Bioorganic Chemical Studies on Wild Edible Mushrooms

THESIS

Bioorganic Chemical Studies on Wild Edible Mushrooms

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Author

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Abstract

Wild edible mushrooms are widely accepted as food and generally consumed worldwide as medicinal products. The chemical investigation of fruiting bodies as well as mycelia of higher fungi to discover new compounds along with their bioactivities is carried out extensively. Search for bioactive metabolites from three kinds of mushrooms *Leucopaxillus giganteus*, *Stropharia rugosoannulata*, and *Entoloma clypeatum* were carried out.

1) Bioorganic chemical studies on wild edible mushroom *Leucopaxillus giganteus*

Evaluation of chemical constituents and nutritional values of *Leucopaxillus giganteus* have been considerably studied. In this study, the chemical exploration of mushroom *L. giganteus* was performed along with Axl and immune checkpoint suppression activities and plant growth regulating activity. Fresh fruiting bodies of *L. giganteus* was extracted with EtOH and then acetone to obtain the crude extracts. The partition method divided them into *n*-hexane, EtOAc, and EtOH soluble parts. The EtOAc soluble part was subjected to silica gel flash column chromatography, which was further purified by HPLC. Structural identification of each compounds was performed by the interpretation of spectroscopic data analysis. As a result, three compounds were isolated and identified as ethyl 2-(2-oxopyrrolidin-1-yl)acetate (**1**), monoethyl succinate (**2**), and 4-ethoxy-2-hydroxy-4-oxobutanoic acid (**3**).

Furthermore, the absolute configuration of a malic acid derivative (**3**) that was previously isolated from the mushroom was determined. In this study, we synthesized stereoisomer of **3** (**4**) to determine the absolute configuration and also other two analogs (**5** and **6**) to study the structure activity relationship. As a result, compound **3** was determined to be *R*, indicating that it was a novel compound.

All the obtained compounds were evaluated their activities against the Axl and immune checkpoint assays and plant growth regulating activity assay. In the first assay, compounds **1** and **2** inhibited Axl, PD-L1, and PD-L2 expressions. Among malic-acid esters (**3**–**6**), only the isolated compound **3** showed the suppression effect on all the gene expressions. In the second assay, compound **1** showed the promotion effect at 10 and 100 nmol/paper on hypocotyl growth, while **2** showing inhibition activity at 1000 nmol/paper against hypocotyl and root growth. The inhibition activity of the novel compound **3** was the strongest among all the compounds tested. The antipode of **3** (**4**) showed much less activity than **3**.

2) Bioorganic chemical studies on culture broth of *Stropharia rugosoannulata*

Stropharia rugosoannulata is a wild edible mushroom which commonly called Saketsubatake in Japanese or wine-cap stropharia in English, and it belongs to the

Strophariaceae family. As an edible mushroom, *S. rugosoannulata* has some functional-food constituents possessing some pharmaceutical properties. As a continuous study, the chemical exploration of the culture broth of *S. rugosoannulata* was conducted along with the evaluation of the antibacterial activity and plant growth regulating activity.

S. rugosoannulata mycelia were cultured for 30 days. Afterwards, the mycelia were separated by filtration to obtain the culture broth, then concentrated under reduced pressure. The concentrated filtrate was partitioned into *n*-hexane, EtOAc, EtOH, and H_2O soluble parts. The EtOAc soluble part was subjected to silica gel flash column chromatography, which was further purified by HPLC. Structural determination of each compounds was performed by interpretation of IR, MS and NMR data. As a result, nine compounds were isolated from the culture broth of *S. rugosoannulata* and identified as (*S*)-4-(hydroxymethyl)-3,4-dihydroquinolin-2(1H)-one (**7**), acetyl-*D*-phenylalanine (**8**), quinoline-4-carboxaldehyde (**9**), quinoline-4-carboxylic acid (**10**), (*R*)-1-phenylethane-1,2-diol (**11**), (1*R*,2*S*)-1-phenylpropane-1,2- diol (**12**), (*R*)-5-((*S*)-1-hydroxyethyl)dihydrofuran-2(3H)-one (**13**), 4-(2-hydroxyethyl)phenol (**14**), and 3,5-dichloro-4-methoxybenzoic acid (**15**). All the compounds showed no antibacterial activity against *Clavibacter michiganensis, Pectobacterium carotovorum,* and *Burkholderia glumae.* However, compound **10** showed the strongest inhibition towards lettuce growth on the plant growth regulation assay.

3) Bioorganic chemical studies on wild edible mushroom *Entoloma clypeatum*

Entoloma clypeatum is known as Harushimeji in Japanese and belongs to *Entolomataceae* family. This wild edible mushroom has been found as a good source of proteins and total carbohydrates with low fat contents. However, there is no report about isolation of bioactive compounds from the fungus. In this study, the chemical exploration of the fresh fruiting bodies of *E. clypeatum* was carried out.

Fresh fruiting bodies of *E. clypeatum* were extracted with EtOH and then acetone to obtain the crude extracts. The partition method divided them into n-hexane, EtOAc, and EtOH soluble parts. The EtOAc soluble part was subjected to silica gel flash column chromatography, which was further purified by HPLC. Structural determination of each compounds was performed by interpretation of MS and NMR data. As a result, six known compounds were isolated and identified as (*R*,*E*)-4-hydroxynon-2-enoic acid (**16**), 9-methoxy-9-oxononanoic acid (**17**) 4-oxononanoic acid (**18**), methyl benzoate (**19**), Cyclo(*D*-Pro-*L*-val) (**20**), and nicotinic acid (**21**). In the plant growth regulating activity assay, compound **16** showed strong promotion activity towards root growth, as well as compound **21** strongly inhibited hypocotyl growth.

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Chapter 1

Introduction

Wild edible mushrooms are widely accepted as food and generally consumed worldwide as medicinal products. Research into chemical composition and biological properties of the wild mushrooms has been widely published. It was previously mentioned that the wild mushrooms are rich sources of protein and carbohydrates, and had low amounts of fat.[1] It was also proved that they contain very useful phytochemicals such as phenolics, tocopherols, ascorbic acid, and carotenoids.[2] Other than that, the phenolic and polysaccharidic fractions of wild edible mushrooms showed the free radical scavenging and antioxidant activity, respectively.[3] Therefore, they are becoming essential in the human diet for their nutritional and pharmacological roles. The effort to find secondary metabolites with significant activity has attracted attention of scientist.

The chemical investigation of fruiting bodies as well as mycelia of higher fungi to elucidate new compounds along with their bioactivities is carried out extensively. The search for bioactive compounds from the fresh fruiting bodies of *Tricholoma flavovirens* resulted in the isolation of the plant growth regulators; 5,7-dimethoxy-2,4-dimethylindole, 5-methoxy-2,4-dimethylindole, and 7-acetamidophthalide.[4] Moreover, 10-dehydroxymelleolide D and 13-hydroxymelleolide K were isolated from the culture broth of *Armilaria* sp. found as plant growth regulators as well.[5] In addition, *Bombyx mori* inoculated with *Cordyceps militaris* produced 3′-deoxynisine and cordycepin that exhibited the toxicity activities against A549, PANC-1, and MCF-7 cancer cells.[6] To find other secondary metabolites, the isolation from three kinds of mushrooms *Leucopaxillus giganteus*, *Stropharia rugosoannulata*, and *Entoloma clypeatum* were carried out.

L. giganteus is a wild edible mushroom which belongs to *Tricholomataceae* Family commonly called giant funnel mushroom in English or Ooichotake in Japanese. This saprophyte mushroom lives in grassland, fields and meadows, and it may produce fairy rings.[7] *L. giganteus* contains high phenolics, ascorbic acid, β-carotene and lycopene that are correlated with antioxidant properties.[8] Additionally, galactomannoglucans from this mushroom have been introduced to work in immunomodulating and antitumor activities.[9] Literature has shown that *L. giganteus* extracts also have antimicrobial activity by inhibiting the growth of *Escherichia coli* and *Proteus mirabilis*, and being bactericide for *Pasteurella multocida*, *Streptococcus pyogenes*, and *Streptococcus agalactiae*.[10] This mushroom has been known to produce a unique low-molecular compound called clitocine which has diverse biological activities such as inhibitory activity against adenosine kinase, insecticidal effect, and antitumor activities.[11-13] The most research proved it as a potent anticancer agent by activating caspase-3, -8, -9, knocking-down of Mcl-1, and inhibiting transcription factor NF-kB.[14-16]

S. rugosoannulata is a wild edible mushroom which commonly called Saketsubatake in Japanese or wine-cap stropharia in English, and it belongs to the *Strophariaceae* family. This mushroom is widespread in northern temperate zones throughout the world. This mushroom is cultivated and widely consumed in the world due to pharmacological activities. *S. rugosoannulata* contains antioxidant polysaccharides against radicals. It is indicated that *S. rugosoannulata* is a valuable source for the utilization of polysaccharides as potential pharmaceuticals.[17] As an edible mushroom, *S. rugosoannulata* has some functional-food constituents possess some pharmaceutical properties, such as anti-fungal activity and thapsigargin toxicity inhibition.[18] This mushroom contains potent suppression compounds for the formation of osteoclast which indicated the use of *S. rugosoannulata* as osteoporosis treatment. Previously, four novel compounds have been isolated from the fruiting bodies of *S. rugosoannulata,* Strophastereols A to D, with ER-stress suppression and anti MRSA activities.[19] Two novel sterols were also discovered from the hexane-soluble of the fresh fruiting bodies.[20] In addition, three compounds have been isolated from hexane-soluble and found to exhibit the inhibition of plant growth activities.[21]

E. clypeatum is known as Harushimeji in Japanese and belongs to *Entolomataceae* family. This wild edible mushroom has been found as a good source of proteins and total carbohydrates with low fat contents. It has been shown to have high monosodium glutamate (MSG) and 5'-nucleotides levels and possess highly umami taste.[22] This entolomatoid fungus has been recorded in the vicinity of Rosaceous and Ulmaceae plants around the spring. Although, this mushroom exhibited slow and unsteady growth, it was successfully isolated via basidiospore isolation.[22] The morphologically uniqueness of the mushroom has been arousing researchers' interest to describe the morphological characters and isolate the pure culture to determine the contribution of this fungal species associated with rosaceous plants.[24, 25] However, there is no report about isolation of bioactive compounds from the fungus.

Lung cancer is the leading cause of cancer morbidity and mortality worldwide. For this reason, molecular targeted drug discovery and drug discovery development against lung cancer are proceeding actively all over the world. Axl, a member of receptor tyrosine kinases (RTKs), has been designated as a strong candidate for targeted therapy of cancer.[26] Similarly, immune-checkpoint (PD-1, PD-L1, and PD-L2) blockade has triggered a clinical reaction of people with lung cancer in latest clinical studies.[27] There is ongoing research to develop possible drugs to target these signaling pathway (Axl and immune checkpoints) and treat cancers. Isolation of other therapy agents of cancer from natural sources is one of the possible contributions for drug discovery. To develop anticancer agents targeting these

signaling pathways (Axl and immune checkpoints), we tried to isolate the potential Axl and immune checkpoints inhibitors from the mushroom.

In this study, the chemical exploration of three kinds of mushrooms *L. giganteus*, *S. rugosoannulata*, and *E. clypeatum* was performed. A number of bioactive compounds were identified by interpretation of spectra data. Moreover, their biological activities were evaluated on Axl and immune checkpoint assay, plant growth regulating assay, and antibacterial assay. Herein, the isolation and structural identification along with biological activities of the compounds are described.

Chapter 2

Bioorganic chemical studies on wild edible mushroom *Leucopaxillus giganteus*

Section 1 Isolation of bioactive compounds from *Leucopaxillus giganteus*

The fresh fruiting bodies were extracted with EtOH and then acetone to obtain the crude extracts. Afterwards, they were combined and concentrated under reduced pressure. The concentrated solutions were partitioned into *n*-hexane, EtOAc, and H_2O soluble parts. Then, the H_2O soluble part was dried and extracted with EtOH (Figure 1).

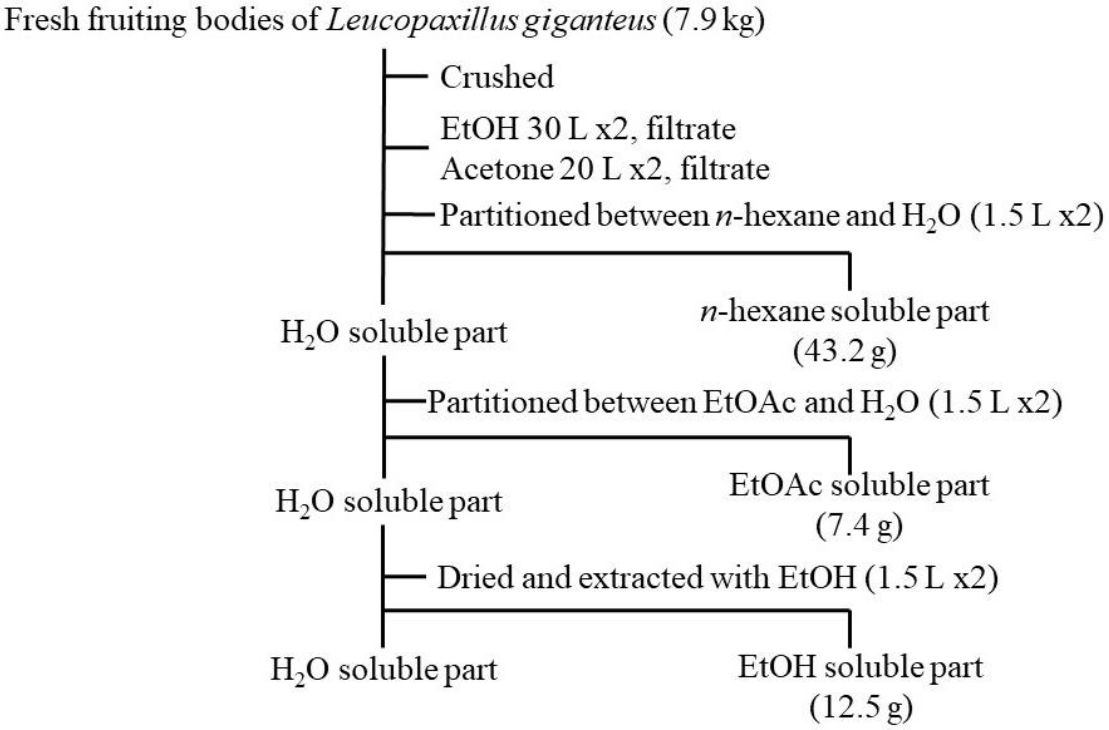


Figure 1 Partition scheme of *L. giganteus* extracts

The EtOAc soluble part was subjected to silica gel flash column chromatography to obtain 12 fractions (1-12). Further purification was conducted by repeated chromatography using flash column chromatography or HPLC. The purification of fraction 4 afforded compounds **1** and **2** (Figure 2).

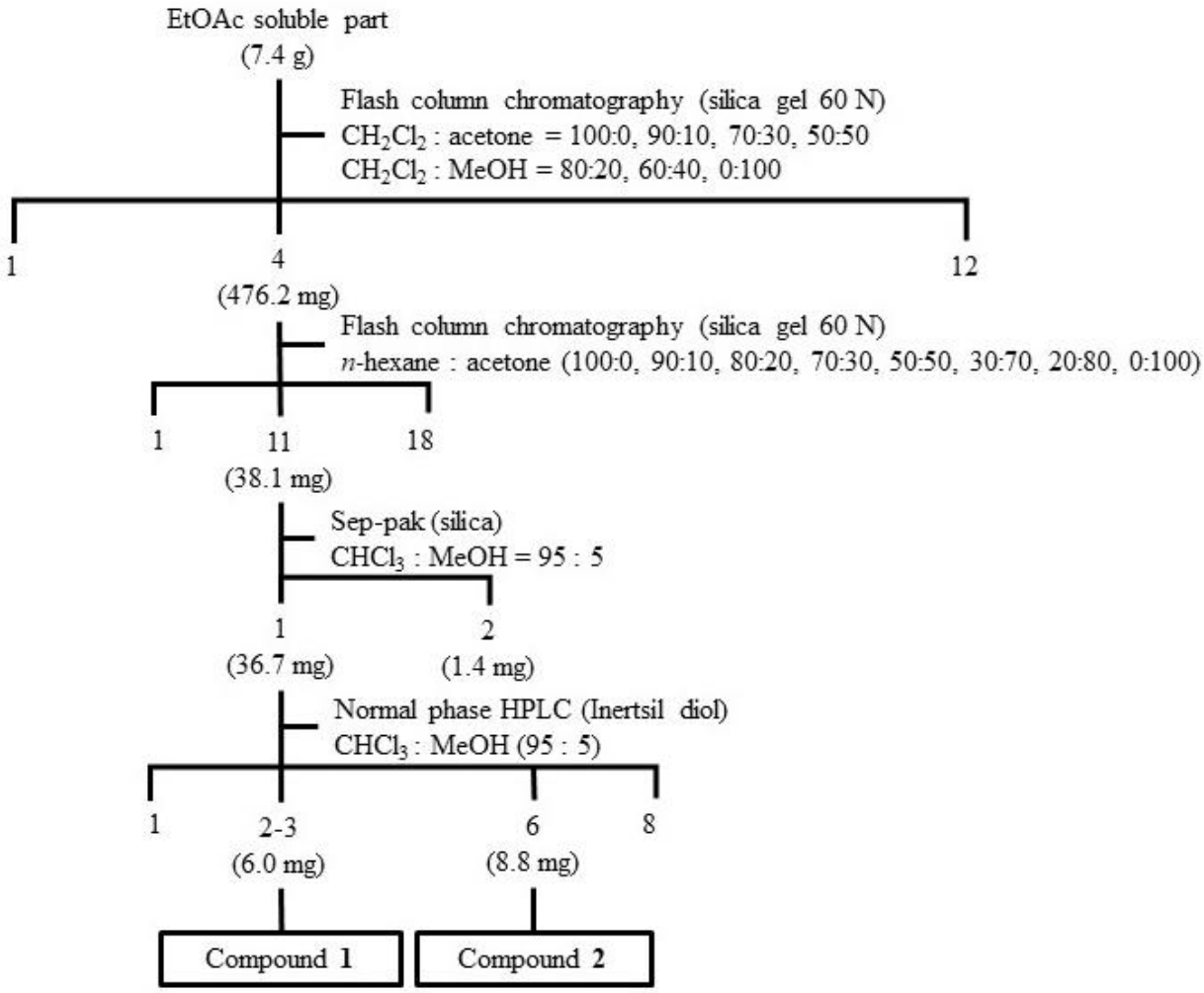


Figure 2 The isolation and purification scheme of compounds from *L. giganteus*

Section 2 Structural analysis of bioactive compounds from *Leucopaxillus giganteus*

2-1 Compound **1**

Compound **1** was isolated as pale yellow oil. Its molecular formula was determined as $C_8H_{13}NO_3$ by HRESIMS *m/z* 172.0999 $[M+H]^+$ (calcd. for $C_8H_{14}NO_3$, 172.0968), indicating the presence of three degrees of unsaturation in the molecule (Figure 4). The planar structure of **1** was elucidated by interpretation of the NMR spectra, including DEPT, COSY, HMBC, and HMQC data. The DEPT experiment indicated the presence of one methyl, five methylenes, and two tetrasubstituted carbons. The presence of pyrrolidine moiety was constructed by COSY correlations (H-2/ H-3; H-3/ H-4) and the HMBC correlations (H-2/C-1, C-3, C-4; H-3/ C-2, C-4; H-4/C-1, C-2, C-3). The ethyl acetate moiety was constructed by a COSY correlation (H-3'/H-4') and the HMBC correlations (H-1'/C-2'; H-3'/C-2', C-4'; H-4'/C-3'). The HMBC correlations (H-1'/C-1, C-4) suggested the ethyl acetate attached on the *N*-site of pyrrolidine (Figures 8 and 9). Compound **1** was identified as ethyl 2-(2-oxopyrrolidin-1-yl)acetate. This compound has been chemically prepared from 2-pyrrolidone and ethyl chloroacetate.[28] It also has been synthesized as a fungicide.[29] However, it was isolated from a natural source for the first time.

Table 1 ^{1}H and ^{13}C NMR assignments for **1**

	Compound **1** ($CDCl_3$)	
Position	δ_C	δ_H (multiplicity, *J* in Hz)
1	175.6	
2	30.3	2.40 (t, *J* = 8.2)
3	17.9	2.07 (m)
4	47.7	3.46 (t, *J* = 7.2)
1'	44.1	4.03 (s)
2'	168.7	
3'	60.4	4.10 (dd, *J* = 14.3, 7.3)
4'	14.2	1.24 (t, *J* = 3.1)

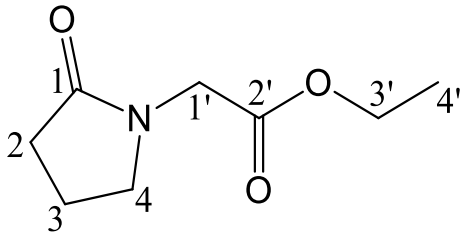


Figure 3 Structure of **1**

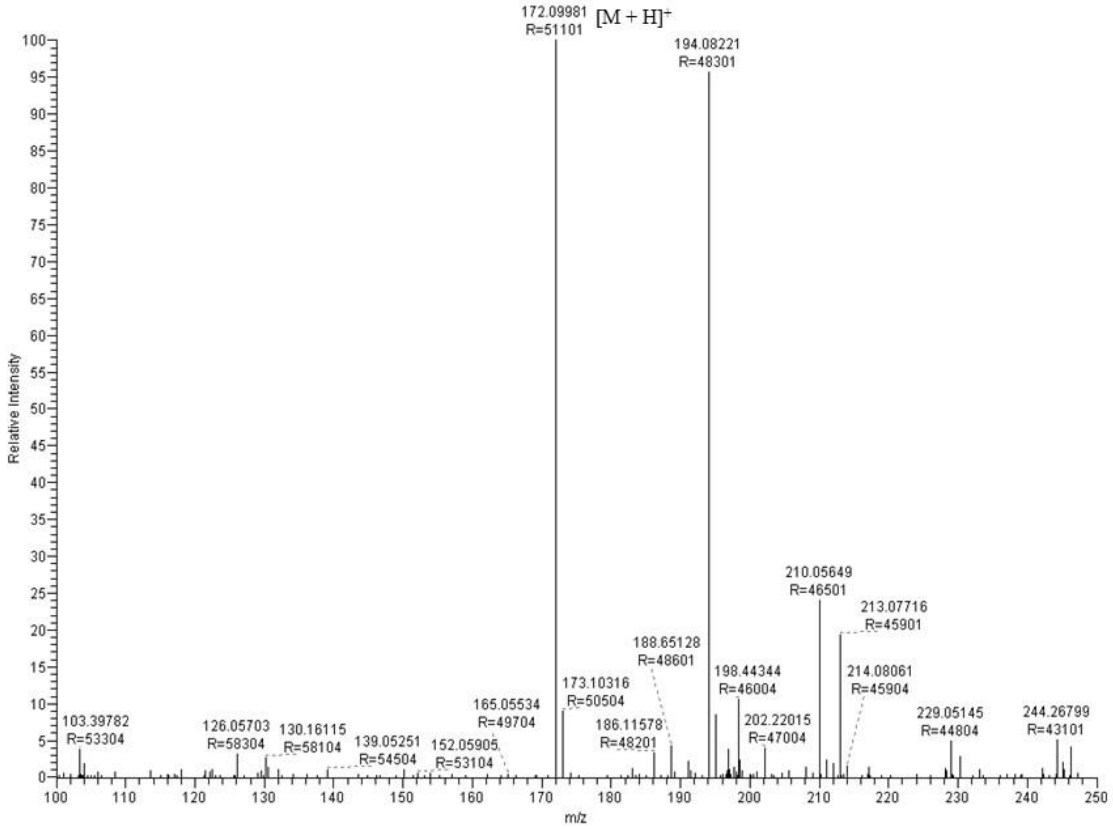


Figure 4 ESIMS spectrum of **1**

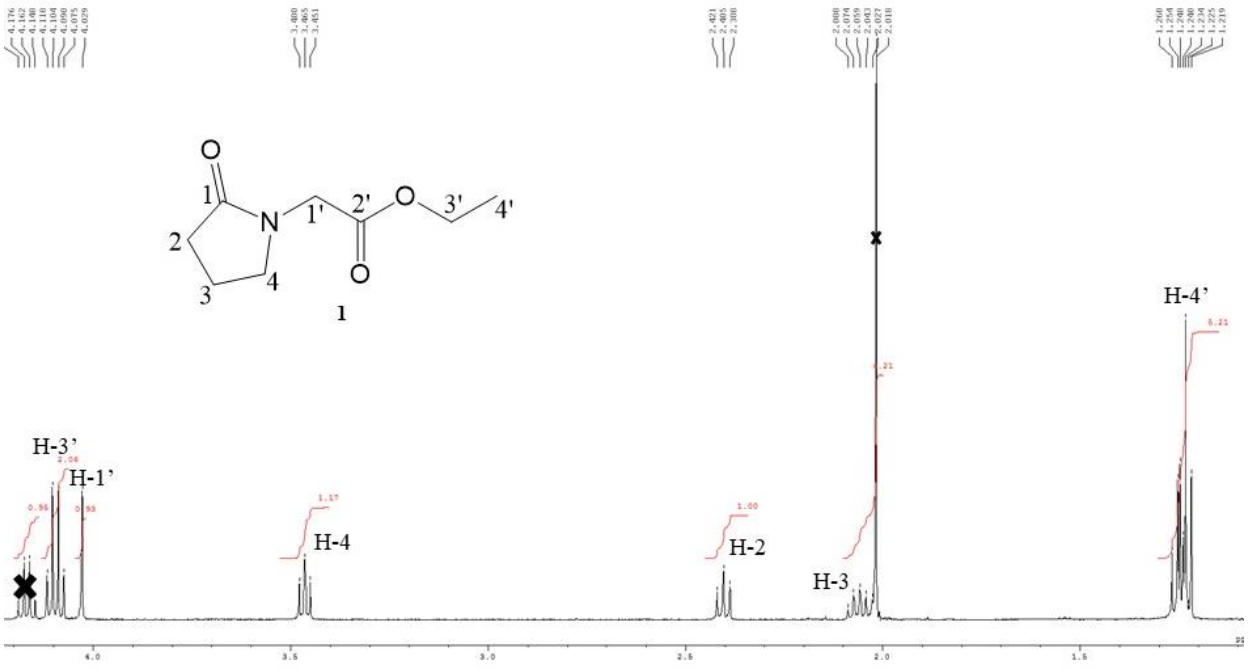


Figure 5 ^{1}H NMR spectrum of **1** ($CDCl_3$)

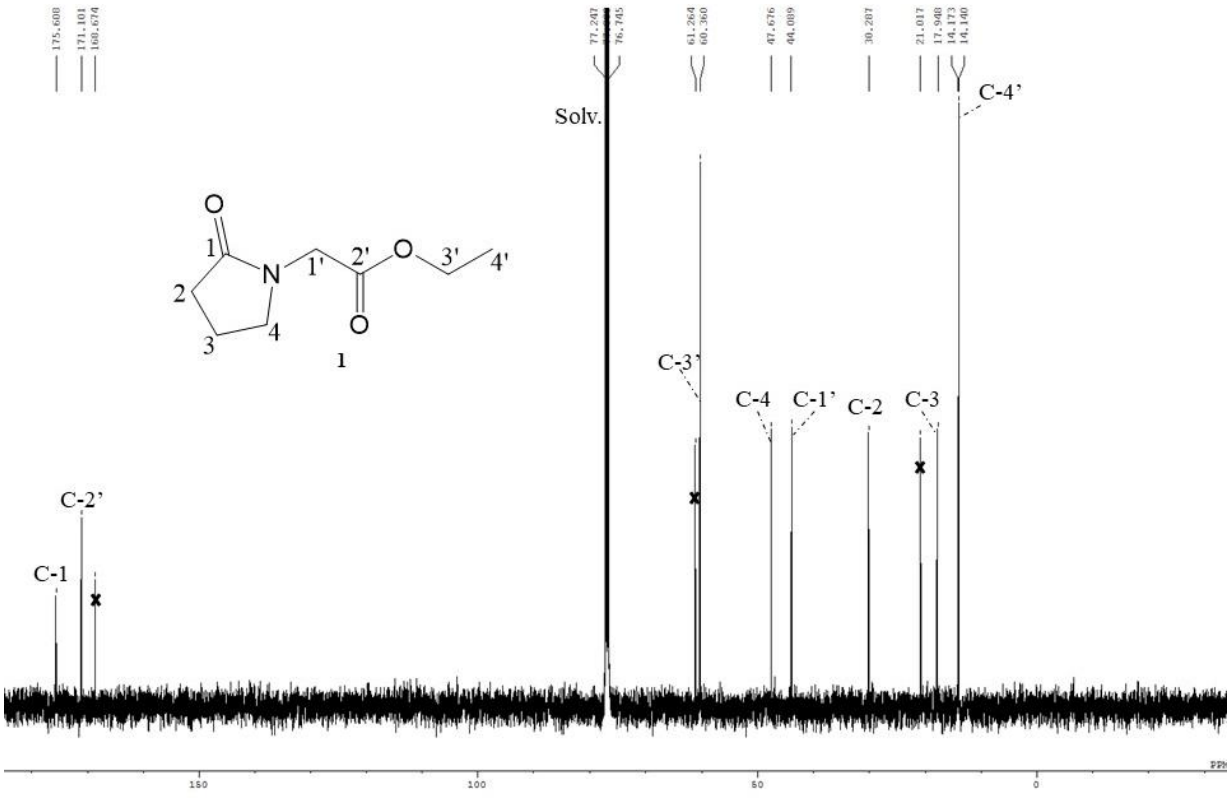


Figure 6 ^{13}C NMR spectrum of **1** ($CDCl_3$)

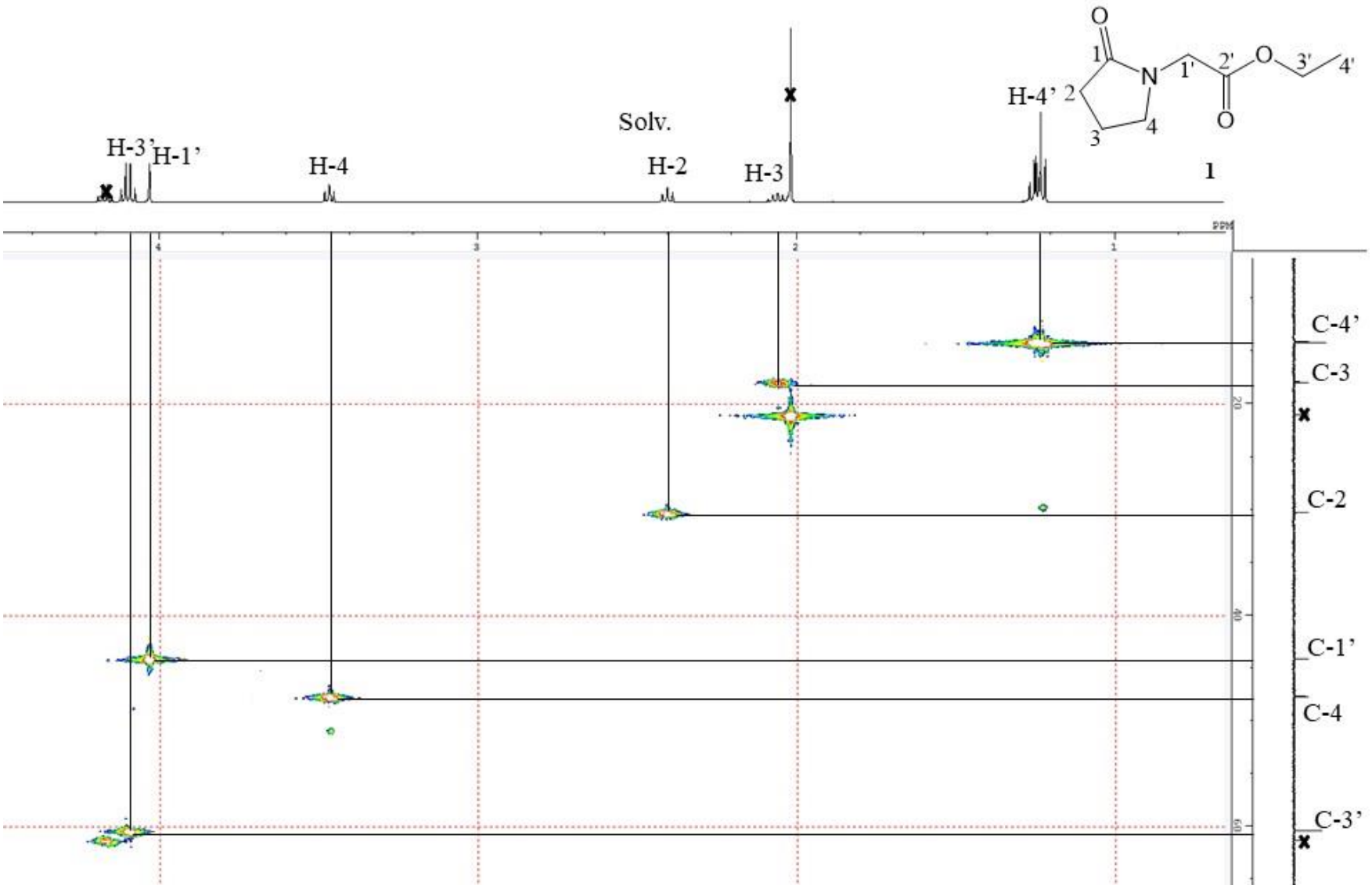


Figure 7 HMQC spectrum of **1** ($CDCl_3$)

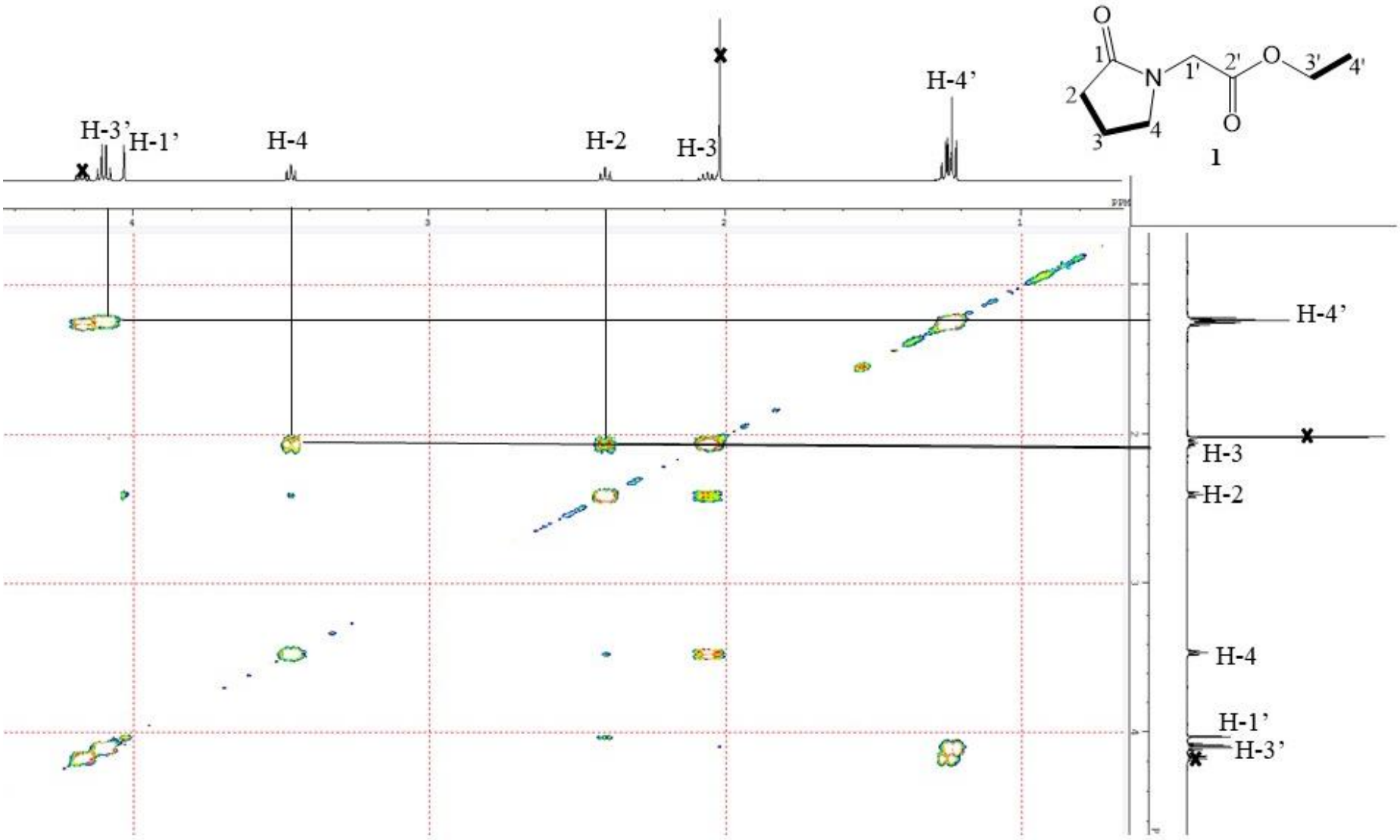


Figure 8 COSY spectrum of **1** ($CDCl_3$)

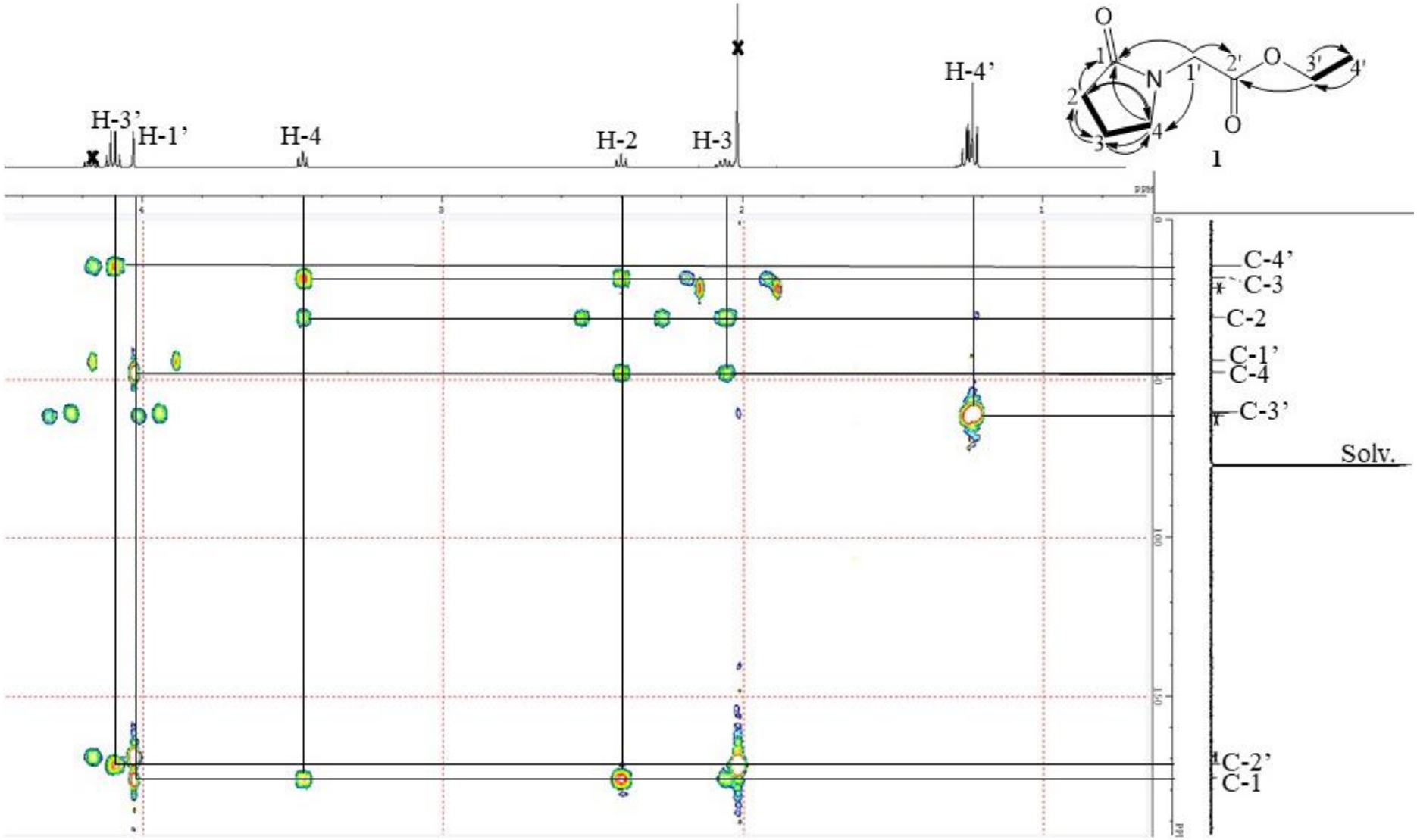


Figure 9 HMBC spectrum of **1** ($CDCl_3$)

2-2 Compound **2**

Compound **2** was isolated as pale yellow oil. Its molecular weight was determined by ESIMS; *m/z* 169 $[M+Na]^+$ (Figure 11). The planar structure of **2** was identified by interpretation of the NMR spectra data and its comparison to the reported one (Table 2). Compound **2** was identified as known compound monoethyl succinate (Figure 10). The chemical exploration of plants, *Ferula syreitschikowii* and *Ranunculus ternatus*, leading to isolation of **2**.[30, 31] For the first time, compound **2** was found to be a chemical constituent of marine fungus, *Cladosporium cladosporioides.*[32] Compound **2** has also been isolated as an acidic inhibitor leading to the growth suppression of the dwarf peas elongation.[33]

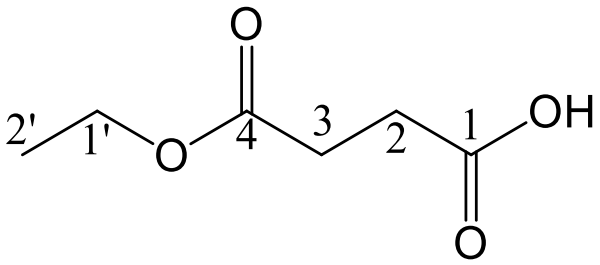


Figure 10 Structure of **2**

Table 2 ^{1}H and ^{13}C NMR assignments for **2**

	Compound **2** ($CDCl_3$)	
Position	δ_C	δ_H (multiplicity, *J* in Hz)
1	177.6	
2	28.8	2.70 (m)
3	28.9	2.60 (m)
4	172.1	
1’	60.8	4.14 (m)
2’	14.1	1.30 (t, *J* = 13.0)

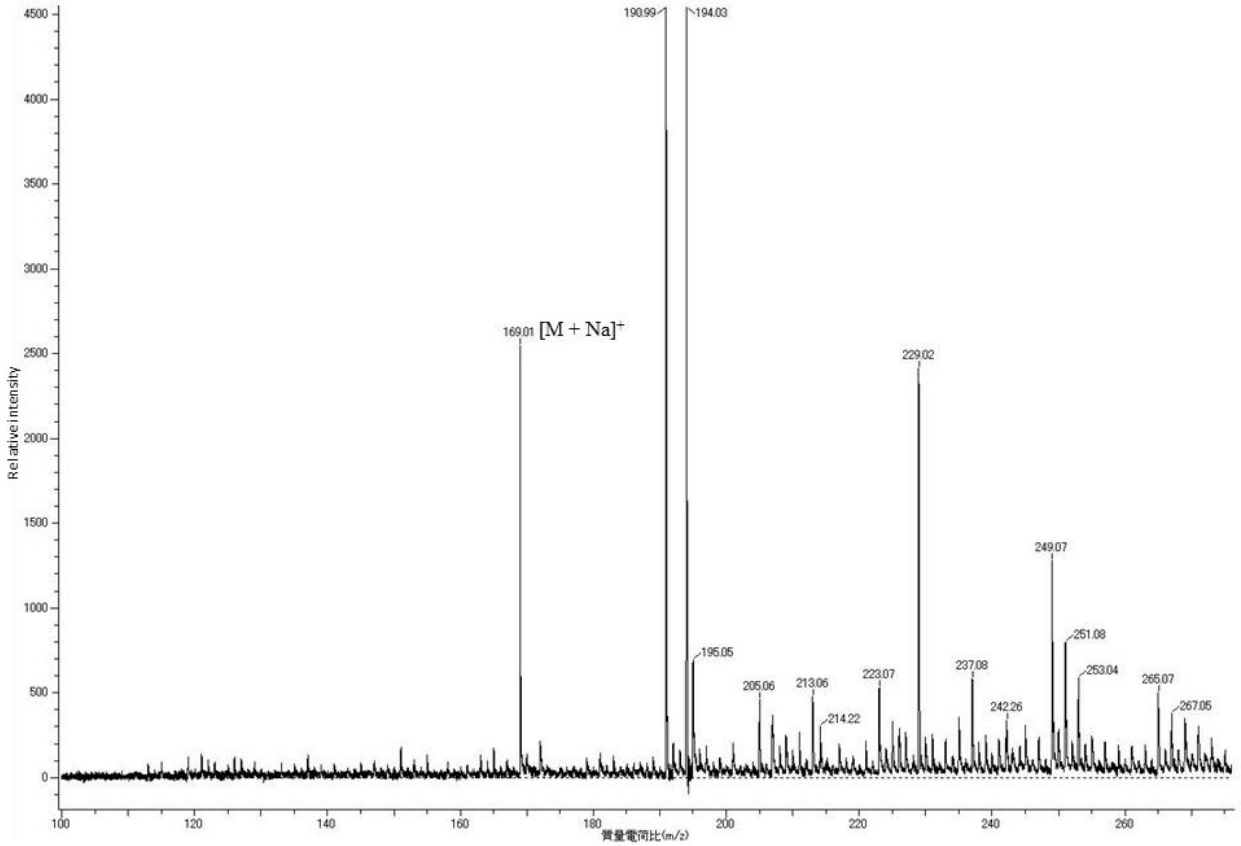


Figure 11 ESIMS spectrum of **2**

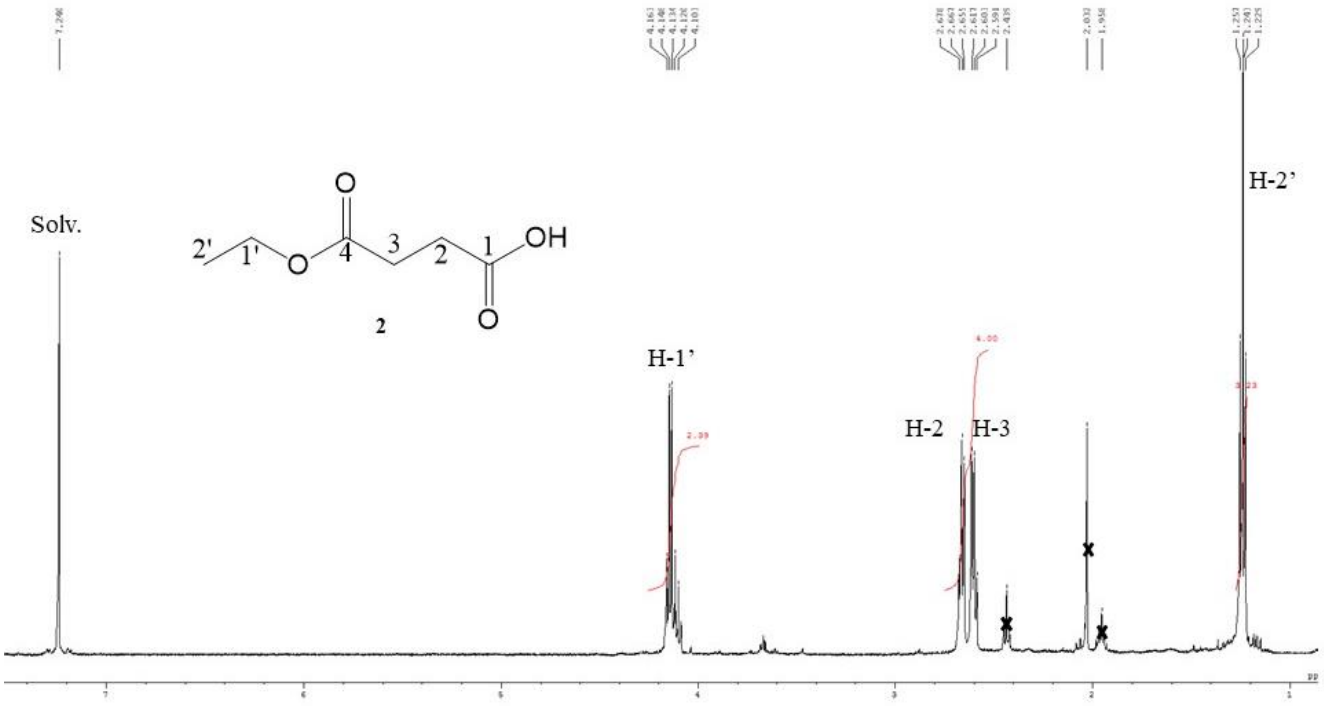


Figure 12 ^{1}H NMR spectrum of **2** ($CDCl_3$)

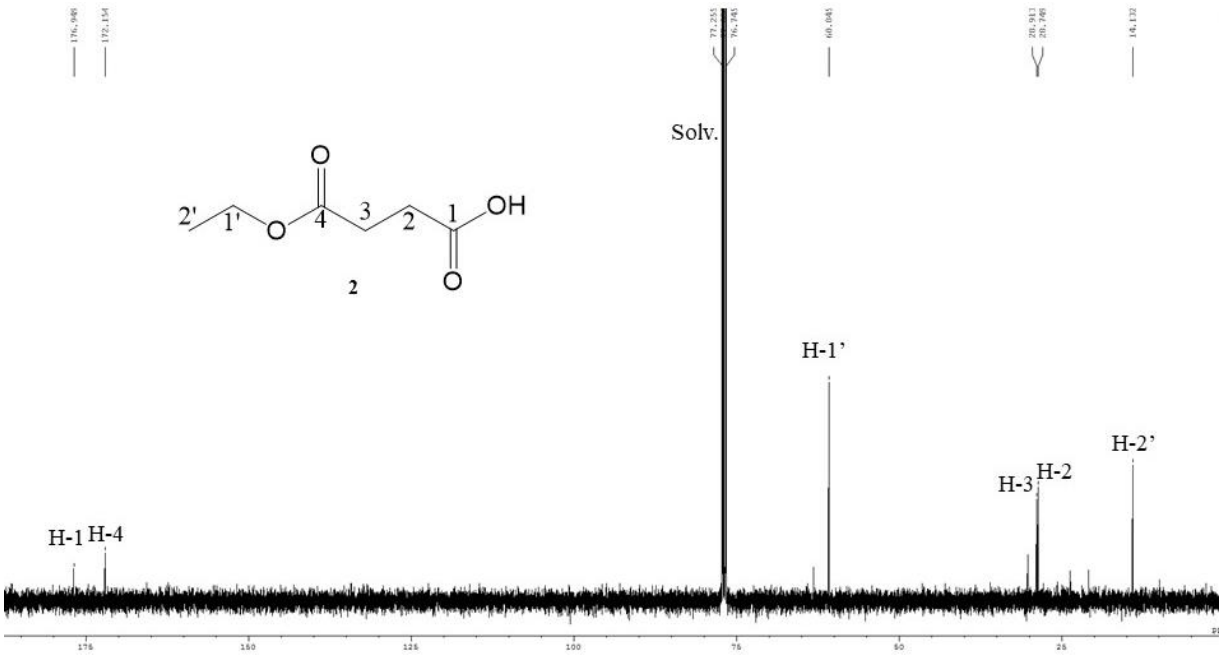


Figure 13 ^{13}C NMR spectrum of **2** ($CDCl_3$)

Section 3 Esterification of malic acid

In the previous study, a malic acid derivative was isolated from the fruiting bodies of *L. giganteus*.[33] The compound that has the same planar structure to the compound (**3**) has been reported as one of chemical constituents from the seeds of *Morinda citrifolia*, the whole plant of *Lobelia chinensis*, and the dried roots of *Ampelopsis japonica.* However, specific rotation and CD data have not been reported in the literatures (Figure 14).[35-37]

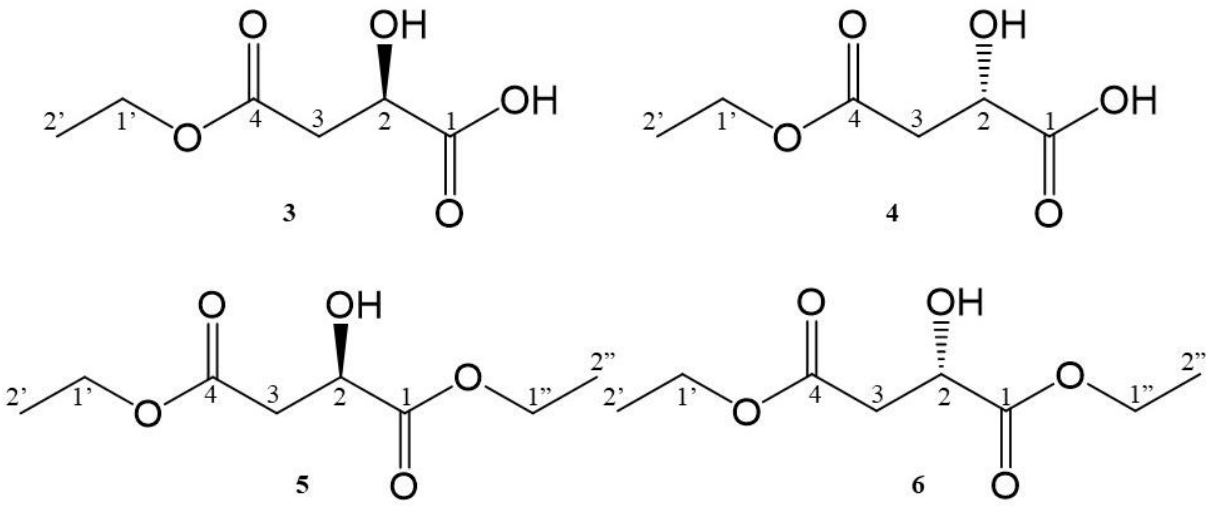


Figure 14 The structures of compounds **3**–**6**

The determination of the absolute configuration of **3** was confirmed by comparing its specific rotation of the synthetic products. Consequently, (*S*) or (*R*)-malic acid were reacted in the presence of HCl for 15 min at room temperature (Figure 15; Table 3). In order to study the structure activity relationship of **3**, the di-esters compounds (**5** and **6**) were chemically prepared.

