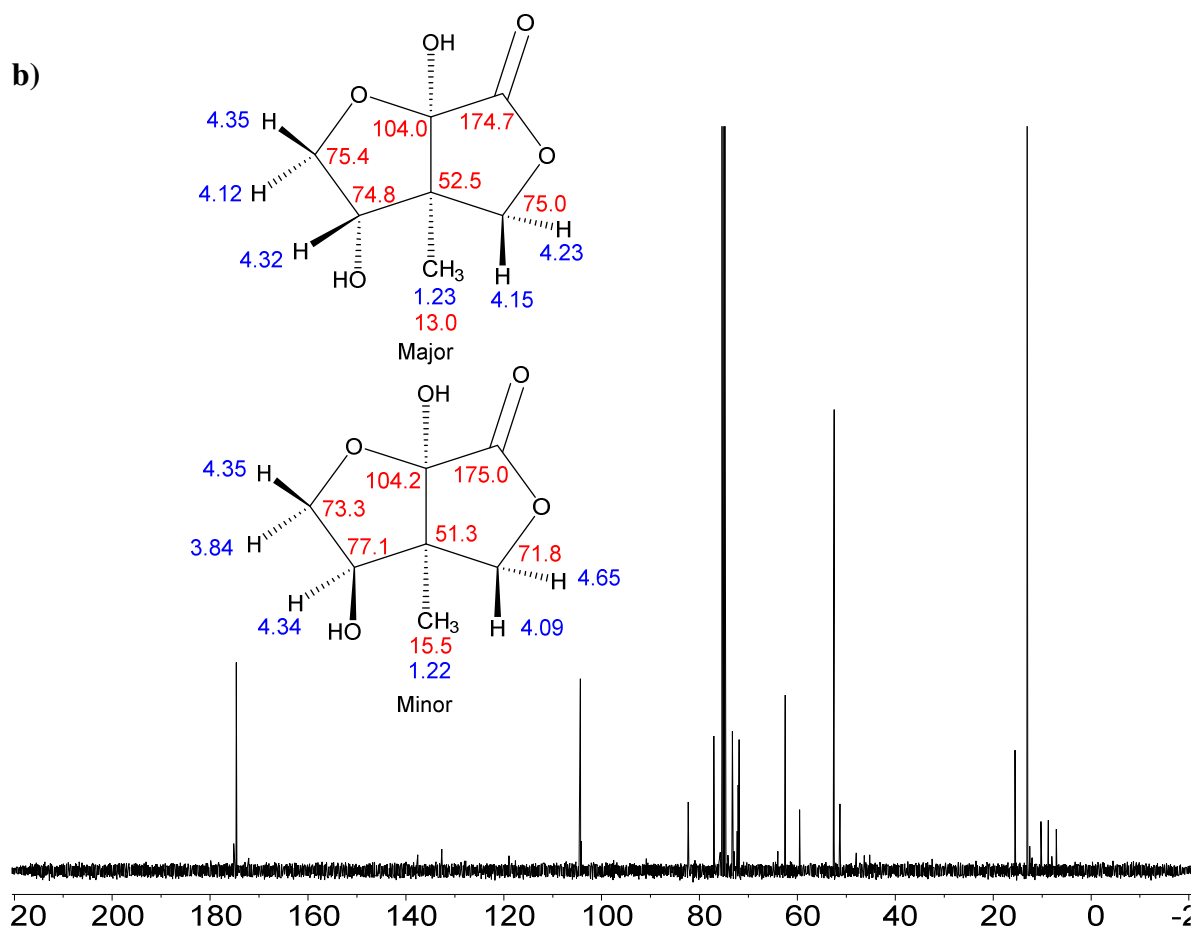


Figure S225 NMR spectra (CDCl_3) of **7j**: a) ^1H , b) ^{13}C and c) Original spectra and after adding 2 eq of *R* configured Pirkle's alcohol.

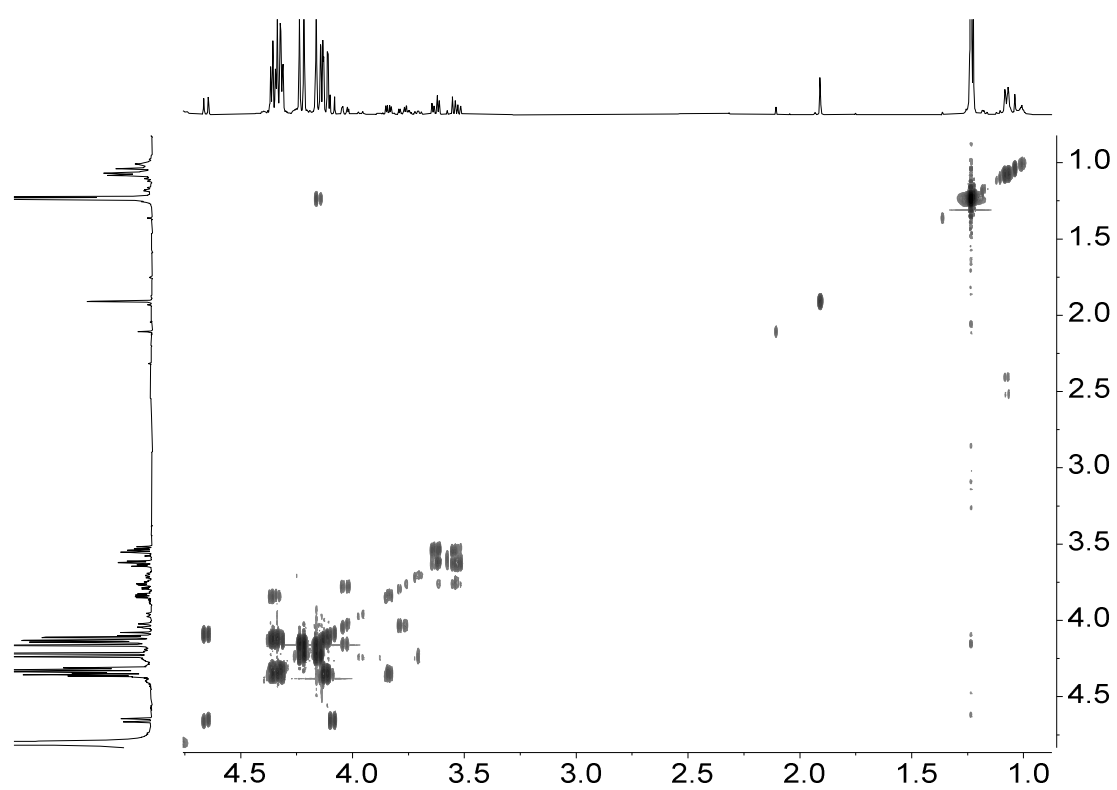
a)



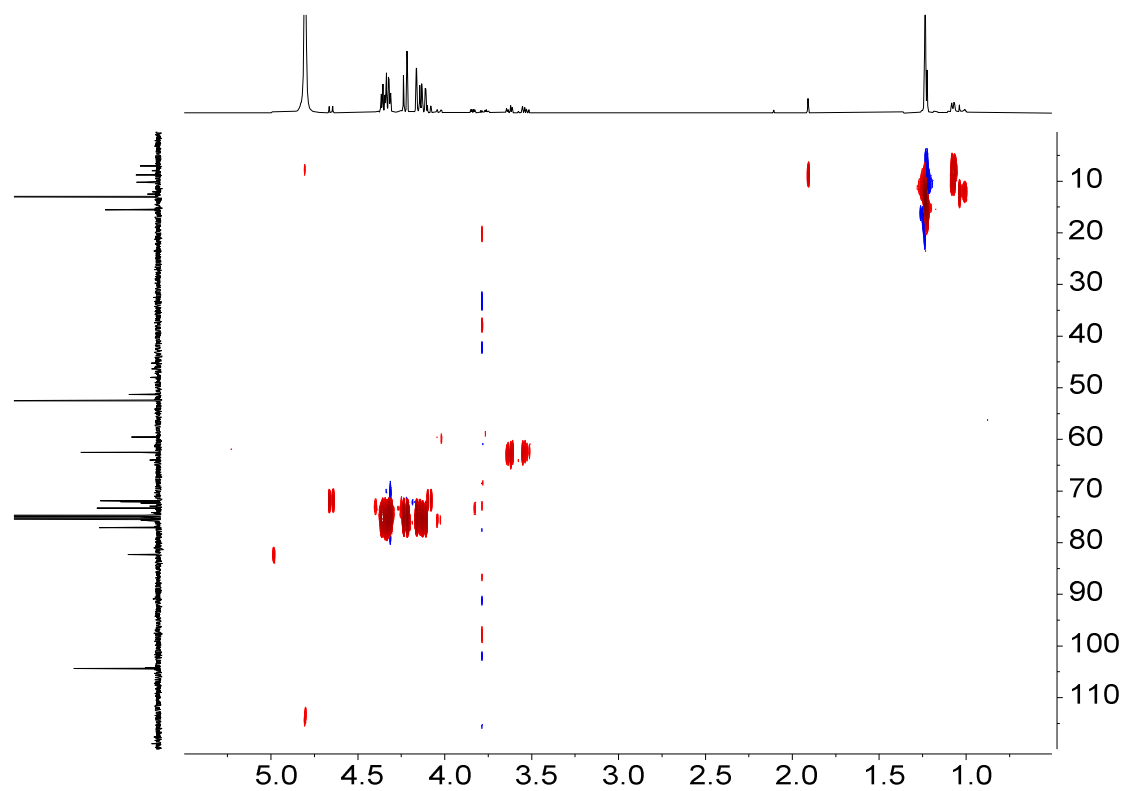
b)

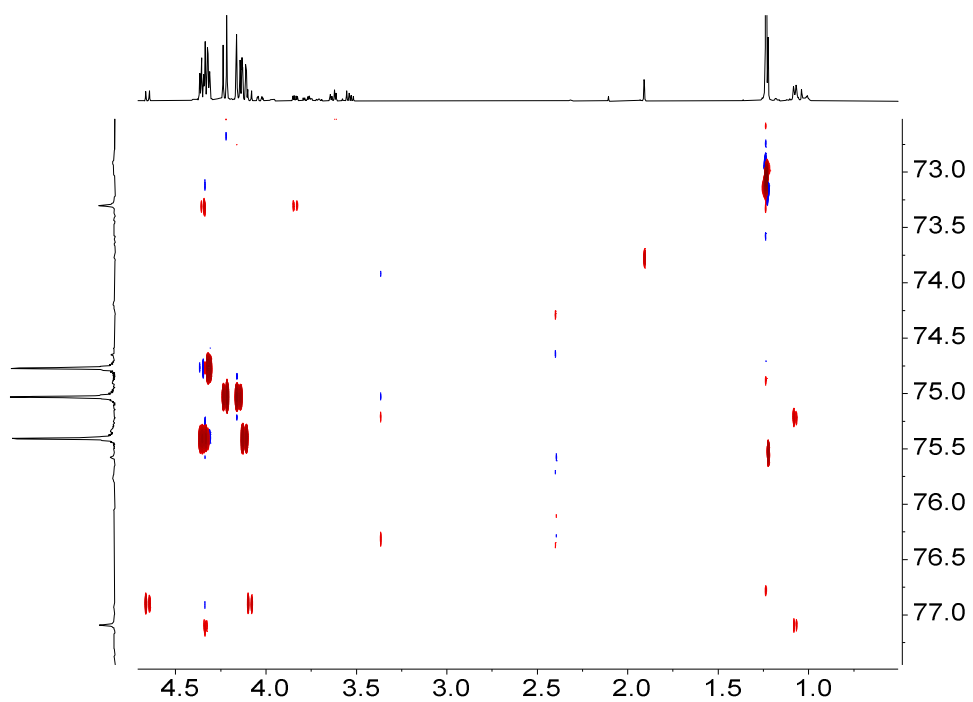


c)

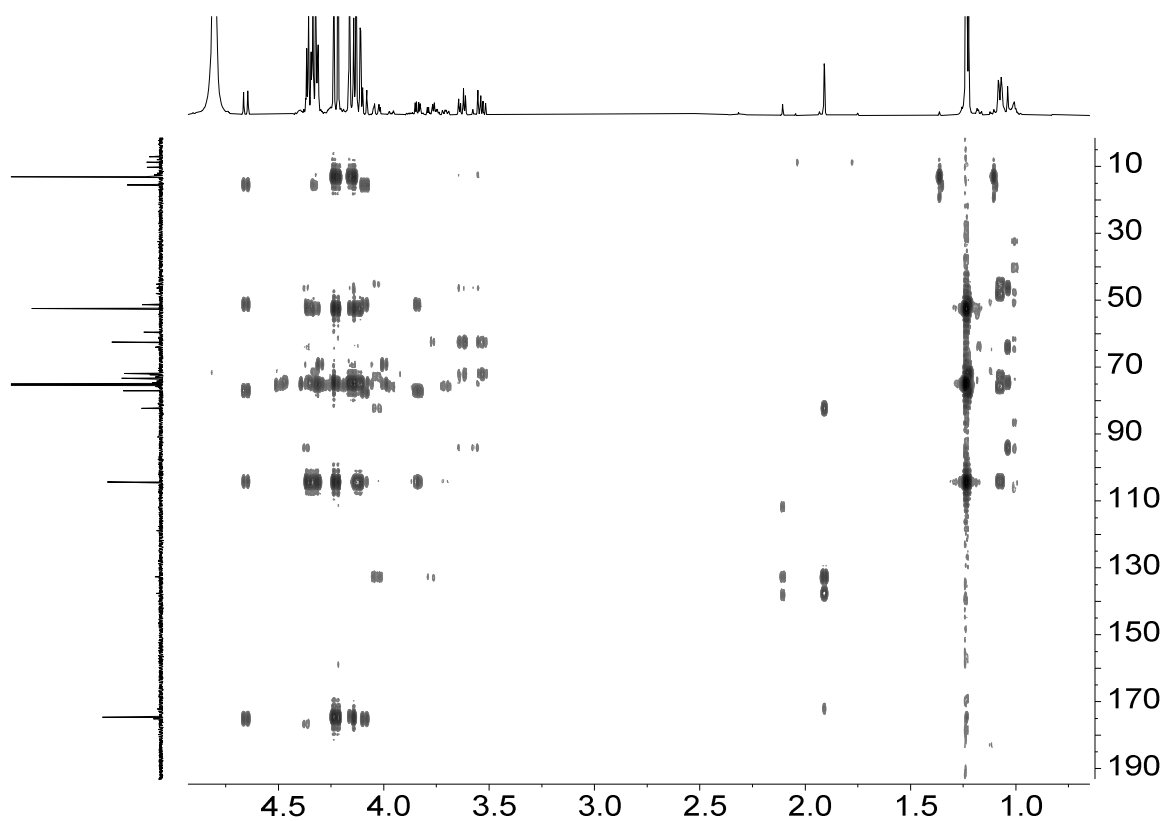


d)





e)



f)

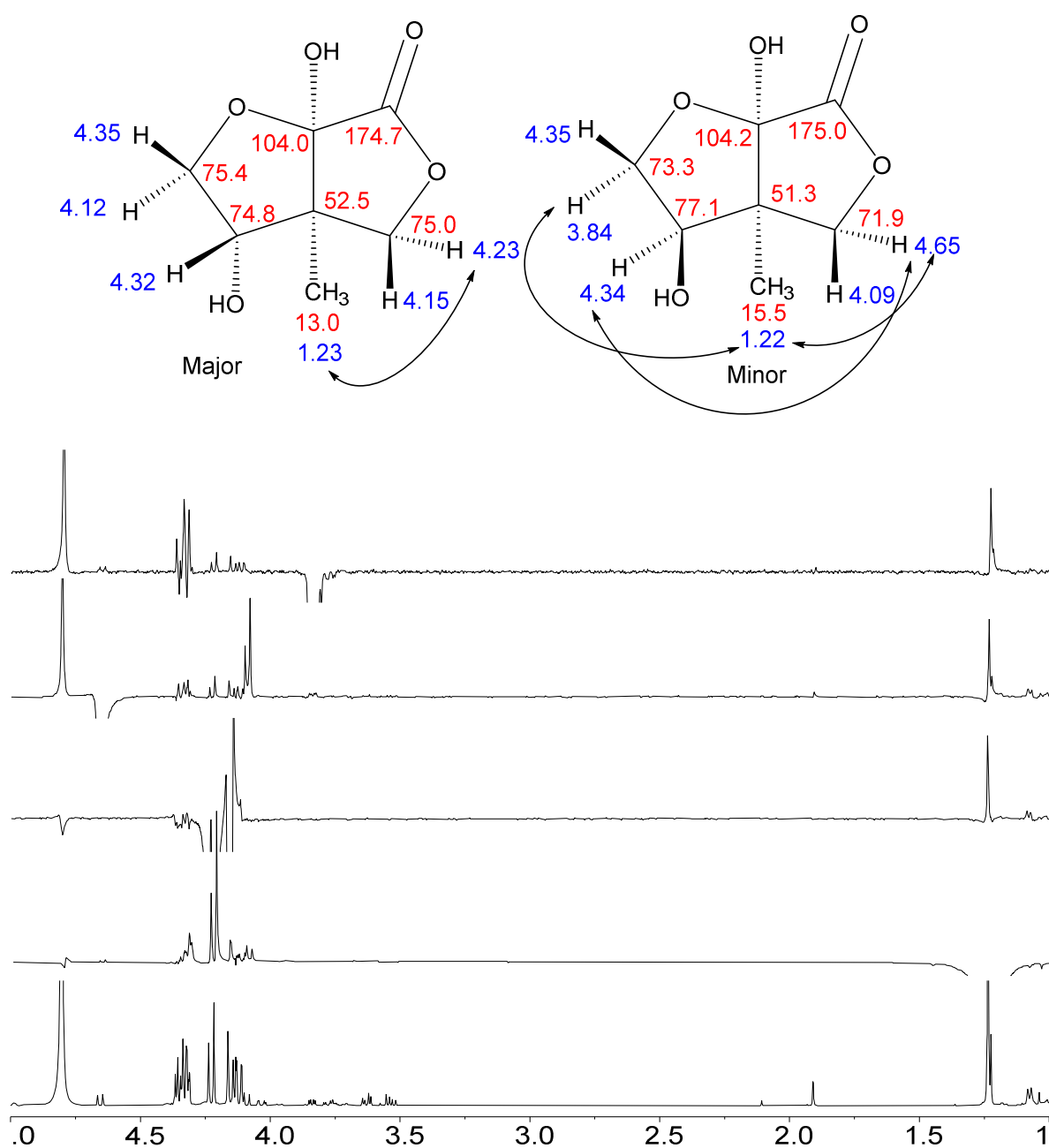
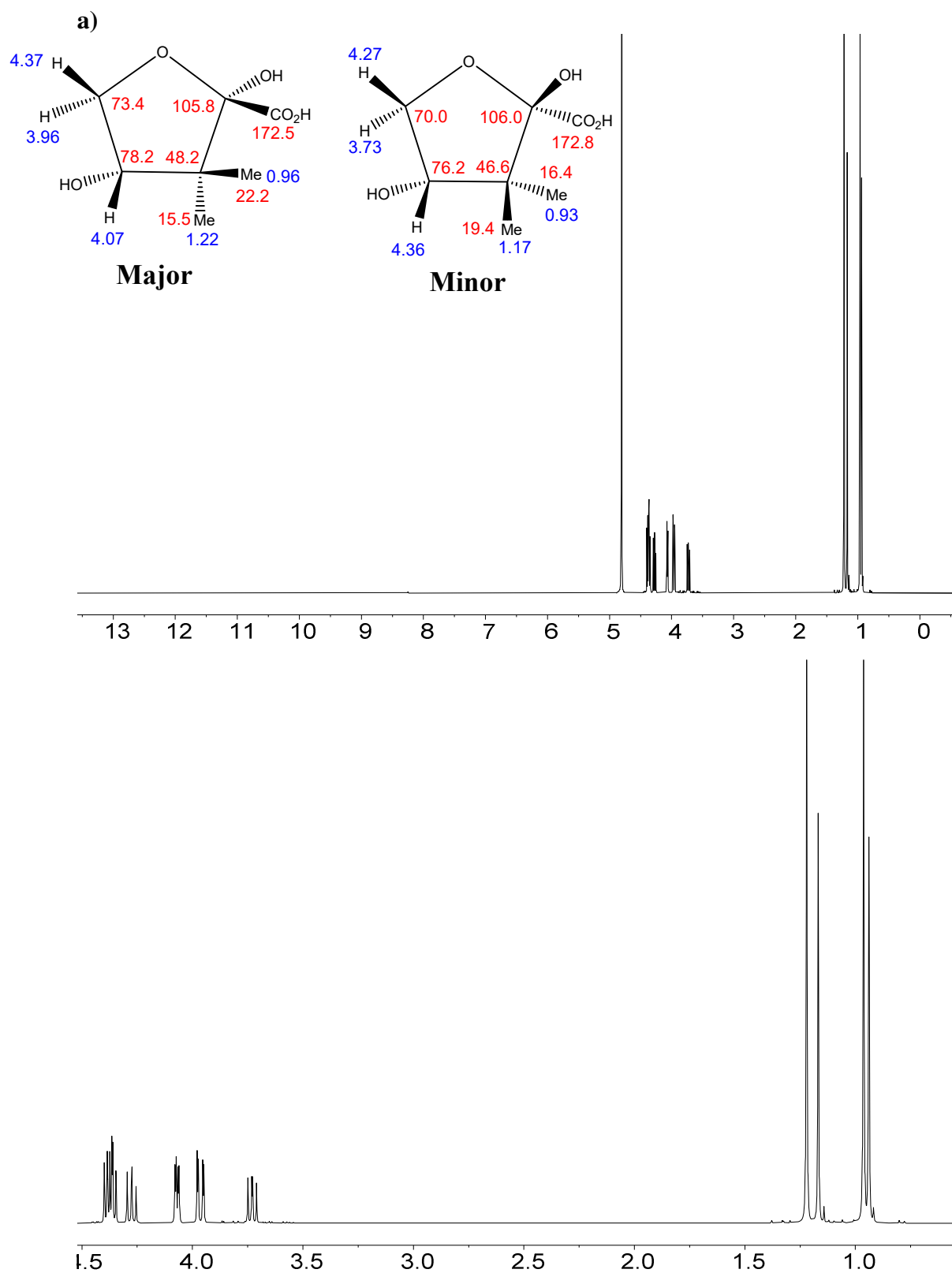
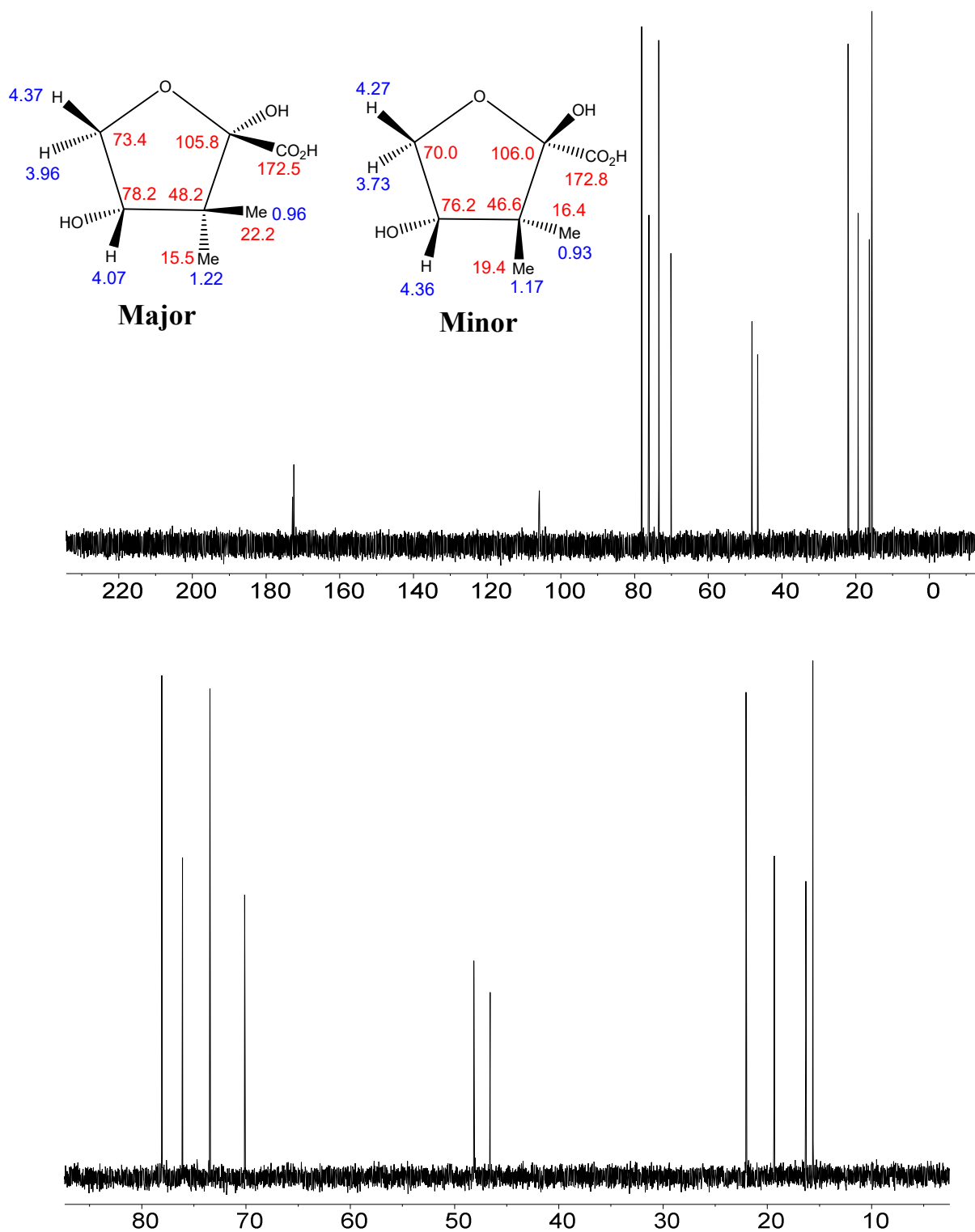


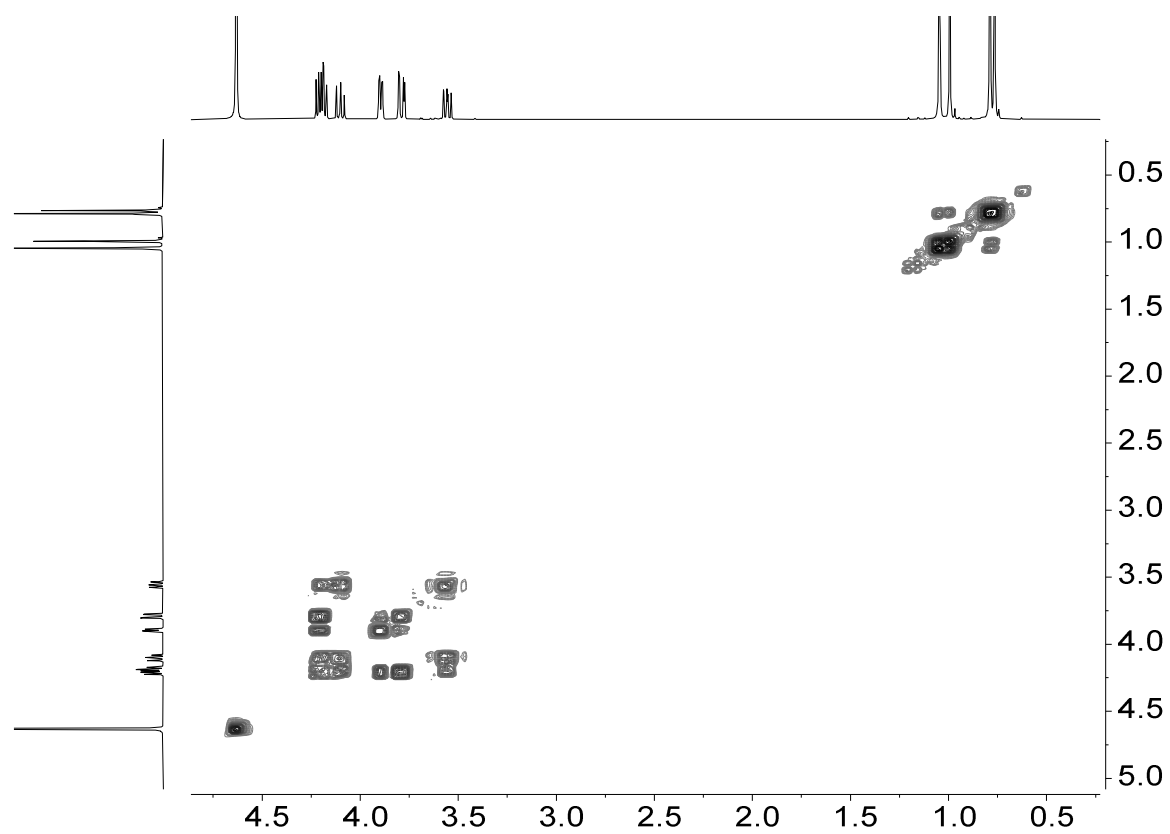
Figure S226 NMR spectra (CDCl₃) of **10a** and **10b**: a) ¹H, b) ¹³C, c) gCOSY d) HSQC, e) HMBC and f) Selective 1D nOe. E171-157-exp104.



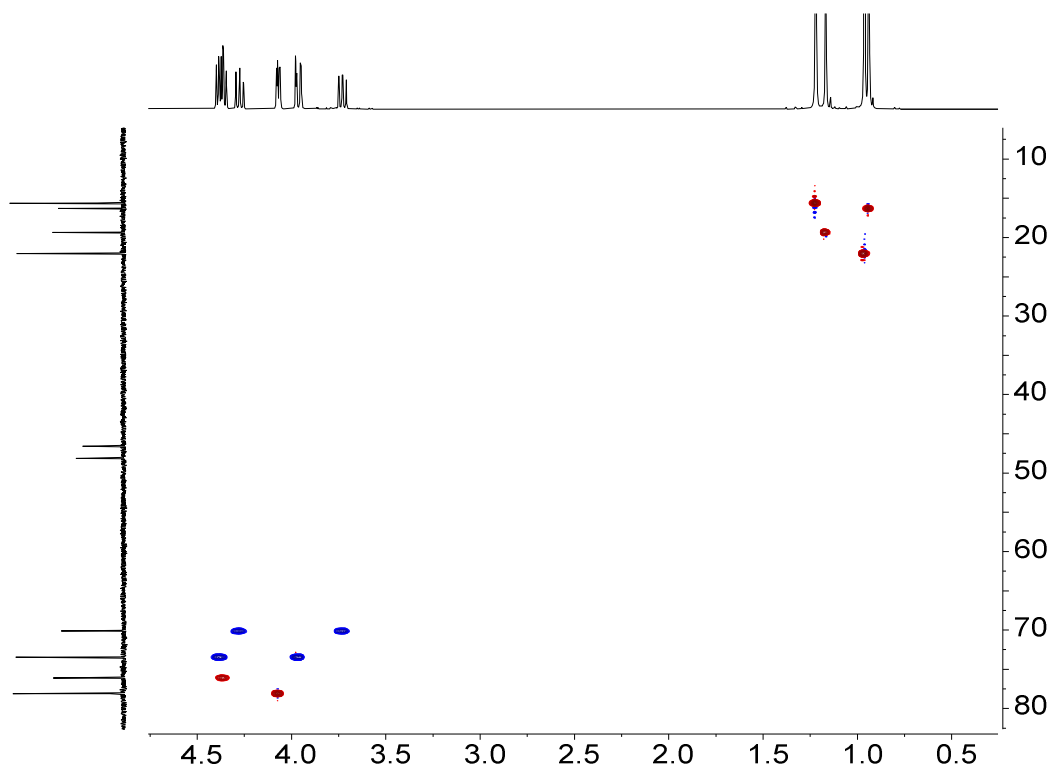
b)



c)



d)



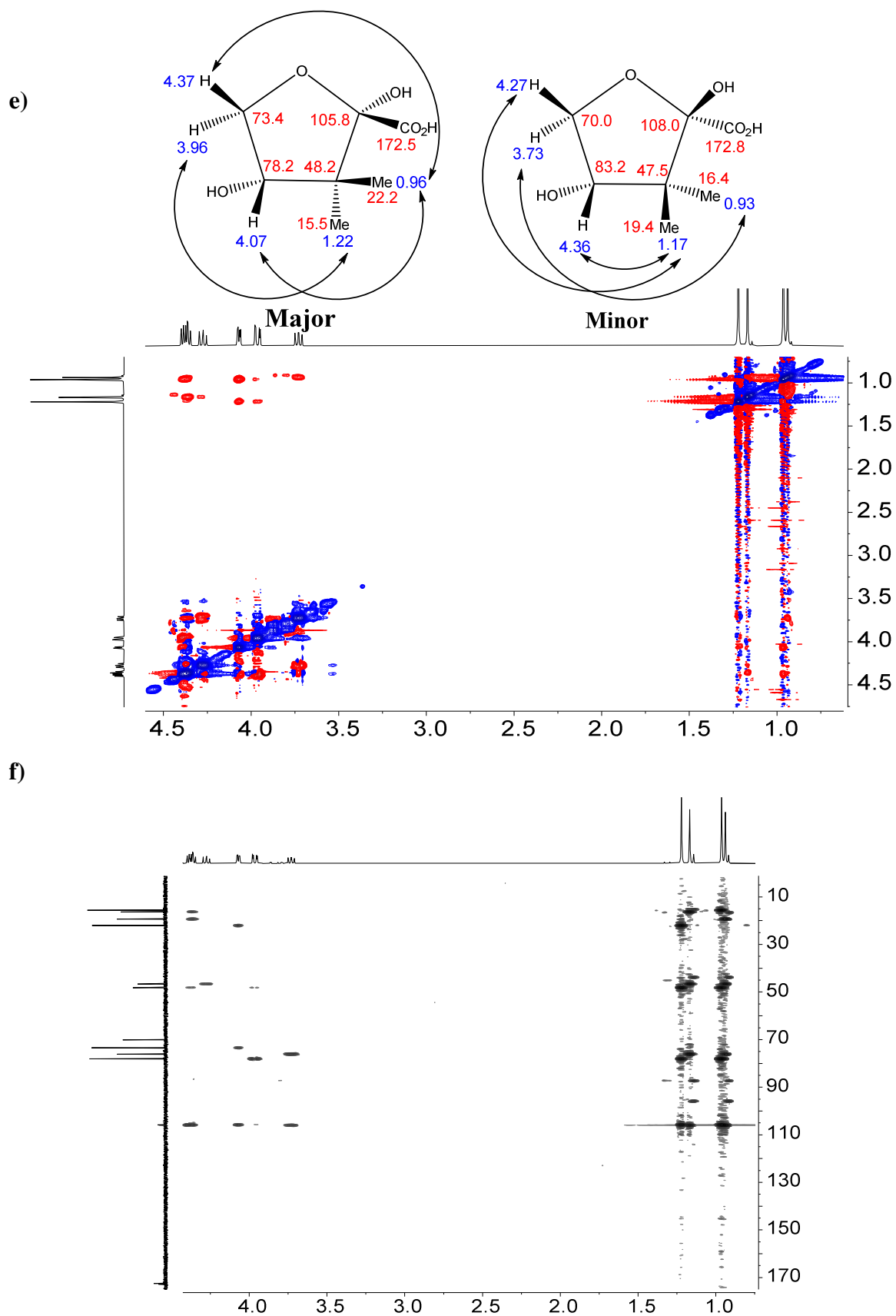
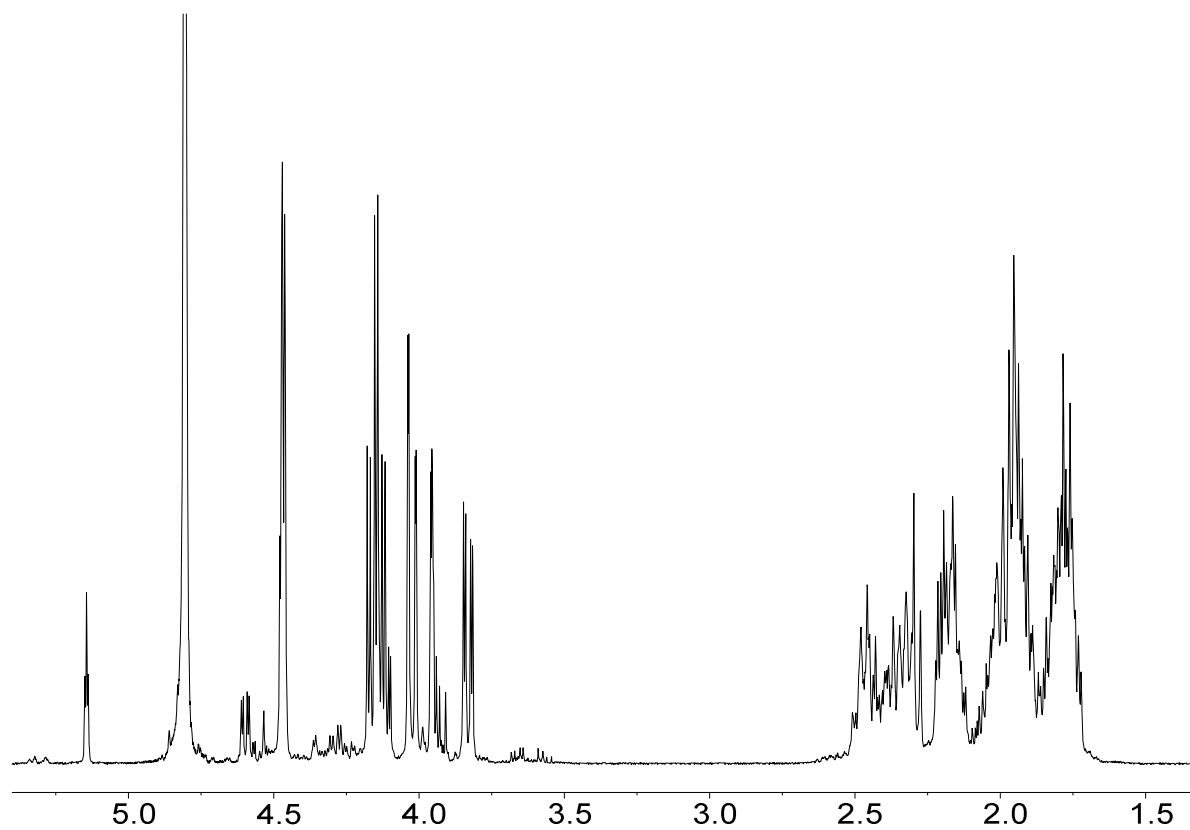
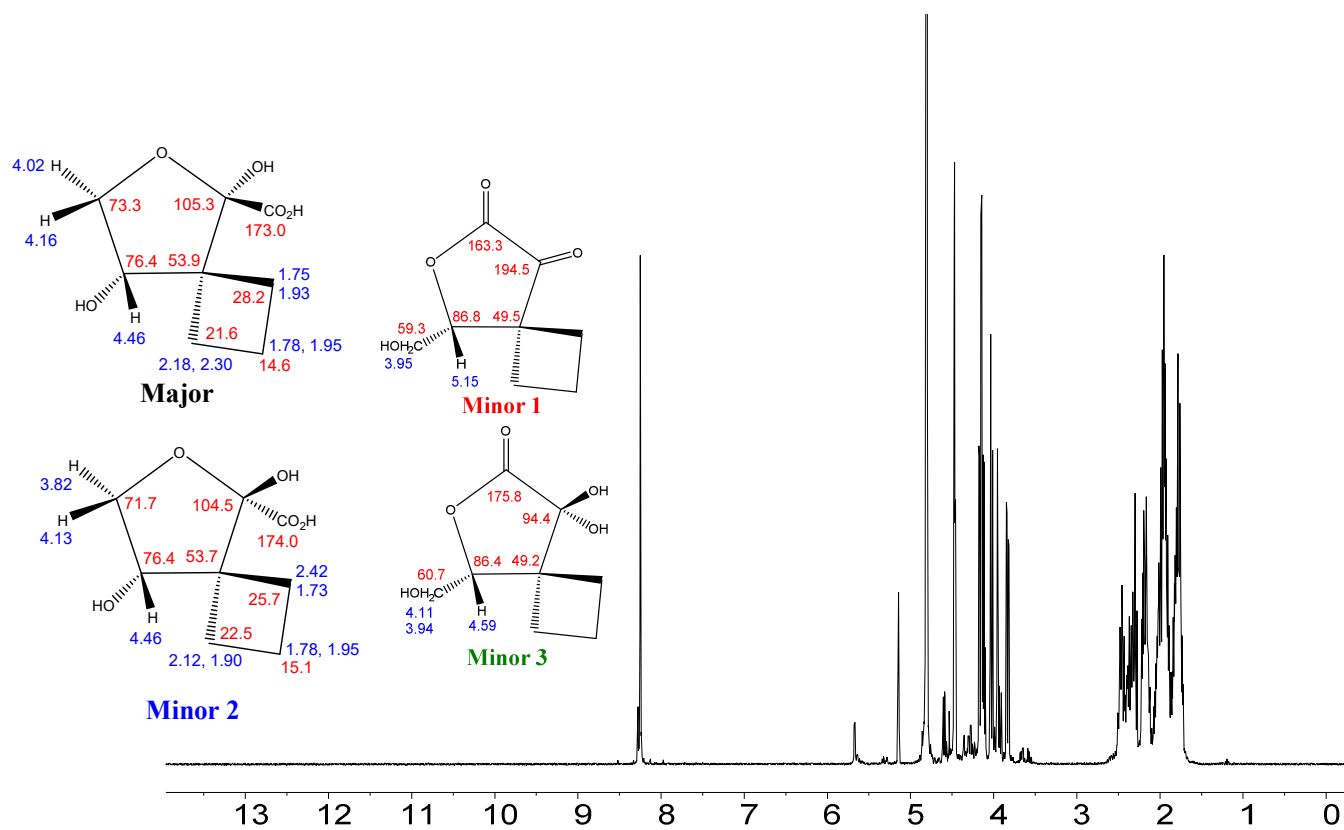
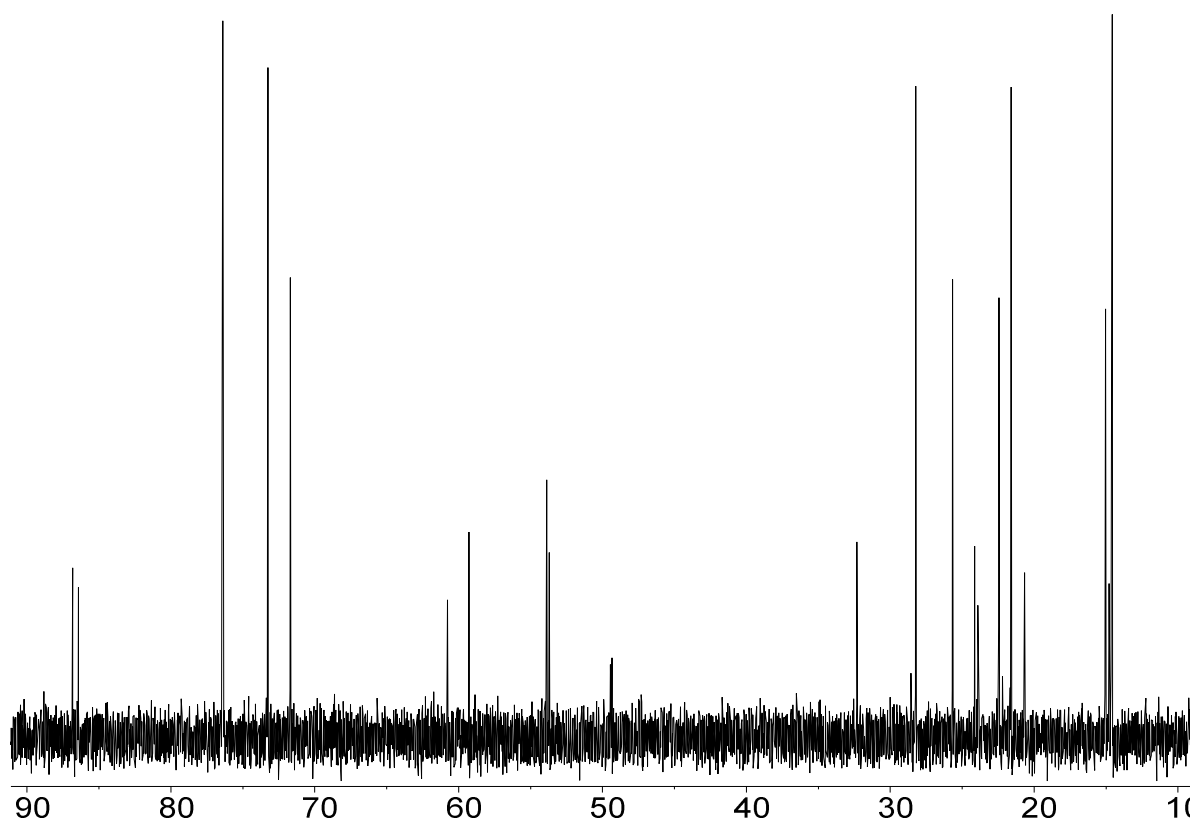
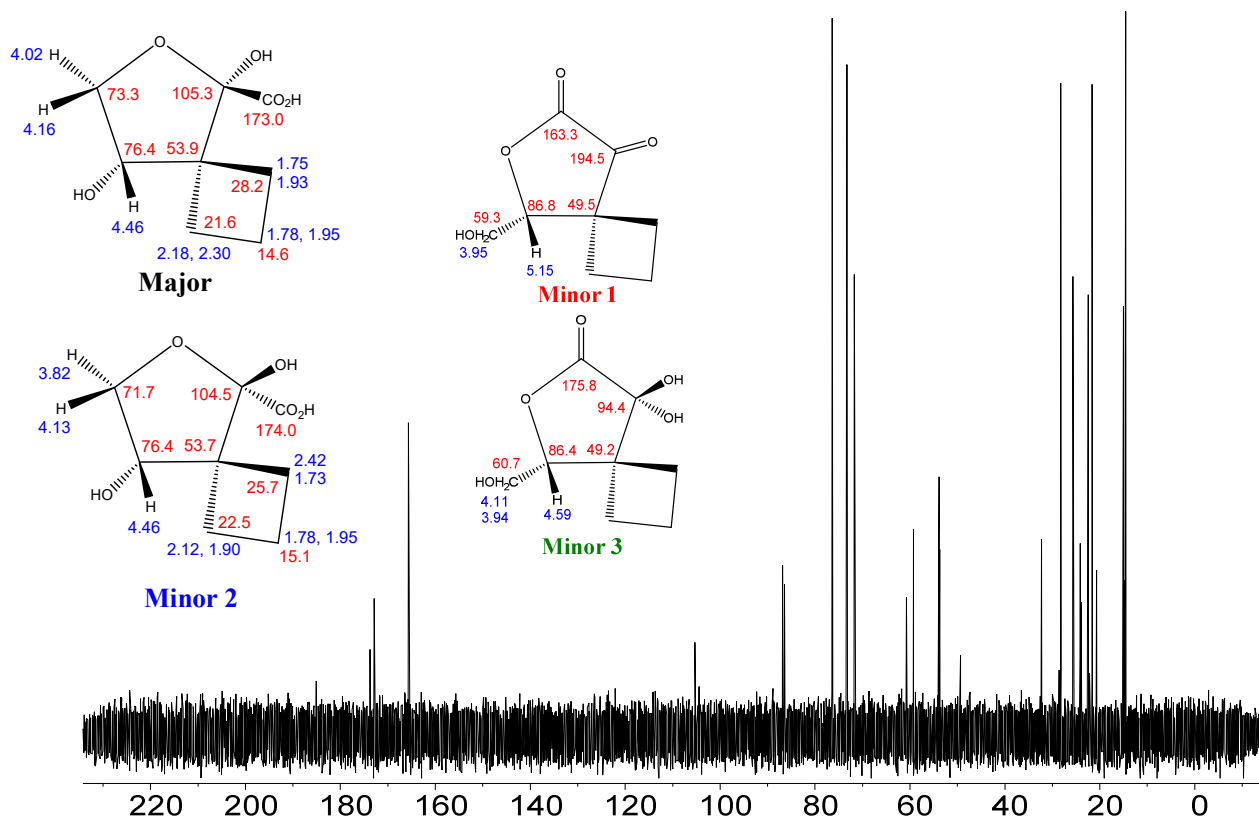


Figure S227 NMR spectra (D_2O) of **9a**: a) 1H , b) ^{13}C , c) gCOSY d) gHSQC e) 2D NOESY and f) HMBC. E178-18-exp9.

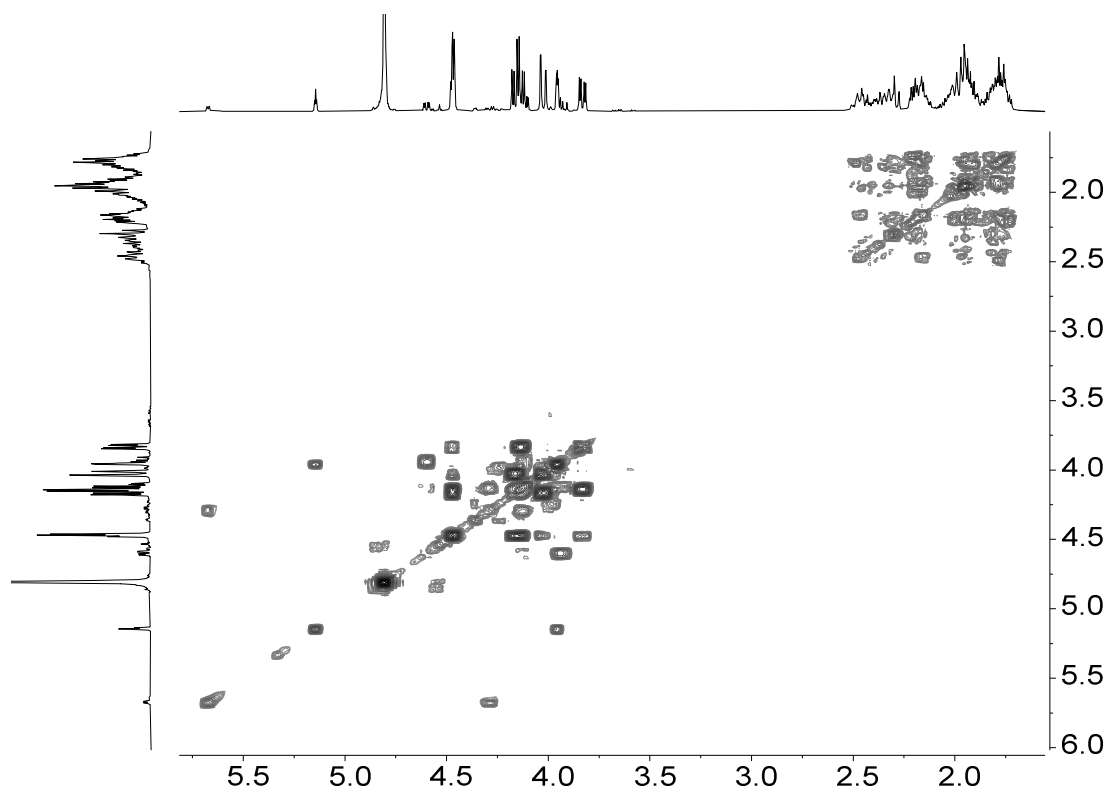
a)



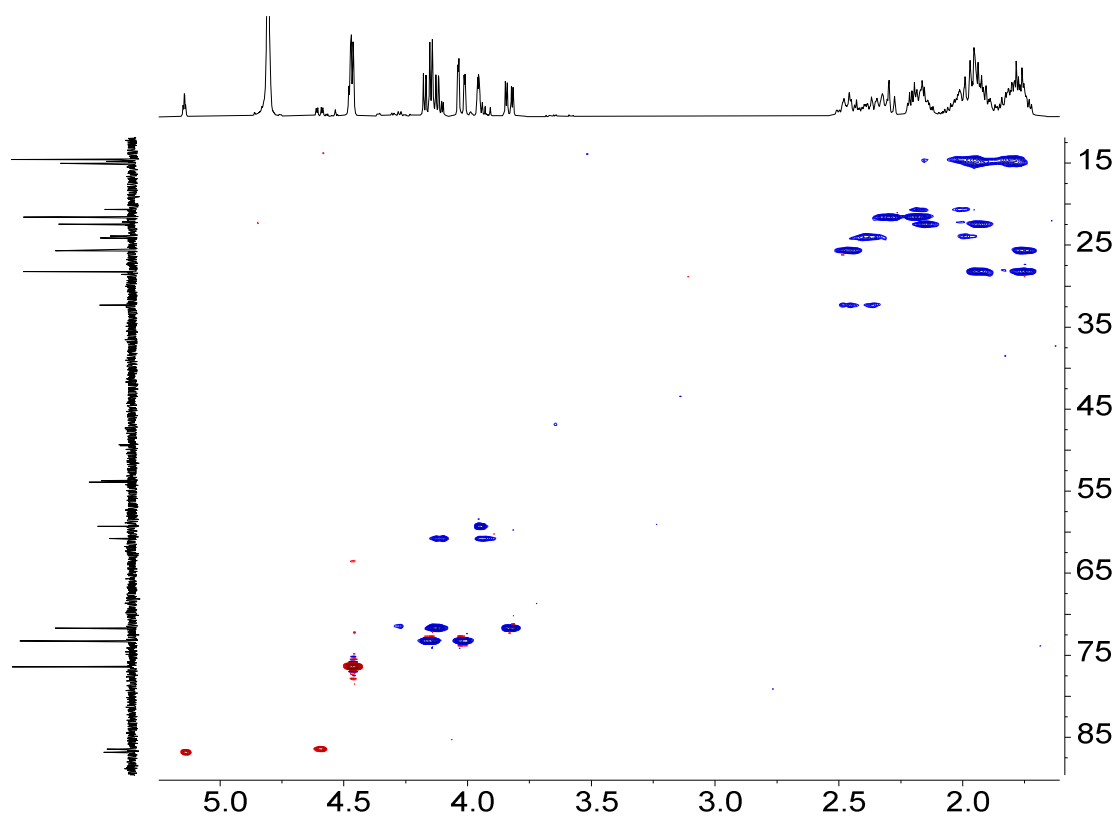
b)



c)



d)



e)

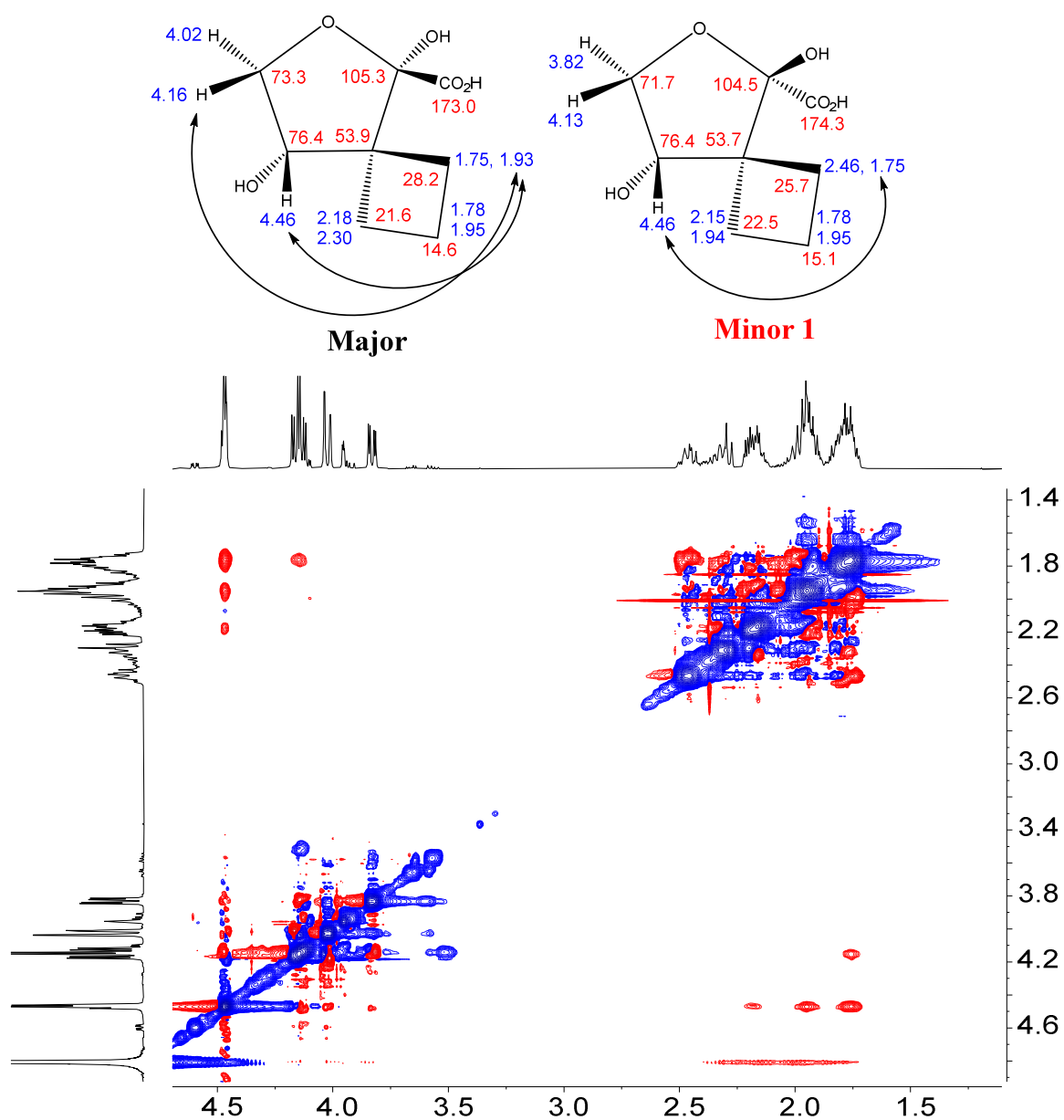
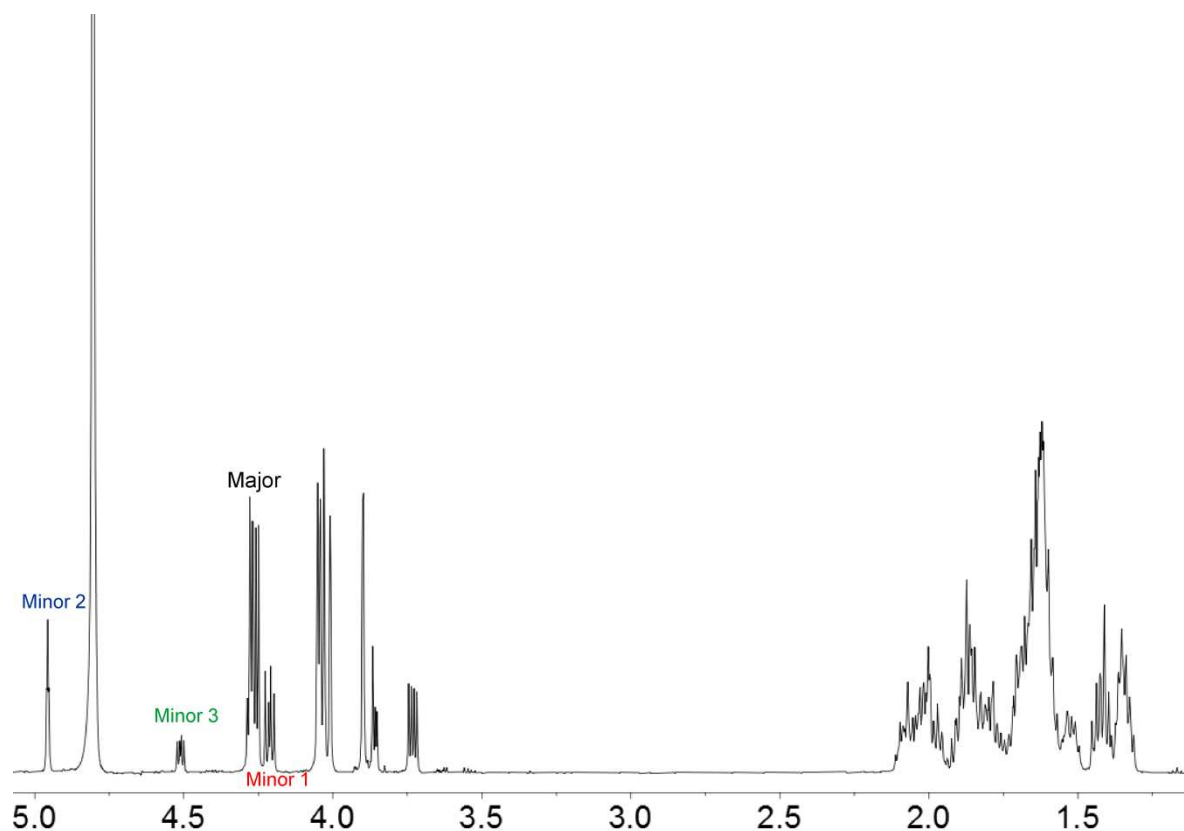
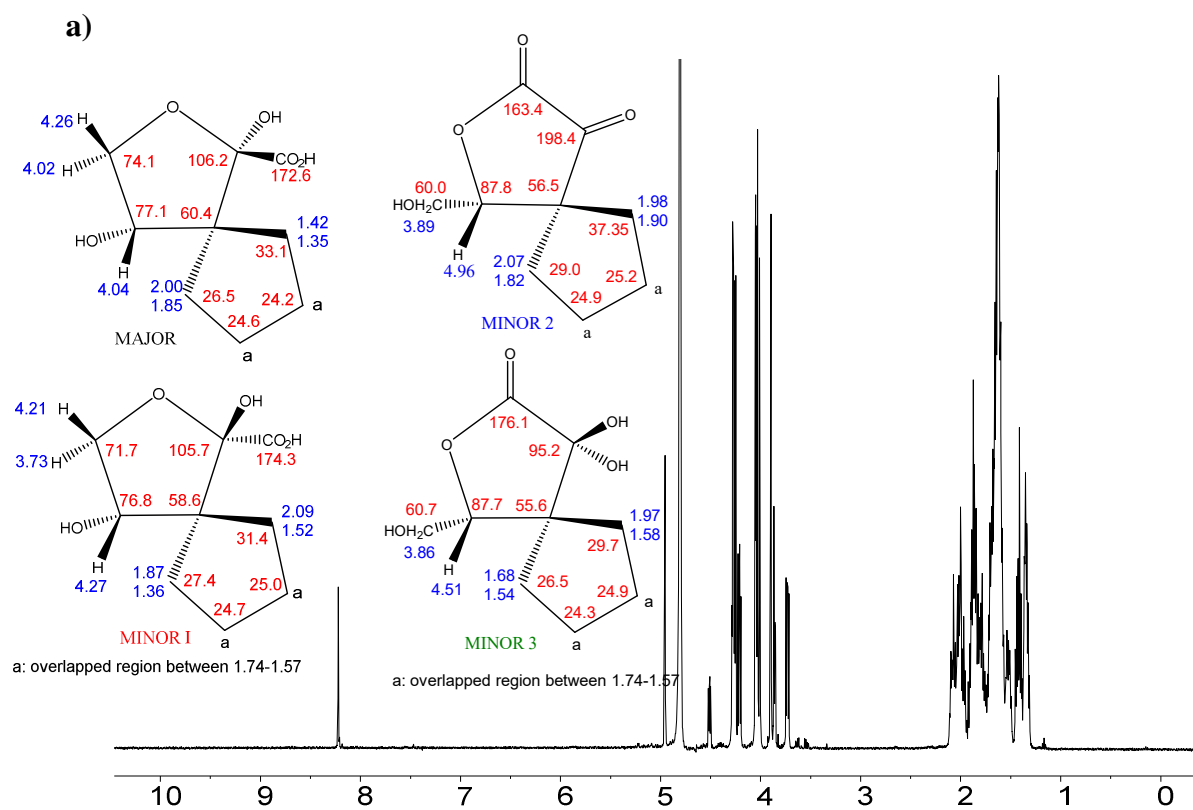
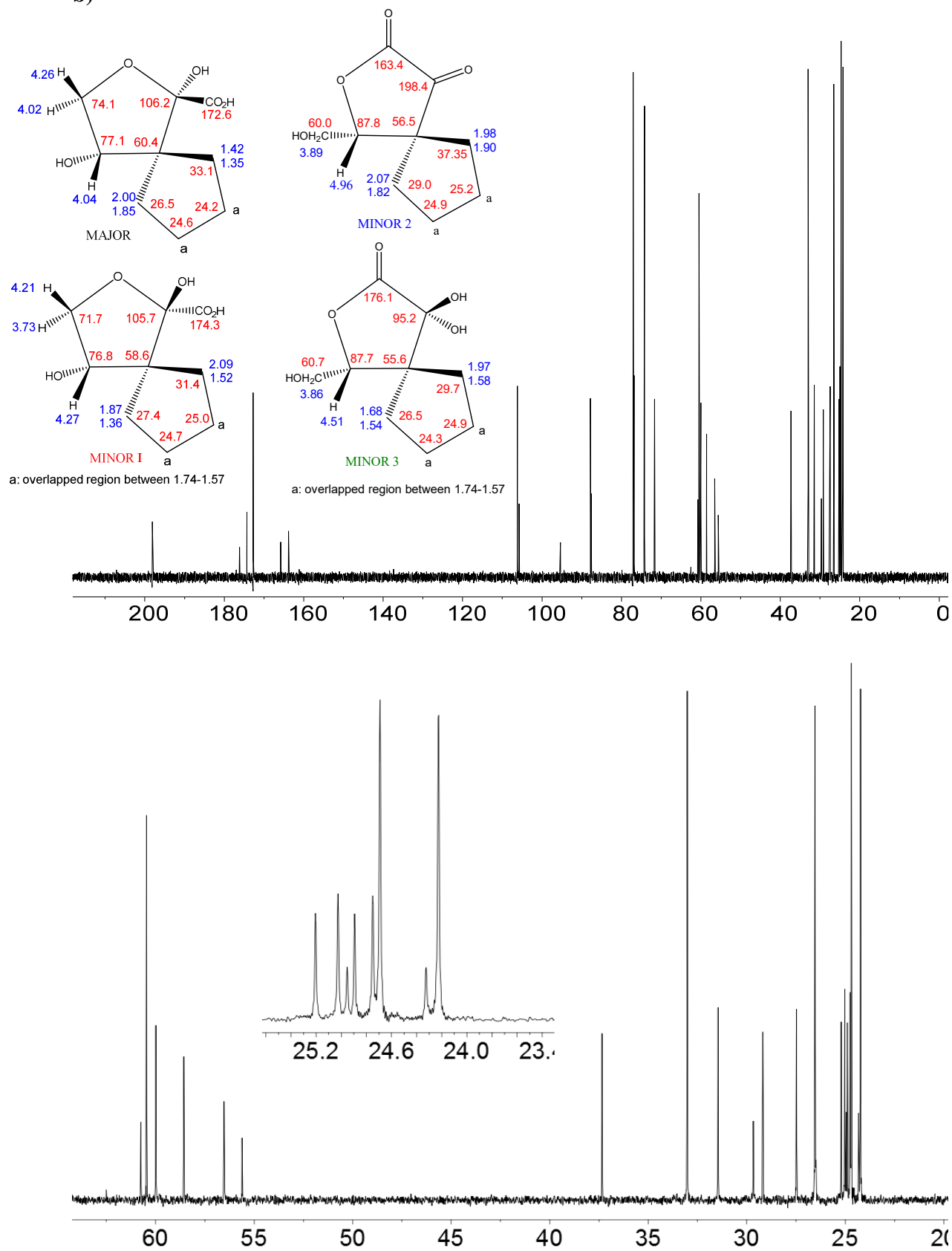


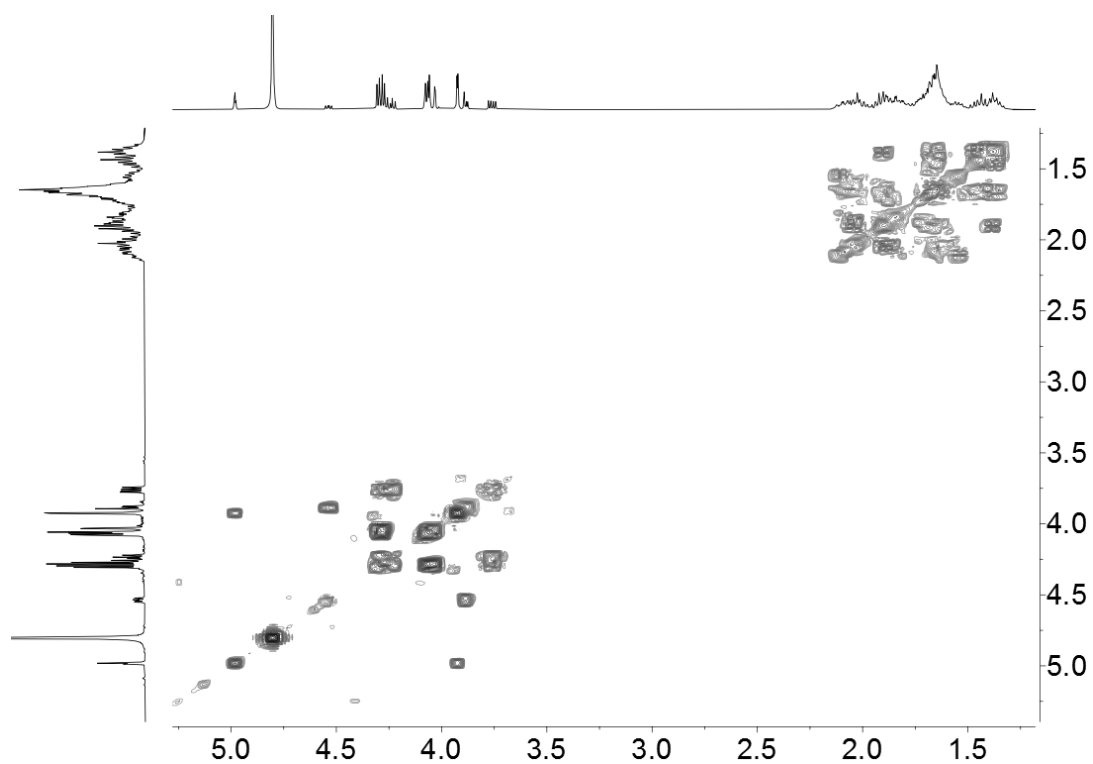
Figure S228 NMR spectra (D₂O) of **9b**: a) ¹H, b) ¹³C, c) gCOSY d) gHSQC and e) 2D NOESY. E178-16-exp8.



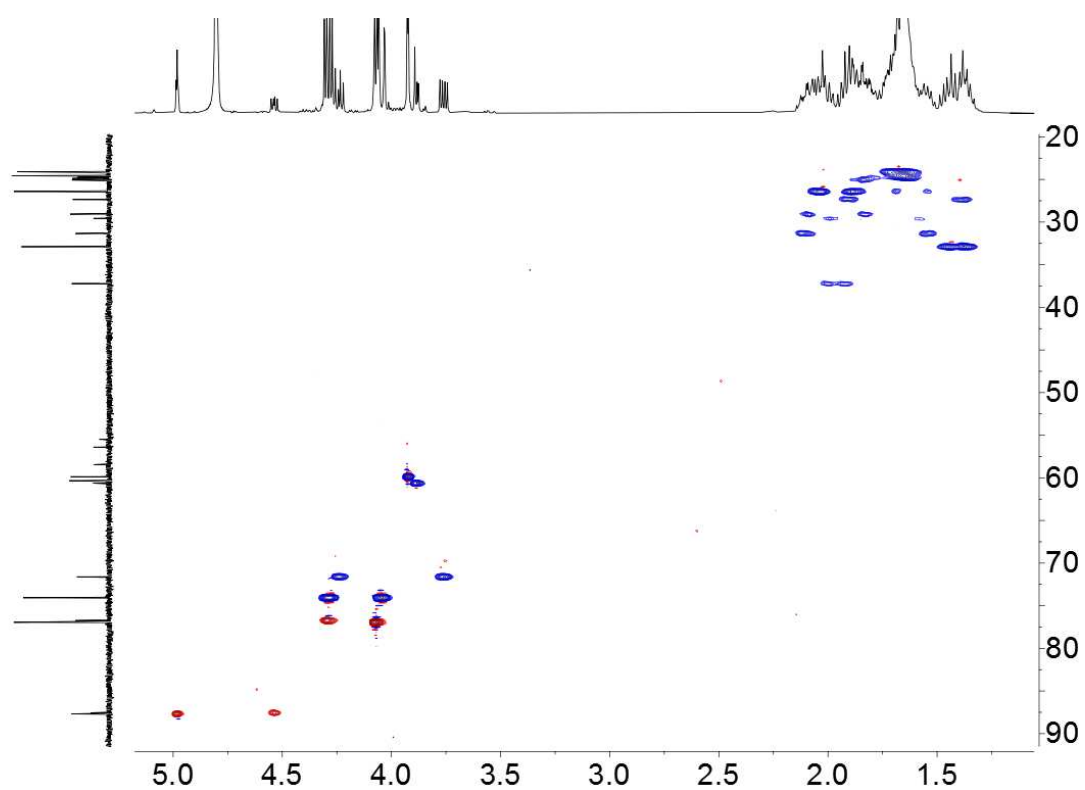
b)



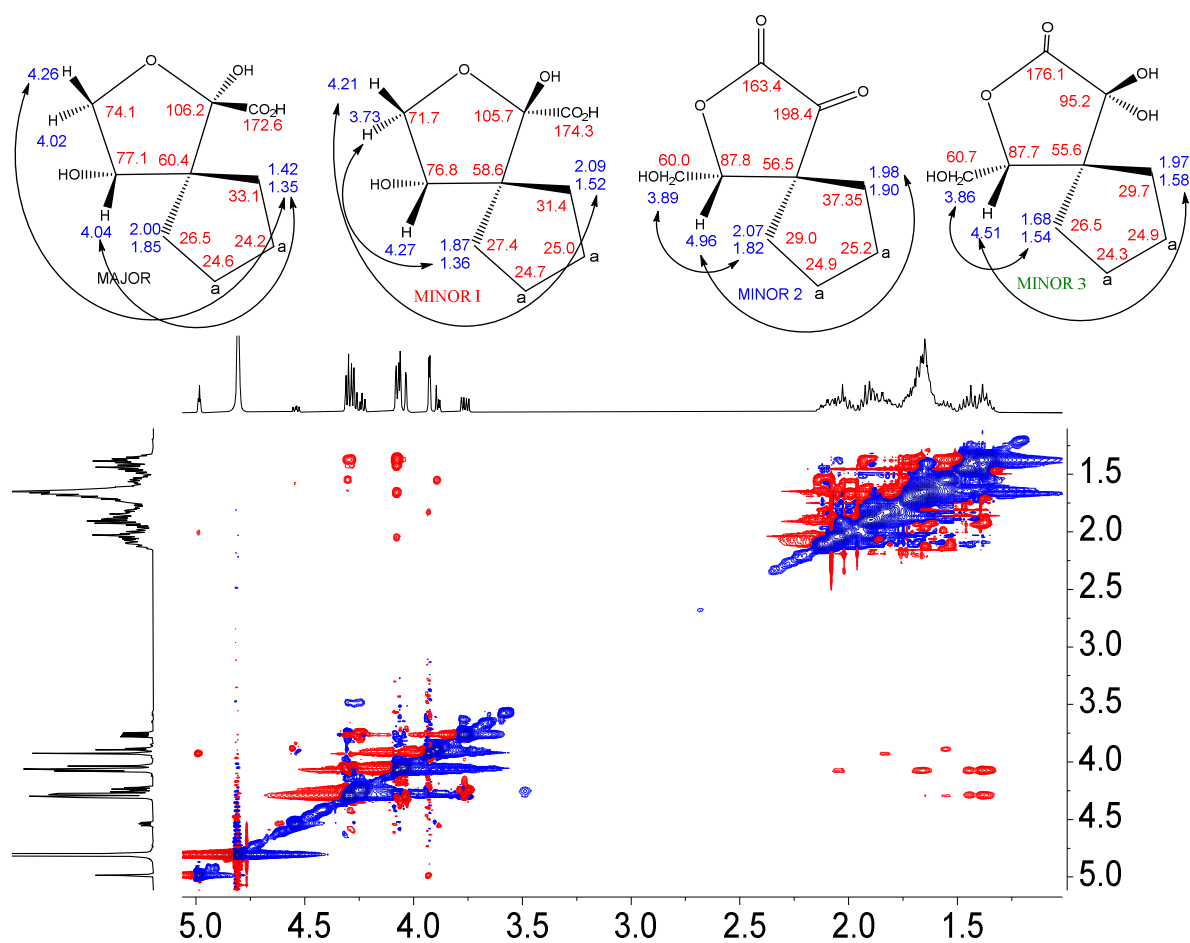
c)



d)



e)



f)

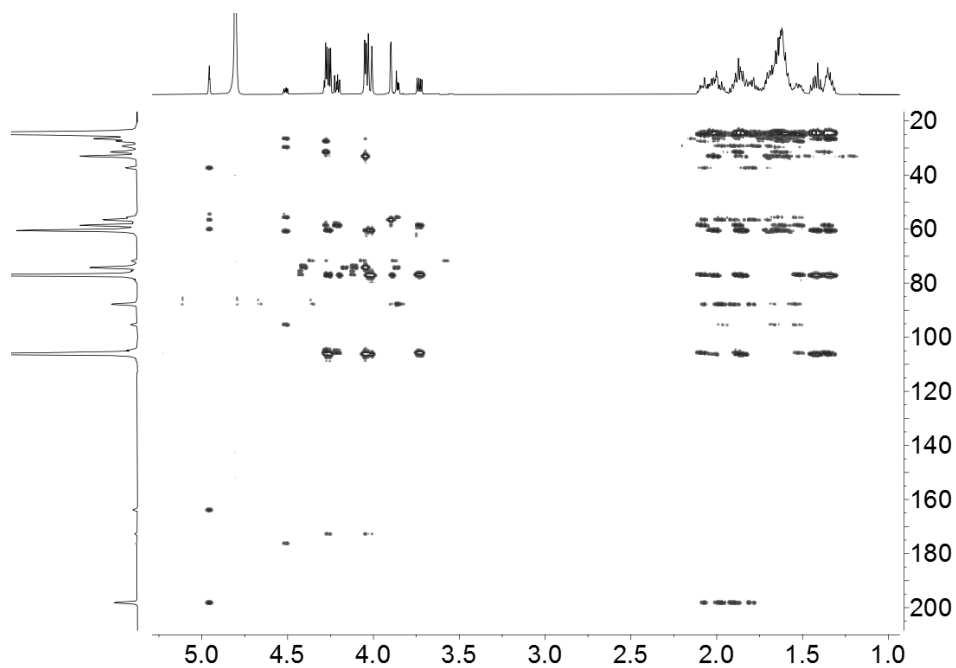
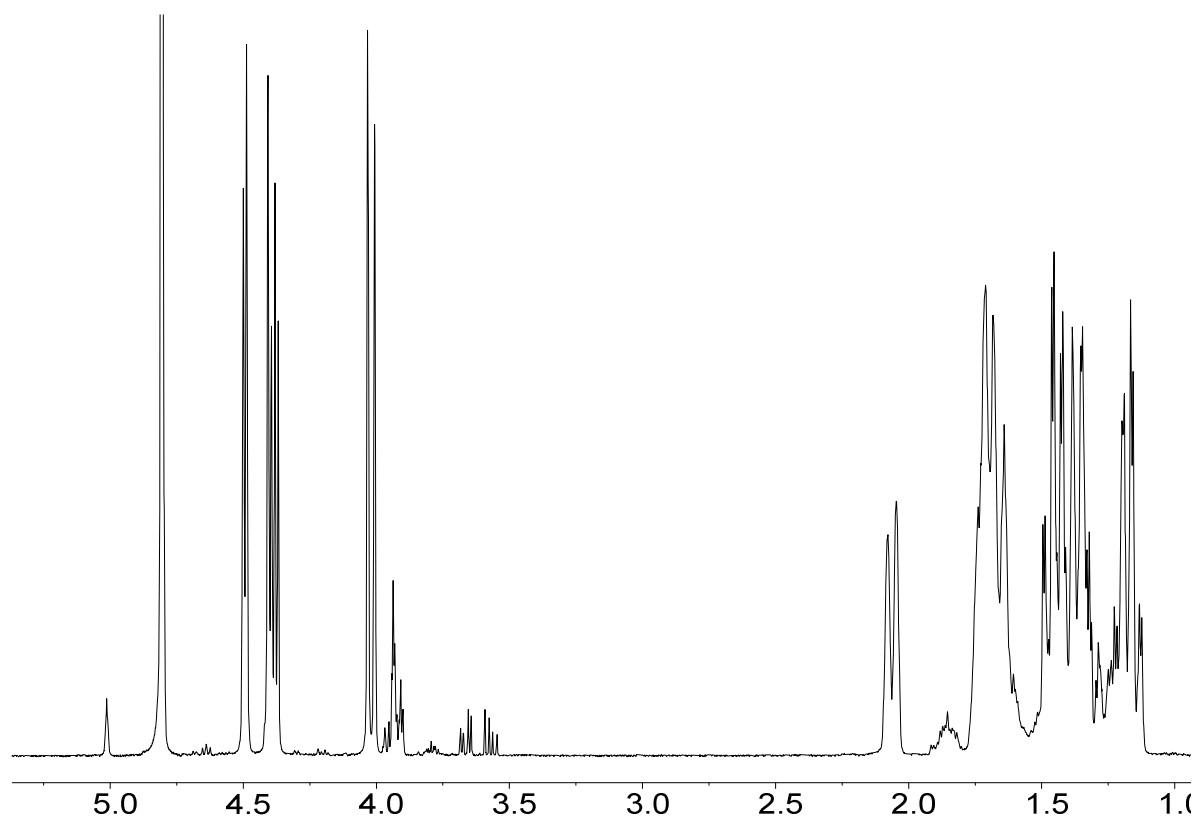
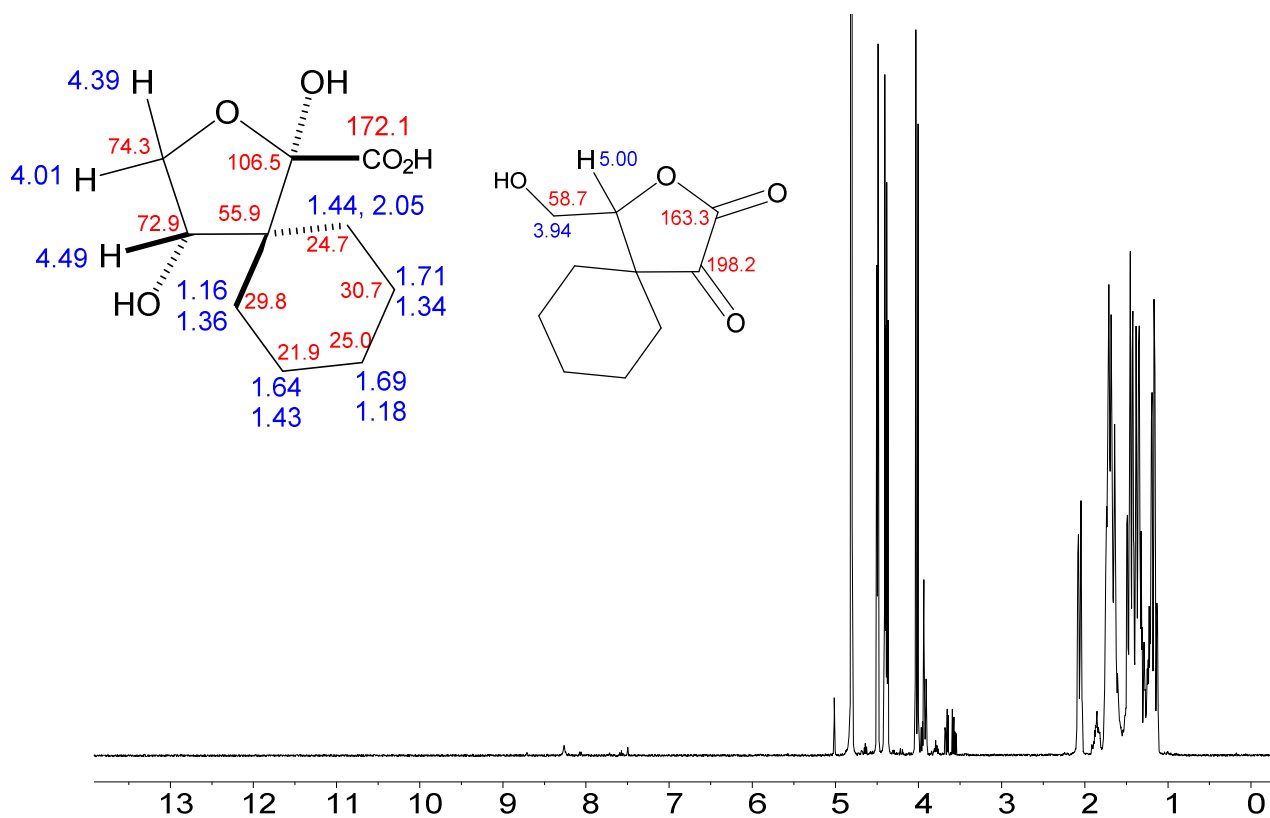
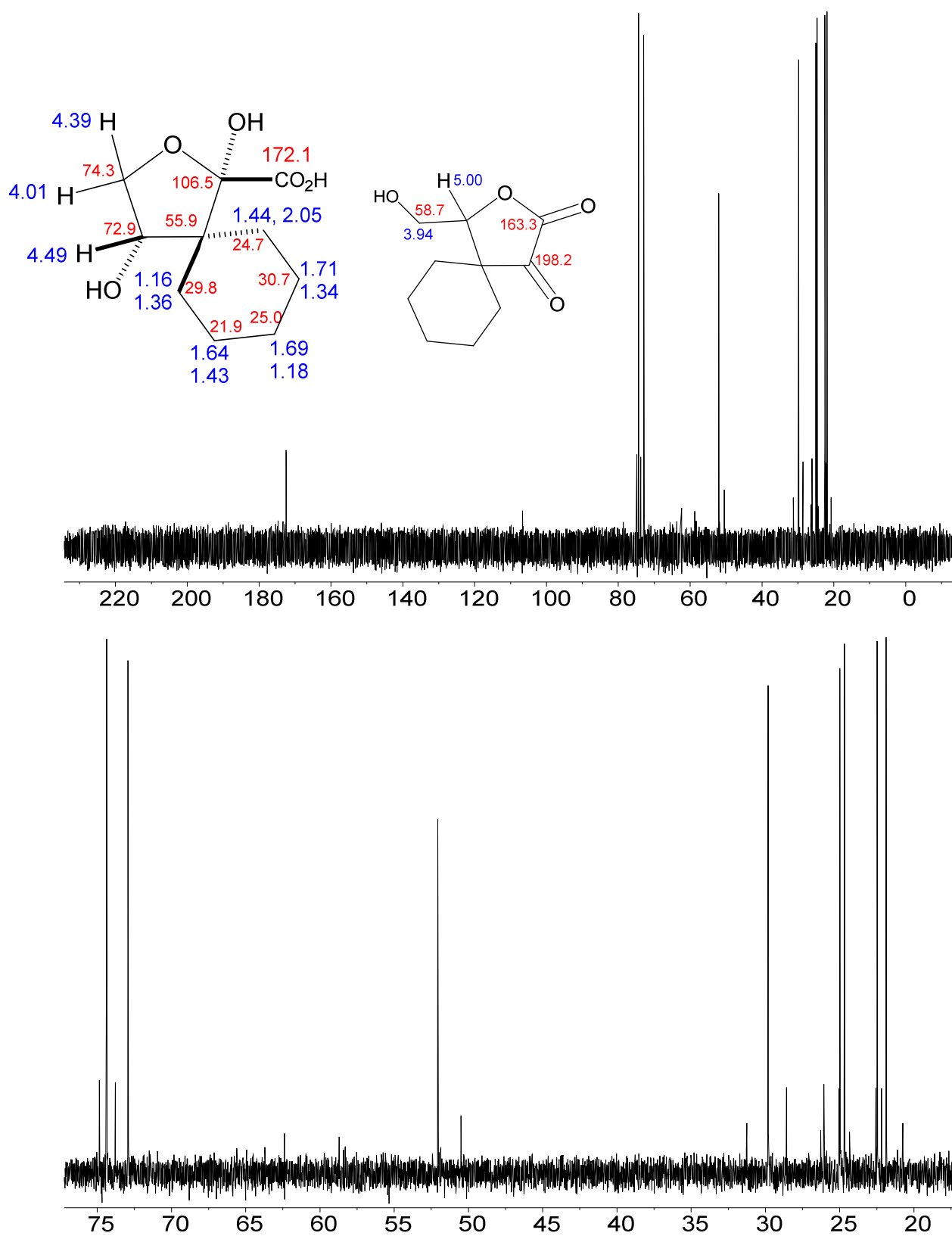


Figure S229 NMR spectra (D₂O) of **9c**: a) ¹H, b) ¹³C, c) gCOSY d) gHSQC, e) 2D NOESY and f) HMBC. E178-23-exp12.

