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New sesquiterpene acid and inositol derivatives from *Inula montana* L.

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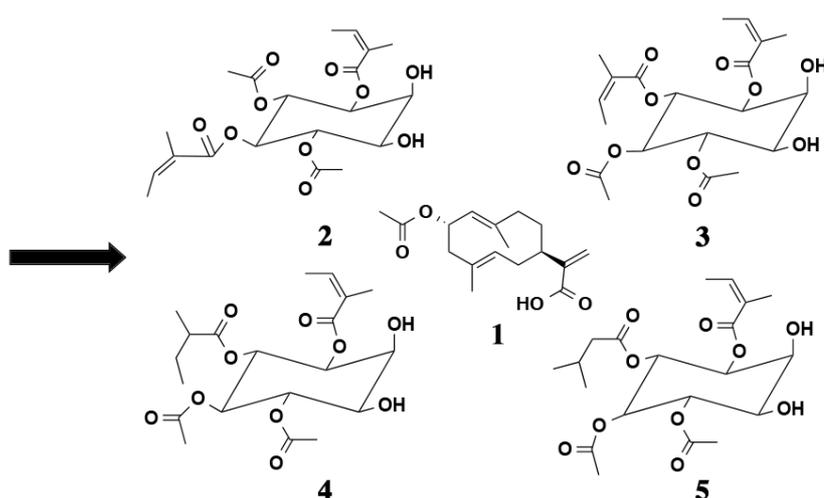
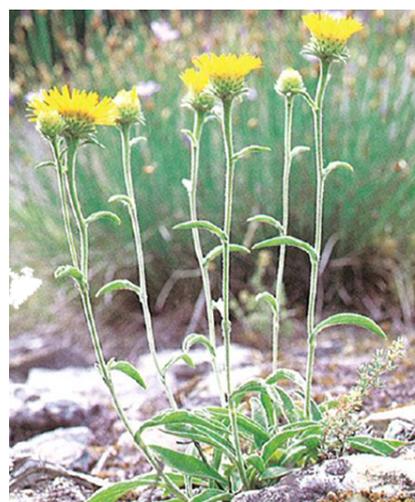
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Keywords: *Inula montana* L., Sesquiterpenes, Inositol derivatives, Anti-inflammatory activity.



Abstract

A phytochemical investigation of the ethanol extract of leaves and flowers of *Inula montana* L. led to the isolation of one new sesquiterpene acid called Eldarin (**1**) and four new inositol derivatives, Myoinositol,1,5-diangelate-4,6-diacetate (**2**), Myoinositol,1,6-diangelate-4,5-diacetate (**3**), Myoinositol-1-angelate-4,5-diacetate-6-(2-methylbutirate) (**4**), Myoinositol-1-angelate-4,5-diacetate-6-isovalerate (**5**) isolated for the first time, along with eleven known compounds described for the first time in *Inula montana*, 1 β -Hydroxyarbusculin A (**6**), Artemorin (**7**), Santamarin (**8**), Chrysosplenol C (**9**), 6-Hydroxykaempferol 3,7-dimethyl ether (**10**), Reynosin (**11**),

Calenduladiol-3-palmitate (12), Costunolide (13), 4-Hydroxy-3,5-dimethoxybenzenemethanol (14), 9 β -Hydroxycostunolide (15) and Hispidulin (16). Structural elucidation has been carried out by spectral methods, such as 1D and 2D NMR, IR, UV and HR-ESI-MS. These compounds have been tested *in vitro* for anti-inflammatory and cytotoxic activity on macrophages RAW 264.7. As a result, compounds 2, 3, 7, 13, 14, 15 and 16 showed a release of NO with IC₅₀ value < 30 μ M on macrophages.

1. Introduction

The *Inula* genus comprises more than one hundred species, mainly distributed in Africa, Asia and Europe, predominantly, in the Mediterranean Area. Compounds isolated from the species of this genus are mono-, sesqui-, di- and triterpenes, flavonoids and inositol derivatives. Cyclic sesquiterpenes are the predominant constituents of *Inula* genus [1–3]. Alcoholic and oil preparations of the flowering aerial parts of this plant are widely used in the traditional medicine of Provence (France) in the treatment of hematomas, as well as *Arnica montana* L. [4]. However, its phytochemical composition has been poorly investigated [5–8]. Only six sesquiterpene lactones, four free flavonoid aglycones and volatile composition have been reported in *Inula montana* L. until now [5,6].

Antioxidant and anti-protozoocidal activities of *Inula montana* extracts have been previously reported as well. No anti-inflammatory activity has been investigated comparing to *Inula cappa* or *Inula wissmanniana* [3,7–9]. In this study, we describe the isolation of sixteen compounds for the first time from *Inula montana* L., including five new ones. The anti-inflammatory activity of the isolated compounds has been also evaluated.

2. Experimental part

2.1 General

Optical rotations were measured using Anton Paar polarimeter (l=10 mm, \varnothing =5 mm, Ref. 279986.05). UV spectra were recorded on Shimadzu UV-1650pc spectrophotometer. IR spectra were recorded on Bruker Vertex 70 FT-IR with ATR module. 1D and 2D NMR spectra were recorded on Bruker AVANCE III 600

spectrometer (^1H 600.13MHz) with tetramethylsilane (TMS) as internal standard at 300K. Chemical shifts (δ) were expressed in ppm with TMS as an internal reference. HR-ESI-MS was performed on SYNAPT G2 HDMS (Waters) spectrometer. UHPLC was performed on Agilent 1290 liquid chromatograph equipped with a Zorbax Eclipse Plus, C18 (100 \times 2.1 mm, 2.8 μm) (Agilent) column. HPLC was performed on Agilent 1200 liquid chromatograph equipped with a Luna C18 (150 \times 4.6 mm, 3 μm) column. Preparative HPLC was performed on Gilson PLC2020 with a Luna C18 (150 \times 21.2 mm, 3 μm) column (Phenomenex). Flash chromatography was performed using a Teledyn Isco CombiFlash Rf 200 (USA). TLC analysis was performed on Silica F₂₅₄ plates (Merck).

The positive control dexamethasone, phosphate tampon, dimethyl sulfoxide, RAW 264.7 cells and Lyposaccharides (LPS) of *E.coli* were purchased from Sigma-Aldrich. Griess reagent was purchased from Promega.

2.2 Plant material

The plant *I. montana* was collected in Merindol (Luberon Natural Regional Park, France) in June 2015 and identified by Dr. Didier Morisot from the University of Montpellier and by Mr. Laurent Michel from Luberon Natural Reserve. Voucher specimens of leaves (IMFF151) and flowers (IMFLF151) were deposited in the Laboratory of Pharmacognosy of Aix Marseille University (Marseille, France).

2.3 Extraction and isolation

The air-dried plant materials: leaves (0.2 kg) and flowers (0.2 kg), were extracted separately with EtOH 95% (v/v) (1.2 L \times 3), by percolation at room temperature for 18 h, to afford crude extracts E2 (leaves, 8.6 g) and L2 (flowers, 23.7 g). After concentration *in vacuo*, each extract was suspended in H₂O and partitioned by petroleum ether and dichloromethane successively, to provide a petroleum ether E2P (2.1 g) and dichloromethane E2D (1.0 g) extracts for leaves and petroleum ether L2P (4.4 g) and dichloromethane L2D (0.7 g) extracts for flowers.

The leaves dichloromethane extract E2D (0.9 g) was subjected to reversed phase silica gel (C18, 100 g) flash chromatography and eluted with ~~water/methanol~~ MeOH-H₂O (0-30 min 35-85% MeOH, 30-31 min 85-100% MeOH, 31-35 min 100% MeOH (v/v), flow rate: 40 mL/min) to afford three fractions (E2D1), (E2D2), (E2D3)

and three compounds **6** (0.3 mg), **7** (19.2 mg) and **8** (0.3 mg). Fraction E2D1 (50.5 mg) was subjected to preparative HPLC (C18, 3 μ m, 150 \times 21.2 mm, flow rate 10.6 mL/min, wavelength 225 nm) using MeOH-H₂O system (52-48, v/v) which gave two compounds: **2** (12 mg) and **3** (12.7 mg). Fraction E2D2 (50.7 mg) was separated by preparative HPLC (C18, 3 μ m, 150 \times 21.2 mm, flow rate 10.6 mL/min, wavelength 210 nm) using MeOH-H₂O system (42-58, v/v) which afforded compounds **9** (0.3 mg), **10** (0.2 mg) and **11** (0.5 mg). Fraction E2D3 (12.7 mg) has been found to be as the mix of two compounds **4** and **5**.

The flowers petroleum ether extract L2P (2.1 g) was subjected to silica gel (24g) flash chromatography and eluted with ~~n-hexane/ethyl acetate~~ n-Hexane-Ethyl acetate (EtOAc) (0-30 min 0-20% EtOAc, 30-42 min 20-100% EtOAc, 42-50 min 100% EtOAc (v/v), flow rate 20 mL/min) to give three fractions (L2P1), (L2P2) and (L2P3). Fraction L2P1 (350 mg) was subjected to silica gel (24g) flash chromatography and eluted with toluene/EtOAc (0 – 20 min 0% EtOAc, 20 – 45 min 0-5% EtOAc, 45 – 50 min 5% EtOAc, 50 – 55 min 5 – 100 % EtOAc, 55 – 62 min 100% EtOAc (v/v), flow rate 20 mL/min) to afford compound **12** (23,2 mg). Fraction L2P2 (48 mg) was separated by HPLC ~~using as eluent water/methanol~~ (C18, 3 μ m, 150 \times 21.2 mm, 0 – 60 min, flow rate 21.2 mL/min, wavelength 210 nm) using MeOH-H₂O system (52-48, v/v) which gave compound **13** (9.4 mg). Fraction L2P3 (50 mg) was separated by HPLC (C18, 3 μ m, 150 \times 21.2 mm, flow rate 21.2 mL/min, wavelength 210 nm) using MeOH-H₂O system (0 – 20 min 40 – 100 % MeOH, 20 – 30 min 100 % MeOH) which gave compound **1** (16.2 mg).

Fraction L2D (0.6 g) was subjected to reversed phase silica gel (C18, 100 g) flash chromatography and eluted with the same conditions as the fraction E2D to afford one fraction (L2D1) and two compounds **14** (15 mg) and **15** (5 mg). The fraction L2D1 was subjected to HPLC (C18, 3 μ m, 150 \times 21.2 mm, 0 – 60 min, flow rate 21.2 mL/min, wavelength 210 nm) using MeOH-H₂O system (50-50, v/v) which gave compound **16** (12.3 mg).

2.3.1 Eldarin (**1**) White amorphous powder; $[\alpha]_D^{25}$ - 40,11 (c 0.53, MeOH); UV λ_{\max} (log ϵ) 205 nm (3.4; MeOH); IR (ATR) ν_{\max} 3440, 3055, 2926, 2858, 1724,

1693, 1621, 1432, 1369, 1241, 1018, 956, 735 cm^{-1} ; ^1H and ^{13}C NMR data, see Table 1; HR-ESI-MS: m/z 293.1748 $[\text{M} + \text{H}]^+$ (calcd. for $\text{C}_{17}\text{H}_{25}\text{O}_4^+$: 293.1747).

2.3.2 Myoinositol,1,5-diangelate-4,6-diacetate (**2**) Yellow oil; $[\alpha]_D^{25}$ - 8,35 (c 1.1, MeOH); UV λ_{max} (log ϵ) 217 nm (3.6; MeOH); IR (ATR) ν_{max} 3472, 3022, 2928, 1751, 1719, 1647, 1457, 1365, 1223, 1138, 1083, 1037 cm^{-1} ; ^1H and ^{13}C NMR data, see Tables 2 and 3; HR-ESI-MS: m/z 429.1756 $[\text{M} + \text{H}]^+$ (calcd. for $\text{C}_{20}\text{H}_{29}\text{O}_{10}^+$: 429.1755).

2.3.3 Myoinositol,1,6-diangelate-4,5-diacetate (**3**) Yellow oil; $[\alpha]_D^{25}$ - 20,04 (c 0.4, MeOH); UV λ_{max} (log ϵ) 217 nm (4.0; MeOH); IR (ATR) ν_{max} ~3373, 2926, 1706, 1645, 1457, 1381, 1352, 1230, 1146, 1084, 1042 cm^{-1} ; ^1H and ^{13}C NMR data, see Tables 2 and 3; HR-ESI-MS: m/z 429.1757 $[\text{M} + \text{H}]^+$ (calcd. for $\text{C}_{20}\text{H}_{29}\text{O}_{10}^+$: 429.1755).

2.3.4 Myoinositol-1-angelate-4,5-diacetate-6-(2-methylbutirate) (**4**) and myoinositol-1-angelate-4,5-diacetate-6-isovalerate (**5**) Yellow oil; IR (ATR) ν_{max} 3473, 2964, 2933, 1746, 1647, 1460, 1366, 1226, 1152, 1036 cm^{-1} ; ^1H and ^{13}C NMR data, see Tables 2 and 3; HR-ESI-MS: m/z 431.1908 $[\text{M} + \text{H}]^+$ (calcd. for $\text{C}_{20}\text{H}_{31}\text{O}_{10}^+$: 431.1912).

Table 1
NMR data of compound **1**. ^1H (600MHz), ^{13}C (150MHz) in CDCl_3 at 300K.

No.	δ_{H}	δ_{13C}
1ax	5.03 (br d 10.6)	128.1
2ax	5.68 (br td 10.6, 5.3)	69.7
3ax	2.18 (br t 11.1)	44.7
3eq	2.58 (dd 11.1, 5.3)	
4	-	134.4
5ax	5.34 (t 7.5)	129.5
6ax	1.94 (td 12.6, 7.5)	32.4
6eq	2.22 (m)	
7ax	2.39 (m)	39.4
8ax	1.86 (br td 13.8, 3.6)	34.6
8eq	1.67 (m)	
9ax	1.77 (br td 14.0, 4.3)	34.6
9eq	2.32 (br d 13.8)	
10		140.3
11		146.0
12		171.8
13	6.29 (br s) 5.66 (br s)	125.4
14	1.86 (s)	22.5
15	1.59 (s)	16.8
2- CH_3CO_2	2.05 (s)	21.5
	-	171.4

δ in ppm, br: broad, s: singlet, d: doublet, t: triplet, q: quadruplet, m: multiplet, (J Hz)

2.4 Anti-inflammatory and cytotoxicity assays

In vitro anti-inflammatory assay is based on the ability of immortalized mouse macrophages RAW 264.7 to generate a strong inflammatory response leading to NO release, after stimulation by antigens of *E. coli*. Mouse macrophages were seeded into 48-well tissue culture plates at the concentration of 1.10^5 cells/ml (200 μ l/well) for 24 hours at 37°C (5% CO₂). At the end of the incubation period, the culture medium was replaced by 200 μ l of medium containing appropriate concentrations of evaluated compound, and cells were incubated at 37°C (5% CO₂) for one hour. Experiments were performed in duplicate and dexamethasone was used as a positive control at the concentrations of 1, 5, 10, 50 and 100 μ M. At the end of the incubation period, pro-inflammatory LPS from *E.coli* were added to the cell cultures (1 μ g/ml). Then the cells were incubated at 37°C (5% CO₂) for 18 hours. NO release was measured in the culture supernatant by the Griess reaction: 50 μ l of the supernatants were transferred into the 96-well tissue culture plate, and 50 μ l of the Griess modified reagent were added into each well. After a 15 min period at room temperature, Optical Density (OD) of each well was read at 540 nm by fluorescence – luminescence reader Infinite M200 Pro (TECAN). Results obtained for wells, treated with evaluated compound, were compared to untreated control wells (DMSO, 100% viability) and converted into the percentage values.

Cell viability was measured to validate this assay: the WST-1 vital dye reagent was used to measure mitochondrial respiration. For this purpose, the culture medium was decanted and 50 μ l of the WST-1 reagent (1/10 dilution in phosphate buffer) were added into each well. After a 30 min incubation period at 37°C (5% CO₂), OD of each well was read at 450 nm by a fluorescence – luminescence reader Infinite M200 Pro (TECAN). The results obtained for wells, treated with evaluated compound, were compared to untreated control wells (DMSO, 100% viability) and converted into the percentage values.

