Investigating metabolism and interspecies interactions in fungi with the use of chemical modifiers and media variation

PhD Thesis

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Abstract

Fungi are incredibly diverse and talented producers of secondary metabolites, representing a reservoir of potential natural products of medicinal, industrial and agricultural value. As well as this, edible fungi such as the truffle forming species are also highly prized and of high economic importance. In this work, a variety of fungal cultures were isolated from wild specimens collected within the UK. The potential of these fungi as sources of novel natural products was investigated utilising an OSMAC approach; fungi were cultivated in a range of liquid, semi-solid and solid media, and the metabolite profiles produced were analysed by HPLC and LCMS. As well as screening the fungi in various media, the effect of a range of small molecule elicitors on the metabolite profiles of the fungi was examined. These substances included epigenetic modifiers, biological precursors and enzyme inhibitors. Treatment with these substances altered the metabolite profiles of the fungi in many cases, resulting in increased amounts of particular metabolites compared to controls, or the production of compounds not detected in control fermentations, including biotransformation products. Attempts were made to isolate and identify metabolites produced by the fungi using a variety of extraction, chromatographic, and spectroscopic techniques. Culture extracts from the laboratory fermentations were tested for antibacterial properties, alongside a series of extractions and chromatographic fractions obtained from several species of wild mushrooms. Many of the samples tested showed antibacterial efficacy against a variety of both Gram-positive and Gram-negative species. Several of the extracts obtained from the wild mushroom species proved to be especially promising, in some cases having stronger antibacterial effects on difficult to treat bacteria than Ampicillin.

Hypocrealean fungi are known to be metabolically talented and rich sources of natural products with bioactive potential. European entomopathogenic and arachnogenous (spider-pathogenic) fungi have been poorly investigated, with all of the chemical work to date conducted on Asian strains. Much of this work was conducted on a spider-pathogenic species. Multigene phylogenetic analysis revealed that the culture fits within the *Gibellula pulchra* clade.

As well as these laboratory experiments, the effect of bacterial co-cultures, epigenetic modifiers, nutritional additives and other small molecule elicitors on mycorrhization of *Quercus robur* by *Tuber aestivum* was studied in a nursery pot-trial. It was found that co-culture with particular bacteria lead to an increase in mycorrhization, which may lead to industrial applications.

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Chapter One

Introduction

1. Overview of natural products discovery and importance

1.1 Natural Products, primary metabolites and secondary metabolites

Natural products can be defined as compounds or substances that are found in the natural kingdom and produced by living things, in the broadest sense. However, in the field of natural products research and chemistry, 'natural product' typically refers specifically to 'specialised' or 'secondary' metabolites, which although they may have important functions, purposes or convey an evolutionary advantage to the producing organism, are not essential in the normal essential biology of life. Primary metabolites are the compounds produced by and shared broadly across the different phyla of living organisms, that are used as the molecular building blocks of life and are essential for biological processes of survival, such as cellular growth, functioning, repair and reproduction. Examples of primary metabolites include amino acids, carbohydrates, nucleic acids and lipids, which form a pool of substances from which increasingly complex substances and materials are formed, such as the proteins involved in cellular structure (e.g. cytoskeleton, cell membrane and cell wall proteins), the enzymes involved in biosynthetic pathways and regulation of cellular function, DNA, RNA, phospholipid membranes [1].

In contrast to primary metabolites, which are widely shared throughout living organisms as the basic and essential building blocks of life, secondary metabolites are narrowly distributed throughout different organisms, their known distribution often being restricted to only one specific species, genera or groups of related organisms [2]. This restricted natural occurrence combined with the number of structural analogues (produced through non-specific biosynthesis) and the immense variety and taxonomic distribution of natural products, has even opened up the possibility to identify an organism or group of organisms based on its chemical profile in the field of chemotaxonomy [3].

Secondary metabolites are small molecules that are produced from the shared, common pool of precursors involved in primary metabolism, which can be highly complex, are incredibly diverse, and serve specialised or unknown functions for the producing organisms [1, 2]. For example, secondary metabolites can be involved in

inter-organism chemical signalling [4-6], survival (for example by reducing predation, herbivory or mycophagy by possessing toxic or poisonous properties) and ‘microbial warfare’; microbial metabolites conferring an advantage on the producing organism through inhibition or killing of competing organisms [7].

1.2 Natural products as a source of pharmaceuticals

1.2.1 Historic natural products derived from plants

Historically, natural products have been an important source of drugs, with many examples of medicinal substances that have been discovered from nature or developed from natural product lead compounds (**Figures 1, 5, 6**). In fact, around 50% of drugs in clinical use are either natural products or compounds which have been derived from them [8].

Plants have been a particularly rich source of natural products with and without known biological activity. Many plant species have been used medicinally since long before their active components were known in traditional herbal or folk medicine. With the advent of modern chemical techniques and instrumentation, the active components of many of these plants have been identified, isolated, and methods for their mass production have been developed. The adage ‘The dose makes the poison’, accredited to Paracelsus, is a core principle in the field of toxicology and analogous to the pharmacological concept of the therapeutic index (the margin between an effective and toxic dose of a drug). Early discoveries of medicinal natural substances often came from poisonous plants, for example, many medicines on the World Health Organisations list of essential medicines are active components of poisonous plants or semi-synthetic derivatives of them [9], likely due to the obvious poisonous effects of these plants leading to their investigation in lower dosages in the treatment of pathology. For example, the anticholinergic tropane alkaloids including atropine and scopolamine (Hyoscine), derived from various species in the Solanaceae (Nightshade) family of plants, such as *Atropa belladonna*, are highly poisonous, yet are some of the oldest used medicinal substances [10, 11]. In fact, recent chemical analysis of human hair found in Menorca has dated the use of plants containing tropane alkaloids as well as ephedrine to the bronze age [12].

The semisynthetic anti-inflammatory analgesic aspirin (acetylsalicylic acid) was originally derived from the acetylation salicylic acid, produced from salicin following discovery of the medicinal properties of the Willow tree (*Salix* spp.) [13, 14]. The analgesic opiates morphine, codeine, and several semi-synthetic opiates, are all derived from *Papaver somniferum* or its latex (opium), a substance with one of the longest histories of use as a medicine [15, 16]. The chemotherapeutic drug Paclitaxel (or Taxol), a taxane, was initially extracted from the Pacific yew tree (*Taxus brevifolia*), and due to extensive overharvesting of the species, was later produced semi-synthetically alongside docetaxel (Taxotere) from 10-deacetylbaccatin extracted from the leaves of the European Yew (*Taxus baccata*) [17, 18].

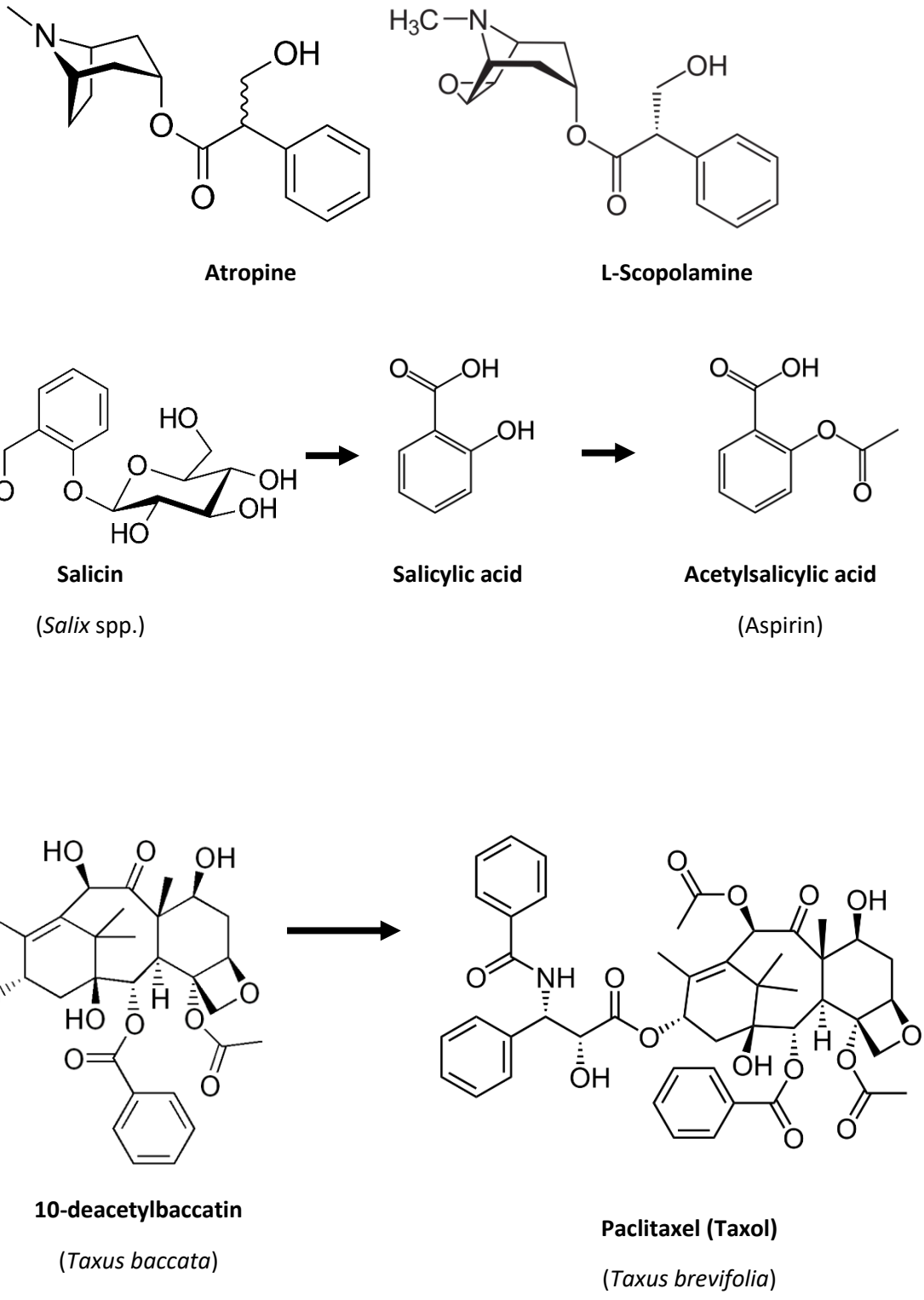


Figure 1. Examples of historic discovery of drugs derived from plants.

1.2.2 Fungal secondary metabolites background

The fungal kingdom is a broad group of incredibly diverse organisms including yeasts, moulds, and organisms which form larger more complex structures such as the mushrooms of the subkingdom Dikarya (sometimes referred to as the 'higher fungi'), which contains the divisions/phyla Ascomycota and Basidiomycota (Figure 2-3). They are distributed globally, and present in almost every environment. They serve essential ecological functions, such as decomposition of organic material, maintaining the health of forests, they serve as an essential food source for mycophagous animals, and are an important source of pharmaceuticals.

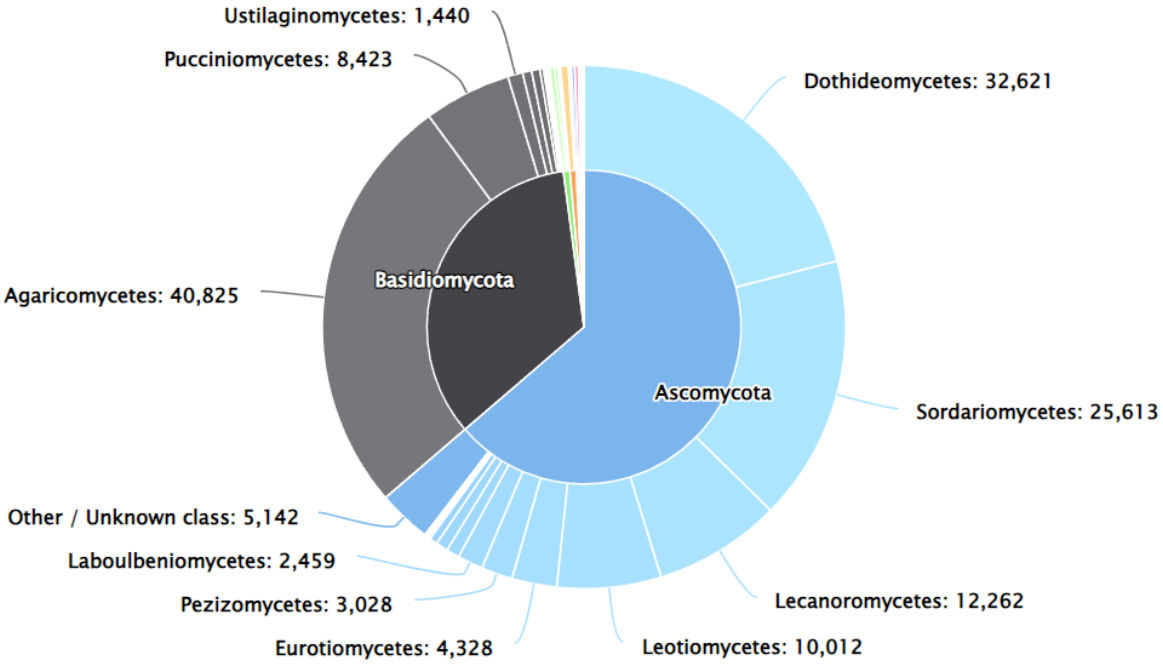


Figure 2. Taxonomic overview of the kingdom Fungi, showing the breakdown of 156,035 known species in the kingdom into the different phyla. Ascomycota contains the most species (99,396 species), followed by Basidiomycota (53,309 species). Produced from Species Fungorum Plus (Accessed 14th August 2024) [2].

According to Species Fungorum, the fungal kingdom includes at least around 156,035 known species, but the total number of species is unknown. In 2007, 1.5 million species was a commonly reported estimate [19], but the number has been revised

frequently following additional studies, with various publications reporting estimates ranging from 3-12 million [20-22].

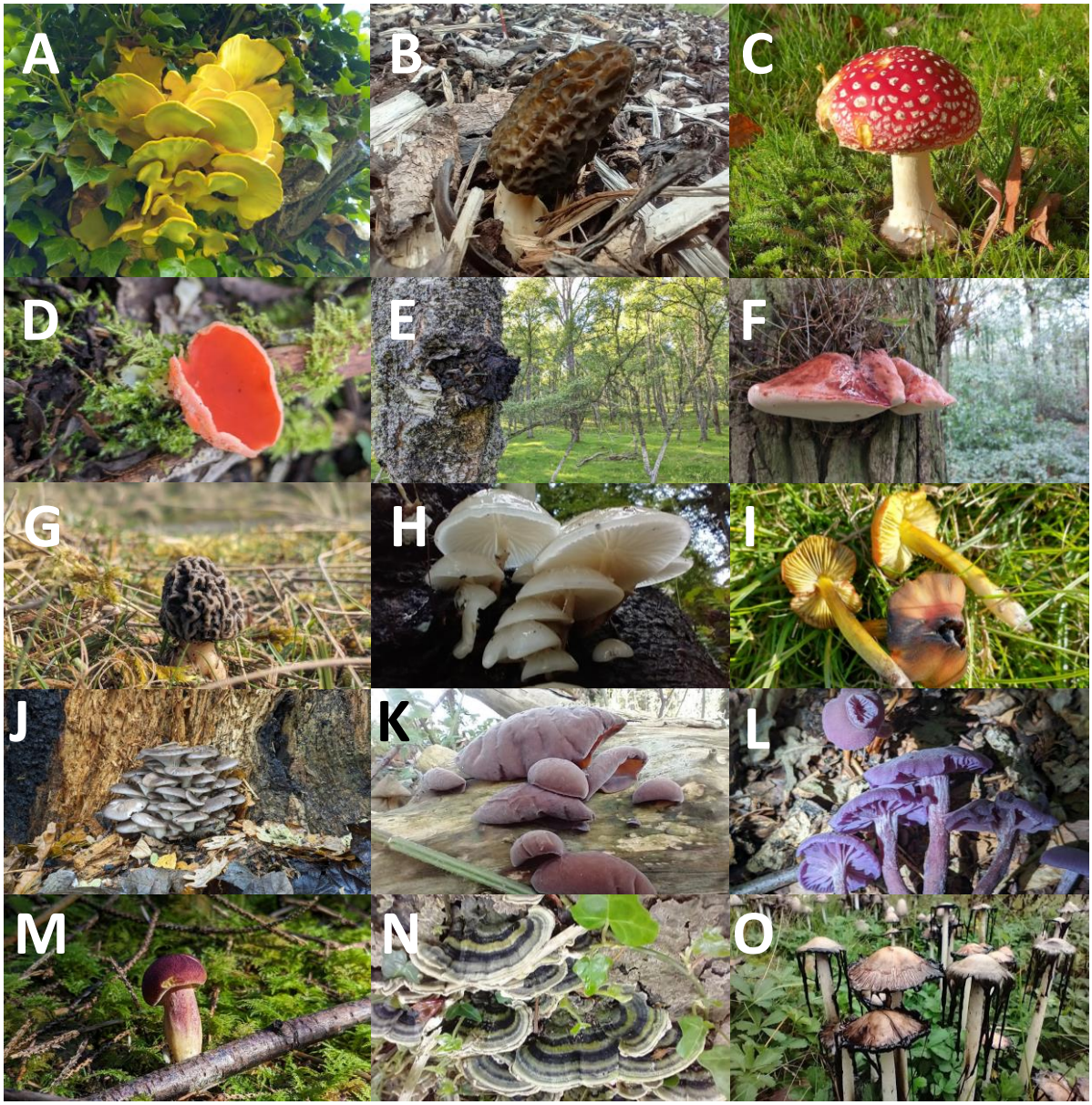


Figure 3. Selected example species showing the morphological diversity of macrofungi that occur in the UK. **A:** *Laetiporus sulphureus.* **B:** *Morchella elata.* **C:** *Amanita muscaria*. **D:** *Sarcoscypha coccinea.* **E:** *Inonotus obliquus*. **F:** *Fistulina hepatica*. **G:** *Morchella* sp. **H:** *Oudemansiella mucida*. **I**: Hygrocybe conica. **J**: *Pleurotus ostreatus*. **K**: *Auricularia auricula-judae*. **L**: *Laccaria amethystina*. **M:** Tricholomopsis rutilans. **N:** Trametes versicolor. **O:** Coprinus comatus.

The phylogenetic diversity of fungi is matched by metabolic diversity; fungi are capable of producing a wide variety of bioactive compounds with diverse and novel structures, mechanisms of action, applications and pharmacology [23-25]. Consequently, exploration of fungal chemistry has historically been and continues to be of high interest to the pharmaceutical industry, in natural products research, and has been

incredibly fruitful [25]. For example, in a recent review of antifungal natural products with novel scaffolds discovered between 2010-2019, between fungi, plants, algae, sponges, actinomycete bacteria and non-actinomycete bacteria, almost 50% were derived from fungi [26]. Many fungal natural products have found use as or served as the lead compounds for the development of new pharmaceuticals and agrochemicals [24]. For example the β-lactam antibiotics and the statins were discovered from *Penicillium* spp. and *Aspergillus terreus* respectively [27-29], the Ergot alkaloids from *Claviceps purpurea* [30]. Even one of the most dangerous mycotoxins, α-amanitin produced by *Amanita phalloides*, has found use in biological research due to its inhibition of RNA polymerases, and antibody conjugates have been investigated in cancer medicine [31]. Fungal products derived from mushrooms and their extracts, for example the Erinacines, nerve-growth factor stimulating cyathin diterpenoids from *Hericium erinaceus* [32], and the Ganoderic acids, triterpenoids with potential anti-cancer properties [33] are also becoming increasingly popular in alternative medicine and the health supplement industry [34-36], as research and understanding of their potential health benefits increases (**Figure 4**).

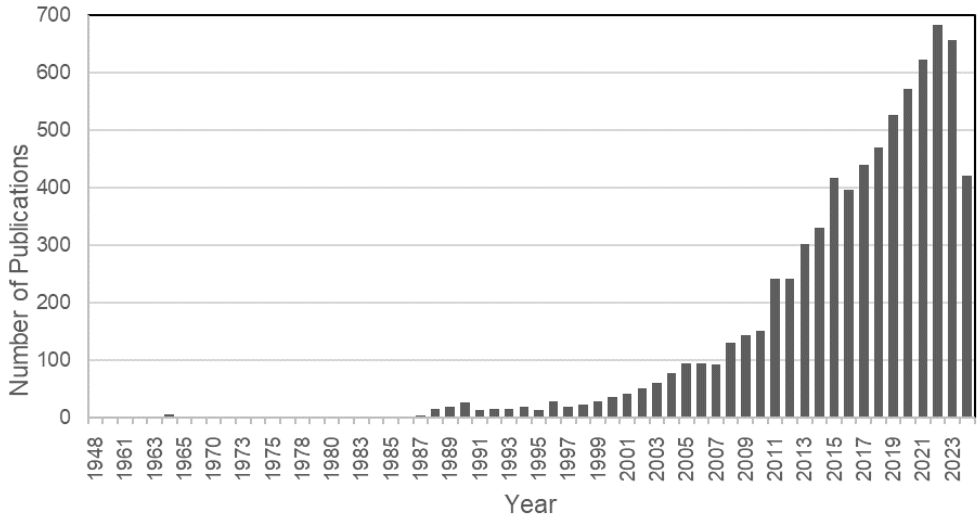
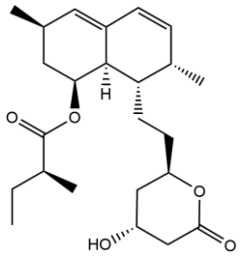


Figure 4. Number of publications (Total 7576) per year between 1948-2023 retrieved from PubMed using the search term "medicinal mushroom".

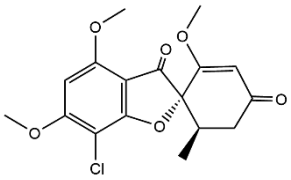
Griseofulvin, an antifungal drug used to treat ringworm, was derived from Penicillium griseofulvin [8]. In the 20th century, certain mushroom-derived metabolites played an important role in our understanding of the pharmacology of the nervous system, for example muscarine (discovered from *Amanita muscaria* along with muscimol and ibotenic acid), aided in the characterisation of the muscarinic acetylcholine receptor through its agonistic action [37, 38], and Psilocybin, a metabolite found in various mushroom species and genera, found widespread use in psychiatry.

Pleuromutilin is an antibiotic terpene originally discovered in the 1950s from the mushroom species *Omphalina mutila* (formerly *Pleurotus mutilus*). It has antibacterial properties through a novel mechanism of action, inhibiting protein systhesis by binding to peptidyl transferase in the ribosomal 50S subunit. Pleuromutilin has been used as a lead compound to generate further antibiotics in the class which have been approved as medicines, including Tiamulin, Retapamulin, Valnemulin, and Lefamulin [24, 39, 40]. Irofulven, an experimental drug which has been under development as a cancer medication, is an analogue of the Illudin family of sesquiterpenes found in *Omphalotus illudens* [24].

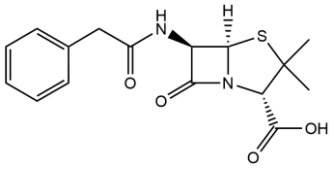
Natural products derived from mushrooms have also proved to be successful leads in the development of agricultural fungicides with a novel mechanism of action as quinone outside inhibitors, for example, Azoxystrobin was developed following the discovery of the strobilurins and the structurally similar Oudemansins from *Strobilurus tenacellus* and *Oudimansiella mucida*, respectively [24, 41, 42].



