SUPPLEMENTARY MATERIAL

Isolation of amylase regulators from the leaves of *Ixeridium dentatum*

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ABSTRACT

Two new compounds, one sesquiterpene lactone (**1**) and one phenylethanoid tautomer (**2**), together with eleven known compounds (**3**–**13**) were isolated from the leaves of *Ixeridium dentatum.* Their structures were determined by extensive spectroscopic methods, including 1D-, 2D-NMR, and mass spectrometry. All compounds were evaluated for their amylase secretion activity in human salivary gland cells after treatment in 40 mM of high glucose. All compounds showed increased amylase secretion activity. Moreover, previously undescribed compounds (**1**–**2)**, luteolin 7-*O*-*β*-D-glucopyranoside (**10**), quercimeritrin (**11**), and quercetin 3-*O*-*β*-D-xylopyranoside (**13**) exhibited significant amylase activity comparable to the positive control.

Keywords *Ixeridium dentatum*; sesquiterpene lactone; phenylethanoid; amylase secretion activity.

Experimental

General

Column chromatography (CC) was carried out with Kieselgel 60 silica gel (70-230 and 230-400 mesh, Merck, Darmstadt, Germany) and YMC RP-silica gel (150μm, Fuji Silysia Chemical, Aichi, Japan). Thin layer chromatography (TLC) was performed using a pre-coated silica-gel 60 F254 (0.25 mm, Merck) and RP-18 F254S plates (0.25 mm, Merck). NMR spectra were recorded using an Agilent 400-MR NMR spectrometer (Santa Clara, CA, USA). HR-ESI-MS were conducted using an Agilent 6530 Accurate-Mass Q-TOF LC/MS spectrometer (Santa Clara, CA, USA).

Extraction and isolation

The dried leaves of *I. dentatum* (5.0 kg) were extracted with MeOH (3 × 10 L, 50°C) under sonication for 4 h to yield 290.0 g extract after solvent evaporation. This extract was suspended in H_2O and successively partitioned with *n*-hexane and EtOAc to obtain the *n*-hexane (ID1, 91.0 g), EtOAc (ID2, 15.1 g) and H_2O (ID3, 178.0 g) extracts after removal of the solvents *in vacuo.*

ID2 was subjected to a silica gel CC and eluting with a gradient of $CHCl_3$: MeOH (10 : 1 → 1 : 1, v/v) to obtain four sub-fractions, ID2A (1.5 g), ID2B (3.3 g) ID2C (2.3 g), and ID2D (2.2 g). The ID2B fraction was chromatographed on a RP-18 CC eluting with MeOH : H_2O (4 : 1, v/v) to give three smaller fractions, ID2B1, ID2B2 and ID2B3. ID2B1 was further chromatographed on HPLC using J'sphere ODS H-80, 250 mm × 20 mm column, 22% aq. MeCN, and a flow rate of 3 mL/min to yield **2** (20.4 mg), **5** (1.4 mg) and **6** (18.5 mg). ID2B3 was also chromatographed on HPLC using J'sphere ODS H-80, 250 mm × 20 mm column, 22% aq. MeCN to afford **10** (7.1 mg) and **13** (18.4 mg).

ID3 was chromatographed on a Diaion HP-20 column eluting with H_2O containing increasing concentrations of MeOH (25, 50 and 75%) to obtain three sub-fraction ID3A (14.5 g), ID3B (13.3 g) and ID3C (23.2 g). The ID3B was chromatographed on a silica gel CC eluting with $CHCl_3$: MeOH (10 : 1, v/v) to give three sub-fractions, ID3B1, ID3B2 and ID3B3. The ID3B1 fraction was chromatographed on HPLC using J'sphere ODS H-80, 250 mm × 20 mm column, 8% aq. MeCN, and a flow rate of 3 mL/min to yield **7** (3.6 mg), **8** (1.5 mg) and **9** (2.4 mg). The ID3B2 fraction was running on the same above condition to give **3** (10.4 mg) and **4** (3.2 mg). The ID2B fraction was chromatographed on a RP-18 CC eluting with MeOH : H_2O (1 : 2, v/v) to afford **1** (5.2

mg), **11** (7.8 mg) and **12** (116.7 mg).

Amylase assay

HSG cells were obtained from the American Type Culture Collection and cultured in RPMI 1640 media (Sigma R1363, 2 mM L-glutamine, without glucose & $NaHCO_3$) supplemented with 10% fetal bovine serum (Gibco), 5 mM glucose (Sigma, G-8270), 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco), 100 μg/mL gentamycin (Gibco), 10 mM HEPES (Sigma, H3375), and sodium bicarbonate (Sigma, S6014) (pH 7.4). After 70% confluency, cells were sub-cultured in RPMI 1640 media containing high glucose (40 mM) for 7 days. Cells were washed, fed with fresh media and treated the isolated compounds (20 μg/mL) every day. At 7^{th} day, media was collected from the cultured plates and placed in an ultrafiltration (Vivaspin 15R, Sartorius, United Kingdom) for collecting the high concentration of proteins from the cell media. They were then spun at a speed of 3000 g for 20 min at 4℃. Protein concentration was measured using the Bradford method (Bradford Assay, BioRad, Hercules, CA, USA). 100 μg Proteins were loaded in the 96 well plates to measure amylase activity.

Amylase activity was assessed using a commercial kit (K711-100, BioVision Inc., Milpitas) and used ethylidene-pNP-G7 as a substrate. Absorbance, which is directly related to a-amylase activity, was measured at 405 nm at 25℃ by using an auto-analyser (Alcyon 300® analyser, Molecular Devices Corp., Sunnyvale, CA, USA).

Protein quantification assay

Protein quantification was determined using a Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, Cambridge, MA). The methodology from the Bio-Rad instruction manual was followed. Briefly, 20 μL of the concentrated media was added to 980 μL of 20% Bio-Rad Protein Assay Dye Reagent then mixed it properly. To detect protein concentration in a sample, a cuvette method using an Eppendorf BioPhotometer (Eppendorf AG, Hamburg, Germany) at a wavelength of 595 nm.

Statistical Analysis

Data are presented as means ± standard deviation (SD). Statistical significance was determined using one-way ANOVA analysis/Tukey HSD post hoc test with a significant set at $p <$

0.01. Statistical test was performed with GraphPad Prism 5.0 software (GraphPad Software Inc, San Diego, CA).

Table S1. Amylase secretion activity of isolated compounds from leaves of *I. dentatum*

Compound	nitrophenol (nmol)
40 mM glucose	0.495 ± 0.005
5 mM glucose	0.800 ± 0.000**
1	1.075 ± 0.065***
2	0.900 ± 0.000***
3	0.735 ± 0.005
4	0.840 ± 0.060**
5	0.845 ± 0.035**
6	0.890 ± 0.020***
7	0.855 ± 0.025**
8	0.875 ± 0.005***
9	0.870 ± 0.030***
10	0.935 ± 0.035***
11	0.910 ± 0.020***
12	0.875 ± 0.015***
13	0.950 ± 0.090***
8-epiisolipidiol-3-*O*-*β*-D-glucopyranoside	0.985 ± 0.025***

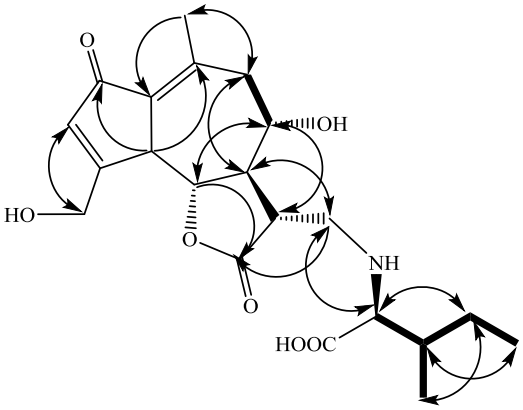
The absorbance at 405 nm was measured using a microplate reader. High glucose (40 mM) were treated in HSG cells to make an *in vitro* model of xerostomia. 5 mM Glucose treatment is for the normal salivary secretion. $^{**}P<0.01$ and $^{***}P<0.001$ indicate statistically significant differences compared to the control (40 mM glucose). 8-epiisolipidiol-3-*O*-*β*-D-glucopyranoside was used as a positive control.

