Mining for Novel Bioactive Fungal Metabolites through Precursor-directed Biosynthesis

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Abstract

Antibiotics have played a crucial role in modern medicine for more than 60 years by effectively treating infectious diseases and reducing the occurrence of infections in immunocompromised patients. Nevertheless, the excessive use of antibiotics has resulted in an increase in antibiotic-resistant bacteria, requiring the development of new antibiotics with unique mechanisms. One promising approach is exploring natural products such as fungal secondary metabolites. Precursor-directed biosynthesis (PDB) involves the use of specific precursor molecules to produce modified metabolites possessing unique activities, potentially useful in medicine, agriculture, and other fields.

PDB uses the adaptive metabolic processes of microorganisms to incorporate unnatural precursors, creating novel derivatives with potentially improved efficacy and reduced toxicity. This approach requires understanding microbial growth, precursor behaviour, and biosynthetic pathways. Advances in synthetic biology and metabolic engineering have further expanded these possibilities.

Aspergillus species, commonly found fungi, can exhibit both advantageous and detrimental effects. Certain species can cause infections and producing harmful mycotoxins, whereas *Aspergillus terreus* is known for its ability to thrive in adverse conditions and produce beneficial secondary metabolites, including lovastatin (a cholesterol-lowering drug) and antibacterial agents like terretonin.

In this study, the incorporation of phenylalanine and its derivatives into the biosynthetic pathways of *A. terreus* was investigated to enhance the production of modified aromatic metabolites. The study examined six phenylalanine derivatives: phenylalanine, dihydroxyphenylalanine, β-phenylalanine, fluoro-phenylalanine, chloro-phenylalanine, and bromo-phenylalanine. Each derivative was isolated and purified through similar extraction protocols, ensuring consistency and comparability in the resulting fractions. The fractions were then subjected to Minimum Inhibitory Concentration (MIC) assays to evaluate their antimicrobial efficacy against *Staphylococcus aureus* and *Escherichia coli*. Further chemical analysis was conducted using solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) to separate and identify the compounds present.

This study demonstrates the potential of *A. terreus* in generating bioactive secondary metabolites through PDB, offering a promising strategy for the development of novel antimicrobial agents. The findings highlight the importance of investigating fungal metabolites and metabolic engineering to enhance the biosynthetic capacity of microorganisms, in response to the demand of new antibiotics due to rising antimicrobial resistance.

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1 Introduction

1.1. Natural Products

Natural products are small organic molecules produced by living organisms such as bacteria, fungi, and plants.[1] Natural products, referred to as secondary metabolites, are generated by organisms in response to their environment or as a defence mechanism against predators to improve their chances of survival.[2] Compared to primary metabolites, which are essential to the organism's survival, secondary metabolites may differ across species, covering a wide range of complex chemical structures.[3] Nevertheless, many of these compounds have complex structures with multiple chiral centres and labile connectivity, making their chemical synthesis difficult.[3] Natural products can be synthesised chemically through both semi-synthesis and total synthesis, making significant contributions to the advancement of organic chemistry through the provision of complex synthetic objectives.[4] Alternatively, biosynthesis and fermentation techniques are suitable for synthesising such compounds in pharmaceutical, agricultural, and other relevant fields.[3]

Natural products have been a prolific source and an inspiration for numerous medical agents with widely divergent chemical structures and biological activities, including antimicrobial, immunosuppressive, anticancer, and anti-inflammatory activities, to develop into treatments that can be used therapeutically for human diseases.[5] Historically, the use of natural products has been documented in the form of traditional medicines, remedies, potions, and oils, with many of these bioactive compounds remaining unidentified.[2] The only medications available to treat human and animal diseases before the 20th century were crude and semi-pure extracts of plants, animals, microbes, and minerals.[6] However, with the rise of the receptor theory of drug action in the 20th century, specific chemical compounds in extracts turned out to play a major role in the biological activity of the drug based on the idea that the effect of the drug in the human body is mediated by their interactions with biological macromolecules, primarily proteins or nucleic acids.[6] As a result, isolated pure chemical substances became a treatment for modern diseases, followed by increased interest in knowing the chemical structure of those compounds responsible for the effects of these medicinal extracts.[6]

Despite the success of traditional natural products in drug discovery, most pharmaceutical companies reduced their natural product operations during the past two decades.[2] One of the primary reasons for this decline is the inadequacy of traditional natural product extract libraries in aligning with high-throughput screening (HTS) methods.[7] The shift towards HTS against defined molecular targets has led many pharmaceutical companies to prioritise 'screen-friendly' synthetic chemical libraries instead.[7] This change was accelerated by the introduction of combinatorial chemistry, which promised simpler, more drug-like screening libraries with wider chemical diversity.[7] Moreover, progress in molecular biology, cellular biology, and genomics has expanded the understanding of molecular targets and pathways implicated in diseases.[2] This enhanced knowledge has shortened the drug discovery timelines and prompted a shift towards the development of compounds that can more precisely target these specific molecular mechanisms.[2] From a commercial perspective, the drive for rapid screening, hit identification, and lead development often favours synthetic libraries due to their perceived

efficiency and patentable nature.[7]

However, emerging trends suggest a renewed interest in natural products in drug discovery due to their potential as a rich source of novel drugs.[6] The structural diversity and wide range of biological activities found in natural products make them ideal candidates for drug development.[6] They can interact with various biological targets and modulate different cellular processes, including enzymes, receptors, and ion channels.[6] Moreover, the safety and efficacy of natural products have contributed to their continuous use in drug development, especially in combating diseases, including cancer, infectious diseases, and neurological disorders.[6] Recent advancements in technology and increased sensitivity in instrumentation required for the rapid identification of novel bioactive natural products have addressed previous challenges associated with natural product research.[7] Breakthroughs in separation and structure-determination technologies have made it easier to study and utilise natural products in drug discovery.[7] In addition, mass spectrometry is used in various stages of the discovery process, ranging from sample selection, screening, and fractionation to identification and dereplication.[1]

1.2. Fungal Natural Products

Fungi account for approximately 30% of microbial natural products, which is growing due to increasing interest in fungal natural products.[3] Many fungi are already ideal hosts for secondary metabolite production, as they grow well on cheap carbon sources.[3] They have also shown great success with traditional strain improvement and process improvement strategies, resulting in a multi-gram per litre yield of the desired pharmaceutical products.[3] Fungi, isolated from various sources, including soil, plants, and marine environments, are a rich source of natural products with diverse chemical structures and biosynthetic pathways, making them attractive for drug discovery.[8] Some of these natural products are effective against human pathogens exhibiting broad-spectrum activity arising from their interactions with different organisms.[9,10]

Historically, actinomycetes are filamentous fungi and have been the primary source of antibiotics in human therapies, regarding them as the most potent natural source of antibiotics available.[11] Alexander Fleming's discovery of penicillin (**1**) in 1928 was a significant event that highlighted the importance of fungal natural products. In addition to penicillin, antimicrobial compounds were derived from diverse chemical classes of fungal metabolites, such as cephalosporin (**2**), echinocandins (**3**), cyclosporine (**4**) and pleuromutilin (**5**).[9] This diversity further reinforces the critical role that fungi play in the development of essential antibiotic therapies.

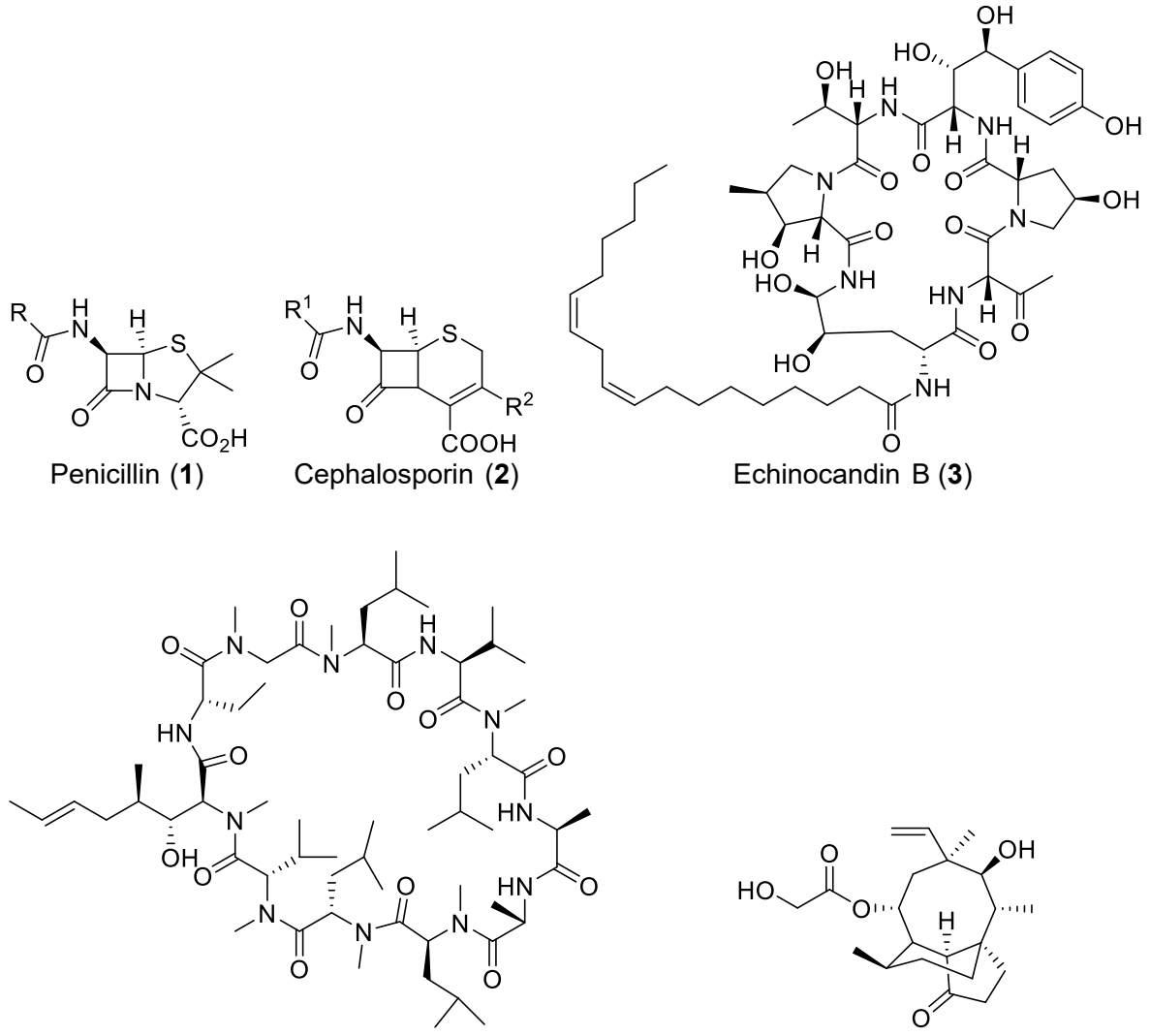


Figure 1.1. Representative structures of antimicrobial fungal metabolites.

A recent study by Grey et al. identified several strains of New Zealand fungi with antimycobacterial activity using a bioluminescence-based assay.[12] The 36 fungal isolates from Manaaki Whenua - Landcare Research's International Collection of Microorganisms from Plants (ICMP) were grown on different media and screened for activity (12 Grey, 2021). Almost all 36 fungi tested exhibited marked antimycobacterial properties, whereas 9 (25%) were identified as unknown species, representing the biodiversity endemic to Aotearoa, New Zealand.[12] This presents compelling statistical proof regarding how growth media affects antibacterial activity and suggests there is still much to be learned about the potential of fungi for drug discovery.[12]

1.3. Precursor-directed Biosynthesis

There are several biological or chemical routes to natural products and their derivatives, but each has its limitations, such as failure of the desired application or unwanted side effects

depending on the target.[13,14] A common approach in this field is semi-synthesis, where the natural product is first extracted from its native producer and then chemically modified.[14] Despite this, the manipulation of living organisms or their components to produce desirable compounds has resulted in the synthesis of novel molecules, providing new leads in drug development and other applications.[13,15] This bioengineering approach not only enhances the yield and specificity of these compounds but also enables the creation of entirely new structures with potentially improved efficacy and reduced toxicity.[16] Advances in synthetic biology and metabolic engineering have further expanded the possibilities, allowing researchers to design and construct metabolic pathways in microorganisms to produce complex natural products that are difficult to obtain through traditional methods.[16] This has opened up new avenues for the discovery and production of pharmaceuticals, agrochemicals, and other valuable bioactive molecules.