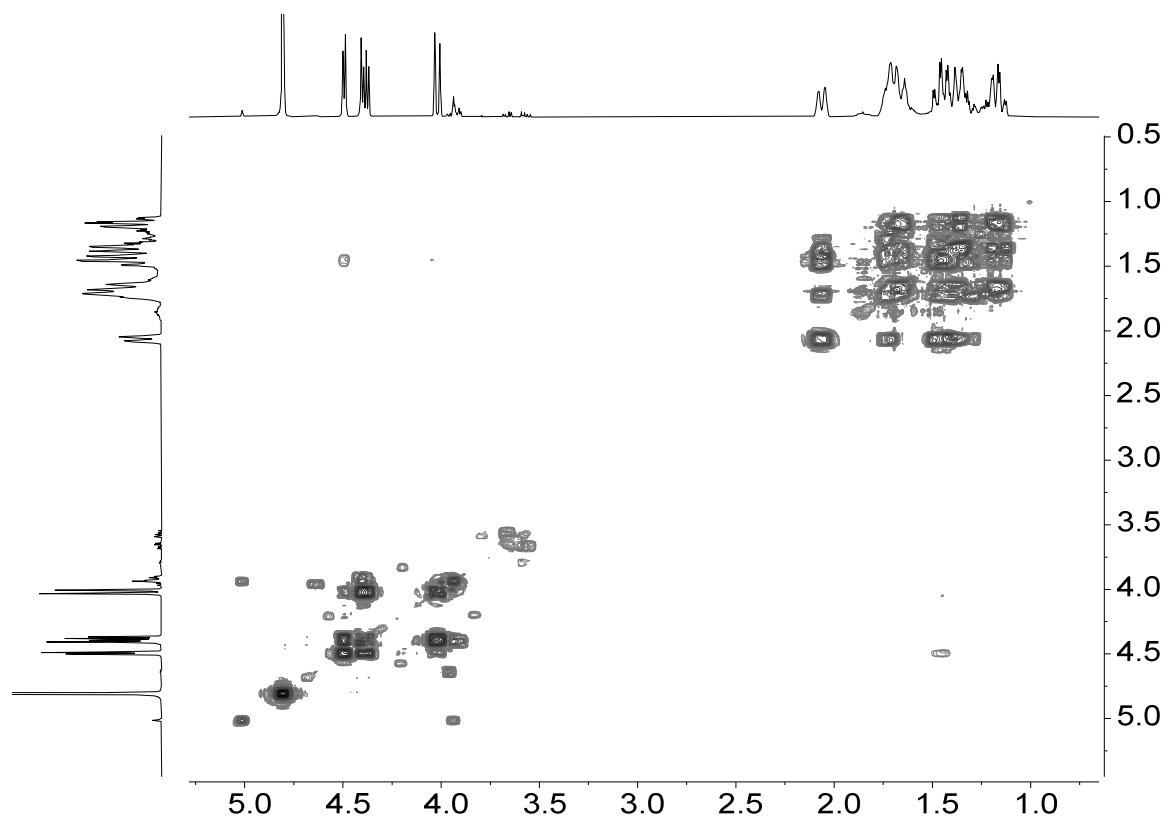
a)



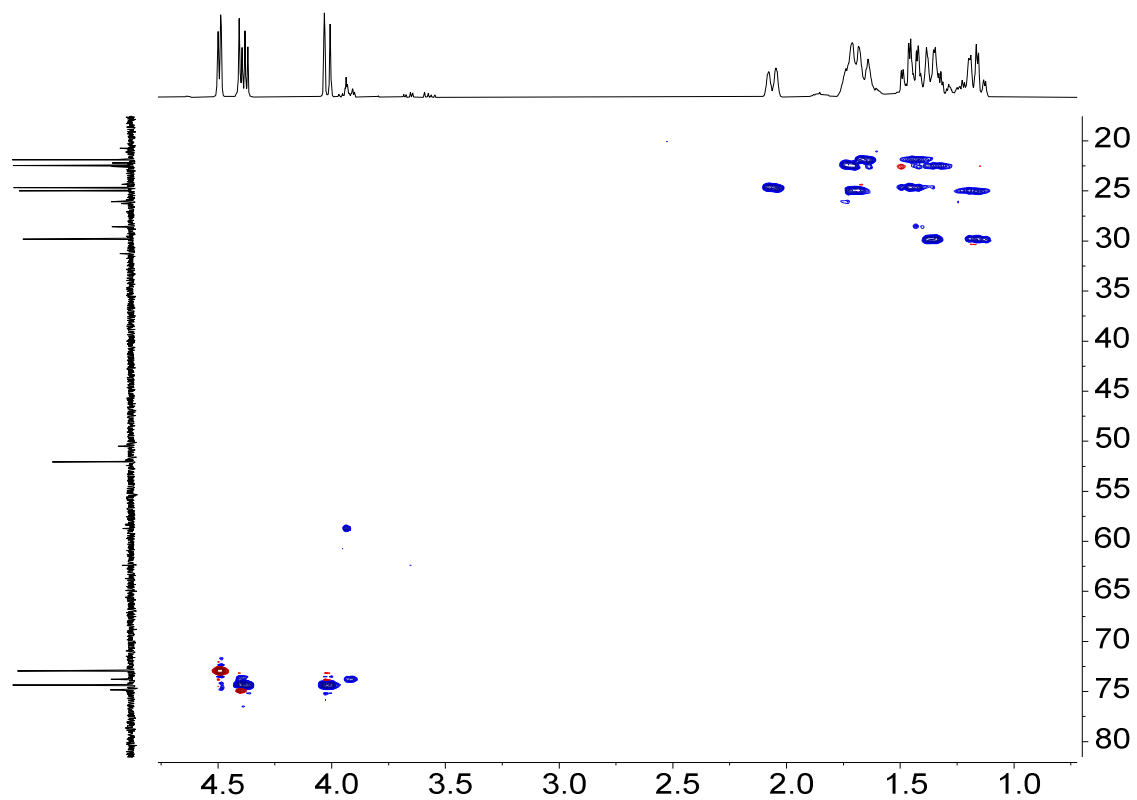
b)



c)



d)



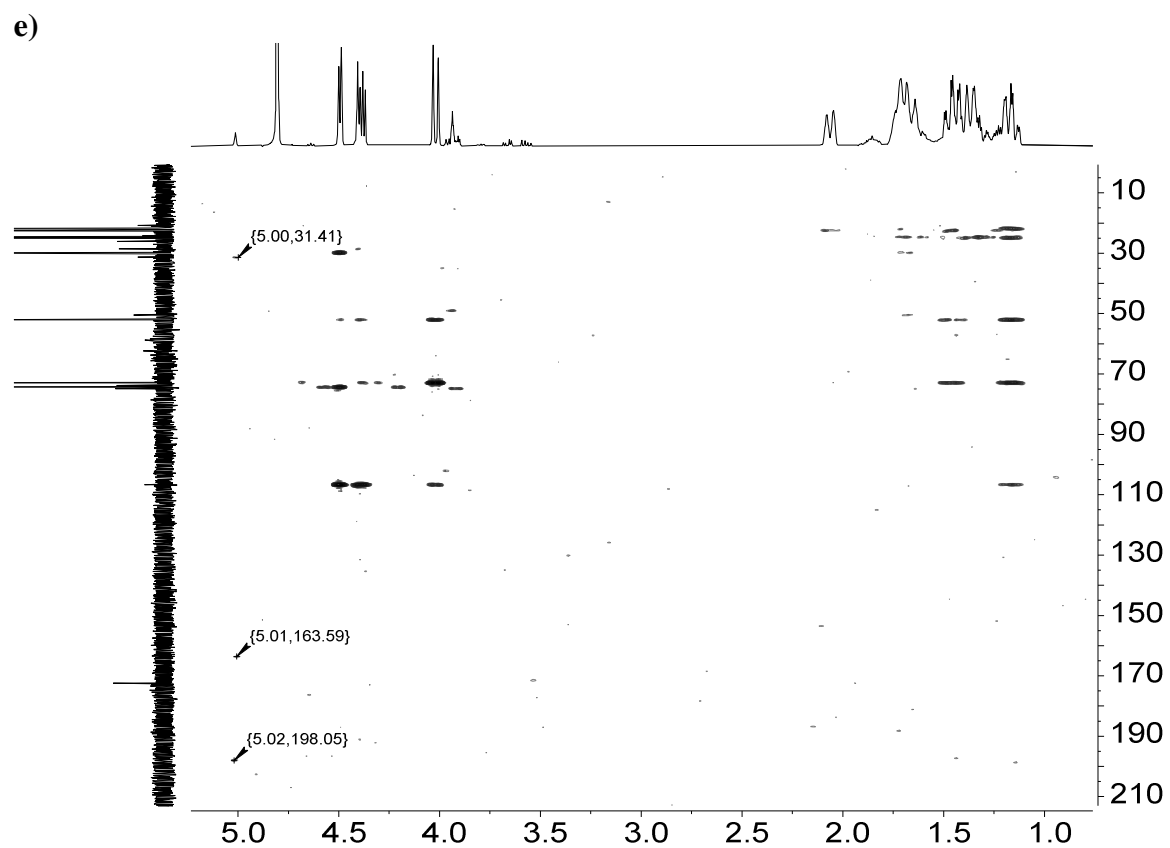
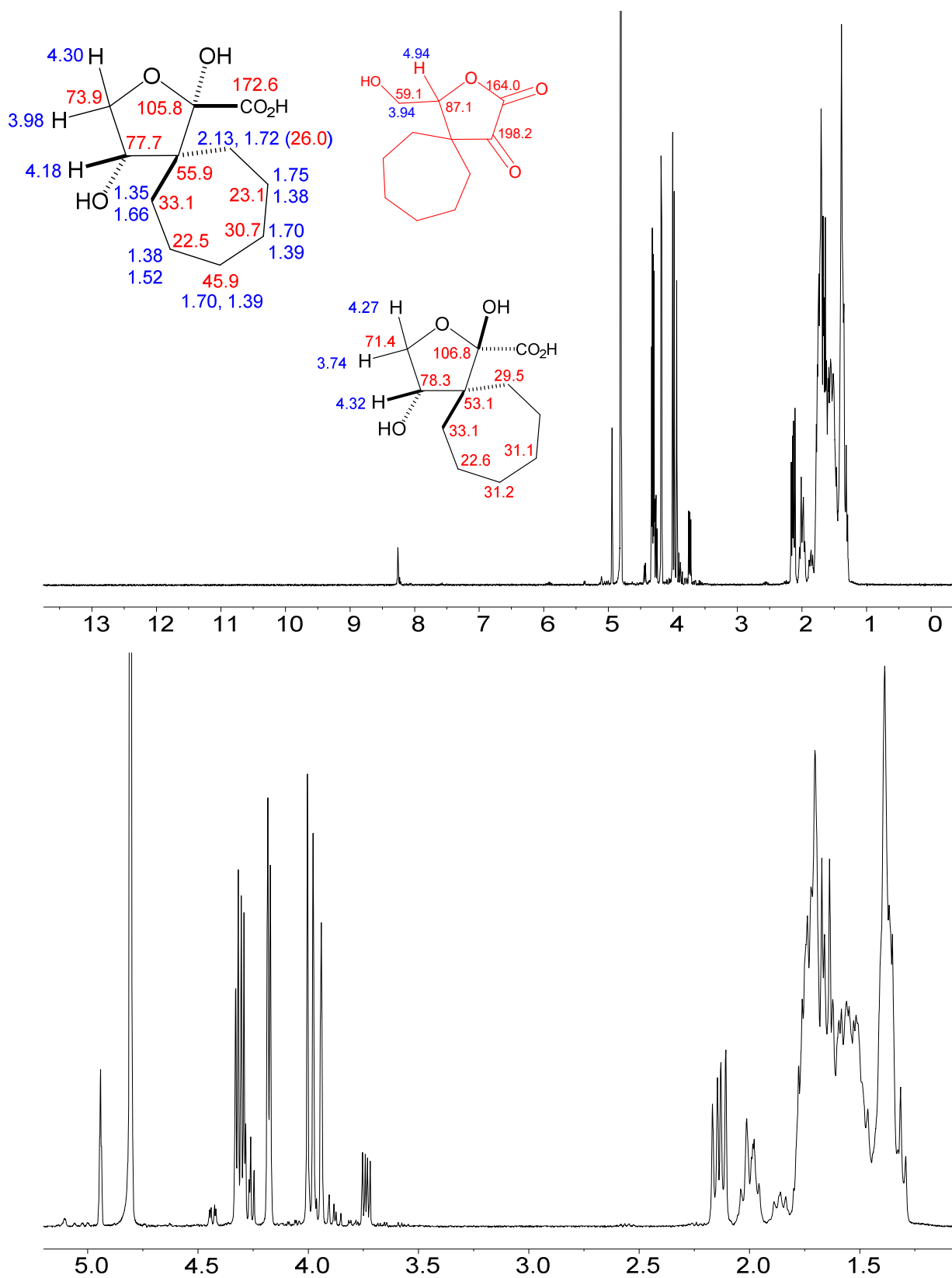
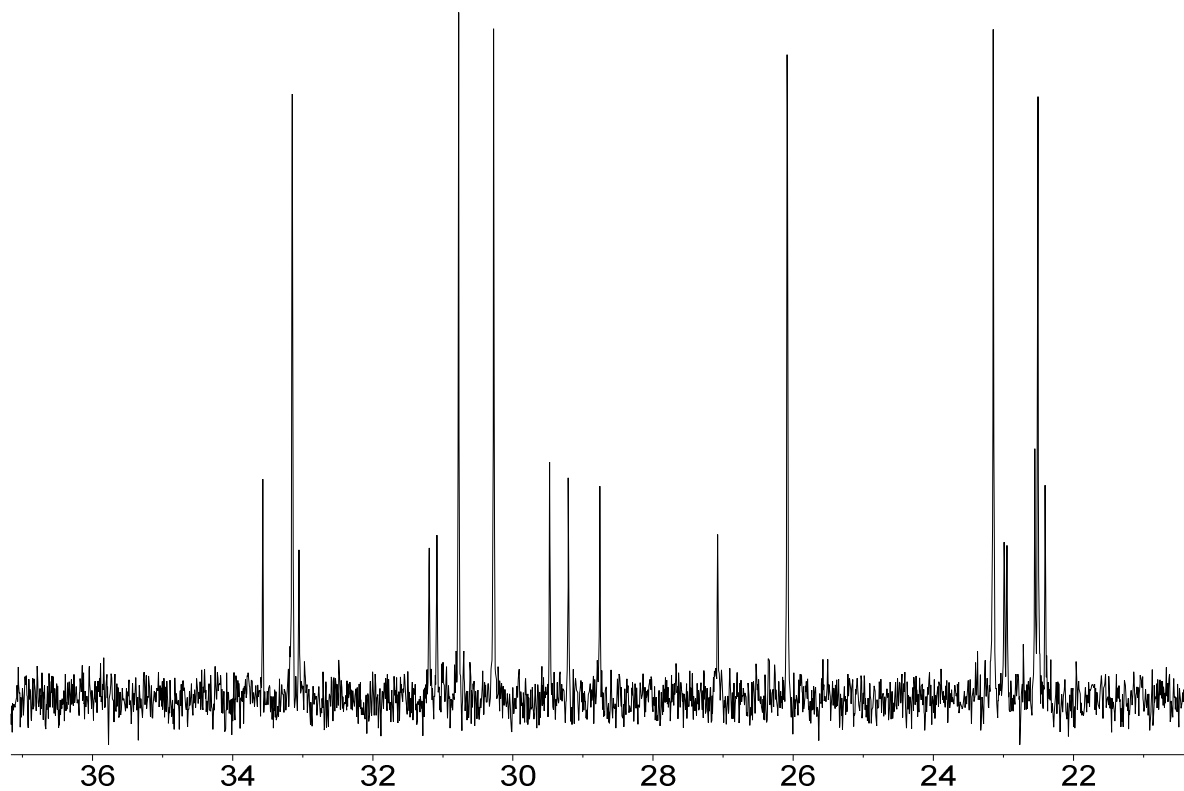
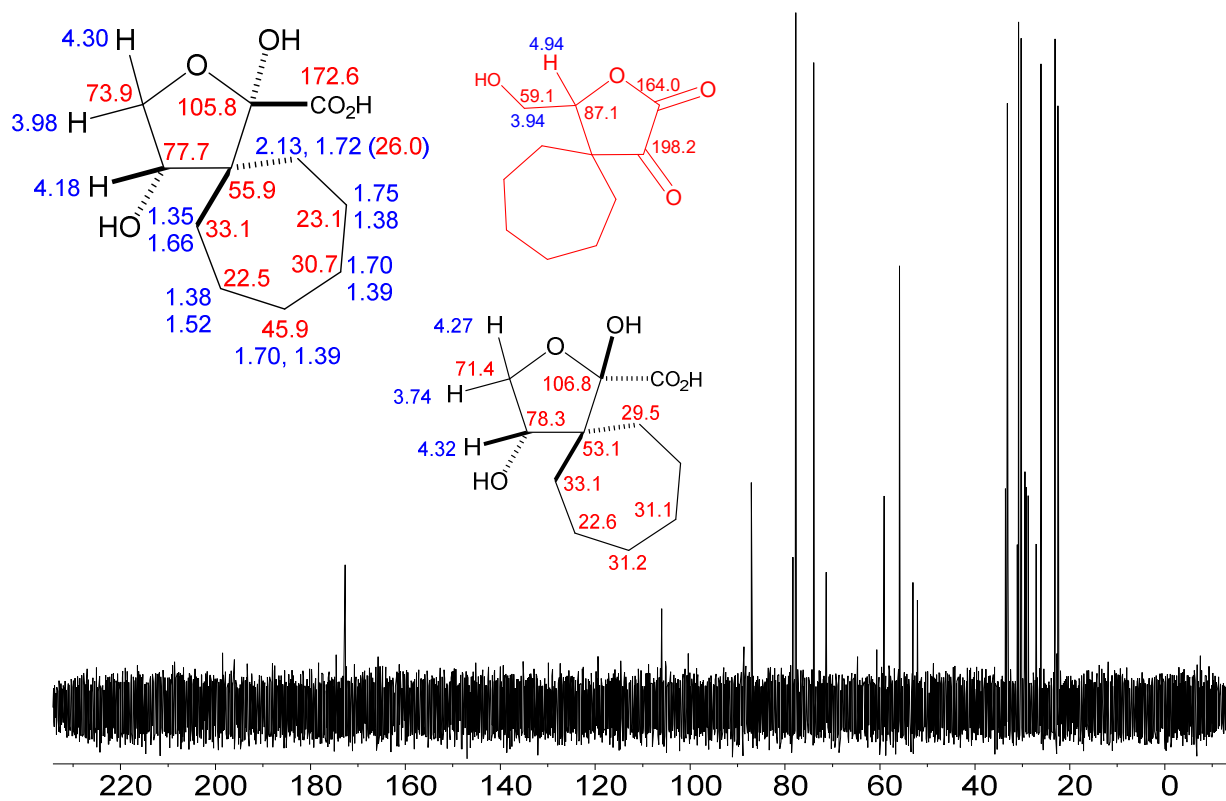


Figure S230 NMR spectra (D₂O) of **9d**: a) ¹H, b) ¹³C, c) gCOSY d) gHSQC, and e) HMBC. E178-147-exp97.

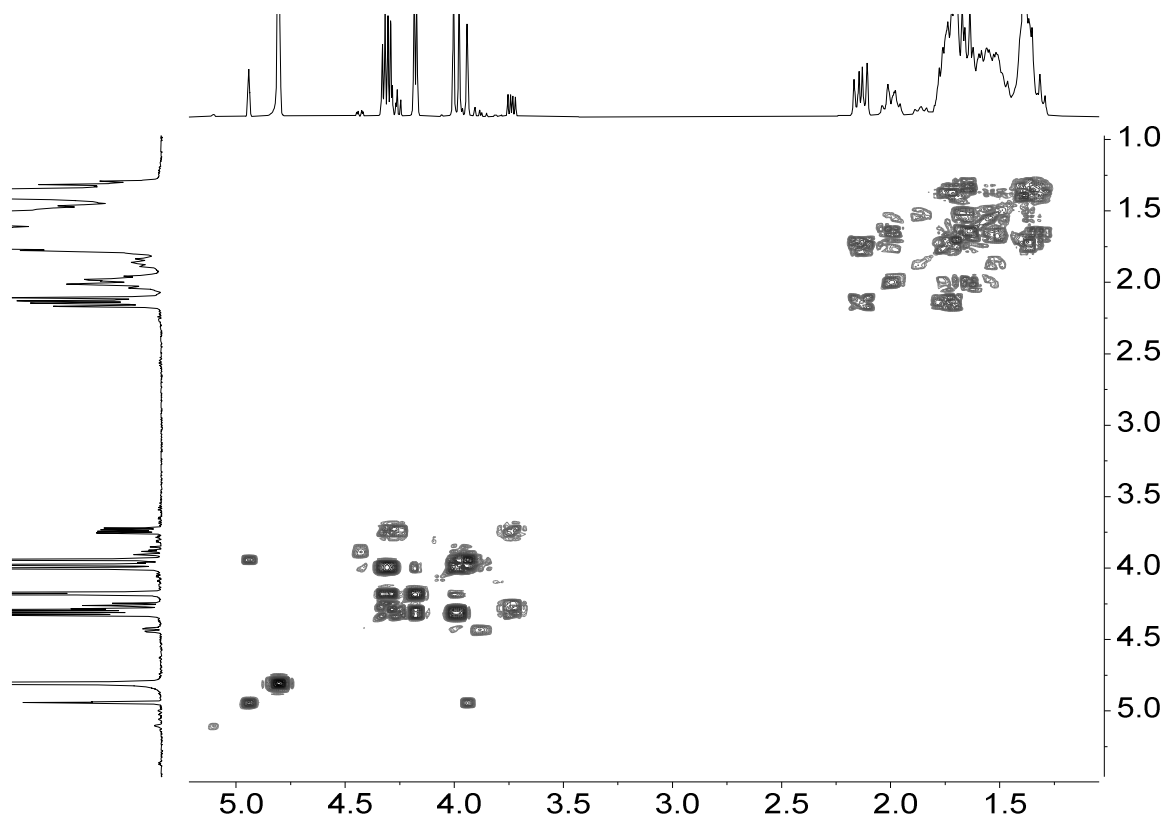
a)



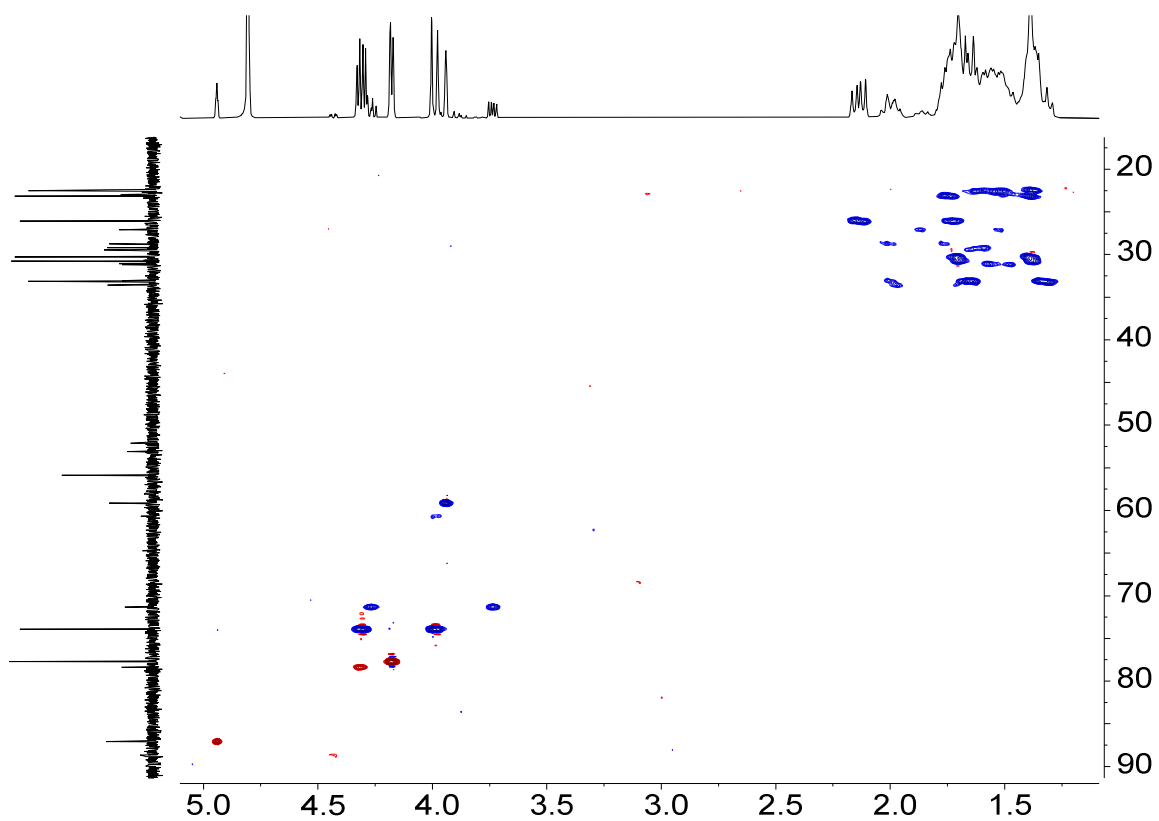
b)



c)



d)



e)

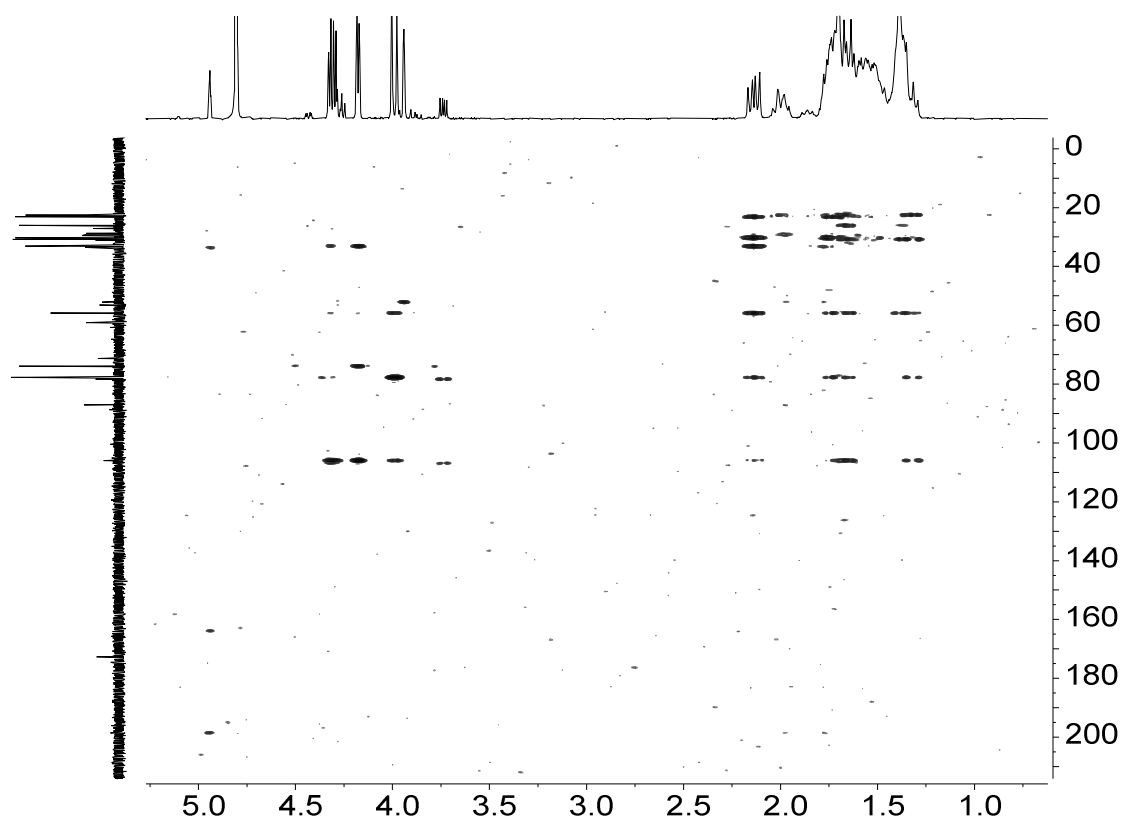
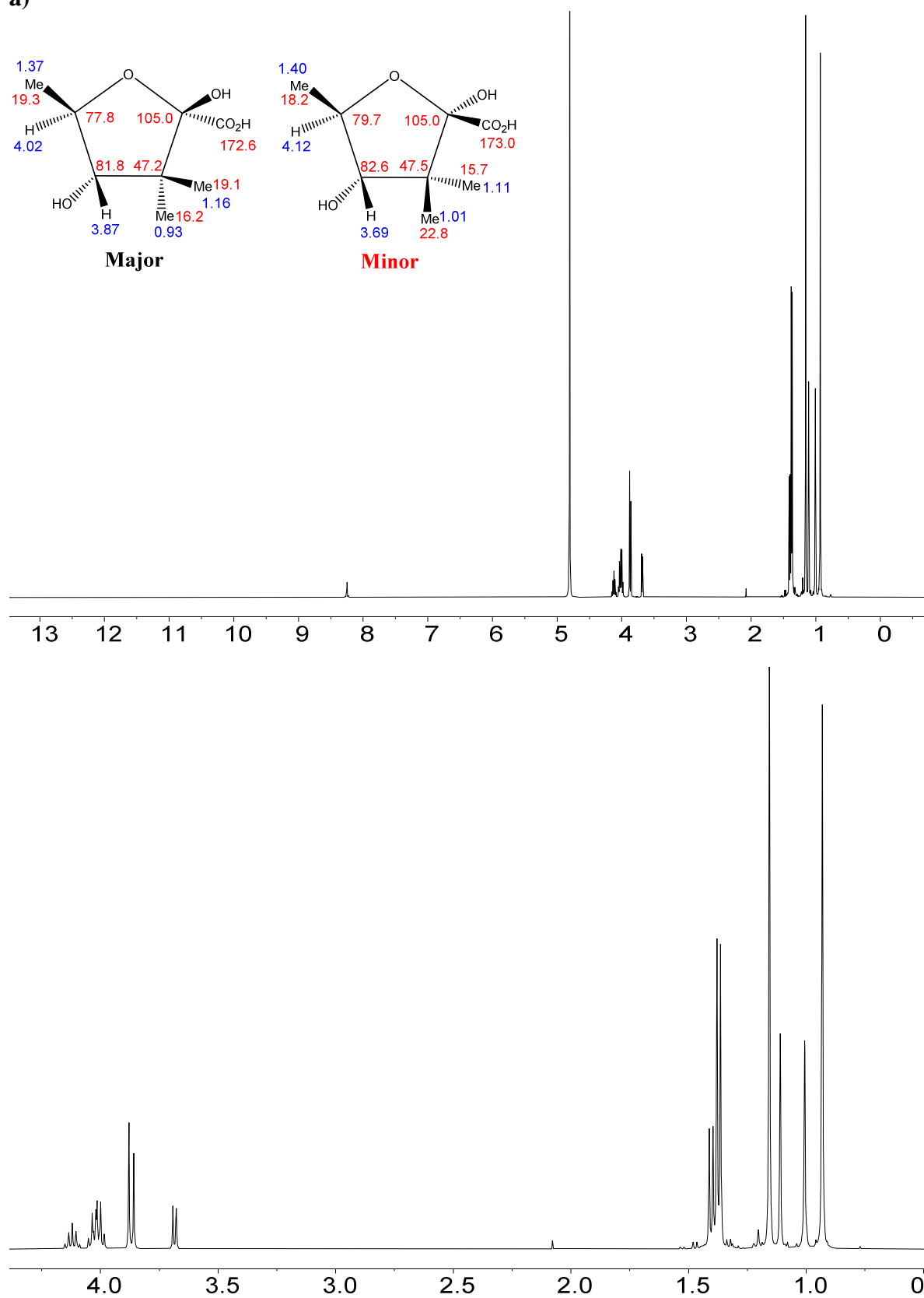
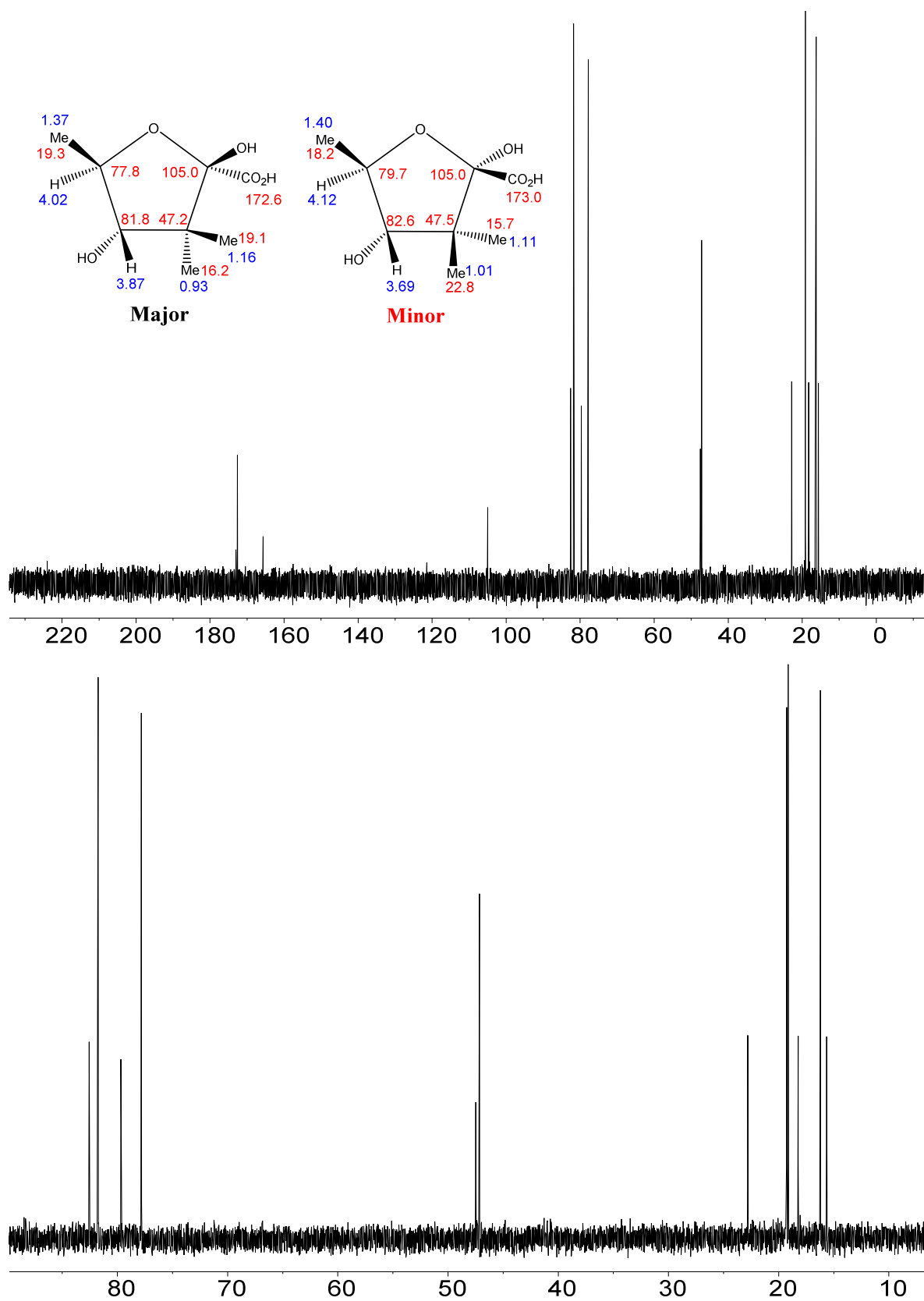


Figure S231 NMR spectra (D₂O) of **9e**: a) ¹H, b) ¹³C, c) gCOSY d) gHSQC, and e) HMBC. E178-141-exp94.

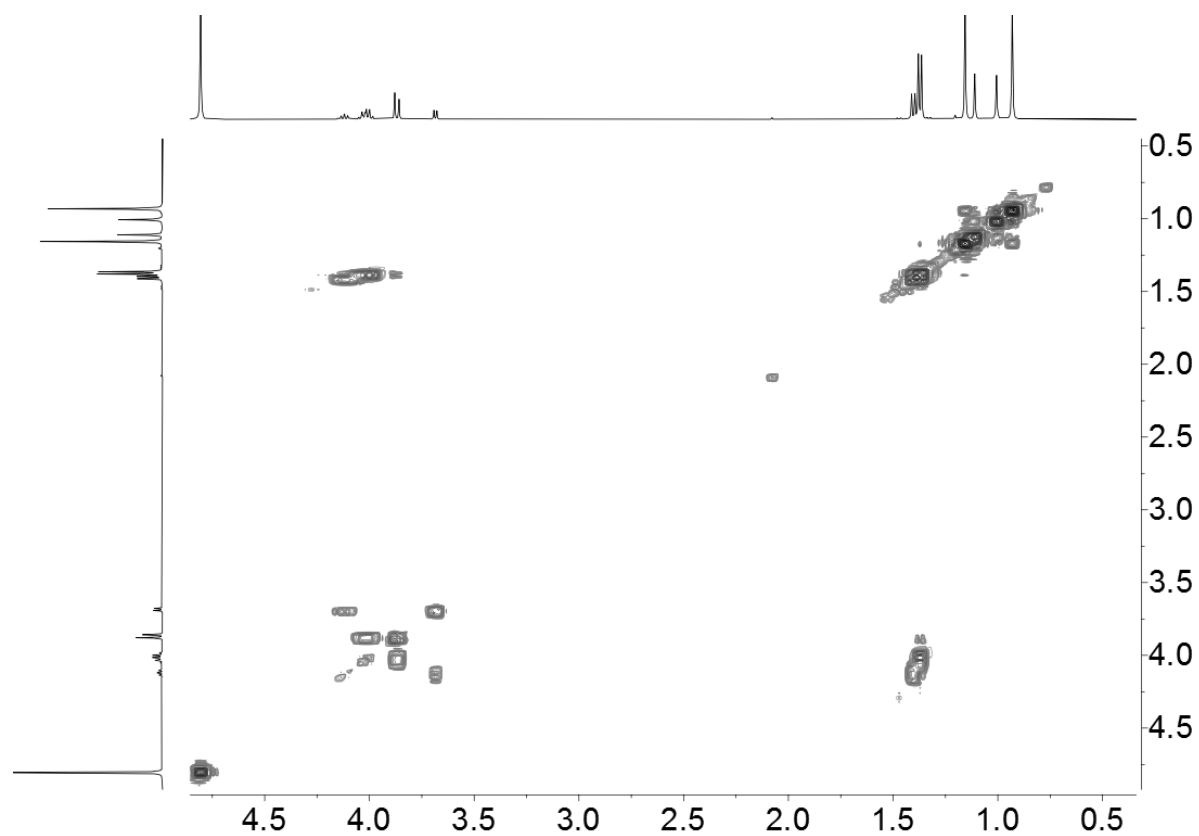
a)



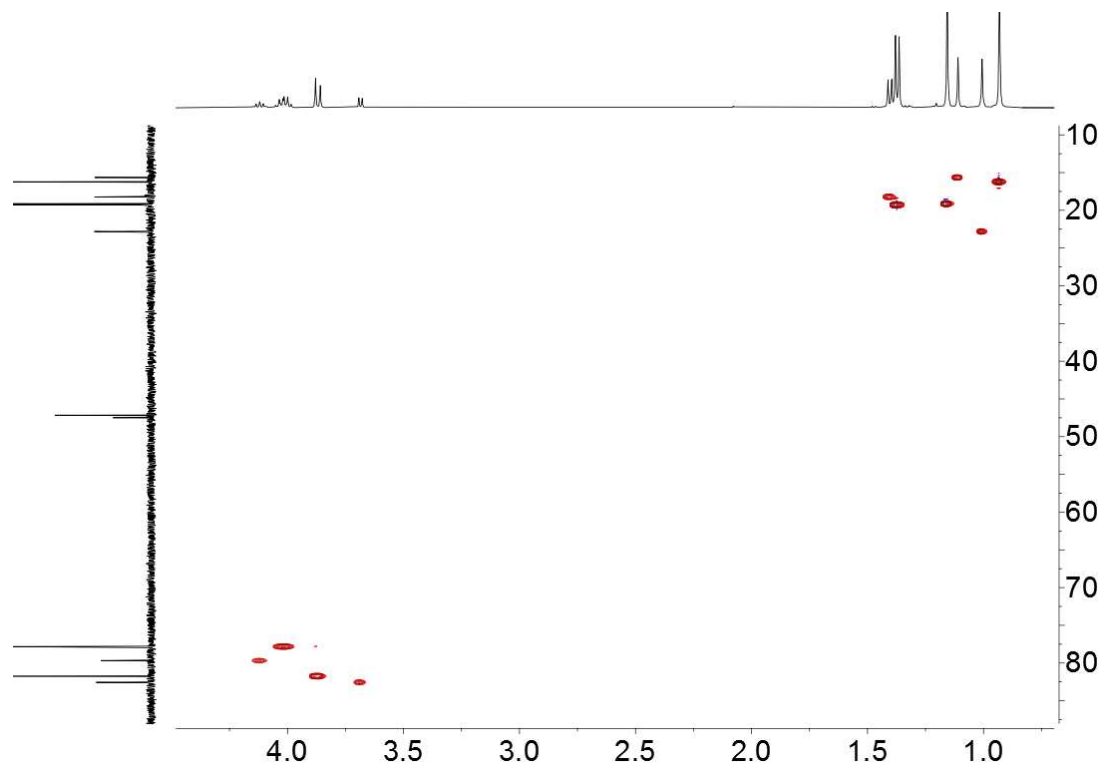
b)



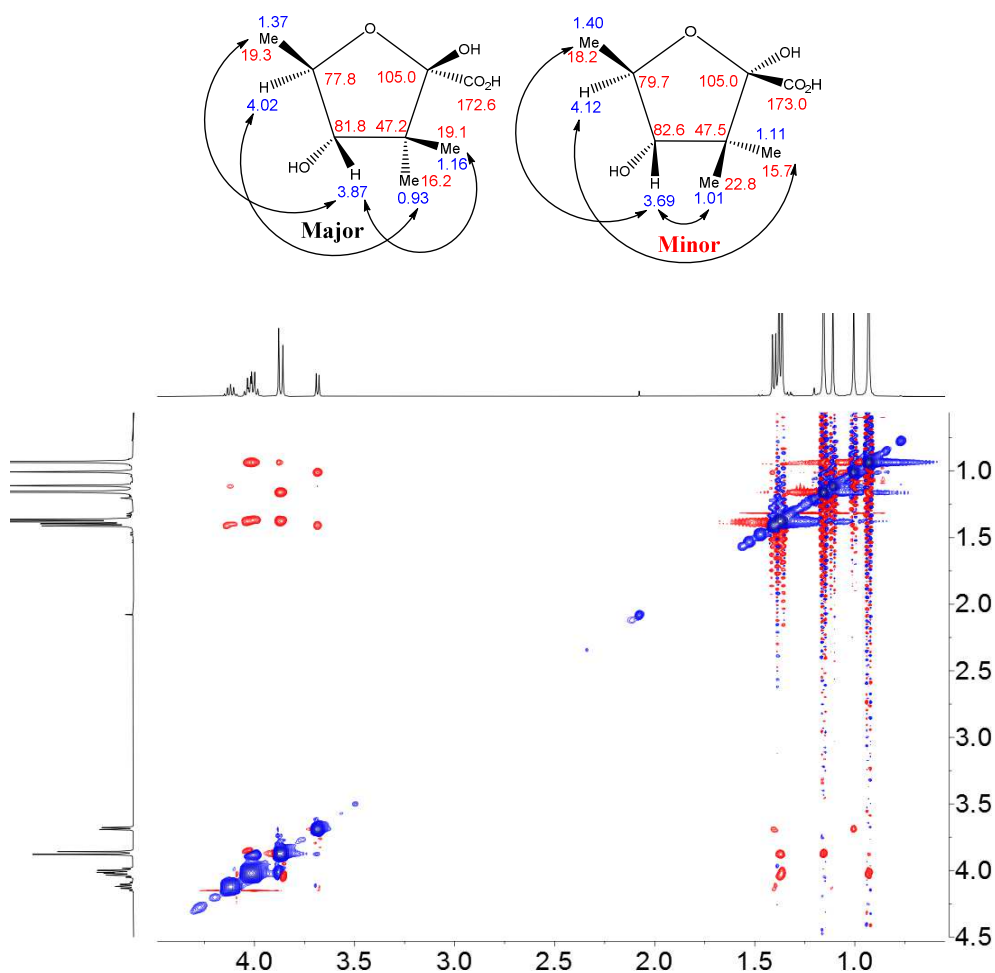
c)



d)



e)



f)

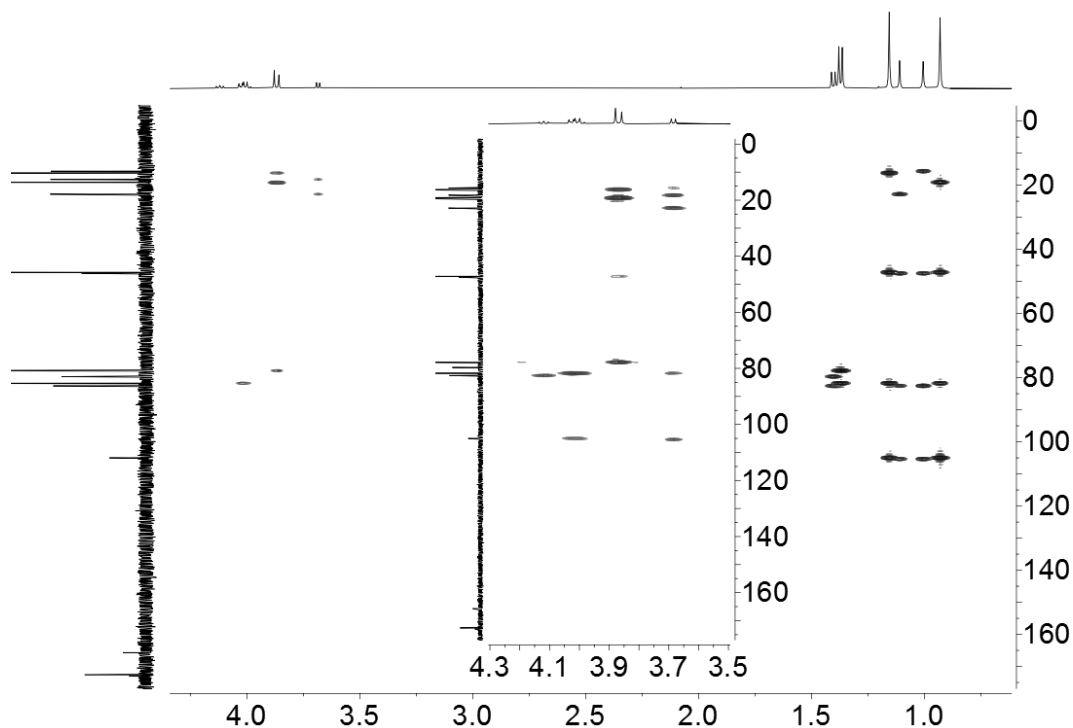
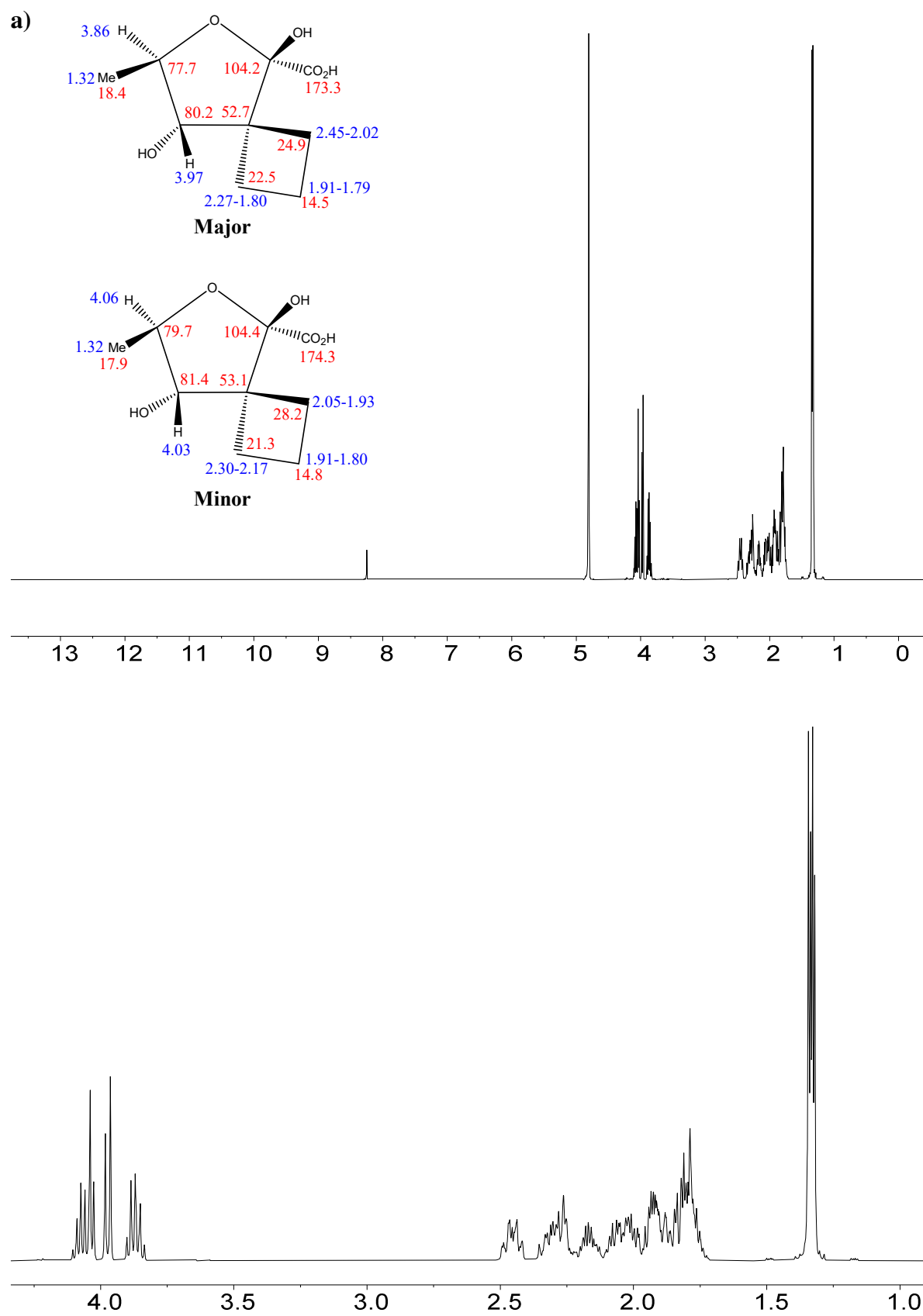
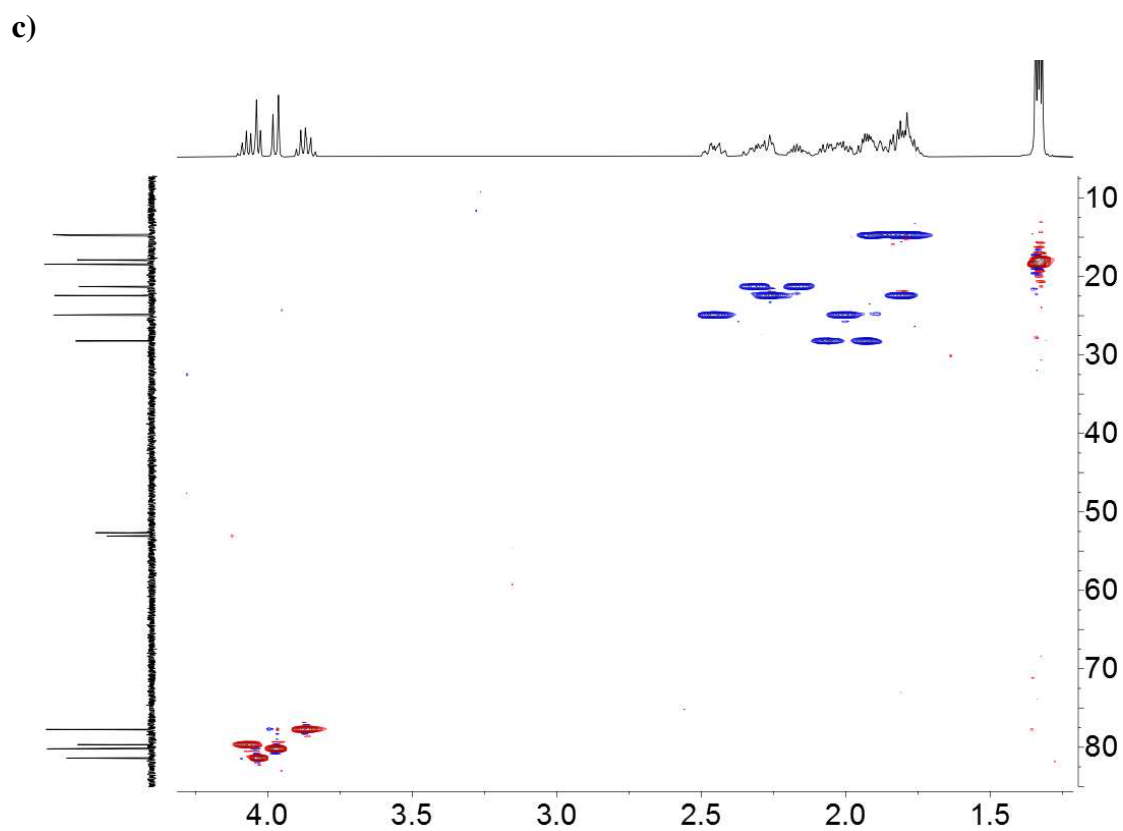
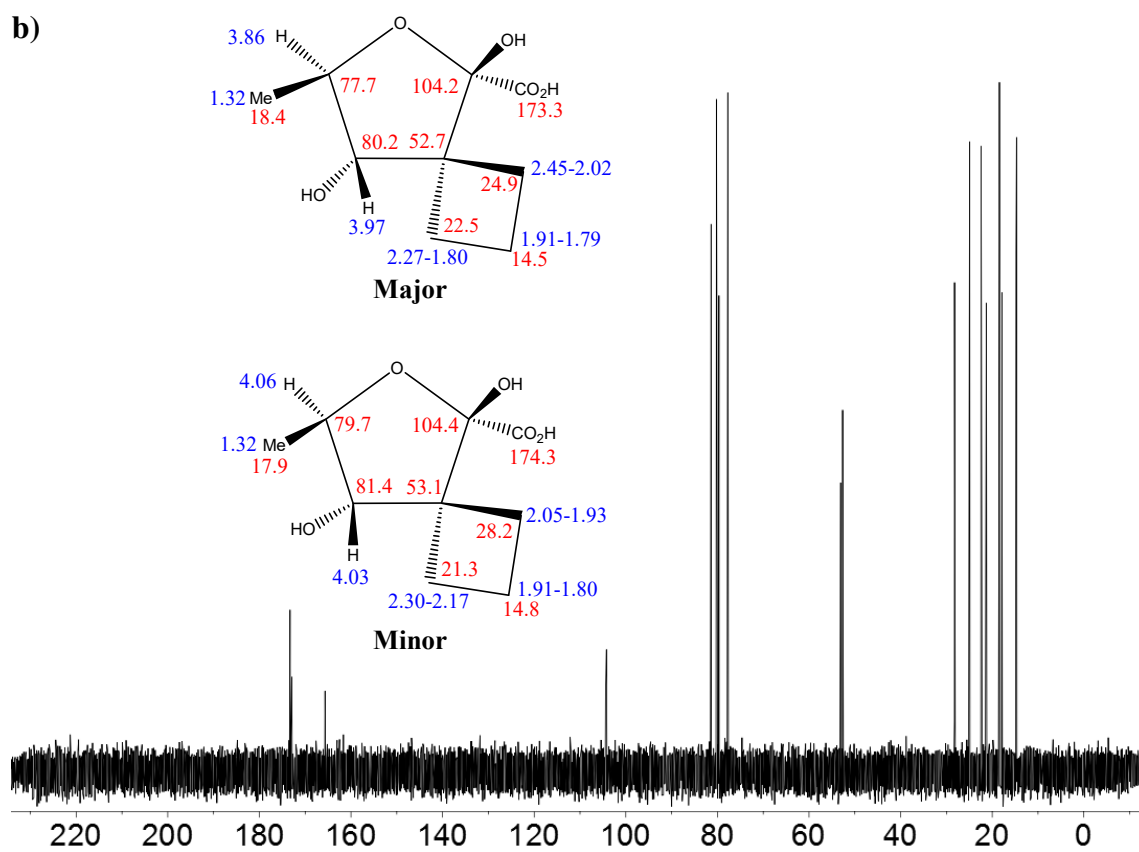
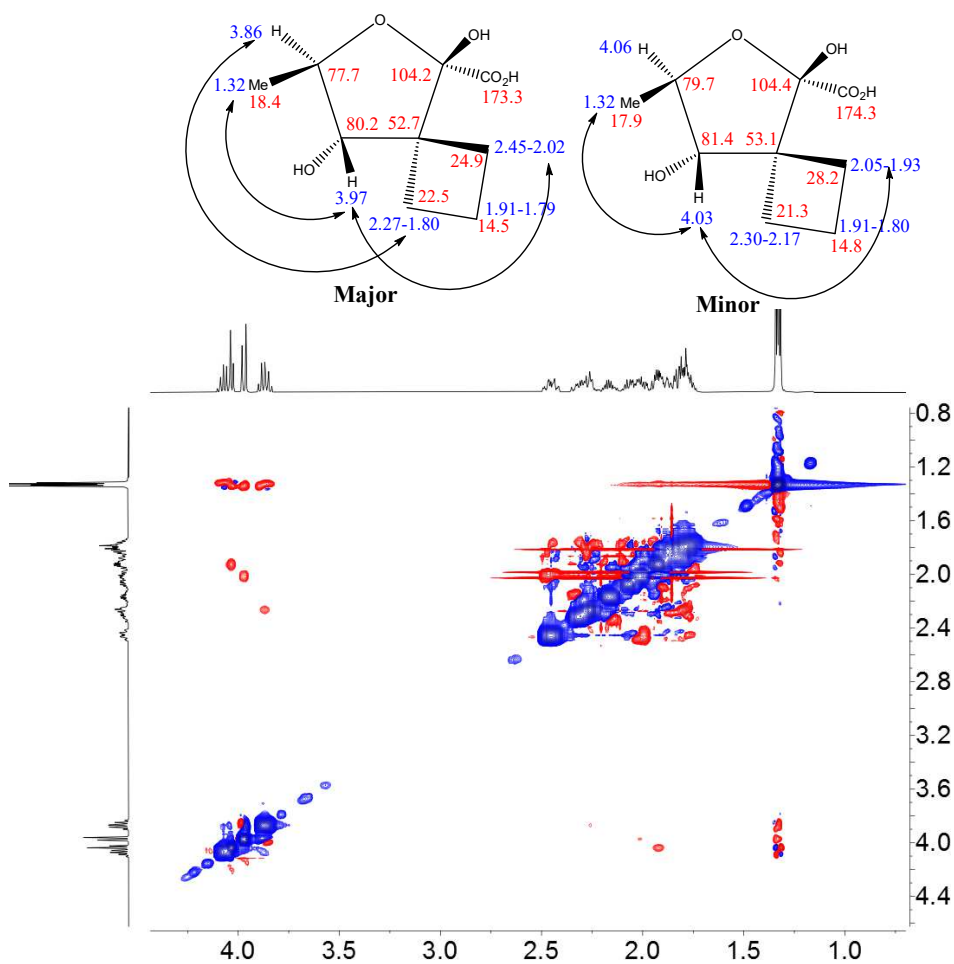


Figure S232 NMR spectra (D₂O) of **9f**: a) ^1H , b) ^{13}C , c) gCOSY d) gHSQC, e) 2D NOESY and f) HMBC. E178-60-exp37.





d)



e)

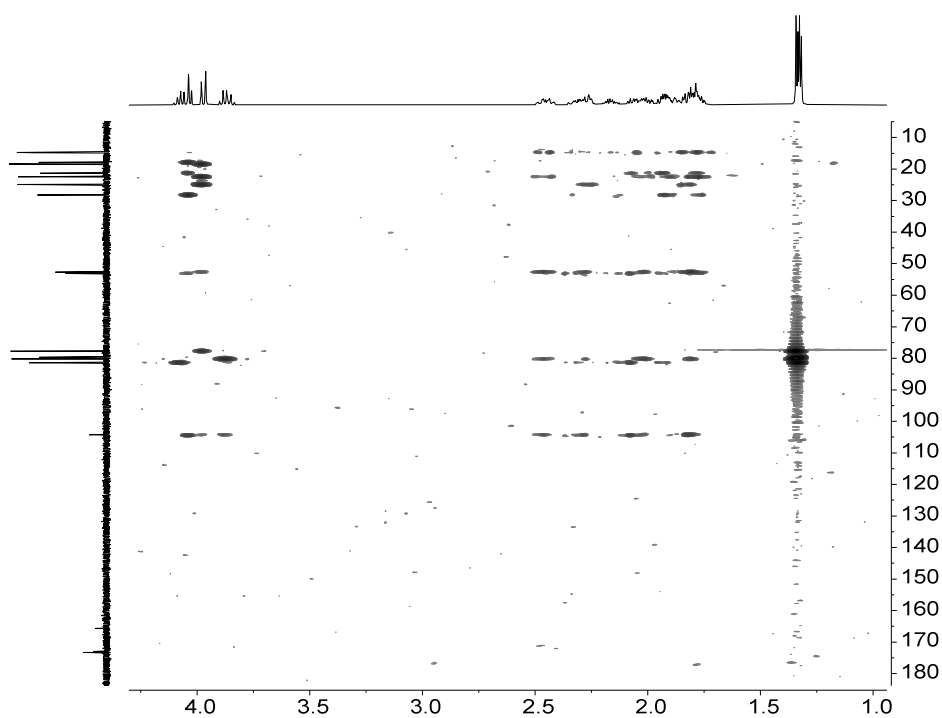
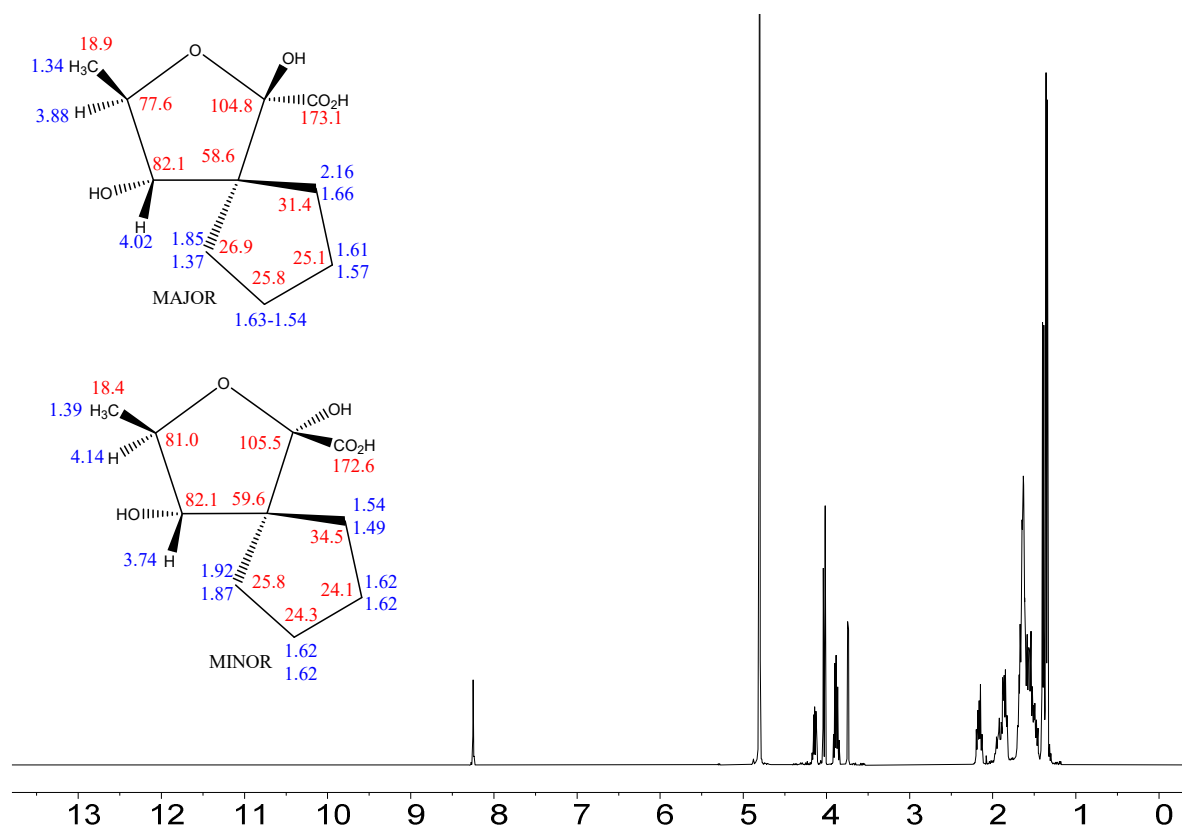
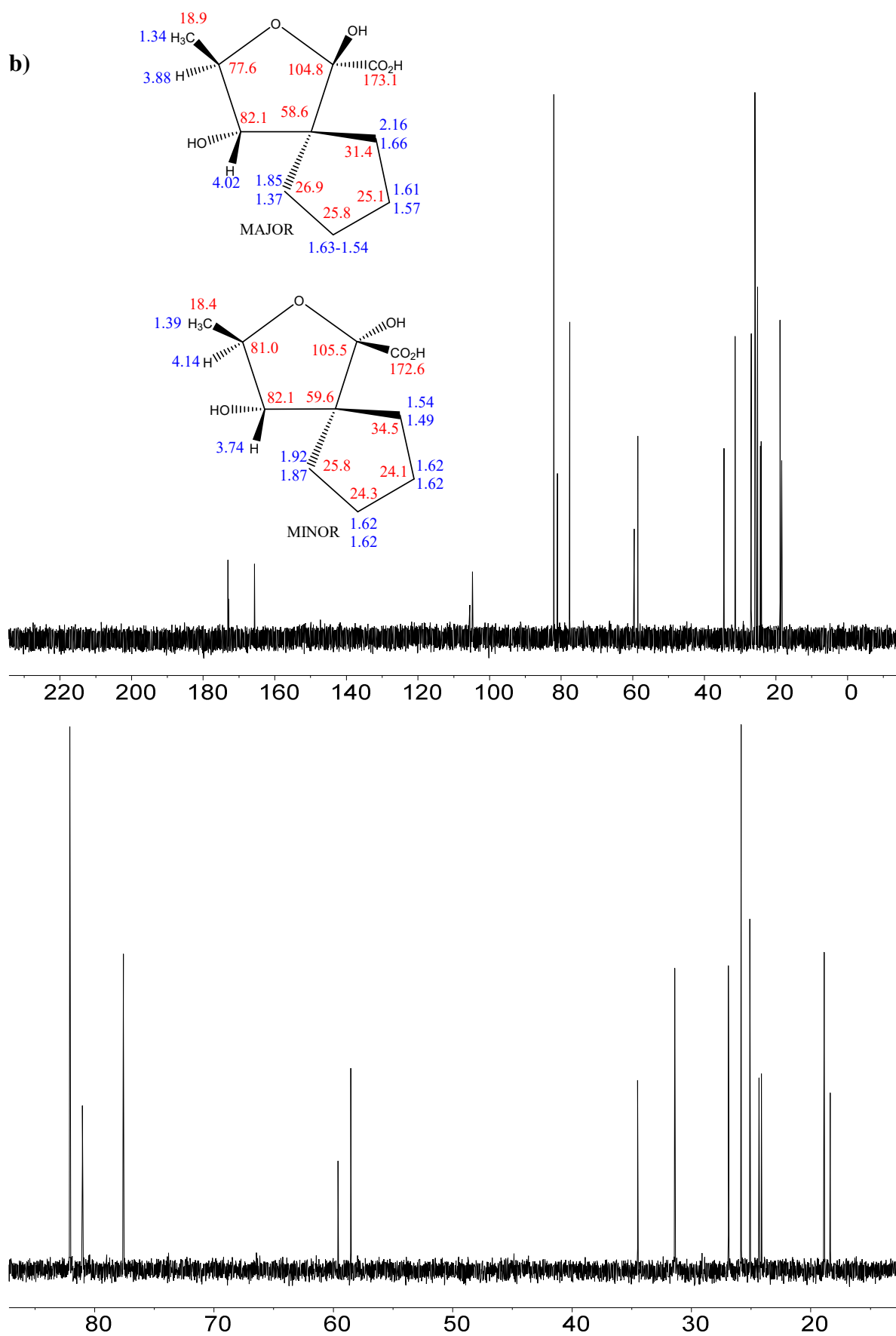


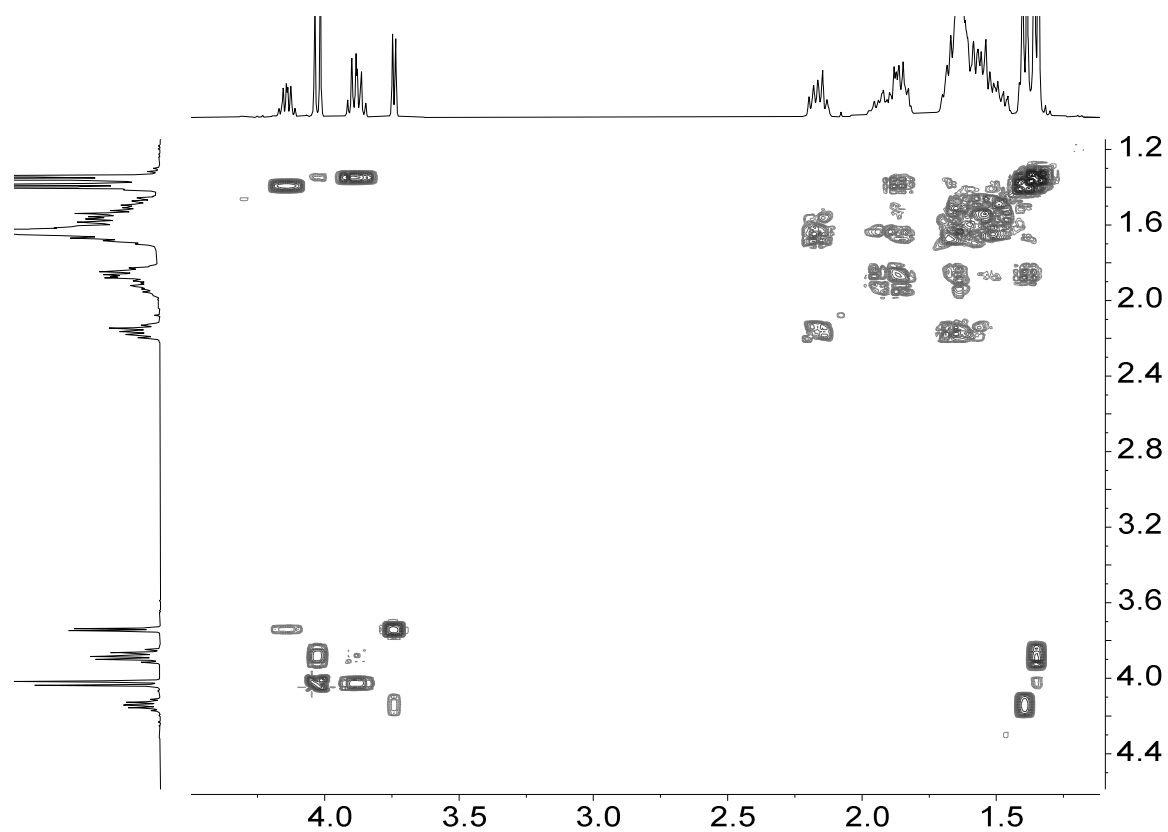
Figure S233 NMR spectra (D₂O) of **9g**: a) ^1H , b) ^{13}C , c) gHSQC, d) 2D NOESY and e) HMBC. E178-77-exp49.

a)

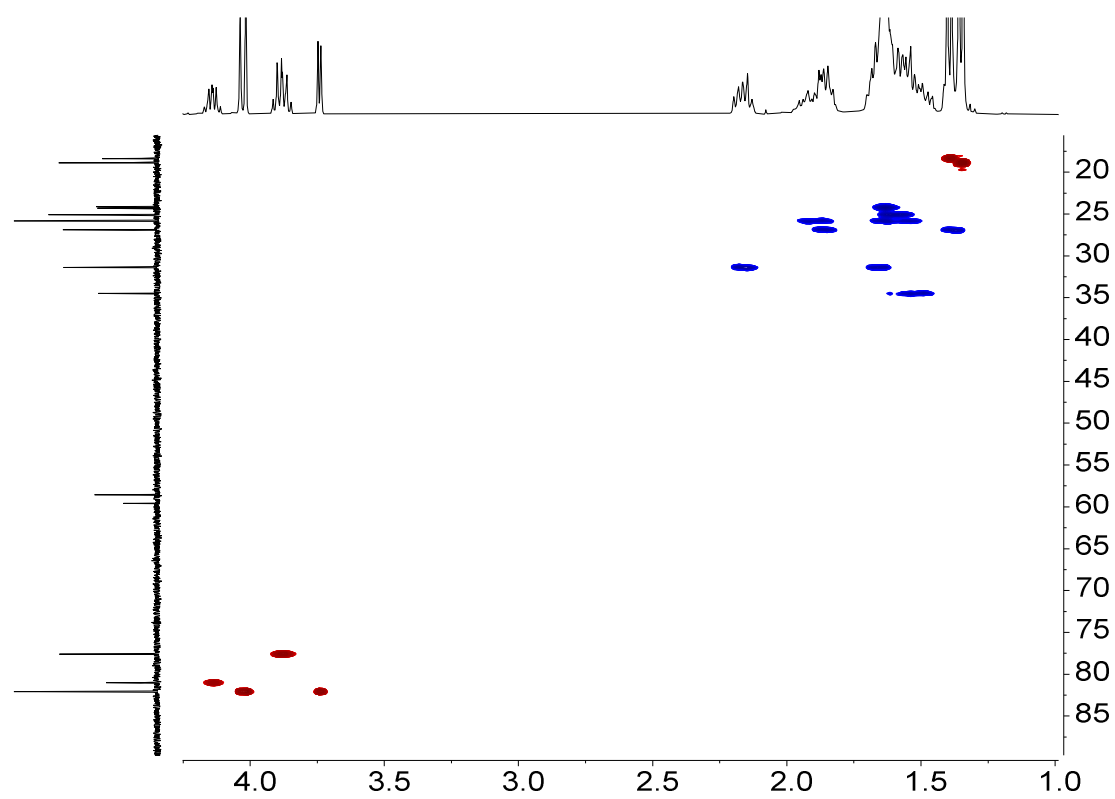




c)



d)



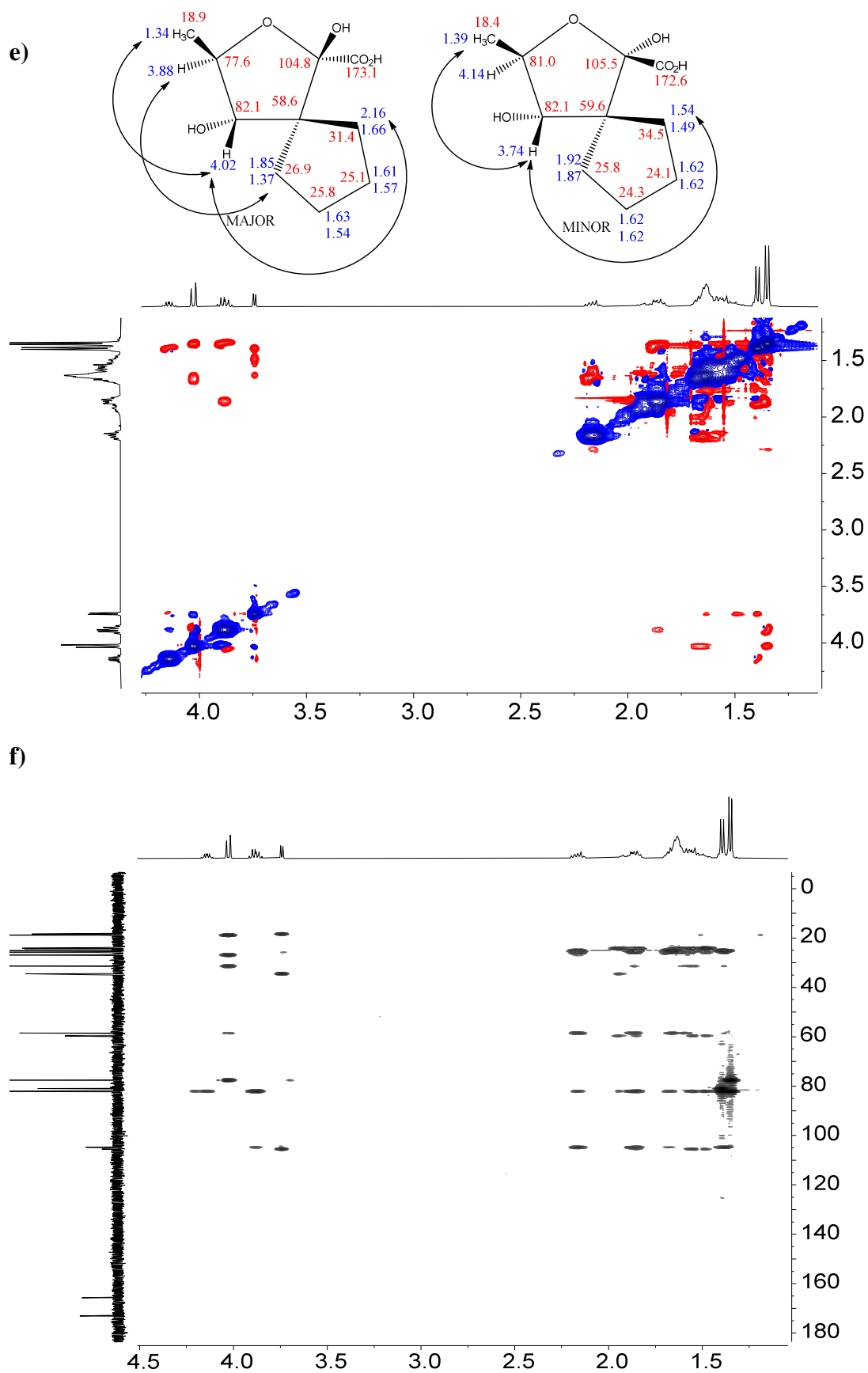
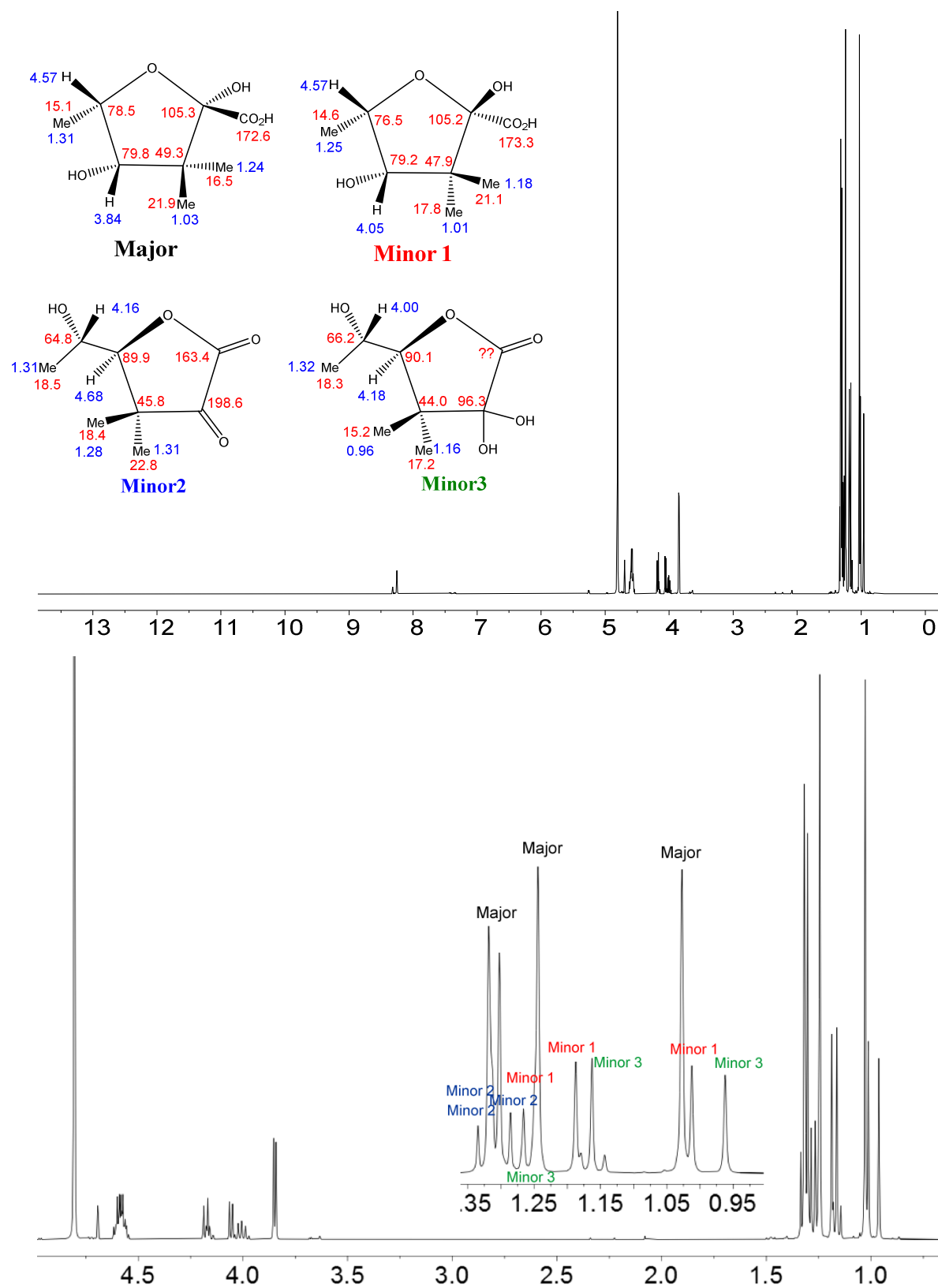
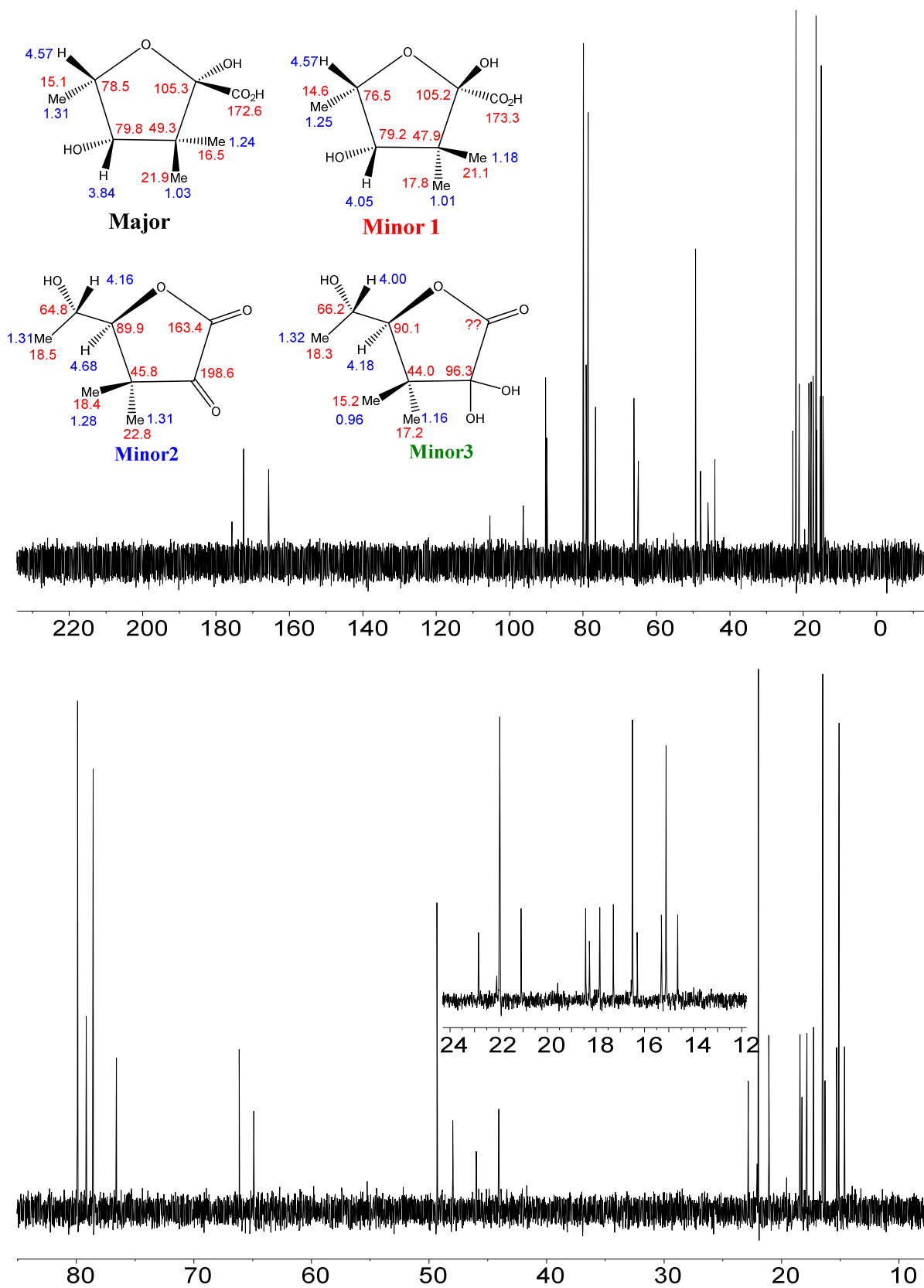


Figure S234 NMR spectra (D₂O) of **9h**: a) ¹H, b) ¹³C, c) gCOSY, d) gHSQC, e) 2D NOESY and f) HMBC. E178-90-exp59.