Table S2. Total protein concentration of isolated compounds from leaves of *I. dentatum*

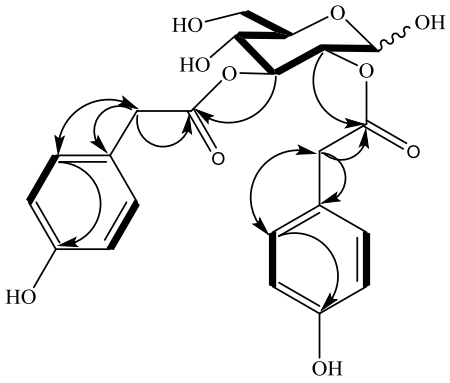
Compound	Conc. (mg/mL)
40 mM glucose	4.313 ± 0.011
5 mM glucose	3.415 ± 0.002
1	3.204 ± 0.009
2	3.111 ± 0.098
3	3.528 ± 0.002
4	3.358 ± 0.065
5	3.303 ± 0.102
6	3.269 ± 0.054
7	3.349 ± 0.013
8	3.312 ± 0.006
9	3.381 ± 0.030
10	3.364 ± 0.002
11	3.381 ± 0.009
12	3.231 ± 0.006
13	3.246 ± 0.117
8-epiisolipidiol-3-*O*-*β*-D-glucopyranoside	3.050 ± 0.054

The absorbance at 595 nm was measured using an Eppendorf BioPhotometer. 8-epiisolipidiol-3-*O*-*β*-D-glucopyranoside was used as a positive control.

Figure S1. The key HMBC and COSY correlations of compounds **1**–**2**.



1



2

Figure S2. Experimental CD spectra of compound **1**.

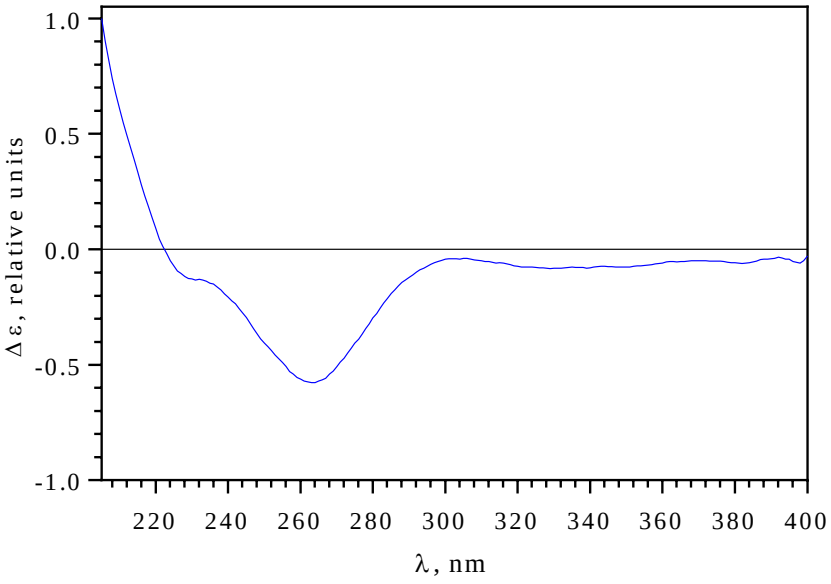
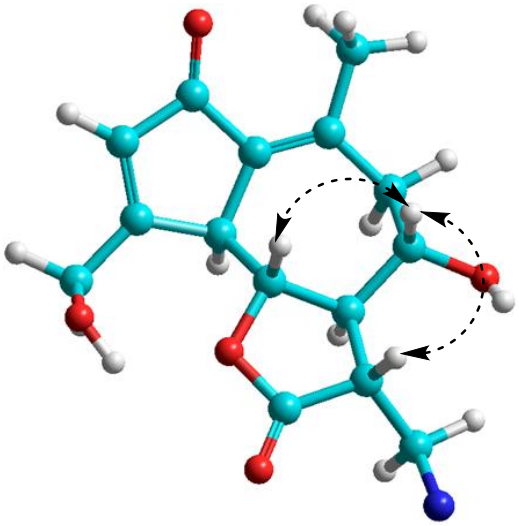


Figure S3. The key NOESY correlation of compound **1**.