Precursor-directed biosynthesis (PDB) is a powerful method for valuable derivatisation of microbial lead structures that involves the incorporation of chemically synthesised substrates for enzymatic transformations.[13,14] PDB is performed by introducing biosynthetic precursor analogues with different functional groups, which the organism can subsequently use to produce modified metabolites through enzymatic processes.[13] The basic idea behind PDB is to redirect metabolic pathways by supplying precursor molecules used as building blocks for target compounds.[17] This method takes advantage of the metabolic versatility of microorganisms to incorporate unnatural building blocks, consequently expanding structural diversity in natural product analogues.[17] By incorporating the precursor related to the desired compound, biosynthetic pathways can be modulated to enhance productivity or generate novel derivatives with improved biological activities.[18]

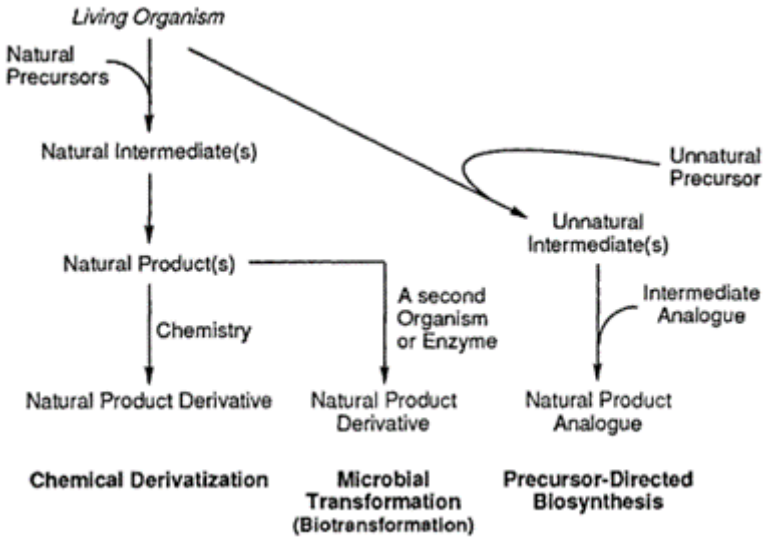


Figure 1.2. An overview of precursor-directed biosynthesis besides other biological derivatisation methods.[13]

However, the precursor-directed biosynthesis (PDB) method relies on a series of prerequisites for its successful implementation. It is necessary to understand the underlying microbial growth, the specific behaviour of the unnatural precursor to be supplied, and, most importantly, the biosynthetic assembly of the secondary metabolite.[13] Information about microbial growth includes how the microorganism processes different substrates and how it responds to external stimuli like the introduction of artificial precursors.[13] Besides, the feeding conditions of the artificial precursors play a decisive role in optimising the synthesis process. This includes the timing and amount of the precursor to be added to the culture to ensure the maximum yield of the desired product.[13] A precursor analogue is often transported into the microbial cells like the natural precursor or can enter the biosynthetic pathway via other transport mechanisms, which allows it to compete with endogenous substrates.[13] Here, such intracellular checkpoints may prevent cells from accumulating undesirable intermediates and byproducts that cannot produce the final product.[18] Therefore, understanding the transport mechanisms for natural and artificial precursors is required. Comprehensive knowledge of how the microorganism modifies the natural precursors and their analogues enzymatically to enter the biosynthetic pathway can also enable the rational design of effective PDB strategies.[13] One way to track the fate of the precursors being degraded into their basic building blocks or biosynthetic intermediates is by incorporating labelled precursors into the growth medium or comparing those to well-known examples.[13] Consequently, having convenient systems for analysing natural and modified metabolites, such as HPLC and LC-MS, is essential to monitor the production of desired products and validate the success of precursor-directed modifications.[13]

As microorganisms have complex biosynthetic pathways capable of introducing modifications in a controlled and efficient way, one of the major advantages of precursor-directed biosynthesis (PDB) is its ability to produce modified metabolites that may be difficult to obtain by chemical synthesis.[3] In the production of new secondary metabolites via PDB, the enzymes in those metabolic pathways are often relatively less selective and can accept alternative analogues or incomplete products from earlier steps.[19] From an economic perspective, PDB offers a cost-effective way to get structurally modified natural products for a fast and efficient evaluation of structure-activity relationships, enhancing those from chemical synthesis.[13] Furthermore, PDB is a practical and straightforward method that does not require specific genetic mutations or enzyme inhibitors, unlike other biological derivatisation methods.[13] PDB is performed directly with the producing organism without requiring screening multiple strains for desired microbial transformations. Therefore, PDB avoids time-consuming processes, which makes it much faster.[13]

1.4. *Aspergillus*

Aspergillus is a filamentous fungus found in diverse habitats, including soil, decaying organic matter, and indoor environments.[20,21] *Aspergillus* consists of about 250 named species, and a few of these species are considered opportunistic pathogens that can cause infections, particularly in immunocompromised individuals.[21,22] Furthermore, certain strains of *Aspergillus* produce mycotoxins, which can contaminate food and feed, posing risks to human and animal health.[21] *Aspergillus* species is also a harmful pathogen to crops, which may even act as a human pathogen to cause aspergillosis.[20,23] Therefore, *Aspergillus* in indoor environments

were shown to be a public health hazard worldwide.[21]

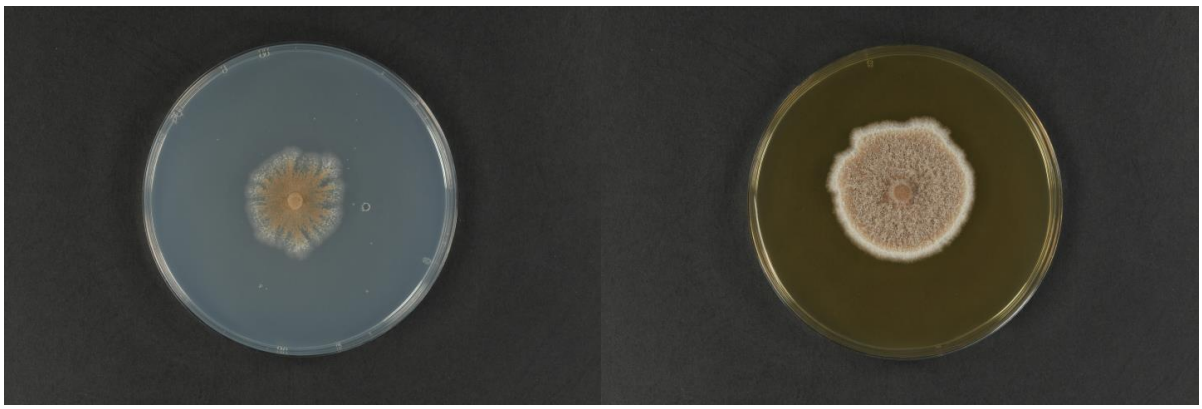


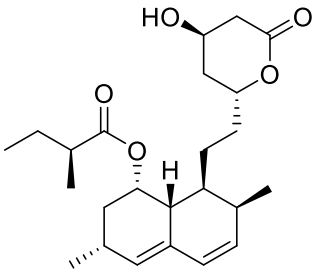
Figure 1.3. *Aspergillus terreus* 477.

However, *Aspergillus* has received attention for its unique features and diverse applications, from pharmaceuticals to biotechnology. Unlike other microbial cell factories, many species of *Aspergillus* tolerate extreme cultivation conditions.[22] For example, *Aspergillus* can be cultivated over a wide range of temperatures (10–50 °C), pH (2–11), salinity (0–34%), water activity (0.6–1) and under oligotrophic or nutrient-rich conditions.[22] *Aspergillus* constantly fights off predators and competitors to live alongside them in their specific environmental niche.[24] They must adapt to these dynamic environments and compete for limited nutrients, allowing them to reproduce and disseminate, which is necessary for survival.[24]

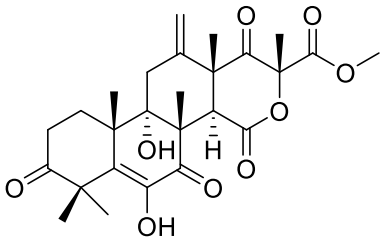
In response to their environment, *Aspergillus* have developed the ability to produce a wide range of secondary metabolites, which can be used in survival strategies.[23] Many of these secondary metabolites have significant biological activities that have potential for industrial applications in various fields, including medicine, agriculture, and others.[25,26] One of the most important secondary metabolites is lovastatin, a cholesterol-lowering drug, of which *Aspergillus terreus* was the initial source.[20] The clinical success of lovastatin highlights the relevance of secondary metabolites derived from natural sources.[20] Furthermore, *A. terreus* is employed in the biotechnological production of enzymes and organic acids due to its ability to secrete these compounds efficiently.[24]

Among its numerous species, *Aspergillus terreus* is a promising prospective source for drug discovery since it is rich in diverse bioactive secondary metabolites.[27] Many new secondary metabolites have been isolated and reported from *A. terreus*, originating from different chemical classes, such as polyketides, alkaloids, and mycotoxins.[27] Lovastatin (**6**), used in cholesterol-lowering treatments, belongs to the family of polyketides. Similarly, terretonin (**7**), a polyketide-derived metabolite, exhibits a distinct chemical scaffold with antibacterial properties. Territrems and territrem B (**8**) represent tremorgenic mycotoxins, characterised by their neurotoxic effects mediated through interactions with acetylcholinesterase.[28] Furthermore, butyrolactone I (**9**) hold a broad scope of biological activities such as antioxidant,

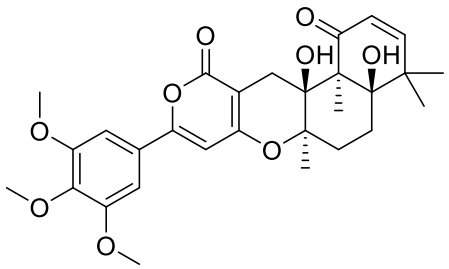
antidiabetic, antitumour, and antiapoptotic.[27]



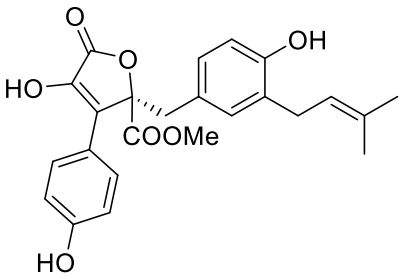
Lovastatin (**6**)



Terretonin (**7**)



Territrem B (**8**)



Butyrolactone I (**9**)

Figure 1.4. Chemical structures of secondary metabolites isolated from *Aspergillus terreus* 477.

The biosynthesis of these secondary metabolites from *A. terreus* has also been reported. The biosynthetic pathway of aspulvinones exemplifies the intricate enzymatic machinery employed by *A. terreus* to synthesise structurally diverse and biologically active secondary metabolites.[29] The aspulvinone skeleton is derived from the shikimate pathway, contributing to the assembly of the basic building blocks for the range of aromatic metabolites and amino acids.[30] The biosynthesis of aspulvinone begins with the synthesis of shikimic acid, which is a precursor for aromatic primary metabolites such as tyrosine and phenylalanine.[31] Phenylalanine is an essential amino acid for the synthesis of other amino acids, proteins, and other vital molecules like neurotransmitters and hormones.[32] The shikimate pathway produces L-phenylalanine (L-Phe) and L-tyrosine (L-Tyr), which undergoes dimeric condensation (**Figure 1.5**).[33] As a proof principle, in this study, the incorporation of phenylalanine and its derivatives into the biosynthetic pathways of *Aspergillus terreus* 477 be investigated to produce modified aromatic metabolites. This approach highlights the potential of metabolic engineering to harness and enhance the natural biosynthetic capabilities of microorganisms, enabling the production of novel compounds with improved or unique biological activities.

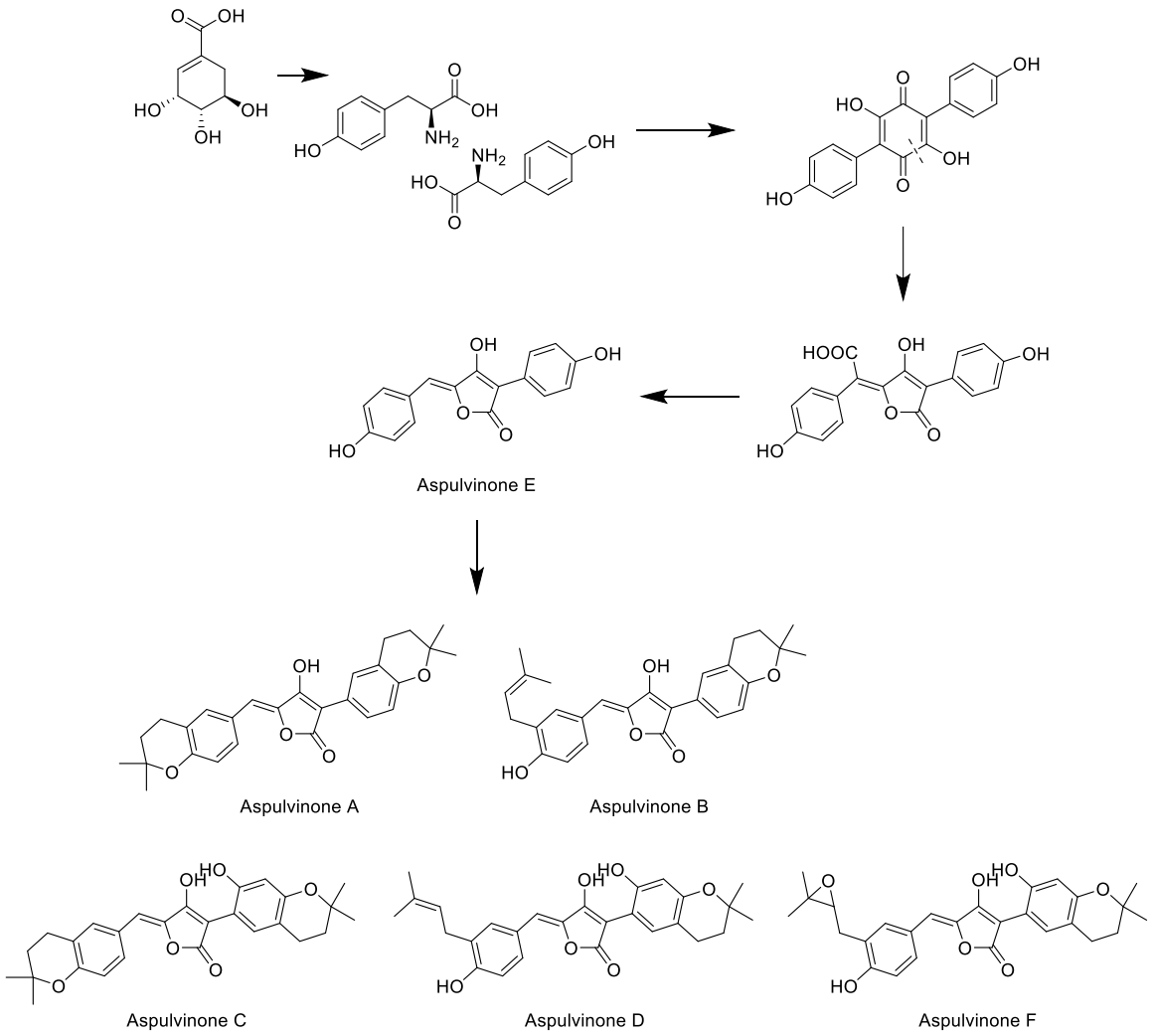


Figure 1.5. Biosynthetic pathway to Aspulvinones from the incorporation of phenylalanine.