Inhibition of NO release and inhibition of cell viability were expressed as percentages comparing to the negative controls:

$$\text{Percentage of NO release or cell viability} = \frac{100 \times (\text{OD of test well} - \text{OD of blank})}{\text{OD of DMSO control} - \text{OD of blank}}$$

The concentrations of the test material causing respectively a 50% decrease of NO release ($IC_{50-NO\ release}$) and a 50% decrease of cell viability ($IC_{50-cell\ viability}$) were calculated through non-linear regression analysis using software Tablecurve Version 2.0. The anti-inflammatory ratio was defined as the ratio between anti-inflammatory activity and toxicity. It was expressed as follows:

$$\text{Anti-inflammatory ratio} = IC_{50\ cell\ viability} / IC_{50\ NO\ release}$$

3. Results and Discussion

One new sesquiterpene acid, called Eldarin (**1**), four new inositol derivatives, Myoinositol,1,5-diangelate-4,6-diacetate (**2**), Myoinositol,1,6-diangelate-4,5-diacetate (**3**), Myoinositol-1-angelate-4,5-diacetate-6-(2-methylbutirate) (**4**), Myoinositol-1-angelate-4,5-diacetate-6-isovalerate (**5**), together with eleven compounds 1 β -Hydroxyarbusculin A (**6**) [10], Artemorin (**7**) [11], Santamarin (**8**) [12], Chrysosplenol C (**9**) [13], 6-Hydroxykaempferol 3,7-dimethyl ether (**10**) [14], Reynosin (**11**) [15], Calenduladiol-3-palmitate (**12**) [16], Costunolide (**13**) [17], 4-Hydroxy-3,5-dimethoxybenzenemethanol (**14**) [18] and 9 β -Hydroxycostunolide (**15**) [19] have been isolated for the first time from leaves and flowers of *I. montana*. Among the known compounds, compounds **6** and **7** have been obtained from *Inula* genus for the first time [1,2]. Known compounds have been identified by comparison of their spectroscopic data with those in the literature. Inositol derivatives with hemiterpenic acid moieties has been already described in *Inula cappa*, but they are not frequently found in genus *Inula* [3, 20].

Previously, six sesquiterpene lactones [4] have been described in *Inula montana* L. by Gonzalez et al. (2001), however, in our study, we haven't isolated any of these reported sesquiterpene lactones. On the other hand, only one flavonoid aglycone, Hispidulin (**16**) [21], reported by Reynaud et al. (1999) has been isolated and identified in our study [6].

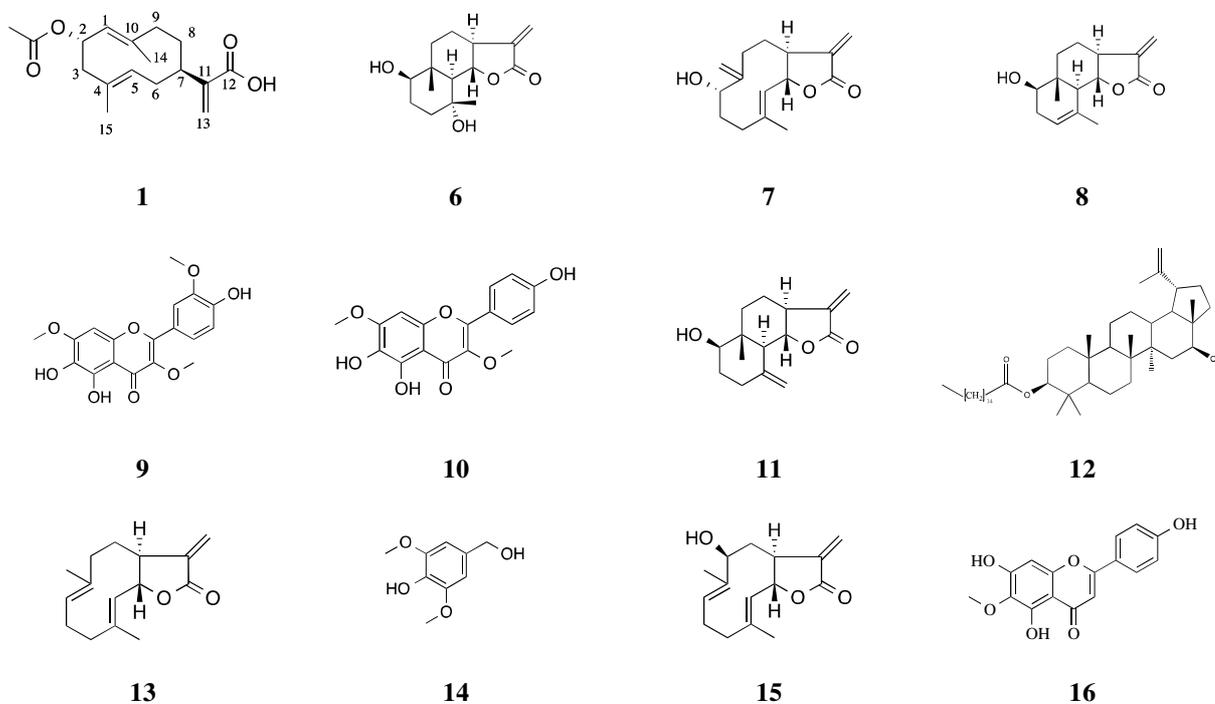


Fig. 1. Structures of the isolated compounds **1**, **6–16** from *I. montana*.

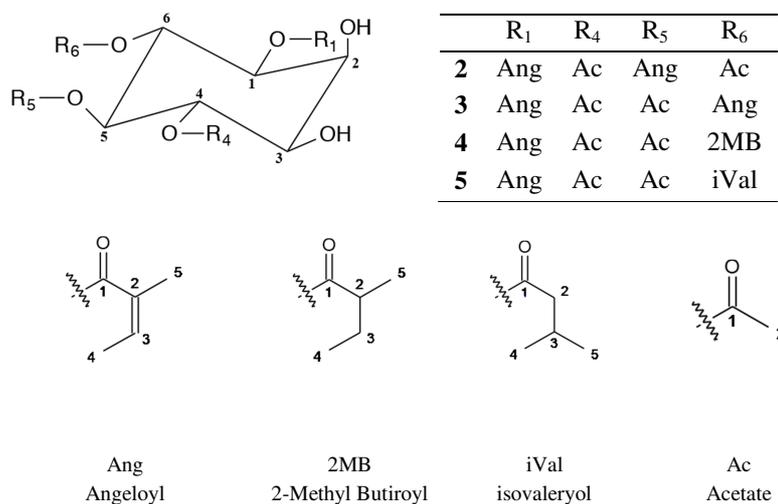


Fig. 2. Structures of the isolated inositol derivatives **2–5** from *I. montana*.

Compound **1** (Fig. 1) has been obtained as a white amorphous powder. Its molecular formula $C_{17}H_{24}O_4$ has been established by positive HR-ESI-MS, which gave a pseudo-molecular ion peak at m/z 293.1748 $[M+H]^+$ (calcd. for $C_{17}H_{25}O_4^+$ 293.1747), with the existence of six degrees of unsaturation. The IR spectrum suggested the presence of carbonyl acid (1693 , 1241 and 3440 cm^{-1}) and acetate group (1724 and 1241 cm^{-1}). The ^{13}C and edited-HSQC spectra gave seventeen carbon resonances with the presence of five quaternary carbons including two carbonyls (171.8 , 170.9 ppm) and three ethylenic carbons (146.0 , 140.3 , 134.4 ppm),

four methines including two olefinic carbons (129.5, 128.1 ppm), one oxygenated sp^3 carbon (69.7 ppm), one sp^3 carbon (39.4 ppm), five methylenes including one methylene sp^2 carbon (125.4 ppm), four methylene sp^3 carbons (44.7, 34.6, 34.6, 32.4 ppm), three methyls (22.5, 21.5, 16.8 ppm). The 1H NMR spectrum indicated the presence of one methylene 6.29 ppm (br s), and 5.66 ppm (br s), one methine bearing by an oxygen 5.68 ppm (br td, J 10.6, 5.3), two ethylenic protons 5.34 (t 7.5) and 5.03 ppm (br d J 10.6), four methylenes 2.58 (dd J 11.1, 5.3) and 2.18 (br t J 11.1), 2.22 (m) and 1.94 (td J 12.6, 7.5), 1.86 (br td J 13.8, 3.6) and 1.67 (m), 2.32 (br d J 13.8) and 1.77 (br td J 14.0, 4.3), one methine 2.39 (m), three methyls 1.86 (s), 1.59 (s) which one from acetate group 2.05 (s). The spectroscopic data suggested a germacrene skeleton: the COSY (H_1-H_2 , H_2-H_3 , H_5-H_6 , H_6-H_7 , H_7-H_8 , H_8-H_9) and the HMBC (H_2-C_{10} , $H_{15}-C_3$, $H_{15}-C_5$, $H_{14}-C_9$, $H_{14}-C_1$) correlations (Fig. 3) had to define a ten carbon ring. The second moiety with based on HMBC correlations ($H_{13}-C_{11}$, $H_{13}-C_{12}$, $H_{13}-C_7$) defined an isobutenic acid connected to the C_7 on the ten carbons ring. An acetate moiety was observed and connected to the C_2 on the ten carbons ring by the presence of the 3J HMBC correlation (H_2-COO). The proton H_{6ax} at 1.94 (td J 12.6, 7.5) show a 3J *trans* coupling constant of 12.6 Hz with H_7 in axial position at 2.39 (m) and the proton H_2 at 5.68 (br td J 10.6, 5.3) show two 3J *trans* coupling constant of 10.6 Hz with H_1 at 5.03 (br d J 10.6) and H_{3ax} at 2.18 (br t J 11.1), so H_2 is in axial position. NOESY spectra showed NOE correlation between H_{2ax} and H_{14} and H_{15} methyls groups. Between the four possible conformations of germacrene moiety described by de Kraker *et al.*, the only possibility is the conformation noted "UU" with the orientation of C-14 and C15 methyls groups to Upside [22]. The other NOE correlations observed between H_1-H_{3ax} , $H_{3ax}-H_5$, H_5-H_{7ax} , H_1-H_5 give the stereochemistry of **1** (Fig. 3). Compound **1** was previously proposed by Zdero *et al.*, as a possible intermediate compound in the transformation of germacrene A acid to the desacetyl derivative of compound **1**, but it has never been isolated [19]. In our study, the structure of compound **1** isolated from *I. montana* is elucidated as shown, and this compound is called Eldarin.

Compound **2** (Fig. 2) was obtained as yellow oil. Its molecular formula $C_{20}H_{28}O_{10}$ was established by positive HR-ESI-MS, which gave a pseudo-molecular

ion peak at m/z 429.1756 $[M+H]^+$ (calcd. for $C_{20}H_{29}O_{10}^+$ 429.1755), with the existence of seven degrees of unsaturation. The IR spectrum suggested the presence of hydroxyl groups (3472 cm^{-1}), carbonyl groups of ester function (1751 , 1719 and 1222 cm^{-1}), double-bond groups (3022 , 1647 cm^{-1}) and methyl groups (1365 cm^{-1}). The ^1H and COSY NMR spectra indicated the presence of myoinositol structure with H-1 axial at 5.02 ppm (dd, J 10.2, 2.6 Hz), H-2 equatorial at 4.23 ppm (t, J 2.6 Hz), H-3 axial at 3.84 ppm (dd J 10.0, 2.6 Hz), H-4 axial at 5.44 ppm (t, J 10.0 Hz), H-5 axial at 5.26 (t, J 10.0 Hz), and H-6 axial at 5.68 ppm (t J 10.0 Hz). In the HMBC spectrum, the two ^1H resonances at 1.93 and 2.01 ppm (each 3H, s) are correlated to two carbonyl carbons at 171.3 and at 172.0 ppm respectively indicate two acetate moieties. Two other ^1H resonances at 1.92 and at 1.96 ppm (each 3H, dq J 7.5, 1.5) correlated to each of two olifenic carbon at 128.4, 140.2 ppm and at 128.6, 140.4 ppm respectively, and the two last ^1H resonances at 1.80 and 1.86 (each 3H, br quint J 1.5) correlated to each of two same olifenic carbons respectively, which indicated the presence of two angelate moieties. The four chemical shifts downfield at 5.02 ppm (H-1), 5.26 ppm (H-5), 5.44 ppm (H-4) and at 5.68 ppm (H-6) were connected to two acetate moieties with H-4 and H-6 respectively by the presence of HMBC (Fig. 3) correlation with carbonyl carbons at 172.0 and 171.3 ppm respectively, and were connected to two angeloyl moieties with H-1 and H-5 by the presence of HMBC correlation with carbonyl carbons at 168.3 and 168.0 ppm respectively. The NOE correlations (Fig. 3) between H-1, H-5 with the methyl groups of angeloyl and H-4, H-6 with the methyl group of acetyl confirmed the structure of compound **2** as myoinositol-1,5-diangelate-4,6-diacetate.

Compound **3** (Fig. 2) was obtained as yellow oil. Its molecular formula $C_{20}H_{28}O_{10}$ was established by positive HR-ESI-MS, which gave a pseudo-molecular ion peak at m/z 429.1750 $[M+H]^+$ (calcd. for $C_{20}H_{29}O_{10}^+$ 429.1755), with the existence of seven degrees of unsaturation. The IR, ^1H and ^{13}C NMR spectra are close to compound **2** with the presence of two angeloyl, two acetate moieties and one myoinositol structure. The main difference observed is a substituent pattern of inositol ring: the HMBC spectrum (Fig. 3) showed that H-5 at 5.22 ppm (t, J 10.0) is connected with an acetate carbonyl at 171.2 ppm, and H-6 at 5.73 ppm (t, J 10.0) is

connected with an angeloyl carbonyl at 167.7 ppm. Thus, compound **3** was established as myoinositol-1,6-diangelate-4,5-diacetate.

Compound **4** (Fig. 1) has been obtained as a mix with compound **5** as yellow oil. The molecular formula $C_{20}H_{30}O_{10}$ was established by positive HR-ESI-MS, which gave a pseudo-molecular ion peak at m/z 431.1908 $[M+H]^+$ (calcd. for $C_{20}H_{31}O_{10}^+$ 431.1912), with an existence of six degrees of unsaturation. The IR, 1H and ^{13}C NMR spectra are close to compound **3** with an exception for compound **4** with the presence of a hydrogenated double bond of one angeloyl moiety. The 1H NMR spectrum showed two methyl resonances at 0.83 ppm (t, J 7.4), 1.03 ppm (d, J 7.0), one methylene with two resonances at 1.58 ppm (m), 1.38 ppm (m) and one methine resonance at 2.29 ppm (m) linked to carbonyl ester at 176.5 ppm corresponding to the 2-methylbutiroyl moiety. The HMBC spectrum (Fig. 3) showed that H-6 at 5.65 ppm (t, J 10.0) is connected with the 2-methylbutiroyl carbonyl at 176.5 ppm, thus, compound **4** was established as myoinositol-1-angelate-4,5-diacetate-6-(2-methylbutirate).

For compound **5**, the 1H NMR spectrum showed the second difference with the spectrum of compound **3** with two methyl resonances at 0.89 ppm (each d, J 6.7), one methylene with two resonances at 2.12 ppm (dd, J 14.9, 7.4), 2.09 ppm (dd, J 14.9, 6.8) and one methine resonance at 1.96 ppm (m) linked to carbonyl ester at 172.9 ppm corresponding of an isovaleroyl moiety. The HMBC spectrum (Fig. 3) showed that H-6 at 5.65 ppm (t, 10.0) is connected with the isovaleroyl carbonyl at 172.9 ppm, thus, compound **5** was established as myoinositol-1-angelate-4,5-diacetate-6-isovalerate.

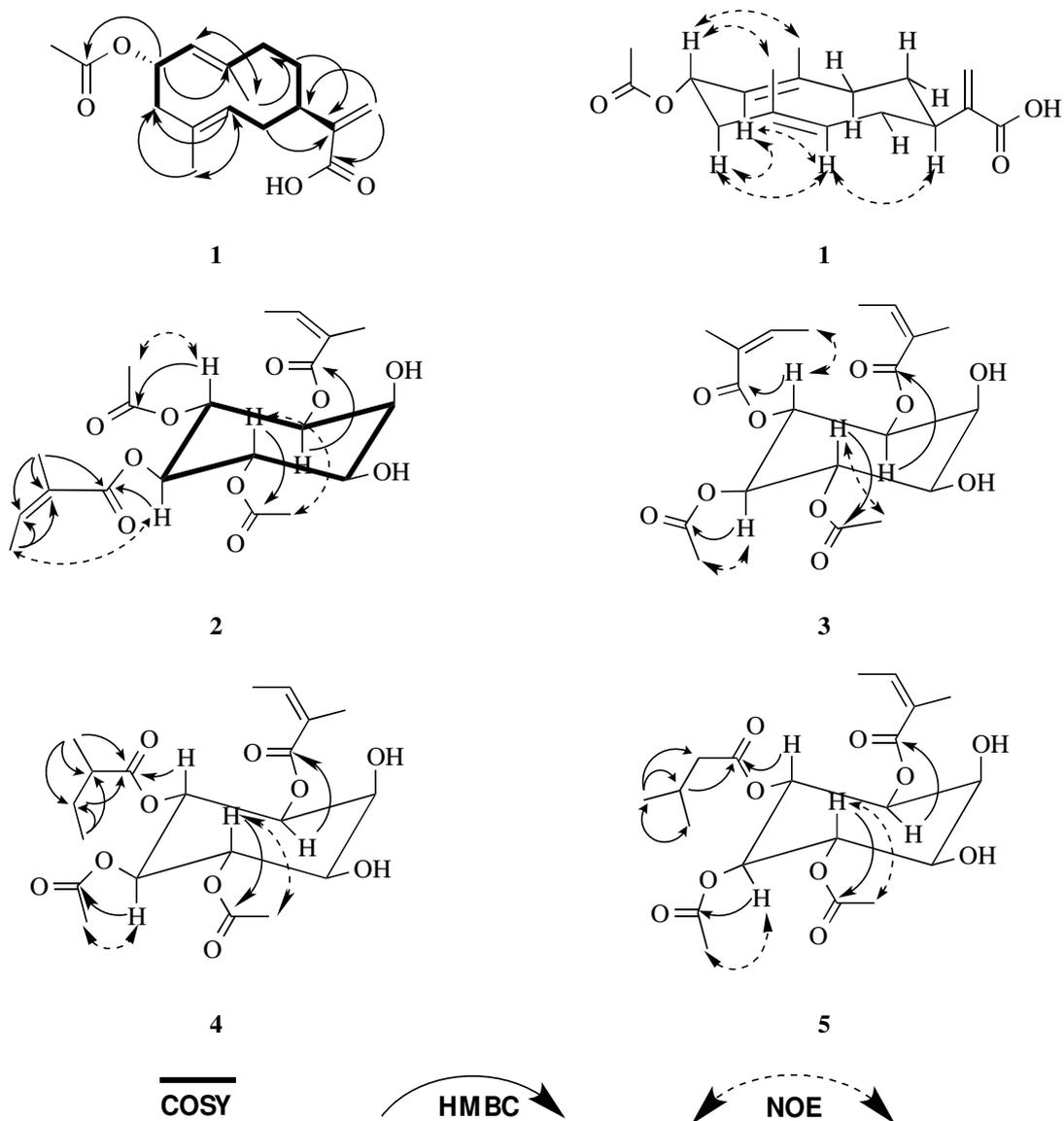


Fig. 3. Keys COSY, HMBC and NOE correlations of compounds 1 - 5.