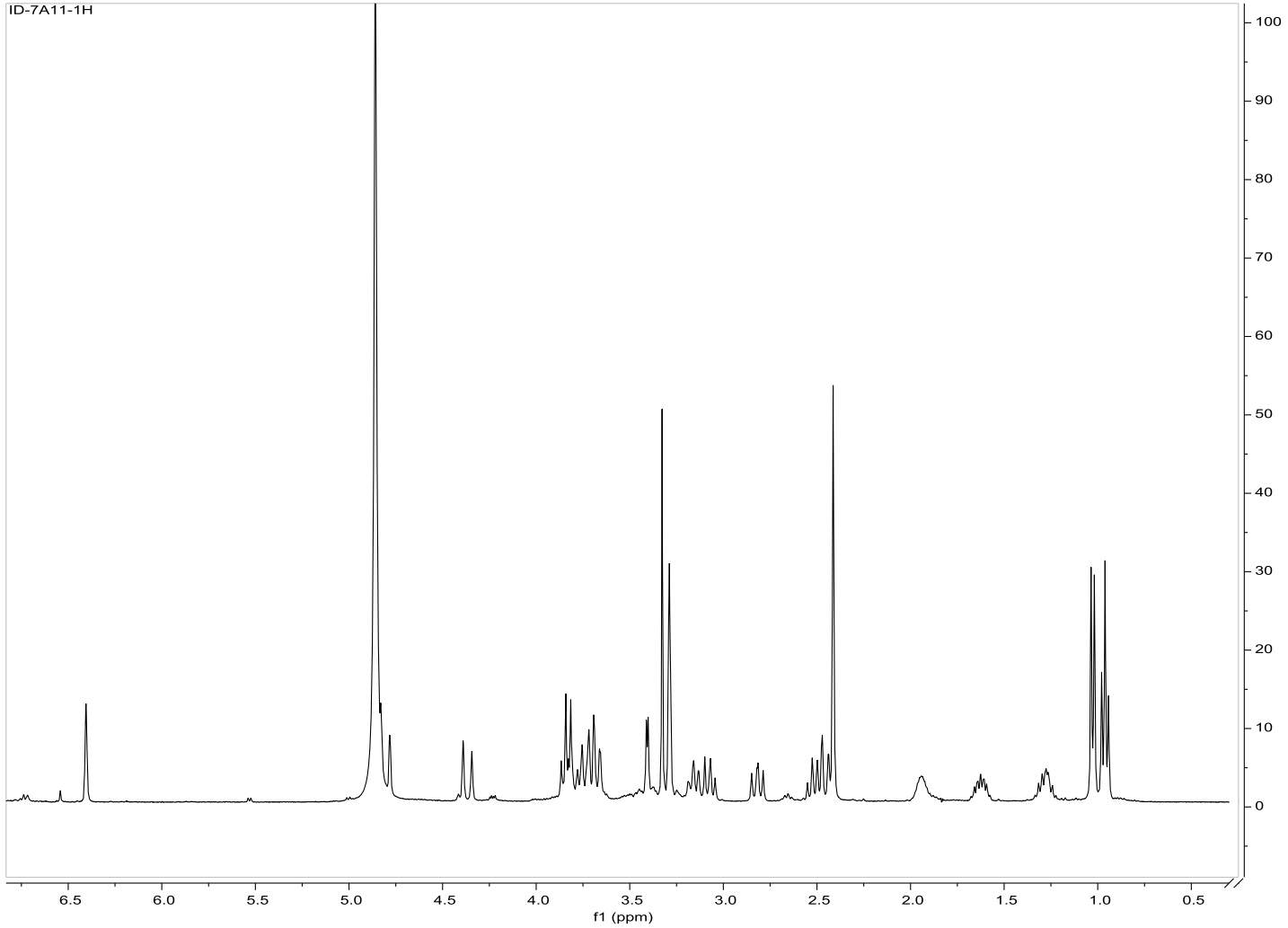


Figure S4 ^{1}H-NMR spectrum of compound **1** (400 MHz, methanol-d_4)

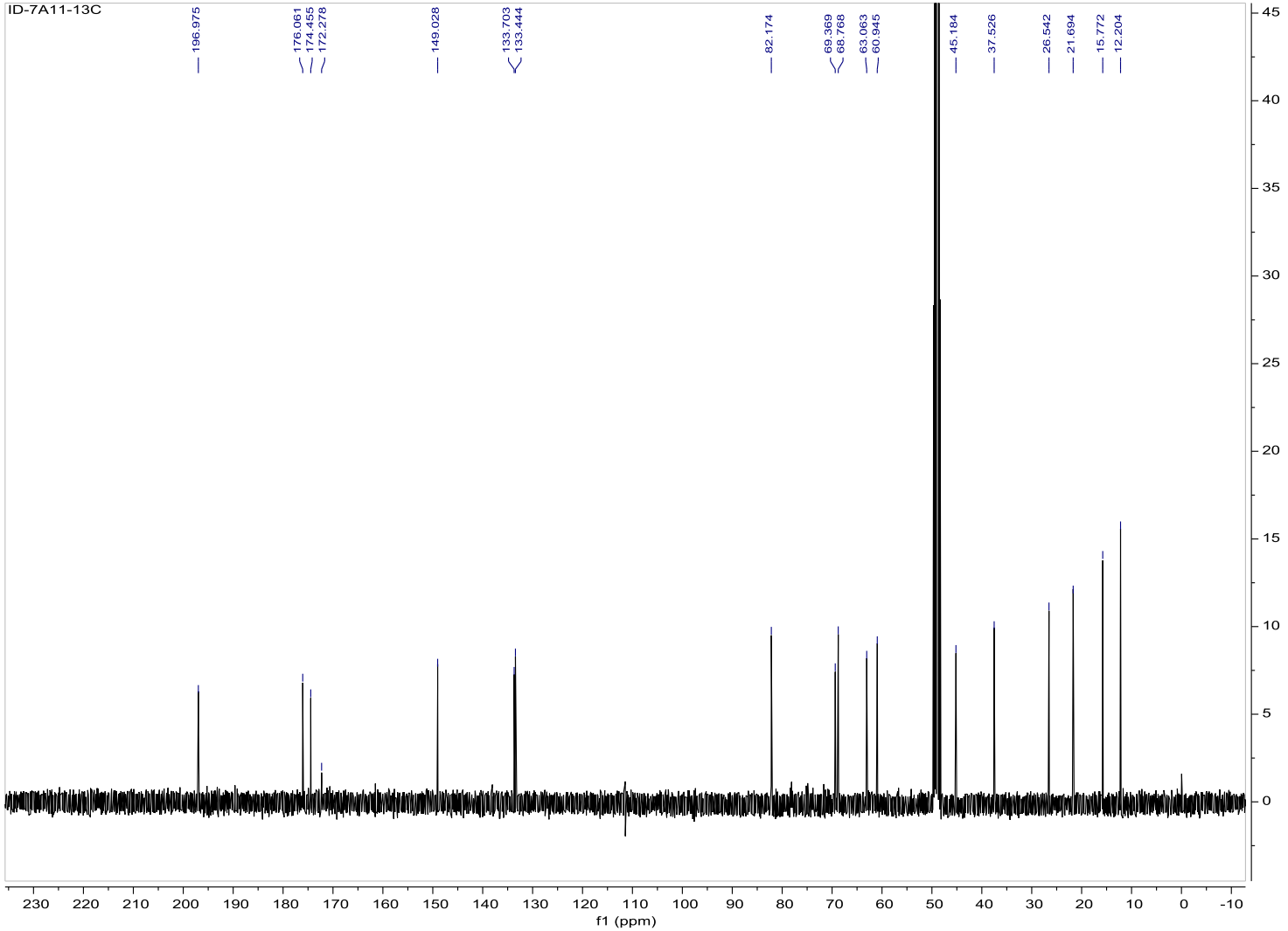


Figure S5 ^{13}C-NMR spectrum of compound **1** (100 MHz, methanol-d_4)

