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New polyoxygenated polyketide from pathogenic fungus *Cylindrocarpon destructans* with α-glycosidase inhibitory activity

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Abstract: Phytochemical investigation on pathogenic fungus *Cylindrocarpon destructans* isolated form *Meconopsisgrandis* plant led to the isolation of one new polyoxygenated polyketides, namely cylindrocarpolide A along with five known compounds. The structures of the isolated compounds were elucidated by 1D and 2D NMR and mass spectroscopic data analysis. The isolated compounds were evaluated for α-glycosidase inhibition activity. The compounds isolated compounds were found to have strong to weak inhibition against the α-glycosidase enzymes.

Keywords: Pathogenic fungus, *Cylindrocarpon destructans*, *Meconopsis grandis*, α-glycosidase.

Introduction

Species of *Cylindrocarpon*Wollenw. are common and may be isolated as soil inhabitants, saprobes on dead plant material, root colonizers or pathogens, or weak pathogens of various herbaceous and woody plants [1]. *Cylindrocarpon* destructans (Zinnsm.) [anamorph of *Neonectria* radicicola] and *C. obtusisporum* have been reported to cause the root rots of various hosts [2,3], and a black foot disease of grapevines [4-6]. *C. destructans* (*C. radicicola*) has frequently been reported to cause decay of woody seedlings, especially conifers, and many other hosts as well [2]. Generally, this fungus is not severe in its pathogenicity and has been regarded in many cases as the infectious wound fungus or the secondary invader.

Cylindrocarpon species have been rarely associated with human disease. They are known to cause post-traumatic keratitis [7,8] and have been implicated in mycetoma following injury [9,10], athlete's foot [11], peritonitisina case of continuous ambulatory peritoneal dialysis [12], localized invasive lesion in a case of AML [13], disseminate disinfection in neutropenic patients [14], Tinea pedis [15], Cutaneous infection [16]. The human-infecting species include *C. cyanescens*, *C. destructans*, *C. lichenicola* and *C. vaginae* [17].

Previous phytochemical studies on *Cylindrocarpon* species have resulted in the isolation of Orsenol, Orsellinic acid, Ilicicolin (A-F), Ascochlorin, fatty acids [18], Cylindrols [19], Cylindrocyclin [20] Colletorine, Colletochlorin and Curvularine derivatives [21].

Only a few metabolites such as radicicol and radicicolin [22] have been reported from *Cylindrocarpon destructans* (*C. radicola*). In this paper we reported the isolation, characterization and biological activities of one new polyketide (**1**) and five compounds (**2-6**) from *Cylindrocarpon destructan*.

Experimental Section

General

Optical rotations were measured with an Abbemat 300 spectrometer. NMR spectra were recorded with a Bruker Avance 400 (400 MHz for ^{1}H and 100 MHz for ^{13}C spectrometer (Bruker Corporation, Switzerland). HRESIMS spectra were recorded using an LTQ-Orbitrap LC-MS spectrometer (Thermo Corporation, USA). UV spectra were recorded on a Blue Star A spectrophotometer. Thin layer chromatography silica gel GF_{254} (Qingdao Marine Chemical, Factory PR China) were used for TLC. Sephadex LH-20 (Amersham Pharmacia) and Silica gel (100-200, 200-

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300, and 300-400 mesh, Qingdao Marine Chemical Factory, Qingdao, PR China) were used for column chromatography. The reagents in the research process were analytical grade from Guangzhou chemical reagent factory.

Fungal material

The fungal strain *Cylindrocarpon destructans* (Gen accession number KC904953) was isolated from fresh roots of *Meconopsisgrandis* (Tibetan Blue puppy) in Tibetan Plateau near to Damxung, Tibet, China. The fungus was identified by using morphological characteristics and International transcribed spacer region. A voucher specimen (DH 29) has been preserved on PDA at 4°C at the school of marine science, Sun Yat-Sen University.