1.5. Dereplication Methods

A reliable dereplication method is required to reduce the detection of known constituents at early screening stages for natural products, given the high rates of rediscovery associated with this process.[25] Traditional methods identify active compounds from a primary screen, involving the regrowth of the microorganisms in larger quantities with extraction and analysis using nuclear magnetic resonance (NMR), which is time-consuming and labour-intensive.[25] Consequently, screening efficiency is improved, and large-scale cultivation is avoided by removing redundant compounds at early screening stages.[25]

High-performance Liquid Chromatography (HPLC) is a versatile and robust technique widely used to isolate natural products.[26] This chromatographic method is highly effective in separating complex mixtures, which is essential in analytical chemistry for the identification, quantification, and purification of individual components.[26] HPLC has become increasingly preferred among analytical techniques, particularly for the separation of natural products in

complex matrices, such as crude extracts for selective detection and quantification.[27] Typically, the isolation of natural products is followed by the evaluation of crude extracts in biological assays to comprehensively characterise their properties.[26] The resolving capacity of HPLC is well-suited for quickly analysing complex samples containing multiple components.[26]

Due to its high sensitivity and fast screening capabilities compared to many other separation and identification techniques, tandem mass spectroscopy (MS/MS) is emerging as a vital dereplication tool for natural product detection in complex extracts.[28] After ions generated by LC-MS analysis are isolated based on their mass intensity (*m/z*), the MS/MS technique could be used to identify the chemical structure of the compounds in a sample by the fragmentation of those ions.[28,36] This MS/MS data would be uploaded onto the Global Natural Product Social Networking (GNPS) platform, which could be used to compare the compounds discovered with those already identified by others.[36] As a result, dereplicating known compounds isolated from fungi has become easier using LC-MS/MS coupled with GNPS.

1.6. Aims

We hypothesise that through enzymatic reactions, the microorganisms will utilise the non-natural precursors to generate modified metabolites that can exhibit unique biological activities that have potential applications in various fields, including medicine, agriculture, and others.[25,28] Previous studies have shown that phenylalanine, when used as a substrate, can potentially enhance the synthesis of different secondary metabolites.[37]

The main aims of this study were to:

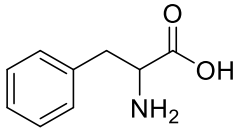
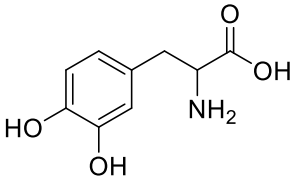
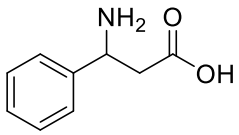
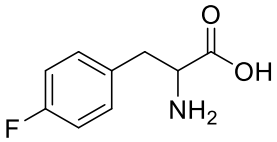
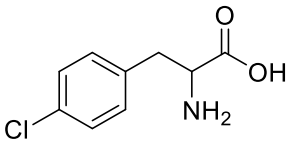
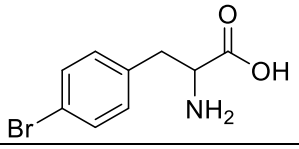
1. Grow fungal isolates of *Aspergillus terreus* 477 by itself and in the presence of phenylalanine and phenylalanine analogues.
2. Investigate the antimicrobial activity of extracts against *E. coli* and *S. aureus*.
3. Fractionate extracts to separate out secondary metabolites from sugars and fatty acids.
4. Investigate the antimicrobial activity of the fractions against *E. coli* and *S. aureus*.
5. Analyse all the crude and subfractions generated by HPLC and LC-MS to narrow down the source of activity.

2 Results and Discussion

2.1 Preparation of Fungal Cultures

Fungal isolate ICMP 477 was incubated with different precursors on plates of potato dextrose agar (PDA) and agar at room temperature for 4 weeks (**Figure 2.1**). The precursors consist of phenylalanine and its derivatives, whose chemical structures are shown in **Table 2.1**. The fully grown plates were then freeze-dried for up to two days to remove all the moisture from the agar in preparation for extraction.

Table 2.1. List of precursors used in this study.

Precursors	Chemical Structure
Phenylalanine	
Dihydroxyphenylalanine (DOPA)	
β-Phenylalanine	
p-Fluoro-phenylalanine	
p-Chloro-phenylalanine	
p-Bromo-phenylalanine	

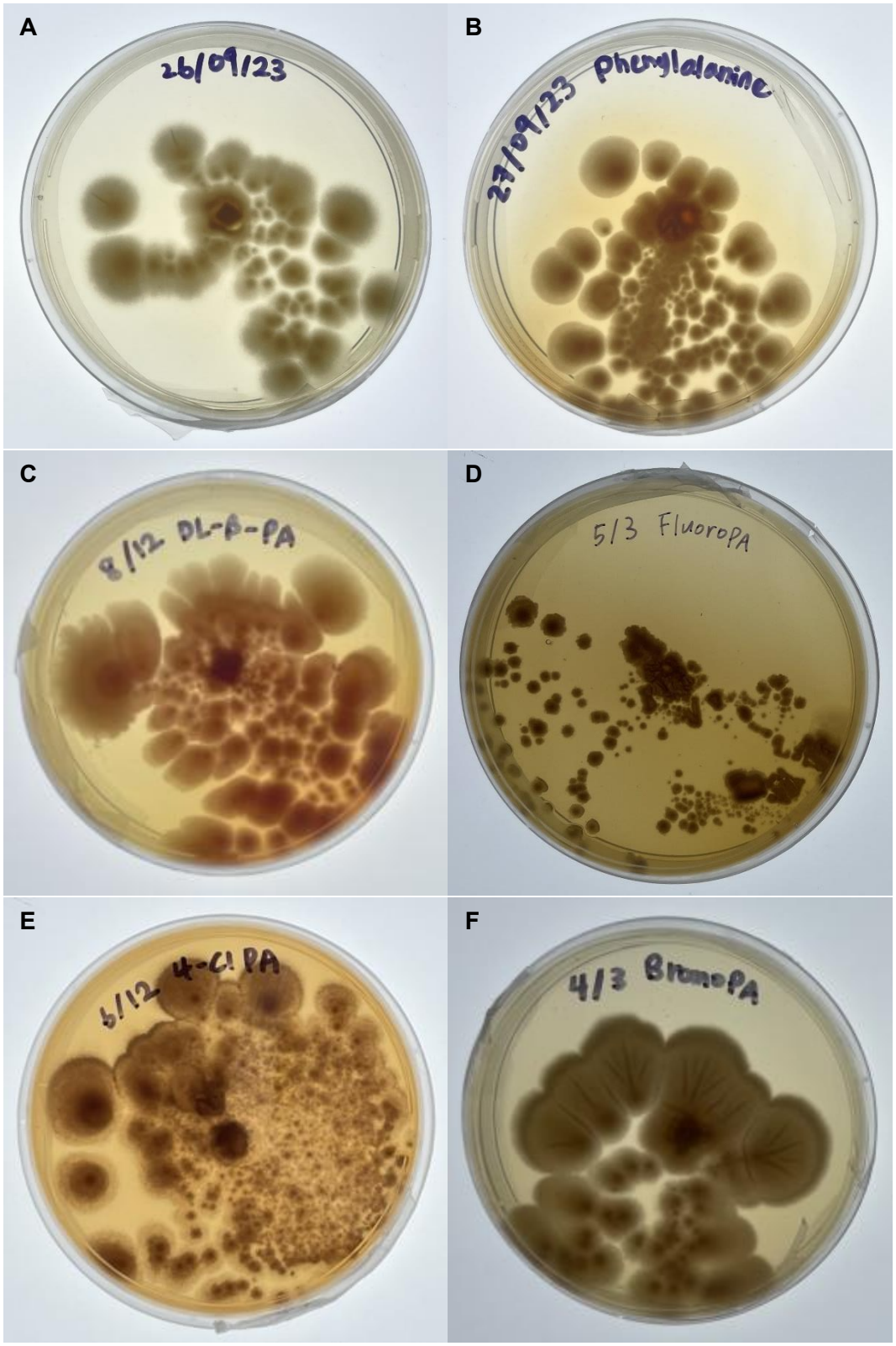


Figure 2.1. Aspergillus Terreus Growth (A) Control. (B) Phenylalanine. (C) Beta-phenylalanine. (D) Fluorophenylalanine. (E) Chlorophenylalanine. (F) Bromophenyalanine.

2.2 Fungal Extraction

To extract bioactive compounds from fungal cultures, the freeze-dried plates containing the fungal material were broken into smaller pieces and submerged in methanol (MeOH), followed by dichloromethane (DCM), ensuring the extraction of any remaining compounds. After sufficient soaking, the solvents were filtered and collected for subsequent drying.

2.3 Antimicrobial Activity Testing of the Crude

The antimicrobial activity of seven fungal extracts, with or without phenylalanine and its derivatives, was assessed using a bioluminescence-based assay (**Table 2.2**). The crude extracts were serially diluted 2-fold across the wells of 96-well plates, with concentrations ranging from 0.016 to 1 mg/mL and final volumes of 50 mL, plated in duplicate (**Figure 2.2**). The antimicrobial activity of fungal extracts with different precursors was studied against two multi-drug resistance bacterial strains, *S. aureus* (Gram-positive) and *E. coli* (Gram-negative). In addition, rifampicin and erythromycin were used as positive bacterial inhibitor standards for Gram-positive and Gram-negative bacteria, respectively.

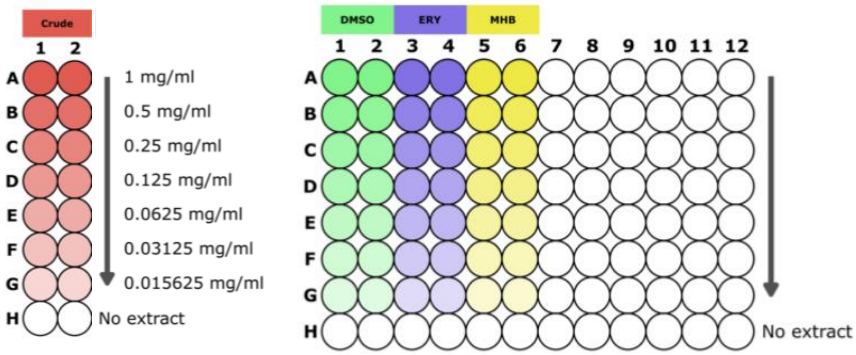


Figure 2.2. Plate layout for MIC testing of crude.

Minimum inhibitory concentrations (MICs) of the crude extracts against *E. coli* and *S. aureus* are shown in **Table 2.2**. Dihydroxyphenylalanine (DOPA) and bromophenylalanine (Br-PA) were the most effective precursors against both *E. coli* and *S. aureus*, with the lowest MIC values. In contrast, beta-phenylalanine (β-PA) was inactive against both bacteria at the concentrations tested. Phenylalanine (PA), chlorophenylalanine (Cl-PA), and fluorophenylalanine (F-PA) exhibited moderate antibacterial activity against *S. aureus* but were inactive against *E. coli* at the concentrations tested. The absence of antibacterial activity in the control against both bacterial species highlighted the important role of the precursors on bacteria exhibiting different minimum inhibitory concentration (MIC) values.

Table 2.2. Activity of crude ICMP 477 extracts against *E. coli* and *S. aureus* in MIC assay.

Additive	**Bacterium**	**Extract MIC (µg/mL)**
		Crude
None (Control)	*E. coli*	>1000
	S. aureus	>1000

PA	*E. coli*	>1000
	S. aureus	500
DOPA	*E. coli*	250
	S. aureus	125
Cl-PA	*E. coli*	>1000
	S. aureus	500
β-PA	*E. coli*	>1000
	S. aureus	>1000
F-PA	*E. coli*	>1000
	S. aureus	500
Br-PA	*E. coli*	250
	S. aureus	125

The minimum inhibitory concentration (MIC) was defined as the lowest concentration at which an extract could cause a minimum 90% (1-log) reduction in area under curve (AUC) values compared to controls containing 4% v/v DMSO. Extract fractions highlighted in red were not active at 1000 µL/mL, and blue was active below 1000 µL/mL. Key: PA, Phenylalanine; DOPA, Dihydroxyphenylalanine.

2.4 Fractionation

All extracts were fractionated using C_8 reversed-phase column chromatography. The crude was dissolved and loaded onto a silica column, eluting with a gradient of solvent starting with a higher proportion of water and gradually increasing the proportion of methanol (**Table 2.3**). Each fraction was collected separately and concentrated by rotary evaporation. For some extracts, extra fractions were collected to ensure that all remaining compounds were eluted from the column or to separate different colour bands observed during the elution.

