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Running Title: Anti-phytopathogenic bacterial compounds from *Agaricus blazei* mycelia

Anti-phytopathogenic bacterial fatty acids from the mycelia of the edible mushroom *Agaricus blazei*

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ABSTRACT

Five compounds including a new compound (**1**) were isolated from mycelia of a mushroom-forming fungus *Agaricus blazei*. Compound **2** was isolated from nature for the first time. Their structures were determined by the interpretation of spectroscopic data. In the bioassay examining growth inhibitory activity against phytopathogenic bacteria *Clavibacter michiganensis*, *Burkholderia glumae*, and *Peptobacterium carotovorum*, all the compounds showed inhibition effects on *C. michiganensis*. Compounds **3** and **4** also showed weak inhibitory activity against growth of *B. glumae.*

Keywords: structural determination, mycelium, *Agaricus blazei*, isolation, phytopathogenic bacterial activity

Higher fungi have a wide variety of forms and functions, and have long been valued not only as food, but also as traditional Chinese medicine. For this reason, many compounds with various bioactive effects have been isolated from mushrooms. In our previous studies, plant growth regulators, 2-azahypoxanthine and imidazole-4-carboxamide were isolated from the culture broth of one of the fairy ring-forming fungi *Lepista sordida*, and hericenones C to H were found from *Hericium erinaceus* as nerve growth factor (NGF) synthesis-promoting compounds (Choi *et al*., 2010a, b; Kawagishi *et al*., 1991; Kawagishi *et al*., 1993). Recently, we also reported erinachromanes A, B and erinaphenol A from the culture broth of *Hericium erinaceus* that showed suppression activity on the growth of lettuce (Wu *et al*., 2019).

Agaricus blazei (Japanese name: Himematsutake) is an edible and medicinal mushroom that belongs to the family Agaricaceae. This mushroom is known as *Cogumelo do Sol* (mushroom of the sun) in Brazil. The fruiting bodies of *A. blazei* have anti-tumor, liver function improving, anti-cancer, and immunity lowering effects (Kimura *et al*., 2004; Kim *et al*., 2005a, b). In our previous papers, we have isolated a lectin, an anti-tumor β-(1→6)-D-glucan-protein complex and several steroids showing cytotoxicity against HeLa cells from the fruiting bodies of this fungus (Kawagishi *et al*., 1988a, b; Kawagishi *et al*., 1989; Kawagishi *et al*., 1990). As for mycelia of the fungus, brefeldin A was isolated as an Erk1/2-activating component (Dong *et al*., 2013). However, compared to the studies on fruiting bodies of *A. blazei*, there are only a few reports that described low-molecular compounds from the mycelia. In this study, we describe the isolation, structural determination, and anti-phytopathogenic bacterial

activity of five compounds from the mycelia.

Experimental

General Experimental procedures

^{1}H NMR spectra (one- and two-dimensional) were recorded on JNM-ECZ500R spectrometer at 500 MHz, and ^{13}C NMR spectra were recorded on the same instrument at 125 MHz (JEOL, Tokyo, Japan). HRESIMS spectra were measured on a LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific). An FT/IR-4100 (Jasco, Tokyo, Japan) instrument was used to record the IR spectra, and the specific rotation values were measured by a Jasco DIP-1000 polarimeter (Jasco, Tokyo, Japan). HPLC separations were performed with a Jasco Chromatography Data Station ChromNAV system using reverse-phase HPLC columns (ODS-P, InertSustain, Tokyo, Japan; CAPCELL PAK C18 AQ, Osaka, Japan). Silica gel plate (Merck F254), ODS gel plate (Merck F254), and silica gel 60 N (Kanto Chemical, Tokyo, Japan) were used for analytical TLC and for flash column chromatography. All solvents used throughout the experiments were obtained from Kanto Chemical Co. (Tokyo, Japan).

Fungal and Bacterial Material

Agaricus blazei Murrill (Iwade strain 101) has been stored in Iwade Mushroom Institute in Japan. *Clavibacter michiganensis* SUPP573, *Burkholderia glumae* SUPP1744, and *Peptobacterium carotovorum* SUPP8 have been deposited in Faculty of Agriculture, Shizuoka University, Japan.

Extraction and Isolation

Dried powder of the mycelia of *A. blazei* (3.2 kg) was extracted with *n*-hexane, EtOAc, EtOH and water (30 L each, three times), successively. The EtOAc soluble part (44.0 g) was fractionated by silica gel flash column chromatography (*n*-hexane; *n*-hexane/EtOAc=90/10, 80/20, 70/30, 65/35, 60/40, 55/45, 50/50, 40/60, 30/70, 20/80, 10/90; EtOAc; MeOH; 1.5 L each) to obtain 20 fractions (Fractions 1 to 20). Fraction 7 (eluted with *n*-hexane/EtOAc = 65/35, 1.04 g) was further separated by normal-phase MPLC over silica gel and gradient eluted with *n*-hexane: EtOAc (from 100:0 to 0:100, obtained fractions 7-1 to 7-7) and EtOAc: MeOH (from 100:0 to 0:100, obtained fractions 7-8 to 7-14). Fraction 7-3 (132.1 mg) was separated by reverse-phase HPLC (ODS-P, MeCN/H_2O=8/2) to afford compound **1** (1.3 mg), **2** (2.7 mg), **3** (0.6 mg) and **4** (1.8 mg). Compound **5** (3.2 mg) was obtained from fraction 7-5 (48.5 mg) by reverse-phase HPLC (CAPCELL PAK C18 AQ, MeOH/H_2O=8/2).

(9*R*,10*E*,12*Z*)-9-acetoxyoctadeca-10,12-dienoic acid (**1**): Yellow oil; IR (neat, ν_{max}): 1715, 2930 cm^{-1}; ^1H and ^{13}C NMR, see **Table 1**; $[\alpha]_D^{28}$ -85 (*c* 0.13, MeOH); ESIMS *m/z* 337 $[M-H]^-$; HRESIMS *m/z* 337.2401 $[M-H]^-$ (calcd. for $C_{20}H_{33}O_4$, 337.2384).

(9*R*,10*E*,12*E*)-9-acetoxyoctadeca-10,12-dienoic acid (**2**): Yellow oil; ^1H and ^{13}C NMR, see **Table 1**; $[\alpha]_D^{28}$ -2.5 （*c* 0.27, MeOH）; ESIMS *m/z* 337 $[M-H]^-$; HRESIMS *m/z* 337.2414 $[M-H]^-$ (calcd. for $C_{20}H_{33}O_4$, 337.2384).

Antibacterial Activity

Each bacterium (*C. michiganensis*, *B. glumae*, and *P. carotovorum*) was taken from the slant using an inoculation loop and suspended in 1 mL of sterile water in 1.5 mL Eppendorf tube, and a suspension of 10^8 colony forming unit (CFU)/mL was made by reference to OD_{600}. YP medium (yeast extract 5 g/L, peptone 10 g/L, agar 15 g/L) in a test tube was autoclaved for 20 min at 121 °C. The medium was left to stand until the temperature reached at about 30°C, and 100 μL of each bacterium suspension was added to the medium, and the mixture was poured into a Petri dish.

40 μL of solution of each compound (0.1, 0.05, and 0.01 μmol in MeOH) were put on a paper disc (8 mm in diameter). After the discs were dried in the air, they were put on the medium as shown in **Figure 3**. MeOH only applied disc was used as control. They were incubated for 3 days to evaluate their antibacterial activity.

Results and discussion

The dried mycelia of *A. blazei* were extracted with *n*-hexane, EtOAc, EtOH and water, successively. The EtOAc soluble part was fractionated by repeated chromatography. As a result, a new compound (**1**) and four known compounds (**2**–**5**) were isolated (**Figure 1**).

Compound **1** was obtained as a yellow oil. The molecular formula was determined as $C_{20}H_{34}O_4$ by HRESIMS (*m/z* 337.2401 $[M-H]^-$; calcd. for $C_{20}H_{33}O_4$, 337.2384), indicating the presence of four degrees of unsaturation in the molecule. The structure of **1** was elucidated by interpretation of NMR data, including DEPT, HMQC, DQF-COSY and HMBC data (**Figure S1-5**, **Table 1**). The ^{13}C NMR, DEPT and HMQC data

established the presence of two methyls, 11 methylenes, five methines and two carboxy groups (δc 170.5 , 177.6). The COSY and HMBC correlations are illustrated in **Figure 2**. The COSY correlations (H-10/H-11, H-11/H-12, H-12/H-13, H-13/H-14, H-12/H-14), HMBC correlations (H-11/C-9, 12, 13; H-12/C-10, 11, 14; H-13/C-11, 14, 15), ^{1}H NMR and ^{13}C NMR data indicated the presence of a conjugated diene from C-10 to C-13 (C-10: δc 130.8, C-11: δc 128.1, C-12: δc 127.4, C-13: δc 133.9) and H-10 to H-13 [H-10: δ_H 5.55 (dd, J =7.5, 15.3), H-11: δ_H 6.50 (dd, J =15.3, 11.1), H-12: δ_H 5.94 (dd, J =11.1, 11.0), H-13: δ_H 5.47 (m)]. The acetoxy group at C-9 was elucidated by the COSY correlations (H-8/H-9, H-9/H-10) and the HMBC correlations (H-8/C-6, 7, 9; H-9/C-7, 8, 9-O$\underline{C}$OCH$_3$, 10, 11; H-10/C-8, 9, 12; 9-OCOC$\underline{H}_3$/9-O$\underline{C}$OCH$_3$). The ^{1}H-NMR and ^{13}C-NMR signals [C-1: δ_C 177.6; C-2: δ_C 33.6, δ_H 2.34 (t, J =7.5); C-3: δ_C 24.6, δ_H 1.63 (m); C-4: δ_C 28.9, δ_H 1.30 (m)], the COSY correlation (H-2/H-3, H-3/H-4), and HMBC correlations (H-2/C-1, 3, 4; H-3/C-1, 2, 4) suggested the presence of one carboxy group with methylenes. The COSY correlations (H-14/H-15, H-17/H-18), the HMBC correlations (H-14/C-12, 13, 15, 16; H-15/C-13, 16, 17; H-16/C-17; H-17/C-16; H-18/C-16, 17), the molecular formula, and the unsaturation degree indicated that this compound is 9-acetoxyoctadeca-10,12-dienoic acid. The coupling constants [C-10: δ_H 5.55 (dd, J =7.5, 15.3); C-11: δ_H 6.50 (dd, J =15.3, 11.1,), C-12: δ_H 5.94 (dd, J =11.1,11.0)] indicated that the double bond is 10*E*, 12*Z*. To determine the absolute configuration of **1**, the specific rotation {$[\alpha]_D^{28}$ -85 (*c* 0.13, MeOH)} was compared with that {$[\alpha]_D^{25}$ +11.4 (*c* 0.4, MeOH)} of the deacylated analog, (9*S*,10*E*,12*Z*)-9-hydroxyoctadeca-10,12-dienoic acid, whose absolute configuration has been