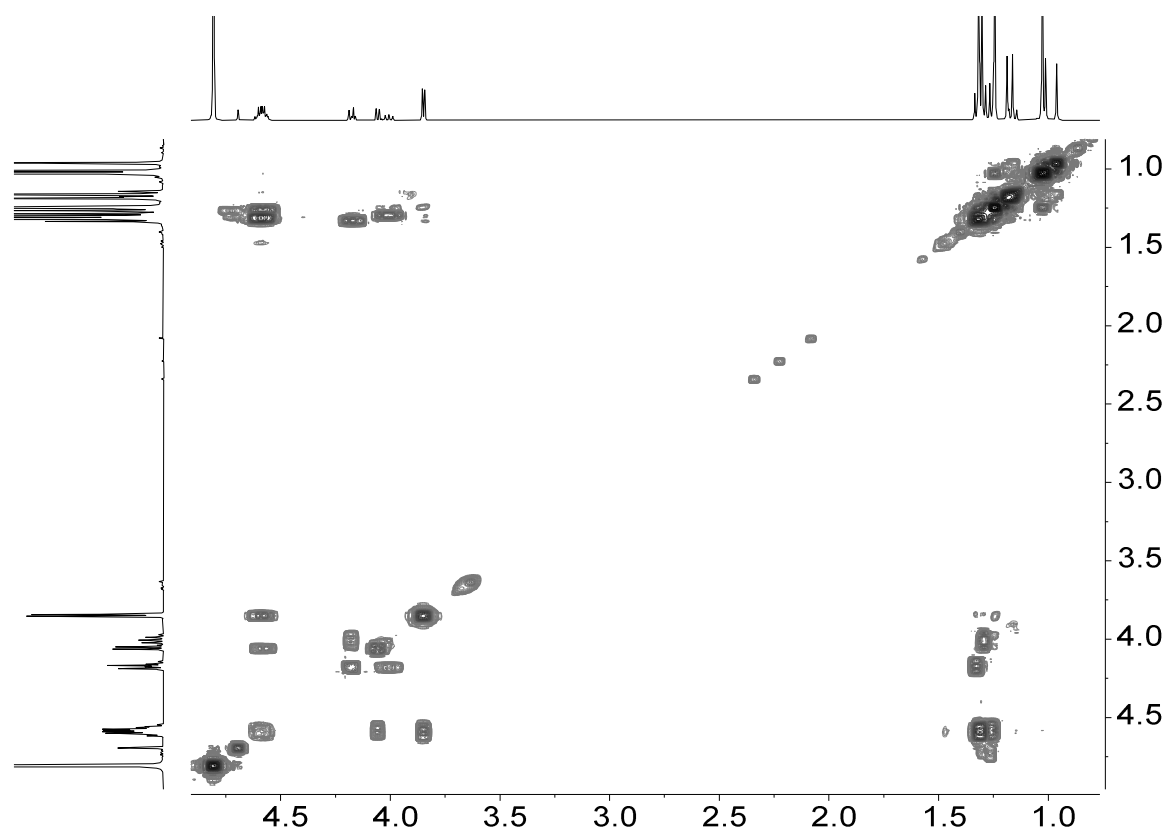
a)



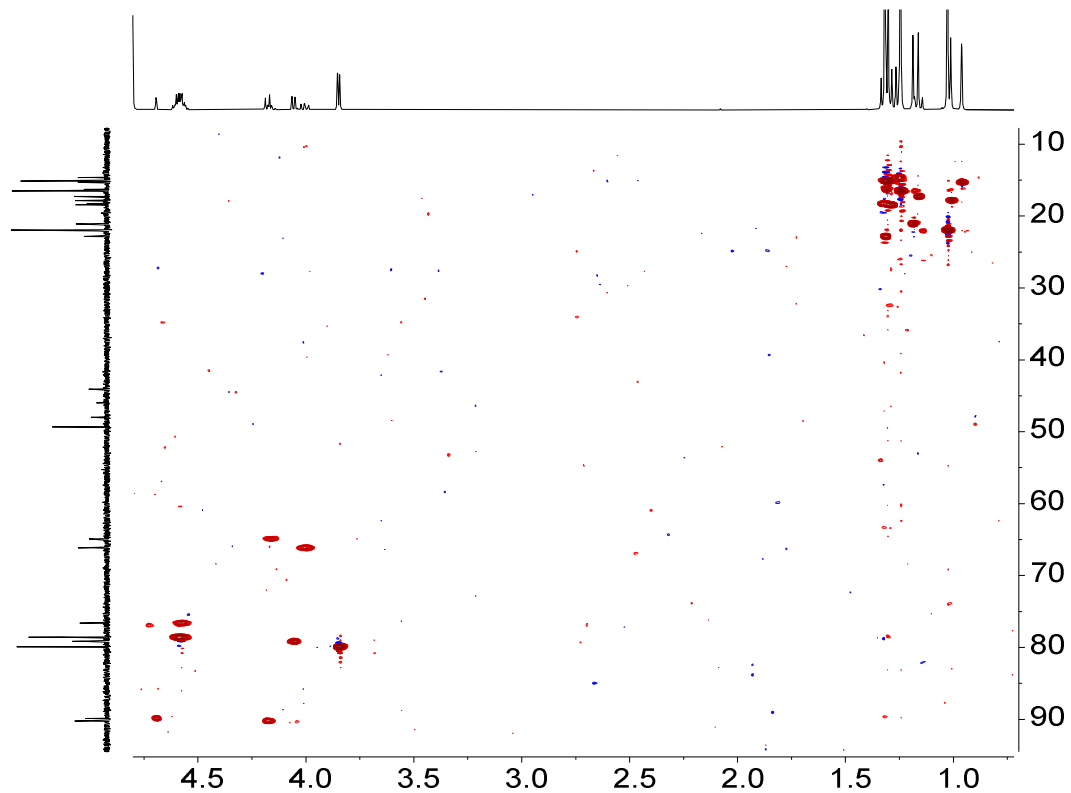
b)



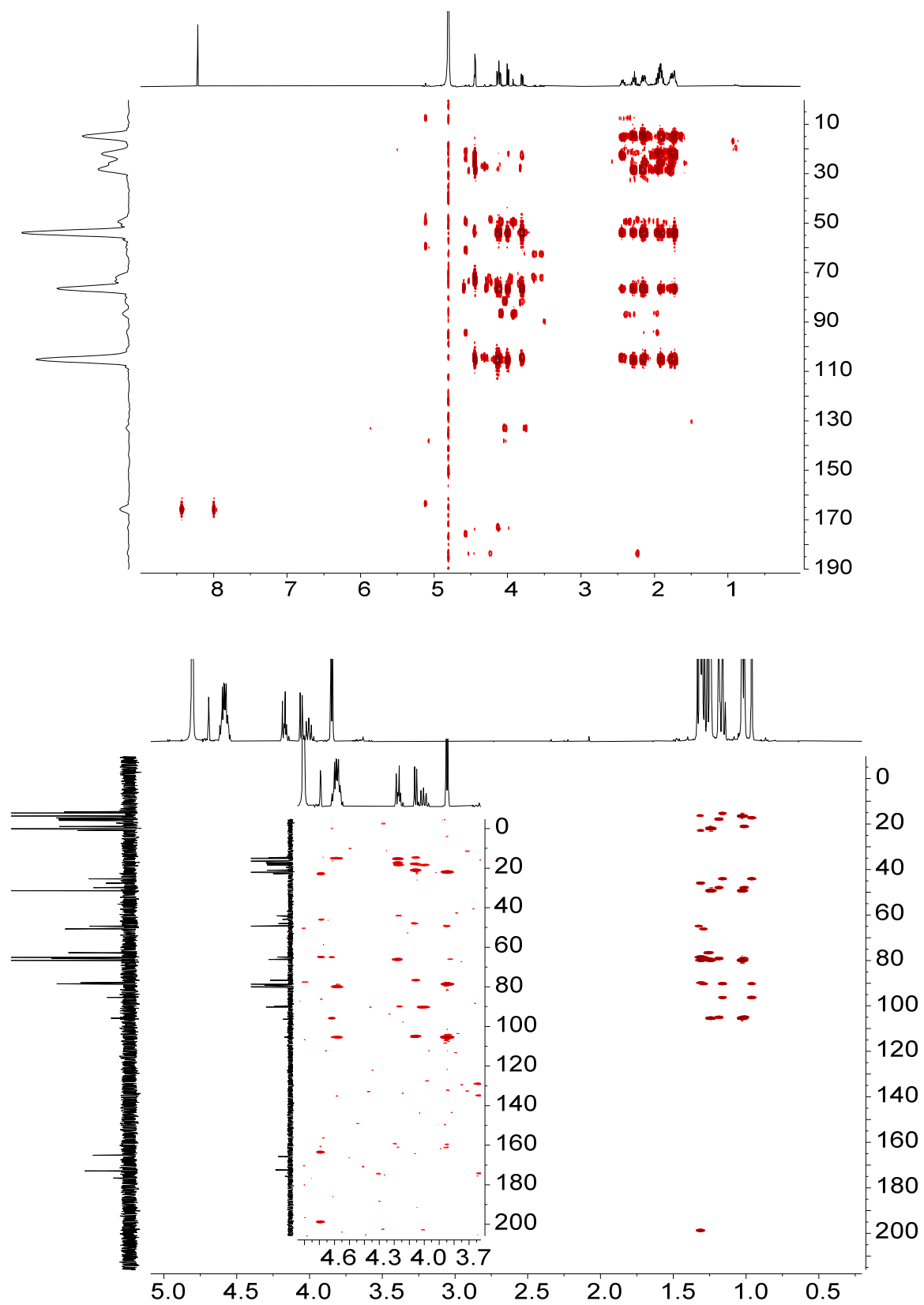
c)



d)



e)



f)

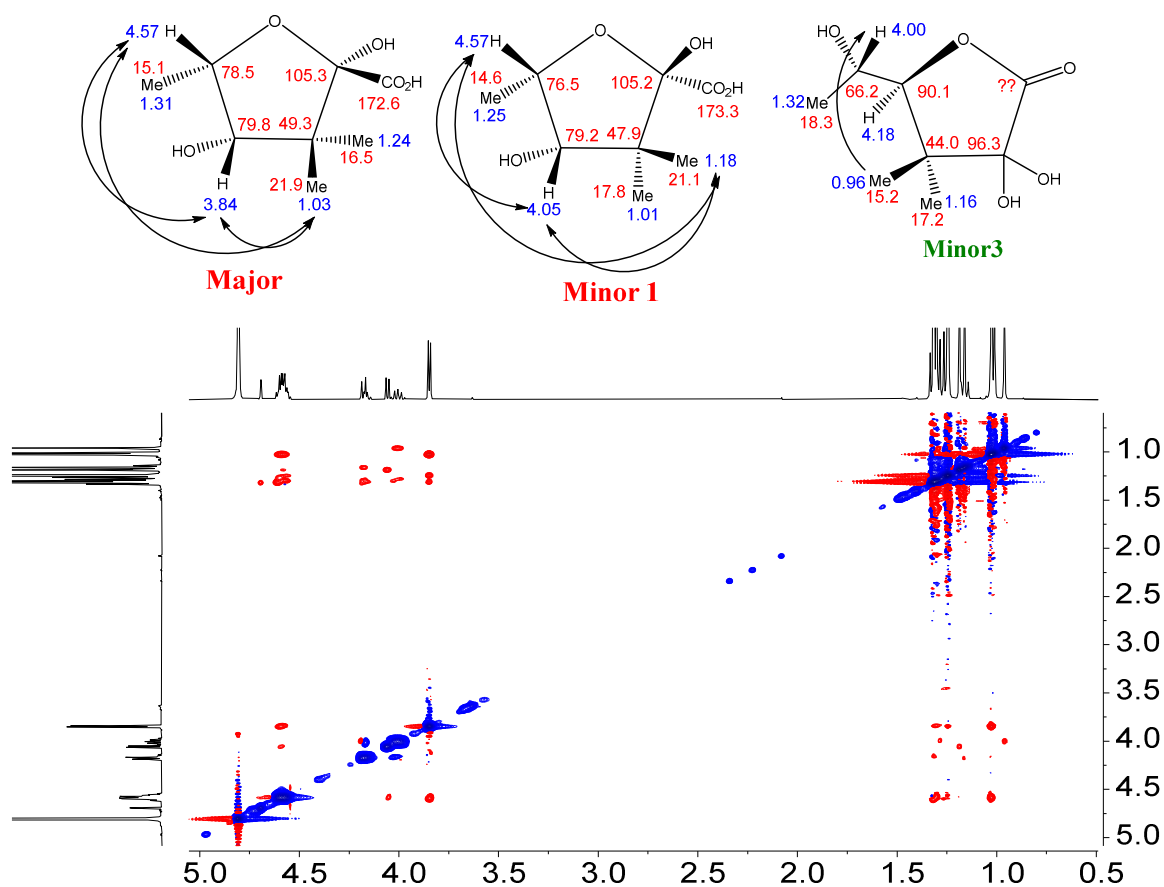
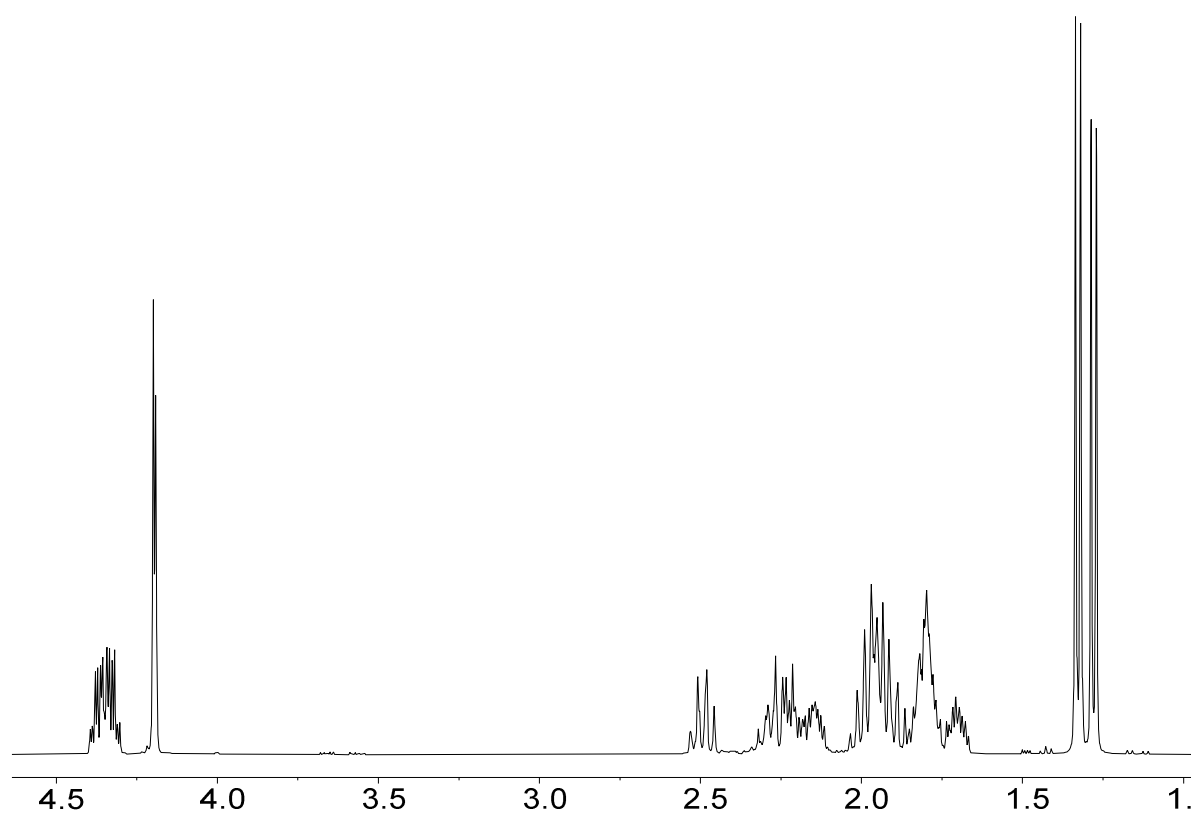
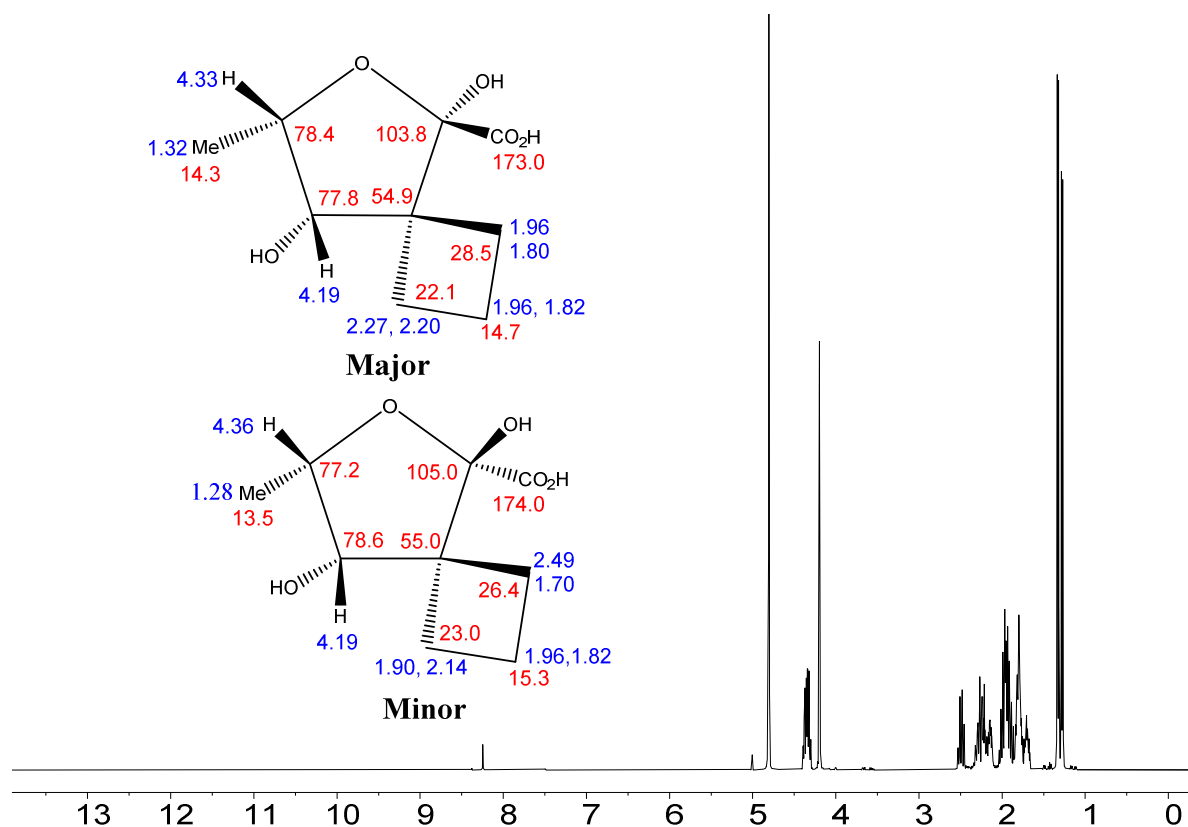
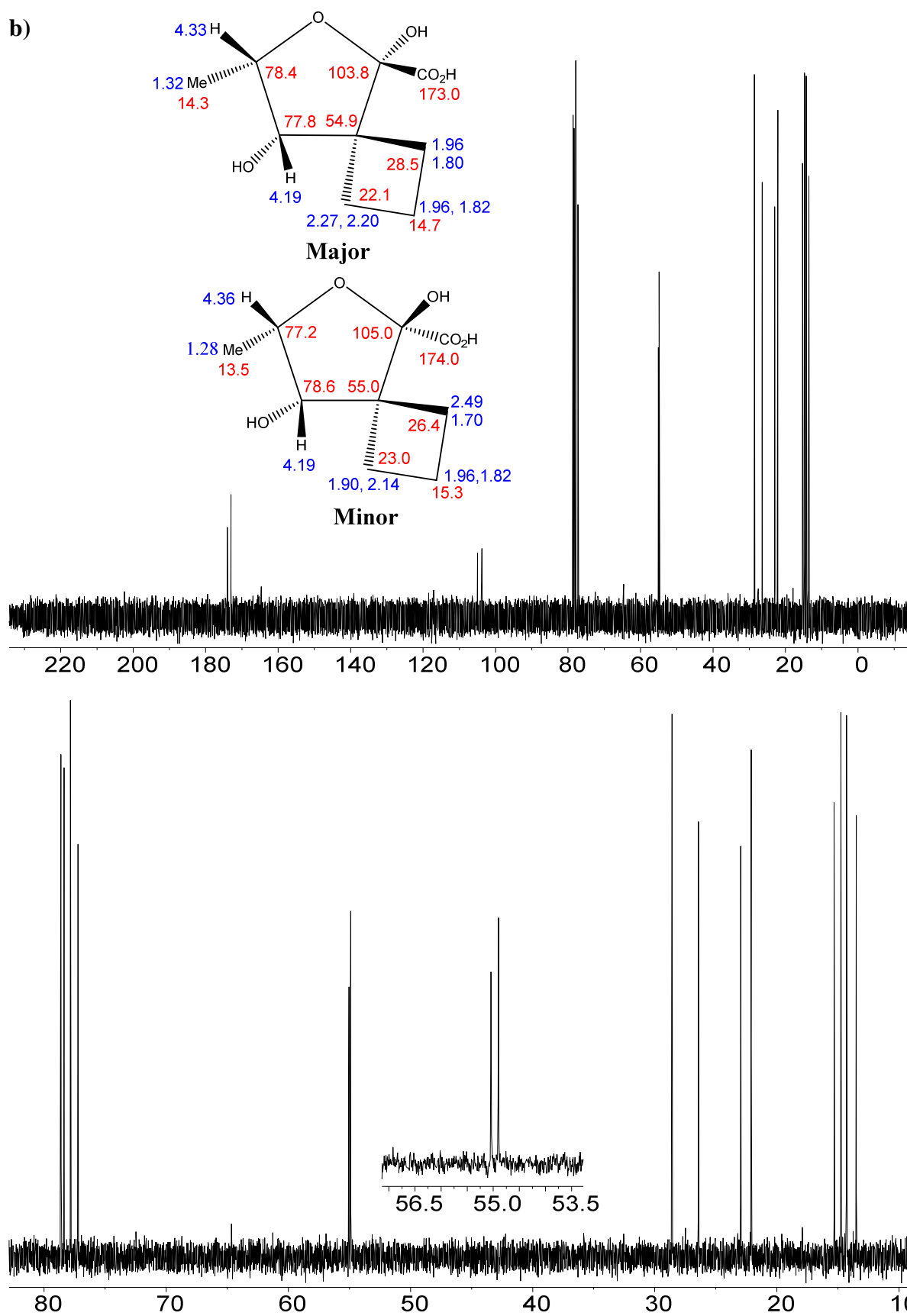


Figure S235 NMR spectra (D₂O) of **9i**: a) ¹H, b) ¹³C, c) gCOSY, d) gHSQC, e) HMBC and f) 2D NOESY. E178-103-exp71.

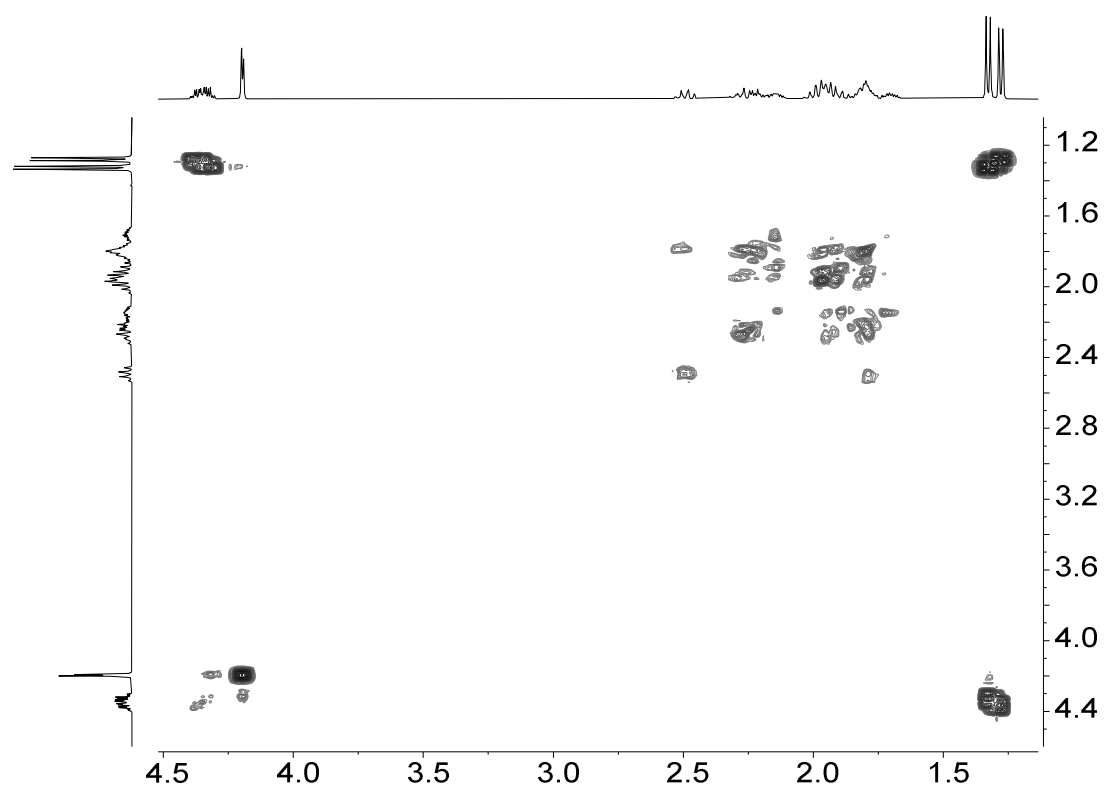
a)



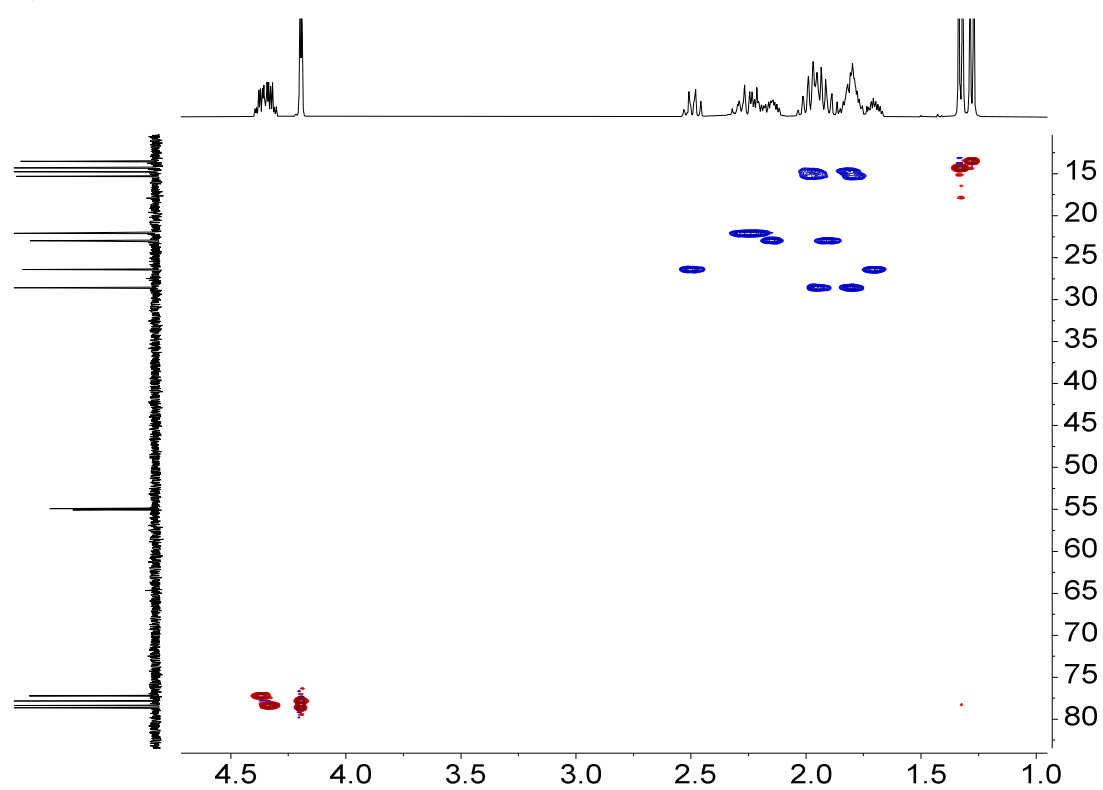
b)



c)



d)



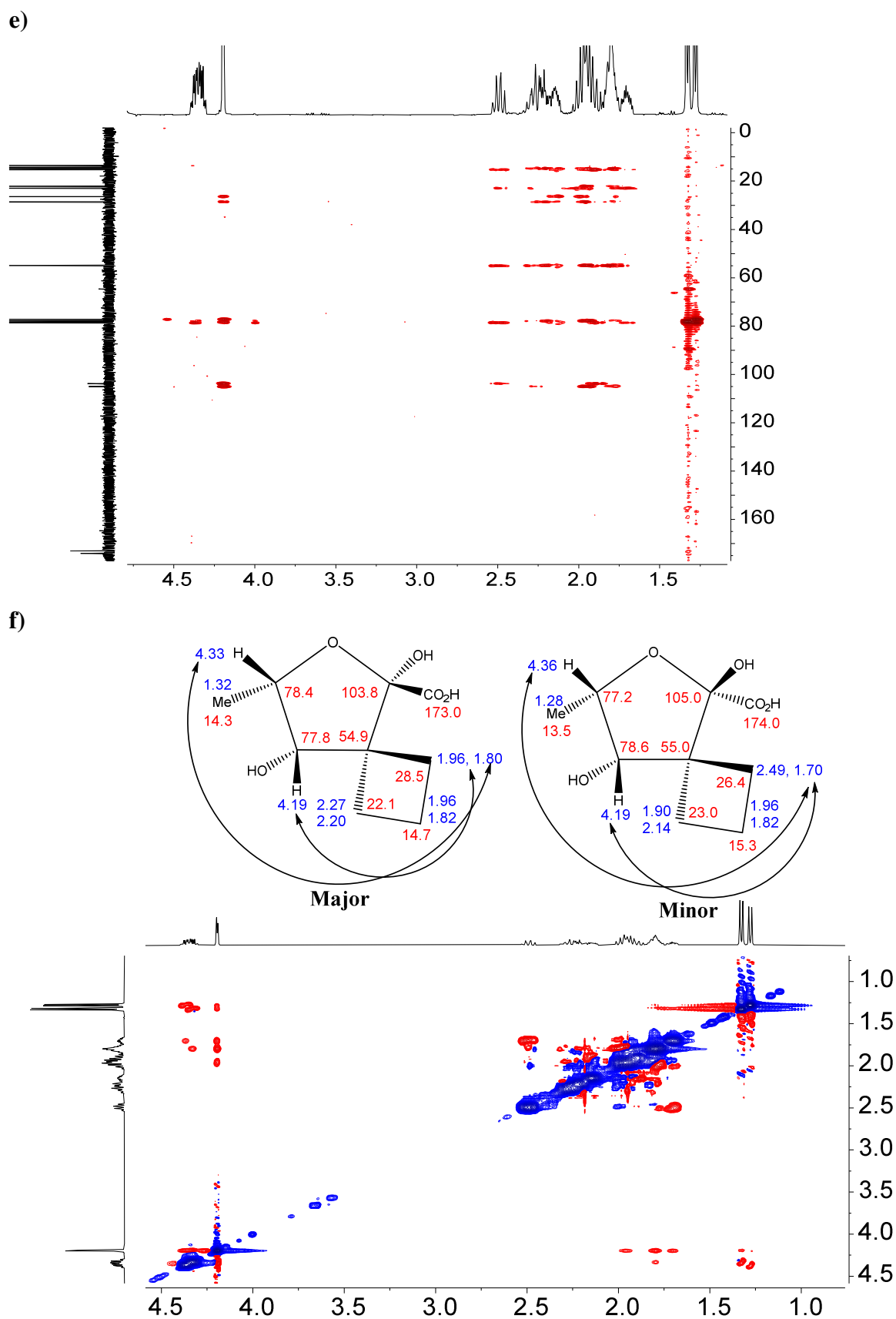
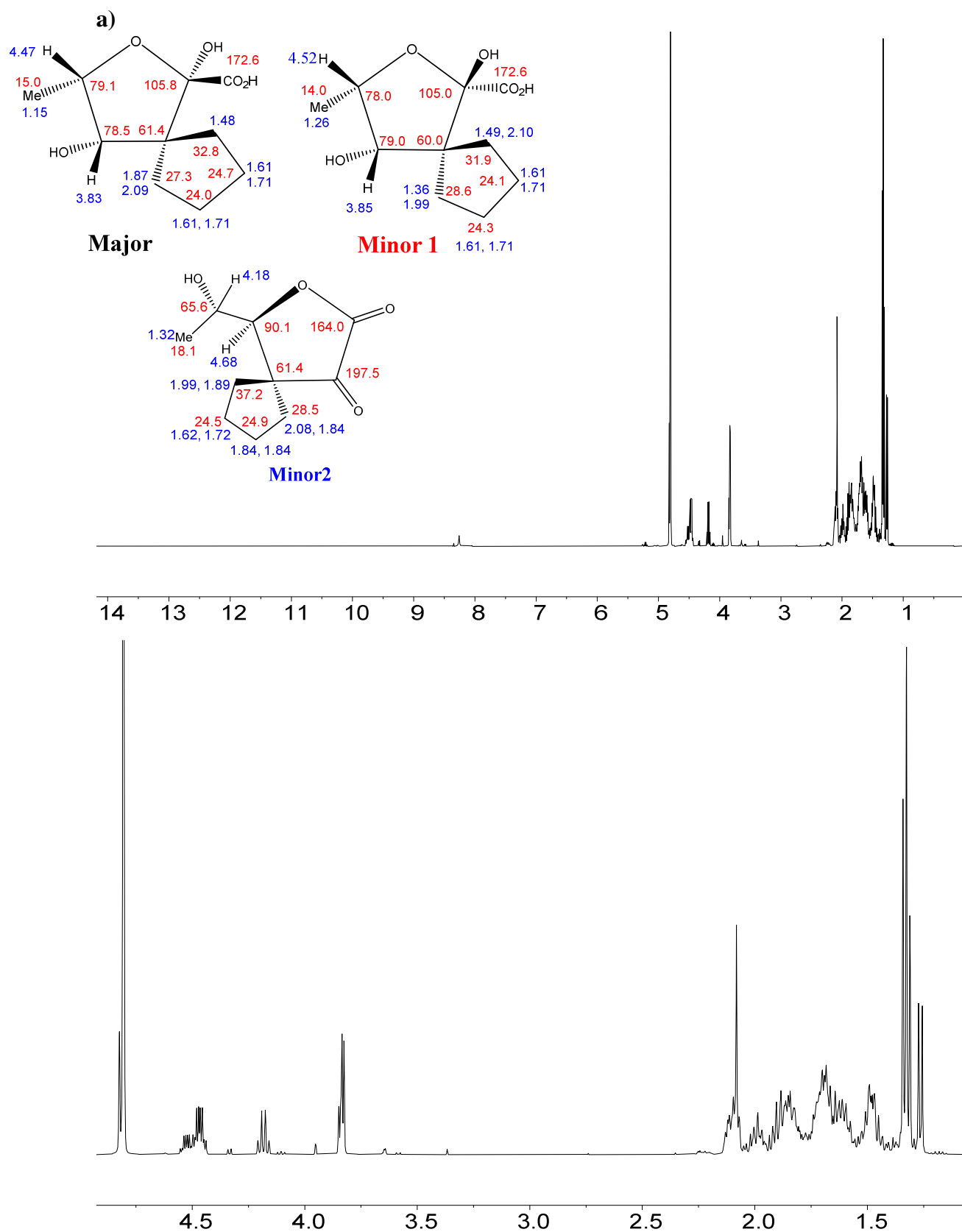
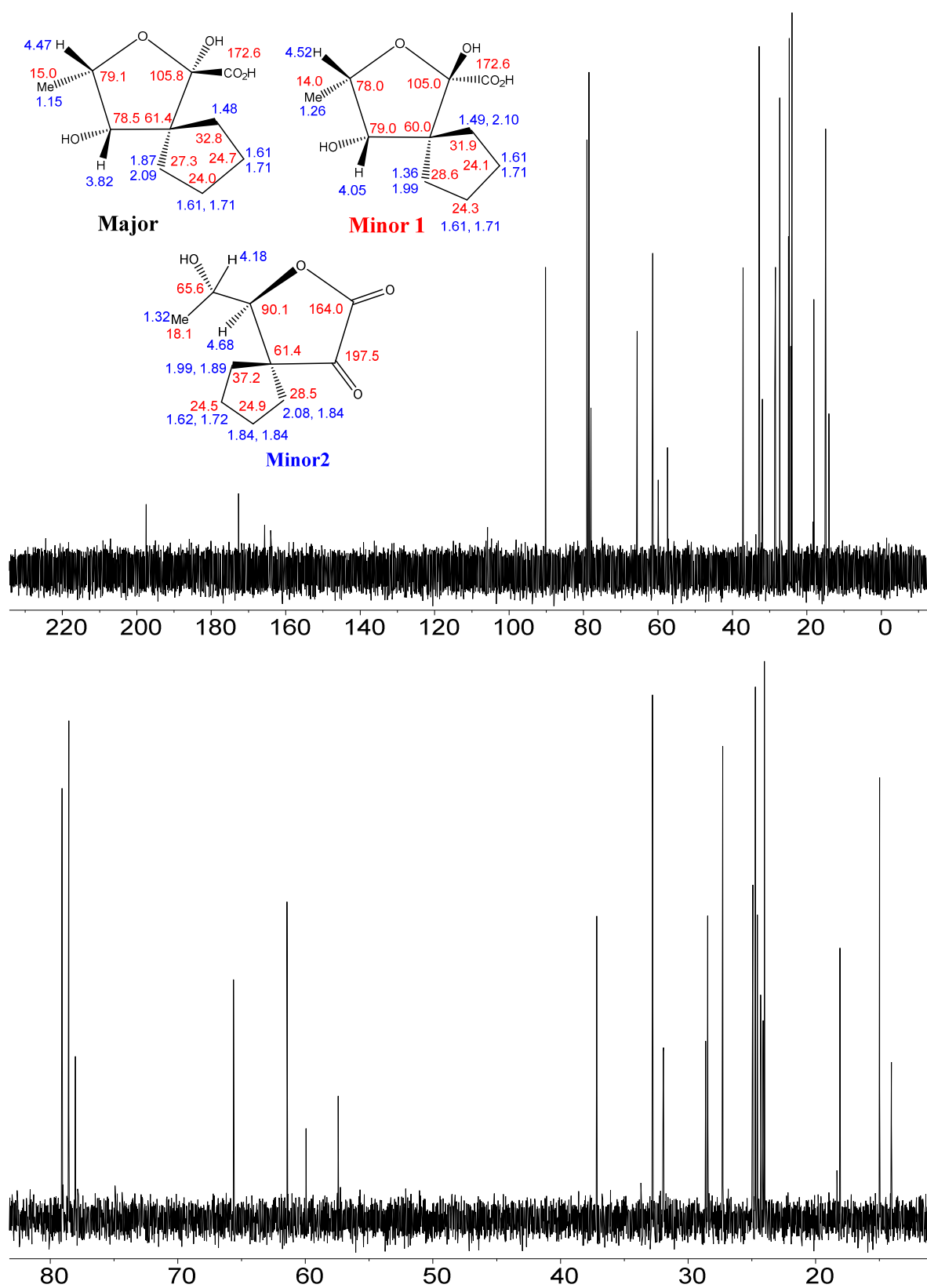


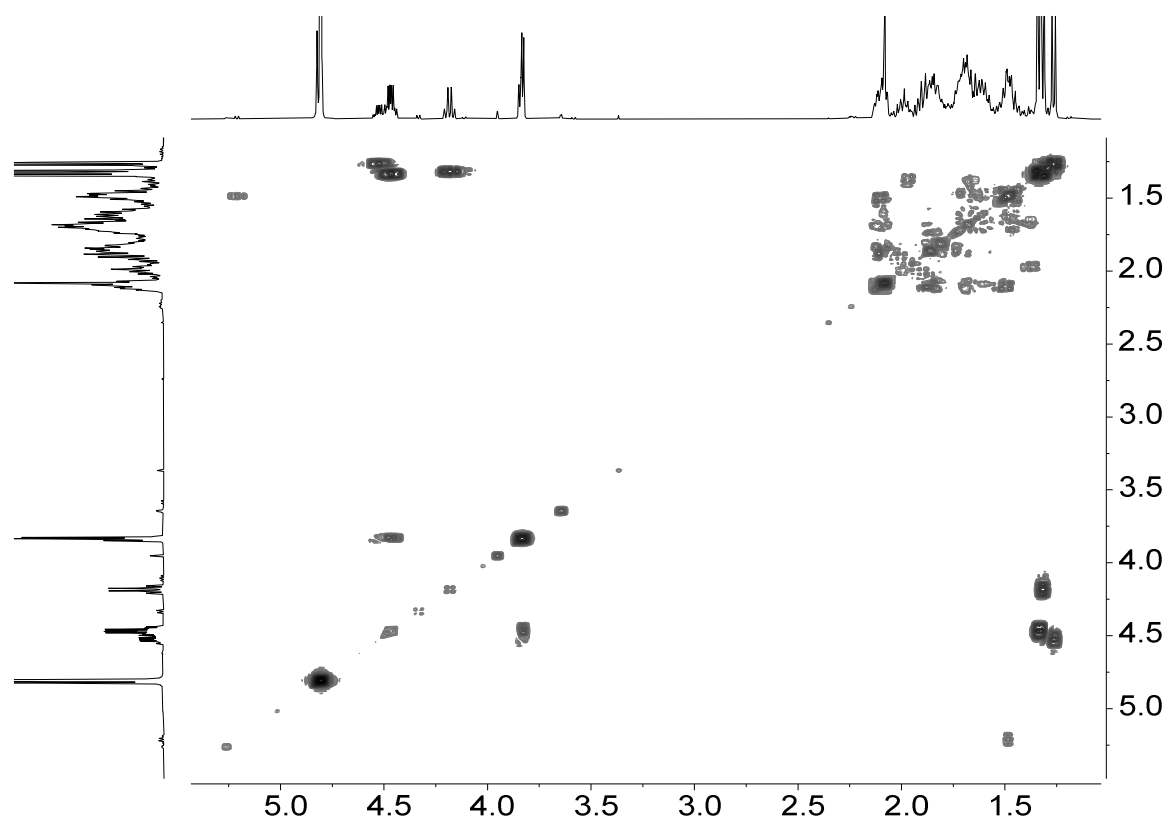
Figure S236 NMR spectra (D₂O) of **9j**: a) ¹H, b) ¹³C, c) gCOSY, d) gHSQC, e) HMBC and f) 2D NOESY. E178-105-exp72.



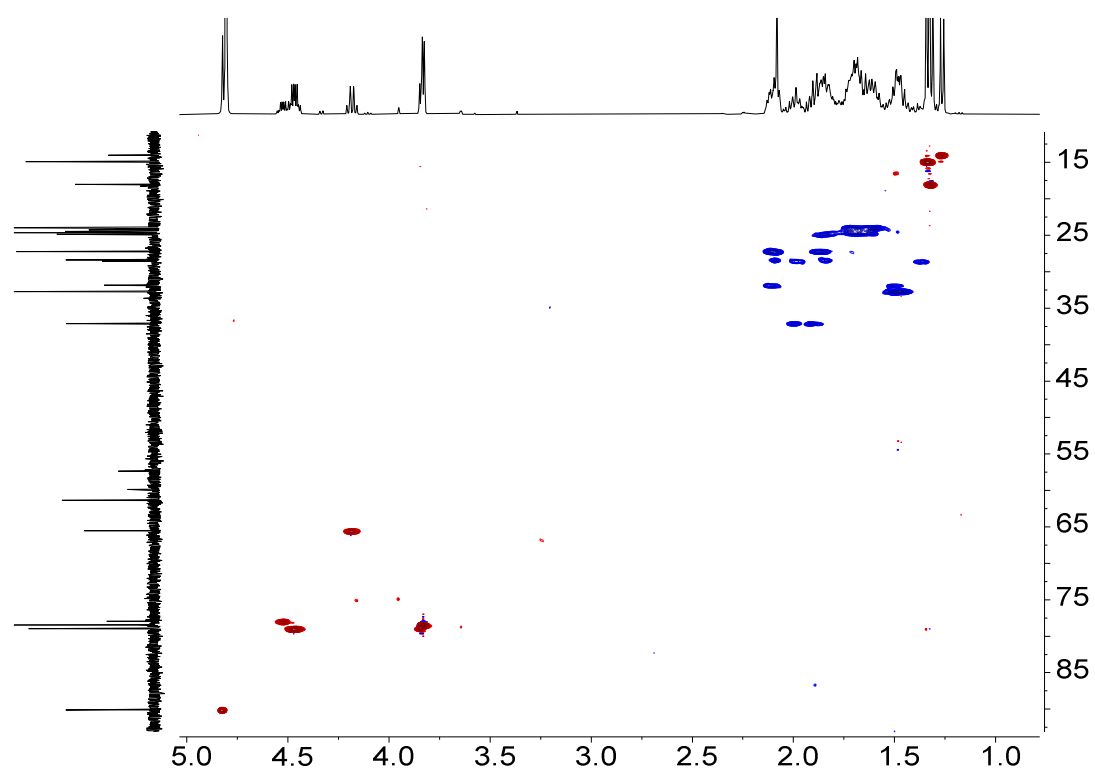
b)



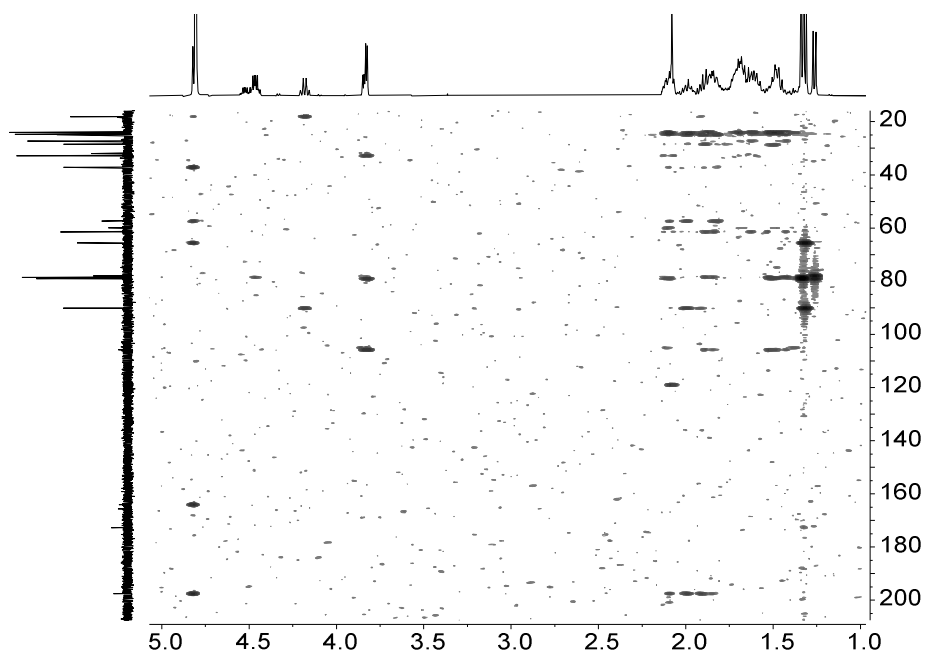
c)



d)



e)



f)

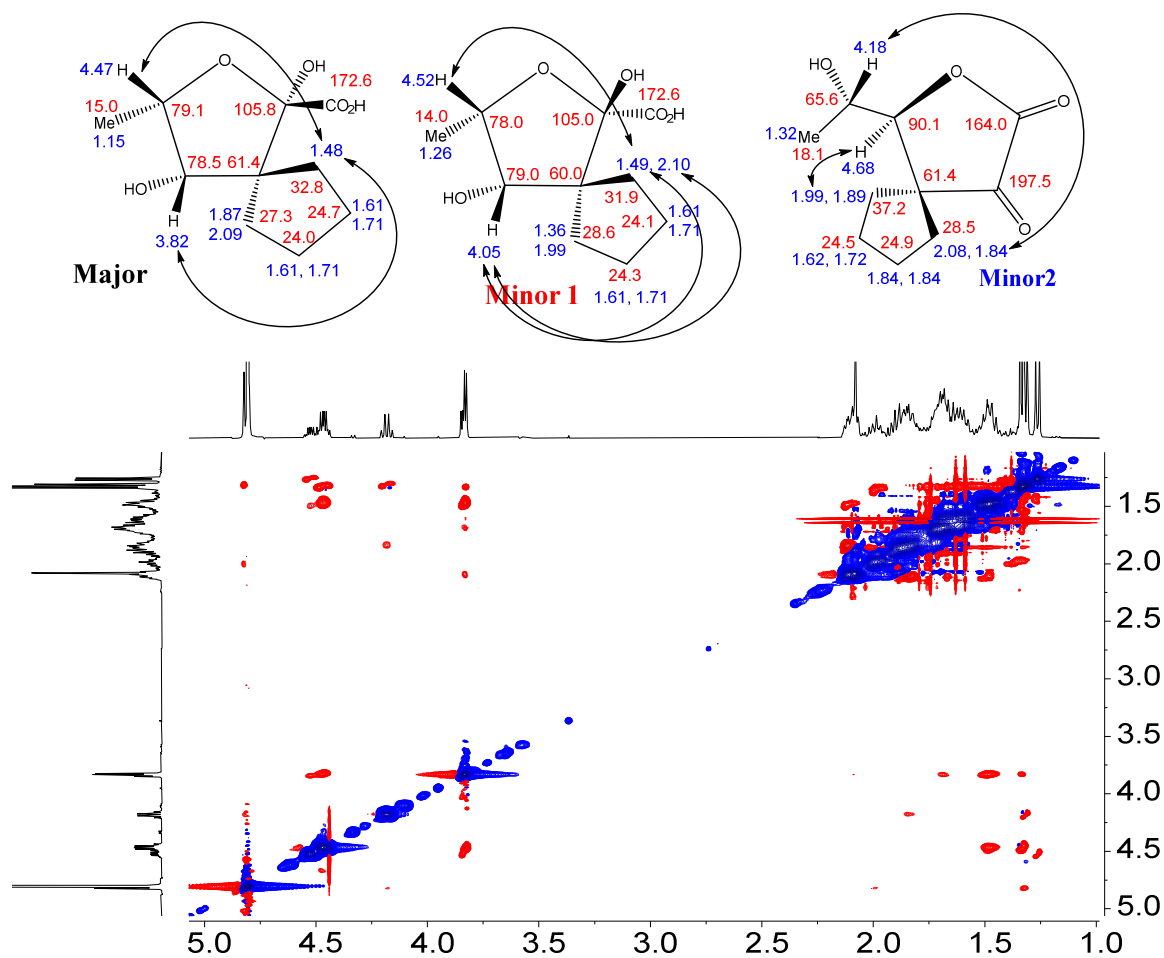
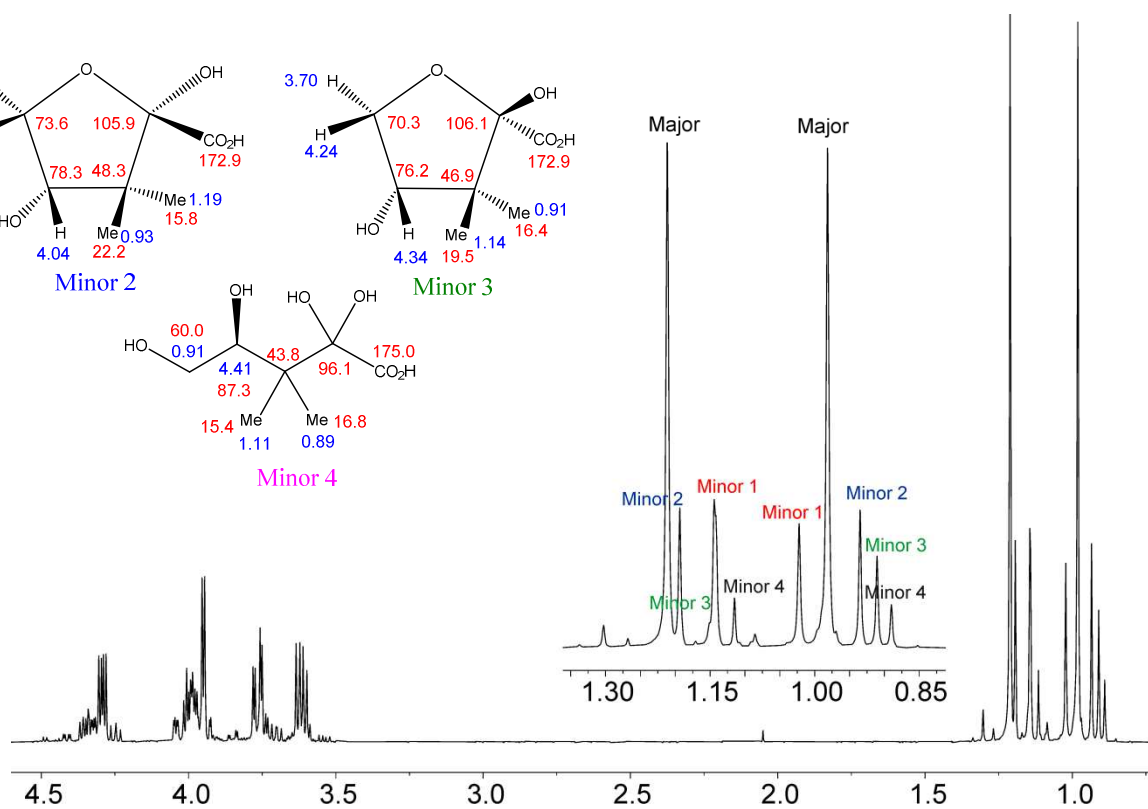
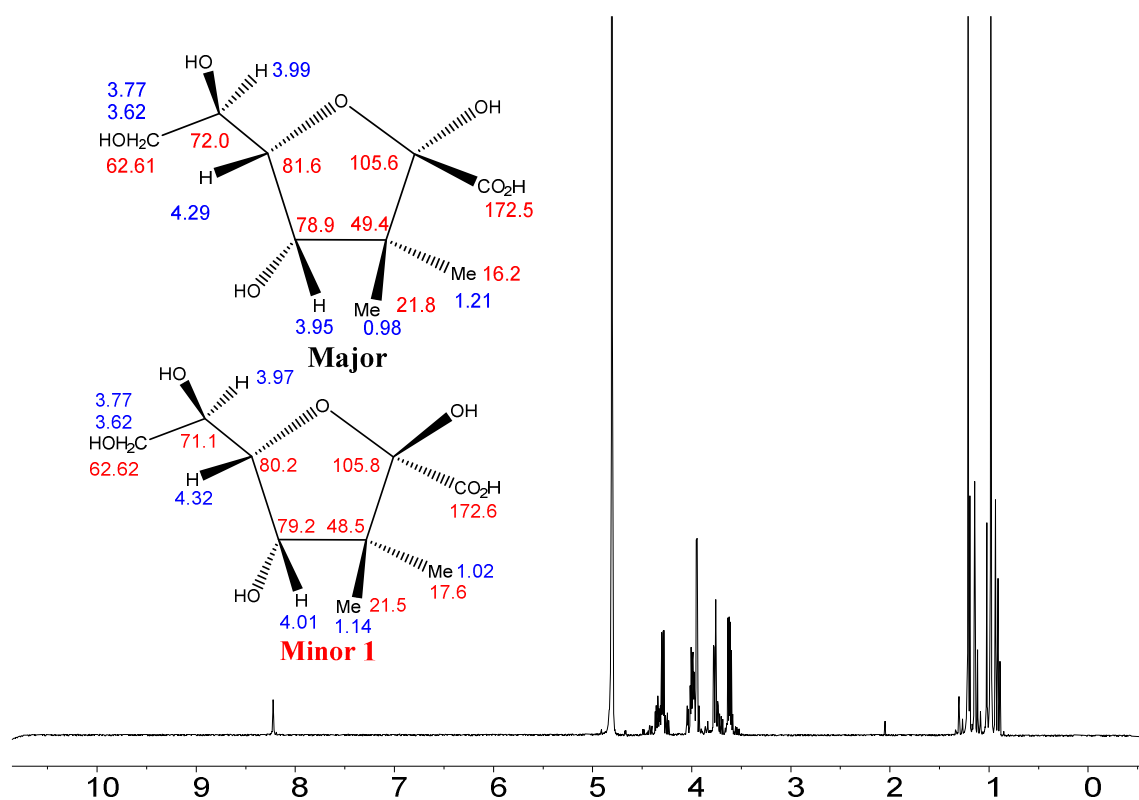
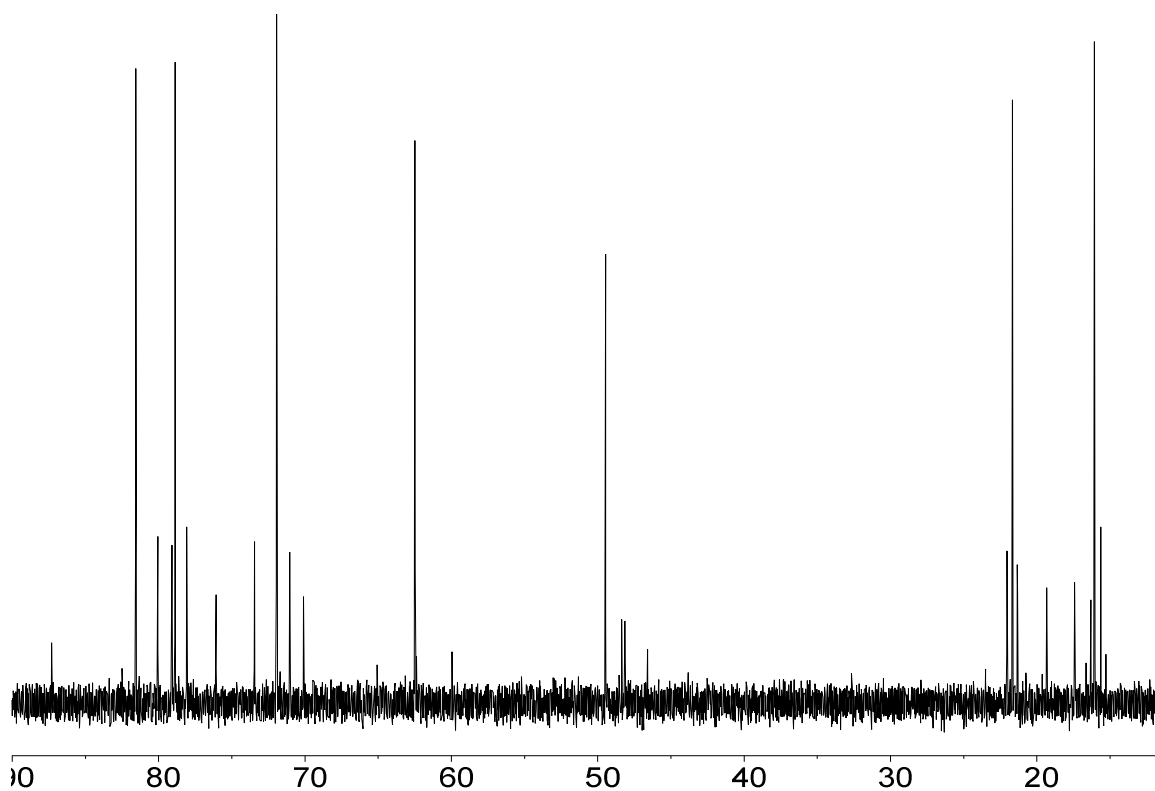
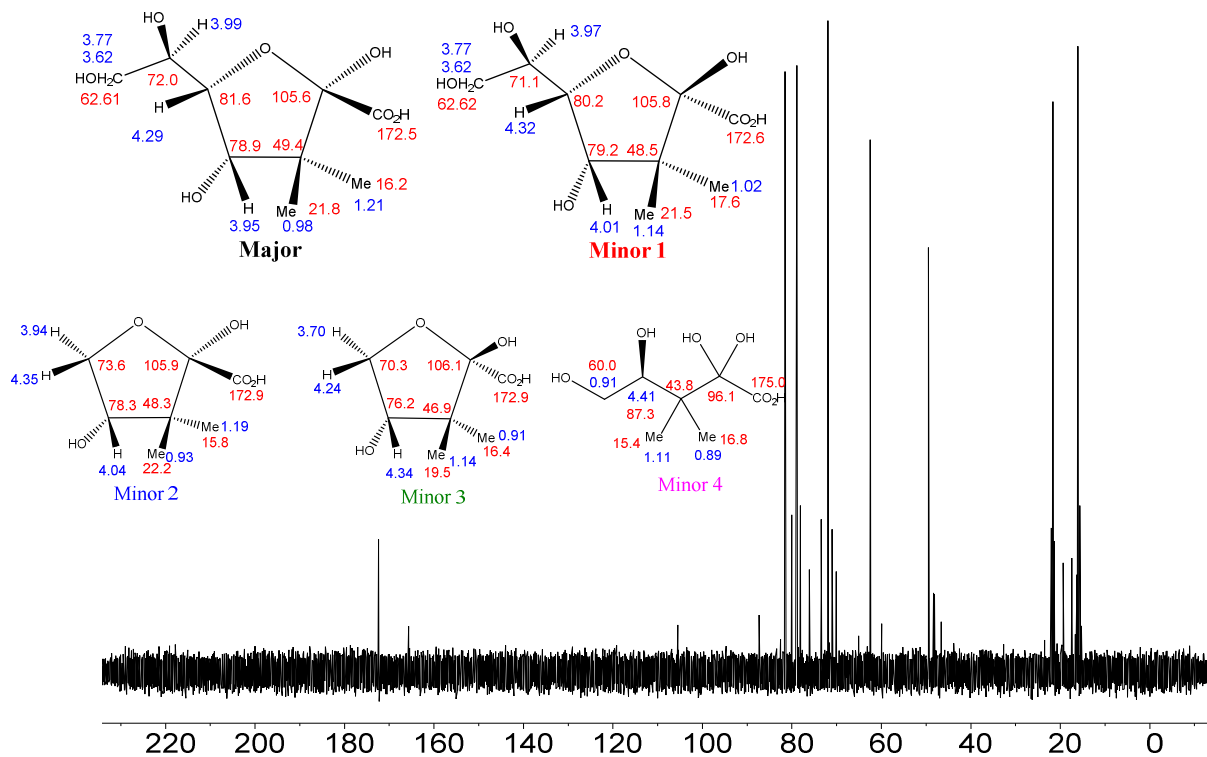


Figure S237 NMR spectra (D₂O) of **9k**: a) ¹H, b) ¹³C, c) gCOSY, d) gHSQC, e) HMBC and f) 2D NOESY. E178-107-exp73.

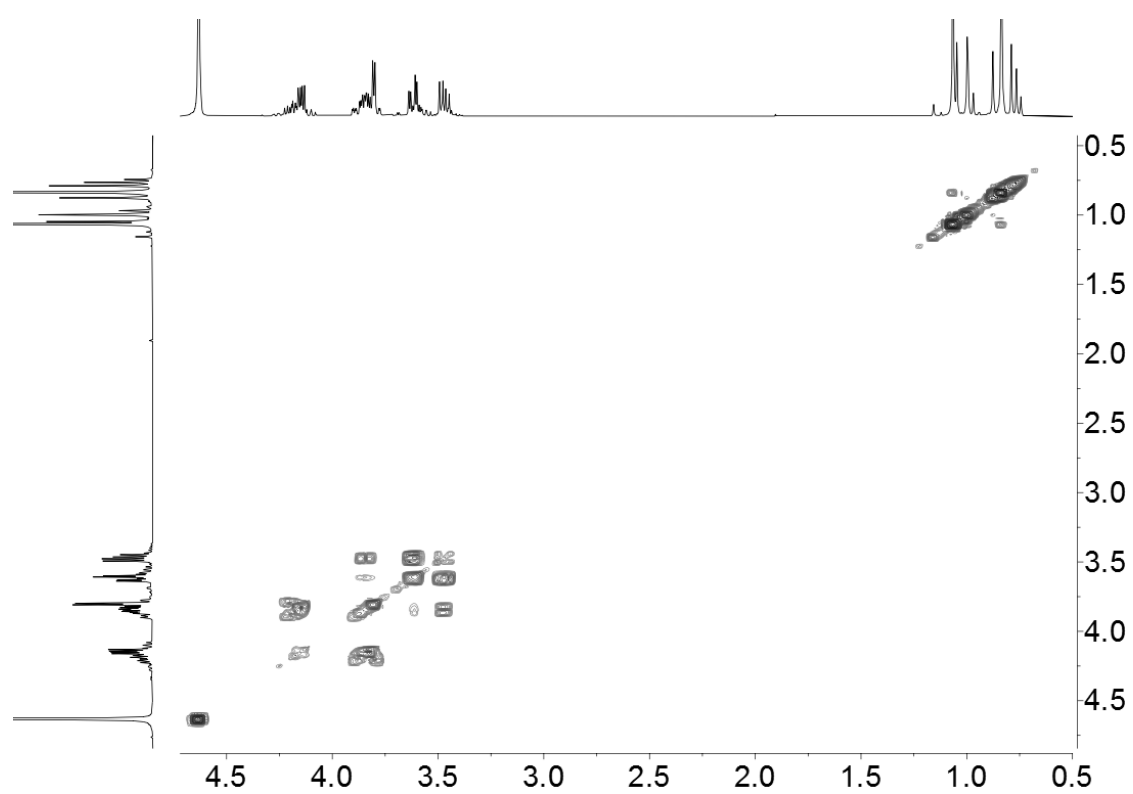
a)



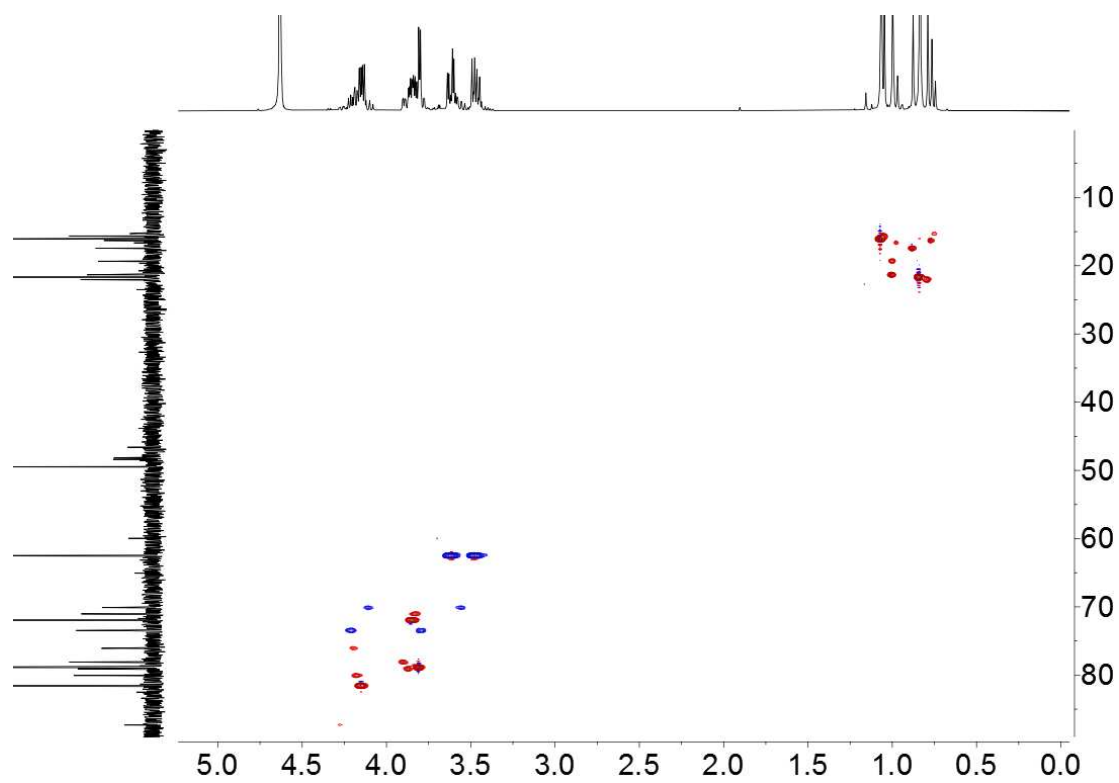
b)



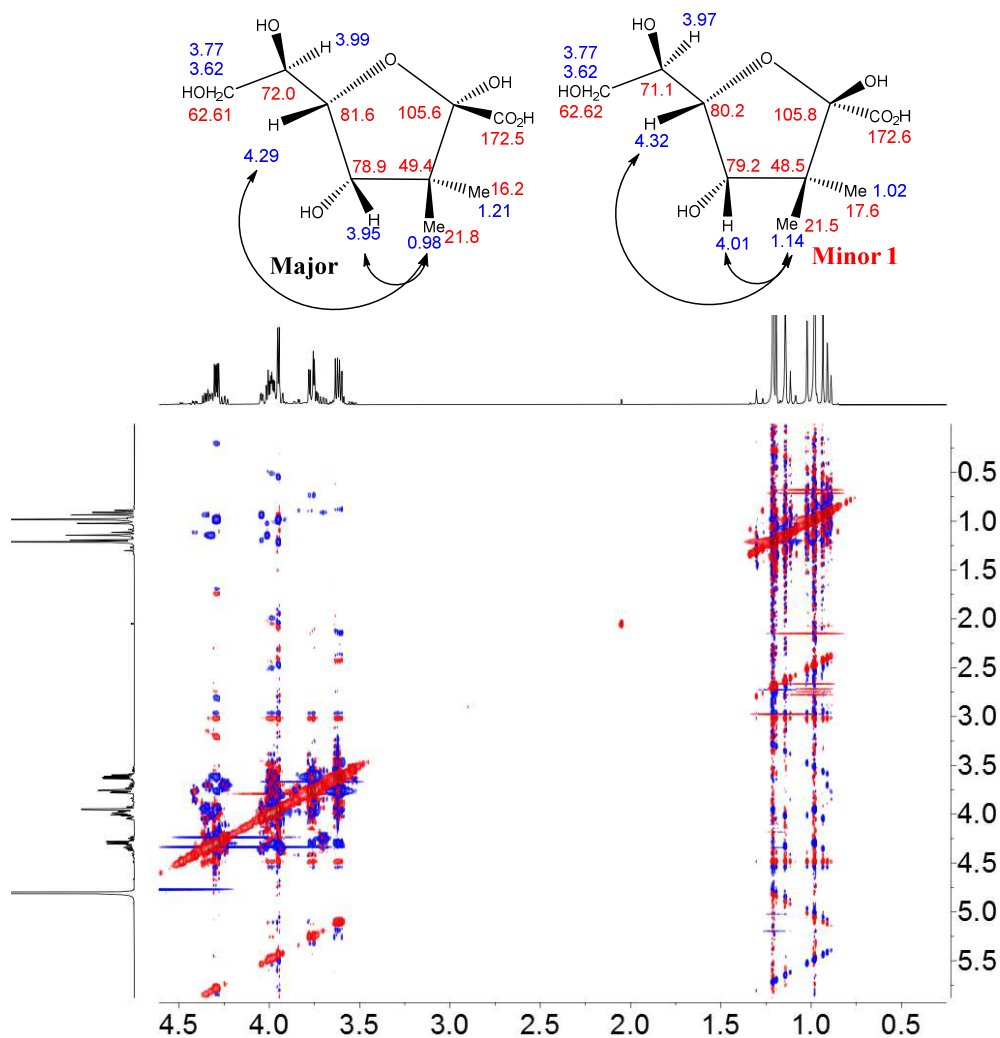
c)



d)



e)



f)

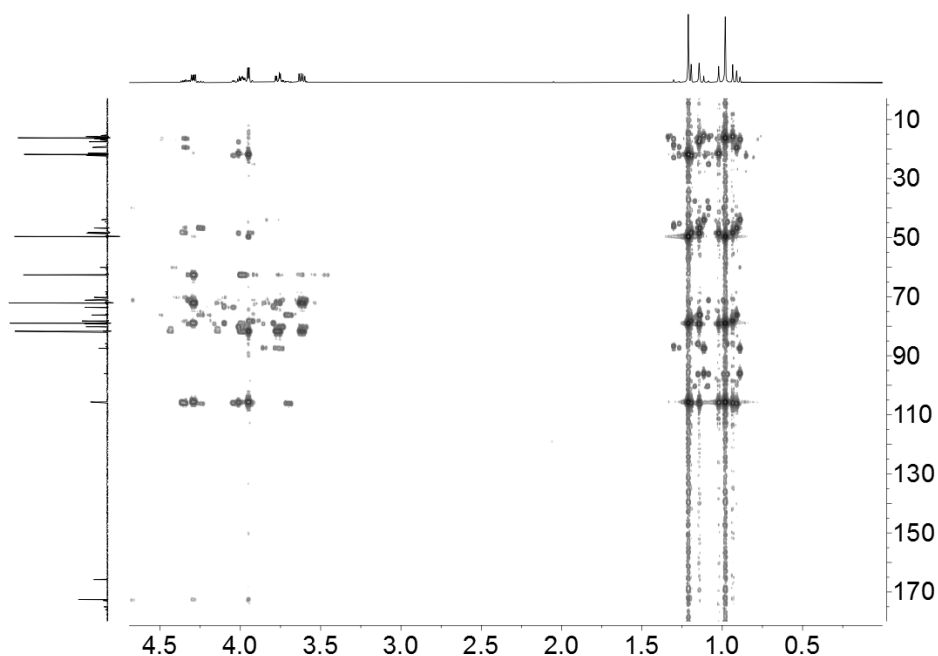


Figure S238 NMR spectra (D_2O) of **9l**: a) ^1H , b) ^{13}C , c) gCOSY, d) gHSQC, e) 2D NOESY and f) HMBC. E178-50-exp30.

a)

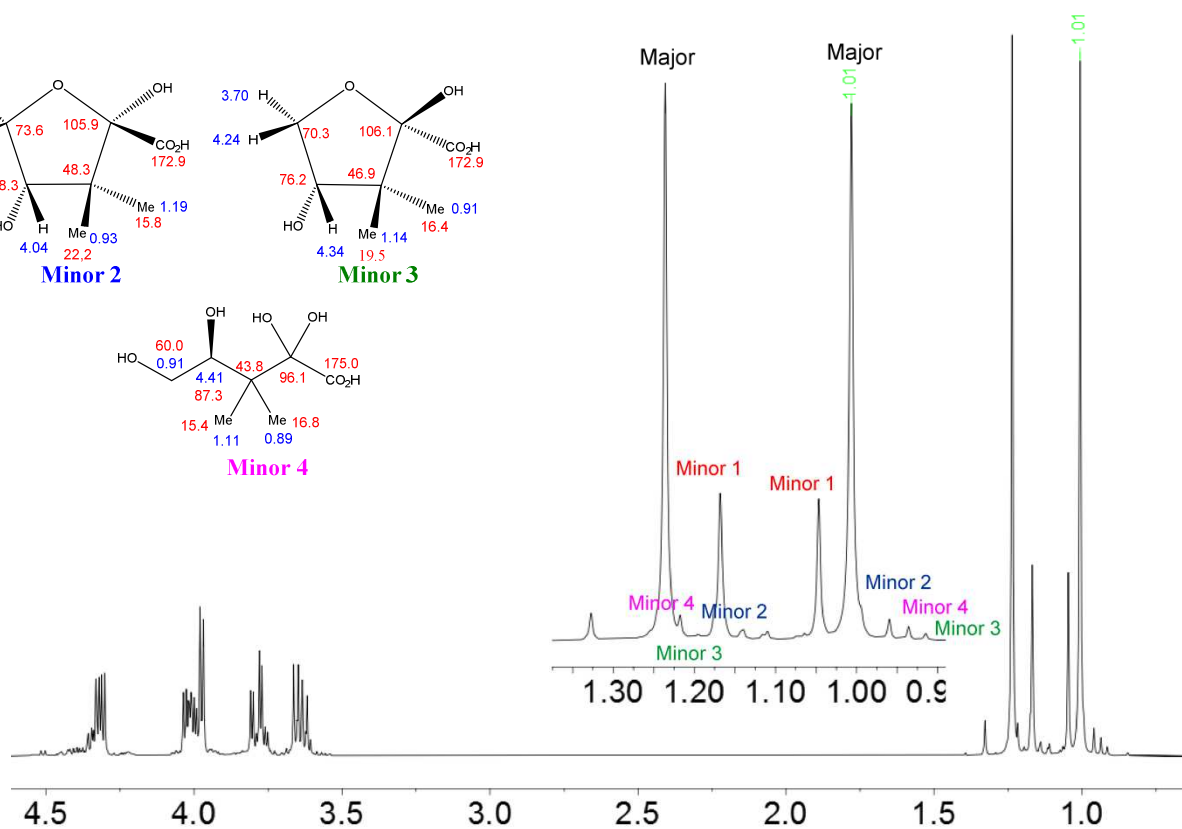
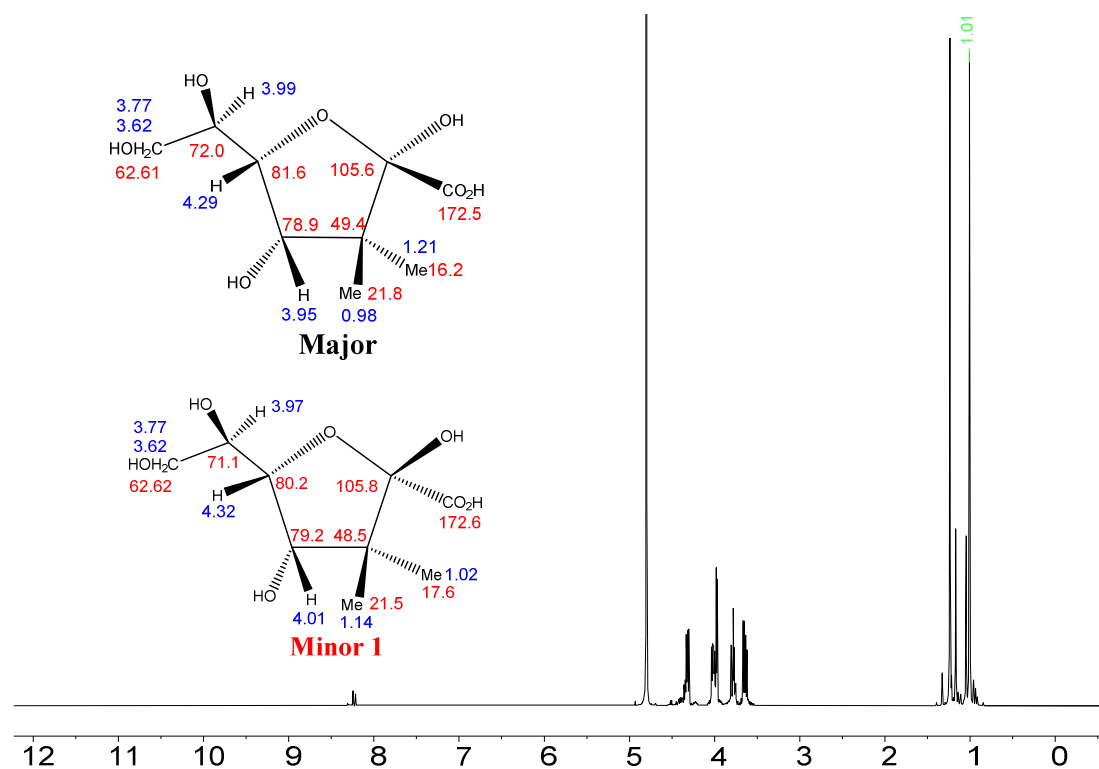
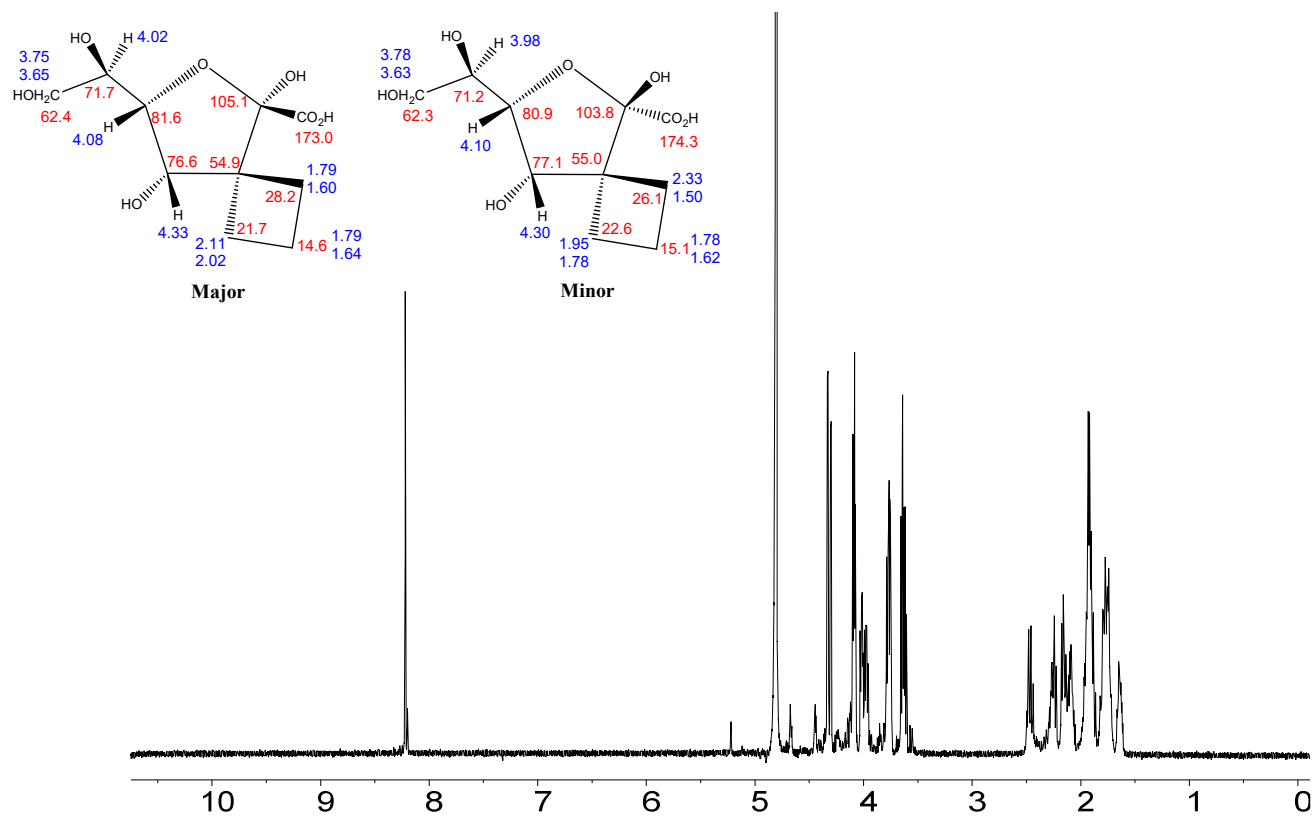
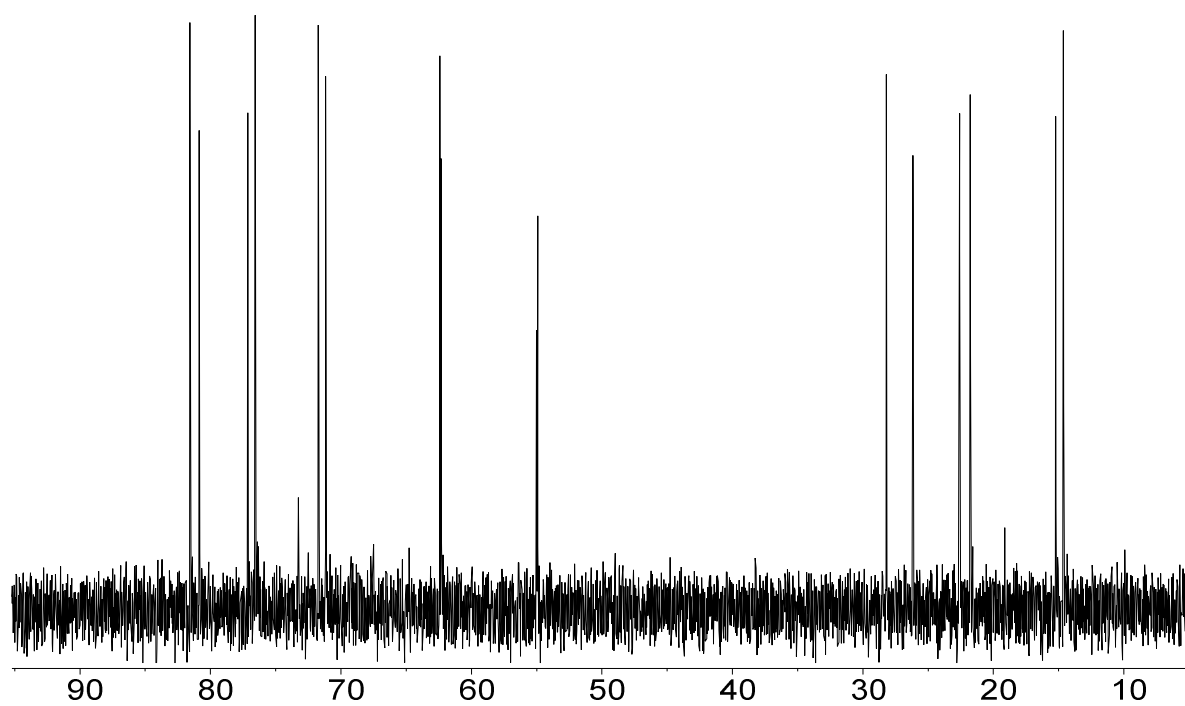
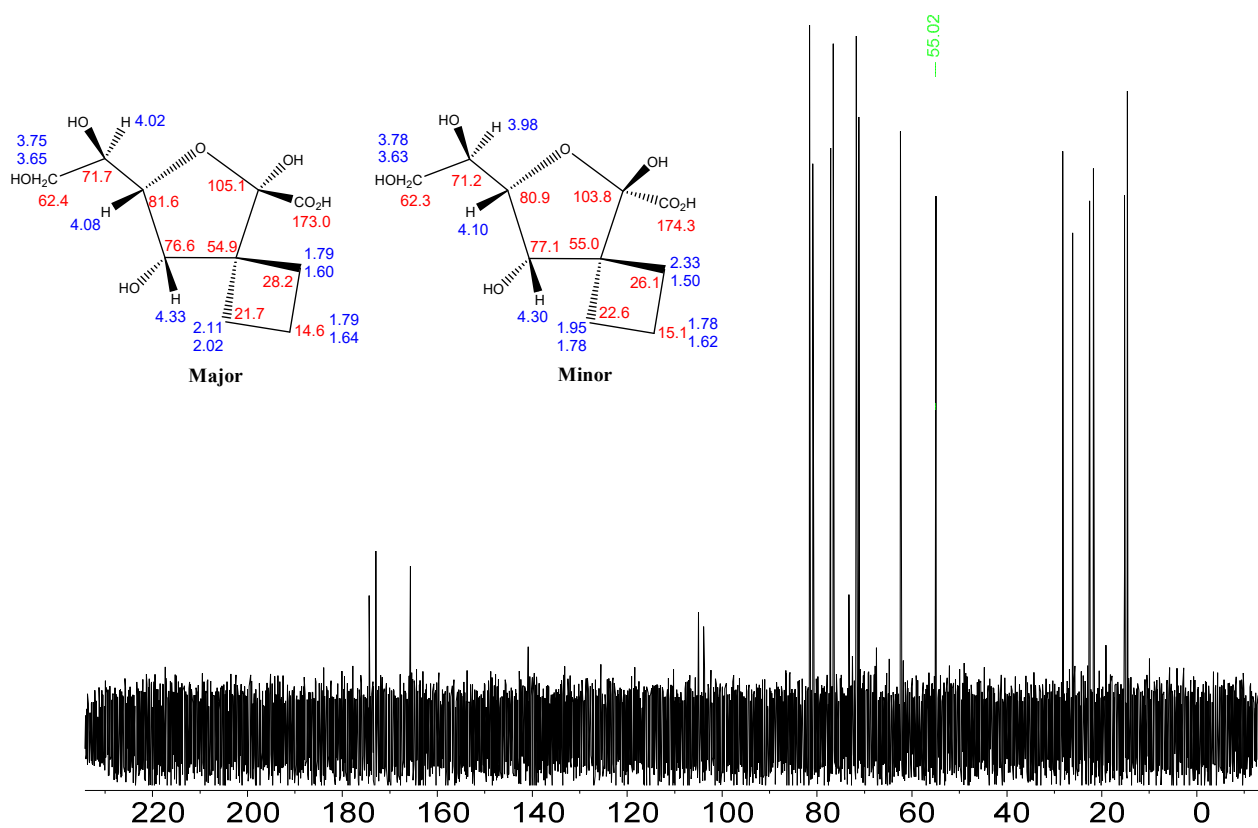


Figure S239 NMR spectra (D₂O) of **9I** (without FSA): a) ¹H. E178-64-exp40.

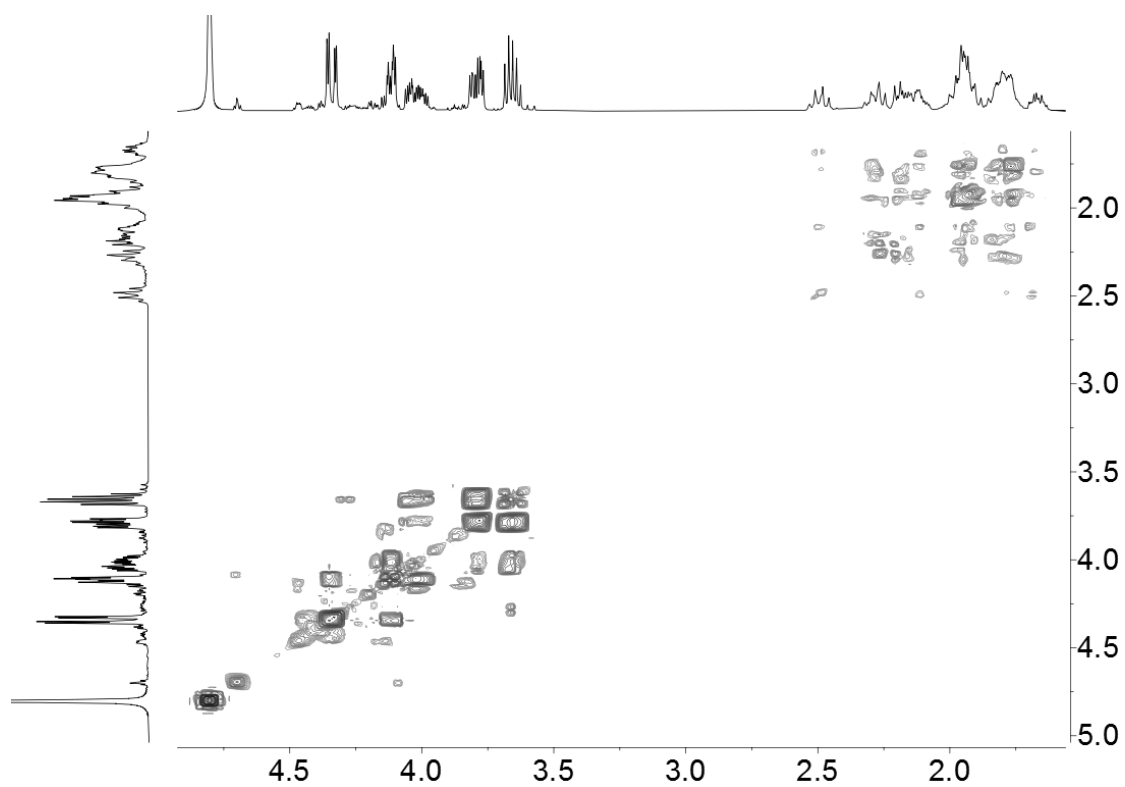
a)



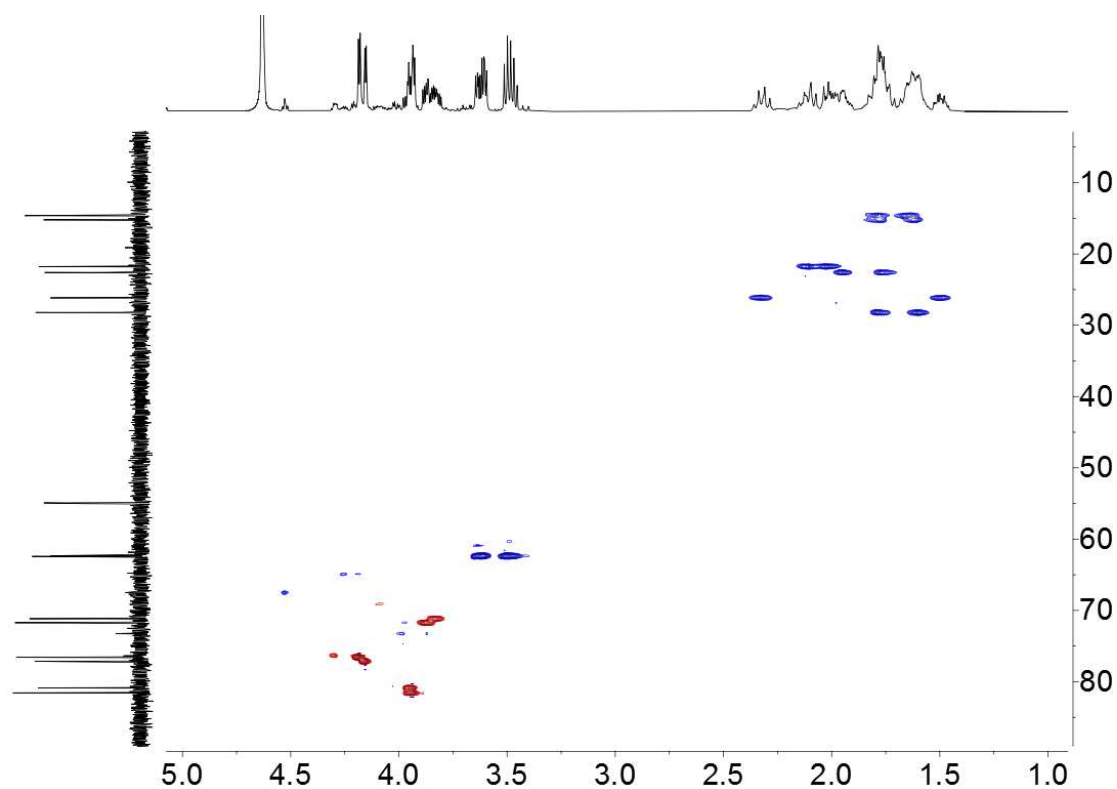
b)



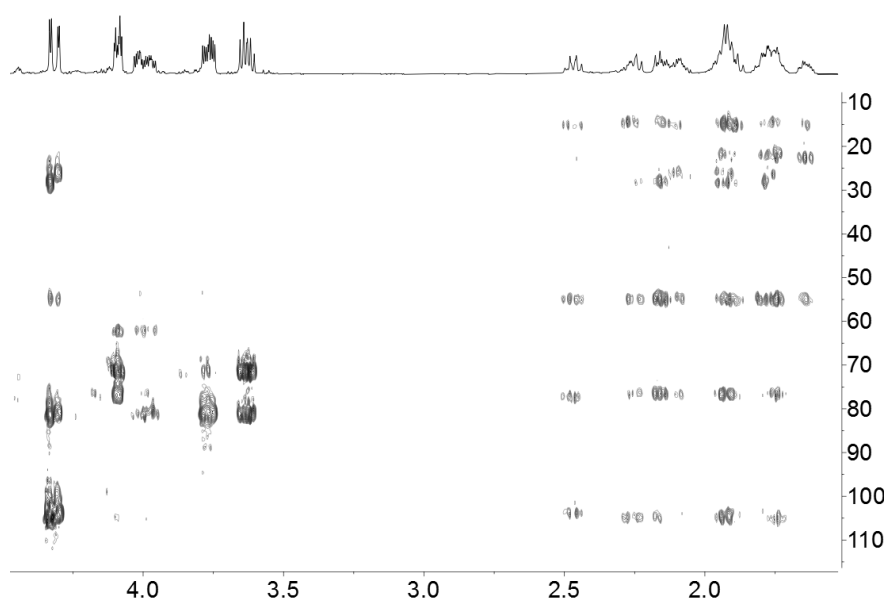
c)



d)



e)



f)

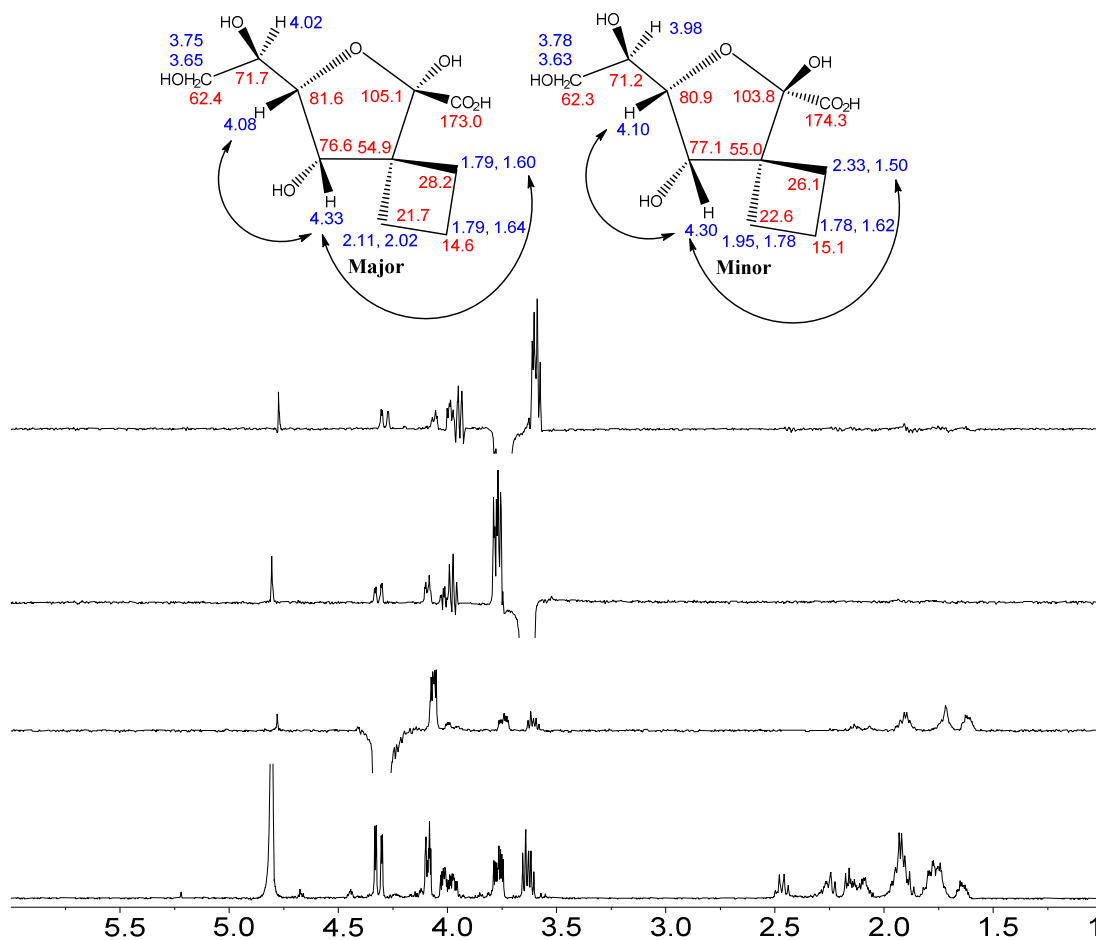
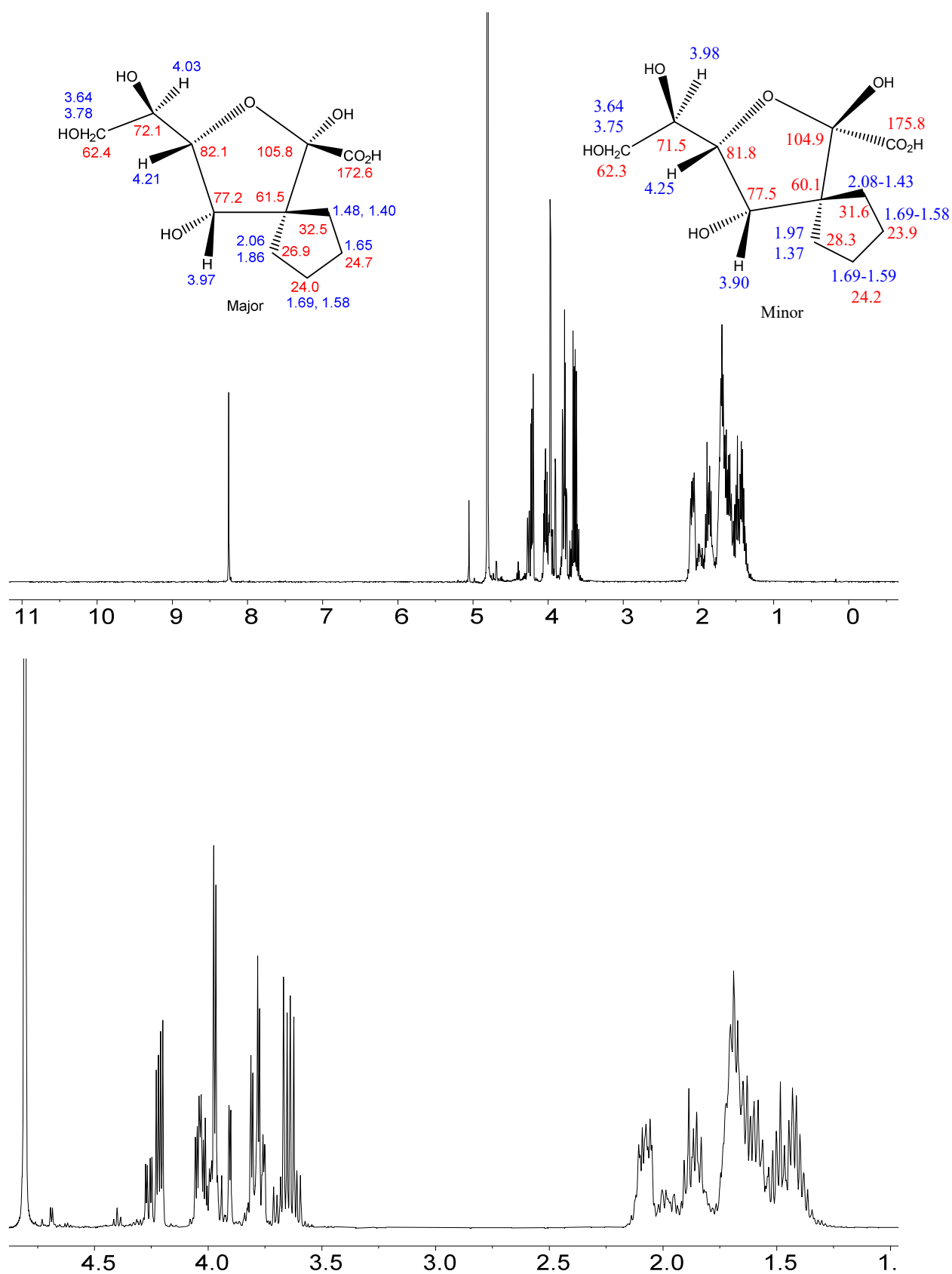
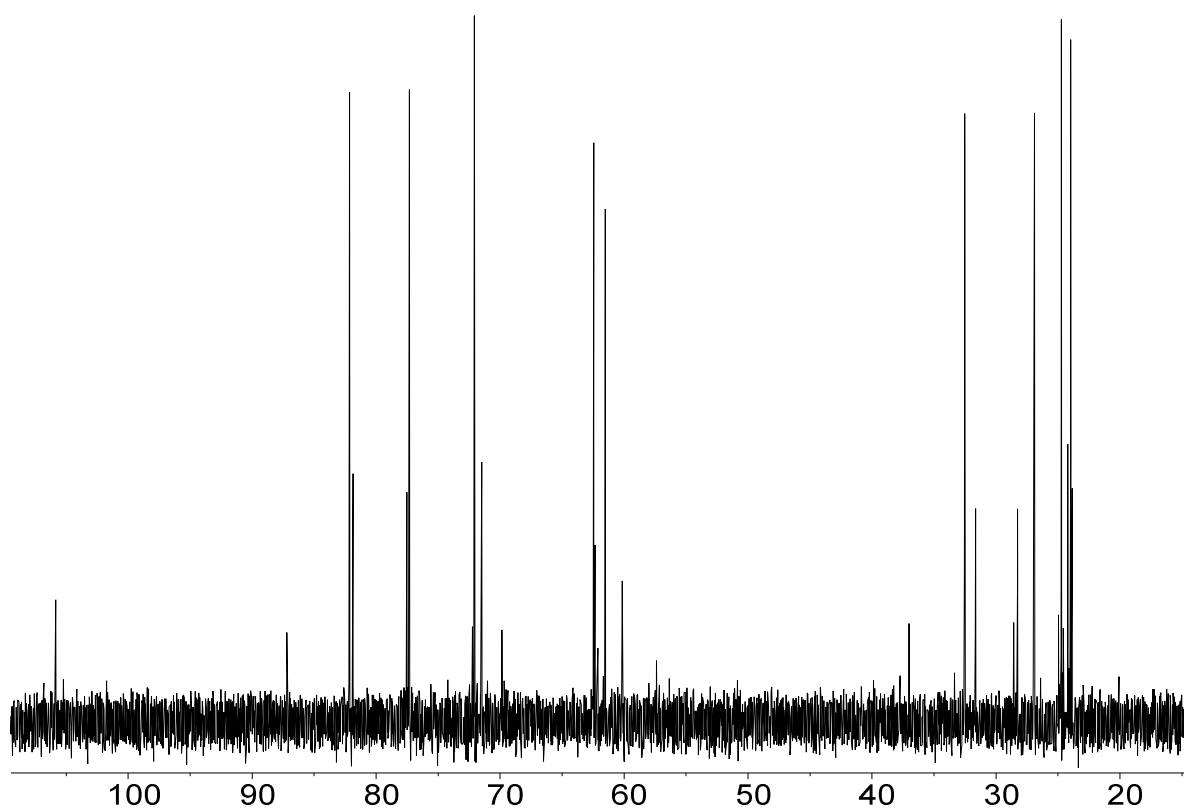
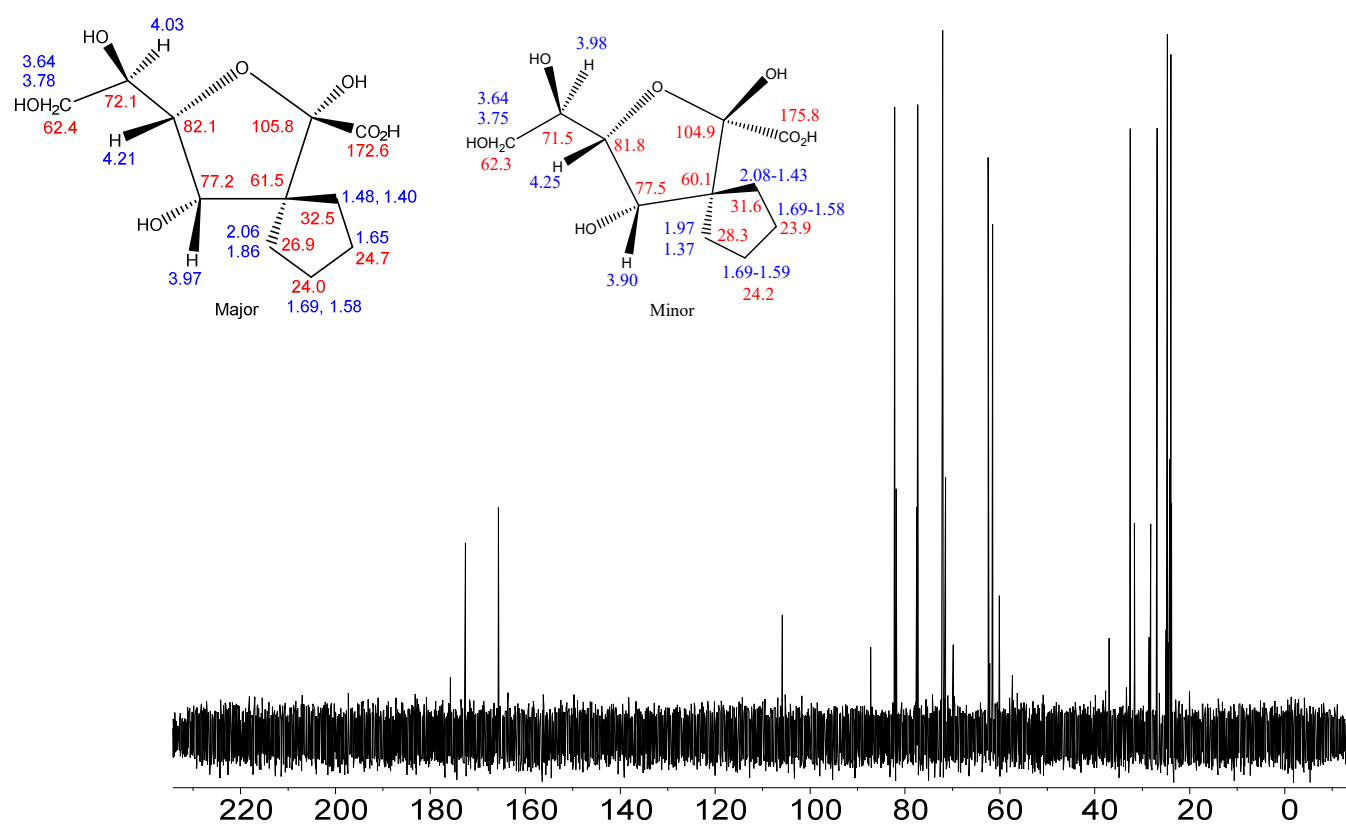


Figure S240 NMR spectra (D₂O) of **9m**: a) ¹H, b) ¹³C, c) gCOSY, d) gHSQC, e) HMBC and f) Selective 1D nOe spectra. E178-54-exp33.