Anti-inflammatory activity and cytotoxicity of nine isolated compounds have been tested *in vitro* comparing to dexamethasone as a positive control. The results are summarized in Table 4. The new inositol derivatives **2** and **3**, sesquiterpene lactones **7**, **13** and **15** and phenolic compounds **14** and **16** showed release of NO with IC_{50} value $< 30 \mu\text{M}$ on macrophages RAW 264.7, while compounds **1** and **12** have not shown neither anti-inflammatory activity, nor cytotoxicity at the concentrations at 51.34 and 22.04 μM respectively. Compound **15** exhibited cytotoxicity with IC_{50} value 1.21 μM . Comparing the anti-inflammatory ratio we can conclude that compounds **2**, **7**, **13**, **14** and **16** present promised anti-inflammatory activity with the anti-inflammatory ratio higher than the positive control dexamethasone.

Table 4Anti-inflammatory, cytotoxic activity and anti-inflammatory ratio of compounds **1-3, 7, 12-16**.

Compounds	NO release IC₅₀ (μM)^a	Toxicity IC₅₀ (μM)	Anti-inflammatory ratio
1	>51.34	>51.34	-
2	3.53 ± 0.97	213.93 ± 24.5	60
3	27.37 ± 6.7	144.1 ± 13.7	5
7	0.16 ± 0.07	18.93 ± 3.1	126
12	>22.04	>22.04	-
13	0.3 ± 0.07	17.78 ± 1.4	59
14	1.63 ± 0.55	178.08 ± 24.1	109
15	0.68 ± 0.09	1.21 ± 0.14	2
16	0.4 ± 0.08	22.98 ± 5.7	57
Dexamethasone ^b	4.33	163.33	38

^a The IC₅₀ values of compounds **1** and **12** have not been detected at the concentration of 51.34 and 22.04 μM respectively.

^b Positive control

Acknowledgements

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Conflict of Interest Statement

The authors have declared that there is no conflict of interest.

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**New sesquiterpene acid and inositol derivatives
from *Inula montana* L.**

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Supporting Information

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Index

For Compound 1:

S1. ¹H NMR Spectrum (600 MHz, CDCl₃) of Compound **1**
S2. ¹³C NMR Spectrum (150 MHz, CDCl₃) of Compound **1**

S3. HSQC Spectrum of Compound **1**

S4. ¹H-¹H COSY Spectrum of Compound **1**

S5. NOESY Spectrum of Compound **1**

S6. IR Spectrum of Compound **1**

S7. HR-ESI-MS Spectrum of Compound **1**

For Compound 2:

S8. ¹H NMR Spectrum (600 MHz, CD₃OD) of Compound **2**

S9. DEPTQ135 Spectrum of Compound **2**

S10. HSQC Spectrum of Compound **2**

S11. HMBC Spectrum of Compound **2**

S12. ¹H-¹H COSY Spectrum of Compound **2**

S13. NOESY Spectrum of Compound **2**

S14. IR Spectrum of Compound **2**

S15. HR-ESI-MS Spectrum of Compound **2**

For Compound 3:

S16. ¹H NMR Spectrum (600 MHz, CD₃OD) of Compound **3**

S17. ¹³C NMR Spectrum (150 MHz, CD₃OD) of Compound **3**

S18. HMBC Spectrum of Compound **3**

S19. ¹H-¹H COSY Spectrum of Compound **3**

S20. NOESY Spectrum of Compound **3**

S21. IR Spectrum of Compound **3**

S22. HR-ESI-MS Spectrum of Compound **3**

For mix of Compound 4 and Compound 5:

S23. ¹H NMR Spectrum (600 MHz, CD₃OD) of mix of Compound **4** and Compound **5**

S24. DEPTQ135 Spectrum of mix of Compound **4** and Compound **5**

S25. HSQC Spectrum of mix of Compound **4** and Compound **5**

S26. HMBC Spectrum of mix of Compound **4** and Compound **5**

S27. ¹H-¹H COSY Spectrum of mix of Compound **4** and Compound **5**

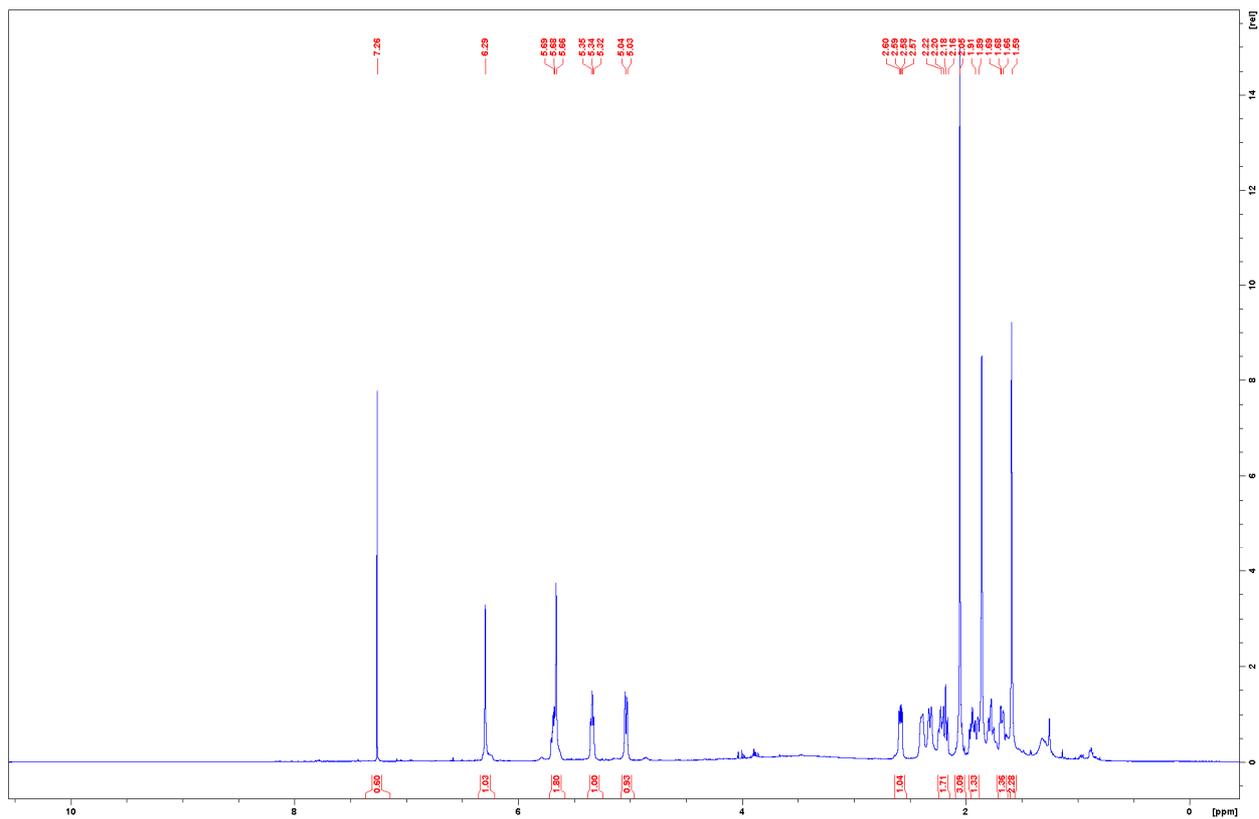
S28. NOESY Spectrum of mix of Compound **4** and Compound **5**

S29. IR Spectrum of mix of Compound **4** and Compound **5**

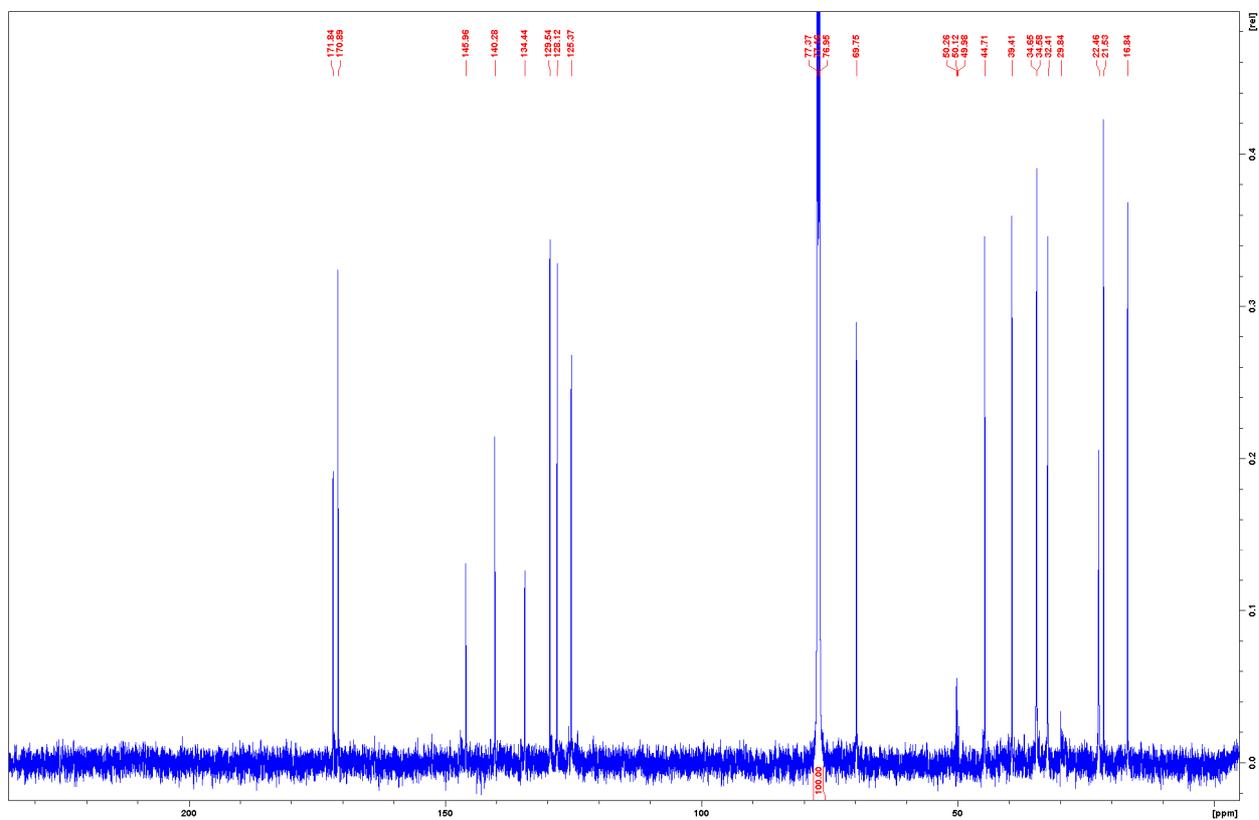
S30. HR-ESI-MS Spectrum of mix of Compound **4** and Compound **5**

For Compound 1:

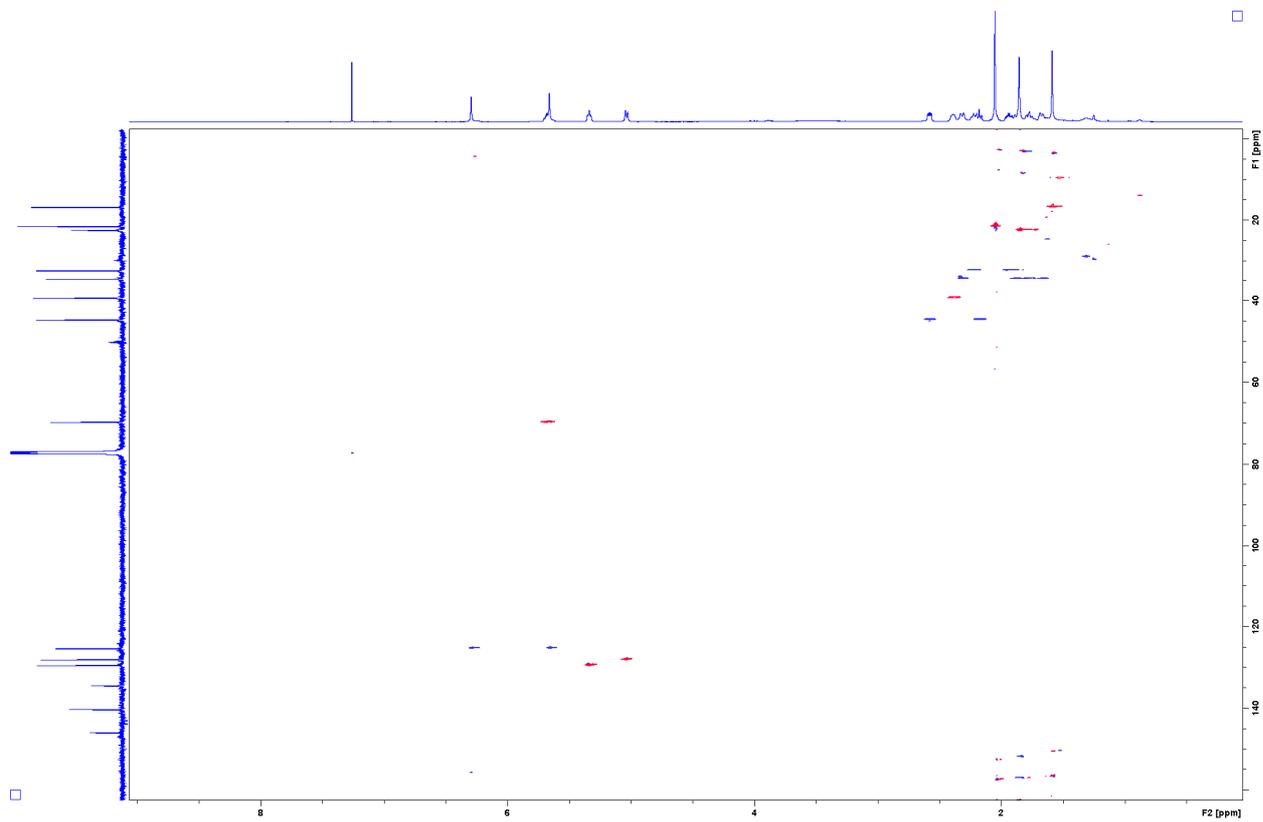
S1. ^1H NMR Spectrum (600 MHz, CDCl_3) of Compound 1



