

Using metabolomic tools to bioprospect antibiofilm agents from Philippine endolichenic fungi

A thesis submitted in fulfillment of the
requirements for the degree of Doctor of Philosophy

by
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A handwritten signature in blue ink, appearing to read "Peter Hoggard", written over the word "Signed:".

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Declaration of Generative Artificial Intelligence Assistance

This thesis made limited use of artificial intelligence tools (OpenAI's ChatGPT v 4.0) for language refinement, including grammar checking and improving clarity. All research design, data analysis, results, and interpretations are entirely my own, and I am fully responsible for the originality and integrity of the work.



Roberth Riggs Rondilla

01 September 2025

Research dissemination

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List of Abbreviations

^{13}C NMR	^{13}C Carbon Nuclear Magnetic Resonance spectroscopy
^1H	Proton Nuclear Magnetic Resonance spectroscopy
ACN	Acetonitrile
BLAST	Basic Local Alignment Search Tool
BPC	Biofilm prevention concentration
COSY	Correlation spectroscopy
<i>d</i>	doublet
DCM	Dichloromethane
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNP	Dictionary of Natural Products
EtOAc	Ethyl acetate
HEK293	Human Embryonic Kidney 293
HMBC	Heteronuclear Multiple-Bond Correlation
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Correlation
HR-LCMS	High resolution-liquid chromatography Mass spectrometry
IC ₅₀	50% inhibition concentration
ITS	Internal Transcribed Spacer
JMod	J-Modulated Spin
<i>m</i>	multiplet
MEB	Malt Extract Broth
MeOH	Methanol
MBIC	Minimum Biofilm Inhibition Concentration
MIC	Minimum Inhibitory Concentration
mins	Minute
MPLC	Medium pressure liquid chromatography
MRSA	Methicillin resistant Staphylococcus aureus
MW	Molecular weight
<i>m/z</i>	mass to charge ratio
MS	Mass spectrometry
NMR	Nuclear Magnetic Resonance Spectroscopy
OPLS-DA	Orthogonal Partial least squares-discriminant analysis
OSMAC	One Strain MAny Compounds
PBS	Phosphate buffer saline
PCA	Principal component analysis
PCR	Polymerase chain reaction

PDA	Potato dextrose agar
PDB	Potato dextrose broth
PLS-DA	Partial least squares-discriminant analysis
PTLC	Preparative thin layer chromatography
<i>q</i>	quartet
<i>s</i>	Singlet
SBHA	Suberoylbishydroxamic acid
SIMCA	Soft Independent Modelling by Class Analogy
<i>t</i>	triplet
TLC	Thin layer chromatography
TOCSY	Total correlation spectroscopy
UV	Ultraviolet

ABSTRACT

The rise of drug-resistant pathogenic bacteria is an alarming threat to global public health. One key factor contributing to antibiotic resistance is the formation of bacterial biofilms, which protects microorganisms from antimicrobial agents and causes genetic mutations. Thus, the discovery of compounds that can inhibit or disrupt biofilm formation is an urgent and promising research direction.

This PhD study investigated endolichenic fungi (ELF) as a novel source of antibiofilm secondary metabolites. ELF are mutualists within the lichen thallus and are known producers of a wide array of bioactive metabolites. A total of ten ELF collected from the Philippines were screened for their antibacterial and antibiofilm activities against methicillin-resistant *Staphylococcus aureus* (MRSA; ATCC 29213) and *P. aeruginosa* (ATCC 43300). Metabolomics-based chemical profiling using liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance spectroscopy (NMR) were employed. Three ELF stood out both with antimicrobial effects and distinct chemical profiles and thus were prioritised for subsequent investigations. These ELF were identified by molecular DNA sequencing as *Daldinia eschscholtzii*, *Hypoxylon lateripigmentum*, and *Xylaria cubensis*. To enhance metabolite production, different culture media (four types) and incubation durations (15 and 30 days) were tested, along with the supplementation of chemical elicitors (5-azacytidine and suberoyl bishydroxamic acid). Optimised culture conditions were established and applied for scaled-up fermentation.

For *X. cubensis*, rice medium with a 30-day incubation was optimal. Chromatographic isolation led to the identification of mellein, a possible mixture of hydroxylated mellein analogues, phloroglucinol (first reported from a fungus), and monolinolein. Mellein inhibited MRSA biofilm formation by 77.83% (prebiofilm) and 71.82% (postbiofilm) at

100 µg/mL. Phloroglucinol showed 93.8% antibacterial and 98.69% pre-biofilm inhibition at the same concentration. Both compounds exhibited no cytotoxicity to HEK293 cells.

H. lateripigmentum was found to have enriched production of metabolites in potato dextrose broth (PDB) for 15 days and malt extract broth for 30 days. Applying the isolation strategy described above, TAN-1612, 3-hydroxypropionic acid, and 2,3-didehydropalitanin were isolated and identified from the PDB culture while TAN-1612, daldinones A, B, and E were obtained from MEB cultures. These compounds were reported here for the first time in this species. TAN-1612 exhibited potent antibacterial activity (MIC = 13.83 µM) and biofilm inhibition (BPC = 12.50 µM), with low cytotoxicity (IC₅₀>200 µM).

D. eschscholtzii produced 2(3H)-benzofuranone, regiolone, tyrosol, and niacin in rice medium, though these exhibited negligible bioactivity (<40% prebiofilm inhibition at 100 µg/mL) which suggested possible synergistic and/or additive effects in crude extracts or parent fractions. Supplementation with chemical elicitors in its PDB culture led to the enhanced production of cytochalasin derivatives and BNT. These compounds, along with daldinone B, showed strong antibiofilm activity in MRSA (>70% prebiofilm inhibition at 100 µg/mL) but with weak cytotoxicity (IC₅₀ range 95-115 µM), warranting further structure-activity relationship (SAR) studies as proposed.

In conclusion, herein this study demonstrated the utility of metabolomics in natural product bioprospecting, from extract prioritisation, culture optimisation, and compound isolation. A total of 25 compounds were isolated and identified from the three Philippine ELF strains. This study identified compounds that have potential to be developed as antibiofilm drugs such as TAN-1612, BNT, and cytochalasins. This work positions Philippine ELF as promising sources of compounds targeting drug-resistant biofilm-forming pathogens.

Keywords: *Endolichenic fungi, biofilm formation inhibition, metabolomics, OSMAC, MRSA*

Chapter 1. Introduction

Since ancient times, one of humankind's most important achievements has been understanding how to treat diseases. Historically, botanicals and herbs are consumed orally or applied topically to cure illnesses. In earlier times, the discovery of new medicines was not solely the result of chance; many also emerged from the systematic and continuous pursuit of remedies within ancient traditional medical practices like Traditional Chinese Medicine and Ayurveda. Then, a systematic process to discover new drugs continuously evolved throughout modern times brought about by technological and scientific advances (Blass, 2021). Since the 19th century, medicine for previously thought incurable diseases was discovered. A classic example would be the discovery of penicillin from *Penicillium notatum*, which revolutionized modern medicine and antibiotic drugs (Fleming, 1944). Another notable breakthrough is the discovery of the carbapenem class of antibiotics in the 1980s, which offered enhanced selectivity Gram-negative bacteria (Craig, 1997).

Despite these significant advances, the global healthcare system continues to face the growing threat of antimicrobial resistance. For instance, methicillin-resistant *Staphylococcus aureus* (MRSA) was identified as early as the 1960s, shortly after methicillin's introduction (Jevons, 1961). Likewise, resistance to meropenem, a carbapenem antibiotic, has been reported in *Pseudomonas aeruginosa* strains (Meletis et al., 2012). Alarming, of the 32 antibiotics in clinical development as of 2019, only six were classified as innovative, highlighting the critical shortfall in novel drug discovery (WHO, 2021). If unaddressed, antimicrobial resistance could result in an estimated 10 million deaths annually by 2050 (O'Neill, 2016). Beyond achieving therapeutic efficacy of drugs, their safety, toxicity, and side effects must also be considered. Allergic reactions caused by cephalosporin antibiotics were recorded for example (Perez-Inestrosa et al., 2005). In

the case of the antiarthritis medication rofecoxib (Vioxx®), it was withdrawn from the market as it was found to increase the risk for cardiovascular diseases (Karha & Topol, 2004). From all these points and examples, it is evident that there is a vital need to identify next-generation therapeutic agents that have minimal side effects.

Known as natural products, molecules from biological sources have been extensively investigated in the drug discovery pipeline. These compounds offer large scaffold diversity and structural complexity (Atanasov et al., 2021). Around 65% of approved anticancer drugs in the market since 1981 were based on natural products (Newman & Cragg, 2020). According to origin, around 70% of the 300,000 NPs enlisted in the latest version of Dictionary of Natural Products®, one of the most comprehensive natural products database, are of plant origin (Chassagne et al., 2019). Only about 10% of NPs in the said database are from the kingdom Fungi following Animalia. Hence, tapping fungal organisms for new drug leads is fitting. In this study, endolichenic fungi, a relatively unexplored group of organisms, were investigated for their antibacterial and antibiofilm activities and how comprehensive analytical technologies, microbial culture approaches, and chromatographic purification can present efficient workflows for the discovery of new pharmaceutical drugs.

1.1 Endolichenic fungi

For many years, lichens were thought of as a complex organism with symbiotic relationship between a filamentous fungi called as the mycobiont and one or more photosynthetic organism, either a green algae or a cyanobacteria, known as the photobiont (Brodo et al., 2001). However, the discovery of a third symbiotic component, a basidiomycetous yeast (Mark et al., 2020; Spribille et al., 2016) has led to a revised definition: “A lichen is a self-sustaining ecosystem formed by the interaction of an inhabitant fungus or mycobiont and an extracellular arrangement of one or more photosynthetic partners and an indeterminate number of other microscopic organisms”

(Hawksworth & Grube, 2020). This expanded definition is supported by studies that have identified diverse bacterial communities (Cernava et al., 2019; Grimm et al., 2021) and viruses (Petrzik et al., 2019; Petrzik et al., 2014) within lichens. In addition, another distinct group of filamentous fungi that mutualistically inhabits a lichen called endolichenic fungi (ELF), has been described (Arnold et al., 2009). Thus, these findings support the emerging concept that lichens function as hosts to diverse microbial communities (Figure 1.1) that contributes to the complexity of the lichen symbiotic system.

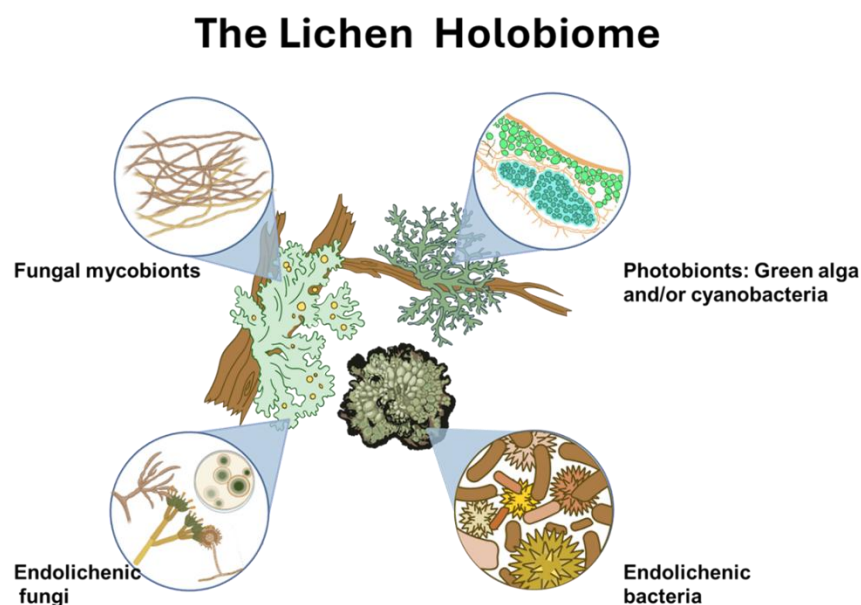


Figure 1.1 Microorganisms that have been discovered living on lichen thalli. Figure partially created in BioRender. Rondilla, R. (2025) <https://BioRender.com/wmyw3tx>

ELF do not cause harm and do not produce sporulating structures within the lichen thalli. They are taxonomically and functionally distinct from the mycobiont, the primary fungal partner in lichens, and from lichenicolous fungi, a group of parasitic fungi known to cause visible symptoms in their lichen hosts (Arnold et al., 2009;

Diederich, Lawrey, & Ertz, 2018). They were also considered facultative than obligate organisms with respect to their lichen hosts, meaning ELF species are not specific to a particular lichen host (Tripathi & Joshi, 2019).

1.1.1 ELF biodiversity

It is estimated that around 13,500 ELF species occur around the world (Singh et al., 2017). For a comprehensive assessment of ELF diversity, traditional culture-independent method and culture-dependent methods such as metabarcoding should be employed as some ELF are unculturable (Chagnon et al., 2016; Suryanarayanan & Thirunavukkarasu, 2017). In a recent review, about 500 ELF have been so far reported of which 135 are identified down to the species level (Chakarwari, Nayaka, & Srivastava, 2020). Predominantly, they belong to the subphylum Pezizomycotina and are phylogenetically-associated with several orders including Ploesporales, Xylariales, and Hypocreales (U'Ren et al., 2010). Therefore, to date, roughly 4% of the expected ELF species had been explored. Furthermore, reported ELF lichen hosts belong mostly to foliose and fruticose lichens representing only 114 known species. This is only a small fraction of the approximately 20,000 accepted lichen species (Lücking, Hodkinson, & Leavitt, 2016). All these points out that a plethora of ELF are waiting to be unraveled for biodiversity studies and other applications. Clearly, studies on ELF diversity are limited and should be pursued to address this huge gap.

1.1.2 Chemical diversity of ELF

As mentioned, ELF is analogous to endophytic fungi particularly in their capacity to produce bioactive secondary metabolites (Patil, Patil, & Maheshwari, 2016; Zheng et al., 2021). The first report of natural products from ELF was published by Paranagama et al. (2007) in *Corynespora* sp. Since then, numerous studies had reported the compounds that can be produced by ELF including alkaloids, chromenones, peptides, quinones,

steroids, terpenoids, and xanthenes which possesses a wide array of biological activities including anti-Alzheimer's, antibacterial, anticancer, antifungal, antioxidant, antiviral, and UV-protectant activities as stated on review papers (Agrawal et al., 2020; Singh et al., 2017; Suryanarayanan & Thirunavukkarasu, 2017; Wethalawe et al., 2021). According to the review of Tripathi and Joshi (2019), starting from 2007 to 2018, 351 ELF-derived metabolites were recorded and 196 of them were new compounds. An updated review by Zhang et al. (2024) brought up the total number of ELF natural products to 519, covering literatures from 2015 to 2023. Of the 407 ELF compounds recorded in the review, 270 (approximately 66%) were newly reported, with polyketides being the dominant class, followed by alkaloids, terpenes, steroids, and other structural groups. Notably, a steady rate of 30 new ELF secondary metabolites has been reported annually since 2015. Most ELF collection and sampling were done in Asia, with China contributing the largest number of reports. This geographic concentration highlights the untapped potential of other biodiverse regions. Accordingly, the present PhD work aimed to explore ELF from the Philippines as it is recognized as a megadiverse region with limited global representation in current ELF research.

To illustrate the wide chemical diversity of ELF metabolites, isolated compounds with antimicrobial activities reported from 2015 to 2024 are listed in Table 1.1 while structures are shown in Figure 1.2. The antimicrobial compounds have diverse chemical structures ranging from diterpenoids, isocoumarins, naphthalene glycoside, polyketides, sesquiterpenoids, terphenyls, and xanthenes. This structural diversity underscores the value of ELF as promising sources for the discovery of novel antimicrobial agents.

Terphenyl derivatives, floricolin A to C, exhibited fungicidal activities (Li et al., 2016). A xanthenone, funiculosone and related molecules mangrovamide J (5) and ravenelin were isolated from the ELF *Talaromyces funiculosus*. The compounds showed antibacterial activities while funiculosone had potency against *C. albicans* (Padhi et al., 2019). Several known compounds such as phomol, 16- α -D-mannopyranosyl

oxyisopimar-7-en-19-oic acid, and 8-methoxy-1-naphthyl- β -glucopyranoside from *Hypoxylon fuscum* were reported to have weak activity against *S. aureus* (Basnet et al., 2019). A chlorinated fused-indole-polyketide named isocoumarindole A from the ELF *Aspergillus* sp. exhibited moderate antifungal activity with an MIC of 32.0 μ g/mL (M. Chen et al., 2019). Acetyl tributyl citrate from *Fusarium proliferatum* displayed moderate inhibition in several bacteria (Tan et al., 2020). The salicylaldehyde derivative, asperglaucin B, demonstrated a variety of good antibacterial activities against several pathogens (Lin et al., 2021). Four eremophilane type of sesquiterpenoids, eremoxylarins D, F, G, and I were isolated from *Xylaria hypoxylon* have potent activity against MRSA (Miral et al., 2023).

Table 1.1 Antimicrobial ELF compounds reported from 2015-2024

ELF species	Lichen host	compound	Bioactivity data	Reference
<i>Floricola striata</i>	<i>Umbilicaria</i> sp.	floricolin A	MIC against <i>Candida albicans</i> : 16 μ g/mL	(Li et al., 2016)
		floricolin B	MIC against <i>C. albicans</i> : 8 μ g/mL	
		floricolin C	MIC against <i>C. albicans</i> : 8 μ g/mL	
<i>Talaromyces funiculosus</i>	<i>Diorygma hieroglyphicum</i>	funiculosone	IC ₅₀ range 23–104 μ g/mL IC ₅₀ range against <i>C. albicans</i> : 35 μ g/mL	(Padhi et al., 2019)
		mangrovamide J	IC ₅₀ range 23–104 μ g/mL	
		ravenelin	IC ₅₀ range 23–104 μ g/mL	
<i>Hypoxylon fuscum</i>	<i>Usnea</i> sp.	phomol	MIC against <i>S. aureus</i> : 51.2 μ M	(Basnet et al., 2019)
		16- α -D-manno-pyranosyl	MIC against <i>S. aureus</i> : 96.5 μ M	

ELF species	Lichen host	compound	Bioactivity data	Reference
		oxyisopimar-7-en19-oic acid		
		8-methoxy-1-naphthyl- β -glucopyranoside	MIC against <i>S. aureus</i> : 89.4 μ M	
<i>Aspergillus</i> sp.	<i>Cetrelia</i> sp.	isocoumarindole A	MIC against <i>C. albicans</i> : 32 μ g/mL	(M. Chen et al., 2019)
<i>Fusarium proliferatum</i>	<i>Parmotrema rampoddense</i>	acetyl tributyl citrate	MIC against <i>S. aureus</i> : 0.78 μ M MIC against <i>Pseudomonas aeruginosa</i> : 0.19 μ M MIC against <i>Klebsiella pneumoniae</i> : 89.4 μ M	(Tan et al., 2020)
<i>Aspergillus chevalieri</i>	<i>Lepraria incana</i>	asperglaucin B	MIC against <i>Pseudomonas syringae</i> pv. <i>actinidiae</i> : 6.25 μ M MIC against <i>Bacillus cereus</i> : 6.25 μ M	(Lin et al., 2021)
<i>Xylaria hypoxylon</i>	<i>Rhizocarpon geographicum</i>	eremoxylarin D	MIC against <i>S. aureus</i> : 6.25 μ g/mL	(Miral et al., 2023)
		eremoxylarin F	MIC against <i>S. aureus</i> : 0.78 μ g/mL MIC against MRSA: 1.56 μ g/mL	
		eremoxylarin G	MIC against <i>S. aureus</i> : 1.56 μ g/mL	
		eremoxylarin I	MIC against <i>S. aureus</i> : 0.39 μ g/mL MIC against MRSA: 1.56 μ g/mL	

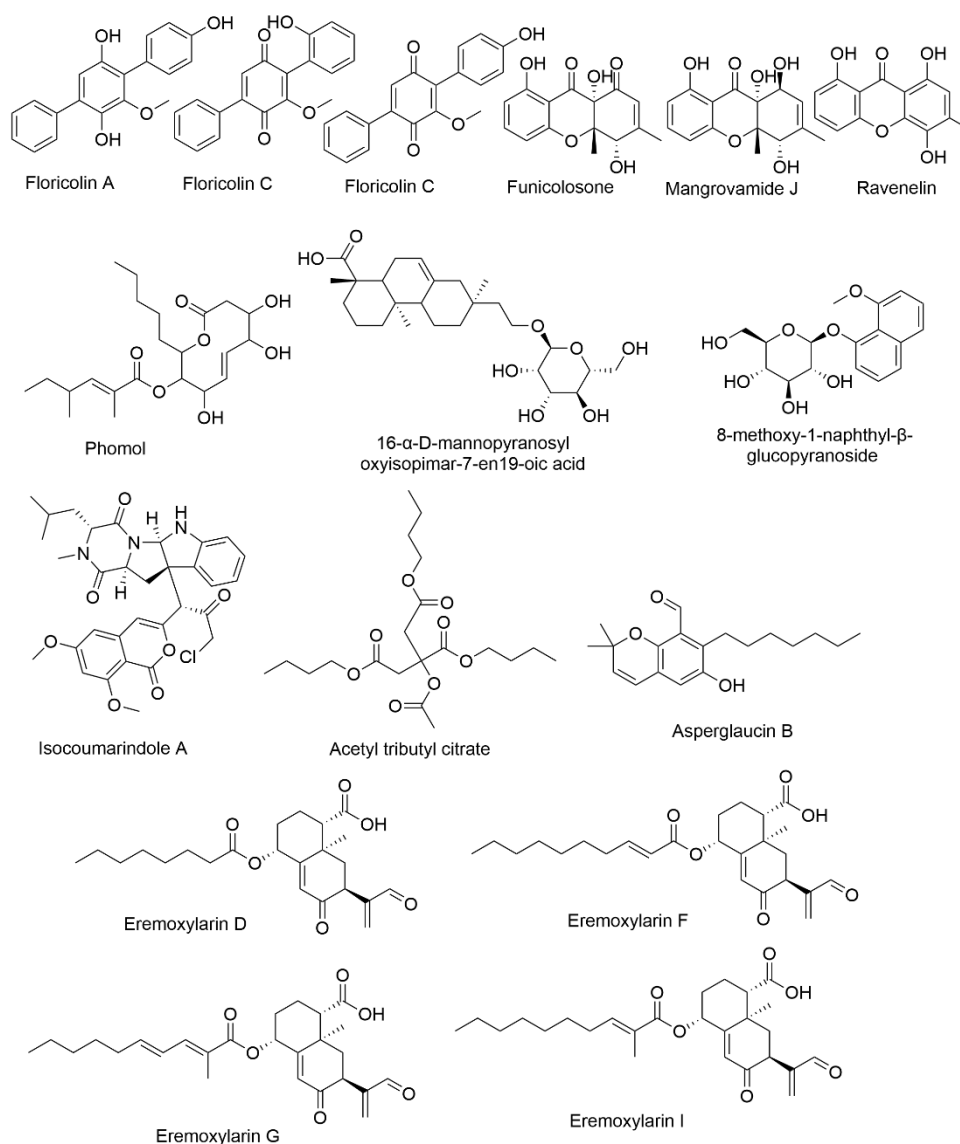


Figure 1.2 Chemical structures of ELF metabolites with antimicrobial activity reported from 2015 to 2024

1.1.3 Comparative insights on ELF and endophytic fungi

Endophytic fungi are mutualists living within the tissues of plants, analogous to lichen-associated ELF. Both belong in the same major taxonomic class (Pezizomycotina) and exhibit similar evolutionary lineage, as demonstrated by several molecular

phylogenetic studies (Arnold et al., 2009; U'Ren et al., 2012; U'Ren et al., 2010). Despite close similarities, ELF and endophytes diverge significantly at the Operational Taxonomic Unit (OTU) level (U'Ren et al., 2012). suggesting potential differences in ecological specialisation and biosynthetic capabilities. This disparity warrants deeper investigation through comparative genomic approaches, which could elucidate not only taxonomic divergence but also the genetic and functional bases of their secondary metabolism.

Endophytes are known to confer various benefits to their plant hosts, including enhanced resistance to abiotic stressors such as drought, salinity, high temperatures, herbivory, and pathogenic microbes (Rodriguez et al., 2009). These protective effects are often mediated by the production of bioactive secondary metabolites. In contrast, specific ecological roles of ELF remain poorly understood and require further investigation (Suryanarayanan & Thirunavukkarasu, 2017; U'Ren et al., 2010). Nevertheless, interest in ELF has grown due to their ability to produce structurally diverse and biologically active compounds.

Endophytes are extensively studied as prolific sources of pharmaceutically valuable secondary metabolites (Adeleke & Babalola, 2021; Gupta et al., 2023; Zheng et al., 2021). A recent review documented 220 new compounds from fungal endophytes over a ten-year period (2011–2021), yielding an average of 22 novel metabolites annually (Wen et al., 2022). By comparison, ELF studies between 2015 and 2023 have reported approximately 30 new compounds per year (Zhang et al., 2024). Although these numbers should be interpreted cautiously given potential disparities in sampling effort, research focus, and methodology, they nonetheless indicate that ELF represent a potentially valuable source of novel chemistry, partly owing to their relative under exploration.

Methodologically, isolation of ELF from host lichens closely follows the established protocols of fungal endophyte isolation. Specific workflows are comparable including field collection, surface sterilisation, cutting of lichen thallus, inoculation onto

culture media, subculturing until culture preservation (Tripathi & Joshi, 2019). As with endophytes, each step is critical, as variations can significantly influence both the isolation success rate and the observed fungal diversity. These procedural similarities underscore the practical accessibility of ELF for culture-based investigations.

Despite their shared taxonomy, evolutionary lineage, and methodological accessibility, ELF present a compelling case for continued exploration in natural product drug discovery, as they remain relatively underexplored compared to well-studied endophytes while their secondary metabolite diversity suggests untapped biosynthetic potential. Furthermore, complementary genome mining strategies will enlighten these biosynthetic potentials. This highlights ELF as a promising reservoir of new natural products, offering distinct advantages for bioprospecting and pharmaceutical development, particularly in understudied geographic regions and lichen hosts.

1.2 Strategies to expand metabolite diversity of ELF

1.2.1 Genomics strategies

As presented in a recent review (Zhang et al., 2024), only 50% of the secondary metabolites produced by ELF are regarded as new chemical entities. The remaining half are known natural products. Identification of new metabolites is perhaps low because most microbial gene clusters are inactive or silent under standard culture conditions (Wiemann & Keller, 2014). Through genome mining, DNA sequences of microorganisms can be identified using *in silico* tools to annotate their biosynthetic gene clusters (BGC) (Biermann, Wenski, & Helfrich, 2022). BGC annotation uncovers the potential metabolites to be produced by the organism by high sequence matching to reference BGCs. AntiSMASH (<https://antismash.secondarymetabolites.org/>) (Blin et al., 2019) and SMURF (<http://www.jcvi.org/smurf/>) (Khaldi et al., 2010) are web-based platforms that are commonly used for BGC annotation. Additionally, “biosynthetic gene similarity

clustering and prospecting engine” (BiG-SCAPE) generates similarity networks based on BGCs to organize them as gene cluster families (Navarro-Muñoz et al., 2020). BiG-SCAPE has been co-developed with “COrE Analysis of Syntenic Orthologues to prioritize Natural product gene clusters” (CORASON) to construct phylogenetic relationships among the BGC clusters.

These advanced genomic tools have been expansively applied in natural product bioprospecting, particularly in uncovering the antibiotic-producing capabilities of *Streptomyces* (Caicedo-Montoya, Manzo-Ruiz, & Ríos-Esteva, 2021) and the diverse biosynthetic potential fungal endophytes (Sagita, Quax, & Haslinger, 2021). However, genomic investigations on ELF remain scarce, with only two published studies to date. The draft genome of *Dothichiza* sp., an ELF isolated from *Usnea longissima*, was reported; however, biosynthetic gene cluster (BGC) annotation was not performed (Wei et al., 2023). More recently, whole genome sequencing was carried with the ELF *Anthostomella pinea* revealed 600 industrially important carbohydrate-active enzymes, 91 functional BGCs, and 164 cytochrome P450 enzyme catalysts (Iacovelli et al., 2024). These limited genomic reports highlight the untapped biosynthetic potential of ELF and emphasize the need for further genomics-based exploration in this understudied fungal group.

1.2.2 OSMAC strategies applied to ELF

Besides genomics-based strategies, cultivation-dependent methods such as the One Strain–Many Compounds (OSMAC) approach offer complementary avenues for enhancing secondary metabolite discovery (Bode et al., 2002). Changing of nutrient regimes and media composition, cultivation parameters (pH, agitation, light, temperature), co-cultivation with other microorganisms, and addition of epigenetic modifiers can be employed. By following this approach, secondary metabolite diversity of fungi and other microorganisms are improved through activation of cryptic BGCs in

standard or normal conditions (Pan et al., 2019; Pinedo-Rivilla, Aleu, & Duran-Patron, 2022; Romano et al., 2018).

OSMAC strategies have been broadly utilized to diversify the metabolic production of endophytic fungi and marine organisms (Hashem et al., 2023; Pinedo-Rivilla, Aleu, & Duran-Patron, 2022). Currently, there are nine published papers showing the applicability of the OSMAC method in ELF context. **Error! Reference source not found.** shows a collection of secondary metabolites produced by ELF when OSMAC was employed. Different thin layer chromatography (TLC) profiles were found on crude extracts of the ELF *Ulocladium* sp. when grown in Czapek's liquid medium versus from a solid rice media. Further isolation work led to the identification of three terpenoids, tricycloalternarenes F-H with ten known tricycloalternares (Wang, Bao, Yang, Guo, et al., 2013). Moreover, when the same fungus was cultured in potato dextrose broth (PDB), ophiobolins P-T and three known sesterterpenes were isolated (Wang, Bao, Yang, Liu, et al., 2013). Ophiobolin T was emphasized to possess cytotoxic and antibacterial activities. When *Myxotrichum* sp. derived from the lichen *Cetraria inslandica* were cultivated in PDB, metabolites including, an austdiol derivative myxodiol A, three fulvic acid derivatives myxotrichin A-C, and a citromycetin derivative, myxotrichin D were obtained (Yuan et al., 2013). However, when *Myxotrichum* sp. were grown in solid rice media, three polyketides myxotritones A-C, together with 7,8-dihydro-7R,8S-dihydroxy-3,7-dimethyl-2-benzopyran-6-one were identified (Yuan et al., 2016). Myxotritone B displayed activity against *Arabidopsis thaliana* root elongation which might be correlated to the defence of its host lichen. Two novel progesteroids, nodulisporisteriods A and B, were isolated from the ELF *Nodulosporium* sp. cultured in rice (Zheng et al., 2013). On the other hand, when the same ELF was grown in PDB, additional ten new progesteroids, nodulisporisteriods C to L were identified (Zhao et al., 2015). The ELF *Xylaria* sp from *Ramalina peruviana* exhibited higher radical scavenging activity when were grown in solid rice media compared to malt extract glucose yeast extract peptone broth (Galinato

et al., 2021). Different morphologies and biofilm inhibition activities were found out in a study involving several ELF grown in PDA, MEA, and SAB (Toure et al., 2022). Fourteen ELF from *Parmotrema tinctorum* were cultured in four different media including beef peptone-dextrose broth (BPDB), malt-yeast extract broth, PDB, and solid oatmeal flakes media. Most ELF displayed significant activities when cultured in BPDB. In particular, *Chaetomium globosum* and *Nodulosporium* sp. exhibited significant potency against plant pathogenic fungi (Paguirigan et al., 2024). From these mentioned studies, it is evident that different culture media influenced the production of structurally unique metabolites and can improve biological activities.

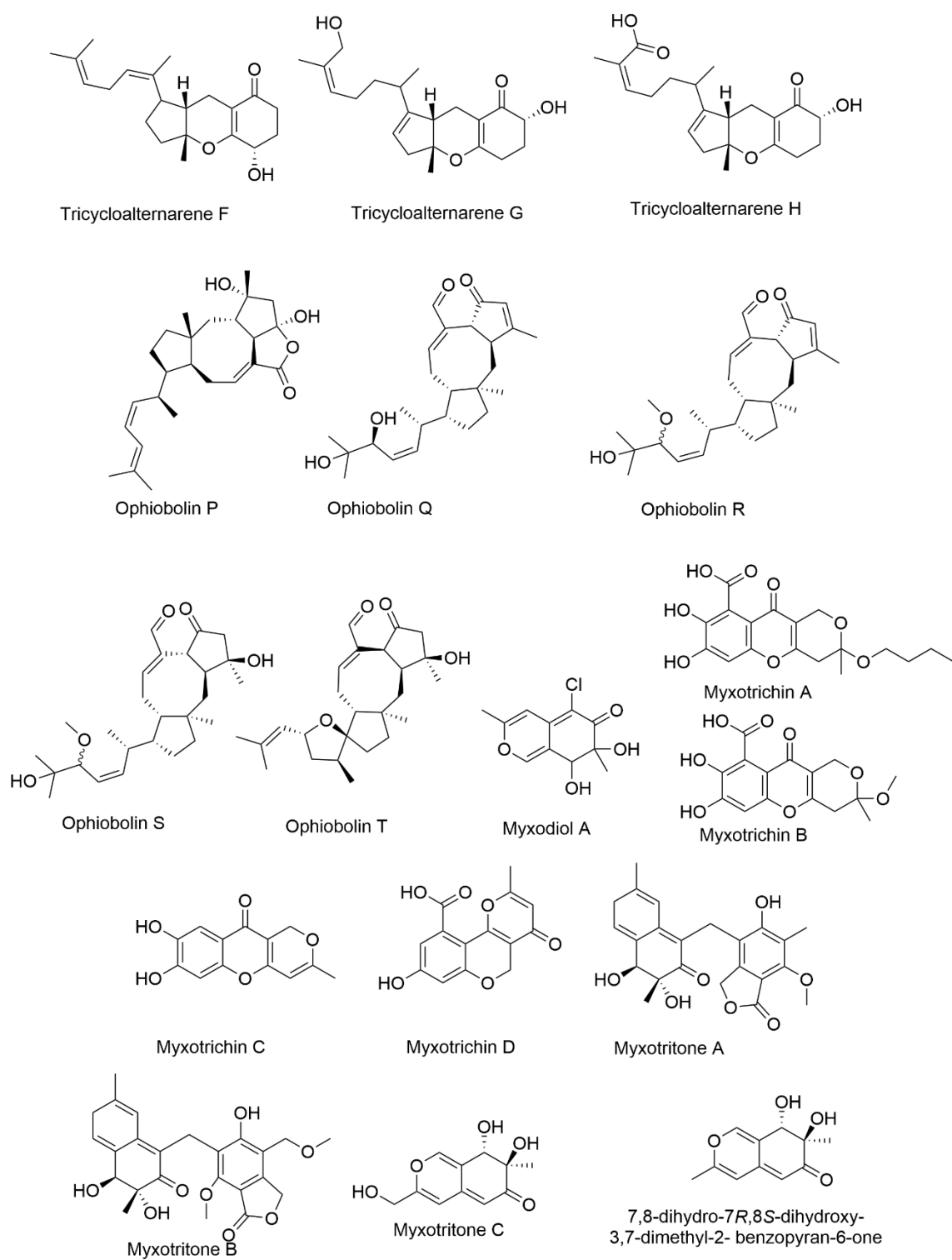


Figure 1.3 Chemical structure of ELF compounds discovered through the OSMAC approach

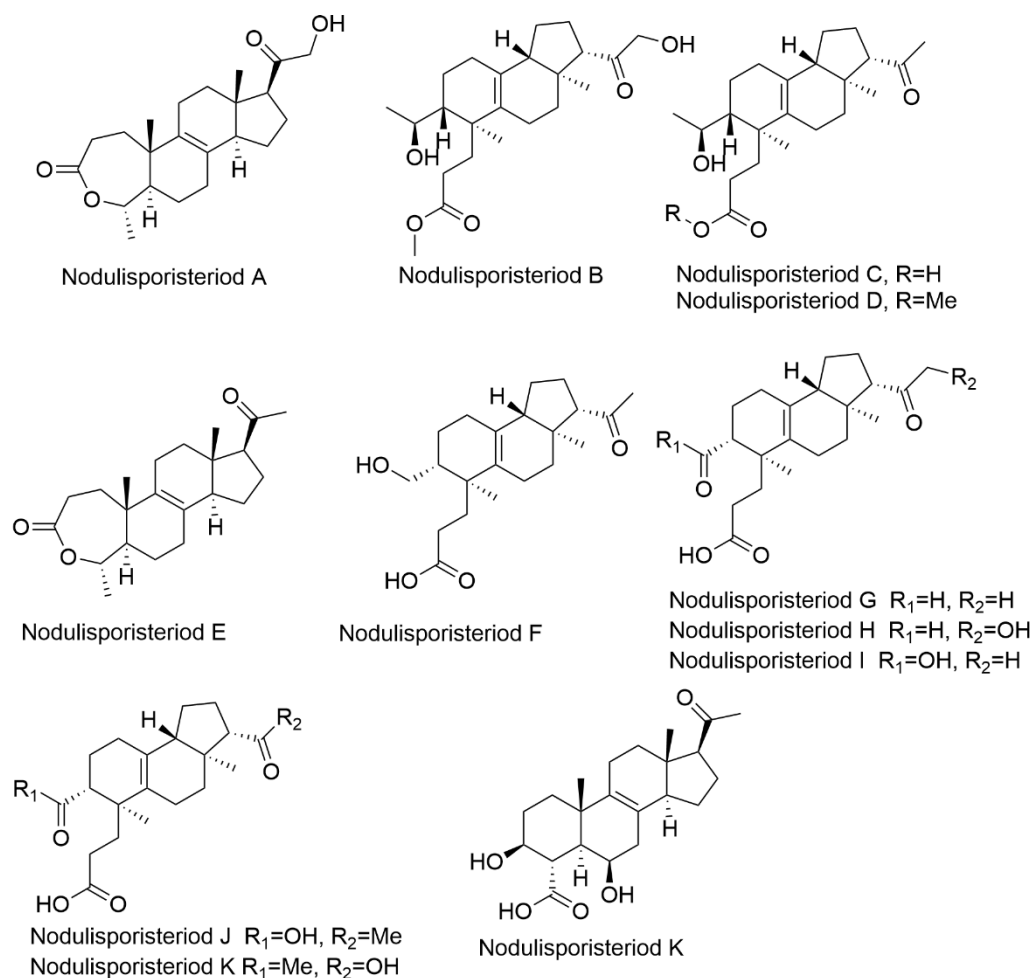


Figure 1.3 cont... Chemical structure of ELF compounds discovered through the OSMAC approach

Besides optimising different culture media, there are other options to apply the OSMAC approach. Modification of specific culture conditions such as the presence of metals or salts, pH, temperature, agitation, incubation duration, light, and oxygen concentration can affect the biosynthesis of interesting metabolites (Pan et al., 2019). For example, addition of ferric ion resulted to the identification of a novel antibacterial cyclodepsipeptide from a red soil-derived strain *Streptomyces* sp. FXJ1.172 (Liu et al., 2016).

Another proven technique is the co-cultivation strategy wherein two or more microorganisms are grown together in a single culture vessel. In this way, it mimics the

complex ecological relationship of microorganisms in nature and often leads to production of new compounds (Bertrand et al., 2014; Peng et al., 2021). One study employed the co-culture of *Streptomyces* sp. and *Bacillus* sp. which led to the production of dentigerumycin E, a new piperazic acid-bearing cyclic peptide with anticancer activities (Shin et al., 2018). In the context of ELF, co-cultivation may help recreate their natural habitat within the lichen thallus, offering valuable insights into interspecies interactions through the production of secondary metabolites. Additionally, it could shed light on the metabolic roles ELF play in lichen symbiosis.

An additional OSMAC approach involves the use of epigenetic modifiers or chemical elicitors that can either activate silent biosynthetic gene clusters or enhance the yield of known metabolites (Seyedsayamdost, 2014). These compounds are synthetic small compound inhibitors of epigenetic enzymes such as DNA methyltransferase (DNMT), histone deacetylase (HDAC), and histone acetyltransferase (HAT) (Xue et al., 2023). For example, treatment with 5-azacytidine, a DNMT inhibitor, significantly enhanced the production of cytochalasin, patulin, and pseurotin A in *Aspergillus clavatus* (Zutz et al., 2013).

Taken altogether, variation in culture conditions, co-cultivation techniques, and epigenetic modification represent effective routes within the OSMAC approach to further expand metabolic diversity. Thus, presents a promising opportunity to pursue for the current research.

1.3 Leveraging the power of metabolomics

Unlocking silent BGCs caused by several culture conditions results to metabolic diversification and thus needs for a comprehensive chemical profiling. Metabolomics can be employed to comprehensively study small molecules (<1500 Da) present in a particular biological system (Fiehn, 2002). Together with the other “omics”-technologies including genomics (studies the genes), transcriptomics (includes RNA), and proteomics

(protein abundances), metabolomics brought up advances not just in natural products but also in disease biomarkers and diagnostics (Bravo-Merodio et al., 2019), crop science (Yang et al., 2021), environmental science (Gruszecka-Kosowska, Ampatzoglou, & Aguilera, 2022), other fields. This is because “omics”-technologies studies large datasets that includes a wide range of molecules in a molecular space which then convey an integrated understanding of the biological system (Kaur, Singh, & Chana, 2021). With respect to natural products drug discovery, genomics and metabolomics are being used either individually or in combination for prioritisation and targeted isolation work (Caesar et al., 2021; van der Hooft et al., 2020; Wolfender et al., 2019). Genomics, provides insights about the genetic potential of a specimen by analyzing the genome of interest and identifying and annotating BGCs responsible for interesting metabolites (Ziemert, Alanjary, & Weber, 2016). On the other hand, metabolomics gives a direct and actual picture of the compounds being expressed by a biological organism thereby revealing a better understanding of affected metabolic pathways and chemical structure information (Prosser, Larrouy-Maumus, & de Carvalho, 2014).

1.3.1 The metabolomics workflow

There are two general approaches of metabolomics depending on the aims of the study. The first is called the targeted metabolomics where a specific compound or set of compounds were analysed and quantified. On the other hand, the untargeted metabolomics approach enables a global profiling of small molecules in the sample. The former is hypothesis-driven while the latter is hypothesis-generating. Usually, untargeted metabolomics approach is employed in natural products for the discovery of interesting compounds (Mishra, Priyanka, & Sharma, 2022) while the targeted method can be used in tandem for validation and quantification (Tebani, Afonso, & Bekri, 2018).

1.3.1.1 Extraction of metabolites

A typical untargeted metabolomics workflow for drug discovery in the context of fungal natural products is shown in Figure 1.4. The first stage is harvesting or extraction of metabolites after fermentation. Ethyl acetate was generally used as the solvent for endophytes and ELF (Nagarajan et al., 2021). However, there are certain studies that utilized other organic solvents such as dichloromethane, methanol, etc. Nevertheless, one should take note the possibility of some metabolites being retained at the mycelium while others being excreted to the fermentation broth (Tripathi & Joshi, 2019). Some groups extract them separately and differences in metabolite profiles were observed using metabolomics (Ibrahim et al., 2020). Furthermore, one should consider using a homogenizer or an ultrasound machine to further enhance the efficiency of metabolite extraction (da Rosa, Sauzem, & Kuhn, 2021). After extraction, solvents can then be removed using a rotary evaporator to obtain crude extracts.

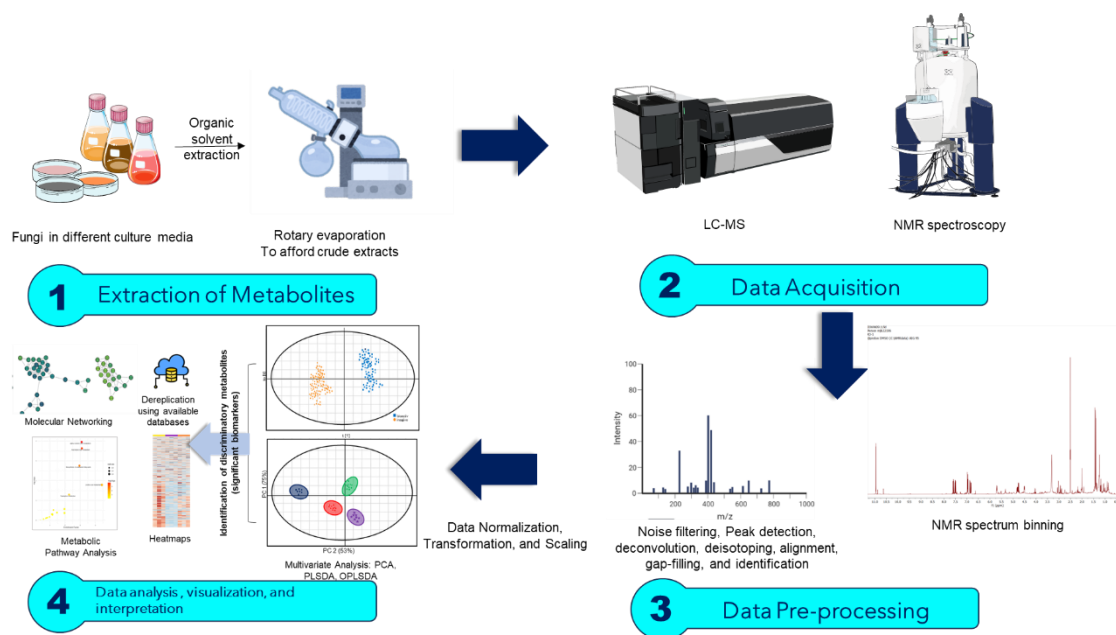


Figure 1.4. Untargeted metabolomics workflow in the context of fungal natural products drug discovery

Acquisition of metabolite profile data is then carried out as the second step in the workflow (Figure 1.4). At this stage, crude extracts are dissolved in appropriate solvents

and injected in analytical instruments such as mass spectrometry (Bravo-Merodio et al.) and nuclear magnetic resonance spectroscopy (NMR). Most often, MS techniques are used in combination with separation techniques such as liquid chromatography (LC-MS), gas chromatography (GC-MS), or capillary electrophoresis (CE-MS). A single extract could possess thousands of complex and diverse secondary metabolites and consequently, there are no single analytical platform that can provide the complete metabolic information (Stuart et al., 2020). MS and NMR are the major analytical techniques used in metabolomics research both of which have their advantages and limitations as discussed in several reviews (Emwas, 2015; Segers et al., 2019; Stuart et al., 2020). Most research studies use a single instrument. However, combining MS and NMR data called “concatenation” are gaining attention as it provides wider metabolome coverage (Forshed, Idborg, & Jacobsson, 2007; Marshall & Powers, 2017; Sampaio, Edrada-Ebel, & Da Costa, 2016; Yusoff et al., 2022; Zanatta et al., 2023).

1.3.1.2 Data acquisition

NMR-based metabolomics

NMR is a spectroscopic technique widely used to identify and elucidate chemical structure of small organic molecules up to large macromolecules. In this technique, a magnetic field and a radiofrequency is applied to a sample that results to resonances at different frequencies for specific nuclei such as ^1H . Despite its low sensitivity compared to MS, NMR plays an important role in the field of metabolomics because it is highly reproducible, simple in terms of sample preparation, non-destructive, and highly quantitative (Nagana Gowda & Raftery, 2017). Since crude extracts contain numerous metabolites, analyzing individual metabolites are impossible. A more practical aim for NMR-based metabolomics is to have an overview of the group of metabolites present in the extract may it be primary metabolites (sugars, amino acids, lipids) or secondary

metabolites (flavonoids, alkaloids, terpenes, etc) (Kim, Choi, & Verpoorte, 2010). As an example, characteristic signals of methyl group of terpenes were able to be associated with the high anti-inflammatory activity of a crude extract from fungal endophyte *Colletotrichum* sp. (Flores-Vallejo et al., 2020). In addition, geographical sources of the medicinal mushroom *Ganoderma lucidum* were distinguished through NMR-based metabolomics approach (Wen et al., 2010).

Mass spectrometry-based metabolomics

MS-based metabolomics have superior sensitivity and wider dynamic range versus NMR-based metabolomics. Because of its sensitivity, thousands of metabolites can be detected in a single crude extract. This complexity then often necessitates prior separation of metabolites of interest. Hence, hyphenated techniques such as LC-MS, GC-MS, and CE-MS become highly efficient instrumentation to study numerous metabolites.. Another problem in MS is the proper ionization of metabolites and ion suppression. GC-MS generally prevents ion suppression since its analytes are already in gaseous phase. However, GC can only detect volatile and low molecular weight compounds (ca. 50-600 Da) (Beale et al., 2018). That is why samples for GC typically needs to be chemically derivatized to be able to be analysed. In this case, LC-MS has the advantage of detecting larger and intact metabolites. Hence LC-MS is the choice for natural products metabolomics (Zhao, Zhang, & Li, 2018). Classically, reverse-phased columns are used to separate non-polar and semi-polar compounds while hydrophilic interaction liquid chromatography (HILIC) gives better resolution for the separation of highly polar compounds (Tang et al., 2016). Furthermore, to enhance resolution, faster analysis, superior MS quality, and greater sensitivity, ultra-performance liquid chromatography (UPLC) columns has been utilized in the recent years (Plumb et al., 2023).

Metabolites should be ionized to be able to measure its mass-to-charge ratio (m/z). Ionization techniques for MS are electrospray ionization, atmospheric pressure chemical

ionization, electron ionization, and matrix-assisted laser desorption ionization. The prevalent technique used in LC-MS metabolomics is the electrospray ionization (Agin et al., 2016). In this method, eluted samples from the LC system are directed via spraying to a vacuum environment with applied voltage that causes nebulized charged droplets. Usually, the generated ions are adducts depending on the mode used either in the positive or negative ion mode. In positive ESI, typical adducts are $[M+H]^+$ and $[M+Na]^+$, where M is the molecular weight of the compound. On the other hand, the common adduct in negative ESI is $[M-H]^-$ (Manohar & Marzinke, 2016). To extend to a more comprehensive analysis and accurate metabolite annotation, MS/MS techniques were employed in the recent decade. In this case, molecular ions are further fragmented to substructures that can be matched with high confidence to an MS/MS database (Suntivich et al., 2022). LC-MS/MS based metabolomics is then an excellent tool for annotation of natural products which will be discussed later in detail.

Application of MS-based metabolomics was successful for fungal natural products research (Nagarajan et al., 2022). To illustrate, LC-MS metabolomics was used as a tool to guide the isolation of compounds with excellent anti-trypanosomal activity from the endophytic fungus *Lasidiplodia theobromae* (Kamal et al., 2017). Furthermore, the fungi *Penicillium setosum* was chemotaxonomically distinguished from other related species by its ability to produce specific metabolites as pinpointed by LC-MS metabolomics (George, Devadasan, & Jisha, 2019).

1.3.1.3 Data pre-processing

After the NMR or MS spectral data had been acquired, a preprocessing step before data analysis should be carried out (Figure 1.4). This initial step is crucial to reduce complexity and including only the important metabolic features from the raw instrumental data. In this process, spectral data from a batch of samples are processed to digital or numerical values that were then aligned for further statistical analysis.

Preprocessing of NMR data is simpler than LCMS data. First, the baseline of NMR spectra should be corrected to eliminate unwanted variability (Euceda, Giskeødegård, & Bathen, 2015). Then, it must be divided or segmented into smaller bins (sometimes called buckets) typically for every 0.04 ppm (Kim, Choi, & Verpoorte, 2010; Lindon, Holmes, & Nicholson, 2001). In each bin, the sum of the total intensities were scaled to minimize variation between samples (Craig et al., 2006). The bucketing step can be done in NMR processing software such as ACD NMR Manager (Advanced Chemistry Development, Toronto, Canada), AMIX-TOOLS (Brucker Biospin GmbH), and MNova (Mestrelab Research, Spain). Since bucketing may lead to loss of spectral resolution, full resolution of NMR data can be done as well. A much bigger data set compared to binning will be generated which can be more informative in several statistical analysis. Peak alignment can also be done when chemical shifts are greatly affected. The NMR data that is aligned or binned is ready for multivariate analysis.

For MS data, pre-processing was generally performed with steps including noise filtering and baseline correction, peak detection (or peak-picking), deconvolution, deisotoping, alignment, gap-filling, and identification (Figure 1.4). Noise filtering separates out instrumental signals from the actual signals arising from the features present in a sample (Castillo et al., 2011). Peak detection identifies the correct signals for the metabolites and avoid false positives (Yi et al., 2016). Selecting the signal cut-off values or threshold must be carefully selected as a high setting can miss low intensity but relevant features or a low setting that will produce large and complex data sets (Barupal et al., 2021). However, Houriet et al. (2022) pointed out that applying a low threshold value can benefit to maximizing quantitative and qualitative data analysis. Next, the deconvolution step allows differentiation of overlapping peaks due to co-eluting compounds. The presence of isotopes results to several peaks for a single metabolite. This can be circumvented by the deisotoping step. Then, alignment refers to matching the same peaks among the samples in the whole batch. Some features might be missed from

the alignment; thus gap-filling is further done to recover missing signals from the raw data or by retention time correction. Identification then deals with obtaining the chemical formula arising from the m/z values of each feature. Compound identification may be extremely challenging specially in untargeted approaches because of diversity of metabolites. Fortunately, the rise of advanced tools and publicly available database greatly aid in metabolite identification.

At a first glance, MS data preprocessing might be daunting. However, there are algorithms and available open-sourced computer programmes to carry out such tasks. Examples of useful softwares include XCMS (Smith et al., 2006), MZMine (Schmid et al., 2023), OpenMS (Röst et al., 2016), MetAlign (Lommen & Kools, 2012), MetaboAnalyst (Pang et al., 2022), and MAVEN (Clasquin, Melamud, & Rabinowitz, 2012).

1.3.1.4 Data analysis, visualization, and interpretation

After pre-processing the raw data, suitable statistical models and chemometrics tools should be employed to produce significant information and scientific discussion from a huge and complex data set (Figure 1.4). There are univariate and multivariate statistical approaches routinely used for metabolomics (Saccenti et al., 2014). The former involves the comparison of single variable between two study groups while the latter analyses the relation of two or more groups at multiple variables. Multivariate analysis is frequently applied for metabolomics analysis as hidden information from biological samples can be unearthed (Lamichhane et al., 2018). Frequently used models include principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), and orthogonal projections to latent squares-discriminant analysis (OPLS-DA).

The metabolomics data to be analysed for multivariate analysis must be scaled (van den Berg et al., 2006). Scaling allows both high and low abundant features or metabolites to have equal weights in the downstream multivariate analysis. Several scaling methods include mean-centring, unit variance, and Pareto. Each scaling method

have their advantages and limitations but Pareto scaling has the compromise between the two extremes (mean-centring and unit variance), and has become a rather good choice (Yang et al., 2015).

Besides data scaling, data normalization and transformation can be done to improve subsequent data analysis and interpretation. Normalization eliminates technical variations in raw data such as instrument drift or sample preparation errors (De Livera et al., 2012). Data-based and sample-based approaches are the main method of choice for normalization of which several computational tools are available (Misra, 2020). Data transformation, meanwhile, applies mathematical operations such as logarithmic and power transformations to reduce data heteroscedasticity and to obtain a more symmetrical data (Sun & Xia, 2024).

Principal Component Analysis

PCA is an unsupervised learning method that shows similarities and differences, presence of outliers, and clustering trends between samples in a data set. The word “unsupervised” means that the data being analysed is not categorized into classes or groups set by the user (Ren et al., 2015). In other words, this model doesn’t require any prior knowledge to classify the metabolomics data set into groups. PCA is usually first carried out with the aim of discovering and exploring any patterns and outliers before applying supervised methods such as PLS-DA and OPLS-DA.

The primary goal of the PCA is to substitute a set of correlated variables with a smaller set of uncorrelated variables known as principal components (PCs). These PCs are designed to preserve most of the information present in the original dataset. The PCs are then visualized in a scores plot (either as line or scatter) arranged in a way that each subsequent PC explains a decreasing amount of variance in the data matrix, i.e., PC1 accounting for the highest variance, PC2 for the second-highest variance, PC3 for the third-highest variance, and so on (Lamichhane et al., 2018).

Another visualization plot produced from PCA is the loadings plot. This plot refers to what the principal component represents in the scores plot. It shows the association between the cores plot trend and the spectral data features (either chemical shifts from NMR data or m/z values from MS data). Specific spectral data information can then be identified that caused the observed trend displayed in the scores plot (Beattie & Esmonde-White, 2021).

As an example, a new natural product was pinpointed as the significant contributor from the clustering observed in the PCA scores plot of LC-MS metabolomics data of 47 microbial strains (Hou et al., 2012). In another study, PCA has discriminated the extracts from three fungal endophyte *Aspergillus* strains signifying uniqueness in their metabolic fingerprints (Sayed et al., 2022).

One downside of PCA is when data are not linearly correlated and thus fails to reveal any patterns or clusters. Hence, PCA can be viewed as a pre-analysis tool to look for trends and outlier points rather than an explicit model for investigating potential biomarkers.

Partial Least Squares-Discriminant Analysis

When a metabolomic data set have categories or groups, supervised models such as PLS-DA can often provide a more meaningful and interpretable output than PCA (Liland, 2011). This model breaks down two matrices (matrix1: containing metabolite information/intensities, matrix2: containing categorical/classifier variables like bioactive and inactive extracts or geographical origins) to define any common variability and generate a visual plot that displays the covariance between the two matrices (Ståhle & Wold, 1987). Significant features can be determined through the loadings plot and variable importance in projection (VIP) scores (Wold, Sjöström, & Eriksson, 2001).

PLS-DA was applied to analyse monoculture and co-culture of fungal endophytes after which 25 metabolites were identified and pinpointed as the cause of variability

between the fungal extracts (Triastuti et al., 2021). Furthermore, through PLS-DA, the discriminatory metabolites responsible for the anti-inflammatory activity exhibited by the endophytic fungi *Colletotrichum* sp. were uncovered (Flores-Vallejo et al., 2020).

PLS-DA is susceptible to overfitting and thus performance tests such as cross-validation, permutation tests, number of misclassifications (NMC), Area Under the Receiver Operating Characteristics (AUROC), Q^2 , and Discriminant Q^2 (DQ^2) should be carried out (Szymańska et al., 2012). Furthermore, there are cases that the discriminatory features determined from the PLS-DA scores plot are actually not related to the classifying variables (Worley & Powers, 2013). Another model called OPLS-DA can thus be applied due to its suitability for this analysis.

Orthogonal Projections to Latent Squares-Discriminant Analysis

OPLS-DA is another supervised model in multivariate analysis. This method reduces model complexity and improves PLS-DA interpretations (Trygg & Wold, 2002) as it uses only a single component as a predictor for the classification. OPLS-DA and PLS-DA have similar power of predictability. However, the former has the benefit of pinpointing which features cause the class separation which were visualized in S-plots (Shyti et al., 2015; Wiklund et al., 2008).

In the study of (Kamal et al., 2017), metabolites causing the observed anti-trypanosomal activity of the endophyte *Lasidiplodia theobromae* were identified through OPLS-DA. Similarly, several peptides and other compounds from the endophytic fungi *Curvularia* sp. were identified as exclusive metabolites produced in different incubation periods (Tawfike et al., 2018). OPLS-DA was also successful in pinpointing discriminating metabolites of mycelium and filtrate extracts from *Xylaria ellisii*. Following metabolomics-guided isolation, these discriminating metabolites were new cyclopeptides exhibiting antibacterial activity (Ibrahim et al., 2020).

Heat maps

Another useful chemometric visualization tool for analysing metabolomic data are heat maps. It is a representation of data of which the values are denoted in colours. Heat maps are usually used in genomics to implicate which genes are highly or lowly expressed (Farrell, 2010). In metabolomics, the relative abundance of ions presents in each sample are represented in a color-coded matrix. Heat maps can be generated from either LC-MS or NMR metabolomics data. The web-based platforms such as XCMS (Smith et al., 2006) and MetaboAnalyst (Pang et al., 2022) have available heat map algorithms for metabolomics data users.

Dereplication in natural products

Dereplication was defined originally as the process of identification the molecules present in a crude extract without prior isolation. In this classical definition, dereplication involves comparison/matching of available information such as spectroscopic data and biological information with compound databases to determine whether known or new compounds are present and to guide subsequent investigation (Dinan, 2005). The concept has since been extended to include the ability to exclude previously studied organisms with known vs unknown compounds in a screening programmes (Colquhoun et al., 2000). Hence, dereplication is an important step in natural products because it minimizes time, effort, and cost to screen out samples of which previously reported compounds are present while giving priority to extracts or fractions with potential chemical novelty (Gerwick & Moore, 2012). In the context of metabolomics, the discriminatory metabolites pinpointed from multivariate analysis can be subjected to dereplication for tentative annotation and prioritisation for further study.

At present, LC-MS is the most commonly employed platform for dereplication in untargeted metabolomics (Gaudêncio et al., 2023). This is due to its exceptional sensitivity and resolution. However, dereplication via NMR spectroscopy are gaining attention in the recent years (Flores-Bocanegra et al., 2022; Garcia-Perez et al., 2020).

Classical dereplication based on MS data is done by searching the compound's accurate mass to chemical structure databases. The Dictionary of Natural Products®, a commercial database, is considered as the most comprehensive natural products database (Sorokina & Steinbeck, 2020) containing 340,000 compounds (accessed February 2025; <http://dnp.chemnetbase.com/>). Another commercially available database exclusive for marine natural products is called MarinLit which includes 42,797 compounds (accessed February 2025; <http://pubs.rsc.org/marinlit/>). The Natural Products Atlas is an open-source database of microbial natural products (from bacteria, cyanobacteria, fungi, and lichens) which to date has 36,727 metabolites (accessed February 2025; <https://www.npatlas.org/>; (Poynton et al., 2025)). Matching features to databases can be carried out automatically using algorithms such as an in-house Microsoft Excel Macro (Kamal et al., 2017; Macintyre et al., 2014; Mazlan, Clements, & Edrada-Ebel, 2020; Sampaio, Edrada-Ebel, & Da Costa, 2016; Santiago et al., 2021; Tawfike et al., 2018; Yusoff et al., 2022; Zanatta et al., 2023).

It is important to take note that accurate mass searches often produce numerous matches due to the presence of isomers. (Mattoli, Gianni, & Burico, 2023). To solve this, tandem MS/MS instruments are used to enable a more accurate identification of compounds and resolving isomers by extracting information from fragmentation patterns of the parent molecule (Nielsen & Larsen, 2015). To date, *in silico* tools were developed such as SIRIUS to rapidly provide chemical structure elucidation *de novo* based on MS/MS data (Dührkop et al., 2019). Other available software include BUDDY (Xing et al., 2023), MetFrag (Ruttkies et al., 2016), among others.

Molecular networking

Molecular networking is another widely utilized complementary dereplication strategy (Yang et al., 2013). Molecules having a common core structure will show very similar MS/MS fragmentation pattern and will be organized in molecular networks or

“molecular families”. Molecular networks based from MS/MS data can be constructed in platforms such as the Global Natural Products Social Molecular Networking (GNPS) (Wang et al., 2016) and Metabolights (Haug et al., 2019). In the GNPS environment, several approaches including feature based-networking, ion-identifying molecular networking, and bioactivity-based molecular networking are developed throughout the years (Qin et al., 2023). To illustrate some applications, molecular networking was used to map the chemical profile of several marine fungi extracts (Oppong-Danquah et al., 2018). It was that revealed that nine molecular clusters were unique in marine fungi co-cultivated with phytopathogenic fungi. Furthermore, the co-cultivation extracts were active against the phytopathogens and thus indicates that co-cultivation induces the production of metabolites that can kill harmful organisms.

Metabolic pathway analysis

The rich information that can be derived from multivariate analysis and subsequent metabolite dereplication/annotation can be collectively analysed in terms of biochemical pathways. In pathway analysis, metabolites are mapped into known or curated biochemical reactions and thus reveals their function in the biological system context (Karnovsky & Li, 2020). Publicly available databases such as Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2011) and BioCyc (Caspi et al., 2012) are commonly used to annotate particular metabolic pathways. Identifying such pathways can be correlated to various biological conditions. For example, mapping in some identified biomarkers of a certain disease into biochemical networks gives out a better understanding about the disease mechanism and possible reactions that can be inhibited for medical treatments. Furthermore, the biochemical reactions involved can then be integrated into the genomics, transcriptomics, or proteomics data. A number of online tools have been put up for mapping metabolomic data into biochemical pathways like in KEGG, MetaboAnalyst (Pang et al., 2022), PaintOmics (Liu et al., 2022), meltDB 2.0 (Kessler et al., 2013), and 3Omics (Kuo, Tian, & Tseng, 2013) to name a few.

1.3.2 Exploring the ELF chemospace using metabolomics tools

Metabolomics had found its way for exploration of ELF chemodiversity. **Error! Reference source not found.** shows ELF compounds which had been identified by the application of several metabolomics techniques. In a particular study, the uniqueness of metabolites produced by the ELF and their lichen host was established using LC-MS based multivariate analysis (Santiago et al., 2021). Furthermore, using metabolomics and dereplication tools, prioritisation was done from nine ELF extracts to arrive in the annotation of methyl xylariate C responsible for the observed antimicrobial activity against *S. aureus* and *E. coli*. Similarly, LC-MS was used to dereplicate bioactive components from 18 ELF extracts that were determined to have different biological activities (Weerasinghe et al., 2022). While many metabolites were annotated, three ELF strains stood out because their major compounds remained unidentified, suggesting the presence of potentially new metabolites for further exploration. In another study, molecular networking was used to rank and select from 40 extracts from 11 ELF strains (Toure et al., 2022). Molecular groups including ceramides and cyclopeptides were pinpointed as the bioactive metabolites against formation of *Candida albicans* biofilm. Moreover, targeted isolation from the prioritised ELF strain, *Preussia persica*, was carried out and led to the identification of an oxygenated fatty acid (8Z)-5,6-epoxy-4-hydroxy-octadec-8-enoic acid with antibiofilm activity. Another study applied molecular networking and LC-MS guided-isolation to identify a cytotoxic cyclic heptadepsipeptide, xylaroamide A, from *Xylaria* sp (Luo et al., 2022). Molecular networking was also applied in the discovery of six naphthol tetramers from *Daldinia childiae* (Kim et al., 2023). Next, crude extracts from the ELF *Chaetomium globosum* and *Nodulisporium* sp. exhibited significant inhibitory effects against plant pathogenic fungi. Molecular networking was then again used to rapidly identify cyclic dipeptides and chaetoglobosins as bioactive metabolites from the said ELF (Paguirigan et al., 2024). The above studies provide clear

evidence that metabolomics can be applied in ELF as a prioritisation tool in screening a collection of extracts or for targeted purification of fractions. Given these excellent examples, power of metabolomics was leveraged throughout this PhD study.

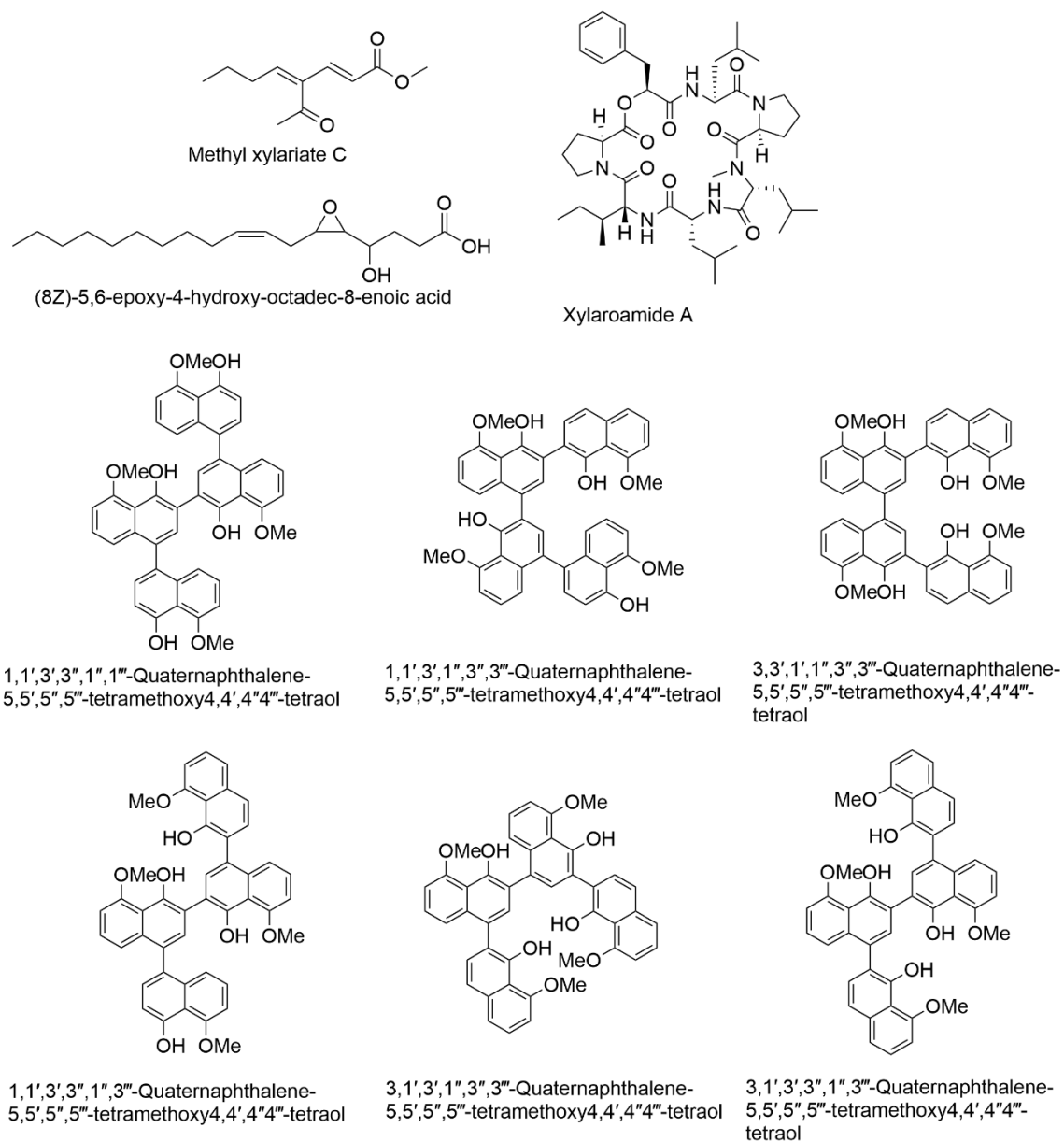


Figure 1.5 Chemical structure of metabolites from ELF discovered using several metabolomics tools

1.4 Antimicrobial resistance and bacterial biofilms

Antibiotic resistance or antimicrobial resistance happens when current antibiotics loses its effectivity to inhibit pathogenic microorganism (Nadeem et al., 2020). It has been estimated that 25% of the recorded illness-related deaths in 2019 were mainly caused by AMR infections (Murray et al., 2022). By 2050, it is predicted that 10 million lives per year will be at stake due to the further rise of AMR (O'Neill, 2016). Aside from deaths, AMR is also a burden in the economics by increasing poverty mostly affecting the poor countries (Bank, 2017). It will also affect the agriculture sector as AMR persist on livestock also (Lekagul et al., 2019). Hence, AMR is really a threat to humanity and actions should be taken to combat this crisis.

Pathogenic microorganisms have evolved to evade inhibition of antibiotics through several biochemical mechanisms. The common pathways include the enzyme-based inactivation of antibiotics, decreased permeability of drugs across the cell membrane, expulsion of the drugs by efflux pumps, and modifications in the target protein of such antibiotics (Giedraitienė et al., 2011). Furthermore, genetic factors such as mutation and horizontal gene transfer are also involved in AMR (von Wintersdorff et al., 2016; Woodford & Ellington, 2007). In a bigger scale, misuse and overuse of antibiotic drugs both in patients and livestock also contribute to the prevalence of AMR cases (Harbarth et al., 2015).

Understanding the causes and mechanisms of AMR serve as guide to bring out strategies to overcome it. First, proper prescribing should be implemented to prevent the overuse and misuse of antibiotics (Finch et al., 2004). Improved and quick surveillance tools should also be developed to monitor the emergence of such infections (Lee et al., 2013). Furthermore, it is also crucial to reduce the use of antibiotics in livestock. The combination of antibiotics has also been discovered as a viable approach to regain the sensitivity of bacteria to antibiotics. By combining two or more agents based on the susceptibility pattern of the infectious microorganisms, a synergistic effect can be

observed, thereby improving the effectiveness of the treatment (Fischbach, 2011). Lastly, the development and discovery of new drugs are always vital for combating bacterial infection. Therapeutic agents acting on a novel target or a new mode of action are key strategies in developing new antibiotics (Ruggieri et al., 2023).

1.4.1 Biofilms

Bacteria were thought to be living independently but it is discovered that they live in biofilms which are bacterial communities living within the extracellular matrix attached to surfaces (Stoodley et al., 2002). Biofilms are made up of exopolysaccharides, extracellular proteins, and extracellular DNA (**Error! Reference source not found.**). These components provide a hydrated and stronger structure that keeps bacteria in proximity allowing cell-to-cell communication and genetic exchange. It also provides protection against harsh conditions including dehydration, nutrient depletion, toxicity to metals, oxidative stress, UV exposure, extreme temperatures, and antimicrobial drugs (Flemming & Wingender, 2010).

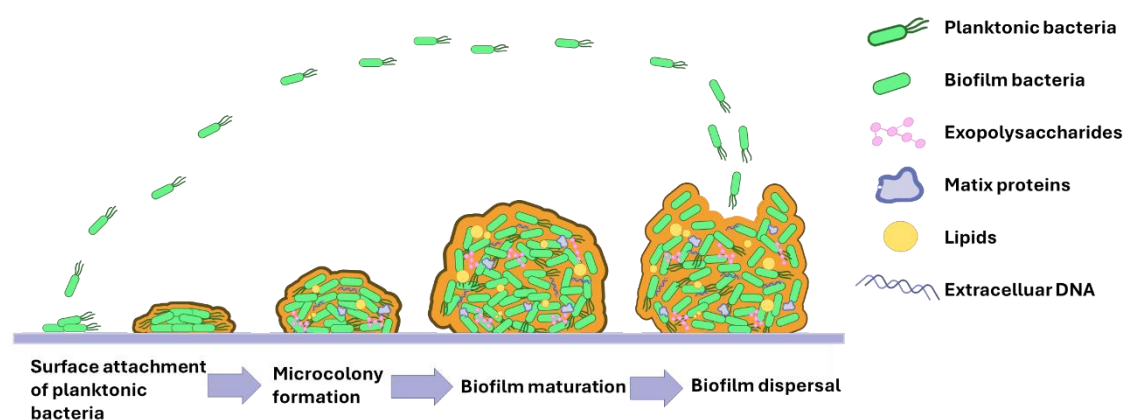


Figure 1.6. The formation and composition of bacterial biofilms

Bacterial biofilms are commonly found in natural and industrial settings. They can easily form on a variety of surfaces, including medical devices and human tissues (Bjarnsholt et al., 2013). In fact, a significant portion of healthcare-associated infections are attributed to the colonization of biofilm-forming pathogens on the surfaces of

implanted medical devices (Dufour et al., 2010). Moreover, infections caused by biofilms often tend to be chronic in nature. Examples of such chronic infections include lung infections in cystic fibrosis patients, chronic bone infection, prostatitis, rhinosinusitis, chronic middle ear infection, chronic wounds, persistent urinary tract infections, endocarditis, chronic gum disease, and tooth decay (Burmølle et al., 2010).

The inherent defence provided by biofilm can create an environment favorable to the emergence of spontaneous mutations. Additionally, the close physical proximity of bacterial cells within biofilm matrix has been suggested to facilitate the accelerated transfer of plasmid DNA (Stewart, 2002). Hence, biofilm is also a key contributor to AMR. It is therefore making sense to use and discover drugs that can inhibit biofilm formation and thus is the primary aim of this study.

Antibiofilm agents capable of inhibiting the formation and/or degrading biofilms have been identified throughout the years. These compounds include natural products, synthetic compounds, enzymes, peptides, chelating agents, polyphenols, and certain antibiotics (Asma et al., 2022; Walsh et al., 2019). There are a number of review articles showing the value of natural products as antibiofilm compounds with different biochemical mechanisms as shown in **Error! Reference source not found.** (Fydrych et al., 2025; Lu et al., 2019; Melander, Basak, & Melander, 2020; Sukmarini, Atikana, & Hertiani, 2024). For example, garlic extract caused *Pseudomonas aeruginosa* more susceptible to tobramycin, enhanced respiratory burst, and increased phagocytosis by polymorphonuclear leukocytes (PMNs) in pulmonary mouse models (Bjarnsholt et al., 2005). The common plant flavonoid, quercetin, was found to be a competitive inhibitor of the sortase A, an essential enzyme for bacterial colonization and biofilm formation (Wang et al., 2018). Lastly, a recent success is the report of a randomized, double-blind clinical trial showing the effects of *Matricaria chamomilla* extract on reducing biofilm formation and gingival bleeding in patients with gingivitis (Goes et al., 2016)

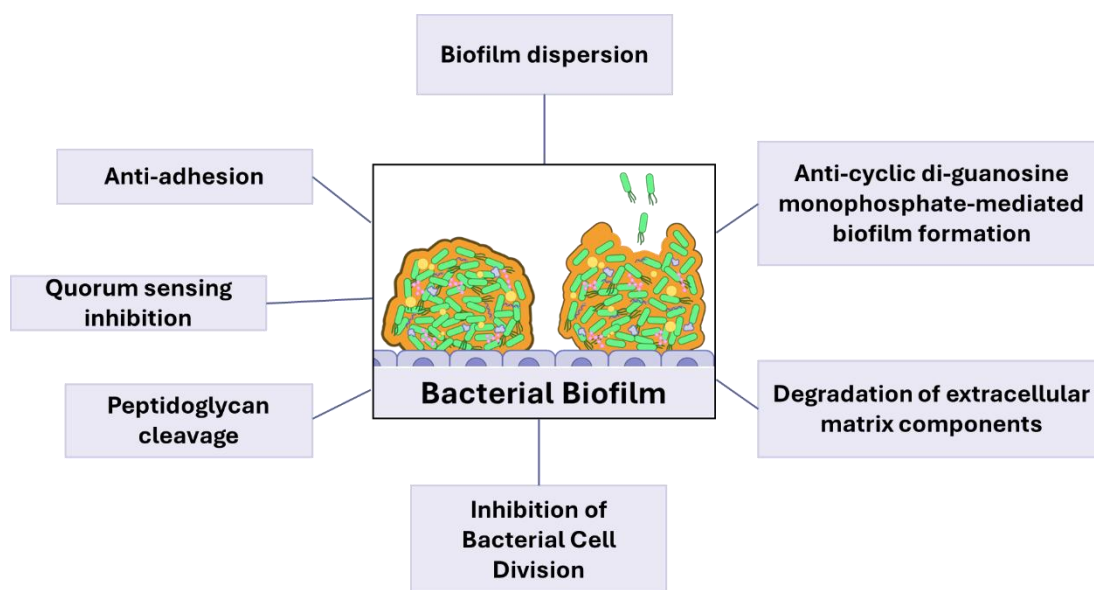


Figure 1.7 Different mechanisms of action against bacterial biofilms

1.4.2 ELF compounds with antibiofilm activities

ELF have also been explored as sources of compounds with antibiofilm activities (Millot et al., 2025). Structures of these examples were shown in **Error! Reference source not found..** Pyridoxatin isolated from the ELF *Tolypocladium cylindrosporum* prevented the formation of *Candida albicans* biofilms (Chang et al., 2015). Similarly, biatriosporin D, a phenolic compound isolated from the ELF *Biatrispora* sp., displayed anti-virulence activity by inhibiting adhesion, hyphal morphogenesis and biofilm formation of *C. albicans* (Zhang et al., 2017). The terphenyl pigment floricolin C from the ELF *Floricola striata* exhibited inhibition of planktonic and biofilm states *C. albicans* through reactive oxygen species accumulation (Zhang et al., 2018). Moreover, four ELF extracts isolated from the lichen host the *Nephroma laevigatum* displayed *C. albicans* antibiofilm activity and antiproliferative activity (Lagarde et al., 2019). In another report, virulence factors such as pyocyanin, protease, elastase, rhamnolipids, and extracellular polysaccharides of

P. aeruginosa PAO1 was reduced by the extract of ELF *Aspergillus quandricinctus*. Several fatty acids were determined by GC-MS as major components of the said fungus (Prateeksha et al., 2020). Lastly, the oxygenated fatty acid, (8Z)-5,6-epoxy-4-hydroxy-octadec-8-enoic acid, with antibiofilm activity to *C. albicans* was isolated using an MS-molecular networking-targeted approach from the ELF *Preussia persica* (Toure et al., 2022).

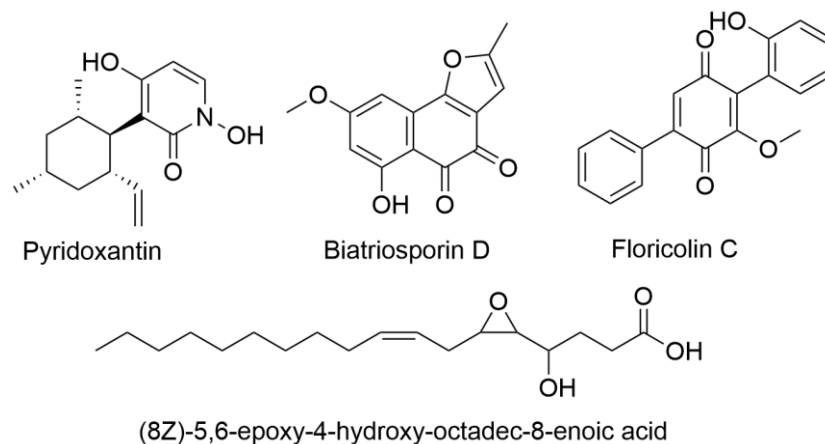


Figure 1.8 Chemical structures of ELF metabolites with reported antibiofilm activities

Collectively, these findings suggest that ELF-derived compounds and extracts hold strong potential as antibiofilm agents, particularly against *C. albicans*. However, the limited application to bacterial pathogens highlights a critical research gap and points out the need to further explore ELF for antibiofilm activity in a broader range of clinically relevant microbes.

1.5 Gaps, aims and objectives of the study

Despite the growing interest in endolichenic fungi ELF as sources of bioactive compounds, their potential for producing antibiofilm agents remains underexplored particularly when guided by metabolomics. Furthermore, most studies focus on standard

culture conditions, overlooking optimisation strategies such as OSMAC and chemical elicitation that can enhance metabolite diversity. Thus, to bridge these gaps, this study aimed to discover and isolate antibiofilm compounds from Philippine ELF through an integrated metabolomics, OSMAC, and targeted isolation workflow. To achieve this, the following specific objectives were accomplished:

1. isolate and culture ELF fungi from Philippine lichen species;
2. apply ^1H -NMR and LC-MS-based metabolomics for chemical profiling;
3. perform antibacterial and antibiofilm assays against MRSA and *P. aeruginosa*;
4. employ the OSMAC strategy in prioritised ELF strains to enhance chemical diversity by varying culture media, incubation periods, and supplementation of chemical elicitors to be used for scaled up production;
5. perform chromatographic purification assisted by metabolomics;
6. structure elucidation of isolated metabolites using various spectroscopic techniques including NMR and MS;
7. assess the cytotoxicity of isolated compounds against human kidney cell lines (HEK293).

Hypothesis: Philippine ELF are producers of diverse bioactive secondary metabolites with antibiofilm activity, and these compounds can be effectively prioritized, enhanced, and isolated through integrative metabolomics tools and OSMAC approaches.

Chapter 2. Methodology

2.1 Collection and identification of lichen hosts

Lichen hosts utilised in this study were collected from Lucban, Quezon, Philippines (14.112507, 121.559789). The lichens were identified as *Parmotrema gardneri* (C.W.Dodge) Sérus. and *Ramalina farinacea* (L.) Ach. and confirmed by Prof. Thomas Edison dela Cruz, mycologist from University of Santo Tomas, Manila, Philippines. *P. gardneri* were picked from *Adonidia merrillii* while *R. farinacea* were from *Cocus nucifera*.

2.2 Isolation of endolichenic fungi

To remove epiphytic fungi, lichen samples were surface-sterilized. Lichen thallus of *P. gardneri* were first cut into fragments (0.1 cm), immersed in the following reagents: 70% ethanol (Green Cross Inc, Philippines) for 90 s, 0.5% bleach (Zonrox, Green Cross Inc, Philippines) for 5 min, and sterile water for 90 s. *R. farinacea* were also cut into fragments (0.1cm) and submerged in the following treatments: 70% ethanol for 90 s, 0.5% bleach for 3 min, and sterile water for 90 s. The lichen fragments were aseptically blot-dried into sterile paper towels and then put into potato dextrose agar (PDA; TM Media India) in 6 plates with 5 fragments each. To verify the efficiency of surface sterilisation, lichen fragments were tissue-printed to a separate PDA plate. These plates were then incubated at room temperature and monitored for 14 days. Any emerging endolichenic fungi were then subcultured to new PDA plates until pure cultures were obtained. Nine ELF were isolated from *P. gardneri* and coded as **P1** to **9** while four ELF isolates were obtained then from *R. farinacea* and were coded as **R1** to **4**. Next, a total of 10 ELF was selected: **P3** to **P6**, **P8** to **P9**, and **R2** to **R5** based on morphologies and transferred to Strathclyde Institute of Pharmacy and Biomedical Sciences (SIPBS), Glasgow, UK.

Voucher specimens were maintained in potato dextrose broth: glycerol (70:30) cryotubes at -80 °C at Natural Products Metabolomics Laboratory, SIPBS.

2.3 Preparation of ELF crude extracts for biological assays and metabolomics profiling

ELF were cultured in PDA media made in-house. In this method, washed, unpeeled, 100 g diced potatoes (British baking potato from Aldi, UK) were boiled in 500 mL of deionised water for 30 minutes. It was then filtered through a cheesecloth and 10 grams of glucose monohydrate (Sigma-Aldrich France) were added to the filtrate. Next, the solution was cooled, and the volume was then made up to 500.0 mL using deionized water. The pH was adjusted to 7.4 using 0.1 M NaOH. Finally, agar (10 g; Sigma-Aldrich France) was added and submitted for autoclaving (121 °C, 15psi, 15mins).

To prepare the crude extracts, each ELF were cultured in three PDA plates in triplicate. The duration of incubation of ELF were based their growth rates: fast-growing ELF for 7 days while slow growers for 15 days at 27 °C. The ELF **P6** is a more slow-growing fungi and were incubated for 25 days.

After the required days of incubation, each extraction replicate was transferred to a 250 mL conical flask and 250 mL of ethyl acetate (Sigma-Aldrich, France) was added. Next, it was homogenized using a homogenizer (Ultraturrax T18 Basic, IKA, Germany) for 30 minutes and allowed to macerate overnight. After this, it was filtered, and the residue was further extracted twice. Then, the organic extracts were combined and dried using a rotary evaporator (Rotavor R3 system, Buchi, Switzerland) at 40 °C. Extracts were not dried completely but transferred to pre-weighed vials. Complete evaporation was done by drying under nitrogen gas (Laboratory Nitrogen gas generator, Peak Scientific) with mild heating (40 °C) on a digital block heater (Stuart SBH130D/3, UK). PDA was also extracted to represent media blank. After crude extracts were obtained, their weights

were noted to calculate appropriate amounts to be used for analysis as described in Table 2. Lastly, crude extracts were stored in the fridge at 4 °C until analysis.

Table 2.1. Amount of crude extracts needed for biological assays and metabolomics analysis

Test	Weight required	Solvent volume	Final concentration
LC-MS	1 mg	1 mL MeOH:ACN	1 mg/mL
¹ H-NMR	5 mg	600 µL DMSO-d ₆	5 mg/600 µL
Biological assay	1 mg	100 µL DMSO	10 mg/mL

2.4 LC-MS Analysis

The method of Macintyre et al. (2014) were followed for the Liquid chromatography-Mass spectrometry (LC-MS) analysis of crude extracts and other samples. Samples including solvent blanks were maintained in an autosampler kept at 4 °C and injected (10 µL) in the Accela HPLC system (ThermoScientific, Germany) using an Accucore™ RP-MS column (5 µm × 100 mm × 3 mm; ThermoScientific UK). The mobile phase used two solvents which are A (water and 0.1% formic acid) and B (ACN and 0.1% formic acid) at a flow rate of 300 µL/min. Then, the gradient programme was as follows: starting at 90% A and 10% B, at 30 min 0% A and 100% B, at 36 min 90% A and 10% B until end at 45 min. Mass spectrometry (Q Exactive Plus Orbitrap Mass Spectrometer, ThermoScientific, Germany) was then carried out over a mass range of 100–2000 m/z in positive and negative ionization modes with spray voltage of 4.5 kV and capillary temperature at 270 °C.

2.4.1 LC-MS Data Processing

Raw LC-MS data were first sliced into positive and negative mode data sets through the MassConvert tool from ProteoWizard (Chambers et al., 2012). Then, each data set were processed in MZmine 3.4 software (Schmid et al., 2023) consisting the

following steps and settings: mass detection (mass detector: centroid; noise level: 10000; minimum time span: 0.2 min; minimum height: 1×10^4 ; m/z tolerance: 0.001 m/z or 5 ppm), deconvolution (chromatographic threshold: 5%), deisotoping (m/z tolerance: 0.001 m/z or 5 ppm, retention time (RT) tolerance: 0.1 absolute (min), maximum charge: 2, representative isotope: most intense), filtering (m/z range: 100–2000, RT: 0–45 min), alignment (m/z tolerance: 0.001 m/z or 5 ppm, m/z weight: 20, RT tolerance: 5 relative %) and gap filling (m/z tolerance: 0.001 m/z or 5 ppm, intensity tolerance: 25%). Lastly, the processed data were exported as a comma-separated value (CSV) file. The CSV files from positive and negative mode were then combined in an in-house Excel Macro for clean-up of peaks from media blank. This CSV file was then imported to SIMCA software for multivariate analysis as described in section 2.8.

2.5 NMR analysis

Samples dissolved in DMSO- d_6 (Sigma) was put in 5 mm NMR tubes (Norell, New York, USA) for acquisition in an NMR spectroscopy equipment (Bruker Ultrashield 400 with liquid nitrogen cryoprobe, Bruker). Pure and semi-pure compounds were also analysed for other NMR experiments (^{13}C Jmod, 2D NMR such as COSY, HSQC, and HMBC) using the same spectrometer. Most NMR spectra were acquired in DMSO- d_6 rather than the more commonly used CDCl_3 for comparison with reported values. This choice was made because residual acidic impurities in CDCl_3 can compromise the stability of certain compounds during solvent recovery, whereas DMSO- d_6 provides a more stable and inert medium for sensitive metabolites.

NMR data was processed in MestRenova software (v14; Mestrelab Research, Spain). Initially, raw NMR fid files were baseline corrected and then properly phased. A selection of NMR spectra was then stacked and processed using the Chemometrics tool

of Mnova. In this step, NMR spectral were binned at the width of 0.04 ppm. Finally, it was imported as a CSV file to be subjected for multivariate analysis.

2.6 Biological assays

A culture of Methicillin-resistant *S. aureus* (ATCC 29213) and *P. aeruginosa* (ATCC 43300) was first prepared by transferring a loopful of respective bacterial strains from a stock culture to an 25mL Luria broth (LB; 1% peptone, 1% sodium chloride, 0.5% yeast extract) enriched with glucose (1%) and incubated (Sciquip) for overnight at 37 °C with shaking. After the incubation, 100 µL of the overnight culture was transferred to a fresh 100 mL LB-glucose, further incubated for 4 hrs at 37 °C with shaking to obtain exponentially growing bacteria. The optical density (OD) of this culture was measured at 600 nm. The bacterial cell concentration was then determined from the OD using the Agilent online cell culture concentration calculator (<https://www.agilent.com/store/biocalculators/calcODBacterial.jsp>). From the obtained concentration, a target cell concentration of 1×10^6 CFU/mL were prepared in LB-glucose. This main bacterial suspension will be then used for subsequent planktonic, prebiofilm and postbiofilm assays. All assays are done in triplicate.

2.6.1 Planktonic assay

In a 96-well plate, 1µL of test samples (crude extracts, negative control DMSO, positive control ciprofloxacin) were added and mixed with 100 µL of main bacterial suspension as prepared from Section 2.7. Bacterial suspension and broth wells were also allotted (see Figure 2.1). To avoid evaporation, plates were wrapped with parafilm. The assay plates were then incubated for 20 hrs at 37 °C with shaking. After 20 hrs, OD at 600 nm were read using a microwell plate reader (SpectraMax M5 reader, Molecular Devices, UK). Afterwards, the plates were further processed for prebiofilm assay as described in section 2.7.2

The biological activity from the planktonic assay were expressed as % inhibition and calculated as follows:

$$\% \text{ inhibition} = \left(1 - \frac{\text{OD}_{\text{Sx}} - \text{OD}_{\text{broth}}}{\text{OD}_{\text{bacteria}} - \text{OD}_{\text{broth}}}\right) \times 100$$

, where OD_{Sx} = OD of test sample

OD_{broth} = OD of broth wells

$\text{OD}_{\text{bacteria}}$ = OD of bacterial suspension wells

	1	2	3	4	5	6	7	8	9	10	11	12
A	broth	broth	bacteria	bacteria	bacteria	bacteria	bacteria	bacteria	CIP-50	bacteria	broth	broth
B	broth	broth	7MB	P4-1	P6-1	P8-3	R2-3	R4-3	CIP-12.5	bacteria	broth	broth
C	broth	broth	15MB	P4-2	P6-2	P9-1	R3-1	R5-1	CIP-6.25	bacteria	broth	broth
D	broth	broth	25MB	P4-3	P6-3	P9-2	R3-2	R5-2	CIP-3.13	bacteria	broth	broth
E	broth	broth	P3-1	P5-1	P6-60	P9-3	R3-3	P5-4	CIP-1.56	bacteria	broth	broth
F	broth	broth	P3-2	P5-2	P8-1	R2-1	R4-1	R5-3	CIP-0.78	bacteria	broth	broth
G	broth	broth	P3-3	P5-3	P8-2	R2-2	R4-2	DMSO	CIP-0.39	bacteria	broth	broth
H	broth	broth	bacteria	bacteria	bacteria	bacteria	bacteria	bacteria	CIP-0.098	bacteria	broth	broth

Figure 2.1. Example of sample plate map in a 96 well plate

Experiments were done twice with three replicates. MIC from dose response curves were calculated using the built-in algorithm in GraphPad Prism v8.0 (<https://www.graphpad.com/support/faq/fitting-bacterial-growth-data-to-determine-the-mic-and-nic/>).

2.6.2 Prebiofilm assay

From the plates from 2.7.1, the process was continued by removing the planktonic media. This was done by pipetting off 120 μL of the planktonic liquid and discarding it in Virkon solution (Rely+On, Lanxess, UK). The wells further washed by adding 100 μL of phosphate buffer saline (PBS; Oxoid, UK) and removing it by pipetting off 120 μL . For *P. aeruginosa*, washing with PBS was done twice. Next, 90 μL of Dulbecco's Modified Eagle Medium 1X (DMEM; Fisher UK) and 10 μL of resazurin (10x solution in PBS; Sigma-Aldrich, France) were added to the wells. The plates were then covered in foil and incubated at 37 °C with shaking. Incubation times were as follows: 90 mins for MRSA

and 150 mins for *P. aeruginosa*. The fluorescence was then measured at 560 nm emission wavelength and 590 nm excitation wavelength using the microplate reader.

The biological activity from the prebiofilm assay were expressed as % inhibition and calculated as follows:

$$\% \text{ inhibition} = \frac{\text{RFU}_{\text{bacteria}} - \text{RFU}_{\text{Sx}}}{\text{RFU}_{\text{bacteria}} - \text{RFU}_{\text{broth}}}$$

, where RFU_{Sx} = RFU of test sample

$\text{RFU}_{\text{broth}}$ = RFU of broth wells

$\text{RFU}_{\text{bacteria}}$ = RFU of bacterial suspension wells

Experiments were done twice with three replicates.

2.6.3 Postbiofilm assay

The postbiofilm assay measures the capability of samples to eradicate or destroy pre-formed biofilms. To do this, 100 μL of main bacterial suspension were seeded to wells and incubated for 24 hrs at 37 °C with shaking. Media was removed after 24 hrs then 1 μL of the test samples were added followed by 100 μL plain LB. This was then incubated for another 20 hrs at 37 °C with shaking. The next day, media were again removed and washed as described from section 2.6.2. The same amount DMEM (90 μL) and resazurin (10 μL) were added to the wells. The plates were then covered in foil and incubated at 37 °C with shaking. Incubation times were as follows: 90 mins for MRSA and 120 mins for *P. aeruginosa*. The fluorescence were then measured at 560 nm emission wavelength and 590 nm excitation wavelength using the microplate reader. The biological activity from the postbiofilm assay were expressed as % inhibition and calculated as shown in Section 2.7.2. Experiments were done twice with three replicates.

2.6.4 Cytotoxicity assays

A preliminary cytotoxicity assay was conducted for the bioactive compounds using Human Embryonic Kidney 293 (HEK293) cells from ATCC (CRL-1573). Cell culture was maintained in 25 cm^2 flasks in medium of DMEM supplemented with 10% fetal bovine

serum (FBS; Invitrogen, UK), 1% penicillin/streptomycin (Invitrogen, UK), and 1% L-glutamine (Invitrogen, UK) inside an incubator at 37 °C, 5% CO₂ (Napco, US). Once a >85% confluency in flasks were reached, the cells were passaged or split by first discarding the medium and detaching the cells by adding 3 mL of TrypLE™ Express (Thermo Fisher, UK) and incubating for 10 mins. The cells were pelleted by centrifuge (2 mins, IEC Medispin, ThermoScientific Germany) and resuspended in cell culture medium for counting in a haemocytometer. A cell count of 3 x10⁴ cells/cm² were utilised for passaging to 25 cm² flasks. A cell count of 40 x 10⁴ cells/mL was then added (100µL) to 96-well plates and was allowed to attach for 48 hrs. Then, media was removed then treated with compounds in varying dilutions (1.25-100 µg/mL). The plates were then further incubated for 24 hrs and then 10 µL of PrestoBlue (Thermo Fisher, UK) was added to each well and incubated for 1 hr. Finally, fluorescence were read using a Hidex Sense plate reader (Hidex Oy, Finland) at 530/595 nm excitation/emission wavelengths.

The biological activity from the cytotoxicity assay was expressed as % inhibition and calculated as follows:

$$\% \text{ inhibition} = \left(1 - \frac{RFU_{Sx}}{RFU_{DMSO}}\right) \times 100$$

,where RFU_{Sx} = fluorescence reading of test samples

RFU_{DMSO} = average fluorescence reading of DMSO control

Experiments were done twice with two replicates. IC₅₀ from the dose response curves were calculated based on GraphPad Prism v8.0.

2.7 Multivariate Analysis

Multivariate analysis of NMR and LCMS data were carried out using SIMCA software (v 17 and 18, Sartorius, Germany). Statistical models such as principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), and orthogonal partial least squares-discriminant analysis (PLS-DA) were employed. For all the models, Pareto scaling was applied. Model validations were primarily checked from

the R2 and Q2 values. Permutation tests (n = 100) were further performed to assess the validity of the PLS-DA and OPLS-DA models, ensuring that the observed class separation was not due to random chance.

To create heatmap, NMR csv files were processed in R (code can be found in S 2). For LC-MS data, its csv file was uploaded in MetaboAnalyst website (<https://www.metaboanalyst.ca>) (Pang et al., 2022).

Furthermore, an in-house Excel Macro were utilized to carry out dereplication and matching of putative compounds from the Dictionary of Natural Products (DNP, version 2018) with mass accuracy filter of 5 ppm.

2.8 Molecular identification of selected ELF

2.8.1 DNA extraction

Three ELF were further selected for molecular identification based on their excellent biological activity and interesting metabolite profiles. A part of mycelium of the three ELF was scraped from a 10-day old culture and transferred to 1.5 mL Eppendorf tubes. Then, 100 μ L of Extraction Solution from the DNA kit (REDEExtract-N-Amp™ Plant PCR Kit, Merck, USA) were added and vortex quickly. The tube was then heated at 95 °C for 10 mins. Next, 100 μ L of the Dilution Solution were mixed to the extract and vortexed. This DNA extract solution was stored at -20 °C until use.

2.8.2 PCR Amplification

A 50 μ L PCR reaction was set as follows: 25 μ L of the PCR Ready mix, 3 μ L each of ITS1 and ITS4 primers (10 pmol/ μ L in TrisEDTA buffer), 4 μ L of DNA extract and 15 μ L of nuclease-free water. ITS1 (5' TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-

TCCTCCGCTTATTGATATGC-3') were ordered from Integrated DNA Technologies (USA). Reaction was carried out in the thermal cycler (MWG AG Biotech Primus 96 plus, Cole-Parmer, UK) with the following conditions: initial denaturation of 95 °C for 3 mins, and 40 cycles of denaturation (95 °C, 30 s), annealing (56 °C, 30 s), and extension (72 °C for 1 min). After 40 cycles, a final extension was done at 72 °C for 10 mins and was hold finally at 4 °C.

2.8.3 PCR products purification

The PCR products were purified using agarose gel electrophoresis. First, 1% agarose (Merck, USA) was prepared with 1x TAE (TrisAcetate EDTA) buffer and allowed to solidify in the agarose plate. TAE buffer (with 10 µL ethidium bromide) was used to fill the tank. Then, 19 µL of Hyperladder 50bp DNA ladder and 10 µL of the PCR products were added to the wells. Three wells were allotted for each ELF. It was then operated for 45 minutes at 50 V. After that, the gel was visualized under UV using a transilluminator. Bands from the three wells for ELF were then sliced and transferred to 1.5mL Eppendorf tubes for extraction.

Tubes containing the agarose gel bands were then processed using the gel band purification kit to dissolve the agarose and extract only the amplified ITS genes using a kit (Illustra GFX PCR DNA kit, GE Healthcare, UK). First, 300 µL of the Capture buffer type 3 from the kit were added to the Eppendorf tubes and mixed by inversion. The tubes were incubated at 60 °C until agarose was dissolved (~10 mins) and the color of the solution was yellow to pale orange. It was centrifuged and the liquid at the bottom was collected and transferred onto an assembled Microspin column and Collection tube. This was further incubated at room temperature for 1 minute and afterwards centrifuged at 16 000 g for 30 s. The liquid collected at the Collection tube was discarded and then a 500 µL of Wash buffer type 1 was added to the Microspin column. The assembly was again centrifuged at 16 000 g for 30 s and the collection tube was discarded. The Microspin column was then assembled to a new 1.5mL Eppendorf tube. Then, 50 µL of Elution

buffer type 4 was added to the center of the spin column, incubated at room temperature for 1 min, and centrifuged at 16 000 g for 1 min. The eluted liquid now contained the purified ITS genes. The Microspin column was discarded, and the ITS gene tubes were stored at -20 °C until submission for Sanger sequencing at Eurofins Genomics GmbH (Ebersberg, Germany) using the TubeSeq Supreme Service.

2.8.4 DNA sequence assembly and identification

The ITS sequence obtained was first trimmed up and aligned using BioEdit software (v 7.2, (Hall, 2004)). Then, the aligned sequences were compared using nucleotide Basic Local Alignment Search Tool (BLAST, blast.ncbi.nlm.nih.gov) programme. Species identification was determined from the lowest expect value (E-value) and the highest similarity percentage of the BLAST search output

2.9 OSMAC methodology

2.9.1 Media optimisation

Two solid and two liquid media were used for media optimisation studies. Jasmine rice (100 g; Green Dragon, Thailand), oats (150 g; Morrisons, UK), potato dextrose broth (PDB, 250 mL; similar preparation with PDA as in section 3.9 but without agar), and malt extract broth (MEB, 250 mL; Oxoid, UK) were prepared in 500 mL Erlenmeyer flasks. Three replicates were prepared for each for two incubation periods (15 days and 30 days). Media were then autoclaved at 121 °C, 15 psi, for 15 min.

Selected ELF strains were then inoculated to the prepared media and incubated at 27 °C for 15 or 30 days. After such, extraction, biological and metabolomics analysis were carried out following 2.3, 2.4, 2.5, 2.6, and 2.6.4.

2.9.2 Chemical elicitors

Suberoylbishydroxamic acid (SBHA; Merck, USA) and 5-azacytidine (5-Aza; Merck, USA) were used for chemical elicitor studies. First, stock solutions were prepared

in sterile water and stored at 4 °C until use. Prior to use, the stock solutions were filter-sterilised. PDB was prepared as usual and appropriate volumes of stock solution of elicitors were added to afford a final concentration of 100 µM (in 250 mL final volume). Selected ELF strains were inoculated and incubated at 27 °C for 15 days. After such, extraction, biological and metabolomics analysis were carried out following 2.3, 2.4, 2.5, 2.6, and 2.6.4.

