# Antiproliferative and Antiplasmodial Compounds from Selected *Streptomyces* species

L. Harinantenaina Rakotondraibe,[a*] Rado Rasolomampianina,[b] Hyun-Young Park,[a] Jie Li,[a] Carla Slebodnick,[c] Peggy J. Brodie,[c] Leah C. Blasiak,[d] Russell T. Hill,[d] Onja Andriambeloson,[b] Yongchun Shen,[f] Edward M. Suh,[f] Maria B. Cassera,[e] Felicite Rejo-Fienena,[b] and David G. I. Kingston[c]

[a]*Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus, Ohio 43210, United States*

[b]*Centre National de Recherches sur l'Environnement, B.P 1739, Antananarivo 101, Madagascar*

[c]*Department of Chemistry and the Virginia Tech Center for Drug Discovery, M/C 0212, Virginia Tech, Blacksburg, Virginia 24061, United States*

[d]*Institute of Marine and Environmental Technology, University of Maryland Center for Environmental Science Baltimore, Maryland, MD 21201, United States*

[e]*Department of Biochemistry and Virginia Tech Center for Drug Discovery, M/C 0308, Virginia Tech, Blacksburg, Virginia 24061, United States*

[f]*Eisai Research Institute, 4, Corporate Drive, Andover, Massachusetts 01810*

*Corresponding authors. L. Harinantenaina Rakotondraibe: Tel: +1 614 292 4733 ; fax: +1 614 292 4733.
*E-mail address:* rakotondraibe.1@osu.edu

## Experimental Section

### 1. General Experimental Procedures.

Optical rotations were recorded on a Perkin-Elmer 343 automatic polarimeter. IR spectra were obtained on a Thermo Scientific Nicolet 6700 FT-IR spectrometer. NMR spectra were recorded in $CD_3OD$ on Bruker Avance 400 or 500 spectrometers. The chemical shifts are given in δ (ppm), and coupling constants (*J*) are reported in Hz. Mass spectra were obtained on an Agilent 6220 LC-TOF-MS in the positive ion mode. Preparative HPLC was performed using: Shimadzu LC-10AT pumps coupled with a semi-preparative Varian Dynamax C-18 column (5 μm, 250x10 mm), a Shimadzu SPD M10A diode array detector (DAD) and a SCL-10A system controller (flow rate 2 mL/min) and Hitachi Primaide equipped with Primaide 1430 diode array detector, Primaide 1210 autosampler, Primaide 1110 pump and Advantage Lancer C18 column (5 μm, 250x4.6 mm), flow rate 1 mL/min. Open column chromatography was performed using silica gel (230-400 mesh, Silicycle Co. USA).

### 1.1. Isolation, Identification and Cultivation of *Streptomyces* strains.

#### 1.1.1. Soil sampling

Soil samples were collected from five sites located in different regions of Madagascar: Ankafobe Forest and Ibity Massif in the central region, Pointe à Larrée and Ambalabe Forests in the central east and Orangéa Forest in the northern region). Nine soil samples were taken at 2 cm or 3 cm of depth for each site, put into sterile plastic bags and were transported to the laboratory. Soil samples were dried for at least 7 days in a desiccator prior to bacterial isolation.

### 1.1.2. Isolation

Three selective isolation methods were used for bacterial isolation: antibiotic selection method using AS1 agar medium (yeast extract 1g, L-alanine 0.2 g, L-arginine 0.2 g, L-asparagine 0.5 g, soluble starch 5 g, NaCl 2.5 g, $Na_2SO_4$ 10 g, agar 15 g, sterile distilled water 1000 mL supplemented with trimethoprim (20 µg/ml), nalidixic acid (50 µg/ml) and cycloheximide (50 µg/ml); heat treatment method by drying soil samples at 100°C for 1 h and Grayson aerosolized sampler (GAS) method by putting reversed AS1 plate, 1 cm from the top of the bottle containing 1 g of soil sample previously shaken and settled for 1 h. A bulb air pump allowed the spores disseminated in the bottle to be inoculated onto AS1 plates. A serial dilution technique was applied for the two first methods. Plates were incubated at 30 °C for 7 to 30 days.

### 1.1.3. Purification, identification and conservation

After incubation, colonies showing *Streptomyces* characteristics (rough, dry and powdery colonies embedded in the agar) were selected and purified onto a new AS1 plate. Pure colonies were preserved in glycerol 20% (v/v) and stored at –80°C. The *Actinomycetes* strain MMS-030-F-01-A-XT (*S.1*) was isolated from the Ankafobe Forest by antibiotic selection method while the strains MMS-0088-N-01-A-XT (*S.2*) and MMS-0085-C-XT (*S.3*) were both isolated from the Ibity Massif using antibiotic selection and GAS methods, respectively.

The bacterial strain *Streptomyces sp.* (*S.4*) XM6014 was isolated from the tissue of a sponge *X. muta* collected at a depth of 22 m in August, 2005 at Conch Reef, Key Largo, Florida, USA (24˚56.829N, 80˚27.409W) by scuba diving (sponge specimen number XM65).

The bacterial strain *Streptomyces sp.* (*S.4*) XM6014 was isolated from the tissue of the sponge *X. muta* as described by Montalvo and coworkers.[1] Briefly, the sponge was washed with filter-sterilized seawater, a 1-$cm^3$ piece was excised, ground in sterile artificial seawater and a dilution series was prepared. The diluted suspension (100 μL) was spread on an ISP2 agar plate. The ISP2 agar medium contained 4 g of yeast extract [AMRESCO, J-850), 10 g of malt extract (SIGMA), 4 g of dextrose (Mallincrodt Chemical works, St Louis and New York), and 20 g of agar (DIFCO) per 1 L of sterile distilled $H_2O$. The plate was incubated for 1 week at room temperature. The four strains were subjected to phylogenetic analyses based on the comparison of 16S rRNA sequences by BLAST searching against the National Center for Biotechnology Information (NCBI) database, and all four strains demonstrated 100% identity with *Streptomyces* sp. The cultured *Streptomyces* strains MMS-030-F-01-A-XT and MMS-0088-N-01-A-XT are deposited at the Centre National de Recherches sur l'Environnement, Antananarivo, Madagascar. The 16S rRNA gene sequences for strains MMS-030-F-01-A-XT (*S.1*), MMS-0088-N-01-A-XT (*S.2*) and MMS-0085-C-XT (*S.3*) are available in the NCBI database under the accession numbers KR534215, KR336605 and KR336606, respectively. The cultured *Streptomyces* strain XM6014 is deposited at the Institute of Marine and Environmental Technology, University of Maryland Center for Environmental Science. The 16S rRNA gene sequence is available in the NCBI database under the accession number KP280056.