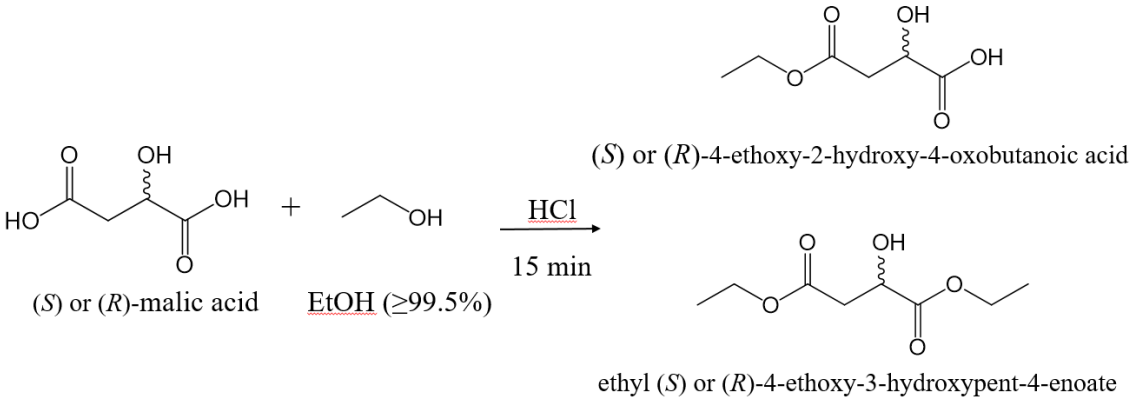


Figure 15 The esterification scheme of (*S*) or (*R*)-Malic acid

Table 3 The conditions of esterification of (*S*) or (*R*)-Malic acid

No	-malic acid	EtOH	HCl	Temperature	Duration
ref.[38]	(*S*)- 10 g	40 g	4 g	boiled	4 H
1	(*S*)- 2 g	10.14 mL	679 µL (on ice)	room temperature	15 min
2	(*S*)- 2 g	10.14 mL	679 µL (on ice)	room temperature	15 min
3	(*R*)- 852.4 mg	4.32 mL	289.4 µL (on ice)	room temperature	4 H

After a repeated purification, two stereoisomers of monoethyl esters **3** and **4** along with diethyl esters **5** and **6** were isolated (Figures 14 and 16). The identification of each compounds was performed by interpretation of the MS and NMR spectra (Table 4; Figures 17-28).

The absolute configuration of **3** was determined by comparing its specific rotation { $[\alpha]_D^{26}$ +13 (*c* 0.25, MeOH) } with that of synthetic one { $[\alpha]_D^{28}$ +12 (*c* 0.25, MeOH) } and its enantiomer (**4**) { $[\alpha]_D^{26}$ -15 (*c* 0.24, MeOH) }. The comparison of specific rotation among the natural compound and synthetic ones suggested that the absolute configuration of the compound **3** was determined to be *R*, Thus, this finding allowed to conclude that compound **3** was a novel compound, (*R*)-4-ethoxy-2-hydroxy-4-oxobutanoic acid (Figure 14).

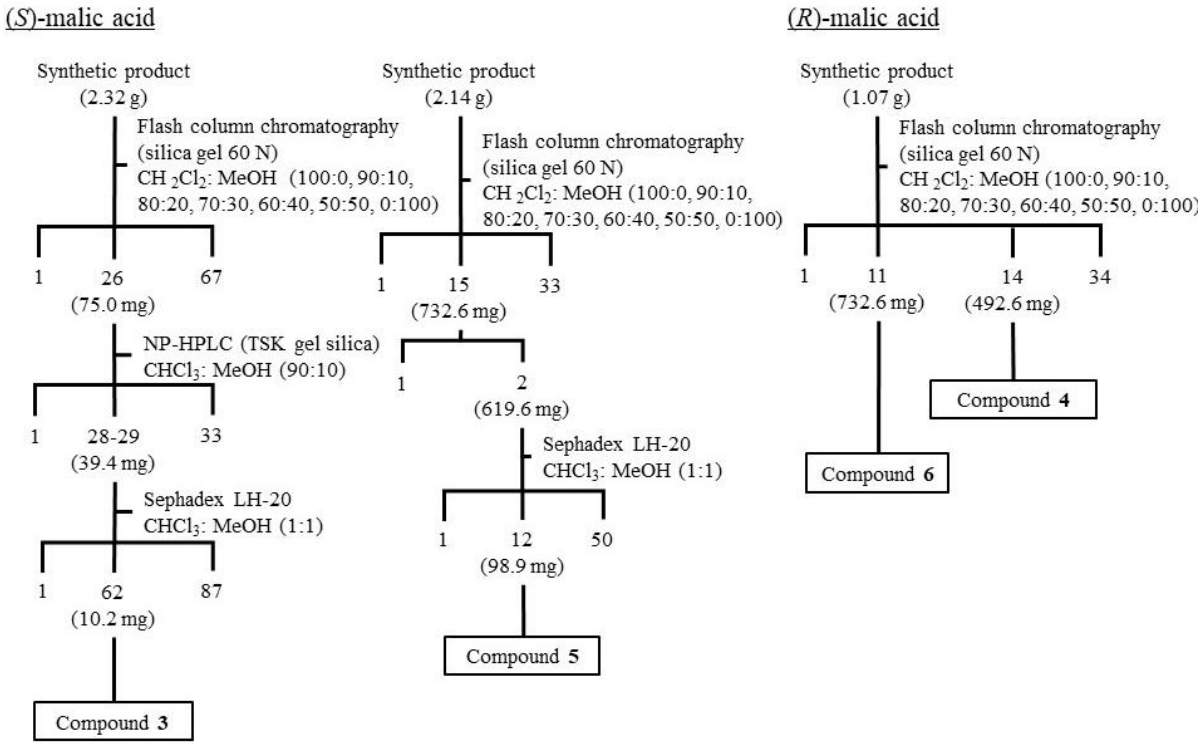


Figure 16 The purification scheme of (*S*) or (*R*)-Malic acid esterification products

Table 4 ^{1}H and ^{13}C NMR assignments for **c**ompounds **3**–**6** (in CD_3OD)

Position	**3**		**4**		**5**		**6**	
	δ_C	δ_H (multiplicity, *J* in Hz)	δ_C	δ_H (multiplicity, *J* in Hz)	δ_C	δ_H (multiplicity, *J* in Hz)	δ_C	δ_H (multiplicity, *J* in Hz)
1	174.8		174.7		174.5		174.5	
2	68.7	4.46 (t, *J* = 6.0 Hz)	68.8	4.46 (t, J = 6.0)	68.6	4.14 (m)	68.6	4.14 (m)
3	40.0	2.65 (dd, *J* = 16.0, 7.5), 2.75 (dd, *J* = 16.0, 4.5)	40.0	2.65 (dd, J = 16.0, 7.3), 2.75 (dd, J = 16.0, 4.5)	40.2	2.69 (dd, J = 16.0, 7.0), 2.77 (dd, J = 15.5, 5.0)	40.2	2.69 (dd, J = 15.5, 7.0), 2.77 (dd, J = 15.5, 5.0)
4	174.1		174.1		172.1		172.1	
1'	62.3	4.19 (m)	62.3	4.19 (m)	62.3	4.19 (m)	62.3	
2'	14.4	1.27 (t, *J* = 7.5)	14.4	1.27 (t, J = 7.3)	14.4	1.27 (t, J = 6.3)	14.4	1.27 (t, J = 6.3)
1''					61.9	4.14 (m)	61.9	4.14 (m)
2''					14.4	1.24 (t, J = 6.0)	14.4	1.24 (t, J = 6.3)

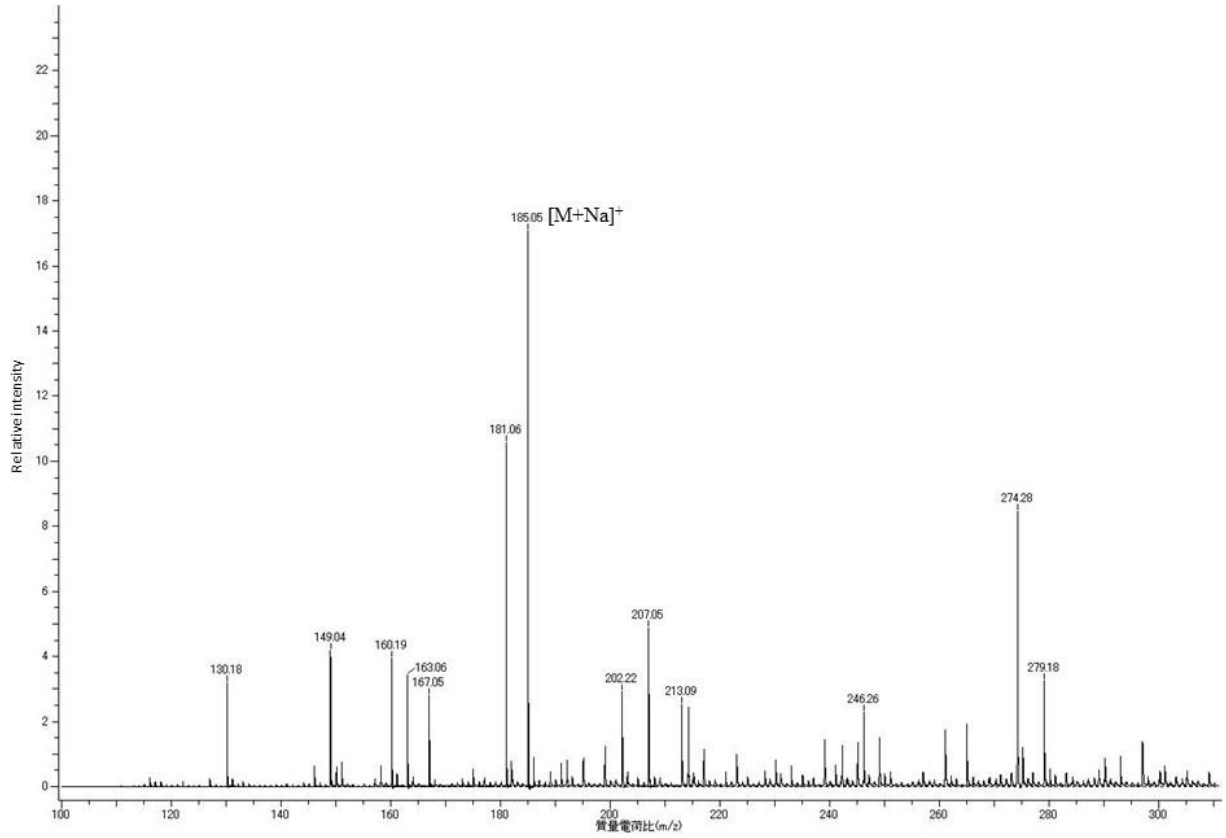


Figure 17 ESIMS spectrum of **3**

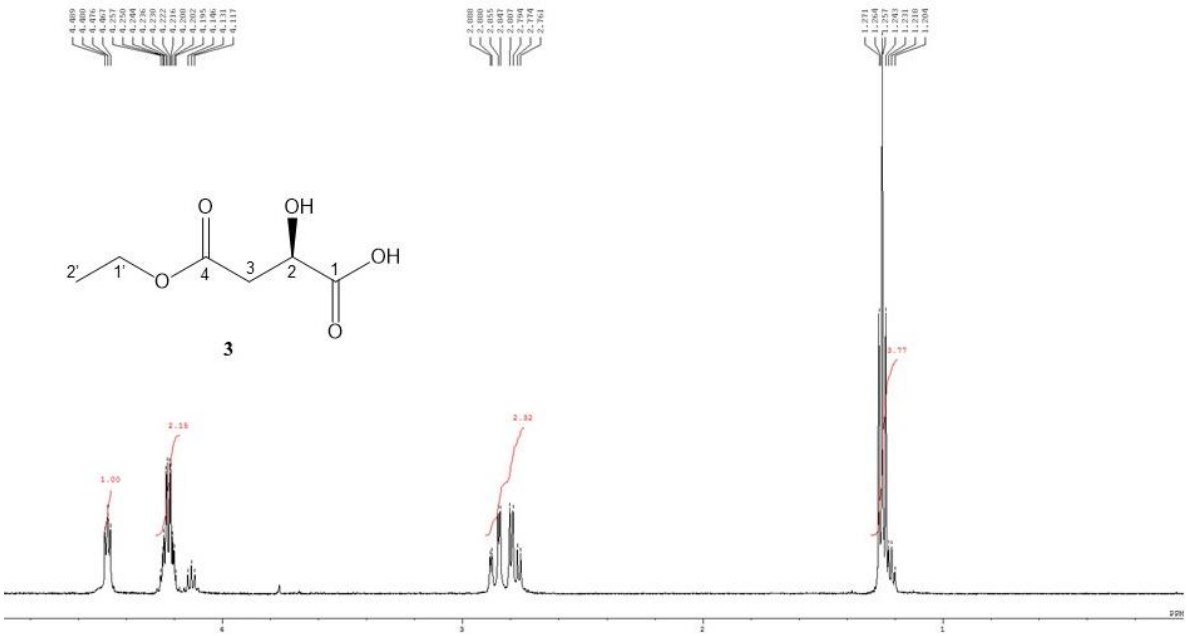


Figure 18 ^{1}H NMR spectrum of **3** (CD_3OD)

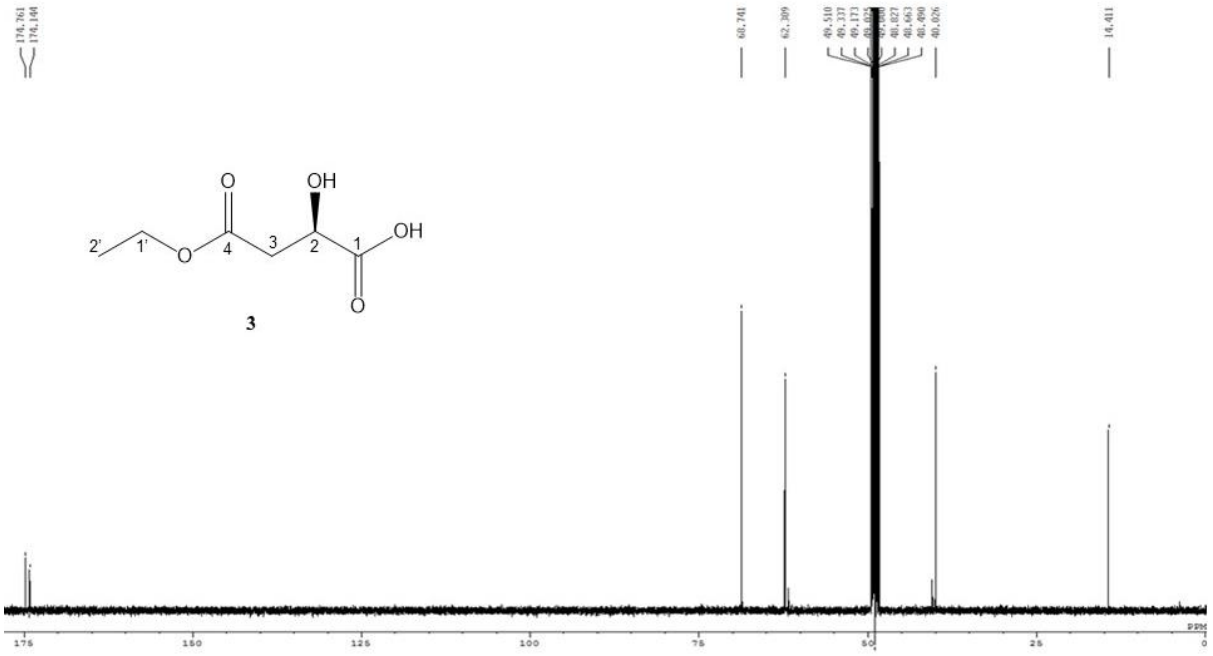


Figure 19 ^{13}C NMR spectrum of **3** (CD_3OD)

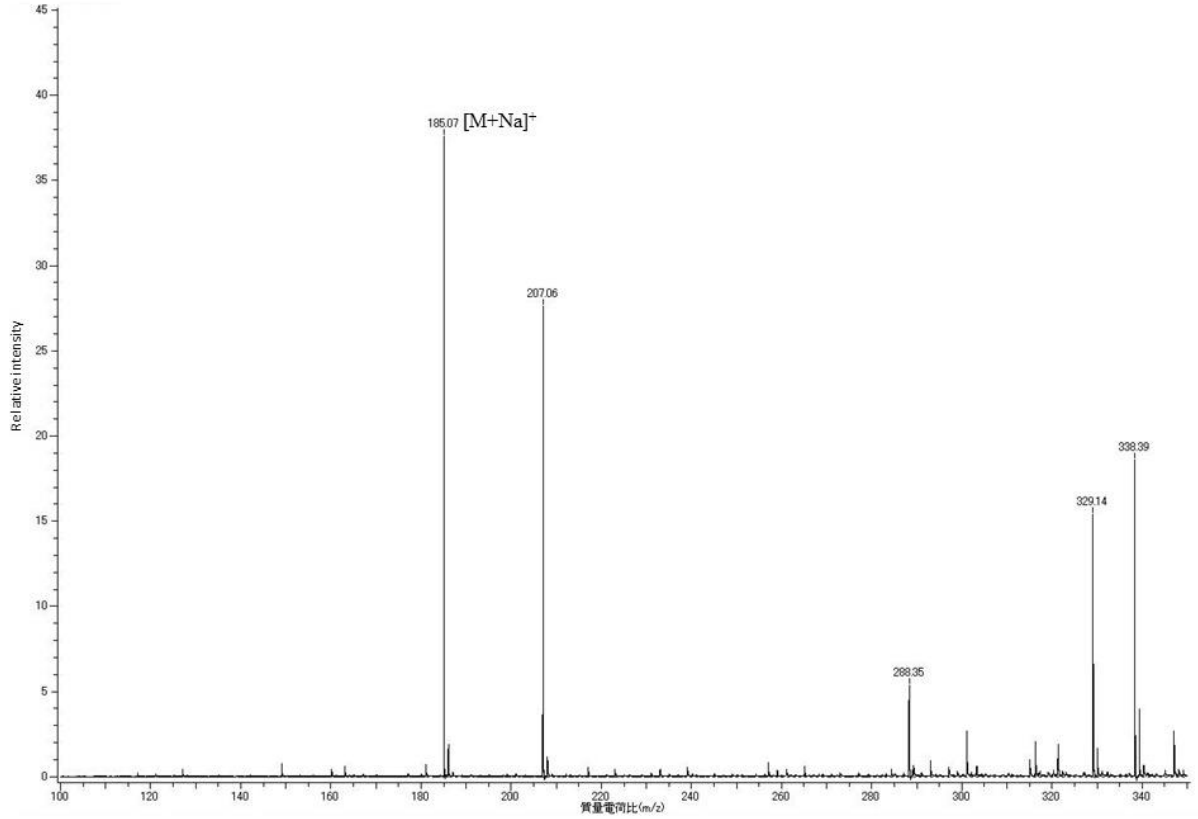


Figure 20 ESIMS spectrum of **4**

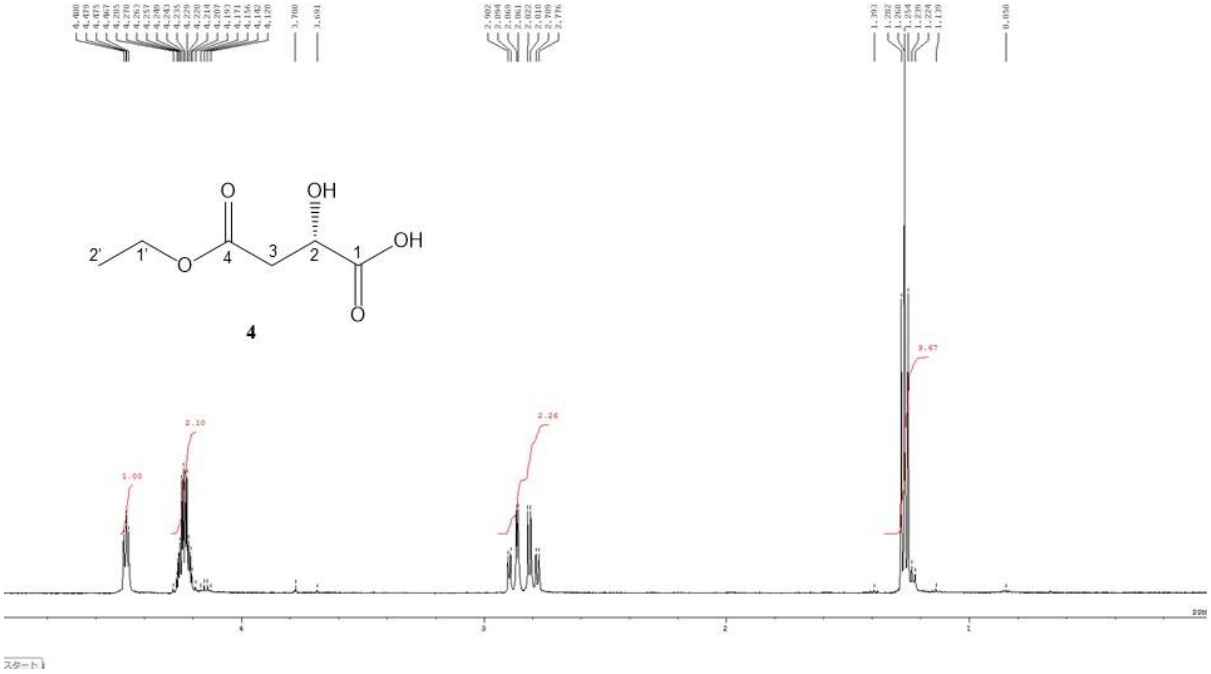


Figure 21 ^{1}H NMR spectrum of **4** (CD_3OD)

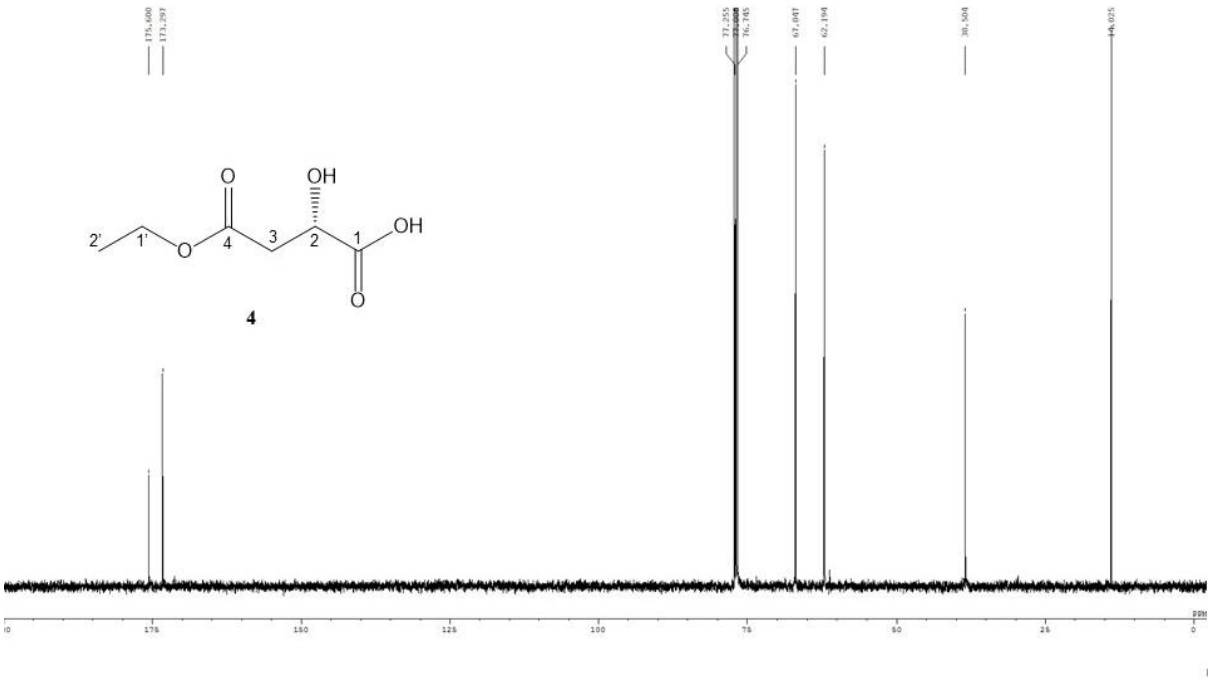


Figure 22 ^{13}C NMR spectrum of **4** (CD_3OD)

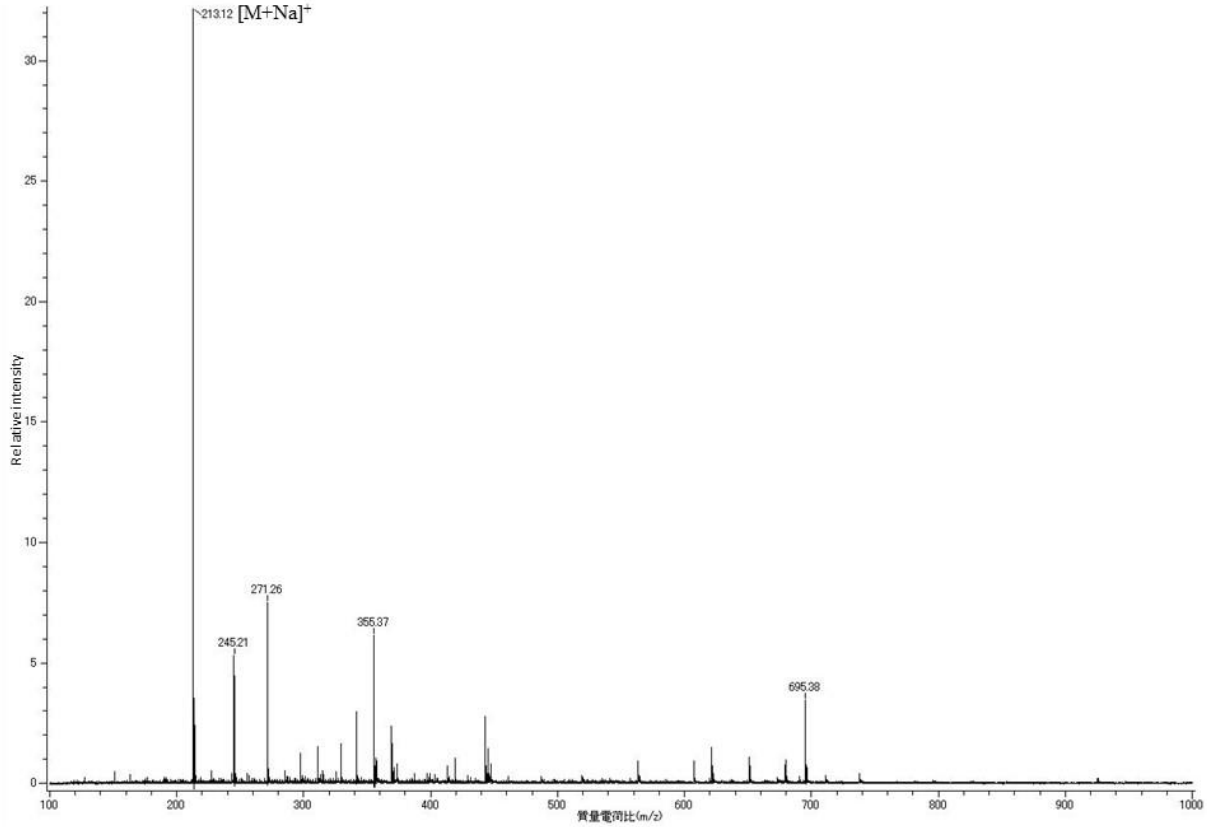


Figure 23 ESIMS spectrum of **5**

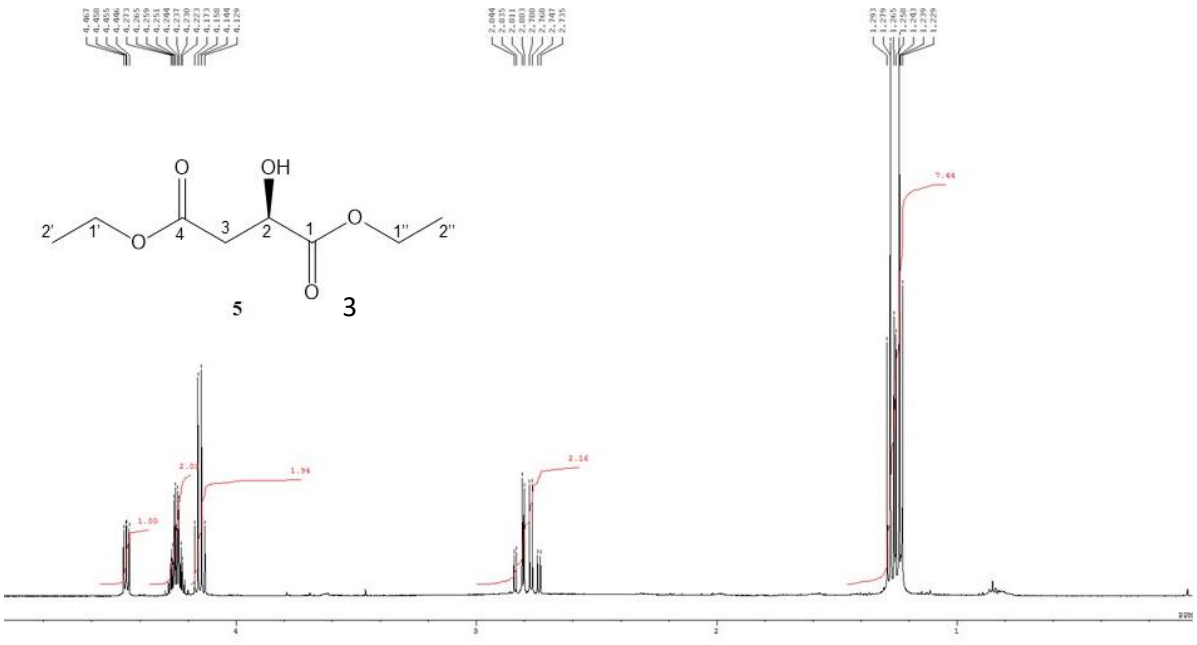


Figure 24 ^{1}H NMR spectrum of **5** (CD_3OD)

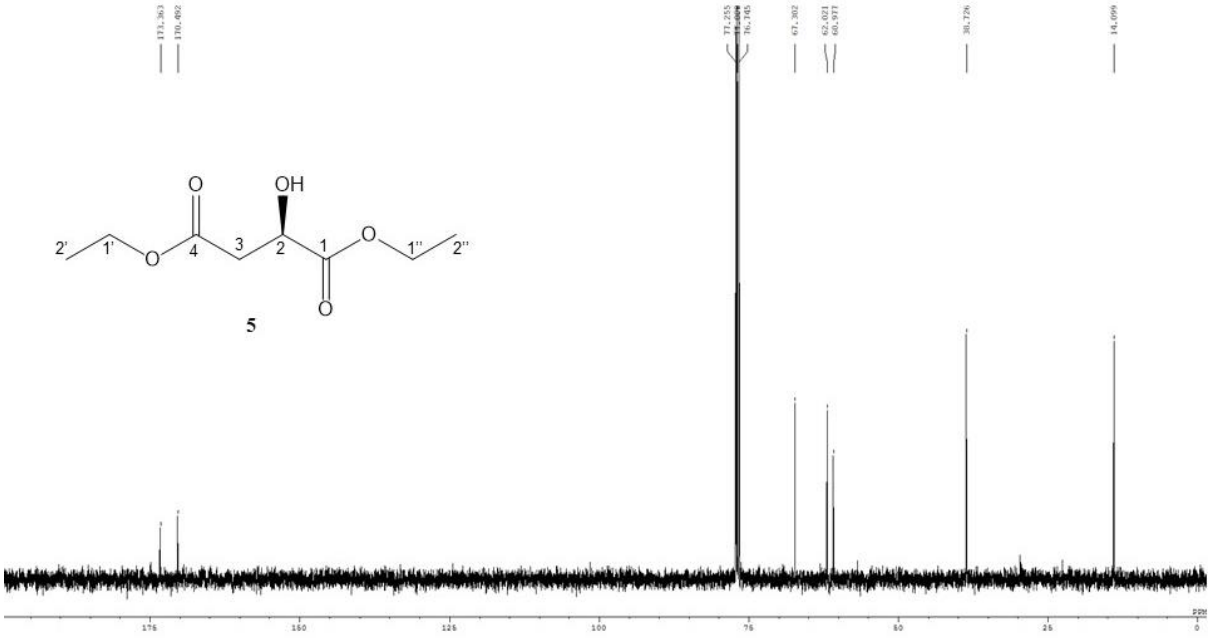


Figure 25 ^{13}C NMR spectrum of **5** (CD_3OD)

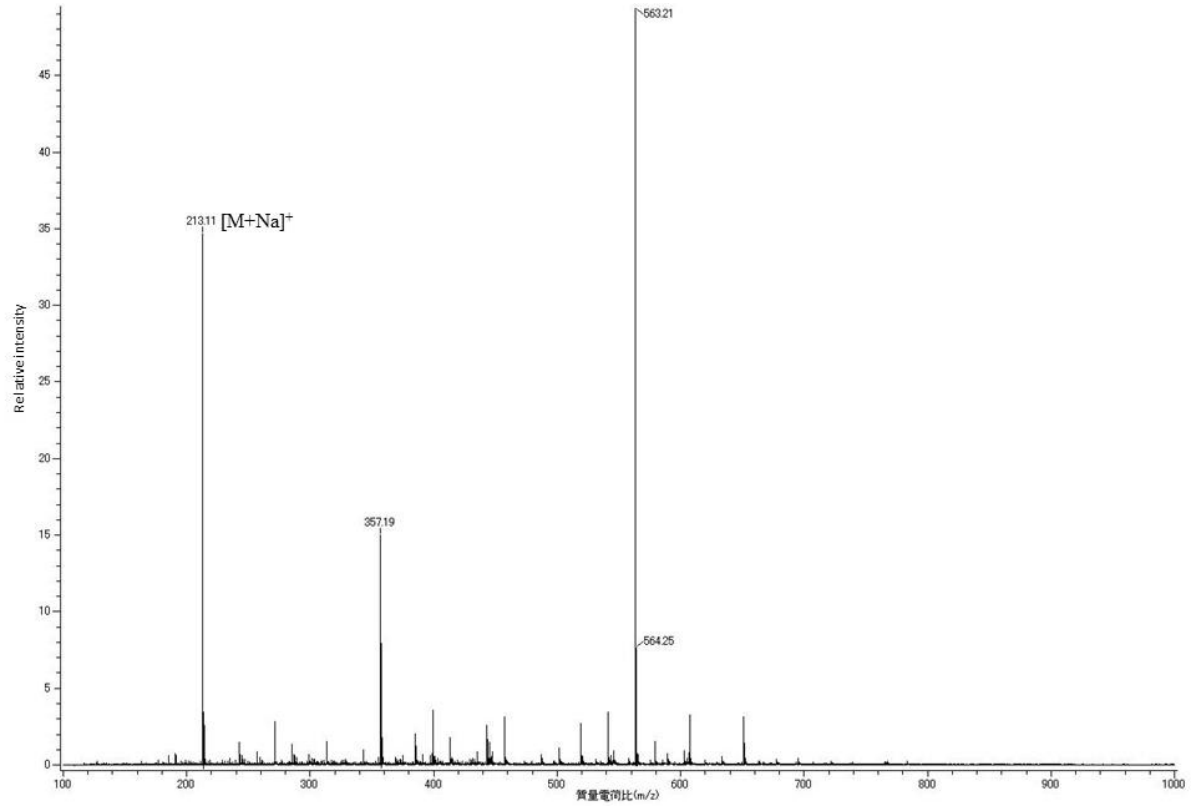


Figure 26 ESIMS spectrum of **6**

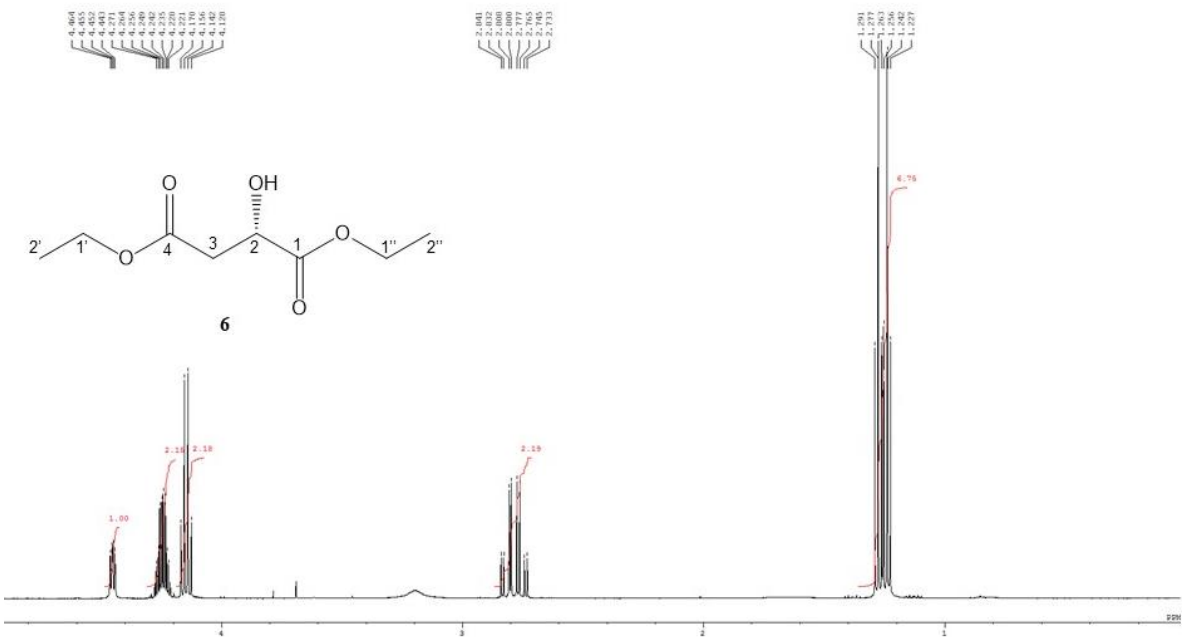


Figure 27 ^{1}H NMR spectrum of **6** (CD_3OD)

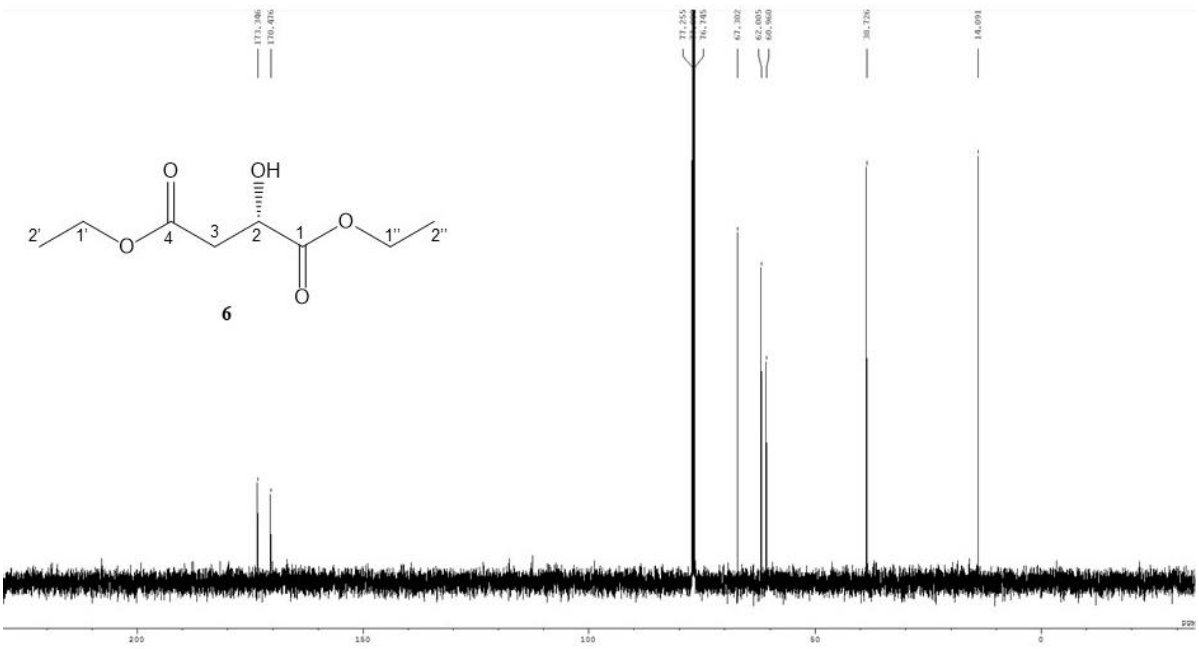


Figure 28 ^{13}C NMR spectrum of **6** (CD_3OD)

Section 4 Biological assay

4-1 Axl and immune checkpoint assay

Each of the natural compounds (**1**–**3**) and synthesized compounds (**4**–**6**) were added to the human A549 alveolar epithelial cell lines. After 24 hours, the mRNA expression of Axl receptor tyrosine kinase and immune checkpoints (PD-L1 and PD-L2) were amplified and then the products were electrophoresed. As shown in Figure 29, compounds **1** and **2** showed inhibition activities of all the three genes expressions, Axl, PD-L1, and PD-L2. Among malic-acid esters (**3**–**6**), compound **4** showed inhibition activities of PD-L1, and PD-L2 expressions, compounds **5** and **6** inhibited Axl and PD-L1 expressions, and only the isolated compound **3** showed the effects of all the gene expressions. Accordingly, it was indicated that the carboxylic acid moiety played an important role in the suppression of PD-L2 and the natural product **3** was the most promising candidate for cancer therapy. To our knowledge, it was the first time that Axl and immune checkpoint inhibitors were isolated from higher fungi.

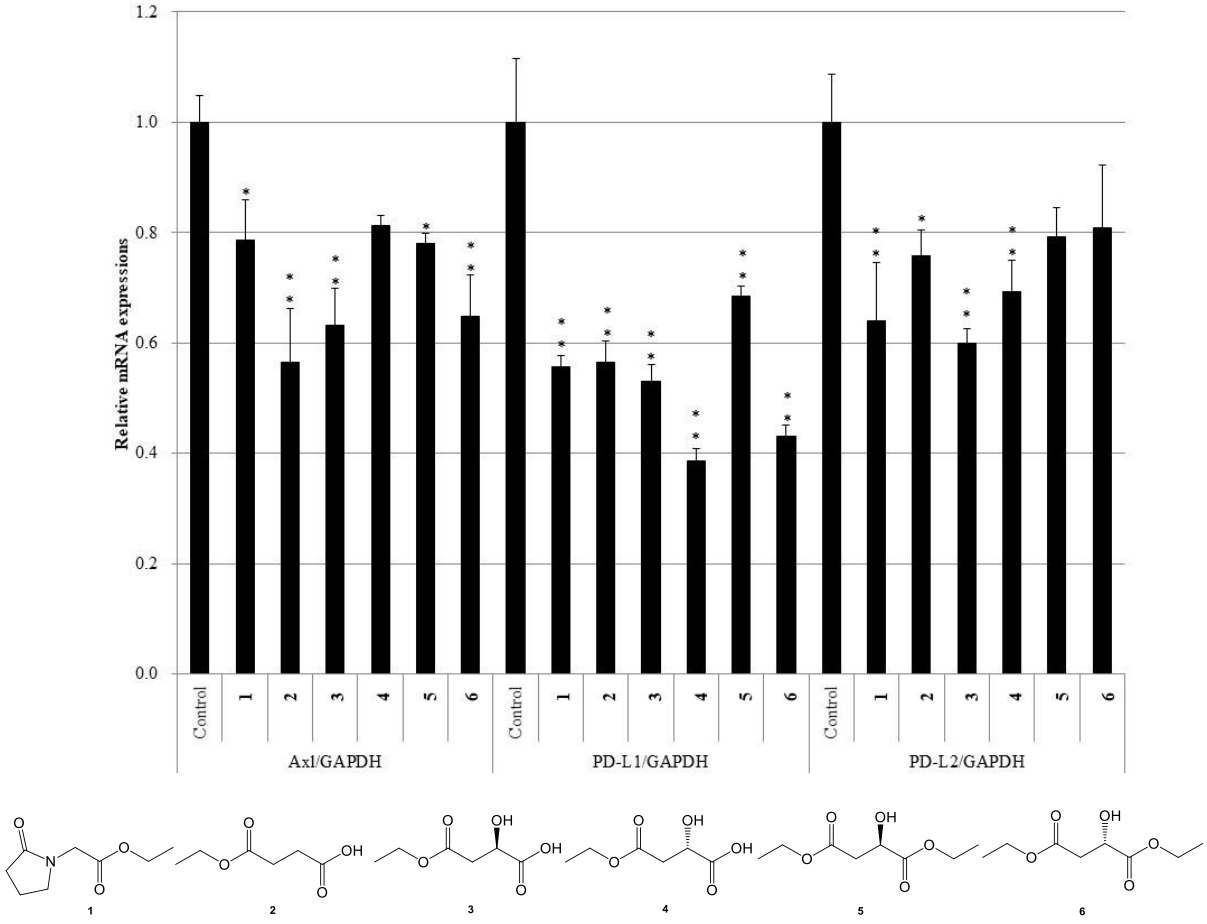


Figure 29 Effect of **1** to **6** on expressions of Axl and immune checkpoints (PD-L1 and PD-L2) on lung cancer cell line A549 cells ($^{*}p < 0.05$, $^{**}p < 0.01$ vs control, $n = 3$)

4-2 Plant growth regulating assay towards lettuce growth

Plant growth regulating activity of **1**–**6** was evaluated using lettuce. 2,4-Dichlorophenoxyacetic acid was used as positive control, which inhibited the hypocotyl and root growth of lettuce dose-dependently. As shown in Figure 30, **1** showed the promotion effect at 10 and 100 nmol/paper against hypocotyl growth, while **2** showing inhibition activity at 1000 nmol/paper against hypocotyl and root growth. In order to study the structure activity relationship, the activity of **3**, its enantiomer (**4**), and di-esters (**5** and **6**) was evaluated (Figure 14). As a result, all compounds showed the inhibition activities with varying doses effects. The inhibition activity of the novel compound **3** was the strongest among all the compounds tested. The antipode of **3** (**4**) showed much less activity than **3**.

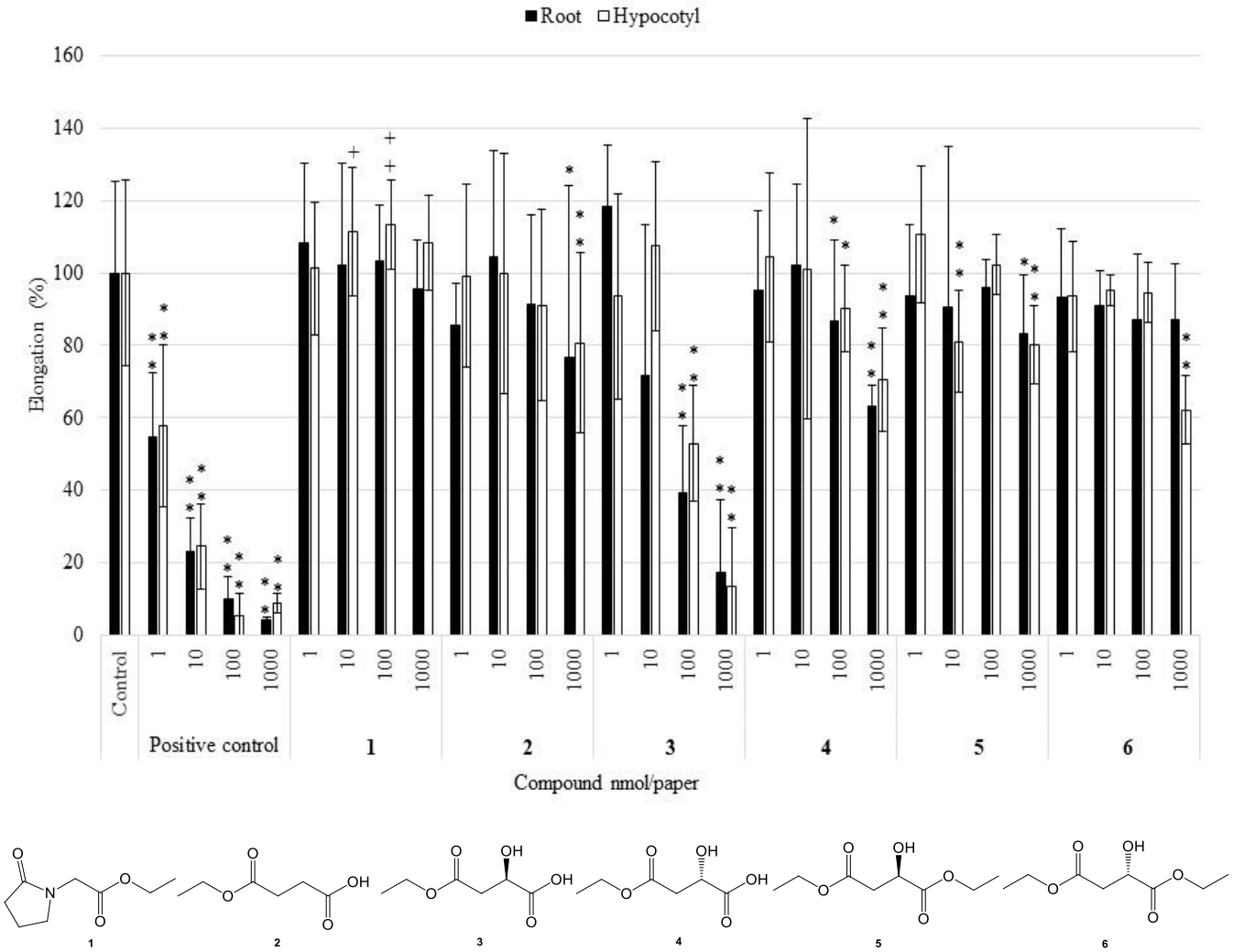


Figure 30 Growth regulating activity against lettuce of compounds **1** to **6** against root or hypocotyl. 2,4-Dichlorophenoxyacetic acid (2,4-D) was used as positive control. Results are the mean ± standard deviation (n = 9). [$^{*}p < 0.05$, $^{**}p < 0.01$ (growth inhibition); $^{+}p < 0.05$, $^{++}p < 0.01$ (growth promotion)].

Chapter 3

Bioorganic chemical studies on culture broth of *Stropharia rugosoannulata*

Section 1 Isolation of bioactive compounds from culture broth of *Stropharia rugosoannulata*

After 30 days' incubation, the culture broth of *S. rugosoannulata* was filtered and concentrated under redued pressure. The concentrated filtrate was partitioned into *n*-hexane, EtOAc, EtOH, and H_2O soluble parts. Then, the H_2O soluble part was dried and extracted with EtOH (Figure 31).

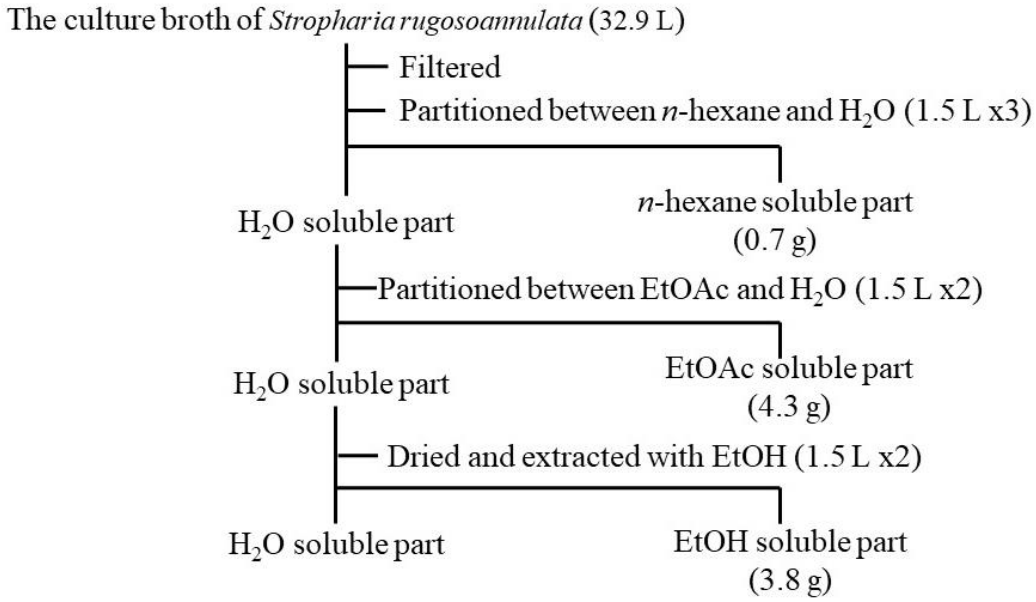


Figure 31 Partition scheme of *S. rugosoannulata* extracts

The EtOAc soluble part was subjected to silica gel flash column chromatography to obtain 16 fractions (1-16). Further purification was conducted by repeated chromatography using flash column chromatography or HPLC. The repeated purification afforded nine known compounds (Figure 32 and 33).