2.10 Purification works

After obtaining best results from the OSMAC approach, selected ELF strains were cultivated upscale using the optimum conditions (media, incubation period, chemical elicitors). After cultivation, it was extracted following Section 2.3. Then, several chromatographic techniques were employed guided by bioassays (Section 2.6) and metabolomics (Sections 2.4 and 2.5) until pure compounds were obtained. The structure of compounds was elucidated using NMR following Section 2.5

2.10.1 Thin layer chromatography

Thin layer chromatography (TLC) of samples was carried out on silica gel 60 plates with fluorescent indicator 254 nm (Merck, USA). Sample extracts were applied using a glass capillary tube (Hawksley, UK). Plates were developed in glass chambers equilibrated properly with organic solvent system of choice. A drop of ammonia solution was added to avoid sample tailing. After TLC run, it was viewed under 254 nm and 365 nm UV lamp (UVGL-55, UVP, UK) to look for UV-active compounds. It was then sprayed with a universal reagent, *para*-anisaldehyde-sulfuric acid inside a safety fume hood and heated to 170°C with a heating gun HL 2010 E (Steinel, Germany) at 170°C to visualize compounds with different colored spots.

The *para*-anisaldehyde-sulfuric acid spray reagent used to view coloured spots indicating the presence of phenols, sugars, steroids and terpenes. It was prepared by adding *p*-anisaldehyde (0.5mL; Sigma-Aldrich, USA), methanol (85 mL; Sigma-Aldrich,

France), glacial acetic acid (Sigma-Aldrich, Germany), and concentrated sulfuric acid (5 mL, added slowly; Fischer Chemical, UK) in that order. The spray reagent was stored in glass bottle covered with aluminum foil and kept in refrigerator prior to use. The reagent was sprayed in TLC plates followed by heating using a heat gun set at 180 °C until colored spots appeared.

2.10.2 Medium pressure and flash chromatography

Fractionation was carried out in VersaFlash Station (Supelco, USA) equipped with modular pumps (Buchi, Switzerland). Crude fungal extracts were first dissolved in minimal solvent to be loaded in Celite S (Merck, UK) and allowed to dried overnight. Normal phase cartridges (VersaPak Silica 40x150mm, Supelco, USA) were used with hexane and ethyl acetate as mobile phases. The linear gradient programmeme starts with 100:0 hexane:ethyl acetate to 0:100 for 60 minutes at 100 mL/min flow rate. Every 50 mL was collected and monitored with TLC using appropriate solvent systems for pooling.

In some cases, a smaller silica cartridge (23 x 53 mm; VersaPak, Supelco, USA) was used and conditions were detailed in specific cases. Another system (Isolera One, Biotage, Sweden) was used for fractions or samples weighing less than 1 gram. Appropriate solvent system was first determined through TLC and then the resulting R_f values were entered into the Isolera One optimizer to confer a recommended gradient programmeme. In most cases, a silica cartridge (Biotage Sfär HP-Sphere, 25-33 µm, Biotage, UK) was used. Specific conditions can be found in the following subsections.

Chapter 3. Biological activity screening and metabolomics profiling of Philippine endolichenic fungi

3.1 ELF from Philippine lichens

In this study, *Parmotrema gardneri* (C.W.Dodge) Sérus. and *Ramalina farinacea* (L.) Ach. were collected from Lucban, Quezon, Philippines. ELF were isolated from these lichen hosts as shown in Figure 3.1 and Figure 3.2. Nine ELF were isolated from *P. gardneri* but only six ELF (**P3 to P6, P8 to P9**) were chosen for further investigation based on their distinct morphologies as recommended by Prof Dr Thomas Edison dela Cruz who also authenticated the lichen host identities. Meanwhile, four ELF (**R2 to R5**) were derived from *R. farinacea* (Figure 3.2). In total, ten ELF strains were used throughout for screening and chemical profiling.

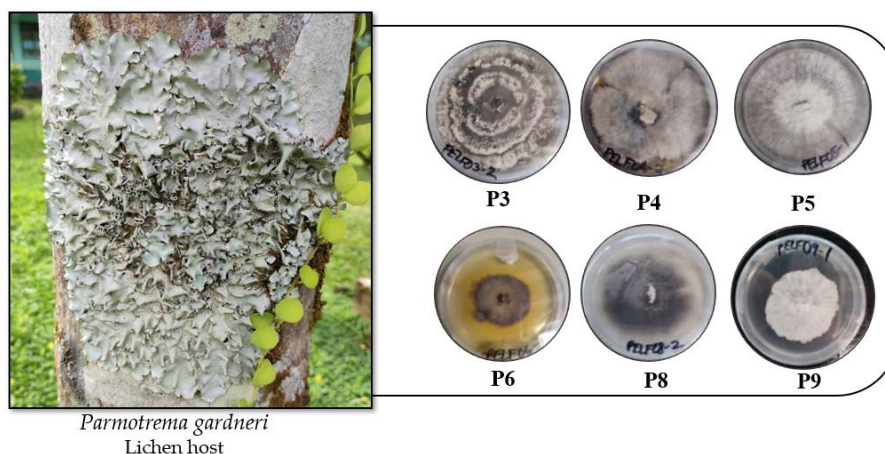


Figure 3.1. Selected ELF isolated from *P. gardneri*. (P = *Parmotrema*; numbers correspond to isolate codes).

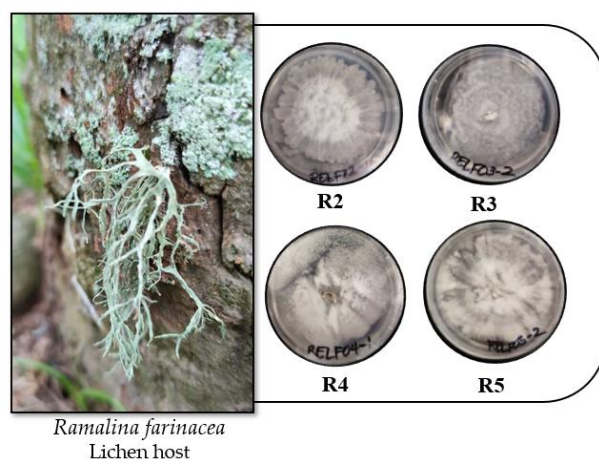


Figure 3.2 ELF isolated from *R. farinacea*. (R = *Ramalina*; numbers correspond to isolate codes).

3.2 Biological activities of ELF crude extracts

The ten ELF species were cultivated in PDA for 7 days for fast-growers (**P3** and **P4**), 15 days for slow-growers (**P5**, **P8**, **P9**, **R2** to **R5**), and a special case of 25 days for **P6**

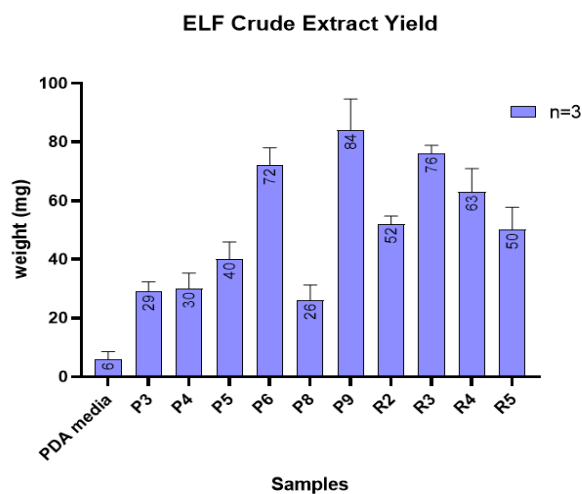


Figure 3.3. Yield of ELF crude extracts for screening studies

which were growing very slow. Ethyl acetate was used to obtain crude extracts. The yields were shown in Figure 3.3. The highest yield of crude extract was produced by **P9**. The ELF crude extracts were examined in three biological assays and metabolomic profiling study.

3.2.1 Planktonic assay

The planktonic assay tests the ability of the crude extracts to inhibit bacterial growth. In this study, Methicillin-resistant *S. aureus* (MRSA) and *P. aeruginosa* were used as test organisms on ELF crude extracts at the final well concentration of 100 µg/mL. Results were expressed as % inhibition and shown in Table 3.1.

Based on Table 3.1, only **P6** have activity against MRSA and *P. aeruginosa* with very weak inhibition of 38.71% and 28.88%, respectively. Despite that only one ELF crude extract showed weak antibacterial activity, their abilities to inhibit biofilm were also tested, which is an important mechanism to combat drug resistance. Biofilm inhibition assay results were discussed next.

3.2.2 Prebiofilm assay

The prebiofilm assay indicates the ability of the test samples to inhibit the formation of biofilm. The same bacterial strains were used as test organisms with ELF crude extracts at the same final well concentration of 100 µg/mL. Results were expressed as % inhibition and shown in Table 3.1

Based on Table 3.1, six ELF crude extracts were able to inhibit the formation of biofilm either for one test bacteria or both while there were also extracts that displayed negative inhibition values. **P3**, **P4**, **P9**, and **R2** exhibited greater than 50% inhibition against *P. aeruginosa*. **P4** have moderate activity (highlighted in orange) while **P6** showed greater than 70% activity (highlighted in red) to both test organisms indicating a potent

activity. This means that these two ELF strains were capable to stop the formation of biofilm by more than half. Moreover, **P6** are noteworthy as this also displayed antibacterial activity from the planktonic assay. On other hand, **R3** displayed moderate inhibition against both bacteria albeit to a lesser extent (~57%).

3.2.3 Postbiofilm assay

The postbiofilm assay procedure can assess the capacity of test samples to eradicate or destroy pre-formed bacterial biofilm. The same bacterial strains were used as test organisms with ELF crude extracts at the same final well concentration of 100 µg/mL. Results were expressed as % inhibition and shown in Table 3.1.

Based on Table 3.1, most crude extracts have excellent inhibition. Moderate activity can be observed for **P4, P5, P6, P8, P9**, and **R2** as they exhibited greater than 50% inhibition to MRSA (highlighted in orange). Only **R3** gave an excellent postbiofilm inhibition that is greater than 70%.

In summary, **P6** was considered as an excellent candidate for strain prioritisation as it displayed consistent dual bioactivities: antibacterial activity, prebiofilm and postbiofilm inhibition. Next is **P4**, as it exhibited both prebiofilm and postbiofilm inhibition. **R3** is also a good ELF strain because of moderate prebiofilm inhibition but with high MRSA postbiofilm activity.

Table 3.1 Biological activities of ELF extracts

ANTIBACTERIAL ACTIVITY, average % inhibition (n=3)					
Sample	MRSA	<i>P. aeruginosa</i>	Sample	MRSA	<i>P. aeruginosa</i>
P3	2.13	0.66	R2	0.74	7.83
P4	-1.19	6.07	R3	9.71	5.98
P5	-17.61	3.75	R4	7.18	10.40
P6	38.71	28.68	R5	3.27	6.72
P8	-1.26	7.72	DMSO	-0.10	7.33
P9	-0.10	10.55	Ciprofloxacin (50µM)	99.12	100.31
PREBIOFILM ACTIVITY, average % inhibition (n=3)					
Sample	MRSA	<i>P. aeruginosa</i>	Sample	MRSA	<i>P. aeruginosa</i>
P3	28.19	57.55	R2	-45.88	62.58
P4	63.23	66.23	R3	57.45	46.26
P5	23.04	3.17	R4	20.41	26.17
P6	96.96	74.79	R5	45.41	-88.96
P8	-18.08	-94.89	DMSO	5.93	-181.10
P9	-22.23	66.25	Ciprofloxacin (50µM)	100.41	99.96
POSTBIOFILM ACTIVITY, average % inhibition (n=3)					
Sample	MRSA		Sample	MRSA	
P3	36.62		R2	57.16	
P4	52.79		R3	82.41	
P5	62.98		R4	36.85	
P6	68.90		R5	43.59	
P8	61.90		DMSO	-17.66	
P9	63.29		Ciprofloxacin (50µM)	97.36	

Testing concentration=100 µg/mL; assays were done twice with three replicates (n=3) Weak inhibition: 30-50% inhibition (coloured in green); Moderate inhibition: 50-70% (coloured in blue); Strong inhibition >70% (coloured in red)

3.3 Metabolomics profiling of ELF crude extracts

3.3.1 NMR-based metabolomics

In this section, metabolomics was applied to ^1H -NMR spectral data of ELF crude extracts. NMR spectra of ELF crude extracts were stacked as shown in Figure 3.4 to give a brief overview. Methylene protons resonating at 0.9- 2.00 ppm (boxed in pink) are present in all crude extracts including the PDA medium. The broad signal indicates long chain CH_2 's which may correspond to long chain fatty acids. Hydroxylated compounds with resonances from 4.0-5.00 ppm (boxed in brown) were also present in **P6** and **R3**. Furthermore, olefinic protons at 5.00 - 6.00 ppm (boxed in red) can be seen in **R3** and **P9**. The olefinic protons may be attributed from unsaturated fatty acids. Lastly, aromatic protons which were resonating at δ 7.00-8.00 ppm (boxed in orange) were prominent in **R3** and to a lesser extent in **P4**, **P6**, and **P9**.

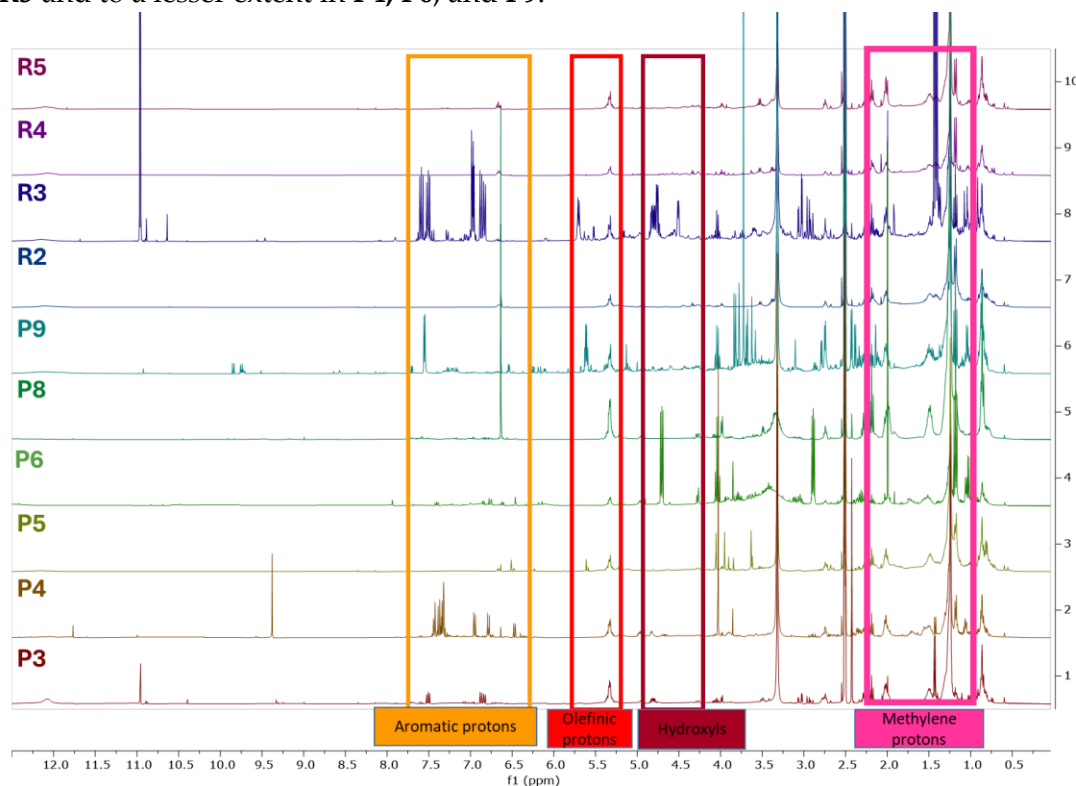


Figure 3.4. Stacked ^1H -NMR spectra of ELF crude extracts. ^1H -NMR recorded at 400MHz in DMSO-d_6 . Boxed in orange-aromatic protons; red-olefinic; brown-hydroxyls; pink-methylene protons

Heatmap were generated in R to give an overview of the diversity of NMR profiles of the ELF crude extracts (Figure 3.5). High intensity chemical shifts were indicated by blue bands. Generally, extraction replicates (as coded by numbers following the ELF codes, e.g., R3-1: 1st extraction replicate) were similar. More intense blue lines can be seen on the NMR data of **P4**, **P6**, **P9**, and **R3**. This points out higher chemical diversity can be correlated to the observed high bioactivities of **P4**, **P6**, and **R3** (pointed in brown arrows).

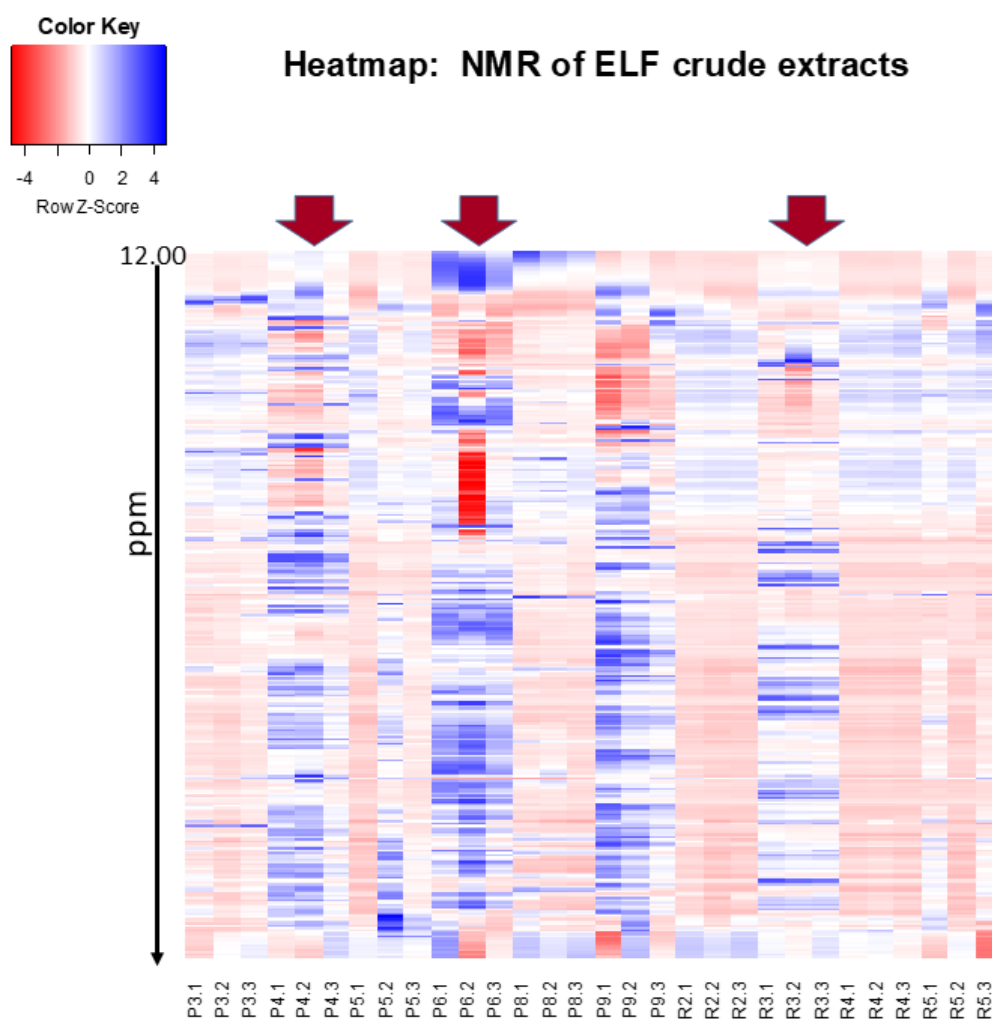


Figure 3.5. Heatmap of NMR spectral data of ELF crude extracts.
Brown arrows indicate bioactive extracts. Heatmap generated in R
(code available in S 2)

Principal Component Analysis (PCA) was first applied to the compiled NMR spectra to investigate similarities and differences of crude extracts in an unsupervised manner. The PCA scores plot was shown in Figure 3.6A. The scores plot have R^2 value of 0.907 and Q^2 of 0.752 indicating good linearity and predictability of the model. Overall, extraction replicates) were precise as they cluster together. **R3** and **P9** were considered outliers as some of their sample replicates were out of the ellipsoid. This indicates highly distinct NMR profile of the two ELF extract samples. **P4**, **P6**, and **P8** were also separated from the rest of the crude extracts clustering together at the zero-point of the axes. This means that the three extracts, **P4**, **P6**, and **P8** also produced some significantly different metabolites from that of the other extracts. The observed excellent bioactivity of P6, P4, and R3 may then be correlated with the discriminating features observed on this PCA. The loadings plot shown at Figure 3.6B shows the chemical shifts (1 to 3 ppm) which makes the distinction between the respective ELF crude extracts.

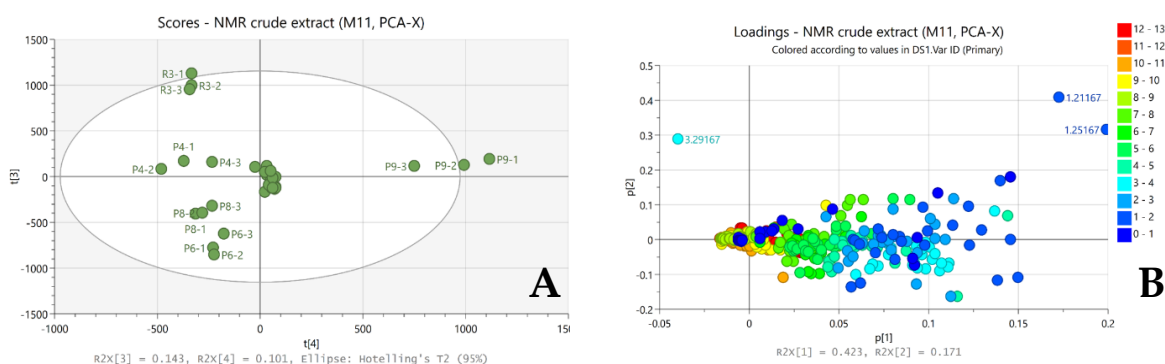


Figure 3.6. A) PCA scores, and B) loadings plots of the NMR spectral data of ELF crude extracts. Each dot in the scores plot represent one replicate (n=3). $R^2=0.91$, $Q^2=0.75$

Furthermore, OPLS-DA were applied to discriminate the bioactive ELF extracts (**P4**, **P6**, and **R3**) from the inactive ones. The corresponding scores plot was shown in Figure 3.7A. The model gave an R^2 of 0.982 and Q^2 of 0.956 which indicates excellent linearity and predictability. As seen from the Figure 3.7A, the bioactive and inactive extracts were clearly distinguished to the right and left, respectively. R^2X_o value at 28.7% is greater than R^2X at 24.5%, which implied that the variance within category is greater than between categories. Additionally, the S-plot (Figure 3.7B) specified that the discriminating chemical shifts for the bioactive extracts were between 1.0-2.0 and 4.01 ppm. In addition, there are more discriminating dots for the bioactive extracts in the S-plot which can mean higher diversity of the extracts' metabolic profile.

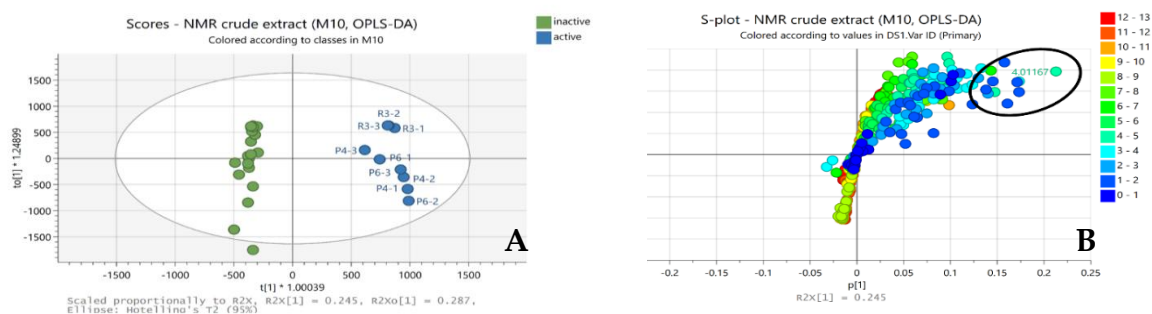


Figure 3.7. A) OPLS-DA scores, and B) S plot of NMR data of ELF crude extracts grouped according to bioactivity. Each dot in the scores plot represent one replicate (n=3). Encircled in black are discriminating chemical shifts for the bioactive extracts. $R^2=0.98$, $Q^2=0.96$

3.3.2 LCMS-based metabolomics

LC-MS based metabolomics were also applied to ELF crude extracts. Because of the high sensitivity of high-resolution mass spectrometry (HRMS), this technique can give a more comprehensive analysis compared to NMR-based metabolomics. To give an overview, an unsupervised PCA was carried out and gave a good fit at R^2 of 0.901 and Q^2 of 0.697. The PCA scores plot were shown in Figure 3.8A. **P4**, **P6**, **R3** and **P8** were differentiated from the rest of the crude extracts lying in the zero-axes region. This means that **P4**, **P6**, **R3** and **P8** have unique LC-MS profiles. **P6** and **P4** were outliers which implies a more distinct metabolic profile. The top discriminating compounds for **P4**, **P6**, and **R3** can be read out from the loadings plot (Figure 3.8B) through the given m/z values. The dereplication of these compounds can be found on Table 3.2.

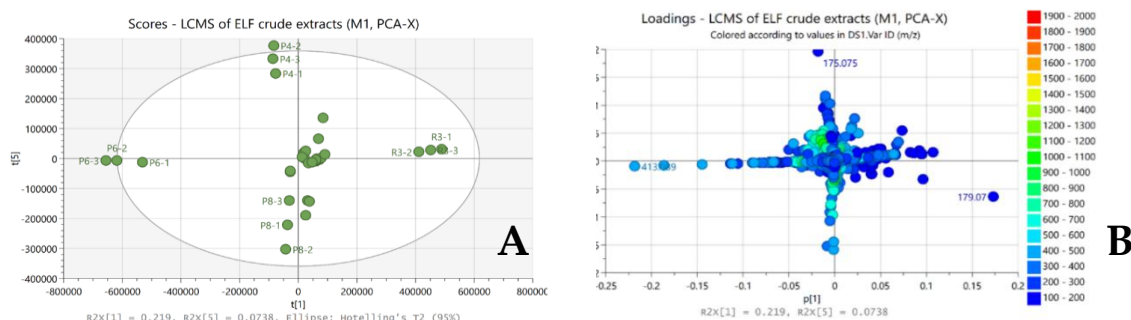


Figure 3.8. A) PCA scores, and B) loadings plot of LCMS data of ELF crude extracts. Each dot in the scores plot represent one replicate ($n=3$). $R^2=0.90$, $Q^2=0.70$

OPLS-DA plot was generated to investigate the similarities and differences of bioactive and inactive ELF extracts and is shown in Figure 3.9A. The model was found to have a good linearity ($R^2 = 0.989$) and predictability ($Q^2=0.973$). The variance between groups is greater than within groups as seen on their $R^2X[1]$ and $R^2Xo[1]$ values at 15.9% and 12.3%, respectively. This then can be further noticed as the bioactive ELF extracts were separated afar from the inactive extracts. Moreover, an S-plot was generated to pinpoint the metabolites that caused the observed bioactivity (encircled in red in Figure 3.9B). The m/z values were ranging from 100 to 500 Da.

To facilitate dereplication, the top 15 metabolites with the lowest p -values ($p < 0.05$) were derived from the S-plot of OPLS-DA model were determined. The statistically significant metabolites were dereplicated and the putative annotations were listed in Table 3.2 while structures were shown in Figure 3.10. In general, the discriminating metabolites belong to naphthalenes, benzopyranones, and isobenzofuranones, etc.

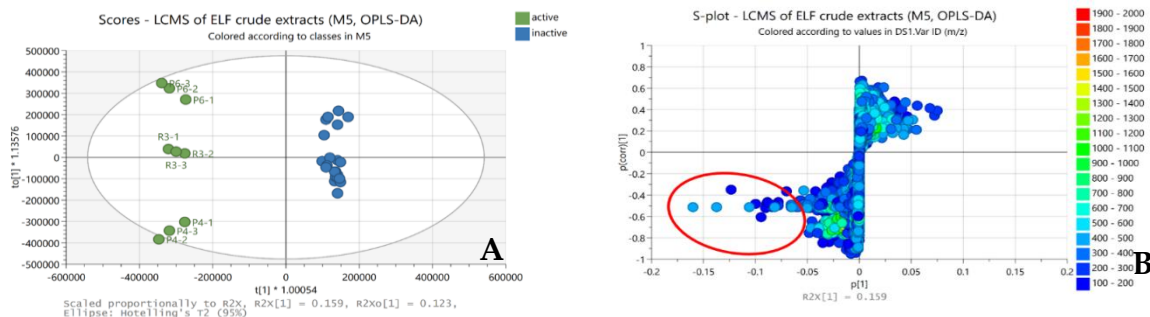


Figure 3.9. A) OPLS-DA scores, and B) S plot of LC-MS data of ELF crude extracts grouped according to bioactivity. Each dot in the scores plot represent one replicate (n=3). Encircled in red are discriminating metabolites of bioactive extracts. $R^2=0.99$, $Q^2=0.97$

Table 3.2 Dereplication of top 15 discriminating bioactive metabolites from 3 ELF strains based on S-plot. Arranged in increasing p-values ($p < 0.05$)

<i>p</i> -value	<i>m/z</i>	Molecular weight (g/mol)	Molecular formula	Dereplication hit	Biological Source
0.000366	179.0703	178.0630	C ₁₀ H ₁₀ O ₃	Mellein	<i>Aspergillus</i> spp., <i>Hypoxylon howieanum</i> , <i>Lasioidiplodia theobromae</i> , <i>Microsphaeropsis</i> sp. <i>Pezicula</i> sp., and other fungi. Also found in the pheromones of some insects. Constit. of <i>Ficus formosana</i> and <i>Garcinia bancana</i>
0.003693	419.2272	418.2199	No hit		
0.00377	175.0755	174.0682	C ₁₁ H ₁₀ O ₂	1,8-Naphthalenediol; Mono-Me ether	<i>Daldinia concentrica</i> , <i>Hypoxylon macrocarpum</i> and <i>Sporothrix</i> sp. (No. 4335)
0.003864	289.1641	288.1568	C ₁₄ H ₂₄ O ₆	Cladosporester C	<i>Cladosporium cladosporioides</i> OUCMDZ-187
0.00399	159.1017	158.0944	C ₈ H ₁₄ O ₃	6-Hydroxy-4-methyl-4-hexenoic acid; (E)-form, Me ester	<i>Penicillium minioluteum</i> ZZ1657
0.004101	193.0860	192.0787	C ₁₁ H ₁₂ O ₃	5-Methylmellein	<i>Aspergillus</i> sp., <i>Fusicoccum amygdali</i> , <i>Hypoxylon illitum</i> , <i>Hypoxylon</i> sp., <i>Lachnellula</i> sp., <i>Microsphaeropsis</i> sp., <i>Nummularia discreta</i> , <i>Pezicula livida</i> , <i>Phomopsis oblonga</i> , <i>Phomopsis oblonga</i> 118, <i>Phomopsis</i> sp. BCC 9789, <i>Pithomyces</i> sp., <i>Plectophomella</i> sp., <i>Valsa ceratosperma</i> , a mangrove

<i>p</i> -value	<i>m/z</i>	Molecular weight (g/mol)	Molecular formula	Dereplication hit	Biological Source
					fungus, and others. Constituent of <i>Semecarpus</i> spp.
0.004122	207.1014	206.0942	C ₁₂ H ₁₄ O ₃	8-Methoxy-5-methylmellein	<i>Hypoxylon investiens</i> BCRC 10F011
0.004225	195.0652	194.0579	C ₁₀ H ₁₀ O ₄	4-Hydroxymellein	<i>Aspergillus ochraceus</i> , <i>Cercospora beticola</i> , <i>Cercospora taiwanensis</i> , <i>Lasiodiplodia theobromae</i> and <i>Xylaria feejeensis</i>
0.00424	367.1765	368.1837	C ₁₉ H ₂₈ O ₇	Pestallic acid I	<i>Pestalotiopsis lespedezae</i> MT521347
0.004249	415.1026	414.0953	C ₂₁ H ₁₈ O ₉	TAN 1612	<i>Penicillium claviforme</i> FL-27337
0.004293	235.0608	236.0681	C ₁₂ H ₁₂ O ₅	Orthosporin	<i>Aspergillus ochraceus</i> , <i>Daldinia concentrica</i> , <i>Drechslera siccans</i> and <i>Rhynchosporium orthosporum</i> . Metab. of mangrove-derived <i>Botryosphaeria</i> sp. SCSIO KcF6 and <i>Daldinia eschscholzii</i>
0.004349	303.0499	302.0426	C ₁₅ H ₁₀ O ₇	Nodulisporone B	<i>Nodulisporium</i> sp. SH-1
0.004452	235.1691	234.1618	C ₁₅ H ₂₂ O ₂	Nigriterpene E	<i>Xylaria nigripes</i>
0.004462	347.1277	346.1204	C ₂₂ H ₁₈ O ₄	Daldinol	<i>Daldinia concentrica</i> , <i>Nodulisporium</i> sp. 7080, and Xylariaceous fungi
Compound dereplication are based on highly accurate mass matching (<5 ppm) against Dictionary of Natural Products, with filtering by fungal biological source. No tandem MS/MS data were acquired; therefore, these are tentative assignments that require confirmation through MS fragmentation data or isolation (cells highlighted in blue correspond to compounds that were subsequently isolated.).					

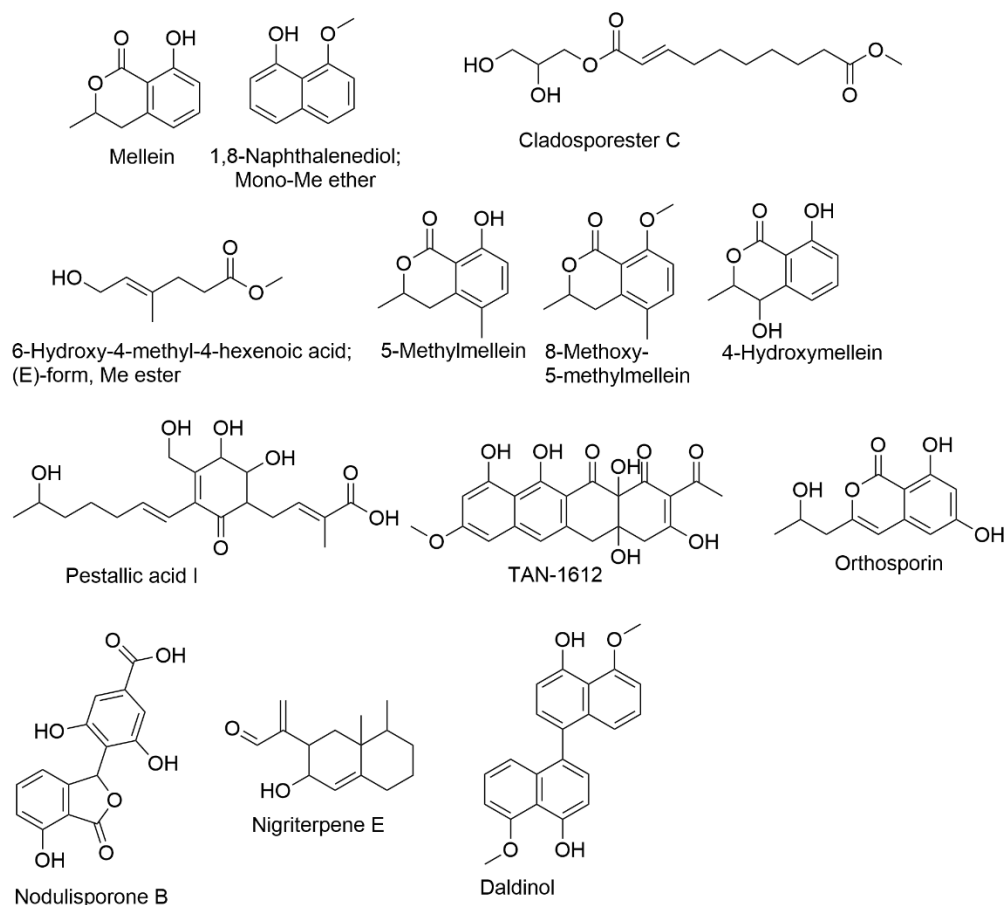


Figure 3.10. Chemical structure of dereplicated metabolites

A heat map, displayed in **Error! Reference source not found.**, was generated in MetaboAnalyst to show the relative abundances of the discriminatory metabolites. It also depicted in which sample extracts these metabolites are concentrated. Orthosporin, 1,8-naphthalenediol, and daldinol were from **P4**. Meanwhile, **R3** contains three mellein derivatives, pestallic acid I, and nigriterpene E. Most of the discriminatory metabolites came from **P6**, which were also structurally diverse. Hence, P6 is the most promising ELF in terms of metabolite diversity.

Moreover, as seen from the reported sources Table 3.2, the dereplicated compounds were commonly isolated from the genera *Daldinia*, *Hypoxylon*, and *Xylaria*. This piece of information can be used as support for the taxonomic identification done

for the selected ELF. Furthermore, the feature with the ion peak at m/z 419.2272 $[M+H]^+$ displayed no hits from the database, which could probably imply new compounds. Overall, these diagnostic metabolites can be targeted for isolation and purification works.

As a summary, because of the excellent biological activities and unique LC-MS and NMR metabolomic profile of P4, P6, and R3, they were prioritized as ELF for further studies including media optimization and isolation work to obtain antibiofilm compounds.

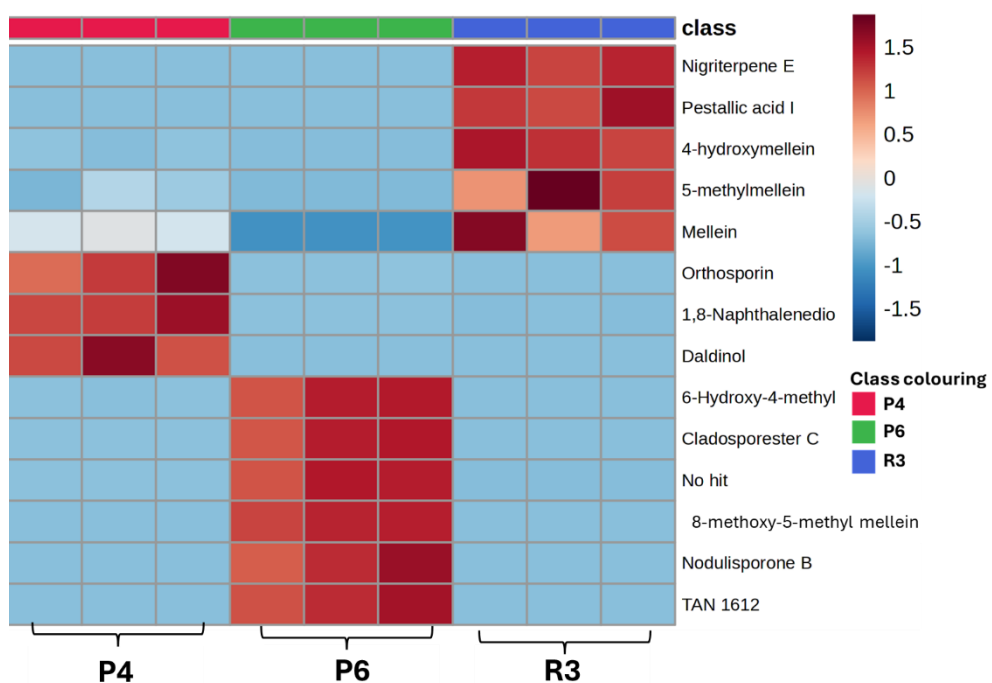


Figure 3.11 Heat map of top 15 discriminatory metabolites. Heatmap was generated in MetaboAnalyst v 6.0.

3.4 Taxonomic identity of prioritized ELF

The three selected ELF (**P4**, **P6**, and **R3**) were identified through molecular methods using their ITS genes. The obtained DNA sequences were aligned and sent for

BLAST searching. The hit with 100% identity was chosen as the identity of each ELF (Table 3.3). These identities will be used from now on for the respective ELF.

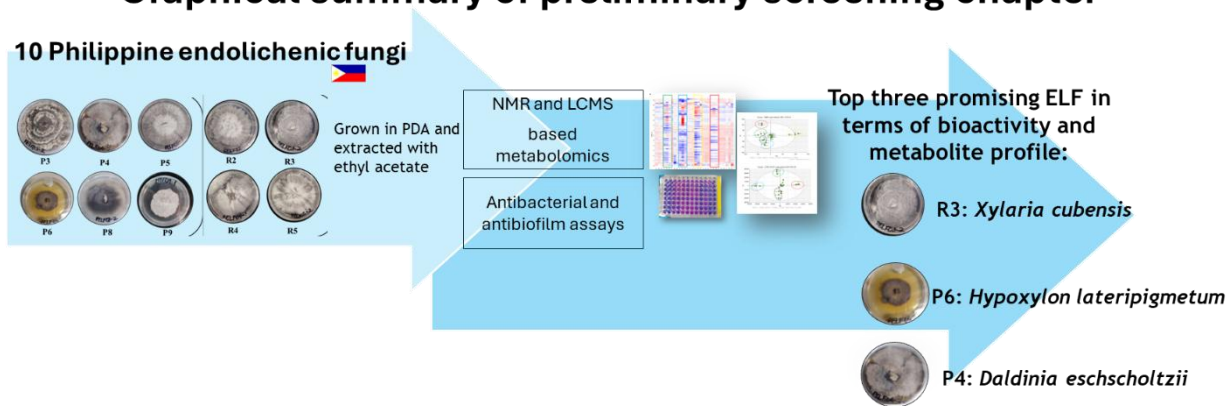
Table 3.3. BLAST search output for the aligned DNA sequences of three selected ELF

ELF Code	Aligned DNA sequence	BLAST search hit	% identity
P4	GGGATCATTACTGAGTTATCTAAACTCCAACCCTATG TGAACCTACCGCCGTTGCCTCGGCGGGCCGCGTTTCG CCCTGTAGTTTACTACCTGGCGGCGCGCTACAGGCC GCCGGTGGACTGCTAAACTCTGTTATATATACGTATCT CTGAATGCTTCAACTTAATAAGTTAAACTTTCAACA ACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCA GCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA GTGAATCATCGAATCTTTGAACGCACATTGCGCCCAT TAGTATTCTAGTGGGCATGCCTGTTGAGCGTCATTTTC AACCCTTAAGCCCCTGTTGCTTAGCGTTGGGAATCTA GGTCTCCAGGGCCTAGTTCCCCAAAGTCATCGGCGG AGTCGGAGCGTACTCTCAGCGTAGTAATACCATTCTC GCTTTTGACAGTAGCCCCGGCGGCTTGCCGTAAACC CCTATATCTTTAGTGGTTGACCTCGAATCAGGTAGGA ATAC	<i>Daldinia eschscholtzii</i>	100.00
P6	TGAACCTTACCGTCGTTGCCTCGGCGTGAGCTGCGG CTACCCGGTAGCTACCCTGTAGCTACCCTGTAGCCGG TTCACGGCCCCGCCGAAGGACAGCTAAACTCTTGTTA ATTACCACTGTATCTCTGAATCGTTAACTAAATACGTT AAAACCTTTCAACAACGGATCTCTTGTTCTGGCATCG ATGAAGAACGCAGCGAAATGCGATAAGTAATGTGA ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGC ACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTATT CGAGCGTCATTTCAACCCTTAAGCCAATGCTGCTTAG TGTTGGGAGCATAACCCTCCTCGGGGGGTATCTCCTTA AAGTTAGTGGCGGAGTTAGGGTACACTCTCAGCGTA GTAATTTCTCTCGCTCGGGTAG	<i>Hypoxylon lateripigmentum</i>	100.00
R3	AACTCCCAAACCCATGTGAACATACCCATTGTTGCCT CGGCAGGTGCGACGCCTACCTTGTAAGTCCCTACCCTG TAGGGCCTGCCCCGGGAGACGCGCGGTAAGCCCCGCC GGCGGCCCCACCAAACTCTGTTTGATATTAAATTCTGA ACTTGTAATAAATAAGTTAAACTTTCAACAACGG ATCTCTTGTTCTGGCATCGATGAAGAACGCAGCGA AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGA ATCATCGAATCTTTGAACGCACATTGCGCCCATAGC ATTCTAGTGGGAGGACCGTTGAGCGTCATTTGAC CCTTAAGCCTTGTTGCTTAGTGTTAGGAGCCTACAG CAATGTAGCTCCTCAAAGTTAGTGGCGGAGTCGGCT CACACTCTAGACGTAGTAATTTTATCTCGCCTATCAG TTAGACCCGATCCCCTGCCGTAAACCCCCCAACT TCTC	<i>Xylaria cubensis</i>	94.80

3.5 Summary

This chapter is fundamentally the start of this PhD project. From the ten ELF brought from the Philippines, three stood out in terms of their prominent chemical diversity as noted by the metabolomics-based profiling using LC-MS and NMR. These ELF were **P4**, **P6**, and **R3**. The same fungi exhibited moderate to strong antibiofilm activities against (60-95% inhibition). Metabolomics tools were able to pinpoint the compounds present within the bioactive fungal extracts. These discriminatory metabolites were dereplicated for annotation and belongs to several natural product class such as naphthalenes, benzopyranones, and isobenzofuranones, etc. Lastly, the three fungi were identified as *Daldinia eschscholtzii*, *Hypoxylon lateripigmentum*, and *Xylaria cubensis* using molecular methods. These three fungi were prioritised for further experiments and investigation as presented in the next following chapters for each of the respective ELF.

Graphical summary of preliminary screening chapter



Chapter 4. *Xylaria cubensis*

4.1 Chapter overview

This chapter focuses on the chemical and antibiofilm activities of the R3 ELF or *X. cubensis*. One Strain Many Compounds (OSMAC) strategy was employed to find the best culture media to maximize the potential of the fungus to produce antibiofilm compounds. Afterwards, isolation and purification work of bioactive metabolites were done. The final section describes the identification of isolated compounds followed by their antibiofilm activities.

4.2 Media optimisation studies of *Xylaria cubensis*

X. cubensis (Mont.) Fr. being a slow growing fungus was cultivated for 15 and 30 days in four different media (oat, rice, potato dextrose broth, and malt extract broth). Ethyl acetate was then used to extract the metabolites, and the yield is shown in Figure

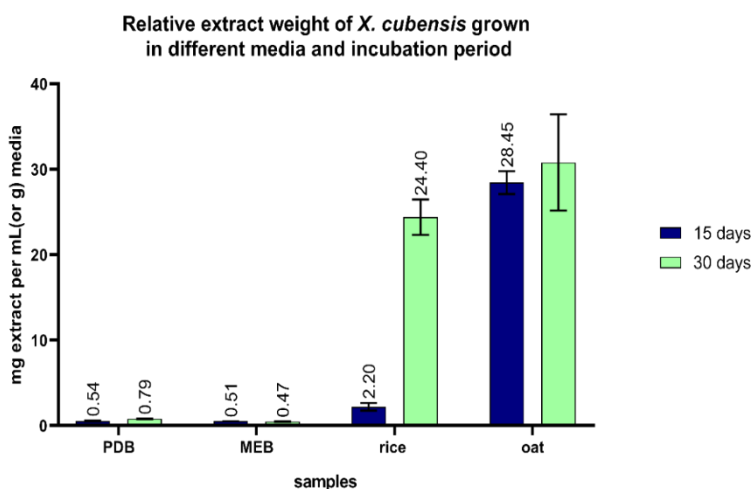


Figure 4.1. Extraction Yield of *X. cubensis* in different media

4.1. Solid media gave higher yields in contrast with the liquid media extracts. The highest yield was afforded by the 30-day oat extract.

4.2.1 Biological assay results

X. cubensis crude extracts were tested for their antibiofilm activities as displayed in Table 4.1. The bioassay results from the preliminary screening assay of the extract obtained from the PDA (Potato Dextrose Agar) media was also included in Table 4.1 to compare the assay results of fungal extracts afforded from the other media used for metabolite production optimisation. There was potency in antibacterial activity in the rice culture fungal extracts while the activity was regarded as excellent in the extract from the 30-day culture. Prebiofilm activity notably increased for all extracts obtained from the various media and were exhibiting more than 50% inhibition. The highest prebiofilm inhibition was observed from the 30-day rice culture extract. On one hand, compared to PDA, there is a decrease in postbiofilm activity in extracts afforded by PDB (Potato Dextrose Broth) culture media while the extracts from other culture media have similar inhibition with no significant statistical difference. Overall, the extracts from rice and MEB cultures incubated for 15 and 30 days were considered as the most active because they afforded strong prebiofilm and postbiofilm inhibition activities. The most remarkable extract would be from the 30-day rice culture as it exhibited strong antibacterial activity as well.

Table 4.1. Biological activities of *X. cubensis* grown in different media.

ANTIBACTERIAL ACTIVITY, average % inhibition (n=3)					
Media	MRSA	<i>P. aeruginosa</i>	Media	MRSA	<i>P. aeruginosa</i>
PDA	9.71	5.98			
PDB blank	-7.38	-13.43	Rice Blank	18.11	3.41
15day-PDB	18.79	-1.70	15day-Rice	45.86	-8.66
30day-PDB	3.32	-8.39	30day-Rice	72.45	16.95
MEB blank	13.04	4.22	Oat Blank	-3.72	4.83
15day-MEB	11.79	2.97	15day-Oat	13.05	8.60
30day-MEB	-1.26	7.23	30day-Oat	9.68	7.59
DMSO	26.11	9.70	Ciprofloxacin (50 µM)	98.60	99.76
PREBIOFILM ACTIVITY, average %inhibition (n=3)					
Media	MRSA	<i>P. aeruginosa</i>	Media	MRSA	<i>P. aeruginosa</i>
PDA	48.42	46.26			
PDB blank	-15.36	-7.95	Rice Blank	0.16	10.07
15day-PDB	84.31	84.99	15day-Rice	72.47	86.57
30day-PDB	61.57	65.06	30day-Rice	97.88	83.53
MEB blank	-13.56	-75.20	Oat Blank	27.30	45.79
15day-MEB	71.08	75.37	15day-Oat	67.40	85.97
30day-MEB	73.67	78.01	30day-Oat	64.15	74.61
DMSO	-11.56	-74.71	Ciprofloxacin (50 µM)	99.80	94.16
POSTBIOFILM ACTIVITY, average %inhibition (n=3)					
Media	MRSA		Media	MRSA	
PDA	87.65				
PDB blank	2.66		Rice Blank	41.62	
15day-PDB	64.13		15day-Rice	90.99	
30day-PDB	68.52		30day-Rice	89.62	
MEB blank	14.07		Oat Blank	-6.17	
15day-MEB	94.17		15day-Oat	89.35	
30day-MEB	94.33		30day-Oat	91.05	
DMSO	-2.97		Ciprofloxacin (50 µM)	94.79	
Testing concentration=100 µg/mL; assays were done twice with three replicates (n=3); Moderate inhibition: 50-70% (coloured in blue); Strong inhibition >70% (colorued in red); Media: PDB-Potato dextrose broth, MEB- Malt extract broth, PDA -potato dextrose agar (media used in the screening)					

4.2.2 Metabolomics profiling of *X. cubensis* extracts

4.2.2.1 NMR metabolomics

X. cubensis extracts were subjected to ^1H -NMR profiling. As shown in Figure 4.2, extracts from oat media are rich in fatty acids as broad signals in the methylene region are obvious (boxed in black). Hence, though oat media are giving high extract yield, it contains mostly of lipids. Distinctive aromatic protons (boxed in orange in Figure 4.2) can be seen on extracts from 15 and 30-day PDB and in 30-day rice cultures. Thus, aromatic compounds were produced in after 30 days of incubation in rice media. Fungal extracts obtained from the rice media were also rich in unsaturated fatty acids as presented by olefinic protons observed around 5.5 to 6.0 ppm (boxed in red in Figure 4.2)

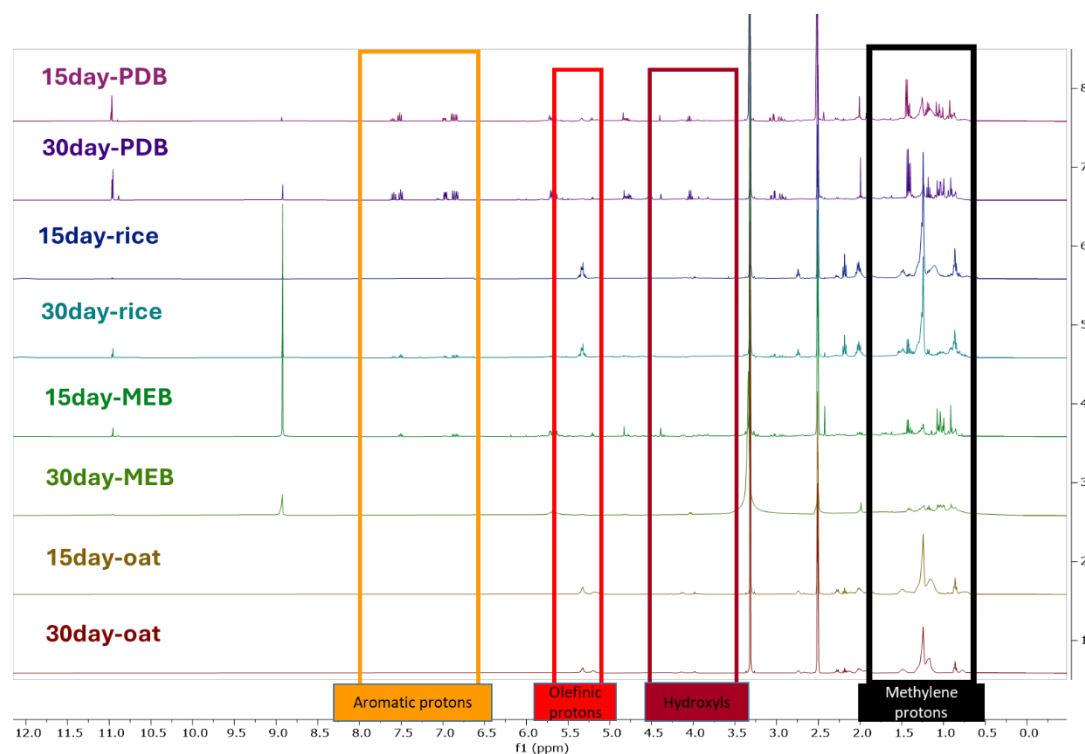


Figure 4.2. Stacked ^1H -NMR spectra of *X. cubensis* extracts grown in different media. PDB-potato dextrose broth. MEB-malt extract broth. ^1H -NMR recorded at 400 MHz in DMSO-d_6

Multivariate analysis was employed for further analysis of their NMR profile. The supervised model, PLS-DA, were utilized. In this model, the media type was used to classify the culture extracts into groups. The model gave excellent reliability based on the R²_Y and Q² values. As seen on the scores plot (Figure 4.3A), the extracts from the solid media (oat and rice), can be found on the lower right hand while extracts from the liquid cultures are clustering in the upper right hand of the plot. This implies differences in chemical profiles of extracts obtained between the solid versus liquid media. One replicate of an extract from the 30-day malt culture was an outlier as it was shown outside the sphere area. Moreover, extracts from 15-day rice cultures were differentiated indicating their unique chemical profiles. In the corresponding loadings plot in Figure 4.3B, extracts of the 15-day rice cultures contained resonances for methylene protons (1-2 ppm) and aromatic protons (6-7 ppm). On the other hand, extracts from 30-day MEB cultures indicated two distinct peaks at 8.89 ppm which could be assigned to an exchangeable proton while the resonance at 5.65 ppm could be either an olefinic or an aromatic proton *ortho* to an -OH substituent.

Next, OPLS-DA model was applied to differentiate the active and inactive fungal extracts. The active samples are placed on the left-hand side of the OPLS-DA scores plot (Figure 4.3C). The discriminatory chemical shifts for the active samples were indicated in the S-plot (Figure 4.3D). The chemical shifts were 8.89 ppm and 5.65 ppm which were also marked in the previous PLS-DA model.

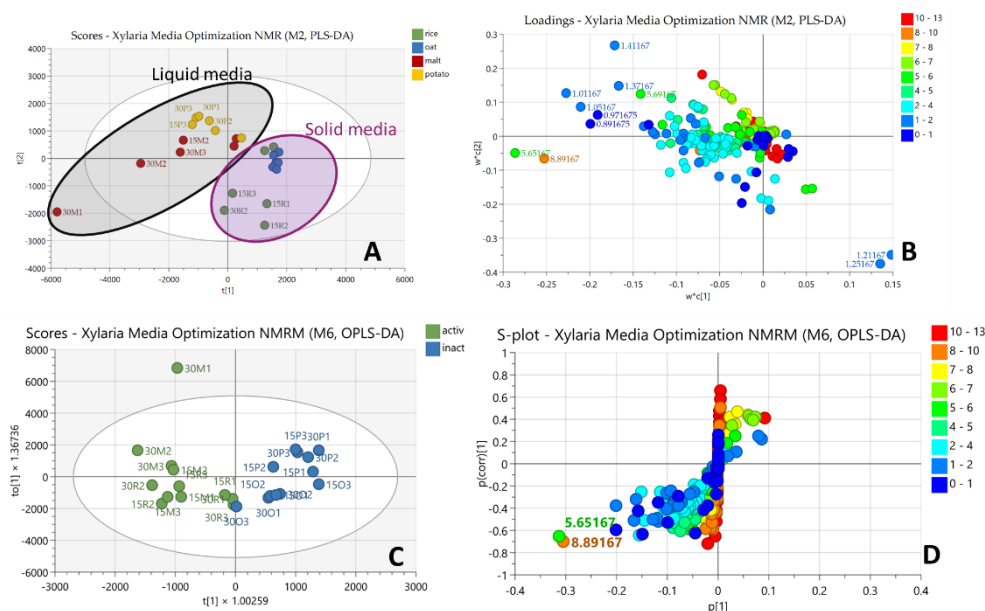


Figure 4.3. ^1H -NMR based multivariate analysis of *X. cubensis* grown in different media. A) PLS-DA Scores plot, B) PLS-DA loadings plot, C) OPLS-DA scores plot, D) OPLS-DA S-plot. Encircled in black in the PLS-DA scores plot are extracts from liquid media while encircled in purple belong to solid media. Each dot in the scores plot represent one replicate ($n=3$). PLS-DA $R^2=0.80$ $Q^2=0.66$; OPLS-DA $R^2=0.542$, $Q^2=0.205$

4.2.2.2 LCMS-based metabolomics

The more sensitive and detailed LC-HRMS based-metabolomics approach was also utilized to analyse the *X. cubensis* extracts obtained from four different culture media of two incubation periods. The PCA scores plot and its corresponding loadings plot were displayed in Figure 4.4A and B, respectively. The results were parallel to the PCA scores plot based on NMR spectral data, the extracts from the solid and liquid media were well-differentiated. Extracts from solid media (rice and oat) can be found on the left-hand side of the scores plot (Figure 4.4A) while extracts from liquid cultures were located on the right-hand side. It can also be noticed that the extracts from PDB and MEB were on the upper and lower sides respectively. The culture extracts from rice media were clustering out from the rest which implicate uniqueness of their chemical profile. Based on the loadings plot (Figure 4.4B), features with molecular weights less than 380 Da can be

found on extracts from liquid cultures while higher molecular weight compounds were afforded by fungal extracts obtained from rice media.

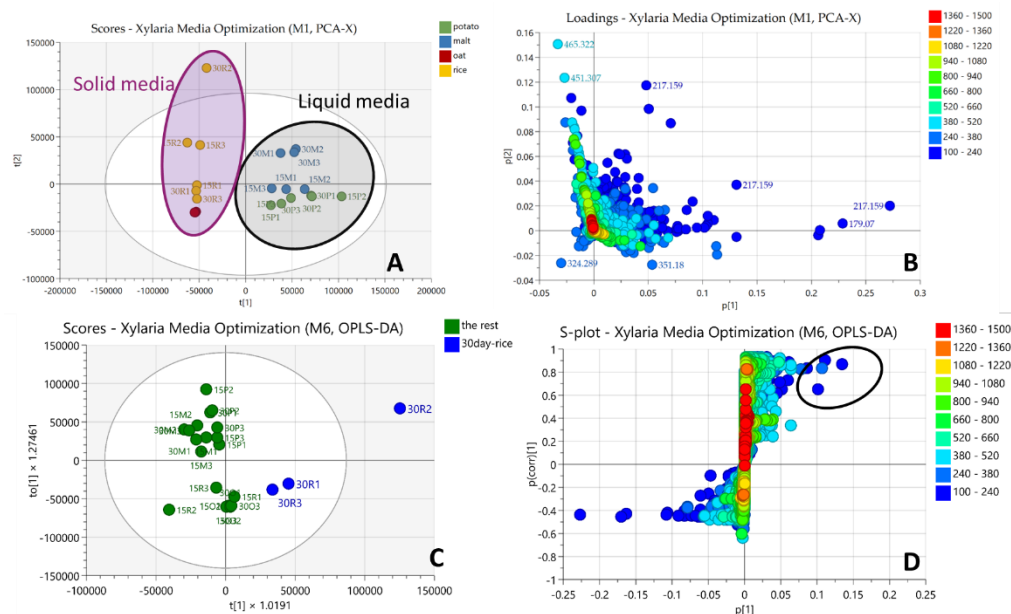


Figure 4.4. LCMS-based Multivariate Analysis of *X. cubensis* grown in different media. A) PCA Scores plot, B) PCA loading plot, C) OPLS-DA Scores plot, D) OPLS-DA S-plot. Encircled in black in the scores plot are extracts from liquid media while encircled in purple belong to solid media. Each dot in the scores plot represent one replicate (n=3). PCA $R^2=0.92$, $Q^2=0.79$. OPLS-DA $R^2=0.66$, $Q^2=0.33$

To determine the active compounds of the most bioactive extract obtained from the 30-day rice culture, OPLS-DA model was applied. The generated scores plot, and S-plot were shown in Figure 4.4C and D. The said extract can be found on the right-hand side of the scores plot. Some variability was observed amongst the active extracts as the replicates were quite distant from one another. The discriminating features were encircled in black in the S-plot (Figure 4.4D). These features were then dereplicated from the Dictionary of Natural Products and the hits were presented in Table 4.2

Testing concentration=100 $\mu\text{g/mL}$; assays were done twice with three replicates (n=3); Moderate inhibition: 50-70% (coloured in blue); Strong inhibition >70% (coloured in red)

Aromatic compounds including 1,8-naphthalenediol, mellein, and a glycosylated mellein were putatively identified. Aromatic proton signals from the ^1H -NMR profiles (Figure 4.2) can then be attributed to these compounds. Moreover, a murolic acid derivative was also pinpointed as a discriminatory metabolite. Congeners of murolic acid have been previously reported from several lichens (Řezanka & Guschina, 2000, 2001). Since this study involves extracts from endolichenic fungi, the latter compound could be indicative of its biosynthesis by the fungal species living inside lichen thallus. Yet, it is important to note that these are tentative dereplication. It is imperative to isolate and identify this murolic acid to prove and support the hypothesis. Finally, these pinpointed discriminatory metabolites should be targeted for fractionation to arrive at pure compounds that were predicted to have excellent biological activities.

Table 4.2. Dereplication of discriminatory metabolites of the bioactive *X. cubensis* extract grown in rice for 30 days. Arranged in increasing p-values ($p < 0.05$)

<i>p</i> -value	<i>m/z</i>	Molecular weight (g/mol)	Molecular formula	Dereplication hit	Biological Source
3.72E-06	443.1203	444.1276	C ₁₆ H ₂₄ O ₇	Xylaroside A	<i>Xylaria</i> sp. PSU-D14
1.50E-05	531.3180	532.3253	C ₂₇ H ₄₈ O ₁₀	Murolic acid-4S,20-dihydro, β -D-glucopyranosyl ester	Constituent of various lichens
4.00E-05	133.0648	132.0575	No hit 1		
5.49E-05	179.0701	178.0628	C ₁₀ H ₁₀ O ₃	Mellein or Isosclerone or Isoochracein	<i>Aspergillus</i> spp., <i>Hypoxylon howieanum</i> , <i>Microsphaeropsis</i> sp., <i>Lasiodiplodia theobromae</i> , <i>Pezicula</i> sp., and other fungi. <i>Aspergillus fumigatus</i> , <i>Cladosporium cladosporioides</i> HDN14-342, <i>Microsphaeropsis</i> sp., <i>Penicillium citrinum</i> , <i>Phaeoacremonium aleophilum</i> , <i>Scytalidium</i> sp., <i>Sclerotinia sclerotiorum</i> , <i>Verticillium dahliae</i> , and the marine-derived fungi. Constituents of the fruit of <i>Juglans mandshurica</i> var. <i>sieboldiana</i> . <i>Daldinia concentrica</i> S 0318, <i>Hypoxylon howieanum</i> strain 525, other <i>Hypoxylon</i> spp. and <i>Virgaria boninensis</i> FKI-6621
6.17E-05	483.2970	484.3040	C ₂₇ H ₄₀ N ₄ O ₄	Ramosine A	Alkaloid from the roots of <i>Paliurus ramosissimus</i>
6.59E-05	355.1035	356.1108	C ₁₆ H ₂₀ O ₉	5-O- α -D-glucopyranosyl-mellein	<i>Aspergillus</i> sp. CMM

<i>p</i> -value	<i>m/z</i>	Molecular weight (g/mol)	Molecular formula	Dereplication hit	Biological Source
9.13E-05	161.0597	160.0524	C ₁₀ H ₈ O ₂	1,8-Naphthalenediol	Found in ascomycete fungi
0.000101	513.3075	514.3148	C ₂₈ H ₄₂ N ₄ O ₅	Microsporin B	<i>Microsporum cf. gypseum</i>
0.00023	229.1078	230.1151	C ₁₁ H ₁₈ O ₅	Hydroxypestalotin	<i>Penicillium spp.</i> , <i>Pestalotiopsis oenotherae</i> and <i>Pestalotia sp.</i>
0.000246	255.1225	254.1152	C ₁₃ H ₁₈ O ₅	Daldiniapyrone	<i>Daldinia concentrica</i>
0.000248	271.1192	272.1264	C ₁₃ H ₂₀ O ₆	Pestalopyrone A	<i>Aspergillus niger</i> FKI-2342
0.000295	227.0924	228.0997	C ₁₁ H ₁₆ O ₅	Xylapyrone D	<i>Xylaria sp.</i> BM9
0.000401	401.1094	402.1166	C ₁₇ H ₂₂ O ₁₁	Apodanthoside	<i>Feretia apodanthera</i>
0.000929	243.0877	244.0950	No hit 2		
0.000941	507.3330	508.3403	No hit 3		
Compound dereplication are based on highly accurate mass matching (<5 ppm) against Dictionary of Natural Products, with filtering by fungal biological source. No tandem MS/MS data were acquired; therefore, these are tentative assignments that require confirmation through MS fragmentation data or isolation (cells highlighted in blue correspond to compounds that were subsequently isolated)					

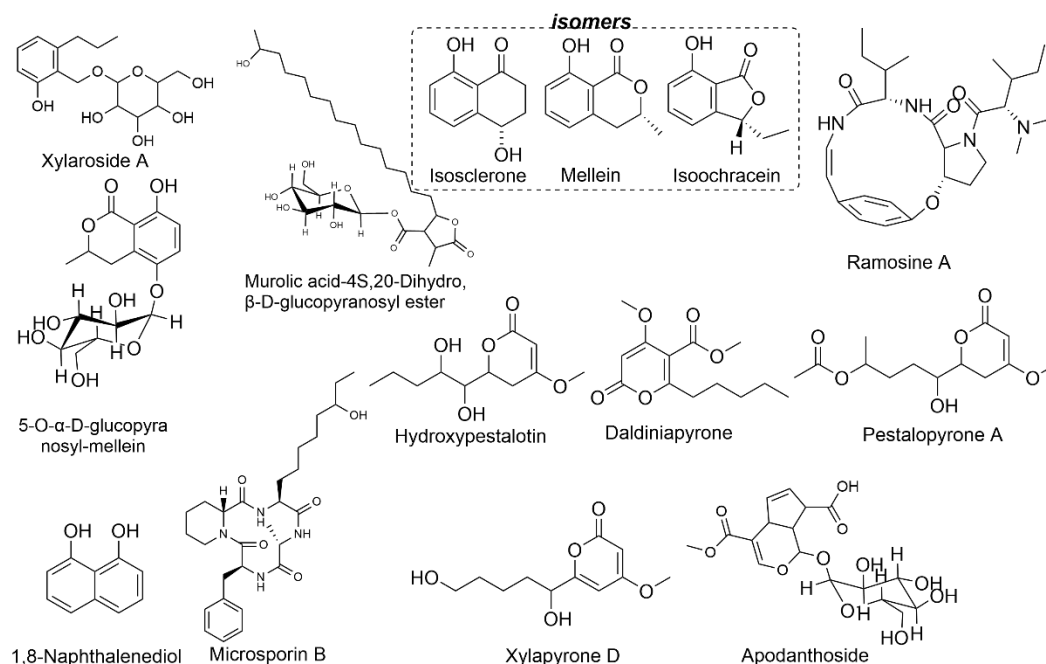


Figure 4.5. Chemical structure of dereplicated discriminatory metabolites from the bioactive extract *X. cubensis* grown in rice media for 30 days

The relative abundance of the discriminatory metabolites was depicted using a heatmap as shown in Figure 4.6. Clearly, these defining features were found almost exclusively in fungal extracts obtained from the 30-day rice culture. Though some compounds (marked with a star) were found to a significantly lesser extent in extracts from the 15-day rice culture. This suggested that these discriminatory compounds build up over time.

As a summary, the 30-day rice culture was the optimum media and incubation period for *Xylaria* to produce its bioactive and distinct chemical profile. The bioassay and metabolomics profiling data results all point out to this evidential hypothesis. Hence, this fermentation conditions were applied in the scaled-up cultivation of *X. cubensis* as described in section 4.3.

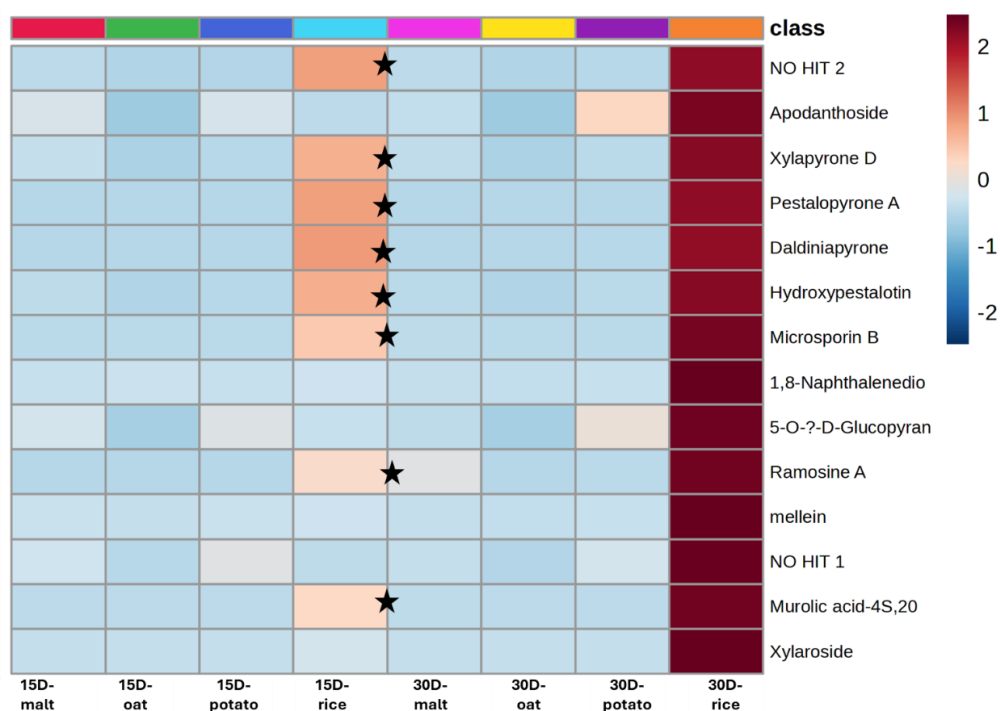


Figure 4.6 Heatmap of top discriminatory metabolites from the bioactive extract *X. cubensis* grown in rice for 30 days. Marked with star are features that are also present in extracts obtained from 15-days rice cultures. Heatmap was generated in MetaboAnalyst v 6.0.

4.3 Scaled up production and solvent partitioning of bioactive metabolites

X. cubensis was scaled up in rice media for 30 days as it afforded the most bioactive extracts while exhibiting a distinct metabolomic profile. *X. cubensis* was then inoculated in twenty (20) 500 mL-flasks containing 100 g rice per flask and incubated for 30 days at 27 °C. After cultivation, it was extracted with ethyl acetate to yield a total crude extract of 17.4 g.

To wash out lipids, the extract was then redissolved with 200 mL of 90:10 methanol/water mixture and partitioned over 200 mL hexane thrice. The polar and non-polar layers were coded as XM and XH, respectively. As seen in Figure 4.7, the olefinic

and methylene protons of the crude extract went to XH while only a considerable amount remained on XM. The aromatic protons, on the other hand, were found enriched in XM. Further chromatographic purification was done on the bioactive fraction XM. The schematic diagram for the purification work was summarized in Figure 4.8.

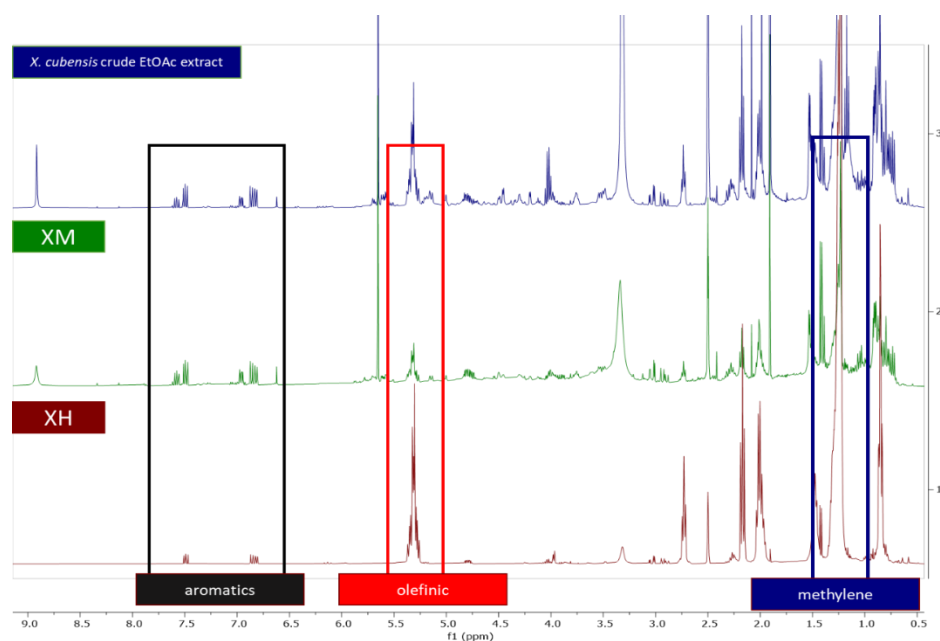


Figure 4.7. Stacked ^1H -NMR spectrum of *X. cubensis* crude extract, XM, and XH. Boxed in blue represents the long chain methylene protons of lipids, red box for olefinic protons, and black box for aromatic protons

4.4 Fractionation

4.4.1 Fractionation results

The Buchi flash system was used to fractionate XM (10 g) over a silica cartridge by linear gradient elution with hexane and ethyl acetate (Table 4.3). The collection volume for each fraction was 50 mL resulting to a total of 80 flasks. Each flask was monitored on TLC to obtain 11 pooled fractions with weights displayed in the schematic diagram in Figure 4.8. TLC summary plates were shown in Figure 4.9. The fractions were coded XM1 to XM11.

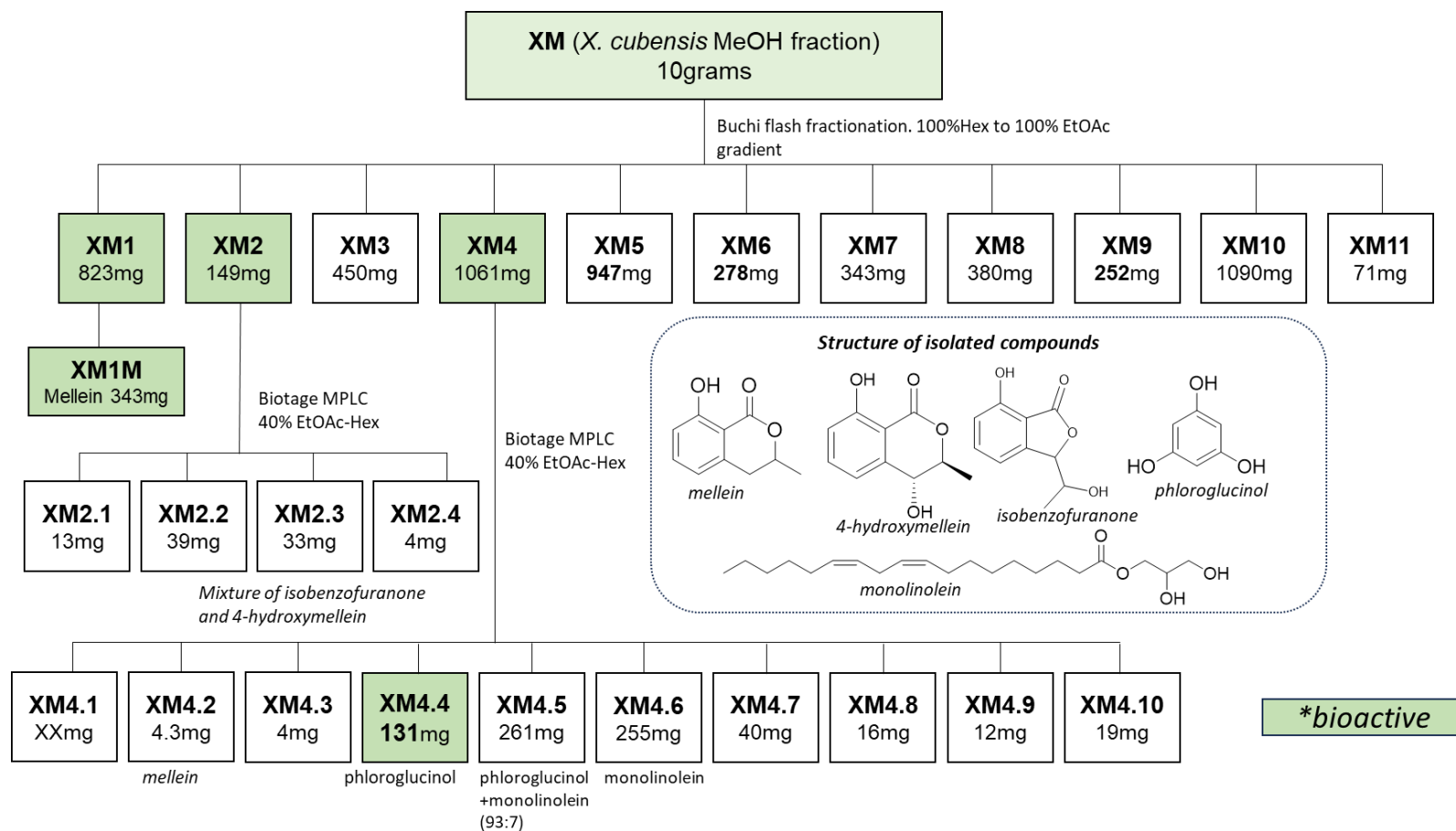


Figure 4.8 Schematic diagram for the isolation works done with *X. cubensis* grown in rice for 30days.

Table 4.3. Parameters for XM fractionation

Stationary phase: VersaPak Silica Cartridge, 40 x 150mm (Sigma)	
Time (min)	Solvent
0-5	100% Hexane
5-60	100%hexane to 100% EtOAc (linear gradient)
60-75	100% EtOAc
75-80	80% EtOAc in methanol (isocratic)
80-85	70% EtOAc in methanol (isocratic)
<i>Flow rate: 100ml/min; collection vol: 50mL</i>	

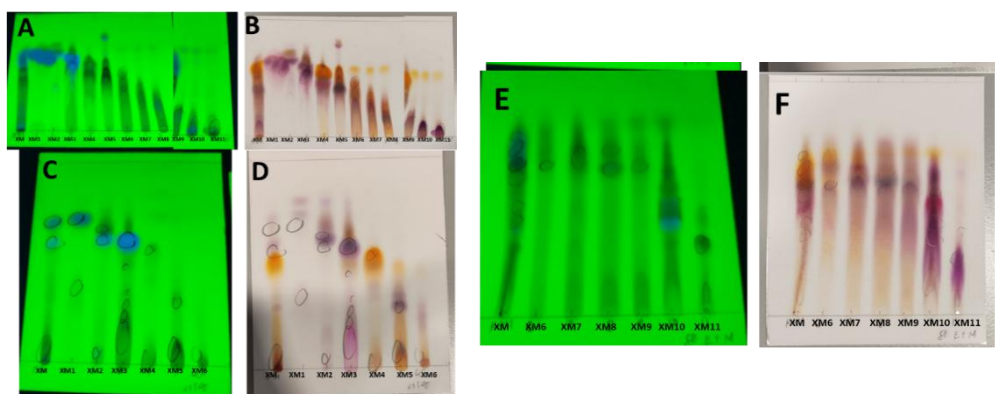


Figure 4.9. TLC summary plate of XM fractions in normal phase silica gel. A and B) Including all fractions XM1-11 using solvent system: 100% EtOAc, C and D) less polar fractions only, XM1-6, using solvent system: 20% hexane/EtoAc, E and F) more polar fractions, XM6-11, using solvent system 80% EtOAc/MeOH. A, C, E) Viewed in UV254nm; C and F) sprayed with anisaldehyde.

4.4.2 Biological assay results

The fractions were screened for biological activities (Table 4.4). Generally, the strong activity of the crude extract was relatively weakened or lost after fractionation. This implied that the compounds in the crude extract were synergistically more effective. The antibacterial activity of the crude extract was lost after the fractionation. Only a substantial inhibition of 50% was exhibited by the first fraction, XM1. All fractions also exhibited weak prebiofilm inhibitory activity except for XM4 with moderate inhibition.

Comparing their postbiofilm activities, XM1 was most active followed by XM2 and XM4 with moderate activity.

Table 4.4 Biological activities of XM fractions

ANTIBACTERIAL ACTIVITY, average % inhibition (n=3)					
Sample	MRSA	<i>P. aeruginosa</i>	Sample	MRSA	<i>P. aeruginosa</i>
Crude XM	72.45	16.96	XM7	15.59	-288.48
XM1	49.75	-280.36	XM8	11.25	-296.19
XM2	13.88	-290.49	XM9	6.24	-319.67
XM3	12.61	-302.18	XM10	-9.41	-428.47
XM4	9.75	-315.13	XM11	8.93	-286.17
XM5	16.63	-285.55	DMSO	6.36	-272.98
XM6	18.64	-287.07	Ciprofloxacin (50 μ M)	84.19	99.06
PREBIOFILM ACTIVITY, average % inhibition (n=3)					
Sample	MRSA	<i>P. aeruginosa</i>	Sample	MRSA	<i>P. aeruginosa</i>
Crude XM	97.88	16.96	XM7	24.67	6.10
XM1	11.90	-1.60	XM8	46.36	23.46
XM2	40.56	19.90	XM9	36.45	55.58
XM3	36.63	-35.87	XM10	49.28	32.07
XM4	54.55	59.98	XM11	19.83	32.77
XM5	45.13	-15.69	DMSO	14.78	-15.89
XM6	45.99	-40.52	Ciprofloxacin (50 μ M)	97.69	96.52
POSTBIOFILM ACTIVITY, average % inhibition (n=3)					
Sample	MRSA		Sample	MRSA	
Crude XM	89.62		XM7	26.18	
XM1	72.79		XM8	5.11	
XM2	55.13		XM9	4.78	
XM3	29.18		XM10	15.57	
XM4	66.45		XM11	20.20	
XM5	11.64		DMSO	-7.28	
XM6	28.54		Ciprofloxacin (50 μ M)	94.79	

Testing concentration=100 μ g/mL; assays were done twice with three replicates (n=3) ; Moderate inhibition: 50-70% (coloured in blue); Strong inhibition >70% (coloured in red)

4.4.3 NMR-based metabolomics

The stacked ^1H -NMR spectra of the 11 fractions are shown in **Error! Reference source not found.** Aromatic protons can be seen on the non-polar fractions (XM1 to 3) and polar fractions (XM8-11). The spectra of XM1, a bioactive fraction, seems to be simpler or less complex than the other fractions, which may indicate its higher purity. The same can be observed for XM4, which have two very striking singlet peaks at 8.89 and 5.65 ppm.

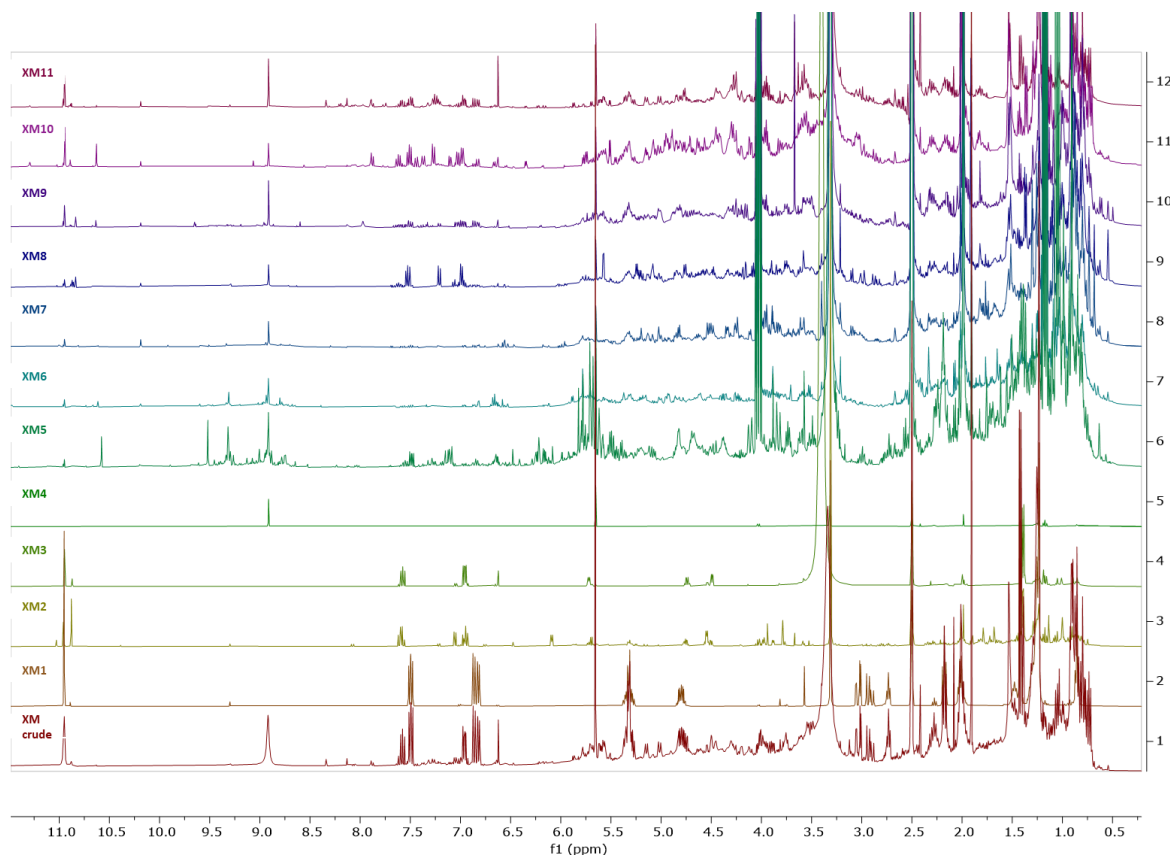


Figure 4.10. Stacked ^1H -NMR spectrum of XM fractions in ascending order. ^1H -NMR recorded at 400MHz in DMSO- d_6

Metabolomics tools were applied to the NMR spectral data of the fractions. As exhibited by the PCA scores plot (Figure 4.11A), there were no distinct grouping pattern. However, XM1 to 4 were closest together. XM10 seems to be very similar to the crude extract (XM). XM5, an inactive fraction, were far away from the rest of the fractions which

means a more unique chemical profile albeit its inactivity. Looking at the loadings plot, it must be the methylene signals between 1-2 ppm that could be responsible for the uniqueness of XM5.

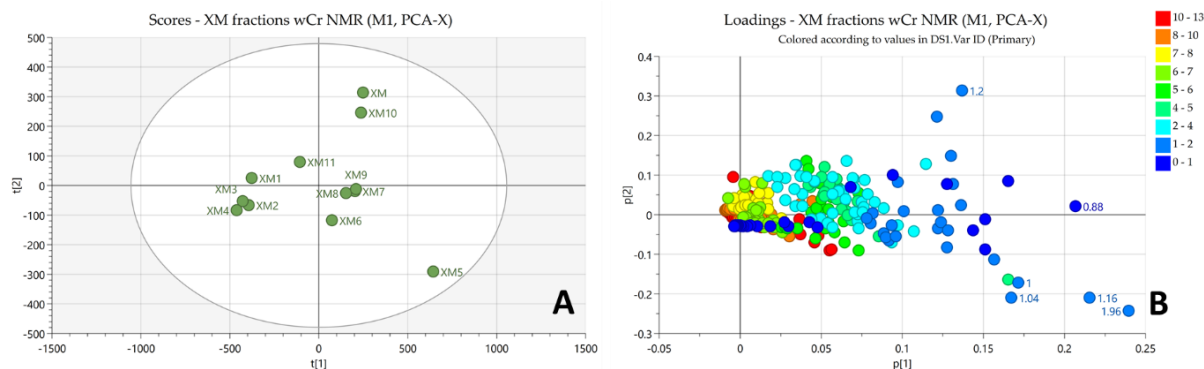


Figure 4.11 ^1H -NMR based multivariate analysis of XM fractions. A) PCA Scores plot, B) PCA loadings plot. $R^2=0.95$, $Q^2=0.81$

4.4.4 LC-MS based metabolomics

Multivariate analysis of the LC-MS data of the fractions were shown in Figure 4.12. The PCA scores plot (Figure 4.12A) displayed no perceivable groupings or patterns. XM2 was positioned on the right-most quadrant of the plot and the distinguishing feature for this fraction was the ion peak at m/z 195.1013 Da for $[\text{M}+\text{H}]^+$. This feature was dereplicated as either 4-hydroxymellein or isobenzofuranone derivative. On the other hand, features with ion peaks at m/z 235.1689 and 217.1583 Da were discriminatory for the inactive XM5 fraction. The latter feature is an $[\text{M}+\text{H}-\text{H}_2\text{O}]^+$ adduct while the former is an $[\text{M}+\text{H}]^+$ of the compound with a molecular mass of 234.1618 g/mol. It was previously dereplicated as nigriterpene E as shown in section 3.3.2.

XM1 and XM4 were regarded as the active extracts in the applied OPLS-DA model. These active fractions were nested on the right-hand quadrants of the OPLS-DA scores plot (Figure 4.12C). S-plot was then used to point out the discriminatory features for the active group. Those were encircled in blue in Figure 4.12D while the top 10 most

statistically significant (lowest p -values) and its hits from the database were summarized in Table 4.5

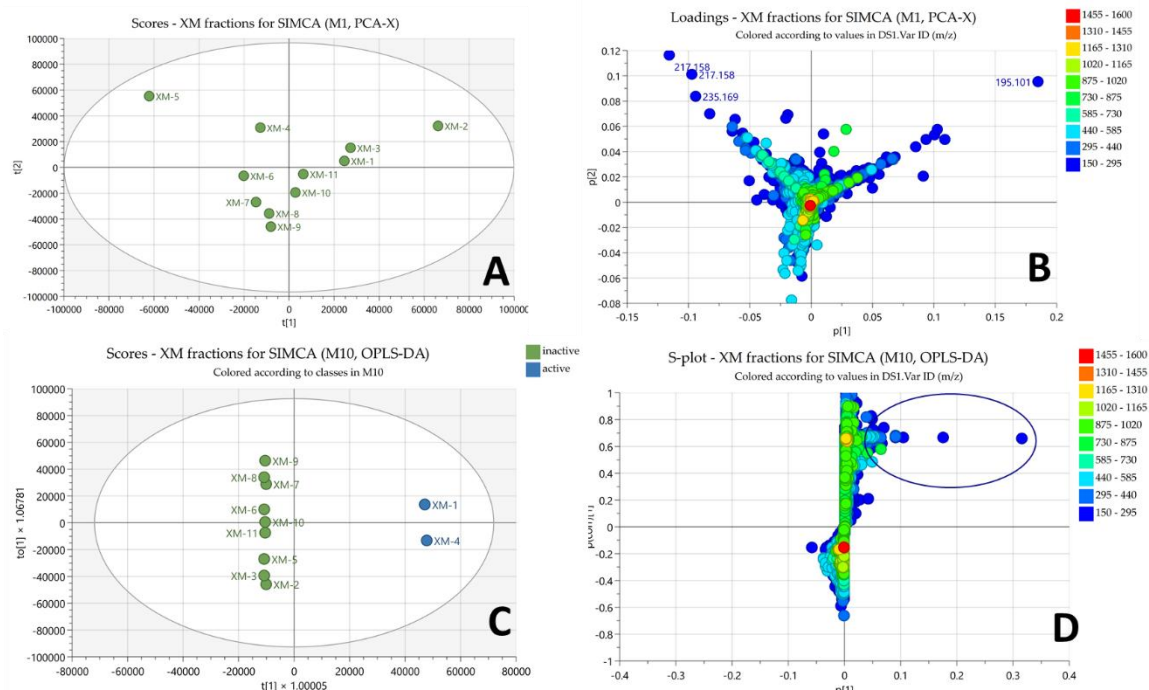


Figure 4.12. LCMS-based multivariate analysis of XM fractions A) PCA Scores plot, B) PCA Loadings Plot, C) OPLS-DA Scores plot, and D) OPLS-DA S-plot. PCA $R^2=0.41$, $Q^2=-0.05$. OPLS-DA $R^2=0.99$, $Q^2=0.71$

Looking at the dereplication table (Table 4.5), the most statistically significant metabolites were unsaturated fatty acids attached to a glycerol unit such as the monolinolein and monolinolenin. Furthermore, the feature with an ion peak at m/z 179.0700 $[M+H]^+$ resulted into the dereplication of several isomeric compounds including isosclerone, mellein, and isoochracein (Figure 4.16). These were included in the table to show the diversity of chemical compounds in the selected fraction. Hence, it was expected that isomers or derivatives will be isolated from the fractions.

Table 4.5 Dereplication of discriminatory metabolites of the bioactive XM1 and 4 fractions. Arranged in increasing *p*-values (*p*<0.05)

<i>p</i> -value	<i>m/z</i>	Molecular weight (g/mol)	Molecular formula	Dereplication hit	Biological Source
0.002308	353.2684	352.2611	C ₂₁ H ₃₆ O ₄	Monolinolenin	<i>Raphanus sativus</i> and <i>Tulipa gesneriana</i>
0.022646	355.2837	354.2764	C ₂₁ H ₃₈ O ₄	Linolenin	<i>Arisaema amurense</i> , <i>Chrysanthemum leucanthemum</i> , and <i>Sclerotinia fructicola</i>
0.0244	162.0628	161.0555	M-H ₂ O+H of <i>m/z</i> 179.07		
0.024503	180.0732	179.0659	M+2H of <i>m/z</i> 179.07		
0.024607	161.0595	160.0522	M-H ₂ O+2H of <i>m/z</i> 179.07		
0.025117	179.0700	178.0627 or	C ₁₀ H ₁₀ O ₃	Isosclerone or Mellein or Isoochracein	<i>Aspergillus fumigatus</i> , <i>Cladosporium cladosporioides</i> HDN14-342, <i>Microsphaeropsis</i> sp., <i>Penicillium citrinum</i> , <i>Phaeoacremonium aleophilum</i> , <i>Scytalidium</i> sp., <i>Sclerotinia sclerotiorum</i> , <i>Verticillium dahliae</i> , and the marine-derived fungi. Constituents of the fruit of <i>Juglans mandshurica</i> var. <i>sieboldiana</i> <i>Aspergillus</i> spp., <i>Hypoxylon howieanum</i> , <i>Microsphaeropsis</i> sp., <i>Lasiodiplodia theobromae</i> , <i>Pezicula</i> sp., and other fungi <i>Daldinia concentrica</i> S 0318, <i>Hypoxylon howieanum</i> strain 525, other <i>Hypoxylon</i> spp. and <i>Virgaria boninensis</i> FKI-6621
0.025476	271.1190	272.1263	NO HIT		
0.025871	403.2491	404.2564	C ₂₄ H ₃₆ O ₅	Lucidone	<i>Ganoderma resinaceum</i>
0.025995	255.1223	254.1151	C ₁₃ H ₁₈ O ₅	Xylapyrone A	<i>Xylaria</i> sp. BM9
0.043789	193.0857	192.0784	C ₁₁ H ₁₂ O ₃	5-Methylmellein	<i>Aspergillus</i> sp., <i>Fusicoccum amygdali</i> , <i>Hypoxylon illitum</i> , <i>Hypoxylon</i> sp., <i>Lachnellula</i> sp., <i>Microsphaeropsis</i> sp., <i>Nummularia discreta</i> , <i>Pezicula livida</i> , <i>Phomopsis oblonga</i> , <i>Phomopsis oblonga</i> 118, <i>Phomopsis</i> sp. BCC 9789, <i>Pithomyces</i>

<i>p</i> -value	<i>m/z</i>	Molecular weight (g/mol)	Molecular formula	Dereplication hit	Biological Source
					sp., <i>Plectophomella</i> sp., <i>Valsa ceratosperma</i> , a mangrove fungus, and others. Constituent of <i>Semecarpus</i> spp.
Compound dereplication are based on highly accurate mass matching (<5 ppm) against Dictionary of Natural Products, with filtering by fungal biological source. No tandem MS/MS data were acquired; therefore, these are tentative assignments that require confirmation through MS fragmentation data or isolation (cells highlighted in blue correspond to compounds that were subsequently isolated)					

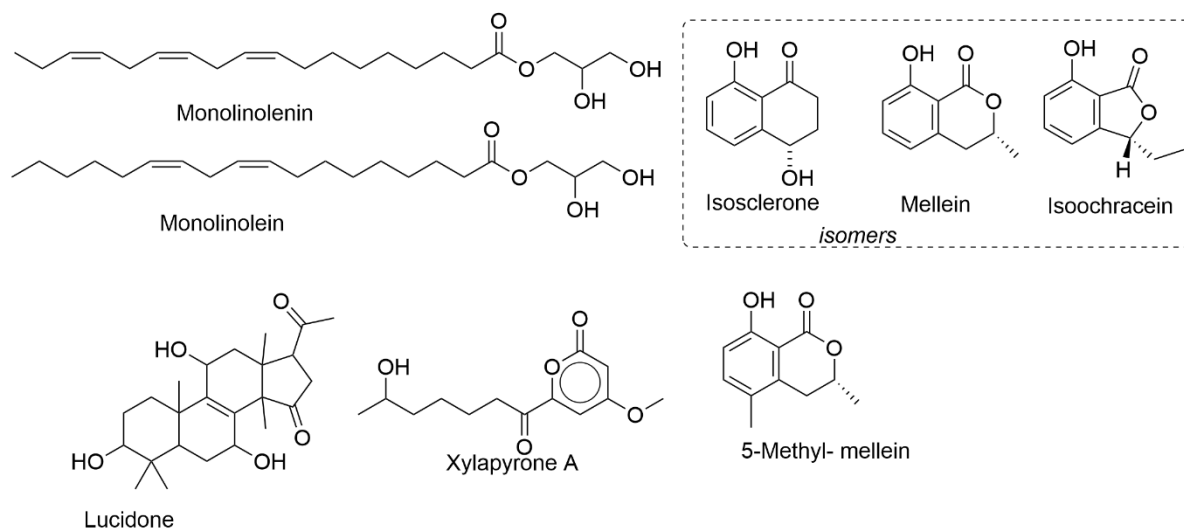


Figure 4.13. Chemical structures of dereplication hits from XM1 and XM4 fractions

4.5 Chromatographic purification of XM fractions

Fraction XM1 was found bioactive in both the antibacterial and postbiofilm assays, hence, XM1 was pursued for further purification work. To eliminate the unwanted fatty acids, XM1 was partitioned over hexane and methanol to afford XM1H and XM1M (343 mg), respectively. The undesirable peaks were less intense in XM1M suggesting a fair removal of the lipids.

Because of its moderate postbiofilm activity and its less complex ^1H -NMR spectra, XM2 was also chosen for further purification work on the Biotage Isolera One Flash system. Chromatographic purification was accomplished over a silica cartridge (Sfar HP-Sphere, 25-33 μm , 10g; Biotage) by stepwise isocratic elution starting with 40% ethyl acetate/hexane for 7 mins, then with 70% ethyl acetate/ 30%hexane for 5 mins and ends with 100% ethyl acetate for another 5 mins at a flow rate of 36 ml/min. The flash chromatography yielded four pooled fractions coded XM2.1 to 2.4. XM2.3 was prioritized for identification because of its distinct resonances exhibited by its NMR spectral data.

XM4, with its moderate inhibition activity in the prebiofilm and postbiofilm assays, was also subjected to chromatographic purification work on a Biotage system over a 50g HP-Sil column. The fraction (1 gram) was eluted starting with 50% ethyl acetate/50% hexane for 12 min, then with 70% ethyl acetate/30% hexane for 5 mins and finally with 100% ethyl acetate for 12 mins. A total of 10 pooled fractions were obtained and coded XM4.1 to 4.10. The major compound of XM4 was found mainly in fractions XM4.4 and XM.4.5. Structure elucidation works were then accomplished for purified fractions XM4.2, XM4.3, XM4.4, and XM4.7 as presented in section 4.6.3 and 4.6.4.