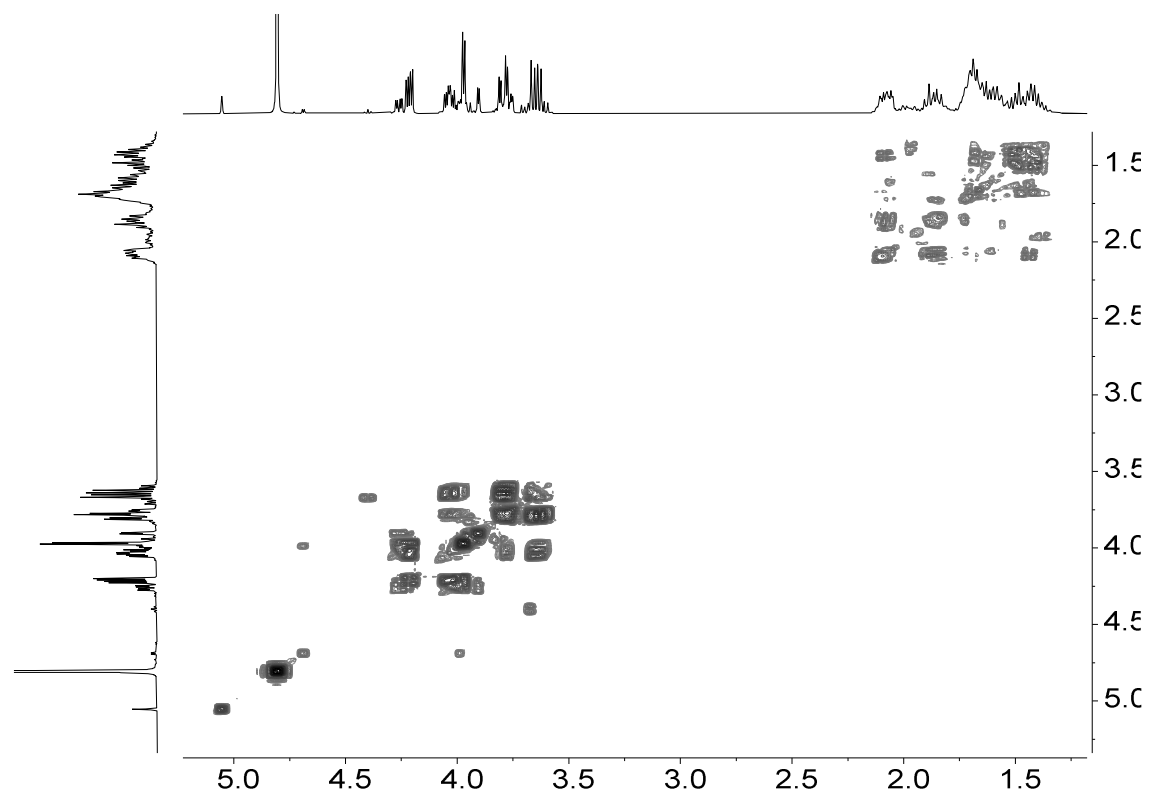
a)



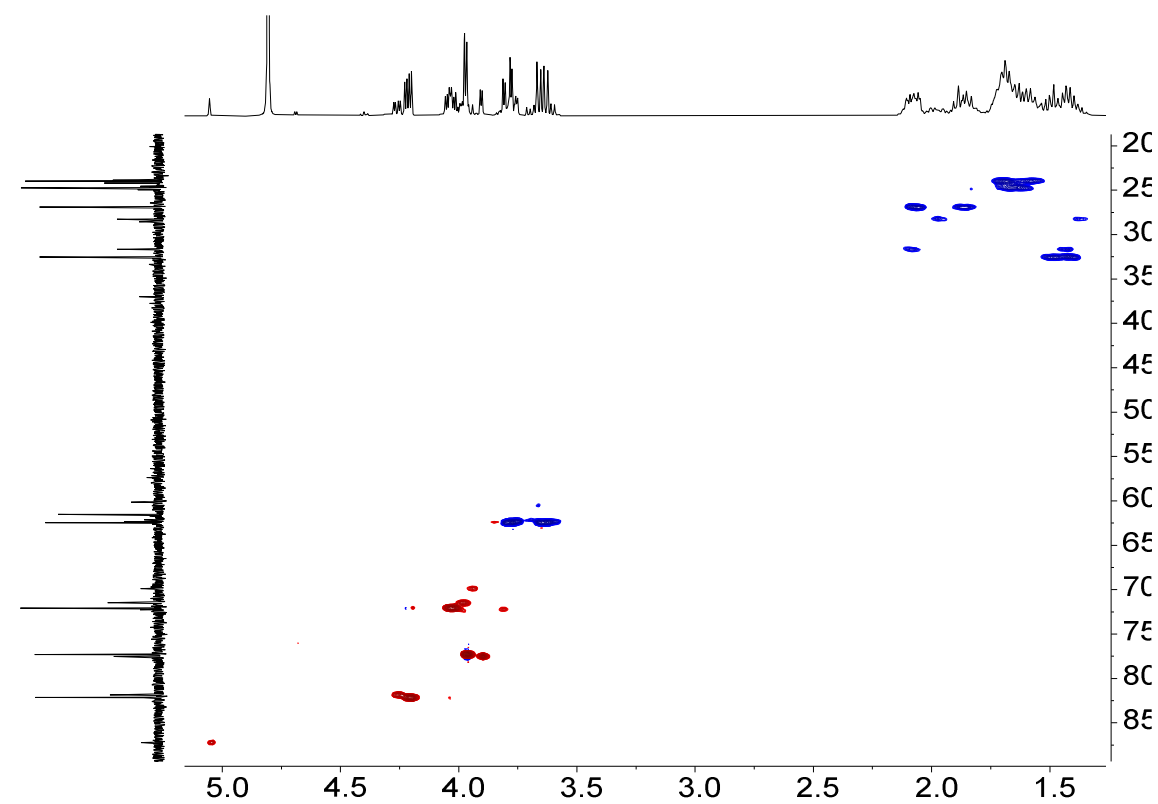
b)



c)



d)



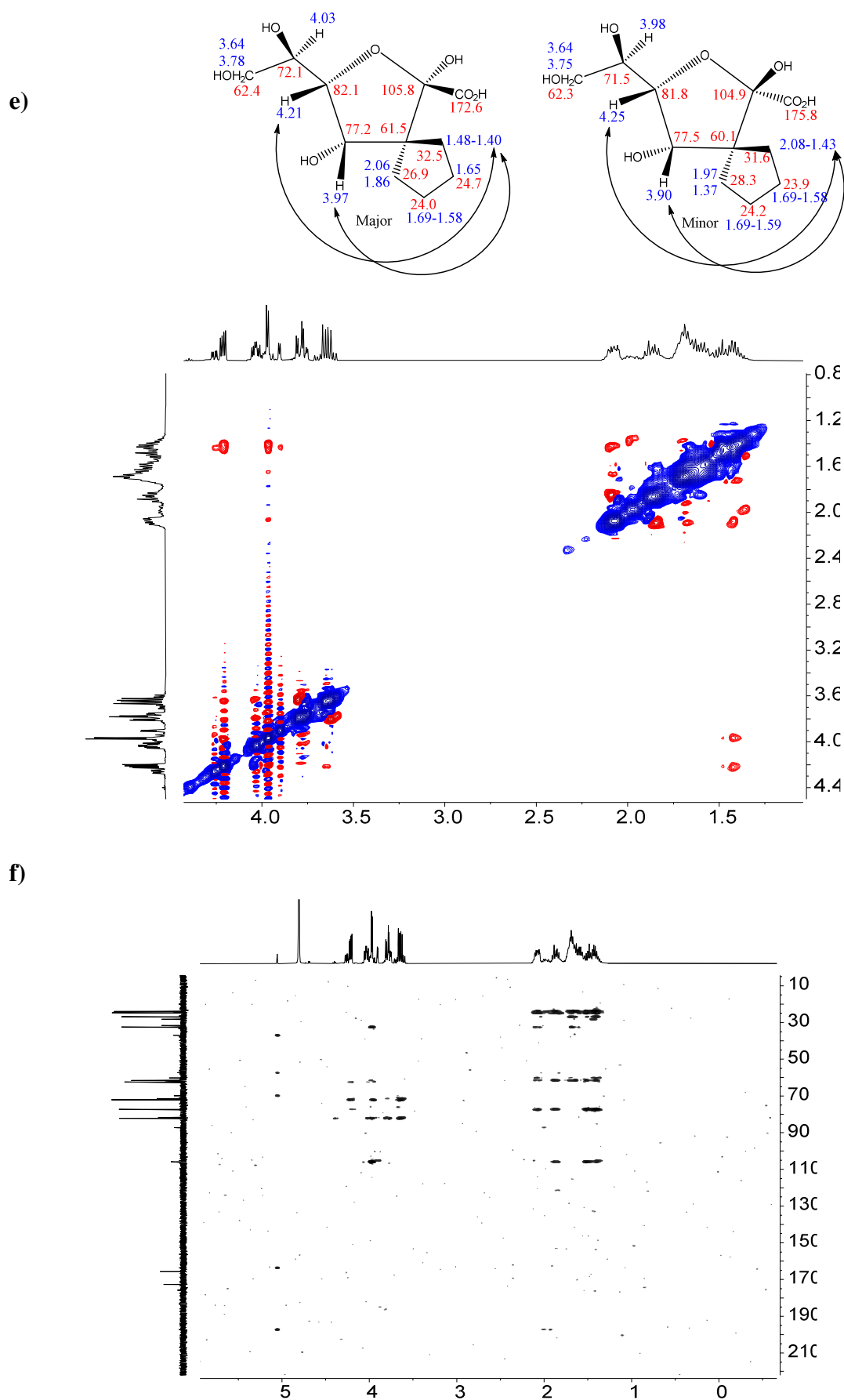
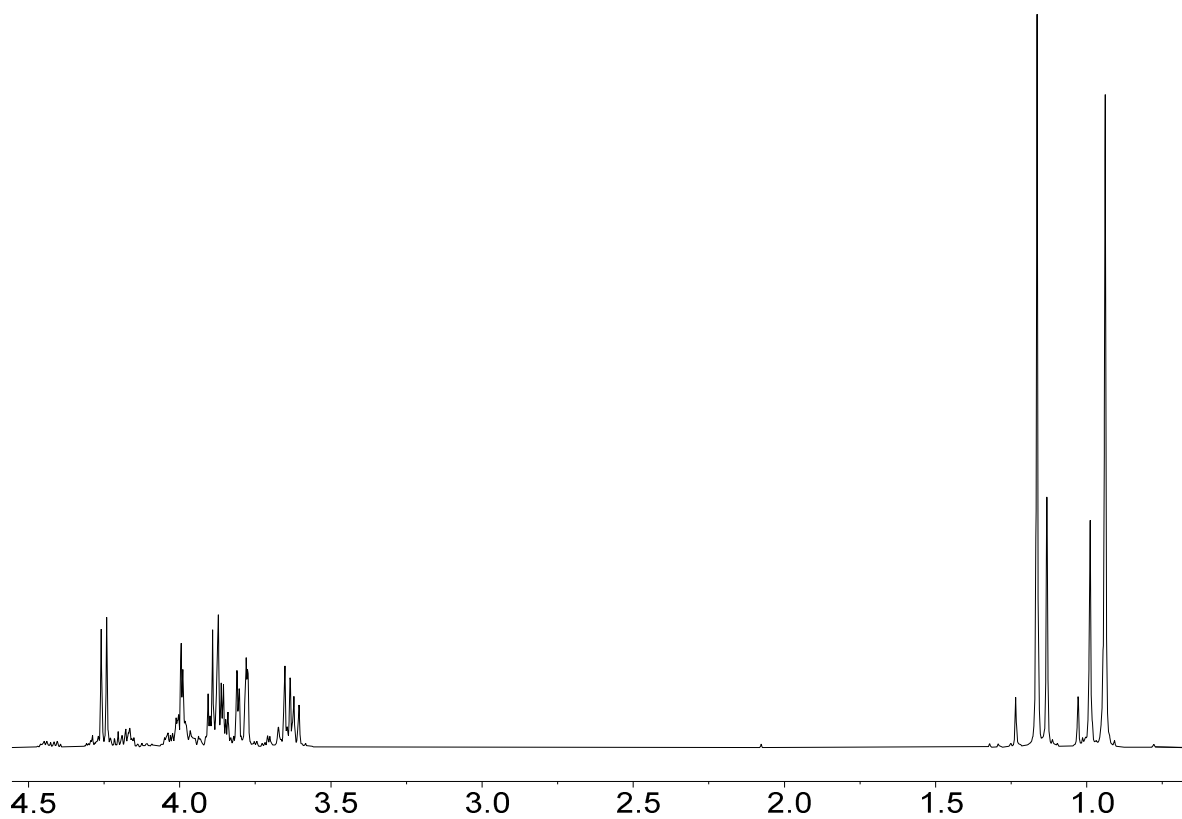
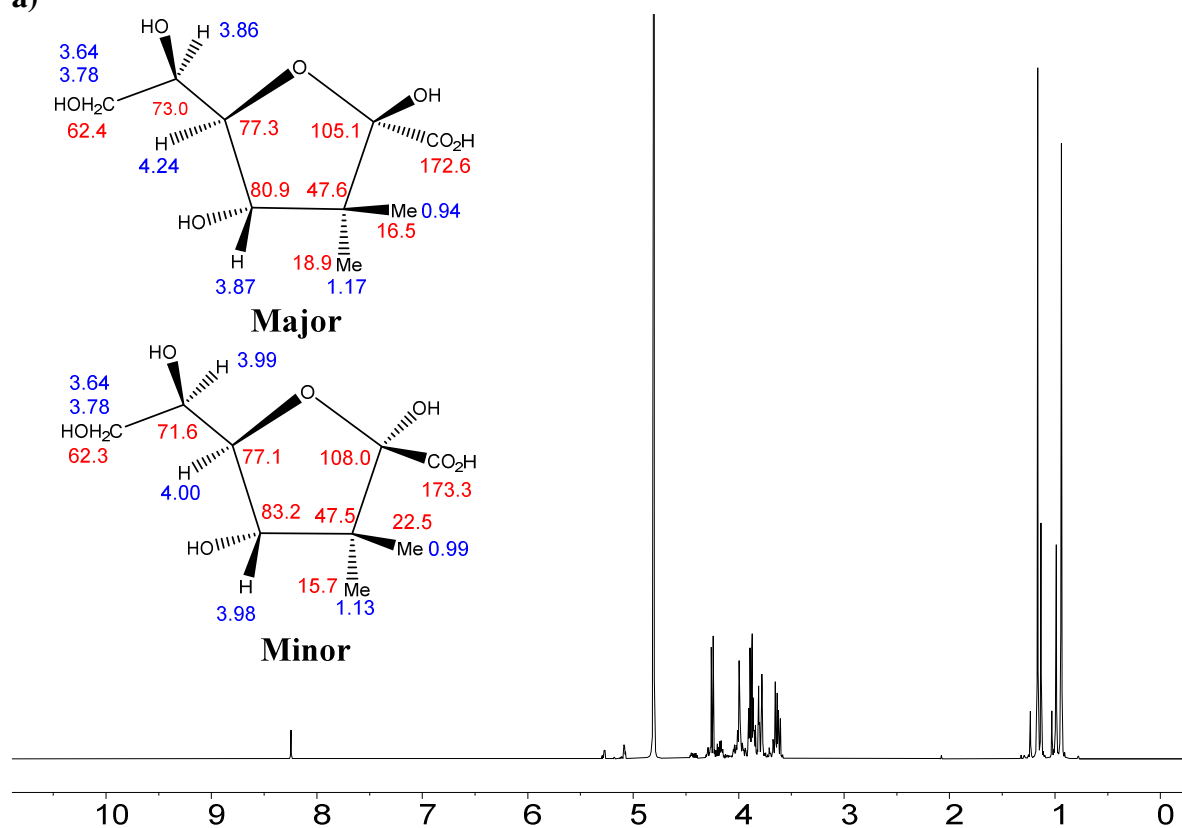
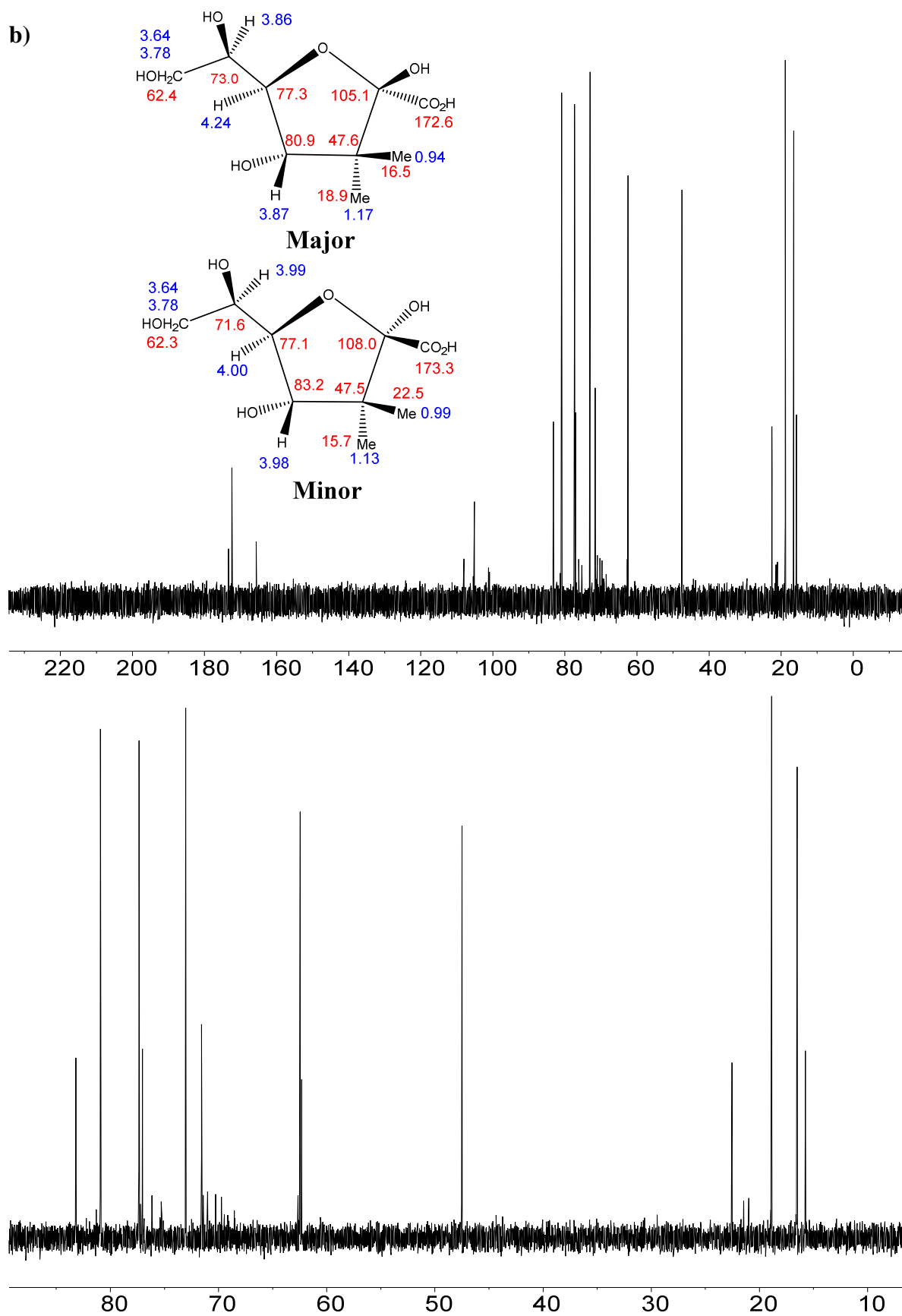


Figure S241 NMR spectra (D₂O) of **9n**: a) ¹H, b) ¹³C, c) gCOSY, d) gHSQC, e) 2D NOESY and f) HMBC. E178-70-exp43.

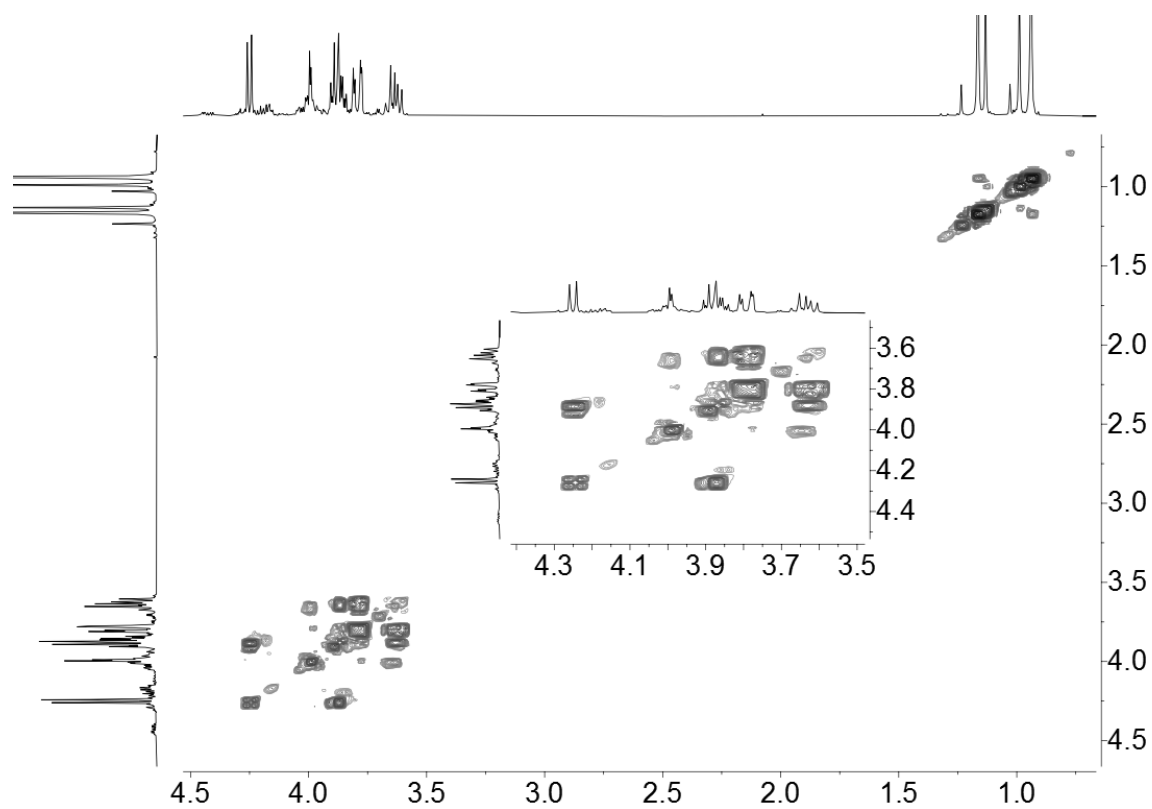
a)



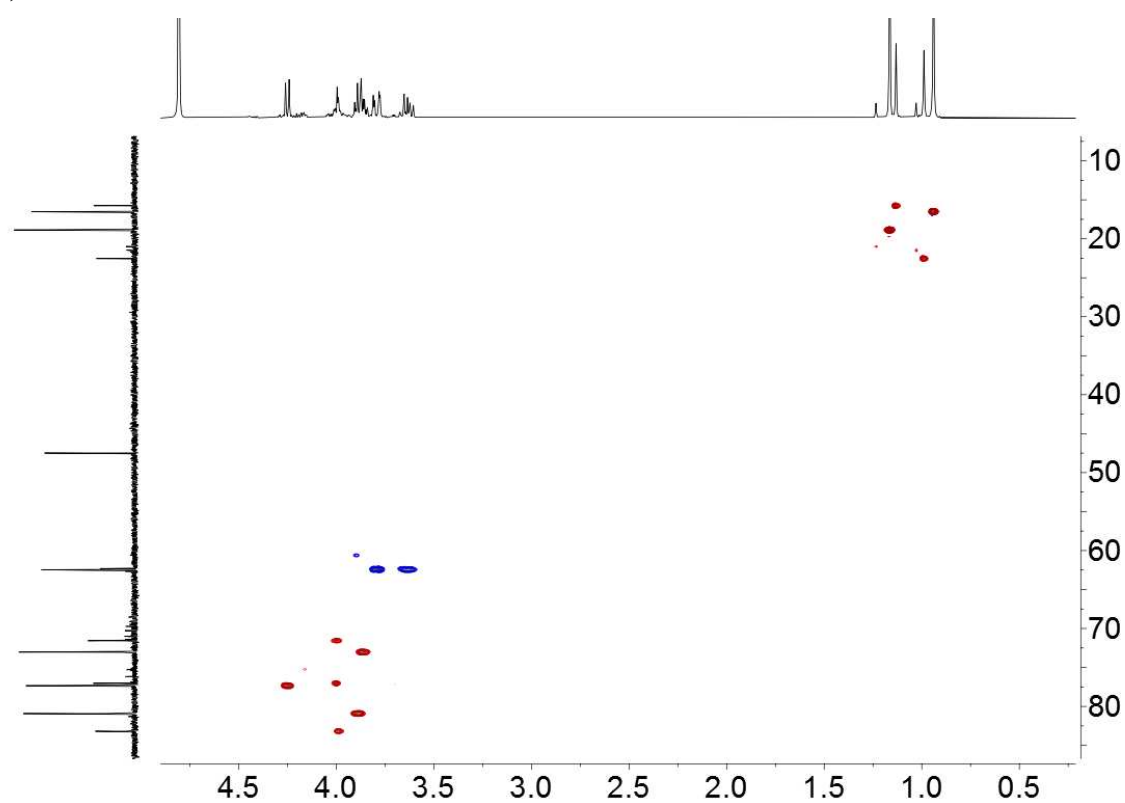
b)



c)



d)



e)

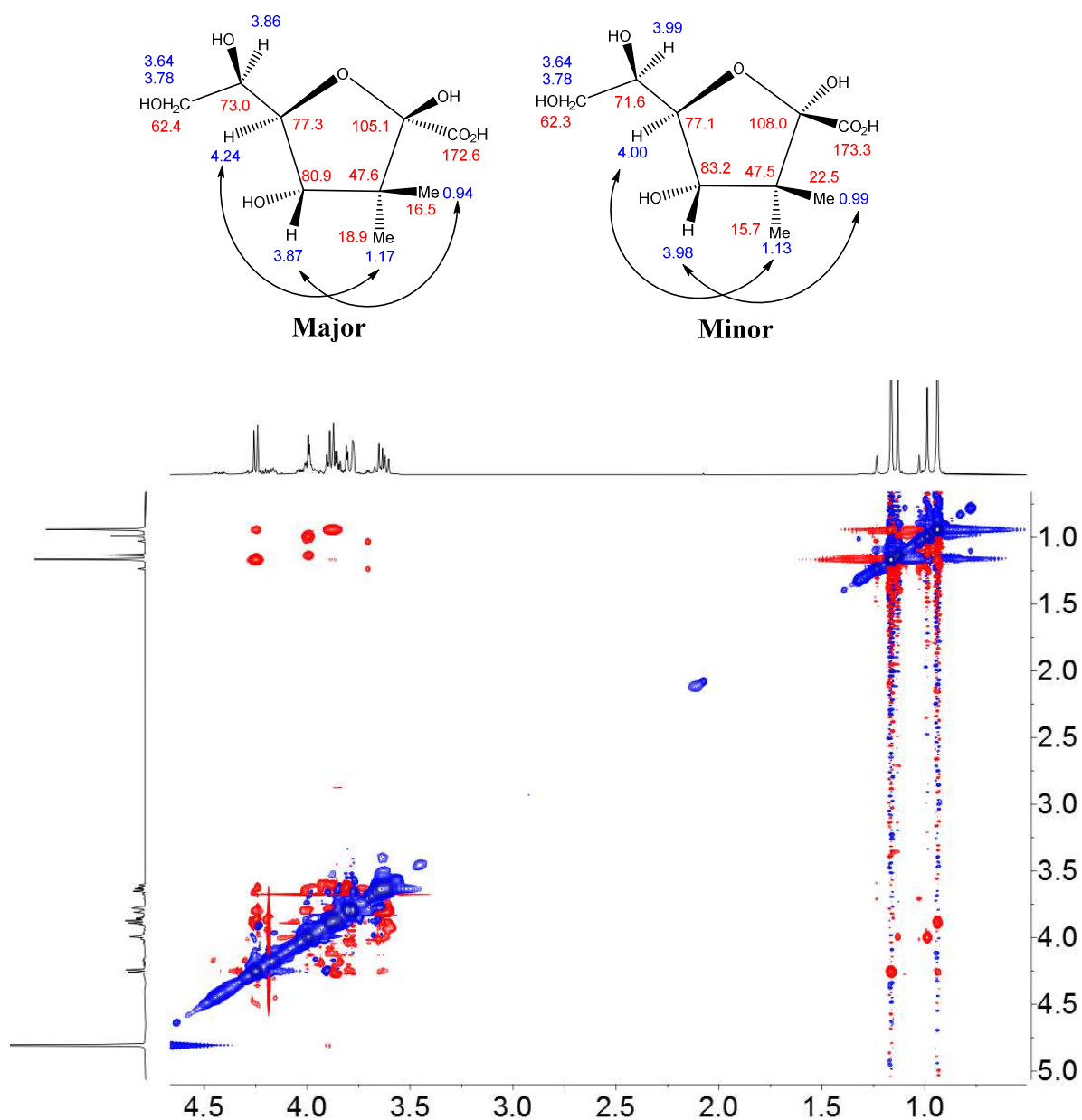
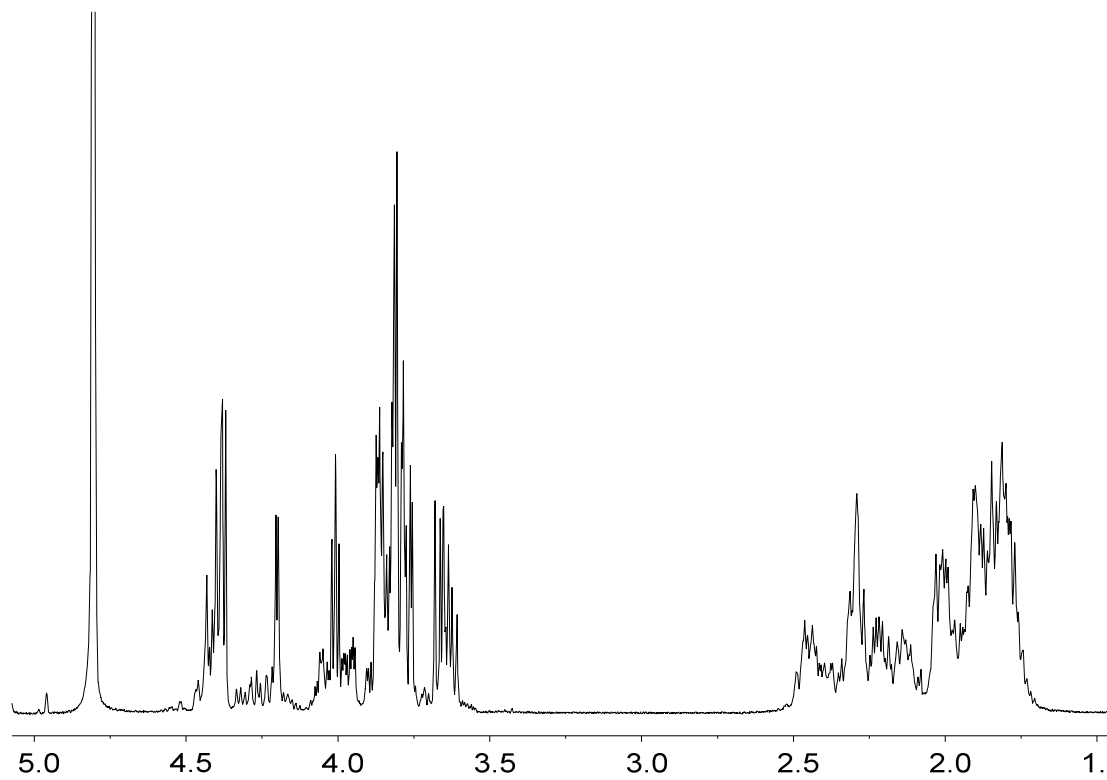
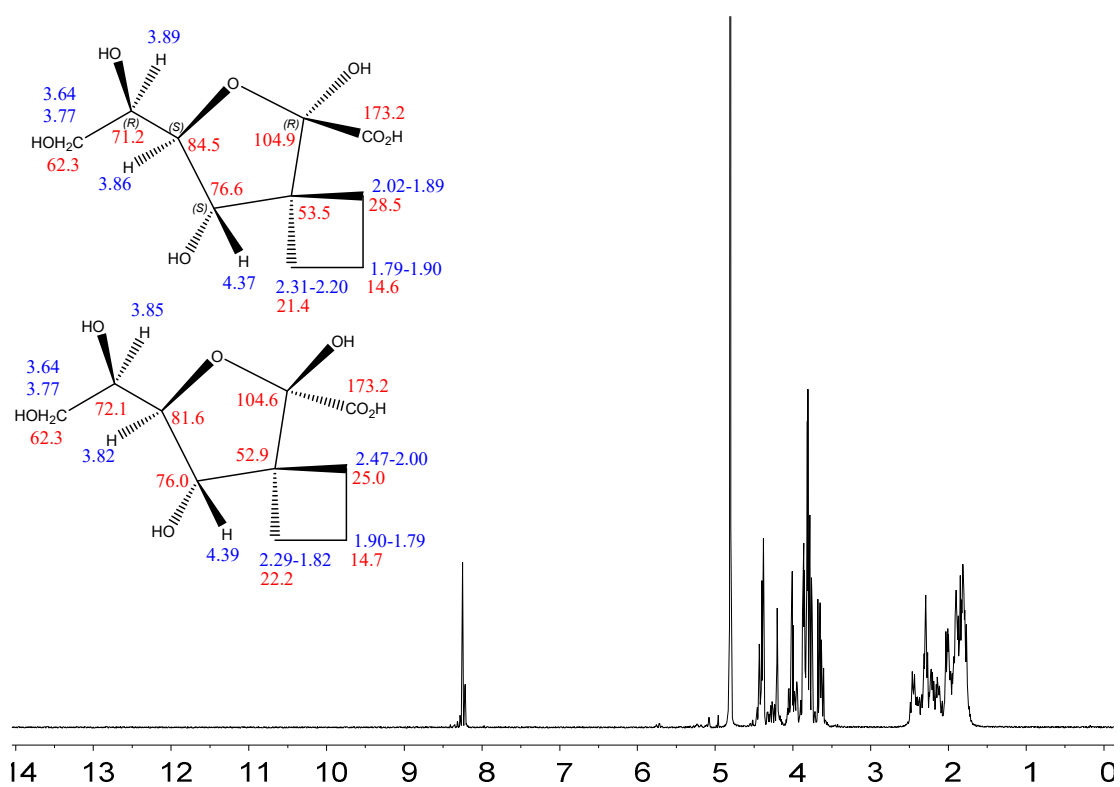
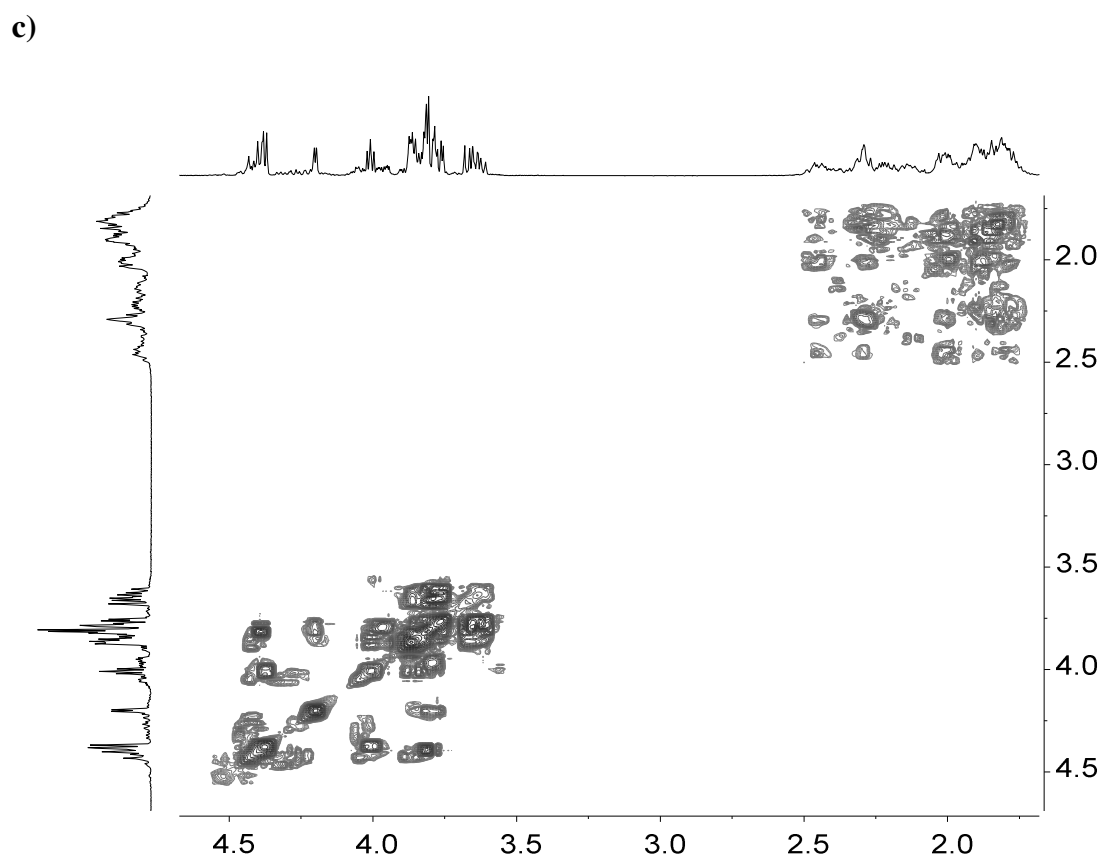
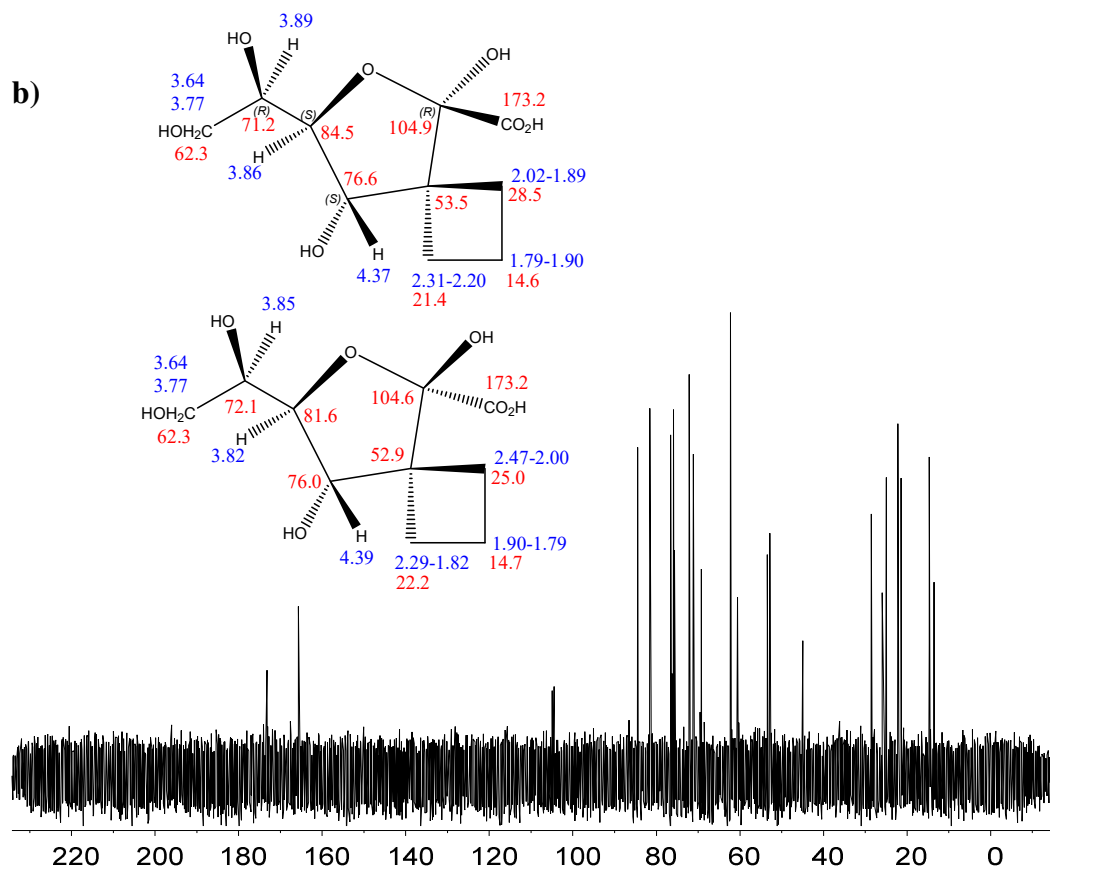


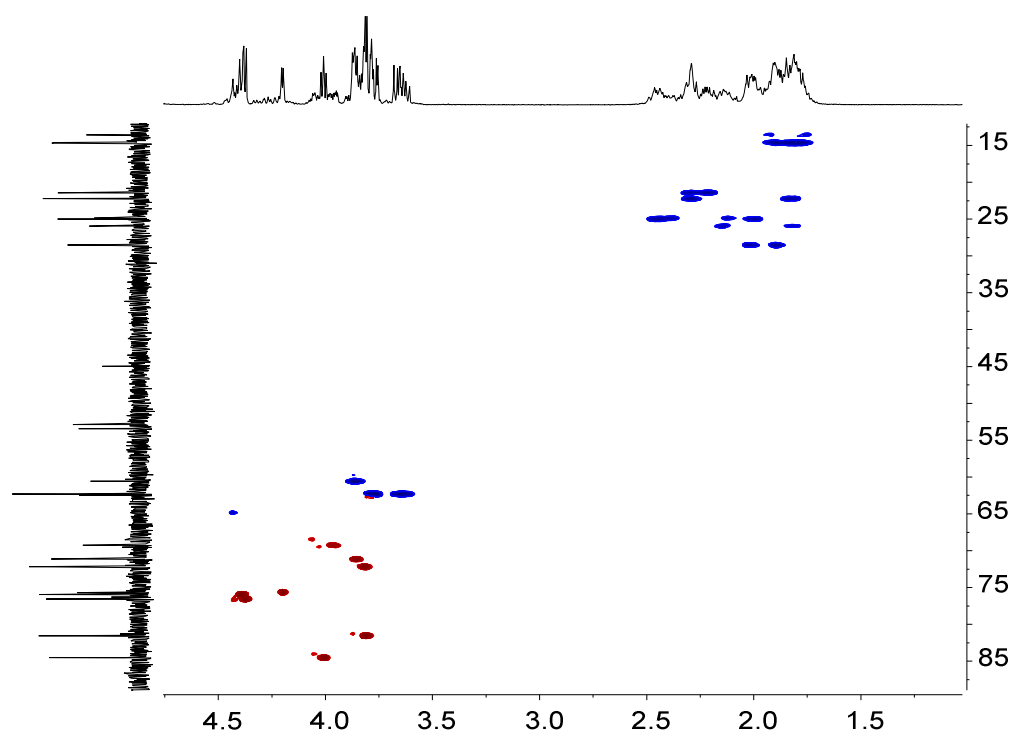
Figure S242 NMR spectra (D₂O) of **9o**: a) ¹H, b) ¹³C, c) gCOSY, d) gHSQC and e) 2D NOESY. E178-68-exp42.

a)

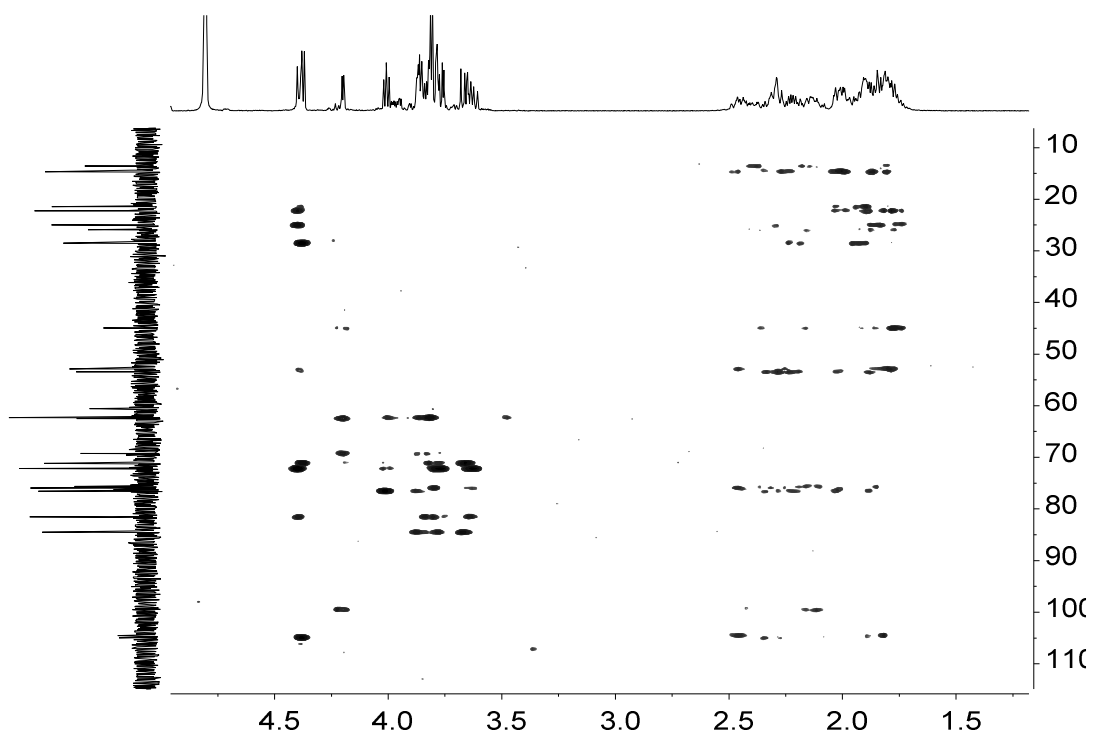




d)



e)



f)

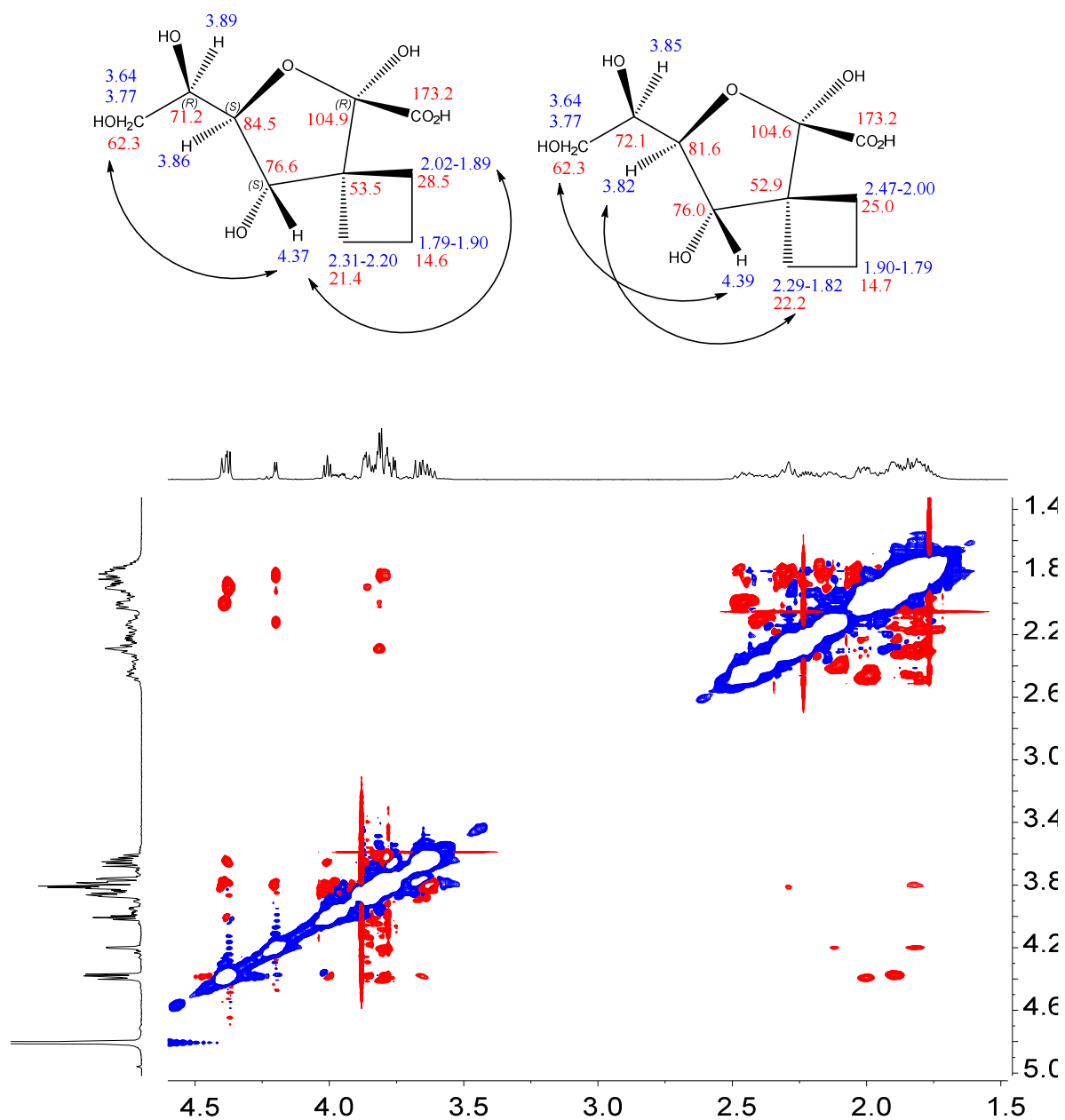
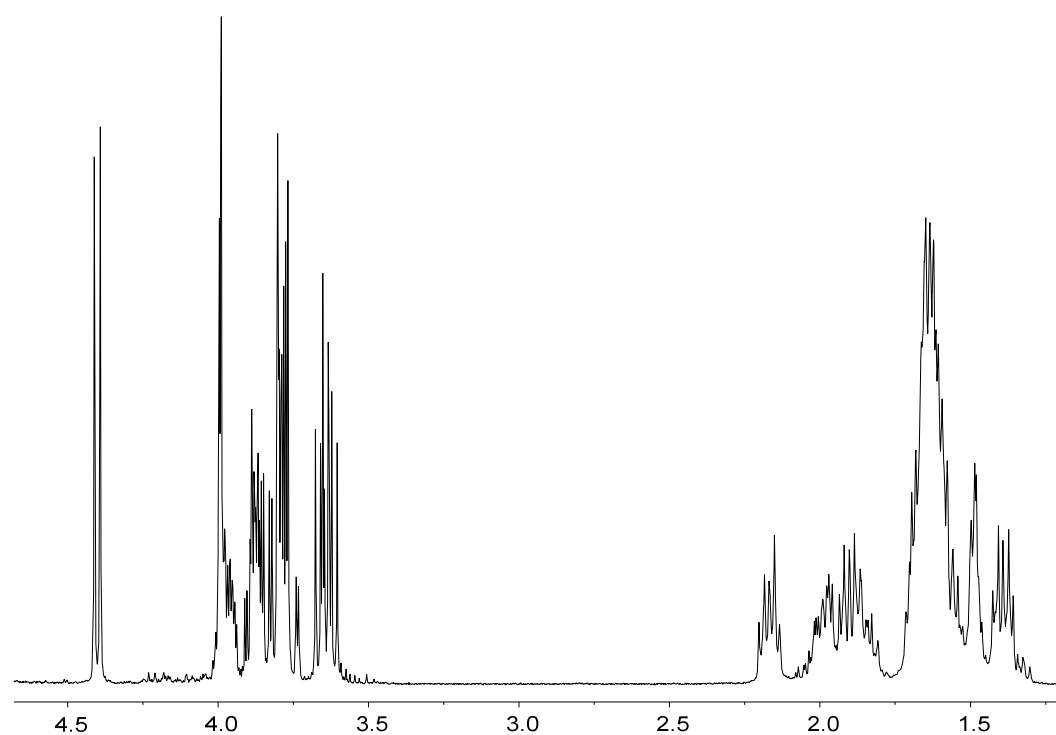
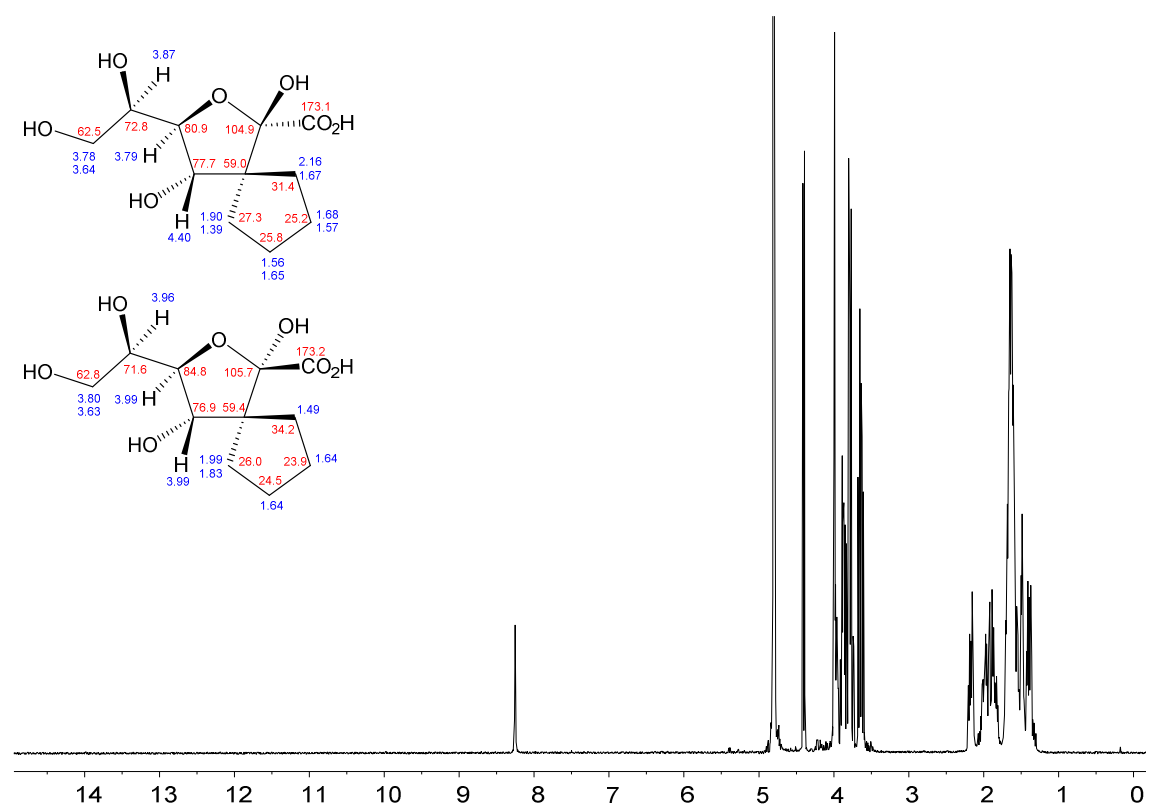
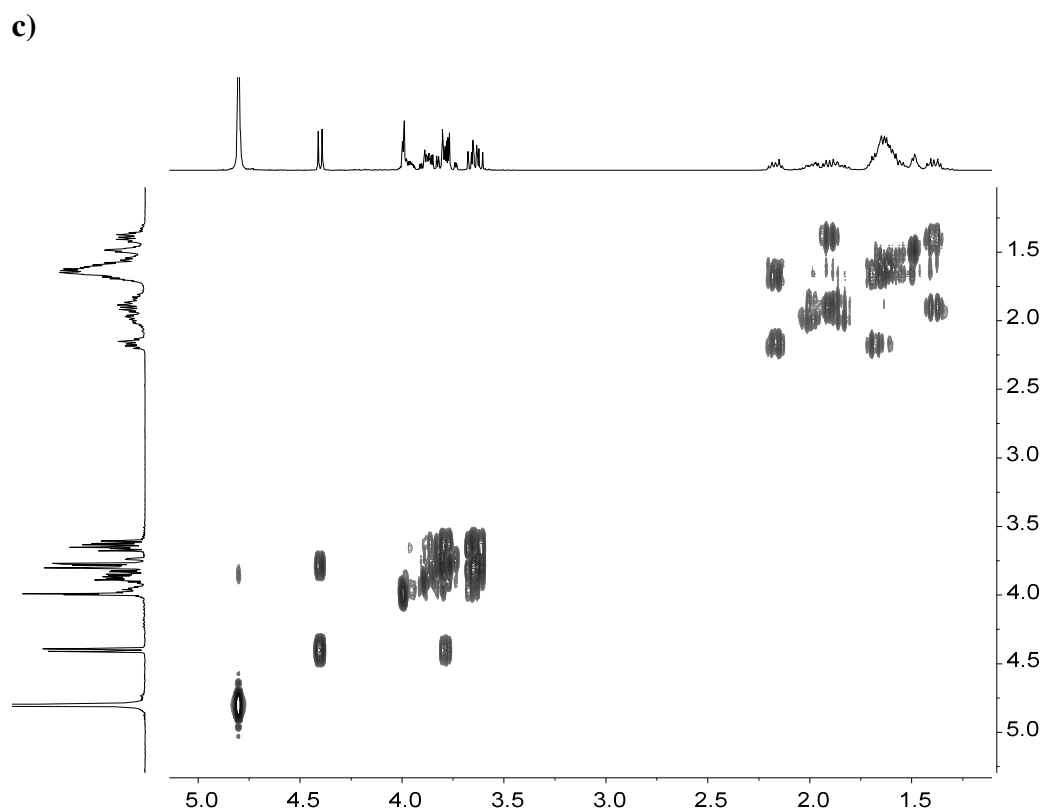
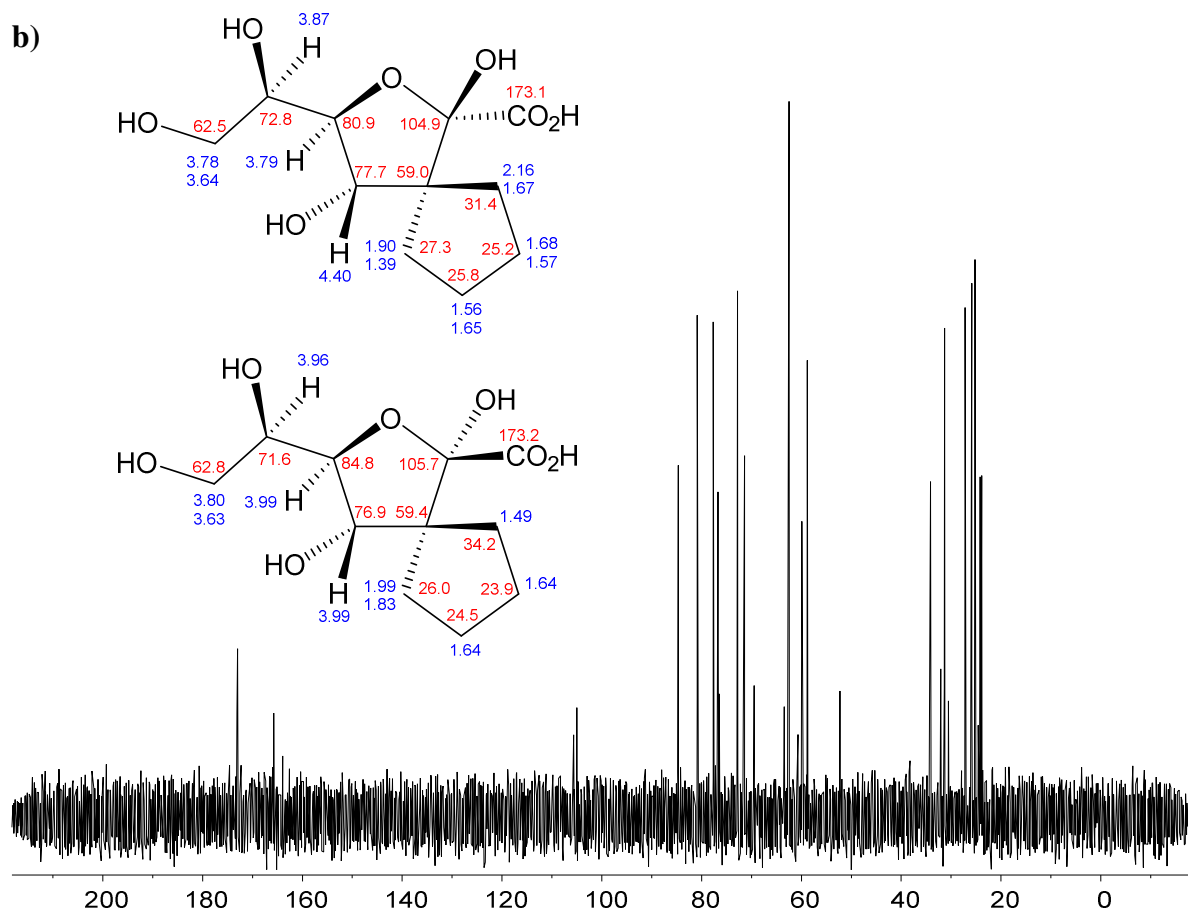


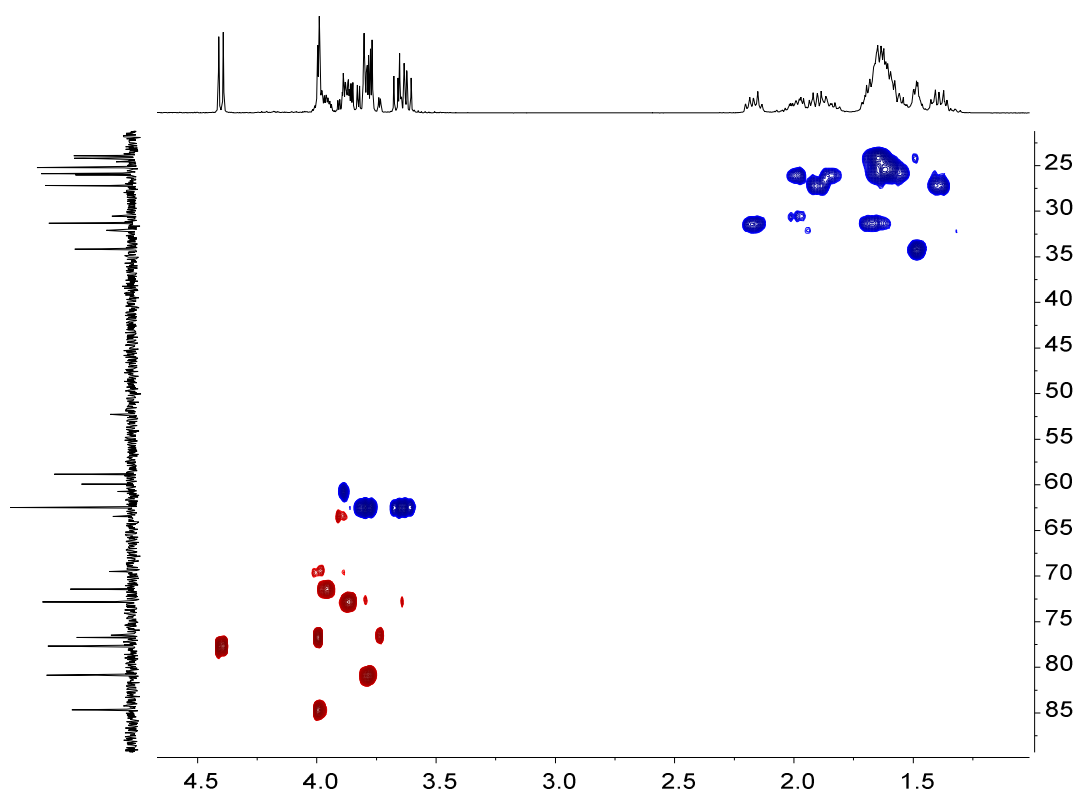
Figure S243 NMR spectra (D₂O) of **9p**: a) ¹H, b) ¹³C, c) gCOSY, d) gHSQC, e) HMBC and f) 2D NOESY. E178-84-exp54.

a)

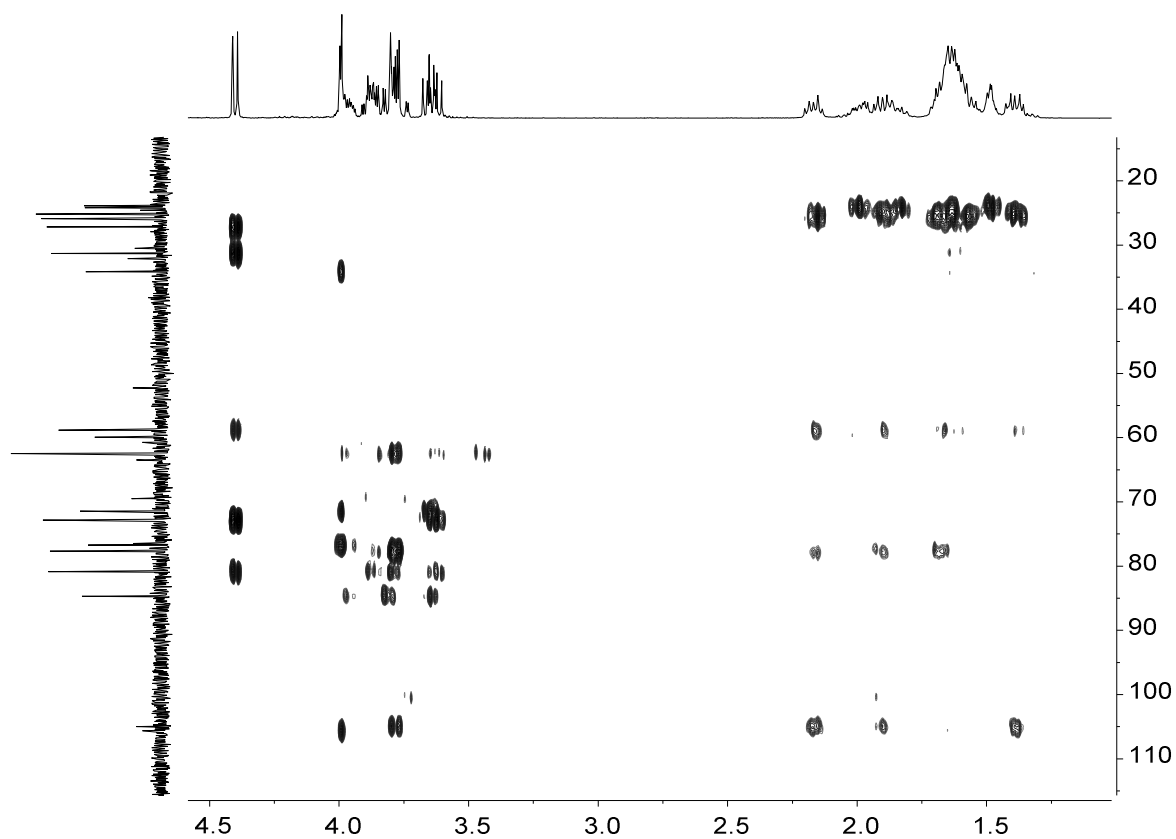




d)



e)



f)

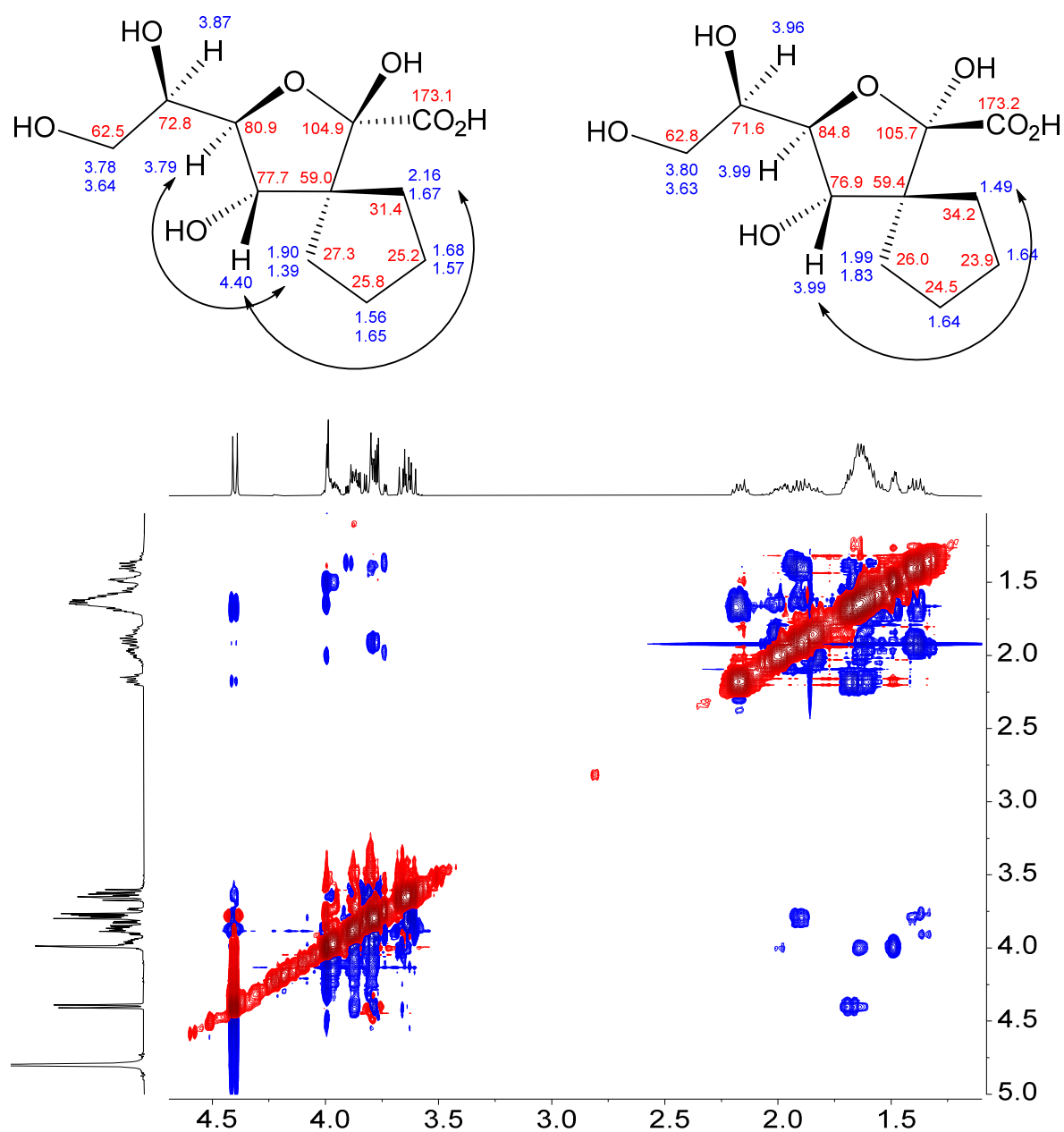


Figure S244 NMR spectra (D_2O) of **9q**: a) ^1H , b) ^{13}C , c) gCOSY, d) gHSQC, e) HMBC and f) 2D NOESY. E178-151-exp100.

6.3.5 Computational Models.

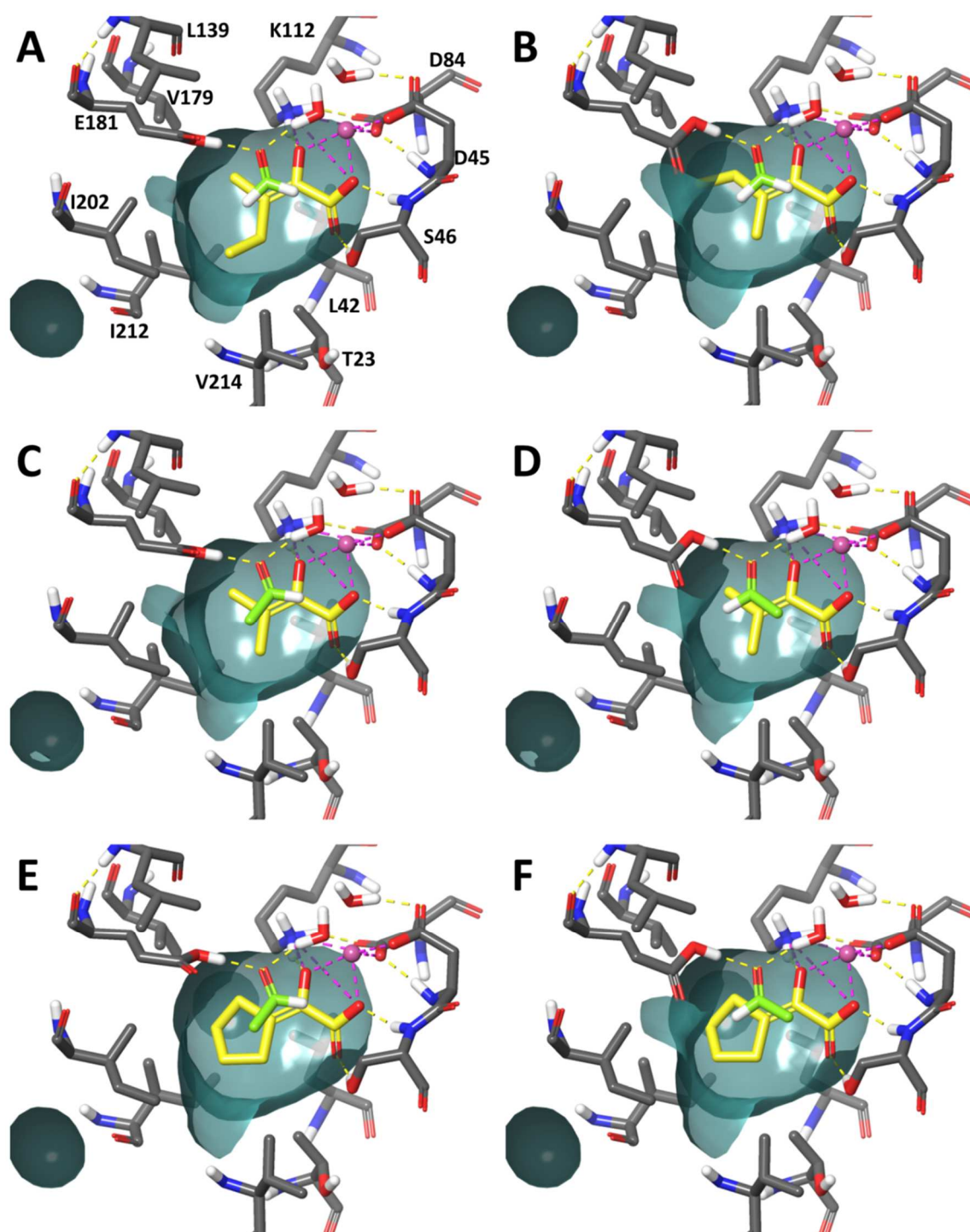


Figure S245 Models of wild-type KPHMT complexed with electrophiles (green C-atoms) (A,B) **2a** and (C-F) **2b**, and nucleophiles (yellow C-atoms) (A) *S*-**1n**, (B) *R*-**1n**, (C,D) **1a** and (E,F) **1r**. Nucleophiles are shown in their enolate form coordinated to the metal cofactor (purple sphere). Electrophile **2b** is shown approaching the enolate from its *re*- (C,E) or *si*-face (D,F). The surface of the active site cavities near to the nucleophile substrates are shown in cyan. Interactions are shown with dashed lines: H-bond in yellow, salt bridges in magenta.

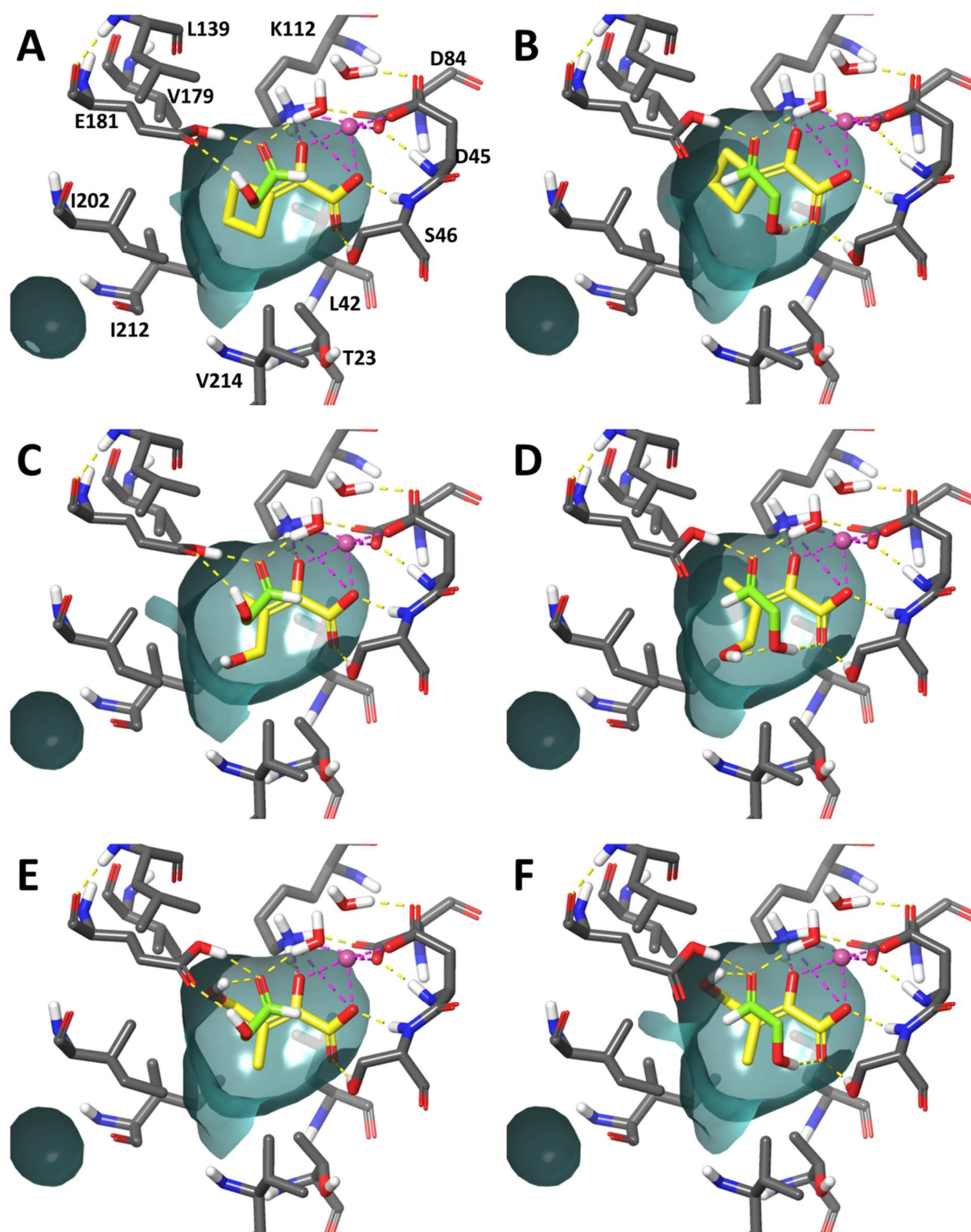
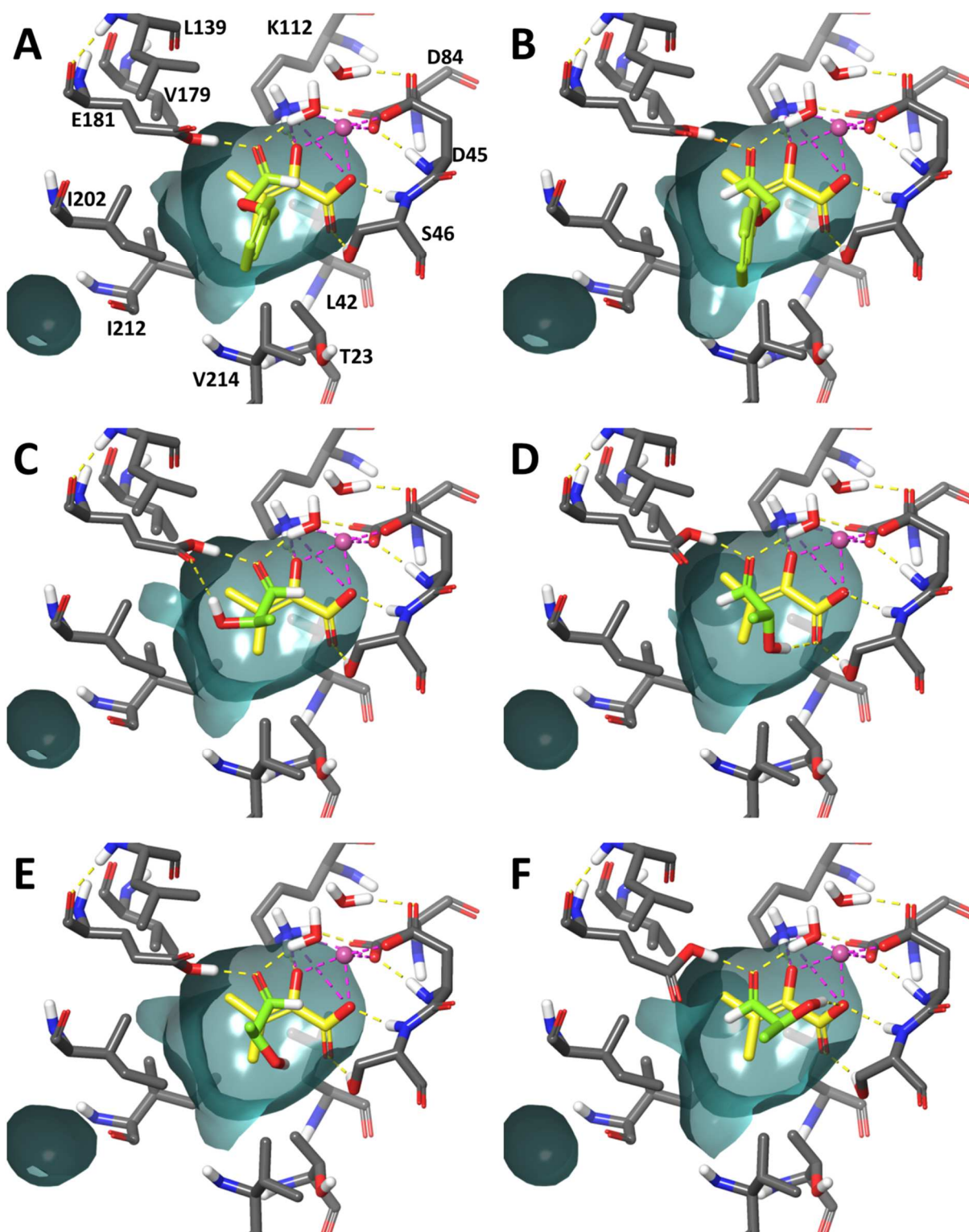


Figure S246 Models of wild-type KPHMT complexed with electrophile (A-F) **2c**, and nucleophiles (A,B) **1q**, (C,D) *S*-**1v** and (E,F) *R*-**1v**. Electrophile **2c** is shown approaching the enolate from its *re*- (A,C,E) or *si*-face (B,D,F). Other settings as in Figure S118.



