Supporting Information

Cupin-domain containing protein is not essential for the alkyl salicylaldehyde formation in *Aspergillus ustus*

Marlies Peter and Shu-Ming Li*

Institut für Pharmazeutische Biologie und Biotechnologie, Fachbereich Pharmazie, Philipps-Universität Marburg, Robert-Koch-Straße 4, 35037 Marburg, Germany.

* Corresponding author, e-mail: shuming.li@staff.uni-marburg.de.

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Experimental section

Sequence analysis

The genome of *Aspergillus ustus* 3.3904 was sequenced and published by Pi *et al*. in 2015.[1] The genomic sequence reported in this study is available in GenBank under the accession number JOMC01000132. To predict biosynthetic gene clusters, initial analysis was carried out with antiSMASH (http://antismash.secondarymetabolites.org/)[2] and 2nd find (https://biosyn.nih.go.jp/2ndfind/). Detailed investigation including annotation of genes and prediction of enzyme functions (Table S1) were conducted using BLASTp program (http://www.ncbi.nlm.nih.gov).

Strains, media, and growth conditions

All strains used and created in this study are listed in Table S2. *Escherichia coli* DH5α and *Saccharomyces cerevisiae* HOD114-2B cells were cultivated as described before.[3] *Aspergillus ustus* 3.3904 was purchased from China General Microbiological Culture Collection Centre (Beijing, China) and cultivated in liquid potato dextrose broth (PDB, Sigma, Germany, Darmstadt) for 3 days at 30 °C for harvesting mycelium for subsequent genomic DNA isolation. *Aspergillus nidulans* strains were grown at 37 °C on LMM agar plates for spore production with the required supplements (1 g/L uracil, 1.2 g/L uridine, 2.5 mg/L riboflavin, 0.5 mg/L pyridoxine). The LMM agar plates were prepared as given in a previous publication.[3] For secondary metabolite production, the *A. nidulans* strains were cultivated in liquid PDB medium at 25 °C. To quantify the metabolite production, three independent cultures per strain were set up in 50 mL PDB medium for quantification.

Genomic DNA isolation

To extract the genomic DNA, liquid PDB medium was inoculated with spores of *A. ustus* 3.3904, *P. roqueforti* FM164 and *A. nidulans* and stand for two days at 37 °C. The media were removed by centrifugation. Subsequent isolation of the genomic DNA was performed as described in a previous publication.[4]

PCR amplification, cloning, and plasmid construction

The generated plasmids and the used primers are listed in Tables S3 and S4, respectively. Primers were synthesised by SeqLab GmBH (Göttingen, Germany). Q5® High-Fidelity Polymerase (New England Biolabs) was used for PCR amplification as given in the manufacturer's instruction.

To construct the expression plasmid pMP024 with a segment of 13,572 bp (bp 10521 – 24091 of JOMC01000132), three fragments of 5241, 5700, and 2630 bp were amplified from the

genomic DNA of *A. ustus* 3.3904. The fragments share overlapping regions of approximately 300 bp to each other and 30 bp to the vector pJN017.[5] The sequence of the transcription factor KIA75503 is directly cloned behind the strong *gpdA* promoter sequence. The three fragments and the SfoI-linearised pJN017 were assembled via homologous recombination in *Saccharomyces cerevisiae* HOD114-2B.[6]

For cloning of the expression construct pMP046, the putative gene coding for PsaCP with a length of 725 bp and additional 500 bp at 5'-UTR and 380 bp at 3'-UTR was amplified via PCR from the genomic DNA of *A. ustus* including 30 bp overhang on both 5' and 3' to the plasmid pMP024. Insertion of *psaCP* into the NotI-linearised expression plasmid pMP024 was carried out by using homologous recombination in *Escherichia coli* DH5α.[7]

To generate the deletion constructs pMP030, pMP038, pMP039, and pMP040, fragments of 5'-UTR and 3'-UTR in lengths of about 620 – 730 bp were amplified from genomic DNA from *P. roqueforti* for *anuC* deletion or *A. ustus* for deletion of *psaOX1*, *psaPS*, and *psaOX2*, respectively. The vector backbone including *ampR* and URA3 was amplified from pJN017 and *afpyrG* from pYH-wA-pyrG, respectively.[8] Assembly of fragments of 5'-UTR, 3'-UTR, vector backbone and *afpyrG* was achieved in yeast as mentioned above.[6]

For cloning of the deletion plasmid pMP043, the same strategy was used as for other deletion constructs described above. 5'-UTR and 3'-UTR of the putative gene ANIA_7635 from *A. nidulans*, the vector backbone including *ampR* and URA3, and *afpyro* from pYWB1[9] were fused via homologous recombination in yeast.[6]

To verify the plasmids, digestion with suitable restriction enzymes was conducted (Figure S1). The final plasmids, both expression and deletion constructs, were linearised by SwaI and transformed into *A. nidulans* protoplasts with a PEG-mediated method according to the protocols reported previously.[8,10]

Verification and cultivation of *A. nidulans* transformants

Selection of transformants was performed directly after transformation with the subsequent cultivation using riboflavin, uracil and uridine, and pyridoxine autotrophy. For the expression strain MP13 a visual control of conidia colour change from green to white provided a first hint of correct integration in the *wA* locus. Genomic DNA was isolated from both expression and deletion strains and the correct integration was verified by PCR. Primers for verification of expression strains are designed to bind upstream or downstream of the *wA* locus and within the desired gene(s). The same approach was used for the deletion strains. Primers bind upstream or downstream of the deleted gene and within the *afpyrG* or *afpyro* gene. Additionally, amplification from upstream to downstream of a deleted gene was carried out to

test its locus and to ensure its absence. The used primers are given in Table S4. The results of transformant verification are illustrated in Figure S1.

The correct transformants were cultivated in liquid PDB media for 14 days at 25 °C for detection of secondary metabolites. Samples of the cultures were extracted with equal volumes of EtOAC for three times and after evaporation dissolved in MeOH for LC-MS analysis.

Large-scale fermentation, extraction, and isolation of secondary metabolites

To isolate compounds **1** – **4**, 6 L of PDB medium were inoculated with spores from *A. nidulans* MP13 and cultivated for 14 days at 25 °C. The mycelia were separated from the medium by filtration and washed with water for three times. Extraction was carried out using equal volume of EtOAc for three times. The crude extract was obtained after evaporation under reduced pressure. 2.6 g crude extract were subjected to silica gel column chromatography (silica gel 60, 0.04 – 0.063 mm, Roth, Karlsruhe) and separation of secondary metabolites was achieved by elution with CH_2Cl_2/MeOH (100:10). The obtained 9 fractions were analysed with LC-MS and further purified using semi-preparative HPLC on an Agilent HPLC 1200 equipped with an XDB-C18 column (9.4 × 250 mm, 5 µm, VDS Optilab Chromatographie Technik GmbH, Bremen, Germany).

Fraction 3 was used to purify compounds **1** and **2**. 32 mg of compound **1** were obtained after isocratic elution (H_2O/CH_3CN = 50/50, flow rate = 2.0 mL/min, λ = 280 nm, t_R = 15.2 min). Further purification of fraction 3 resulted in 9 mg of compound **2** (H_2O/CH_3CN = 55/45, flow rate = 2.0 mL/min, λ = 310, 280 nm, t_R = 18.7 min). Compound **3** (16 mg) was isolated from fraction 2 by isocratic elution (H_2O/CH_3CN = 10/90, flow rate = 2.0 mL/min, λ = 340, 270 nm, t_R = 10.5 min). Fraction 4 was purified with isocratic elution (H_2O/CH_3CN = 68/32, flow rate = 2.0 mL/min, λ = 270 nm, t_R = 7 min), leading to the isolation of compound **4** (10 mg).

Cultivation of *A. nidulans* LO8030 with compounds 1 – 3

Compounds **1** – **3** were dissolved in DMSO to give 100 mM stock solutions. The *A. nidulans* strain LO8030 was cultivated in 4 flasks with 10 mL PDB media at 25 °C for 2 days. 2.5 µL of **1**, **2**, or **3** were added at day 3 (25 µM). After cultivation for another 1, 3, and 5 days samples were extracted with EtOAc and analysed on LC-MS. Additionally, compounds **1** – **3** were incubated with PDB medium and treated in the same procedure as described above to test their stability.

LC-MS analysis

For LC-MS monitoring of secondary metabolites, an Agilent 1260 HPLC equipped with VDSpher PUR100 C18-M-SE column (150 × 2.0 mm, 3 µm, VDS optilab Chromatographie Technik GmbH) and a Bruker microTOF QIII mass spectrometer was used as reported in a

previous publication.[3] Elution profile A corresponds to a method with a linear gradient of acetonitrile in water (5 – 100%) within 30 minutes. Elution profile B uses a linear gradient of acetonitrile in water (5 – 100%) within 10 minutes. A flow rate of 0.3 mL min^{-1} was used. For determination of the exact masses, positive ion mode electrospray ionization (ESI) was selected with a capillary voltage set to 4.5 kV and a collision energy of 8.0 eV. 5 mM sodium formate was used in each run for mass calibration. The masses were scanned in the range of *m/z* 100.000 – 1500.000. Evaluation of the data was conducted by using Compass DataAnalysis 4.2 software (Bruker Daltonik, Bremen, Germany).

NMR analysis

The NMR spectra of the purified compounds **1** – **4** were recorded on a JOEL ECA-500 MHz spectrometer (JOEL, Tokyo, Japan) with $CDCl_3$ as solvent. Analysis of all spectra was performed by using MestReNova 14.0.0 (Mestrelab Research).

Structural elucidation

The isolated compounds **1** – **4** were determined by comprehensive interpretation of their MS and NMR data (Figures S3–19). Compound **1** was assigned to 6-propyl salicyl alcohol by comparison of its NMR data with the reported data.[11] A singlet for two protons of the methylene group (C-7) was observed at δ_H 4.91 ppm in the ^1H NMR spectra and δ_C at 60.2 ppm in the $^{13}C\{^1H\}$ NMR spectra.[12] The structures of compounds **2** and **3** were deduced from **1** as alkylated salicylic acid and alkylated salicylaldehyde, respectively. A singlet for one proton of the aldehyde group of **3** was observed at δ_H 11.94 ppm and δ_C at 195.4 ppm.[12] Compound **4** showed signals for a dihydro-γ-pyrone moiety comparable to those described previously.[13] Signals for three methylene groups and one methyl group in the high field of the ^1H NMR (δ_H 0.91 – 2.21 ppm) and another methylene group at δ_H 3.83 ppm can be assigned to δ_C at 13.7 – 37.2 ppm and δ_C at 58.9 ppm, respectively, with HSQC correlations. Interpretation of the ^1H-^1H COSY spectra revealed the presence of a propyl side chain and a hydroxyethyl group at C-3. The HMBC data confirmed the connection of the hydroxyethyl group at C-3 and the propyl group at C-7 to the dihydro-γ-pyrone moiety.

Physicochemical properties

6-Propyl salicyl alcohol (**1**): white powder. UV (deduced from HPLC analysis) λ_{max} 230, 280 nm. ^1H NMR (500 MHz; $CDCl_3$): δ 7.09 (m, 1H), 6.73 (d, *J* = 8.2, 1H), 6.70 (d, *J* = 7.6, 1H), 4.91 (s, 2H), 2.55 (m, 2H), 1.52 (m, 2H), 0.94 (t, *J* = 7.3, 3H). ^{13}C NMR (125 MHz; $CDCl_3$): δ 156.7, 141.1, 128.9, 122.9, 121.8, 114.6, 60.2, 35.4, 24.9, 14.1. HRMS (ESI) m/z: $[M+Na]^+$ Calcd for $C_{10}H_{14}O_2Na$, 189.0886; found 189.0893.

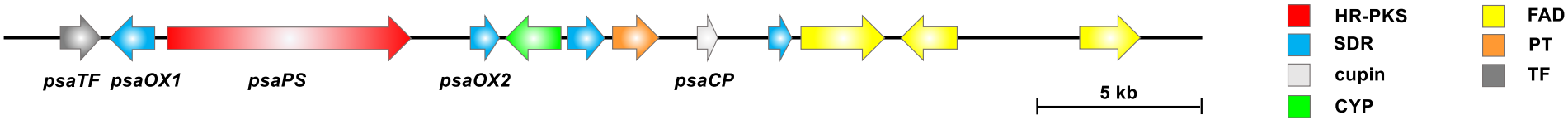
6-Propyl salicylic acid (**2**): white powder. UV (deduced from HPLC analysis) λ_{max} 248, 284, 314 nm. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.36 (m, 1H), 6.87 (d, *J* = 8.3, 1H), 6.77 (d, *J* = 7.6, 1H), 2.96 (m, 2H), 1.64 (m, 2H), 0.98 (t, *J* = 7.3, 3H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 175.5, 163.8, 147.5, 135.5, 123.0, 116.0, 110.6, 38.6, 25.3, 14.4. HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{10}H_{13}O_3$, 181.0859; found 181.0856.