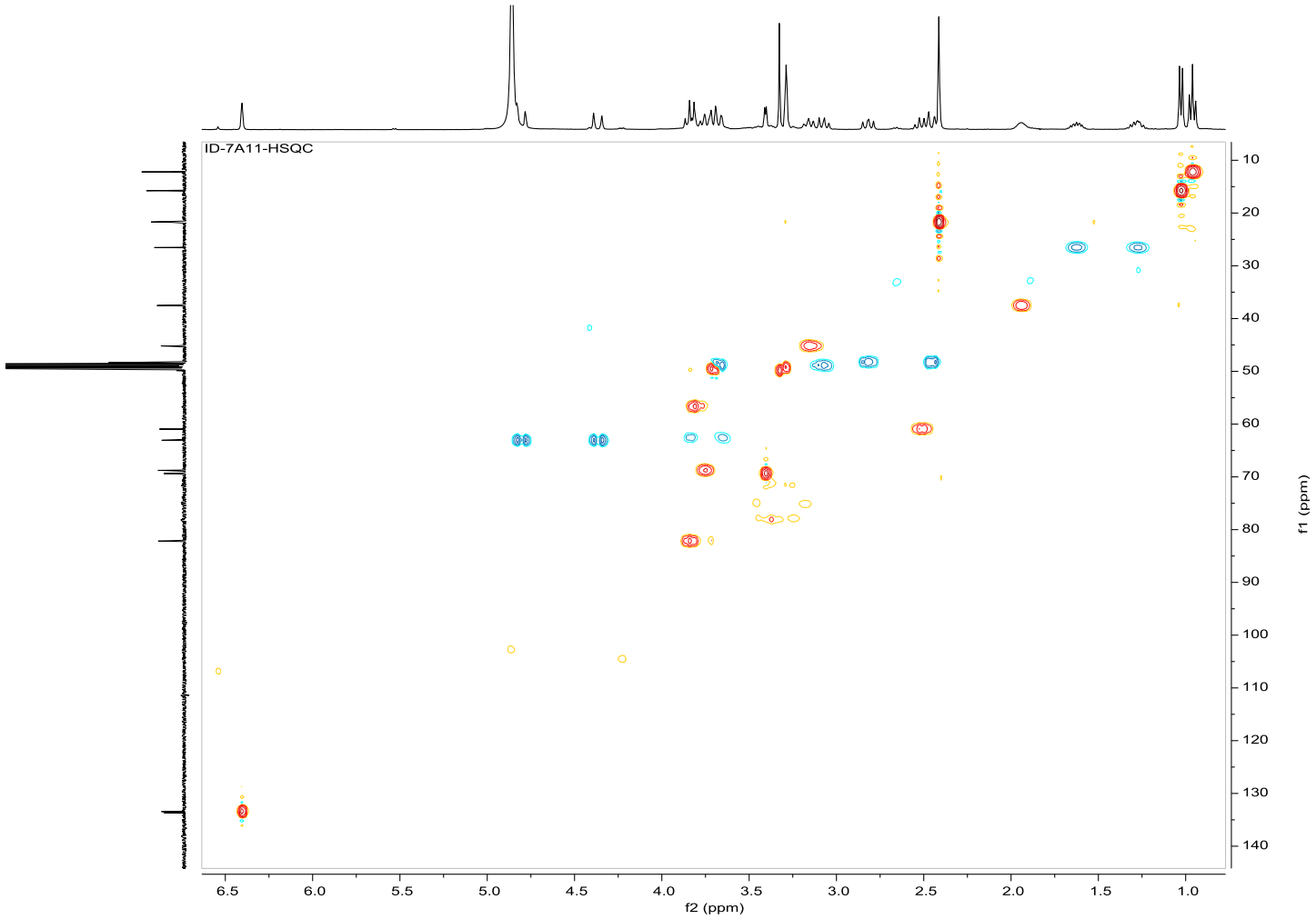


Figure S6 HSQC spectrum of compound **1** (methanol-d_4)

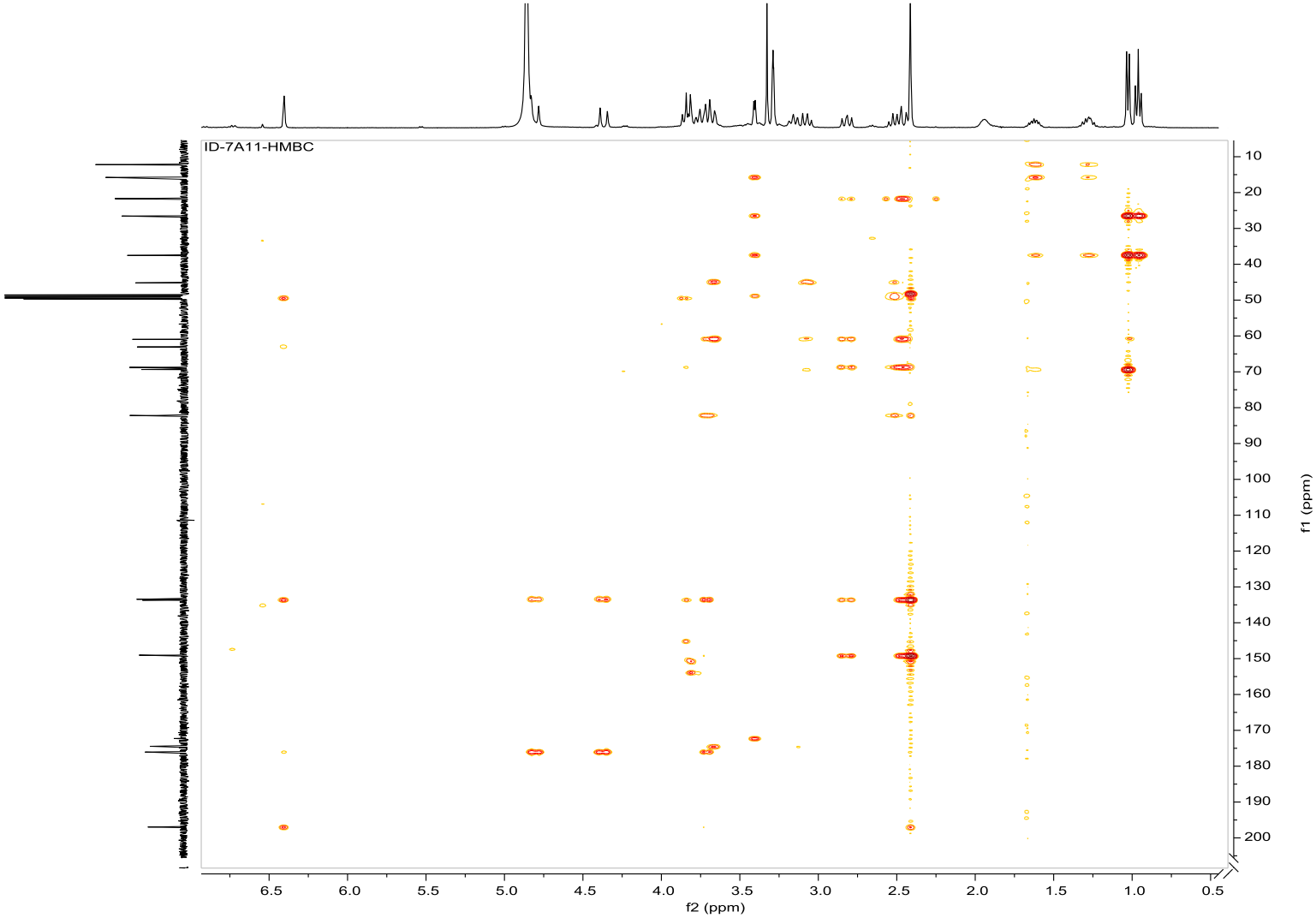


Figure S7 HMBC spectrum of compound **1** (methanol-d_4)