4.6 Structure elucidation of isolated compounds

4.6.1 XM1M as mellein

Mellein

Code: XM1M

Weight: 343 mg (3.4% yield)

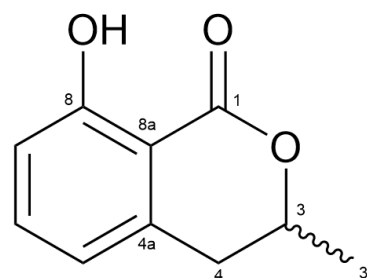
From: XM1

Purity: 80%

Physical description: yellow powder

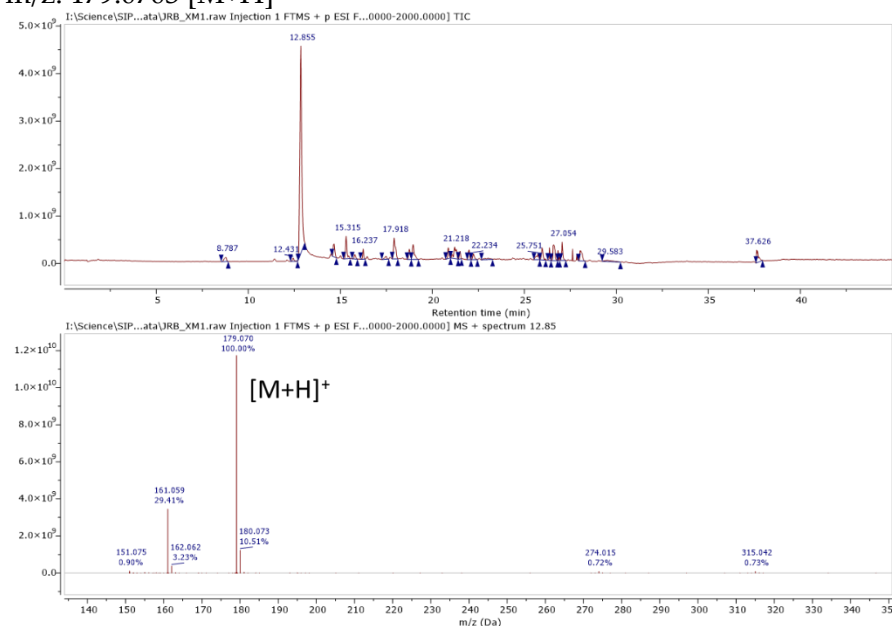
Molecular formula: $C_{10}H_{10}O_3$

Molecular weight: 178.1846 g/mol



retention time: 12.86 min

m/z: 179.0703 $[M+H]^+$



Formula	Calculated Mass	Target Mass	Double Bond Equivalence	Absolute Error (ppm)	Error (mDa)	Error (ppm)	Fitness
1 C10 H10 O3	179.07027	179.06971	6.0	3.15	-0.56	-3.15	1.000

Figure 4.14 Chemical and spectroscopic data of XM1M as mellein

XM1M was obtained as a yellow powder. It has a molecular formula of $C_{10}H_{10}O_3$ based on the HR-ESIMS ion with an ion peak at m/z of 179.0703 $[M+H]^+$ (DBE=6) (Figure 4.14). The 1H and ^{13}C NMR data shown in Table 4.6 recorded in DMSO- d_6 indicated the presence of one ester carbonyl (δ_c 169.4, C-1), six aromatic carbons (δ_c 108.4, C-8a; 177.8, C-5; 135.8, C-6; 114.9, C-7; 161.5, C-8; 140.8, C-4a), one oxygenated methine carbon (δ_c 75.6, C-3), one methylene carbon (δ_c 33.0, C-4) and one methyl carbon (δ_c 19.8, C-3a). The aromatic protons exhibited an ABC system with peaks at δ_H 6.82 (1H, dd, 7.35, 1.01), 7.50 (1H, dd, 8.40, 7.40), and 6.87 (1H, dt, 8.58, 1.05) corresponding to H-5, H-6, and H-7, respectively. A deshielded proton at δ_H 10.97 were assigned to the phenolic hydroxyl proton. The deshielding effect was due to the six-membered intramolecular H-bond formed by the phenolic proton and the proximal carbonyl group. The methine, methylene, and methyl protons were assigned due to their respective integration values and COSY correlations. Its 1D and 2D NMR spectra are included in Figure S 1 to Figure S 4. The NMR data were closely comparable (Table 4.6) with the literature values which further supports the elucidation of XM1M as mellein. The identification of mellein answered now the question of which isomer was pinpointed from the dereplication in Table 4.5

Table 4.6. 1H and ^{13}C NMR data comparison of XM1M from reported values

Atom no	XM1M, this study		mellein (Schulz et al., 1995)	
	δ_H in ppm, in ppm (J in Hz), 400 MHz in DMSO- d_6	δ_c in ppm in ppm002C100 MHz in DMSO- d_6	δ_H in ppm, in ppm (J in Hz), 500 MHz in CDCl $_3$	δ_c in ppm in ppm 125 MHz in CDCl $_3$
1	-	169.4	-	169.6
3	4.80 (1H, dqd, 11.26, 6.29, 3.43)	75.6	4.73 (1H, m)	76.11
3a	1.43 (1H, dd, 6.31)	19.8	1.53 (3H, d, 6.3)	20.75
4	2.98 (2H, m)	33.0	2.93 (2H, d, 7.3)	34.56

4a	-	140.8	-	139.41
5	6.82 (1H, 7.35, 1.01)	117.8	6.69 (1H, dd, 7.4, 0.8)	117.92
6	7.50 (1H, dd, 8.40, 7.40)	135.8	7.41 (1H, dd, 8.0)	136.13
7	6.87 (1H, dt, 8.58, 1.05)	114.9	6.89 (1H, d, 8.1)	116.18
8	-	161.5	-	162.15
8-OH	10.97 (1H, s)	-	11.04 (1H, s)	-
8a	-	108.4	-	108.29

Mellein was first isolated from *Aspergillus melleus* (Nishikawa, 1933). This compound was commonly isolated from fungi but there were also sometimes derived from plants, insects, and bacteria. It is a 3,4-dihydroisocoumarin natural product originating from the polyketide pathway (Reveglia, Masi, & Evidente, 2020). From this core structure, many derivatives have been later isolated and reported. For example, 5-carboxymellein, (*R*)-(-)-methoxycarbonyl mellein, 8-methoxymellein, (*R*)-5-methylmellein including a new derivative akolitserin were obtained from the endophytic fungi *X. cubensis* (Fan et al., 2014). The current study is the first to report the isolation of mellein from *X. cubensis*.

In-depth NMR analysis of ^1H -NMR of XM2.3 suggested the plausible identification of these two isomers: 7-hydroxy-3-(1-hydroxyethyl)isobenzofuran-1(3*H*)-one and 4-hydroxymellein. While this represents a plausible structural assignment, it is also possible that XM2.3 contains mixtures of enantiomers of these isomers. A way to prove this would be further purification and absolute stereochemical analysis but was impossible due to limited amounts (32 mg) of this fraction. Nevertheless, the structural features of the isobenzofuranone and hydroxymellein isomers are interpreted in the following text.

The overlapped peak assignments were shown in Figure 4.16. Based on integration values of corresponding resonances for the respective compounds in ^1H -NMR; the ratio of the mixture was at 6:4 (A:B).

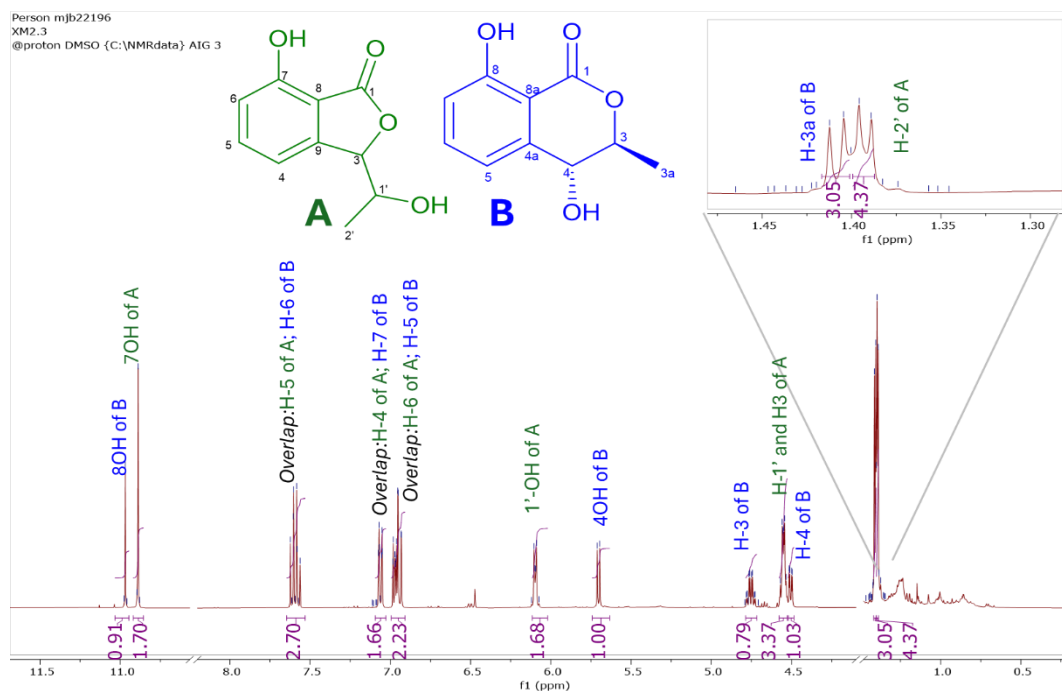


Figure 4.16 ^1H -NMR of XM2.3 showing the overlap of peaks of possibly 7-hydroxy-3-(1-hydroxyethyl)isobenzofuran-1(3H)-one (A; coloured in green) and 4-hydroxymellein (B; coloured in blue). Recorded in 400MHz in DMSO- d_6

The more abundant feature of XM2 has detected at the ion peak at m/z of 195.1016 $[\text{M}+\text{H}]^+$ (Figure 4.15). From this, the calculated molecular weight is 194.094 g/mol, which corresponds to a molecular formula of $\text{C}_{10}\text{H}_{10}\text{O}_4$ (DBE=6). Combined analysis of the ^1H and ^{13}C NMR data (Table 4.7) recorded in DMSO- d_6 , showed the presence of one ester carbonyl (δ_c 168.1, C-1), six aromatic carbons (δ_c 143.7, C-9; 116.5, C-4; 136.6, C-5; 118.7, C-6; 160.5; C-7; 106.9, C-7), two oxygenated methines (δ_c 67.3, C-3; δ_c 79.8, C-1'), and a methyl group (δ_c 17.7, C-2'). Two exchangeable protons were also observed: one is a deshielded proton at δ_H 89 (s), (7-OH) like in mellein, and a hydroxyl proton attached to a methine unit at δ_H in ppm, 6.10 (m), (1'-OH). Because of the aromatic protons' *ortho* coupling constants and COSY correlations (Figure S 5Figure S 8), it was deduced to

follow an ABC ring system arrangement like that of mellein. Collectively, this isomer was elucidated as 7-hydroxy-3-(1-hydroxyethyl)isobenzofuran-1(3*H*)-one. Table 4.7 showed that the NMR data closely agreed with the literature values.

Table 4.7. ¹H and ¹³C NMR data comparison of 7-Hydroxy-3-(1-hydroxyethyl)isobenzofuran-1(3*H*)-one in XM2.3 from reported values

	7-Hydroxy-3-(1-hydroxyethyl)isobenzofuran-1(3<i>H</i>)-one in XM2.3. <i>This study</i>		7-Hydroxy-3-(1-hydroxyethyl)isobenzofuran-1(3<i>H</i>)-one (Rahman & Gray, 2005)	
	δ_{H} in ppm, (<i>J</i> in Hz) 400 MHz in DMSO- <i>d</i> ₆	δ_{C} in ppm, 100 MHz in DMSO- <i>d</i> ₆	δ_{H} in ppm, (<i>J</i> in Hz) 400 MHz in CDCl ₃	δ_{C} in ppm, 100 MHz in CDCl ₃
1	-	168.1		168.6
3	4.55 (1H, m)	67.3	4.62 (m)	69.4
4	7.06 (1H, dt, 7.48,0.91)	116.5	7.03 (br d, 8.0)	116.4
5	7.59 (1H, dt, 8.46, 7.42)	136.6	7.55 (t, 8.0)	137.1
6	6.97 (1H, dd. 8.43, 1.05)	118.7	7.01 (d, 8.0)	118.1
7	-	160.5	-	162.3
7-OH	10.89 (1H, s)	-	Not reported	-
8	-	106.9	-	106.9
9	-	143.7	-	141.4
1'	4.55 (1H, m)	79.8	11.00 (s)	-
1'-OH	6.10 (1H, m)	-	4.62 (m)	80.1
2'	1.39 (3H, d, 2.71)	17.7	1.53 (d, 6.2)	18.1

4-Hydroxymellein follows the same substitution pattern for mellein as discussed in section 4.6.1. However, instead of a methylene proton, an oxygenated methine unit at δ_{H} 4.51 (dd, *J*=6.24 and 2.12 Hz, H-4) was on C-4 at δ_{C} 65.2. A hydroxyl moiety was attached to this methine at δ_{H} 5.70 (d, *J*=6.27 Hz, 4-OH) as seen on the COSY (S9) correlations. Therefore, this led to the elucidation of 4-hydroxymellein. The *trans* orientation of the two oxygenated methines were construed from the large coupling

constants of 6.56 and 6.24 Hz. The correlations from COSY and HMBC spectra as shown in Figure S 9Figure S 12, respectively supported to the structure elucidation of this compound. Close comparison with the reported NMR data further settled the structure elucidation work.(Table 4.8).

Table 4.8 ^1H and ^{13}C NMR data comparison of 4-hydroxymellein with reported values

Atom no	4-hydroxymellein from XM2.3. <i>This study</i>		(3S,4R) 4-hydroxy mellein (Rivera-Chávez et al., 2015)	
	δ_{H} in ppm, (<i>J</i> in Hz) 400MHz in DMSO- d_6	δ_{C} in ppm, 100 MHz in DMSO- d_6	δ_{H} in ppm, (<i>J</i> in Hz) 400MHz in MeOH- d_4	δ_{C} in ppm 100MHz in MeOH- d_4
1	-	160.5		171.0
3	4.75 (1H, dd, 6.56, 2.14)	78.3	4.72 (1H, qd, 2.1, 6.6)	79.9
3a	1.41 (3H, m)	15.8	1.47 (3H, d, 6.6)	16.4
4	4.51 (1H,dd, 6.24, 2.12)	65.2	4.55 (1H, d, 2.1)	67.6
4-OH	5.70 (1H, d, 6.27)	-	-	-
4a	-	142.4	-	143.1
5	6.95 (1H, dd, 7.16, 0.79)	118.7	6.96 (1H, d, 8.4)	119.8
6	7.59 (1H, dd, 8.45, 7.42)	136.4	7.56 (1H, dd, 7.4,8.4)	137.7
7	7.06 (1H, dt, 7.48, 0.91)	116.7	6.99 (1H, d, 7.4)	118.5
8	-	169.0	-	162.9
8-OH	10.97 (1H, s)	-	-	-
8a	-	107.4	-	108.4

The isobenzofuranone derivative was first isolated from the curry plant *Murraya koenigii* (Rahman & Gray, 2005). It was also isolated from several fungi including *Microcera larvarum* (Cadelis et al., 2020) and *Lasiodiplodia venezuelensis* (Pellissier et al., 2021). Meanwhile, 4-hydroxymellein, was first isolated from *Aspergillus ochraceus* (Cole et al., 1971). It was also isolated from other fungal taxa including from the endophyte *Xylaria* sp. (Tansuwan et al., 2007) and *Xylaria feejeensis* (Rivera-Chávez et al., 2015). Cadelis et al. (2020) isolated both isomers as well from the enthomopathogenic fungi

Microcera larvarum. However, this is the first time that 7-hydroxy-3-(1-hydroxyethyl)isobenzofuran-1(3H)-one and 4-hydroxymellein were reported from *Xylaria cubensis*.

4.6.3 XM4.4 as phloroglucinol

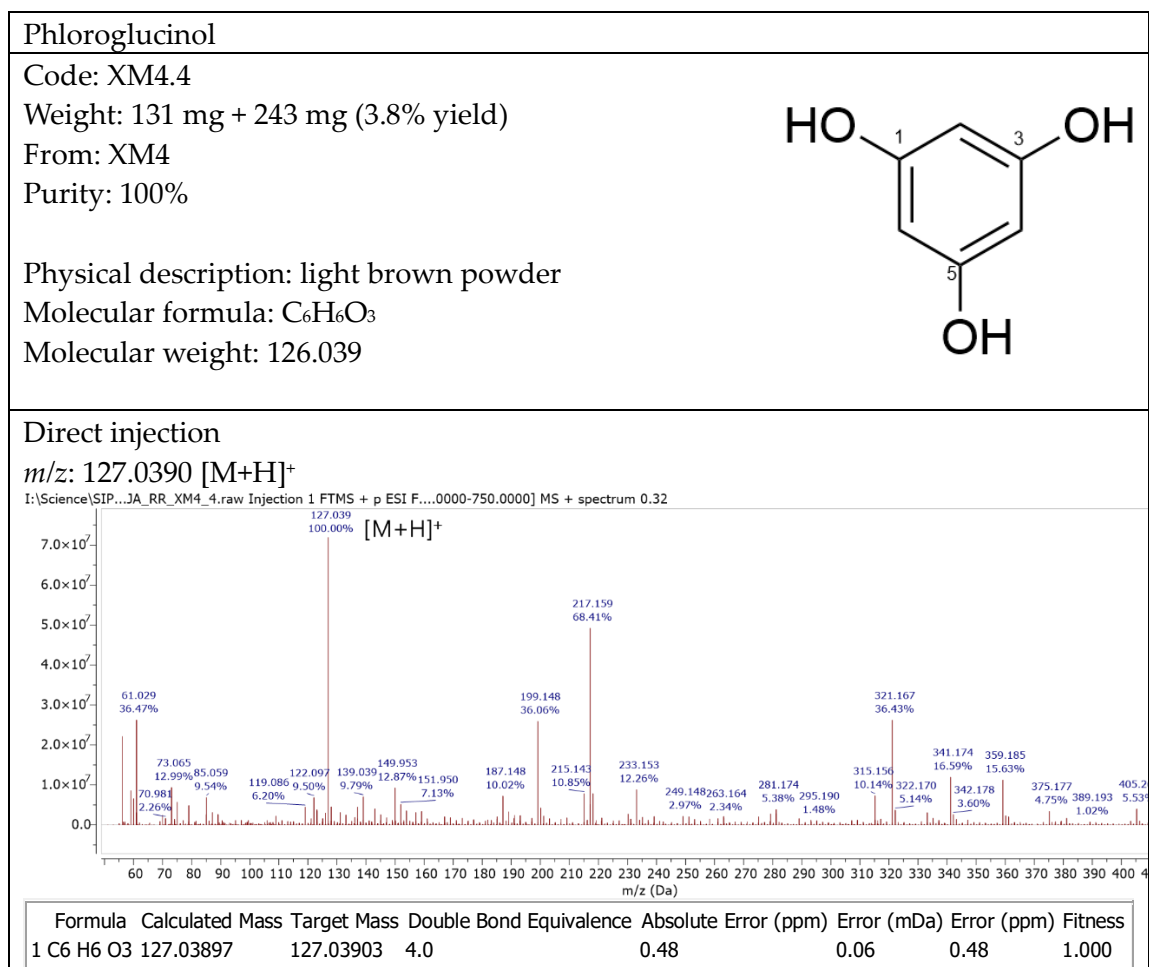


Figure 4.17 Chemical and spectroscopic data of XM4.4 as phloroglucinol

XM4.4 was obtained as a light orange powder with a base peak at m/z 127.0390 Da [M+H]⁺ corresponding to the formula C₆H₆O₃ (DBE=4) and molecular weight of 126.039 g/mol. The ¹H and ¹³C data (

Table 4.9, Figure S 13 (Figure S 14) shows only two peaks conforming to one aromatic proton at δ_{H} 5.66 (s) and one exchangeable proton at δ_{H} 8.92 (s) having equal integration values. Each peak corresponds to three chemically equivalent protons to make up for the computed molecular formula. The structure was confirmed to be 1,3,5-trihydroxybenzene or phloroglucinol by close comparing to literature data (Table 4.9).

Table 4.9 ^1H and ^{13}C NMR data comparison of XM4.4 from reported values

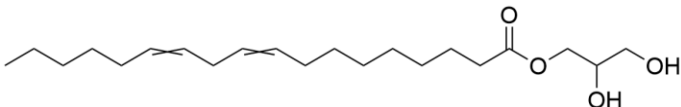
Atom no	XM4.4, <i>this study</i>		Phloroglucinol (Marshall, Cable, & Botting, 2010)	
	δ_{H} in ppm, (J in Hz), 400MHz in DMSO- d_6	δ_{C} in ppm 100 MHz in DMSO- d_6	δ_{H} in ppm, (J in Hz) 400MHz in CDCl_3 + DMSO- d_6	δ_{C} in ppm, 100MHz in CDCl_3 + DMSO- d_6
1,3,5	-	158.2	-	158.8
2,4,6	5.66 (3H, s)	93.4	5.82 (3H, s)	94.8
1-OH, 3-OH, 5-OH	8.92 (3H, s)	-	-	-

The two ^1H -NMR peaks of phloroglucinol had persistently appeared throughout the course of this investigation with *X. cubensis*. In the media optimisation stage (section 4.2.2.1, Figure 4.3), these peaks were pinpointed as discriminatory peaks of the active extracts. In the fractionation stage, the peaks were also seen specifically at XM4 (see 4.4.3, Figure 4.10). These instances illustrated the effectivity of NMR-based metabolomics as a guiding tool for targeted isolation.

Phloroglucinol is a polyphenolic compound that mostly occurred in plant species, brown and red algae, and bacteria. Formylated congeners of phloroglucinol was reported in *Eucalyptus* plant species (Eschler et al., 2000). Around 500 derivatives of phloroglucinol was reported from the plant genus *Hypericum* alone (Bridi, Meirelles, & von Poser, 2018). Several species of brown algae were found to contain phloroglucinol and its oligomers

called phlorotannins (Shibata et al., 2004). The biosynthesis of phloroglucinol was delineated finally through a biosynthetic investigation in *Pseudomonas fluorescens* Pf-5 (Achkar et al., 2005). The compound has not been reported in the fungal kingdom. However, heterodimer derivatives of phloroglucinol have been isolated from the sponge-derived *Aspergillus* sp. SCSIO 41018 (Guo et al., 2019). The aforementioned study did not isolate phloroglucinol itself but rather considered it as a part in their plausible biosynthesis. The current study was the first report of the isolation of phloroglucinol as a fungal metabolite. Thus, this study provides evidence of phloroglucinol production by fungal species.

4.6.4 Identification of XM4.7 as monolinolein/linoleoyl glycerol

Monolinolein or Linoleoyl glycerol	
Code: XM4.7	
Weight: 33 mg (0.2% yield)	
From: XM4	
Purity: 95%	
Physical description: orange powder	
Molecular formula: C ₂₁ H ₃₈ O ₄	
Molecular weight: 354.2834 g/mol	
retention time: 25.82 min	

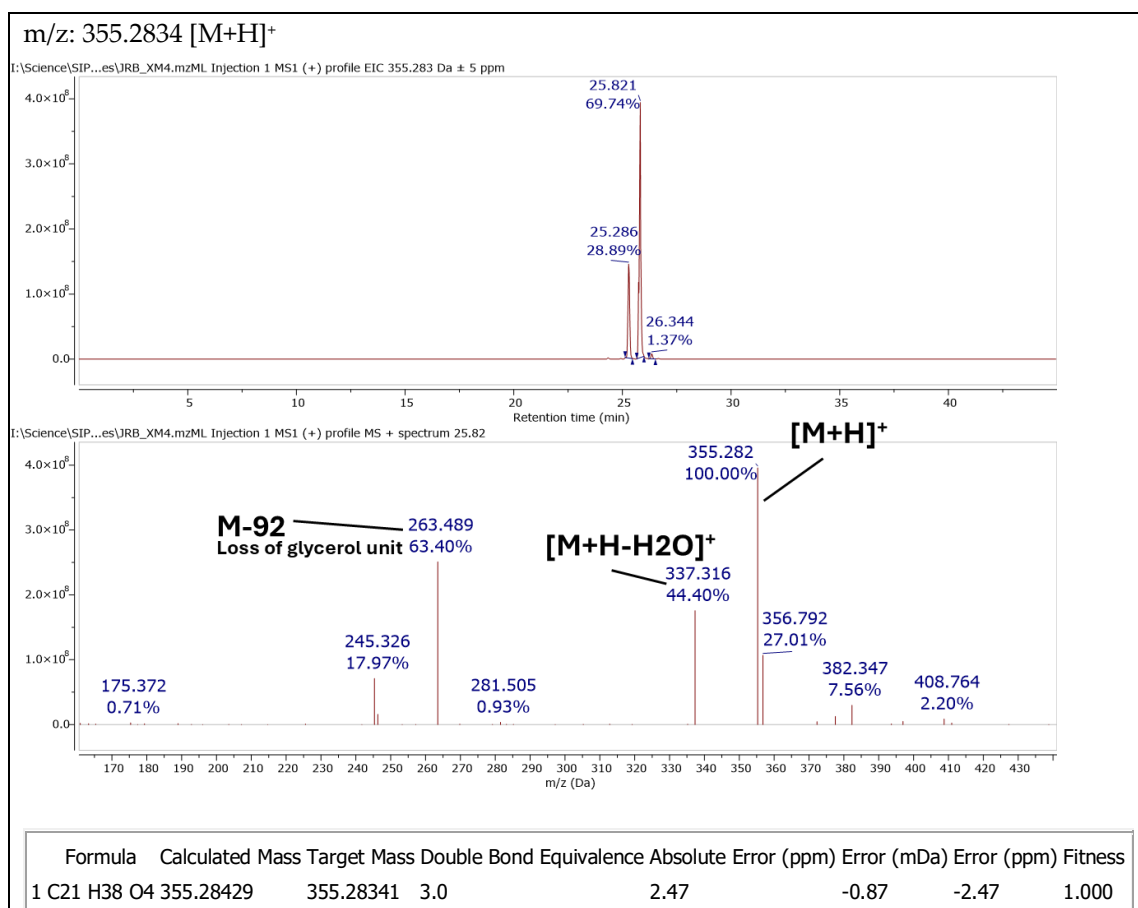
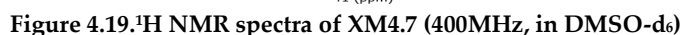


Figure 4.18 Chemical and spectroscopic data of XM4.7 as monolinolein

The major peak in XM4 was located at 25.82 min and by which it has a molecular ion peak m/z of 355.283 for $[M+H]^+$. Its calculated molecular formula is C₂₁H₃₈O₄ (DBE=3; Figure 4.18). This feature was also a discriminatory metabolite and was pinpointed from the dereplication in Table 4.5. It was identified as linoleoyl glycerol or monolinolein, having two double bounds and a carbonyl group which equates to the DBE of 3. Furthermore, it gave an ion peak at m/z of 263.236 which were calculated for a loss of a glycerol unit. This supports the fact there is a glycerol backbone.



groups were overlapping at δ_{H} 1.28. Some integration values do not match the number of protons and hence must be disregarded.

The unsaturated fatty acid was also isolated and identified before in our group with more detailed elucidation including 2D NMR experiments. As shown Figure 4.20, their ^1H -NMR spectra matched which further confirmed the structure elucidation of monolinolein. It was previously reported from an orchid plant (Stoessl, Fisch, & Arditti, 1980). To the best of our knowledge, this is the first time that monolinolein was isolated from *Xylaria cubensis*.

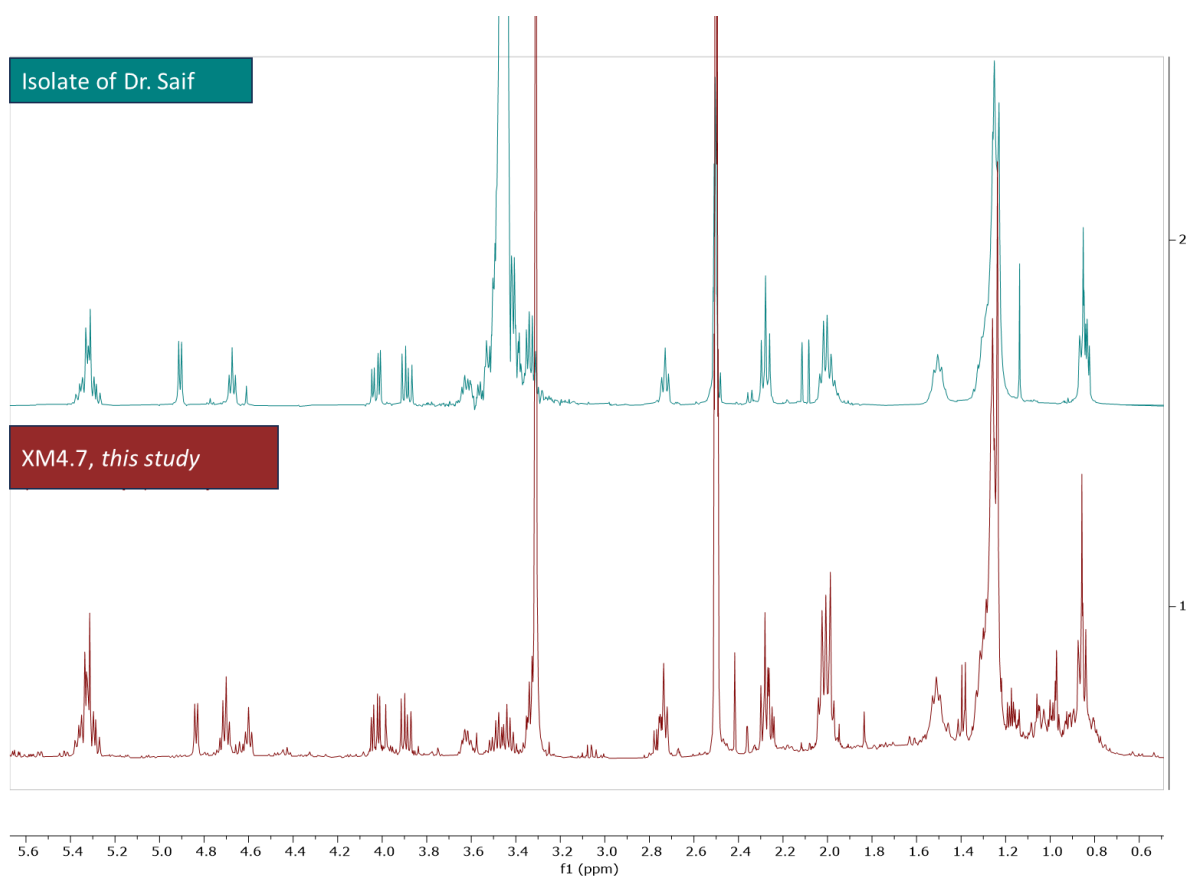


Figure 4.20 Stacked ^1H -NMR spectra of XM4.7 (red) and same molecule from a sample (in blue green) isolated by Dr. Saif Jaber of Natural Products Metabolomics group.

4.7 Biological activities of isolated compounds from *X. cubensis* grown in rice

The five isolated compounds were tested again for their biological activities. Compounds were tested at 100 µg/mL followed by half-fold dilution to compute for MIC, BPC, and MBIC values against MRSA. They were not tested against *P. aeruginosa* because their mother fractions were inactive against it. The results were shown in Table 4.10. Phloroglucinol seems to be active as it gives >70% inhibition in the antibacterial and prebiofilm activity. Mellein is also active at prebiofilm and postbiofilm inhibition assays at the highest concentration tested (100 µg/mL). Albeit, their MIC, BPC, and MBIC values are not strong enough at lower dilutions and thus are not comparable to the potency of positive control ciprofloxacin.

Table 4.10 Biological activities of compounds from *X. cubensis* against MRSA

Sample code	Identity, %purity	Antibacterial activity		Prebiofilm activity		Postbiofilm activity	
		%Inhibition @100 µg/mL	MIC (µM)	%Inhibition @100 ug/mL	BPC (µM)	%Inhibition @100 ug/mL	MBIC (µM)
XM1M	mellein, 80%	43.02	>100	77.83	>100	71.82	>100
XM2.3	7-hydroxy-3-(1-hydroxy-ethyl)isobenzofuran-1(3H)-one, 60%	30.36	>100	32.59	>100	-1.87	>100
XM4.4	phloroglucinol, 100%	93.8	>100	98.69	>100	1.65	>100
XM4.7	monolinolein, 95%	26.25	>100	9.74	>100	19.96	>100
Ciprofloxacin		97.65	2.03	99.86	1.56	94.47	10.52
DMSO		6.73	>100	-13.79	>100	0.4	>100

MIC- Minimum Inhibitory Concentration; the smallest concentration of sample to give 100% inhibition;

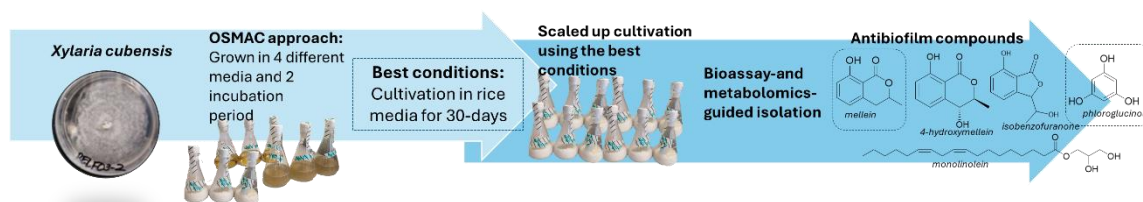
BPC- Biofilm Prevention Concentration; the least concentration of sample to give 90% inhibition in the prebiofilm assay

MBIC- Minimum Biofilm Inhibition Concentration; the least concentration of sample to give 90% inhibition in the postbiofilm assay

Assays were done twice with three replicates (n=3) Testing concentration=100 µg/mL; Moderate inhibition: 50-70% (coloured in orange); Strong inhibition >70% (coloured in red). Dose response curves of active compounds can be found in Supporting Information

4.8 Chapter summary

This chapter investigated the secondary metabolite production of *Xylaria cubensis* employing the OSMAC approach in four different media (rice, oat, PDB, MEB) and two incubation periods (15 and 30 days). Cultivation in 30-day rice media resulted in the highest bioactivity and distinct chemical diversity, including isocoumarins and aromatic compounds specific from this parameter. Based on these results, large-scale cultivation was performed under these conditions, followed by targeted chromatographic isolation. Three compounds were isolated and identified, including mellein, phloroglucinol, and monolinolein using NMR and MS data. Mellein and monolinolein had previously been highlighted as discriminatory metabolites in active fractions, demonstrating the value of metabolomics in guiding purification. Phloroglucinol was also consistently dereplicated using NMR-based metabolomics and is here reported for the first time from a fungal species. In addition, one fraction appeared to contain two isocoumarin isomers. Among the isolated compounds, mellein (77% prebiofilm inhibition) and phloroglucinol (93.89% antibacterial and 98.69% prebiofilm inhibition) exhibited notable biological activities, though potency declined at lower concentrations. Overall, the chapter emphasizes the usefulness of metabolomics in media selection and isolation of target compounds for *X. cubensis*.



Graphical summary of *Xylaria* chapter

Chapter 5. *Hypoxylon lateripigmentum*

5.1 Chapter overview

This chapter is on ELF P6, which was later taxonomically identified by molecular technique as *Hypoxylon lateripigmentum*. OSMAC was applied by varying the culture media and incubation period. Two culture conditions were used for scaling up and followed by chromatographic purification of target bioactive compounds. Several compounds were finally identified that has potent antibiofilm activity.

5.2 Media optimisation studies of *Hypoxylon lateripigmentum*

Hypoxylon lateripigmentum J. Fourn., Kuhnert & M. Stadler is slow growing and thus, was cultivated for 15 and 30 days in four different media (oat, rice, potato dextrose broth (PDB), and malt extract broth (MEB)) for media optimisation. Ethyl acetate was then used to extract the metabolites, and the yield was shown in Figure 5.1. Cultivation in solid media gave higher yields in contrast with the liquid media extracts. The highest yield was afforded by the 30-day oat extract.

Relative extract weight of *H. lateripigmentum* grown in different media and incubation period

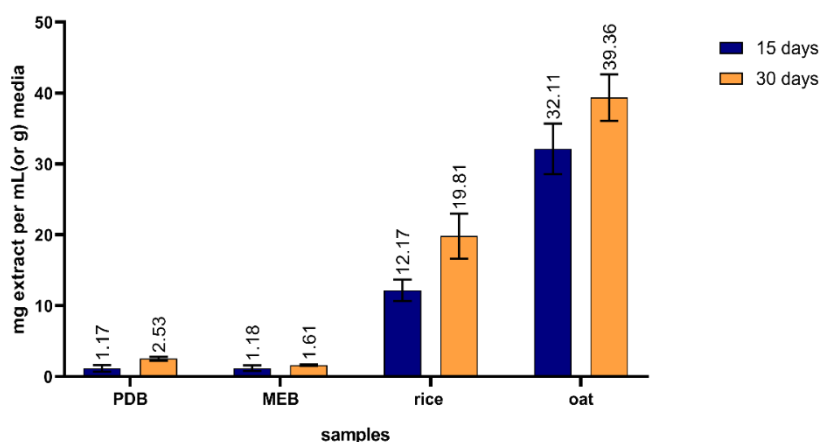


Figure 5.1. Extraction Yield of *H. lateripigmentum* in different media

5.2.1 Biological assay results

Bioassays were conducted on the culture extracts of *H. lateripigmentum* grown under various conditions (Table 5.1). The table also includes results from the preliminary screening assay using extracts from Potato Dextrose Agar (PDA) to serve as the baseline medium for comparing fungal extracts afforded from other media. As shown in the table, the bioactivity varied between each media and cultivation periods. The 15-day PDB and 15-day rice culture extracts exhibited strong antibacterial activity, but their 30-day extracts have moderate inhibition. Strong antibacterial activity was also showed by MEB culture extracts both at 15 and 30-days. Notably, only the 15-day PDB extract demonstrated strong dual prebiofilm inhibition against both test organisms. In contrast, rice and MEB extracts displayed strong prebiofilm activity specifically against MRSA. Oat-based extracts exhibited prebiofilm inhibition against *Pseudomonas*, but only weak activity against Gram-positive bacteria. Strong postbiofilm inhibition against MRSA was observed in the 15-day rice and 30-day oat extracts, although these values were lower compared to the PDA extract. At this stage, it is not possible to definitively identify the optimal culture conditions, as several media and time points demonstrated promising bioactivity. Therefore, in the following sections in this chapter, metabolomics analysis was employed to guide the selection of the most favourable conditions.

Table 5.1. Biological activities of *H. lateripigmentum* grown in different media.

ANTIBACTERIAL ACTIVITY, average % inhibition (n=3)					
Media	MRSA	<i>P. aeruginosa</i>	Media	MRSA	<i>P. aeruginosa</i>
PDA	38.71	28.68			
PDB blank	-7.38	-13.43	Rice Blank	18.11	3.41
15day-PDB	73.47	17.88	15day-Rice	77.93	23.58
30day-PDB	68.43	-12.97	30day-Rice	58.80	35.26
MEB blank	13.04	4.22	Oat Blank	-3.72	4.83
15day-MEB	74.23	7.86	15day-Oat	57.20	2.37
30day-MEB	70.79	24.04	30day-Oat	40.49	3.48
DMSO	26.11	9.70	Ciprofloxacin (50 µM)	98.60	99.76
PREBIOFILM ACTIVITY, average % inhibition (n=3)					
Media	MRSA	<i>P. aeruginosa</i>	Media	MRSA	<i>P. aeruginosa</i>
PDA	96.96	74.79			
PDB blank	-15.36	-7.95	Rice Blank	50.16	70.07
15day-PDB	98.77	85.08	15day-Rice	95.36	-71.12
30day-PDB	98.33	-361.76	30day-Rice	93.21	-219.58
MEB blank	-13.56	-75.20	Oat Blank	27.30	45.79
15day-MEB	56.61	-404.22	15day-Oat	43.64	84.77
30day-MEB	80.96	-388.99	30day-Oat	32.52	88.00
DMSO	-11.56	-74.71	Ciprofloxacin (50 µM)	99.80	100.91
POSTBIOFILM ACTIVITY, average % inhibition (n=3)					
Media	MRSA		Media	MRSA	
PDA	87.92				
PDB blank	2.66		Rice Blank	41.62	
15day-PDB	34.10		15day-Rice	77.61	
30day-PDB	35.12		30day-Rice	29.79	
MEB blank	1.32		Oat Blank	-6.17	
15day-MEB	6.35		15day-Oat	69.17	
30day-MEB	57.79		30day-Oat	72.15	
DMSO	-2.97		Ciprofloxacin (50 µM)	91.84	

Testing concentration=100 µg/mL; assays were done twice with three replicates (n=3); Moderate inhibition: 50-70% (coloured in blue); Strong inhibition >70% (coloured in red); Media: PDB-Potato dextrose broth, MEB- Malt extract broth, PDA -potato dextrose agar (media used in the preliminary screening stage)

5.2.2 Metabolomics profiling

5.2.2.1 NMR-based profiling

Culture extracts of *H. lateripigmentum* were subjected for ^1H -NMR. **Error! Reference source not found.** shows the stacked ^1H -NMR spectra of the extracts. There is a noticeable presence of aromatic protons (boxed in orange) in the malt extracts. Broad methylene proton signals, boxed in black, indicate the presence of fatty acids across all extracts. Looking at the spectra of oat extracts, lipids and fatty acids were the main components. Consequently, oat extracts will be less interesting to consider in further analysis. There are two distinct sharp doublets at 4.7 ppm and 2.7 ppm in all culture extract except in 30-MEB cultures. These peaks seem decreasing in concentration from 15

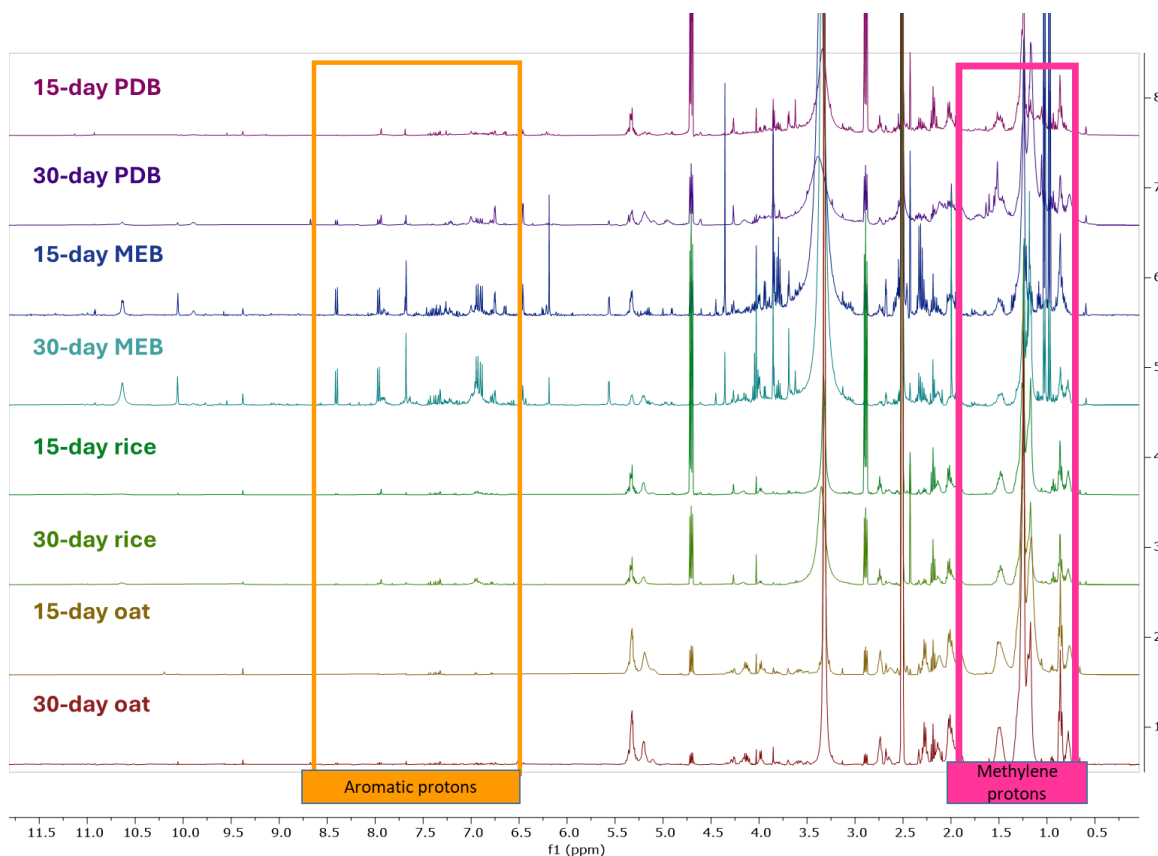


Figure 5.2. Stacked ^1H -NMR spectra of *H. lateripigmentum* grown in different media. ^1H -NMR recorded in 400 MHz in DMSO-d_6 to 30 days.

A heatmap based on their NMR spectra illustrated the overall chemodiversity of *H. lateripigmentum* extracts as shown on Figure 5.3. Strong blue bands are clearly present on 15-day PDB, 30-day PDB, and 30-day MEB (boxed in black). The oat extracts, on the other hand, display lower band intensity, reflecting a less chemically diverse profile. Although oat extracts showed notable post-biofilm inhibition and higher yield, the limited chemodiversity suggested that the compounds present may be less promising for further exploration. A similar observation applies to the rice extracts.

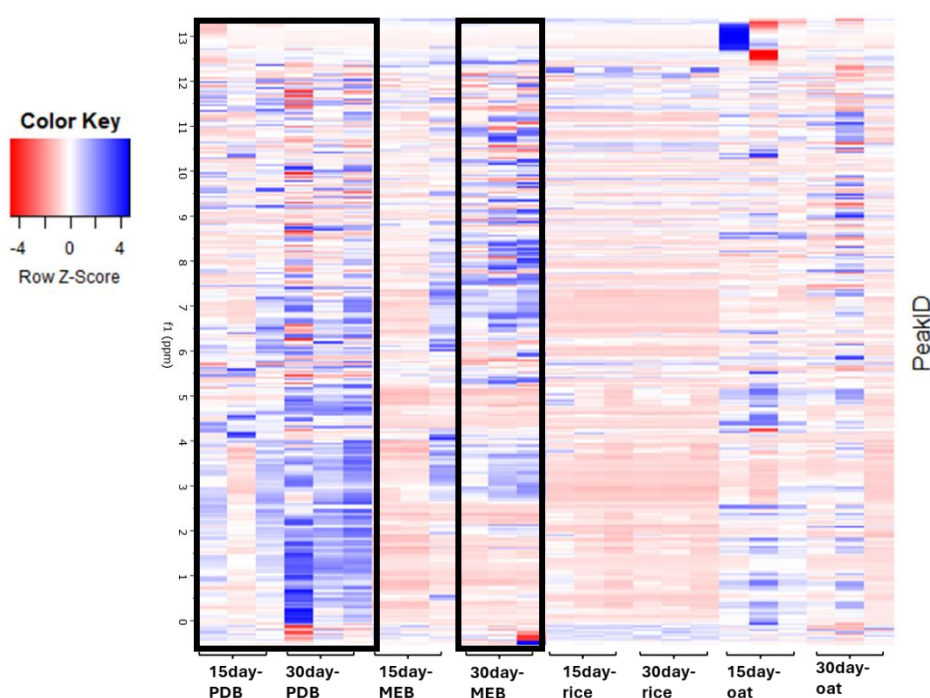


Figure 5.3. Heatmap based on ^1H -NMR profiles of *H. lateripigmentum* extracts grown in different media. Boxed in black are culture extracts with higher diversity as indicated with strong blue bands. Heatmap generated in R (code available in S 2)

Next, multivariate analysis was applied to the NMR spectra. The PLS-DA scores and loadings plot were shown in Figure 5.4A and B, respectively. The solid and liquid media samples were not seen in the same region. The 30-day potato extract clustered out

from the rest which could mean uniqueness of metabolites in this sample. These metabolites have discriminating chemical shifts at 2-4 ppm as denoted in the loadings plot. The two distinct peaks (4.7 and 2.7 ppm) in the stacked spectra in **Error! Reference source not found.** was determined to be discriminating features (encircled in the loadings plot Figure 5.4B) of the 15-day rice culture extract. The coefficient plot shown in Figure 5.4C revealed the impact or influence of the culture media on the occurrence of these two discriminating chemical resonances. For extracts obtained from PDB and rice cultures, the presence of resonances at 4.7 and 2.7 ppm were positively correlated while they were absent in cultures grown in MEB and oat.

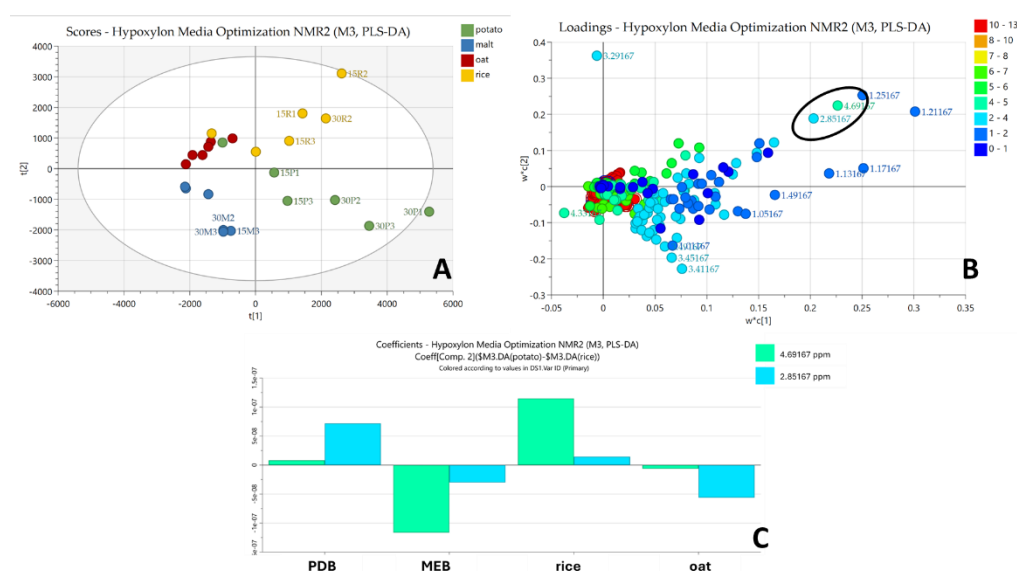


Figure 5.4. ¹H-NMR based multivariate analysis of *H. lateripigmentum* grown in different media. A) PLS-DA Scores plot, B) PLS-DA loadings plot, C) PLS-DA coefficient plot. Each dot in the scores plot represent one replicate (n=3). Encircled in the loadings plot are the chemical shifts distinct in the ¹H-NMR spectra of PDB and rice extracts. R²=0.79, Q²=0.61.

5.2.2.2 LC-MS-based profiling

LC-MS-based profiling was also employed on extracts of *H. lateripigmentum* obtained from different media at two growth periods. The PCA scores plot and loadings

plot were shown in Figure 5.5A and B. Extracts from solid media were clustered together, which suggests that their chemical profiles were similar. A more diverse chemistry can be observed in extracts of cultures grown in PDB and MEB which are both liquid media. Again, the 30-day PDB culture extract were producing a more unique set of metabolites. This sample was rich in compounds ranging from 380 to 660 Da. The 15-day PDB culture extract also contained similar compounds as seen on the loadings plot. In addition, lower molecular weight compounds (199-380 Da) can also be observed on this extract.

Looking back, extracts from the 15-day PDB cultures gave better biological activity than extracts obtained from the 30-day PDB culture though it can be observed that the latter has a more distinctive chemical profile as seen in Figure 5.5A. Smaller metabolites from the 15-day PDB culture extract were being predicted as the bioactive components while those with higher MWs being produced at the 30th day were being hypothesised as inactive. Extracts obtained from the oat media were also bioactive but is less interesting in terms of their chemical diversity and thus the culture conditions will not be further used for the scale-up. The extract from the 15-day PDB culture were then selected as the optimum condition for scale-up.

To annotate the bioactive metabolites from the 15-day PDB culture extract, an OPLS-DA model was utilized. The scores and S-plot were exhibited in Figure 5.5C and D. The active samples appeared on the right quadrants of the scores plot. It can also be noticed that 30-days PDB culture extracts were separated from the inactive cluster which indicated that it is chemically different from the rest of the group.

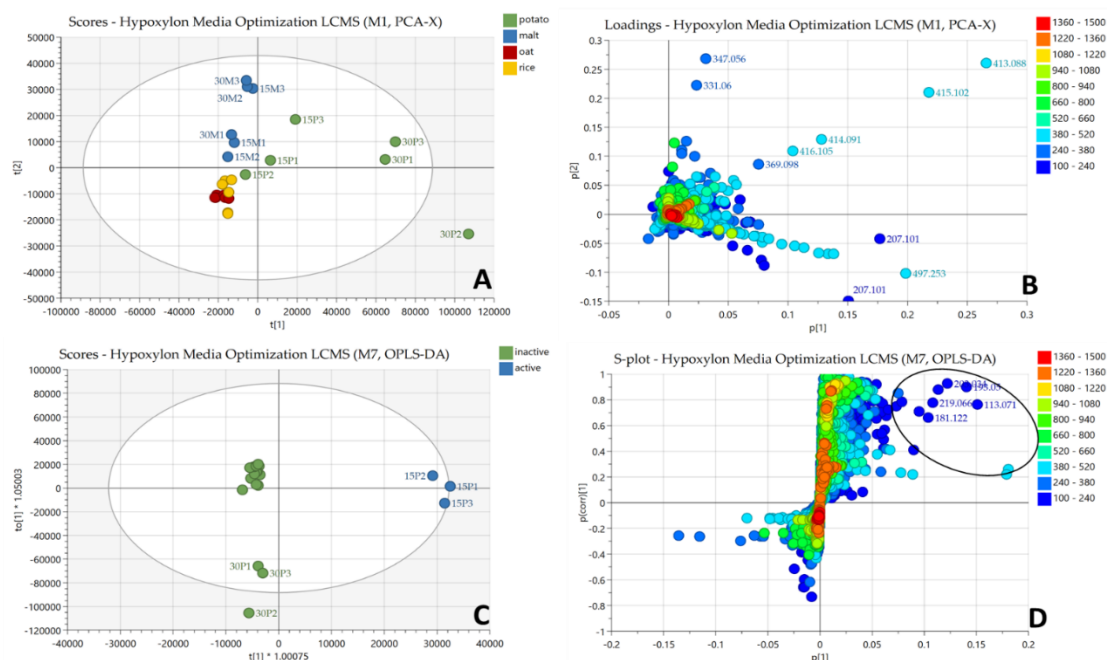


Figure 5.5. LCMS-based multivariate analysis of *H. lateripigmentum* grown in different media. A) PCA Scores plot, B) PCA loadings plot, C) OPLS-DA Scores plot, D) OPLS-DA S-plot. Each dot in the scores plot represent one replicate (n=3). PCA: R²=0.93, Q²=0.79; OPLS-DA R²=0.99, Q²=0.87

The most statistically significant metabolites ($p < 0.05$) that caused the excellent bioactivity were encircled in black in the S-plot (Figure 5.5D). These features were then dereplicated using an in-house Excel macro with Dictionary of Natural Products database. The hits were listed at Table 5.2 while the structures were given in **Error! Reference source not found.**

The chemical features of the compound hits were diverse that included anthraquinones, depsidones, naphthols, etc. TAN-1612 and 8-methoxy-5-methylmellein

has been previously annotated in the preliminary screening work (see Table 3.2). Some of the dereplicated metabolites have been earlier reported from the genus *Hypoxylon*. This includes 5-methylmellein (Anderson, Edwards, & Whalley, 1983), 8-methoxy-5-methylmellein (Chang et al., 2014), and 1,8-dimethoxynaphthalene (J. Zhang et al., 2020). Furthermore, lichenic acid compounds were also annotated such as norsolorinic acid (Anderson, Thomson, & Wells, 1966), decarboxyalectoronic acid (Elix, Adler, & Wardlaw, 1996), and superpicrolichenic acid (Elix, Calanasan, & Archer, 1991). These compounds could be targeted for isolation works to indicate whether endolichenic fungi could be the 'true' producers of these metabolites being used to assist the host in their chemical defence. However, while high resolution mass spectrometry could detect such compounds at nano-level concentrations, the feasibility of isolating lichenic acids would depend on their relative abundance in the extract. Albeit it was still of great advantage detecting these discriminatory metabolites at nano-level concentrations.

Table 5.2. Dereplication of discriminatory metabolites of the bioactive *H. lateripigmentum* extract grown in potato dextrose broth for 15 days. Arranged in increasing *p*-values (*p*<0.05)

<i>p</i> -value	<i>m/z</i>	Molecular weight (g/mol)	Molecular formula	Dereplication hit	Biological Source
7.31E-08	323.0560	324.0633	C ₁₈ H ₁₂ O ₆	Asperxanthone	<i>Aspergillus</i> sp. MF-93 and <i>Phomopsis amygdali</i>
8.13E-08	399.0722	400.0794	C ₂₀ H ₁₆ O ₉	Versiconal acetate	<i>Aspergillus flavus</i> and <i>Aspergillus parasiticus</i> , <i>Bipolaris sorokiniana</i> ,
1.12E-06	193.0857	192.0785	C ₁₁ H ₁₂ O ₃	5-Methylmellein	<i>Aspergillus</i> sp., <i>Fusicoccum amygdali</i> , <i>Hypoxylon illitum</i> , <i>Hypoxylon</i> sp., <i>Lachnellula</i> sp., <i>Nummularia discreta</i> , <i>Pezicula livida</i> , <i>Phomopsis oblonga</i> , <i>Phomopsis oblonga</i> 118, <i>Pithomyces</i> sp., <i>Plectrophomella</i> sp., <i>Valsa ceratosperma</i> .
3.47E-06	415.1017	414.0944	C ₂₁ H ₁₈ O ₉	TAN-1612	<i>Penicillium claviforme</i> FL-27337
3.83E-06	207.1013	206.0941	C ₁₂ H ₁₄ O ₃	8-Methoxy-5-methylmellein	<i>Hypoxylon investiens</i> BCRC 10F0115
5.05E-06	189.0909	188.0836	C ₁₂ H ₁₂ O ₂	1,8-Dimethoxynaphthalene	<i>Daldinia concentrica</i> , <i>Sporothrix</i> sp. No. 4335 and the marine-derived <i>Hypoxylon rubiginosum</i> FS521 and <i>Daldinia eschscholtzii</i> HJ001
1.97E-05	369.0977	370.1050	C ₂₀ H ₁₈ O ₇	Norsolorinic acid	Constituents of lichens <i>Lecidea piperis</i> and <i>Solorina crocea</i> . Produced by <i>Aspergillus versicolor</i> and <i>Aspergillus parasiticus</i> , <i>Emericella corrugata</i> , <i>Emericella navahoensis</i> and a marine-derived <i>Aspergillus versicolor</i> SH0105
6.41E-05	429.0819	430.0892	C ₂₁ H ₁₈ O ₁₀	Purpurquinone D	<i>Aspergillus</i> sp. 16-5C
0.001014	496.2424	497.2497	C ₂₂ H ₃₅ N ₅ O ₈	Thr-ser-lys-tyr	Not reported

0.001147	497.2529	496.2457	C ₂₉ H ₃₆ O ₇	Penicilone A	<i>Penicillium janthinellum</i> HK1-6
0.001209	469.2218	468.2145	C ₂₇ H ₃₂ O ₇	Decarboxyalectoronic acid	Constituent of the lichen <i>Hypotrachyna gracilescens</i>
0.001943	499.2686	498.2613	C ₂₉ H ₃₈ O ₇	Superpicrolichenic acid	<i>Pertusaria truncata</i>
0.002269	230.1172	229.1099	C ₁₄ H ₁₅ NO ₂	Penipaline C	<i>Penicillium paneum</i> SD-44
0.002788	473.2894	472.2821	C ₂₈ H ₄₀ O ₆	Lithocarin B	<i>Diaporthe lithocarpus</i> A740

Compound dereplication are based on highly accurate mass matching (<5 ppm) against Dictionary of Natural Products, with filtering by fungal biological source. No tandem MS/MS data were acquired; therefore, these are tentative assignments that require confirmation through MS fragmentation data or isolation (cells highlighted in blue correspond to compounds that were subsequently isolated)

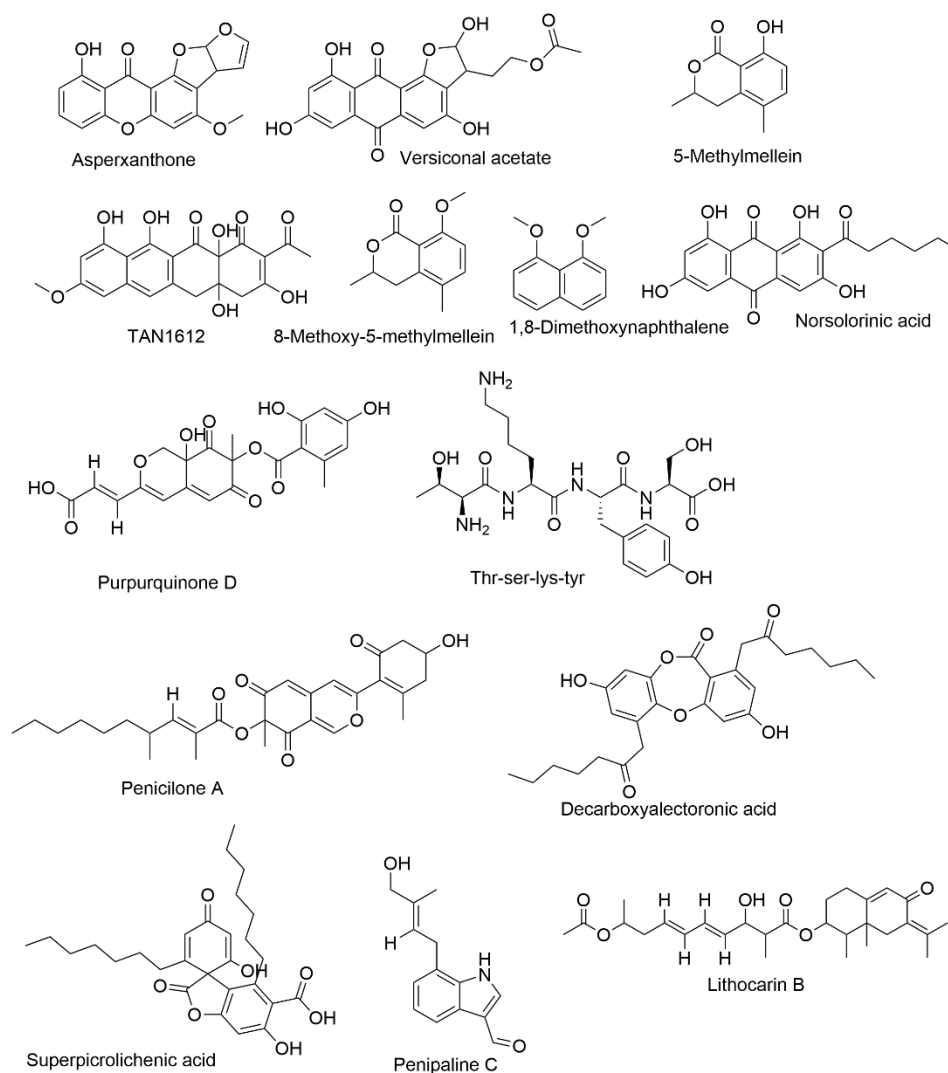


Figure 5.6. Chemical structure of discriminatory metabolites from the bioactive extract *H. lateripigmentum* grown in potato dextrose broth for 15 days

The heatmap illustrated in Figure 5.7 depicted the relative abundance of the discriminatory metabolites pinpointed from the crude extract obtained from the 15-day PDB culture. Notably, the intensity of the discriminatory metabolites was very high in the extracts from the 30-day PDB culture compared with that of the 15-day PDB culture extract, suggesting metabolite accumulation increased over time. However, as shown in Table 5.1, the antibacterial activity of extracts from the 15-day PDB culture were higher

than 30-day PDB extract. This discrepancy may be due to the antibacterial compound(s) not being among the annotated features listed in Table 5.2. It is possible that these compound(s) were not ionizable or outside the measurement range in our mass spectrometry methodology and therefore not being detected.

Additionally, the heatmap in Figure 5.7 shows the presence of three notable metabolites—TAN-1612, purpuquinone D, and norsolorinic acid—in the 30-day MEB culture extract. This extract also exhibited strong antibacterial and prebiofilm activity (Table 5.1). Thus, 30-day cultivation in MEB may also serve as a viable alternative for scale-up production. Despite the higher metabolite yield at 30 days, the 15-day PDB culture extract is sufficient to produce bioactive metabolites with both antibacterial and prebiofilm activities. Therefore, the 15-day cultivation is a more practical and time-efficient option for scaling up.

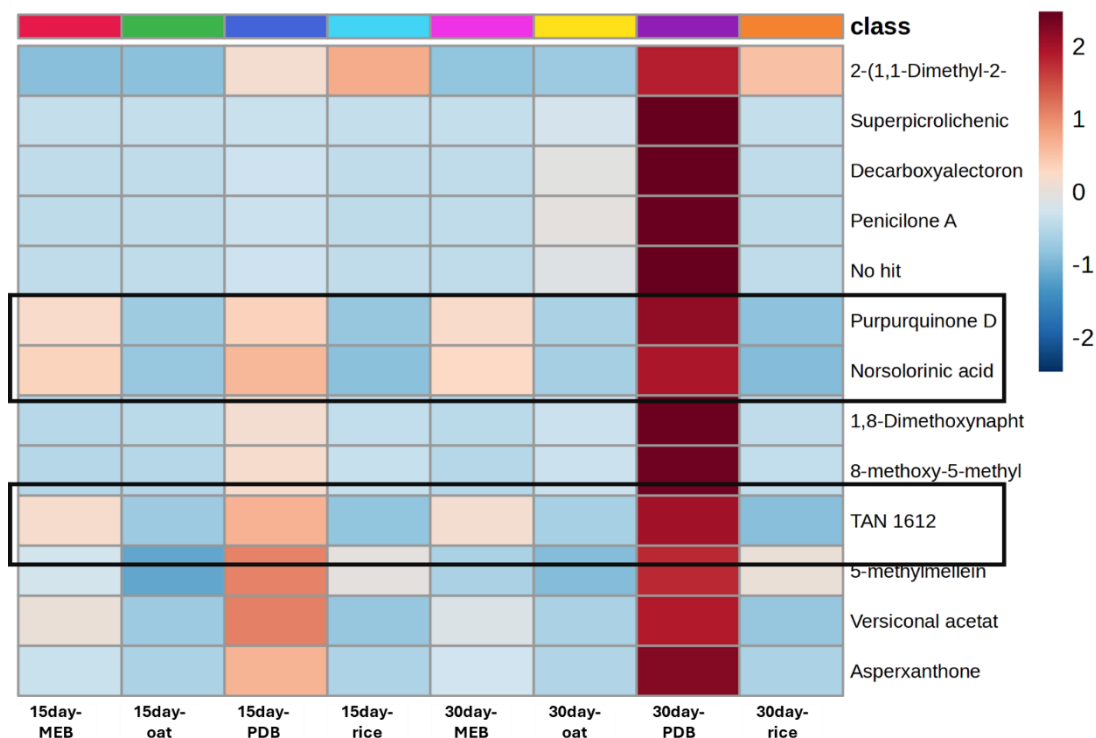


Figure 5.7 Heatmap of top discriminatory metabolites from the bioactive extract *H. lateripigmentum* grown in potato dextrose broth for 15 days. Boxed in black are features that are present in 2 or more media such as MEB and PDB. Heatmap was generated using MetaboAnalyst v6.0

5.2.3 Summary of OSMAC works

The 15-day PDB and 30-day MEB culture conditions of *Hypoxylon lateripigmentum* were determined to be the best conditions for scale-up based on their strong bioactivities and distinct metabolomic profiles. The 15-day PDB extract exhibited potent antibacterial and dual prebiofilm inhibition against *MRSA* and *P. aeruginosa*, making it the most promising candidate for further work. Metabolomic profiling analyses revealed distinct chemical profiles specially for 15-day PDB and 30-day MEB culture extracts. Through LC-MS-based metabolomics, key bioactive compounds such as TAN-1612, purpuquinone D, and norsolorinic acid were annotated on the three fungal extracts. Meanwhile, ^1H -NMR-based profiling unravelled that the chemical shifts 4.7 and 2.8 ppm were distinct in the 15 and 30-day PDB culture extracts while completely absent in 30-day MEB extracts. The 30-day MEB extract, while cultivated for a longer period, also

displayed strong antibacterial and prebiofilm activity. It exhibited a different metabolite profile, with the absence of the distinct peaks observed in the PDB extract, indicating a separate set of bioactive compounds. The selection of these two conditions for scale up aimed to maximize both chemical diversity and biological potency, providing a comprehensive basis for downstream isolation and structural elucidation as detailed in 5.2.3 and 5.4.

5.3 *Hypoxylon lateripigmentum* grown in potato dextrose broth

5.3.1 Scaled up production and solvent partitioning of bioactive metabolites

H. lateripigmentum was scaled up using its optimised media conditions on PDB media. In particular, 250 mL x 20 PDB flasks for a total of 5.0 L were prepared, inoculated, and incubated for 15 days at 27 °C. The resulting fungal cultures were extracted with ethyl acetate to afford a total crude extract of 9.67 grams.

The extract was then reconstituted in 100 mL of 90:10 methanol/water mixture and partitioned over 100 mL hexane thrice to remove lipids. The polar and non-polar layers were coded as HM and HH, respectively. The ¹H-NMR spectra, shown in Figure 5.8, clearly depicted that lipids were effectively removed and was mainly present in HH extract only. This is because of the peaks around 1.2-1.5 ppm (boxed in black; peaks for methylene groups of lipids) can be found in HH. From then, purification works was focused on the bioactive HM extract. The schematic diagram was shown in Figure 5.9.

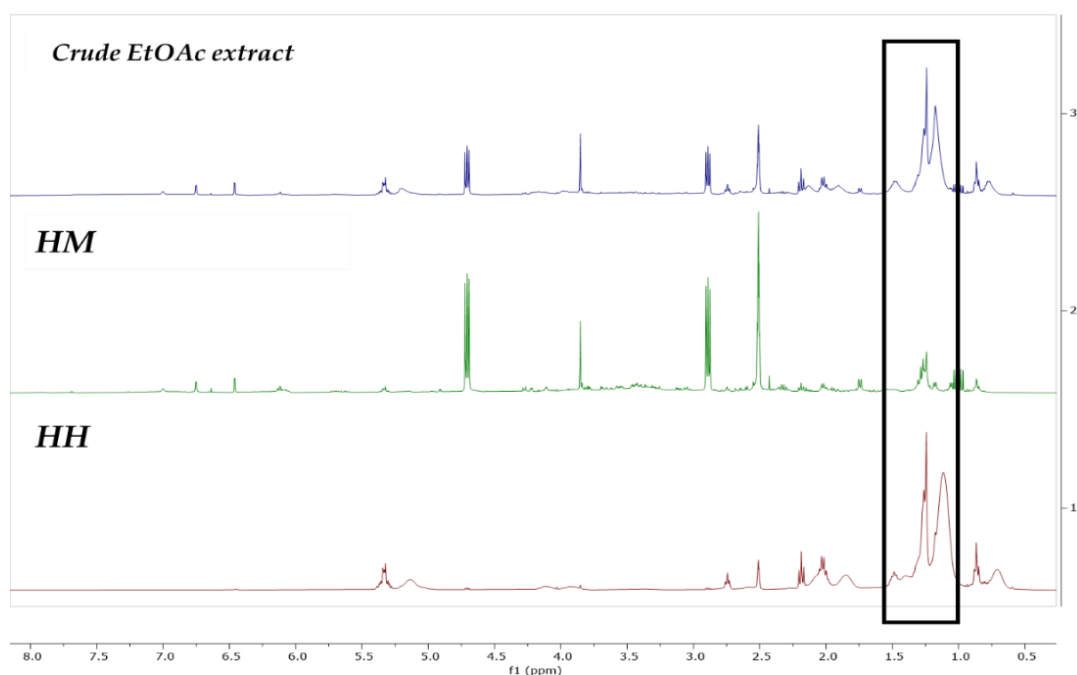


Figure 5.8. ^1H -NMR spectrum of *H. lateripigmentum* crude extract, HM, and HH. Boxed in black represents the long chain methylene protons of lipids. ^1H -NMR recorded in 400 MHz in DMSO-d_6

5.3.2 Fractionation

A fractionation step was carried out with HM. As HM were being prepared for fractionation, a dark brown precipitate was obtained and isolated. This precipitate, coded as HP (600mg), was insoluble in acetone. HP was established as a pure compound through NMR analysis. The structure identification of HP was detailed in section 5.3.4.1

HM was fractionated in Buchi system over a silica cartridge in a linear gradient elution programme of hexane and ethyl acetate (Table 5.3). Fifty (50) mL collection was done and monitored in TLC to pool similar fractions. A total of eight pooled fractions were acquired with weights displayed in Figure 5.9. TLC summary plates shown in Figure 5.10. The fractions were coded HM1 to HM8

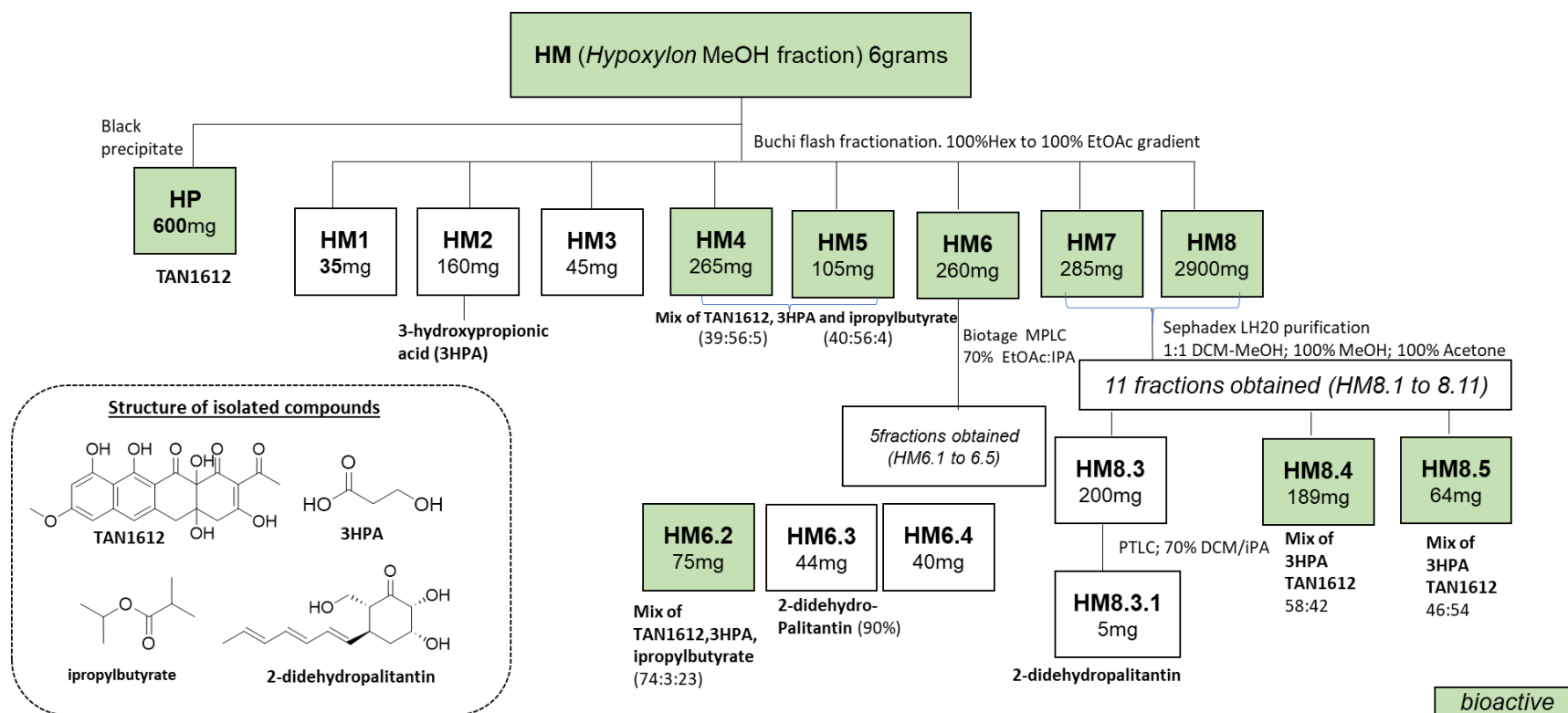


Figure 5.9 Schematic diagram summary for the isolation works done with *H. lateripigmentum* grown in PDB for 15 days

Table 5.3. Parameters for HM fractionation in Buchi

Stationary phase: VersaPak Silica Cartridge, 40 x150mm (Sigma)	
Time (min)	Solvent
0-5	100% Hexane
5-60	100% hexane to 100% EtOAc (linear gradient)
60-70	100% EtOAc
70-80	50% EtOAc in Acetone (isocratic)
80-85	100% Acetone
85-90	80% Acetone in Methanol (isocratic)
Flow rate: 100ml/min; collection vol: 50mL	

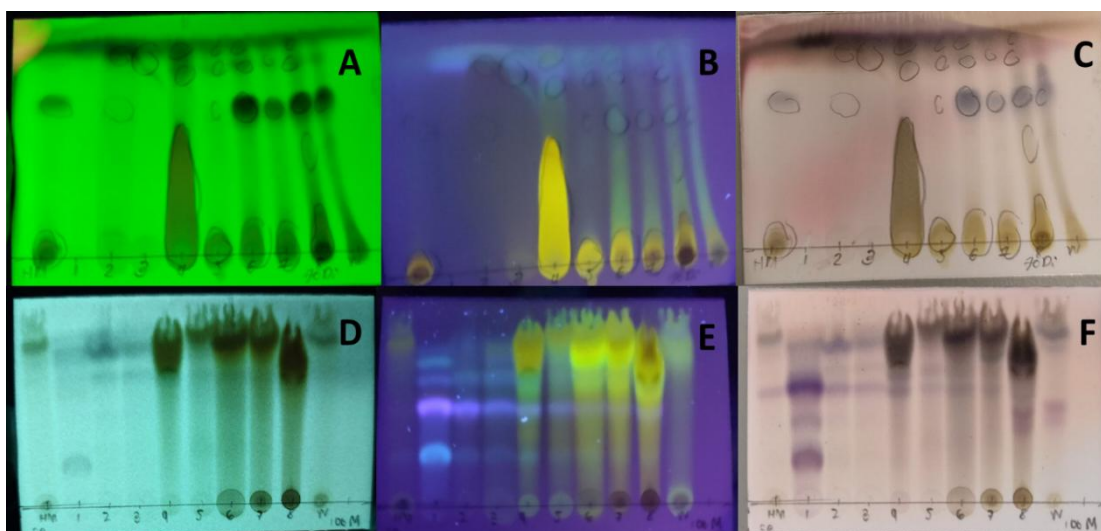


Figure 5.10. TLC summary of HM fractions in normal phase silica gel (A-C) and reverse phase (D-F). A and D) UV254nm; B and E) UV365nm; C and F) sprayed with anisaldehyde. Solvent system for normal phase TLC: 7:3 DCM/isopropanol; Solvent system for reverse phase TLC: 100% methanol

The eight fractions together with HM were tested for bioassays and its results were shown in Table 5.4. The strong antibacterial and prebiofilm activities of the HM were retained in fractions HM4 to HM8. It implied that polar metabolites could be the main reason for the observed bioactivities. HM do not possess any postbiofilm activity but HM1, HM4, and HM7 showed moderate inhibition.

Table 5.4 Biological activities of HM fractions

ANTIBACTERIAL ACTIVITY, average % inhibition (n=3)					
Sample	MRSA	<i>P. aeruginosa</i>	Sample	MRSA	<i>P. aeruginosa</i>
Crude HM	92.09	13.35	HM6	85.62	15.22
HM1	48.87	24.96	HM7	91.11	7.22
HM2	21.01	7.45	HM8	91.64	22.17
HM3	18.52	10.92			
HM4	95.38	14.62	DMSO	10.27	3.21
HM5	97.44	18.95	Ciprofloxacin (50 μ M)	97.78	96.52
PREBIOFILM ACTIVITY, average % inhibition (n=3)					
Sample	MRSA	<i>P. aeruginosa</i>	Sample	MRSA	<i>P. aeruginosa</i>
Crude HM	99.99	84.36	HM6	99.92	70.26
HM1	38.02	70.19	HM7	99.93	87.94
HM2	20.69	23.31	HM8	99.94	85.46
HM3	21.41	47.03			
HM4	99.92	61.68	DMSO	3.06	-14.03
HM5	99.71	71.40	Ciprofloxacin (50 μ M)	99.45	96.77
POSTBIOFILM ACTIVITY, average %inhibition (n=3)					
Sample	MRSA		Sample	MRSA	
Crude HM	34.18		HM6		65.87
HM1	14.12		HM7		61.95
HM2	2.37		HM8		51.20
HM3	3.96				
HM4	50.05		DMSO		0.29
HM5	18.27		Ciprofloxacin (50 μ M)		87.07

Testing concentration=100 μ g/mL; assays were done twice with three replicates (n=3);
 Moderate inhibition: 50-70% (coloured in blue); Strong inhibition >70% (coloured in red)

The fractions were subjected for ^1H -NMR analysis and their stacked spectrum were showed in Figure 5.11. The sharp two peaks at 4.6 and 2.8 ppm from the HM parent fraction appeared on almost all fractions starting from HM2. These two peaks were also seen constantly even at different media (section 5.2.2.1). Their intensities were decreasing as the polarity of the fractions increased (from HM1 to HM8). HM2 and HM3 were

almost similar and were of higher purity. These can be considered pure compounds though they are inactive. HM4 and HM5 still contains those two characteristic peaks but to a lesser extent. These fractions contain minor impurities such as the two doublets at 1.0 ppm.

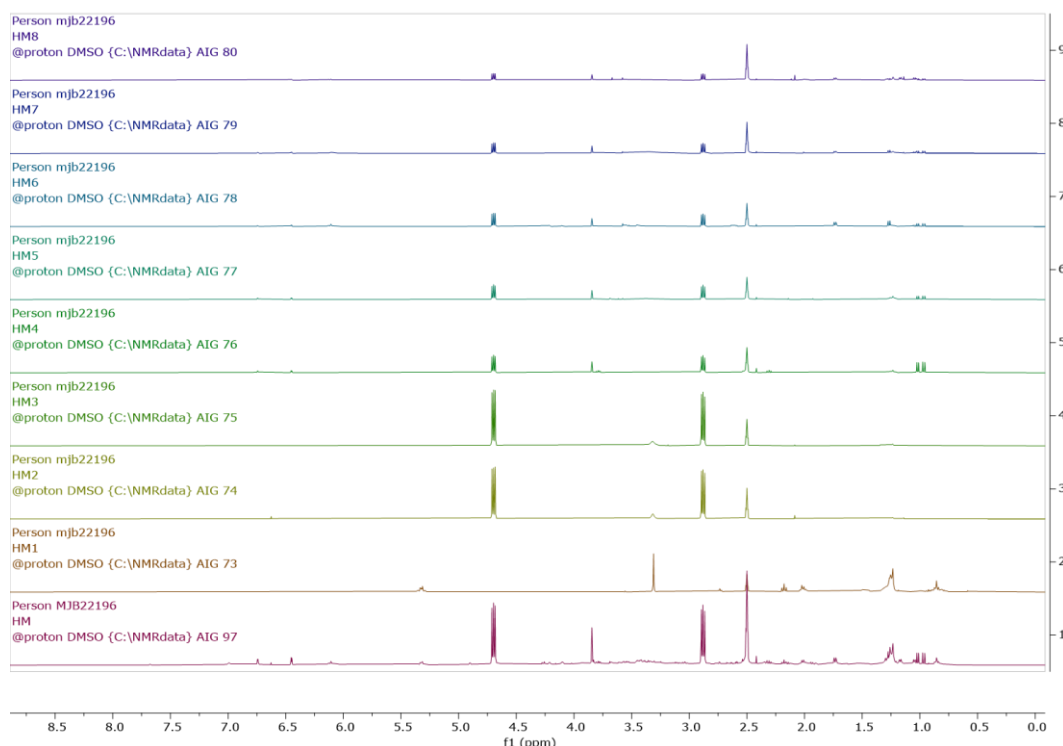


Figure 5.11. Stacked ^1H -NMR spectrum of HM fractions in ascending order. ^1H -NMR recorded in 400 MHz in DMSO-d_6

To further investigate the chemical profiles of the respective fractions, NMR-and LC-MS analyses were employed. Figure 5.12 illustrated the trends in HM fractions based on NMR. From the PCA scores plot, it is visible that the bioactive fractions (HM4 to 8) were clustering together and were distant from HM1 and HM2 and 3. This pattern can also be seen on their TLC profiles (Figure 5.10). Looking at the corresponding loadings plot at Figure 5.12B, the characteristic chemical shifts for HM1 were 1.21 and 1.26 ppm which were most probably lipid molecules. This is expected since HM1 is the first fraction

eluate or the most non-polar. On the other hand, the 4.7 ppm and 2.8 ppm were the distinct signals for HM2 and HM3 fraction. Upon probing, HM2 was pure, and its structure elucidation will be described later (section 5.3.4.2). Lastly, the bioactive fractions, HM4 to 8, clustered near the origin which reflects similar chemical fingerprint. Overall, the NMR-based metabolomics approach successfully distinguished and characterized the fractions in relation to their biological properties.

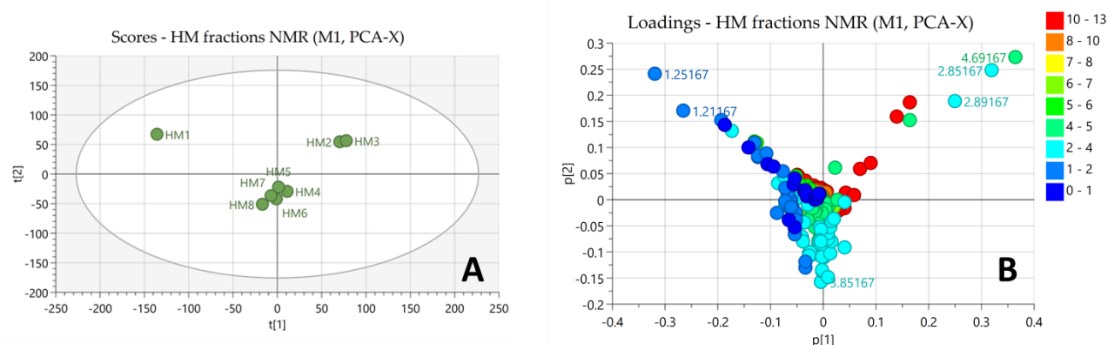


Figure 5.12. ^1H -NMR based multivariate analysis of HM fractions. A) PCA scores plot, and B) PCA loadings plot. $R^2=0.94$, $Q^2=0.71$

The LC-MS based chemical profiling analysis was displayed in Figure 5.13. The PCA scores plot (Figure 5.13A) did not have any clear clusters but the bioactive fractions, HM4 to 8, were all positioned on the right quadrants of the plot (encircled in red). The plot did not have the same pattern with that of the NMR spectral dataset. The corresponding loadings plot (Figure 5.13B) showed higher chemical diversity for HM4 to 8. This illustrated the differences between the said fractions.

A closer examination of the OPLS-DA scores plot (Figure 5.13C) showed that the active fractions were positioned on the left quadrants, with HM4 and HM5 located in lower left quadrant, distinct from the clustered HM6 to HM8 in upper left quadrant. This suggests that HM4 and HM5 may contain a different set of metabolites compared to the other active fractions. However, these differences cannot be resolved from this model

alone. The S-plot revealed the discriminating metabolites on the active fractions instead. Those were encircled in blue in Figure 5.13D. The most statistically significant (lowest p -values) features were dereplicated using the Dictionary of Natural Products. Hits were summarized in Table 5.5 and **Error! Reference source not found.** Most of the dereplicated hits were already annotated in media optimisation stage (see Table 5.2). Hence, the tools utilized were effectively predicting the isolation of these metabolites.

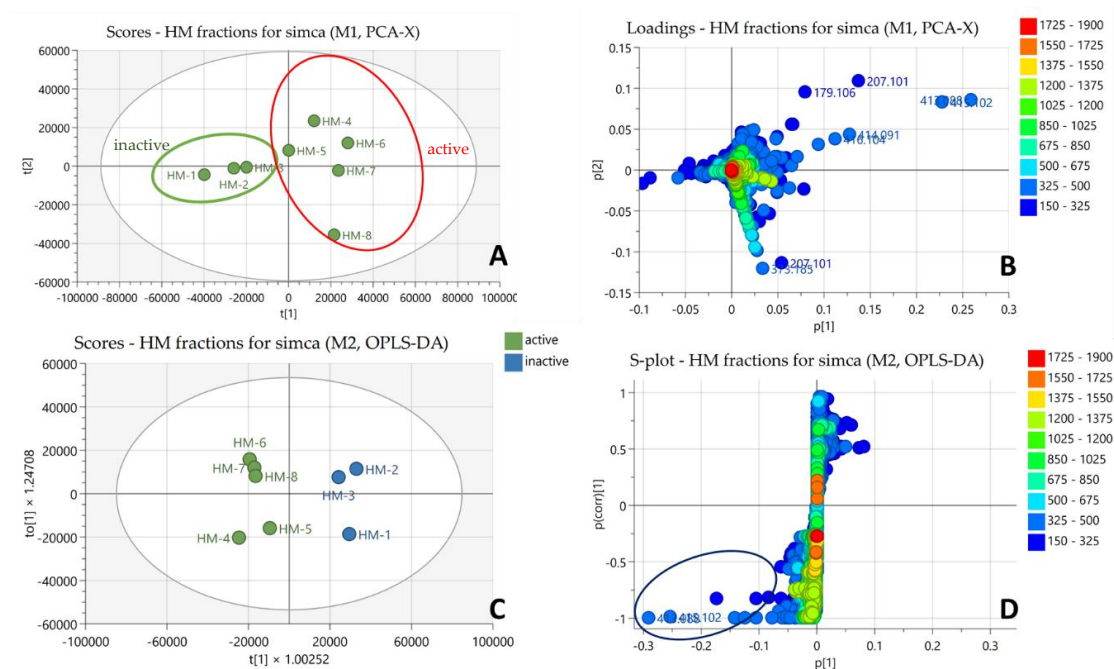


Figure 5.13. LCMS-based multivariate analysis of HM fractions A) PCA Scores plot, B) PCA Loadings Plot, C) OPLSDA Scores plot, D) OPLSDA S-plot. Encircled in red are the active HM fractions while green is the inactive. Encircled in black in the S-plot were the discriminatory metabolites for the bioactive fractions. PCA: $R^2=0.60$, $Q^2=0.08$; OPLS-DA $R^2=0.96$, $Q^2=0.85$

Table 5.5 Dereplication of discriminatory metabolites from the bioactive HM5 to 8 fractions, derived from the S-plot

<i>p</i> -value	<i>m/z</i>	Molecular weight (g/mol)	Molecular formula	Dereplication hit	Biological Source
2.97E-05	369.0975	370.1048	C ₂₀ H ₁₈ O ₇	Norsolorinic acid	Constituents of lichens <i>Lecidea piperis</i> and <i>Solorina crocea</i> . Produced by <i>Aspergillus versicolor</i> and <i>Aspergillus parasiticus</i> , <i>Emericella corrugata</i> , <i>Emericella navahoensis</i> and a marine-derived <i>Aspergillus versicolor</i> SH0105
3.17E-05	429.0819	430.0892	C ₂₁ H ₁₈ O ₁₀	Purpurquinone D	<i>Aspergillus</i> sp. 16-5C
0.000108	416.1045	M+2H of TAN-1612	C ₂₁ H ₁₈ O ₉	TAN-1612	<i>Penicillium claviforme</i> FL-27337
0.000117	415.1015	414.094			
0.002834	399.072	400.0792	C ₂₀ H ₁₆ O ₉	Versiconal acetate	<i>Aspergillus flavus</i> , <i>Aspergillus parasiticus</i> , and <i>Bipolaris sorokiniana</i> ,
0.01193	395.0771	396.0843	C ₂₁ H ₁₆ O ₈	Preussomerin M	Isol. from a mangrove-derived <i>Lasiodiplodia theobromae</i>
0.033316	179.1064	178.0991	C ₁₁ H ₁₄ O ₂	Trienylfuranone B	<i>Hypoxylon submonticulosum</i> DAOMC 242471 on <i>Rubus idaeus</i>
0.04055	208.1042	M+2H of 8-methoxy-5-Methylmellein	C ₁₂ H ₁₄ O ₃	8-Methoxy-5-methylmellein	<i>Hypoxylon investiens</i> BCRC 10F0115
0.041124	207.1012	206.0939			
0.044419	189.0907	188.0834	C ₁₂ H ₁₂ O ₂	1,8-Dimethoxynaphthalene	<i>Daldinia concentrica</i> , <i>Sporothrix</i> sp. No. 4335 and the marine-derived <i>Hypoxylon rubiginosum</i> FS521 and <i>Daldinia eschscholtzii</i> HJ00
Compound dereplication are based on highly accurate mass matching (<5 ppm) against Dictionary of Natural Products, with filtering by fungal biological source. No tandem MS/MS data were acquired; therefore, these are tentative assignments that require confirmation through MS fragmentation data or isolation (cells highlighted in blue correspond to compounds that were subsequently isolated)					

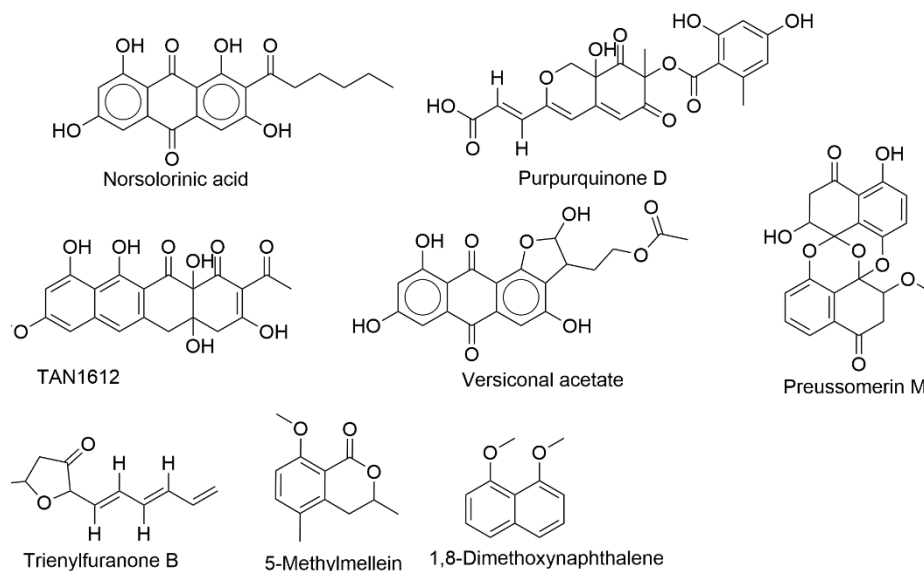


Figure 5.14 Chemical structures of hits from HM5 to 8 fractions

5.3.3 Chromatographic purification of HM fractions

HM6 was selected for further purification because of its strong bioactivity. It was fractionated on the Biotage™ MPLC system over a silica cartridge (10 g Sfär HP-Sphere, 25-33 μm , Biotage™). A sequential isocratic elution started at 40% ethyl acetate/hexane for 28 mins, followed by 100% ethyl acetate for 22 mins then 50% ethyl acetate/acetone for 10 mins. Eluates were collected followed by TLC monitoring to pool similar fractions. After this, five fractions were obtained and were coded HM6.1 to 6.5. The major peaks from HM6 can be found on HM6.2 and 6.3. Thus, the composition of these samples was investigated in detail in section 5.3.4

The active fractions HM7 and 8 were also selected for further purification. They were combined since their NMR profile were similar. The combined fraction was subjected to a Sephadex LH-20 open column chromatography using the stepwise isocratic elution with 1L of 50% dichloromethane/methanol followed by 1L of 100% methanol, and 500 mL 100% acetone. A total of 11 fractions were pooled after TLC

monitoring and coded as HM8.1 to 8.11. HM8.3 was further purified by preparative TLC to afford HM8.3.1 to give a pure compound identified in detail in section 5.3.4.4

5.3.4 Structure elucidation of isolated compounds

5.3.4.1 HP as TAN-1612

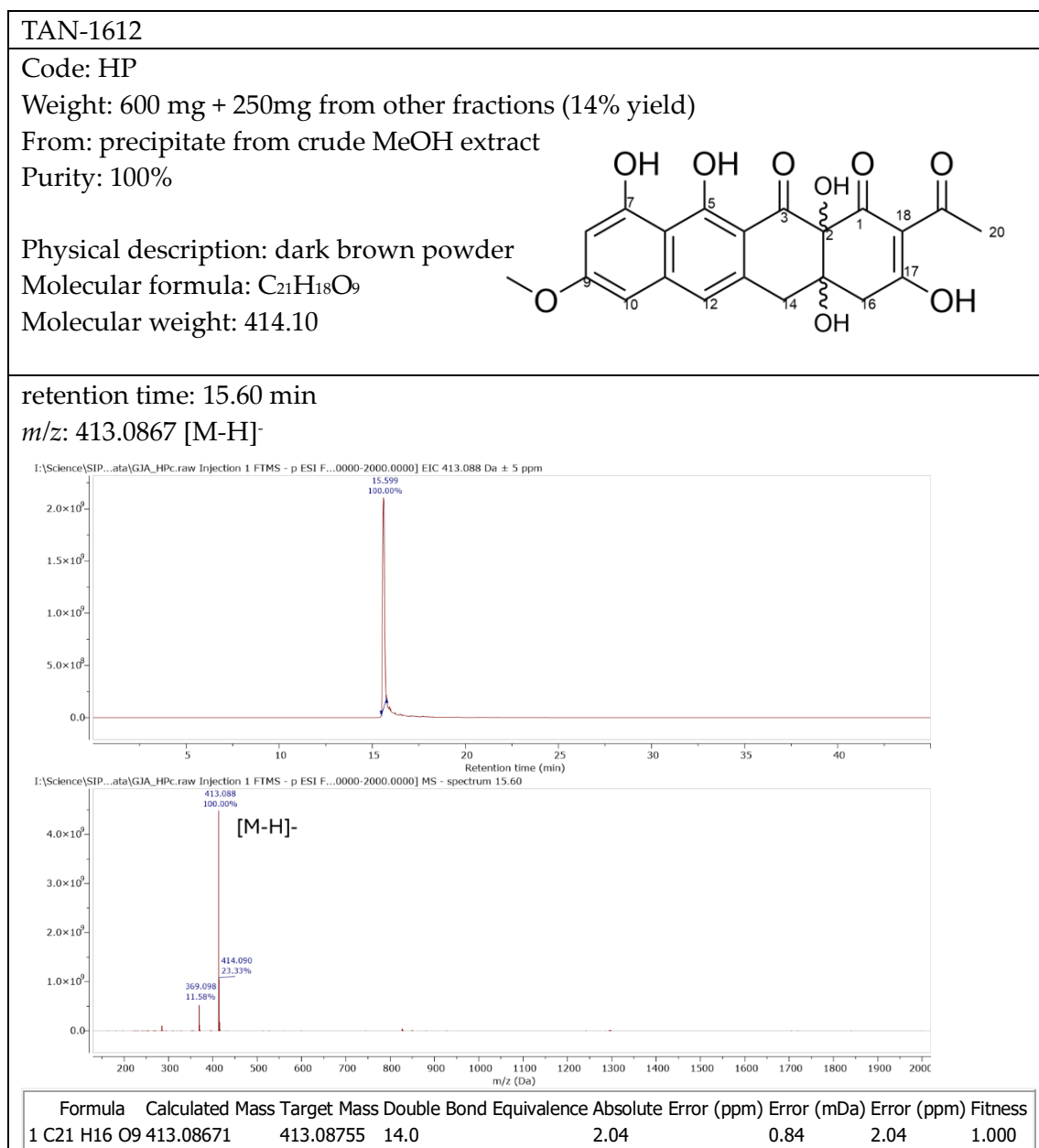


Figure 5.15 Chemical and spectroscopic data of HP as TAN-1612

HP was obtained as dark brown precipitated before fractionation of the crude extract (Figure 5.15). It has an ion peak of 413.0867 Da which was an $[M-H]^-$ corresponding to the molecular formula of $C_{21}H_{16}O_9$ (DBE=14). Combined analysis of 1H and ^{13}C NMR experiments (Table 5.6, Figure S 15Figure S 18), together with the metabolomics dereplication process, led to its structure elucidation as TAN-1612, a tetracycline analogue precursor (Baldera-Aguayo, Lee, & Cornish, 2022). In brief, there are three aromatic protons of which two protons are meta-coupling (δ_H in ppm, 6.55, d, 2.3, H-8, and δ_H in ppm, 6.60 d, 2.3, H-10). The other aromatic proton is a singlet at δ_H in ppm, 6.95 (1H, s, H-12). A methoxy group was present at δ_H 3.93 since its integration is 3.00 and is attached to a ^{13}C signal of 55.1 ppm. Deshielded exchangeable protons can be seen at δ_H 15.14(s) and δ_H in ppm, 9.53 (s) which were assigned for 5-OH and 7-OH, respectively. The two methylene groups (δ_H 3.45 (d, $J=15.6$ Hz), H-14a and δ_H 3.03 (d, $J=15.9$ Hz) H-14b; δ_H 2.94, m, H-16) and the terminal methyl group (δ_H 2.94, H-20) were assigned based on their HMBC correlations (Figure S 18). The NMR data closely agrees with the reported values of TAN-1612 (Table 5.6) (Li et al., 2011).

Furthermore, TAN-1612 were determined to be present in other fractions including HM4, HM5, HM6.2, HM8.4, and HM8.5 as evident in their 1H -NMR spectrum (Figure 5.16). However, impurities can be seen on these fractions. The impurities were most likely the same compound. The relative percentage of TAN-1612 in the fractions were computed by q NMR. This brings an additional 250 mg of TAN-1612 for a total of 850mg yield. The 14% yield (600 mg + 260 mg in 6-gram crude extract) was quite high and thus can be considered as a major compound of this extract. The titre production of TAN-1612, in this case, is calculated to be 170 mg per litre of PDB media.

Table 5.6 ^1H and ^{13}C NMR data comparison of HP from reported values of TAN-1612

Atom no	HP, This study		TAN-1612 (Li et al., 2011)	
	δ_{H} in ppm, (J in Hz) 400MHz in CDCl_3	δ_{C} in ppm 100MHz in CDCl_3	δ_{H} in ppm, (J in Hz) 500Mhz in CDCl_3	δ_{C} in ppm 125Mhz in CDCl_3
1	-	189.9	-	190.6
2	-	71.3	-	72
3	-	195.7	-	196.4
4	-	105.6	-	106.3
5	-	159.4	-	167.5
5-OH	15.14 (1H, s br)	-	15.03 (s br, 1H)	-
6	-	107.4	-	108.1
7	-	159.4	-	160.1
7OH	9.53 (1H, s)	-	9.47 (s, 1H)	-
8	6.55 (1H, d, 2.33)	101.2	6.49 (d, 1H, 1.95)	101.9
9	-	164.1	-	167.5
9-OCH ₃	3.93 (3H, s)	55.1	3.88 (s, 3H)	55.8
10	6.60 (1H, d, 2.35)	100.0	6.55 (d, 1H, 1.95)	100.7
11	-	140.7	-	141.4
12	6.95 (1H, s)	117.9	6.89 (s, 1H)	118.6
13	-	132.6	-	133.3
14	3.45 (1H, d, 15.67) 3.03 (1H, d, 15.90)	38.1	2.97 (d, 1H, 15.80) 3.39 (d, 1H, 16.15)	38.8
15	-	79.0	-	79.7
16	2.94 (2H, m)	40.2	2.87 (d, 1H, 19.10) 2.94 (d, 1H, 19.10)	40.7
17	-	194.2	-	195
18	-	110.1	-	110.8
19	-	202.1	-	202.8
20	2.71 (3H, s)	27.4	2.66 (s, 3H)	28.1

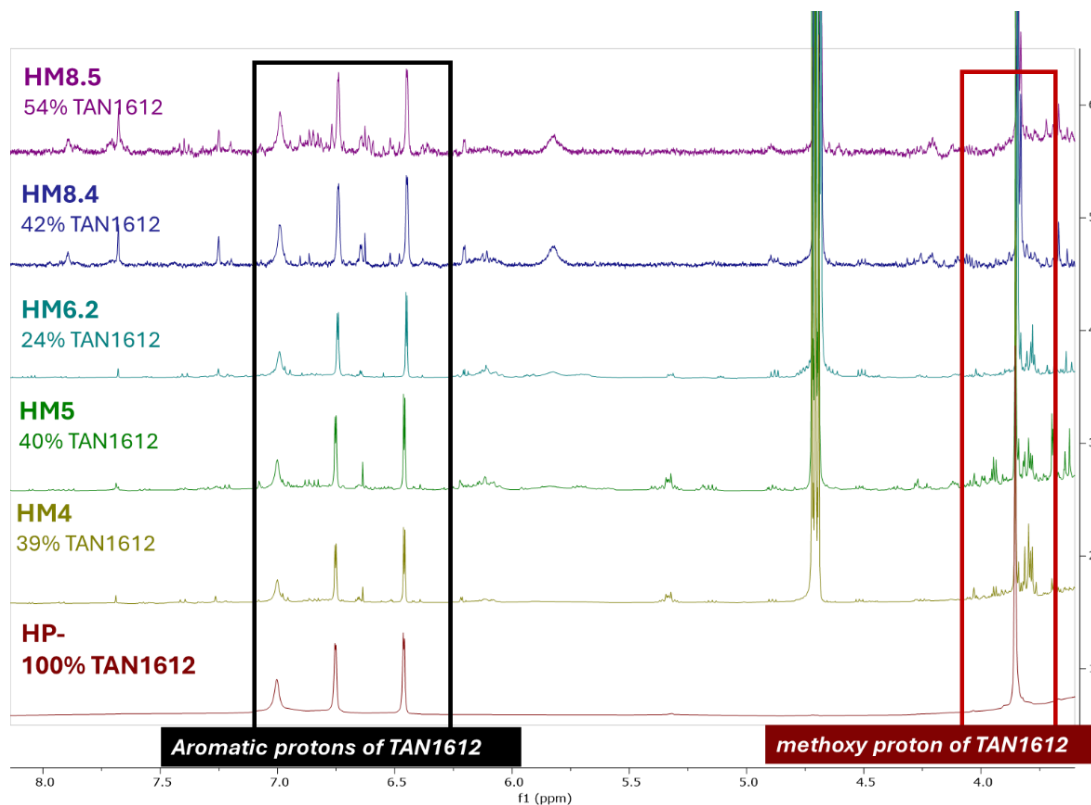


Figure 5.16 ^1H NMR of fractions that also contains TAN-1612. Boxed in black are the aromatic proton peaks while boxed in red are the methoxy proton of TAN-1612. Relative purity of TAN-1612 in each fraction was indicated. ^1H -NMR recorded in 400 MHz in DMSO-d_6

The isolation of TAN-1612 was expected because it has been defined as a target bioactive metabolite from the preliminary screening (Chapter 3), the media optimisation stage (Section 5.2), and the first fractionation step (Section 5.3.2). Thus, it is not surprising that it was purified and finally elucidated. TAN-1612 is a polycyclic aromatic polyketide that is structurally related to tetracyclines. It was originally isolated from the fungus *Penicillium claviforme* FL-27337 (Ishimaru, Tsuboya, & Saijo, 1994). Its tautomeric form was found to be produced by *Aspergillus niger* and exhibited neuropeptide Y and neurokinin-1 receptor antagonist activity (Kodukula et al., 1995; Shu et al., 1995). Herein, TAN-1612 production by *Hypoxylon lateripigmentum* will be reported for the first time.