The *Streptomyces* strain XM6014 was cultured in ISP2 liquid medium (25 mL) at 25 °C for 7 days. Stocks of the isolated bacterial strain were stored at −80 °C in culture medium containing 20% glycerol (v/v). For the purpose of large-scale fermentation, two pieces of 0.25 $cm^2$ of ISP2 agar containing *S.4*

culture was first seeded in a 500 mL flask containing 250 mL of ISP2 broth. This seed culture was incubated at room temperature on a shaker (MAXQ 3000 Shaker, Thermoscientific) at 180 rpm for 7 days. Five milliliters of the seed broth was added in each 10 flasks (500 mL), each containing 250 mL ISP2 broth. The new cultures were incubated at room temperature for 15 days. At the end of the fermentation period, the broth was extracted with 6 L of EtOAc and the solvent was removed under vacuum to yield 386 mg of extract.

### 1.1.4. Antiproliferative Bioassays

Antiproliferative activity against the drug-sensitive A2780 human ovarian cancer cell line was performed at Virginia Polytechnic Institute and State University as previously described,[2] while the protocol for A2058 melanoma, and H522-T1 nonsmall cancer lung is described in our previous literature.[2]

### 1.1.5. Antiplasmodial Bioassays

The effect of each fraction and pure compound on parasite growth of the Dd2 strain was measured in a 72 h growth assay in the presence of drug as described previously with minor modifications.[3-5] Briefly, ring stage parasite cultures (200 μL per well, with 1% hematocrit and 1% parasitemia) were then grown for 72 h in the presence of increasing concentrations of the drug in a 5.05% $CO_2$, 4.93% $O_2$, and 90.2% $N_2$ gas mixture at 37 °C. After 72 h in culture, parasite viability was determined by DNA quantitation using SYBR Green I (50 μL of SYBR Green I in lysis buffer at 0.4 μL of SYBR Green I/mL of lysis buffer). The half-maximum inhibitory concentration ($IC_{50}$) calculation was performed with GraFit

software using a nonlinear regression curve fitting. $IC_{50}$ values are the average of three independent determinations with each determination in duplicate and are expressed ± SEM.

### 1.1.6. Extraction and dereplication

#### 1.1.6.1. Extraction

The ethanol extract of the fermented microbial samples (*S.1*-*S.4*) were evaporated and shipped to Virginia Tech for antiproliferative and antimalarial bioassay. The extracts obtains from *S.1* and *S.2* showed antiproliferative activity with $IC_{50}$ values of 2 μg/mL and 3.5 μg/mL, respectively, while *S.3* and *S.4* displayed antimalarial activity ($2.5<IC_{50}<5$ μg/mL and $IC_{50}$~10 μg/mL, respectively). Liquid-liquid partition of the extracts using ethyl acetate (EA) and water afforded EA fractions with improved activity.

#### 1.1.6.2. Dereplication

The $^{1}$H NMR spectrum of the bioactive microbial EA fraction obtained from *S.1* was recorded in order to screen the known possible metabolites responsible of the activity. The presence of ionophores was evidenced by the presence of characteristic $^{1}$H-NMR signals at: δ 0.7 ppm, t, *J* = 7.1 Hz (secondary methyl group), and δ 1~1.30 ppm, t, *J* = 7.1 Hz ($\underline{CH_3}$-$CH_2$-) and at δ 3.70~4.99 ppm (oxygen-bearing methine multiplet signals of 1,4-tetrahydofuran and the presence of the molecular ion peaks ascribable to **1 - 4** in the mass spectroscopic data. Toyacamycin was identified from the $^{1}$H NMR signals at 8.45 (s, H-8), 8.24 (s, H-2), 6.93 (br. s, $NH_2$), 6.05 (1H, d, *J* = 5.5 Hz, H-1′), 5.25 (2H, br. s, 3′-OH and 5′-OH), 4.35 (1H, t, *J* = 5.5, H-2′), 4.12 (1H, brt, *J* = 4.6, H-3′), 3.92 (1H, q, *J* = 3.7 Hz, H-4′), 3.66 (1H, dd, *J* =

12, 3.7, H-5′a), 3.56 (1H, *J* = 12, 3.7, H-5′b) and UV spectrum.

#### 1.1.6.3. Isolation

##### 1.1.6.3.1. Ionophores from *S.1*.

In order to isolate larger amount of compounds **1** - **4** for biological assay, bigger-scale fermentation of *S.1* was performed. Silica gel column chromatography of the ethyl acetate extract using hexanes and ethyl acetate (1:1) yielded compounds **1** (4.3 mg), **2** (1.2 mg), **3** (7 mg) and **4** (3.1 mg).

##### 1.1.6.3.2. Toycamycin (5) from *S.2*.

The bioactive ethyl acetate obtained from liquid-liquid partition was subjected to HPLC (solvent system: methanol and water gradient) and preparative TLC (hexanes and ethyl acetate 1:1) to afford compound **5** (6 mg).

##### 1.1.6.3.3. Piperafizin (6) from *S.3*.

Silica gel column chromatography eluted with hexanes ethyl acetate gradient, starting from 4:1 of the bioactive ethyl acetate obtained from liquid-liquid partition yielded compound **6** (2 mg).

