

# Supplementary Materials

## Chemical Investigation of Diketopiperazines and N-Phenethylacetamide Isolated from *Aquimarina* sp. MC085 and Their Effect on TGF- $\beta$ -Induced Epithelial–Mesenchymal Transition

Myong Jin Lee <sup>1,†</sup>, Geum Jin Kim <sup>2,3,†</sup>, Myoung-Sook Shin <sup>1</sup>, Jimin Moon <sup>2</sup>, Sungjin Kim <sup>1</sup>, Joo-Won Nam <sup>2</sup>, Ki Sung Kang <sup>1,\*</sup> and Hyukjae Choi <sup>2,3,\*</sup>

<sup>1</sup> College of Korean Medicine, Gachon University, Seongnam 13120, Korea; myongene@naver.com (M.J.L.); ms.shin@gachon.ac.kr (M.-S.S.); qkrnsld@naver.com (S.K.)

<sup>2</sup> College of Pharmacy, Yeungnam University, Gyeongsan 38541, Korea; kimgumjin@naver.com (G.J.K.); hyp1112@yu.ac.kr (J.M.); jwnam@yu.ac.kr (J.-W.N.)

<sup>3</sup> Research Institute of Cell Culture, Yeungnam University, Gyeongsan 38541, Korea

\* Correspondence: [kkang@gachon.ac.kr](mailto:kkang@gachon.ac.kr) (K.S.K.); [h5choi@yu.ac.kr](mailto:h5choi@yu.ac.kr) (H.C.); Tel.: +82-31-750-5402 (K.S.K.); +82-53-810-2824 (H.C.)

† These authors contributed equally to this work.

## General Experimental Procedure

**Figure S1.** LR-ESI-MS data of **1**

**Figure S2.**  $^1\text{H}$  NMR spectrum (250 MHz) of **1** in  $\text{DMSO-}d_6$

**Figure S3.**  $^{13}\text{C}$  NMR (63 MHz) spectrum of **1** in  $\text{DMSO-}d_6$

**Figure S4.** HMQC spectrum of **1** in  $\text{DMSO-}d_6$

**Figure S5.** HMBC spectrum of **1** in  $\text{DMSO-}d_6$

**Figure S6.**  $^1\text{H}$  NMR spectrum (600 MHz) of **1** in  $\text{CD}_3\text{OD}$

**Figure S7.**  $^{13}\text{C}$  NMR (150 MHz) spectrum of **1** in  $\text{CD}_3\text{OD}$

**Figure S8.** C3 Marfey's analysis of **1**

**Figure S9.** LR-ESI-MS data of **2**

**Figure S10.**  $^1\text{H}$  NMR spectrum (250 MHz) of **2** in  $\text{CD}_3\text{OD}$

**Figure S11.**  $^{13}\text{C}$  NMR (63 MHz) spectrum of **2** in  $\text{CD}_3\text{OD}$

**Figure S12.** C3 Marfey's analysis of **2**

**Figure S13.** LR-ESI-MS data of **3**

**Figure S14.**  $^1\text{H}$  NMR spectrum (250 MHz) of **3** in  $\text{CDCl}_3$

**Figure S15.**  $^{13}\text{C}$  NMR (63 MHz) spectrum of **3** in  $\text{CDCl}_3$

## **General Experimental Procedure**

Optical rotation was measured using a Jasco DIP-1000 polarimeter (Tokyo, Japan). Nuclear magnetic resonance (NMR) spectra were recorded using a 250 MHz Bruker NMR spectrometer (DMX 250) and 600 MHz Varian NMR spectrometer (VNS-600, Palo Alto, CA, USA) at the Core Research Support Center for Natural Products and Medical Materials (CRCNM). Low-resolution electrospray ionization MS (LR-ESI-MS) was performed using an Agilent 6120 single-quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) with a C3 column (Agilent SB-C3 Zorbax, 5  $\mu$ m, 4.6  $\times$  150 mm). Isolation of the compounds was carried out using a Waters 1525 binary high-performance liquid chromatography (HPLC) pump with a Waters 996 photodiode array (PDA) with a reversed-phase HPLC (RS Tech, Hector-M 5 $\mu$ m C18, 250  $\times$  4.6 mm).

## **Fermentation of *Aquimarina* sp. MC085 and Preparation of Extracts**

A bacterial strain *Aquimarina* sp. MC085 (GenBank accession no. MG016025) was incubated at 25°C with shaking incubator at 150 rpm in 5 L of SYP media. After 7 days, the broth media of strain MC085 was extracted twice with ethyl acetate and the combined extract were evaporated.

### Fractionation and Isolation of Compounds 1-3

The dried crude extract (1,116 mg) was separated into seven fraction by silica-gel small column chromatography using step-gradient eluent mixture of dichloromethane and methanol (from 100:0 to 0:100). Fraction 5 (210.5 mg) was isolated by reversed phase HPLC (HECTOR-M C18 5 $\mu$ , 250  $\times$  4.6 mm) with a stepwise gradient of MeOH : H<sub>2</sub>O (from 25 : 75 to 50 : 50) to afford *cyclo*(L-Pro-L-Leu) (**1**, 3.5 mg), *cyclo*(L-Pro-L-Ile) (**2**, 7.0 mg), and *N*-phenethylacetamide (**3**, 2.5 mg).

### Acid Hydrolysis and C3 Marfey's Analysis of Compounds 1 and 2

Each compound (100  $\mu$ g) was dissolved in 6 N HCl (400  $\mu$ L) in 4 mL-glass vials, respectively. These vials were incubated at 110  $^{\circ}$ C for 4 hours with stirring. The resulting acid hydrolysates were dried under N<sub>2</sub> gas stream. The dried acid hydrolysates were resuspended in deionized water. After removal of water under N<sub>2</sub> gas stream, the hydrolysates were dissolved in 40  $\mu$ L of MeOH and treated with 60  $\mu$ L of 1.0 M NaHCO<sub>3</sub> and 25  $\mu$ L of 1% L-FDAA (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) in acetone. The reaction mixtures in each vial were incubated at 40  $^{\circ}$ C for 1 hour with stirring, and the reaction was quenched with addition of 60  $\mu$ L of 1 N HCl. The resulting solutions (10  $\mu$ L) were injected to LC-ESI-MS; Agilent Zorbax SB-C<sub>3</sub> column, 5  $\mu$ m, 150  $\times$  4.6 mm, 50  $^{\circ}$ C, 1.0 mL/min, solvent A(deionized water), solvent B [95% MeOH + 5% of (95% CH<sub>3</sub>CN + 5% formic acid)], A:B=70:30  $\rightarrow$  45:55 (95 min)  $\rightarrow$  0:100 (97

min) → 0:100 (104 min) → 70:30 (105 min) → 70:30 (110 min). The reaction products with FDAA-derivatized hydrolysates were analyzed on a negative mode of a LC-MS system. The retention time of the DAA-derivatives of constituting amino acids were compared with those of the authentic standards (DAA-L-Pro : 15.5 min, DAA-D-Pro : 19.0 min, DAA-L-*allo*-Ile : 39.5 min, DAA-L-Ile : 41.8 min, DAA-L-Leu : 42.6 min, DAA-D-*allo*-Ile : 61.7 min, DAA-D-Ile : 64.5 min, DAA-D-Leu : 65.0 min).