6-Propyl salicylaldehyde (**3**): pale yellow oil. UV (deduced from HPLC analysis) λ_{max} 228, 270, 340 nm. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 11.94 (1H, s), 10.30 (s, 1H), 7.40 (m, 1H), 6.82 (d, *J* = 8.5, 1H), 6.73 (d, *J* = 7.5, 1H), 2.89 (m, 2H), 1.68 (m, 2H), 0.99 (t, *J* = 7.3, 3H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 195.4, 163.5, 147.3, 137.5, 121.4, 118.0, 116.1, 33.9, 26.4, 14.0. HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{10}H_{13}O_2$, 165.0910; found 165.0909.

(**4**): white powder. UV (deduced from HPLC analysis) λ_{max} 224, 270 nm. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 5.33 (s, 1H), 4.57 (ddt, *J* = 12.8, 8.5, 4.3, 1H), 3.83 (m, 2H), 2.46 (dd, *J* = 16.8, 12.9, 1H), 2.40 (dd, *J* = 16.8, 4.3, 1H), 2.21 (m, 2H), 2.06 (m, 1H), 1.90 (m, 1H), 1.57 (m, 2H), 0.94 (t, *J* = 7.3, 3H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 193.1, 177.6, 104.5, 77.0, 58.9, 41.2, 37.2, 36.8, 19.9, 13.7. HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{10}H_{17}O_3$, 185.1172; found 185.1164.

Supplementary Tables

Table S1. Sequence identities of proteins encoded by *psa* cluster from *A. ustus* and ANIA_7635 from *A. nidulans* with those of known clusters



	A. ustus **PsaOX1** **300 aa**	***A. ustus*** **PsaPS (KIA75504)** **2388 aa**	***A. ustus*** **PsaOX2 (KIA75505)** **278 aa**	***A. ustus*** **PsaCP (KIA75508)** **189 aa**	***A. nidulans*** **ANIA_7635** **144 aa**
srd cluster *Neurospora crassa*	59%, SrdE, (XP_965604) 290 aa	46%, SrdA, (XP_965600) 2382 aa	49%, SrdC, (XP_965602) 272 aa	49%, SrdD (XP_965603) 196 aa	39%, SrdD, (XP_965603) 196 aa
vir cluster *Trichoderma virens*	57%, VirD, (XP_013952635) 287 aa	41%, VirA, (XP_013952638) 2381 aa	44%, VirB, (XP_013952637) 249 aa	54%, VirC (XP_013952636) 194 aa	43%, VirC, (XP_013952636) 194 aa
fog cluster *Aspergillus ruber*	58%, FogD, (XP_040639027) 286 aa	43%, FogA, (XP_040639024) 2403 aa	48%, FogB, (XP_040639025) 273 aa	54%, FogC (XP_040639026) 203 aa	41%, FogC, (XP_040639026) 203 aa
anu cluster *Penicillium roqueforti*	63%, AnuB, (CDM34451) 284 aa	65%, AnuA, (CDM34450) 2360 aa	74%, AnuF, (CDM34455) 276 aa	75%, AnuC (CDM34452) 190 aa	41%, AnuC, (CDM34452) 190 aa
str cluster *Stachybotrys* sp.	54%, StrD, (BDX35604) 288 aa	44%, StrA, (BDX35601) 2426 aa	48%, StrB, (BDX35602) 273 aa	54%, StrC (BDX35603) 207 aa	38%, StrC, (BDX35603) 207 aa

Accession numbers are given in parenthesis.

Table S2. Strains used in this study

Strain	Genotype	Created with plasmid	Reference
E. coli DH5α	F- *endA1 glnV44 thi-1 recA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17($r_K^-m_K^+$), λ^-	-	[14]
S. cerevisiae HOD114-2B	*MATα ura3-52 his3Δ1 leu2-3112*	-	[6]
P. roqueforti FM164	wildtype	-	[15]
A. ustus 3.3904	wildtype	-	CGMCCC
A. nidulans			
LO8030	*pyroA4, riboB2, AfpyrG89, nkuA::argB*, deletion of secondary metabolite clusters: (AN7804-AN7825)Δ, (AN2545-AN2549)Δ, (AN1039-AN1029)Δ, (AN10023-AN10021)Δ, (AN8512- AN8520)Δ, (AN8379-AN8384)Δ, (AN9246-AN9259)Δ, (AN7906-AN7915)Δ, (AN6000-AN6002)Δ.	-	[16]
BK06	*wA*-PKS::*gpdA*(p) + 500bp 3'UTR-*Afribo* in LO8030	-	[10]
BK08	*wA*-PKS*::gpdA*(p)-annullatin cluster (PROQFM164_S03g001173 − PROQFM164_S03g001183 + 502 bp 3'UTR):*Afribo* in LO8030	-	[17]
MP13	*wA*-PKS::*gpdA*(p)-*psaTF-psaOX1-psaPS-psaOX2*:*Afribo* in LO8030	pMP024	This study
MP15	Δ*anuC*::*AfpyrG* in BK08	pMP030	This study
MP22	Δ*psaPS*::*AfpyrG* in MP13	pMP039	This study
MP23	Δ*psaOX1*::*AfpyrG* in MP13	pMP038	This study
MP24	Δ*psaOX2*::*AfpyrG* in MP13	pMP040	This study
MP26	ΔANIA_7635::*AfpyroA* in MP13	pMP043	This study
MP31	wA-PKS::gpdA(p)- *psaTF-psaOX1-psaPS-psaOX2*-Afribo-500 bp 5'-UTR-*PsaCP*-380 bp 3'-UTR in LO8030	pMP046	This study

CGMCCC: China General Microbiological Culture Collection Center (Beijing, China)

Table S3. Plasmids used in this study

Plasmid	Genotype	Description	Reference
pJN017	*URA3, wA* flanking, *gpdA(p), AfriboB, Amp*	standard-vector for heterologous expression in *A. nidulans* LO8030	[5]
pMP024	*URA3, wA* flanking, *gpdA(p), psaTF-psaOX1-psaPS-psaOX2*, *AfriboB, Amp*	Heterologous expression of *psaTF*, *psaOX1*, *psaPS*, and *psaOX2* in *A. nidulans* LO8030	This study
pMP030	*URA3,* 622 bp 5'-UTR and 609 bp 3'-UTR PCR fragment of *anuC, AfpyrG, Amp*	Deletion of *anuC* in BK08	This study
pMP038	*URA3,* 700 bp 5'-UTR and 635 bp 3'-UTR PCR fragment of *psaOX1, AfpyrG, Amp*	Deletion of *psaOX1* in MP13	This study
pMP039	*URA3,* 730 bp 5'-UTR and 728 bp 3'-UTR PCR fragment of *psaPS, AfpyrG, Amp*	Deletion of *psaPS* in MP13	This study
pMP040	*URA3,* 696 bp 5'-UTR and 623 bp 3'-UTR PCR fragment of *psaOX2, AfpyrG, Amp*	Deletion of *psaOX2* in MP13	This study
pMP043	*URA3,* 764 bp 5'-UTR and 783 bp 3'-UTR PCR fragment of ANIA_7635, *AfpyroA, Amp*	Deletion of ANIA_7635 in MP13	This study
pMP046	*URA3, wA* flanking, *gpdA(p), psaTF-psaOX1-psaPS-psaOX2, AfriboB,* 500 bp 5'-UTR, *psaCP*, 380 bp 3'-UTR, *Amp*	Heterologous expression of *psaTF*, *psaOX1*, *psaPS*, *psaOX2,* and *psaCP* in *A. nidulans* LO8030	This study

Table S4. Oligonucleotide primers used in this study

Primers	Sequence (5‘-3‘)	Description
MP22F1for	CTAACAGCTACCCCGCTTGAGCAGACATCACCGGCATGATGTCGGTTTCGCTTCC	Cloning *psaTF*, *psaOX1*, *psaPS*, and *psaOX2* in pJN017 = pMP024
MP22F1rev	TGACATGAGAGCCAGATCCATCTC	
MP22F2for	GCTACATTCGACTTCAGCGATACC	
MP22F2rev	GAGTGTCGTAAACCAGGTGGTG	
MP22F3for	GTGTAGATGGGATGGTTTGCTTG	
MP24F3rev	GCCTCAACACCATATTTTAATCCCATGTGGGCCTAAAACTGCTGAACCTGCAAGG	
SSt81	GCGAGCCTTCCATAGTTACG	Verification of MP13
MP22F1midrev	GCGTTCCAGTTTCCGGAATTG	
MP24SDRoutfor	CACCTTGCAGGTTCAGCAG	
JN015ctrl_01	GCACTCTGGAAACGAACTCC	
MPDelAmpfor	CAGGGGATAACGCAGG	Amplification of the backbone for cloning of deletion plasmids
MPDelURArev	ACACAGGAAACAGCTATGAC	
MPpyrGfor	GAGAGTTATTCTGTGTCTG	Amplification of *afpyrG* as selection marker for deletion
MPpyrGrev	ATTCTGTCTGAGAGGAG	
MPAfpyrofor	GTAATGTAAGGTCAGTTCGAGACCATC	Amplification of *afpyro* as selection marker for deletion
MPAfpyrorev	GATCGTAACTCCGTAGGATGTGTACC	
MPanuCupfor	TTCGTAATCATGGTCATAGCTGTTTCCTGTGTCAGATCAGCATTGTCTCCATTCG	Amplification of the upstream and downstream regions of *anuC* for pMP030; together with MPDelAmpfor, MPDelURArev, MPpyrGfor, and MPpyrGrev
MPanuCuprev	ACACAACATATTTCGTCAGACACAGAATAACTCTCGCGGACATGGCAATTTTCCC	
MPanuCdwfor	GGCATCACGCATCAGTGCCTCCTCTCAGACAGAATTCACCGCAAGTGAAGCCATC	
MPanuCdwrev	TTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGGAACAGCGACAAGGCTAGAC	
BK177	ACATTGGTCGCAGCTATTGC	Verification of MP15; proving absence of the gene with MPanuCupfor and MPanuCdwrev
MPpyrG5’rev	GCTAGAATGGGGTAGACAGGC	
MPpyrG3’for	CAGTGCCTCCTCTCAGACAG	
MPanuBdwfor	GCATCACGCATCAGTGCCTCCTCTCAGACAGAATGCGGTACAATGGCTAACATCC	
MP43upfor	TGAATTCGTAATCATGGTCATAGCTGTTTCCTGTGTGTCACGTAACGTAACGGCA	Amplification of the upstream and downstream regions of ANIA_7635 for pMP043; together with MPDelAmpfor, MPDelURArev, MPpyrofor, and MPpyrorev
MP43uprev	TATCAGATGGTCTCGAACTGACCTTACATTACCATTTTCGAAGTTTGGTCTCTGG	
MP43dwfor	TAGACGTCAAGGTACACATCCTACGGAGTTACGATCCAAAGTGCTGTCCATTCGC	
MP43dwrev	TTGCTCACATGTTCTTTCCTGCGTTATCCCCTGCAGGTCGTTGAGAAGATGATGG	
MPpyro5’rev	AGATGGTCTCGAACTGACCTTAC	Verification of MP26; for 5’: MP43upfor with MPpyro5’rev
MPpyro3’for	GTACACATCCTACGGAGTTACGATC	
MP43dwextrev	GATATGTGCGGAGAAATTGTTGAGG	

Table S4. Oligonucleotide primers used in this study (continued)