###### 1.1.6.3.3.1. X-ray structure of Piperafizin (6)

A colorless plate was cut (0.33 x 0.28 x 0.14 $mm^3$), mounted, and centered on the goniometer of an Oxford Diffraction Gemini A Ultra diffractometer operating with MoKα radiation. The data collection routine, unit cell refinement, and data processing were carried out with the program CrysAlisPro.[12] The Laue symmetry and systematic absences were consistent with the orthorhombic space group *Pbca*. The structure was solved by direct methods and refined using SHELXTL NT.[13] The

final refinement model involved anisotropic displacement parameters for all non-hydrogen atoms and a riding model for all hydrogen atoms.

**1.1.6.3.3.2. Crystal data:** Colorless crystals; $C_{19}H_{16}N_2O_2$, Mr =304.34, tetragonal, *P*-$42_1c$, a= 20.3660(2) Å, b= 20.3660(2) Å, c= 7.40781(18) Å, α= 90.00, β= 90.00, γ = 90.00, V= 3072.56(9) $Å^3$, 16968 reflections, 210 parameters. The atomic coordinates and equivalent isotropic displacement parameters, as well as a full list of bon distances and angles, and the structure factor table are deposited as supplementary material at the Cambridge crystallographic Data Centre (Deposition No. CCDC 980560). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 1223 336 033 or e-mail: deposit@ccdc.cam.ac.uk).

**1.1.6.3.4. Xestostreptin from *S.4*.**

The ethyl acetate fraction obtained from liquid-liquid partition of *S.4* was subjected to $C_{18}$ HPLC using MeOH and $H_2O$ (solvent system: 20% aqueous MeOH for 10 min, from 20% to 50% aqueous MeOH at 10 min to 15 min, 50% to 100% from 15 min to 25 min and then keep 100% MeOH until 30 min) to yield compound 7 (5 mg, *RT* 8.5 min).

**1.1.6.3.4.1. Xestostreptin:** Amorphous white powder; $[\alpha]_D^{21}$ +12 ( *c* 0.1, MeOH); IR $\nu_{max}$ $cm^{-1}$: 2924, 1723, 1686, 1621, 1461, 1286, 1128 $cm^{-1}$; $^1H$ NMR (400 MHz, $CDCl_3$), and $^{13}C$ NMR (100 MHz, $CDCl_3$), see Table 1; HRESIMS *m/z* 199.1068 $[M+H]^+$ (required for $C_9H_{15}N_2O_3^+$, *m/z* 199.1077)

## References and Notes

1. Montalvo, N. F.; Davis, J.; Vicente, J.; Pittiglio, R.; Ravel, J.; Hill, R. T., Integration of culture-based and molecular analysis of a complex sponge-associated bacterial community. *PLoS One* **2014,** 9, (3), e90517/1-e90517/8, 8 pp.

2. Harinantenaina, L.; Brodie, P. J.; Maharavo, J.; Bakary, G.; TenDyke, K.; Shen, Y.; Kingston, D. G. I., Antiproliferative homoscalarane sesterterpenes from two Madagascan sponges. *Bioorg. Med. Chem.* **2013,** 21, (11), 2912-2917.

3. Bennett, T. N.; Paguio, M.; Gligorijevic, B.; Seudieu, C.; Kosar, A. D.; Davidson, E.; Roepe, P. D., Novel, rapid, and inexpensive cell-based quantification of antimalarial drug efficacy. *Antimicrobial agents and chemotherapy* **2004,** 48, (5), 1807-10.

4. Smilkstein, M.; Sriwilaijaroen, N.; Kelly, J. X.; Wilairat, P.; Riscoe, M., Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. *Antimicrobial agents and chemotherapy* **2004,** 48, (5), 1803-6.

5. Harinantenaina, L.; Bowman, J. D.; Brodie, P. J.; Slebodnick, C.; Callmander, M. W.; Rakotobe, E.; Randrianaivo, R.; Rasamison, V. E.; Gorka, A.; Roepe, P. D.; Cassera, M. B.; Kingston, D. G. I., Antiproliferative and Antiplasmodial Dimeric Phloroglucinols from Mallotus oppositifolius from the Madagascar Dry Forest(1). *Journal of Natural Products* **2013,** 76, (3), 388-393.

### Graphical Abstract

# SUPPORTING INFORMATION II

# Antiproliferative and Antiplasmodial Compounds from Selected *Streptomyces* species

L. Harinantenaina Rakotondraibe,[a*] Rado Rasolomampianina,[b] Hyun-Young Park,[a] Jie Li,[a] Carla Slebodnick,[c] Peggy J. Brodie,[c] Leah C. Blasiak,[d] Russell T. Hill,[d] Onja Andriambeloson[b], Yongchun Shen,[f] Edward M. Suh,[f] Maria B. Cassera,[e] Felicite Rejo-Fienena,[b] and David G. I. Kingston[c*]

*[a]Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus, Ohio 43210, United States*, *[b]Centre National de Recherches sur l'Environnement, B.P 1739, Antananarivo 101, Madagascar*, *[c]Department of Chemistry and the Virginia Tech Center for Drug Discovery, M/C 0212, Virginia Tech, Blacksburg, Virginia 24061, United States*, *[d]Institute of Marine and Environmental Technology, University of Maryland Center for Environmental Science Baltimore, Maryland, MD 21201, United States*, *[e]Department of Biochemistry and Virginia Tech Center for Drug Discovery, M/C 0308, Virginia Tech, Blacksburg, Virginia 24061, United States*, *[f]Eisai Research Institute, 4, Corporate Drive, Andover, Massachusetts 01810*

