Fungal Polyketide Synthase Product Chain-Length Control by Partnering Thiohydrolase

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Table S1: H NMR (500 MHz, CD_3OH) and ^{13}C NMR (125 MHz, CD_3OH) data for **3**, **4**, **5** and **6.** **S33**

Table S2: Primers used in this study. **S34**

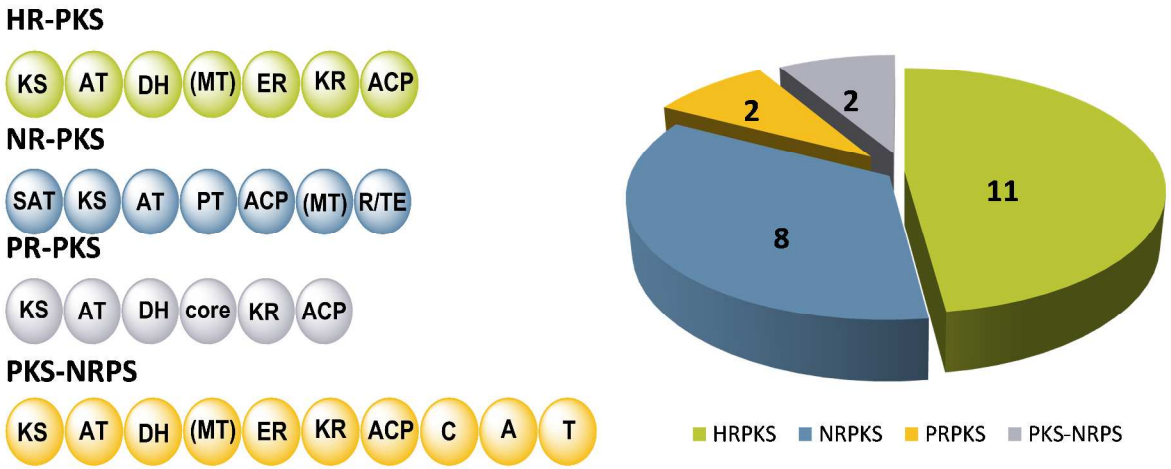


Figure S1. Breakdown of PKS and PKS-NRPS obtained from genome mining in *E. brefeldianum*

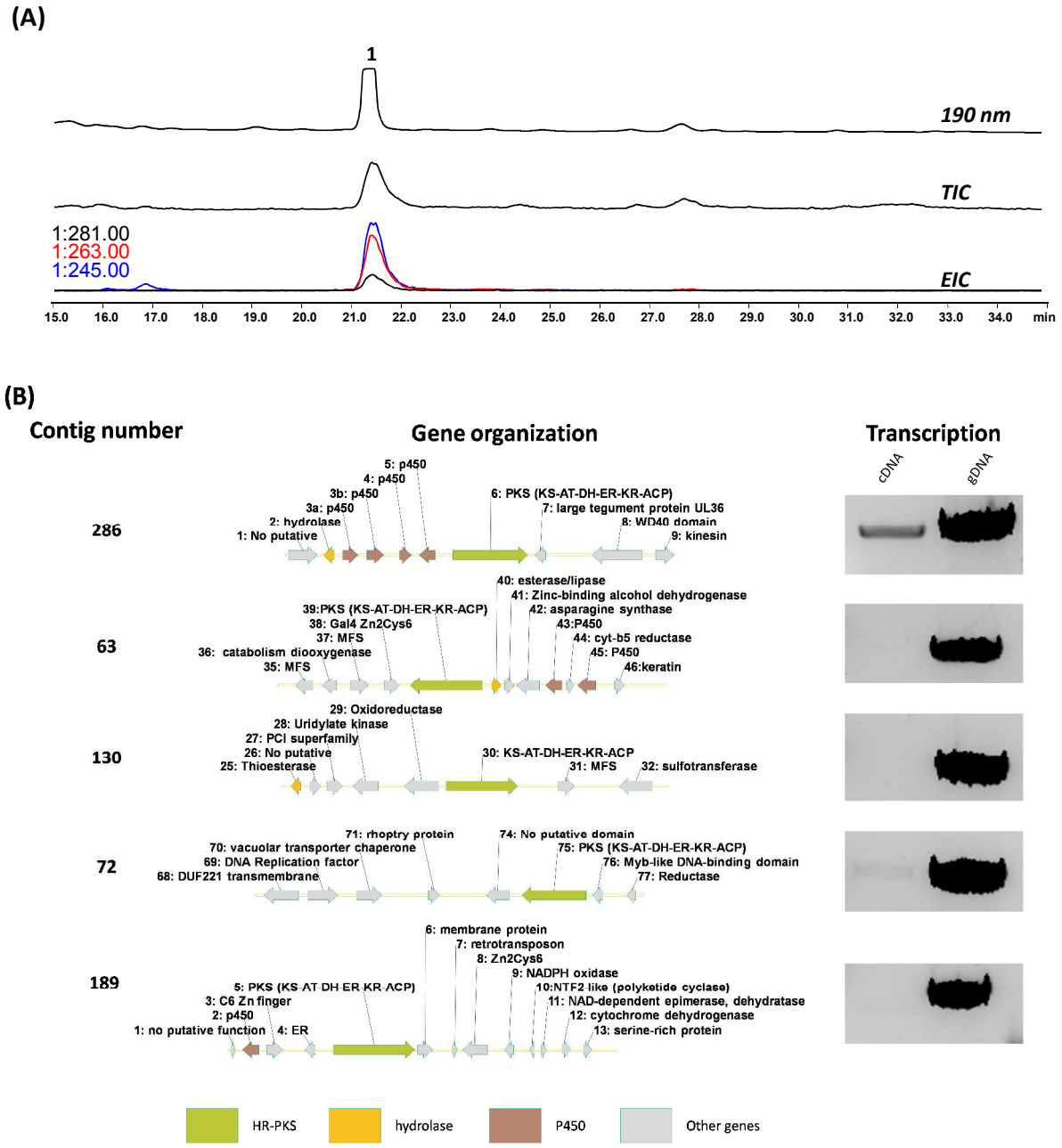


Figure S2. Production of BFA correlated with transcription of Contig_286 HRPKS. (A) LC-MS profile of *E. brefeldianum* ATCC58665 culture grown in MEM media after three days. This media was used to optimize production of BFA and reduce production of other metabolites. The cells for mRNA extraction were collected from the same culture. (B) Gene organization of the HR-PKS-containing contigs (Domain organization: KS-AT-DH-ER-KR-ACP) in *E. brefeldianum* and transcriptional analyses on the PKS genes from each contig.

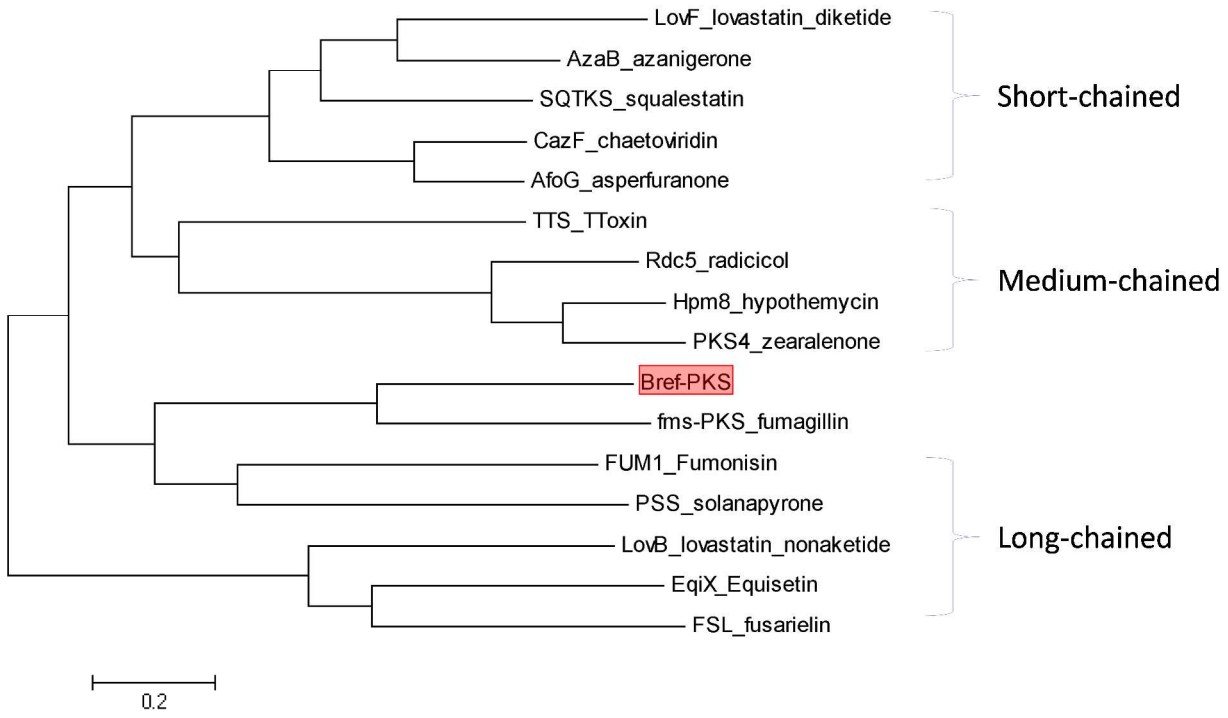


Figure S3. Phylogenetic tree containing BrefPKS and HRPKSs of known products. The alignment was performed on Mega 5.05.

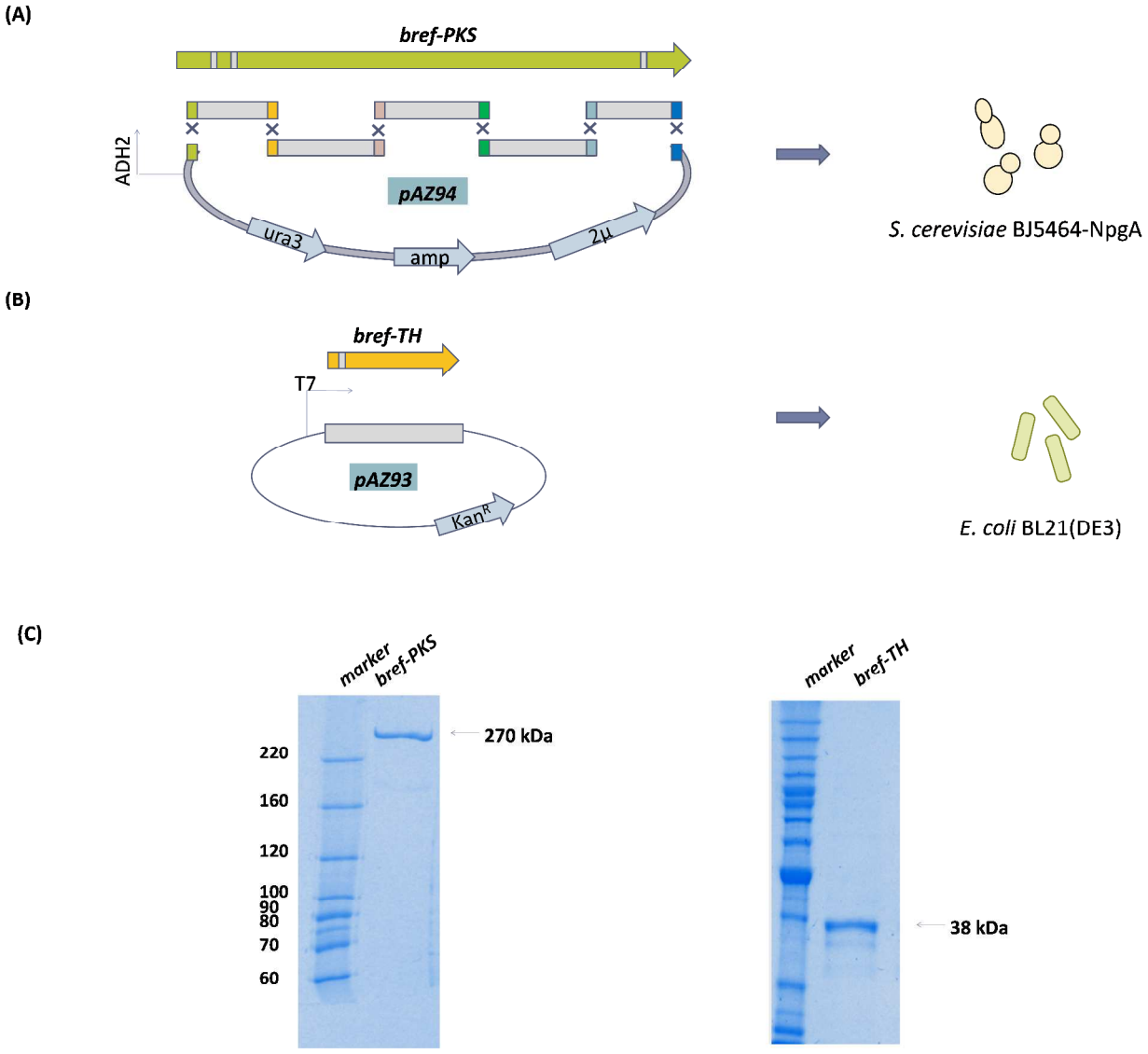


Figure S4. Expression of Bref-PKS and Bref-TH. Construction of expression plasmids (A) pAZ94 (Bref-PKS) for *S. cerevisiae* expression and (B) pAZ93 (Bref-TH) for *E. coli* expression. All fragments were amplified from cDNA. (C) The proteins were expressed and purified and the elution samples were run on SDS gel. Bref-PKS was run on 6% acrylamide, while Bref-TH was run on 12% acrylamide.

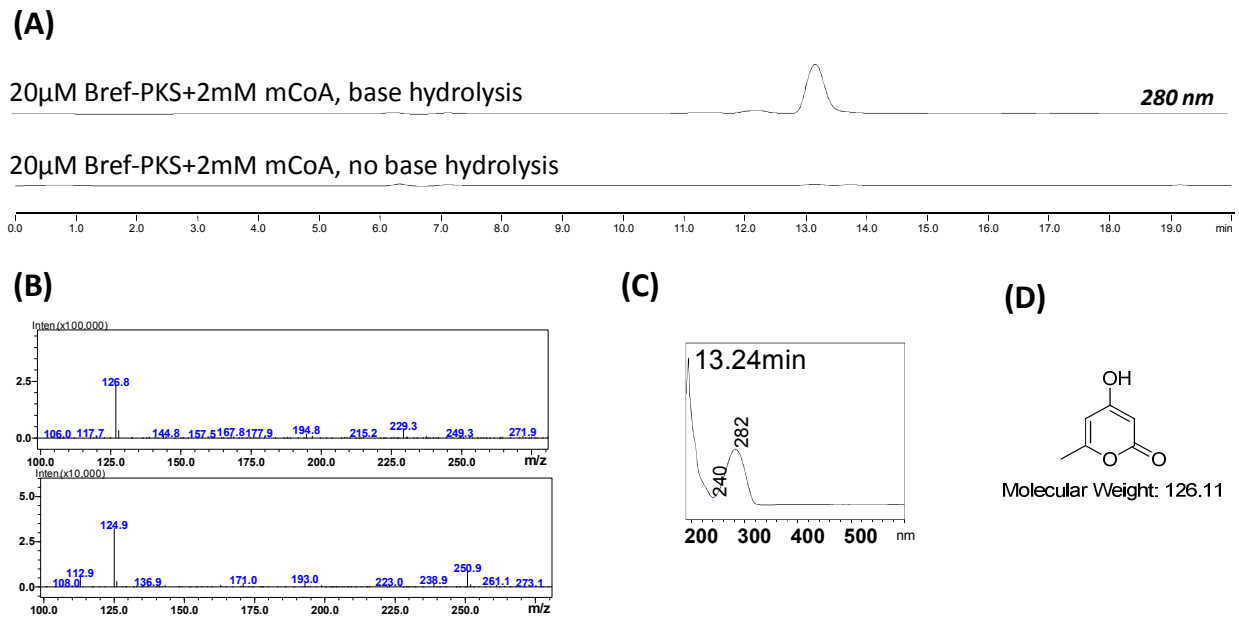


Figure S5. Minimal PKS assay on Bref-PKS. (A) HPLC analysis comparing the base-hydrolyzed and non-hydrolyzed control. The peak at 13.24 min has a mass (B) and a UV profile (C) consistent with the triketide pyrone as shown in (D).