155 determined (Naidu *et al*., 2007). All the data allowed us to conclude that **1** was
156 (9*R*,10*E*,12*Z*)-9-acetoxyoctadeca-10,12-dienoic acid (**Figure 1**). This is a new
157 compound.

158 Compound **2** was identified as (10*E*,12*E*)-9-acetoxyoctadeca-10,12-dienoic acid by
159 the interpretation of its spectral data including the DQF-COSY and HMBC (**Table 1**,
160 **Figure S6 - S11**). Racemate of **2** has already been synthesized, however, no spectral
161 data is available. The isolated **2** is optically active and absolute configuration of **2** was
162 determined by comparison of its specific rotation {$[\alpha]_D^{28}$ -2.5 (*c* 0.27, MeOH)} with
163 that of the deacylated analog, (9*S*,10*E*,12*E*)-9-hydroxyoctadeca-10,12-dienoic acid
164 {$[\alpha]_D^{25}$ +15.1 (*c* 0.8, MeOH)}, indicating that **2** was (9*R*,10*E*,12*E*)-9-acetoxyoctadeca-
165 10,12-dienoic acid (Naidu *et al*., 2007; **Figure 1**, **Figure S7-11**). This is the first time
166 to isolate the compound from nature. There are no reports of biological activity of the
167 racemate.

168 Compounds **3** to **5** were respectively identified as (9*Z*,11*E*)-13-oxooctadeca-9,11-
169 dienoic acid, (9*E*,11*E*)-13-oxooctadeca-9,11-dienoic acid and (*S*,9*Z*,11*E*)-13-
170 hydroxyoctadeca-9,11-dienoic acid by the comparison of their spectroscopic data with
171 those reported (Yoshikawa *et al*., 1996; Qi *et al*., 2020; **Figure S12-17**). Compound **3**
172 was isolated from the Ghanaian endophytic fungus, *Penicillium herquei* strain BRS2A-
173 AR and possessed antimicrobial and cytotoxic activities (Hayibor *et al*., 2019).
174 Compound **4** was first isolated from the seed oil of *Malpighia emarginata* (Phillips *et*
175 *al*., 1970), and showed inhibitory activity against the growth of EL4 mouse lymphoma
176 cells and stimulating activity against white blood cells in the blood (Kodaka *et al*., 2019;

177 Henricks *et al*., 1991). Compound **5** was first isolated as a plaque lipid component of
178 the human aortic vessel wall, and isolated from the fruiting bodies of this fungus as a
179 stimulator of peroxisome proliferator-activated receptors and leukocytes in the blood
180 (Buchanan *et al.,* 1985; Osaki *et al*., 1994; Delerive *et al*., 2000).

181 Compounds **1** to **5** were tested for growth inhibitory activity against *Clavibacter*
182 *michiganensis*, *Burkholderia glumae* and *Peptobacterium carotovorum*. All of these are
183 typical bacteria that cause enormous damage to vegetables and grains grown in Japan.
184 *C. michiganensis* causes ulcer disease and is a parasite of tomatoes. It is a Gram-
185 positive bacterium, which is rare among plant pathogens (Fatmi *et al*. 2017). *B. glumae*
186 infects rice only under natural conditions, and the disease caused by this bacterium is a
187 serious global-scale problem that causes yield reduction of rice (Ham *et al*. 2011). The
188 disease develops rot during seedling growth (Pedraza *et al*. 2018). *P. carotovorum*
189 causes soft rot on many crops including Chinese cabbage, lettuce, leeks, and potatoes
190 (Oskiera *et al.* 2017). As a result, compounds **3** and **4** showed weak inhibitory activity
191 against the growth of *B. glumae* at all the concentrations (**Figure S18**, **Table S1**). On
192 the other hand, all the compounds inhibited the growth of *C. michiganensis* at all
193 concentrations (**Figure 3**, **Table 2**). The inhibitory zone caused by **2** was much wider
194 than that by **1**. Both the compounds are *cis-trans* isomers each other, indicating that
195 *trans* configuration at C-12 in **2** strengthened the inhibitory activity against the growth
196 of *C. michiganensis.* In addition, **5** showed stronger activity than **3**. The difference of
197 their structures is that **5** has a hydroxy group at C-13 while C-13 of **3** is a carbonyl
198 group. The result suggested that the hydroxyl group at C-13 of **5** played an important

role in the stronger inhibitory activity of the compound.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Author contribution

H. Ka. conceived the project. H. Ka. and J. Wu designed the chemical experiments. R. O. and J. Wa. performed the experiments. Y. T. provided the bacteria strains and designed the bioassay. J. Wa., J. Wu, J.-H.C and H. Ka. wrote the manuscript. All authors contributed to discussions.

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Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

Disclosure statement

No potential conflict of interest was reported by the authors.

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List of Figures and Tables.

Table 1. NMR data for **1** and **2** in $CDCl_3$.

	1			**2**		
Position	δ_C	type	δ_H (type, multiplicity, *J* in Hz)	δ_C	type	δ_H (type, multiplicity, *J* in Hz)
1	177.6	C		178.3	C	
2	33.6	CH_2	2.34 (t, 7.5)	33.7	CH_2	2.34 (t, 7.4)
3	24.6	CH_2	1.63 (m)	24.6	CH_2	1.63 (m)
4	28.9	CH_2	1.30 (m)	28.8*	CH_2	1.29 (m)
5	29.05*	CH_2	1.30 (m)	28.9*	CH_2	1.29 (m)
6	29.12*	CH_2	1.30 (m)	29.0*	CH_2	1.29 (m)
7	25.1	CH_2	1.31 (m)	25.1	CH_2	1.34 (m)
8	34.5	CH_2	1.58, 1.64 (m)	34.5	CH_2	1.53, 1.66 (m)
9	74.8	CH	5.28 (ddd, 7.2, 7.2, 7.5)	74.7	CH	5.23 (m)
10	130.8	CH	5.55 (dd, 7.5, 15.3)	128.6	CH	5.47 (dd, 7.8, 15.3)
11	128.1	CH	6.50 (dd, 15.3,11.1)	133.2	CH	6.20 (dd, 15.3,10.4)
12	127.4	CH	5.94 (dd, 11.1,11.0)	129.2	CH	5.99 (dd, 10.4, 15.0)
13	133.9	CH	5.47 (m)	136.6	CH	5.72 (m)
14	27.8	CH_2	2.17 (m)	32.6	CH_2	2.07 (m)
15	29.22*	CH_2	1.38 (m)	29.1*	CH_2	1.38 (m)
16	31.4	CH_2	1.31 (m)	31.4	CH_2	1.28 (m)
17	22.5	CH_2	1.31 (m)	22.5	CH_2	1.29 (m)
18	14.0	CH_3	0.89 (t, 7.0)	14.0	CH_3	0.88 (t, 7.3)
9-O$\underline{C}$OCH_3	170.5	C		170.5	C	
9-OCO$\underline{C}H_3$	21.4	CH_3	2.05 (s)	21.4	CH_3	2.04 (s)

* interchangeable among one another

Table 2. Diameter of inhibition zone by positive control (ampicillin) and compounds **1-5** against *Clavibacter michiganensis*.

