Supplemental Material for

Photochemical Oxazole-Nitrile Conversion Downstream of Rhizoxin Biosynthesis and its Impact on Antimitotic Activity

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Experimental

General: NMR spectra were recorded on Bruker Avance DRX 500 and DPX 300 instruments. Spectra were referenced to the residual solvent signals. HRESI-MS were recorded on a Finnigan MAT 95XL sector field mass spectrometer with a compatible ion source. IR and UV spectra were obtained using a Jasco FT/IR 4100 spectrometer and a Specord 200 photometer (Analytik Jena AG, Germany), respectively. Analytical HPLC was performed on a Shimadzu HPLC system consisting of an autosampler, high-pressure pumps, column oven and DAD. HPLC conditions: C18 column (Grom Sil 100 ODS 0AB, 3 µm, 250x4.6 mm) and gradient elution (MeCN/0.1% TFA(H_2O) 25/75 5 min, in 35 min to MeCN/0.1% TFA(H_2O) 80/20, in 5 min to 100% MeCN), flow rate 1 mL min^{-1}. Preparative HPLC was performed on a Shimadzu HPLC system with a UV detector. LC-MS measurements were performed using an Exactive Orbitrap High Performance Benchtop LC-MS Mass Spectrometer with an electrospray ion source (Thermo Fisher Scientific, Bremen). HPLC conditions: C18 column (Betasil C18 3µm 150x2.1 mm) and gradient elution (MeCN/0.1 % (v/v) HCOOH (H_2O) 5/95 for 1 min, going up to 98/2 in 15 min, then 98/2 for another 3 min; flow rate 0.2 mL min^{-1}; injection volume: 3 µL).

Creating a Δ*rhiG*-AT1 knockout mutant of *B. rhizoxinica*: For the generation of a Δ*rhiG*-AT1 mutant in *B. rhizoxinica*, a single crossover strategy was used. The final single crossover construct (pNB8) contained a kanamycin cassette and is based on the suicid vector pK19.[1] To generate a homologous region to the targeted gene, a PCR amplicon was generated using the following primers: AT1_F/AT1_R. The product of the PCR was ligated into pGEM-T Easy® (Promega) yielding pNB1. Subsequently, pNB1 and pK19 were separately digested with *Bam*HI. The 0.6 kb fragment from pNB1 was ligated into pK19 yielding pNB8. *B. rhizoxinica* cells were transformed with pNB8.

Transformants were selected on kanamycin (50 µg mL^{-1}) containing nutrient agar plates (Merck). After three-five days of cultivation (30 °C, 110 rpm) single colonies appeared and were checked by PCR for the successful recombination event (see control primers in table S2). The selection process was repeated until a mutant was identified, showing an integrated kanamycin cassette (picture S1). The expected size of the control PCR products was 0.8 kb.

Mutants containing a disrupted *rhiG* were cultivated in 100 mL VK medium (composed of 1% corn starch, 0.5% glycerol, 1% gluten meal, 1% dried yeast, 1% corn steep liquor, and 1% $CaCO_3$, pH 6.5) containing kanamycin (50 µg mL^{-1}) in 300 mL shaking flasks. After four days of cultivation (30 °C, 110 rpm) liquid cultures were extracted and metabolic profiles were analyzed by LC-MS. For further information see *Extraction*.

Complementation of *B. rhizoxinica*:pNB8: For complementation of the *rhiG*-AT1 knockout, an expression vector containing the intact gene *rhi*G was used. The primers tAT_N_F and tAT_N_R were used for gene amplification and introduction of *Nco*I sites up- and downstream of the gene. After the ligation into the *Eco*RV site of pGEM®-T Easy (Promega), yielding pNB11, this construct was digested with *Nco*I and the insert cloned into the *Nco*I digested pHKT2[2] resulting pNB12. *E. coli* Top10 competent cells were transformed with pNB12 and cultivated on trimethoprim containing LB agar plates (1500 µg mL^{-1}). Subsequently, pNB12 was introduced into competent *B. rhizoxinica*:pNB8 cells. After cultivation on kanamycin (50 µg mL^{-1}) and trimethoprim (100 µg mL^{-1}) containing nutrient agar plates (Merck) the colonies were checked by PCR. Subsequently, the transformants were cultured in 100 mL VK medium (containing 50 µg mL^{-1} kanamycin and 100 µg mL^{-1} trimethoprim) in 300 mL shaking flasks. After four days of cultivation (30 °C, 110 rpm), the samples were analyzed for rhizoxin production (picture S1). For further information see *Extraction*.

Extraction: After four days of cultivation (30 °C, 110 rpm), 100 mL ethyl acetate were added to the liquid cultures and allowed to stand overnight at room temperature. The organic phases were separated, dried over sodium sulphate and concentrated under reduced pressure. The organic residue was redissolved in 1 mL acetonitrile and filtered. Afterwards the solution was diluted 1:1 with acetonitrile. For monitoring the metabolic profiles, 2 µL of each sample were used for LC-ESI-MS analysis.

Large-scale cultivation, stable isotope labelling, and extraction: Bacteria were isolated from *Rhizopus microsporus* HKI-315 as described before.[3] 14 L of VK medium, distributed equally in 28 Erlenmeyer flasks (1L), were inoculated with the bacterial preculture. Cultivation took place at 30 °C, 180 rpm for 4 days. The entire fermentation broth was exhaustively extracted with ethylacetate, and the combined extracts were concentrated under reduced pressure.
For labelling experiments three Erlenmeyer flasks (500 mL) containing each 100 mL of VK medium were inoculated with the bacterial preculture. At the time of inoculation either 50 mg of U-^{13}C-^{15}N-glycine or 50 mg of U-^{13}C-^{15}N-serine (dissolved in sterile water) were added. The third culture was supplemented with water instead, serving as a control. After two hours 20 mg of labelled amino acids and after four hours a further 10 mg of labelled compound were fed. Cultivation took place at 30 °C, 180 rpm for 4 days. For extraction ethylacetate was used.

Purification procedure: The crude extract was fractionated by size exclusion chromatography on Sephadex LH-20 (eluent methanol). Final purification of compounds **7** and **8** was achieved by two steps of preparative RP-HPLC: 1. Nucleosil 100-7 250x40, flow rate 30 mL min^{-1}, 2. Eurospher 100-5 250x20, flow rate 12 mL min^{-1}, gradient: MeCN / H_2O 25:75 for 5 min, leading to MeCN / H_2O 80:20 in 35 min, then 83% MeCN for 10 minutes, UV detection at 304 nm.

Photooxidation of pure rhizoxin derivatives: A crude mixture of rhizoxins (2 mg, rhizoxin S2 and WF-1360F) was dissolved in CH_2Cl_2 and irradiated with a 40W lamp at −78 °C (or at room temperature, respectively) for 1 h while oxygen was bubbled through the solution. Methylene blue was used as a sensitizer. After evaporation of the solvent, the crude product was redissolved in MeOH and analyzed by HPLC-DAD. Compounds were identified by comparison to authentic standards.