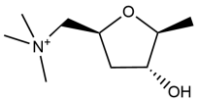
Lovastatin



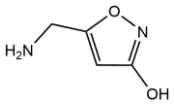
Griseofulvin



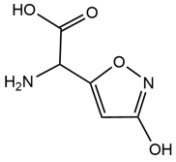
Penicillin G



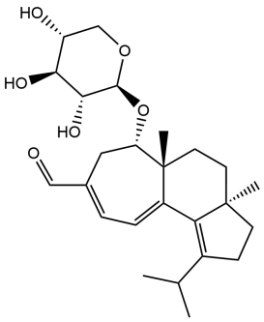
Muscarine



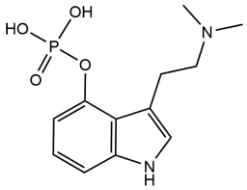
Muscimol



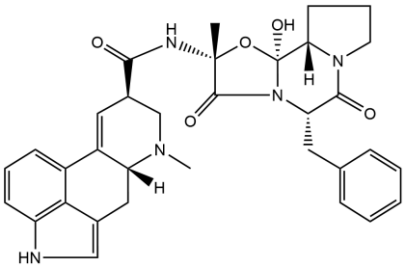
Ibotenic acid



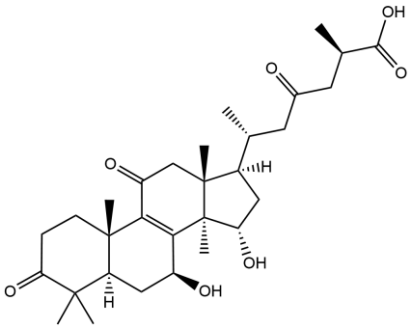
Erinacine A



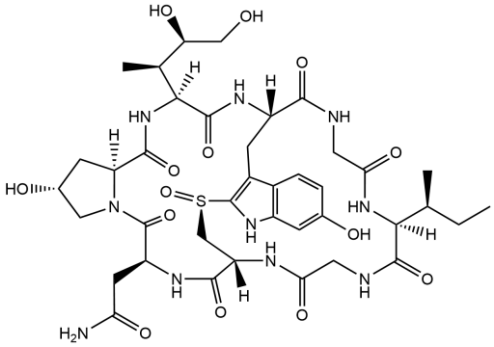
Psilocybin



Ergotamine



Ganoderic acid A



α-Amanitin

Figure 5. Examples of historic discovery of drugs derived from Fungi.

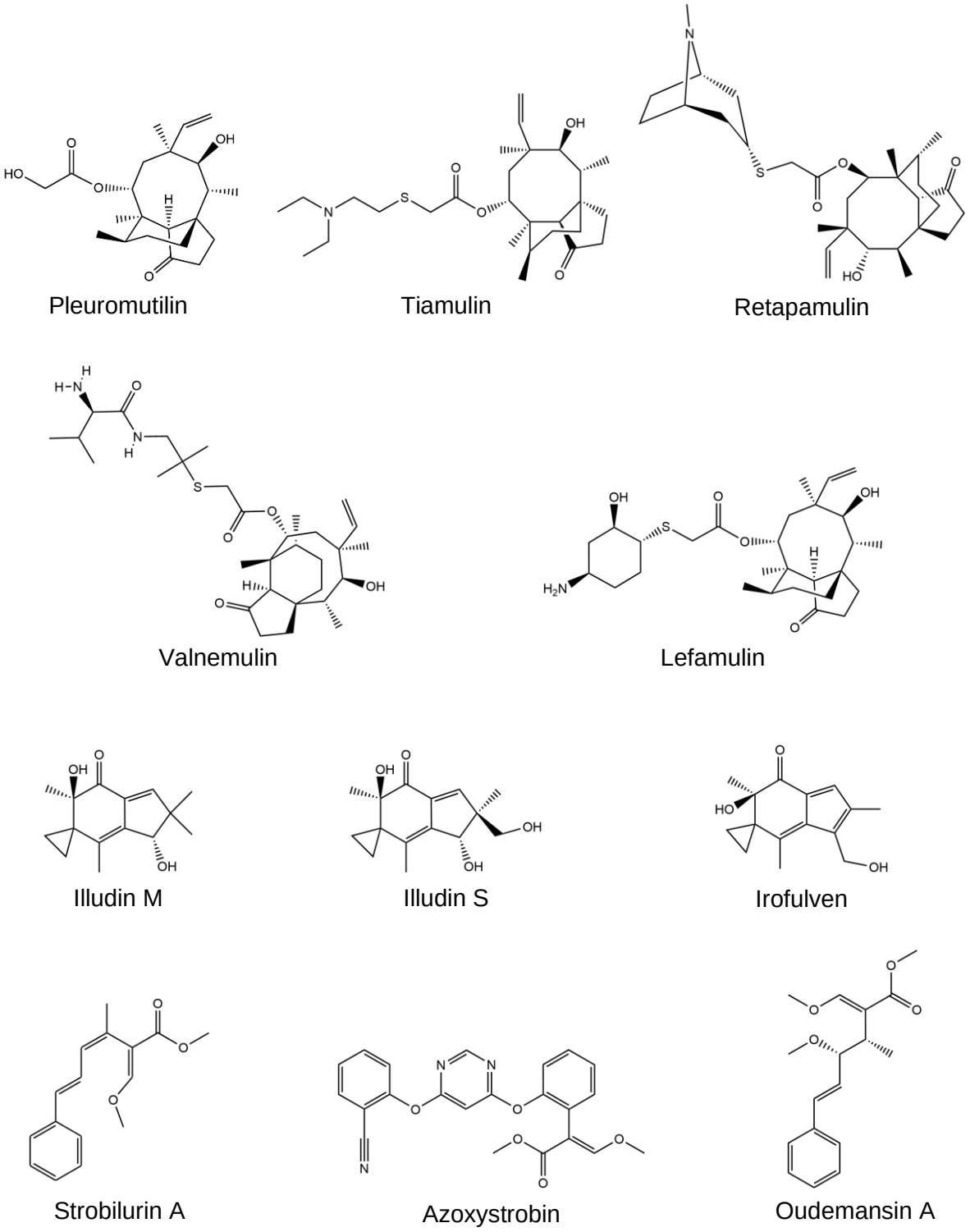


Figure 6. Examples of important pharmaceuticals and agrochemicals developed from mushroom metabolites.

Many of these metabolites are alkaloids, a major class of natural products with a high degree of structural diversity, many of which have bioactivity and have been utilised themselves as medicines, or as starting material in the production of semi-synthetic derivatives [43].

Some of the most famous alkaloids derived from fungi include the Ergot alkaloids such as the ergolines, derived from the Hypocrealean genus *Claviceps*, in particular *Claviceps purpurea*, from which many drugs in use today were derived [44, 45], having an impressive range of therapeutic applications. These include Ergotamine and Ergometrine, which were once used in obstetrics to induce uterine contractions and treat haemorrhage, and Ergotamine is also used as a treatment for migraines. Bromocriptine is utilised as a treatment for pituitary tumours, Parkinson's disease and hyperprolactinaemia. Hydergine, due to its action in increasing cerebrovascular blood flow and modulation of neurotransmitter systems has found use in the treatment of dementia and recovery from stroke.

There are several known simple indole alkaloids of plant or fungal origin which are closely structurally related to serotonin and are considered as psychedelics. Although they are capable of producing powerful perceptual changes, alterations of cognitive processes, and other physiological effects through via full or partial agonism of the 5-HT_{2A} receptor, they are generally not considered as physiologically harmful or addictive [46]. In the fungal kingdom, psilocybin may be the most famous example, and is produced by many mushroom species across several genera including certain *Inocybe* spp., *Pluteus salicinus*, several *Panaeolus spp*., but in particular mushrooms of the *Psilocybe* genus [47, 48] (**Figure 7**), many of which are also known to produce multiple analogues of psilocybin, such as baeocystin and norbaeocystin.

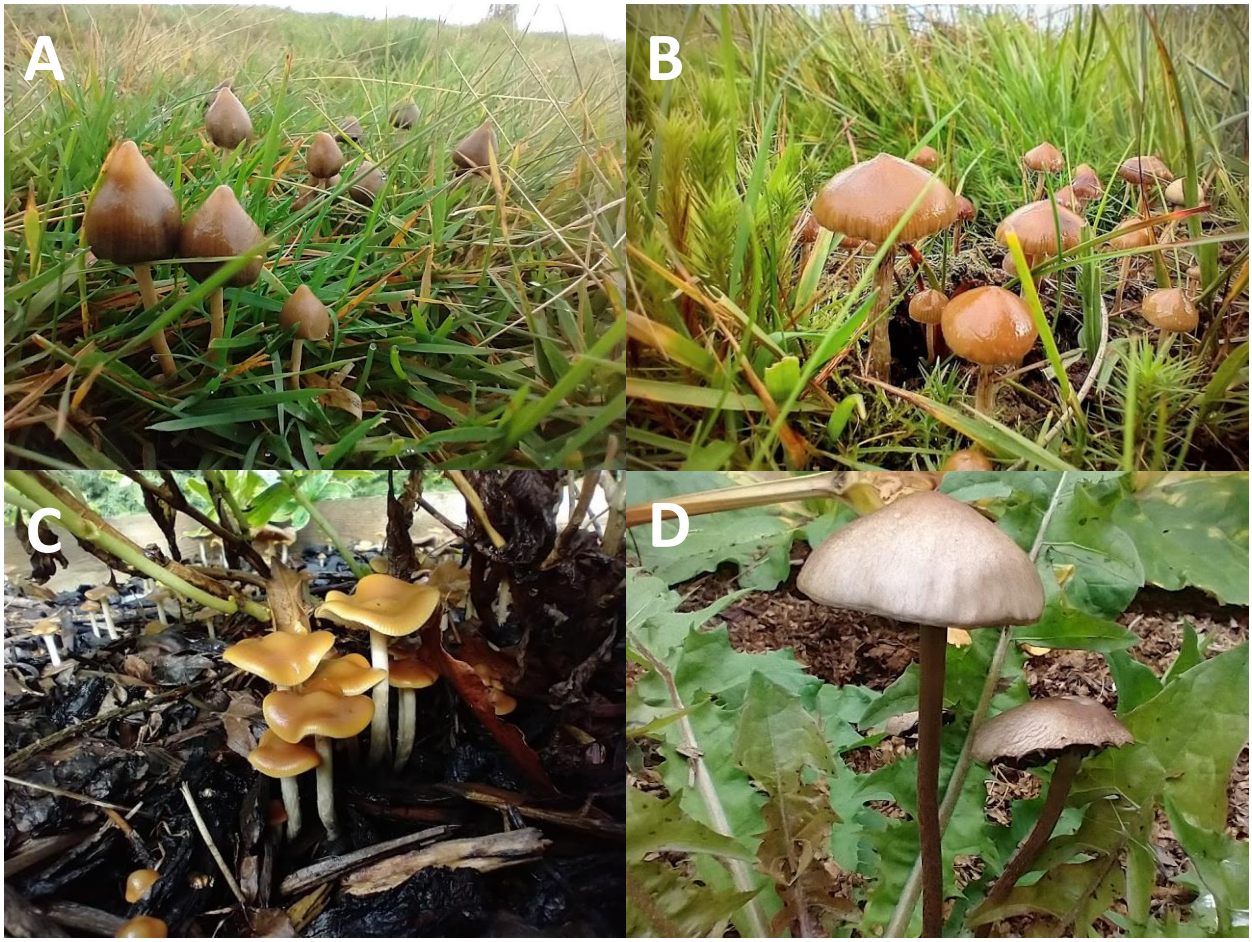


Figure 7. Examples of British psilocybin-containing mushroom species. A: *Psilocybe semilanceata*. **B:** *Psilocybe fimetaria*. **C:** *Psilocybe cyanescens*. **D:** *Panaeolus cinctulus*, All images photographed by the author.

Psilocybin was discovered and its structure elucidated from *Psilocybe Mexicana* in the 1950s [49], though psilocybin-containing mushrooms had been used by indigenous cultures in central and South America in ritualistic, religious and/or healing ceremonies since the pre-colombian era [50]. Following it's discovery, in 1960 Sandoz Pharmaceuticals began to produce and distribute a pharmaceutical form called Indocybin™, containing 2mg of psilocybin per tablet [50], promoting it's potential applications in psychiatry. Recently, it has been investigated alongside conventional therapy as a treatment for major depressive disorder, showing significant antidepressant potential in initial studies after a single dose [51], which subsequent follow up studies have shown may persist for at least 12 months [52]. As well as this, the drug has shown potential in the treatment of end-of-life anxiety [53], as well as being widely used as a recreational drug.

1.3 Issues in natural products discovery

Despite the historic and continuing success in natural product discovery from fungi, the potential for fungi to produce bioactive secondary metabolites is still considered to be greatly underestimated [54]. This is especially true considering that new species continue to be discovered, and that there are estimated to be several million undiscovered species [20]. As well as this potential abundance of undiscovered fungi ,which will certainly contain species capable of producing novel metabolites, even many well-known species are underexplored chemically.

Therefore, it is considered that the fungi still represent a largely untapped source of natural products [55]. However, there are significant challenges faced in the discovery of new natural products, including a high rediscovery rate of already known compounds. This underlines the importance of utilising methods to reduce the likelihood of rediscovery through a process known as dereplication, during which a metabolite under analysis is identified as a known compound early on, before attempting to isolate the metabolite in sufficient yield and purity for structural elucidation, which is often a challenging, time-consuming, costly series of steps that require considerable skill and expertise in several areas, and may still not produce enough pure material [23].

Another issue faced is the lack of access to or the difficulty in obtaining and isolating cultures of novel microbial species and strains. Many of the known species have already been chemically investigated, and although it may still be possible to discover more natural products from them, the easiest novel metabolites to purify (the so called low-hanging fruit) may have already been discovered. In order to facilitate the discovery of novel microbes to study, researchers are increasingly searching for them in more unusual and difficult to access environments, such as mangroves [56, 57], tropical rainforests and the deep sea [57-61]. As well as this, more unusual groups of organisms are being targeted, for example the largely unexplored entomopathogenic fungi [62].

Finally, genome mining techniques have demonstrated in multiple microbial species that the biosynthetic gene clusters (BGCs) responsible for the production of secondary metabolites are often expressed only at a low level (below detection), or are not expressed at all, they are silent or 'cryptic' under laboratory conditions under which the

specific factors and conditions required for BGC expression and metabolite production (which may be unknown) are absent [63].

1.3.1 One Strain Many Compounds (OSMAC) approaches

Secondary metabolite production in laboratory cultivation has several prerequisites; the BGCs responsible for their biosynthesis must be active, and the organism under investigation must have the necessary biosynthetic precursors and environmental factors required. The metabolites produced in a given medium and set of environmental parameters may not be truly representative of the true metabolic potential of a given organism. Consequently researchers have pursued media and environmental manipulation as a way to maximise the diversity or yield of metabolites produced and to determine the factors required for their biosynthesis, so that sufficient yields can be obtained for structural elucidation, bioassays, and large-scale production. This is a traditional and still successful method utilised in natural products discovery from microbial species known as the One Strain Many Compounds (or OSMAC) approach [59, 60, 64].

By varying the available nutritional components in the culture medium, for example carbon sources (Various sugars, glycerol, crude extracts such as malt extract, potato extract), nitrogen sources (for example by addition of various isolated amino acids, concentrations of crude nitrogen sources such as peptone, yeast extract etc), as well as pH, light exposure, temperature, availability of oxygen and turbidity to list a few, it is possible to affect the metabolism of the species and cause it to produce different metabolites under different conditions. For example, by varying the temperature, pH, and salinity of the culture media, the marine derived fungus *Arthrinium saccharicola* was shown to grow better in freshwater medium at 30°C, pH 6.5, but produce more metabolites and have greater antibacterial effect at 25°C at pH 4.5. The fungus displayed the same pattern when varying the available carbon and nitrogen sources; growing better in a high nitrogen medium but producing a greater antibacterial effect in a higher carbon medium [65]. Trace element content (for example the halogens, zinc, magnesium, Nickel) has also been manipulated to alter metabolic profiles. For example, compared to the original medium, *Aspergillus flavipes* produced 6 known cytochalasans as well as 2 novel compounds when Sodium

Bromide was included in the culture medium [66], and Streptomyces sp. WU20 produced new antimicrobial metabolites when nickel was added [67]. Microorganisms are typically grown in liquid fermentation, but it has been shown that cultivation on solid media can also result in a different metabolic profile [59], for example, several metabolites were produced by *Talaromyces wortmannii* differentially between liquid PDB and a solid rice based medium, including novel anthelmintic polyketides [68].

1.3.2 The use of epigenetic modifiers to aid in natural product discovery

Epigenetic regulation includes a fundamental set of mechanisms which cause heritable and reversible changes in the utilisation of DNA without changing the DNA sequence. For example, DNA methylation by DNA methyltransferase (DNMT) is a gene silencing mechanism. Histone modifications also regulate gene expression, for example histone acetylation by Histone Acetyltransferase (HAT) allows transcription factors to bind and express genes, while Histone deacetylation by Histone Deacetylase (HDAC) can reduce gene expression. Epigenetic regulation is involved in differentiation, maintenance of DNA integrity, response to environmental factors, and are now believed to be factors in the pathophysiology of a number of diseases, thus presenting new drug targets for the likes of cancer and neurodegenerative diseases [69]. In relation to plants and animals, epigenetic research on fungi is comparatively limited, even more so in the Dikaryon phylum (the mushroom and truffle forming genera within Basidiomycota and Ascomycota) which includes all edible and medicinal mushrooms of economic importance. At the time of writing, the majority of epigenetic research has been conducted using animals, plants, prokaryotes and Ascomycete moulds, for example the model organism *Neurospora crassa* [70]. Fungi are known for their ability to adapt to changes in environmental stimuli, and epigenetic regulation is believed to be important in this phenotypic plasticity [71].

However, fungi share the same epigenetic machinery as animals, meaning that the epigenetic modifier drugs approved as human medicines such as 5-Azacytidine (5-aza), a DNA methyltransferase inhibitor, Vorinostat (SAHA), a Histone deacetylase inhibitor (**Figure 8**), can also be used to alter epigenetic regulation in fungi, in some cases resulting in the activation of secondary metabolite BGCs silent under normal laboratory conditions. Indeed, microbial epigenetics has already proven to be a

promising avenue of research with not only potential for drug discovery [63, 72], but also in industrially-important strain improvement [73], and improving our understanding of host-pathogen interactions [74]. The use of epigenetic modifiers to increase the production of secondary metabolites in microorganisms has already proven particularly fruitful [70, 72].

A number of histone modifications are currently known to occur, including acetylation, methylation, phosphorylation and glycosylation. Histone acetylation is carried out by Histone acetyltransferases (HAT), and deacetylation by Histone deacetylases (HDAC). Histone acetylation is involved in the transition of heterochromatin to euchromatin, in which gene transcription is on, as acetylation allows transcription factors to bind and initiate transcription [69], so targeting HDAC via addition of an inhibitor such as SAHA can result in increased metabolite production in fungal fermentation. There is evidence that histone modifications and DNA methylation are linked, and that for some sites histone modifications are necessary for demethylation [75]. The HDAC inhibitor Trichostatin A has been shown to reduce genome methylation, meaning that histone acetylation itself can lead to changes in methylation patterns and gene expression [76]. For these reasons, a histone deacetylase (HDAC) inhibitor such as vorinostat (SAHA) or suberoyl bis-hydroxamic acid (SBHA) may increase the demethylation caused by 5-aza, potentially altering transcription of more genes than under 5-aza alone. There is evidence to support the efficacy of the combined HDAC and DNMT inhibition approach for fungal natural products discovery, for example treatment of the entomopathogenic fungus *Gibellula formosana* with SBHA and RG-108 (DNMT inhibitor) resulted in the production and isolation of several novel secondary metabolites, including ergosterol analogues and isariotin analogues [77].

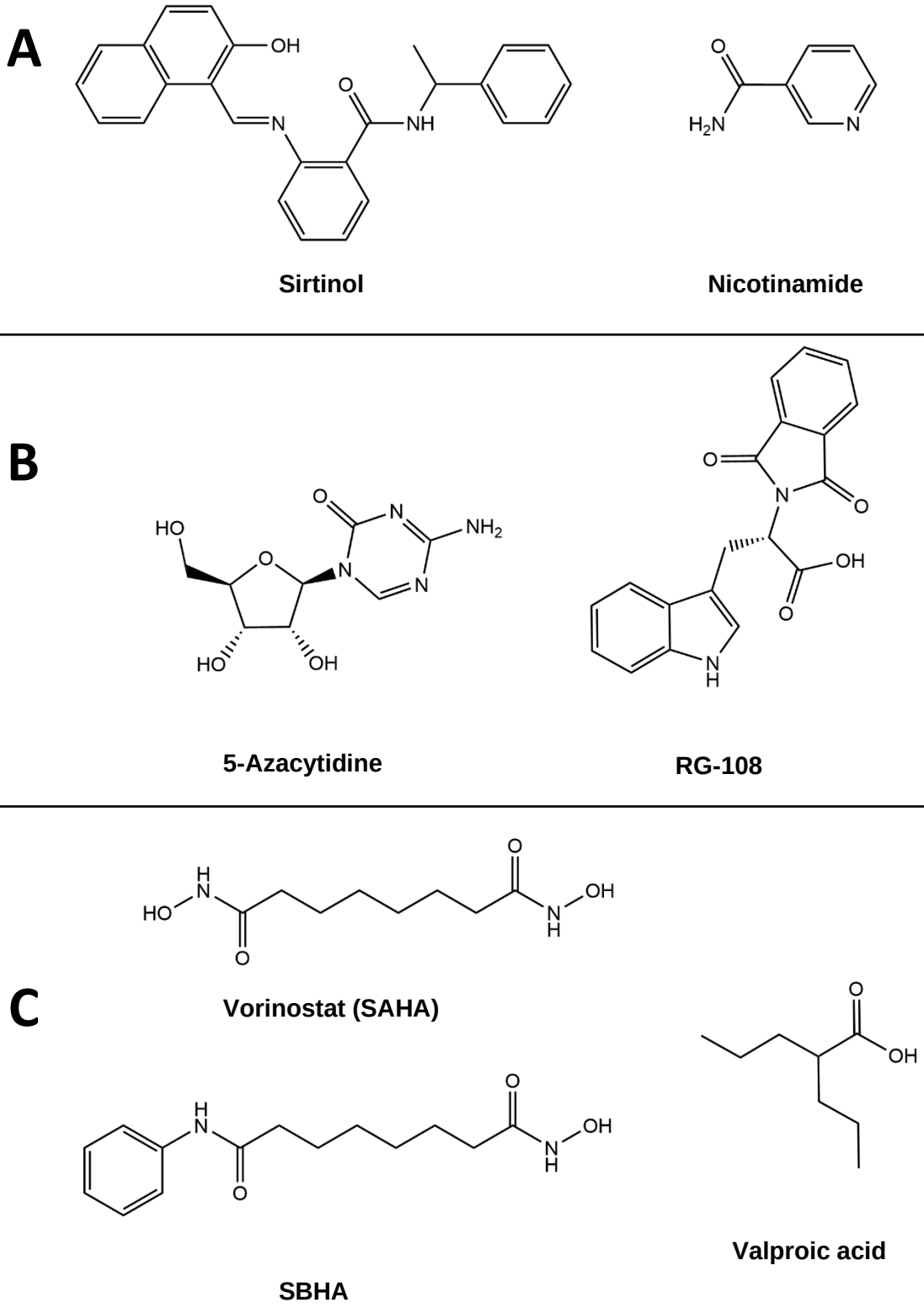


Figure 8. Structures of selected epigenetic modifiers with different mechanisms of action. **A:** Sirtuin inhibitors – Sirtinol and Nicotinamide. **B:** DNA Methyltransferase (DNMT) inhibitors – 5-Azacytidine (5-aza) and RG-108. **C:** Histone Deacetylase (HDAC) inhibitors – Vorinostat (SAHA), Suberoyl bis-hydroxamic acid (SBHA) and Valproic acid (VPA).

1.4 Aims and overview of the research reported in this project

Natural products research has been and continues to be incredibly important in the discovery of a plethora of diverse new substances, which serve as medicines and agrochemicals, as well as help us to better our understanding of biochemistry; that of the producing organism, the organisms and environment with which it interacts and lives, and that which is relevant to the treatment of pathology. Despite the historic success in the field, including in the discovery and development of fungal metabolites into useful and industrially important products, there still remains much work to be done, and significant problems to overcome, such as the previously mentioned challenges faced in novel natural product discovery.