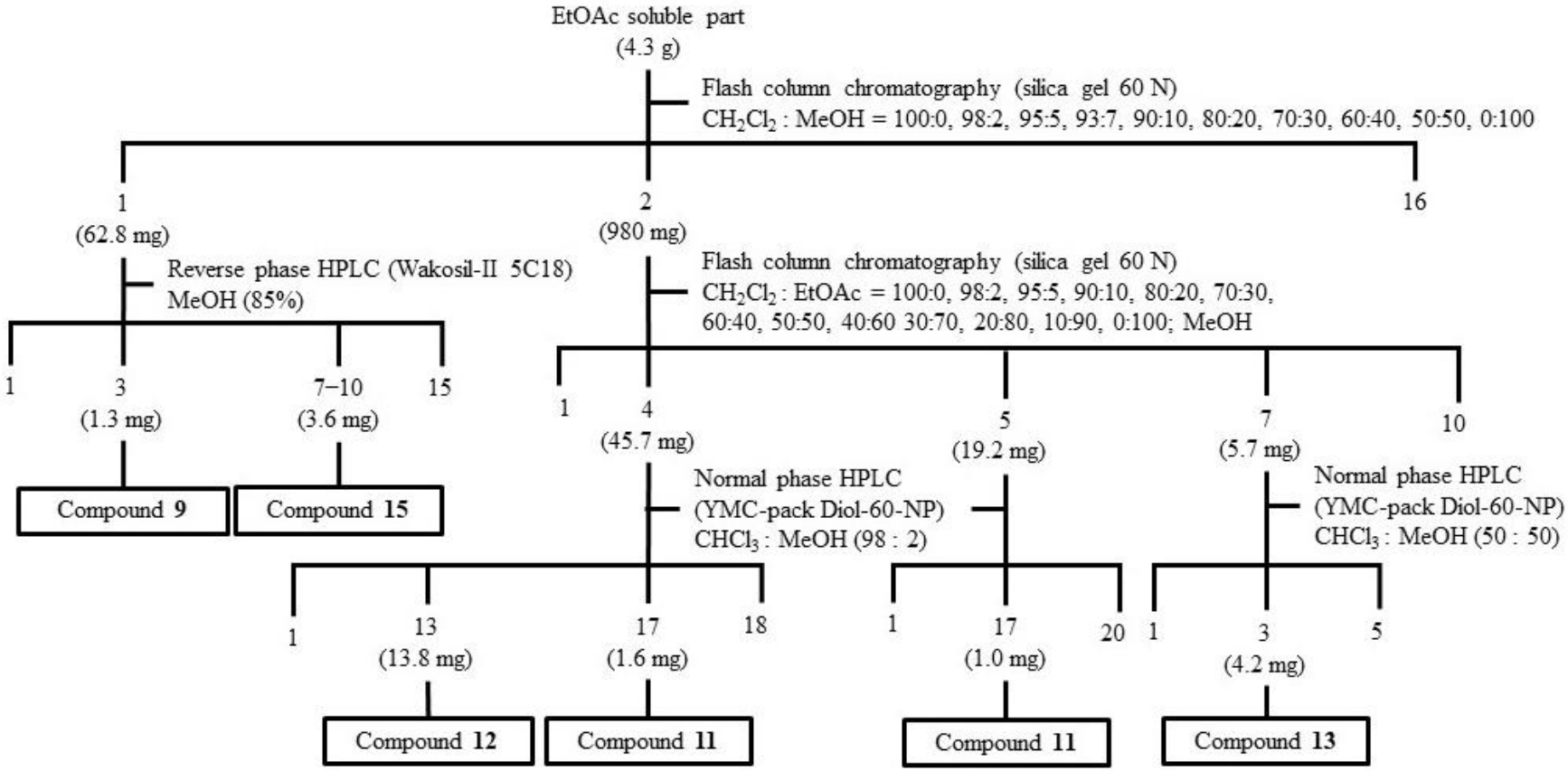


Figure 32 The isolation and purification scheme of compounds from *S. rugosoannulata* (1)

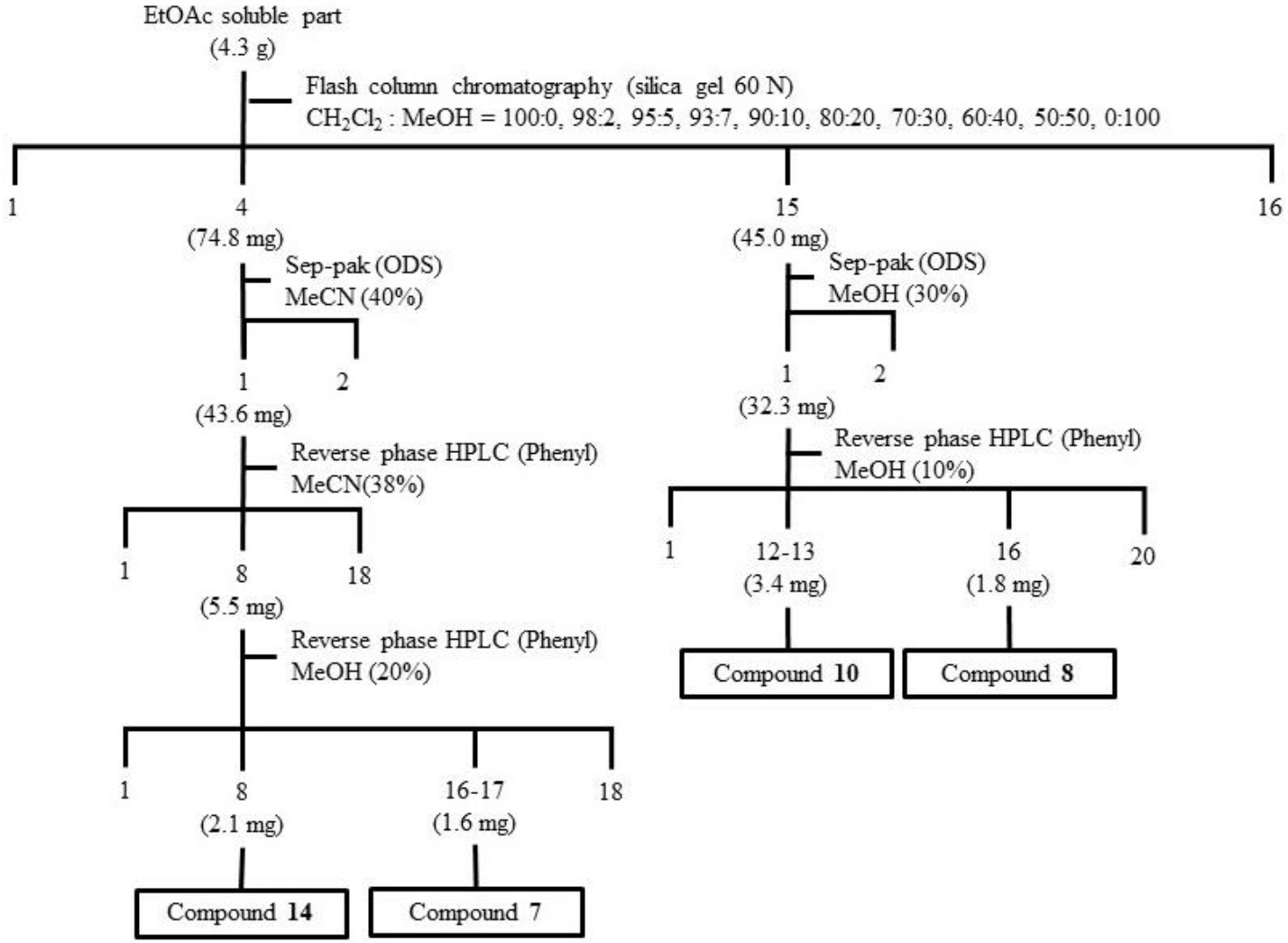


Figure 33 The isolation and purification scheme of compounds from *S. rugosoannulata* (2)

Section 2 Structural analysis of bioorganic compounds from *Stropharia rugosoannulata*

2-1 Compound **7**

Compound **7** was isolated as pale yellow oil. Its molecular formula was determined as $C_{10}H_{11}NO_2$ by HRESIMS *m/z* 200.0686 $[M+Na]^+$ (calcd. for $C_{10}H_{11}NO_2Na$, 200.0682), indicating the presence of six degrees of unsaturation in the molecule (Figure 35). The IR spectrum showed a hydroxyl group (3251 cm^{-1}), an amine group (2924 cm^{-1}) and a carbonyl group (1699 cm^{-1}) (Figure 36). The planar structure of **7** was elucidated by interpretation of the NMR spectra, including DEPT, COSY, HMBC, and HMQC data. The DEPT experiment indicated the presence of two methylenes, five methines, two quaternary carbons, and one tetrasubstituted carbon. The ^1H and ^{13}C NMR data indicated the presence of two aromatic groups (δ_H 6.86 (d, J = 8.0), 7.03 (t, J = 7.8), 7.21 (t, J = 7.0) and δ_C 128.0, 122.7, 124.1, 109.7, 141.0, respectively) (Table 5; Figures 37 and 38). The COSY and HMBC spectra (H-6/H-7; H-7/H-8; H-8/H-9 and H-6/C-9, C-10; H-7/C-5, C-8; H-8/C-6, C-9, C-10; H-9/C-5, C-7, respectively) suggested the presence of a benzene ring. The HMBC correlations of H-3/C-2, C-4, C-5; H-4/C-3, C-5 confirmed the structure corresponding to carbostyril derivative (Figures 40 and 41). The hydroxymethyl moiety was constructed by a COSY correlations (H-3/H-4; H-4/H-1') and the HMBC correlations (H-3/C-11; H-4/C-11; H-11/C-3, C-2). The absolute configuration of **7** was determined by comparing its specific rotation { $[\alpha]_D^{25}$ -31 (*c* 0.15, MeOH) } with that of its reported analogue { $[\alpha]_D^{20}$ -14 (*c* 3.0, MeOH) }.[39] The planar structure of **7** has been reported as a chemically prepared constituent.[40] However, there is no specific rotation and CD data have been reported in the literatures, therefore, the absolute configuration of **7** has not been determined yet. This the first time for **7** to be determined its absolute configuration. Accordingly, compound **7** was identified as (*S*)-4-(hydroxymethyl)-3,4- dihydroquinolin-2(1H)-one (Figure 34). In this study compound **7** was isolated as a natural compound for the first time.

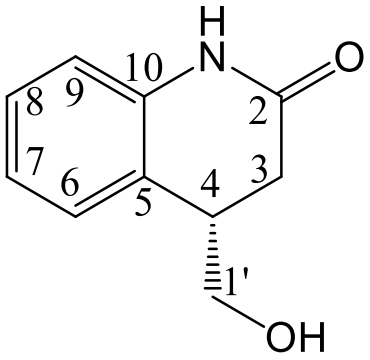


Figure 34 Structure of **7**

Table 5 ^{1}H and ^{13}C NMR assignments for **7**

	Compound **7** ($CDCl_3$)	
Position	δ_C	δ_H (multiplicity, J in Hz)
1		
2	180.7	
3	33.2	2.05 (m)
		2.24 (m)
4	44.7	3.60 (t, J = 7.0)
5	129.4	
6	128.0	7.03 (t, J = 7.8)
7	122.7	6.86 (d, J = 8.0)
8	124.1	7.03 (t, J = 7.8)
9	109.7	7.21 (t, J = 7.0)
10	141.0	
1’	60.9	3.89 (t, J = 5.8)

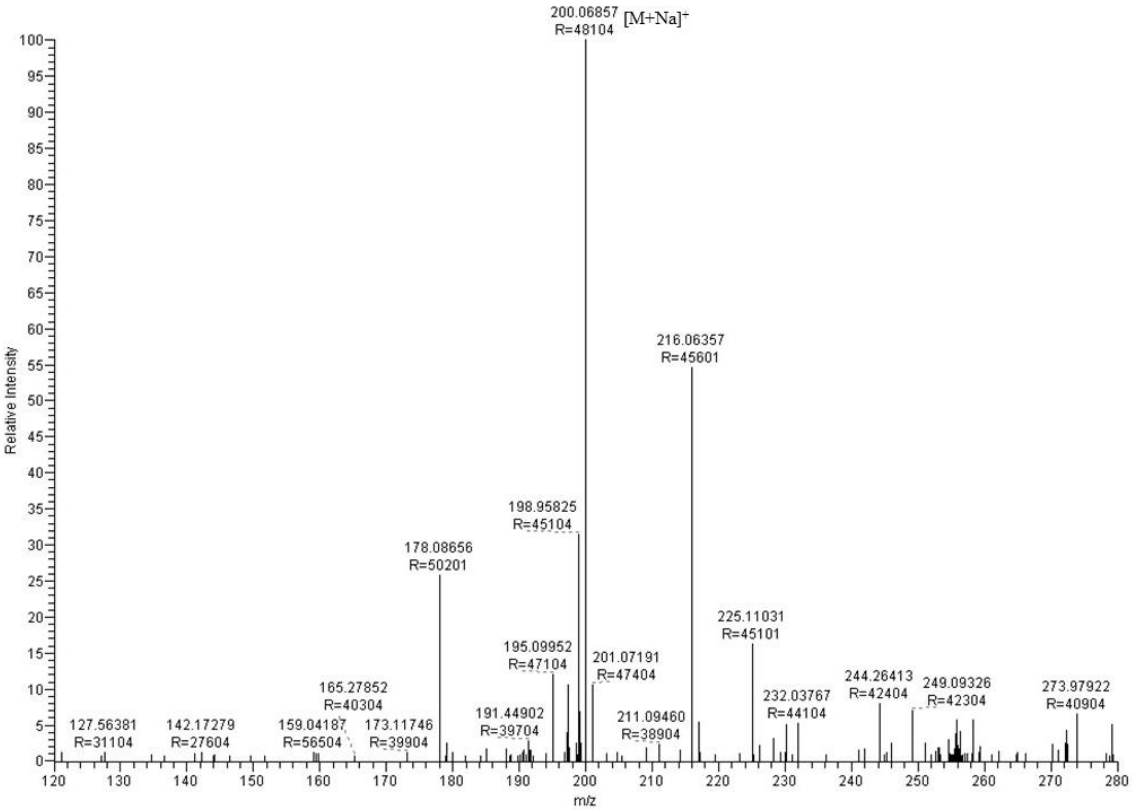


Figure 35 ESIMS spectrum of **7**

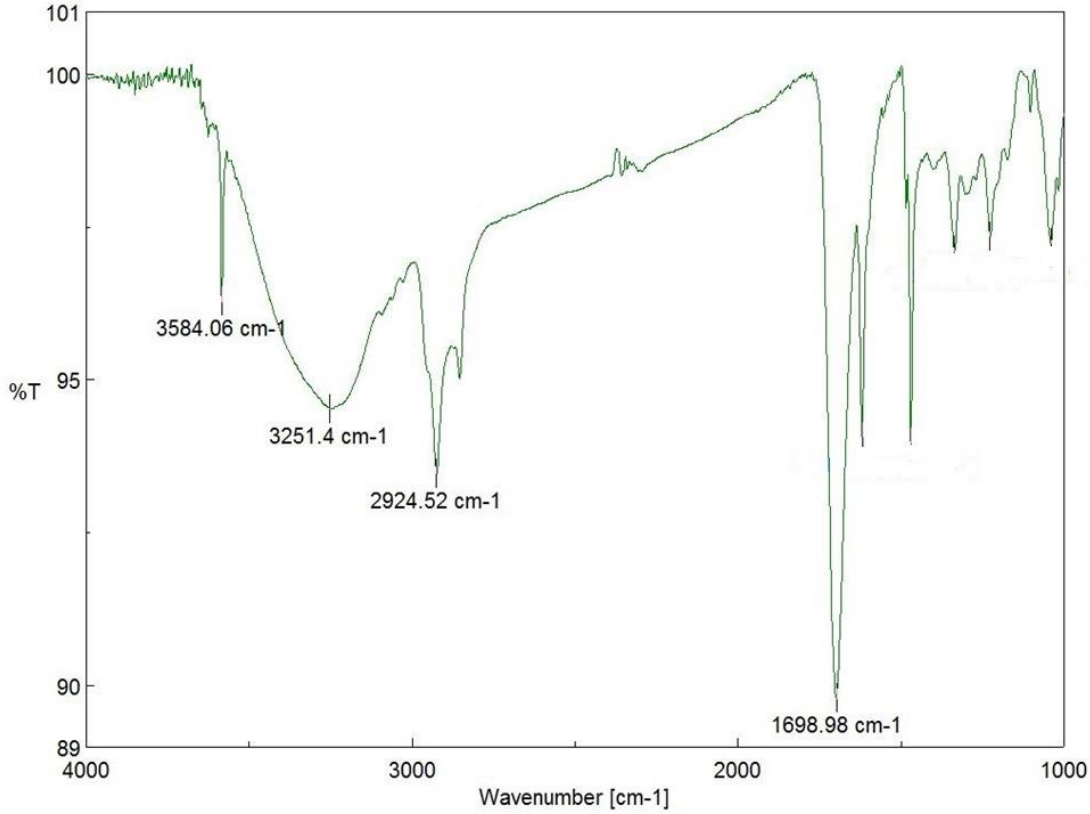


Figure 36 IR spectrum of **7**

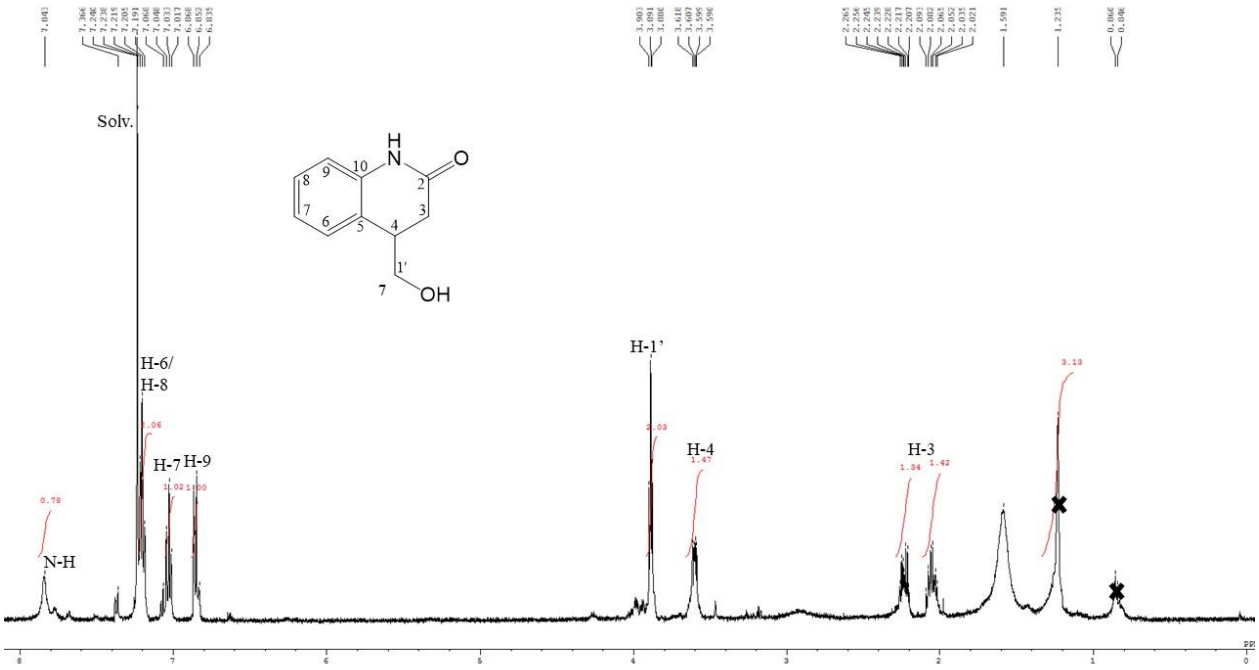


Figure 37 ^{1}H NMR spectrum of **7** ($CDCl_3$)

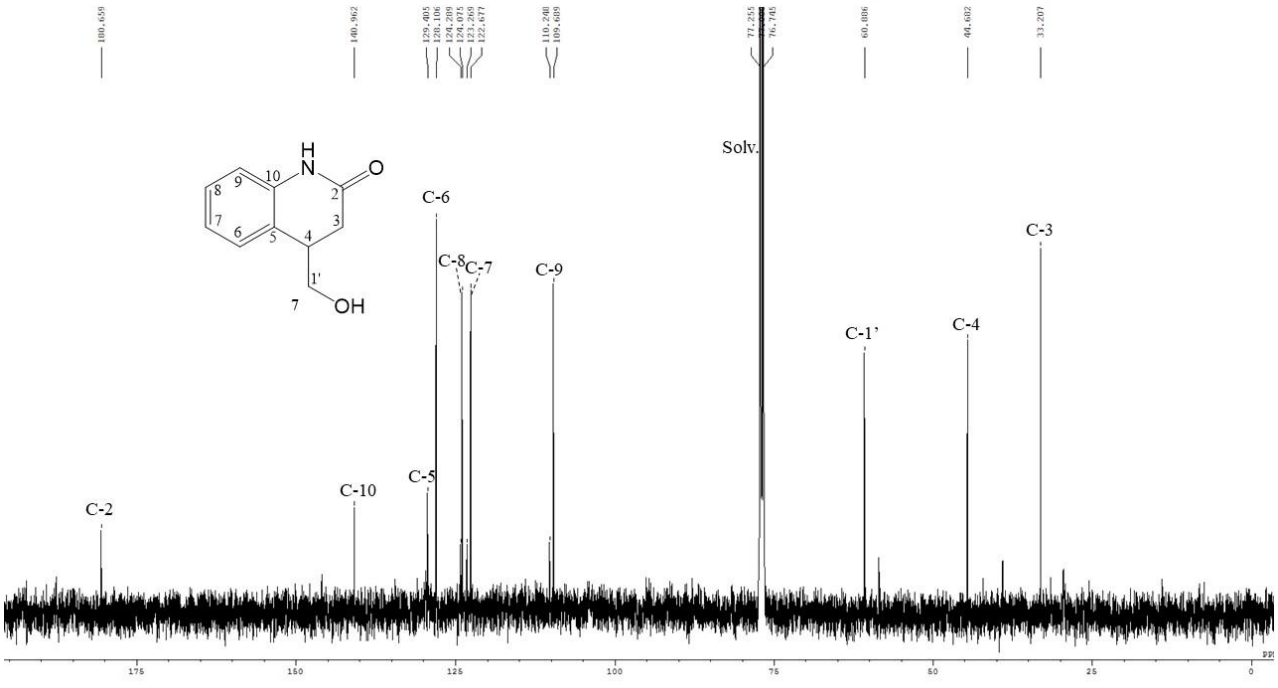


Figure 38 ^{13}C NMR spectrum of **7** ($CDCl_3$)

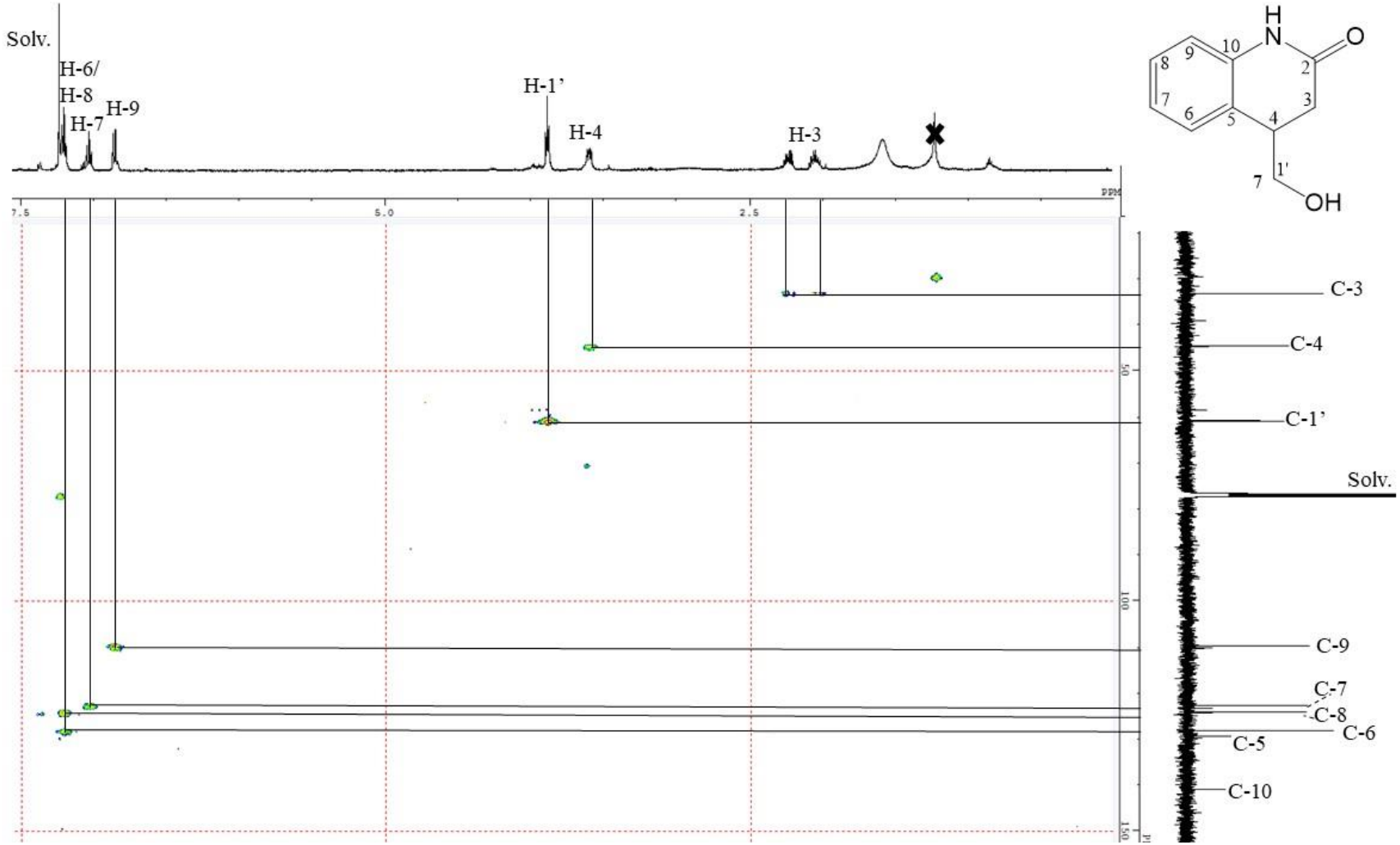


Figure 39 HMQC spectrum of **7** ($CDCl_3$)

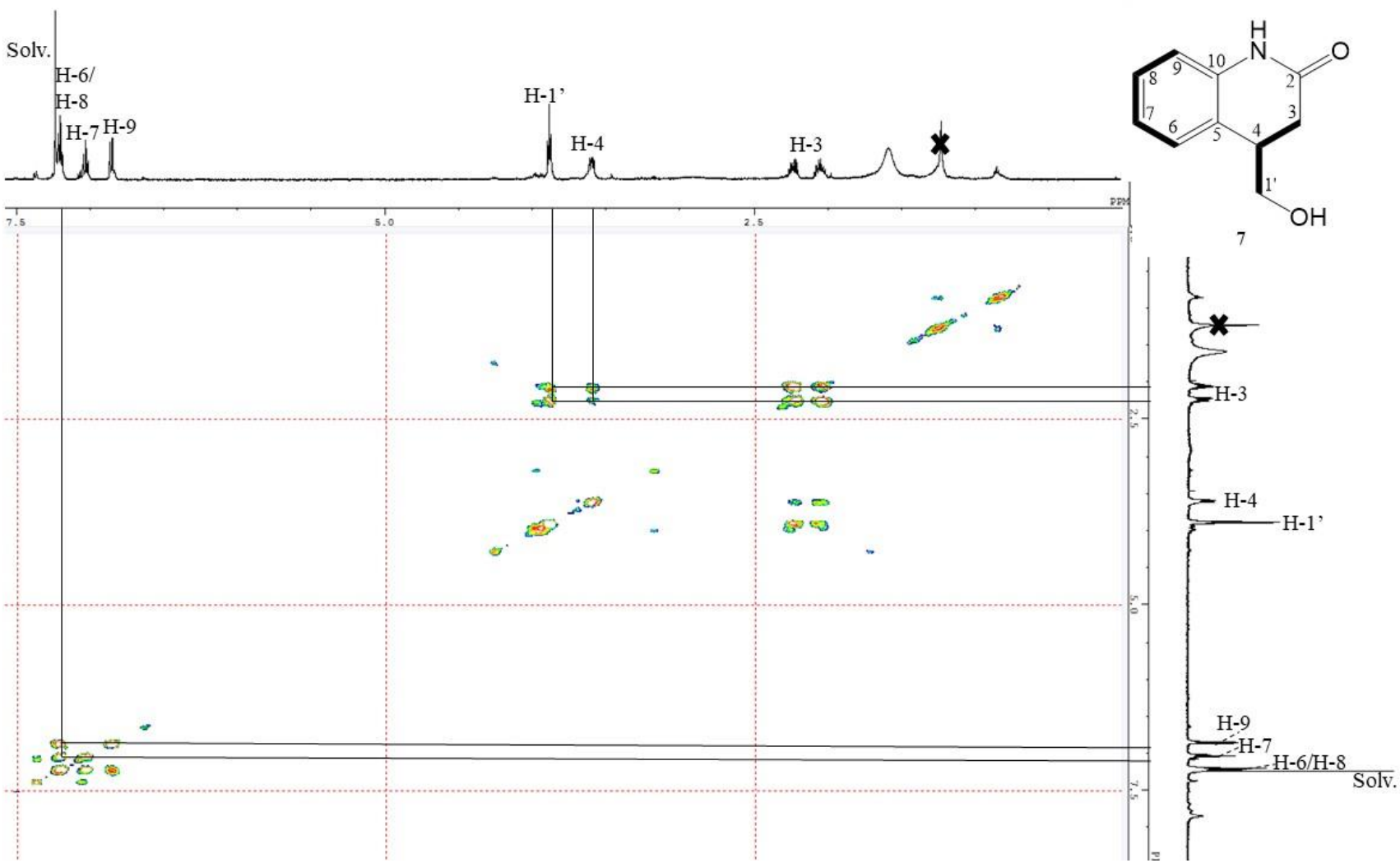


Figure 40 COSY spectrum of **7** ($CDCl_3$)

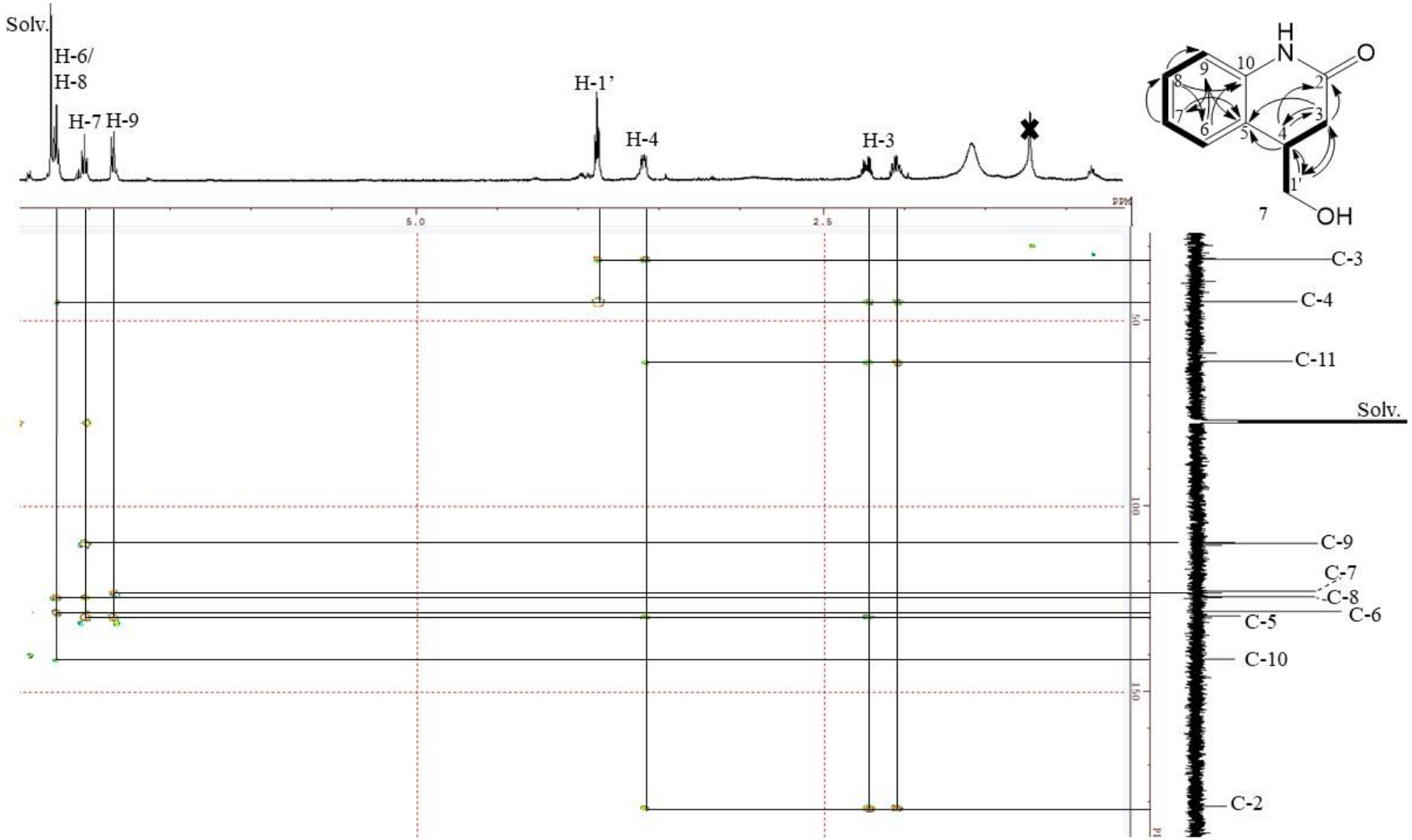


Figure 41 HMBC spectrum of **7** ($CDCl_3$)

2-2 Compound **8**

Compound **8** was isolated as a colorless crystal. Its molecular weight was determined by ESIMS; *m/z* 206 $[M-H]^-$ (Figure 43). The interpretation of NMR spectra indicated that compound **8** was a known compound *N*-acetyl phenylalanine (Figures 44 and 45; Table 6). The absolute configuration of **8** was measured as $[\alpha]_D^{28}$ -17 (*c* 0.22, EtOH) and compared to the reported one { $[\alpha]_D^{25}$ -48 (*c* 4.0, EtOH) }.[41] Compound **8** was identified as acetyl-*D*-phenylalanine (Figure 42). In this study, compound **8** was isolated as a natural compound for the first time.

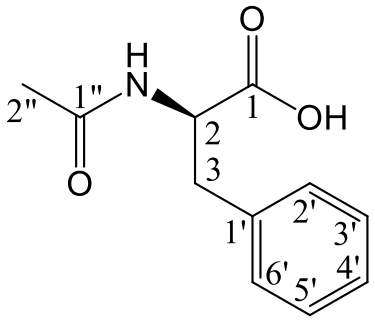


Figure 42 Structure of **8**

Table 6 ^1H and ^{13}C NMR assignments for **8**

	Compound **8** (CD_3OD)	
Position	δ_C	δ_H (multiplicity, *J* in Hz)
1	172.0	
2	57.5	4.47 (t, *J* = 8.5)
3	39.0	2.91 (dd, *J*=13.5, 8.5)
		3.21 (dd, *J*=13.5, 4.5)
1’	139.6	7.19 (m)
2’	129.2	7.19 (m)
3’	130.4	7.19 (m)
4’	127.4	7.19 (m)
5’	130.4	7.19 (m)
6’	129.2	7.19 (m)
1”	172.0	
2”	22.6	1.88 (s)

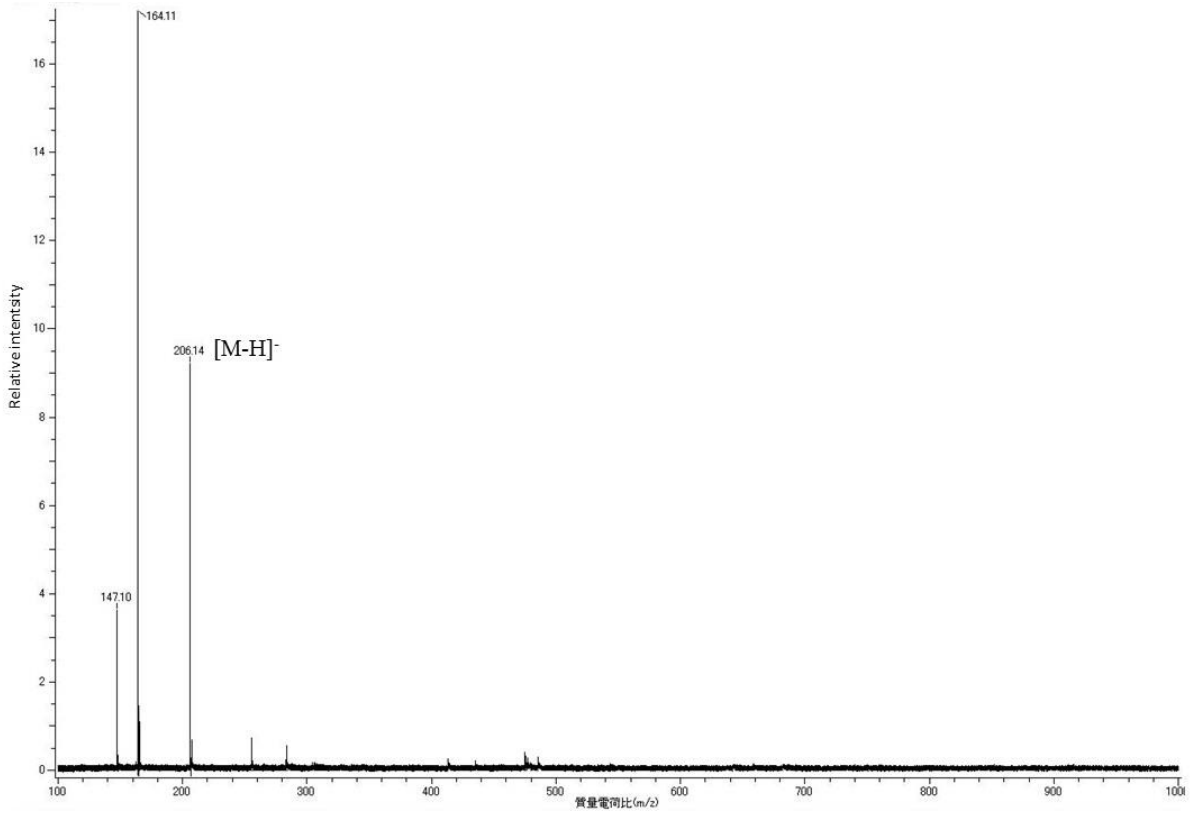


Figure 43 ESIMS spectrum of **8**

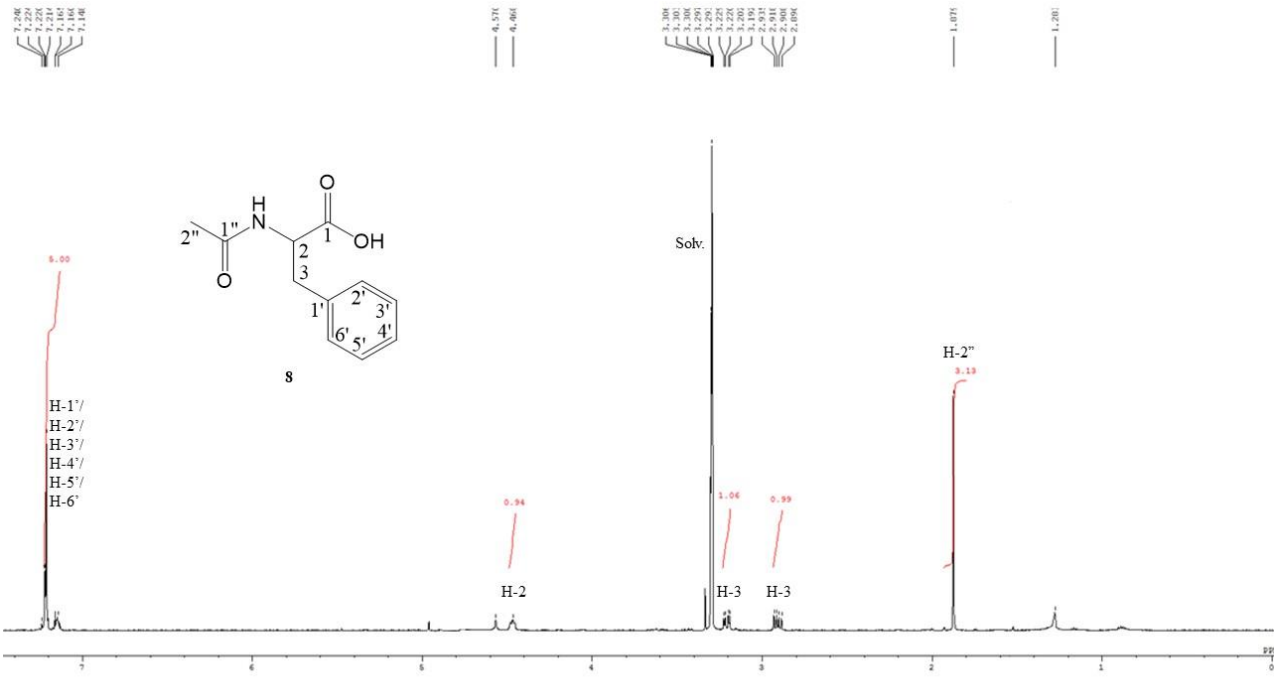


Figure 44 ^{1}H NMR spectrum of **8** (CD_3OD)

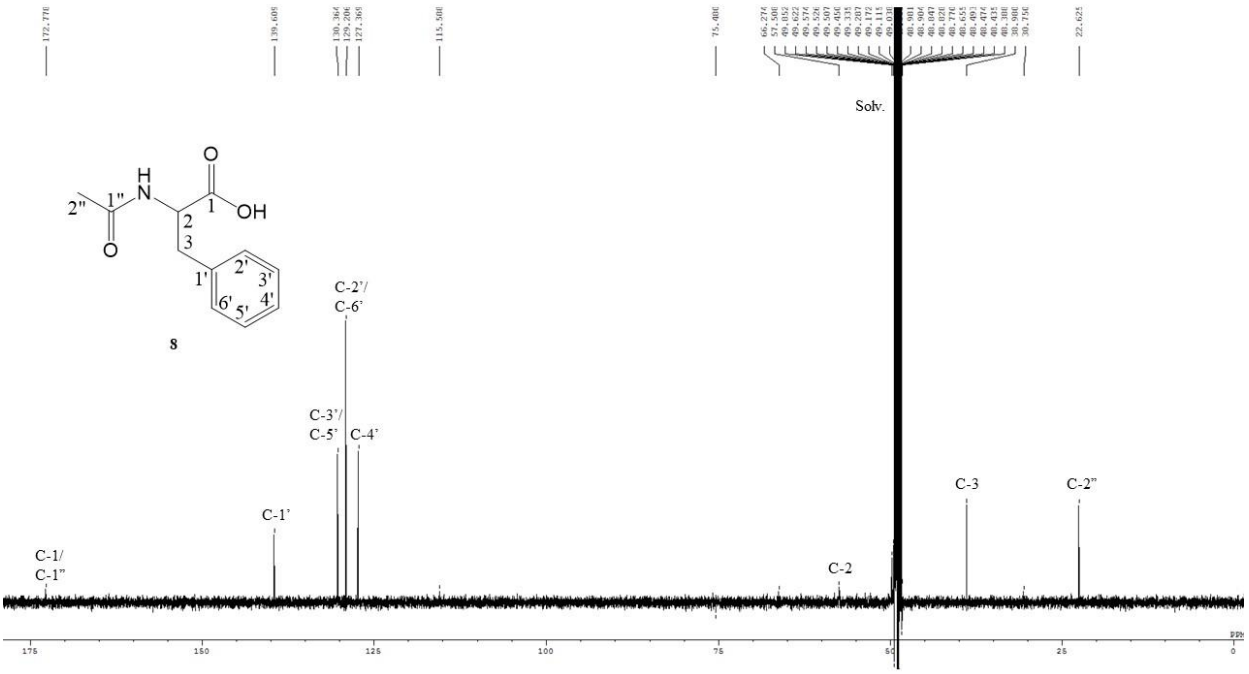


Figure 45 ^{13}C NMR spectrum of **8** (CD_3OD)

2-3 Compound **9**

Compound **9** was isolated as a pale yellow oil. Its molecular formula was determined as $C_{10}H_7NO$ by HRESIMS *m*/*z* 158.0603 $[M+H]^+$ (calcd. for $C_{10}H_8NO$, 158.0600), indicating the presence of eight degrees of unsaturation in the molecule (Figure 47). The planar structure of **9** was elucidated by interpretation of the NMR spectra, including DEPT, COSY, HMBC, and HMQC data. The DEPT experiment indicated the presence of six methines, three quaternary carbons, and one tetrasubstituted carbon. The quinoline structure was constructed by the COSY correlations (H-5/H-6; H-6/H-7; H-7/H-8 and H-2/H-3) and the HMBC correlations (H-2/C-8a; H-3/C-4a, C-8a; H-5/C-7, C-8a' H-6/C-4a, C-8; H-7/C-4a, C-8a; H-8/ C-4a, C-6) (Figures 51 and 52). The presence of aldehyde group at C-4 was suggested by the HMBC correlations (H-1'/C-4, C-4a) (Figure 52). Compound **9** was identified as quinoline-4-carboxaldehyde (Figure 46). Previously, **9** has been isolated as a constituent from leaves of *Ruta chalepensis*. However, there is no report about the isolation of quinoline-4-carboxaldehyde from fungus. The antimicrobial assay showed that quinoline-4-carboxaldehyde strongly inhibited the growth of *Clostridium perfringens* and weakly inhibited the growth of *Escherichia coli.* Also, this compound has been evaluated its potential for managing populations insect pest. [42, 43]

Table 7 ^1H and ^{13}C NMR assignments for **9**

	Compound **9** ($CDCl_3$)	
Position	δ_C	δ_H (multiplicity, *J* in Hz)
1		
2	150.3	9.20 (d, *J* = 4.5)
3	125.8	7.80 (d, *J* = 4.5)
4	137.0	
4a	124.0	
5	124.4	9.01 (d, *J* = 8.5)
6	129.5	7.73 (t, *J* = 7.5)
7	130.3	7.82 (d, *J* = 7.5)
8	129.8	8.22 (d, *J* = 8.0)
8a	149.2	
1'	192.8	10.5 (s)

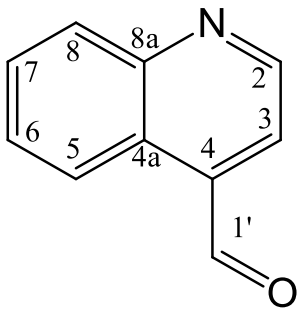


Figure 46 Structure of **9**

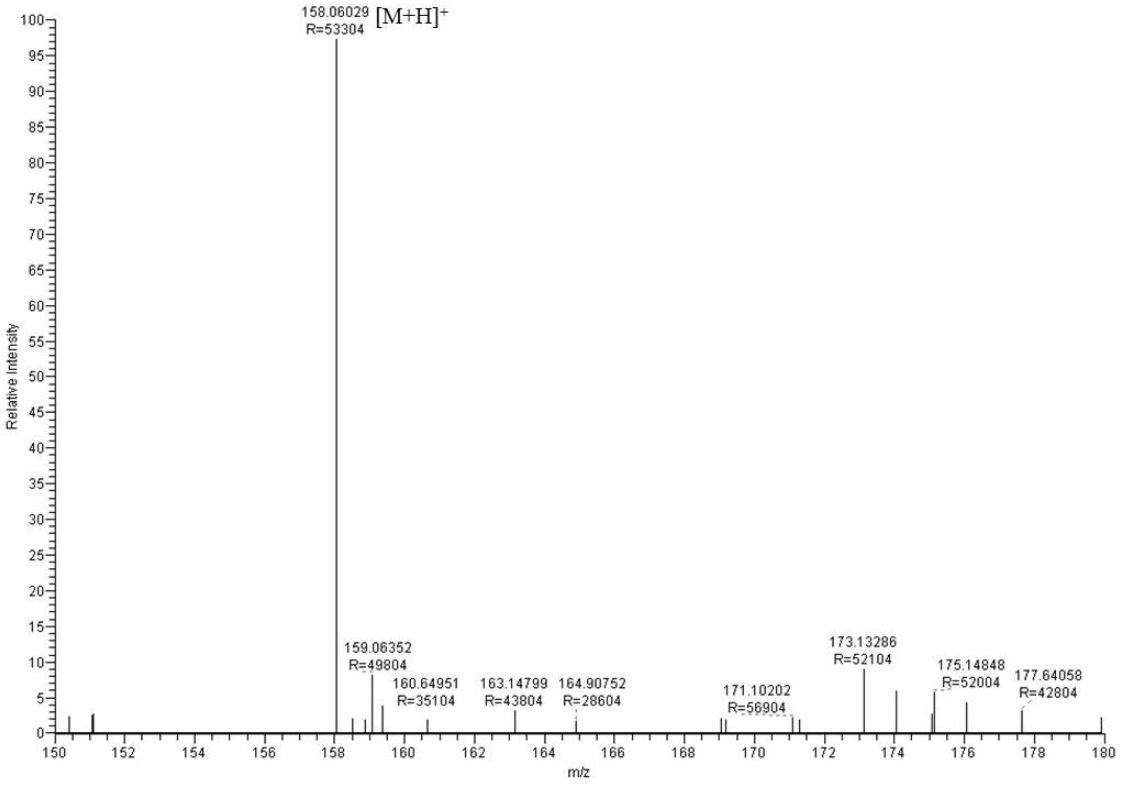


Figure 47 ESIMS spectrum of **9**

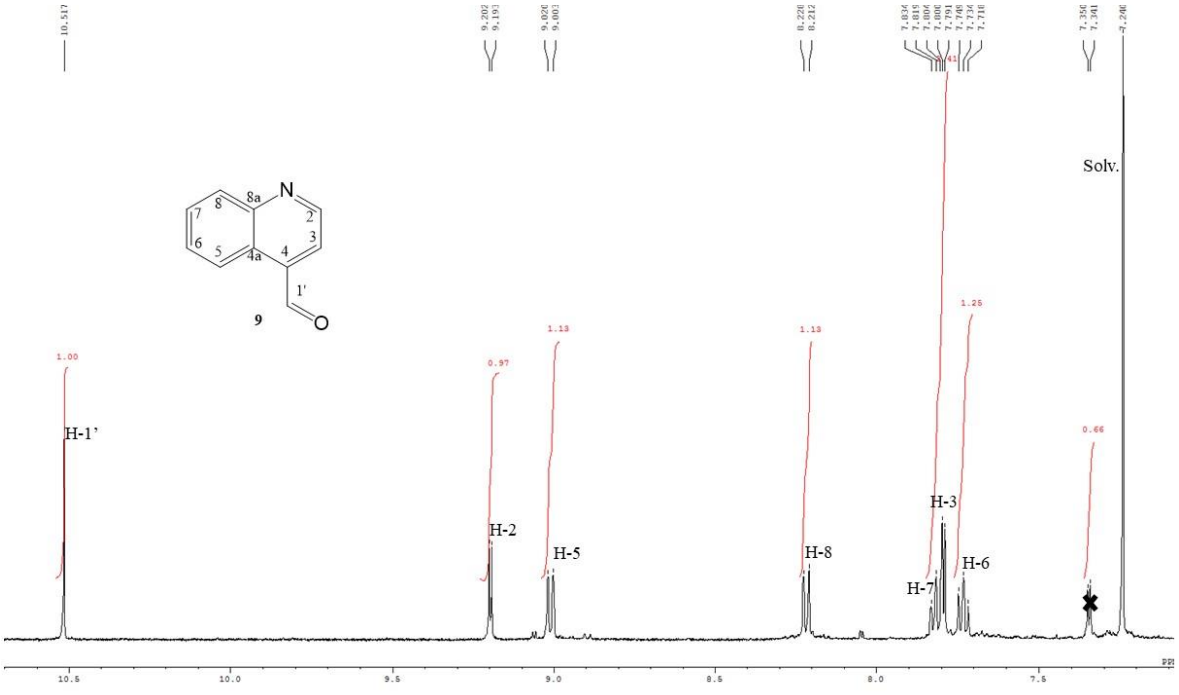


Figure 48 ^{1}H NMR spectrum of **9** ($CDCl_3$)