### **Cell Culture and Treatment**

A549 (Human lung carcinoma epithelial) cells were incubated in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum and 1% penicillin–streptomycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. The cells were then treated with compounds **1-3**, respectively, in medium for 6 hours. This was followed by treatment with TGF-β (5 ng/mL) for 42 hr in the presence and absence of compounds at indicated concentrations.

### **Cell Viability Assay**

An EZ-Cytox assay kit from DoGenBio (Seoul, Korea) was used for cell viability. Cells ( $5 \times 10^4$  cells/well) were split in a 96-well plate. After 24hours, compounds **1-3** were added to serum free medium for 6 hr before TGF-β (5 ng/mL) stimulation. EZ-Cytox reagent was added to the plate and incubated for 1hr at 37°C. The absorbance at 450 nm was recorded using an enzyme-linked immunosorbent assay (ELISA) plate reader.

### **Western Blotting Analysis**

A549 cells were treated with compounds 1-3 as previously above. After 6 hours, 5 ng/mL of TGF- $\beta$  was treated with or without each peptide for 42 hours. Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.25% deoxycholate, 1% NP-40, and 1 mM EDTA) and protein concentration was determined by BCA reagent (ThermoScientific, Rockford, IL, USA) as prescribed by the manufacturer's instructions. Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, USA). After transfer, membranes were blocked with 5% nonfat dried milk/TBST (20 mM Tris pH 7.4, 150 mM NaCl, and 0.2% Tween-20) for 1 hr and then incubated overnight at 4°C with primary antibody. The antibodies used included anti-smad2/3, phosphorylated smad2/3, N-cadherin, E-cadherin, Vimentin, Snail,  $\beta$ -catenin and GAPDH from Cell Signaling Technology (USA). Following wash with TBST, the membranes were probed with HRP-labeled secondary antibodies. Finally, an enhanced chemiluminescence detection kit (Fisher Scientific, Rockford, IL, USA) was used to visualize the immunoreactive proteins.

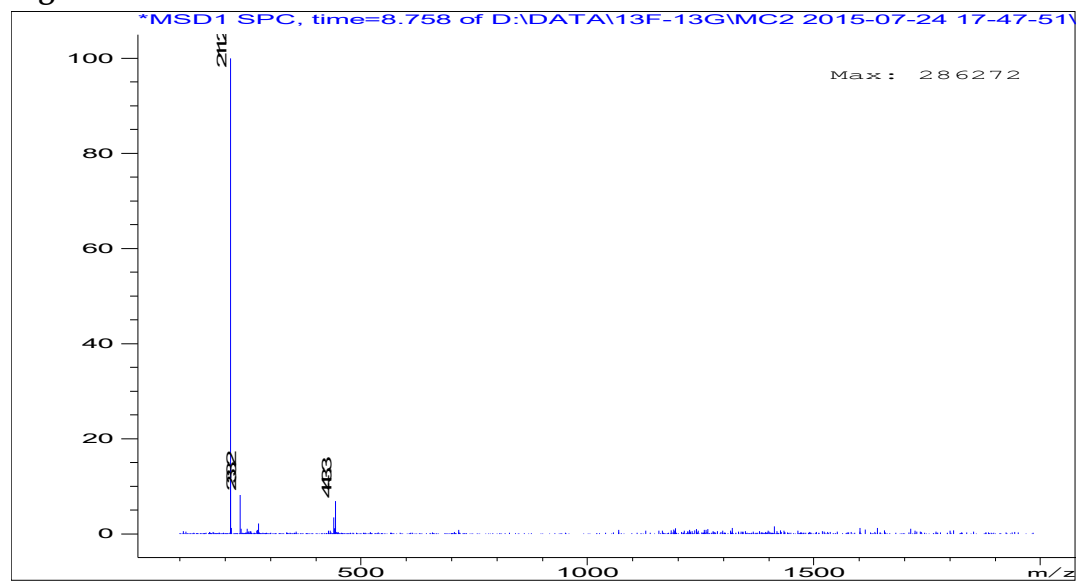
### **Gelatin Zymography**

Cell supernatants of serum-free cultures were concentrated using Amicon Ultra-4 Centrifugal

Filter Devices (Millipore, Billerica, MA, USA). MMP-2 activity was determined by Zymogram-PAGE System (Komabiotek, Seoul, Korea) with the manufacturer's