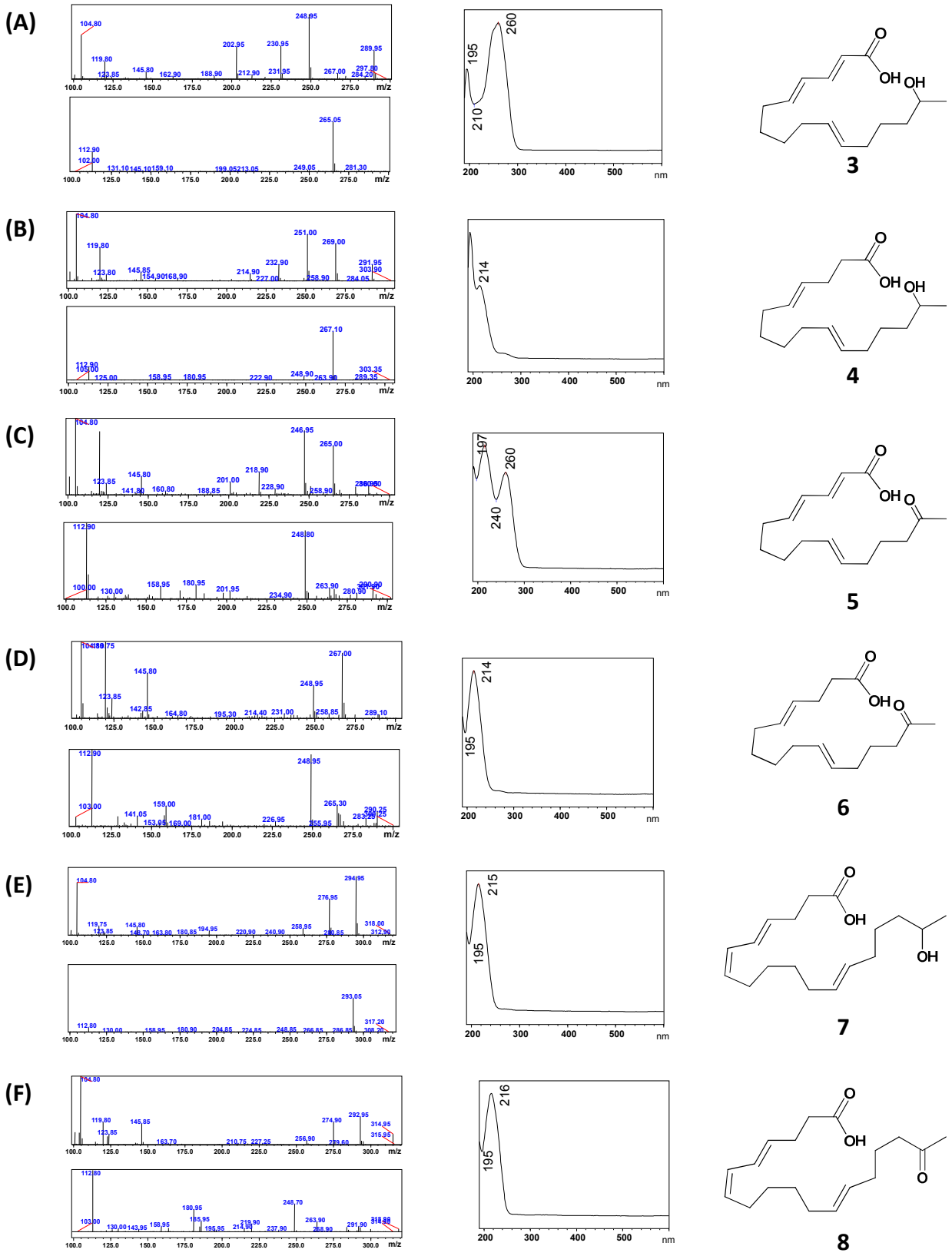


Figure S6. MS and UV spectra of compounds 3 to 8 (A-F).

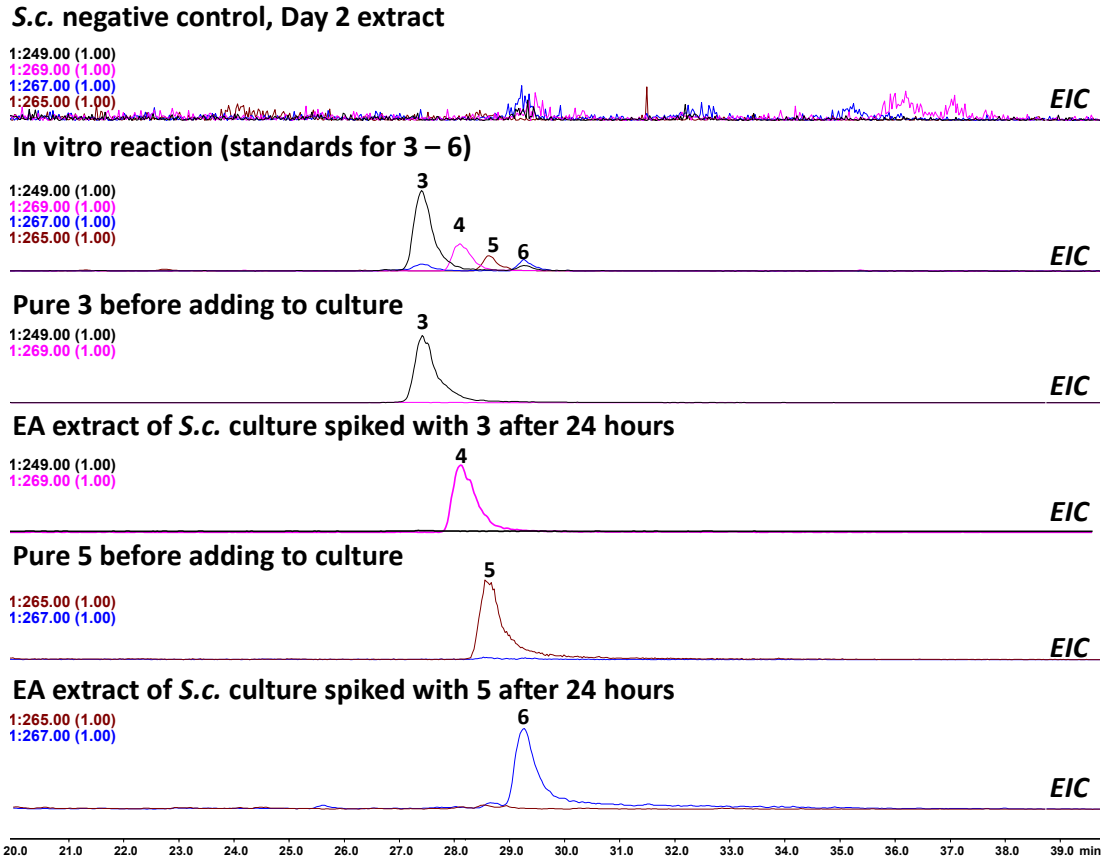


Figure S7. Yeast biotransformation of compounds 3 and 5 to 4 and 6, respectively. In order to confirm that **3** and **5** were indeed converted to **4** and **6** by yeast endogenous enzymes, we grew two 2-ml culture of *S. cerevisiae* BJ5464-NpgA (empty host) on YPD for 24 hours. Purified **3** and **5** were then added to each culture and incubated O/N with shaking at 28°C. After 24 hours, 500µL of each culture was extracted with EA+acid and dried subsequently. The extract was analyzed on LC/MS, showing that **3** and **5** were indeed nearly fully converted to **4** and **6**, respectively. This explains why only **4** and **6** were left in the yeast culture harboring *bref-PKS* and *bref-TH* after more than three days of culturing.

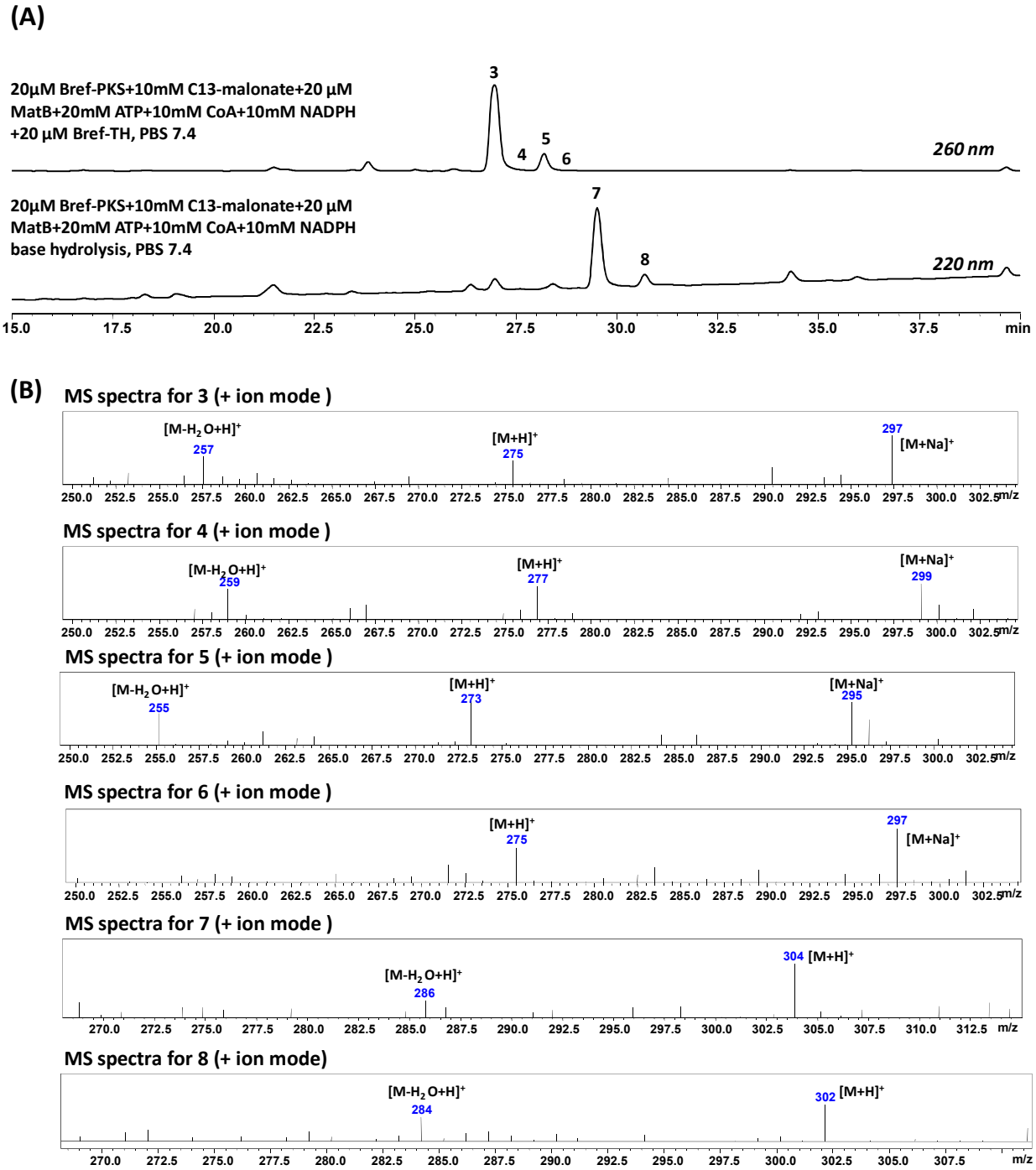


Figure S8. C13-labeling of the product polyketides using the MatB regeneration system. (A) HPLC chromatogram of the in vitro reaction with Bref-hydrolase and with base hydrolysis. **(B)** MS spectra for compounds **3** to **8**. Comparison of each MS spectrum to Figure S5 shows the +8 mu increase for compounds **3** to **6**, and +9mu increase for **7** and **8**.

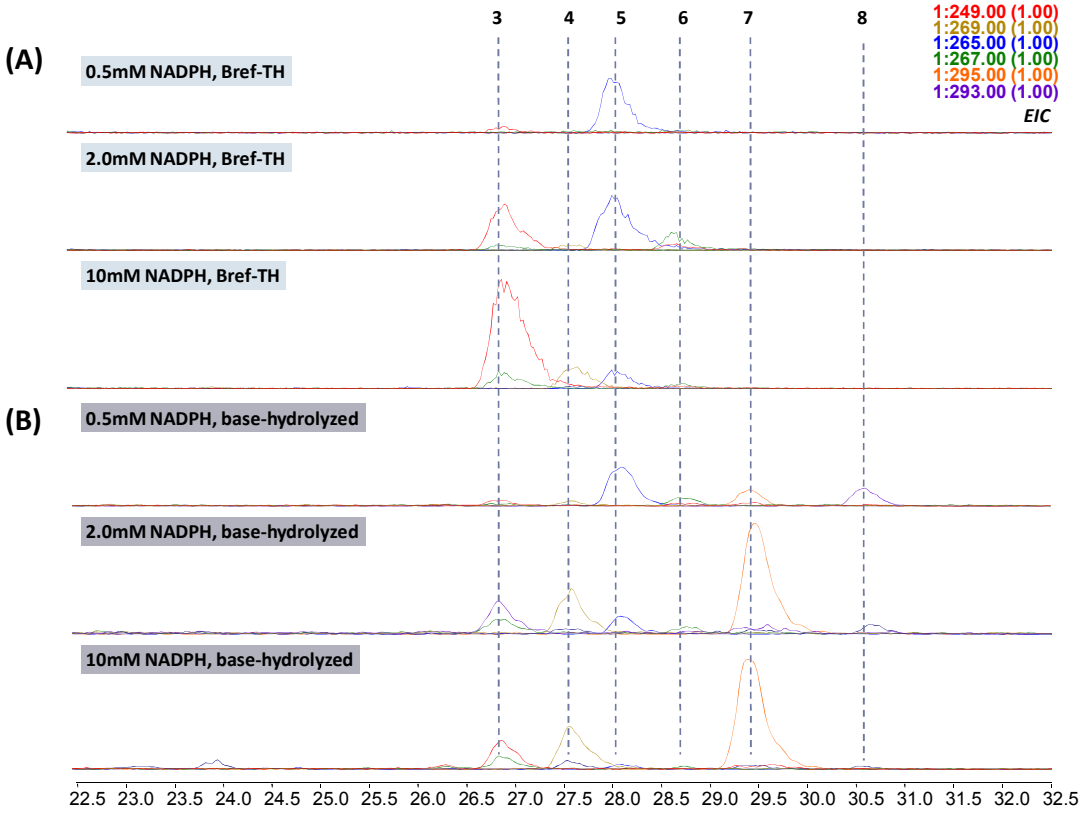


Figure S9. Effect of NADPH on the product profile. *Bref*-PKS was incubated with 2mM mCoA and increasing concentrations of NADPH from 0.5 mM to 10 mM. In (A), Bref-TH was added to offload the product, while in (B) base hydrolysis was performed instead prior to extraction. Notice that at lower concentrations, **5** was the dominant product, regardless of the releasing mechanism.