The aims of this research were to collect and isolate cultures from a variety of fungal species native to the UK, including species with known medicinal potential that have already been chemically investigated, but also underexplored or neglected species on which little to no chemical investigation has been done to date, building a culture collection to explore chemically through laboratory fermentations and chromatographic methods. The influence of media composition on metabolite profiles was investigated, in addition to the effects of various epigenetic modifiers, in order to identify the most promising species and media composition for further research and attempts at novel metabolite purification at a larger scale, as well as to identify species which respond to epigenetic modification through the production of new metabolites in laboratory conditions.

Following these screening experiments, one of the fungi, a spider-pathogenic fungus in the *Gibellula pulchra* clade, was selected as a promising species for more in depth experimentation through further media and epigenetic manipulation experiments as well as with the addition of other elicitor compounds and metabolic precursors.

Hundreds of extracts were obtained from these experiments, a selection of which were screened for antibacterial efficacy alongside extracts of wild mushrooms, and the most promising leads were followed through chemical fractionation methods in attempts to isolate and identify the responsible compounds.

Finally, this project aimed to examine unknowns concerning the biology of ectomycorrhizal fungi through fieldwork, culture isolation and fermentation attempts, and in nursery pot-trials, where the effect of inter-species interactions with several

bacterial strains on colonisation of the host plant was examined, alongside the effects of addition of elicitor compounds.

Chapter Two

Screening selected UK native fungi in several media with epigenetic modifiers and other chemical elicitors

2.1 Overview

In this research, cultures from a variety of British fungi were isolated and their chemistry was examined utilizing a small scale fermentation screening approach.

The fungi were fermented in liquid and semi-solid media, with and without the addition of various epigenetic modifiers and chemical elicitors, in order to characterise their metabolic profiles under these conditions, identify responders to epigenetic modification, and identify candidates for larger scale fermentations aimed at isolating novel secondary metabolites with potential bioactivity.

2.1.1 Fungal species utilised

Cultures of several fungal species were isolated from wild samples collected within the UK (Figure 9-10, collection locations Appendix 7), of the total cultures isolated, 14 isolated cultures were screened in this manner (*Fomitopsis pinicola, Fomes fomentarius, Trametes versicolor, Inonotus obliquus, Tremella mesenterica, Onygena equina*, *Gibellula pulchra*, *Tuber borchii*, *Ganoderma resinaceum, Morchella elata, Beauveria bassiana,* and 3 additional unidentified spider-pathogenic fungi).

Several of these fungi are considered as ‘medicinal mushrooms’, for example *Fomes fomentarius, Trametes versicolor, Inonotus obliquus* and *Ganoderma resinaceum*, and have been previously studied chemically [34, 35, 78]. However, others can be considered as neglected species, as they appear to have had no previous chemical investigation to date, despite having been known for a long time and there being evidence that they may have significant potential in novel secondary metabolite discovery. For example, *Onygena equina* (Figure 10), a species which lives on the horns and hooves of dead undulates, has not been investigated, though through genomic analysis and chemical analysis of other species within the Onygenales, it has been revealed that the order possesses great potential for producing diverse metabolites (Figure 11).

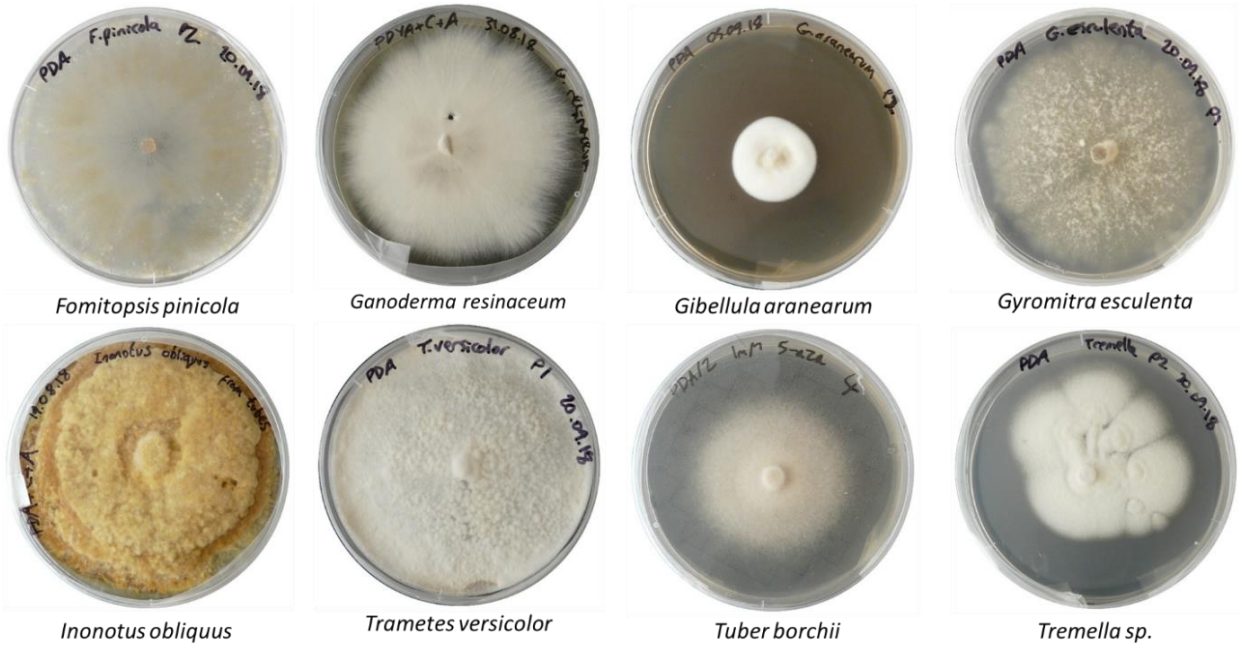


Figure 9. Example fungal cultures isolated and used in this work growing on agar plates

Figure 10. *Onygena equina* fruit bodies (White arrows) growing on the decaying horn of a sheep (*Ovis aries*) in moorland in Northern England. Several specimens were collected for culture isolation and utilised in chemical screening.

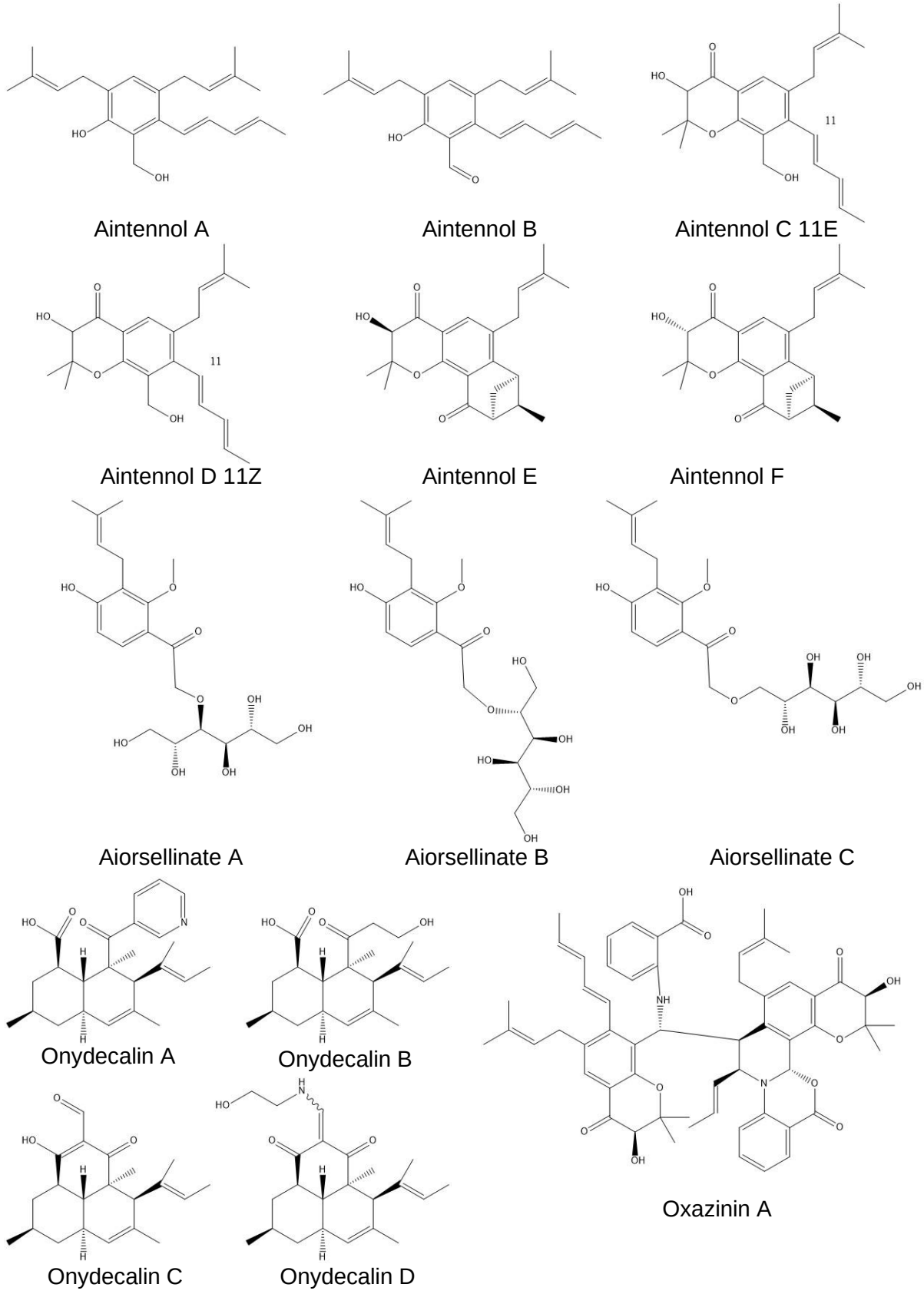


Figure 11. Structures of known compounds from the Onygenales family of fungi

2.1.2 Approaches utilised

Fermentations were initially conducted on a small-scale (10mL), and the fungi were screened in the presence of 5-Azacytidine (DNA methyltransferase inhibitor), Vorinostat/SAHA (a HDAC inhibitor), Valproic acid (HDAC inhibitor), Sirtinol (Sirtuin inhibitor), and Nicotinamide (Sirtuin inhibitor). In later experiments, the combination of multiple compounds with differing mechanisms of action was tested to determine if there were any synergistic effects from, for example 5-Azacytidine + SAHA, SAHA + Sirtinol, 5-Azacytidine + SAHA + Sirtinol. Aswell as the epigenetic modifiers, various other additives were tested including tryptophol, tryptamine, tryptophan, and estrone.

High performance liquid chromatography was utilised as the primary method to determine any effects on the metabolite profiles of the fungi treated with the chemical additives. After screening, the most promising cultures were utilised in larger scale fermentations, in order to try and obtain and purify a sufficient metabolite yield for structural elucidation and bioassays.

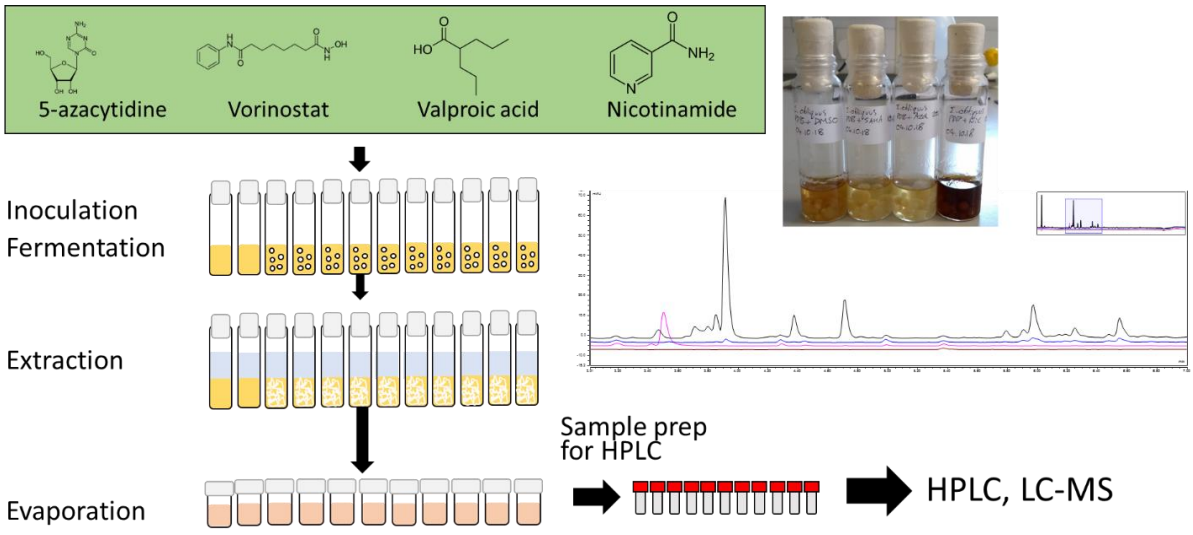


Figure 12. Schematic overview of the experimental process used in the screening experiments

2.2 Experimental methods.

2.2.1 Culture isolation methods.

Specimens of fungal fruiting bodies were collected and returned to the laboratory (Collection details in Appendix 7), and identified by examination of their ecological and morphological characteristics (habitat, substrate, macroscopic morphology, microscopic morphology (for example analysis of spores). After identification and return to the laboratory, attempts to isolate cultures were made within 24 hours of collection, inside a laminar flow cabinet.

In general, culture isolation was conducted by cleaning the outside of the specimen with 70% isopropyl alcohol (When possible – some species such as *Onygena equina* and spider-pathogenic fungi were too small and delicate) and opening the specimen via taring or cutting into it using a sterile scalpel. Small pieces of tissue were removed from inside the fungi and placed onto Potato Dextrose Agar (PDA) plates containing a combination of Chloramphenicol and Ampicillin to prevent bacterial contamination. The initial plates were sealed with parafilm and incubated at 25^{o}C until sufficient mycelium had grown from the excised tissue samples to transfer to a second pate, at this point small sections/wedges of agar were cut from the edge of the growing colonies and transferred to new agar plates. This transfer process was repeated several times in order to remove the new growth from the original sample, which is a potential source of contamination.

Some isolations were conducted by germinating spores on agar plates rather than excising tissue from within the fungus, for example with dried specimens, donated spore prints, and very small specimens where tissue excision was not possible.

Plates were discarded in the event of no growth, or contamination by yeasts, bacteria or common fungal contaminant species such as *Aspergillus*, *Trichoderma* or *Penicillium*.

2.2.2 Preparation of inoculum

Liquid inoculum was prepared by aseptically removing small pieces of mycelium from the growing agar cultures and transferring into autoclave-sterilised liquid media (for example Potato Dextrose Broth or Yeast Malt Glucose Broth) in small conical flasks. The inoculated flasks were incubated (25°C) and shaken at 120-150rpm until sufficient mycelial biomass had grown in the liquid media. The liquid cultures were then gently homogenised using a sterile potter homogenizer prior to use.

Figure 13. Photograph showing an example of a small-scale screening experiment with *Fomitopsis pinicola* in PDB.

2.2.3 Small-scale epigenetic screening methods

For the smallest scale (10mL) fermentations, 10mL of liquid media was added to 30mL glass tubes, and cellulose stoppers/filters were added to allow gas exchange and prevent contamination (**Figure 12)**. The tubes were then autoclaved to sterilise. After sterilisation, the tubes were inoculated by transferring homogenised fungal inoculum (0.1mL) via pipette.

2.2.4 Preparation and addition of chemical additives

Stock solutions of the various chemicals were prepared in DMSO to give a concentration of 10mM, and then sterilised by filtration through 0.2µm syringe filters into sterile glass vials, Eppendorf tubes or cryotubes, which were used immediately or stored at -20°C until further use. Chemical additives were added to fungal fermentations at the point of inoculation, to give a final concentration of 100µM.

2.2.5 Extraction of small-scale screening fermentations

Fermentations were sampled at various timepoints for extraction, to allow monitoring of metabolite profiles over time. The initial small-scale fermentation samples were transferred into a potter homogenizer and homogenized, then the homogenized material was returned to the glass fermentation tubes and 20mL of ethyl acetate was added. Chemical-resistant lids were placed onto the tubes, which were then shaken on a shaker platform for several hours. After shaking, the mixtures were allowed to sit until the ethyl acetate layer had separated from the aqueous layer, at which point the ethyl acetate was collected, dried using magnesium sulphate, filtered, and then evaporated under reduced pressure until no solvent or water remained.

2.2.6 Large scale extraction methods

For larger-scale fermentations, a more thorough extraction procedure was utilised to facilitate purification and allow comparisons between metabolites present in the biomass and in the fermented liquid media. Firstly, biomass was separated from the liquid media. For volumes under 500mL, this was done via vacuum filtration or centrifugation in 50mL falcon tubes (6000rcf, 10 minutes, 4°C). For volumes greater than 500mL, the biomass was separated from the liquid media via large-scale centrifugation in 1000mL centrifuge bottles (3000rcf, 10 minutes, 4°C). After centrifugation the supernatant was decanted off the biomass into a separate glass container. In the event of solid material on the surface of the supernatant, the liquid was filtered via vacuum filtration.

Supernatant samples were extracted with an equal volume of ethyl acetate, magnetically stirred at room temperature, at a speed great enough to fully mix the two layers. The extractions were stirred magnetically for several hours, then removed from the stirrer and transferred into a separatory funnel and the layers were allowed to separate. The aqueous layer was then returned to the extraction vessel and extracted again 2 more times, or until the non-polar layer did not change colour during extraction. Water was removed from the ethyl acetate by mixing with a sufficient quantity of $MgSO_4$, which was then removed by filtration and washed with a small amount of ethyl acetate. The ethyl acetate was then removed under reduced pressure using rotary evaporators (40°C), and then placed on a high vacuum apparatus for several hours to remove traces of solvent.

Biomass samples from fermentations over 500mL were homogenised via blending, and 500mL acetone was added to the biomass in a duran bottle. The acetone/biomass mixture was then sonicated (30 minutes, 40°C), and the acetone collected via filtration. The biomass acetone extraction was then repeated 2 more times, or until the acetone was no longer coloured after filtration. The acetone was then transferred into a round-bottom flask and the acetone was removed under vacuum (40°C) until only water and insoluble material remained. The contents of the flasks were then transferred into separatory funnels, and the flasks were rinsed into the funnels using 100mL dH_2O and 100mL EtoAc. The separatory funnels were shaken to mix the contents and the tap was opened regularly to vent the pressure buildup. After shaking for several minutes, the layers were allowed to separate, and the EtoAc was collected in a beaker. The EtoAc extraction was repeated another 2 times, or until the EtoAc was no longer coloured after filtration. Water was removed from the pooled EtoAc by mixing with $MgSO_4$ and filtering, and the $MgSO_4$ was washed with a small volume of EtoAc. The EtoAc was then removed under vacuum using a rotary evaporator (40°C). For these larger samples, the EtoAC extract residue was transferred from the flasks into a separatory funnel by repeatedly adding small volumes of 90% methanol 10% dH_2O and transferring them, until 100mL had been used. Next, small volumes of Hexane were added to the flasks, swirled, and transferred into the separatory funnel until 100mL had been used. The contents of the separatory funnels were then mixed by shaking and regularly venting the pressure buildup. The layers were then allowed to separate and were split into different vessels, and then water was removed by adding

$MgSO_4$ and filtering into round bottom flasks, and rinsing the $MgSO_4$ with a small amount of methanol or hexane. The solvents were then removed under reduced pressure, and placed onto the high-vacuum for several hours to remove traces of solvents. Extracts were transferred from the round-bottom flasks into pre-weighed 15mL glass scintillation vials, by repeatedly adding to the flasks small volumes of methanol, swirling, and pipetting into the vials through a small piece of cotton placed into a glass Pasteur pipette. The volume of methanol used was kept as low as possible to transfer the extracts. The vials were then heated to 40°C and placed under an argon stream until all of the methanol had evaporated, then the vials were placed under a high vacuum to remove traces of solvent. When fully dry, the vials were re-weighed and the initial weight of the empty vial was subtracted to the final weight, to give the extraction yields, which were used to produce solutions of known concentration for analysis by HPLC, LCMS and anti-microbial assays.

Large-scale fermentations were also sampled (10mL) regularly in order to monitor the changes in the metabolite production over time. Biomass obtained from 10mL time-point samples was extracted by sonicating in acetone (20mL) for 30 minutes (40°C). The acetone was then collected by filtration and the biomass extracted in this way 2 more times. Acetone extracts were pooled and the acetone was removed under reduced pressure on a rotary evaporator, leaving a mixture of water and insoluble residue. The contents of the flasks were then transferred into a separatory funnel, and the flask rinsed into the funnel with 50mL dH_2O and 50mL Ethyl acetate. The separatory funnel was shaken vigorously several times to mix the two layers, opening the tap regularly to vent the pressure. The Ethyl acetate layer was collected, and the EtoAc extraction of the aqueous layer was repeated 2 more times. The EtoAc extracts were pooled together and water was removed by mixing with $MgSO_4$ and filtering, causing the EtoAc to become clear. The EtoAc was then removed under vacuum using a rotary evaporator (40°C), leaving a residue in the flask.

Supernatant samples were extracted with an equal volume of ethyl acetate, magnetically stirred at room temperature, at a speed great enough to fully mix the two layers. The extractions were stirred magnetically for several hours, then removed from the stirrer and transferred into a separatory funnel and the layers were allowed to separate. The aqueous layer was then returned to the extraction vessel and extracted again 2 more times, or until the non-polar layer did not change colour during extraction.

Water was removed from the ethyl acetate by mixing with a sufficient quantity of $MgSO_4$, which was then removed by filtration and washed with a small amount of ethyl acetate. Finally, the ethyl acetate was evaporated under reduced pressure.

2.2.7 Chemical fractionation methods

After analysis of the crude supernatant and biomass extracts by HPLC, selected extracts were subjected to automated flash chromatography (CombiFlash, Teledyne) in attempts to isolate individual compounds and semi-purified fractions.

Extracts were dissolved in small volumes of methanol and transferred into round-bottom flasks containing a small amount of TELOS material. The methanol was then removed under reduced pressure whilst rotating the flask, leaving the extract mixed into the TELOS material. The samples were then added into plastic combiflash cartridges containing a silica frit and compressed using a plastic rod. Sand was added to the top of the cartridge to occupy any remaining space.

Extracts were separated using pre-filled C18 flash chromatography columns using a gradient starting with high aqueous conditions (Solvent A, water), and finishing with 100% organic solvent (Solvent B) (methanol, acetonitrile, or both in succession). As the percentage of solvent B increased over the run, the UV absorbance of the eluent was monitored at several wavelengths, and was collected into glass test tubes racks. After the separation, the tubes containing the fractions were transferred into round-bottom flasks, separating the peaks from the UV chromatogram into the different flasks. Solvents were removed under reduced pressure, and the residues were analysed by HPLC/LCMS to determine purity and attempt to identify the metabolites present. Additional rounds of fractionation were conducted in attempts to further purify the components of the metabolite fractions.

Sufficiently pure compounds, in a great enough yield, were subjected to NMR spectroscopy in attempts to obtain NMR spectra for structural elucidation.

2.2.8 Analysis of extracts by HPLC

Extracts were analysed via HPLC at 210nm, 254nm, 270nm and 360nm using a C18 Accucore 2.1µm column. Solvent A was water + 0.1% formic acid, and solvent B was Acetonitrile + 0.1% Formic acid.

2.2.9 Sample preparation for HPLC

After evaporation of all solvent, the crude extract residues from 10ml screening fermentations were dissolved in 1ml of 80% HPLC-grade MeOH/20% MilliQ H_2O and filtered through 0.2µm PTFE syringe filters into 1.5ml HPLC vials for analysis.

2.2.10 Chromatographic method

Samples were analysed using a Thermo Scientific Dionex Ultimate 3000 UHPLC system fitted with an Thermo Scientific Accucore^{TM} 100mm x 2.1mm C18 column with 2.6µm particle size.