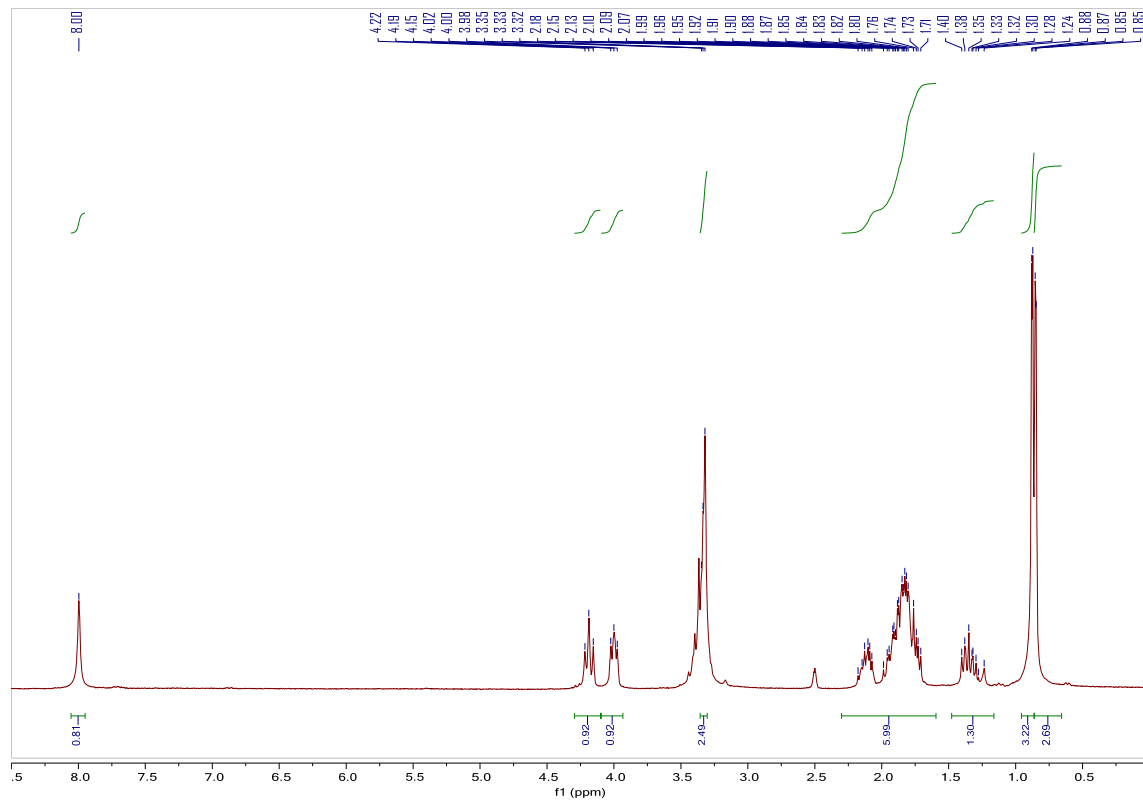
instructions. In brief, after electrophoresis, the gel was washed to remove the SDS for 30 min and then incubated overnight at 37°C with developing buffer. Then the gel was stained with Coomassie Blue R-250 and destained with methanol and acetic acid. The gelatinolytic activities were then visualized as a white band and the gels were scanned.

**Figure S1.** LR-ESI-MS data of **1**

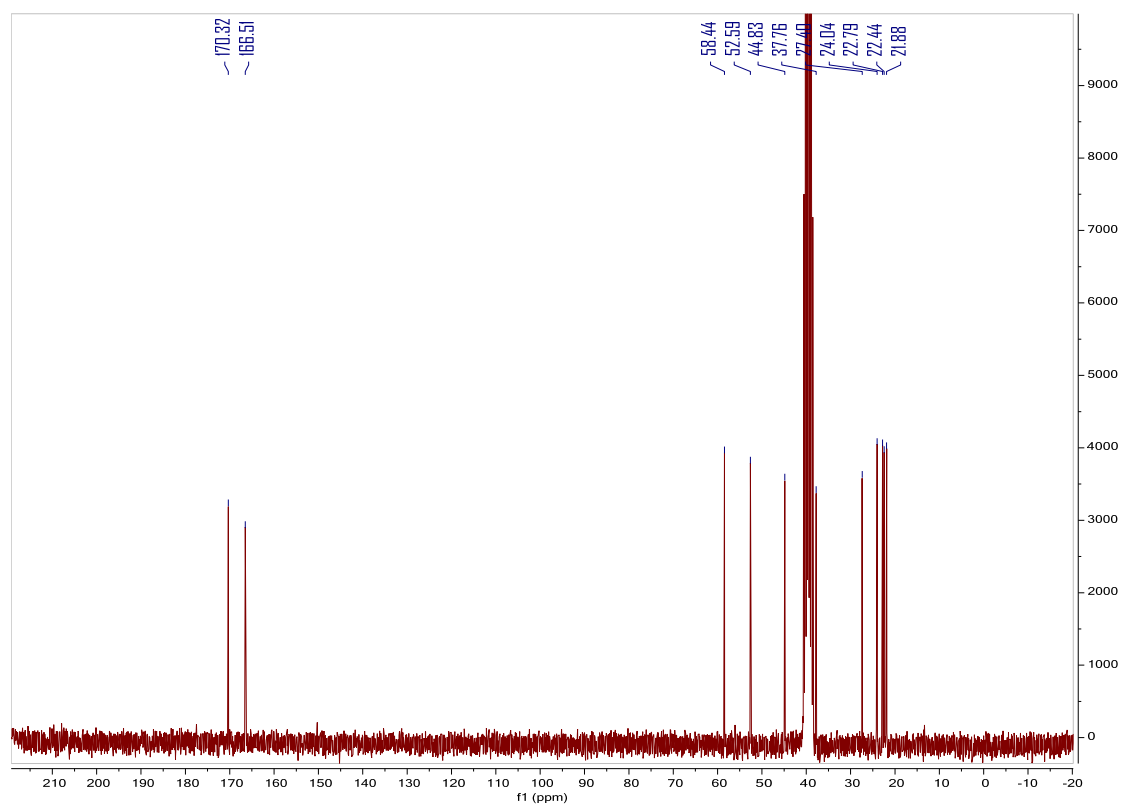




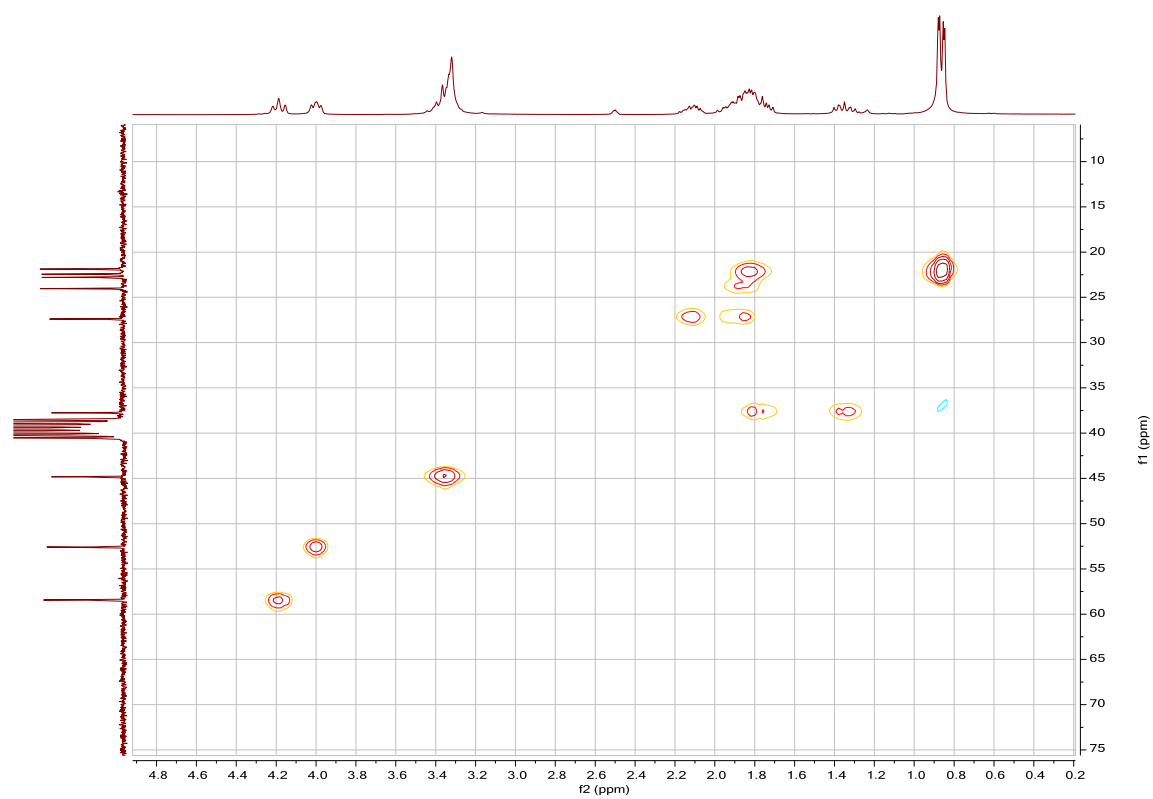
**Figure S2.**  $^1\text{H}$  NMR spectrum (250 MHz) of **1** in  $\text{DMSO-}d_6$