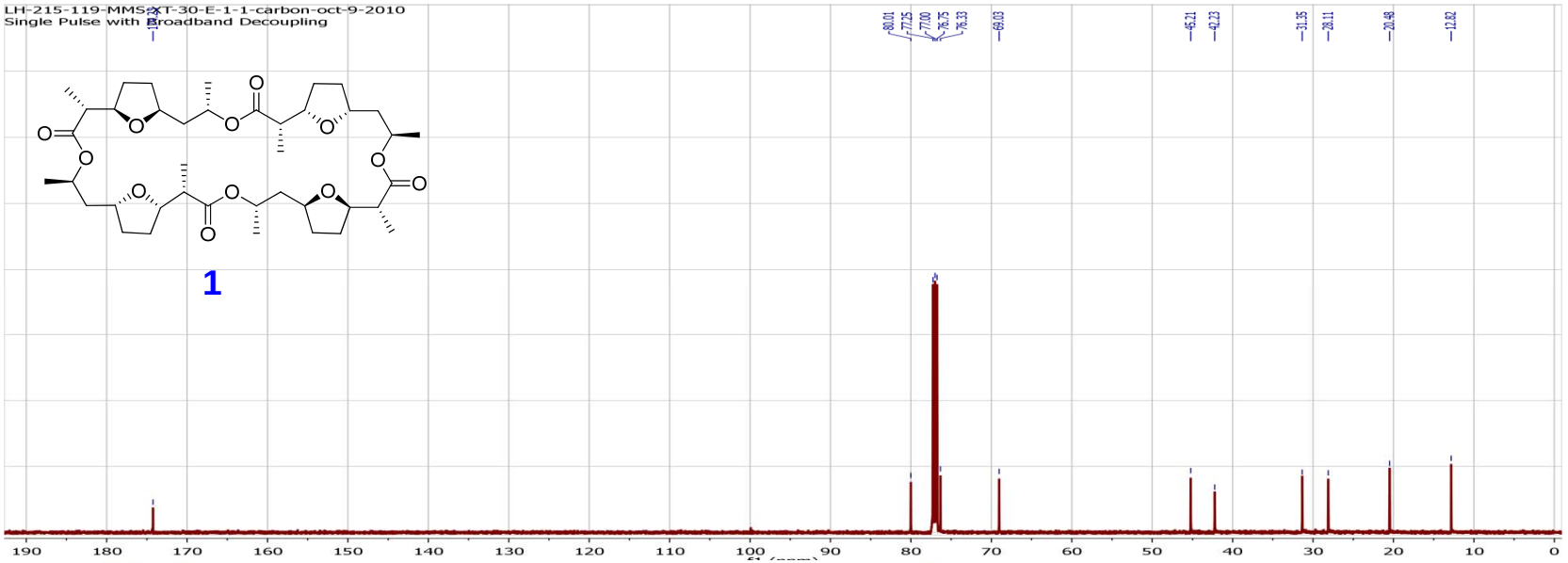
# Antiproliferative and Antiplasmodial Compounds from Selected *Streptomyces* species

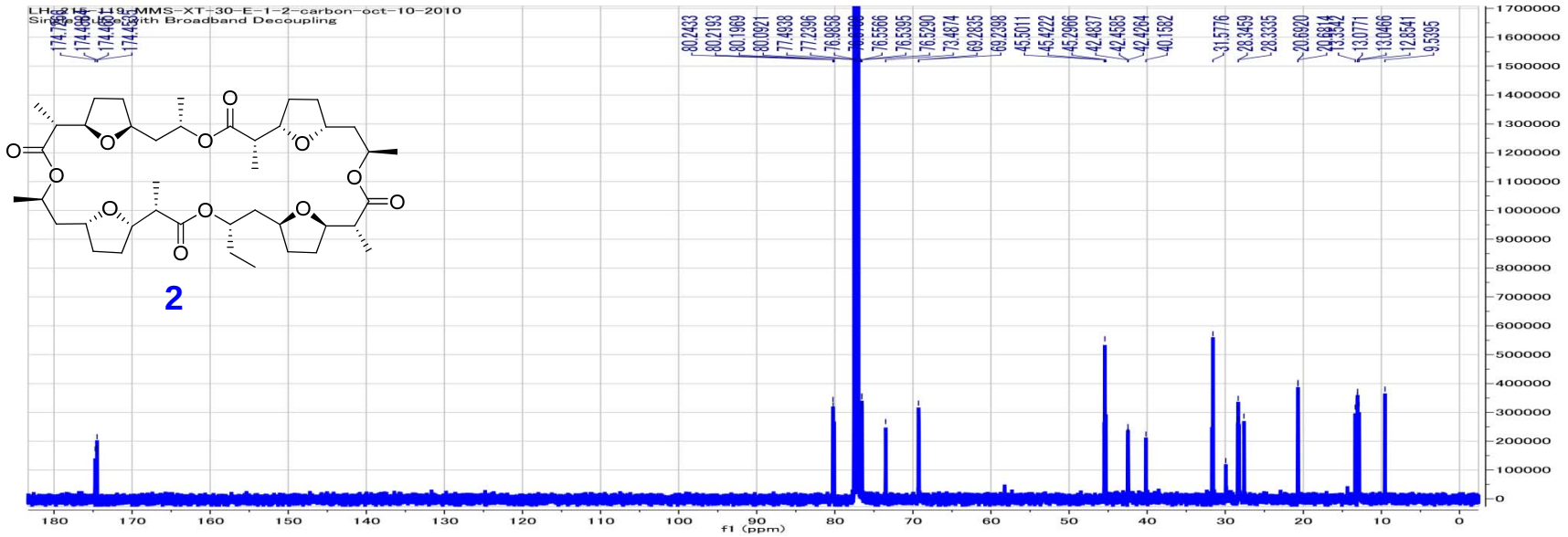
L. Harinantenaina Rakotondraibe,[a*] Rado Rasolomampianina,[b] Hyun-Young Park,[a] Jie Li,[a] Carla Slebodnick,[c] Peggy J. Brodie,[c] Leah C. Blasiak,[d] Russell T. Hill,[d] Onja Andriambeloson[b], Yongchun Shen,[f] Edward M. Suh,[f] Maria B. Cassera,[e] Felicite Rejo-Fienena,[b] and David G. I. Kingston[c*]