TAN-1612 was considered as an excellent biosynthetic scaffold for tetracycline synthesis because of its chemical structural similarity to tetracycline. Additionally, heterologous expression and production in other fungal hosts was ideal since it is fungal metabolite. The enzymatic reaction mechanisms involved in the biosynthesis of TAN-1612 was elucidated in *Aspergillus niger* ATCC 1015 (Li et al., 2011). Furthermore, the authors have co-expressed the biosynthetic enzymes to *Saccharomyces cerevisiae* BJ5464-NpgA to produce around 10 mg/L titre of TAN-1612. Another study bioengineered *S. cerevisiae* with the co-expression of efflux pumps to alleviate the toxicity of TAN-1612 to the yeast itself (Baldera-Aguayo, Lee, & Cornish, 2022). Table 5.7 displays the yield of TAN-1612 in comparison from previous reports. Remarkably, *H. lateripigmentum* demonstrated significantly higher titre and production rate than the engineered yeast strains. This highlights a more efficient and natural alternative for TAN-1612 production. Hence, this study presents a more effective and natural approach in the production of TAN-1612. Additionally, the purification process in this study was more practical, eliminating the need for chromatographic steps.

Table 5.7 Comparison of TAN-1612 production from different strains and conditions

Parameter	This work	(Li et al., 2011)	(Baldera-Aguayo, Lee, & Cornish, 2022)
Strain	<i>H. lateripigmentum</i>	<i>S. cerevisiae</i> BJ5464-NpgA	<i>S. cerevisiae</i> #1474
Culture method	Potato dextrose broth with 2% glucose, 27 °C	Yeast extract peptone (YPD) medium with 1% glucose, 28 °C	Complete synthetic media (CSM) without glucose, 30 °C
Incubation period	15 days	2 days	4 days
Extraction and purification method	Ethyl acetate extraction, solvent partitioning	Ethyl acetate extraction, Sephadex LH20 purification	Ethyl acetate extraction, Sephadex LH20 purification
Titre (mg/L of media)	170	10	32.5
TAN-1612 production per day (mg/day)		5	8.13

5.3.4.2 3-hydroxypropanoic acid (3HPA)

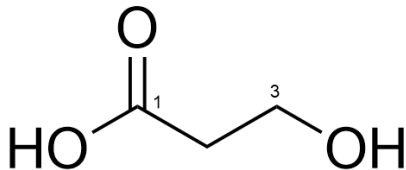
3-hydroxypropanoic acid (3HPA), 3-hydroxypropionic acid, β -lactic acid, hydracrylic acid	
Code: HM2 and HM3	
Weight: 195mg + 375mg from other fractions (9.5% yield)	
From: HM2, HM3, and other fractions	
Purity: 100% in HM2 and HM3	
Physical description: yellow powder	
Molecular formula: C ₃ H ₆ O ₃	
Molecular weight: 90.08 g/mol	
Undetectable in the MS measuring range	

Figure 5.17 Chemical and spectroscopic data of HM2 and HM3

HM2 and HM3 (Figure 5.17) were both obtained as a yellow powder. Their TLC profiles were different (Figure 5.10) but their ¹H-NMR spectra were similar (Figure 5.11). The structure was established as 3-hydroxypropionic acid (3HPA) via combined ¹H and ¹³C NMR analysis (Table 5.8, S19-S21). Its ¹H NMR spectrum contains only two peaks which are also consistently present in the other fractions (Section 5.3.2). The two peaks (δ_{H} 4.71, t, $J=5.83$ Hz, H-2; and 2.89, t, $J=6.10$ Hz, H-3) in the spectra have equal integration and multiplicities (triplets) which mean that they correlate to each other. A methylene group, H-3, is attached to a carbon with δ_{C} 70.33 indicating an oxygen-bearing methylene carbon. On the other hand, the signal of H-2 at δ_{H} 4.70 is quite deshielded compared to H-3, suggesting that it is adjacent to an electron withdrawing group. In this case, it is the carboxyl group of the molecule. The overall structure was confirmed since its experimental signals were closely comparable with those from the literature (Table 5.8)(Schwarz et al., 2004)

Table 5.8. ^1H and ^{13}C NMR comparison of 3HPA from reported values

Atom no	This study		3-Hydroxypropionic acid (Schwarz et al., 2004)	
	δ_{H} in ppm, (J in Hz) 400MHz in DMSO- d_6	δ_{C} in ppm, 100MHz in DMSO- d_6	δ_{H} in ppm, (J in Hz) 500MHz in CDCl_3	δ_{C} in ppm, 125MHz in CDCl_3
1	-	171.5	-	171.7
2	4.71 (2H, t, 5.83)	30.6	4.61 (2H, t, 6.2)	30.8
3	2.89 (2H, t, 6.10)	70.4	2.95 (2H, t, 6.2)	69.8

3HPA was detected in HM2 and HM3 with absolute purity. On the other hand, it was also present with some impurities in other fractions such as HM4, HM5, HM6.2, HM8.4, and HM8.5 by looking at its ^1H -NMR shown in Figure 5.18. The concentration of 3HPA in each fraction was computed relative to identified impurities. The amount have 3HPA from these fractions has a sum of 375 mg. Thus, a total of 570 mg of 3HPA was

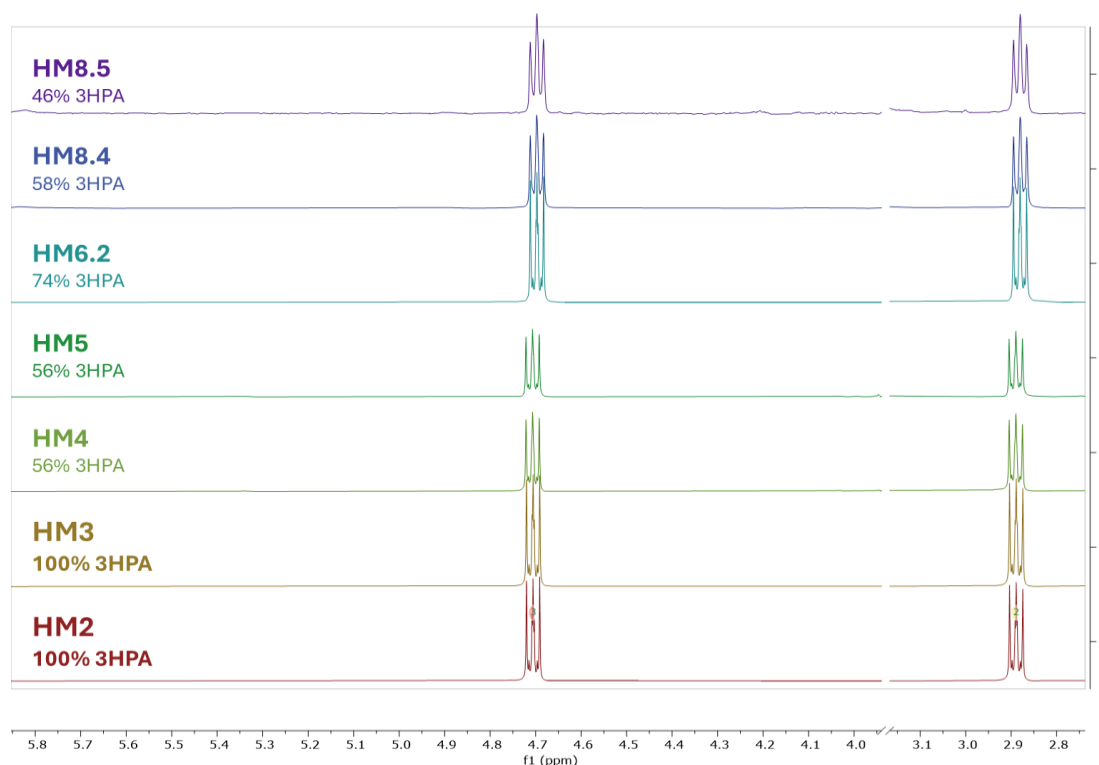


Figure 5.18 ^1H NMR (400MHz, DMSO- d_6) of fractions that also contains 3HPA. Relative purity of 3HPA in each were indicated. ^1H -NMR recorded in 400 MHz in DMSO- d_6

collected from the crude extract giving around 10% yield in the crude extract or a 114 mg/L of PDB media.

The compound 3HPA was first reported as a fungal metabolite in *Phomopsis phaseoli*, where it also exhibited selective nematocidal activity against *Meloidogyne incognita* (Schwarz et al., 2004). It also occurred in Egyptian propolis (Hegazi & El Hady, 2002), Herba Pogostemonis (Kim et al., 2012), stems of *Commiphora wightii* (Bhatia et al., 2015), a rare ginger *Amomum muricarpum* (Barbosa et al., 2017), and *Hibiscus cannabinus* leaves (Oh et al., 2021). To the best of current knowledge, this study will be the first to report the occurrence of 3HPA in *Hypoxyton lateripigmentum*.

3HPA is an organic acid related to lactic acid and is used as a key precursor in the manufacture of plastics, pharmaceuticals, and agriculture products. Traditional chemical synthesis using compounds like acrylic acid and 1,3-propanediol are costly, toxic, and environmentally harmful (Liang et al., 2019). Microbial fermentation of renewable feedstocks offers a more sustainable and promising alternative for 3HPA production (Kumar, Ashok, & Park, 2013; Matsakas et al., 2018). Several microorganisms have been genetically modified for increased production of 3HPA with titres up to 71.9 g/L (Chu et al., 2015). A recent study demonstrated the multidimensional engineering of the bacteria *Escherichia coli* for an efficient biosynthesis of 3HPA starting from glucose with a titre yield of 52.73 g/L (Zhang et al., 2023). This is far lower than the titre yield by *H. lateripigmentum* which is only around 0.114 g/L. Nevertheless, this current work presents a promising natural producer that does not require extensive genetic modification.

5.3.4.3 HM4 and other fractions contains isopropyl isobutyrate

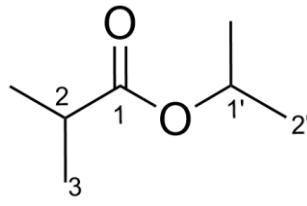
isopropyl isobutyrate (IPB), isopropyl 2-methylpropanoate	
Code: HM4, etc	
Weight: 18mg from different fractions (0.3% yield)	
From: HM4, HM5, and other HM6.2	
Purity: 5% in HM4	
Physical description: yellow powder	
Molecular formula: C ₇ H ₁₄ O ₂	
Molecular weight: 130.10 g/mol	
	
Undetectable in the MS measuring range	

Figure 5.19 Chemical and spectroscopic data of HM4 with IPB

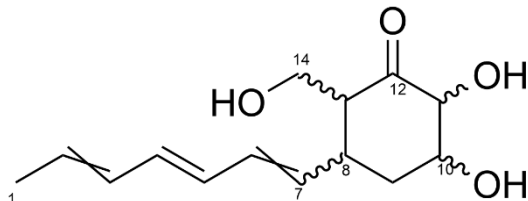
Isopropyl isobutyrate (IPB) was identified as a minor component in HM4, HM5, and HM6.2 (5%, 4%, and 2%, respectively). It could not be found in the LC-MS data because it has low molecular weight that is below the measuring range of 150 Da (Figure 5.19), but ¹H-NMR was able to identify its characteristic signals. Its full NMR data are shown in Figure S 23 to Figure S 26. Its ¹H and ¹³C NMR data closely agrees with its reported values (Table 5.9), thus confirming the elucidation of IPB.

Table 5.9 ¹H and ¹³C NMR comparison of IPB from reported values

Atom no	IPB, This study		Isopropyl isobutyrate (Alshakova, Gabidullin, & Nikonov, 2018)	
	δ_{H} in ppm, (<i>J</i> in Hz) 400MHz in DMSO-d ₆	δ_{C} in ppm, 100MHz in DMSO-d ₆	δ_{H} in ppm, (<i>J</i> in Hz) 400MHz in CDCl ₃	δ_{C} in ppm 125MHz in CDCl ₃
1	-	176.1	-	176.7
2	2.33 (1H, m)	46.8	2.47 (1H, m)	34.1
3	0.98 (6H, d, 7.04)	12.2	1.13 (6H, d, 6.2)	18.9
1'	3.80 (1H, m)	67.5	4.97 (1H, hept, 6.2)	67.2
2'	1.03 (6H, d, 6.33)	19.8	1.21 (6H, d, 6.2)	21.7

IPB is a carboxylic acid ester compound which has been noted for its sweet, citrus, and fruity taste profile (Yannai, 2012). It has been detected but not quantified in pineapple (*Ananas comosus*) fruits (Duke, 1994). The roots of Aconiti Radix (a product used in Traditional Chinese Medicine) consisted of volatile components such as IPB (Wang et al., 2014). This will be the first time of IPB report of occurrence from fungi.

5.3.4.4 HM6.3 and HM8.3.1 as 2,3-didehydropalitantin

2,3-didehydropalitantin	
Code: HM6.3 and HM8.3.1	
Weight: 73mg total (1.2% yield)	
From: HM6.2 and HM8.3	
Purity: 90% in HM6.2; 100% in HM8.3.1	
Physical description: yellow needles	
Molecular formula: C ₁₄ H ₂₀ O ₄	
Molecular weight: 252.14	
retention time: 10.22 min	
<i>m/z</i> : 253.1434 [M+H] ⁺	

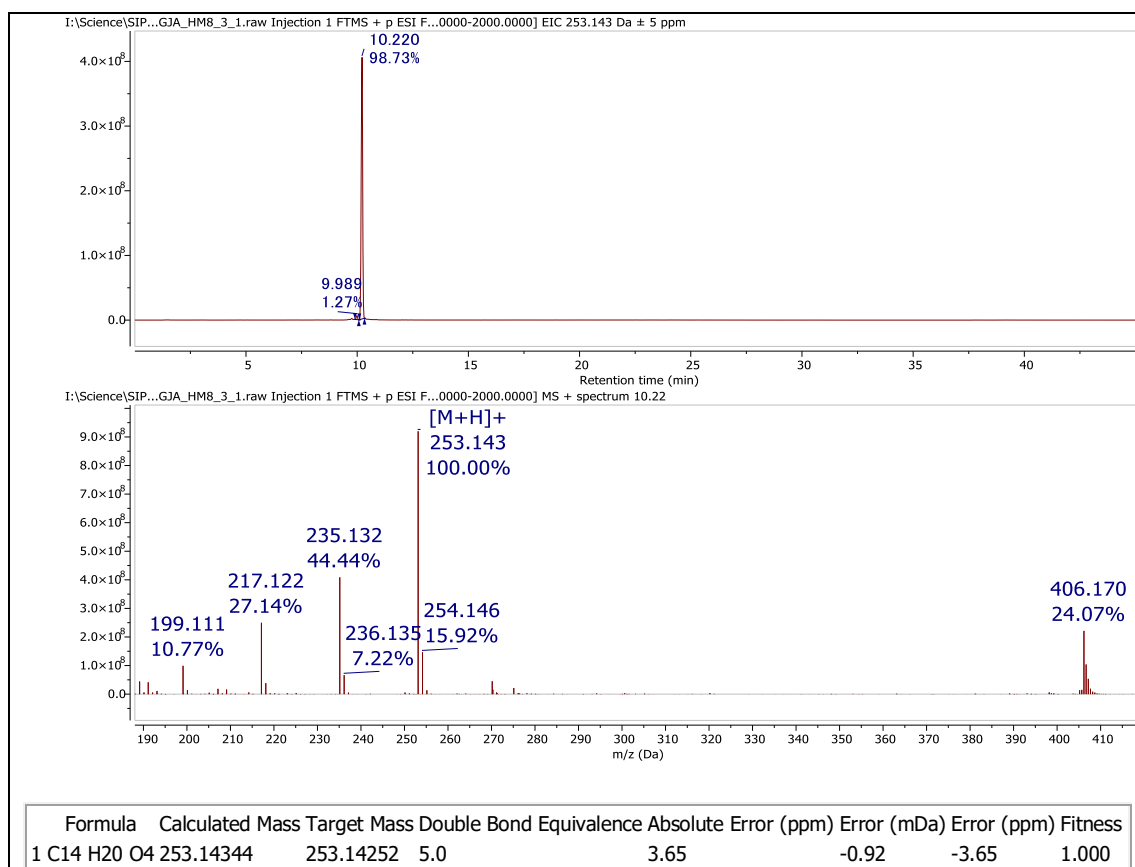


Figure 5.20 Chemical and spectroscopic data of HM8.3.1

HM8.3.1 was purified by preparative TLC from HM8.3 fraction. It was obtained as yellow needles. It has a base peak of 253.1452 $[M+H]^+$ which was calculated for 252.14 g/mol with a molecular formula of C₁₄H₂₀O₄ (DBE=5) as shown in Figure 5.20. Combined ¹H and ¹³C NMR analysis led its structure elucidation to be 2,3-didehydropalitantin (Table 5.10). A total of 6 olefinic protons were present of which 4 are overlapping in δ_H in ppm, 6.10-6.14 ppm. The other two olefinic protons are separated from the overlapped peaks and has a *trans* orientation due to large coupling constants (δ_H 5.77, dt, $J=14.6$, 6.8 Hz, H-2 and 5.63, dt, $J=14.1$, 8.9 Hz, H-7). The rest were aliphatic protons including two methine protons (δ_H 4.22, ddd, $J=6.4$, 3.3, 1.2 Hz, H-10; 4.11 d, $J=5.04$ Hz, H-11) attached to oxygenated carbons (δ_C 77.9, C-10; and 72.6, C-11). These vicinal methine protons exhibit a typical axial/equatorial coupling pattern, as indicated by a 3J of approximately

5 Hz. Additionally, a pair of diastereotopic methylene protons was observed at 3.56 ppm (ddd, $J = 11.4, 6.5, 5.3$ Hz, H-14') and 3.45 ppm (ddd, $J = 10.7, 5.3, 2.5$ Hz, H-14''), which are bonded to an oxygen-bearing carbon, resonating at δ_c 57.6 (C-14). The terminal methyl group was also implicated at δ_H 1.75 (3H, d, $J=6.81$ Hz, H-1). Its 2D NMR data (Figure S 27 to Figure S 29) together with its close comparison with reported values (Table 5.10) confirm its structure (Lee & Oh, 2006).

Table 5.10 ^1H and ^{13}C NMR comparison of 2,3-didehydropalitanin from reported values

AtomNo	This study		2,3-Didehydropalitanin (Lee & Oh, 2006)	
	δ_H in ppm, (J in Hz) 400Mhz in DMSO- d_6	δ_c in ppm 100Mhz in DMSO- d_6	δ_H in ppm, (J in Hz) 400Mhz in CD_3OD	δ_c in ppm 100Mhz in CD_3OD
1	1.75 (3H, d, 6.81)	18.6	1.74 (3H, d, 6.8)	
2	5.71 (1H, dt, 14.6, 6.8)	129.8	5.68 (1H, dq, 14.8, 6.8)	
3	6.18 – 6.04 (4H,m)	132.2-130.7	6.00-6.20 (1H, m)	131.8
4				132.6
5				132.0
6				129.7
7	5.63 (1H, dd, 14.1, 9.0)	136.4	5.55 (1H, dd, 14.4, 9.2)	134.3
8	2.61 (1H, m)	39.4	2.74 (1H, m)	39.3
9'	1.91 (1H, t, 13.1)	37.3	1.94 (2H, m)	36.7
9''	1.77 (1H, t, 4.0)			
10	4.22 (1H, ddd, 6.4, 3.3, 1.1)	77.9	4.26 (1H, dd, 6.0, 3.2)	77.6
10-OH	4.64 (1H, d, 6.4)	-	-	
11	4.11 (1H, d, 5.0)	72.6	4.28 (1H, d, 3.2)	72.6
11-OH	4.83 (1H, d, 2.9)	-	-	-
12	-	209.5	-	209.5
13	2.37 (1H, dddd, 15.1, 11.3, 7.6, 5.8)	55.2	2.41 (1H, m)	54.9
14'	3.56 (1H, ddd, 11.4, 6.5, 5.3)	57.6	3.68 (2H, m)	58.1
14''	3.45 (1H, ddd, 10.7, 5.3, 2.5)			
14-OH	4.11 (1H, d)	-	-	

2,3-Didehydropalitantin was identified in HM8.3.1. This compound was also identified in HM6.3 but with 90% relative purity as shown in Figure 5.21. The total yield of this compound was 73 mg from the 6-gram crude extract.

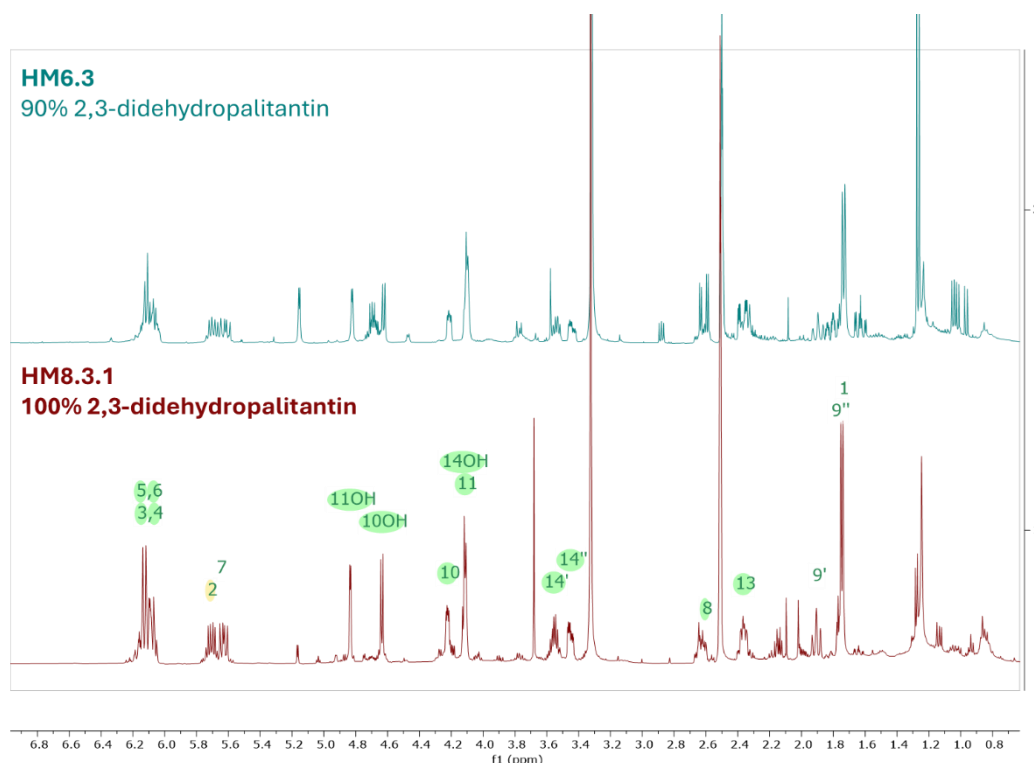


Figure 5.21 ^1H -NMR of fractions that also contains 2,3-didehydropalitantin. ^1H -NMR recorded in 400 MHz in DMSO-d_6

2,3-Didehydropalitantin was first reported from the epiphytic fungus *Paraphaeosphaeria* sp. It has a configuration of 8*R*, 10*S*, 11*S*, 13*R* (Lee & Oh, 2006). Its 10*R* enantiomer, named as dendrodochol A, was isolated from the sea-cucumber derived fungus *Dendrodochium* sp. (Xu et al., 2014). HM8.3.1 was assigned as in the former enantiomer because their coupling constants are equivalent. This cyclohexanol polyketide was a congener of palitantin isolated from *Penicillium palitans* (Bowden, Lythgoe, & Marsden, 1959). The antifungal and antibiotic derivative, frequentin, was

obtained from *Penicillium frequentans* (Curtis, Hemming, & Smith, 1951). Herein, this work reports the occurrence of 2,3-didehydropalitantin in *H. lateripigmentum*.

5.3.5 Biological activities of isolated compounds

Four different compounds were so far identified from isolation works in *H. lateripigmentum* grown in PDB. The compounds TAN-1612, 3HPA, and 2,3-didehydropalitantin were obtained from different fractions in varying purity. Their biological activities against MRSA were evaluated and the results was summarized in Table 5.11. Fractions containing IPB was not included since it was just a minor impurity.

HP is 100% pure TAN-1612 which exhibited excellent activity. It has low MIC and BPC values indicating a remarkable bioactivity, albeit quite far from the values of the positive control ciprofloxacin. The other fractions (HM8.4, HM8.5, HM6.2) that contain less TAN-1612 exhibited higher MIC and BPC values than the pure ones. Although, the bioactivity data do not seem directly proportional with the relative concentration of TAN-1612 in these fractions. On the other hand, 3HPA and 2,3-didehydropalitantin are completely inactive. All the samples were tested inactive against *P. aeruginosa* (data not included).

Table 5.11 Biological activities of compounds from *H. lateripigmentum* grown in PDB against MRSA

Sample code	Identity, %purity	Antibacterial activity		Prebiofilm activity		Postbiofilm activity	
		%Inhibition @100 ug/mL	MIC (μM)	%Inhibition @100 ug/mL	BPC (μM)	%Inhibition@100 ug/mL	MBEC (μM)
HP	TAN-1612, 100%	88.87	13.83	100.05	12.50	71.82	>100
HM8.5	TAN-1612, 54% 3HPA, 46%	96.34	53.00	100.43	50.00	0.23	>100
HM8.4	TAN-1612, 42% 3HPA, 58%	94.42	31.00	100.18	25.00	35.36	>100
HM6.2	TAN-1612, 24% 3HPA, 74%	98.89	50.96	100.00	50.00	31.01	>100
HM2	3HPA, 100%	21.01	>100	20.69	>100	-1.87	>100
HM8.3.1	2,3-didehydropalitan-tin, 100%	-7.95	>100	47.79	>100	2.37	>100
Ciprofloxacin		97.65	2.03	99.86	1.56	94.47	10.52
DMSO		6.73	>100	-13.79	>100	0.4	>100

Assays were done twice with three replicates (n=3); Moderate inhibition: 50-70% (coloured in blue); Strong inhibition >70% (coloured in red);
MIC- Minimum Inhibitory Concentration; the smallest concentration of sample to give 100% inhibition; computed from a series of sample dilutions in GraphPad Prism
BPC- Biofilm Prevention Concentration; the smallest concentration of sample to give 90% inhibition in the prebiofilm assay
MBIC- Minimum Biofilm Inhibition Concentration; the smallest concentration of sample to give 90% inhibition in the postbiofilm assay
Dose response curves of active compounds can be found in Supporting Information

5.4 *Hypoxylon lateripigmentum* grown in malt extract broth

5.4.1 Scaled up production and solvent partitioning of bioactive metabolites

Following the findings from the OSMAC studies in Section 5.2, noteworthy results were also observed when *H. lateripigmentum* was cultivated in MEB for 30 days. This extract has a comparable yield to that of the 15-day PDB cultures while also having strong antibacterial and prebiofilm activity against MRSA (Table 5.1). However, the metabolite profile of 30-day MEB culture extracts differ remarkably, as this extract was

devoid of 3HPA, as revealed by ^1H -NMR metabolomics profiling (Figure 5.4) while another set of metabolites such as the daldinones are being produced. To validate this finding from the profiling stage and explore the production of other bioactive secondary metabolites, a scaled-up cultivation of *H. lateripigmentum* was carried out. The fungus was cultured in 14 flasks, each containing 250 mL MEB, for 30 days at 27 °C. Ethyl acetate was used for extraction to afford a crude extract weighing 6.50 grams.

The crude extract was partitioned over hexane and 90%methanol: water to remove unwanted lipids. The hexane and methanol layer were coded as LH and LM, respectively. Looking at their stacked ^1H NMR spectra (Figure 5.22), methylene protons (boxed in black) can be found in LH together with minute aromatic protons. The major aromatic protons present in the crude extract were concentrated in LM. Preliminary bioassay indicated inactivity in LH and good activity with LM thus this was focused for the proceeding chromatographic fractionation (Figure 5.23).

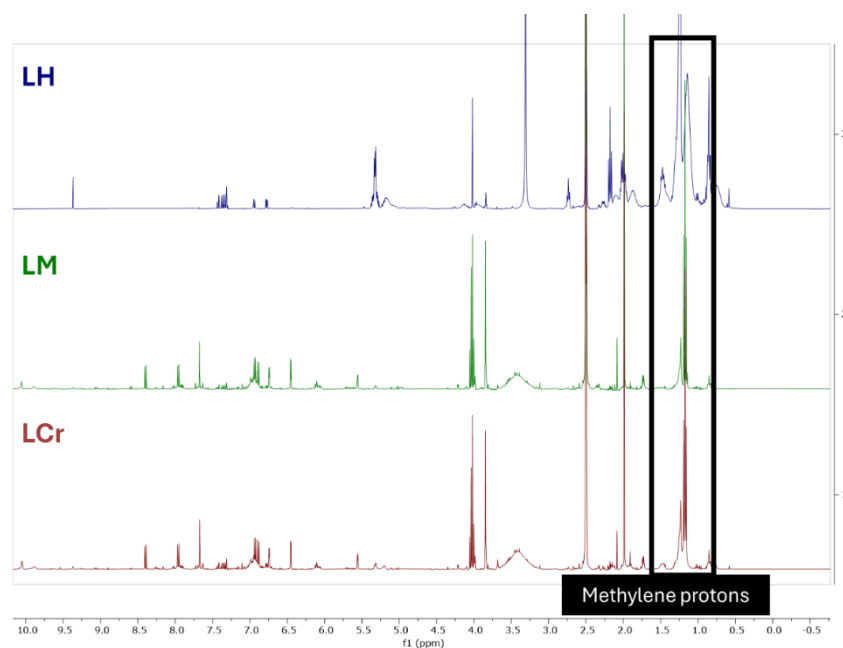


Figure 5.22. Stacked ^1H -NMR spectrum of *H. lateripigmentum* crude extract (LCr), LM, and LH. Boxed in black represents the long chain methylene protons of lipids. ^1H -NMR recorded in 400 MHz in DMSO-d_6

5.4.2 Fractionation

The LM crude methanol extract was fractionated in Buchi system over a silica gel cartridge in a linear gradient programme of hexane and ethyl acetate followed by different mixtures of organic solvents (Table 5.12). Fifty (50) mL eluates were collected and monitored via TLC to pool similar fractions. A total of eleven pooled fractions (coded LM1 to LM11) were acquired with weights displayed in Figure 5.9. TLC summary plate was shown in Figure 5.24.

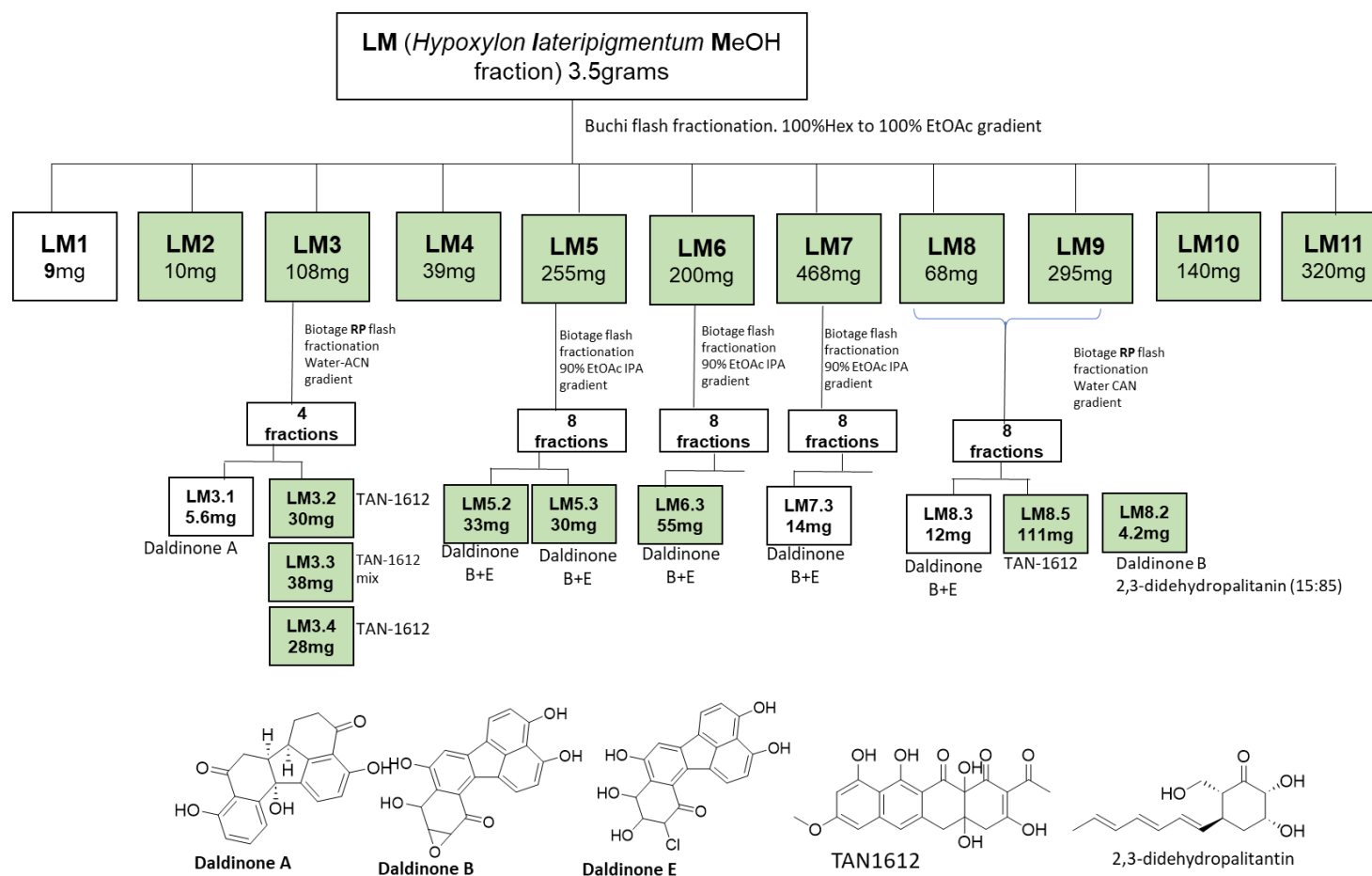


Figure 5.23 Schematic diagram summary for the isolation works done with *H. lateripigmentum* grown in MEB for 30 days

Table 5.12. Parameters for LM fractionation in Buchi

Stationary phase: VersaPak Silica Cartridge, 40x150mm (Sigma)	
Time (min)	Solvent
0-60	100% Hexane to 100% EtOAc (linear gradient)
60-70	100% EtOAc
70-80	50% EtOAc in Acetone (isocratic)
80-85	100% Acetone
85-90	90% Acetone in Methanol (isocratic)
Flow rate: 100ml/min; collection vol: 50mL	

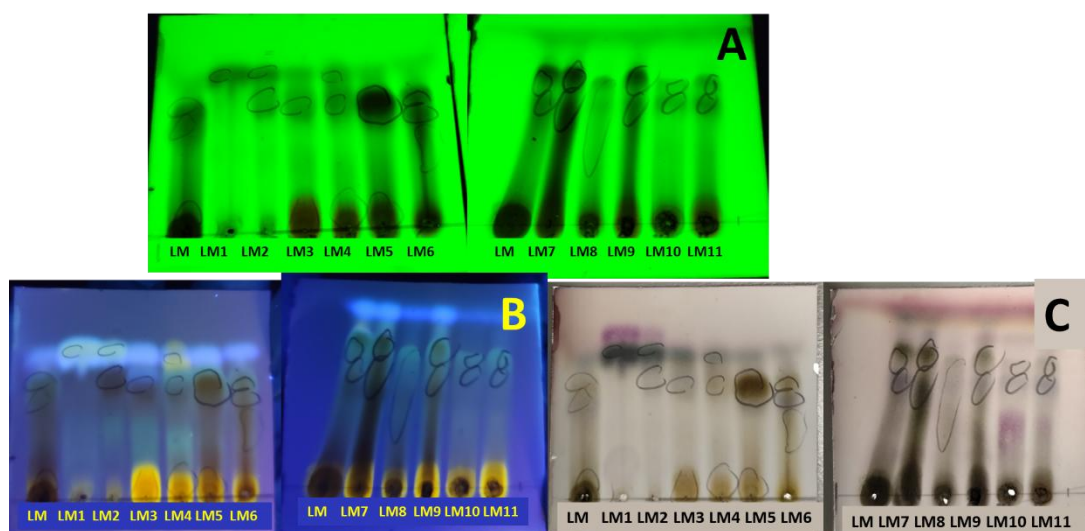


Figure 5.24 TLC summary plates of LM fractions in normal phase silica gel (A-C). A) UV254nm, B)UV365nm, C) sprayed with anisaldehyde. Solvent system: 7:3 EtOAc/isopropanol

The fractions were tested for antibacterial and antibiofilm activities. The results were summarized in Table 5.13. Generally, almost all fractions were active against MRSA in the antibacterial and prebiofilm assays. In these assays, all fractions exhibited strong activity (>70%) except for LM1 which has moderate antibacterial activity. Furthermore, the moderate postbiofilm inhibition activity of the LM fraction was also displayed by LM2, LM3, LM7, and LM10.

The $^1\text{H-NMR}$ of LM fractions were shown in Figure 5.26. The major aromatic peaks in the parent fraction LM were found in fraction LM4 to 10. Furthermore, by applying PCA, LM1 and LM2 were separated out from the rest of the fractions (Figure

5.25). Particularly, in the loadings plot, the discriminating peaks for LM1 and LM2 were around 1.24 ppm. These peaks can be assigned to methylene protons of lipids. This is not surprising since LM1 and LM2 are the first fractions of which non-polar compounds like lipids usually elute.

Table 5.13 Biological activities of LM fractions

ANTIBACTERIAL ACTIVITY, average %inhibition (n=3)					
Sample	MRSA	<i>P. aeruginosa</i>	Sample	MRSA	<i>P. aeruginosa</i>
Crude LM	88.55	-21.42	LM7	89.36	3.37
LM1	60.51	9.74	LM8	90.97	2.07
LM2	91.68	0.70	LM9	85.79	-28.32
LM3	91.61	-3.90	LM10	88.57	-12.79
LM4	89.72	-11.91	LM11	94.36	1.91
LM5	91.59	3.56	DMSO	12.12	7.13
LM6	90.41	4.74	Ciprofloxacin (50 µM)	97.51	99.70
PREBIOFILM ACTIVITY, average %inhibition (n=3)					
Sample	MRSA	<i>P. aeruginosa</i>	Sample	MRSA	<i>P. aeruginosa</i>
Crude LM	99.97	-134.45	LM7	99.96	-464.68
LM1	80.65	53.87	LM8	100.05	-252.46
LM2	99.06	-41.49	LM9	100.00	-342.92
LM3	99.98	-9.35	LM10	100.07	-311.41
LM4	99.83	-193.65	LM11	99.99	-140.52
LM5	99.97	-170.64	DMSO	-1.23	-120.43
LM6	100.01	-389.60	Ciprofloxacin (50 µM)	99.81	98.88
POSTBIOFILM ACTIVITY, average %inhibition (n=3)					
Sample	MRSA		Sample	MRSA	
Crude LM	57.79		LM7	66.59	
LM1	-23.80		LM8	13.36	
LM2	59.47		LM9	35.70	
LM3	62.82		LM10	59.84	
LM4	46.62		LM11	-3.49	
LM5	40.23		DMSO	-2.43	
LM6	27.83		Ciprofloxacin (50 µM)	96.91	

Testing concentration=100 µg/mL; assays were done twice with three replicates (n=3); Moderate inhibition: 50-70% (coloured in blue); Strong inhibition >70% (coloured in red);

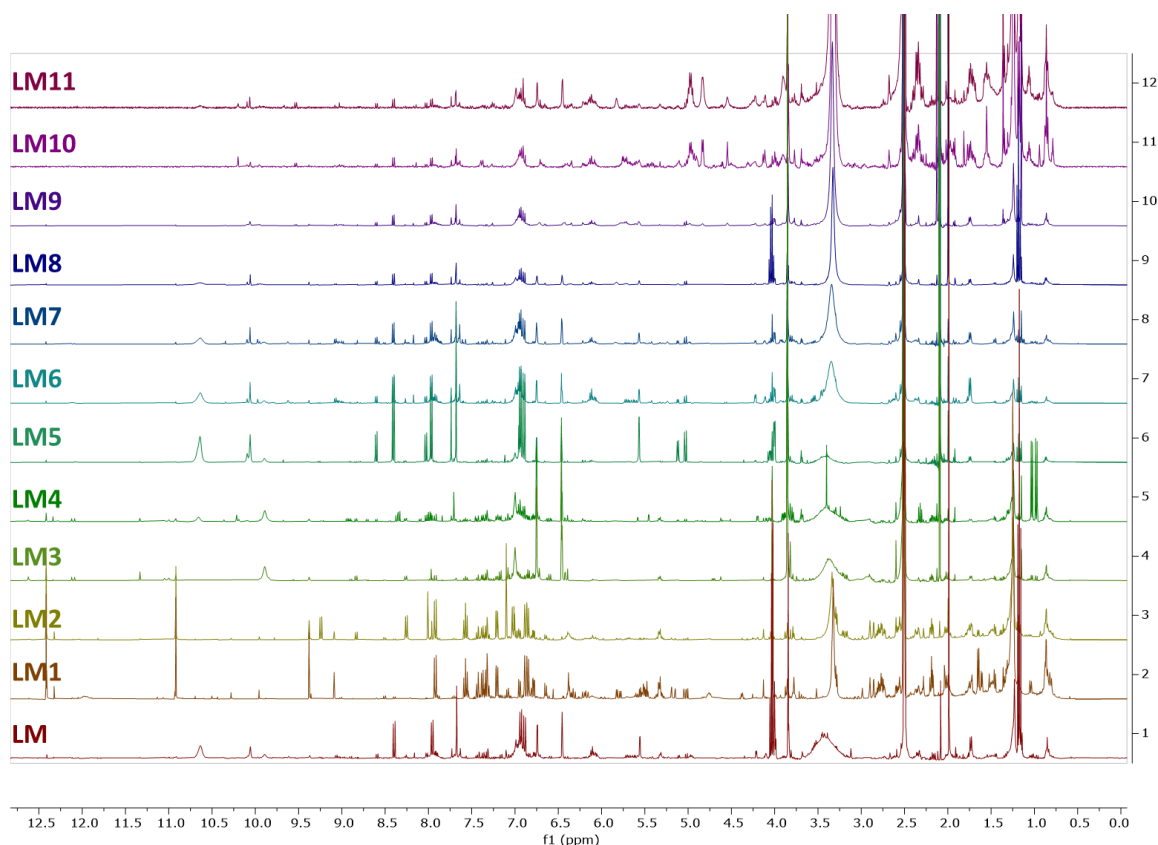


Figure 5.26 Stacked ^1H -NMR spectrum of LM fractions in ascending order. ^1H -NMR recorded in 400 MHz in DMSO-d_6

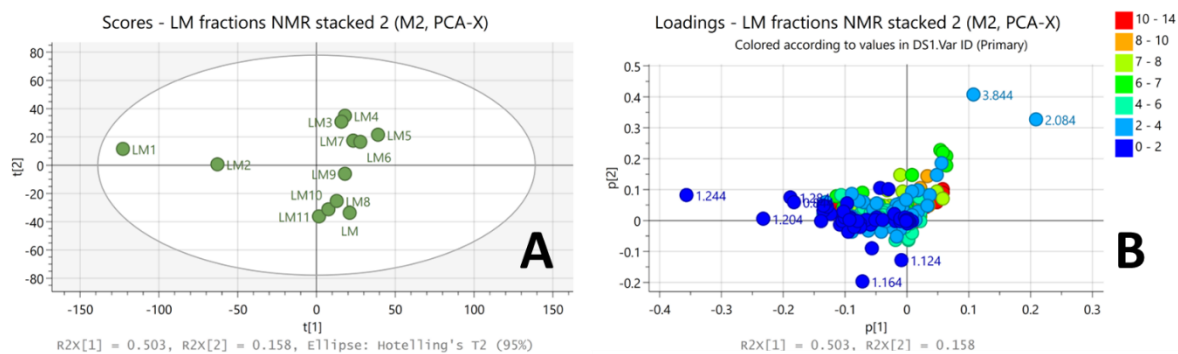


Figure 5.25 ^1H -NMR based multivariate analysis of LM fractions. A) PCA scores plot B) PCA Loadings plot. $R^2=0.94$, $Q^2=54$

The fractions were also analysed for LC-MS based chemical profiling studies. A similar pattern was observed in the PCA scores plot wherein LM1 and LM2 were

separated (Figure 5.13). Additionally, LM3 and LM4 were grouped out from the rest of the fractions. This implied a unique set of metabolites in this pair of fractions while LM5 to 11 might have a similar set of compounds.

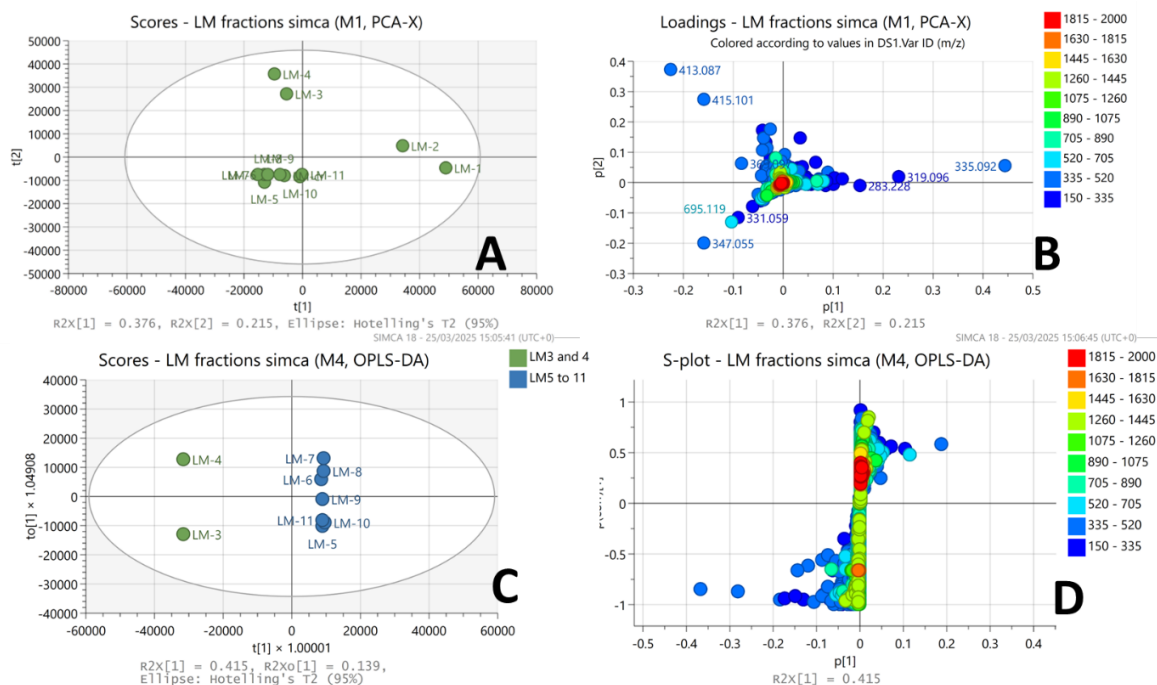


Figure 5.27 . LCMS-based multivariate analysis of LM fractions A) PCA Scores plot, B) PCA Loadings Plot, C) OPLS-DA Scores plot, and D) OPLS-DA S-plot. PCA: $R^2=0.92$, $Q^2=0.43$; OPLS-DA $R^2=0.99$, $Q^2=0.98$

To annotate the discriminatory metabolites, OPLS-DA model was employed. In this model, LM1 and LM2 were excluded since the former has moderate bioactivity while the latter was regarded as like LM1 from the previous findings in the NMR-based metabolomics. On top of that, the amount of LM1 and LM2 (Figure 5.23) were very minute to be selected for further purification work. Hence, in the OPLS-DA model used, LM3 and 4 were grouped together and can be found on the left quadrants of the of the scores plot while the remaining fractions can be found in the right-hand side (Figure 5.27C). The discriminatory metabolites from each group were derived from the S-plot. The dereplication was summarized in Table 5.14 and **Error! Reference source not found..** Most of the annotated hits are structurally related such as the daldinone A and B, truncatone, and bulgarein. TAN-1612 was dereplicated once again in this fungus.

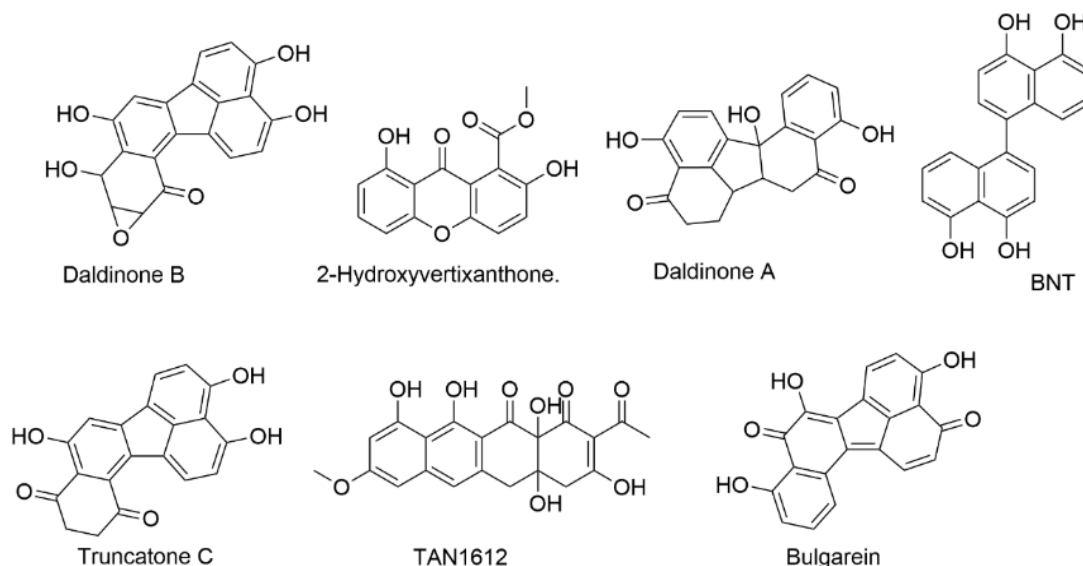


Figure 5.28 Chemical structure of discriminatory metabolites from LM fractions

5.4.3 Purification of compounds from LM fractions

Although LM3 and 4 are active fractions, LM3 was chosen over the latter for further purification because the former has higher amount. LM3 was fractionated in the Biotage MPLC system over a C18 cartridge (KP-C18-HS, 12 g, 50 μ m; Biotage). The stepwise gradient elution programme started 10% ACN in water (+0.1% formic acid) for 5 min followed by an increase from 10% to 100%ACN in water for 60 minutes and finally an isocratic step of 100% ACN for 5 minutes. Four subfractions were obtained and were coded LM3.1 to 3.4. LM3.1, LM3.2, and LM3.4 were identified in detail in section 5.4.4

LM5 was purified separately in the Biotage™ MPLC system over a normal phase silica cartridge (Sfär HP-Sphere, 10g, 25-33 μ m; Biotage™). A stepwise elution programme started with 90% EtOAc:IsoPrOH for 20 min followed by 70% EtOAc for 20min then finally at 50% EtOAc for 10 min. Eight pooled subfractions were obtained from the parent fractions and were coded LM5.1 to 5.8. The similar parameters were

applied to purify LM6 and eight pooled fractions were afforded (code: LM6.1 to LM6.8). Structure elucidation was focused in LM5.2, LM5.3, and LM6.3.

LM7 was purified as well using the same system above but with a stepwise elution programme which started with 95% EtOAc:IsoPrOH for 20 min then 90%EtOAc for 20min and finally with 50% EtOAc for 5 min. Four subfractions were obtained and were coded LM7.1 to LM7.4. Structure elucidation was focused on LM7.3

LM8 and LM9 were pooled together because they have similar TLC and NMR profiles. The combined fraction was purified in the Biotage™ MPLC system over a C18 cartridge (KP-C18-HS, 12 g, 50 µm; Biotage™). The stepwise gradient elution programme started 10% ACN in water (+0.1% formic acid) for 5 min followed by a linear gradient increase from 10% to 100%ACN in water for 60 minutes and finally an isocratic step of 100% ACN for 5 minutes. Eight fractions were obtained and were coded as LM8.1 to 8.8. Three fractions including LM8.3, LM8.5, and LM8.2 were picked for structure identification.

5.4.4 Structure elucidation of isolated compounds

5.4.4.1 LM3.1 as daldinone A

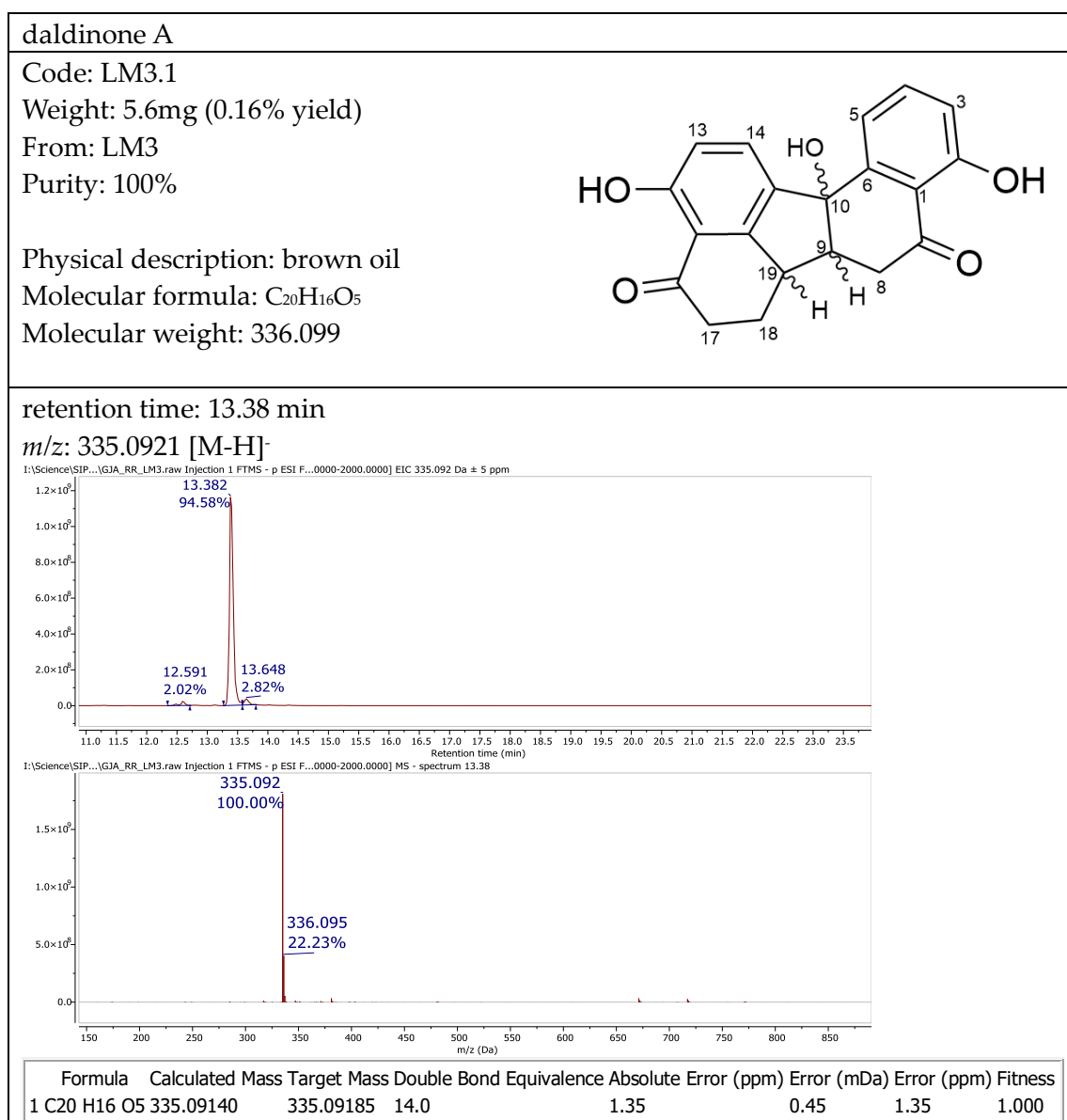


Figure 5.29 Chemical and spectroscopic data of LM3.1

LM3.1, daldinone A, was obtained as a brown oil and its molecular formula was found to be C₂₀H₁₆O₅ (DBE=13) based on the ion peak m/z of 335.092 Da [M-H]⁻ as shown

in Figure 5.29. Its ^1H -NMR (Figure S 30) and COSY (Figure S 31) spectra were used for structure elucidation. One dimensional ^{13}C -NMR was not carried out because of very low yield (5 mg). In the ^1H -NMR spectrum, five aromatic protons were present of which three were in an ABC system (δ_{H} 7.57, t, $J=8.0$ Hz, H-5; 7.21, dd, $J=7.79$, 1.07 Hz, H-4; 6.88, dd, $J=8.45$, 0.98 Hz, H-3) while two followed an *ortho* coupling (δ_{H} 7.92, d, $J=8.38$ Hz, H-12; and 6.85, dd, $J=8.28$, 1.05 Hz, H-13). Two exchangeable deshielded protons were distinguishable (δ_{H} 12.41, s, 3-OH; and 10.92, s, 12-OH). COSY was able to establish the spin system connecting the aliphatic protons. From the above data, the structure of LM3.1 was confirmed together with comparison with literature values (Table 5.15) (Quang et al., 2002).

Table 5.15 ^1H NMR data comparison of LM3.1 from reported values of daldinone A

Atom No	This study	(Quang et al., 2002)
	δ_{H} in ppm, (J in Hz) in DMSO d_6 400MHz	δ_{H} in ppm, (J in Hz) in CDCl_3 600MHz
2-OH	12.41 (1H, s)	12.4 (1H, s)
3	6.88 (1H, dd, 8.45, 0.98)	6.91 (1H, dd, 1.1, 8.2)
4	7.21 (1H, dd, 7.79, 1.07)	7.51 (1H, dd, 8.0, 8.2)
5	7.57 (1H, t, 8.03)	7.15 (1H, dd, 1.1, 8.0)
8a	3.28 (1H, overlap)	3.30 (1H, dd, 5.8, 17.6)
8b	2.88 (1H, dd, 17.6, 2.0)	2.93 (1H, dd, 2.2, 17.6)
9	2.56 – 2.54 (1H, m)	2.66 (1H, ddd, 2.2, 5.8, 10.4)
10-OH	6.38 (1H, s)	-
12	7.92 (1H, d, 8.38)	7.82 (1H, d, 8.2)
13	6.85 (1H, dd, 8.28, 1.05)	6.92 (1H, dd, 1.1, 8.2)
14-OH	10.92 (1H, s)	11.0 (1H, s)
17a	2.80 (1H, dt, 8.6, 4.1)	2.74 (1H, ddd, 2.8, 4.9, 17.6)
17b	2.61 – 2.57 (1H, m)	2.58 (1H, dd, 4.6, 17.9)
18a	1. 1.76 (1H, d, 4.4)	1.81 (1H, dd, 3.9, 17.6)
18b	2.35 – 2.31 (1H, m)	2.44 (1H, ddd, 2.5, 4.6, 17.6)
19	2.75 (1H, dd, 14.0, 4.5)	2.80 (1H, dd, 4.7, 10.4)

Daldinone A was originally reported from *Daldinia concentrica* (Quang et al., 2002). It was also isolated from a soil fungus *Hypoxylon* sp. DWS T-P-6 grown in rice

media for 40 days (Shi et al., 2016) and from the stromata of *Hypoxylon cohaerens* (Quang et al., 2005). This compound has been proposed as chemotaxonomic markers of *Hypoxylon* sect. *Annulata* taxa (Quang et al., 2005). Interestingly, the initial description of *H. lateripigmentum* from Martinique as a novel species did not produce daldinone A, but instead synthesized BNT and azaphilones (Kuhnert et al., 2014). In contrast, in the present study, daldinone A was afforded from the extracts of *H. lateripigmentum* grown in MEB culture for 30 days, along with BNT (as annotated in Table 5.5), while azaphilones were not observed. This discrepancy is likely attributable to differences in culture media and possibly the geographic origin of the fungal isolates. Indeed, as discussed in Section 5.3, daldinone A was not detected when the fungus was cultivated in PDB, highlighting the influence of media composition on secondary metabolite production. Furthermore, a recent study reported new azaphilones from a specimen of *H. lateripigmentum* collected in Iran (Lambert et al., 2025), reinforcing the idea that metabolite profiles can vary across isolate origins. This is the first report of daldinone A production by *H. lateripigmentum*, suggesting that its secondary metabolite production is highly responsive to cultivation conditions and varies from strain origin.

5.4.4.2 Mixtures of Daldinone B and E in several fractions

Daldinone B	Daldinone E																																
Code: in several fractions-LM5.2, LM5.3, LM6.3, LM7.3, LM8.3, LM8.2	Code: in several fractions-LM5.2, LM5.3, LM6.3, LM7.3, LM8.3, LM8.2																																
Weight: 109mg (3.1% yield)	Weight: 35mg (1% yield)																																
From: LM	From: LM																																
Purity: 60 to 85%	Purity: 15-40%																																
Physical description: brown oil	Physical description: brown oil																																
Molecular formula: C ₂₀ H ₁₂ O ₆	Molecular formula: C ₂₀ H ₁₂ ClO ₆																																
Molecular weight: 348.063 g/mol	Molecular weight: 384.032 g/mol																																
retention time: 10.31 min <i>m/z</i> : 347.0550 [M-H] ⁻	retention time: 10.88 min <i>m/z</i> : 383.0317 [M-H] ⁻																																
1: Science\SDP... \GCIA_RR_LMS.raw Injection 1 FTMS - p ESI F... 0000-2000.0000] EIC 347.035 Da ± 5 ppm 1: Science\SDP... \GCIA_RR_LMS.raw Injection 1 FTMS - p ESI F... 0000-2000.0000] MS - spectrum 10.28 <table><thead><tr><th>Formula</th><th>Calculated Mass</th><th>Target Mass</th><th>Double Bond Equivalence</th><th>Absolute Error (ppm)</th><th>Error (mDa)</th><th>Error (ppm)</th><th>Fitness</th></tr></thead><tbody><tr><td>1 C₂₀ H₁₂ O₆</td><td>347.05501</td><td>347.05510</td><td>16.0</td><td>0.24</td><td>0.08</td><td>0.24</td><td>1.000</td></tr></tbody></table>	Formula	Calculated Mass	Target Mass	Double Bond Equivalence	Absolute Error (ppm)	Error (mDa)	Error (ppm)	Fitness	1 C ₂₀ H ₁₂ O ₆	347.05501	347.05510	16.0	0.24	0.08	0.24	1.000	1: Science\SDP... \GCIA_RR_LMS.raw Injection 1 FTMS - p ESI F... 0000-2000.0000] EIC 383.032 Da ± 5 ppm 1: Science\SDP... \GCIA_RR_LMS.raw Injection 1 FTMS - p ESI F... 0000-2000.0000] MS - spectrum 10.88 <table><thead><tr><th>Formula</th><th>Calculated Mass</th><th>Target Mass</th><th>Double Bond Equivalence</th><th>Absolute Error (ppm)</th><th>Error (mDa)</th><th>Error (ppm)</th><th>Fitness</th></tr></thead><tbody><tr><td>1 C₂₀ H₁₂ O₆ Cl</td><td>383.03169</td><td>383.03202</td><td>15.0</td><td>0.86</td><td>0.33</td><td>0.86</td><td>0.999</td></tr></tbody></table>	Formula	Calculated Mass	Target Mass	Double Bond Equivalence	Absolute Error (ppm)	Error (mDa)	Error (ppm)	Fitness	1 C ₂₀ H ₁₂ O ₆ Cl	383.03169	383.03202	15.0	0.86	0.33	0.86	0.999
Formula	Calculated Mass	Target Mass	Double Bond Equivalence	Absolute Error (ppm)	Error (mDa)	Error (ppm)	Fitness																										
1 C ₂₀ H ₁₂ O ₆	347.05501	347.05510	16.0	0.24	0.08	0.24	1.000																										
Formula	Calculated Mass	Target Mass	Double Bond Equivalence	Absolute Error (ppm)	Error (mDa)	Error (ppm)	Fitness																										
1 C ₂₀ H ₁₂ O ₆ Cl	383.03169	383.03202	15.0	0.86	0.33	0.86	0.999																										

Figure 5.30 Chemical and spectroscopic data of LM fractions with daldinone B and E mixtures

A set of subfractions obtained from the purification of several LM fractions were found to have similar ^1H -NMR spectra (Figure 5.31). Through combined NMR and MS (Figure 5.30) analysis, daldinone B and daldinone E were identified on these fractions. Their relative ratio, as shown in the stacked spectra in Figure 5.31, were calculated based on their integration values. Daldinone E is a chlorinated derivative of daldinone B. The presence of chlorine was revealed from its HR-MS data. There is a presence of a M+2 peak corresponding to the ^{37}Cl isotope. The ratio of base peak and the M+2 peak was 3:1 (Figure 5.30) which is the typical abundance of isotopes of chlorinated compounds.

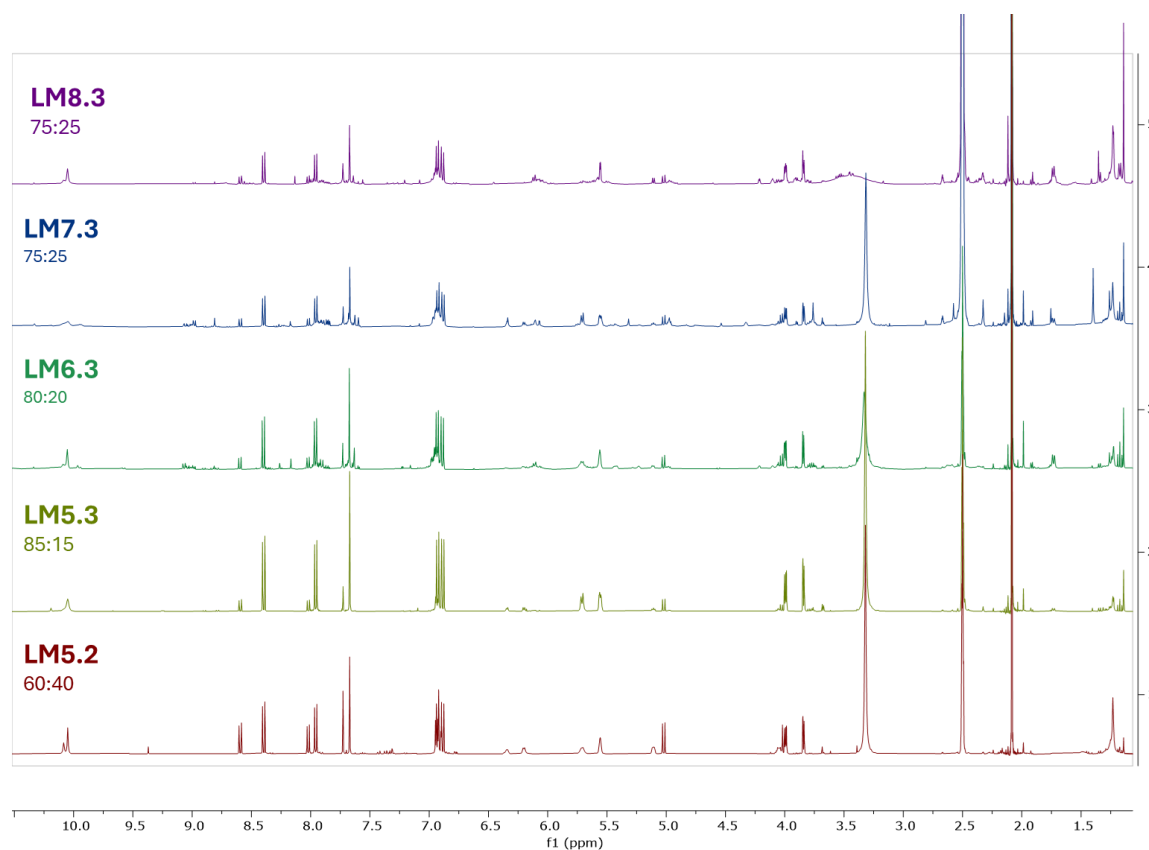


Figure 5.31 Stacked ^1H -NMR of fractions that contain Daldinone B and Daldinone E. Relative ratio were also indicated as Daldinone B:E. ^1H -NMR recorded in 400 MHz in DMSO-d_6

Several purification steps were carried out to separate the daldinone B and E but was unsuccessful. Figure 5.32 shows the ^1H -NMR peak assignment of each compound in the mixture by comparing from the individually extracted spectra. COSY analysis, displayed in Figure 5.33, also revealed the spin systems in the aromatic and aliphatic regions of these compounds. Finally, the structures were confirmed by comparison with literature values (Table 5.16)(Du, King, & Cichewicz, 2014).

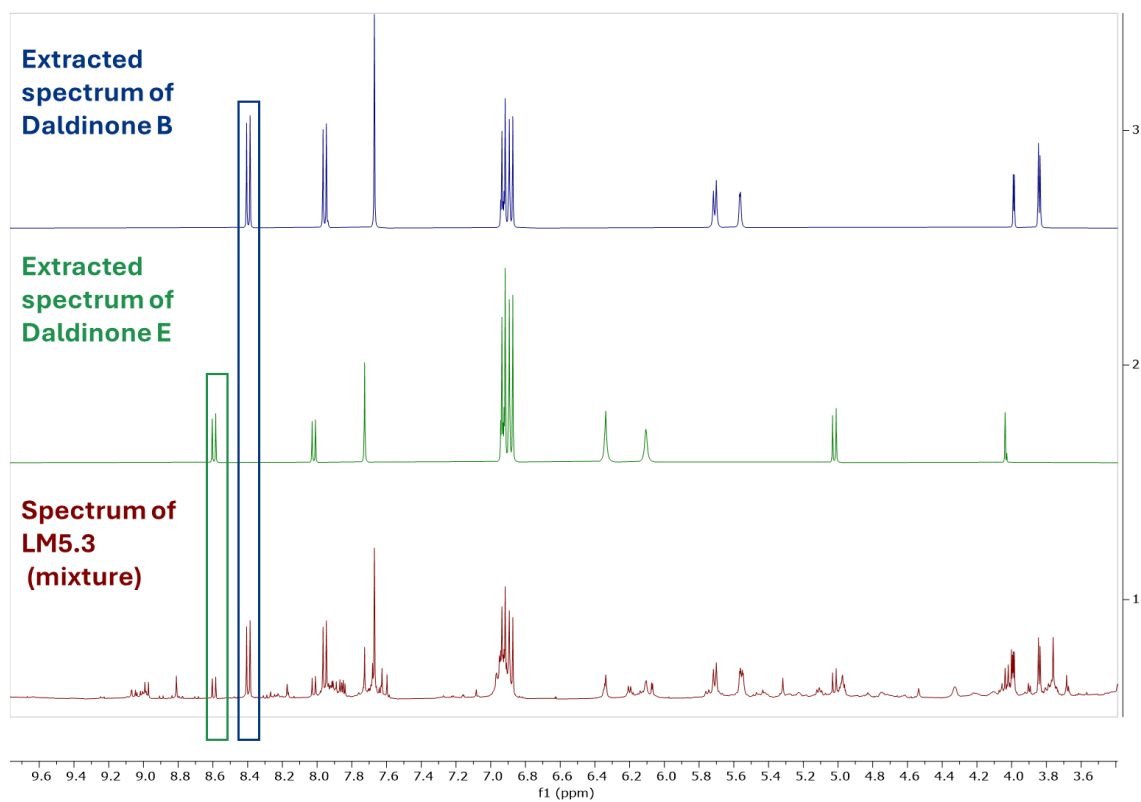


Figure 5.32 ^1H -NMR spectra showing the assignment of peaks of daldinone B and E in the original mixture (boxed in blue and green, respectively). ^1H -NMR recorded in 400 MHz in DMSO-d_6

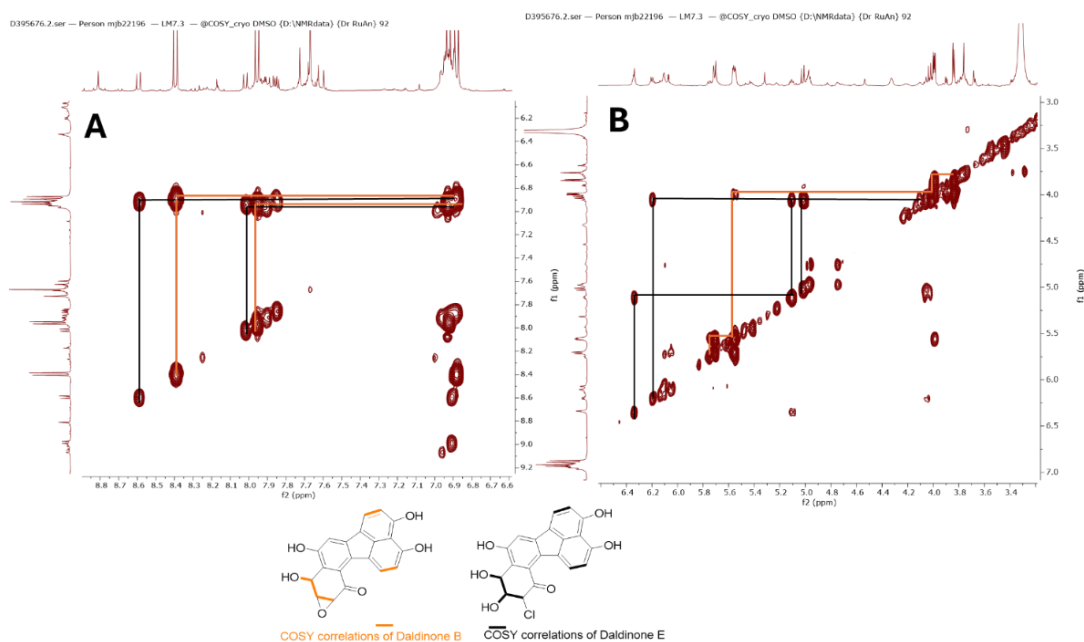


Figure 5.33 COSY correlations of daldinone B and E (coloured in orange and black, respectively). A) aromatic region B) aliphatic region

Table 5.16 ¹H NMR data comparison from reported values of daldinone B and E

Atom no	daldinone B		daldinone E	
	This study	(Du, King, & Cichewicz, 2014)	This study	(Du, King, & Cichewicz, 2014)
	δ_{H} in ppm, (<i>J</i> in Hz) DMSO <i>d</i> ₆ 400MHz	δ_{H} in ppm, (<i>J</i> in Hz) DMSO <i>d</i> ₆ 400MHz	δ_{H} in ppm, (<i>J</i> in Hz) in DMSO <i>d</i> ₆ 400MHz	δ_{H} in ppm, (<i>J</i> in Hz) in DMSO <i>d</i> ₆ 400MHz
1	-	-	-	-
2	5.56 (1H,dd, 6.29, 2.45)	5.56 (br s)	5.11 (1H, d, 6.71)	5.14, (1H, br dd, 3.8, 5.0)
3	3.99 (1H,dd, 4.25, 2.48)	3.99 (dd, 4.1,2.6)	4.03 (1H, d, 7.11)	4.33, (1H, br dd, 3.8, 5.5)
4	3.84 (1H,dd, 4.27, 0.75)	3.84 (d,4.1)	5.02 (1H, d, 8.37)	5.70 (1H, d, 2.1)
8	7.67 (1H, s)	7.67 (s)	7.73 (1H, s)	7.70, s
12	8.40 (1H, d, 8.01)	8.39 (d,8.0)	8.59 (1H, d, 8.10)	8.78, (1H, d, 8.0)
13	6.88 (1H, d, 8.05)	6.85 (d, 8.0)	6.88 (1H, d, 8.05)	6.90, (1H, d, 8.0)
17	6.92 (1H, d, 7.71)	6.89 (d, 8.0)	6.92 (1H, d, 7.71)	6.92, (1H, d, 8.0)
18	7.96 (1H, d, 7.66)	7.94 (d, 8.0)	8.02 (1H, d, 7.67)	7.96, (1H, d, 8.0)
2-OH	5.71 (1H, d, 6.69)	5.60 (d, 5.0)	6.34 (1H, d, 2.55)	5.60, (1H, d, 5.0)
3-OH	-	-	6.20 (1H, d, 5.37)	5.91, (1H, d, 3.8)

Daldinone B was also originally isolated from *Daldinia concentrica* (Quang et al., 2002). It was later discovered in another *Daldinia* species but its structure was revised (Du, King, & Cichewicz, 2014). The original and revised structures were shown in Figure 5.34 with the corrected position of the ketone group. Furthermore, the same authors reported the isolation of daldinone E as well from the same fungus. In their study, *Daldinia* sp. was cultivated in Cheerios® media with the supplementation of the epigenetic modifier suberoylanilide hydroxamic acid (SAHA). SAHA, a known chemical elicitor, induced the production of the novel chlorinated daldinone derivative, daldinone E. This metabolite was proven to be a natural product and not as a mere artifact. The reported yield was 0.0058% and 0.0034% for the daldinone B and E while in this study, the computed yields were 3.1% and 1%, respectively. These values were higher than the reported (Table 5.17). Hence, cultivation of *H. lateripigmentum* in MEB was more efficient in terms of the production of these daldinone derivatives. This finding is particularly noteworthy given their previously reported strong DPPH radical scavenging activities (IC_{50} values of 3.6 and 3.1 μ M, respectively)(Du, King, & Cichewicz, 2014).

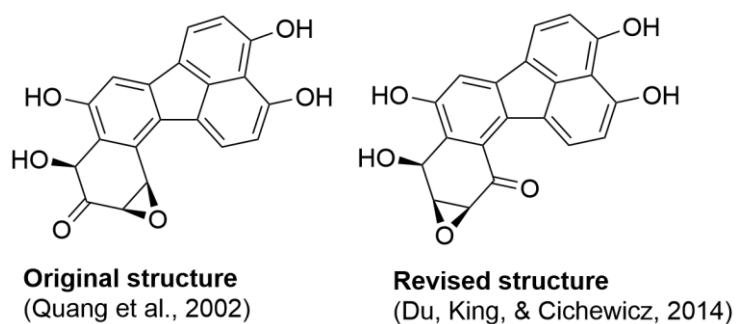


Figure 5.34 Original and revised structures of daldinone B

Table 5.17 Comparison of yields of daldinone derivatives

Culture method	Cheerios with 3% sucrose and 800 μ M SAHA, room temp, 30 days (Du, King, & Cichewicz, 2014)	MEB, 27 °C, 30 days, this work
Daldinone B titre (mg/L of media)	6.30	31
Daldinone E titre (mg/L of media)	3.75	10

5.4.4.3 TAN-1612

TAN-1612 was once more identified in several fractions including LM3.2, LM3.4, and LM8.5. The distinguishing peaks for TAN-1612 in reference to HP (check section 5.3.4.1) was evident on their ^1H -NMR spectra shown in Figure 5.35. The total amount of TAN 162 isolated from the crude extract is 169mg or about 5% yield (titre: 48 mg/L of MEB). This was less than the yield in the previous section which had a 14% yield (titre: 170 mg/L). This implied that PDB, in this case, was a better media compared to MEB with respect to the production of the bioactive compound TAN-1612.

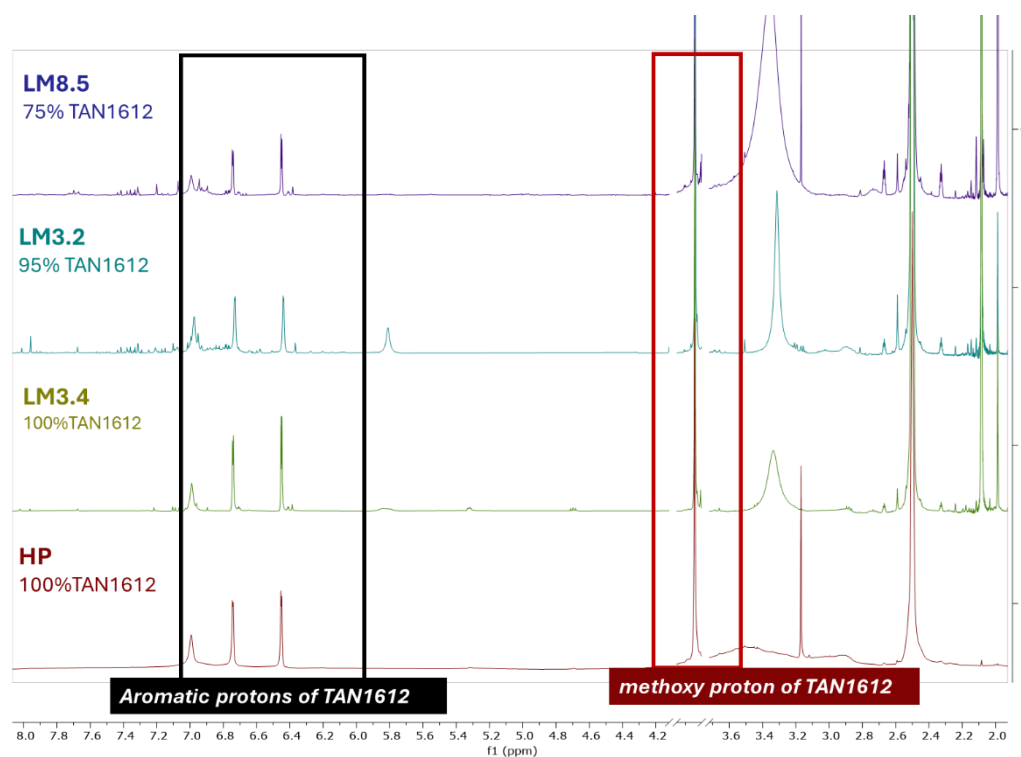


Figure 5.35 Stacked ^1H -NMR spectra of fractions containing TAN-1612. Relative purity was computed. Boxed in black are the distinguishing aromatic proton peaks while boxed in red are the methoxy proton of TAN-1612. ^1H -NMR recorded in 400 MHz in DMSO-d_6

5.4.4.4 2,3-didehydropalitantin in LM8.2

Fractionation of LM8 afforded the fraction LM8.2 which contains 2,3-didehydropalitantin and daldinone B. Its ^1H -NMR spectrum was displayed in Figure 5.36. By qNMR computation, LM8.2 is 85% 2,3-didehydropalitantin. This brings to about 4 mg or 0.1% yield of this compound in this extract. This is remarkably less than the yield

when this fungus was grown in PDB. Hence, PDB gave better yield for this compound similar to the production of TAN-1612.

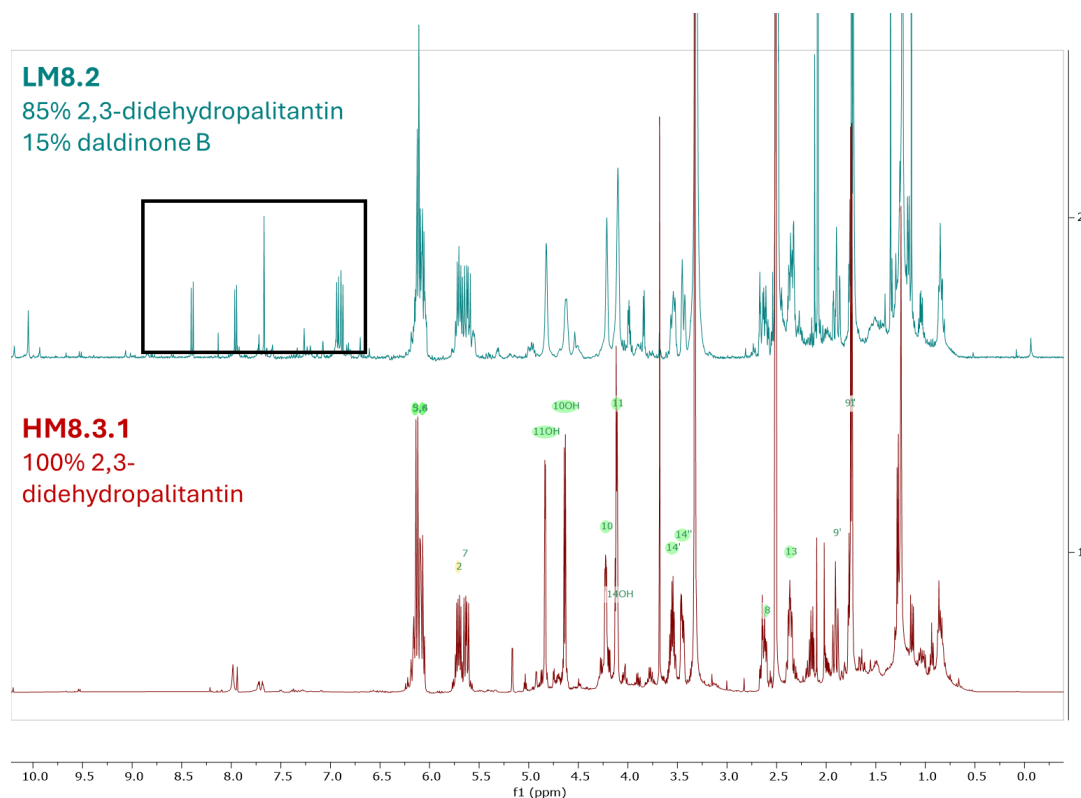


Figure 5.36 Stacked ^1H -NMR spectra of LM8.2 and HM8.3.1 (400MHz, DMSO-d_6). LM8.2 contains 15% daldinone B with distinguishing peaks boxed in black. Green numbers in the bottom spectra corresponds to the assignments of 2,3-didehydropalitantin. ^1H -NMR recorded in 400 MHz in DMSO-d_6

5.4.5 Biological activities of isolated compounds

The antibacterial and antibiofilm activities of the isolated compounds were reported in Table 5.18. The isolated compounds were evaluated against MRSA only since their parent fractions were inactive to *P. aeruginosa*. Among the tested compounds, TAN-1612 exhibited the lowest MIC and BPC, that is statistically significant in comparison to the positive control ciprofloxacin. Thus, its bioactivity is not comparable with ciprofloxacin. As observed from previous results (Table 5.11), TAN-1612 have potent antibacterial and antibiofilm activity. Moreover, its MIC and BPC values were consistent with those obtained in earlier bioassays. The mix of daldinone B/E fractions (LM6.3,

LM5.2, LM5.3) exhibited >90% prebiofilm inhibition at higher concentrations but their BPC are >100 μ M. The same manner can be noticed for daldinone A. Hence, these compounds are potent only at higher concentrations.

Table 5.18 Biological activities of isolated compounds from *H. lateripigmentum* grown in MEB against MRSA

Sample code	Identity, %purity	Antibacterial activity		Prebiofilm activity		Postbiofilm activity	
		%Inhibition @100 μ g/mL	MIC (μ M)	%Inhibition @100 μ g/mL	BPC (μ M)	%Inhibition @100 μ g/mL	MBIC (μ M)
LM3.1	daldinone A, 100%	72.75	75.91	91.46	>100	-18.39	>100
LM3.2	TAN-1612, 95%	92.31	21.35	100.10	15.10	-1.95	>100
LM3.4	TAN-1612, 100%	94.30	20.62	100.07	30.19	-6.14	>100
LM8.5	TAN-1612, 75%	94.10	94.21	100.05	60.40	-3.30	>100
LM5.3	daldinone B, 85%	78.90	>100	100.00	>100	74.97	>100
LM6.3	daldinone B, 80%	39.63	>100	90.40	>100	45.16	>100
LM5.2	daldinone B, 60%	36.30	>100	91.31	>100	71.92	>100
LM8.2	2,3-didehydro-palitantin, 85%	40.81	>100	-3.76	>100	-2.81	>100
Ciprofloxacin		97.65	2.03	99.86	1.56	94.47	10.52
DMSO		6.73	>100	-13.79	>100	0.4	>100

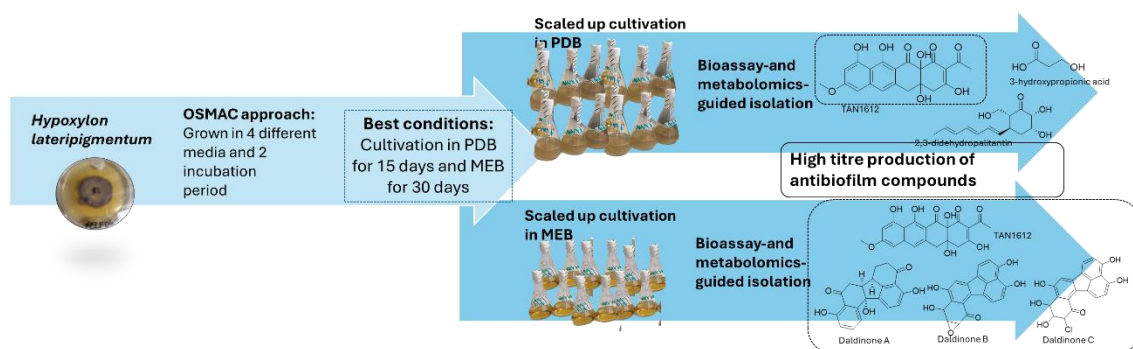
Assays were done twice with three replicates (n=3); Moderate inhibition: 50-70% (coloured in blue); Strong inhibition >70% (coloured in red);
 MIC- Minimum Inhibitory Concentration; the smallest concentration of sample to give 100% inhibition; computed from a series of sample dilutions in GraphPad Prism
 BPC- Biofilm Prevention Concentration; the smallest concentration of sample to give 90% inhibition in the prebiofilm assay
 MBIC- Minimum Biofilm Inhibition Concentration; the smallest concentration of sample to give 90% inhibition in the postbiofilm assay
 Dose response curves of active compounds can be found in Supporting Information

5.5 Chapter summary

This chapter focuses on the bioactive metabolite production of *Hypoxylon lateripigmentum*. Through variation in cultivation media and incubation periods, it was

discovered that growing the fungus in PDB for 15 days and MEB for 30 days yielded bioactive extracts, particularly effective against MRSA prebiofilm inhibition assays. Metabolomic profiling analyses revealed that 15-day PDB extracts had distinct metabolites associated with potent antibacterial and antibiofilm activities, while longer cultivations (e.g., 30 days) produced chemically richer but less active extract. After scale up in PDB for 15 days and subsequent fractionation steps, four compounds were isolated and identified, including TAN-1612, 3-hydroxypropionic acid, isopropyl butyrate, and 2,3-didehydropalitanin. TAN-1612, a tetracycline derivative, was the major compound and displayed excellent antibacterial ($\text{MIC} = 13.83 \mu\text{M}$) and prebiofilm activities ($\text{BPC} = 12.50 \mu\text{M}$). *H. lateripigmentum* was also grown in MEB for 30 days which led to the identification of TAN-1612 and daldinone compounds. 3HPA was not isolated from this extract, confirming the observations from the $^1\text{H-NMR}$ profiling. These isolated compounds were reported for the first time from *H. lateripigmentum*. Furthermore, this work found out that 15-day cultivation in PDB is better than the 30-day cultivation in MEB for the production of the antibiofilm compound TAN-1612. Although, both conditions were significantly higher than the reported production yields of other microorganisms in the literature. The same scenario was observed in terms of daldinone derivatives production in MEB. The overall results showed the enriched secondary metabolite production of the Philippine ELF *H. lateripigmentum*.

Graphical summary of *Hypoxylon* chapter



Chapter 6. *Daldinia eschscholtzii*

6.1 Chapter overview

This chapter is on *Daldinia eschscholtzii*, one of the three ELF prioritised in the preliminary screening work completed in Chapter 3. OSMAC strategy was employed to optimise the media and culture conditions. The influence of chemical elicitors to the production of bioactive metabolites were also investigated. Scaled up work was done with the chemical elicitor treatment to finally isolate antibiofilm metabolites.

6.2 Media optimisation studies of *Daldinia eschscholtzii*

D. eschscholtzii was incubated for 7 and 15 days in four media (oat, rice, potato dextrose broth (PDB), and malt extract broth (MEB)). Subsequently extraction with EtOAc, afforded crude culture extracts. Figure 6.1 present the extract weights. Oat culture yielded the most amount of fungal crude extract while the least yield can be observed in PDB culture extracts dextrose. Yields increased in all media from day 7 to day 15.

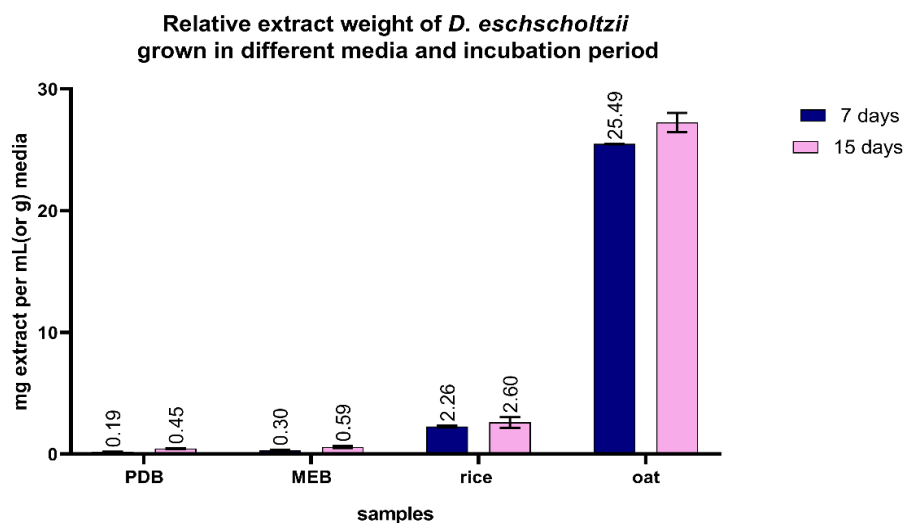


Figure 6.1. Extraction Yield of *D. eschscholtzii* in different media

6.2.1 Biological assay results

The results of the bioassays conducted in *D. eschscholtzii* crude extracts afforded from different media were displayed in Table 6.1. The table also includes results from the preliminary screening assay using culture extracts from Potato Dextrose Agar (PDA) to serve as the baseline medium for comparing fungal extracts afforded from other media. None of the samples exhibited potent antibacterial activity. However, five of them have potency in the antibiofilm assays. This includes culture extracts from 7-day PDB, 7-day MEB, 15-day MEB, 15-day rice, and 15-day oat media. These extracts have higher prebiofilm activity in comparison to the culture extract obtained from the PDA medium. Thus, the prebiofilm inhibition activity was enhanced in these media. The rice culture extracts, both 7 and 15 days, have prebiofilm activity against *P. aeruginosa*. However, the rice media blank itself has activity against the said bacteria. Hence, the observed activity of the fungal extracts from the rice culture could be attributed to the rice media itself. This seems to be an experimental error since blank extracts of rice media in previous optimisation stages were inactive (Table 4.1 and Table 5.1). The 15-day oat culture extract and the oat blank extract were also active against *P. aeruginosa*. On one hand, the postbiofilm activity of the fungal extract observed in PDA was absent in most other extracts, except in the 15-day rice culture extract. Although, its postbiofilm activity was still considered moderate at 50 to 70% inhibition.

Table 6.1 Biological activities of *D. eschscholtzii* grown in different media.

ANTIBACTERIAL ACTIVITY, average % inhibition (n=3)					
Media	MRSA	<i>P. aeruginosa</i>	Media	MRSA	<i>P. aeruginosa</i>
PDA	-1.19	6.07			
PDB blank	-6.65	-12.54	Rice Blank	27.22	7.07
7 day-PDB	-5.64	-0.35	7 day-Rice	25.39	1.02
15 day-PDB	22.92	9.15	15 day-Rice	31.33	1.22
MEB blank	12.74	4.37	Oat Blank	-2.82	6.57
7 day-MEB	-13.08	7.67	7 day-Oat	11.49	4.90
15 day-MEB	33.30	8.65	15 day-Oat	6.92	-3.12
Ciprofloxacin (50µM)	99.99	99.92	DMSO	26.11	9.68
PREBIOFILM ACTIVITY, average % inhibition (n=3)					
Media	MRSA	<i>P. aeruginosa</i>	Media	MRSA	<i>P. aeruginosa</i>
PDA	63.23	66.23			
PDB blank	-15.36	-5.44	Rice Blank	32.69	65.81
7 day-PDB	95.14	-264.70	7 day-Rice	64.34	65.91
15 day-PDB	45.29	-181.14	15 day-Rice	88.21	70.54
MEB blank	-13.56	-94.21	Oat Blank	-9.99	52.02
7 day-MEB	97.57	-281.49	7 day-Oat	50.67	-97.43
15 day-MEB	84.92	-205.83	15 day-Oat	84.84	66.58
Ciprofloxacin(50µM)	100.01	94.89	DMSO	-11.56	-101.01
POSTBIOFILM ACTIVITY, average% inhibition (n=3)					
Media	MRSA		Media	MRSA	
PDA	60.06				
PDB blank	2.66		Rice Blank		-1.62
7 day-PDB	-6.81		7 day-Rice		46.79
15 day-PDB	-3.54		15 day-Rice		62.84
MEB blank	1.32		Oat Blank		-6.17
7 day-MEB	-7.40		7 day-Oat		21.78
15 day-MEB	3.45		15 day-Oat		-1.31
Ciprofloxacin (50µM)	92.15		DMSO		-6.80

Testing concentration=100 µg/mL; assays were done twice with three replicates (n=3); Moderate inhibition: 50-70% (coloured in blue); Strong inhibition >70% (coloured in red); incubation period:15-15day, 30-30day; Media: P-Potato dextrose broth, M- Malt extract broth, R-rice, O-oat, PDA -potato dextrose agar (media used in the screening)

6.2.2 Metabolomics Profiling

6.2.2.1 NMR-based profiling

The fungal extracts were then analysed for ^1H -NMR profiling. As shown in Figure 6.2, the culture extract obtained from oat media was very rich in fatty acids as broad signals in the methylene region were observed (boxed in pink). Hence, though oat media were giving higher extract yields, it contained mainly of lipids. This has also been observed on *Xylaria* and *Hypoxylon* grown in oat media, as discussed in Chapters 4 and 5, respectively. Aromatic protons were prominent in extracts obtained from cultures incubated for 7 and 15-day in MEB media. These resonances can also be observed on 15-day rice and 7-day PDB culture extracts. Thus, aromatic compounds were produced later in the growth phase in rice medium while the compounds were being depleted over time in PDB culture. The presence of olefinic protons which can be attributed to unsaturated

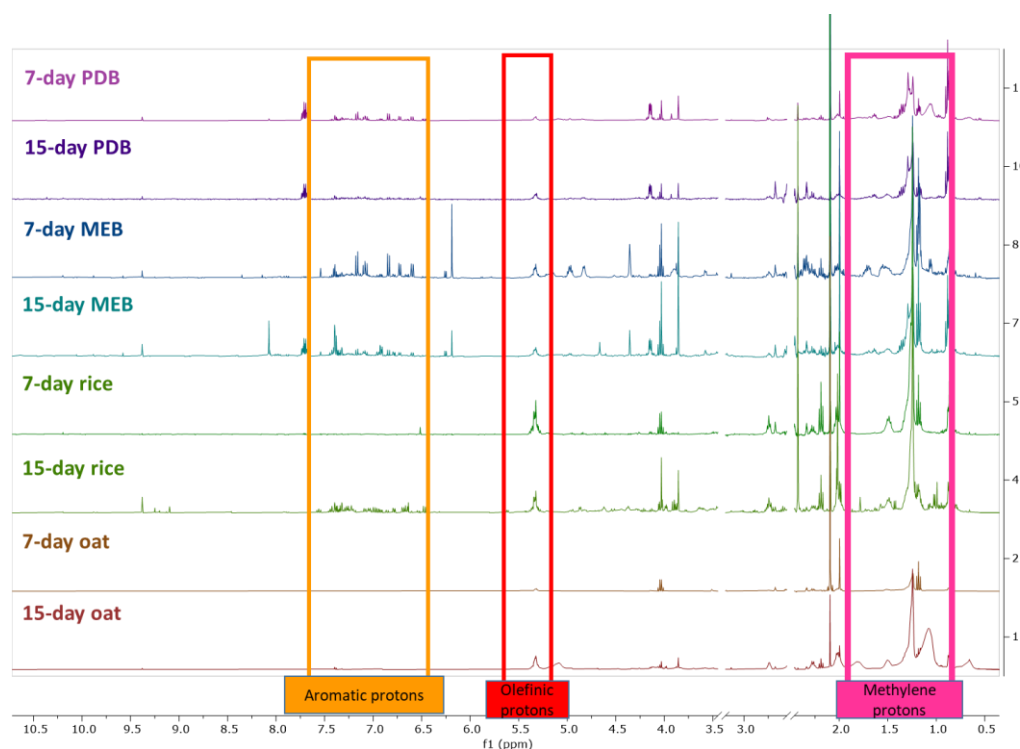


Figure 6.2. Stacked ^1H -NMR spectra of *D. eschscholtzii* grown in different media. ^1H -NMR recorded in 400 MHz in DMSO-d_6

fatty acid molecules are seen prominently in culture extracts of fungi grown on rice media.

A heatmap was generated from their NMR spectral data (Figure 6.3) to depict the overall chemodiversity. Less intense blue bands can be found on extracts obtained from the oat and MEB media, which indicates low chemical diversity. Extracts prepared from the MEB media exhibited strong prebiofilm inhibition but the heatmap implied low metabolite diversity. Meanwhile stronger blue bands were observed on extracts from the 15-day rice culture while the excellent bioactivity of this extract can be attributed to their chemical diversity.