Table 2.3. Composition of the solute used to elute each fraction.

Extract Fraction	Water (%)	Methanol (%)	Typical Compounds Present
1	100	0	Sugars
2	75	25	Amino acids, peptides, and proteins
3	50	50	
4	25	75	
5	0	100	Fatty acids and sterols

2.5 High-performance Liquid Chromatography (HPLC) Analysis

The crude extract samples were processed through solid-phase extraction (SPE), dissolved in 50:50 acetonitrile with formic acid (FA/ACN) and injected into the HPLC system at a flow rate of 1mL/min using specified solvent gradients detailed in **Table 2.4**.

Table 2.4. Solvent gradient over time during a single 45-minute run by HPLC

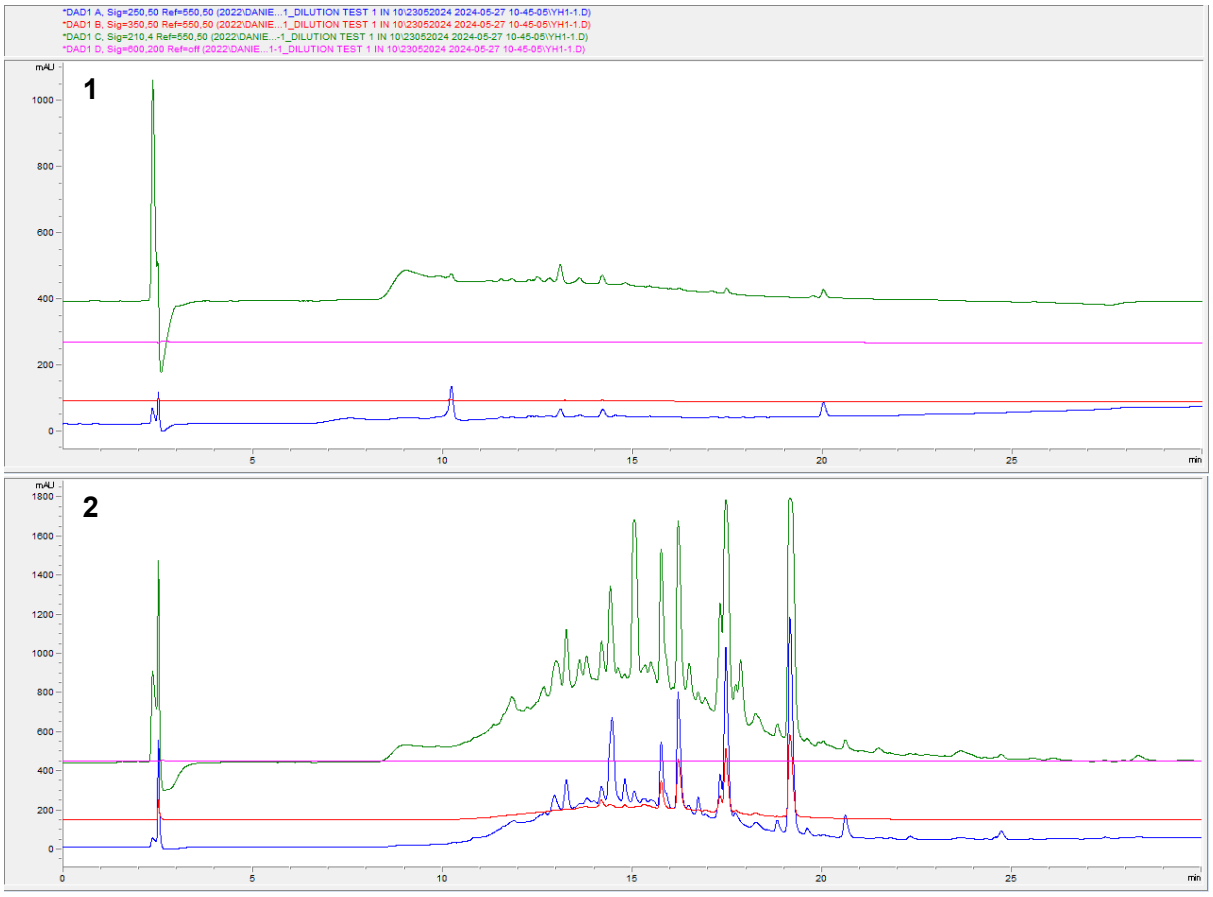
Time (min)	0.1% FA/water (%)	0.1% FA/ACN (%)
0	95	5
3	95	5
28	40	60

37	5	95
40	5	95
41	95	5
45	95	5

The HPLC profiles of seven fungal extracts with or without additives are presented in **Figures 2.3 - 2.6**. The patterns of each extract were established according to the relative retention times (RRTs) and peak areas (RPAs). The comparison of this data revealed distinct clustering of the extracts based on the type of substrate used, suggesting significant metabolic changes resulting from different substrates.

Crude (**Figure 2.3**)

No distinct peaks were observed in the crude extract of the control. In comparison, three small peaks were observed in β-PA additive suggesting the presence of three novel compounds or the enhancement of previously dormant compounds. In all others there was a visible change in the fungal metabolic profile. The largest change was observed in PA where several new peaks, not present in the control were observed. For the DOPA extract, there were one or two distinct peaks that stood out. Both the Cl-PA and F-PA extracts showed the production of a dominant peak at different retention times in comparison to each other, with a comparable peak height to PA.



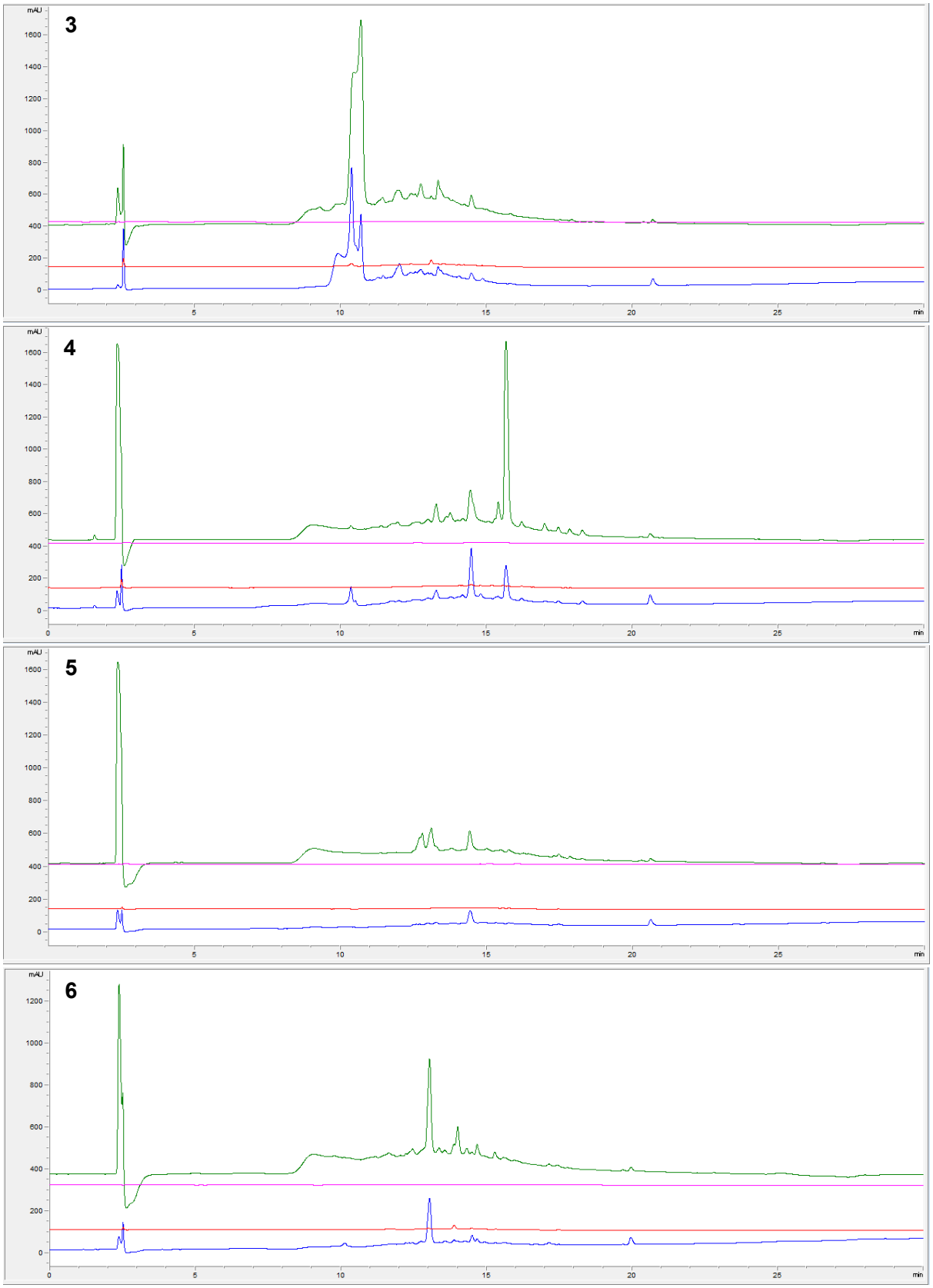
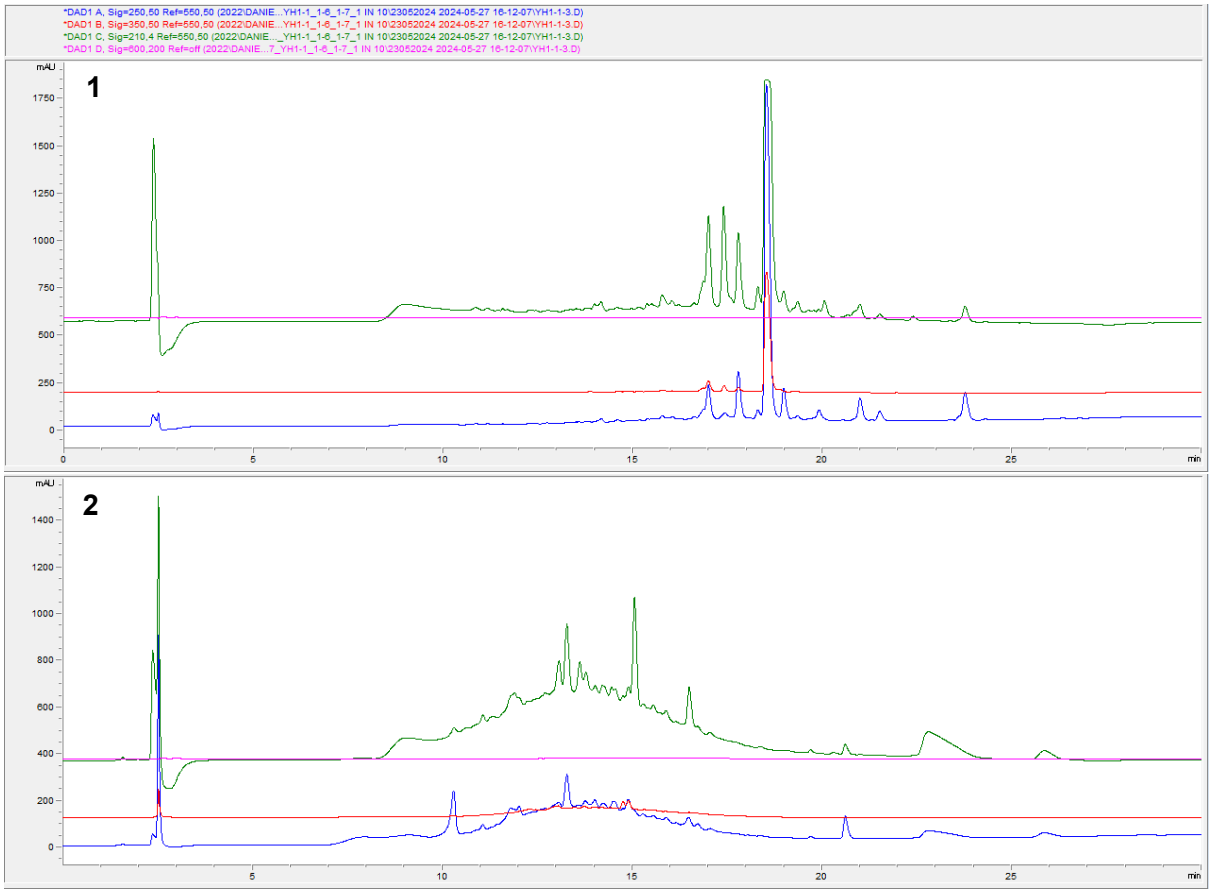