Fermentation, extraction and fractionation

The fungal strain was cultured for 6 days at 28 ˚C in Petri dishes containing Potato dextrose agar. The agar supported mycelia were then cut and transferred to 1000 mL Erlenmeyer flasks containing 500 mL potato dextrose broth (12 gm of PDB dissolved in 500 mL of 3% saline water) and then incubated at 28 ˚C for 5 days with continuous shaking on a shaker at 150 rpm. Then 10 mL of the fungal broth were added into rice medium (110 bottles each 1000 mL Erlenmeyer flasks, each containing 60 g rice in 80 mL of 3% saline water) and were incubated for 30 days under static conditions and light. After incubation, the mycelia cultivated rice medium were crushed and extracted three times with methanol. The methanol extract was concentrated with a rotary evaporator to get methanolic crude which was then suspended in 20% MeOH-H_20. The suspension was then fractionated with n-hexane, chloroform, ethyl acetate to get the corresponding n-hexane (15 g), Chloroform (26 g) and ethyl acetate (44 g) fractions. The n-hexane fraction was further divided into 6 subfractions (D-I) by silica gel CC with gradient elution of pet ether/CH2Cl2 (from 100:00 to 30:70). The chloroform extract was separated into 7 sub-fractions (J-P) by silica gel column chromatography by a gradient elution of petroleum ether/CH_2Cl_2 (from 90:10 to 00:100) and then CH_2Cl_2/MeOH (from 100:00 to 00:100). Ethyl acetate fraction was further fractionated over silica gel column chromatography with gradient elution of pet ether Ether/EtOAc (100:00–00:100) and then EtOAc/MeOH (100:00 to 00:100) to get 9 fractions (Q-Y).

Table 1. ^{1}H (400 MHz) and ^{13}C (100MHz) NMR data for Compound **1**.

Position	δH (*J* in Hz)	δC	HMBC Correlation
1			
2		151.9	
3	7.57 (1H, s)	109.6	2, 3a, 7a
3a		125.7	
4		118.3	
5		123.1	
6		127.2	
7		141.0	
7a		142.5	
8	10.55 (1H, s)	189.5	4
9		135.5	
10		118.7	
10a		113.2	
11		171.1	
12			
13	5.12 (2H,s)	66.9	10a, 11, 13a
13a		138.9	
14		150.7	
15		132.4	
16	9.61 (1H, s)	195.4	10
17	2.71 (3H, s)	11.9	9, 14, 15
18	2.14 (3H, s)	8.7	5, 6, 7
19	2.46 (3H, s)	10.2	4, 5, 6

Purification of the compounds

Fr. G was further re-chromatograhed over silica gel CC eluted with Pet-ether: CH_2Cl_2(100:00 to 00:100) to get five subfractions (G-1 to G-5). Fraction G-2 (32 mg) was further purified by Sephadex LH-20 with Pet-ether/CH_2Cl_2/MeOH (1:1:1) to afford colorless oil of compound **2** (12 mg). Fraction N was subjected to silica gel CC eluted with CH_2Cl_2/MeOH (100:00 to 70:30) to get five subfractions (N1-N5). Fr. N-3 (was further separated by Sephadex LH-20 eluted with CH_2Cl_2/MeOH (1:1) to get three fractions (N-3-1 to N-3-3). Fr. N-3-2 (21 mg) was further purified by HPLC (75 % CH_3CN-H_2O flow rate 1.0 ml /min; $C_{18,}$ 10×250 mm, 5 um) to get two pure Compounds（**3** (8 mg) and **4** (6 mg) as a white powder**.** Fr. P (28 mg) was purified by Sephadex LH-20 with CH_2Cl_2: MeOH (v/v; 1:1) and then by semi-preparative HPLC (65 % MeOH-H_2O flow rate 1.5 ml /min; $C_{18,}$ 10×250 mm, 5 um) to afford a compound **5** (15 mg) as yellow needles.

Fraction **R** (23 mg) was subjected to series of silica gel chromatographic separation by gradient elution with pet ether/EtOAc and then EtOAc/MeOH and then to HPLC (70% MeOH-H_2O, flow rate 1.0 ml/min; C18; 10×250 mm, 5 um) to get a pure compound **6** (13 mg) as light yellow prisms.