MPSDRaupfor	TGAATTCGTAATCATGGTCATAGCTGTTTCCTGTGTGAGGGGACTGGCACGGATA	Amplification of the upstream and downstream regions of *psaOX1* for pMP038; together with MPDelAmpfor, MPDelURArev, MPpyrGfor, and MppyrGrev; Verification of MP23: 5' with MPSDRaupfor and MPpyrG5'rev; 3' with pyrG3'for and MPPKSuprev; gene absence with MPSDRaupfor and MPSDRadwrev
MPSDRauprev	CAACATATTTCGTCAGACACAGAATAACTCTCGTCTGTATCCTTGCACTGACACT	
MPSDRadwfor	GGCATCACGCATCAGTGCCTCCTCTCAGACAGAATCTGAATTCCAGAGCCAGAGC	
MPSDRadwrev	CCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGGGTGACTTGCGGCGAATG	
MPPKSupfor	CGTAATCATGGTCATAGCTGTTTCCTGTGTCCTGACTTGAAATACACCAATTCCG	Amplification of the upstream and downstream regions of *psaPS* for pMP039; together with MPDelAmpfor, MPDelURArev, MPpyrGfor, and MppyrGrev; Verification of MP22: 5' with MPSDRadwfor and MPpyrG5'rev; 3' with pyrG3'for and MPSDRbuprev; gene absence with MPPKSupfor and MPPKSdwrev
MPPKSuprev	CAACATATTTCGTCAGACACAGAATAACTCTCGTAGAAACTGTCGACGTTGAAGC	
MPPKSdwfor	ATCACGCATCAGTGCCTCCTCTCAGACAGAATGTGTAGATGGGATGGTTTGCTTG	
MPPKSdwrev	CTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGCTGGCCATTTGAGCGAGGA	
MPSDRbupfor	ATTCGTAATCATGGTCATAGCTGTTTCCTGTGTGATATGGCAACTTACTTGGCGC	Amplification of the upstream and downstream regions of *psaOX2* for pMP040; together with MPDelAmpfor, MPDelURArev, MPpyrGfor, and MppyrGrev; Verification of MP24: 5' with MP22F3for and MPpyrG5'rev; 3' with pyrG3'for and JN015ctrl_01; gene absence with MPSDRbupfor and MPSDRbdwrev
MPSDRbuprev	ACAACATATTTCGTCAGACACAGAATAACTCTCGTGCCATGATGTAAATGGTGTC	
MPSDRbdwfor	GCATCACGCATCAGTGCCTCCTCTCAGACAGAATGATTACAATGTCAACAGCGCG	
MPSDRbdwrev	TTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGGGCCTAAAACTGCTGAACCTG	
MP46cupinfor	AATCCTATAAATTGGGAAAACTAGAGACCCCGGTCGCGTTGTTGAAACGGGATTG	Cloning of *psaCP* in pMP024
MP46cupinrev	TCGCCCTTTGTCATAGTAAAGTGATTCGCGTCATGCCTTGTACGCTGCAACTCAT	
MP46cupinfor NotI	GCGGCCGCGCGTTGTTGAAACGGGATTG	Verification of MP31, proving presence of *psaCP*; amplification of 5' with SSt81 and MP22F1midrev; 3' with MP24SDRoutfor and JN015ctrl_01
MP46cupinrev NotI	GCGGCCGCCCTTGTACGCTGCAACTCAT	

Supplementary Figures

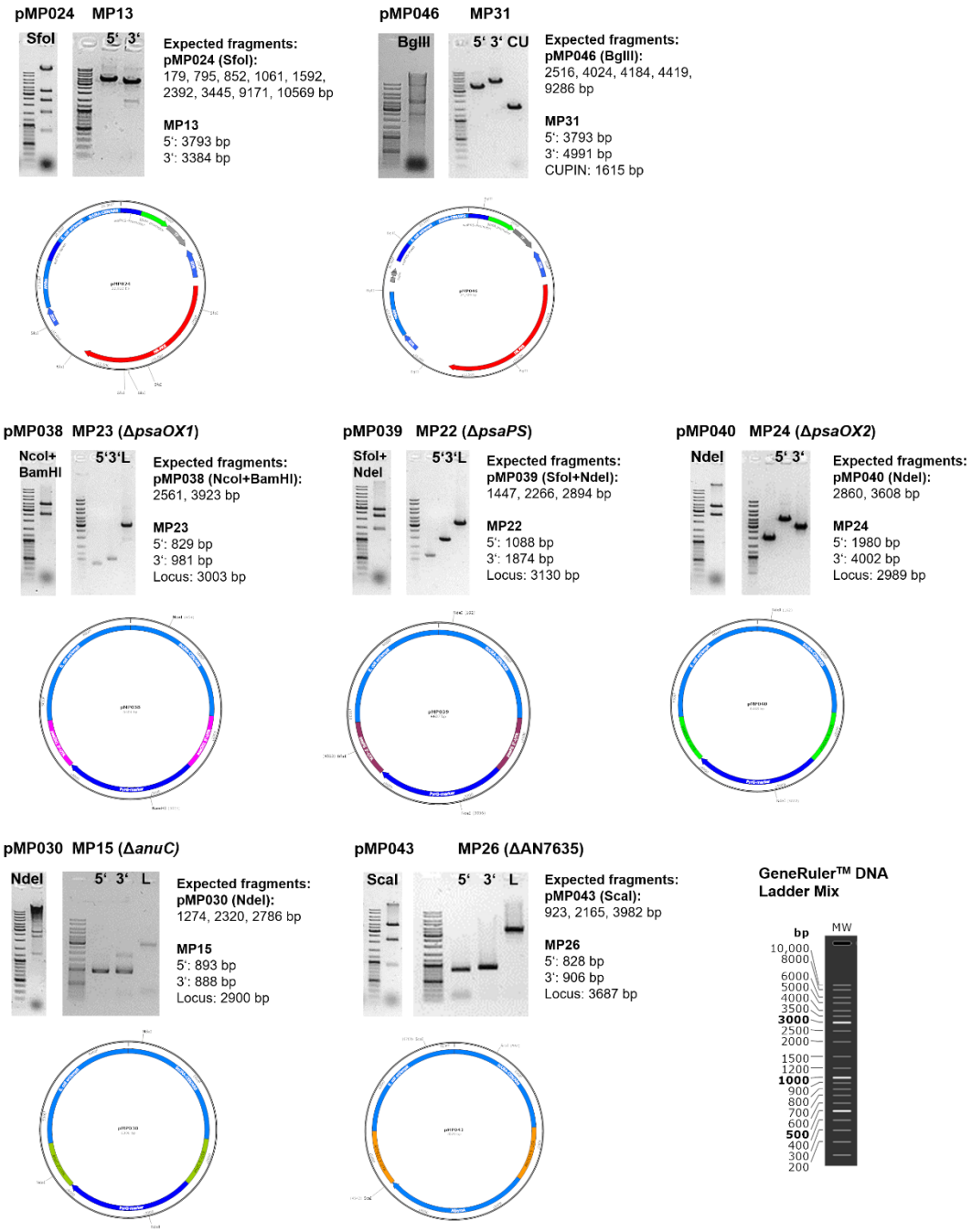


Figure S1. PCR verification of plasmids for heterologous expression and deletion constructs as well as of the obtained mutants.

To prove the presence of the target genes, PCR verifications were performed with genomic DNA of the heterologous expression (HE) *A. nidulans* transformants. The primers were designed to bind outside of the *wA* locus and inside the desired gene(s). To verify the deletion strains, the correct site-specific integration (corresponding 5'- and 3'-regions) and amplification of the locus were tested, to prove the absence of the gene and the presence of the inserted selection marker. The GeneRuler DNA Ladder mix from Thermo Fisher served as size standard for DNA fragments. The primers are given in Table S4.

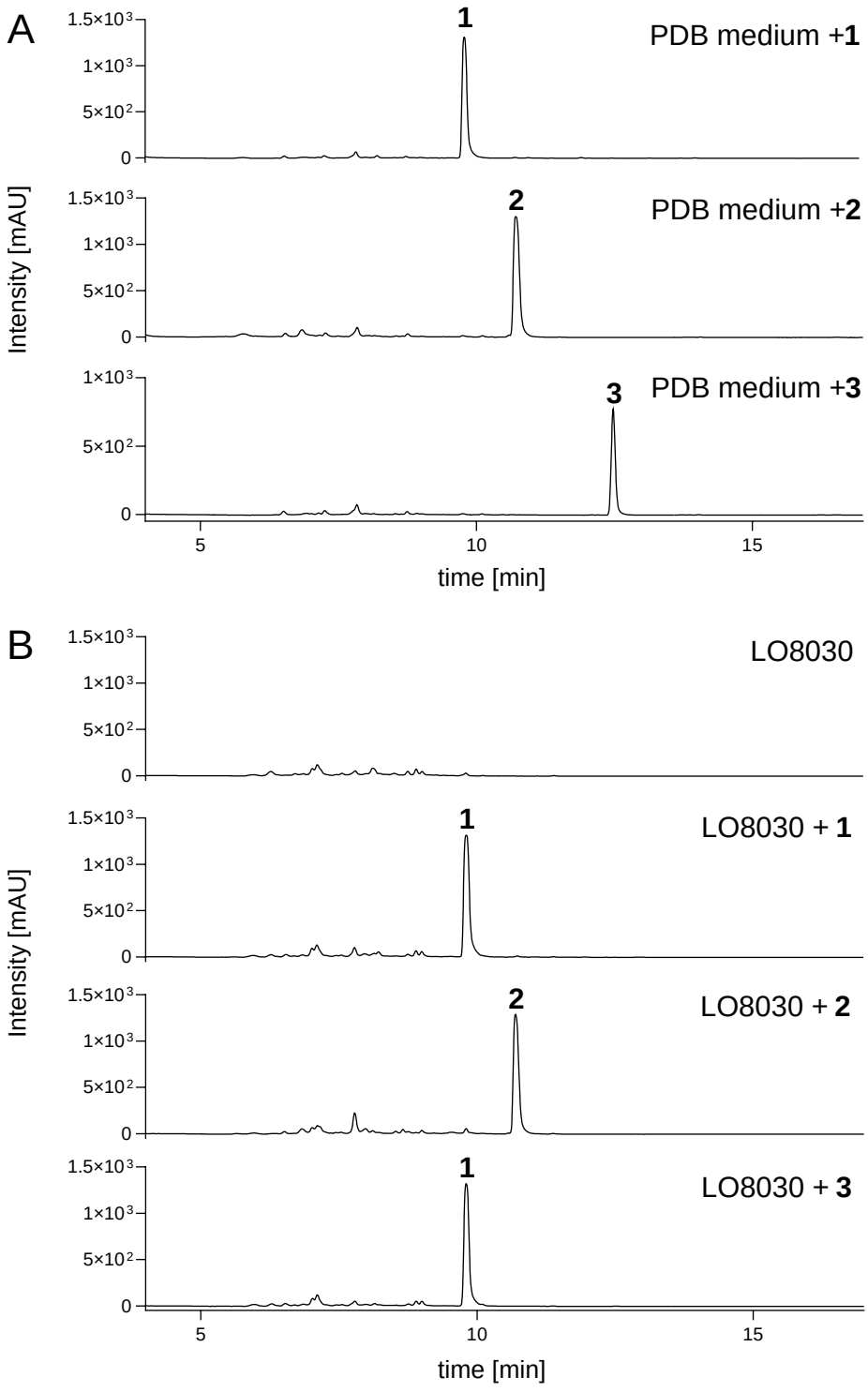


Figure S2. LC-MS analysis of compounds **1** – **3** after incubation in PDB medium or cultivation with *A. nidulans* LO8030.

Incubation of PDB medium with compounds **1** – **3** (A) and cultivation of *A. nidulans* LO8030 with compounds **1** – **3** for 1 day. The analysis was carried out with elution profile B and UV absorptions are illustrated at 270 – 340 nm.

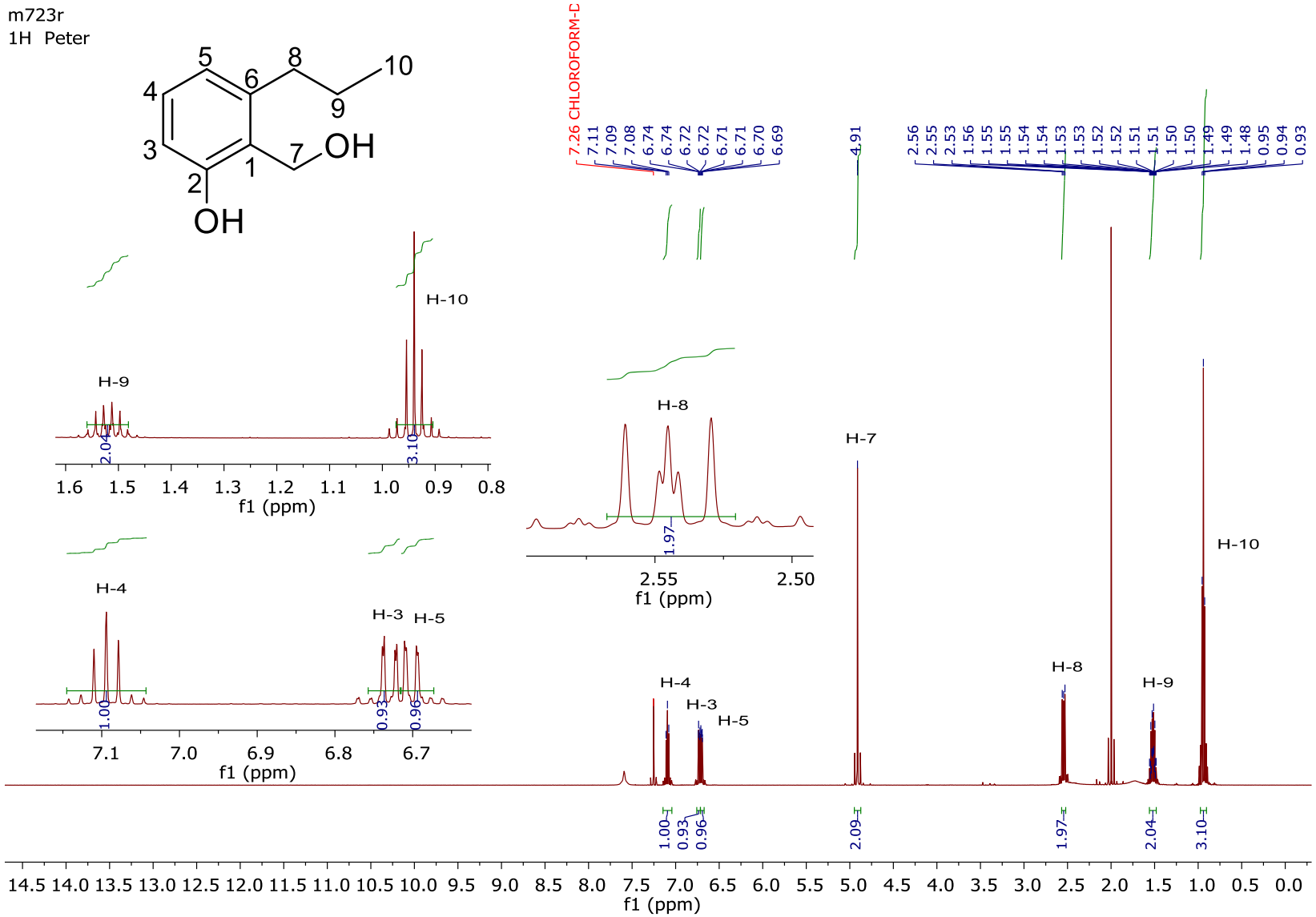


Figure S3. ^{1}H NMR spectrum of **1** in $CDCl_3$ (500 MHz).