Photooxidation in liquid cultures: To perform this experiments the *Burkholderia* mutants were cultured in MGY medium (M9 minimal medium supplement with 1.25 g L^{-1} yeast extract and 10 g L^{-1} glycerol) to exclude nitrile formation processes because of VK media compounds. Cultivation took place in 50 mL MGY medium containing kanamycin (50 µg mL^{-1}) in 100 mL shaking flasks. After four days of cultivation (30 °C, 110 rpm) liquid cultures were extracted and exposed to light. Subsequently, the metabolic profiles were analyzed by LC-MS.

Table S1. Photooxidation in liquid cultures

Experiment	Light Source	Exposure time	Observation / Comments
_1	no light	-	growing concentration of rhizoxin nitrile derivatives with increasing exposure time
_2	daylight lamp	2 h	
_3	daylight lamp	4 h	

Figure S1. Photooxidation in liquid cultures

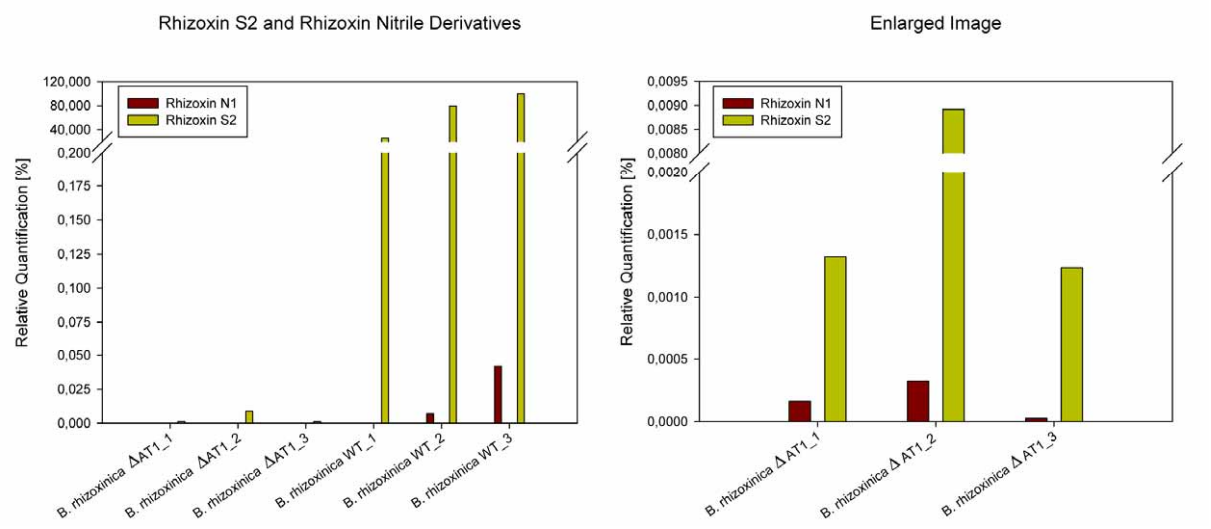


Table S2. Plasmid list

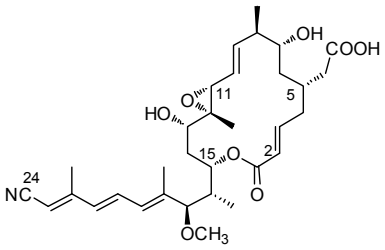
Name	Size [kb]	Description	Reference
20G04	46.8	Amp^R, Neo^R, ColE1 ori, SV40 ori,	[4]
pGEM®-T Easy	3.0	Amp^R, f1 origin, *lacZα*	Promega
pK19	2.7	Kan^R, LacZα , *aph*II	[1]
pHKT2	6.8	Tp^R, pBBR1Tp containing 1.6 kbp gfp *Kpn*I/*Xba*I fragment from pASV2[5]	[2]
Single crossover construct			
pNB1	5.7	Amplified 0.5 kb large PCR product ; additional *Bam*HI sites; cloned into pGEM®-T Easy	This study
pNB8	6.7	*Bam*HI fragment of pNB1 cloned into pK19	This study
Complementation constructs			
pNB11	5.2	Amplified *rhi*G cloned into pGEM®-T Easy, additional *Nco*I sites	This study
pNB12	8.1	*Nco*I fragment of pNB11 cloned into pHKT2	This study

Table S3. Primer list

Name	Sequence	PCR product [kb]
Primers to construct single crossover plasmid		
AT1_F	GGATCCCGAGCTTGATGCGCTGATTC	0.6
AT1_R	GGATCCCTTGCCGTAAACGCAACTCACTG	
Control primers for single crossover in *B. rhizoxinica*		
Kan_Pri1	ATGATTGAACAAGATGGA	0.8
Kan_Pri2	AGAAGAACTCGTCAAGAA	
Primers to construct complementation plasmids *B. rhizoxinica*:pNB8 mutant		
tAT_N_F	5'-ATATATCCATGGTGCCGCGATCAGAGCAGTAG-3'	2.1
tAT_N_R	5'-AGAGAGCCATGGCGGACGTGCACCAGACATTC-3'	

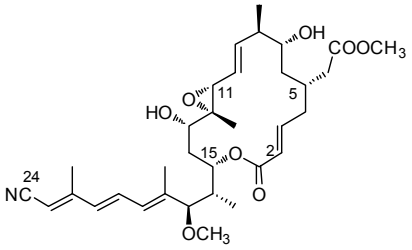
Physicochemical data

rhizoxin N1 (7)



White amorphous powder. ^{1}H NMR (300 MHz, CD_3OD) ^{13}C NMR (75 MHz, CD_3OD) see table S3. (+)-ESI-MS *m/z* 594 $[M+Na]^+$, *m/z* 572 $[M+H]^+$ HRESI-MS: *m/z* $[M+Na]^+$ = 594.3057 (calcd. for $C_{32}H_{45}NO_8Na$ 594.3043) IR (ATR, solid film): $\tilde{\nu}$ = 3357, 2969, 2930, 2873, 2207, 1698, 1685, 1596, 1570, 1498, 1407, 1387, 1268, 1241, 1206, 1055, 1014, 970, 760, 694 cm^{-1} . UV (MeOH): λ_{max} (lgε) = 303 (4.38) nm.

rhizoxin N2 (8)



White amorphous powder. ^{1}H NMR (300 MHz, CD_3OD) ^{13}C NMR (75 MHz, CD_3OD) see table S3. (+)-ESI-MS *m/z* 608 [M+Na] HRESI-MS: *m/z* $[M+Na]^+$ = 608.3211 (calcd. for $C_{33}H_{47}NO_8Na$ 608.3199) IR (ATR, solid film): $\tilde{\nu}$ = 3440, 2979, 2925, 2855, 2208, 1713, 1599, 1573, 1438, 1376, 1261, 1202, 1151, 1085, 1049, 1009, 976 cm^{-1} . UV (MeOH): λ_{max} (lgε) = 303 (4.62) nm.

