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細菌的十一異戊基二烯焦磷酸合成酶之抑制劑的合成與評測

Synthesis and Evaluation of Bacterial Undecaprenyl Diphosphate Synthase Inhibitors

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細菌的十一異戊基二烯焦磷酸合成酶之抑制劑的合成與評測

Synthesis and Evaluation of Bacterial Undecaprenyl Diphosphate Synthase Inhibitors

本論文係陳曉萱君（R06B46007）在國立臺灣大學生化科學研究所完成之碩士學位論文，於民國 108 年 06 月 18 日承下列考試委員審查通過及口試及格，特此證明。

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其實有太多太多的感謝，真的無法以短短幾行字簡單帶過，只好將一切點滴記在心頭。

陳曉萱　　謹誌於
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摘要

耐甲氧西林金黃色葡萄球菌(methicillin-resistant *Staphylococcus aureus*)等具多重抗藥性的金黃色葡萄球菌，是種致命且需要新的抗生素治療的醫院感染細菌。十一異戊基二烯焦磷酸合成酶 (undecaprenyl diphosphate synthase, UPPS) 將八當量的異戊烯基焦磷酸 (isopentenyl pyrophosphates, IPP) 與一當量的法尼基焦磷酸 (farnesyl pyrophosphate, FPP) 聚合，形成十一異戊基二烯焦磷酸 (undecaprenyl diphosphate, UPP)，是用於合成細菌細胞壁的肽聚醣的必要前驅物，因此十一異戊基二烯焦磷酸合成酶可作為新抗生素的標靶。基於十一異戊基二烯焦磷酸合成酶之結構和先前的研究，我們設計了一系列吡咯烷酮的衍生物，並使用法尼基焦磷酸的螢光衍生物MANT-O-GPP的活性測試法來測試它們對大腸桿菌及金黃色葡萄球菌之十一異戊基二烯焦磷酸合成酶的抑制作用，其中具有鹵素或苯基的化合物對抑制十一異戊基二烯焦磷酸合成酶更有效，而最小抑菌濃度的測試結果表明它們具有抑制枯草桿菌 (*Bacillus subtilis*) 的活性，根據酵素動力學和結構模擬，這些化合物對十一異戊基二烯焦磷酸合成酶而言是混合型的抑制劑。

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ABSTRACT

The multiple antibiotic-resistant *Staphylococcus aureus*, such as methicillin-resistant *Staphylococcus aureus* (MRSA), is a fatal nosocomial infection that needs new antibiotics. Undecaprenyl diphosphate synthase (UPPS) condenses a farnesyl pyrophosphate (FPP) with eight isopentenyl pyrophosphates (IPP) to form undecaprenyl diphosphate (UPP) for the biosynthesis of peptidoglycan essential for bacterial cell wall, so it is a potential drug target for antibiotic. Based on UPPS structure and previous research, we designed a series of 4-carboxy-1-(4-styrylcarbonylphenyl)-2-pyrrolidinone derivatives and used a fluorescent analog of FPP, MANT-O-GPP, to test their inhibition on *E. coli* and MRSA UPPS. The compounds with halogen or benzene group were more potent to inhibit UPPS. Then, the EC_{50} test showed that they have anti-bacterial activities to *Bacillus subtilis*. According to the enzyme kinetics and modeling, these compounds were mixed inhibitors of UPPS.

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ABBREVIATIONS

DMSO, dimethyl sulfoxide;

Da, Dalton:

EA, Ethyl acetate;

EC_{50}, Half maximal effective concentration

*Ec*UPPS, *Escherichia coli* undecaprenyl pyrophosphate synthase;

EtBr, ethidium bromide;

FPP, farnesyl pyrophosphate;

IPP, isopentenyl pyrophosphate;

UPP, undecaprenyl pyrophosphate;

UPPS, undecaprenyl pyrophosphate synthase;

Hepes, 4-2(-hydroxyethyl)-1-piperazineethanesulfonic acid;

IC_{50}, half maximal inhibitory concentration;

IPTG, isopropyl-β-thiogalactopyranoside;

MANT-O-GPP,

(2E,6E)-8-O-(N-methyl-2-aminobenzoyl)-3,7-dimethyl-2,6-octandien-1-pyrophosphate;

mp, melting temperature;

MRSA, methicillin-resistant *Staphylococcus aureus*;

Ni-NTA, nickel nitrilo-tri-acetic acid;

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NMR, nuclear magnetic resonance;

PCR, polymerase chain reaction;

*Sa*UPPS, *Staphylococcus aureus* undecaprenyl pyrophosphate synthase;

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis;

TLC, thin-layer chromatography;

Tris, tris(hydroxymethyl)aminomethane

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1 INTRODUCTION

1.1 Pathogens

Pathogens are microorganisms which can infect humans and cause diseases and death, such as bacteria, fungi, viruses[1]. Among them, the most common are bacteria. Pathogenic bacteria often infect humans with compromised immunity. One of the most terrifying bacteria is *Mycobacterium tuberculosis* which caused tuberculosis and killed about 2 million people a year, mostly in sub-Saharan Africa[2]. Other significant bacterias are Streptococcus and Pseudomonas which cause pneumonia, and Shigella, Campylobacter, and Salmonella which cause foodborne illnesses[3-4]. Pathogenic bacteria also cause diseases such as tetanus, typhoid fever, diphtheria, syphilis, and leprosy[5].

1.2 Antibiotics and resistance

Bacteria can be killed by antibiotics. The first commercialized antibiotic, penicillin, was discovered by Alexander Fleming[6] in 1928. It is a β-lactam interrupts the formation of peptidoglycan cross-linkages in the bacterial cell wall[7]. However, a few years later, a β-lactamase emerged in some bacteria to destroy and abolish the effect of penicillin. In 1960, scientists developed its derivatives, such as methicillin and carbapenem, which are less active toward β-lactamase [8-9]. Unfortunately, one year later, methicillin-resistant *Staphylococcus aureus* (MRSA) appeared and again brought a

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deadly threat to humans[8]. In 1992, scientists have noticed antibiotics resistance crisis[10]. As reported, bacteria had resistance to antimicrobial agents because of chromosomal changes or the exchange of genetic materials via plasmids and transposons. *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *staphylococci* which cause respiratory and cutaneous infections, and members of the *Enterobacteriaceae* and *Pseudomonas* families, organisms which cause diarrhea, urinary infection, and sepsis, are resistant to all of the older antibiotics. The extensive use of antibiotics in the community and hospitals make this crisis even more serious. In 2008, Rice recommended "the ESKAPE bugs" referred to the six common antibiotic-resistant bacteria *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumanni*, *Pseudomonas aeruginosa,* and *Enterobacter* species in hospitals[11]. Then, scientists sought other targets to fight resistant bacteria. For example, linezolid approved for commercial use in 2000 is an antibiotic used to treat Gram-positive bacteria that are resistant to other antibiotics. It binds to the 50S subunit of the prokaryotic ribosome and prevents the initiated complex forming for protein synthesis forming[12]. Other targets including the enzymes or elements participating cell wall biosynthetic pathways are being explored[13-17].

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1.3 Undecaprenyl pyrophosphate synthase as a potential antibiotic target

Undecaprenyl pyrophosphate synthase, UPPS, catalyzes consecutive condensation of eight molecules of isopentenyl diphosphate (IPP) with farnesyl diphosphate (FPP) to form UPP. It belongs to a prenyltransferase family which transfers prenyl groups to acceptors and participates in isoprenoid biosynthetic pathways[18]. UPP then acts as a lipid carrier for bacterial peptidoglycan biosynthesis[19-21]. This pathway of peptidoglycan synthesis is shown in Figure 1[22]. Due to its pivotal role in cell wall biosynthesis, UPPS has been suggested as a potential antibiotic target[23-32].

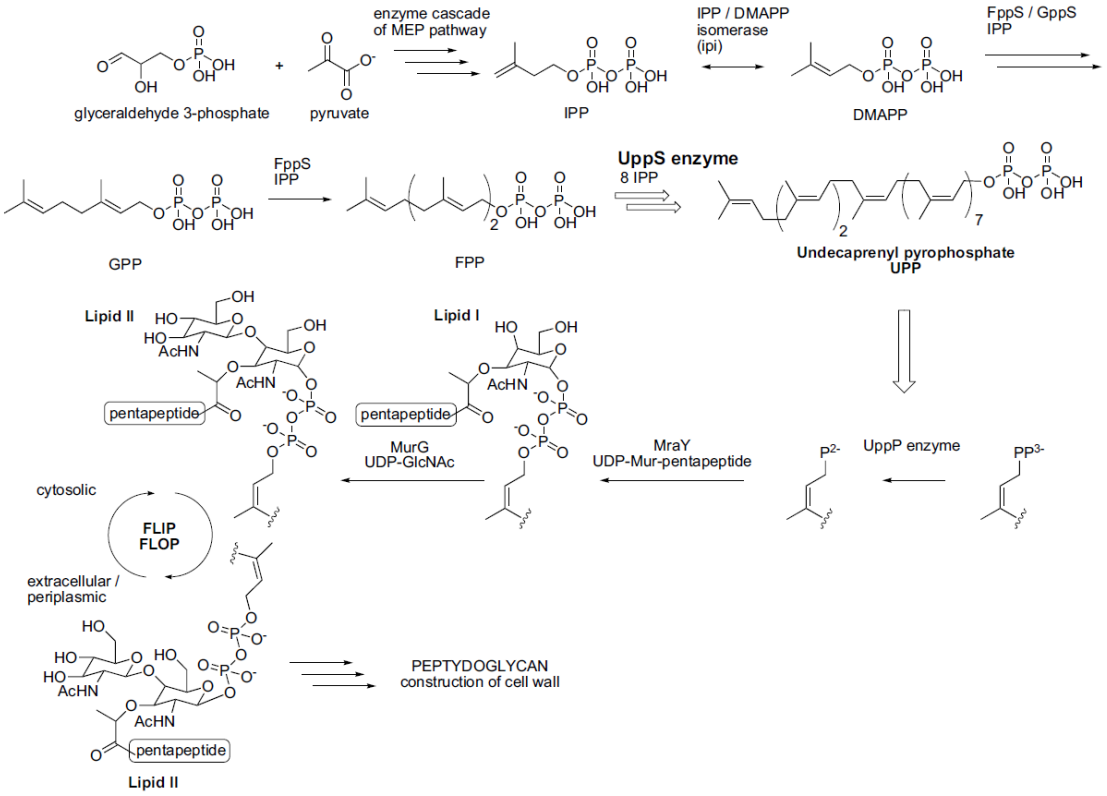

Figure 1. The pathway of peptidoglycan synthesis.

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1.4 Purpose of study

Based on the rationale, we wanted to design inhibitors against UPPS and evaluated them. A previous postdoctor in our laboratory, Dr. Vathan Kumar, discovered a hit **VK-278** he synthesized to inhibit UPPS. Following his discovery, I synthesized its anaologues and measured their inhibition on UPPS. We chose *E. coli* and *S. aureus* UPPS as working subjects because *S. aureus* is a Gram-positive resistant species and *E. coli* is a Gram-negative bacterium for comparison. We used a fluorescent analogue of FPP, MANT-O-GPP, to monitor the activity of UPPS because of its fluorescent increase at 420 nm during chain elongation[33-34]. We also investigated their types of inhibition with steady-state kinetic measurements at different substrate and inhibitor concentrations and docking by iGEMDOCK. Then, the compounds with better inhibition on UPPS enzymes were tested for inhibiting bacterial growth.

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2 MATERIALS AND METHODS

2.1 Chemicals

4- aminoacetophenone, itaconic acid, 4-bromobenzaldehyde, 4-cyanobenzaldehyde, 3-cyanobenzaldehyde, 3-nitrobenzaldehyde, 4-biphenylcarboxaldehyde, and 3,4-dichlorobenzaldehyde were purchased from AK Scientific (Union City, USA). Benzaldehyde, 4-chlorobenzaldehyde, and 4-carboxybenzaldehyde were purchased from Acros Organics (New Jersey, USA). 4-fluorobenzaldehyde was purchased from Alfa Aesar (Ward Hill, USA). GenepHlow^{TM} Gel/PCR kit was purchased from Geneaid (Taiwan). TLC, pET-32 Xa/LIC Vector Kit, and Ni-NTA were purchased from Merck (Darmstadt, Germany). Thrombin was purchased from GE Healthcare (Chicago, United States). MANT-O-GPP was synthesized previously in our laboratory. IPP was purchased from Echelon Biosciences (Salt Lake City, USA).

2.2 Synthesize the inhibitor of UPPS

2.2.1 Synthesis of 1-(4-Acetylphenyl)-4-carboxy-2-pyrrolidinone (1)

A mixture of 1 g (7.40 mmol) 4-aminoacetophenone and 1.2 g (8.89 mmol) itaconic acid was stirred and heated (110-130 ℃) under reflux for 18 hours. The progress of the reaction was monitored by TLC. After cooling to room temperature, 10 ml methanol was added to the reaction mixture. The reaction mixture was sonicated and

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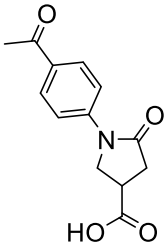
heated to dissolve in methanol, then cooled to room temperature to wait for recrystallization. Crystallization was filtered and washed with EA to yield the product. The product was dissolved in the DMSO-d6 to test NMR by Bruker AVIIIHD 400MHz FT-NMR in the department of chemistry, National Taiwan University (Taiwan) and was dissolved in the methanol for mass measurement by Bruker UPLC-MS in the College of Life Science (TechComm, National Taiwan University, Taiwan) to confirm the product. Then, the product was measured its melting temperature by Fargo MP-1D Melting Point Apparatus in our lab.

1-(4-Acetylphenyl)-4-carboxy-2-pyrrolidinone (1)



White solid, Yield : 49.8 %, mp 180-181 °C, ^{1}H NMR (400 MHz, DMSO-d6) δ: 2.54 (s, 3H), 2.70-2.85 (m, 2H), 3.33-3.40 (m, 1H), 3.99-4.12 (m, 2H), 7.80, 7.96 (2d, J=8.9 Hz, 4H), ^{13}C NMR (100 MHz, DMSO-d6) δ: 26.5, 35.0, 35.3, 49.8, 118.4, 129.2, 132.1 ,143.1, 172.6, 174.0, 196.6, MS m/z : $[M+H]^+$ = 248.09 (calcd. for $C_{13}H_{13}NO_4$ 248.09)

2.2.2 General procedure of 4-Carboxy-1-(4-styrylcarbonylphenyl)-2-pyrrolidinones (2a-j).

1-(4-Acetylphenyl)-4-carboxy-2-pyrrolidinone **1** 0.1g (0.40 mmol) in 5 ml ethanol treated with 500 μl of 50% NaOH under magnetically stirred condition at room temperature was reacted with benzaldehyde (0.5 mmol). The mixture was stirred

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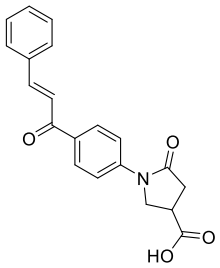
magnetically until complete consumption of the starting material **1**. The progress of the reaction was monitored by TLC. After the reaction was completed, ethanol was removed under reduced pressure. The residue was dissolved in 10 ml ddH_2O. The solution was transferred to a separatory funnel and extracted with EA. The aqueous layer was collected and added 100 ml ice, then acidified with aq HCl to pH 1-2. The yellow precipitate was filtered and then washed with water[35]. The products were dissolved in the DMSO-d6 for NMR measurement and in the methanol for mass measuremen to confirm the product. Then, the products were measured their melting temperature.

4-Carboxy-1-(4-styrylcarbonylphenyl)-2-pyrrolidinone (2a)



Light yellow solid, Yield : 70.0 %, mp 209-210 ℃, ^{1}H NMR (400 MHz, DMSO-d6) δ: 2.76-2.83 (m, 2H), 3.37-3.40 (m, 1H), 4.02-4.07 (m, 2H), 7.44-7.46 (m, 3H), 7.85-7.89 (m, 4H), 7.73, 7.75 (2d, J=15.6 Hz, 2H), 8.19 (d, J=9.0 Hz, 2H) 12.79 (s, 1H), ^{13}C NMR (100 MHz, DMSO-d6) δ: 35.0, 35.4, 49.8, 118.5, 121.9, 128.8, 128.9, 129.6, 130.5, 132.6921, 134.7, 143.2, 143.6, 172.6, 174.0, 187.7, MS m/z : $[M+H]^+$ = 336.12 (calcd. for $C_{20}H_{17}NO_4$ 336.12)

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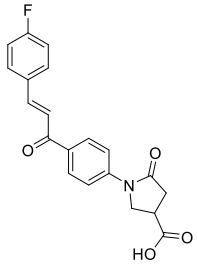
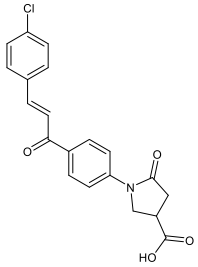
1-[4-(4-fluorostyrylcarbonyl)phenyl]-4-carboxy-2-pyrrolidinone (2b)



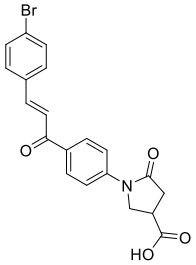
Yellow solid, Yield : 42.4 %, mp 256-257 ℃, ^{1}H NMR (400 MHz, DMSO-d6) δ: 2.41-2.52 (m, 2H), 2.76 (m, 1H), 3.17-3.49 (m, 2H), 7.85 (d, J=15.6 Hz, 2H) 7.25-7.30 (m, 2H), 7.63 (d, J= 15.6 Hz, 1H), 7.85 (d, J= 15.6 Hz, 1H), 7.91-7.99 (m, 4H), ^{13}C NMR (100 MHz, d6-DMSO) δ: 36.1, 41.6, 44.4, 111.4, 116.1, 116.3, 122.7, 125.7, 131.1, 131.2, 131.4, 132.2, 140.5, 153.3, 174.2, 175.7, 186.2, MS m/z : $[M+H]^+$ = 354.11 (calcd. for $C_{20}H_{16}FNO_4$ 354.11)

1-[4-(4-chlorostyrylcarbonyl)phenyl]-4-carboxy-2-pyrrolidinone (2c)



Yellow solid, Yield : 20.5 %, mp 232-233 ℃, ^{1}H NMR (400 MHz, MeOD) δ: 2.58-2.78 (m, 2H), 3.13-3.16 (m, 1H), 3.42-3.62 (m, 2H), 6.73 (m, J=7.7 Hz, 2H), 7.42 (d, J=4Hz, 2H), 7.63-7.77 (m, 4H), 7.96 (d, J=7.7 Hz, 2H), ^{13}C NMR (100 MHz, MeOD) δ: 34.6, 42.5, 45.0, 112.6, 123.8, 127.3, 130.1, 130.8, 132.6, 135.4, 136.9, 142.4, 154.7, 175.4, 176.8, 189.6 , MS m/z : $[M+H]^+$ = 370.08 (calcd. for $C_{20}H_{16}ClNO_4$ 370.08)

1-[4-(4-Bromostyrylcarbonyl)phenyl]-4-carboxy-2-pyrrolidinone (2d)



Dark yellow solid, Yield : 75.0 %, mp 232-233 ℃, ^{1}H NMR (400 MHz, DMSO-d6) δ: 2.72-2.87 (m, 2H), 3.34-3.41 (m, 1H), 4.02-4.15 (m, 2H), 7.64-7.71 (m, 3H), 7.83-7.86 (m, 4H), 7.99 (d, J=15.6 Hz, 1H), 8.19 (d, J=8.9 Hz, 2H) 12.76 (s, 1H), ^{13}C NMR (100 MHz, d6-DMSO) δ: 35.1, 35.4, 49.8, 118.5,

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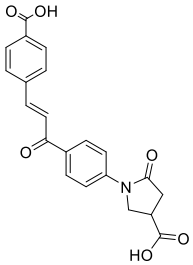
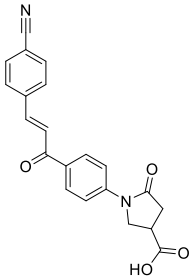
122.7, 123.9, 129.6, 130.8, 131.8, 132.6, 134.0, 142.2, 143.3, 172.7, 174.1, 187.5, MS m/z : $[M+H]^+$ = 413.03 (calcd. for $C_{20}H_{16}BrNO_4$ 413.03)