Fraction T was re-chromatographed over Silica gel CC by gradient elution with EtOAc/MeOH (100:00 to 00:100) to get 7 sub-fractions (T-1 to T-7). Fr. T-6 was applied to semi-preparative HPLC with (80% CH_3CN-H_2O flow rate 1.5 ml /min; $C_{18,}$ 10×250 mm, 5 um) to yield a pure brown amorphous powder of compound **1** (8 mg)**.**

CylindrocarpolideA (1)

Brown amorphous powder. UV (MeOH)λ_{max} (logε): 235 (2.57), 257, 264 (2.41), 274 (2.32), 312 (2.18); HRESIMS *m/z:* 381.0935 $[M+H]^+$ (calcd. for $C_{21}H_{16}O_{7,}$ 381.0929);
^{1}H NMR and ^{13}C NMR (CD_3COCD_3), see Table 1.

Assay for α-Glucosidase Inhibitory Activity

An assay of α-glucosidase inhibitory activity was performed using a reported method, with slight modifications [23]. All the assays were performed using 10 mM KH_2PO_4-K_2HPO_4 buffers, pH 7.0. The enzyme solution was prepared to give 2.0 Units/mL in 2 mL buffer solution. The reaction mixture contained phosphate buffer, pH 7.0 (150 µL), 20 µL of enzyme solution, and 20 µL DMSO or inhibitor (test sample dissolved in DMSO (10 µmol/mL)), and 20 µL of substrate (*p*-nitrophenyl glycoside, 1.5 mg/mL). The inhibitors were pre-incubated with the enzyme at 37 °C for 20 min, and the substrate was then added. The reaction was monitored spectrophotometrically by measuring the absorbance at 400 nm for 1-min intervals. Calculations were performed according to the equation η (%) = [(B − S)/B] × 100% (B stands for the assay medium with DMSO; S stands for the assay medium with inhibitor). All measurements were done in triplicate from two independent experiments. The reported IC_{50} was the average value of two independent experiments.

Results and discussion

The fungus *Cylindrocarpon* sp. DH 29 was cultured in solid rice medium for 30 days. The $CHCl_3$ and EtOAc fraction were repeatedly fractionated and purified by using silica gel column chromatography, Sephadex LH-20, reverse phase silica column and HPLC to obtain one new compound (**1**) and five known compounds (**2-6**) Figure **1**.