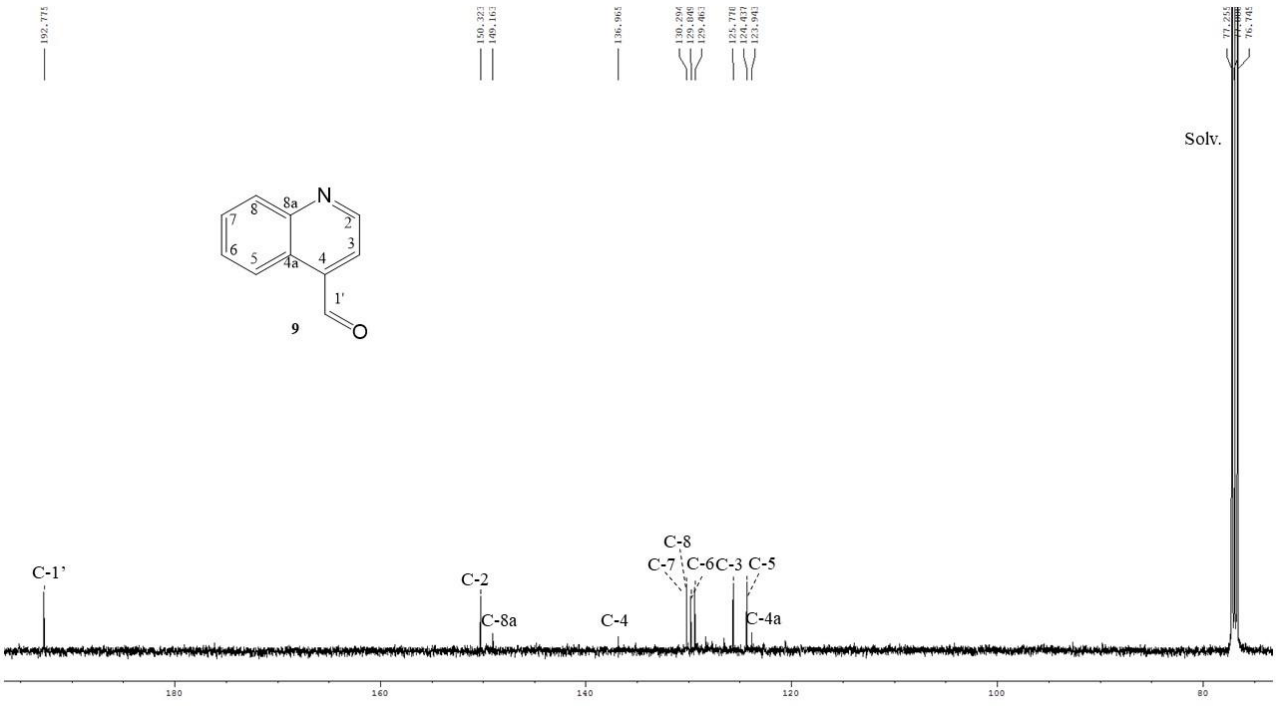


Figure 49 ^{13}C NMR spectrum of **9** ($CDCl_3$)

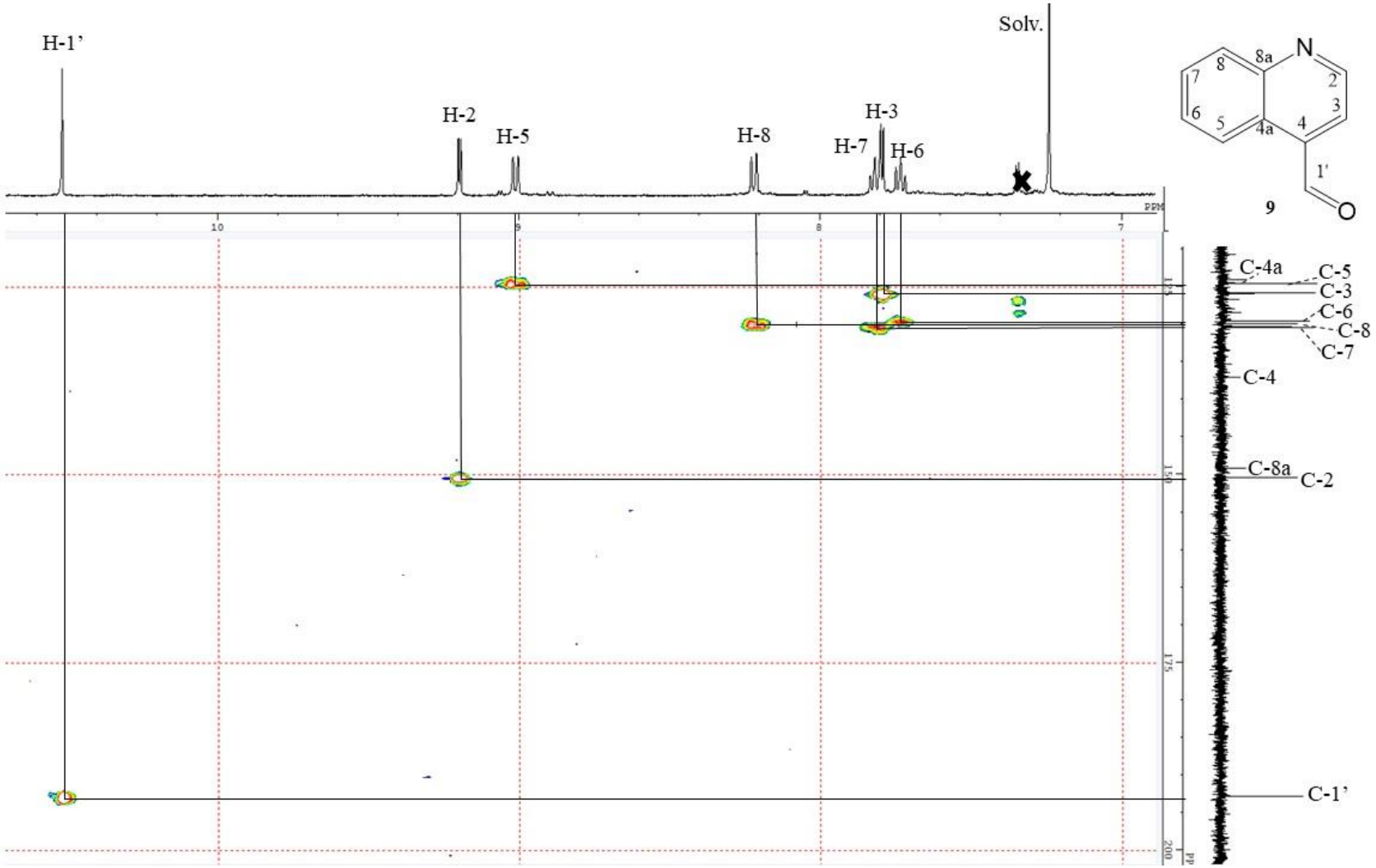


Figure 50 HMQC spectrum of **9** ($CDCl_3$)

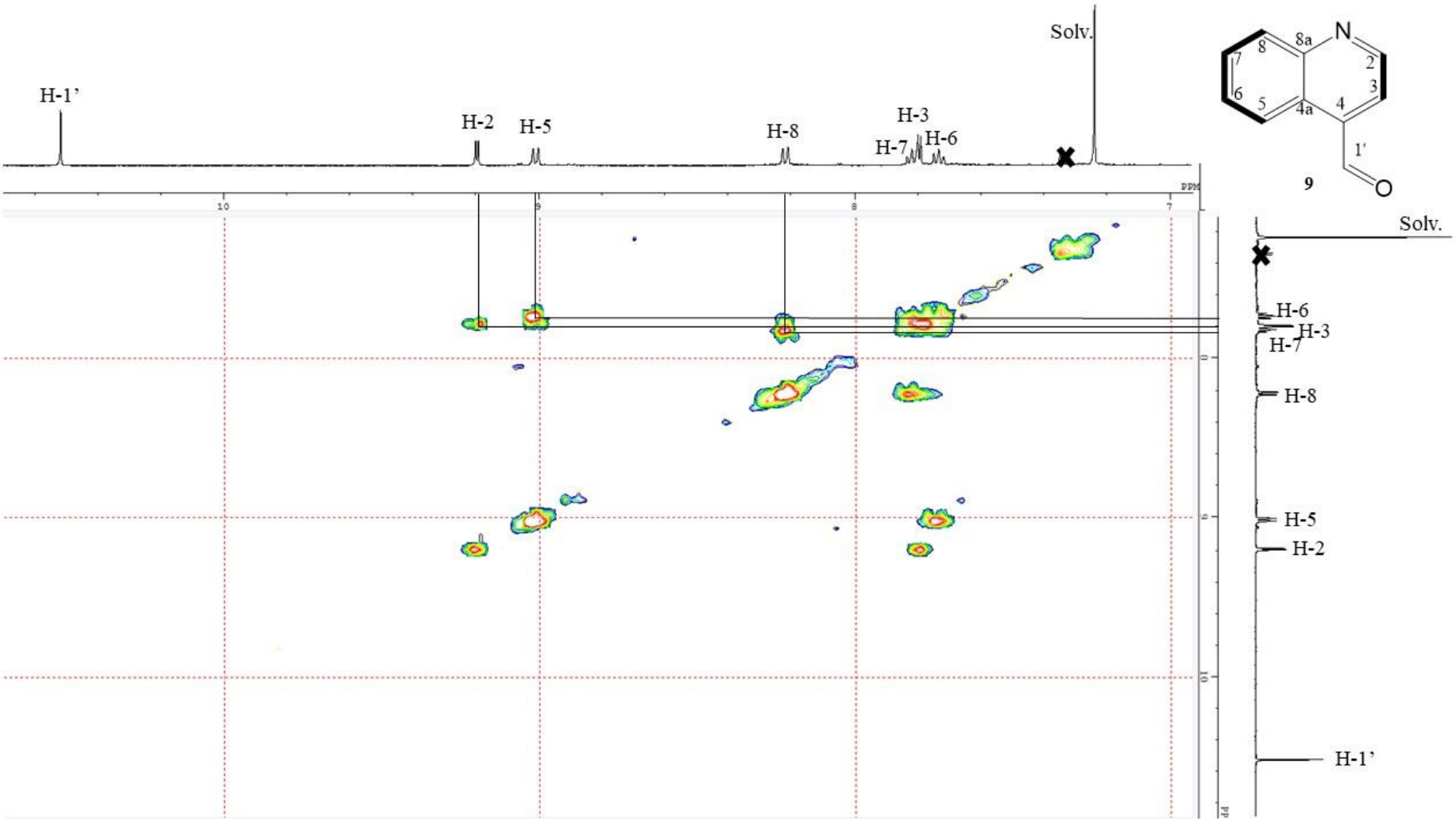


Figure 51 COSY spectrum of **9** ($CDCl_3$)

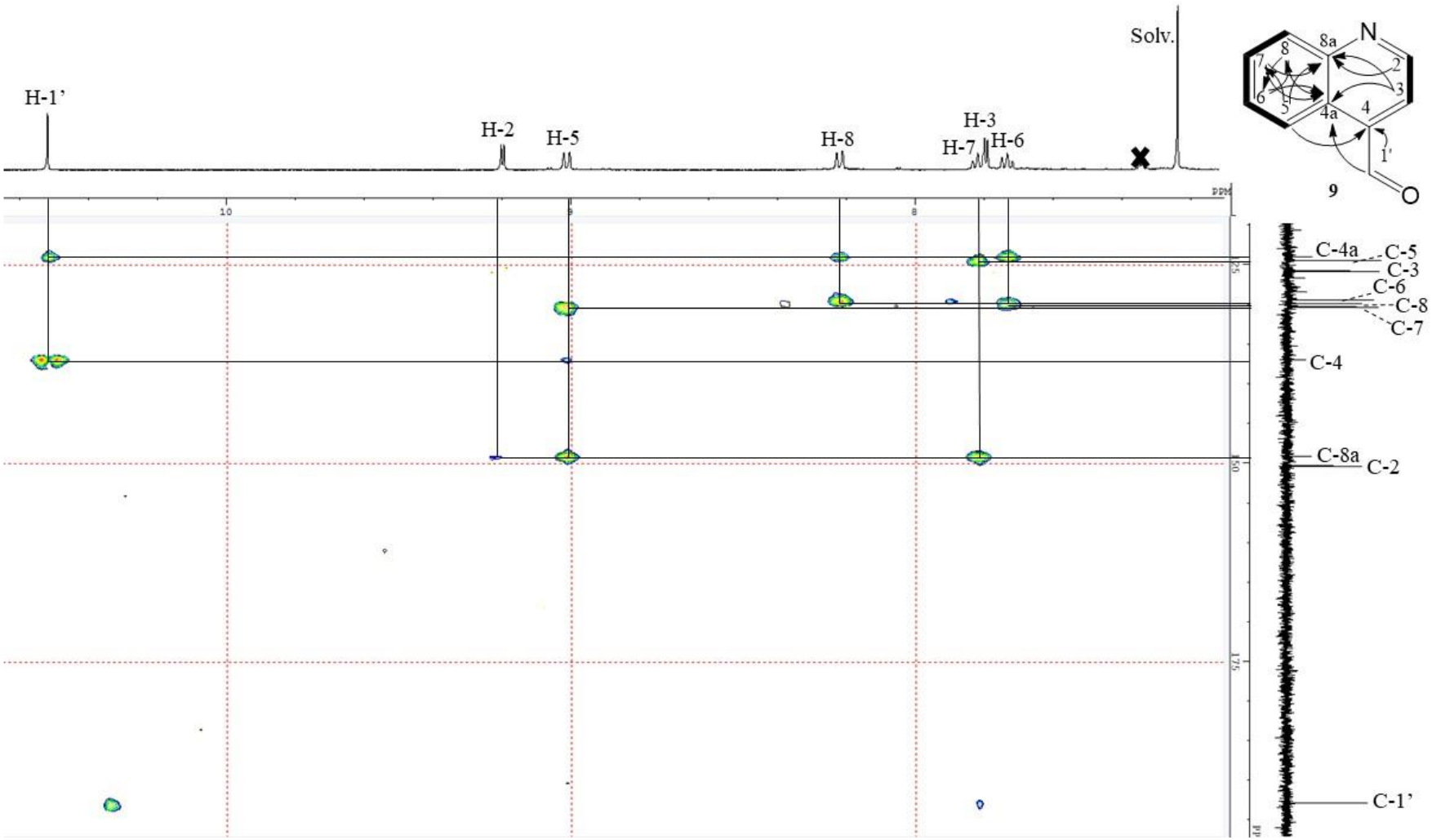


Figure 52 HMBC spectrum of **9** ($CDCl_3$)

2-4 Compound **10**

Compound **10** was isolated as a white amorphous. Its molecular formula was determined as $C_{10}H_7NO_2$ by HRESIMS *m/z* 174.0546 $[M+H]^+$ (calcd. for $C_{10}H_8NO_2$, 174.0550), indicating the presence of eight degrees of unsaturation in the molecule (Figure 54). The planar structure of **10** was elucidated by interpretation of the NMR spectra, including DEPT, COSY, HMBC, and HMQC data. The DEPT experiment indicated the presence of six methines, three quaternary carbons, and one tetrasubstituted carbon. The NMR experiment showed similar data to quinoline-4-carboxaldehyde, except the absence of ^{1}H NMR signal at H-1' (Table 8; Figure 55). This data suggested the presence of carboxyl group by ^{13}C NMR signal δ_C 175.6 (Table 8; Figure 56). The HMBC correlations (H-3/C-1') indicated the group attached at C-4 (Figure 59). Thus, compound **10** was identified as quinoline-4-carboxylic acid. This compound has been isolated from various species and genera of myxobacteria.[43] However, there is no study about the isolation of quinoline-4-carboxylic acid from mushroom has been reported. The bioactivities of **10** has been evaluated on antifungal assay which showed moderate activity against a variety of yeasts and fungi. [44] Meanwhile, it was found that **10** has a potent antidiabetic activity.[45]

Table 8 ^{1}H and ^{13}C NMR assignments for **10**

	Compound **10** (CD_3OD)	
Position	δ_C	δ_H (multiplicity, *J* in Hz)
1		
2	151.0	8.82 (d, *J* = 5.0)
3	120.3	7.63 (d, *J* = 4.5)
4	149.2	
4a	126.5	
5	128.0	8.53 (d, *J* = 8.0)
6	128.0	7.59 (t, *J* = 7.5)
7	130.9	7.75 (t, *J* = 15.5)
8	129.2	8.02 (d, *J* = 11.5)
8a	149.2	
1'	175.6	

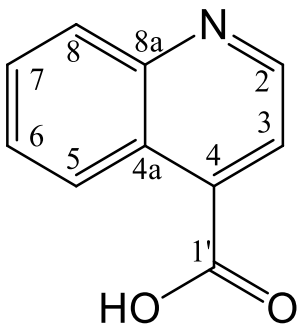


Figure 53 Structure of **10**

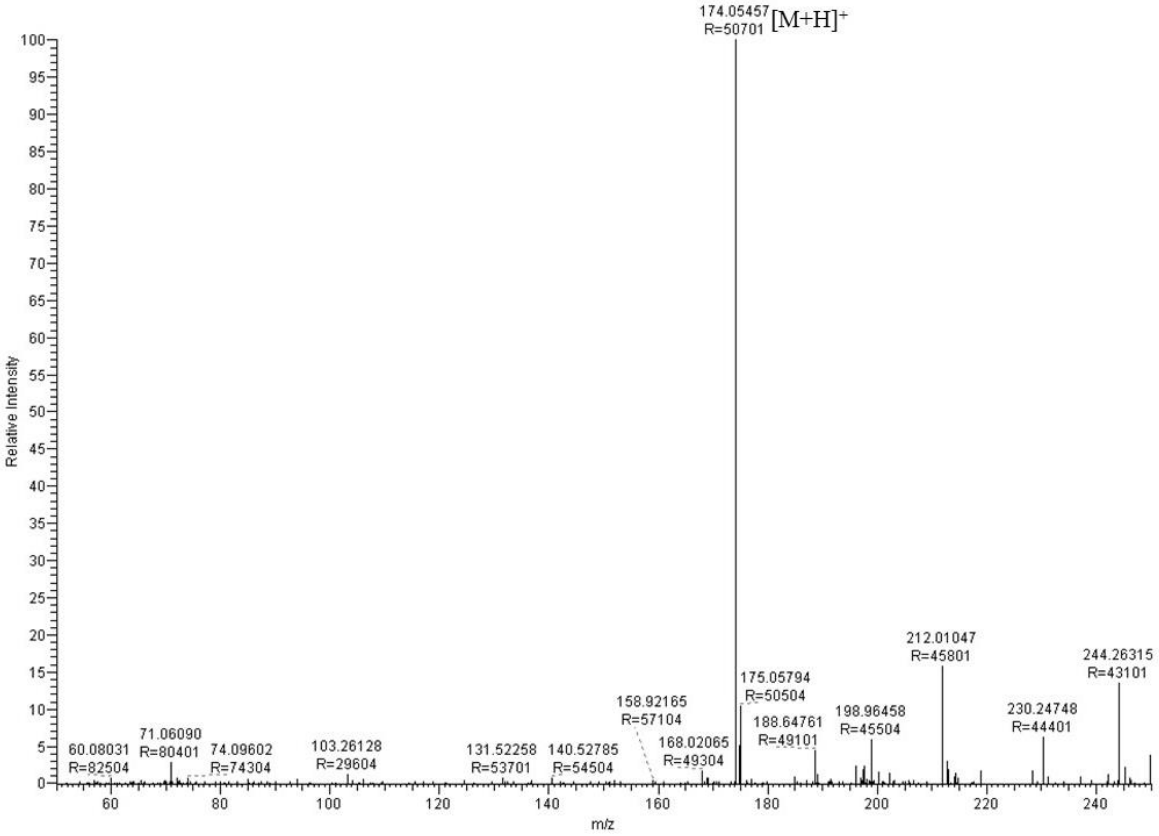


Figure 54 ESIMS spectrum of **10**

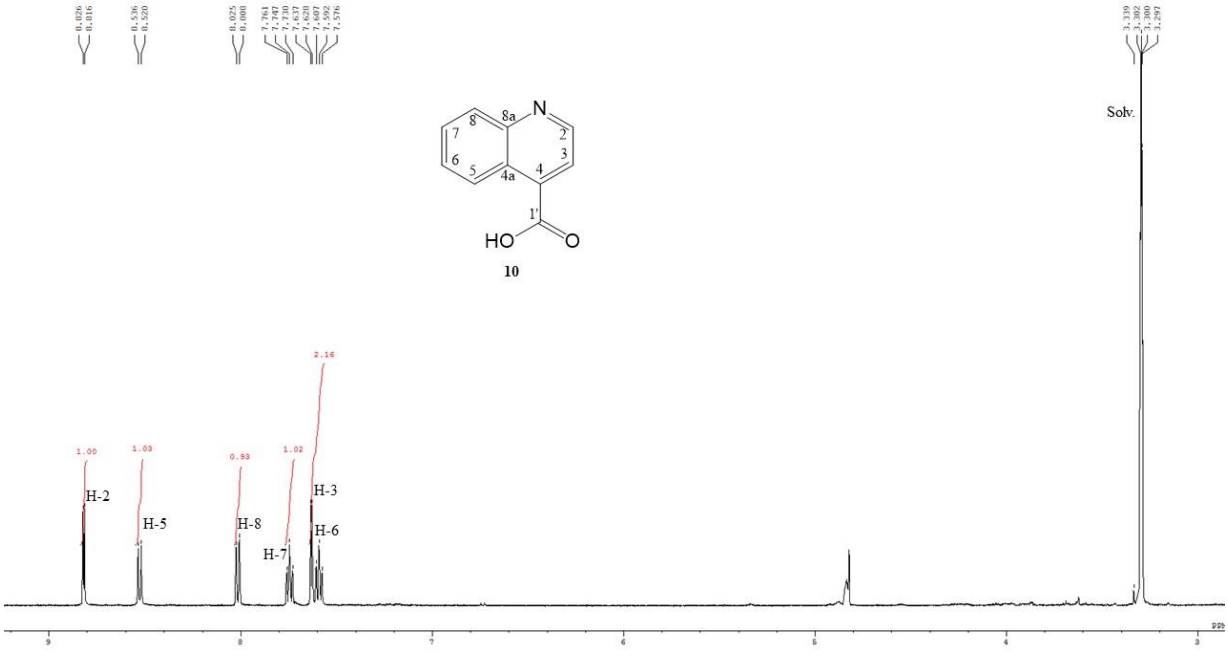


Figure 55 ^{1}H NMR spectrum of **10** (CD_3OD)

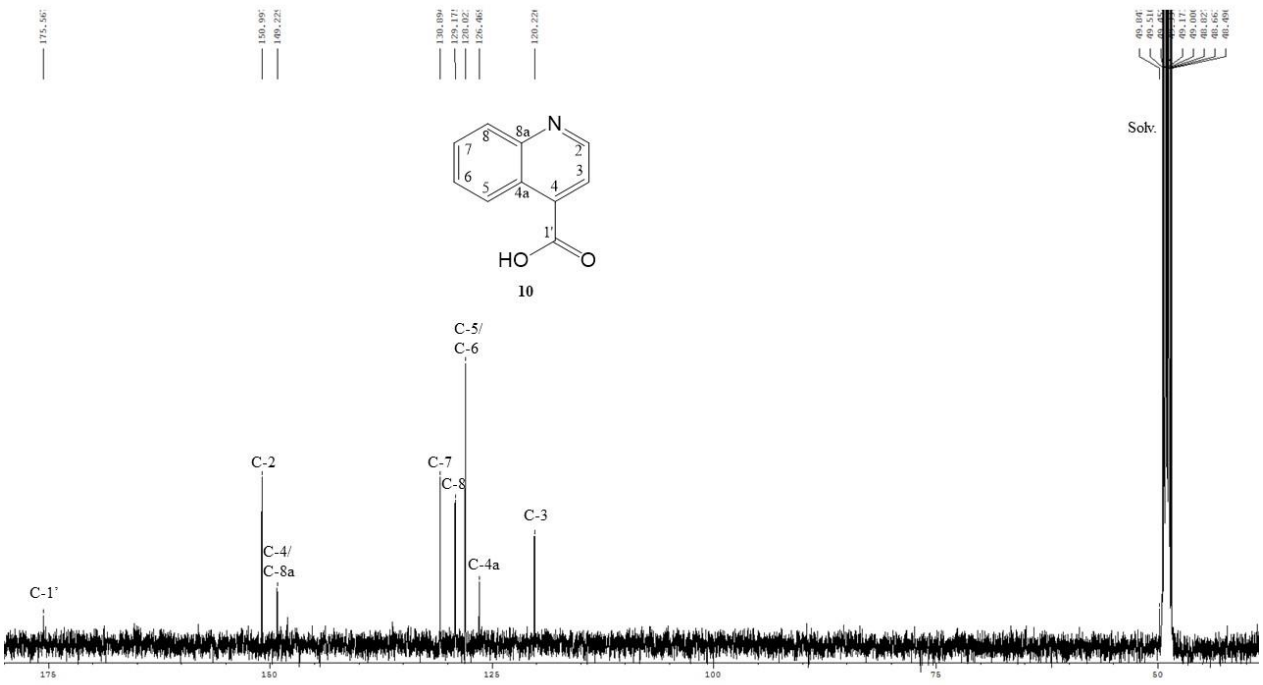


Figure 56 ^{13}C NMR spectrum of **10** (CD_3OD)

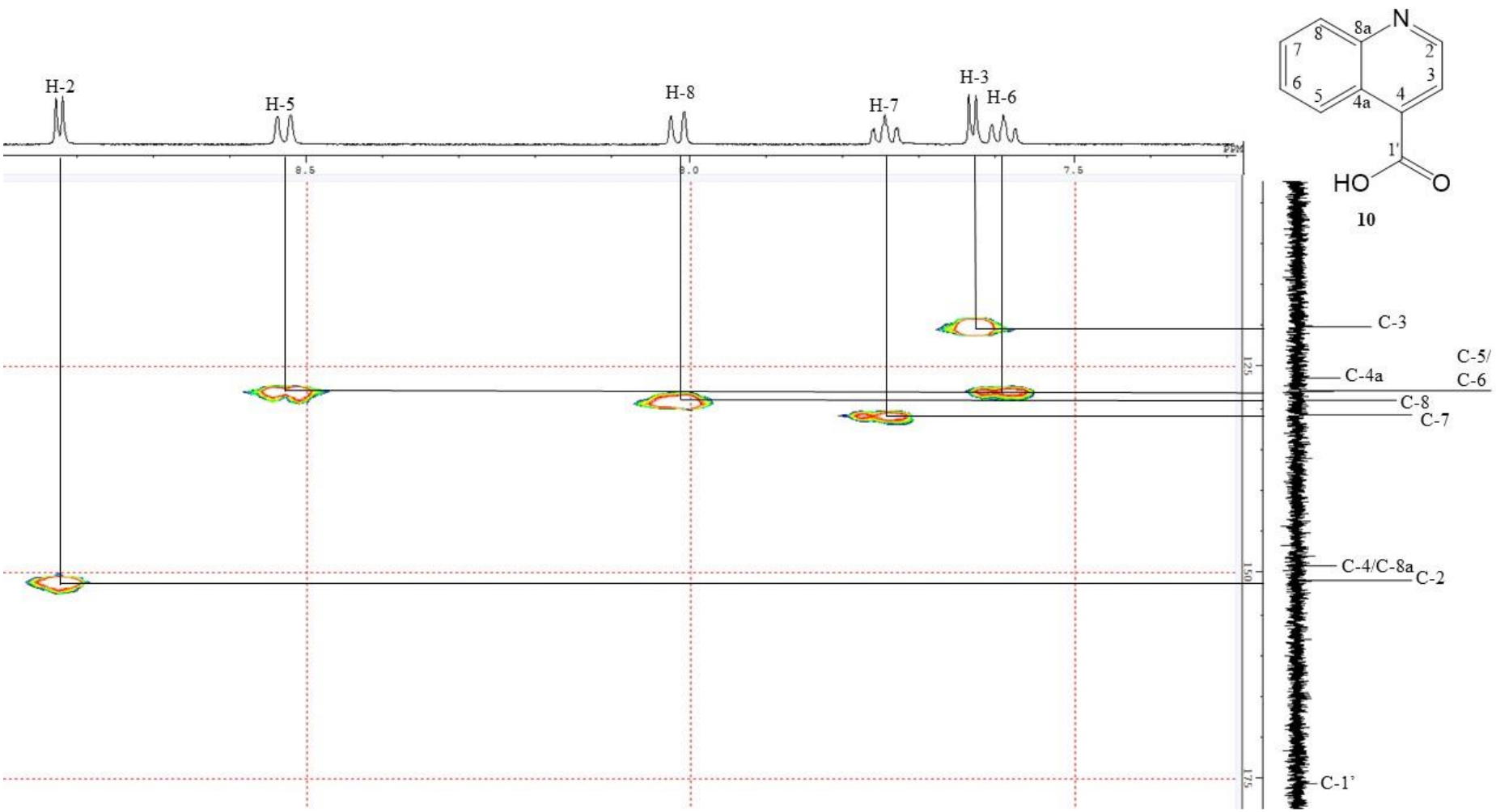


Figure 57 HMQC spectrum of **10** (CD_3OD)

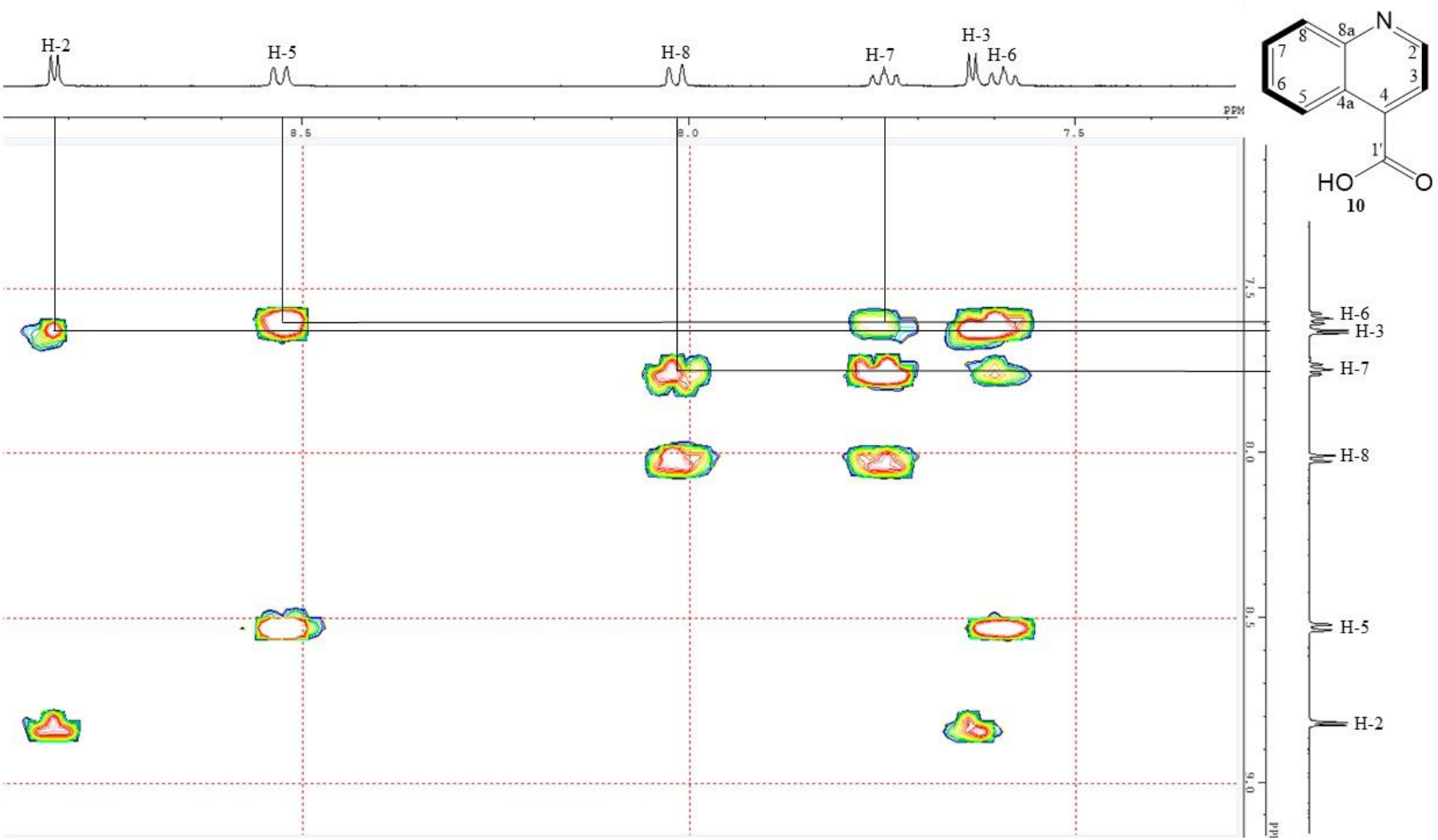


Figure 58 COSY spectrum of **10** (CD_3OD)

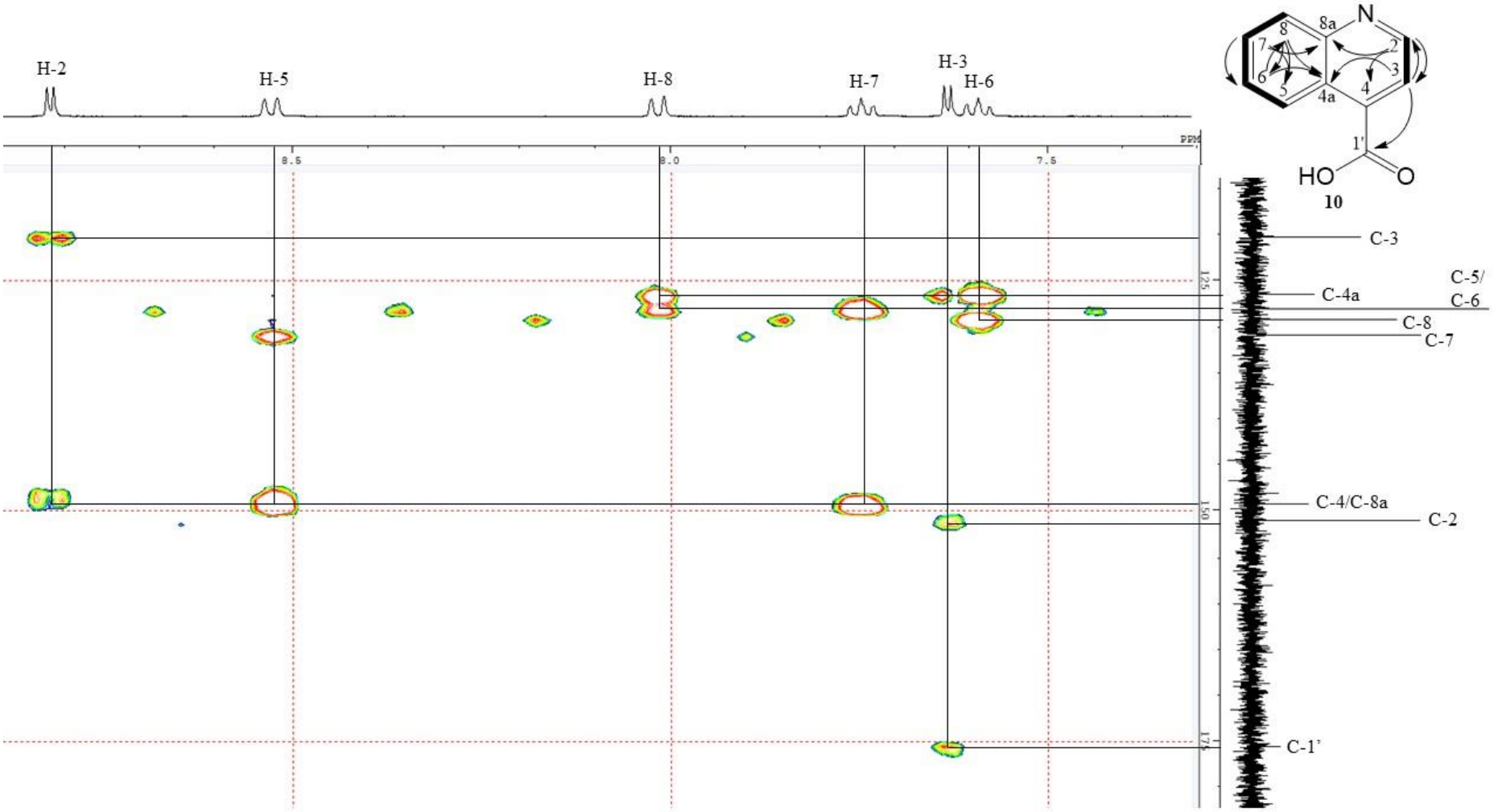


Figure 59 HMBC spectrum of **10** (CD_3OD)

2-5 Compound **11**

Compound **11** was isolated as a colorless oil. Its molecular formula was determined as $C_8H_{10}O_2$ by HRESIMS *m/z* 161.0578 $[M+Na]^+$ and 137.0603 $[M-H]^-$ (calcd. for $C_8H_{10}O_2Na$, 161.0573; $C_8H_9O_2$, 137.0597), indicating the presence of four degrees of unsaturation in the molecule (Figures 61 and 62). Compound **11** was identified by the interpretation of ESIMS and NMR spectra and their comparison to its reported analogue.[46] Also, the optical rotation of **11** was measured as $[\alpha]_D^{25}$ -43 (*c* 0.10, $CHCl_3$) and compared to the reported one { $[\alpha]_D$ -64 (*c* 0.82, $CHCl_3$) }.[47] Compound **11** was identified as a known compound (*R*)-1-phenylethane-1,2-diol (Figure 60). In this study, compound **11** was isolated as a natural compound for the first time.

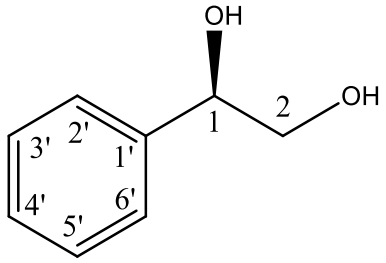


Figure 60 Structure of **11**

Table 9 ^1H and ^{13}C NMR assignments for **11**

Compound **11** ($CDCl_3$)		
Position	δ_C	δ_H (multiplicity, *J* in Hz)
1	74.5	4.82 (dd, *J* = 8.0, 3.5)
2	68.1	3.76 (dd, *J* = 11.0, 3.0)
		3.62 (dd, *J* = 11.5, 6.5)
1’	140.6	
2’	126.1	7.33 (m)
3’	128.6	7.33 (m)
4’	128.1	7.33 (m)
5’	128.6	7.33 (m)
6’	126.1	7.33 (m)

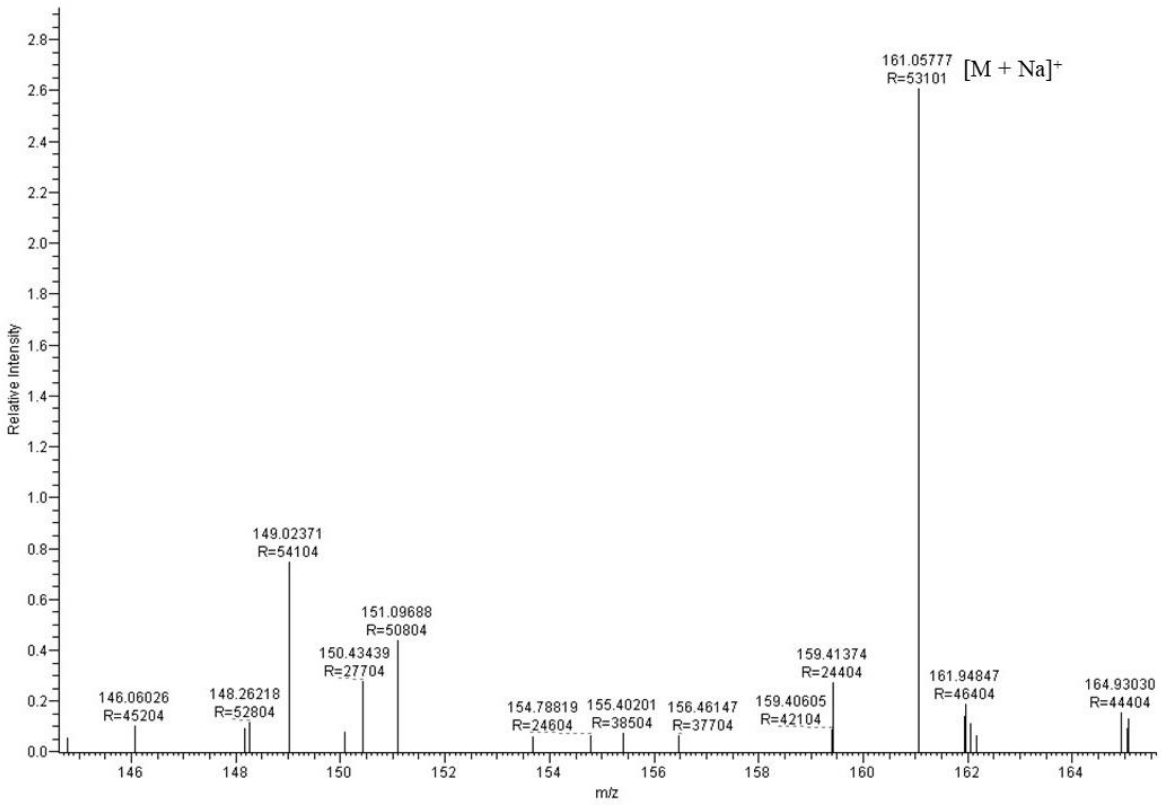


Figure 61 ESIMS spectrum of **11 (**[M+Na]$^{+}$)

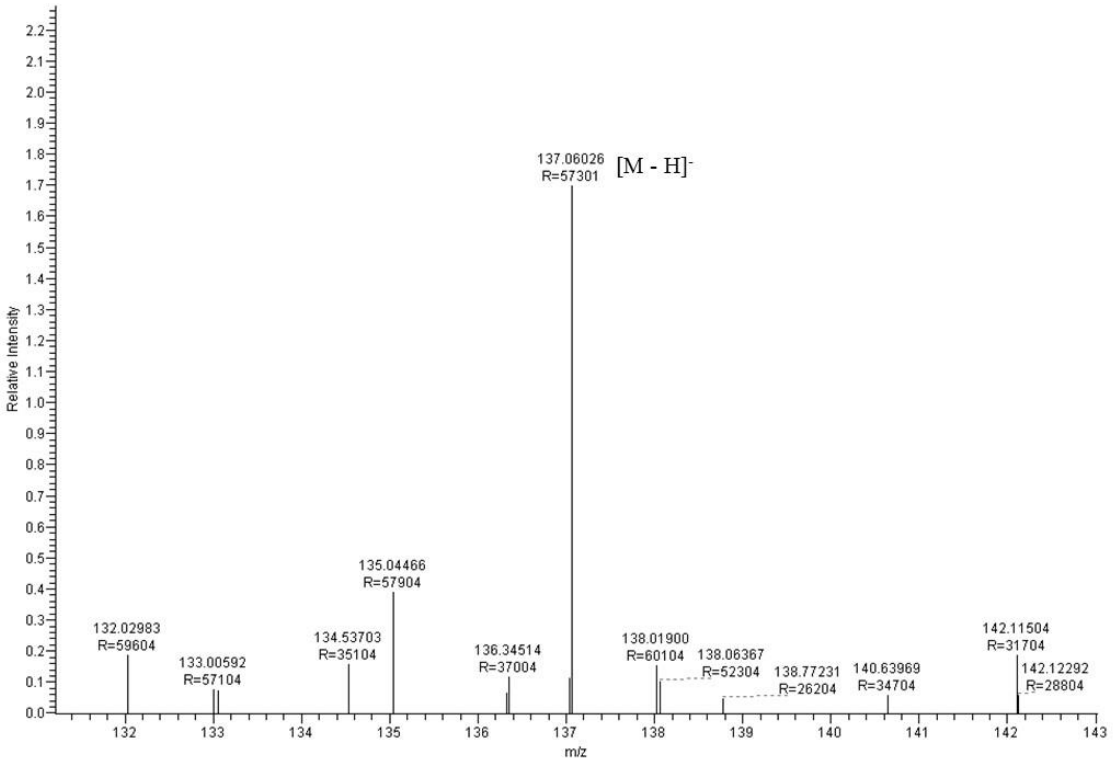


Figure 62 ESIMS spectrum of **11 (**[M-H]$^{-}$)

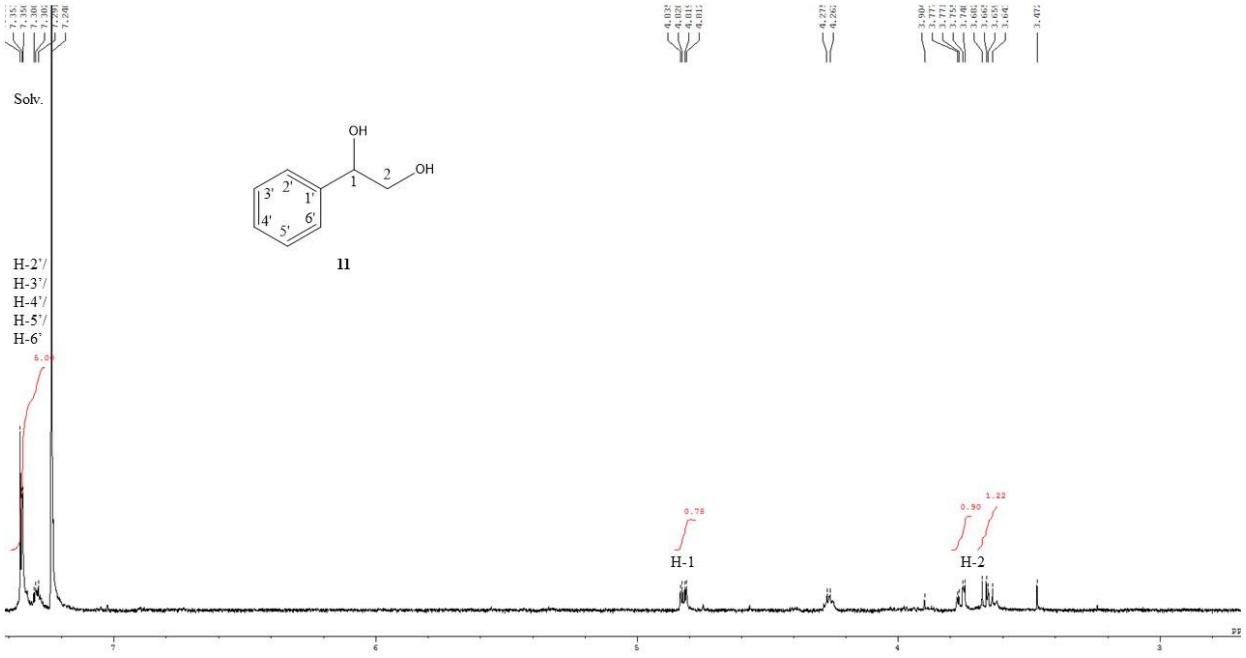


Figure 63 ^{1}H NMR spectrum of **11** ($CDCl_3$)

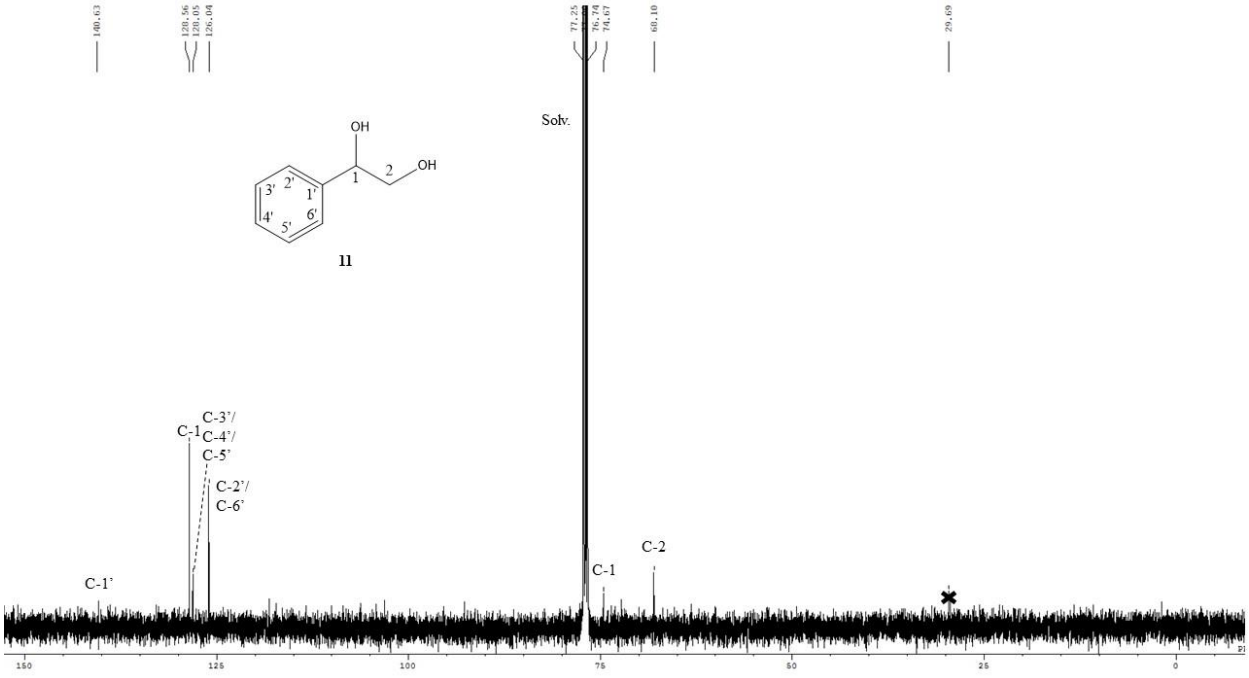


Figure 64 ^{13}C NMR spectrum of **11** ($CDCl_3$)

2-6 Compound **12**

Compound **12** was isolated as a colorless oil. Its molecular formula was determined as $C_9H_{12}O_2$ by HRESIMS *m/z* 175.0774 $[M+Na]^+$ (calcd. for $C_9H_{12}O_2Na$, 175.0735), indicating the presence of four degrees of unsaturation in the molecule (Figure 66). The planar structure of **12** was elucidated by interpretation of the NMR spectra, including DEPT, COSY, HMBC, and HMQC data. The DEPT experiment indicated the presence of one methyl, seven methines and one quaternary carbon. The NMR experiments showed compound **12** has similar data to compound **11**, except the presence of the NMR signals for δ_H 1.07 (d, *J*=6.5) and δ_C 17.3 indicated the presence of a methyl group (Table 10; Figures 67 and 68). Also, the presence of the hydroxylated methine was suggested by the ^{1}H NMR signal at δ_H 4.00 (m) (Table 10; Figure 67). The optical rotation of **12** was measured as $[\alpha]_D^{26}$ -20 (*c* 0.57, $CHCl_3$) and compared to the reported one { $[\alpha]_D^{25}$ -35 (*c* 1.0, $CHCl_3$) }.[48] Compound **12** was identified as a known compound (*1R*,*2S*)-1-phenylpropane-1,2-diol (Figure 65). It has been reported that compound **12** was isolated from the fungus *Trametes* sp. and the wood-decaying fungus species, *Xylaria* cf. *cubensis*.[48, 49]

Table 10 ^{1}H and ^{13}C NMR assignments for **12**

	Compound **12** ($CDCl_3$)	
Position	δ_C	δ_H (multiplicity, *J* in Hz)
1	77.5	4.66 (d, *J* = 4.5)
2	71.3	4.00 (m)
3	17.3	1.07 (d, *J*=6.5)
1’	140.3	
2’	126.6	7.29 (m)
3’	128.4	7.29 (m)
4’	127.9	7.29 (m)
5’	128.4	7.29 (m)
6’	126.6	7.29 (m)

