SUPPLEMENTAL INFORMATION:

Complexity generation in fungal polyketide biosynthesis: a spirocycle-forming P450 in the concise pathway to the antifungal drug griseofulvin

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Supplemental Materials and Methods

Strains and Culture Conditions. *P. aethiopicum* IBT 5753 was obtained from the IBT culture collection (Kgs. Lyngby, Denmark) and maintained on YMEG-agar (4g/L yeast extract, 10g/L malt extract, 4g/L dextrose, 16g/L agar) or glucose minimal media with 10 mM ammonium tartrate as sole nitrogen source (GMM-NH_4^+)[1] at 28 °C. *Saccharomyces cerevisiae* strain BJ5464-NpgA (*MATα ura3-52 his3-Δ200 leu2- Δ1 trp1 pep4::HIS3 prb1Δ1.6R can1GAL*) was used as the yeast expression strain. *Escherichia coli* BL21 (DE3) (Novagen) was used as the *E.coli* expression strain.

General Molecular Biology Experiments. General molecular cloning techniques were done as described elsewhere.[2] PCR was performed using Phusion® DNA Polymerase (New England Biolabs). DNA restriction enzymes were used as recommended by the manufacturer (New England Biolabs). DNA sequences of the gene constructs were confirmed by Sanger sequencing by Laragen, Inc. *E. coli* TOP10 (Invitrogen) and XL1-Blue (Stratagene) were used for cloning, following standard recombinant DNA techniques.[2] RNA extraction was performed using a RiboPure Yeast Kit (Ambion) and ImProm-II™ Reverse Transcription System for RT-PCR (Promega) was used to synthesize complementary DNA (cDNA) from total RNA. Transformation of *S. cerevisiae* BJ5464 transformation was done using the *SC* EasyComp Transformation kit (Invitrogen).

General protocol for protein expression in *E.coli*. Expression plasmid p*ET*28-*gsfB*, p*ET*30-*gsfC*, p*ET*24-*gsfD*, p*ET*24-*gsfI* or p*ET*28-*gsfE* was transformed into electrocompetent *E.coli* BL21 (DE3) and the cells were grown in 500 mL LB at 37°C and 250 rpm. When the OD_{600} reading reached 0.5, the cultures were cooled to 16 °C and protein expression was induced by addition of 60 µM IPTG and grown for additional 16 hours prior to His-tag-fusion protein purification (*vide infra*).

Heterologous expression of GsfA in *S.cerevisiae*. The two fragments of the cDNA for *gsfA*, was amplified using primer pairs GsfA-*Nhe*I-F and GsfA-*Afl*II-R and GsfA-*Afl*II-F and GsfA-*EcoR*V-R and cut with the appropriate restriction enzymes. The two fragments were simultaneously ligated into YepLac195 vector (ura3 selection marker), linearized by digestion with *Nhe*I and *Sma*I, such that the *gsfA* cDNA is flanked upstream by ADH2 promoter and downstream by an in-frame C-terminal His-tag and ADH2 terminator. The resulting YepLac195-GsfA plasmid was transformed for propagation in *E. coli* XL1and verified by sequencing. BJ5464-NpgA cells harboring YepLac195-GsfA were grown in YPD (10 g/L yeast extract, 20 g/L peptone) supplemented with 1% dextrose and incubated at 28 °C with shaking for 72 hours prior to His-tag fusion protein purification (*vide infra*).

General His-tag fusion protein purification. *S.cerevisiae* BJ5464-NpgA or *E.coli* BL21 cells expressing the desired protein were pelleted and resuspended in Buffer A (50 mM Tris-HCl pH 7.9, 500 mM NaCl) with 20 mM imidazole prior to lysing by sonification. The cell lysate was subjected to centrifugation at 27000g for 30 min for *E.coli* or 40000g for 1 hour for *S.cerevisiae*. Nickel-NTA-agarose resin was then added to the clarified lysate and the mixture was gently stirred at 4 °C overnight. The desired protein was then purified using gravity-flow column chromatography with increasing concentrations of imidazole (20-250 mM) in Buffer A. Purified protein was concentrated and buffer was exchanged into Buffer B (50 mM Tris-HCl, 2 mM EDTA, 50 mM NaCl, pH 8.0) using an Amicon Ultra-15 Centrifugal Filter Unit and stored in 10% glycerol.

LCMS Analysis of *In vitro* assay and knockout mutant metabolic extracts. LC-MS was conducted with a Shimadzu 2010 EV Liquid Chromatograpy Mass Spectrometer using both positive and negative electrospray ionization monitoring the *m*/*z* range 200-800 and photodiode array monitoring the 290 nm wavelength. Samples were separated on a Phenomenex Luna 5µ 100 x 2 mm C18 reverse-phase column using a flow rate of 0.1 mL/min on a linear gradient of 5-95% solvent B in 30 min followed by isocratic 95% solvent B for another 15 min (solvent A: water with 0.1% (v/v) formic acid, solvent B: acetonitrile with 0.1% (v/v) formic acid).

Purification and Characterization of Norlichexanthone (5) from *S.cerevisiae*. *S. cerevisiae* strain BJ5464-NpgA harboring YepLac195-GsfA plasmid was inoculated to 4 mL Yeast Synthetic Drop-Out medium without uracil. The cells were grown for 36 hours with constant shaking at 28 °C. The seed culture was inoculated 1 L YPD (10 g yeast extract, 20 g peptone and 950 mL Milli-Q water) supplemented with 1% dextrose and grown for an additional 72 hours at 28 °C with constant shaking. The cells were harvested by centrifugation (3750 rpm, 10 minutes, 4 °C), extracted twice with equal volume of ethyl acetate and was evaporated to dryness. The extract was redissolved, loaded into a Sephadex LH-20 column (300 mm x 30 mm) and eluted using 1:1 ratio of chloroform and methanol. The fractions containing the desired compound, as ascertained using thin-layer chromatography and LCMS, were pooled and dried. The dried extract was resuspended in minimal volume of methanol, centrifuged and injected to the HPLC equipped with a Phenomenex Luna 5µ 250 x 10mm C18 reverse-phase column at a flow rate 2.5 mL/min and using a linear gradient of 40-80% solvent B over 30 min (Solvent A: water with 0.1% trifluoroacetic acid (TFA), solvent B: acetonitrile with 0.1% TFA). NMR characterization (1D ^{1}H and ^{13}C, 2D HSQC and HMBC) of the purified compound was then performed on a Bruker AV500 NMR (500 MHz) equipped with 5mm dual cryoprobe at the UCLA Molecular Instrumentation Center**.**

Fungal Transformation and Gene Deletion in *P. aethiopicum*. Polyethylene glycol (PEG)-mediated transformation was done essentially as described previously.[3] Briefly, conidia from *P.aethiopicum* was inoculated to 250 mL liquid GMM-NH_4^+ for 13 hours at 28 °C, 250 rpm for germination. The harvested germlings were then digested with 3 mg/mL lysing enzyme (Sigma-Aldrich) and 2 mg/mL Yatalase (Takara Bio) to obtain the protoplasts, which were then transformed with the linear knockout cassette. The linear knockout cassettes containing the glufosinate resistance gene *bar* was constructed as described elsewhere[3, 4] and using primers listed in Table S1 (See Supplemental Information Table S1). After PEG-mediated transformation, the protoplasts were inoculated into GMM-NH_4^+ media supplemented with 1.2 M sorbitol, agar and 10mg/mL of glufosinate as selective agent. Genomic DNA from the transformants was isolated using Carlson lysis buffer and chloroform extraction followed by precipitation of DNA in the aqueous phase by addition of equal volume of isopropanol. PCR screening was performed as described previously[3] using primers listed in Table S1.

Biotransformation of 11 to 14. *S. cerevisiae* BJ5464 transformed with *pESC-gsfF*/*AtCPR* was inoculated in 5 mL synthetic leucine dropout synthetic media (SDM, -Leu) and was grown overnight at 28 °C, 250 rpm. The overnight culture was inoculated to 500 mL synthetic leucine dropout media with galactose (SGMM, -Leu) for induction. The cells were grown at 28 °C, 250 rpm for 36 hours after induction and were pelleted by centrifugation at 4 °C. The cell pellet was resuspended in 50 mL SGMM, -Leu and 11 was added to a final concentration of 1 µM. The culture was grown at 28 °C, 250 rpm for 24 hours after feeding. Thereafter 1 mL of whole cell

culture was taken, extracted twice with ethyl-acetate and dried to completeness before being subjected to LCMS analysis as described above.

Yeast microsome isolation and *in vitro* assay of GsfF. Yeast microsome extraction was adapted from the method described by Ralston *et al* and Barriuso *et al*.[5, 6] The overnight culture of BJ5464-NpgA harboring p*ESC-gsfF*/*AtCPR*, grown in the same manner as was done for the biotransformation of 11 to 14, was inoculated to 20 mL SDM, -Leu and shaken for an additional 24 hrs prior to transfer to 500 mL synthetic leucine dropout media with galactose (SGMM, -Leu) for induction. The cells were grown at 28 °C, 250 rpm for 24 hours after induction and were pelleted by centrifugation at 4 °C. The cell pellet was then resuspended in 100 mL TES buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA and 0.6 mM sorbitol). The cells were pelleted again, resuspended in 100 mL TES buffer with 10 mM β-mercaptoethanol and incubated at 25 °C for 10 minutes. The cells were then pelleted and resuspended in 2.5 mL extraction buffer (TES buffer supplemented with 1% bovine serum albumin and 2 mM β-mercaptoethanol and 1mM phenylmethylsulfonyl fluoride (Sigma-Aldrich)). Zirconia/silica beads (0.5 mm in diameter, Biospec Products) were added until skimming the surface of the cell suspension. Cell walls were disrupted manually by hand-shaking in a cold room for 10 min at 30 s intervals separated by 30 s intervals on ice. Cell extracts were transferred to a 50 mL centrifuge tube, the Zirconia/silica beads were washed three times with 5 mL of extraction buffer, and the washes were pooled with the original cell extracts. Finally, microsomes were obtained by differential centrifugation at 10,000*g* for 10 min at 4°C to remove cellular debris followed by centrifugation at 100,000*g* for 70 min at 4°C. The microsomal pellets were weighed prior to resuspension in 1.5 mL of TEG-M buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 20% glycerol, and 1.5 mM 2-mercaptoethanol) and stored frozen at -80 °C. The GsfF in vitro assay was performed by addition of microsomal protein to a final concentration of 0.42 mg/mL to 50 μM griseophenone B and 2mM NADPH in a 100 μL reaction. The reaction was incubated at 28 °C overnight and was extracted with ethyl acetate. The organic phase was dried and redissolved in 10 μL methanol prior to injection to LC-MS and analyzed as the same manner as above.

Table S1: Primers used for this study

Primer name	**Sequence (5'-> 3')**	**Notes**
gsfB-KO-P1	ctatgaagacccttccacctggcta	Screening of mutants and cloning of KO cassette
gsfB-KO-P2	acgcactcgttgctatgtcaaaca	cloning of KO cassette
gsfB-KO-P3	cctgcccgtcaccgagatttagaacgagcaacctgtcaaggatg	
gsfB-KO-P4	cttcaatatcatcttctgtcgacgtcgcctgctaagggaatctgacgggcaa	
gsfB-KO-P5	cttgagcgcctcctgagagtt	
gsfB-KO-P6	tccgacgacaggttccatcactct	Screening of mutants and cloning of KO cassette
gsfC-KO-P1	catccttgacaggttgctcgtt	Screening of mutants and cloning of KO cassette
gsfC-KO-P2	cgacacgttcttgccctctcgaa	cloning of KO cassette
gsfC-KO-P3	ctgcccgtcaccgagatttagtaccttactcgtcagccagtct	
gsfC-KO-P4	cttcaatatcatcttctgtcgac-acaactctcaggaggcgctc	
gsfC-KO-P5	tccatacccccgacacctcc	
gsfC-KO-P6	tcgaccagtctctcggcgta	Screening of mutants and cloning of KO cassette
gsfD-KO-P1	acgacttgccatcggcacca	Screening of mutants and cloning of KO cassette
gsfD-KO-P2	ctttctgggcatgttaggcgaa	cloning of KO cassette
gsfD-KO-P3	ctgcccgtcaccgagatttagaggctggatcggtattgagc	
gsfD-KO-P4	cttcaatatcatcttctgtcgacg-gactaagaatatcacgaggt	
gsfD-KO-P5	gattacgccaagccataaggca	
gsfD-KO-P6	gacacagtctgcactatgtcgaata	Screening of mutants and cloning of KO cassette
gsfE-KO-P1	gcaagttctagtacccgcgc	Screening of mutants and cloning of KO cassette
gsfE-KO-P2	gtctgagtcggtacgctcg	cloning of KO cassette
gsfE-KO-P3	tgcccgtcaccgagatttaggaagccagcactgagcactgc	
gsfE-KO-P4	tcaatatcatcttctgtcgaccgacaagtttagacagctgggg	
gsfE-KO-P5	gccttgagattcttggtctggg	
gsfE-KO-P6	gcacctgggcaaattgaatgg	Screening of mutants and cloning of KO cassette

Table S1 (continued): Primers used for this study

Primer name	Sequence (5'-> 3')	Notes
gsfF-KO-P1	ggaccttaacccgactaagaatatc	Screening of mutants and cloning of KO cassette
gsfF-KO-P2	aaggtccagctgatctcatgaatgtg	cloning of KO cassette
gsfF-KO-P3	ctgcccgtcaccgagatttagcataggtcaaccatagtcgggtg	
gsfF-KO-P4	cttcaatatcatcttctgtcgac gtgctgacatcgtcacagattgctc	
gsfF-KO-P5	Ataggatggcatcgcgtataagg	
gsfF-KO-P6	cttagcttcaggctctacgcg	Screening of mutants and cloning of KO cassette
gsfH-KO-P1	gtatggtccctcgggtgc	Screening of mutants and cloning of KO cassette
gsfH-KO-P2	ggatacattttcttcgtcggc	Cloning of KO cassette
gsfH-KO-P3	tgcccgtcaccgagatttaggtggactagacatcctgatcc	
gsfH-KO-P4	tcaatatcatcttctgtcgacaccaaggtagtgtcgaatcc	
gsfH-KO-P5	gtggcccaggaaattggg	
gsfH-KO-P6	gcttggatcgggctcg	Screening of mutants and cloning of KO cassette
gsfK-KO-P1	gtggtggcggttctcag	Screening of mutants and cloning of KO cassette
gsfK-KO-P2	gacctcaggcaaggagac	Cloning of KO cassette
gsfK-KO-P3	tgcccgtcaccgagatttaggctcgatgaggaactgtggcag	
gsfK-KO-P4	tcaatatcatcttctgtcgaccgtctaccacaacctaccgc	
gsfK-KO-P5	ggaaacccacccagtcttgc	
gsfK-KO-P6	ggccgagcgaaatgacgg	Screening of mutants and cloning of KO cassette
gsfA-*Nhe*I-F	aaaatggctagcatgacttccgctaaggtctta	Cloning of YepLac195-gsfA
gsfA-*Afl*II-R	gcctcttaagaaccacgcttccgactcc	
gsfA-*Afl*II-F	aattcttaagaggcttgaggatgcagaagcc	
gsfA- *EcoR*V-R	gatatcacgcacctcgatgccaaggtctt	
gsfB- F	aaattcatatggcgtccaatacaagtcggt	Cloning of pET28-gsfB
gsfB- R	caatggatccacacatgccgaaatcgatgtttctga	
gsfC- F	ggtattgagggtcgcatgactcttgaccaaattagtcggatac	Cloning of pET30-gsfC
gsfC_R	agaggagagttagagccttatgacgtcgctaagctggc	

Table S1 (continued): Primers used for this study

Primer name	**Sequence (5'-> 3')**	**Notes**
gsfD-F	agctacatatgtctacccctgagcaatggatcca	Cloning of pET24-gsfD
gsfD- R	aaatttggatccctccctctttaaccttgcct	
gsfE- F	tttacatatgccaaaaacagctttcatcactggc	Cloning of pET28-gsfE
gsfE- R	aatgaattcatttaggtatcaaccccagctgtc	
gsfF- F	accctcactaaagggcggccgcatgactgttttgtttattct	Cloning of pESC-Leu-AtCPR/GsfF
*gsfF*_R	cttatcgtcgtcatccttgtaatctagacctcggactgtaactttaacc	
gsfI-F	aaaaagctagcgcgattcctcaatcttgtac	Cloning of pET24-gsfI
gsfI-R	ataatgaatcccatttggagatcccct	

Table S2: Mass and UV-Vis spectra and NMR data of **5** in $(CD_3)_2CO$ on 500 MHz Bruker NMR spectrometer

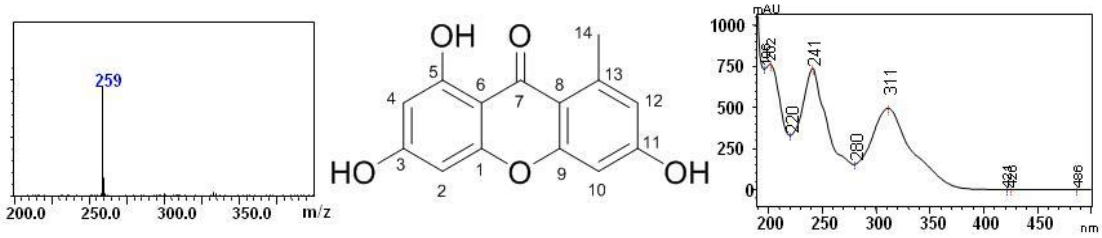
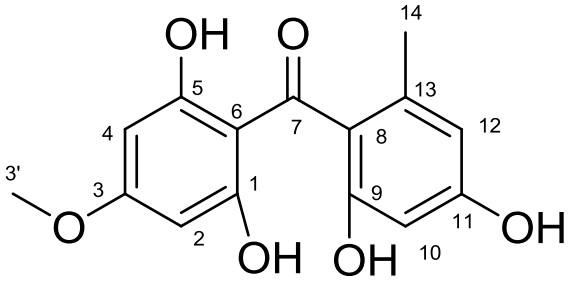
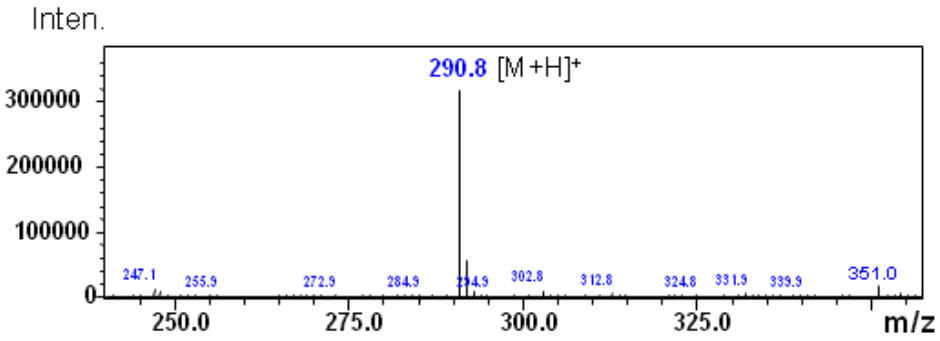
Norlichexanthone (**5**) (M.W. 258.2 g/mol)			
			
Assignment	**^{13}C ppm**	**^{1}H ppm, integration, mult, J_{HH} (Hz)**	**HMBC signals**
C1	158.026		H2
C2	93.946	6.321, 1H, t, 2 Hz	H4
C3	164.889		H4
C4	98.686	6.189, , 1H, t, 2 Hz	H2, O5-H
C5	164.563		H4, H14, O5-H
C6	104.183		H4, H14, O5-H
C7	182.984		H2
C8	112.558		H12, H14
C9	160.214		H10
C10	101.967	6.721, 1H, s	H12, H14
C11	160.231		H10, H12
C12	116.889	6.708, 1H, s	H10, H14
C13	144.368		H10, H14
C14	23.426	2.776, 3H, s	H12
O5-H		13.452, 1H, s	

Table S3: Mass and UV-Vis spectra of **6**.

