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Studies on Antibacterial Secondary Metabolites from Marine-Derived *Streptomyces* sp. MS101

해양방선균 *Streptomyces* sp. MS101 유래의 항균활성물질 연구

2014 년 8 월

서울대학교 대학원

농생명공학부 응용생명화학전공

박 하 영

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지도 교수 오 기 봉

이 논문을 농학석사 학위논문으로 제출함
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Abstract

Studies on Antibacterial Secondary Metabolites from Marine-Derived *Streptomyces* sp. MS101

Park, Hayoung
Department of Agriculture Biotechnology
The Graduate School
Seoul National University

Microorganisms such as fungi and bacteria secrete antibiotics to kill of inhibit the growth of other microorganisms. Genus *Streptomyces*, belonging to Gram-positive bacteria, comprises the main portion of microorganism which produces natural antibiotics.

To investigate bioactive compounds from marine actinomycetes, 373 isolates were obtained from near-shore

sediment sample collected in Micronesia in December 2011. A marine actinomycete with antibacterial activity was adopted for this study. By 16S rRNA gene sequence analysis, this strain was identified as a novel strain of *Streptomyces* genus. It named as *Streptomyces* sp. MS101.

The isolate, labeled as strain MS101, was cultured in 6 normal actinomycete liquid media. Significant antibacterial activity was observed in GTYB medium. Therefore, *Streptomyces* sp. MS101 was fermented in large scale (98 L) in GTYB media at 28℃ with shaking at 130 rpm for 2 weeks. The culture broth was concentrated, extracted with MeOH, and sequentially fractionated with organic solvents. From the EtOAc fraction, compound **1-4** were isolated by variety chromatographic methods, such as flash column chromatography, open column chromatography, and high performance liquid chromatography.

Structure elucidation of compound **1-4** was achieved by mass spectrometry and nuclear magnetic resonance spectroscopy. On the basis of the combined spectroscopic analysis, compound **1-4** were elucidated as Benzenacetamide, Sch 538415, Deoxynybomycin, and Cyclo(L-Pro-L-Phe), respectively. These compounds were already reported previously by other researchers.

To determine the bioactivity of the purified compounds, minimum inhibitory concentration (MIC) assay and enzyme

inhibition assay were performed. In the antimicrobial activity assays, compounds **2** and **3** showed significant antibacterial activities against both Gram-positive and Gram-negative bacteria with MIC values in the range from 0.20 to 6.25 μg/mL. However, compound **1-4** did not show antifungal activity. Inhibitory activity toward several target enzymes related to antimicrobial activities (isocitrate lyase, sortase A) was evaluated. Compounds **1**, **3**, and **4** have weak inhibitory activities to isocitrate lyase. Compound **2** has weak inhibitory activities to sortase A of *Streptococcus mutans.*

These findings indicate that the relatively under investigated marine-derived *Streptomyces* have great potential for the discovery of bioactive small molecules.

Keywords: marine actinomycete, *Streptomyces,* natural product, antibacterial activity, enzyme inhibition

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List of Abbreviation

Aq.	aqueous
Amp	ampicillin
BLAST	basic local alignment search tool
$CDCl_3$	deuterated-chloroform
CFU	colony forming unit
COSY	correlation spectroscopy
CV	column volume
DC	dichloromethane, methylene chloride
DEPT	distortionless enhancement by polarization transfer
DIP	direct insertion probe
DMSO	dimethyl sulfoxide
DW	distilled water
EIMS	electron impact mass spectrometry (low resolution)
ESIMS	electro spray ionization mass spectrometry (low resolution)
EtOAc	ethyl acetate
FCC	flash (column) chromatography
GTYB	glucose, tryptone, yeast extract, beef extract mixed media
GTYB70	glucose, tryptone, yeast extract, beef extract, sea salt (70% of sea) mixed media
HPLC	high performance liquid chromatography
HRESIMS	high resolution electro spray ionization mass spectrometry

HMBC	heteronuclear multiple bond correlation
HSQC	heteronuclear single quantum coherence
ICL	isocitrate lyase
IC_{50}	the half maximal inhibitory concentration
LCMS	liquid chromatography mass spectrometry
MeOH	methanol
MIC	minimal inhibitory concentration
MS	mass spectrometry
n-Hx	normal-hexane
NCBI	national center for biotechnology information
NP	normal phase
NMR	nuclear magnetic resonance
$O.D._{600}$	optical density at wavelength 600 nm
OPC	open column chromatography
pHMB	*p*-hydroxymecuribenzoic acid
R_f	retention factor
RI	refractive index
RP	reverse phase
RPM	revolution per minute
SrtA	sortase A
TLC	thin layer chromatography

Ⅰ. Introduction

The term "antibiotic," defined in 1942 by Selman A. Waksman, was originally referred to any microbial product antagonistic to the growth of another microorganism (1). Today, "antibiotic" describes any compound that kills (bactericidal) or inhibits the growth (bacteriostatic) of bacteria.

Of the 12 antibacterial classes, nine are derived from a natural product template. The molecular architectures of the β-lactams (penicillins, cephalosporins, carbapenems, monobactams), polyketides (tetracycline), phenylpropanoids (chloramphenicol), aminoglycosides (streptomycin), macrolides (erythromycin), glycopeptides (vancomycin), streptogramins (pristinamycin), and, most recently, the lipopeptides (daptomycin) and glycylcyclines (tegicycline) are borrowed from natural products. The other three classes the sulfonamides, quinolones (ciprofloxacin), and oxazolidinones (linezolid) have no precedence in Nature. Their design is purely synthetic and not influenced by structural elements of known metabolites (2).

Soil is a natural reservoir for microorganisms and their antimicrobial products(3). Historically, the study of natural products has focused upon the elaborate biosynthetic pathways of terrestrial plants and microorganisms. In the late 1960s,

however, the search for novel metabolites took a new direction as the realm of exploration expanded to include plants and animals in the sea (4). Marine microbes have shown tremendous potential as producers of novel natural products with important biomedical roles (5). The production of natural products by marine organisms appears to be a response to various ecological pressures within the environment (6).

Actinomycetes are Gram-positive bacteria that are wide spread in nature and play a pivotal role in the production of bioactive metabolites (7). Among the genera of actinomycetes, the genus *Streptomyces* is represented in nature by the largest number of species and varieties, which differ greatly in their morphology, physiology, and biochemical activities. Interestingly, the majority of the antibiotic-producing actinomycetes are found among these species, which led to a growing economic importance for this group of organisms (8).

Marine *Streptomyces* occur in different biological sources such as fishes, molluscs, sponges, seaweeds and mangroves, besides seawater and sediments (9). Isolation of *streptomyces* from marine sediments and suggested that the isolated *streptomyces* may be valuable for the production of antibiotics (10).

Streptomyces have been the most fruitful source of microorganisms for all types of bioactive metabolites that have important applications in human medicine and in agriculture

(11). Active molecules of *Streptomyces* species are generally extracellular and their isolation in highest purity from the complex fermentation broth needs the application of a combination of various separation steps such as solvent extraction, chemical precipitation, ion exchange chromatography, HPLC purification (12).

Natural products having novel structures have been observed to possess useful biological activities. For more than two decades, clinicians and public health officials have faced hospital acquired methicillin-resistant *Staphylococcus aureus* (MRSA), which also bears resistance too many antibiotics. During much of this time, vancomycin has been the therapeutic answer to MRSA, but that paradigm has changed. Vancomycin-resistant strains have emerged clinically (13). Search for new antibiotics effective against multi-drug resistant pathogenic bacteria is presently an important area of antibiotic research.

Also, search for inhibitor that targets bacterial protein is important. There are lots of synthetic inhibitors but, in many cases, these inhibitors are not pharmacologically suitable for testing *in vivo* because of their toxicity and low activity. Therefore, the potent of natural product compounds as inhibitor on pathogen protein is important. Isocitrate lyase (ICL) plays a major role in virulence bacteria. ICL is an enzyme that transforms isocitrate into glyoxylate in the glyoxylate cycle (14). It is well known that the glyoxylated cycle operates in

bacteria, fungi, some protein and plants (15-17). Since expression of glyoxylate cycle genes is detected during specific stages of the interaction between host and pathogen in a variety of human-pathogenic bacteria and fungi, the development of specific inhibitors against ICL is an attractive prospect (18-20). Sortase A plays a major role in Gram-positive bacteria, it has been known for some time that cell-surface proteins can covalently attach to peptidoglycan (21). It also plays a key role in the adhesion to and invasion of hosts (22). Especially, *Streptococcus mutans* is a Gram-positive oral bacterium responsible for human dental caries (23). Sortase A enzyme is responsible for sorting and anchoring surface proteins to the cell wall of *S. mutans* (24). The adherence of bacteria to the dental surfaces is the first step in the development of the complex biofilm community that constitutes dental plaque (25). Therefore, this enzyme could be a target for the prevention of dental caries.

In this study, the main purpose is identifying compounds which show antibacterial activity. The purposes of this study more in details are to isolate and identify MS101 strain, to screen proper culture media for *Streptomyces* sp. MS101, and to purify antibacterial secondary metabolites which inhibit the growth of *Proteous hauseri*, Gram-negative bacteria, from *Streptomyces* sp. MS101 fermented media. After isolating antibacterial secondary metabolites, structure elucidation will

be performed. Some bioactivities, antimicrobial activity against bacterial strain and fungal strain will be checked. In continuation for the discovery of inhibitors of enzymes from natural products, enzyme inhibitory activities about *Candida albicans* isocitrate lyase (ICL) and *Streptococcus mutans* sortase A (SrtA) will be evaluated.

Ⅱ. Materials and Methods

1. Isolation of marine actinomycetes

The strain MS101 was isolated from near-shore sediment collected in Micronesia in December 2011. To isolate MS101 from marine samples, two different media were used: (i) HV agar medium consisted of 1 g of humic acid dissolved in 10 mL of 0.2 N NaOH, 0.5 g of Na_2HPO_4, 1.7 g of KCl, 0.05 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.02 g of $CaCO_3$, B vitamins (0.5 mg each of thiamine-HCl, riboflavin, niacin, pyridoxine-HCl, inositol, Ca-pantothenate, p-aminocenzoic acid and 0.25 mg of biotin), 18 g of agar, and 1 L of distilled water (26), (ii) Actinomycete isolation agar medium consisted of 22 g of Himedia Actinomycete Isolation Agar (Himedia, Mumbai, India), 5 mL of glycerol, and 1 L of distilled water. 50 mg of cycloheximide was added to both of the media after sterilization .

2. Identification of MS101

Total genomic DNA of MS101 was extracted by using a i-genomic BYF DNA mini kit (intron biotech, Korea). A region of 1518 base pair from the 16S rRNA gene was amplified by using the primers F27 (5' -AGAGTTTGATCMGGCTCAG-3') and R1525 (5' -AAGGAGGTGWTCCARCC-3') (27). Amplified 16S rRNA gene was cleaned by using PCR product Purification kit (intron biotech, Korea) and cloned into pGEM-T Easy vector (Promega Corp., Madison, Wisconsin, USA) following manufacturer' s instruction. Sequence of 16S rRNA gene was analyzed by an Applied Biosystem model 373A automatic sequencer and a BigDye Terminator Cycle Sequencing kit (Perkin-Elmer applied Biosystems).

The 16S rRNA gene sequences are matched with sequences in the nucleotide database using the BLAST algorithm with default parameters within NCBI (National Center for Biotechnology Information).

For the construction of phylogenetic trees, the sequence was aligned with Clustal X v. 1.83 and analyzed with BioEdit v. 7.2.3 (28, 29). Trees were derived from neighbour-joining method by MEGA5.2 program (30).

3. Medium screening for cultivation of MS101

To compare antimicrobial activities, six different sea salt added media were used. GTYB70 medium consisted of 10 g of glucose, 2 g of tryptone, 1 g of yeast extract, 1 g of beef extract, 23 g of sea salt, and 1 L of distilled water (31). Bennett70 medium consisted of 10 g of glucose, 2 g of NZ-amine, 1 g of yeast extract, 1 g of beef extract, 23 g of sea salt (70% of sea), and 1 L of distilled water (32). SYP70 medium consisted of 10 g of starch, 4 g of yeast extract, 2 g of peptone, 23 g of sea salt, and 1 L of distilled water. SPY medium consisted of 10 g of glucose, 3 g of yeast extract, 5 g of peptone, 23 g of sea salt, and 1 L of distilled water (33). M2 medium consisted of 4 g of glucose, 4 g of yeast extract, 10 g of malt extract, and 1 L of distilled water (34). YPM70 medium consisted of 4 g of mannitol, 2 g of yeast extract, 2 g of peptone, 23 g of sea salt, and 1 L of distilled water (35). After first medium screening, to confirm whether sea salt could be added or not, second medium screening was conducted. Sea salt added GTYB70 and sea salt-free GTYB media were used. GTYB medium consisted of 10 g of glucose, 2 g of tryptone, 1 g of yeast extract, 1 g of beef extract, and 1 L of distilled water (31). All media were sterilized by autoclaving at 121℃ for 20 minutes.

MS101 was cultured in 100 mL of media at 24~28℃ with

shaking at 130 rpm for 2 weeks. The fermented culture was filtered by filter paper (300 mm, qualitative, Advantec, Japan) to separate the mycelia from the fermented media. The filtrate was extracted with EtOAc as same volumes for three times. The EtOAc extract was evaporated *in vacuo* at 34℃ and then the residue was dissolved in DMSO and stored at −20℃.

4. Purification of compound 1-4

1) Fermentation of MS101

MS101 was cultured in GTYB media (196 × 0.5 L) at 24~28℃ with shaking at 130 rpm for 2 weeks.

The fermented culture (98 L) was filtered by filter paper (300 mm, qualitative, Advantec, Japan) and concentrated under reduced pressure. The dried residue was repeatedly extracted with MeOH for desalting.

2) Organic solvent partitioning of MS101 extract

The dried MeOH extract (402.23 g) was dissolved in H_2O again. Liquid-liquid partitioning was conducted between H_2O and n-Hx first, H_2O and EtOAc serially. Each solvent was partitioned as same volume for three times and then each solvent layer was dried under reduced pressure.

3) Reverse phase flash column chromatography

An aliquot of EtOAc layer (8.9546 g) was separated by C_{18} RP FCC using a sequential mixture of MeOH and H_2O as eluents (five fractions in gradient, 10%, 40%, 70%, 90% aq. MeOH 2 CV, and MeOH 5 CV), acetone, and finally EtOAc.

Chromatography condition was column size Φ 4.3 × 11 cm (Pyrex), ODS-A resin 25 g (YMC*GEL, pore size 12 nm, particle size 75 μm).

4) Normal phase open column chromatography (EtOAc)

The 10%, 40%, and 70% aq. MeOH fractions (1.1297 g) which showed antibacterial activity were separated by silica NP OPC (first OPC, fOPC). Mobile phase was the mixtures of EtOAc, n-Hx, and MeOH (isocratic elution, 12: 5: 1, v/v/v). Finally, resin was washed with EtOAc and MeOH mixture as the ratio of (12: 1, v/v), (6: 1, v/v), (3: 1, v/v), (1: 1, v/v), and MeOH.

Chromatography condition was column size Φ 2 × 110 cm (DURAN), silica resin 25 g, flow rate 3~5 mL/min, collection volume 10 mL.

5) TLC bio-autography overlay assay

fOPC 3, 4, and 5 fractions (521.9 mg) which showed antibacterial activity were conducted in TLC bio-autography overlay assay (36, 37). *Proteous hauseri* NRBC 3851 was incubated in 5 mL LB liquid media at 37℃ for 16 hours. After incubation, bacteria were spreaded on the LB agar plate. After that, TLC in saturated chamber was performed. The spot patterns detected by UV wavelength 365 nm were observed and R_f of spots were checked. TLC plate was faced down onto the inoculated LB agar media and incubated at 37℃ for 16 hours. After incubation, the inhibition zones which formed side of TLC plate on the agar surface were observed.

6) Normal phase open column chromatography (DC)

fOPC 3, 4, and 5 fractions (521.9 mg) which showed antibacterial activity were separated by silica NP OPC (second OPC, sOPC). Mobile phase was the mixtures of DC and MeOH. Chromatography condition was column size Φ 1 × 35 cm (Pyrex), silica resin 16 g, flow rate 3~5 mL/min, collection volume was determined by UV 365 nm patterns. Mobile phase was ran as stepwise gradient elution with the mixtures of DC and MeOH (from 100: 0 to 50: 50, v/v). Finally resin was washed by MeOH 10 CV.

7) High performance liquid chromatography

sOPC 2 and 3 fractions (144.7 mg) were separated by HPLC. HPLC were conducted on a TRILUTION LC control software with a 321 pump, a UV/VIS−151 detector (Gilson, Middleton, WI, USA), and a Shodex RI−101 refractive index detector (Showa Denko, Tokyo, Japan). Semi−preparative column (Agilent ZORBAX Eclipse Plus RX silica 4.6 × 250 mm) with guard column was used. Sample was solved in DC. Mobile phase was mixture of EtOAc, n−Hx, and MeOH (13: 17: 1, v/v/v). Flow rate was 3 mL/min. Running time was 60 minutes. UV wavelength 365 nm was used.

6. Structure elucidation of compound 1-4

EIMS analysis was performed by DIP method. EIMS spectra were recorded in DC solutions on JMS-600 (JEOL Ltd. Tokyo, Japan) spectrometer. Measurements on all compounds were taken with an electron ionizing voltage of 70 eV. Search for expected structure of compounds was performed in the NIST library. Mass spectra were provided by the National Center for Inter-University Research Facilities, Seoul, Korea.

ESIMS spectra at the low resolution were recorded in MeOH solutions on LC-MS/MS 8030 plus (Shimadzu Corp., Japan). Mass spectra were provided by the Pesticide Chemistry and Toxicology Laboratory, Seoul, Korea.

HRESIMS (positive-ion mode) spectra were recorded in MeOH solutions on Q Exactive MS (Thermo Fisher Scientific, Bremen, Germany). Mass spectra were provided by the National Instrumentation Center for Environmental Management, Seoul, Korea.

1D and 2D NMR spectra (^{1}H, ^{13}C, $^{1}H-^{1}H$ COSY, DEPT, HSQC, and HMBC) were recorded in $CDCl_3$ solutions on Bruker AVANCE 600 spectrometer (Bruker BioSpin Ltd., Germany). Both proton and carbon NMR spectra were measured at 600 MHz, Cryoprobe (z-gradient, 5mm TXI). NMR spectra were provided by the National Center for Inter-University Research Facilities, Seoul, Korea.

7. Bioactivity of compound 1-4

1) Antimicrobial activity assay

① Antibacterial activity assay

The following 6 microorganisms, obtained from the stock culture collection at American Type Culture Collection (ATCC) (Rockville, MD, U.S.A) and NITE Biological Resource Center (NBRC) (Tokyo, Japan) were used in this study: *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Kocuria rhizophila* NBRC 12708, *Salmonella enterica* ATCC 14028, *Proteus hauseri* NBRC 3851, and *Escherichia coli* ATCC 25922. Bacteria were grown overnight in Luria Bertani (LB) broth at 37℃.

The antibacterial activity was determined by the two-fold microtiter broth serial dilution method. Dilution of compound dissolved in DMSO were added to each well of 96-well microtiter plate containing fixed volume of m Plate Count Broth (mPCB, Difco). The concentration of compounds ranges from 100 to 0.10 μg/mL. Each well was inoculated with an overnight culture of bacteria (5×10^5 CFU/mL), and incubated at 37℃ for 16 hours. The minimal inhibitory concentration (MIC) value was determined as the lowest concentration of test compounds that inhibits bacterial growth (38). Ampicillin was used as a reference compound.