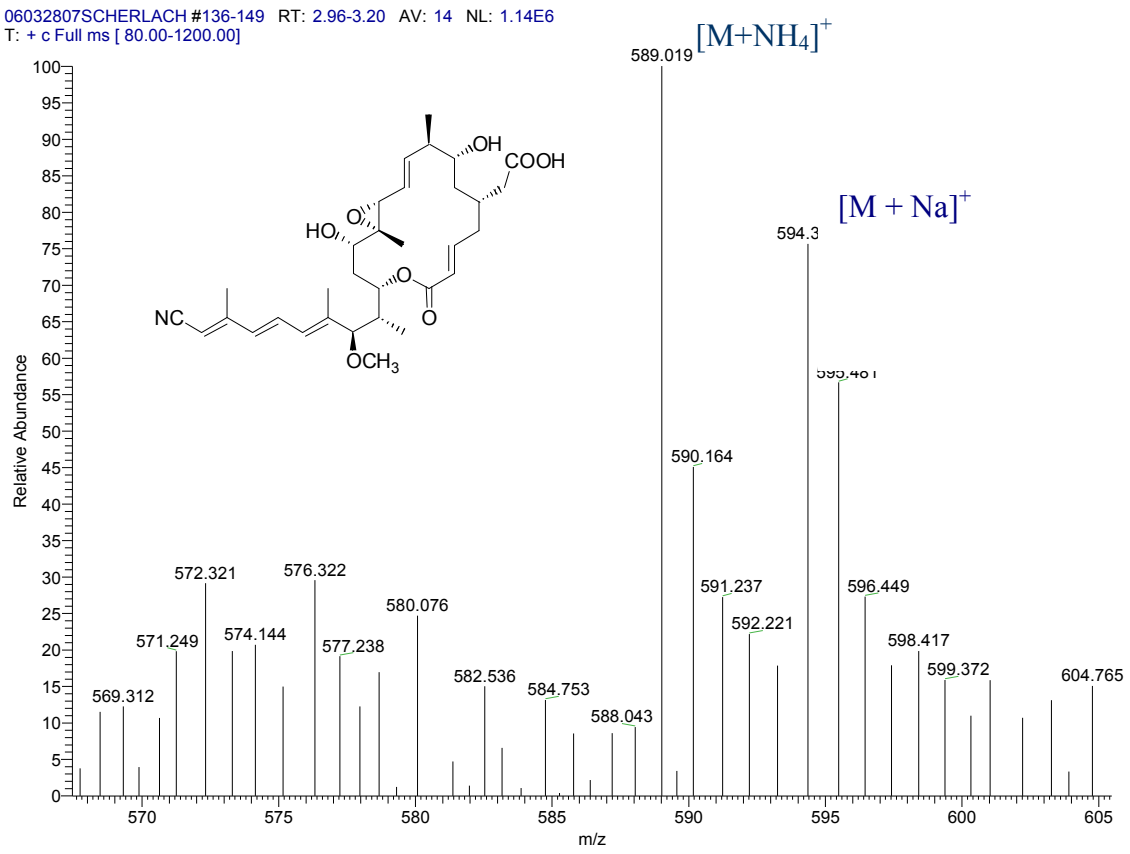
Table S4. 1D NMR data for rhizoxin N1 and N2

position	rhizoxin N1 **(7)** δ_H (J [Hz])	**r**hizoxin N1 **(7)** δ_C	**r**hizoxin N2 **(8)** δ_H (J [Hz])	**r**hizoxin N2 **(8)** δ_C
1	**-**	**167.0**	**-**	**166.8**
2	**5.74 d (15.6)**	**125.7**	**5.77 d (15.6)**	**126.2**
3	**6.85 ddd (15.5, 7.5)**	**148.8**	**6.77 ddd (15.6, 8.4, 7.2)**	**147.6**
4	**2.47 m**	**37.5**	**2.47 m***	**36.8**
	2.15 m		**2.13 m**	
5	**2.28 m***	**33.5**	**2.28 m**	**33.0**
5a	**2.30 - 2.10 (2H) m***	**45.6****	**2.55 m***	**41.2**
			2.40 m	
5b	**-**	**n. d.**[+]	**-**	**174.8**
6	**1.70 m**	**40.0**	**1.70 m**	**39.2**
	1.10 m		**1.10 m**	
7	**3.10 m**	**73.8**	**3.10 m**	**73.9**
8	**2.00 m**	**46.8**	**2.00 m**	**46.8**
8a	**1.03 d (6.6)**	**17.8**	**1.03 d (6.6)**	**17.7**
9	**5.43 dd (15.7, 9.2)**	**142.6**	**5.44 dd (15.7, 9.2)**	**142.3**
10	**5.15 dd (15.7, 8.2)**	**127.1**	**5.16 dd (15.7, 8.2)**	**127.3**
11	**2.97 d (8.2)**	**63.4**	**2.98 d (8.2)**	**63.4**
12	**-**	**66.2**	**-**	**66.2**
12a	**1.29 s**	**11.2**	**1.29 s**	**11.2**
13	**2.90 dd (10.7, 2.7)**	**79.6**	**2.92 dd (10.9, 2.6)**	**79.4**
14	**2.00 m**	**34.1**	**2.00 m**	**34.1**
	1.75 m		**1.75 m**	
15	**4.73 dd (9.5, 3.5)**	**74.7**	**4.74 dd (9.6, 3.6)**	**74.9**
16	**2.05 m**	**40.8**	**2.05 m**	**40.8**
16a	**0.98 d (6.8)**	**10.3**	**0.98 d (6.8)**	**10.4**
17	**3.36 d (8.5)**	**90.3**	**3.36 d (8.5)**	**90.2**
17-OCH_3	**3.16 s**	**56.7**	**3.16 s**	**56.7**
18	**-**	**143.7**	**-**	**143.6**
18a	**1.88 s**	**12.2**	**1.88 s**	**12.2**
19	**6.17 d (11.1)**	**129.5**	**6.18 d (11.0)**	**129.5**
20	**6.99 dd (15.2, 11.0)**	**132.5**	**6.99 dd (15.2, 11.0)**	**132.4**
21	**6.42 d (15.3)**	**133.7**	**6.42 d (15.2)**	**133.7**
22	**-**	**159.0**	**-**	**158.9**
22a	**2.21 s**	**16.6**	**2.21 s**	**16.6**
23	**5.42 s**	**98.1**	**5.42 s**	**98.1**
24	**-**	**118.8**	**-**	**118.8**
5b-OCH_3	**-**	**-**	**3.67 s**	**52.0**