Mobile phases (**Mobile phase A**: MilliQ H_2O + 0.1% formic acid, **Mobile Phase B**: HPLC-grade Acetonitrile + 0.1% formic acid) were prepared fresh on the day of each run. Before running the samples the system was allowed sufficient time to heat the column oven to 40°C, the mobile phase lines were primed for 10 minutes, the injection needle was washed. At the start of each run the column was washed by pumping 100% solvent B at 0.5ml/min. Mobile phase starting conditions were pumped through the system. After preparing the system and washing the column, the proportion of mobile phase A was gradually increased until the method starting conditions were reached, and the UV absorption of the eluent and the pressure of the system were monitored until both reached a stable baseline.

Samples were ran using a gradient method starting at 10% mobile phase B/80% mobile phase A, holding for 1 minute, then increasing to 100% mobile phase B over 7 minutes, maintaining 100% mobile phase B for 4 minutes, then reducing back to starting conditions over 0.1 minute and holding starting conditions for 4 minutes to re-equilibrate the column between samples.

All sequences were started with 2 injections of blank 80% HPLC-grade MeOH/20% MilliQ H_2O prior to the first extract sample, and another blank injection between each extract sample in the run.

The following UV wavelengths were monitored: 210nm, 254nm, 270nm and 360nm.

2.2.11 Analysis of chromatographic data

HPLC data was interpreted using ACD Spectrus Processor. Chromatograms of extracts from fermentations in different media, and those with and without chemical modifiers added were stacked, and their UV absorbance profiles were compared to determine differences between the various groups.

In order to confirm which peaks resulted from the fermentation media ingredients and chemical modifiers added, media extract controls and chemical additive controls were included to determine their contribution to the chromatograms.

2.3 Results and Discussion

2.3.1 Effect of fermentation condition and duration on the metabolite profile of Gibellula pulchra

Gibellula pulchra fermented in either shaken (120rpm), shaken for half of the fermentation then static for the second half, or completey static, displayed significant differences in the abundance of particular metabolites produced (Figure 14). For example, in completely static fermentation, an increased abundance of metabolites was observed in the 3 - 3.5 minute region compared with the other conditions (Figure 14, Blue), while the shaken then static fermentation resulted in a significant increase in abundance of a peak at 5.5 minutes (Figure 14, Brown). Constant shaking resulted in the production of more polar metabolites, as well as several peaks in the 4-4.5 minute region not seen in the other fermentations (Figure 14, Green).

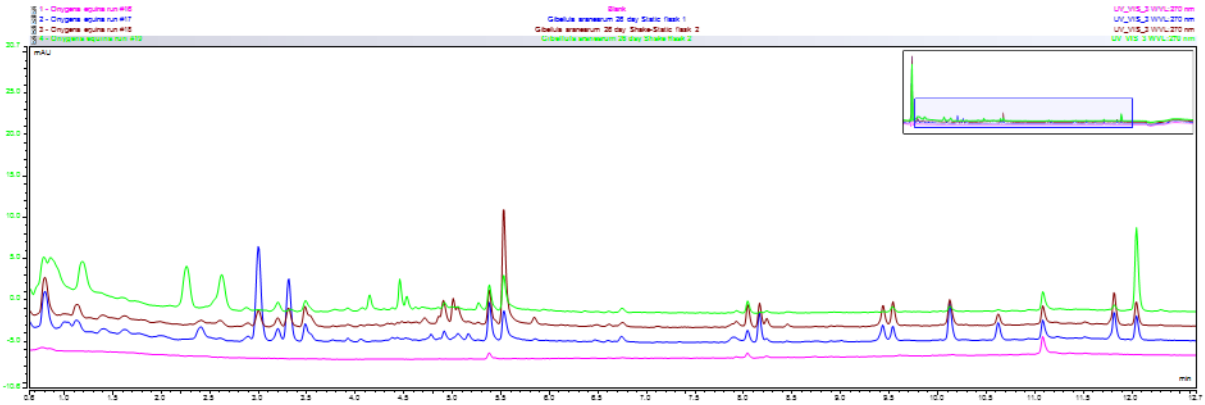


Figure 14. HPLC chromatograms (270nm) of extracts from *Gibellula pulchra* showing differences in metabolite profile under different conditions. Flasks incubated under constant shaking (Green), no shaking (Blue), and shaken for half of the fermentation then static for the second half (Brown).

In extracts of shaken fermentations sampled on day 12, only 1 major peak was present in the chromatogram, whereas 19 day fermentations showed a significant increase in the number of peaks observed (Figure 15)

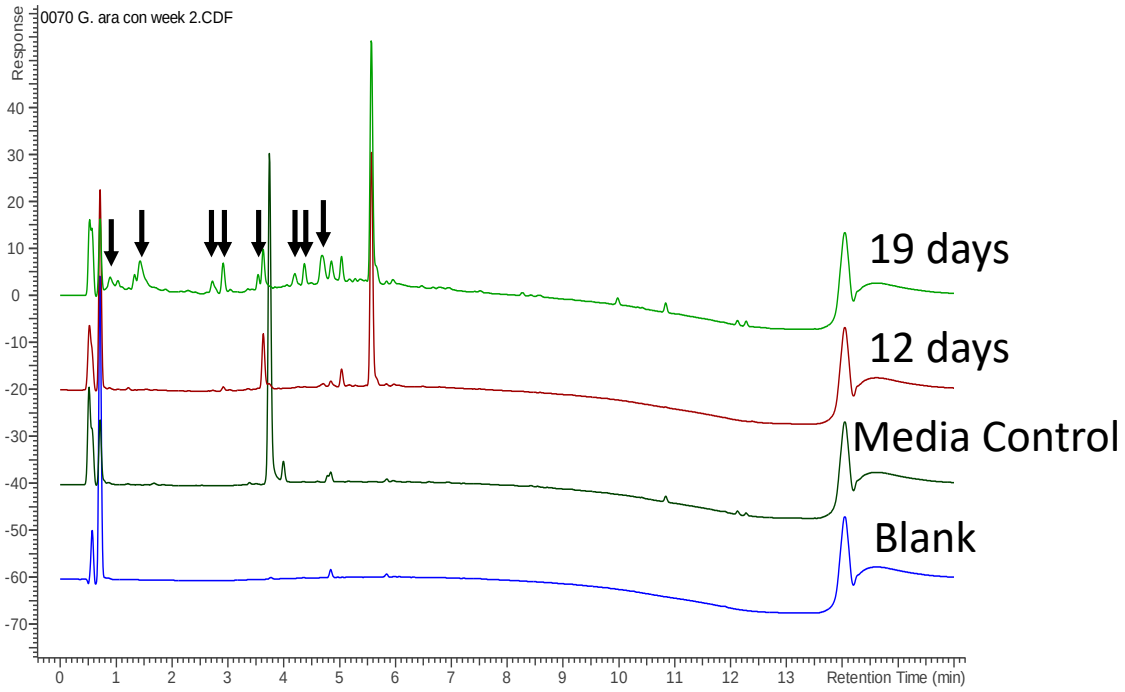


Figure 15. HPLC chromatograms (254nm) showing *Gibellula* pulchra fermentation extracts at 12 days and 19 days. At 19 days, several metabolites are present (arrows) which are not in the 12 day sample.

2.3.4 Small-scale epigenetic and chemical screening results

The initial screening experiments utilised four chemical additives (5-Azacytidine, Vorinostat, Valproic acid, Nicotinamide). Colour changes were often observed between the different treatments compared to controls, as well as changes in morphology and growth. For example, cultures treated with SAHA or Azacytidine often had a much darker brown colour then control cultures treated with DMSO. Some species displayed reduced biomass when treated with epigenetic modifiers, particularly *Tuber borchii*. The epigenetic modifiers did not have any significant effect on some of the species, however the HPLC chromatograms showed clear differences between some of the treated cultures compared with controls. For example, addition of SAHA to the fermentation medium of *Fomitopsis pinicola* resulted in several peaks which were not present in the control culture (**Figure 16**).

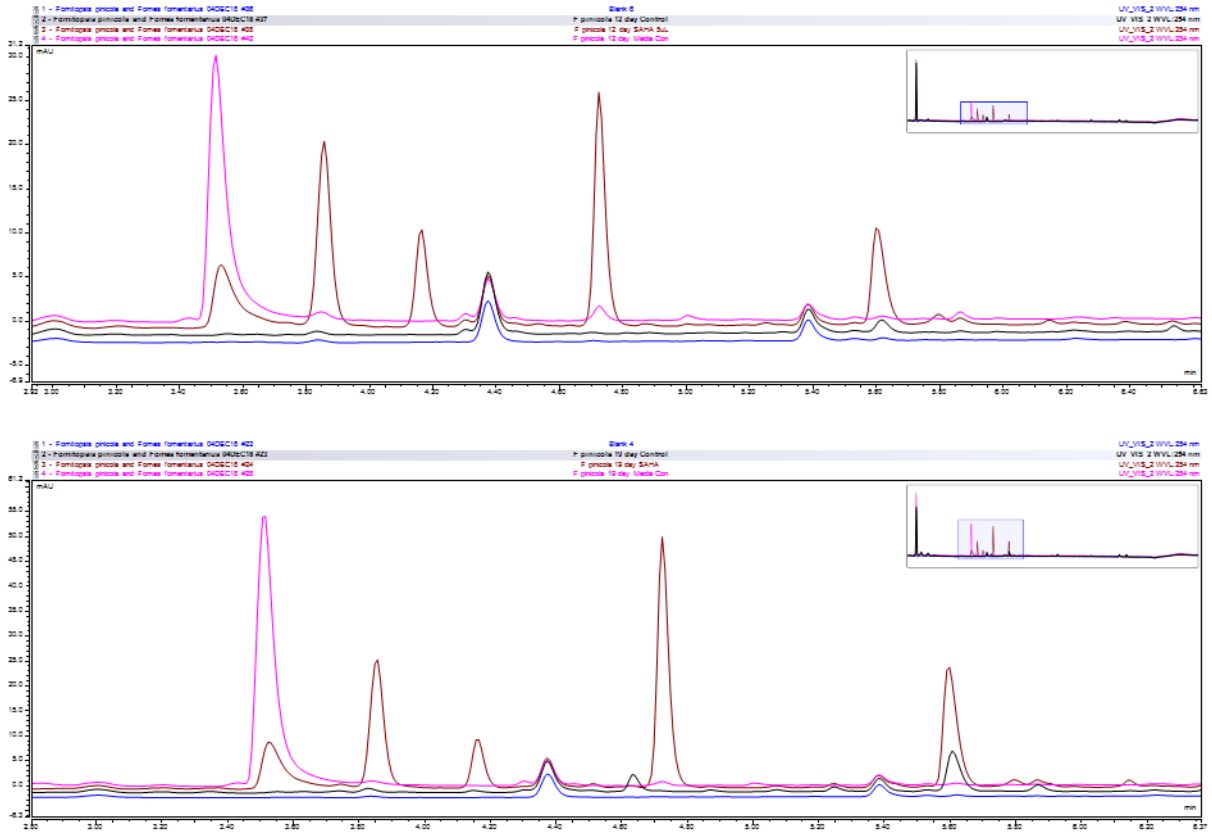


Figure 16. HPLC chromatograms (254nm) of *F. pinicola* 12-day (top) and 19 day fermented cultures (Bottom), showing MeOH Blank (Blue), untreated control culture (Black), SAHA-treated culture (Brown) and Media control (Pink).

Cultures of *Fomes fomentarius* showed little metabolite production or effect of the presence of the addition of the epigenetic modifiers, the most prominent differences resulting from addition of Vorinostat to the culture medium (**Figure 17**)

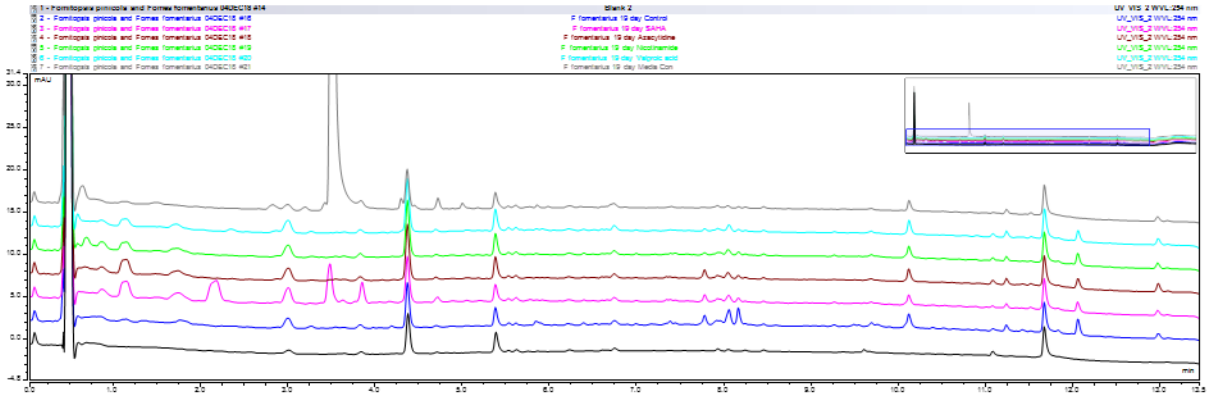


Figure 17. HPLC chromatograms (254nm) of *Fomes fomentarius* extracts (19 day fermentation) showing few peaks and only slight differences between the groups, with Vorinostat (Pink) having the greatest effect

Fermentations of *Gibellula pulchra* showed a greater number of metabolites present in the extracts compared with other species that were screened, as well as some significant differences in the groups treated with the epigenetic modifiers (**Figures 18 and 19)**.

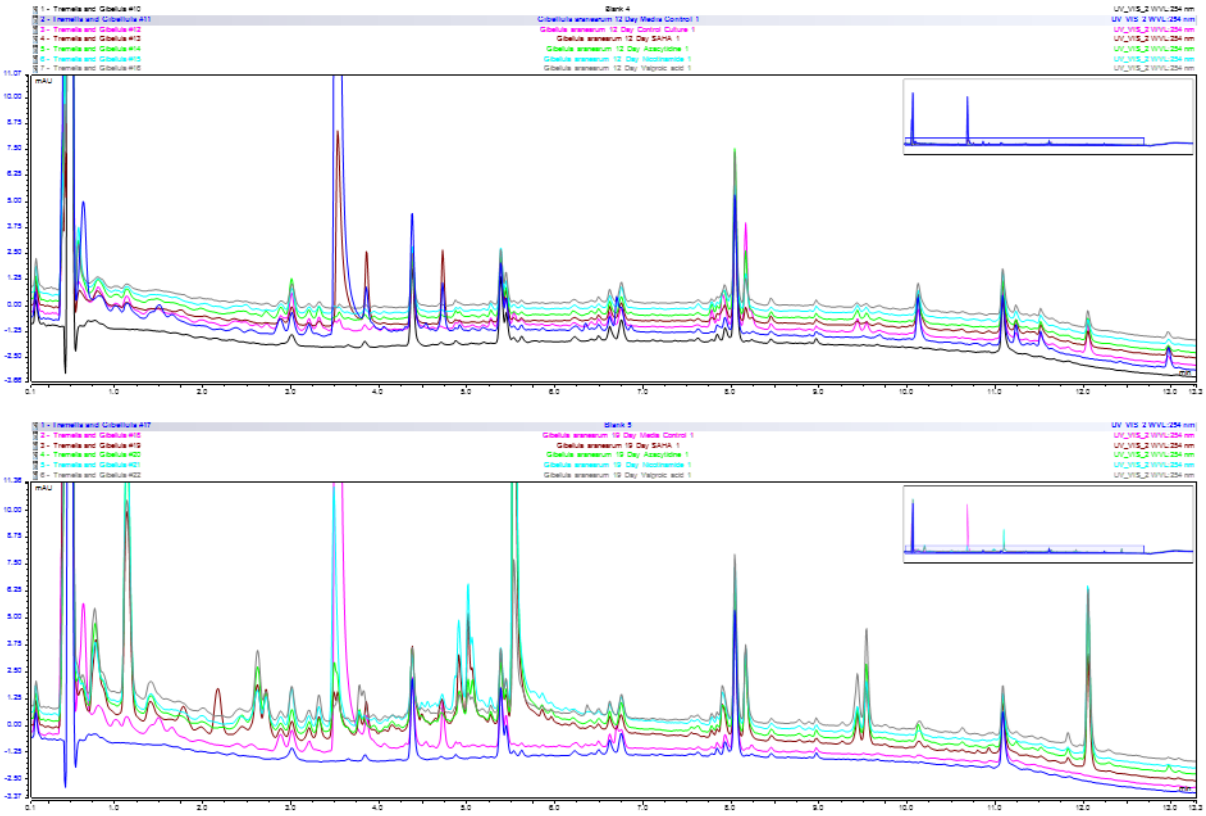


Figure 18. HPLC chromatograms (254nm) of *Gibellula* isolate 1. treated with various epigenetic modifiers after 12 day (Top) and 19 day fermentations (Bottom). Blue = MeOH Blank, Pink = Media control, Brown = SAHA, Green = Azacytidine, Light Blue = Nicotinamide, Grey = Valproic acid.

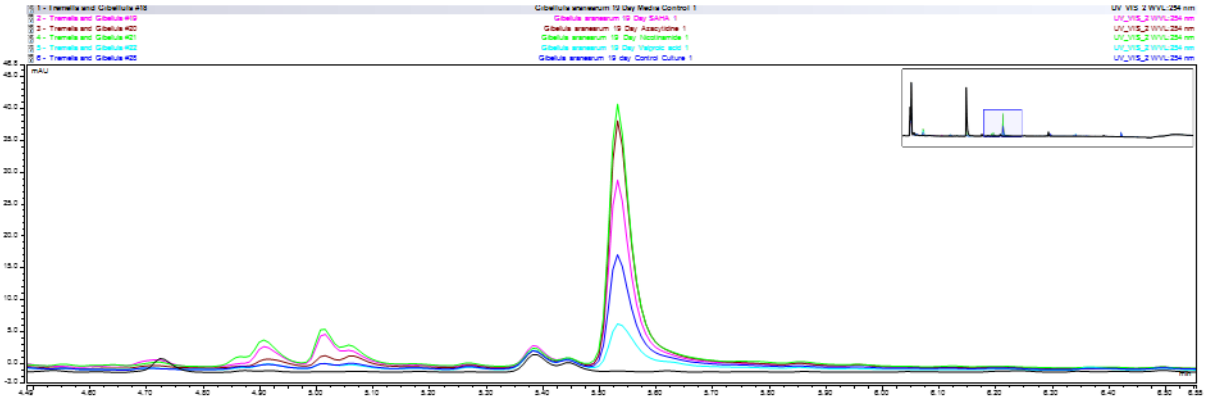


Figure 19. HPLC chromatograms (254nm) showing altered metabolite abundance in *Gibellula pulchra* fermentation extracts treated with different epigenetic modifiers. The peak at 5.65 min is increased in cultures treated with Nicotinamide (Green), 5-Azacytidine (Brown), and SAHA (Pink) compared with untreated control (Blue), while it is reduced in the valproic acid group (Light blue). Black = Media control.

Treatment with the epigenetic modifiers did not appear to have a significant effect on the metabolic profiles of *Tremella mesenterica* compared with untreated control cultures (**Figure 20).**

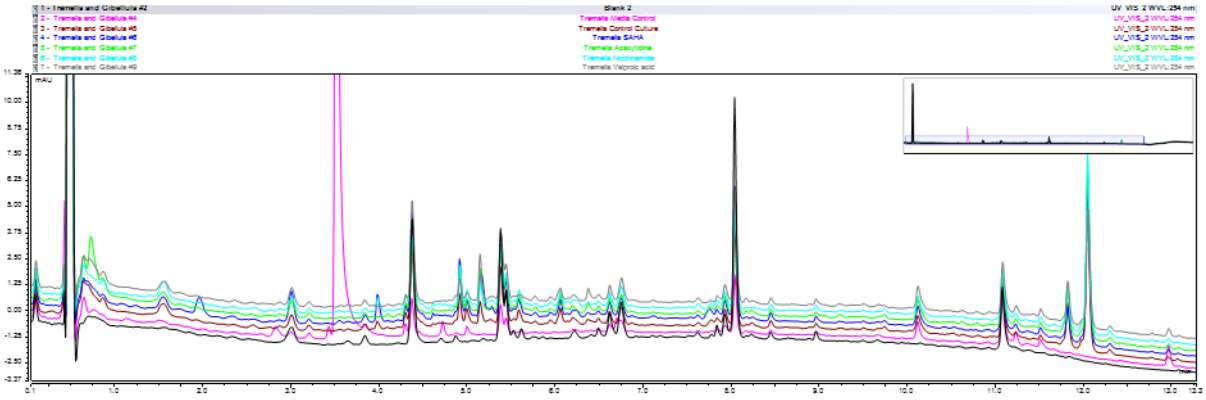


Figure 20. HPLC chromatograms (254nm) of *Tremella mesenterica* grown in the presence of the epigenetic modifiers. Pink = media control, Brown = Control culture, Dark Blue = SAHA-treated, Green = Azacytidine-treated, Light Blue = Nicotinamide-treated, Grey = Valproic acid-treated

Inonotus obliquus culture extracts displayed simple metabolic profiles with few peaks present, of the epigenetic modifiers Vorinostat had the greatest effect, resulting in the production of some peaks not seen in the controls (**Figure 21**).

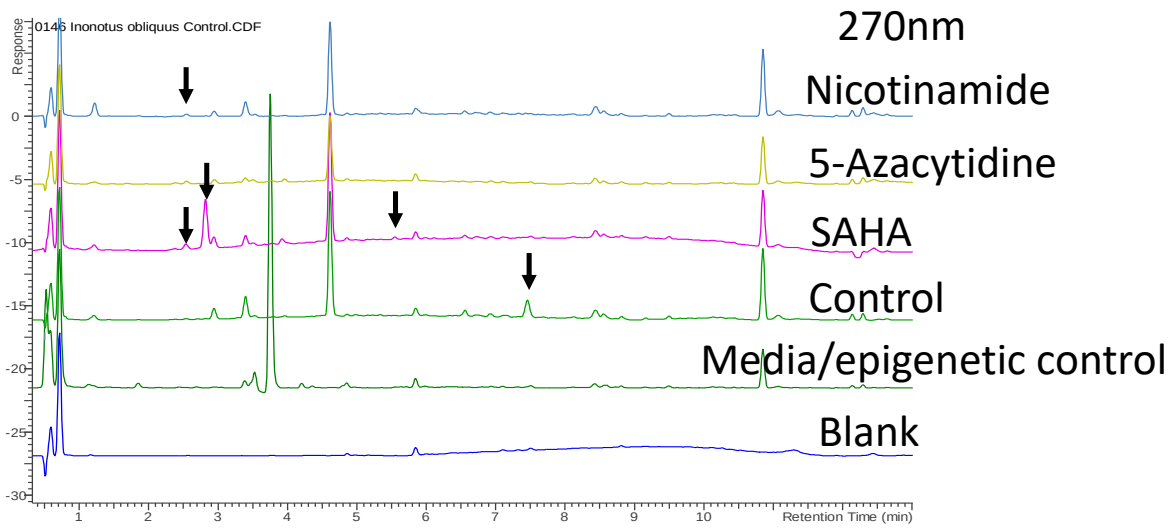


Figure 21. HPLC Chromatograms (270nm) showing the effect of various epigenetic modifiers on *Inonotus obliquus* culture extract composition. Arrows indicate differences between the groups.