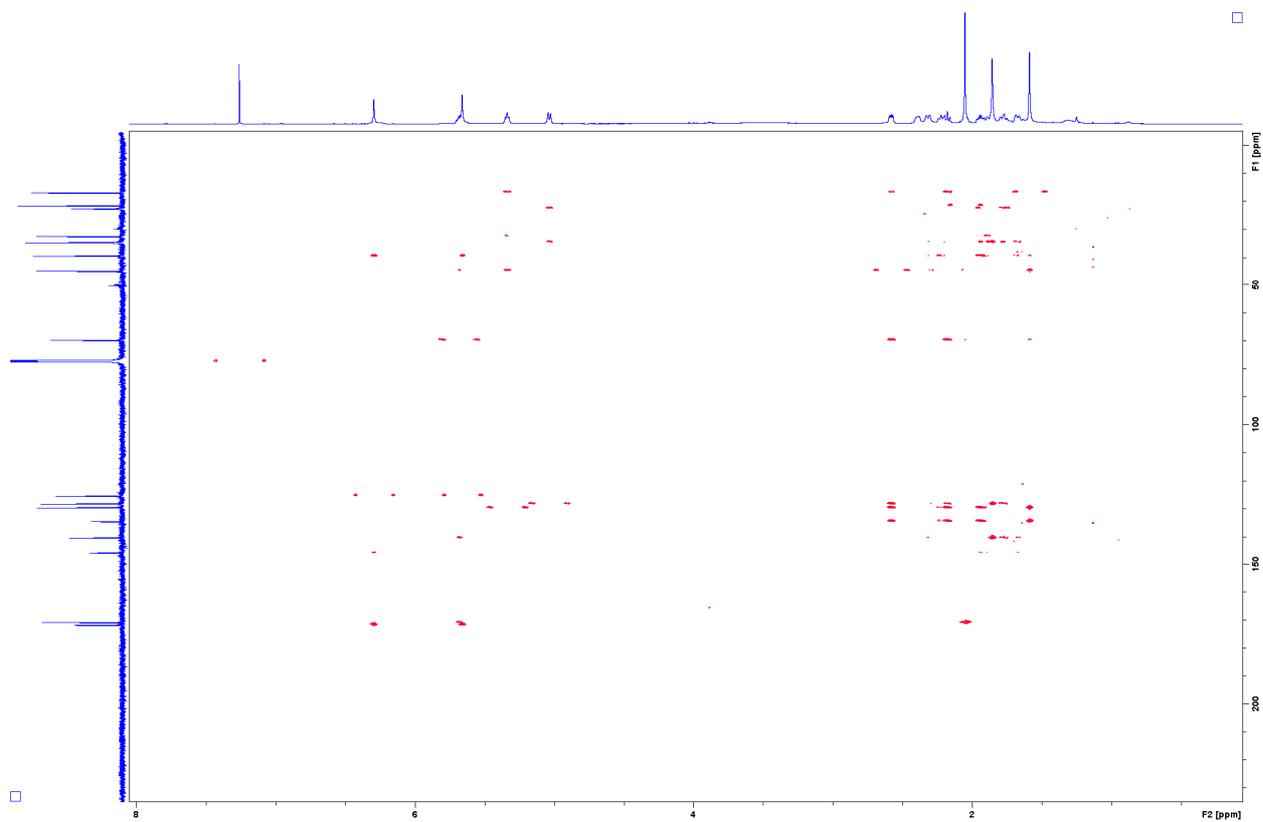
S2. ^{13}C NMR Spectrum (150 MHz, CDCl_3) of Compound 1



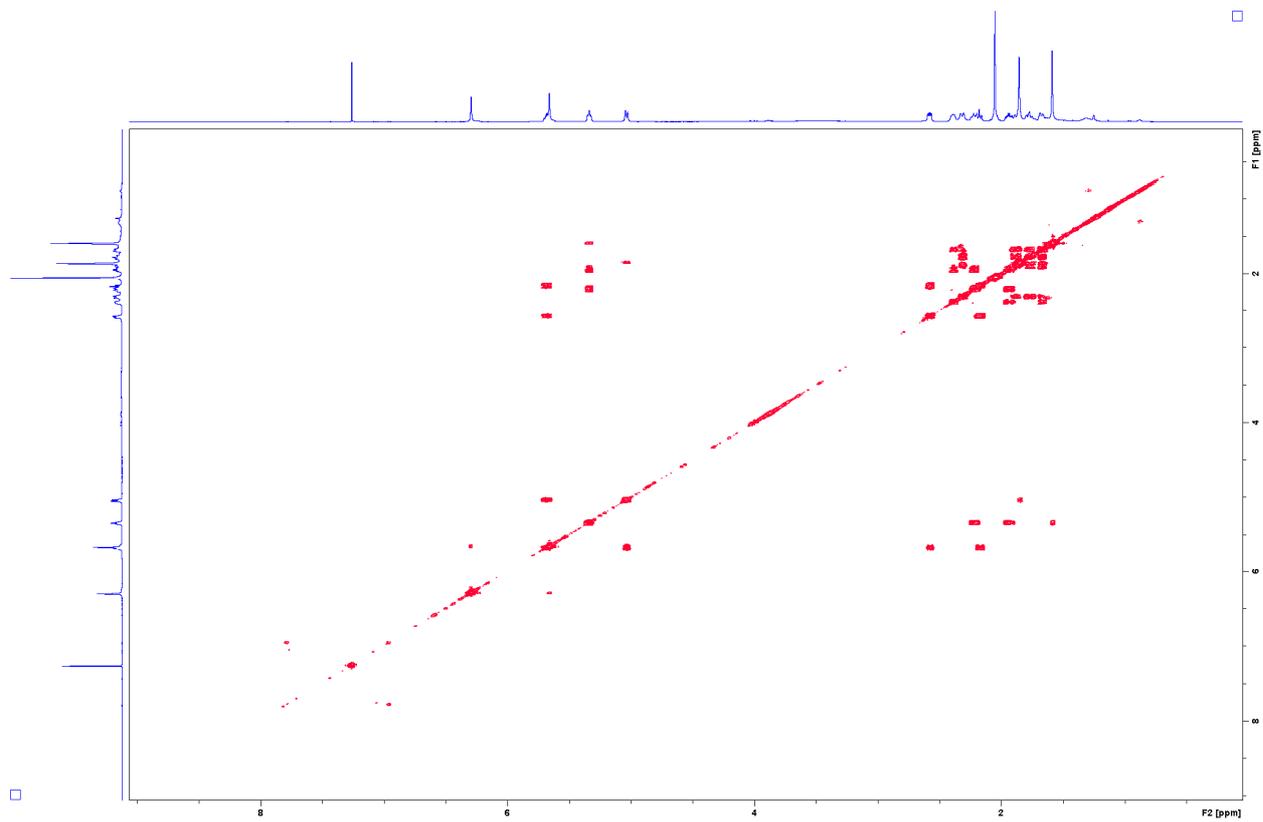
S3. HSQC Spectrum of Compound 1



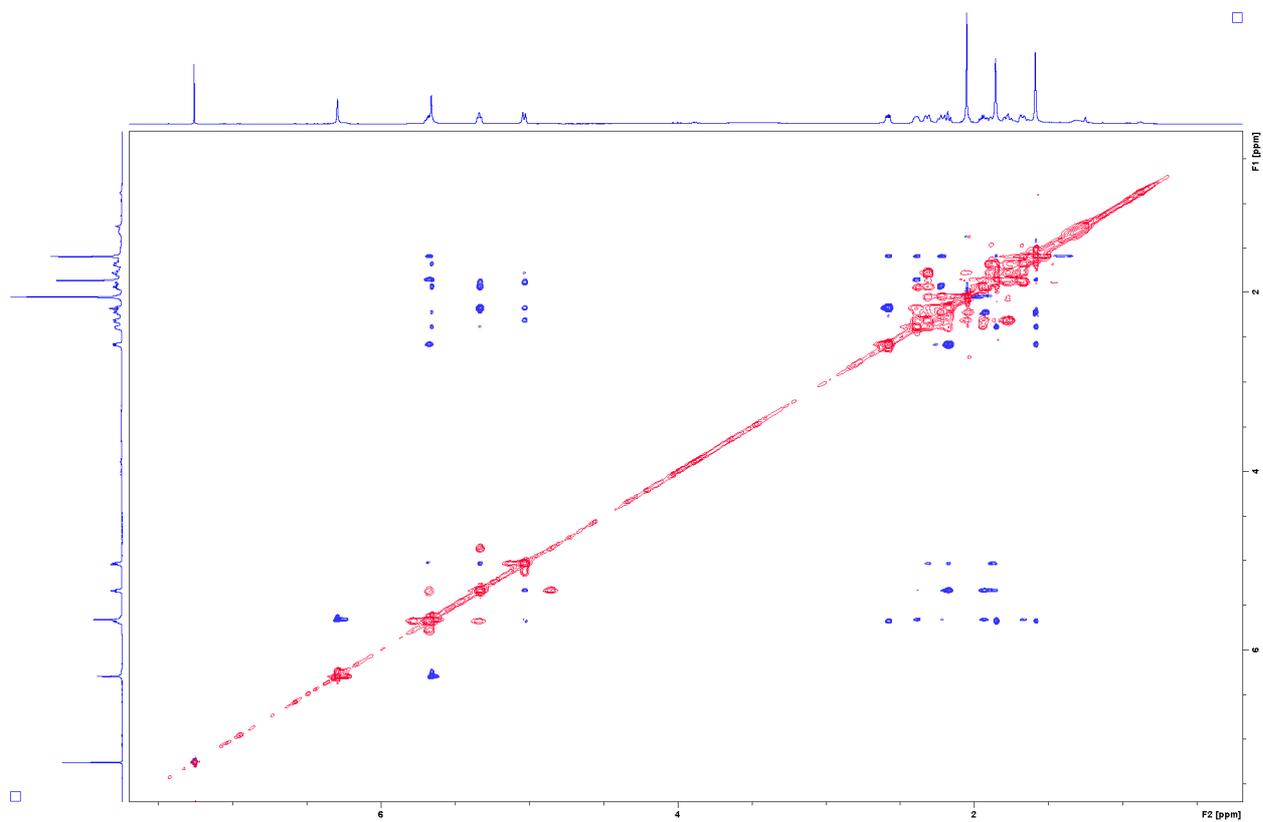
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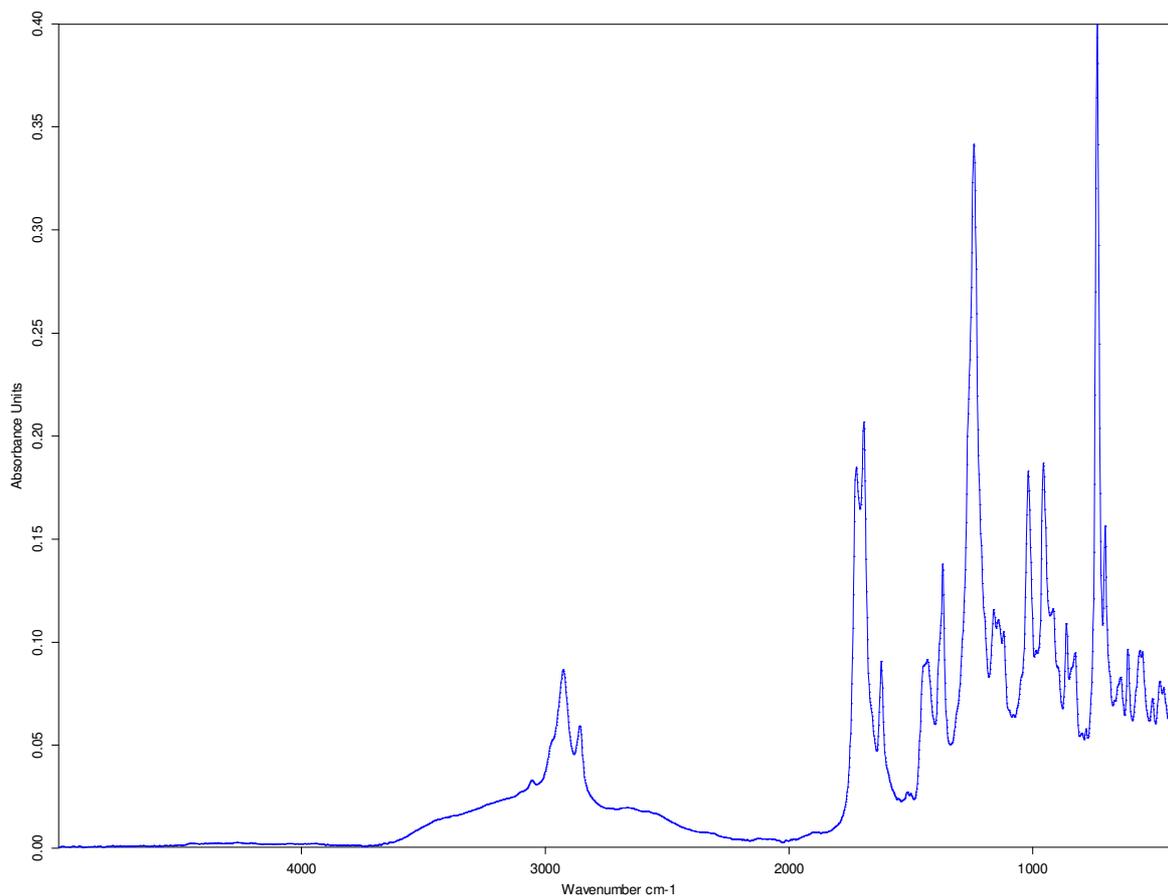
S4. ^1H - ^1H COSY Spectrum of Compound 1