② Antifungal activity assay

Aspergillus fumigates HIC 6094, *Trichophyton rubum* IFO 9185, *Trichophyton mentagrophytes* IFO 40996, and *Candida albicans* ATCC 10231 as microorganisms were used in antifungal activity assay. *A. fumigates*, *T. rubum*, and *T. mentagrophytes* were grown in Potato Dextrose Agar (PDA, Acumedia Manufacturers, Inc., Maryland) medium at 28℃ for 2 weeks. *C. albicans* was grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) broth at 28℃ for 12 hours. The fungal activities of compounds were determined by following broth dilution method M27-A2, which was proposed by the National Committee for Clinical Laboratory Standards (39). Each compound dissolved in DMSO was added to each well of 96 well microtiter plate containing fixed volume of Potato Dextrose Broth (PDB, Difco) to prepare serial two-fold dilution. The concentration of compounds ranges from 100 to 0.10 μg/mL. In the case of *A. fumigates*, *T. rubum*, and *T. mentagrophytes*, about 10^4 spore/mL was contained in one hundred microliters of the broth. In the case of *C. albicans*, about 10^2 spore/mL was contained in one hundred microliters of the broth. The MIC values were determined after incubation at 28℃ for 48 hours. Amphotericin B was used as a reference compound (40).

2) Enzyme inhibition assay

① *Candida albicans* isocitrate lyase (ICL) inhibition assay

The cloning and purification of ICL from the genomic DNA *Candida albicans* ATCC 10231 were carried out as described previously (41). Briefly, *C. albicans* strain were grown in YPD medium. Genomic DNA of *C. albicans* was prepared with Wizard genomic DNA purification Kit (Promega, USA). Based on the nucleotide sequence for *C. albicans ICL1* (Genbank accession number AF222905), two synthetic primers (5'-A**GAATTC**CTACCATGCTTACACTCC-3', forward primer; 5'-CTTC**GTCGAC**TCAAAATTAAGCCTTG-3', reverse primer) were designed to carry the EcoR1 and Sal1 recognition sites, respectively (bold). PCR reaction consisted of 4 minutes at 94℃ followed by 30 cycles of 1 minute at 94℃, 1 minute at 48℃, and 1 minute at 72℃ followed by a single cycle at 1 minute at 72℃. The resulting PCR product was cloned in the pBAD/Thio-TOPO vector according to the instructions of manufacturer. The *E. coli* TOP10 was chosen for transformation. The positive transformants were grown in LB medium containing ampicillin (50 μg/mL) at 37℃ for 16 hours ($O.D._{600}$ = 1~2 Abs). The pre-incubated cells (2 mL) were inoculated into 400 mL of LB broth containing ampicillin and incubated with shaking at 37℃ until $O.D._{600}$ reached 0.5 Abs approximately. At this point L-arabinose was added to the

medium to a final concentration of over 0.02% and an additional incubation was performed at 25℃ for 8 hours to induce the expression of ICL proteins. Growth at the low resulted in improved solubility of the recombinant system are fused to His-Patch(HP)-thioredoxin at the N-terminal for the simplified purification (42). Since HP-thioredoxin has a Ni-binding property at pH 7.2, the expressed ICL could easily be purified by Ni-NTA affinity column chromatography (Qiagen, Hilden, Germany).

The enzyme activity of the purified ICL was determined by the method of Dixon and Kornberg (43). A 1 mL aliquot of the reaction mixture contained 20 mM sodium phosphate buffer (pH 7.0), 1.27 mM *threo*-DL (+) isocitrate, 3.75 mM $MgCl_2$, 4.1 mM phenylhydrazine, and 2.5 μg/mL purified ICL. The reaction was performed at 37℃ for 30 minutes with and without prescribed concentration of inhibitor dissolved in DMSO (final concentration, 1%). The formation of glyoxylate phenyl-hydrazone was followed spectrophotometrically at 324 nm. The effect of inhibitor on ICL was calculated as a percentage, relative to solvent-treated control, and the IC_{50} values were calculated using nonlinear regression analysis (percent inhibition *versus* concentration). 3-Nitropropionate is an ICL inhibitor used as positive control.

② *Streptococcus mutans* sortase A(SrtA) inhibition assay

The cloning and purification of sortase A from the genomic DNA *Streptococcus mutans* OMZ65 were carried out as described previously (44). Briefly, *S. mutans* OMZ65 was isolated from the human oral cavity. Recombinant $SrtA_{\Delta 40}$ derived from *S. mutans* OMZ65 was purified from *Escherichia coli* extract by metal chelate-affinity chromatography, and the enzyme activities were determined from the increased fluorescence intensity upon cleavage of the synthetic peptide substrate containing the LPETG motifs (45).

Reactions were performed in 100 μL containing 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 5 mM $CaCl_2$, 1 μg of fluorescent peptide (H_2N-Abz-LPXTG-Dap(Dnp)-NH_2), 1mM penta-Gly, 24 μg of recombinant $SrtA_{\Delta 24}$, and the test sample. Each test compound was dissolved in DMSO and diluted with sterilized DW (final concentration, 0.5% DMSO, which was found to have no effect on the enzyme activity when the concentration was <1%). Reactions were carried out for 1 hour at 37℃. Sample fluorescence was measured using emission and excitation wavelengths of 420 and 317 nm, respectively (FLx 800, BioTek Instruments Inc., VT, USA). pHMB (Sigma-Aldrich), a known sortase A inhibitor, was used as a positive control (46). pHMB was dissolved 0.5 mg/ml in DW. 10 mM NaOH was added to increase solubility of pHMB.

Ⅲ. Results

1. Isolation of marine actinomycetes

373 of marine microorganisms were isolated from 77 marine samples. Among them, actinomycete strain MS101 was isolated from near-shore sediment (**Figure 1**).

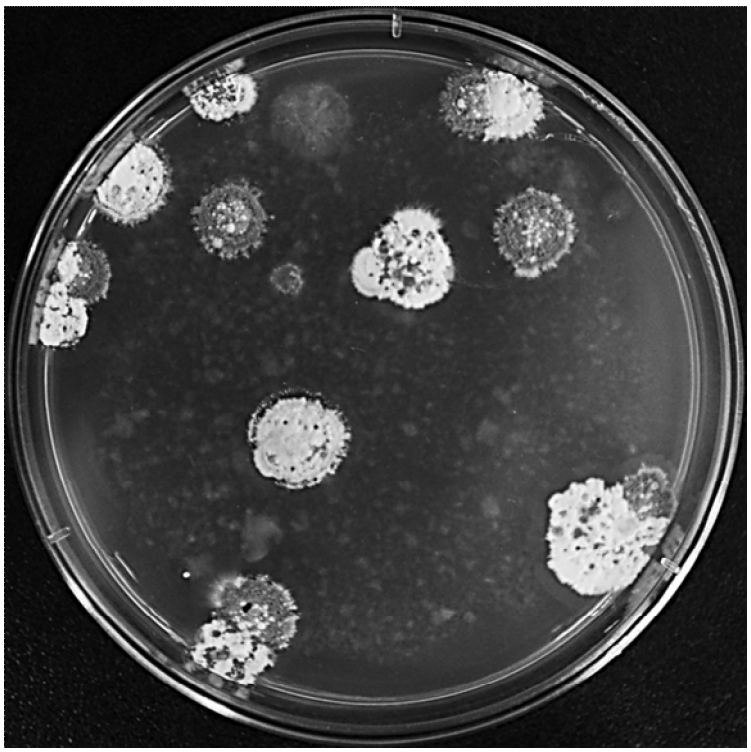


Figure 1. Morphology of *Streptomyces* sp. MS101

2. Identification of MS101

According to 16S rRNA gene sequence analysis, 1518 base pairs were confirmed (**Appendix**). Based on that result, BLAST search was performed against NCBI database. The result shows that MS101 16S rRNA gene sequence was not matched with any bacterial sequence perfectly. However, *Streptomyces cellostaticus* strain CSSP188 (GenBank accession No. NR043339), *Streptomyces* sp. NEAU-YX9 (GenBank accession No. KC306504), *Streptomyces avermitilis* NCIMB 12804 (GenBank accession No. NR114521), *Streptomyces fimbriatus* NBRC 13549 (GenBank accession No. AB184444), and *Streptomyces* sp. Sm22 (GenBank accession No. AJ308571) shares 99% identity with MS101 rRNA fragment (**Figure 2**). It suggests that MS101 should be novel strain of *Streptomyces* genus. Therefore, MS101 was named as *Streptomyces* sp. MS101.

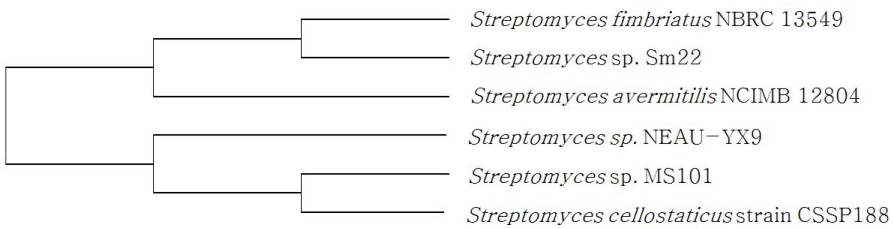


Figure 2. Phylogenetic tree of *Streptomyces* sp. MS101

3. Medium screening for cultivation of MS101

At the first media screening, EtOAc extract from GTYB70 exhibited significant antibacterial activity (Table 1. (a)). The second media screening, EtOAc extract from salt-free GTYB media exhibited more significant antibacterial activity than salt-added GTYB media (Table 1. (b)).

Table 1. Antibacterial activities of EtOAc extract from medium.

(a)

Medium	MIC (μg/mL)	
	Gram(+) bacteria	Gram (−) bacteria
	[a]*S. aureus*	[b]*P. hauseri*
GTYB70	500	125
Bennett70	500	250
SYP70	500	125
GPY70	>500	>500
M2 70	>500	125
YPM70	>500	125
[c]Amp	0.78	0.78

(b)

Medium	MIC (μg/mL)	
	Gram (+) bacteria	Gram (−) bacteria
	[a]*S. aureus*	[b]*P. hauseri*
GTYB70	>500	62.5
GTYB	500	31.25
[c]Amp	0.78	0.78

[a]*Staphylococcus aureus* ATCC 6538p, [b]*Proteous hauseri* NBRC 3851

[c]Ampicillin was used as positive control

Each medium was cultured at 28℃ with shaking at 130 rpm for 2 weeks after spore suspension. (a) Tested on salt added six medium (b) Tested on salt added and salt−free GTYB media

4. Purification of compound 1−4

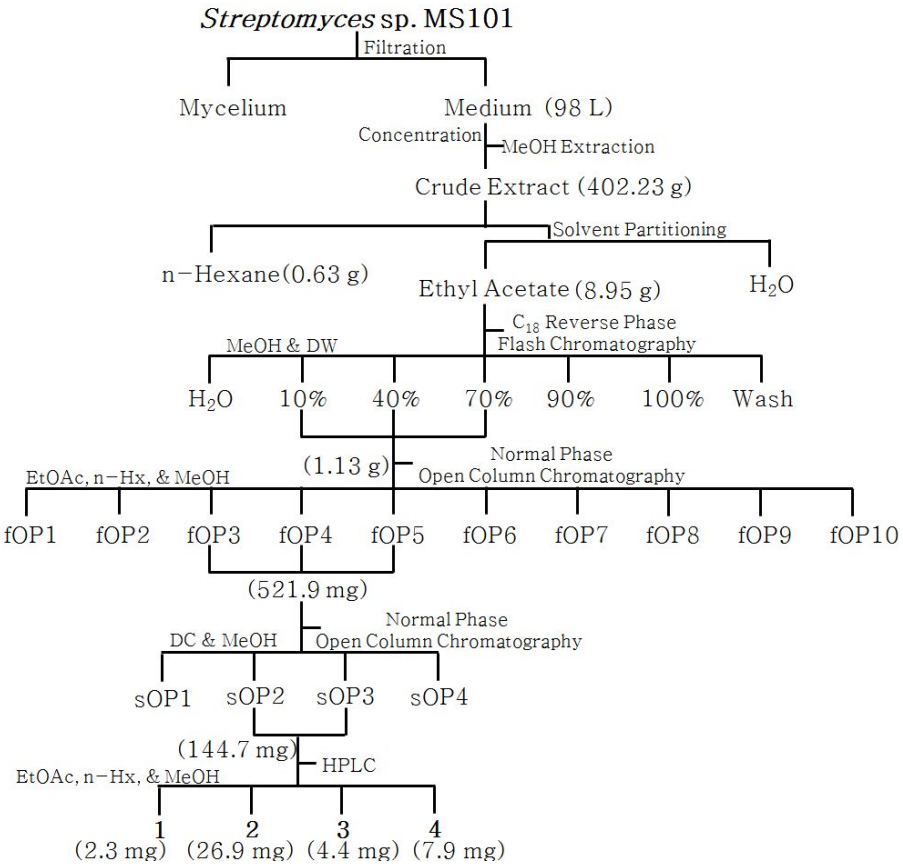


Figure 3. Isolation procedure of compound 1−4

1) Fermentation of MS101

MS101 culture media was gathered upto 98 L (0.5 L × 196). The fermented media was filtered and concentrated under reduced pressure. The dried residue was repeatedly extracted with MeOH for desalting. MeOH extract was gathered upto 402.23 g.

2) Organic solvent partitioning of MS101 extract

Liquid-liquid partitioning from MeOH extract, aliquots of n-Hx layer (632.3 mg) and aliquots of EtOAc layer (8.95 g) were yielded. Aliquots of EtOAc layer exhibited stronger inhibitory activity than aliquots of n-Hx layers (Table 2).

Table 2. Antibacterial activities of organic solvent layer

Organic layer	MIC (μg/mL)	
	Gram (+) bacteria	Gram (−) bacteria
	[a]*S. aureus*	[b]*P. hauseri*
n-Hx	500	125
EtOAc	>500	31.25
[c]Amp	1.56	0.78

[a]*Staphylococcus aureus* ATCC 6538p, [b]*Proteous hauseri* NBRC 3851
[c]Ampicillin was used as positive control

3) Reverse phase flash column chromatography

EtOAc extract (8.95 g) was subjected to C_{18} RP FCC. As a result, 10% aq. MeOH fraction (134.5 mg), 40% aq. MeOH fraction (693.3 mg), and 70% aq. MeOH (301.9 mg) showed the most significant inhibitory activities among all the other fractions (Table 3).

Table 3. Antibacterial activities and weight of each fraction after flash column chromatography

RP-FCC Fraction	Weight (mg)	MIC (μg/mL)
		[a]*P. hauseri*
H_2O (1)	6606.4	>200
H_2O (2)	253.4	>200
H_2O (3)	94.9	>200
10% aq. MeOH	134.5	50
40% aq. MeOH	693.3	25
70% aq. MeOH	301.9	12.5
90% aq. MeOH	280	200
MeOH	372.8	50
Wash	212.8	>200
[b]Amp	-	0.78

[a]*Proteous hauseri* NBRC 3851

[b]Ampicillin was used as positive control

4) Normal phase open column chromatography (EtOAc)

FCC 10% aq. MeOH, 40% aq. MeOH, and 70% aq. MeOH fractions (1.13 g) were subjected to NP OPC (first OPC, fOPC). As a result, fOPC 3, 4, and 5 fractions (521.9 mg) and fOPC 8, 9, and 10 fractions (192.4 mg) showed the most significant inhibitory activities out of all the other fractions (Table 4).

Table 4. Antibacterial activities and weight of each fraction after open column chromatography

First NP-OPC	Weight (mg)	MIC (μg/mL)
		[a]*P. hauseri*
fOPC 1	36.4	>200
fOPC 2	80.2	>200
fOPC 3	136.8	12.5
fOPC 4	140.3	6.25
fOPC 5	244.8	12.5
fOPC 6	40.8	50
fOPC 7	63.6	100
fOPC 8	84.7	3.13
fOPC 9	86.0	12.5
fOPC 10	21.7	12.5
[b]Amp	-	0.78

[a]*Proteous hauseri* NBRC 3851

[b]Ampicillin was used as positive control

5) TLC bio-autography overlay assay

To confirm which spot has antibacterial activity on TLC plate, TLC bio-autography overlay assay was conducted. As a result, inhibition zones were formed at R_f value 0.20 mainly and R_f value 0.36 slightly using EtOAc, n-Hx, and MeOH (12: 3: 1, v/v/v) as solvent. With solvent condition changed, especially DC and MeOH (8: 1, v/v), only one inhibition zone was formed at R_f value 0.66. In this condition, inhibition spot was located at the top in TLC with UV wavelength 365 nm detect. It indicates that it can be refined more easily. Two antibacterial zones with EtOAc, n-Hx, and MeOH solvent, R_f values 0.20 and 0.36, were integrated at R_f value 0.66 with changed solvent condition (Supplementary Figure 1).

6) Normal phase open column chromatography (DC)

fOPC 3, 4, and 5 fractions (521.9 mg) were subjected to NP OPC (second OPC, sOPC). As a result, sOPC 2 and 3 fractions (144.7 mg) had R_f value 0.66 in NP TLC using DC and MeOH (8: 1, v/v) as solvent (Supplementary Figure 2).

7) High performance liquid chromatography

sOPC 2 and 3 fractions (144.7 mg) were separated by HPLC. As conducted chromatographic condition, compound **1−4** were flowed out at retention time 21, 24, 46, and 50 minutes, respectively (**Figure 4**). After conducting one more purification step with a changed chromatographic condition, compound **1−4** were yielded 3.4, 26.9, 4.4, and 7.9 mg, respectively.

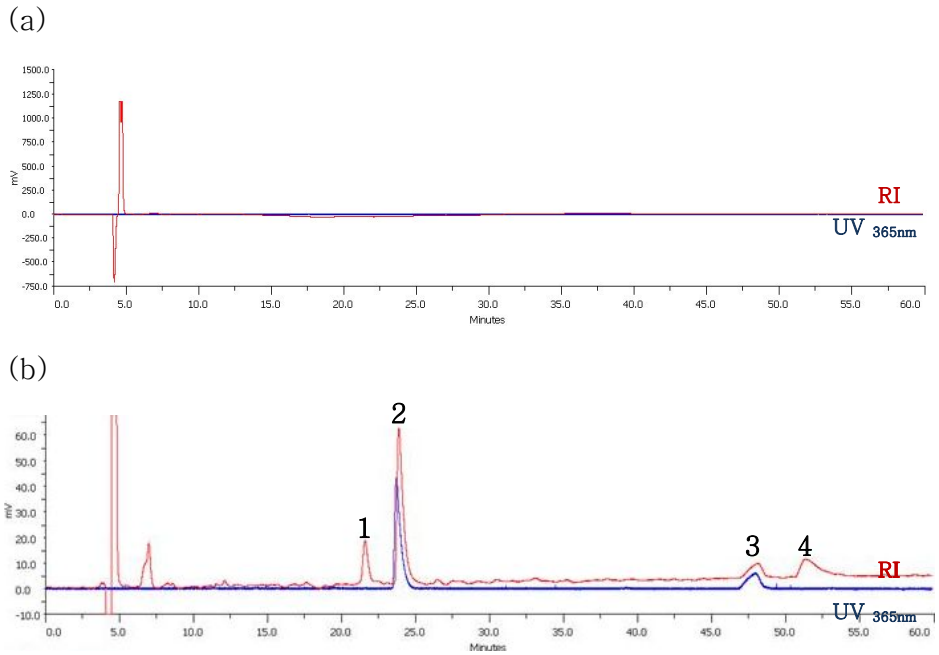


Figure 4. HPLC chromatogram of compound **1−4**

Semi−preparative HPLC was used. (silica column, solvent condition EtOAc: n−Hx: MeOH = 13: 17: 1, v/v/v, isocratic elution, flow rate 3 mL/min, running time 60 minutes, detected by RI and UV wavelength 365 nm) (a) DC control (b) sOPC 2 and 3 fractions in DC

5. Structure elucidation of compound 1−4

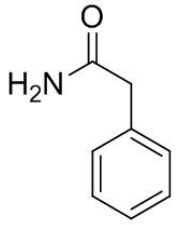
1) Structural analysis of compound 1

EIMS analysis with DIP method revealed fragmentation pattern of compound **1** (**Supplementary Figure 3**). The result of library search on NIST, Mass spectrum of compound **1** highly matches to mass spectrum of Benzenacetamide (CAS No. 103−81−1). According to International Union of Pure and Applied Chemistry (IUPAC), the molecular formula of Benzenacetamide is C_8H_9NO and the molecular weight is 135.1632.