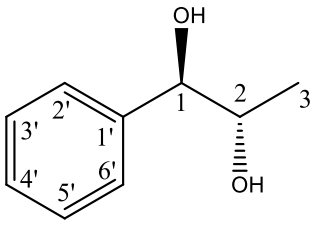


Figure 65 Structure of **12**

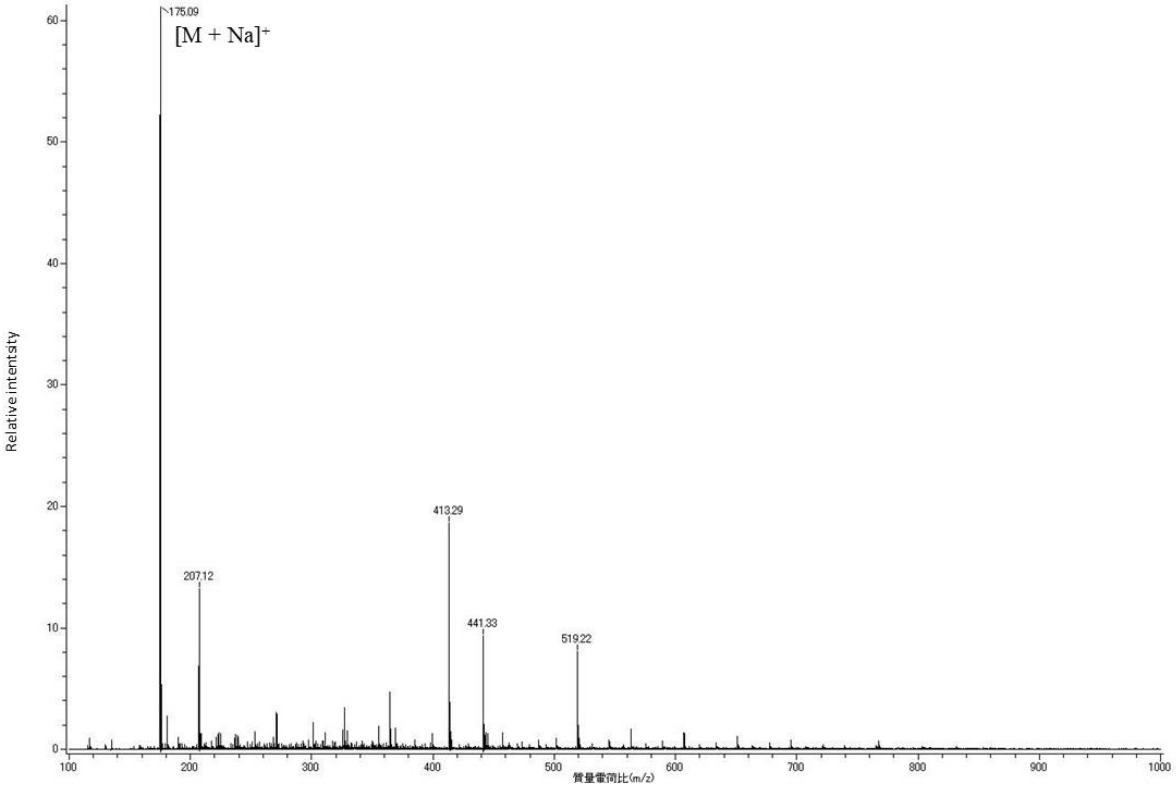


Figure 66 ESIMS spectrum of **12**

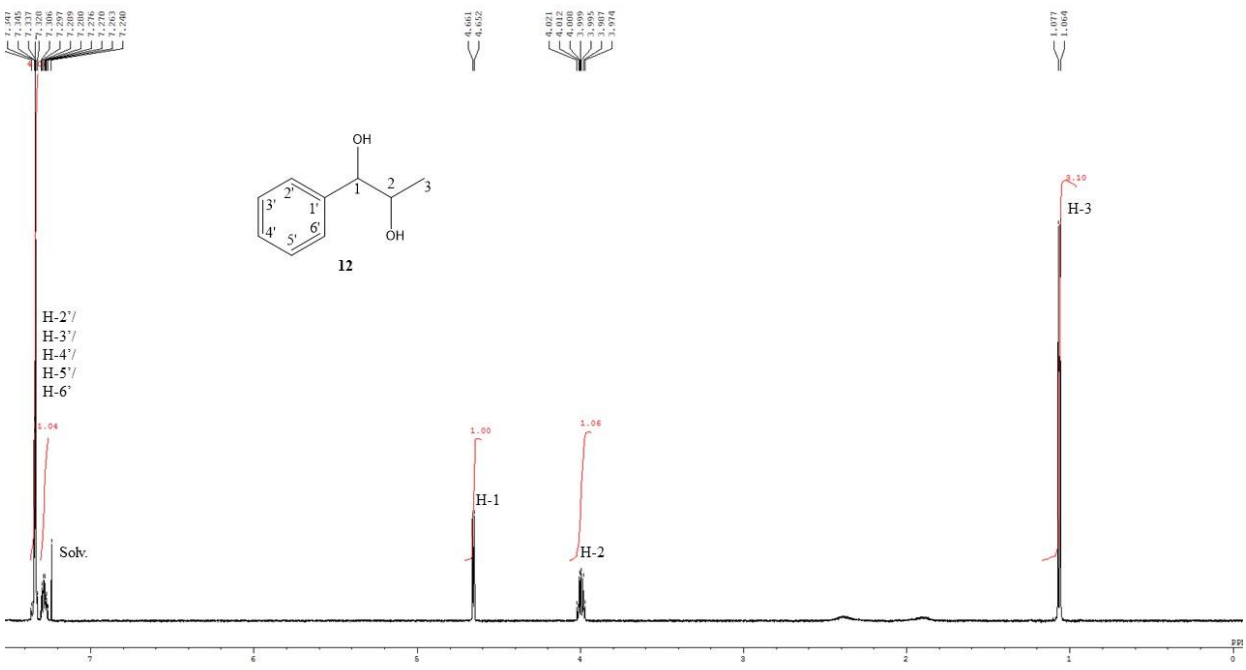


Figure 67 ^{1}H NMR spectrum of **12** ($CDCl_3$)

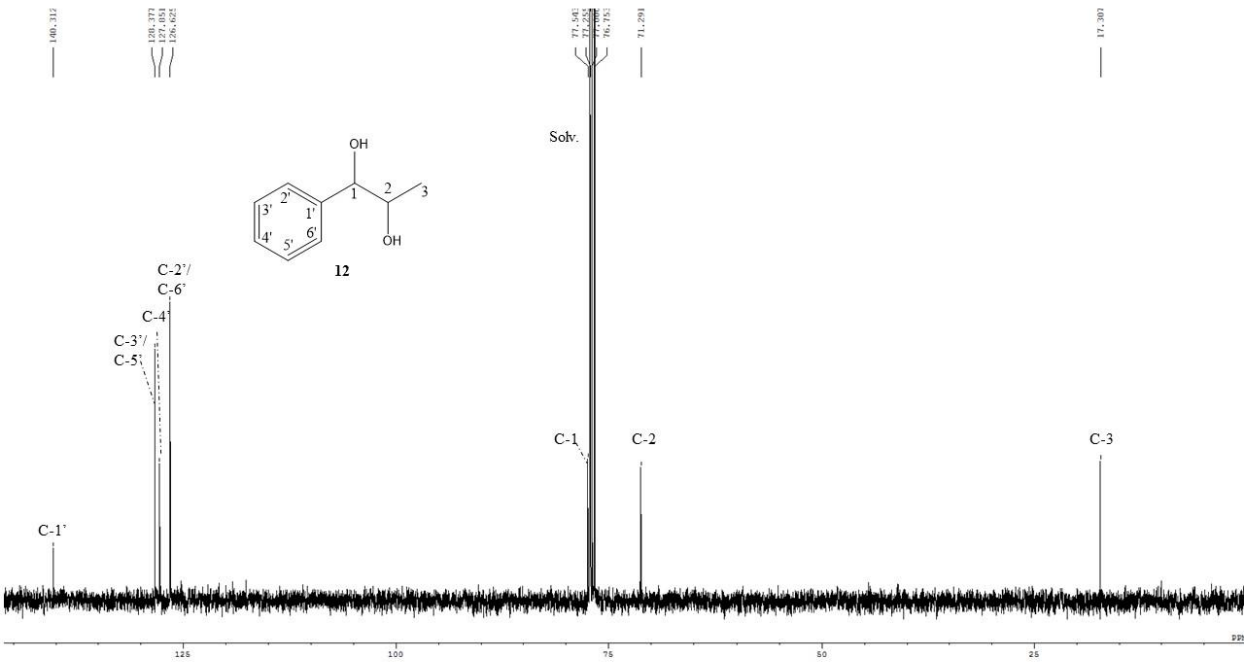


Figure 68 ^{13}C NMR spectrum of **12** ($CDCl_3$)

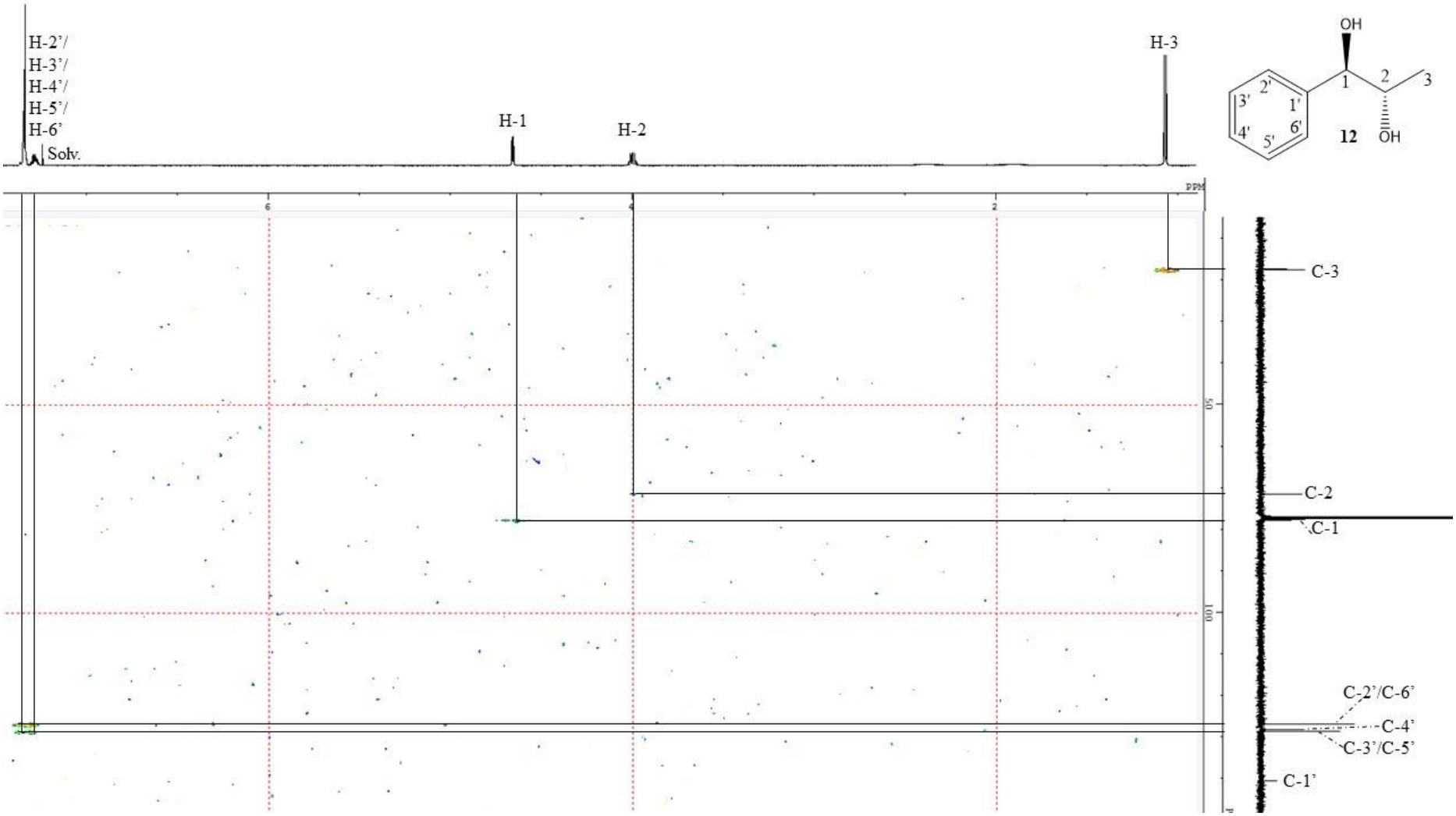


Figure 69 HMQC spectrum of **12** ($CDCl_3$)

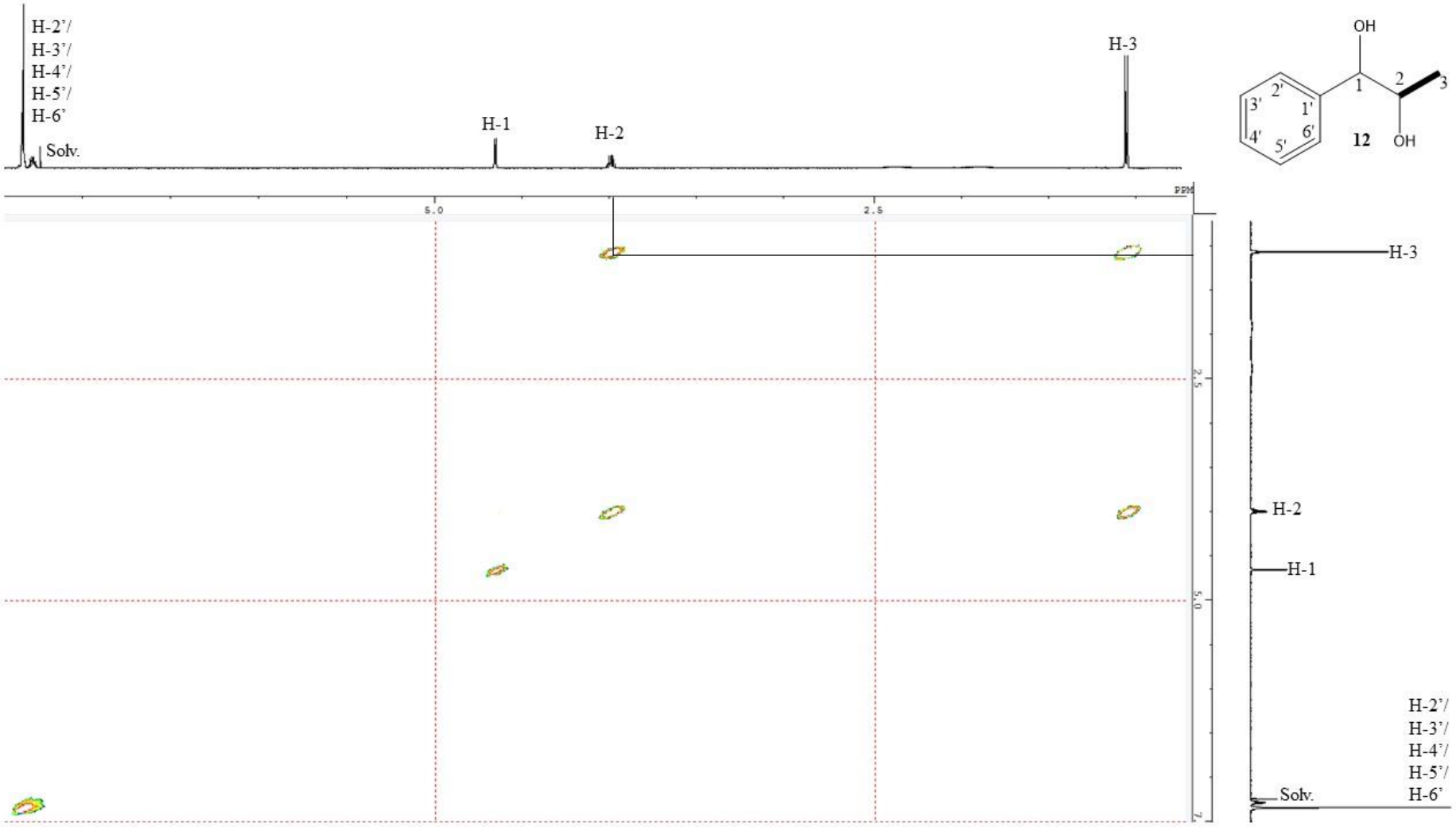


Figure 70 COSY spectrum of **12** ($CDCl_3$)

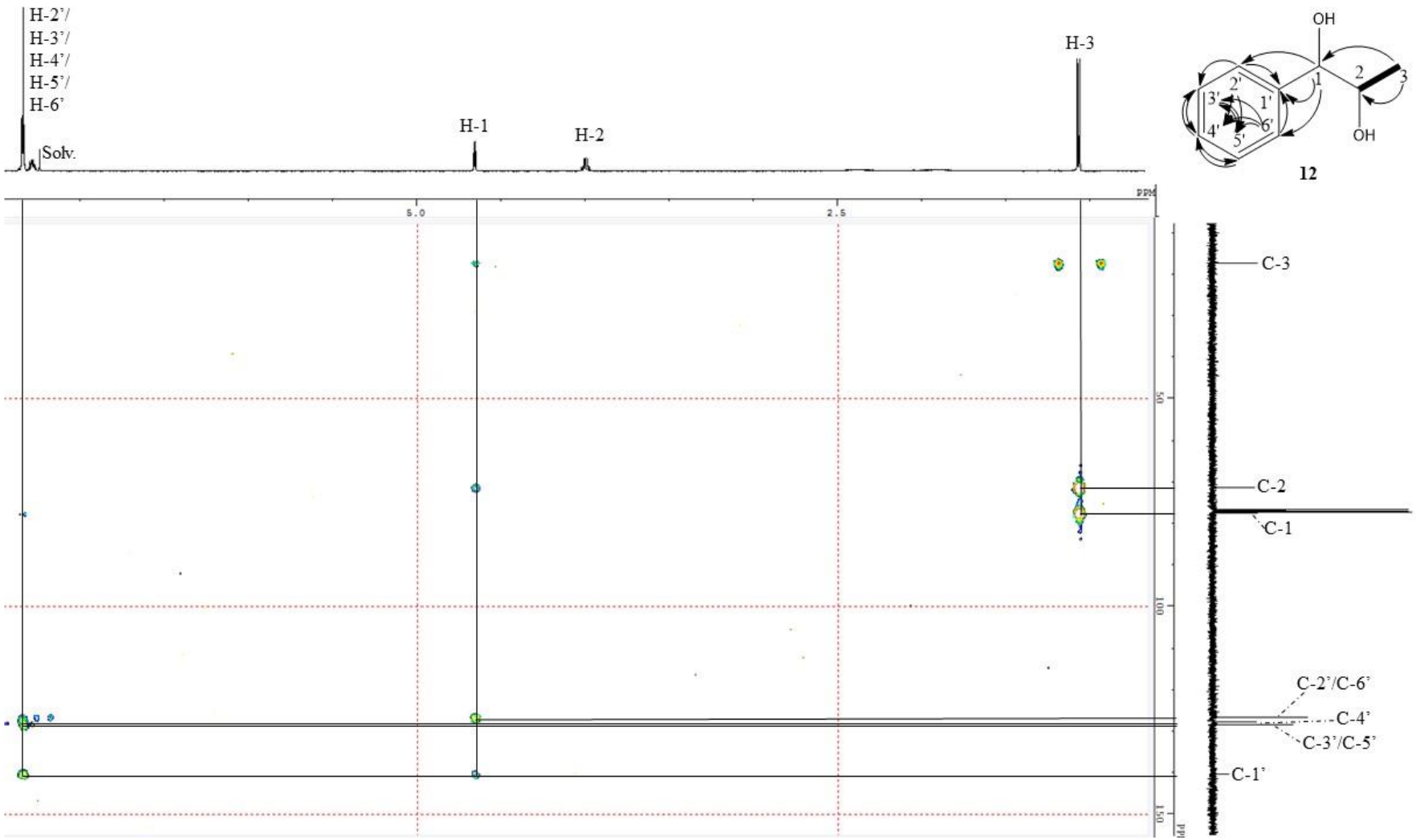


Figure 71 HMBC spectrum of **12** ($CDCl_3$)

2-7 Compound **13**

Compound **13** was isolated as pale yellow oil. Its molecular formula was determined as $C_6H_{10}O_3$ by HRESIMS *m/z* 131.0686 $[M+H]^+$ (calcd. for $C_6H_{11}O_3$, 174.0703), indicating the presence of one degree of unsaturation in the molecule (Figure 73). The planar structure of **13** was elucidated by interpretation of the NMR spectra, including DEPT, COSY, HMBC, and HMQC data. The DEPT experiment indicated the presence of one methyl, two methylenes, two methines, and one tetrasubstituted carbon. The COSY correlations (H-2/H-3; H-3/H-4) and HMBC correlations (H-2/C-1, C-3, C-4; H-3/C-1, C-2, C-4; H-4/C-1) suggested the structure corresponding to dihydrofuran-2-(3*H*)-one (Figures 77 and 78). The ^{1}H NMR signal of δ_H 4.10 (m) indicated the presence of a hydroxylated methine at C-5 (Figure 74; Table 11) and the HMBC correlations of H-3/C-5; H-4/C-5 and H-5/C-4 confirmed it attached at C-4 (Figure 78). The COSY correlation of H-5/H-6 and the HMBC correlations of H-5/C-6; H-6/C-4 and H-6/C-5 confirmed a methyl group attached at C-5 (Figures 77 and 78). The absolute configuration of **13** was determined by comparing its specific rotation, $[\alpha]_D^{25}$ -7 (*c* 0.42, in $CHCl_3$) with that of the previously reported compound { $[\alpha]_D^{20}$ -9 (*c* 4.0, in $CHCl_3$) }.[50] Therefore, compound **13** was identified as (4*R*,5*S*)-5-hydroxyhexan-4-olide (Figure 72). This compound has been isolated from marine derived endophatic fungus, *Penicillium commune*, and the whole plant of *Osmunda japonica*. Compound **13** was reported having antifeeding activities for the larvae of the yellow butterfly, *Eurema hecabe mandarina.*[51, 52]

Table 11 ^{1}H and ^{13}C NMR assignments for **13**

	Compound **13** ($CDCl_3$)	
Position	δ_C	δ_H (multiplicity, *J* in Hz)
1	177.4	
2	28.6	2.53 (m)
3	21.0	2.15 (m)
4	83.4	4.39 (m)
5	67.4	4.10 (m)
6	17.7	1.18 (d, *J* = 6.5)

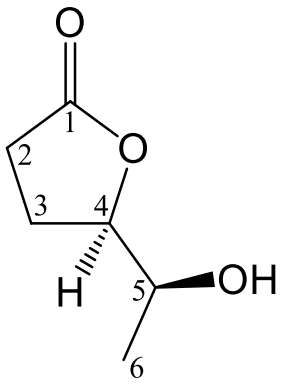


Figure 72 Structure of **13**

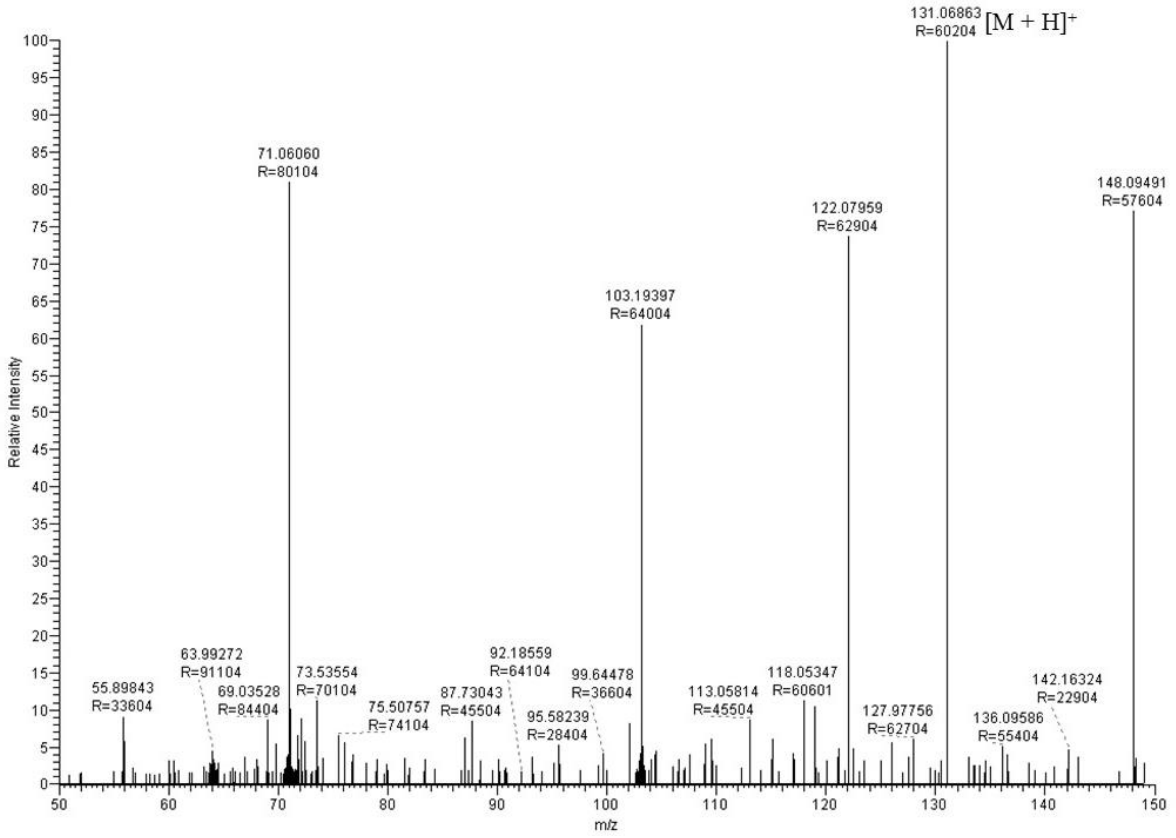


Figure 73 ESIMS spectrum of **13**

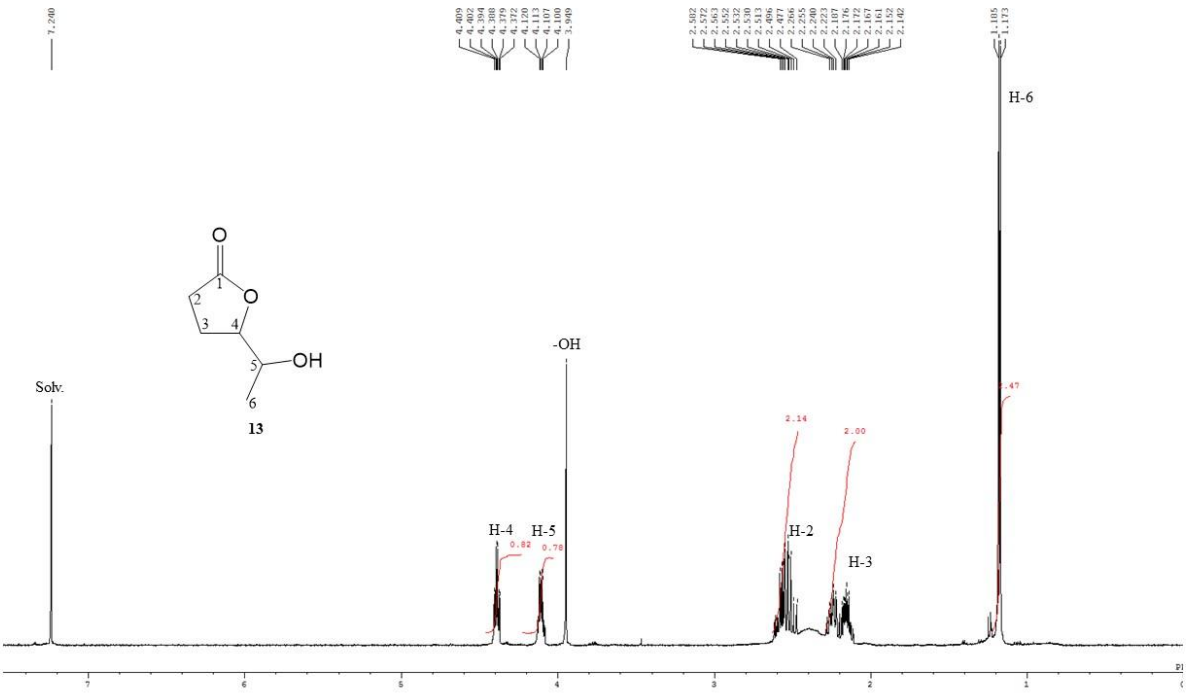


Figure 74 ^{1}H NMR spectrum of **13** ($CDCl_3$)

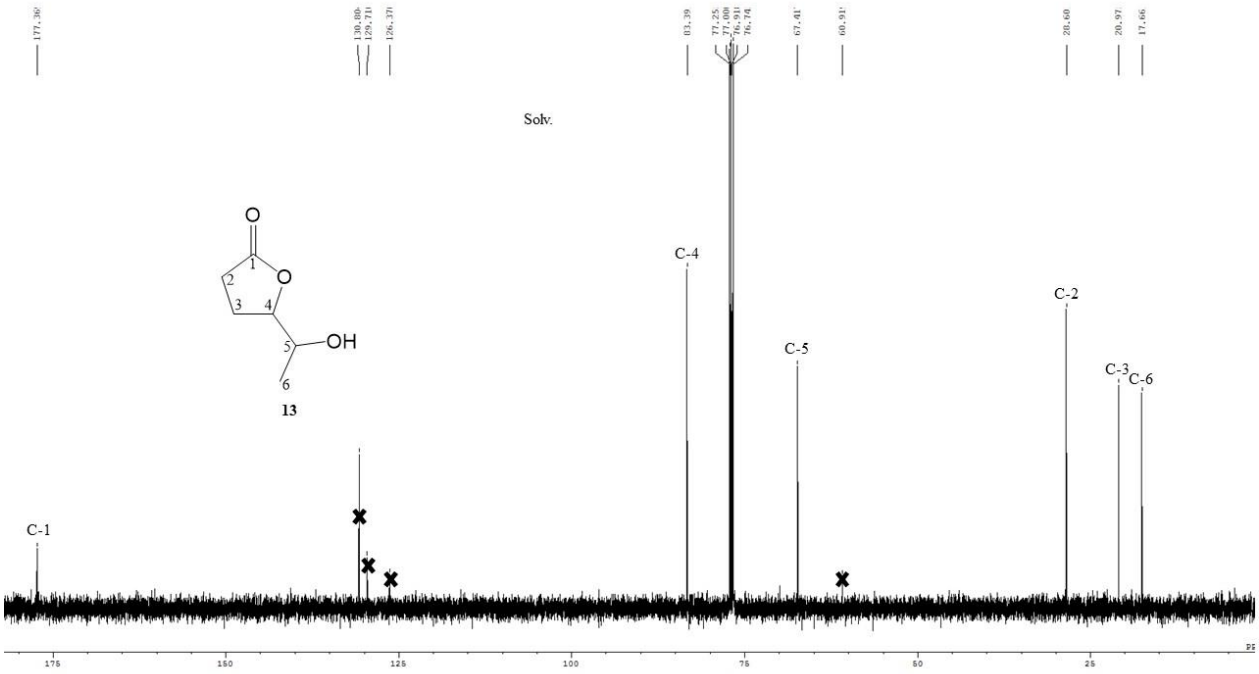


Figure 75 ^{13}C NMR spectrum of **13** ($CDCl_3$)

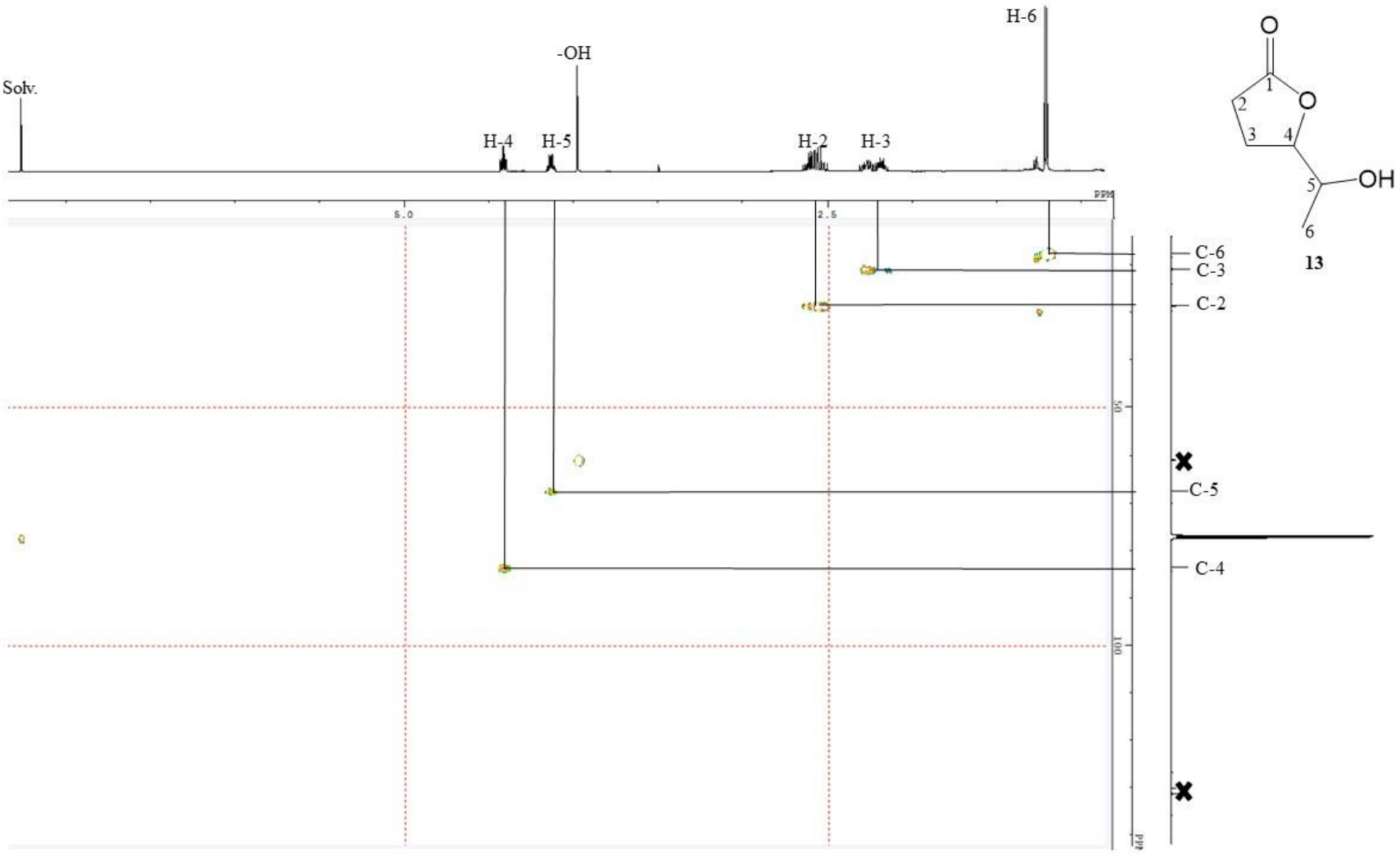


Figure 76 HMQC spectrum of **13** ($CDCl_3$)

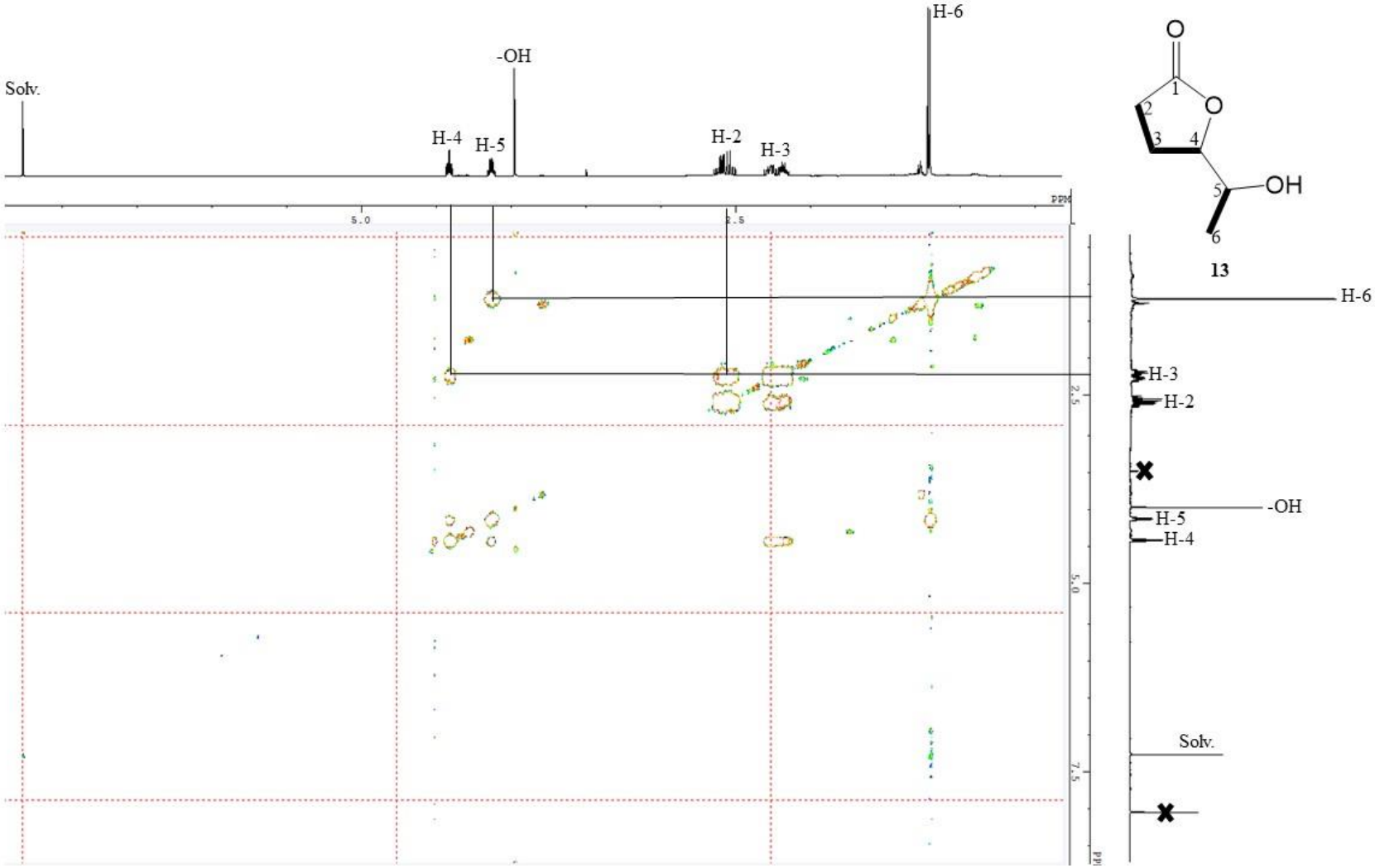


Figure 77 COSY spectrum of **13** ($CDCl_3$)

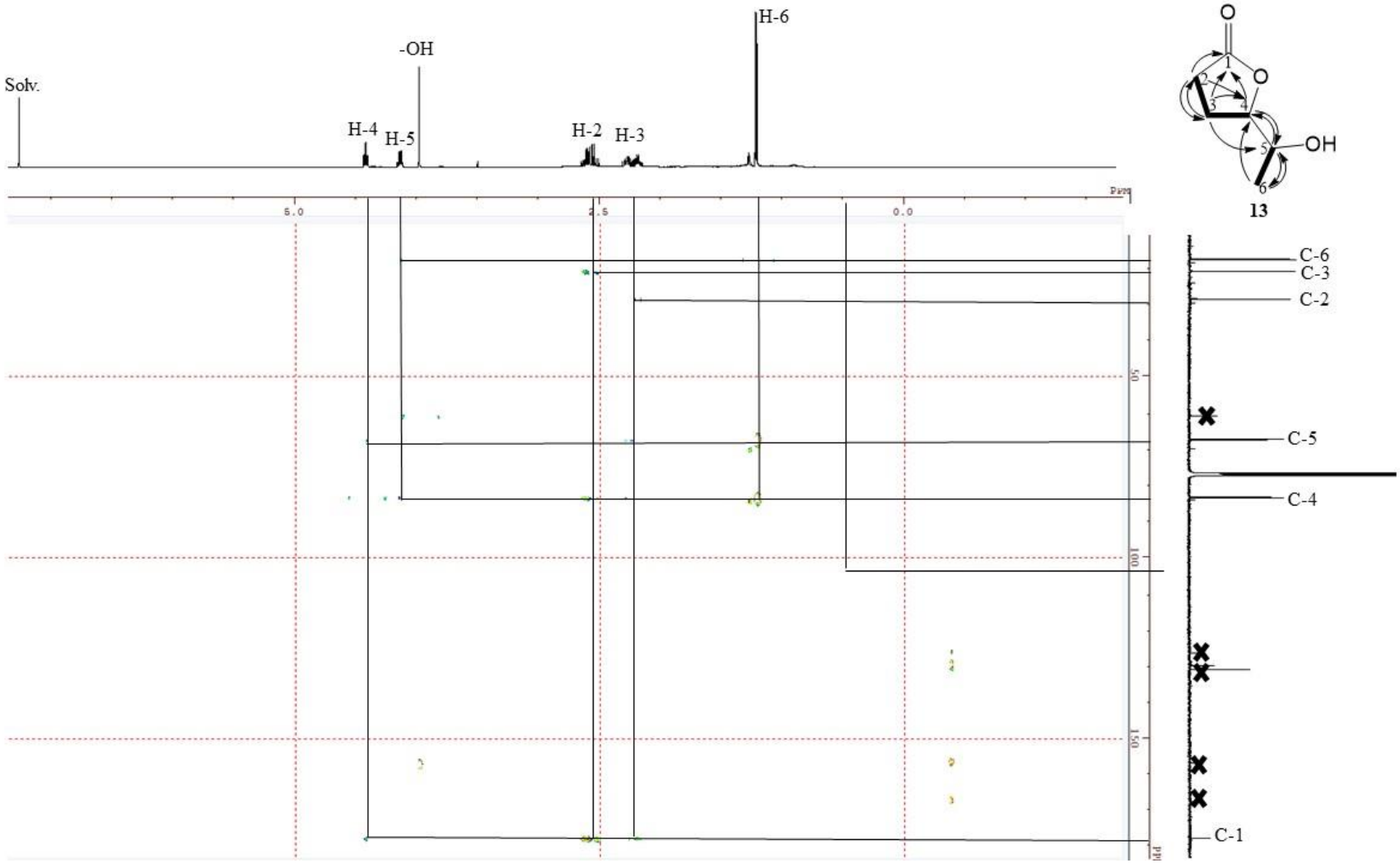


Figure 78 HMBC spectrum of **13** ($CDCl_3$)

2-8 Compound **14**

Compound **14** was isolated as a white amorphous. Its molecular weight was determined by ESIMS; *m/z* 161 $[M+Na]^+$ (Figure 80). Compound **14** was identified by the interpretation of mass and NMR spectra as known compound 4-(2-hydroxyethyl)phenol or tyrosol (Figure 79). This compound has been isolated from the endophytic fungus, *Diaporthe lithocarpus*. Compound **14** was examined against murine leukemia P-388 cells and various bacteria, but no activities exhibited. [53]

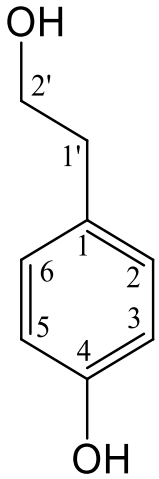


Figure 79 Structure of **14**

Table 12 ^{1}H and ^{13}C NMR assignments for **14**

	Compound **14** (CD_3OD)	
Position	δ_C	δ_H (multiplicity, *J* in Hz)
1	131.0	
2	130.9	7.02 (d, *J* = 8.5)
3	116.1	6.69 (d, *J* = 9.0)
4	156.8	
5	116.1	6.69 (d, *J* = 9.0)
6	130.9	7.02 (d, *J* = 8.5)
1’	39.4	2.73 (t, *J* = 5.0)
2’	64.6	3.67 (t, *J* = 7.0)

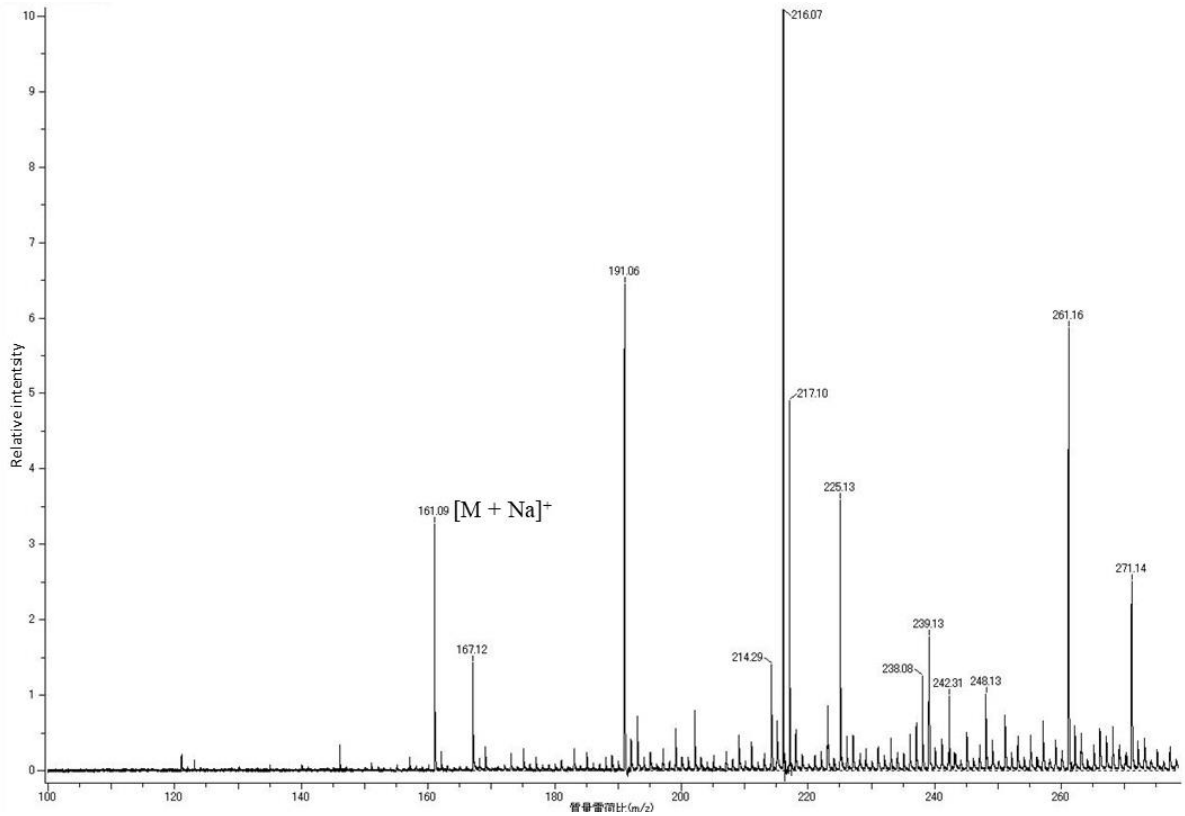


Figure 80 ESIMS spectrum of **14**

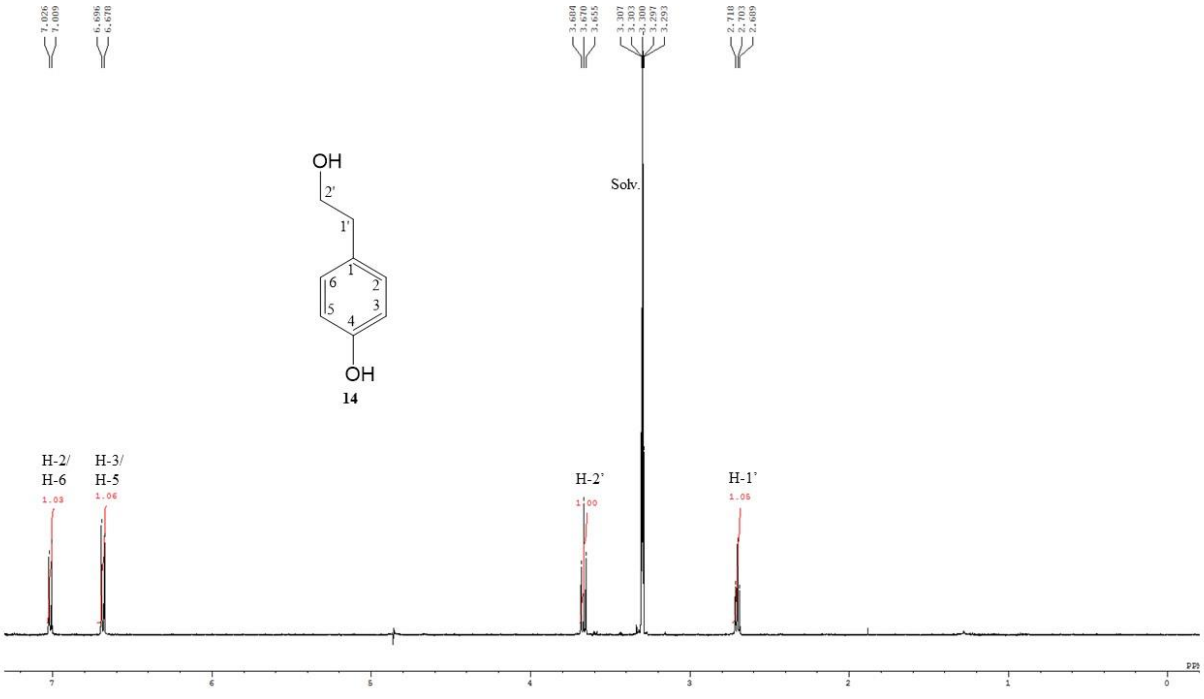


Figure 81 ^{1}H NMR spectrum of **14** (CD_3OD)

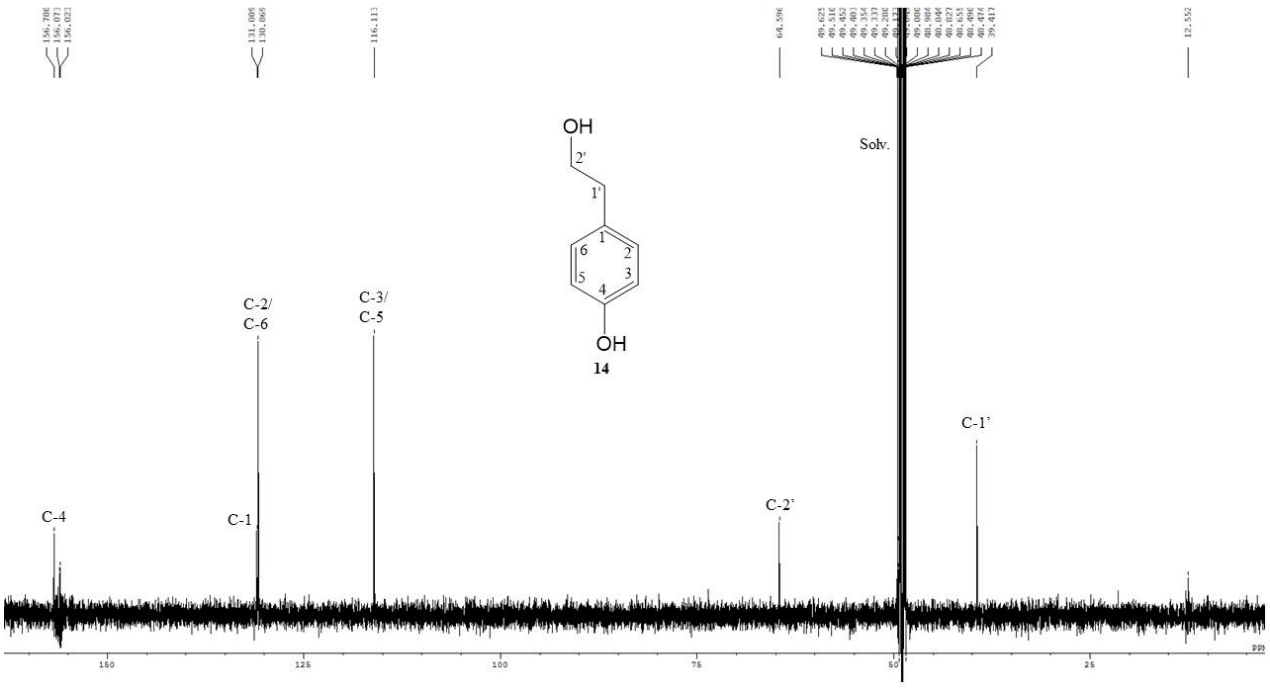


Figure 82 ^{13}C NMR spectrum of **14** (CD_3OD)

2-9 Compound **15**

Compound **15** was isolated as a white amorphous. Its molecular weight was determined by ESIMS; *m/z* 219 $[M-H]^-$ (Figure 85). The planar structure of **15** was elucidated by interpretation of the NMR spectra, including DEPT, COSY, HMBC, and HMQC data. The DEPT experiment indicated the presence of one methyl, two methines, four quaternary carbons, and one tetrasubstituted carbon. The NMR comparison to the reported one indicated compound **15** was a known compound 3,5-dichloro-4-methoxybenzoic acid (Figure 83). This compound has been isolated from an endophytic fungus, *Penicilium* sp.. Compound **15** was subjected to bioactive assays including antifungal and cytotoxicity assays, both assays showed that it exhibited inhibition activity against pathogenic fungus and cytotoxic activity against KB cell.[54]

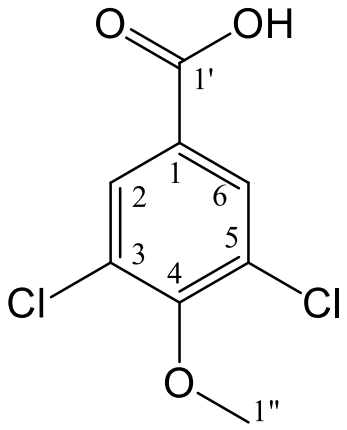


Figure 83 Structure of **15**

Table 13 ^{1}H and ^{13}C NMR assignments for **15**

	Compound **15** (CD_3OD)	
Position	δ_C	δ_H (multiplicity, *J* in Hz)
1	130.4	
2	131.3	8.00 (s)
3	131.1	
4	156.8	
5	131.1	
6	131.3	8.00 (s)
1’	167.8	
1”	61.3	3.92 (s)