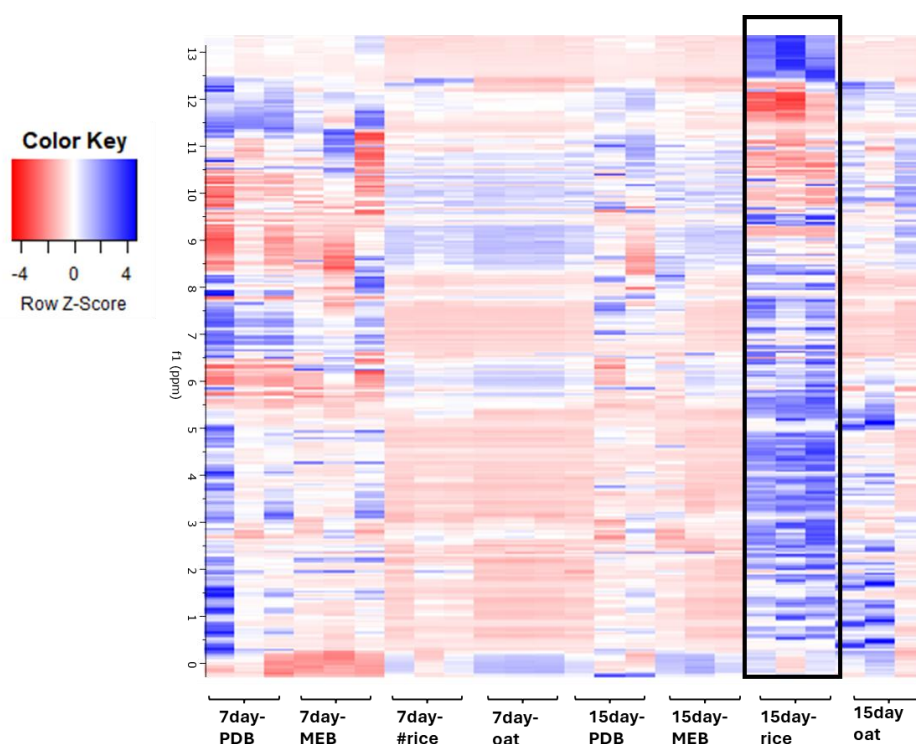


Figure 6.3. Heatmap of ¹H-NMR profiles of *D. eschscholtzii* grown in different media. Extract with intense blue bands, were boxed in black. Heatmap is generated in R. Heatmap generated in R (code available in S 2)

Multivariate analysis was then applied to reveal similarities and differences between the NMR profiles of the culture extracts. Principal component analysis (PCA),

which is an unsupervised approach, was applied first to see overall differences. The scores plot and its corresponding loadings plot were displayed in Figure 6.4A and 6.4B respectively. Looking at the scores plot, fungal extracts from the 7-days PDB, 15-days rice, and 15-days oat cultures were clustered out from the rest of the samples. Notably, these extracts also displayed strong prebiofilm inhibition. Although not all extraction replicates clustered tightly, suggesting some variability within replicates. Furthermore, the chemical shifts that caused the separation can be found in its loadings plot in Figure 6.4B. The signals were mostly from 0 to 2 ppm, which could imply the presence of methylene protons of lipid units or short-length alkyl chains.

The supervised model, PLS-DA, was also employed. As seen on the scores plot (Figure 6.4C), the 15-day rice extracts were well separated out together with the 15-days oat culture extract. One replicate of 7-day PDB was an outlier. This pattern can also be observed in the PCA scores plot (Figure 6.4A). Again, the same distinct chemical shifts

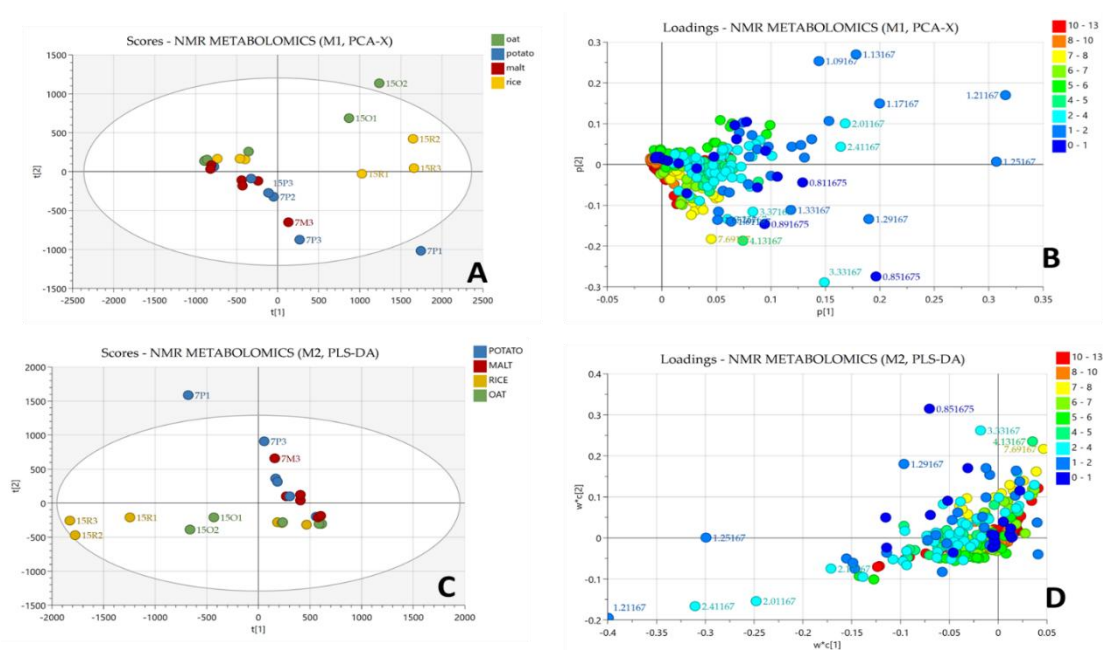


Figure 6.4. ¹H-NMR based multivariate analysis of *D. eschscholtzii* grown in different media. A) PCA Scores plot, B) PCA loading plot, C) PLS-DA Scores plot, D) PLS-DA Loadings plot. Each dot in the scores plot represent one replicate (n=3). PCA: R²=0.94, Q²=0.74; PLS-DA: R²=0.63, Q²=0.28,

were indicated in the loadings plots as with the previous applied model. Hence, the PCA model revealed differences between the *D. eschscholtzii* extracts from various media.

6.2.2.2 LC-MS-based profiling

D. eschscholtzii extracts were also analysed with LC-HRMS for a more comprehensive metabolite profiling analysis. PCA scores plot were shown in Figure 6.5A. Just like with the NMR-based metabolomics, the same culture extracts (in 15 days-oat and 15-days rice) clustered out from the rest while the same one replicate in 7-day PDB was an outlier. By inspecting the loadings plot (Figure 6.5B), extracts obtained from 15 days-oat and 15-days rice were rich with compounds ranging from m/z 100 to 380 Da. On the other hand, PDB culture extracts were rich with compounds affording ion peaks at 380 to 520 Da, slightly of higher molecular weights than those afforded by extracts from the 15 days-oat and 15-days rice culture.

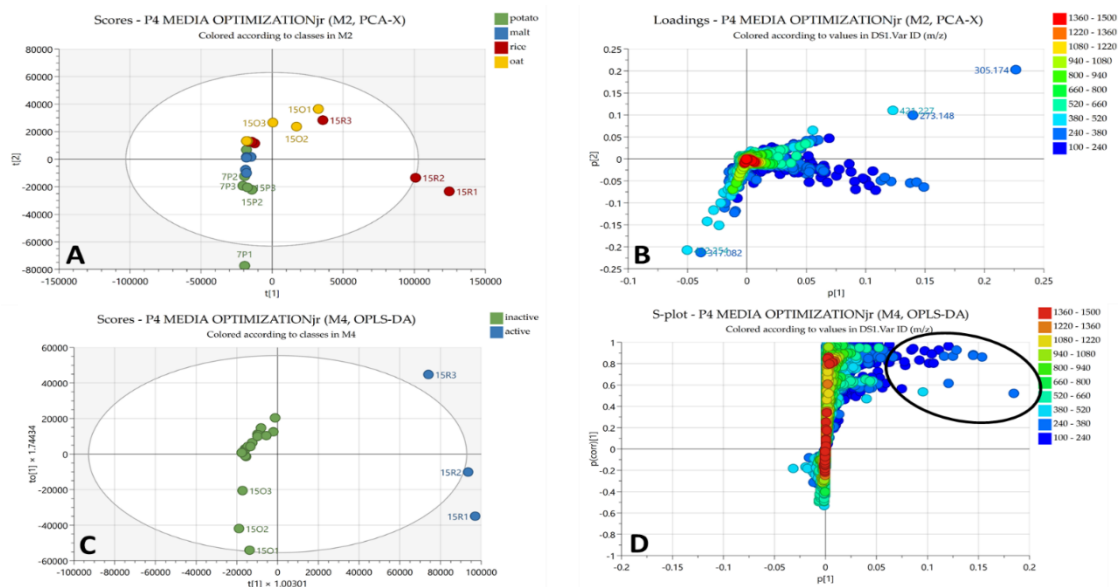


Figure 6.5. LCMS-based multivariate analysis of *D. eschscholtzii* grown in different media. A) PCA Scores plot, B) PCA loading plot, C) OPLS-DA Scores plot, D) OPLS-DA S-plot. Each dot in the scores plot represent one replicate ($n=3$). PCA: $R^2=0.83$, $Q^2=0.65$; OPLS-DA $R^2=0.97$, $Q^2=0.65$

The 15-day rice culture extract was identified as the most promising due to its consistent antibiofilm activity and distinct metabolomic profile across all MVA models. To pinpoint discriminating metabolites from this sample, OPLS-DA was employed (Figure 6.5C). The 15-day rice extracts were distributed over the upper and lower right quadrants of the scores plot. Meanwhile, the culture extract obtained from the oat media incubated for 15-days were still segregated from the rest of the samples, indicating the uniqueness of this extract. The discriminating metabolites of 15-day rice culture extracts were encircled in black in the S-plot (Figure 6.5D). Dereplication was subsequently performed to annotate these compounds.

The putative identification of the discriminatory metabolites of *D. eschscholtzii* grown in rice for 15 days were summarized in Table 6.2 while structures were illustrated in **Error! Reference source not found.**. Based on the dereplication, the compound hits were previously reported metabolites from the same species or the genus. Several unsaturated fatty acids were also dereplicated of which their olefinic and methylene signals were indicated on their NMR spectra (Figure 6.4). The aromatic compounds hits including orthosporin, diaporthin, regiolone, 1,8-naphthalenediol, and xylarenone were supported as well by the chemical shifts observed at 7 to 8 ppm in the NMR spectra of 15-days rice extract. Hence, these compounds can be targeted for isolation.

The heatmap in Figure 6.7 depicts the relative abundance of the discriminatory metabolites in different culture conditions. The abundance of these compounds was higher in the 15-days rice culture extracts, but it can also be found in other media. Hence, these metabolites were enriched when *D. eschscholtzii* was grown in rice media for 15 days.

Table 6.2. Dereplication of top 15 discriminatory metabolites of the bioactive *D. eschscholtzii* grown in rice for 15 days

<i>p</i> -value	<i>m/z</i>	Molecular weight (g/mol)	Molecular formula	Dereplication hit	Biological Source
5.64E-11	213.1487	212.1414	C ₁₂ H ₂₀ O ₃	Helicascolide C	<i>Daldinia eschscholtzii</i> and <i>Helicascus kanaloanus</i> ATCC18591
9.74E-09	235.061	236.0683	C ₁₂ H ₁₂ O ₅	(+)-Orthosporin	<i>Daldinia concentrica</i> , <i>Daldinia eschscholtzii</i> , etc
1.11E-08	161.0599	160.0527	C ₁₀ H ₈ O ₂	1,8-Naphthalenediol	Found in ascomycete fungi
2.18E-07	179.0704	178.0631	C ₁₀ H ₁₀ O ₃	Regiolone	<i>Asteromyces cruciatus</i> 763, <i>Botrytis fabae</i> , <i>Daldinia eschscholtzii</i> HJ004, <i>Discula</i> sp., <i>Microsphaeropsis</i> sp., <i>Phaeoacremonium aleophilum</i> , and the freshwater sp. from strain G730 of the order <i>Helotiales</i> . Constituent of <i>Engelhardtia roxburghiana</i> , <i>Juglans regia</i> , and <i>Juglans mandshurica</i> var. <i>sieboldiana</i>
3.86E-07	251.0914	250.0842	C ₁₃ H ₁₄ O ₅	Diaportin	<i>Daldinia concentrica</i> , and etc
4.40E-07	269.2475	268.2402	C ₁₇ H ₃₂ O ₂	3-Heptadecenoic acid	not reported
1.01E-06	267.2319	266.2247	C ₁₇ H ₃₀ O ₂	6,9-Heptadecadienoic acid	not reported
1.02E-06	255.232	254.2247	C ₁₆ H ₃₀ O ₂	2-Hexadecenoic acid	not reported
1.10E-06	285.2425	284.2352	C ₁₇ H ₃₂ O ₃	2-Hydroxy-15-methyl-9-hexadecenoic acid	not reported

<i>p</i> -value	<i>m/z</i>	Molecular weight (g/mol)	Molecular formula	Dereplication hit	Biological Source
1.92E-05	193.0862	192.079	C ₁₁ H ₁₂ O ₃	Xylarenone.	<i>Xylaria</i> sp. PSU-A80
2.85E-05	175.0755	174.0682	C ₁₁ H ₁₀ O ₂	1,8-Naphthalenediol; Mono-Me ether	<i>Daldinia concentrica</i> , <i>Hypoxylon macrocarpum</i> and <i>Sporothrix</i> sp. (No. 4335)
0.000474	273.1485	272.1412	C ₁₇ H ₂₀ O ₃	1,8,13,16-Heptadecatetraene-4,6-diyne-3,11,12-triol	Not reported
0.002057	421.2269	422.2342	C ₂₃ H ₃₄ O ₇	4-Hydroxysporotricale	<i>Sporotrichum laxum</i> (<i>Phanerochaete prunosum</i>) CBS 578.63
0.003436	305.1744	304.1671	C ₁₈ H ₂₄ O ₄	Solanapyrone B	<i>Alternaria solanii</i> and from an unidentified marine fungus
0.008913	189.0912	188.0839	C ₁₂ H ₁₂ O ₂	1,8-Naphthalenediol; Di-Me ether	<i>Daldinia concentrica</i> and <i>Sporothrix</i> sp. (No. 4335)
Compound dereplication are based on highly accurate mass matching (<5 ppm) against Dictionary of Natural Products, with filtering by fungal biological source. No tandem MS/MS data were acquired; therefore, these are tentative assignments that require confirmation through MS fragmentation data or isolation (cells highlighted in blue correspond to compounds that were subsequently isolated)					

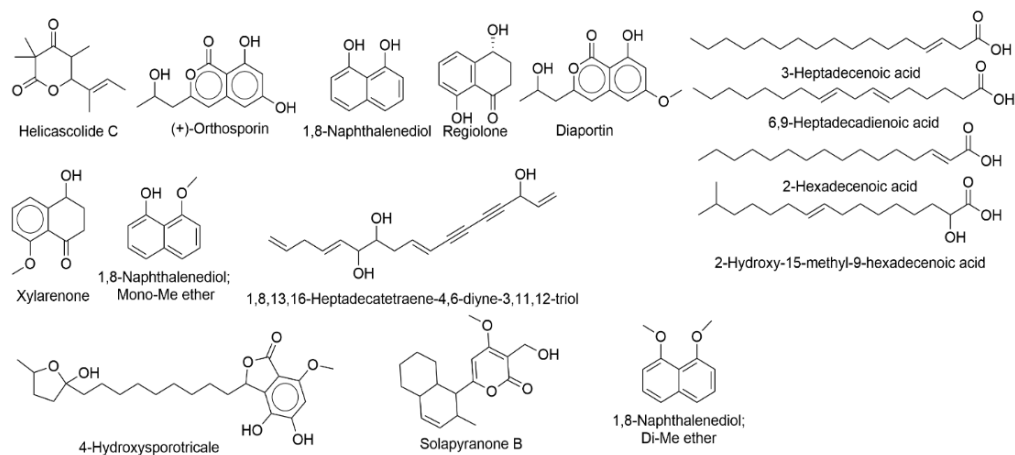


Figure 6.6. Chemical structure of hits from the discriminatory metabolites of *D. eschscholtzii* grown in rice media for 15 days

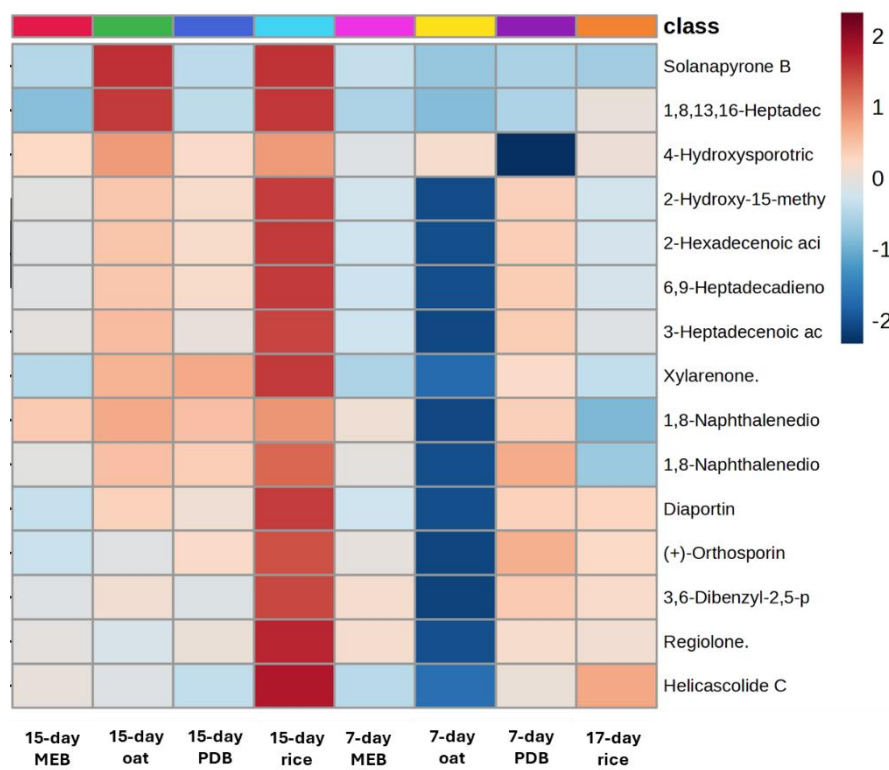


Figure 6.7 Heatmap of top discriminatory metabolites from the bioactive extract *D. eschscholtzii* grown in rice for 15 days. Heatmap was generated in MetaboAnalyst v 6.0

6.2.3 Summary of OSMAC works

The OSMAC approach was applied in *D. eschscholtzii* by growing the ELF in four different media and two duration periods. It resulted in varied biological activities and distinct metabolome profiles. Remarkably, the 15-day rice extract has strong prebiofilm and moderate postbiofilm inhibition against MRSA while exhibiting distinct metabolomics profile. The structure of the discriminatory metabolites from this bioactive extract correlate with its NMR data. Hence, these metabolites can be targeted for isolation work

6.3 Isolation works in *Daldinia eschscholtzii* grown in rice

6.3.1 Scaled up production

Given the remarkable bioactivity of 15-day rice extracts of *D. eschscholtzii*, the fungus was scaled up by cultivating it in 20 flasks containing 100 g of rice at 27 °C. After 15 days, the culture was extracted with EtOAc, affording crude extract weighing 3 grams. The crude extract was partitioned over hexane and methanol:water (9:1) to separate the nonpolar from the polar metabolites. The bioactive methanolic extract (code: DM, 2.1 g) was fractionated on the Biotage™ MPLC system over a silica cartridge (10g Sfär HP-Sphere, 25-33 µm, Biotage™). A linear gradient elution was carried from 37% to 100% EtOAc:hexane for 60 min. It afforded 17 pooled fractions coded as M1 to M17 with weights listed in Table 6.3. The TLC summary plate was shown in Figure 6.8. Only three fractions were further selected for purification based on their strong prebiofilm activity and high yield. Figure 6.9 illustrates the chromatographic purification workflow.

Table 6.3 Weight of 17 fractions obtained from DM fractionation

Fraction code	Amount (mg)	Fraction code	Amount (mg)
M1	171.9	M10	79.7
M2	169.7	M11	144
M3	22.3	M12	24.1
M4	109.9	M13	93.9
M5	98.2	M14	102.6
M6	212.4	M15	11.4
M7	31.3	M16	35.1
M8	35.6	M17	171.7
M9	119.8	Total 1633.6mg (75% recovery)	

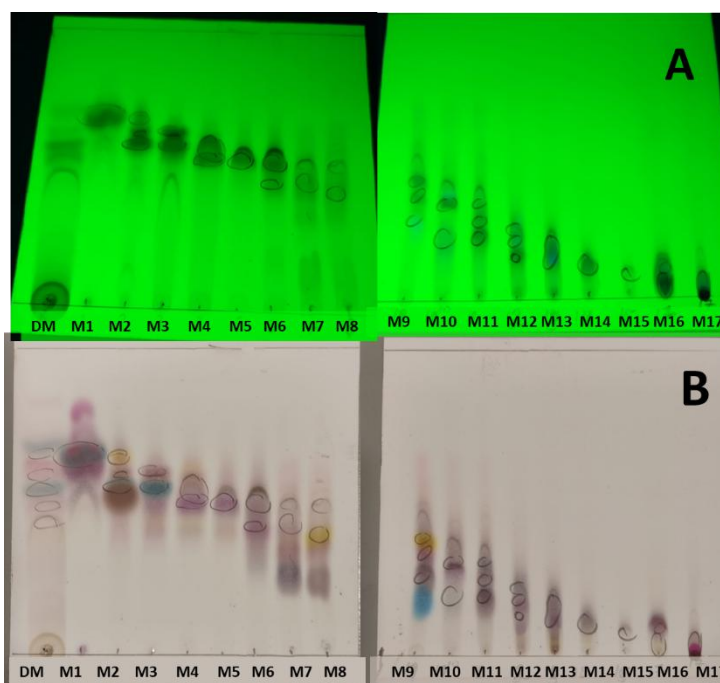


Figure 6.8 TLC summary plate of DM fractions in normal phase silica gel. A) Visualisation in UV254nm; B) plates sprayed with anisaldehyde. Solvent system: 100% ethylacetate

6.3.2 Fractionation

The bioactivity of the 17 fractions was assessed (Table 6.4). The antibacterial activities of the fractions are very weak like its parent fraction (DM). Strong biofilm inhibition against MRSA was observed on fractions M3, M7, and M12. Meanwhile, the

moderation inhibition of the parent fraction (DM), can be tracked down on fractions M3, M4, and M9.

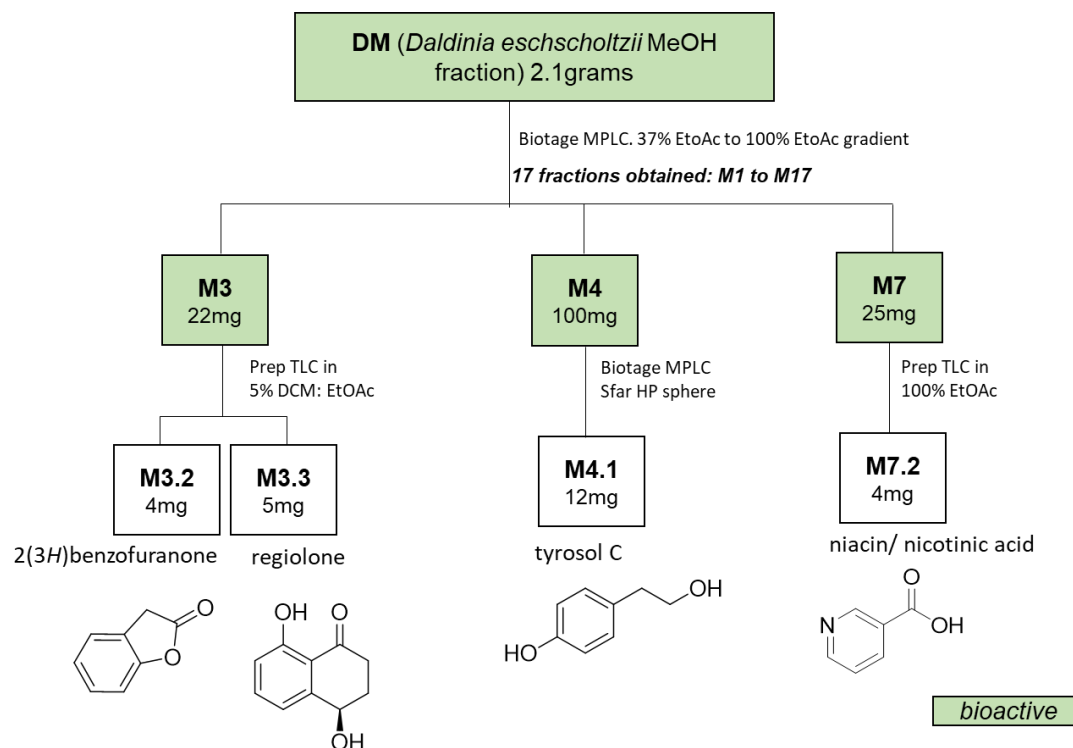


Figure 6.9 Schematic diagram summary for the isolation works done with *D. eschscholtzii* grown in rice for 15 days

Table 6.4 Biological activities of DM fractions

ANTIBACTERIAL ACTIVITY, average % inhibition (n=3)					
Sample	MRSA	<i>P. aeruginosa</i>	Sample	MRSA	<i>P. aeruginosa</i>
DM	31.33	1.22	M10	-27.57	-201.53
M1	-4.86	-223.29	M11	-20.71	-209.72
M2	15.24	-192.64	M12	-11.48	-201.30
M3	6.18	-211.08	M13	46.31	-209.71
M4	19.95	-167.03	M14	40.11	-209.12
M5	29.20	-182.96	M15	6.00	-212.24
M6	-26.45	-185.34	M16	-4.72	-193.90
M7	23.95	-175.98	M17	31.80	-245.71
M8	-18.27	-197.05	DMSO	-2.88	-201.62
M9	-24.70	-199.33	Ciprofloxacin (50 µM)	100.16	99.15
PREBIOFILM ACTIVITY, average % inhibition (n=3)					
Sample	MRSA	<i>P. aeruginosa</i>	Sample	MRSA	<i>P. aeruginosa</i>
DM	88.21	70.54	M10	-75.16	-18.51
M1	33.65	88.47	M11	53.23	-28.33
M2	60.45	-31.22	M12	79.41	-18.94
M3	79.32	-29.67	M13	48.97	-25.07
M4	12.16	-2.95	M14	23.51	-21.85
M5	43.59	-27.02	M15	29.90	-25.97
M6	69.22	-10.35	M16	40.90	-29.75
M7	83.07	-26.97	M17	93.27	74.91
M8	63.37	-23.91	DMSO	-43.21	-21.08
M9	54.25	-29.91	Ciprofloxacin (50 µM)	99.80	99.86
POSTBIOFILM ACTIVITY, average inhibition (n=3)					
Sample	MRSA		Sample	MRSA	
DM	54.70		M10	37.83	
M1	28.64		M11	34.49	
M2	-15.95		M12	54.26	
M3	51.71		M13	43.85	
M4	63.14		M14	30.41	
M5	39.42		M15	52.76	
M6	33.82		M16	43.56	
M7	16.64		M17	61.22	
M8	27.77		DMSO	12.30	
M9	51.18		Ciprofloxacin (50 µM)	90.60	

Testing concentration=100 µg/mL; assays were done twice with three replicates (n=3); Moderate inhibition: 50-70% (coloured in blue); Strong inhibition >70% (coloured in red);

The fractions were subjected to ^1H -NMR profiling. Fractions that exhibited strong to moderate inhibition in the prebiofilm and postbiofilm assays (M2, M3, M4, M6, M7, M8, M9, M11, M12, and M17) were selected for presentation in a simplified and more easily interpretable stacked ^1H -NMR spectrum displayed in Figure 6.10. Prominent aromatic peaks can be observed in fractions M2 to M4. More deshielded aromatic peaks at resonances 7.5 to 9.0 ppm can be found in fractions M7 and M8.

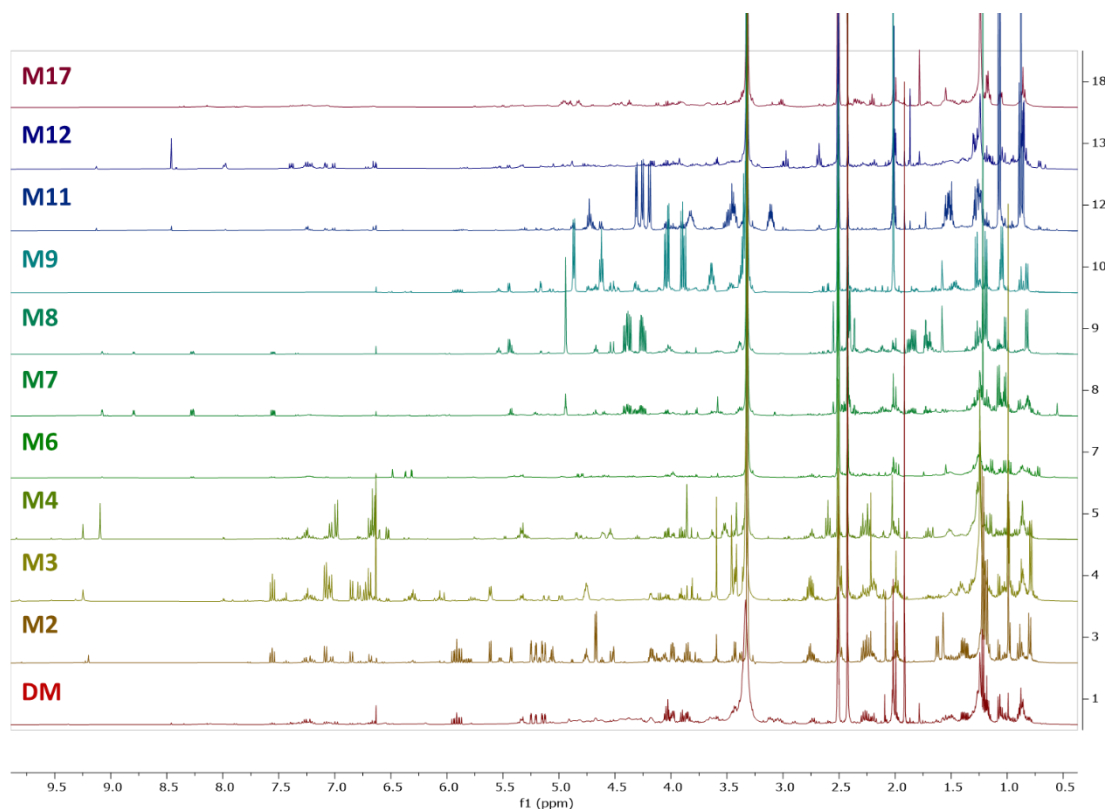


Figure 6.10 Stacked ^1H -NMR of fractions from DM. Only the fractions with strong and moderation prebiofilm inhibition were shown. ^1H -NMR recorded in 400 MHz in $\text{DMSO}-d_6$

^1H -NMR based multivariate analysis was carried out on the 17 fractions from DM. There were no visible differences between fractions based to the PCA scores plot (Figure 6.11A). R^2 and Q^2 values were low that supports the implication of non-significant variances between the fractions. OPLS-DA model was employed by grouping the fractions into two based on their bioactivity: (1) fractions with strong-to moderate activity and (2) inactive fractions. The scores and S-plots for this model were shown in Figure 6.11C and D.

The active fractions were positioned on upper and lower right quadrants of the scores plot. M3 appeared partially distant from the other active fractions indicating greater variability within this fraction compared to the rest of the group. The S-plot pinpoints resonances at 1.21 ppm and 0.97 ppm contributed most to the separation. These chemical shifts likely correspond to methylene and methyl protons of lipid compounds.

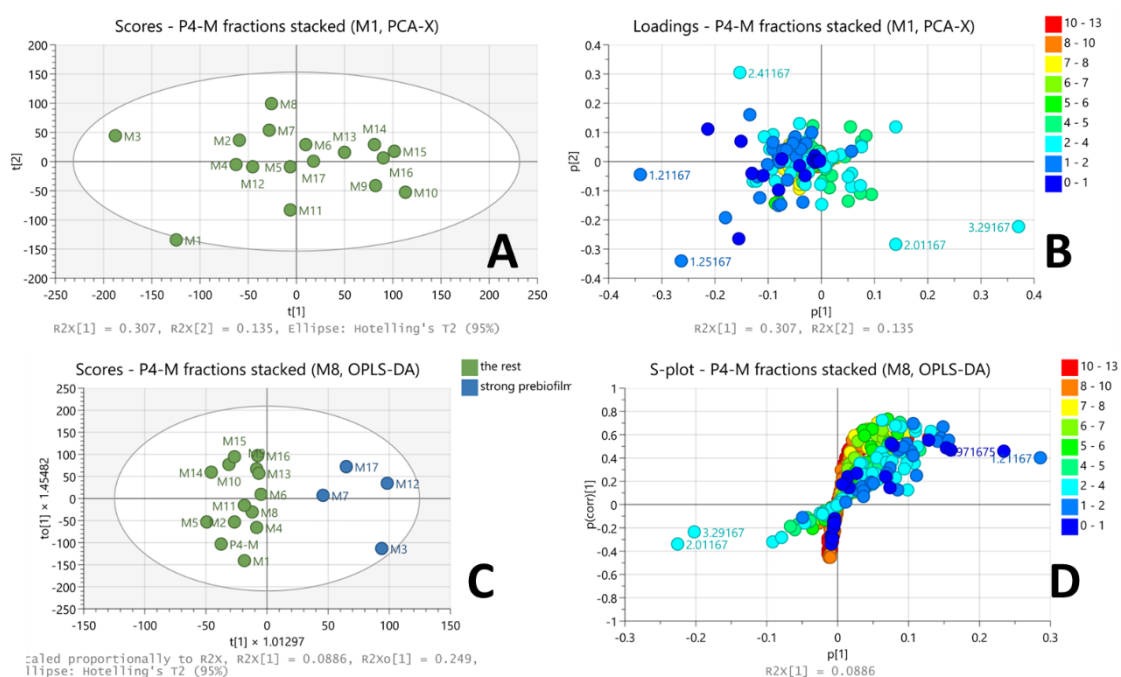


Figure 6.11 ^1H -NMR-based multivariate analysis of DM fractions. A) PCA scores plot, B) PCA Loadings plot, C) OPLS-DA scores plot, D) OPLS-DA S-plot. PCA: $R^2=0.04$, $Q^2=0.08$; OPLS-DA $R^2=0.86$, $Q^2=0.14$

Next, the fractions were also analysed for multivariate analysis based on their LC-MS spectral data. As depicted in the PCA scores plot (Figure 6.12A), fractions M3 and M12 had clustered out from the rest of the fractions. These fractions have strong preboil activity. This implies the chemical uniqueness of these bioactive fractions. The discriminating feature of M3, as labelled in the loadings plot (Figure 6.12B), has an m/z of 269.2477 Da ($[\text{M}+\text{H}]^+$). This was later annotated as a lipid, 9Z-heptadecenoic acid (see Table 6.5).

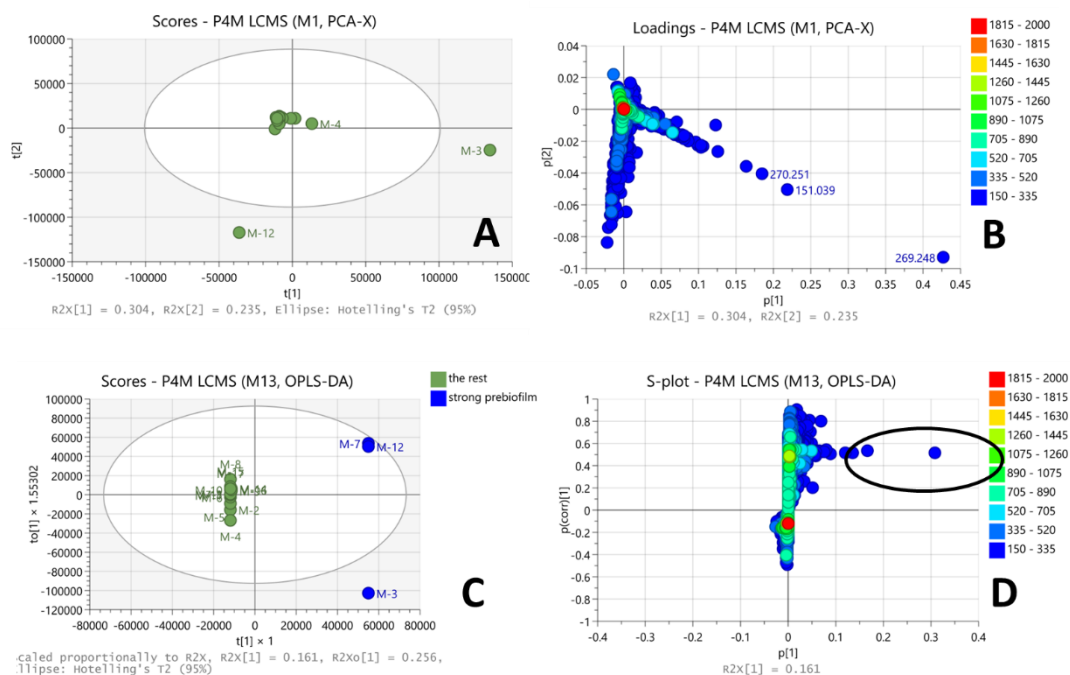


Figure 6.12 LCMS-based multivariate analysis of DM fractions. A) PCA Scores plot, B) PCA loading plot, C) OPLS-DA Scores plot, D) OPLS-DA S-plot. PCA: $R^2=0.54$, $Q^2=-0.21$; OPLS-DA $R^2=0.99$, $Q^2=0.81$

To identify the discriminating metabolites in the active fractions, OPLS-DA was applied. The fractions with strong prebiofilm inhibition (M3, M7, and M12) were grouped together and can be found on the upper and lower quadrants of the scores plot shown in Figure 6.12C. M7 and M12 were clustering together indicating that they have more similarities than with the other fractions. M3 was at the lower right quadrant and was separated from the other two fractions which means larger differences among them. The bioactive discriminatory features were encircled on the S-plot (Figure 6.12D). Its dereplication results were listed in Table 6.4 and their chemical structures were shown in **Error! Reference source not found..**

Two lipids were putatively annotated as 9Z-heptadecenoic acid and 9,12,15-octadecatrienoic acid. The NMR-based metabolomics profiling data supports this because chemical shifts for lipids were also identified as discriminatory features for the bioactive

fractions. Regiolone, which was described in the preliminary screening and OSMAC phases as a target metabolite, was again annotated.

Table 6.5 Dereplication of discriminatory metabolites from selected DM fractions (M3,M7, and M12)

<i>p</i> -value	<i>m/z</i>	Molecular weight (g/mol)	Molecular formula	Dereplication hit	Biological Source
7.71E-05	186.0775	187.0847	C ₈ H ₁₃ NO ₄	Emanidin	<i>Diaporthe</i> sp. SNB-GSS10 and <i>Phomopsis</i> sp. D15a2a
0.0001	157.0508	158.0581	C ₇ H ₁₀ O ₄	Terredionol	<i>Aspergillus terreus</i> and <i>Beauveria amorph</i>
0.0228	191.0703	190.063	C ₁₁ H ₁₀ O ₃	Altechromone A	<i>Aloe ferox</i> , <i>Polygonum cuspidatum</i> and <i>Rheum</i> sp. (rhubarb). Produced by an <i>Alternaria</i> sp., <i>Papulaspora immersa</i> and <i>Talaromyces flavus</i> and a marine-derived fungal strain 222
0.0264	194.1176	193.1104	No hit		
0.0266	187.0965	188.1038	No hit		
0.0277	183.1015	182.0942	C ₁₀ H ₁₄ O ₃	2-(Hydroxymethyl)-3-propylphenol	<i>Daldinia concentrica</i> S0318 and <i>Phomopsis</i> sp. E02011
0.0317	207.1381	206.1308	C ₁₃ H ₁₈ O ₂	(+)-Aureonitol	<i>Chaetomium globosum</i> growing on tomatoes
0.0319	279.2321	278.2248	C ₁₈ H ₃₀ O ₂	9Z,12Z,15Z-octadecatrienoic acid (α-linolenic acid)	various sources
0.0320	171.1169	170.1097	C ₁₃ H ₁₄	2,8,10-Tridecatriene-4,6-diyne	<i>Aethusa cynapium</i> , <i>Peucedanum verticillare</i> , <i>Pimpinella magna</i> and <i>Pimpinella anisun</i>
0.0321	189.1275	188.1202	C ₁₃ H ₁₆ O	<i>E</i> -7-Phenyl-4-hepten-3-one	<i>Phellinus tremulae</i>

<i>p</i> -value	<i>m/z</i>	Molecular weight (g/mol)	Molecular formula	Dereplication hit	Biological Source
0.0331	287.2582	286.2509		No hit	
0.0335	270.2509	269.2436		[M+2H] ⁺ of <i>m/z</i> 269.248 Da	
0.0335	269.2477	268.2404	C ₁₇ H ₃₂ O ₂	9-Heptadecenoic acid	Minor constituent of several animal and vegetable fats. Constituent of <i>Amphimedon complanata</i> . Prod. by <i>Sporothrix flocculos</i>
0.0374	179.0701	178.0628	C ₁₀ H ₁₀ O ₃	Regiolone	<i>Asteromyces cruciatus</i> 763, <i>Botrytis fabae</i> , <i>Daldinia eschscholtzii</i> HJ004, <i>Discula</i> sp., <i>Microsphaeropsis</i> sp., <i>Phaeoacremonium aleophilum</i> , and the freshwater sp. from strain G730 of the order <i>Helotiales</i> . Constituent of <i>Engelhardtia roxburghiana</i> , <i>Juglans regia</i> , and <i>Juglans mandshurica</i> var. <i>sieboldiana</i> .

Compound dereplication are based on highly accurate mass matching (<5 ppm) against Dictionary of Natural Products, with filtering by fungal biological source. No tandem MS/MS data were acquired; therefore, these are tentative assignments that require confirmation through MS fragmentation data or isolation (cells highlighted in blue correspond to compounds that were subsequently isolated)

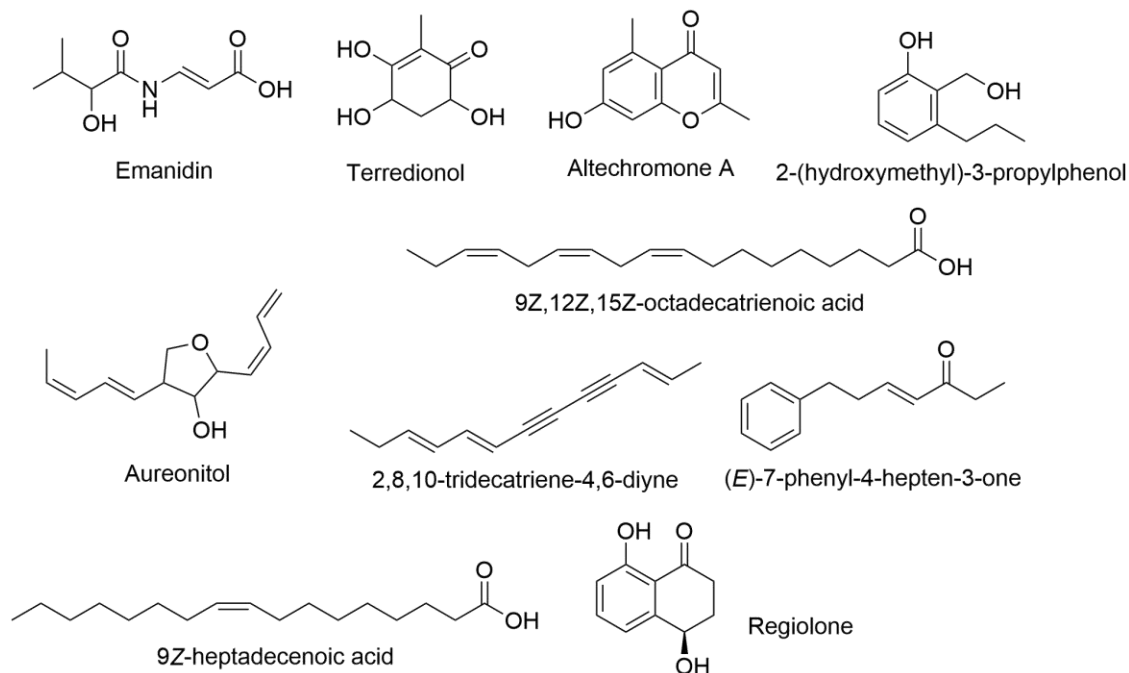


Figure 6.13 Chemical structure of discriminatory metabolites from selected DM fractions (M3, M7, and M12)

6.3.3 Purification of compounds from DM fractions

Fractions M3, M4, and M7 were selected for further purification based on bioactivity and yield. M3 and M7 showed strong prebiofilm inhibition, along with M12. However, M12 was not selected due to its low yield (25 mg, Table 6.3) and complex ^1H -NMR spectrum (Figure 6.10). Although M3 and M7 also had low yields, their ^1H -NMR spectra were simpler, making them more suitable for purification. Furthermore, M4 was chosen for its moderate postbiofilm inhibition, higher yield, and simpler ^1H -NMR spectrum.

M3 was purified using preparative TLC using a DCM:EtOAc (5:95) solvent system. Four fractions were obtained (M3.1 to 3.4). M3.2 and 3.3 afforded the highest yields and were therefore subjected for structure elucidation, as described in sections 6.3.4.1 and 6.3.4.2.

The purification of M4 was carried out in a BiotageTM MPLC system over a normal phase silica cartridge (Sfär HP-Sphere, 25-33 μm , 10g; BiotageTM). A stepwise elution

programme started with 60% EtOAc and 40% hexane for 10 min followed by 65% EtOAc for 10 min, then finally at 90% EtOAc for 15 min. Two subfractions were obtained (M4.1 and M4.2).

M7 was purified by preparative TLC with 100% EtOAc as the eluting solvent. Three subfractions were obtained. The major peaks in ^1H -NMR of M7 can be predominantly found in M7.2, which were later identified as niacin (6.3.4.4).

6.3.4 Structure elucidation of isolated compounds

6.3.4.1 M3.2 as 2(3H)-benzofuranone

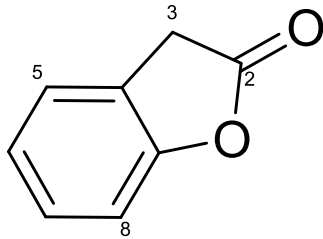
2(3H)-benzofuranone/2-coumaronone/isophthalide	
Code: M3.2	
Weight: 4mg (0.20% yield)	
From: M3	
Purity: 90%	
Physical description: colourless oil	
Molecular formula: $\text{C}_8\text{H}_6\text{O}_2$	
Molecular weight: 134.13 g/mol	
Undetectable in the MS measuring range	

Figure 6.14 Chemical and spectroscopic data of M3.2

M3.2 was obtained as a colourless oil. Combined investigation of ^1H and 2D NMR (Figure S 32 to Figure S 34) of M3.2 led to its identification as 2(3H)-benzofuranone. An ABCD type of benzene substitution was observed based on the pattern of aromatic peaks. Two aromatic protons were shielded because of the electron donating effects of oxygen to its *ortho* (δ_{H} 6.78, dd, $J = 8.02, 1.22$, H-8) and *para* (δ_{H} 6.62, td, $J = 7.40, 1.25$, H-6) positions. A

methylene proton, adjacent to the carbonyl carbon, was assigned to the singlet at δ_{H} 3.45 (H-3). Its NMR data was closely comparable (Table 6.6) to its reported values which further proved its elucidation.

Table 6.6 ^1H and ^{13}C NMR data comparison of M3.2 from reported values of 2(3*H*)-benzofuranone

Atom no	M3.2, this study,		2(3 <i>H</i>)-benzofuranone, (Seath et al., 2017)	
	δ_{H} in ppm, (<i>J</i> in Hz) 400MHz in DMSO d_6	δ_{C} in ppm 100MHz in DMSO d_6	δ_{H} in ppm, (<i>J</i> in Hz) 400 MHz, CDCl_3	δ_{C} in ppm 101 MHz, CDCl_3
2	-	173.2	-	173.6
3	3.45 (2H, s)	36.2	3.66 (2H, s)	32.5
4	-	122.5	-	122.6
5	7.08 (1H, dd, 7.39, 1.73)	131.3	7.26 (1H, m)	128.4
6	6.72 (1H, td, 7.40, 1.25)	119.0	7.06 (1H, t, 7.5)	123.6
7	7.05 (1H, dd, 7.66, 1.82)	128.2	7.19 (1H, m,)	124.1
8	6.78 (1H, dd, 8.02, 1.22)	115.2	7.03 (1H, d, 8.0)	110.3
9	-	155.9	-	154.2

The chemical structure of 2(3*H*)-benzofuranone or 2-coumaronone contains a benzene ring fused to a γ -butyrolactone. It has been reported as a volatile component of several plants such as *Ficus hirta* oil (Du et al., 2018), *Salvadora persica* extracts (Al-Sohaibani & Murugan, 2012), *Anabasis salsa* (Datkhayev et al., 2024), and *Saussurea lappa* root extracts (Mu, Ru, & Li, 2025). It has also been reported from fungi such as *Sordariomycetes* sp. (Jinfeng et al., 2016) and an entomopathogenic fungus *Beuvaria brongniartii* (Fan et al., 2008). In all the studies mentioned above, the compound able to be detected through gas chromatography-mass spectrometry (GC-MS). This study will be the first to report its occurrence from the fungus *Daldinia eschscholtzii*.

6.3.4.2 M3.3 as regiolone

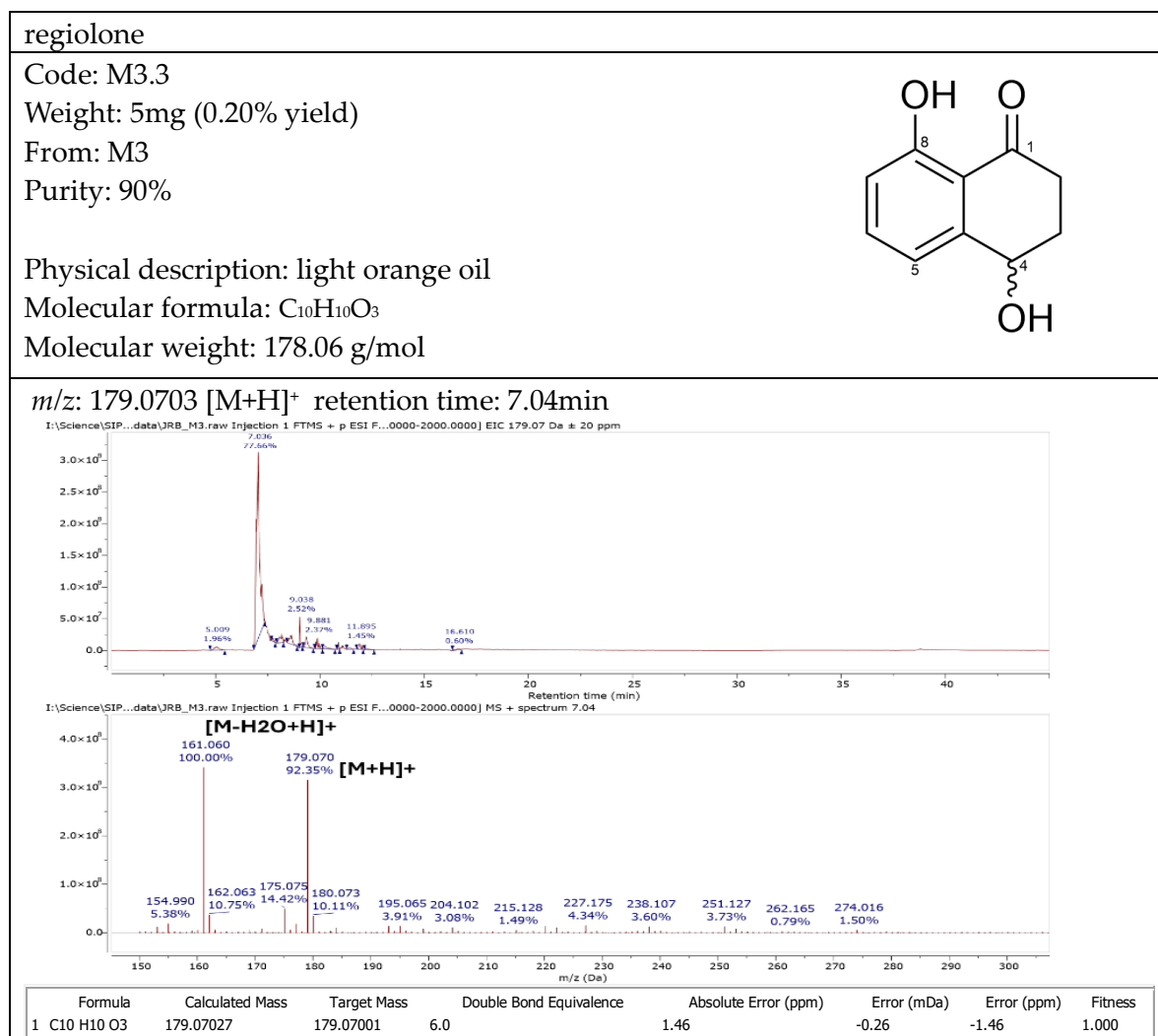


Figure 6.15 Chemical and spectroscopic data of M3.3

M3.3 has an ion peak of 179.0703 [M+H]⁺ which was computed for a molecular formula of C₁₀H₁₀O₃ (DBE=6) as shown in Figure 6.15. This feature was pinpointed as discriminatory metabolite in the media optimisation stage and was putatively annotated as regiolone (see Table 6.2, highlighted in blue). Its ¹H-NMR data (Figure S 35 to Figure S 37) corresponded to its chemical structure. Briefly, the most deshielded peak at 12.40 ppm was assigned as the phenolic hydroxyl proton (8-OH) because it forms an

intramolecular H-bond with the oxygen of the neighbouring carbonyl group. There are three aromatic protons following an ABC pattern according to their coupling pattern (δ_{H} in 7.55, t, $J=7.93$ Hz, H-6; 7.08, dt, $J=7.56, 1.08$ Hz, H-5; 6.84, d, $J=8.31, 0.82$, H-7). There are two coupling methylene protons as indicated in their integration values and its COSY spectrum (S36). Furthermore, the methylene protons are coupling with a methine (δ_{H} 4.75, dd, $J= 15.27, 4.79$ Hz, H-4) bonded to an oxygenated carbon (δ_{C} in ppm 66.4, C-4). Moreover, the large coupling constant ($J = 15.27$ and 4.79 Hz), corresponding to axial-axial and axial-equatorial coupling, respectively, of this methine supported its identification as regiolone and not its enantiomer, isosclerone with coupling constants of $J= 6.8$ and 4.2 Hz (Fujimoto et al., 1986). Altogether, its NMR data closely agreed with the reported values of regiolone as shown in Table 6.7 (Liu et al., 2007).

Table 6.7 ^1H and ^{13}C NMR data comparison of M3.3 from reported values of regiolone

Atom no	M3.3, <i>this study</i> δ_{H} in ppm, (J in Hz) 400MHz in DMSO d_6	δ_{C} in ppm 100MHz in DMSO d_6	regiolone, (Liu et al., 2007) δ_{H} in ppm, (J in Hz) in 400MHz, δ_{C} in ppm CDCl ₃ (100MHz, CDCl ₃)
1	-	206.2	-
2	2.74 (2H, m)	35.7	2.97 (1H, ddd, 18.0, 8.4, 4.8) and 2.62 (1H, ddd, 18.0, 8.4, 4.8)
3	2.20 (1H, m) and 1.98 (1H, q, 4.02)	32.0	2.31 (1H, m) and 2.16 (1H, m)
4	4.75 (1H, dd, 15.27, 4.79)	66.4	4.88 (1H, dd, 7.2, 4.0)
4-OH	5.61 (1H, d, 5.78)	-	-
4a	-	149.4	-
5	7.08 (1H, dt, 7.56, 1.08)	117.7	6.99 (1H, d, 7.6)
6	7.55 (1H, t, 7.93)	137.2	7.46 (1H, t, 8.4, 7.6)
7	6.84 (1H, d, 8.31, 0.82)	116.2	6.89 (1H, d, 8.4)
8	-	162.3	-
8-OH	12.4 (1H, s)	-	12.4 (1H, s)
8a	-	147.4	-

Regiolone is tetralone derivative first isolated from the stem barks of *Juglans regia* (Talapatra et al., 1988). It has also been isolated in several fungal species including *D. eschscholtzii* (Liao et al., 2019). Its *S*-enantiomer called isosclerone was reported from the fungus *Sclerotinia sclerotium* as a phytotoxic metabolite (Morita & Aoki, 1974). The absolute configuration of the enantiomers were assigned as (-)-regiolone and (+)-isosclerone (Evidente et al., 2011).

6.3.4.3 M4.1 as tyrosol

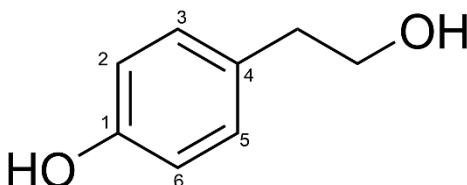
tyrosol/tyrosol C/ <i>para</i> -hydroxyphenethyl alcohol	
Code: M4.1	
Weight: 12mg (0.60% yield)	
From: M4	
Purity: 65%	
Physical description: light brown powder	
Molecular formula: C ₈ H ₁₀ O ₂	
Molecular weight: 138.07 g/mol	
<i>Undetectable in the MS measuring range</i>	

Figure 6.16 Chemical and spectroscopic data of M4.1

MPLC purification of fraction M4 afforded M4.1. Impurities can still be observed from its ¹H-NMR spectrum (Figure S 38 to Figure S 41), but a prominent aromatic compound was able to be identified with around 65% purity. M4.2 also have this compound but contains more impurities. Thus, the elucidation work was focused on the spectrum of M4.1. In the spectrum, two aromatic peaks are *ortho*- and *meta*-coupled (δ_{H} 6.99, dd, $J=8.50, 2.18$ Hz, H-3,5; 6.66, dd, $J=8.57, 3.00$ Hz, H-2,6). Furthermore, both have equal integration values. Together, it points out to an AA'BB' spin system in a benzene ring indicating a *para* substitution. A hydroxyethyl substituent was elucidated from the two methylene protons present (δ_{H} 3.52, td, $J=7.26, 5.11$ Hz, H-1'; 2.60, t, $J=7.26$ Hz, H-2') of which the latter is attached to an oxygen-bearing carbon atom (δ_{C} 62.6, C-1'). This compound was identified as tyrosol with ¹H-NMR values closely matched with the literature (Table 6.8) (Capasso et al., 1992).

Table 6.8 ^1H and ^{13}C NMR data comparison of M4.1 from reported values of tyrosol

Atom no	M4.1, <i>this study</i>	tyrosol, (Capasso et al., 1992)		
	δ_{H} in ppm, (<i>J</i> in Hz) 400MHz in DMSO d_6	δ_{C} in ppm 100MHz in DMSO d_6	δ_{H} in ppm, (<i>J</i> in Hz) in 270MHz, MeOH- d_4	δ_{C} in ppm (68 MHz, MeOH- d_4)
1	-	155.4	-	156.7
1-OH	9.09 (1H, s)	-	-	-
2,6	6.66 (2H, dd, 8.57, 3.00)	114.9	6.67 (2H, d, 8.0)	116.0
3,5	6.99 (2H, dd, 8.50, 2.18)	129.6	6.98 (2H, d, 8.0)	130.8
4	-	129.4	-	130.9
1'	3.52 (2H, td, 7.26, 5.11)	62.6	3.63 (2H)	64.5
2'	2.60 (2H, t, 7.28)	38.2	2.66 (2H)	39.3

Tyrosol is the major phenolic compound in olive oils (Owen et al., 2000; Romero & Brenes, 2012) and wines (Dudley et al., 2008; Gris et al., 2011). It has also been reported to occur in several fungi such as *Ophiostoma crassivaginata* (Ayer & Trifonov, 1995), *Papulaspora immersa* (Borges Coutinho Gallo et al., 2010), *Colletotrichum gloeosporioides* (Masi et al., 2018), *Daldinia loculata* (Nadeau & Sorensen, 2011) and *Daldinia eschscholtzii* (Kongyen et al., 2015).

6.3.4.4 M7.2 as niacin

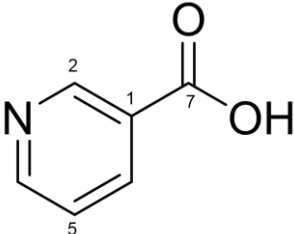
Niacin/nicotinic acid	
Code: M7.2	
Weight: 4mg (0.20% yield)	
From: M7	
Purity: 90%	
Physical description: white powder	
Molecular formula: C ₆ H ₅ NO ₂	
Molecular weight: 123.03 g/mol	
Undetectable in the MS measuring range	

Figure 6.17 Chemical and spectroscopic data of M7.2

Upon purification of M7 in preparative TLC, a pure compound was obtained, as confirmed by its ^1H -NMR spectrum (Figure S 42). The NMR spectrum showed four peaks located at the aromatic region. These protons displayed higher proton resonances relative to those of a typical benzene ring, suggesting the presence of a heteroatom within the aromatic system. The pattern of peaks indicated a mono-substituted, six-membered heteroaromatic ring, consistent with a pyridine derivative. Based on this, the structure was proposed to be niacin or nicotinic acid, a pyridine carboxylic acid. The most deshielded proton resonance at δ_{H} in ppm, 9.07 was assigned to H-2, due to the electron-withdrawing effects of the adjacent nitrogen atom. The remaining three aromatic protons (δ_{H} 8.79, dd, $J=4.8, 1.7$ Hz, H-4; 8.27, dt, $J=7.9, 2.0$ Hz, H-6; 7.54, ddd, $J=7.9, 4.8, 0.9$ Hz, H-5) formed a spin system as shown in the COSY spectrum (Figure S 43) including a *meta* coupling with H-2. Additionally, an HMBC correlation between H-6 and a carbon with resonance of δ_{C} 166.90 (C-7) (see Figure S 44) supported the presence of a carboxyl group. As indicated in Table 6.9, the structure was confirmed by closely-matched comparison from its reported values (Kim, Ryu, & Shim, 2018).

Table 6.9 ^1H and ^{13}C NMR data comparison of M7.2 from reported values of niacin

Atom no	M7.2, This study		niacin, (Kim, Ryu, & Shim, 2018)	
	δ_{H} in ppm, (J in Hz) 400MHz in DMSO d_6	δ_{C} in ppm 100MHz in DMSO d_6	δ_{H} in ppm, (J in Hz) in 500MHz, MeOH- d_4	δ_{C} in ppm 100 MHz, MeOH- d_4
1	-	127.2	-	131.9
2	9.07 (1H, dd, 2.2, 0.9)	149.7	9.02(1H, s)	149.9
3-N	-	-	-	-
4	8.79 (1H, dd, 4.8, 1.7)	152.8	8.64 (1H, d, 3.67)	153.3
5	7.54 (1H, ddd, 7.9, 4.8, 0.9)	123.3	7.48 (1H, dd, 7.7, 5.1)	125.6
6	8.27 (1H, dt, 7.9, 2.0)	136.6	8.32 (1H, dt, 7.9, 1.8)	137.8
7	-	166.9		170.3

Niacin (also known as nicotinic acid or vitamin B3) is an essential nutrient involved in numerous physiological processes. It is a precursor to nicotinamide adenine dinucleotide (NAD⁺) and its phosphorylated form (NADP⁺), which acts as cofactors in many metabolic reactions. Bacteria and plants biosynthesise niacin from tryptophan (Foster & Moat, 1980; Tarr & Arditti, 1982). Occurrence in the fungal kingdom has been documented. Several edible mushrooms have been identified as rich sources of dietary nicotinic acid (Mallavadhani et al., 2006). The endophytic fungus *Gibberella moniliformis* JS1055 produced niacin when it was grown in rice media (Kim, Ryu, & Shim, 2018). Co-culture of *Phomopsis asparagi* with another *Phomopsis* species in rice media afforded the isolation of niacin alongside some new metabolites (Wu et al., 2024). In the present study, cultivation of *D. eschscholtzii* in rice media also yielded niacin. These observations suggest that rice-based fungal cultivation may favour the biosynthesis of this essential nutrient. However, further systematic investigations are necessary to substantiate this hypothesis.

6.3.5 Biological activities of isolated compounds

The four compounds were evaluated for their biological activities against MRSA. As shown in Table 6.10, none of the individual compounds exhibited strong inhibitory activity. In contrast, the fractions from which these compounds were isolated showed strong to moderate activity, suggesting that the bioactivity may not be attributed to a single constituent. These findings raise a possibility that the observed prebiofilm activity of the parent fractions result from synergistic interactions among multiple metabolites.

Table 6.10 Biological activities of compounds from *D. eschscholtzii* grown in rice against MRSA

Sample code	Identity, %purity	Antibacterial activity		Prebiofilm activity		Postbiofilm activity	
		%Inhibition @100 µg/mL	MIC (µM)	%Inhibition @100 µg/mL	BPC (µM)	%Inhibition@100 µg/mL	MBIC (µM)
M3.2	2(3H)-benzofuranone, 90%	42.92	>100	5.72	>100	-5.60	>100
M3.3	regiolone, 90%	-12.79	>100	34.70	>100	4.13	>100
M4.1	tyrosol C, 65%	23.07	>100	10.23	>100	-5.00	>100
M7.2	niacin, 90%	-7.64	>100	9.82	>100	-4.28	>100
Ciprofloxacin		97.65	2.03	99.86	1.56	94.47	10.52
DMSO		6.73	>100	-13.79	>100	0.4	>100

Assays were done twice with three replicates (n=3); Moderate inhibition: 50-70% (coloured in orange); Strong inhibition >70% (coloured in red). *All isolated compounds were found inactive*

MIC- Minimum Inhibitory Concentration; the smallest concentration of sample to give 100% inhibition; computed from a series of sample dilutions in GraphPad Prism

BPC- Biofilm Prevention Concentration; the smallest concentration of sample to give 90% inhibition in the prebiofilm assay

MBIC- Minimum Biofilm Inhibition Concentration; the smallest concentration of sample to give 90% inhibition in the postbiofilm assay

6.3.6 Summary

Section 6.3 presents the isolation work with *D. eschscholtzii* following the optimum culture media and incubation period. The fungus was scaled up in rice media for 15 days and fractionated to afford four metabolites: 2(3H)-benzofuranone, regiolone, tyrosol, and niacin. However, none of these compounds displayed significant biological activities. The isolated compounds particularly regiolone as one of the defined target metabolites could have acted synergistically and/or additively to exhibit the observed biological activities in their parent extracts. Given the lack of bioactivity in the initial set of isolated compounds, further exploration of the biosynthetic capacity of *D. eschscholtzii* was pursued by introducing chemical elicitors in subsequent experiments to potentially induce the production of novel or more potent metabolites.

6.4 The influence of chemical elicitors to *Daldinia eschscholtzii*

OSMAC strategies were employed in this study by varying both the culture media and incubation period. This approach proved effective in identifying optimised conditions that promoted the diversification of secondary metabolites. To further enhance metabolite production, epigenetic modification can also be explored as a viable complementary strategy (Bind et al., 2022). For this purpose, *D. eschscholtzii* was selected due to its fast growth. Its cultivation in PDB for 15 days was chosen, as the extracts from this condition exhibited moderate prebiofilm activity (Table 6.4). At this point, the aim was to evaluate whether the introduction of epigenetic modifiers or chemical elicitors to *D. eschscholtzii* could influence the production of its bioactive metabolite, particularly those that may have been diminished or lost during purification, as observed in Section 6.3.5.

Two known elicitors, suberoyl bishydroxamic acid (SBHA) and 5-azacytidine (Aza), was added to PDB media. The fungus was grown also in plain PDB to serve as a control. After 15 days of incubation, cultures were extracted with EtOAc. The resulting crude extracts, derived from SBHA- and Aza-treated PDB cultures (hereafter referred to as SBHA and Aza extracts, respectively), were subjected to bioassay and metabolomics profiling

6.4.1 Biological assay results

The results of the bioassays performed on *D. eschscholtzii* crude extracts were presented in Table 6.11. All the extracts were inactive against *P. aeruginosa* since their activities were below 50%. There was a statistically significant increase in antibacterial activity against MRSA for extracts obtained with SBHA- and Aza-elicited cultures. However, the antibacterial activities were still classified as moderate (50–70% inhibition). The SBHA-elicited culture extract exhibited strong prebiofilm inhibitory activity, while the Aza-elicited culture extract demonstrated moderate inhibition. Furthermore, both values were statistically significant compared to the culture extracts obtained from the PDB media with

no elicitors, indicating that the addition of chemical elicitors enhanced bioactivity. Finally, no significant improvement in postbiofilm inhibitory activity was observed from extracts in either of the cultures with any of the chemical elicitors added to the media.

Table 6.11 Biological activities of *D. eschscholtzii* grown in PDB with elicitors

Antibacterial activity, average % inhibition (n=3)		
sample	MRSA	<i>P. aeruginosa</i>
plain PDB (control)	22.92	9.15
SBHA	51.43 ^a	15.42
Aza	62.74 ^a	18.81
Ciprofloxacin (50 µM)	98.12	99.77
DMSO	-14.54	0.83
Prebiofim activity, average % inhibition (n=3)		
sample	MRSA	<i>P. aeruginosa</i>
plain PDB (control)	45.29	-181.14
SBHA	85.26 ^a	20.38
Aza	68.72 ^a	-16.58
Ciprofloxacin (50 µM)	98.12	99.86
DMSO	-14.54	-9.48
Postbiofilm activity, average % inhibition (n=3)		
sample	MRSA	
plain PDB (control)	-3.54	
SBHA	-0.85	
Aza	2.92	
Ciprofloxacin (50 µM)	90.25	
DMSO	-11.11	

^a-statistically significant (P<0.05) vs the control group. Testing concentration=100 µg/mL; assays were done twice with three replicates (n=3); Moderate inhibition: 50-70% (coloured in orange); Strong inhibition >70% (coloured in red);

6.4.2 Metabolomics Profiling

6.4.2.1 NMR-based profiling

The stacked ^1H -NMR spectra of the crude extracts were shown in Figure 6.18. Notably, the aromatic proton signals were more prominent in the extracts from elicited PDB cultures. Similar trends can be observed on the olefinic region and methylene proton regions, suggesting increased chemical complexity. To further support these findings, a heatmap was generated and is shown in Figure 6.19. The heatmap reveals a higher chemical diversity in extracts obtained in cultures grown with elicitors compared to those without, with the extracts afforded by SBHA-elicited cultures displaying the most pronounced overall increase.

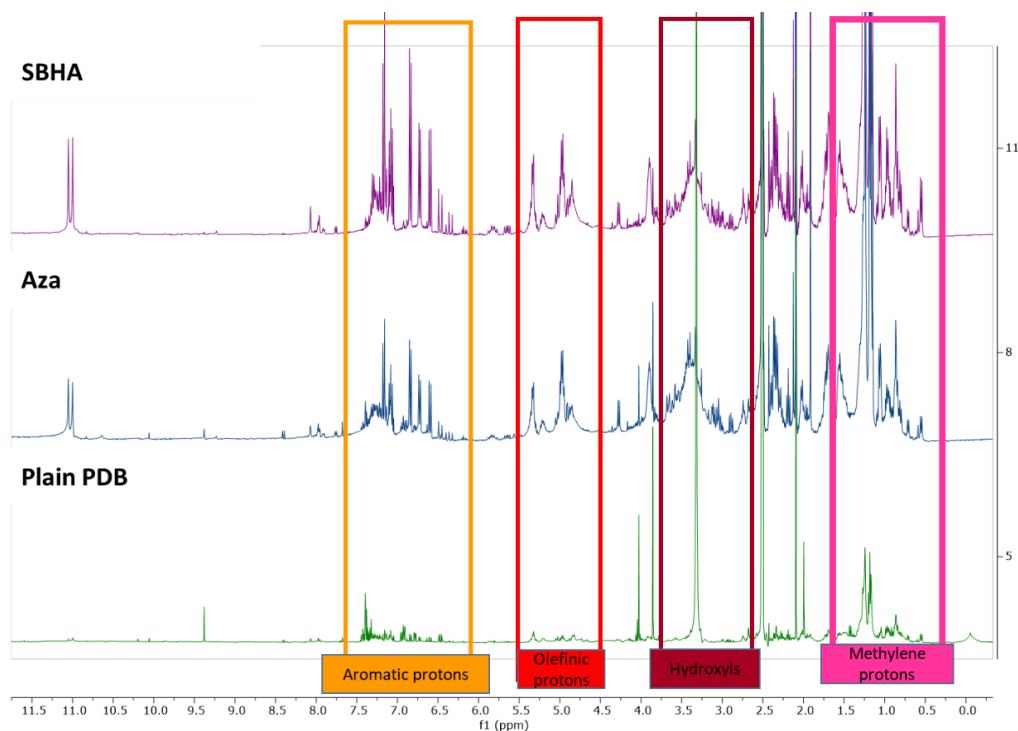


Figure 6.18. ^1H -NMR spectra of *D. eschscholtzii* extracts grown with and without SBHA and Aza elicitors. ^1H -NMR recorded in 400 MHz in DMSO-d_6

Heatmap based on NMR profiles of *D. eschscholtzii* extracts

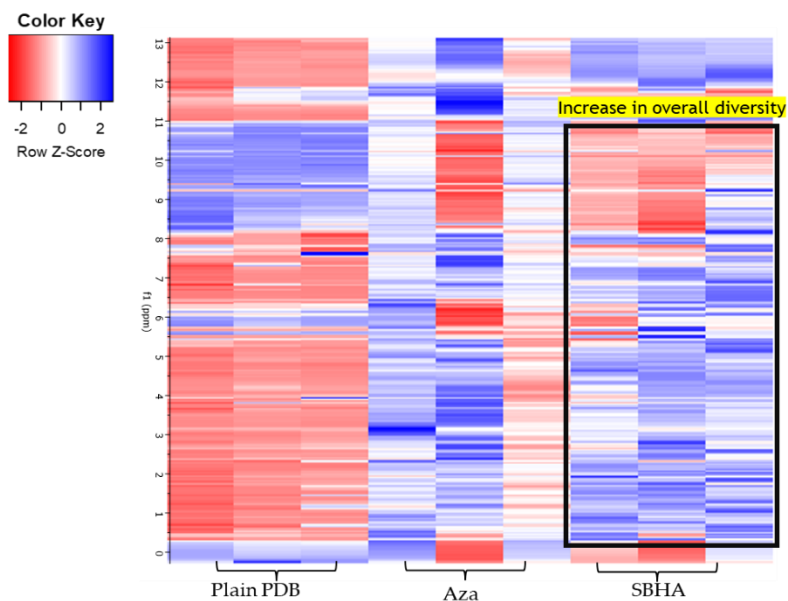


Figure 6.19. ^1H -NMR-based heat map comparing the metabolite profiles of *D. eschscholtzii* extracts grown with and without SBHA and Aza elicitors. Heatmap generated in R (code available in S 2)

Multivariate analysis was applied to determine similarities and differences between the NMR profiles of the extracts. PCA scores plot and its corresponding loadings plot were displayed in Figure 6.20A and B, respectively. According to the scores plot, extracts obtained from elicited cultures were clearly separated from unelicited PDB cultures, which was positioned on the opposite side of the scores plot. The loadings plot revealed a greater dispersion of data points corresponding to extracts from elicited cultures, particularly positioned on the right quadrants. These dots, representing chemical shifts primarily in the 1 to 4 ppm range, suggested the presence of hydroxyl-bearing protons at 3 to 4 ppm and aliphatic proton signals such as methylene and methyl groups at 1 to 3 ppm. The supervised PLS-DA model (Figure 6.20C and D) displayed a similar clustering pattern to that of the PCA, reinforcing the observed distinction between extracts from elicited and non-elicited PDB cultures. This trend is also evident in the heatmap shown in Figure 6.19. Collectively,

the ^1H -NMR-based multivariate analyses indicated that the application of epigenetic elicitors induced *D. eschscholtzii* to produce a more chemically diverse set of metabolites.

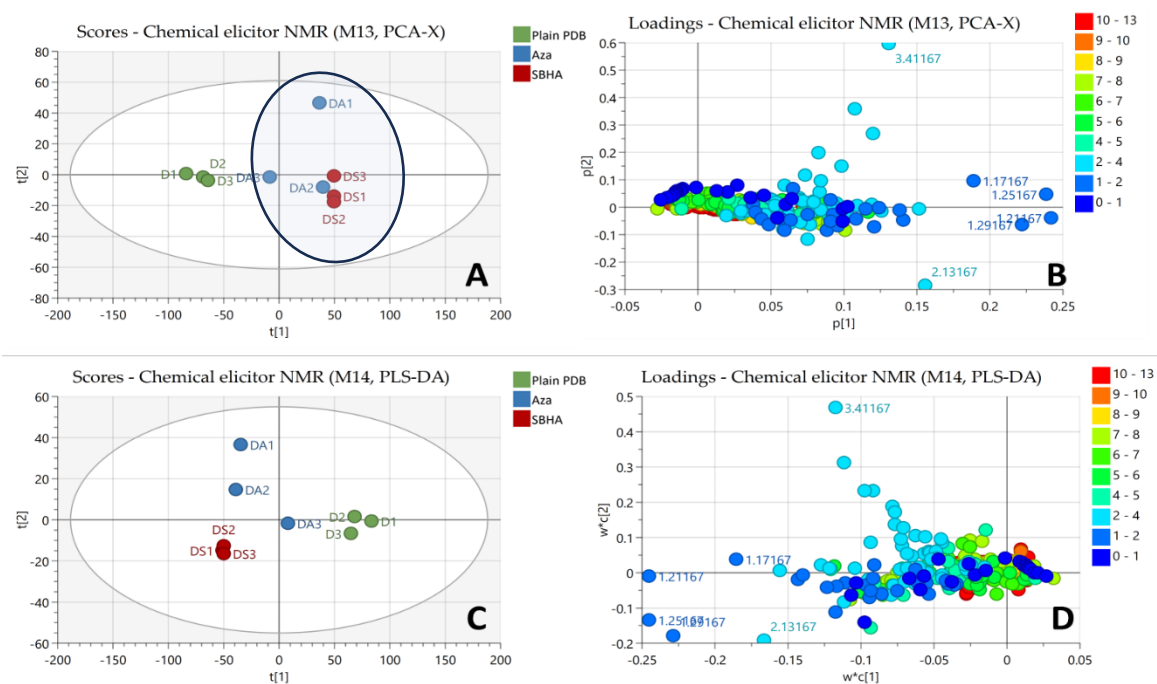


Figure 6.20. ^1H -NMR-based multivariate analysis of *D. eschscholtzii* extracts grown with and without elicitors A) PCA Scores and B) PCA loadings plot, C) PLS-DA Scores and D) PLS-DA Loadings plot. Dots in the scores plot represent a technical replicate ($n=3$). PCA: $R^2=0.96$ $Q^2=0.78$; PLS-DA: $R^2=0.80$ $Q^2=0.57$

6.4.2.2 LC-MS-based profiling

Multivariate analyses were applied to the LC-MS data of the fungal extracts. As shown in Figure 6.21A, the PCA scores plot revealed a clear separation between the elicited extracts and those from plain PDB culture extract, consistent with the NMR-based analysis. Both the unsupervised and supervised models exhibited similar clustering patterns (Figure 6.21A to D). Examination of the corresponding loadings plots indicated comparable feature diversity between the elicited and unelicited cultures. Markedly, extracts obtained from the

elicited cultures showed features with higher m/z values (~900 Da), which were absent in extracts from unelicited PDB cultures.

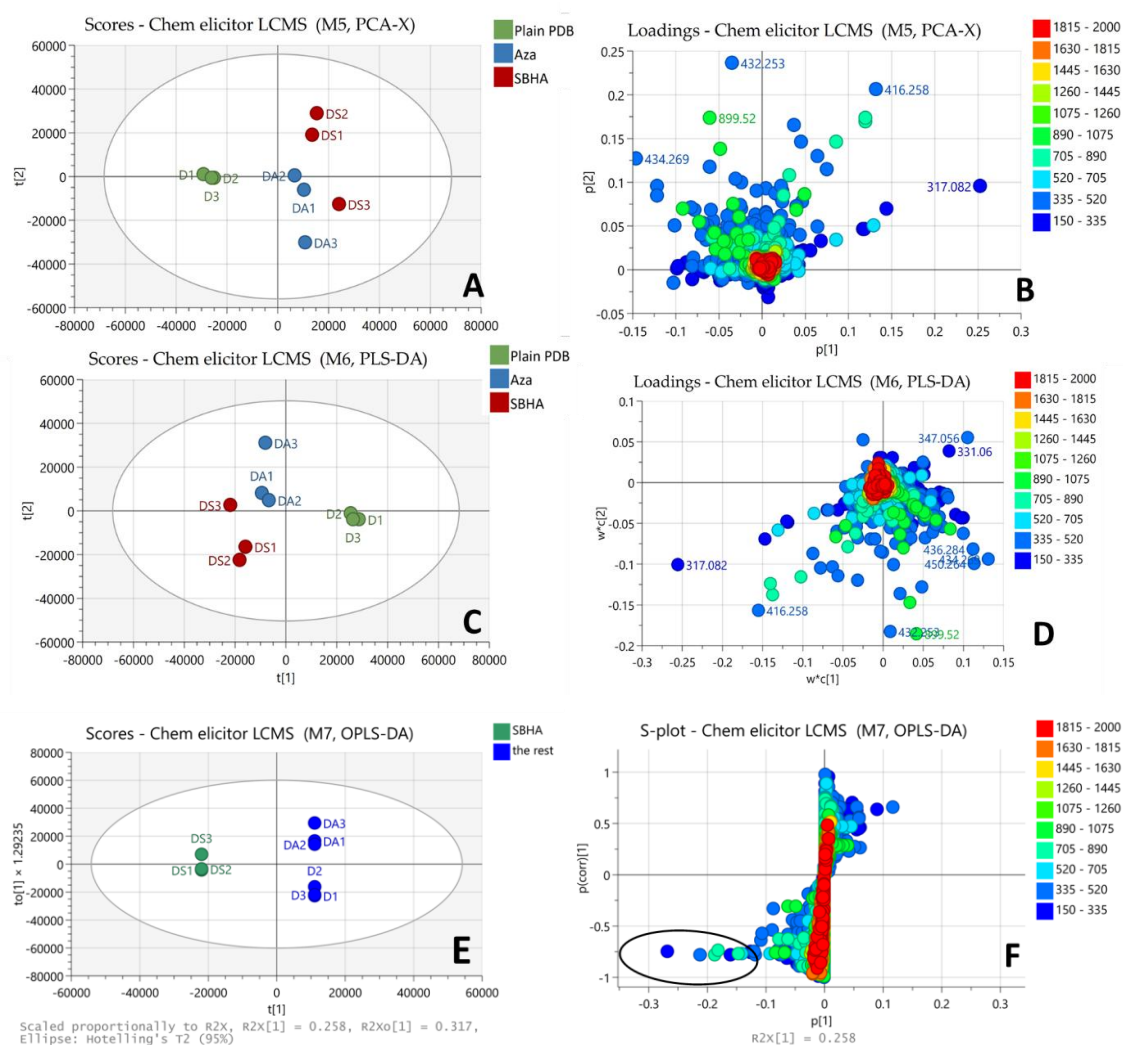


Figure 6.21. LCMS-based multivariate analysis of *D. eschscholtzii* extracts grown with and without elicitors A) PCA Scores, B) PCA Loadings plot, C) PLS-DA Scores, and D) PLS-DA Loadings plot, E) OPLS-DA scores plot, F) OPLS-DA S-plot. Dots in the scores plot represent a technical replicate (n=3). PCA: R²=0.68 Q²=0.38; PLS-DA: R²=0.99 Q²=0.89; OPLS-DA R²=0.99, Q²=0.91

To pinpoint the discriminatory metabolites of extracts afforded by SBHA-elicited cultures, an OPLS-DA model was employed. The SBHA extracts were located at the left

quadrants of the scores plot while extracts from Aza-elicited and non-elicited PDB media are on the opposite side as shown in Figure 6.21A. Key discriminatory features were encircled in the corresponding S-plot (Figure 6.21B) and were subsequently dereplicated using an in-house Excel macro in conjunction with the Dictionary of Natural Products database.

The putative identities of the discriminatory metabolites of *D. eschscholtzii* grown in PDB treated with SBHA were summarized in Table 6.12, while structures were showed in **Error! Reference source not found..** Many of the features were assigned as adducts or fragment ions of a 2M parent ion. For example, the discriminatory ion peaks with m/z of 320.0996 Da, 635.1713 Da, and 636.1745 Da were determined to be $[M+2H]^+$, $[2M-H]^-$, and $[2M]^-$, respectively. These were features associated with 1,1'-binaphthalene-4,4',5,5'-tetrol (BNT) with a molecular weight of 318.0891 g/mol. Alvertoxin and truncatone D are structurally related with BNT as they are naphthol dimers. Furthermore, several ion peaks were determined to be adducts of a cytochalasin and their isomers. These cytochalasin isomers do not have their conventional or trivial names and are listed only by their IUPAC names in the database. For clarity and ease of presentation, the derivatives were named according to the nomenclature used in the original literature reports (Buchanan, Hashimoto, & Asakawa, 1995; Buchanan, Hashimoto, & Asakawa, 1996). This approach was adapted hereafter to denote respective cytochalasin derivatives.

Table 6.12. Dereplication of discriminatory metabolites of the bioactive *D. eschscholtzii* grown in PDB with the chemical elicitor SBHA

<i>p</i> -value	<i>m/z</i>	Molecular weight (g/mol)	Molecular formula	Dereplication hit	Reported source
0.000874	319.0964	318.0891	C ₂₀ H ₁₄ O ₄	1,1'-binaphthalene-4,4',5,5'-tetrol (BNT)	<i>Daldinia concentrica</i> and many <i>Hypoxylon</i> spp.
0.000969	320.0996	[M+2H] ⁺ of BNT			
0.001623	635.1713	[2M-H] ⁻ of BNT			
0.001683	636.1745	[2M] ⁻ of BNT			
0.002066	331.0610	332.0683	C ₂₀ H ₁₂ O ₅	Altertoxin VI	<i>Alternaria tenuissima</i> QUE1Se
0.002125	498.3791	497.3719	No hit		
0.003318	333.0767	334.0840	C ₂₀ H ₁₄ O ₅	Truncatone D	<i>Annulohypoxylon cf. truncatum</i>
0.034921	417.2616	[M-2H+H ₂ O] ⁺ of MW 433.258	C ₂₈ H ₃₅ NO ₃	cytochalasin isomers:	Produced by several <i>Daldinia eschscholtzii</i> strains
0.035767	416.2580	[M-H+H ₂ O] ⁺ of MW 433.258		Cytochalasin 6 Cytochalasin 9 cytochalasin 11	
0.039456	867.5300	[2M+H] ⁺ of MW 433.258		Neocytochalasin	
Compound dereplication are based on highly accurate mass matching (<5 ppm) against Dictionary of Natural Products, with filtering by fungal biological source. No tandem MS/MS data were acquired; therefore, these are tentative assignments that require confirmation through MS fragmentation data or isolation (cells highlighted in blue correspond to compounds that were subsequently isolated)					

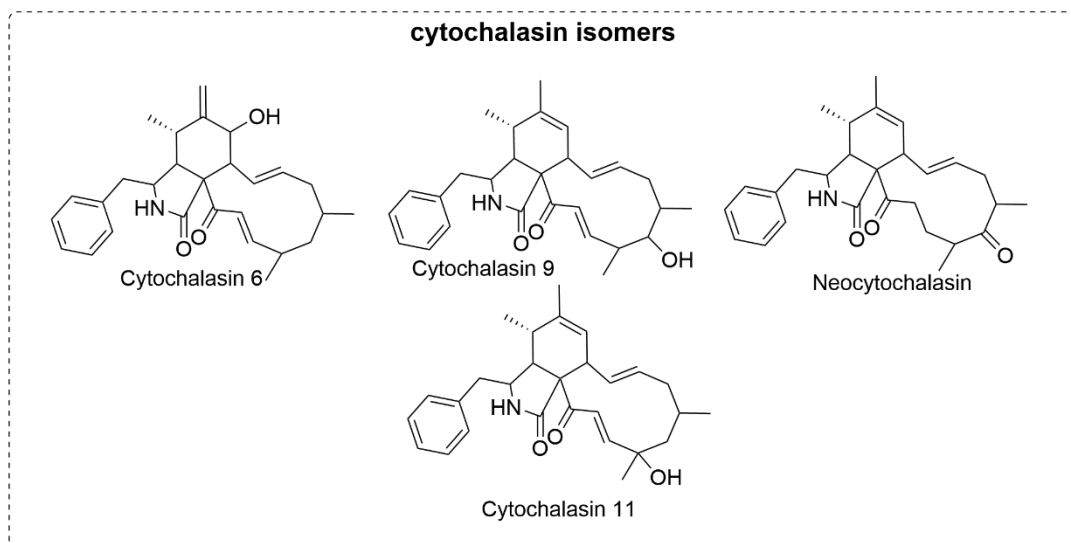
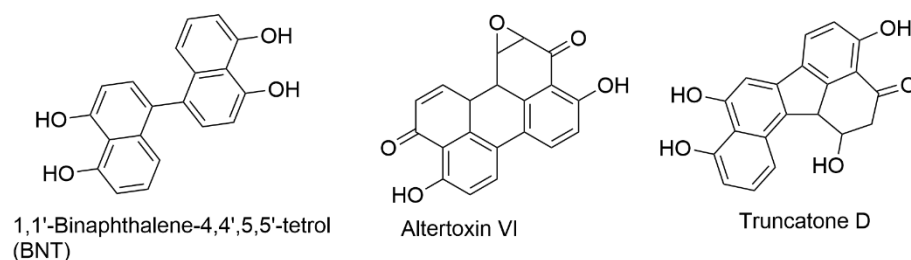


Figure 6.22. Chemical structures of dereplicated discriminatory metabolites from *D. eschscholtzii* grown in PDB with SBHA. The underlined metabolites were later isolated

6.4.3 Summary

The LC-MS-based metabolomic profiling supported the results obtained from NMR, emphasizing that elicitors, particularly SBHA-elicited cultures, stimulated the production of unique metabolites in *D. eschscholtzii*. These metabolites were either absent or present only in low concentrations in extracts of unelicited PDB cultures. Bioassay results confirmed that these elicited compounds contributed to antibacterial and antibiofilm activities.

6.5 Isolation works in *Daldinia eschscholtzii* grown in PDB with SBHA

6.5.1 Scaled up production and solvent partitioning of bioactive metabolites

From the previous section, it was found out that the presence SBHA improved significantly the metabolite diversity and bioactivity of *D. eschscholtzii*. Thus, the fungus was scaled up in SBHA-supplemented PDB media (35 flasks x 250 mL PDB + SBHA for a total of 8.75 L; @27 °C). EtOAc was used for extraction, affording a crude extract weighing at 10.3 grams.

To discard non-polar lipids, the crude extract was partitioned over hexane and 90% aqueous methanol. The non-polar and polar extracts were coded as DSH (1.24g) and DSM (7.57g), respectively. As shown in their stacked spectra at Figure 6.23, the methylene proton signals are broader more prominent in DSH indicating higher concentration of lipids in this extract. Aromatic proton peaks were more pronounced in DSM than DSH. Furthermore, bioassays revealed potency in DSM while DSH was inactive. Thus, DSM was chosen for further purification work.

6.5.2 Fractionation

DSM (7.57 g) was fractionated using a Buchi flash chromatography system over silica cartridge following a linear gradient elution programme detailed in Table 6.13. The 150 eluates were monitored with TLC to pool similar fractions. A total of 10 fractions were obtained and were coded as DSM1 to DSM10. Their weights are shown in the summary schematic diagram in Figure 6.24. The TLC summary plates can be seen in Figure 6.25.

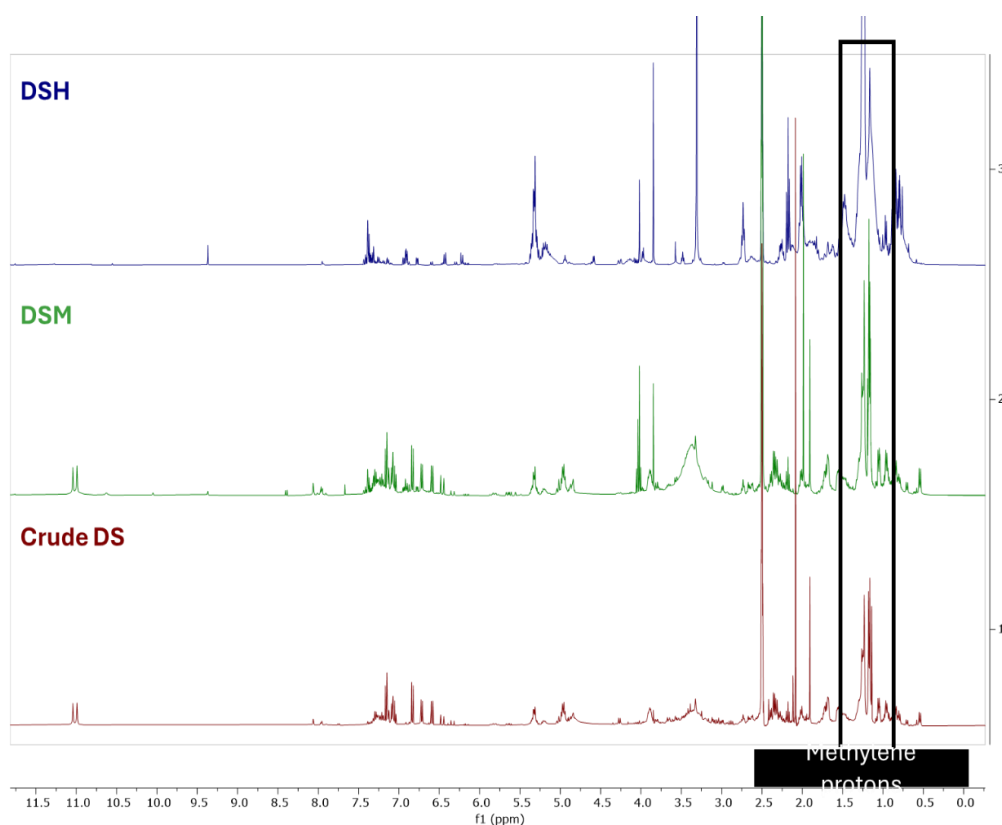


Figure 6.23 Stacked ^1H -NMR spectrum of DS crude extract, DSM, and DSH. Boxed in black represents the long chain methylene protons of lipids. ^1H -NMR recorded in 400 MHz in DMSO-d_6

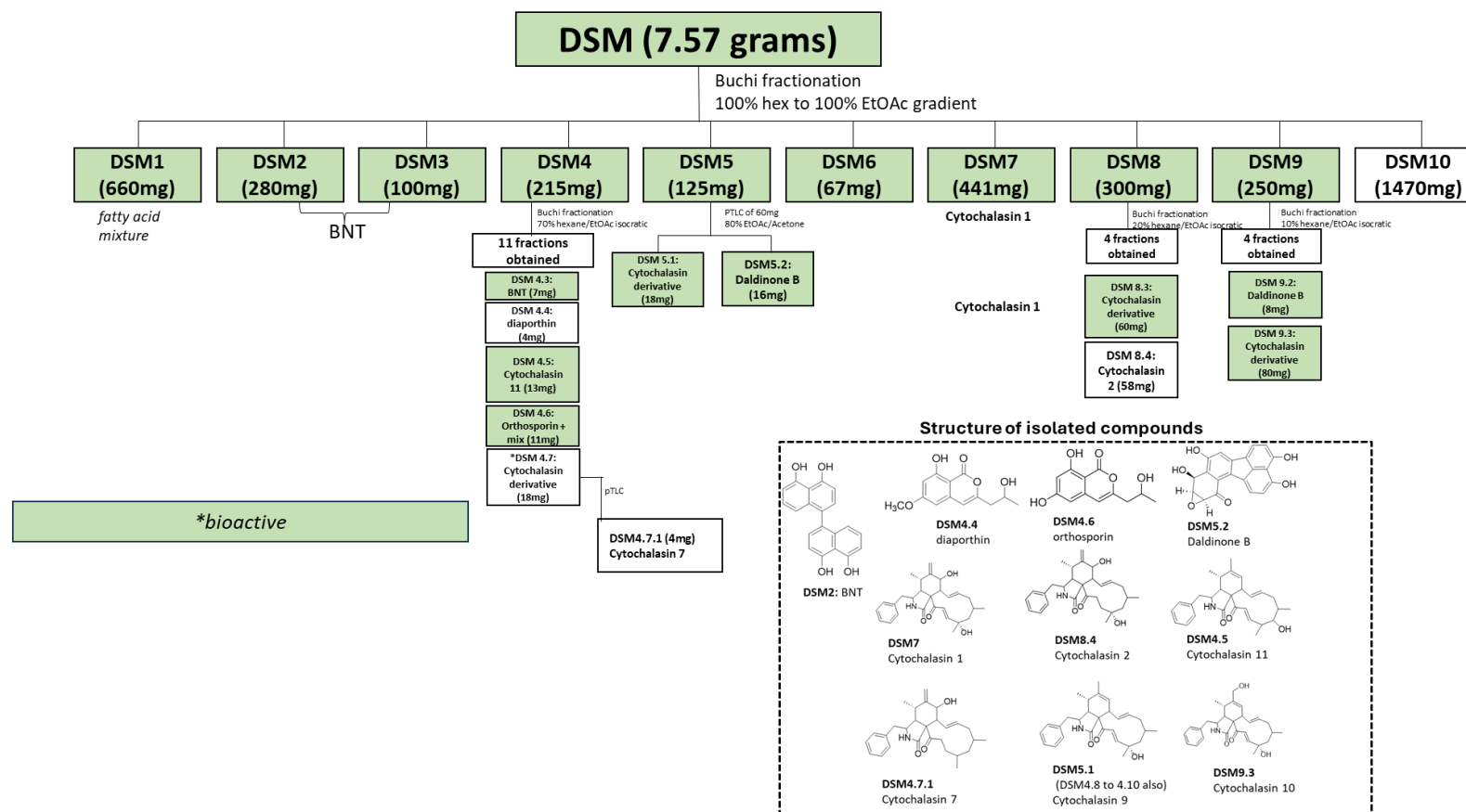


Figure 6.24 Schematic diagram summary for the isolation works done with *D. eschscholtzii* in PDB + SBHA for 15 days

Table 6.13 Parameters for the DSM fractionation in Buchi

Stationary phase: VersaPak Silica Cartridge, 40 x 150mm (Sigma)	
Time (min)	Solvent
0-60	100% Hexane to 100% EtOAc (linear gradient)
60-70	90% EtOAc-10% methanol
70-80	80% EtOAc-20% methanol (isocratic)
Flow rate: 100ml/min; collection vol: 50mL	

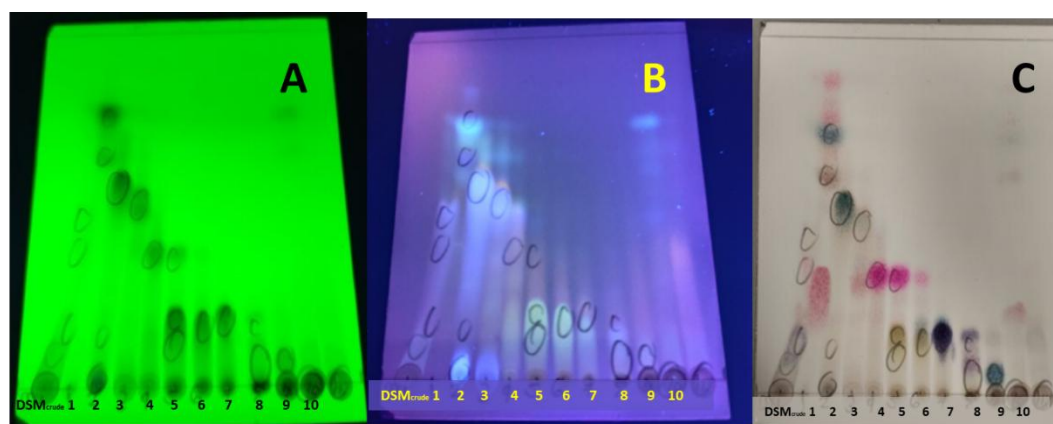


Figure 6.25 TLC summary plate of DSM fractions in normal phase silica gel (A-C). A) UV 254nm; B) UV 365nm C) sprayed with anisaldehyde. Solvent system 2:8 DCM/Ethyl acetate

The biological activities of DSM fractions were evaluated against MRSA and *P. aeruginosa*. The results of the are summarized in Table 6.14. All the fractions exhibited weak activity against *P. aeruginosa*. Some fractions have higher antibacterial activity than the crude DSM. Particularly, fractions DSM4 to DSM7 have strong antibacterial inhibition. Almost all the fractions exhibited strong prebiofilm inhibition similar to the parent DSM extract. Meanwhile, only DSM1 demonstrated strong postbiofilm inhibition.

Table 6.14 Biological activities of DSM fractions

ANTIBACTERIAL ACTIVITY, average inhibition (n=3)					
Sample	MRSA	<i>P. aeruginosa</i>	Sample	MRSA	<i>P. aeruginosa</i>
Crude DSM	52.45	9.96	DSM7	71.89	8.36
DSM1	14.75	3.48	DSM8	33.35	9.19
DSM2	62.55	15.96	DSM9	46.90	-12.96
DSM3	56.47	2.07	DSM10	-3.75	8.23
DSM4	93.41	7.70			
DSM5	81.88	12.50	DMSO	10.27	7.13
DSM6	86.35	6.39	Ciprofloxacin (50 μ M)	97.77	99.70
PREBIOFILM ACTIVITY, average inhibition (n=3)					
Sample	MRSA	<i>P. aeruginosa</i>	Sample	MRSA	<i>P. aeruginosa</i>
Crude DSM	83.88	9.96	DSM7	100.02	8.36
DSM1	85.27	3.48	DSM8	94.01	9.19
DSM2	99.79	15.96	DSM9	90.64	-12.96
DSM3	99.95	2.07	DSM10	67.63	8.23
DSM4	99.96	7.70			
DSM5	96.17	12.50	DMSO	6.74	11.82
DSM6	99.66	6.39	Ciprofloxacin (50 μ M)	99.73	98.95
POSTBIOFILM ACTIVITY, average inhibition (n=3)					
Sample	MRSA		Sample	MRSA	
Crude DSM	-0.85		DSM7	56.80	
DSM1	78.47		DSM8	4.45	
DSM2	44.32		DSM9	-0.25	
DSM3	34.29		DSM10	17.76	
DSM4	32.67				
DSM5	54.47		DMSO	20.33	
DSM6	37.24		Ciprofloxacin (50 μ M)	90.12	

Testing concentration=100 μ g/mL; assays were done twice with three replicates (n=3); Moderate inhibition: 50-70% (coloured in blue); Strong inhibition >70% (coloured in red);

The stacked ^1H -NMR of spectra of the fractions were displayed in Figure 6.26. The spectrum of DSM2 was particularly clean, with sharp, well-resolved peaks, suggesting that this fraction likely resembles a pure compound. Given that DSM2 exhibited bioactivity, structural elucidation was subsequently undertaken (Section 6.5.4.1). DSM3 displayed a spectrum similar to that of DSM2, implying the presence of structurally related or identical compounds. DSM1 mostly contains unsaturated lipids

because of resonances at 5.5 to 6.0 ppm (for olefinic protons) and 1.0 to 2.0 ppm (long chain methylene protons). On the other hand, fractions DSM4 through DSM8 showed more complex and diverse spectra, indicating the presence of at least six metabolites. To explore the chemical similarities and differences among these more complex fractions, further analysis using metabolomics was employed.