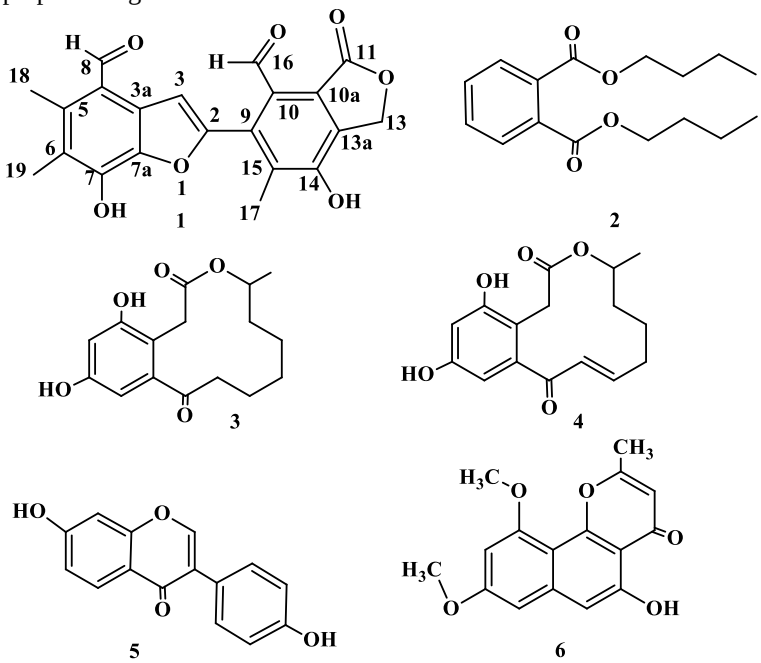

Figure 1. Structures of compounds **1-6**

Compound **1** was isolated as brown amorphous solid. Its molecular formula was established as $C_{21}H_{16}O_7$ by HRESIMS analysis (m/z 381.0935 $[M+H]^+$). The ^{1}HNMR spectrum displayed a pair of singlets at δ 10.55 and 9.61 attributed to two aldehyde moieties. The resonance observed at δ 5.12 (2H, s) was characteristics of oxymethylene protons. The HMBC correlations of oxymethylene protons with the ester carbon signal at δ 171.1 and with the aromatic carbons C-11 and C-13a suggested the presence of lactone functionality fused with the aromatic ring. The singlet 3H proton signals at δ 2.71, 2.46, and 2.14 suggested the presence of three methyl groups. The singlet observed at δ 7.57 was attributed to aromatic proton. The proton NMR data was backed up by ^{13}C NMR spectrum that displayed peaks at δ 189.5 and 195.4 (characteristics of aldehyde moieties), 151.9-109.6 (aromatic carbon), 171.1 (ester carbonyl moiety) 66.9 (oxymethylene carbon) and 11.9, 10.2 and 8.7 (methyl carbons). Analysis of ^{13}C and DEPT experiment revealed the presence of three methine carbons including two aldehyde carbons, one

oxygenated methylene, three methyl and 14 quaternary carbons which include one keto group and 13 fully substituted aromatic carbons. In order to determine H-H correlation COSY experiments (Fig. **S5**) were performed. No COSEY correlation was observed in compound 1, showing that the protons are not adjacent to each other. H-C connectivities were confirmed by HSQC experiment that showed connectivity of respective protons with the corresponding carbons. (Fig. **S6,** see Supplementary Materials at the bottom). The HMBC correlations (Fig. **S7**) of H-3 to C-2, C-3a and C-7a, H-13 to C-11, C-10a and C-13a, H-8 to C-8, H-16 to C-10 and the J^2 and J^3 correlation of CH_3-17, CH_3-18 and CH_3-19 with the aromatic carbon further confirmed the structure of compound **1**. After analysis of the chromatographic data of compound **1** and its close resemblance with reported literature [24] the structure of the compound was named as *Cylindrocarpolide A*.

The known compounds **2-6** were identified by comparison of their spectral data with the literature available and include *dibutyl phthalate* (**2**) [25], *curvularin* (**3**) *dehydrocurvularin* (**4**) [26] *daidzein* (**5**) [27] and *flavasperone* (**6**) [28].

The α-glucosidase inhibiting activity of the isolated compounds was screened *in vitro* along with the acarbose as positive control shown in **Table 2**. The compounds **1**, **5** and **6** showed potent inhibition with IC_{50} values of 23.4 ± 0.3, 36.5 ± 0.5 and 52.6 ± 0.6 respectively. Compounds **3** and **4** showed moderate anti-diabetic activity with IC_{50} values of 82.5 ± 0.8 and 66.3 ± 0.5. Compound **2** did not show inhibition activity at all.

Table 2. α-glucosidase inhibitory activity of compounds **1-6** [a].

Compounds	IC_{50} (µM)
1	23.4 ± 0.3
2	>200
3	82.5 ± 0.8
4	66.3 ± 0.5
5	36.5 ± 0.5
6	52.6 ± 0.6
Acarbose [b]	573.6 ± 2.2

[a] IC_{50} values are shown as mean ±SD from two independent experiments; [b] Positive control.

The results indicate that the number and position of hydroxyl groups also effect the inhibition activity as shown by the difference in inhibition of compound **3**, **4**, **5** and **6**. All these findings showed that the compounds may have special interaction in the form of H-bonding with the enzyme. Hence the presence of acidic and hydroxyl groups enhances the activity due to the formation of H-bonding between the substrate and the enzyme. The position of OH group as found in the C-ring of compound **5** also effect the inhibition. Methylation of phenolic OH groups as in compound **6** have a negative effect on the inhibition activity of these compounds. Double bond also enhances the activity as shown by comparing the activity of compounds **3** and **4**. Sterically hindered moieties around the binding groups also weaken the interaction between the compounds and the enzyme.

Acknowledgements

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Supplementary material

All the supporting information including NMR and Mass spectra of new compounds are available in the supplementary data.

Conflict of interest

The authors declare no conflict of interest.

Conclusion

Phytochemical studies of the fungus *Cylindrocarpon destructans* DH 29 were carried out using latest chromatographic and spectroscopic techniques. One new and five known compounds were obtained from the cultured rice medium of the mentioned fungus.

The α-glucosidase inhibiting activity of the isolated compounds were screened *in vitro* along with the acarbose as a positive control. The isolated compounds showed strong to weak α-glycosidase inhibitory activity.