μmol/paper disc[a]	Diameter of inhibition zone (mm)					
	ampicillin	**1**	**2**	**3**	**4**	**5**
0.1	26	10	18	16	14	16
0.05	22	9	16	11	11	14
0.01	9	na [b]	11	9	na	10

[a] paper disc (8 mm in diameter)
[b] no activity

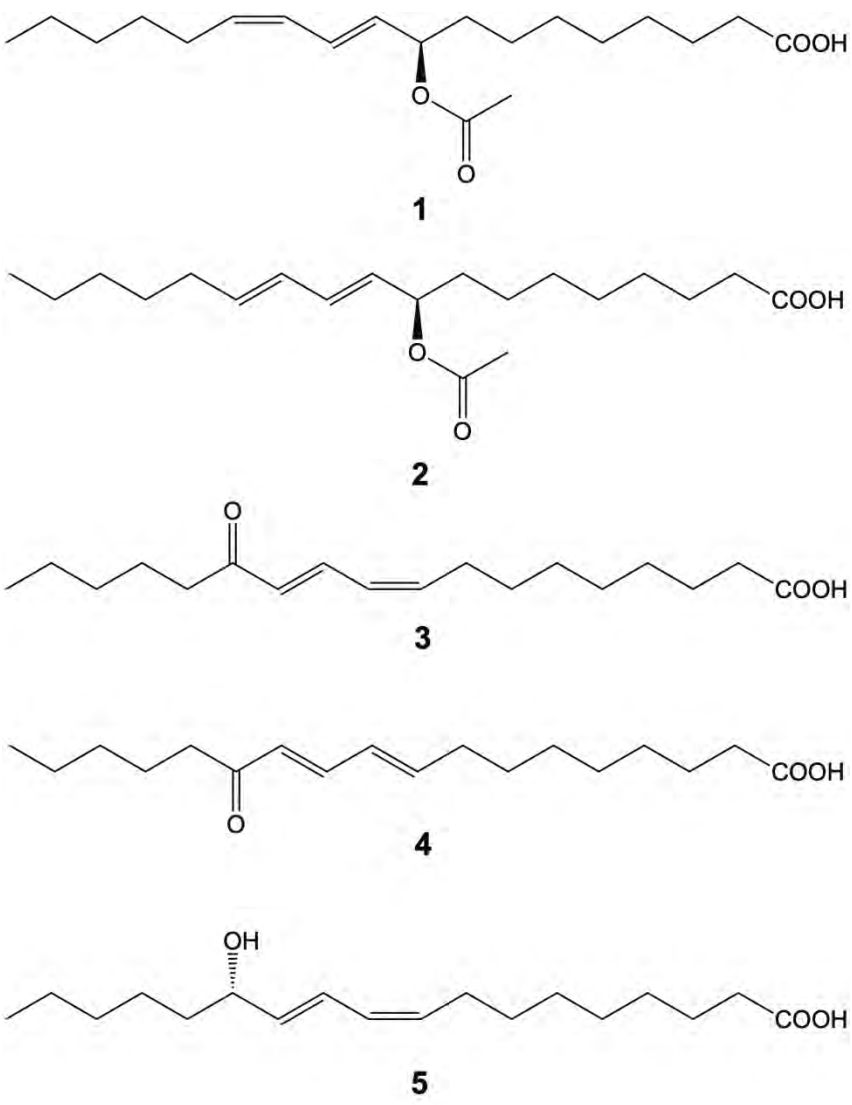

Fig. 1 Wang *et al.*

Figure 1

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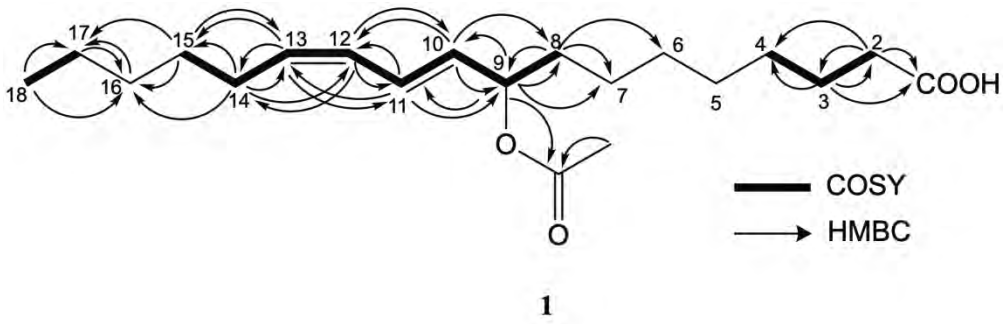

1

Fig. 2 Wang *et al*.

Figure 2

174x78mm (300 x 300 DPI)

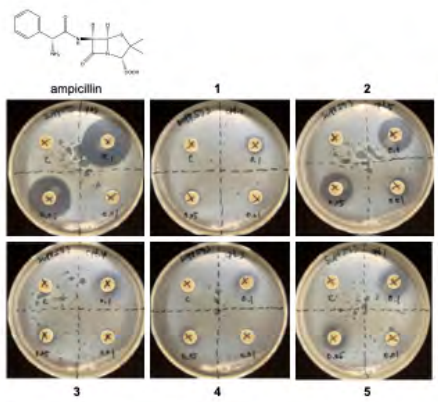

Fig. 3 Wang *et al.*

Figure 3

338x190mm (54 x 54 DPI)

Supplementary Material

REGULAR PAPER

Anti-phytopathogenic bacterial fatty acids from the mycelia of the edible mushroom *Agaricus blazei*

Junhong Wang,[1] Jing Wu,[2] Ryuhei Ogura,[2] Hajime Kobori,[3] Jae-Hoon Choi,[1,2,4] Hirofumi Hirai,[1,2,4] Yuichi Takikawa,[1,2] and Hirokazu Kawagishi[2]*

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List of Supplementary Material

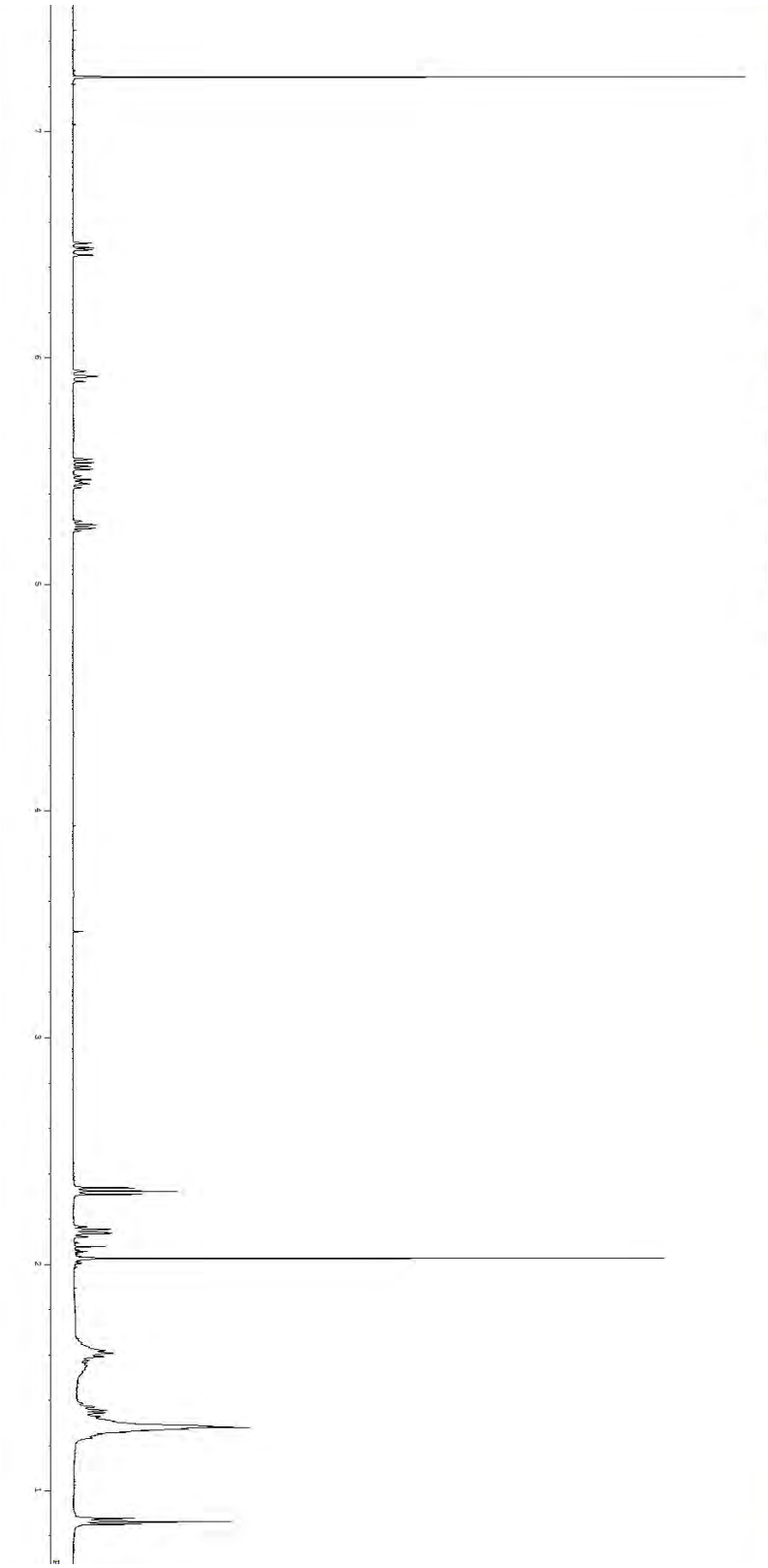


Figure S1. ^{1}H NMR spectrum of **1** ($CDCl_3$, 500 MHz).