*** Partial overlapping of signals**
**** Chemical shift deduced from HMQC coupling**
[+] Not detectable due to low concentration

Figure S2. Mass spectra of unlabelled (**a**) and labelled (**b**) rhizoxin N1

a



b

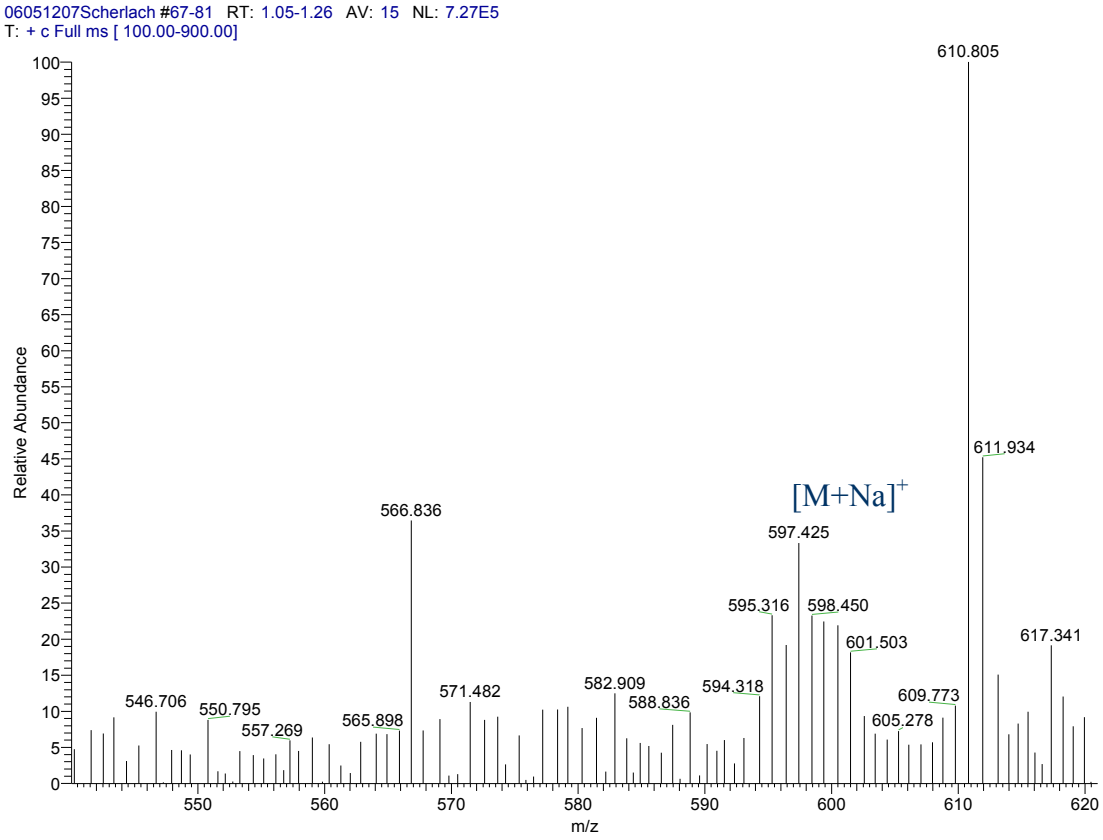


Figure S3. ^{1}H NMR of **7**

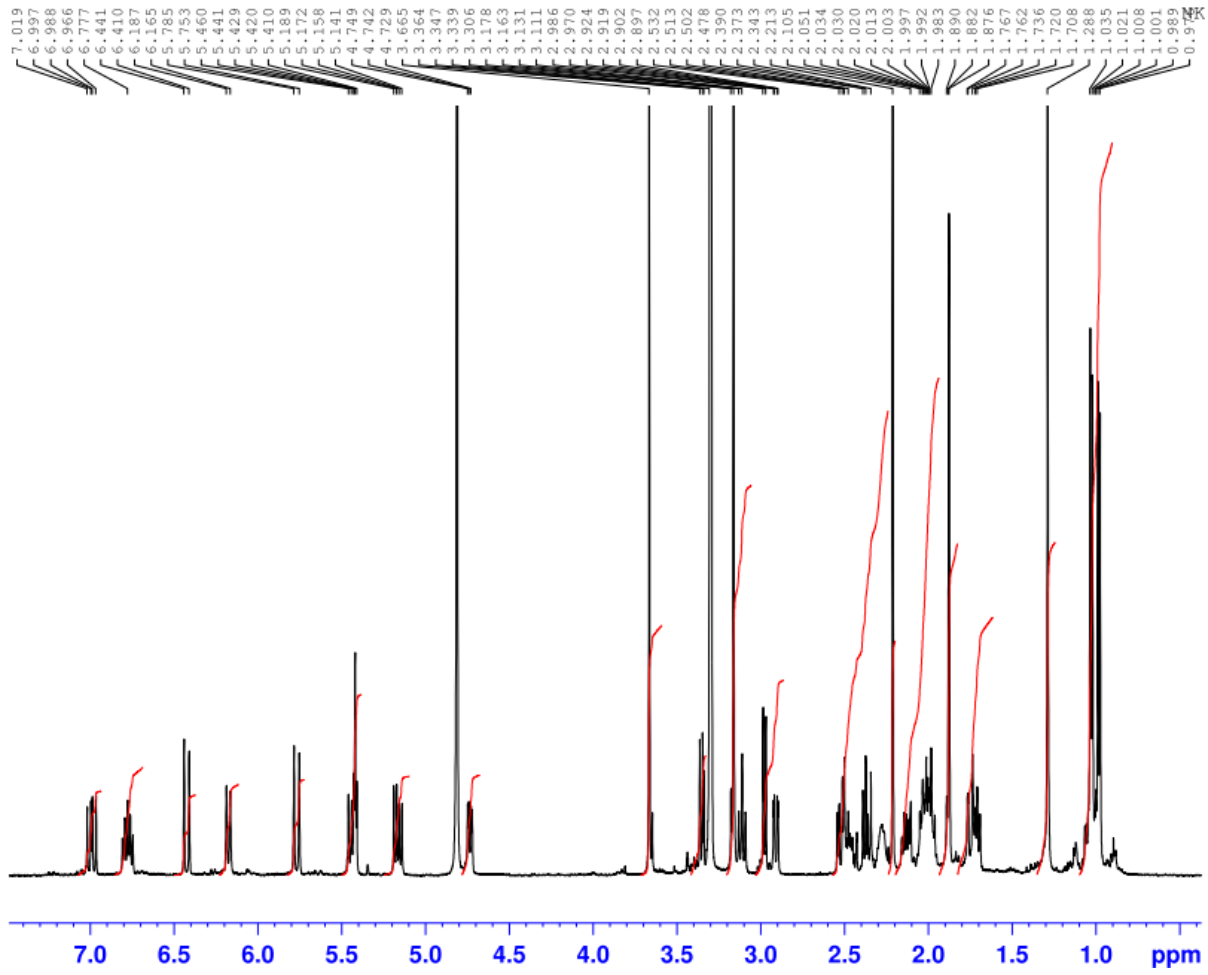


Figure S4. ^{13}C NMR of **7**