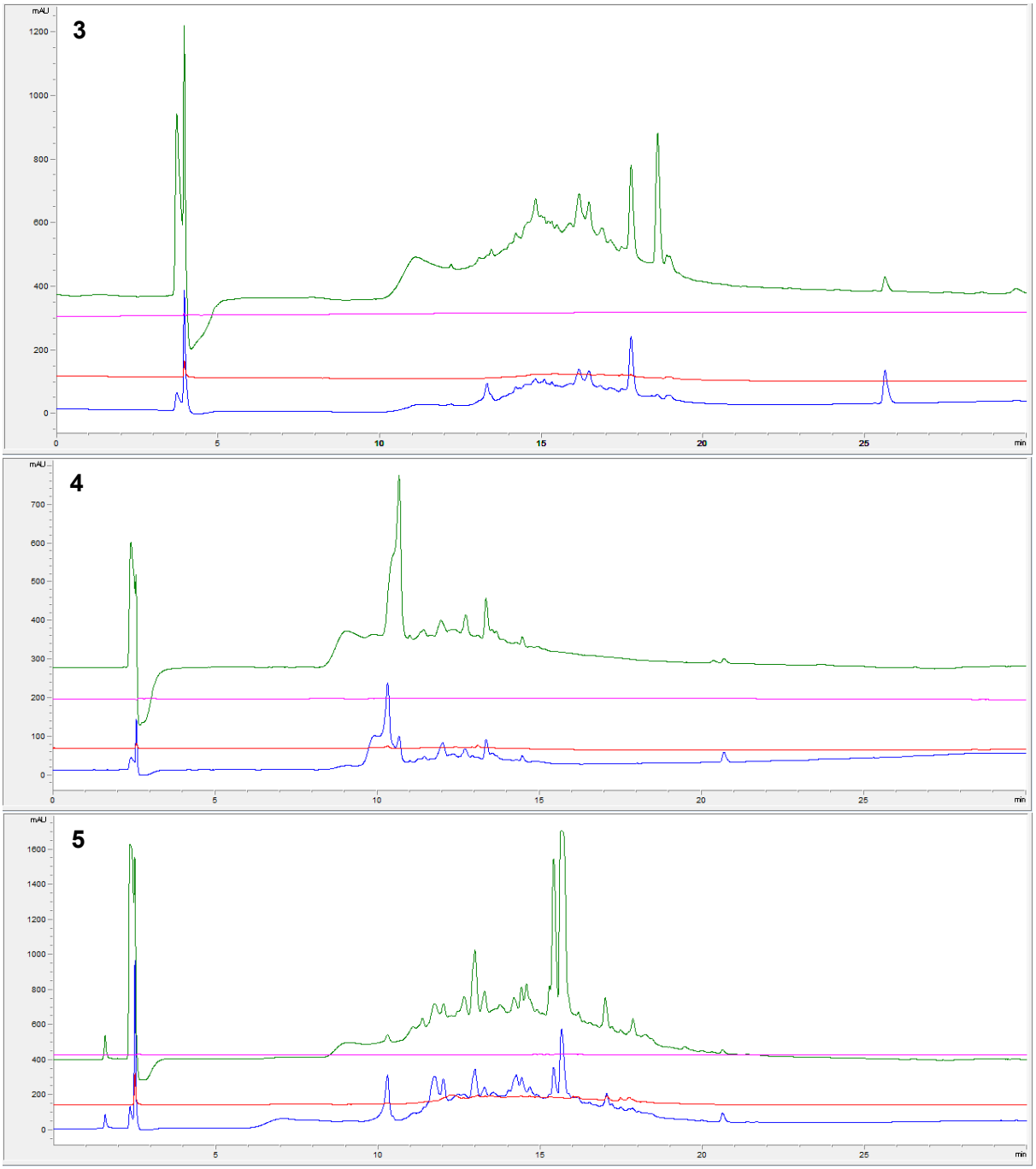
Figure 2.3. HPLC chromatograms of ICMP 477 extracts with (1) no substrate, (2) PA, (3) DOPA, (4) Cl-PA, (5) β-PA, and (6) F-PA in crude. (HPLC eluent: 0-45 min, 5-95% 0.1%

FA/ACN and 0.1% FA/water, flow rate: 1 mL/min).

Fraction 3 (**Figure 2.4**)

Compared to the control, all extracts containing an additive exhibited the presence of numerous compounds due to the peaks, which are not present in the control. These peaks appeared to be shifted towards a shorter retention time. The fractions for DOPA were collected separately based on the colour of the bands as fraction 3 and 3.1. Both exhibited similar peak patterns, with variations in the presence of certain peaks at distinct retention times. The Cl-PA exhibited a distinct doublet that was not observed in the control. β-PA and F-PA showed a similar profile of peaks with varying magnitudes. Similarly, the production of one or two distinct compounds was observed in the Br-PA, which was not readily apparent in the control.

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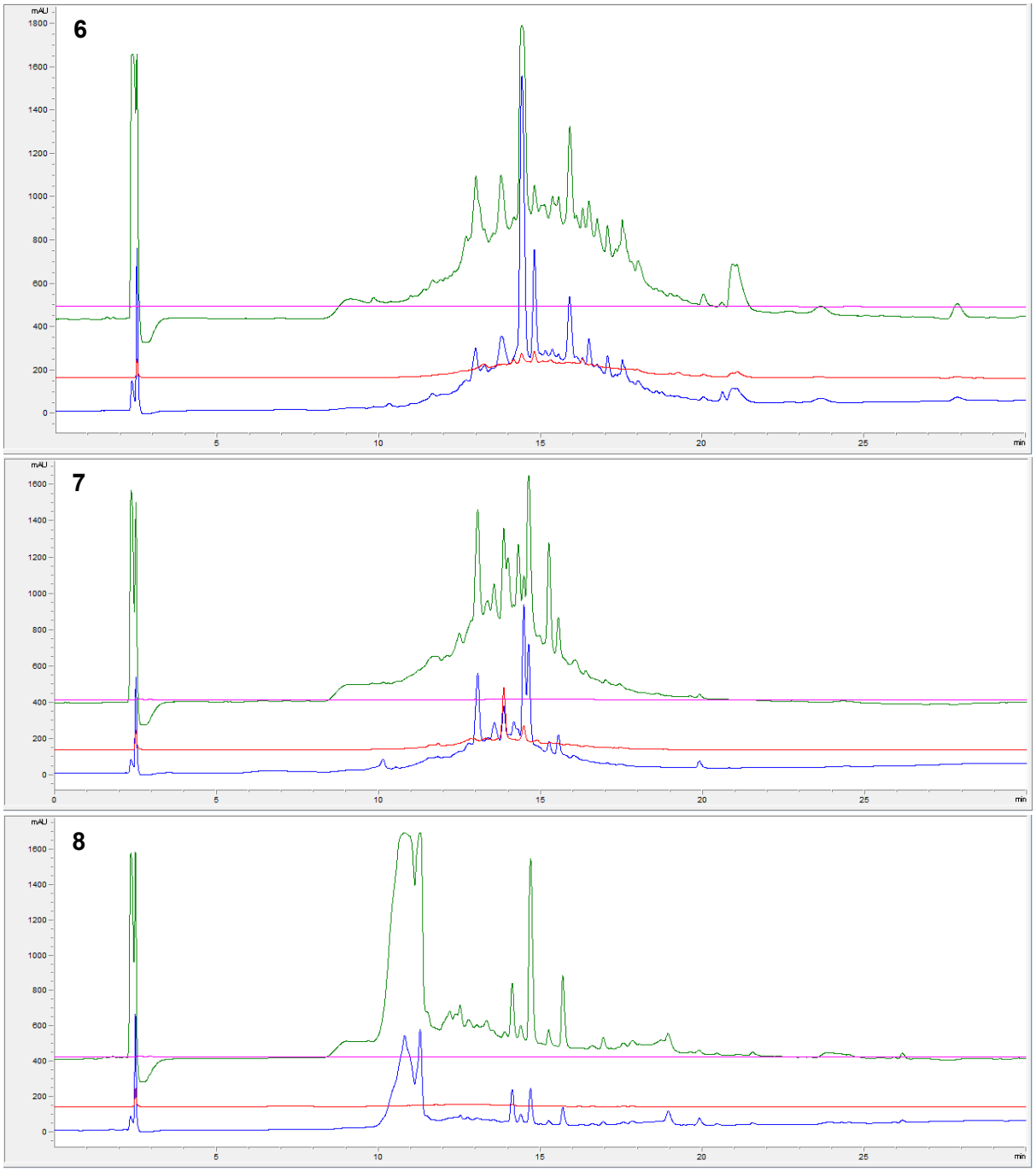
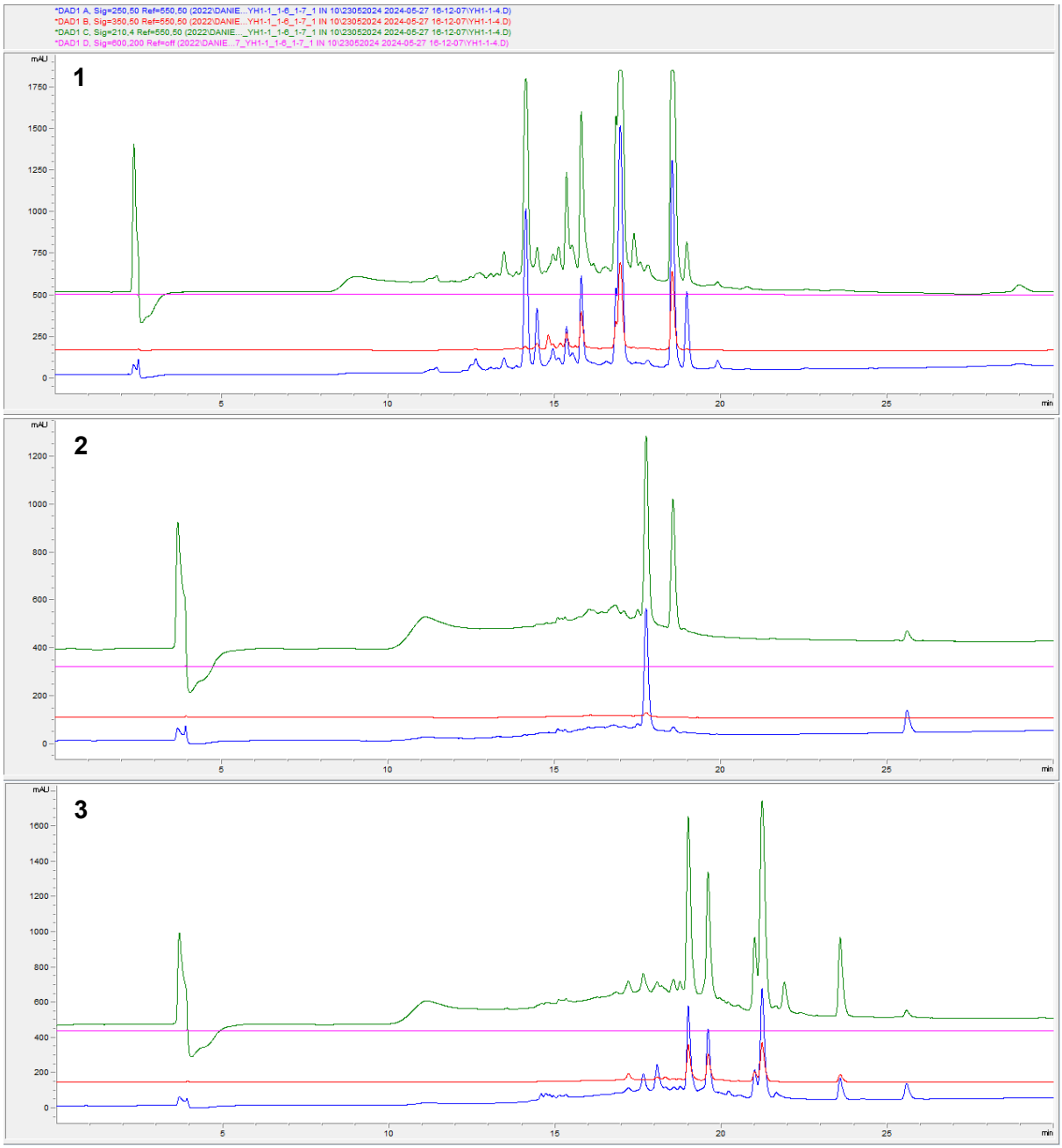


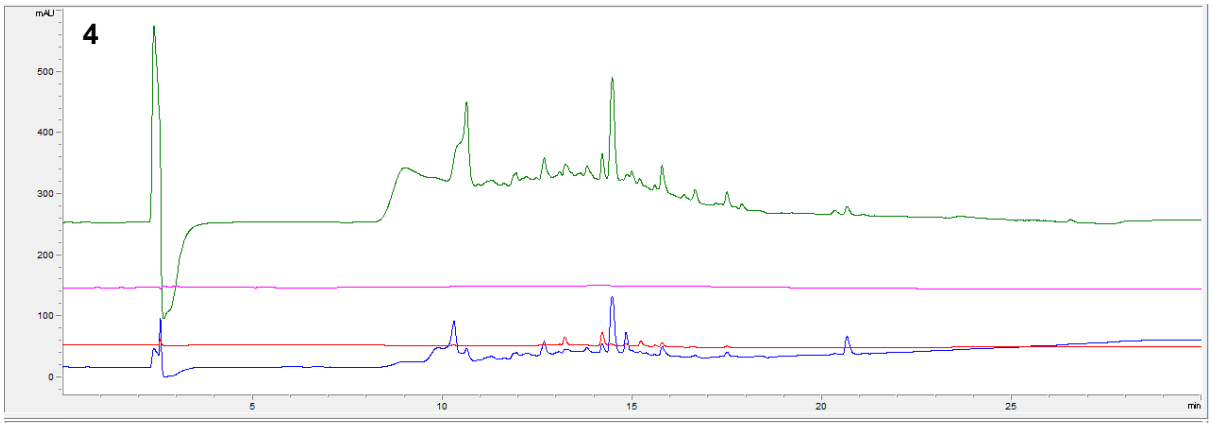
Figure 2.4. HPLC chromatograms of ICMP 477 extracts with (1) no substrate, (2) PA, (3) PA (orange band), (4) DOPA, (5) Cl-PA, (6) β-PA, (7) F-PA, and (8) Br-PA in fraction 3. (HPLC eluent: 0-45 min, 5-95% 0.1% FA/ACN and 0.1% FA/water, flow rate: 1 mL/min).