^{1}H and ^{13}C NMR data assigned for this compound by $^{1}H-^{1}H$ COSY, DEPT, HMBC, and HSQC NMR (**Supplementary Figure 4−9**). The ^{1}H NMR spectrum of compound **1** (**Table 5**) showed singlet at δ_H 3.63, one doublet at δ_H 7.25, and two triplet at 7.29, 7.35, respectively. A singlet at δ_H 3.63 representing methylene resonance. One doublet at δ_H 7.25, and two triplets at 7.29 and 7.35 representing one aromatic ring. As the ^{13}C NMR spectrum of compound **1** (**Table 5**), a total of six carbon resonances were observed including one amide carbonyl carbon, one methylene carbon, and four aromatic carbons.

Expected structure of compound **1** (**Figure 5**) was matched with Benzenacetamide.

Table 5. ^{1}H and ^{13}C NMR assignments for compound 1



compound 1		
position	δ_C	δ_H
1	133.8, C	
2	129.5, CH	7.25 (d, 7.8)
3	129.2, CH	7.35 (t, 7.8)
4	127.8, CH	7.29 (t, 7.8)
5	129.2, CH	7.35 (t, 7.8)
6	129.5, CH	7.25 (d, 7.8)
7	43.1, CH_2	3.63 (s)
8	174.8, C	

Data were obtained in $CDCl_3$ solutions.
Data were measured at 600 MHz.

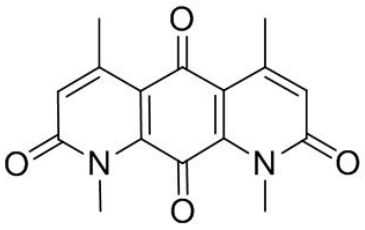
2) Structural analysis of compound 2

LCMS analysis interfaced with ESI indicated the presence of a protonated molecular ion $(M+H)^+$ at m/z 299 (data not shown). Elemental composition analysis revealed the molecular formula of compound **2** to be $C_{16}H_{14}N_2O_4$ based on HRESIMS data (Calculated: m/z 299.1032 for $C_{16}H_{15}N_2O_4$. Found: m/z 299.1026) (**Supplementary Figure 11**).

^1H and ^{13}C NMR data assigned for compound **2** by $^1H-^1H$ COSY, DEPT, HMBC, and HSQC NMR (**Supplementary Figure 12-17**). The ^1H NMR spectrum of compound **2** (**Table 6**) was simple with three singlets at δ_H 2.50, 3.65 and 6.60. It representing a double-bond attached CH_3, a nitrogen attached $N-CH_3$ and a vinyl CH, respectively. In the ^{13}C NMR spectrum (**Table 6**), a total of nine carbon resonances were observed including three carbonyls, one vinyl methine, three vinyl/aromatic quaternary carbons, one nitrogen attached methyl, and one double bond attached methyl carbon. Among the three carbonyls, two signals at δ_C 178.7 and 181.3 represented typical 1, 4-quinone carbonyl carbons, while the other signal at δ_C 161.3 was considered as an amide carbonyl carbon. The resonance at δ_C 34.0 was assigned to a nitrogen attached methyl group.

Expected structure of compound **2** (**Figure 6**) was matched with Sch 538415.

Table 6. ^{1}H and ^{13}C NMR assignments for compound 2



compound 2		
position	δ_C	δ_H
2	162.8, C	
3	126.4, CH	6.60 (s)
4	148.9, C	
4a	117.8, C	
5a	117.8, C	
5	148.9, C	
6	126.4, CH	6.60 (s)
7	162.8, C	
8a	142.8, C	
9	178.7, C	
9a	142.8, C	
10	181.3, C	
1−Me	34.0, CH_3	3.68 (s)
4−Me	22.6, CH_3	2.50 (s)
5−Me	22.6, CH_3	2.50 (s)
8−Me	34.0, CH_3	3.68 (s)

Data were obtained in $CDCl_3$ solutions.
Data were measured at 600 MHz.

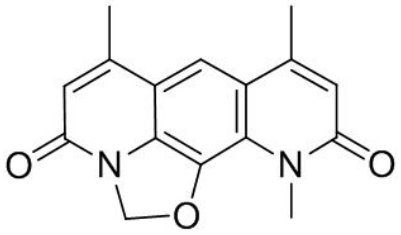
3) Structural analysis of compound 3

LCMS analysis interfaced with ESI indicated the presence of a protonated molecular ion $(M+H)^+$ at m/z 283 (data not shown). Elemental composition analysis revealed the molecular formula of compound **3** to be $C_{16}H_{14}N_2O_3$ based on HRESIMS data (Calculated: m/z 283.1077 for $C_{16}H_{15}N_2O_3$. Found: m/z 283.1077) (**Supplementary Figure 19**).

^1H and ^{13}C NMR data assigned for compound **3** by $^1H-^1H$ COSY, DEPT, HMBC, and HSQC NMR (**Supplementary Figure 20-25**). The ^1H NMR spectrum of compound **3** (**Table 7**) was seven singlets at δ_H 2.50, 2.53, 3.95, 6.40, 6.47, 6.53 and 7.46. It representing two of double bond attached CH_3, a nitrogen attached $N-CH_3$, a nitrogen and oxygen attached $-CH_2$, two of vinyl CH, a aromatic CH, respectively. In the ^{13}C NMR spectrum (**Table 7**), a total of 16 carbon resonances were observed including two amide carbonyl carbons, two vinyl methines, eight vinyl/aromatic quaternary carbons, one nitrogen attached methyl carbon, two double bond attached methyl carbons, and one nitrogen and oxygen attached methylene carbon.

Expected structure of compound **3** (**Figure 7**) was matched with Deoxynybomycin (CAS No. 27259-98-9).

Table 7. ^{1}H and ^{13}C NMR assignments for compound 3



compound 3		
position	δ_C	δ_H
2	161.8, C	
3	120.7, CH	6.53 (s)
4	146.8, C	
4a	120.4, C	
5a	120.7, C	
5	147.6, C	
6	121.2, CH	6.47 (s)
7	158.8, C	
8a	131.8, C	
9	135.8, C	
9a	125.6, C	
10	113.3, CH	7.46 (s)
11	86.0, CH_2	6.40 (s)
1−Me	32.5, CH_3	3.95 (s)
4−Me	20.2, CH_3	2.50 (s)
5−Me	17.9, CH_3	2.53 (s)

Data were obtained in $CDCl_3$ solutions.
Data were measured at 600 MHz.

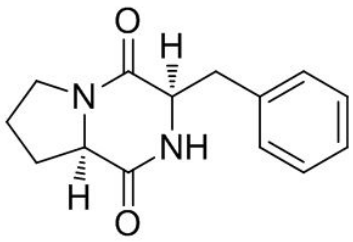
3) Structural analysis of compound 4

LCMS analysis interfaced with ESI indicated the presence of a protonated molecular ion $(M+H)^+$ at m/z 245. Elemental composition analysis revealed the molecular formula of compound **4** to be $C_{14}H_{16}N_2O_2$ based on HRESIMS data (Calculated: m/z 245.2912 for $C_{14}H_{17}N_2O_2$. Found: m/z 245.1285) (**Supplementary Figure 27**).

^1H and ^{13}C NMR data assigned for compound **4** by $^1H-^1H$ COSY, DEPT, HMBC, and HSQC NMR (**Supplementary Figure 28-33**). The ^1H NMR spectrum of compound **4** (**Table 8**) consisted of many singlets, doublets, and triplets. Proton resonances at δ_H (1.92, 2.01), (2.02, 2.33), (2.78, 3.64), and (3.58, 3.63) represent four methylene signal. Proton resonances at δ_H 4.08 and 4.27 represent two methines signal. Proton resonances at δ_H 7.23, 7.29, and 7.35 represent three aromatic protons. In the ^{13}C NMR spectrum (**Table 8**), a total of 12 carbon resonances were observed including two amide carbonyl carbons, four methylene carbons, two methines, and four vinyl/aromatic quaternary carbons.

Expected structure of compound **4** (**Figure 8**) was matched with Cyclo(L-Pro-L-Phe) (CAS No. 3705-26-8).

Table 8. ^{1}H and ^{13}C NMR assignments for compound **4**



compound 4		
position	δ_C	δ_H
2	45.5, CH_2	3.58 (1H, m), 3.63 (m)
3	22.6, CH_2	1.92 (m), 2.01 (m)
4	28.4, CH_2	2.02 (m), 2.33 (m)
5	59.2, CH	4.08 (dd, 6.5, 7.5)
6	169.4, C	
8	56.2, CH	4.27 (d, 11.0)
9	165.1, C	
10	36.8, CH_2	2.78 (dd, 11.0, 14.0), 3.64 (m)
11	135.9, C	
12	129.3, CH	7.23 (d, 7.8)
13	129.1, CH	7.35 (t, 7.8)
14	127.6, CH	7.29 (t, 7.8)
15	129.1, CH	7.35 (t, 7.8)
16	129.3, CH	7.23 (d, 7.8)

Data were obtained in $CDCl_3$ solutions.
Data were measured at 600 MHz.

6. Bioactivity of compound 1-4

1) Antimicrobial activity assay

Compound **2** and **3** displayed antibacterial activities against most tested strain excluding *Escherichia coli* ATCC 25922, with MIC averages ranging between 0.20-25 μg/mL. Especially, compound **3** displayed significant antibacterial activity against Gram-positive bacteria, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, and *Kocuria rhizophila* NBRC 12708; Gram-negative bacteria, *Salmonella enterica* ATCC 14028 and *Proteus hauseri* NBRC 3851, with MIC value of 1.56, 0.2, 1.56, 0.78, and 0.39 μg/mL, respectively. compound **2** has strong antibacterial activity against Gram-positive bacteria, *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633; Gram-negative bacteria, *Salmonella enterica* ATCC 14028 and *Proteus hauseri* NBRC 3851 with MIC value of 6.25, 3.13, 3.13, and 3.13 μg/mL, respectively. Compound **1** and **4** have no activities against any tested strain (**Table 9**). Amp or DMSO was used as positive or negative control (DMSO data not shown).

All compounds did not have antifungal activities against any tested strain (data not shown).

2) Enzyme inhibition assay

The effect of isolated compound **1−4** on *C. albicans* ICL and *S. mutans* SrtA were evaluated *in vitro*. The inhibitory potencies of the tested compounds were shown in IC_{50} value, the concentration of an inhibitor that was required for 50 percent inhibition of an enzyme *in vitro* (**Table 10**). Compounds **1**, **3**, and **4** inhibited ICL activity, with IC_{50} values of 75.59, 57.80, and 74.80 μg/mL, respectively. In the case of compound **2**, reddish−orange color was shown due to chromophore. At wavelength 254 nm, which was spectrometry condition of ICL inhibition assay, the initial value was so high that data was not determined. Compound **1−4** were compared to that of a known ICL inhibitor, 3−nitropropinate (IC_{50} 2.09 μg/mL). Compound **2** was found to be *S. mutans* SrtA inhibitor, with IC_{50} values of 72.06 μg/mL. Compound **1−4** were compared to that of a known *S. mutans* SrtA inhibitor, pHMB (IC_{50} 1.46 μg/mL). DMSO was used as negative control of ICL and SrtA (data not shown).

Table 9. Antibacterial activities of compound 1–4

compound	MIC (μg/mL)					
	Gram (+) bacteria			Gram (−) bacteria		
	A	B	C	D	E	F
1	>100	>100	>100	>100	>100	>100
2	6.25	3.13	25	3.13	3.13	>100
3	1.56	0.20	1.56	0.78	0.39	>100
4	>100	>100	>100	>100	>100	>100
[a]Amp	0.20	0.78	0.39	3.13	0.78	3.13

A: *Staphylococcus aureus* ATCC 25923, B: *Bacillus subtilis* ATCC 6633,
C: *Kocuria rhizophila* NRBC 12708, D: *Salmonella enteriaca* ATCC 14028,
E: *Proteus hauseri* NRBC 3851, F: *Escherichia coli* ATCC 25922
[a]Amp(ampicillin) was used as positive control

Table 10. Enzyme inhibitory activities of compound 1–4

compound	IC_{50} (μg/mL)	
	C. albicans ICL	*S. mutans* SrtA
1	75.59	>100
2	[a]ND	72.06
3	57.80	>100
4	74.80	>100
[b]3−NP	2.09	–
[c]pHMB	–	1.46

[a]Not Determined
[b]3−Nitropropionate was used as positive control of inhibitor of ICL
[c]*p*−Hydroxymecuribenzoic acid was used as positive control of inhibitor of SrtA

Ⅳ. Discussion

Actinomycete MS101 was collected and isolated from near-shore sediment of Micronesia in December 2011. According to 16S rRNA gene sequence analysis result, actinomycete strain MS101 was identified as a novel strain of *Streptomyces* genus. It was named as *Streptomyces* sp. MS101.

Streptomyces sp. MS101 was cultured in GTYB media and four compounds were isolated by various chromatographic methods. Compound **1-4** were yielded 3.4, 26.9, 4.4, and 7.9 mg, respectively.

As the results of structure analysis, four compounds were identified. Compound **1** was known as Benzenacetamide. Compound **2** was known as Sch 538415, a diazaanthraquinone derivatives. Compound **3** was known as Deoxynybomycin. Compound **4** was known as Cyclo(L-Pro-L-Phe), a diketopiperazine(DKP) derivates.

The result of bioactivity assay, compounds **2** and **3** exhibited significant antibacterial activity with MIC value 0.20-6.25 μg/mL. Compounds **1, 3,** and **4** had inhibitory activities against ICL of *Candida albicans*. Compound **2** had inhibitory activity against sortase A of *Streptococcus mutans.*

Compound **2**, identified as Sch538415, was a natural product that had recently been isolated and characterized. It was first

reported in 2003, isolated from an bacterial microbe (47). It exhibited inhibitory activity in the bacterial acyl carrier protein synthase (AcpS) and exhibited antibacterial activity. In 2006, the antineoplastic activities were also reported. It exhibited significant growth inhibitory activity when evaluated against the murine P388 lymphocytic leukemia cell line and a minipanel of human tumor cell lines (48). The synthesis of Sch 538415 was first developed in 2010 (49).

Compound **3**, isolated from *Streptomyces* sp. MS101, was identified as Deoxynybomycin. Deoxynybomycin was a derivative of nybomycin which was first chemically prepared (50, 51), and then found as a natural product of *Streptomyces hyalinum,* in 1970 (52). As the name suggests, it exhibited significant antibacterial activity. Furthermore, according to research in 2000, Deoxynybomycin was a selective anti-tumor agent induced apoptosis and inhibited topoisomerase I but not topoisomerase II (53).

Compound **4** was identified as Cyclo(L-Pro-L-Phe). According to previous research, it exhibited antimicrobial and antifouling activity (54, 55).

This study confirmed that actinomycetes *Streptomyces* sp. MS101, which is isolated from near-shore sediment, is a natural producer of the promising anticancer and antibacterial drug candidate Sch 538415 and Deoxynybomycin.

In the case of Sch 538415, as a natural product, there has

been nothing reported in academia that a secondary metabolite from any plants, fungi, and other microorganisms. However, there were only two strains of bacteria reported in academia. One strain that was reported in 2003, was a unidentified bacterium. Other strain that was reported in 2006, was *Streptomyces* sp. isolated from riverbank soil. Comparing report written in 2006 to MS101 strain in this study, while the former one produces 3.5 mg per 375 L (9.3 μg/L), MS101 produces 26.9 mg per 98 L (274.5 μg/L). MS101 is nearly 30 times more productive than *Streptomyces* sp. that was reported in 2006. It suggests that a *Streptomyces* species which produce Sch 538415 more effectively was isolated and identified in this study.

Both MS101 and *Streptomyces* sp. that was reported in 2006 inhabited in aquatic ecosystem in common, though it was different in the point that one was isolated from near-shore and the other was isolated from riverbank soil. However, the effectiveness of producing Sch 538415 had significant difference. For the further study, it is possible to figure out the mechanism producing Sch 538415 more effectively by comparing gene of both bacterial strain with different effectiveness. If this study is successively conducted, the efficient mechanisms to produce (diaza-)anthraquinone derivates which has significant bioactivity such as anticancer and antibacterial activity are expected to study.

fOPC 8, 9, and 10 fractions gathered in this study showed significant antibacterial activity. However, it was not used since the quantity of these were comparatively lower than the one of fOPC 3, 4, and 5 fractions. Therefore, if the quantity of fOPC 8, 9, and 10 is sufficiently secured in GTYB media, it is anticipated for it to isolate other bioactive compounds.

In the condition of GTYB culture media, anthraquinone, nybomycin, diketopiperazine derivates which were known for its outstanding bioactivity were produced from MS101. Therefore, it is predicted to figure out whether it produces compounds effectively or can produce new compounds by changing carbon sources and nitrogen sources of culture media.