The studies also reveal a SAR (structure-activity relationship) between the structural features of compounds and the corresponding glycosidase enzymes but more research is suggested to establish the exact chemistry. It is strongly recommended that future investigation on this fungus should be carried out to explore its hidden medicinal values.

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Supplementary materials

New polyoxygenated polyketide from pathogenic fungus *Cylindrocarpon destructans* with α-glycosidase inhibitory activity

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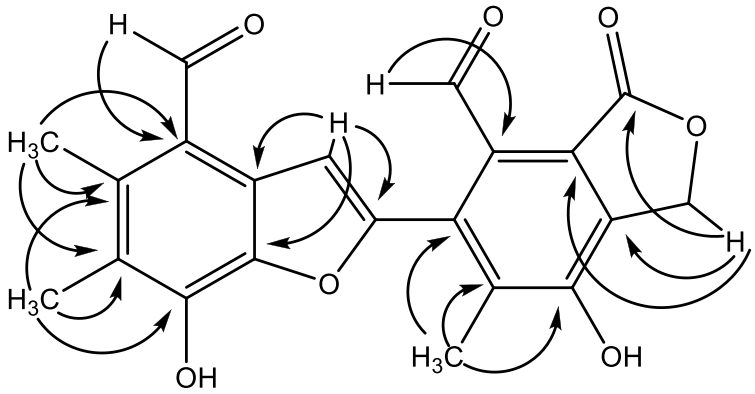