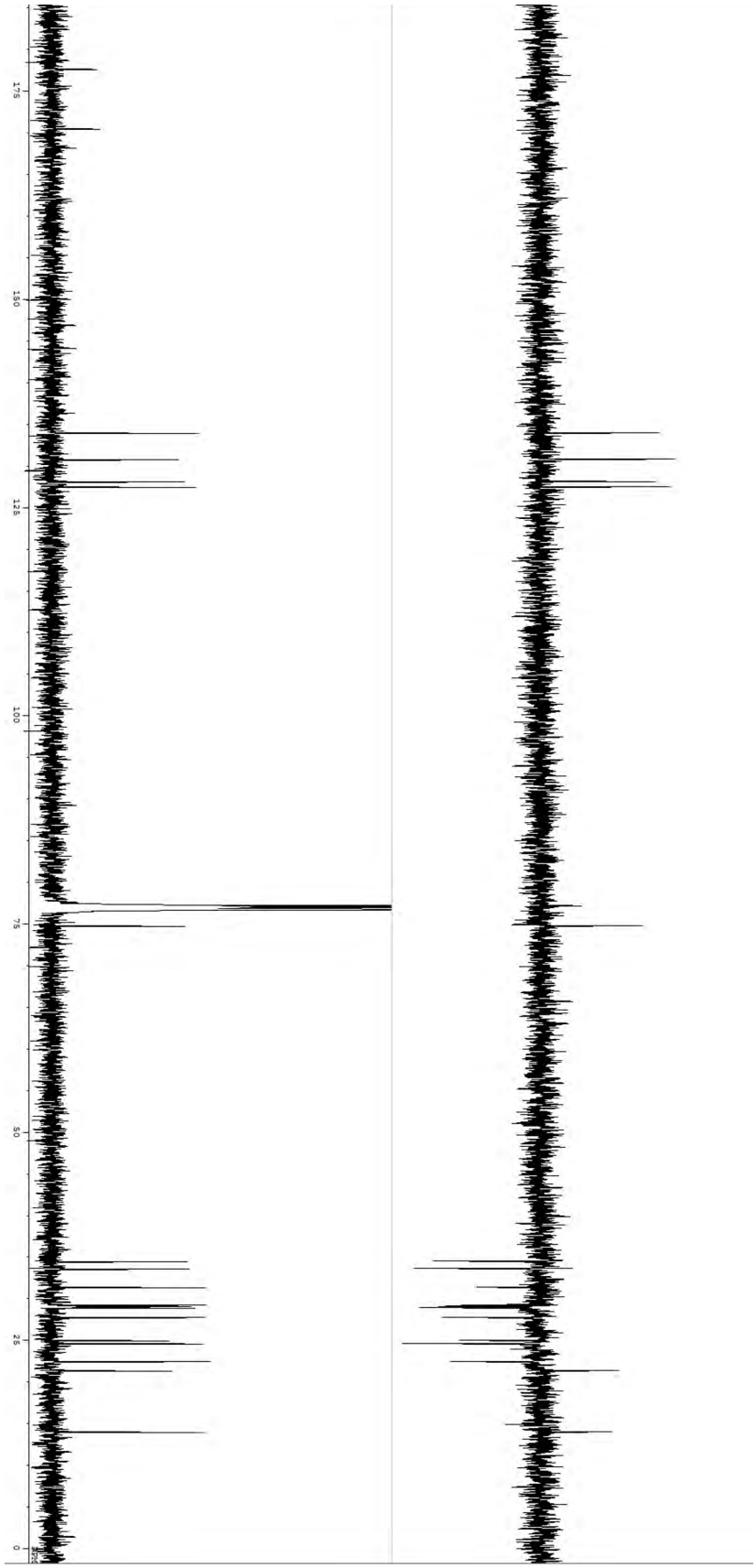


Figure S2. ^{13}C NMR and DEPT spectra of **1** ($CDCl_3$, 125 MHz).

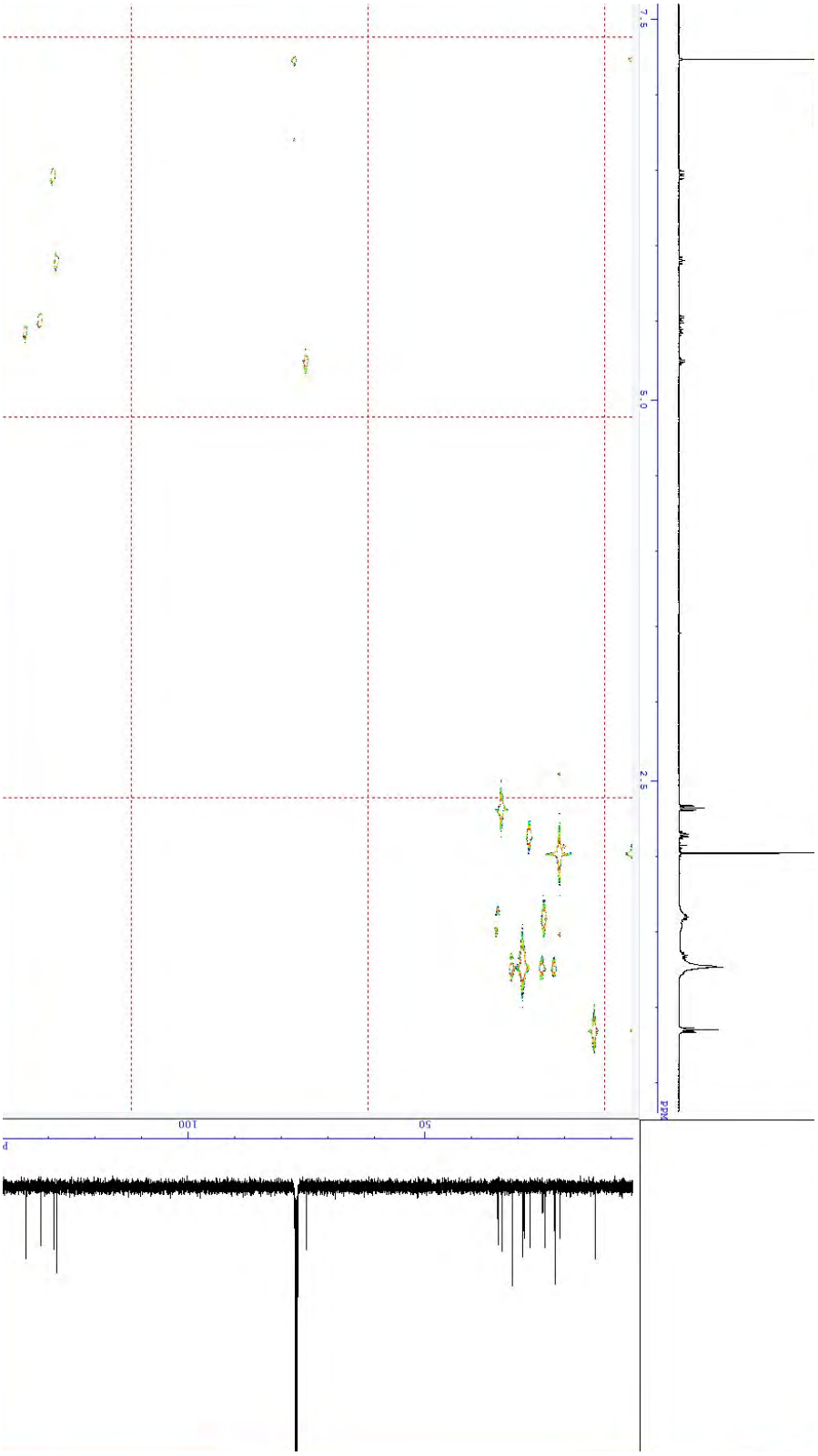


Figure S3. HMQC spectrum of **1** ($CDCl_3$).

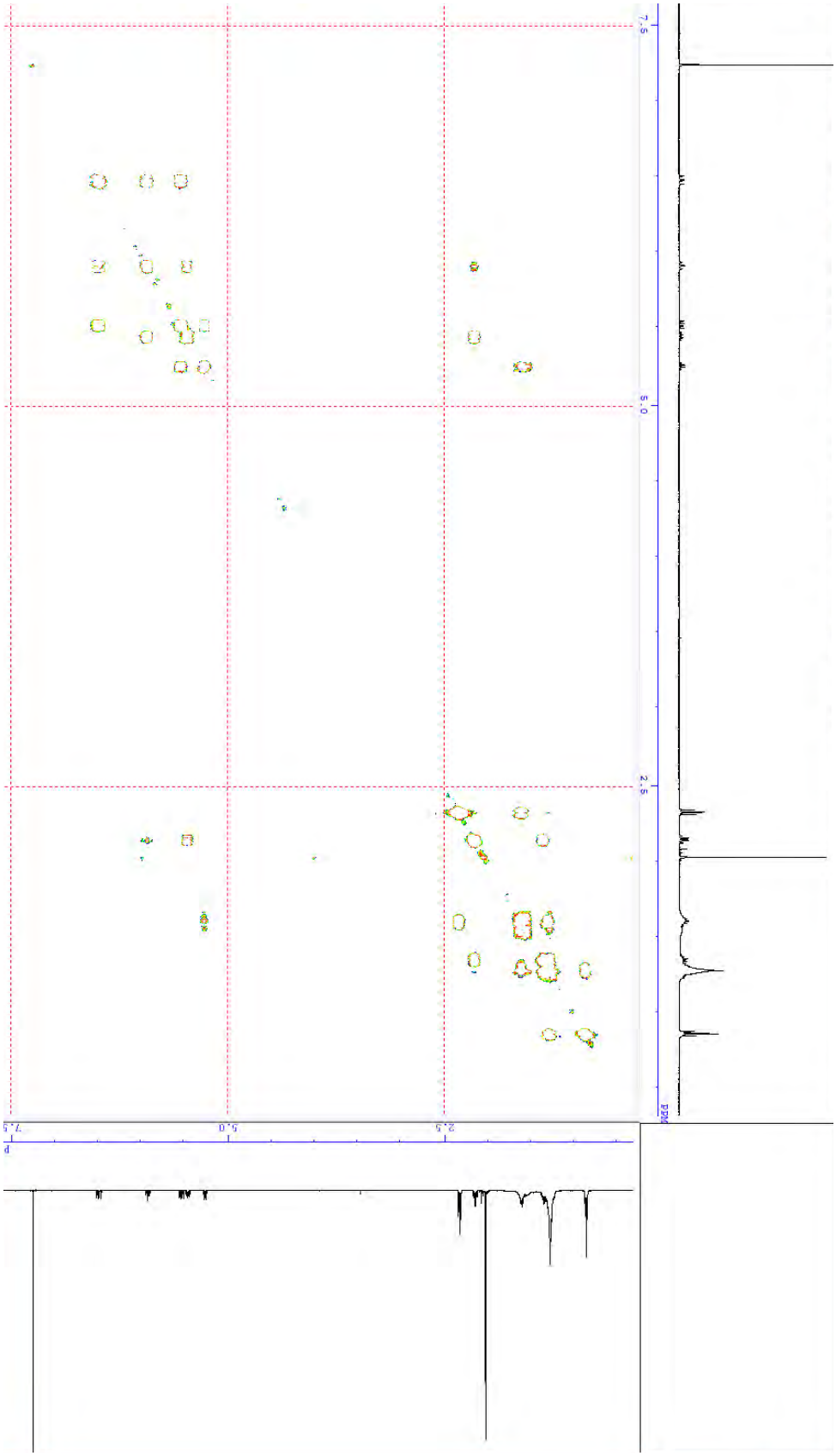


Figure S4. ^{1}H-^{1}H COSY spectrum of **1** ($CDCl_3$).