1-[4-(4-carboxystyrylcarbonyl)phenyl]-4-carboxy-2-pyrrolidinone (2e)



Yellow solid, Yield : 38.0 %, mp 308-309 ℃, ^{1}H NMR (400 MHz, MeOD) δ: 2.52-2.72 (m, 2H), 3.04-3.07 (m, 1H), 3.37-3.58 (m, 2H), 6.72 (d, J=8.8 Hz, 2H), 7.42 (d, J=8.5 Hz, 2H), 7.63-7.78 (m, 4H), 7.96 (d, J=8.8 Hz, 2H), ^{13}C NMR (100 MHz, MeOD) δ: 35.9, 43.4, 45.4, 112.6, 124.0, 127.2, 130.2, 130.9, 132.6, 135.5, 1369, 142.4, 154.9, 176.8, 178.3, 189.6, MS m/z : $[M+H]^+$ = 380.11 (calcd. for $C_{21}H_{17}NO_6$ 380.11)

1-[4-(4-cyanostyrylcarbonyl)phenyl]-4-carboxy-2-pyrrolidinone (2f)



Yellow solid, Yield : 30.9 %, mp 250-251 ℃, ^{1}H NMR (400 MHz, DMSO-d6) δ: 2.67-2.79 (m, 2H), 3.03-3.12 (m, 1H), 4.01-4.05 (m, 2H), 7.73 (d, J=15.6 Hz, 1H), 7.86, 7.91 (2d, J=8.3 Hz, 4H), 8.08-8.10 (m, 3H), 8.20 (d, J=8.6 Hz, 2H), ^{13}C NMR (100 MHz, DMSO-d6) δ: 36.6, 51.2, 112.2, 118.3, 118.6, 125.3, 129.4, 129.8, 132.0, 132.7, 139.3, 141.2, 143.8, 174.0, 175.2, 187.5, MS m/z : $[M+H]^+$ = 361.11 (calcd. for $C_{21}H_{16}N_2O_4$ 361.11)

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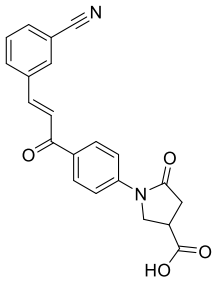
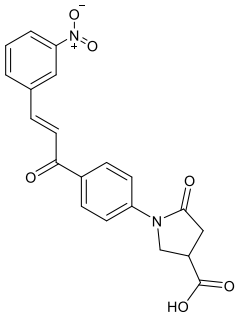
1-[4-(3-cyanostyrylcarbonyl)phenyl]-4-carboxy-2-pyrrolidinone (2g)



Light yellow solid, Yield : 34.6 %, mp 270-271 °C, ^{1}H NMR (400 MHz, DMSO-d6) δ: 2.62-2.67 (m, 1H), 2.78-2.83 (m, 2H), 3.95-4.10 (m, 2H), 7.62-7.74 (m, 2H), 7.85-7.87 (m, 3H), 8.09-8.21 (m, 4H), 8.48 (s, 1H), ^{13}C NMR (100 MHz, DMSO-d6) δ: 37.4, 37.6, 52.1, 112.1, 118.2, 118.5, 124.2, 129.7, 130.1, 131.9, 133.3, 133.6, 136.1, 140.9, 144.1, 174.8, 175.3, 187.4, MS m/z : $[M+H]^+$ = 361.11 (calcd. for $C_{21}H_{16}N_2O_4$ 361.11)

1-[4-(3-nitrostyrylcarbonyl)phenyl]-4-carboxy-2-pyrrolidinone (2h)



Light yellow solid, Yield : 30.2 %, mp 224-225 °C, ^{1}H NMR (400 MHz, DMSO-d6) δ: 2.72-2.85 (m, 2H), 3.29-3.37 (m, 1H), 4.02-4.14 (m, 2H), 7.72-7.63 (m, 1H), 7.81-7.88 (m, 3H), 8.17 (d, J=15.6 Hz, 1H), 8.23-8.27 (m, 3H), 8.33 (d, J=7.6 Hz, 1H), 8.77 (s, 1H) , ^{13}C NMR (100 MHz, DMSO-d6) δ: 35.4, 35.6, 50.1, 118.5, 123.0, 124.6, 124.7, 129.8, 130.3, 132.3, 135.0, 136.6, 141.0, 143.5, 148.4, 172.9, 174.2, 187.5, MS m/z : $[M+H]^+$ = 381.11 (calcd. for $C_{20}H_{16}N_2O_6$ 381.11)

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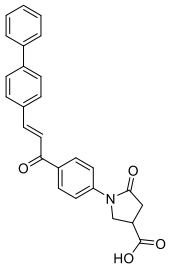
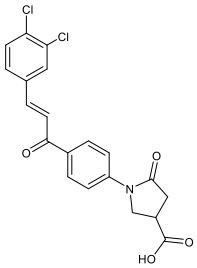
1-(4-(3-([1,1'-biphenyl]-4-yl)acryloyl)phenyl)-5-oxopyrrolidine-3-carboxylic acid (2i)



Yellow solid, Yield : 63.7 %, mp 280-281 ℃, ^{1}H NMR (400 MHz, DMSO-d6) δ: 2.72-2.86 (m, 2H), 3.21-3.47 (m, 1H), 4.03-4.14 (m, 2H), 6.67-6.69 (d, J=8.60 Hz),7.38-7.50 (m, 3H), 7.64-7.79 (m, 5H), 7.85-8.01 (m, 5H), 8.21 (d, J=8.7 Hz, 1H), ^{13}C NMR (100 MHz, DMSO-d6) δ: 35.3, 35.5, 50.02, 118.5, 121.8, 126.7, 127.0, 128.0, 129.0, 129.5, 129.6, 131.0, 132.7, 133.9, 139.2, 142.0, 143.1, 172.8, 174.2, 187.6, MS m/z : $[M+H]^+$ = 412.15 (calcd. for $C_{26}H_{21}NO_4$ 412.15)

1-[4-(3,4-dichlorostyrylcarbonyl)phenyl]-4-carboxy-2-pyrrolidinone (2j)



Yellow solid, Yield : 20.7 %, mp 208-209 ℃, ^{1}H NMR (400 MHz, DMSO-d6) δ: 2.44-2.58 (m, 2H), 2.82-2.88 (m, 1H), 3.26-3.47 (m, 2H), 6.70 (d, J=8.8 Hz, 2H), 7.58 (d, J=15.5 Hz, 1H), 7.69-7.74 (m, 1H), 7.83-7.88 (m, 1H), 7.98-8.05 (m, 3H), 8.25 (d, J=1.8 Hz, 1H), ^{13}C NMR (100 MHz, DMSO-d6) δ: 34.8, 41.0, 43.8, 111.0, 124.6, 125.2, 128.8, 129.8, 130.8, 131.2, 131.7, 132.0, 136.1, 138.6, 153.0, 173.4, 174.9, 185.5, MS m/z : $[M+H]^+$ = 404.05 (calcd. for $C_{20}H_{15}Cl_2NO_4$ 404.05)

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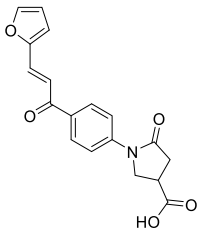
1-(4-(3-(furan-2-yl)acryloyl)phenyl)-5-oxopyrrolidine-3-carboxylic acid (2k)



Brown solid, Yield : 52.5 %, mp 195-196 ℃, ^{1}H NMR (400 MHz, DMSO-d6) δ: 2.72-2.87 (m, 2H), 3.34-3.40 (m, 1H), 4.01-4.14 (m, 2H), 6.68-6.69 (m, 1H), 7.09-7.10 (d, J=3.36 Hz, 1H), 7.55(s, 2H), 7.83-7.85 (d, J=8.88 Hz, 2H), 7.90 (d, J=1.16 Hz, 1H), 8.09-8.11 (d, J=8.8 Hz, 2H), 12.77 (s, 1H), ^{13}C NMR (100 MHz, DMSO-d6) δ: 35.0, 35.3, 49.8, 113.1, 116.9, 118.6, 129.3, 130.1, 132.6, 143.1, 146.1, 151.2, 172.6, 174.0, 187.1, MS m/z : $[M+H]^{+}$ = 326.10 (calcd. for $C_{20}H_{15}Cl_2NO_4$ 326.10)

2.3 *Sa*UPPS cloning

The gene of SaUPPS was synthesized by Bio Basic Inc. (Canada). The forward primer 5'-GGTATTGAGGGTCGCGAATTCGAGAACCTGTACTTCCAGGG-3' (froward) and the backward primer 5'-AGAGGAGAGTTAGAGCCCTCGAGTTATTCCTCGCTCAGGCC-3' for PCR reactions to amplify the gene were prepared by MISSION BIOTECH Inc. (Taiwan). Thirty cycles of PCR reactions were performed using a thermocycler (Biometra) with the denaturing temperature at 94 ℃ for 30 s, melting temperature at 66 ℃ for 30 s, and the annealing temperature at 72 ℃ for 1 min. The PCR product was subjected to electrophoresis on 1% agarose gel with EtBr in TAE buffer. The correct band on the gel

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was cut and the DNA was purified by GenepHlow^{TM} Gel/PCR kit. The product was treated with T4 DNA Polymerase and annealed to pET32Xa/LIC vector by incubation at 22 °C for 5 min. The recombinant *Sa*UPPS plasmid was transformed to *E.coli* DH5α competent cells and spread on LB agar plate containing 100 μg/mL ampicillin. An Ampicillin-resistant colony was selected and added to 5 mL fresh LB medium containing 100 μg/mL ampicillin and incubated at 37 °C overnight. The sequence of *Sa*UPPS in the plasmid was confirmed by MISSION BIOTECH Inc. (Taiwan)

2.4 Purification of His-tagged *Ec*UPPS or *Sa*UPPS and removal of the tag

The plasmid containing *Ec*UPPS or *Sa*UPPS gene and pET32Xa/LIC vector was transformed to *E.coli* BL21 (*DE*3) and spread on LB agar plate containing 100 μg/mL ampicillin. A single colony was picked and added to 5 mL fresh LB medium containing 100 μg/mL ampicillin and stirred at 37 °C overnight. The culture was transferred to 800 ml fresh LB medium containing 100 μg/mL ampicillin and stirred at 37 °C. The cells were grown to OD_{600} = 0.6 and the protein expression was induced with 1 mM IPTG. After 4 hours, the culture was centrifuged at 6000 rpm for 15 min. The supernatant was discarded and the cell paste was collected. The cell paste was suspended in 50 ml lysis buffer (pH7.5) containing 10 mM Tris-HCl, 500 mM NaCl, 10 mM imidazole, and 2 μM 2-mercaptoethanol. The cells were disrupted with a French pressure cell press

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(AIM-AMINCO spectronic Instruments). The cell lysate was centrifuged at 16000 rpm for 30 min at 4 °C to remove the cell debris. The cell-free extract was mixed with Ni-NTA resin which had been equilibrated with the lysis buffer. The mixture was shaken for 0.5-1 hour at 4 °C and then loaded into a column. After the Ni-NTA column was washed with the washing buffer (the lysis buffer plus 25 mM imidazole for 20-fold resin volume), the His-tagged *Ec*UPPS or *Sa*UPPS was eluted with 20 mL elution buffer (the lysis buffer plus 250 mM imidazole). The His-tagged protein-containing fractions were collected, concentrated and added with 10 μl thrombin to digest His-tag, then the mixture was put in a dialysis bag and dialyzed against the buffer containing 20 mM Tris-HCl, 150 mM NaCl, and 2 mM $CaCl_2$ overnight at 4 °C. The mixture in the bag was passed through a Ni-NTA column to collect the flow through as the purified tag-free *Ec*UPPS or *Sa*UPPS. SPS-PAGE was used to analyze the expression and purification effect of *Ec*UPPS or *Sa*UPPS.

2.5 Kinetic measurements

2.5.1 General procedure

All reactions were in 100 μL solutions with 100 mM Hepes-KOH buffer (pH 7.5), 50 mM KCl, 0.5mM $MgCl_2$, and 0.1% Triton X-100 with 0.1μM *Ec*UPPS or 0.01 μM *Sa*UPPS at 25 °C. The fluorescence change of MANT-O-GPP every 10 s in a total

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period of 10 min was monitored by using a Hybrid Multi-Mode Reader (BioTeK Synergy™ H1) utilizing an excitation wavelength of 352 nm and an emission wavelength of 420 nm.

2.5.2 Extinction coefficient of MANT-O-GPP elongated product formation

The standard curve of the total fluorescence change versus the consumed MANT-O-GPP was used to calculate the extinction coefficient of MANT-O-GPP elongated product formation, which was used to calculate the initial rate of the UPPS reactions. To obtain this standard curve, 0.008, 0.016, 0.031, 0.063, 0.125, 0.25, 0.5 μM MANT-O-GPP were reacted with 30 μM IPP to yield difference levels of fluorescence increase. This plot was linear and the slope was used to give the extinction coefficient by excel.

2.5.3 Kinetic constant measurements

The kinetic constants were determined in 100 μL mixture with 0.1μM *Ec*UPPS or 0.01 μM *Sa*UPPS and different substrate concentrations by monitoring their fluorescence changes. To measure the K_m and k_{cat} of IPP for *Ec*UPPS, 2 μM MANT-O-GPP was used to saturate the enzyme and 1.88, 3.75, 7.5, 15, 30, 60, 120μM IPP was used. To test Km and k_{cat} of MANT-O-GPP for *Ec*UPPS, 90 μM IPP was reacted with 0.03, 0.06, 0.125, 0.25, 0.5, 1, 1.5, 2 μM MANT-O-GPP. To test the K_m and k_{cat} of IPP for *Sa*UPPS, 2 μM MAN-O-GPP reacted with 0.11, 0.23, 0.47, 0.94, 1.88,

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3.75, 7.5, 15 μM IPP. To test the K_m and k_{cat} of MANT-O-GPP for *Sa*UPPS, 10 μM IPP reacted with 0.02, 0.03, 0.06, 0.125, 0.25, 0.5, 1, 1.5 μM MANT-O-GPP. The plots of initial rates versus substrate concentrations were analyzed by GraphPad Prism computer program. The data were fitted by non-linear regression of the Michaelis-Menten equation (eq.1) to obtain K_m and V_{max} values, then k_{cat} was calculated from V_{max}/[E].

$$V_0 = V_{max} [S] / (K_m + [s]) \quad \text{eq.1}$$

2.5.4 EcUPPS and SaUPPS inhibition assays

For measuring the IC_{50} values of compounds **2a-j** and **VK-278** on *Ec*UPPS or *Sa*UPPS, 0.1 μM *Ec*UPPS or 0.01 μM *Sa*UPPS was used in a reaction mixture containing MANT-O-GPP, IPP at the concentration of K_m, and various concentrations of the compound ranging from 0 to 100 μM. Stock solutions of compounds **2a-j** and **VK-278** were 10 mM in DMSO[34]. IC_{50} values were obtained by fitting the plots of initial rates versus the concentrations of compounds **2a-j, VK-278** with Eq.2.

$$A(I) = A(0) \times [I] / ([I] + K_m) \quad \text{eq.2}$$

In this equation, A(I) is the enzyme activity with an inhibitor concentration of I, A(0) is the enzyme activity without inhibitor, and I is the concentration of inhibitor.

2.5.5 Measure the inhibited type of compounds

To test the inhibition type of compound **2d**, different concentrations of substrates and the compound were used to monitor the fluorescence changes. For the inhibition

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type of compound **2d** with respect to IPP in *Sa*UPPS, 2 μM MANT-O-GPP and 0.3, 0.6, 1.2, 2.4, 4.8 μM IPP were reacted without or with compound **2d** in IC_{50} or 1/2 IC_{50}. For the inhibition type of compound **2d** with respect to MANT-O-GPP in *Sa*UPPS, 30 μM IPP and 0.25, 0.5, 1, 1.5, 2 μM MANT-O-GPP were reacted without or with compound **2d** in 1/2 IC_{50} or IC_{50}. The initial rates of different substratre concentrations were calculated from the extinction coefficient by excel. Then, the plots of reciprocal of initial rates versus reciprocal of substrate concentrations were used to determine the inhibition patterns and the K_i values.

2.6 Antibacterial experiments

EC_{50} of the compounds were chosen to present their antibacterial activity. *B. subtilis* was chosen to represent gram-positive bacteria and *E. coli* Rosetta was chosen to represent gram-negative bacteria. For *E. coli*, a single colony was picked to culture in 3 mL fresh LB medium with 100 μg/mL chloramphenicol overnight at 37 ℃ with shaking at 190 rpm. For *B. subtilis*, a single colony was picked to culture in 3 mL fresh LB medium overnight at 37 ℃ with shaking at 190 rpm. The overnight culture was diluted 100-fold into fresh LB medium and incubated 3 h at 37 ℃ with shaking at 190 rpm. Then, the 3 h culture was diluted 400-fold into fresh LB medium and added with 60 μL of different concentrations of compound **2d** dissolved in 100 % DMSO. After

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incubation for 16-20 h at 37 ℃ with shaking at 190 rpm, their OD_{600} values were measured by Hybrid Multi-Mode Reader.

2.7 Docking compound in *Sa*UPPS

The molecular docking was performed using the iGemDOCK to predict how *Sa*UPPS interacts with compound **2d**. Compound **2d** was docked to the structures of *Sa*UPPS with FPP (PDB ID 4H8E[24]). Then, the docking results were analyzed to study the interaction between *Sa*UPPS and compound **2d** and compare with the interaction between *Sa*UPPS and FPP.

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3 RESULT

3.1 Synthesis of pyrrolidinone derivatives

3.1.1 1-(4-Acetylphenyl)-4-carboxy-2-pyrrolidinone (1)

In the beginning, I adopted the method of Ausra et al. reported in 2007[35] to synthesize compound **1**. In this method, compound **1** should precipitate in water after adding aq HCl to pH1, but I did not get the same result. Then, I changed the approach to synthesize compound **1**. To prevent compound **1** from dissolving in water, the mixture was heated to melt and reacted themselves without water. Products were dissolved in methanol with heat after reactions and cooled to be recrystallized. Although some products remained in methanol, this approach could be used to get purified compound **1**.

3.1.2 4-Carboxy-1-(4-styrylcarbonylphenyl)-2-pyrrolidinones derivatives (2a-j)

The synthesis of compound **2a-j** was based on aldol condensation, but the synthesis at room temperature for overnight failed to produce products when compound **1** reacted with NaOH and benzaldehyde at the same time. I then added NaOH to deprotonate compound **1** 30 min before adding various benzaldehydes to successfully make compound **2a-j**. Although compound **2a-j** are hydrophobic, they could be dissolved in ddH_2O with aq NaOH because of their carboxyl group. On the other hand, benzaldehydes could be dissolved in EA, but not in ddH_2O. Based on these different

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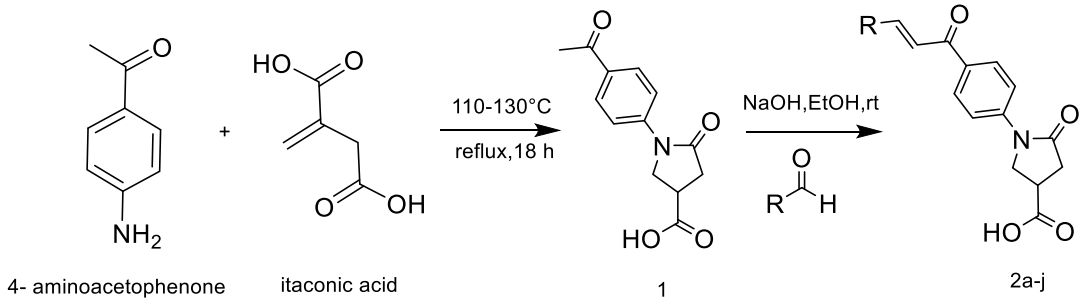
properties, compound **2a-j** were separated from the unreacted benzaldehydes with EA and water by the separatory funnel. Then, the collection of aqueous layers was added HCl to protonate the carboxylate anion, so compounds **2a-j** were precipitated and filtered out. The total synthetic scheme is shown in Scheme 1. Although this method could yield purified compound **2a-j**, it could not be used to yield the compounds with hydrophilic groups. For example, the compound with the hydroxyl group did not precipitate even after adding aq HCl to pH < 1.