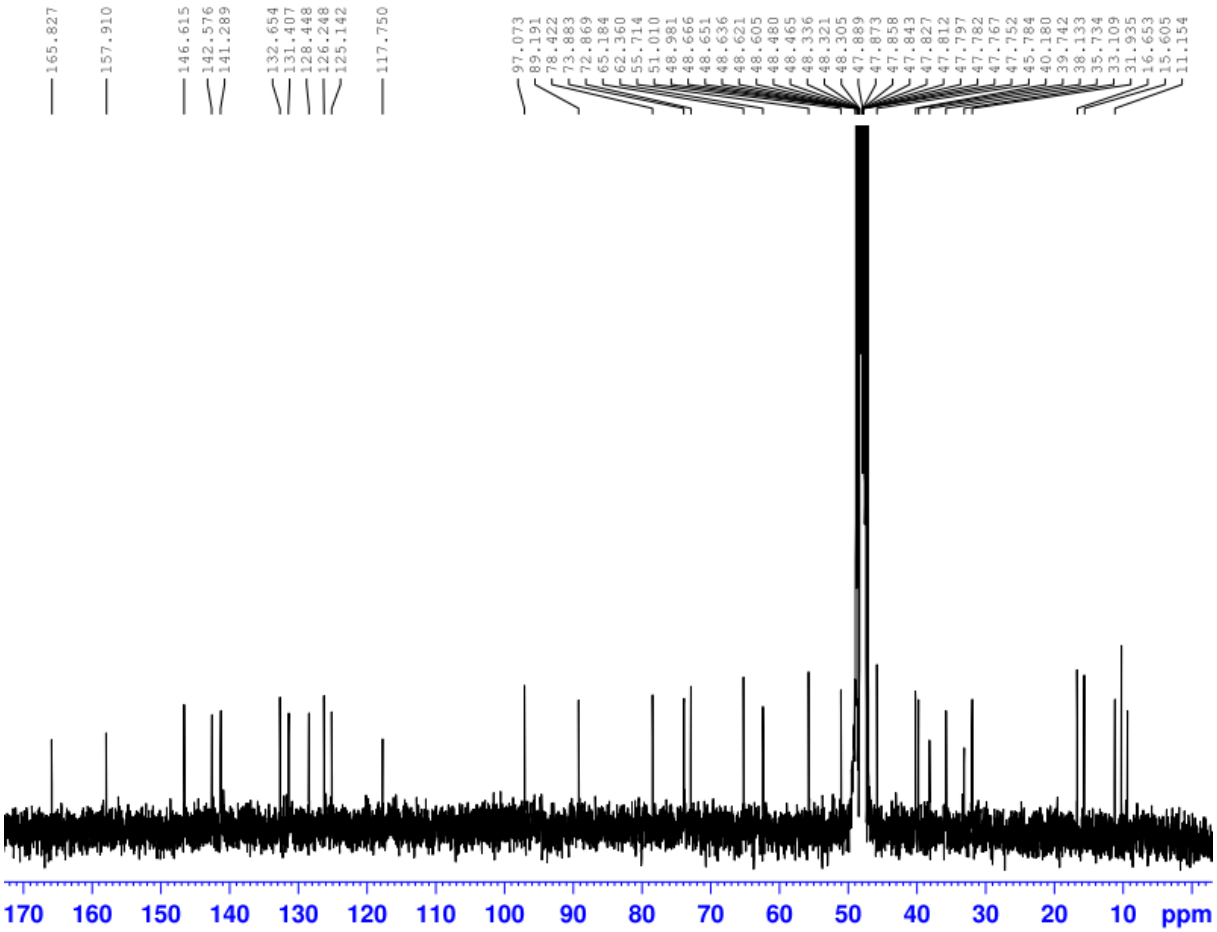


Figure S5. HSQC NMR of **7**

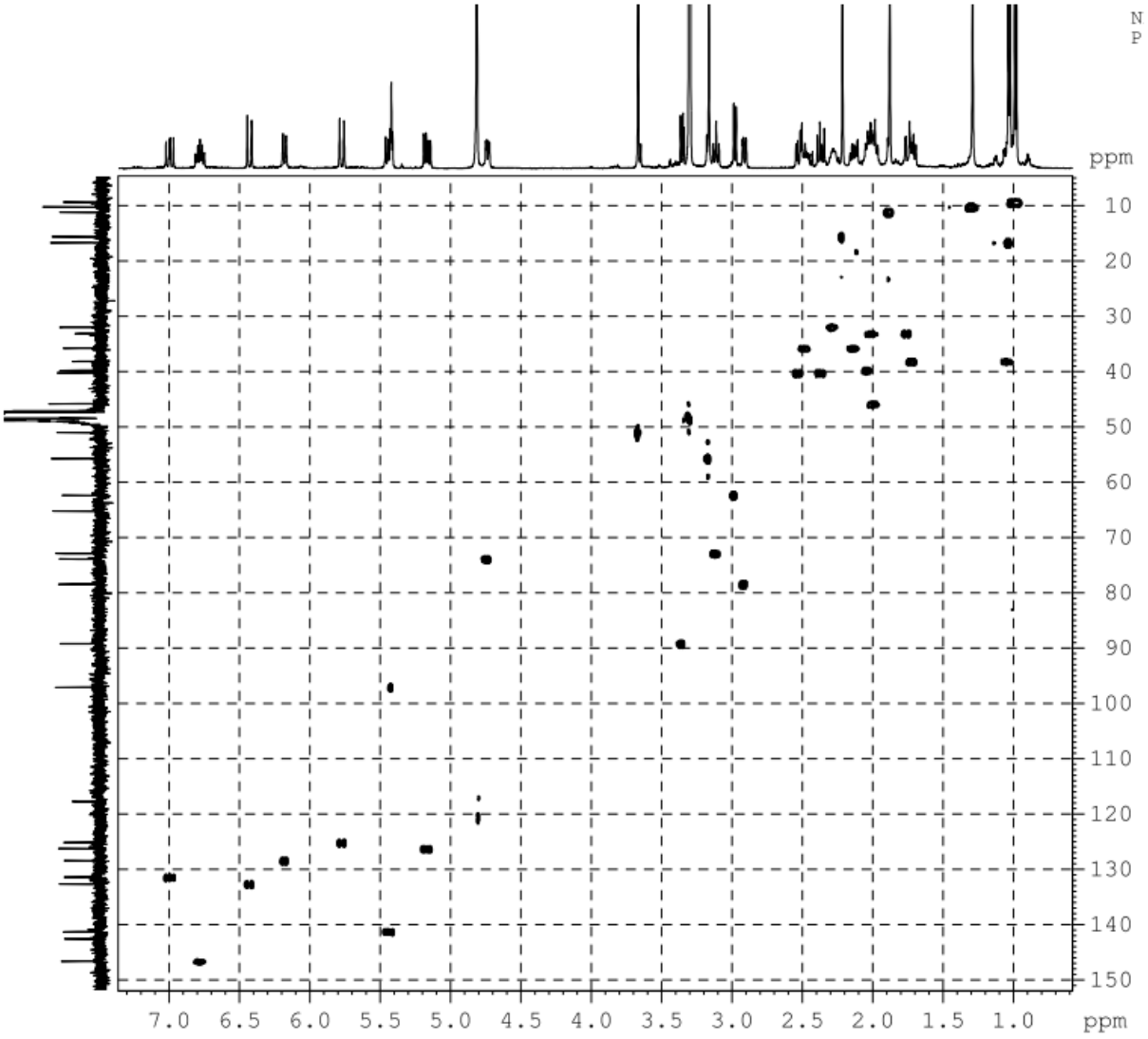


Figure S6. HMBC NMR of **7**

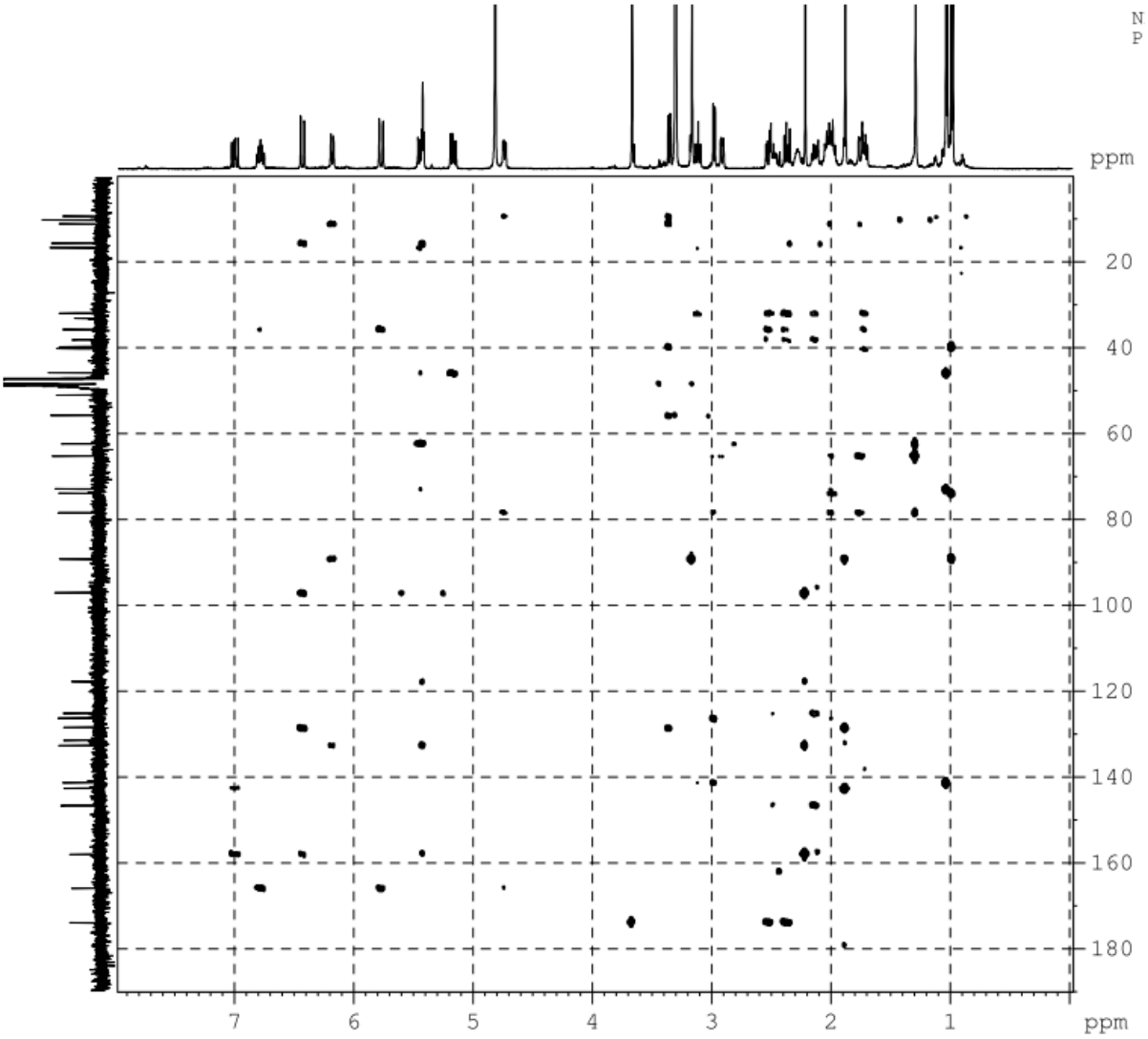