S5. NOESY Spectrum of Compound 1



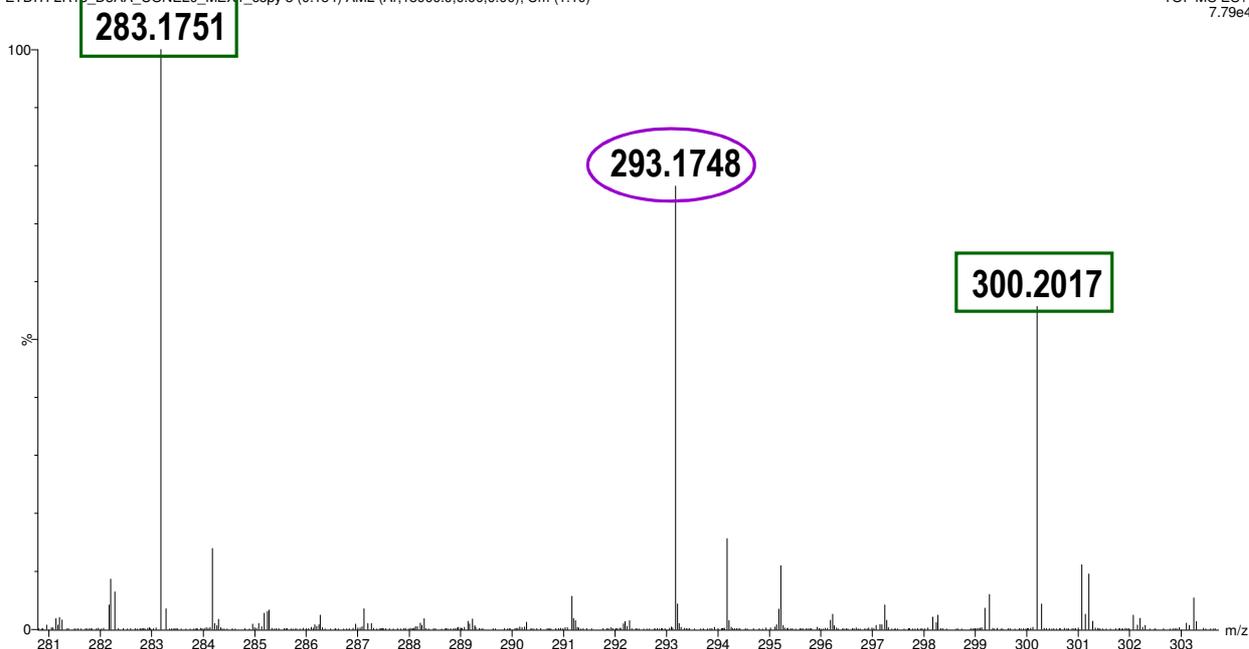
S6. IR Spectrum of Compound 1



S7. HR-ESI-MS Spectrum of Compound 1

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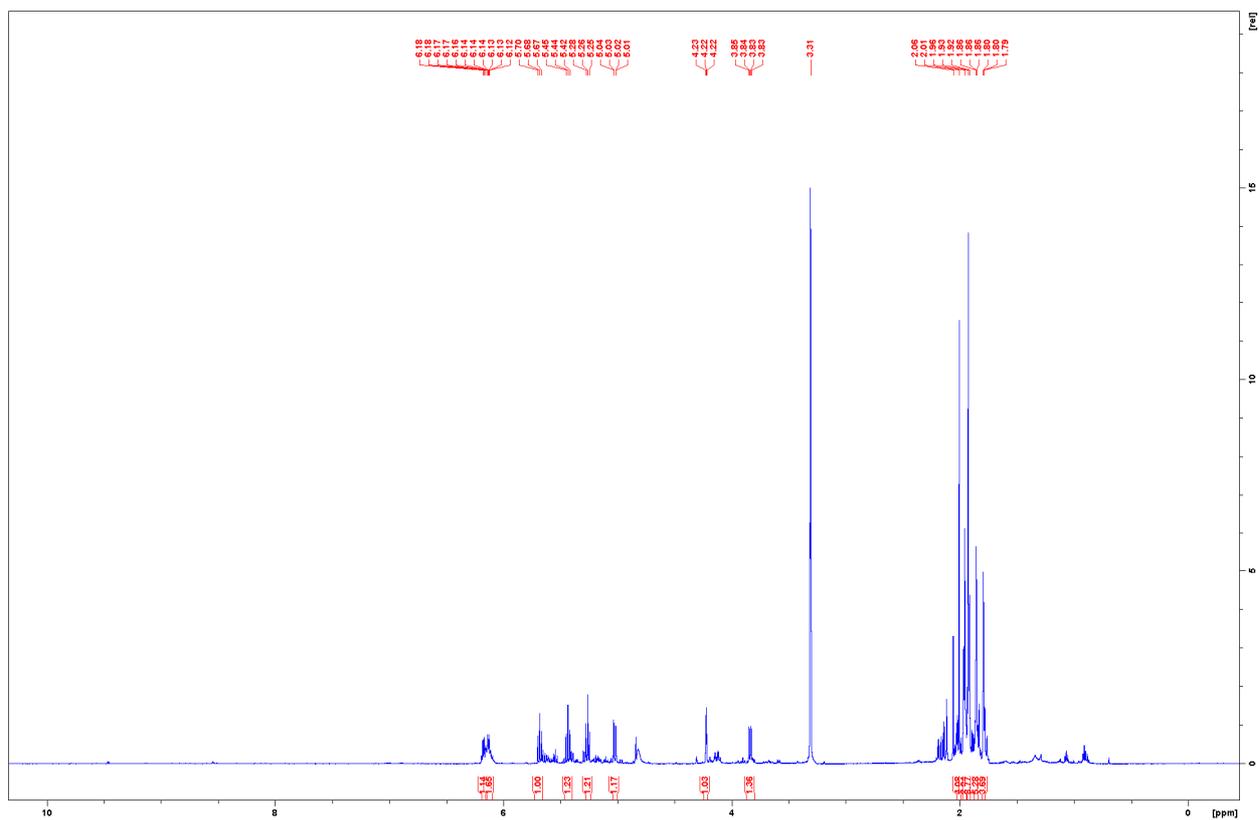
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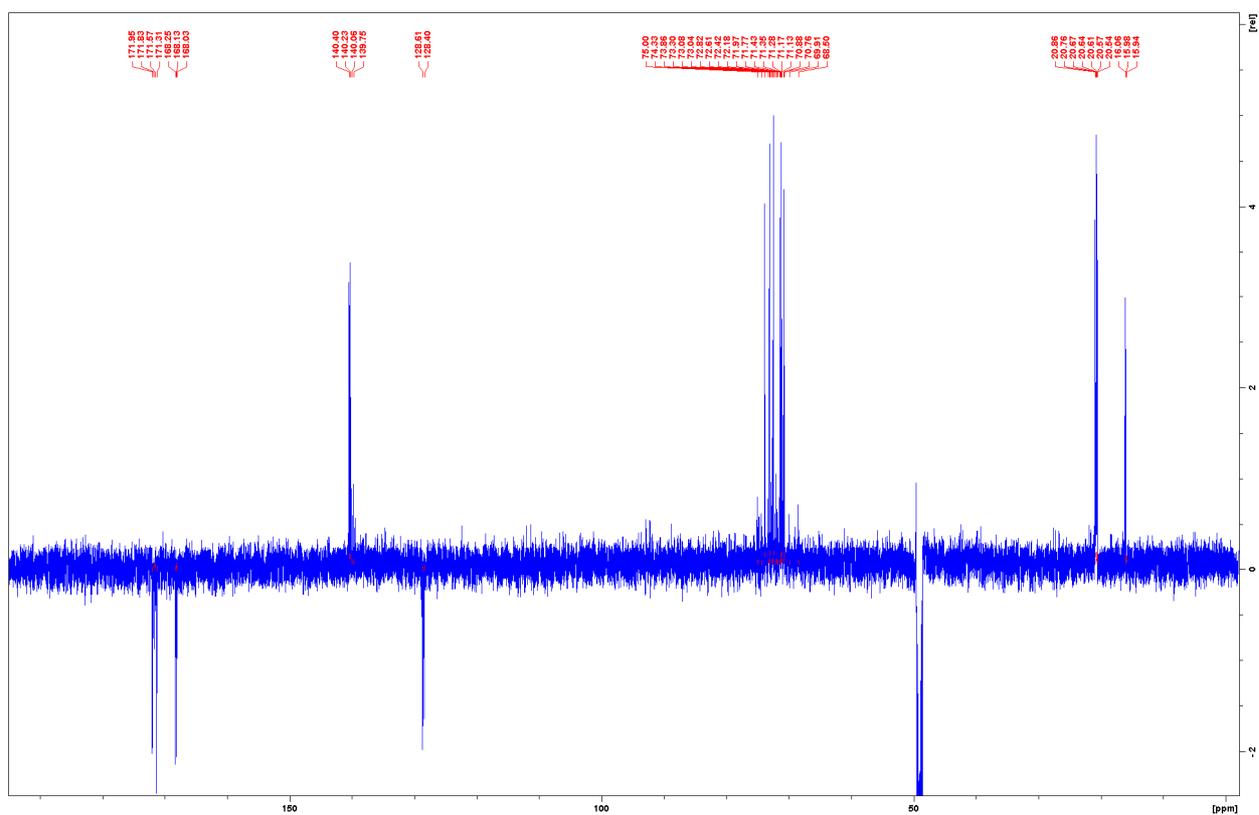
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For Compound 2:

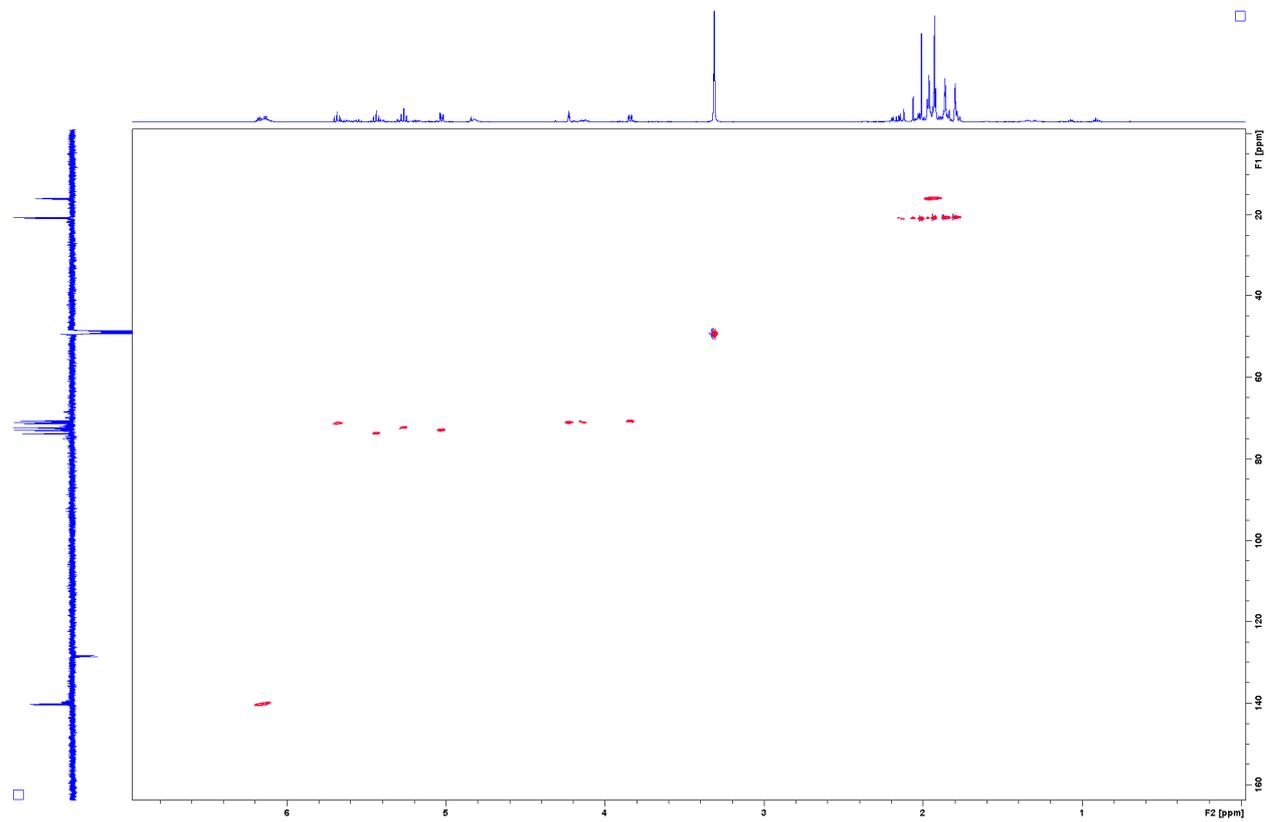
S8. ¹H NMR Spectrum (600 MHz, CD₃OD) of Compound 2