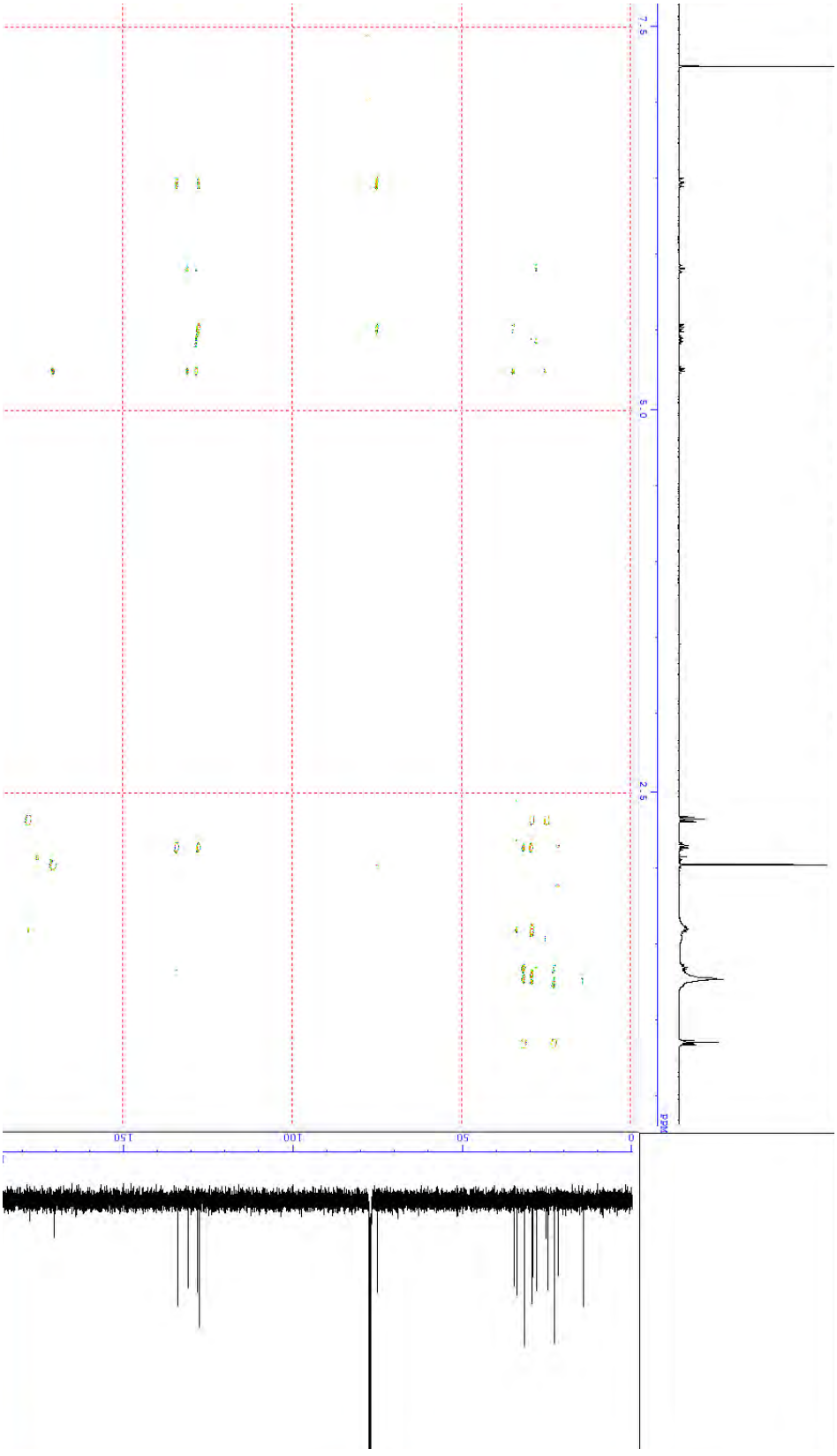
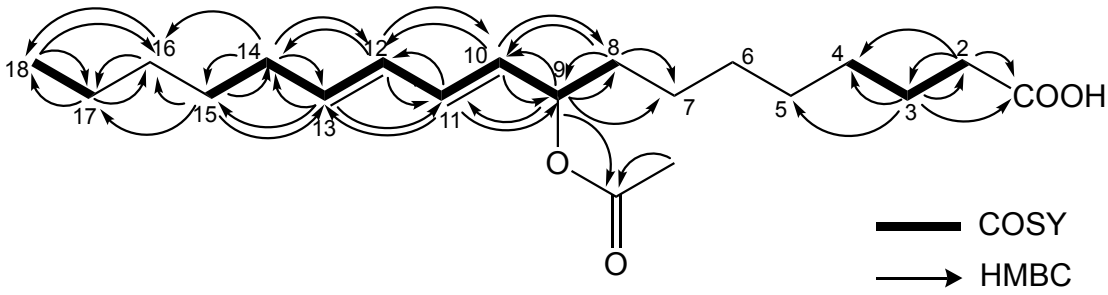


Figure S5. HMBC spectrum of **1** ($CDCl_3$).



2

Figure S6. $^{1}H-^{1}H$ COSY and HMBC correlations for **2**.

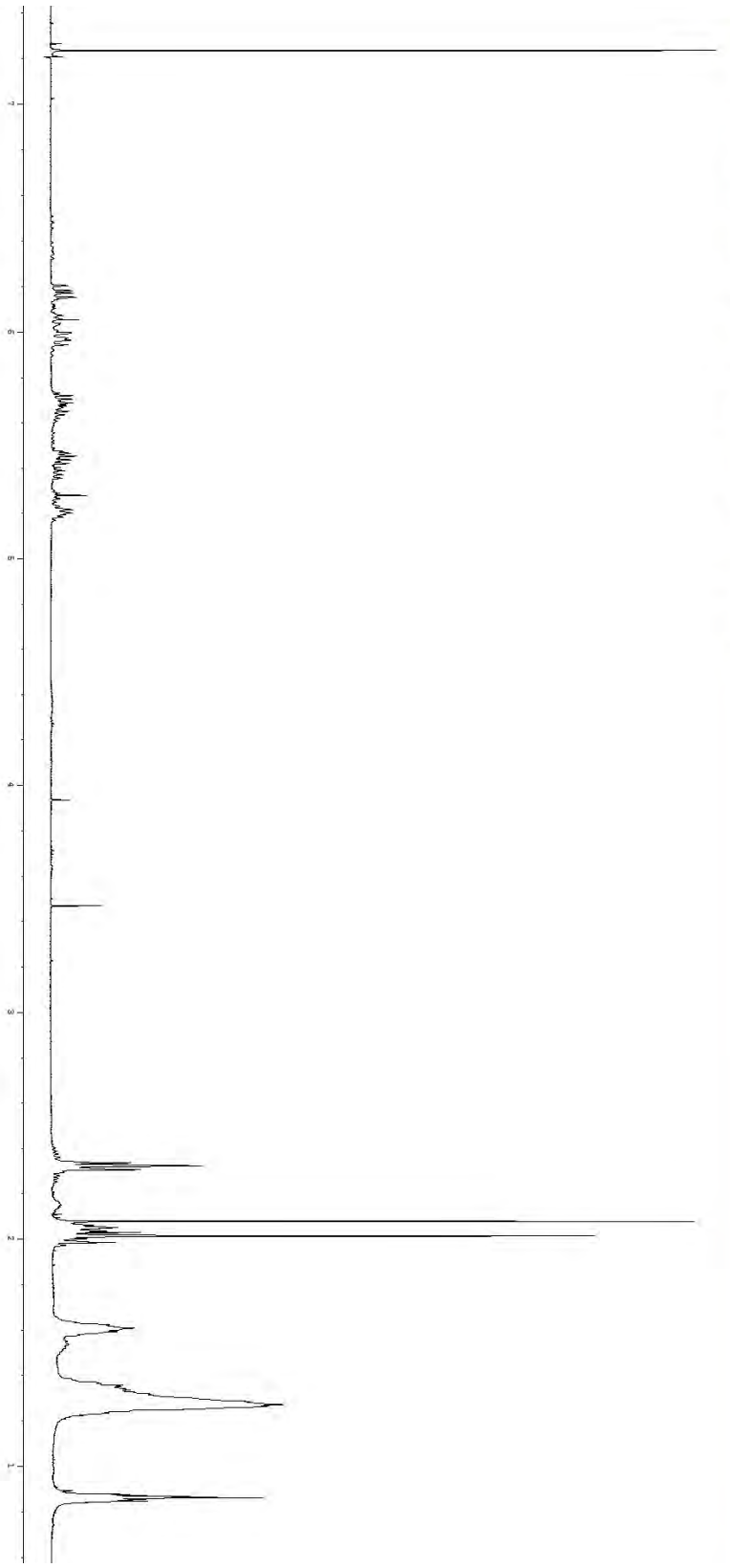


Figure S7. ^{1}H NMR spectrum of **2** ($CDCl_3$, 500 MHz).

Figure S8. ^{13}C NMR and DEPT spectra of **2** ($CDCl_3$, 125 MHz).