Griseophenone D (**6**)(M.W. 290.3)





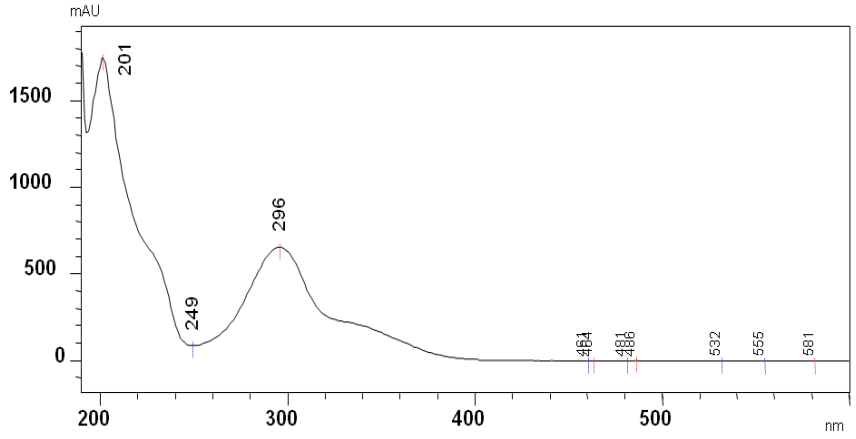


Table S4: Mass and UV-Vis spectra and NMR data of **7** in $(CD_3)_2CO$ on 500 MHz Bruker NMR spectrometer

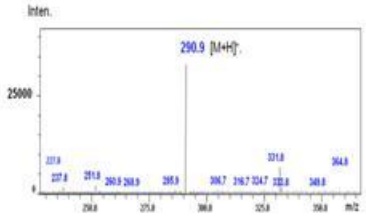
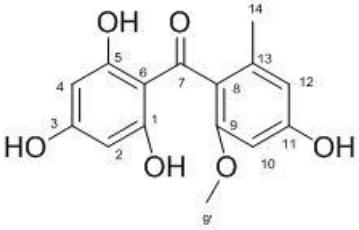
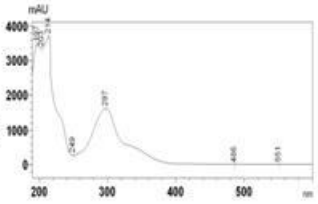
Griseophenone E (**7**)(M.W. 290.3 g/mol)			
			
Assignment	**^{13}C ppm**	**^{1}H ppm, integration, mult, J_{HH} (Hz)**	**HMBC signals**
C1, C5	166.54		H2, H4
C2, C4	95.78	5.86, 2H, s	H2, H4
C3	165.66		H2, H4
C6	106.88		H2, H4
C7	199.92		
C8	125.41		H14, H10, H12
C9	158.32		H9', H10
C9'	55.96	3.64, 3H,s	
C10	97.45	6.31,1H, d, 1.5 Hz	
C11	159.36		H10
C12	109.62	6.29, 1H, d, 1.5Hz	H14, H10, H12
C13	136.40		H14
C14	19.17	2.08, 3H,s	H12

Table S5: Mass and UV-Vis spectra and NMR data of **8** in $(CD_3)_2CO$ on 500 MHz Bruker NMR spectrometer

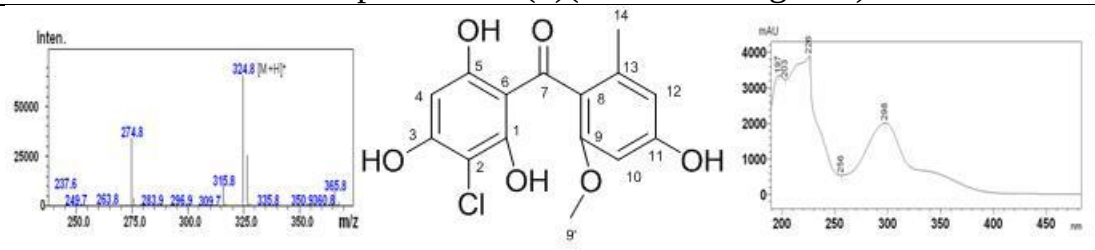
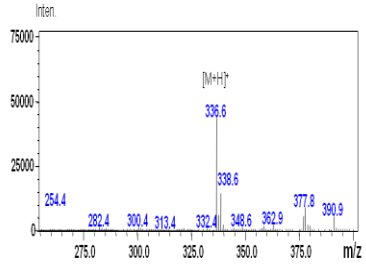
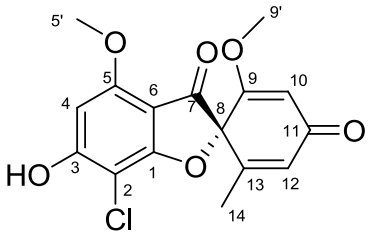
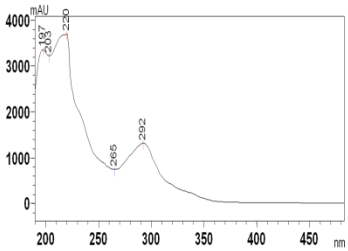
Griseophenone F (**8**)(M.W. 324.7 g/mol)			
			
Assignment	**^{13}C ppm**	**^{1}H ppm, integration, mult, J_{HH} (Hz)**	**HMBC signals**
C1	161.38		
C2	99.95		H4
C3	161.57		H4
C4	95.81	6.08 , 1H, s	
C5	162.21		
C6	107.27		H4
C7	200.51		H4
C8	124.97		H14, H12, H10
C9	158.46		H9', H10
C9'	55.98	3.64 , 1H, s	
C10	97.63	6.32 , 1H, d, 1.8 Hz	
C11	159.55		H12
C12	109.66	6.31, 1H, d, 1.8 Hz	H14, H10
C13	136.61		H14
C14	19.18	2.09, 3H, s	H12

Table S6: Mass and UV-Vis spectra and NMR data of **9** in $(CD_3)_2CO$ on 500 MHz Bruker NMR spectrometer

Desmethyl-dehydrogriseofulvin B (**9**)(M.W. 336.7 g/mol)



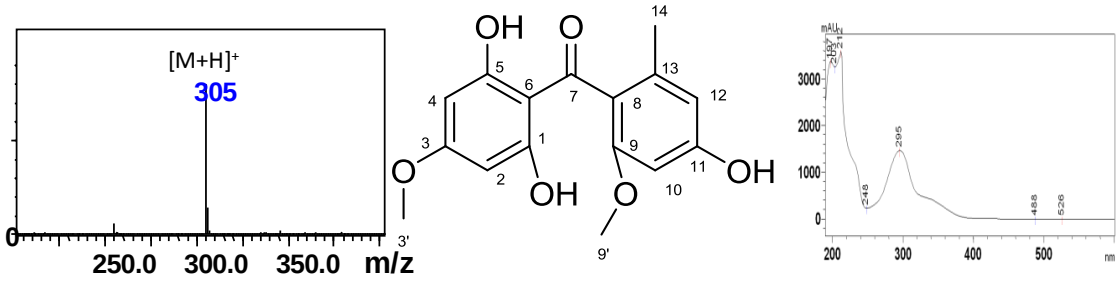




Assignment	^{13}C ppm	^{1}H ppm, integration, mult, J_{HH} (Hz)	HMBC signals
C1	170.3114		
C2	95.4402		H4
C3	165.0561		H4
C4	94.6581	6.795 ,1H, s	
C5	157.9998		H5', H4
C5'	55.6593	3.868, 3H, s	
C6	102.9		H4
C7	187.4717		H4
C8	88.4008		H14, H10, H12
C9	167.8811		H9', H10
C9'	56.0094	3.707,3H, s	
C10	103.1345	5.681 ,1H, d, 1.1 Hz	
C11	185.3387		H10
C12	128.6774	6.149,1H, t, 1.1 Hz	H10, H14
C13	147.2017		H14
C14	15.4892	1.773 ,3H, d, 1.1 Hz	H12

Table S7: Mass and UV-Vis spectra and NMR data of **10** in $(CD_3)_2CO$ on 500 MHz Bruker NMR spectrometer

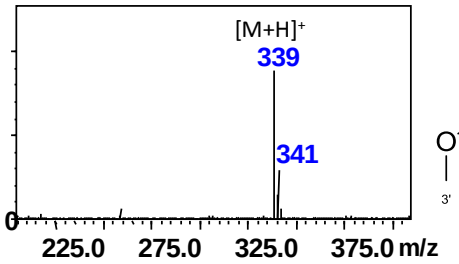
Griseophenone C (**10**) (M.W. 304.1 g/mol)

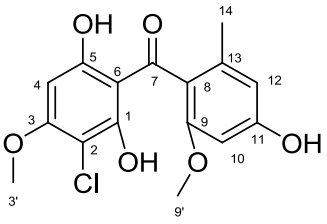


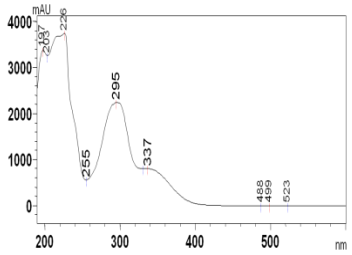
Assignment	^{13}C ppm	^{1}H ppm, integration, mult, J_{HH} (Hz)	HMBC signals
C1, C5	165.3484		H(2,4)
C4, C2	94.18	5.93, 2H, s	H(2,4)
C3	167.5571		H3', H(2, 4)
C3'	55.94	3.81, 3H, s	
C6	107.5		H(2,4)
C7	200.56		
C8	125.31		H12, H14
C9	158.36		H9'
C9'	55.8272	3.64, 3H, s	
C10	97.3642	6.33, 1H, d, 1.8 Hz	H12
C11	159.41		H10, H12
C12	109.59	6.31, 1H, d, 1.8 Hz	H10,H14
C13	136.41		H14
C14	19.1588	2.08, 3H, s	H9'

Table S8: Mass and UV-Vis spectra and NMR data of **11** in $(CD_3)_2CO$ on 500 MHz Bruker NMR spectrometer.

Griseophenone B (**11**) (M.W. 338.1 g/mol)



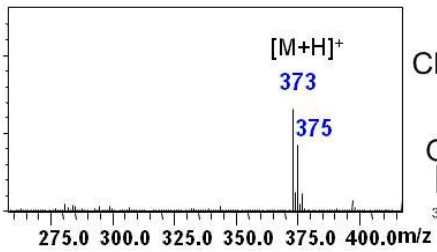


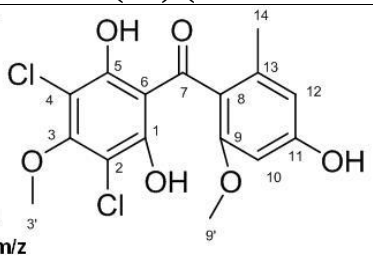


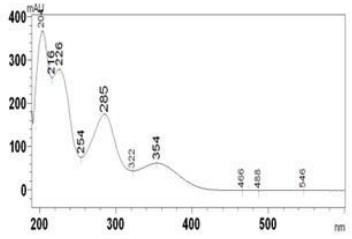
Assignment	^{13}C ppm	^{1}H ppm, integration, mult, J_{HH} (Hz)	HMBC signals
C1	159.1		
C2	99.72		H4
C3	161.52		H3',H4
C3'	55.93	3.905, 3H, s	
C4	91.79	6.164, 1H, s	
C5	162.33		
C6	106.8		H4
C7	200.05		H4
C8	123.96		H10,H12, H14
C9	157.64		H9'
C9'	55.11	3.628, 3H, s	
C10	96.48	6.323, 1H, d, 1.8 Hz	H9', H12
C11	158.79		H10, H12
C12	108.84	6.315, 1H, d 1.8 Hz	H10, H14
C13	135.83		H14
C14	18.3164	2.08, 3H, s	H12

Table S9: Mass and UV-Vis spectra and NMR data of **12** in $(CD_3)_2CO$ on 500 MHz Bruker NMR spectrometer.

Griseophenone G (**12**) (M.W. 372.0 g/mol)



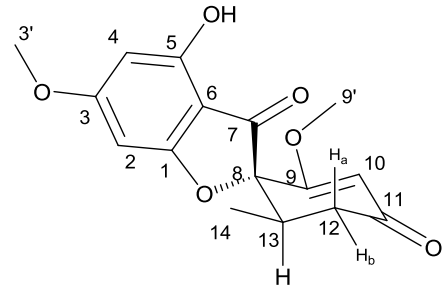


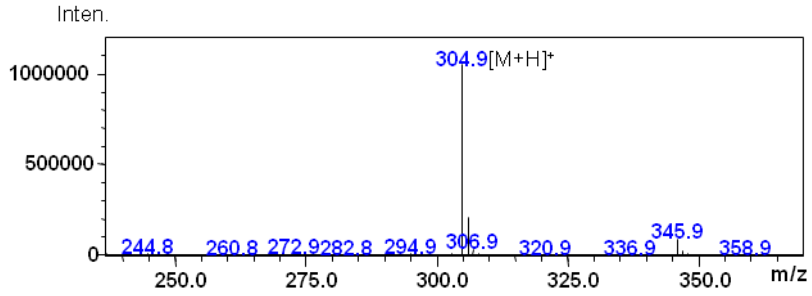


Assignment	^{13}C ppm	^{1}H ppm, integration, mult, J_{HH} (Hz)	HMBC signals
C1 and C5	160.2667		
C2 and C4	158.044		
C3	158.98		H3’
C3'	56.025	3.626 , 3H, s	
C6	110.781		
C7	201.7137		
C8	124.185		H12, H14
C9	159.223		H9’, H10
C9'	61.21	3.963, 3H, s	
C10	97.39	6.341, 1H, s	H12
C11	107.909		H10, H11
C12	110.049	6.341 , 1H,s	H10, H14
C13	137.428		H14
C14	19.358	2.12 , 3H, s	H12

Table S10: Mass and UV-Vis spectra of **13**.

Desmethyl-dechloro-griseofulvin (**13**) (M.W. 304.7 g/mol)





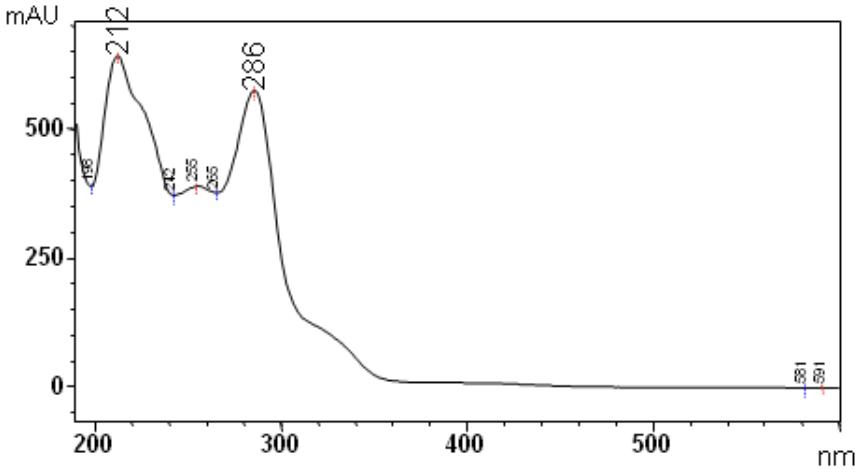


Table S11: Mass and UV-Vis spectra and NMR data of **14** in $(CD_3)_2CO$ on 500 MHz Bruker NMR spectrometer.

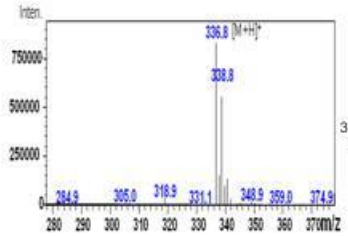
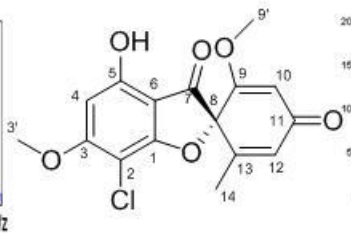
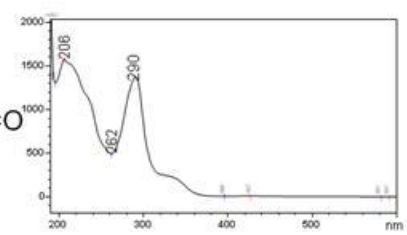
Desmethyl-dehydro-griseofulvin A (**14**) (M.W. 336.7 g/mol)			
  			
Assignment	**^{13}C ppm**	**^{1}H ppm, integration, mult, J_{HH} (Hz)**	**HMBC signals**
C1	169.42		
C2	104.52		H4
C3	165.69		H3', H4
C3'	57.69	4.01, 3H, s	
C4	95.59	6.42, 1H, s	
C5	158.46		H4
C6	96.15		H4
C7	190.24		H4
C8	89.31		H10, H12, H14
C9	168.62		H9', H10
C9'	57.09	3.72, 3H, s	
C10	104.24	5.7, 1H, d, 1.05 Hz	
C11	186.2		H10
C12	129.82	6.17, 1H, t, 1.3 Hz	H10, H14
C13	147.87		H14
C14	16.53	1.79, 3H, d, 1.2 Hz	H12

Table S12: Mass and UV-Vis spectra and NMR data of **15** in $(CD_3)_2CO$ on 500 MHz Bruker NMR spectrometer.

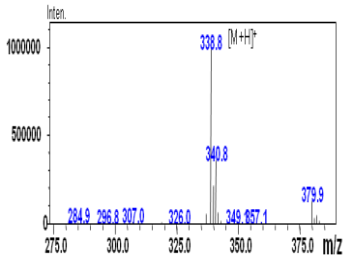
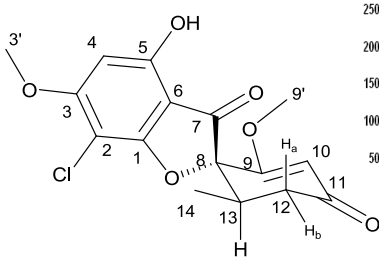
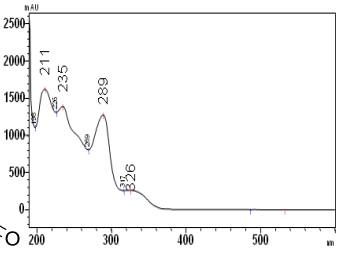
Desmethyl-griseofulvin (**15**) (M.W. 338.7 g/mol)			
  			
Assignment	**^{13}C ppm**	**^{1}H ppm, integration, mult, J_{HH} (Hz)**	**HMBC signals**
C1	168.327		
C2	95.204		H4
C3	164.704		H3', H4
C3'	56.763	3.99, 3H, s	
C4	94.197	6.393, 1H, s	
C5	156.209		H4
C6	104.181		H4
C7	192.884		H4
C8	90.499		H10,H14
C9	170.4		H9', H10
C9'	56.404	3.69, 3H, s	
C10	104.443	5.554, 3H, s	
C11	194.957		H10, H12a, H12b,
C12	39.769	H12a-2.35, 1H, dd, 3.6 Hz, 14 Hz H12b,-2.80, 1H, dd(overlap)	H10, H13
C13	36.235	2.80, 1H, m (overlap)	H12, H14
C14	13.573	0.92, 3H, d, 6.2 Hz	H13

Table S13: Mass and UV-Vis spectra and NMR data of **16** in $(CD_3)_2CO$ on 500 MHz Bruker NMR spectrometer.

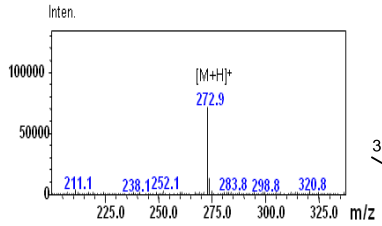
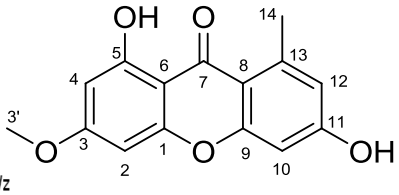
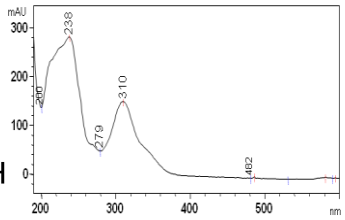
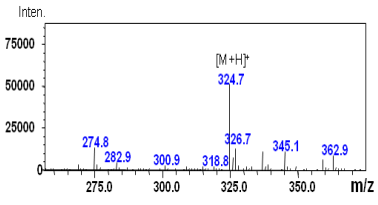
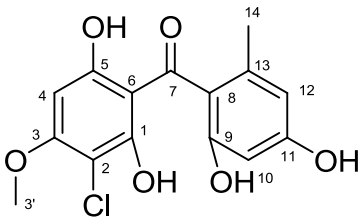
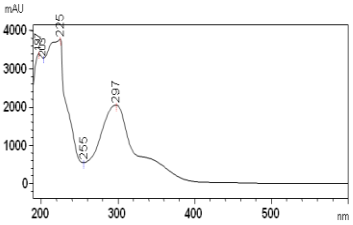
Griseoxanthone C (**16**) (M.W. 272.3 g/mol)			
  			
Assignment	**^{13}C ppm**	**^{1}H ppm, integration, mult, J_{HH} (Hz)**	**HMBC signals**
C1	157.87		H2
C2	92.48	6.42, 1H, d, 2 Hz	H4
C3	166.98		H4, H3'
C3'	56.27	3.92, 3H, s	
C4	97.50	6.260, , 1H, d, 2 Hz	H2, O5-H
C5	163.80		H4, H14, O5-H
C6	104.33		H4, H14, O5-H
C7	183.08		H2
C8	112.50		H12, H14
C9	160.30		H10
C10	101.50	6.786, 1H, s	H12, H14
C11	164.58		H10, H12
C12	117.05	6.736, 1H, s	H10, H14
C13	144.39		H10, H14
C14	23.42	2.770, 3H, d, 1.5 Hz	H12
O5-H		13.489, 1H, s	

Table S14: Mass and UV-Vis spectra and NMR data of **17** in $(CD_3)_2CO$ on 500 MHz Bruker NMR spectrometer.

Griseophenone H (**17**) (M.W. 324.7 g/mol)







Assignment	^{13}C ppm	^{1}H ppm, integration, mult, J_{HH} (Hz)	HMBC signals
C1	157.09		
C2	100.6		H4
C3	162.07		H3', H4
C3'	56.8	3.91 ,3H, s	
C4	92.83	6.21,1H, s	
C5	162.63		H4
C6	108.49		H4
C7	200.73		
C8	122.55		H12, H14
C9	159.29		H10
C10	101.17	6.26, 1H, d, 1.8 Hz	
C11	160.15		H10, H12
C12	109.84	6.25, 1H, d, 1.8 Hz	H10,H14
C13	138.13		H14
C14	19.72	2.1,3H, s	H12

Table S15: Mass and UV-Vis spectra and NMR data of **18** in $(CD_3)_2CO$ on 500 MHz Bruker NMR spectrometer.