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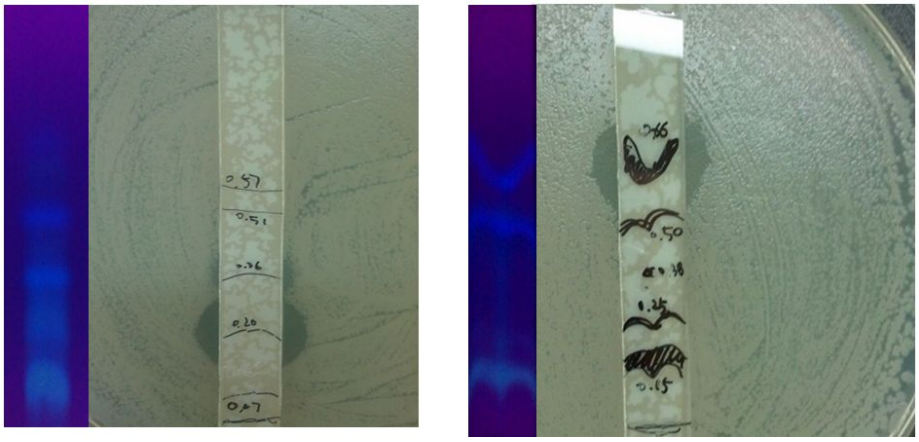
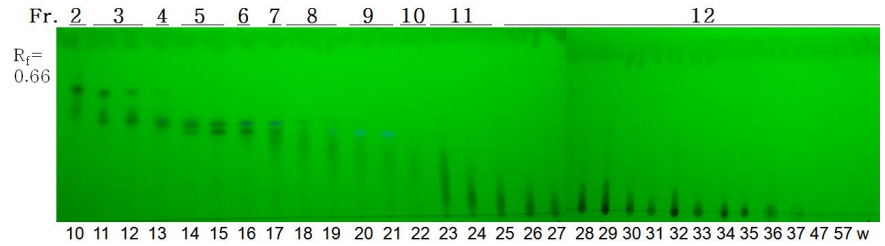
(a) (b)

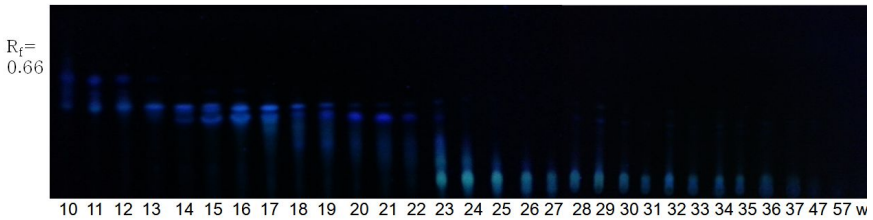


Supplementary Figure 1. TLC bio-autography overlay assay fOPC 3, 4, and 5 sample, NP TLC, UV wavelength 365 nm detect, *Proteus hauseri* NBRC 3851, 37℃, 12 hours incubation (a) EtOAc, MeOH and n-Hx (12: 1: 3, v/v/v) (b) DC and MeOH (8: 1, v/v)

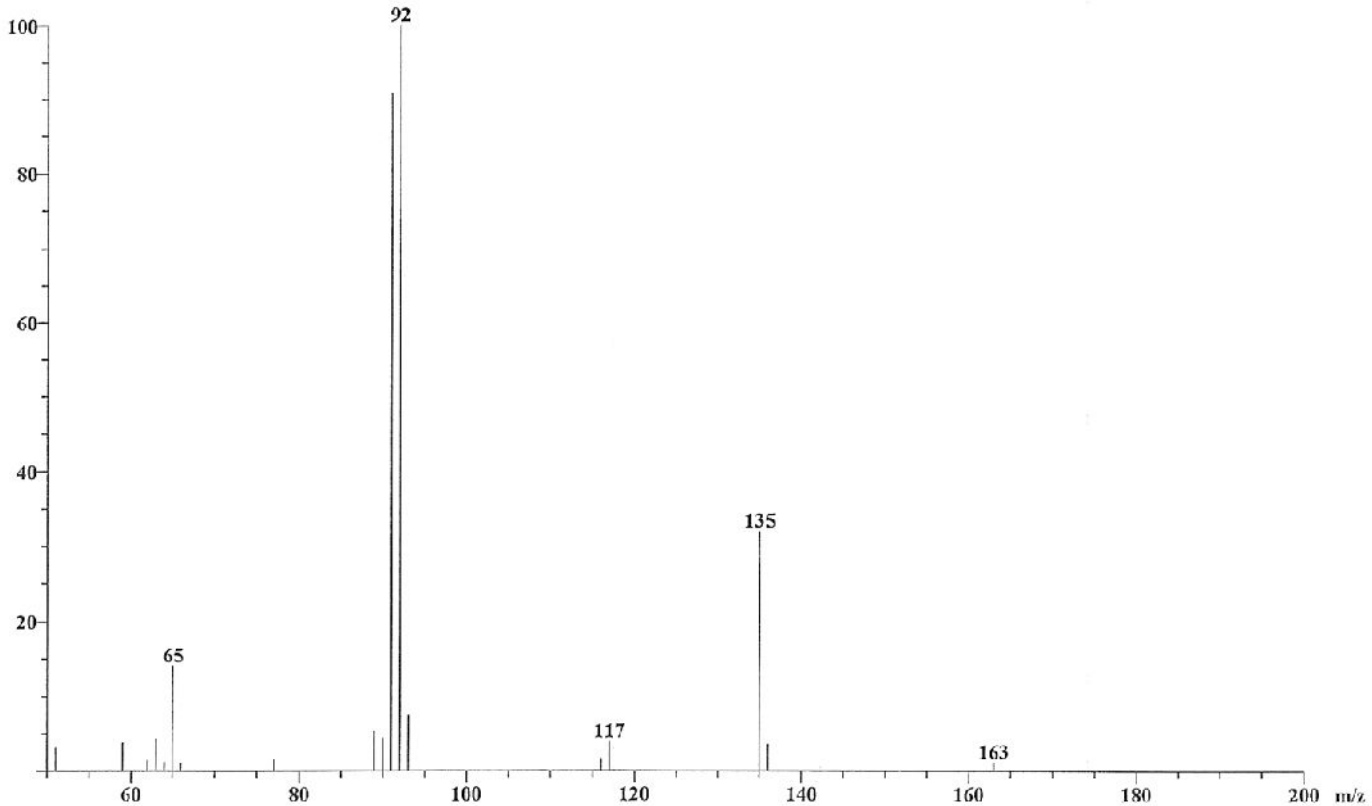
(a)



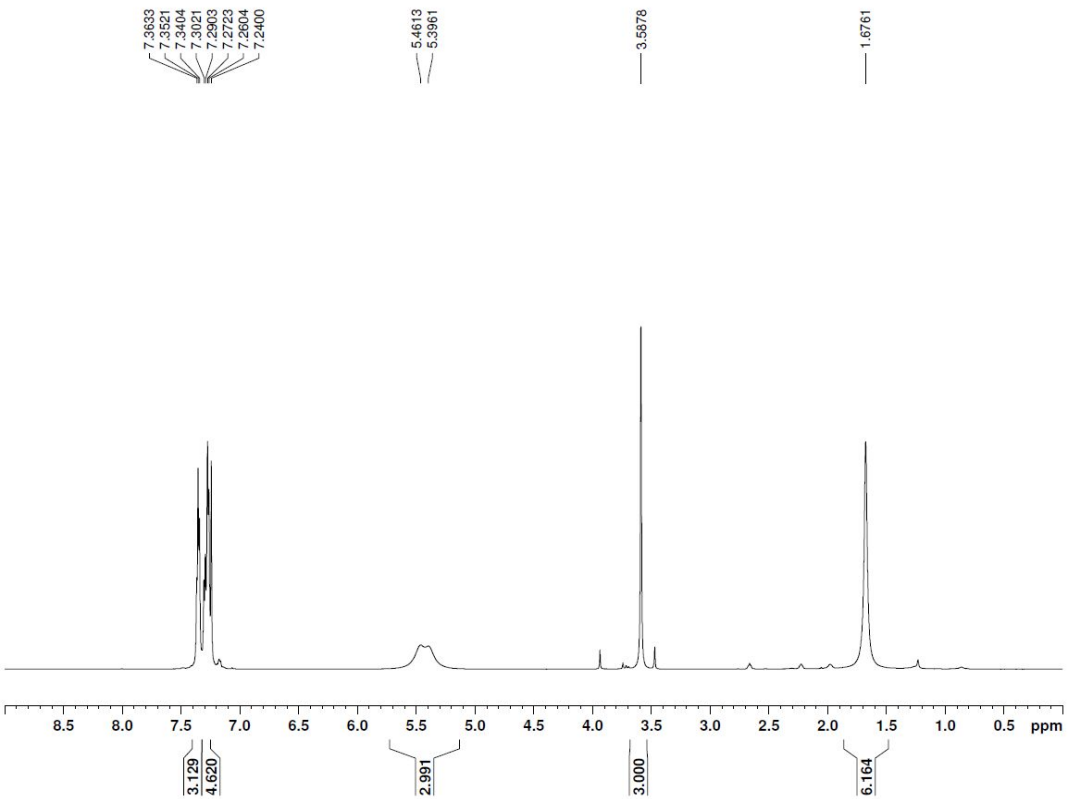
(b)



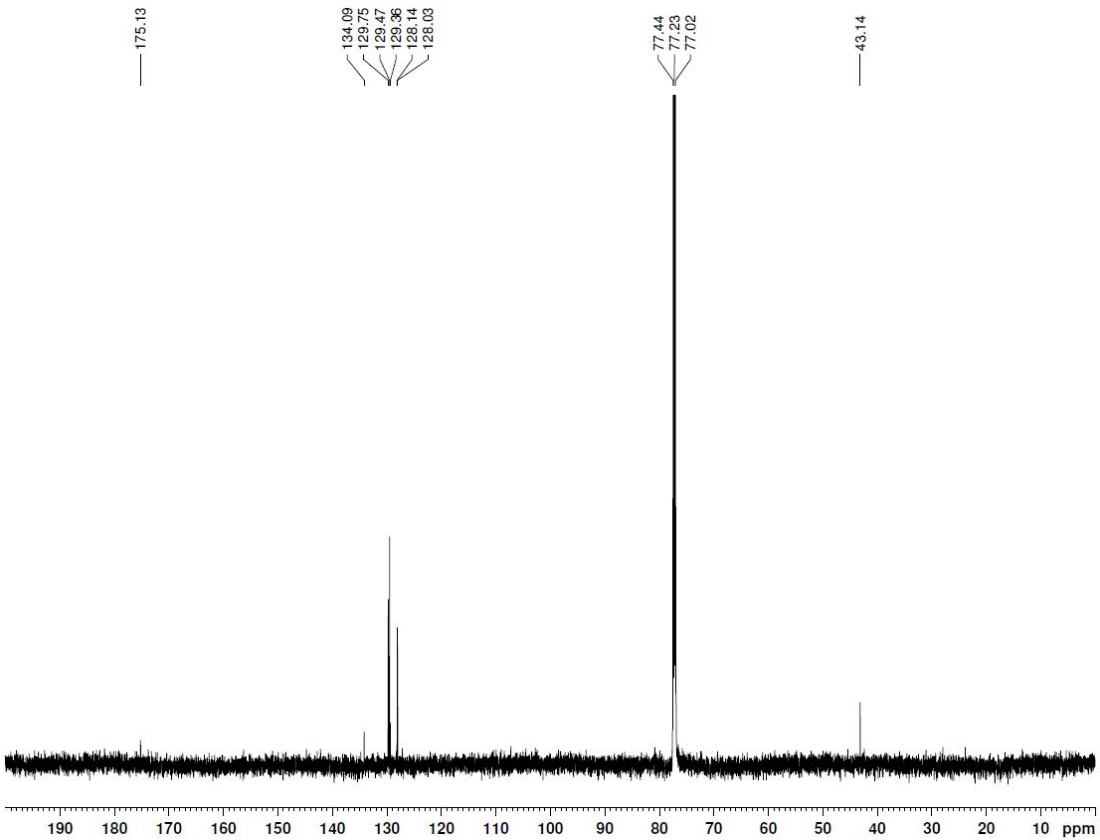
Supplementary Figure 2. TLC pattern of NP OPC (DC) fOPC 3, 4 and 5 sample, NP TLC, Solvent DC: MeOH, (8: 1, v/v) (a) UV wavelength 254 nm detect (b)UV wavelength 365 nm detect



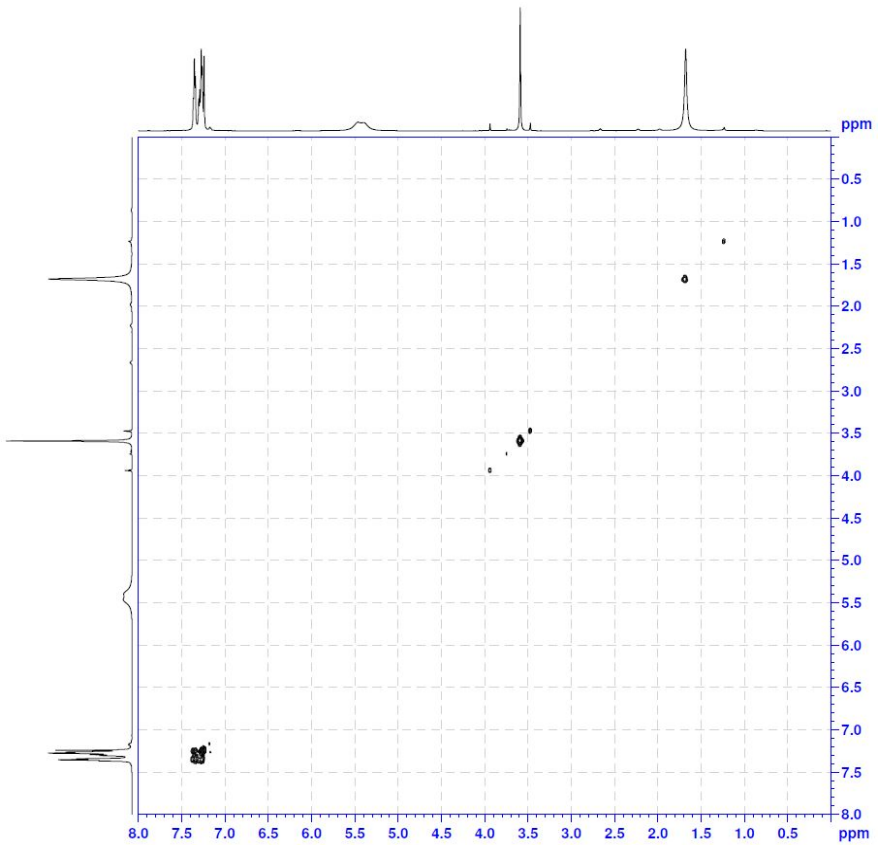
Supplementary Figure 3. EIMS spectrum of compound 1



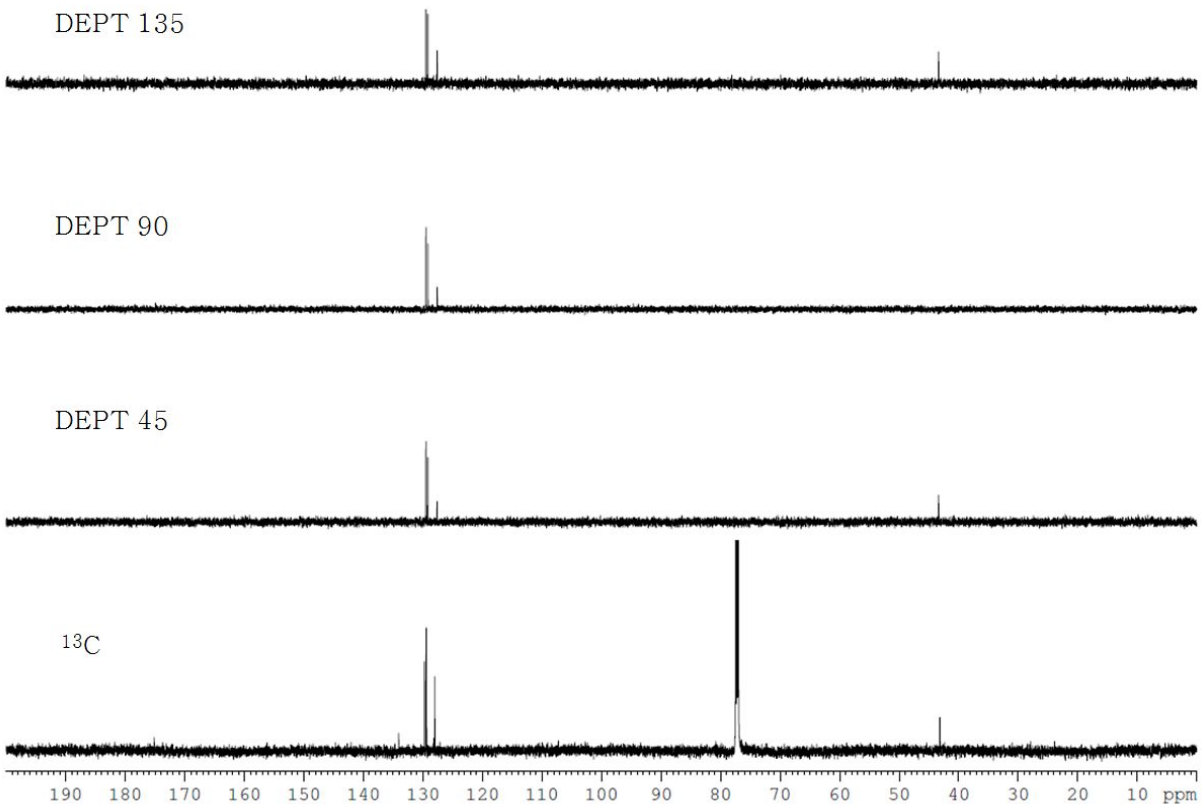
Supplementary Figure 4. ^{1}H NMR spectrum of compound 1



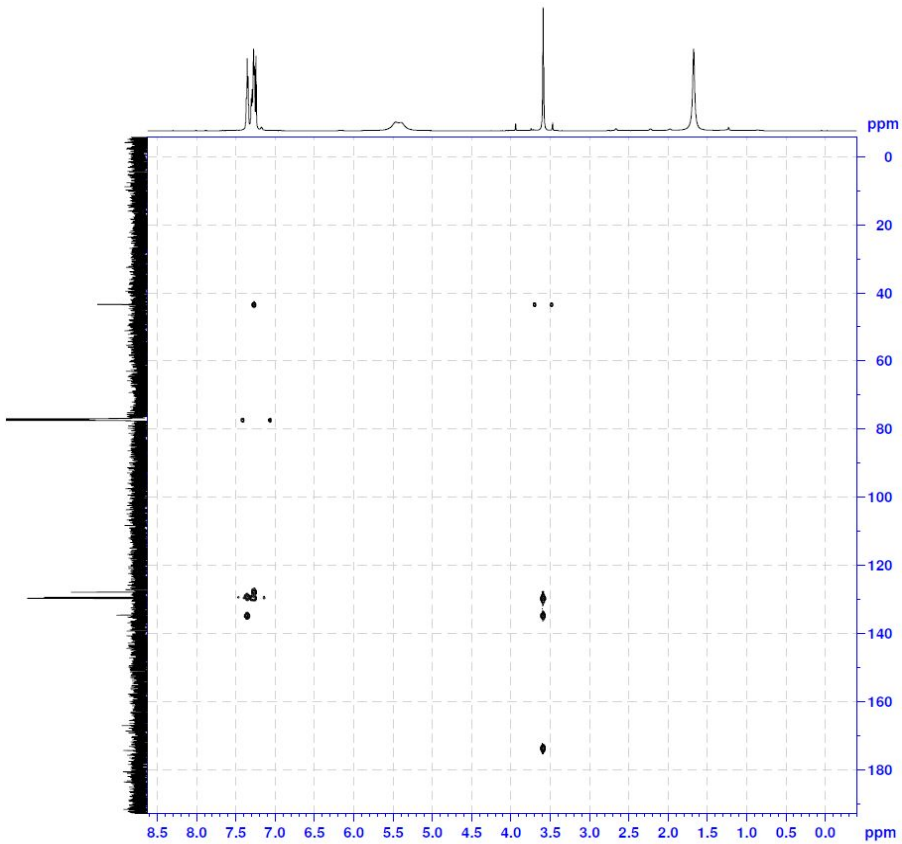
Supplementary Figure 5. ^{13}C NMR spectrum of compound **1**