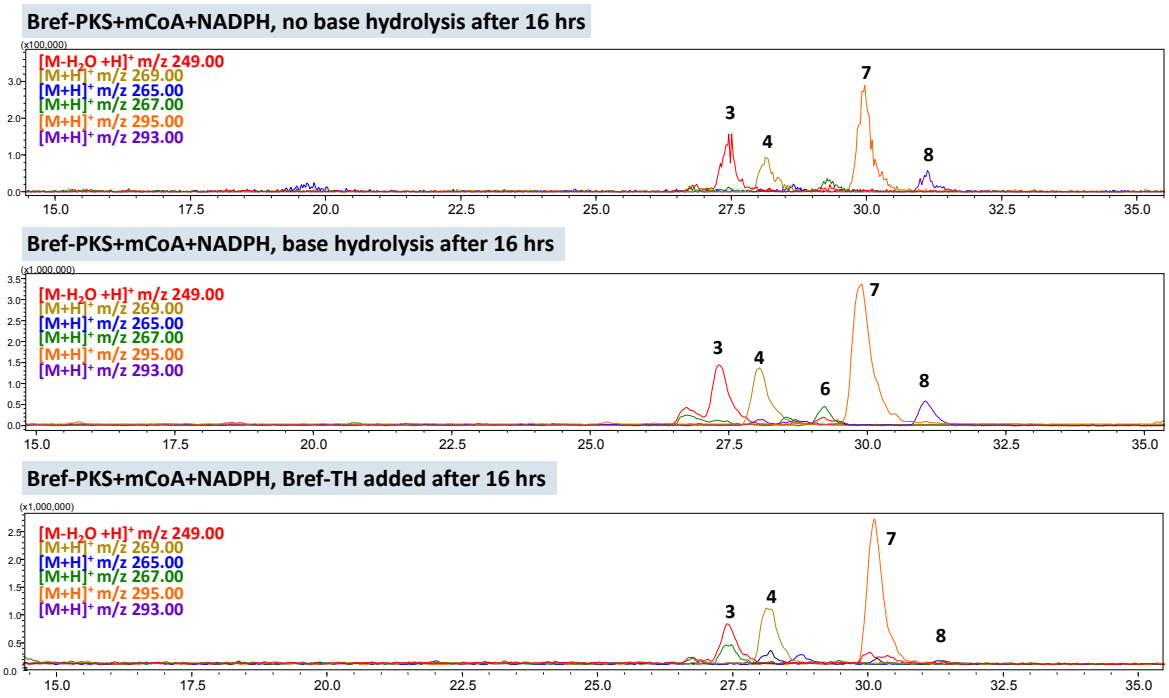
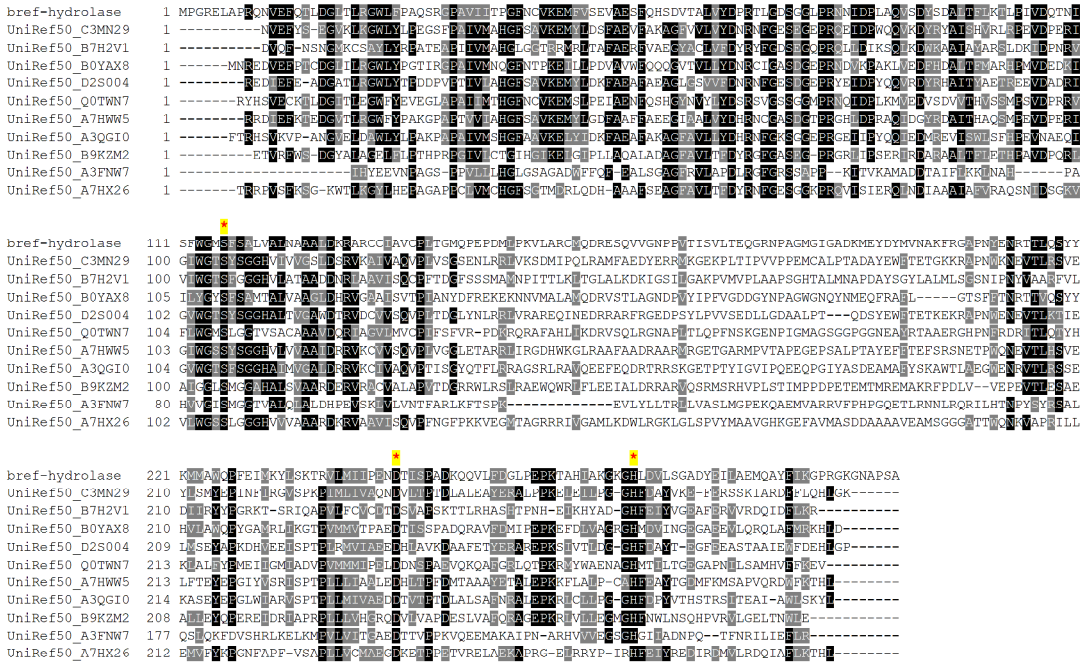


Figure S10. Effect of base hydrolysis and post-addition of Bref-TH on product release. EIC spectra for specific product masses in the non-hydrolase in vitro reactions with and without base hydrolysis. Base hydrolysis is found to facilitate the release of the polyketides. Addition of equimolar concentration of Bref-TH after 16 hours of in vitro reaction yielded the same product profile. Therefore, in the absence of an immediate releasing partner, the polyketide chain stalled on the ACP, leading to a longer chained product.

(A)



(B)

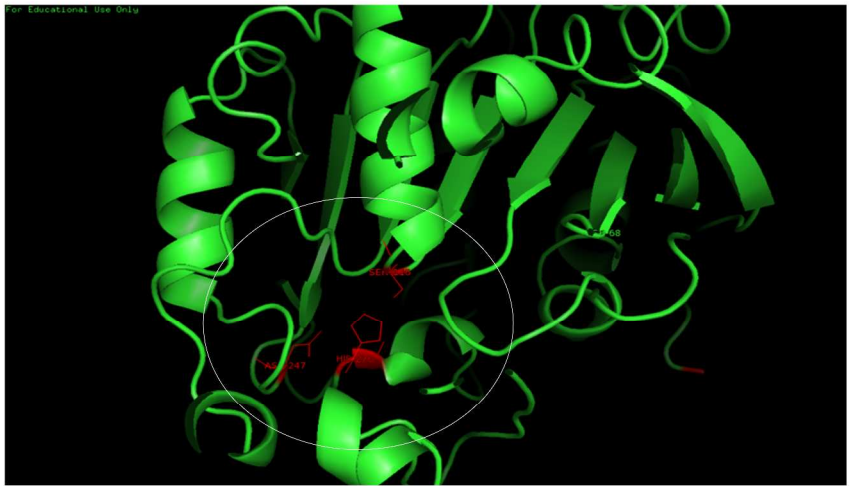


Figure S11. Modeling of Bref-TH to known hydrolases allows identification of its active site residues. (A) Sequence alignment of Bref-TE to other structurally-related hydrolases. The catalytic triad of Ser-116, Asp-247 and His-276 was conserved. (B) The model shows how the three residues are within catalytic distances of each other.

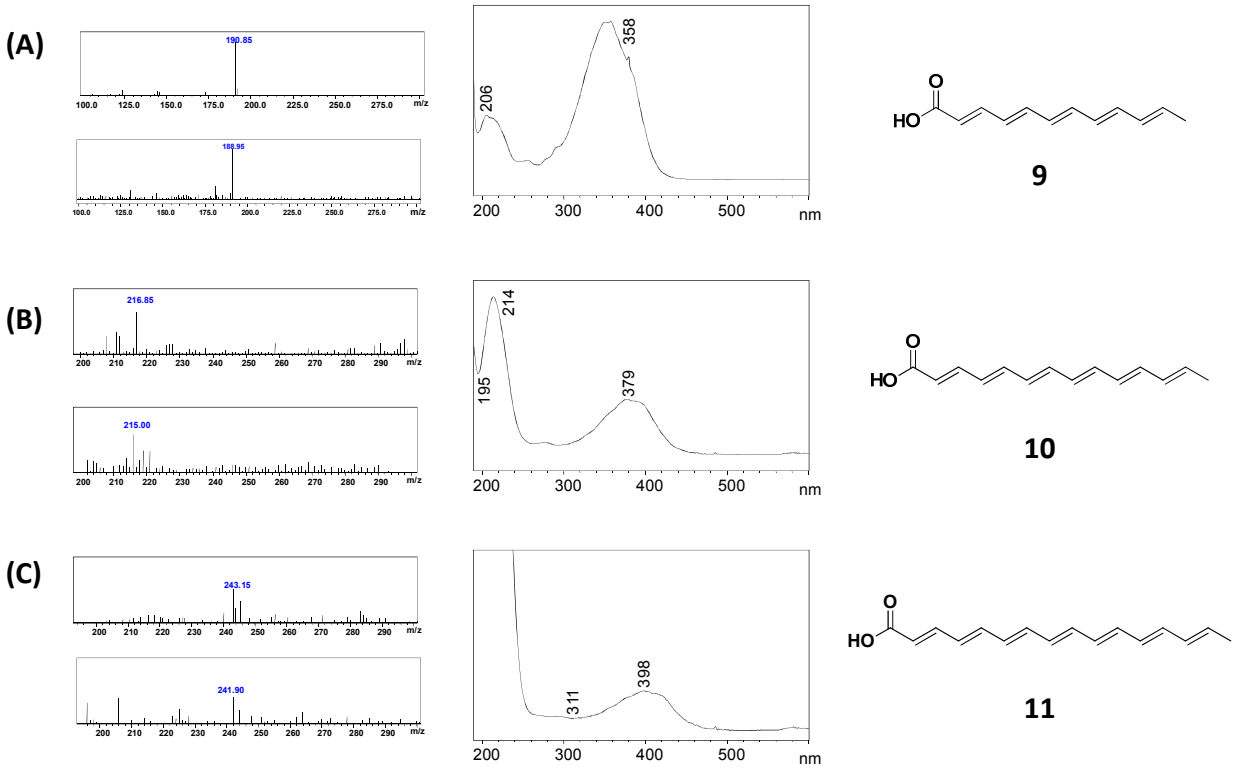


Figure S12. MS and UV spectra of compounds **9** to **11** (A-C).

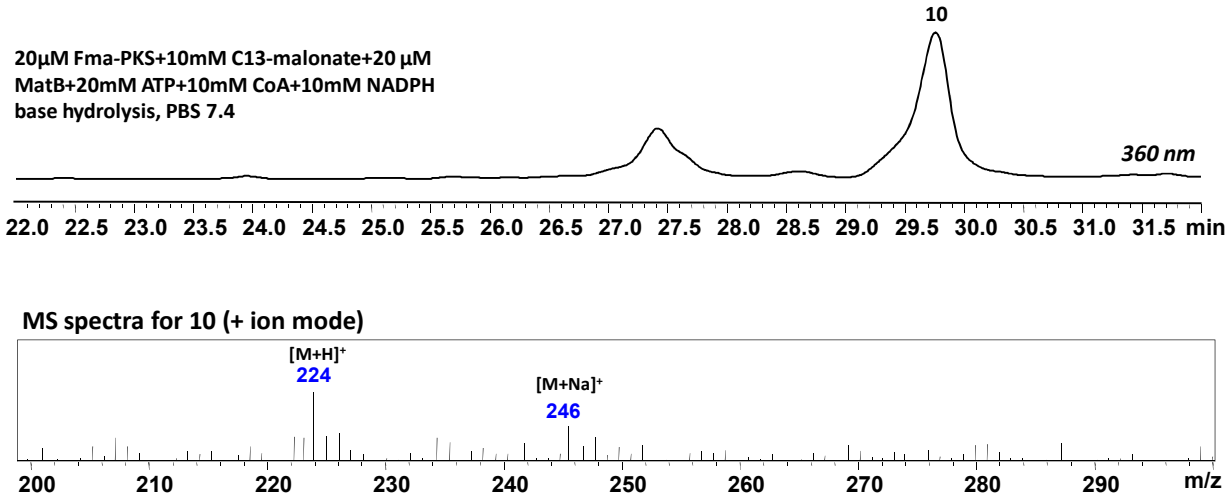


Figure S13. HPLC trace and positive ion mass spectra for the in vitro reaction of Fma-PKS with MatB regeneration system for C13-labeling of the heptaketide product, **10**. A mass shift of +7 mu was observed.

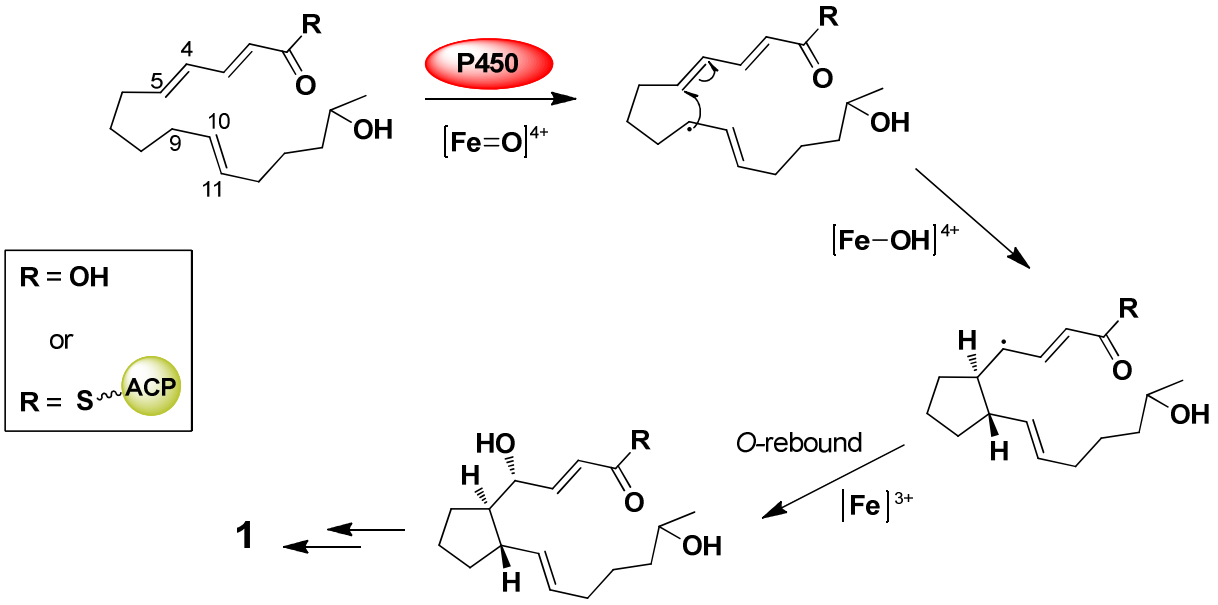


Figure S14. Proposed mechanism for the formation of the cyclopentane ring. We propose that a C9 radical initiated by a P450 enzyme could perform the alkene addition to the C4-C5 double bond to form the cyclopentane moiety. This is followed by an oxygen rebound which hydroxylated C4 and resulted in the final structure observed in BFA (1).