Ganoderma resinaceum culture extracts showed little difference from the media control, with few metabolites produced. Treatment with the epigenetic modifiers appeared to inhibit the production of the primary metabolites produced (**Figure 22).**

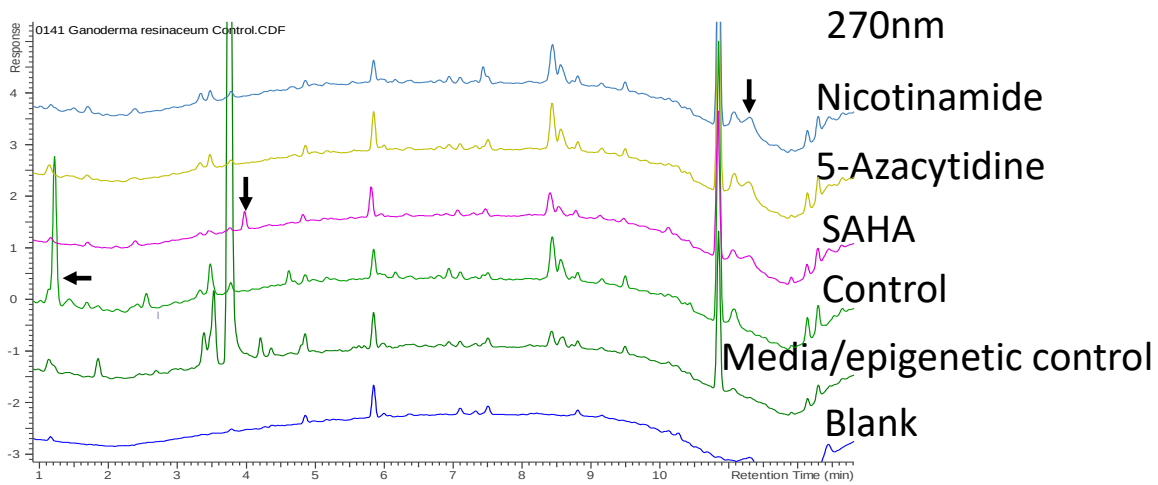


Figure 22. HPLC Chromatograms (270nm) showing the effect of various epigenetic modifiers on *Ganoderma resinaceum* culture extract composition. Arrows indicate differences between the groups.

Trametes versicolor responded with new peaks in the HPLC chromatograms to Nicotinamide, 5-azacytidine and Vorinostat, with different peaks present in each group (**Figure 23).**

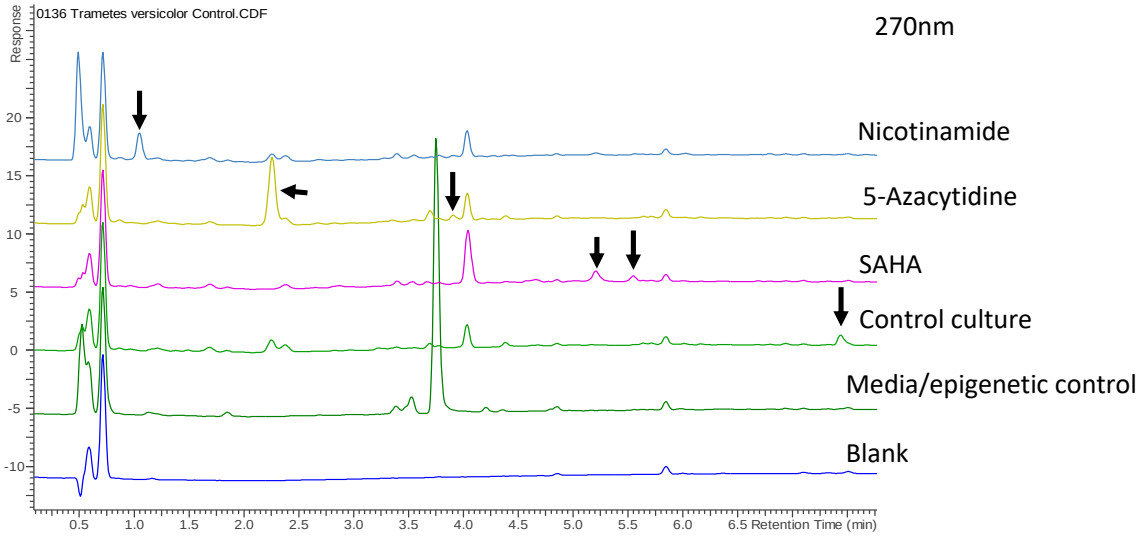


Figure 23. HPLC Chromatograms (270nm) showing the effect of various epigenetic modifiers on *Trametes versicolor* culture extract composition. Arrows indicate differences between the groups.

In a screening experiment employing a range of non-epigenetic modifier small molecules, several compounds were identified as having a significant effect on the metabolic profiles of *Gibellula pulchra*. Treatment with Estrone resulted in significant production of several peaks not seen or present at a much lower concentration in control cultures (**Figure 24**).

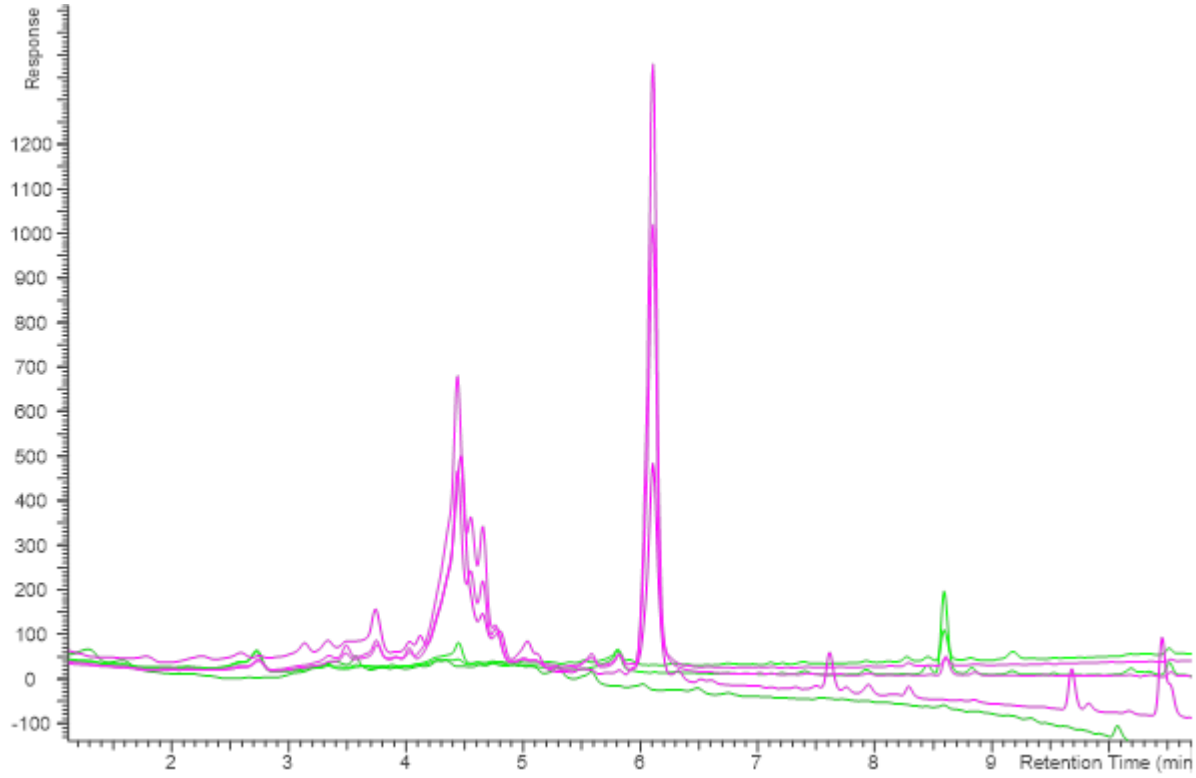


Figure 24. Estrone-treated (Pink) vs control (Green) *Gibellula* fermentation chromatograms (Triplicate)

Treatment with 5-iodotubercidin appeared to increase the production of a number of metabolites that were present in the control cultures at a lower concentration (**Figure 25**), and treatment with tryptamine resulted in the production of two major peaks not seen in the controls, and apparent increased production of several peaks present at a lower concentration in the controls (**Figure 26**).

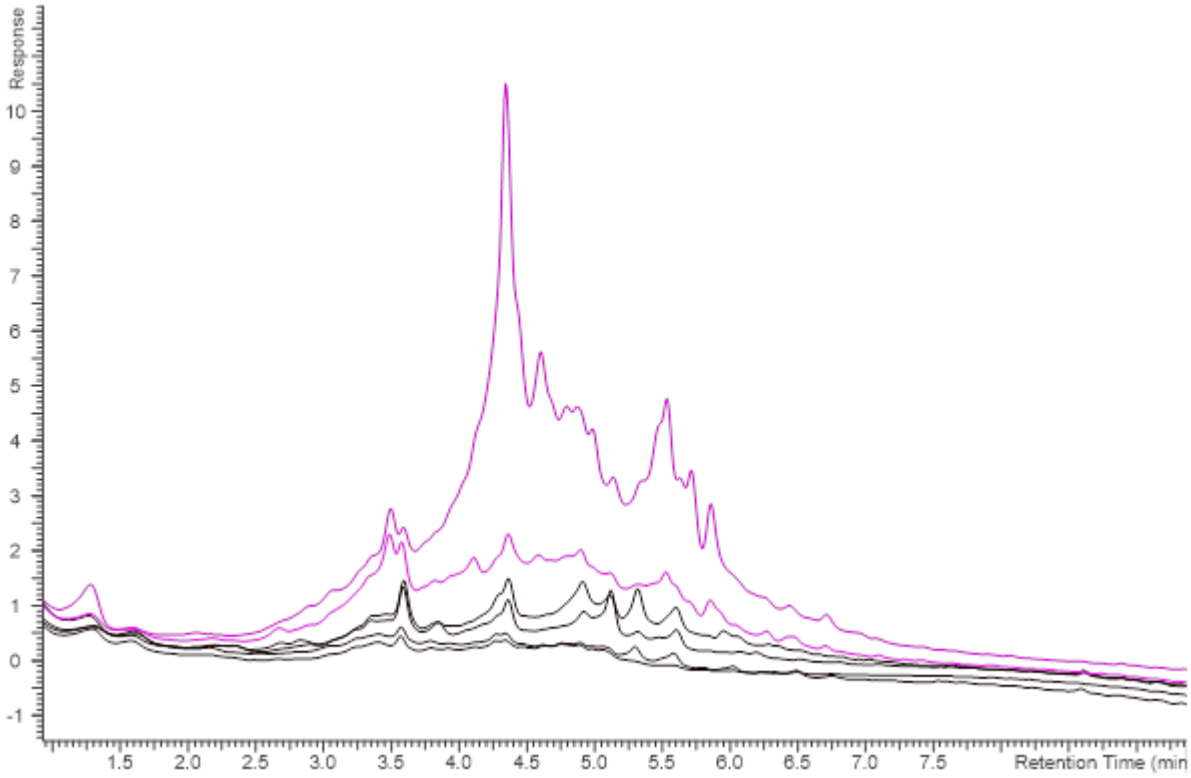


Figure 25. HPLC chromatograms (360nm) of 5-iodotubercidin-treated (Pink) and control (Black) *Gibellula pulchra* fermentation extracts, showing increased metabolite abundance in the treated fermentations

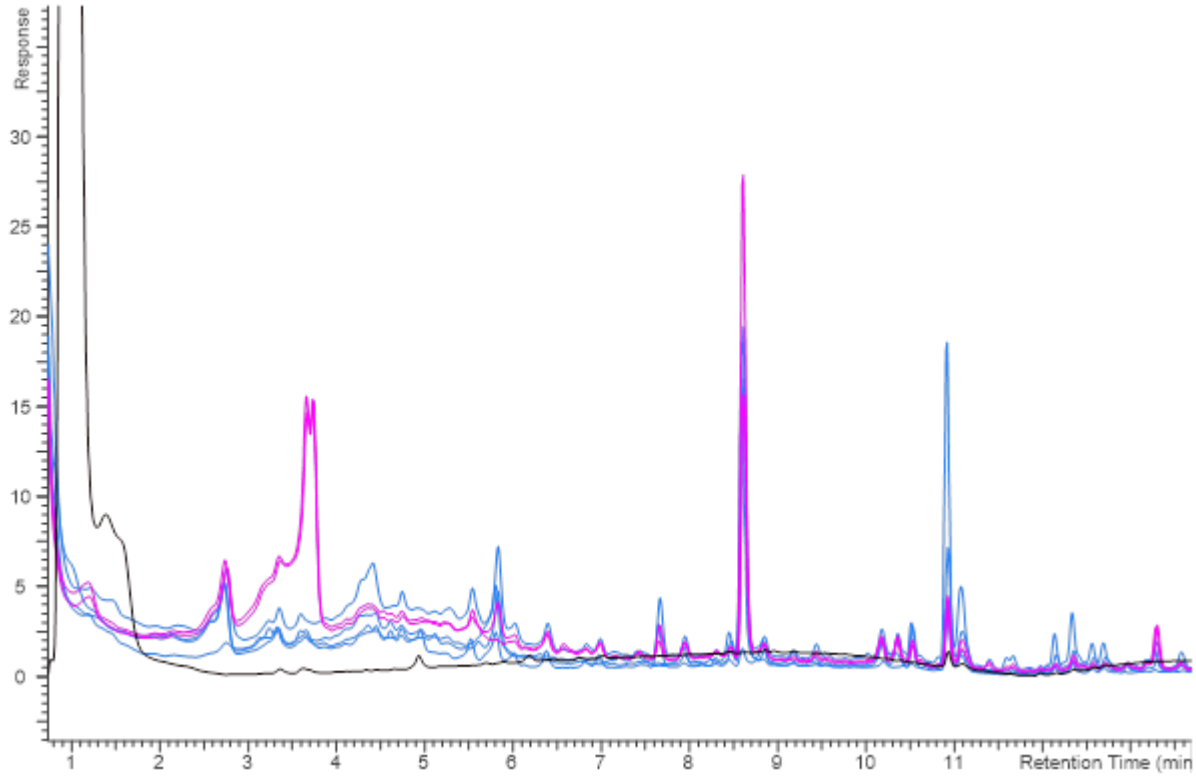


Figure 26. HPLC chromatograms (270nm) of extracts from *Gibellula pulchra* control cultures (Blue, n=4), tryptamine-treated cultures (Pink, n=2) and tryptamine control (black). In the tryptamine-treated fermentations, additional peaks can be seen in the 3-4 minute region which do not correspond to tryptamine itself (Black), which eluted from the column significantly earlier.

2.4 Large-scale *Onygena equina* fermentation results

2.4.1 Scale up of *Onygena equina* fermentations

In the initial screening experiments, *Onygena equina* produced a complex mixture of metabolites, and was one of the species which responded the strongest to the presence of Vorinostat in the culture medium, with significantly increased amounts of metabolites observed in the Vorinostat-treated cultures (**Figure 27**). Considering that it is an unusual fungus as it occurs specifically on the horns and hooves of dead undulates, and no natural products had yet been isolated from the species, it was selected as one of the fungal species to be grown on a larger scale in the presence of Vorinostat, as well as other epigenetic modifiers alone and in combination. Additionally, other members of the Onygenales family have proven to produce a range of structurally interesting metabolites (**Figure 11**). The fungus was also grown in various media (YM, Q6 and ZM) on a larger scale to test for differences in metabolite

profiles produced in the various different media, and samples were collected regularly to monitor the production of metabolites over time to determine which was the best media and to aid in isolation attempts.

Of the several media tested, ZM proved to produce the greatest variety of metabolites and the highest extraction yields, so it was used for subsequent fermentations (**Figures 28-31**).

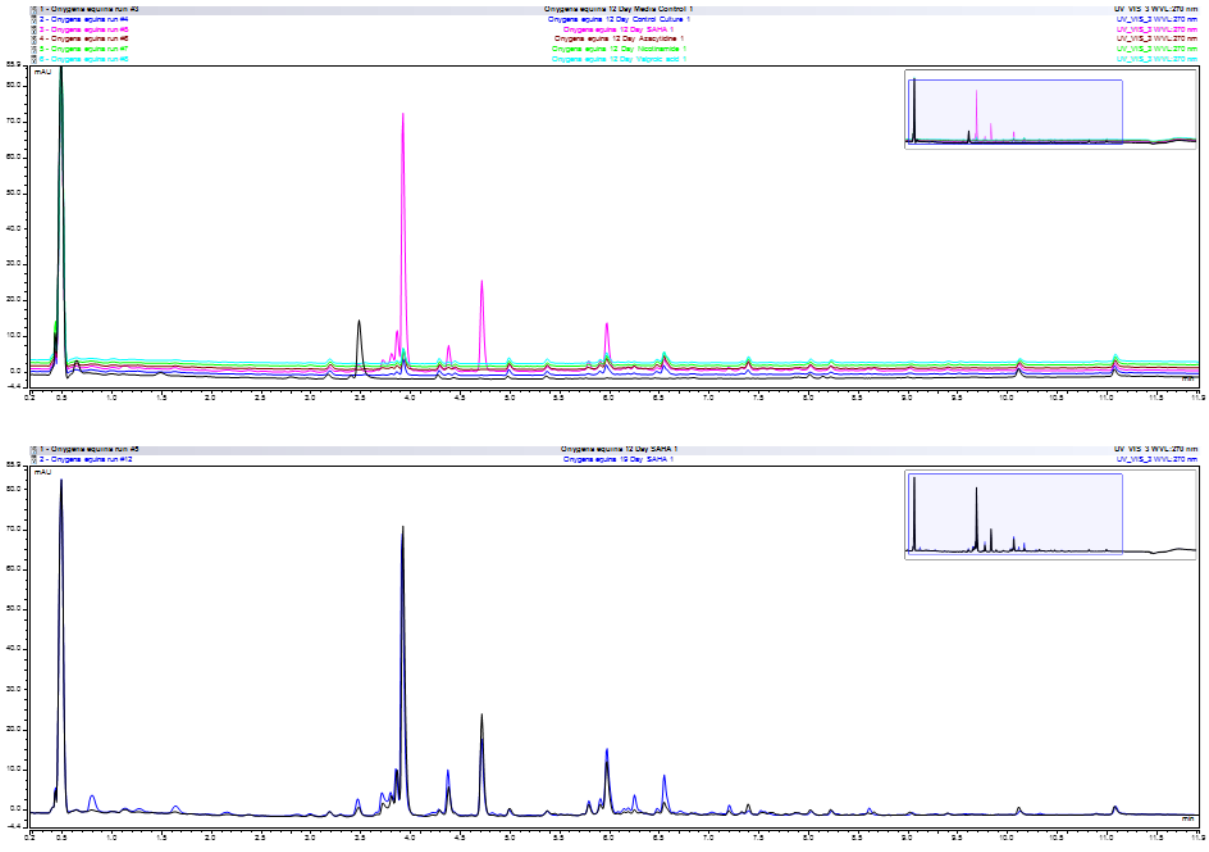


Figure 27. Top: HPLC Chromatograms (270nm) of *Onygena equina* PDB fermentation extracts grown in the presence of various epigenetic modifiers (Pink = Vorinostat, light blue = Valproic acid, Green = Nicotinamide, Brown = 5-Azacytidine, Dark blue = untreated control, Black = PDB media control extract). Although all of the modifiers had some effect, the most pronounced effect was seen from the addition of Vorinostat/SAHA (Pink), which significantly increased the production of several metabolites detected in the other groups, as well as inducing the production of compounds not detected in the other groups. **Bottom:** SAHA-treated cultures extracted at 12 days (Black) and 19 days (Blue), showing little difference between the extraction timepoints.

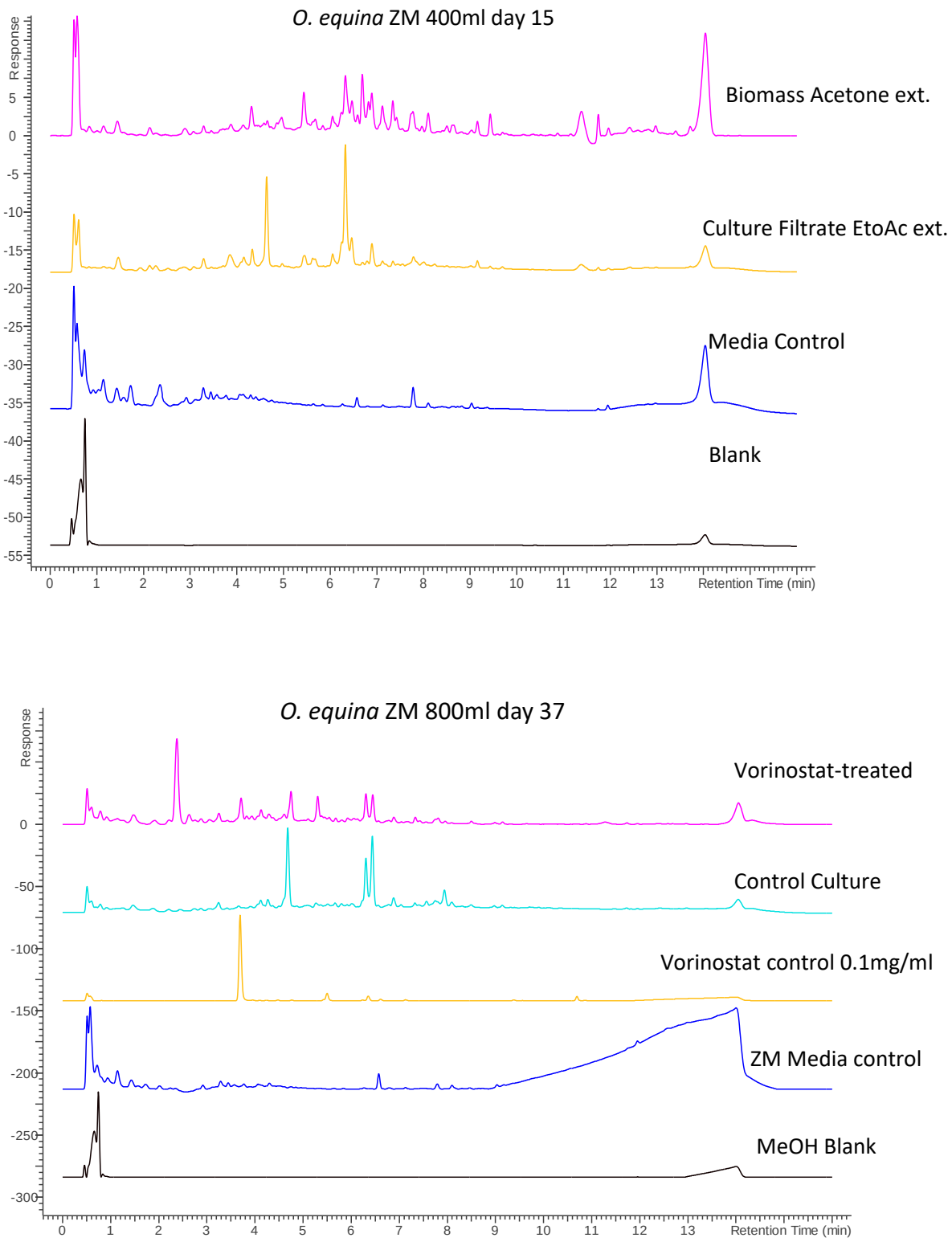


Figure 28. Top: Comparison of biomass and supernatant extracts of *Onygena equina* grown in ZM medium (400mL) after 15 days. **Bottom:** HPLC Chromatograms of culture filtrate ethyl acetate extracts (37 days) from Vorinostat-treated and untreated control fermentations (800mL)

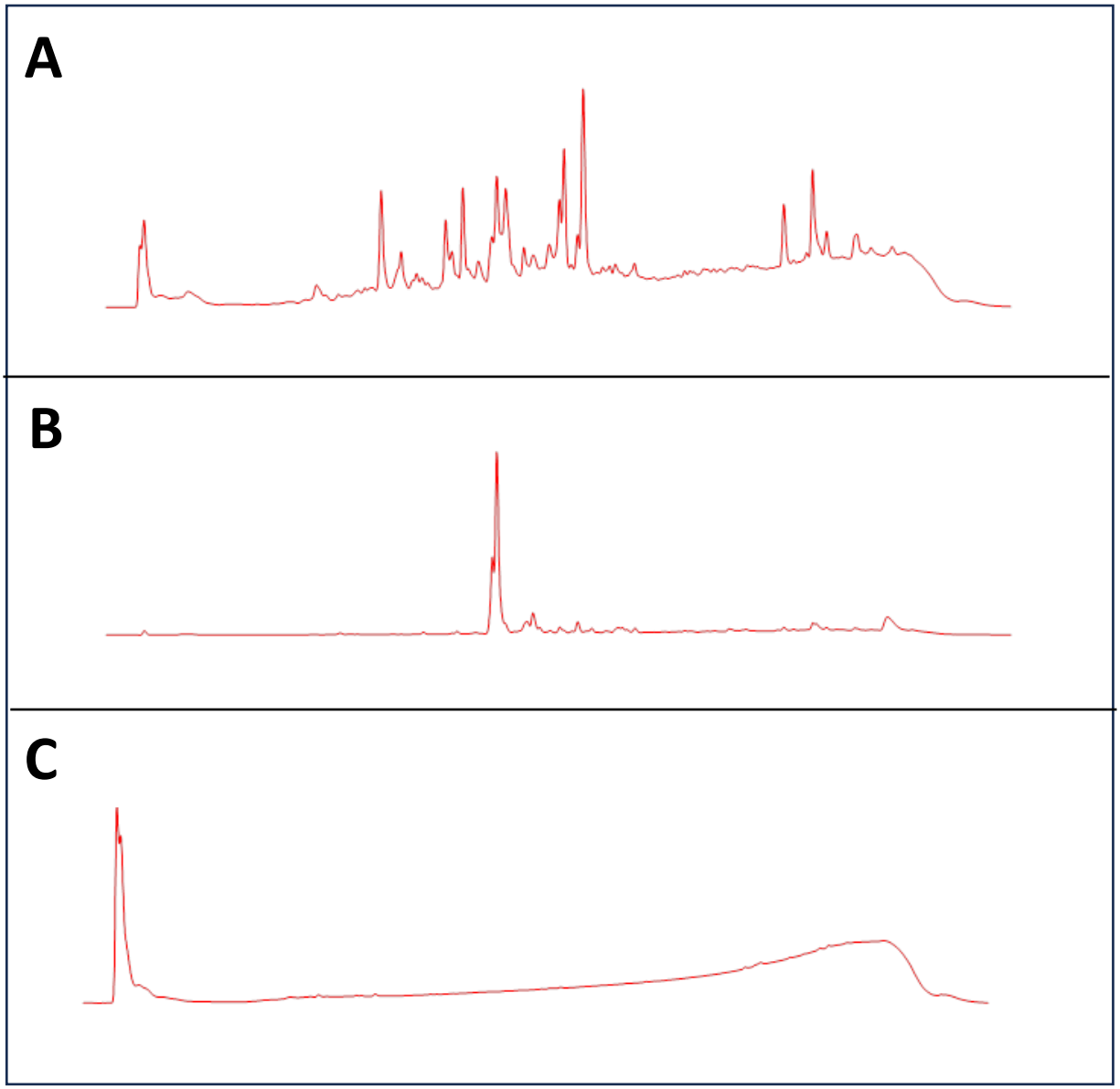


Figure 29. HPLC Chromatograms (254nm) of various *Onygena equina* extracts obtained from the extraction of a film of biomass grown on the surface of a static fermentation in ZM media. An initial Acetone extract was extracted with ethyl acetate (A), then Hexane (B) and finally methanol (C). While the ethyl acetate extract contained a complex mixture of components, the hexane extract was far less complex, and the methanol extract consisted mostly of highly polar material.