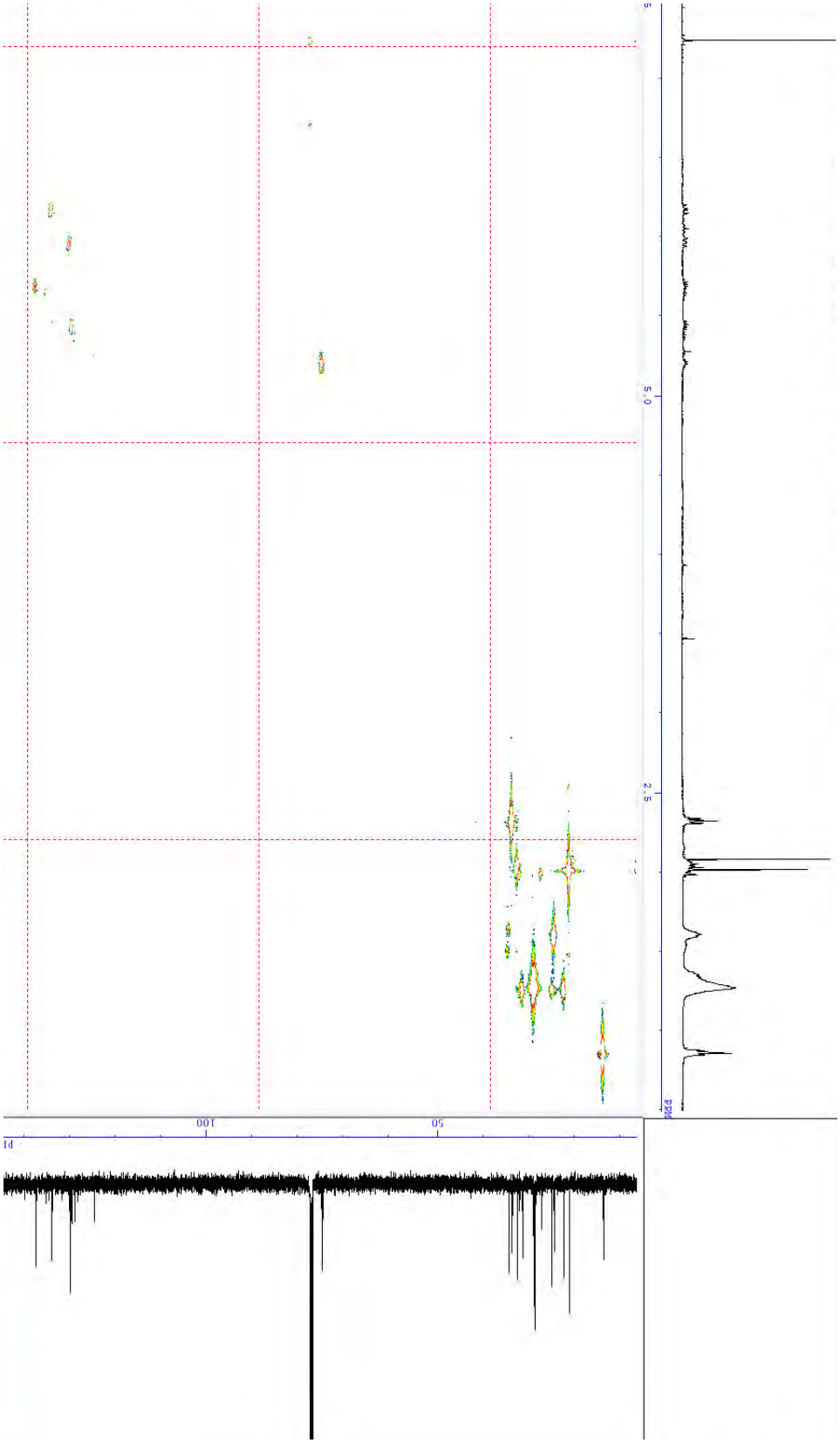


Figure S9. HMQC spectrum of **2** ($CDCl_3$).

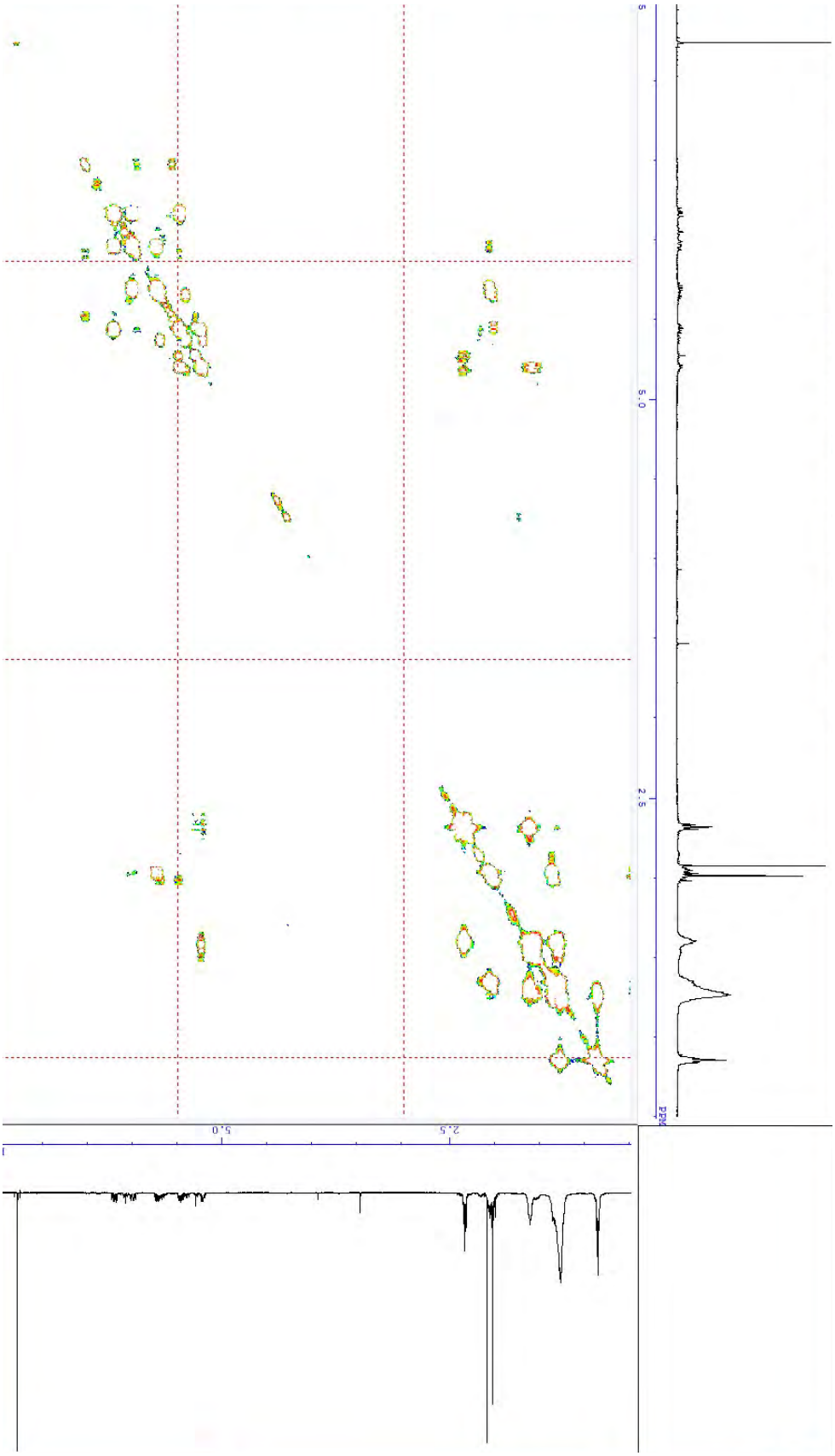


Figure S10. ^{1}H-^{1}H COSY spectrum of **2** ($CDCl_3$).