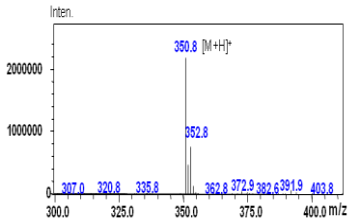
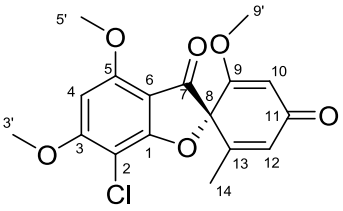
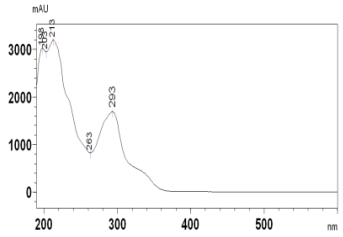
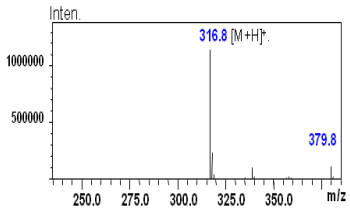
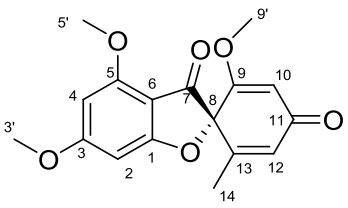
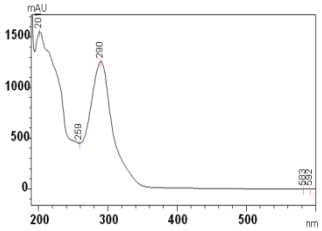
Dehydro-griseofulvin (**18**) (M.W. 350.8 g/mol)			
			
Assignment	**^{13}C ppm**	**^{1}H ppm, integration, mult, J_{HH} (Hz)**	**HMBC signals**
C1	170.303		
C2	97.4693		H4
C3	165.9245		H3', H4
C3'	57.9741	4.125,3H,s	
C4	91.8759	6.789 ,1H, s	
C5	159.4749		H5', H4
C5'	56.9964	4.000 ,3H,s	
C6	104.2393		H4
C7	186.9645		H4
C8	89.3667		H14, H10, H12
C9	168.5715		H9', H10
C9'	57.0895	3.714 ,3H,s	
C10	104.9241	5.700,1H, d, 1.3 Hz	
C11	186.2087		H10
C12	129.8836	6.169, 1H, t, 1.4 Hz	H10, H14
C13	147.7481		H14
C14	16.4739	1.772,3H, d, 1.37 Hz	H12

Table S16: Mass and UV-Vis spectra and NMR data of **19** in $(CD_3)_2CO$ on 500 MHz Bruker NMR spectrometer.

Dehydro—dechlorogriseofulvin (**19**)(M.W. 316.3 g/mol)			
  			
Assignment	**^{13}C ppm**	**^{1}H ppm, integration, mult, J_{HH} (Hz)**	**HMBC signals**
C1	177.1366		H2
C2	94.2726	6.266, 1H, d,1.7 Hz	H4
C3	169.0983		H2,H3', H4
C3'	56.9068	3.9773, 3H,s	
C4	90.1736	6.4462 ,1H, d, 1.7 Hz	H2
C5	160.5407		H5', H4
C5'	56.5022	3.909, 3H,s	
C6	104.0223		H4
C7	189.0155		H4
C8	88.9061		H14, H10, H12
C9	171.6062		H9', H10
C9'	56.8816	3.699, 3H,s	
C10	104.0091	5.664 ,1H, d, 1.3 Hz	H12
C11	186.3979		H10
C12	129.8836	6.130, 1H, t, 1.4 Hz	H10, H14
C13	148.4656		H14
C14	16.4971	1.741, 3H, d, 1.5 Hz	H12

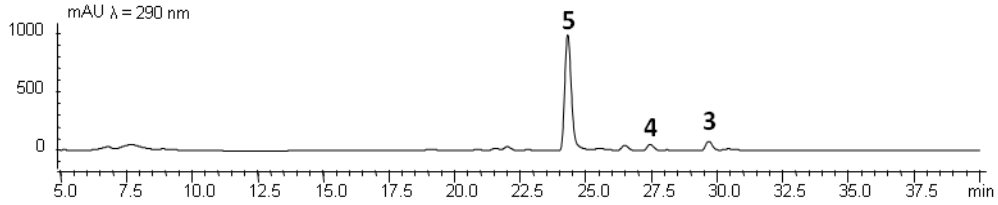


Figure S1: Feeding of norlichexanthone (**5**) to Δ*gsfA* mutant. Chromatogram from the LCMS analysis from the extracts of Δ*gsfA* mutant fed with 0.1 mg/mL of **5**, showing that feeding of **5** did not restore the production of griseofulvin(**1**) or dechlorogriseofulvin (**2**). Also shown in the trace are viridicatumtoxin (**3**) and tryptoquialanine (**4**).

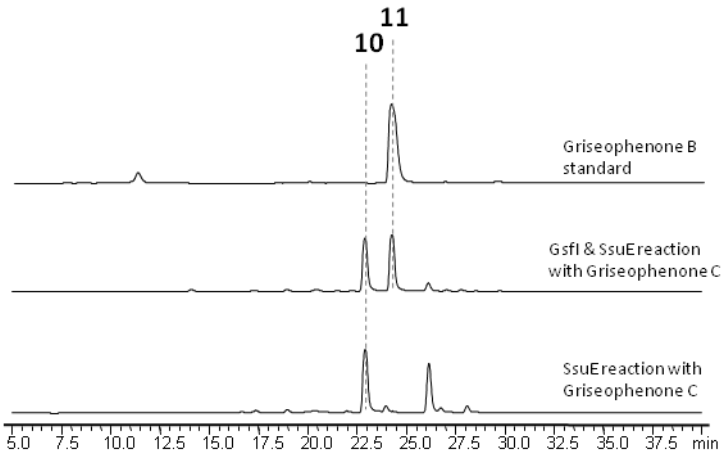


Figure S2: *In vitro* assay of GsfI using griseophenone C (**10**) as substrate. GsfI, heterologously expressed and purified from *E.coli* BL21 cells was incubated together with the NADPH-dependent flavin reductase SsuE and griseophenone C in 100 mM sodium phosphate buffer (pH 7.4) and 50 mM NaCl showing the conversion of **10** to griseophenone B

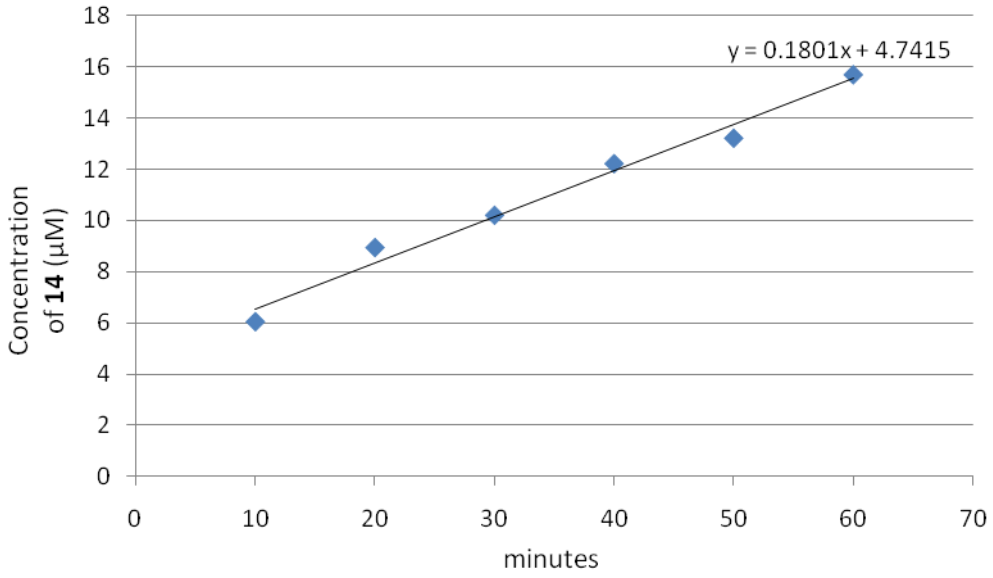


Figure S3: Time course-*in vitro* assay of GsfF from 0-60 minutes using 50 µM griseophenone B (**10**) and 1mM NADPH as substrates and 0.412 mg/mL microsomal protein in 100 mM Tris-HCl (pH 7.5) showing a turnover rate of 0.437 µM/ min·mg of micromal protein. The concentration of protein from the microsomes was determined by comparison with bovine serum albumin (BSA) standard curve. The amount of product desmethyl-dehydrogriseofulvin (**14**) was quantified by comparison with a standard curve using purified **14** from Δ*gsfD* fermentation.

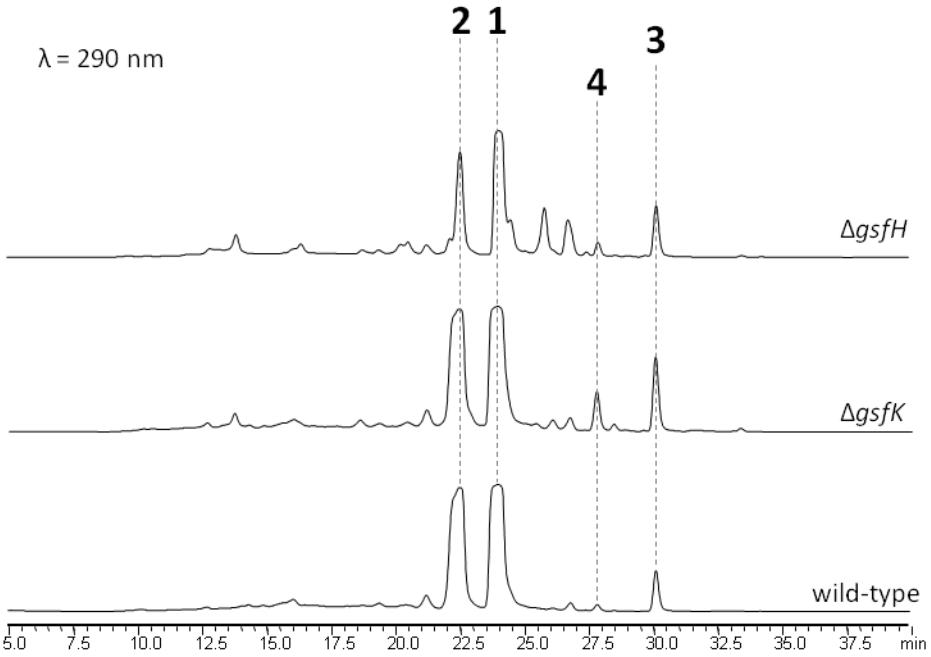
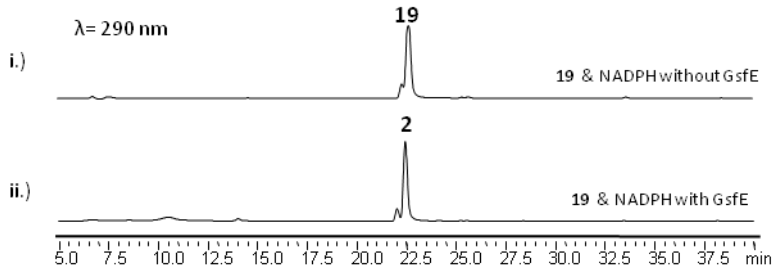
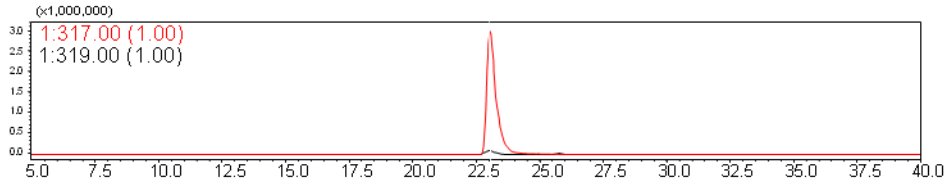


Figure S4: Metabolic profile of Δ*gsfH* and Δ*gsfK* mutants showing the production of griseofulvin (**1**) and dechlorogriseofuvlin (**2**).

a.)



b.)



c.)

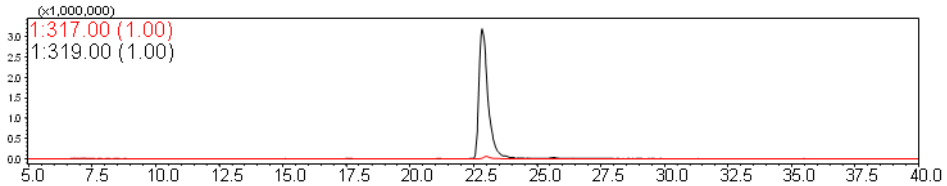


Figure S5: *In vitro* assay of GsfE using dechloro-dehydrogriseofulvin (**19)** as substrate. a,) i.) Chromatogram (λ = 290 nm) from extract of **19** incubated overnight with GsfE only in 100 mM Tris-HCl (pH 7.5). ii.) .) Chromatogram (λ = 290 nm) from extract of **19** incubated overnight with GsfE and 1 mM NADPH in 100 mM Tris-HCl (pH 7.5) showing conversion to dechlorogriseofulvin (**2**). b.) Extracted ion chromatogram of (a, i.) showing **19** (m/z = 317, $[M+H]^+$). c.) Extracted ion chromatogram of (a, ii.) showing **2** (m/z = 319, $[M+H]^+$).

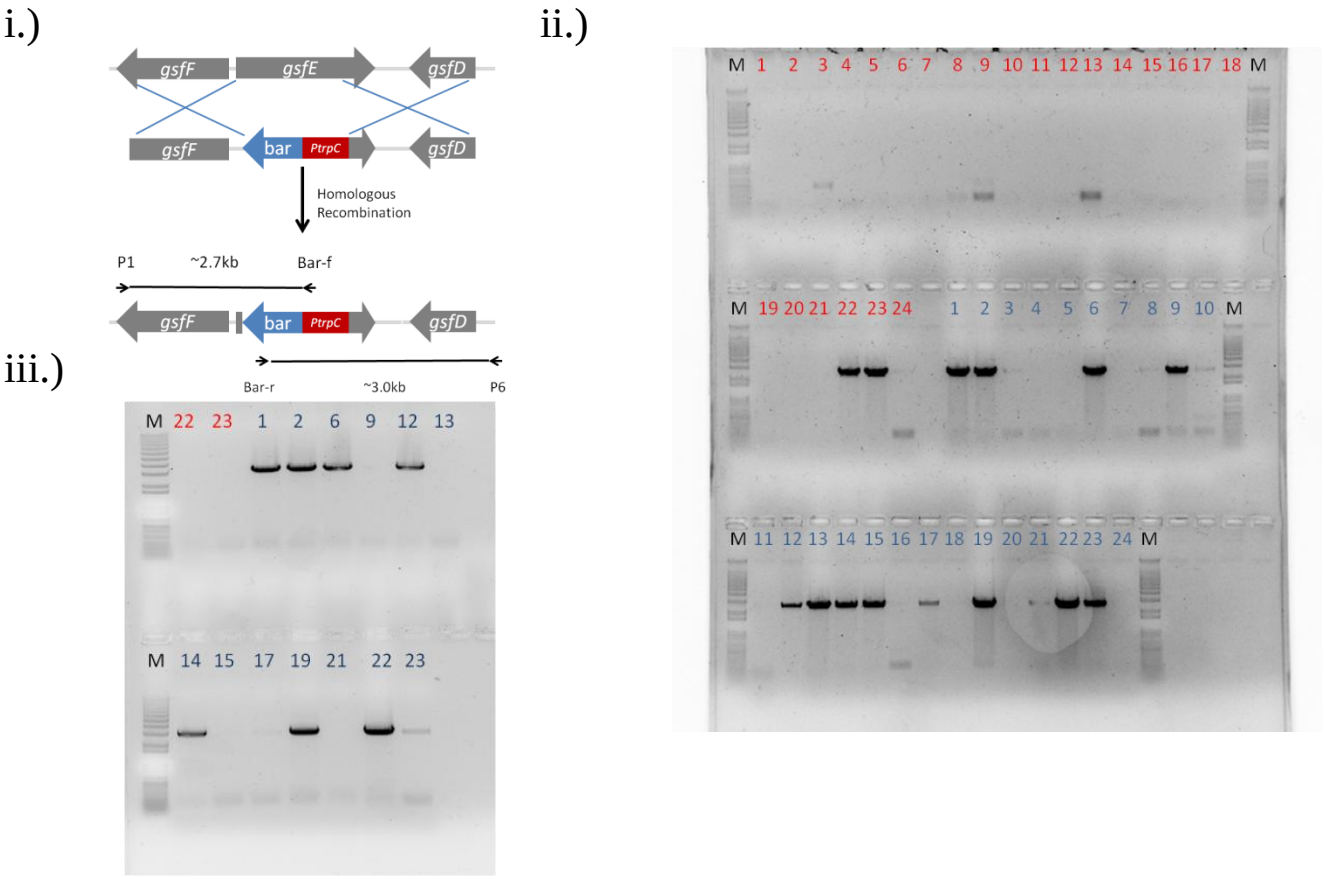


Figure S6: Δ*gsfE* mutant screening shown as example of PCR screening method. i.) Linear knockout cassette showing the positions of the primer binding site after successful disruption of the targeted gene. ii.) Amplification of genomic DNA from 48 Δ*gsfE* transformants (plate I, 1-24 in red and plate II, 1-24 in blue) using primer pair Δ*gsfE*-KO-P1 and bar-f showing expected ~2.7 kb amplicon. iii.) Amplification of genomic DNA from Δ*gsfE* transformants that passed the screening from (ii.) using primer pairs Δ*gsfE*-KO-P6 and bar-r.

i.) ii.)

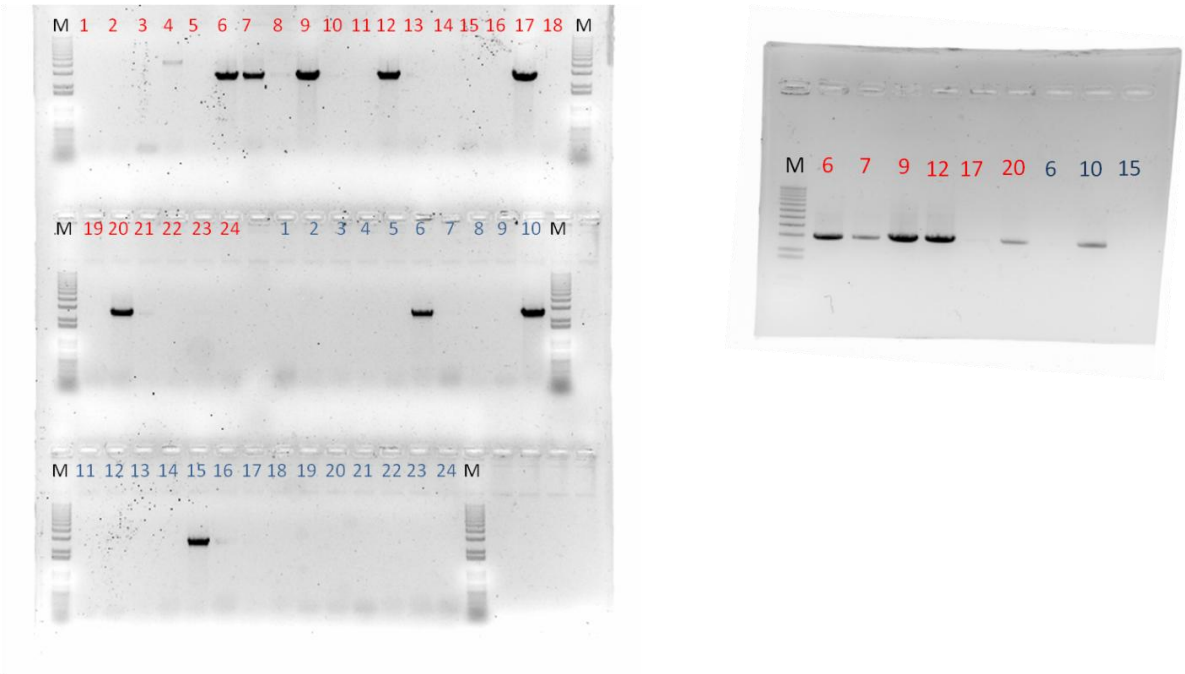
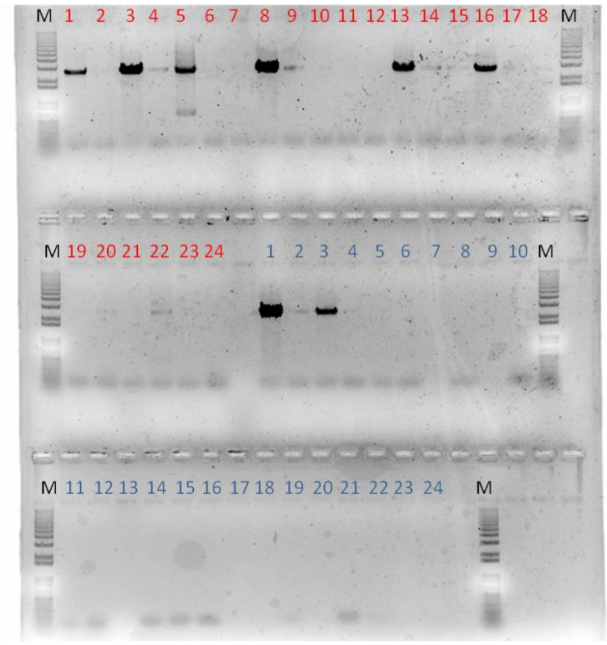


Figure S7: Δ*gsfH* mutant screening. i.) Amplification of genomic DNA from 48 Δ*gsfH* transformants (plate I, 1-24 in red and plate II, 1-24 in blue) using primer pair Δ*gsfH*-KO-P1 and bar-f showing expected ~2.7 kb amplicon. ii.) Amplification of genomic DNA from Δ*gsfH* transformants that passed the screening from (i.) using primer pairs Δ*gsfH*-KO-P6 and bar-r.

i.)



ii.)