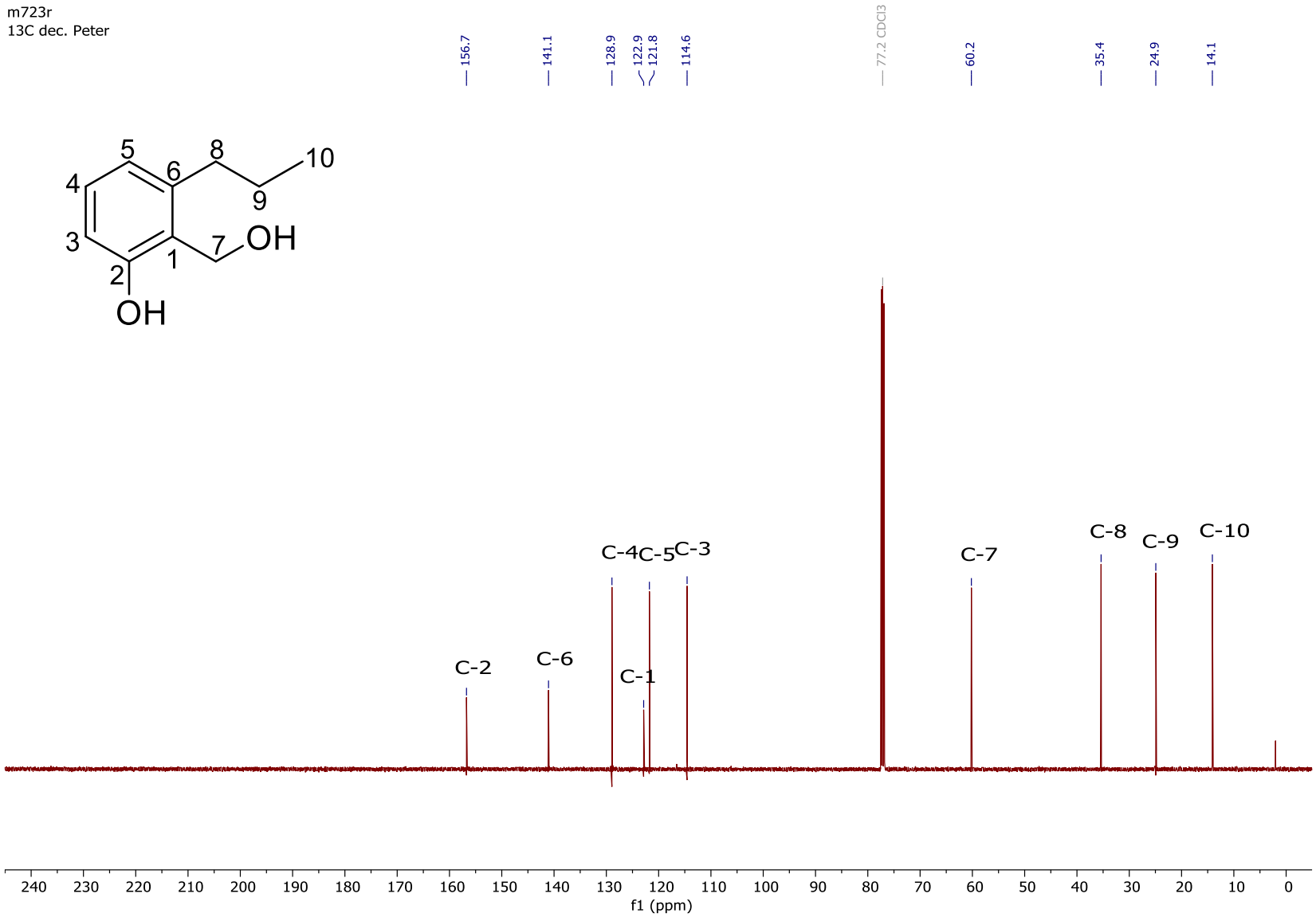


Figure S4. ^{13}C NMR spectrum of **1** in $CDCl_3$ (125 MHz).

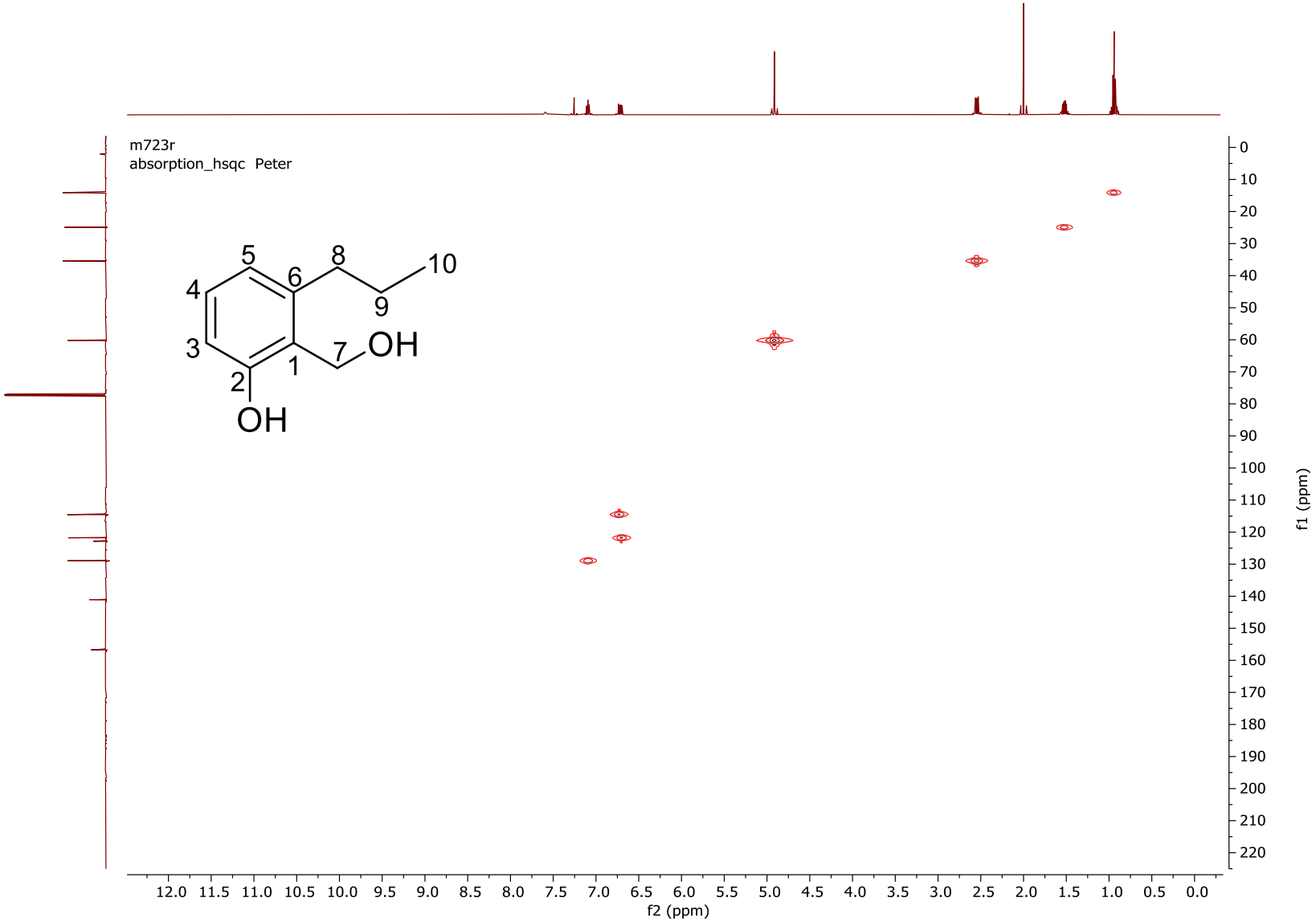


Figure S5. HSQC spectrum of **1** in $CDCl_3$.

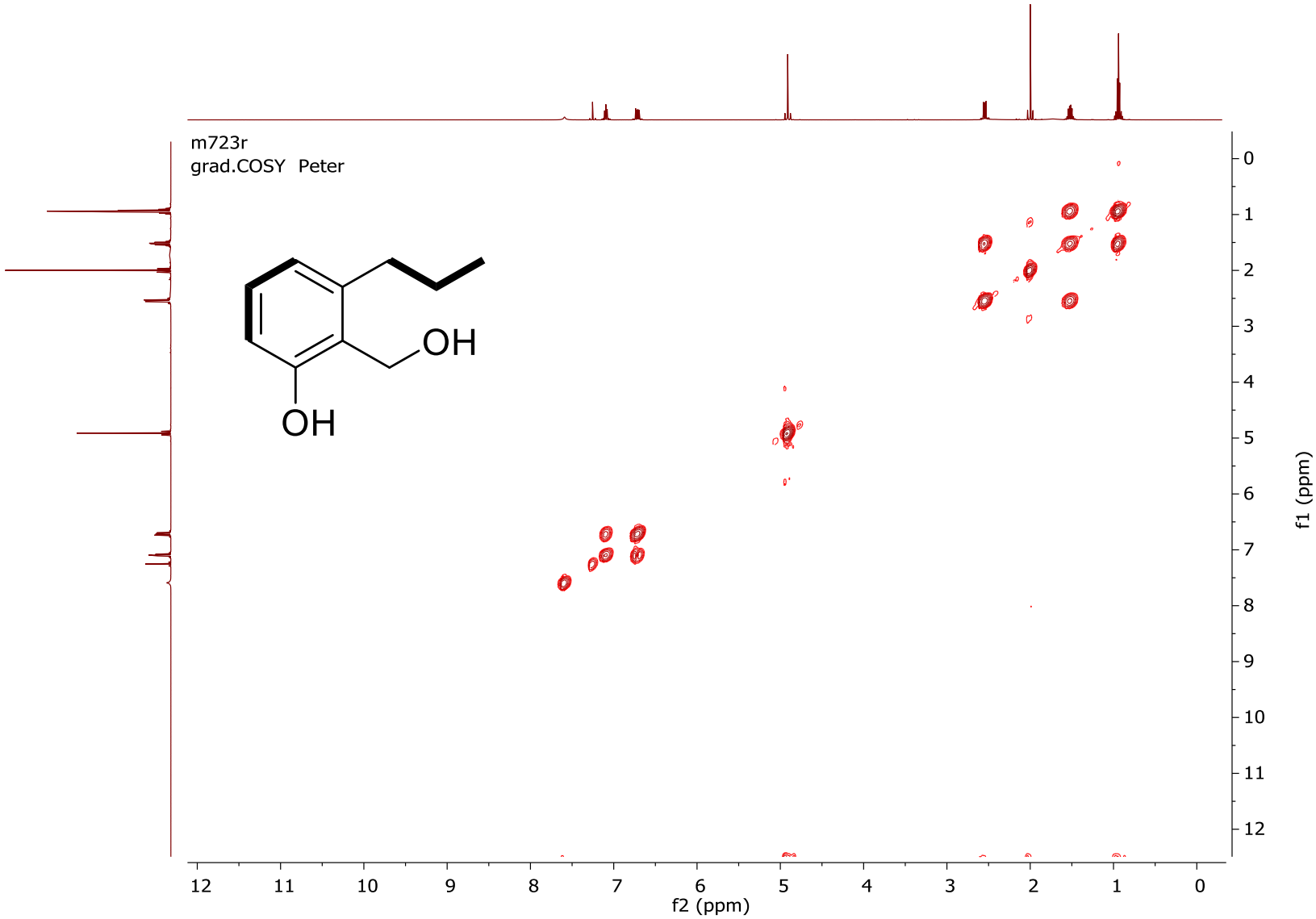


Figure S6. ^{1}H-^{1}H COSY spectrum of **1** in $CDCl_3$.