Scheme 1. Synthesis of the pyrrolidinone derivatives

3.2 Purification of UPPS

We chose the UPPS in *E. coli* and *S. aureus* to represent gram-negative and gram-positive bacteria, respectively. The plasmid for His-tagged *Ec*UPPS has been previously constructed in our laboratory. I cloned the gene of *Sa*UPPS into pET32Xa/LIC vector to form the plasmid. Then, the plasmids were transformed in *E. coli* BL21 (*DE*3) to overexpress UPPS. After the first purification step with Ni-NTA column, Factor Xa was added to cleave the His-tag. However, the FXa cleavage

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efficiency was quite low, so I cleaved the tag with thrombin. After the enzyme was successfully cleaved by thrombin; UPPS were further purified with a second Ni-NTA column and were collected in flow throught. The 10 % SDS-PAGE analysis is shown in figure 2. In this figure, the *Ec*UPPS and *Sa*UPPS with His-tag both had a band close to 48 kDa. After adding thrombin, 15 kDa His-tag and other residues were cut. The finally purified *Ec*UPPS and *Sa*UPPS without His-tag both had a band between 28-35 kDa. These results were consistent with the theoretical values.

3.3 Kinetic constant of UPPS

Because our compounds had absorption at 360 nm which is the detective wavelength in EnzChek pyrophosphate assay kit, MANT-O-GPP was chosen to measure the activity of UPPS. When MANT-O-GPP reacted with IPP to undergo chain elongation by *Ec*UPPS or *Sa*UPPS, its emission at 420 nm increased. The fluorescence change of MANT-O-GPP can be converted to reaction velocity by using its linear standard curve; this method can be used to measure the kinetics of UPPS[33-34]. The linear standard curve of MANT-O-GPP was determined in Figure 3. As shown in Figure 4 and 5, the K_m of MANT-O-GPP and IPP were 0.67 and 24.33 μM, respectively, for *Ec*UPPS. The K_m of MANT-O-GPP and IPP were 0.32 and 0.38 μM, respectively, for *Sa*UPPS. *Sa*UPPS had higher k_{cat} than *Ec*UPPS (Table 1).

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3.4 Compound 2a-j inhibit UPPS activity

To test the inhibition of compound **2a-j** on UPPS, we added different concentrations of compound **2a-j** to IPP and MANT-O-GPP in the concentrations of their K_m. The compound **VK-278** was also tested with the same method (Figure 6, 7). Their IC_{50} values are shown in Table 2. According to these results, we could get there conclusions. First, the compounds with halogen and benzene group were more potent to inhibit the activity of UPPS. Compounds **2b-d** with halogen more easily enter the activity site of UPPS due to their higher inductive effects and lower steric hindrance. On the other hand, the compound **2i** with benzene group and FPP the substrate of UPPS both were hydrophobic, so the compound **2i** was more suitable in the active site of UPPS. Second, comparing compound **2f t**o **2g** or **VK-278** to **2h**, we found that the positions of substituents affected their inhibition. The para-substituted compounds were more potent to inhibit the activity of UPPS than the meta-substituted compounds. Third, based on the properties of halogen, we tried to synthesize the compound with more halogen. Compound **2j** having two chlorines were the most potent to inhibit the activity of UPPS and had low micromolar IC_{50} value.

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3.5 Antibacterial activity of compound 2d, i, j

To test whether these compounds could inhibit the growth of bacteria, their EC_{50} values were measured. Three of compounds were displayingmore potent to inhibit the activity of UPPS were tested on *E. coli* first. Although these compounds could be dissolved in DMSO, they had to be added into LB medium. Since the survival of *E. coli* was little affected by 2 % DMSO[36], these compounds were dissolved in LB medium with 2% DMSO as the final concentration. Under this situation, the maximum concentration of the compounds was 800 μM. After treating each compound overnight, *E. coli* grew well as the control without the compound. *E. coli* was one of the gram-negative bacteria which had the outer membrane, so these compounds were difficult to cross the cell wall[37]. Then, we tested whether these compounds could inhibit gram-positive bacteria. Due to the biosafety Level of *S. aureus*, we chose *B. subtilis* to represent the gram- positive bacteria. Compound **2d** in 800 μM and compound **2i, 2j** in 400 μM could inhibit over 90 % *B. subtilis* growth (Figure 8). The values of EC_{50} are listed in Table 3. These results show that these compounds could inhibit the growth of *B. subtilis*, so they may also inhibit the growth of other gram-positive bacteria. In addition, we could find that Compound **2i** has more potential to inhibit the growth of *B. subtilis* than compound **2j** which was opposite to IC_{50} values. This unexpected result may due to the hydrophobic of compound **2i** which makes it cross cell membrane easier.

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3.6 Compound 2d was a mixed inhibitor

To investigate the inhibition types of these compounds, we chose compound **2d** which had better yield during synthesis and more potent to inhibit UPPS activity to react with different concentrations of substrates. The lineweaver-burk plot shown in Figure 9 revealed mixed inhibition pattern (three lines intersect at the second quadrant and close to the y-axis) against *Sa*UPPS with respect to both IPP and MANT-O-GPP. Based on these results, compound **2d** was supposed to bind at a different location from those for binding of IPP and MANT-O-GPP. Based on this result, the values of Ki of IPP and MANT-O-GPP were determined as 29.0 ± 1.8 and 30.2 ± 1.5 μM, respectively. Then, the values of Ki' of IPP and MANT-O-GPP were determined as 26.5 ± 7.1 and 34.5 ± 8.7 μM, respectively.

3.7 Compound 2d docked in the activity site of UPPS with FPP

To predict the compound binding location, iGEMDOCK computer program was used to dock compound **2d** and UPPS containing FPP. The best docking results are shown in Figure 10. Although compound **2d** seemed to be docked in the active site of UPPS, the residues interacting with compound **2d** were different from those with FPP. The compound **2d** did not interact with the p-loop (G-N-G-R motif) which is used to recognize FPP and catalysis (D33 in *Sa*UPPS)[38-39], confirming its non-competition in

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binding. On the other hand, the compound **2d** and FPP both interacted with R84 on the loop (F77-R84) which controls channel opening to release the products, so compound **2d** could bind to the UPPS whether or not the UPPS has already bound with FPP.

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4 DISCUSSION

UPPS catalyzes the condensation of IPP and FPP to form UPP which is a carrier for peptidoglycans synthesis, so it is a key enzyme for the synthesis of bacterial cell wall[19]. Because of its role, UPPS could be a valid target for antibiotic[23-28]. In fact, it has been postulated that several antibiotics without known targets may inhibit UPPS[25, 37]. Therefore, we designed and synthesized a series of 1-(4-Acetylphenyl)-4-carboxy-2-pyrrolidinone derivatives to inhibit the activity of UPPS and thus the growth of bacteria.

We tried to mainly synthesize the derivatives with electron-withdrawing substituents on the benzaldehydes. Compound **2a-j** were synthesized successfully. Using the same approach, we intended to synthesize the compounds with electron-donating groups such as 4-diethylaminobenzaldehyde but the products were either with low yield or impure. We also tried to use other aldehydes to replace benzaldehydes, but the products were also impure and difficult to be purified by precipitation. Therefore, the approach still requires modification.

Although the EnzChek pyrophosphate assay kit was commonly used to assay the activity of UPPS[27, 40], our compound had absorbance at 360 nm, the same as the detected wavelength in this assay. The other assay method by using [^{14}C]IPP to monitor the activity[16, 33, 41] is tedious and expansive. As a result, we used MANT-O-GPP which

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was an analog of FPP with fluorescence to evaluate the activity of UPPS[33-34]. Before measuring the IC_{50} values of compounds, the K_m of UPPS had to be determined. Although the K_m value of IPP for *Sa*UPPS was different from reported probably due to the different substrates used. Based on the K_m of IPP and MANT-O-GPP, we used those concentrations to measure the IC_{50} of compounds. According to the inhibition assay, compound **2d**, **2i**, **2j,** and **2k** were more potent to inhibit UPPS activity. Compound **2d** and **2j** with halogens have inductive effects and low steric hindrance, so their structures suited to the active site of UPPS. Then, compound **2j** was more potent to inhibit UPPS because it had one more halogen on benzene to withdraw electrons. On the other hand, compound **2i** was highly hydrophobic like FPP, so it could fit into the active site of UPPS. These compounds had low micromolar ranges of IC_{50}, so they may have the potential to inhibit the growth of bacteria.

To investigate the antibacterial activity of these compounds, we test their EC_{50} values for *E. coli* and *B. subtilis*. The EC_{50} values of compound **2d**, **2i**, and **2j** for *E. coli* were too high to be determined probably because of the thick cell wall of *E. coli*. This situation was beneficial to the treatment for *S. aureus* because most of *E. coli* were good for human bodies. Due to the high risk of growing *S. aureus*, we chose *B. subtilis* and the EC_{50} values of compound **2d**, **2i**, and **2j** were approximately 100-300 μM, higher than the IC_{50} values in inhibiting UPPS. The inefficiency may be due to the difficulty of

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the compounds across the cell wall, which needs to be further tested. Moreover, their solubility in water can be improved by using various acids to salify them[37].

Based on the results of the inhibition and the docking experiments, we assume that these compound were the mixed-type or non-competitive type of inhibitors against UPPS. But the docking models were speculations, they might not be correct. However, the actual interactions between the compounds and UPPS have to be ensured by crystallographic structural studies.

In conclusion, although the antibacterial activities of the tested compounds were not good enough for the treatment of bacterial infectious diseases, they were effective to inhibit UPPS activity with low micromolar IC_{50}. This series of compounds could serve as a starting point for a new class of antibiotics after optimization.

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TABLE

Table 1. The kinetic constants of *Ec*UPPS and *Sa*UPPS.

Protein	MANT-O-GPP K_m (μM)	IPP K_m (μM)	k_{cat} (s^{-1})
*Ec*UPPS	0.67 ± 0.077	24.33 ± 3.33	0.020 ± 0.001
*Sa*UPPS	0.31 ± 0.03	0.41 ± 0.04	0.085 ± 0.002

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Table 2. The IC_{50} of compounds against *Ec*UPPS and *Sa*UPPS.

Compound	R group	IC_{50} for *Ec*UPPS (μM)	IC_{50} for *Sa*UPPS (μM)
2a	phenyl	34.7 ± 1.0	55.5 ± 1.0
2b	4-fluorophenyl	22.5 ± 1.0	27.2 ± 1.1
2c	4-chlorophenyl	16.7 ± 1.0	17.9 ± 1.1
2d	4-bromophenyl	10.8 ± 1.1	15.5 ± 1.0
2e	4-carboxyphenyl	63.5 ± 1.1	88.4 ± 1.0
2f	4-cyanophenyl	32.1 ± 1.0	39.3 ± 1.1
2g	3-cyanophenyl	51.9 ± 1.0	70.2 ± 1.0
VK-278	4-nitrophenyl	12.7 ± 1.0	20.1 ± 1.1
2h	3-nitrophenyl	36.2 ± 1.0	41.3 ± 1.1
2i	biphenyl	4.4 ± 1.1	5.0 ± 1.0
2j	3,4-dichlorophenyl	1.7 ± 1.0	6.4 ± 1.0
2k	furanyl	11.0 ± 1.0	18.7 ± 1.0

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Table 3. The EC_{50} values of compound 2d, i, and j.

Compound	EC_{50} for *E. coli* (μM)	EC_{50} for *B. subtilis* (μM)
2d	> 800	305.8 ± 1.0
2i	> 800	109.1 ± 1.1
2j	> 800	208.9 ± 1.1

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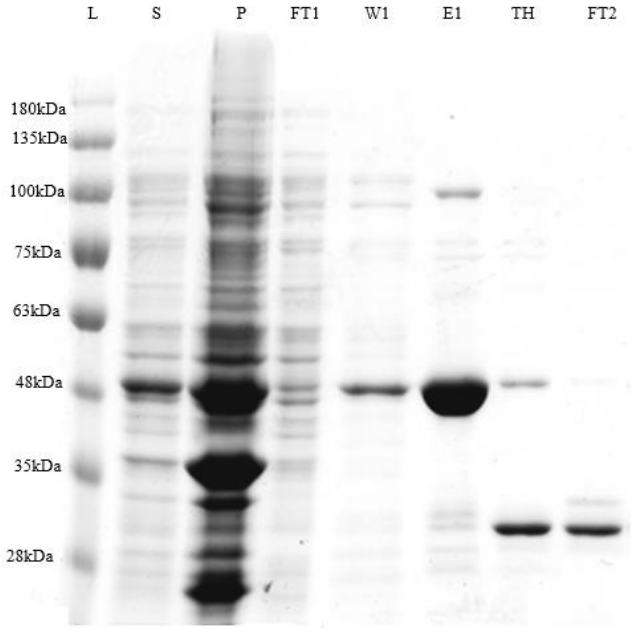
FIGURE

(A)



(B)

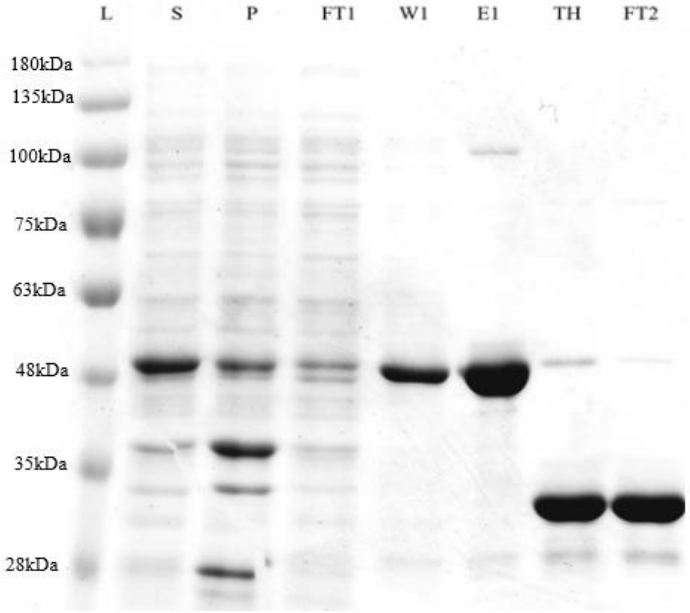


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Figure 2. SDS-PAGE analysis of the purified *Ec*UPPS and *Sa*UPPS.

(A) SDS-PAGE analysis of *Ec*UPPS after different steps of purification. (B) SDS-PAGE analysis of *Sa*UPPS after different steps of purification. L: prestained protein ladder; S: Supernatant; P: Pallet; FT1: flow through with 10 mM imidazole buffer from the first Ni-NTA column; W1 : washed with 25 mM imidazole; E1: eluted with 250 mM imidazole; TH : E1 treated with Thrombin at 4°C overnight; FT2 : TH pass Ni-NTA column;

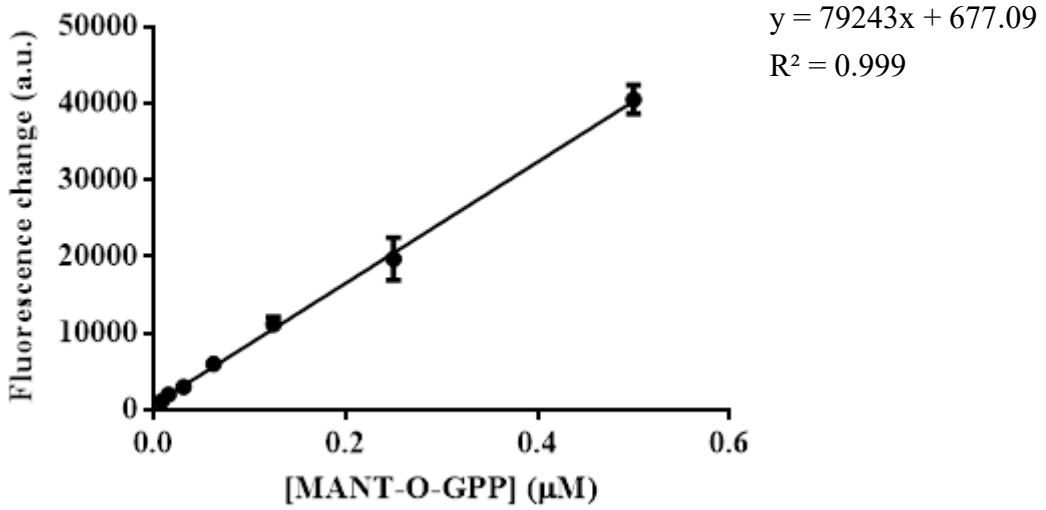

Figure 3. The linear standard curve of MANT-O-GPP converts to the product.

The plot of the total fluorescence change vs. the different concentrations of MANT-O-GPP used for converting to product catalyzed by 0.01 μM of UPPS 10mins. The extinction coefficient of MANT-O-GPP converted to the product was determined to be 79243 a.u./1 μM (the slope of the fitted line). The conversion was assumed to be 100%.

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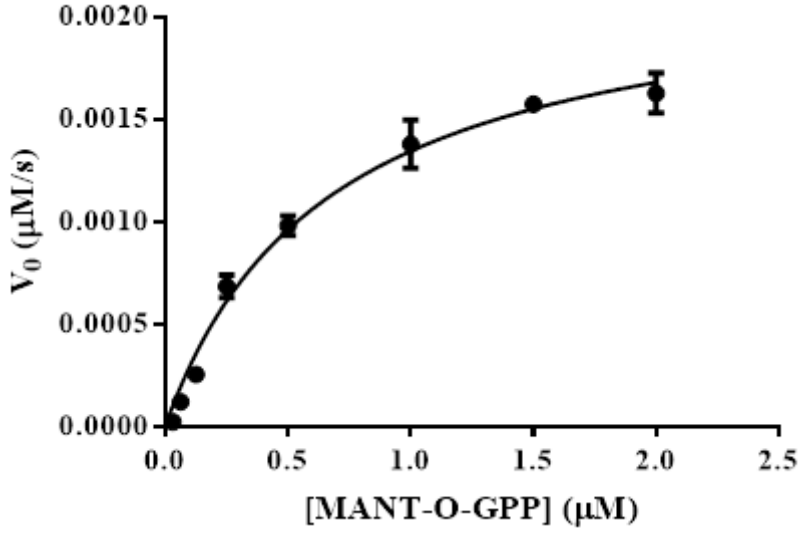
(A)



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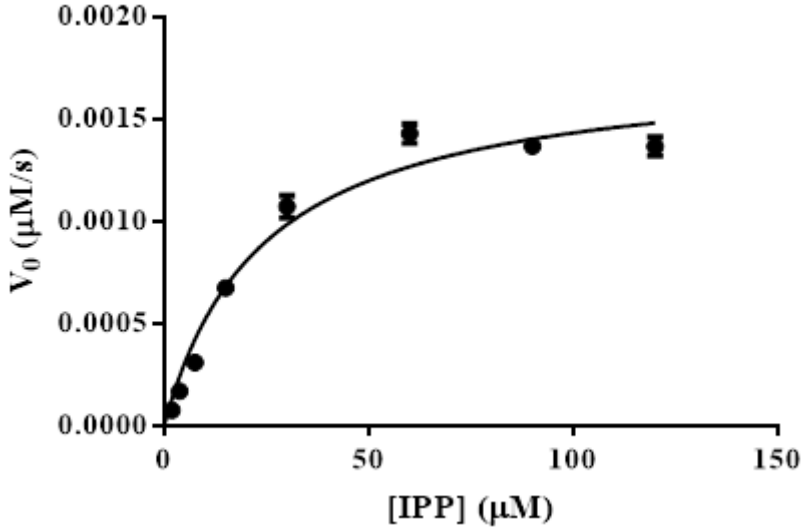

Figure 4. The kinetic constant of *Ec*UPPS.