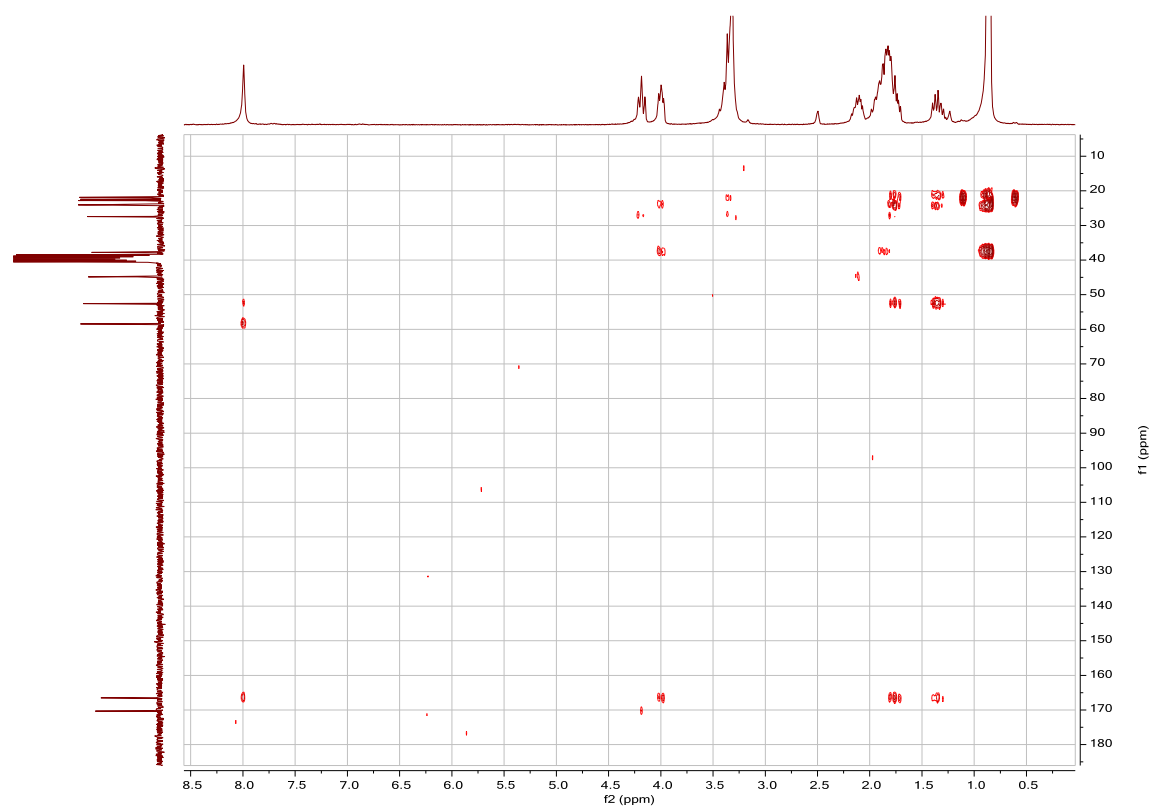
**Figure S3.**  $^{13}\text{C}$  NMR (63 MHz) spectrum of **1** in  $\text{DMSO-}d_6$



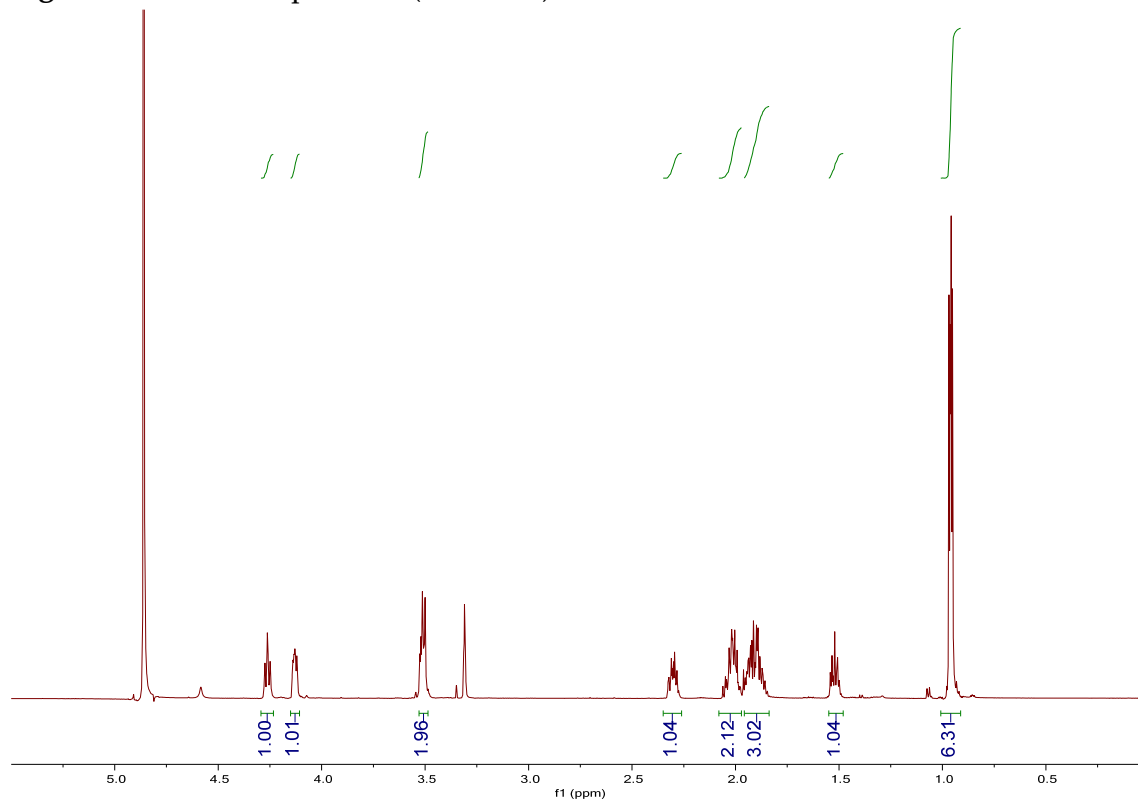
**Figure S4.** HMQC spectrum of **1** in DMSO- $d_6$