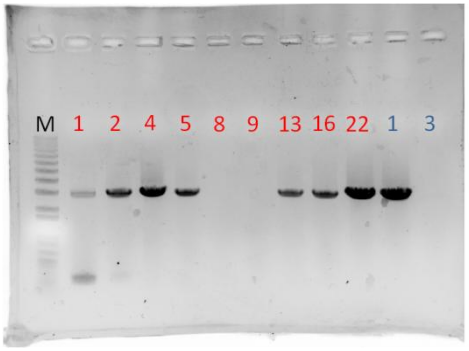


Figure S8: Δ*gsfK* mutant screening. i.) Amplification of genomic DNA from 48 Δ*gsfK* transformants (plate I, 1-24 in red and plate II, 1-24 in blue) using primer pair Δ*gsfK*-KO-P1 and bar-f showing expected ~2.7 kb amplicon. ii.) Amplification of genomic DNA from Δ*gsfK* transformants that passed the screening from (i.) using primer pairs Δ*gsfK*-KO-P6 and bar-r.

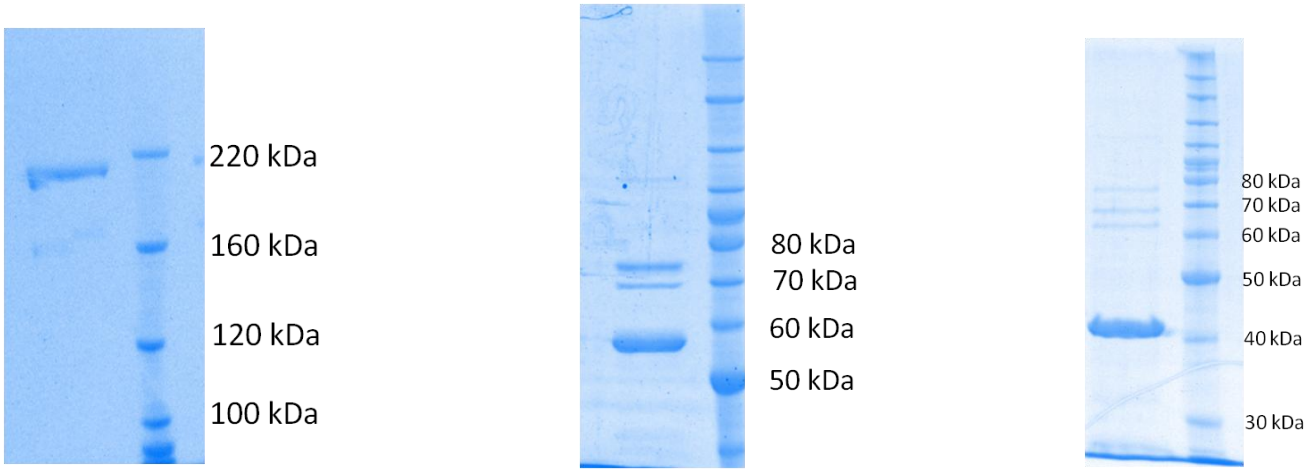


Figure S9: SDS-PAGE gels of the PKS GsfA (left, expected size 185 kDa), halogenase GsfI (middle, expected size 58 kDa) and the dehydrogriseofulvin reductase GsfE (right, expected size 41 kDa)

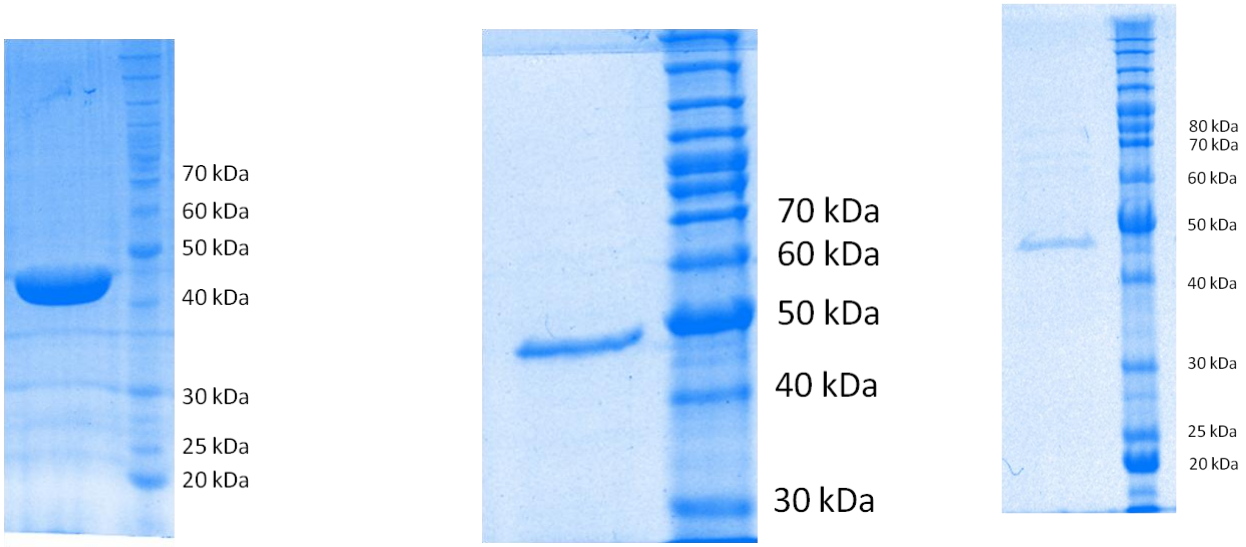


Figure S10: SDS-PAGE gel of GsfB (left, expected size 47 kDa) , GsfC (middle, 49 kDa) and GsfD (right, 47 kDa)

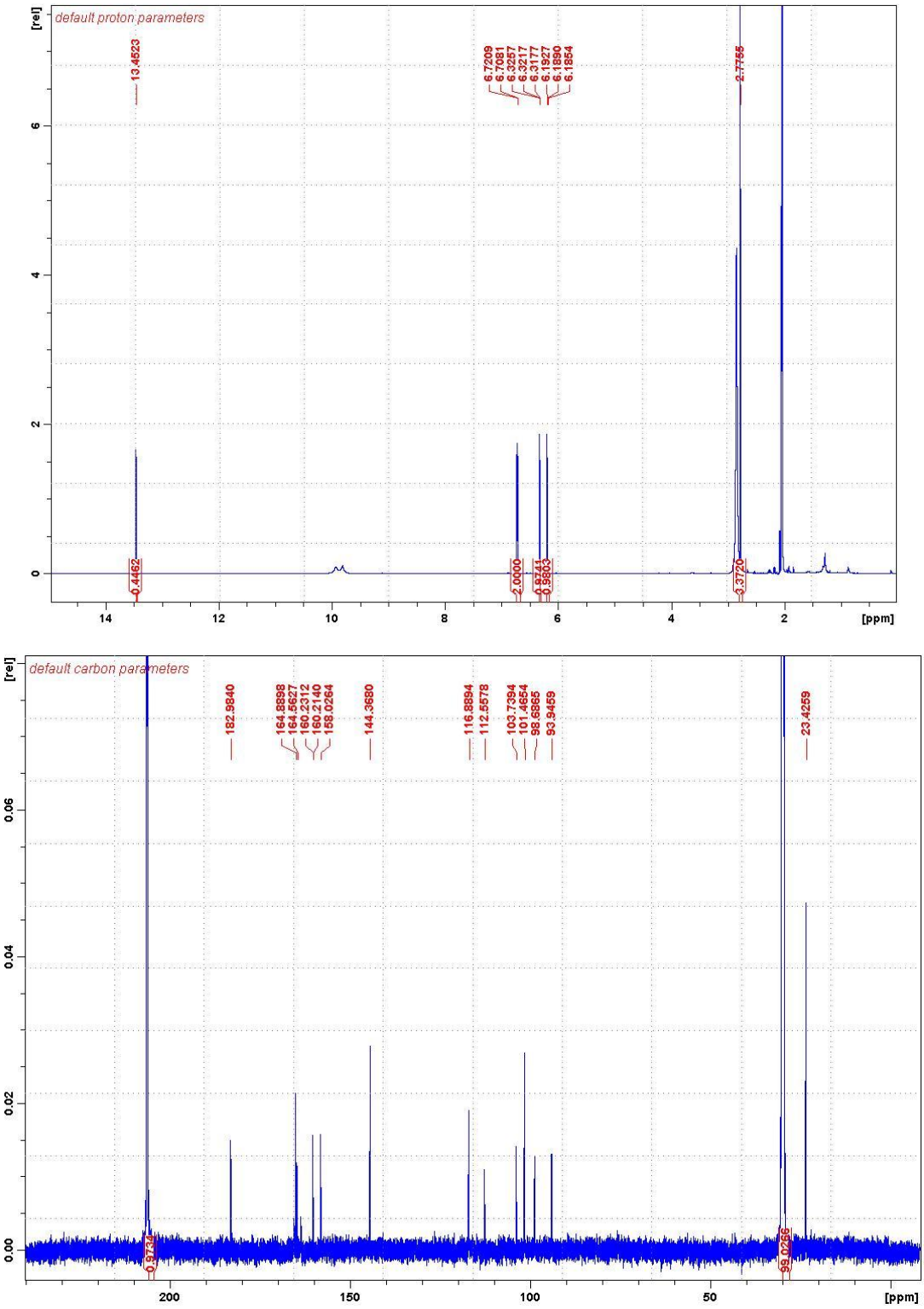


Figure S11: 1D ^{1}H (top) and ^{13}C NMR (bottom)spectrum of **5** in $(CD_3)_2CO$ (500 MHz).

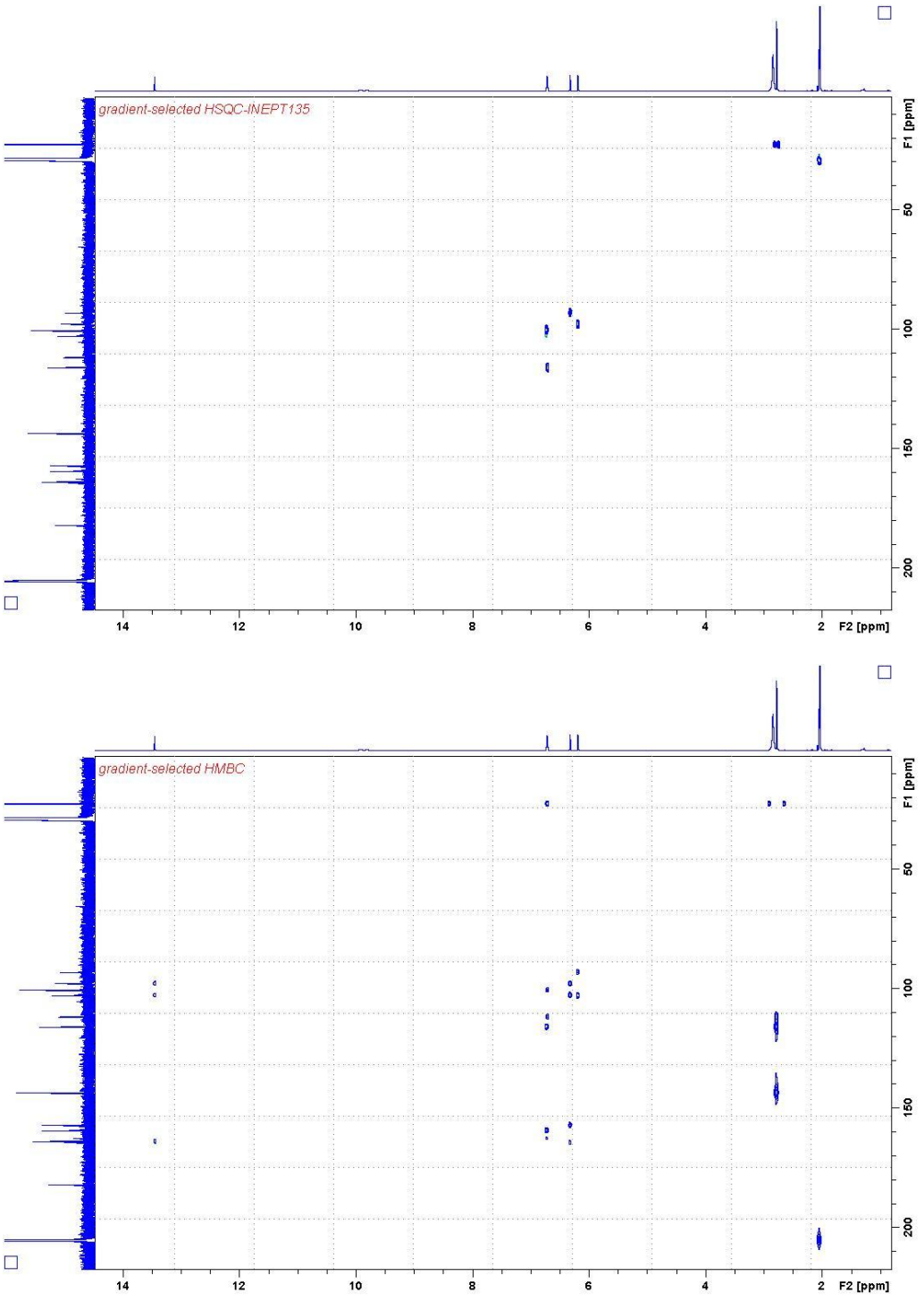


Figure S12: 2D HSQC (top) and HMBC (bottom) of **5** in $(CD_3)_2CO$ (500 MHz).

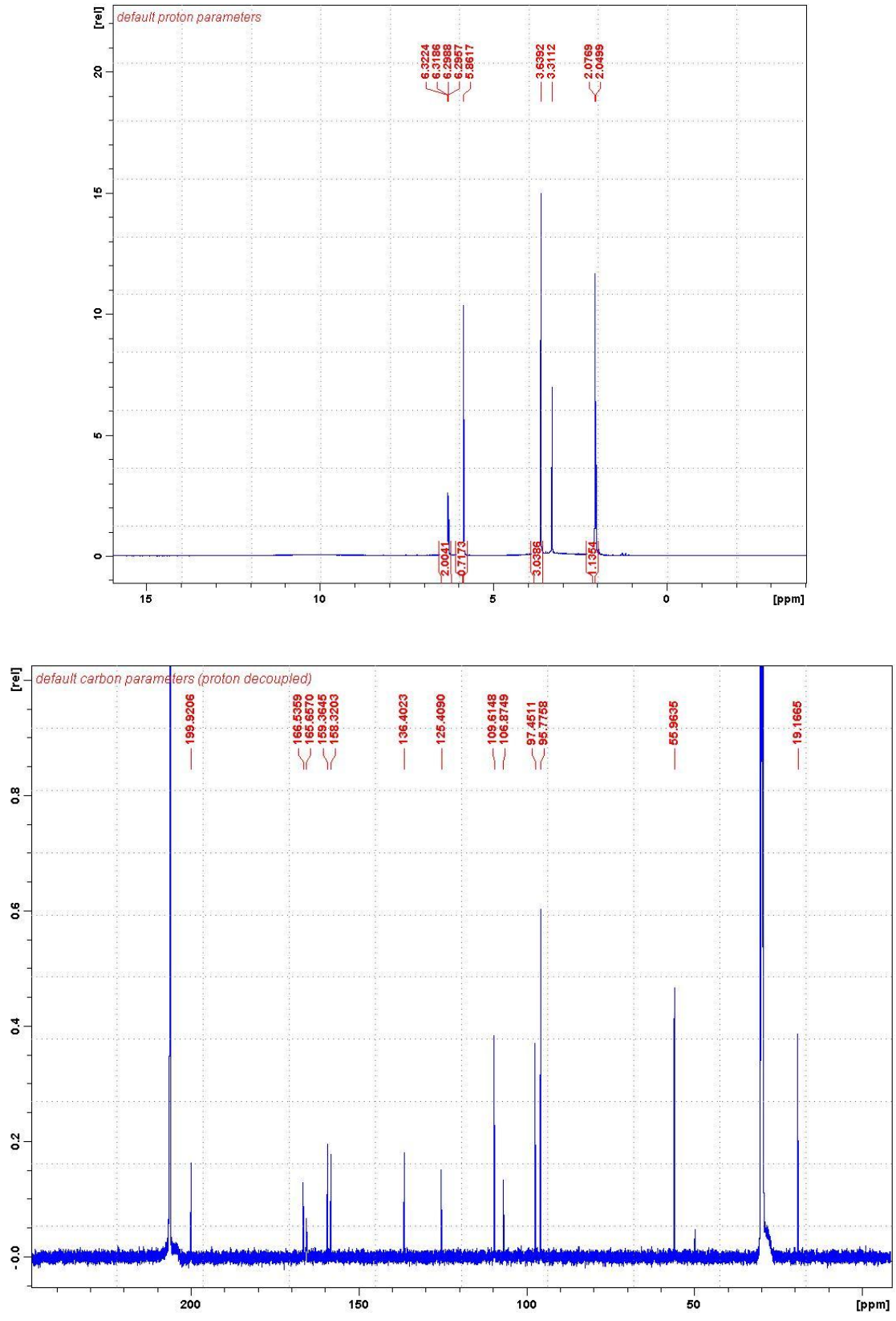


Figure S13: 1D ^{1}H (top) and ^{13}C NMR (bottom)spectrum of **7** in $(CD_3)_2CO$ (500 MHz).

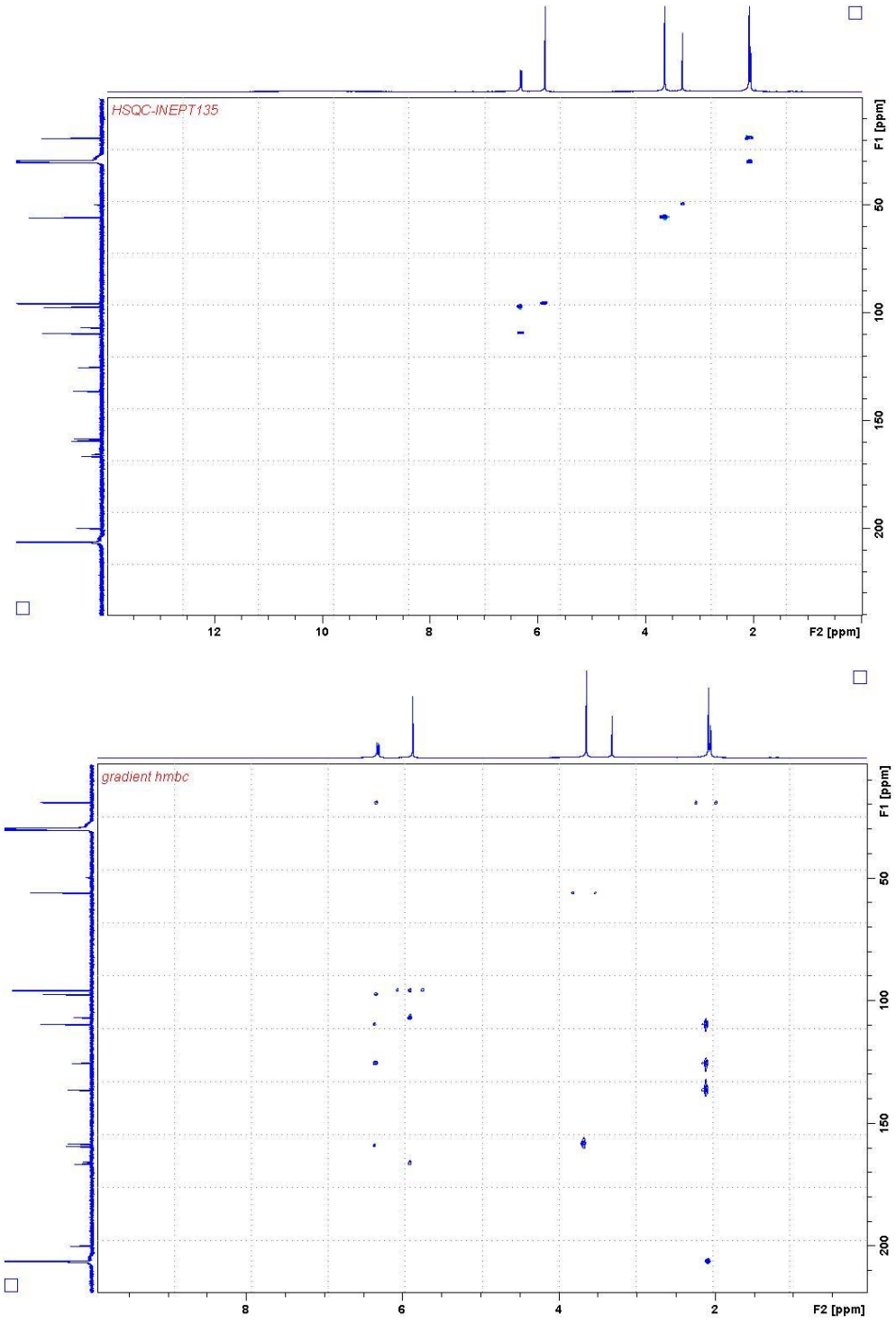


Figure S14: 2D HSQC (top) and HMBC (bottom) of **7** in $(CD_3)_2CO$ (500 MHz).