## Table of content

# $^{13}$C-NMR spectra of compounds 1 and 2

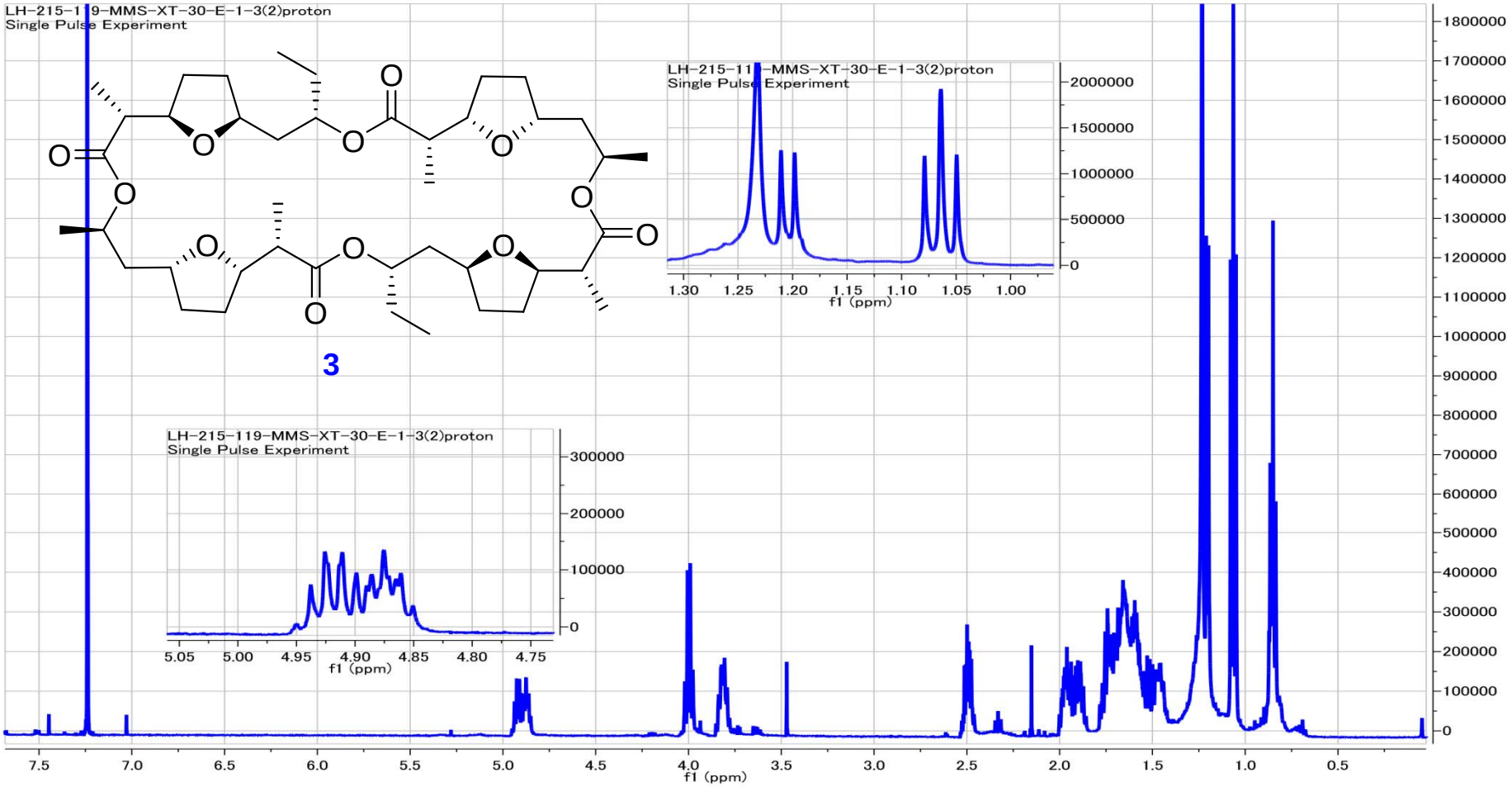




# $^{1}$H NMR spectrum (500 MHz, Chloroform-d) of 