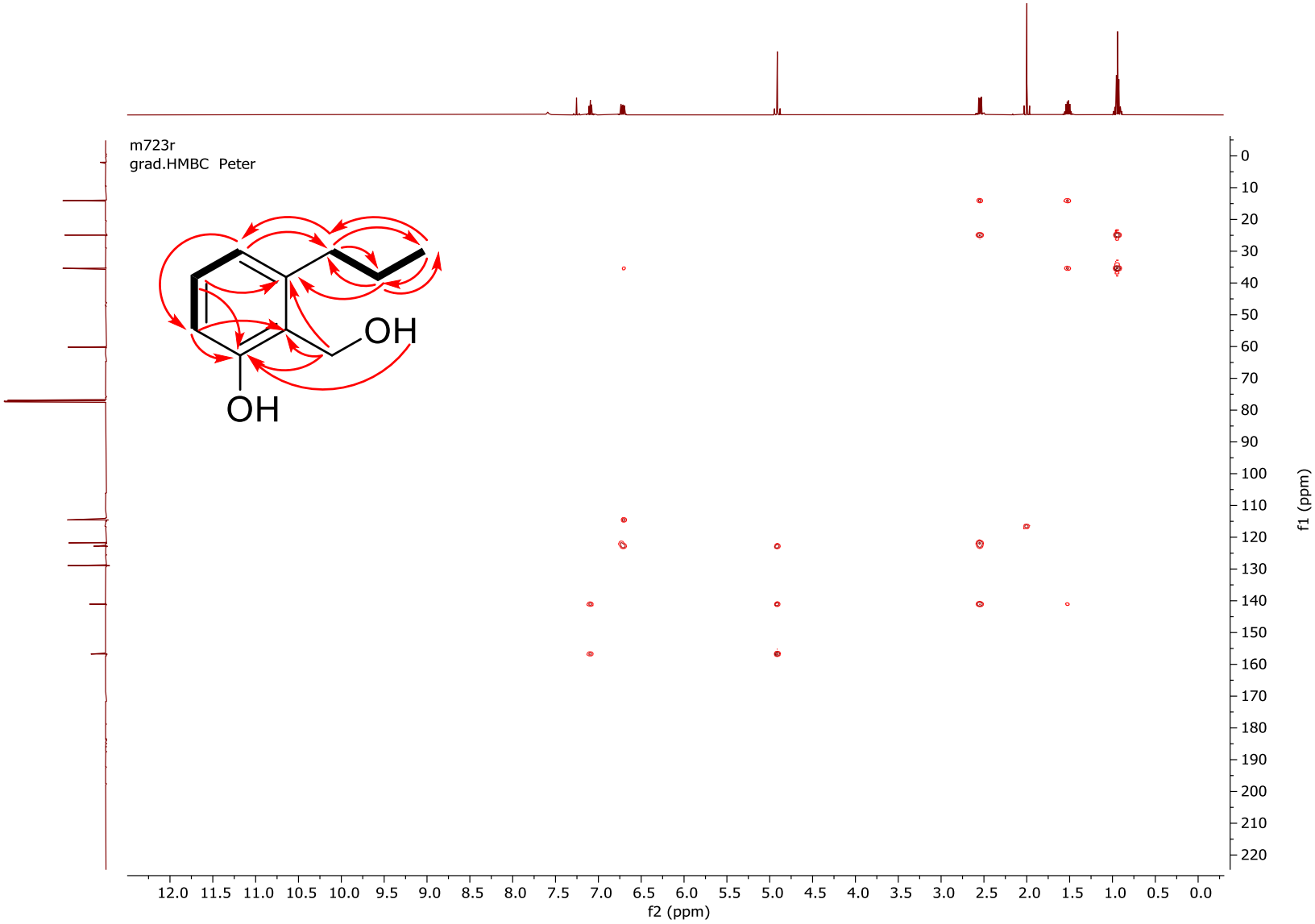


Figure S7. HMBC spectrum of **1** in $CDCl_3$.

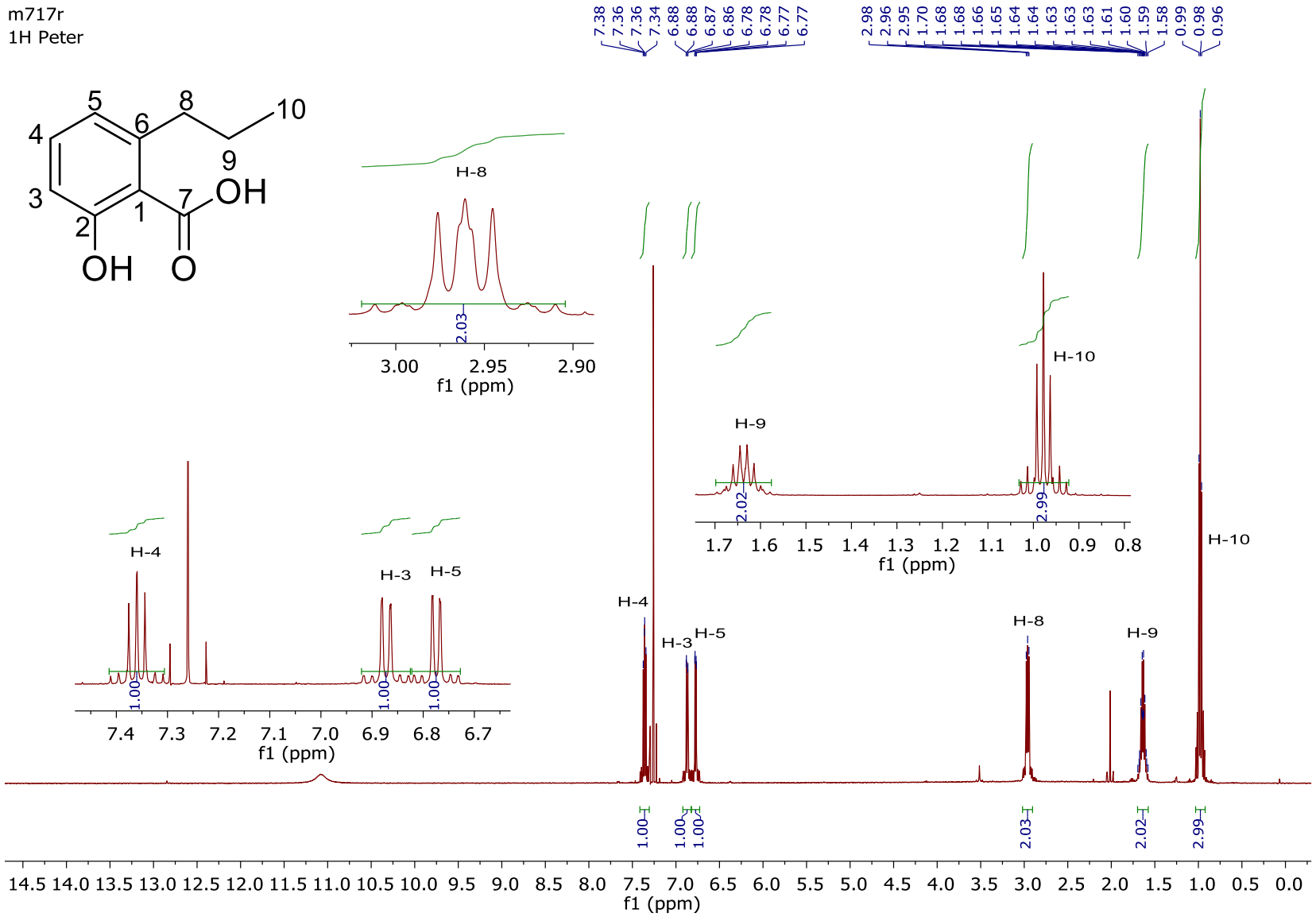


Figure S8. ^{1}H NMR spectrum of **2** in $CDCl_3$ (500 MHz).

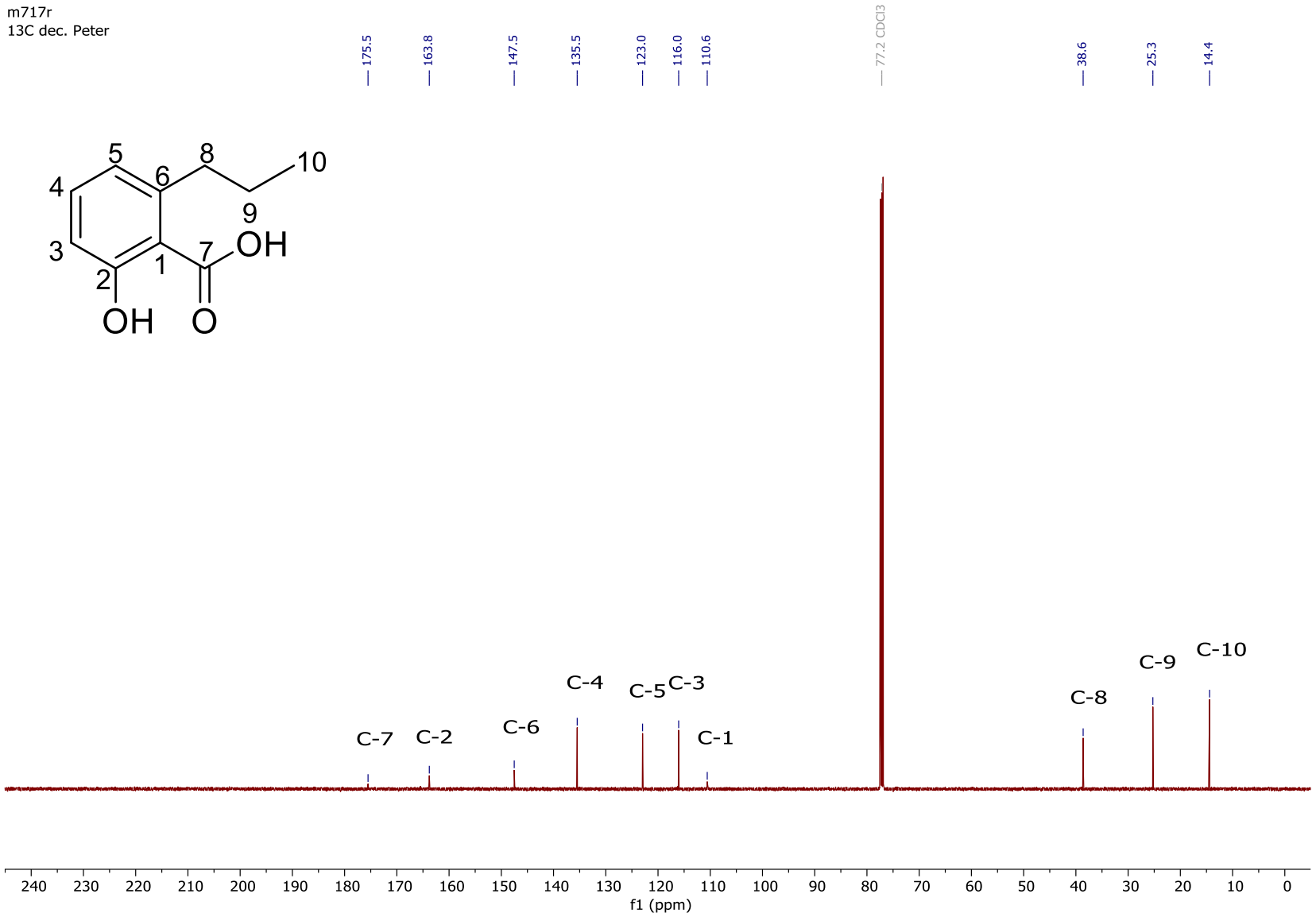


Figure S9. ^{13}C NMR spectrum of **2** in $CDCl_3$ (125 MHz).