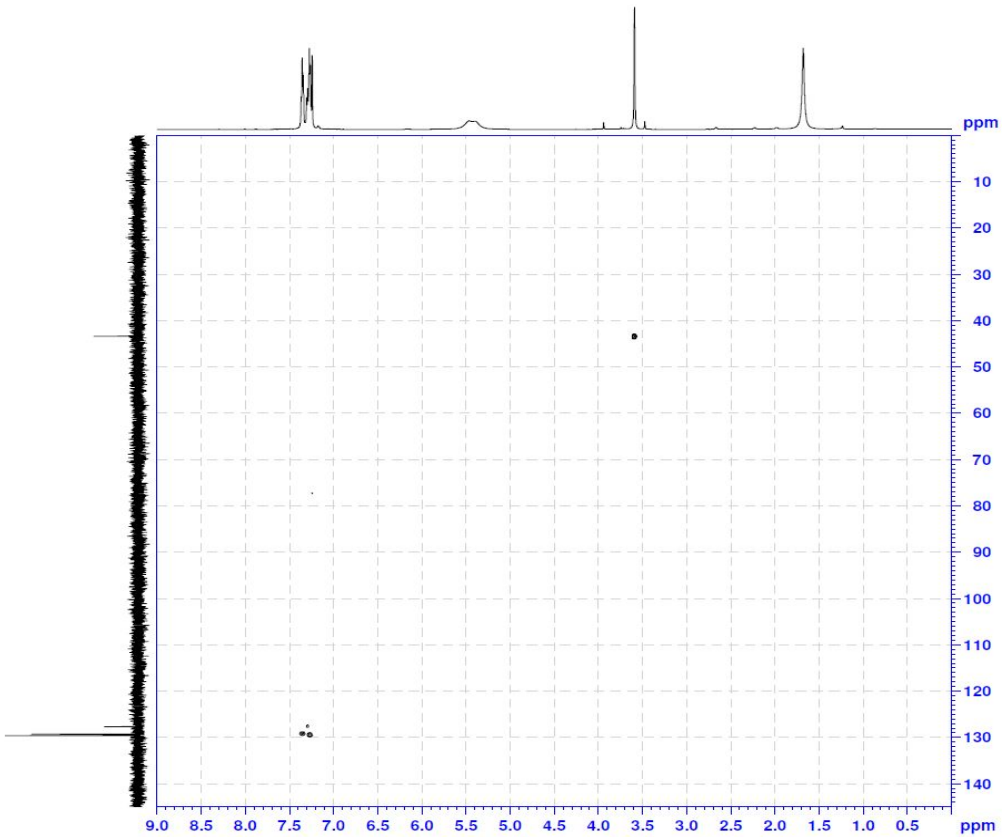
Supplementary Figure 6. $^{1}H-^{1}H$ COSY spectrum of compound **1**



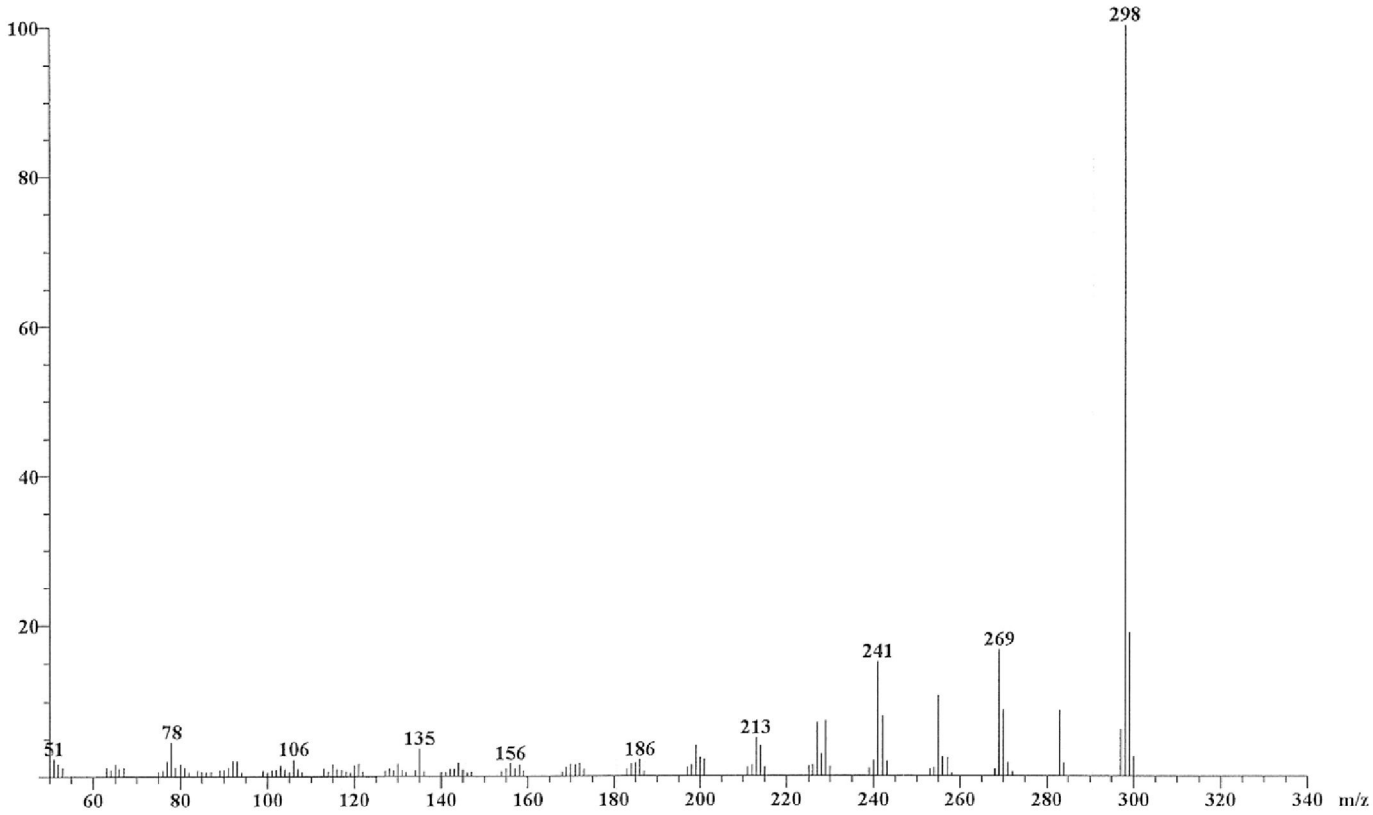
Supplementary Figure 7. DEPT spectrum of compound **1**



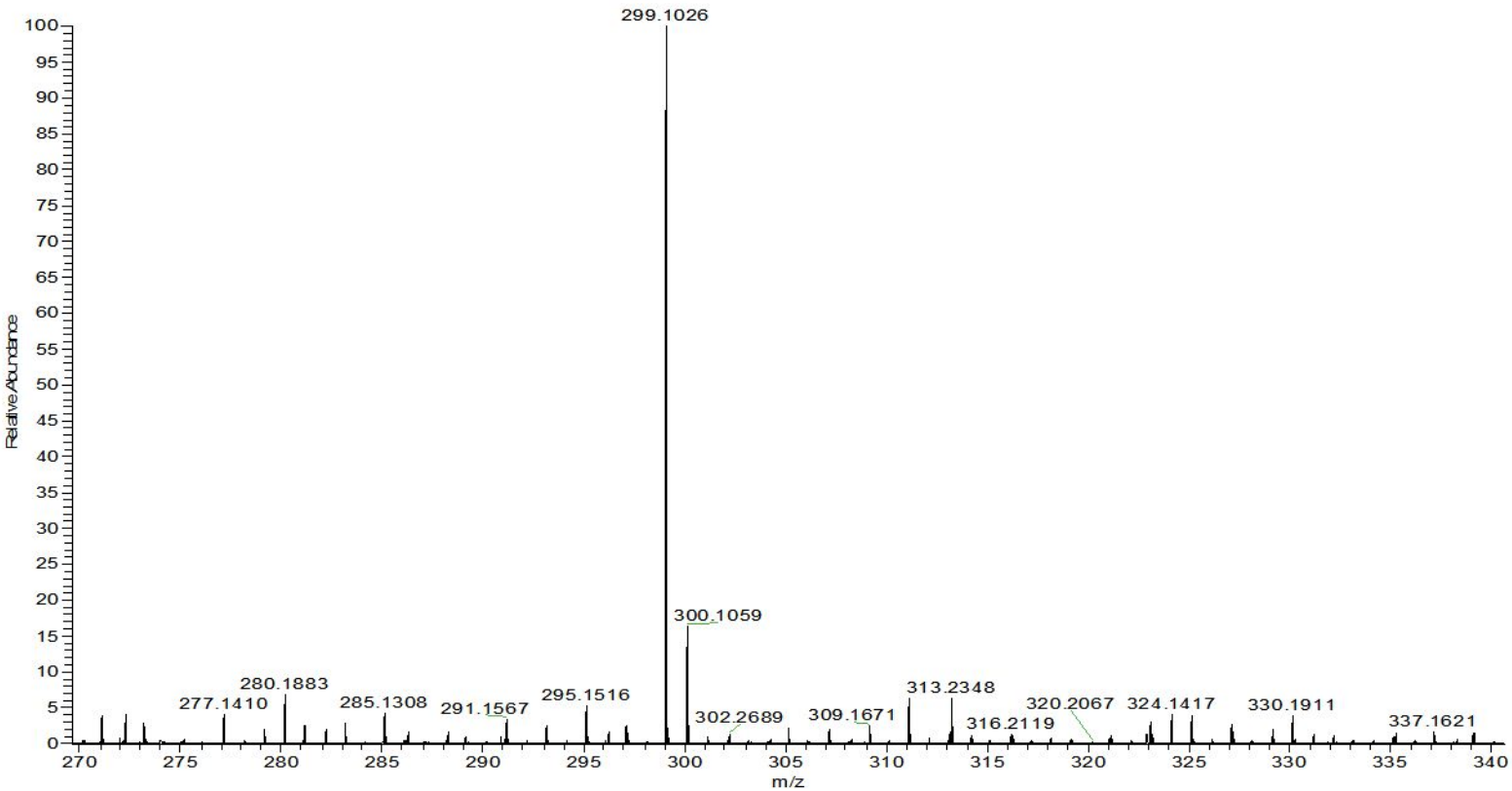
Supplementary Figure 8. HMBC spectrum of compound **1**



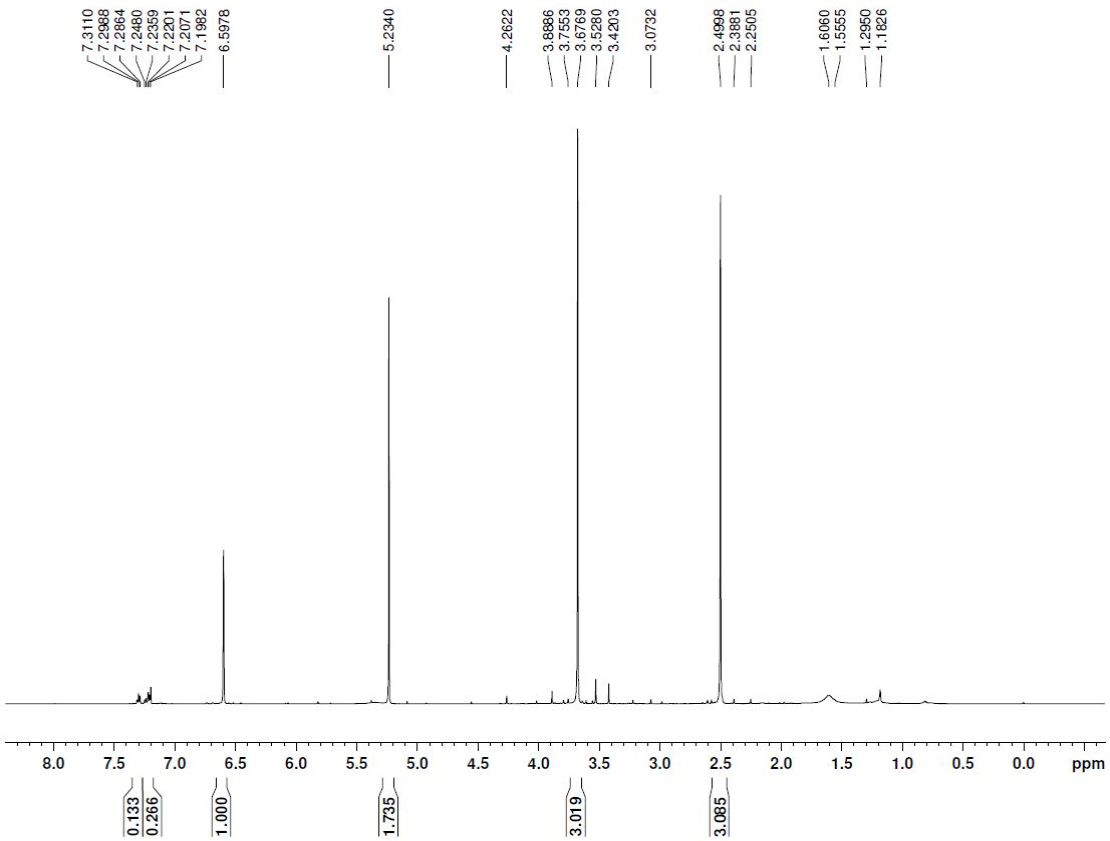
Supplementary Figure 9. HSQC spectrum of compound **1**



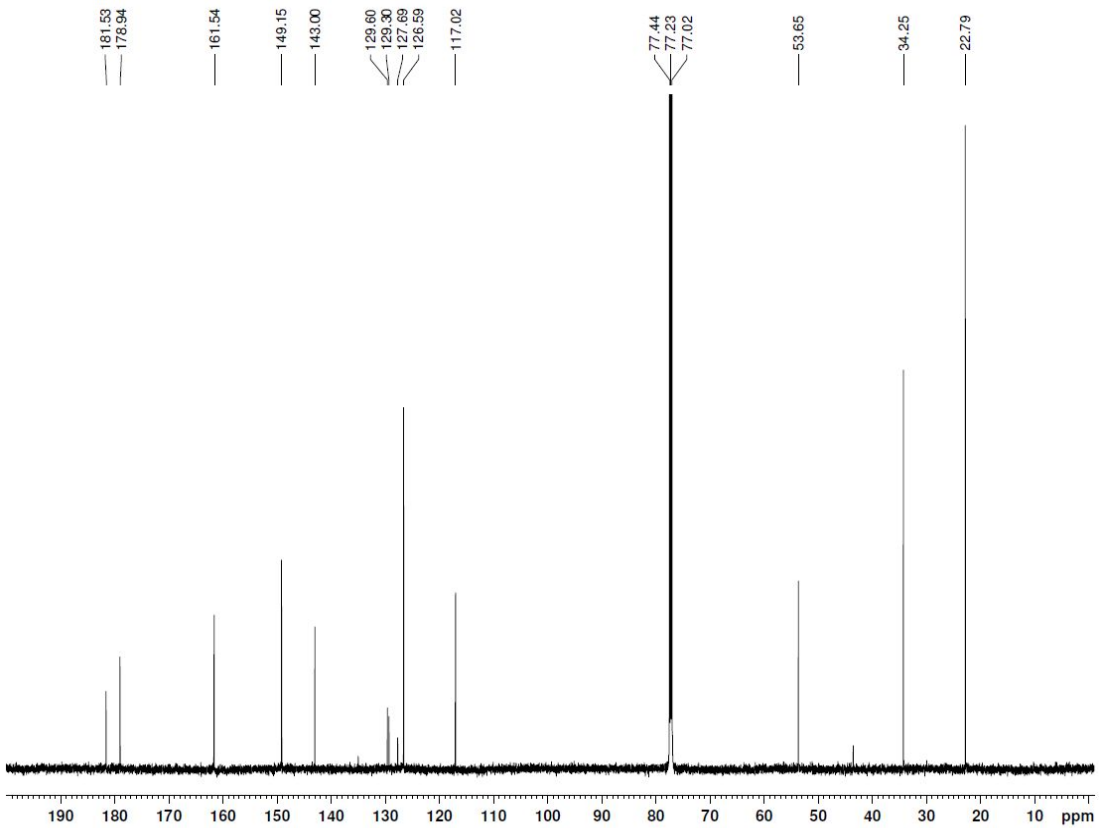
Supplementary Figure 10. EIMS spectrum of compound 2



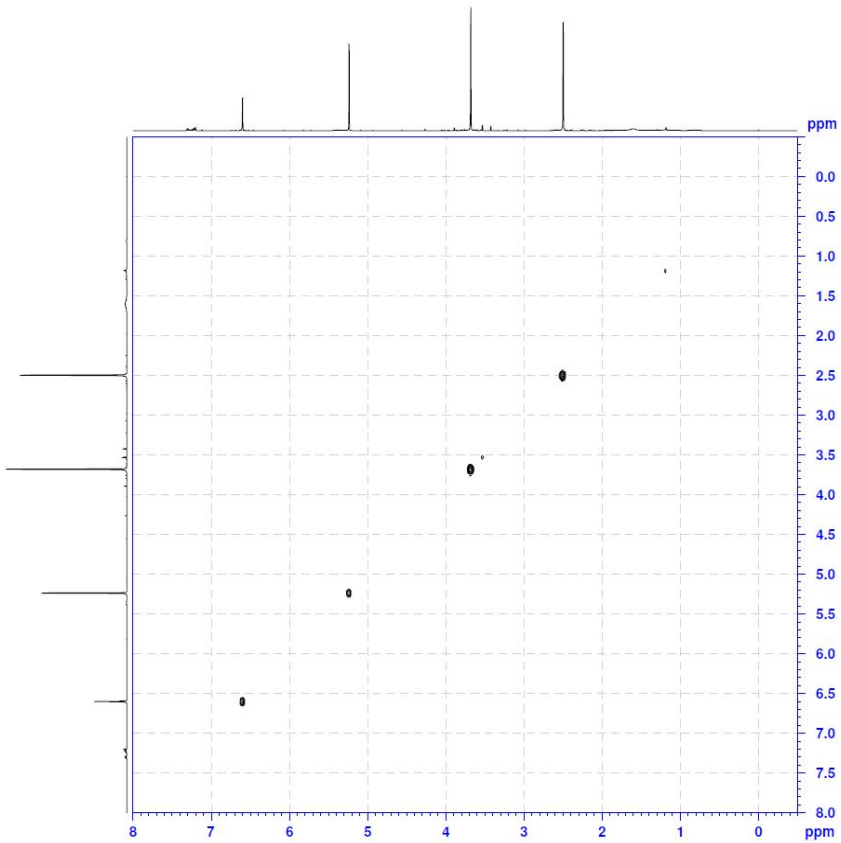
Supplementary Figure 11. HRESIMS spectrum of compound 2