3

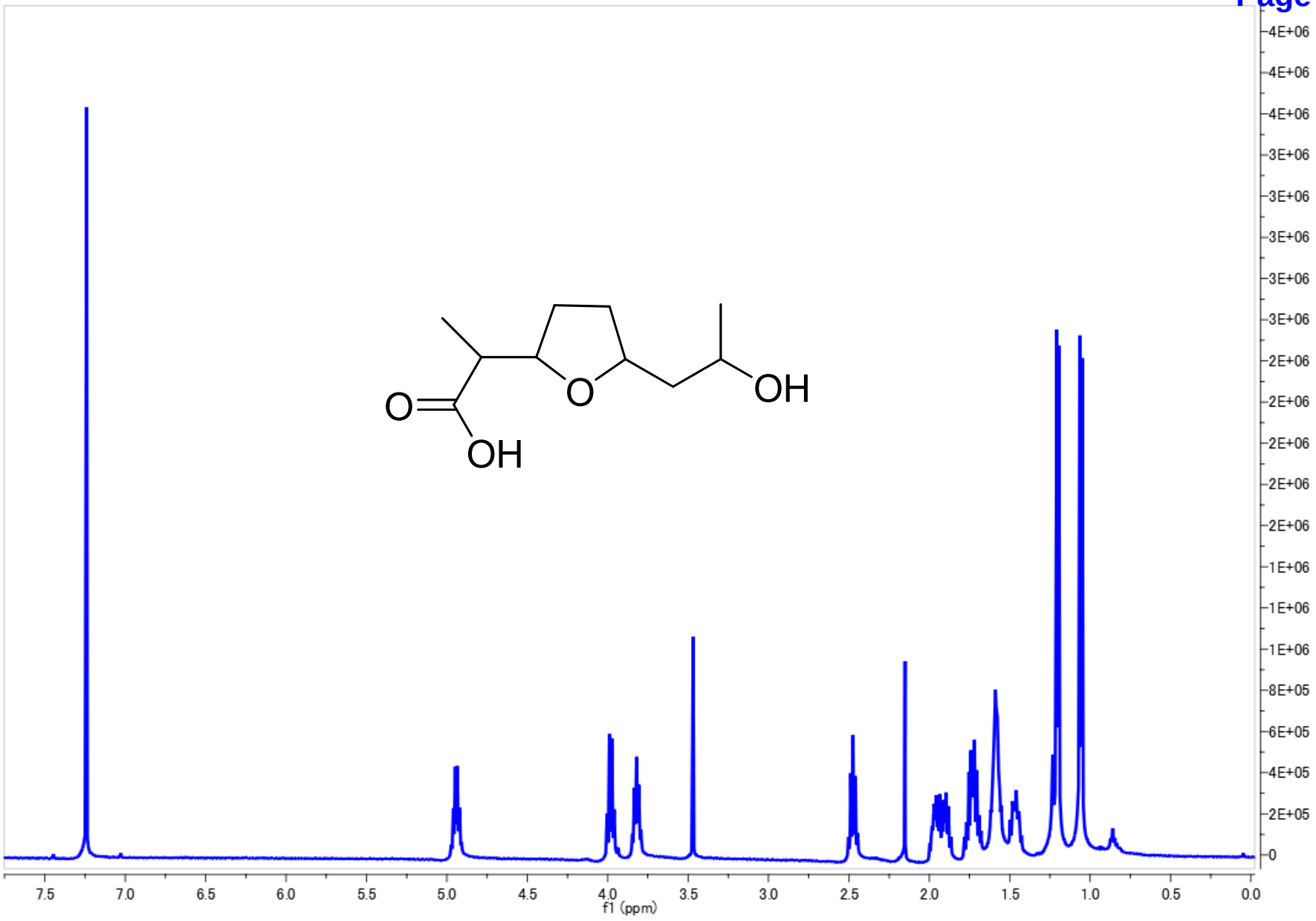





3

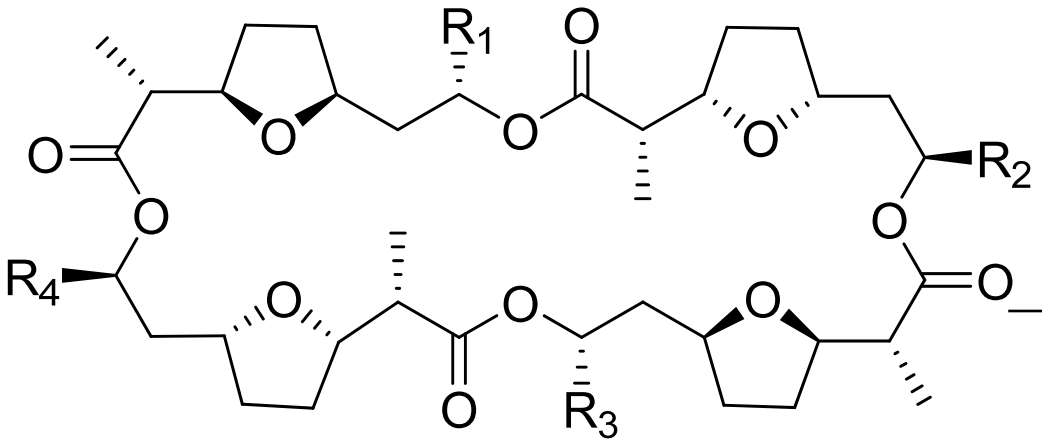
# $^{1}$H NMR spectrum (500 MHz, Chloroform-d) of 4