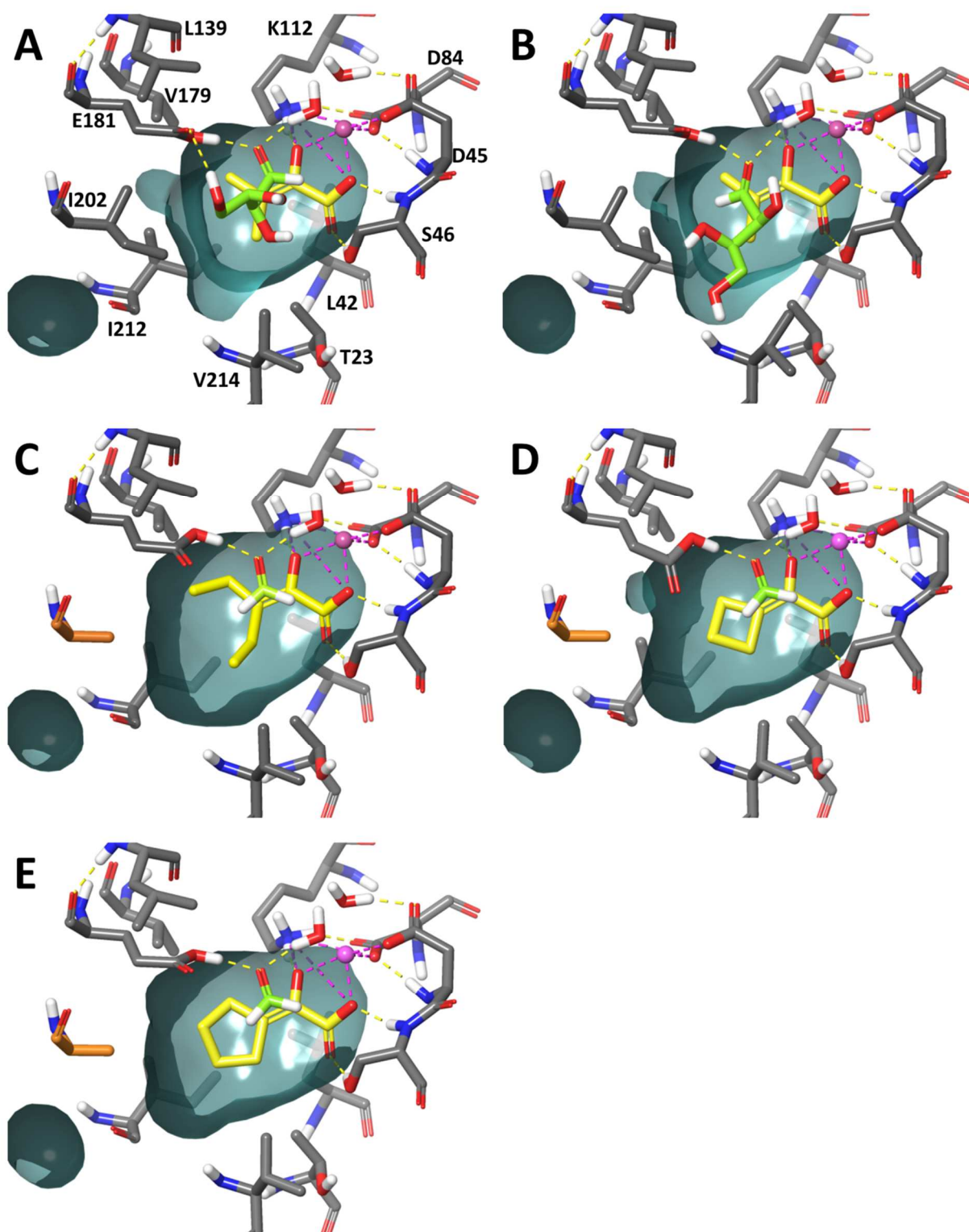


Figure S248 Models of (A,B) wild-type KPHMT complexed with electrophile **2g** and nucleophile **1a**, and of (C-E) KPHMT I202A complexed with electrophile **2a** and nucleophiles (C) **1o**, (D) **1q** and (E) **1r**. Electrophile **2g** is shown approaching the enolate from its *re*- (A) or *si*-face (B). The mutated residue is shown in orange, other settings as in Figure S118.

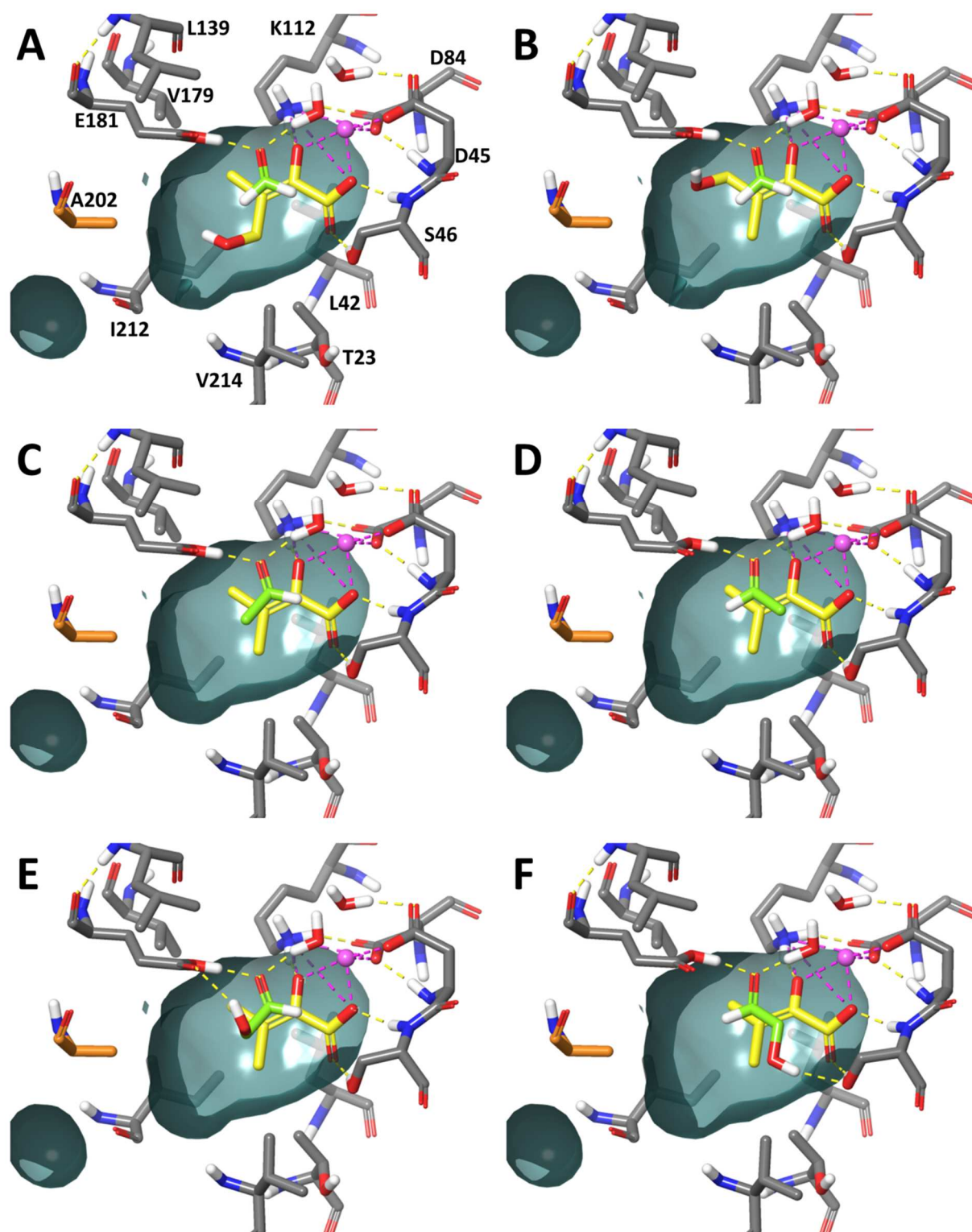


Figure S249 Models of KPHMT I202A complexed with electrophiles (A-B) **2a**, (C,D) **2b** and (E,F) **2c**, and nucleophiles (A) *S*-**1v**, (B) *R*-**1v** and (C-F) **1a**. Electrophiles **2b** and **2c** are shown approaching the enolate from its *re*- (C,E) or *si*-face (D,F). The mutated residue is shown in orange, other settings as in Figure S118.

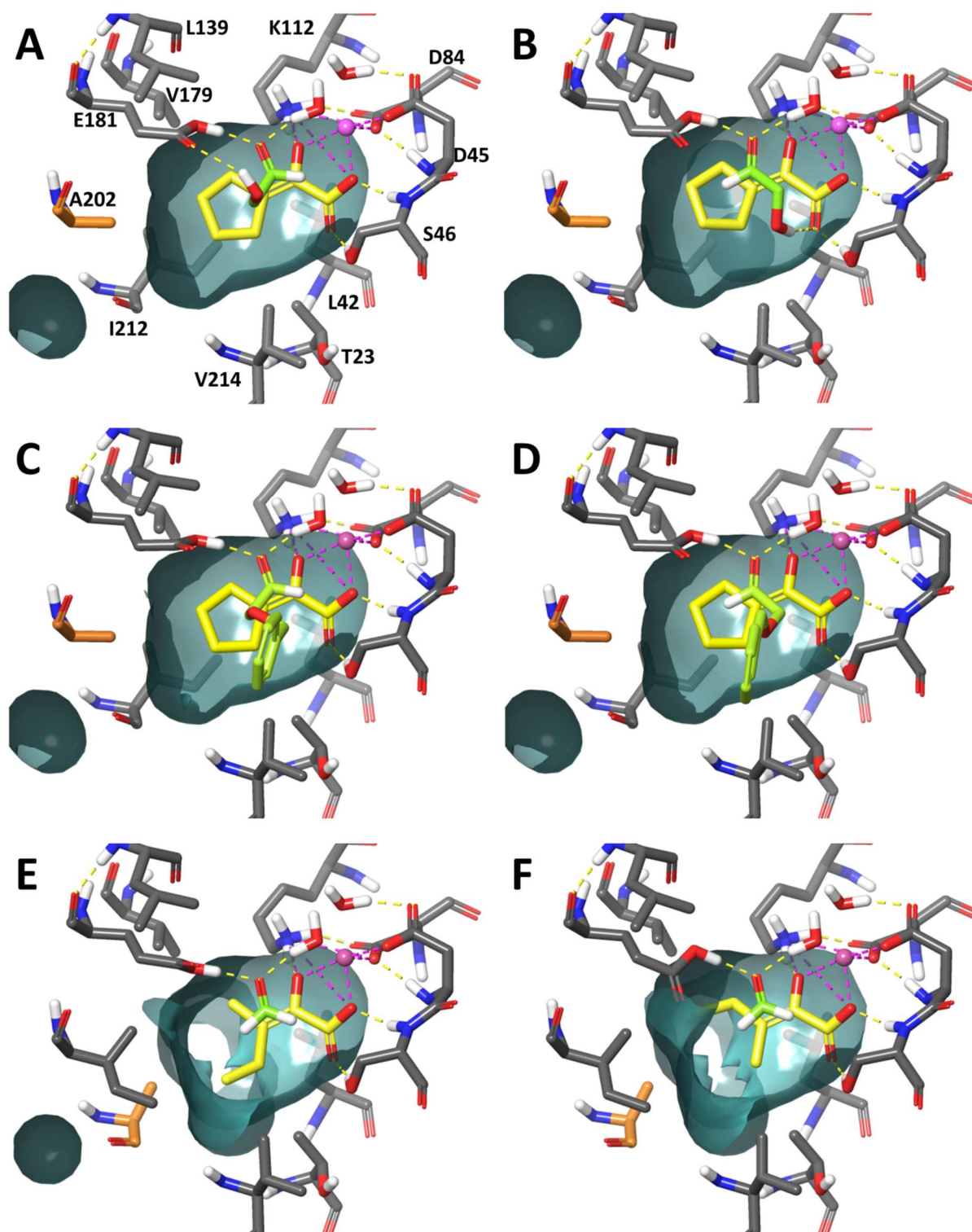


Figure S250 Models of (A-D) KPHMT I202A complexed with electrophiles (A,B) **2c** and (C,D) **2d**, and nucleophile (A-D) **1r**, and of KPHMT I212A complexed with electrophile **2a** and nucleophiles (E) (*S*)-**1n** and (F) (*R*)-**1n**. Electrophiles **2c** and **2d** are shown approaching the enolate from its *re*- (A,C) or *si*-face (B,D). The mutated residues are shown in orange, other settings as in Figure S118.

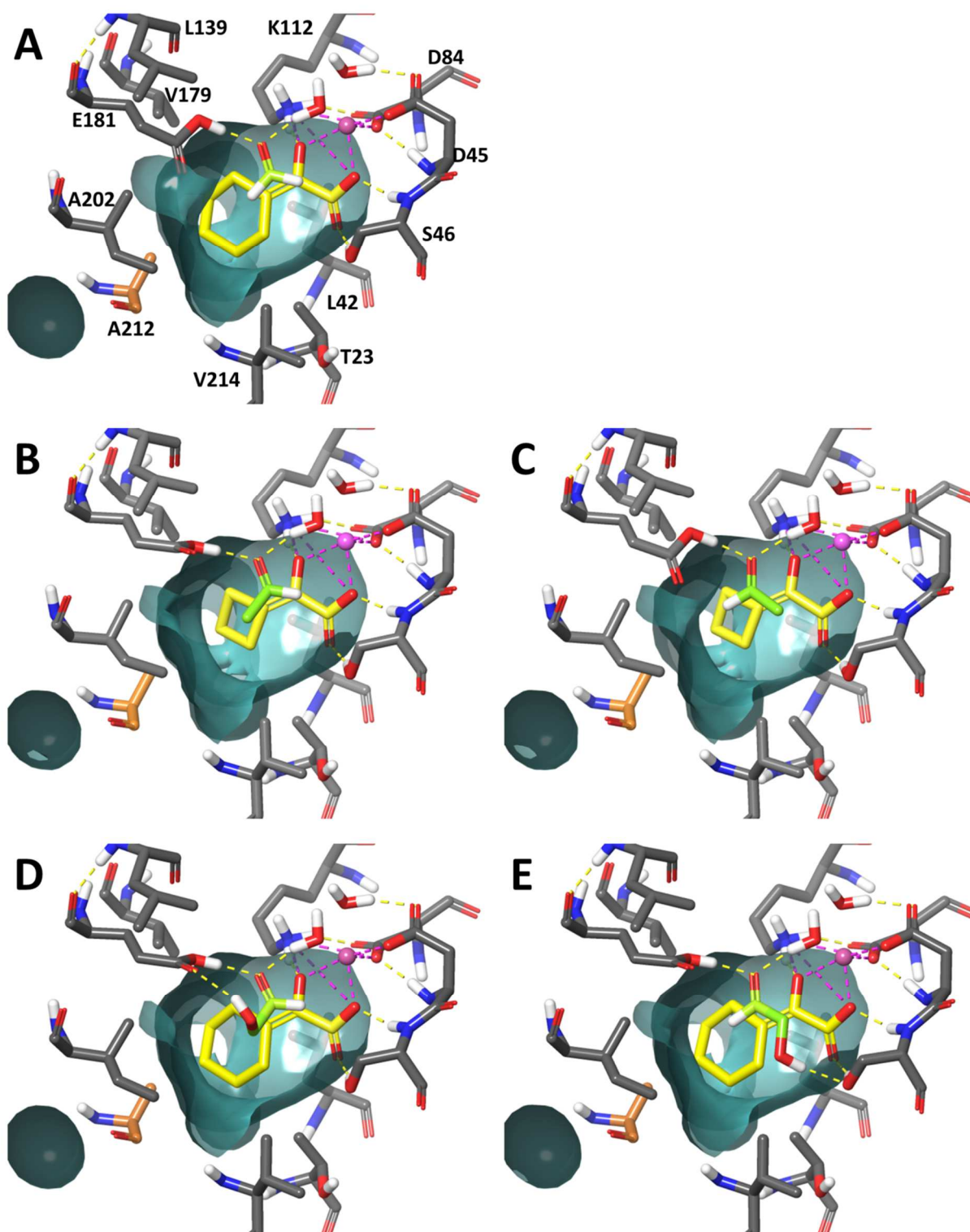


Figure S251 Models of KPHMT I212A complexed with electrophiles (A) **2a**, (B,C) **2b** and (D,E) **2c**, and nucleophiles (A,D,E) **1s** and (B,C) **1q**. Electrophiles **2b** and **2c** are shown approaching the enolate from its *re*- (B,D) or *si*-face (C,E). The mutated residue is shown in orange, other settings as in Figure S118.

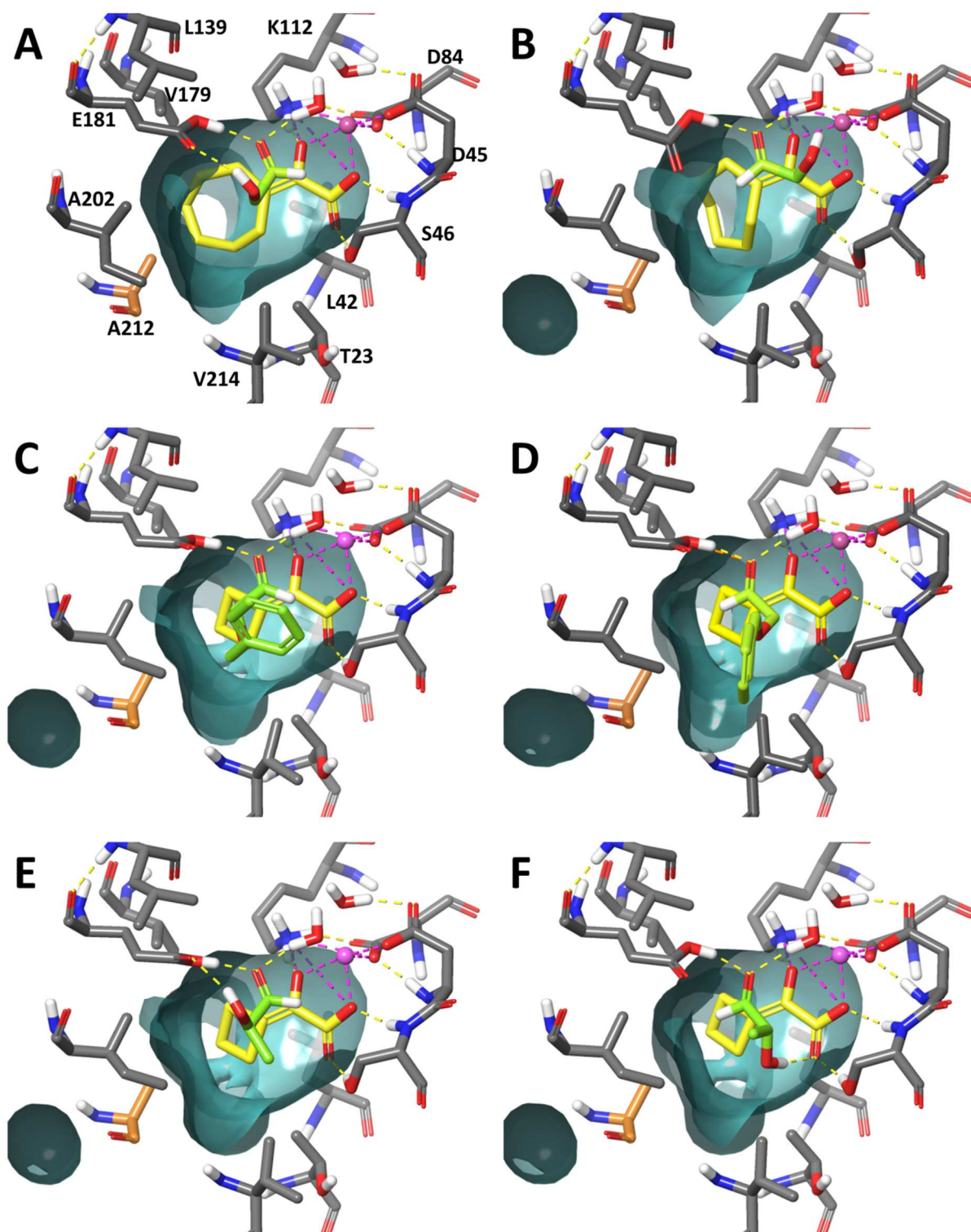


Figure S252 Models of KPHMT I212A complexed with electrophiles (A,B) **2c**, (C,D) **2d** and (E-F) **2e**, and nucleophiles (A,B) **1t** and (C-F) **1q**. Electrophiles **2c**, **2d** and **2e** are shown approaching the enolate from its *re*- (A,C,E) or *si*-face (B,D,F). The mutated residue is shown in orange, other settings as in Figure S118.

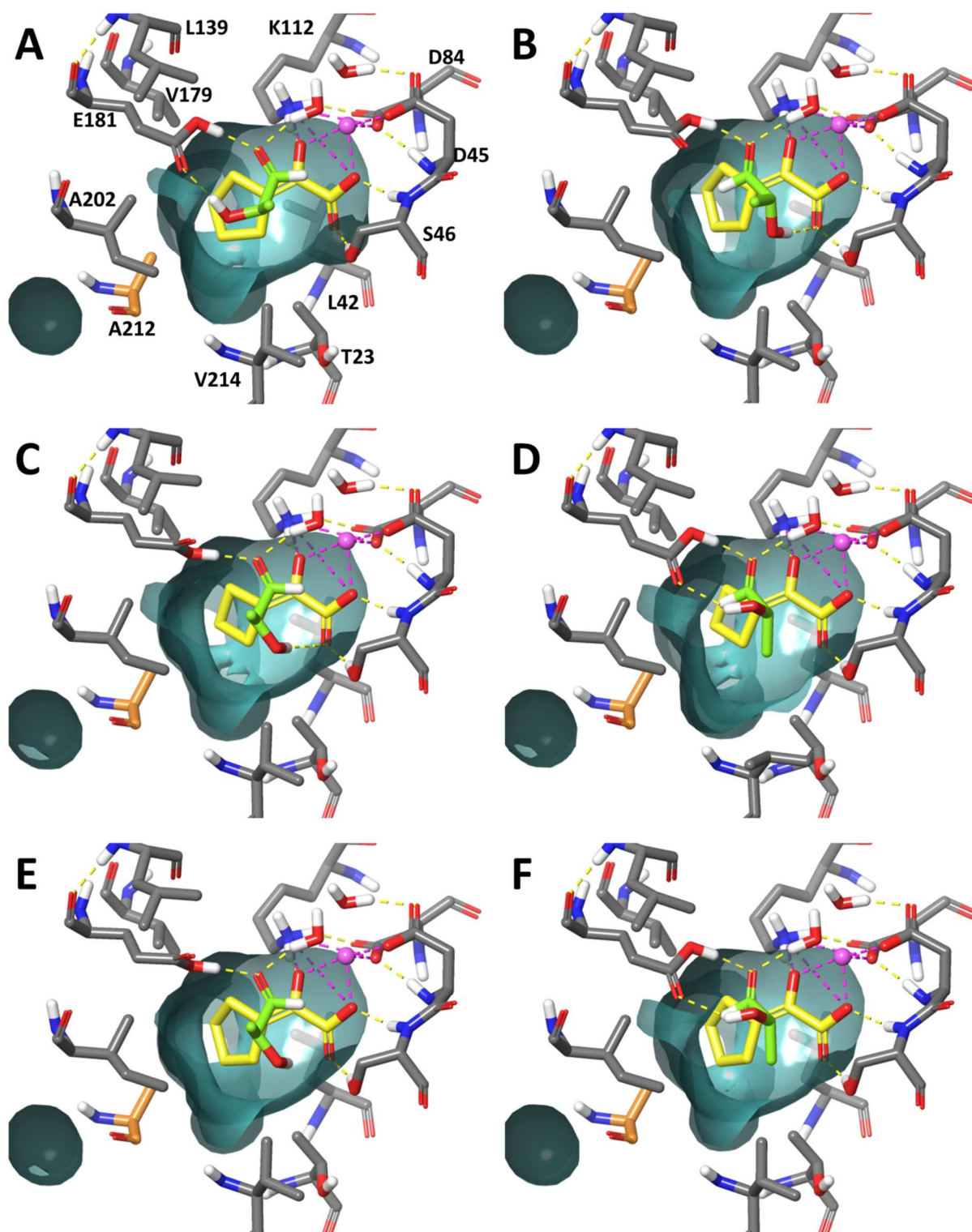


Figure S253 Models of KPHMT I212A complexed with electrophiles (A,B) **2e** and (C-F) **2f**, and nucleophiles (A,B,E,F) **1r** and (C,D) **1q**. Electrophiles **2e** and **2f** are shown approaching the enolate from its *re*- (A,C,E) or *si*-face (B,D,F). The mutated residue is shown in orange, other settings as in Figure S118.

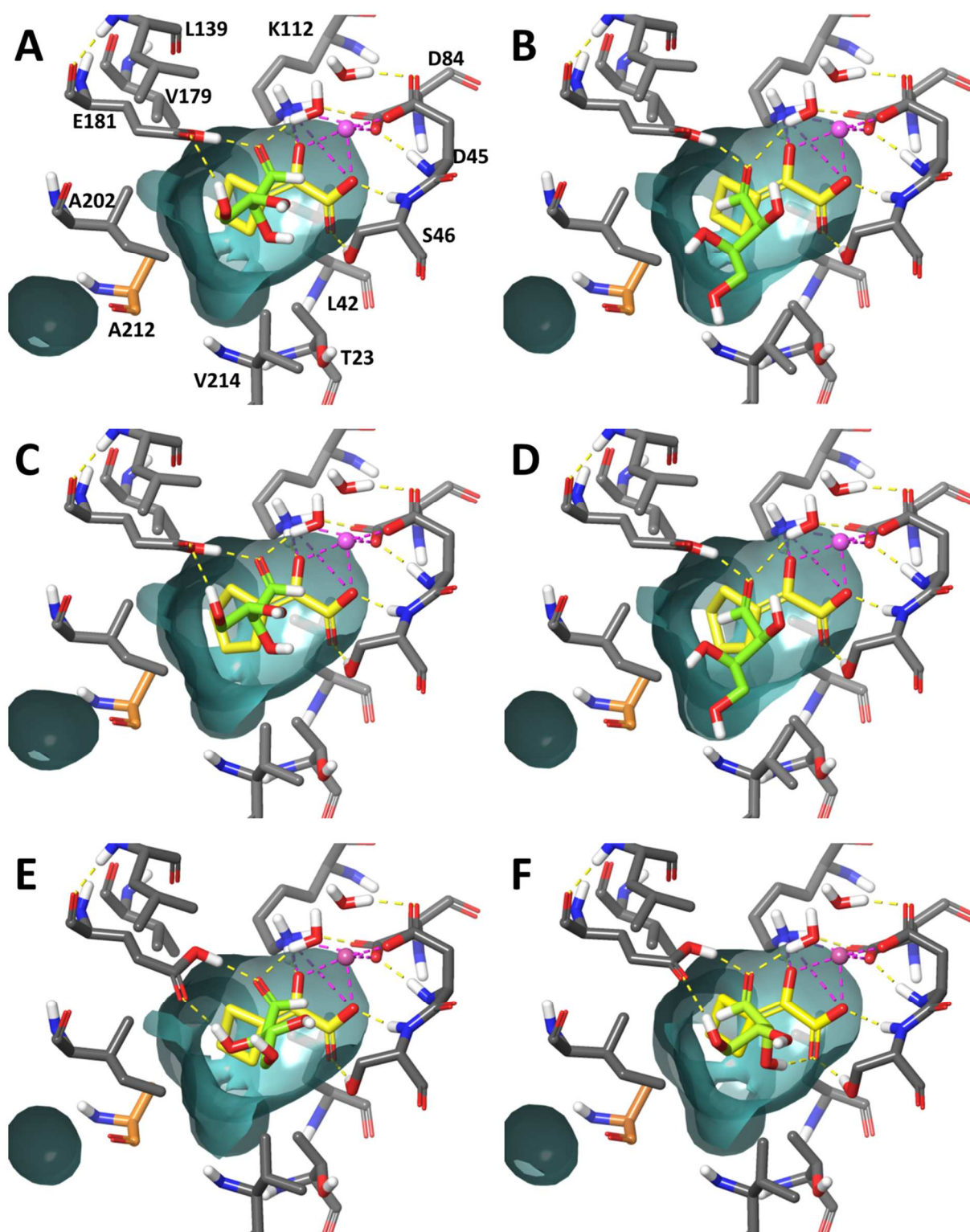


Figure S254 Models of KPHMT I212A complexed with electrophiles (A-D) **2g** and (E,F) **2h**, and nucleophiles (A,B,E,F) **1q** and (C,D) **1r**. Electrophiles **2g** and **2h** are shown approaching the enolate from its *re*- (A,C,E) or *si*-face (B,D,F). The mutated residue is shown in orange, other settings as in Figure S118.

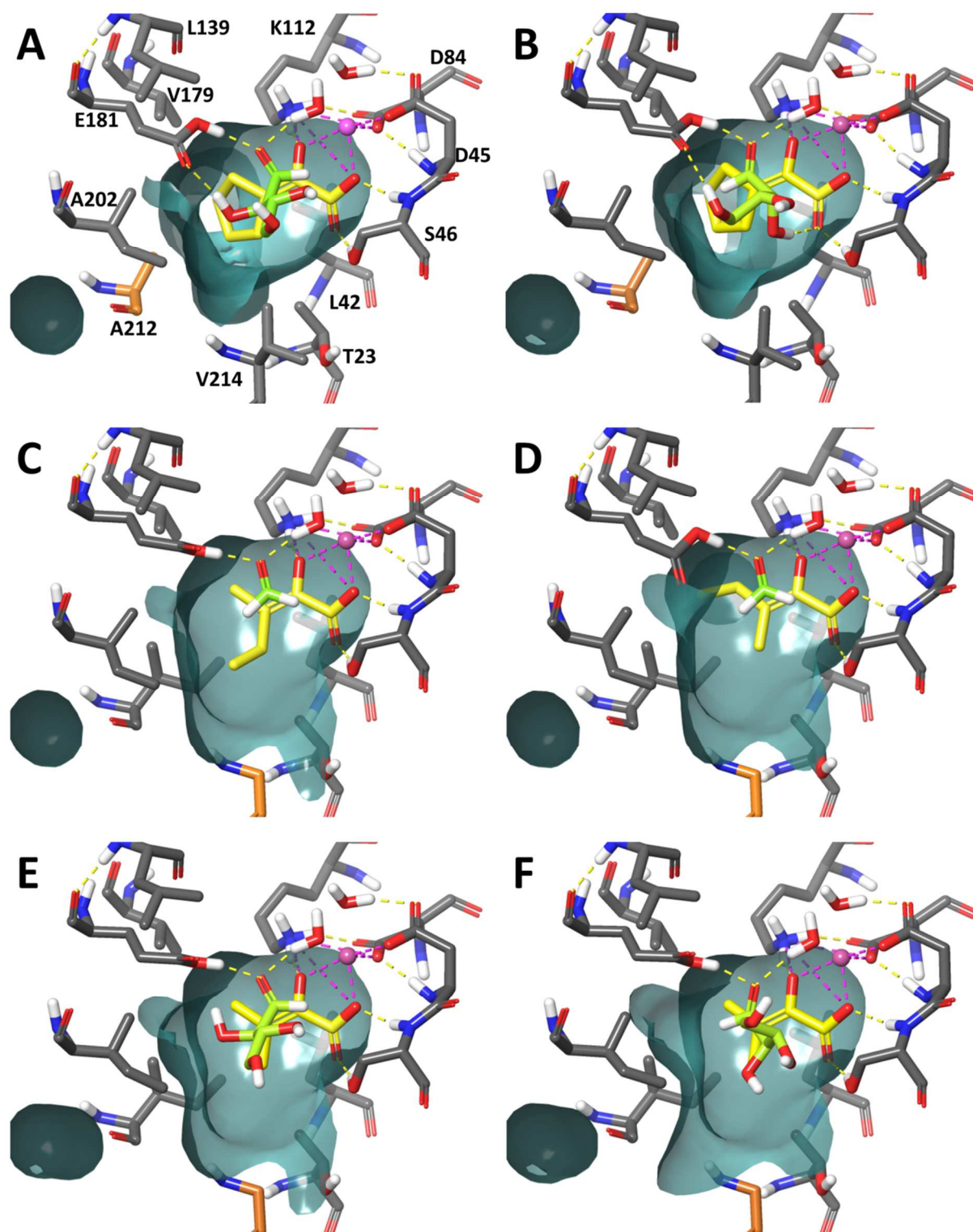


Figure S255 Models of (A,B) KPHMT I212A complexed with electrophile **2h** and nucleophile **1r**, and of KPHMT V214G complexed with electrophiles (C,D) **2a** and (E,F) **2h**, and nucleophiles (C) (*S*)-**1n**, (D) (*R*)-**1n** and (E,F) **1a**. Electrophile **2h** is shown approaching the enolate from its *re*- (A,E) or *si*-face (B,F). The mutated residues are shown in orange, other settings as in Figure S118.

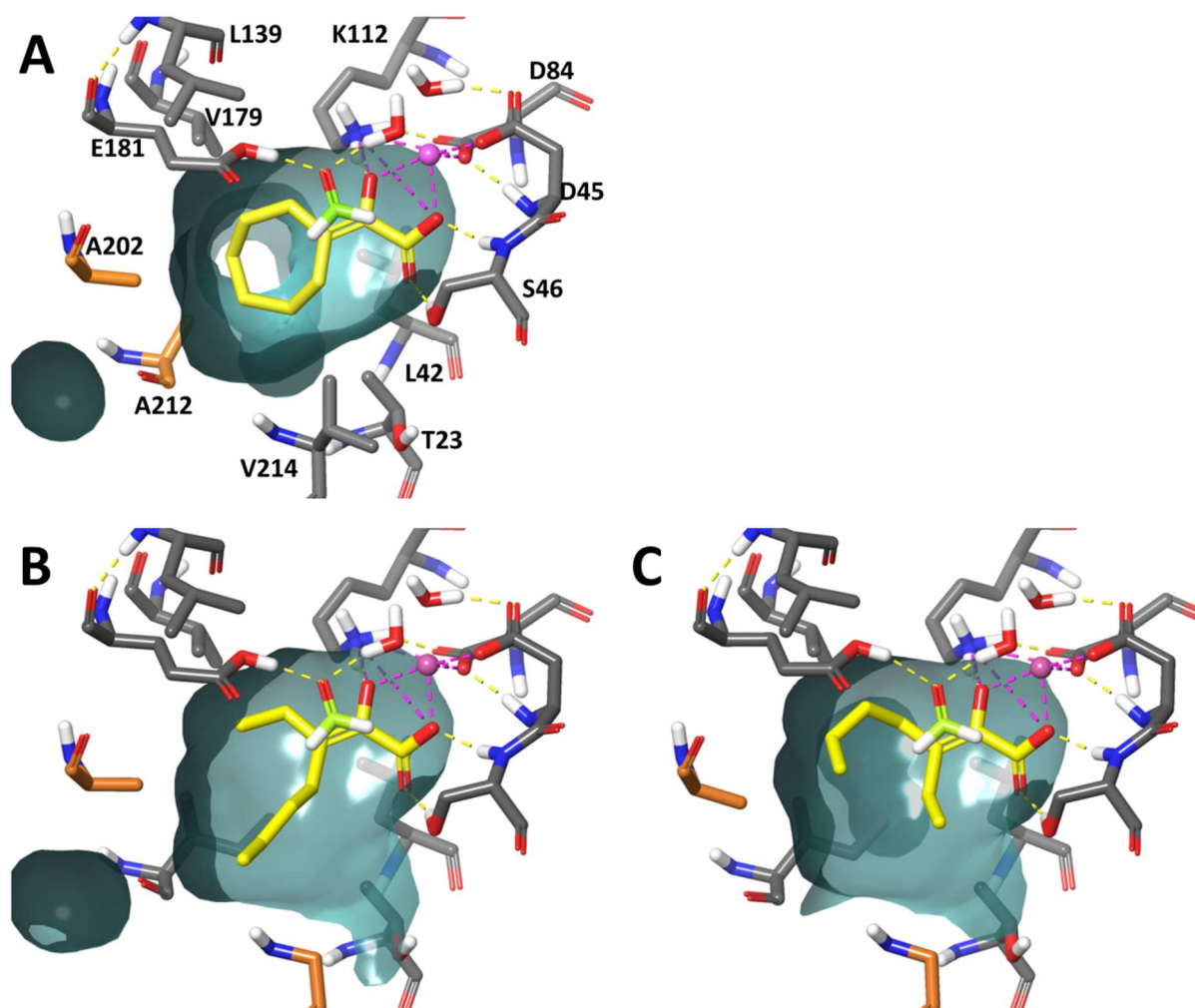


Figure S256 Models of (A) KPHMT I202A/I212A complexed with electrophile **2a** and nucleophile **1t**, and of KPHMT I202A/V214G complexed with electrophile **2a** and nucleophiles (B) *(S)*-**1p** and (C) *(R)*-**1p**. The mutated residues are shown in orange, other settings as in Figure S118.

6.3.6 Computational Methods (description).

Protein complexes were modeled with the package Schrödinger Suite 2020-2,¹ through its graphical interface Maestro.² The Protein Preparation Wizard^{3,4} included in Maestro was used to prepare the protein structures by removing solvent molecules and ions (except the essential cation and the two coordinated waters), adding hydrogens, setting protonation states⁵ and running a restrained minimization using the OPLS3 force-field.⁶ The program MacroModel³ with the same force field and GB/SA water solvation conditions⁷ was used for further molecular mechanics calculations. The program QSite⁸⁻¹⁰ was used for the QM/MM calculations.

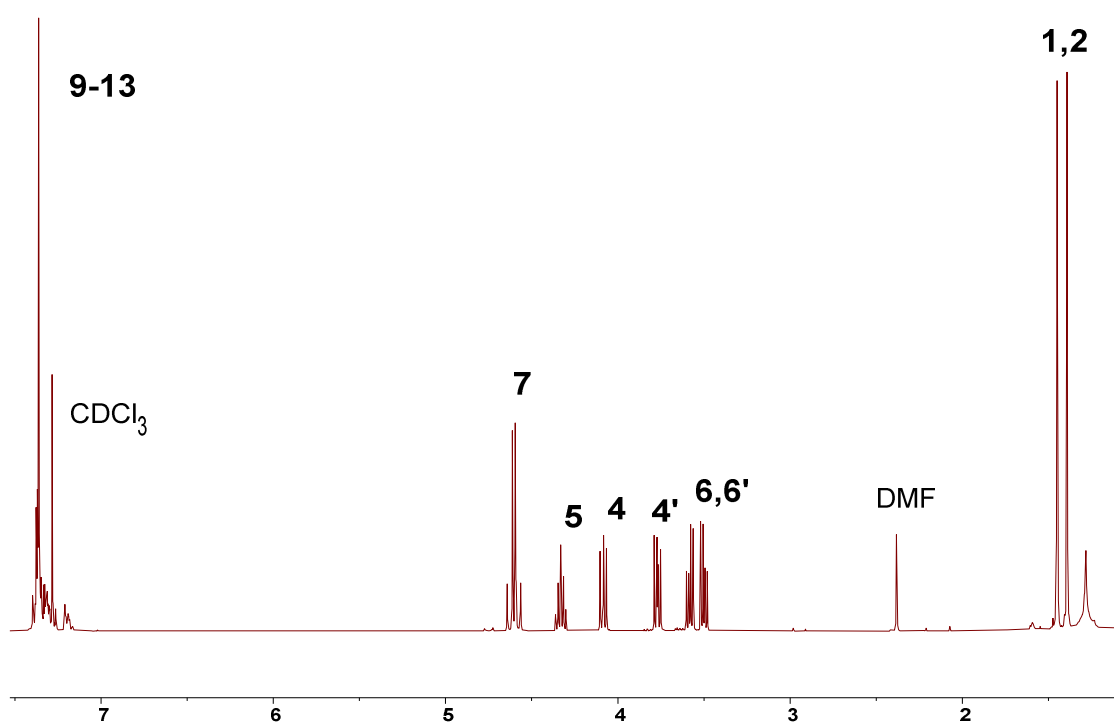
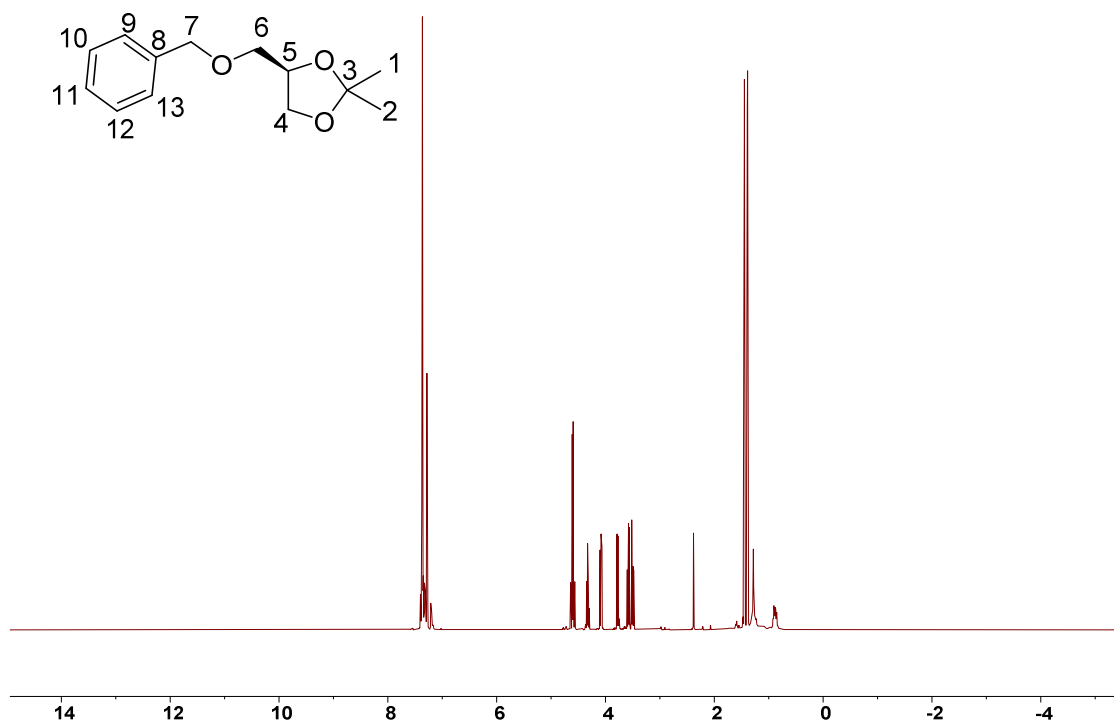
Molecular models of the pre-reactive nucleophilic enolate intermediates and the electrophiles bound into the active site of the KPHMT proteins were generated starting from the crystal structure of KPHMT in complex with ketopantoate (PDB 1M3U),¹¹ following a similar protocol to that previously described.^{12,13} The KPHMT structure is decameric but each monomer has its own active site which is not participated by residues of any other monomer, thus only chain A was considered for our calculations. Mutations were introduced within Maestro, followed by restrained minimization. The structure of the ketopantoate molecule bound into the active site of the KPHMT crystal structure was modified using Maestro, to build the corresponding nucleophile molecules as their corresponding enolates. The electrophiles were manually placed in the active site of each enzyme and adequately oriented to satisfy the conditions of (i) having its C-atom at approximately 2.4 Å of the reactive enolate C-atom of the nucleophile, and (ii) forming hydrogen bond interactions with the protonated E181 residue and a metal-coordinated water (residue E181 has been postulated as the catalytic base that abstracts the C3 proton of the nucleophile, to form the enolate, and could also participate on the protonation of the alcoxide derived from the electrophilic carbonyl, once the C-C bond is formed). The structures of the complexes were minimized with QSite at the DFT B3LYP/6-31G** level of theory, establishing a distance constraint between the two reactive C-atoms to keep them at a distance of 2.4 Å. The QM/MM boundary was defined by placement of hydrogen caps between the C α and C β atoms of residues D45 and D84 of KPHMT. The Mg²⁺ cation and the two coordinated waters, as well as the nucleophile enolate and the electrophile molecules, were also included as part of the QM region. All residues with atoms within 6 Å of the QM region were simultaneously optimized using the OPLS 2005 force-field,^{14,15} while residues which were further away were kept frozen. A distance

dependent dielectric ($\epsilon = 4$) was used for calculating the electrostatic component of the MM energy. Electrophiles **2b-2h** were modeled approaching the nucleophile enolates from their *re*- and *si*-faces. Electrophiles **2b-2h** were modeled approaching the nucleophile enolates from their *re*- and *si*-faces. Furthermore, to find the best bound conformations for the more flexible electrophiles (**2c-2h**) a conformational search was performed using the mixed MCMM/LMCS method¹⁶ implemented in MacroModel to find the best poses for the C2-substituents, while the rest of the system was kept frozen. Then, the best conformers detected by this search were QM/MM optimized as above.

6.4 Data from chapter 3.3

6.4.1 NMR spectra of compounds C11–16

a)



b)

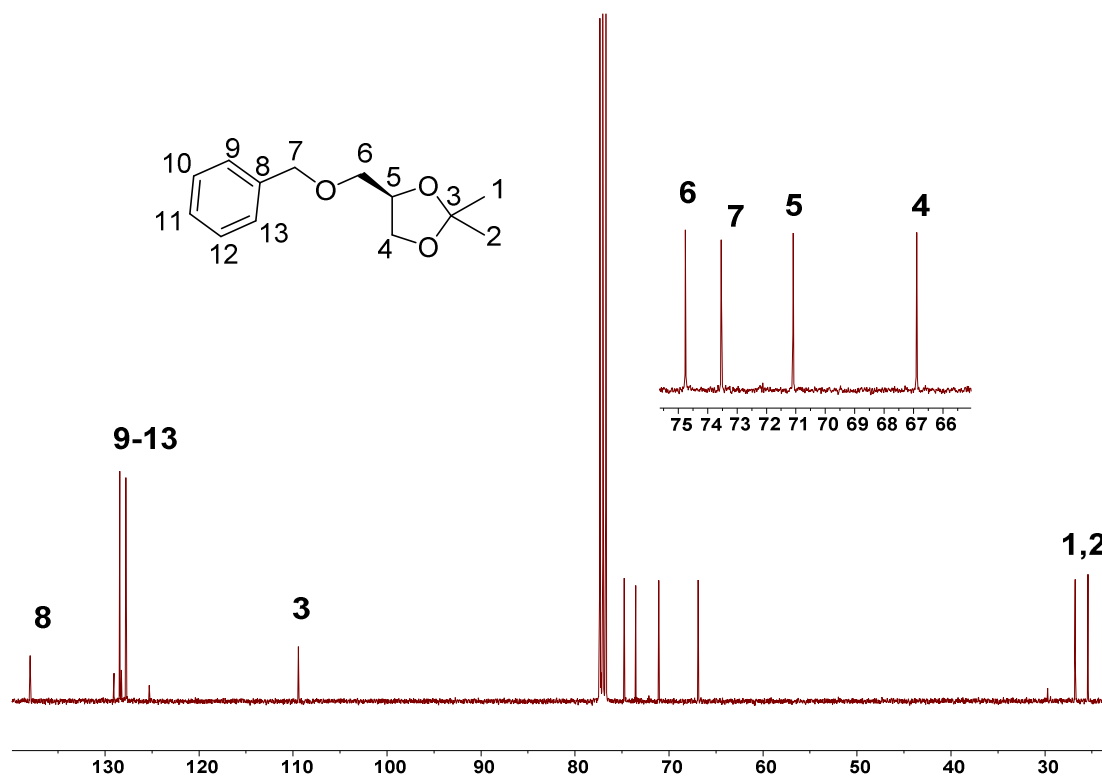


Figure S257 NMR spectra (CDCl₃) of **C11b**: a) ^1H and b) ^{13}C .

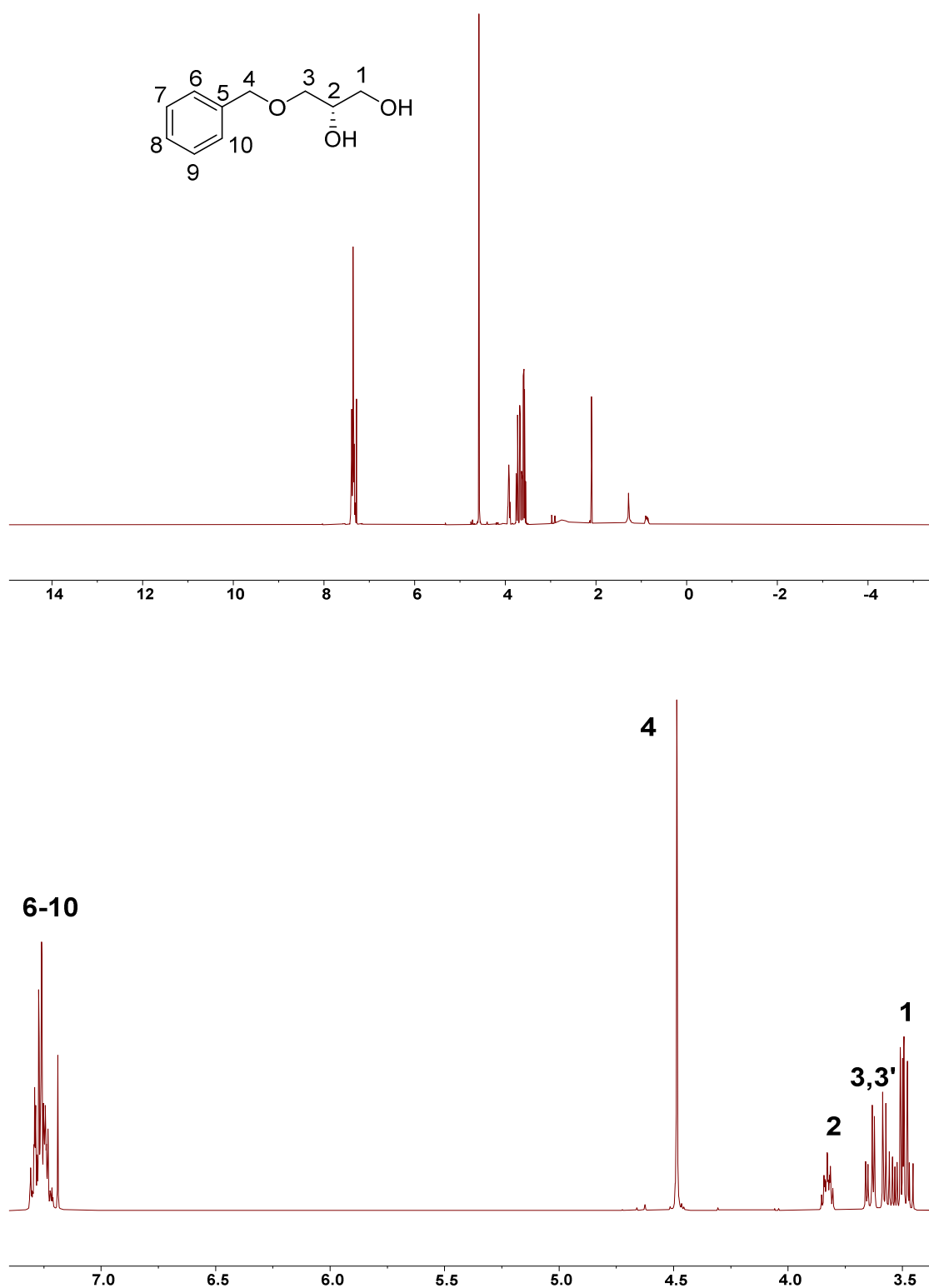


Figure S258 ^1H NMR spectra (CDCl_3) of **C11c**.

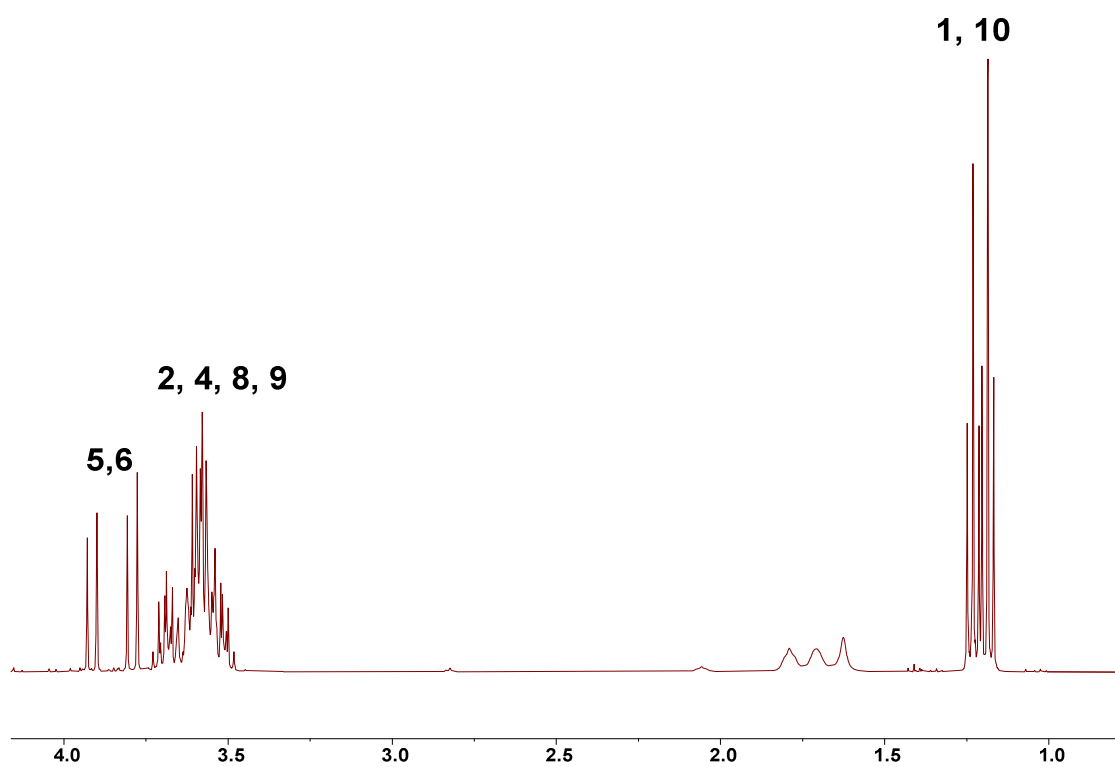
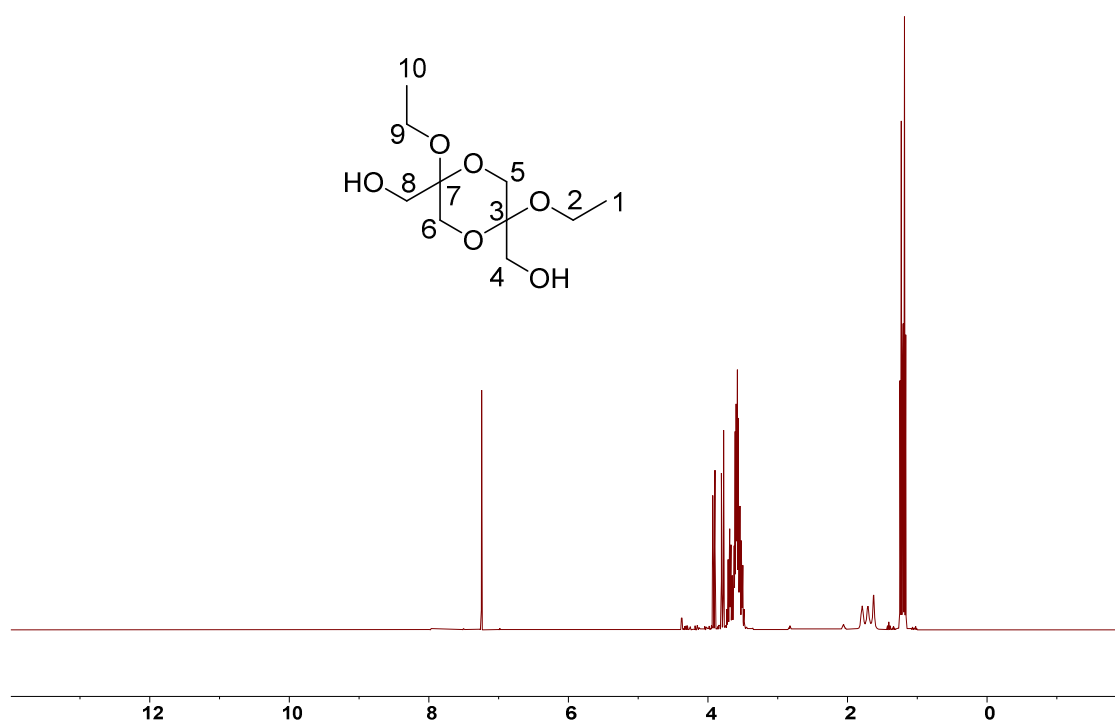


Figure S259 ^1H NMR spectra (CDCl_3) of **C13b**.

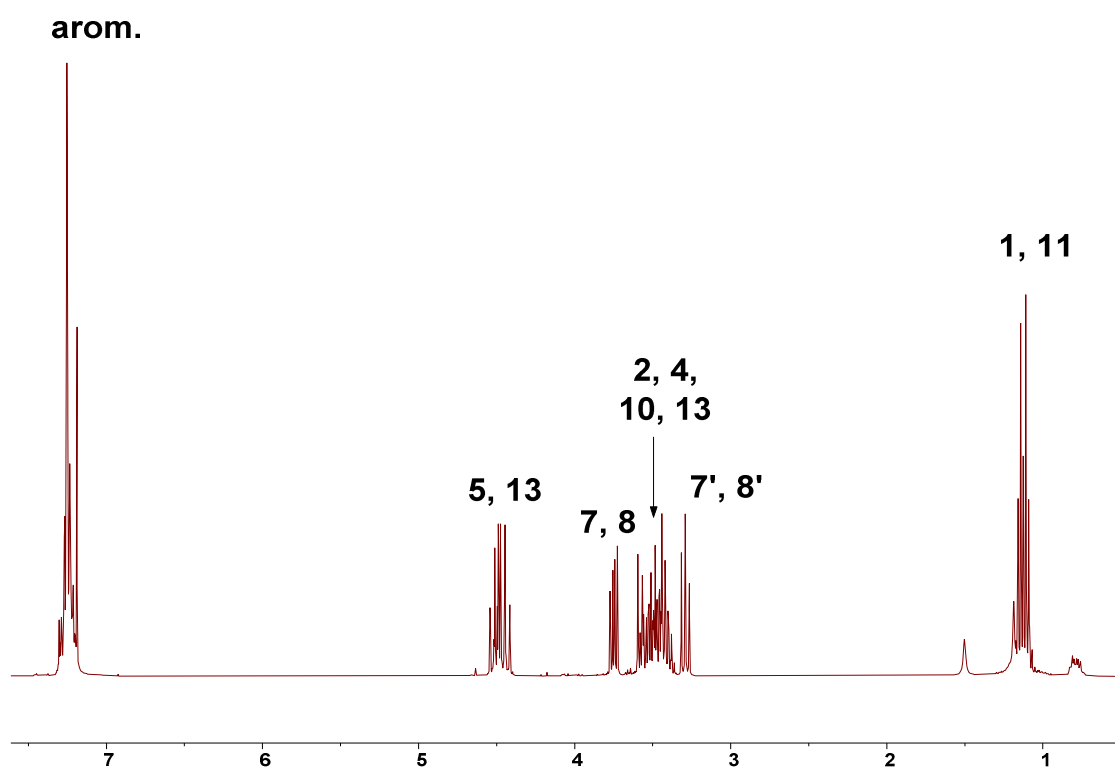
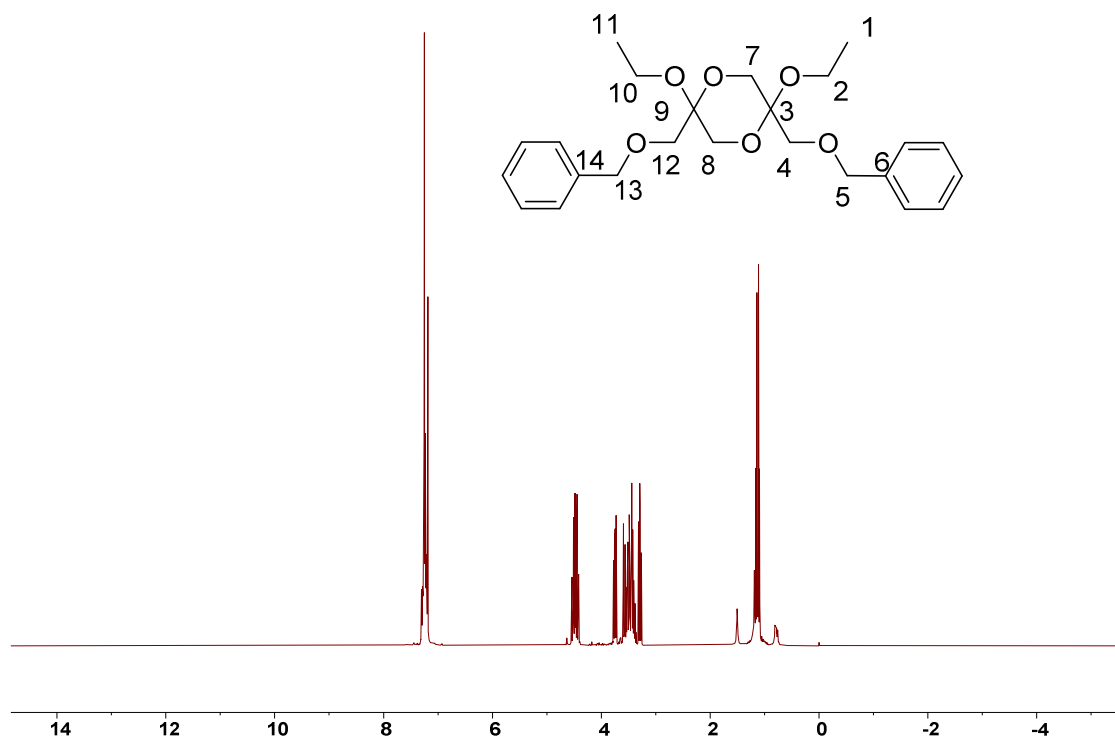
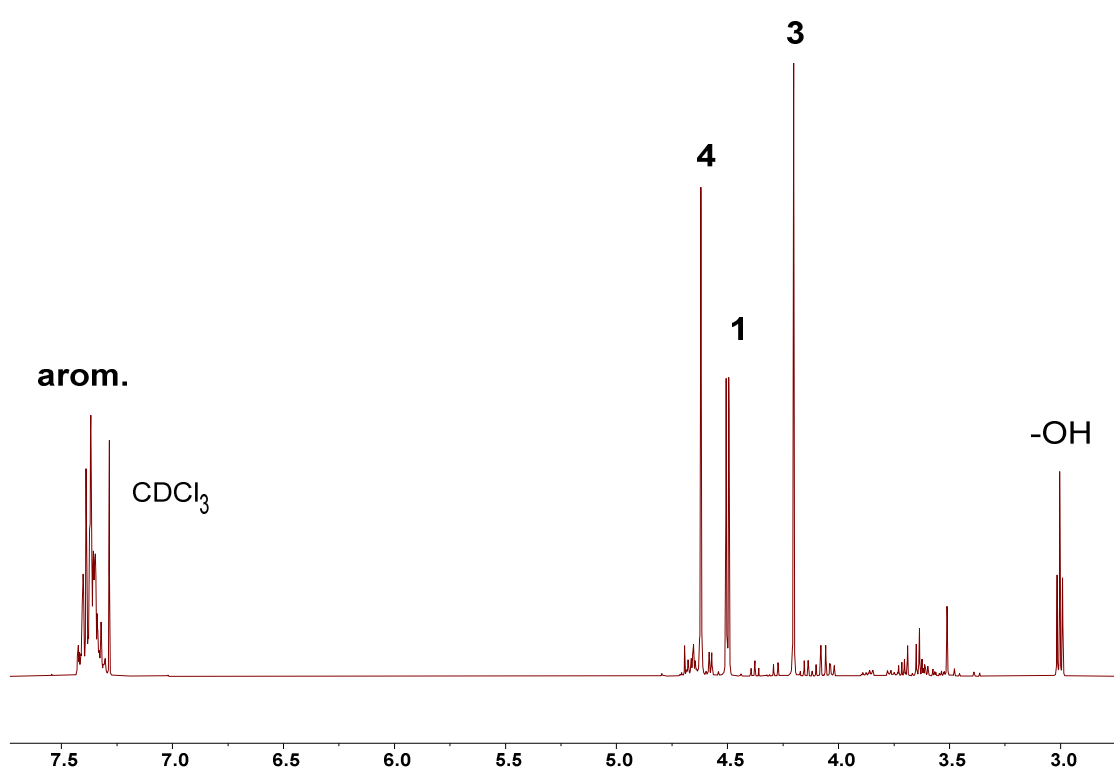
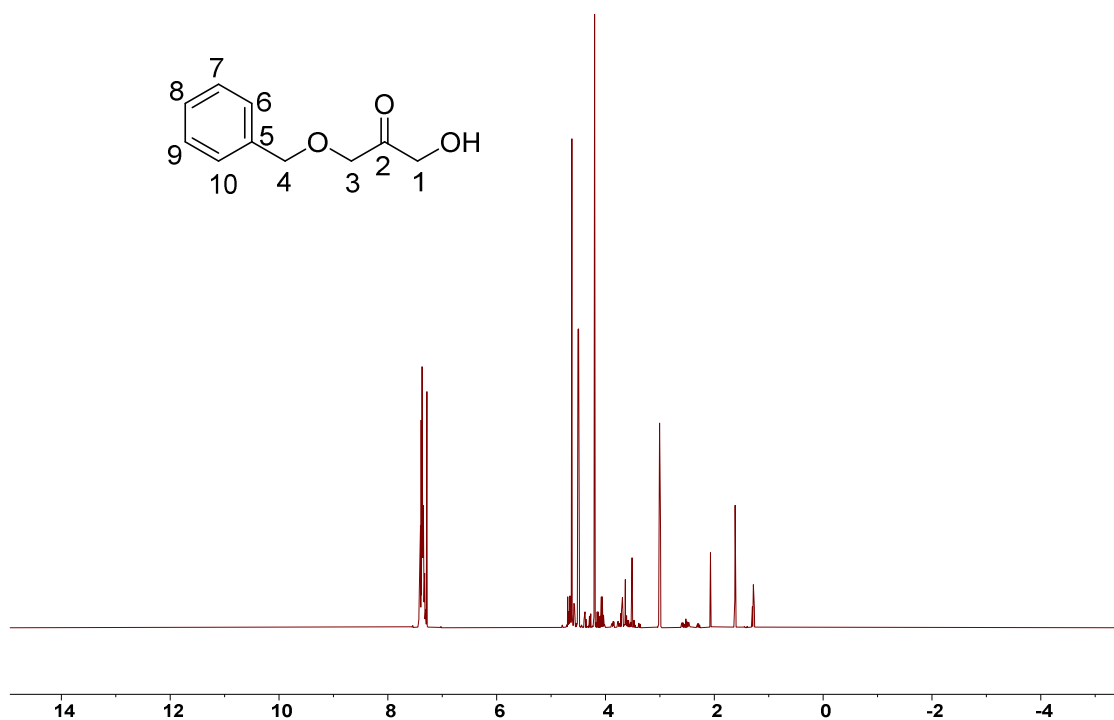


Figure S260 ^1H NMR spectra (CDCl_3) of **C13c**.

a)



b)

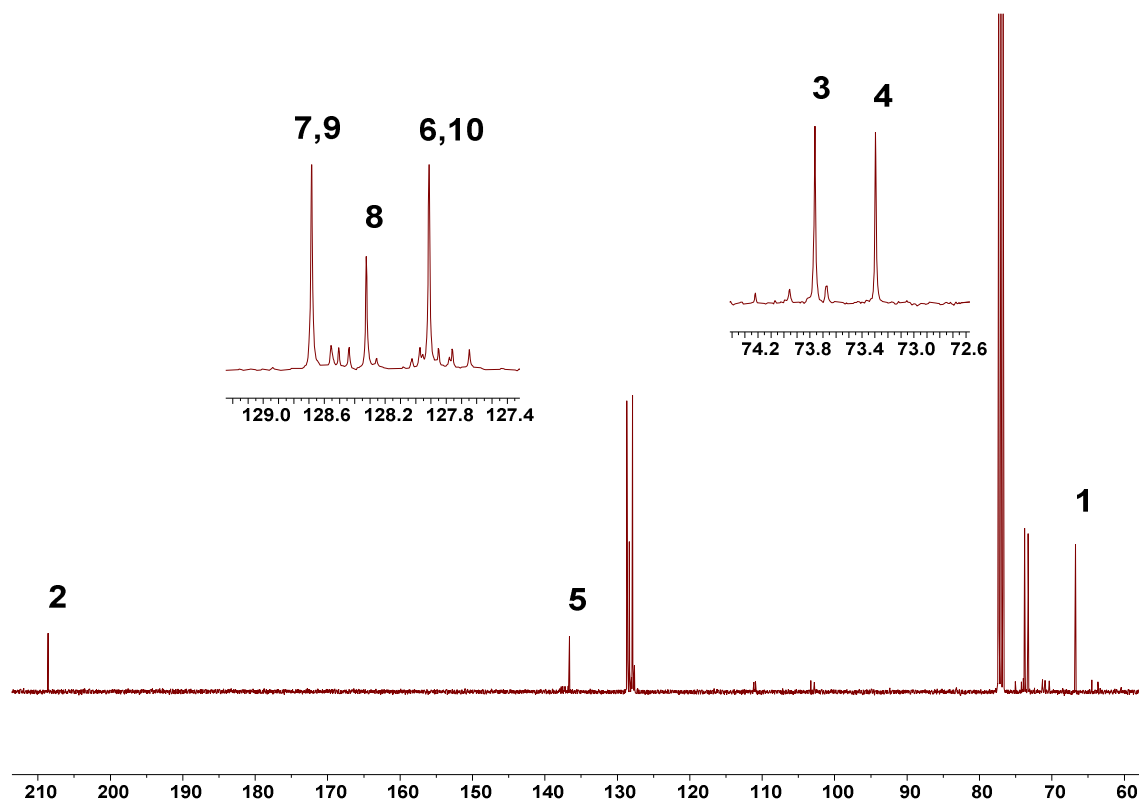
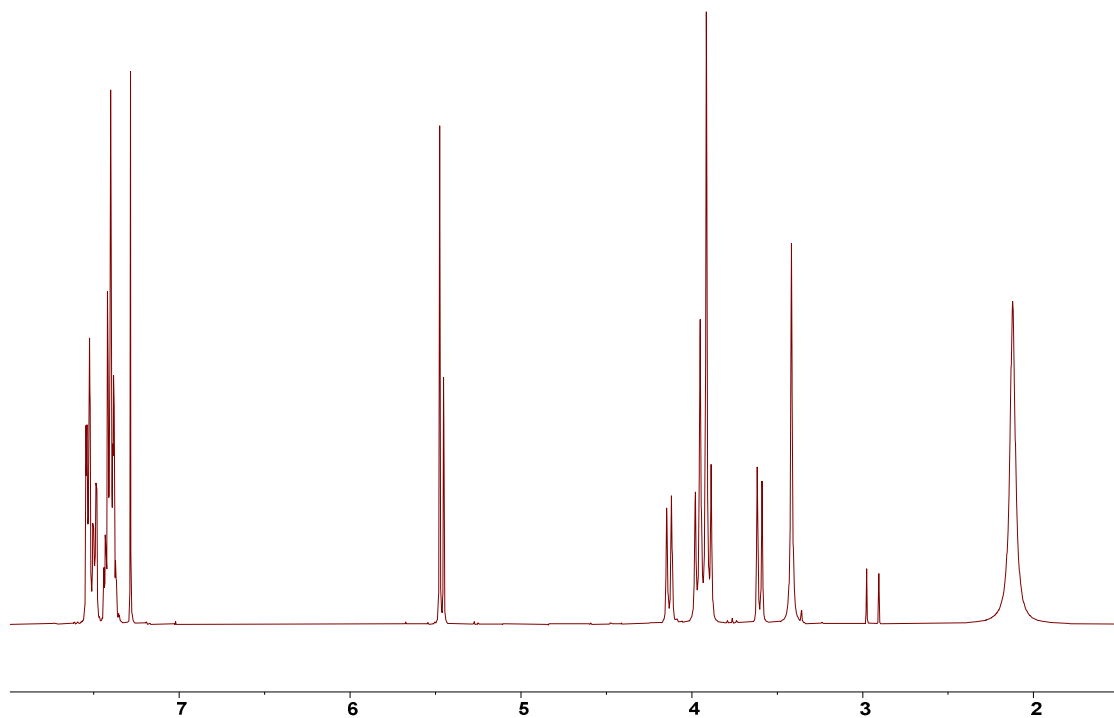
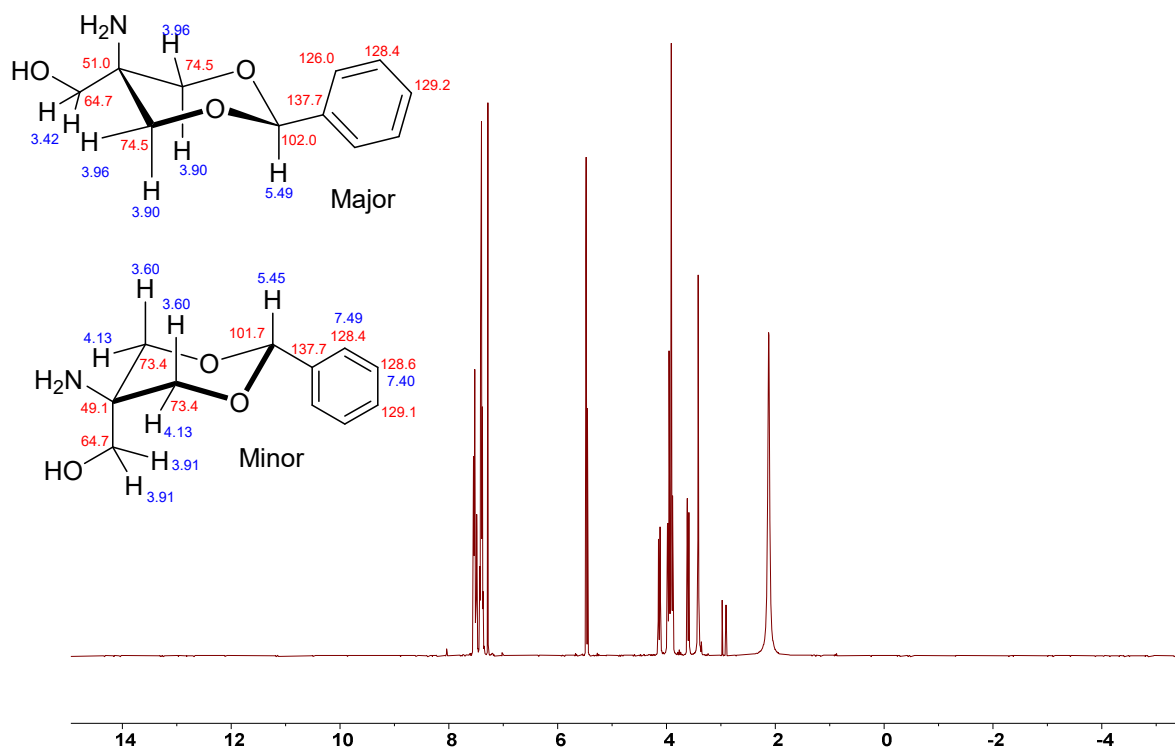
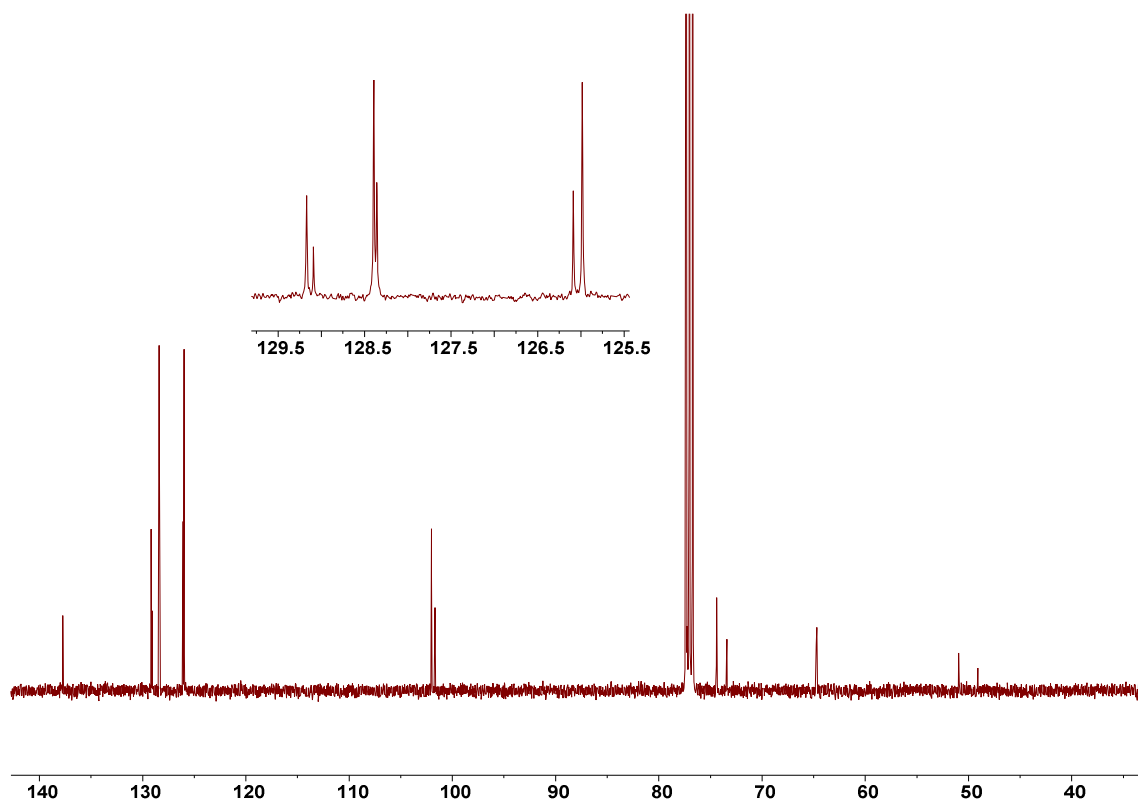


Figure S261 NMR spectra (CDCl_3) of **C13d**: a) ^1H and b) ^{13}C .