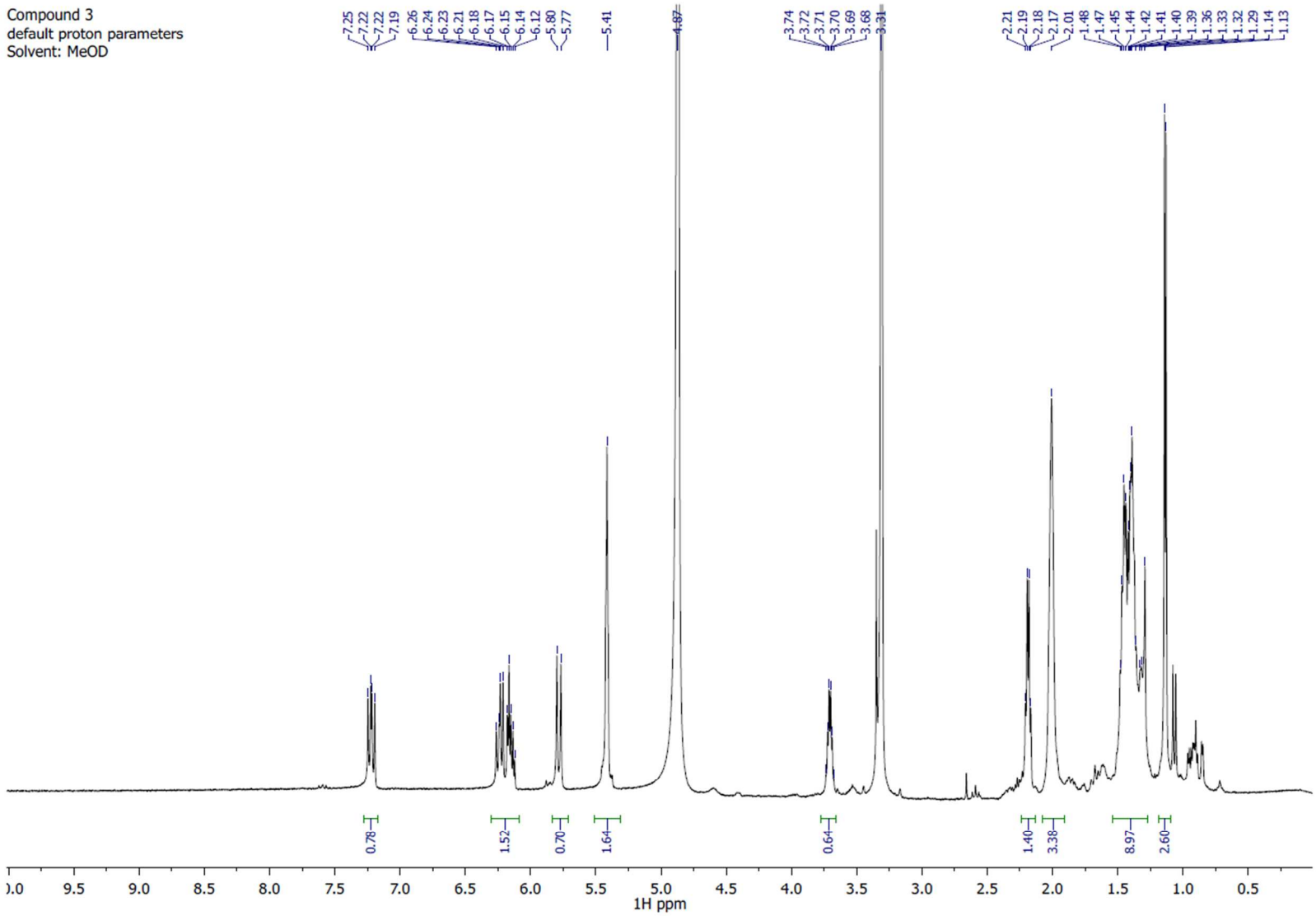


Figure S15A. 1H spectrum for compound **3**. Measured in CD_3OD at 500 MHz.

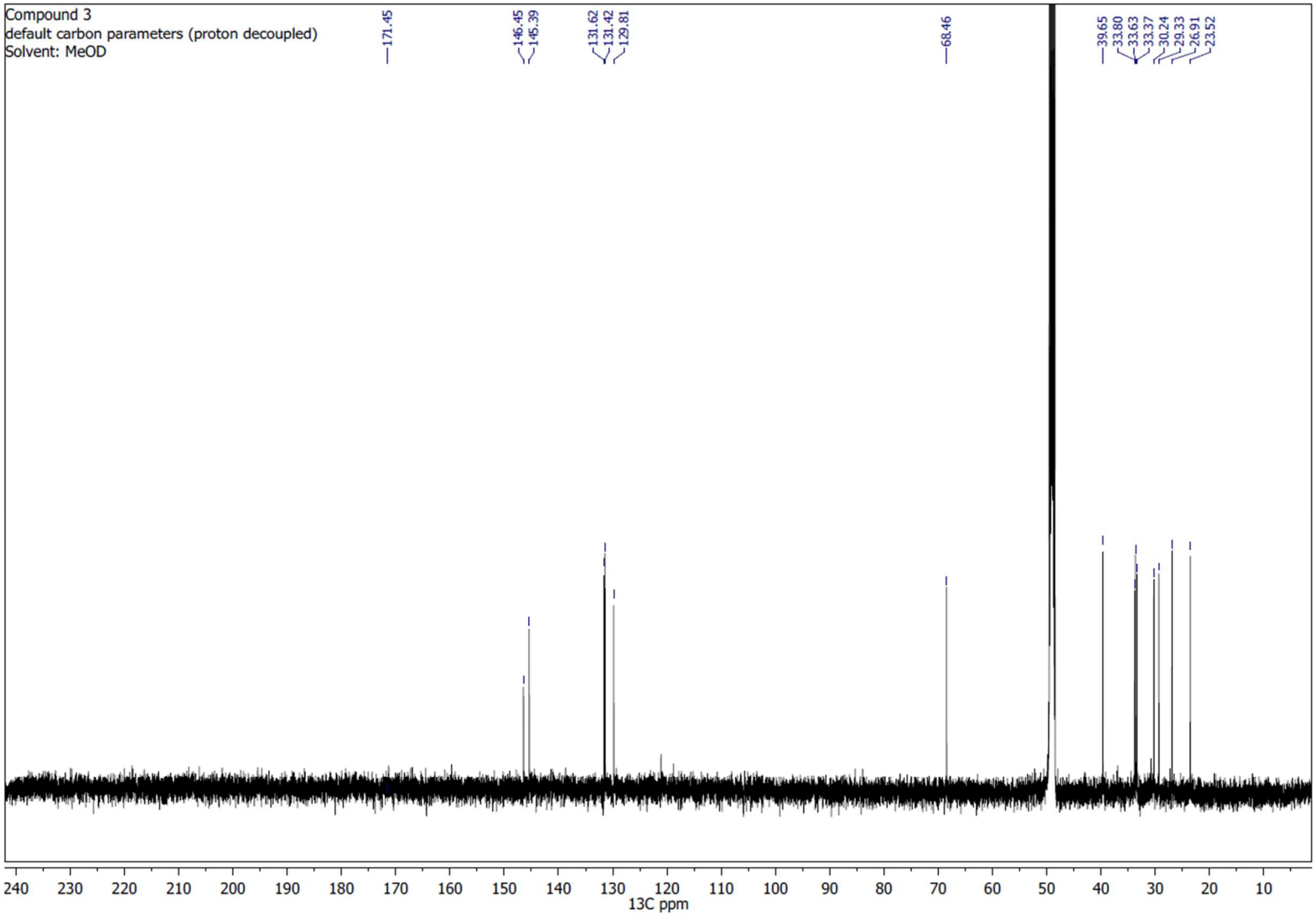


Figure S15B. 13C spectrum for compound **3.** Measured in CD_3OD at 125 MHz.