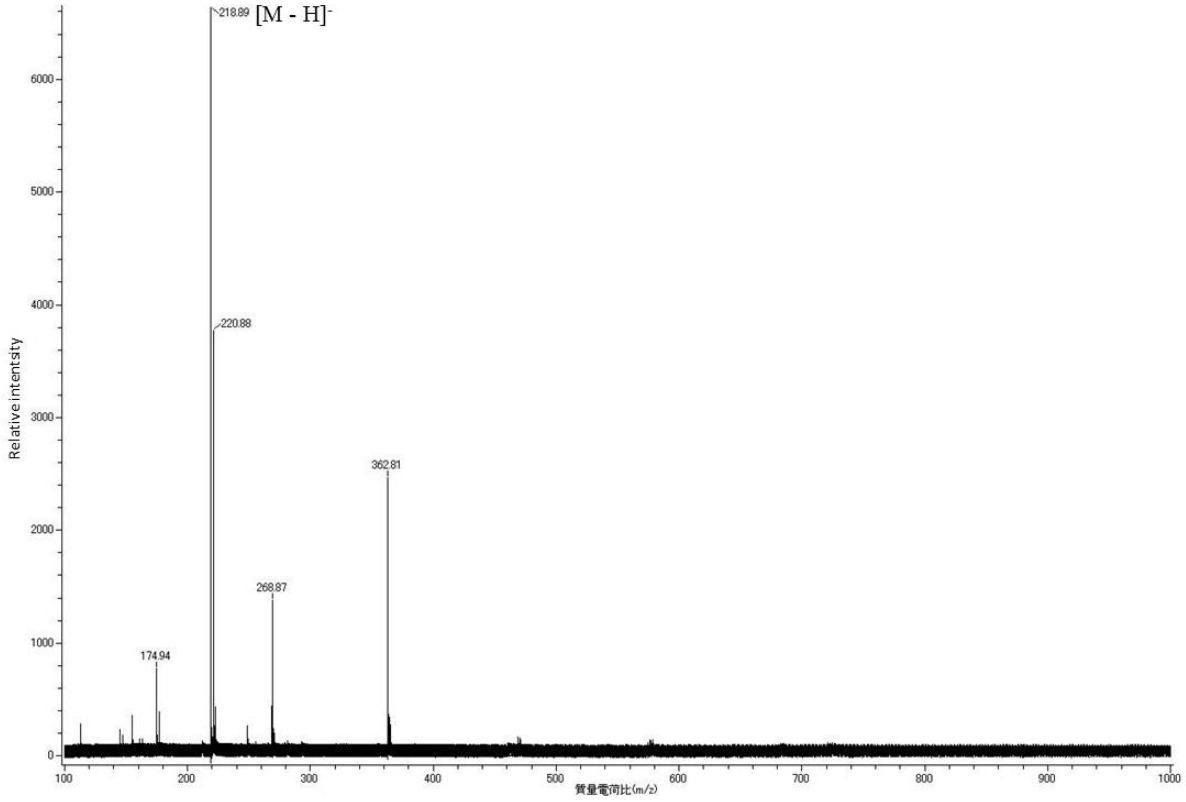


Figure 84 ESIMS spectrum of **15**

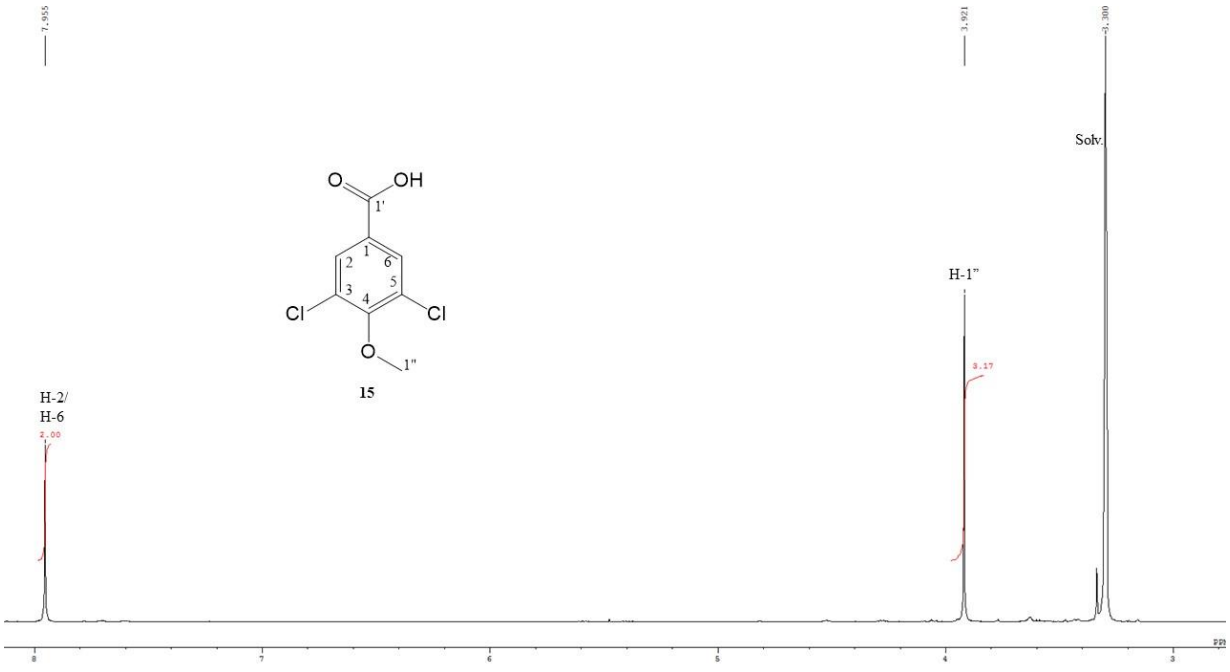


Figure 85 ^{1}H NMR spectrum of **15** (CD_3OD)

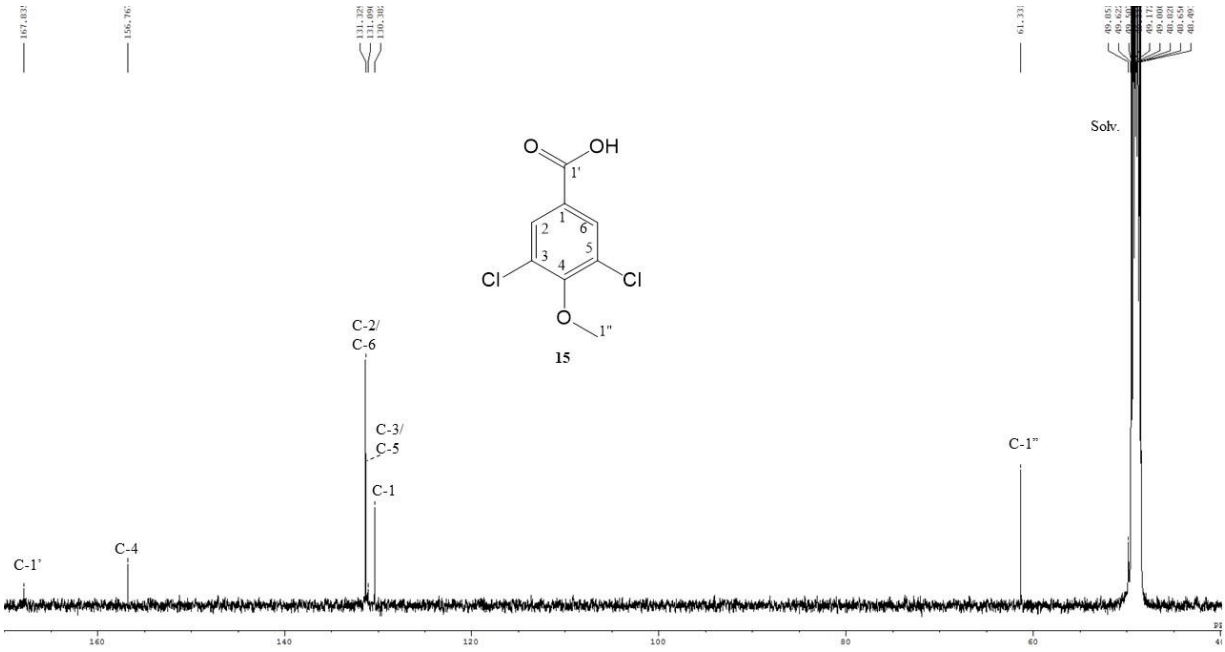


Figure 86 ^{13}C NMR spectrum of **15** (CD_3OD)

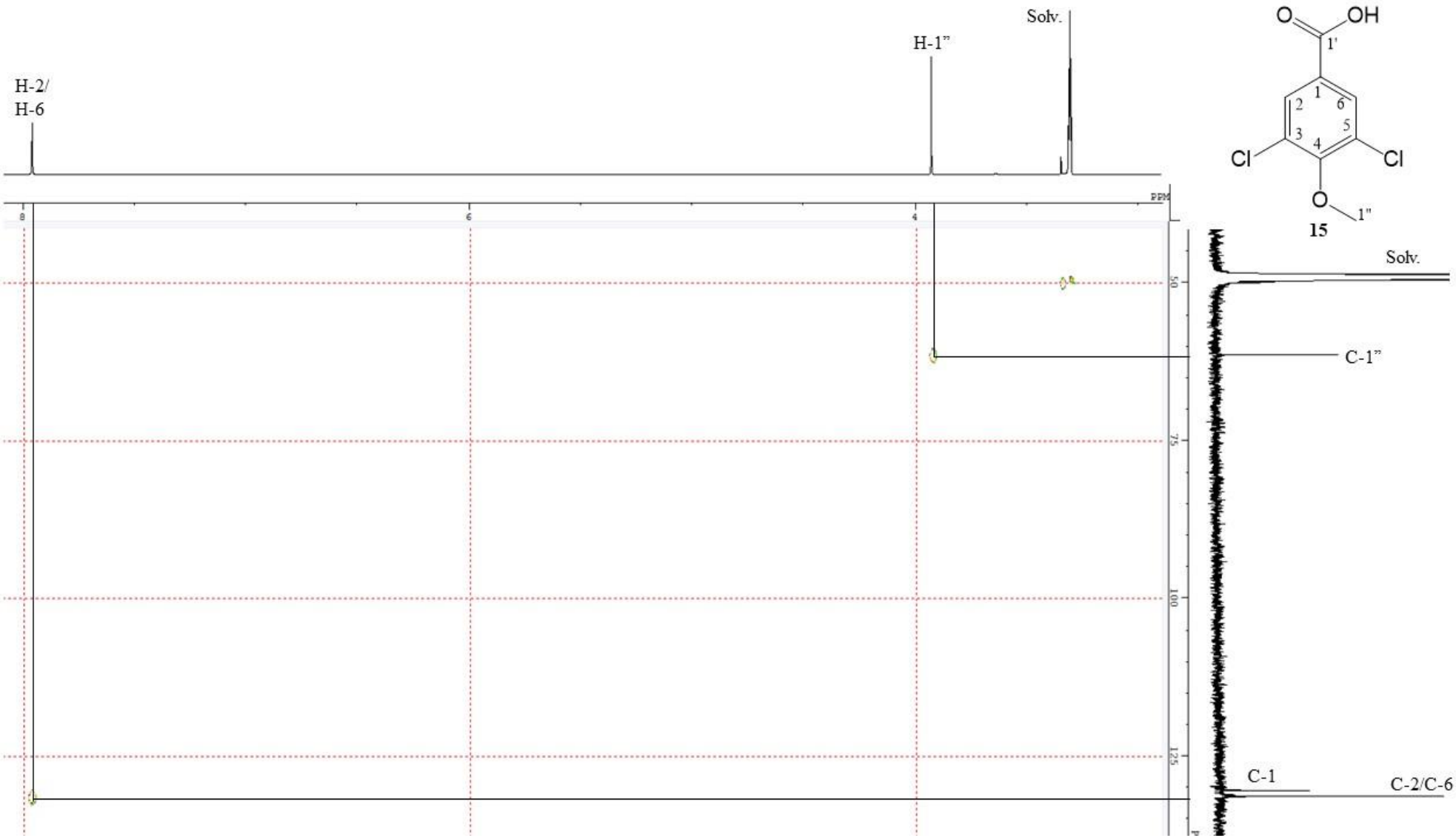


Figure 87 HMQC spectrum of **15** (CD_3OD)

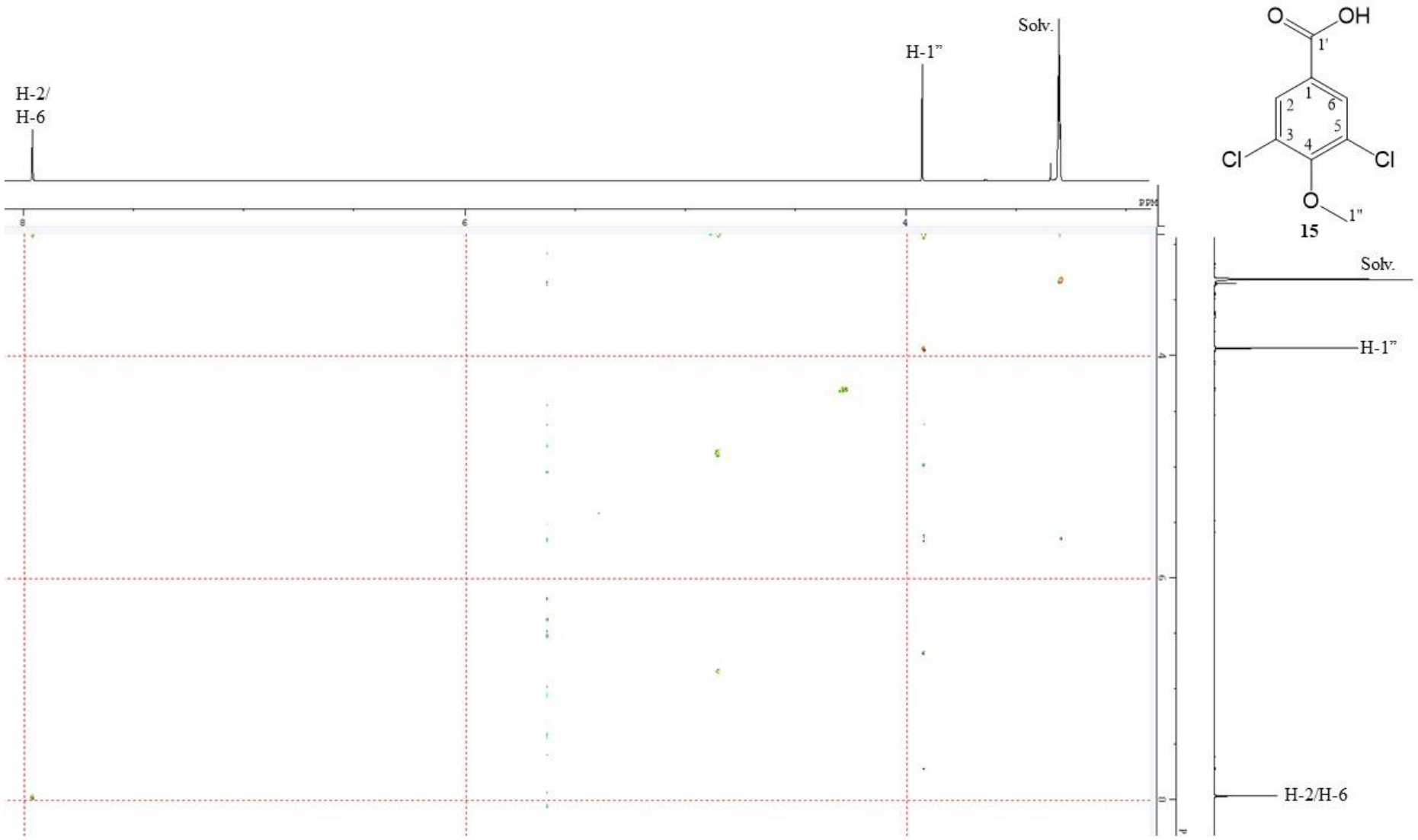


Figure 88 COSY spectrum of **15** (CD_3OD)

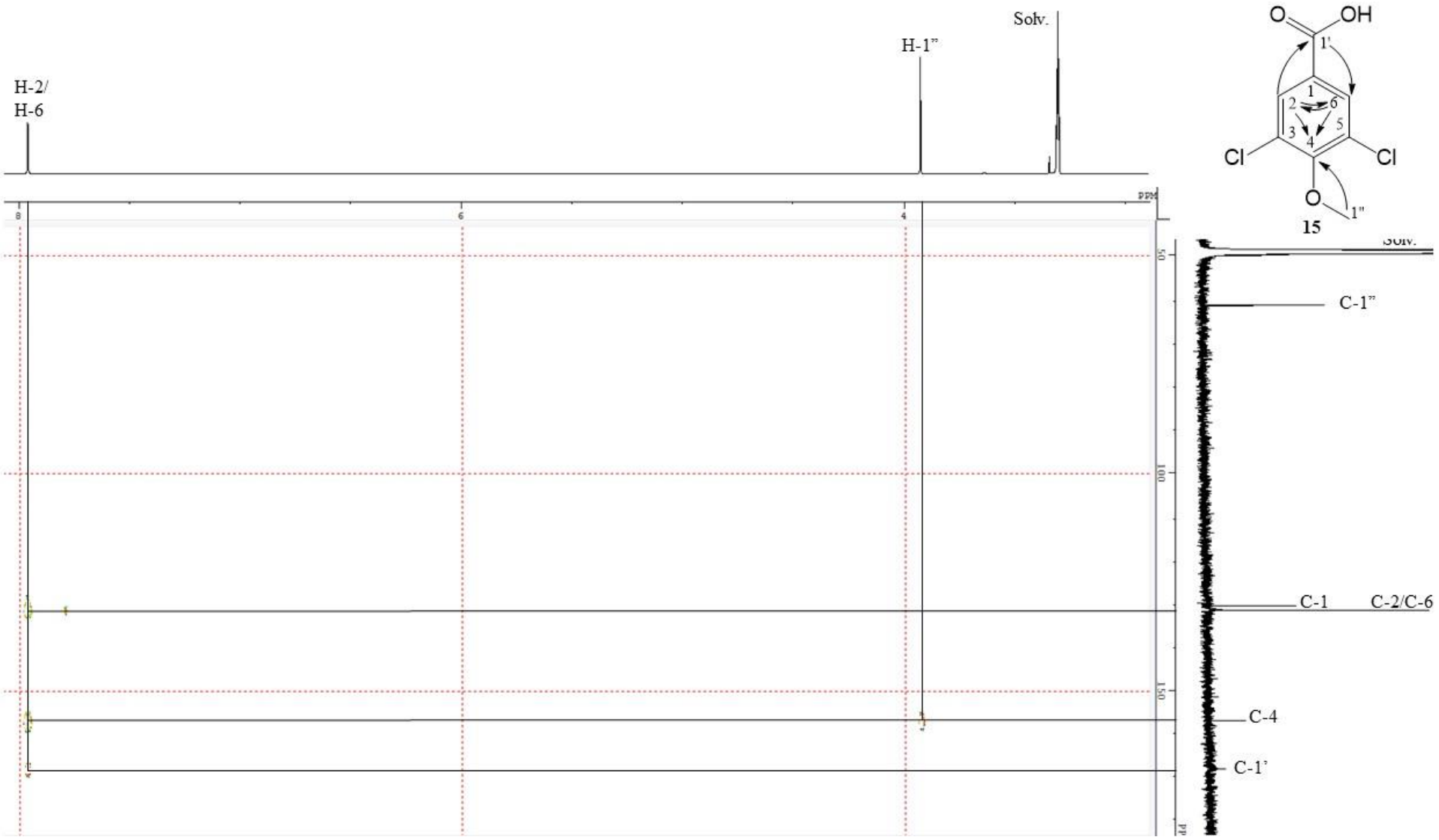


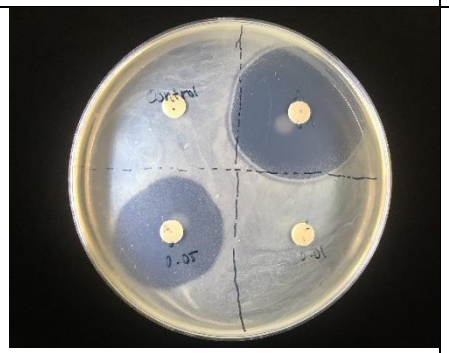
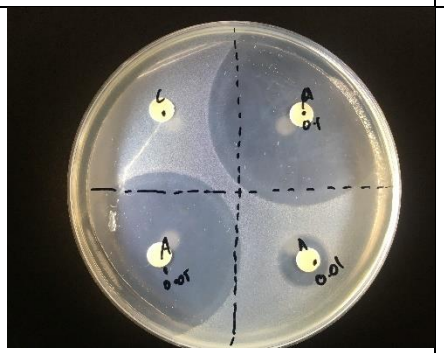
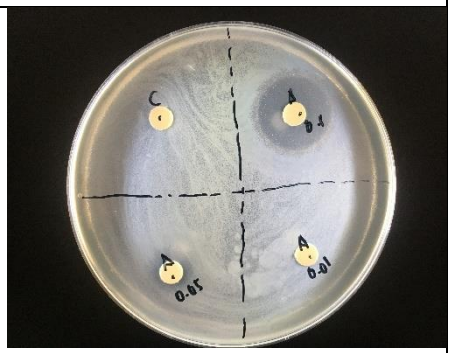
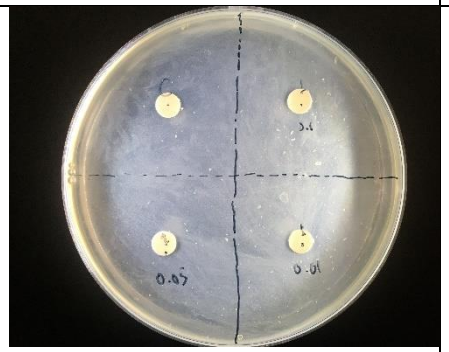
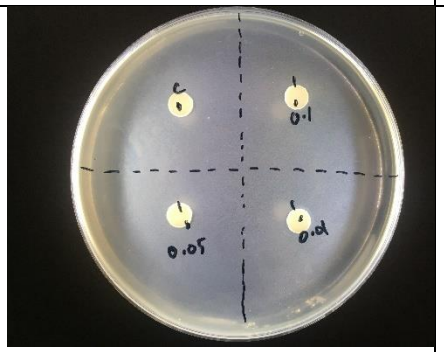
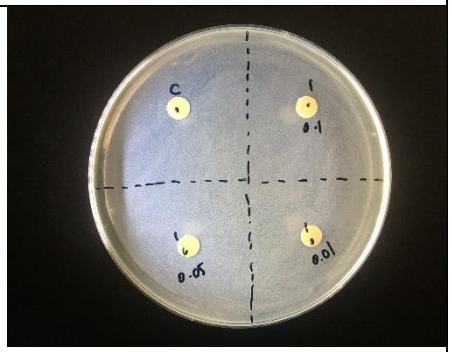
Figure 89 HMBC spectrum of **15** (CD_3OD)

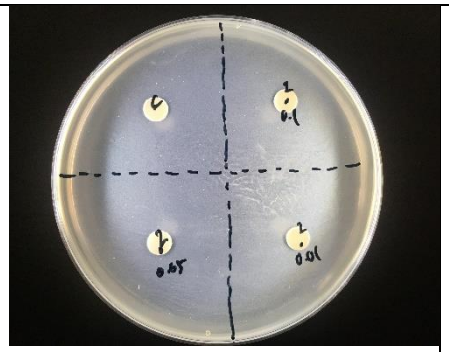
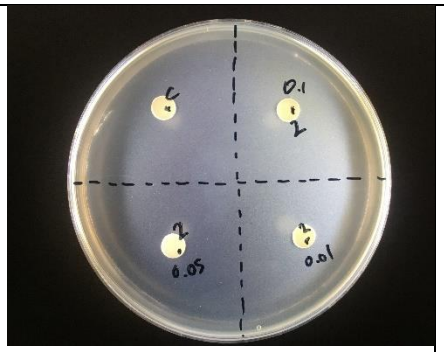
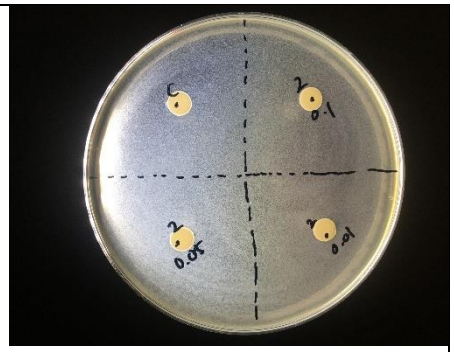
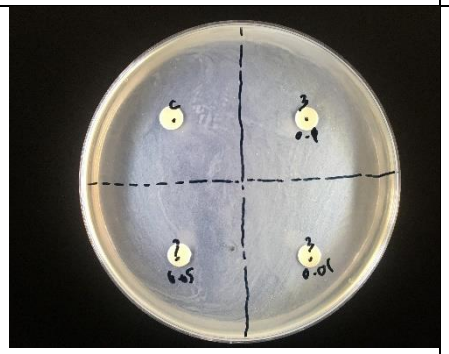
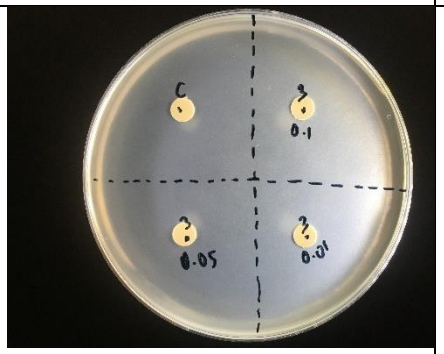
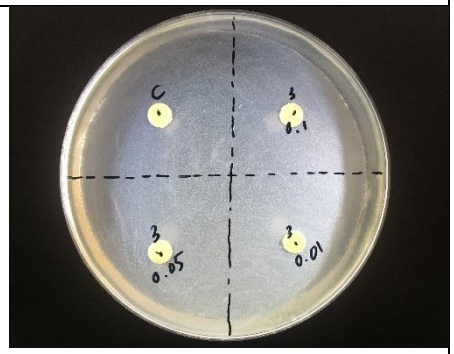
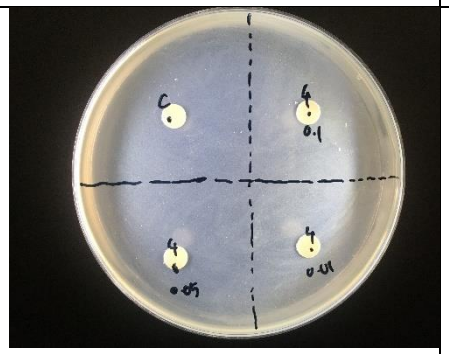
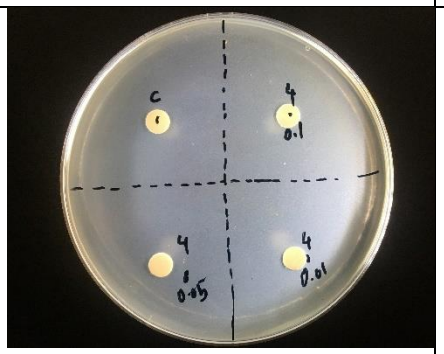
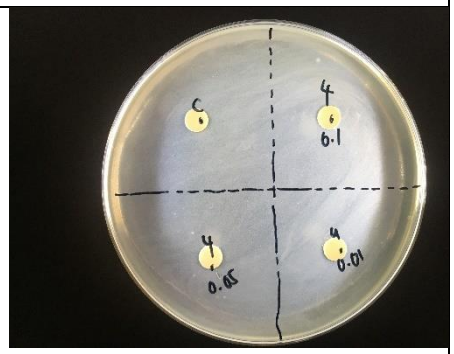
Section 3 Biological assay

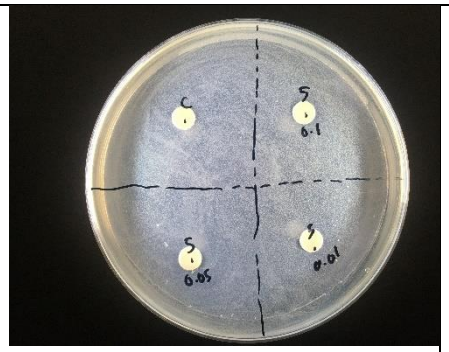
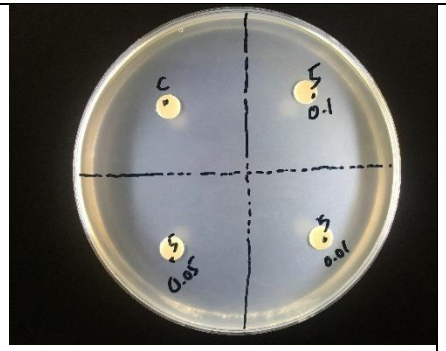
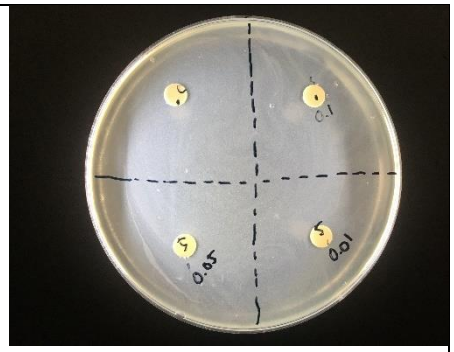
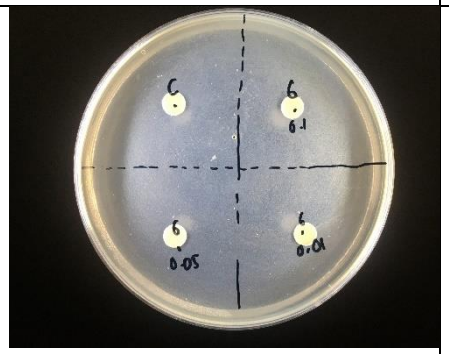
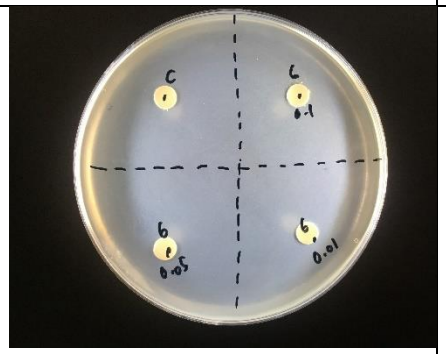
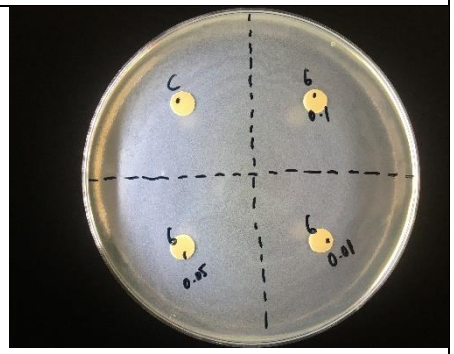
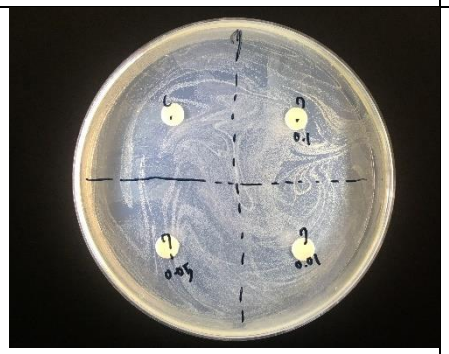
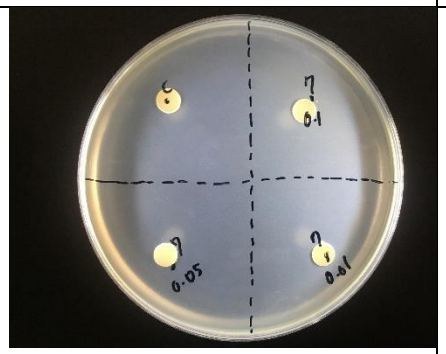
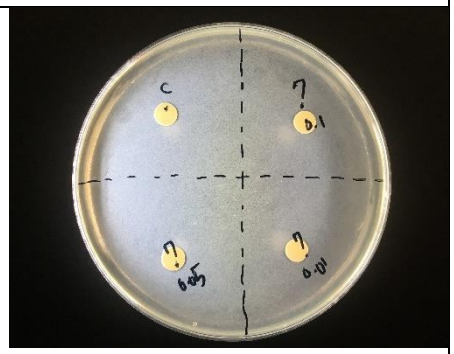
3-1 Antibacterial assay

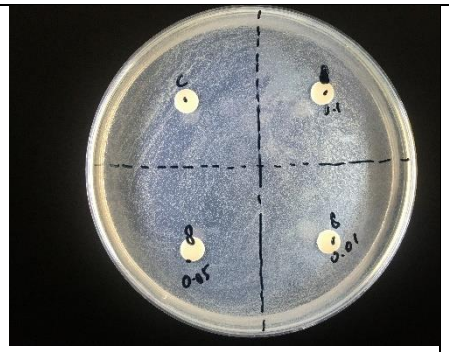
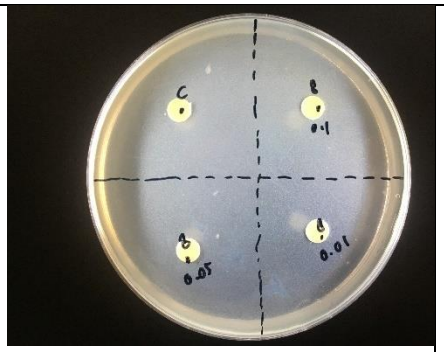
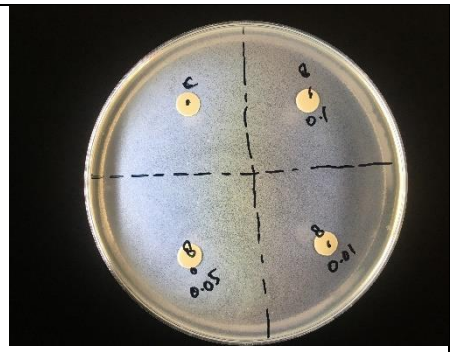
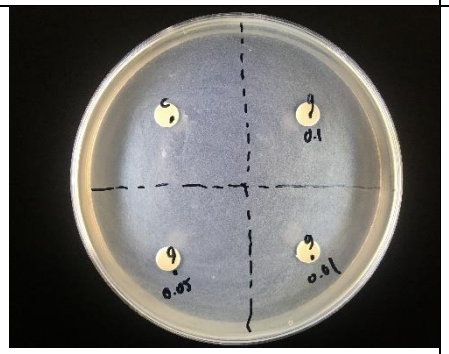
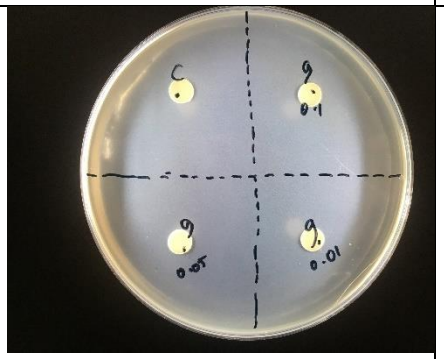
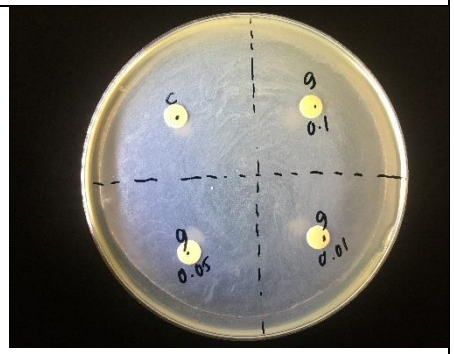
Antibacterial activity of compounds **7−15** was evaluated with Ampicillin used as positive control. Each of the two isolated compounds (**7−15**) was added to paper disks (0.01, 0.05, and 0.5 mmol/paper) and then placed to the yeast medium agar containing bacteria *Clavibacter michiganensis*, *Pectobacterium carotovorum*, and *Burkholderia glumae.* After 7 days, the inhibition area was measured. As a result, there is no inhibition activity found (Table 14).

Table 14 Antibacterial effect of compounds **7−15**

Compound	*C. michiganensis*	*P. carotovorum*	*B. glumae*
Ampicillin			
7			

8			
9			
10			

11			
12			
13			

14			
15			

3-2 Plant growth regulating assay towards lettuce growth

Plant growth regulating activity of **7**–**15** was evaluated using lettuce. 2,4-Dichlorophenoxyacetic acid was used as positive control, which inhibited the hypocotyl and root growth of lettuce dose-dependently. As shown in Figure 90, all the compounds exhibited activity towards lettuce growth. Compounds **8** and **13** showed weak inhibition activity against hypocotyl growth at 100 and 1000 nmol/paper, respectively. Compound **7** showed inhibition activity towards root growth at 100 nmol/paper and both root and hypocotyl at 1000 nmol/paper. Although compounds **14** and **15** have significance inhibition activity, they also showed promotion activity towards root at 1 nmol/paper and hypocotyl at 100 nmol/paper, respectively. The quinoline compounds **9** and **10** exhibited inhibition effect on lettuce growth. Compound **9** inhibited root growth at 100 and 1000 nmol/paper, while compound **10** inhibited strongly both root and hypocotyl growth at 1000 nmol/paper. The strong inhibition activity of **10** indicated that the presence of carboxylic acid group has an important role to suppress the growth of lettuce. The diol compounds **11** and **12** exhibited inhibition effect on lettuce growth as well. Compound **11** exhibited significance inhibition activity of root growth at 100 and 1000 nmol/paper, while **12** showing inhibition activity at 1000 nmol/paper. The similar structure of both compounds indicated that the presence of methyl group attenuates the inhibition activity on lettuce growth.

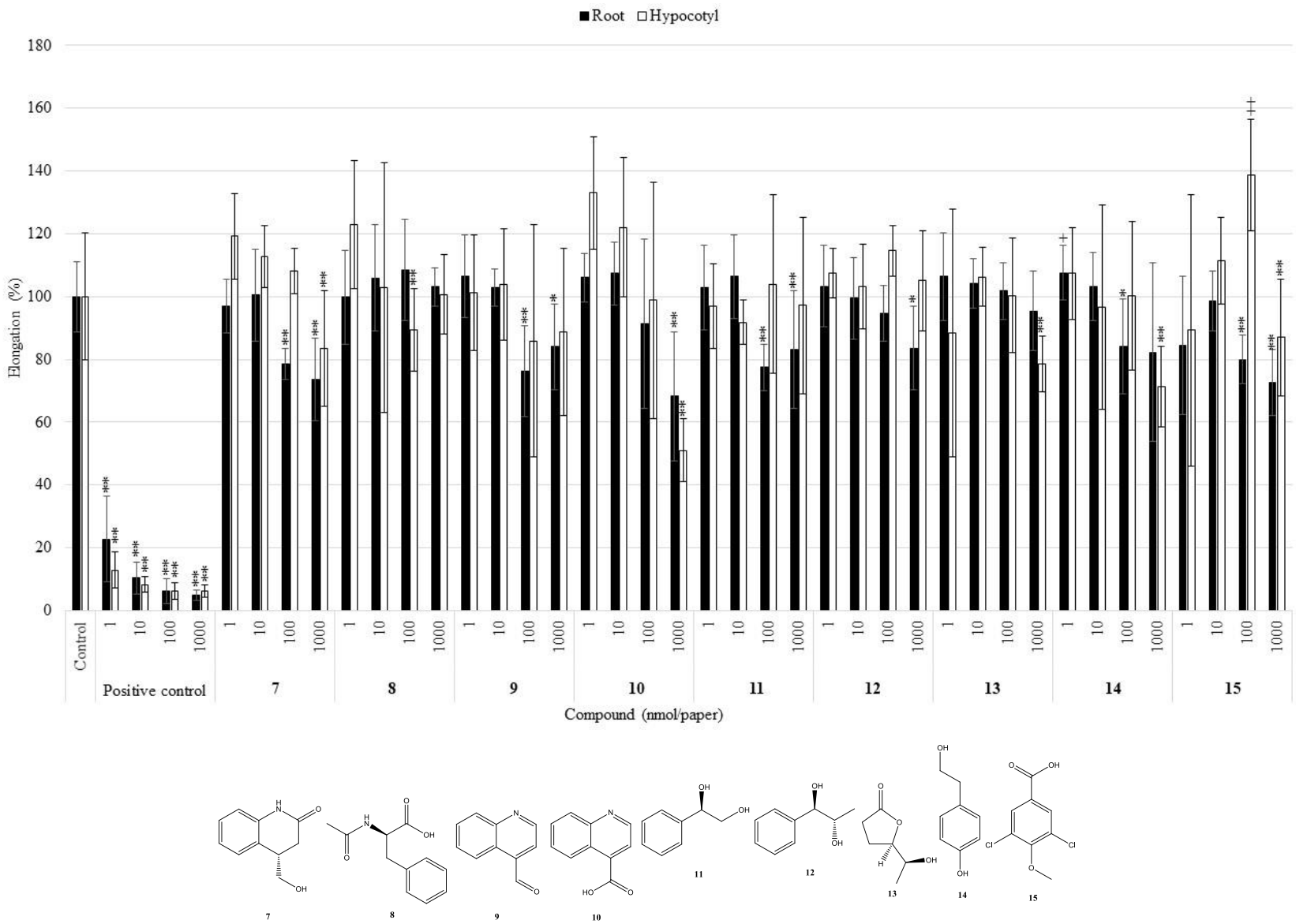


Figure 90 Growth regulating activity against lettuce of compounds **7** to **15** against root or hypocotyl. 2,4-Dichlorophenoxyacetic acid (2,4-D) was used as positive control. Results are the mean ± standard deviation (n = 9). [$^{*}p < 0.05$, $^{**}p < 0.01$ (growth inhibition); $^{+}p < 0.05$, $^{++}p < 0.01$ (growth promotion)].

Chapter 4

Bioorganic chemical studies on wild edible mushroom *Entoloma clypeatum*

Section 1 Isolation of bioactive compounds from *Entoloma clypeatum*

The fresh fruiting bodies were extracted with EtOH and then acetone to obtain the crude extracts. Afterwards, they were combined and concentrated under reduced pressure. The concentrated solutions were partitioned into *n*-hexane, EtOAc, EtOH, and H_2O soluble parts. Then, the H_2O soluble part was dried and extracted with EtOH (Fig. 91).

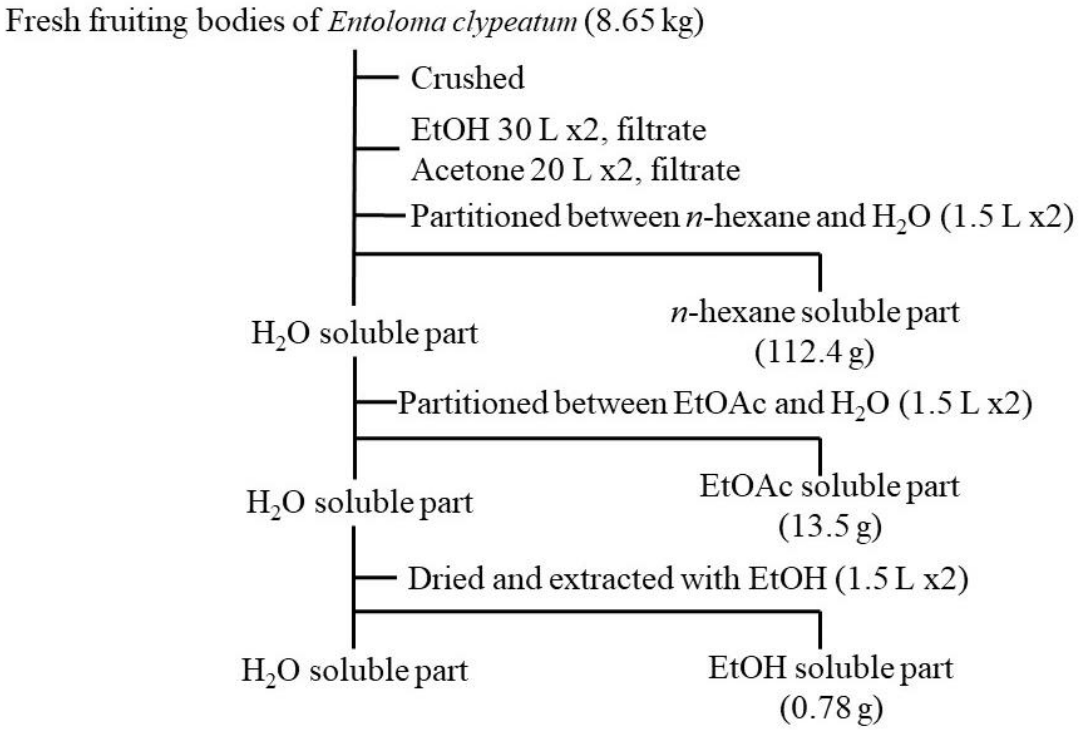


Figure 91 Partition scheme of *E. clypeatum* extracts

The EtOAc soluble part was subjected to silica gel flash column chromatography to obtain 12 fractions (1-12). Further purification was conducted by repeated chromatography using flash column chromatography or HPLC. The purification of fractions 2 and 3 afforded compounds **16** and **21** (Figures 92 and 93).

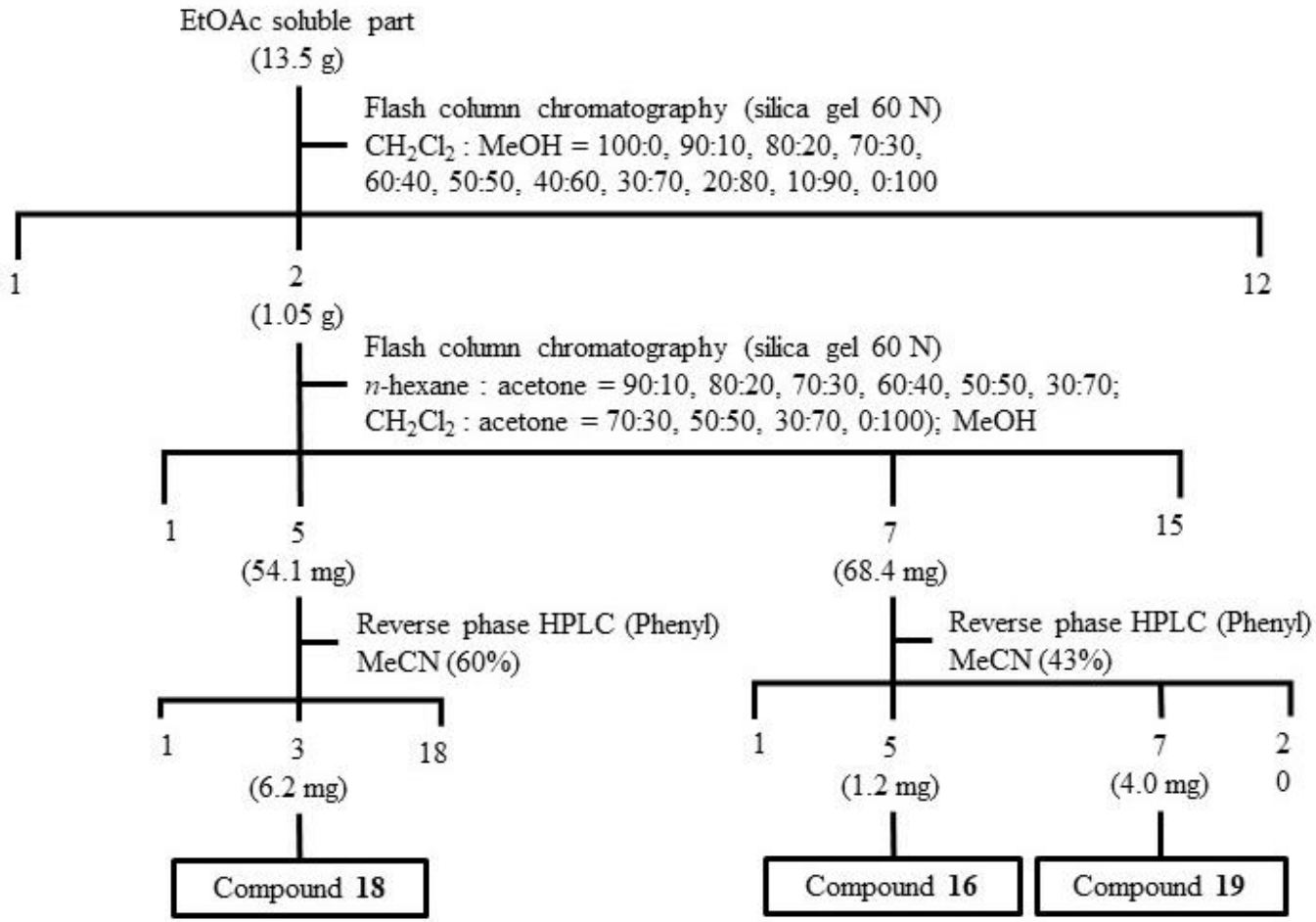


Figure 92 The isolation and purification scheme of compounds from *E. clypeatum* (1)

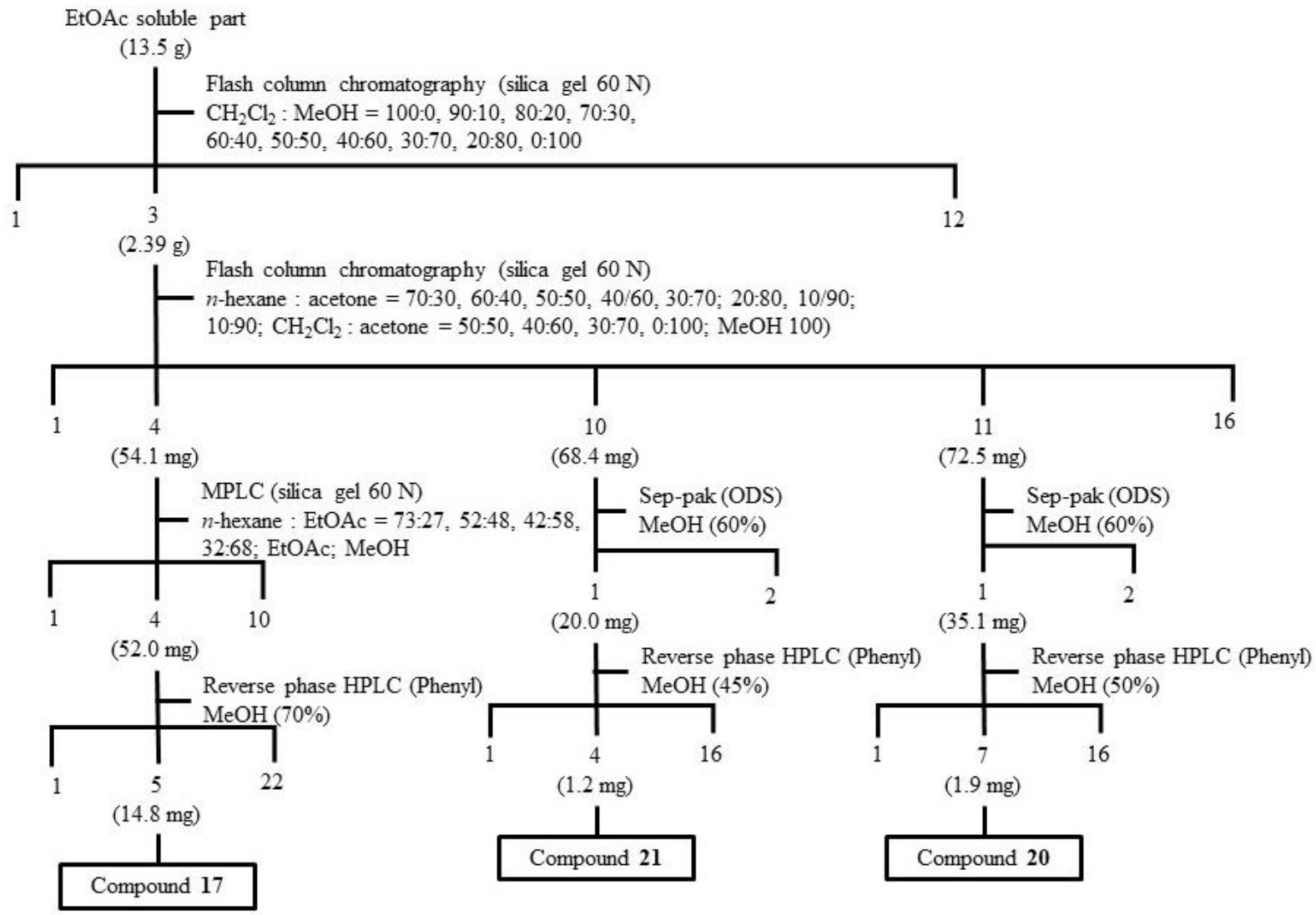


Figure 93 The isolation and purification scheme of compounds from *E. clypeatum* (2)

Section 2 Structural analysis of bioactive compounds from *Entoloma clypeatum*

2-1 Compound **16**

Compound **16** was isolated as a colorless oil. Its molecular weight was measured by ESIMS; *m/z* 171 $[M-H]^-$ (Figure 95). The planar structure of **16** was elucidated by interpretation of the NMR spectra, including DEPT, COSY, HMBC, and HMQC data. The DEPT experiment indicated the presence of one methyl, four methylenes, three methines, and one tetrasubstituted carbon. The ^{1}H NMR signals at δ_H 6.05 (dd, J = 16, 14.5) and δ_H 7.05 (dd, J = 15.5, 11.0) indicated the presence of unsaturated acid (Figure 96; Table 15). The COSY correlations (H-2/H-3; H-3/H-4; H-4/H-5) and the HMBC correlations (H-2/C-1, C-4; H-3/C-1, C-4) constructed the 4-hydroxy-2-unsaturated acid. The presence of the hydrocarbon chain was confirmed by the COSY correlations (H-5/H-6; H-6/H-7; H-7/H-8; H-8/H-9) and the HMBC correlations (H-6/C-7, C-8; H-7/C-8; H-8/C-7) (Figures 99 and 100). The absolute configuration of **16** was determined by comparing its specific rotation { $[\alpha]_D^{26}$ -24 (*c* 0.18, $CHCl_3$) } with that of its reported one { $[\alpha]_D^{23}$ -28 ($CHCl_3$) }.[55] Compound **16** was identified as (*R*,*E*)-4-hydroxynon-2-enoic acid. This compound has been isolated from the stem bark of *Ailianthus altissima* and reported it has no anti-inflammatory activity.[56] However, it was isolated from mushroom for the first time.