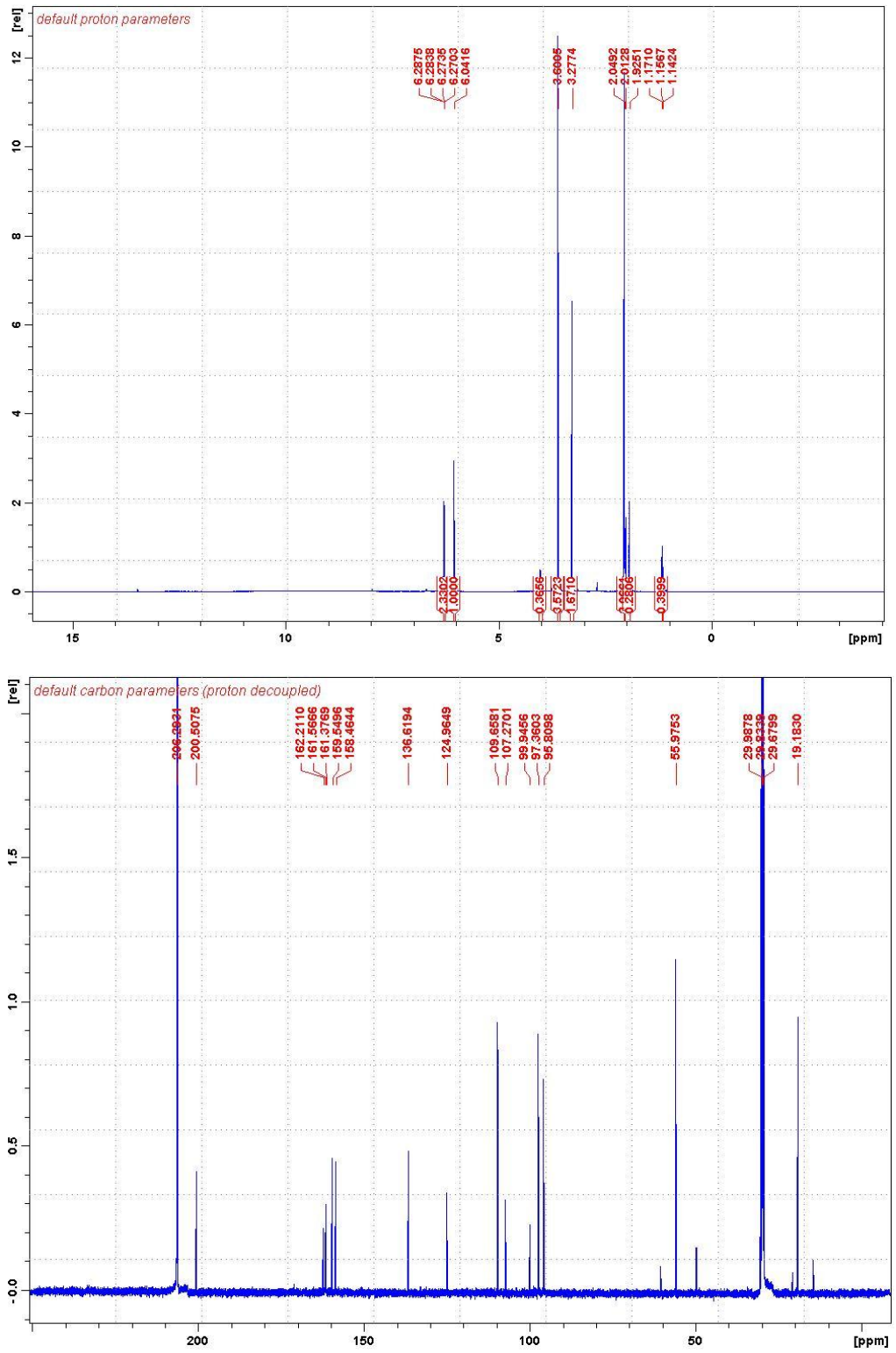


Figure S15: 1D ^{1}H (top) and ^{13}C NMR (bottom)spectrum of **8** in $(CD_3)_2CO$ (500 MHz).

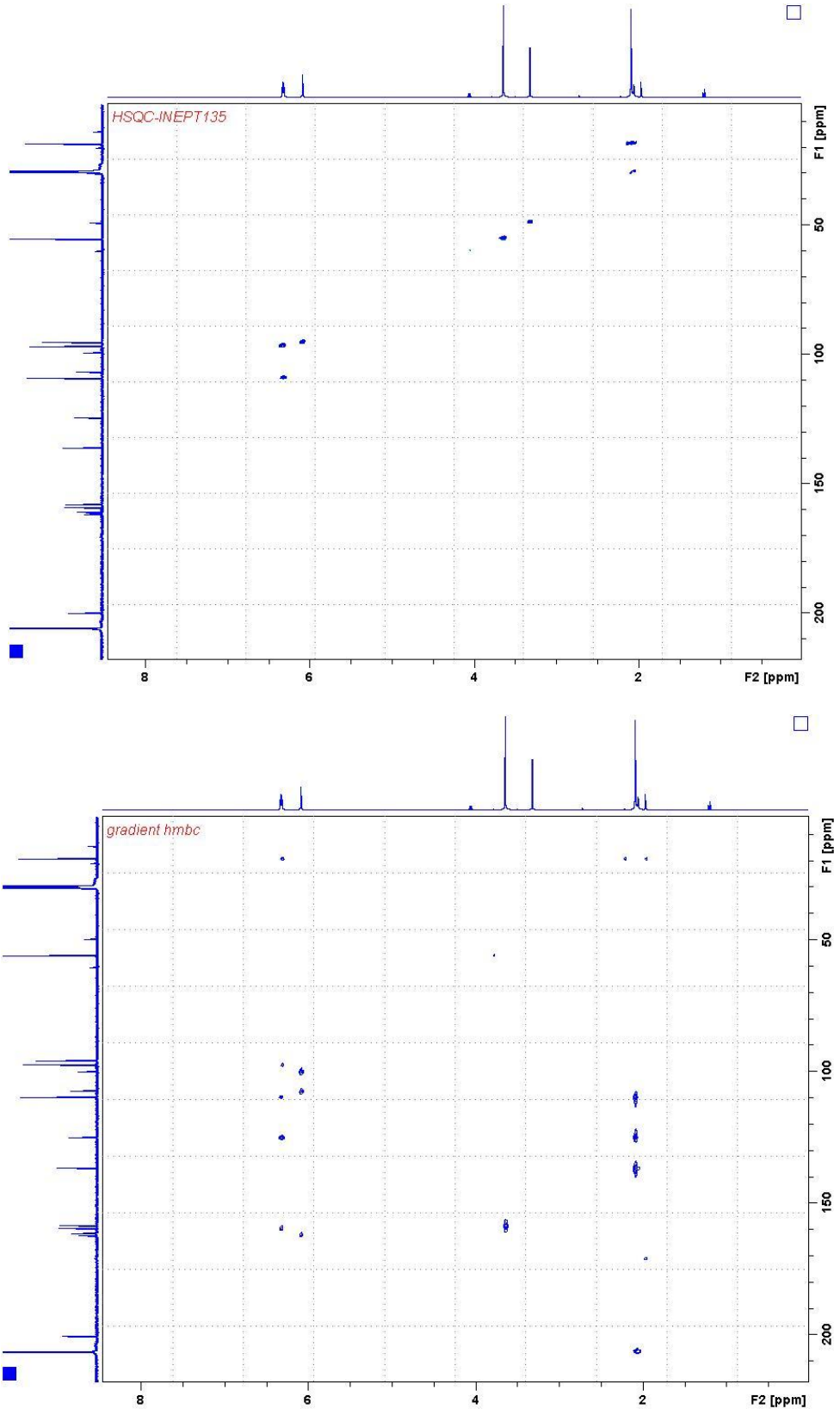


Figure S16: 2D HSQC (top) and HMBC (bottom) of **8** in $(CD_3)_2CO$ (500 MHz).

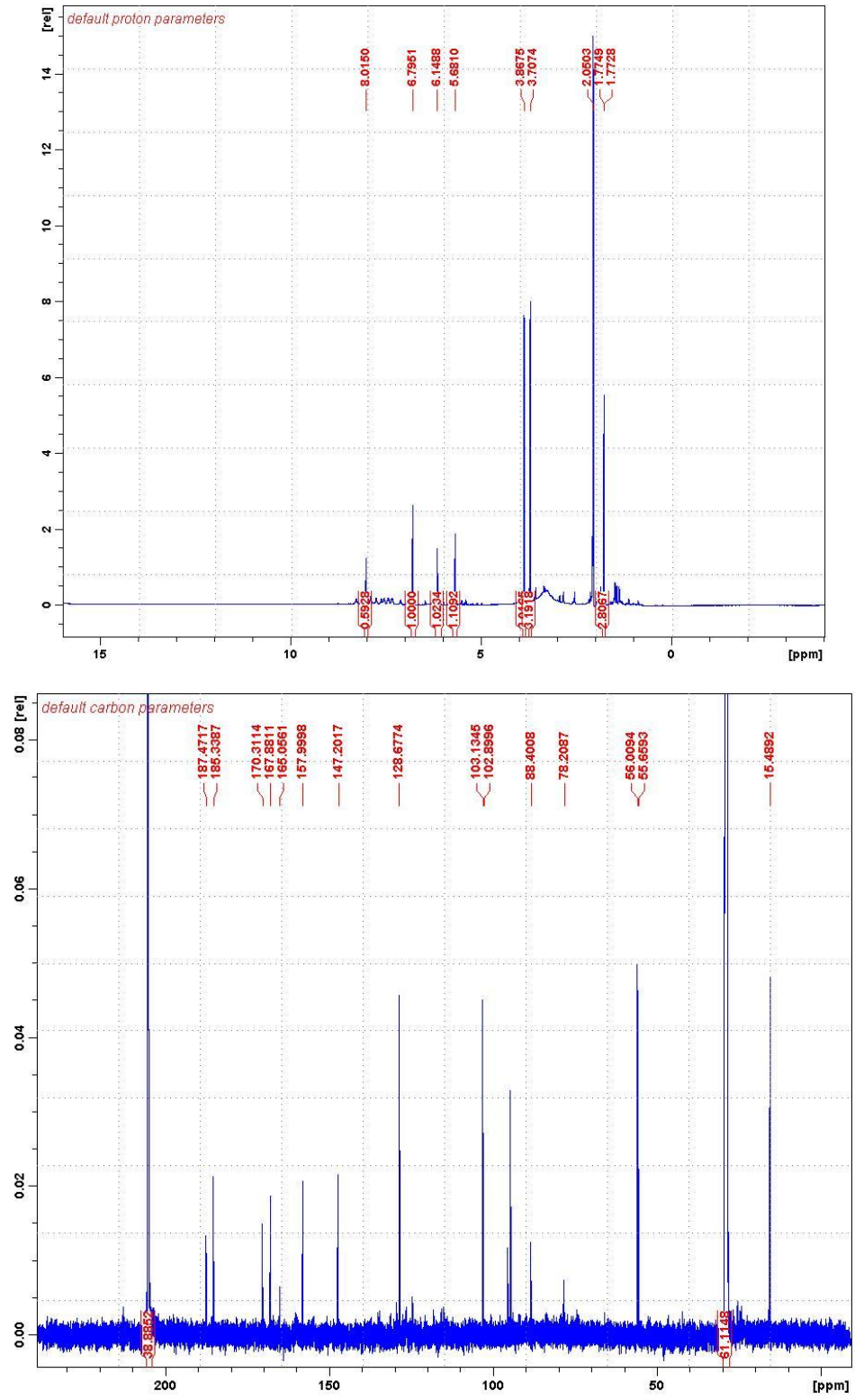


Figure S17: 1D ^{1}H (top) and ^{13}C NMR (bottom)spectrum of **9** in $(CD_3)_2CO$ (500 MHz).

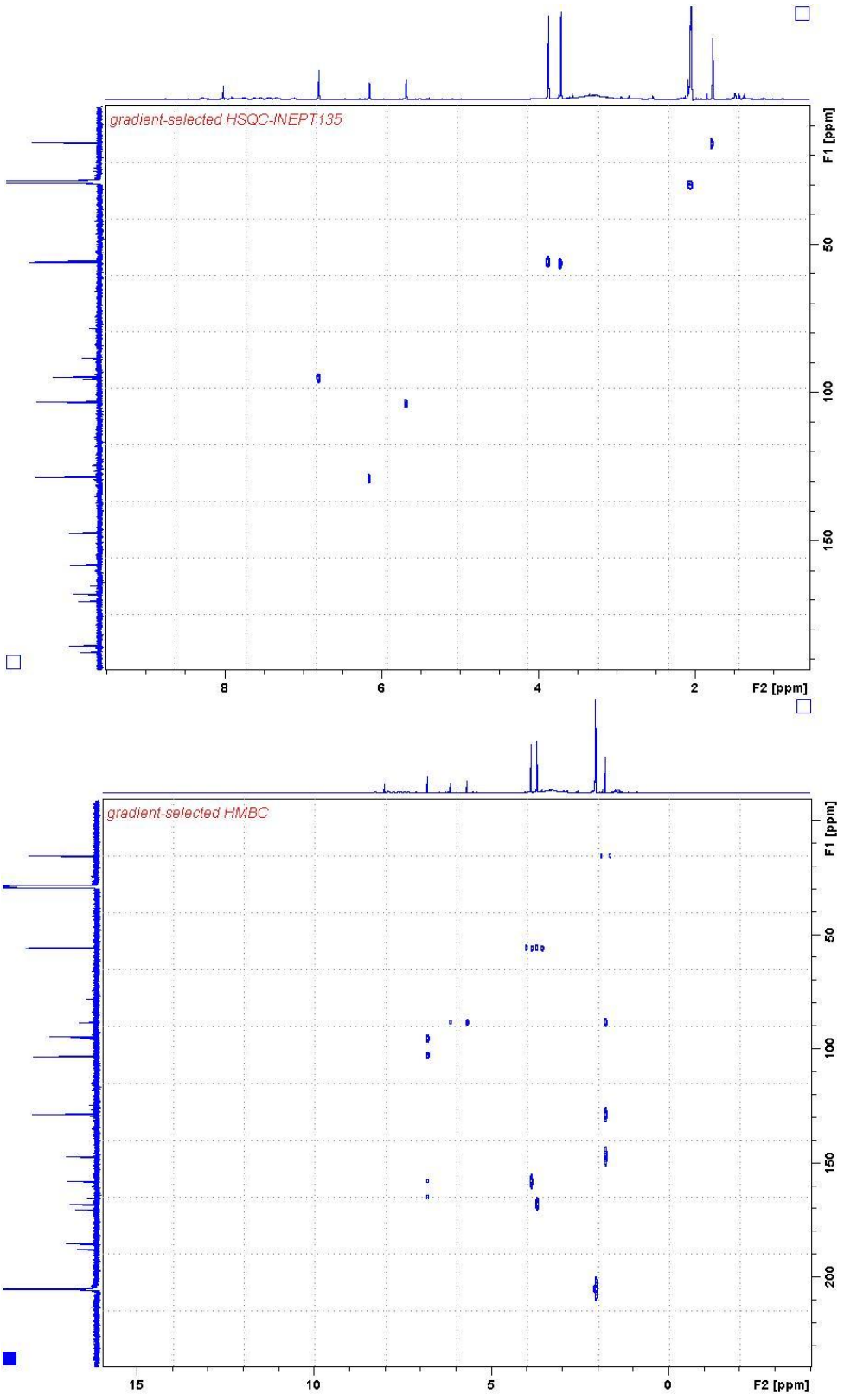


Figure S18: 2D HSQC (top) and HMBC (bottom) of **9** in $(CD_3)_2CO$ (500 MHz).

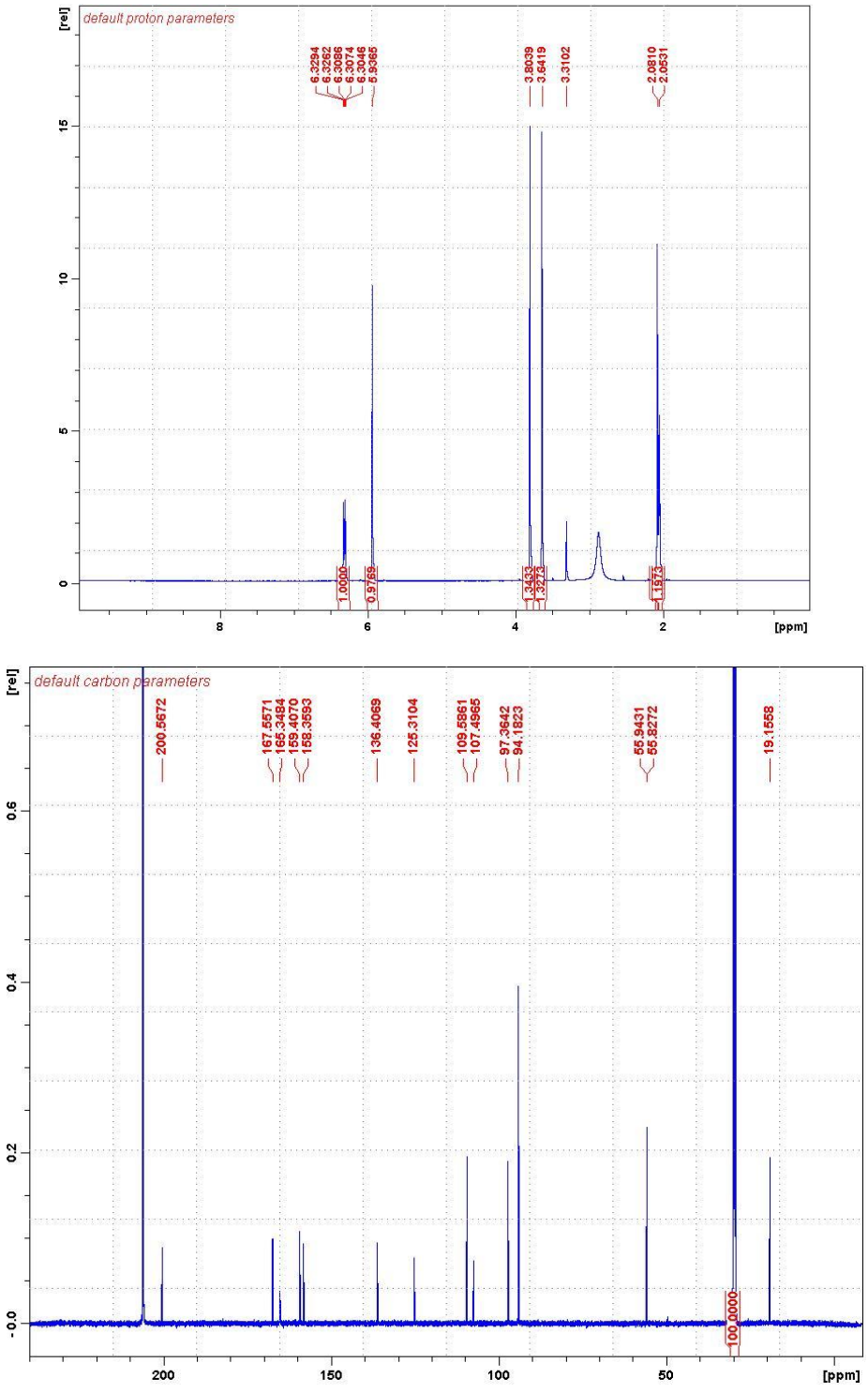


Figure S19: 1D ^{1}H (top) and ^{13}C NMR (bottom)spectrum of **10** in $(CD_3)_2CO$ (500 MHz).

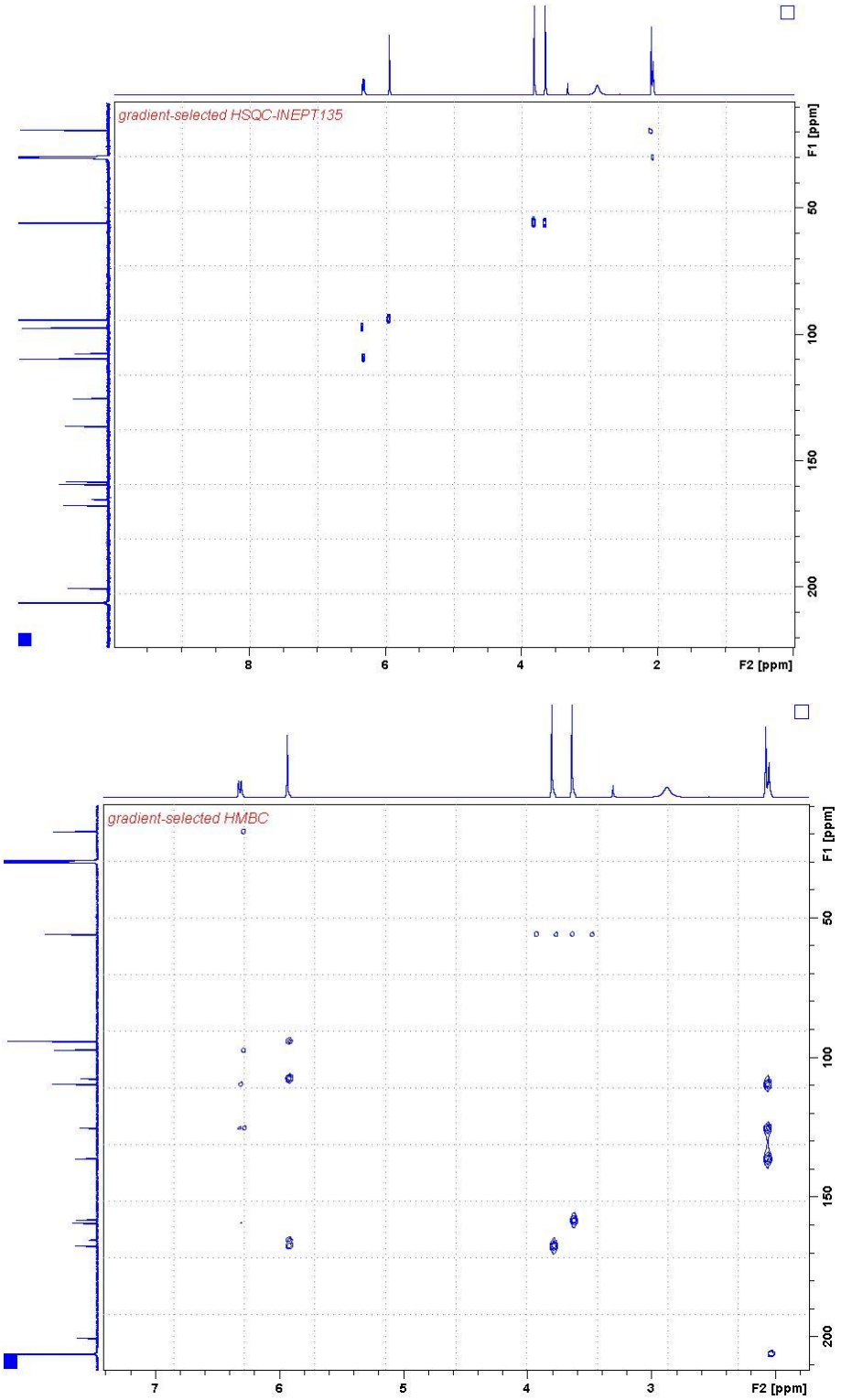


Figure S20: 2D HSQC (top) and HMBC (bottom) of **10** in $(CD_3)_2CO$ (500 MHz).