(A) The plot of V_0 vs. [MANT-O-GPP] was fitted with Michaelis-Menten equation to yield the K_m of MANT-O-GPP and k_{cat} of *Ec*UPPS. (B) The plot of V_0 vs. [IPP] for the K_m of IPP and k_{cat} of *Ec*UPPS.

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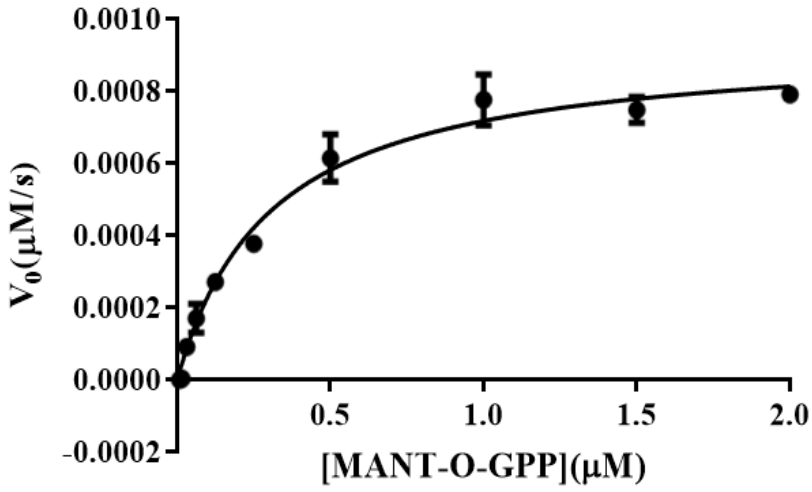
(A)



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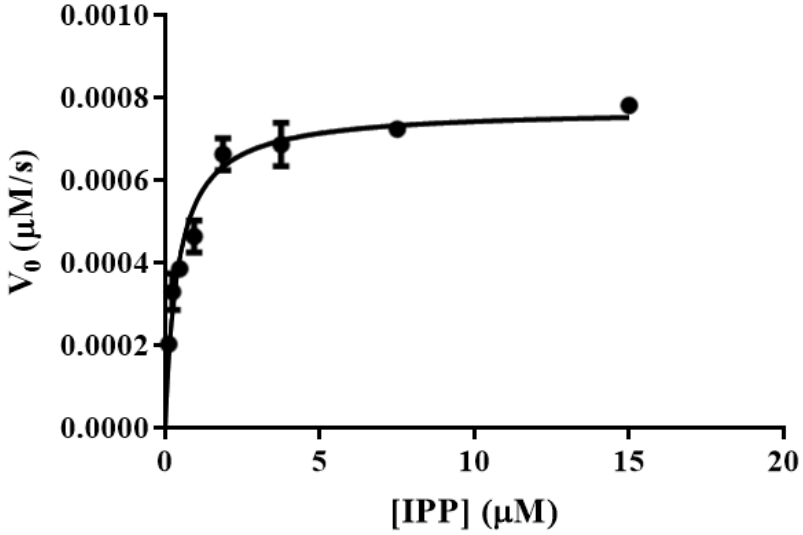

Figure 5. The kinetic constant of *Sa*UPPS.

(A) The plot of V_0 vs. [MANT-O-GPP] was fitted with Michaelis-Menten equation to yield the K_m of MANT-O-GPP and k_{cat} of *Ec*UPPS. (B) The plot of V_0 vs. [IPP] for the K_m of IPP and k_{cat} of *Ec*UPPS.

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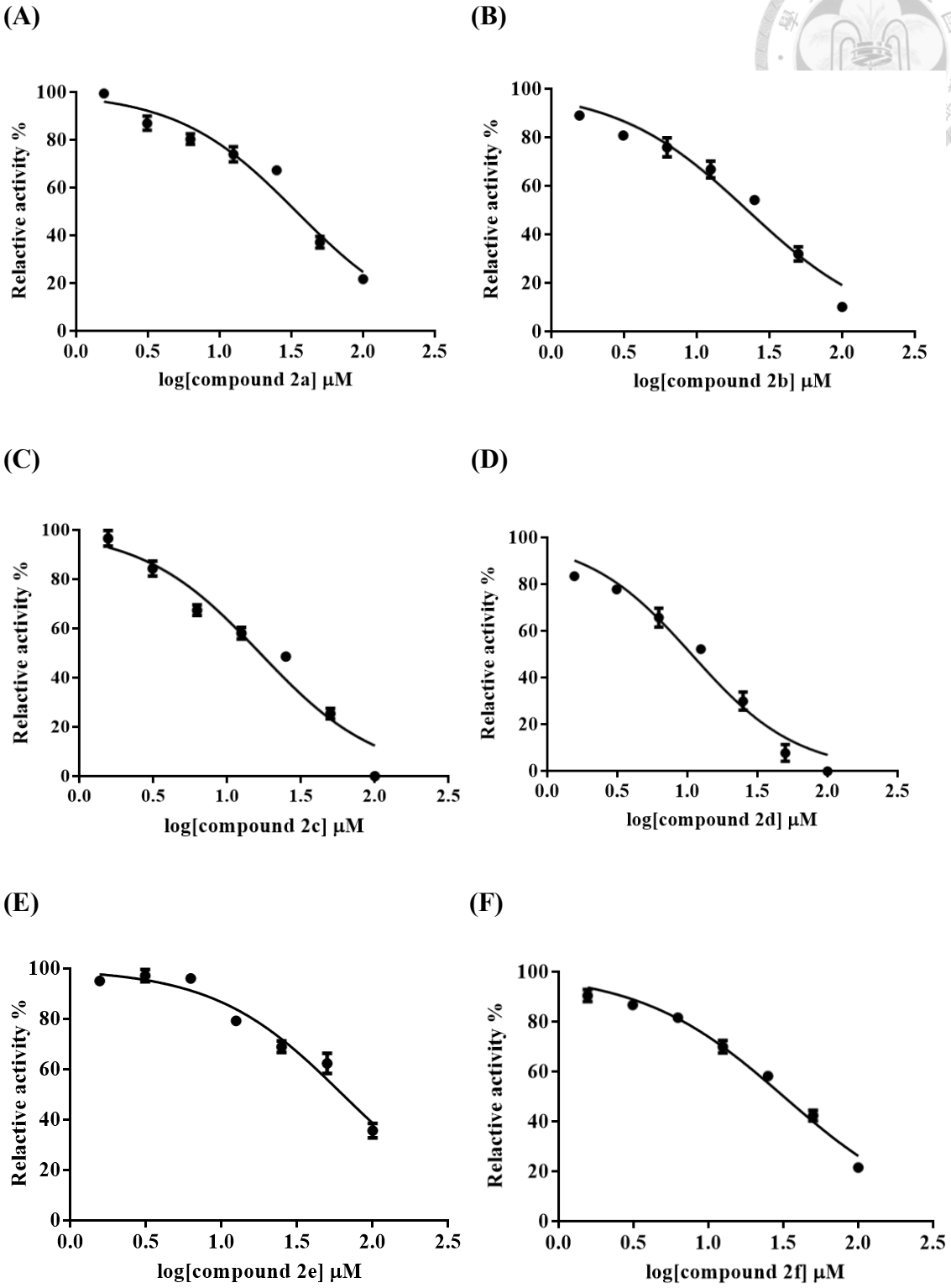

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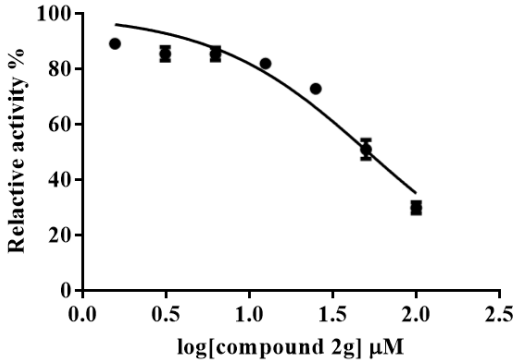
(G)



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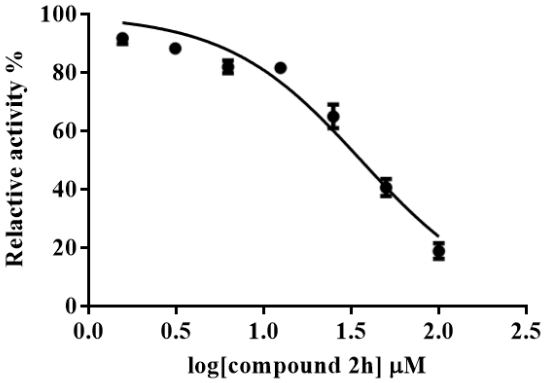
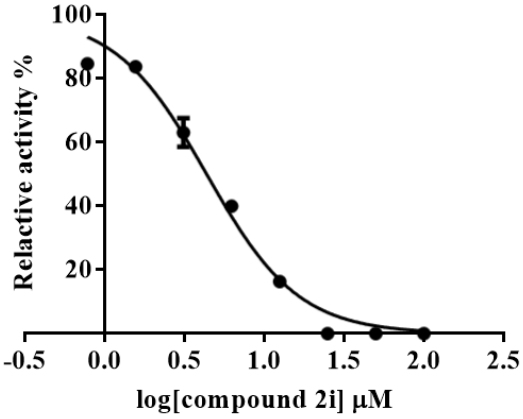
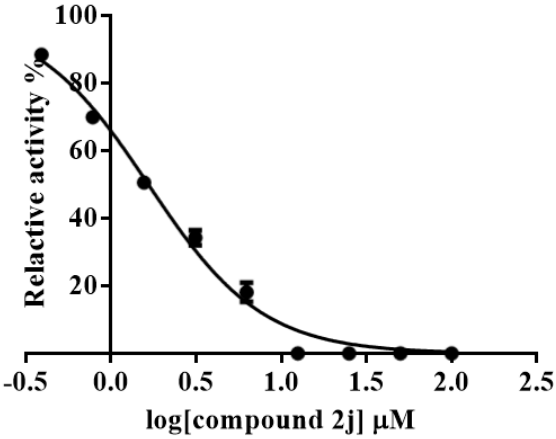


(I)



(J)



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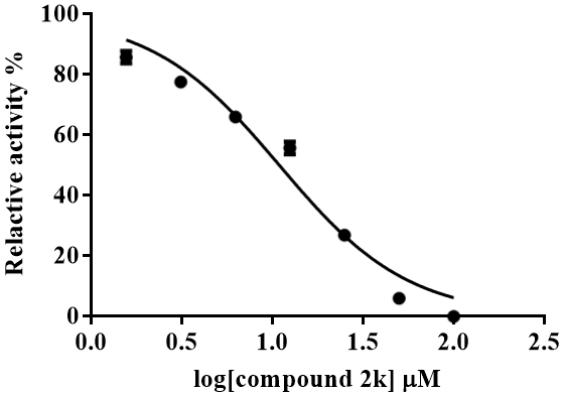

(L)

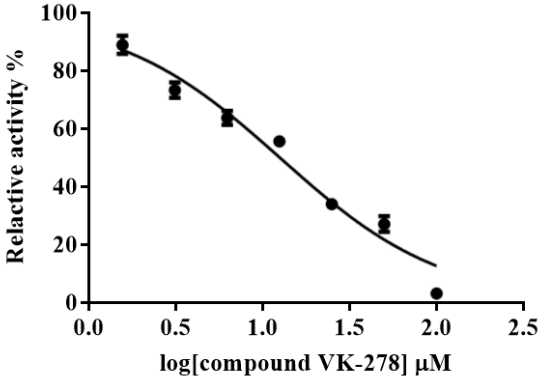

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Figure 6. The inhibition assay for *Ec*UPPS.

These plots are *Ec*UPPS activities relative to the control without any inhibitor vs. the logarithm values of compound concentrations for (A) **2a**, (B) **2b**, (C) **2c**, (D) **2d**, (E) **2e**, (F) **2f**, (G) **2g**, (H) **2h**, (I) **2i**, (J) **2j**, (K) **2k,** and (L) **VK-278**.

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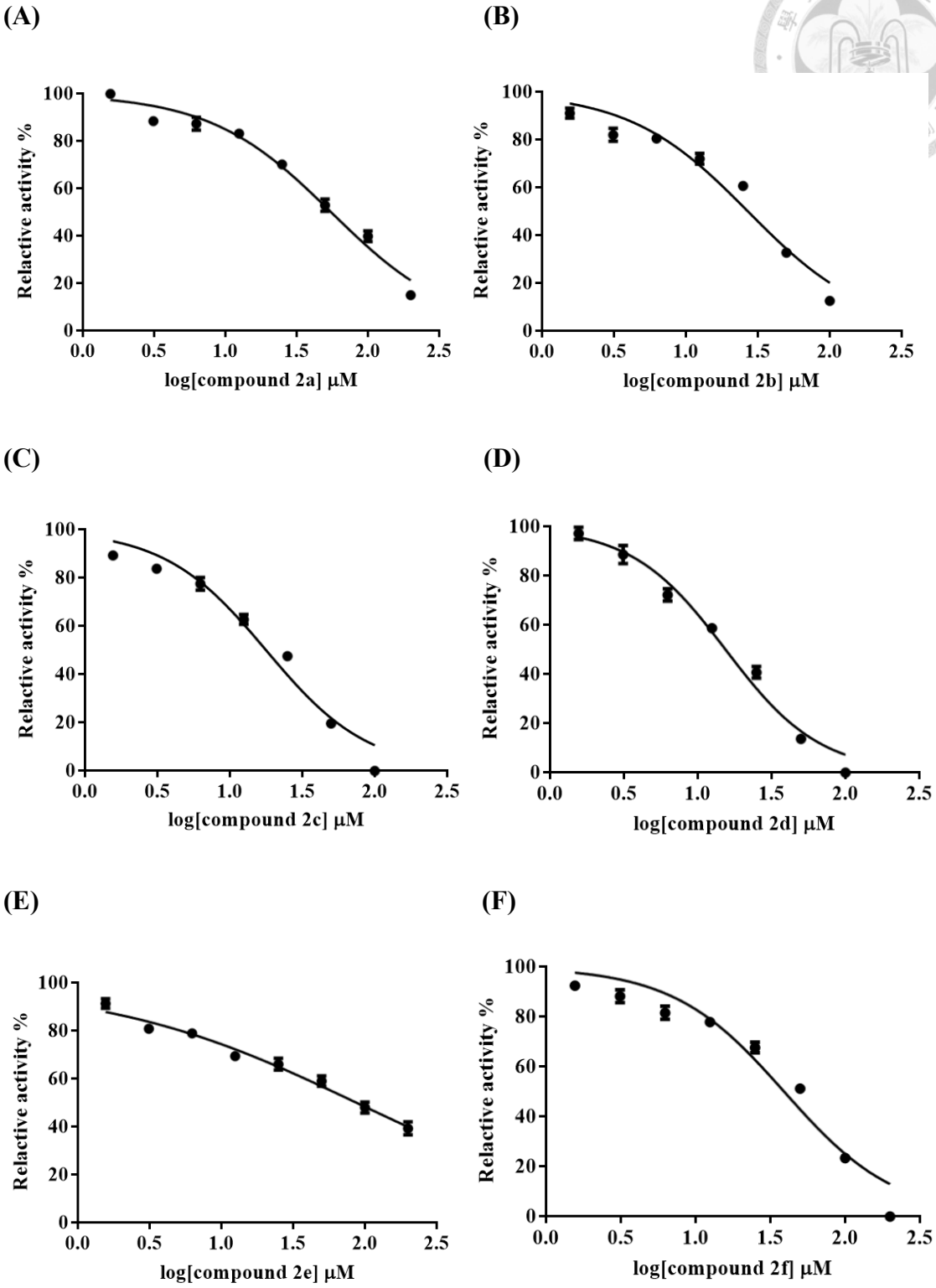

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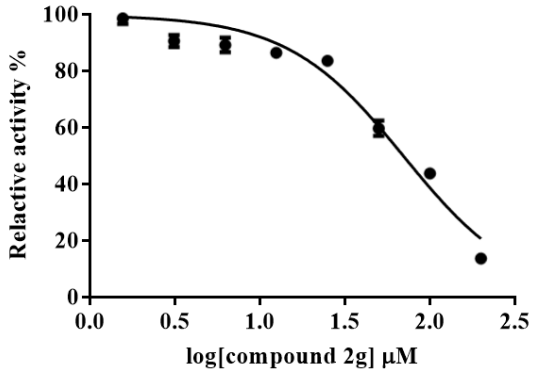
(G)



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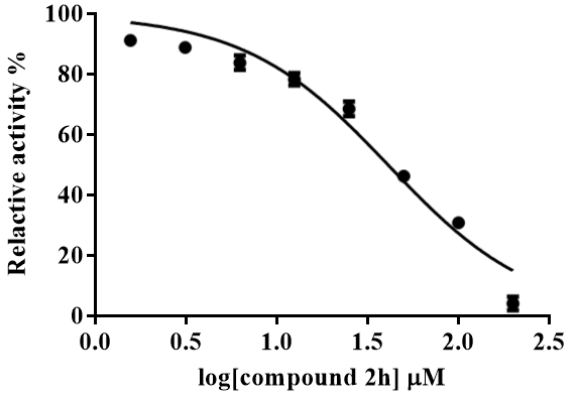

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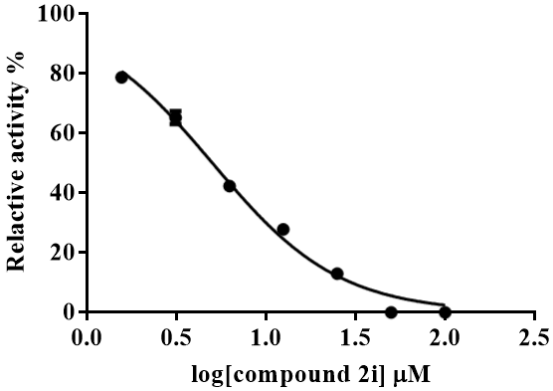
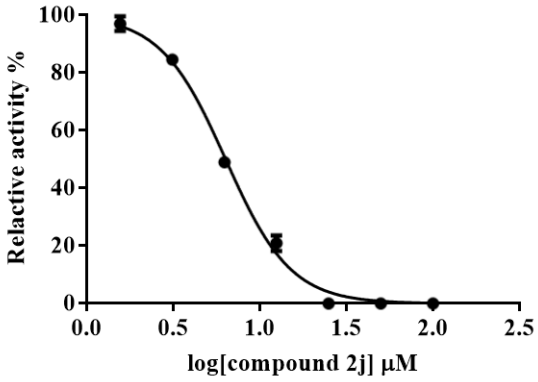


(J)



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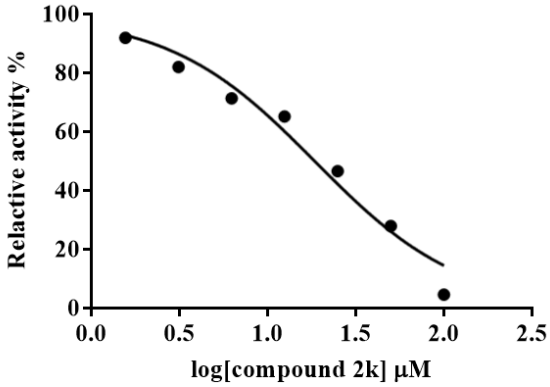

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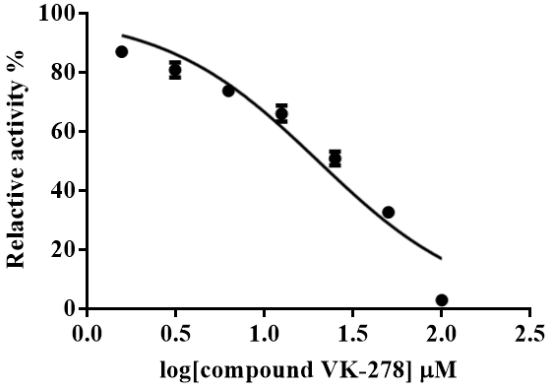

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Figure 7. The inhibition assay for *Sa*UPPS.