Figure **S1**. Key HMBC H-C correlation of compound **1**

Figure S2. ^{1}H NMR spectrum of compound 1 in Acetone-d6

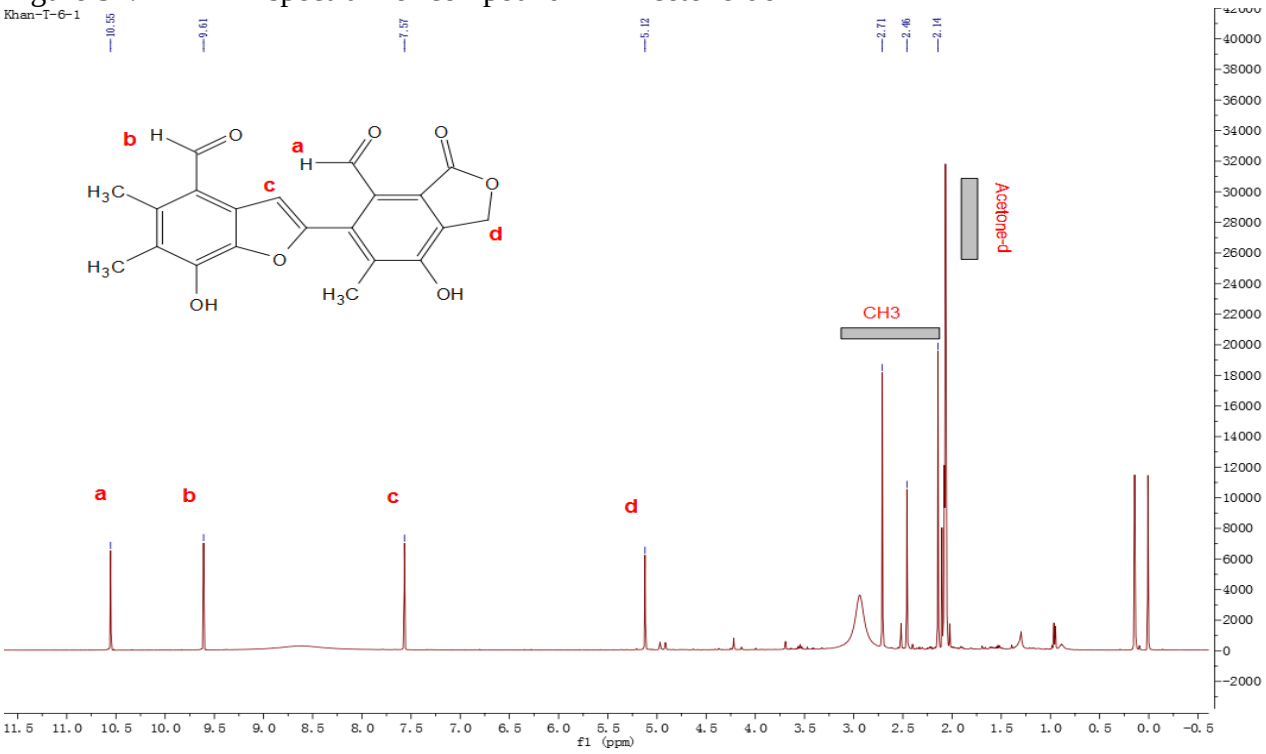

Figure S3. ^{13}C NMR spectrum of compound 1 in Acetone-d6