Fraction 4 (**Figure 2.5**)

Overall, fraction 4 of the extracts containing an additive (2-8) might contain a subset of the compounds found in the crude extract (1), exhibiting similar major peaks. New peaks at a retention time of 11 min were detected in Br-PA, suggesting the production of different metabolites in response to Br-PA from the control. Moreover, PA showed a shift in the peaks

towards a longer retention time than the control. PA was collected into two separate fractions, labelled as fractions 4 and 4.1, consistent with the band colours observed in fraction 4. Two distinct peaks were observed in fraction 4, with a noticeable shift towards a longer retention time in fraction 4.1. Besides these two peaks, fraction 4.1 exhibited additional peaks with longer retention times, which were absent in fraction 4.2. In DOPA, the majority of the peaks in control disappeared, leaving a single clear peak and a newly emerged (relatively broad) peak. Cl-PA exhibited a higher number and intensity of peaks as like in the control, indicating a greater presence of constituents. For β-PA, it had the closest peak profile to the control than any other additives. In F-PA, several peaks observed in the control group were absent, with the remaining peaks showing a reduced magnitude relative to the control group.

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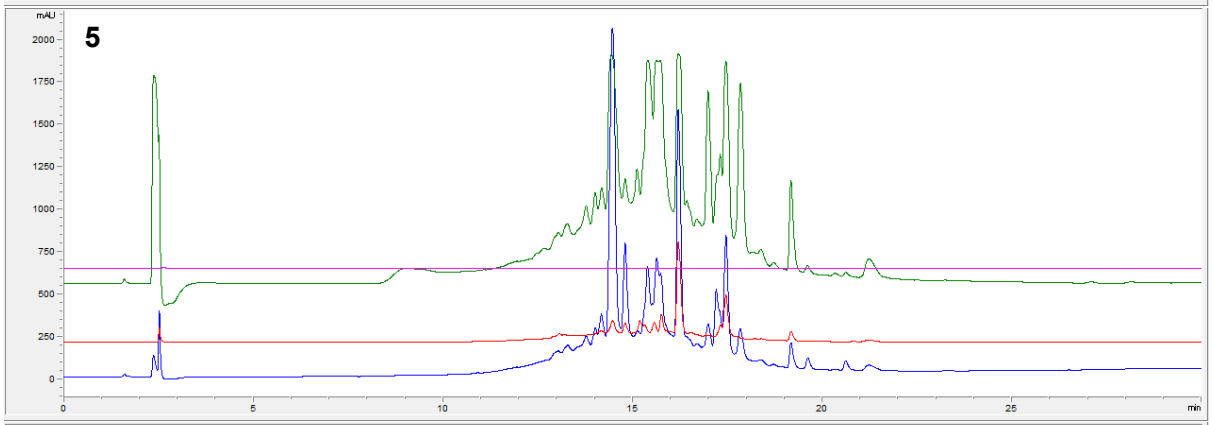
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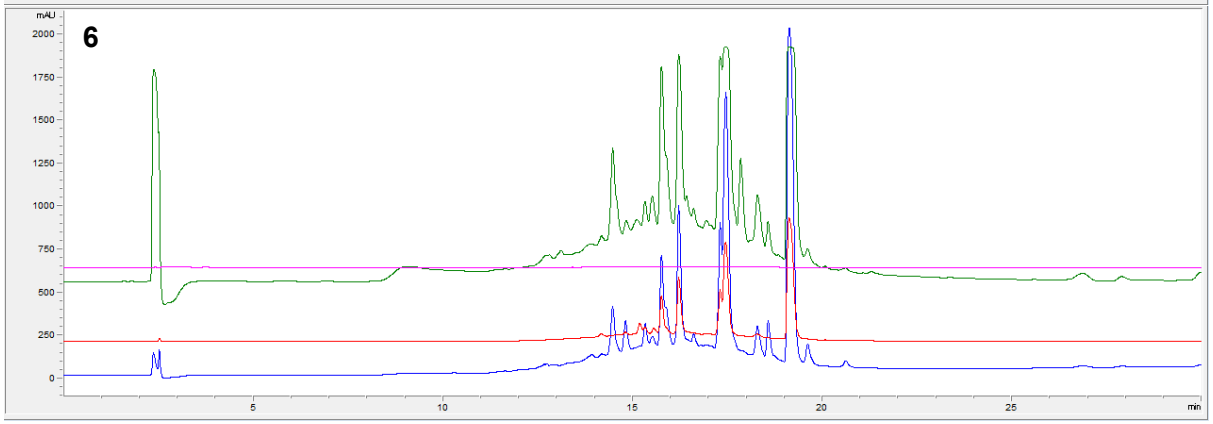
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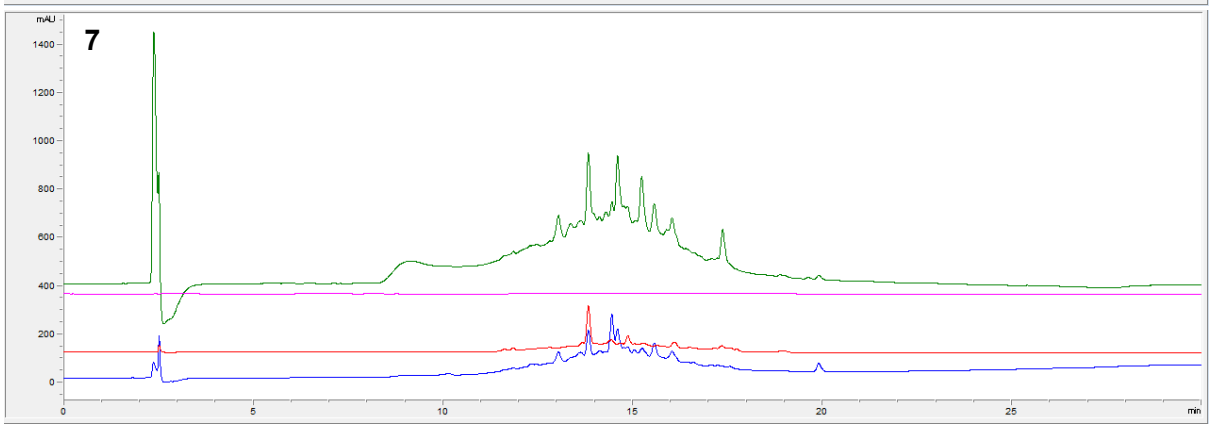
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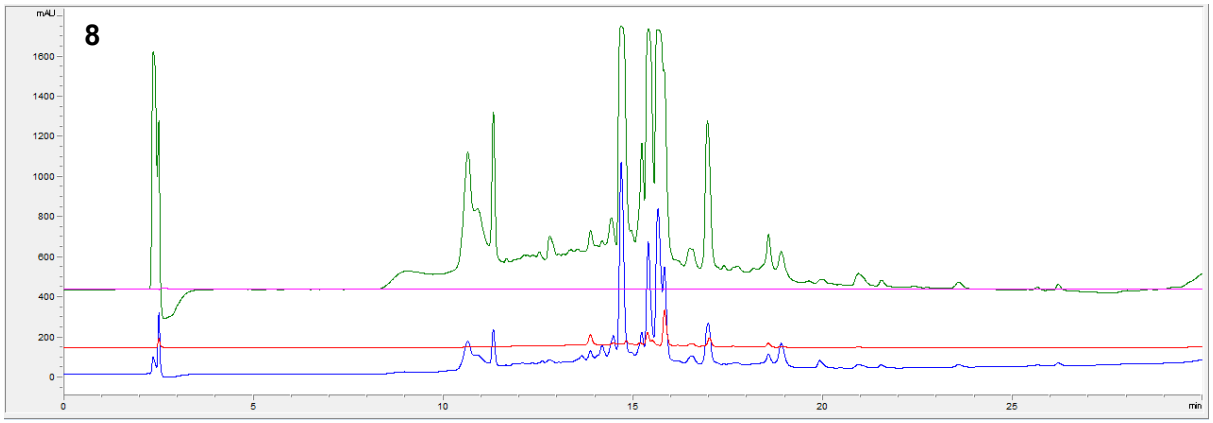
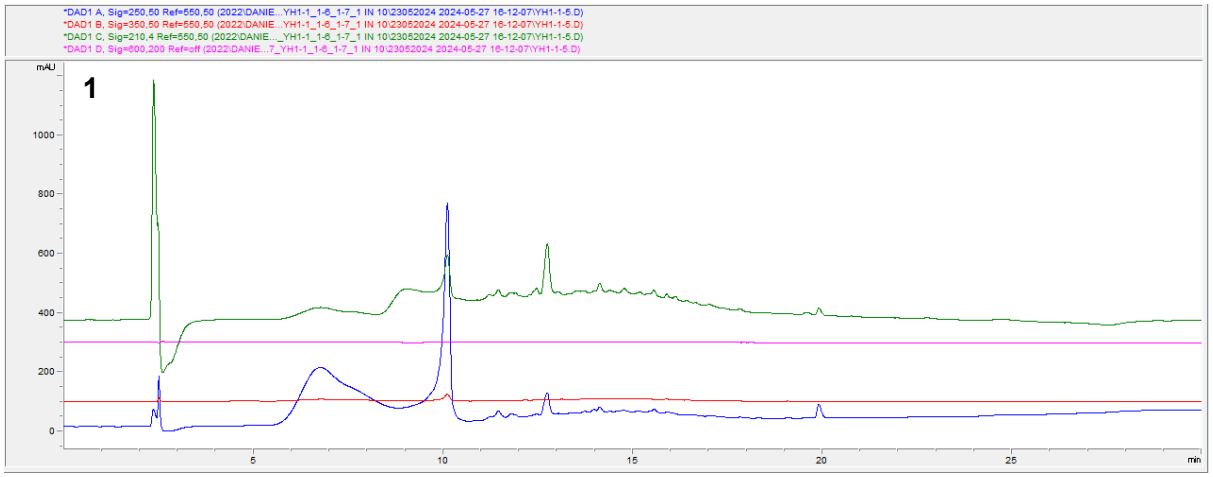
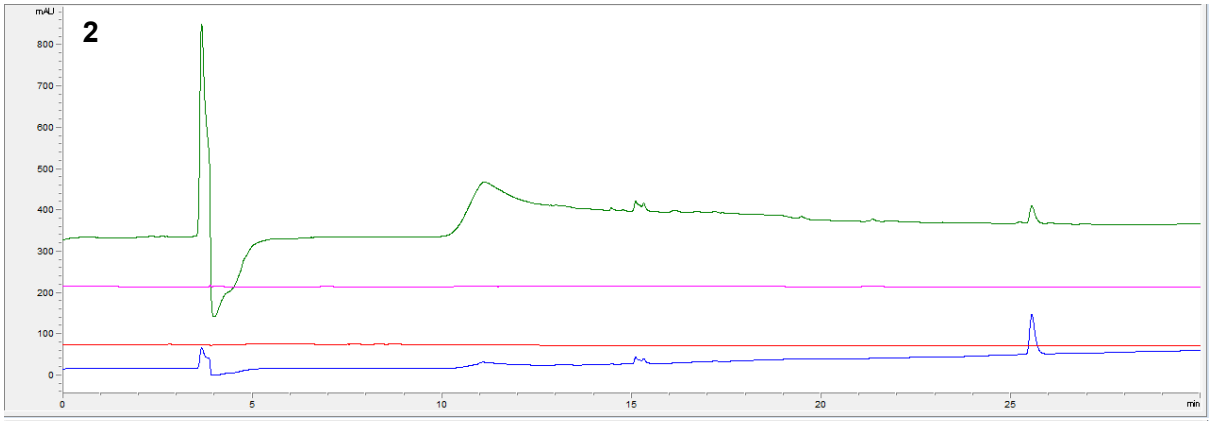


Figure 2.5. HPLC chromatograms of ICMP 477 extracts with (1) no substrate, (2) PA, (3) PA (brown band), (4) DOPA, (5) Cl-PA, (6) β-PA, (7) F-PA, and (8) Br-PA in fraction 4. (HPLC eluent: 0-45 min, 5-95% 0.1% FA/ACN and 0.1% FA/water, flow rate: 1 mL/min).

Fraction 5 (**Figure 2.6**)

Fraction 5 exhibited two clearly identifiable peaks, which were significantly absent in all the additives. PA, DOPA, and F-PA did not demonstrate significant distinctions from the control, showing only minimal additional peaks. In Br-PA, a new peak with minor intensity was observed between the two existing peaks in the control. The Cl-PA and β-PA extracts had increased contents of the compounds than the control, mostly with a longer retention time.