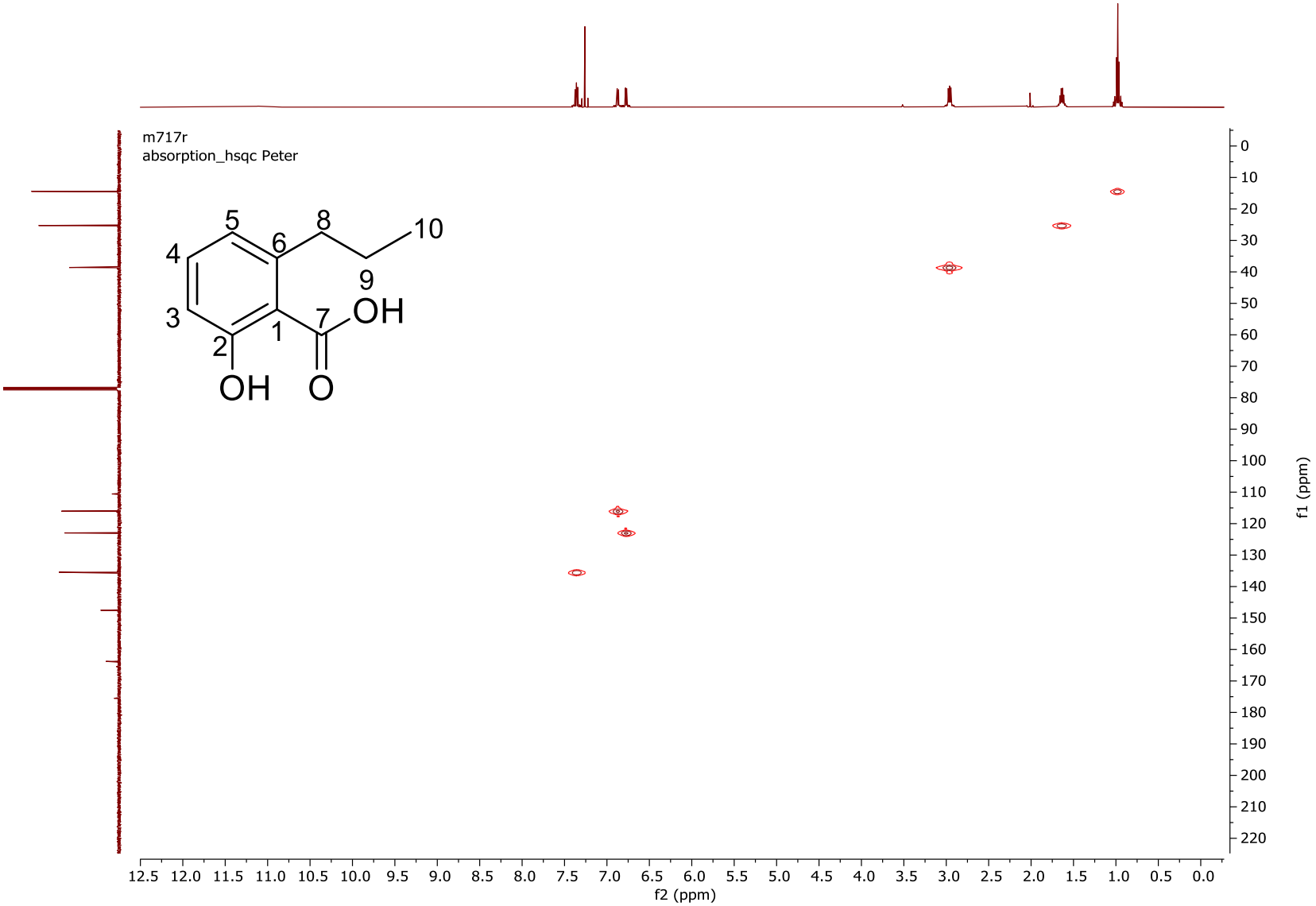


Figure S10. HSQC NMR spectrum of **2** in $CDCl_3$.

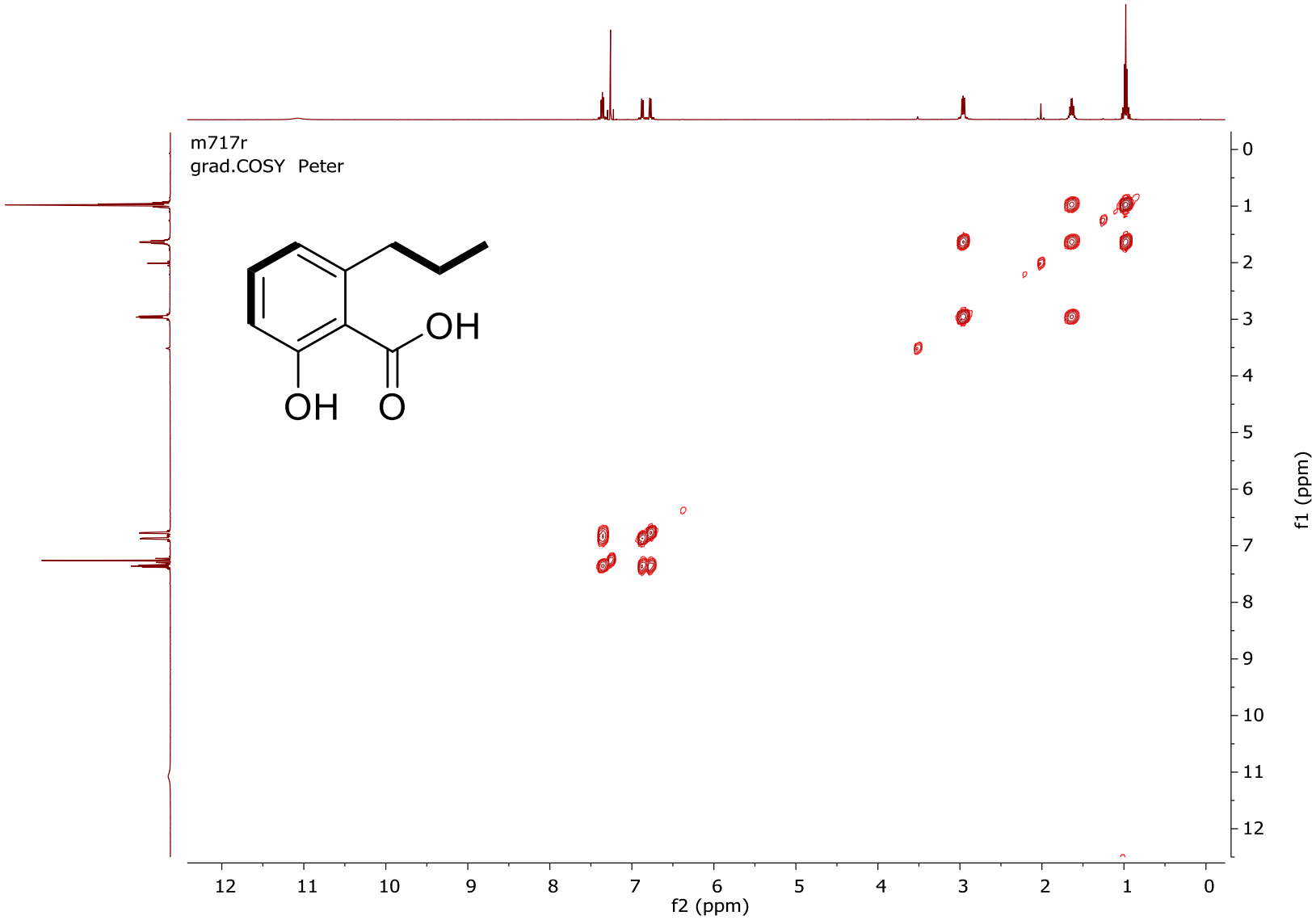


Figure S11. ^{1}H-^{1}H COSY NMR spectrum of **2** in $CDCl_3$.

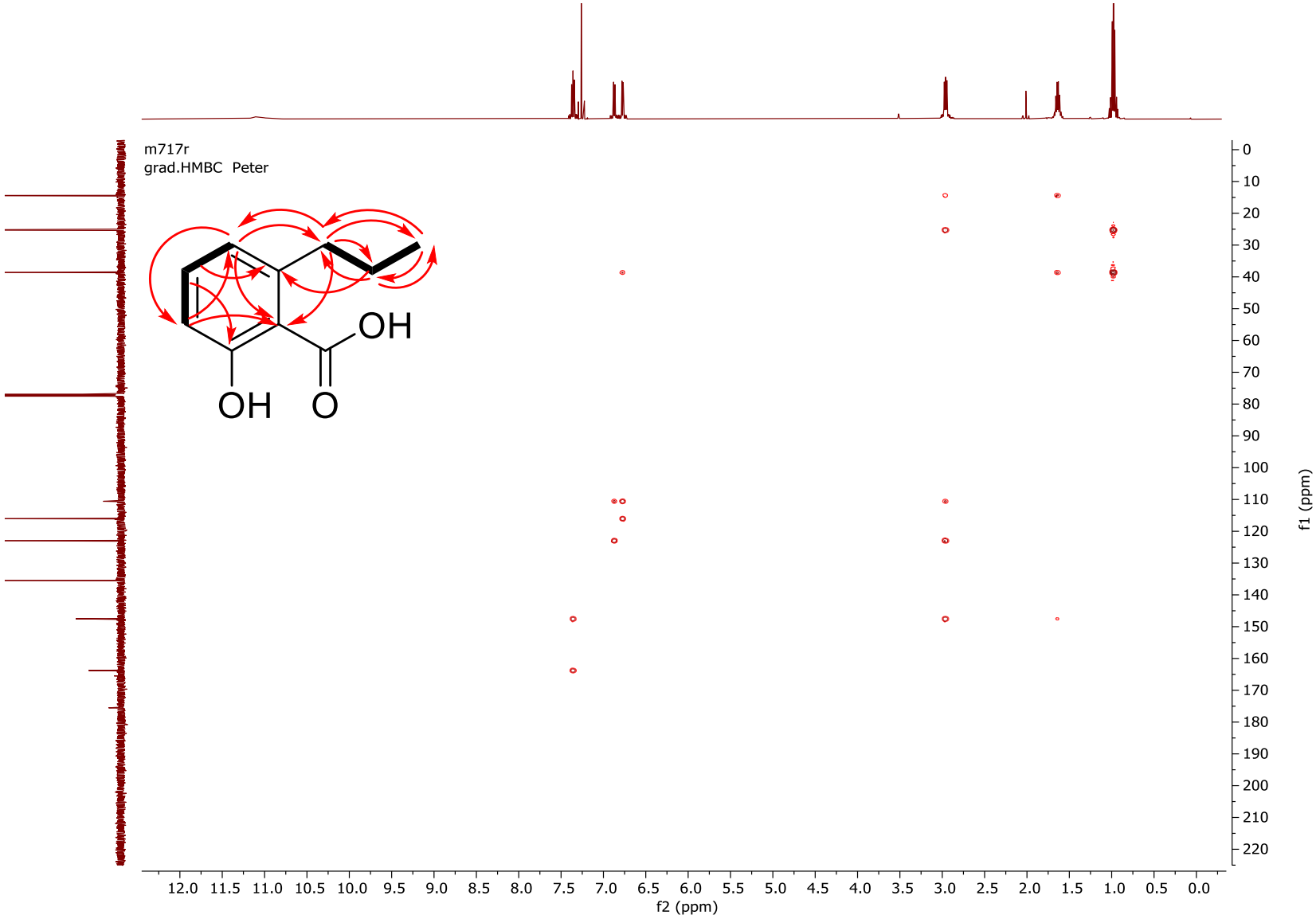


Figure S12. HMBC NMR spectrum of **2** in $CDCl_3$.