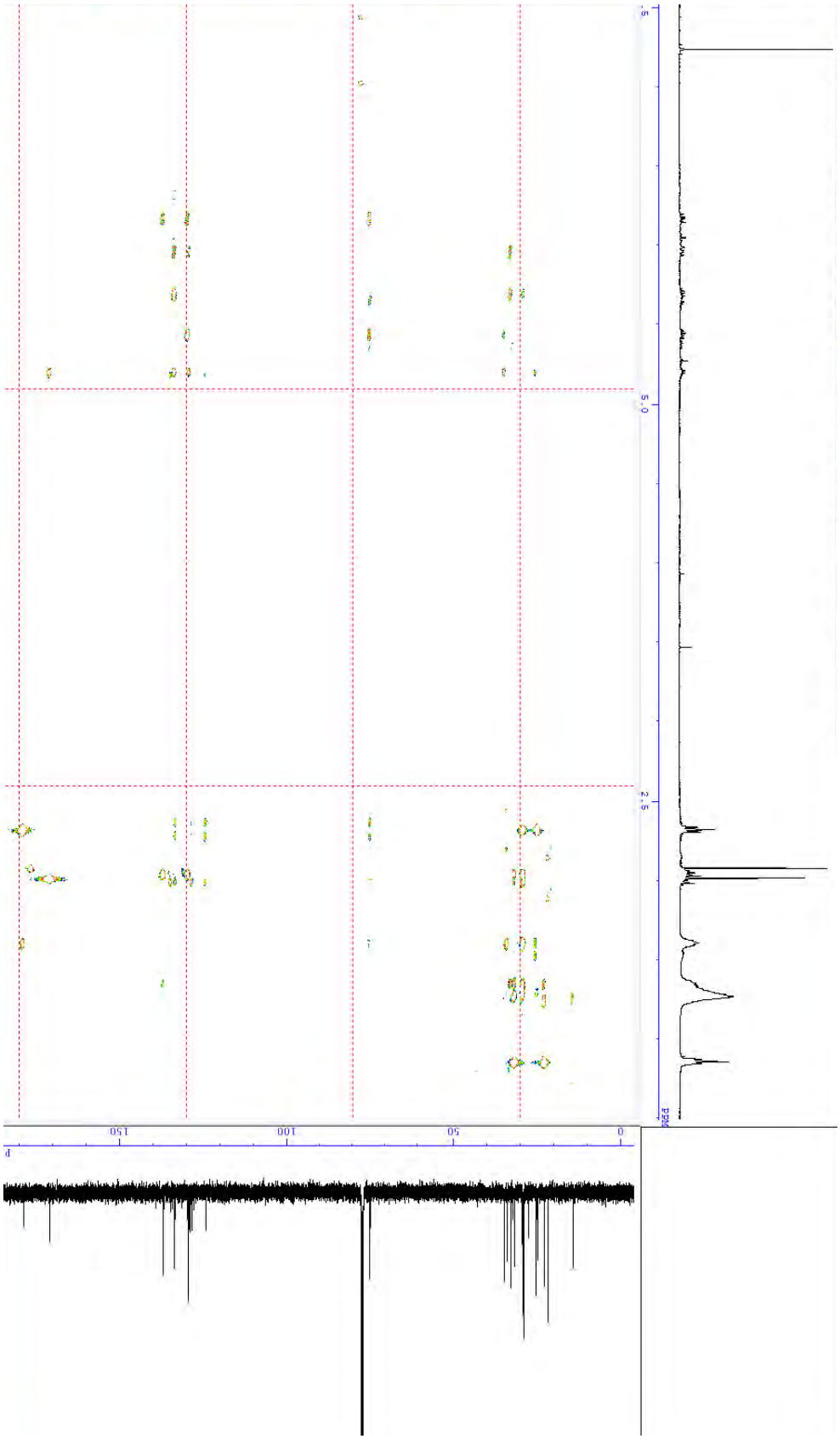


Figure S11. HMBC spectrum of **2** ($CDCl_3$).

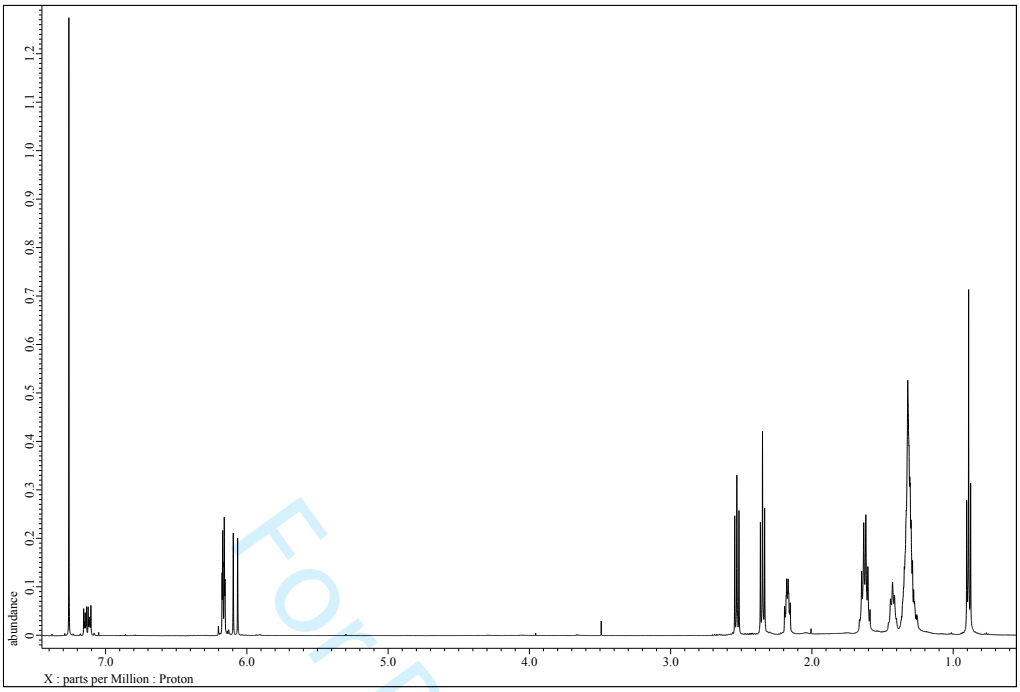


Figure S12. ^{1}H NMR spectrum of **3** ($CDCl_3$, 500 MHz).

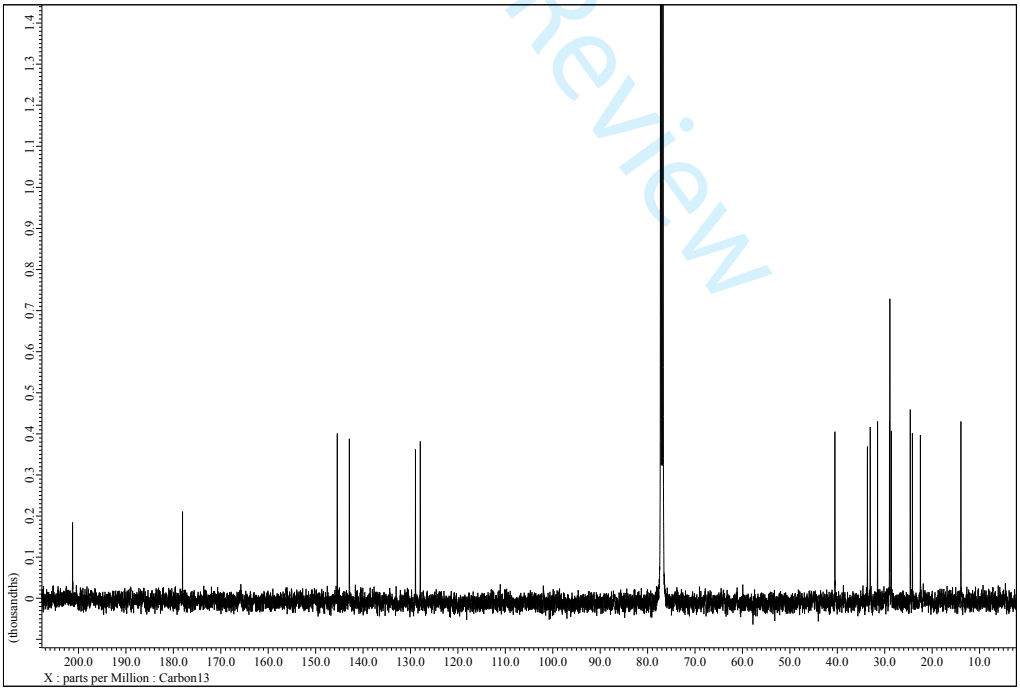


Figure S13. ^{13}C NMR spectrum of **3** ($CDCl_3$, 125 MHz).

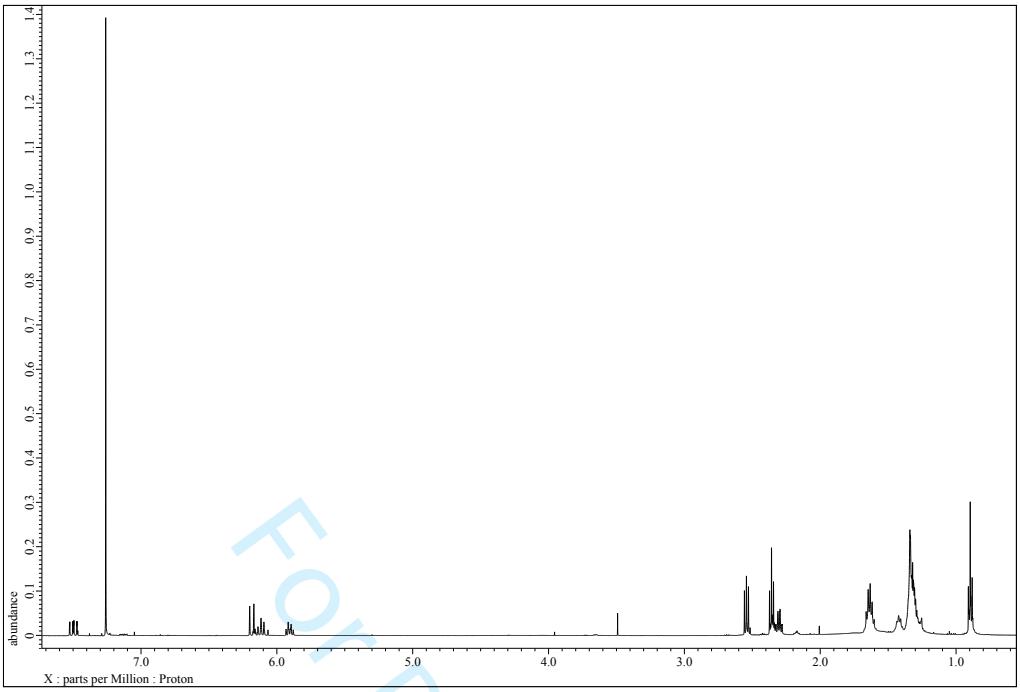


Figure S14. ^{1}H NMR spectrum of **4** ($CDCl_3$, 500 MHz).