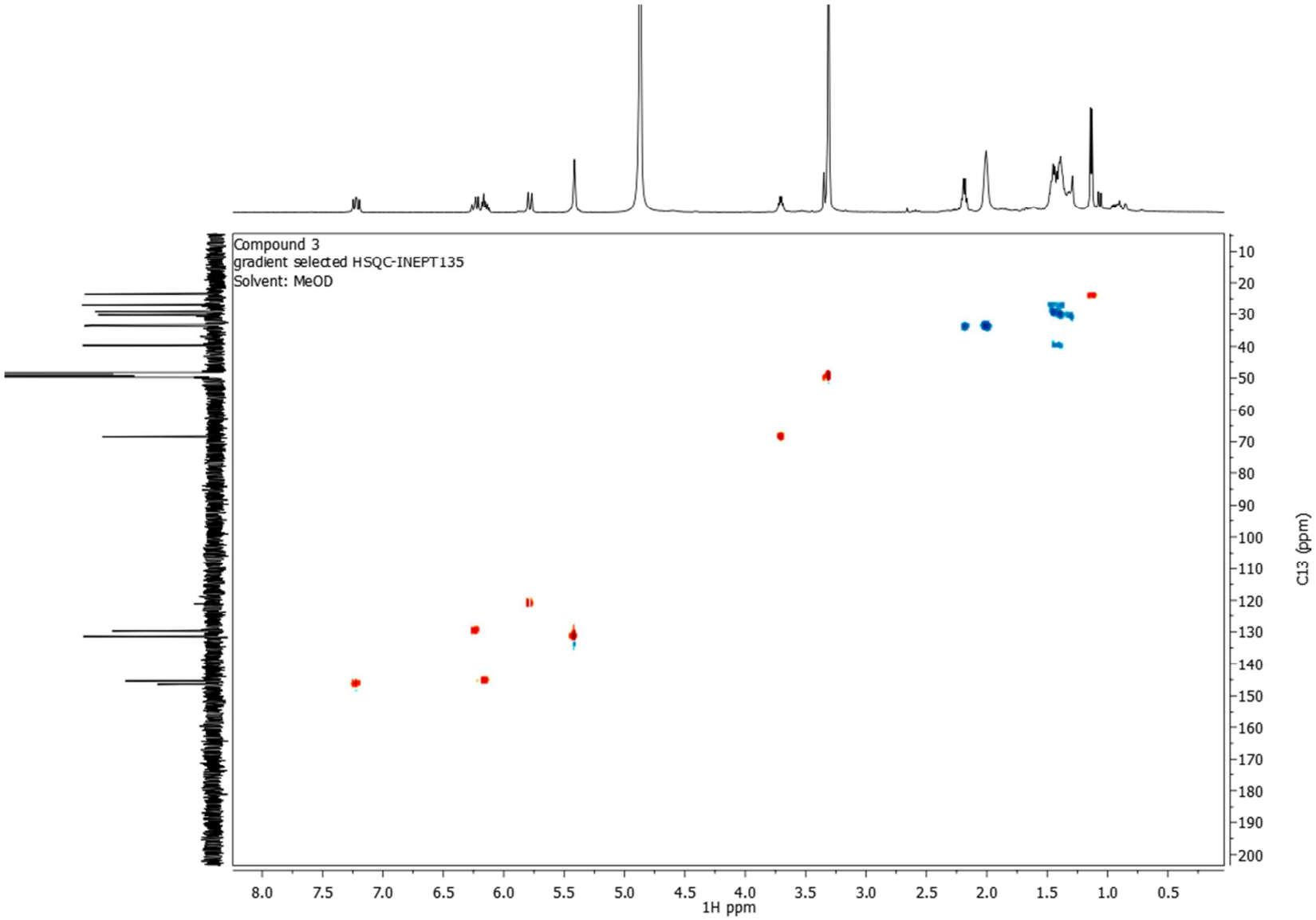


Figure S15C. HSQC-INEPT135 spectrum for compound **3.**

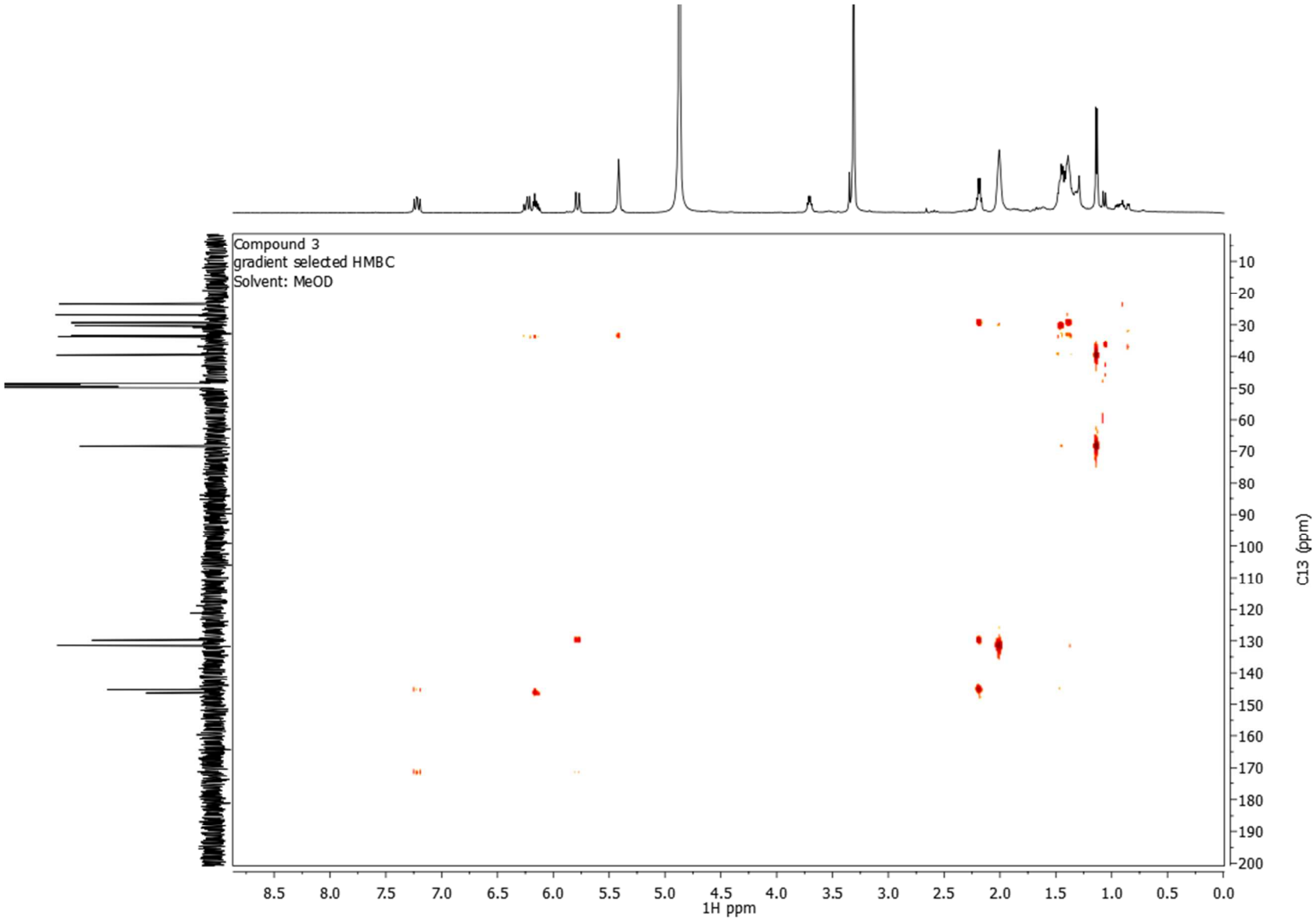


Figure S15D. HMBC spectrum for compound **3.**

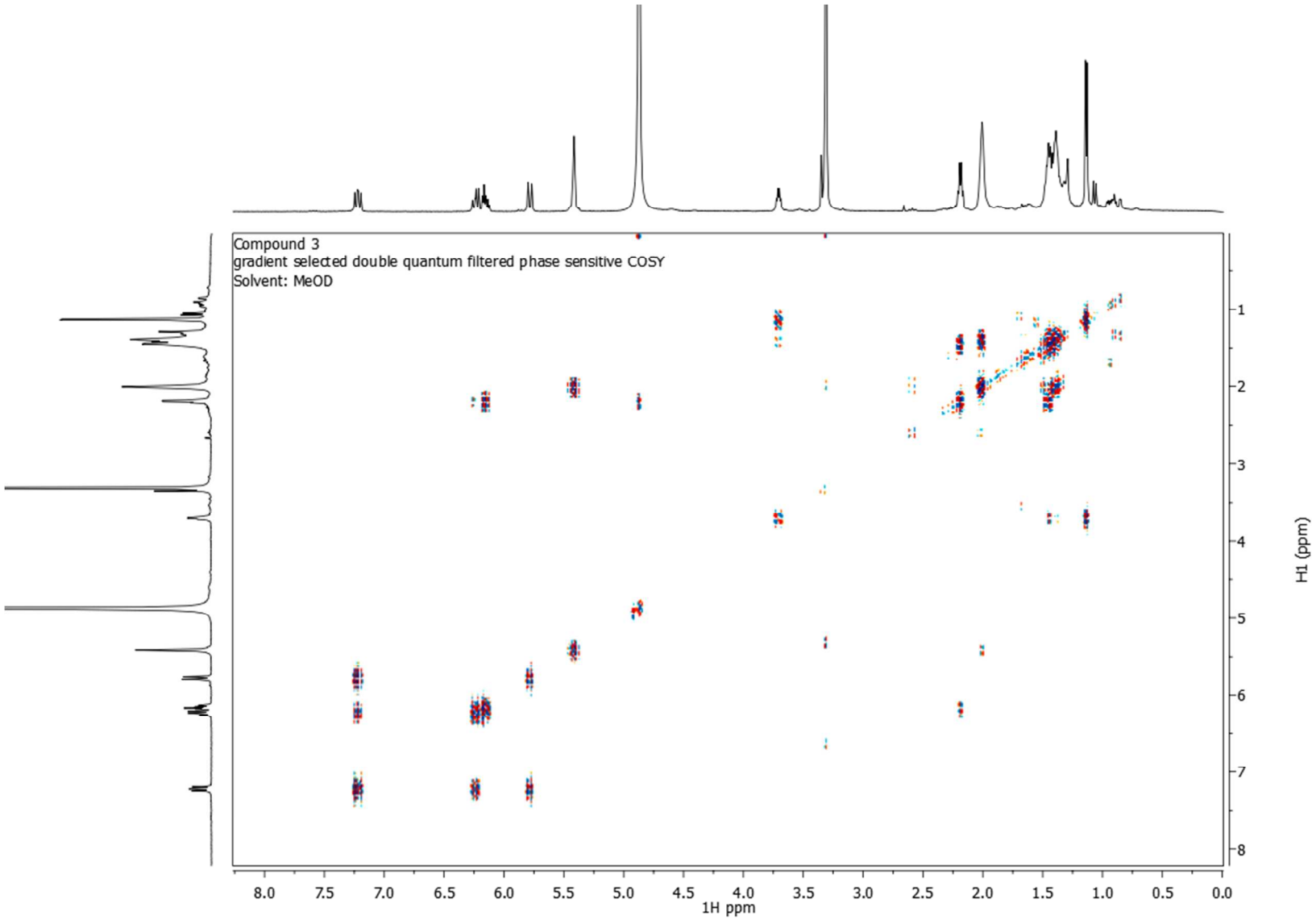


Figure S15E. COSY spectrum for compound **3.**

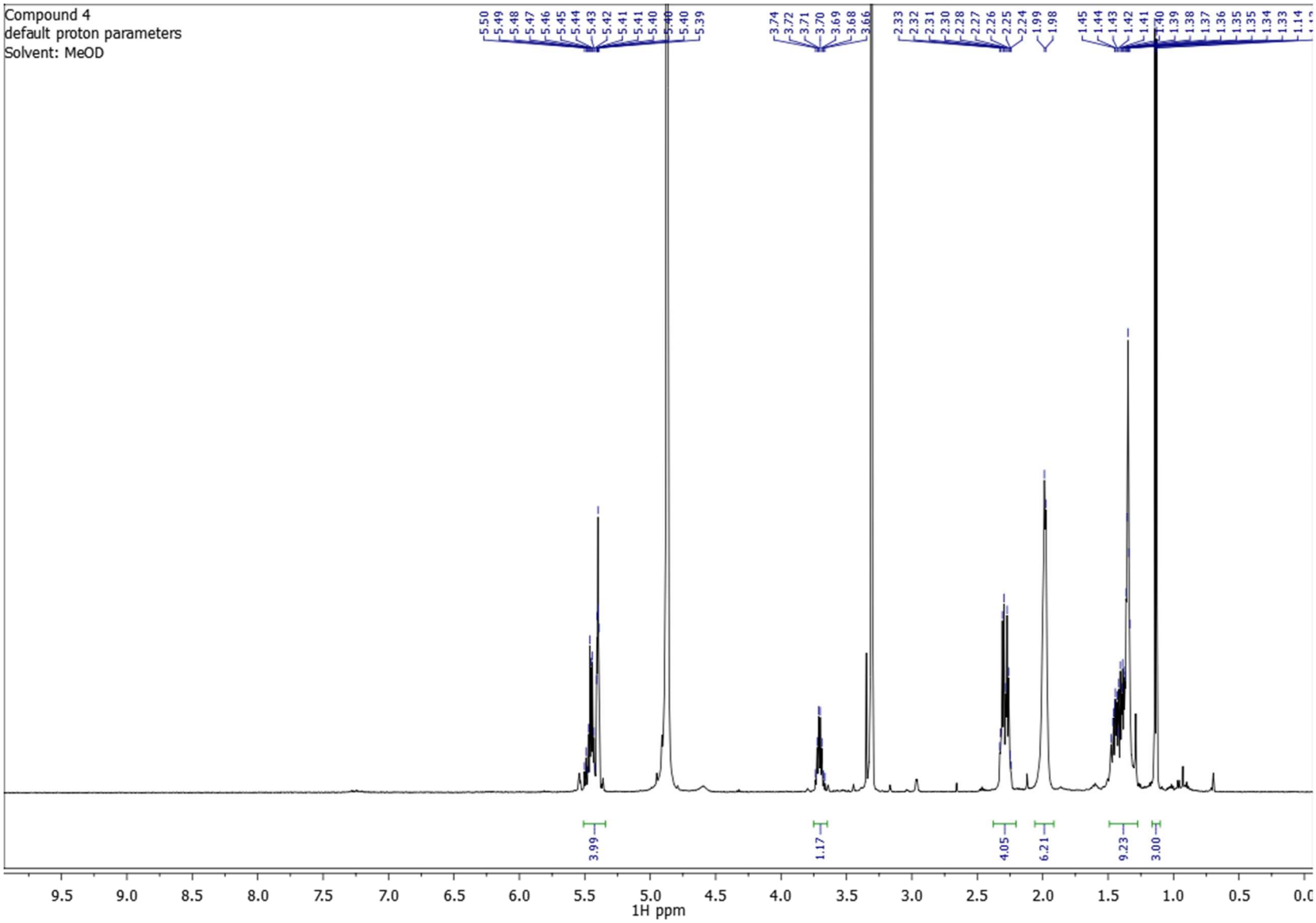


Figure S16A. 1H spectrum for compound **4**. Measured in CD_3OD at 500 MHz.

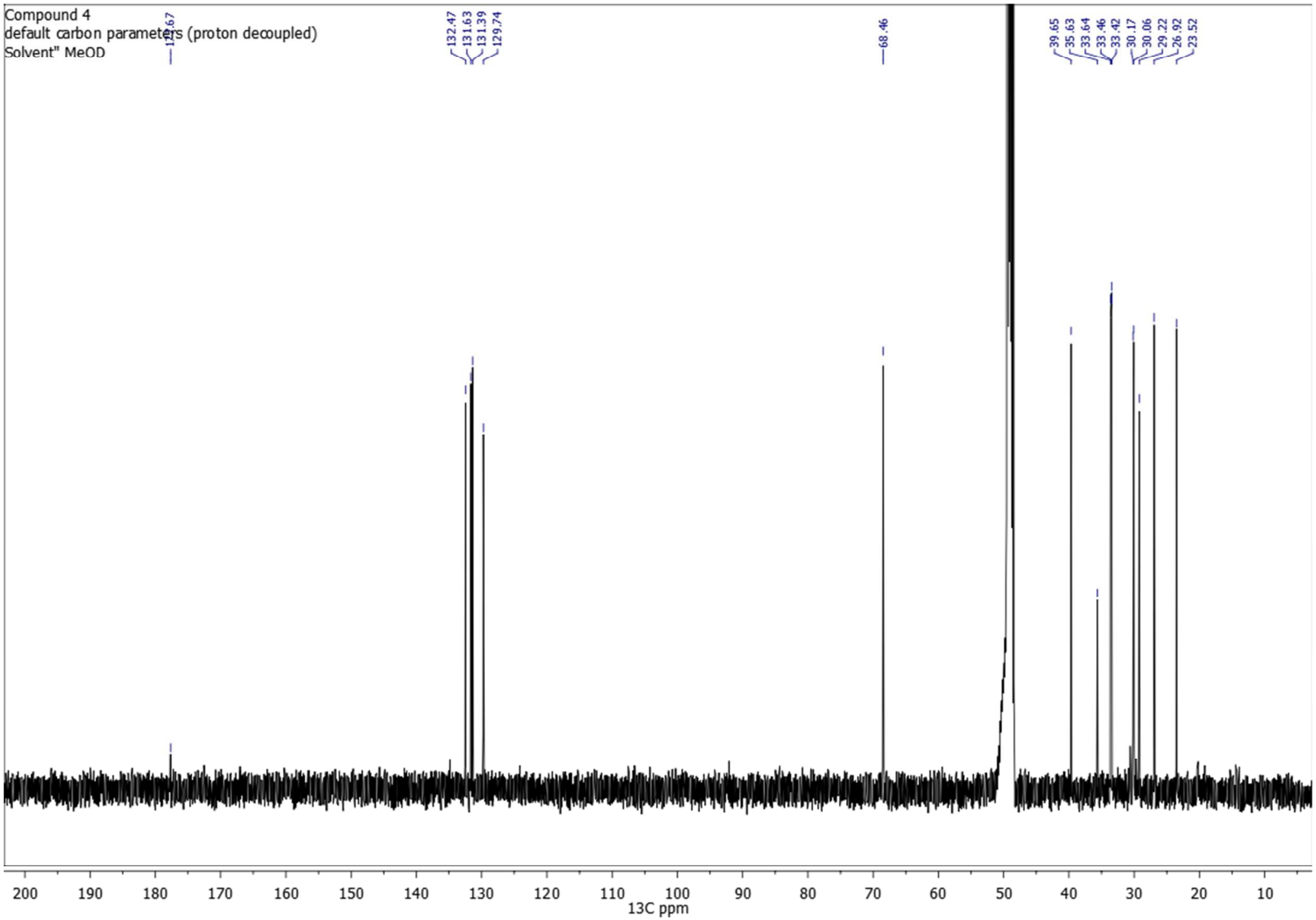


Figure S16B. 13C spectrum for compound **4.** Measured in CD_3OD at 125 MHz.