These plots are *Sa*UPPS activities relative to the control without any inhibitor vs. the logarithm values of compound concentrations for (A) **2a**, (B) **2b**, (C) **2c**, (D) **2d**, (E) **2e**, (F) **2f**, (G) **2g**, (H) **2h**, (I) **2i**, (J) **2j**, (K) **2k,** and (L) **VK-278**.

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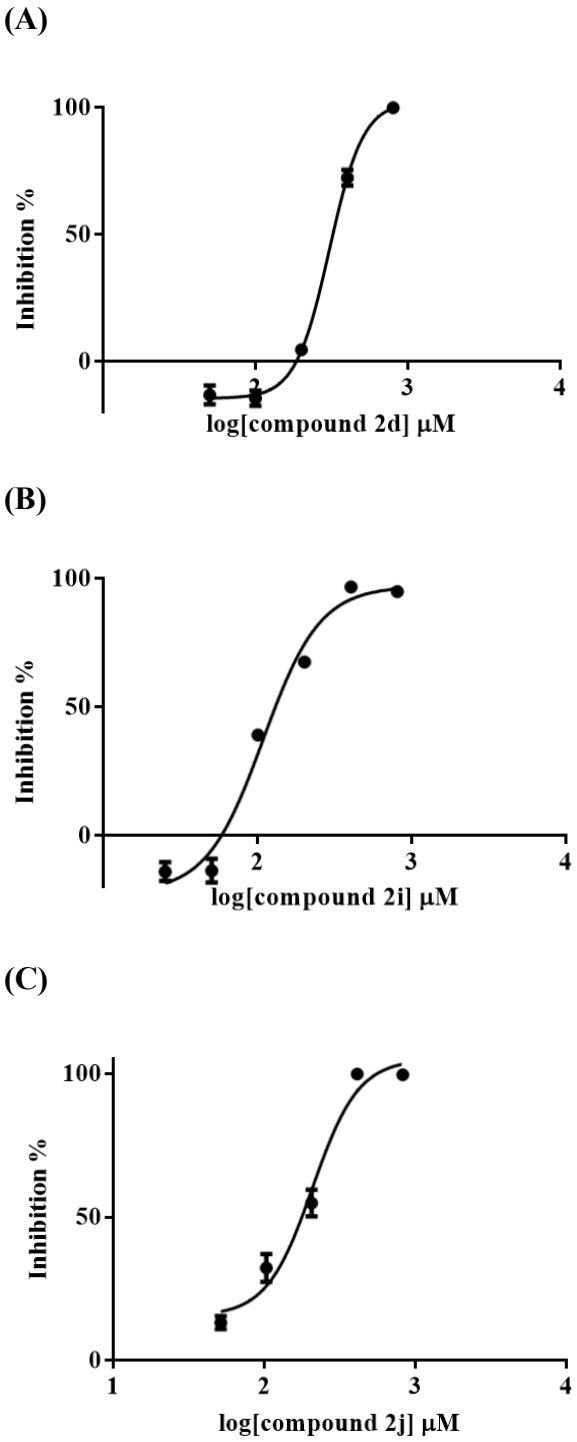

Figure 8. The EC_{50} of compound 2d, I and j against *B. subtilis.*

These plots are cell numbers relative to the control without any inhibitor vs. the logarithm of compound concentrations for (A) **2d**, (B) **2i**, and (C) **2j**.

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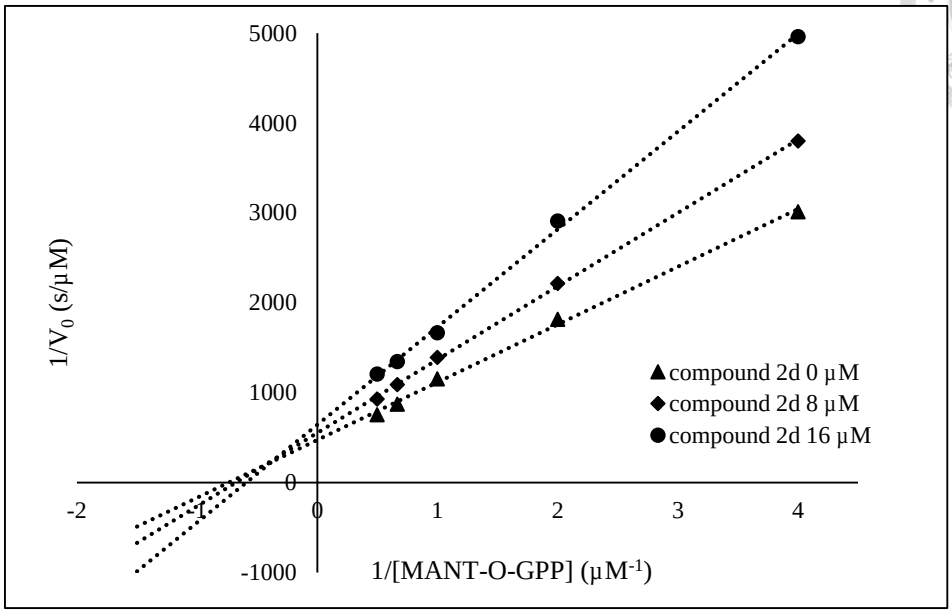
(A)



(B)

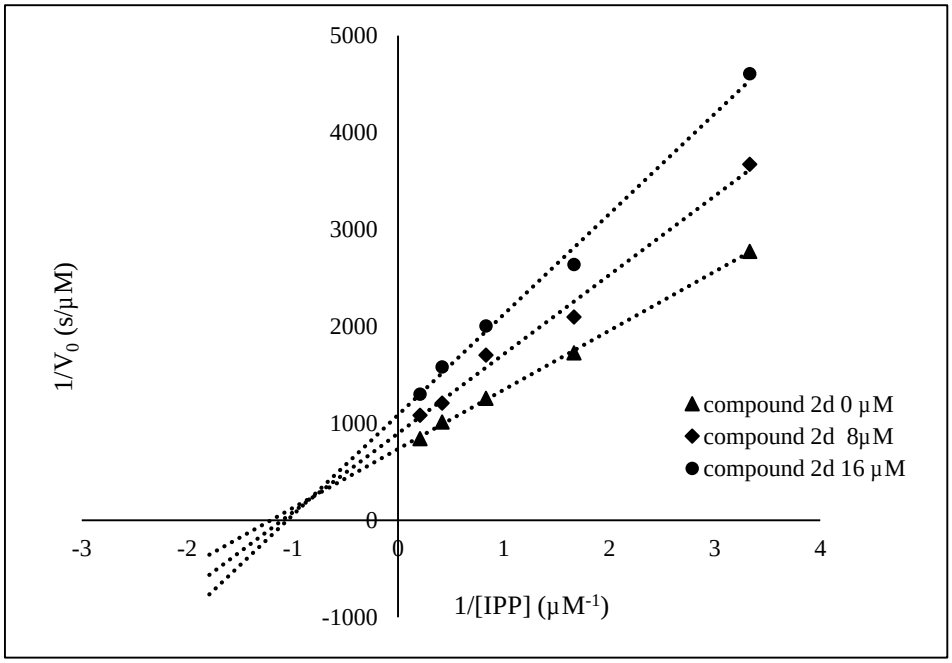


Figure 9. Compound 2d is a mixed inhibitor of *Sa*UPPS.

The lineweaver-burk plots of *Sa*UPPS $1/V_0$ vs. 1/[MANT-O-GPP] (A) and 1/[IPP] (B) for compound **2d**.

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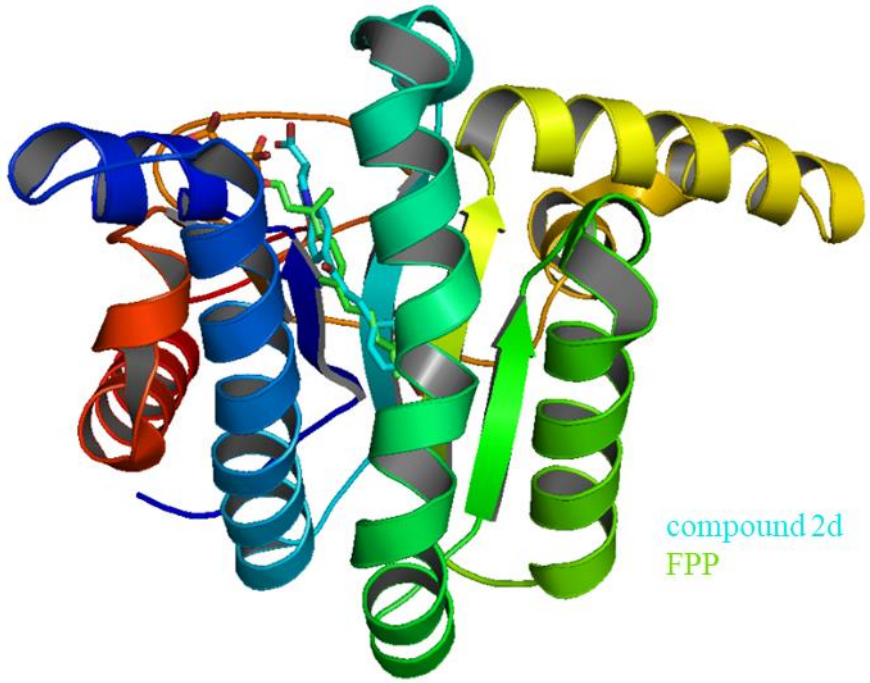
(A)



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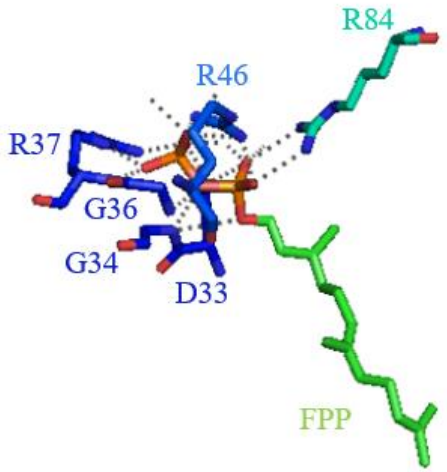

(C)

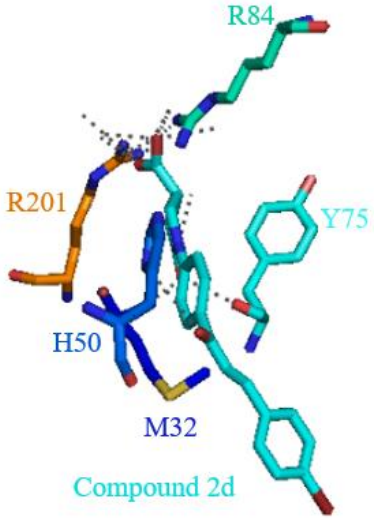

Figure 10. Docking of compound 2d in *Sa*UPPS with FPP.

(A) The docking model of compound **2d** in *Sa*UPPS with FPP.

(B) The interactions between *Sa*UPPS and FPP.

(C) The interactions between *Sa*UPPS and compound **2d**.

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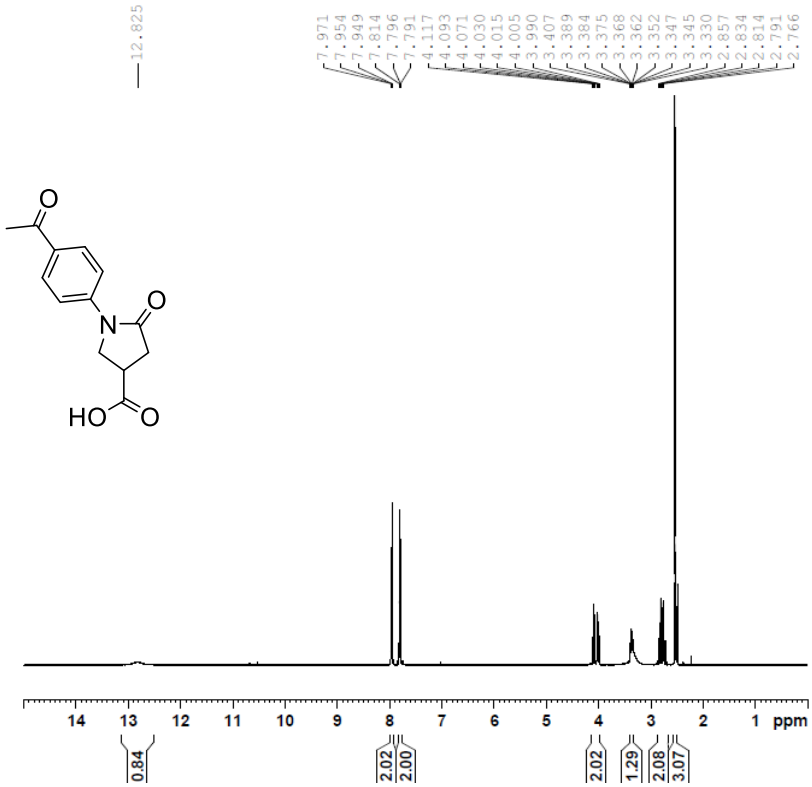
SPECTURM

^{1}H (top) and ^{13}C (bottom) spectura of compound 1



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PROCNO 1

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FIDRES 0.537281 Hz
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RG 165.3
DW 56.800 usec
DE 14.47 usec
TE 298.9 K
D1 1.00000000 sec
TD0 1
SFO1 400.1328009 MHz
NUC1 1H
P1 15.50 usec
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F2 - Processing parameters
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WDW EM
SSB 0
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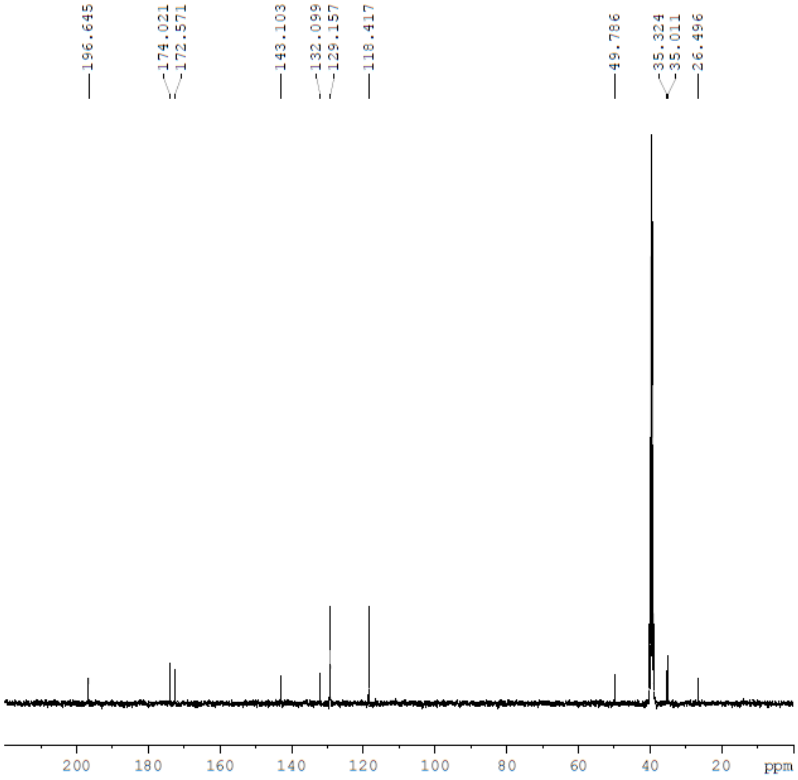
GB 0
PC 1.00



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PROCNO 1

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NS 123
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FIDRES 0.866977 Hz
AQ 1.1534336 sec
RG 212.49
DW 17.600 usec
DE 6.50 usec
TE 298.9 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1
SFO1 100.6258487 MHz
NUC1 13C
P1 10.50 usec
PLW1 47.29999924 W
SFO2 400.1316005 MHz
NUC2 1H
CPDPRG[2 waltz16
PCPD2 90.00 usec
PLW2 10.89999962 W
PLW12 0.32330000 W
PLW13 0.16236000 W

F2 - Processing parameters
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WDW EM
SSB 0
LB 3.00 Hz
GB 0
PC 1.40

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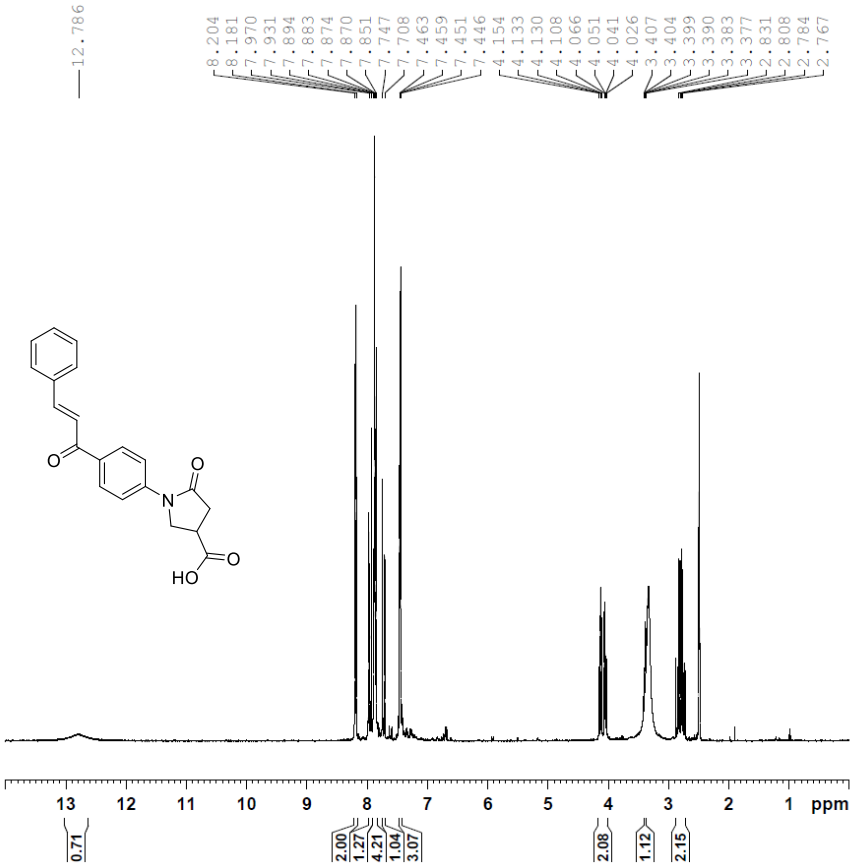
^{1}H (top) and ^{13}C (bottom) spectura of compound 2a



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EXPNO 11111
PROCNO 1

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TD 32768
SOLVENT DMSO
NS 20
DS 0
SWH 8802.817 Hz
FIDRES 0.537281 Hz
AQ 1.8612224 sec
RG 31.38
DW 56.800 usec
DE 14.47 usec
TE 300.4 K
D1 1.00000000 sec
TD0 1
SFO1 400.1328009 MHz
NUC1 1H
P1 15.50 usec
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F2 - Processing parameters
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WDW EM
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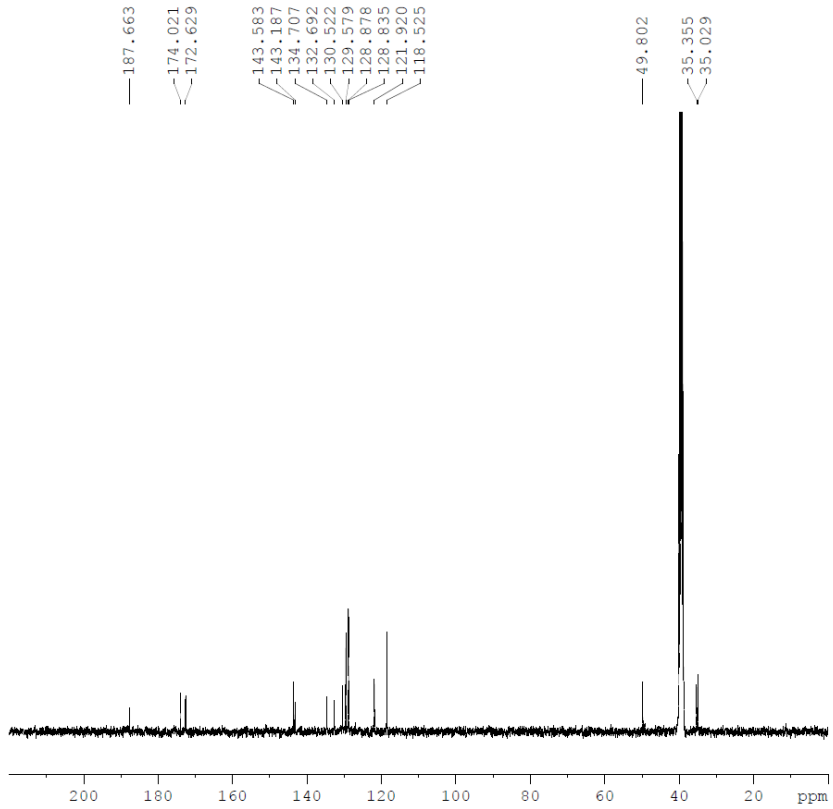
GB 0
PC 1.00