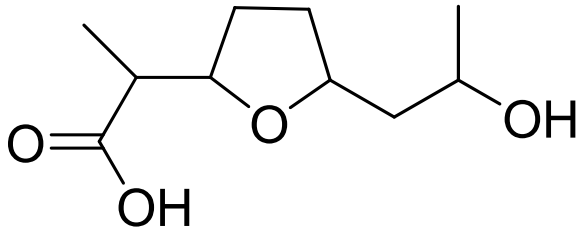




# Structures and bioligical activity of compounds 1 - 4





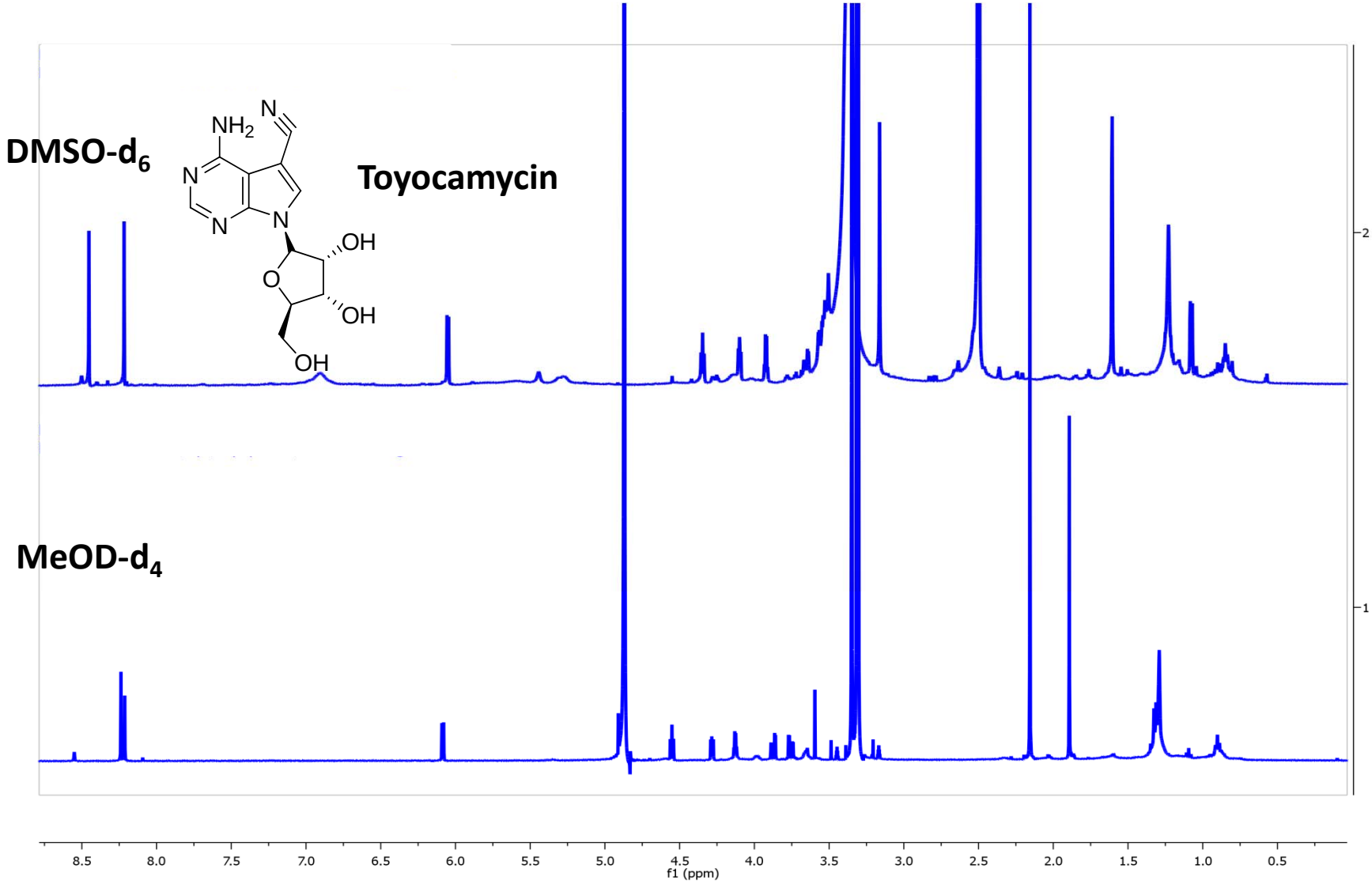




**4: ±-Nonactic acid**

| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | A2058, Melanoma ($IC_{50}$ μM) | H522-T1, NSCL, ($IC_{50}$ μM) | A2780, ($IC_{50}$ μM) |
|---|---|---|---|---|---|---|---|
| **1: Nonactin** | Me | Me | Me | Me | 0.192 | 0.146 | 0.12 |
| **2: Monactin** | Me | Me | Et | Me | 0.0167 | 0.0125 | 0.1 |
| **3: Dinactin** | Et | Me | Et | Me | 0.0168 | 0.0134 | 0.2 |

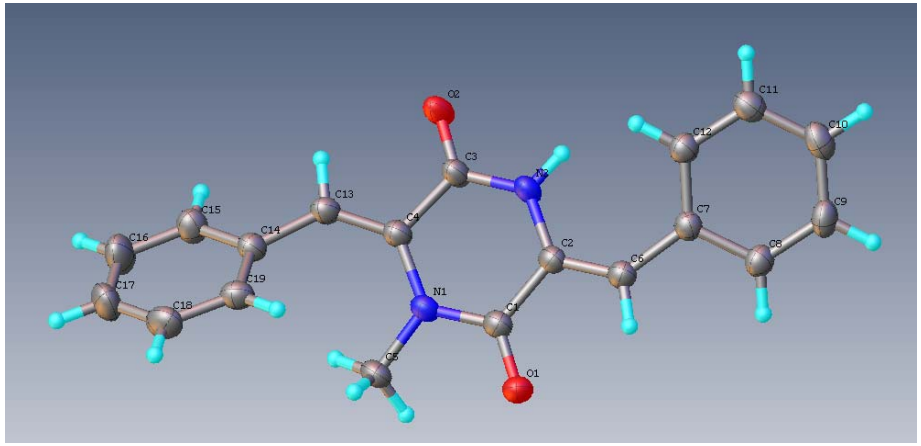
# $^{1}$H NMR spectra (500 MHz, DMSO-d6 and Methanol-d4) of Toyacamycin (5)