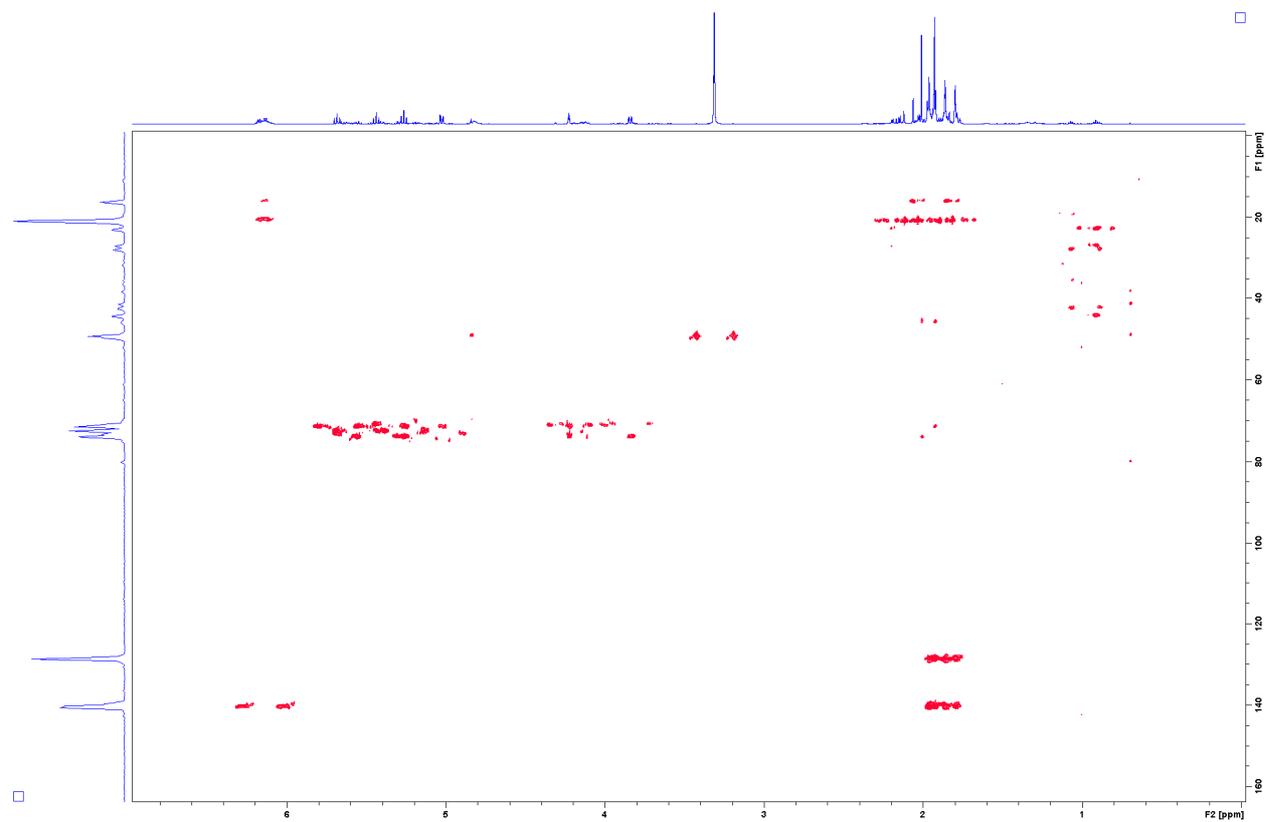
S9. DEPTQ135 Spectrum of Compound 2



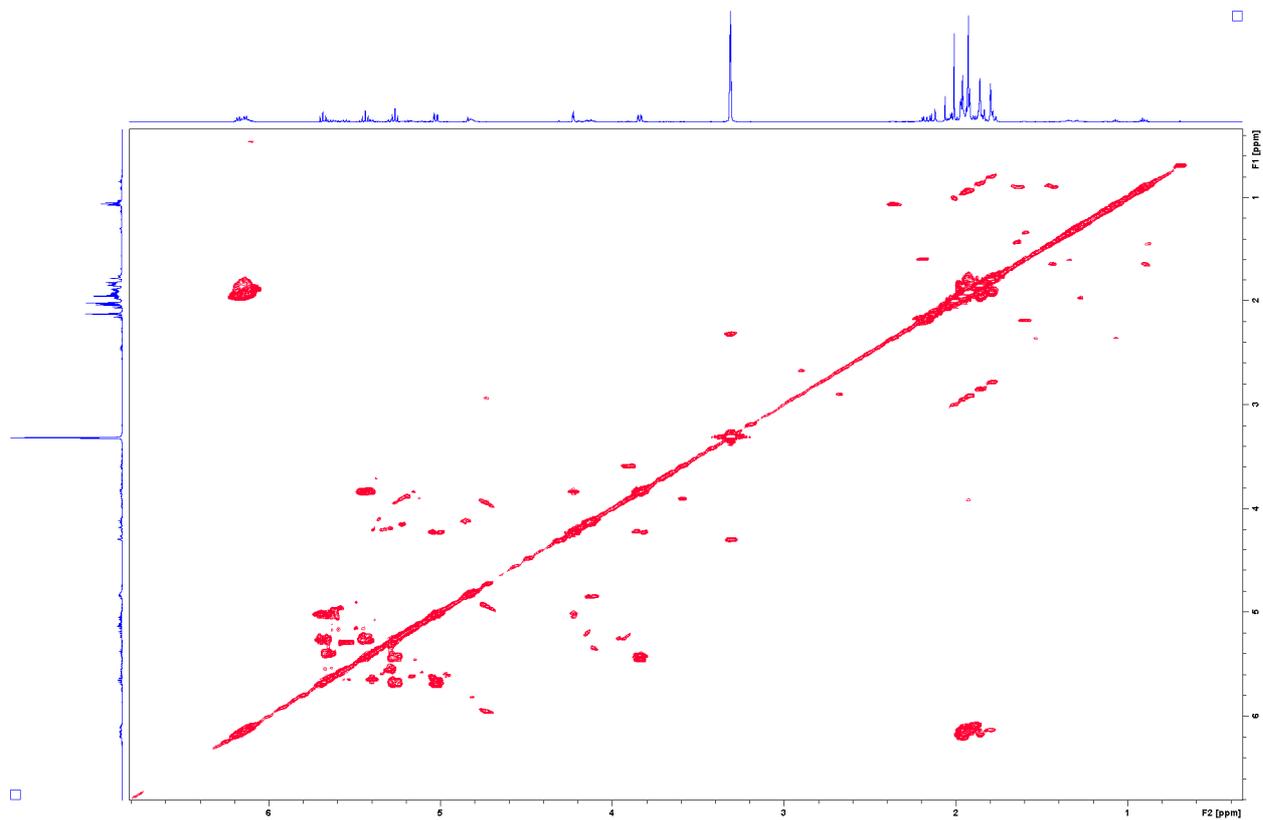
S10. HSQC Spectrum of Compound 2



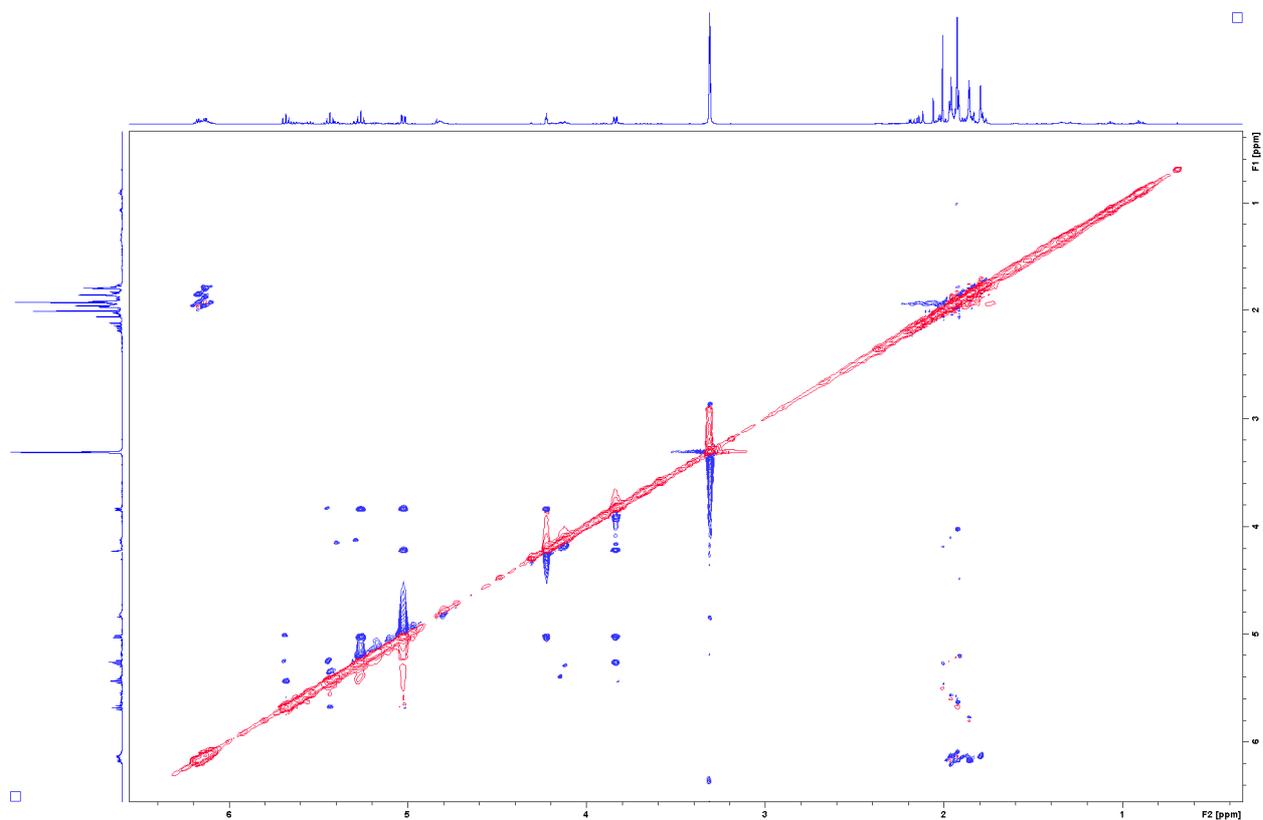
S11. HMBC Spectrum of Compound 2



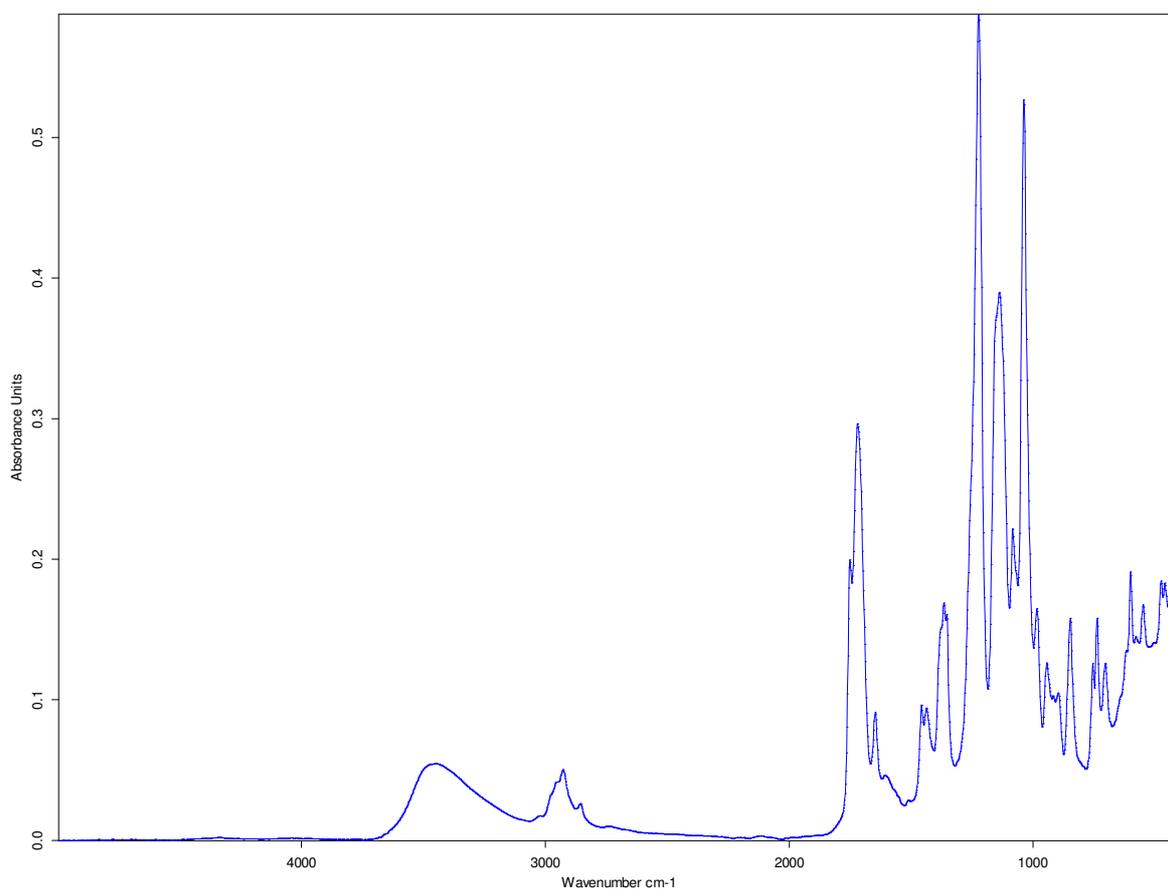
S12. ^1H - ^1H COSY Spectrum of Compound 2



S13. NOESY Spectrum of Compound 2



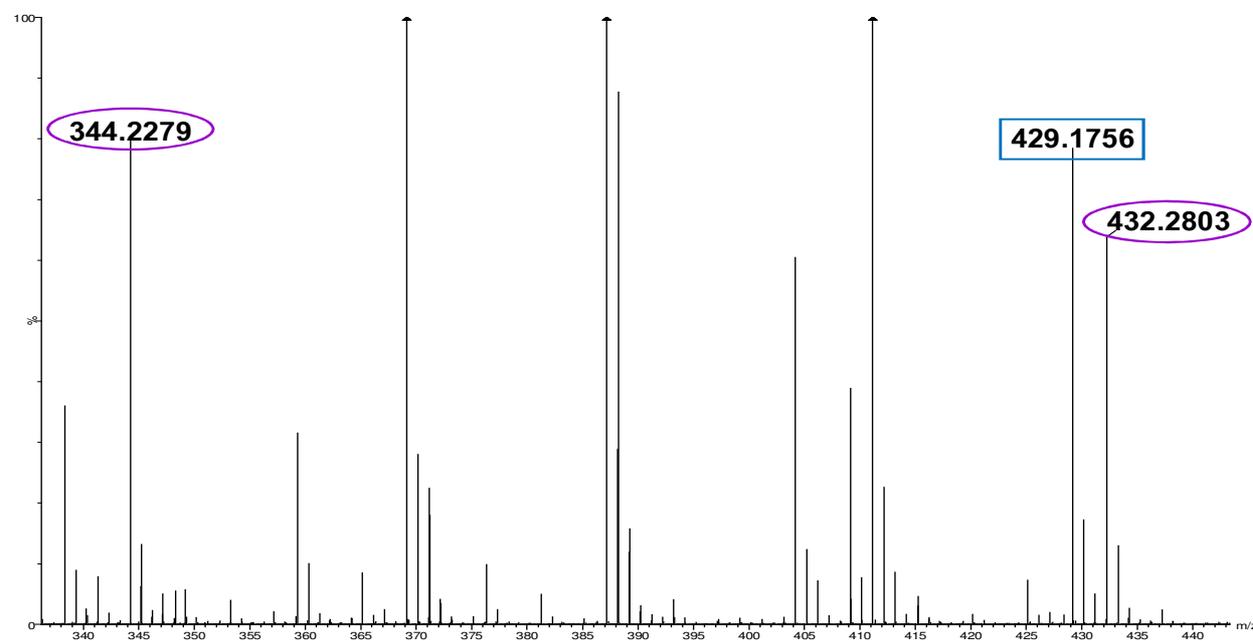
S14. IR Spectrum of Compound 2



S15. HR-ESI-MS Spectrum of Compound 2

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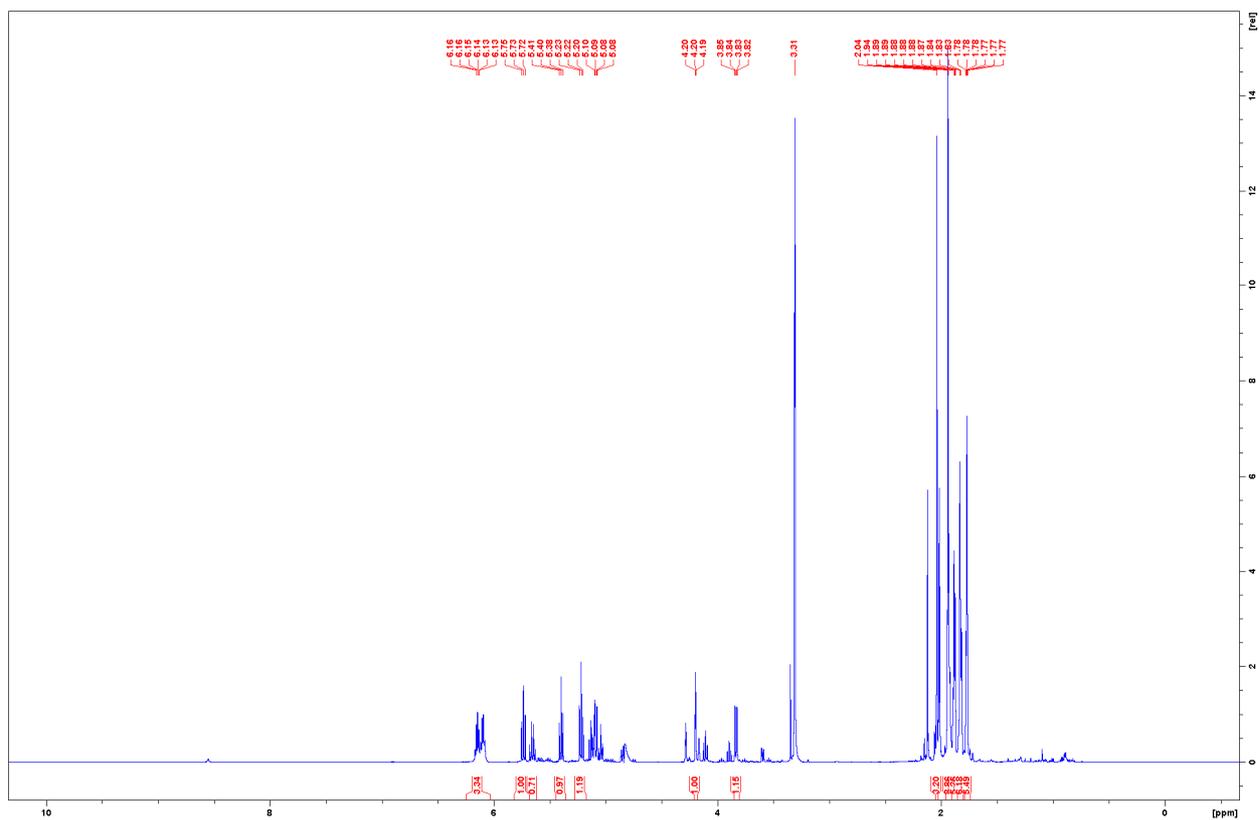
TOF MS ES+
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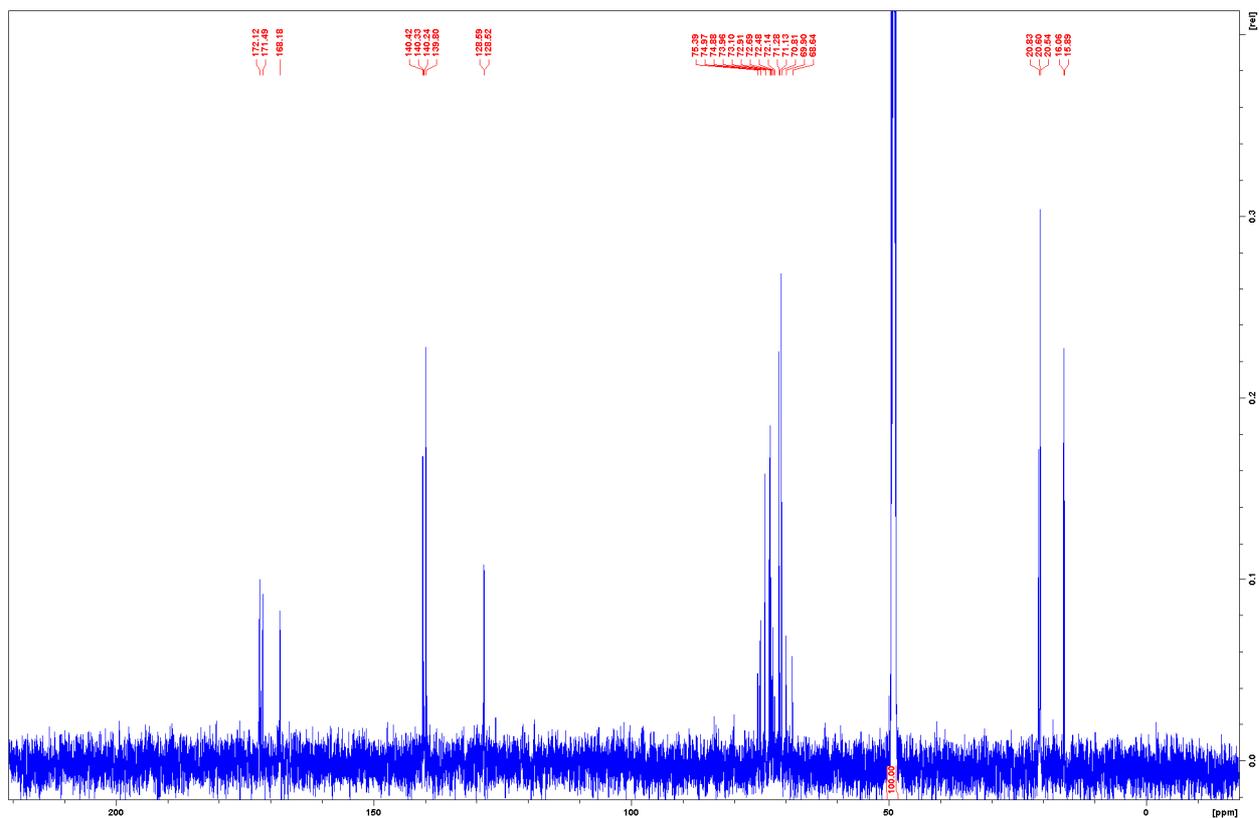
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Experimental values	1	429.1757	+ 0.2	+ 0.5
	2	429.1757	+ 0.2	+ 0.5
	3	429.1756	+ 0.1	+ 0.2

For Compound 3:

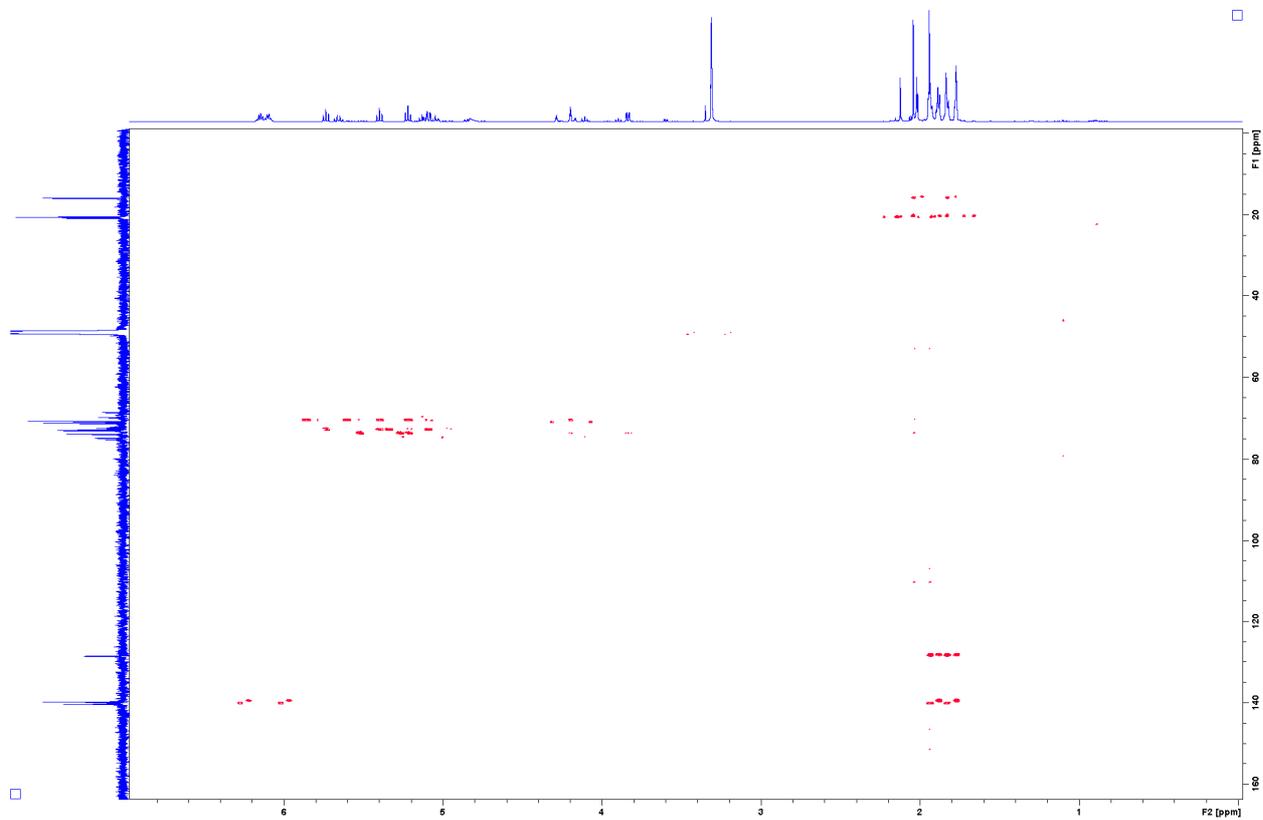
S16. ^1H NMR Spectrum (600 MHz, CD_3OD) of Compound 3