**Figure S5.** HMBC spectrum of **1** in DMSO- $d_6$



**Figure S6.**  $^1\text{H}$  NMR spectrum (600 MHz) of **1** in  $\text{CD}_3\text{OD}$



**Figure S7.**  $^{13}\text{C}$  NMR spectrum (150 MHz) of **1** in  $\text{CD}_3\text{OD}$

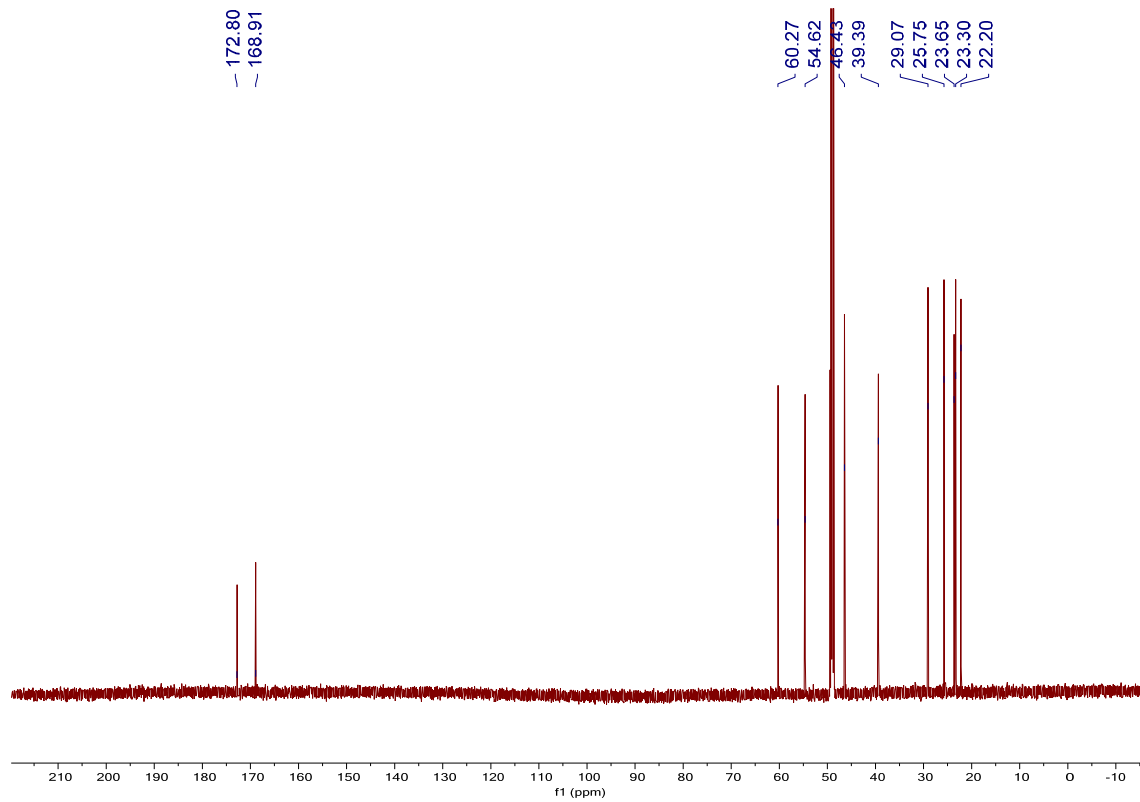
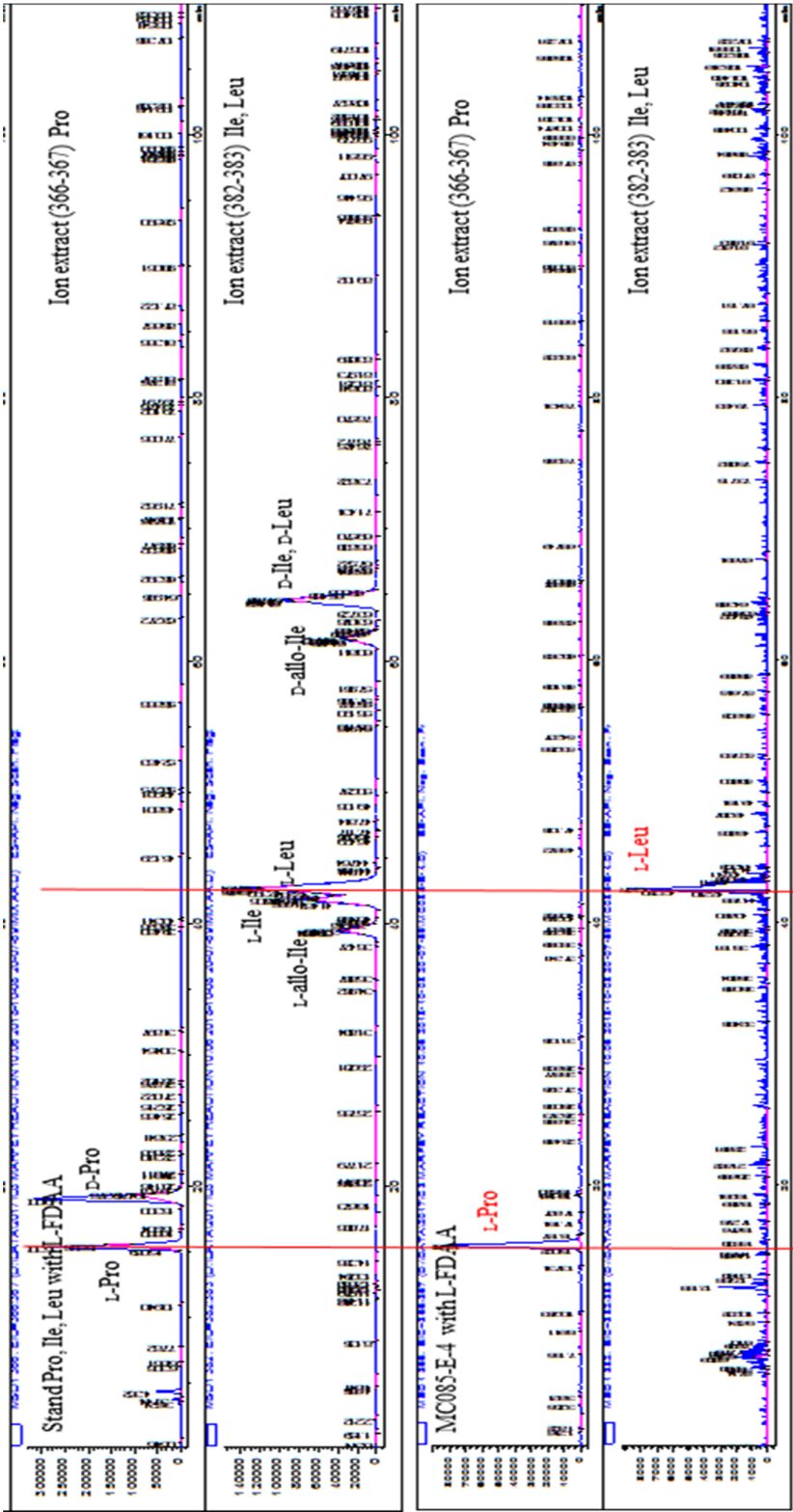
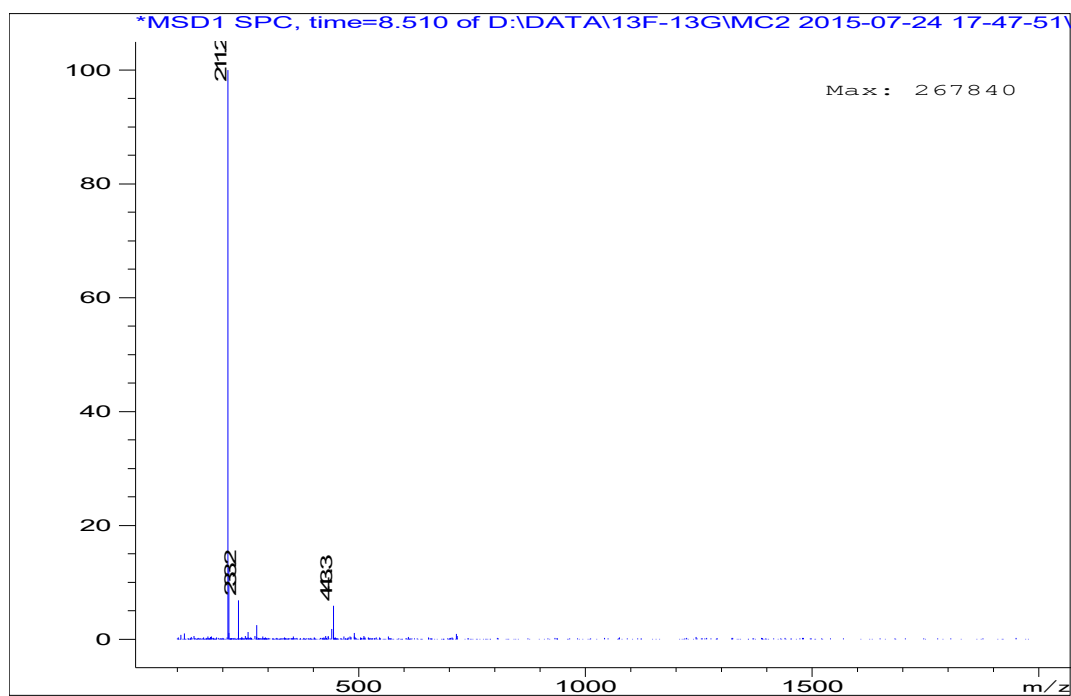