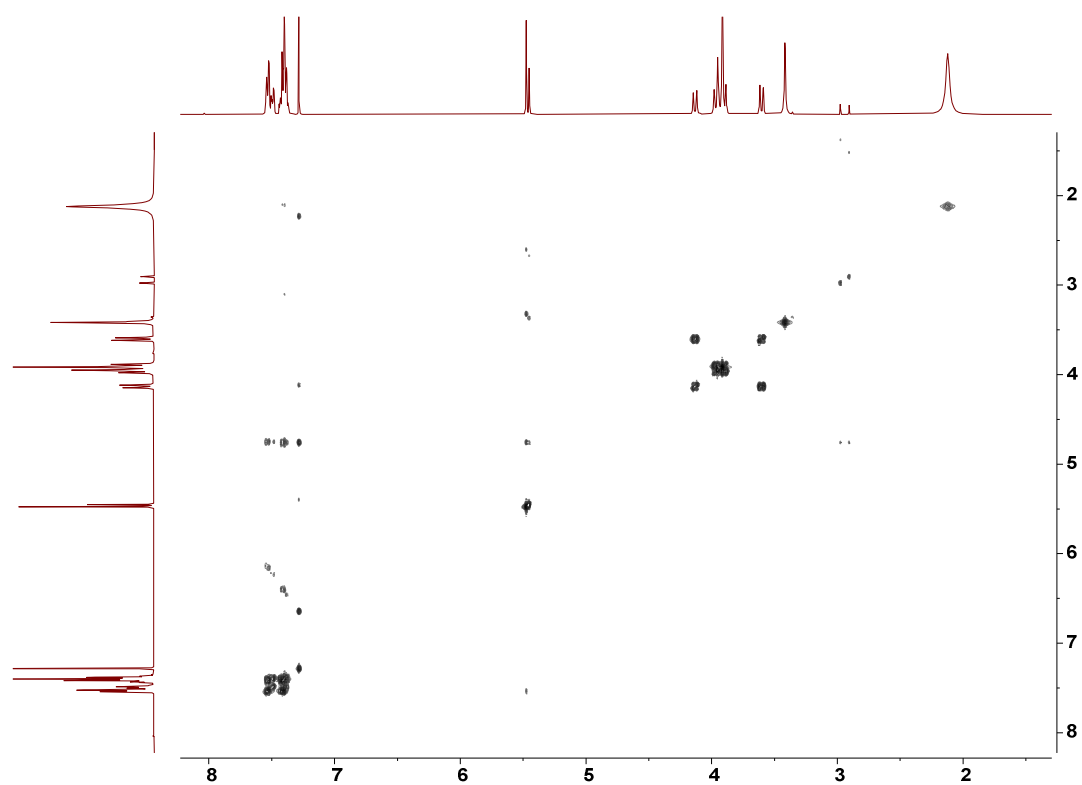
a)



b)



c)



d)

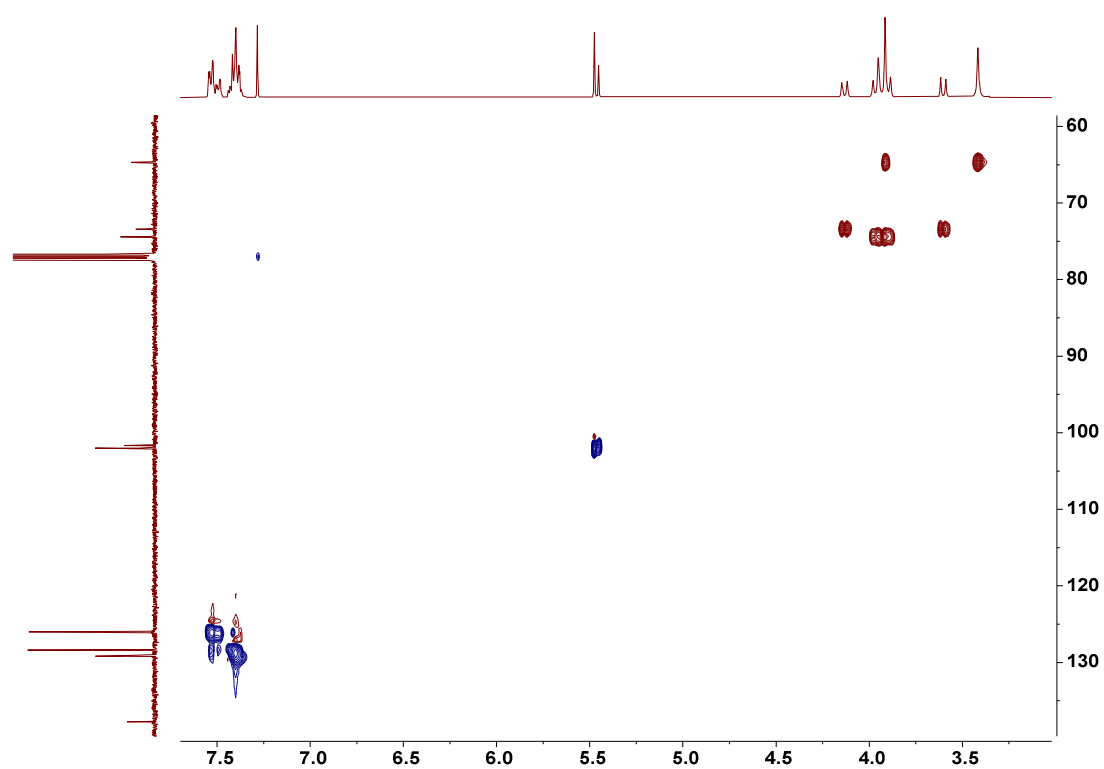
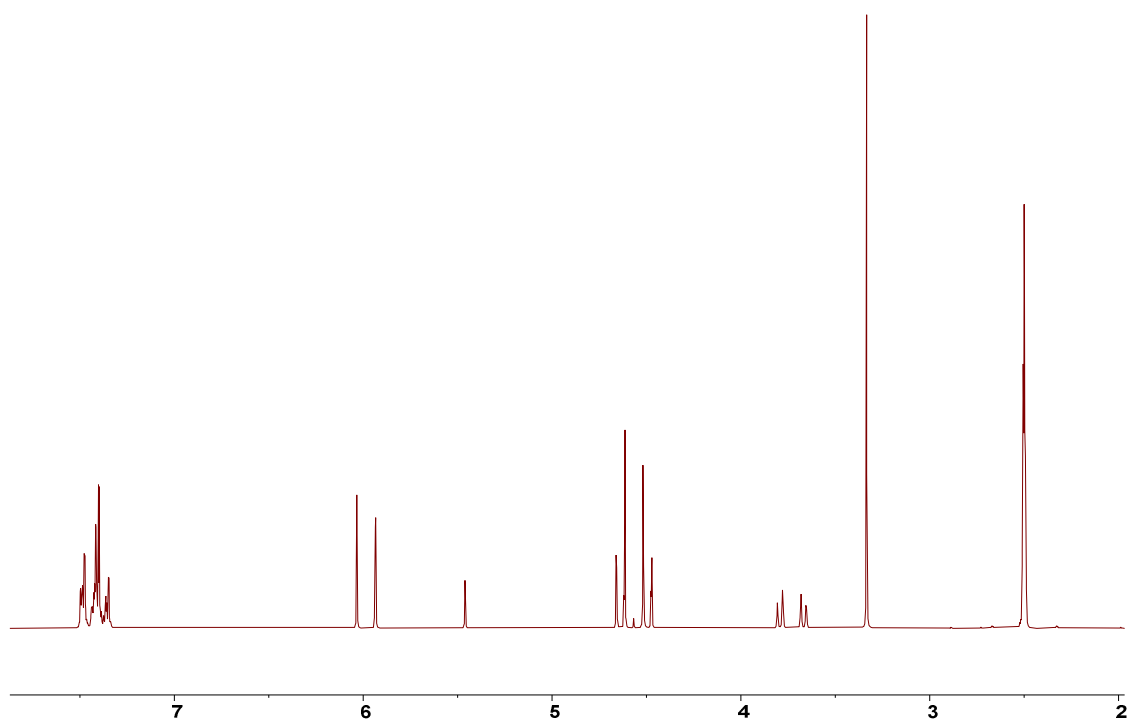
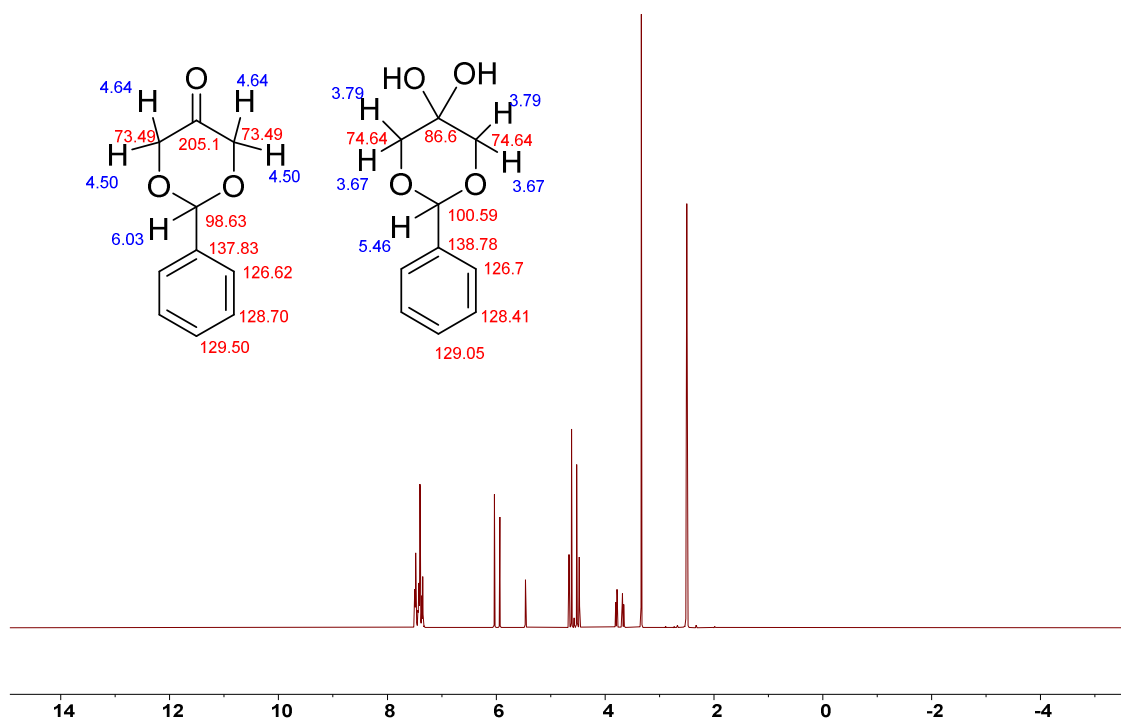
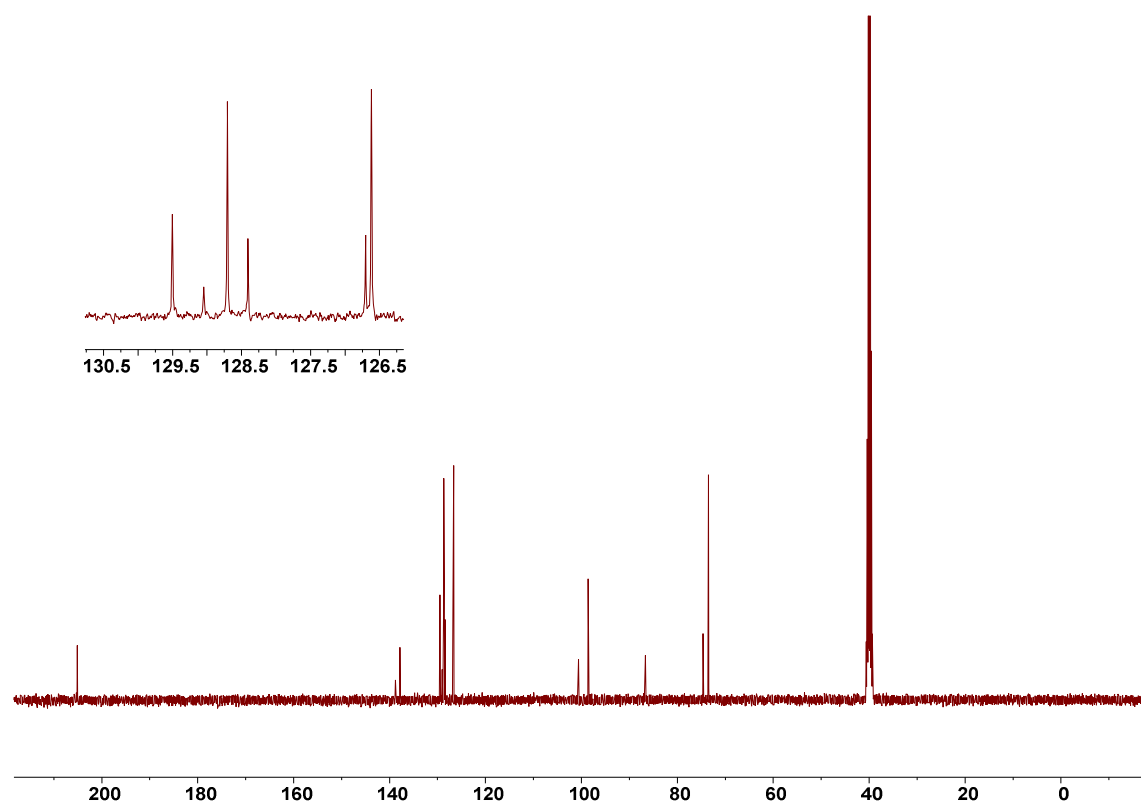


Figure S262 NMR spectra (CDCl_3) of **C14b**: a) ^1H , b) ^{13}C , c) gCOSY and d) gHSQC.

a)



b)



c)

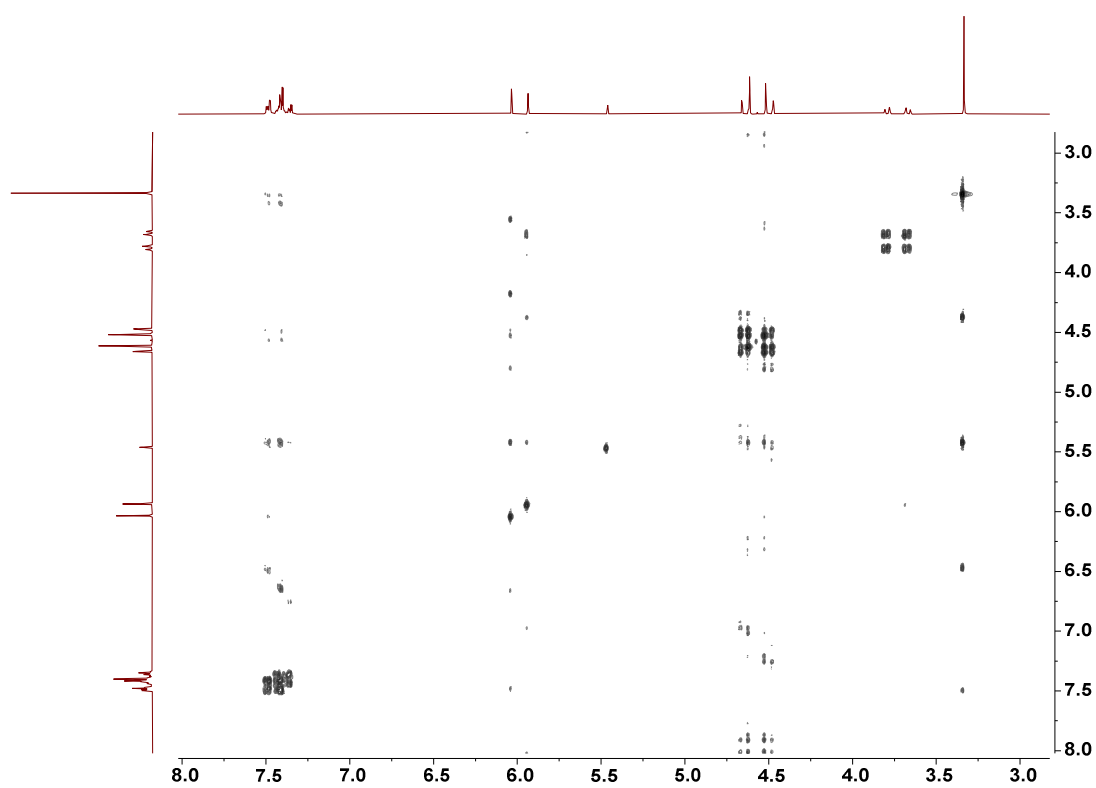
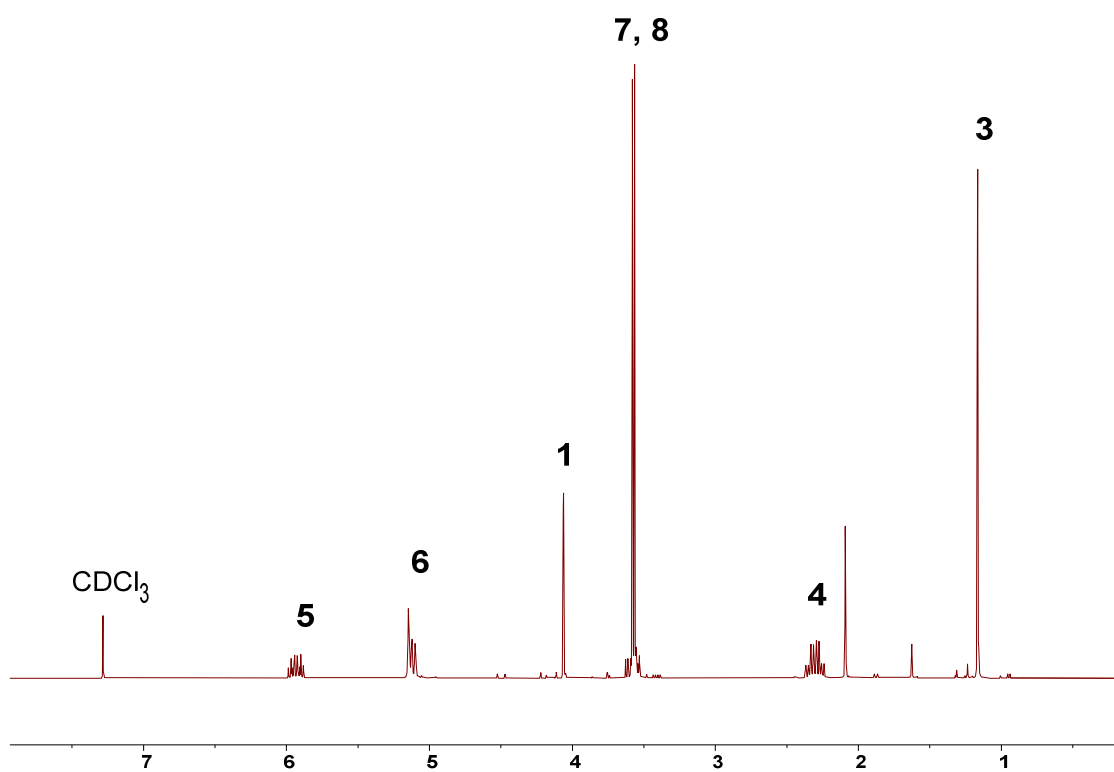
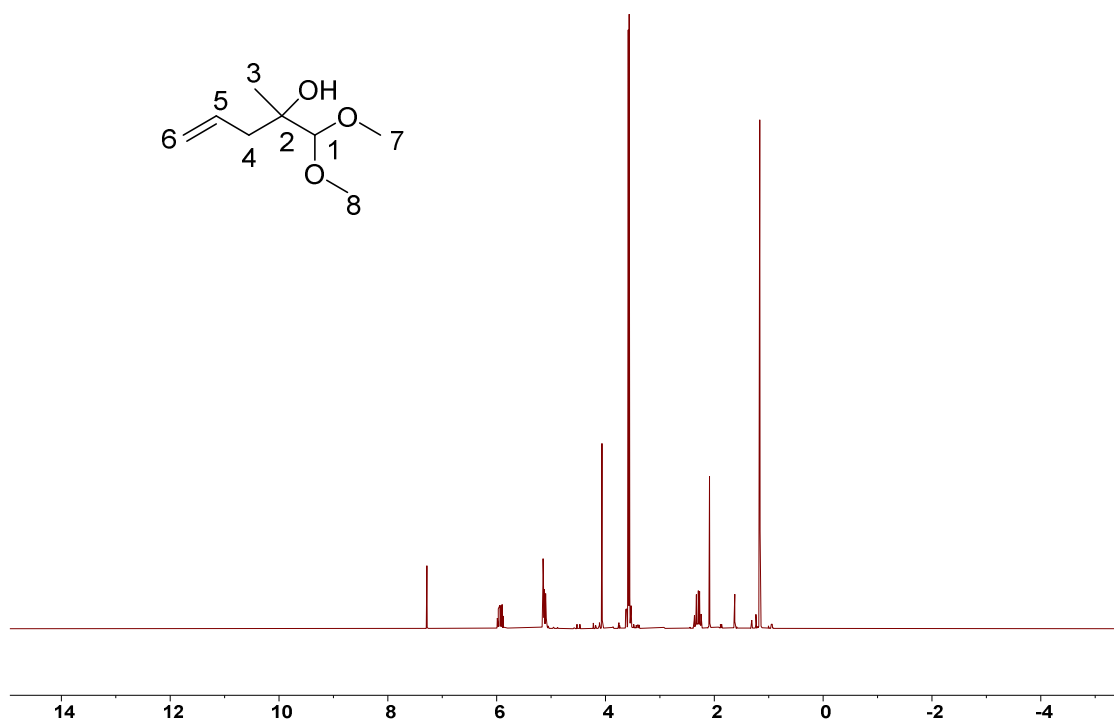


Figure S263 NMR spectra (DMSO) of **C14c**: a) ^1H , b) ^{13}C and c) gCOSY.

a)



b)

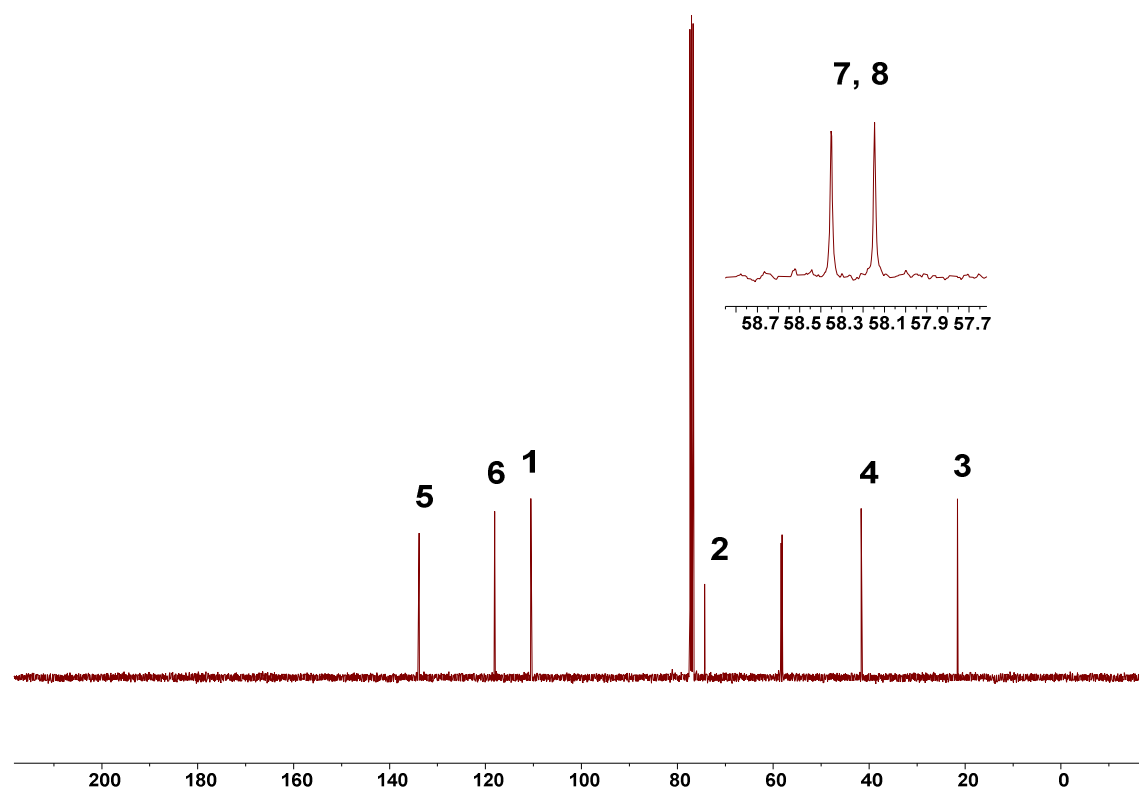
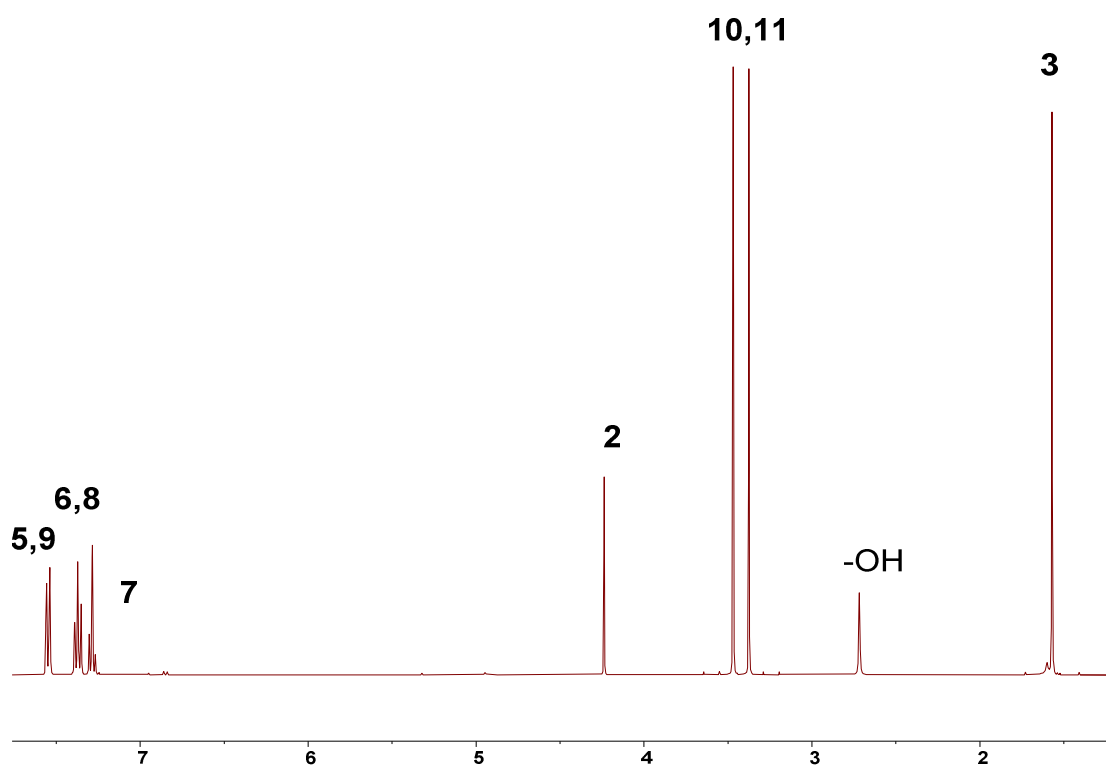
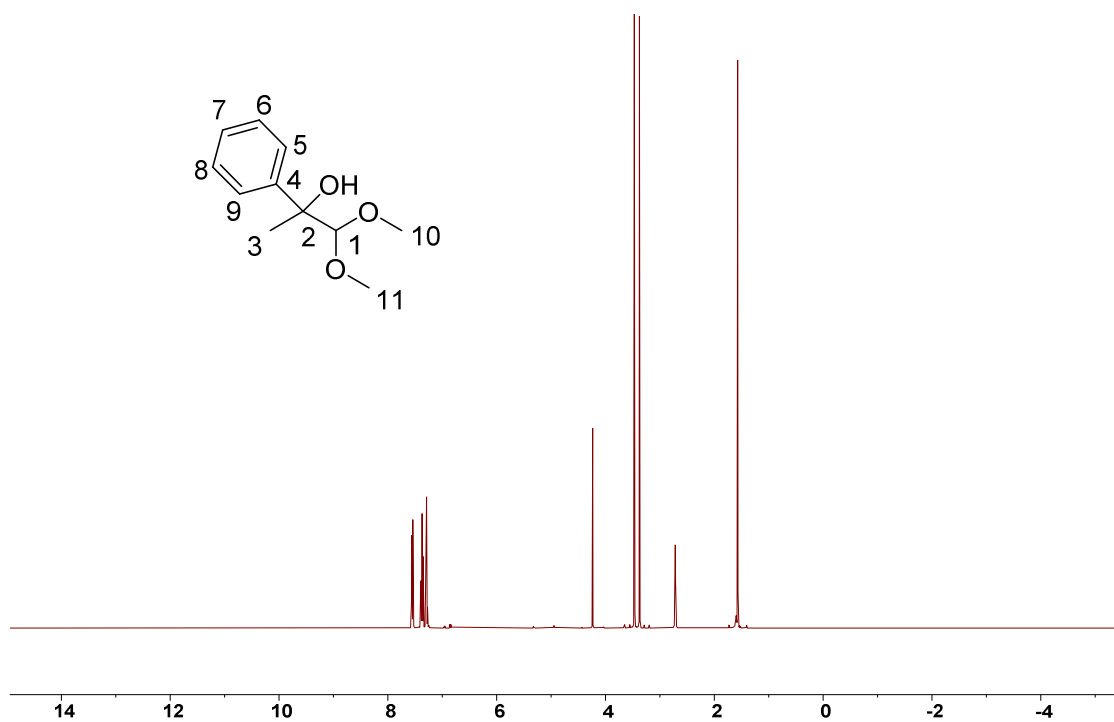


Figure S264 NMR spectra (CDCl_3) of **C15b**: a) ^1H and b) ^{13}C .

a)



b)

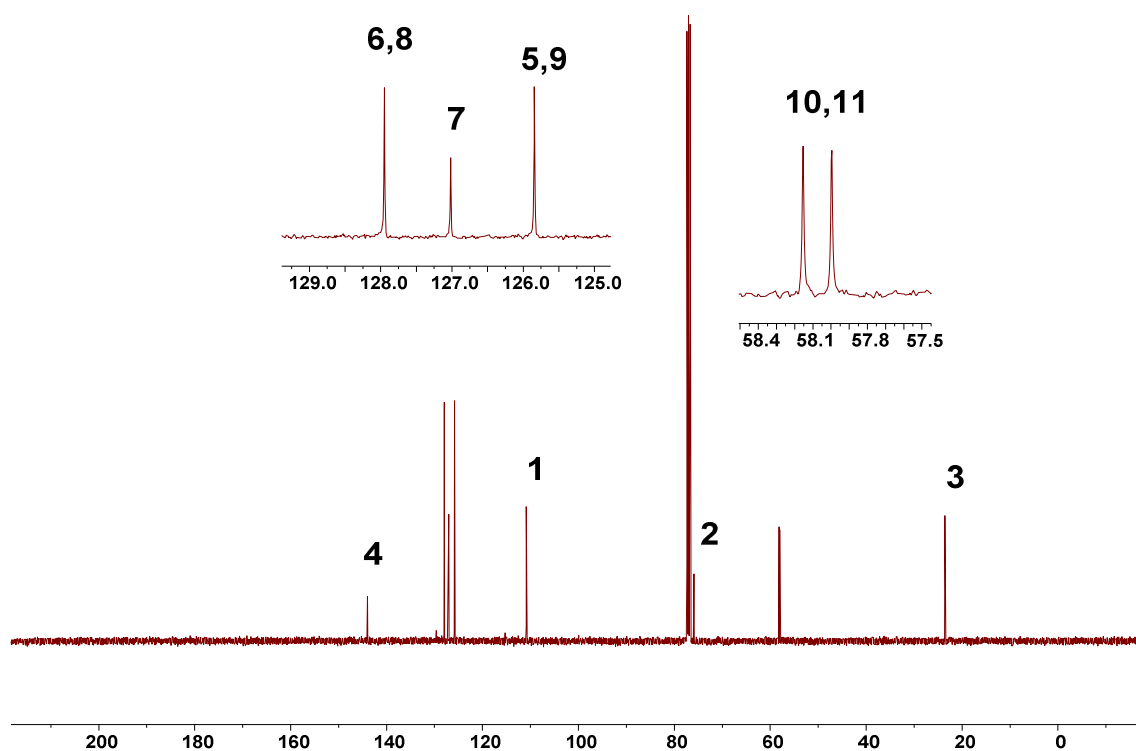
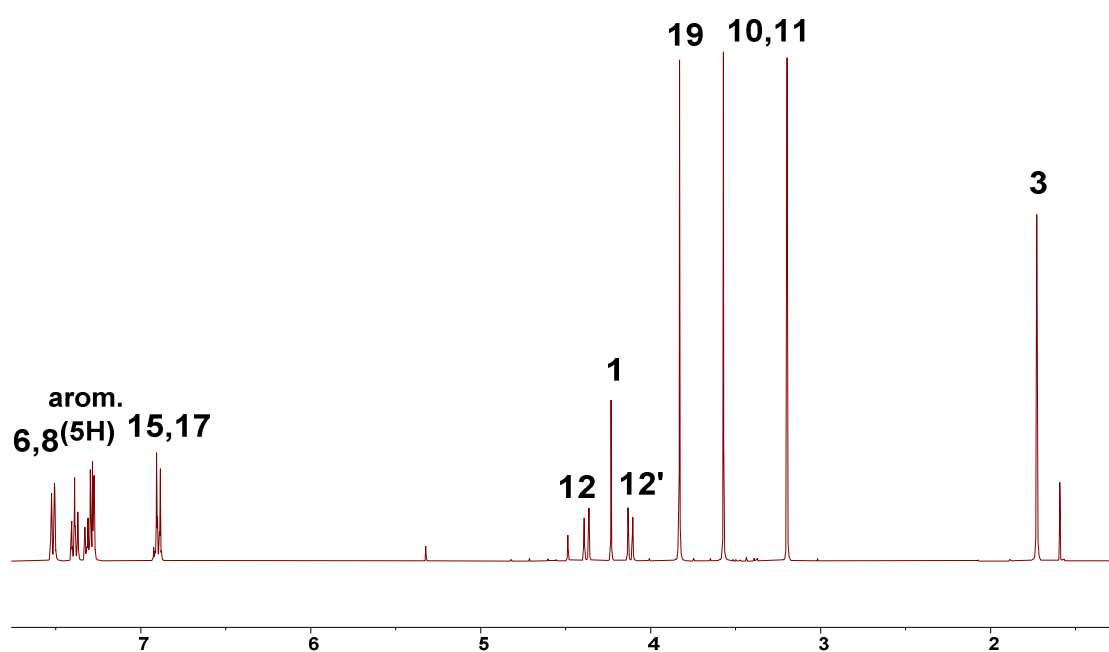
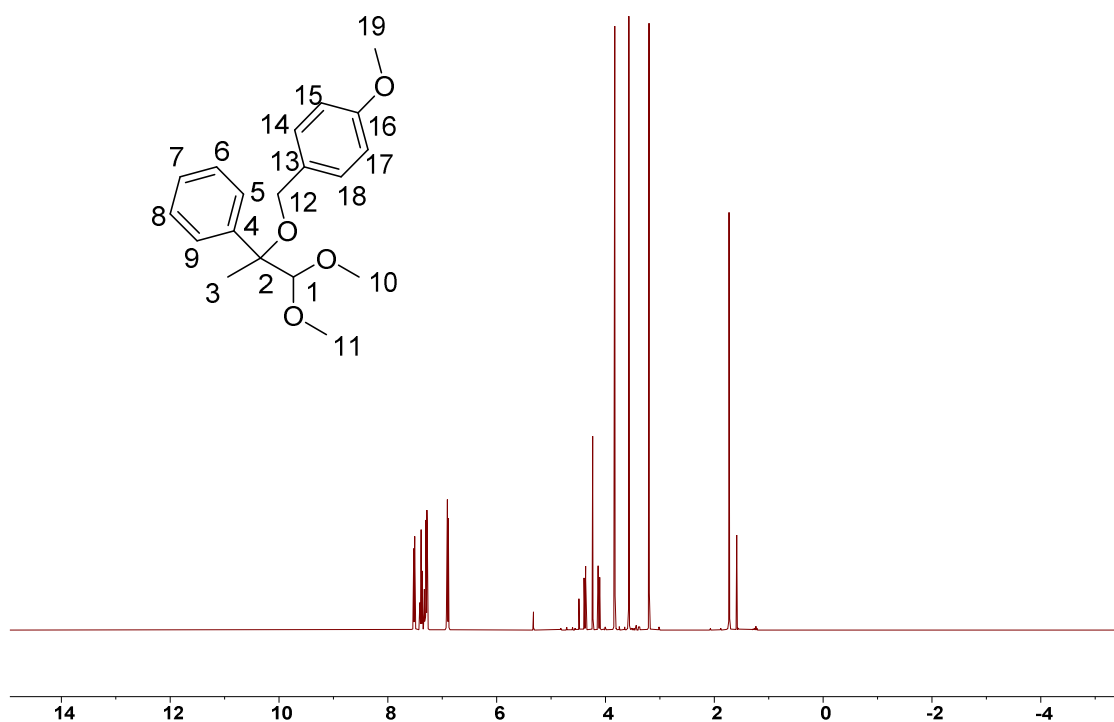


Figure S265 NMR spectra (CDCl_3) of **C15c**: a) ^1H and b) ^{13}C .

a)



b)

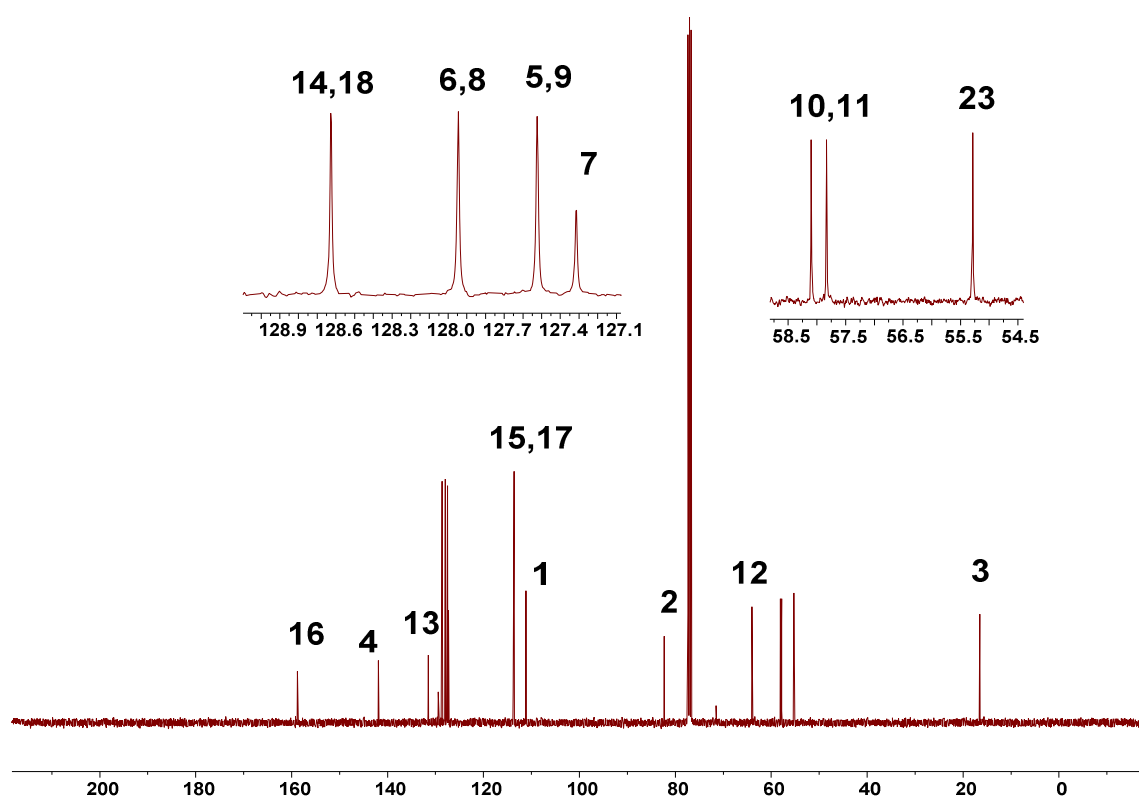
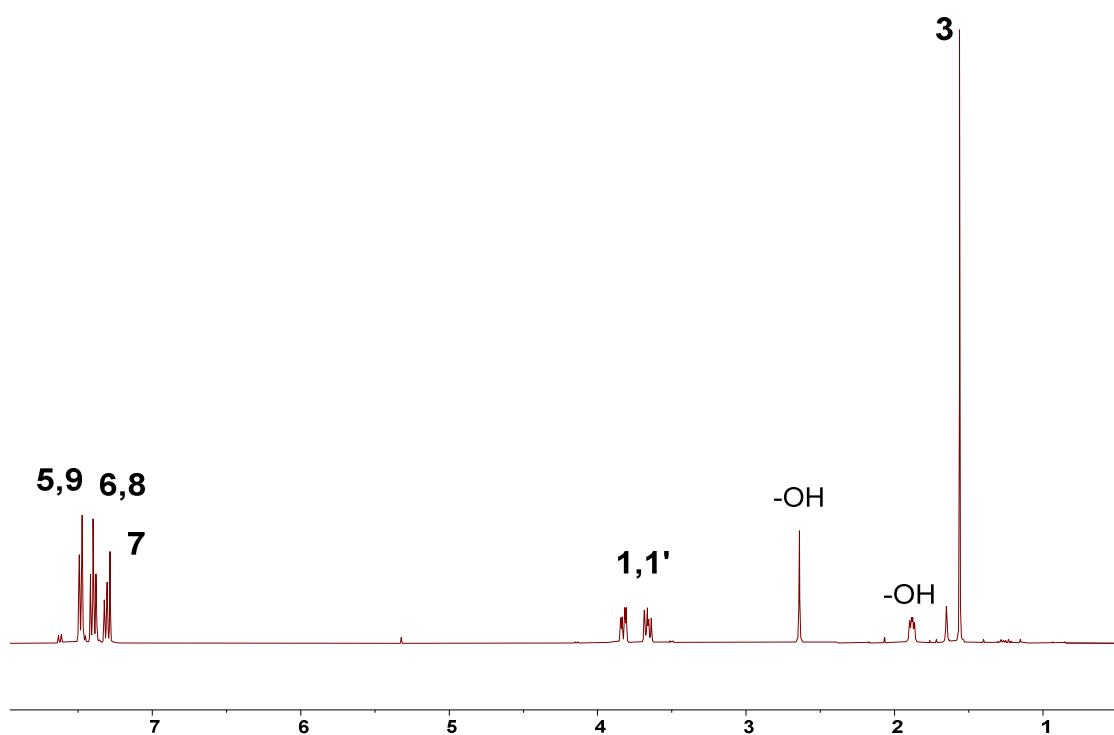
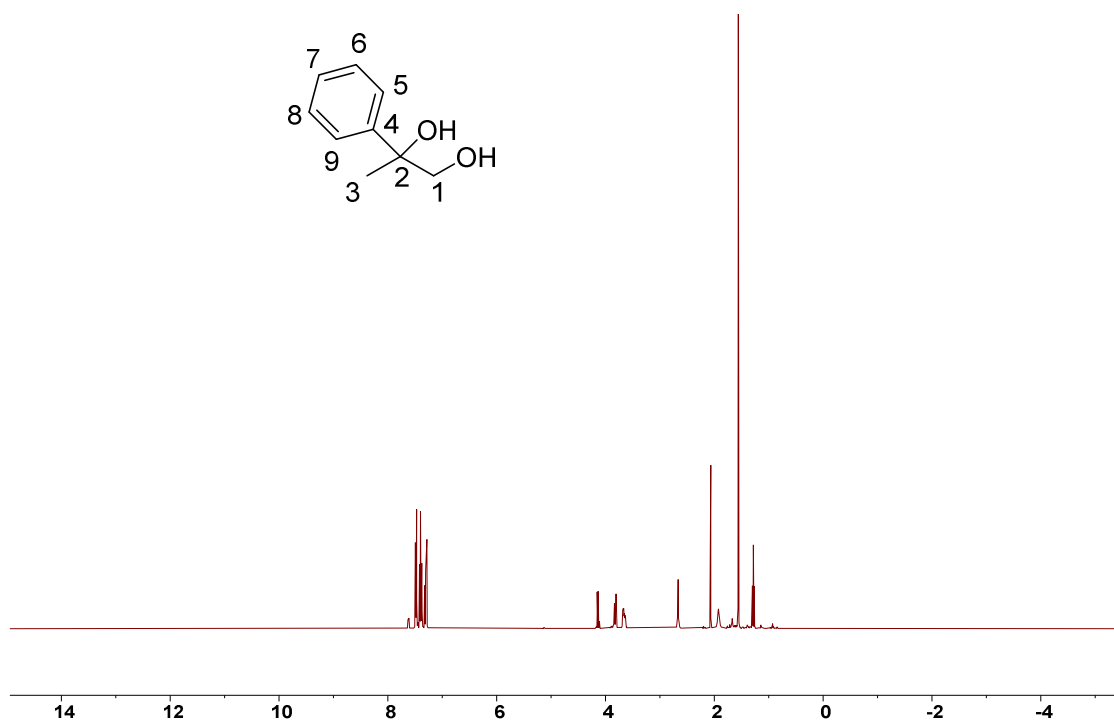


Figure S266 NMR spectra (CDCl_3) of **C15f**: a) ^1H and b) ^{13}C .

a)



b)

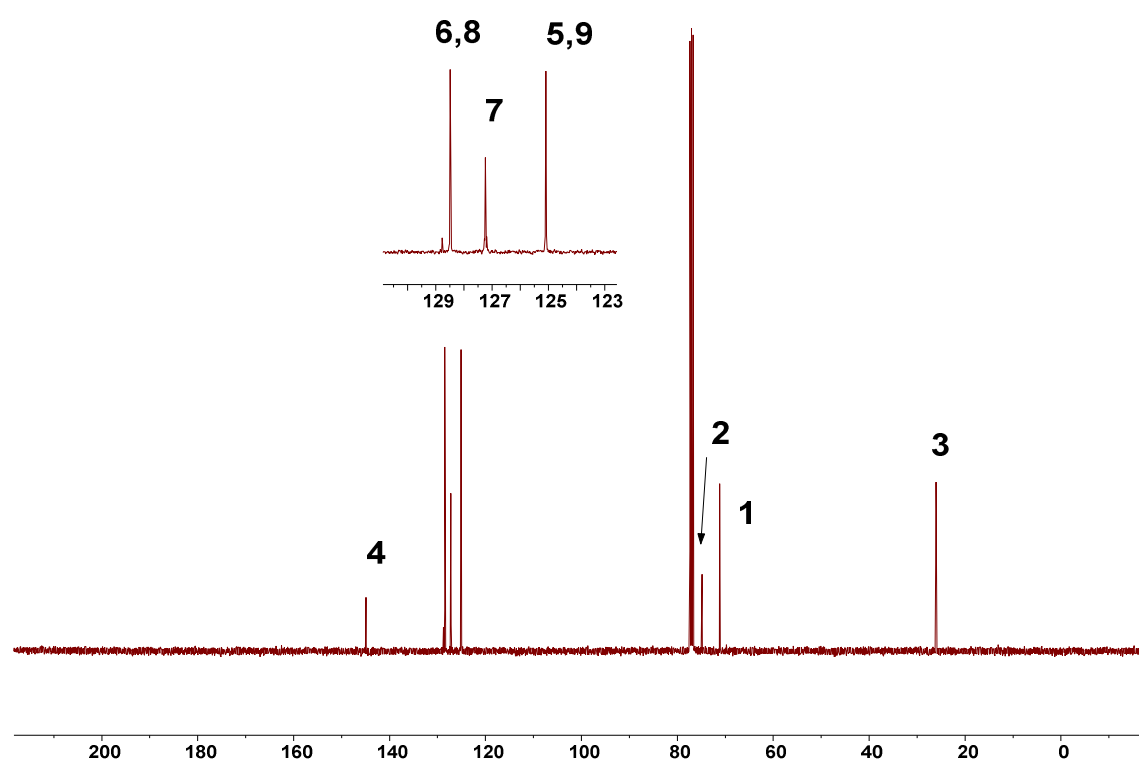
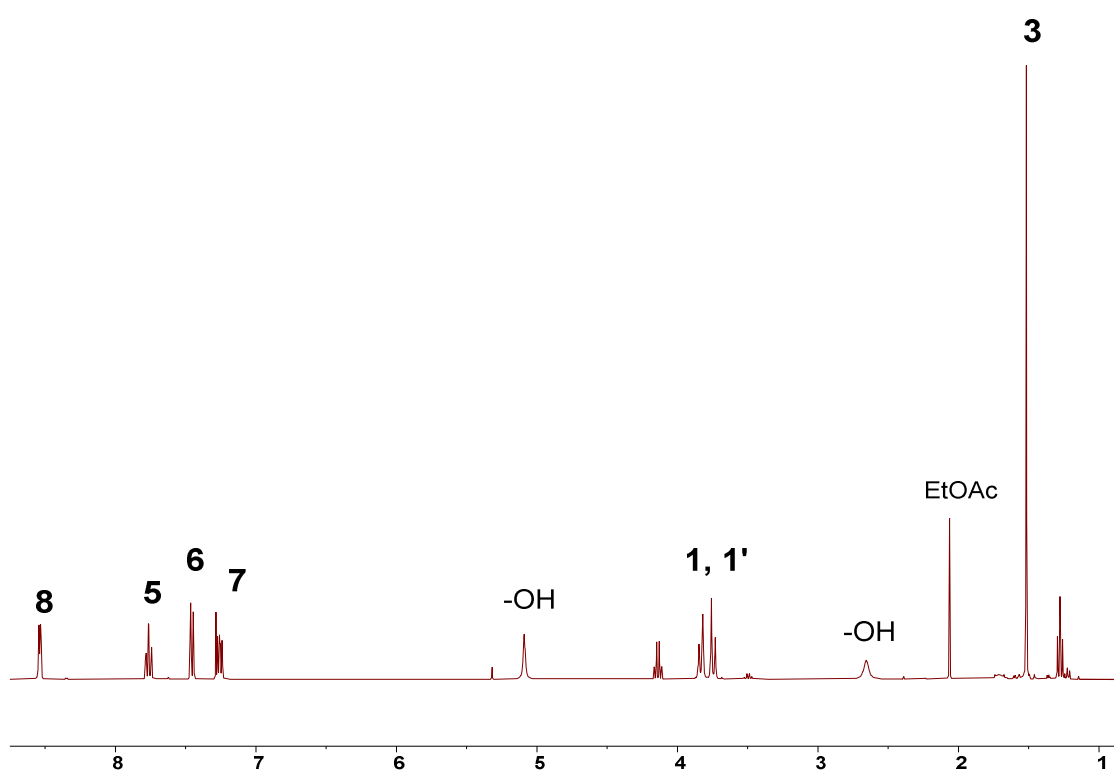
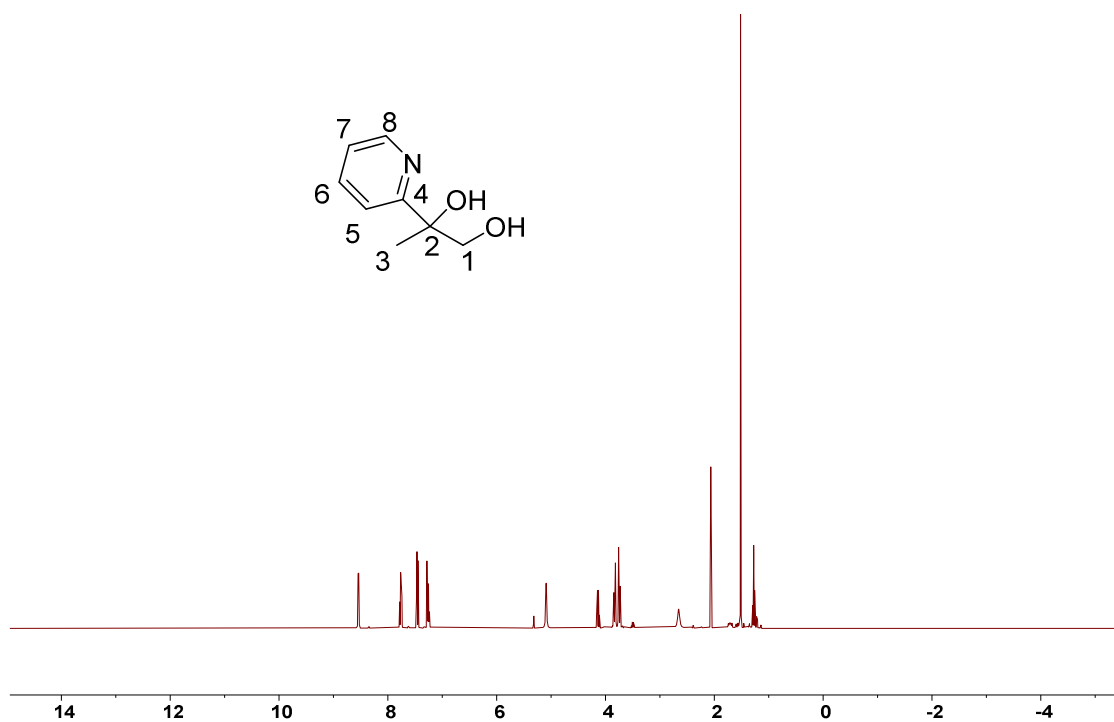


Figure S267 NMR spectra (CDCl_3) of **C16b**: a) ^1H and b) ^{13}C .

a)



b)

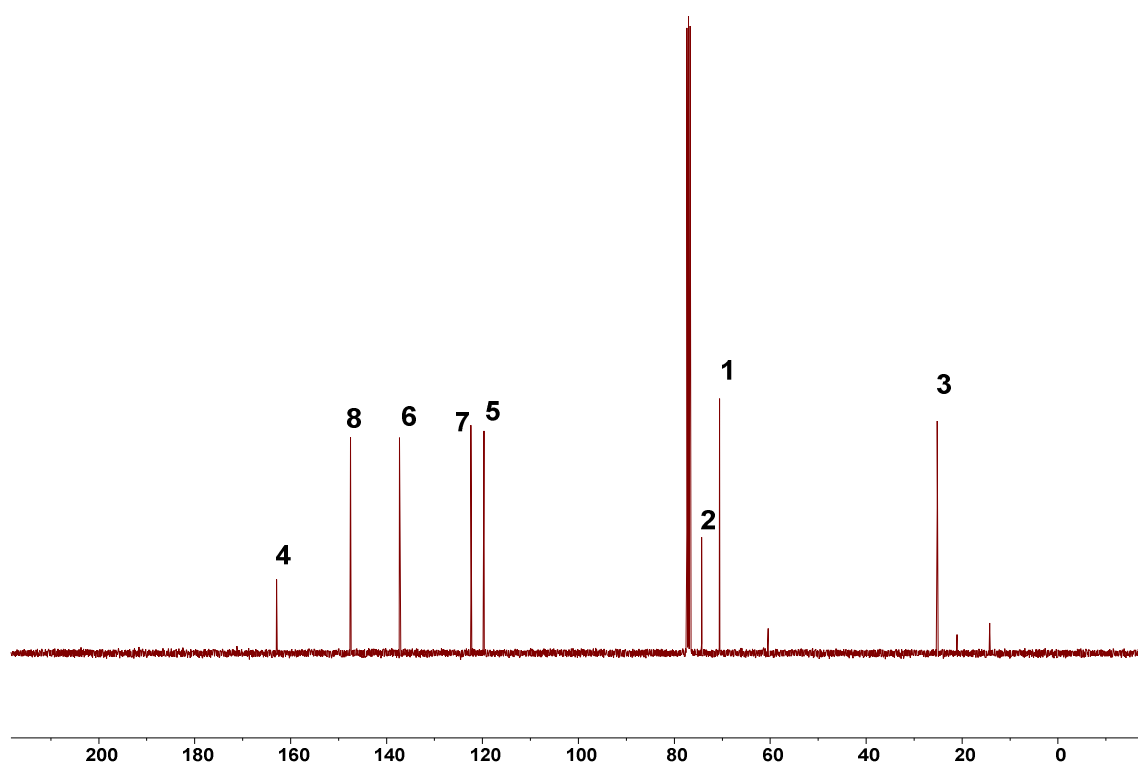
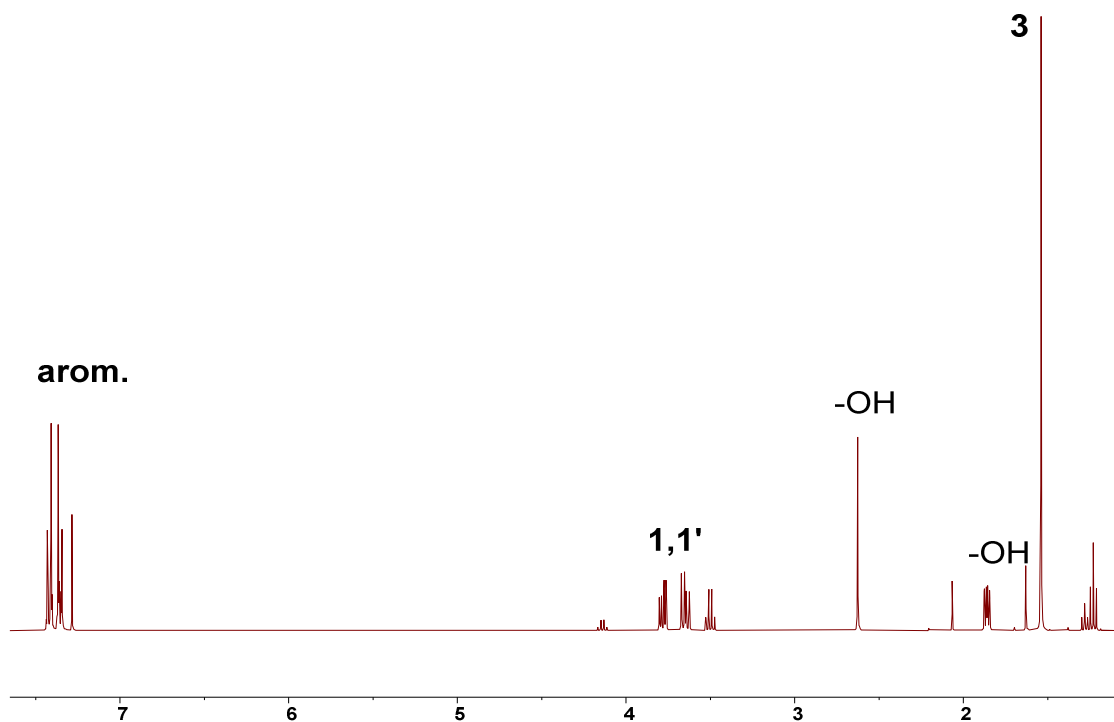
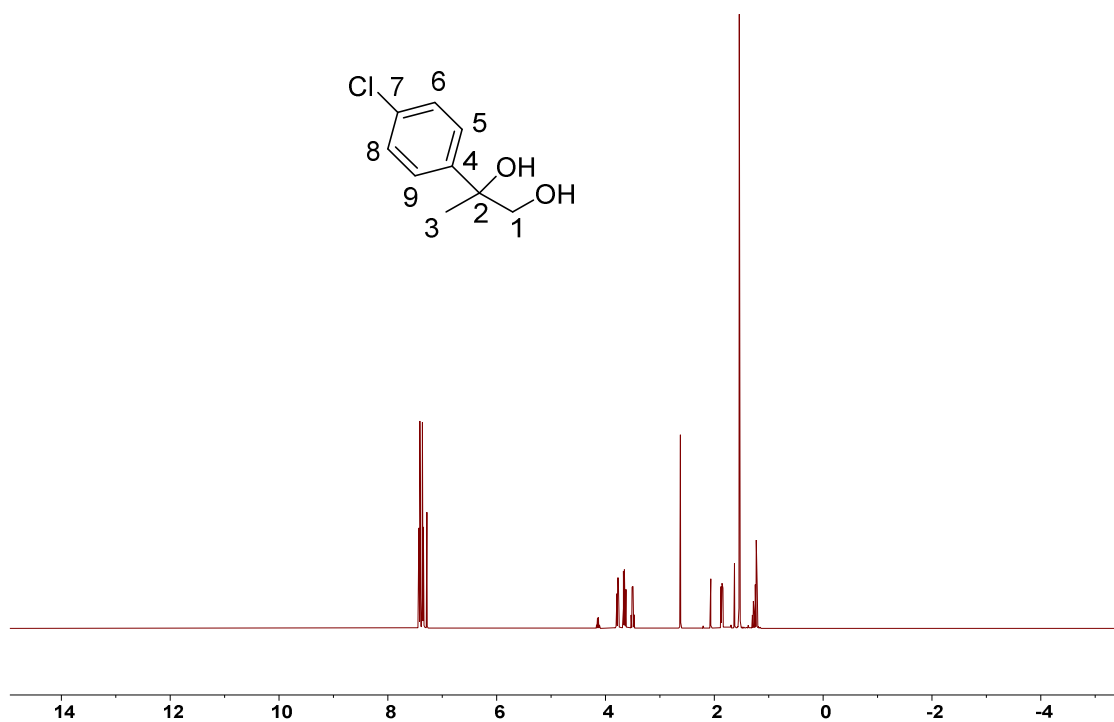


Figure S268 NMR spectra (CDCl_3) of **C16c**: a) ^1H and b) ^{13}C .

a)



b)

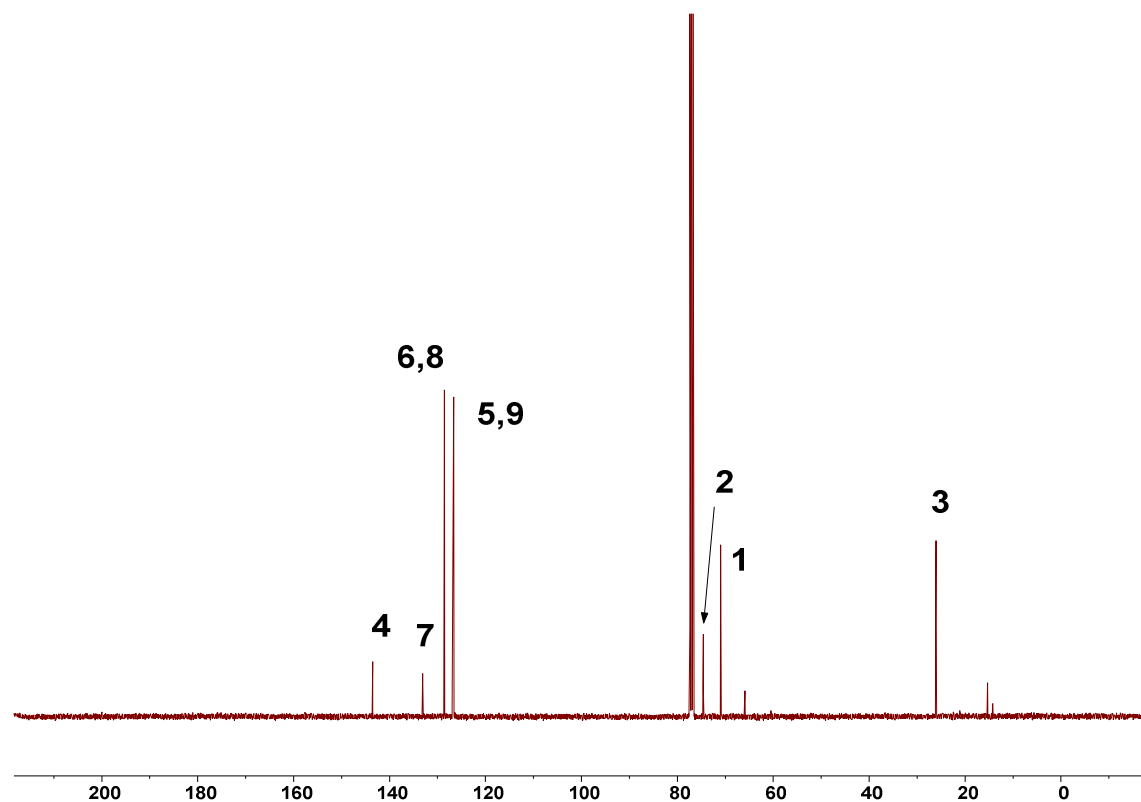
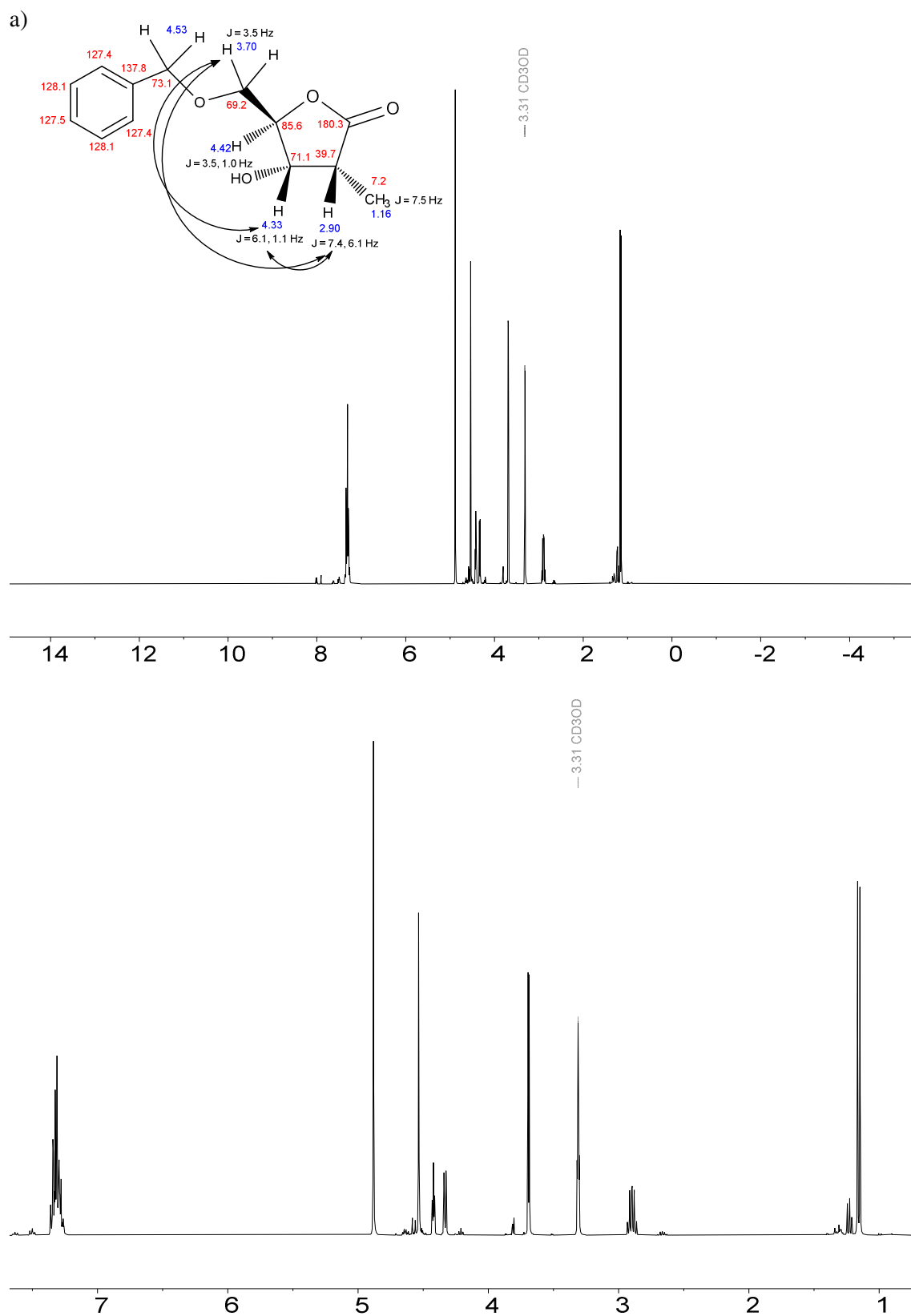
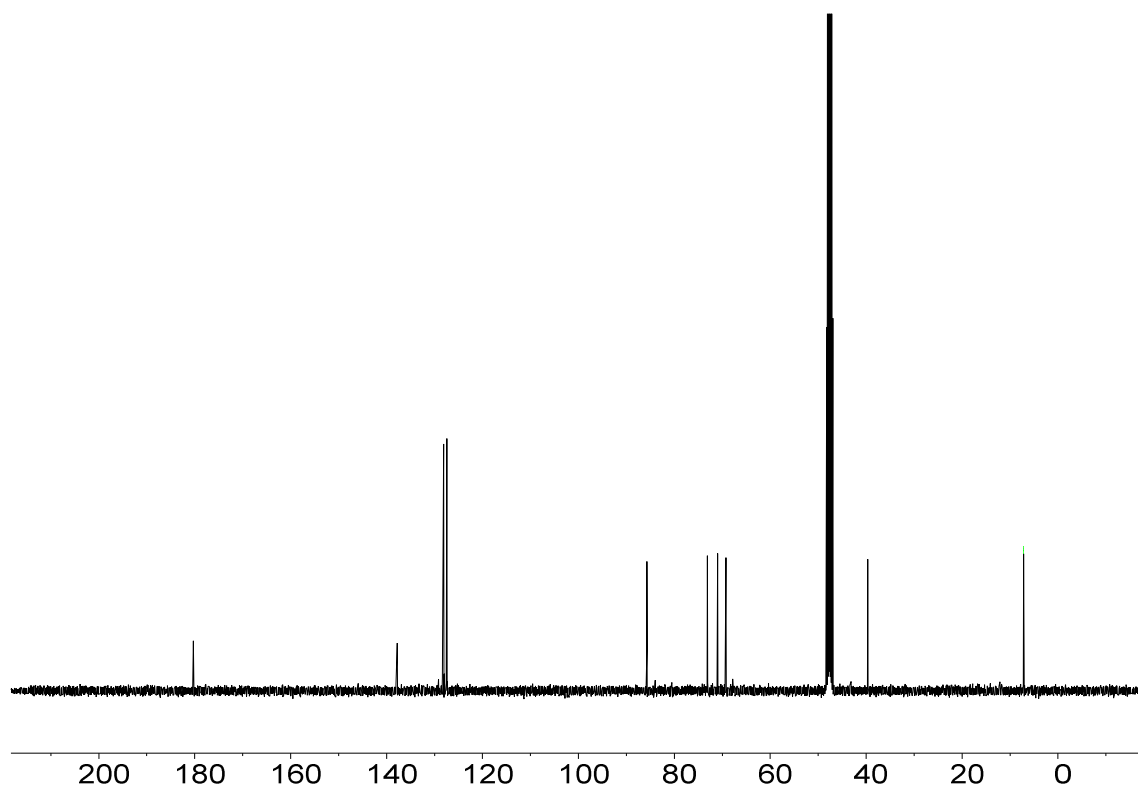


Figure S269 NMR spectra (CDCl_3) of **C16d**: a) ^1H and b) ^{13}C .

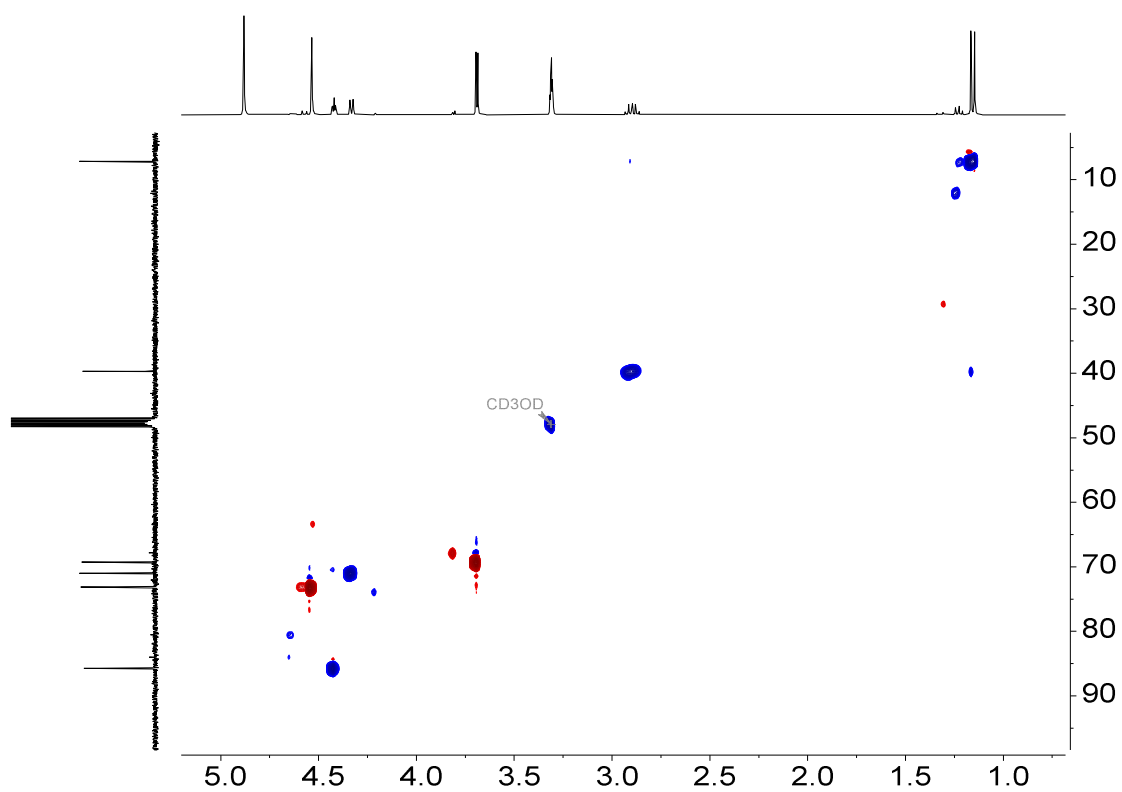
6.4.2 NMR spectra of compounds 11 and 12



b)



c)



d)

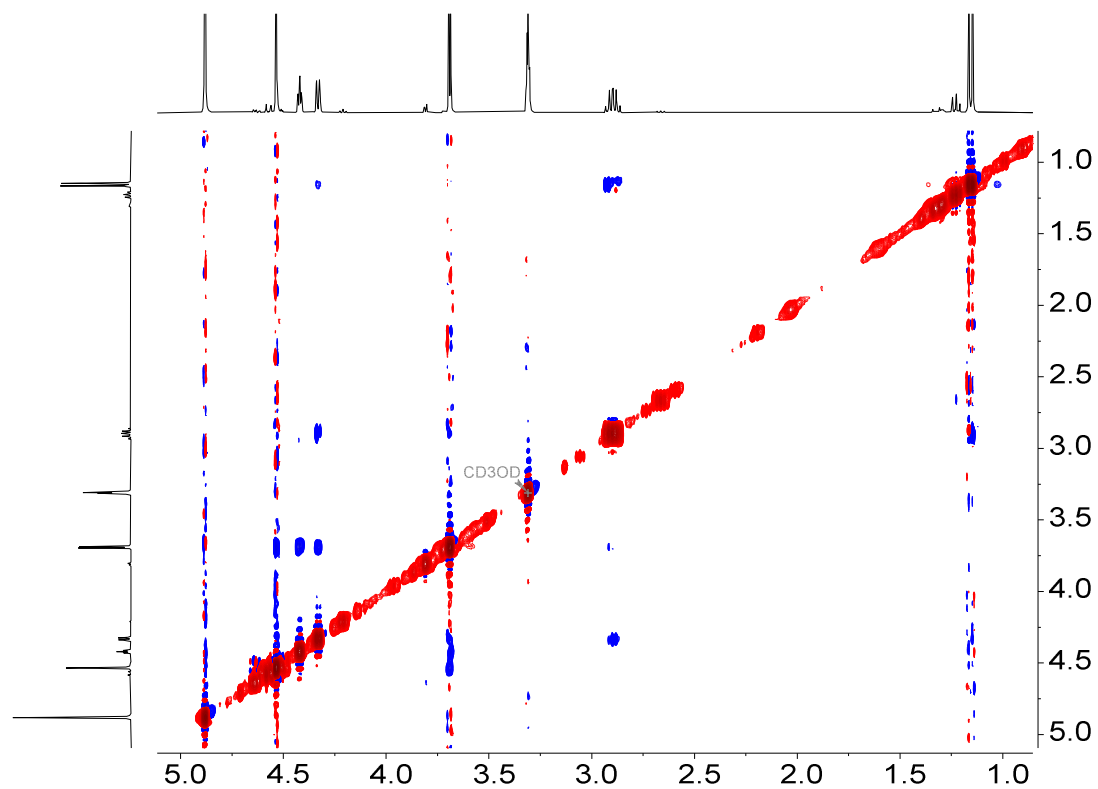
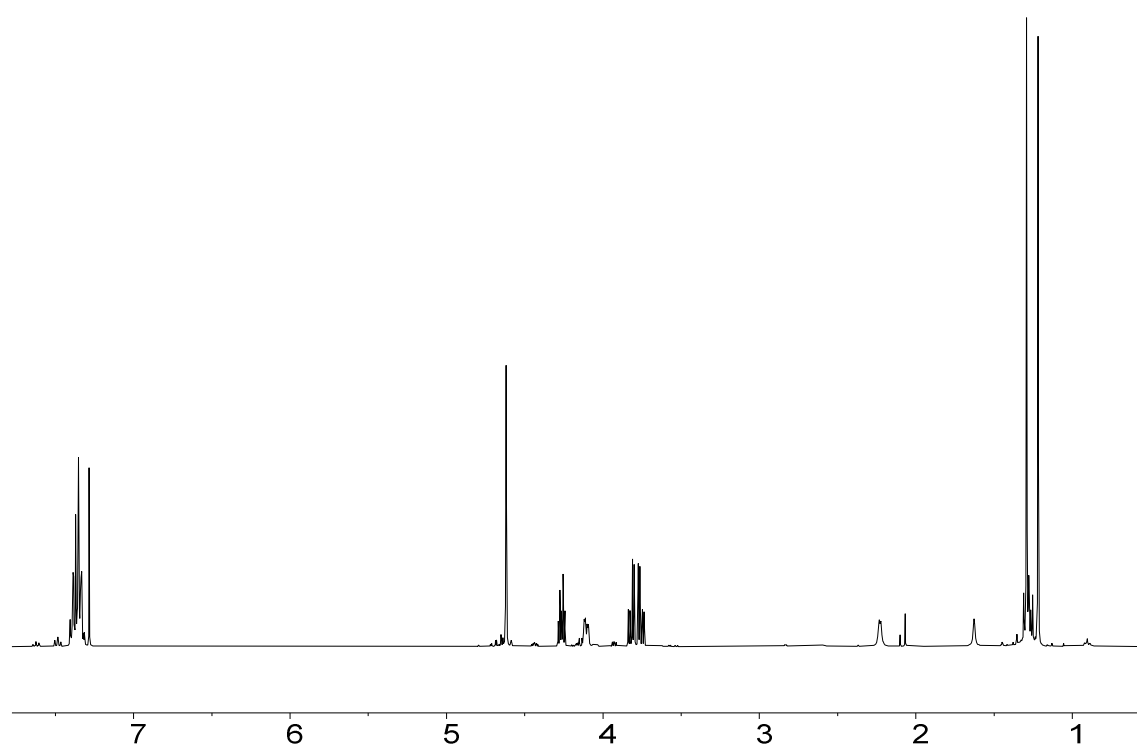
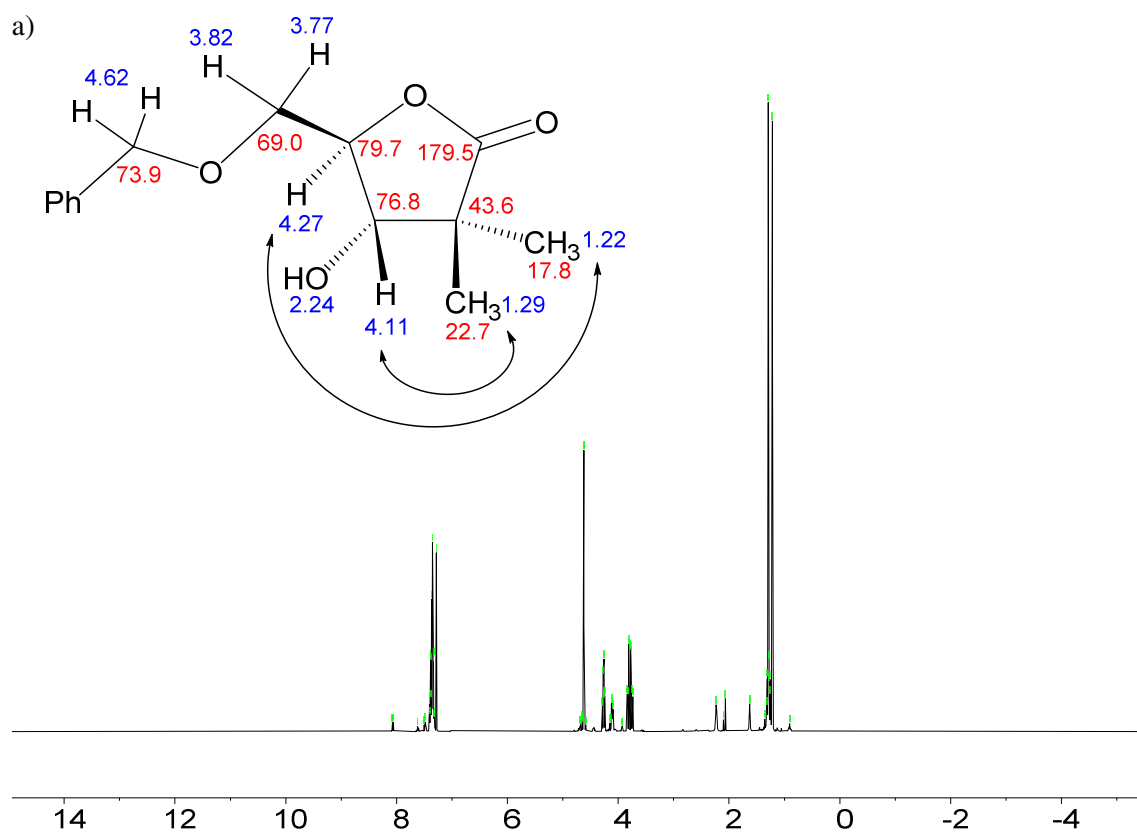
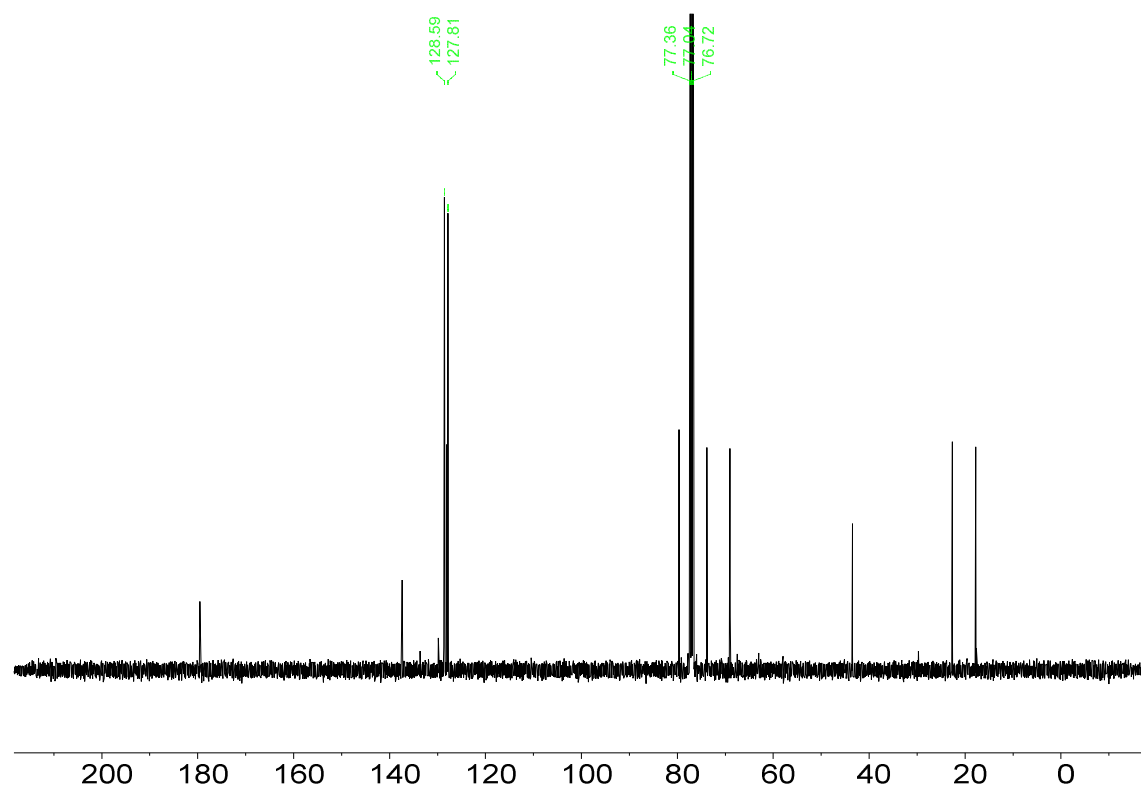


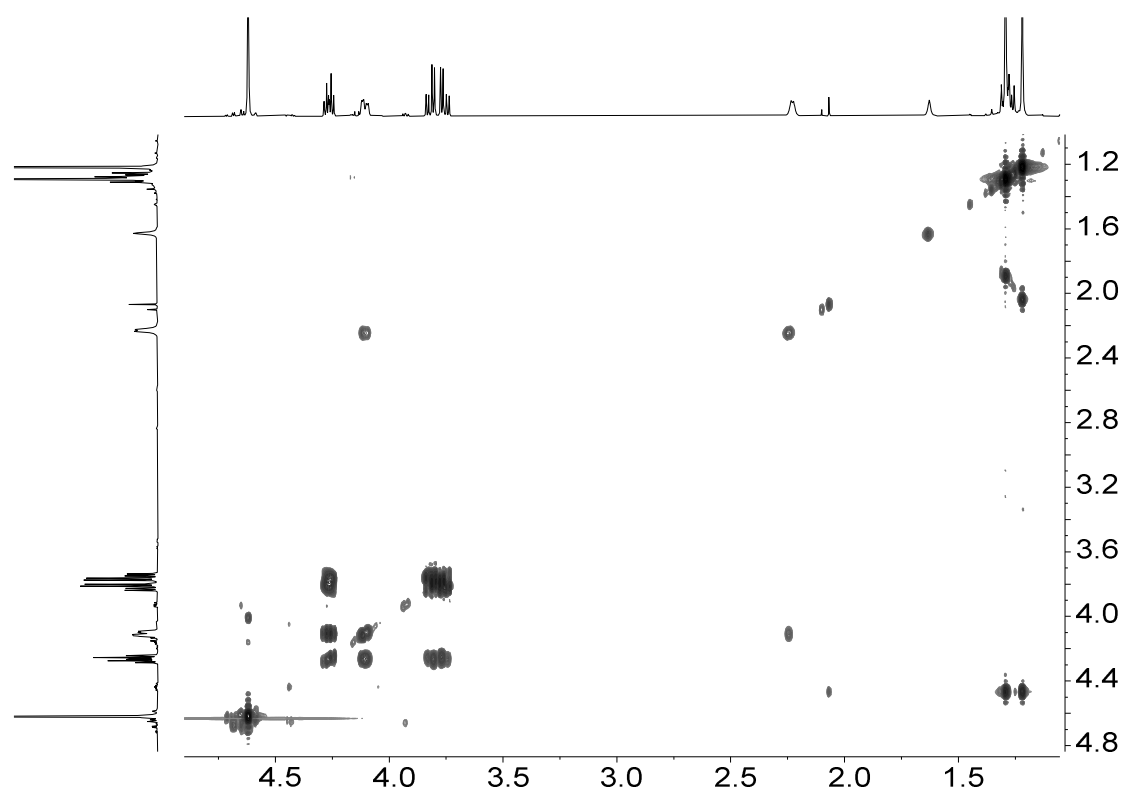
Figure S270 NMR spectra (MeOD) of **11a**: a) ^1H , b) ^{13}C , c) gHSQC d) 2D NOESY.



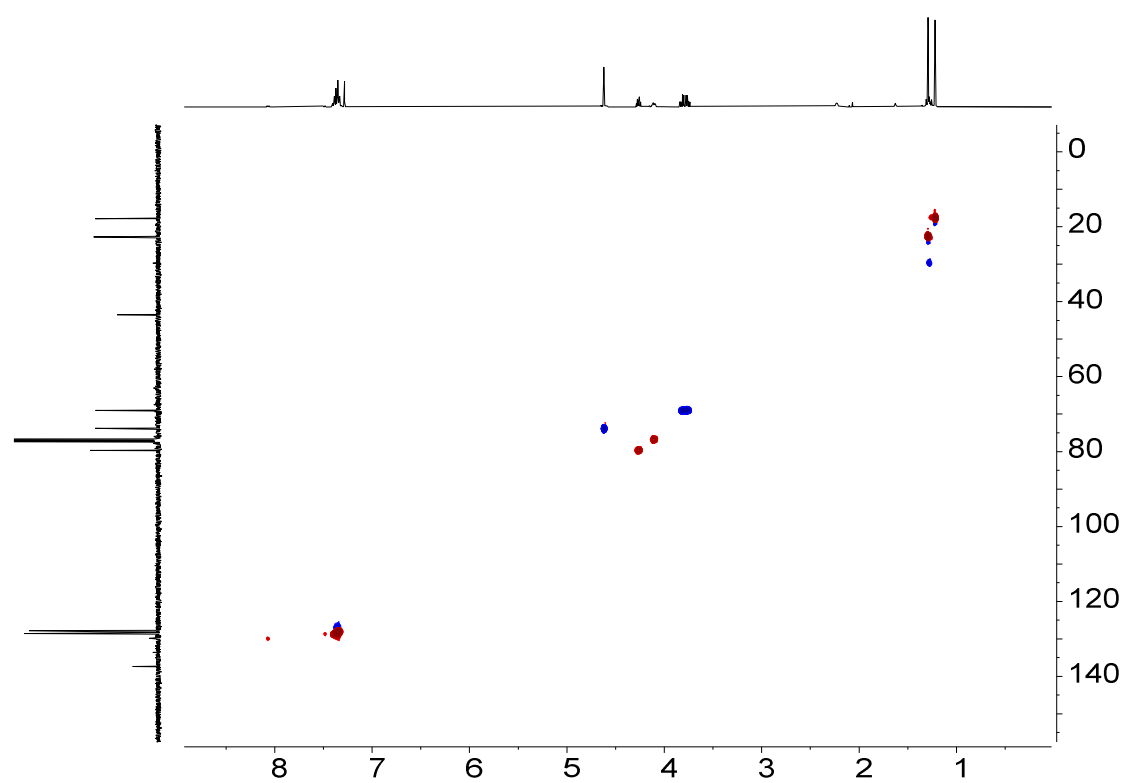
b)



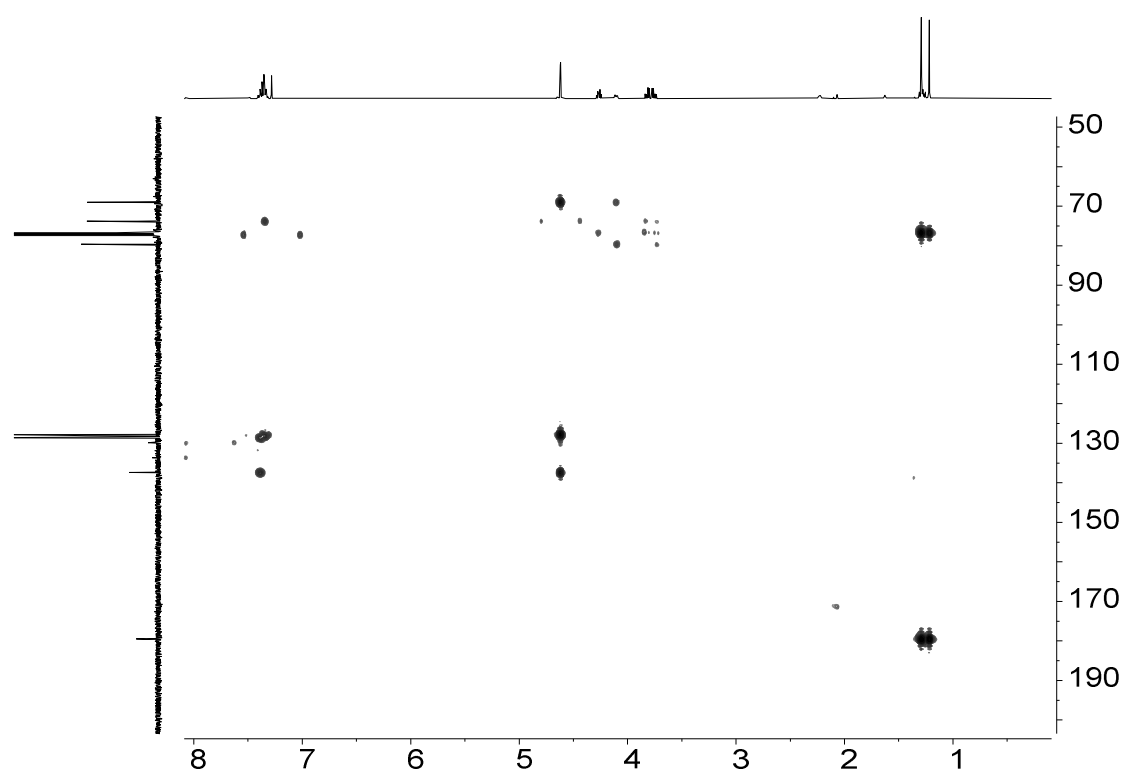
c)



d)



e)



f)

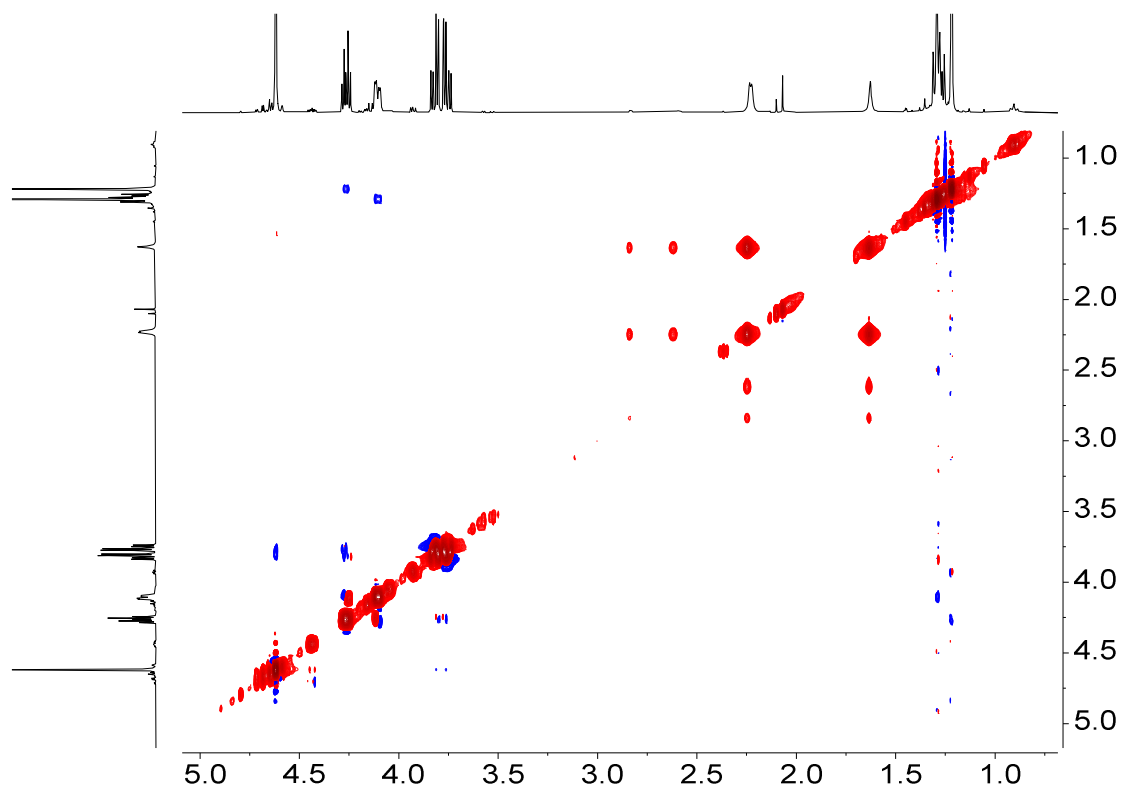
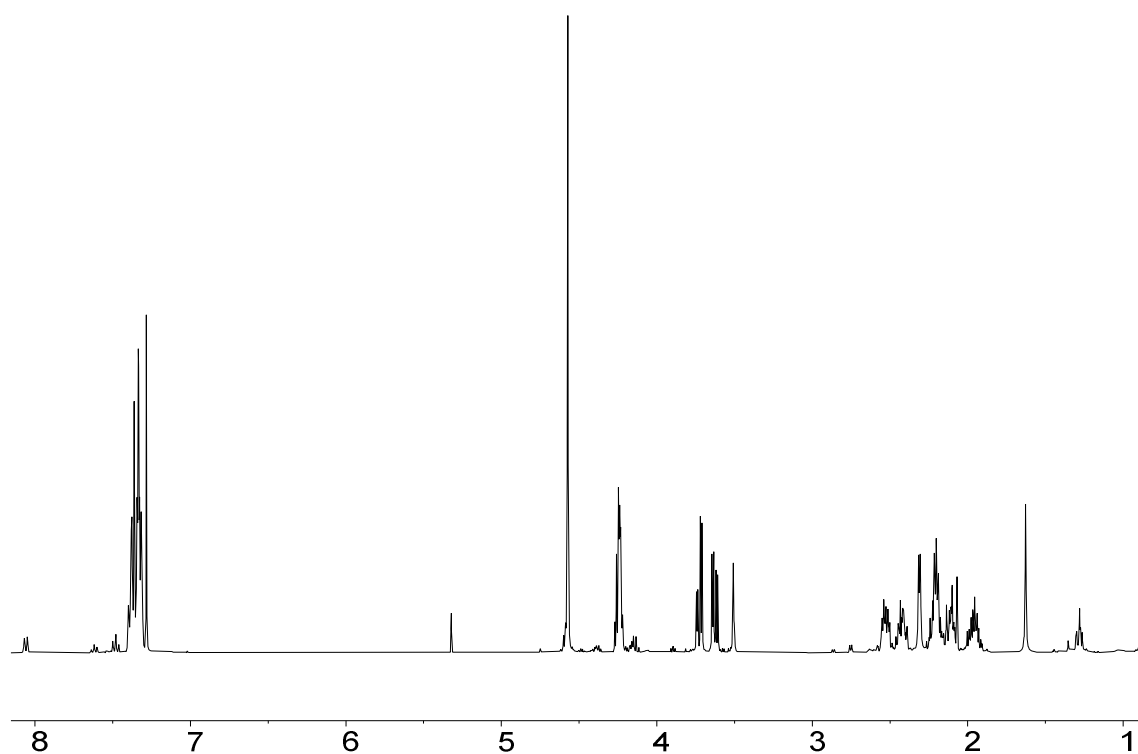
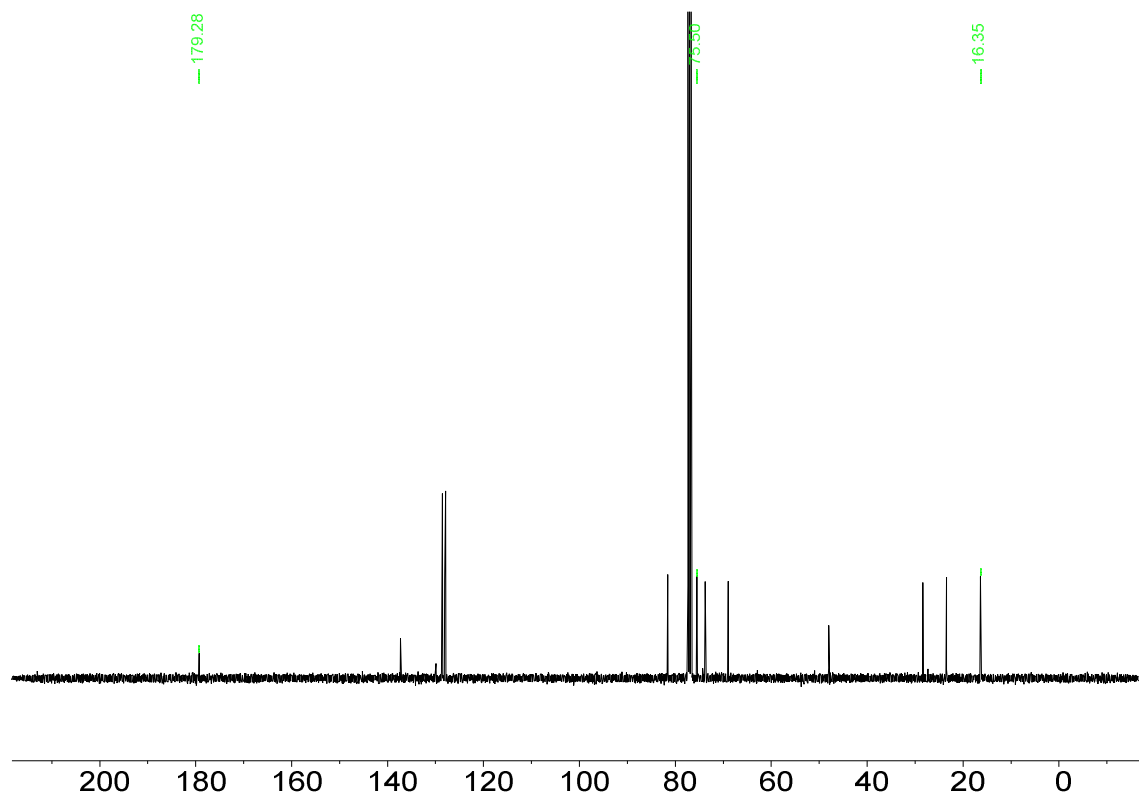


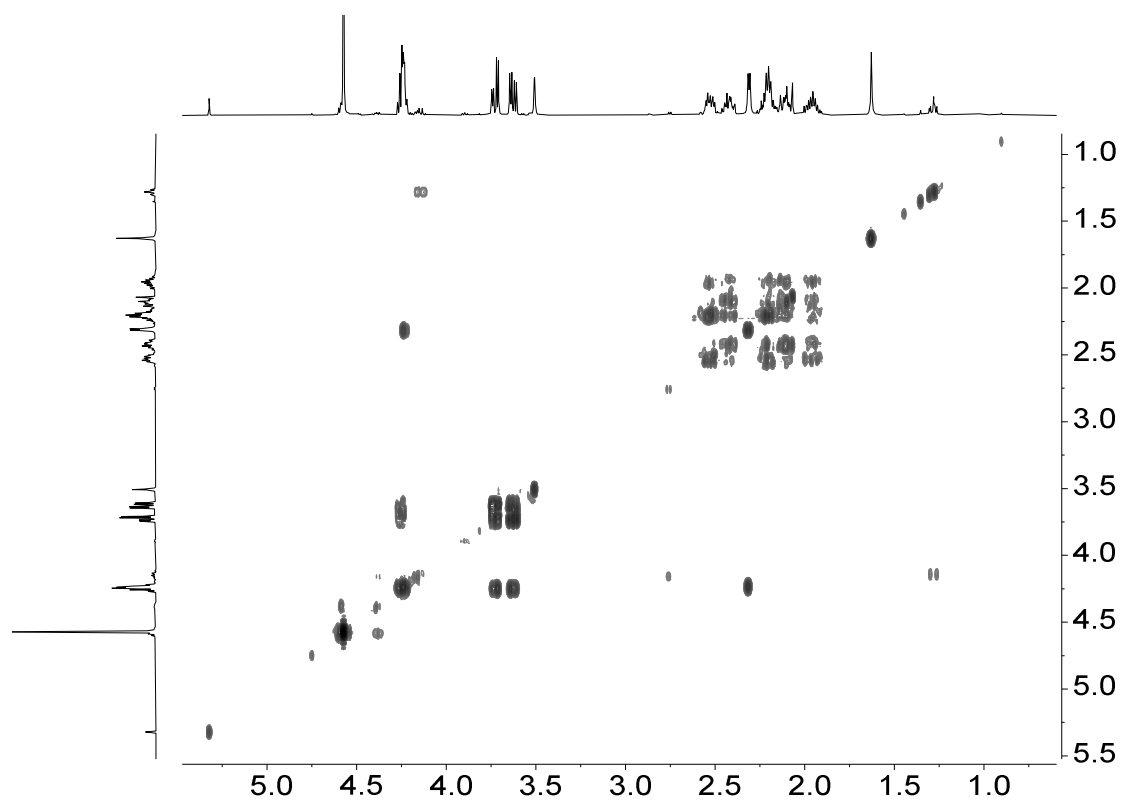
Figure S271 NMR spectra (CDCl_3) of **11b**: a) ^1H , b) ^{13}C , c) gCOSY, d) gHSQC e) HMBC and f) 2D NOESY.