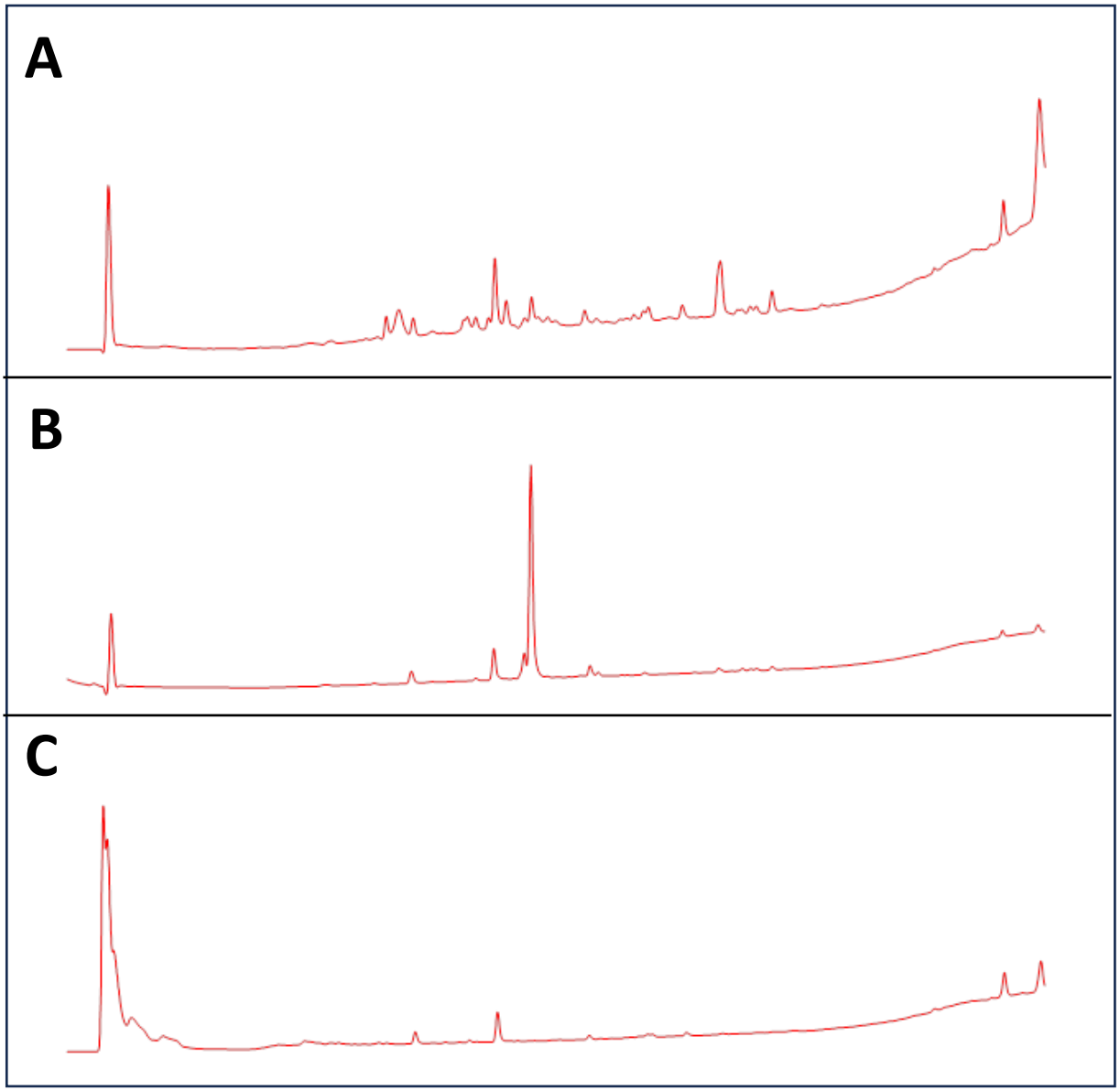


Figure 30. HPLC Chromatograms (254nm) of various *Onygena equina* extracts obtained from the extraction of the submerged biomass grown in a static fermentation in ZM media. The initial Acetone extract was extracted with ethyl acetate (A), then hexane (B) and finally methanol (C). The metabolite profiles are similar to the surface-grown biomass (Figure 20).

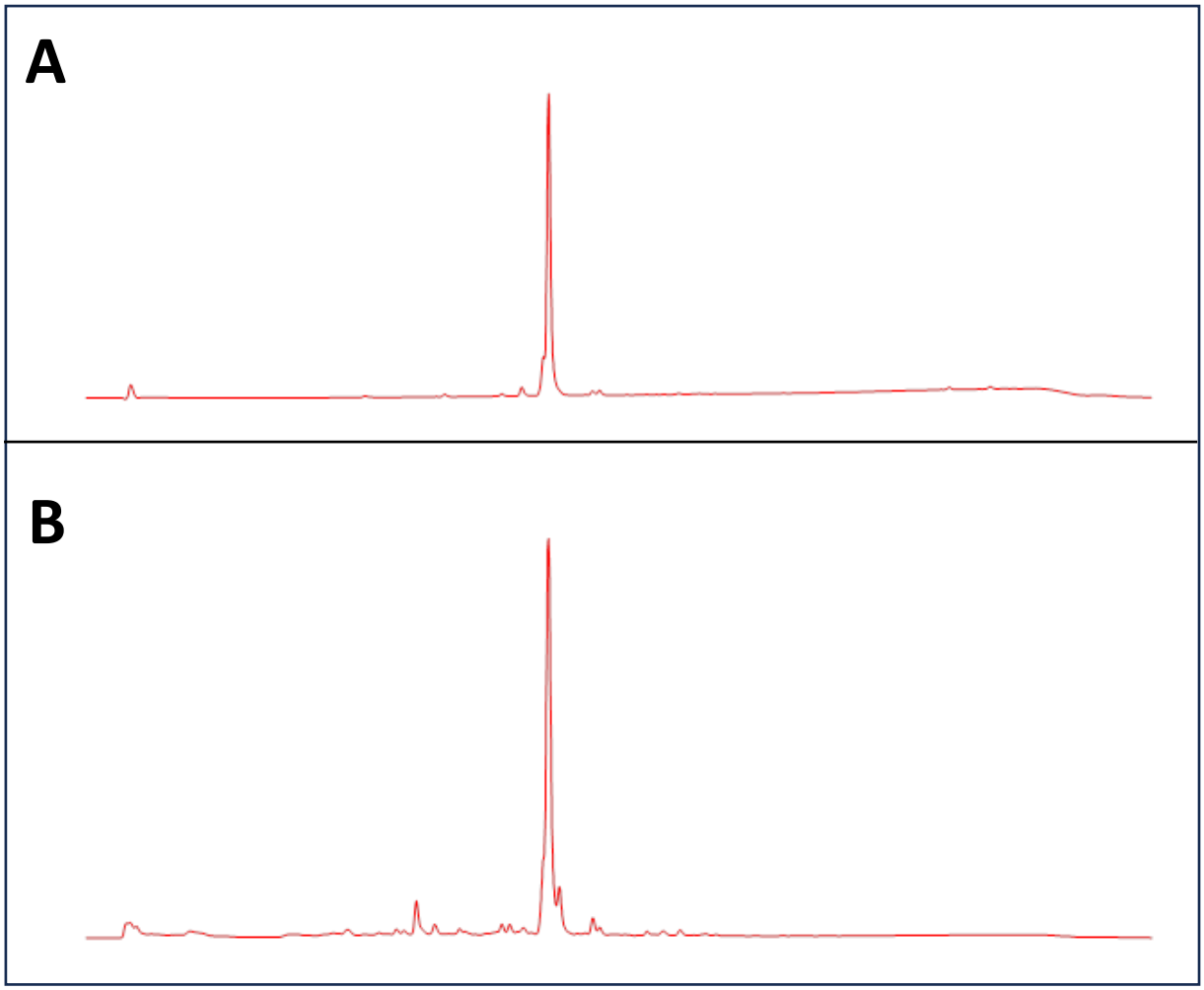


Figure 31. HPLC Chromatograms (254nm) of Hexane (A) and Methanol extracts (B) of the media filtrate. The metabolite profiles of the media filtrate extracts were far less complex than the biomass extracts.

Chapter 3

Studies of UK-native entomopathogenic fungi

3.1 Background and overview of Hypocrealean entomopathogenic fungi phylogeny and biology

Entomopathogenic fungi are species which specialise in parasitising insects. Most species are Ascomycetes within the order Hypocreales, which contains many fungal parasites of animals, plants, as well as human pathogens. There are also entomopathogenic species within the Mucoromycota and Zoopagomycotina. Some of the most well-known entomopathogenic fungi are species in Cordycipitaceae and Ophiocordycipitaceae, which contain many genera and known species (Figure 32).

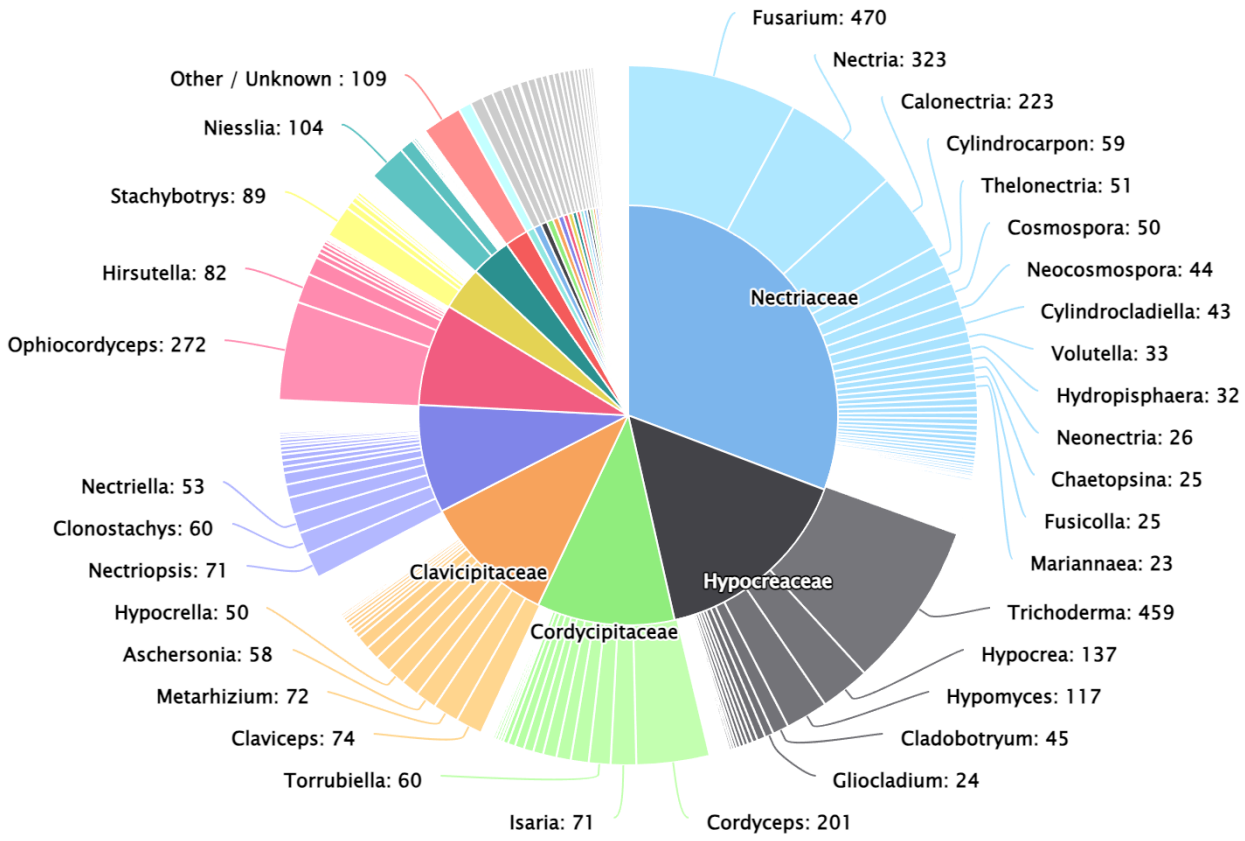


Figure 32. Taxonomic overview of the Order Hypocreales, showing the grouping of 5999 known species from 482 genera into 23 Families. Produced from Species Fungorum Plus (Accessed 14th August 2024)

Entomopathogenic fungi can be highly specialised in the insect species which they occur on, however there are also certain generalist species which have a lower host-specificity, for example *Zoophthora radicans* (Entomophthorales) is known to occur on at least 80 species within Diptera, Coleoptera, Lepidoptera, and Homoptera [79]. Some of these generalist species of entomopathogenic fungi such as *Beauveria bassiana* (**Figure 33**) and *Metarhizium anisopliae*, have been explored as bio-insecticides/pesticides against various insect species including ticks [80-82], coleopterans [83], the Khapra beetle (*Trogoderma granarium*) and the red flour beetle (*tribolium castaneum*) [84] due to their low host specificity.

Figure 33. *Beauveria bassiana* on a Shield bug (*Halyomorpha* sp.) imaged via stereomicroscopy.

Ophiocordyceps sinensis specialises on moth pupae and is only found in the Tibetan plateau. It has anti-inflammatory properties [85], and a history of use in Traditional Chinese Medicine (TCM), where the wild collected or cultivated fungus as well as extracts produced from it, are consumed as a medicinal substance or health supplement [86]. The mycelium of the species is grown on an industrial scale in China for use in supplement manufacture, but it took several decades of research in order to grow the fruiting body in a laboratory/manufacturing setting [87]. *Cordyceps militaris* is a widely distributed species which specialises on moth pupae, similar to

Ophiocordyceps sinensis. *C. militaris* is also used in TCM as well as in dietary supplements, again being produced on an industrial scale for these purposes [86, 88]. It is also possible to produce the fruiting body in laboratory conditions, typically utilising a rice-based substrate. *Ophiocordyceps unilateralis*, a species which specialises on ants in tropical regions, has gained media attention in recent years through being featured in documentaries, and is one of the better-studied entomopathogenic fungi.

Figure 34. Examples of several British Hypocrealean Fungi. A – *Cordyceps militaris* growing from moth pupa – A famous entomopathogenic ascomycete which parasitises moth pupae, also cultivated on a large scale in Asia as an alternative to *Ophiocordyceps sinensis*, which is potentially under threat from over-harvesting as the fruitbody cannot be easily cultivated. **B-C – *Tolypocladium*** sp. *growing on Elaphomyces* sp. (False truffle).

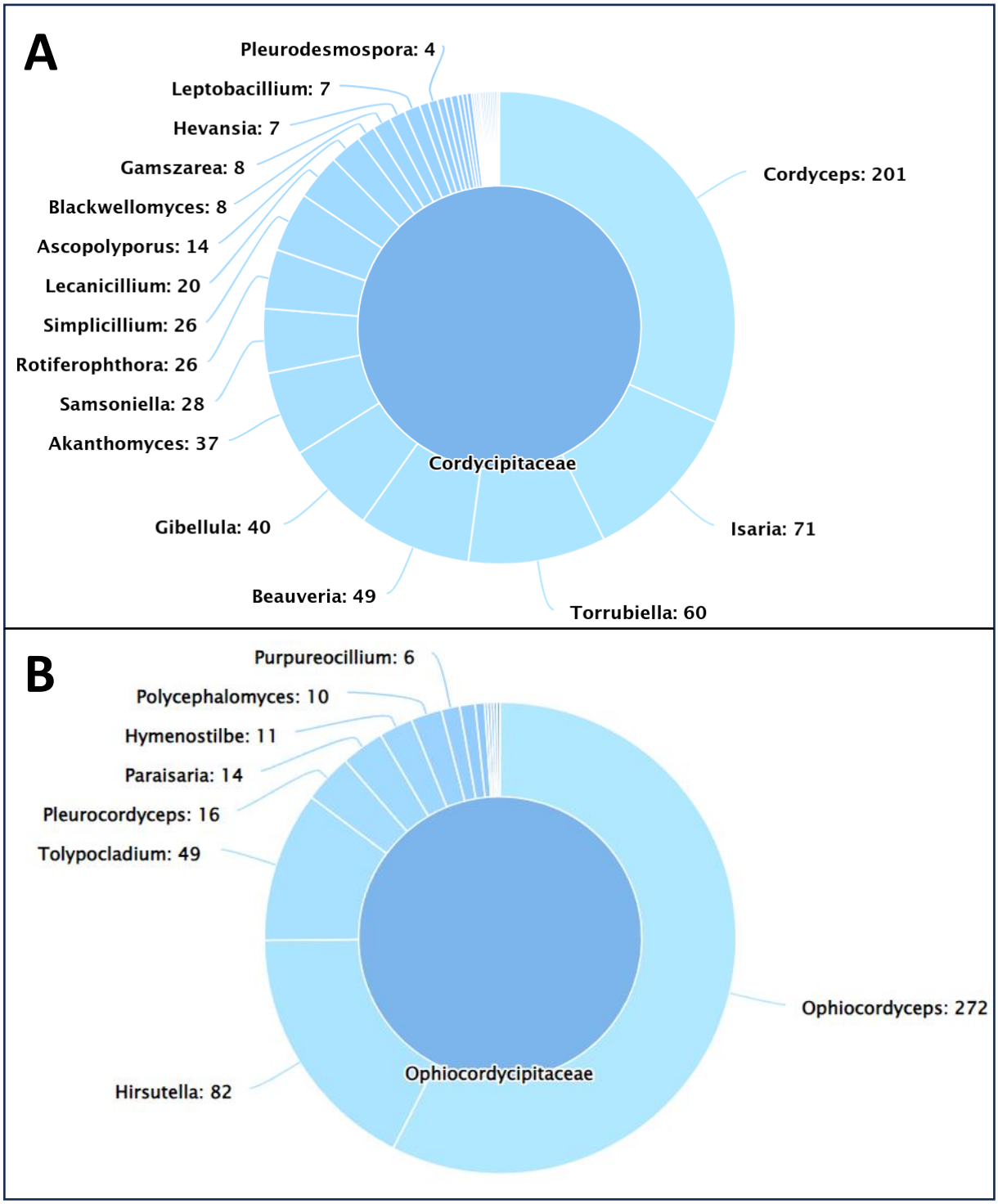


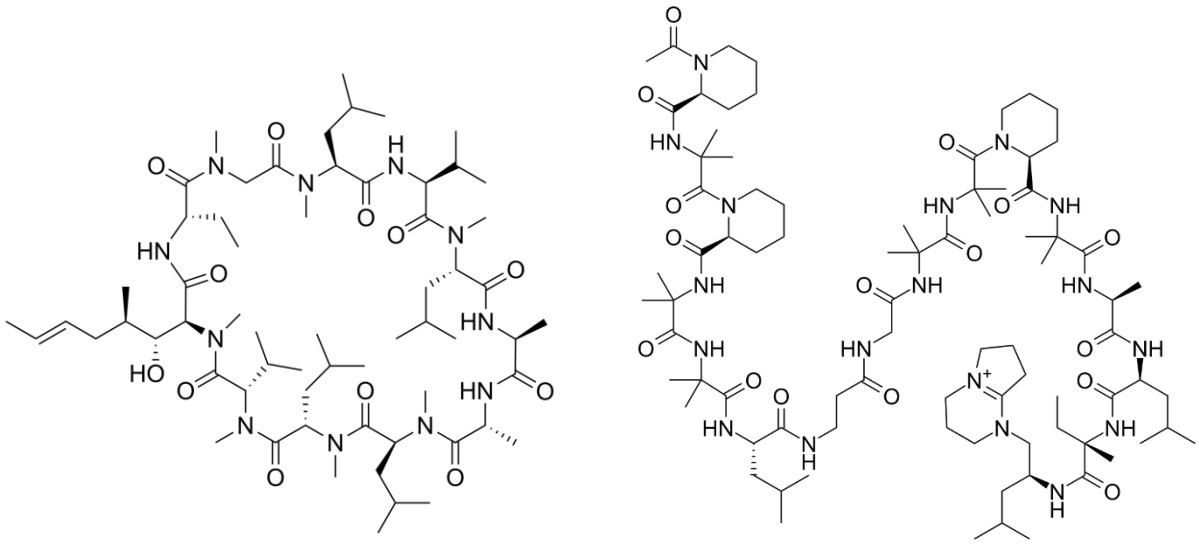
Figure 35. A: Taxonomic overview of the Family Cordycipitaceae, showing the grouping of 637 species into 35 genera. **B:** Taxonomic overview of the Family Ophiocordycipitaceae, showing the grouping of 473 species into 15 genera. From Species Fungorum Plus (Apr 2024). DOI:10.15468/ts7wsb

3.2 Secondary metabolites of Hypocrelean fungi

Hypocrealean Fungi (HF) are known to be rich sources of structurally diverse secondary metabolites with varied bioactivities [89-93]. Several species have a long history of use in Chinese herbal medicine, and are becoming increasingly popular in the west (e.g. *Ophiocordyceps sinensis, Cordyceps militaris*) [35, 94, 95].