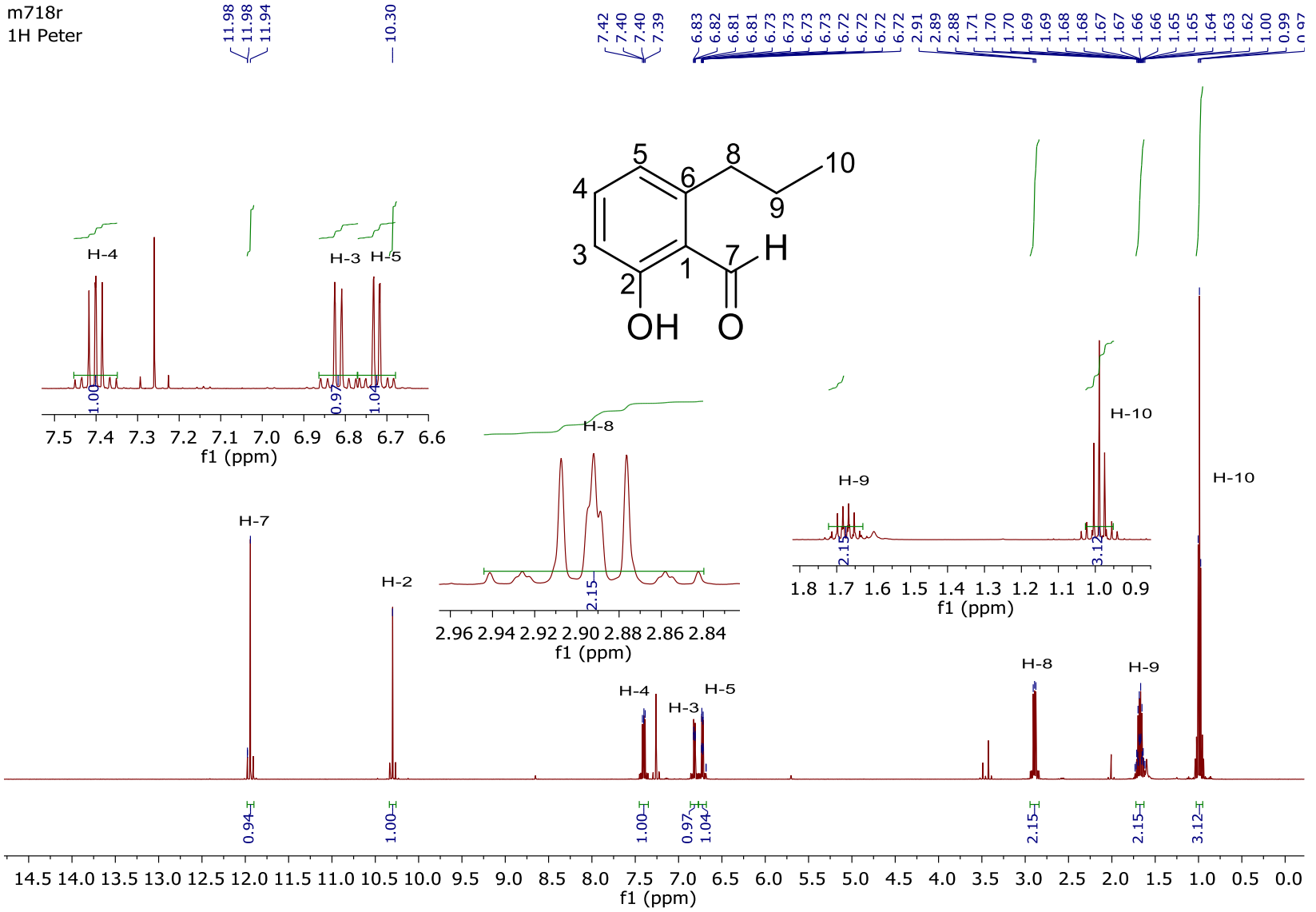


Figure S13. ^{1}H NMR spectrum of **3** in $CDCl_3$ (500 MHz).

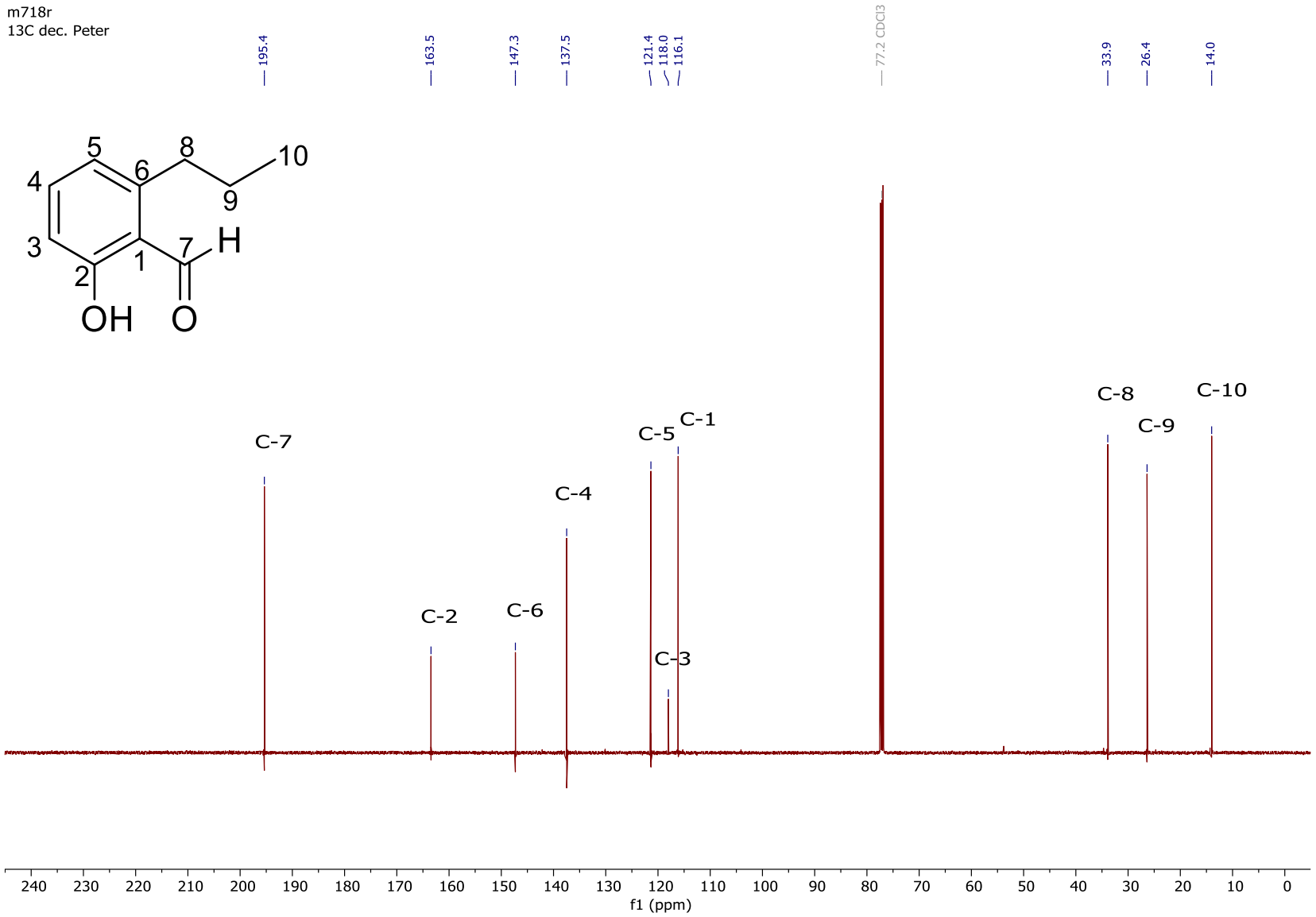


Figure S14. ^{13}C NMR spectrum of **3** in $CDCl_3$ (125 MHz).

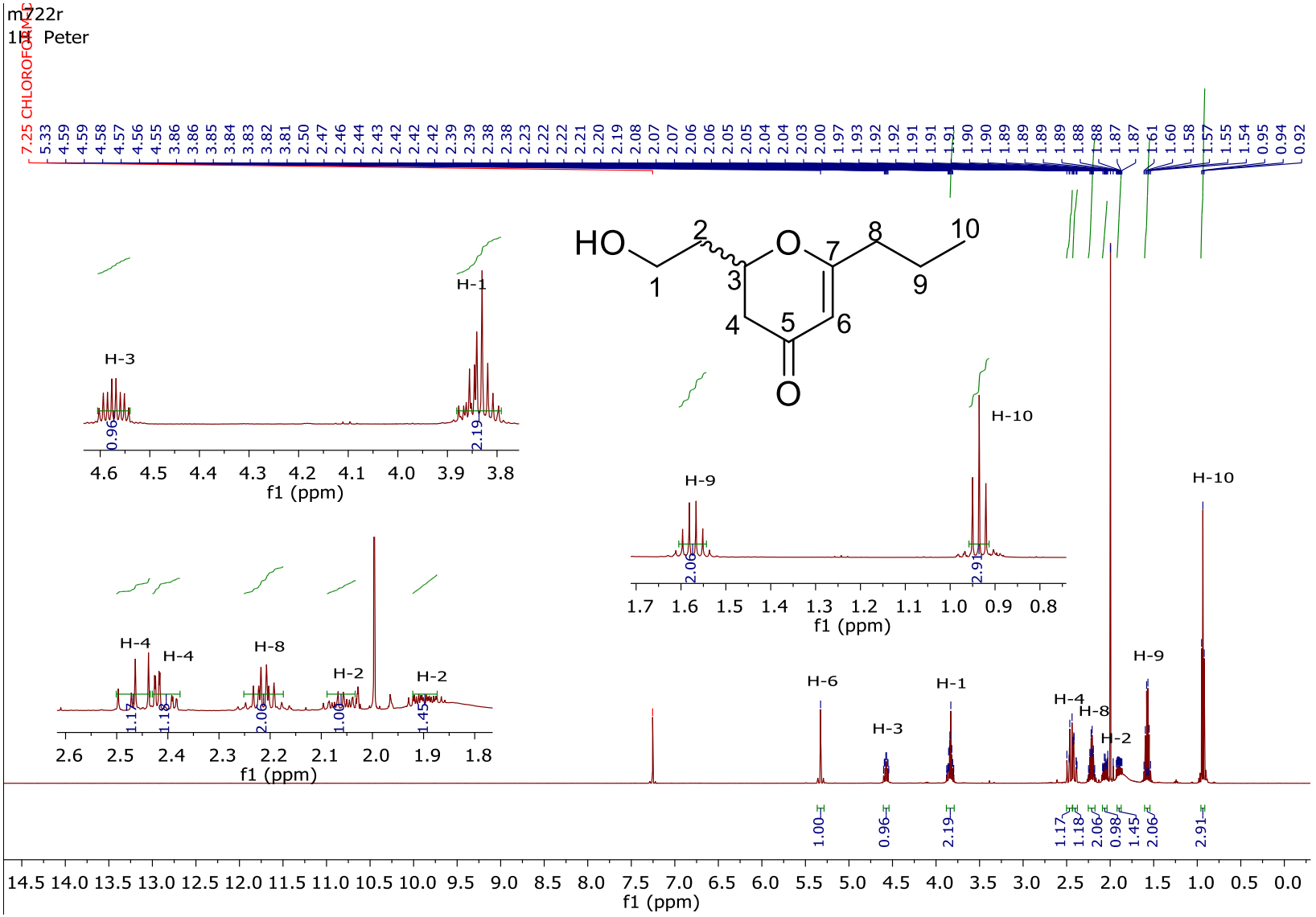


Figure S15. ^{1}H NMR spectrum of **4** in $CDCl_3$ (500 MHz).

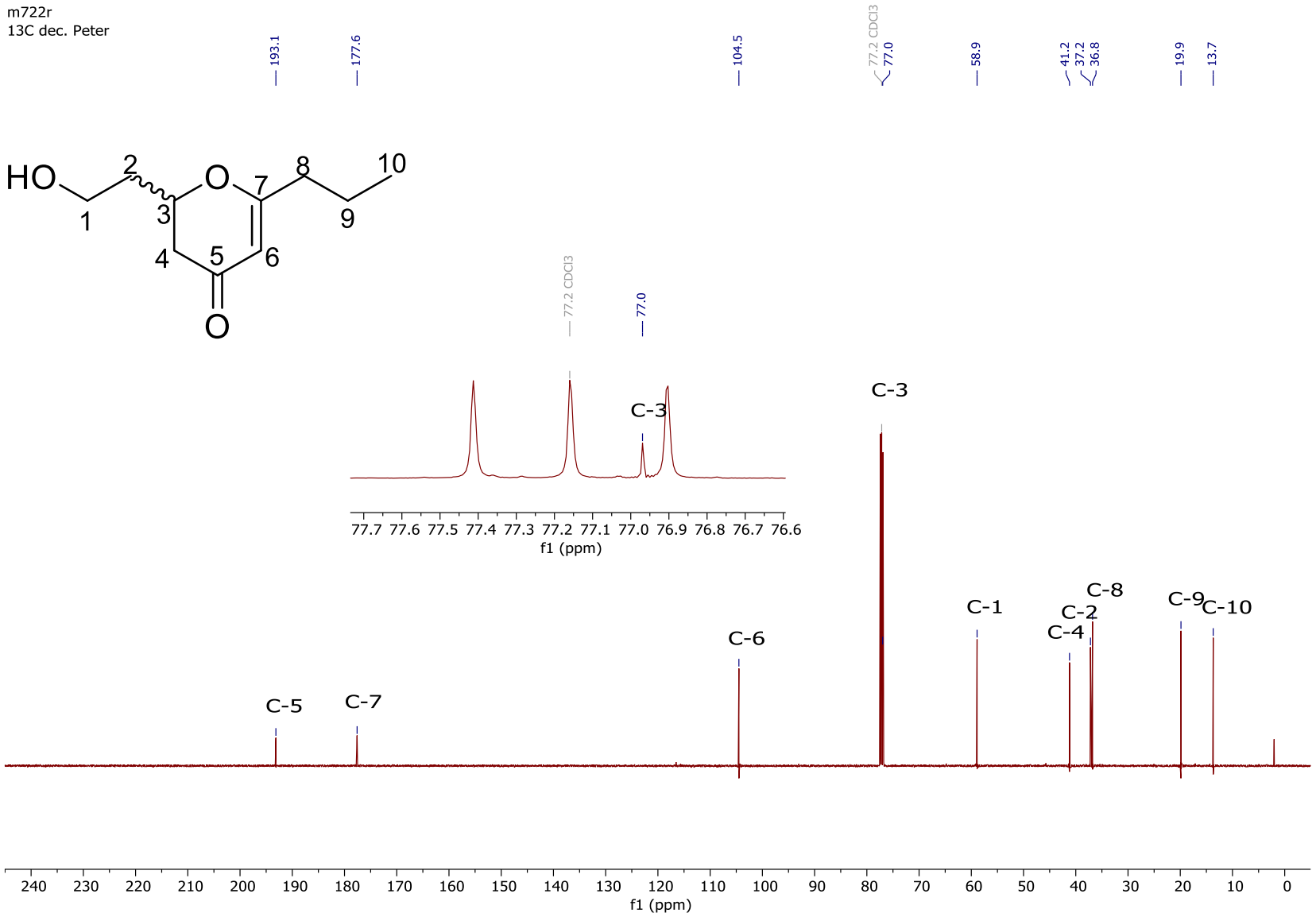


Figure S16. ^{13}C NMR spectrum of **4** in $CDCl_3$ (125 MHz).