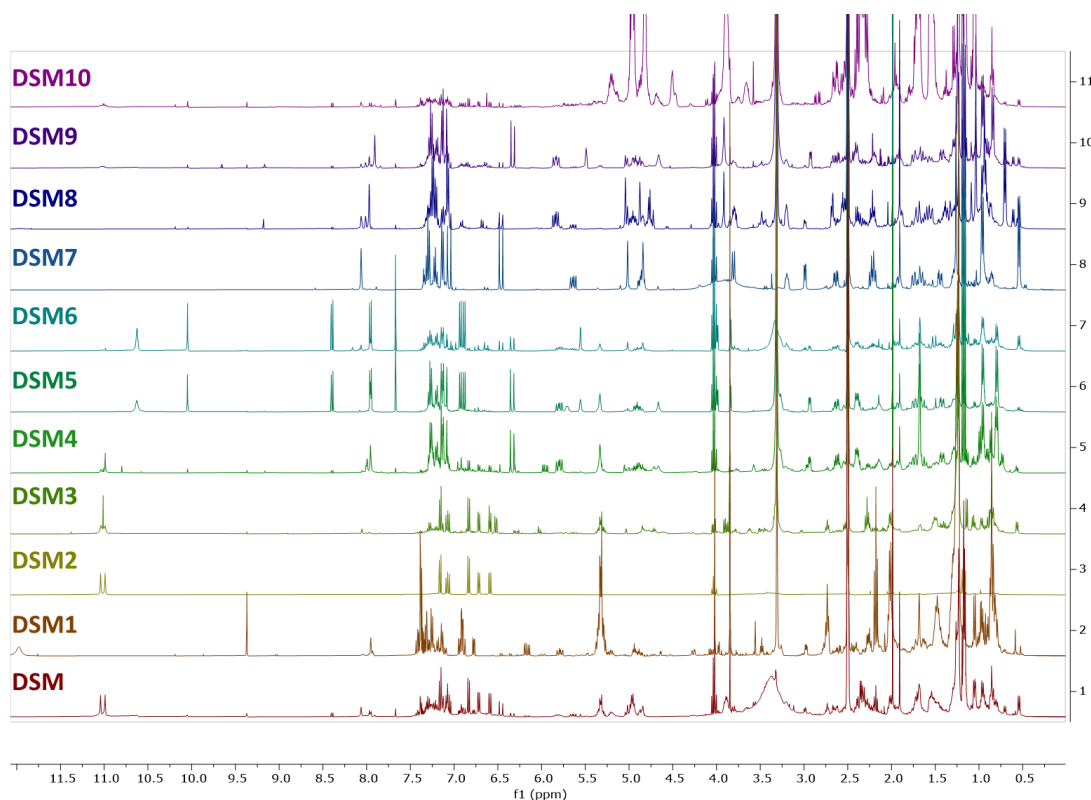


Figure 6.26 Stacked ^1H -NMR spectrum of DSM fractions in ascending order. ^1H -NMR recorded in 400 MHz in DMSO-d_6

Multivariate analyses were applied in their ^1H -NMR spectral data. PCA distinctly separated DSM2, as illustrated in the scores plot in Figure 6.27A. The distinguishing chemical shifts for DSM2 fraction according to the loadings plot at Figure 6.27B were green dots representing aromatic protons (6 to 8 ppm) and red dots highly deshielded protons at 10 to 13 ppm. In contrast, the first and last fractions, DSM1 and DSM10, were

clearly separated (encircled in blue) from the rest of the fractions that were clustering together. For further investigation using an OPLS-DA model, fractions exhibiting strong antibacterial bioactivity (DSM4 to DSM7, refer to Table 6.14) were grouped together against the less active and inactive fractions. (Figure 6.27C). The fractions exhibiting strong antibacterial activity were positioned on the right quadrants of the scores plot while the less active fractions were distributed over the upper and lower left quadrants. The chemical shifts that contributed to the separation of the bioactive group can be derived from the S-plot plot (Figure 6.27D) which were protons on the methyl region (0–1 ppm), methine region (4–5 ppm), and aromatic region (7–8 ppm). This indicates the involvement of both aliphatic and aromatic compounds in the observed bioactivity.

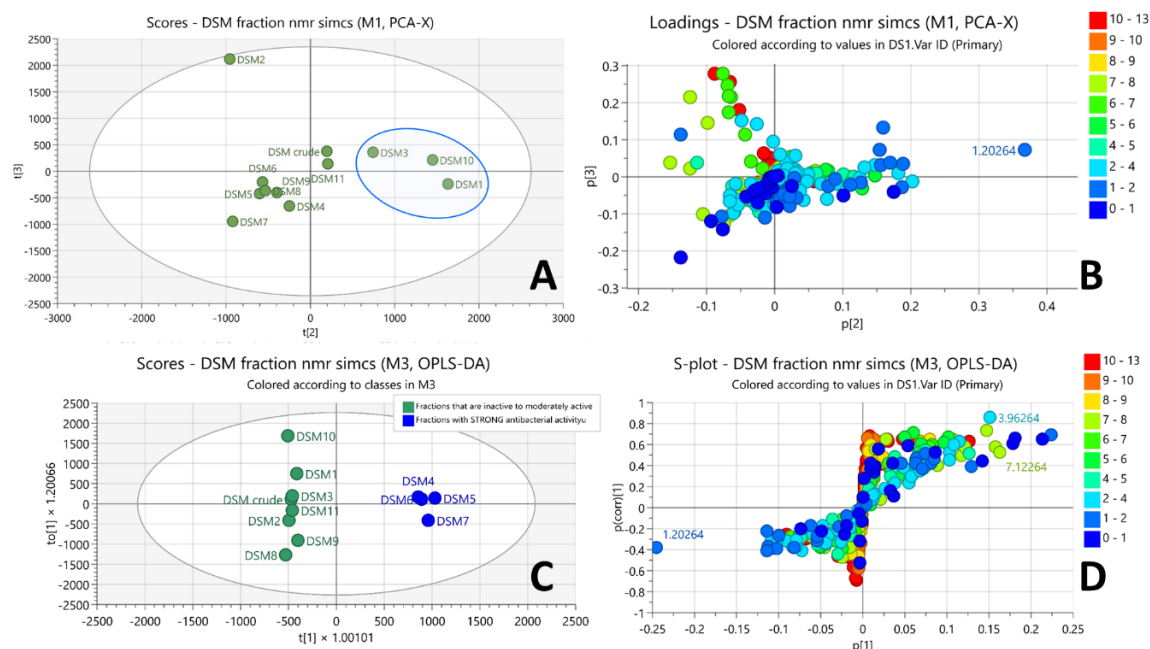


Figure 6.27 ^1H -NMR-based multivariate analysis of DSM fractions. A) PCA scores plot, B) PCA Loadings plot, C) OPLS-DA scores plot, D) OPLS-DA S-plot. PCA: $R^2=0.96$ $Q^2=0.56$; OPLS-DA: $R^2=0.99$ $Q^2=0.77$

LCMS-based metabolomics was also employed to achieve higher analytical sensitivity. In the PCA scores plot shown in Figure 6.28A, the fractions were consecutively grouped into clusters: DSM1 to 3 (encircled in red), DSM4 to 6 (encircled

in blue), and then the remaining fractions encircled in green. The OPLS-DA model (Figure 6.28C) applied had stratified the fractions based on their antibacterial activities based on Table 6.14. The active fractions in the antibacterial assay can be seen at the upper and lower right quadrants of the scores plot. The discriminating metabolites from these fractions can be derived from the S-plot with its dereplication hits listed in Table 6.15. The structure of the hits was shown in **Error! Reference source not found..**

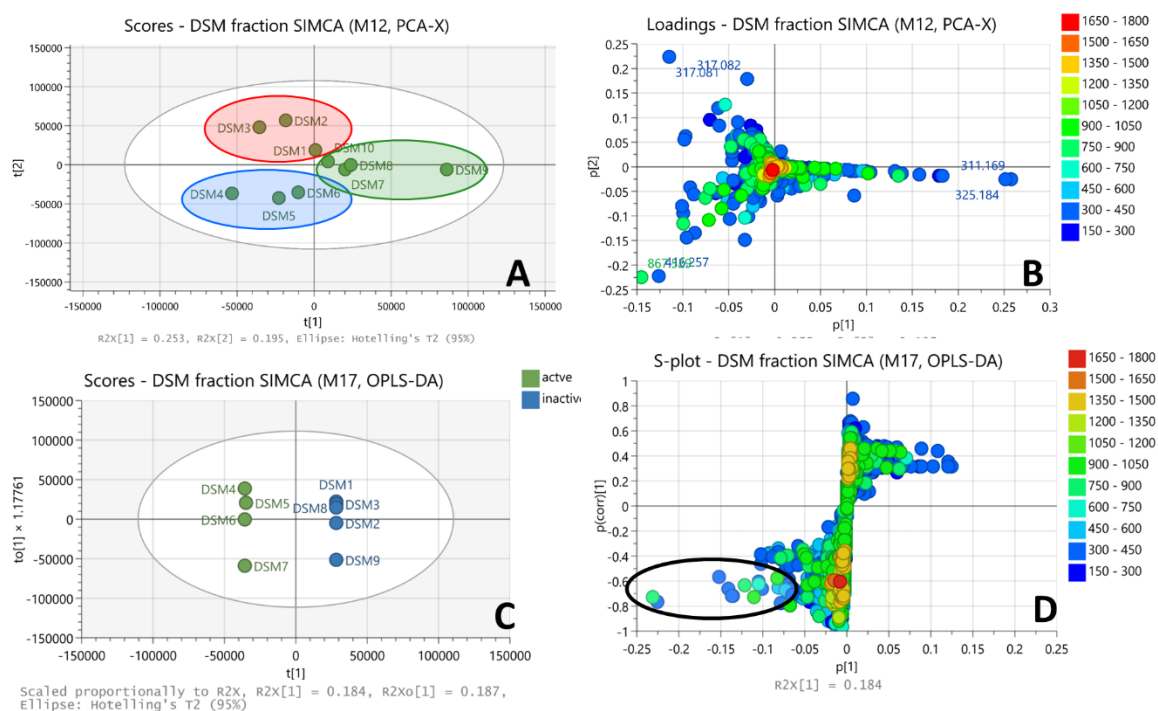


Figure 6.28 LC-MS based multivariate analysis of DSM fractions. A) PCA Scores plot, B) PCA loadings plot, C) OPLSDA Score plot, D) OPLSDA S-plot. Samples in the OPLSDA model are grouped according to their antibacterial activity. Encircled in black in the S-plot is the discriminating metabolites for the active fractions. PCA R2=0.87, Q2=0.09; OPLS-DA R2=0.99, Q2=0.99

Most of the discriminatory metabolites were annotated as derivatives of cytochalasins including some isomers consistent to the findings in the profiling stage (Section 6.4.2). The top three features in Table 6.15 was found to be adducts of a single molecule with the MW 435.2489 Da that was a cytochalasin derivative. This discriminating metabolite found at ion peak m/z 416.2580 was already annotated at the previous screening stages with chemical elicitors (refer to Section 6.4.2.2 and Table 6.12). Daldinone B was annotated again as well. Although the final features listed in the table did not meet the statistical significance threshold (p -value), it was included due to its eventual identification in the later chromatographic purification work. The likely explanation for its high p -value is its broader distribution across multiple fractions while not exclusively included in one defined group of the OPLS-DA model.

Table 6.15 Dereplication of discriminatory metabolites from active DSM fractions

<i>p</i> -value	<i>m/z</i>	MW (g/mol)	MF	Putative identity	Reported source
0.016115	416.257	[M+H-H ₂ O] ⁺ of MW 433.2690	C ₂₈ H ₃₅ NO ₃	cytochalasin isomers: Cytochalasin 6 Cytochalasin 9 Cytochalasin 11	Prod. by several <i>Daldinia eschscholtzii</i> strains
0.025187	867.529	[2M+H] ⁺ of 433.2690		neocytochalasin	
0.027349	911.523	[2M+FA-H] ⁻ of 433.2690			
0.029396	418.273	[M+H-H ₂ O] ⁺ of 435.2840	C ₂₈ H ₃₇ NO ₃	Cytochalasin 7	Isol. from <i>Daldinia</i> <i>eschscholtzii</i>
0.066787	871.561	[2M+H] ⁺ of 435.2840			
0.05069	331.059	[M+H-H ₂ O] ⁺ of Daldinone B	C ₂₀ H ₁₂ O ₆	Daldinone B	Prod. by <i>Daldinia</i> <i>concentrica</i>
0.05107	347.056	348.063			
0.071274	695.119	[2M-H] ⁻ of Daldinone B			
0.087531	432.252	[M+H-H ₂ O] ⁺ of 449.2640	C ₂₈ H ₃₅ NO ₄	cytochalasin isomers: Cytochalasin 1 Cytochalasin 10	Metab. of <i>Daldinia</i> <i>eschscholtzii</i>
Compound dereplication are based on highly accurate mass matching (<5 ppm) against Dictionary of Natural Products, with filtering by fungal biological source. No tandem MS/MS data were acquired; therefore, these are tentative assignments that require confirmation through MS fragmentation data or isolation (cells highlighted in blue correspond to compounds that were subsequently isolated)					

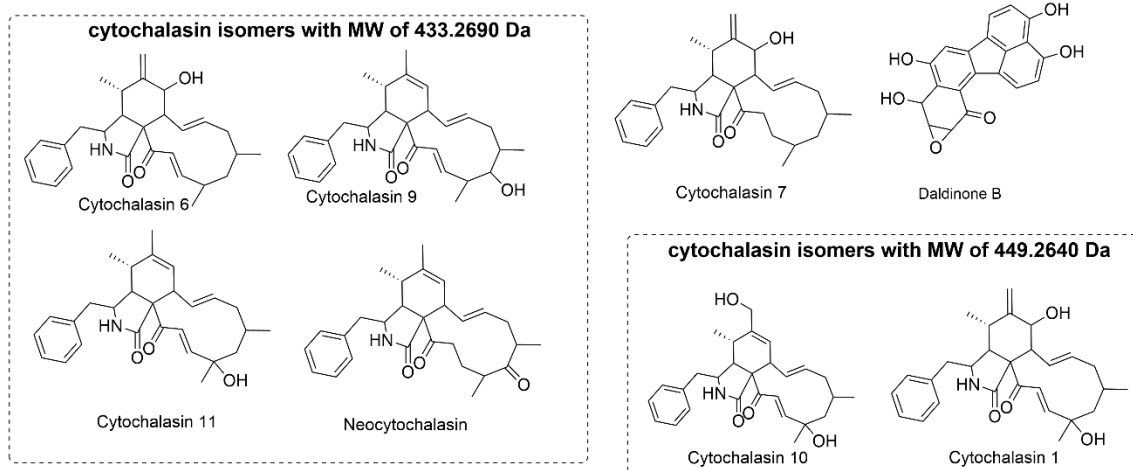


Figure 6.29 Chemical structure of dereplication hits for the discriminatory metabolites of active DSM fractions. The underlined metabolites were later isolated

6.5.3 Chromatographic purification of active compounds from DSM fractions

The fractions selected for chromatographic fractionation were DSM2 and DSM4 to 9 because of their potent biological activities. The $^1\text{H-NMR}$ spectrum of DSM2 and DSM7 indicated high purity, and thus additional purification was unnecessary.

DSM4, having strong antibacterial and prebiofilm activity, was purified in the Buchi flash chromatography using a silica cartridge (23 x 53mm, 11g, VersaPak, Supelco®, USA). The stepwise elution with EtOAc and Hexane started with 30% EtOAc for 10 min to 100% EtOAc in 20 min at a flowrate of 10 mL/min. The purification afforded 11 pooled fractions coded as DSM4.1 to DSM4.11. Based on their $^1\text{H-NMR}$ spectra, DSM4.3 to DSM4.10 were considered pure or semi pure (60-100%) compounds. DSM4.7 was further chromatographed using preparative TLC in 15% DCM:85% EtOAc to obtain compound DSM4.7.1. Structure elucidation was carried out for DSM4.3 to DSM4.10 and DSM4.7.1

Fractions DSM5 and DSM6 have similar TLC profiles (Figure 6.25) and $^1\text{H-NMR}$ spectral data (Figure 6.26). The former has a higher yield compared to the latter. For this

reason, DSM5 was prioritised for further purification by preparative TLC using 8:2 of EtOAc and acetone as the solvent system. This afforded the pure compounds DSM5.1 and DSM 5.2 which were both identified later in Sections 6.5.4.4 and 6.5.4.3, respectively.

DSM8 gave strong prebiofilm inhibition thus was selected for Biotage™ MPLC chromatographic purification over a silica cartridge (Sfär HP-Sphere, 25-33 µm, 10g; Biotage™). A stepwise elution started with 80% EtOAc/ 20% hexane for 17 min, 100% EtOAc for 6 min, and finally with 80% EtOAc/ 20% acetone for another 10 min. This afforded four subfractions (DSM8.1 to DSM8.4). Based on ¹H-NMR, DSM8.1 and 8.2 were still mixtures while DSM8.3 is identical to DSM7 and DSM8.4 is a different compound. Thus, the structure of DSM8.4 was later identified.

DSM9 gave strong prebiofilm inhibition as well and was chosen for further purification via a Buchi flash chromatography over a VersaPak silica cartridge (23 x 53mm, 11g, Supelco®, USA). The stepwise elution started with 90% EtOAc/ 10% hexane for 30 min, to 100% EtOAc for 15 min, then finally with 80% EtOAc/ 20% acetone for 15 min. Four fractions (DSM9.1 to 9.4) were obtained DSM9.2 and DSM9.3 were identified.

6.5.4 Structure elucidation of isolated compounds

6.5.4.1 DSM2 as BNT

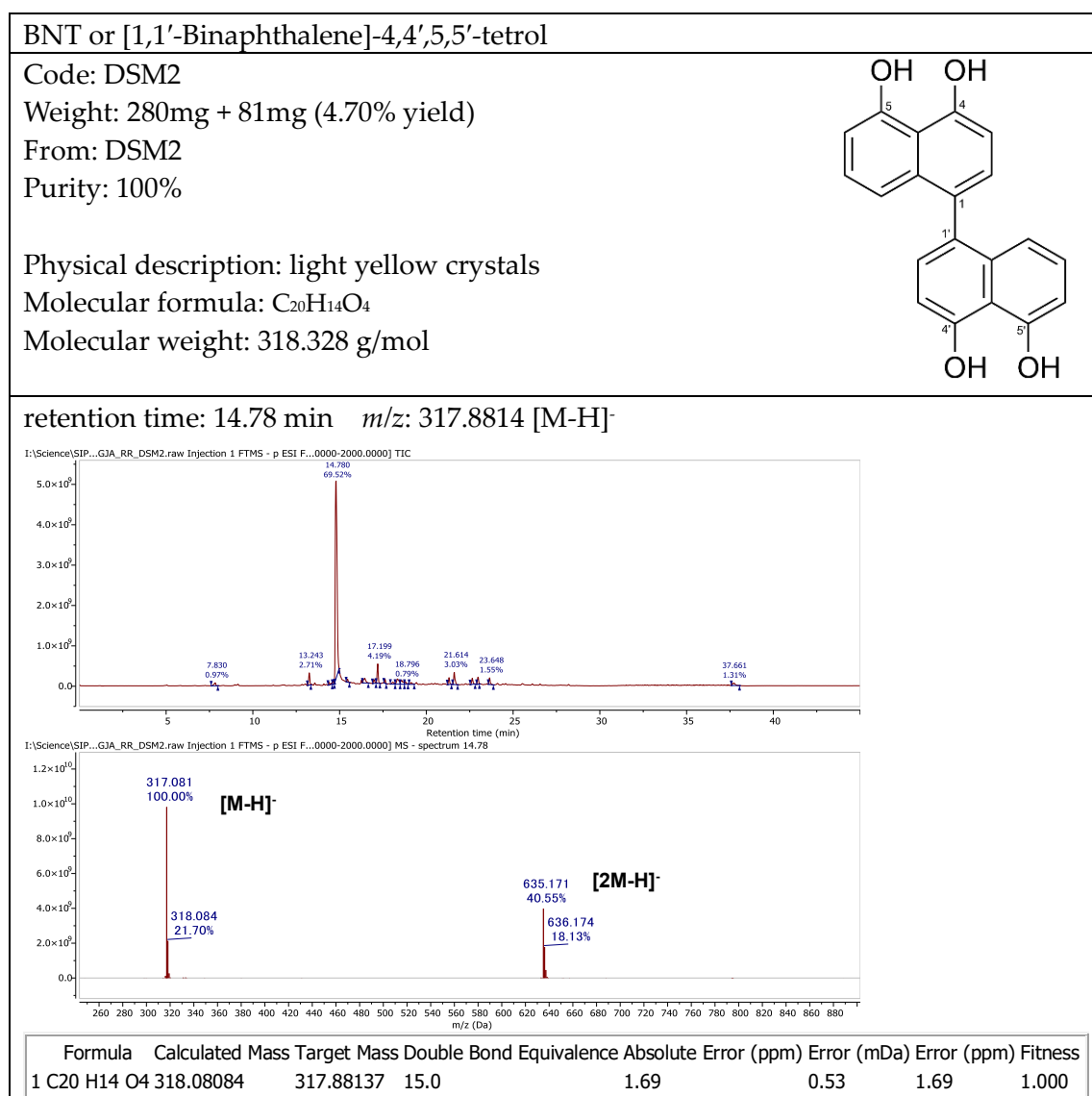


Figure 6.30 Chemical and spectroscopic data of DSM2

DSM2 was obtained as light-yellow crystals from the first fractionation of DSM. HR-MS revealed an ion peak of m/z 317.881 [M-H]⁻ calculated for C₂₀H₁₄O₄ (Figure 6.30). The presence of 10 signals in the ¹³C NMR spectrum (Figure S 46) suggested a symmetric

dimeric structure, accounting for all 20 carbon atoms. The ^1H NMR spectrum displayed five distinct sharp peaks of equal integration in the aromatic region (Figure S 45). Two deshielded peaks at 11.04 ppm and 10.99 ppm were present. This indicated a total of seven unique proton environments, which, when doubled due to symmetry, is proportional to the 14 protons required by the molecular formula. This further supported a dimeric structure. A naphthol monomer can be identified based on the coupling arrangements of the aromatic protons. Combined ^1H , ^{13}C -NMR, and HMBC (Figure S 47) led to the identification of DSM2 as BNT or [1,1'-binaphthalene]-4,4',5,5'-tetrol. Its NMR data closely matched with the reported values from the literature (Zhou et al., 2021).

BNT was found on DSM3 and DSM4.3 as well with yields of 75 mg and 6 mg of BNT, respectively. A total of 280mg of BNT was afforded from DSM2. Thus, a total of 361 mg was isolated from the crude extract at a yield of 4.7%. Hence, this suggested that BNT is a major metabolite in the extract.

Table 6.16 ^1H and ^{13}C NMR data comparison of DSM2 from reported values of BNT

Atom no	This study	(Zhou et al., 2021)		
	δ_{H} in ppm, (J in Hz) 400 MHz in DMSO- d_6	δ_{C} in ppm 100 MHz in DMSO- d_6	δ_{H} in ppm, (J in Hz) 500MHz DMSO- d_6	δ_{C} in ppm 125MHz, DMSO- d_6
4OH, 4'OH	11.02 (2H, s)	-	11.04 (4H, s)	-
5OH, 5'OH	10.99 (2H, s)			
1,1'	-	129.8	-	129.8
2,2'	7.16 (2H, d, 7.7)	129.2	7.17 (2H, d, 7.7),	129.2
3,3'	6.83 (2H, d, 7.7)	108.6	6.84 (2H, d, 7.7)	108.6
4,4'	-	154.1	-	154.1
5,5'	-	154.8	-	154.8
6,6'	6.59 (2H, dd, 8.5, 1.0)	117.9	6.60 (2H, dd,8.5,0.9)	117.9
7,7'	7.07 (2H, dd, 8.5, 7.6)	127.3	7.11 – 7.06 (2H, m, 2H)	127.3
8,8'	6.72 (2H, dd,7.6, 1.1)	108.9	6.73 (1H, d,7.9)	108.9
4a,4a'	-	114.9	-	114.9
8a,8a',	-	136.2	-	136.2

BNT was first discovered as a pigment of *Daldinia concentrica* (Allport & Bu'lock, 1958). This naphthalene derivative was found to be ubiquitous in all the members of the genus *Annulohypoxylon*, *Daldinia*, *Hypoxylon*, *Thamnomycetes*, and *Phylacia* (Stadler & Fournier, 2006). Since then, it has been recognized as chemotaxonomic markers of those fungi belonging to the Hypoxyloideae lineage of *Xylariaceae* family (Stadler et al., 2007; Stadler & Hellwig, 2005). Hence, it was not surprising that BNT was identified from *D. eschscholtzii*.

6.5.4.2 DMS4.6 and 4.4 as orthosporin and diaporthin

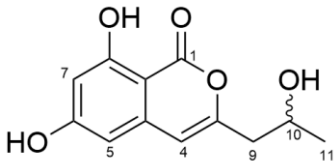
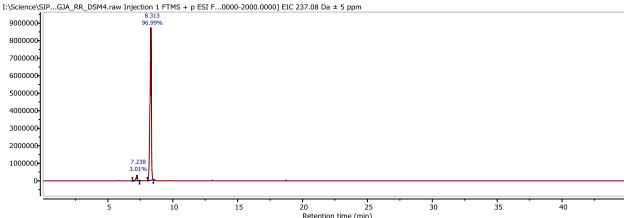
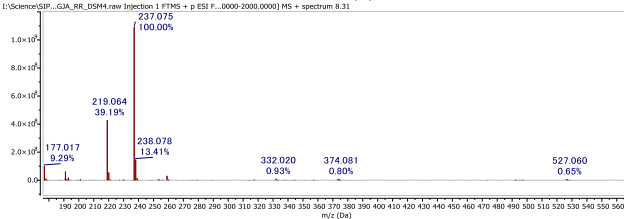
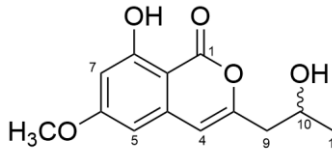
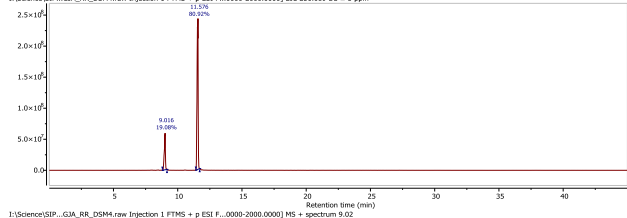
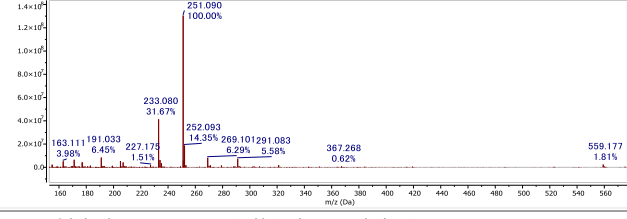
orthosporin code: DSM4.6 Weight: 11mg (0.14% yield) From: DSM4 Purity: 60% Physical description: light orange oil Molecular formula: C₁₂H₁₂O₅ Molecular weight: 236.076 g/mol Retention time: 8.13 min <i>m/z</i> 237.0758 [M+H]⁺    <table><tr><th>Formula</th><th>Calculated Mass</th><th>Target Mass</th><th>Double Bond Equivalence</th><th>Absolute Error (ppm)</th><th>Error (mDa)</th><th>Error (ppm)</th><th>Fitness</th></tr><tr><td>1 C12 H12 O5</td><td>237.07575</td><td>237.07479</td><td>7.0</td><td>4.05</td><td>-0.96</td><td>-4.05</td><td>0.986</td></tr></table>	Formula	Calculated Mass	Target Mass	Double Bond Equivalence	Absolute Error (ppm)	Error (mDa)	Error (ppm)	Fitness	1 C12 H12 O5	237.07575	237.07479	7.0	4.05	-0.96	-4.05	0.986	diaporthin code: DSM4.4 Weight: 4mg (0.05% yield) From: DSM4 Purity: 30% Physical description: light brown oil Molecular formula: C₁₃H₁₄O₅ Molecular weight: 250.08 g/mol retention time: 11.576 min <i>m/z</i>: 251.0914 [M+H]⁺    <table><tr><th>Formula</th><th>Calculated Mass</th><th>Target Mass</th><th>Double Bond Equivalence</th><th>Absolute Error (ppm)</th><th>Error (mDa)</th><th>Error (ppm)</th><th>Fitness</th></tr><tr><td>1 C13 H14 O5</td><td>251.09140</td><td>251.09051</td><td>7.0</td><td>3.54</td><td>-0.89</td><td>-3.54</td><td>1.000</td></tr></table>	Formula	Calculated Mass	Target Mass	Double Bond Equivalence	Absolute Error (ppm)	Error (mDa)	Error (ppm)	Fitness	1 C13 H14 O5	251.09140	251.09051	7.0	3.54	-0.89	-3.54	1.000
Formula	Calculated Mass	Target Mass	Double Bond Equivalence	Absolute Error (ppm)	Error (mDa)	Error (ppm)	Fitness																										
1 C12 H12 O5	237.07575	237.07479	7.0	4.05	-0.96	-4.05	0.986																										
Formula	Calculated Mass	Target Mass	Double Bond Equivalence	Absolute Error (ppm)	Error (mDa)	Error (ppm)	Fitness																										
1 C13 H14 O5	251.09140	251.09051	7.0	3.54	-0.89	-3.54	1.000																										

Figure 6.31 Chemical and spectroscopic data of DSM4.4 and 4.6

Two structurally related compounds, orthosporin and diaporthin, were identified from DSM4.6 and DSM4.4, respectively. They were obtained in very minute amounts while having impurities (30-60% pure). MS data were shown in Figure 6.31. Cytochalasins were most probably the impurities in this case. But still, ^1H NMR analysis was able to identify their structure. In the ^1H -NMR of DSM4.6 (Figure S 48), there are two aromatic protons that were meta coupling based on their J-values (δ_{H} 6.32, $J=2.16$ Hz, H-5; and 6.37, d, $J=2.16$ Hz, H-17). An olefinic singlet can be found at δ_{H} 6.54. An oxygenated methine (δ_{H} 3.90, m, H10) is in between a methylene (δ_{H} 2.51, m, H-9) and a methyl group (δ_{H} 1.14, d, $J=6.15$ Hz, H-11). These three formed the aliphatic side chain of orthosporin. A similar ^1H -NMR pattern (Figure S 49) was observed for DSM4.4, except for the appearance of a methoxy proton at δ_{H} in ppm, 3.85 with an integration of 3H. The OH at C-6 of orthosporin was replaced with the methoxy group in diaporthin. Both of their ^1H -NMR data was closely comparable with the reported values in the literature (Harris & Mantle, 2001; Ichihara et al., 1989).

Table 6.17 ¹H NMR data and comparison from reported values of orthosporin and diaporthin

Atom no	orthosporin		diaporthin	
	This study, δ_{H} in ppm, (<i>J</i> in Hz) 400MHz in DMSO- d ₆	(Ichihara et al., 1989), 400MHz in acetone -d ₆	This study, δ_{H} in ppm, (<i>J</i> in Hz) 400MHz in DMSO-d ₆	(Harris & Mantle, 2001), 500MHz in acetone -d ₆
4	6.49 (1H, s)	6.41 (1H, s)	6.54 (1H, s)	6.48 (1H, s)
5	6.32 (1H, d, 2.16)	6.36 (1H, d, 2.1)	6.51 (1H, d, 2.31)	6.45 (1H, d, 2.3)
6-OH <i>or</i> - OCH3	10.81 (1H, s)	3.86 (1H, s)	3.85 (3H, s)	3.90 (1H, s)
7	6.37 (1H, d, 2.16)	6.40 (1H, d, 2.1)	6.60 (1H, d, 2.30)	6.53 (1H, d, 2.4)
8-OH	11.00 (1H, s)	-	11.01 (1H, s)	none
9	2.51 (2H, m)	2.56 (1H, dd, 14.3, 7.6) 2.61 (1H, dd, 14.3, 5.8)	2.51 (2H, m)	2.57 (1H, dd, 14.3, 7.8) 2.62 (1H, dd, 14.3, 4.9)
10	3.99 (1H, m)	4.17 (1H, m)	3.98 (1H, m)	4.16 (1H, m)
11	1.14 (3H, d, 6.27)	1.22 (3H, d, 6.1)	1.14 (3H, d, 6.15)	1.23 (3H, d, 6.3)

The two compounds were first reported as phytotoxins from *Endothia parasitica* (Boller et al., 1957; Hallock et al., 1988) and *Drechslera siccanis* (Hallock et al., 1988). Several *Daldinia* species has been reported to produce these metabolites including *D. concentrica* (Lee et al., 2006) and *D. eschscholtzii* (Barnes et al., 2016; Liao et al., 2019). Orthosporin was produced through the polyketide pathway (Lee et al., 2006) while diaporthin was produced by methylation of orthosporin (Harris & Mantle, 2001). In this study, the detection of diaporthin and orthosporin in *D. eschscholtzii* cultures even in the presence of a chemical elicitor implied that the specific polyketide biosynthetic pathway was conserved.

6.5.4.3 DSM5.2, DSM6, and DSM9.2 has daldinone B

daldinone B

Code: DSM5.2, DSM6, DSM9.2

Weight: 56 mg (0.74 % yield)

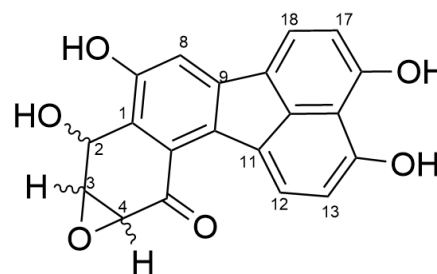
From: several fractions

Purity: 30 to 80%

Physical description: brown oil

Molecular formula: $C_{20}H_{12}O_6$

Molecular weight: 348.063 g/mol



retention time: 10.28 min m/z : 347.0550 $[M-H]^-$

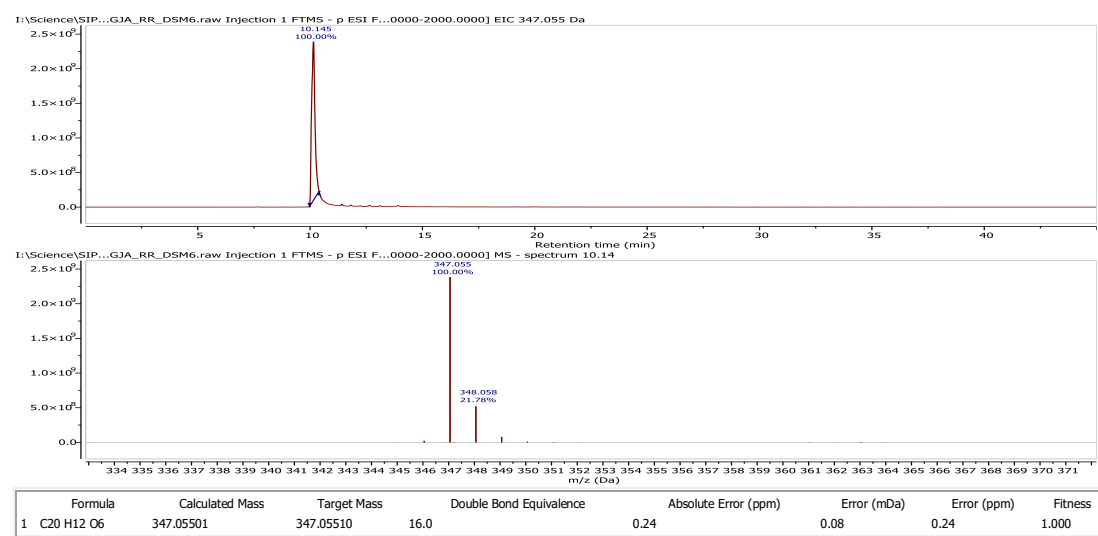


Figure 6.32 Chemical and spectroscopic data of DSM5.2, DSM6, and DSM9.2

Daldinone B was identified once again. Previously, it was isolated from *Hypoxylon lateripigmentum* grown in MEB (Chapter 5). This time, it was identified from the culture extracts of *D. eschscholtzii*, specifically in subfractions DSM5.2, DSM6, and DSM9.2 (Figure 6.32). Their stacked spectrum was shown in Figure 6.33. The purity of daldinone B is 80% in DSM5.2, 60% in DSM6, and 35% in DSM9.2. The total yield of daldinone B from the crude extract is 56 mg which is around a yield of 0.74%.

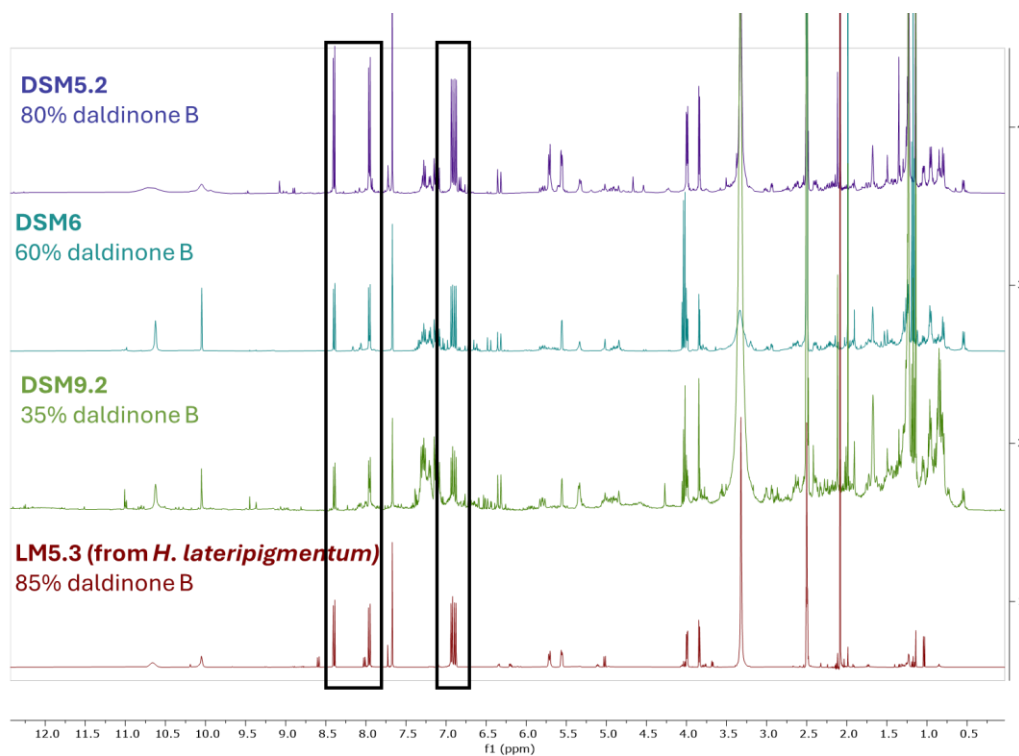


Figure 6.33 Stacked ^1H -NMR spectrum of DSM5.2, DSM6, DSM9.2 and LM5.3. Relative purity of daldinone B in each sample were specified. Boxed in black are the characteristic aromatic protons of daldinone B

Daldinone B has been previously reported from *Daldinia concentrica* (Quang et al., 2002) and from a *Daldinia* species grown in Cheerios[®] media treated with the chemical elicitor suberoyl anilide hydroxamic acid (SAHA) (Du, King, & Cichewicz, 2014). Interestingly, in this work, *D. eschscholtzii* was grown in PDB with the supplementation of the elicitor SBHA. These findings suggest that chemical elicitors can possibly induce the biosynthesis of daldinone B across different *Daldinia* species. Daldinone E, a

chlorinated congener of daldinone B, was previously identified in the SAHA-treated *Daldinia* sp., but was not detected nor isolated in our case. This may be attributed to differences in elicitor structure and activity, or alternatively, to the very low abundance of daldinone E in the culture extract, rendering it undetectable. Further investigation is required to test these hypotheses and to clarify the role of specific elicitors in the biosynthesis of daldinone derivatives.

6.5.4.4 Cytochalasin derivatives

DSM7: cytochalasin 1 code: DSM7 Weight: 441mg (5.82% yield) From: DSM7 Purity: 100% Physical description: light orange powder Molecular formula: $C_{28}H_{35}NO_4$ Molecular weight: 449.26 m/z : 432.2521 $[M+H-H_2O]^+$ rt: 13.10min	DSM5.1: cytochalasin 9 code: DSM5.1 Weight: 18mg (0.24% yield) From: DSM5 Purity: 100% Physical description: brown oil Molecular formula: $C_{28}H_{35}NO_3$ Molecular weight: 433.26 m/z : 416.2572 $[M+H-H_2O]^+$ rt: 20.21min	DSM4.5: cytochalasin 11 code: DSM4.5 Weight: 13mg (0.17% yield) From: DSM4 Purity: 100% Physical description: brown oil Molecular formula: $C_{28}H_{35}NO_3$ Molecular weight: 433.26 m/z : 416.2573 $[M+H-H_2O]^+$ rt: 20.21min
DSM9.3: cytochalasin 10 code: DSM9.3 Weight: 80mg (1.06% yield) From: DSM9 Purity: 100% Physical description: dark red oil Molecular formula: $C_{28}H_{35}NO_4$ Molecular weight: 449.26 m/z : 432.2523 $[M+H-H_2O]^+$ rt: 14.25min	DSM4.7.1: cytochalasin 7 code: DSM5.1 Weight: 4mg (0.05% yield) From: DSM4 Purity: 100% Physical description: light yellow oil Molecular formula: $C_{28}H_{37}NO_3$ Molecular weight: 435.28 m/z : 436.2831 $[M+H]^+$ rt: 19.02min	DSM8.4: cytochalasin 2 code: DSM8.4 Weight: 58mg (0.77% yield) From: DSM8 Purity: 100% Physical description: light orange powder Molecular formula: $C_{28}H_{37}NO_4$ Molecular weight: 451.26 m/z : 434.2723 $[M+H-H_2O]^+$ Rt: 12.79min

Mass spectrum shown in Supporting information

Figure 6.34 Chemical and spectroscopic data of cytochalasin derivatives

A total of six cytochalasin derivatives were identified from the purified fractions of DSM. Chemical structures of the isolated cytochalasins were shown in Figure 6.35. The metabolomic analysis previously carried out (sections 6.4.2.2 and 6.5.2) provided initial hints of the structure of these derivatives. The nomenclature was followed throughout as with Table 6.15 based on the original reports (Buchanan, Hashimoto, & Asakawa, 1995; Buchanan, Hashimoto, & Asakawa, 1996). Mass spectrum of each identified cytochalasin was shown in supporting information.

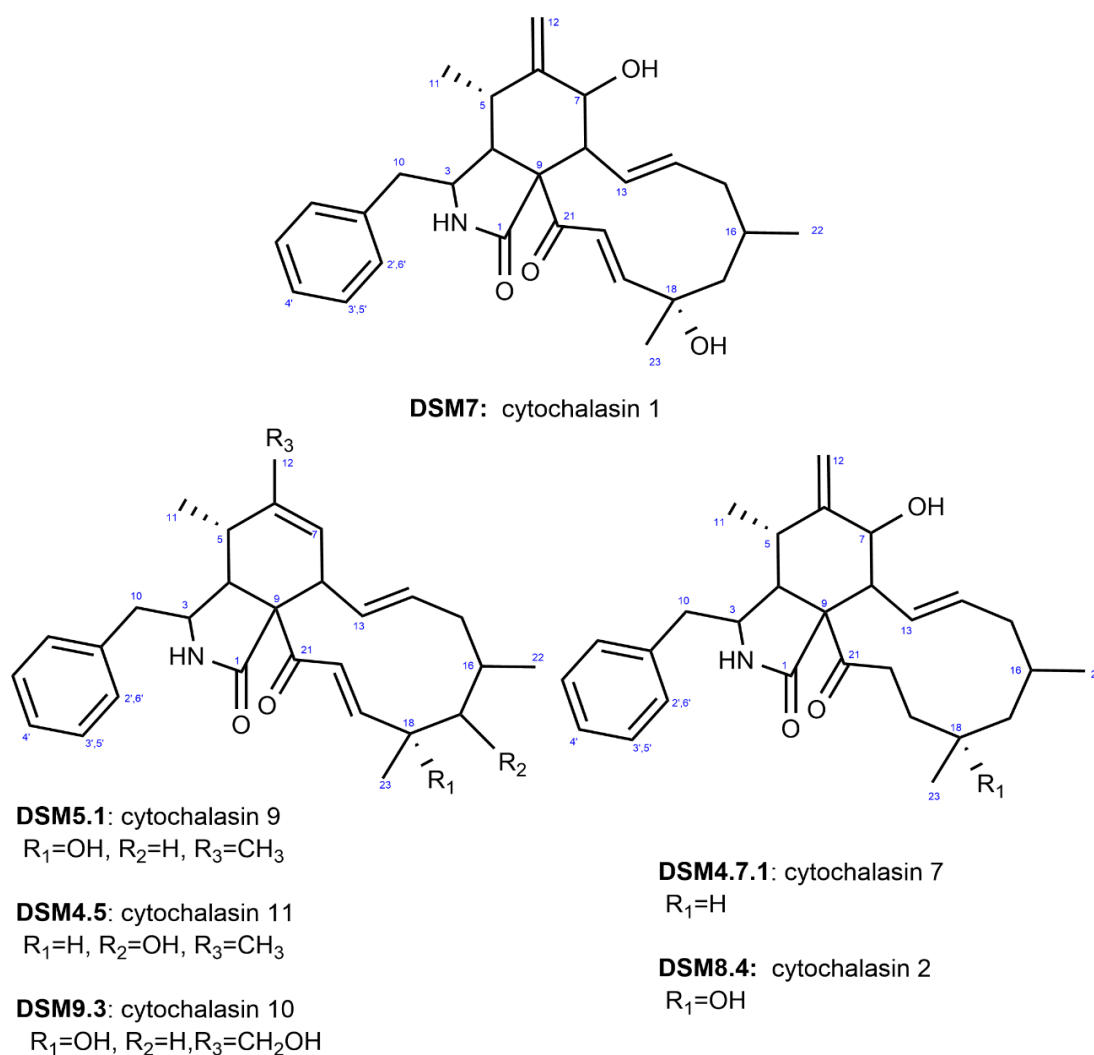


Figure 6.35 Chemical structure of isolated cytochalasin derivatives

The cytochalasin derivative obtained with highest yield was cytochalasin 1, with a total of 441 mg (5.82% yield) from the DSM7 fraction afforded during initial fractionation. This compound can therefore be considered as the major or parent cytochalasin derivative in the extract, while the remaining compounds are regarded as minor derivatives. Similarly, this derivative was one of the major cytochalasin analogue isolated by Buchanan, Hashimoto and Asakawa (1995) from *D. eschscholtzii* collected from nature with a yield of 375 mg (1.10%).

DSM7 has a molecular formula of $C_{28}H_{35}NO_4$ (DBE=12) calculated from the ion peak at m/z 432.2521 which is a $[M+H-H_2O]^+$ fragment ion. The 1H -NMR spectrum revealed the presence of an amide peak (δ_H 8.08, d, $J=1.78$, 2-NH), benzyl side chain (δ_H 7.06, m, H-2',6'; 7.30, t, $J=7.45$ Hz, H-3',5'; 7.22, m, H-4'), exomethylene protons (δ_H 5.02 (s) and 4.86 (m), H-12), two olefinic protons in an enone system (δ_H 7.06, d, $J=15.62$ Hz, H-20; and δ_H in ppm, 6.47, d, $J=15.65$ Hz, H-19), and two additional olefinic protons (δ_H 5.60, dd, $J=15.22, 9.61$ Hz, H-13; and 4.88, m, H-14). These two pairs of olefinic protons are inside a 11-membered macrocyclic system (called endocyclic double bonds). Furthermore, the large coupling constants (15.6 Hz) indicated that olefinic protons were in a *trans* configuration. There are three methyl groups present, one of which is a methyl group (δ_H 1.27, m, H-23) attached to a tertiary carbon bearing an oxygen (δ_C 71.8, C-18). The remaining two methyl groups are next to methine carbons as revealed from their coupling constants (δ_H 0.96, d, $J=6.86$ Hz, H-23; and 0.54, d, $J=6.68$ Hz, H-11). There are two hydroxyl groups which are attached at C-7 (δ_C 71.82) and C-18 (δ_C 71.77). Further analysis of the ^{13}C and 2D NMR data (Figure S 51 to Figure S 54) led to the structure elucidation of cytochalasin 1 as shown in Figure 6.35. Moreover, data closely agreed with the reported NMR data values for this derivative (Table 6.18 and Table 6.19) (Buchanan, Hashimoto, & Asakawa, 1995; Buchanan, Hashimoto, & Asakawa, 1996).

The NMR data of DSM8.4 was shown in Figure S 70Figure S 73. Comparing from DSM7 which has two endocyclic double bonds, DSM8.4 showed only one. It was found out that the double bond in the enone system was reduced to two methylene protons (δ_{H} , 1.37, 2H, m, H-19; and 3.74 (1H) & 1.74 (1H), m, H-20). The structure was confirmed to be cytochalasin 2 (Figure 6.35). DSM4.7.1 followed the same spectral pattern of DSM8.4 except for the absence of -OH group at C-18. Instead, a methine unit was at C-18 (δ_{C} 31.0). The protons in the macrocycle formed a continuous spin system as shown in the COSY and 1D-TOCSY experimental data (Figure S 75-Figure S 78). Hence, DSM4.7.1 was identified as cytochalasin 7. Both congeners closely conform with the reported NMR data (Table 6.18 and Table 6.19) (Buchanan, Hashimoto, & Asakawa, 1995; Buchanan, Hashimoto, & Asakawa, 1996).

The NMR data of DSM5.1, DSM4.5, and DSM9.3 does not show any exomethylene protons at C-12 (Figure S 55 to Figure S 69). Instead, an aliphatic carbon can be found at C-12 while an endocyclic double bond was at C-6 and C-7. The ^{13}C -NMR values are presented at Table 6.19. According to their ^1H -NMR data, these three compounds also have two endocyclic double bonds in the macrocyclic ring like DSM7. Comprehensive investigation led to the identification of DSM5.1 as cytochalasin 9, DSM4.5 as cytochalasin 11, and DSM9.3 as cytochalasin 10. The three are isomers, as shown in their MS data (Figure S 56Figure S 59Figure S 65). Meanwhile their structural differences are displayed in Figure 6.35. Cytochalasin 9 and 11 differs only in the position of -OH: at C-18 and -17, respectively. For cytochalasin 10, a -CH₂OH instead of a methyl group was in C-12. The ^1H and ^{13}C NMR data of three isomers closely matches with the literature as displayed in Table 6.18 and Table 6.19 (Buchanan, Hashimoto, & Asakawa, 1995; Buchanan, Hashimoto, & Asakawa, 1996).

Table 6.18 ¹H-NMR data and comparison of reported values of cytochalasin derivatives (400MHz, DMSO-d₆)

Atom No	DSM5.1		DSM4.5		DSM9.3		DSM7		DSM8.4		DSM4.7.1	
	This study	Ref ^a	This study	Ref ^a	This study	Ref ^a	This study	Ref ^b	This study	Ref ^a	This study	Ref ^a
2-NH	7.96 (1H, s)	5.62 (br,s)	8.00 (1H, s)	5.56 (br s)	7.91 (1H, s)	5.87 (br,s)	8.08 (1H, d, 1.78)	6.29 (br,s)	7.97 (1H, d, 1.55)	5.89 (br,s)	8.02 (1H, s)	5.45 (br,s)
3	3.26 (1H, m)	3.25 (m)	3.26 (1H, m)	3.25 (m)	3.26 (1H, m)	3.44 (m)	3.24 (1H, m)	3.32 (m)	3.20 (1H, td, 4.73,2.3)	3.31 (m)	3.22 (1H, m)	3.30 (m)
4	2.94 (1H, dd, 6.01, 2.62)	3.13 (dd,5.3,3.3)	2.97 (1H, dd, 5.92, 2.45)	3.19 (dd, 5.6, 3.2)	2.93 (1H, dd, 5.88,2.74)	3.15 (dd,5.9,2.9)	3.00 (1H, dd, 6.19, 1.88)	3.17 (dd,5.6,2.4)	2.68 (1H, dd, 6.19, 2.07)	2.96 (dd,6.0,2.3)	2.43 (1H, m)	2.99 (dd,6.0, 2.4)
5	2.18 (1H, m)	2.45 (qd, 7.1,5.3)	2.13 (1H, m)	2.44 (qd, 7.3, 5.6)	2.21 (1H, dd, 9.40,4.83)	2.50 (m)	2.51 (m)	2.79 (br qd, 6.6,5.6)	2.55 (1Hm)	2.83 (br qd, 6.0)	2.61 (1H, m)	2.84 (qd, 6.8, 6.0)
7	5.33 (1H, m)	5.46 (m)	5.34 (1H, ddd, 4.09,2.81, 1.67)	5.46 (m)	5.33 (1H, m)	5.69 (m)	3.80 (d, 9.78)	4.06 (br d, 10.0)	3.80 (1H,dd, 10.29,5.70)	4.12 (br d, 10.0)	3.77 (1H, dd, 10.05, 6.51)	4.10 (br d, 10.0)
8	2.39 (1H, d, 7.33)	2.58 (m)	2.40 (1H, m)	2.57 (m)	2.41 (1H, m)	2.63 (m)	2.22 (1H, m)	2.45 (t,10.0)	2.22 (1H, t)	2.46 (t,10.0)	2.29 (1H, t, 9.46)	2.47 (dd, 10.0, 10.0)
10	2.63 (1H, dd, 13.24,4.93) 2.38 (1H, m)	2.79 (dd,13.4, 4.6) 2.48 (dd, 13.4,9.0)	2.61 (1H, dd, 13.10,4.7) 2.40 (1H, m)	2.78(dd, 13.4, 4.9) (dd, 13.4, 9.0)	2.62 (1H, dd, 10.95,5.4) 2.41 (1H, m)	2.73 (dd,13.4, 5.4) 2.52(dd, 13.4,5.4)	2.64 (1H, dd, 13.03,4.8) 2.22 (1H, m)	2.64 (dd,13.4, 5.9) 2.54 (dd, 13.4,5.9)	2.55 (1H,m) 2.38 (1H,dd,1 3.20,7.31)	2.64 (dd,13.4, 5.4) 2.47 (dd, 13.4,7.8)	2.43 (1H, dd, 13.32,7.1) 2.58 (1H, d, 4.39)	2.65 (dd,13.4, 5.1) 2.45 (dd, 13.4,8.3)

Atom No	DSM5.1		DSM4.5		DSM9.3		DSM7		DSM8.4		DSM4.7.1	
	This study	Ref ^a	This study	Ref ^a	This study	Ref ^a	This study	Ref ^b	This study	Ref ^a	This study	Ref ^a
11	0.79 (3H,d, 7.20)	1.18 (d, 7.1)	0.80 (3H,d, 7.19)	1.17 (d, 7.3)	0.84 (3H,d, 7.25)	1.20 (d, 7.1)	0.54 (3H,d, 6.68)	0.94 (d, 6.6)	0.70 (3H,d, 6.66)	0.99 (d, 6.8)	0.74 (3H,d, 6.41)	1.01 (d, 6.8)
12	1.68 (3H, s)	1.75 (br s)	1.66 (3H, s)	1.74 (br s), Me	3.92 (2H, s)	4.10 (br s) 4.07 (br s)	5.02 (1H, s) 4.86 (1H, m)	5.26 (br s) 5.07 (br s)	5.04 (1H, d, 1.47) 4.86 (1H, m)	5.25 (br s) 5.06 (br s)	5.06 (1H, d, 1.35) 4.90 (1H, s)	5.26 (br s) 5.08 (br s)
13	5.80 (1H,ddd, 15.34,9.89, 1.51)	5.90 (ddd,15. 4,10.0,1.6)	5.80 (1H,ddd, 15.54,9.8 8,1.29)	5.93 (br dd, 15.4, 10.0)	5.84(1H, ddd, 15.37,9.9 2,1.55)	5.90 (ddd,15. 6,10.0,1.4)	5.60 (1H,dd, 15.22,9.6 1)	5.76 (ddd,15. 4,10.0,1.6)	5.84 (1H,ddd, 15.34,9.3 8,1.35)	6.06 (ddd,15. 4,10.0,1.2)	5.81(1H, ddd, 15.46,9.4 3,1.48)	6.05 (ddd,15. 4,10.0,1.5)
14	4.91 (1H,ddd, 15.31,10.9 6,4.25)	5.05 (ddd,15. 4,11.2,4.4)	4.84 (1H,ddd, 18.80,9.3 9,4.79)	4.98 (ddd, 15.4, 8.8, 6.8)	4.95 (1H,m)	5.07 (ddd,15. 6,11.0,4.4)	4.88 (1H, m)	5.2 (ddd,15. 4,11.0,4.9)	4.96 (1H, m)	5.35 (ddd,15. 4,11.0,4.6)	4.99 (1H,m)	5.4 (ddd,15. 4,11.0,4.4)
15	1.91 (1H, m) 1.66 (1H, m)	2.02 (m)	1.78 (2H, m)	1.93 (m, 2H)	1.91 (1H, m) 1.66 (1H, m)	2.02 (m) 1.82 (br q, 11.0)	1.92 (1H, m) 1.66 (1H, m)	2.02 (m)	1.89 (1H, dt) 1.58 (1H, m)	2.03 (m) 1.80 (br q, 11.0)	1.93 (1H, m) 1.59 (1H, m)	2.04 (m) 1.78 (br q, 11.0)
16	1.27 (1H, m)	1.82 (br q, 11.2)	1.39 (1H, m)	1.54 (m)	1.27 (1H, m)	1.34 (m)	1.27 (1H, m)	1.82 (br q, 11.0)	0.94 (1H, m)	1.13 (m)	1.27 (1H, m)	1.42 (m)
17	1.74 (1H, m) 1.42 (1H, m)	1.97 (br dd, 13.1,3.0)	3.57 (1H, td, 4.39, 1.91)	3.88 (m)	1.75 (1H, dd,12.95, 3.95) 1.43 (1H, dd,13.08, 3.01)	1.95 (br dd, 13.2,2.8) 1.64 (dd,13.2, 3.9)	1.76 (1H, m) 1.46 (1H, m)	1.93 (br dd, 13.2,3.0) 1.65 (dd,13.2, 3.9)	1.70 (1H, m) 0.86 (1H, m)	1.93 (br d, 13.6) 1.09 (br ddd,13.6, 5.05,1.5)	1.50 (1H, m) 1.05 (1H, m)	1.17 (dt, 14.5,4.5) 1.68 (br d,1.45)

Atom No	DSM5.1		DSM4.5		DSM9.3		DSM7		DSM8.4		DSM4.7.1	
	This study	Ref ^a	This study	Ref ^a	This study	Ref ^a	This study	Ref ^b	This study	Ref ^a	This study	Ref ^a
18	-	-	4.72 (1H, dd, 4.70)	2.67 (m)	-	-	-	-	-	-	1.44 (1H, m)	1.56 (m)
19	6.34 (1H, d, 15.60)	6.56 (d,15.9)	5.96 (1H, dd, 16.10, 7.27)	6.19 (dd, 16.1, 7.2)	6.33 (1H, d, 14.15)	6.58 (d,15.6)	6.47 (1H, dd, 15.65)	6.59 (d,15.9)	1.34 (2H, m)	1.65 (m)	1.19 (2H, ov)	1.33 (m) 1.42 (m)
20	7.10 (1H, d, 15.61)	7.31 (d,15.9)	6.94 (1H, dd, 16.08,1.34)	7.16 (d d, 16.1, 1.5)	7.11 (1H, d, 15.64)	7.23 (d,15.6)	7.06 (1H, d, 15.62)	7.08 (d,15.9)	3.47 (1H,m) 1.34 (1H,m)	3.70 (ddd,18.8,8.8,1.7) 1.82 (dt, 18.8, 1.7)	3.22 (1H, ov) 1.21 (1H, ov)	3.56 (br dd, 17.0, 8.0) 1.78 (m)
22	0.96 (3H, d, 6.97)	1.05 (d, 7.1)	0.86 (3H, d, 7.22)	1.00 (d, 7.1)	0.96 (3H, d, 6.94)	1.05 (d, 7.1)	0.96 (3H, d, 6.86)	1.05 (d, 7.1)	0.94 (3H, m)	1.03 (d, 6.8)	0.91 (3H, d, 6.77)	0.97 (d, 6.8)
23	1.27 (3H, m)	1.38 (s)	0.99 (3H, d, 6.89)	1.12 (d, 6.8)	1.27 (3H, m)	1.39 (s)	1.27 (3H, m)	1.38 (s)	1.03 (3H, s)	1.19 (s)	0.81 (3H, d, 6.88)	0.89 (d, 7.1)
2'/6'	7.14 (2H, m)	7.12 (m)	7.14 (2H, m)	7.12 (m)	7.13 (2H, m)	7.12 (m)	7.06 (2H, m)	7.12 (m)	7.08 (2H, m)	7.08 (m)	7.14 (2H, m)	7.08 (m)
3'/5'	7.28 (2H, t, 7.69)	7.30 (m)	7.28 (2H, t, 7.69)	7.30 (m)	7.26 (2H, t, 7.69)	7.29(m)	7.30 (2H, t, 7.45)	7.31 (m)	7.25 (2H, m)	7.30 (m)	7.28 (2H, m)	7.30 (m)
4'	7.20 (1H, m)	7.23 (m)	7.20 (1H, m)	7.23 (m)	7.20 (1H, m)	7.22 (m)	7.22 (1H, m)	7.23 (m)	7.21 (1H, m)	7.24 (m)	7.20 (1H, m)	7.23 (m)

^a(Buchanan, Hashimoto, & Asakawa, 1995) ^b(Buchanan, Hashimoto, & Asakawa, 1996)-both references recorded samples dissolved in CDCl₃ at 600MHz

Table 6.19 ¹³C NMR data and comparison of reported values of cytochalasin derivatives (100MHz, DMSO-d₆)

Atom No	DSM5.1		DSM4.5		DSM9.3		DSM7		DSM8.4		DSM4.7.1	
	This study	Ref ^a	This study	Ref ^a	This study	Ref ^a	This study	Ref ^b	This study	Ref ^a	This study	Ref ^a
1	173.4	174.1	173.9	174.4	173.6	174.3	173.48	174	173.9	174.1	174.5	174
3	53.8	54.8	53.8	54.8	53.9	54.8	52.57	53.1	52	52.6	51.9	52.6
4	47.03	48.6	46.7	48.6	47	48.6	44.27	45.4	45.1	46.2	40.5	46.4
5	34.6	34	34.6	34.8	33.9	34	31.45	31.5	31.7	31.7	45.2	31.8
6	140.4	143.2	140.4	140.1	144	143.2	151.39	148.7	151.3	148.8	151.7	148.6
7	125.5	125.5	125.4	125.6	125.3	128.5	71.82	71.5	71.3	71.6	71.1	71.5
8	49.6	50	49.6	49.9	49.6	49.3	51.37	51.7	51.3	51.3	51.1	51.3
9	68.6	68.4	69	68.8	68.5	68.7	64.03	63.5	64.2	63.8	68.6	64
10	43.41	45.1	43.9	45.1	44	44.9	43.41	43.8	42.5	43.8	42.2	44
11	13.4	13.5	13.3	13.4	12.2	12.2	13.16	12.9	13.1	12.9	13	13
12	20	20	20	19.9	61.8	63.2	112.02	113.9	112.5	114.2	112.5	114.3
13	129.3	128.5	129.3	128.5	129.3	128.2	129.88	127.5	130.8	128.3	130.5	127.5
14	134	135	134.4	135	134.2	135.2	134.32	137.2	132.4	135.6	132.9	136.9
15	43.2	42.9	41.2	40.9	43.2	43	43.68	43.2	43.3	42.9	42.7	42.6
16	28.7	29.3	33.3	33.3	28.8	29.2	28.55	28.8	30.2	30.4	28.7	27.2
17	55.1	54.6	78.5	79.4	55.1	54.7	55.81	54.6	44.7	45	38	37.9
18	71.8	73.1	43	43.2	71.8	73.1	71.77	72.8	71.6	73.1	31	31
19	153.3	152.3	148.5	148.6	152.8	153.1	155.31	154.7	31.5	31.1	26.5	27.1
20	132.5	132.1	133.3	133.6	132.5	131.7	131.63	130.5	34.5	34.3	36.6	37
21	200	200	200	197.8	200.1	199.6	199.37	198.9	212.6	211.1	207.5	211.2
22	26.1	25.7	18.1	17	26.1	25.7	26.16	25.6	25.9	25.4	25.9	25.7
23	26.4	25.6	12.3	11.6	26.4	25.4	26.47	25.1	29.2	28	21	20.4
1'	137.4	137.3	137.4	137.3	137.5	137.1	137.62	136.9	137.1	136.6	137.1	136.7
2'/6'	130.1	129.1	130.1	129.1	130.3	129.3	130.01	129.2	130.5	129.4	130.6	129.4

Atom No	DSM5.1		DSM4.5		DSM9.3		DSM7		DSM8.4		DSM4.7.1	
	This study	Ref ^a	This study	Ref ^a	This study	Ref ^a	This study	Ref ^b	This study	Ref ^a	This study	Ref ^a
3'/5'	128.5	128.8	128.5	128.8	128.6	128.8	128.76	128.7	128.6	128.7	128.5	128.8
4'	126.8	126.9	126.8	126.9	126.8	126.9	126.42	126.9	126.8	127	126.8	127

^a(Buchanan, Hashimoto, & Asakawa, 1995) ^b(Buchanan, Hashimoto, & Asakawa, 1996)-both references recorded samples dissolved in CDCl₃ at 150 MHz

Cytochalasins are a diverse family of fungal secondary metabolites. They have a distinct perhydro-isoindolone core structure that is heavily substituted and typically fused to a macrocyclic ring. The macrocycle varies widely among different cytochalasins, with examples including carbocyclic, lactone, and cyclic carbonate forms (Scherlach et al., 2010). Specific amino acid contained into the isoindole moiety results to more structural diversity. It can bear different substituents such as a benzyl group (as seen in the cytochalasins obtained in this work), a *para*-methoxybenzyl group, an (indol-3-yl)methyl group, a 2-methylpropyl group (characteristic of aspochalasins), or a simple methyl group (found in alachalasins) (Binder et al., 1973; Scherlach et al., 2010). Differences in ring size and substitution patterns directs the biological properties of cytochalasins. To date, over 100 related compounds or derivatives have been identified from a wide range of fungal sources, including both ascomycetes and basidiomycetes.

The cytochalasins identified in the present study were first reported by Buchanan and co-workers (1995, 1996) who, along with BNT and daldinone B, have since been recognized as chemotaxonomic markers of the *D. eschscholtzii* species complex (Narmani et al., 2019; Stadler et al., 2014). Buchanan and et al (1995, 1996) had described a total of sixteen congeners of cytochalasins isolated from the stromata (fruiting bodies) of wild *Daldinia eschscholtzii* growing on *Quercus acutissima* (oak trees). In contrast, only six cytochalasin derivatives were isolated in the present study. The reduced structural diversity observed may be attributed to differences in fungal source and cultivation conditions, as Buchanan et al. extracted secondary metabolites from fruiting bodies of naturally occurring *D. eschscholtzii* collected on oak trees, while the present study utilized laboratory-grown fungus. Furthermore, cytochalasin production in the present work was only observed when *D. eschscholtzii* was supplemented with SBHA in its PDB culture. Although this led to a higher yield of specific cytochalasins (i.e., cytochalasin 1 at 5.82% yield), it was accompanied by reduced analogue diversity. This suggests that chemical elicitation played a key role in activating or enhancing the biosynthetic pathway

responsible for cytochalasin production. Additionally, *D. eschscholtzii* is regarded as a complex taxon with different subspecies and that production pattern of cytochalasins vary between species isolated from different geographic environment (Stadler et al., 2014; Stadler, Wollweber, et al., 2001). Hence, it would be valuable to further investigate the molecular and phylogenetic characteristics of the Philippine ELF *D. eschscholtzii* strain in comparison with other geographic isolates.

6.5.5 Biological activities of isolated compounds

The identified compounds were evaluated for their bioactivities. The results are shown in Table 6.20. BNT was the only compound with potent antibacterial and prebiofilm inhibitory activity. Its MIC was 20.22 μM while its BPC was 39.31 μM . The values were the lowest among this set of compounds but still far from the values of ciprofloxacin. Interestingly, BNT exhibited strong antibacterial activity at lower concentrations (12.5–25 $\mu\text{g/mL}$ or 1.5–1.8 μM), while only moderate activity (60%) was observed at the highest tested concentration (100 $\mu\text{g/mL}$ or 2.5 μM). This atypical dose–response behaviour is shown in the MIC curve (U-shaped) shown in Table S 1. On the other hand, most of the compounds have strong antibacterial and prebiofilm activity at the highest testing concentration (100 $\mu\text{g/mL}$) only such as the cytochalasins. Orthosporin exhibited better activity than diaporthin although both possess cytochalasin impurities. Consistent from previous sections (Table 5.18), daldinone B exhibited strong prebiofilm inhibition. Among the cytochalasin derivatives, only three displayed inhibition activities. This includes cytochalasins 1, 9, and 11. The other derivatives did not possess any inhibitory activity against MRSA. These indicates the potential of BNT, cytochalasins, and daldinone B as antibiofilm agents.

Table 6.20 Biological activities of isolated compounds from DSM fractions against MRSA

Sample code	Identity, %purity	Antibacterial activity		Prebiofilm activity		Postbiofilm activity	
		%Inhibition @100 µg/mL	MIC (µM)	%Inhibition @100 µg/mL	BPC (µM)	%Inhibition @100 µg/mL	MBIC (µM)
DSM2	BNT, 100%	60.57	20.22 ^a	99.95	39.31 ^a	4.07	>100
DSM5.2	daldinone B, 80%	63.15	>100	80.72	>100	12.73	>100
DSM4.6	orthosporin, 60%	98.44	>100	99.86	>100	-4.35	>100
DSM4.4	diaporthin, 30%	46.33	>100	22.72	>100	1.87	>100
DSM7	cytochalasin 1, 100%	90.83	>100	92.20	>100	24.36	>100
DSM5.1	cytochalasin 9, 100%	98.43	>100	99.69	>100	-8.66	>100
DSM4.5	cytochalasin 11, 100%	98.24	>100	100.04	>100	14.75	>100
DSM9.3	cytochalasin 10, 100%	57.00	>100	24.89	>100	8.74	>100
DSM8.4	cytochalasin 2, 100%	40.37	>100	-10.58	>100	4.48	>100
DSM4.7.1	cytochalasin 7, 100%	nt	nt	nt	nt	nt	nt
ciprofloxacin		97.65	2.03 ^a	99.86	1.56 ^a	94.47	10.52 ^a
DMSO		6.73	>100	-13.79	>100	0.4	>100

Assays were done twice with three replicates (n=3); Moderate inhibition: 50-70% (coloured in blue); Strong inhibition >70% (coloured in red).

MIC- Minimum Inhibitory Concentration; the smallest concentration of sample to give 100% inhibition; computed from a series of sample dilutions in GraphPad Prism

BPC- Biofilm Prevention Concentration; the smallest concentration of sample to give 90% inhibition in the prebiofilm assay

MBIC- Minimum Biofilm Inhibition Concentration; the smallest concentration of sample to give 90% inhibition in the postbiofilm assay

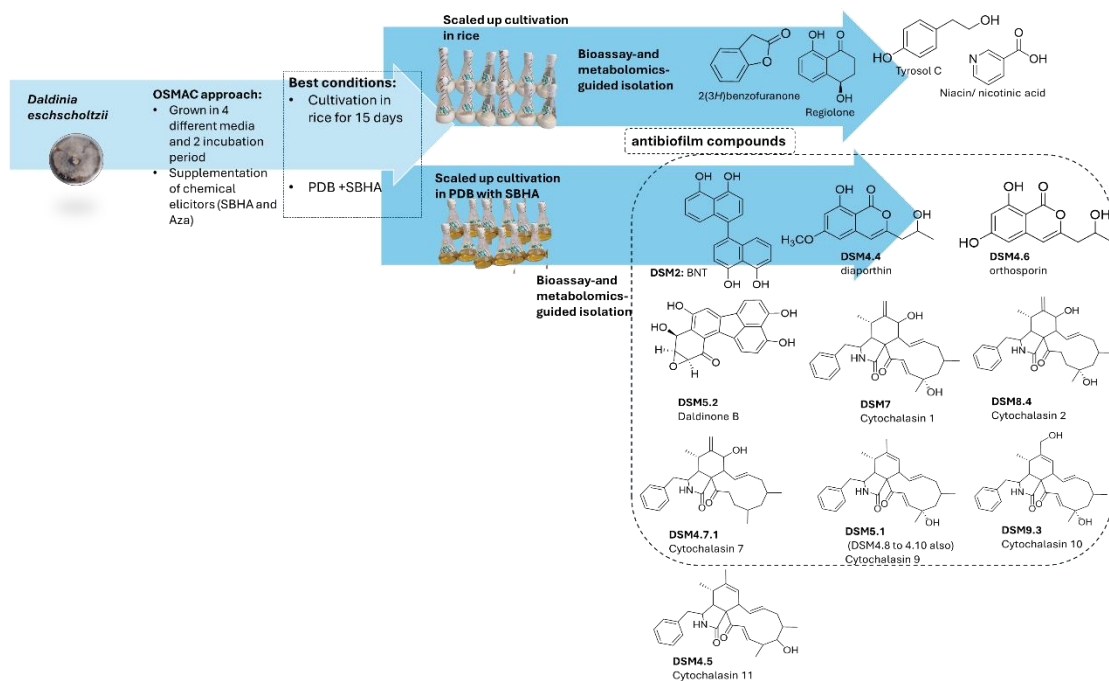
nt- not tested because of insufficient amount

^aDose-response curves were shown in Supporting Information

6.6 Chapter summary

This chapter presents the comprehensive study of *Daldinia eschscholtzii*, focusing on its metabolic and antibiofilm potential under varied culture conditions. Using the OSMAC, the fungus was cultivated in different media and incubation durations, leading

to diverse metabolic profiles and biological activities, particularly in pre- and post-biofilm inhibition assays against MRSA. Among the conditions, the extract obtained from the 15-day rice culture exhibited the highest prebiofilm inhibition and notable chemical diversity, as indicated by both of their NMR and LC-MS-based metabolomics profiles. Subsequent fractionation to the identification of compounds, included 2(3*H*)-benzofuranone, regiolone, tyrosol, and niacin. However, individual compounds did not display the strong bioactivities observed in the crude or semi-purified fractions, suggesting possible synergistic and/or additive effects. Further enhancement of secondary metabolite production was achieved by incorporating two chemical elicitors (SBHA and Aza) into the growth media. The SBHA-treated PDB cultures showed significantly improved metabolite diversity and antibiofilm activities. Multivariate analyses pinpointed distinct chemical signatures associated with the elicited extracts. Several known metabolites including cytochalasins and binaphthyl derivatives were dereplicated and targeted for isolation work. Six cytochalasin derivatives, BNT, daldinone B, orthosporin, and diaporthin were obtained after several chromatographic steps. BNT, daldinone B, and four cytochalasin derivatives exhibited potent antibiofilm inhibition. Overall, this chapter underscores the utility of media optimisation and chemical elicitation to enhance the yield of secondary metabolites with antibiofilm activities in *D. eschscholtzii*.

Graphical summary of *Daldinia* chapter

Chapter 7. General Discussion, Conclusion, and Future works

7.1 Applications of metabolomics in natural products bioprospecting

7.1.1 For strain prioritisation

The natural products drug discovery workflow starts with a library of crude extracts from different organisms with the aim of selecting the strains with promising potency based on predefined goal, e.g., bioactive compounds. Metabolomics are recognized as valuable tools in this pipeline by profiling the chemical diversity of extracts. It can be used as guide to the discovery of new/known compounds associated for the observe pharmacological activities. It is complementary tool with bioassays for strain selection (Hou et al., 2012; Macintyre et al., 2014; Wolfender et al., 2019). Thus, the decision is based on aspects of chemical and biological importance.

This study fundamentally began with ten ELF strains collected from the Philippines. A metabolomics profiling and panel of biological assays were carried out to screen “talented” ELF strains as presented in Chapter 3. From the three biological assays, it was found that only three ELF displayed significant potency. **P6**, **P4**, and **R3** stood out for their remarkable biological activities against MRSA. But more interestingly, these three ELF also stood out in their metabolomics profile. Distinct blue bands were clearly seen in the heat map generated from their ¹H-NMR spectra (Figure 3.5). These ELF also separated from the rest of the crude extracts in the unsupervised multivariate analysis model, principal component analysis (PCA), based on their ¹H-NMR and LC-MS data (Figure 3.6 and Figure 3.8). Hence, the metabolomics tools consistently agree with the biological assays. These are strong basis for selection of **P4**, **P6**, and **R3** for further drug discovery work. Prioritising these three fungi would save time, effort, and economic resources as they exhibited promising potentials with respect to both biological effects and chemical diversity.

Furthermore, discriminatory metabolites for targeted chromatographic isolation work were determined based on Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) model, which were dereplicated using an in-house Macro with Dictionary of Natural Products database. At this point, putative annotation/tentative identification gave hints on the type compounds that did assist on the chromatographic isolation and purification parameters. This piece of information can be also considered as a decision-making tool to prioritise strains producing new chemical entities to avoid redundant isolation of known compounds giving similar biological activity.

Examples of published literatures have demonstrated how multivariate analysis and metabolomics tools can guide strain selection based on novelty and bioactivity potential. In one particular study, three strains out of 77 invertebrate-associated bacterial symbionts stood out by affording interesting secondary metabolites as indicated by multivariate analysis and a dereplication process (Macintyre et al., 2014). Another example involved a panel of 44 pigment-producing endophytic fungi isolated from *Cresencia alata* and 13 of them exhibited anti-inflammatory principles. Based on their ¹H-NMR-based metabolomics profiling approach, terpenes were found to be associated with the observed biological activities (Flores-Vallejo et al., 2020). This PhD work aligns with these earlier published reports that have illustrated the usefulness of metabolomics for prioritisation in early stages of natural products drug discovery. In addition, like this current PhD work, one published study on ELF from various hosts utilized heat maps as well as PCA and PLS-DA plots based on the LC-HRMS spectral data of their crude extracts illustrated that the lichen host and its associated ELF produced distinct secondary metabolites (Santiago et al., 2021). From the same study by Santiago et al., (2021), methyl xylariate C and other compounds were pinpointed from ELF fractions as discriminatory metabolites related to its dual inhibition against *S. aureus* and *E. coli*.

Herein, the importance of metabolomics in bioprospecting was evidently demonstrated. In this study, ten Philippine ELF strains were screened and only three

were found to have excellent biological activities while also holds chemical uniqueness. Using molecular methods, the three fungi **P4**, **P6**, and **R3** were identified as *Daldinia eschscholtzii*, *Hypoxylon lateripigmentum*, and *Xylaria cubensis*, respectively. From the dereplication table (Table 3.2), the reported biological source of the hits is similar from the results of molecular identification. This also confirms the usefulness of metabolomics and chemical profiling in the polyphasic approach for fungi taxonomical systematics (Gannibal, 2022; Kozlovsky, Zhelifonova, & Antipova, 2009; Smedsgaard & Nielsen, 2005)

7.1.2 A complementary tool in the OSMAC approach

After prioritising three ELF, the one strain-many compounds (OSMAC) strategy was employed. In this methodology, culture media and incubation period were optimised for each ELF. PDB and MEB were the liquid culture media used while oat and rice were the solid media utilised. Two incubation periods of 7 and 15 days for *D. eschscholtzii* while 15 and 30 days for *X. cubensis* and *H. lateripigmentum* were employed. The impact of chemical elicitors to *D. eschscholtzii* was also examined. All this aimed to find an optimised culture condition that will produce secondary metabolite diversity and potency in bioactivities.

The OSMAC strategy successfully induced distinct metabolomic responses and bioassay results in all three ELF, underscoring the flexibility of their secondary metabolism in response to nutritional changes. Solid media such as oat promoted higher fungal extract quantities. Yet, culture extracts obtained from oat media were often rich in lipids and offered less chemically diversity, as reflected in their NMR profiles. In the context of drug discovery, lipids are commonly considered undesirable due to their tendency to form complex mixtures of structurally similar molecules that may degrade under certain conditions (Ibarguren, López, & Escribá, 2014). Furthermore, fatty acids are

known to disrupt various non-cell-based assays, often producing false positives during early screening stages (Balunas et al., 2006). Consequently, it made oat-based culture extracts less interesting to pursue despite their high yields. On the contrary, fungal extracts obtained from malt extract broth (MEB) and potato dextrose broth (PDB) cultures were mainly enriched with aromatic metabolites albeit their lower yields. The trend was observed in the three investigated ELF in this study. Overall, the findings tell that maximizing the extract quantity does not necessarily maximize chemical diversity.

The observed chemical variation can be correlated with the composition of each media. Oats are richer in proteins and fats, while rice is carbohydrate-dense (Beloshapka et al., 2016; Liu, 2011; Rasane et al., 2015) likely explaining the fatty acid accumulation in culture extracts obtained from oat media as indicated by their ^1H NMR spectral data (refer to Figure 4.2, **Error! Reference source not found.**, Figure 6.2) and the presence of glycosylated metabolites in fungal extracts from rice media, as particularly observed with *X. cubensis* (Table 4.2). Meanwhile, the complex carbon sources such as maltose and nitrogen sources as in peptone found in MEB appeared to support production of biosynthetically diverse aromatic compounds. The compositional differences among these media influence fungal metabolic pathways by providing distinct nutrient regimes that can modulate the secondary metabolite profiles of fungal extracts.

Additionally, incubation duration periods representing respective fungal growth phases influenced both metabolite composition and bioactivity. For example, aromatic metabolites in *D. eschscholtzii* declined between the 7th and 15th day of incubation in PDB culture, and correspondingly, the extract obtained from the 7-day PDB culture exhibited strong prebiofilm activity, whereas the extract after 15-days of incubation period was inactive. On the other hand, in *H. lateripigmentum*, aromatic compounds were enriched between the 15th and 30th day of cultivation in MEB, while the extract obtained from the 30-days culture showed stronger prebiofilm activity. These findings support previous

reports that fungal secondary metabolism varies with the growth developmental phase (Calvo et al., 2002).

The use of epigenetic modifiers was also investigated and applied to *D. eschscholtzii* cultivation. This experiment still falls under the OSMAC strategies. Chemical elicitors or epigenetic modifiers were known to enhance secondary metabolite expression without altering genes or causing heritable characters (Berger et al., 2009). Instead, these compounds suppress or inhibit the activity of enzymes such as DNA methyltransferase (DNMT) and histone deacetylase (HDAC) and thus promotes the progression of other biosynthetic pathways (Seyedsayamdost, 2014). In this study, 5-azacytidine (5-Aza) and suberoylbishydroxamic acid (SBHA) belonging to DNMT and HDAC inhibitor classes, respectively, were supplemented to the PDB cultures of *D. eschscholtzii*. The fungal extracts from elicited medium demonstrated enhancement of MRSA biofilm inhibition activity and increased metabolic diversity (Section 6.4). BNT ([1,1'-binaphthalene]-4,4',5,5'-tetrol) and cytochalasin derivatives were annotated (and later isolated and structurally identified) from fungal extracts obtained from elicitor-treated media. This finding is consistent with previous reports demonstrating that chemical elicitors can significantly upregulate cytochalasin biosynthesis in various fungal species. For instance, in *Aspergillus clavatus*, Zutz et al. (2013) reported that the production of cytochalasin E was significantly increased upon supplementation with 5-Aza. Similar enhancements were also observed with HDAC inhibitors such as valproic acid and sodium butyrate.

Though several studies have reported the occurrence of BNT and cytochalasins in *D. eschscholtzii* (Buchanan, Hashimoto, & Asakawa, 1995; Buchanan, Hashimoto, & Asakawa, 1996; Narmani et al., 2019; Stadler et al., 2014; Stadler, Wollweber, et al., 2001), these metabolites were not detected under standard PDB cultivation conditions in our study. Key biosynthetic pathways such as the polyketide synthase–nonribosomal peptide synthetase (PKS–NRPS) hybrid pathway involved in cytochalasin production

(Skellam, 2017) and melanin biosynthesis pathway responsible for BNT production (Stadler, Baumgartner, et al., 2001) were most likely silent under standard plain PDB conditions. The pathways must have been activated upon SBHA treatment and thus underscores the utility of chemical elicitation in awakening cryptic gene clusters. Future transcriptomic analyses could confirm whether previously described biosynthetic gene clusters (e.g. *ccsA* and *ccsR* genes of *A. clavatus* (Qiao, Chooi, & Tang, 2011)) are indeed upregulated in response to SBHA treatment. Collectively, these results emphasize that optimal bioactivity does not always coincide with maximal extract yield and highlight the value of integrating metabolomics with OSMAC for effective decision-making. The metabolomics tools effectively distinguished the metabolite diversity caused by the OSMAC experiments carried out in this study. These findings clearly show the critical role of metabolomics as a complementary tool for a comprehensive evaluation of biochemical impacts brought about by the OSMAC strategy.

Numerous studies have demonstrated the combined application of metabolomics and OSMAC methodologies, consistent with the observations from this current work. For example, PCA identified the influence of culture medium (Czapek vs Nutrient broth) and agitation (static vs. shaking) to metabolic profile of the crude extracts from *Xylaria* sp. (Vieira et al., 2021). The work of da Silva et al. (2023) utilized volcano plots and molecular networking to highlight the chemical classes related to antibacterial activity from several extracts of the fungal endophyte *Penicillium* sp. 5MP2F4 against *S. aureus*. Le Loarer et al. (2024) applied the OSMAC strategy to three marine-derived Actinobacteria strains and found that extending the culture duration generally led to denser and more complex molecular networks, indicating increased metabolomic richness. In contrast, variations in medium composition, particularly the use of A1 medium, selectively enhanced the production of unique metabolite clusters associated with potent cytotoxic and antiplasmodial activities of the actinobacterial extracts. A comprehensive metabolomic profiling revealed that treatment of *Aspergillus nidulans*

with epigenetic modifiers such as suberoylanilide hydroxamic acid (SAHA), 5-azacytidine, and nicotinamide induced distinct and complex shifts in secondary metabolism, with each elicitor activating different sets of silent biosynthetic gene clusters (Albright et al., 2015). These perturbations led to the production of novel metabolite families, including previously undetected polyketides and nonribosomal peptides. Together, these studies emphasize the power of integrating metabolomics with OSMAC and epigenetic modulation strategies to systematically uncover metabolic potential and bioactive compound diversity across diverse microbial taxa.

7.1.3 For targeted isolation of bioactive compounds

Another key application of metabolomics in this study was its use in the targeted isolation of bioactive compounds. In this approach, the fractions afforded after chromatography were subjected to ^1H -NMR and LC-MS based metabolomics analyses. By integrating bioassay data with metabolomic profiles, this approach enabled the prioritisation of specific fractions for further purification, thereby streamlining the isolation process and enhancing the efficiency of compound structure elucidation. Furthermore, pure compounds were elucidated through several NMR experiments and have been validated through comparison with reference NMR spectral values.

In this study, a bioassay-metabolomics-guided isolation was employed for the purification of antibiofilm compounds. Bioactive fractions were selected for further purification based on dereplication results, particularly those containing metabolite features previously annotated in the parent crude fungal extract. For instance, in *H. lateripigmentum*, TAN-1612 was annotated in the preliminary screening stage (Table 3.2), then it was once more annotated in several growth media in the media optimisation experiments (Table 5.2), and it was tracked down in several fractions on scaled up production (Table 5.5, Table 5.14). TAN-1612 was then purified, structurally confirmed,

and found to retain the strong antibiofilm activity. In contrast, compounds such as daldinone A and B were only annotated after fractionation, while daldinone E was not detected during the earlier stages of screening. This discrepancy may be attributed to their extremely low abundance while certain metabolites were only enriched after the purification stage. As observed, the final isolated weights of these compounds were also very minimal.

In *X. cubensis*, the compound mellein was consistently annotated across the stages of preliminary screening, media optimisation, and fractionation. Mellein was subsequently purified and structurally elucidated by 2D NMR. In addition, phloroglucinol was also isolated from the active fractions. This is the first report of phloroglucinol being isolated in a fungal species. However, this compound was not annotated/dereplicated in the LC-MS-based metabolomics due to its molecular weight of 126.11 g/mol that was falling outside the mass detection range settings of 150 to 1500 Da of the optimised mass spectrometry parameter in screening fungal metabolites (Macintyre et al., 2014). Interestingly, the ^1H -NMR resonances (8.9 and 5.6 ppm) of phloroglucinol were observed and recognized as discriminatory chemical shifts during the media optimisation stage (refer to Section 4.4.3 and Figure 4.3D). This finding put emphasis on the value of ^1H -NMR-based metabolomics as a complementary technique to LC-MS, particularly in detecting low-molecular-weight or non-ionisable metabolites that may have been disregarded in mass spectrometry analyses.

For *D. eschscholtzii*, regiolone was the only compound consistently annotated in the three stages of the study mentioned earlier. BNT and several cytochalasin derivatives (including their structural isomers) were annotated when the fungus was cultured in a media treated with the chemical elicitor SBHA (see Table 6.12 and Table 6.15). The exact structures of these isomers were elucidated only after further chromatographic purification and comprehensive structural elucidation through 1D- and 2D-NMR

experiments. These findings underscore the critical role of NMR in validating metabolite annotations with high confidence.

Narrowing down the chemical space early in the workflow enabled a focused and efficient isolation of compounds most likely responsible for the observed biofilm inhibition activities. Importantly, only a limited number of metabolite features were consistently annotated across all stages, highlighting the challenges associated with metabolite abundance, and/or detection sensitivity. These results emphasize the value of combining multiplatform analytical equipment such as LC-MS and NMR for the reliable identification of bioactive secondary metabolites.

Several recent studies have employed similar metabolomics-guided isolation strategies. For instance, PCA and OPLS-DA models based on LC-MS data of the antibacterial fractions of *Bacillus velezensis* 7NPB-3B enabled the purification of a new metabolite together with four known cyclic dipeptides (Singh et al., 2024). The presence of naphthol tetramers was suggested by the feature-based molecular networking analysis of the ELF extract and fractions of *Daldivia childei* 047219. This was then confirmed by targeted isolation, eventually ending up with six new naphthol tetramers with weak anti-inflammatory activities (Kim et al., 2023). In the study by Toure et al. (2022), an LC-MS/MS-based molecular networking workflow was combined with bioassay results to construct a molecular network linking anti-biofilm activity to specific metabolite families, which enabled the targeted isolation of oxygenated fatty acids including a new compound from the ELF *Preussia persica*. Similarly, Buedenbender et al. (2018) demonstrated the utility of NMR-based heteronuclear single-quantum correlation–total correlation spectroscopy (HSQC–TOCSY) fingerprinting for the prioritisation of polyketide- and peptide-producing microbial isolates, offering a complementary strategy to LC-MS by enabling rapid dereplication and structural insight directly from crude extracts.

Collectively, these studies validate the effectiveness of a bioassay-metabolomics-guided approach in streamlining the discovery and structural elucidation of bioactive secondary metabolites. The consistent annotation and successful isolation of key compounds highlight the robustness of integrating LC-MS and NMR techniques, ultimately enhancing the reliability and depth of metabolite identification in complex natural product extracts.

7.2 Antibiofilm agents from Philippine ELF

7.2.1 The target pathogens: MRSA and *P. aeruginosa* biofilms

The primary aim of this PhD research is to discover antibiofilm compounds from Philippine ELF. The focus on secondary metabolites with biofilm-inhibitory activity stemmed from the protective nature of bacterial biofilms, which shield pathogens from antimicrobial treatments. In addition, biofilms create an environment conducive to spontaneous mutations and the development of drug resistance (Flemming & Wingender, 2010; Stewart, 2002), often leading to chronic and persistent infections.

Throughout this study, test samples (extracts, fractions, and purified compounds) were tested against MRSA (ATCC 43300) and *Pseudomonas aeruginosa* (ATCC 27853). These bacterial strains are known biofilm-formers and are utilized as testing strains for biofilm susceptibility experiments (Barbarossa et al., 2025; Cao et al., 2017). Furthermore, the strains are clinically relevant due to their established roles in biofilm-associated infections. The MRSA ATCC 43300 strain expresses virulence factors such as phenol-soluble modulins that contribute to robust biofilm formation and persistent colonisation of medical devices, making it a valuable testing organism (Kaur, Harjai, & Chhibber, 2016). Similarly, *P. aeruginosa* ATCC 27853 is known for its strong biofilm-forming capacity and is frequently implicated in chronic and nosocomial infections (Reynolds & Kollef, 2021), including ventilator-associated pneumonia (Parker et al., 2008) and

infections in cystic fibrosis patients (Langan, Kotsimbos, & Peleg, 2015), stressing its importance in testing novel therapeutic strategies.

In this study, it was generally observed that test samples have specific prebiofilm inhibition against MRSA only. In contrast, bioactivity against *P. aeruginosa* was observed exclusively with extracts or fractions derived from *H. lateripigmentum* (Table 5.1 and Table 5.4). This disparity may be attributed to the morphology and biofilm matrix composition differences between the two bacterial test strains. *P. aeruginosa* have pili and flagella that are important structural features for motility, surface attachment, and biofilm formation (Samrot et al., 2021). MRSA is a Gram-positive coccus that produces intercellular adhesin (PIA), a polycationic polysaccharide with β -1,6-linked *N*-acetylglucosamine units (Aboelnaga et al., 2024). On the other hand, *P. aeruginosa* is a Gram-negative rod-shaped bacterium that secretes at least three distinct exopolysaccharides: alginate, Psl (a mannose-rich polymer), and Pel (composed of α -1,4-linked galactosamine and *N*-acetylgalactosamine) (Franklin et al., 2011; Nizer et al., 2024). These polysaccharides serve different structural and protective roles, contributing to the biofilm's robustness of each strain. Consequently, *P. aeruginosa* biofilms are more difficult to inhibit and eradicate due to their complex, multifactorial defences. Indeed, one study demonstrated that *P. aeruginosa* exhibits increased antibiotic tolerance under biofilm conditions compared to its planktonic form (Thöming & Häussler, 2022). Therefore, the limited activity of the extracts against *P. aeruginosa* is consistent with the well-documented strong resistance of this organism.

7.2.2 Performance and relevance of antibiofilm assays

This study assessed three aspects of biological activities: antibacterial, prebiofilm, and postbiofilm inhibitory activity. The antibacterial activity refers to the capability of the extracts to inhibit bacterial growth. This was determined by measuring the optical

density of bacterial cultures after exposing to the test organisms. However, this assay primarily indicate growth inhibition only and do not distinguish between bacteriostatic (growth-inhibiting) and bactericidal (killing) effects (Pankey & Sabath, 2004). Hence, it would be remarkable as well to test whether the extracts exhibiting strong antibacterial activity such as TAN-1612 can kill MRSA. The Minimum Inhibitory Concentration (MIC) values were reported for the pure compounds which pertains to the definition of Clinical & Laboratory Standards Institute (CLSI) guidelines as the lowest concentration of a compound to inhibit bacterial growth (Weinstein et al., 2018). TAN-1612 exhibited low MIC values (13.83 μ M), indicative of promising antibacterial activity. Although its MIC are higher than that of ciprofloxacin, a well-established fluoroquinolone antibiotic, the observed biological activity highlights their potential as lead structures for further optimisation and development.

Following the removal of planktonic cells, adherent bacterial biofilms were quantified to determine the prebiofilm inhibitory activity, which reflects the test samples' ability to prevent the initial formation and/or establishment of biofilms. In parallel, postbiofilm inhibition (evaluated in MRSA only) was assessed in a separate experimental setup. Here, biofilms were first allowed to mature for 24 hrs before being treated with the various test samples. Subsequent quantification of biofilm indicated the disruptive or eradication effects of test samples on established biofilms. For isolated compounds, Biofilm Prevention Concentration (BPC) and Minimum Biofilm Inhibition Concentration (MBIC) values were reported, corresponding to the lowest concentration achieving >90% biofilm inhibition in prebiofilm and postbiofilm assays, respectively. These values were defined based on proposed pharmacodynamic endpoints by Coenye (2023) while recognizing that standard interpretive criteria for biofilm assays are currently lacking, unlike the well-established MIC breakpoints as defined by CLSI.

A single test sample can exhibit multiple and/or distinct bioactivities. For instance, the *D. eschscholtzii* culture extract obtained from rice media incubated for 15-

days displayed strong prebiofilm inhibition, moderate postbiofilm activity, but no measurable antibacterial effect against MRSA (Table 6.1). Meanwhile, the cytochalasins possessed strong antibacterial and prebiofilm activities (Table 6.20) only. These differences suggest that the active compounds within each fungal extract may act through specific mechanisms of action. Antibiofilm agents are known to interfere with various stages of biofilm formation including peptidoglycan degradation, biofilm matrix disruption, inhibition of quorum sensing, prevention of surface adhesion, or interference with bacterial cell division, to name a few (Roy et al., 2018; Srinivasan et al., 2021). Hence, elucidating the specific mechanism(s) underlying the observed bioactivities could provide deeper insights into the therapeutic potential of these metabolites and guide their optimisation as antibiofilm agents.

The antibiofilm activity of the test samples was assessed using a resazurin-based microtiter plate assay. Biofilm viability was measured to calculate its % inhibition. There are different methods to quantify biofilm with respective advantages and limitations (Azeredo et al., 2017). For example, crystal violet staining is a widely adopted method for but comes with several limitations. Particularly, it measures only total biomass without providing information on cell viability. In contrast, redox-based dyes such as resazurin, offer a more informative measure of biofilm viability (Sandberg et al., 2009). Resazurin is a non-fluorescent blue dye that is reduced by metabolically active cells into resorufin, a highly fluorescent compound, thus serving as an indicator of metabolic activity within the biofilm (Guerin et al., 2001). As noted by Coenye (2023), although no standardized protocols currently exist for biofilm susceptibility testing, viability-based methods such as resazurin-based assays, ATP quantification, or plate count methods are increasingly preferred over biomass-only approaches like crystal violet staining. Therefore, the use of a resazurin-based assay in this study aligns with these emerging recommendations for more accurate evaluation of antibiofilm activity.

7.2.3 Bioactivity loss during compound purification: challenges during isolation work

A notable and rather persistent observation across this study was the loss of biological activity following chromatographic purification across multiple fungal extracts. For instance, while the fraction XM2 derived from *X. cubensis* exhibited moderate postbiofilm inhibition in MRSA (Table 4.4, Figure 4.8), activity was markedly diminished after the successive purification step. A similar trend was observed with fractions M3, M4, and M7 obtained from *D. eschscholtzii* cultivated on rice (Figure 6.9, Table 6.4, Table 6.10). Furthermore, fraction DSM9 derived from *D. eschscholtzii* grown in PDB with SBHA also showed reduced or loss of prebiofilm activity after chromatographic fractionation or purification steps (Figure 6.24, Table 6.14, Table 6.20). Purified compounds from extracts of *H. lateripigmentum* grown in PDB were also found inactive against *P. aeruginosa*, despite that their parent extract and fractions exhibiting substantial effects against the microorganism. These results suggest that the purification process may inadvertently disrupt synergistic interactions or degrade some bioactive components, ultimately leading to diminished or completely lost of bioactivity.

Such observations reflect a broad challenge in natural products research. Crude extracts often comprise complex mixtures of chemically diverse constituents. The traditional and "gold standard" of natural product discovery, bioassay-guided fractionation, aims to isolate bioactive compounds by sequentially testing subfractions for activity until pure compounds are obtained. This approach has successfully led to the discovery of several clinically important drugs, including camptothecin and taxol (Wall & Wani, 1996). Despite numerous success, several studies have emphasized that some observed bioactivities arise from complex mixtures of compounds exhibiting additive, synergistic, or antagonistic effects (Stermitz et al., 2000; Vaou et al., 2022; Wagner & Ulrich-Merzenich, 2009). The loss of activity upon fractionation, therefore, represents a significant challenge in the natural products drug discovery pipeline. Consistent with

these reports, this study also encountered such limitations, as noted in the previous paragraph.

In this study, metabolomics was subsequently applied after the first fractionation to characterize fungal extract composition, identify the target metabolites, and prioritisation of fractions for purification (as discussed in Section 7.1.3). While this strategy provided a comprehensive chemical context for interpreting bioactivity trends and effective prioritisation tool, several cases still exhibited loss of activity, highlighting the need for more integrative strategies that go beyond compound identification.

The concept of synergy-directed fractionation, as demonstrated by Junio et al. (2011), further supports the idea that some natural product activities arise from non-linear interactions among their individual components. Their work with *Hydrastis canadensis* showed that neither the major nor minor compounds were active on their own, but combinations produced significant biological effects (Junio et al., 2011). A similar situation may explain some of the activity losses observed in this study, particularly for extracts like XM2, and the compounds from *H. lateripigmentum*.

7.2.4 Bioactive metabolites from Philippine ELF: Antibiofilm activity and cytotoxicity

Chromatographically purified and semi-purified compounds were finally tested in the above-mentioned biological assays and were reported on several tables (Table 4.10, Table 5.11, Table 5.18, Table 6.10, Table 6.20). The compounds with notable antimicrobial and/or antibiofilm activities were mellein and phloroglucinol from *X. cubensis*; TAN-1612, daldinone A and B/E from *H. lateripigmentum*; and BNT, orthosporin, and cytochalasin derivatives from *D. eschscholtzii*. The cytotoxicity to Human Embryonic Kidney (HEK) 293 cells of the bioactive compounds were evaluated. The results are summarized in Table 7.1.

The antibiofilm compounds were evaluated for cytotoxicity using HEK293 cells, a well-characterized, non-cancerous human kidney-derived cell line commonly employed in *in vitro* toxicological studies (Y. Chen et al., 2019; Roma et al., 2023; Stepanenko & Dmitrenko, 2015). This early screening assay provides critical insight into potential safety risks (Dorato & Buckley, 2007), enabling the identification and elimination of toxic compounds prior to preclinical translation. As shown in Table 7.1, compounds such as BNT and some cytochalasin derivatives exhibited low IC_{50} values, indicating cytotoxicity despite their antibiofilm effects against MRSA. In contrast, TAN-1612, mellein, phloroglucinol, daldinone A, and daldinone B did not demonstrate any toxicity toward HEK293 cells ($IC_{50} > 200 \mu M$).

Table 7.1 Cytotoxicity of selected bioactive compounds from Philippine ELF

Sample code	Identity, %purity	Bioactivities against MRSA	HEK293 cell % inhibition @100 µg/mL ^a	IC ₅₀ against HEK293 cells (µM) ^b
XM1M	mellein, 80%	Strong prebiofilm and post biofilm activity	-6.16	>200
XM4.4	phloroglucinol, 100%	Strong antibacterial and prebiofilm activity	3.04	>200
HP	TAN-1612, 100%	Strong antibacterial, prebiofilm and post biofilm activity	26.66	>200
LM3.1	daldinone A, 100%	Strong antibacterial and prebiofilm activity	5.61	>200
LM5.3	daldinone B, 85%	Strong prebiofilm activity	44.67	>200
DSM2	BNT, 100%	Strong antibacterial and prebiofilm activity	91.78	112.7
DSM 4.6	orthosporin, 60%	Strong antibacterial and prebiofilm activity	94.07	>200
DSM 4.5	cytochalasin 11, 100%	Strong antibacterial and prebiofilm activity	93.98	113.10
DSM 5.1	cytochalasin 9, 100%	Strong antibacterial and prebiofilm activity	74.37	>200
DSM7	cytochalasin 1, 100%	Strong antibacterial and prebiofilm activity	86.34	99.05
DSM9.3	cytochalasin 10, 100%	Weak prebiofilm activity	78.25	110.8
DSM 8.4	cytochalasin 2, 100%	Inactive	6.91	>200
Triton X (positive control)			93.23	11.60
DMSO (control)			0.00	>200

Moderate inhibition: 50-70% (coloured in blue); Strong inhibition >70% (coloured in red);
^aassay was done twice with two replicates
^bIC₅₀ was computed from eight concentrations ranging from ca 2-200 µM.

Mellein, isolated from *Xylaria cubensis*, exhibited notable prebiofilm and postbiofilm inhibition against MRSA, with >70% inhibition observed at the highest tested concentration (100 µg/mL). It was not evaluated against *P. aeruginosa* due to the lack of activity of the parent fractions against the test strain that is consistent with previous reports indicating its minimal antivirulence and antibiofilm activity against *Pseudomonas* species (Zhou et al., 2017). Nonetheless, its MRSA-specific antibiofilm inhibition

supplements previously documented biological activities such as phytotoxicity (Parisi et al., 1993), cytotoxicity against leukaemia cell lines (Tawfike et al., 2019), ixodicidal activity (Morales-Sánchez et al., 2021), among others as listed in a recent comprehensive review (Reveglia, Masi, & Evidente, 2020).

On the other hand, the congener 4-hydroxymellein, isolated also from *X. cubensis*, did not inhibit biofilm formation suggesting that hydroxylation at C-4 diminishes bioactivity. Interestingly, a related derivative, 6-methoxymellein, was reported to inhibit biofilms formed by the dermatophytic fungus *Trichophyton rubrum* (Hagag et al., 2024). This observation points to the potential for C-6 modification, *i.e.*, methoxylation, to enhance mellein's antibiofilm properties, particularly against MRSA, while maintaining low cytotoxic profile as indicated in the HEK293 viability assays (Table 7.1). These findings warrant further structure–activity relationship (SAR) studies to explore mellein derivatives as potential antibiofilm agents.

Phloroglucinol demonstrated strong antibacterial inhibition of 93.80% against MRSA at the highest testing concentration of 100 µg/mL. This result agrees with the reported MIC of phloroglucinol of 10.24 mg/mL against the same MRSA strain (Shen et al., 2023). Furthermore, it also displayed strong prebiofilm inhibition of 98.69%. It also exhibited no toxicity against HEK293 which suggested that this compound is another good candidate as an antibiofilm agent. These findings contributed to the diverse bioactivities of phloroglucinol and its derivatives (Khan et al., 2022; Peron et al., 2024; Singh et al., 2010).