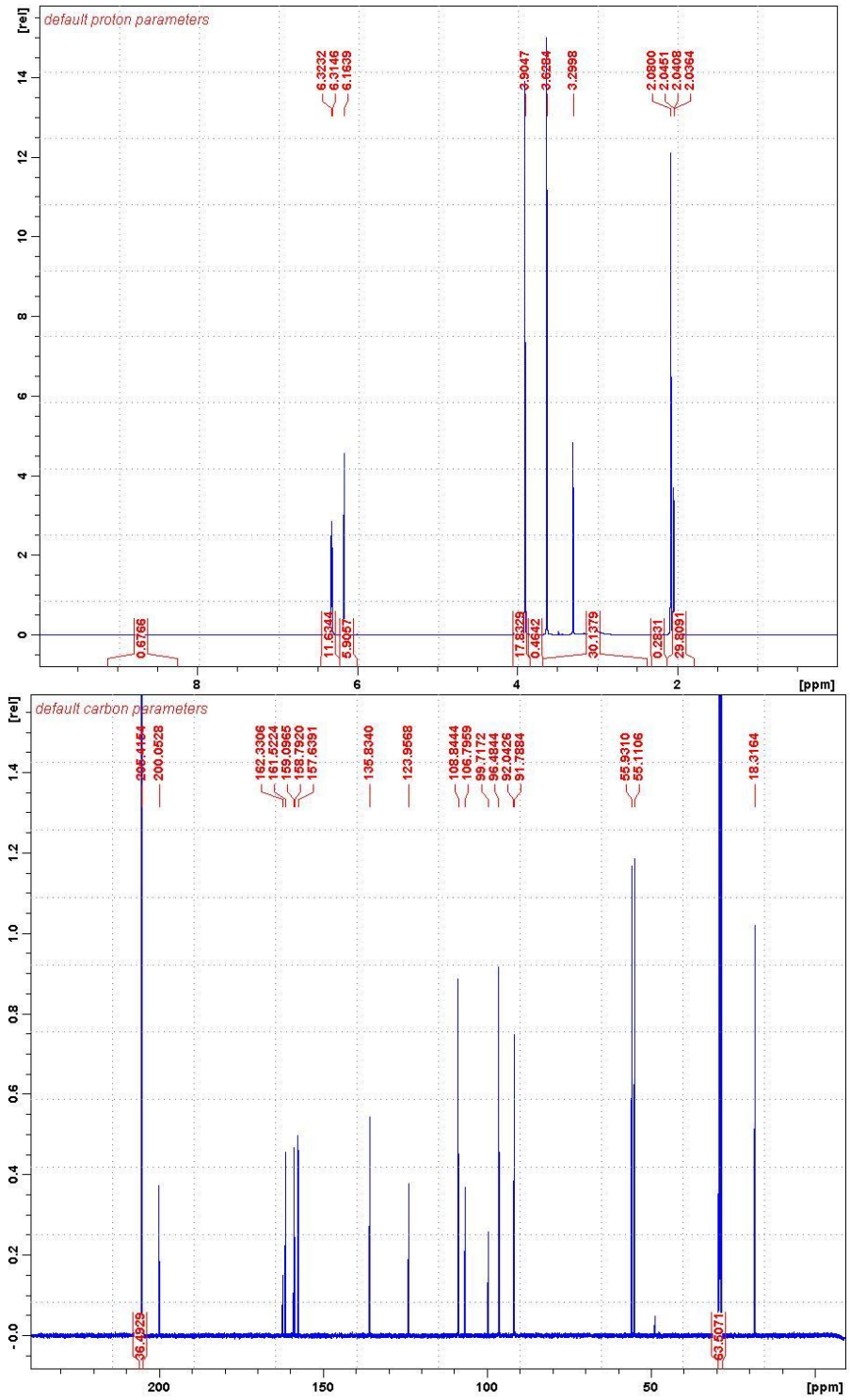


Figure S21: 1D ^{1}H (top) and ^{13}C NMR (bottom)spectrum of **11** in $(CD_3)_2CO$ (500 MHz).

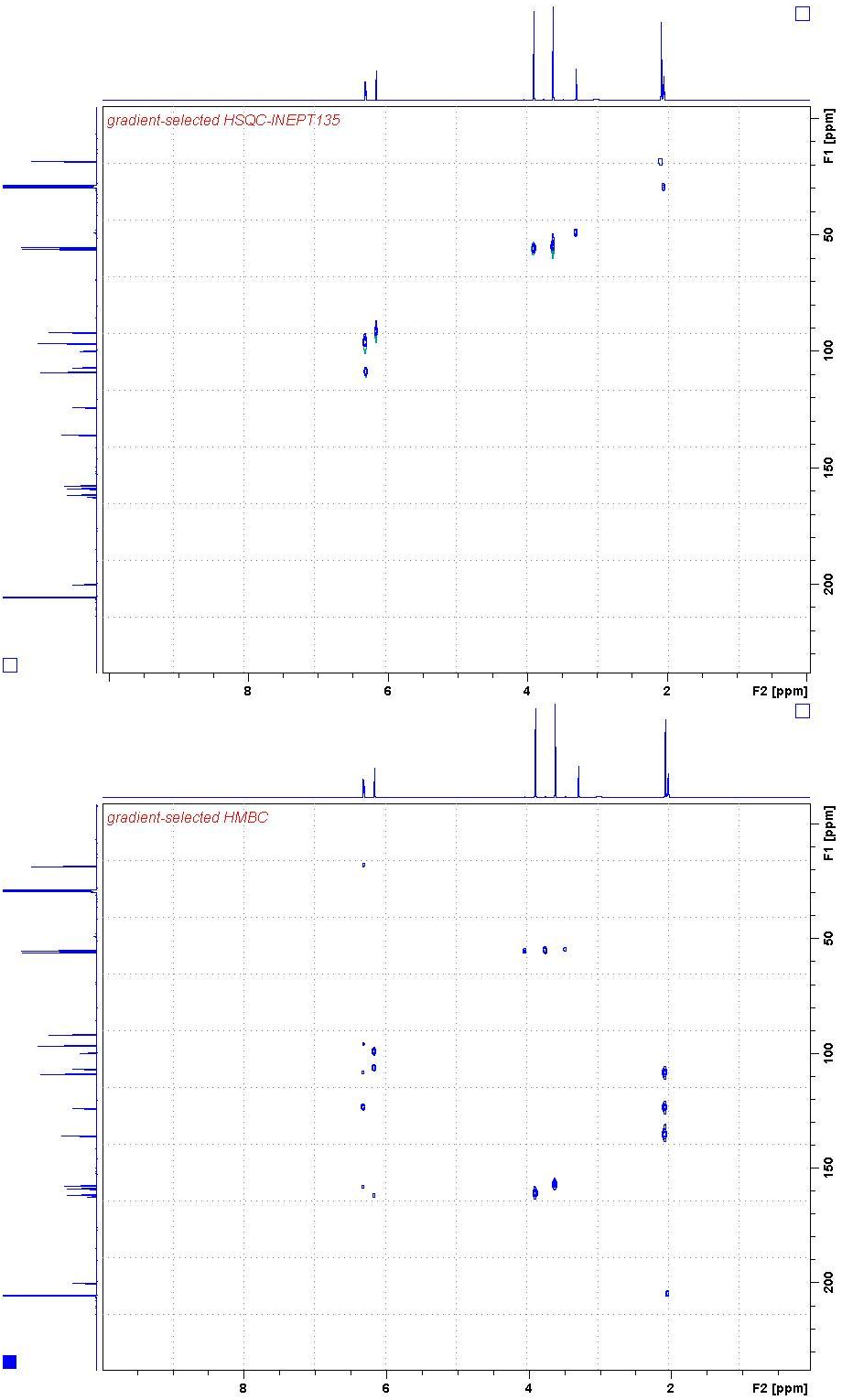


Figure S22: 2D HSQC (top) and HMBC (bottom) of **11** in $(CD_3)_2CO$ (500 MHz).

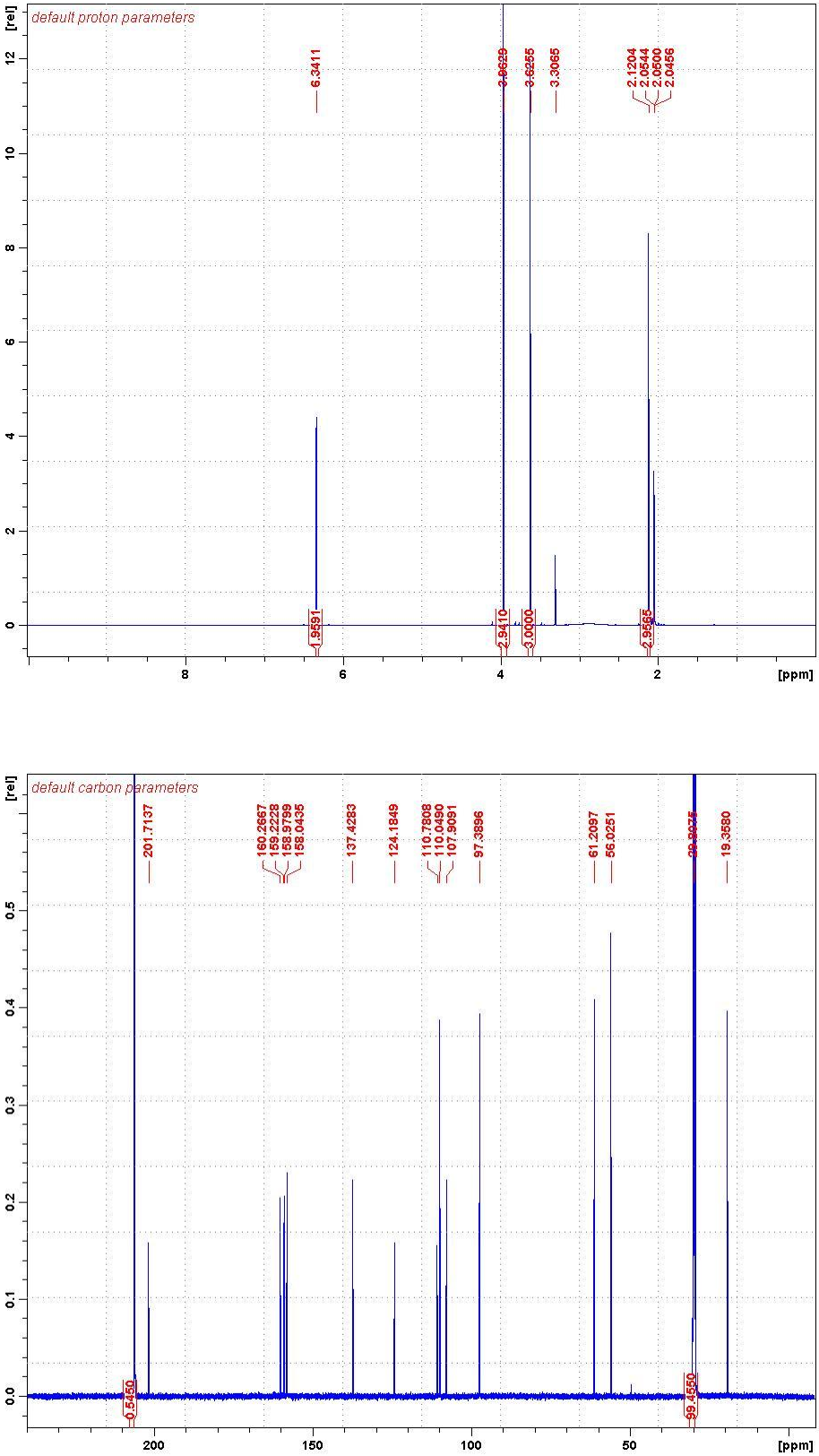


Figure S23: 1D ^{1}H (top) and ^{13}C NMR (bottom)spectrum of **12** in $(CD_3)_2CO$ (500 MHz).

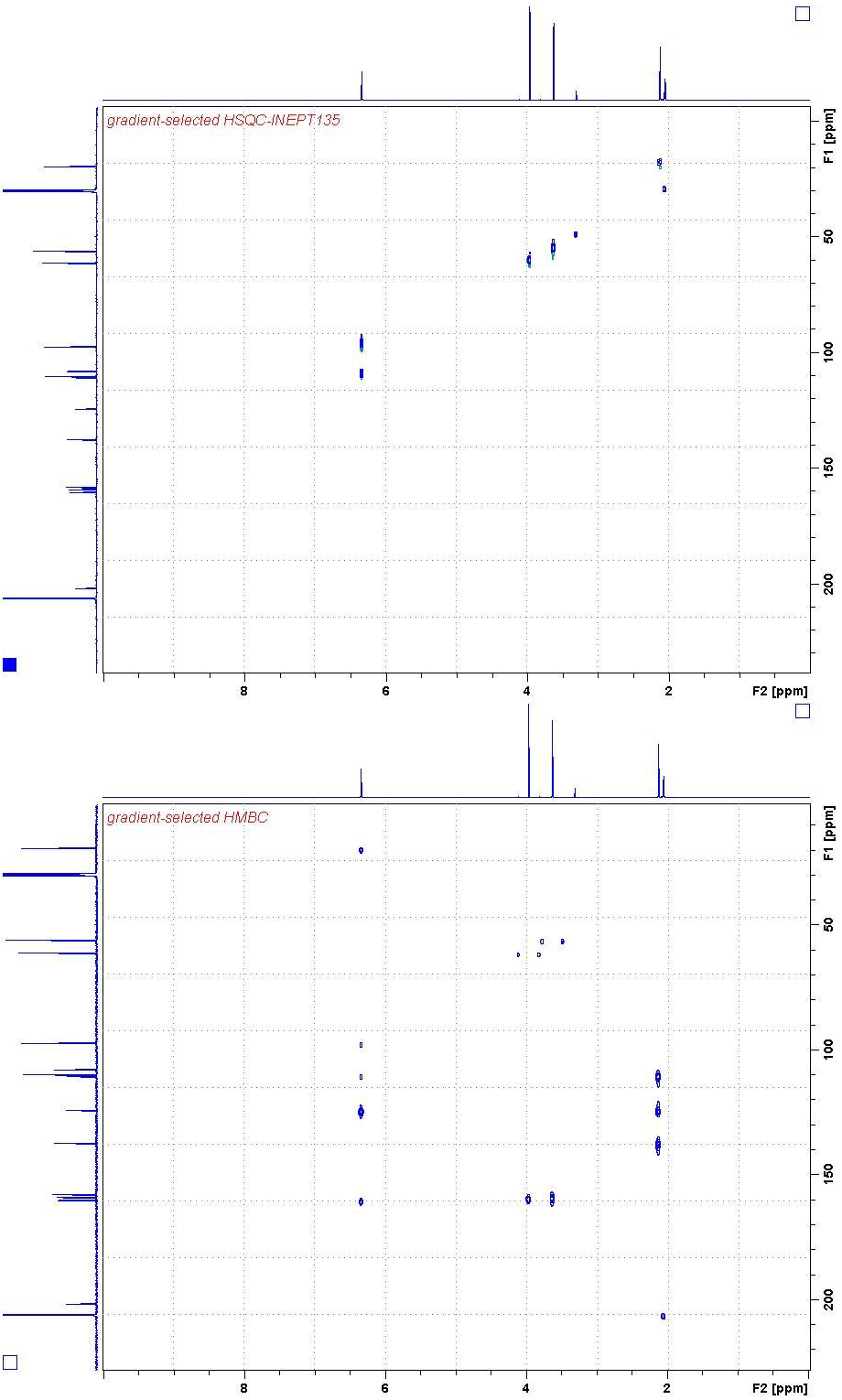


Figure S24: 2D HSQC (top) and HMBC (bottom) of **12** in $(CD_3)_2CO$ (500 MHz).

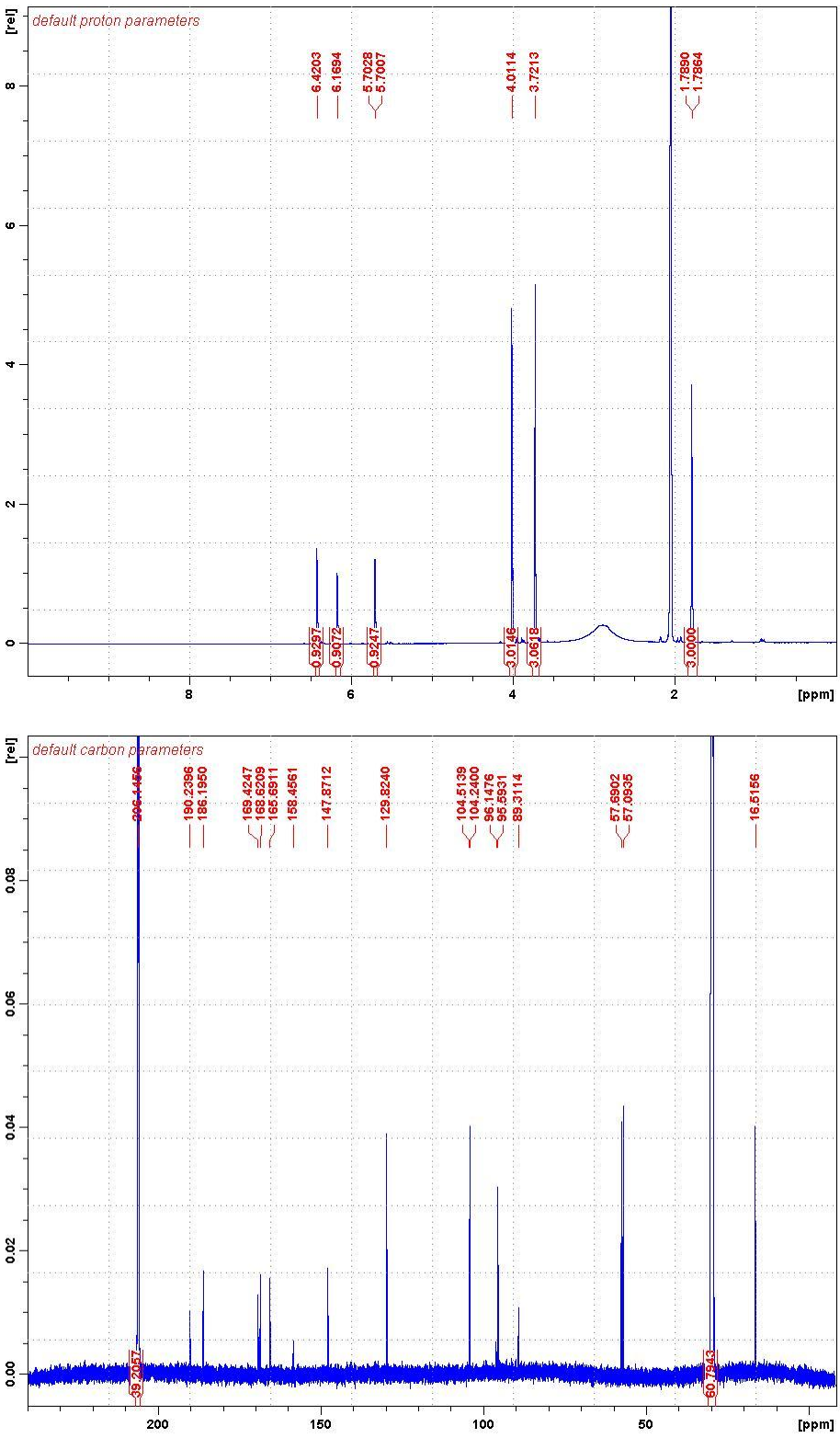


Figure S25: 1D ^{1}H (top) and ^{13}C NMR (bottom)spectrum of **14** in $(CD_3)_2CO$ (500 MHz).