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EXPNO 11112
PROCNO 1

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SOLVENT DMSO
NS 604
DS 4
SWH 28409.092 Hz
FIDRES 0.866977 Hz
AQ 1.1534336 sec
RG 212.49
DW 17.600 usec
DE 6.50 usec
TE 297.5 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1
SFO1 100.6258487 MHz
NUC1 13C
P1 10.50 usec
PLW1 42.50000000 W
SFO2 400.1316005 MHz
NUC2 1H
CPDPRG[2 waltz16
PCPD2 90.00 usec
PLW2 9.89999962 W
PLW12 0.29363999 W
PLW13 0.14747000 W

F2 - Processing parameters
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PC 1.40

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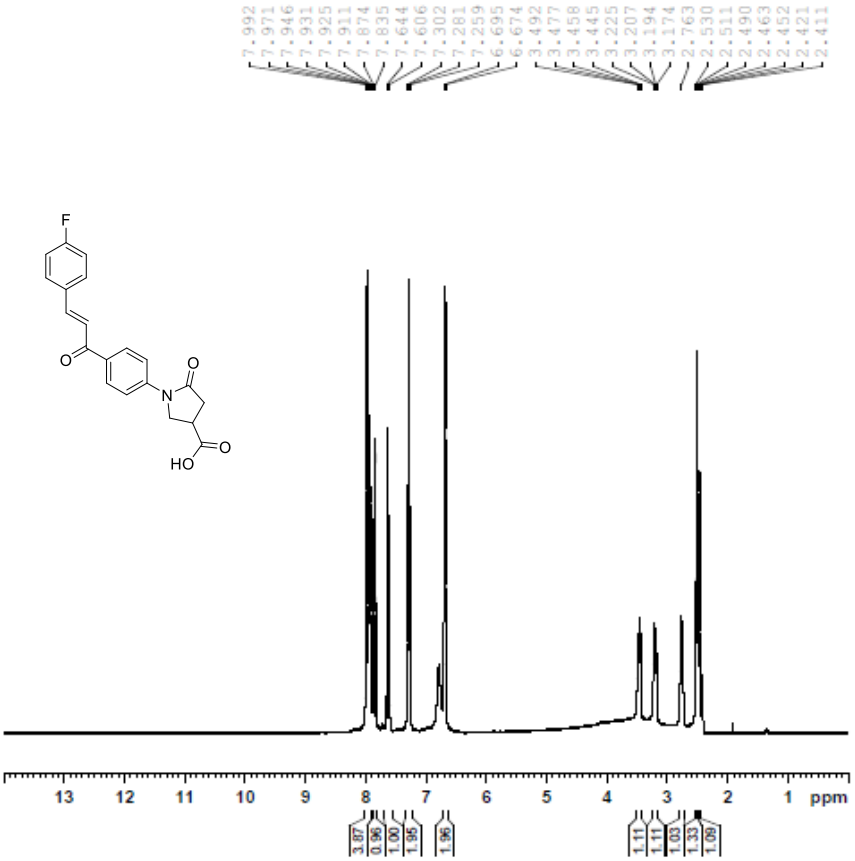
^{1}H (top) and ^{13}C (bottom) spectra of compound 2b



Current Data Parameters
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EXPNO 1
PROCNO 1

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TD 32768
SOLVENT DMSO
NS 20
DS 0
SWH 8802.817 Hz
FIDRES 0.537281 Hz
AQ 1.8612224 sec
RG 118.08
DW 56.800 usec
DE 14.47 usec
TE 298.9 K
D1 1.00000000 sec
TD0 1
SFO1 400.1328009 MHz
NUC1 1H
P1 15.50 usec
PLW1 10.89999962 W

F2 - Processing parameters
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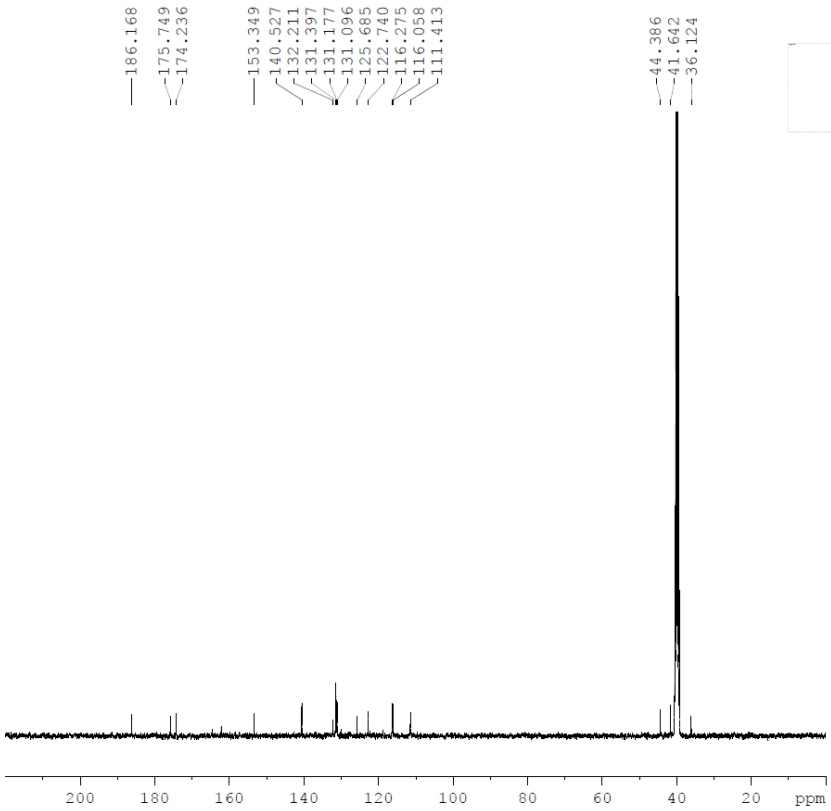

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EXPNO 12
PROCNO 1

F2 - Acquisition Parameters
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TD 65536
SOLVENT DMSO
NS 500
DS 4
SWH 28409.092 Hz
FIDRES 0.866977 Hz
AQ 1.1534336 sec
RG 212.49
DW 17.600 usec
DE 6.50 usec
TE 297.8 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1
SFO1 100.6258487 MHz
NUC1 13C
P1 10.50 usec
PLW1 42.50000000 W
SFO2 400.1316005 MHz
NUC2 1H
CPDPRG[2 waltz16
PCPD2 90.00 usec
PLW2 9.89999962 W
PLW12 0.29363999 W
PLW13 0.14747000 W

F2 - Processing parameters
SI 32768
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WDW EM
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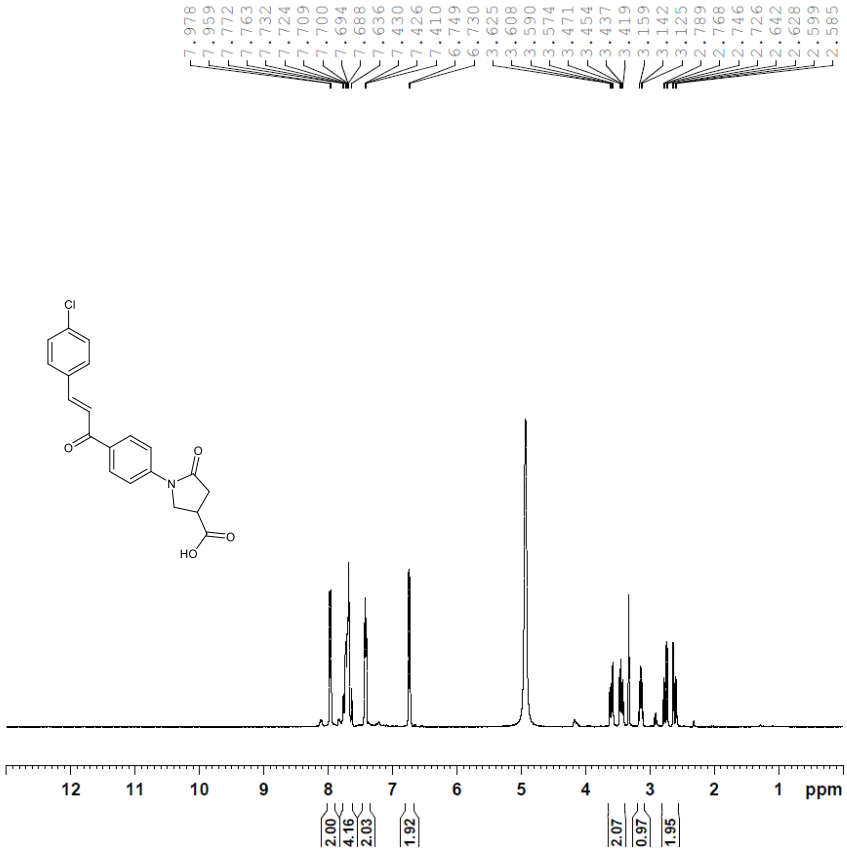
LB 3.00 Hz
GB 0
PC 1.40

doi:10.6342/NTU201901421

^{1}H (top) and ^{13}C (bottom) spectra of compound 2c



Current Data Parameters
NAME compound 2c
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
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Time 12.59 h
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PULPROG zg30
TD 32768
SOLVENT MeOD
NS 20
DS 0
SWH 8802.817 Hz
FIDRES 0.537281 Hz
AQ 1.8612224 sec
RG 118.08
DW 56.800 usec
DE 14.47 usec
TE 298.6 K
D1 1.00000000 sec
TD0 1
SFO1 400.1328009 MHz
NUC1 1H
P1 15.50 usec
PLW1 10.89999962 W

F2 - Processing parameters
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WDW EM
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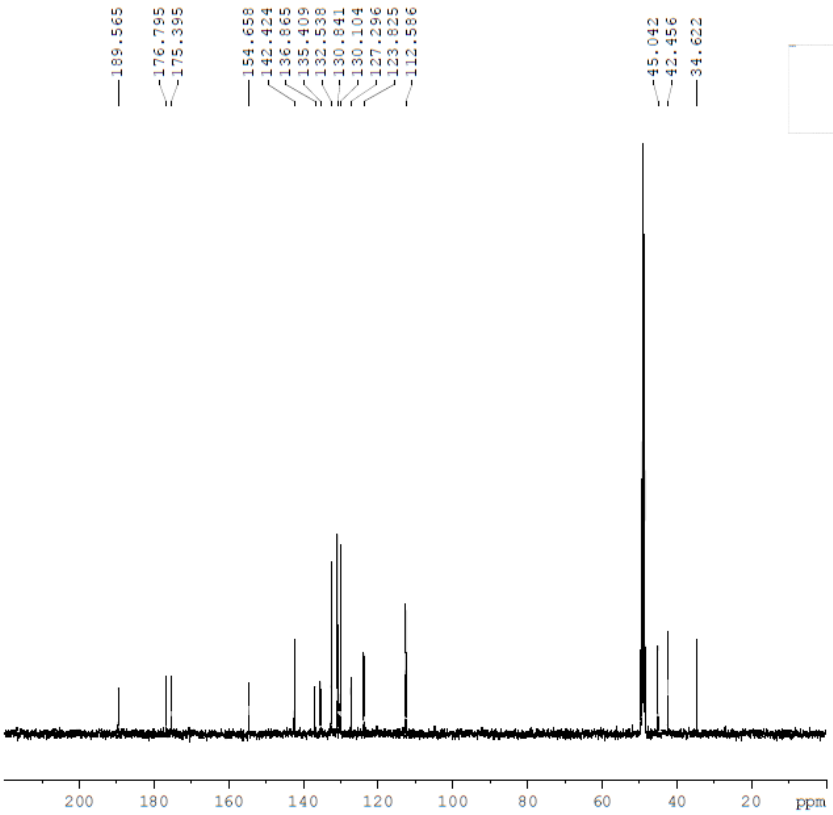
GB 0
PC 1.00



Current Data Parameters
NAME compound 2c
EXPNO 3
PROCNO 1

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Time 13.24 h
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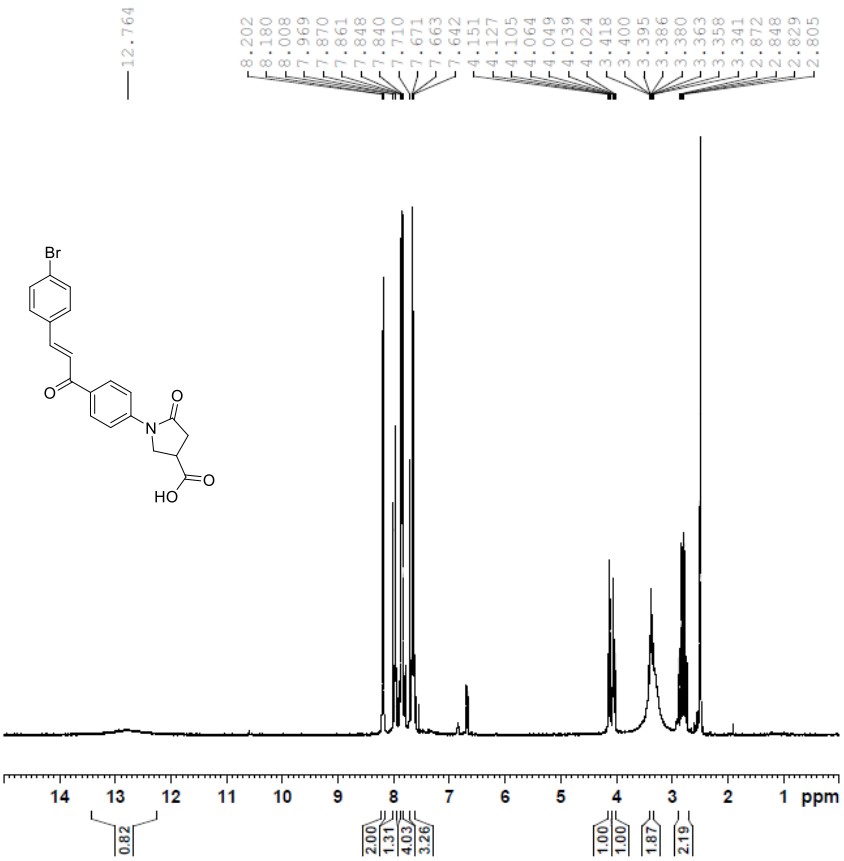
PULPROG zgpg30
TD 65536
SOLVENT MeOD
NS 2
DS 4
SWH 28409.092 Hz
FIDRES 0.866977 Hz
AQ 1.1534336 sec
RG 212.49
DW 17.600 usec
DE 6.50 usec
TE 299.3 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1
SFO1 100.6258487 MHz
NUC1 13C
P1 10.50 usec
PLW1 47.29999924 W
SFO2 400.1316005 MHz
NUC2 1H
CPDPRG[2 waltz16
PCPD2 90.00 usec
PLW2 10.89999962 W
PLW12 0.32330000 W
PLW13 0.16236000 W

F2 - Processing parameters
SI 32768
SF 100.6126328 MHz
WDW EM
SSB 0
LB 3.00 Hz
GB 0
PC 1.40

doi:10.6342/NTU201901421

^{1}H (top) and ^{13}C (bottom) spectura of compound 2d



Current Data Parameters
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EXPNO 111
PROCNO 1

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PULPROG zg30
TD 32768
SOLVENT DMSO
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SWH 8802.817 Hz
FIDRES 0.537281 Hz
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RG 188.2
DW 56.800 usec
DE 14.47 usec
TE 298.6 K
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TD0 1
SFO1 400.1328009 MHz
NUC1 1H
P1 15.50 usec
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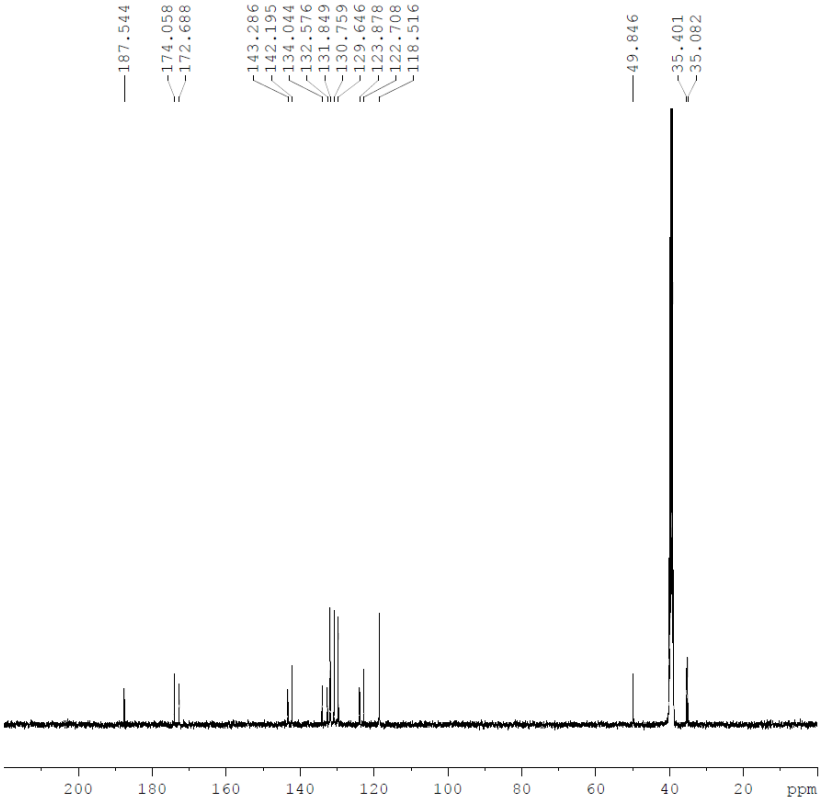

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EXPNO 113
PROCNO 1

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SOLVENT DMSO
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FIDRES 0.866977 Hz
AQ 1.1534336 sec
RG 212.49
DW 17.600 usec
DE 6.50 usec
TE 297.8 K
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D11 0.03000000 sec
TD0 1
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NUC1 13C
P1 10.50 usec
PLW1 42.50000000 W
SFO2 400.1316005 MHz
NUC2 1H
CPDPRG[2 waltz16
PCPD2 90.00 usec
PLW2 9.89999962 W
PLW12 0.29363999 W
PLW13 0.14747000 W