TAN-1612 is the only compound in this study that demonstrated both significant antibacterial and prebiofilm inhibition activity against MRSA. As shown in Table 5.11, it exhibited MIC and BPC of 13.83 µM and 12.50 µM, respectively—the lowest among all tested compounds. Although these values are not as low as those of the positive control, TAN-1612 stands out as a lead structure for further optimisation, particularly because it

was non-toxic to normal cell lines (Table 7.1). TAN-1612 is structurally related to the well-established tetracycline class of antibiotics (structures illustrated in Figure 7.1). Hence, the tetracycline core must be a key pharmacophore to exhibit antimicrobial properties. A tautomeric form of TAN-1612 at C-3 and C-5, BMS-19258, has been reported to exhibit neuropeptide Y and neurokinin-1 receptor antagonist activity (Kodukula et al., 1995; Shu et al., 1995). A recent patent (Strauss et al., 2024) described a carboxamide derivative (at C-18) of TAN-1612, known as TAN-1652 (or athrotainin), which also demonstrated similar MIC against *S. aureus* and showed no cytotoxicity against HEK293, Caco-2, and KB3-1 cell lines. This study therefore identifies TAN-1612 as a potent, non-toxic, antibacterial and antibiofilm compound derived from *H. lateripigmentum*. As discussed in Chapter 5 (Section 5.3.4.1), *H. lateripigmentum* is a highly efficient producer of TAN-162, with a titre of 170 mg/L in PDB culture medium and significantly surpassed the reported titres attained by engineered microbial hosts (Baldera-Aguayo, Lee, & Cornish, 2022; Li et al., 2011). These findings position TAN-1612 as a promising lead compound for the development of next-generation antibiotics targeting MRSA, combining biofilm inhibition, non-toxicity, and high production efficiency in *H. lateripigmentum*.

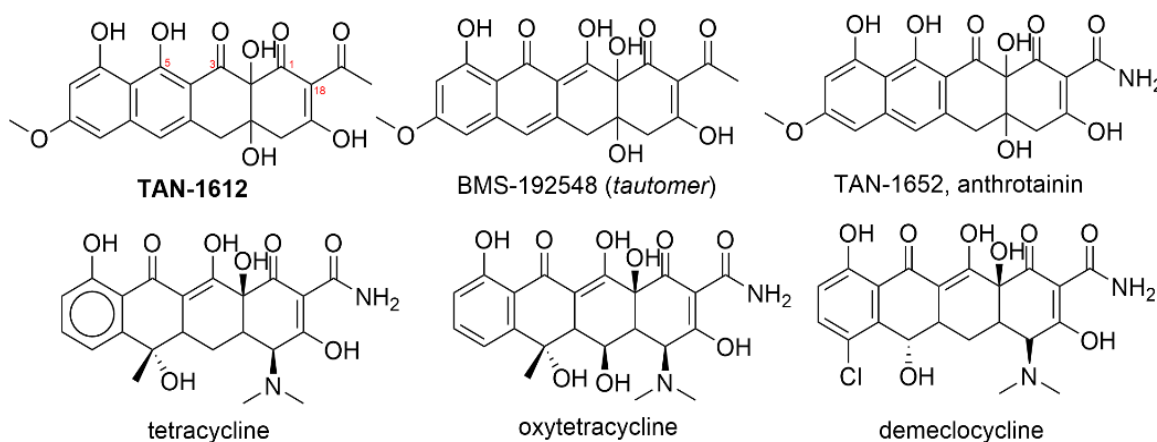


Figure 7.1 Chemical structure of TAN-1612 and related tetracycline antibiotics

Daldinone A, along with a mixture of daldinone B and E obtained from *H. lateripigmentum* cultured in MEB demonstrated strong antibacterial and pre-biofilm inhibition activity against MRSA at the highest tested concentration of 100 µg/mL (Table

5.18). Importantly, these compounds did not exhibit toxicity against HEK293 cells (Table 7.1). Further purification of the daldinone B/E mixture is warranted to identify the specific analogue responsible for the observed bioactivity. Previous studies have reported the antimicrobial properties of daldinone A against *Bacillus subtilis* and *Yarrowia lipolytica* Stadler et al. (2007) and a 20 mm inhibition zone against *P. aeruginosa* (Souza et al., 2024). Furthermore, daldinone B and E displayed radical scavenging activities but showed no substantial activities against MRSA and *Klebsiella pneumoniae*, and no cytotoxicity against human pancreatic carcinoma (MIA PaCa-2) cell line (Du, King, & Cichewicz, 2014). Daldinones A and B were also reported to show moderate nematocidal activities (Quang, Hashimoto, & Asakawa, 2006). The findings from the current study underscore the previously unreported antibiofilm potential of daldinone analogues and support their further investigation as candidate antimicrobial agents.

BNT was identified as a major metabolite of *D. eschscholtzii* cultivated in PDB supplemented SBHA. It possessed strong antibacterial and prebiofilm activities against MRSA with an MIC of 20.22 μ M and BPC of 39.31 μ M. Interestingly, BNT displayed an unusual U-shaped dose-response curve (refer to Table S 1) for its antibacterial activity wherein it exhibited strong inhibition at lower concentrations and moderate activity at higher concentrations. This phenomenon is called hormesis and be graphically seen as either an U-, J-, or inverted U-shaped dose response curves (Calabrese et al., 2010). Reports suggested that hormesis is an adaptive mechanism of bacteria to strengthen their resistance to antibiotics (Iavicoli et al., 2021) and such phenomenon can be observed on known antibiotics such as ciprofloxacin (Linares et al., 2006). Despite the potency, it caused weak toxicity to HEK293 with an IC₅₀ of 112.1 μ M (Table 7.1). These are previously unreported bioactivities of BNT. Beyond its antimicrobial and antiproliferative effects, BNT has also been reported to possess antioxidant properties (Quang et al., 2004) and protective effects to pancreatic β -cells against apoptosis (Lee et al., 2019). These findings present BNT as a bioactive yet a potentially cytotoxic fungal metabolite, suggesting that

structural modification or targeted delivery strategies may be necessary to harness its antibacterial potential while minimizing off-target toxicity.

DSM4.6, a partially purified fraction containing approximately 60% orthosporin, exhibited strong antibacterial and pre-biofilm inhibition activity against MRSA (Table 6.20). It remains uncertain whether the observed bioactivities can be attributed solely to orthosporin, as the fraction also contained a cytochalasin derivative. The pure cytochalasins obtained in the other fractions have found to exhibit similar biological activities, suggesting that the cytochalasin impurity in DSM4.6 may have contributed to the results. Purification of DSM4.6 was not pursued due to low yield at 11mg with still at least 2 compounds to isolate. As such, additional material is needed to enable further purification and accurately identify the compound(s) responsible for the observed activity. Orthosporin has been widely studied, and along with its 6-methoxy derivative, diaporthin, is known for its phytotoxic properties (Harris & Mantle, 2001). Both compounds, isolated from *Diaporthe terebinthifolii* LGMF907, exhibited bacteriostatic effects against a panel of pathogens including MRSA (de Medeiros et al., 2018). However, several studies reported that orthosporin did not display any antimicrobial activities (Barnes et al., 2016; Wu et al., 2019; X.-Q. Zhang et al., 2020). These contradictory findings support the need for further investigation into the antimicrobial potential of orthosporin using a pure compound in standardized testing conditions.

Three cytochalasins (cytochalasin 1, 9, and 11) from *D. eschscholtzii* were also found to have strong antibacterial and prebiofilm inhibition activity at the highest concentration (Table 6.20). Cytochalasins 2 and 10 showed very weak antibacterial activity, with cytochalasin 10 also being inactive in the prebiofilm assay. A detailed comparison of their structures suggests that the presence of a double bond between C19 and C20 within the macrocyclic ring is critical for biofilm inhibition, as cytochalasin 2, which lacks this feature, was inactive. This hypothesis could be further substantiated by evaluating cytochalasin 7, another analogue without the C19–C20 double bond; however,

it was not tested due to limited availability (4 mg). The substituent at C-12 also seems relevant: while the presence of either an exomethylene (cytochalasin 1) or a methyl group

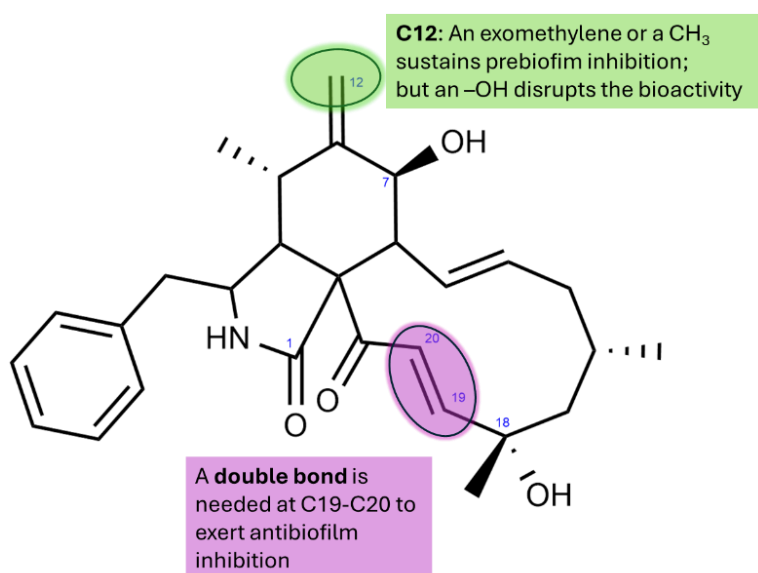


Figure 7.2 A cytochalasin structure highlighting important structural features to inhibit biofilm formation of MRSA

(as in cytochalasins 9 and 11) did not weaken the activity, the introduction of a hydroxyl group at C-12 (as in cytochalasin 10) appeared to reduce biofilm inhibition. Figure 7.2 depicts the cytochalasin scaffold and highlights probable pharmacophores based on this analysis. Nevertheless, more comprehensive studies including a broader set of structural analogues of cytochalasins are necessary to confirm these preliminary hypotheses.

Several studies have examined the biofilm inhibition of cytochalasin analogues. For instance, Yuyama et al. (2018) evaluated 21 cytochalasin derivatives (including the cytochalasins 1, 9, 10, and 11) using a crystal violet-based antibiofilm assay against *S. aureus* (strain: DSM1104). Their results are consistent with the current study: cytochalasin 1 showed the most potent activity, achieving 85% biofilm inhibition at 254 µg/mL, quite similar to our findings. A comparable trend was observed for cytochalasin 9. The study also noted the importance of the olefinic bond at C19-C20 as they observed poor

inhibition when an epoxide was in that position. However, in contrast to the present results, the authors reported weak activity for cytochalasin 11 and stronger activity for cytochalasin 10. These discrepancies may be attributed to differences in bacterial strain or biofilm quantification methods, highlighting the need for further validation among these assays.

As shown in Table 7.1, the cytochalasins exhibited weak antiproliferative activities against HEK293 cells with IC_{50} ranging from 90-120 μ M. Cytochalasin 2 did not exhibit any toxicity which further supports the hypothesis that the olefinic bond between C19 and C20 is crucial for biological effects. Cytochalasins are expected to display toxicity because they are known to interfere various cellular processes by disrupting actin filaments. The name cytochalasin was derived from Greek words “kytos” and “chalsis” which means “cell” and “relaxation”, respectively, referring to this mode of action (Cooper, 1987). Interestingly, many cytochalasins exhibit higher cytotoxicity toward cancer cells than to normal cells (Gao et al., 2018; Zhu et al., 2015). Hence, it will be sensible to evaluate the toxicity of these analogues to several cancer cell lines. If similar selectivity is observed with the current derivatives, these cytochalasin analogues may represent promising candidates for anticancer drug development.

Taken together, the observed antibacterial, antibiofilm, and potential cytotoxic effects of these cytochalasins contribute to their expanding pharmacological profile. Cytochalasins have been reported to possess a diverse range of bioactivities, including cytotoxic, antiviral, immunosuppressive, nematocidal, and antimicrobial properties (Scherlach et al., 2010; Tammam et al., 2025; Zhu et al., 2021). The findings of this study thus support their continued exploration as multi-functional therapeutic agents and provide a foundation for further SAR-based optimisation and development.

7.3 Summary and Conclusions

The primary aim of this study is to discover antibiofilm agents from Philippine ELF by combining metabolomics, OSMAC, and targeted purification techniques using high-throughput chromatography. Compounds that can inhibit biofilm were specifically sought due to the critical role of bacterial biofilms to confer protection against antibiotic treatments, thereby contributing to the development of drug resistance. Philippine ELF were tapped as a source of antibiofilm compounds, given the limited number of studies that have systematically explored their potential for drug discovery particularly at combating bacterial biofilms.

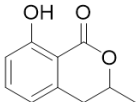
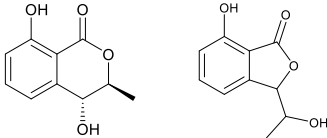
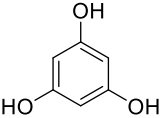
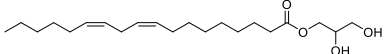
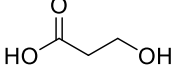
This study highlights the power of integrating metabolomics with natural products bioprospecting to accelerate the discovery of bioactive secondary metabolites. A systematic workflow has been applied beginning with strain prioritisation, followed by OSMAC approach, scale up, targeted isolation, and biological assays to arrive at a diverse range of fungal metabolites with promising antibacterial and antibiofilm properties, particularly against MRSA.

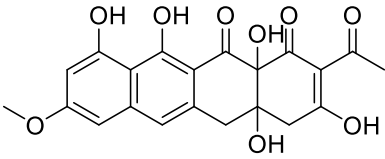
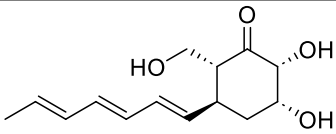
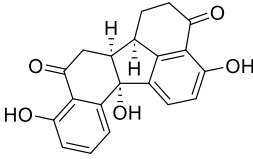
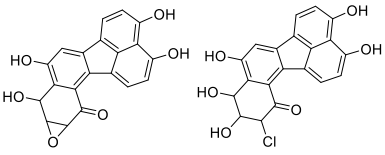
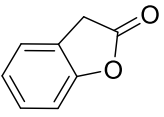
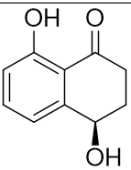
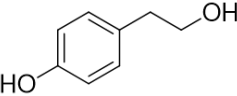
Metabolomics played a central role throughout this research study. In the preliminary screening stage (Chapter 3), ^1H -NMR and LC-MS-based metabolomic profiling effectively prioritised three ELF strains based on their chemical diversity with associated bioactivity potentials. These were identified using molecular methods as *Daldinia eschscholtzii*, *Hypoxylon lateripigmentum*, and *Xylaria cubensis*.

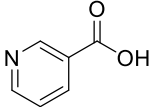
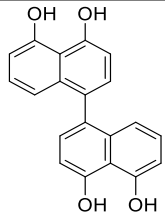
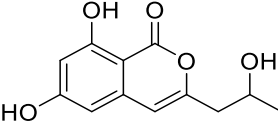
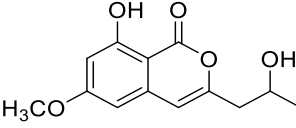
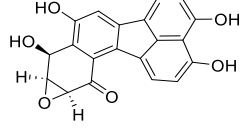
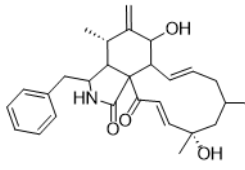
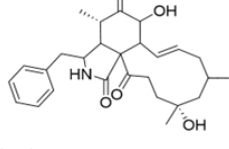
The OSMAC strategy, incorporating variation in culture media, incubation periods, and chemical elicitors, further expanded the chemical space of these strains which allowed the identification of optimal culture conditions for secondary metabolite production. Several metabolite classes with antibiofilm activities were uniquely produced under specific culture conditions, demonstrating the influence of growth conditions on fungal metabolic expression. For *X. cubensis*, cultivation in 30-day rice

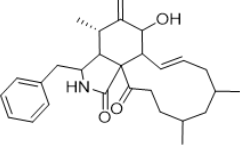
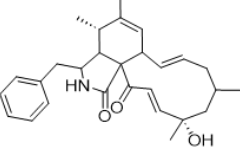
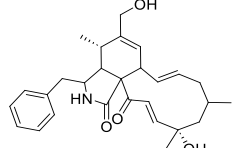
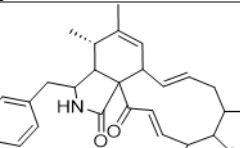
media resulted in the highest bioactivity and chemical diversity. It also afforded phloroglucinol, produced in different media, and of which we are reporting for the first time as a fungal metabolite. TAN-1612 and 2,3-didehydropalitantin were generally detected in the potato dextrose broth (PDB) and malt extract broth (MEB) cultures of *H. lateripigmentum*, albeit with varying yields. Additionally, 3-hydroxypropionic acid was specifically produced in its PDB culture, whereas daldinones A, B, and E were found exclusively in MEB cultures. These compounds were previously unreported in *H. lateripigmentum*. Below in Table 7.2 is a summary of the isolated compounds from respective ELF's investigated with their structures and bioactivity.

Table 7.2 Summary of isolated compounds from ELF and their chemical and biological profiles

Source and growth media	Isolated compound Structure, conventional name, molecular weight	First report in the Genus (Yes or No?)	Observed Bioactivities
<i>X. cubensis</i> grown in rice, 30 days, 27 °C	 mellein, 178.18 g/mol	No	Strong prebiofilm and post biofilm activity, non-cytotoxic
	mixture:  4-hydroxymellein, 194.10 g/mol isobenzofuranone, 194.10 g/mol	No	inactive
	 phloroglucinol, 126.03 g/mol	Yes	Strong antibacterial and prebiofilm activity, non-cytotoxic
	 monolinolein, 354.28 g/mol	Yes	inactive
	 3-hydroxypropionic acid, 90.08 g/mol	Yes	inactive
<i>H. lateripigmentum</i> grown in PDB 15 days, 27 °C			

Source and growth media	Isolated compound Structure, conventional name, molecular weight	First report in the Genus (Yes or No?)	Observed Bioactivities
<i>H. lateripigmentum</i> grown in PDB 15 days, 27 °C and in MEB, 30 days, 27 °C	 TAN-1612, 414.10 g/mol	Yes	Strong antibacterial, prebiofilm and post biofilm activity, non-cytotoxic
	 2,3-didehydropalitanin, 252.14 g/mol	Yes	inactive
<i>H. lateripigmentum</i> grown in MEB, 30 days, 27 °C	 daldinone A, 336.10 g/mol	no	Strong antibacterial and prebiofilm activity, non-cytotoxic
	mixture:  daldinone B daldinone E 348.06 g/mol 384.03g/mol	no	Strong prebiofilm activity, non-cytotoxic
	 2(3H)benzofuranone , 134.13 g/mol	yes	inactive
<i>D. eschscholtzii</i> grown in rice, 30 days, 27 °C	 regiolone, 178.06 g/mol	no	inactive
<i>D. eschscholtzii</i> grown in rice, 30 days, 27 °C	 tyrosol, 138.07 g/mol	no	inactive

Source and growth media	Isolated compound Structure, conventional name, molecular weight	First report in the Genus (Yes or No?)	Observed Bioactivities
<i>D. eschscholtzii</i> grown in PDB + SBHA, 15 days, 27 °C	 niacin, 123.03 g/mol	yes	inactive
	 BNT or [1,1'-Binaphthalene]-4,4',5,5'-tetrol, 318.328 g/mol	no	Strong antibacterial and prebiofilm activity, weak cytotoxicity
	 orthosporin, 236.076 g/mol	no	Strong antibacterial and prebiofilm activity, weak cytotoxicity
	 diaporthin, 250.08 g/mol	no	inactive
	 daldinone B, 348.06 g/mol	no	Strong prebiofilm activity, non-cytotoxic
	 cytochalasin 1, 449.26 g/mol	no	Strong antibacterial and prebiofilm activity, weak cytotoxicity
<i>D. eschscholtzii</i> grown in PDB + SBHA, 15 days,	 cytochalasin 2, 451.26 g/mol	no	inactive

Source and growth media	Isolated compound Structure, conventional name, molecular weight	First report in the Genus (Yes or No?)	Observed Bioactivities
27 °C	 cytochalasin 7, 435.28 g/mol	no	<i>not tested due to limited amount</i>
	 cytochalasin 9, 433.26	no	Strong antibacterial and prebiofilm activity, non-cytotoxic
	 cytochalasin 10, 449.26	no	Weak prebiofilm activity, weak cytotoxicity
	 cytochalasin 11, 433.26 g/mol	no	Strong antibacterial and prebiofilm activity, weak cytotoxicity

However, the rice culture of *D. eschscholtzii* predominantly yielded inactive pure compounds such as 2(3*H*)-benzofuranone, tyrosol C, regiolute, and niacin (Table 7.2). Although regiolute was consistently amongst the identified bioactive target metabolites in the different stages of the study, it was deemed inactive in its purified form. Supplementation with the chemical elicitor suberoylbishydroxamic acid (SBHA) activated otherwise presumably cryptic genes, resulting in the production of 1,1'-binaphthalene-4,4',5,5'-tetrol (BNT), multiple cytochalasin derivatives, and daldinone B (Table 7.2). While these metabolites have been previously reported from unelicited cultures of *D. eschscholtzii*, this study is the first to demonstrate their induction under laboratory conditions in SBHA-treated PDB culture media. These findings underscore

the effectiveness of the OSMAC strategy in activating cryptic metabolite pathways in ELF and highlight its previously underutilized application in ELF drug discovery.

The targeted isolation of compounds employing a metabolomics-guided approach led to the identification of several antibiofilm agents, including TAN-1612, mellein, phloroglucinol, BNT, and three cytochalasin derivatives. Preliminary cytotoxicity screening against HEK293 cells was carried out to uncover their potential as safe therapeutic agents. Among them, TAN-1612 emerged as a particularly promising lead compound due to its potent antibacterial (MIC=13.85 μ M) and antibiofilm (BPC=12.50 μ M) activities combined with non-cytotoxicity. BNT also demonstrated strong antibacterial (MIC=20.22 μ M) and antibiofilm (BPC=39.31 μ M) activities, although it showed weak cytotoxicity toward HEK293 cells (IC₅₀=121.56 μ M). Furthermore, preliminary SAR analysis of the cytochalasin derivatives suggested that specific structural features such as macrocyclic double bonds and substitutions at critical positions may play modulate both antibiofilm activity and cytotoxicity. These findings offer a valuable starting point for the rational design or semi-synthetic studies of fungal-derived antibiofilm agents.

Despite these successes, the study also encountered challenges inherent in natural products research. A recurring limitation was the loss of bioactivity during purification, like the case of *D. eschscholtzii* cultured in rice, presumably due to the disruption of synergistic and/or additive interactions present in crude extracts. These findings reinforce the need for more integrative and systems-level approaches to capture and preserve biologically relevant compound interactions.

In conclusion, this PhD thesis demonstrated the feasibility and advantages of combining metabolomics, OSMAC, and targeted isolation strategies in natural products bioprospecting. This study marks the first report of several secondary metabolites isolated and identified from these Philippine ELF strains, highlighting their untapped

potential for bioactive compound discovery. The Philippine ELF strains investigated herein represent a rich and underexplored source of bioactive compounds. The insights gained not only expand the pharmacological profile of ELF-derived metabolites but also offer an operational workflow for future bioprospecting efforts targeting antimicrobial resistance and biofilm-associated infections.

7.4 Future works

Building on the insights founded from this work, several avenues for future research are proposed:

1. **Tandem MS/MS and molecular networking.** To improve the confidence of metabolite identification, tandem MS/MS analysis should be used. In particular, utilisation of Global Natural Products Social (GNPS) molecular networking (Wang et al., 2016) would enable the organisation of MS/MS spectra into molecular families, facilitate dereplication against community-curated spectral libraries, and enhance the prioritisation of potentially new ELF metabolites. This approach would provide a more robust framework for chemical annotation and strengthen the reliability of metabolomic-driven natural product discovery.
2. **Functional roles of ELF metabolites.** Beyond its therapeutic values and chemotaxonomic roles, secondary metabolites were mainly produced by fungi for several biological functions. For instance, melanin pigments like 1,8-dihydroxynaphthalene (the monomer of BNT) were produced by black fungi *Knufia petricola* and *Cryomyces antarcticus* for UV-protection (Catanzaro et al., 2024). Indeed, BNT, daldinones, and other pigments were stored in granules accumulating on the fruiting bodies of several *Hypoxylon* species growing in the environment (Kuhnert et al., 2021). Cytochalasins were presumed to exhibit non-

specific chemical defence systems in the fruiting bodies of the fungi belonging to Hypoxyloideae taxa (Stadler et al., 2006).

Laser Desorption Ionization-Mass Spectrometry Imaging (LDI-MSI) can be employed to locate cytochalasins, BNT, and other metabolites inside their lichen hosts. In a particular study, LDI-MS was used to spatially map the distribution of specialized metabolites in lichen reproductive and vegetative parts. Remarkably, metabolites known to deter snail herbivores were found to be concentrated in the reproductive structures, suggesting strategic compartmentalisation as a protection on these vital structures (Gadea et al., 2020). Hence, similar chemical ecology studies on ELF-host interactions may provide further insights into the natural roles of these metabolites (such as BNT for photoprotection, or cytochalasins for antibacterial defence) within the lichen thallus.

3. **Co-culture of ELF strains.** Cultivation of ELF with one another in a single vessel offers a promising strategy to further expand their secondary metabolite profiles, analogous to the OSMAC approaches employed in this study. Co-culture mimics natural microbial ecosystems by forming artificial microbial communities, which can trigger competitive or symbiotic interactions that activate otherwise silent biosynthetic gene clusters (Bertrand et al., 2014; Knowles et al., 2022; Peng et al., 2021). In the context of ELF, co-cultivation may simulate their native environment within the lichen thallus and provide insights into interspecies interactions through secondary metabolite production. This approach could uncover novel compounds or enhance yields of bioactive metabolites not observed in monoculture. This could also offer new perspectives on the metabolic contributions of ELF in lichen symbiosis.

Another option would be co-cultivation of the inactive ELF strains with MRSA. In this strategy, ELF strains will be challenged to compete with MRSA

pathogenicity through production of antagonistic secondary metabolites. From there, targeted isolation can be employed to afford previously untapped bioactive compounds from monoculture systems.

4. **Integration with other omics technologies.** To gain a more comprehensive understanding of ELF secondary metabolism, future research should integrate genomics, transcriptomics, and proteomics alongside metabolomics. Genomic analysis enables the identification of biosynthetic gene clusters (BGCs) responsible for secondary metabolite production, as well as genetic adaptations to environmental stimuli. Transcriptomics, in turn, provides insight into gene expression dynamics by identifying which BGCs are upregulated or downregulated under specific culture conditions (Verma et al., 2024). Proteomics provides another critical additional layer, revealing which biosynthetic enzymes are actually expressed and functionally active under given conditions. This can help distinguish between transcriptionally silent but translationally active pathways, or vice versa, and clarify post-transcriptional regulatory mechanisms.

BGCs responsible for the biosynthesis of TAN-1612 have been characterized in other fungal species (Baldera-Aguayo, Lee, & Cornish, 2022; Li et al., 2011). Hence, genomics or transcriptomics analyses could be applied to *H. lateripigmentum* to determine whether the same or alternative biosynthetic pathways are involved in this ELF. Likewise, transcriptomic profiling of *D. eschscholtzii* could clarify whether the BGCs associated with cytochalasin biosynthesis (Qiao, Chooi, & Tang, 2011; Tammam et al., 2025) are transcriptionally silent but activated only upon treatment with chemical elicitors. Consequently, proteomic profiling could determine whether the cytochalasin BGC is indeed translated into functional enzymes, complementing transcriptomic

data. Once BGCs were identified and confirmed in each ELF strain, targeted genetic manipulation including gene overexpression strategies can be employed to enhance the production of these specific antibiofilm metabolites while suppressing unwanted byproducts (gene knockout). Heterologous expression can be explored as well in which the BGCs will be introduced into a different organism that can be grown efficiently like *Escherichia coli* in large bioreactors (Ongley et al., 2013). These biotechnological techniques would make the transition from bench-scale to industrial-scale fermentation more feasible, cost-effective, and selective.

Collectively, a multi-omics framework would enable a more accurate and mechanistic understanding of secondary metabolism (Sahana et al., 2025), making the transition from bench-scale discovery to industrial-scale fermentation more feasible, cost-effective, and selective.

5. **Application of extensive metabolomics tools to investigate metabolite interactions in crude extracts.** Future studies could utilise recently developed comprehensive metabolomics tools to assess potential synergistic, additive, or antagonistic interactions among metabolites within complex mixtures. For example, selectivity ratio plots can be generated to correlate metabolite abundance with biological activity, thereby identifying potential synergists or additives within bioactive fractions (Britton et al., 2018). *Simplify* was another tool that can predict the interaction and additive effects of known active components in a mixture (Caesar et al., 2019). More recently, the same group introduced *Interaction Metabolomics* (Vidar et al., 2023), which removes the prerequisite of prior knowledge of structure or concentration of active compounds. This method uses Compound Interaction Terms (CITs), calculated as the product of ion intensities between all pairs of detected features, to statistically identify synergistic interactions directly from untargeted metabolomics data. This

approach was successfully applied to correlate the synergistic activity of capsaicinoids in *Capsicum chinense* habanero peppers (Vidar et al., 2023), demonstrating its potential to reveal hidden bioactive interactions in complex natural product mixtures. These emerging tools provide a suitable approach for systematically identifying functional interactions among metabolites, thereby enhancing the detection of biologically relevant compounds.

6. **Antibiofilm mechanistic studies.** Future work can include targeted mechanistic assays to elucidate the specific modes of action of the identified antibiofilm compounds, particularly TAN-1612. Potential mechanisms include peptidoglycan degradation, disruption of the extracellular biofilm matrix, inhibition of quorum sensing, interference with bacterial surface adhesion, or impairment of cell division (Roy et al., 2018; Srinivasan et al., 2021). Identifying the precise mode of action will be crucial for determining the clinical relevance and potential applications of these compounds. For instance, if TAN-1612 inhibits biofilm formation by preventing bacterial adhesion to surfaces, it could be developed as an antimicrobial coating for medical devices (Ramos et al., 2011). Additionally, previous studies have shown that certain cinnamic acid derivatives specifically inhibit quorum sensing in *Chromobacterium violaceum*, thereby enhancing its susceptibility to antibiotics such as tobramycin (Cheng et al., 2020). If TAN-1612 exerts a similar quorum sensing inhibition effect, it could potentially be used in combination with conventional antibiotics to overcome antibiotic resistance.
7. **SAR-guided semisynthetic modification of promising compounds.** Based on the preliminary SAR insights from this study, targeted chemical synthesis can be designed to enhance its biological effects. Several studies have reported similar

SAR analysis in cytochalasin derivatives extracted from fungal extracts and their respective antibiofilm activities (Lambert et al., 2023; Yuyama et al., 2018) but no semisynthesis has been pursued to date. A comprehensive SAR-guided semisynthetic studies can be done to optimise the bioactivity of cytochalasin derivatives including enhanced biofilm inhibition and reduced cytotoxic effects. Lastly, stereospecific synthesis should be done to validate the exact stereochemistry as well of the isolated cytochalasin derivatives.

8. **Expanded cytotoxicity profiling.** Future studies should extend cytotoxicity screening by incorporating a panel of cancer cell lines to evaluate potential selective anticancer activity, particularly for non-toxic metabolites such as TAN-1612. This is of interest given that other tetracycline-related compounds have demonstrated cytotoxic effects against acute myeloid leukaemia (HL-60) cells (Song et al., 2014). A similar approach could be applied to the cytochalasin derivatives identified in this study, as structurally related analogues have shown selective cytotoxicity toward various tumour cell lines (Gao et al., 2018; Zhu et al., 2015).

These directions represent logical extensions of the present work and hold promise for advancing both the scientific understanding and therapeutic applications of endolichenic fungal metabolites.

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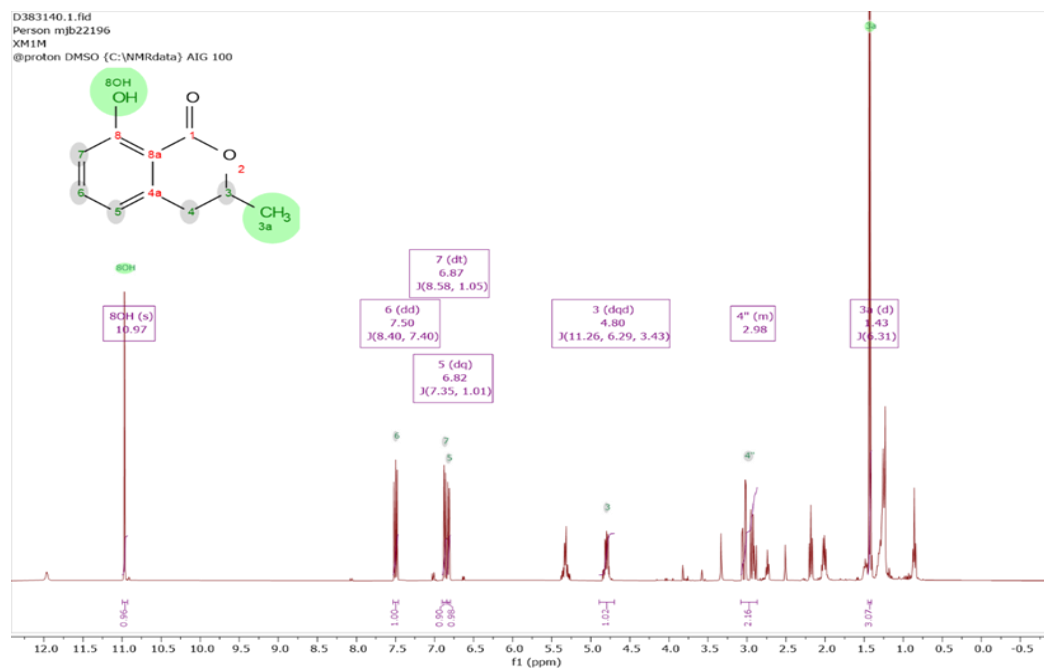
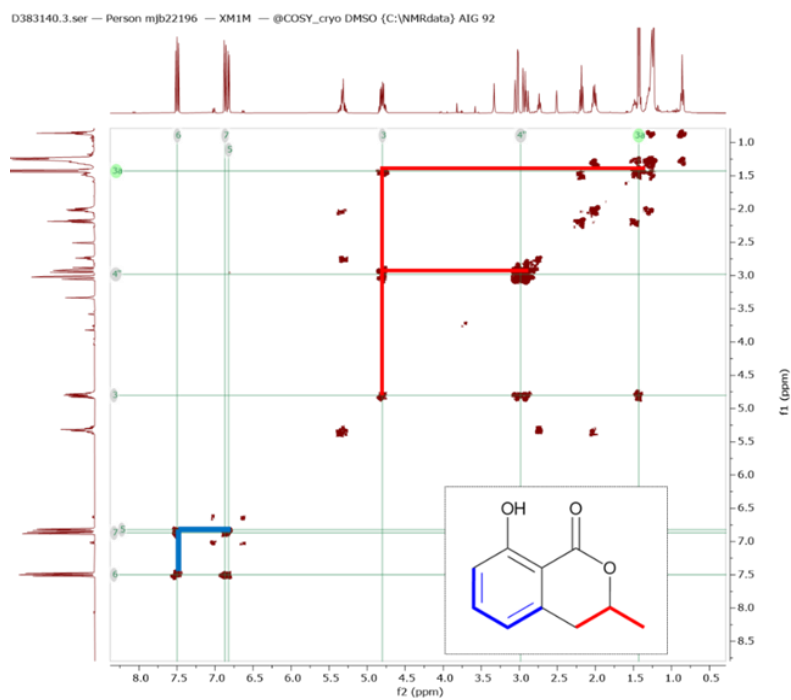
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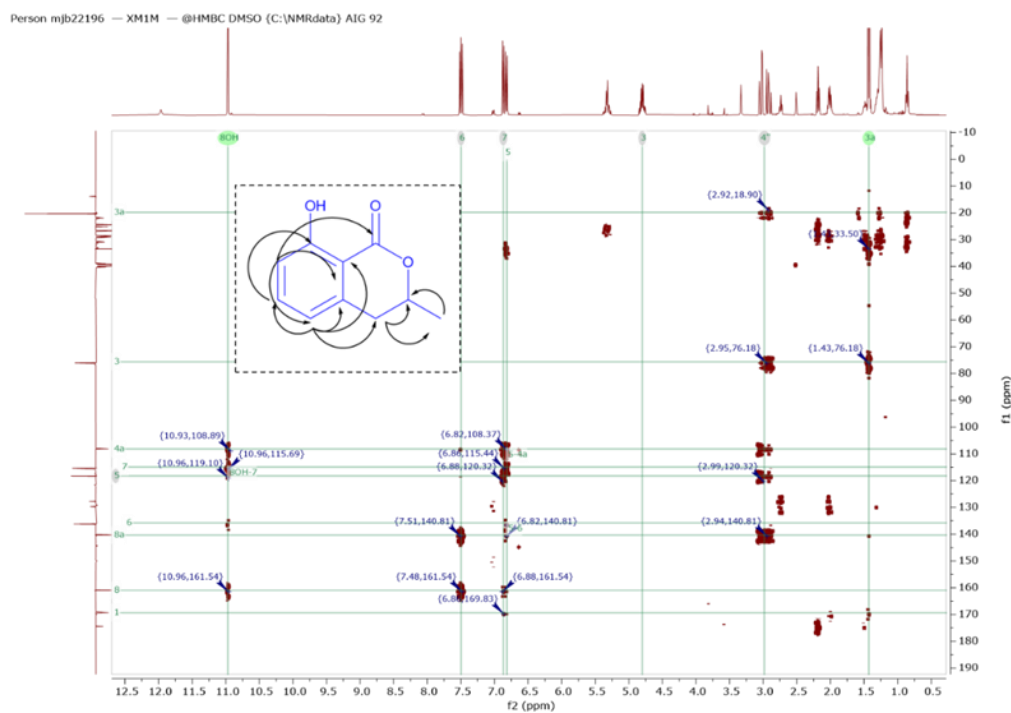
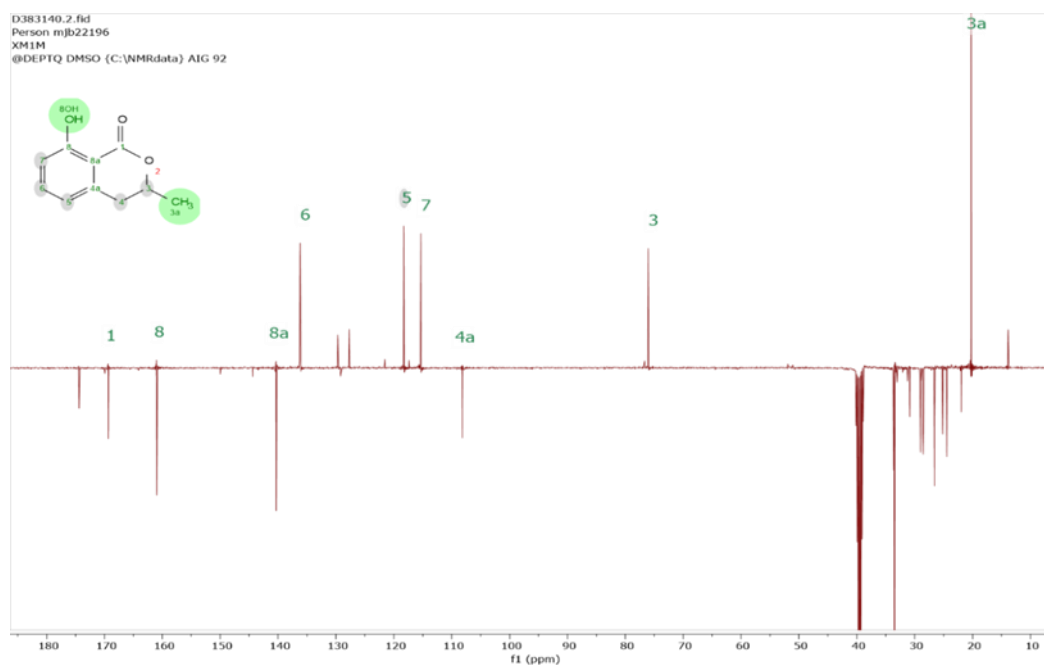
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Supporting Information

Figure S 1. ¹H-NMR spectra of XM1M as mellein (400MHz, DMSO-d₆)Figure S 2. COSY ¹H-NMR spectrum of XM1M as mellein



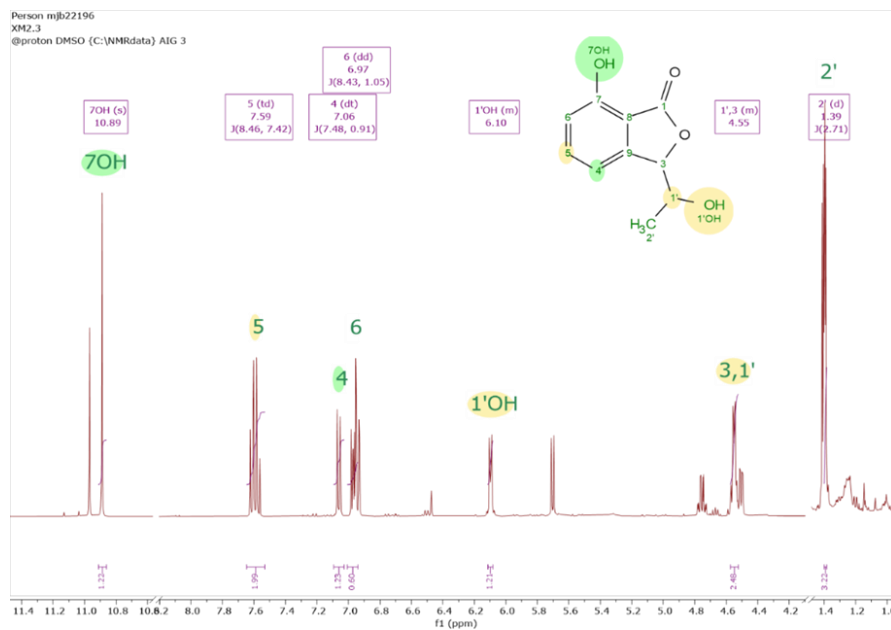


Figure S 6. ^1H NMR of XM2.3 showing the assignments for 7-Hydroxy-3-(1-hydroxyethyl)isobenzofuran-1(3H)-one. (400MHz, DMSO- d_6)

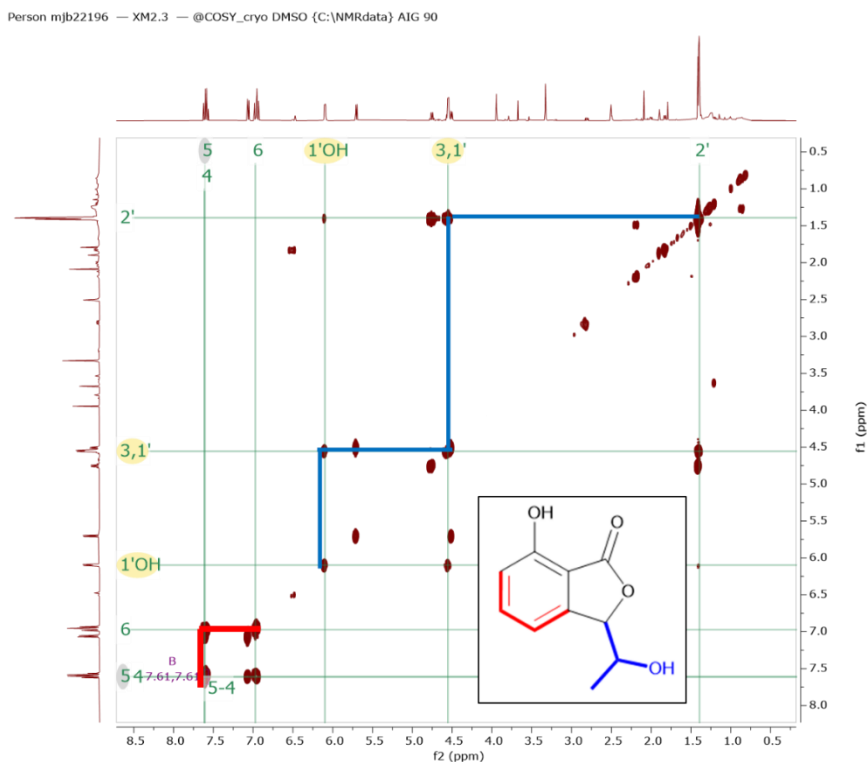


Figure S 5. COSY ^1H -NMR spectrum of 7-Hydroxy-3-(1-hydroxyethyl)isobenzofuran-1(3H)-one in XM2.3

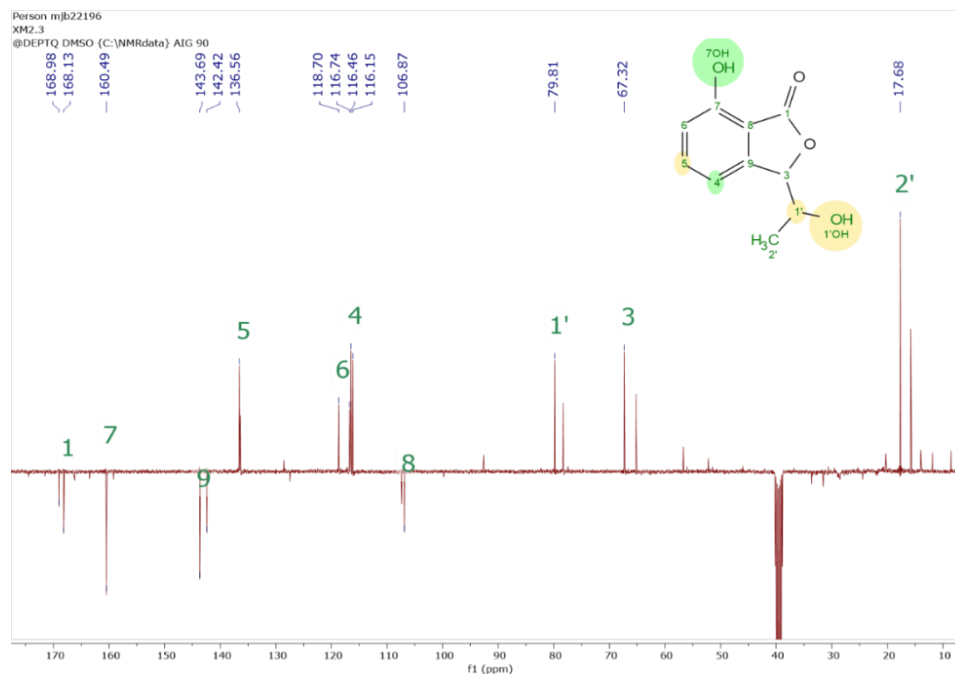


Figure S 7. ^{13}C -NMR of XM2.3 showing the assignments for the compound 7-Hydroxy-3-(1-hydroxyethyl)isobenzofuran-1(3H)-one. (100MHz, DMSO- d_6)

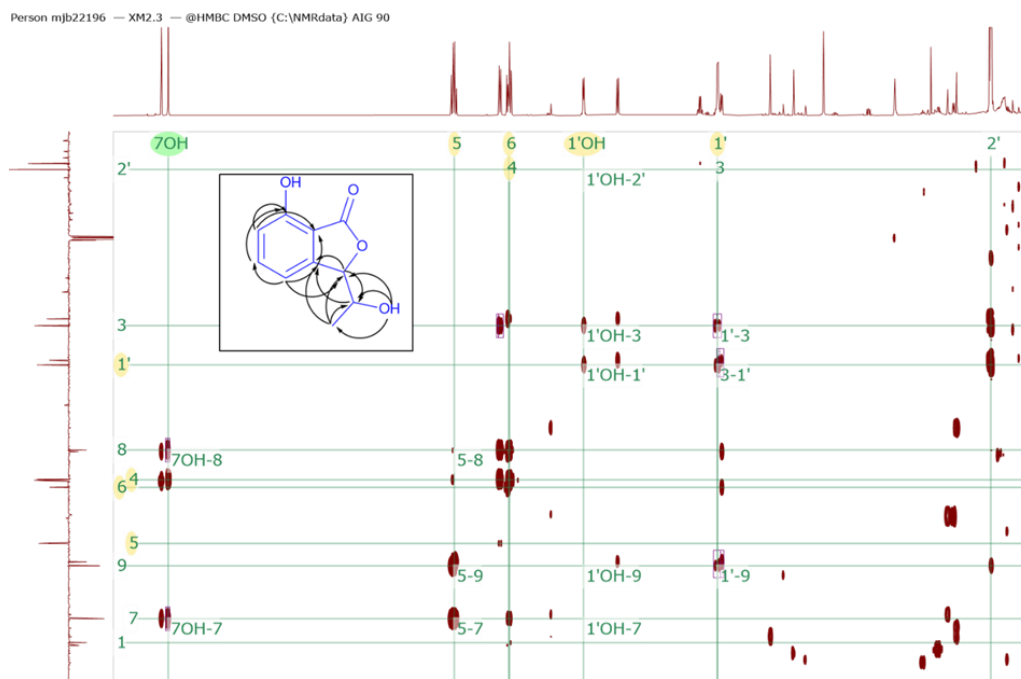


Figure S 8. HMBC of 7-Hydroxy-3-(1-hydroxyethyl) isobenzofuran-1(3H)-one in XM2.3

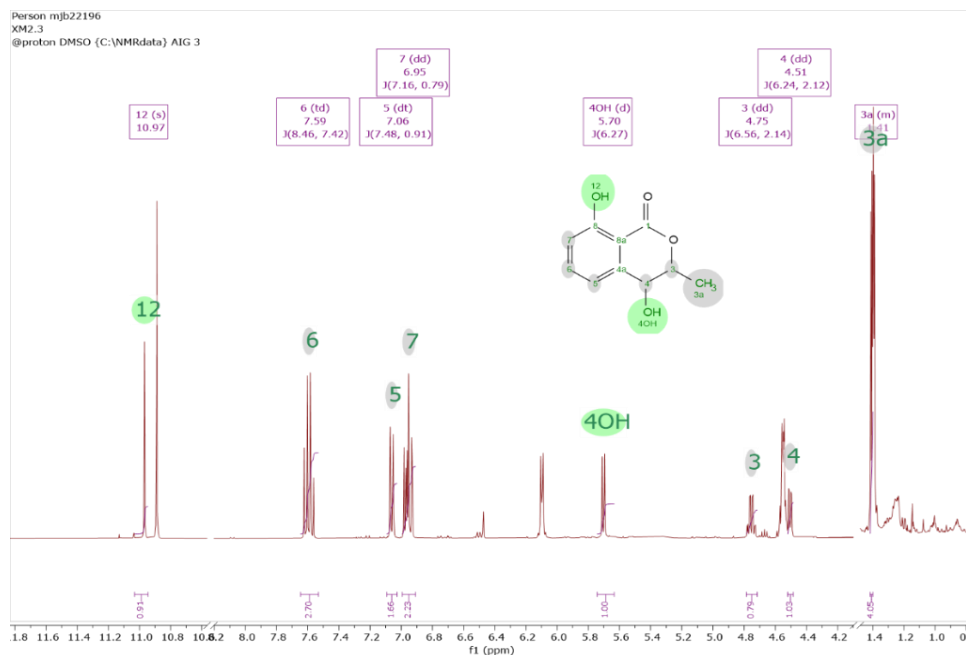


Figure S 9. ^1H -NMR of XM2.3 showing the assignments for 4-hydroxymellein (400MHz, DMSO- d_6)

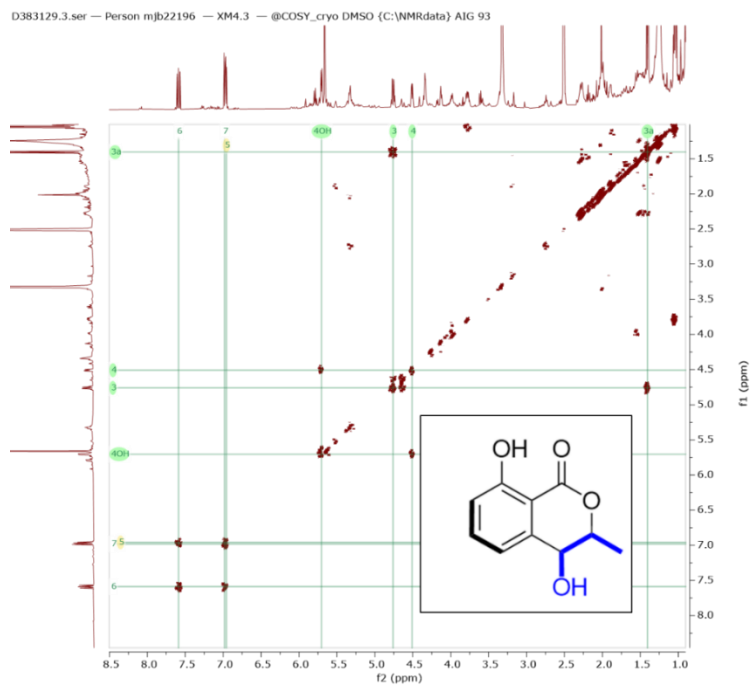


Figure S 10. COSY ^1H -NMR of 4-hydroxymellein (400MHz in DMSO- d_6)

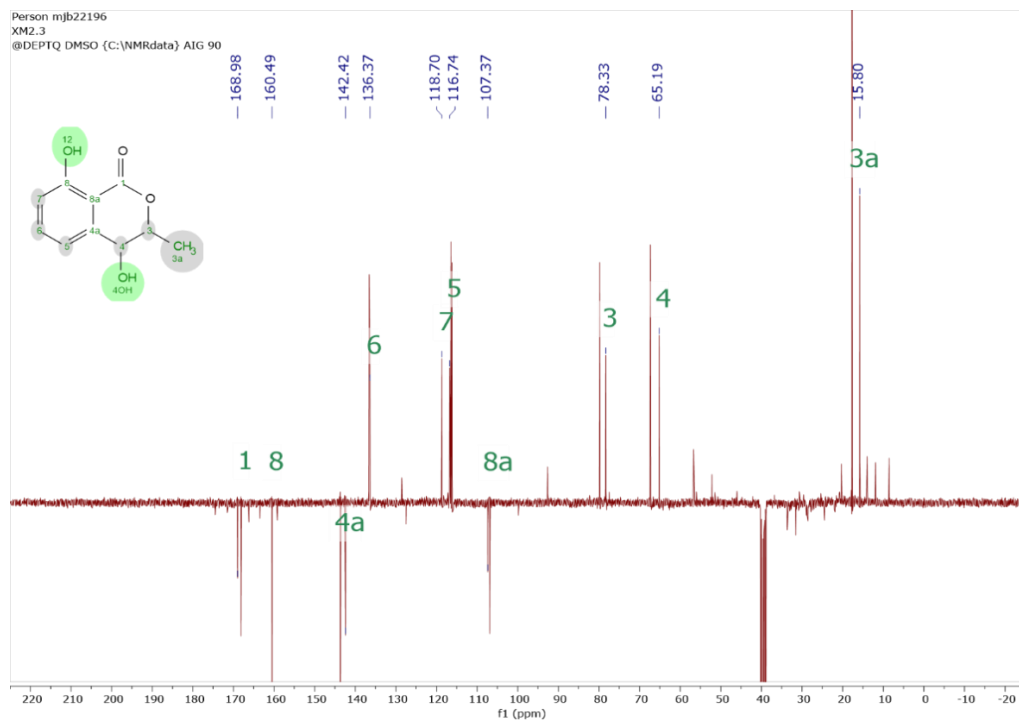


Figure S 12. DEPTQ ^{13}C -NMR spectra of XM2.3 showing the assignments for the compound **4-hydroxymellein**. (100MHz, DMSO-d₆)

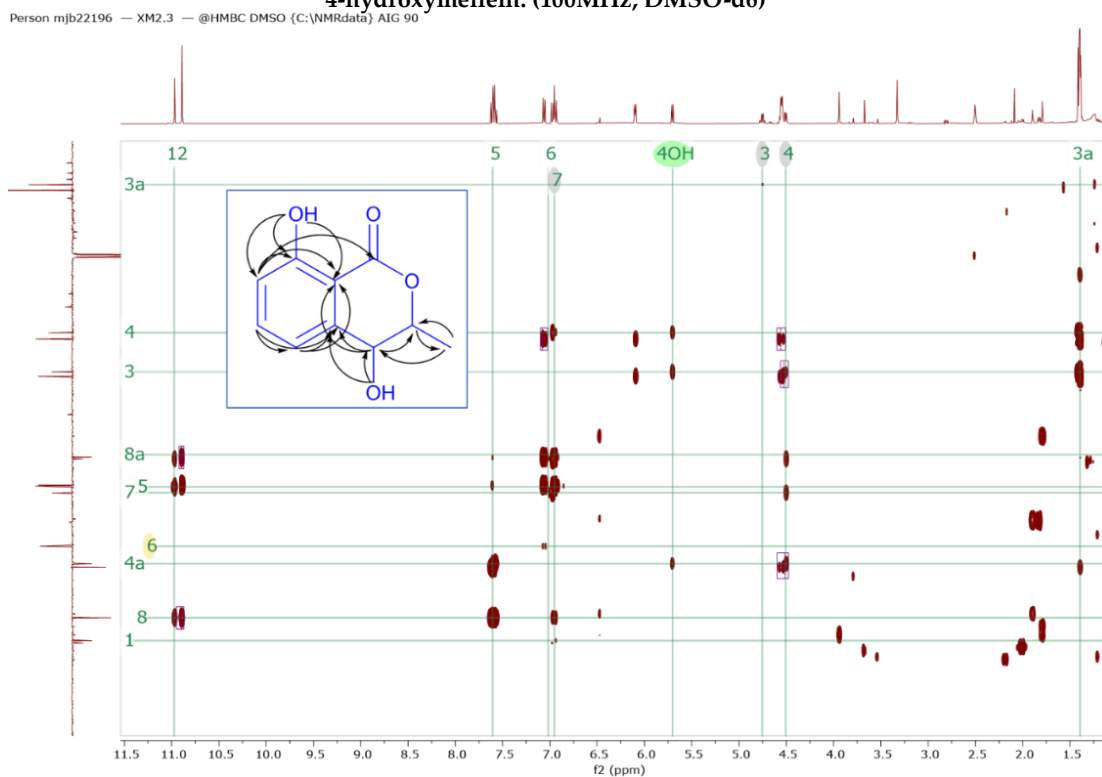


Figure S 11. HMBC of 4-hydroxymellein

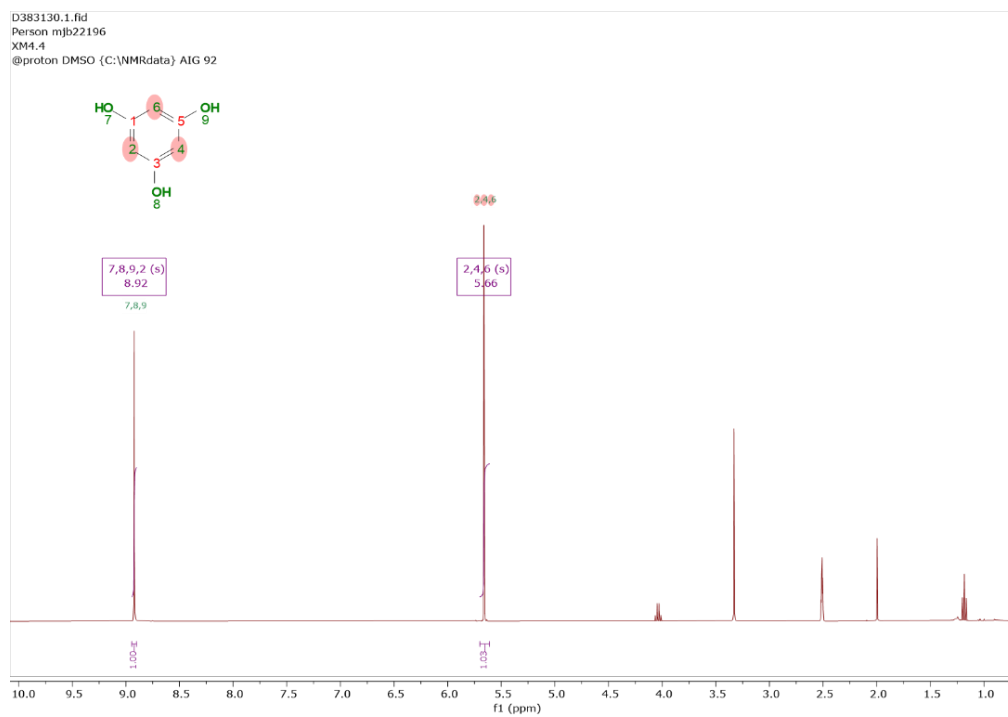


Figure S 14. ^1H -NMR of XM4.4 as phloroglucinol (400MHz, DMSO-d₆)

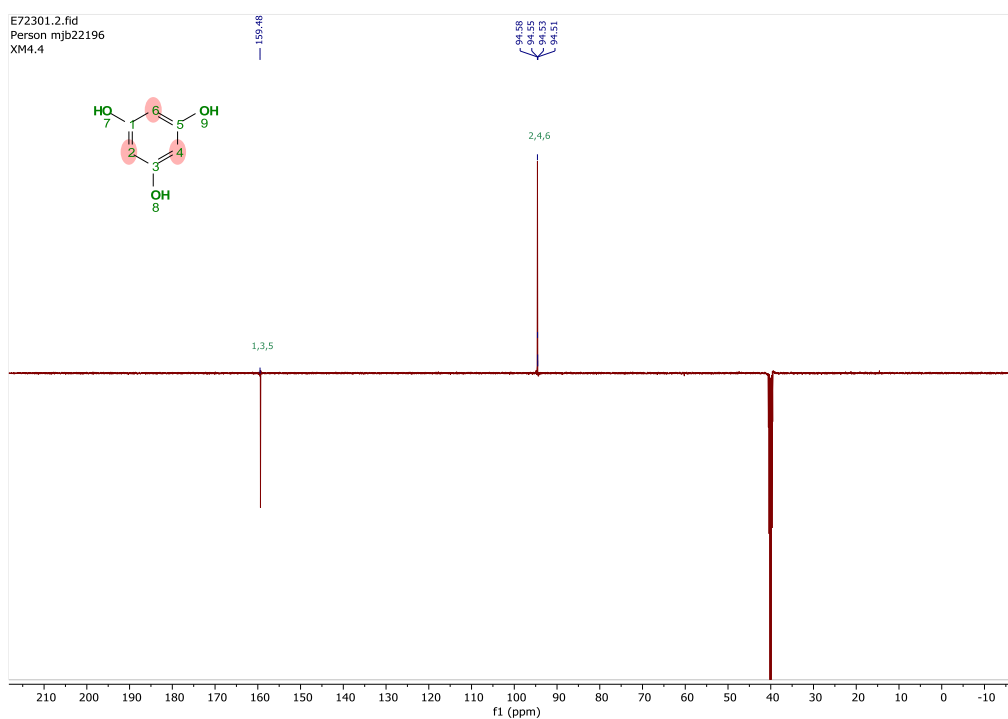


Figure S 13. DEPTQ ^{13}C -NMR spectra of XM4.4 as phloroglucinol. (100MHz, DMSO-d₆)

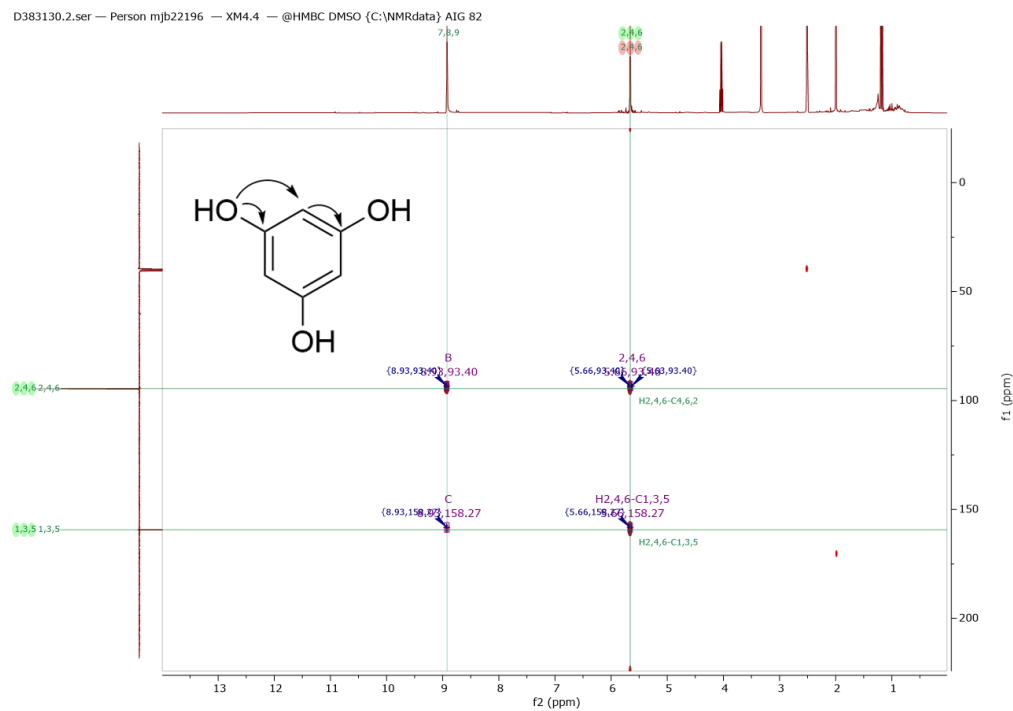
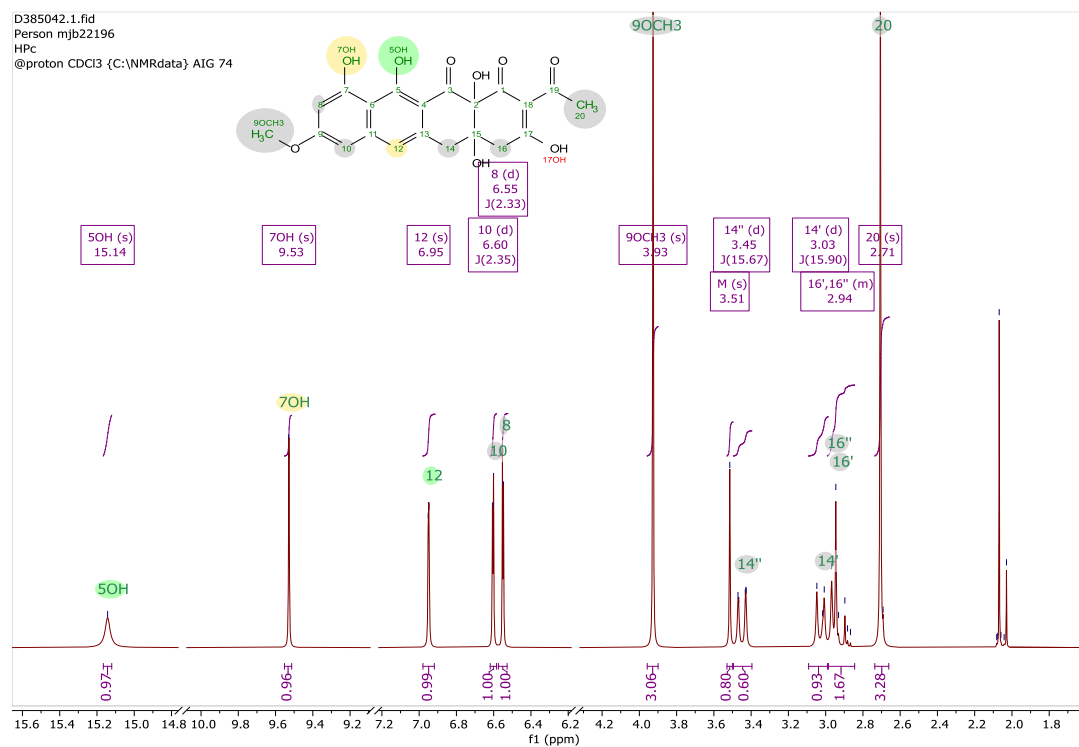
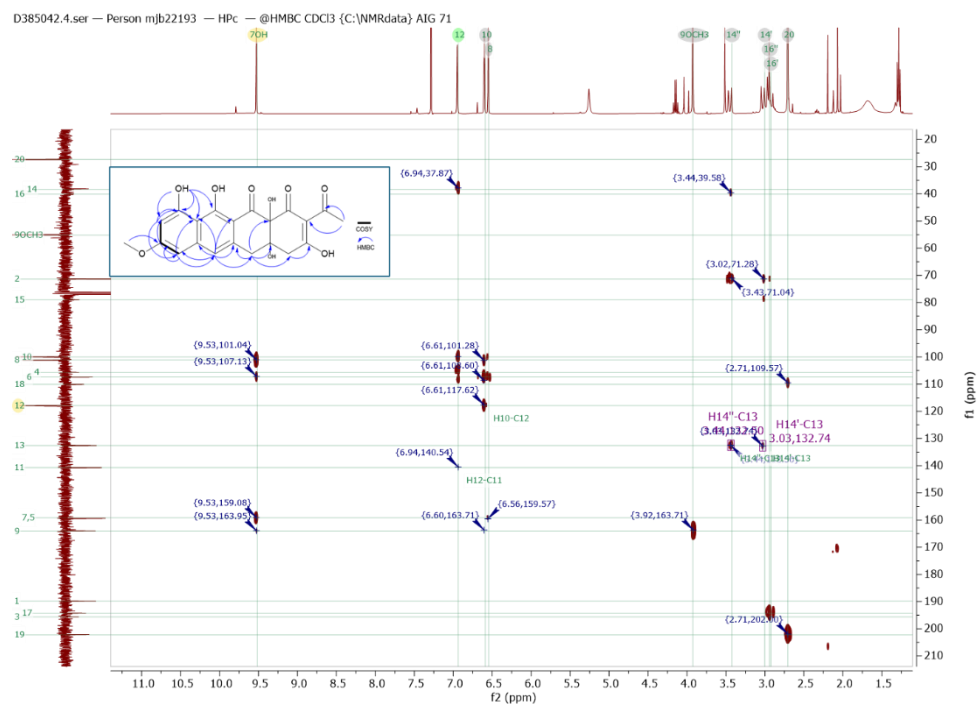
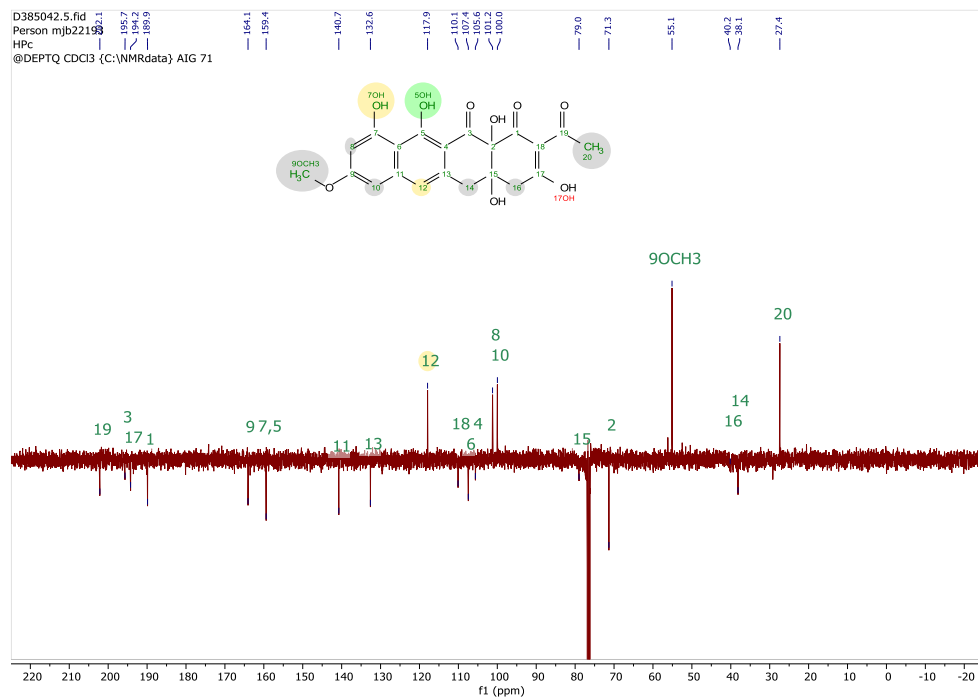
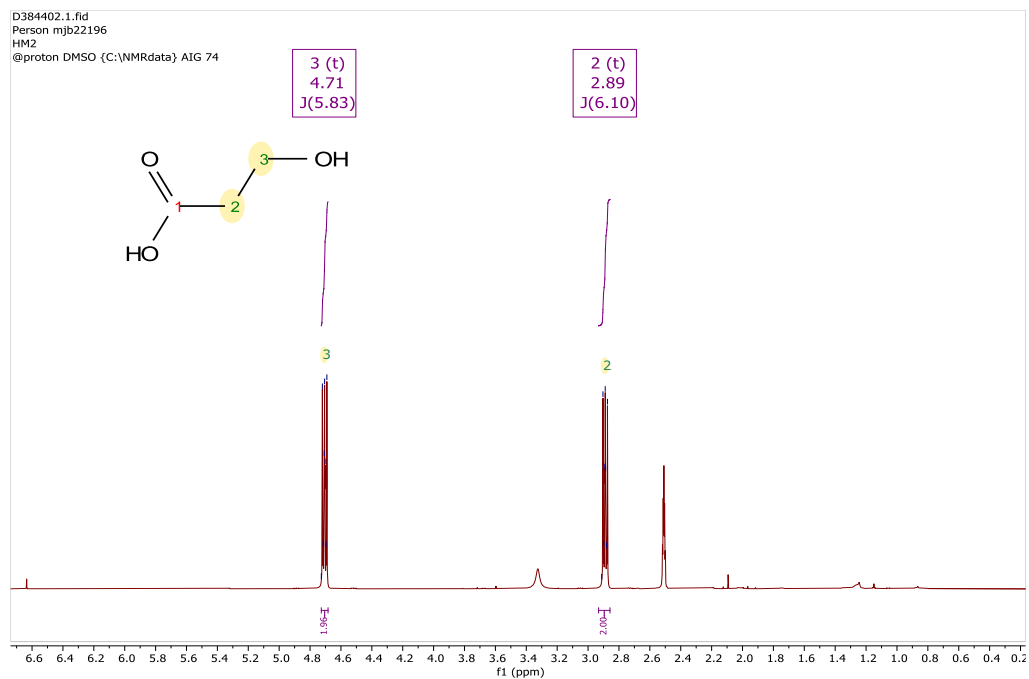
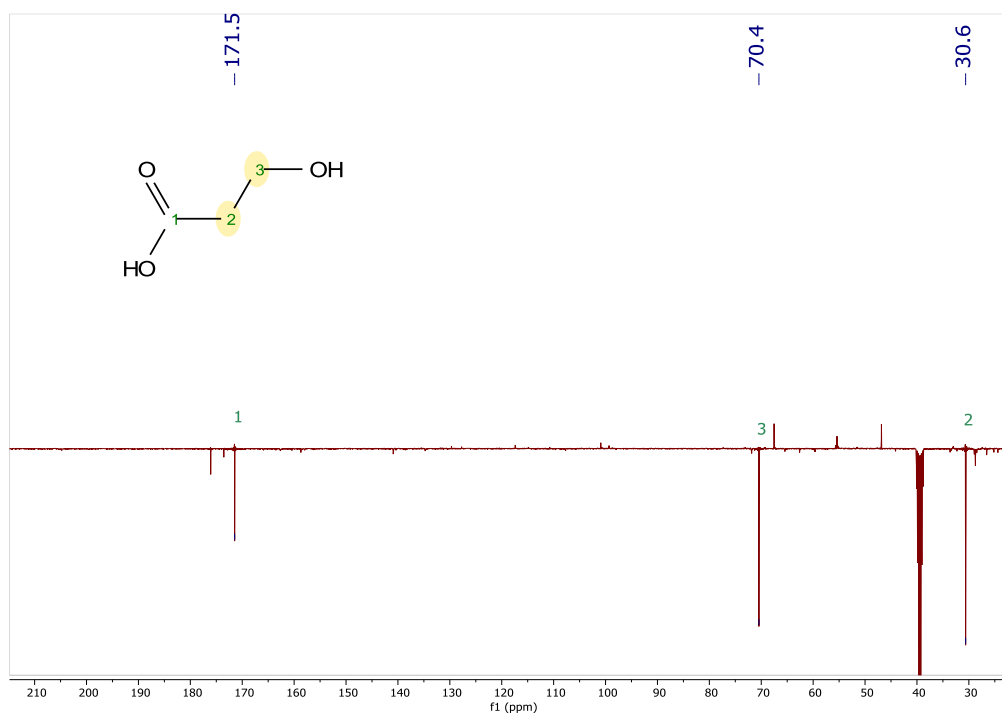


Figure S 16. HMBC of XM4.4 as phloroglucinol

Figure S 15. ^1H -NMR of HP showing the assignments as TAN1612 (400MHz, CDCl_3)



Figure S 19. ^1H -NMR of 3HPA in HM2 (400MHz, DMSO-d₆)Figure S 20. DEPTQ ^{13}C -NMR spectra of HM2 as 3HPA. (100MHz, DMSO-d₆)

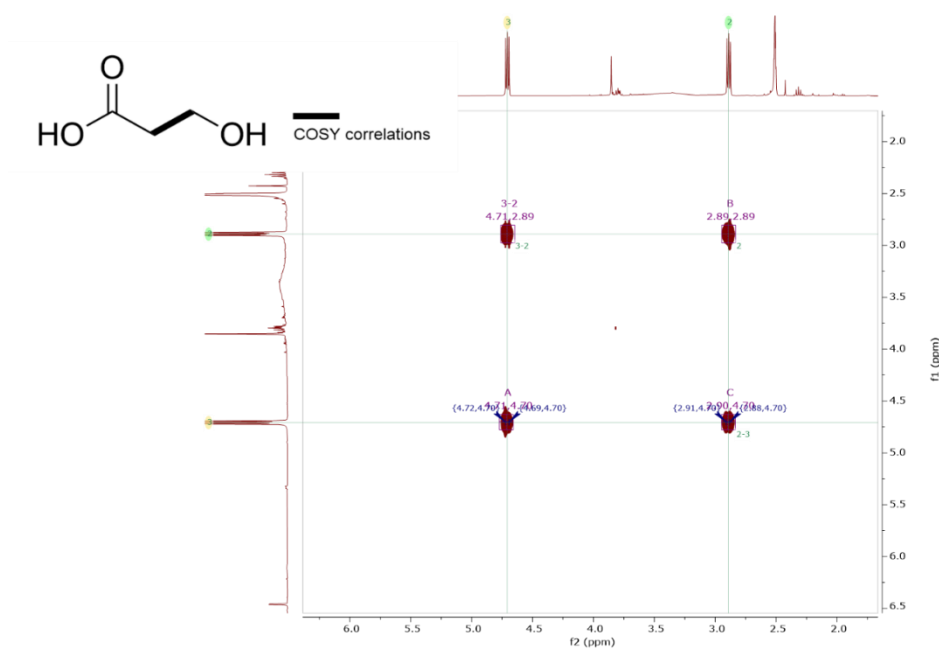
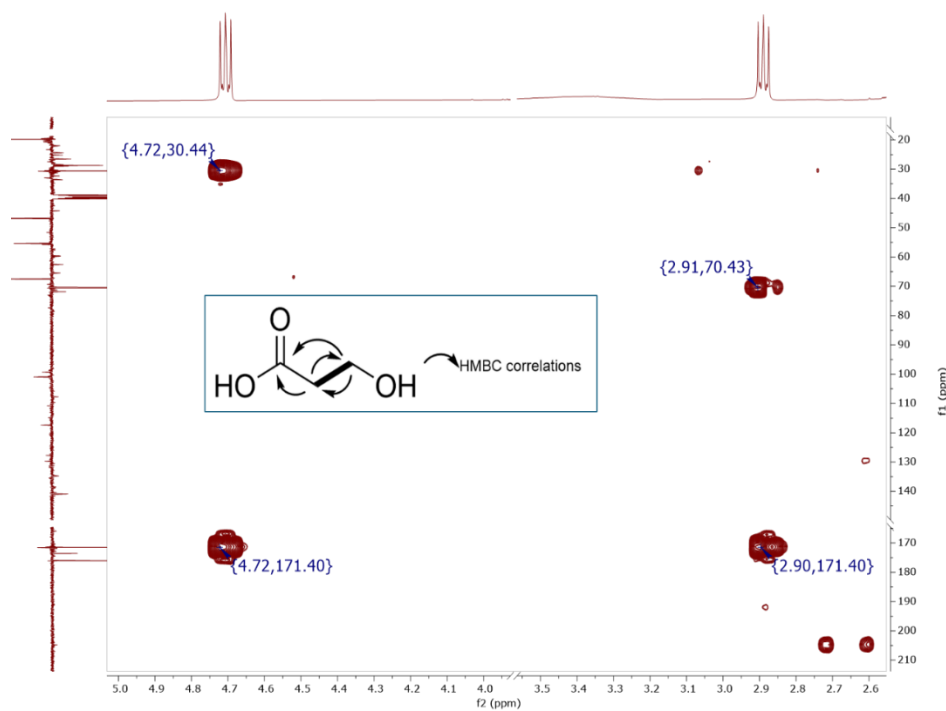
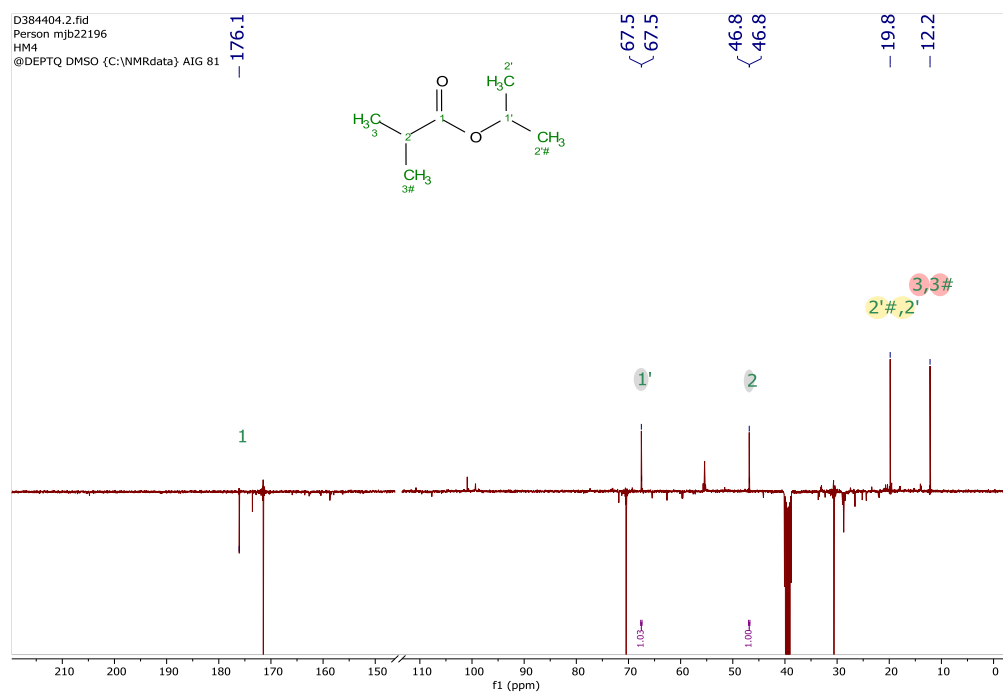
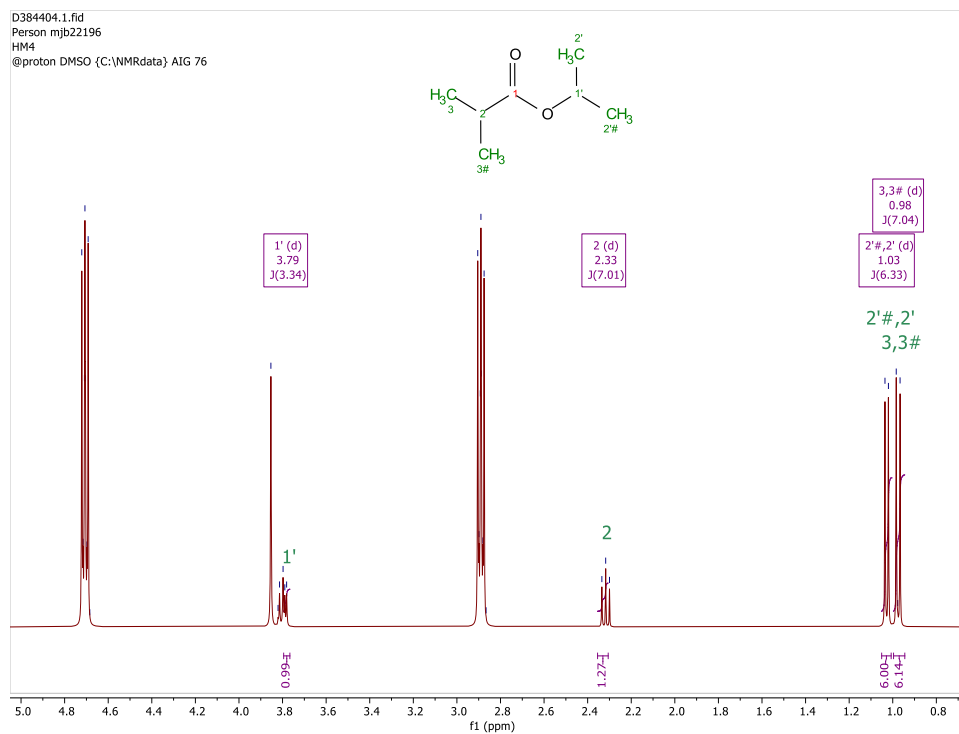
Figure S 21. COSY ^1H -NMR of 3HPA in HM2 (400MHz, DMSO-d₆)

Figure S 22. HMBC of HM2 as 3HPA



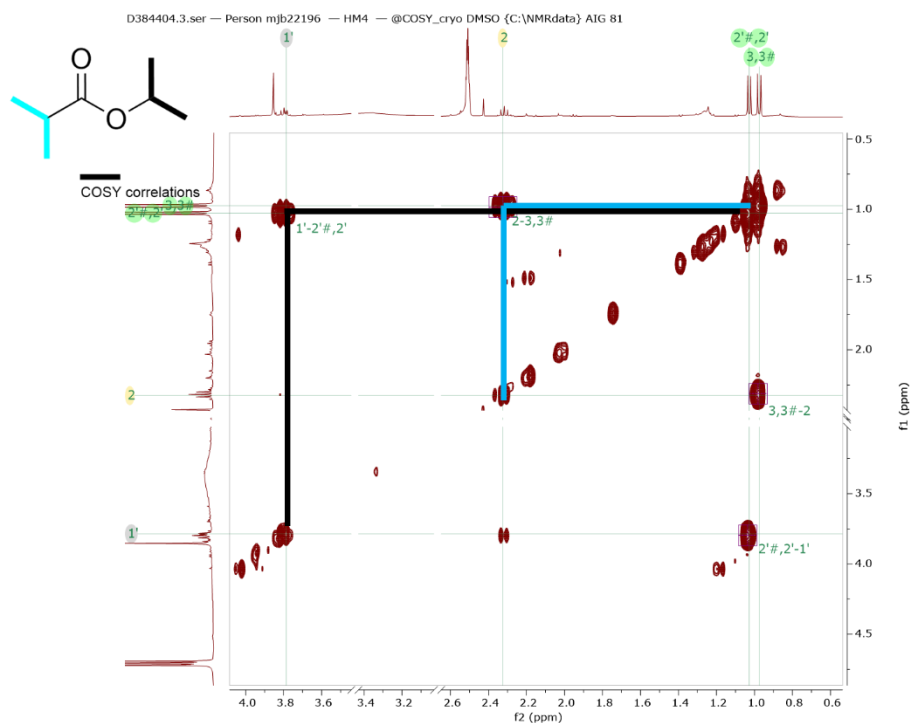


Figure S 25.COSY of HM4 as IPB (100MHz, DMSO-d6)

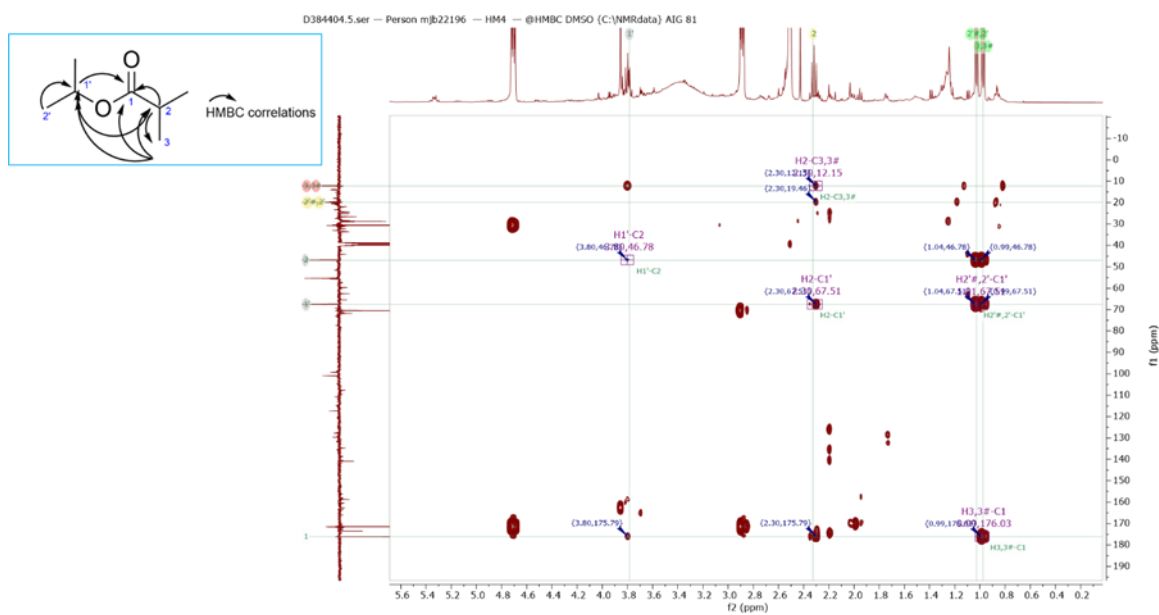


Figure S 26. S1. HMBC of HM4 as IPB

Figure S 28. COSY ^1H -NMR of HM8.3.1 as 2,3-didehydropalitantin (400MHz, DMSO- d_6)

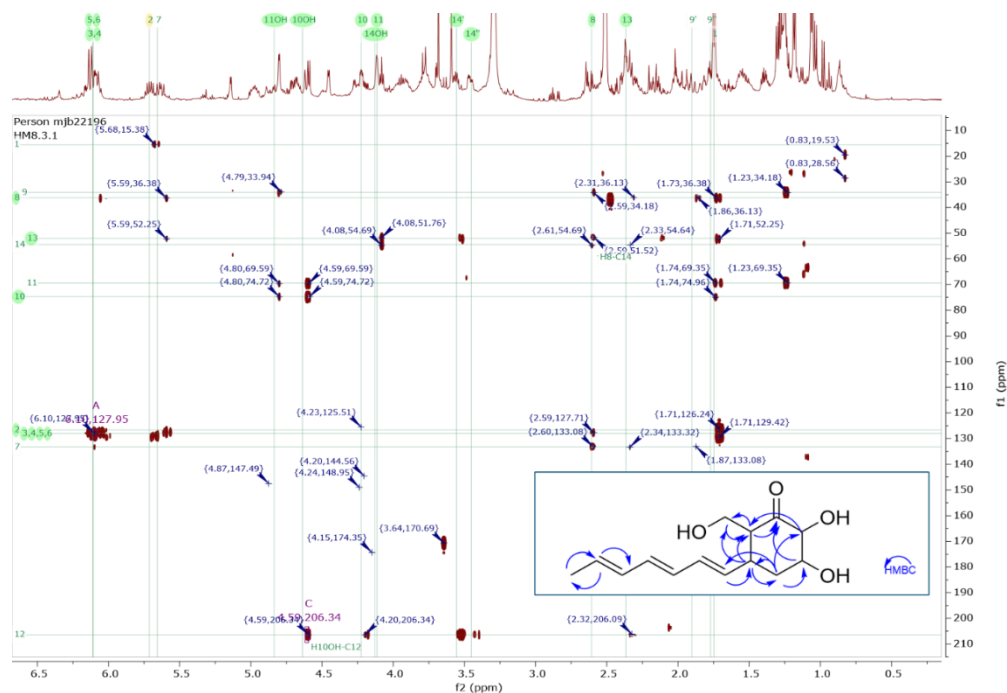


Figure S 29. HMBC of HM8.3.1 as 2,3-didehydropalitatin

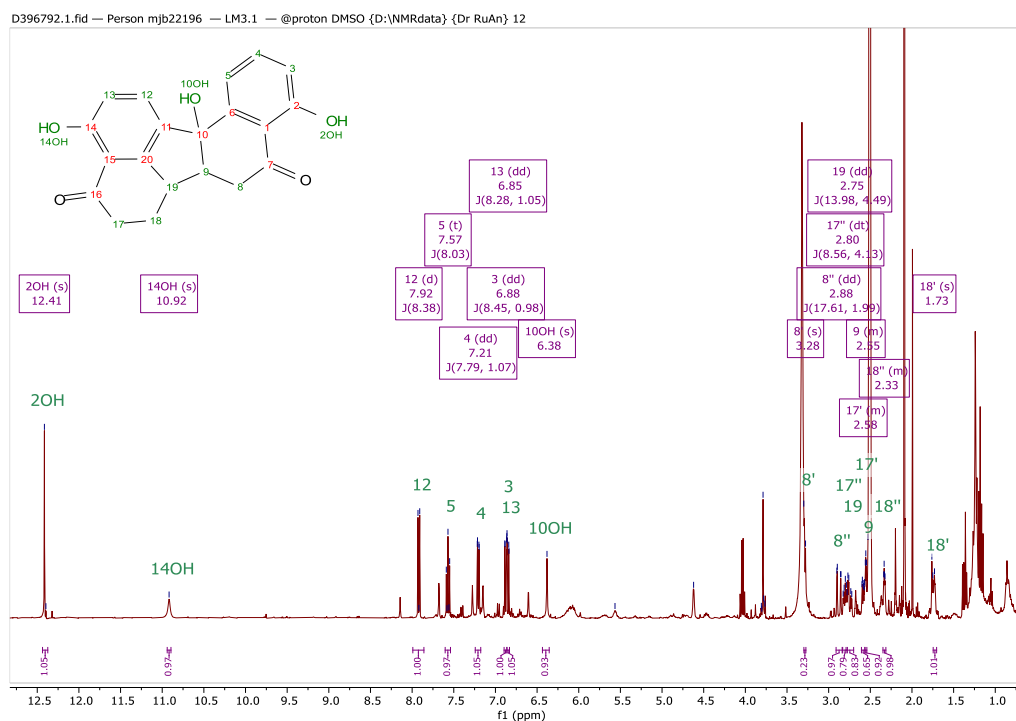
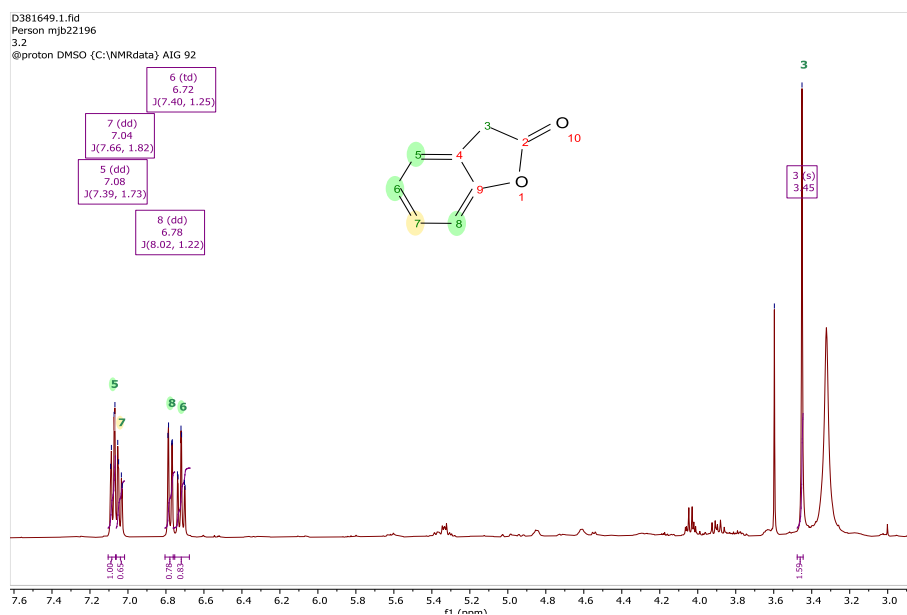
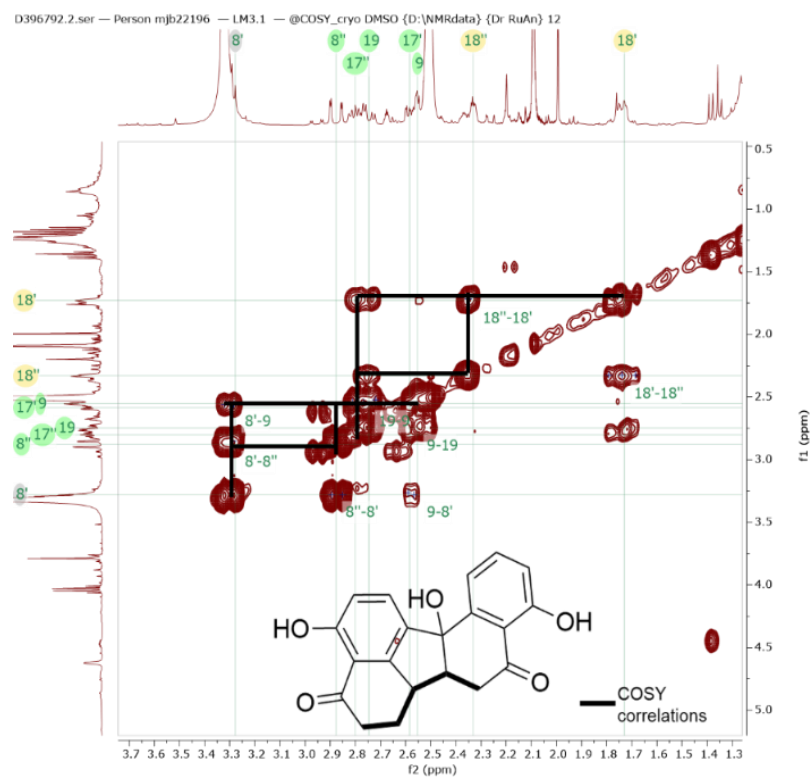


Figure S 30. ¹H-NMR of LM3.1 showing the assignments for daldinone A (400MHz, DMSO-d₆)



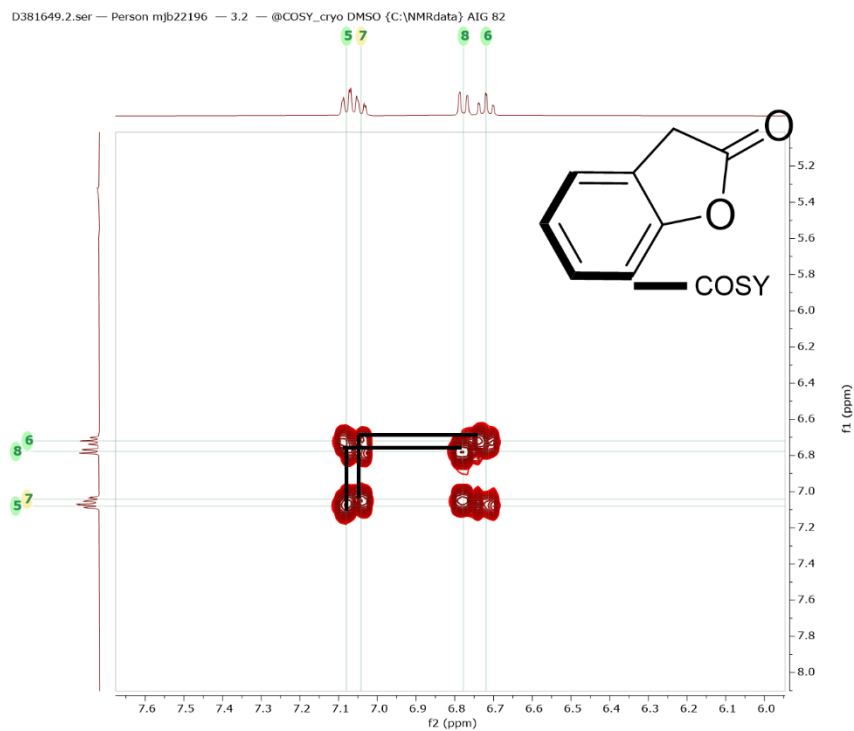


Figure S 33. COSY of M3.2 as 2(3H)benzofuranone

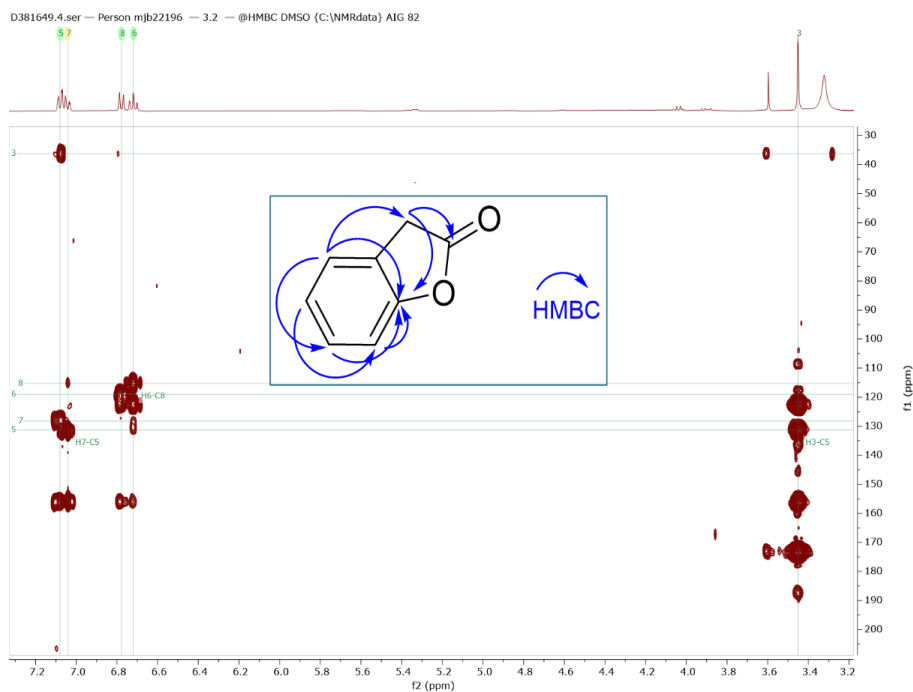
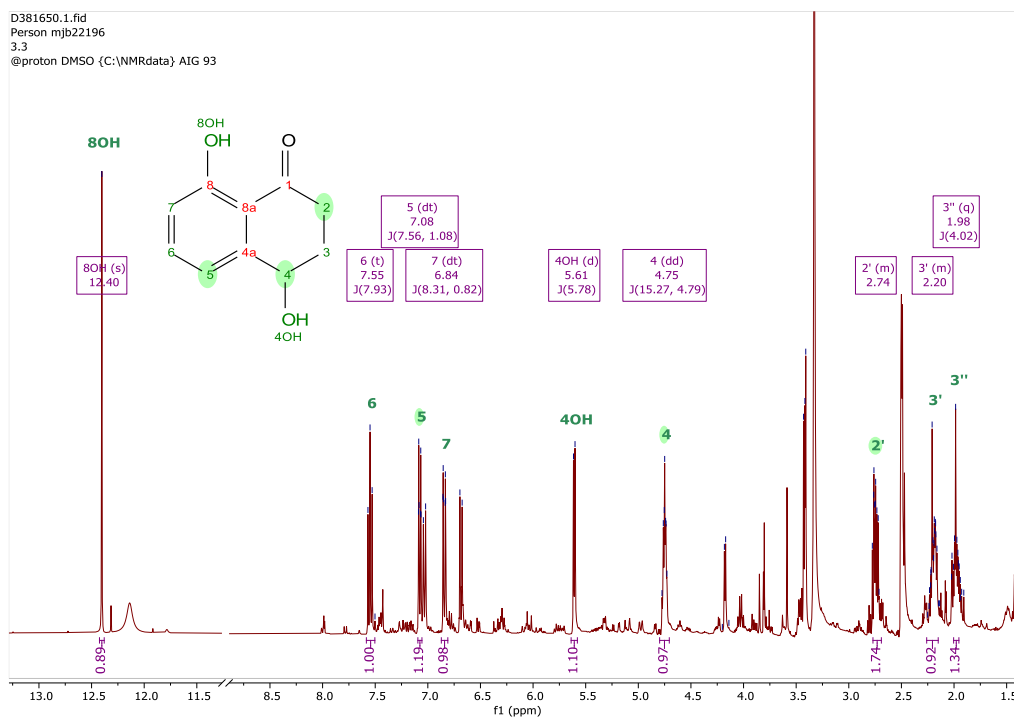
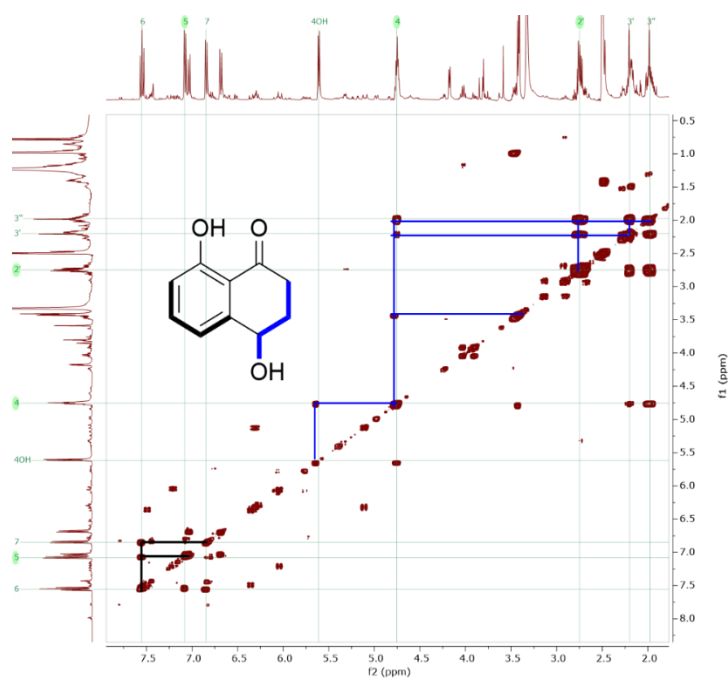