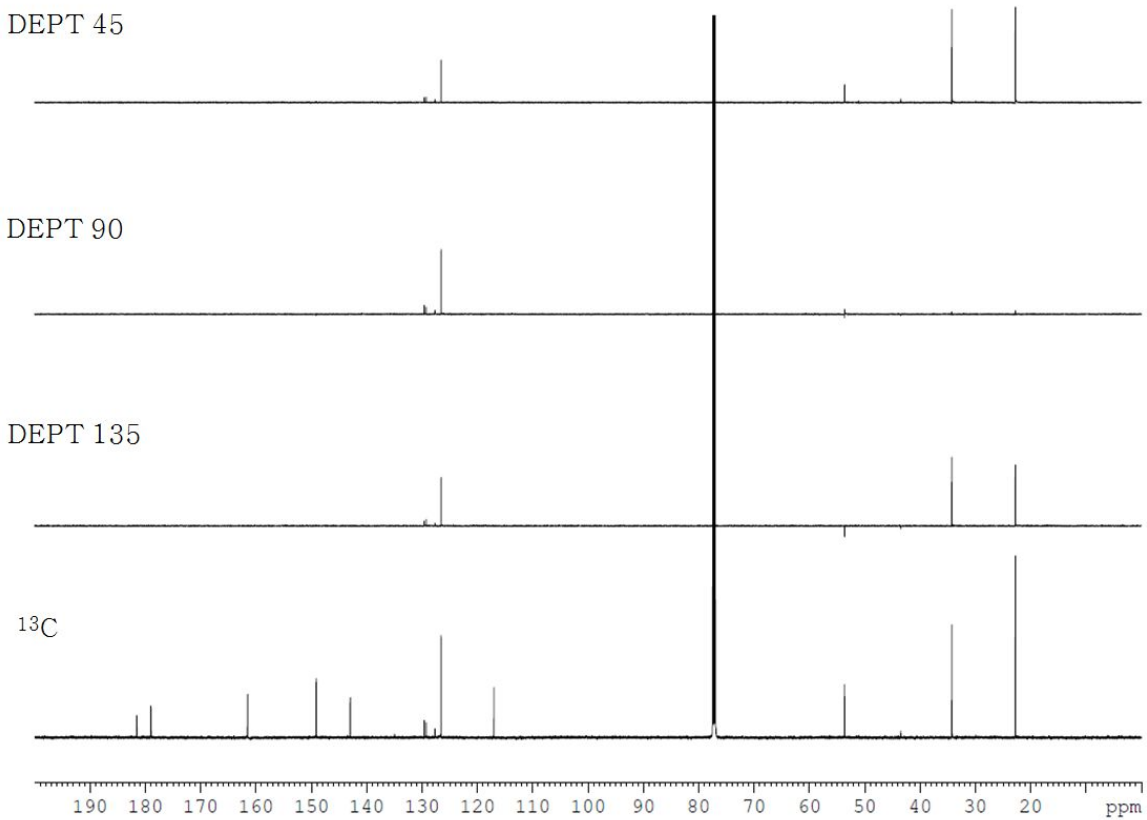
Supplementary Figure 12. ^{1}H NMR spectrum of compound 2



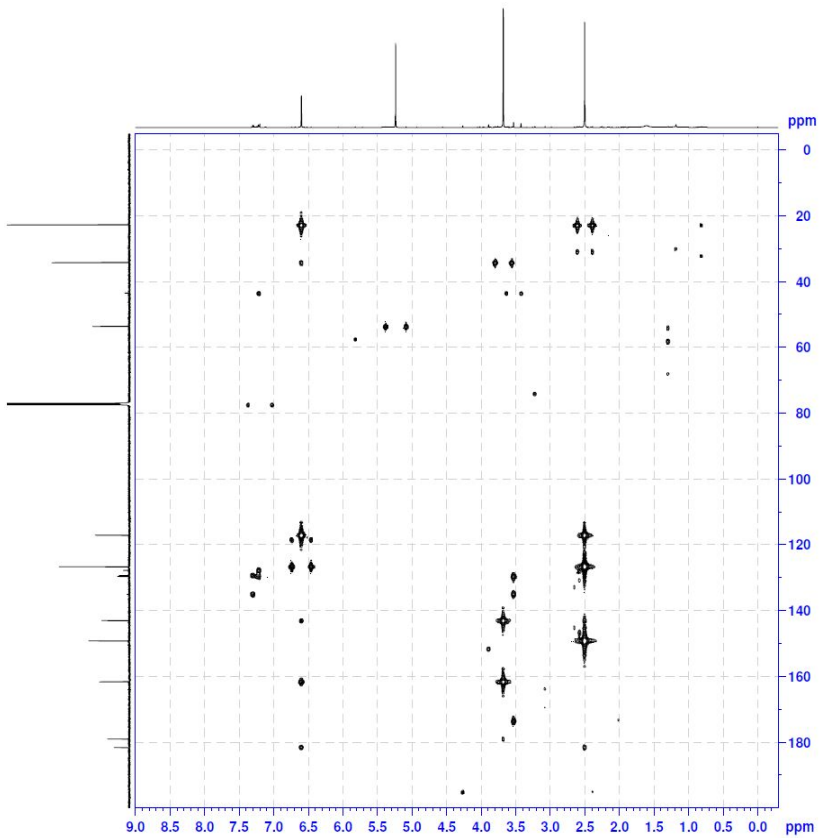
Supplementary Figure 13. ^{13}C NMR spectrum of compound 2



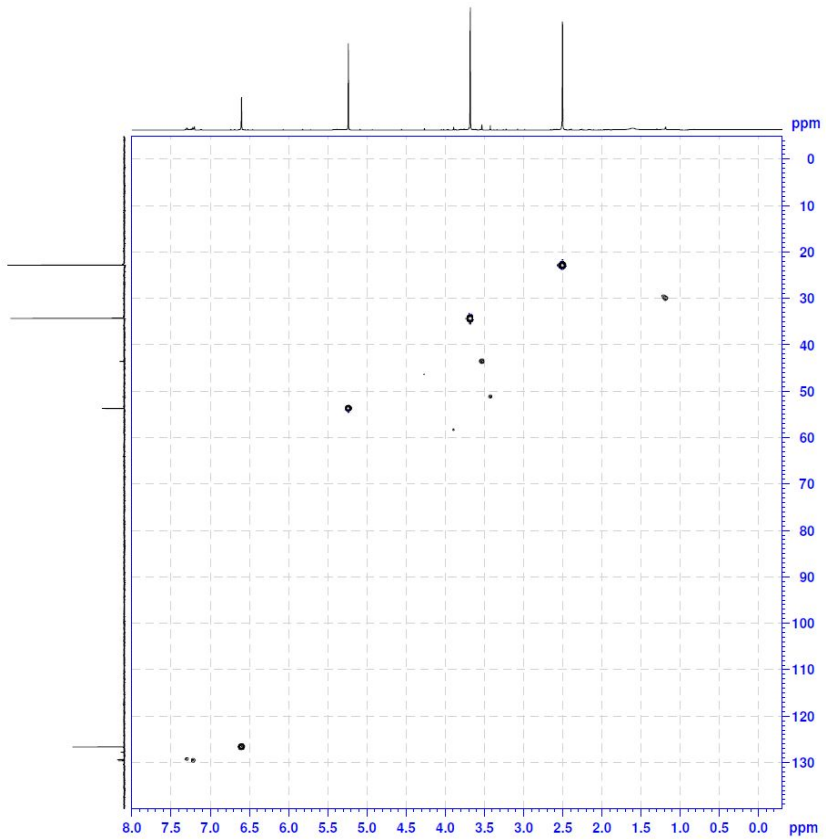
Supplementary Figure 14. $^{1}H-^{1}H$ COSY spectrum of compound 2



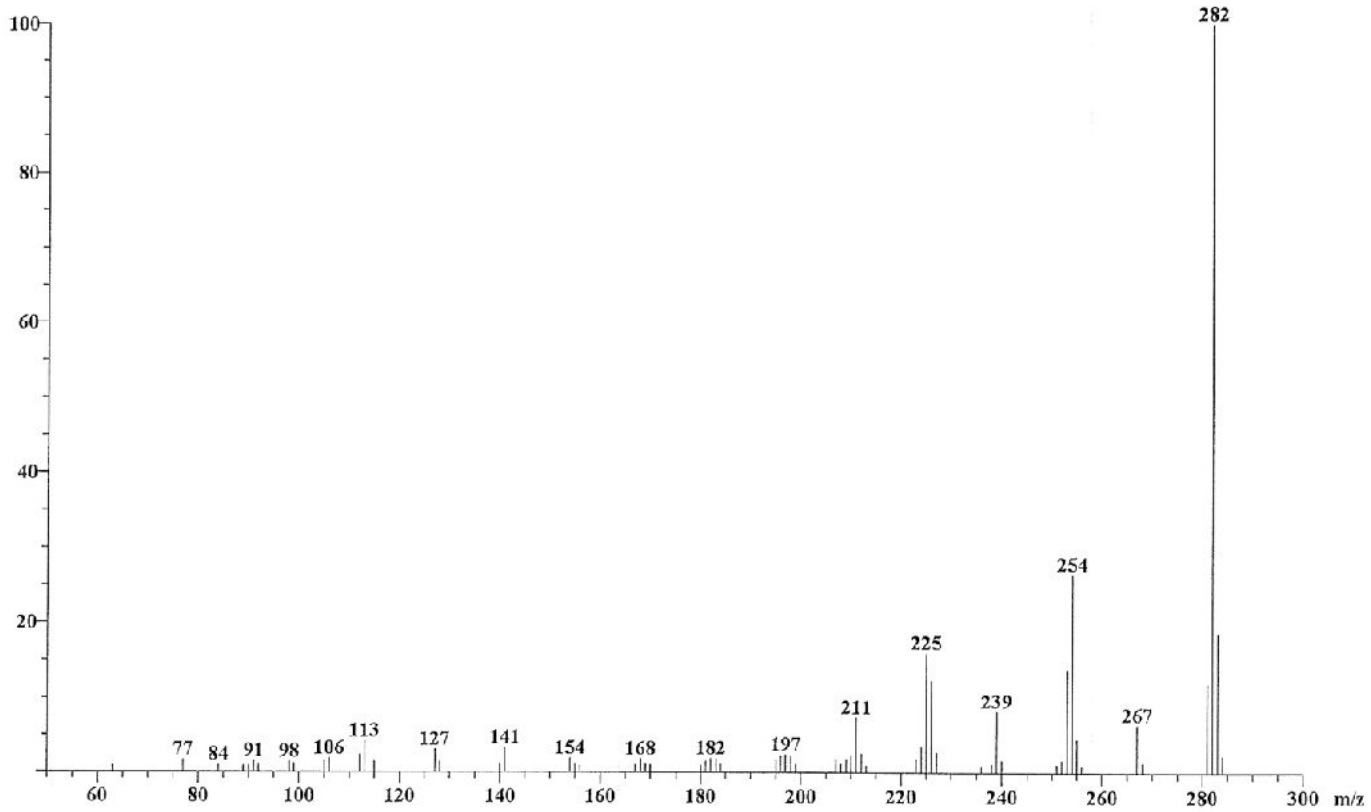
Supplementary Figure 15. DEPT spectrum of compound **2**



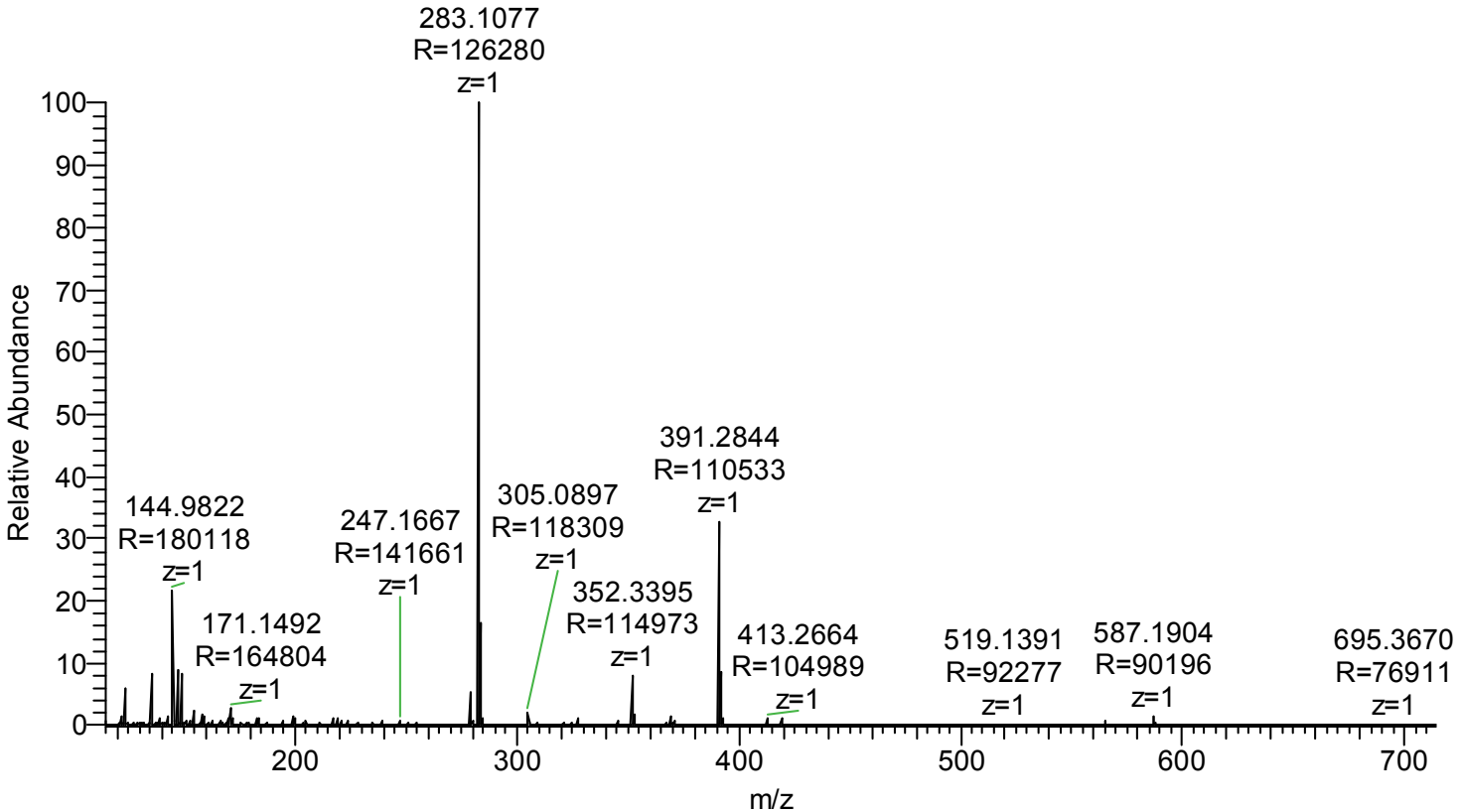
Supplementary Figure 16. HMBC spectrum of compound **2**



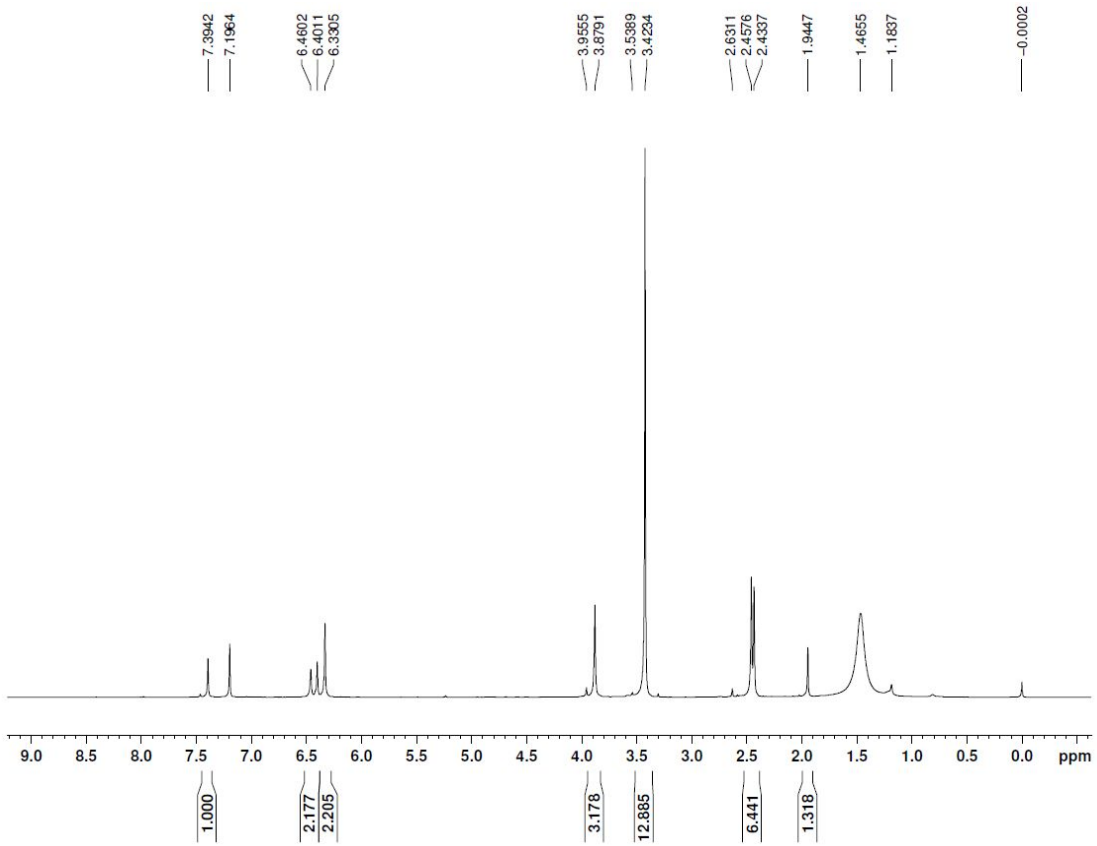
Supplementary Figure 17. HSQC spectrum of compound **2**



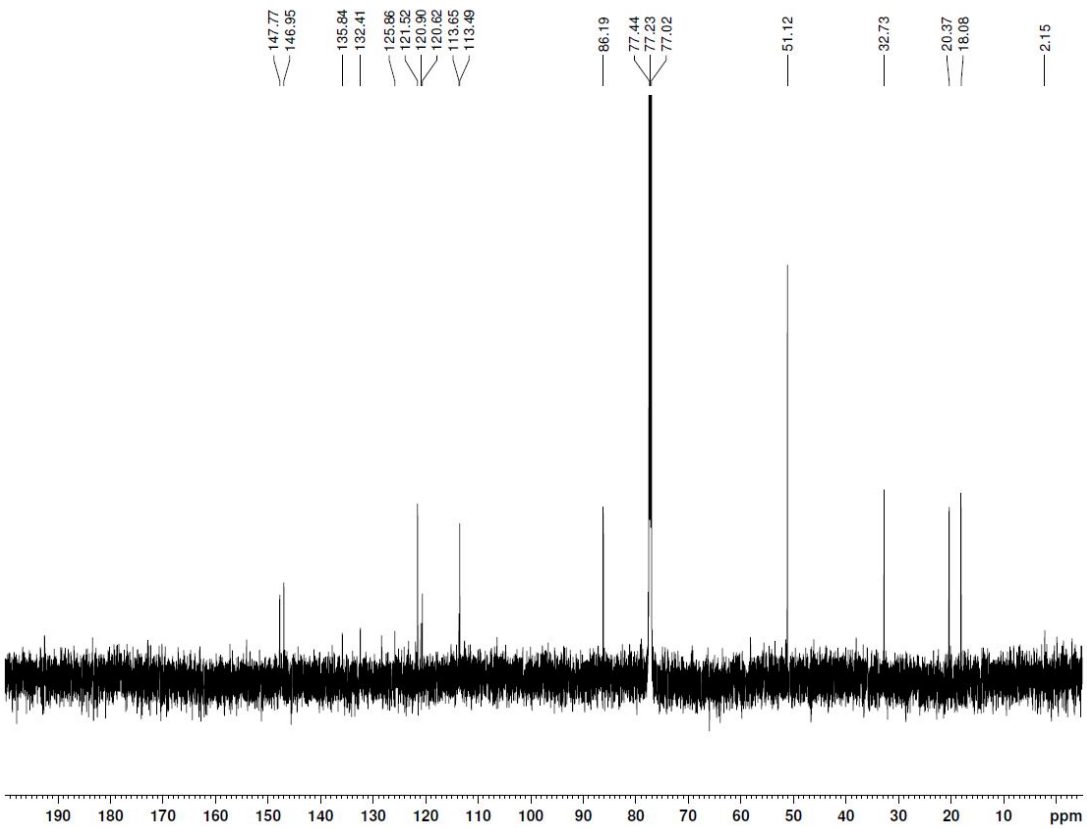
Supplementary Figure 18. EIMS spectrum of compound 3