F2 - Processing parameters
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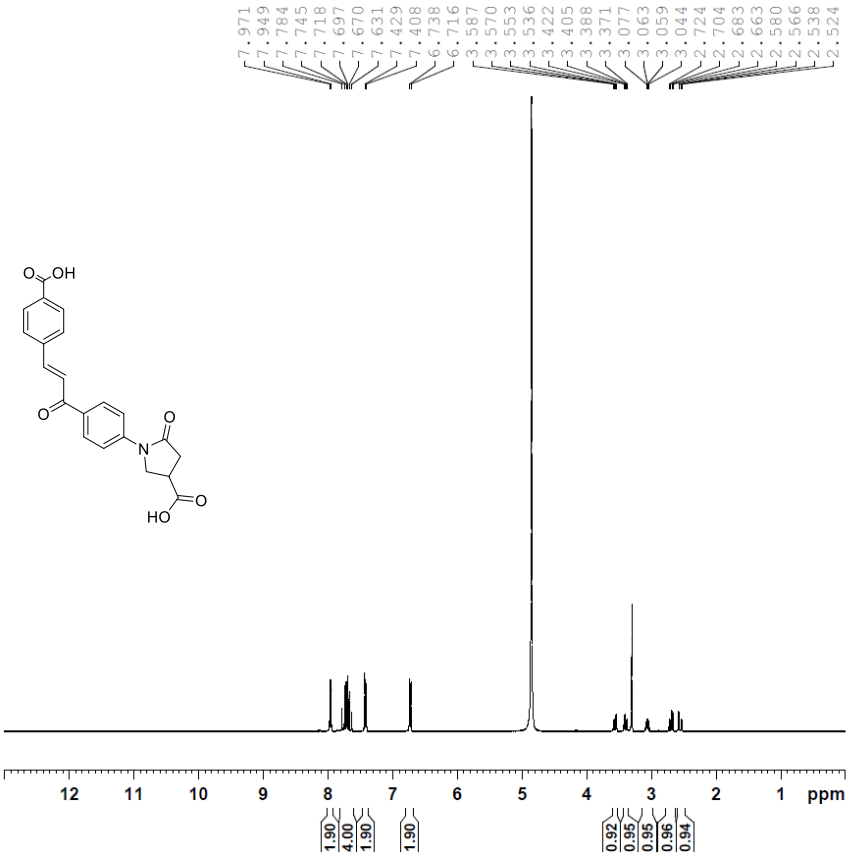
LB 3.00 Hz
GB 0
PC 1.40

doi:10.6342/NTU201901421

^{1}H (top) and ^{13}C (bottom) spectura of compound 2e



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EXPNO 11
PROCNO 1

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TD 32768
SOLVENT MeOD
NS 20
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SWH 8802.817 Hz
FIDRES 0.537281 Hz
AQ 1.8612224 sec
RG 188.2
DW 56.800 usec
DE 14.47 usec
TE 299.0 K
D1 1.00000000 sec
TD0 1
SFO1 400.1328009 MHz
NUC1 1H
P1 15.50 usec
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F2 - Processing parameters
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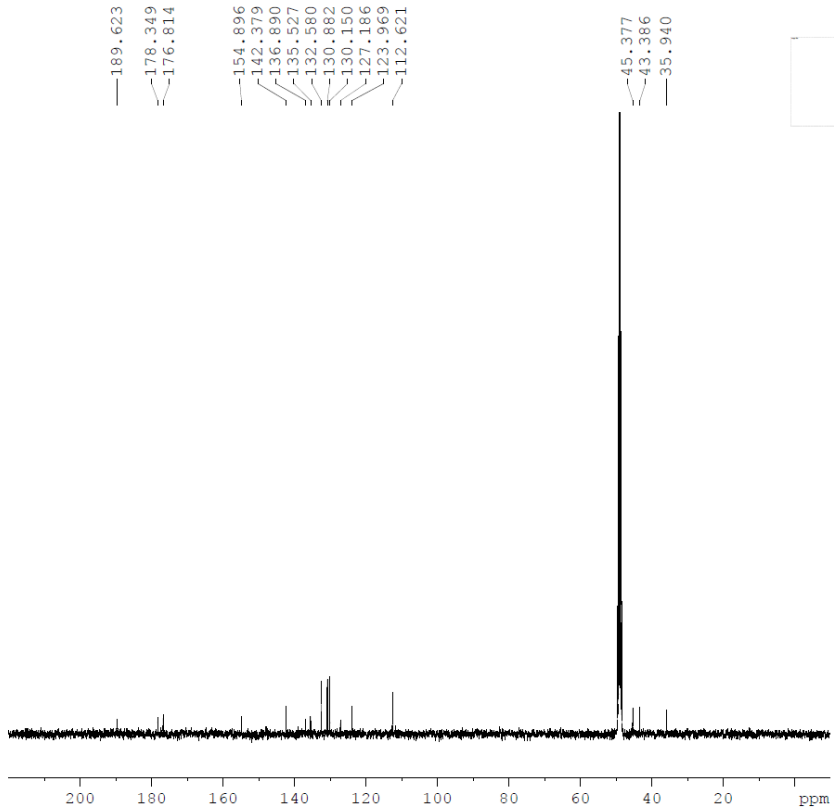

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SOLVENT MeOD
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FIDRES 0.866977 Hz
AQ 1.1534336 sec
RG 212.49
DW 17.600 usec
DE 6.50 usec
TE 299.3 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1
SFO1 100.6258487 MHz
NUC1 13C
P1 10.50 usec
PLW1 47.29999924 W
SFO2 400.1316005 MHz
NUC2 1H
CPDPRG[2 waltz16
PCPD2 90.00 usec
PLW2 10.89999962 W
PLW12 0.32330000 W
PLW13 0.16236000 W

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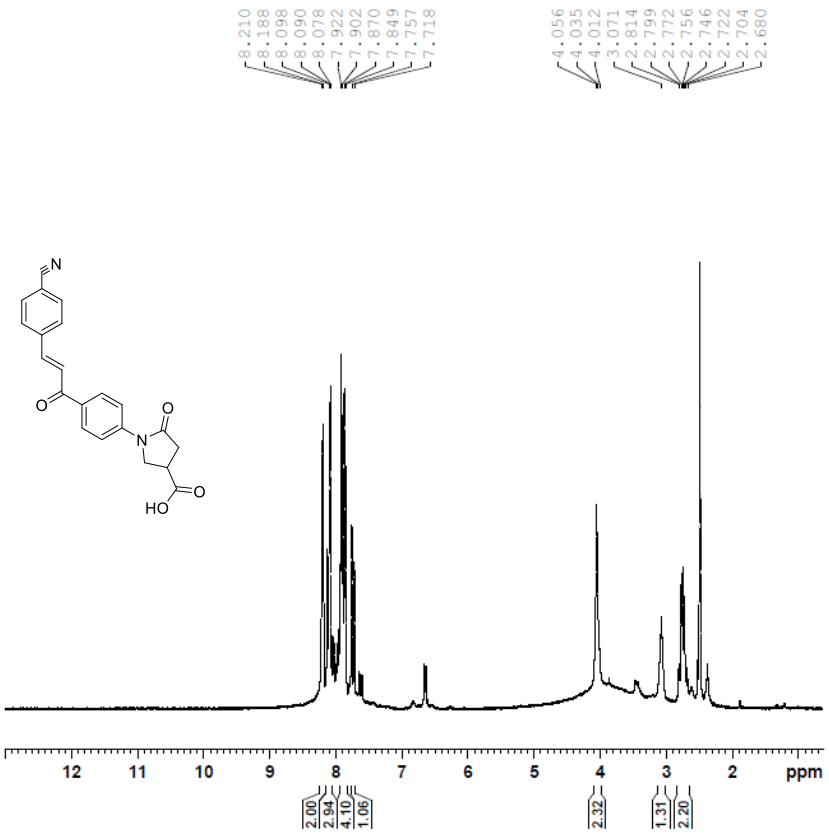
^{1}H (top) and ^{13}C (bottom) spectra of compound 2f



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FIDRES 0.537281 Hz
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RG 165.3
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DE 14.47 usec
TE 297.1 K
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TD0 1
SFO1 400.1328009 MHz
NUC1 1H
P1 15.50 usec
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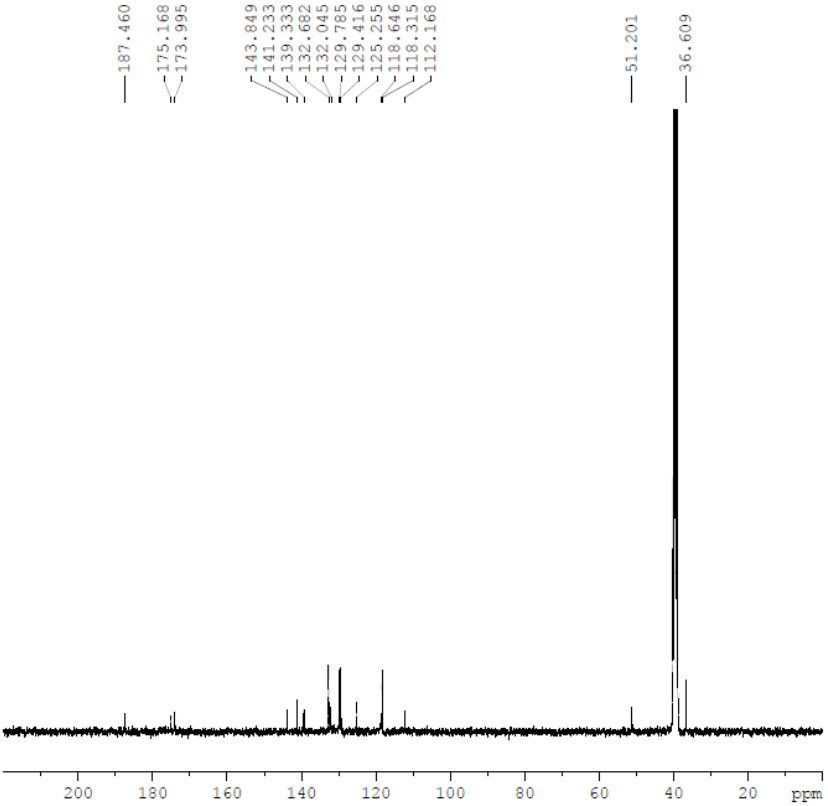

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PULPROG zgpg30
TD 65536
SOLVENT DMSO
NS 700
DS 4
SWH 28409.092 Hz
FIDRES 0.866977 Hz
AQ 1.1534336 sec
RG 212.49
DW 17.600 usec
DE 6.50 usec
TE 297.6 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1
SFO1 100.6258487 MHz
NUC1 13C
P1 10.50 usec
PLW1 42.50000000 W
SFO2 400.1316005 MHz
NUC2 1H
CPDPRG[2 waltz16
PCPD2 90.00 usec
PLW2 9.89999962 W
PLW12 0.29363999 W
PLW13 0.14747000 W

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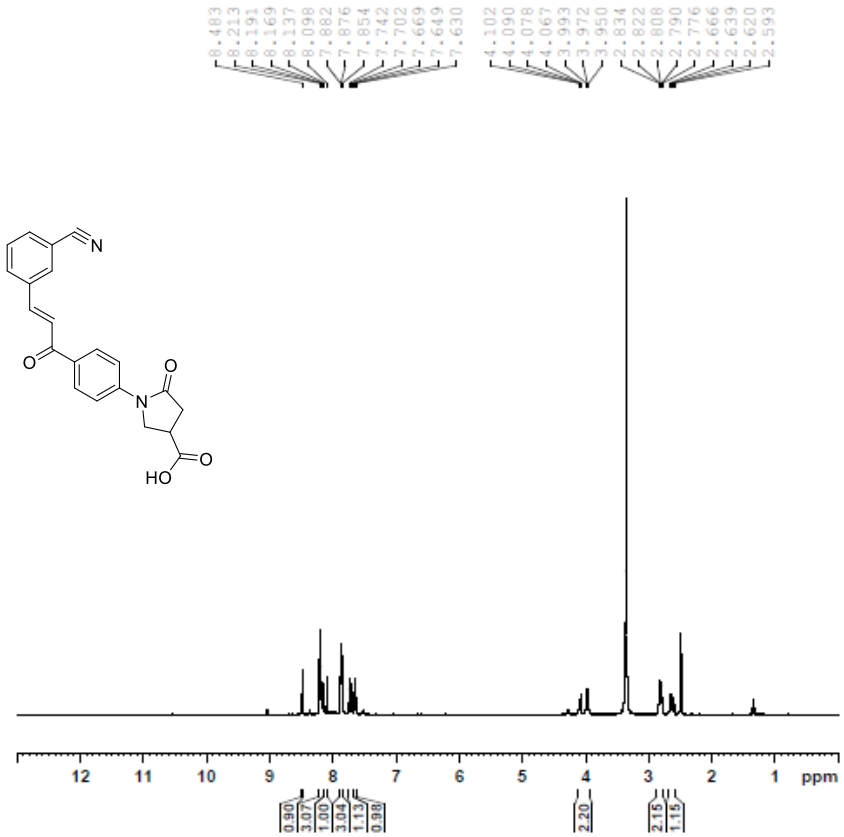
^{1}H (top) and ^{13}C (bottom) spectra of compound 2g



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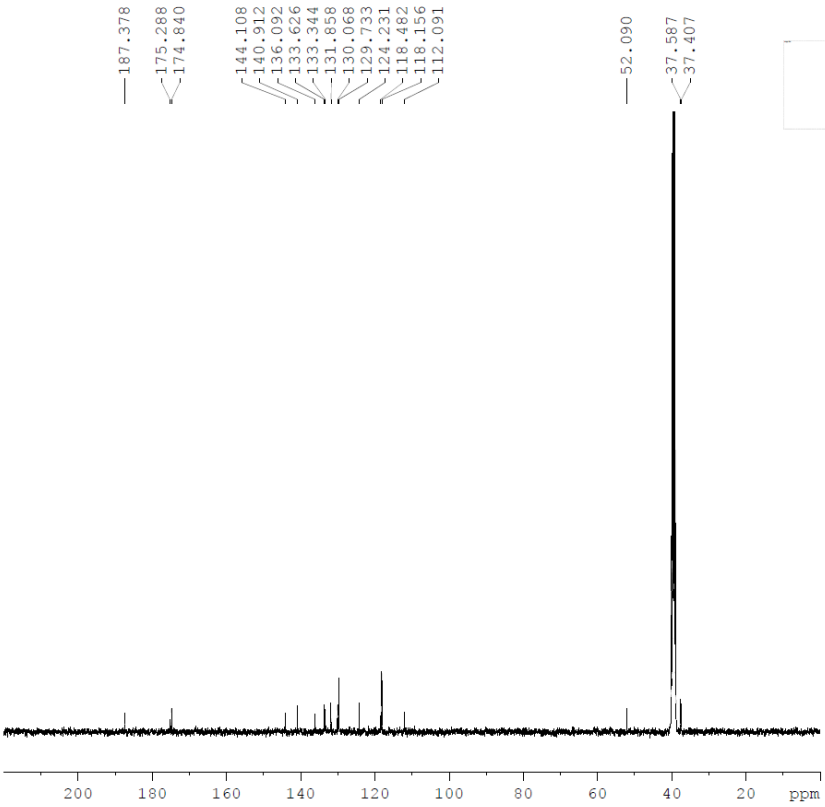
SOLVENT DMSO
NS 20
DS 0
SWH 8802.817 Hz
FIDRES 0.537281 Hz
AQ 1.8612224 sec
RG 165.3
DW 56.800 usec
DE 14.47 usec
TE 297.6 K
D1 1.00000000 sec
TD0 1
SFO1 400.1328009 MHz
NUC1 1H
P1 15.50 usec
PLW1 9.89999962 W

F2 - Processing parameters
SI 131072
SF 400.1300080 MHz
WDW EM
SSB 0
LB 0 Hz
GB 0
PC 1.00



Current Data Parameters
NAME compound 2g
EXPNO 1112
PROCNO 1

F2 - Acquisition Parameters
Date_ 20190505
Time 11.18 h
INSTRUM spect
PROBHD Z108618_0411 (
PULPROG zgpg30
TD 65536
SOLVENT DMSO
NS 600
DS 4
SWH 28409.092 Hz
FIDRES 0.866977 Hz
AQ 1.1534336 sec
RG 212.49
DW 17.600 usec
DE 6.50 usec
TE 298.0 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1
SFO1 100.6258487 MHz
NUC1 13C
P1 10.50 usec
PLW1 42.50000000 W
SFO2 400.1316005 MHz
NUC2 1H
CPDPRG[2 waltz16
PCPD2 90.00 usec
PLW2 9.89999962 W
PLW12 0.29363999 W
PLW13 0.14747000 W

F2 - Processing parameters
SI 32768
SF 100.6128186 MHz
WDW EM
SSB 0
LB 3.00 Hz
GB 0
PC 1.40

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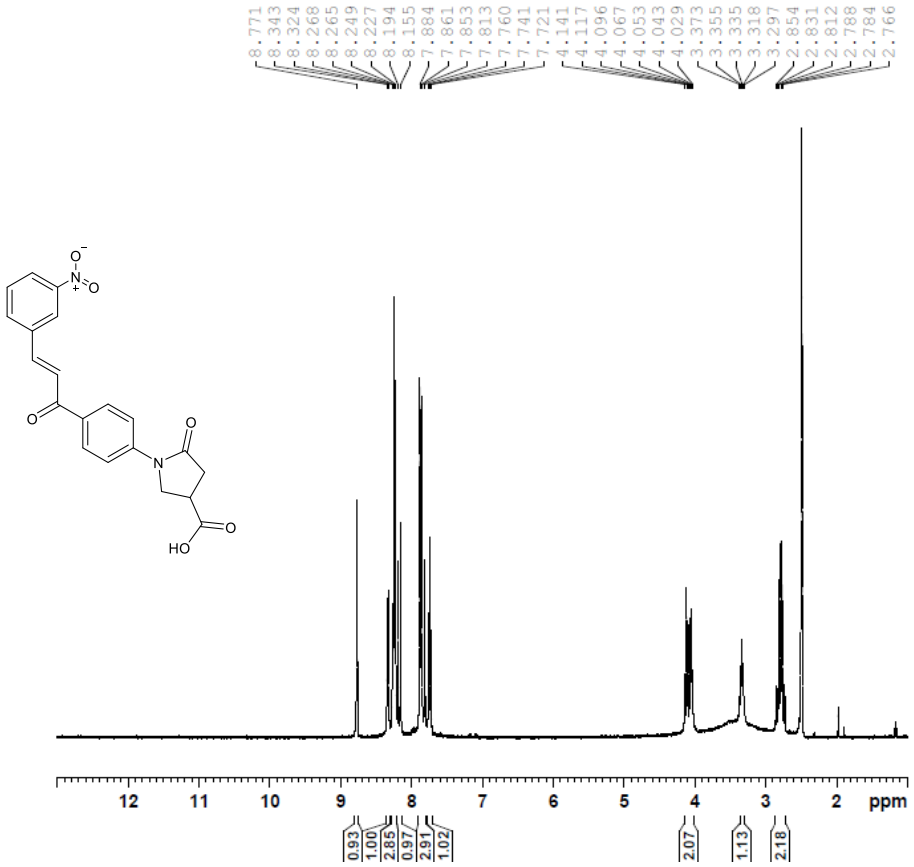
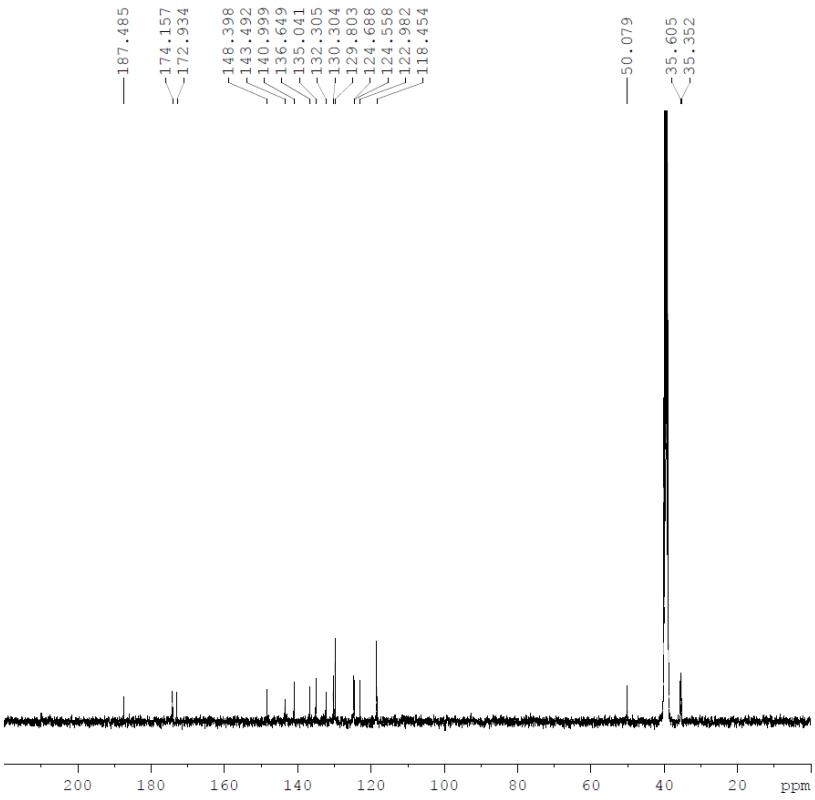
^{1}H (top) and ^{13}C (bottom) spectra of compound 2h