Several important pharmaceuticals have been derived from Hypocrealean fungi, the best known example is perhaps cyclosporin (**Figure 36**). Cyclosporin A is a cyclic peptide composed of 11 amino acids, produced by the nonribosomal peptide synthase (NRPS) Cyclosporin synthetase [96]. Cyclosporin is an immunosuppressant drug used to prevent organ rejection after transplant, as well as to treat several other immune-related conditions such as rheumatoid arthritis and lupus [97, 98]. Cyclosporin was originally isolated in 1970 at Sandoz from cultures of *Tolypocladium inflatum,* isolated from soil samples collected in Norway and Wisconsin [99, 100], and was approved by the FDA in 1983 [101]. It was later determined that *Tolypocladium inflatum* is the soil-inhabiting asexual state (anamorph) of what was then known as *Cordyceps subsessilis* [102], the sexual state (teleomorph) of the species, which is entomopathogenic, specifically targeting beetle larvae [96].

As well as the Cyclosporins, another group of peptides known as Efrapeptins **(Figure 36)** are known to be produced by some *Tolypocladium* spp., such as *Tolypocladium geodes* [103]. The Efrapeptins have insecticidal, antifungal and mitochondrial ATPase inhibitory properties [103].



Cyclosporin A

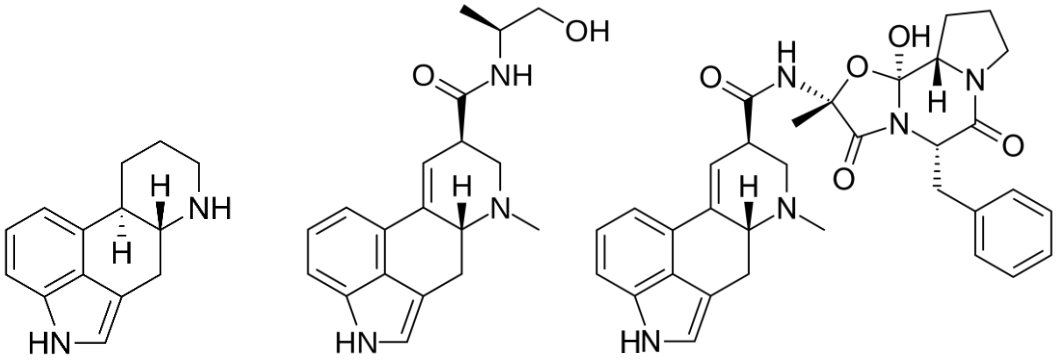
Tolypocladium inflatum

Efrapeptin F

Tolypocladium geodes

Figure 36. Structures of Ciclosporin A, an immunosuppressant drug originally isolated from the Hypocrealean fungus *Tolypocladium inflatum*, and Efrapeptin F, isolated from *Tolypocladium geodes*

The genus *Claviceps* (Clavicipitaceae), specifically *Claviceps purpurea* (Ergot), has been another rich source of potent bioactive natural products [45]. Ergot parasitises various grains and grasses, producing a sclerotium from the seed in the autumn which releases spores that go on to infect more plants the following year. *Claviceps* spp. have been used medicinally in crude form since long before any active constituents were known or isolated [44]. The species contains a complex mixture of potent ergoline alkaloids which have various effects on the body and has occasionally been the cause of mass-outbreaks of ergotism (ergot toxicity), due to contamination of grain used to produce food. The principal active component ergotamine, as well as ergometrine, several semi-synthetic derivatives (including methylergometrine, dihydroergotoxine mesylate (Hydergine), cabergoline and bromocriptine) have found use as pharmaceuticals to treat a variety of conditions [30, 45].



Ergoline skeleton Ergometrine Ergotamine

Figure 37. *Claviceps purpurea* (Ergot) has been one of the richest sources of bioactive natural products in the fungal kingdom, several semi-synthetic pharmaceuticals with a range of indications have been derived from it.

3.3 Spider pathogenic (Araneogenous) fungi

The known species within the genera *Gibellula* and *Akanthomyces* are all specific pathogens of spiders (*Araneae*), however there are also spider-pathogenic fungi within other genera such as *Cordyceps*, *Ophiocordyceps*, *Hevansia* and *Torrubiella* [104]. Much of the research on the metabolites of spider-pathogenic fungi to date has been done on species from the *Gibellula*, *Akanthomyces* and *Torrubiella* genera, and in recent years the spider-fungi have proved to be a promising source of novel natural products. Several novel natural products have been discovered from *Gibellula* (**Figure 37**), *Akanthomyces*, and other genera [91, 93, 105, 106].

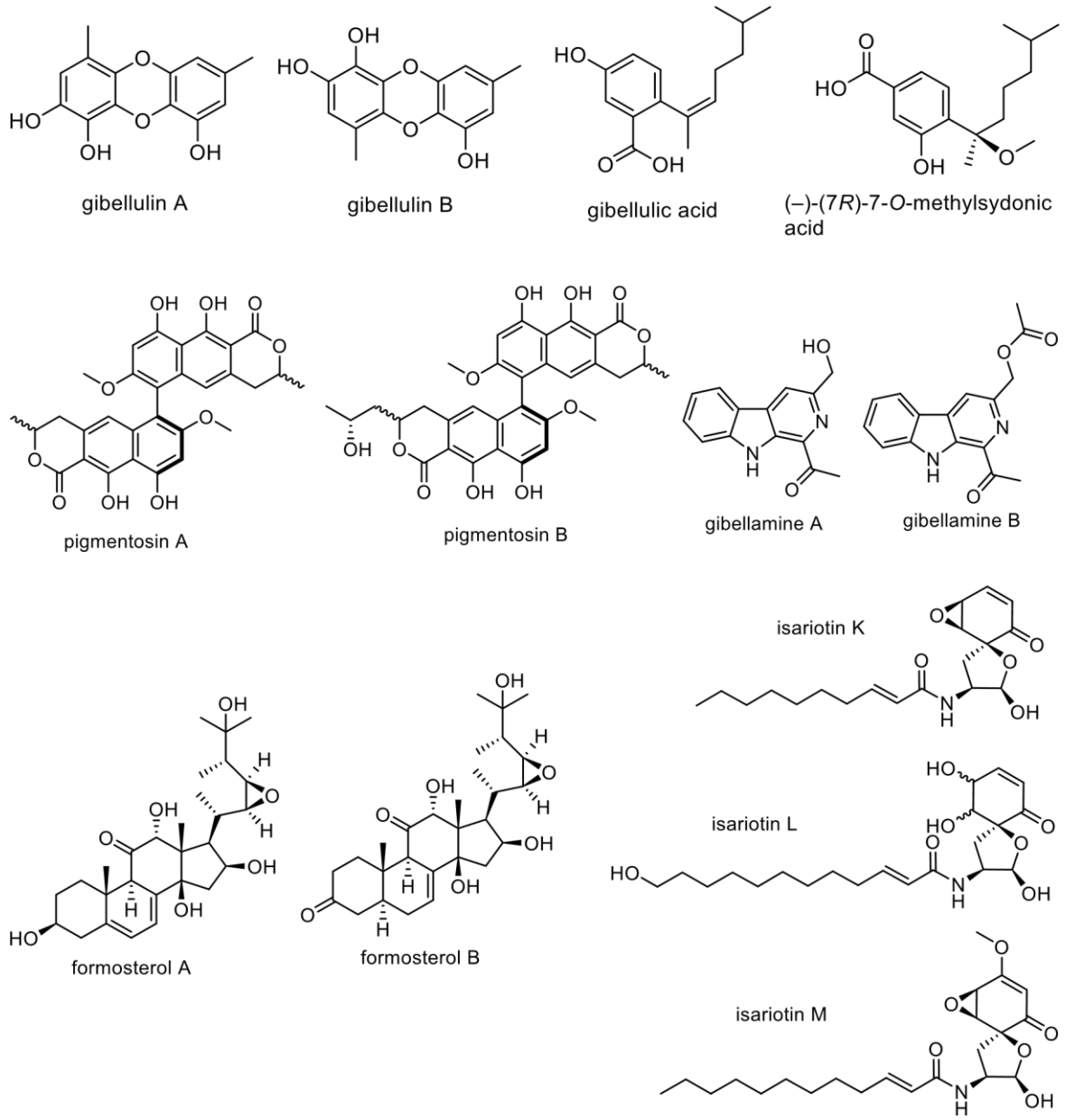


Figure 37 – Example compounds isolated from *Gibellula* spp. Note: Figure produced by Lloyd Philips as part of a laboratory project investigating the metabolites of *Gibellula* strains, under the supervision of the author.

3.4 The use of epigenetic modifiers to produce novel metabolites in *Gibellula* spp.

Epigenetic manipulation with small molecules is a promising method to alter the metabolite production of fungi, and has been shown in some species to induce the production of novel secondary metabolites or increase the production of constitutive secondary metabolites [72, 107].

Several compounds have been induced in *Gibellula formosana* using epigenetic modifiers [77], though it is not currently known how metabolite profiles and responses to epigenetic manipulation vary between different *Gibellula* species and strains of the same species. The only published example of secondary metabolite induction with epigenetic modifiers in the *Gibellula* genus is with *Gibellula formosana*, where application of the HDAC inhibitor suberoyl bis-hydroxamic acid (SBHA), and the DNA methyltransferase inhibitor, RG-108, significantly increased the production and diversity of secondary metabolites produced in fermentation. From the treated fermentation, two novel highly oxidised ergosterol analogues (formosterols A and B) were isolated, as well as five novel isariotins, and six known compounds [77].

However, *Gibellula formosana* was originally isolated from a moth (lepidopteran) host [108]. Although in most collections of *Gibellula* specimens the spider host species is often unidentifiable (as the fungus usually covers too much of the cadaver for identification), like many entomopathogenic fungi, the genus *Gibellula* is highly specific in the animal species on which it occurs, in that none of the phylogenetically confirmed *Gibellula* species are known to occur on hosts other than spiders.

Recently, it has been suggested that 31 *Gibellula* species be considered as accepted, following a comprehensive literature review, morphological analysis of the genus, and multi-locus phylogenetic analysis, including all available sequences on NCBI-GenBank deposited as *Gibellula,* (excluding 25 strains which were only identified to genus level, and sequences deemed too divergent) as well as sequence data from 14 new collections from Atlantic Rainforest sites in Brazil [109]. *Gibellula formosana* MT924519 and *Gibellula curvispora* JQ342826 were excluded from the analysis on the basis that the available sequences were deemed as too divergent from the entire dataset.Indeed, running an NCBI-Blast analysis of the 18S rRNA, ITS1, 5.8SrRNA, ITS2 and 28S rRNA partial and complete sequence available for *Gibellula formosana*

(Accession number AB100360.1) (Accessed 19/08/2024), did not return any other *Gibellula* sequences in the top 100 alignments (sorted by score). The most significant alignments (99-100% identity) are from *Isaria tenuipes* (Currently accepted as *Cordyceps tenuipes*), suggesting that *Gibellula formosana* should not be accepted as a *Gibellula* species and is instead a strain of *Isaria tenuipes*.

Furthermore, the isariotin family of metabolites, six of which were isolated from *Gibellula formosana* fermentation treated with SBHA and RG-108, are not known to occur in any other *Gibellula* species, and are instead highly characteristic metabolites of *Isaria (Cordyceps) tenuipes*

3.5 Introduction to the present work

From the araneogenous fungi, an increasing number of natural products has so far been isolated in recent years, with the majority of the research conducted on strains from Thailand, where there is a much greater biodiversity and abundance of plants, microbial, and arthropod species including spiders than in Europe. To date, no work has been published on the chemistry of any collection of spider-pathogenic fungi outside of East-Asia, likely due to the lower number of species, lower abundance of specimens in temperate regions, and the difficulty in finding specimens. Nevertheless, the published research to date warrants chemical and phylogenetic investigation of more species and strains originating from more diverse geographical regions. It has been shown that different strains of *Akanthomyces novoguineensis* all collected within Ton-Nga-Chang Wildlife Sanctuary, Thailand, vary significantly in their ability to produce several metabolites under laboratory conditions [110], so it is reasonable to assume that fungal cultures obtained across much greater regions, from different species living in different environments and parasitizing different spider species are likely to produce different secondary metabolites.

In this project, several specimens of native British spider-pathogenic fungi were obtained (collection details Appendix 7), cultures were isolated and the metabolite profiles of several araneogenous strains were characterised in different media, with and without the addition of epigenetic modifiers, including DNA methyltransferase inhibitors, HDAC inhibitors and sirtuin inhibitors. As well as this, a range of non-epigenetic additives were trialled and their effects on the metabolic profiles of the fungi

were examined. In the initial small scale screening experiments, tryptamine was identified as one of the compounds which resulted in the production of several additional peaks compared to untreated cultures. As well as this, another related species (*Gibellula gamsii*) is known to be capable of producing the beta-carbolines Gibellamine A and Gibellamine B. For these reasons tryptamine was added to several large-scale fermentations of one of the spider fungi isolates, in order to determine if its addition could lead the production of beta-carbolines and other structurally related compounds via biotransformation.

3.6 Experimental methods

3.6.1 Details of the strains used

In this work, entomopathogenic fungi were collected from two sites in Norfolk, as well as this several specimens were donated by individuals and sent to the University from elsewhere in the country. All entomopathogenic specimens used in this work originated within the UK. In total, 5 spider-associated fungi were collected (**Figure 38 & 39**), as well as 2 Earwig (*Forficula auricularia*)-associated fungi (**Figure 40**), and one stinkbug-associated fungus (**Figure 33**). It was not possible to identify the species of spiders which the fungi were growing on, as the spiders were almost completely covered in mycelium and synnemata from the fungi.

3.6.2 Morphology of the original specimens

Upon collection or receipt of the samples, each specimen was examined microscopically and photographed using a stereo microscope (**Figures 38-40**). Small samples of synnema were removed using sterile needles and placed into lactophenol cotton blue solution, then examined under the Zeiss Axioplan microscope and photographed (Not shown). Additionally, the specimens were photographed using an electron microscope (**Figure 41**).

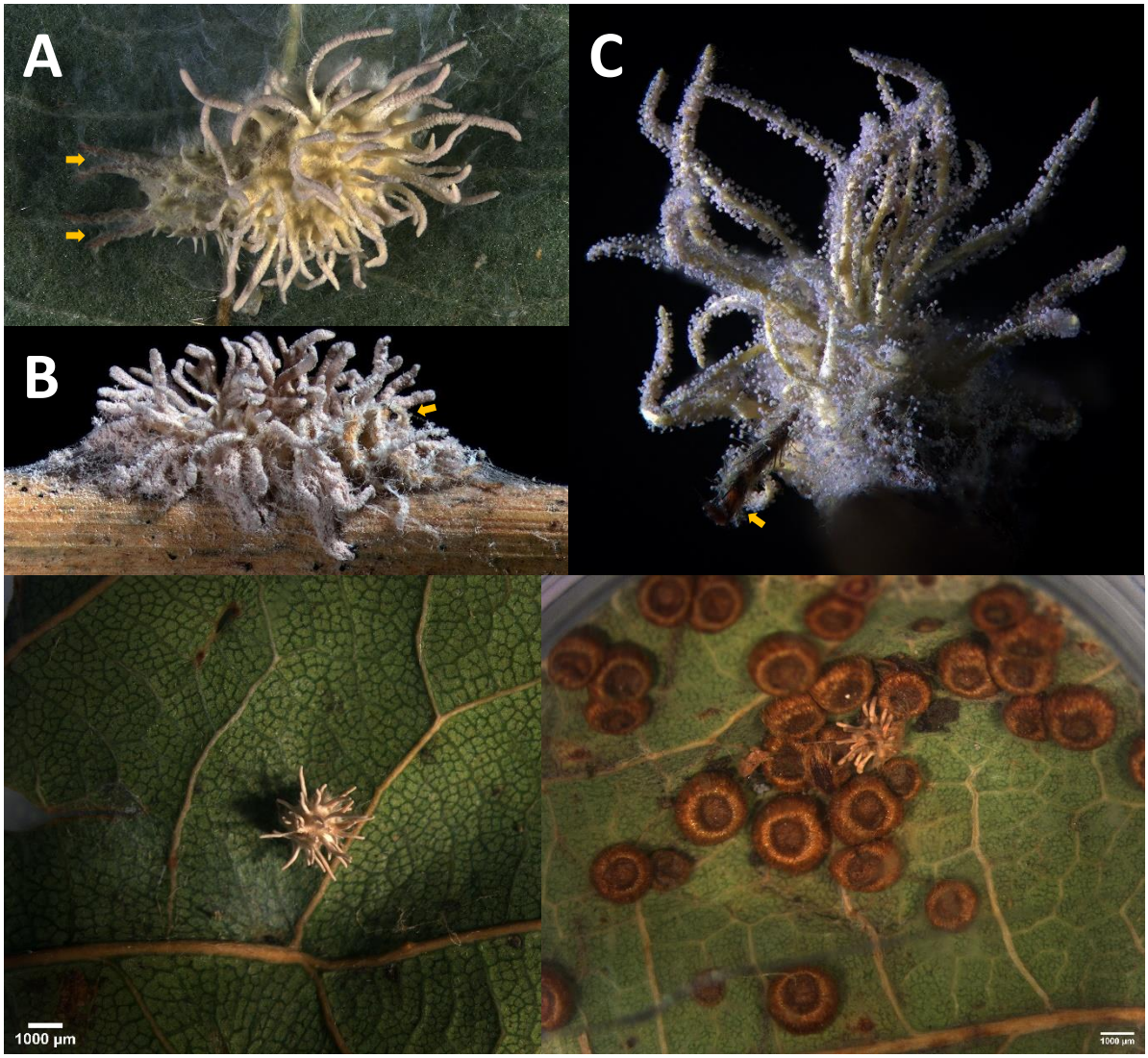


Figure 38. Several UK arachnogenous fungi on spider hosts, with abundant synnemata (conidiophore-bearing structures). **A**. Specimen on the underside of *Robus fruticosus*, from Devon. **B**. Specimen on *Phragmites* sp., Norfolk **C**. Specimen found on *Phragmites* species, Norfolk. Yellow arrows point to visible parts of the arachnid hosts.

Figure 39. Arachnogenous fungus specimens 3 (Top) and 4 (Bottom), shown attached to the underside of leaves, as is usually the case with spider-pathogenic fungi

Figure 40. Earwig (*Forficula Auricularia*) specimens 1 and 2, again seen attached to the underside of leaves. The fungus (unidentified) can be seen emerging from the joints of the body.

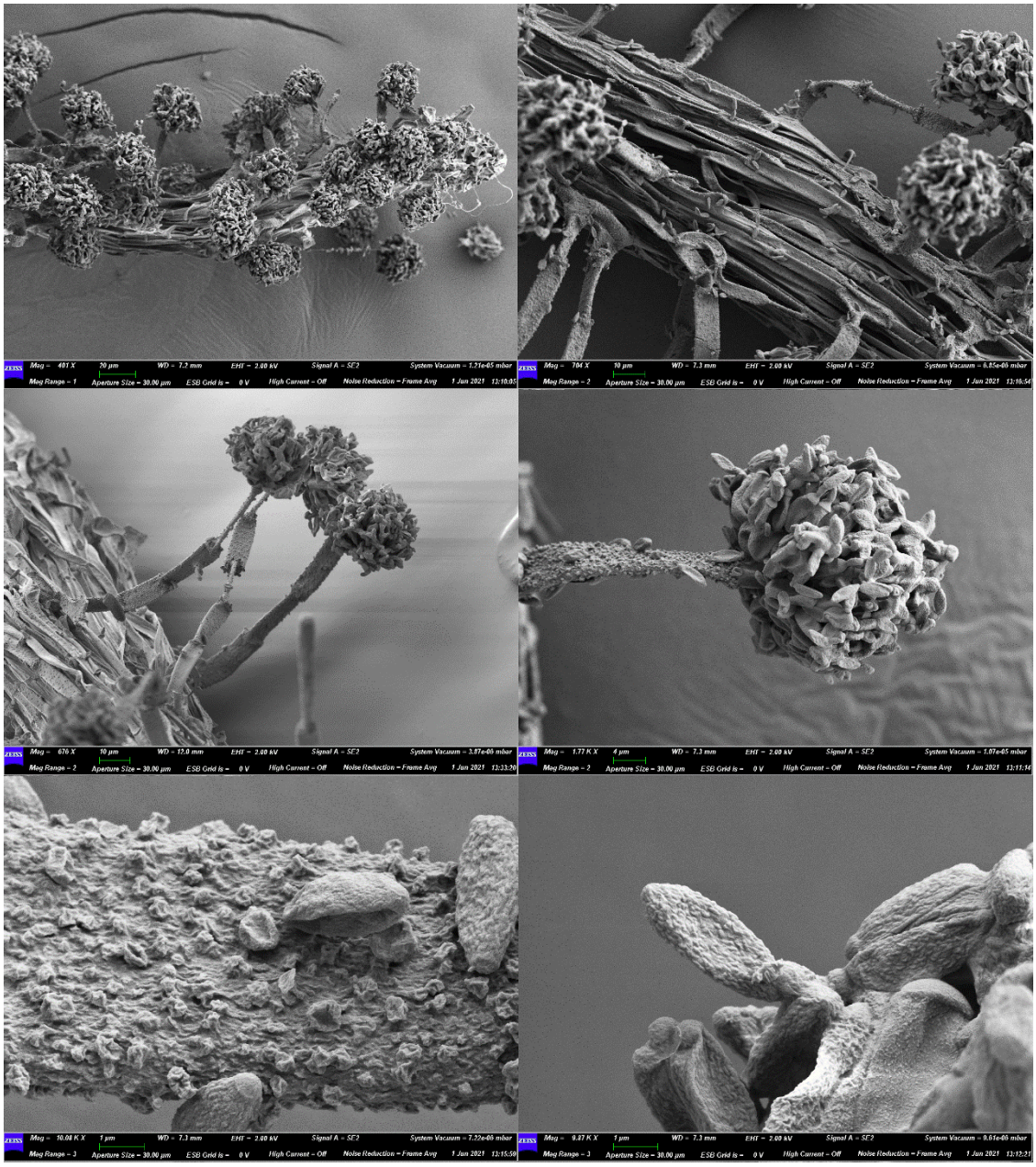


Figure 41. Scanning Electron Microscopy showing various morphological features of *Gibellula* sample 1.

3.6.3 Culture isolations

Several techniques were used in attempts to isolate cultures from the specimens. Synnemata tips were removed asceptically and inoculated onto PDA plates containing chloramphenicol and ampicillin. Plates were also inoculated by touching sterile inoculation loops against the synnemata, to collect conidiospores, which were then spread onto PDA plates with antibiotics. Finally, specimens still attached to plant

material were affixed to the inside of a petri dish lid using blu-tack, and the lids were placed over PDA plates + antibiotics, allowing conidiospores to fall onto the agar and germinate.

Plates were checked for growth regularly, individual colonies were transferred to subsequent PDA plates by cutting agar wedges from the growing edge of the colonies and placing upside-down onto the new plates.