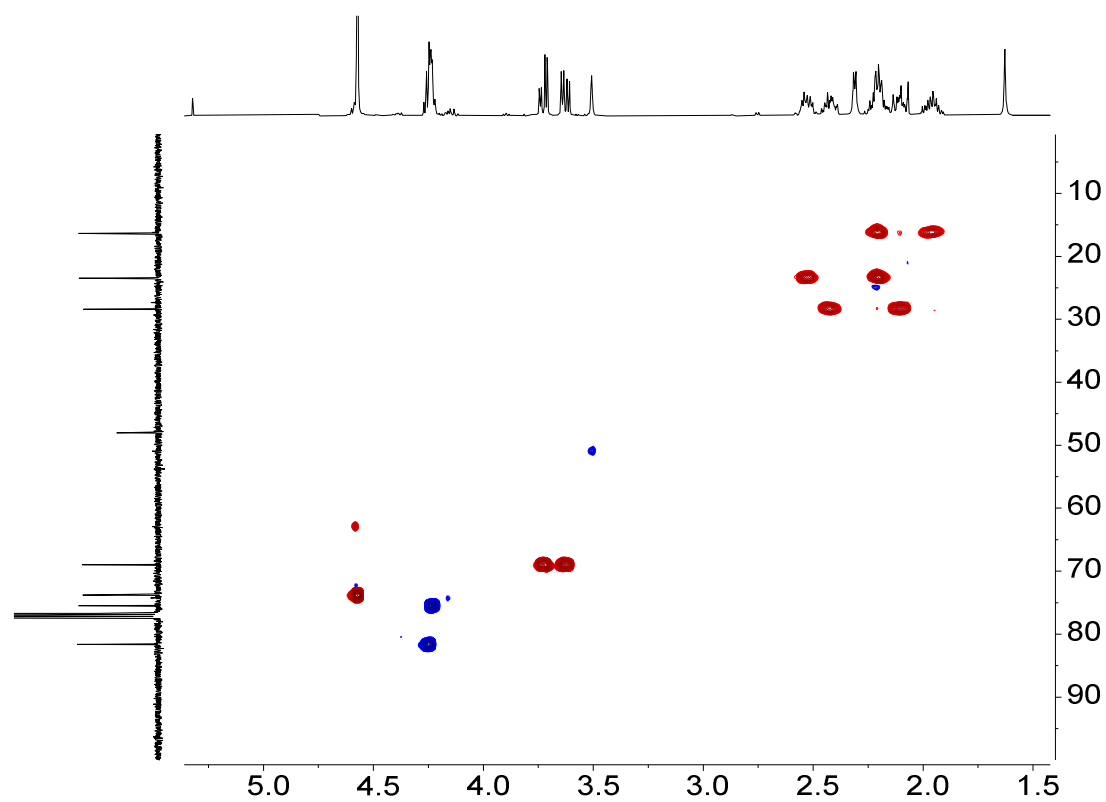
b)



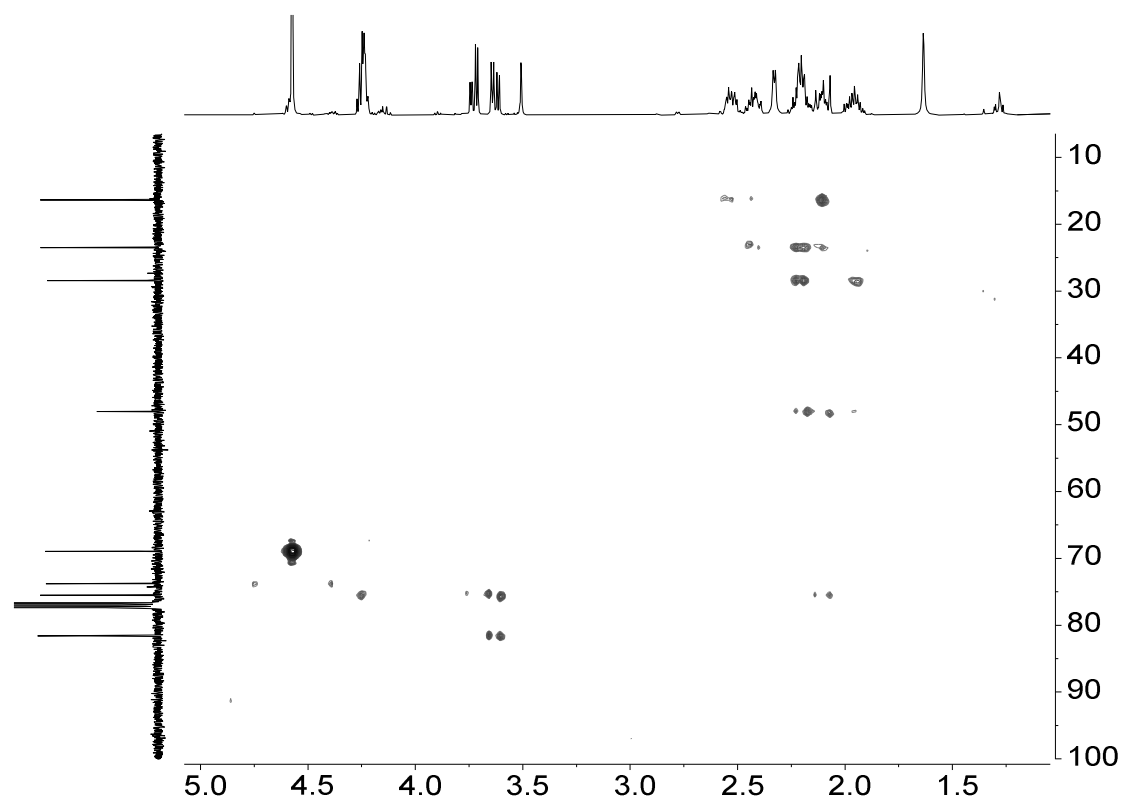
c)



d)



e)



f)

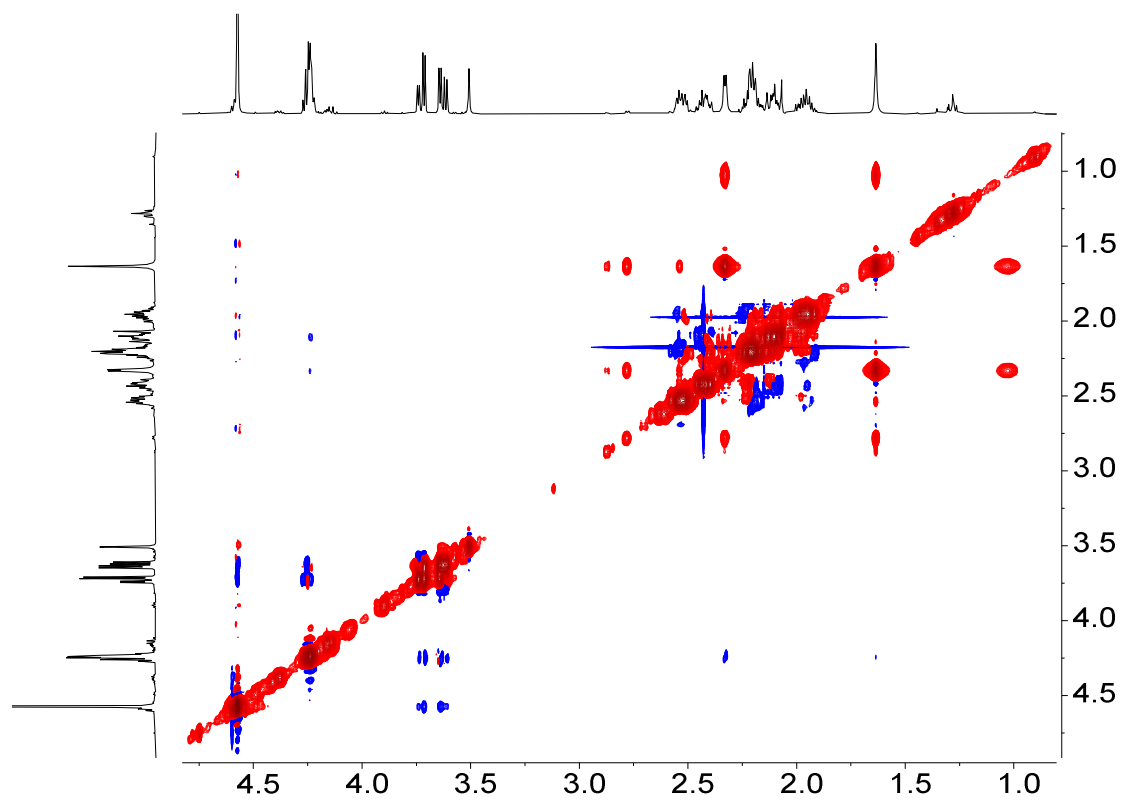
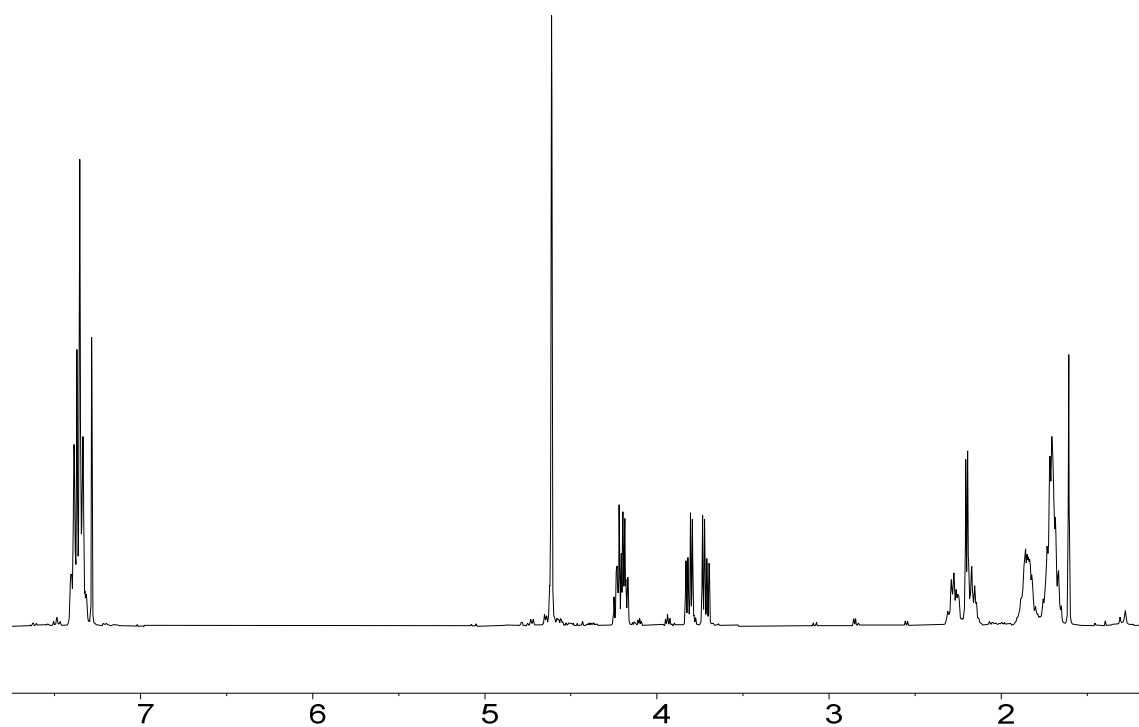
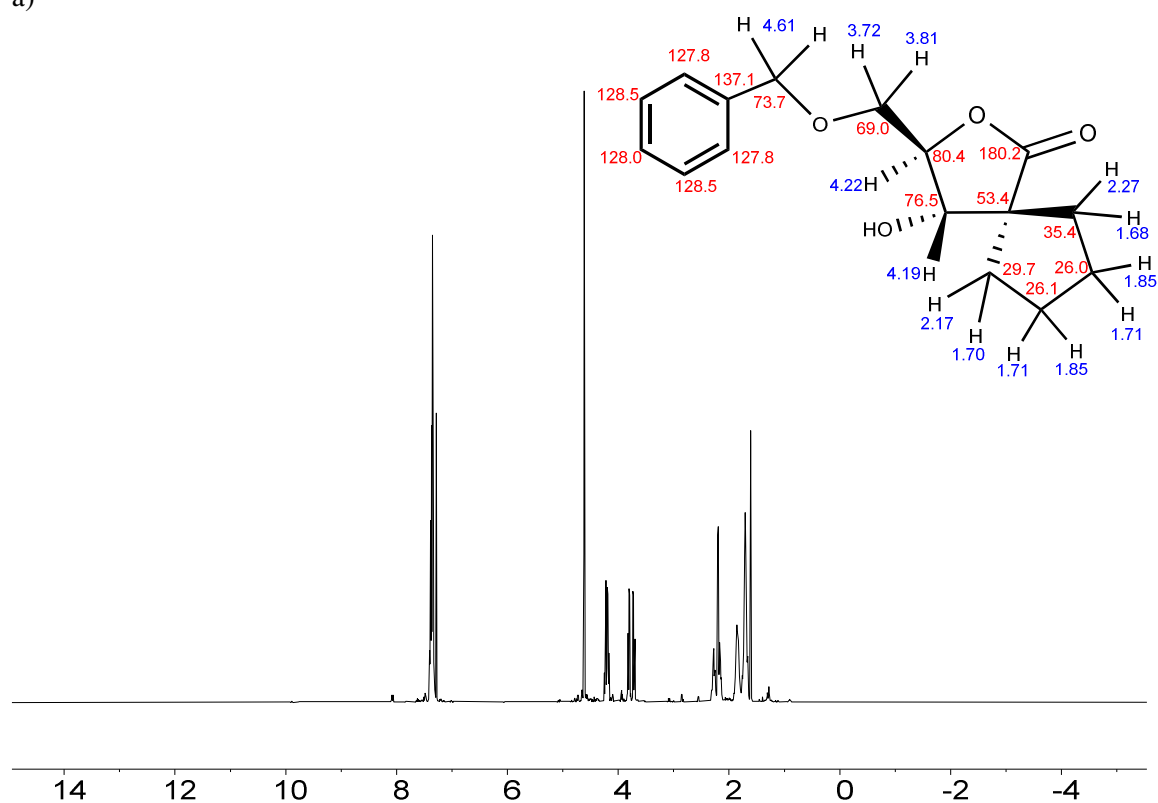
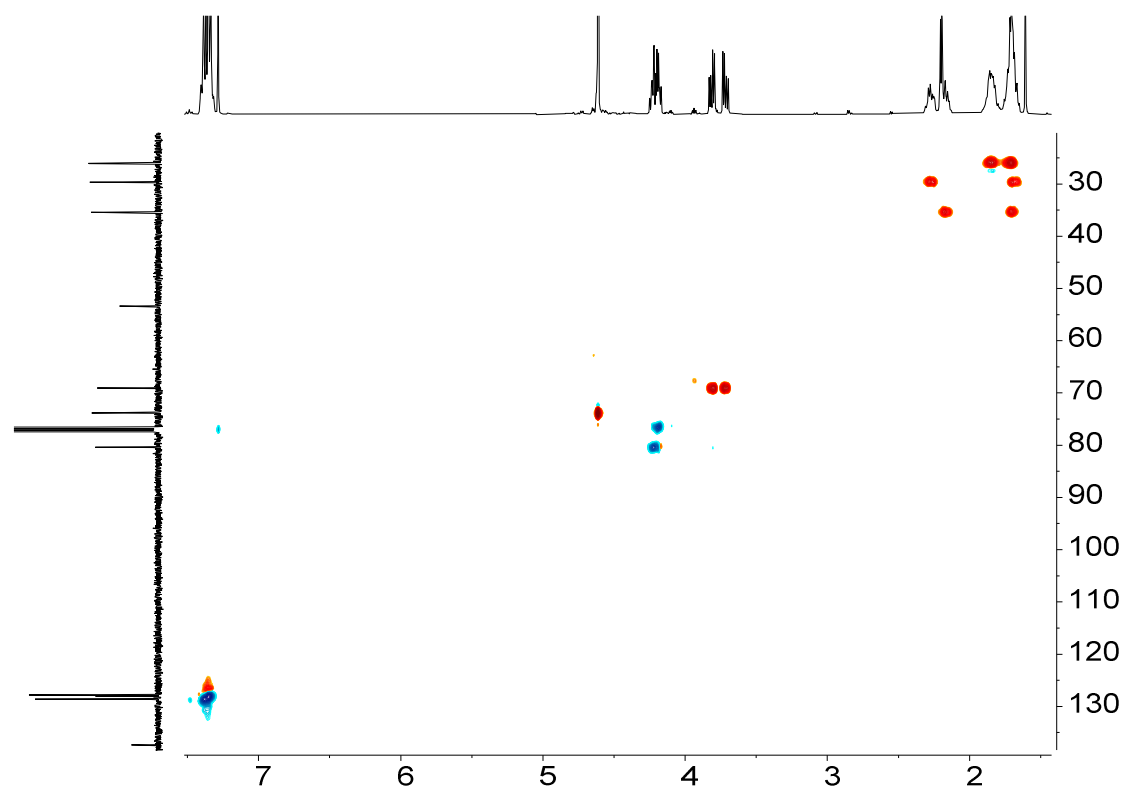


Figure S272 NMR spectra (CDCl₃) of **11c**: a) ¹H, b) ¹³C, c) gCOSY, d) gHSQC e) HMBC and f) 2D NOESY.

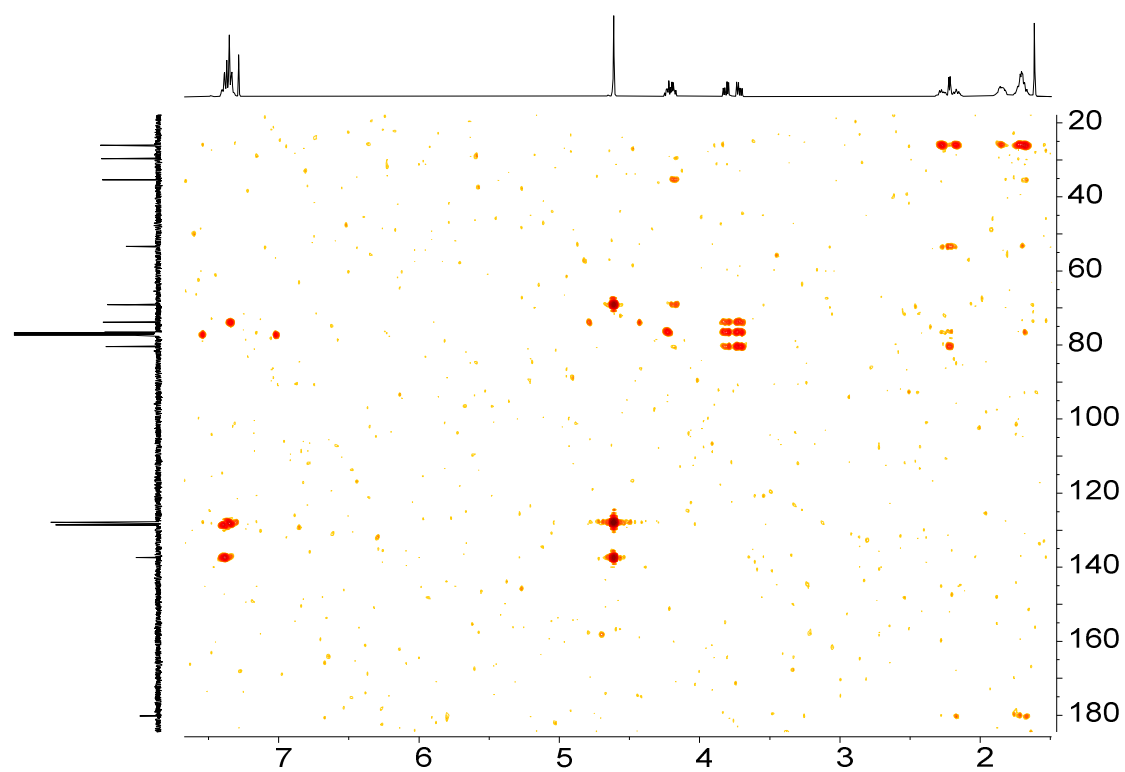
a)



d)



e)



f)

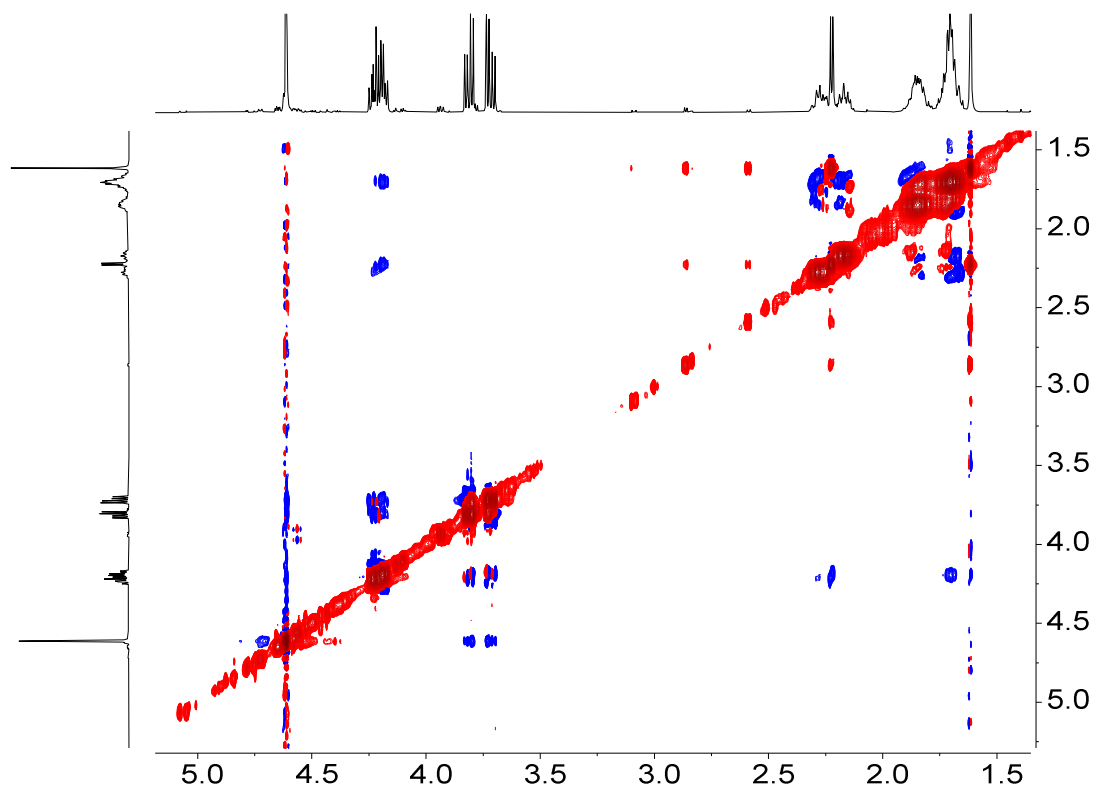
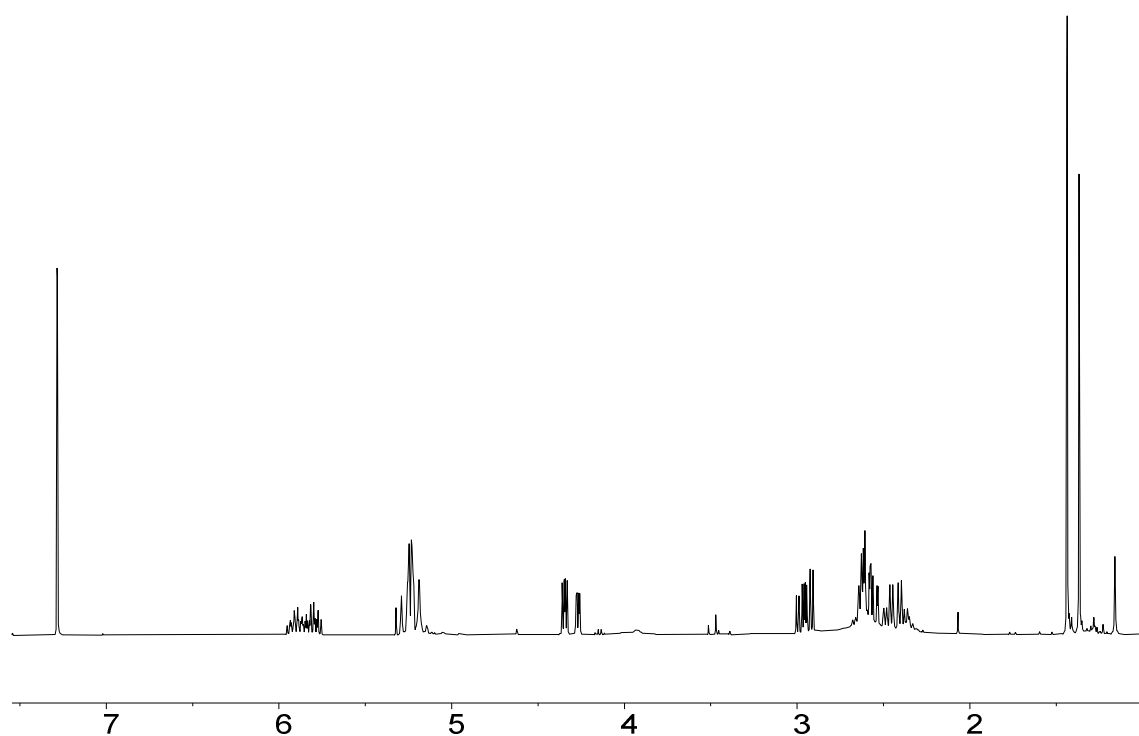
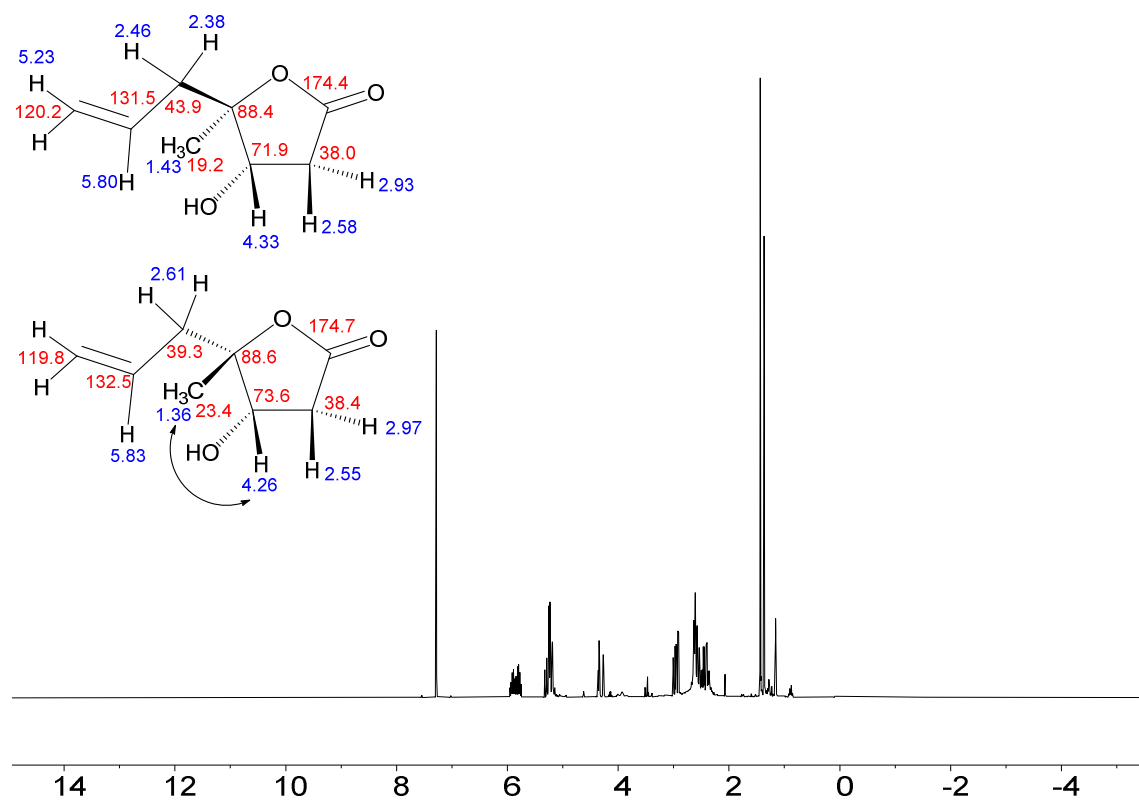
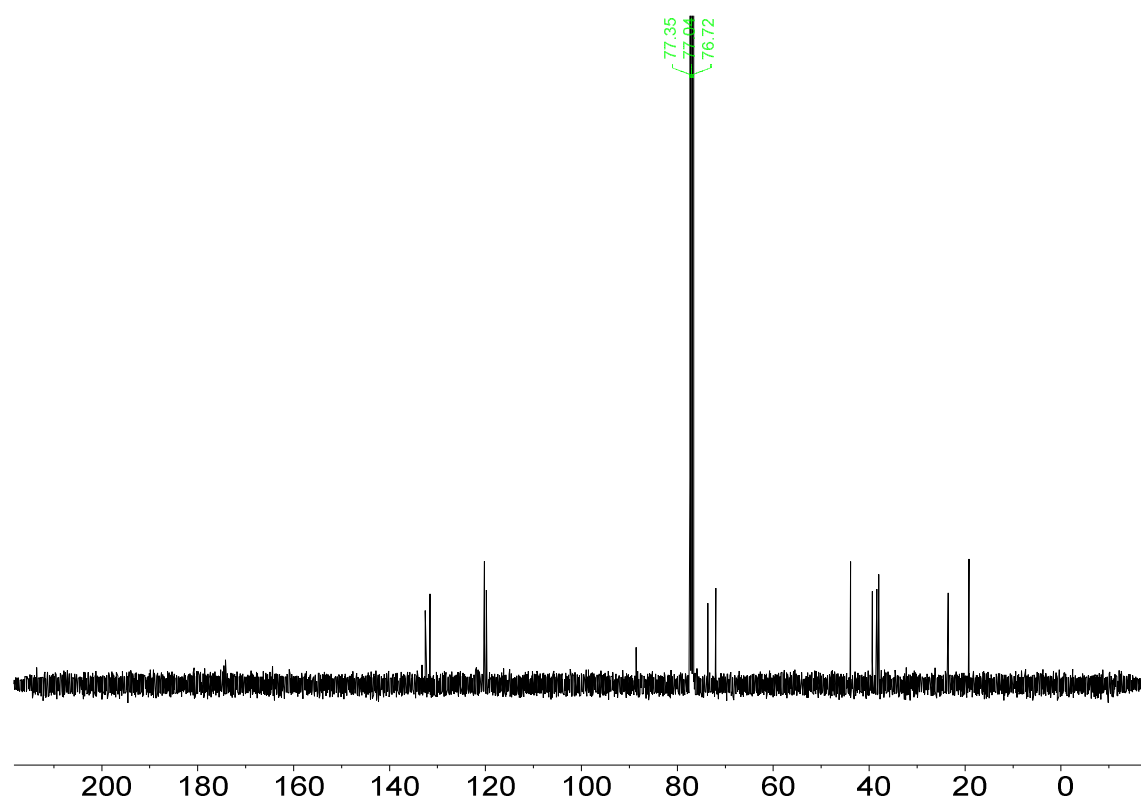


Figure S273 NMR spectra (CDCl_3) of **11d**: a) ^1H , b) ^{13}C , c) gCOSY, d) gHSQC e) HMBC and f) 2D NOESY.

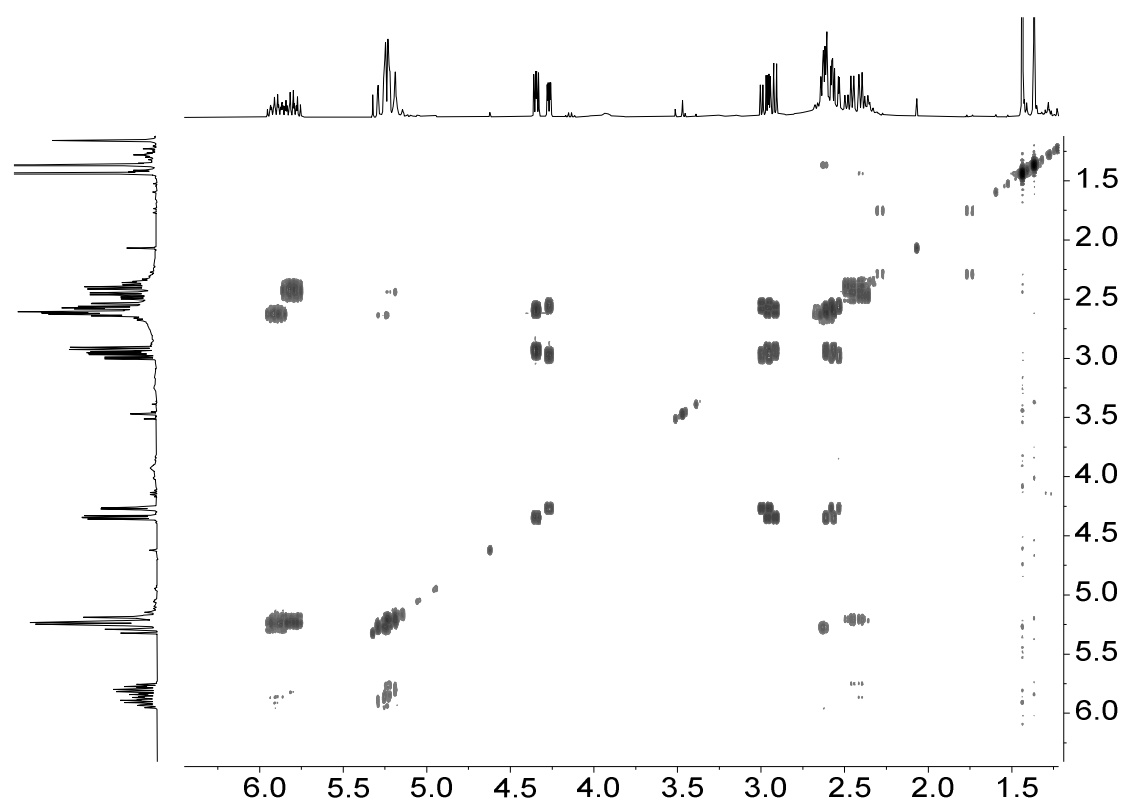
a)



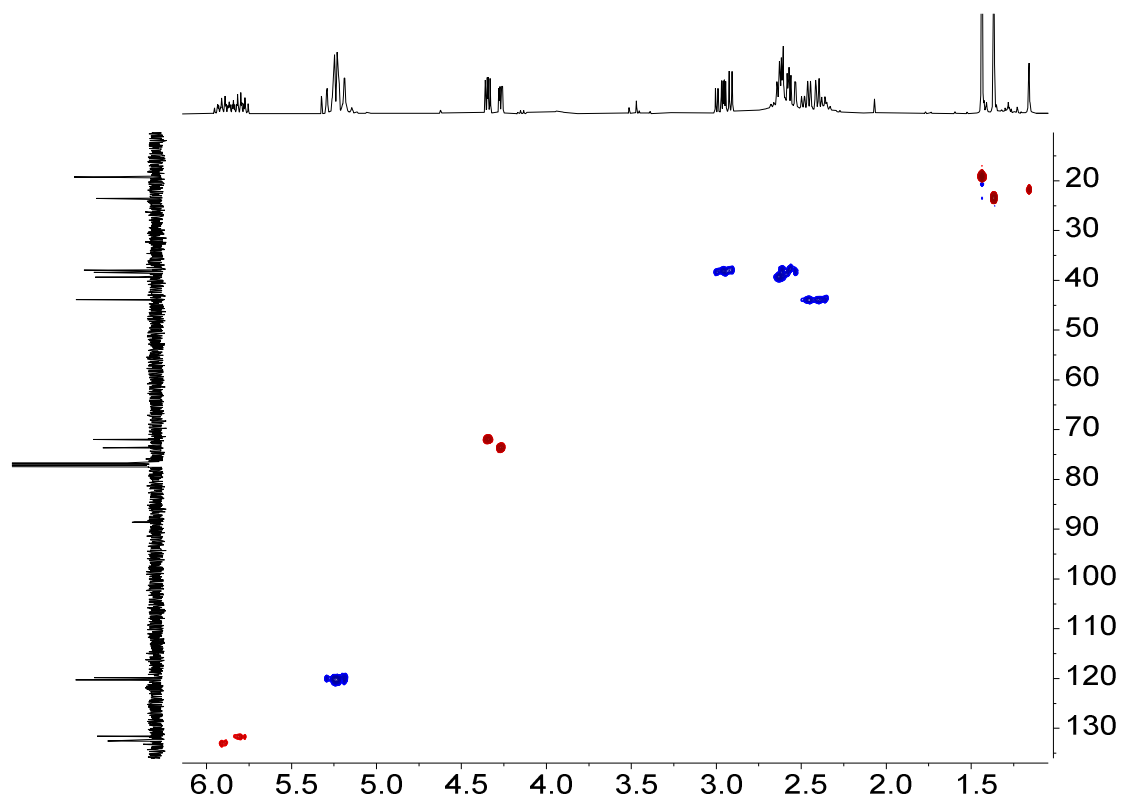
b)



c)



d)



e)

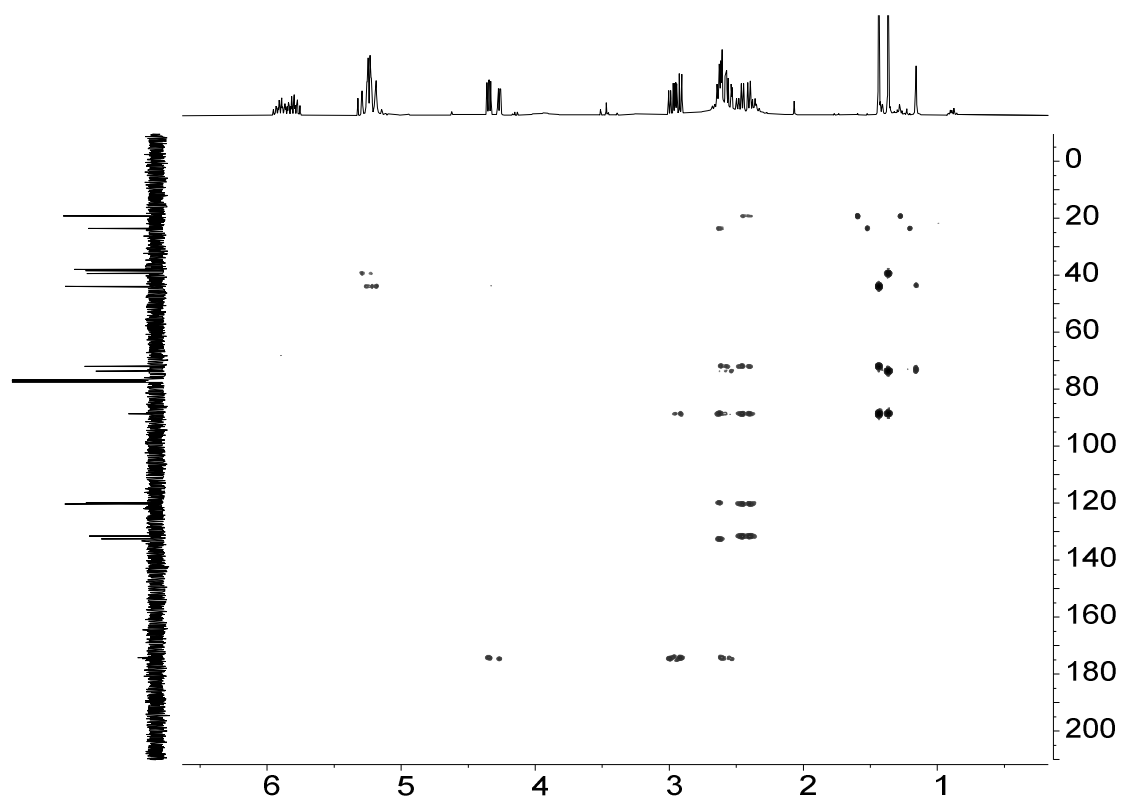
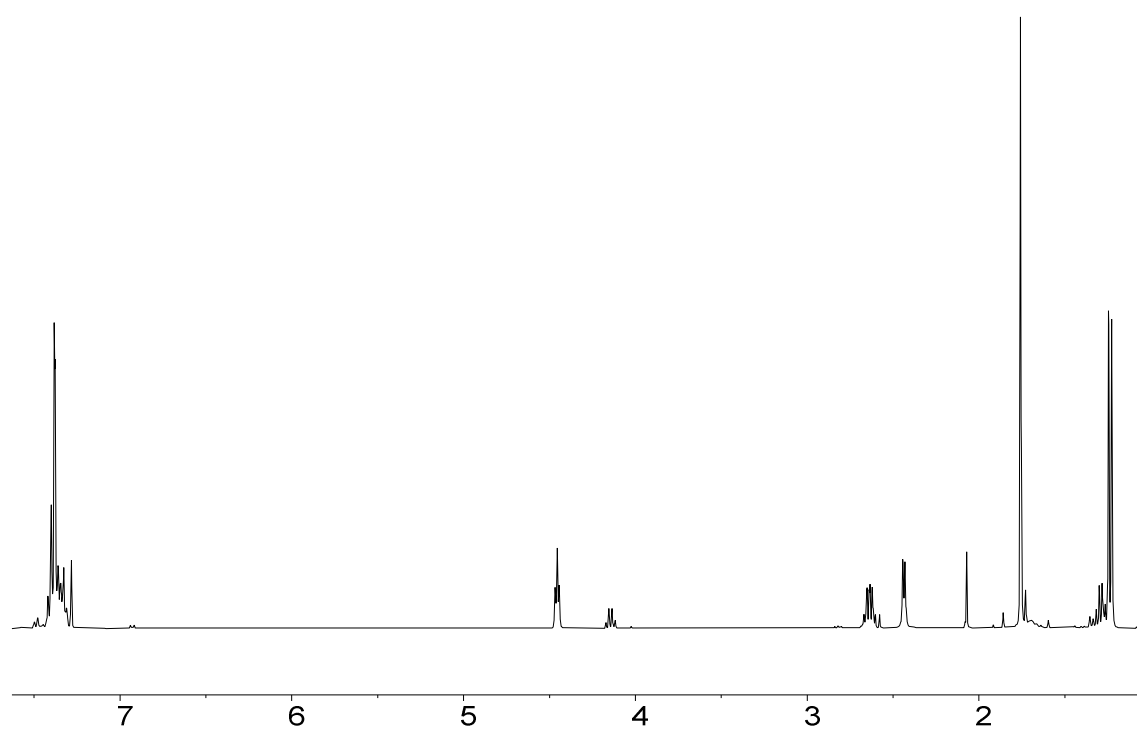
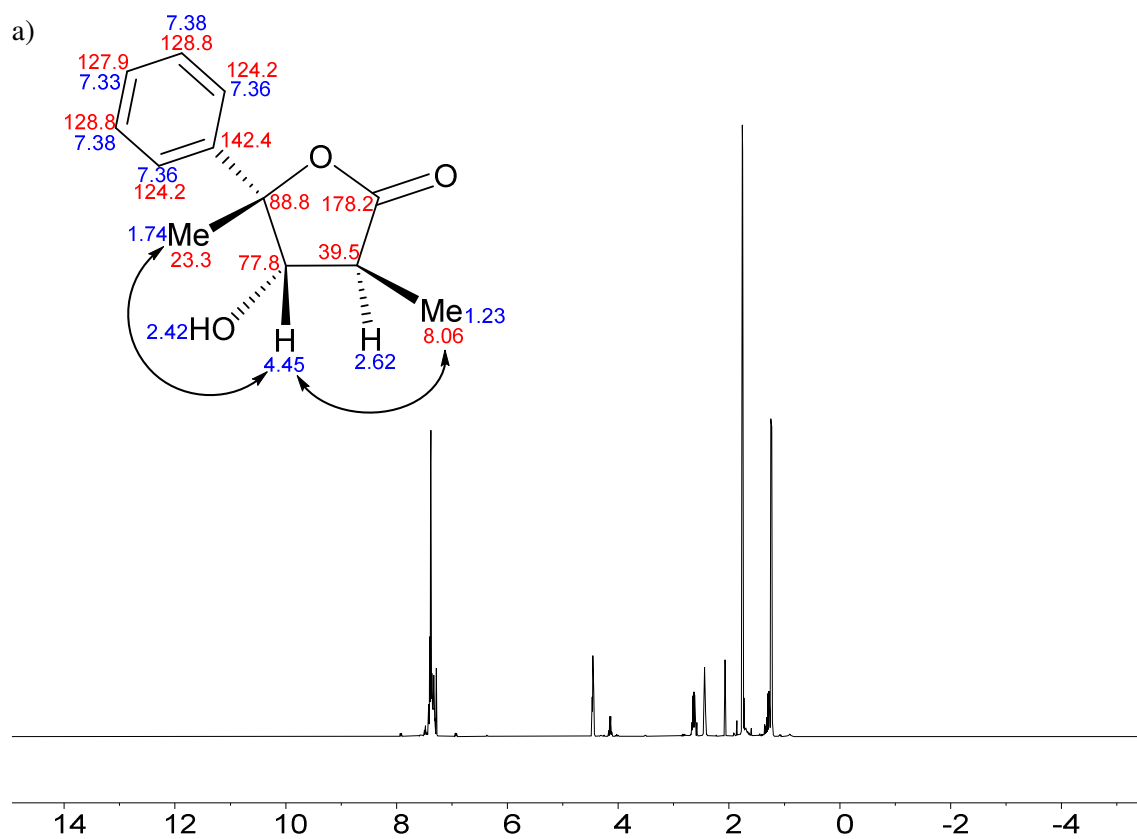
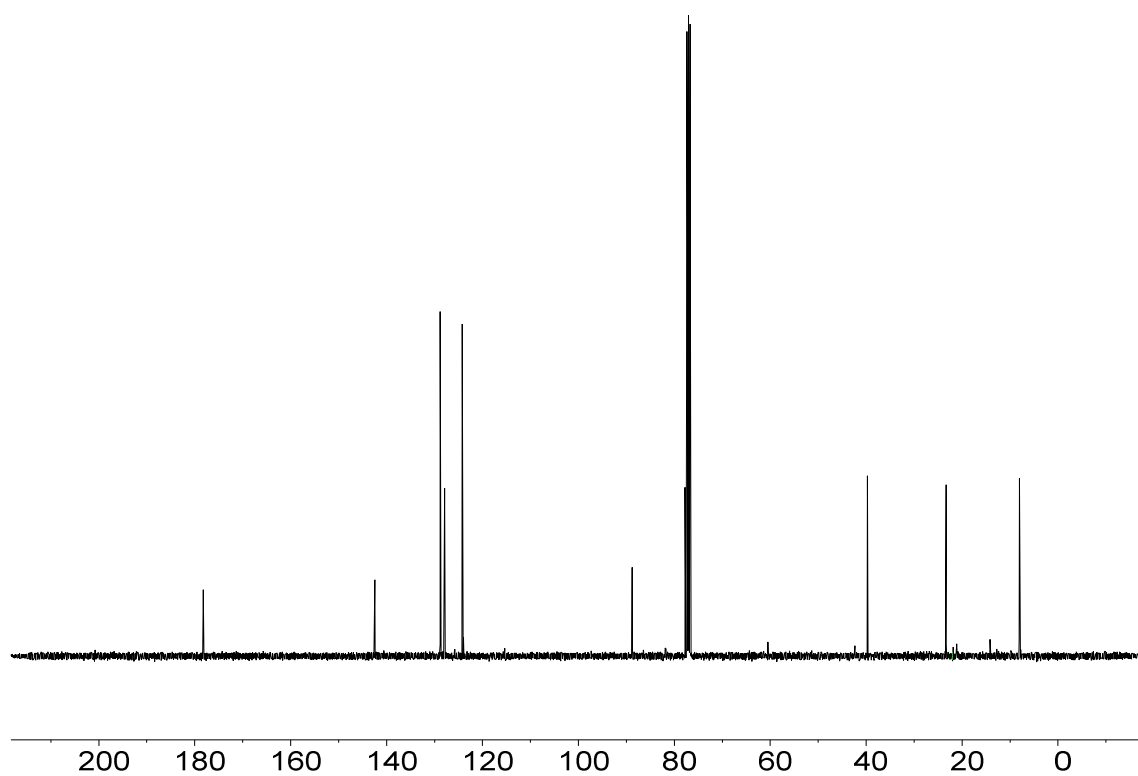


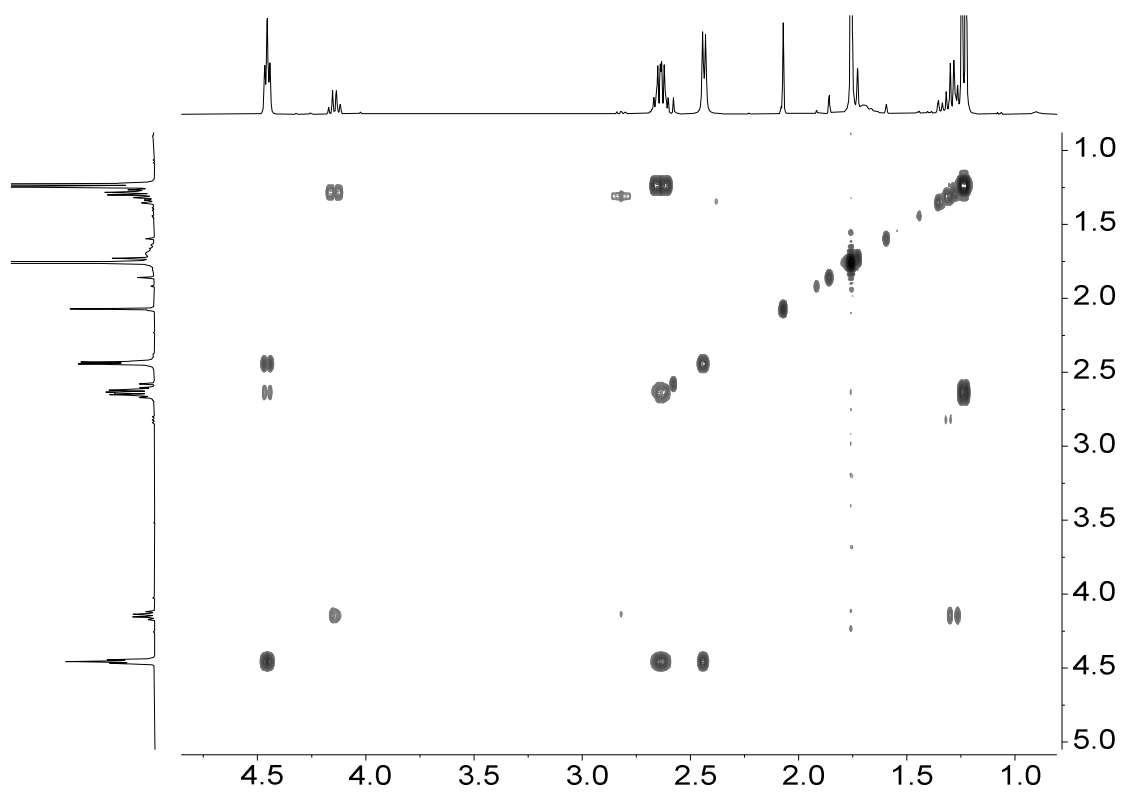
Figure S274 NMR spectra (CDCl_3) of **12a**: a) ^1H , b) ^{13}C , c) gCOSY, d) gHSQC e) HMBC.



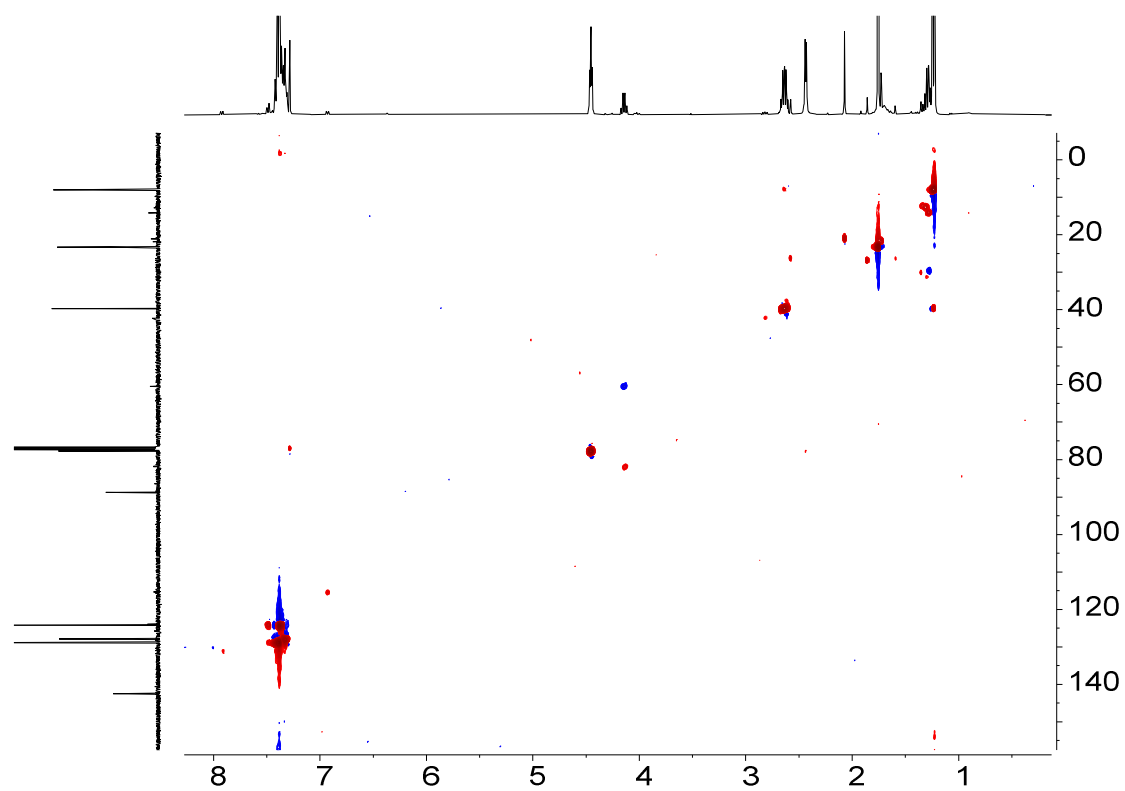
b)



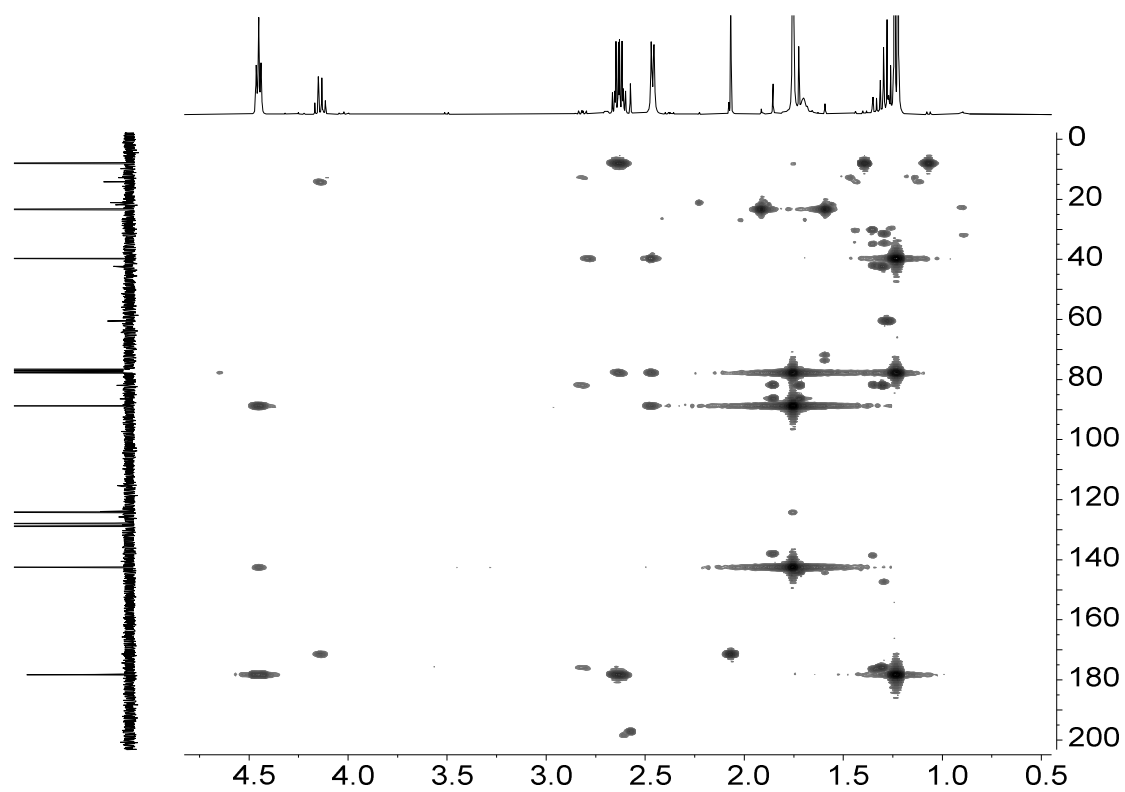
c)



d)



e)



f)

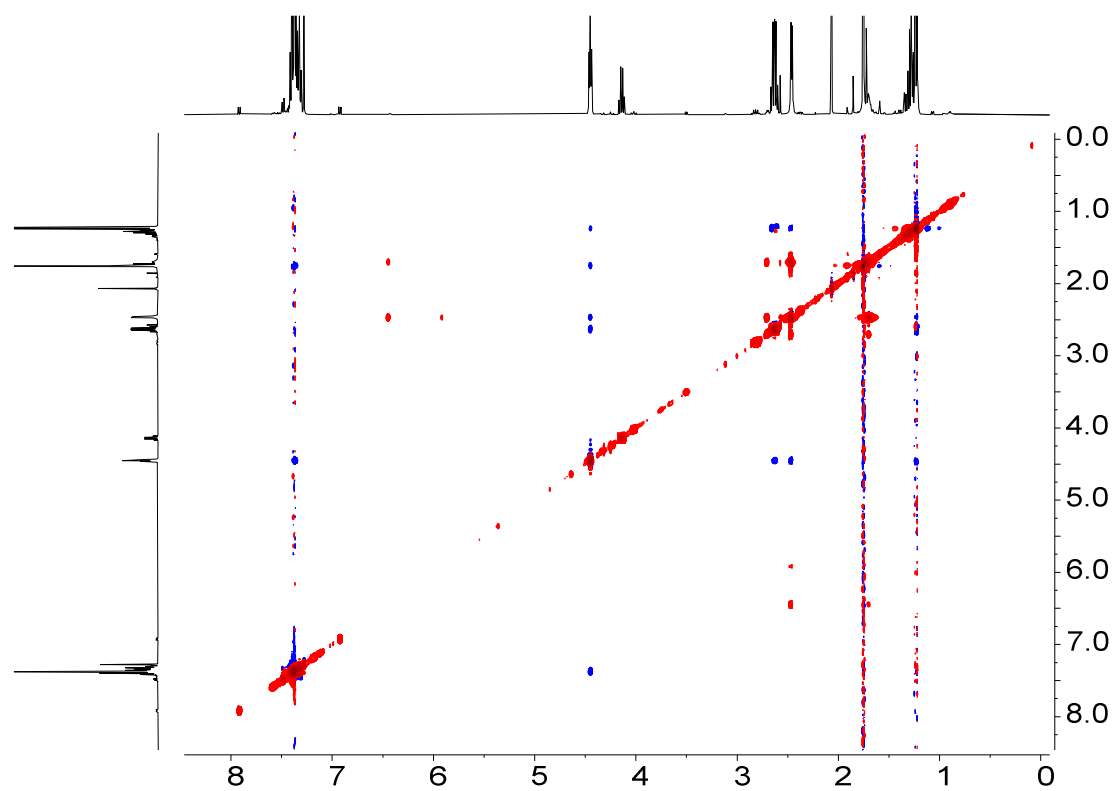
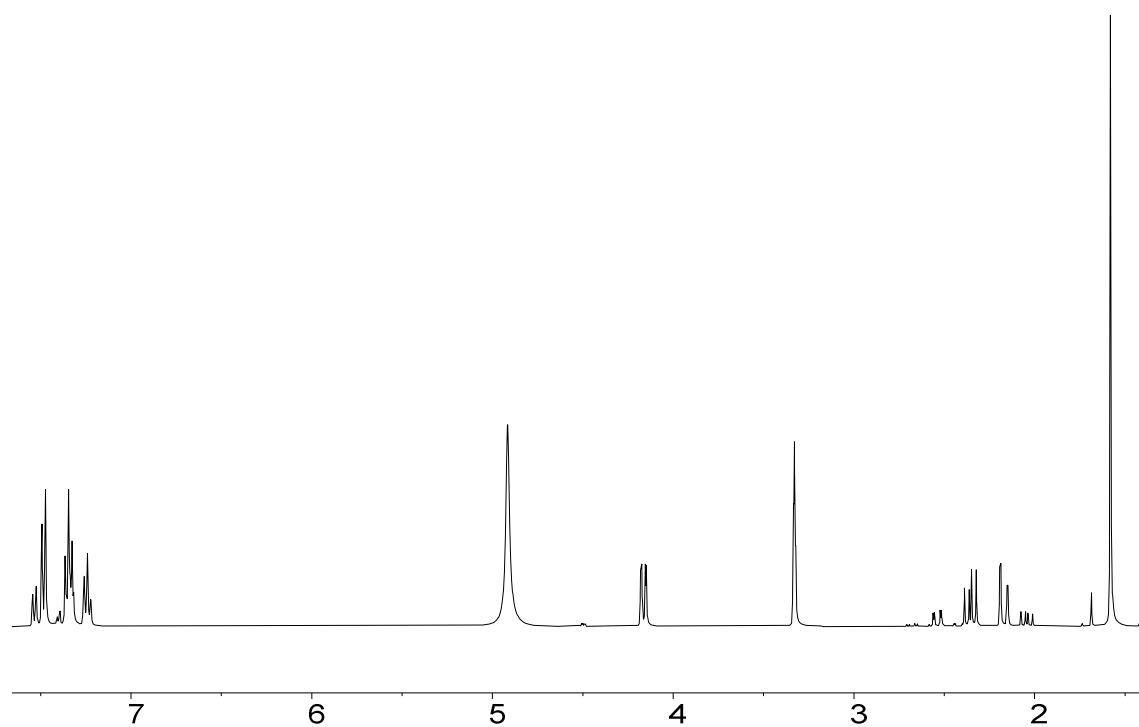
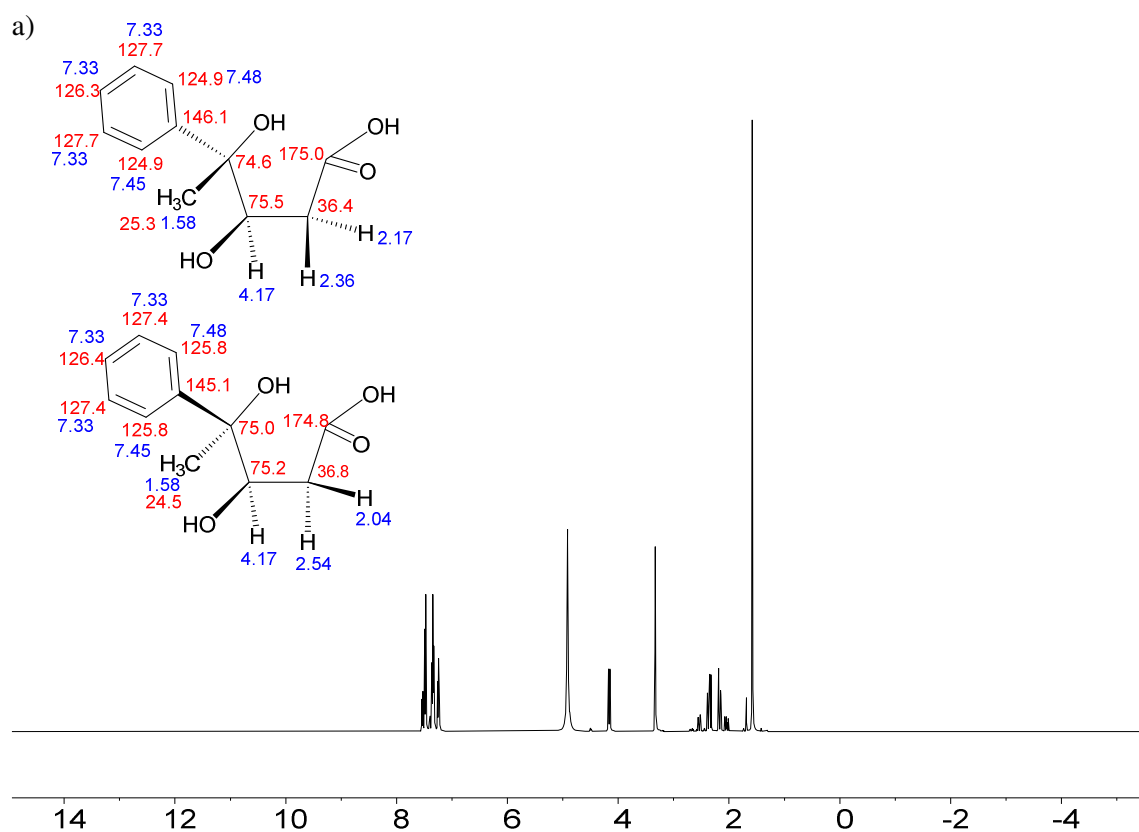
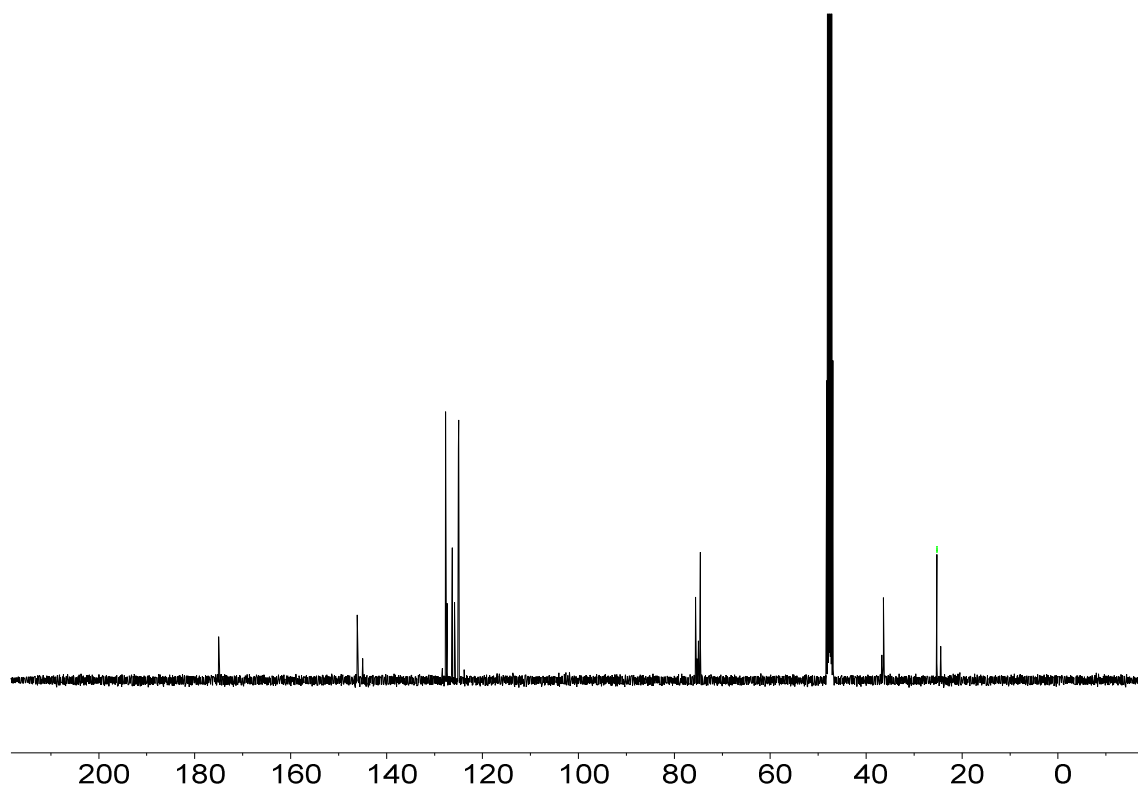


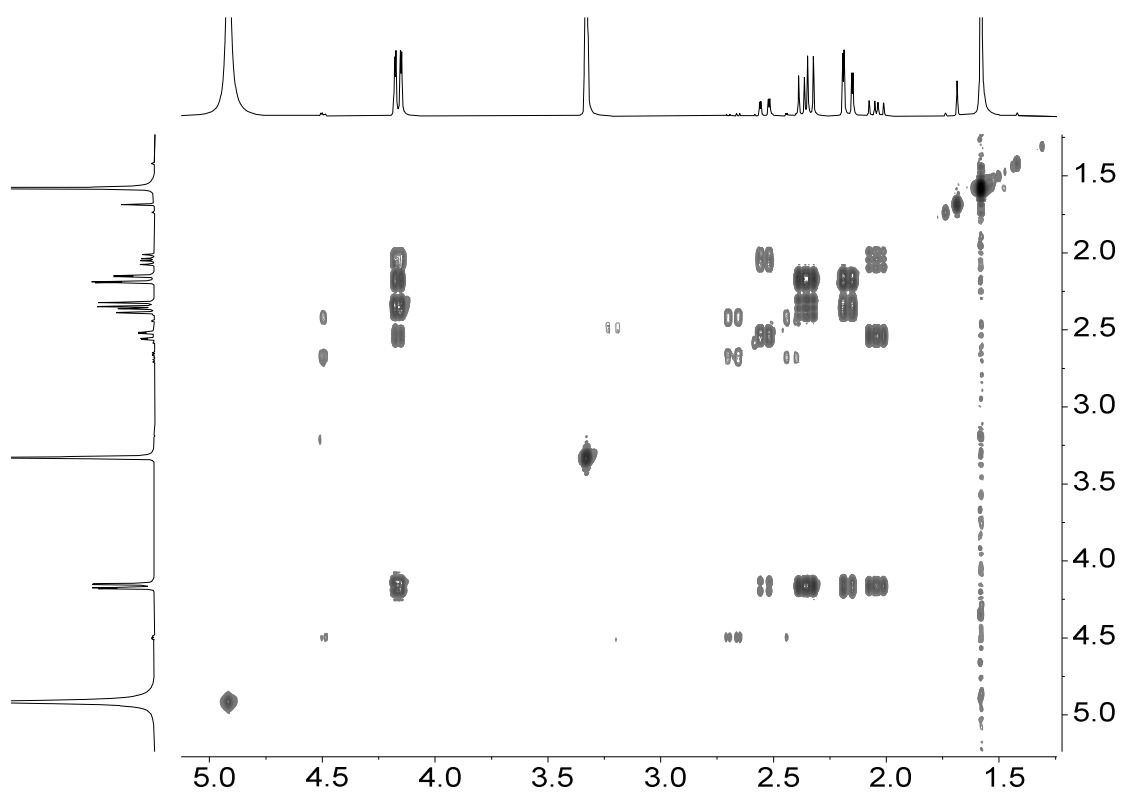
Figure S275 NMR spectra (CDCl₃) of **12b**: a) ¹H, b) ¹³C, c) gCOSY, d) gHSQC e) HMBC and f) 2D NOESY.



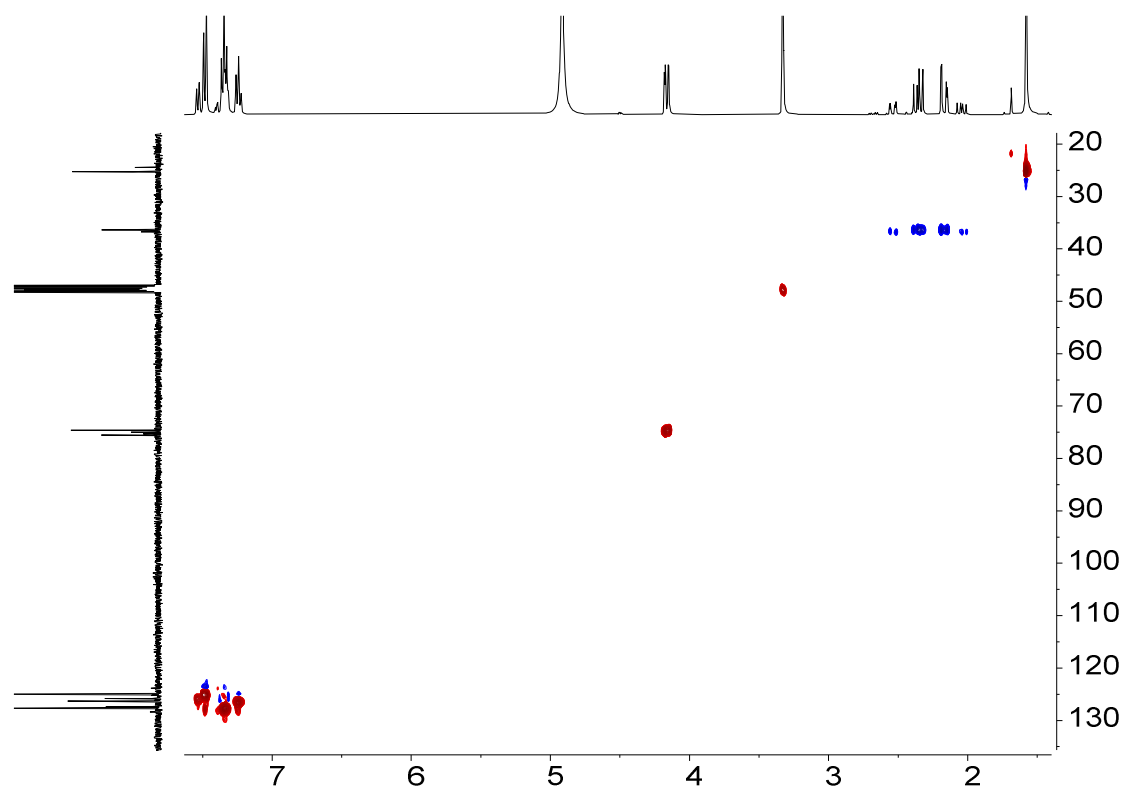
b)



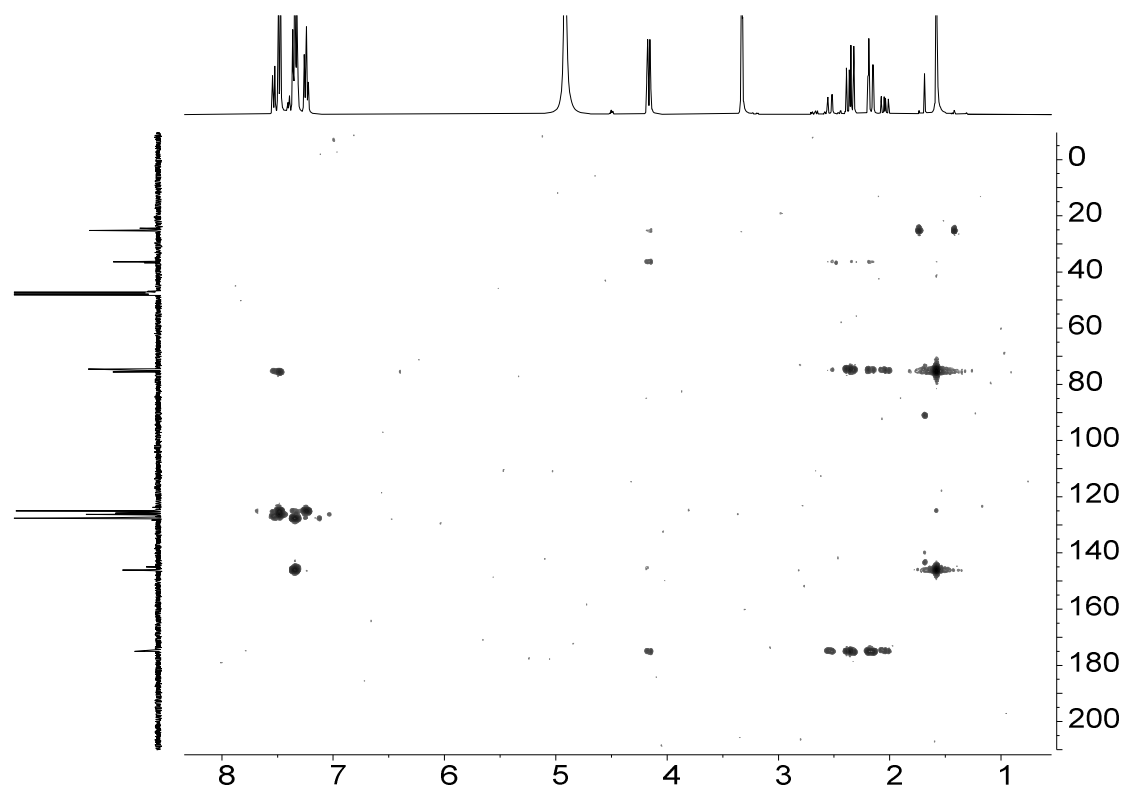
c)



d)



e)



f)

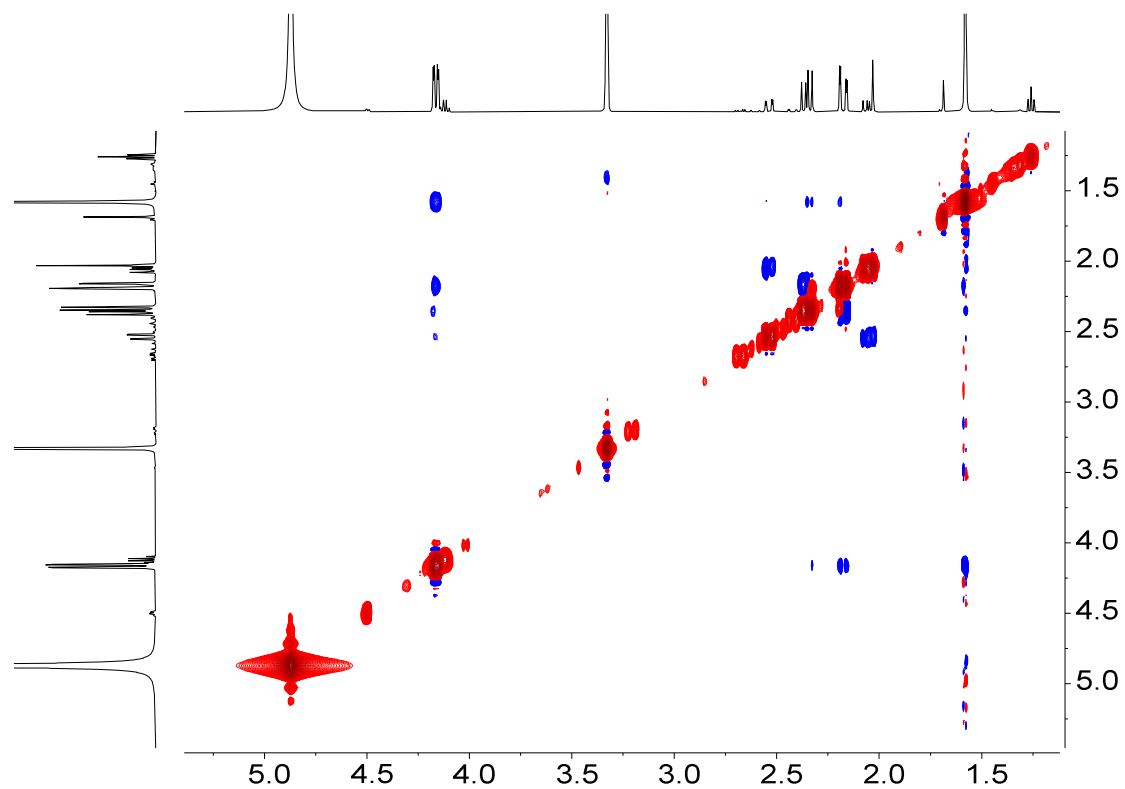
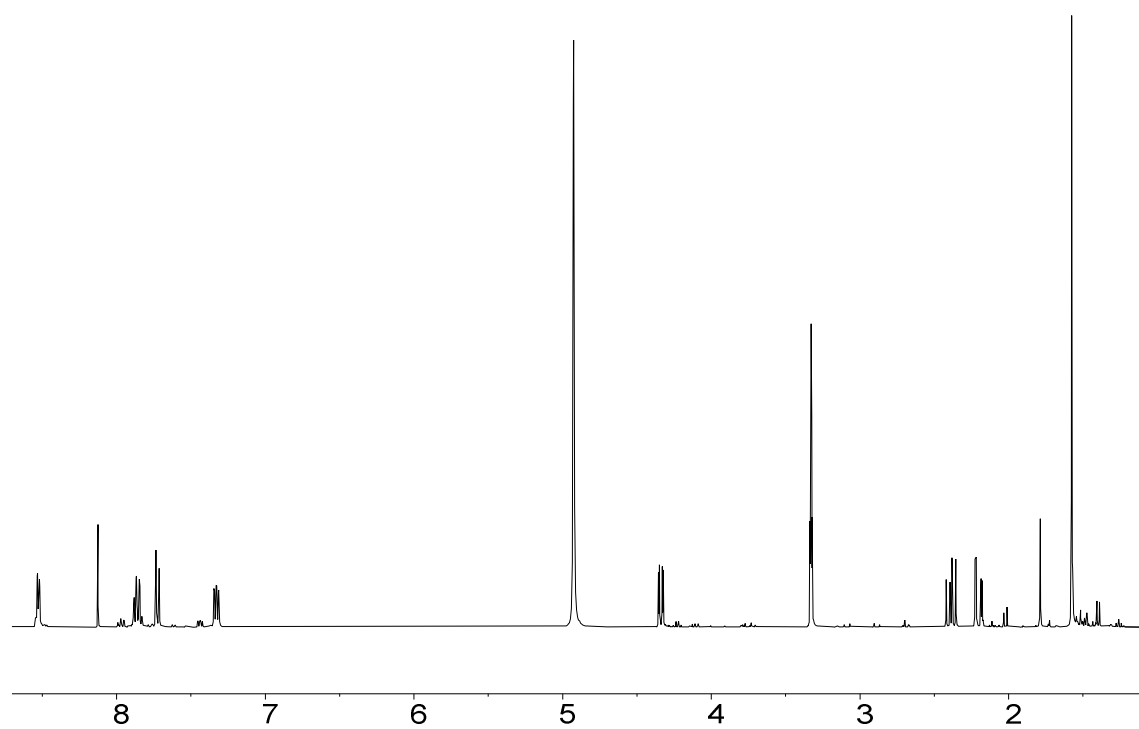
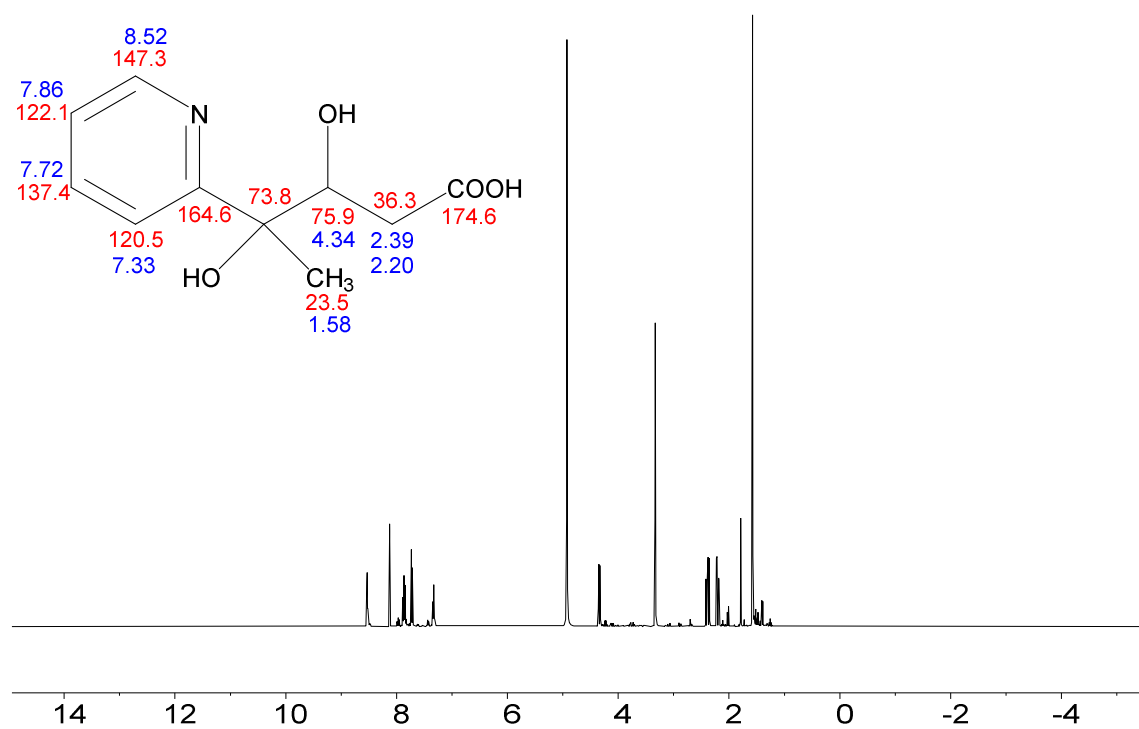


Figure S276 NMR spectra (MeOD) of **12c**: a) ^1H , b) ^{13}C , c) gCOSY, d) gHSQC e) HMBC and f) 2D NOESY.

a)



b)

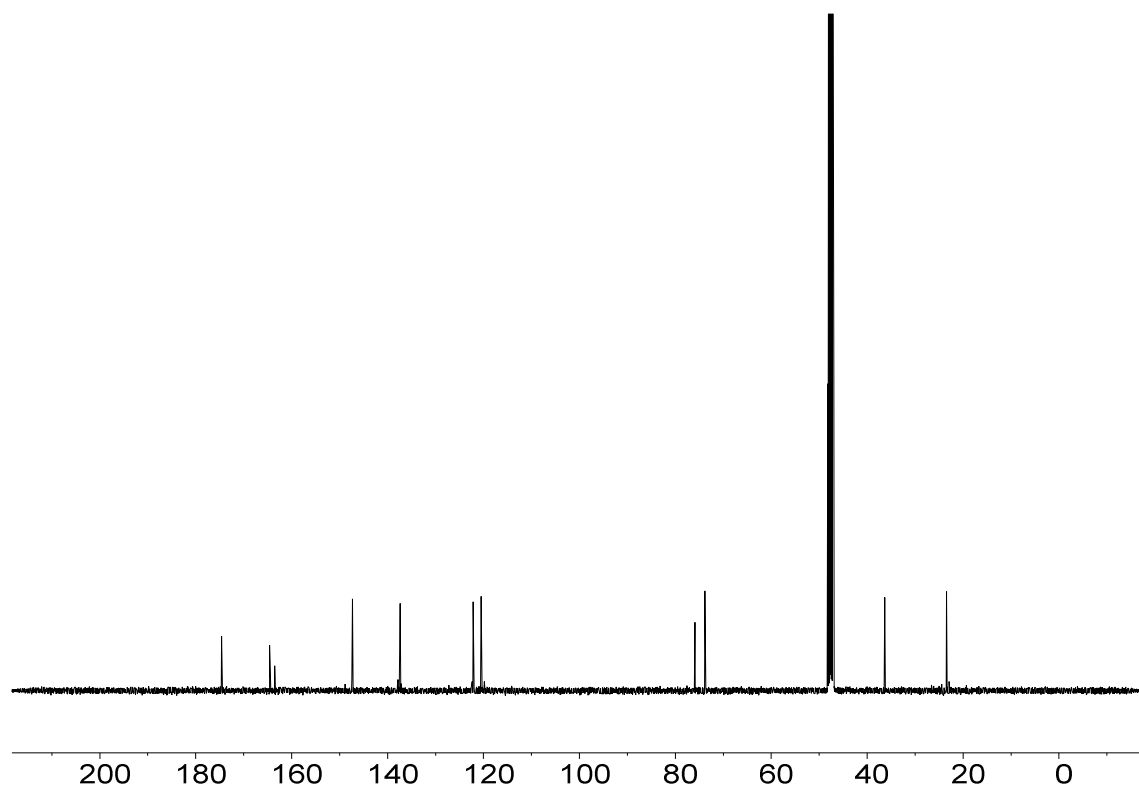
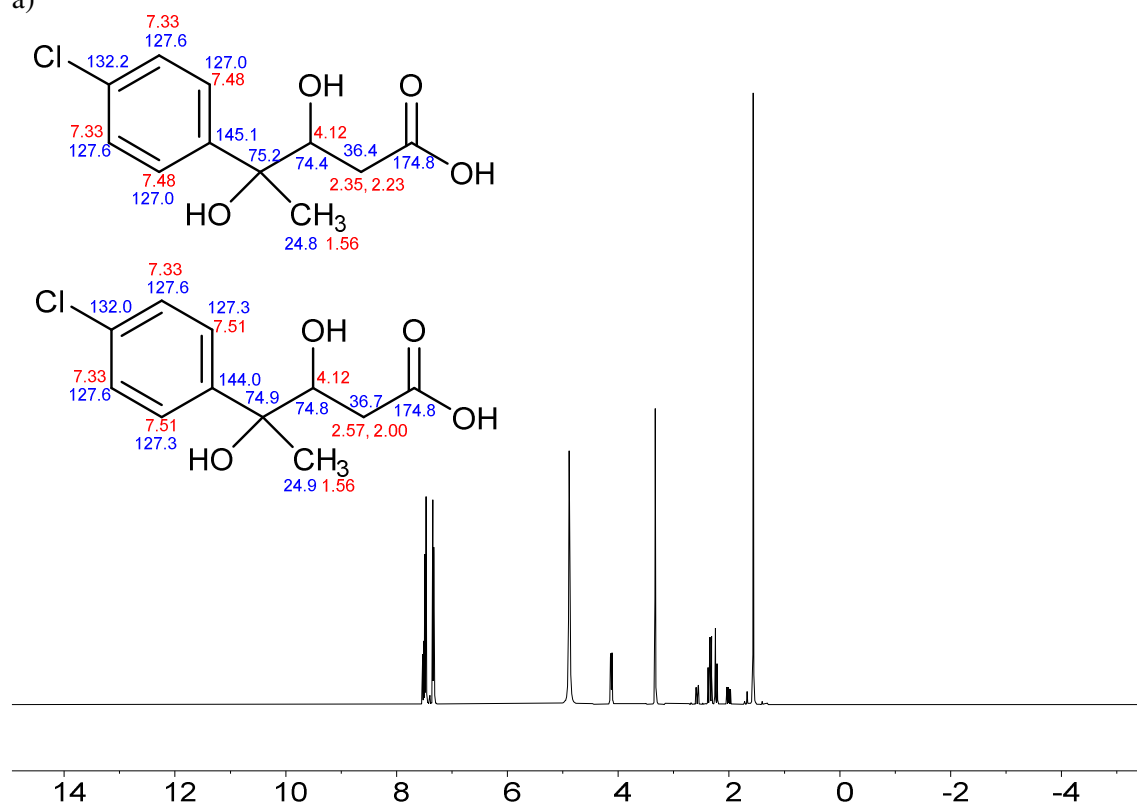
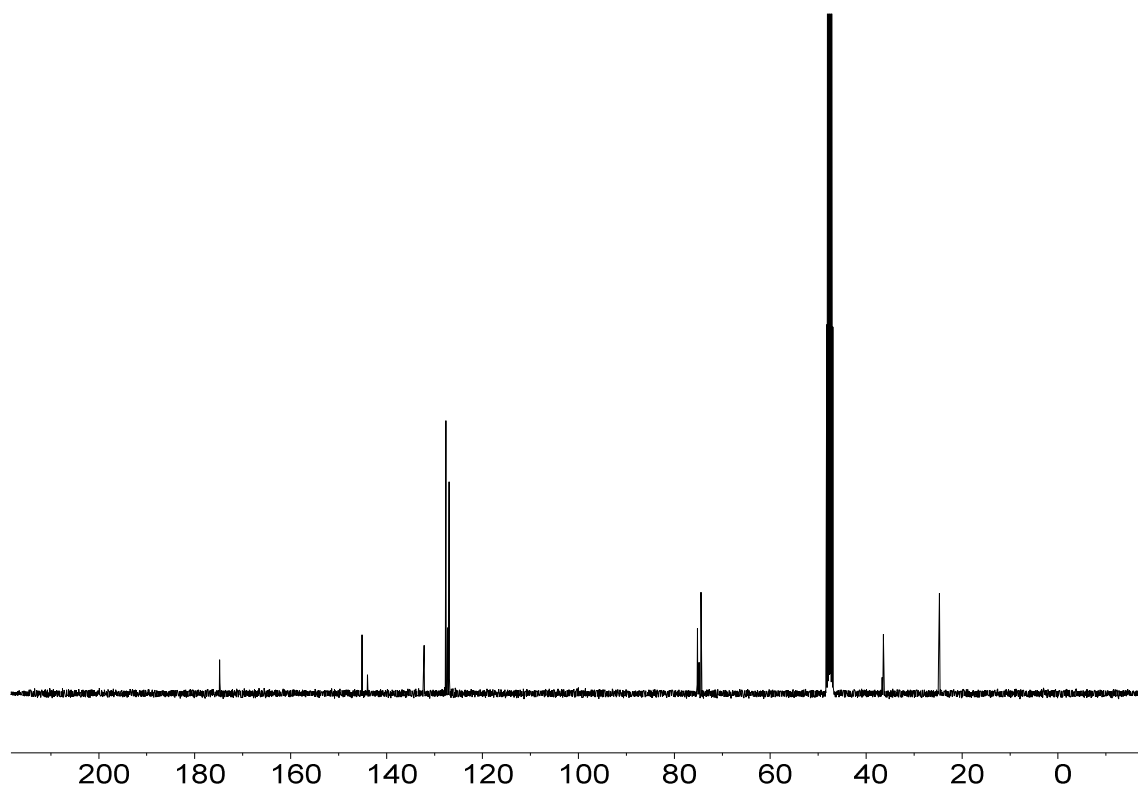


Figure S277 NMR spectra (MeOD) of **12d**: a) ^1H and b) ^{13}C .