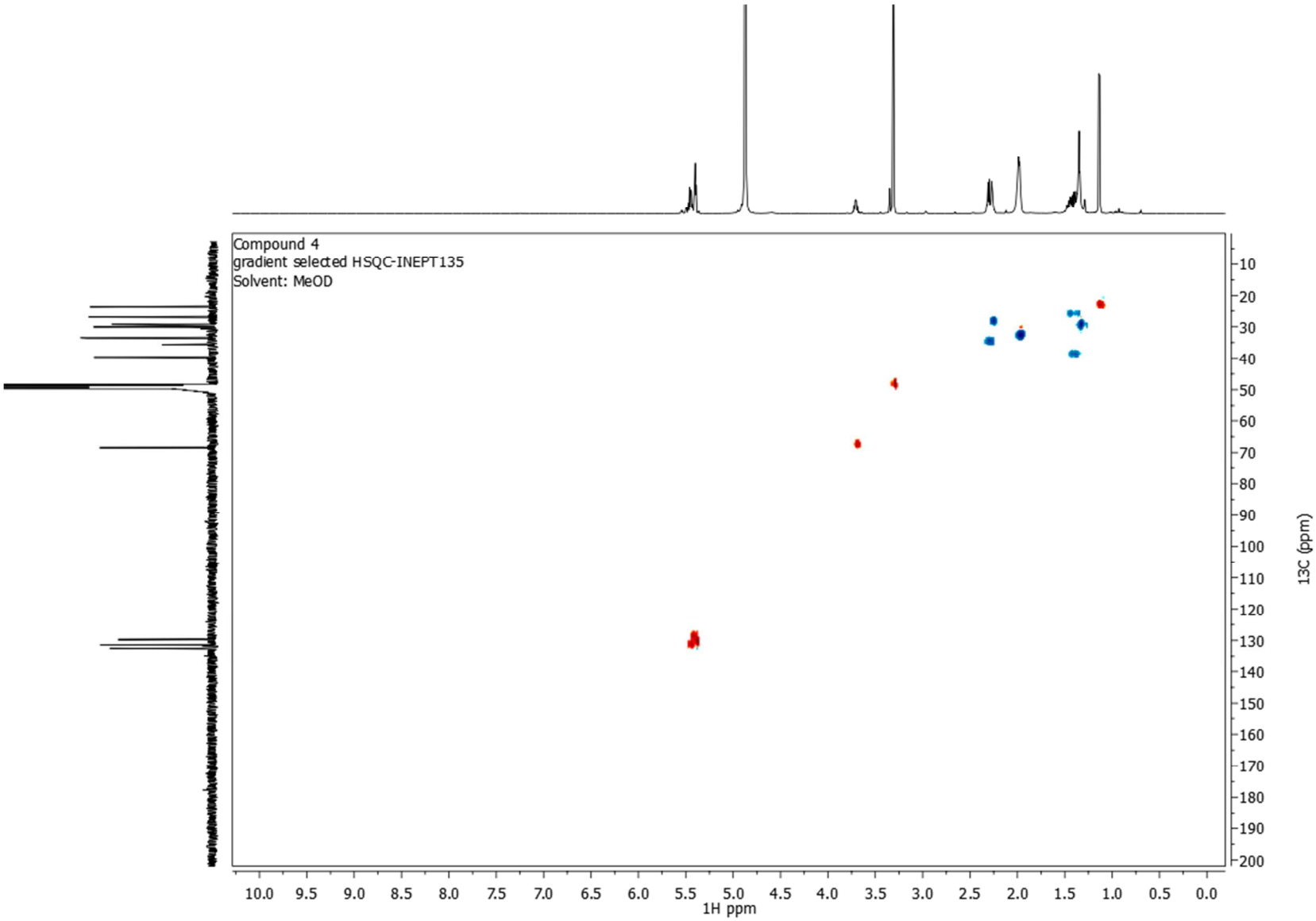


Figure S16C. HSQC-INEPT135 spectrum for compound **4.**

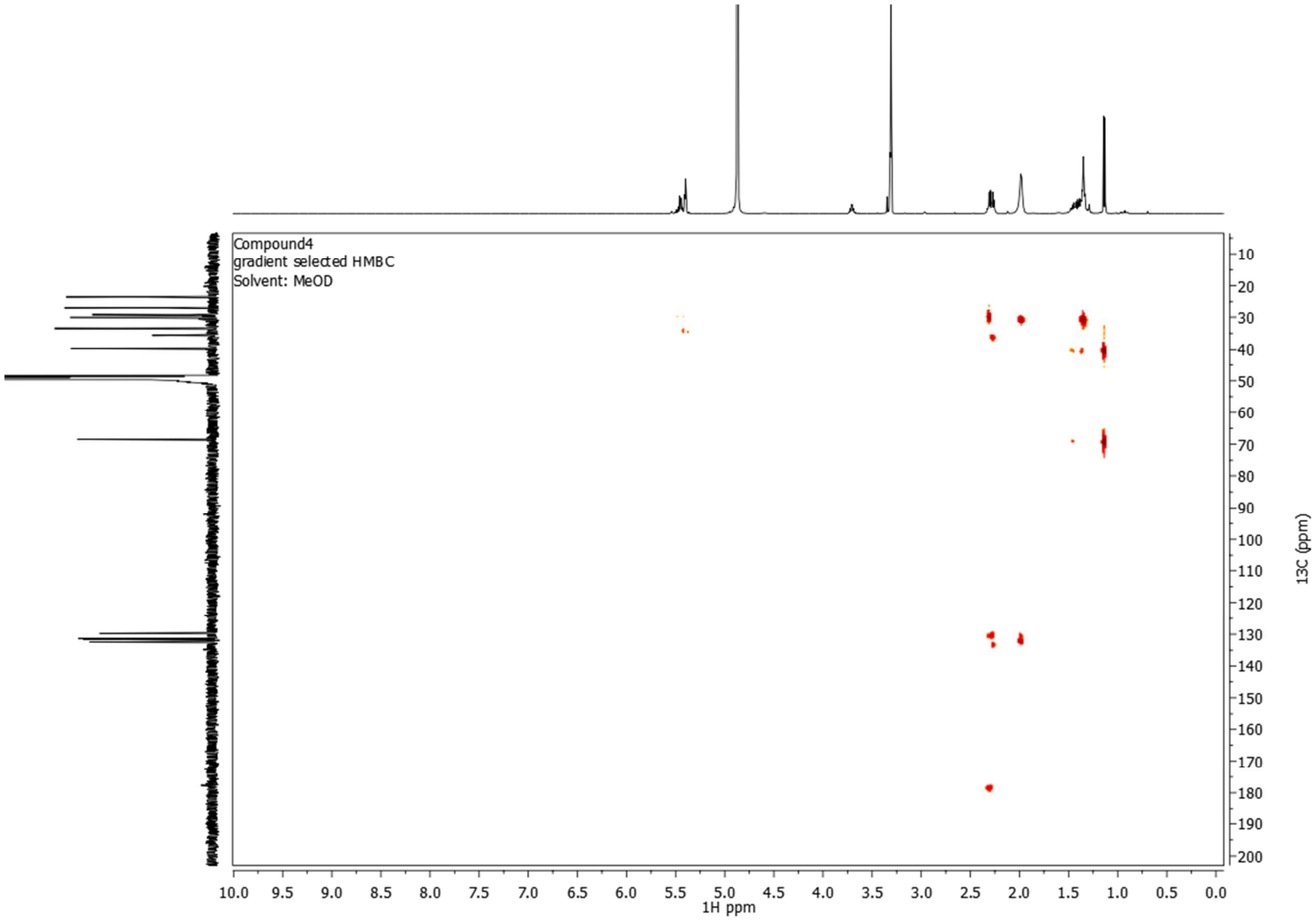


Figure S16D. HMBC spectrum for compound **4.**

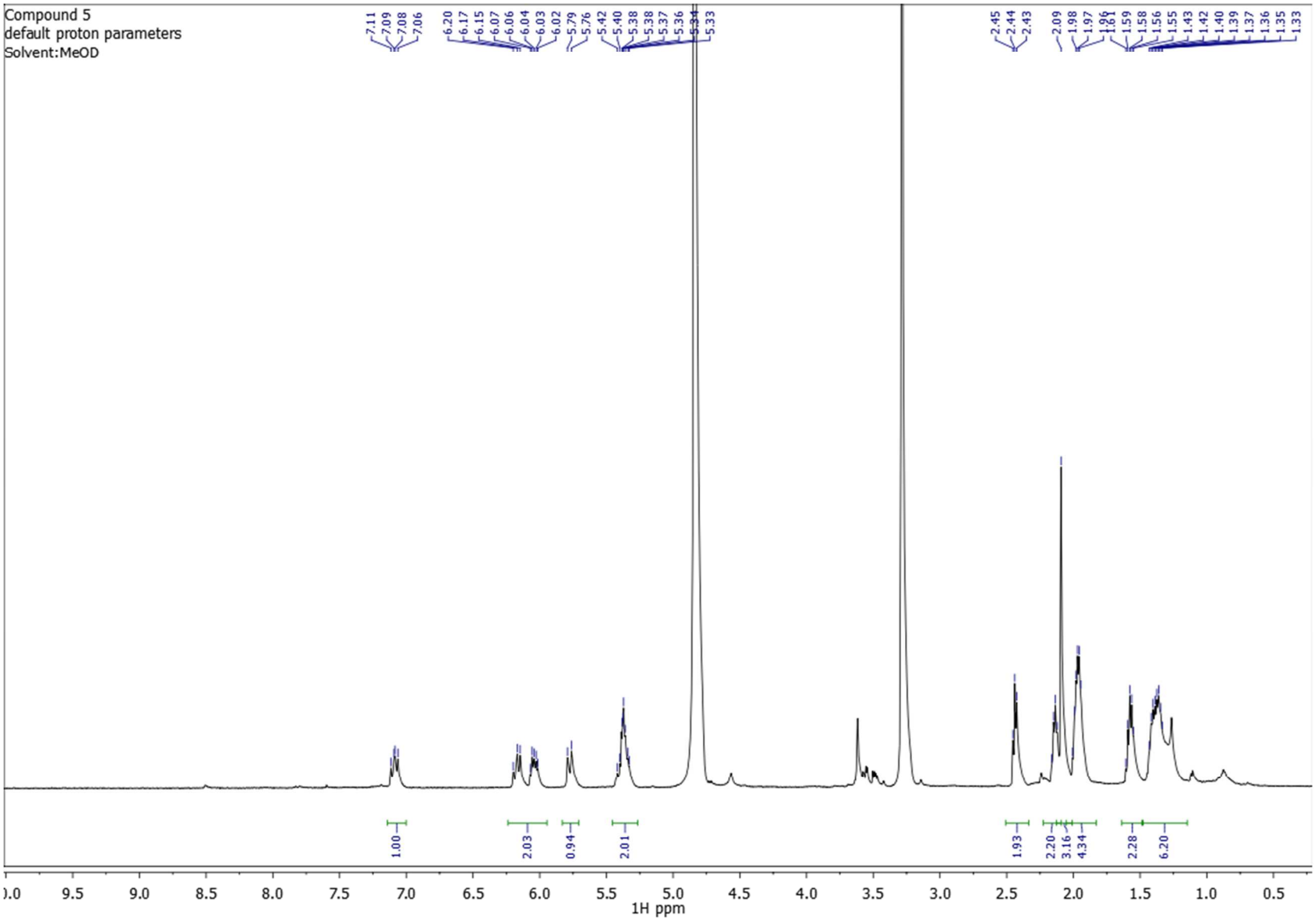


Figure S17A. 1H spectrum for compound **5**. Measured in CD_3OD at 500 MHz.

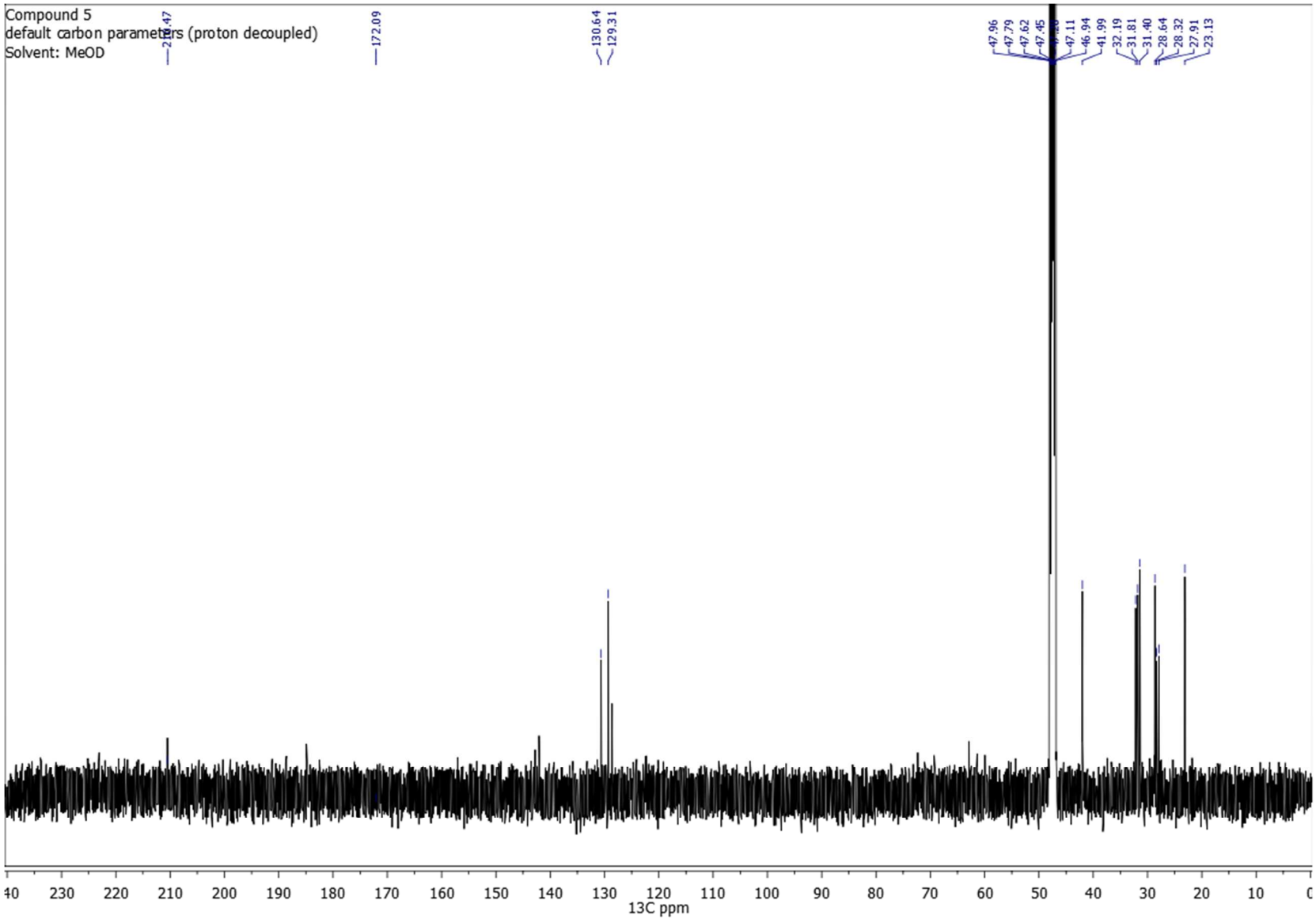


Figure S17B. 13C spectrum for compound **5.** Measured in CD_3OD at 125 MHz.