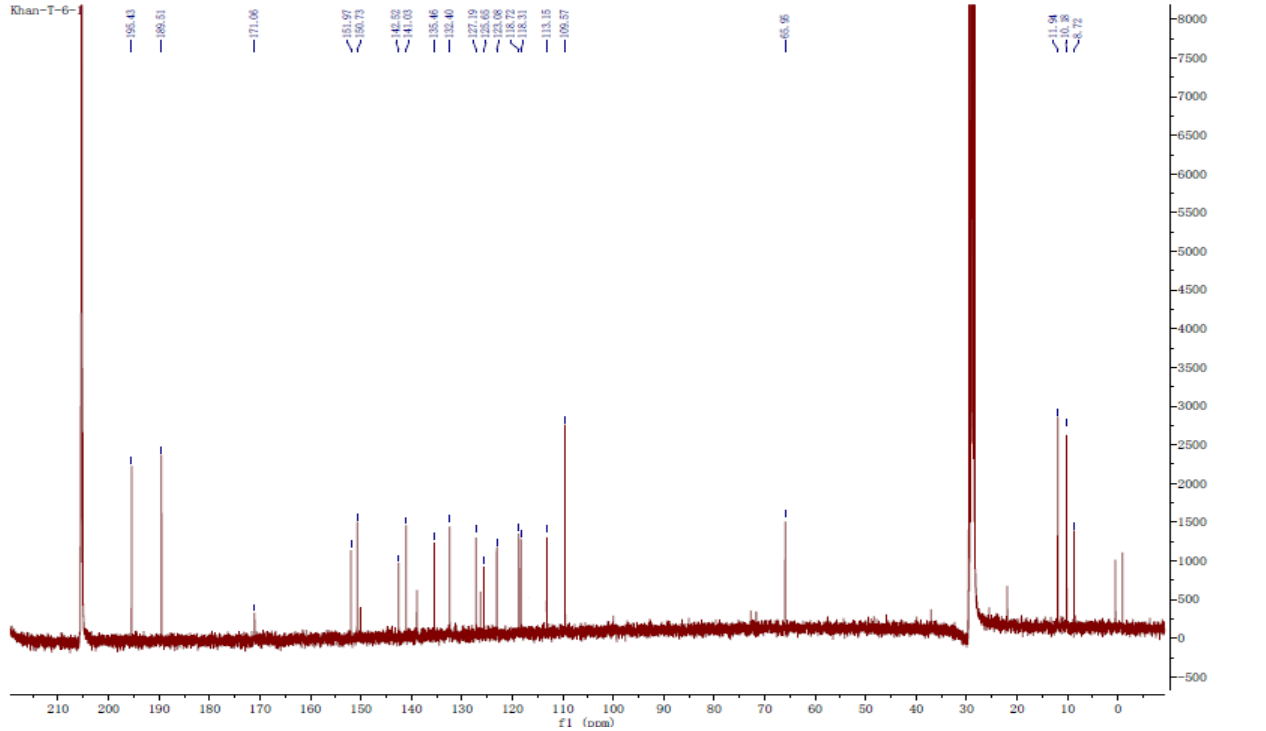

Figure S4. DEPT-135 spectrum of compound 1 in Acetone-d6

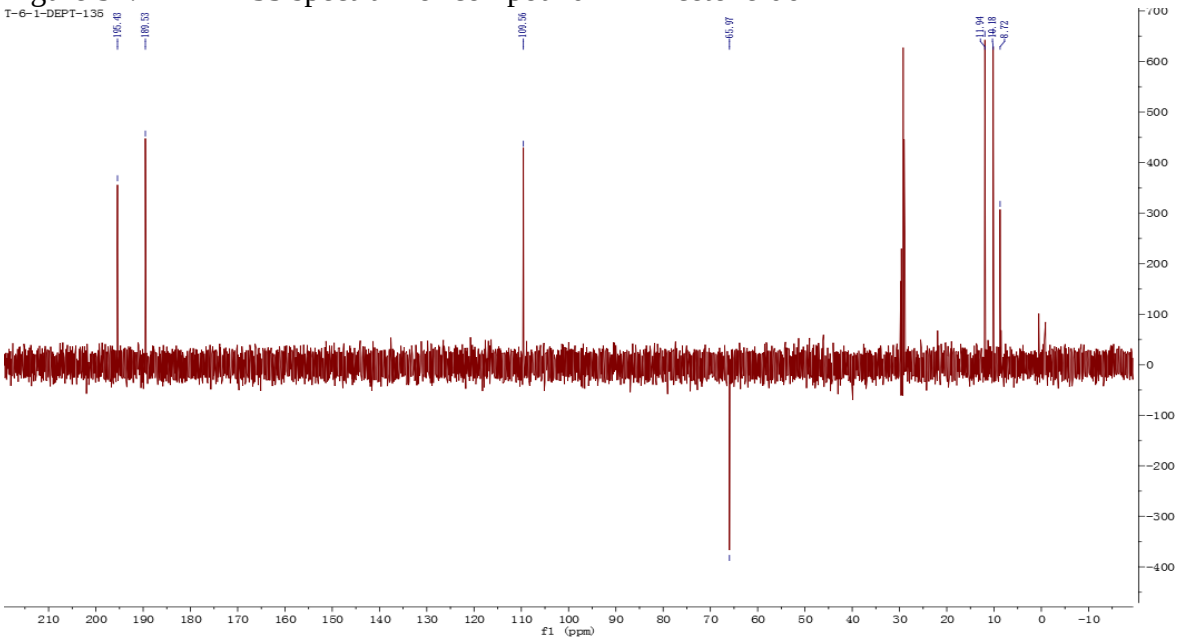

Figure **S5**. The ^{1}H-^{1}H COSEY spectrum of compound **1**