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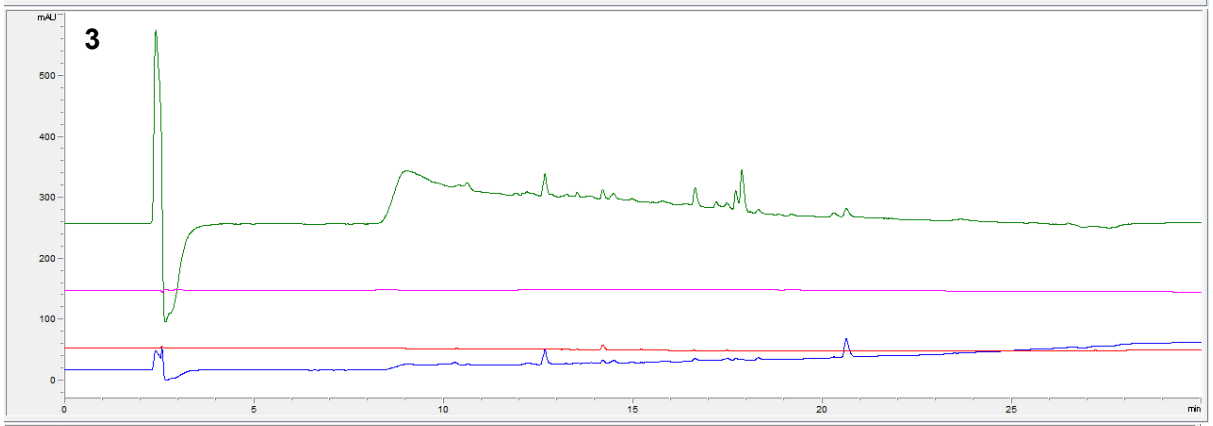
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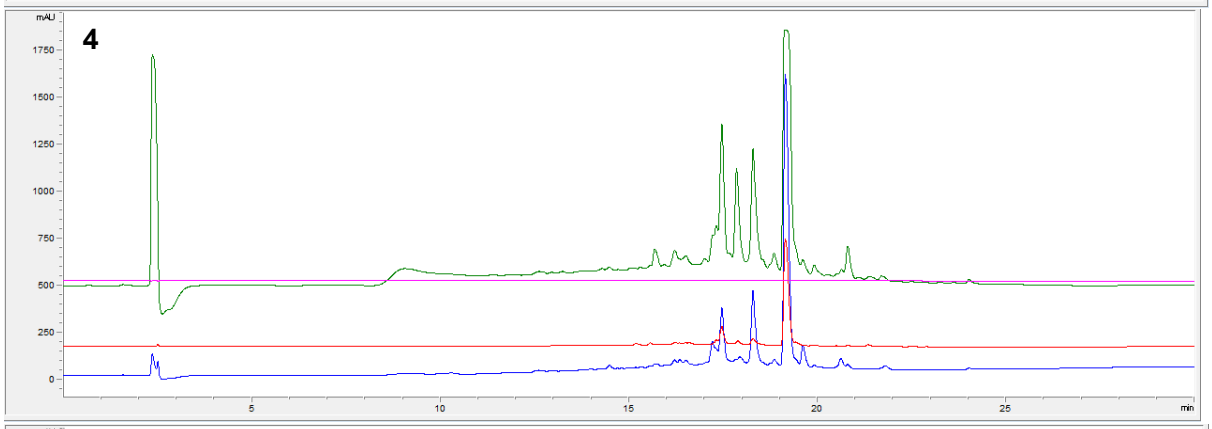
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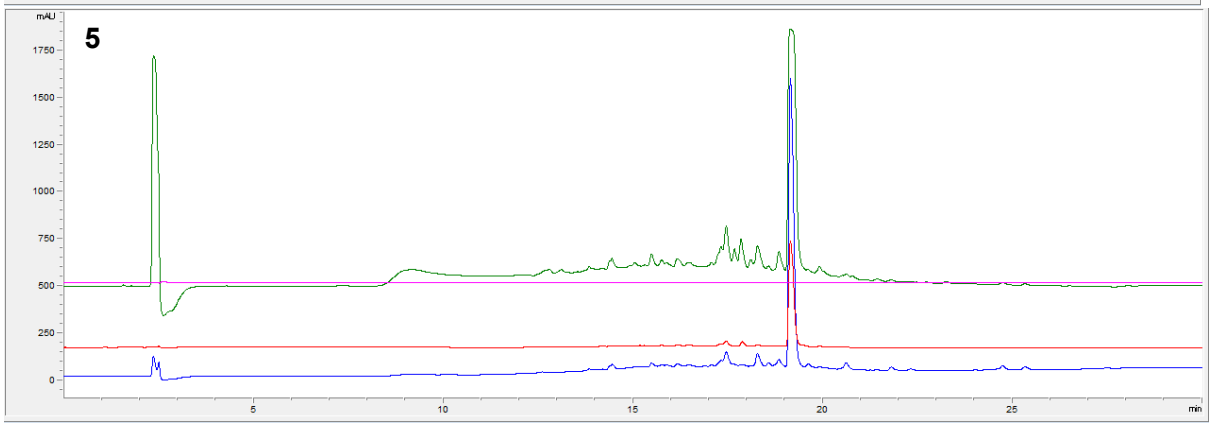
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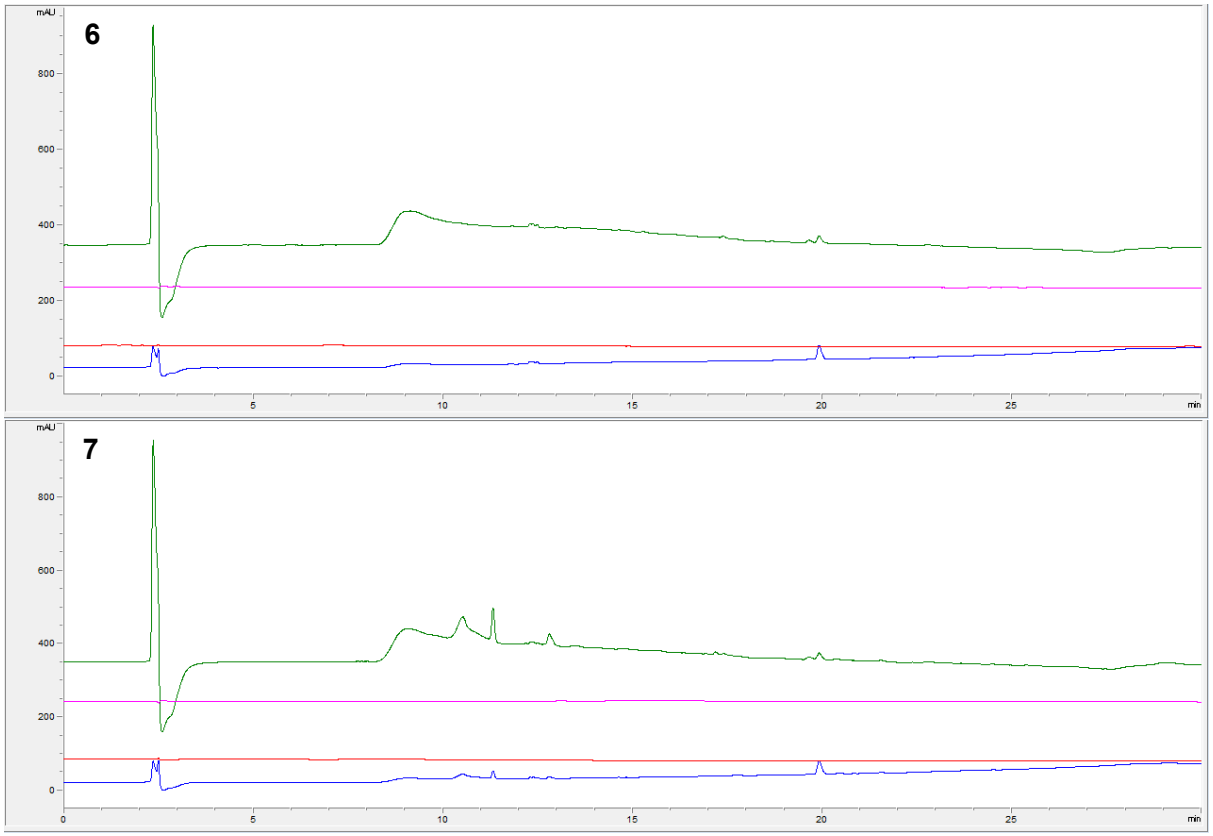

Figure 2.6. HPLC chromatograms of ICMP 477 extracts with (1) no substrate, (2) PA, (3) DOPA, (4) Cl-PA, (5) β-PA, (6) F-PA, and (7) Br-PA in fraction 5. (HPLC eluent: 0-45 min, 5-95% 0.1% FA/ACN and 0.1% FA/water, flow rate: 1 mL/min).

2.6 Antimicrobial Activity Testing of the Fractionated Extracts

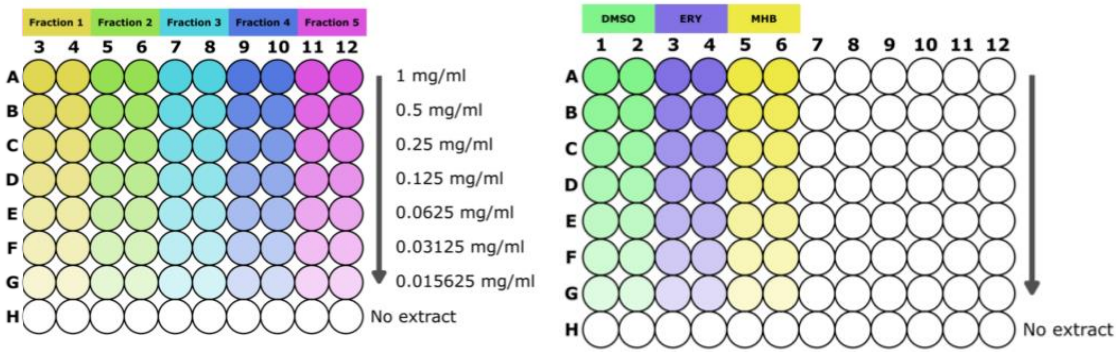

Figure 2.7. Plate layout for testing the fractionated extracts.

The antimicrobial activity of the fractions was tested in a similar manner to the crude (**Figure 2.7**, **Table 2.5**). All seven extracts exhibited antibacterial activity against *S. aureus* at a concentration of up to 250 µg/mL, while the control was only effective against both *E.coli* and *S. aureus* in fraction 5. The highest MICs observed were 125 µg/mL by the control, β-phenylalanine (β-PA), fluorophenylalanine (F-PA), and bromophenylalanine (Br-PA) against

S. aureus. In fraction 4, all extracts except for phenylalanine (PA) exhibited antibacterial activity against *S. aureus* at a concentration of up to 500 µg/mL. Results of the antibacterial activity of the seven fungal extracts show that the majority of these extracts possessed antimicrobial activity against *S. aureus* in Fractions 4 and 5.

Moreover, only dihydroxyphenylalanine (DOPA) was active against *E. coli*, whereas the control, phenylalanine (PA), dihydroxyphenylalanine (DOPA), chlorophenylalanine (Cl-PA), and β-phenylalanine (β-PA) were active against *S. aureus* in at least one other fraction. The data showed retention of the antibacterial activity in the latter fractions, supporting the significance of testing the fractions, especially fractions 3 to 5, in fungal extracts to discover antimicrobial agents. Fractions obtained through techniques like column chromatography are more purified and concentrated, thereby enhancing the sensitivity and specificity in detecting antimicrobial agents that are in low concentrations and masked by other non-active substances in the crude extract. Sugars in fractions 1 and 2 are primarily involved in fundamental cellular functions and generally lack antimicrobial properties, whereas secondary metabolites found in fractions 3 to 5 are more likely to disrupt microbial cell walls, interfere with microbial enzymes, or inhibit nucleic acid synthesis in pathogens, responsible for antimicrobial activities.

Table 2.5. Activity of fractionated ICMP 477 extracts against *E. coli* and *S. aureus* in MIC assay.

Additive	Bacterium	Extract MIC (µg/mL)				
		F1	F2	F3	F4	F5
None (Control)	*E. coli*	>1000	>1000	>1000	1000	125
	S. aureus	>1000	>1000	250	250	125
PA	*E. coli*	>1000			>1000	>1000
	S. aureus	>1000			1000	250
DOPA	*E. coli*	>1000	500	1000	>1000	>1000
	S. aureus	>1000	500	1000	250	250
Cl-PA	*E. coli*	> 1000	1000	1000	>1000	>1000
	S. aureus	> 1000	> 1000	500	500	250
β-PA	*E. coli*	>1000	>1000	>1000	>1000	>1000
	S. aureus	> 1000	>1000	> 1000	250	125
F-PA	*E. coli*	>1000	1000	>1000	>1000	>1000
	S. aureus	> 1000	1000	1000	250	125
Br-PA	*E. coli*	>1000	>1000	>1000	>1000	>1000
	S. aureus	>1000	>1000	1000	500	125

The minimum inhibitory concentration (MIC) was defined as the lowest concentration at which an extract could cause a minimum 90% (1-log) reduction in area under curve (AUC) values compared to controls containing 4% v/v DMSO. Extract fractions highlighted in red were not active at 1000 µL/mL, yellow active at 1000 µL/mL, and blue active below 1000 µL/mL. Key: PA, Phenylalanine; DOPA, Dihydroxyphenylalanine; F1-5, Fraction 1-5 collected from the C_8 reversed-phase column.

2.7 Liquid Chromatography-Mass Spectrometry (LC-MS)

A number of compounds found in active fungal extracts detected by HPLC indicate the presence of bioactive metabolites, potentially novel or known compounds with antimicrobial properties, which can be further characterised using Liquid Chromatography-Mass Spectrometry (LC-MS). Due to time and limited access to equipment, LC-MS data was not acquired at the time of thesis writing.

2.8 Sub-fractionation

LH-20 column chromatography was employed to further purify and isolate compounds from the fractionated extracts obtained after initial extraction and the fractionation process. This technique was chosen for its ability to separate compounds based on their molecular size and polarity in a gentle manner, preserving the integrity of the compounds of interest. By using a solvent system of 5% methanol/dichloromethane, LH-20 chromatography aimed to resolve complex mixtures obtained from the phenylalanine derivatives and control samples, allowing for a more refined analysis of individual components.