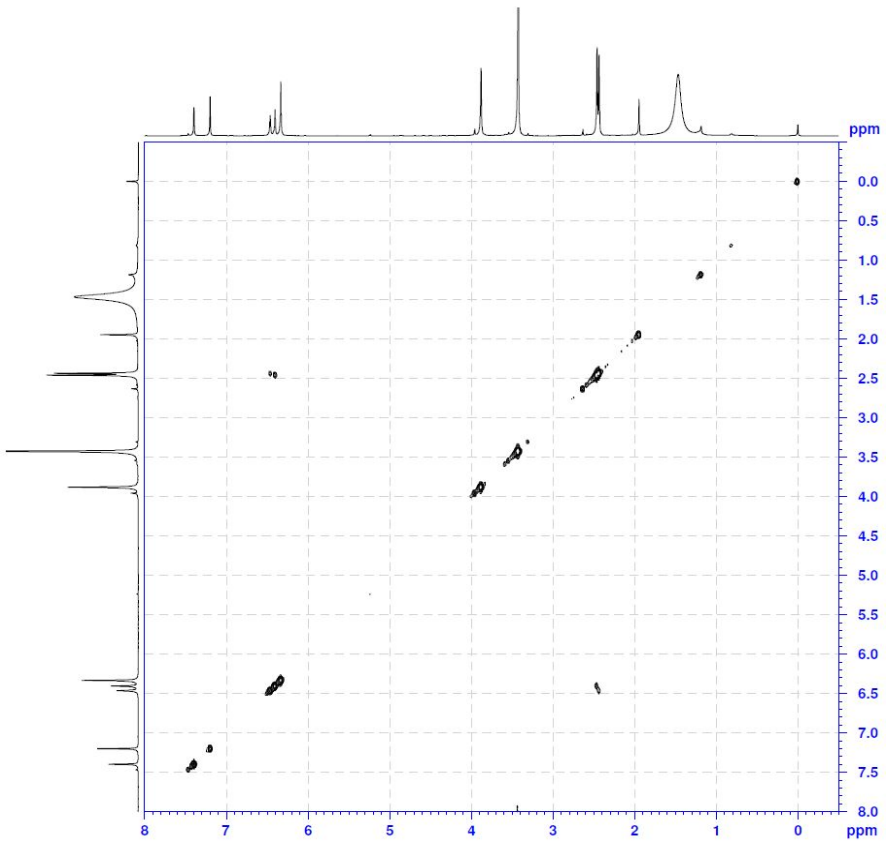
Supplementary Figure 19. HRESIMS spectrum of compound 3



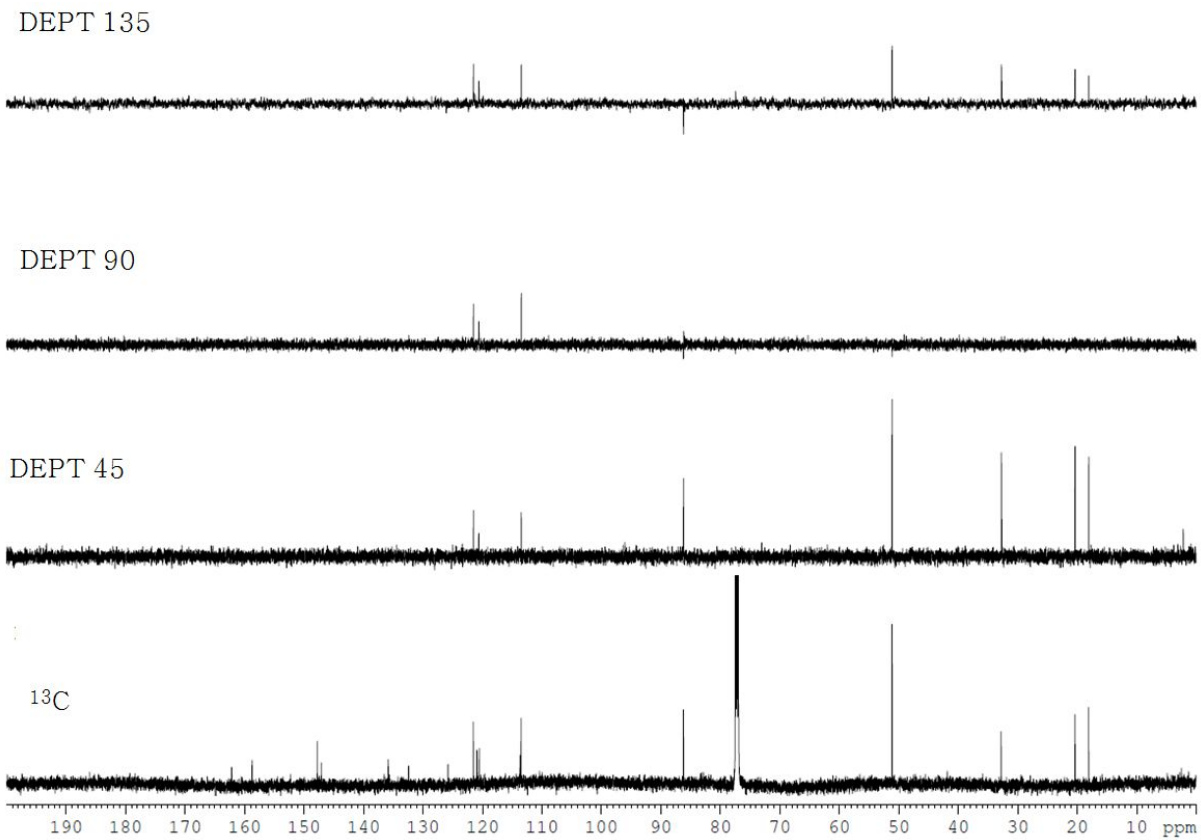
Supplementary Figure 20. ^{1}H NMR spectrum of compound 3



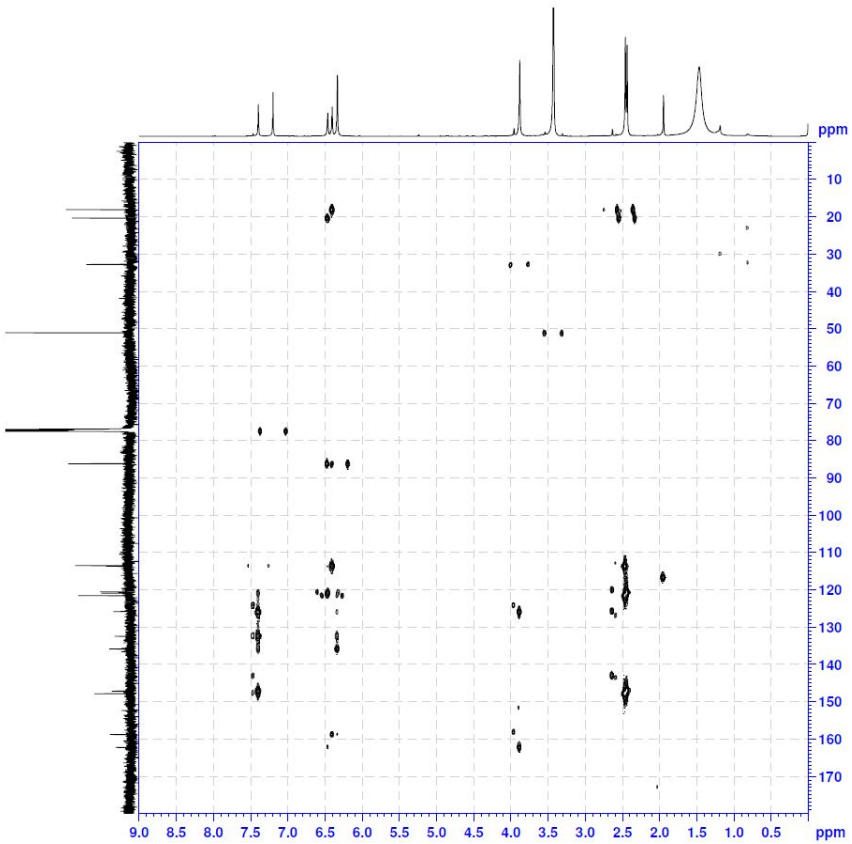
Supplementary Figure 21. ^{13}C NMR spectrum of compound 3



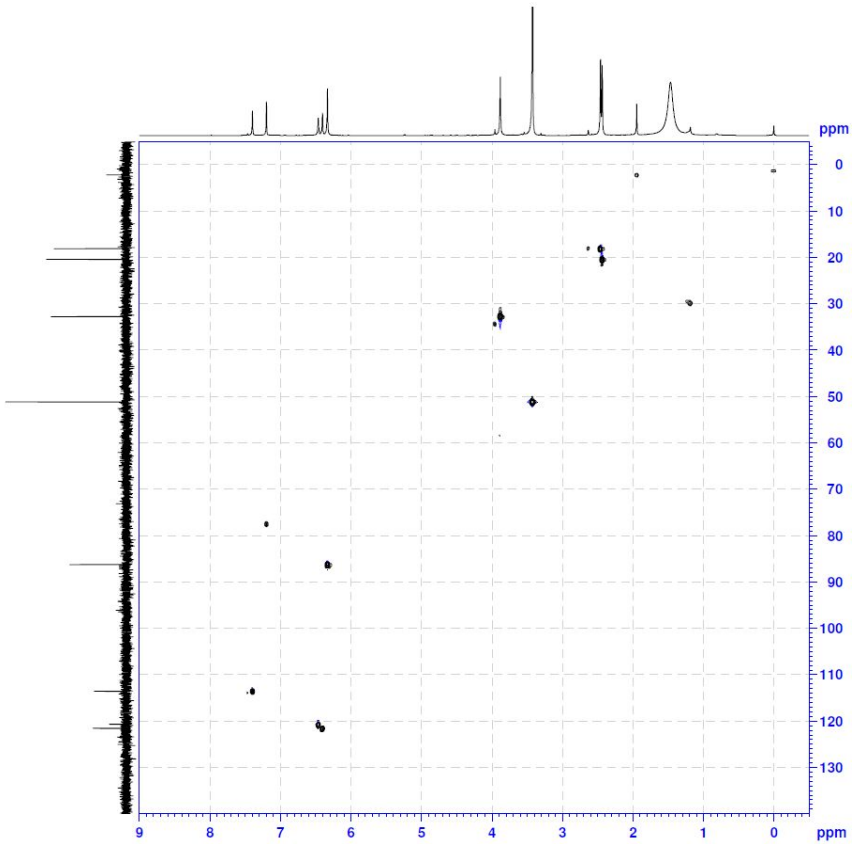
Supplementary Figure 22. $^{1}H-^{1}H$ COSY spectrum of compound **3**



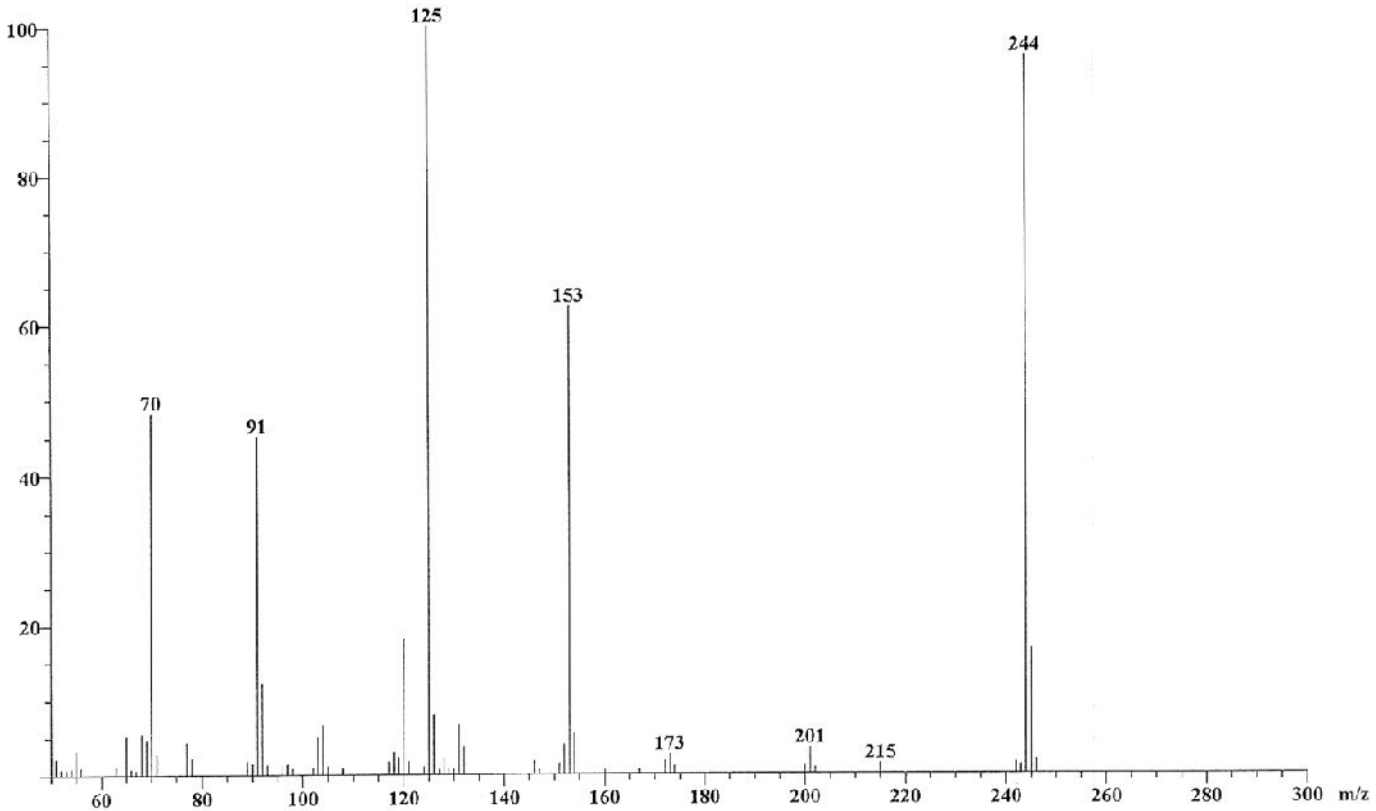
Supplementary Figure 23. DEPT spectrum of compound **3**



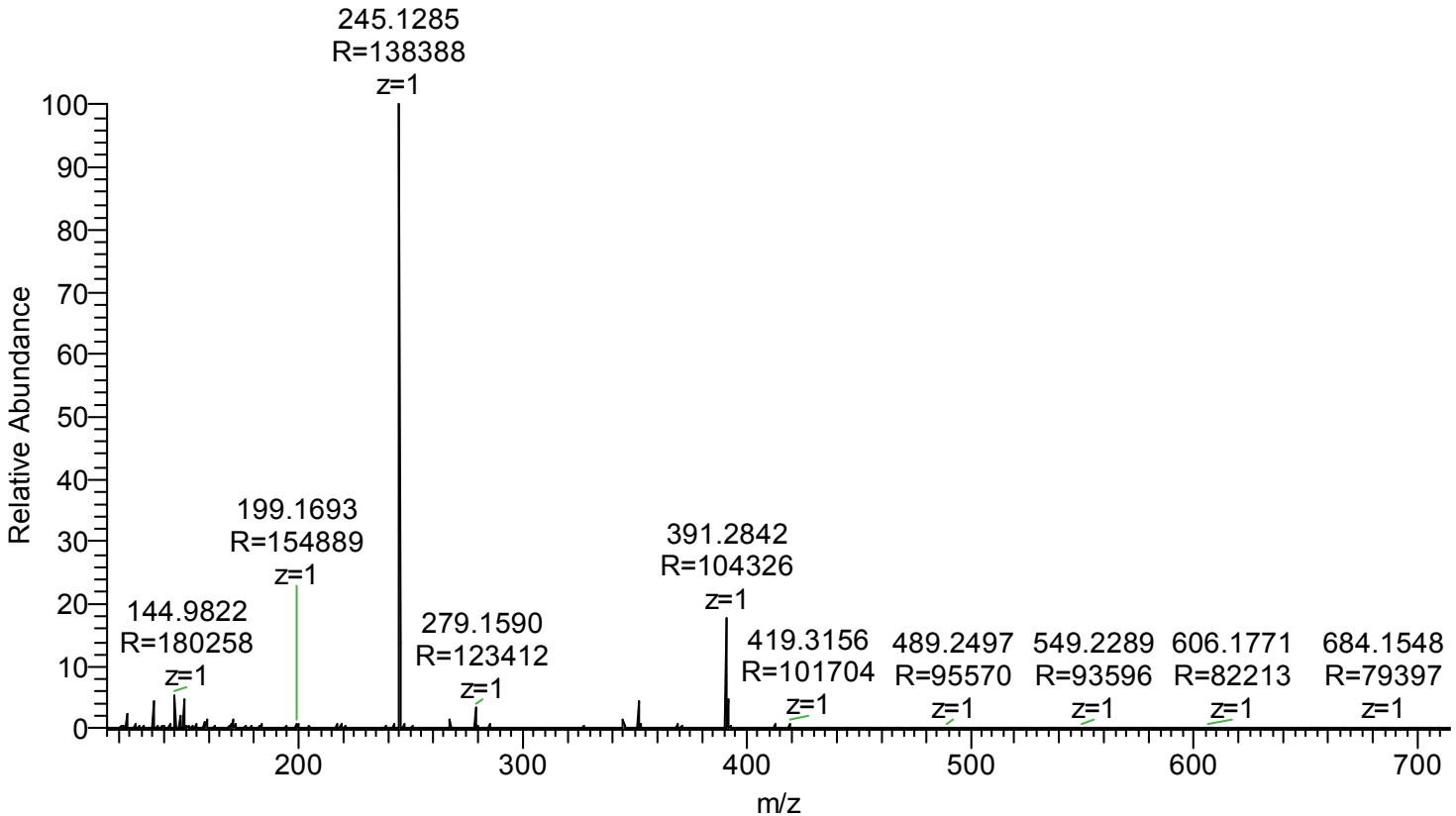
Supplementary Figure 24. HMBC spectrum of compound **3**