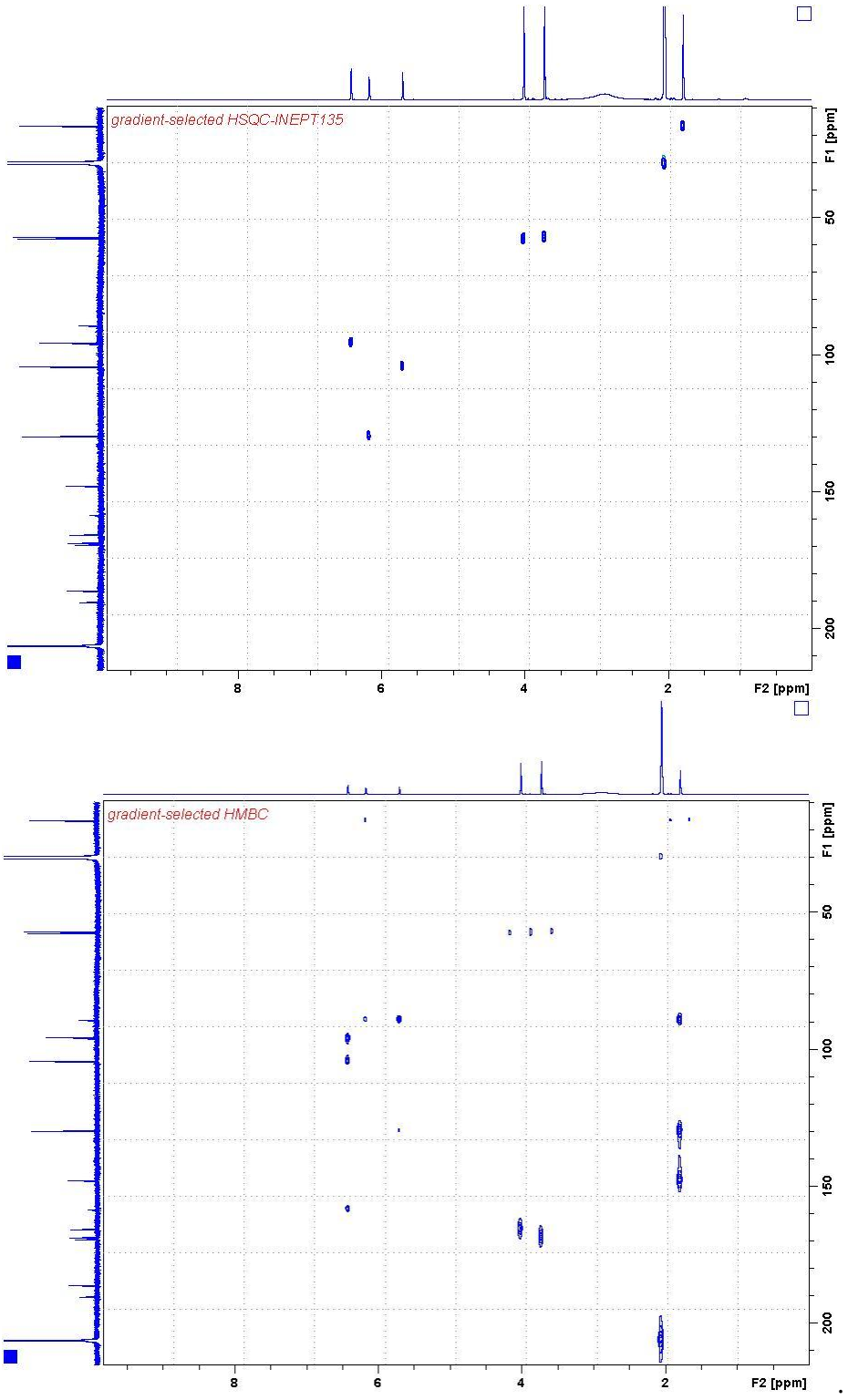


Figure S26: 2D HSQC (top) and HMBC (bottom) of **14** in $(CD_3)_2CO$ (500 MHz).

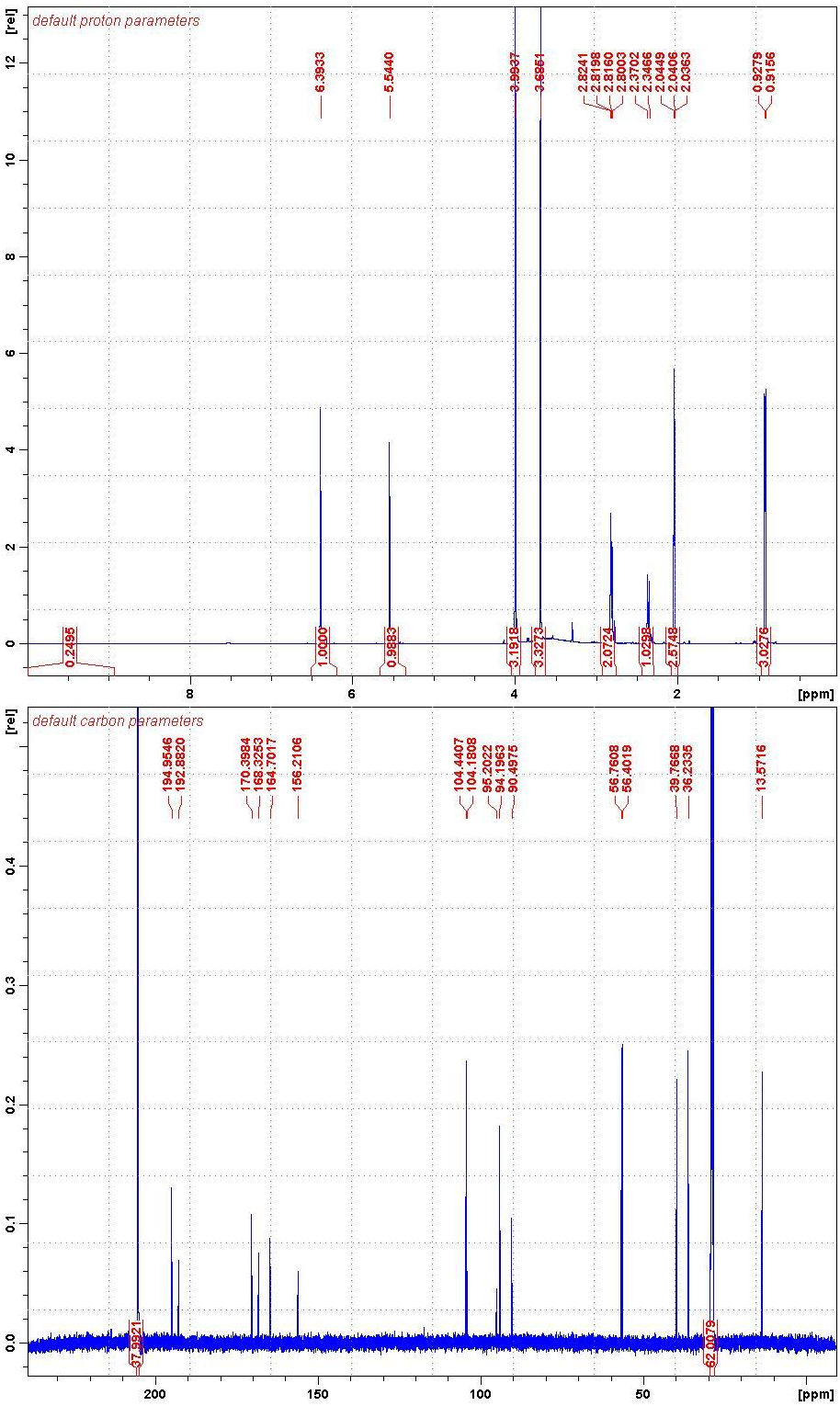


Figure S27: 1D ^{1}H (top) and ^{13}C NMR (bottom)spectrum of **15** in $(CD_3)_2CO$ (500 MHz).

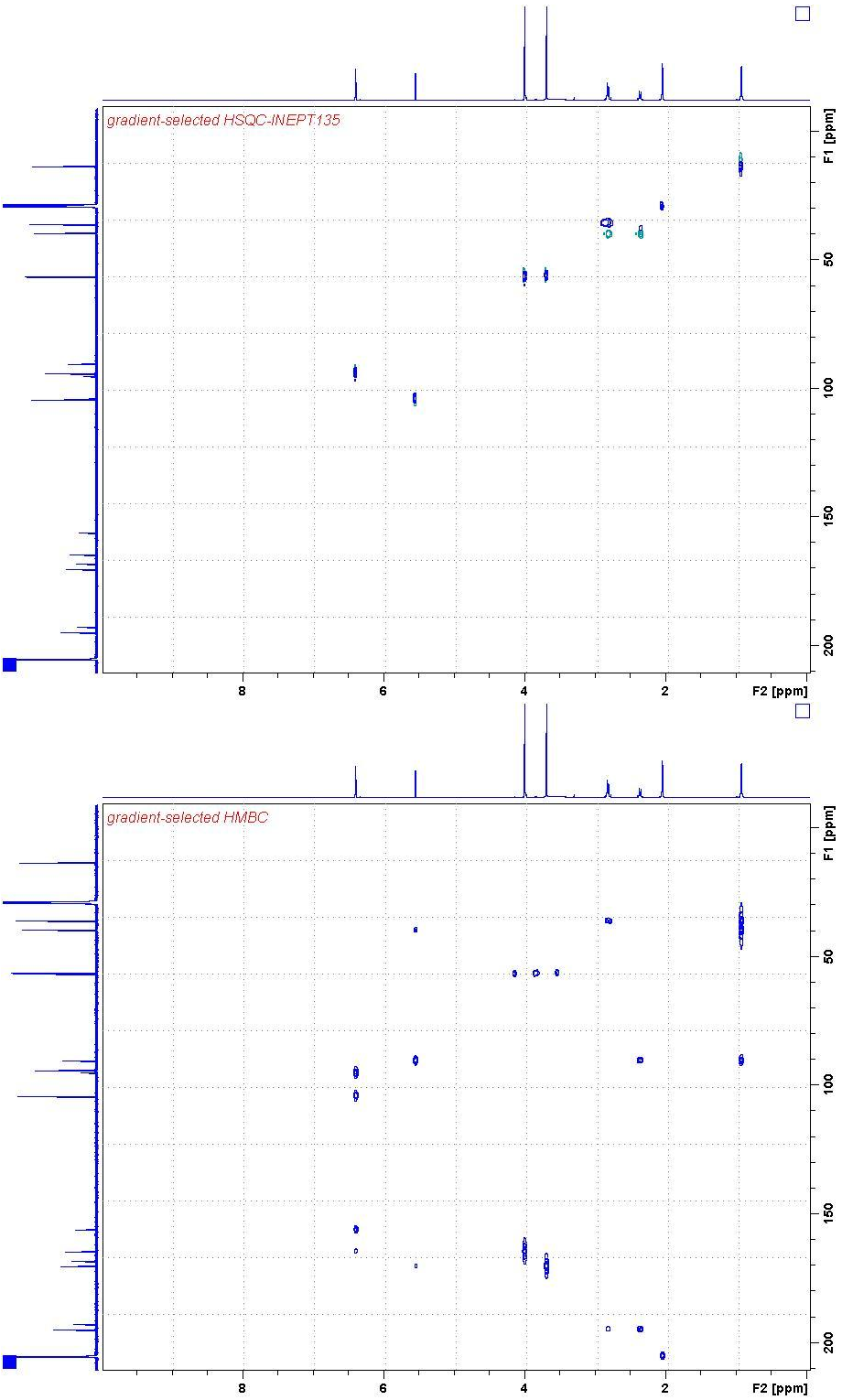


Figure S28: 2D HSQC (top) and HMBC (bottom) of **15** in $(CD_3)_2CO$ (500 MHz).

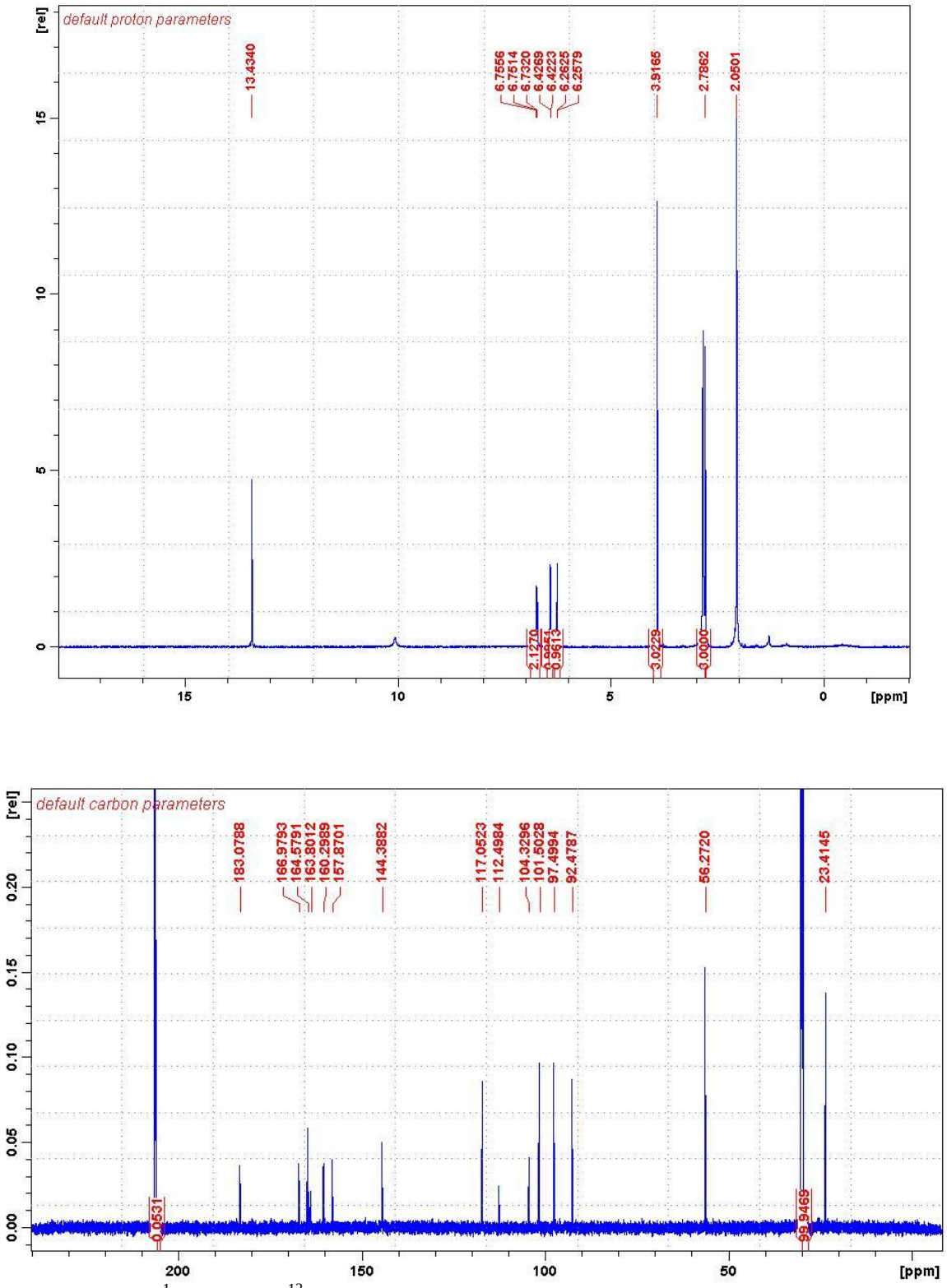


Figure S29: 1D ^{1}H (top) and ^{13}C NMR (bottom)spectrum of **16** in $(CD_3)_2CO$ (500 MHz).

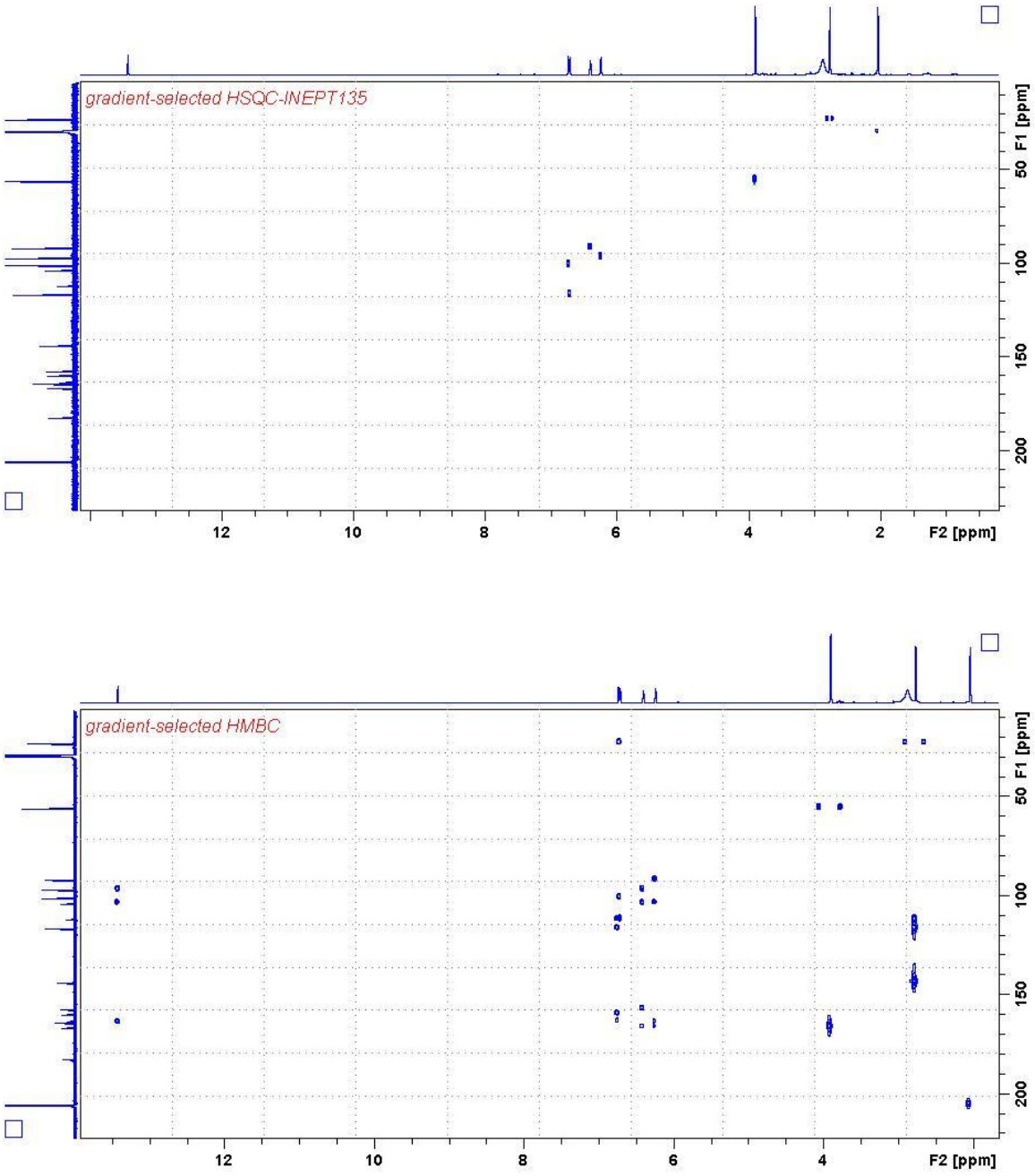


Figure S30: 2D HSQC (top) and HMBC (bottom) of **16** in $(CD_3)_2CO$ (500 MHz).

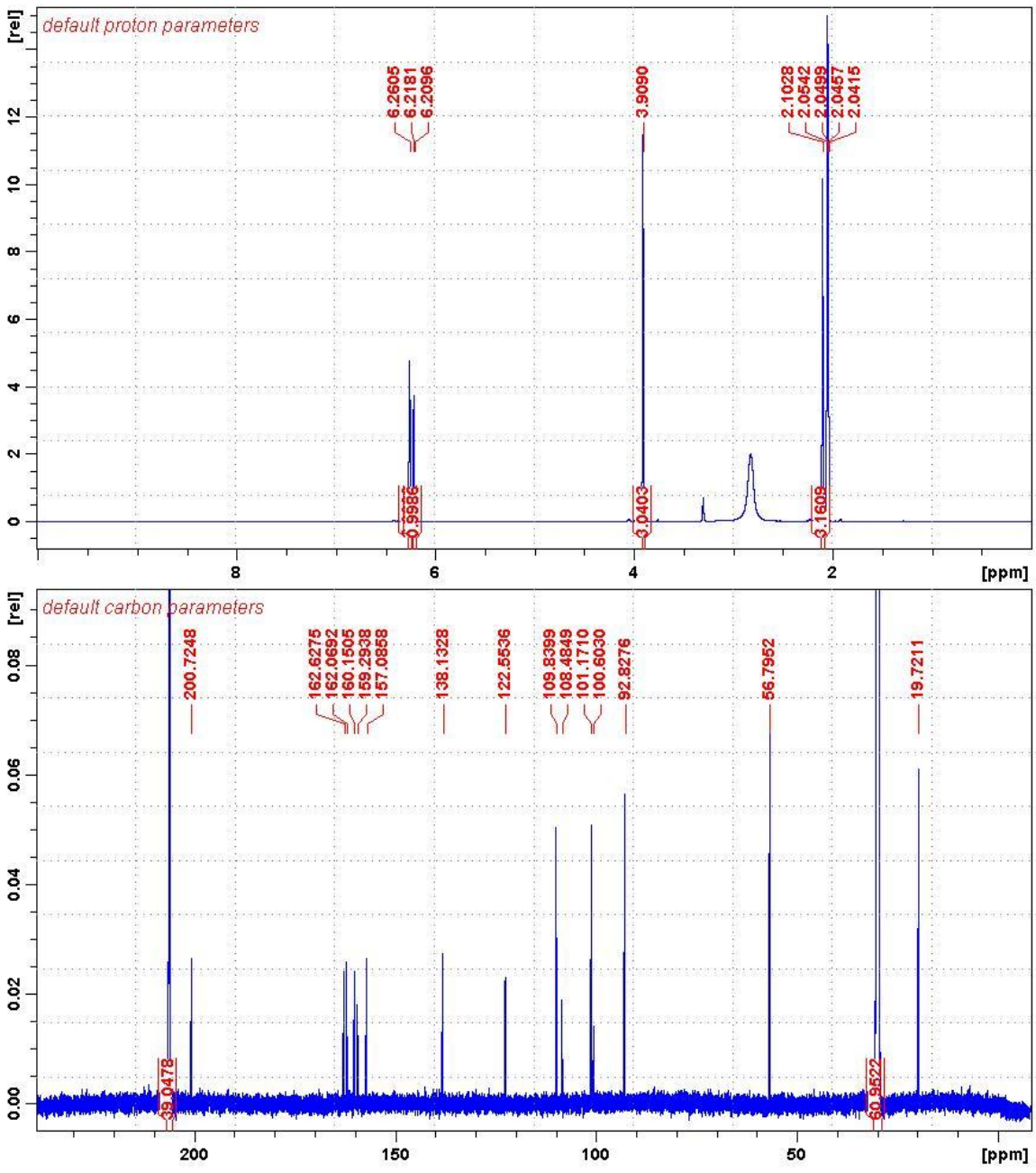


Figure S31: 1D ^{1}H (top) and ^{13}C NMR (bottom)spectrum of **17** in $(CD_3)_2CO$ (500 MHz).