The NMR data comparing the control and various phenylalanine derivatives revealed distinct chromatographic profiles indicative of different compound distributions in the crude and each fraction. Such differences highlight the effectiveness of LH-20 chromatography in separating and analysing phenylalanine derivatives, facilitating the identification and potential characterisation of individual compounds based on their chromatographic behaviour.

Phenylalanine

Phenylalanine was identified in fraction 3 and an interesting compound was identified in fraction 3.1, which was also detected in fraction 5 of the control. Fractions 4.1 (F1) and 5 (G1) were subjected to additional purification using LH-20, resulting in the isolation of subfractions containing tyrosine and other compounds of interest.

Dihydrophenylalanine (DOPA)

Fraction 2-3 and 5-6 were combined and were subjected to additional purification using LH-20, resulting in the isolation of subfractions containing a compound of interest with sugars and fatty acids.

β-phenylalanine

Fraction 5 was subjected to additional purification using LH-20, resulting in the isolation of subfractions containing a compound of interest, which was also detected in fraction 5 of Cl-PA.

Chloro-phenylalanine

Fractions 4 and 5 were subjected to additional purification using LH-20. In the subfractions isolated from fraction 4 resulted in the isolation of tryptophan and a compound of interest.

Fluoro-phenylalanine

Fractions 2-4 were subjected to additional purification using LH-20, resulting in the isolation of subfractions containing a compound of interest.

Bromo-phenylalanine

Fractions 3-5 were subjected to additional purification using LH-20, resulting in the isolation of subfractions containing a compound of interest.

2.9 Conclusion

Antibiotics have played a crucial role in modern medicine by treating infectious diseases and preventing infections in individuals with compromised immune systems. Nevertheless, the excessive utilisation of antibiotics has resulted in a significant rise in antibiotic-resistant bacteria, thus necessitating the development of novel antibiotics with distinct modes of action. One potential approach for discovering novel antibacterial compounds involves investigating natural products found in fungal secondary metabolites. Fungi synthesise a diverse array of secondary metabolites from a limited number of precursor molecules. Precursor-directed biosynthesis (PDB) involves the incorporation of non-natural precursor molecules into the growth medium of microorganisms to generate modified metabolites with distinct biological properties.

In this study, ICMP 477 isolates were cultivated on media, with different phenylalanine derivatives as precursors. Subsequently, a bioluminescence assay was utilised to examine the antimicrobial activity of these cultures. High-performance liquid chromatography (HPLC) was utilised to track the changes in the profile of these fungi. Overall, there was an increase in the production of secondary metabolites when an additive was used, with some precursors producing novel compounds while others enhanced the production of compounds that were also present in the control. Future work will include the structure elucidation of these compounds using NMR spectroscopy and liquid chromatography-mass spectrometry (LC-MS) with the Global Natural Products Social Molecular Networking (GNPS) platform.

This study highlights the potential of sourcing bioactive metabolites via precursor-directed biosynthesis to activate fungal secondary metabolism in the search for novel antimicrobial compounds.

3 Experimental Procedures

3.1 Materials

3.1.1 Culture Media

Table 3.1. Culture media used to culture fungi

Medium	Composition (per L)	Description
Agar		Solidifying agent for use in preparing microbiological culture media.
Potato Dextrose Broth (PDB)	4.0 g potato starch 20.0 g dextrose	A base for the cultivation of yeasts and moulds.

3.1.2 Reagents

Table 3.2. Reagents used in this study

Reagent	Supplier
Acetonitrile (ACN)	Thermo Fisher
Dichloromethane (DCM)	ECP Ltd
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
Ethyl acetate	VWR
Formic acid (FA)	Supelco
Methanol	ECP Ltd

3.1.3 Equipment

Table 3.3. Equipment used in this study

Equipment	Supplier
AVIII-400	Bruker
Innova 44 Shaking Incubator	New Brunswick Scientific
HPLC System (coupled to Q-TOF) • Degasser 1100 series • Capillary pump 1100 series • µ.WPS 1100 series • Thermostat 1290 Infinity • Colcom 1100 series • DAD 1100 series	Agilent Technologies
MilliQ Water Purification System	Merck Millipore
Libra S22 Spectrophotometer	Biochrom
Rotavap	Buchi
Scanvac CoolSafe Freeze Dryer	Labogene
SPD SpeedVac*	Thermo Savant
Victor™ X Light Plate Reader	Perkin Elmer

3.1.4 Microorganisms

Table 3.4. Bacteria strains used in this study

Bacterium	Strain	Description
Escherichia coli	ATCC 25922 Lux	ATCC 25922 with a chromosomal copy of the luxCDABE operon integrated using the p16sluxABCDE plasmid
Staphylococcus aureus	Xen36	ATCC 49525 with a stable copy of a modified luxABCDE operon at a single integration site on a native plasmid

Table 3.5. ICMP isolates used in this study

ICMP isolate	Species	District isolated
477	*Aspergillus terreus*	Auckland

3.2 Methods

3.2.1 General Experimental

MS/MS spectra were acquired on an Orbitrap Exploris spectrometer. Using standard pulse sequences, ^{1}H and ^{13}C NMR spectra were recorded at 298 K on a Bruker Avance 400 spectrometer at 400 and 100 MHz, respectively. Flash column chromatography was carried out using C_8 reversed–phase (40–63 mm) solid support. Gel filtration flash chromatography was carried out on Sephadex LH–20. Thin-layer chromatography was conducted on DC–plastikfolien Kieselgel 60 F254 plates.

3.2.2 Cultivation of ICMP Isolates

To produce a subculture of the original culture, PDA plates were inoculated with *Aspergillus terreus* ICMP 477 alone and in the presence of 1mg/mL phenylalanine derivatives by cutting pre-prepared *Aspergillus terreus* PDA plates into 1 cm^3 pieces and placing them on potato dextrose agar (PDA). These plates were sealed with parafilm and incubated at room temperature for 2 weeks.

The media precursors include phenylalanine, dihydroxyphenylalanine (DOPA), β-phenylalanine, fluoro-phenylalanine, chloro-phenylalanine, and bromo-phenylalanine.

3.2.3 Extraction and Isolation

PDA Control

40 fully grown PDA plates were freeze-dried (40.24 g, dry weight) and extracted using

MeOH (2 × 500 mL) for 3 hours, followed by CH_2Cl_2 (2 × 500 mL) overnight. The extracts were filtered and combined to be concentrated under reduced pressure, giving a crude extract (4.11 g). Preliminary fractionation was carried out by C_8 reversed-phase column chromatography, eluting with a gradient of H_2O/MeOH to afford six fractions (A1 – F1). An extra fraction was collected using the same gradient of solute as fraction 5, which was 100% methanol (MeOH).

Fractions C (50% MeOH) and F (100% MeOH) were further purified by LH-20 column chromatography using 5% MeOH/CH_2Cl_2, which generated four fractions and six fractions, respectively.

Phenylalanine

48 fully grown PDA plates were freeze-dried (42.38 g, dry weight) and extracted using MeOH for 3 hours, followed by CH_2Cl_2 overnight. The extracts were filtered and combined to be concentrated under reduced pressure, giving a crude extract (6.52 g). Preliminary fractionation was carried out by C_8 reversed-phase column chromatography, eluting with a gradient of H_2O/MeOH to afford seven fractions (A2 – G2). Fractions 3 and 4 were collected in two separate sections based on the colour of the band, resulting in additional fractions designated as fractions 3.1 and 4.1.

Fractions F (75% MeOH) and G (100% MeOH) were further purified by LH-20 column chromatography using 5% MeOH/CH_2Cl_2, which generated three fractions and four fractions, respectively.

Dihydrophenylalanine (DOPA)

44 fully grown PDA plates were freeze-dried and extracted using MeOH for 3 hours, followed by CH_2Cl_2 overnight. The extracts were filtered and combined to be concentrated under reduced pressure, giving a crude extract (4.35 g). Preliminary fractionation was carried out by C_8 reversed-phase column chromatography, eluting with a gradient of H_2O/MeOH to afford six fractions (A3 – F3). An extra fraction was collected using the same gradient of solute as fraction 5, which was 100% methanol (MeOH).

Fractions B3 – C3 (25% + 75% MeOH) and E3 (100% MeOH) were further purified by LH-20 column chromatography using 5% MeOH/CH_2Cl_2, which generated five fractions and four fractions, respectively.

Chloro-phenylalanine

47 fully grown PDA plates were freeze-dried (24.09 g, dry weight) and extracted using MeOH for 3 hours, followed by CH_2Cl_2 overnight. The extracts were filtered and combined to be concentrated under reduced pressure, giving a crude extract (3.01 g). Preliminary fractionation was carried out by C_8 reversed-phase column chromatography, eluting with a gradient of H_2O/MeOH to afford six fractions (A4 – F4). An extra fraction was collected using the same gradient of solute as fraction 5, which was 100% methanol (MeOH).

Fractions D4 (75% MeOH) and E4 (100% MeOH) were further purified by LH-20 column chromatography using 5% MeOH/CH_2Cl_2, which generated four fractions and four fractions, respectively.

β-phenylalanine

44 fully grown PDA plates were freeze-dried and extracted using MeOH for 3 hours, followed by CH_2Cl_2 overnight. The extracts were filtered and combined to be concentrated under reduced pressure, giving a crude extract (5.07 g). Preliminary fractionation was carried out by C_8 reversed-phase column chromatography, eluting with a gradient of H_2O/MeOH to afford six fractions (A5 – F5). An extra fraction was collected using the same gradient of solute as fraction 5, which was 100% methanol (MeOH).

Fraction E5 (100% MeOH) were further purified by LH-20 column chromatography using 5% MeOH/CH_2Cl_2, which generated five fractions.

Fluoro-phenylalanine

40 fully grown PDA plates were freeze-dried (31.91 g, dry weight) and extracted using MeOH for 3 hours, followed by CH_2Cl_2 overnight. The extracts were filtered and combined to be concentrated under reduced pressure, giving a crude extract (4.36 g). Preliminary fractionation was carried out by C_8 reversed-phase column chromatography, eluting with a gradient of H_2O/MeOH to afford five fractions (A6 – E6).

Fractions B6 – D6 (25 – 75% MeOH) were further purified together by LH-20 column chromatography using 5% MeOH/CH_2Cl_2, which generated six fractions.

Bromo-phenylalanine

42 fully grown PDA plates were freeze-dried (29.34 g, dry weight) and extracted using MeOH for 3 hours, followed by CH_2Cl_2 overnight. The extracts were filtered and combined to be concentrated under reduced pressure, giving a crude extract (10.13 g). Preliminary fractionation was carried out by C_8 reversed-phase column chromatography, eluting with a gradient of H_2O/MeOH to afford five fractions (A7 – E7).

Fraction C7 – E7 (50, 75, and 100% MeOH) were further purified by LH-20 column chromatography using 5% MeOH/CH_2Cl_2, which each generated five, four, and four fractions.

3.2.4 MIC and MBC Testing of Fractionated Extracts

The dried crude extract and extracts received from fraction A – G were dissolved in 50 mg/mL dimethyl sulfoxide (DMSO) and then diluted into Mueller Hinton broth II (MHB) to achieve a 1 mg/mL concentration. MHB (96 µL) was added to all the wells along the top row of the 96-well plate, and each extract (4 µL) was added to those MHB. MHB (50 µL) was then added to the remaining wells except the top row. The extract solution (50 µL) was transferred from the top row to the bottom rows and discarded, serially diluting two-fold down the plate. Aliquots of bacteria (50 µL), *S. aureus* ATCC 29213 and *E. coli* ATCC 25922, at an OD_{600} of 0.001 (approximately 1×10^6 CFU/mL) were added to all the wells to give a maximum concentration of 1 mg/mL and a minimum concentration of 16 µg/mL. For the positive control, DMSO (4 µL) was diluted to the same concentration as the extract, followed

by the addition of antibiotics, rifampicin and erythromycin, diluted to 5 times the MIC of *S. aureus* and *E.coli*, respectively. MHB (100 µL) was added as a negative sterile broth control, and the same doubling dilution process was repeated for both controls.

Absorbance was measured at 600 nm using an Enspire plate reader at 0, 2, 4, 6, and 24 hours to determine the minimum inhibitory concentration (MIC). Between those times, the plates were incubated at 37 °C with shaking at 100 RPM. After 24 hours, 10 µL of liquid from all wells showing inhibition of bacterial growth was pipetted onto fresh MHA plates. Once all liquid had evaporated, the plates were incubated at 37 °C overnight, and the minimum bactericidal concentration (MBC) was measured.

3.2.5 Solid-phase Extraction (SPE), High-performance Liquid Chromatography (HPLC)

Solid-phase extraction (SPE) on each sample was performed starting with a conditioning step. The Oasis HLB (Hydrophilic-Lipophilic Balance) cartridges were calibrated with methanol and 0.1% formic acid (FA). The crude samples were slowly applied to those SPE cartridges, ensuring the rate was at 1 mL/min. After washing the cartridges with 0.1% FA, 50:50 FA/ACN was used to elute and collect the analyte. Finally, the eluate was concentrated down in the SpeedVac at 35 °C for 45 minutes and reconstituted using 50:50 FA/ACN. The final eluate was vortexed thoroughly and centrifuged for 3 minutes, followed by 1:10 dilution. 50 µL of each sample was injected into the HPLC system at a flow rate of 1 mL/min with a gradient of 0.1% FA/ACN and 0.1% FA/water.

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