Figure S8. C3 Marfey's analysis of 1

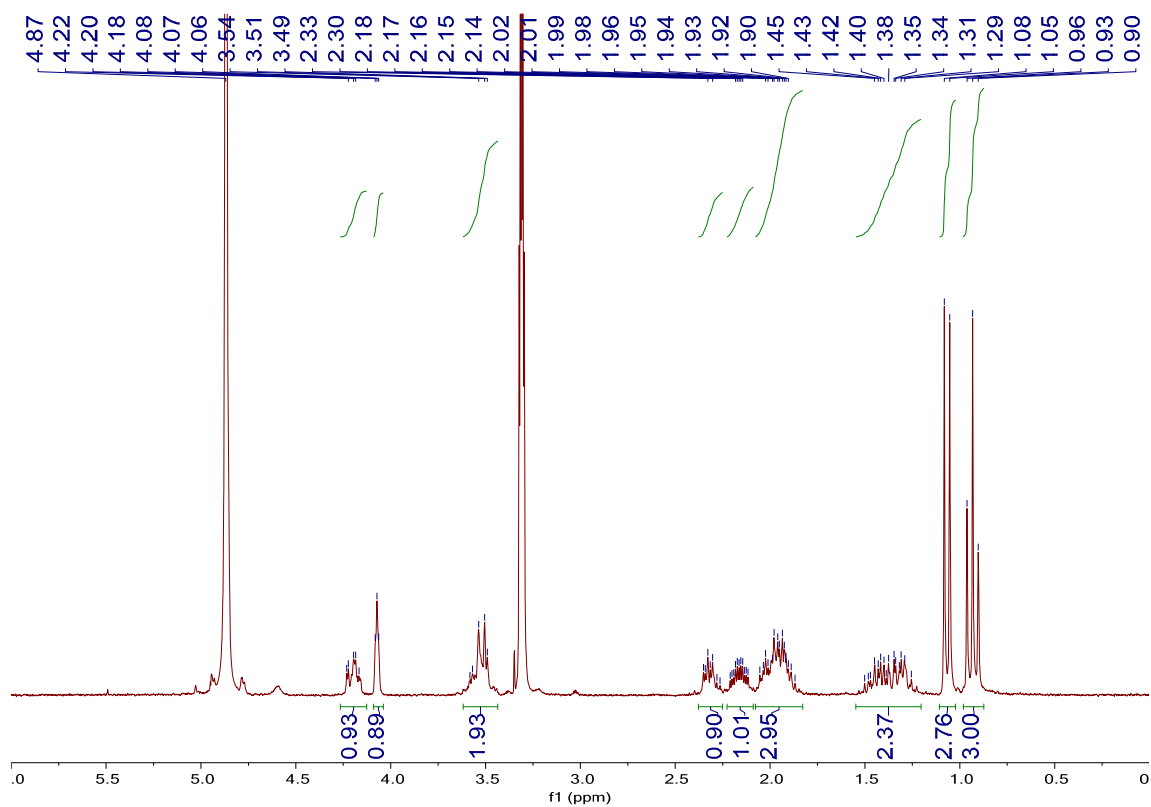


**Figure S9.** LR-ESI-MS data of **2**





**Figure S10.**  $^1\text{H}$  NMR spectrum (250 MHz) of **2** in  $\text{CD}_3\text{OD}$



**Figure S11.