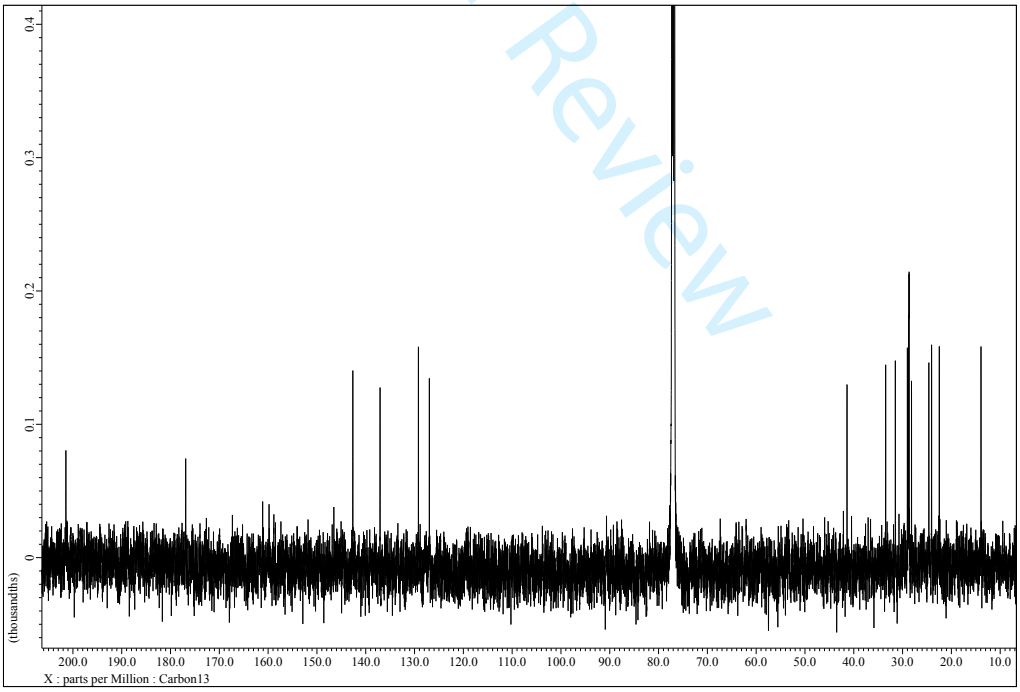


Figure S15. ^{13}C NMR spectrum of **4** ($CDCl_3$, 125 MHz).

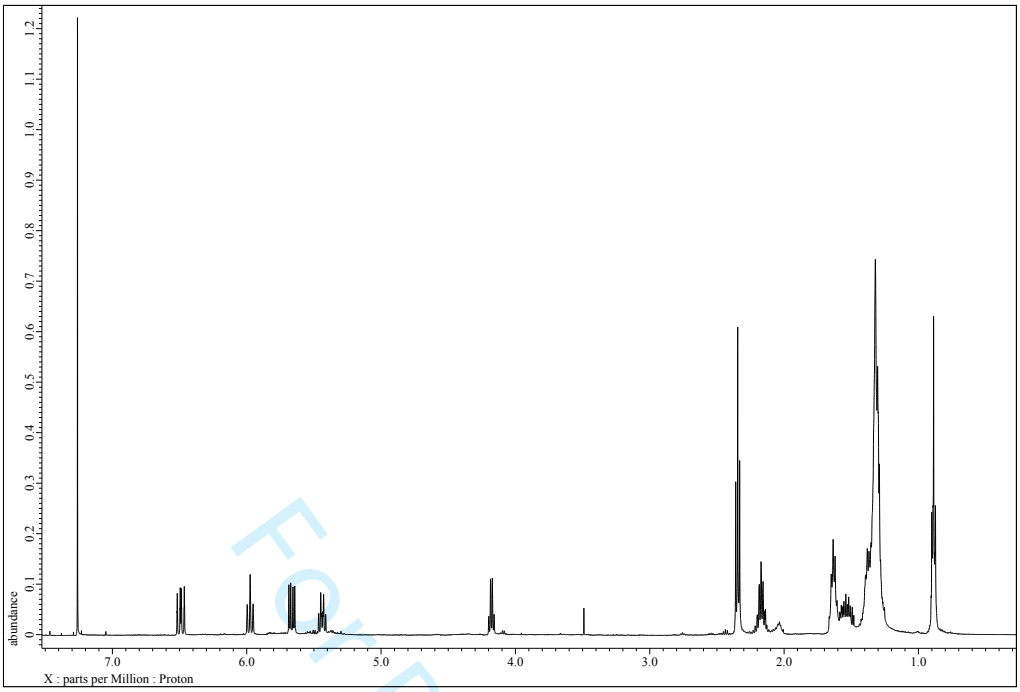


Figure S16. ^{1}H NMR spectrum of **5** ($CDCl_3$, 500 MHz).

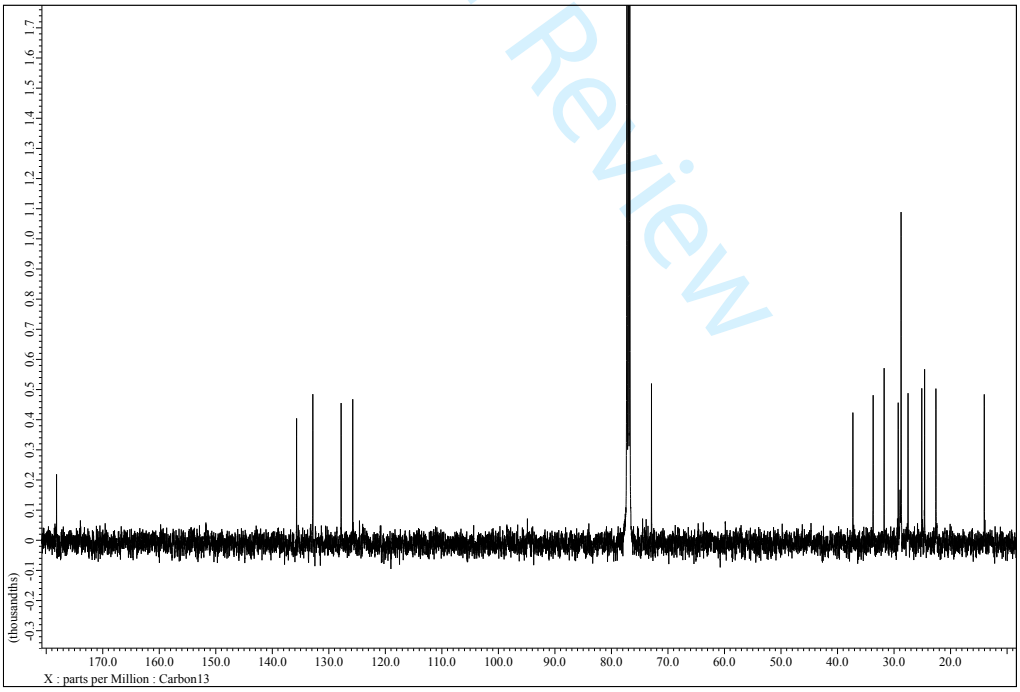


Figure S17. ^{13}C NMR spectrum of **5** ($CDCl_3$, 125 MHz).

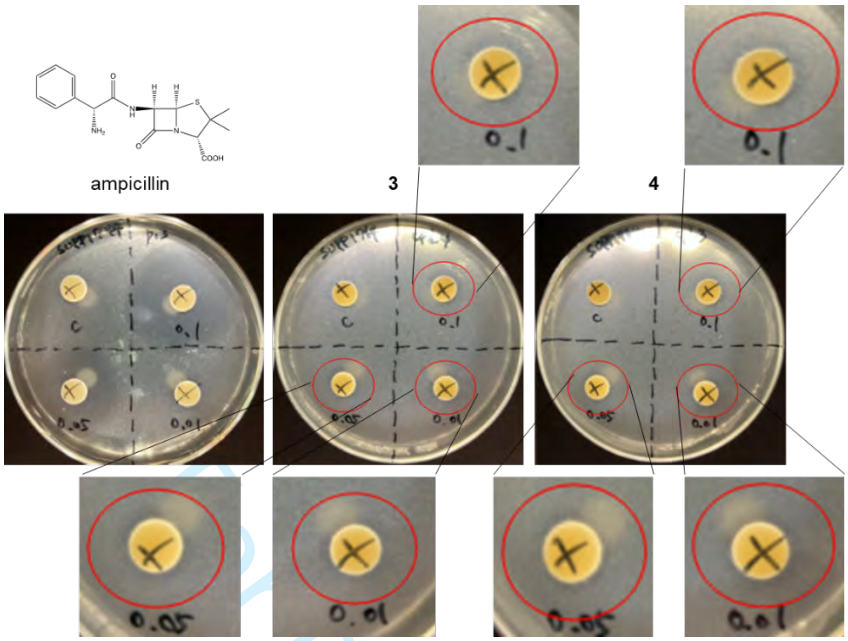
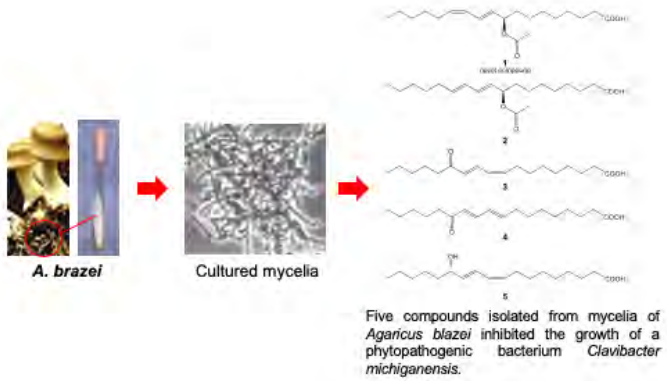


Figure S18. Inhibitory activity of **3**, **4** against *Burkholderia glumae* (positive control, ampicillin).

Table S1. Diameter of inhibition zone by positive control (ampicillin) and compounds **3**, **4** against *Burkholderia glumae*.

μmol/paper disc*	Diameter of inhibition zone (mm)		
	ampicillin	**3**	**4**
0.1	36	12	11
0.05	28	10	10
0.01	12	9	9

*paper disc (8 mm in diameter)



Graphical Abstract

338x190mm (54 x 54 DPI)