Figure S7. ^{1}H NMR of **8**

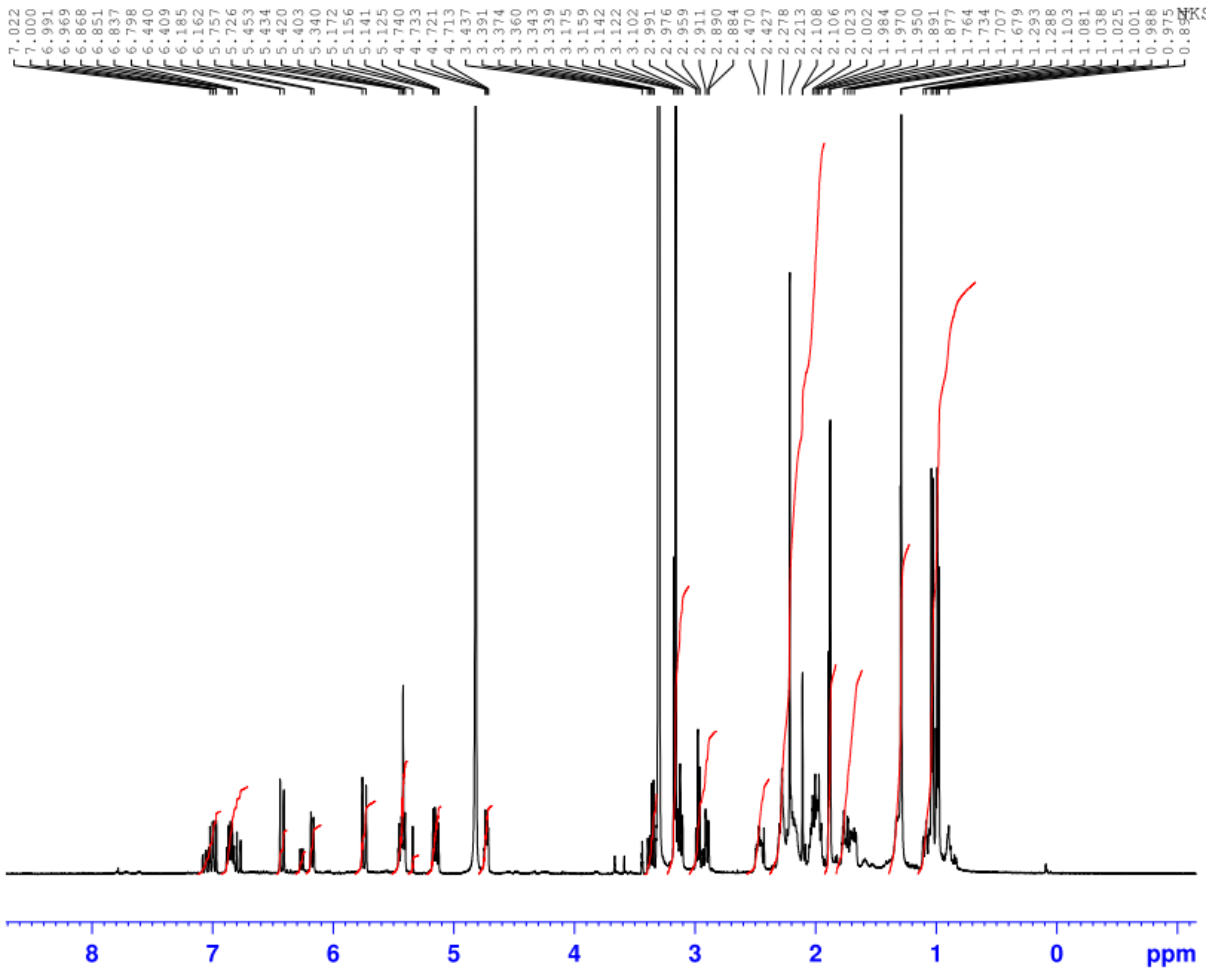


Figure S8. ^{13}C NMR of **8**

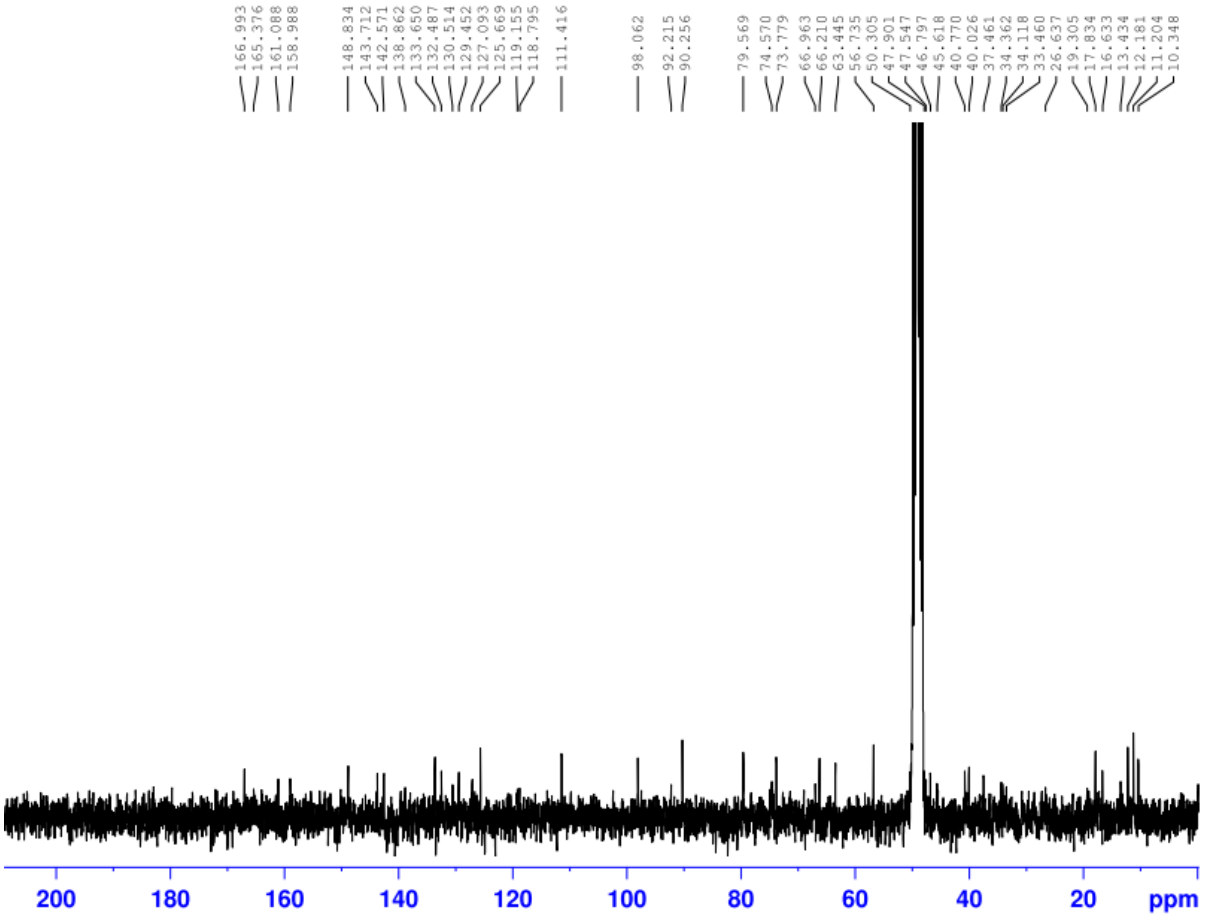


Figure S9. HSQC NMR of **8**