**  $^{13}\text{C}$  NMR spectrum (63 MHz) of **2** in  $\text{CD}_3\text{OD}$

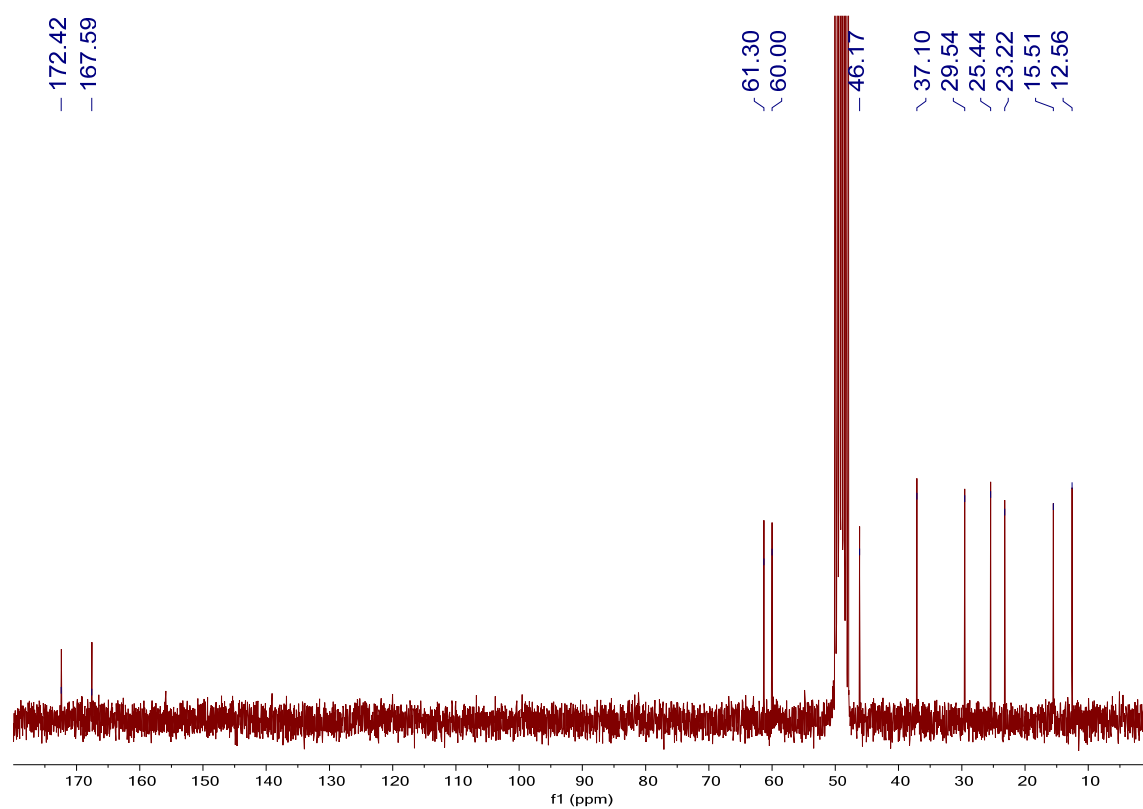
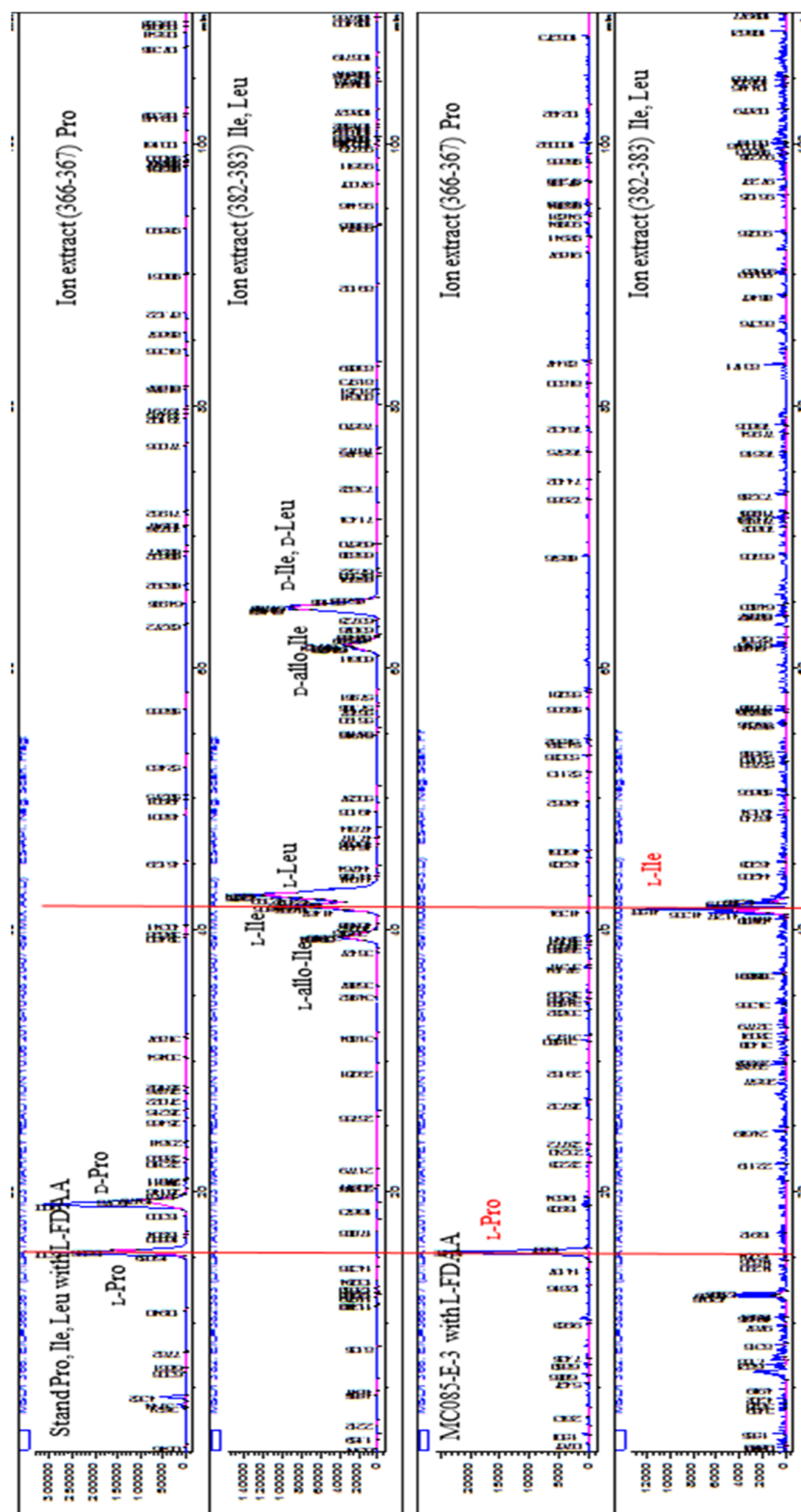
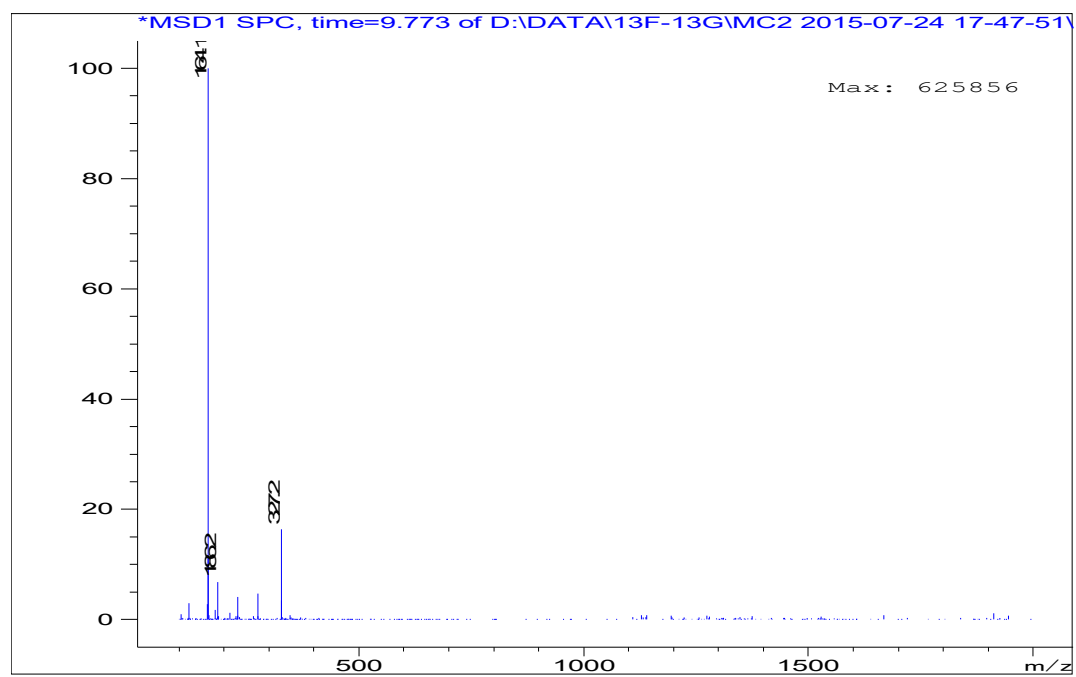