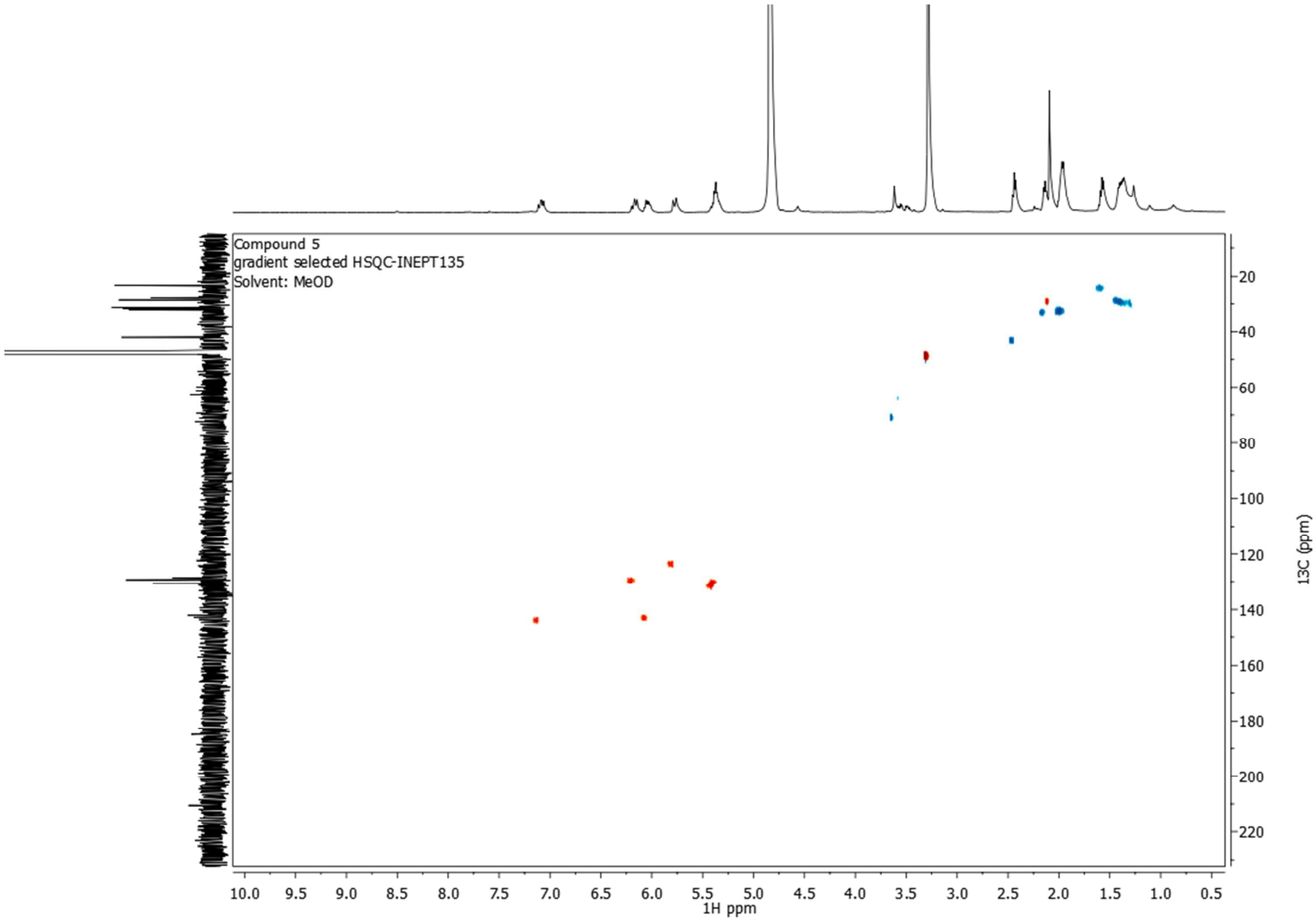


Figure S17C. HSQC-INEPT135 spectrum for compound **5.**

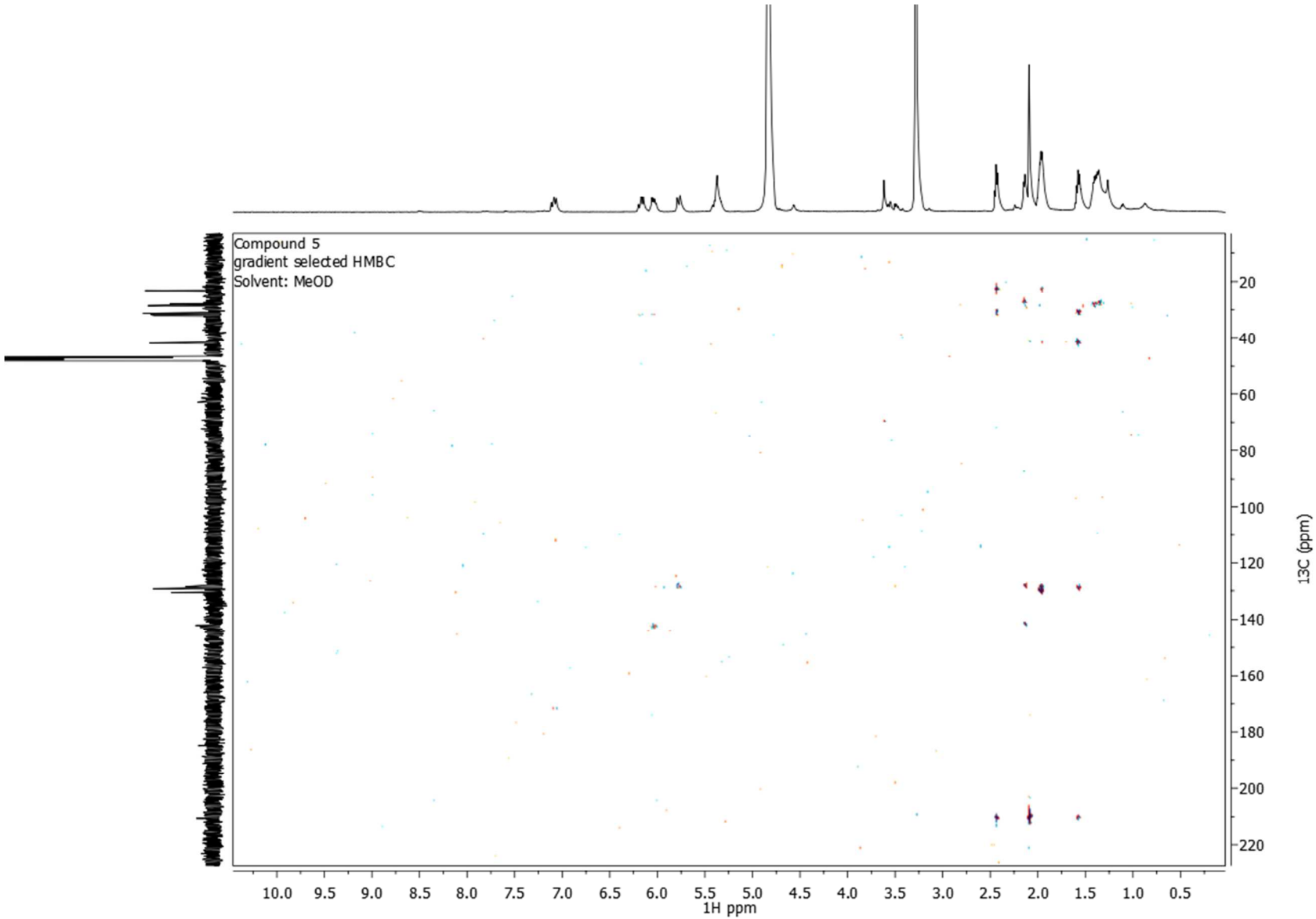


Figure S17D. HMBC spectrum for compound **5.**

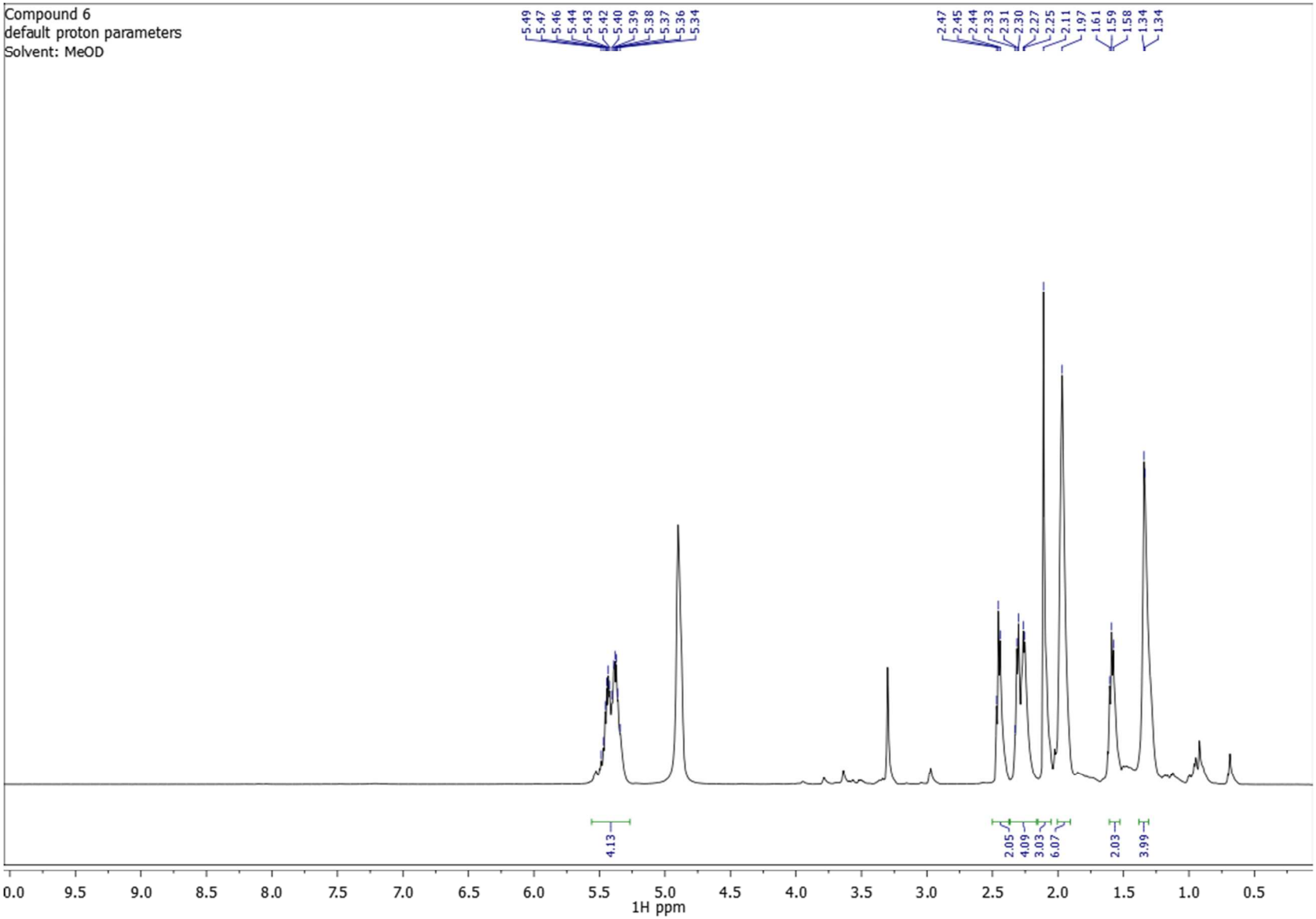


Figure S18A. 1H spectrum for compound **6**. Measured in CD_3OD at 500 MHz.

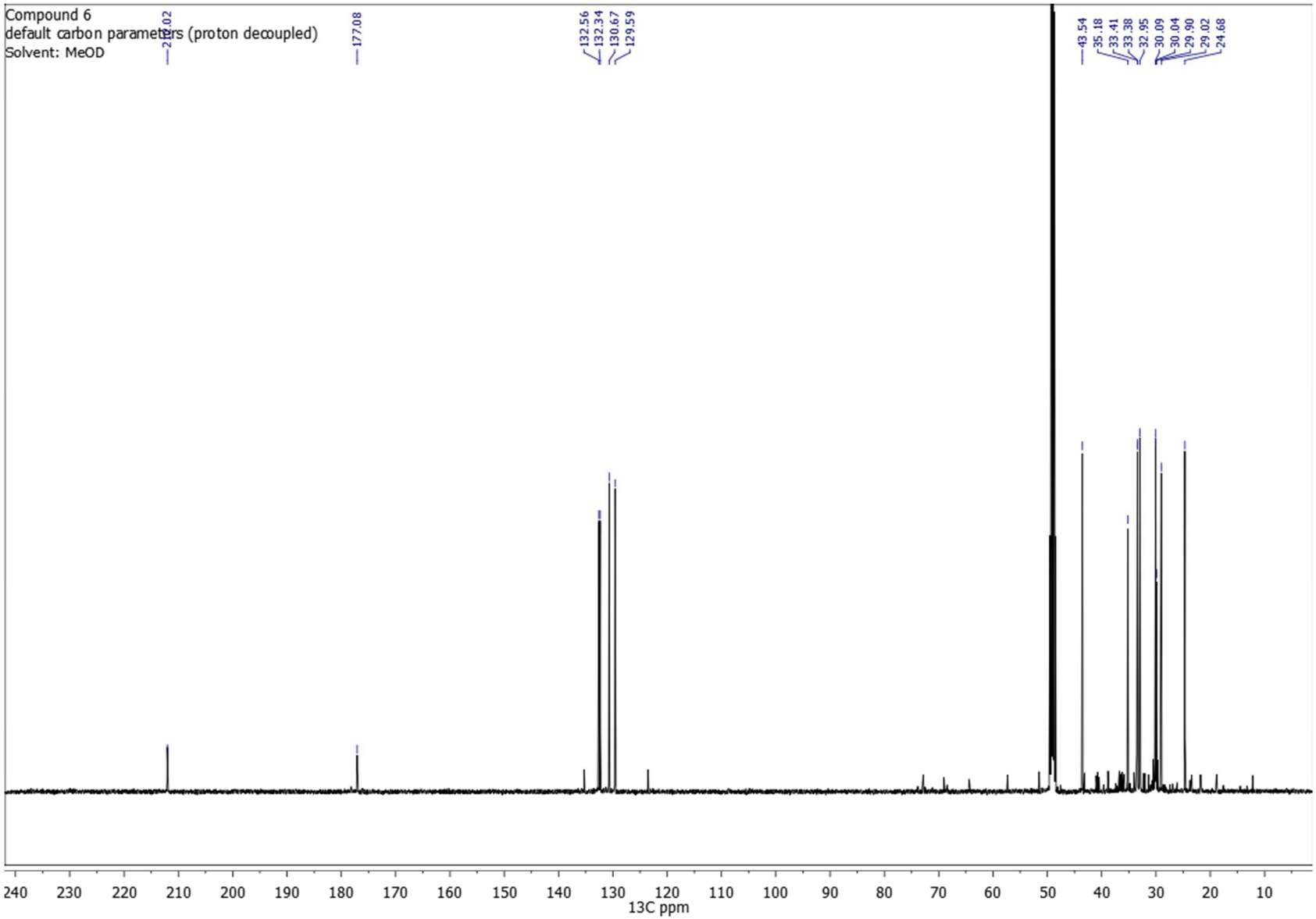


Figure S18B. 13C spectrum for compound **6.** Measured in CD_3OD at 125 MHz.