Table 15 ^{1}H and ^{13}C NMR assignments for **16**

	Compound **16** ($CDCl_3$)	
Position	δ_C	δ_H (multiplicity, J in Hz)
1	169.5	
2	118.9	6.05 (dd, J = 16, 14.5)
3	152.7	7.05 (dd, J = 15.5, 11.0)
4	71.0	4.34 (m)
5	36.5	1.57 (m)
6	24.8	1.32 (m)
7	31.6	1.32 (m)
8	22.5	1.32 (m)
9	14.0	0.88 (t, J = 7.0)

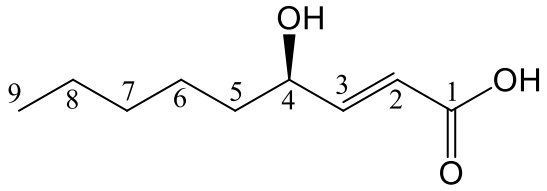


Figure 94 Structure of **16**

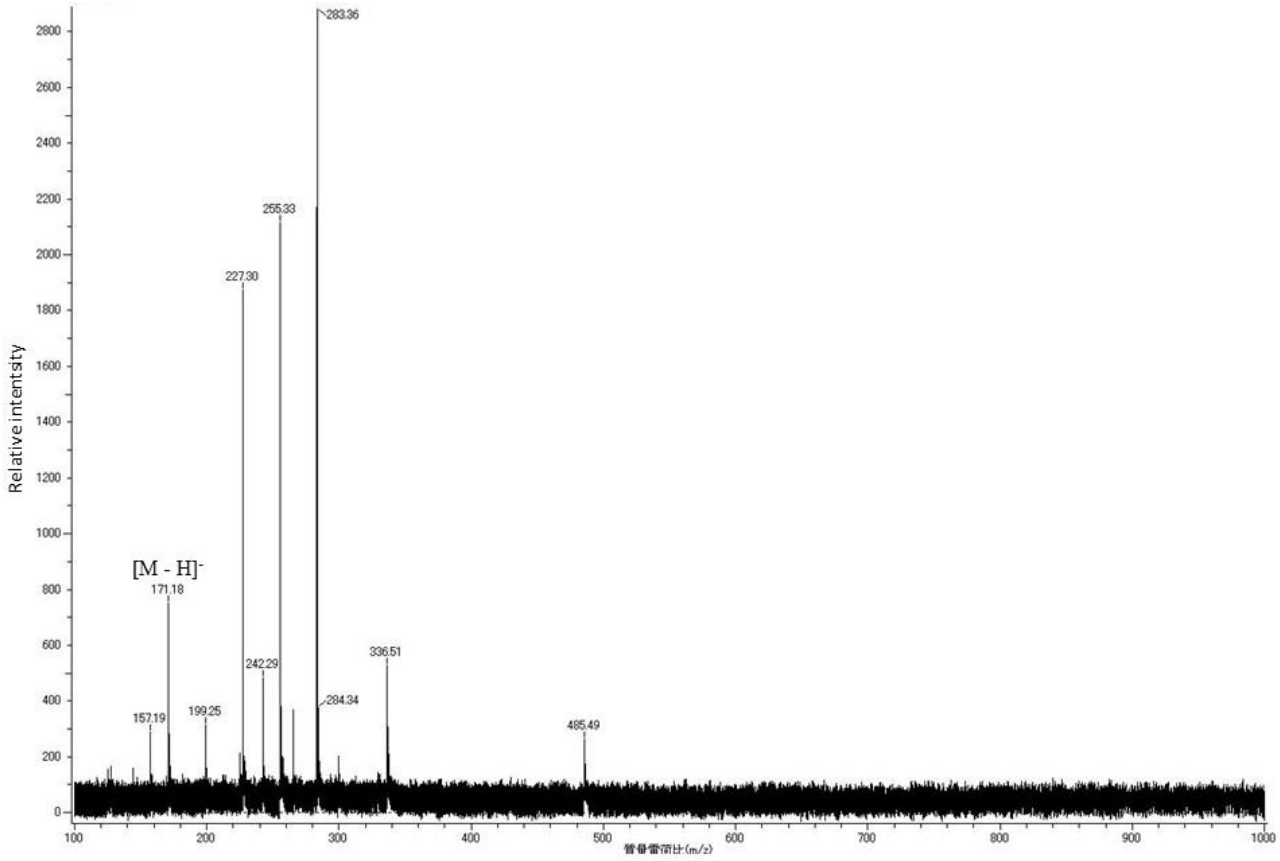


Figure 95 ESIMS spectrum of **16**

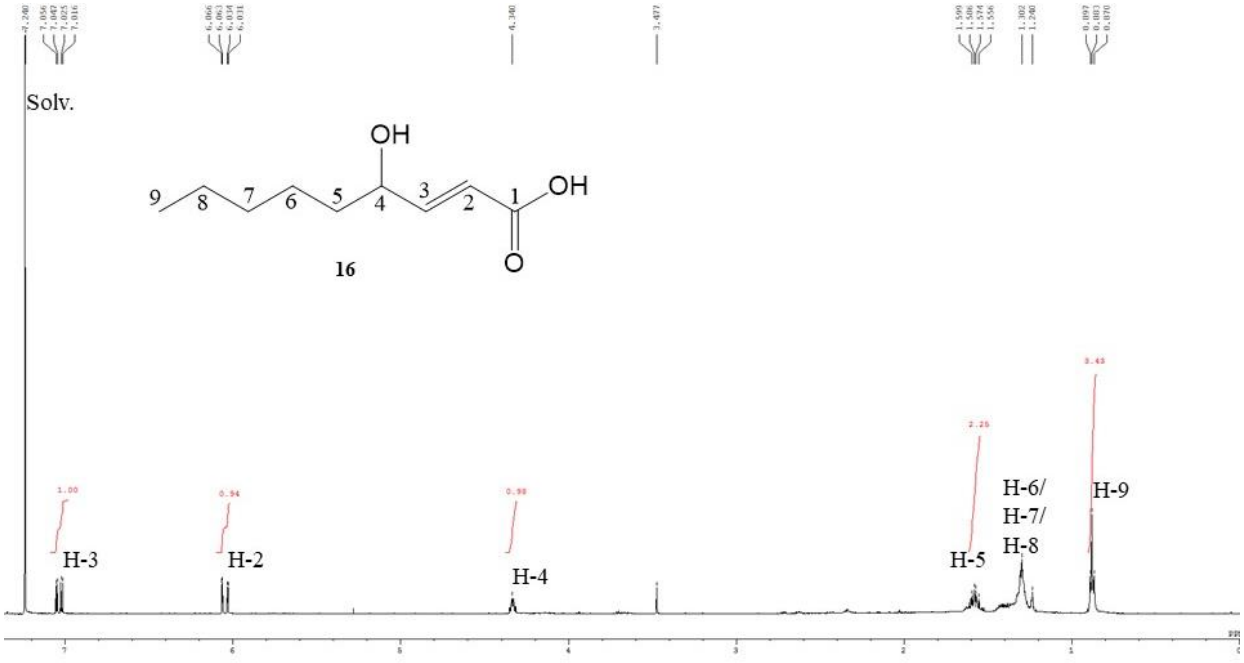


Figure 96 ^{1}H NMR spectrum of **16** ($CDCl_3$)

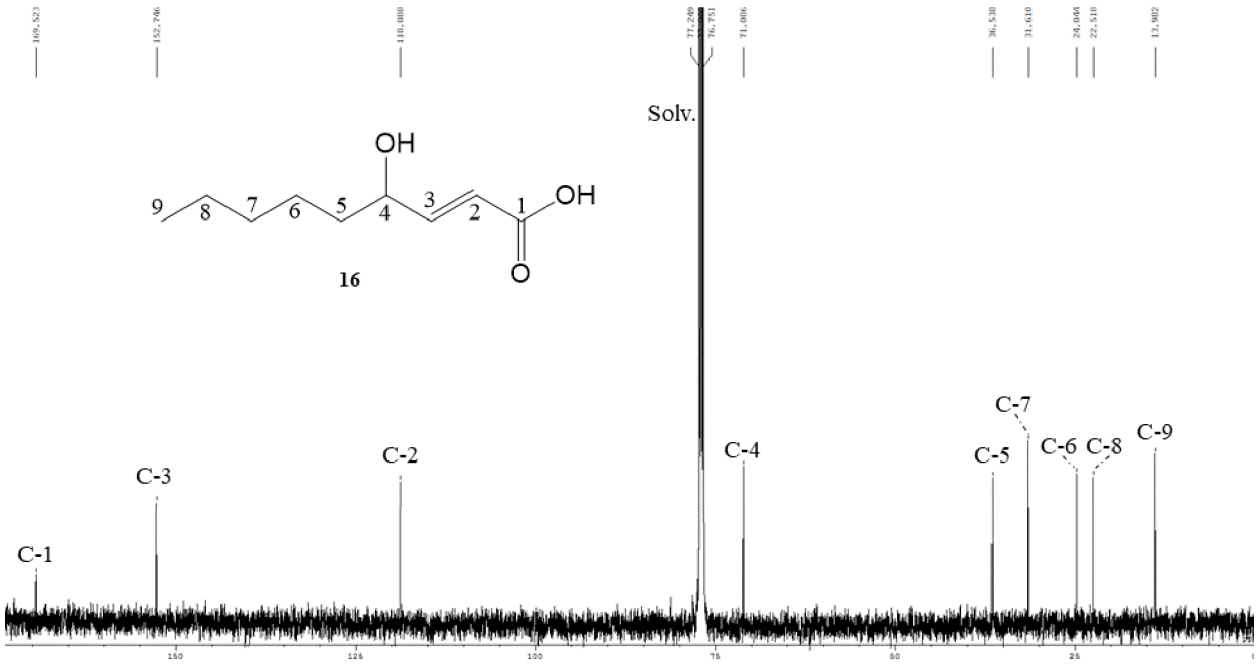


Figure 97 ^{13}C NMR spectrum of **16** ($CDCl_3$)

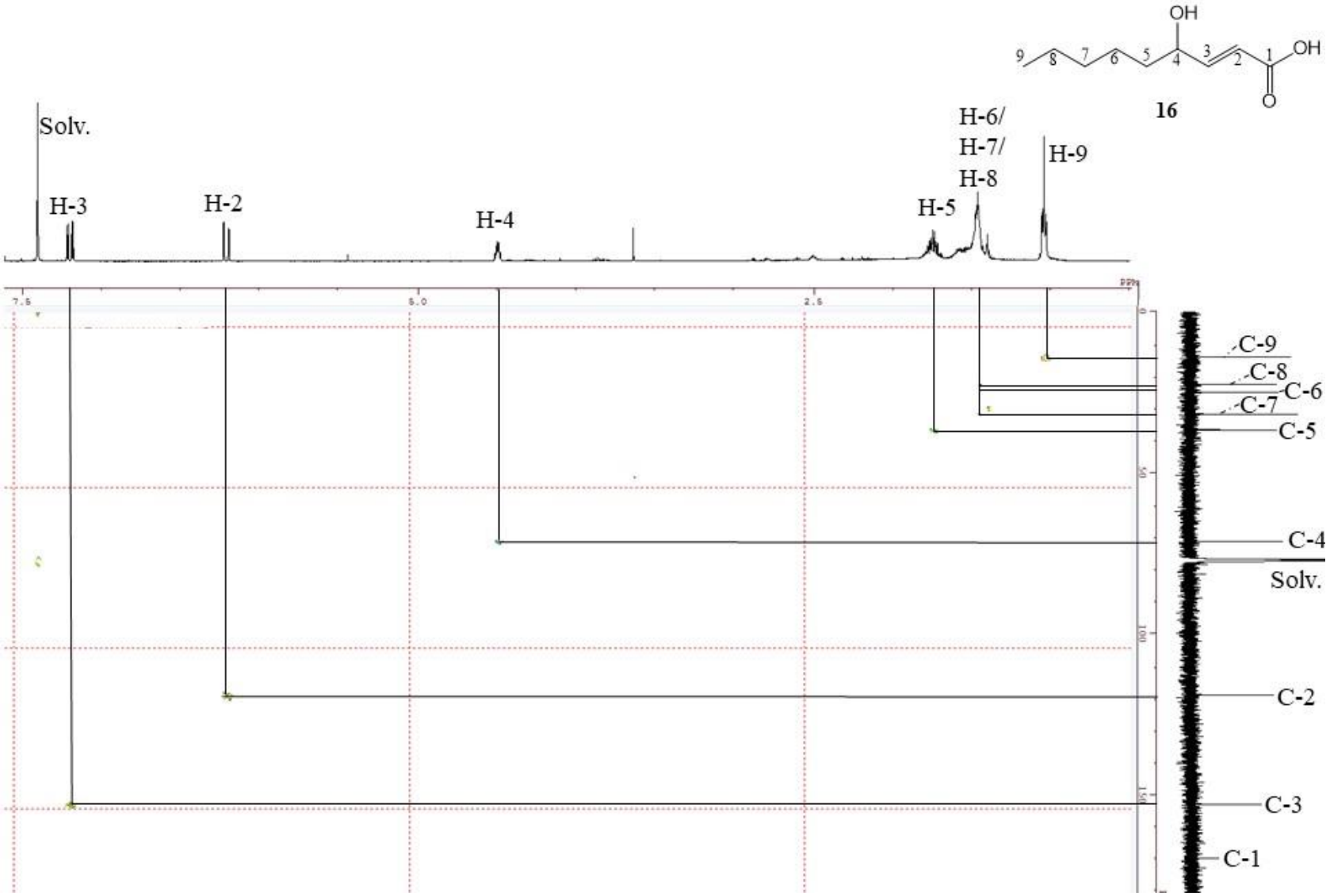


Figure 98 HMQC spectrum of **16** ($CDCl_3$)

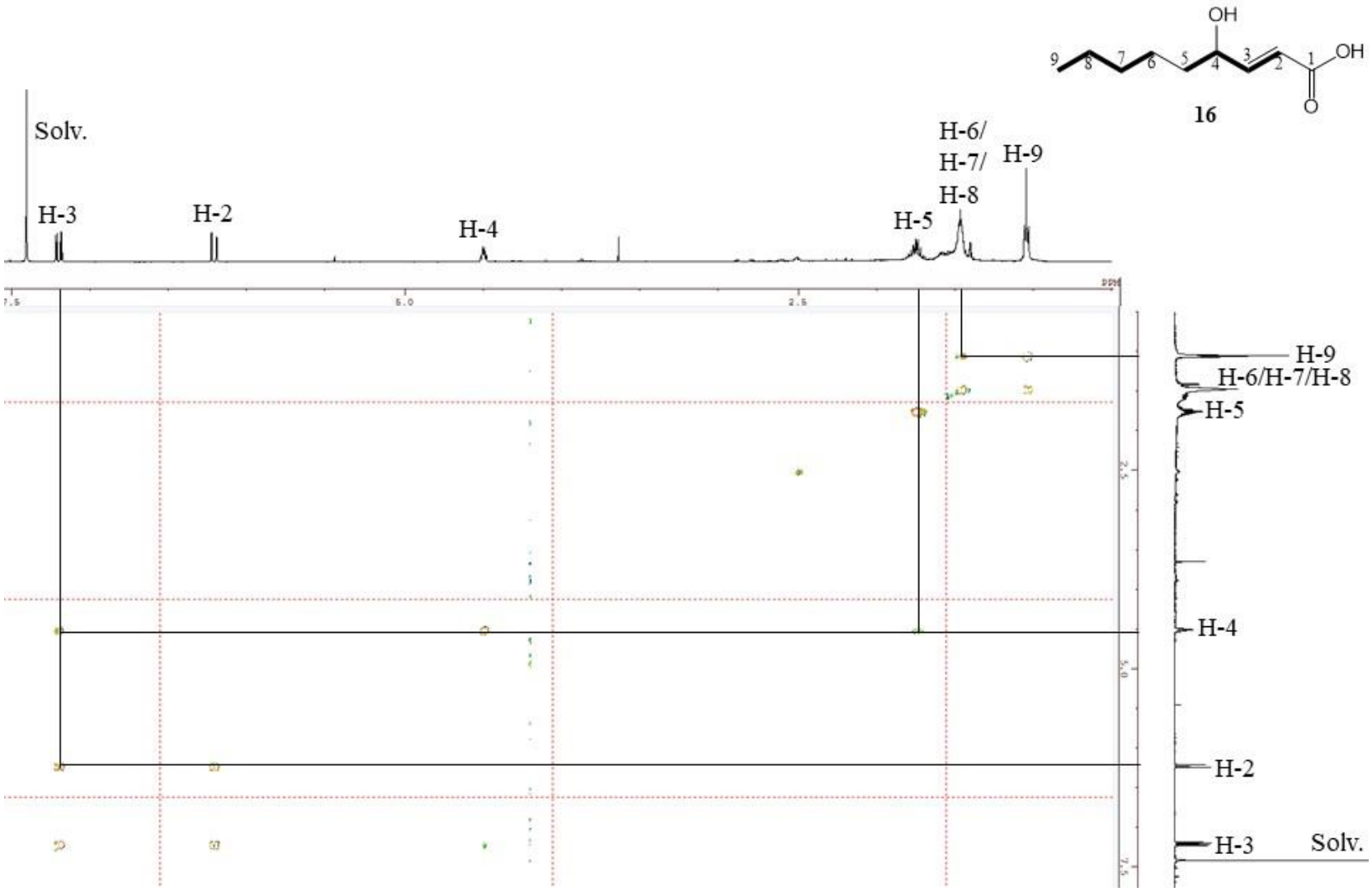


Figure 99 COSY spectrum of **16** ($CDCl_3$)

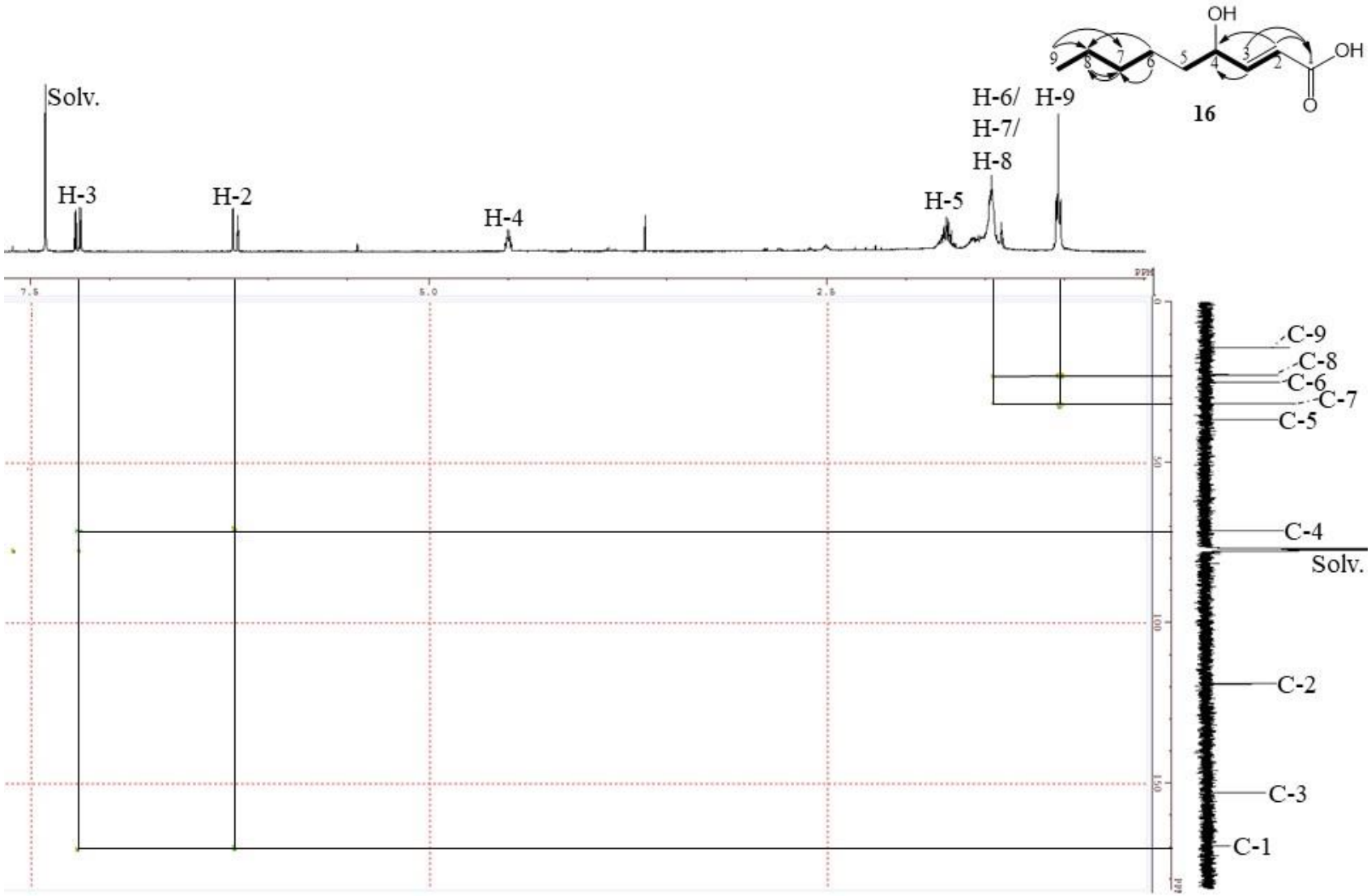


Figure 100 HMBC spectrum of **16** ($CDCl_3$)

2-2 Compound **17**

Compound **17** was isolated as a colorless oil. Its molecular formula was determined as $C_{10}H_{18}O_4$ by HRESIMS *m/z* 225.1088 $[M+Na]^+$ (calcd. for $C_{10}H_{18}O_4Na$, 225.1103), indicating the presence of two degrees of unsaturation in the molecule (Figure 102). The planar structure of **17** was identified by interpretation of the NMR spectra data and its comparison to the reported one (Table 16). Compound **17** was identified as 9-methoxy-9-oxonanoic acid that has been isolated as an antifungal from leaves of wild rice (*Oryza officinalis*).[57] However, it was isolated from mushroom for the first time.

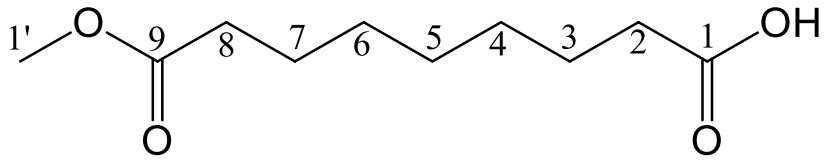


Figure 101 Structure of **17**

Table 16 ^1H and ^{13}C NMR assignments for **17**

	Compound **17** ($CDCl_3$)	
Position	δ_C	δ_H (multiplicity, *J* in Hz)
1	179.4	
2	34.0	2.31 (m)
3	24.8	1.60 (m)
4	28.9	1.30 (m)
5	28.9	1.30 (m)
6	28.9	1.30 (m)
7	24.9	1.60 (m)
8	33.9	2.31 (m)
9	174.3	
1’	51.5	3.63 (s)

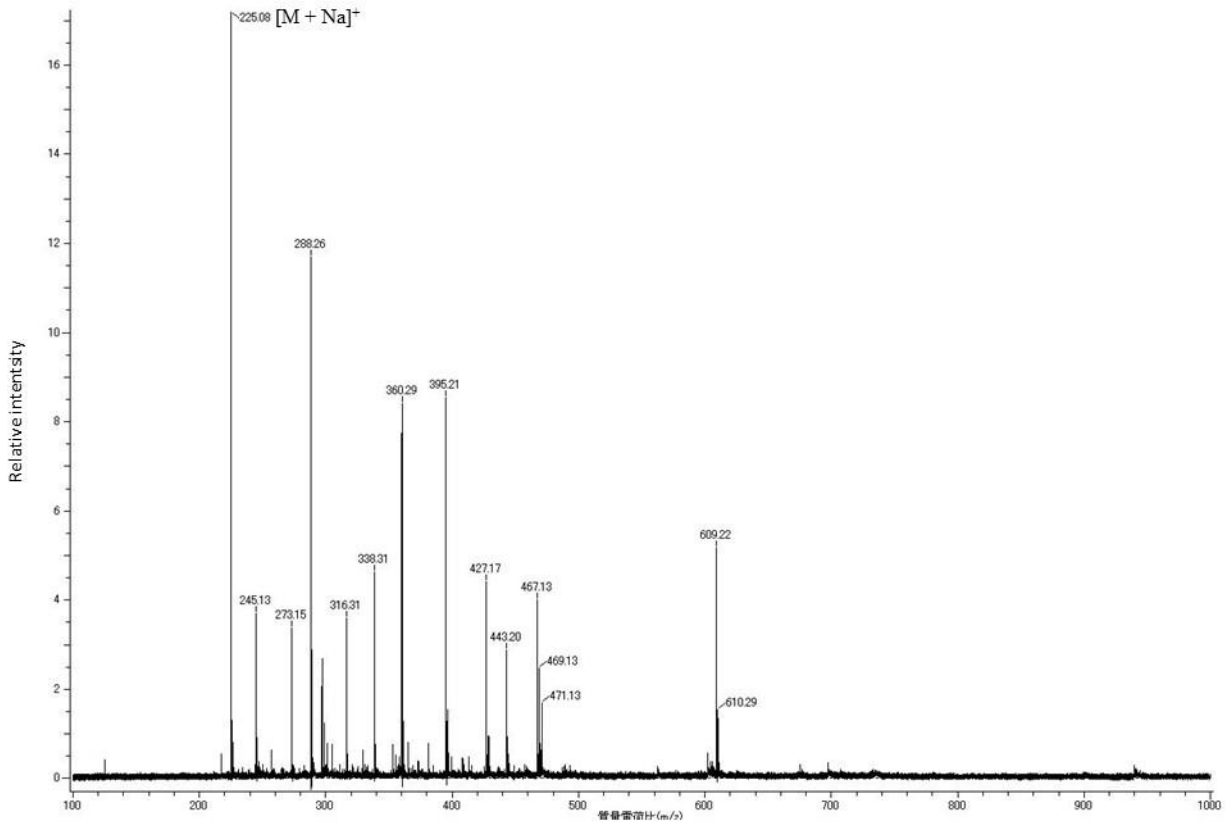
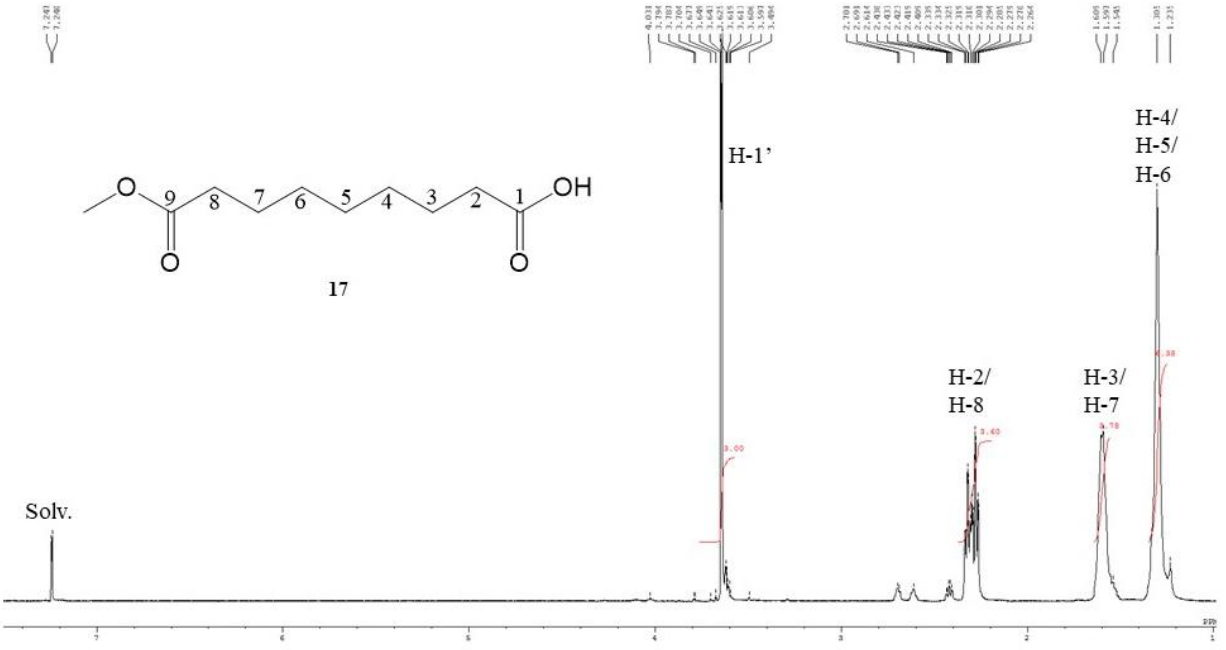


Figure 102 ESIMS spectrum of **17**



17

Figure 103 ^{1}H NMR spectrum of **17** ($CDCl_3$)

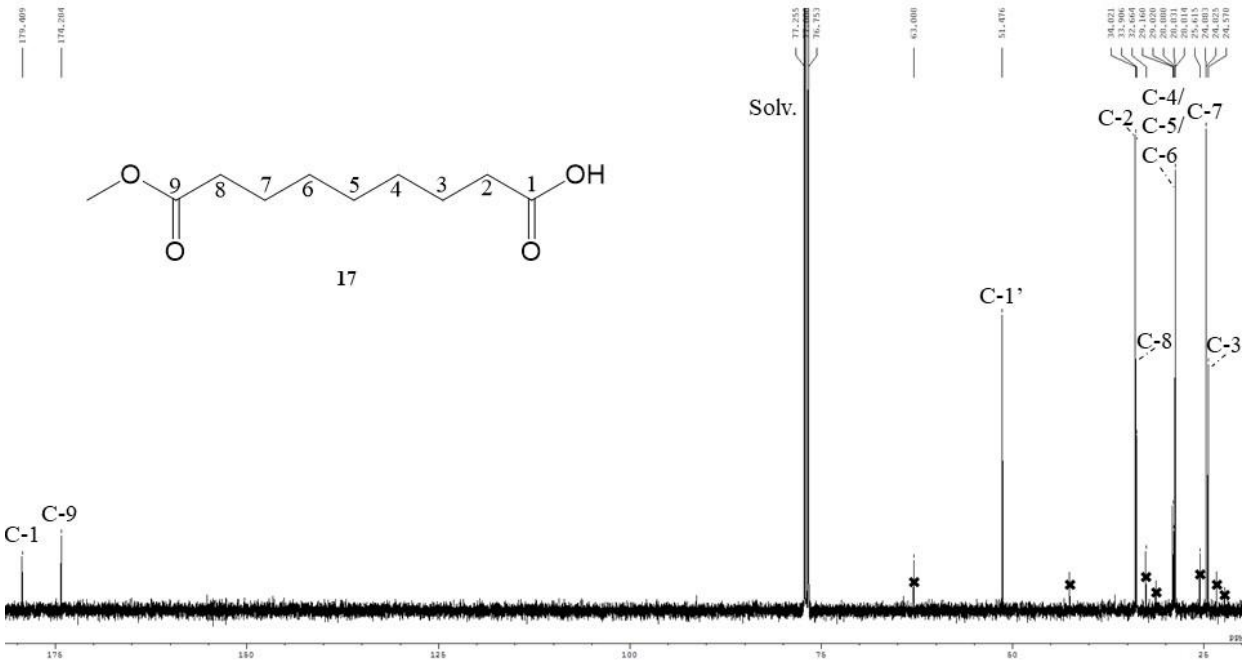


Figure 104 ^{13}C NMR spectrum of **17** ($CDCl_3$)

2-3 Compound **18**

Compound **18** was isolated as colorless crystal. Its molecular weight was measured by ESIMS; *m/z* 195 $[M+Na]^+$ (Figure 106). The planar structure of **18** was elucidated by interpretation of the NMR spectra, including DEPT COSY, HMBC, and HMQC data. The DEPT experiment indicated the presence of one methyl, six methylenes, and two tetrasubstituted carbons. The presence of levulinic acid moiety was constructed by the HMBC correlations of H-2/C-1, C-3, C-4 and H-3/C-1, C-2, C-4. The COSY correlations (H-5/H-6; H-6/H-7; H-7/H-8; H-8/H-9) and the HMBC correlations (H-5/C-4, C-6; H-6/C-8; H-7/C-8; H-9/C-7, C-8) suggested the presence of hydrocarbon chain attached at C-5 (Figures 110 and 111). Compound **18** was identified as 4-oxononanoic acid that has been isolated from the edible mushroom *Cortinarius caperatus* and reported it has growth regulatory activities towards plants.[58]

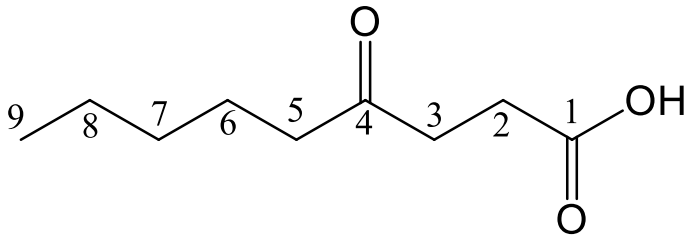


Figure 105 Structure of **18**

Table 17 ^{1}H and ^{13}C NMR assignments for **18**

Compound **18** ($CDCl_3$)		
Position	δ_C	δ_H (multiplicity, *J* in Hz)
1	178.1	
2	27.7	2.61 (t, *J* = 6.5)
3	36.7	2.70 (t, *J* = 6.0)
4	209.0	
5	42.7	2.42 (t, *J* = 7.5)
6	23.5	1.57 (m)
7	23.4	1.57 (m)
8	31.3	1.26 (m)
9	13.9	0.85 (t, *J* = 6.5)

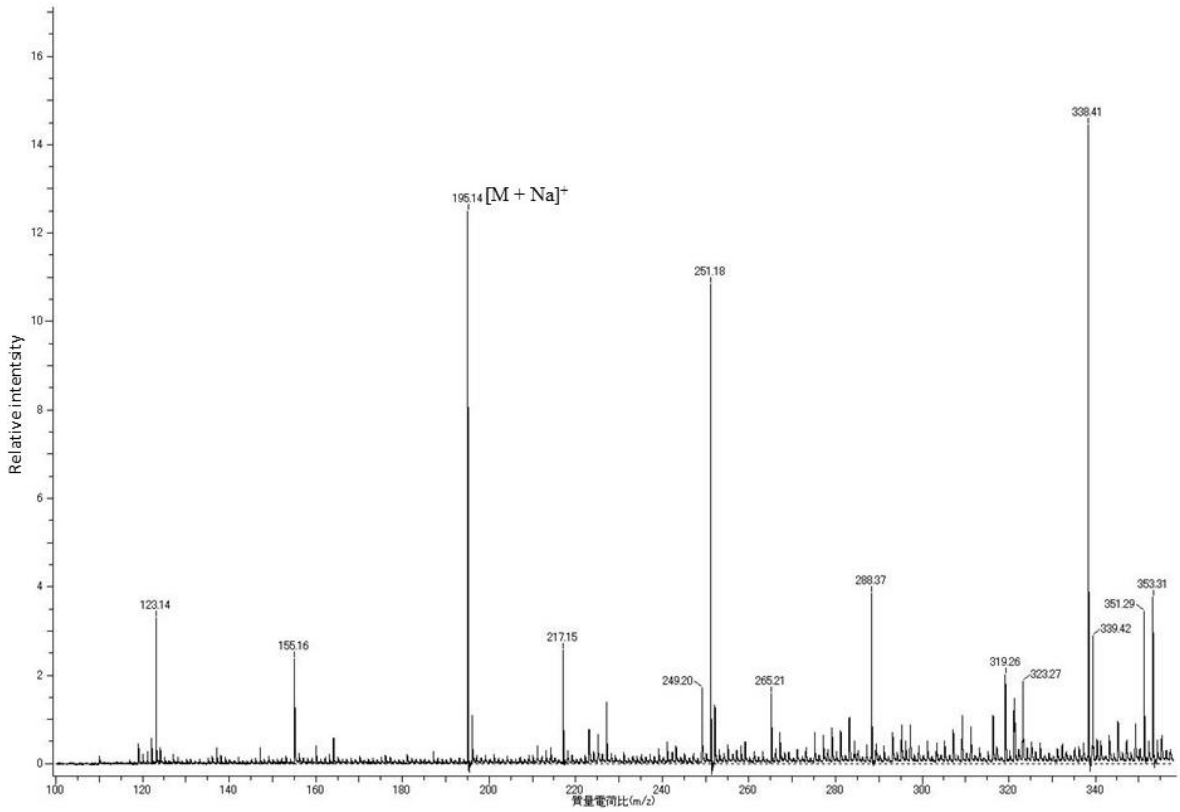


Figure 106 ESIMS spectrum of **18**

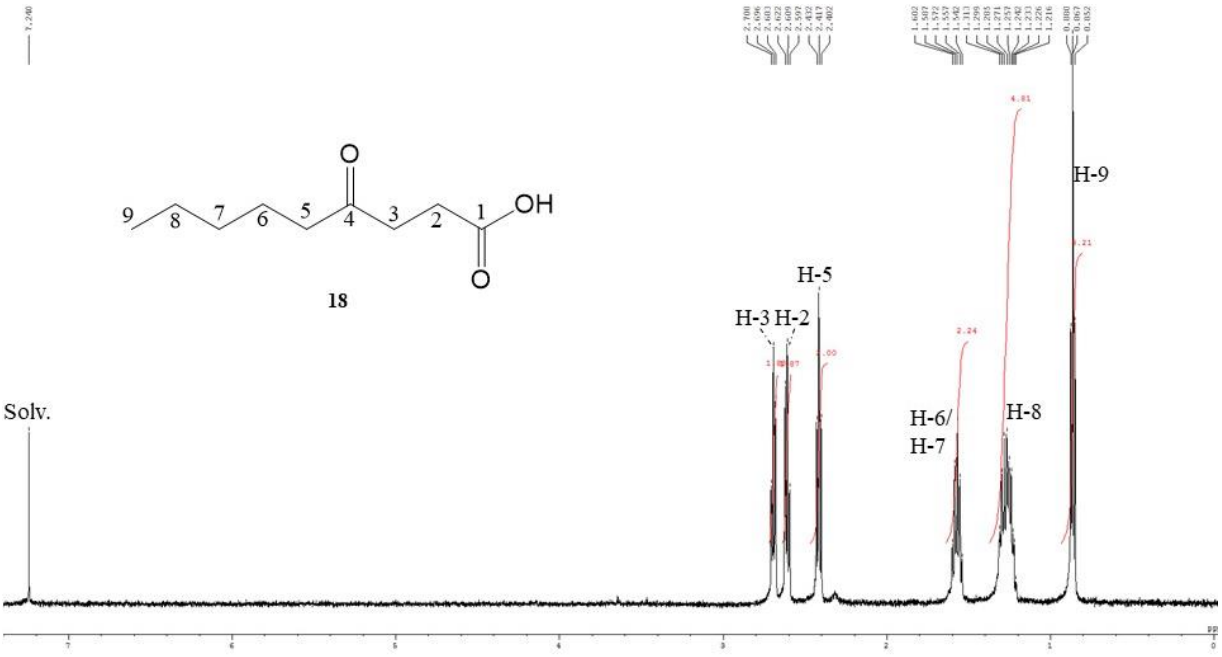


Figure 107 ^{1}H NMR spectrum of **18** ($CDCl_3$)

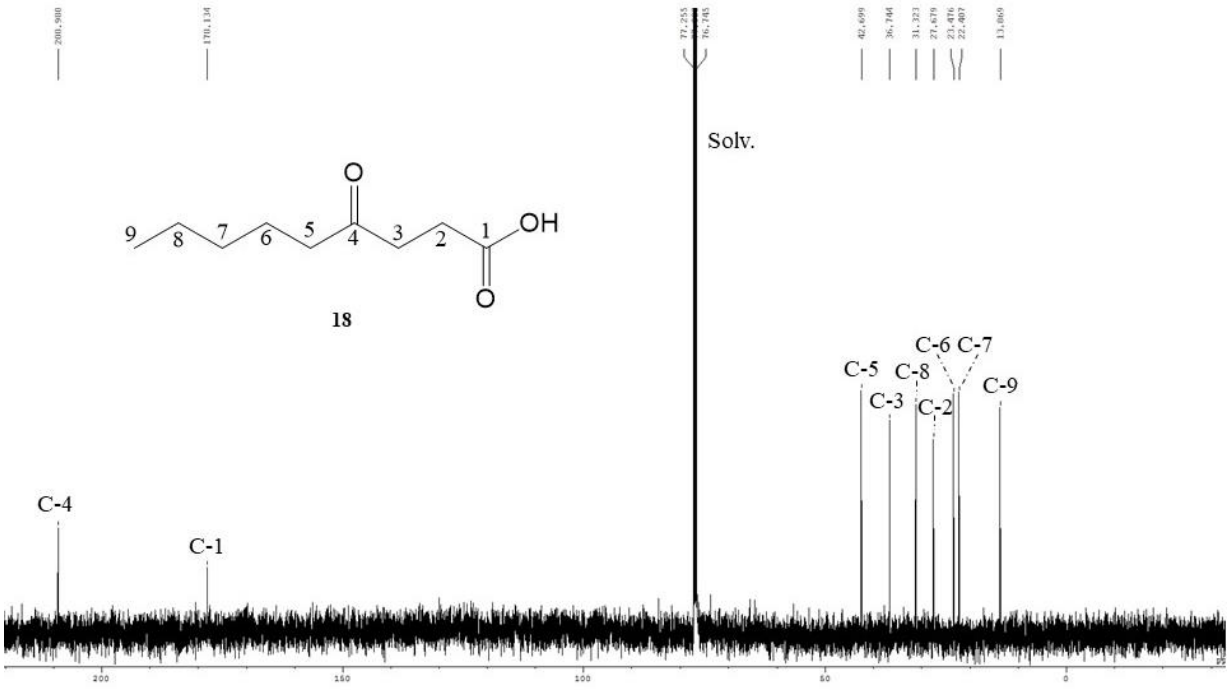


Figure 108 ^{13}C NMR spectrum of **18** ($CDCl_3$)

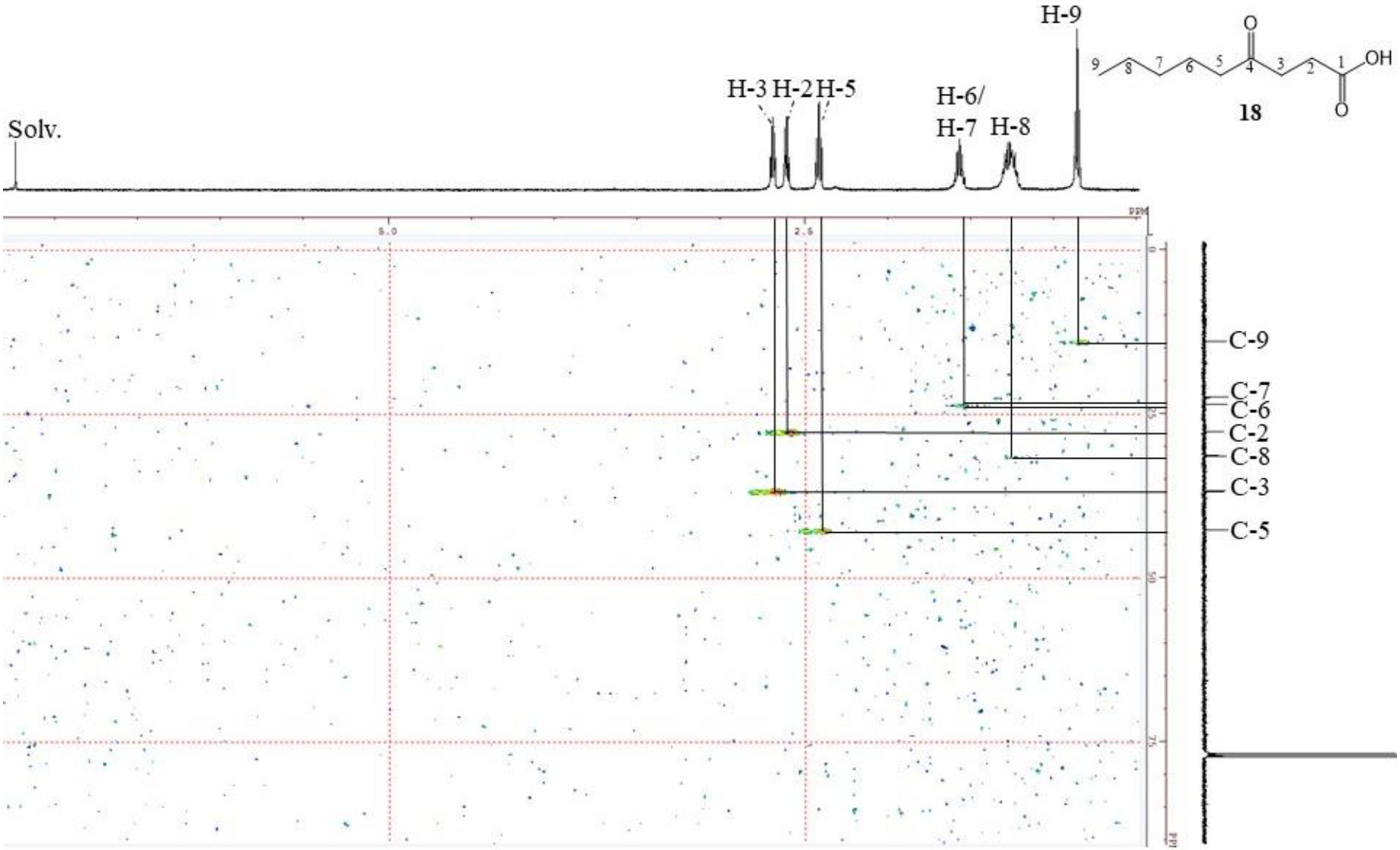


Figure 109 HMQC spectrum of **18** ($CDCl_3$)

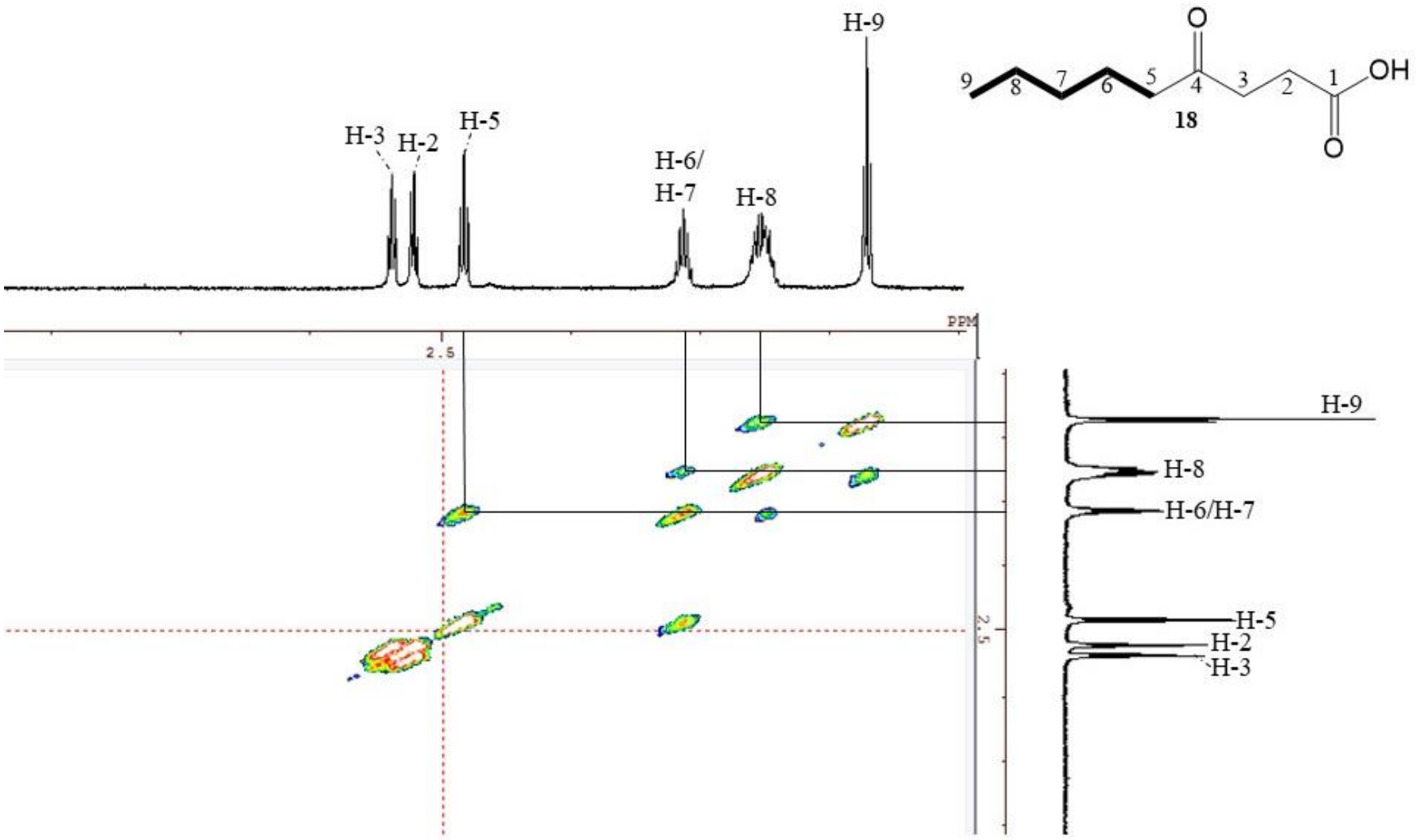


Figure 110 COSY spectrum of **18** ($CDCl_3$)

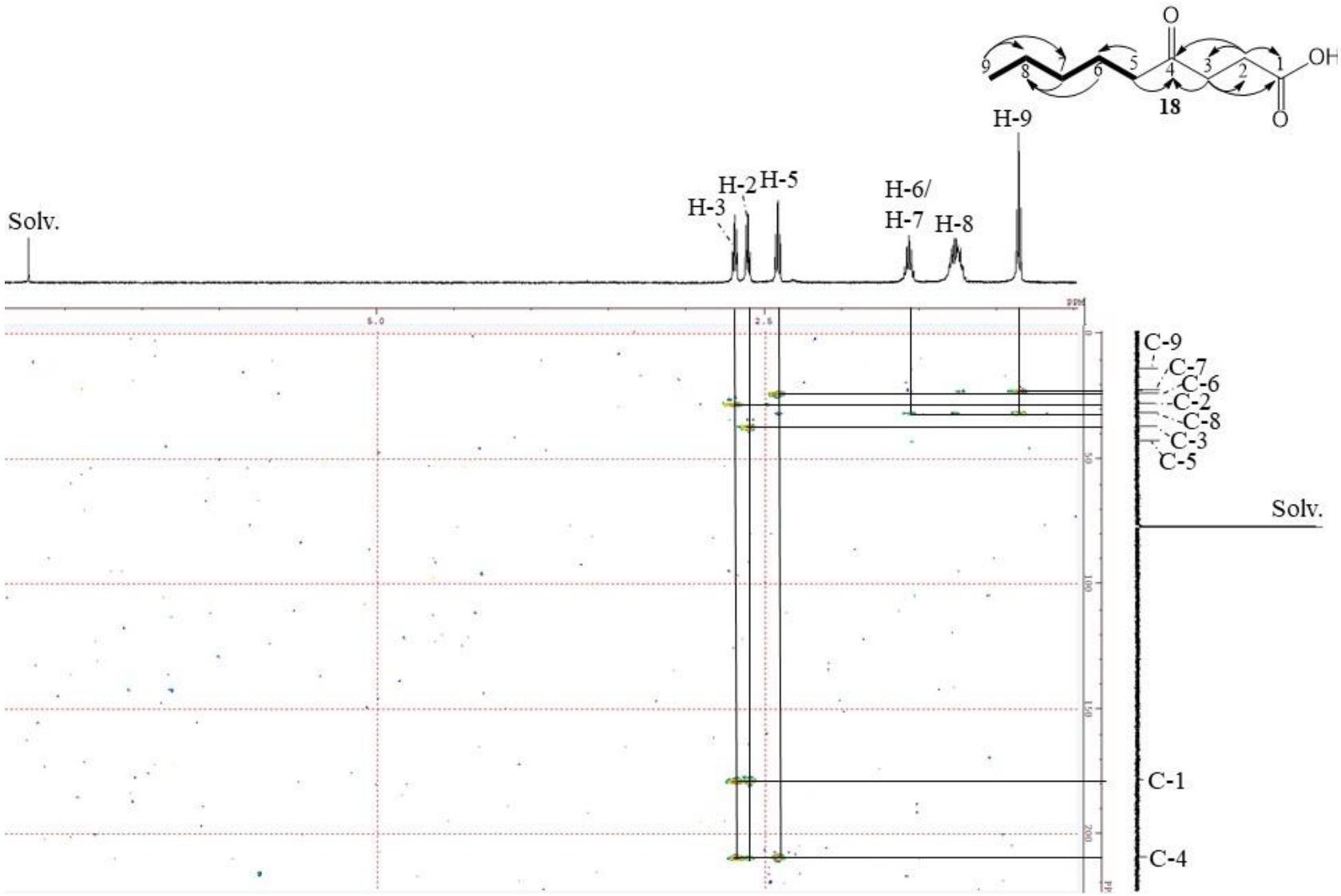


Figure 111 HMBC spectrum of **18** ($CDCl_3$)

2-4 Compound **19**

Compound **19** was isolated as colorless liquid. Its molecular formula was determined as $C_8H_8O_2$ by HRESIMS *m*/*z* 159.0440 $[M+Na]^+$ (calcd. for $C_8H_8O_2Na$, 159.04637), indicating the presence of five degrees of unsaturation in the molecule (Figure 113). The planar structure of **19** was identified by interpretation of the NMR spectra data and its comparison to the reported one (Table 18). Compound **19** was identified as methyl benzoate that has been isolated from the edible mushroom *Cortinarius caperatus* and reported it has growth regulatory activities towards plants.[57]