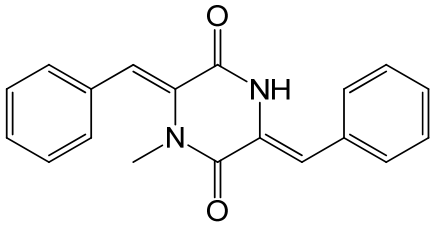




## Ortep drawing structure of compound 6

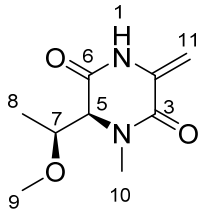




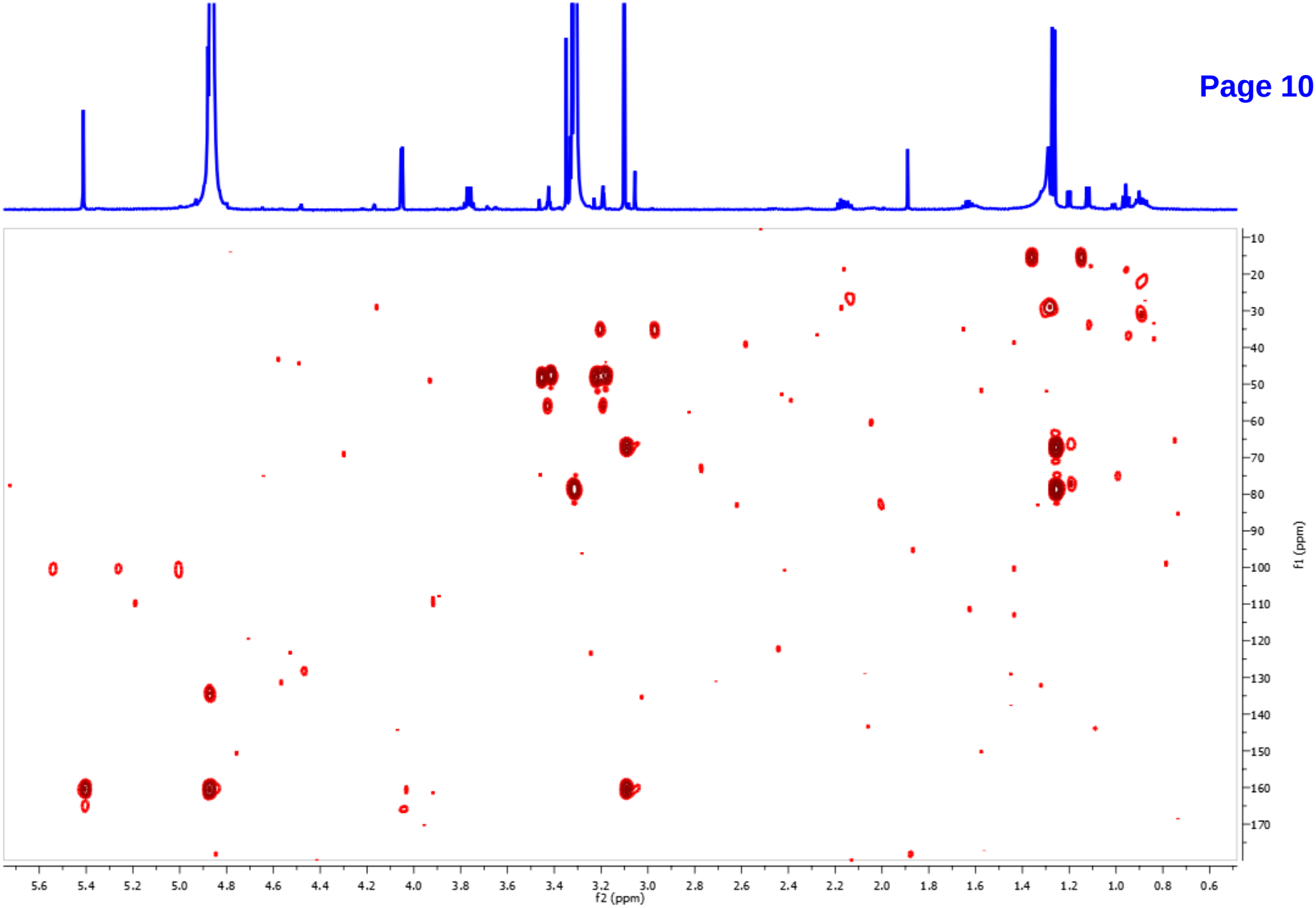

Piperafizine A

# HSQC spectrum of compound 7



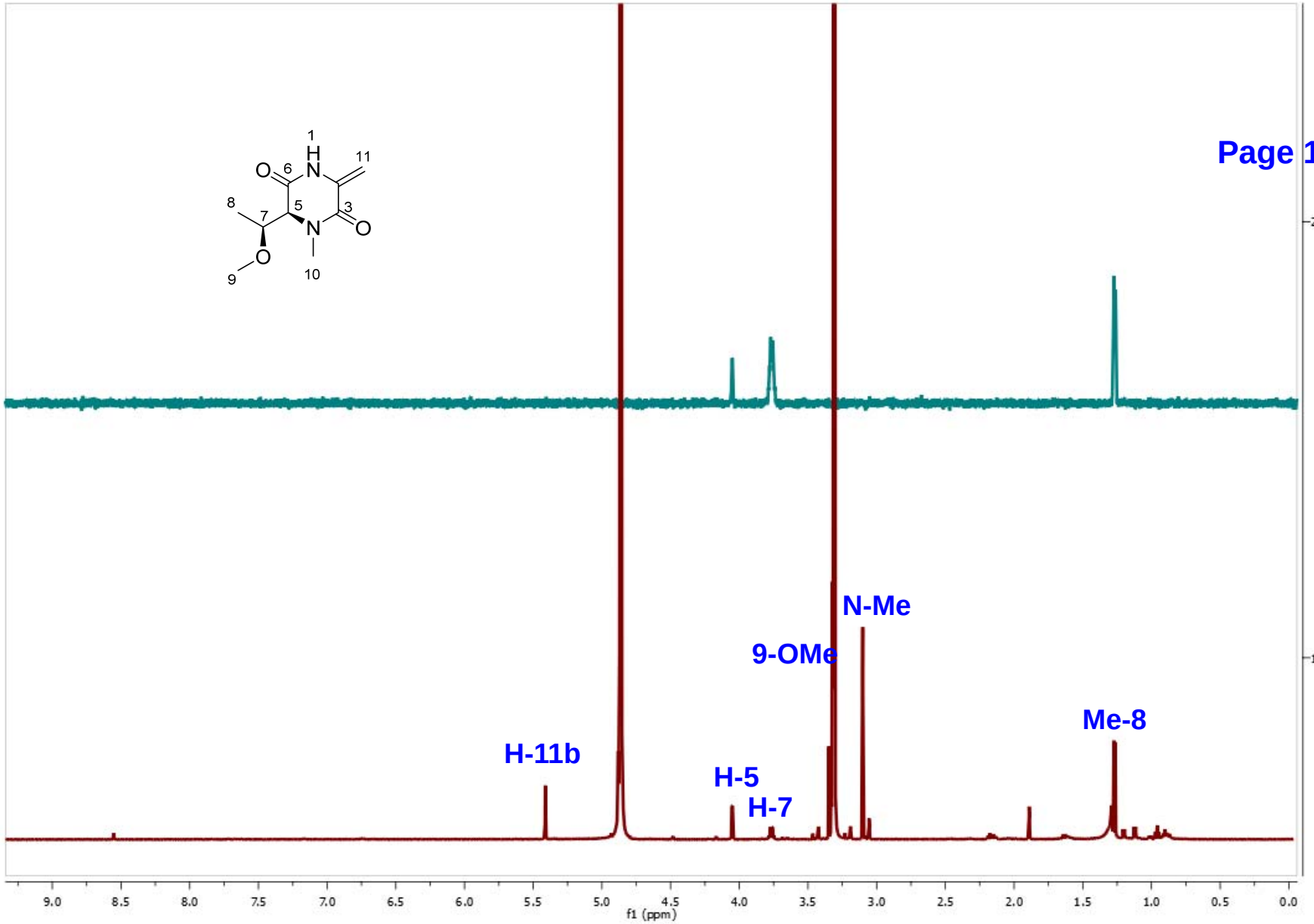
# HMBC spectrum of compound 7





# Selective 1D TOCSY of compound 7 (excitation of one of the signal of H-7 at δ 3.76)





**Selective nOe of compound 7 (irradiation of one of the exocyclic methylene signals H-11b at δ 5.41)**