Current Data Parameters
NAME compound 2h
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
Date_ 20180130
Time 16.32 h
INSTRUM spect
PROBHD Z108618_0411 (
PULPROG zg30
TD 32768
SOLVENT DMSO
NS 20
DS 0
SWH 8802.817 Hz
FIDRES 0.537281 Hz
AQ 1.8612224 sec
RG 212.49
DW 56.800 usec
DE 14.47 usec
TE 298.0 K
D1 1.00000000 sec
TD0 1
SFO1 400.1328009 MHz
NUC1 1H
P1 15.50 usec
PLW1 10.89999962 W

F2 - Processing parameters
SI 131072
SF 400.1300078 MHz
WDW EM
SSB 0
LB 0 Hz
GB 0
PC 1.00



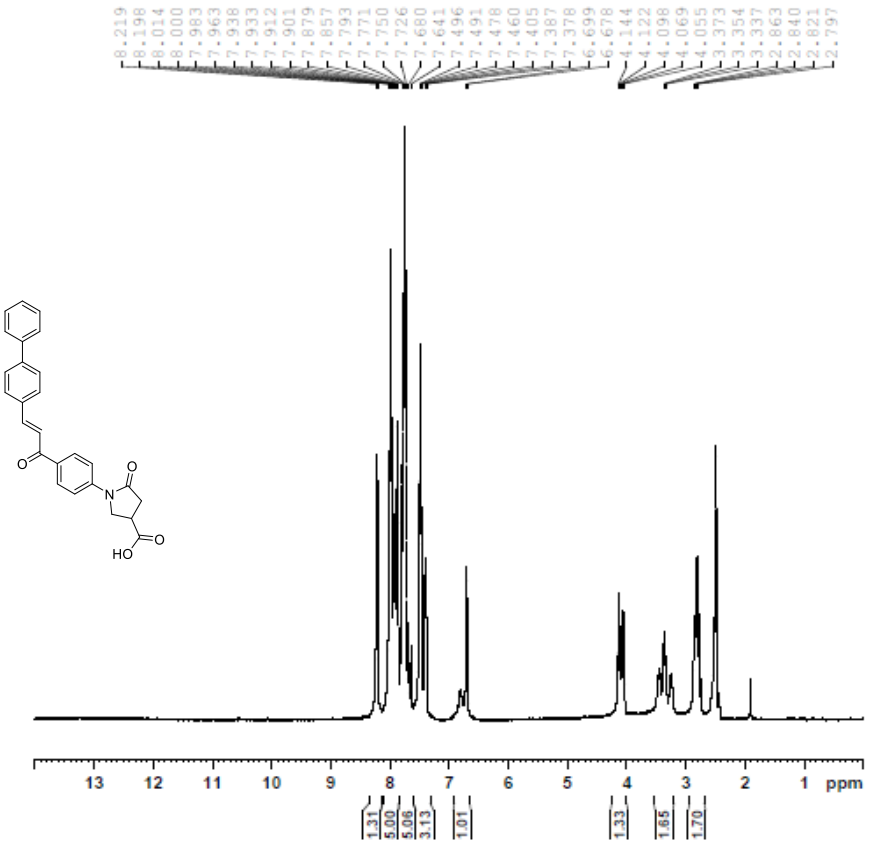
Current Data Parameters
NAME compound 2h
EXPNO 3
PROCNO 1

F2 - Acquisition Parameters
Date_ 20190509
Time 14.51 h
INSTRUM spect
PROBHD Z108618_0411 (
PULPROG zgpg30
TD 65536
SOLVENT DMSO
NS 750
DS 4
SWH 28409.092 Hz
FIDRES 0.866977 Hz
AQ 1.1534336 sec
RG 212.49
DW 17.600 usec
DE 6.50 usec
TE 297.7 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1
SFO1 100.6258487 MHz
NUC1 13C
P1 10.50 usec
PLW1 42.50000000 W
SFO2 400.1316005 MHz
NUC2 1H
CPDPRG[2 waltz16
PCPD2 90.00 usec
PLW2 9.89999962 W
PLW12 0.29363999 W
PLW13 0.14747000 W

F2 - Processing parameters
SI 32768
SF 100.6128202 MHz
WDW EM
SSB 0
LB 3.00 Hz
GB 0
PC 1.40

doi:10.6342/NTU201901421

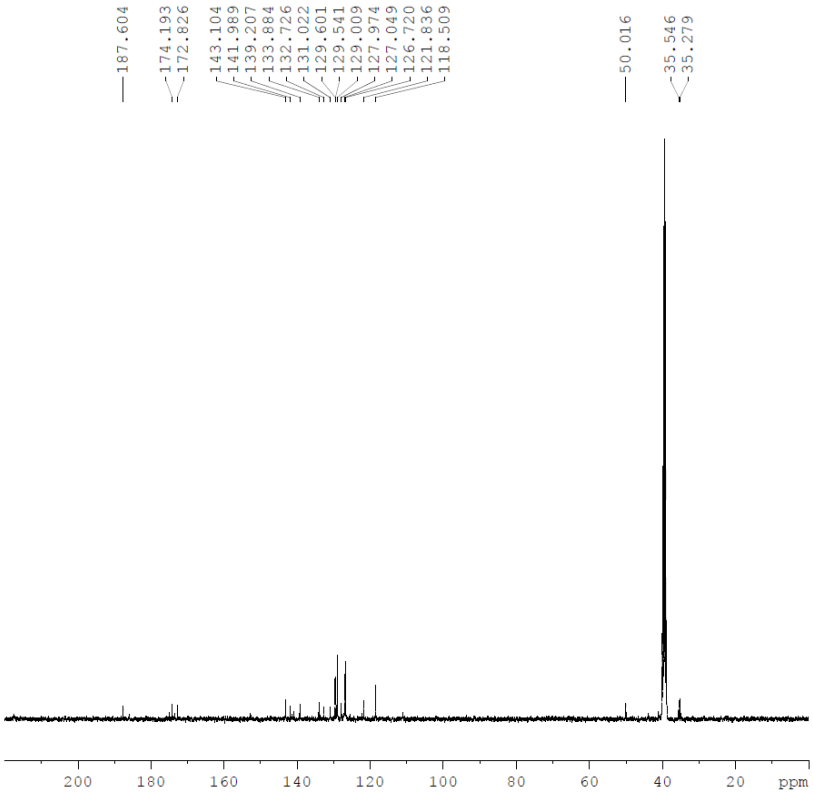
^{1}H (top) and ^{13}C (bottom) spectra of compound 2i



Current Data Parameters
NAME compound 2i
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
Date_ 20180313
Time 15.12 h
INSTRUM spect
PROBHD Z108618_0411 (
PULPROG zg30
TD 32768
SOLVENT DMSO
NS 20
DS 0
SWH 8802.817 Hz
FIDRES 0.537281 Hz
AQ 1.8612224 sec
RG 118.08
DW 56.800 usec
DE 14.47 usec
TE 298.9 K
D1 1.00000000 sec
TD0 1
SFO1 400.1328009 MHz
NUC1 1H
P1 15.50 usec
PLW1 10.89999962 W

F2 - Processing parameters
SI 131072
SF 400.1300087 MHz
WDW EM
SSB 0
LB 0 Hz
GB 0
PC 1.00



Current Data Parameters
NAME compound 2i
EXPNO 22
PROCNO 1

F2 - Acquisition Parameters
Date_ 20190510
Time 14.27 h
INSTRUM spect
PROBHD Z108618_0411 (
PULPROG zgpg30
TD 65536
SOLVENT DMSO
NS 205
DS 4
SWH 28409.092 Hz
FIDRES 0.866977 Hz
AQ 1.15343336 sec
RG 212.49
DW 17.600 usec
DE 6.50 usec
TE 297.5 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1
SFO1 100.6258487 MHz
NUC1 13C
P1 10.50 usec
PLW1 42.50000000 W
SFO2 400.1316005 MHz
NUC2 1H
CPDPRG[2 waltz16
PCPD2 90.00 usec
PLW2 9.89999962 W
PLW12 0.29363999 W
PLW13 0.14747000 W

F2 - Processing parameters
SI 32768
SF 100.6128183 MHz
WDW EM
SSB 0
LB 3.00 Hz
GB 0
PC 1.40

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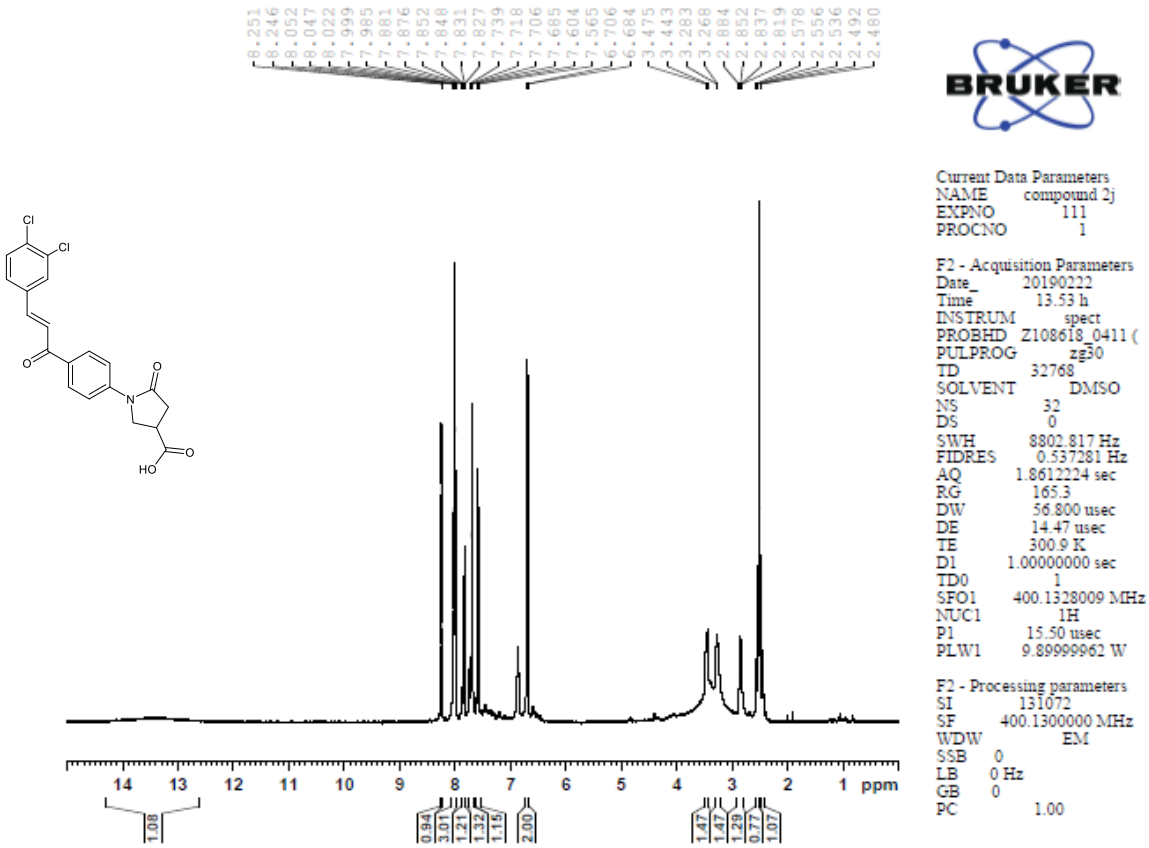
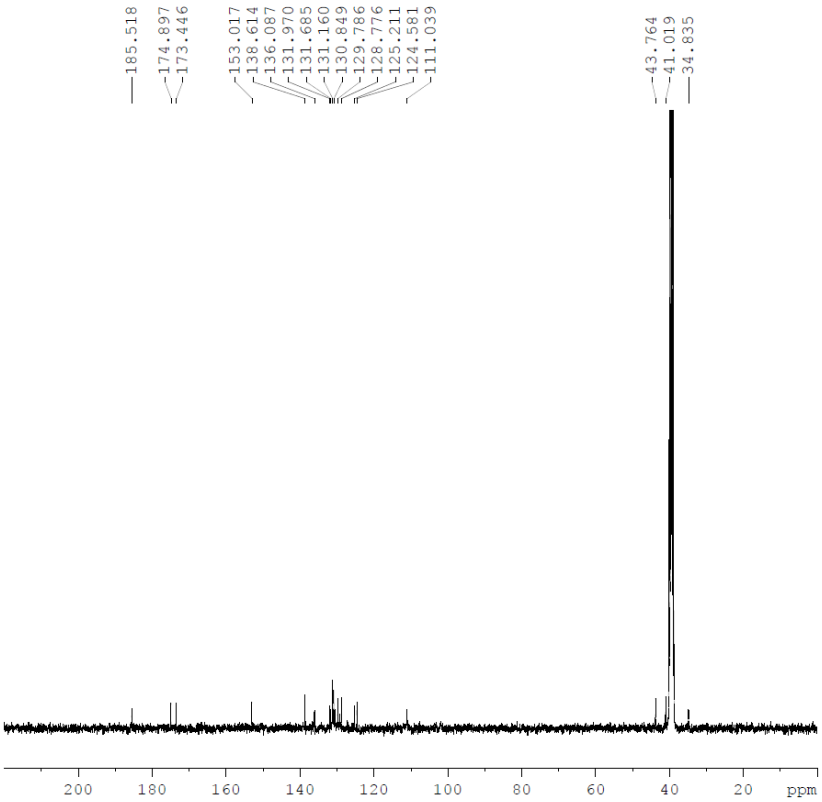
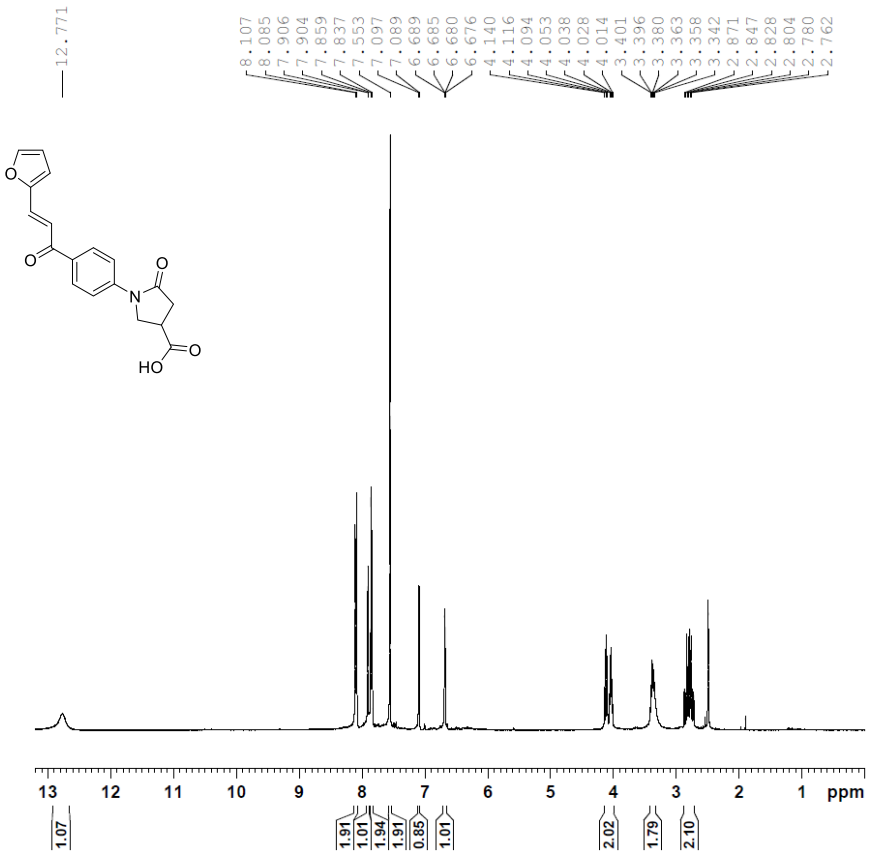
^{1}H (top) and ^{13}C (bottom) spectura of compound 2j





doi:10.6342/NTU201901421

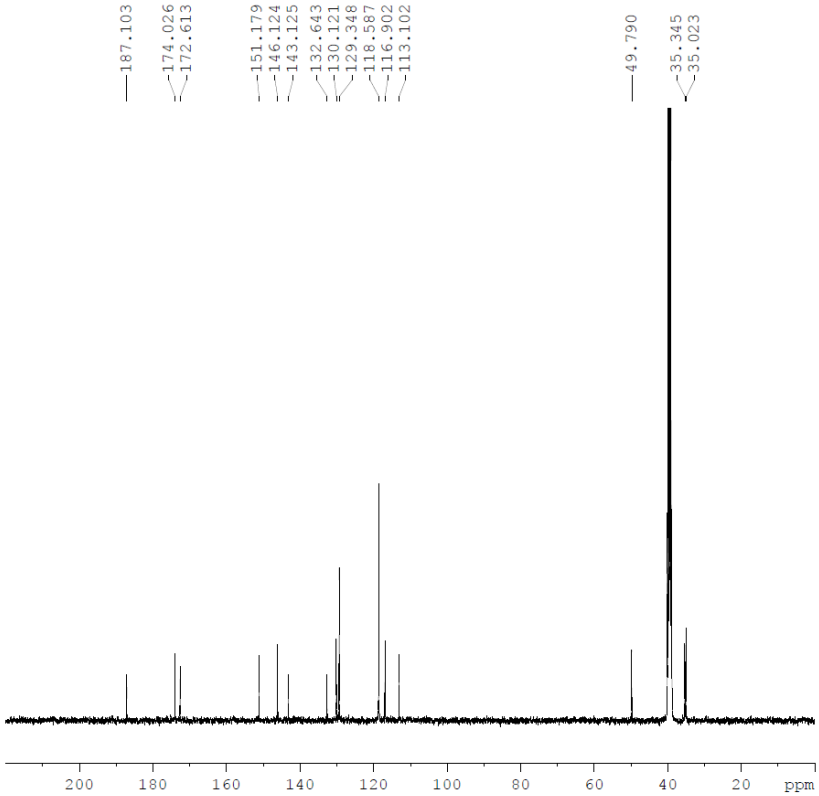
^{1}H (top) and ^{13}C (bottom) spectra of compound 2k



Current Data Parameters
NAME compound 2k
EXPNO 11111
PROCNO 1

F2 - Acquisition Parameters
Date_ 20190611
Time 16.09 h
INSTRUM spect
PROBHD Z108618_0411 (
PULPROG zg30
TD 32768
SOLVENT DMSO
NS 20
DS 0
SWH 8802.817 Hz
FIDRES 0.537281 Hz
AQ 1.8612224 sec
RG 133.5
DW 56.800 usec
DE 14.47 usec
TE 297.0 K
D1 1.00000000 sec
TD0 1
SFO1 400.1328009 MHz
NUC1 1H
P1 15.50 usec
PLW1 9.89999962 W

F2 - Processing parameters
SI 131072
SF 400.1300074 MHz
WDW EM
SSB 0
LB 0 Hz
GB 0
PC 1.00



Current Data Parameters
NAME compound 2k
EXPNO 11112
PROCNO 1

F2 - Acquisition Parameters
Date_ 20190611
Time 16.51 h
INSTRUM spect
PROBHD Z108618_0411 (
PULPROG zgpg30
TD 65536
SOLVENT DMSO
NS 718
DS 4
SWH 28409.092 Hz
FIDRES 0.866977 Hz
AQ 1.1534336 sec
RG 212.49
DW 17.600 usec
DE 6.50 usec
TE 297.7 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1
SFO1 100.6258487 MHz
NUC1 13C
P1 10.50 usec
PLW1 42.50000000 W
SFO2 400.1316005 MHz
NUC2 1H
CPDPRG[2 waltz16
PCPD2 90.00 usec
PLW2 9.89999962 W
PLW12 0.29363999 W
PLW13 0.14747000 W

F2 - Processing parameters
SI 32768
SF 100.6128180 MHz
WDW EM
SSB 0
LB 3.00 Hz
GB 0
PC 1.40

doi:10.6342/NTU201901421