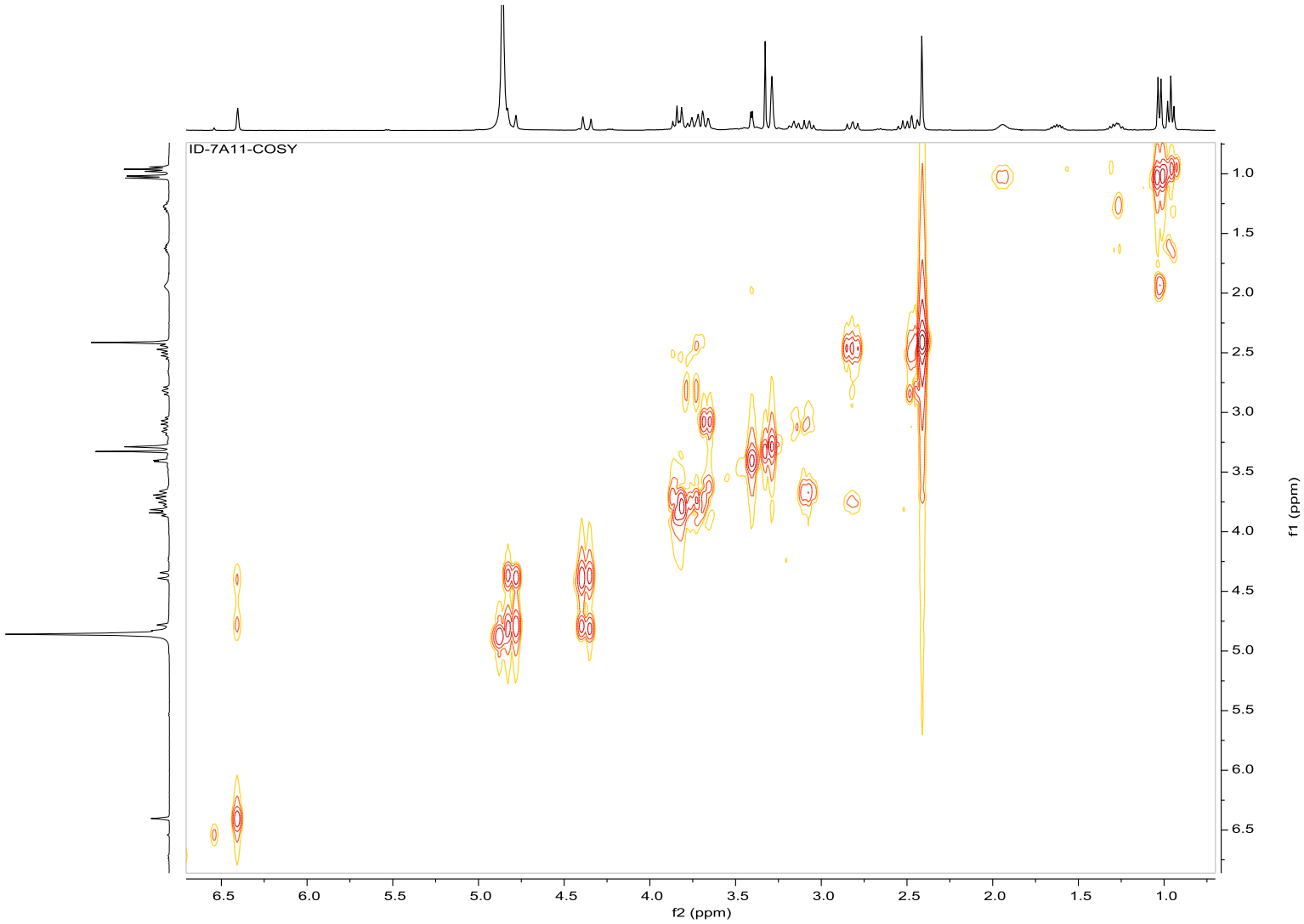


Figure S8 COSY spectrum of compound **1** (methanol-d_4)

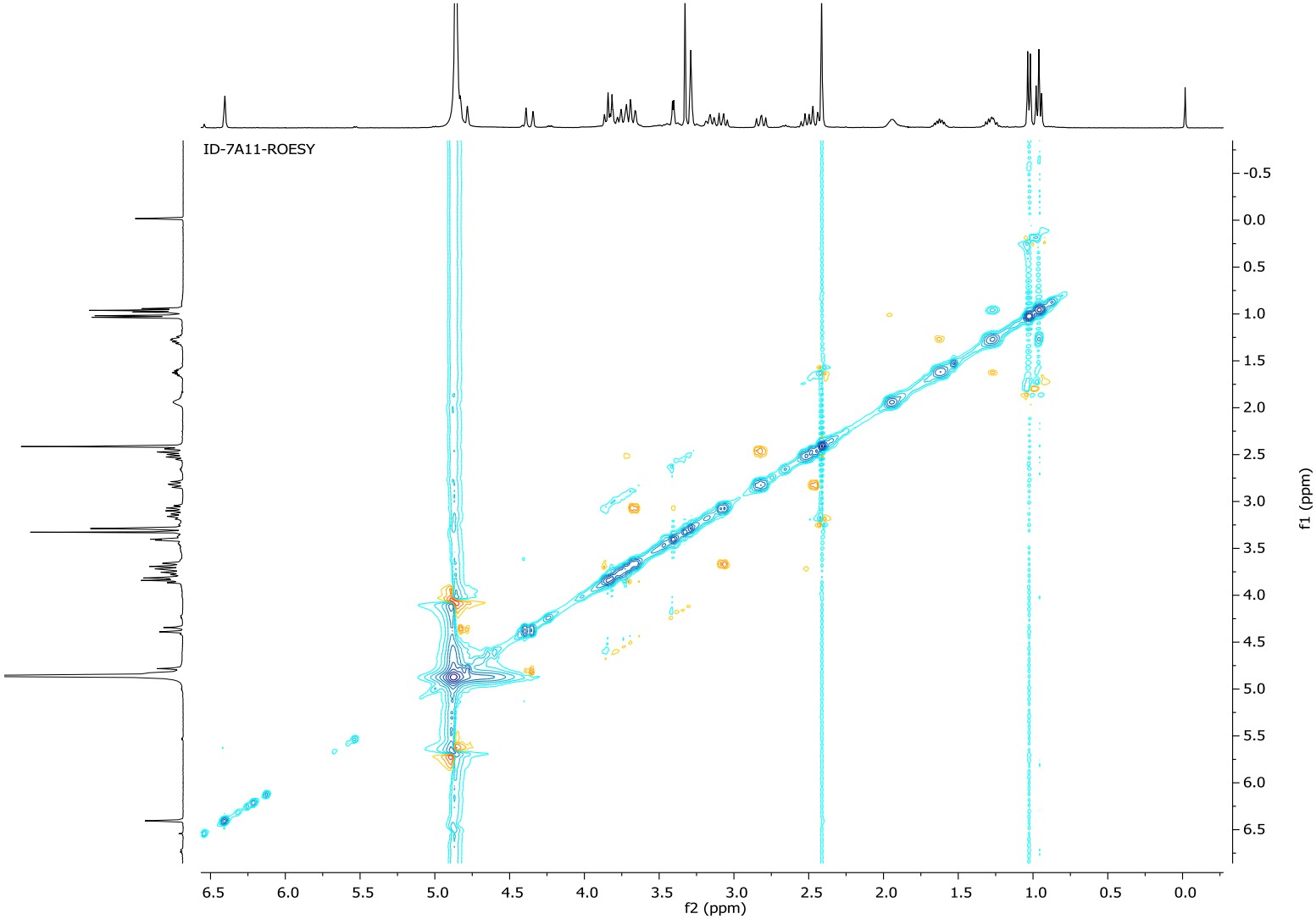


Figure S9 ROESY spectrum of compound **1** (methanol-d_4)