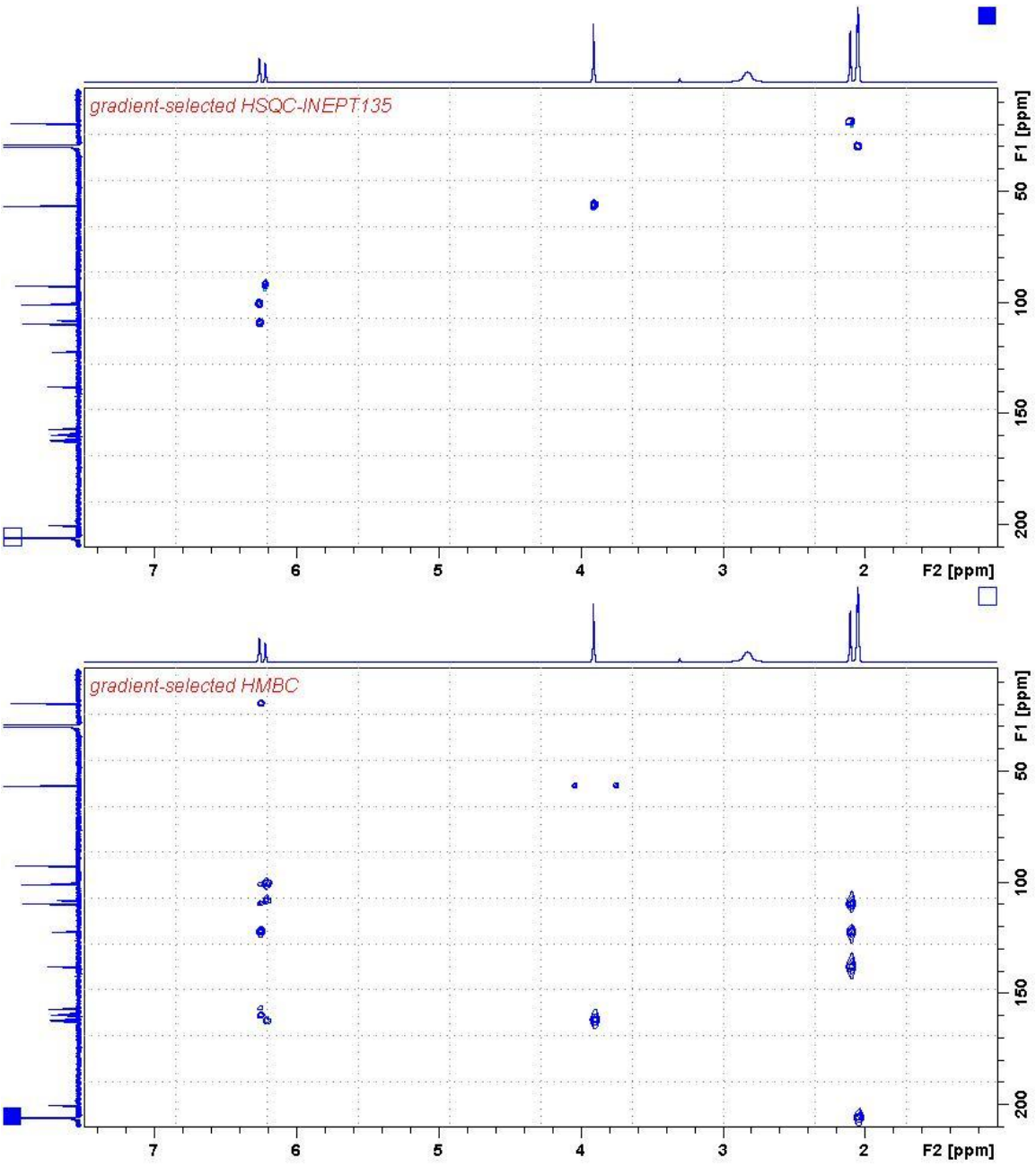


Figure S32: 2D HSQC (top) and HMBC (bottom) of **17** in $(CD_3)_2CO$ (500 MHz).

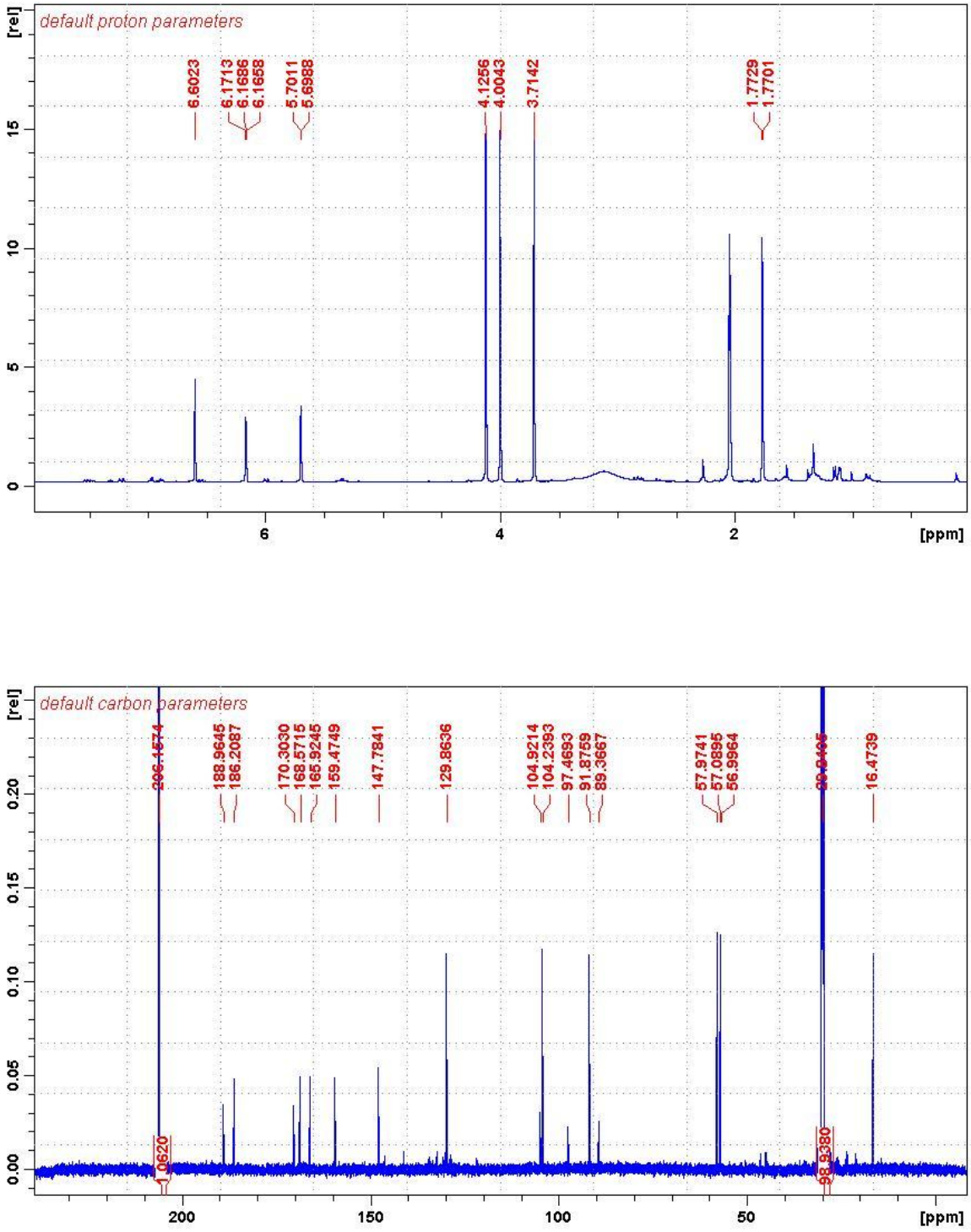


Figure S33: 1D ^{1}H (top) and ^{13}C NMR (bottom)spectrum of **18** in $(CD_3)_2CO$ (500 MHz).

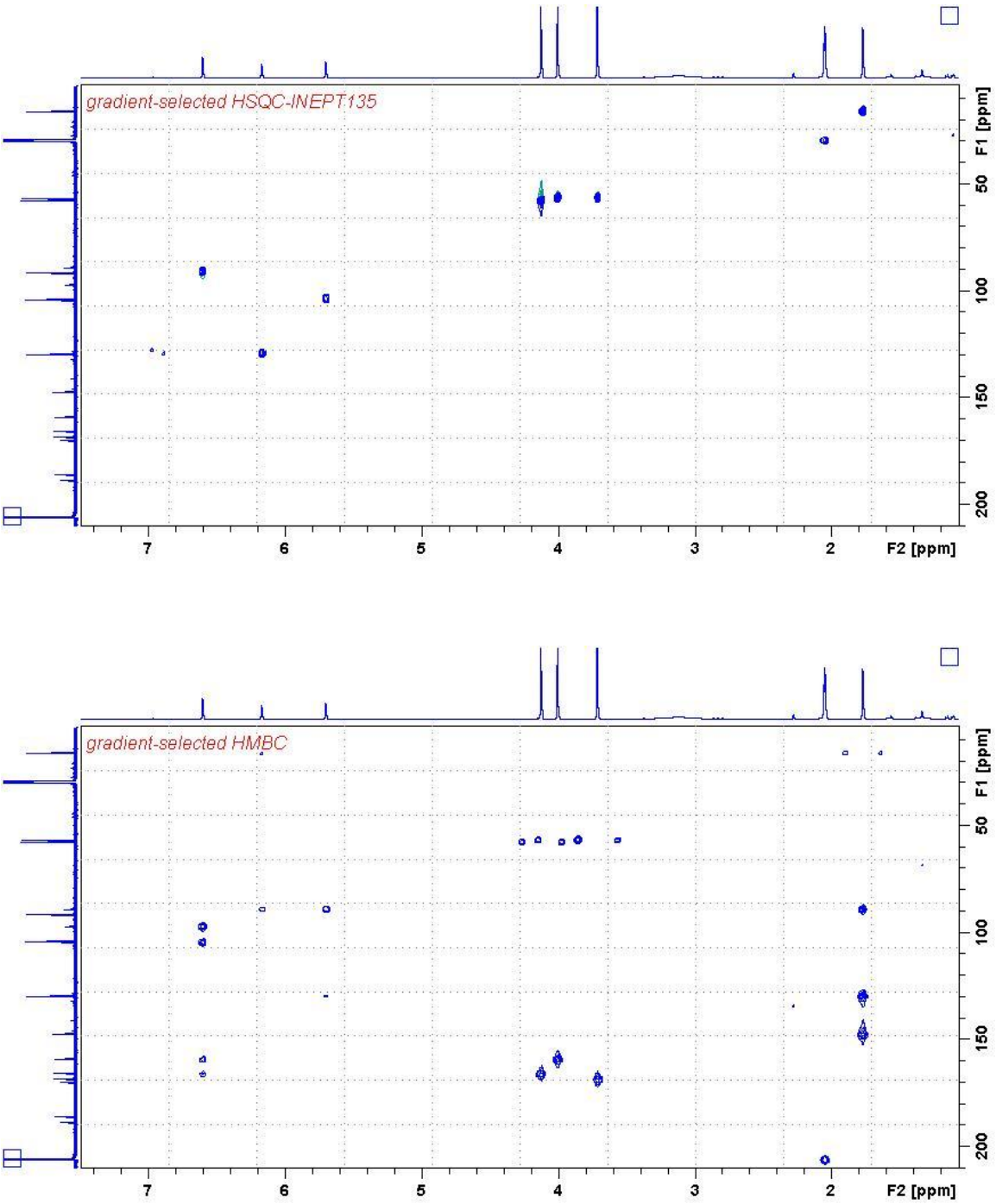


Figure S34: 2D HSQC (top) and HMBC (bottom) of **18** in $(CD_3)_2CO$ (500 MHz).

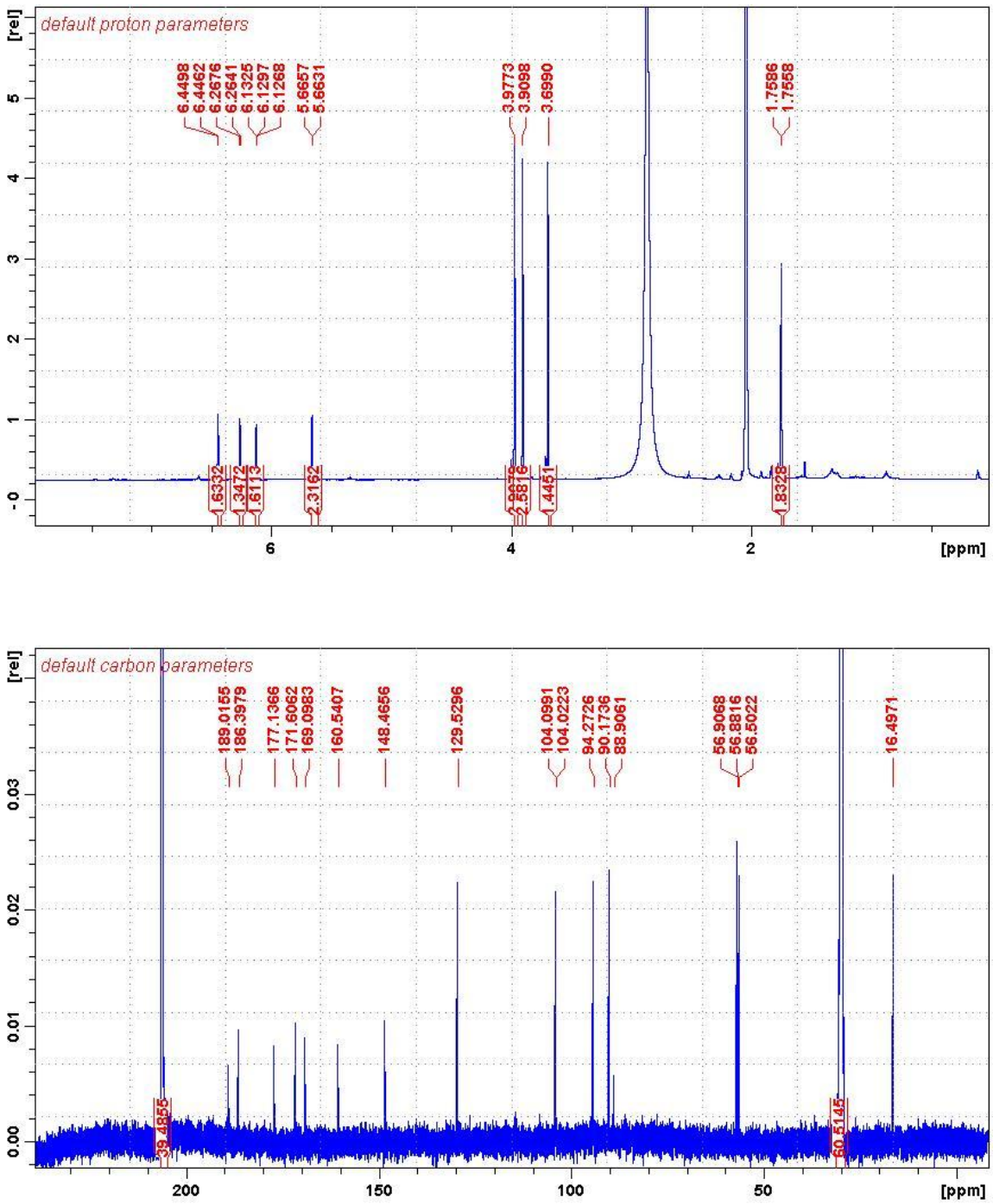


Figure S35: 1D ^{1}H (top) and ^{13}C NMR (bottom)spectrum of **19** in $(CD_3)_2CO$ (500 MHz).

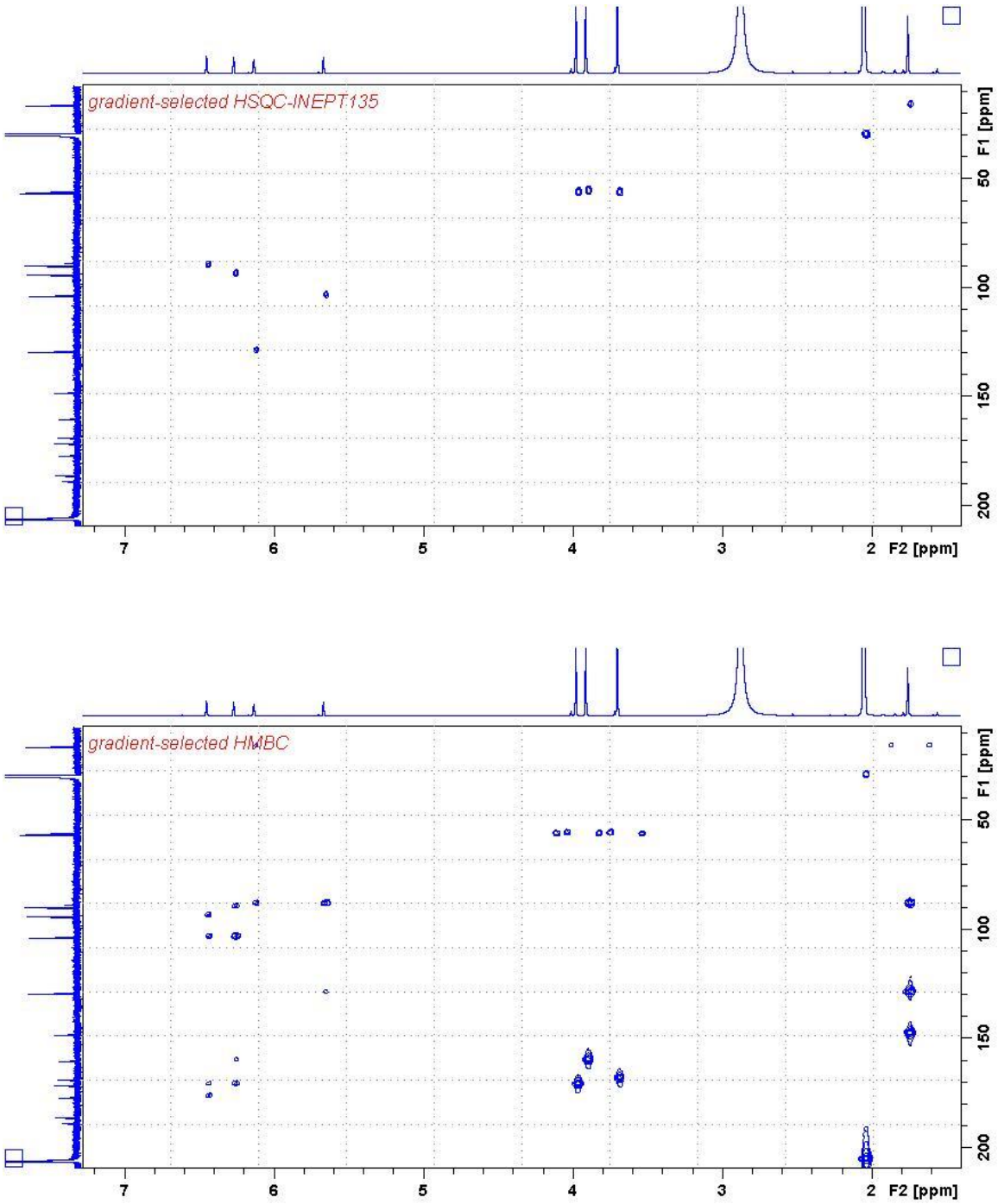


Figure S36: 2D HSQC (top) and HMBC (bottom) of **19** in $(CD_3)_2CO$ (500 MHz).

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4. Szewczyk, E., Nayak, T., Oakley, C. E., Edgerton, H., Xiong, Y., Taheri-Talesh, N., Osmani, S. A., and Oakley, B. R. (2007) Fusion PCR and gene targeting in *Aspergillus nidulans*, *Nat Protocols 1*, 3111-3120.
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6. Ralston, L., Kwon, S. T., Schoenbeck, M., Ralston, J., Schenk, D. J., Coates, R. M., and Chappell, J. (2001) Cloning, heterologous expression, and functional characterization of 5-epi-aristolochene-1,3-dihydroxylase from tobacco (*Nicotiana tabacum*), *Arch Biochem Biophys 393*, 222-235.