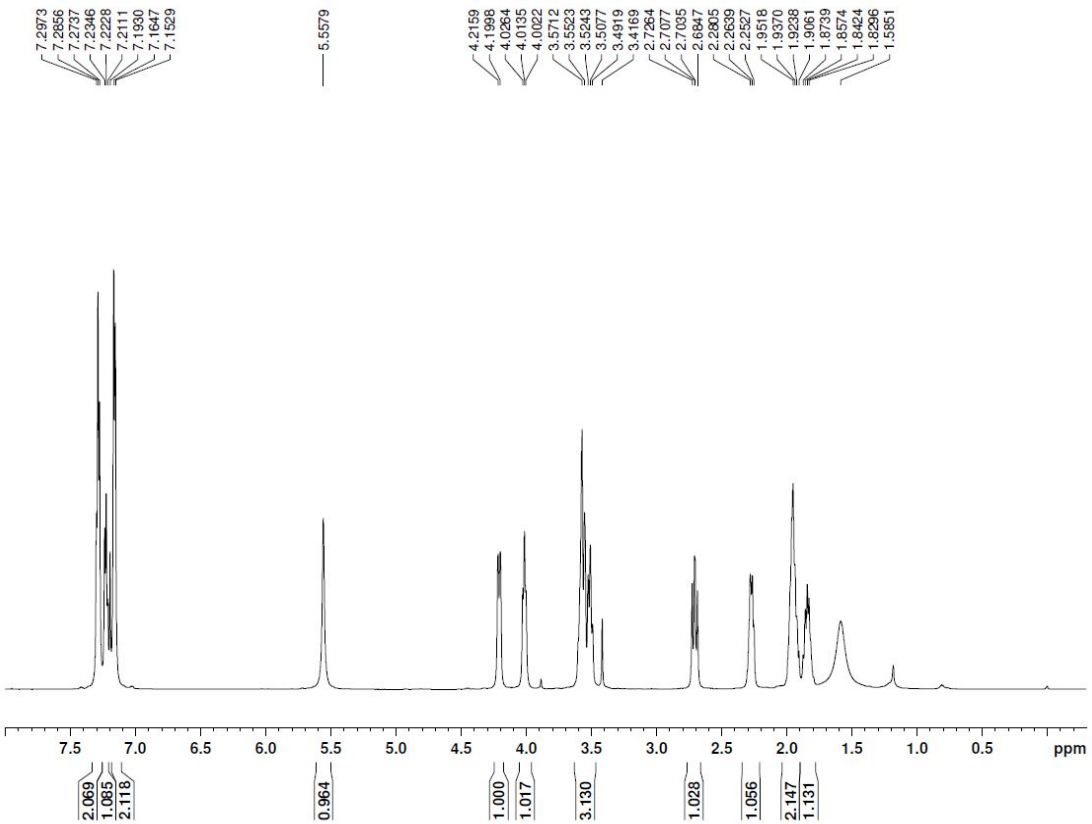
Supplementary Figure 25. HSQC spectrum of compound **3**



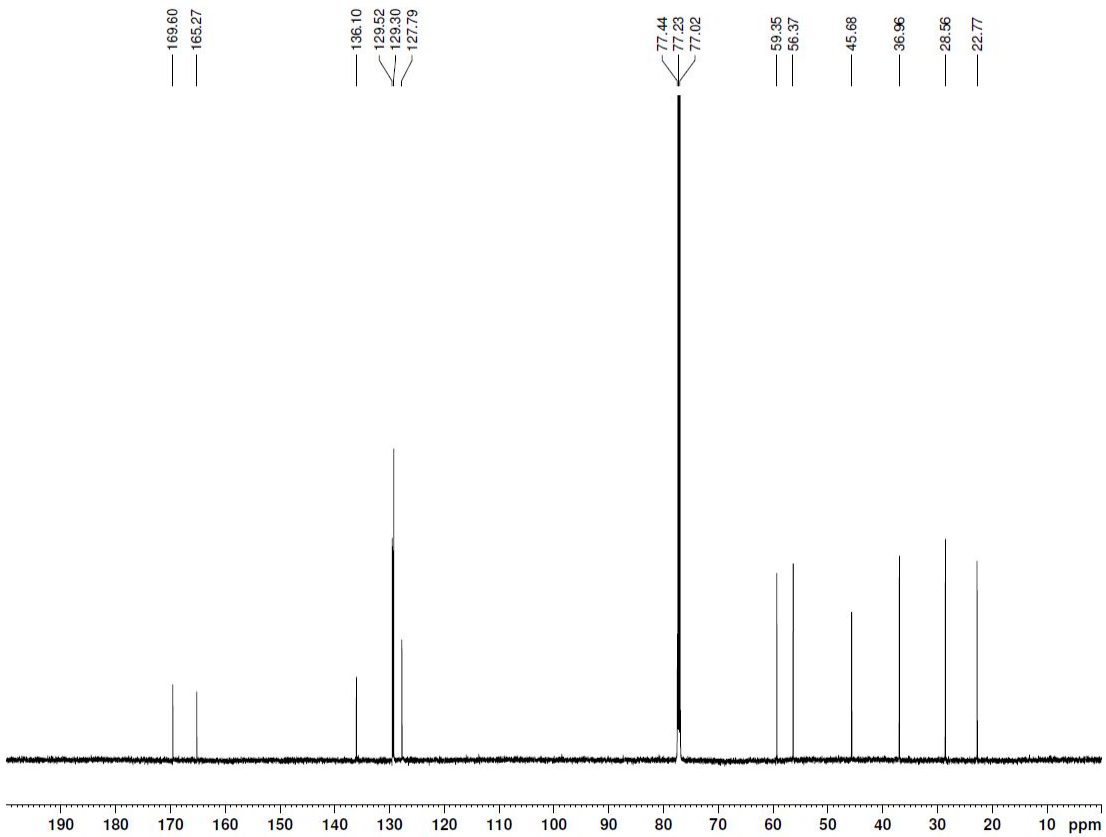
Supplementary Figure 26. EIMS spectrum of compound 4



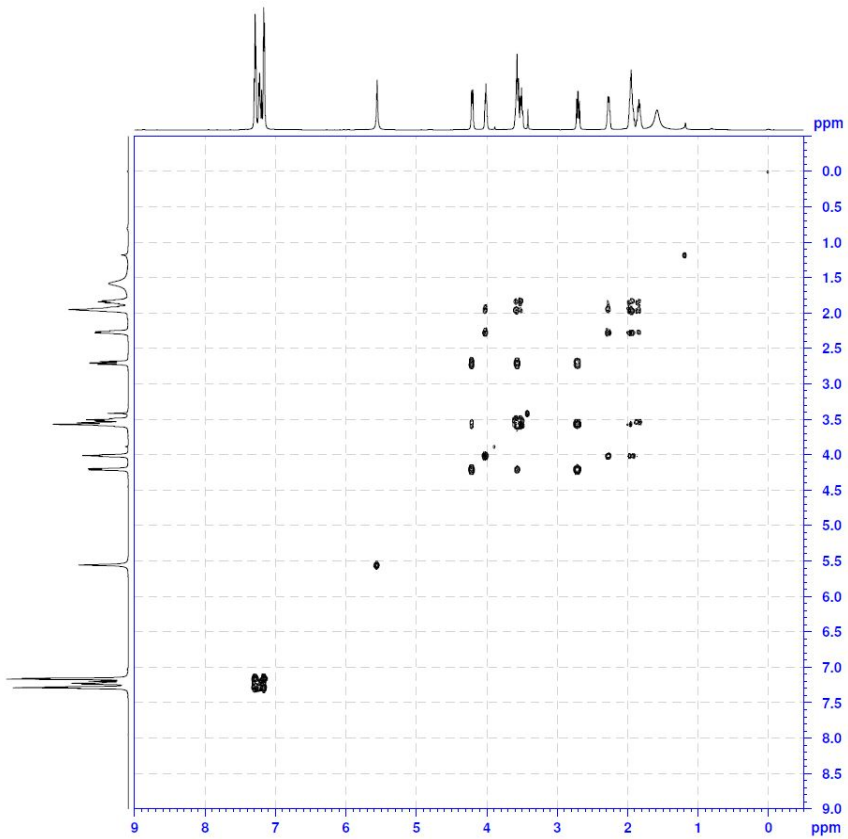
Supplementary Figure 27. HRESIMS spectrum of compound 4



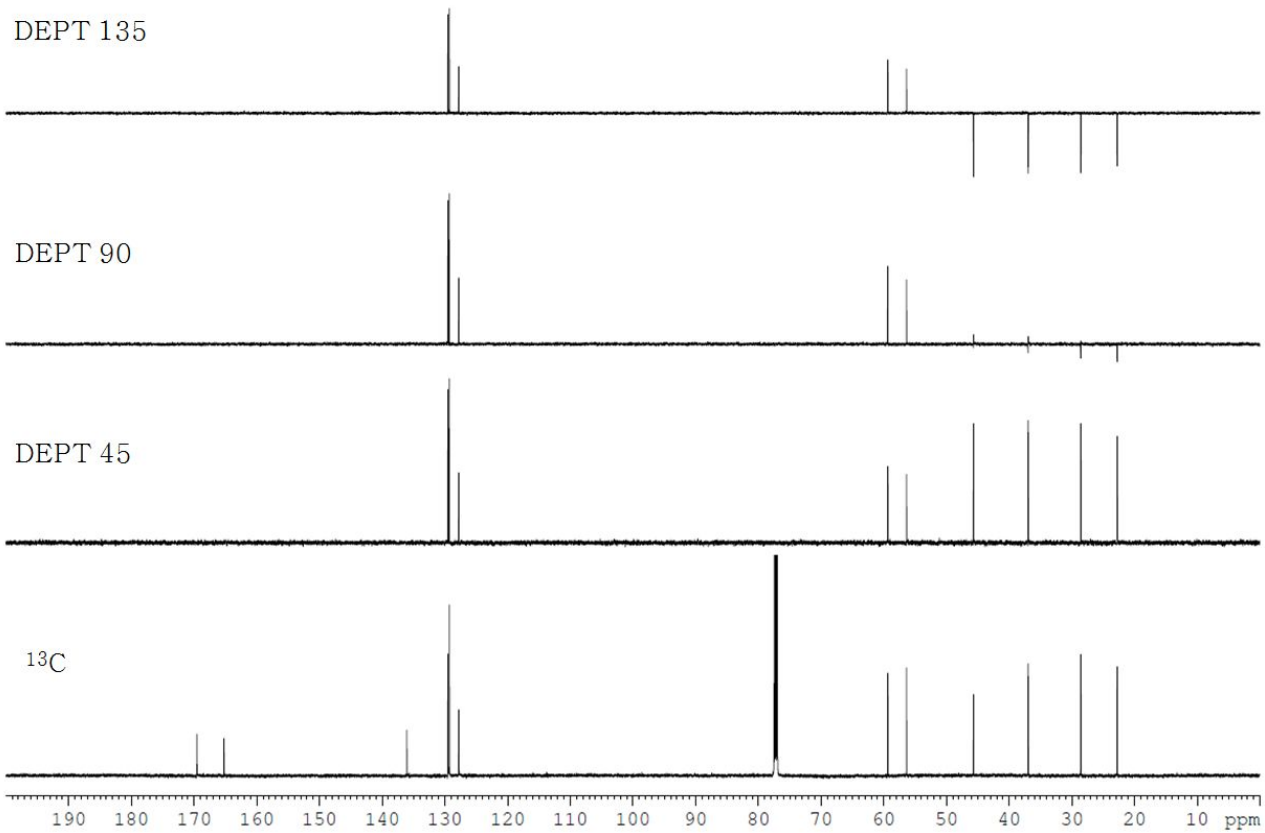
Supplementary Figure 28. ^{1}H NMR spectrum of compound **4**



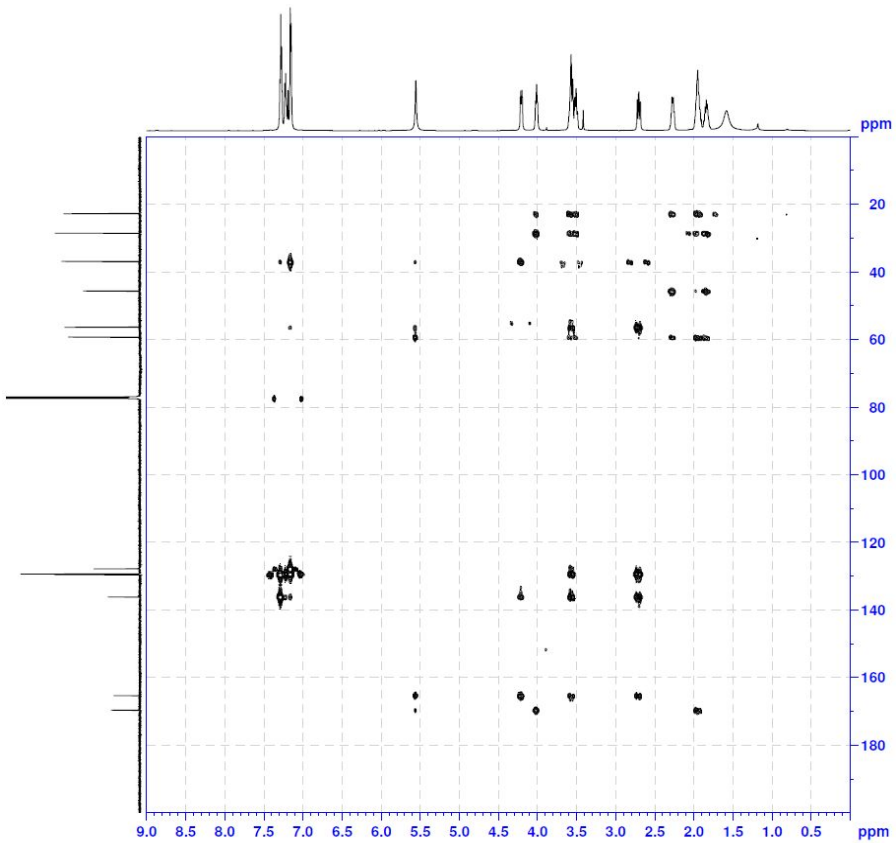
Supplementary Figure 29. ^{13}C NMR spectrum of compound 4



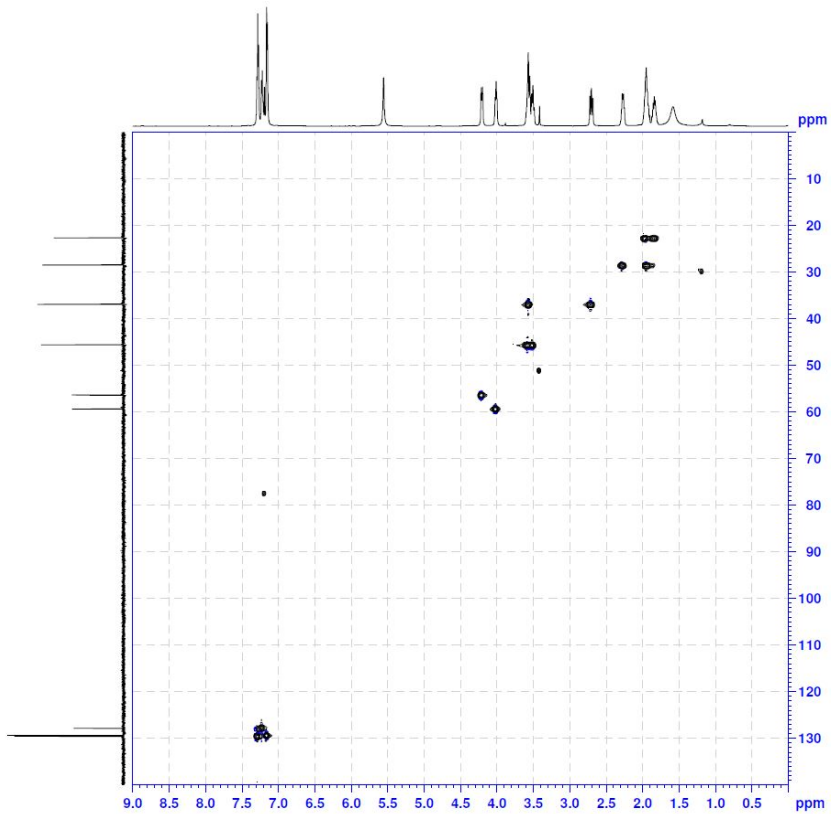
Supplementary Figure 30. $^{1}H-^{1}H$ COSY spectrum of compound **4**



Supplementary Figure 31. DEPT spectrum of compound **4**



Supplementary Figure 32. HMBC spectrum of compound **4**



Supplementary Figure 33. HSQC spectrum of compound **4**

Appendix

1518 base pairs were sequenced by 16S rRNA gene sequence of *Streptomyces* sp. MS101.

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TGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACAC
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ATCACCTCCTTA

초 록

해양방선균 *Streptomyces* sp. MS101 유래의 항균활성물질 연구

박 하 영

곰팡이, 세균, 기타 미생물들은 다른 미생물을 죽이거나 생장을 저해하기 위해 환경으로 항생물질을 분비한다. 그람 양성균에 속하는 *Streptomyces* 속은 현재까지 알려진 천연 유래 항생제의 대부분을 차지하고 있다.

본 연구에서는 해양방선균으로부터 생리활성물질 개발연구를 위하여 2011년 10월 마이크로네시아에서 채취한 해안 퇴적물로부터의 시료를 사용하였다. 분리된 해양미생물 중 항세균활성을 가지는 marine actinomycete MS101에 주목하여 16S rRNA 유전자 염기서열 분석을 수행 한 결과, *Streptomyces* 속에 속하는 새로운 균주를 획득한 것을 확인하고, 이를 *Streptomyces* sp. MS101로 명명하였다.

방선균의 배양 시 일반적으로 사용되는 6가지 배지를 선택하여 *Streptomyces* sp. MS101을 배양시켜 본 결과 GTYB배지에서 항세균활성이 높게 나오는 것을 확인하였다. 따라서 *Streptomyces*

sp. MS101을 2 주에 걸쳐 28℃에서 130 rpm으로 GTYB배지에 배양하여 총 98 L를 얻어냈다. 배양 후 균주의 배양 여액으로부터 메탄올 조추출물을 얻은 후, 이것으로부터 진공 실리카 플래쉬 크로마토그래피, 오픈 컬럼 크로마토그래피, HPLC를 통해 총 4 가지의 화합물을 순수분리하였다. 이들 물질에 대해 질량분석법, 핵자기공명법을 이용하여 구조분석을 실시하였다. 그 결과 화합물 **1**을 Benzenacetamide로, **2**는 Sch 538415로, **3**은 Deoxynybomycin로, **4**는 Cyclo(L-Pro-L-Phe)로 동정하였으며, 이들 화합물은 기지물질임을 확인하였다.

분리된 화합물에 대해 항미생물활성을 측정하였다. 화합물 **1, 4**는 항세균활성을 보이지 않았으나 화합물 **2, 3**은 0.20-6.25 μg/mL의 MIC 값의 범위로 그람 양성 및 음성 세균에 대하여 강한 항세균활성을 보이는 것을 확인하였다. 그러나 이 화합물들은 항진균활성을 보이지 않았다. 또한 순수 분리된 화합물이 항미생물 활성과 연관된 특정 단백질에 대한 저해제로서의 역할을 하는 지 확인하기 위하여 isocitrate lyase, sortase A 단백질에 대한 저해 활성 실험을 수행하였다. 그 결과 화합물 **1, 3, 4**는 isocitrate lyase에 대해 약한 저해 활성을 보였으며, 화합물 **2**는 *Streptococcus mutans*의 sortase A에 대하여 약한 저해활성을 가지는 것을 확인 하였다.

본 연구에서는 해양 퇴적물로부터 *Streptomyces* 속에 속하는 새로운 균주를 확보하였고, 분리 된 *Streptomyces* sp. MS101로부터 항암제와 항생제로 사용 할 수 있는 후보물질인 Sch 538415, Deoxynybomycin 을 생산 하는 것을 확인하였다.

주요어: 해양방선균, *Streptomyces,* 천연물, 항세균활성, 효소 저해

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