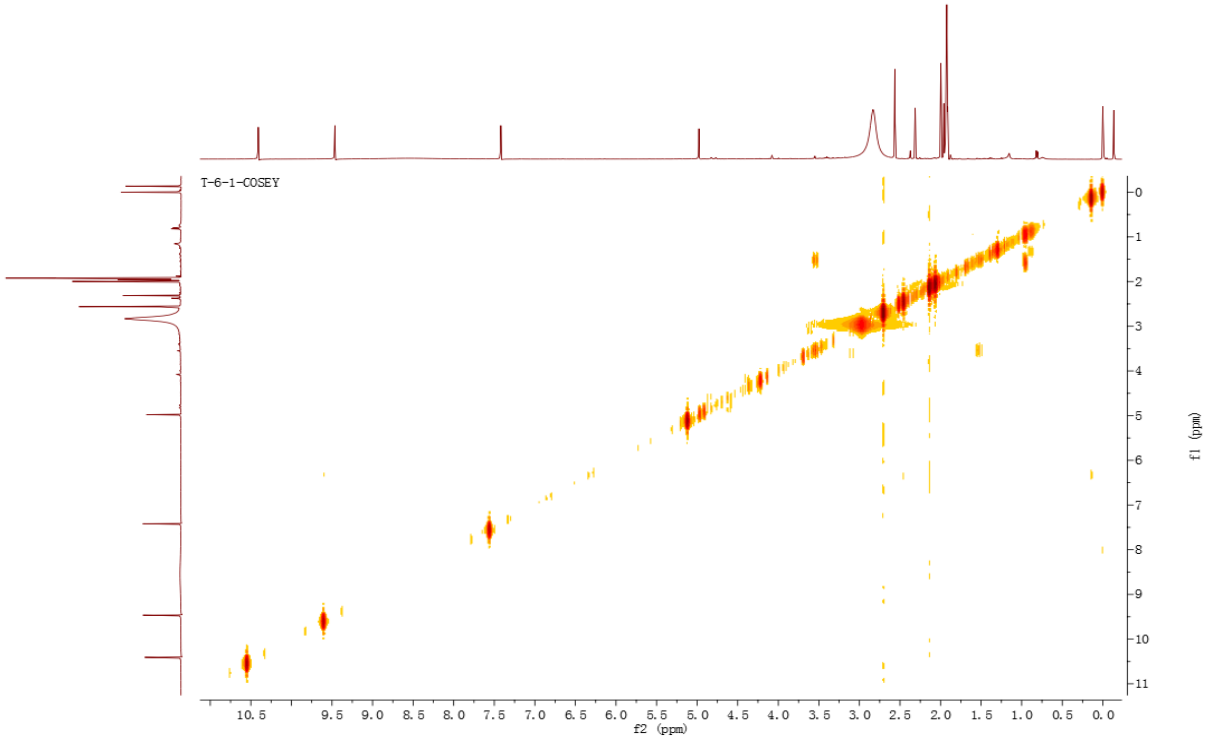

Figure **S6**. HSQC Spectrum of compound **1** in Acetone-d6

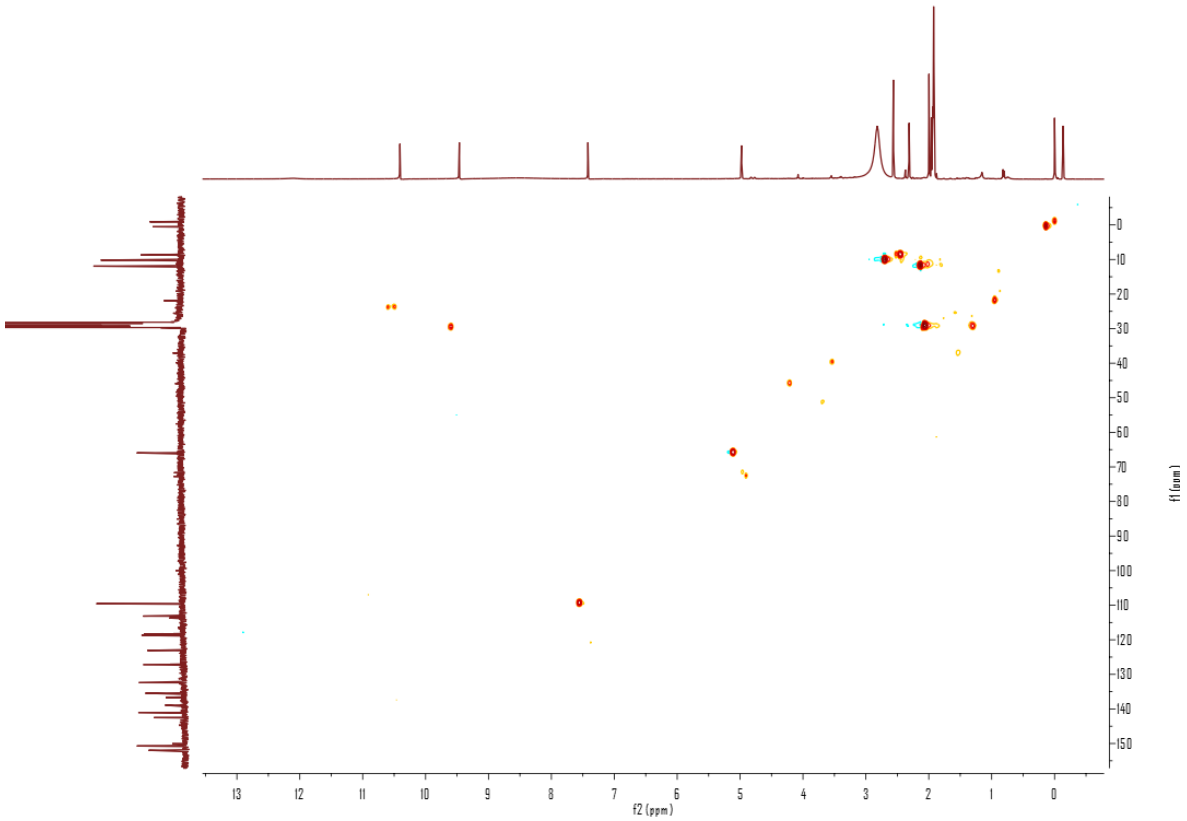

Figure **S7**. HMBC Spectrum of compound **1** in Acetone-d6