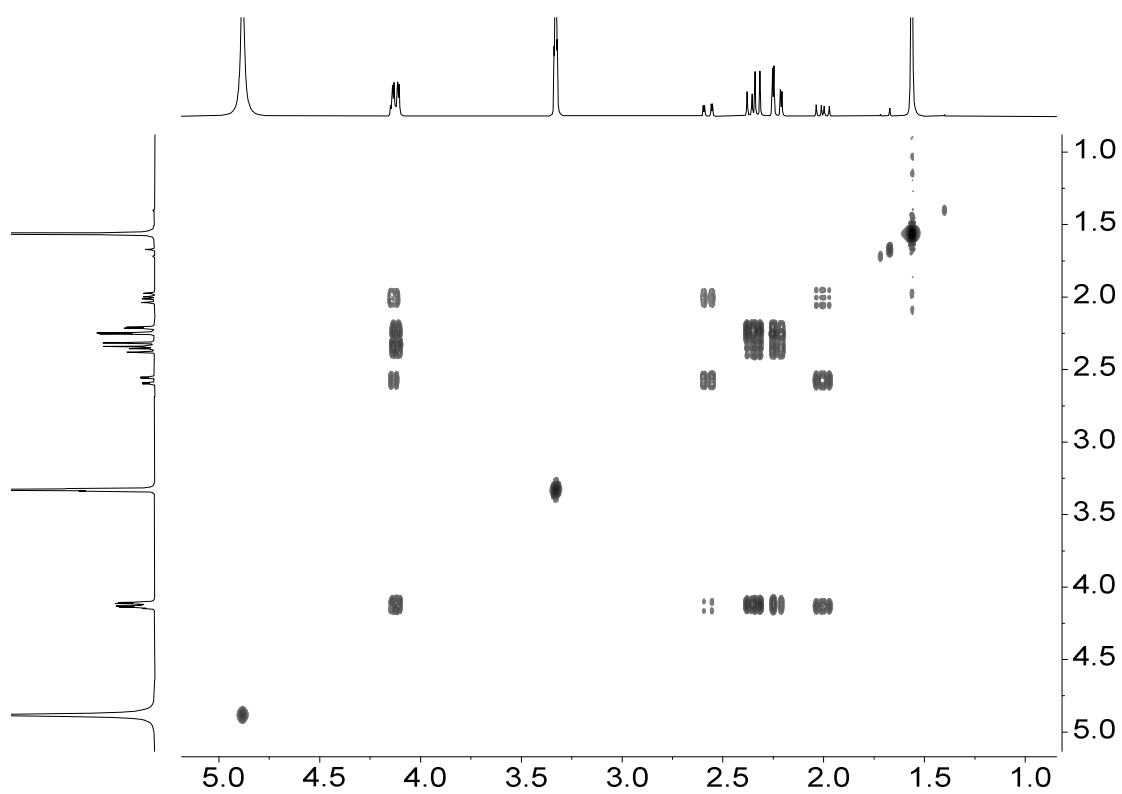
a)



b)



c)



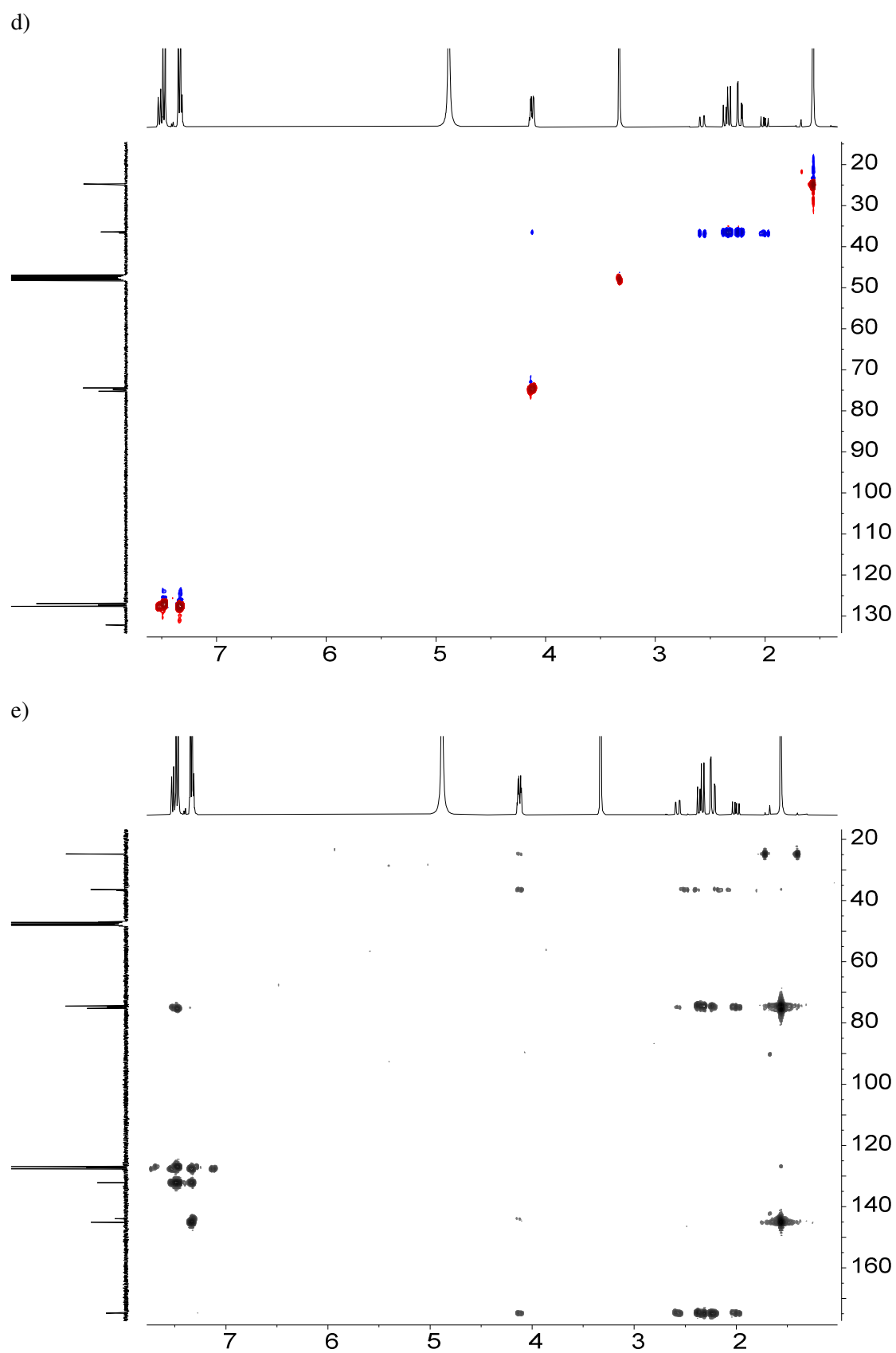
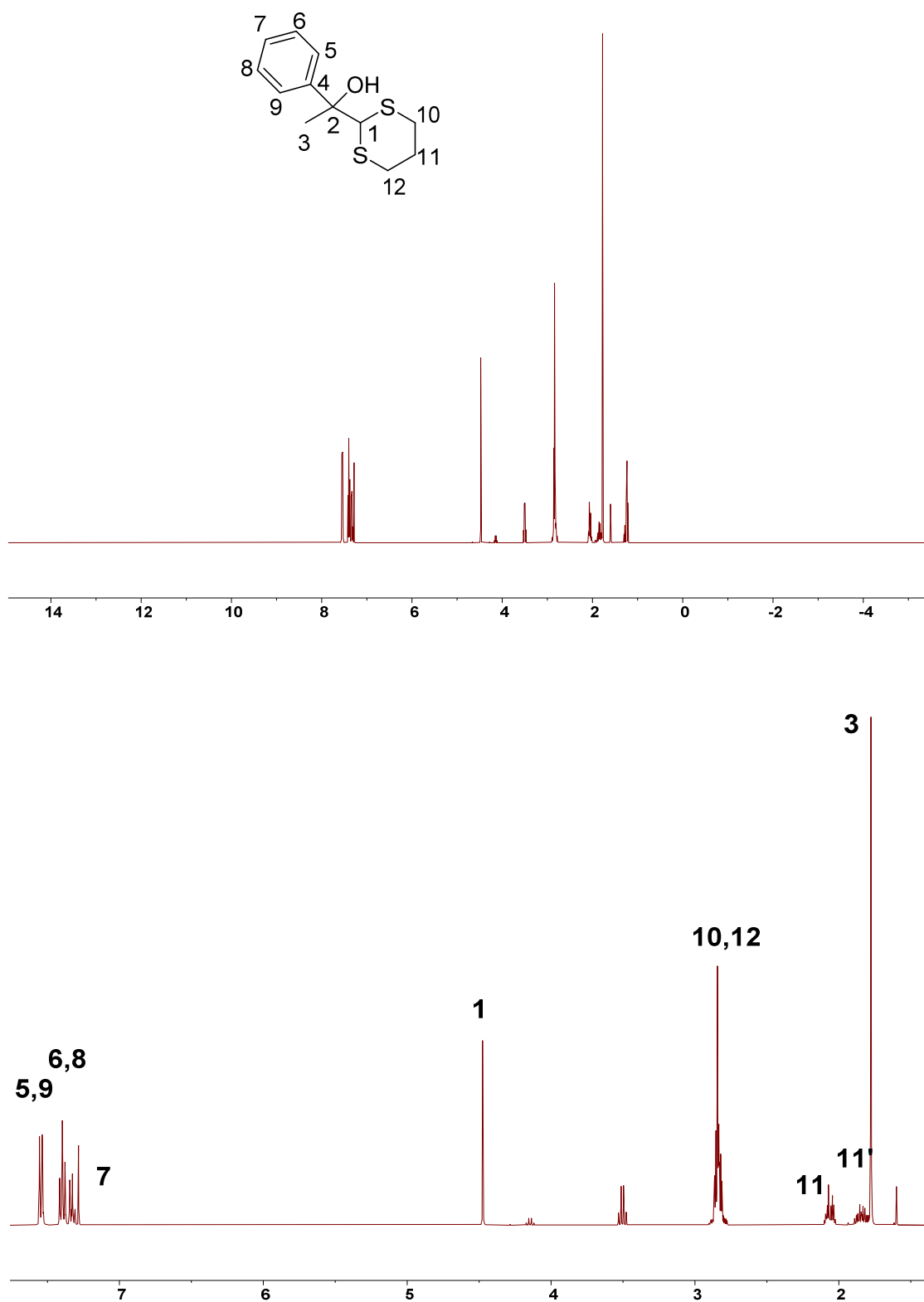


Figure S278 NMR spectra (MeOD) of **12e**: a) ^1H , b) ^{13}C , c) gCOSY, d) gHSQC e) HMBC.

6.4.3 NMR spectra of compounds C17–21

a)



b)

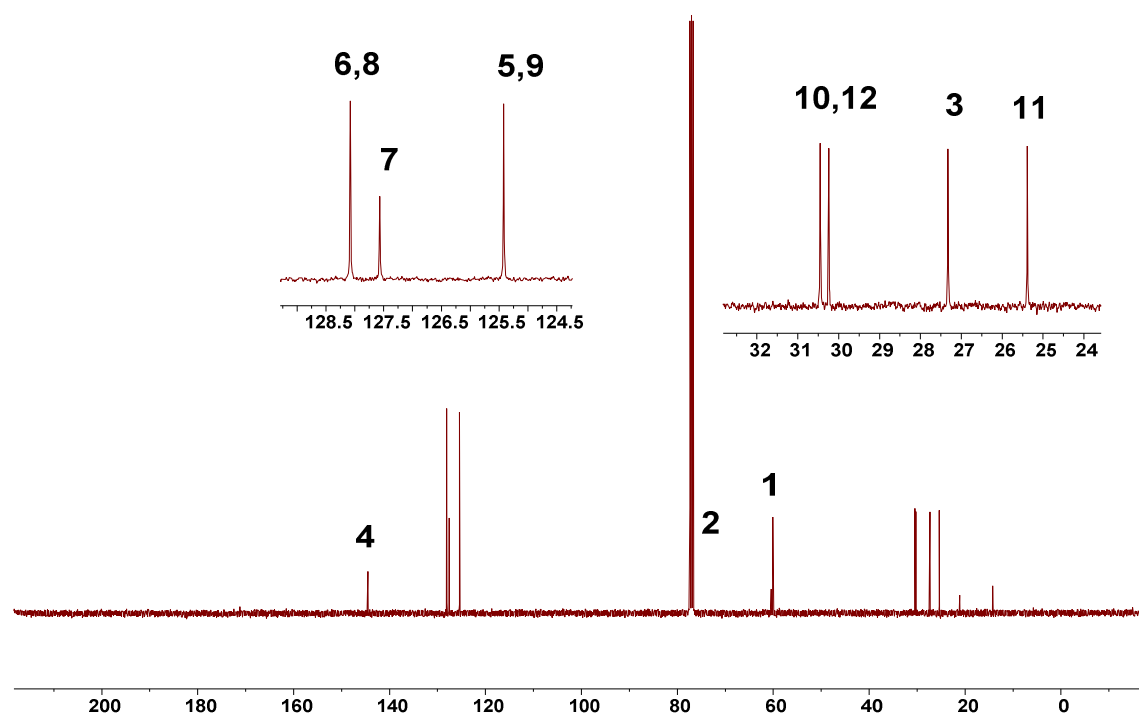
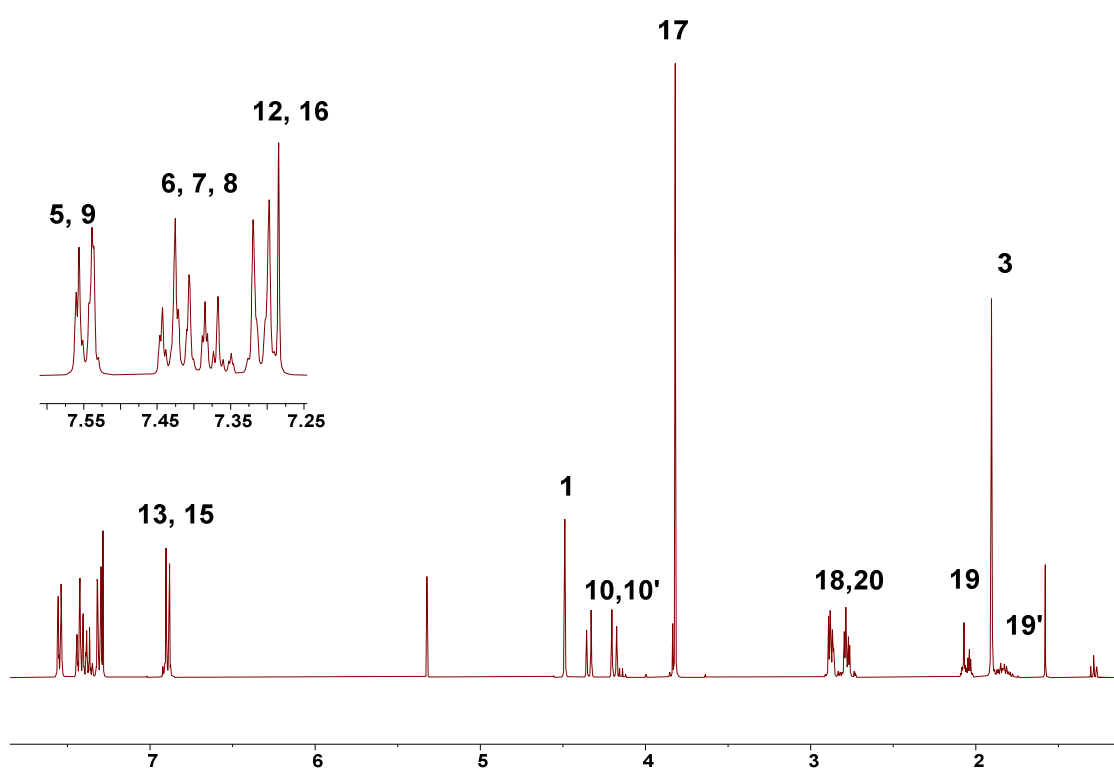
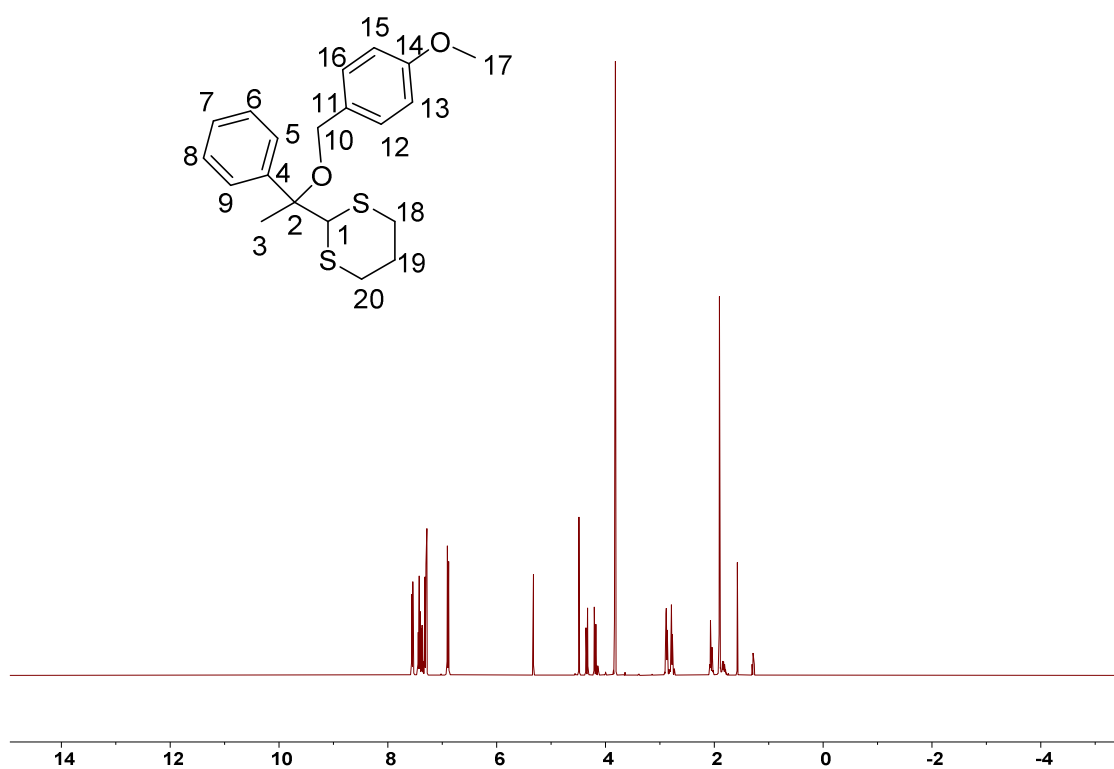


Figure S279 NMR spectra (CDCl_3) of **C17b**: a) ^1H and b) ^{13}C .

a)



b)

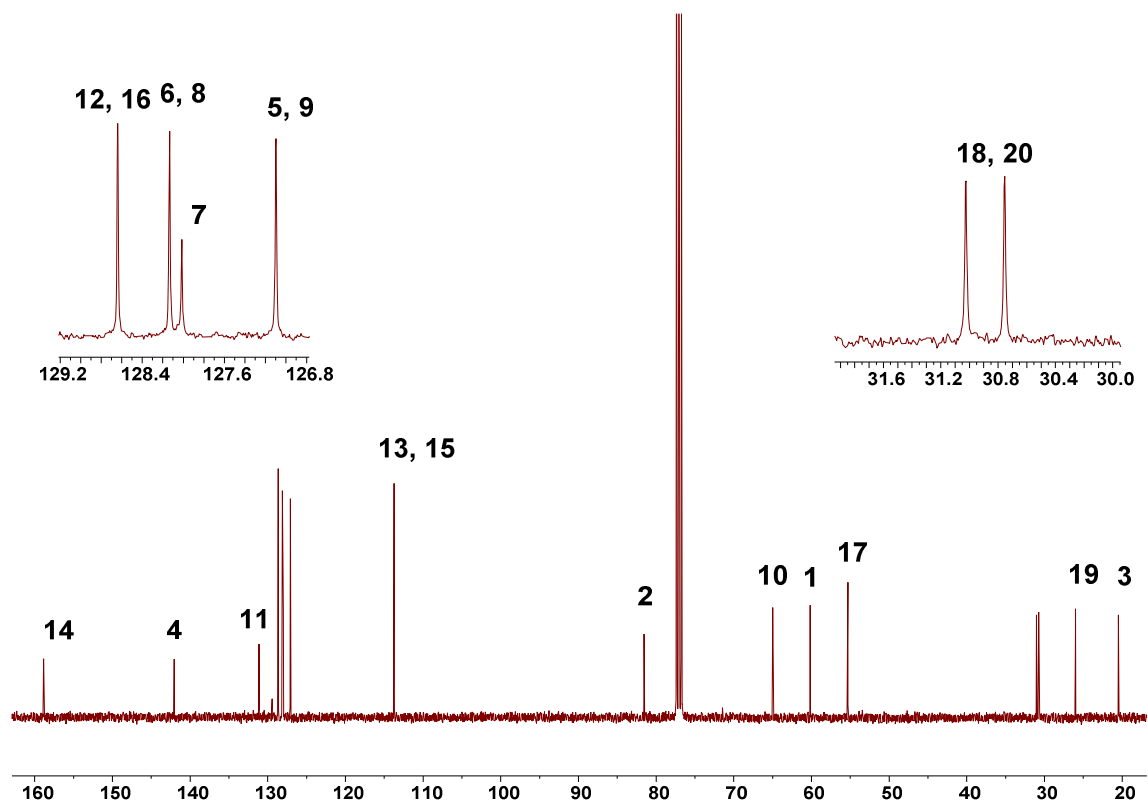
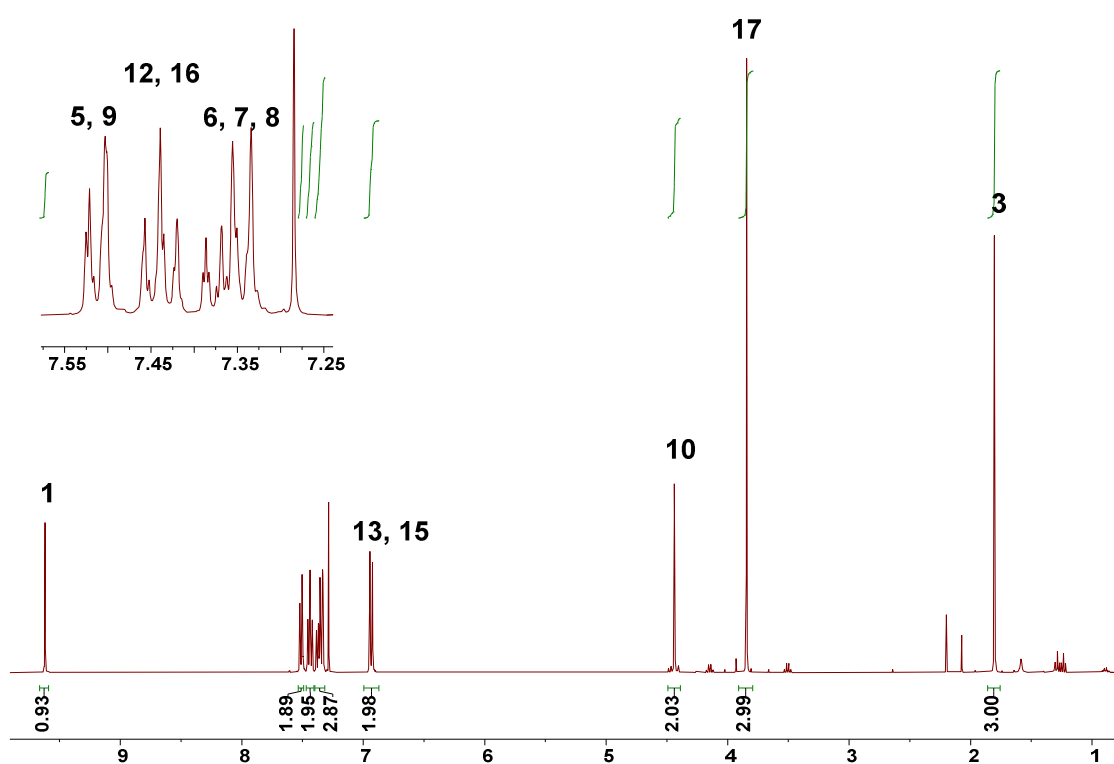
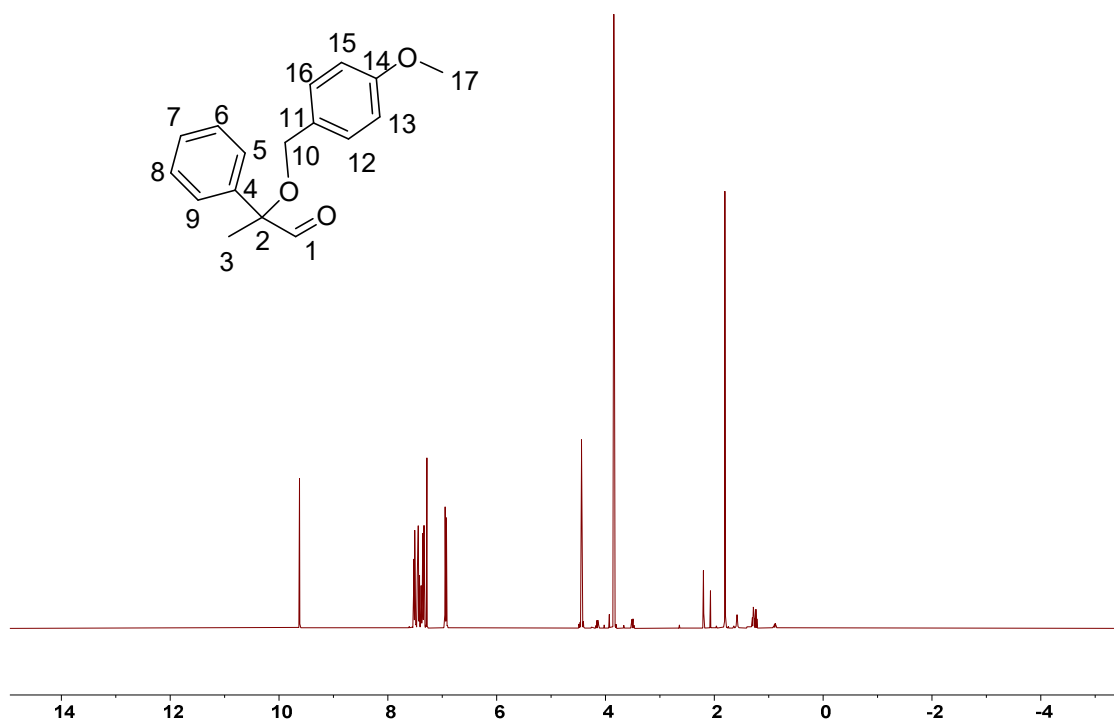


Figure S280 NMR spectra (CDCl_3) of **C17c**: a) ^1H and b) ^{13}C .

a)



b)

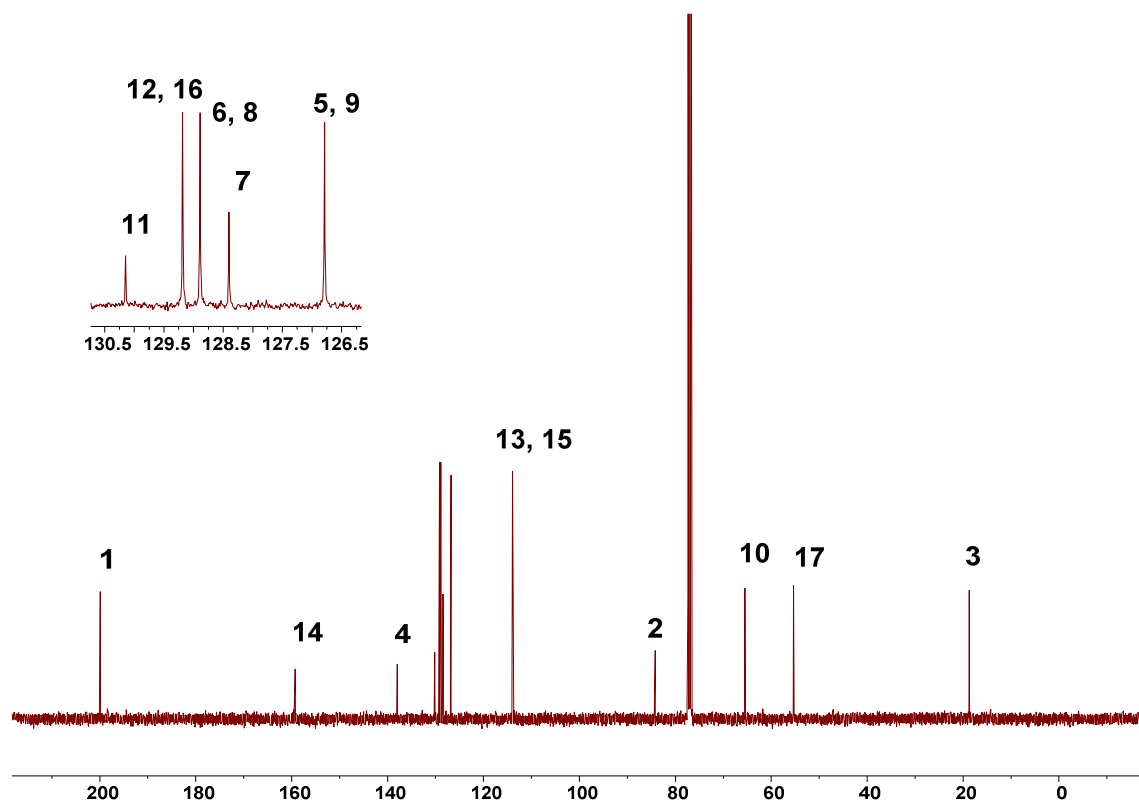
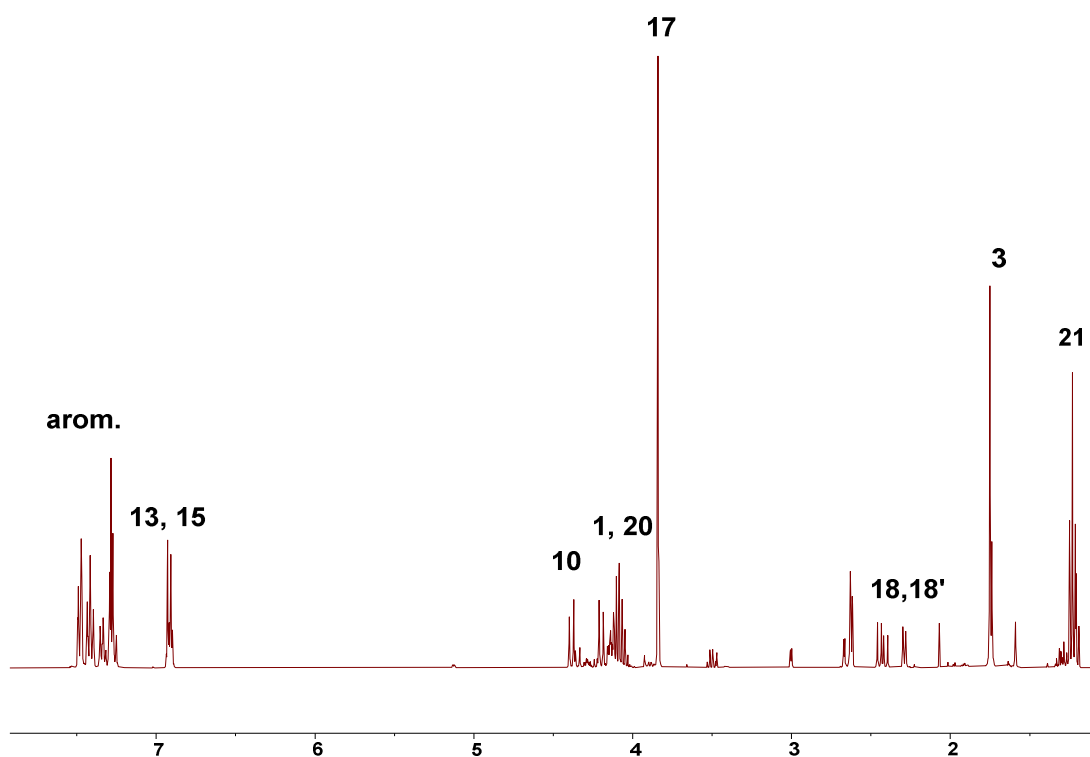
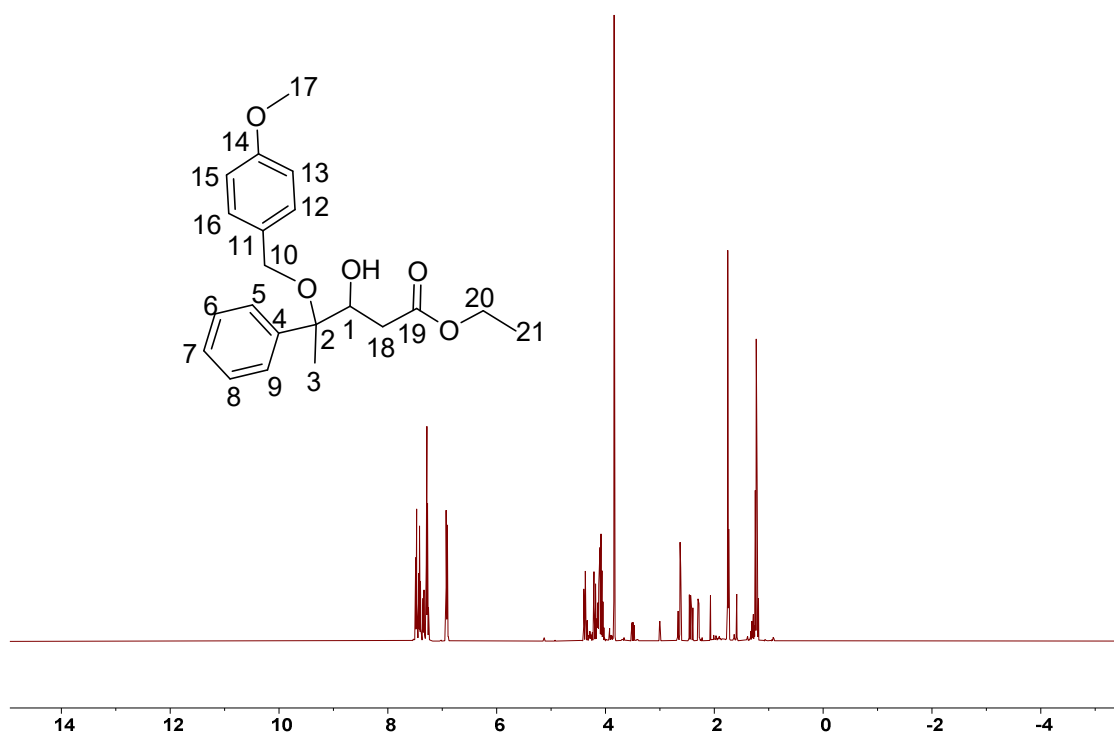


Figure S281 NMR spectra (CDCl_3) of **C18**: a) ^1H and b) ^{13}C .

a)



b)

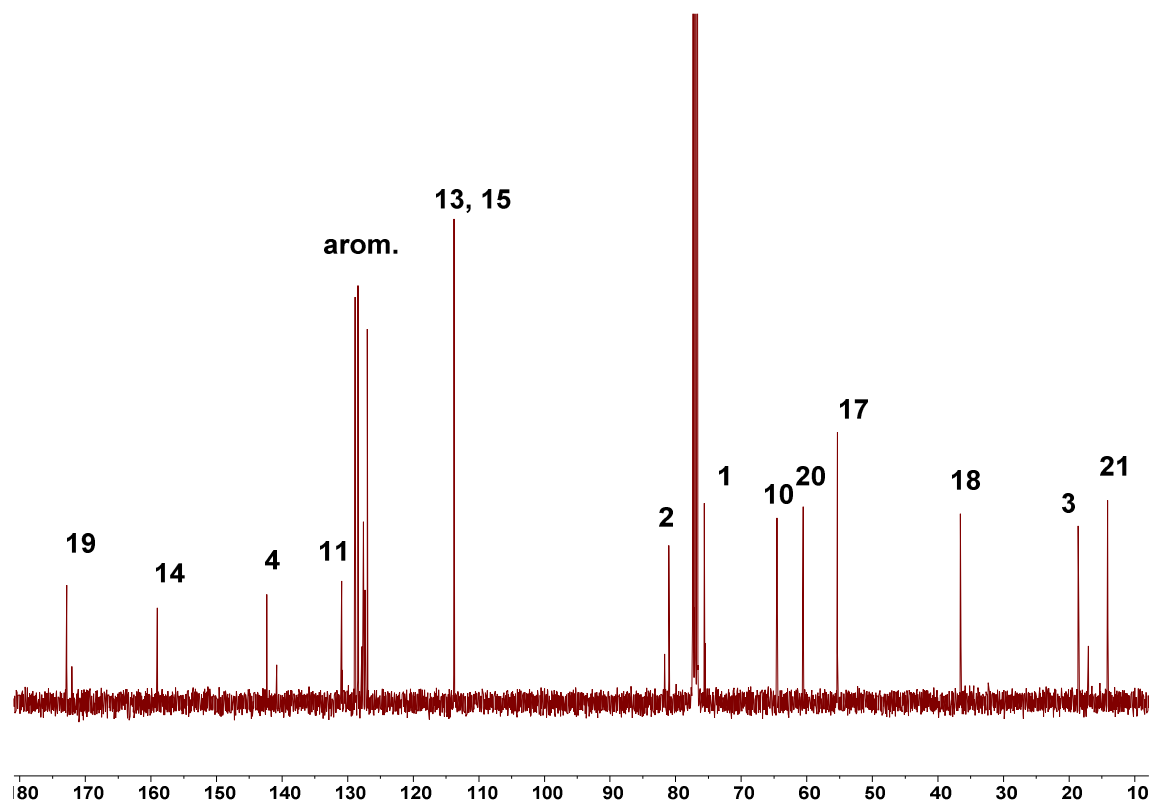
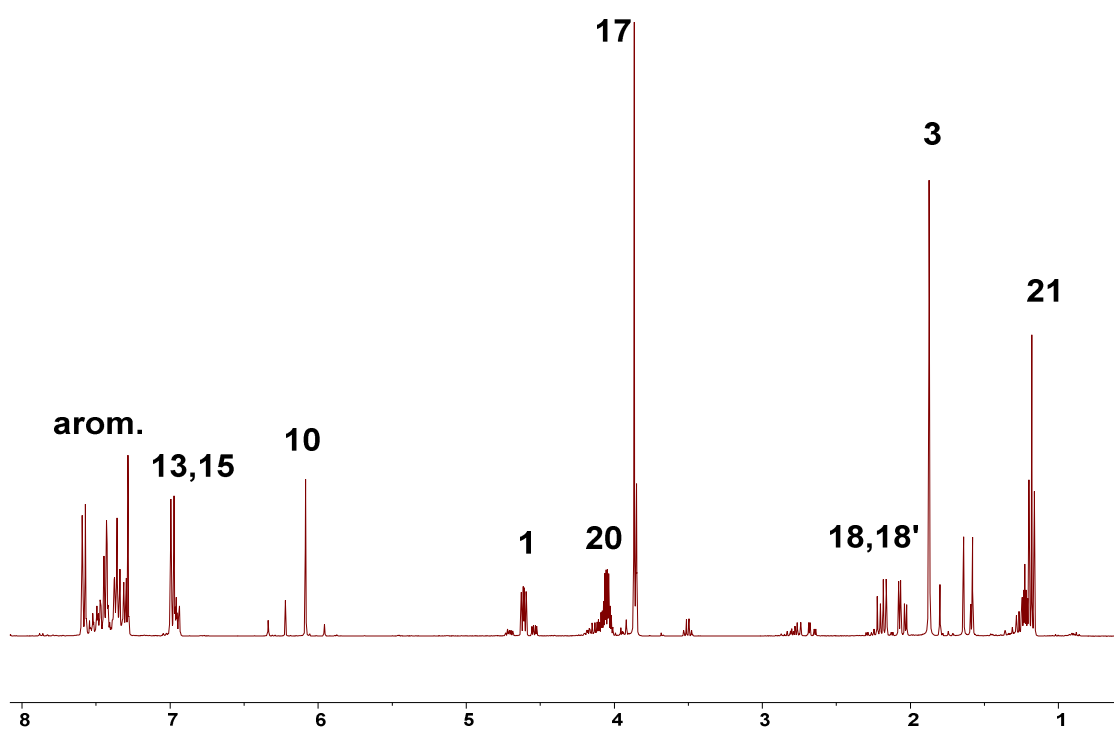


Figure S282 NMR spectra (CDCl_3) of **C19a**: a) ^1H and b) ^{13}C .

The chemical structure shows a benzofuran derivative with a 4-methoxyphenyl group and a 2-methoxyethyl ester side chain. The atoms are numbered 1 through 21. The ¹H NMR spectrum (400 MHz, CDCl₃) displays peaks from 0 to 10 ppm. Key features include a singlet at ~7.2 ppm (aromatic H-6), a doublet at ~7.4 ppm (aromatic H-5), a multiplet between 6.8-7.1 ppm (aromatic H-7,8,9), a singlet at ~6.9 ppm (aromatic H-4), a singlet at ~6.7 ppm (aromatic H-3), a singlet at ~5.5 ppm (aromatic H-2), a singlet at ~4.7 ppm (OCH₃-17), a quartet at ~4.1 ppm (CH₂-18), a singlet at ~3.9 ppm (OCH₃-20), a triplet at ~2.3 ppm (CH₂-19), and a doublet at ~1.2 ppm (CH₃-21).



b)

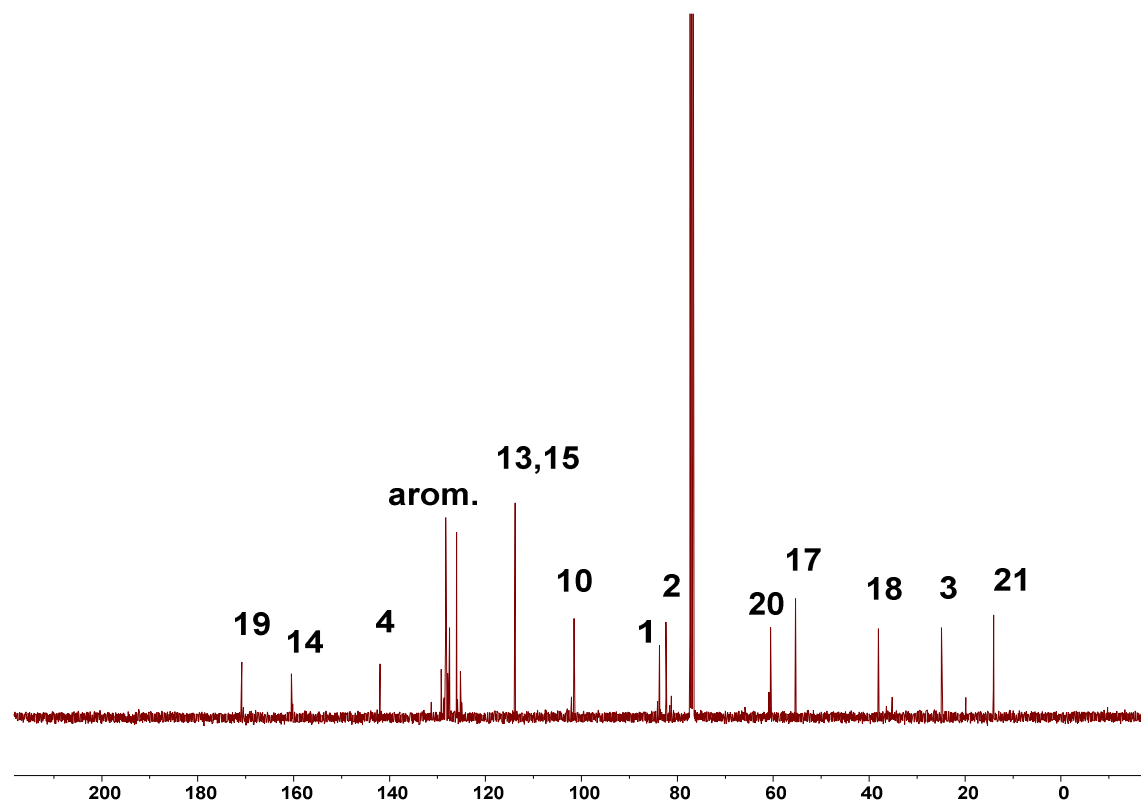
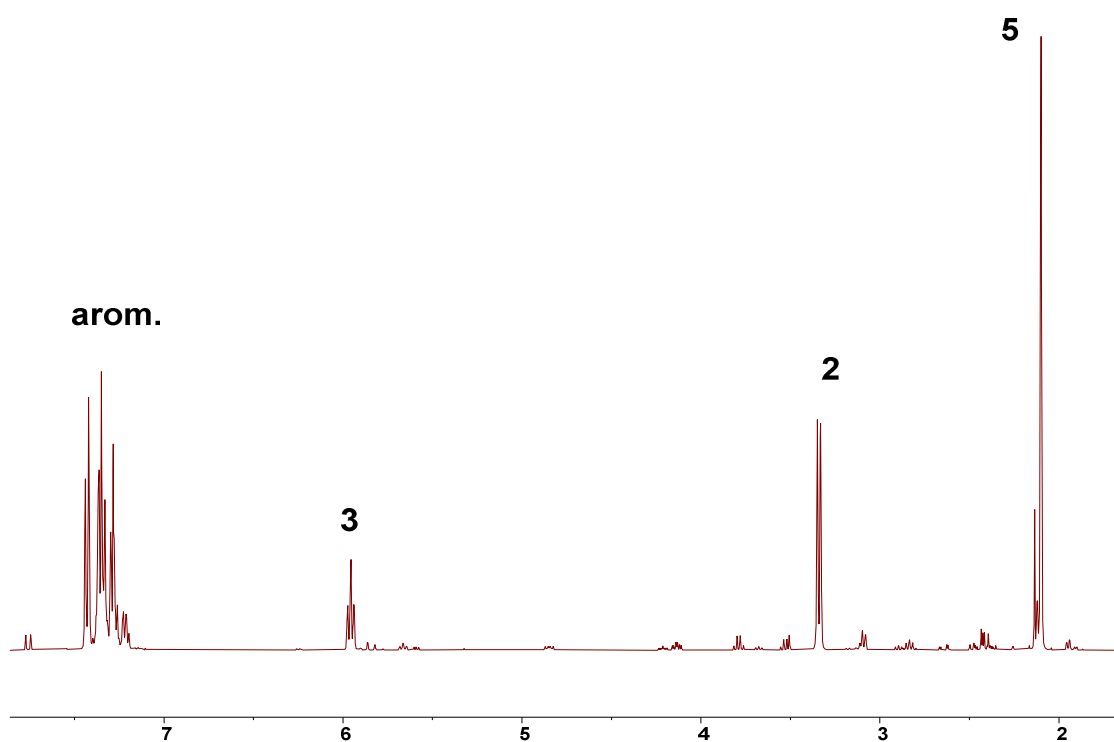
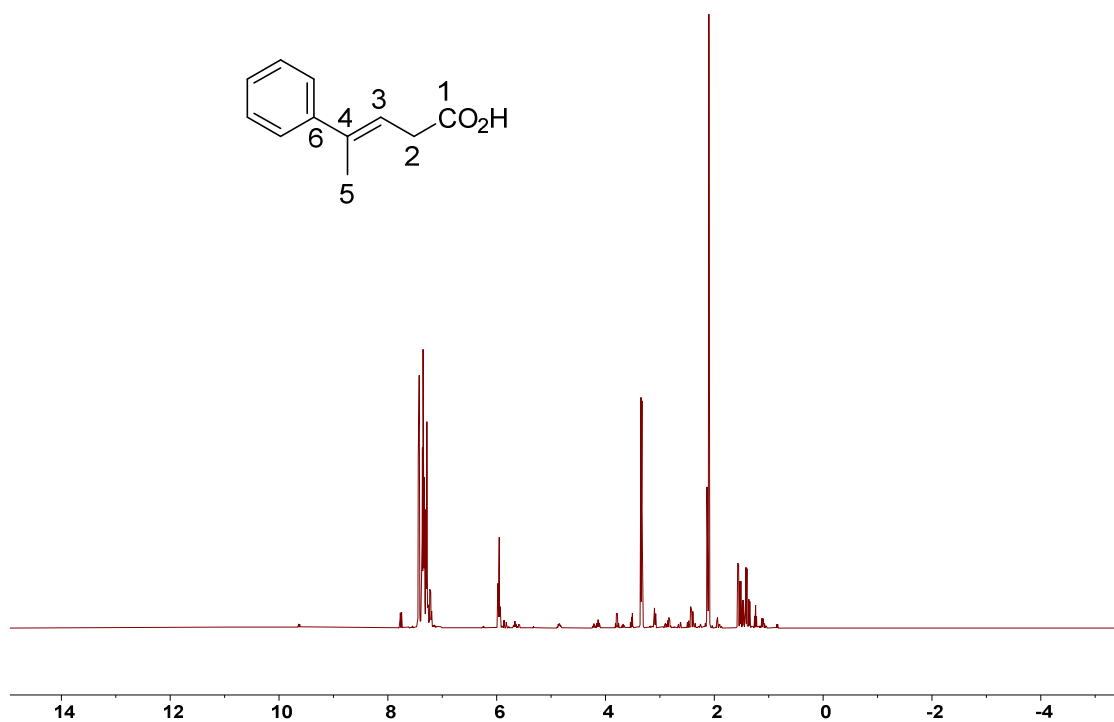


Figure S283 NMR spectra (CDCl₃) of **C19b**: a) ¹H and b) ¹³C.

a)



b)

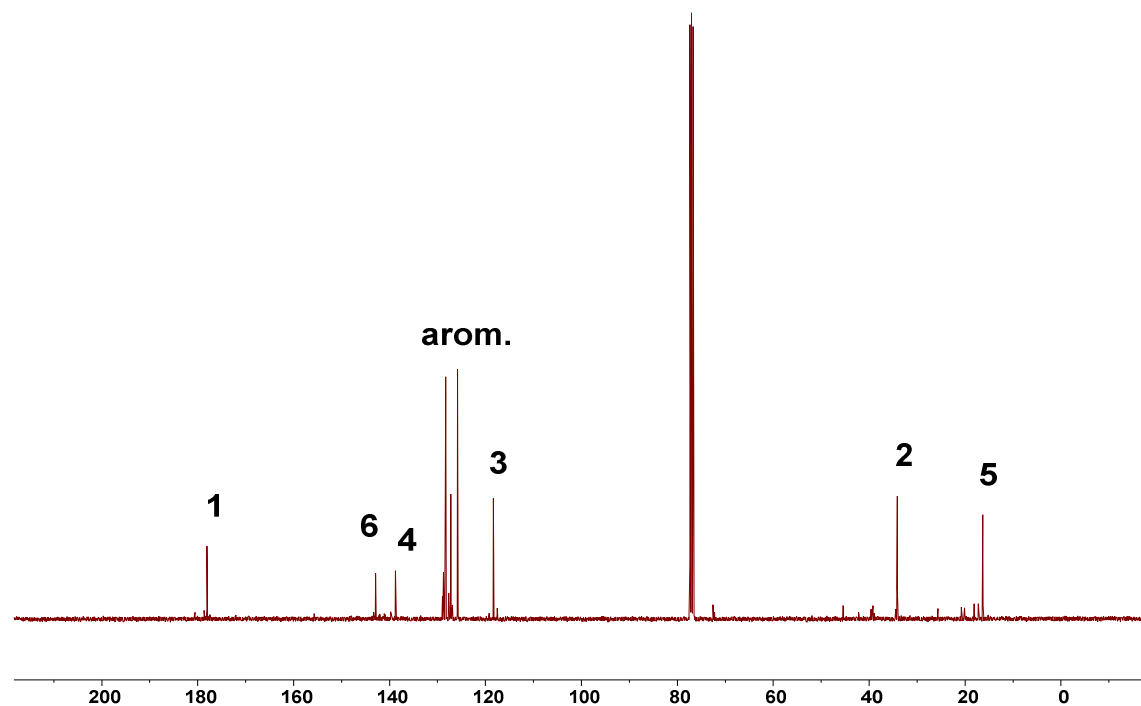
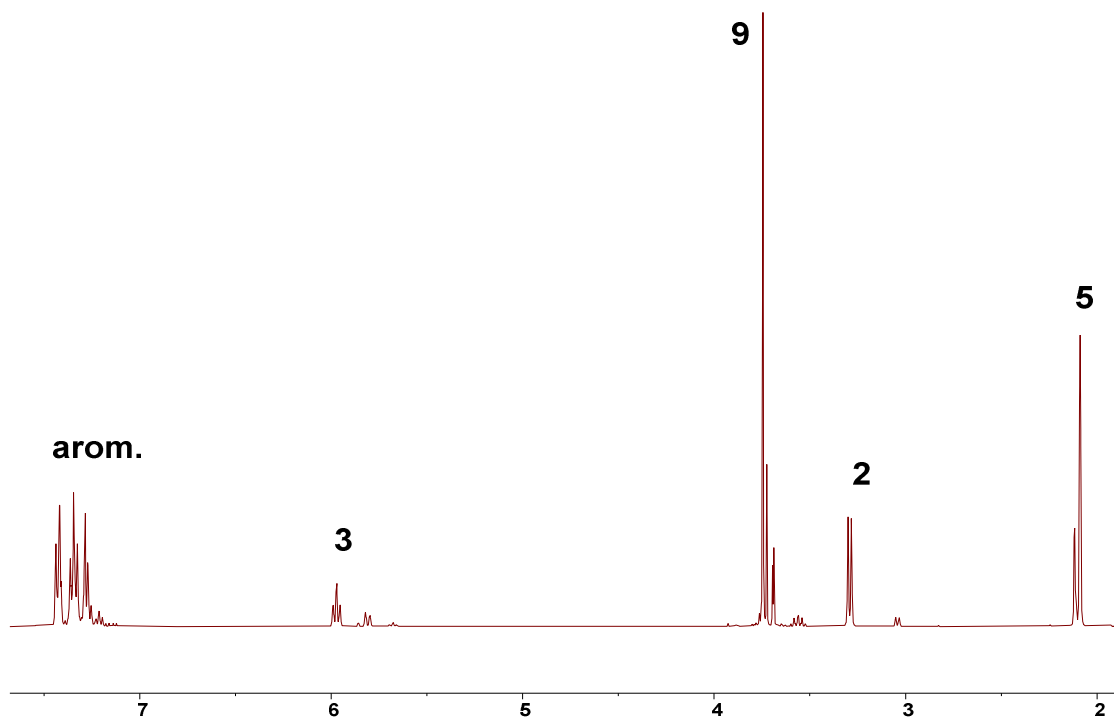
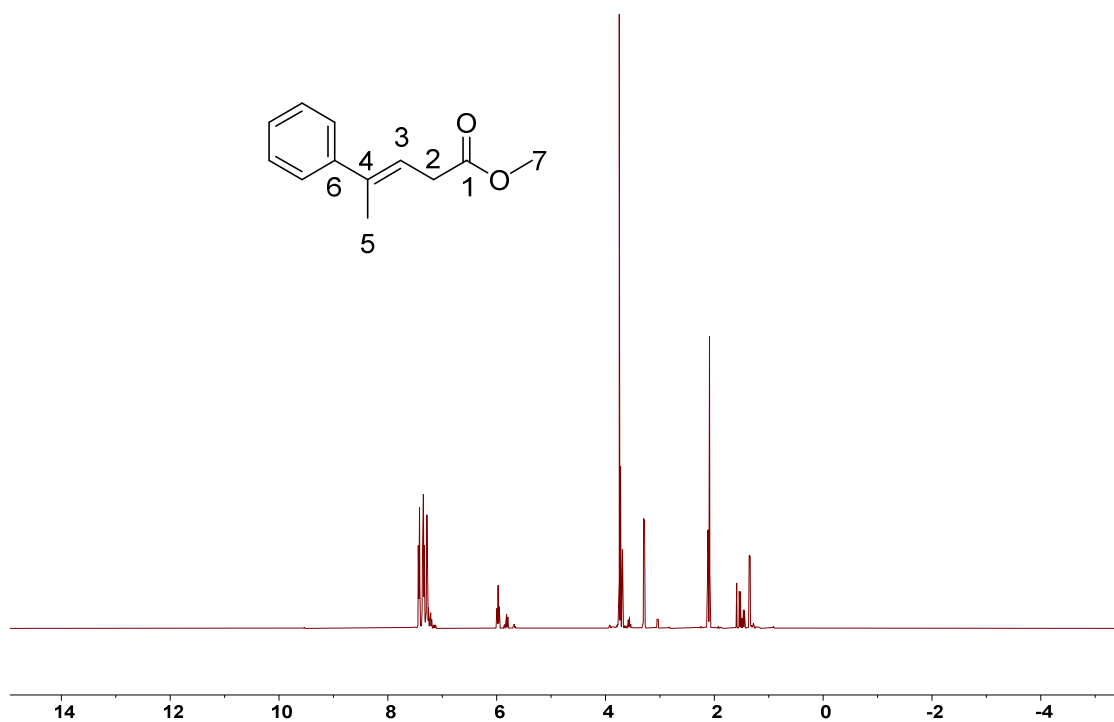


Figure S284 NMR spectra (CDCl_3) of **C20b**: a) ^1H and b) ^{13}C .

a)



b)

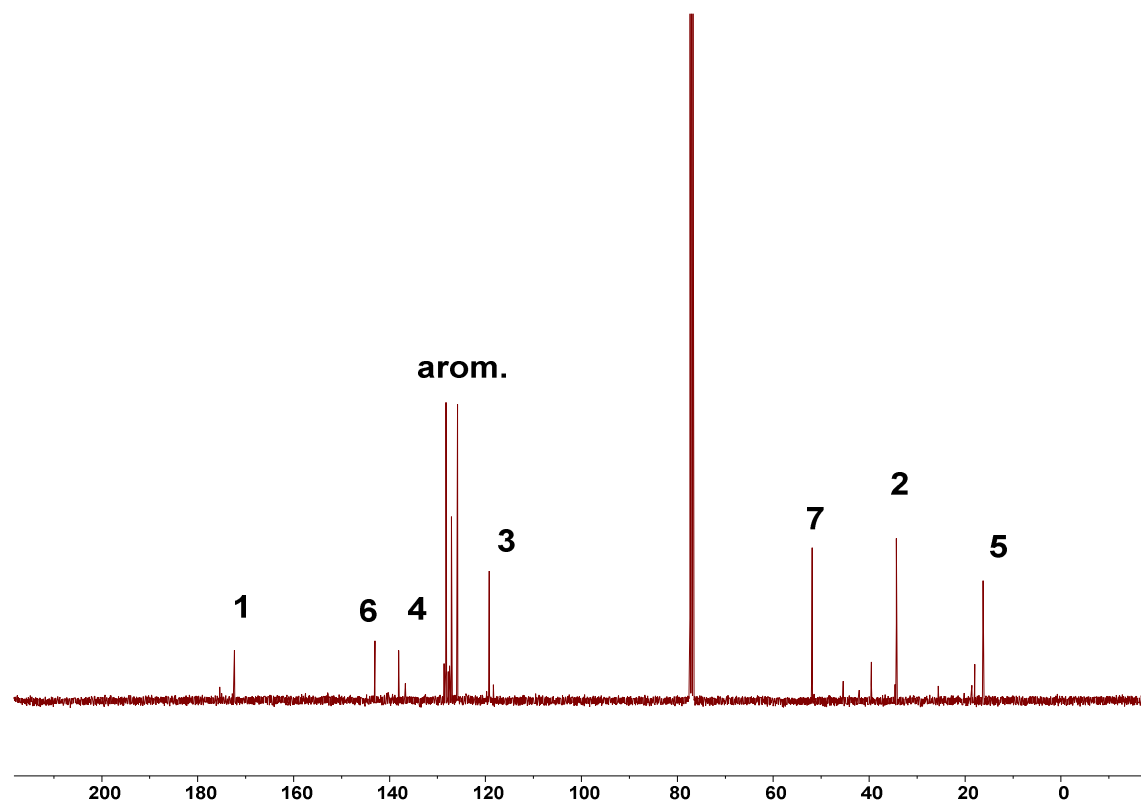
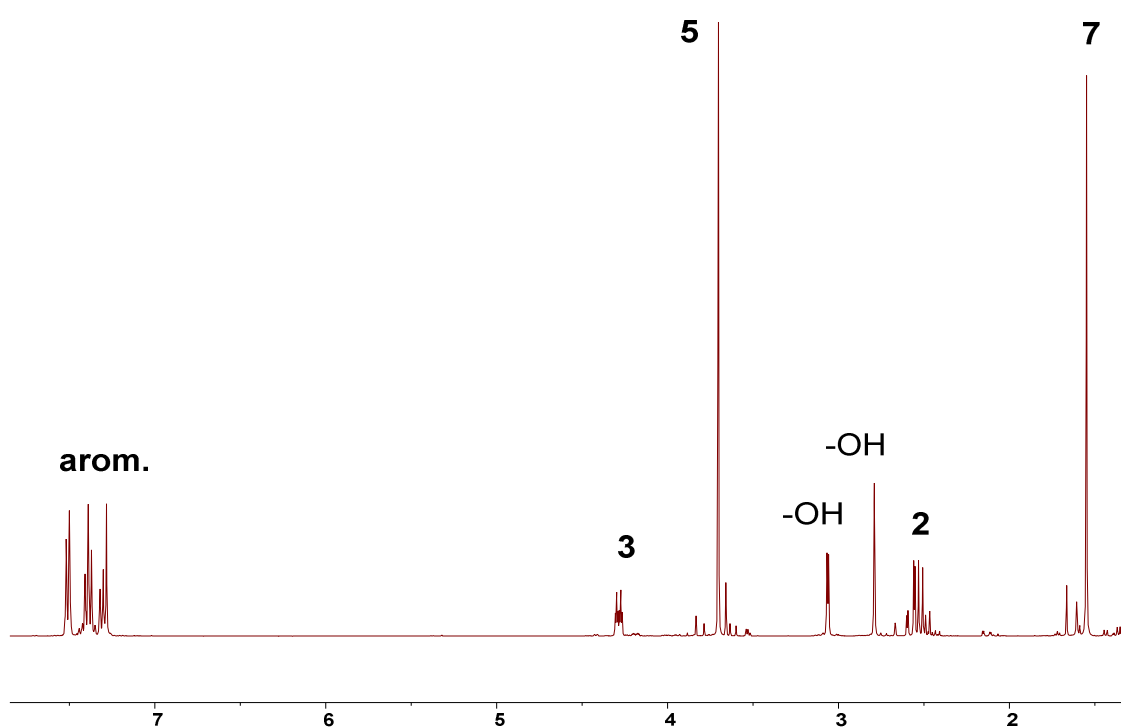
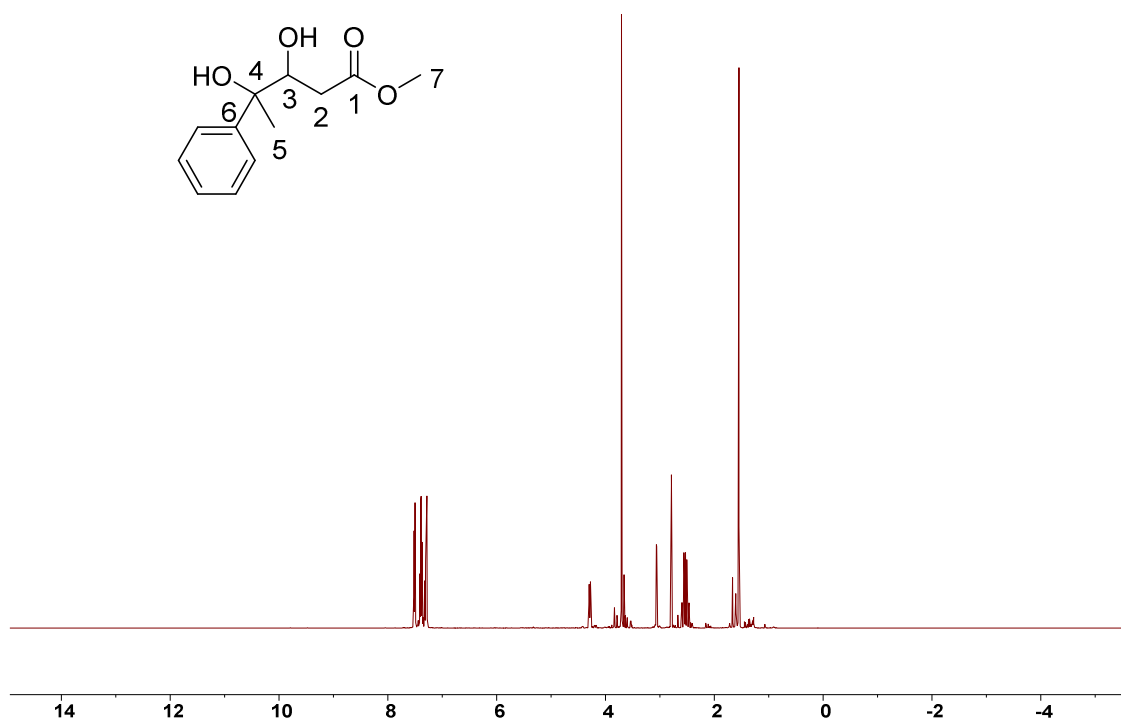


Figure S285 NMR spectra (CDCl_3) of **C20c**: a) ^1H and b) ^{13}C .

a)



b)

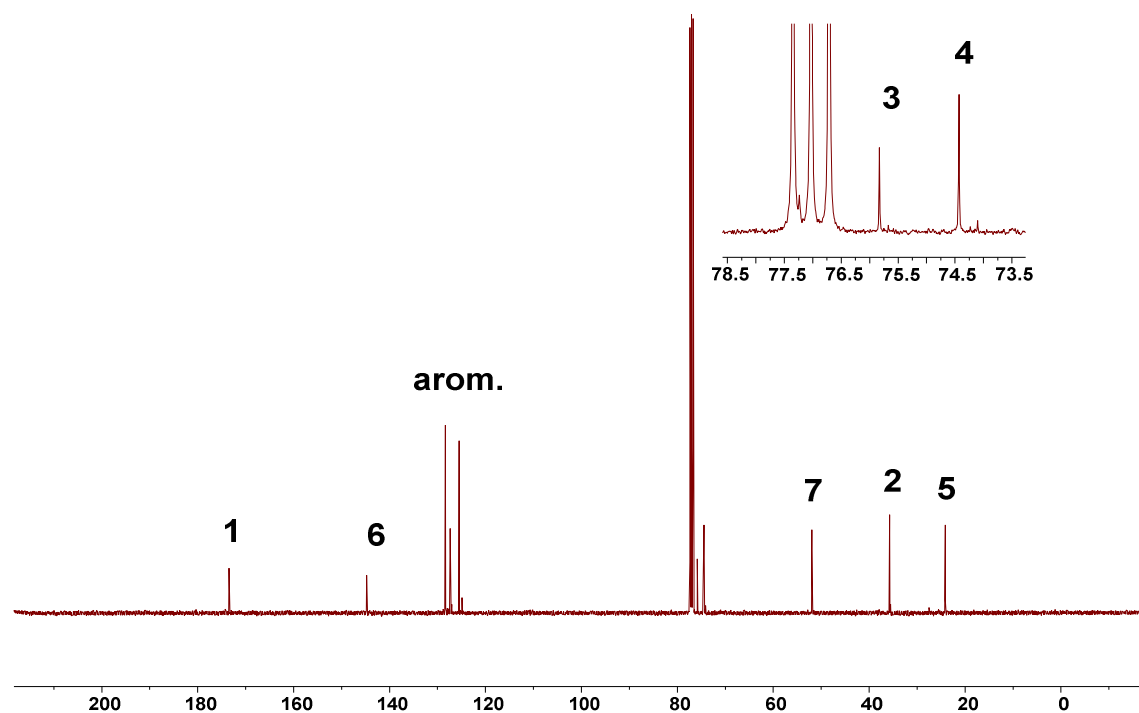


Figure S286 NMR spectra (CDCl_3) of **C20d**: a) ^1H and b) ^{13}C .

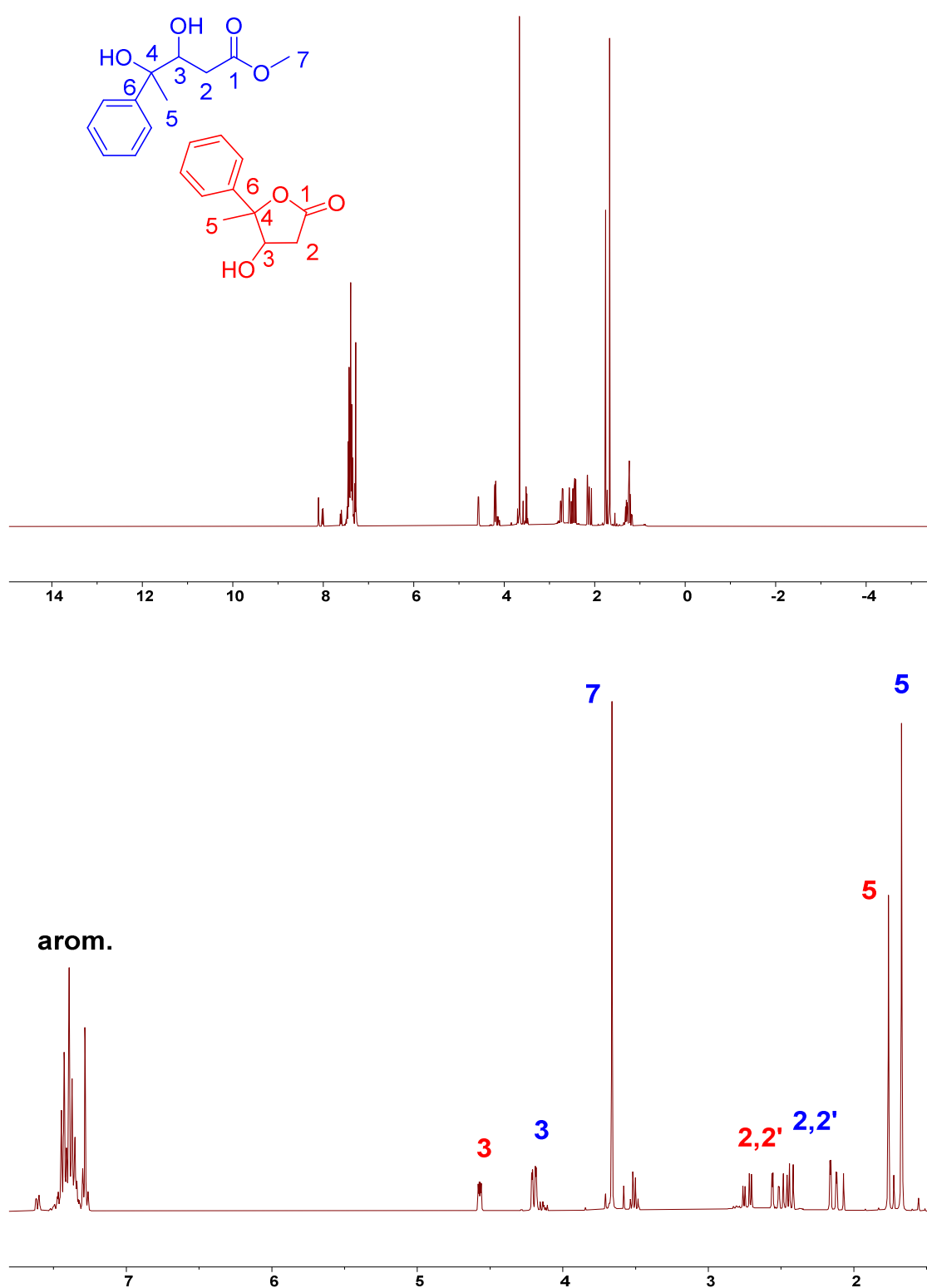
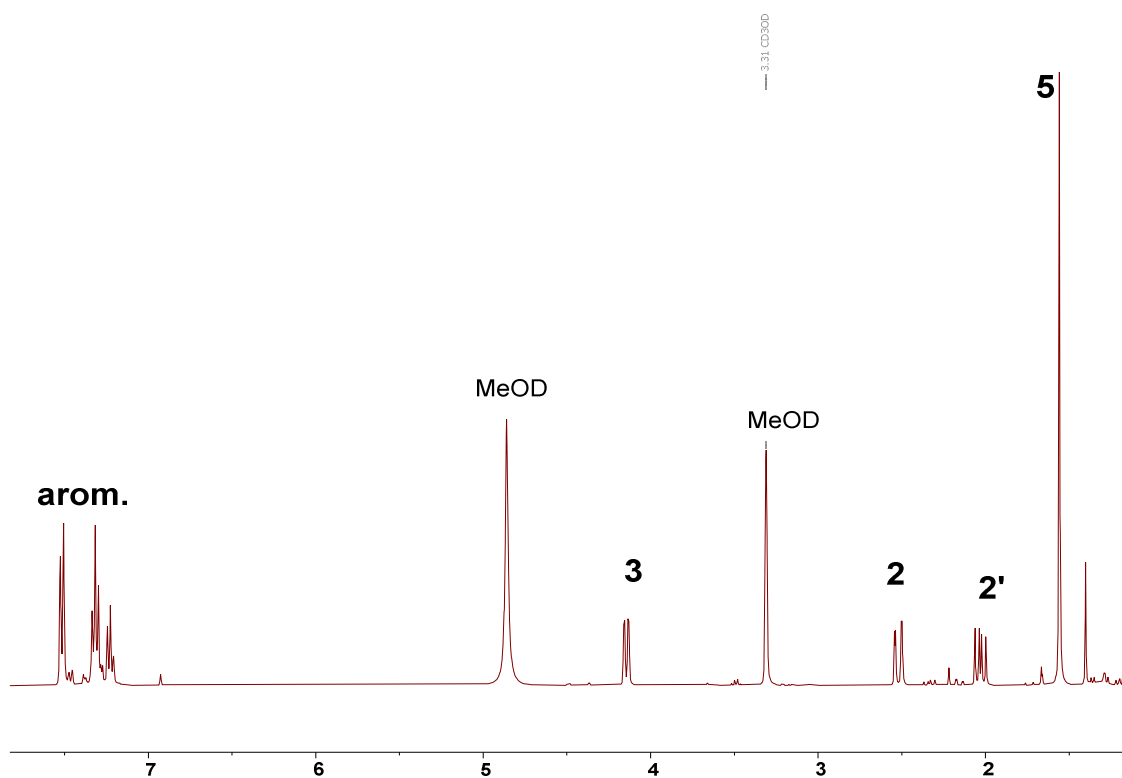
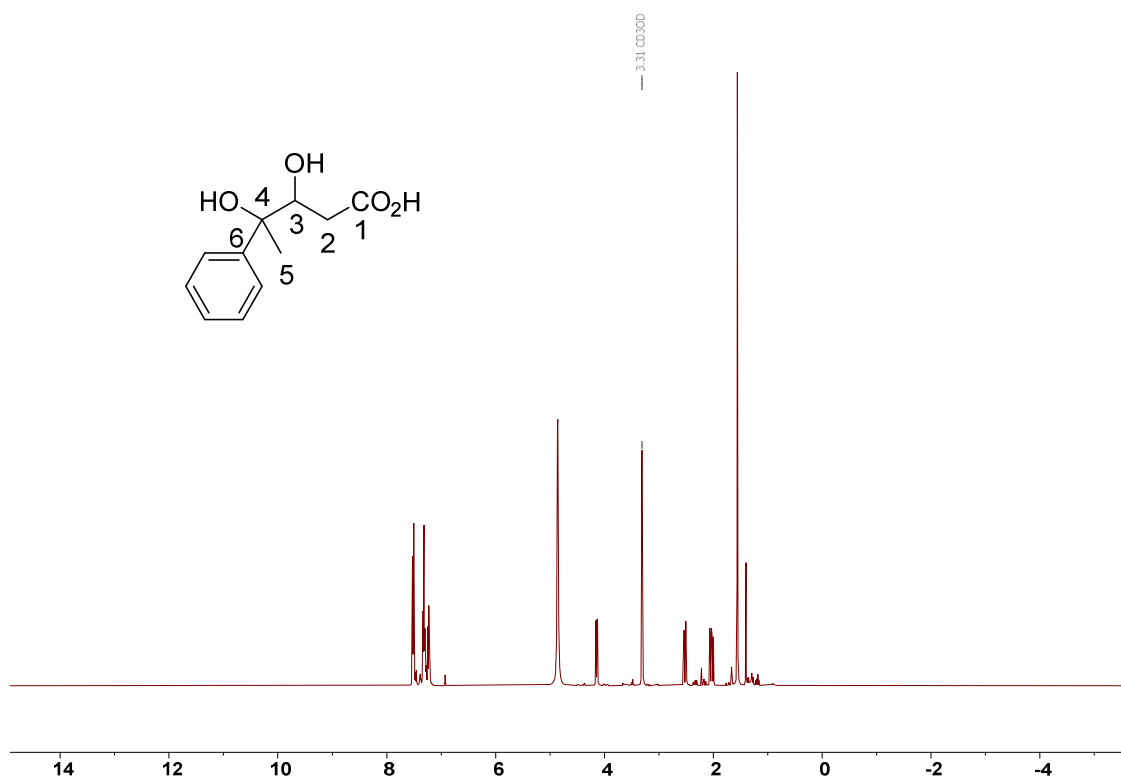


Figure S287 ^1H NMR spectra (CDCl_3) of **C20f** and **C20g**.

a)



b)

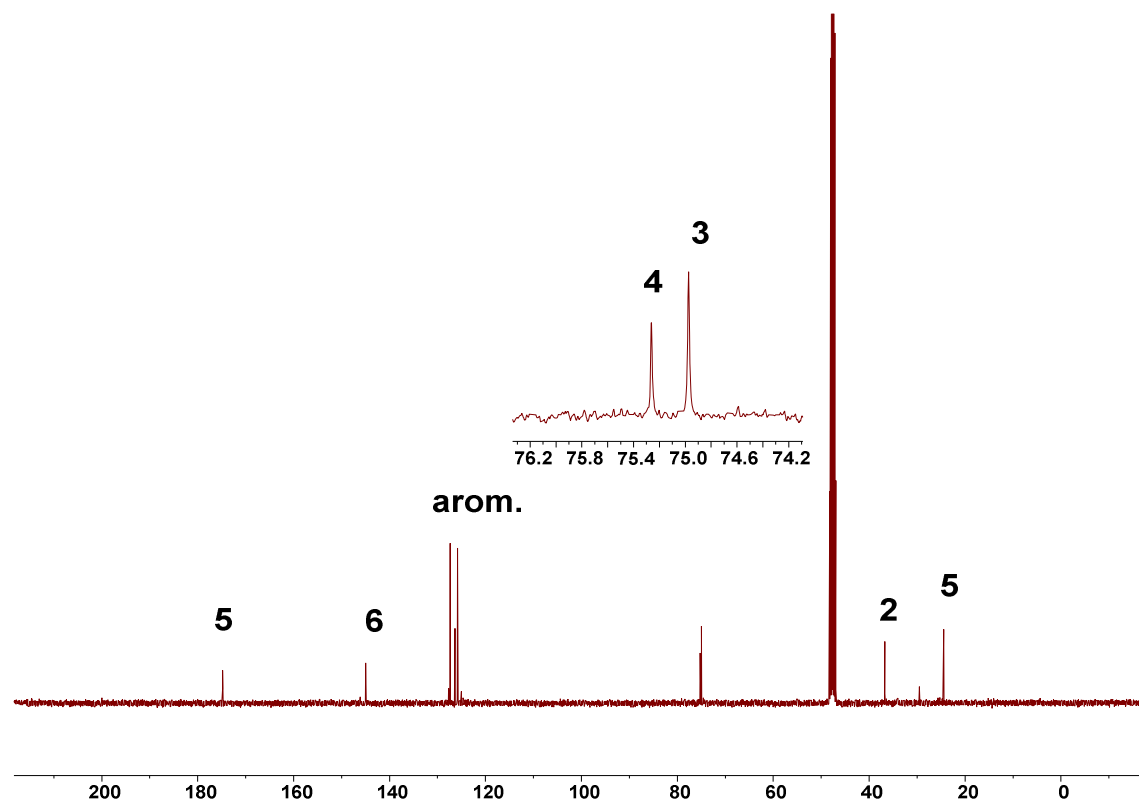
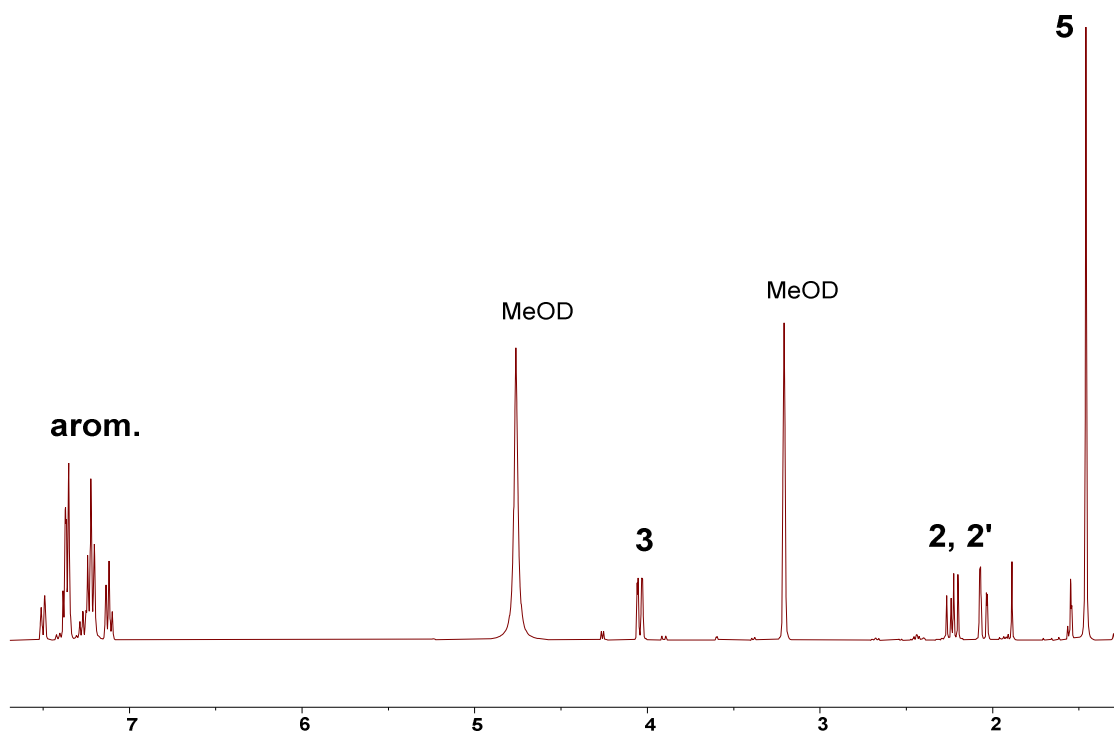
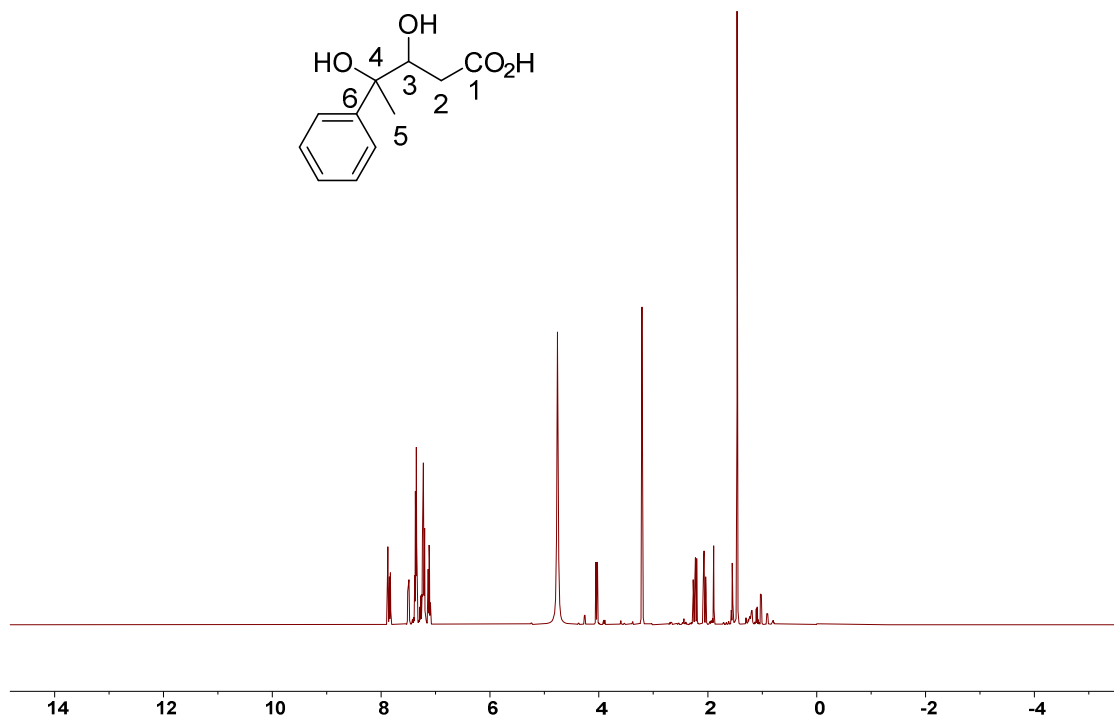
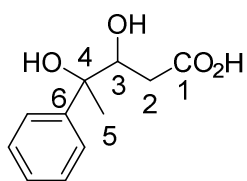


Figure S288 NMR spectra (MeOD) of **C21a**: a) ^1H and b) ^{13}C .

a)



b)

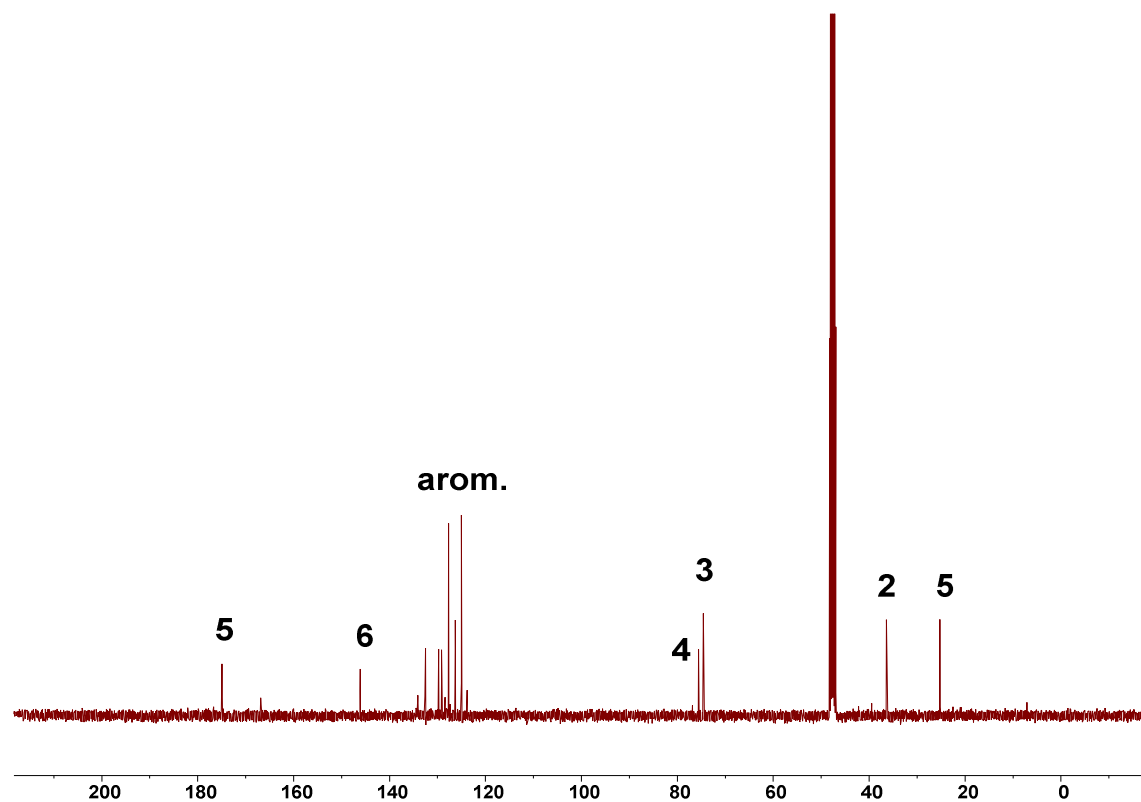


Figure S289 NMR spectra (CDCl_3) of **C21b**: a) ^1H and b) ^{13}C .

6.5 BIBLIOGRAPHY

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