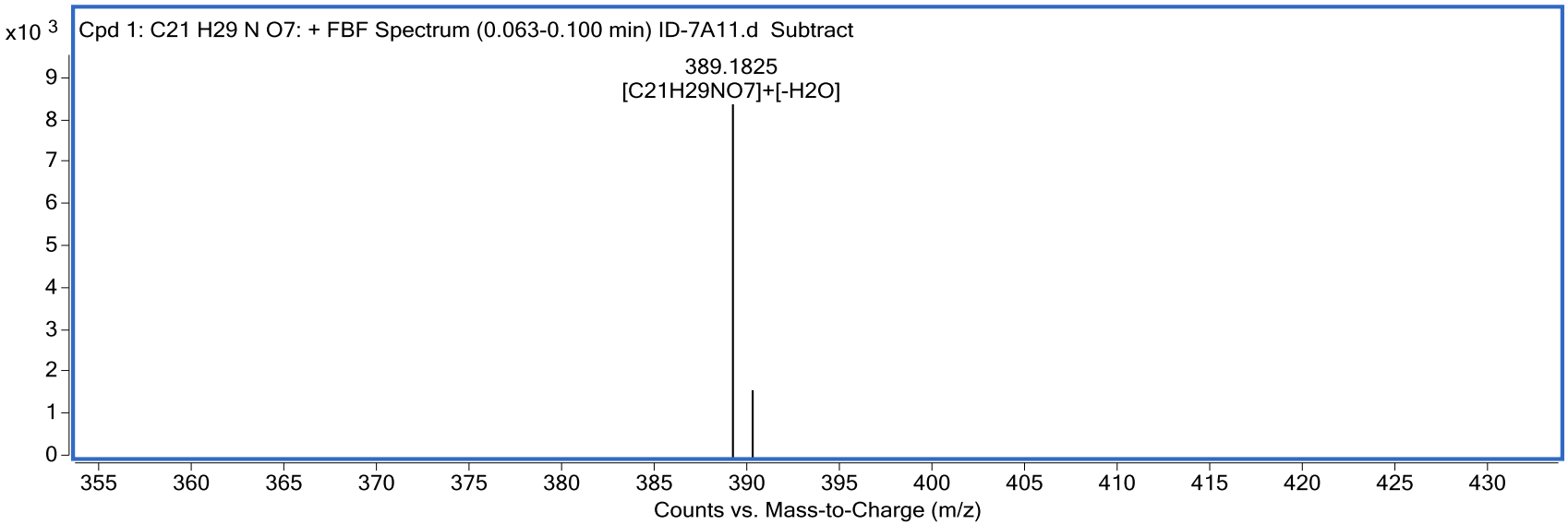


Figure S10 HR-ESI-MS of compound **1**

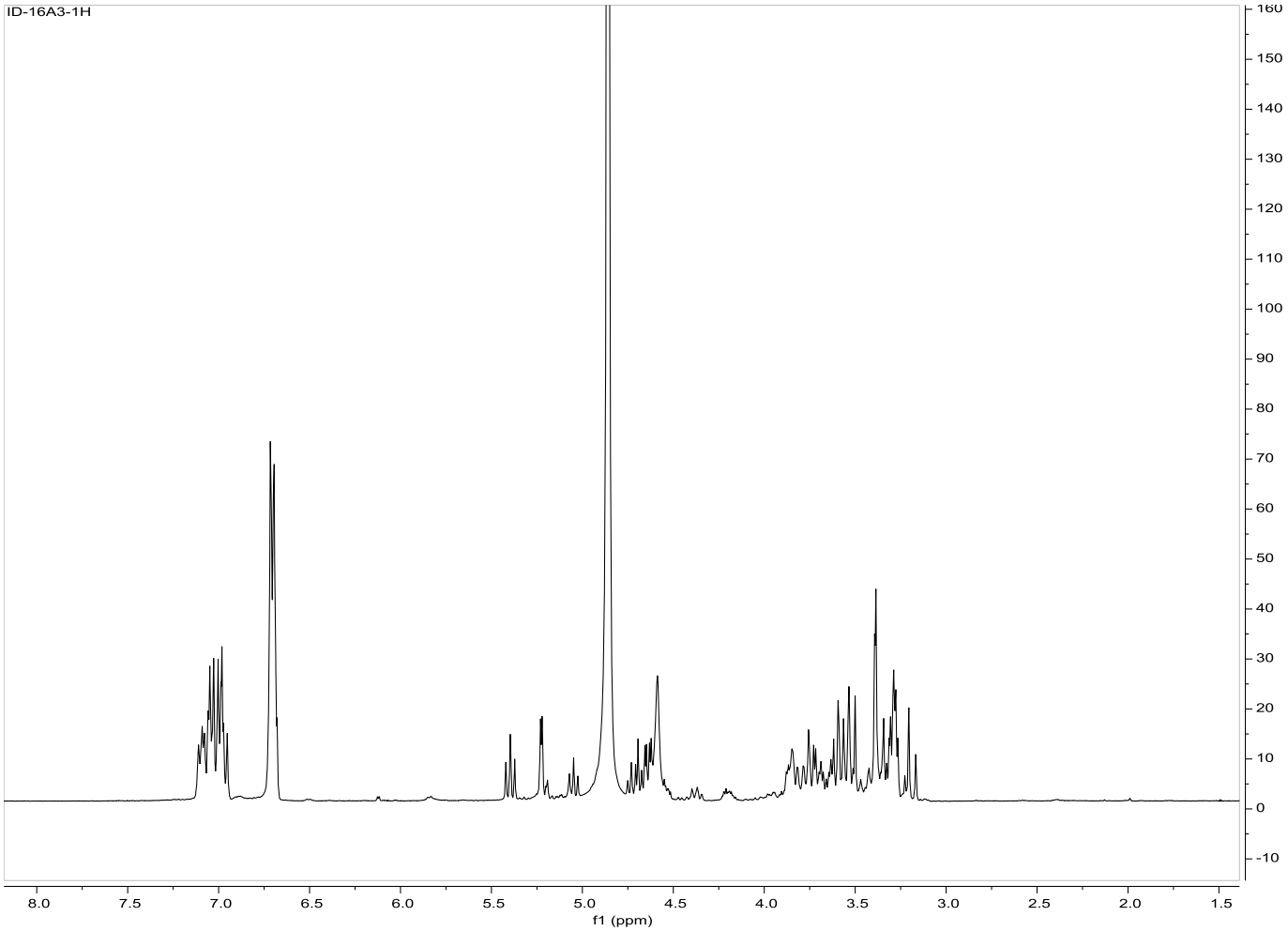


Figure S11 ^{1}H-NMR spectrum of compound **2** (methanol-d_4)