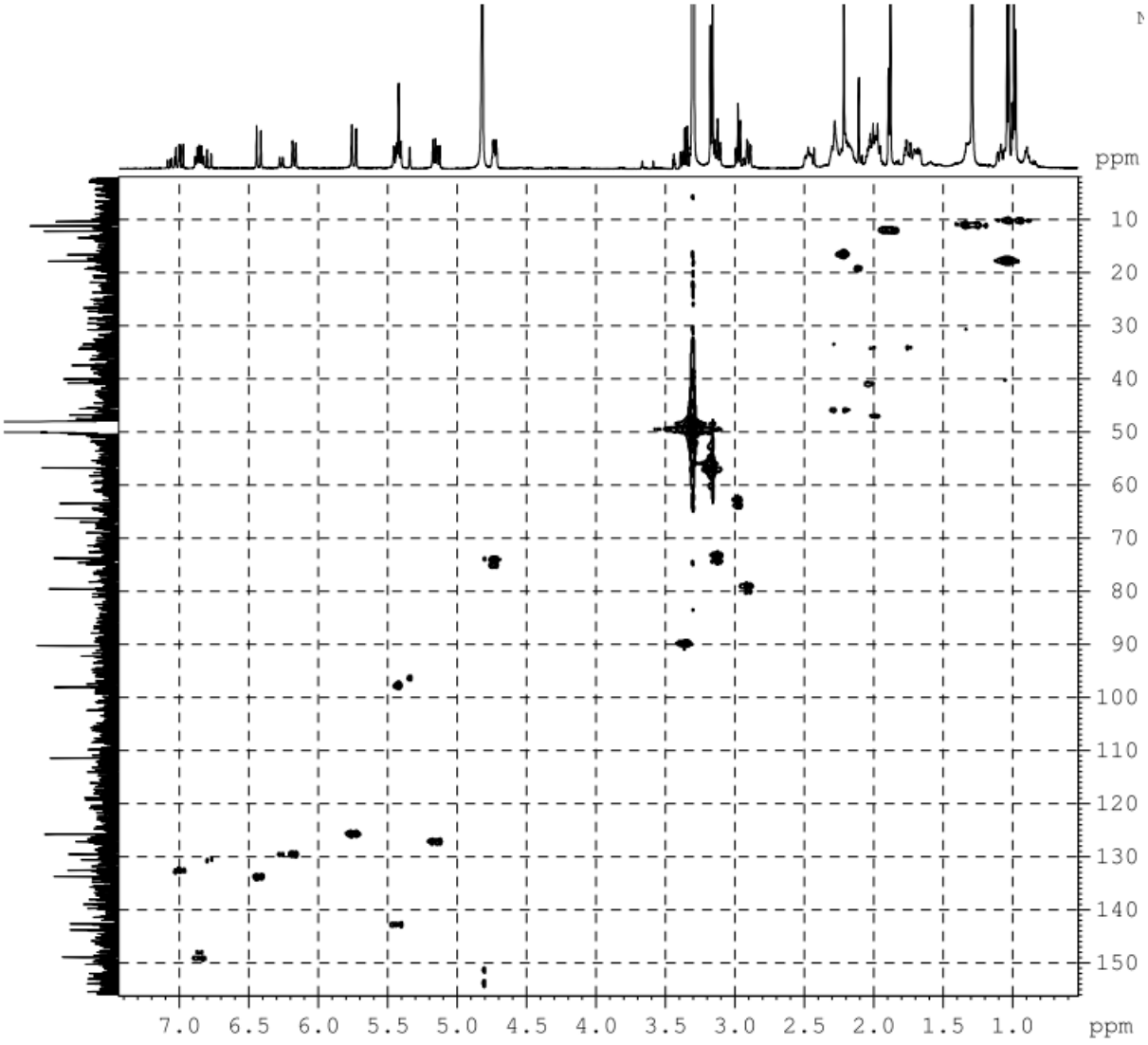
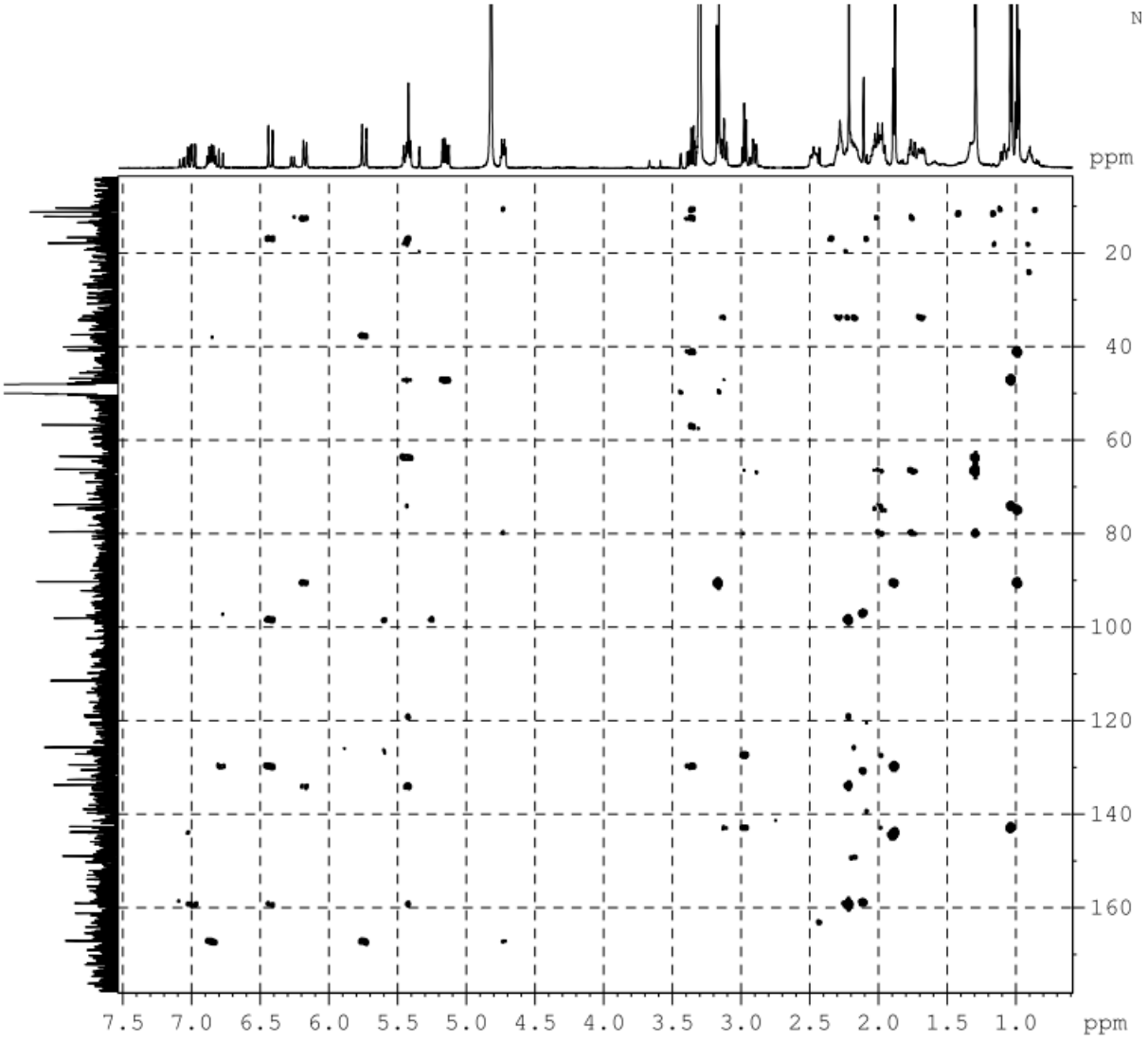


Figure S10. HMBC NMR of **8**



References

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