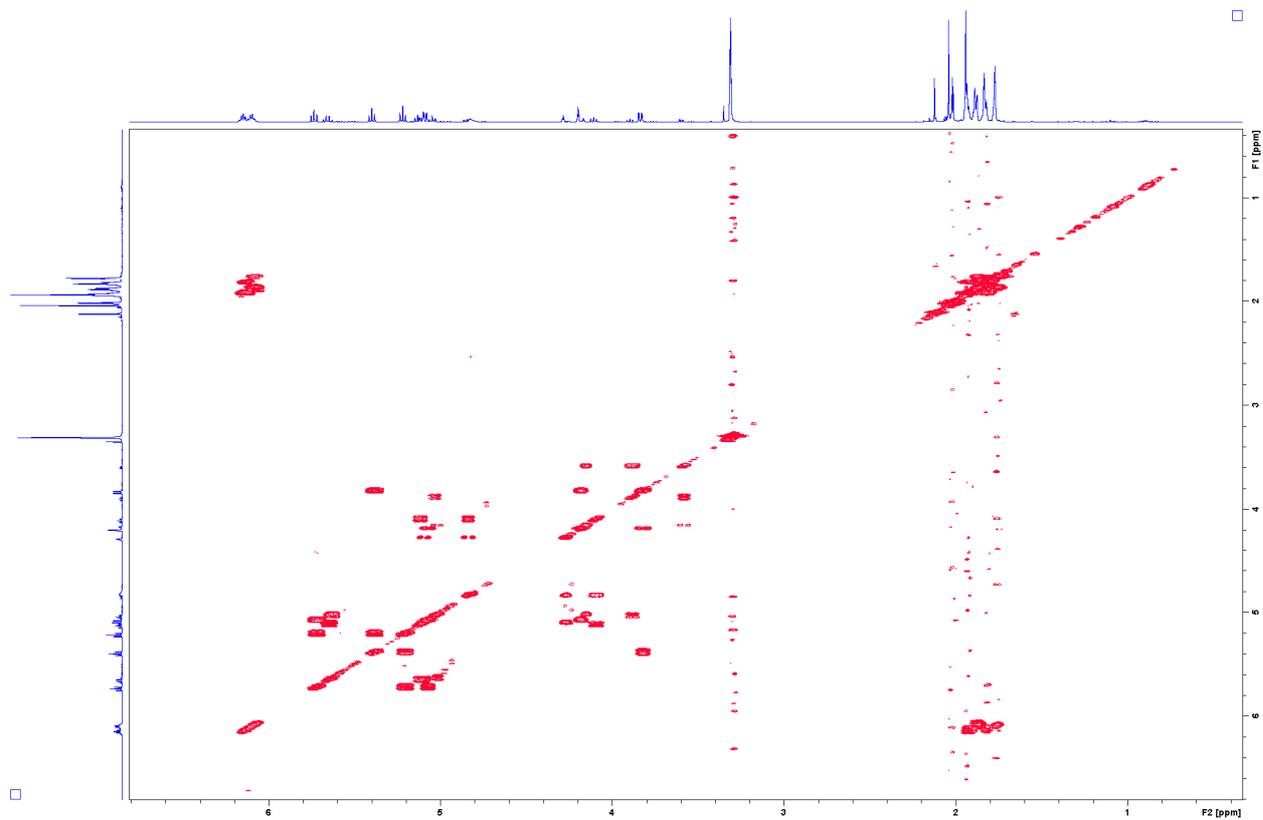
S17. ^{13}C NMR Spectrum (150 MHz, CD_3OD) of Compound 3



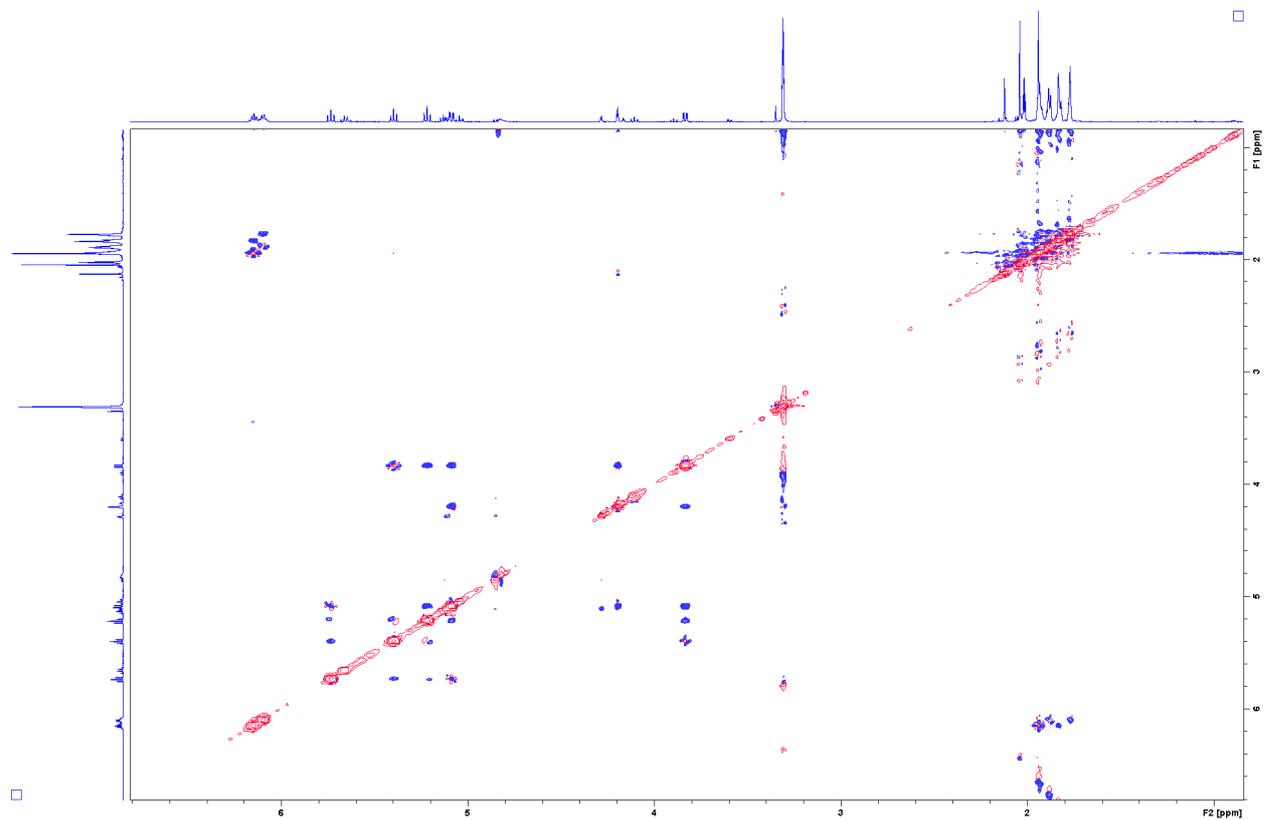
S18. HMBC Spectrum of Compound 3



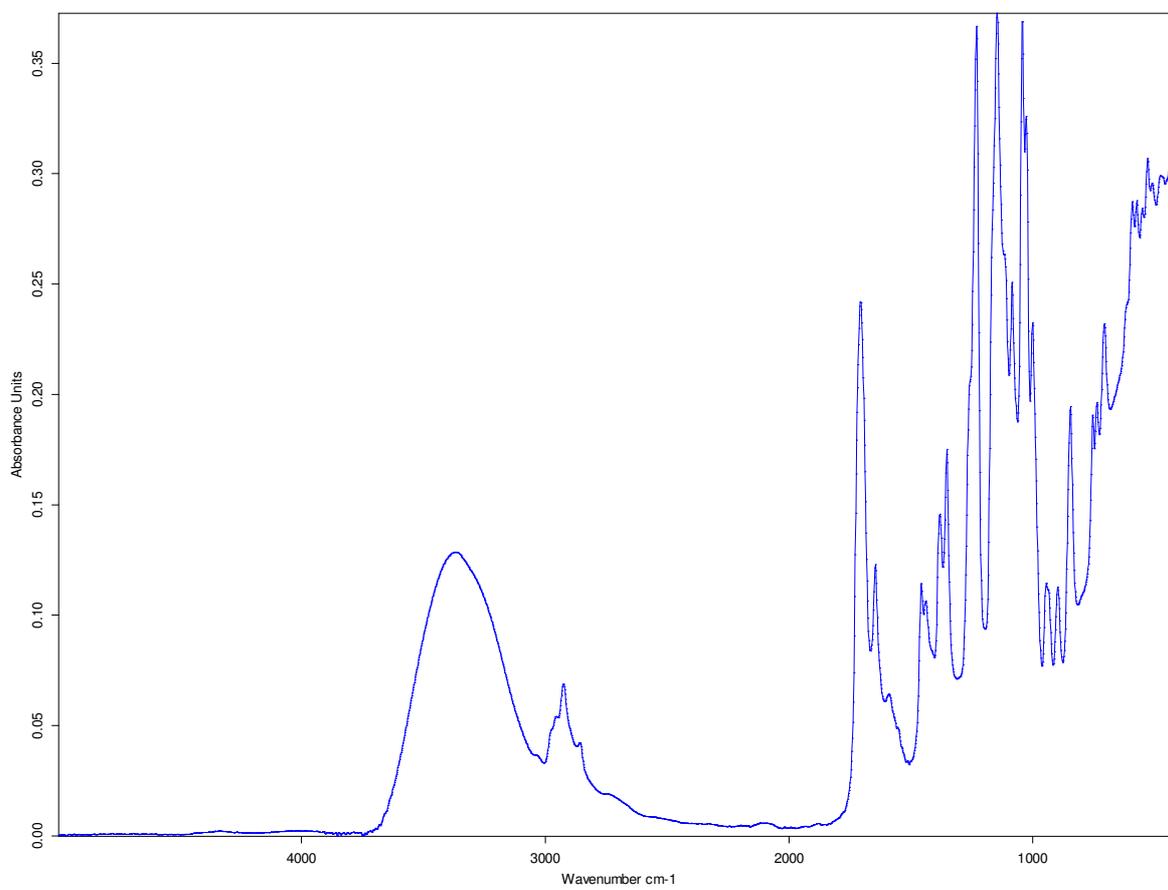
S19. ^1H - ^1H COSY Spectrum of Compound 3