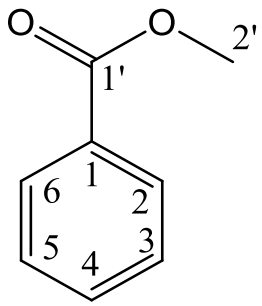


Figure 112 Structure of **19**

Table 18 ^1H and ^{13}C NMR assignments for **19**

Compound **19** ($CDCl_3$)		
Position	δ_C	δ_H (multiplicity, *J* in Hz)
1	129.4	
2	128.7	7.28 (m)
3	127.4	7.20 (m)
4	133.3	7.26 (m)
5	127.4	7.20 (m)
6	128.7	7.28 (m)
1’	177.1	
2’	41.0	3.60 (s)

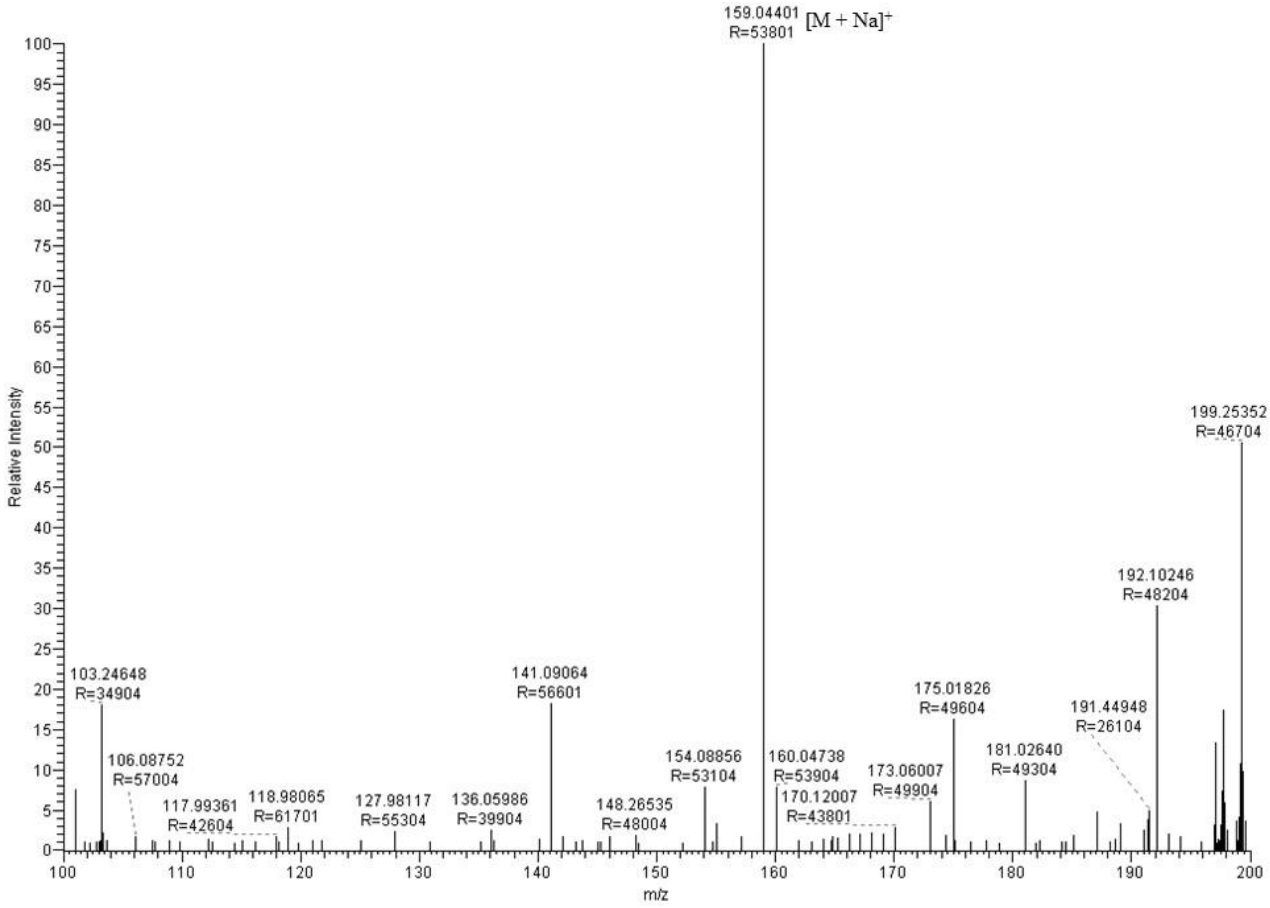


Figure 113 ESIMS spectrum of **19**

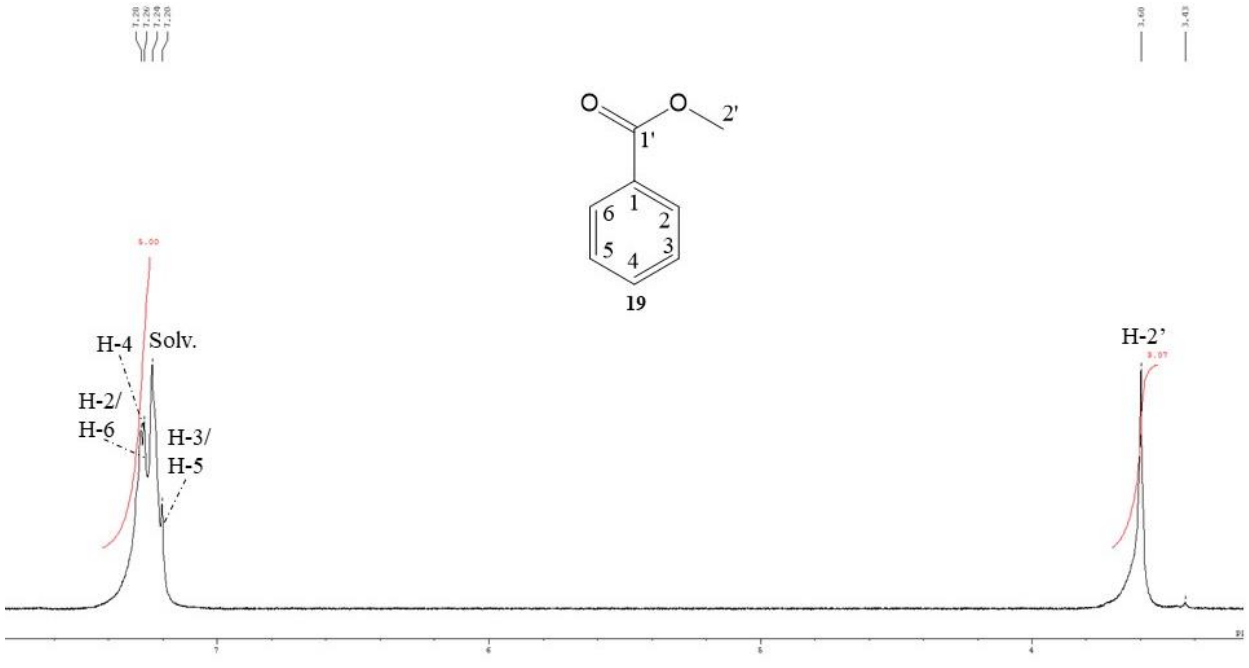


Figure 114 ^{1}H NMR spectrum of **19** ($CDCl_3$)

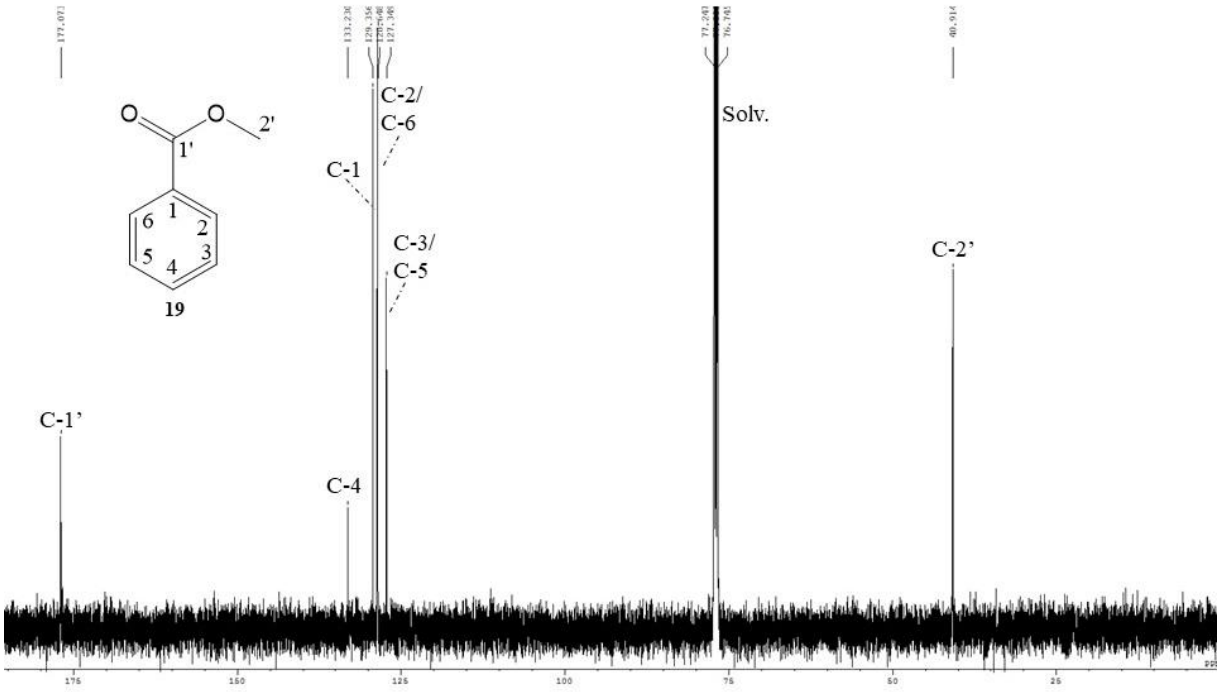


Figure 115 ^{13}C NMR spectrum of **19** ($CDCl_3$)

2-5 Compound **20**

Compound **20** was isolated as colorless crystal. Its molecular weight was measured by ESIMS; *m/z* 197 $[M+H]^+$ (Figure 117). The planar structure of **20** was identified by interpretation of the NMR spectra data and its comparison to the reported one (Table 19). The absolute configuration of **20** was determined by comparing its specific rotation { $[\alpha]_D^{26}$ -57 (*c* 0.19, MeOH) } with that of its reported one { $[\alpha]_D$ -22 (*c* 0.20, MeOH) }. Compound **20** was identified as *cyclo*(D-Pro-L-Val) that has been isolated from the edible mushroom *Aspergillus* sp. as a specific *β*-glucosidase inhibitor.[59]

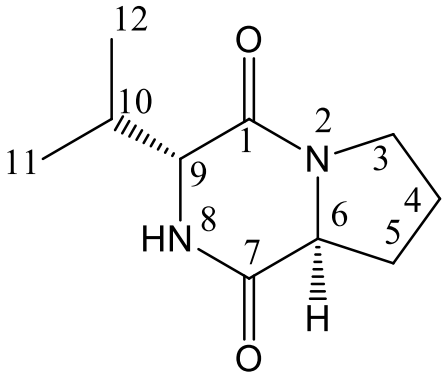


Figure 116 Structure of **20**

Table 19 ^{1}H and ^{13}C NMR assignments for **20**

Compound **20** ($CDCl_3$)		
Position	δ_C	δ_H (multiplicity, *J* in Hz)
1	164.8	
2		
3	45.2	3.52 (m), 3.61 (m)
4	19.3	1.90 (m)
5	22.4	2.05 (m), 2.35 (m)
6	60.4	3.91 (m)
7	170.1	
8		
9	58.8	4.06 (m)
10	28.5	2.61 (m)
11	16.0	0.89 (d, *J* = 10.0)
12	16.0	1.05 (d, *J* = 10.0)

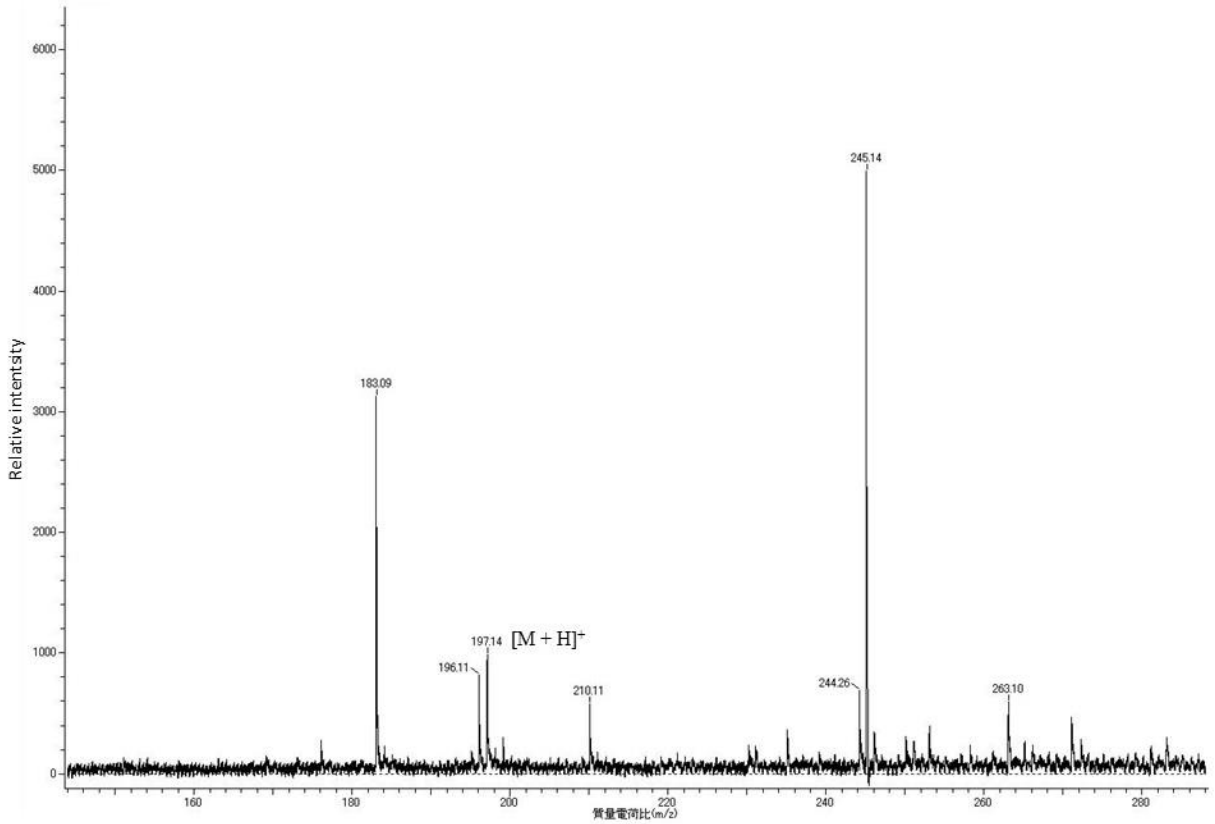
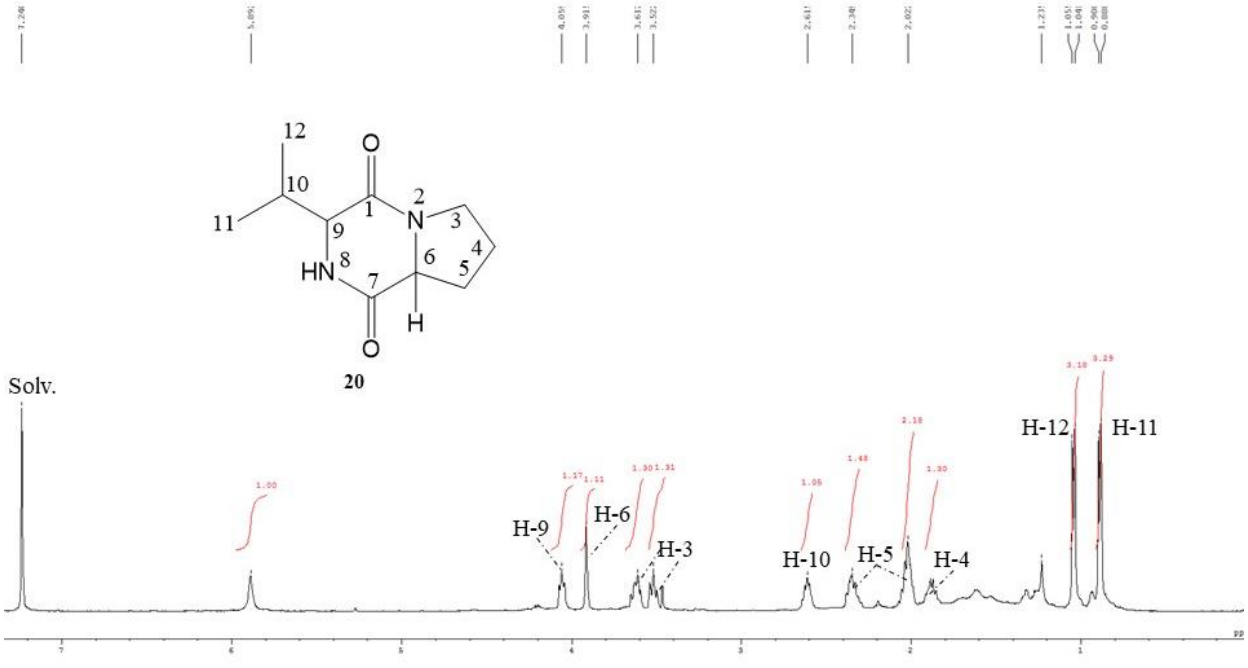


Figure 117 ESIMS spectrum of **20**



20

Figure 118 ^{1}H NMR spectrum of **20** ($CDCl_3$)

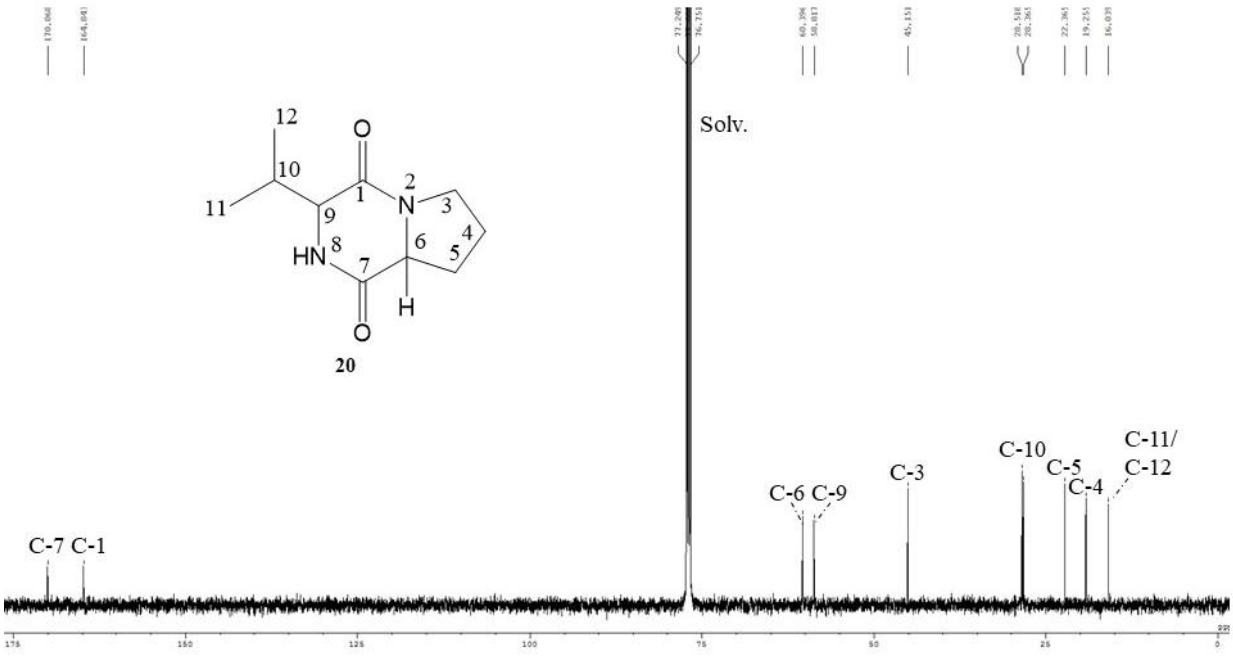


Figure 119 ^{13}C NMR spectrum of **20** ($CDCl_3$)

2-6 Compound **21**

Compound **21** was isolated as a white amorphous. Its molecular weight was determined by ESIMS; *m/z* 122 $[M-H]^-$ (Figure 121). The planar structure of **21** was identified by interpretation of the NMR spectra data and its comparison to the reported one (Table 20). Compound **21** was identified as 3-pyridinecarboxylic acid or nicotinic acid (Figure 120) by NMR spectra (Table 20; Figures 122 and 123). Compound **21** is a commercialy available substance and has been isolated from many natural products, including an edible mushroom *Astraeus odoratus*. Compound **21** is a common compound which has been used to help in the fight against various diseases, such as elavated fasting glucose, diabetes, metabolic syndrome, and the treatment of dyslipidemia.[60]

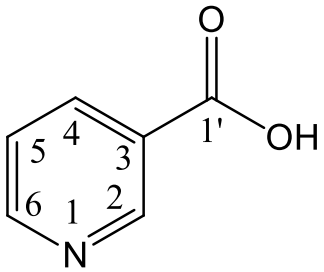


Figure 120 Structure of **21**

Table 20 ^{1}H and ^{13}C NMR assignments for **21**

	Compound **21** (CD_3OD)	
Position	δ_C	δ_H (multiplicity, *J* in Hz)
1		
2	149.5	9.01 (d, *J*= 2.1)
3	131.5	
4	137.3	8.27 (m)
5	125.1	7.53 (dd, *J*= 4.9, 7.9)
6	152.8	8.68 (dd, *J*= 1.5, 4.9)
1’	169.8	

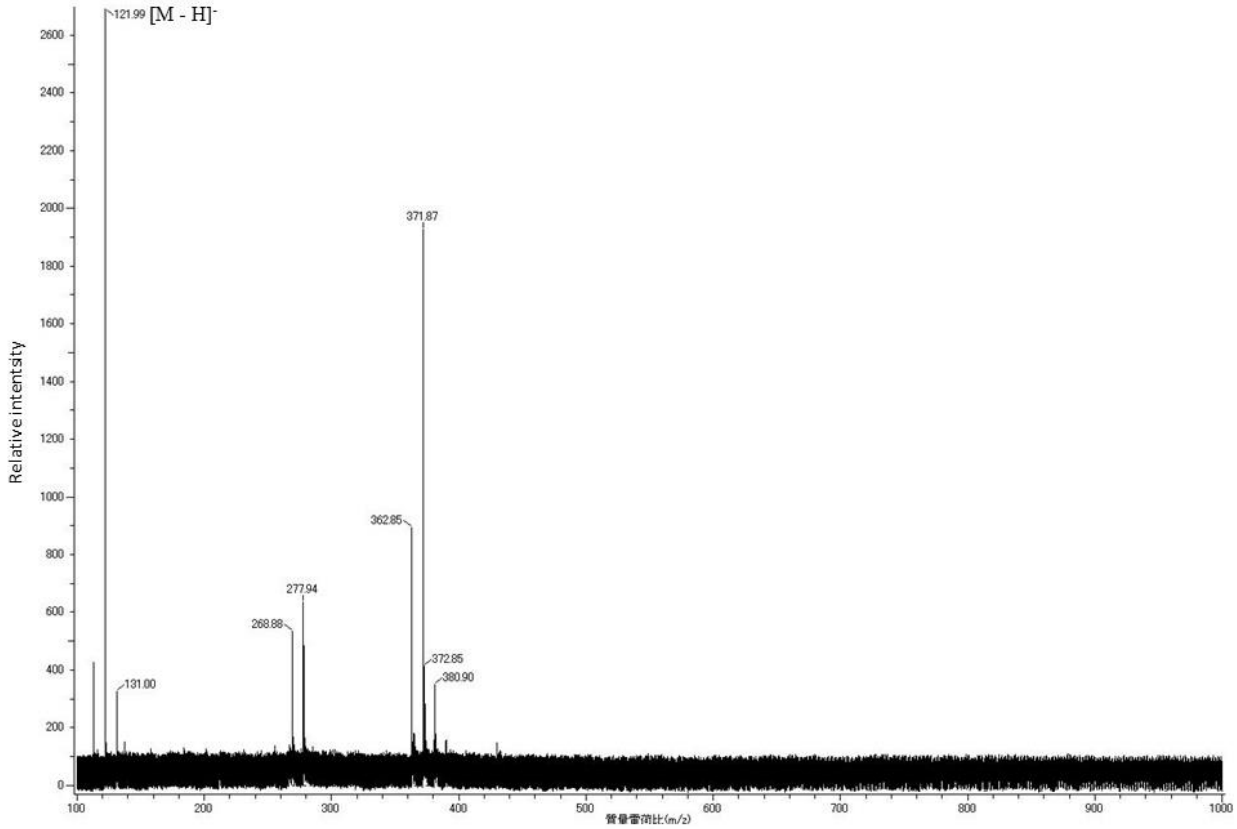


Figure 121 ESIMS spectrum of **21**

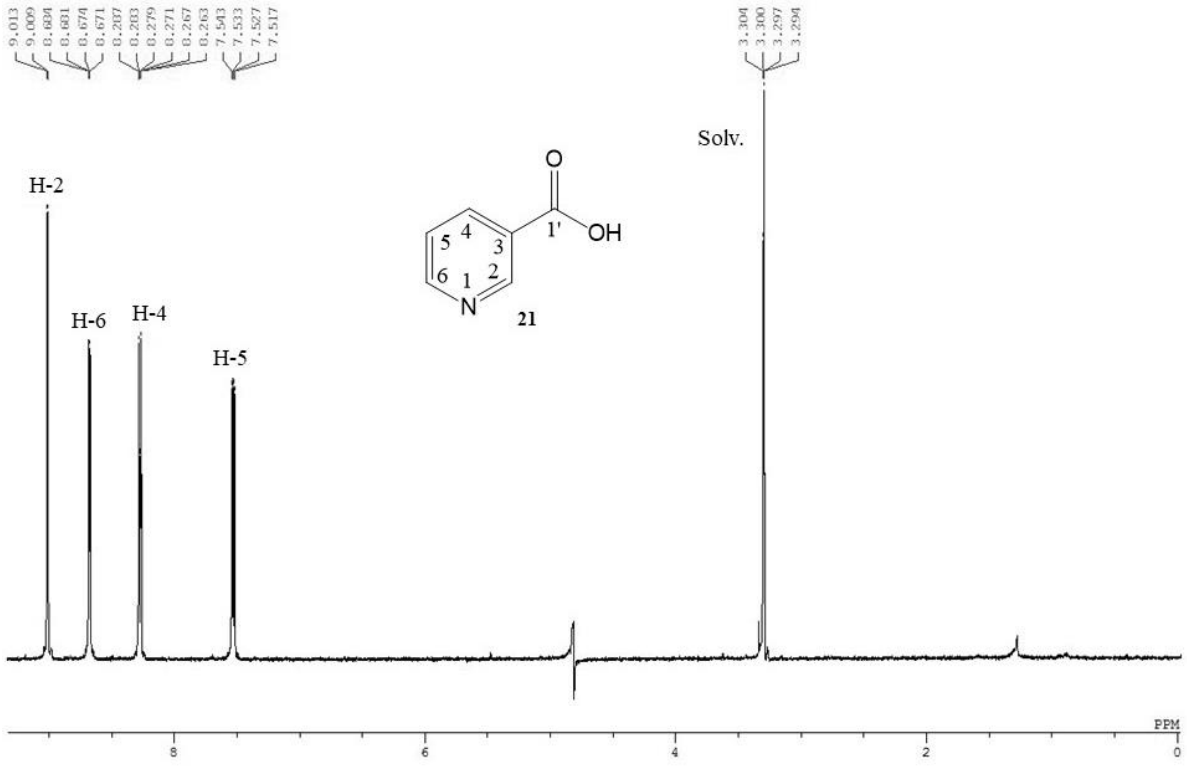


Figure 122 ^{1}H NMR spectrum of **21** (CD_3OD)

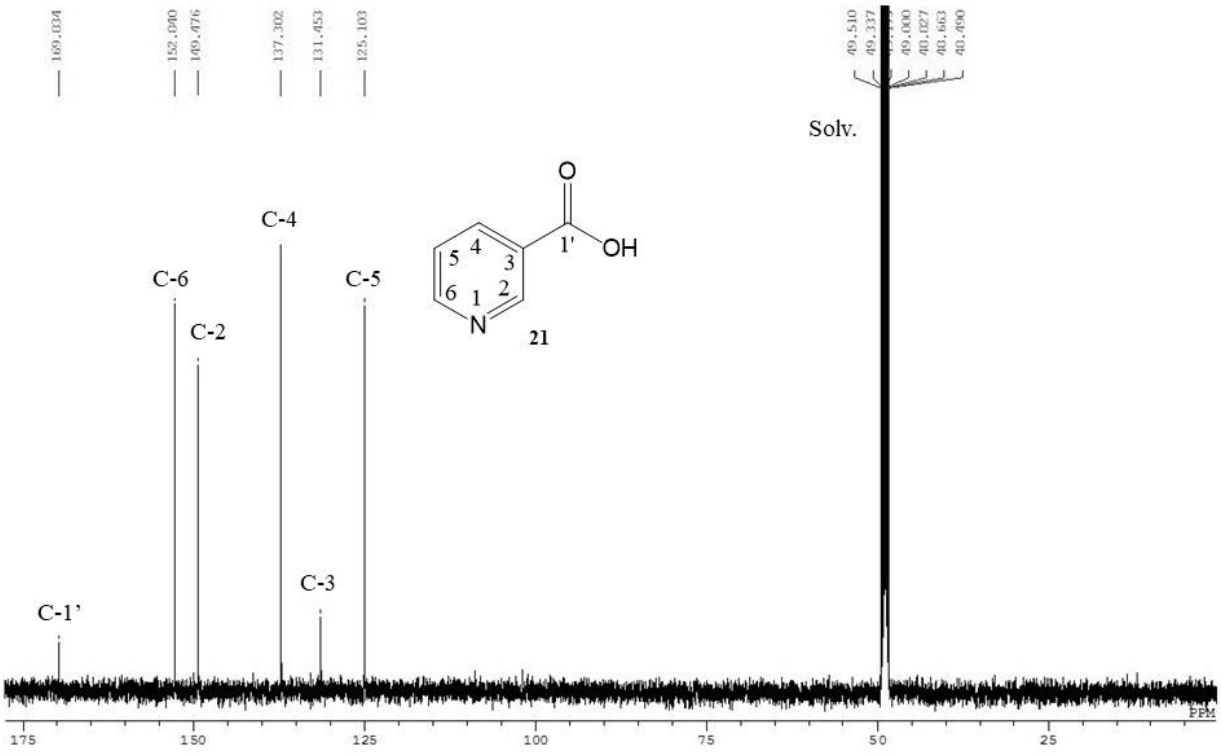


Figure 123 ^{13}C NMR spectrum of **21** (CD_3OD)

Section 3 Biological assay

3-1 Plant growth regulating activity towards rice seedlings

Plant growth regulating activity of **16**–**21** was evaluated towards rice seedlings. 2,4-Dichlorophenoxyacetic acid was used as positive control, which inhibited the hypocotyl and root growth of lettuce dose-dependently. As shown in Figure 124, **16** showed a significance promotion activity at 100 μM towards root growth, while **18** showing inhibition activity at 1 μM against root growth and 100 μM against hypocotyl growth. Interestingly, compound **19** exhibited both activities, it promoted the root growth at 1 and 10 μM as well as inhibited the hypocotyl growth at 100 μM. Compounds **20** and **21** showed strong inhibition activity against hypocotyl growth, especially **21** inhibited at 10 and 100 μM.

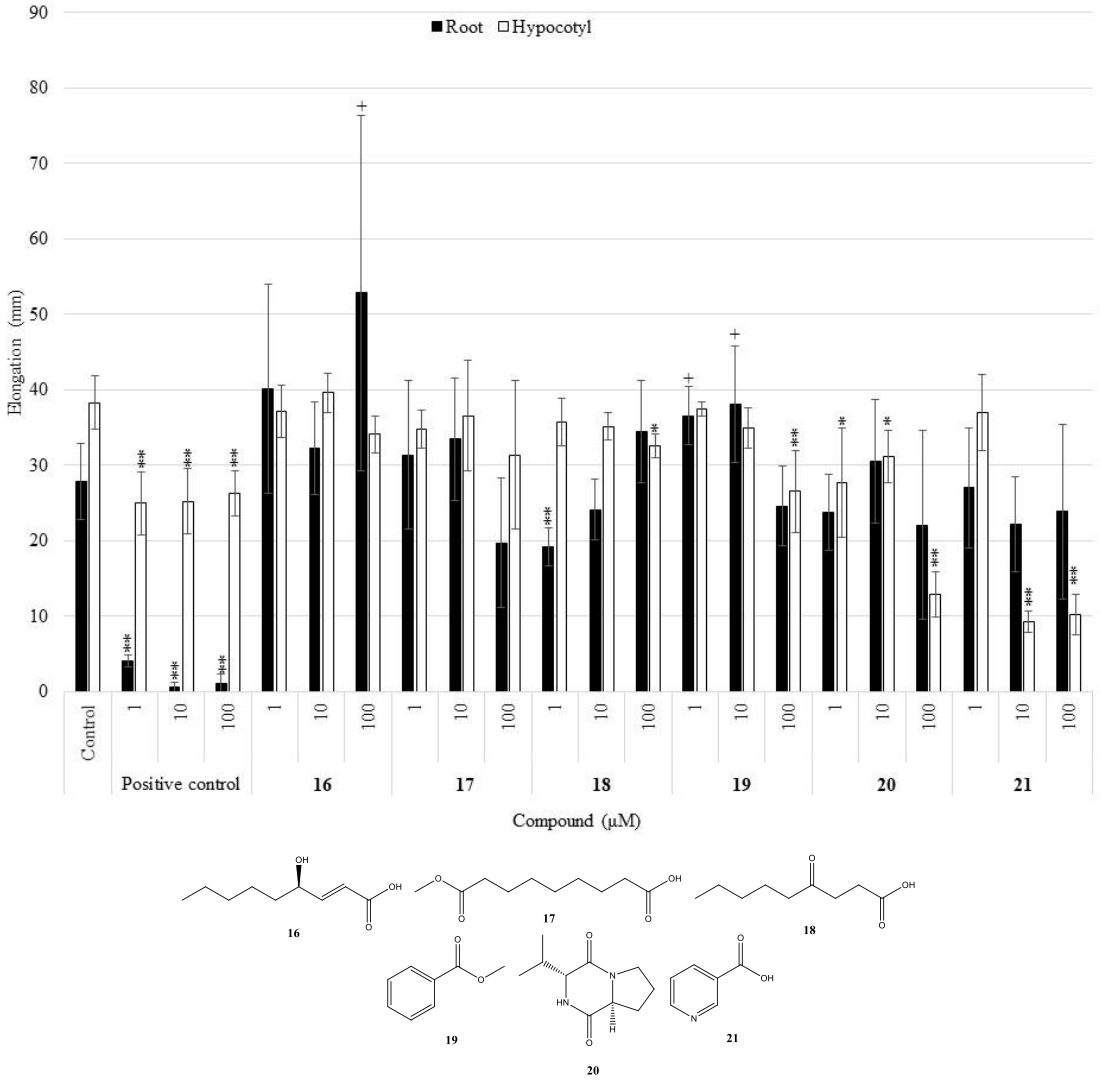


Figure 124 Growth regulating activity towards rice seedlings of compounds **16** to **21** against root or hypocotyl. 2,4-Dichlorophenoxyacetic acid (2,4-D) was used as positive control. Results are the mean ± standard deviation (n = 5). [$^{*}p < 0.05$, $^{**}p < 0.01$ (growth inhibition); $^{+}p < 0.05$, $^{++}p < 0.01$ (growth promotion)].

Chapter 5
Materials and Methods

Section 1 General equipment

The equipment used in this experiment including purification of substances and structural identification instruments. NMR spectra were measured on JEOL JMN-EX-270 FT NMR spectrometer (Jeol Ltd., Tokyo, Japan) and JEOL lambda-500 FT NMR spectrometer (Jeol Ltd., Tokyo, Japan). Molecular weight of compounds was measured by JEOL JMS-DX320, JEOL JMS-DX320HF, and JMS-T100LC mass spectrometer (Jeol Ltd., Tokyo, Japan). IR spectra were evaluated on a FTIR-4100 (Jasco, Tokyo, Japan). The specific rotation values were measured with a Jasco DIP-1000 polarimeter (Jasco Co., Tokyo, Japan). HPLC separation was performed with a Jasco Gulliver system (Jasco Co., Tokyo, Japan) using two reverse-phase HPLC columns (Wakosil-II 5C18 HG Hrep, 20×250 mm, Wako, Osaka, Japan; Phenyl, 20×250 mm, GL Sciences Inc., Tokyo, Japan) and two normal phase HPLC columns (Inertsil Diol, 20×250 mm, GL Sciences Inc., Tokyo, Japan; YMC-pack Diol-60-NP, 20×250 mm, YMC Co., Ltd., Kyoto, Japan). MPLC separation was performed with a Smart Flash AKROS Operation Manual (Yamazen Corp., Japan) using a silica gel column (2.3 x 12.3 cm, 16 g). ODS cartridges (Waters Sep-Pak®, Japan) were used in the pro-processing. Silica gel plate (TLC Silica gel 60 F254, Merck KGaA, Darmstadt, Germany) and silica gel 60N (Kanto Chemical Co.,Inc., Tokyo, Japan) were used for analytical TLC and for flash column chromatography, respectively.

Section 2 Isolation of bioactive compounds from *Leucopaxillus giganteus*

7.9 kg of the fresh fruiting bodies were extracted with EtOH (30 L, 2 times) and then acetone (20 L, 2 times) to obtain the crude extracts. Afterwards, they were combined and concentrated under reduced pressure. The concentrated solutions were partitioned into *n*-hexane, EtOAc, EtOH, and H_2O soluble parts. The EtOAc soluble part (14.8 g) was subjected to silica gel flash column chromatography (CH_2Cl_2; 90/10, 70/30, 50/50 CH_2Cl_2/acetone; 80/20, 60/40 CH_2Cl_2/MeOH; MeOH 1.0 L each) to obtain 12 fractions (1 to 12). Fraction 4 (476.2 mg) was subjected to silica gel flash column chromatography (*n*-hexane; 90/10, 80/20, 70/30, 50/50, 30/70, 20/80 *n*-hexane /acetone; acetone; MeOH 200 ml each) to obtain 18 fractions (4-1 to 4-18). Fraction 4-11 (38.1 mg) was further fractionated by normal phase HPLC (Inertsil Diol, UV 240 nm, 5 mL/min, $CHCl_3$/MeOH = 95/5) to give compounds **1** (6.0 mg) and **2** (8.8 mg).

Section 3 Isolation of bioactive compounds from culture broth of *Stropharia rugosoannulata*

S. rugosoannulata NBRC 31871 was deposited at the culture collection of the Forestry and Forest Products Research Institute. The culture medium (24 g/L) of *S. rugosoannulata* was prepared containing potato dextrose broth (Difco). The medium was packed in each glass bottle (7.2 g/300 mL shake flask) and autoclaved (120 °C, 1.2 atm, 20 min). The preincubated mycelia of *S. rugosoannulata* (10 pcs) were inoculated to the bottle and incubated under the conditions (22°C, shaking with 120 rpm) for seven days in an incubator (NR-30, Tietech, Japan). *S. rugosoannulata* was further incubated for 30 days in the jar fermentor (Mitsuwa Frontech Corp., Japan). The culture broth of *S. rugosoannulata* (32.9 L) was filtered and concentrated under reduced pressure. The concentrated filtrate was partitioned between *n*-hexane and water, EtOAc and water, and EtOH and water. The EtOAc soluble part (4.3 g) was subjected to silica gel flash column chromatography (CH_2Cl_2; 98/2, 95/5; 93/7; 90/10; 80/20; 70/30; 60/40; 50/50 CH_2Cl_2/MeOH; MeOH; 1.0 L each) to obtain 16 fractions (1 to 16). Fraction 1 (62.8 mg) was purified by reverse phase HPLC (Wakosil-II 5C18, UV 210 nm, 5 mL/min, 85% MeOH) to give compounds **9** (1.3 mg) and **15** (3.6 mg). Fraction 2 (980 mg) was subjected to silica gel flash column chromatography (CH_2Cl_2; 98/2, 95/5; 93/7; 90/10; 80/20; 70/30; 60/40; 50/50; 40/60; 30/70; 20/80; 10/90 CH_2Cl_2/EtOAc; EtOAc; MeOH; 0.5 L each) for further purification to give 10 fractions (2-1 to 2-10). Fraction 2-4 (45.7 mg) and fraction 2-5 (19.2 mg) were further purified by normal phase HPLC (YMC-pack Diol-60-NP, UV 250 nm, 5 mL/min, 98/2 $CHCl_3$/MeOH) to afford compounds **12** (13.8 mg) from fraction 2-4 and **11** (2.6 mg) from both fraction. Fraction 2-7 (5.7 mg) was also further purified by normal phase HPLC (YMC-pack Diol-60-NP, UV 250 nm, 0.5 mL/min, 50/50 $CHCl_3$/MeOH) to obtain compound **13** (4.2 mg). Fraction 4 (74.8 mg) was separated by ODS cartridges (40% MeCN) to give 2 fractions (4-1 and 4-2). Fraction 4-1 (43.6 mg) was purified by reverse phase HPLC (Phenyl, UV 210 nm, 5 mL/min, 38 % MeCN) to give 18 fractions (4-1-1 to 4-1-18). Fraction 4-1-8 (5.5 mg) was further purified by reverse phase HPLC (Phenyl, UV 210 nm, 0.5 mL/min, 20% MeOH) to give compounds **7** (1.6 mg) and **14** (2.1 mg). Fraction 15 (45.0 mg) was separated by ODS cartridges (30% MeOH) to give 2 fractions (15-1 and 15-2). Fraction 15-1 (32.3 mg) was fractionated by reverse phase HPLC (Phenyl, UV 210 nm, 5 mL/min, 10% MeOH) to obtain compounds **10** (3.4 mg) and **8** (1.8 mg).

Section 4 Isolation of bioactive compounds from *Entoloma clypeatum*

8.65 kg of the fresh fruiting bodies were extracted with EtOH (1.5 L, 2 times) and then acetone (1.5 L, 2 times) to obtain the crude extracts. Afterwards, they were combined and concentrated under reduced pressure. The concentrated solutions were partitioned into *n*-hexane, EtOAc, EtOH, and H_2O soluble parts. The EtOAc soluble part (13.5 g) was subjected to silica gel flash column chromatography (CH_2Cl_2; 90/10, 80/20, 70/30, 60/40, 50/50, 40/60, 30/70, 20/80, 10/90 CH_2Cl_2/MeOH; MeOH 1.0 L each) to obtain 12 fractions (1 to 12). Fraction 2 (1.05 g) was subjected to silica gel flash column chromatography (90/10, 80/20, 70/30, 60/40, 50/50, 30/70 *n*-hexane/acetone; 70/30, 50/50, 30/70 CH_2Cl_2/acetone; acetone; MeOH 0.5 L each) to obtain 15 fractions (2-1 to 2-15). Fraction 2-5 (54.1 mg) was further fractionated by reverse phase HPLC (Phenyl, UV 210 nm, 5 mL/min, 60% MeCN) to give compounds **18** (6.2 mg). Fraction 2-7 (68.4 mg) was also fractionated by reverse phase HPLC (Phenyl, UV 210 nm, 5 mL/min, 43% MeCN) to give compounds **16** (1.2 mg) and **19** (4.0 mg). Fraction 3 (2.39 g) was subjected to silica gel flash column chromatography (70/30, 60/40, 50/50, 40/60, 30/70, 20/80, 10/90 *n*-hexane/acetone; 50/50, 40/60, 30/70 CH_2Cl_2/acetone; acetone; MeOH 0.5 L each) to obtain 16 fractions (3-1 to 3-16). Fraction 3-4 (54.1 mg) was subjected to silica gel MPLC (73/27, 52/48, 42/58, 32/68 *n*-hexane/EtOAC; EtOAc; MeOH) to give 10 fractions (3-4-1 to 3-4-10). Fraction 3-4-4 (52.0 mg) was further fractionated by reverse phase HPLC (Phenyl, UV 210 nm, 5 mL/min, 70% MeOH) to give compounds **17** (14.8 mg). Fraction 3-10 (68.4 mg) was separated by ODS cartridges (60% MeOH) to give 2 fractions (3-10-1 and 3-10-2). Fraction 3-10-1 (20.0 mg) was fractionated by reverse phase HPLC (Phenyl, UV 210 nm, 5 mL/min, 45% MeOH) to give compounds **21** (1.2 mg). Fraction 3-11 (72.5 mg) was separated by ODS cartridges (60% MeOH) to give 2 fractions (3-11-1 and 3-11-2). Fraction 3-11-1 (35.1 mg) was fractionated by reverse phase HPLC (Phenyl, UV 210 nm, 5 mL/min, 50% MeOH) to give compounds **20** (1.9 mg).

Section 5 Esterification of malic acid

(*R*) or (*S*)-Malic acid (0.9 g or 2 g) and EtOH (10.1 mL) were reacted in the presence of 1M HCl (679 μL) for 15 min at room temperature.[37] The reaction mixture (1.1 g or 2.0 g) was subjected to silica gel flash column chromatography followed by Sephadex LH-20 gel (GE Healthcare, Uppsala, Sweden; $CHCl_3$/MeOH = 1/1, 35×500 mm, 100 g). As a result, two stereoisomers of monoethyl esters **3** (18.6 mg, 1.8 % yield) and **4** (10.2 mg, 0.4 % yield) along with diethyl esters **5** (12.0 mg, 1.0 % yield) and **6** (98.9 mg, 3.5% yield) were isolated.

Section 6 Biological assay

6-1 Axl and immune checkpoint assay

The human A549 alveolar epithelial cell line was purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured in DMEM, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and 100 U/mL penicillin plus 100 U/mL streptomycin. All cells were cultured at 37˚C in 75 cm^2 flasks in an atmosphere composed of 5% CO_2 and 95% air. Confluent cells were passaged after 5–7 days.

A549 cells in 0.1% BSA-DMEM were seeded in 24-well plates. *L. giganteus* compounds (20 μg/mL) were added to the wells, and the plates were incubated for 24 hours. Total RNA was extracted using Sepasol®-RNA Ⅰ SuperG (Nacalai) following the instructions of the manufacturer. One microgram of total RNA was denatured at 65˚C for 10 min, and then reverse-transcribed using ReverTra Ace Reverse Transcriptase (TOYOBO) and oligo (dT) primer in a volume of 20 μl according to the manufacturer's protocol. The cDNA was amplified by PCR and the conditions were as follows: 94˚C, 1 min; 60˚C, 1 min and 72˚C, 1 min for 28–35 cycles. The sequences of the primers are described as forward and reverse sequence (5′ > 3′) as GGAGCGAGATCCCTCCAAAAT and GGCTGTTGTCATACTTCTCATGG for GADPH gene, TGCCATTGAGAGTCTAGCTGAC and TTAGCTCCCAGCACCGCGAC for Axl gene, GGACAAGCAGTGACCATCAAG and CCCAGAATTACCAAGTGAGTCCT for PD-L1 gene, ACCGTGAAAGAGCCACTTTG and GCGACCCCATAGATGATTATGC for PD-L2 gene, respectively. PCR products were electrophoresed on a 1.5 % agarose gel and then stained with ethidium bromide solution.

Data are expressed as the mean ± standard error of the mean (SEM). The statistical difference was calculated by analysis of variance with *post hoc* analysis using Fisher's predicted least significant difference test. All statistics were performed using the StatView 5.0 package (Abacus Concepts, Berkeley, CA, USA). $P<0.05$ was considered as statistically different.

6-2 Plant growth regulation assay towards lettuce growth

Lettuce seeds (*Lactuca sativa* L. cv. Cisko; Takii Co., Ltd., Tokyo, Japan) was used in this study for bioactivity examination. Suitable amount of lettuce seeds was put on filter paper (Advantec No. 2, ϕ 55 mm; Toyo Roshi Kaisha, Ltd., Japan), soaked in distilled water in a Petri dish (ϕ 60×20 mm), and incubated in a dark growth chamber at 20˚C for 24 hours. Compounds **1**–**15** and 2,4-dichlorophenoxyacetic acid (2,4-D, positive control) were dissolved in mL of

MeOH (1, 10, 10^2 and 10^3 nmol/mL) and allowed permeating on filter paper (ϕ 55 mm) in a Petri dish (ϕ 60×20 mm). After the sample solution had been dried, 1 mL of distilled water was poured on the sample-loaded paper or intact filter paper (control). The pre-incubated lettuces (n = 9 in each Petri dish) were transferred onto the filter paper and incubated in a dark growth chamber at 20˚C for 3 days. The length of the root and the hypocotyl were measured using a digimatic caliper (Mitutoyo Coporation CD-15AXR, Japan).

6-3 Antibacterial assay

Compounds **7**–**15** were evaluated the antibacterial activity towards three kinds of bacteria *Clavibacter michiganensis*, *Pectobacterium carotovorum*, and *Burkholderia glumae.* Ampicillin was used as positive control which inhibited the growth of bacteria dose-dependently. Each test compound solution and ampicillin were dissolved in MeOH (0.1, 0.05, and 0.01 μmol/10 μL) and allowed permeating on sterilized paper disks (Advantec ϕ 8 mm). Each air-dried paper disk containing the compounds were placed directly onto the bacteria-containing yeast peptone medium. Plates were further incubated at 28˚C for 7 days. After the incubation, the activity was evaluated by observation of the inhibition zones.

6-4 Plant growth regulating activity towards rice seedlings

Rice seeds (Oryza sativa L. cv. Nipponbare) were sterilized in ethanol for 5 min and then 1% sodium hypochlorite for 30 min on a plastic container (18.5 X 14.5 X 4.5 cm). The seeds were washed completely in sterile water and germinated for 2 d at 30˚C with intensive light. Compounds **16**–**21** and 2,4-dichlorophenoxyacetic acid (2,4-D, positive control) were dissolved in 500 μL of MeOH (1, 10, 100 μM) into glass jars (ϕ 5.5 X 10 cm). After the sample solution had been dried, the seedlings (n = 5) were planted into glass jars containing 5 mL distilled water. Afterward incubated for 5 d at 30˚C with 12 h intensive light. The elongations were measured using a digimatic caliper (Mitutoyo Coporation CD-15AXR, Japan).

Conclusion

In this research, bioactive metabolites from three kinds of mushrooms *Leucopaxillus giganteus*, *Stropharia rugosoannulata*, and *Entoloma clypeatum* were isolated.

1) Bioorganic chemical studies on wild edible mushroom *Leucopaxillus giganteus*

Three compounds, ethyl 2-(2-oxopyrrolidin-1-yl)acetate (**1**) and monoethyl succinate (**2**), and 4-ethoxy-2-hydroxy-4-oxobutanoic acid (**3**) were isolated from the fresh fruiting bodies of *L. giganteus.* In this study, the absolute configuration of a malic acid derivative (**3**) that was previously isolated from the mushroom was determined. The stereoisomers of **3** (**4**) was synthesized to determine the absolute configuration and other two analogs (**5** and **6**) to study the structure activity relationship. As a result, compound **3** was determined to be *R*, indicating that it was a novel compound.

All the obtained compounds were evaluated their activities against the Axl and immune checkpoint assays and plant growth regulating assay. In the first assay, compounds **1** and **2** inhibited Axl, PD-L1, and PD-L2 expressions. Among malic-acid esters (**3**–**6**), only the isolated compound **3** showed the suppression effects on all the gene expressions. In the second assay, the inhibition activity of the novel compound **3** was the strongest among all the compounds tested. The antipode of **3** (**4**) showed much less activity than **3**.

2) Bioorganic chemical studies on culture broth of *Stropharia rugosoannulata*

Nine compounds were isolated from the culture broth of *S. rugosoannulata* and identified as (*S*)-4-(hydroxymethyl)-3,4-dihydroquinolin-2(1H)-one (**7**), acetyl-*D*-phenylalanine (**8**), quinoline-4-carboxaldehyde (**9**), quinoline-4-carboxylic acid (**10**), (*R*)-1-phenylethane-1,2-diol (**11**), (1*R*,2*S*)-1-phenylpropane-1,2- diol (**12**), (*R*)-5-((*S*)-1-hydroxyethyl)dihydrofuran-2(3H)-one (**13**), 4-(2-hydroxyethyl)phenol (**14**), and 3,5-dichloro-4-methoxybenzoic acid (**15**). All the compounds showed no antibacterial activity against *Clavibacter michiganensis, Pectobacterium carotovorum,* and *Burkholderia glumae.* However, compound **10** showed the strongest inhibition towards lettuce growth on the plant growth regulation assay.

3) Bioorganic chemical studies on wild edible mushroom *Entoloma clypeatum*

Six known compounds were isolated and identified as (*R*,*E*)-4-hydroxynon-2-enoic acid (**16**), 9-methoxy-9-oxononanoic acid (**17**) 4-oxononanoic acid (**18**), methyl benzoate (**19**), Cyclo(*D*-Pro-*L*-val) (**20**), and nicotinic acid (**21**). Compound **21** showed the strongest inhibition activity towards rice seedlings growth.

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