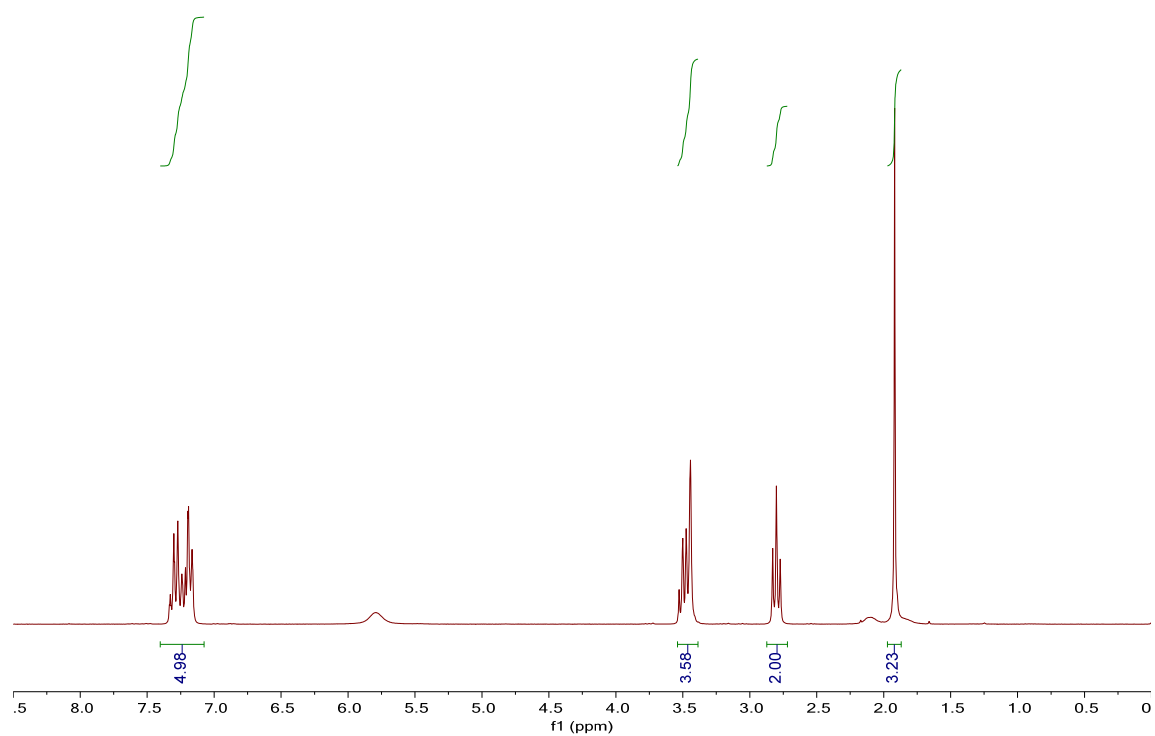
Figure S12. C3 Marfey's analysis of 2



**Figure S13.** LR-ESI-MS data of 3



**Figure S14.**  $^1\text{H}$  NMR spectrum (250 MHz) of **3** in  $\text{CDCl}_3$



**Figure S15.**  $^{13}\text{C}$  NMR spectrum (63 MHz) of **3** in  $\text{CDCl}_3$