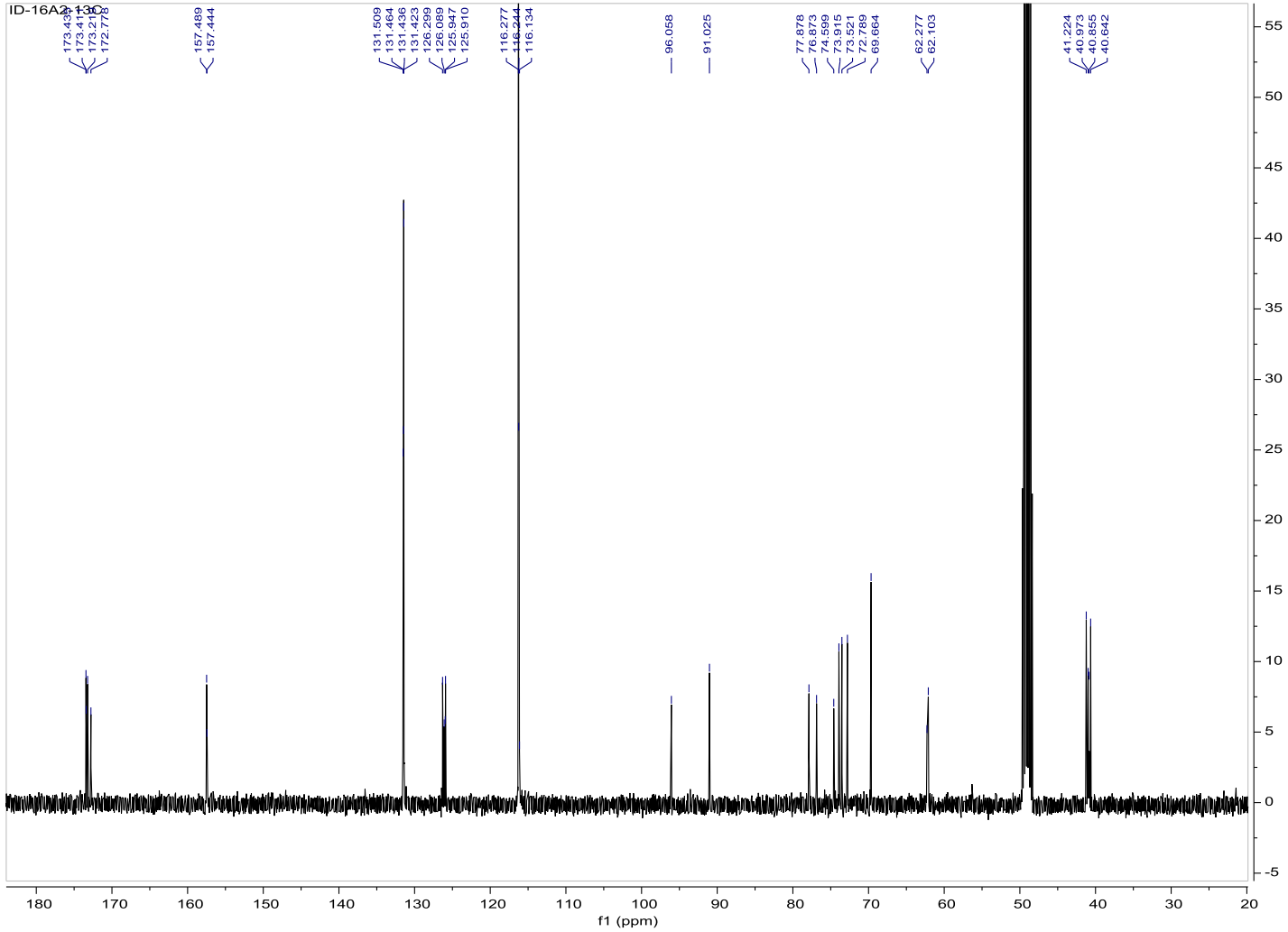


Figure S12 ^{13}C-NMR spectrum of compound **2** (methanol-d_4)

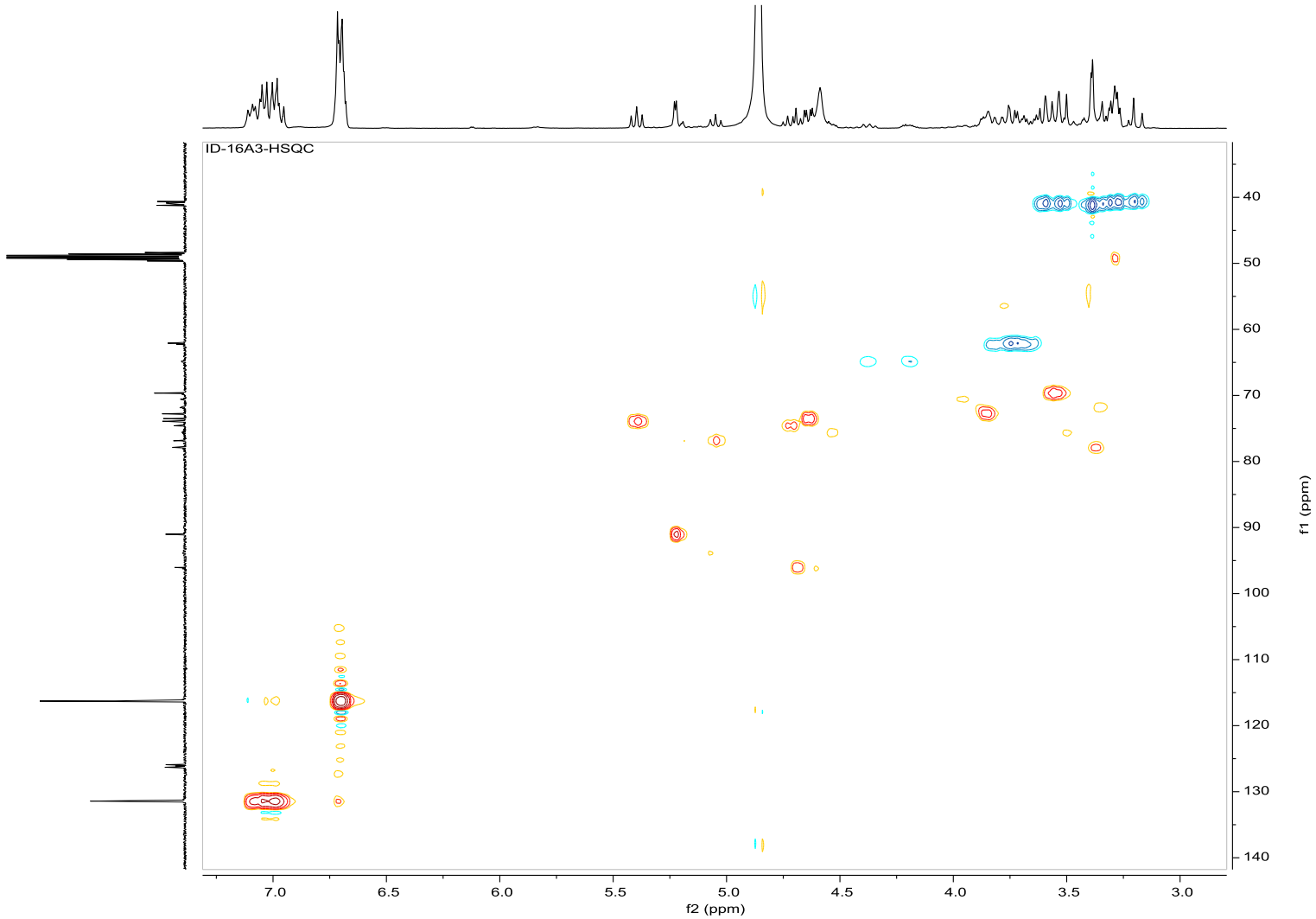


Figure S13 HSQC spectrum of compound **2** (methanol-d_4)