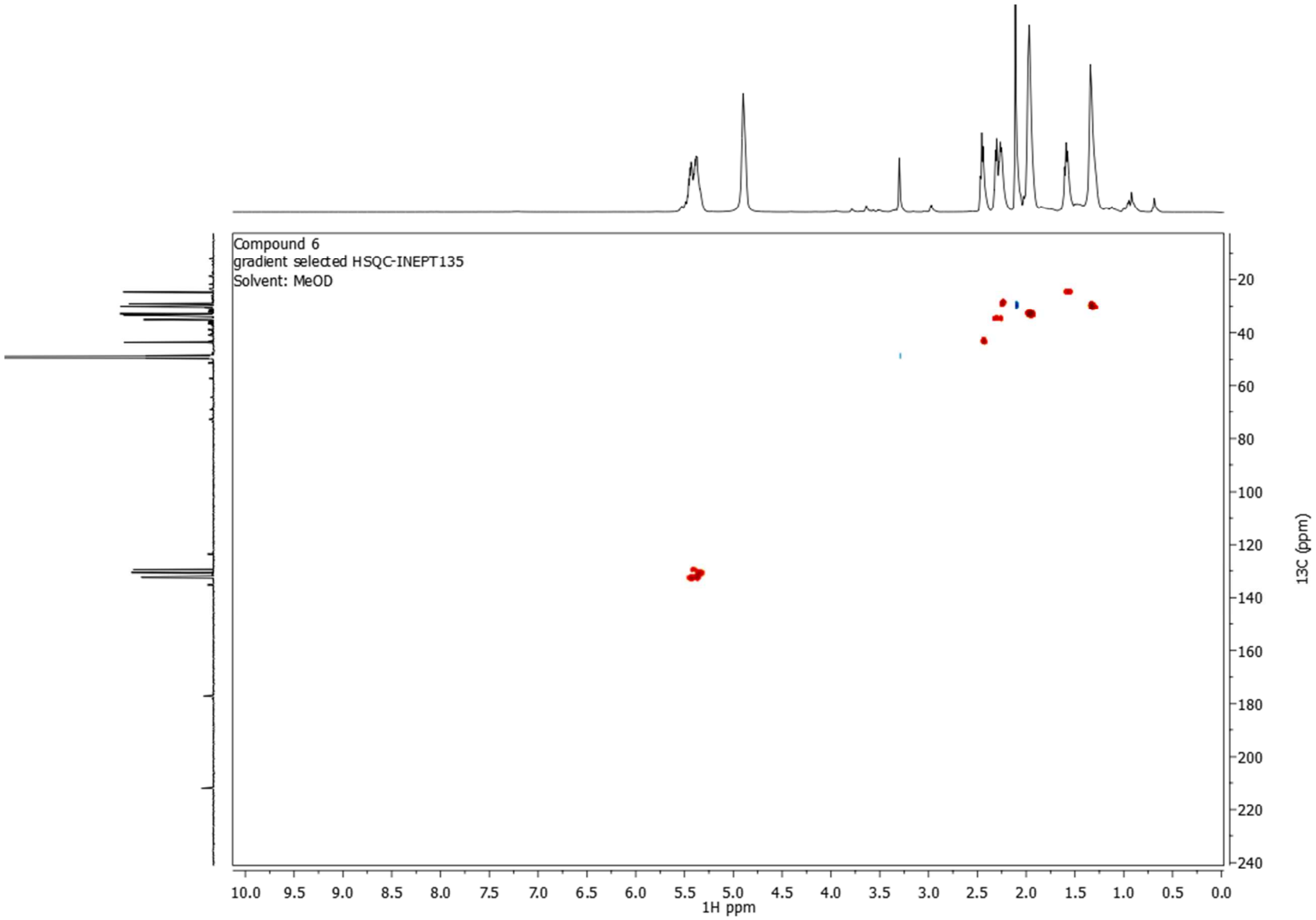


Figure S18C. HSQC-INEPT135 spectrum for compound **6.**

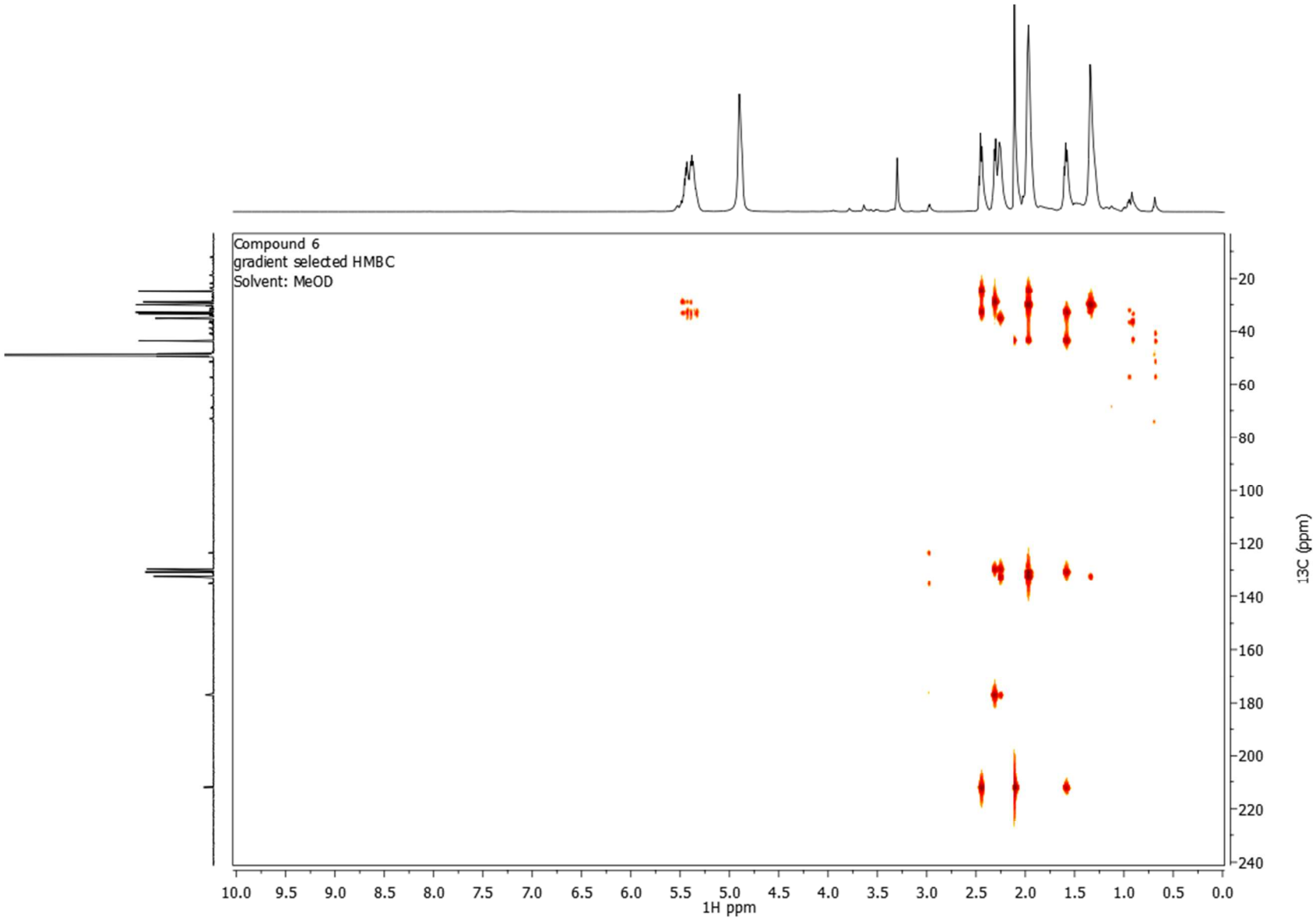


Figure S18D. HMBC spectrum for compound **6.**

Table S1. ^{1}H NMR (500 MHz, CD_3OH) and ^{13}C NMR (125 MHz, CD_3OH) data for **3**, **4**, **5** and **6**

	3		**4**		**5**		**6**	
No	**^{13}C NMR δ [ppm]**	**^{1}H NMR δ [ppm] (area, m, J_{hh} [Hz])**	**^{13}C NMR δ [ppm]**	**^{1}H NMR δ [ppm] (area, m, J_{hh} [Hz])**	**^{13}C NMR δ [ppm]**	**^{1}H NMR δ [ppm] (area, m, J_{hh} [Hz])**	**^{13}C NMR δ [ppm]**	**^{1}H NMR δ [ppm] (area, m, J_{hh} [Hz])**
1	171.3	-	177.8	-	171.2	-	177.1	-
2	121.1	5.78 (CH, d, 15.3)	35.6	2.31 (CH_2, d, 6.0)	122.4	5.82 (CH, d, 15.3)	35.2	2.32 (CH_2, d, 6.1)
3	146.5	7.22 (CH, dd, 15.3, 10.6)	29.2	2.27 (CH_2, m)	144.4	7.13 (CH, dd, 14.9, 10.9)	29.0	2.27 (CH_2, m)
4	129.8	6.23 (CH, dd, 15.1, 10.6)	129.7	5.44 (CH, dt, 3.8, 1.9)	129.3	6.22 (CH, dd, 15.1, 10.6)	129.6	5.44 (CH, dt, 3.8, 1.9)
5	145.4	6.16 (CH, m)	132.5	5.45 (CH, m)	143.6	6.10 (CH, m)	132.6	5.38 (CH, m)
6	33.8	2.19 (CH_2, m)	33.6	1.99 (CH_2, m)	33.4	2.19 (CH_2, m)	33.4	1.99 (CH_2, m)
7	29.3	1.45 (CH_2, m)	30.2	1.35 (CH_2, m)	29.9	1.41 (CH_2, m)	30.1	1.36 (CH_2, m)
8	30.2	1.38 (CH_2, m)	30.1	1.35 (CH_2, m)	30.2	1.41 (CH_2, m)	30.0	1.36 (CH_2, m)
9	33.6	2.01 (CH_2, m)	33.4	1.99 (CH_2, m)	33.00	1.99 (CH_2, m)	33.4	1.99 (CH_2, m)
10	131.4	5.41 (CH, m)	131.6	5.40 (CH, m)	130.6	5.42 (CH, m)	132.3	5.40 (CH, m)
11	131.6	5.41 (CH, m)	131.4	5.40 (CH, m)	128.6	5.46 (CH, m)	130.7	5.40 (CH, m)
12	33.4	2.01 (CH_2, m)	33.5	1.99 (CH_2, m)	33.8	2.14 (CH_2, m)	32.9	1.99 (CH_2, m)
13	26.9	1.42 (CH_2, m)	26.9	1.46 (CH_2, m)	24.7	1.60 (CH_2, m)	24.2	1.60 (CH_2, m)
14	39.7	1.44,1.39 (CH_2, m)	39.7	1.50,1.42 (CH_2, m)	43.5	2.48 (CH_2, t, 7.3)	43.5	2.46 (CH_2, t, 7.3)
15	68.5	3.71 (CH, dd, 11.6, 6.0)	68.5	3.71 (CH, dd, 12.0, 6.2)	212.3	-	212.0	-
16	23.5	1.14 (CH3, d, 6.2)	23.5	1.14 (CH_3, d, 6.2)	29.5	2.11 (CH_3, s)	29.1	2.09 (CH_3, s)

Table S2. Primers used in this study.		
Primer name	**Sequence (5' --> 3')**	**Note**
Primers for cloning		
Bref hydrointron for EcoRI	aagaattcatgcctggtcgggaacttgc	Bf286 hydrolase for pHis8 EcoRI for
Bref hydrointron rev1 NotI	aagcggccgcctacgcactcggtgcgtttc	Bf286 hydrolase for pHis8 NotI rev
AZ3	ctagcgattataaggatgatgatgataagactagtatggcgcctcacaactccct	Bf286 HR-PKS for XW55 P1for
AZ4	tactcttgacggagccaatatacagtggcgcctctggcttcctagatgcgctgaa	Bf286 HR-PKS for XW55 P1rev
AZ5	ggtgtcgcgtcagtgttcagcgcatctaggaagccagaggcgccactgtatattg	Bf286 HR-PKS for XW55 P2for
AZ6	cttgggcttgatgccgtcgaccttgttgacctgggccagatccacttttccaccg	Bf286 HR-PKS for XW55 P2rev
AZ7	atctctttacgtcgccggtggaaaagtggatctggcccaggtcaacaaggtcgac	Bf286 HR-PKS for XW55 P3for
AZ8	ggcgaaggtcaaaccagcagatttgagcaggtcagcgtaagagccattgtcgaac	Bf286 HR-PKS for XW55 P3rev
AZ9	tggagaggctgagcagttcgacaatggctcttacgctgacctgctcaaatctgct	Bf286 HR-PKS for XW55 P4for
AZ10	gtaacgcattgcgcccgaaacattgtcccatccgaatgtcgtagccacgtggact	Bf286 HR-PKS for XW55 P4rev
AZ11	tggccacgtcaagccagtccacgtggctacgacattcggatgggacaatgtttcg	Bf286 HR-PKS for XW55 P5for
AZ12	atttaaattagtgatggtgatggtgatgcacgtgtacagccgtcaaacgagcga	Bf286 HR-PKS for XW55 P5rev
AZ40	aactatcaactattaactatatcgtaatacatgcctggtcgggaacttgc	Bf286 hydrolase with XW51 overlap
AZ41	acctctcccttctgcaataattccaatcccctacgcactcggtgcgtttc	Bf286 hydrolase with XW51 overlap
Primers for mutagenesis		
AZ42	gaccaaacaaatatatcgttctgggggatgGctttttccgccctcgttgcactcaatgca	E. brefeldianum hydrolase S116A mutagenesis for
AZ43	ttgctgcttgtctgctggtgatatggtaGcattttccggaatgatcatgagcacccttgt	E. brefeldianum hydrolase D247A mutagenesis rev
AZ45	atgcctggtcgggaacttgctccccgtcaaaatgttgagttccaaactctcgacggtctc	E. brefeldianum hydrolase mutagenesis for
AZ46	cgcactcggtgcgtttccttttcctcgtgggcctttgataaagtacgcttgcatctcagc	E. brefeldianum hydrolase mutagenesis rev
AZ49	gtagtctgctccgctcaaaacgtcgagaGCacccttccctttcgcgatgtgagcggtctt	E. brefeldianum hydrolase H276A mutagenesis rev
Primers for RT-PCR		
HR-PKS and hydrolases		
Bref 286 HR-KS RTf	tccaactgctcgtgctctct	Bref 286 PKS, 600 bp region with intron for
Bref 286 HR-KS RTr	accttgggcttgatgccgtc	Bref 286 PKS, 600 bp region with intron rev
Bf72 HR-75 RTf	cagatcgatacgcgagcaag	Bref 72 PKS, 600 bp region with intron for

Bf72 HR-75 RTr	cagacggagatctgcacgag	Bref 72 PKS, 600 bp region with intron rev
Bf130 HR RTf	ctgacgggtcctagcatgac	Bref 130 PKS, 600 bp region with intron for
Bf130 HR RTr	gggcttcttaaagtcggcat	Bref 130 PKS, 600 bp region with intron rev
Bf63for RTPCR	agataagcaggagcccatcg	Bref 63 PKS, 600 bp region with intron for
Bf63rev RTPCR	ttttgccatcggcgttgaca	Bref 63 PKS, 600 bp region with intron rev
Bf189 HR RTf	ccagacgctagcatccggc	Bref 189 PKS, 600 bp region with intron for
Bf189 HR RTr	ggattttctccttgatctggaaaccc	Bref 189 PKS, 600 bp region with intron rev
AZ188	atgaggattctctgtctccatgg	RT-PCR check on Contig_63 esterase for
AZ189	ctatgcctgagctatagcccag	RT-PCR check on Contig_63 esterase rev
AZ190	atggccgacccagcctcc	RT-PCR check on Contig_130 thioesterase for
AZ191	ctacagcttctgctgaccctc	RT-PCR check on Contig_130 thioesterase rev
Bref_286 genes		
Bref Hydrolase RTf	atgcctggtcgggaacttgc	RT-PCR Bref_286 hydrolase, 600bp region for
Bref Hydrolase RTr	ctacgcactcggtgcgtttc	RT-PCR Bref_286 hydrolase, 600bp region rev
AZ30	atgttcgacatttacgactactcac	E. brefeldianum, 1.7kb P450-3a for
AZ31	tcactttaccagtaccctgactc	E. brefeldianum, 1.7kb P450-3a rev
AZ32	atgtatcaccttatcccgtttgcc	E. brefeldianum, 1.6kb P450-3b for
AZ33	ctagaactttctagtggtaagccg	E. brefeldianum, 1.6kb P450-3b rev
AZ34	atggttcaggagctgactgaagaa	E. brefeldianum, 1.5kb P450-4 for
AZ35	ctattgcttctgttggtgtcgc	E. brefeldianum, 1.5kb P450-4 rev
AZ36	atgttggcactttgggtattagctg	E. brefeldianum, 1.5kb P450-5 for
AZ37	ctacttaactagaacacgcacacc	E. brefeldianum, 1.5kb P450-5 rev
AZ89	atgcccggccgaccaaccgt	WD for RT-PCR for
AZ90	ctgcctggtagtgtgtcgattgg	WD for RT-PCR rev
AZ202	atgtctgactcgttgatgaaacgag	Gene 1 RT in Contig 286 for
AZ203	gttctttaccagtgacttcgaagac	Gene 1 RT in Contig 286 rev
AZ204	atgtcaggatcatcggagccttac	Gene 7 RT in Contig 286 for
AZ205	tcaggaaacaccggggtttatgg	Gene 7 RT in Contig 286 rev
AZ206	atggcacaccccagcatctc	Gene 9 RT in Contig 286 for