Figure S 34. HMBC of M3.2 as 2(3H)benzofuranone

Figure S 35. ^1H -NMR of M3.3 as regiolone (400MHz, DMSO- d_6)Figure S 36. COSY ^1H -NMR of M3.3 as regiolone

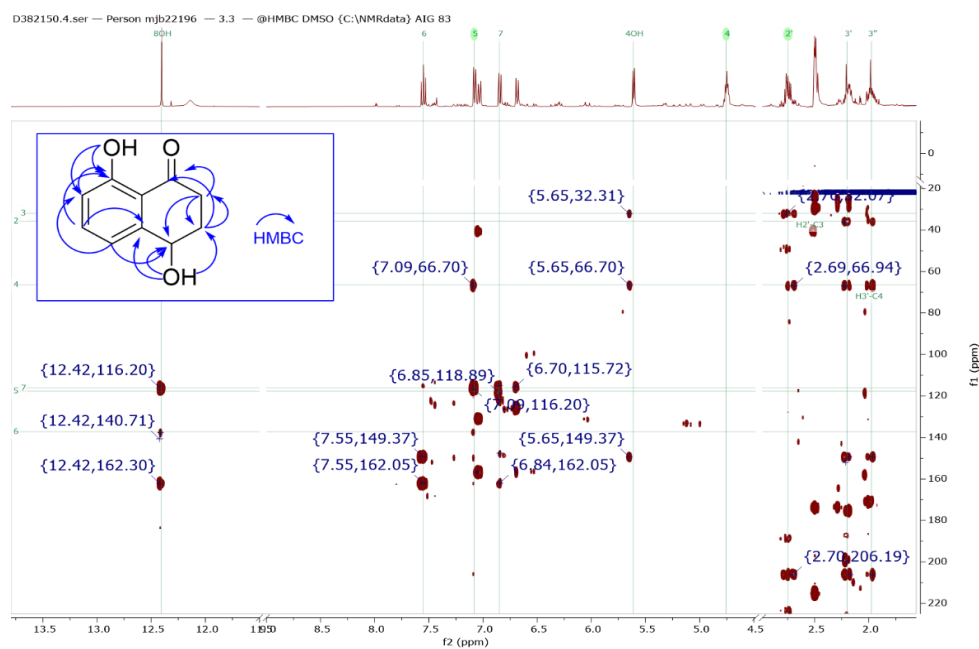
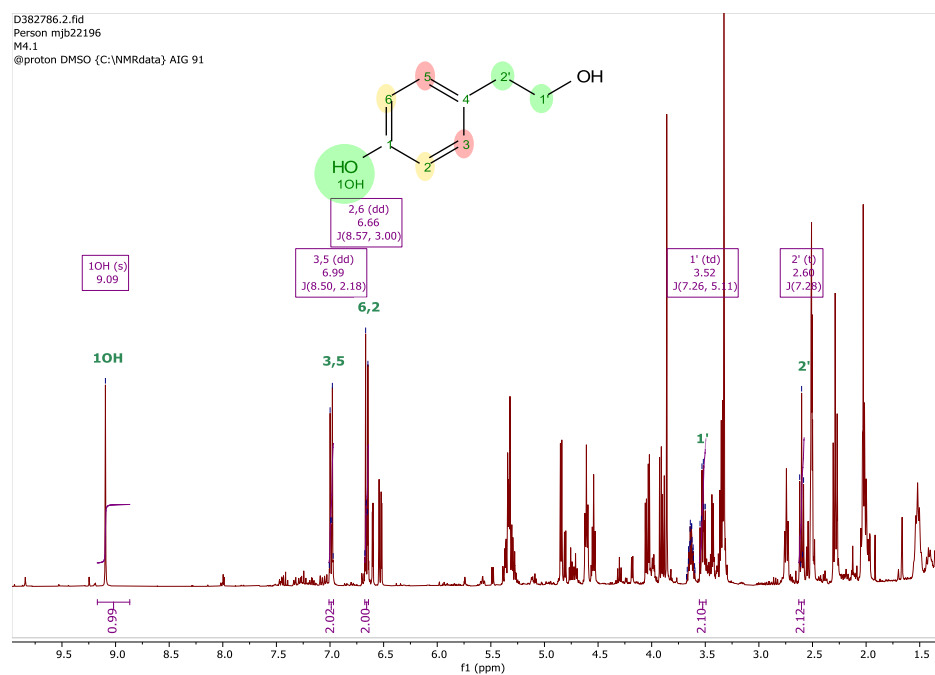


Figure S 37. HMBC of M3.3 as reglone

Figure S 38. ¹H-NMR of M4.1 as tyrosol (400MHz, DMSO-d₆)

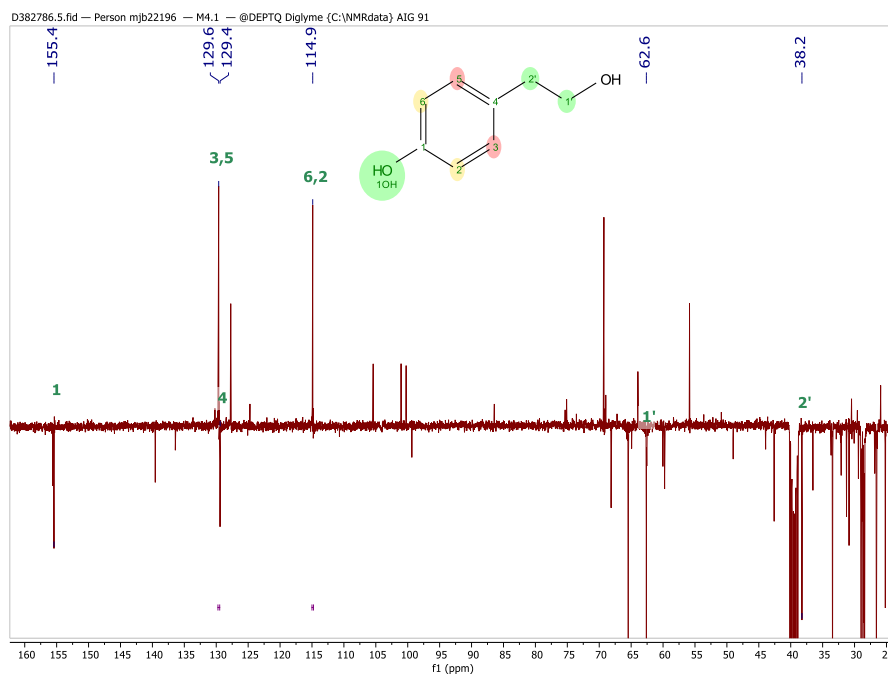
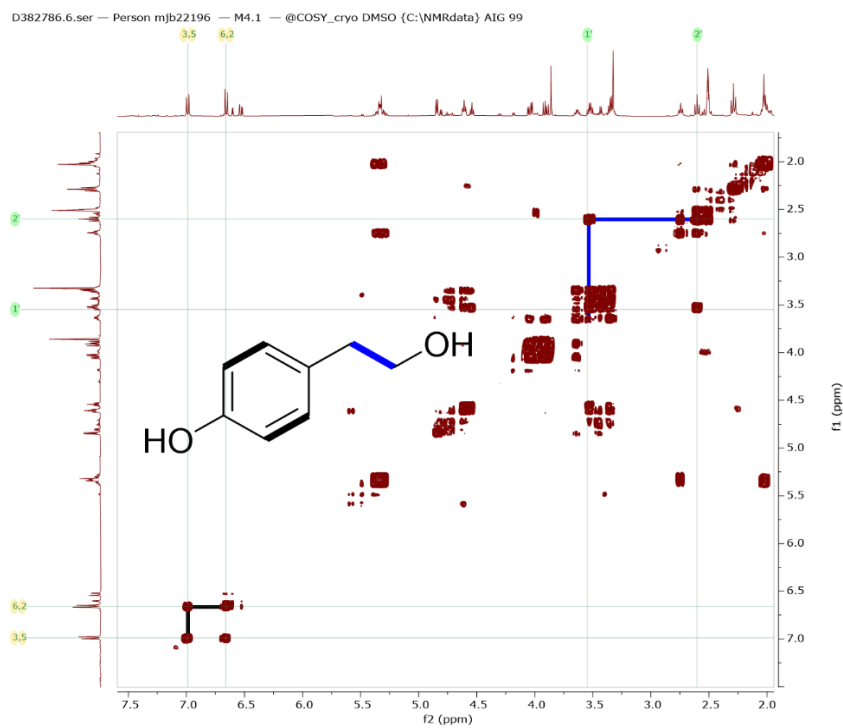
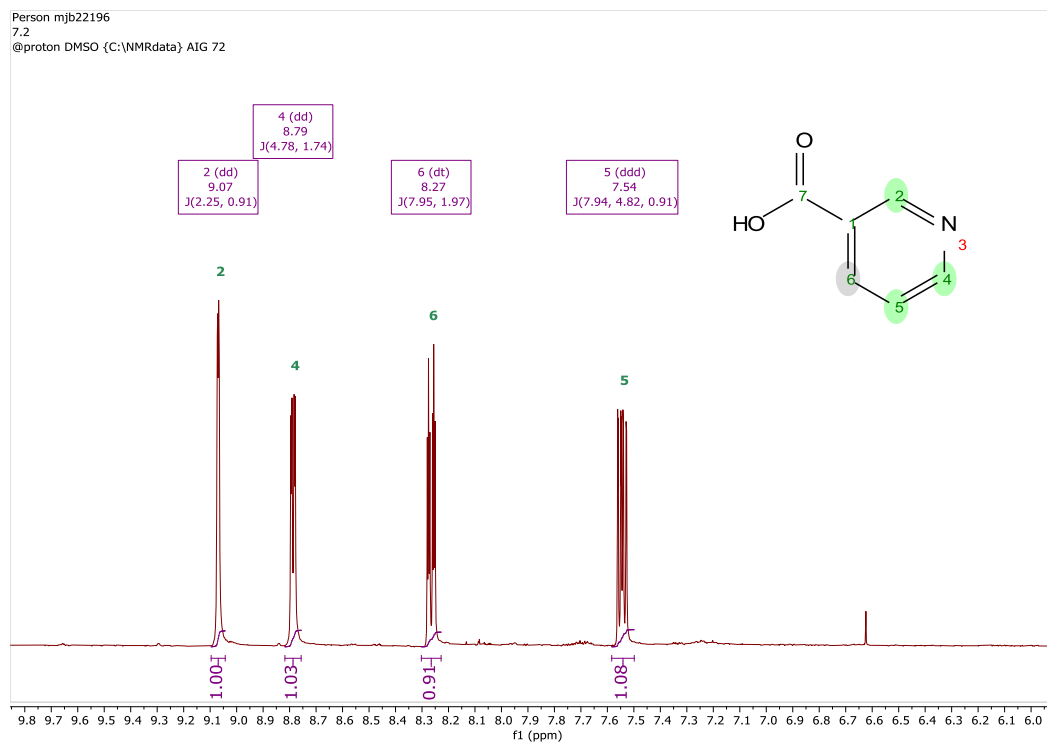




Figure S 41. HMBC of M4.1 as tyrosol

Figure S 42. ^1H -NMR of M7.2 as niacin (400MHz, DMSO- d_6)

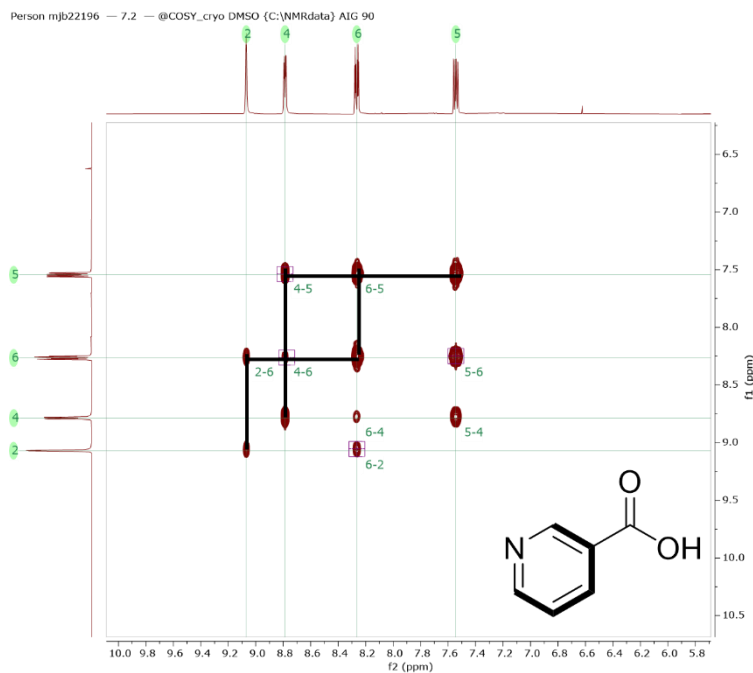
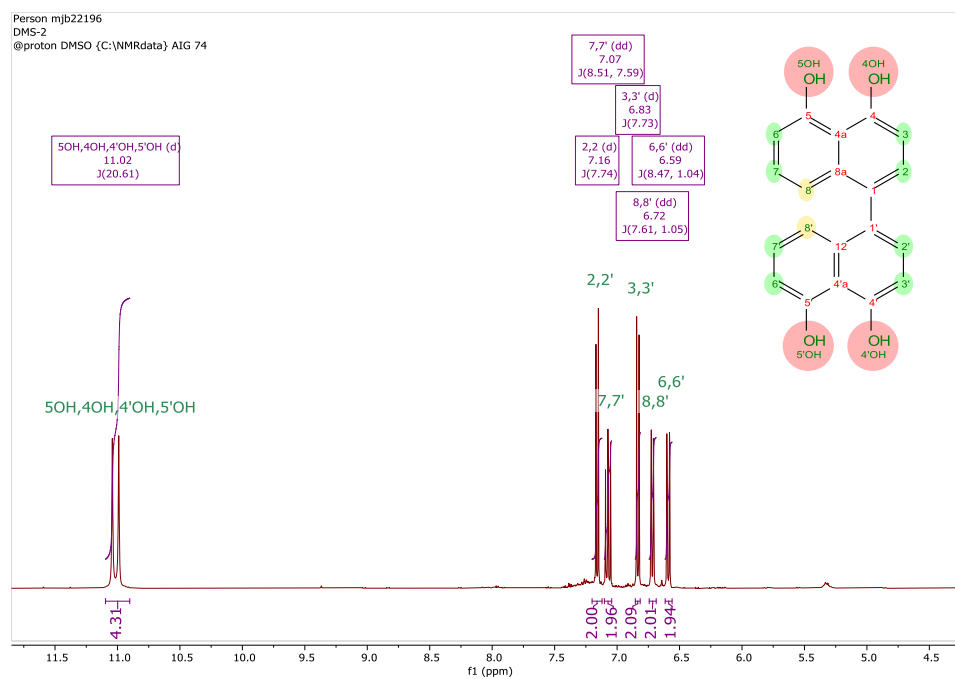
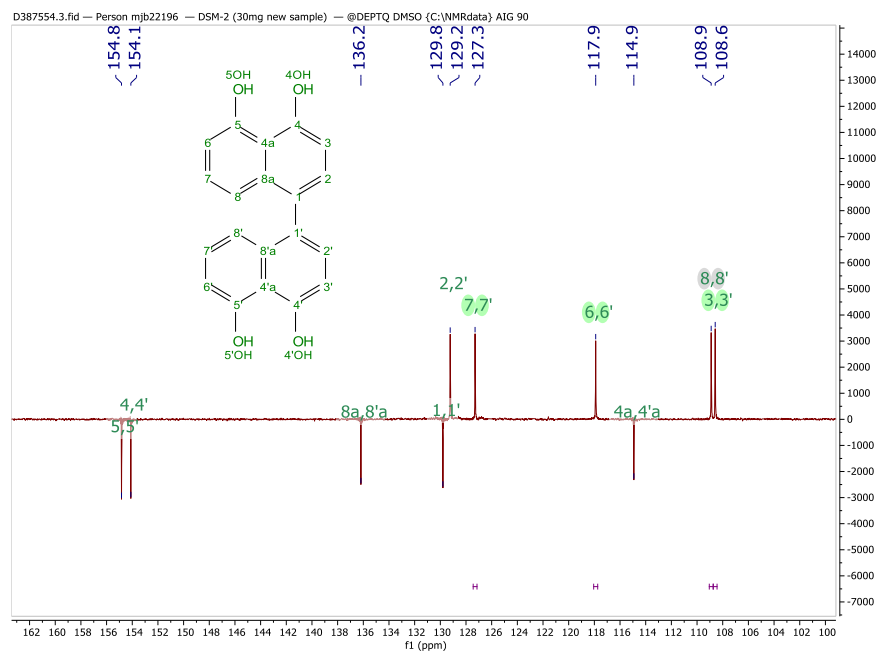
Figure S 43. COSY ^1H -NMR of M7.2 as niacin

Figure S 44. HMBC of M7.2 niacin

Figure S 45. ^1H -NMR of DSM2 as BNT (400MHz, DMSO- d_6)Figure S 46. DEPTQ ^{13}C -NMR spectra of DSM2 as BNT (100MHz, DMSO- d_6)

D387554.5.ser — Person mjb22196 — DSM-2 (30mg new sample) — @HMBC DMSO {C:\NMRdata} AIG 90

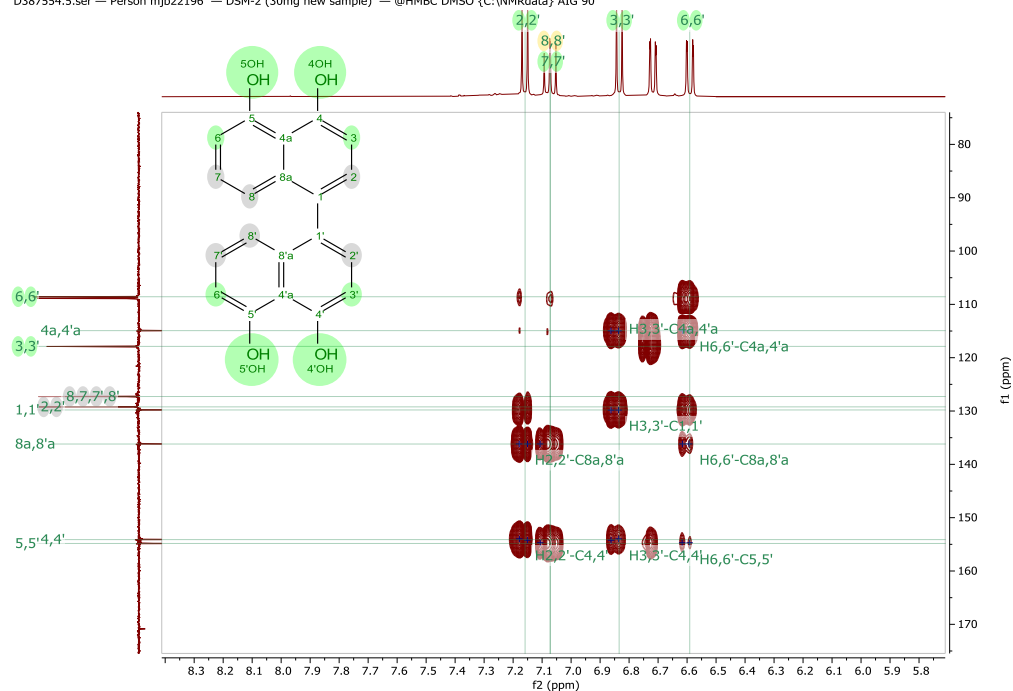


Figure S 47. HMBC of BNT

D391129.1.fid — Person mjb22196 — DSM4.6 — @proton DMSO {D:\NMRdata} AIG 96

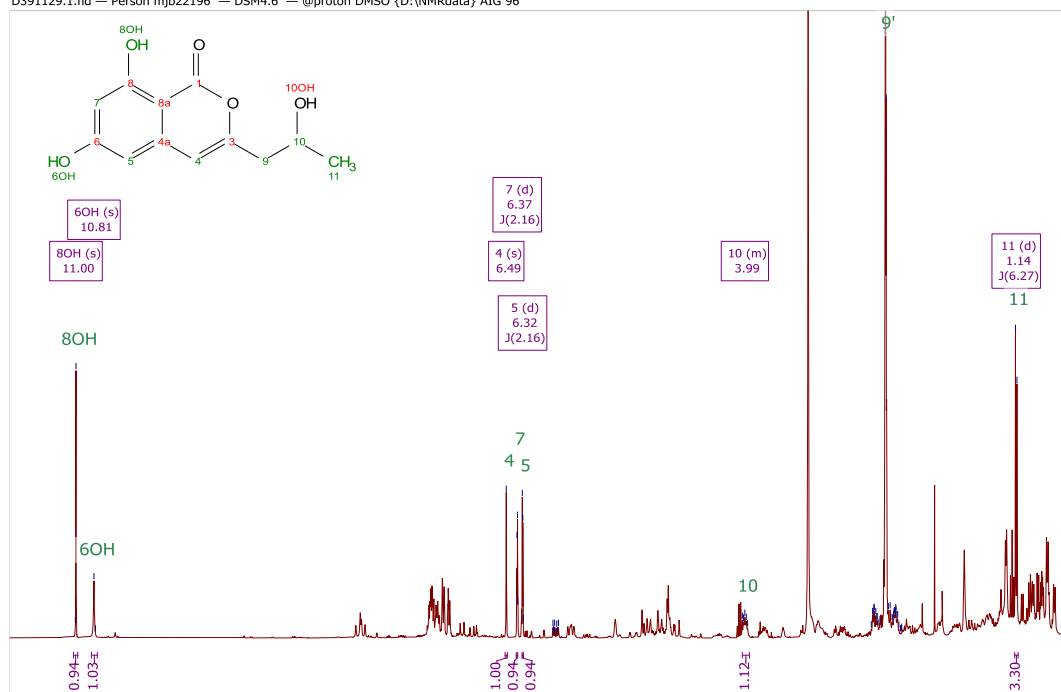


Figure S 48. ^1H -NMR of DSM4.6 as orthosporin (400MHz, DMSO-d₆)

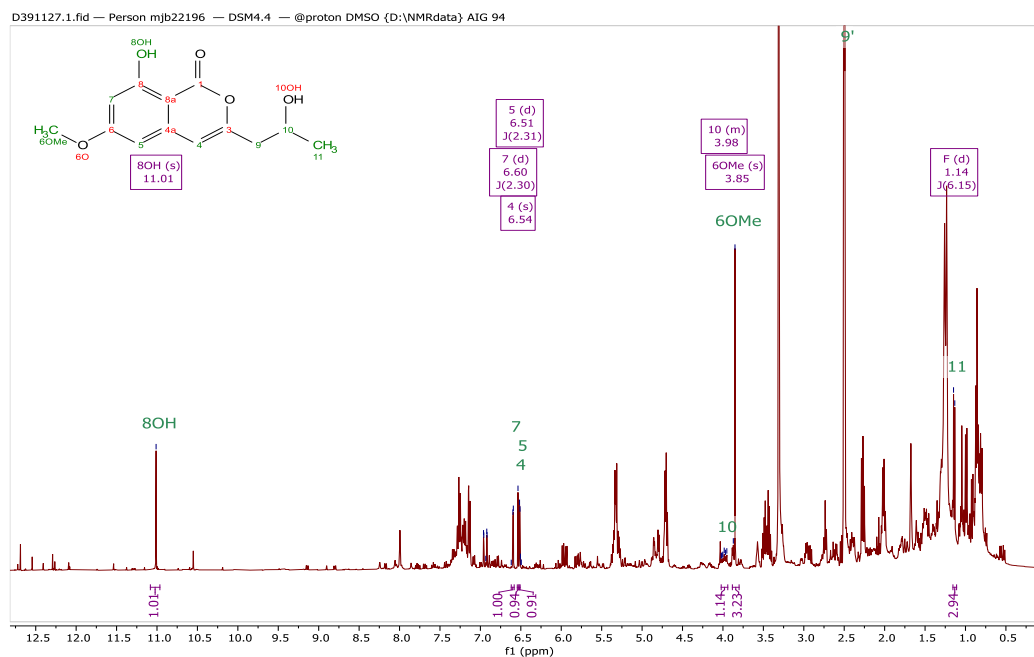


Figure S 49. ¹H-NMR of DSM4.4 as diaporthin (400MHz, DMSO-d₆)

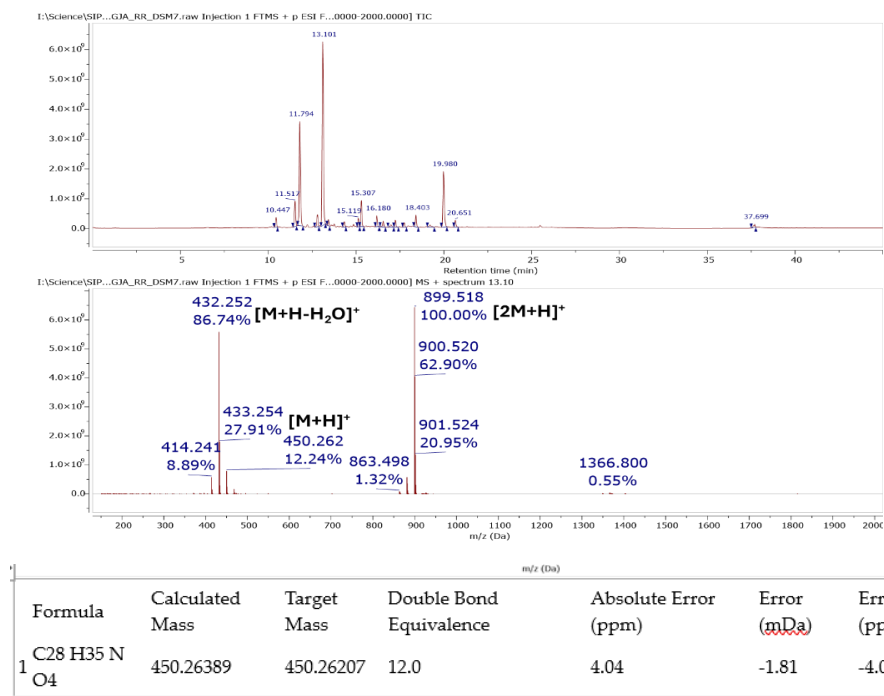


Figure S 50. TIC and MS data of DSM7

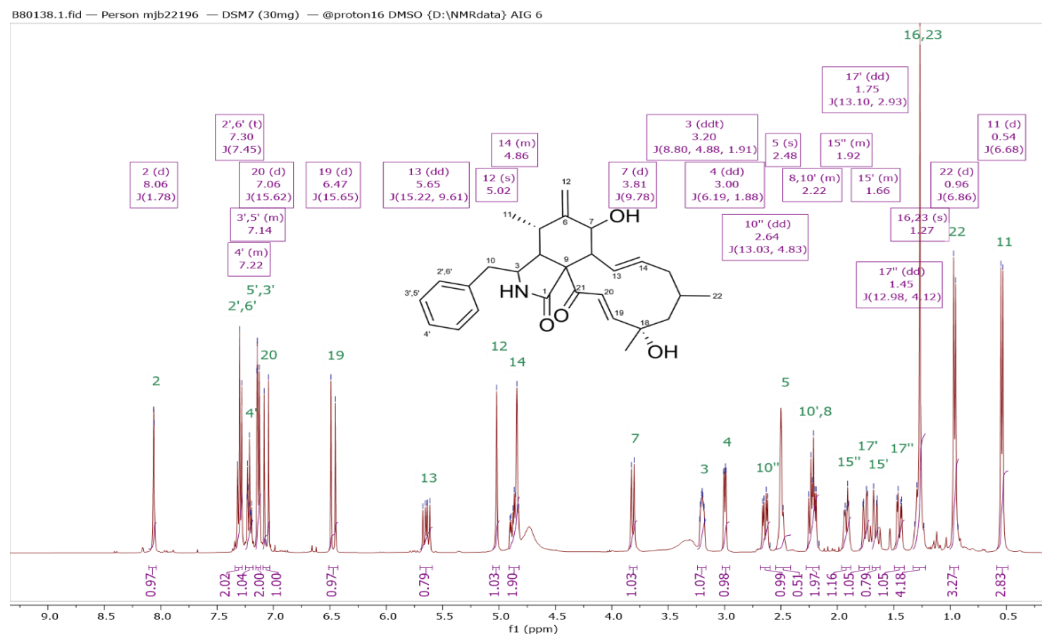


Figure S 52. ^1H -NMR of DSM7 as cytochalasin 1 (400MHz, DMSO- d_6)

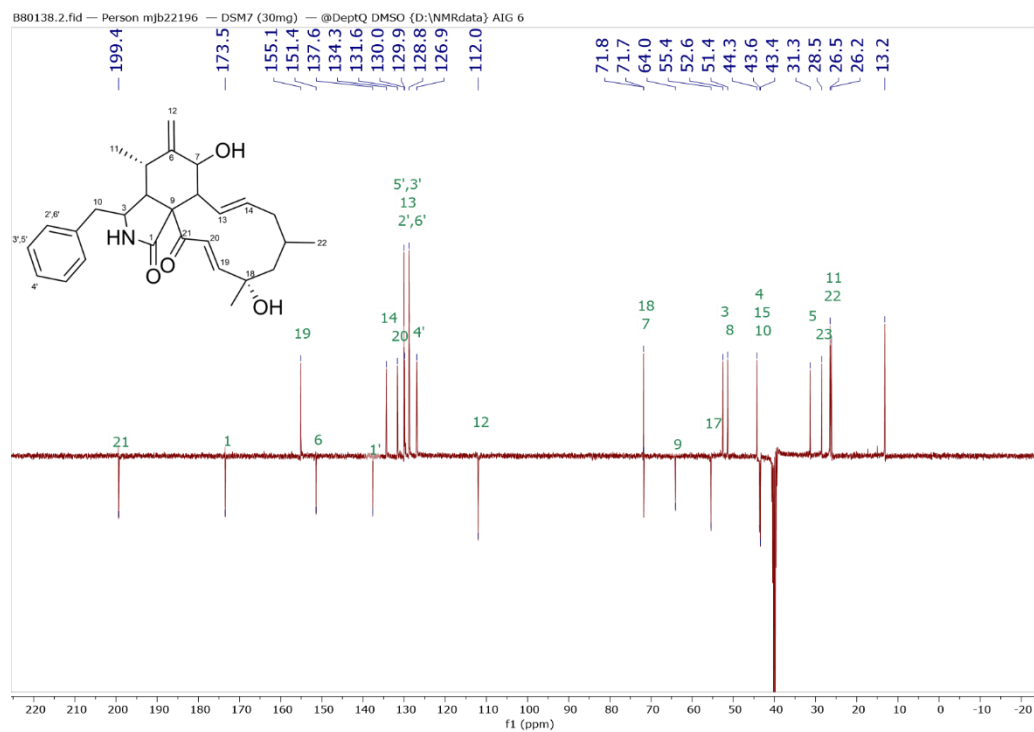


Figure S 51. S2. DEPTQ ^{13}C -NMR of DSM7 as cytochalasin 1 (100MHz, DMSO- d_6)

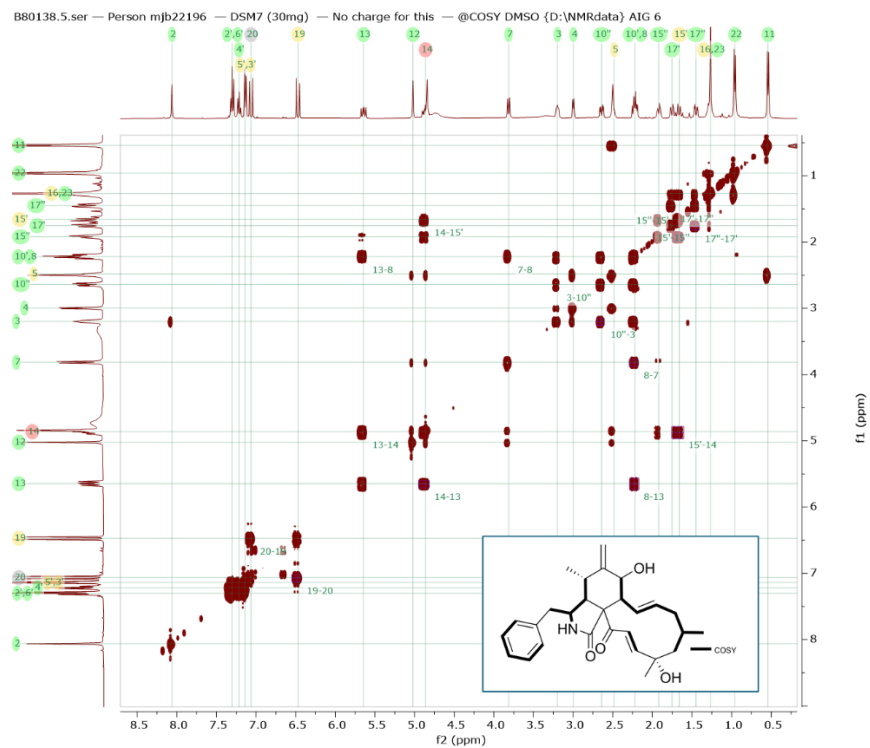
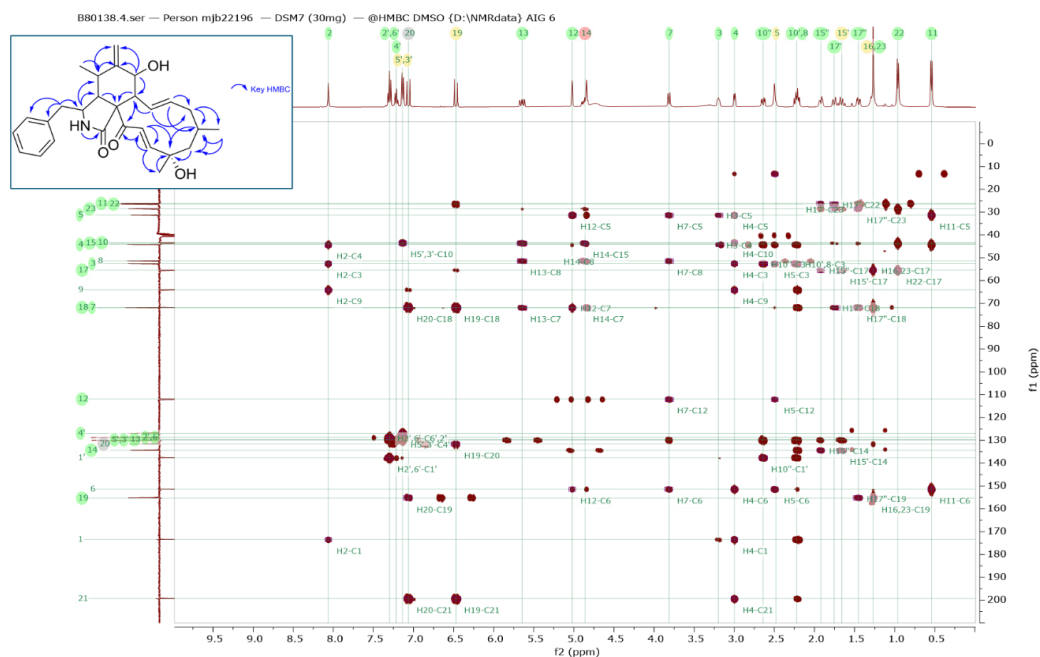
Figure S 53. COSY ^1H -NMR of DSM7 as cytochalasin 1

Figure S 54. HMBC of DSM7 as cytochalasin 1

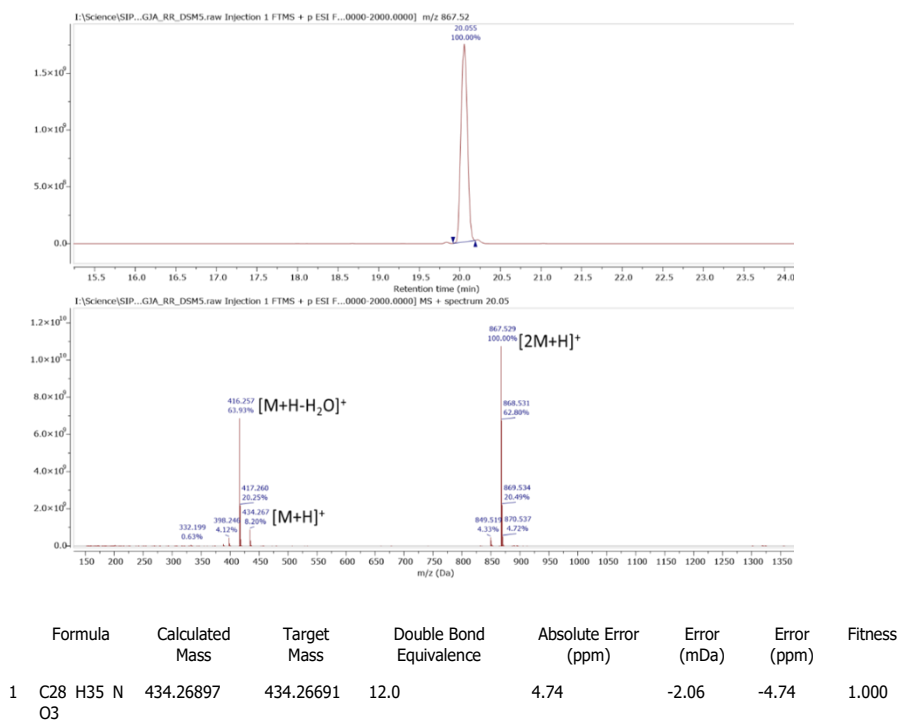
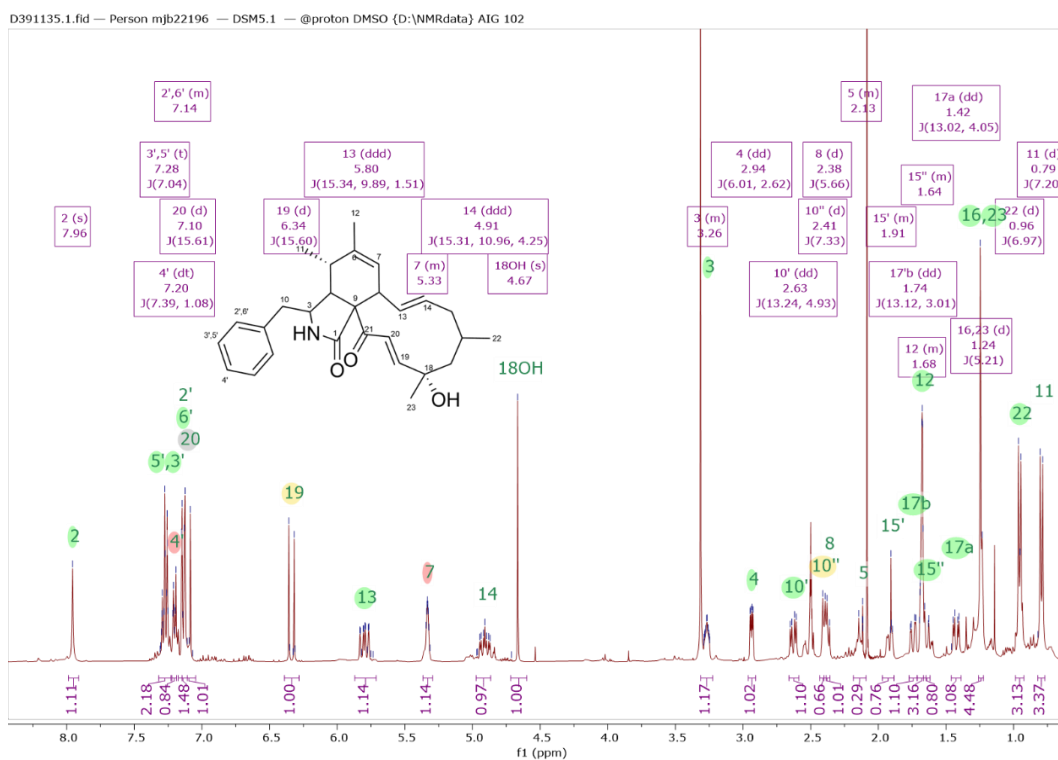
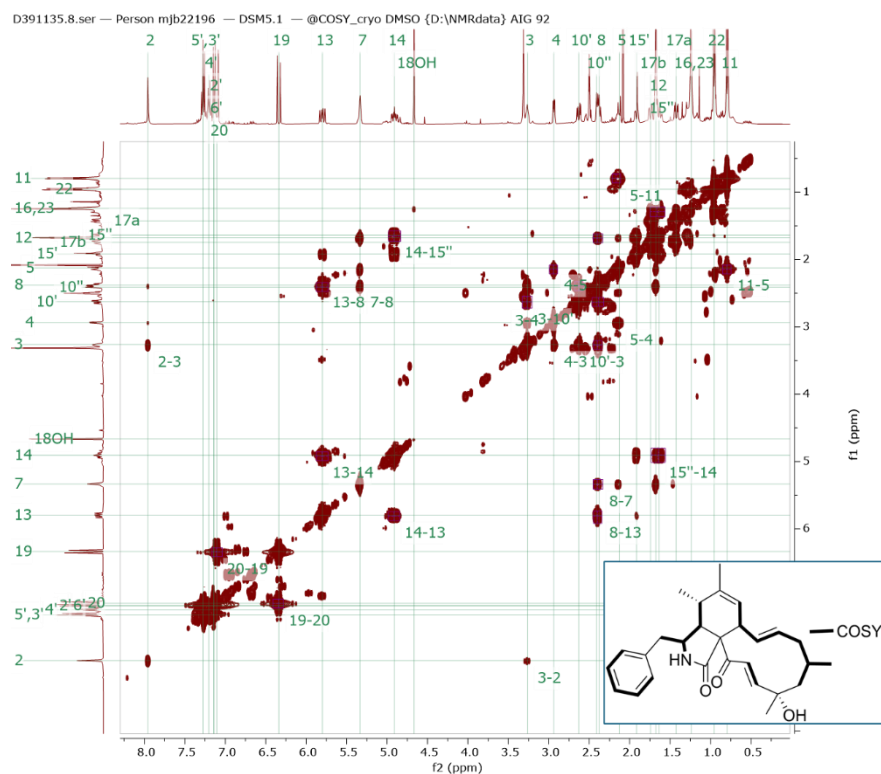
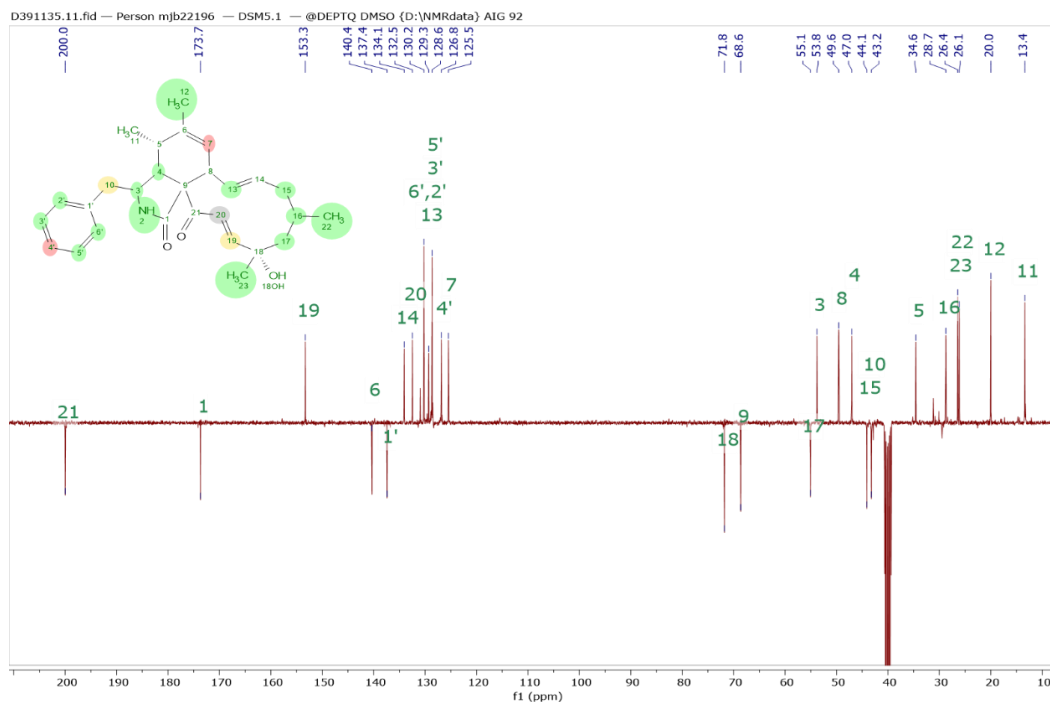
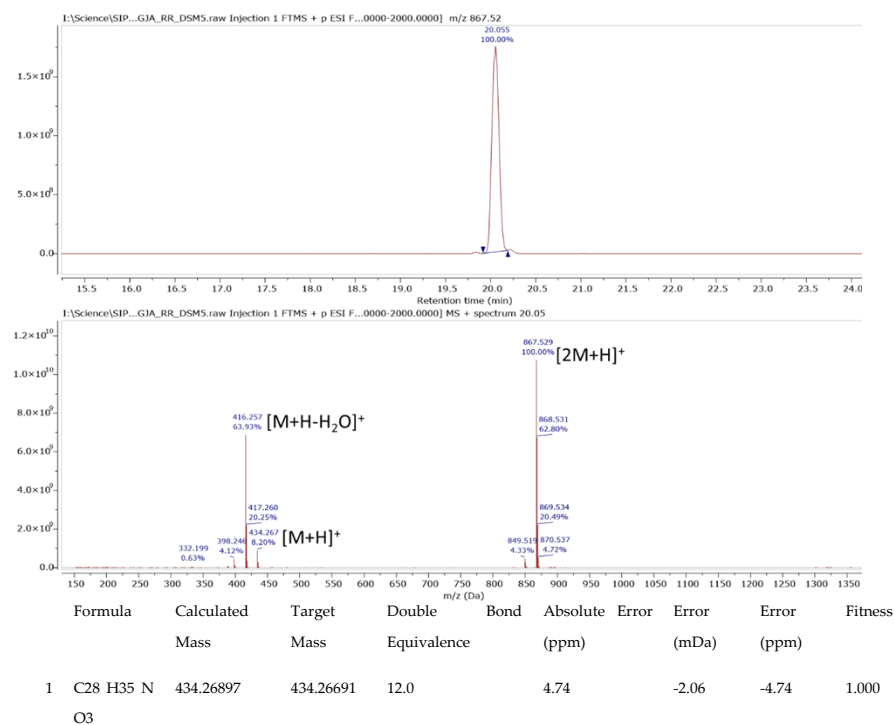


Figure S 56. TIC and MS of DSM5.1 as cytochalasin 9







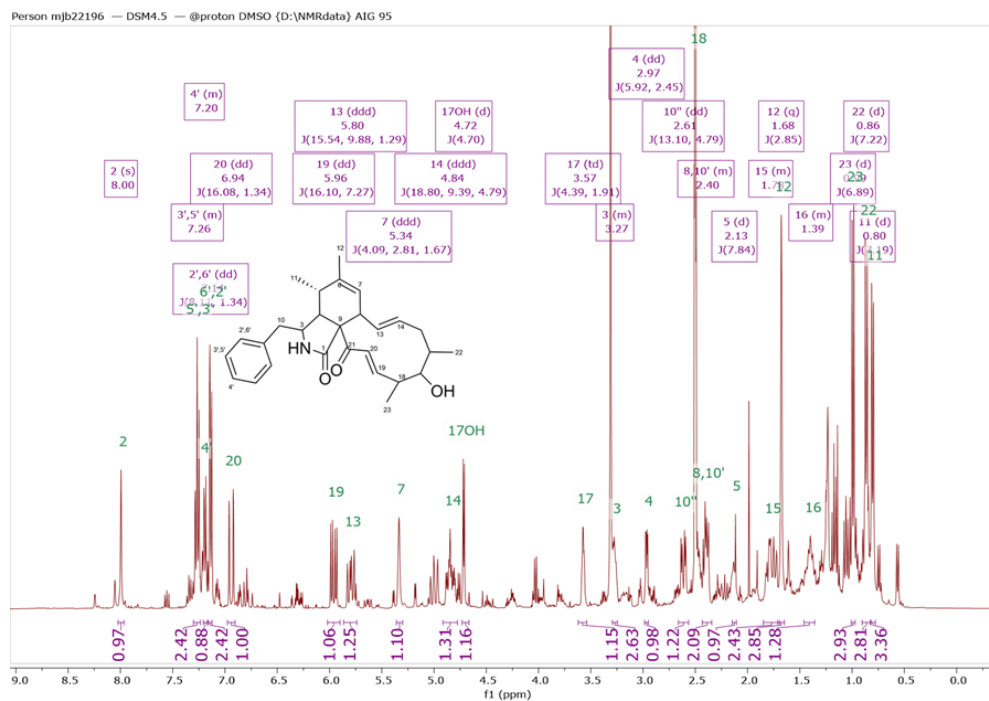


Figure S 61. ^1H -NMR of DSM4.5 as cytochalasin 11 (400MHz, DMSO- d_6)

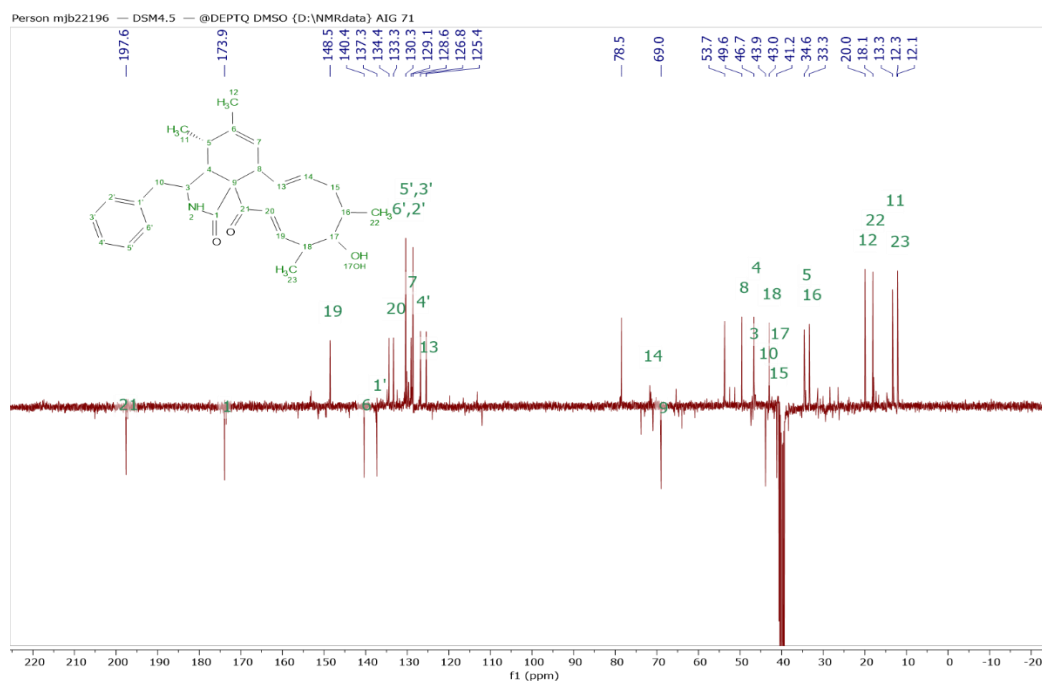


Figure S 62. DEPTQ ^{13}C -NMR of DSM4.5 as cytochalasin 11 (100MHz, DMSO- d_6)

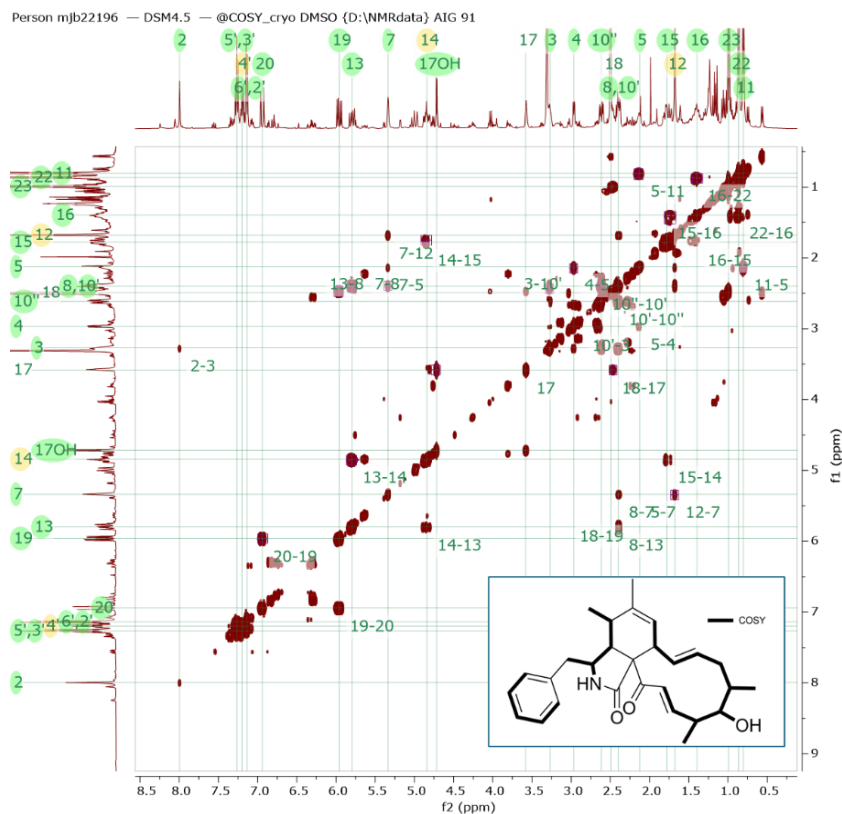
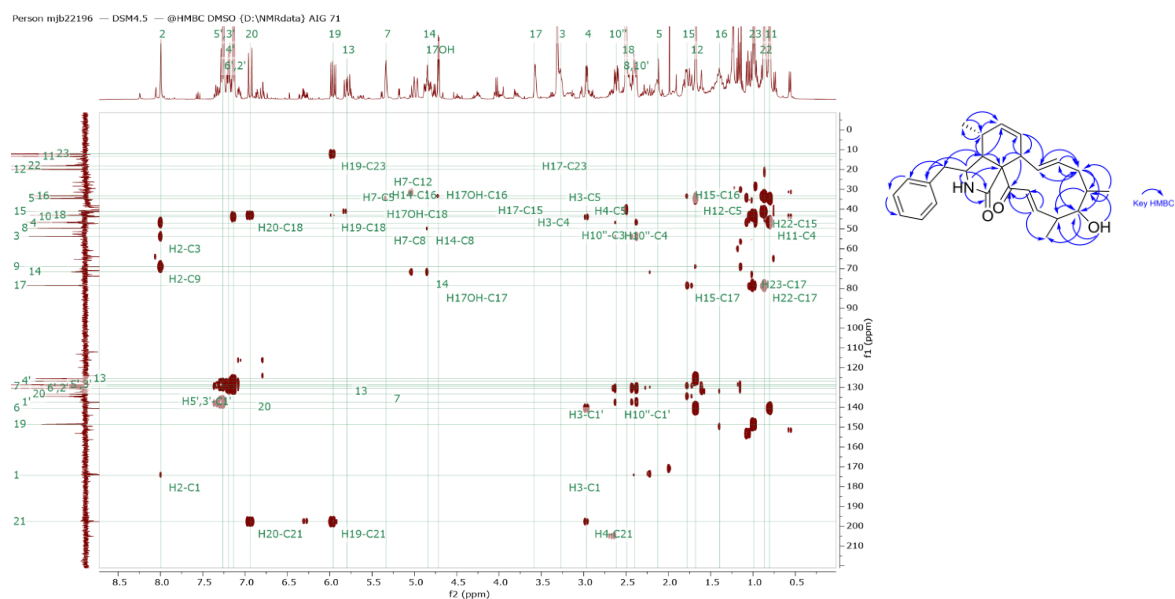
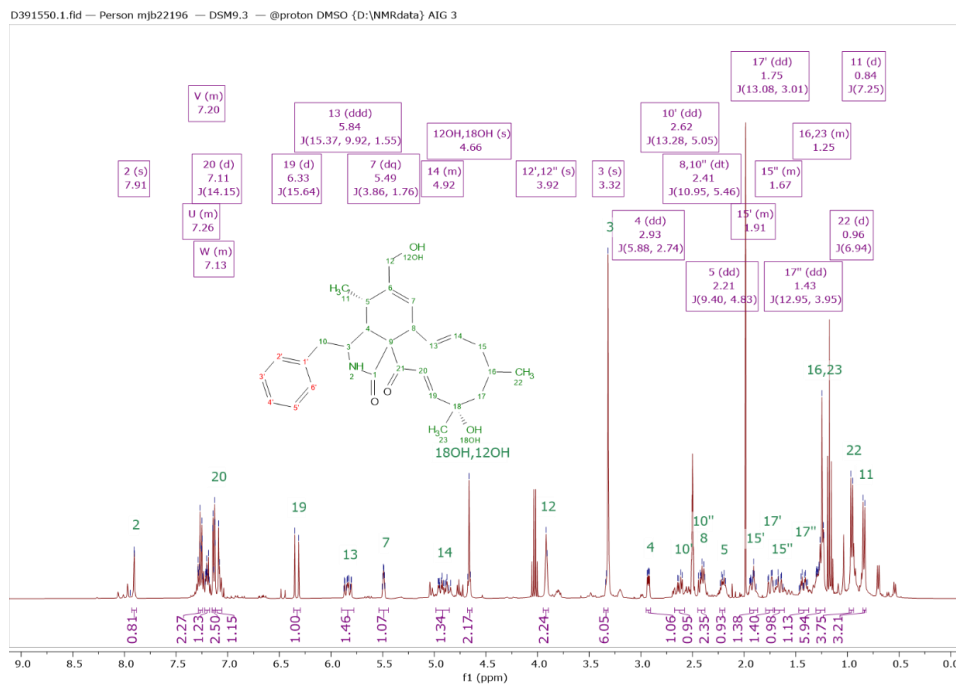
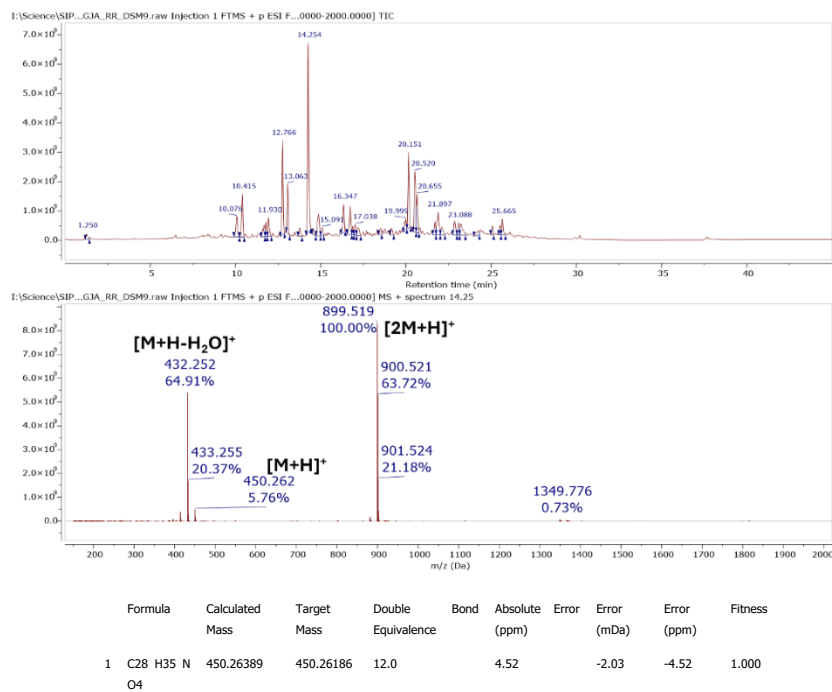
Figure S 63. COSY ^1H -NMR of DSM4.5 as cytochalasin 11

Figure S 64. HMBC of DSM4.5 as cytochalasin 11



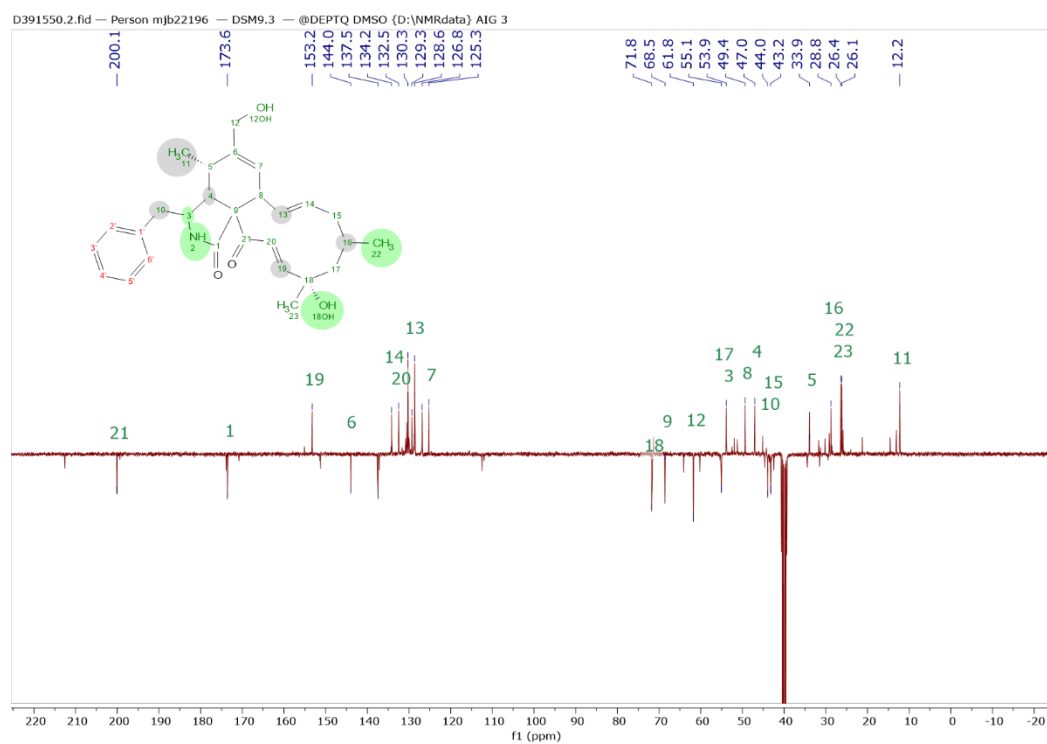


Figure S 67. DEPTQ ^{13}C -NMR of DSM9.3 as cytochalasin 10 (100MHz, DMSO-d₆)

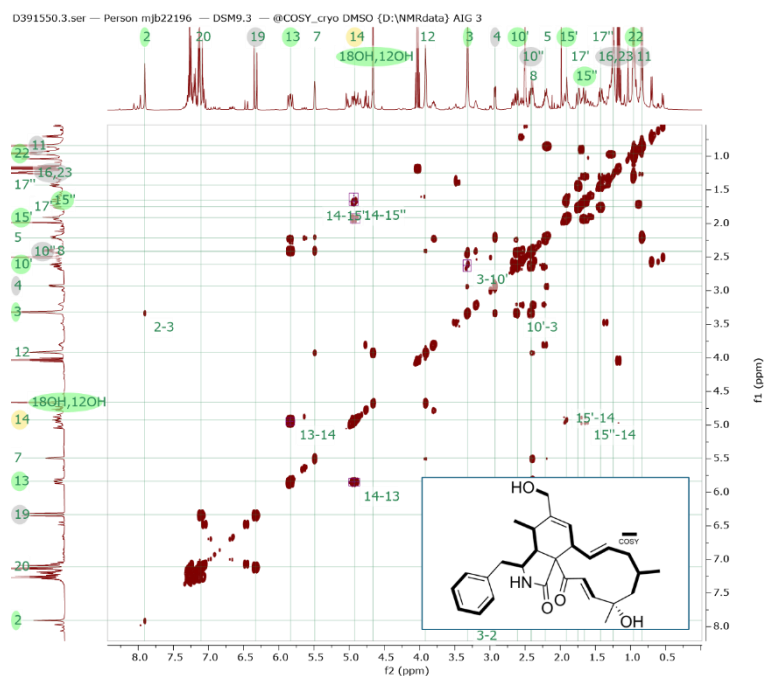


Figure S 68. COSY ^1H -NMR of DSM9.3 as cytochalasin 10

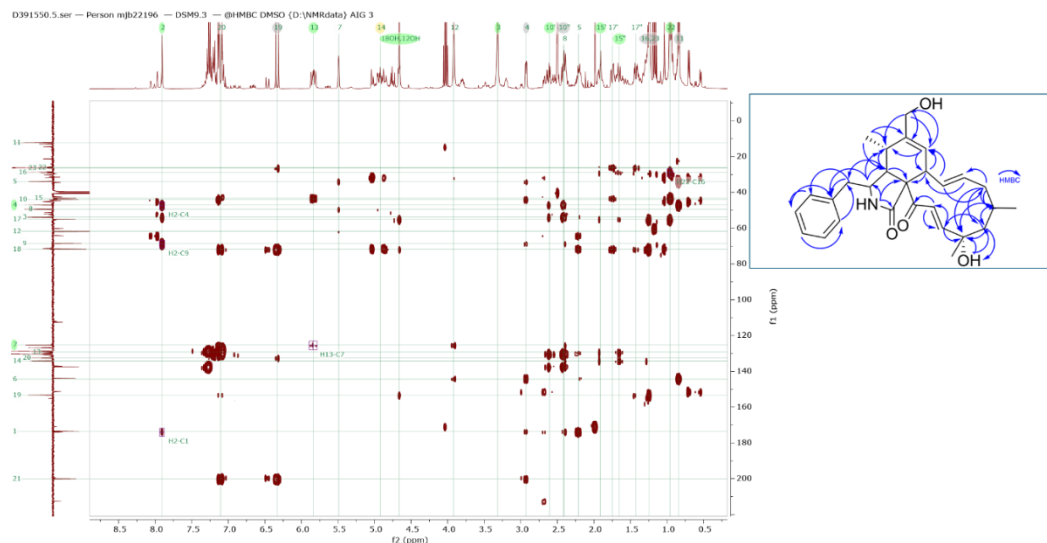
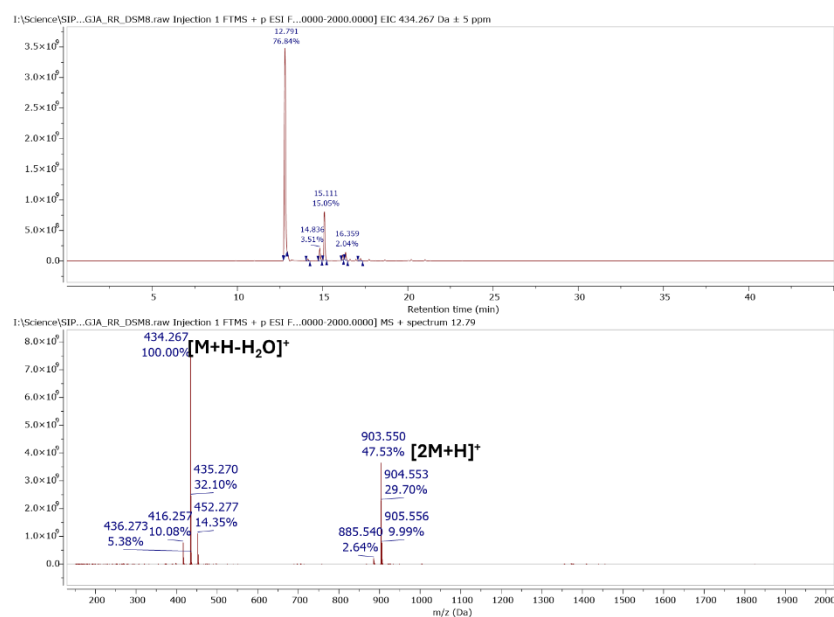


Figure S 69. HMBC of DSM9.3 as cytochalasin 10



Formula	Calculated Mass	Target Mass	Double Bond Equivalence	Absolute Error (ppm)	Error (mDa)	Error (ppm)	Fitness
1 C ₂₈ H ₃₇ N O ₄	452.27954	452.27740	11.0	4.74	-2.14	-4.74	1.000

Figure S 70. TIC and MS of DSM8.4 as cytochalasin 2

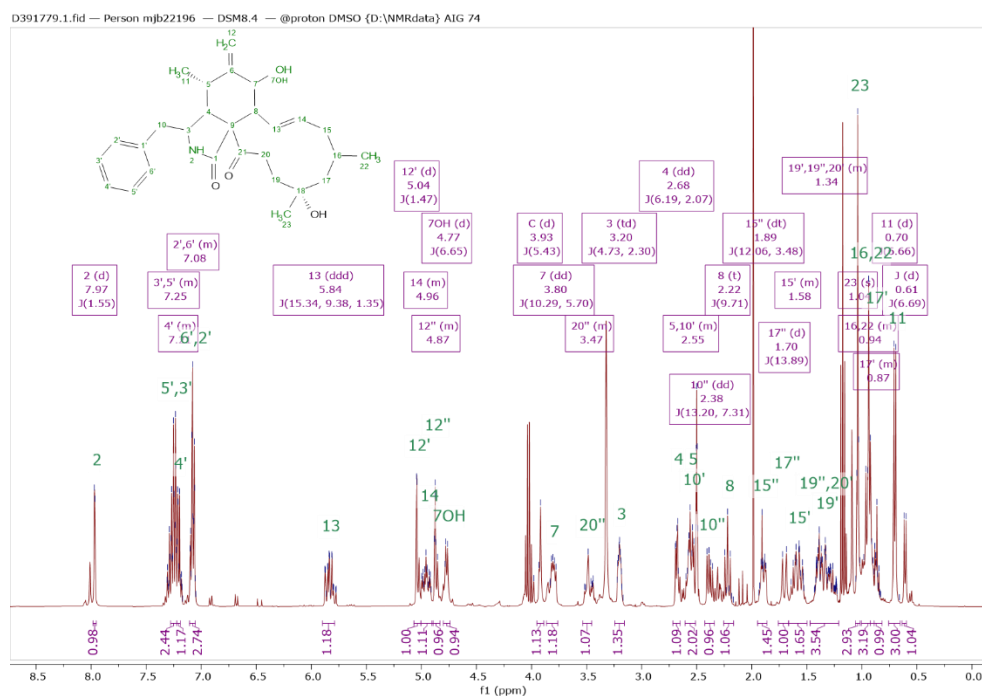


Figure S 71. ¹H-NMR of DSM8.4 as cytochalasin 2(400MHz, DMSO-d₆)

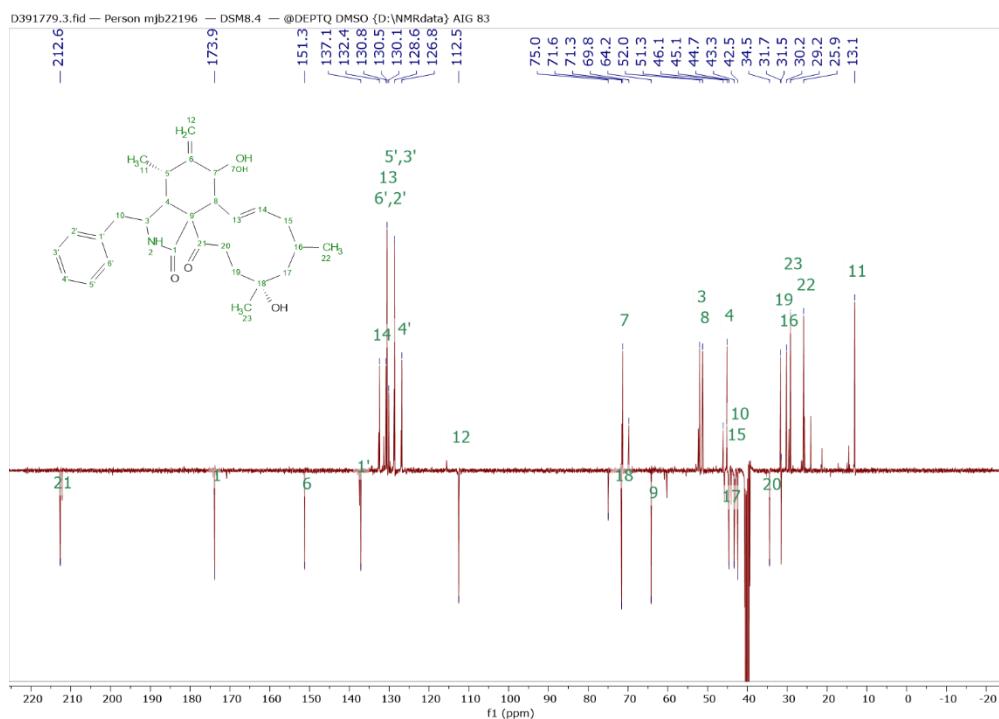


Figure S 72. DEPTQ ¹³C-NMR of DSM8.4 as cytochalasin 2 (100MHz, DMSO-d₆)



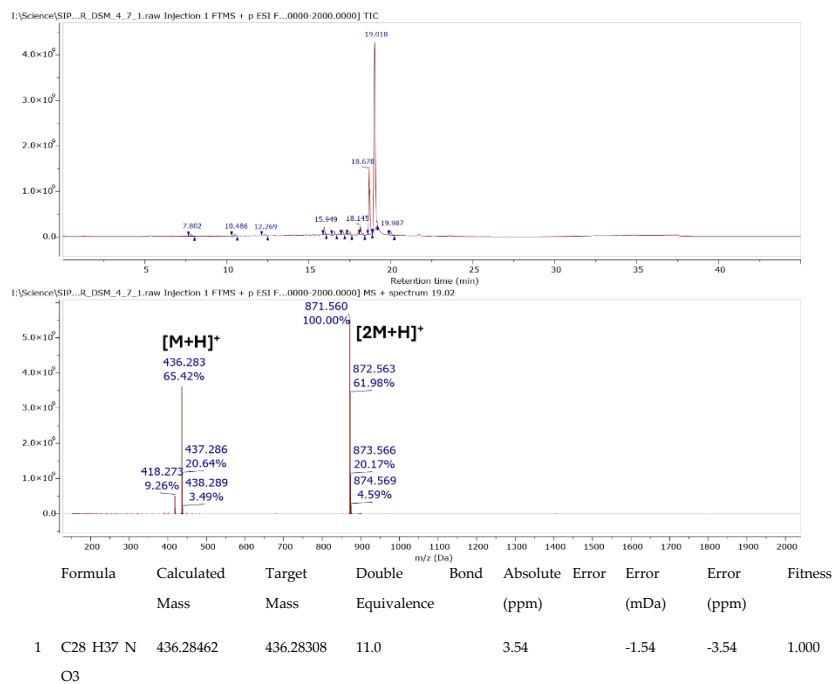


Figure S 76. TIC and MS of DSM4.7.1 as cytochalasin 7

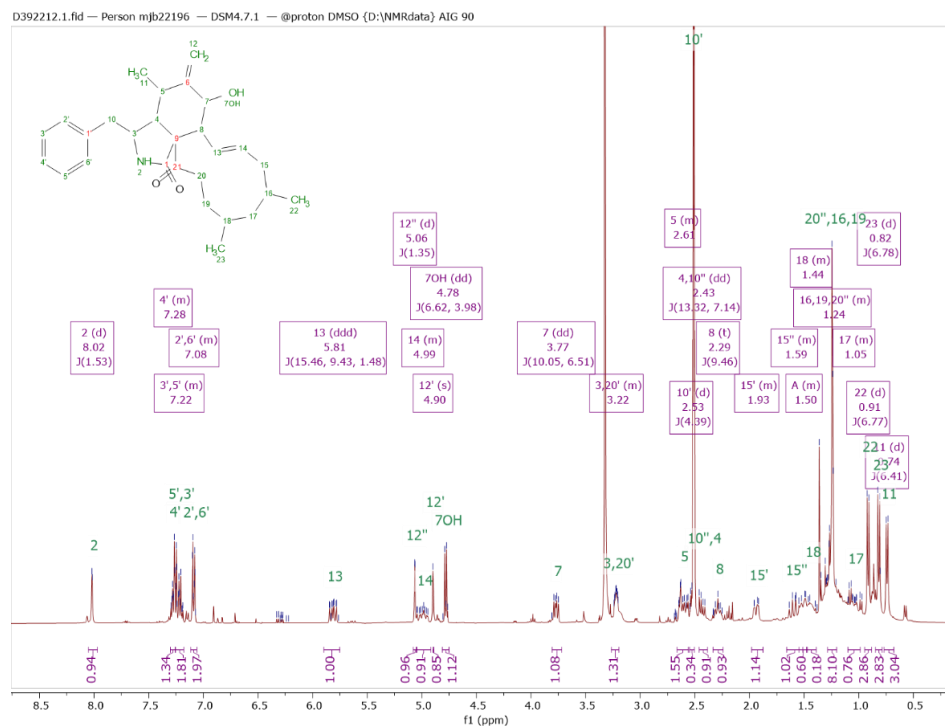


Figure S 75. 1H NMR of DSM4.7.1 as cytochalasin 7 (400MHz, DMSO-d6)

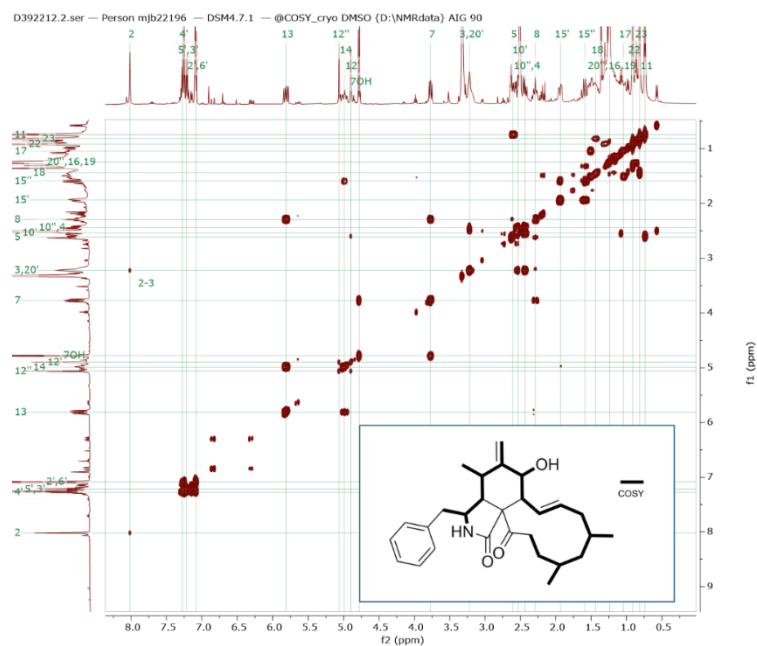


Figure S 78. COSY ^1H -NMR of DSM4.7.1 as cytochalasin 7

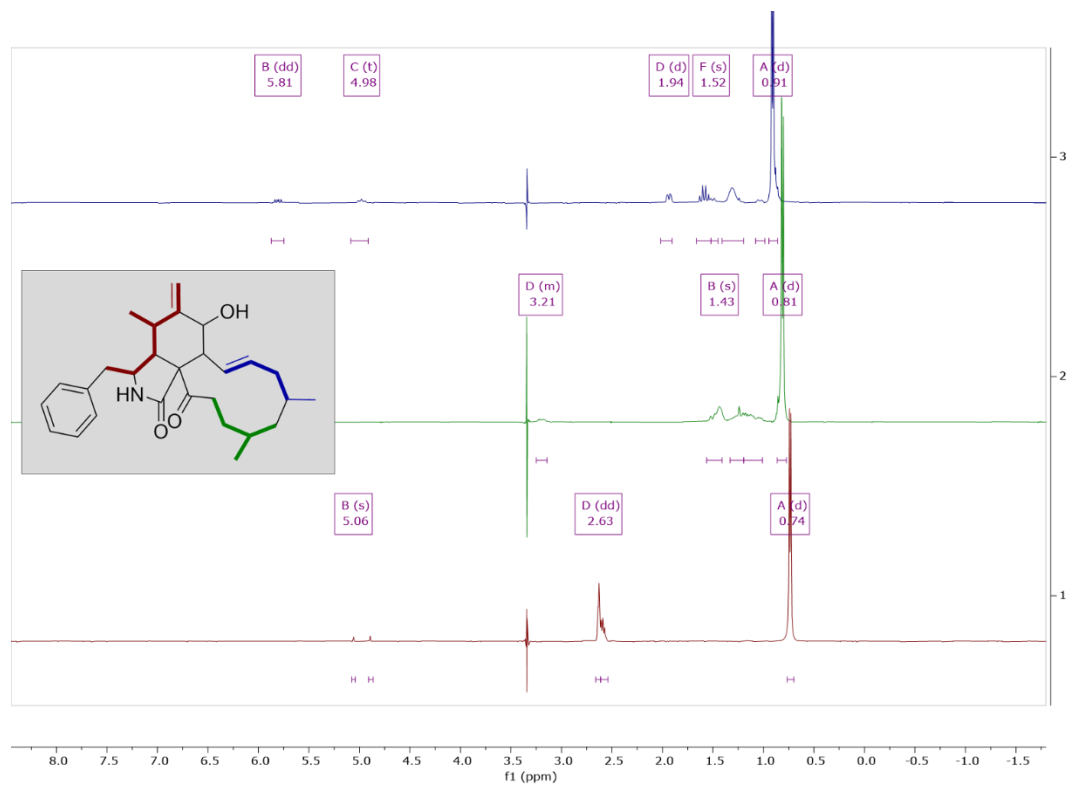
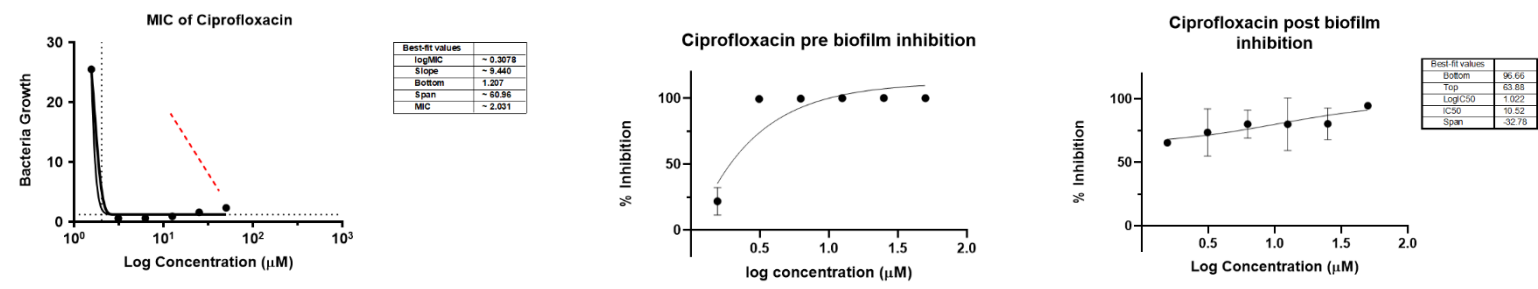


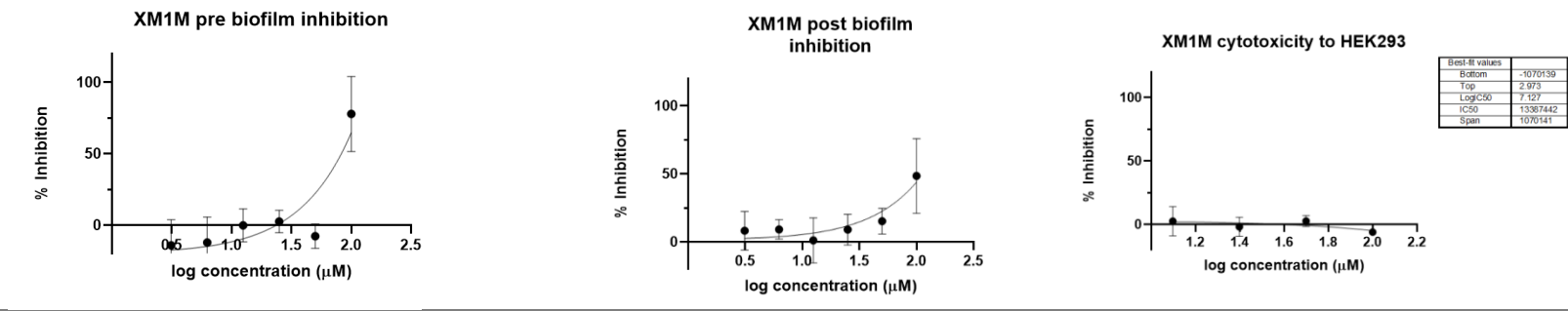
Figure S 77. 1D TOCSY of DSM4.7.1 as cytochalasin 7

Table S 1. Dose response curves of active compounds

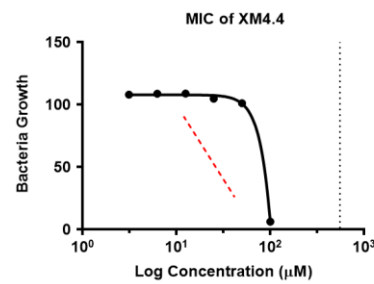
CIPROFLOXACIN- positive control in antimicrobial assays



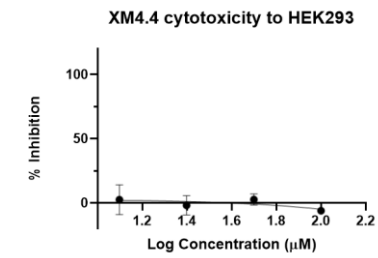
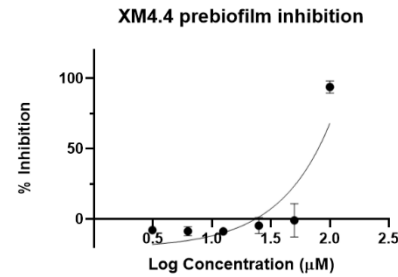
XM1M mellein



XM4.4 phloroglucinol

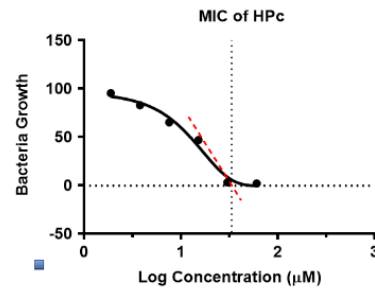


Best-fit values	
logMIC	~2.743
Slope	~8.866
Bottom	~-26862
Span	~26959
MIC	~562.9

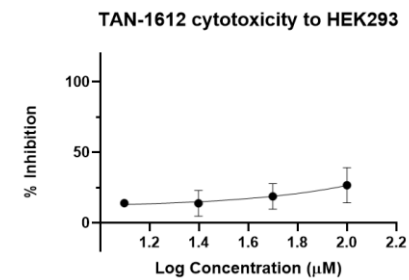
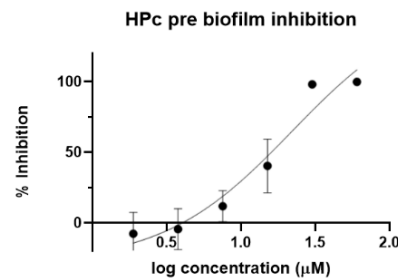


Best-fit values	
Bottom	~-1070139
Top	2.973
LogIC50	7.127
IC50	13367442
Span	1076141

HPc- TAN-1612

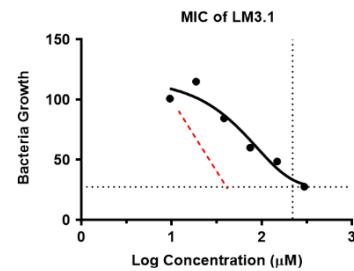


Best-fit values	
logMIC	1.823
Slope	3.361
Bottom	-0.7584
Span	96.32
MIC	33.36

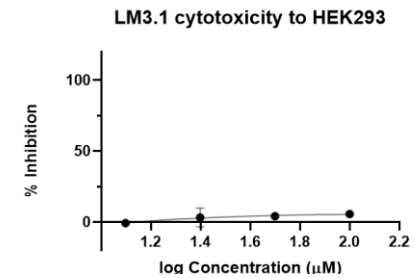
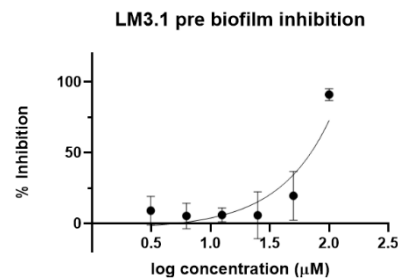


Best-fit values	
Bottom	298764
Top	10.98
LogIC50	6.285
IC50	1926939
Span	-298753

LM3.1 daldinone A

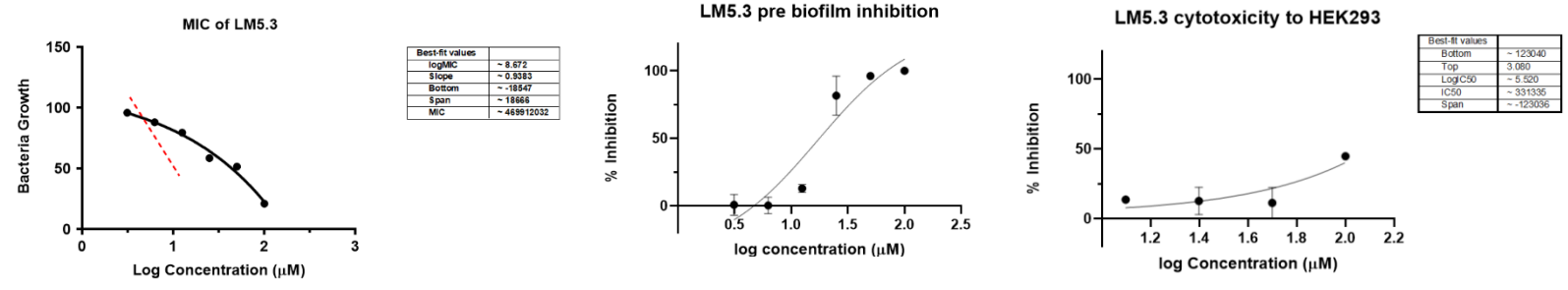


Best-fit values	
logMIC	2.341
Slope	2.364
Bottom	27.48
Span	89.28
MIC	219.5

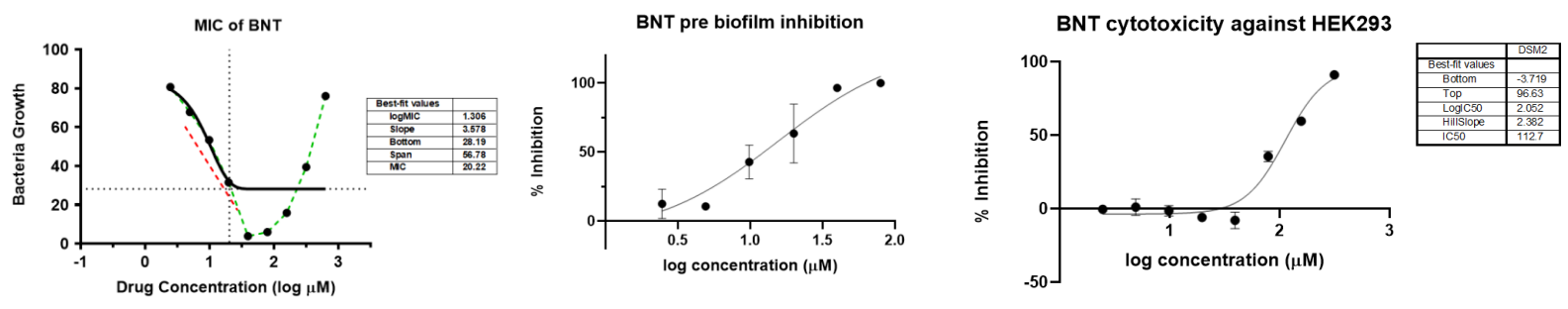


Best-fit values	
Bottom	6.293
Top	~-947.7
LogIC50	~-1.034
IC50	~-0.09249
Span	~-954.0

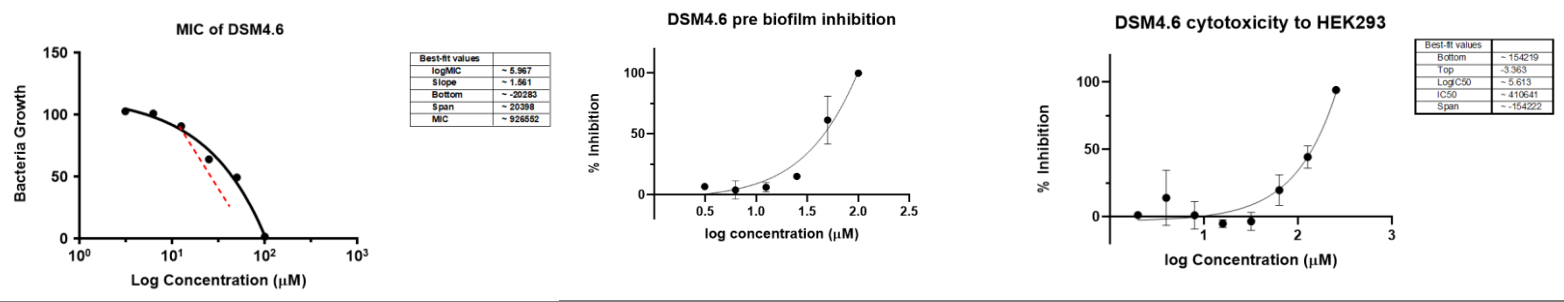
LM5.3 daldinone B:E (85:15)



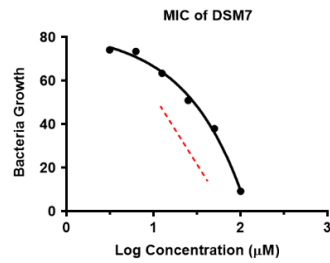
DSM 2: BNT- UNUSUAL U-SHAPED dose-response curve



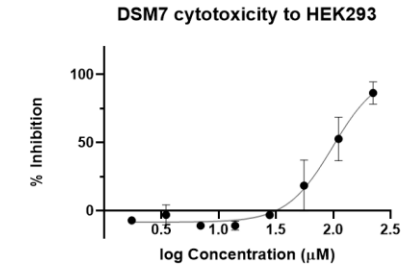
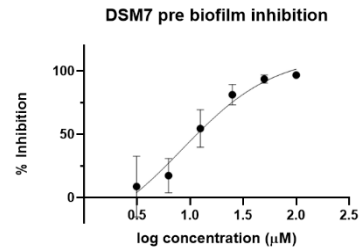
DSM4.6 orthosporin, 60%



DSM 7 cytochalasin 1

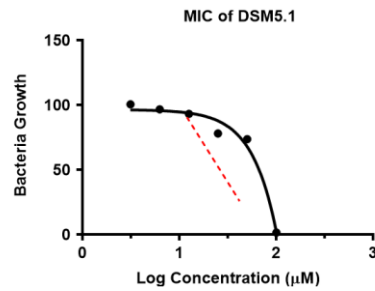


Best-fit values	
logMIC	~ 6.224
Slope	~ 1.468
Bottom	~ 14451
Span	~ 12534
MIC	~ 167502

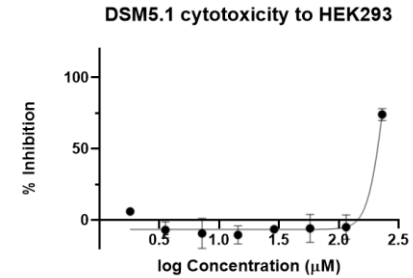
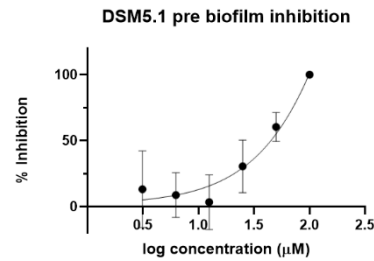


Best-fit values	
Bottom	-8.586
Top	102.3
LogC50	1.996
HillSlope	2.167
IC50	99.05

DSM5.1 cytochalasin 9

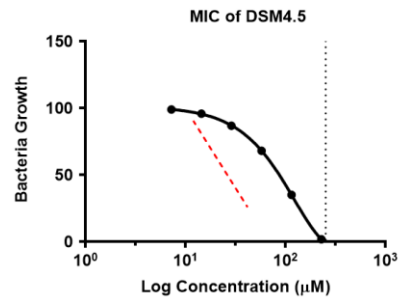


Best-fit values	
logMIC	~ 3.955
Slope	~ 3.957
Bottom	~ 79067
Span	~ 79163
MIC	~ 9011

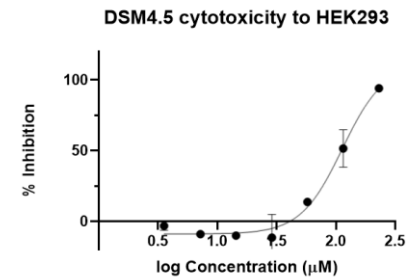
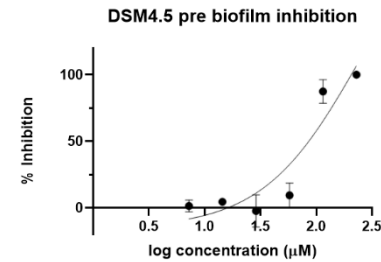


Best-fit values	
Bottom	-6.642
Top	~ 170.3
LogC50	~ 2.375
HillSlope	~ 6.470
IC50	~ 237.2

DSM4.5 cytochalasin 11

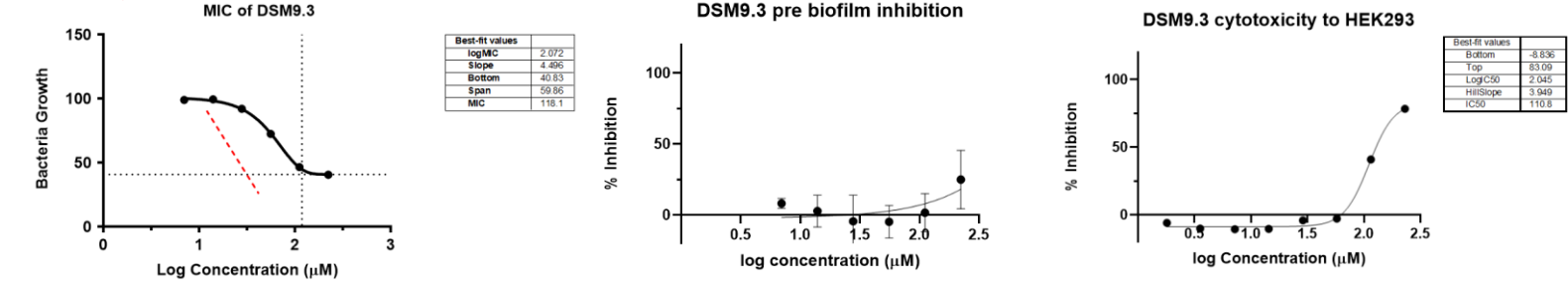


Best-fit values	
logMIC	2.402
Slope	3.146
Bottom	-6.395
Span	109.8
MIC	252.5

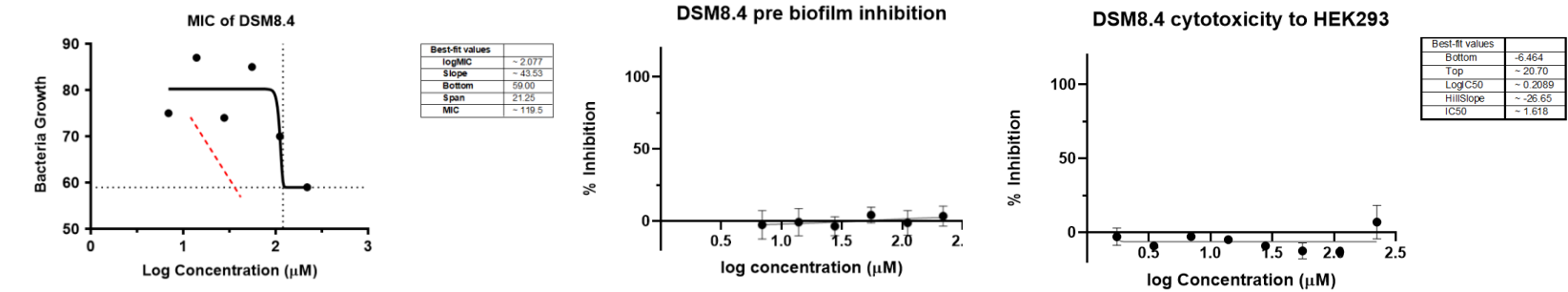


Best-fit values	
Bottom	-9.042
Top	111.3
LogC50	2.063
HillSlope	2.472
IC50	113.1

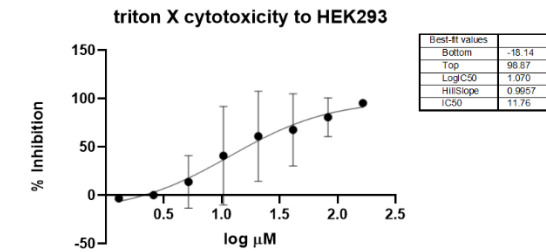
DSM9.3 cytochalasin 10



DSM8.4 cytochalasin 2



triton X-positive control for cytotoxicity assay



S 2 R Code to generate heatmaps

```

>setwd ("pathfile") ## Set the working directory

> list.files()

> data<-read.csv("data.csv files) ## load your .csv file into R and name it as data

>rownames(data)<-data$PeakID ##convert the rownames of "data" to "PeakID"

> data<-data[,2:10] ##remove unwanted columns in "data" n= Col#+number of samples

> data_matrix<-data.matrix(data) ##convert the data frame to a data matrix and name it as
"data_matrix"

>library("gplots") ##load "gplots" package

>hv<-heatmap.2(data_matrix, col=redblue(256), scale="row", margins=c(5,2),
dendrogram="column",

                Rowv=FALSE, Colv=FALSE, xlab= "Extract", ylab= "PeakID", labRow="",
                main = "Heatmap: Extract vs PeakID",
                key= TRUE, symkey=TRUE, keysize=1.2, density.info="none", trace="none")

##heatmap

>hv<-heatmap.2(data_matrix, col=redblue(256), scale="row", margins=c(5,2),
dendrogram="column",

                Rowv=FALSE, Colv=TRUE, xlab= "Extract", ylab= "PeakID", labRow="",
                main = "Heatmap: Extract vs PeakID",
                key= TRUE, symkey=TRUE, keysize=1.2, density.info="none", trace="none")

##heatmap

```