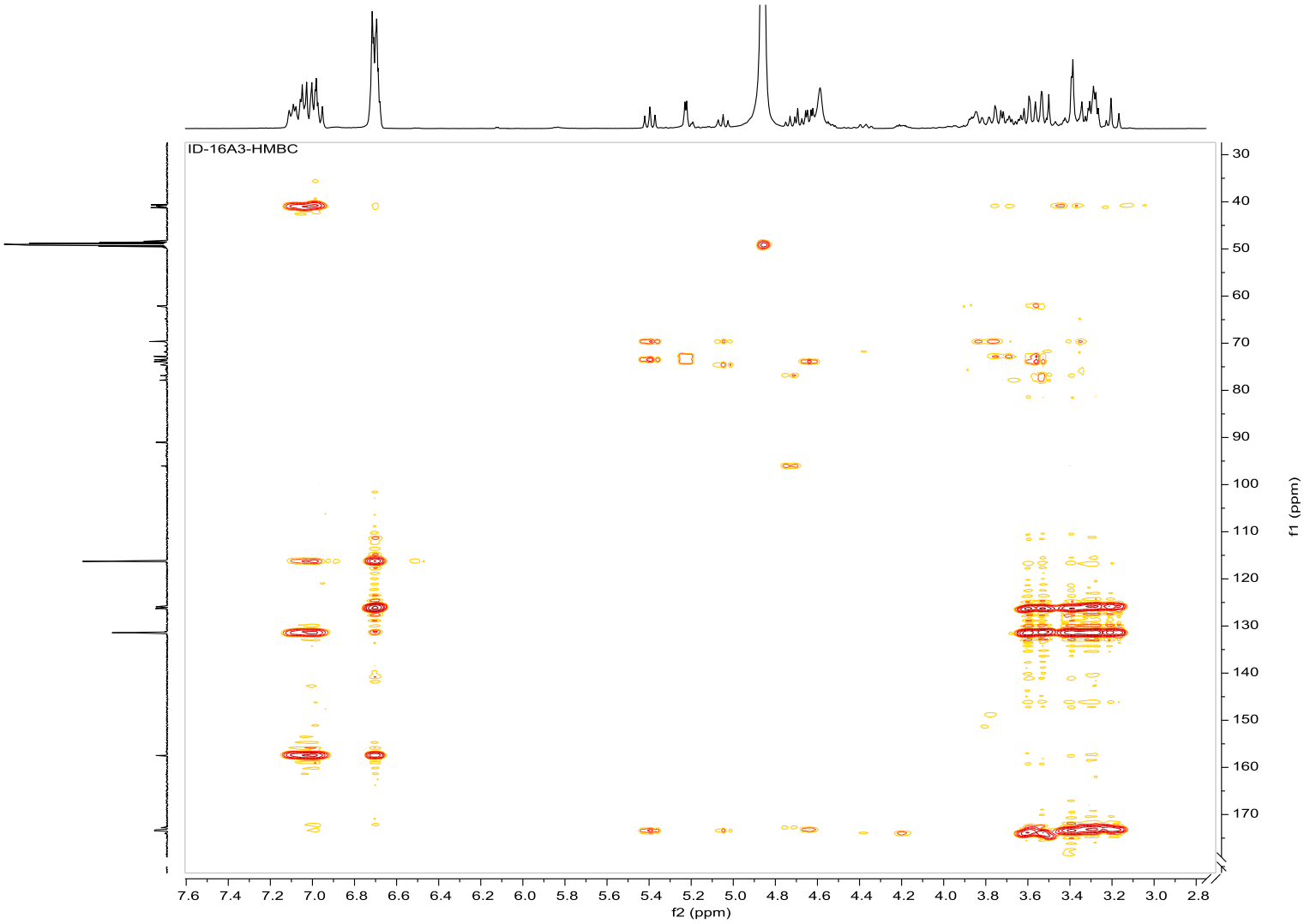


Figure S14 HMBC spectrum of compound **2** (methanol-d_4)

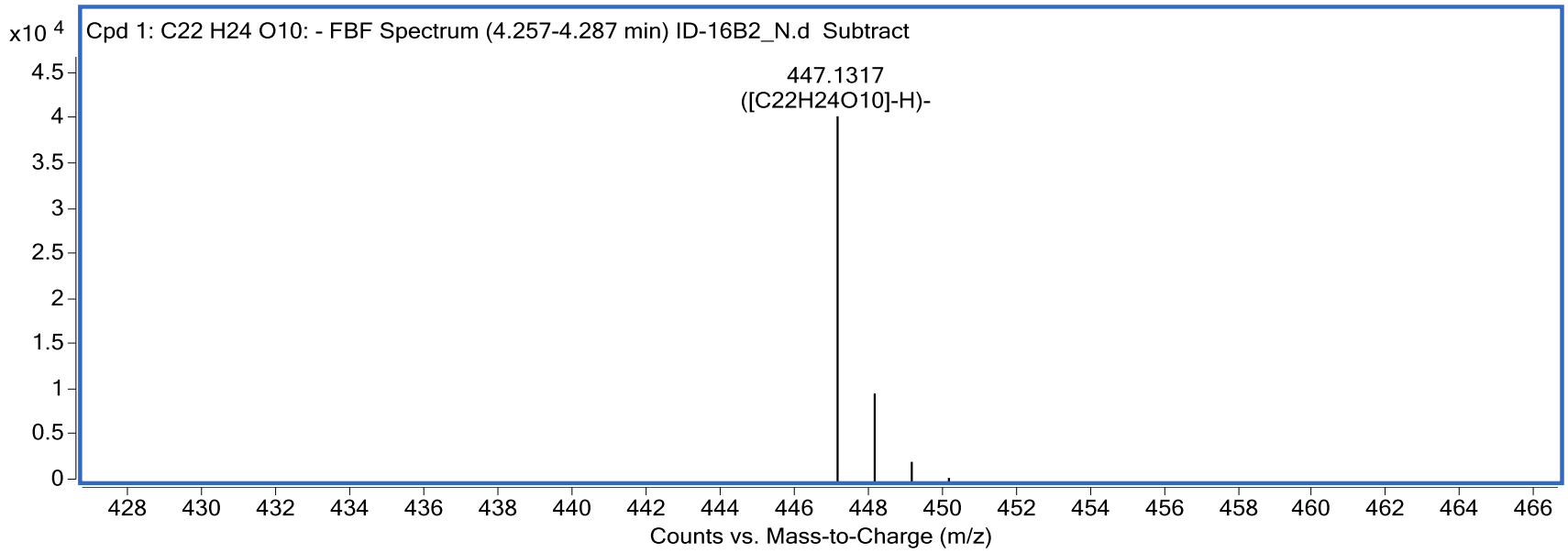


Figure S15 HR-ESI-MS of compound **2**