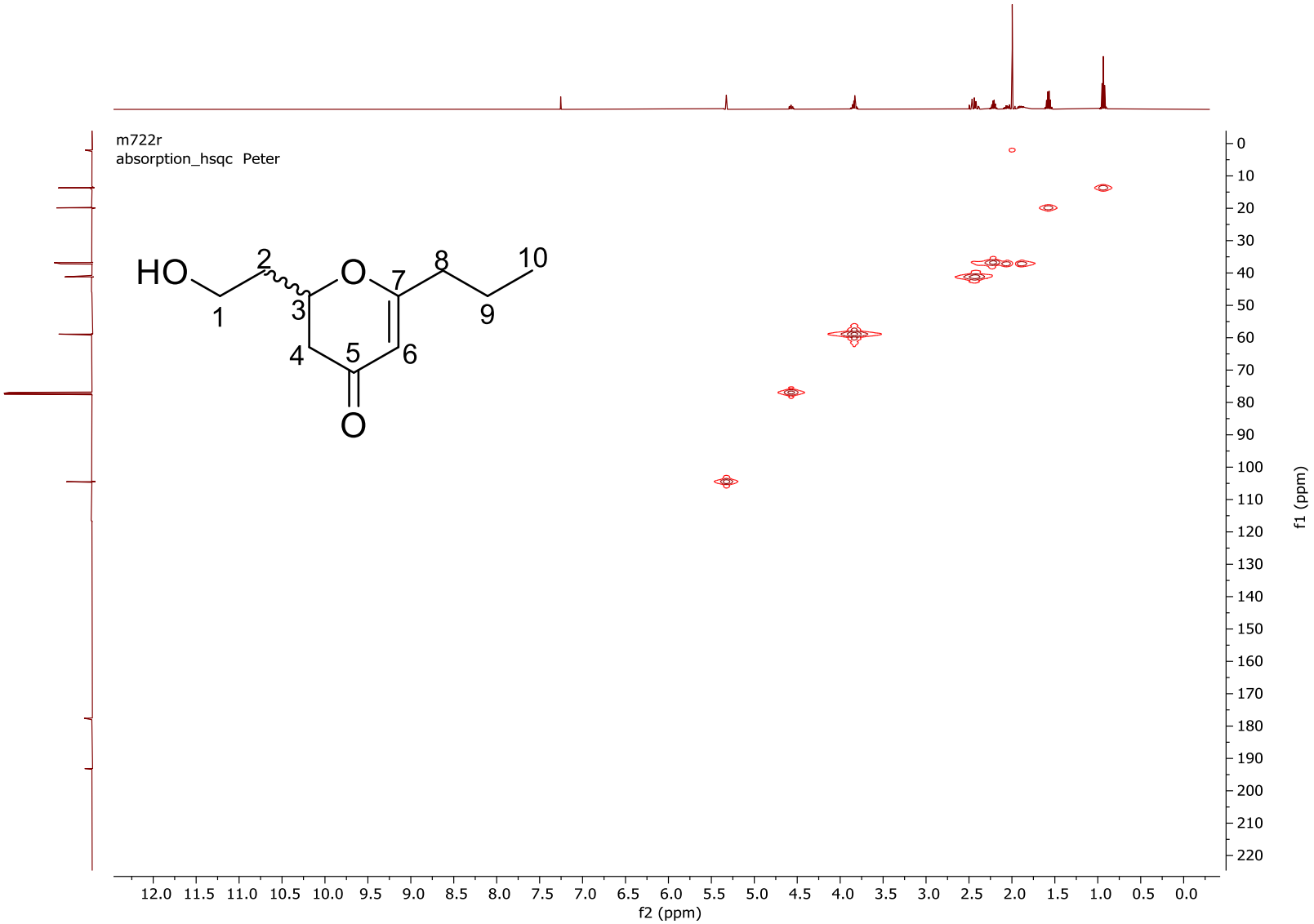


Figure S17. HSQC spectrum of **4** in $CDCl_3$.

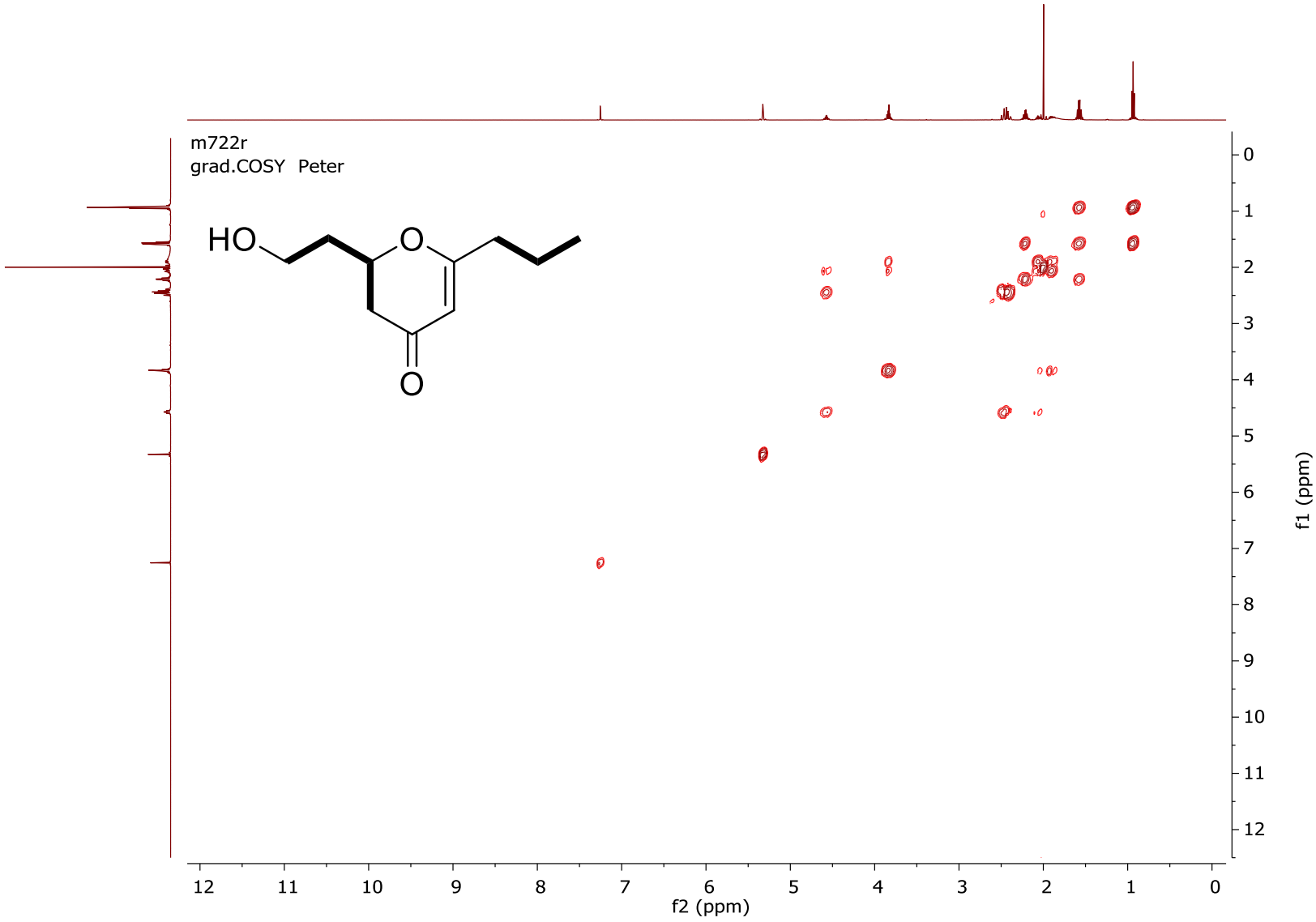


Figure S18. ^{1}H-^{1}H COSY NMR spectrum of **4** in $CDCl_3$

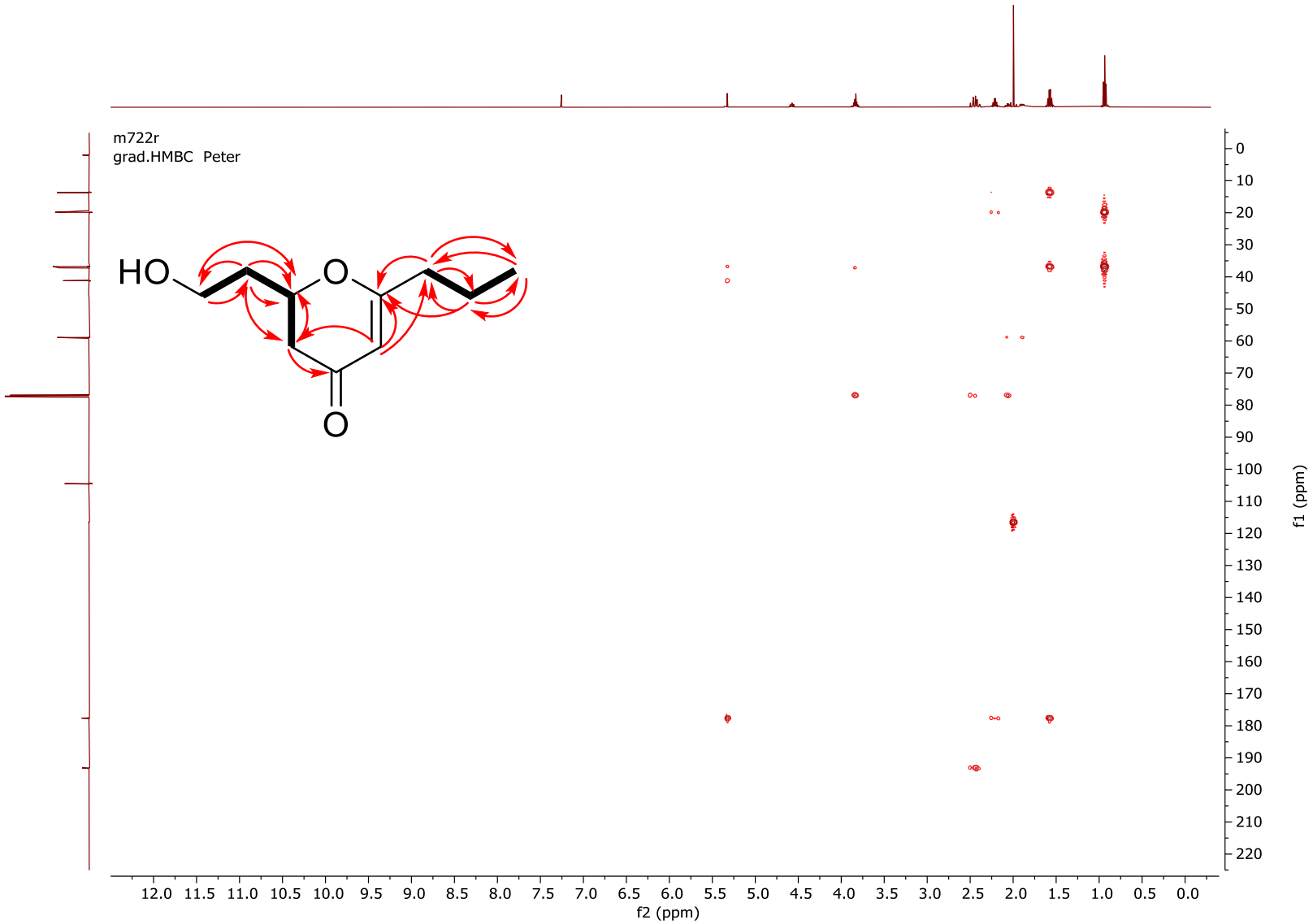


Figure S19. HMBC spectrum of **4** in $CDCl_3$

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