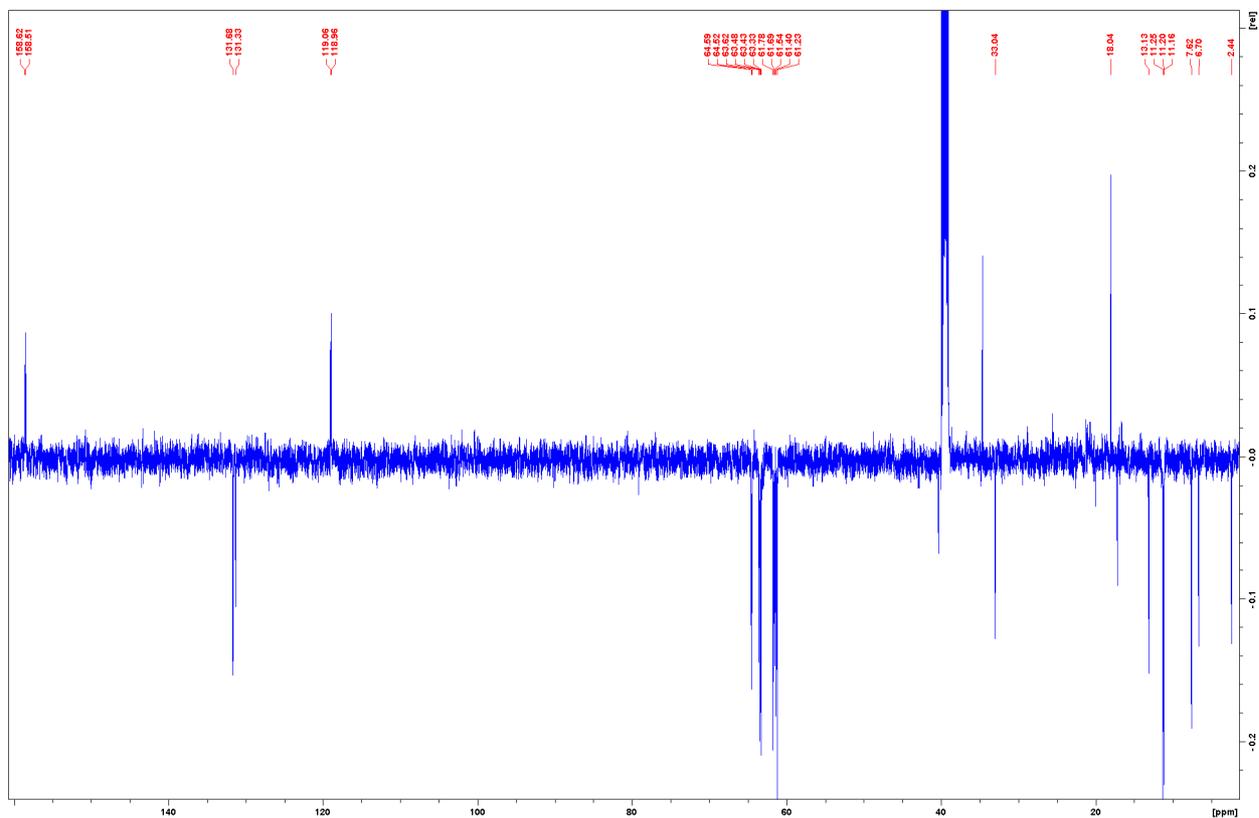


S20. NOESY Spectrum of Compound 3

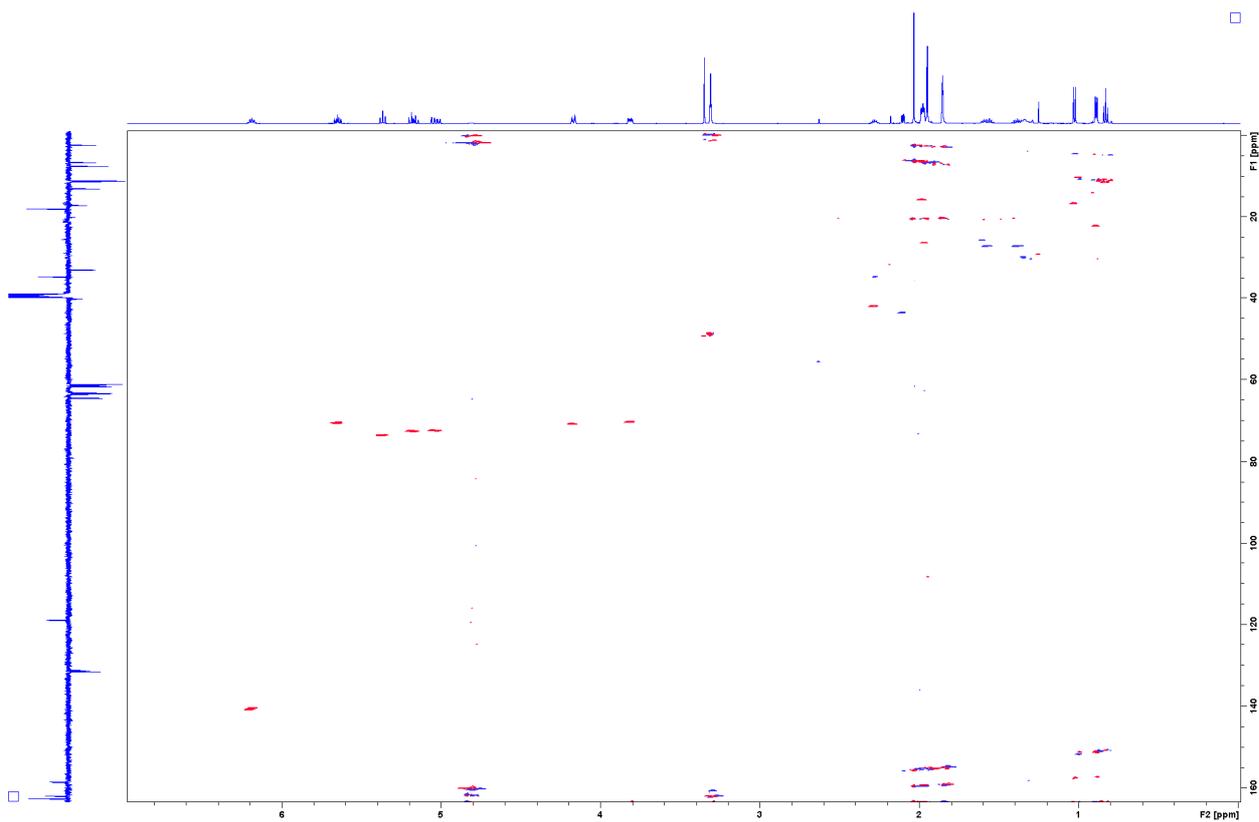


S21. IR Spectrum of Compound 3

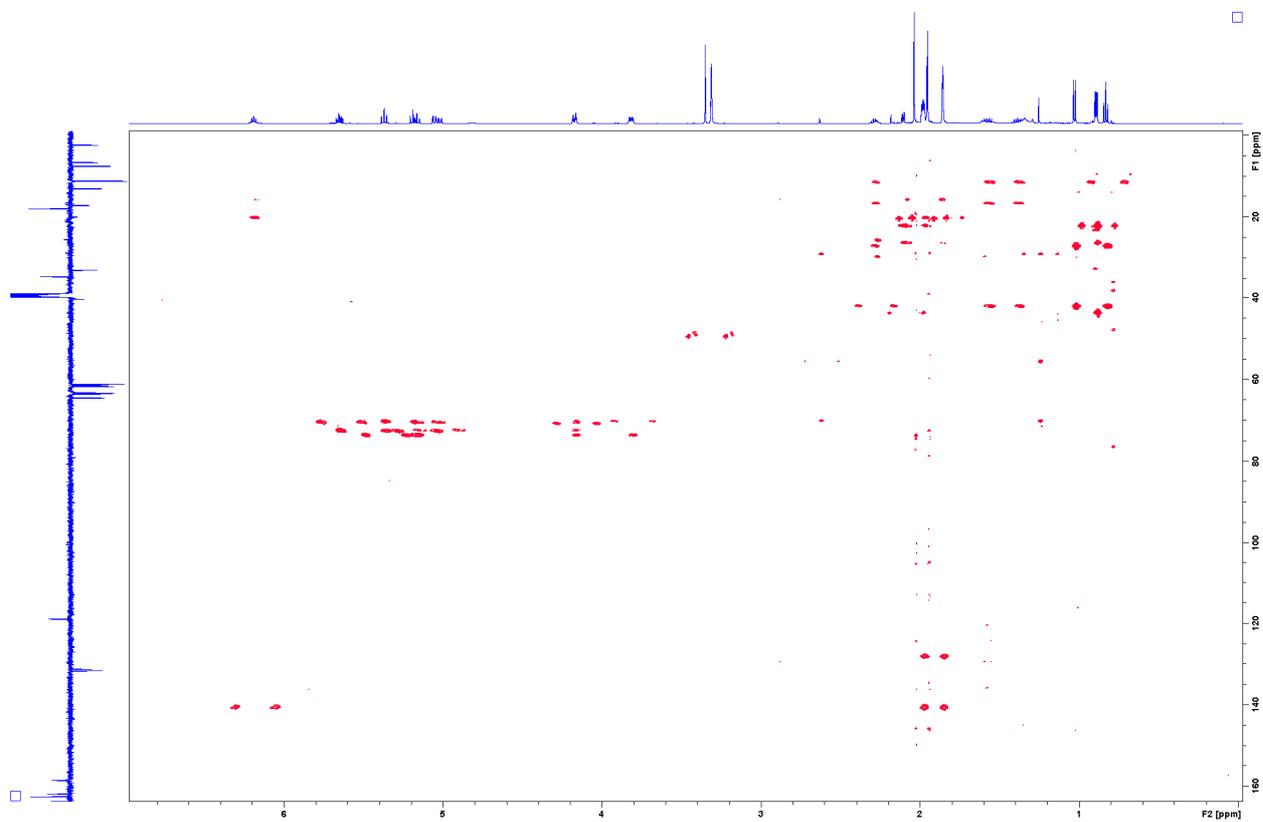




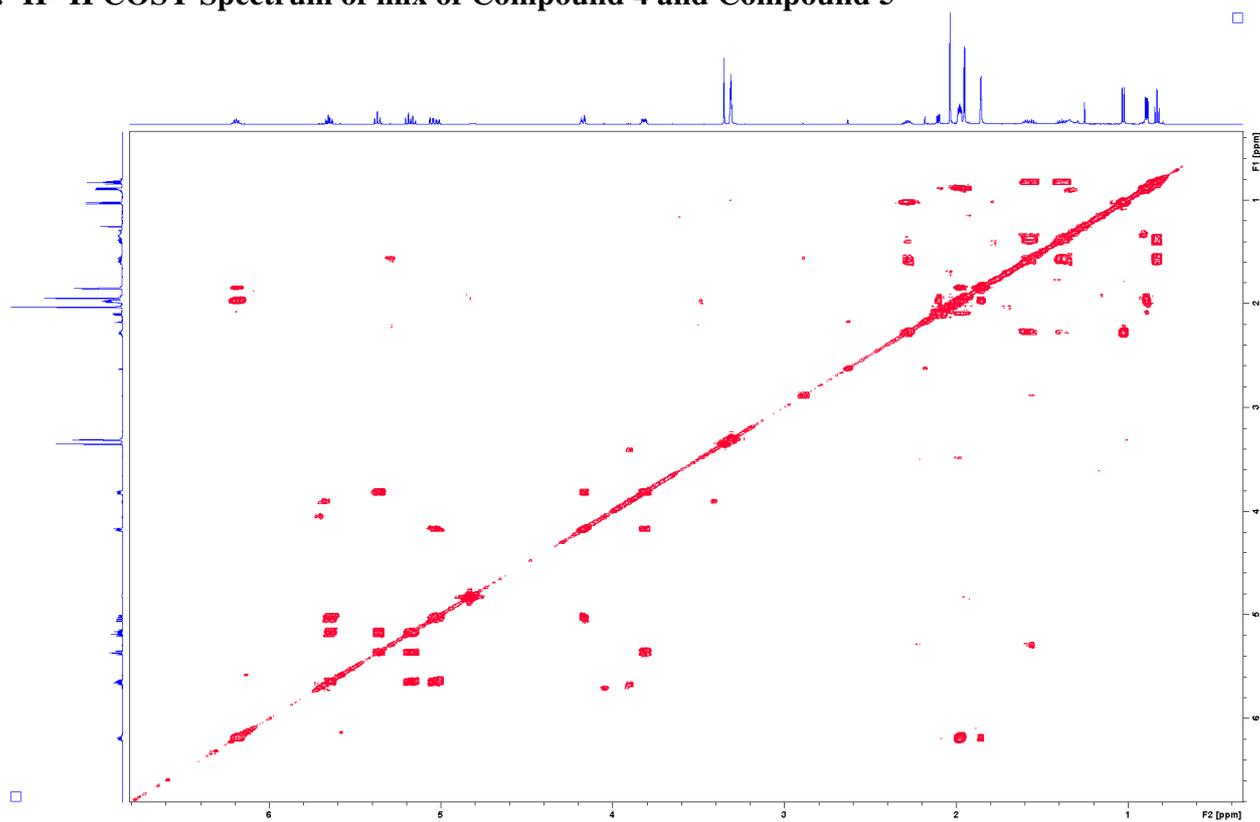
S25. HSQC Spectrum of mix of Compound 4 and Compound 5



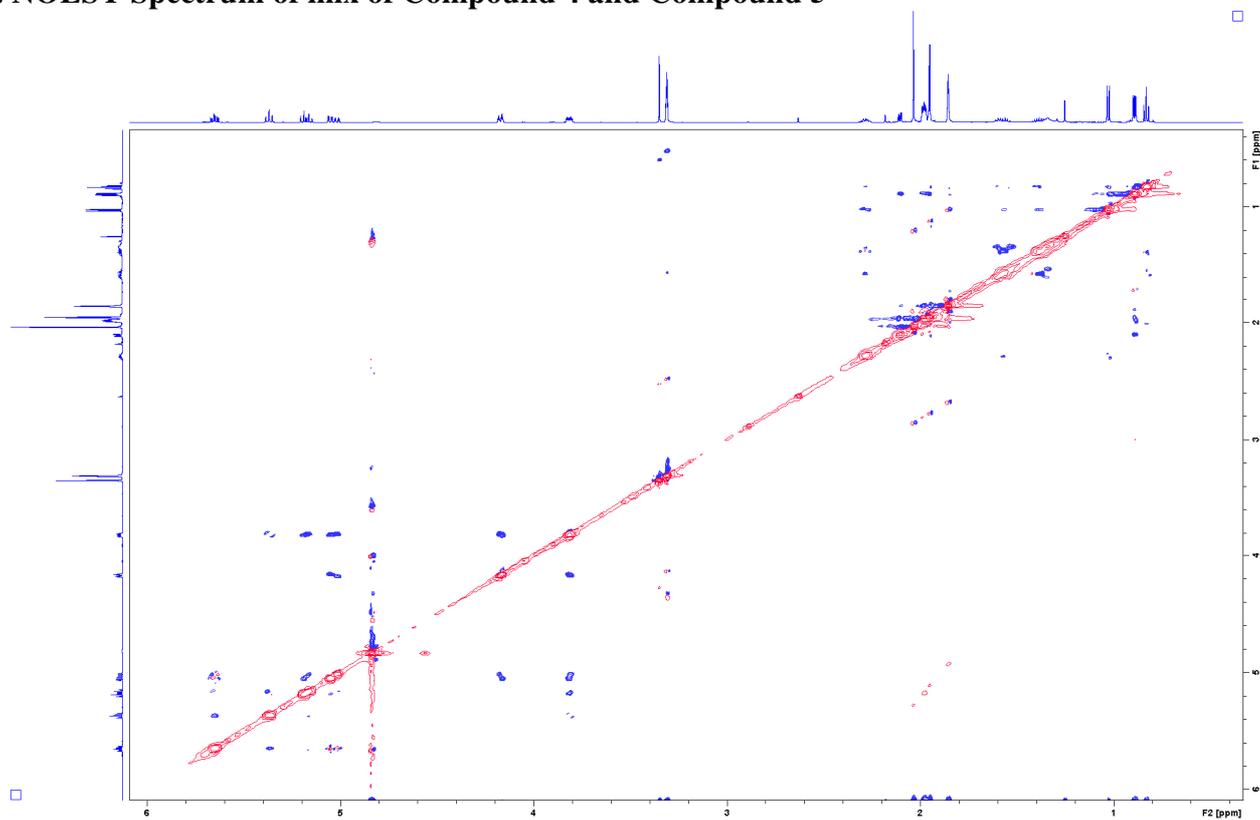
S26. HMBC Spectrum of mix of Compound 4 and Compound 5



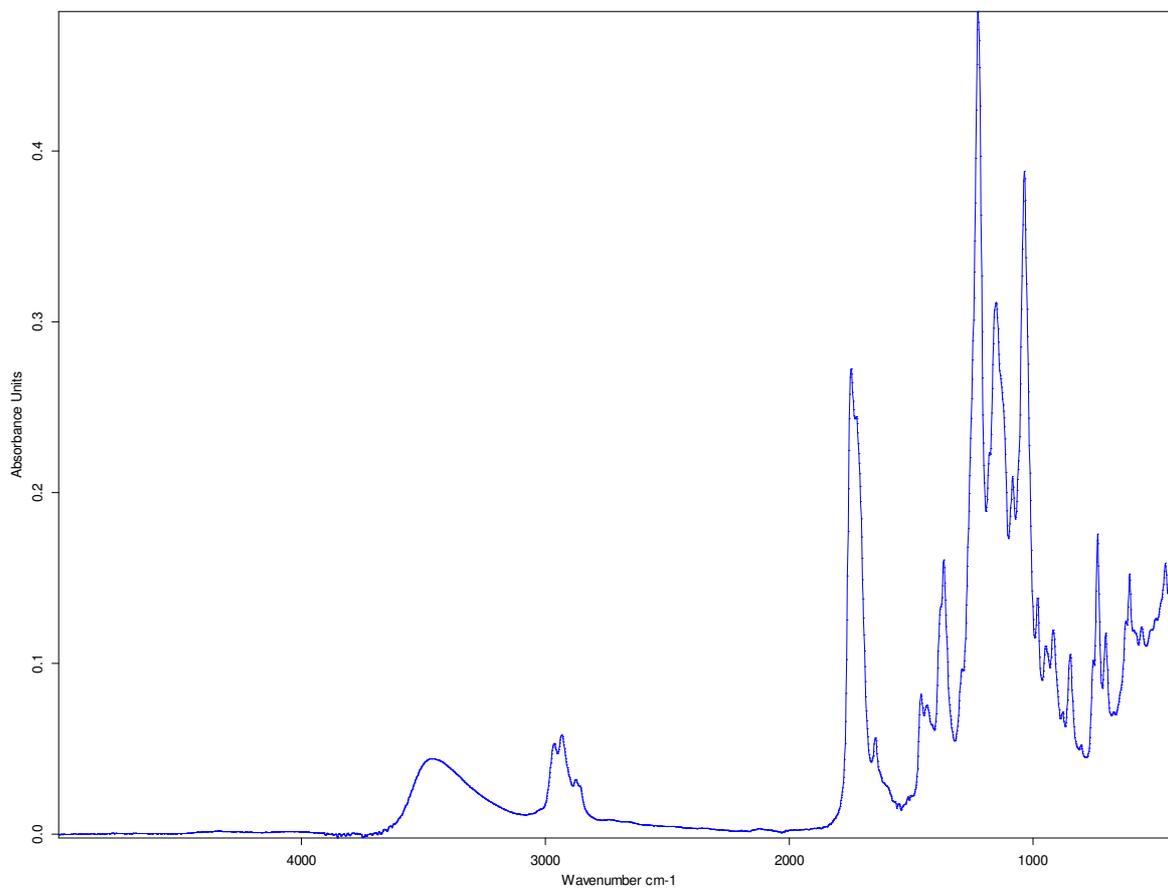
S27. ^1H - ^1H COSY Spectrum of mix of Compound 4 and Compound 5



S28. NOESY Spectrum of mix of Compound 4 and Compound 5

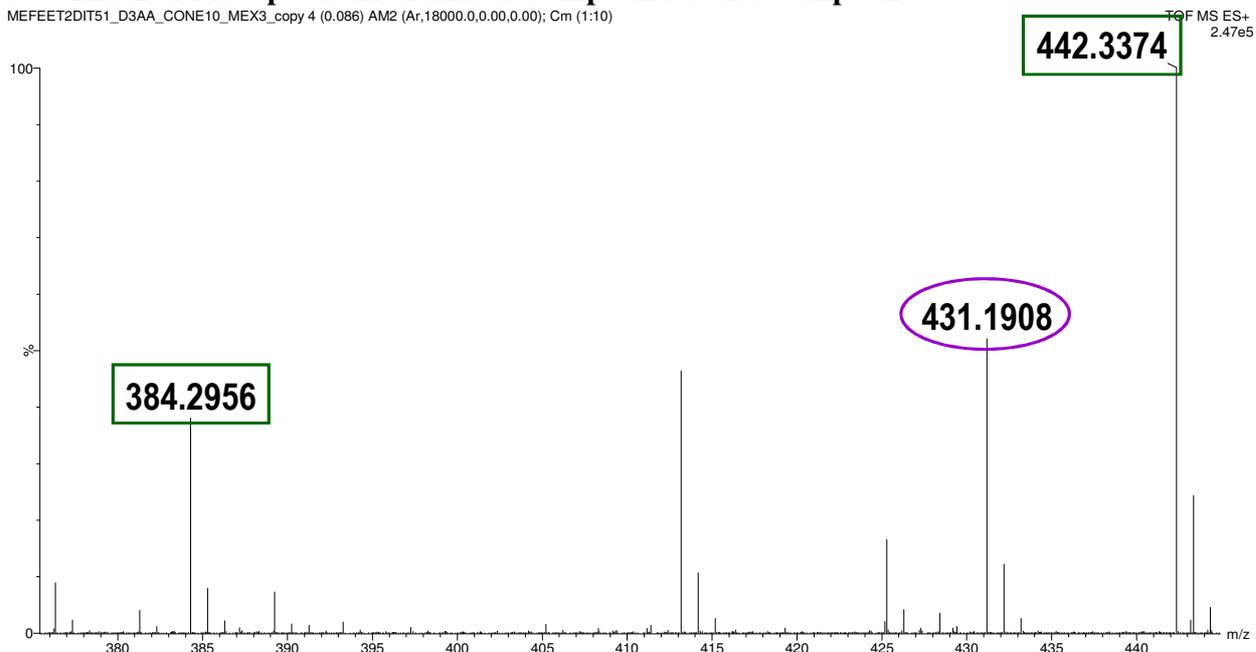


S29. IR Spectrum of mix of Compound 4 and Compound 5



S30. HR-ESI-MS Spectrum of mix of Compound 4 and Compound 5

MEFEET2DITS1_D3AA_CONE10_MEX3_copy 4 (0.086) AM2 (Ar,18000.0,0.00,0.00); Cm (1:10)



		m/z	error (mDa)	error (ppm)
Teoretical values		431.1912	-	-
Experimental values	1	431.1907	-0.5	-1.2
	2	431.1907	-0.5	-1.2
	3	431.1908	-0.4	-0.9