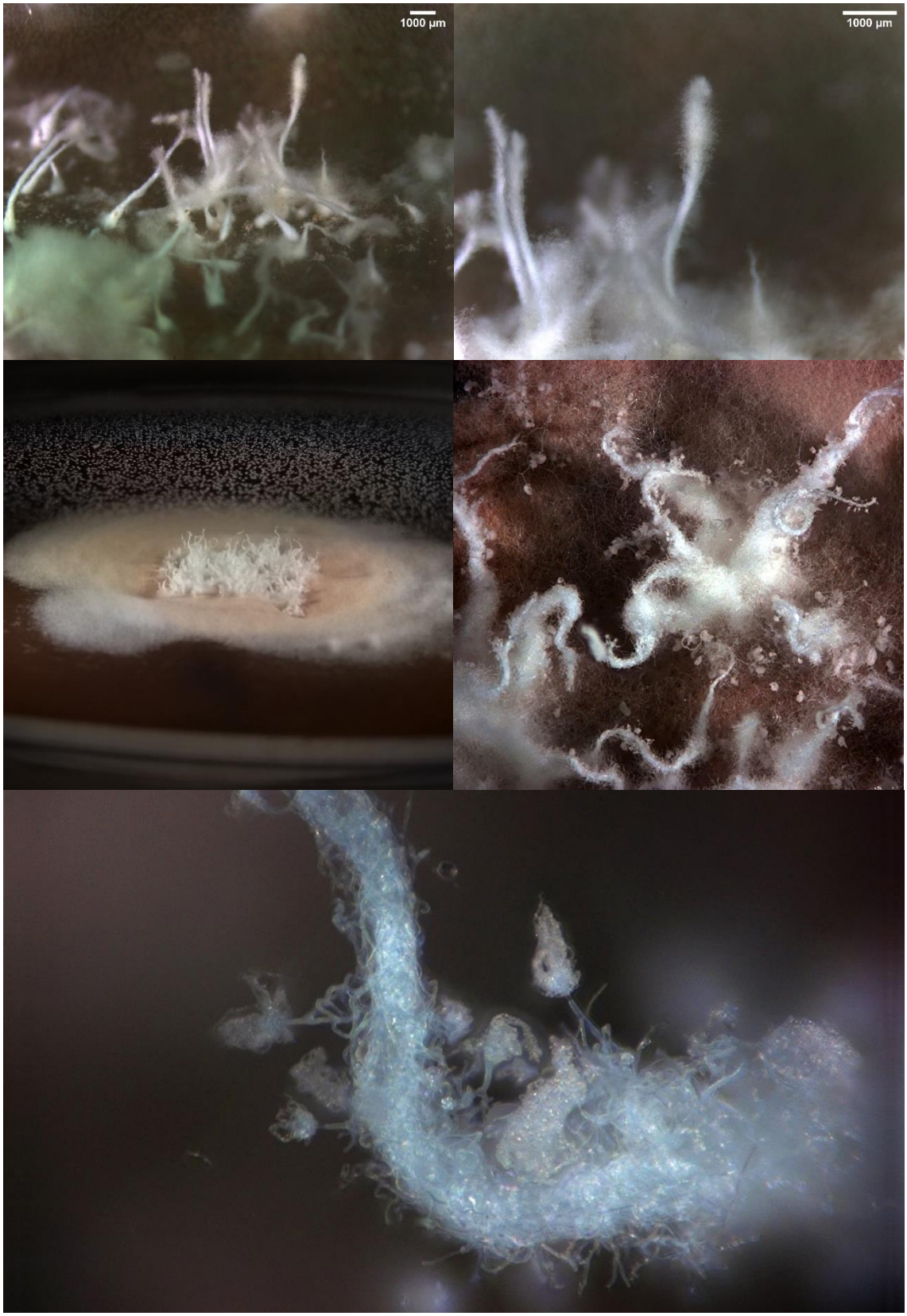


Figure 42. Stereo microscopy showing the formation of synnemata in cultures of *Gibellula pulchra* grown on PDA, a rare occurrence in *in vitro* cultivation of spider-pathogenic fungi.

3.6.4 DNA work and phylogenetic analysis

Note: The phylogenetic analysis used to identify the *Gibellula pulchra* strain isolated and used in this research was conducted by Wilawan Kuephadungphan.

DNA extraction, PCR amplification and product purification were conducted as previously described [111], but in brief, the primers used were ITS1F and ITS4 (for the ITS region), LR5 and LROR (for LSU), 983F and 2218R (for TEF1) and RPB1-Ac and fRPB1-Cr for RPB1.

The multigene phylogenetic analysis was performed by Wilawan Kuephadungphan using protocols previously described [112].

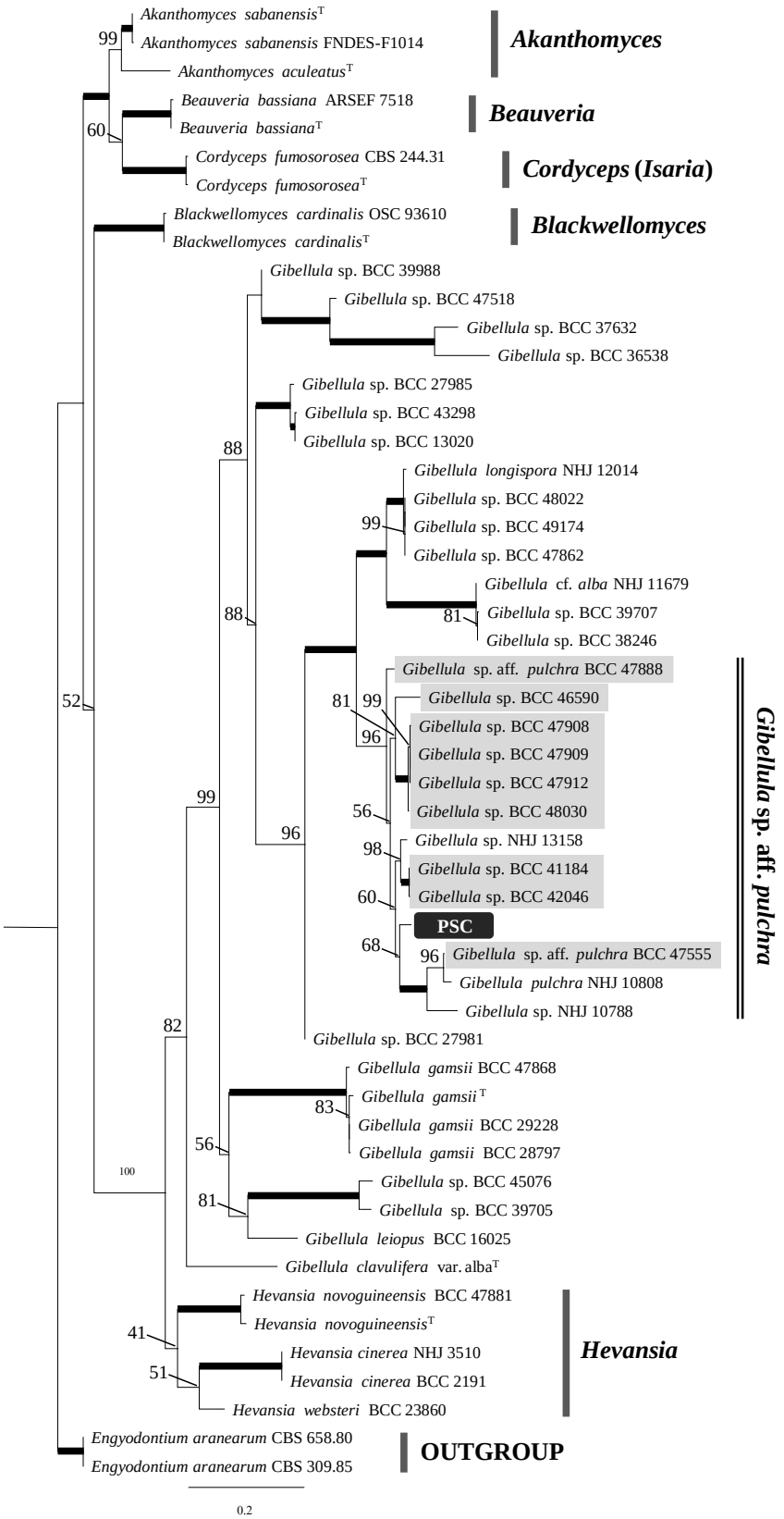


Figure 43. RAxML tree based on the concatenated four gene dataset (ITS, LSU, EF1-α and RPB1) showing the relationship among *Gibellula* and its related genera. Bootstrap proportion of ≥50% are provided above corresponding nodes and 100% in a thicker line. The type strains are marked with a superscript T (^{T}). 'PSC' represents the strain isolated in this work. The phylogenetic analysis and this figure were produced by Wilawan Kuephadungphan and included here with her permission.

Arachnogenous fungi, and entomopathogenic fungi in general, appear to be very rare in both the UK and Europe (In comparison to the greater biodiversity seen in more tropical regions such as Thailand and South America.

They may in fact be more common than it appears, as the fact that it is extremely difficult to find specimens due to their small size, habitat in dense foliage, and tendency to develop on the underside of plant material means that they may be under-recorded in fungal databases. Additionally, many finds are likely not recorded, collected, and explored scientifically. There are also limited numbers of people trying to find these European entomopathogenic fungi, many finds appear to be accidental.

There is also less apparent interest in the mycology and mycological chemistry of UK/Europe-Native entomopathogenic fungi (and non-pathogenic fungi such as mushroom forming species), as compared with Asia, and most mycological research conducted in the UK is in the field of medical mycology, relating to human pathogen research. The amount of metabolic research conducted on European spider pathogenic fungi to the present day appears to be limited. That is to say, to the knowledge of the author, this is the first chemical research conducted using European spider pathogenic species. All arachnogenous fungi research to date has been conducted using Asian strains and species, mostly from Thailand.

Due to these issues, and the lack of collaboration/crossover between mycology and entomology/arachnology, this is a neglected research topic and there is presently very little known about the biology, ecology, lifecycle and secondary metabolism of these fungi.

Recent genetic analyses using modern techniques such as multi-gene phylogenetic analyses are suggesting that there are in fact many more species of entomopathogenic fungi than currently thought. This may be especially true for Europe/ the UK, where identification of species has been done based on morphological examination alone, with no genetic or chemotaxonomic work included. Many species of *Gibellula* look very similar and are difficult to distinguish, and many of the records in the UK are likely incorrectly identified.

Due to the common methods utilised in culture isolation from these fungi (Such as obtaining and working with single spore colonies, from which the genetic and chemical

work is conducted), it is possible that many of these under-explored fungi are in fact multi-species complexes, as is the case with *Cordyceps sinensis*. The modern approach of obtaining totally pure isolates from conidiospores may be mistakenly concluding that the fungi on the wild-collected specimens is one species, when in fact it may be a complex of several symbiotic and/or parasitic fungi and bacteria that result in the typical morphology seen in the wild.

Indeed, in this research several observations were made which suggest that this might be the case. On agar plates where entire synemata were smeared over the agar rather than single conidiospore colonies, several distinct fungal species formed colonies on the agar, and many more synnemata were observed, including synnemata producing conidiophores. Additionally, the other, darker fungal colonies grew on both the agar and directly on/in the *Gibellula* agar grown synnemata. Pure isolates of the G*ibellula* culture rarely produced synnemata (**Figure 29)**, and the vast majority were sterile (not producing conidiospores.

Many agar colonies formed raised masses of hyphae, sometimes in rings around the initial inoculation point, other times sparsely throughout the colony or at the edge of the colony (Not shown). These masses appear to be primitive synnemata that are unable to grow to full synnemata, potentially due to the lack of a necessary factor in the single spore-isolate.

The chemical, morphological, and genetic work done so far has proven that this is an area of research with great potential for the discovery of novel natural products, including those with bioactivity that have been discovered so far.

3.6.5 Chemical investigation of the isolates

In order to examine the effect of different nutritional compositions in the growth media on metabolite profiles, the fungi were grown in several different liquid and solid media (PDB, YM, YMB, ZM, Q6, Supplemented Brown Rice, PDA, MEA, SDA). Most experiments were conducted in either PDB, YM, ZM or Q6. Fermentations were sampled (10mL) every two days until completion, the glucose concentration and pH of these regular samples were recorded, the samples were centrifuged and filtered to separate the biomass from the media filtrate, and the biomass and media filtrates were

extracted using the methods previously described. Metabolite profiles in the extracts were monitored via LCMS (**Figure 31)**, and the remaining fermentations were harvested and extracted when the metabolite profiles no longer changed, or the peaks of interest began to get smaller.

In this series of experiments, the effects of addition of various epigenetic modifiers alone and in combination were tested (**Figures 33-37**). Epigenetic modifiers employed included 5-Azacytidine, Vorinostat, Sirtinol, and a combination of all three epigenetic modifiers.

3.7 Results

3.7.1 Comparison of metabolite profiles between different media

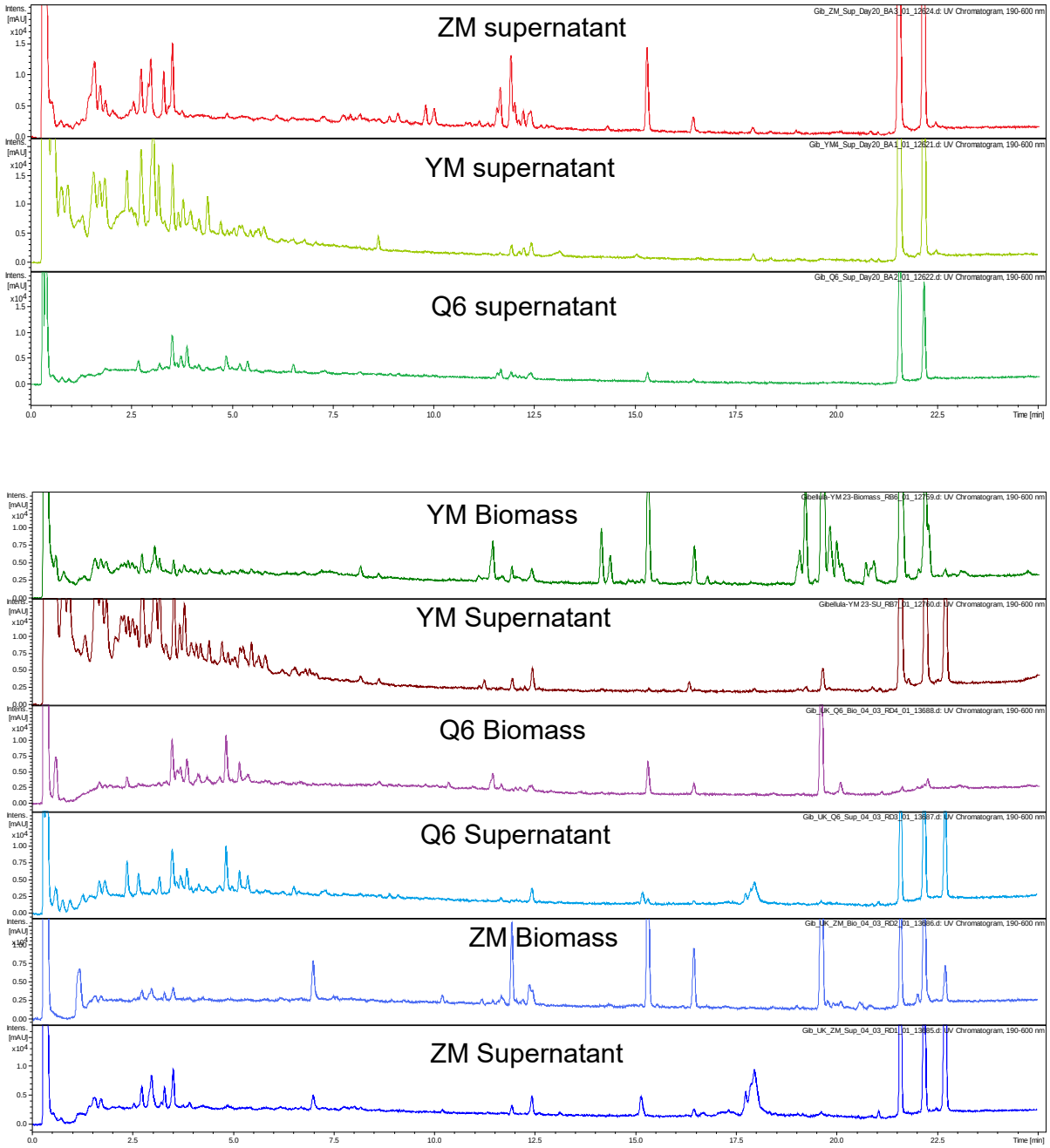


Figure 44. Total absorbance chromatograms (190-600nm) comparing biomass and supernatant extracts of *Gibellula pulchra* fermented in YM, Q6, and ZM liquid medium

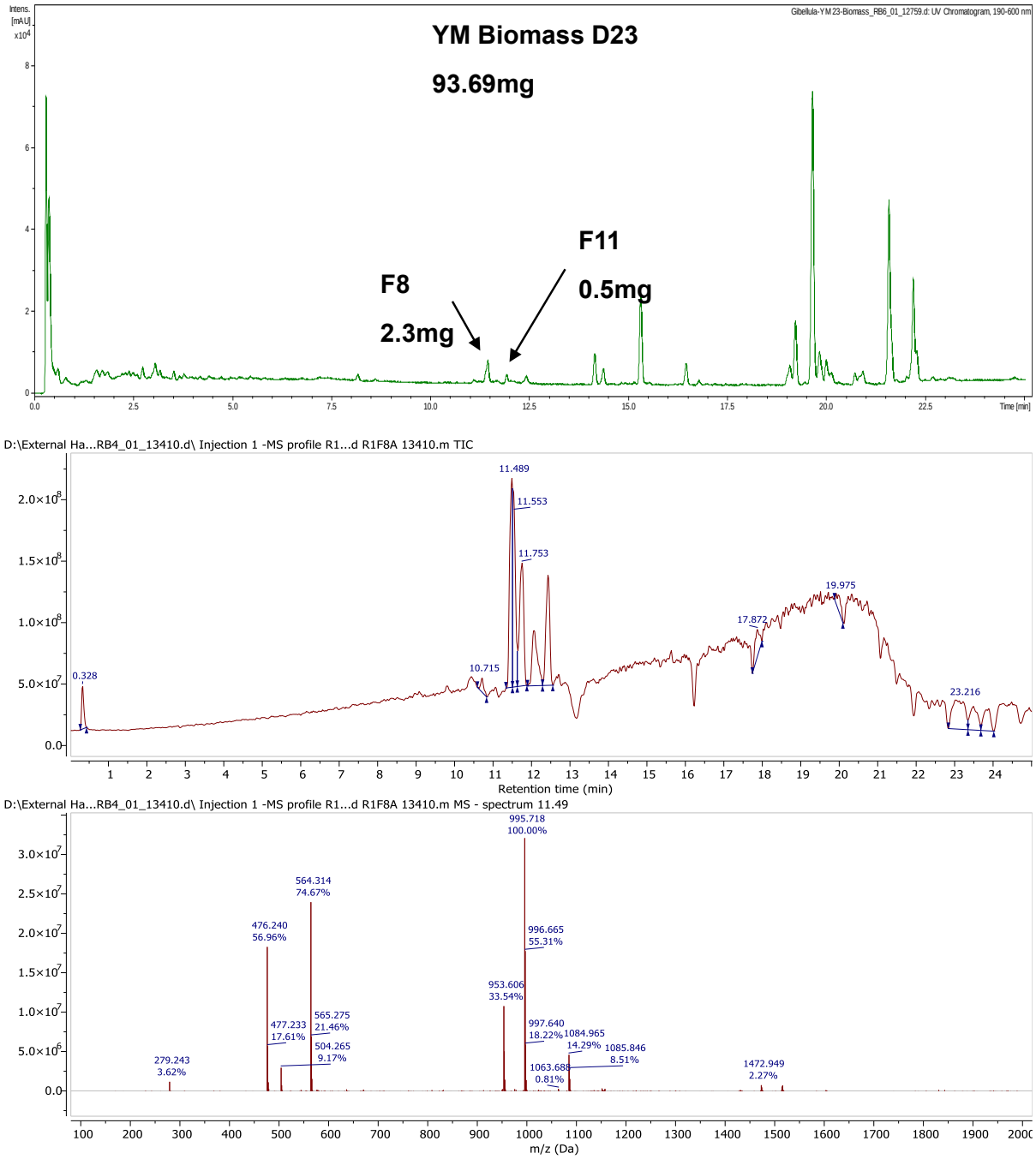


Figure 45. Top: HPLC chromatogram (190-600nm) of a *Gibellula pulchra* biomass extract (93.69mg) grown in YM medium (1L) for 23 days, showing two fractions (F8 and F11) obtained via preparative HPLC. **Middle:** HREIMS Total ion chromatogram of Fraction 8, with 4 major peaks (11-13min). **Bottom:** HREIMS spectrum of the major peak at 11.4 min. NMR spectra obtained from F11 is in Appendix 6.

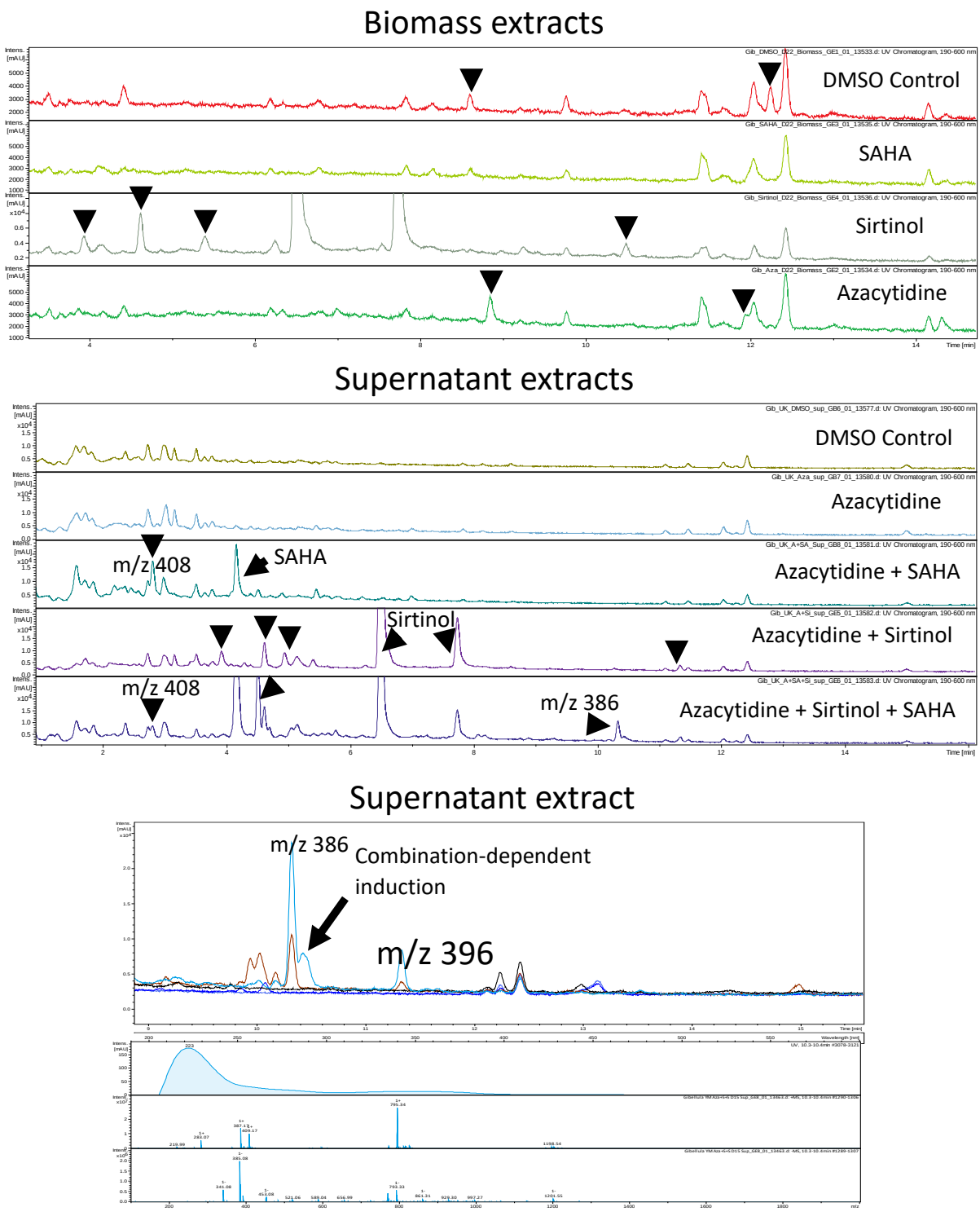


Figure 46. HPLC chromatograms (190-600nm) showing the effect of various epigenetic modifiers (SAHA, Sirtinol, Azacytidine, alone and in combination) on the metabolic profile of *Gibellula pulchra* biomass and supernatant extracts. Arrows indicate epigenetic modifier and induced peaks. Combination-dependent induction was observed in supernatant extract from cultures treated with all three epigenetic modifiers.

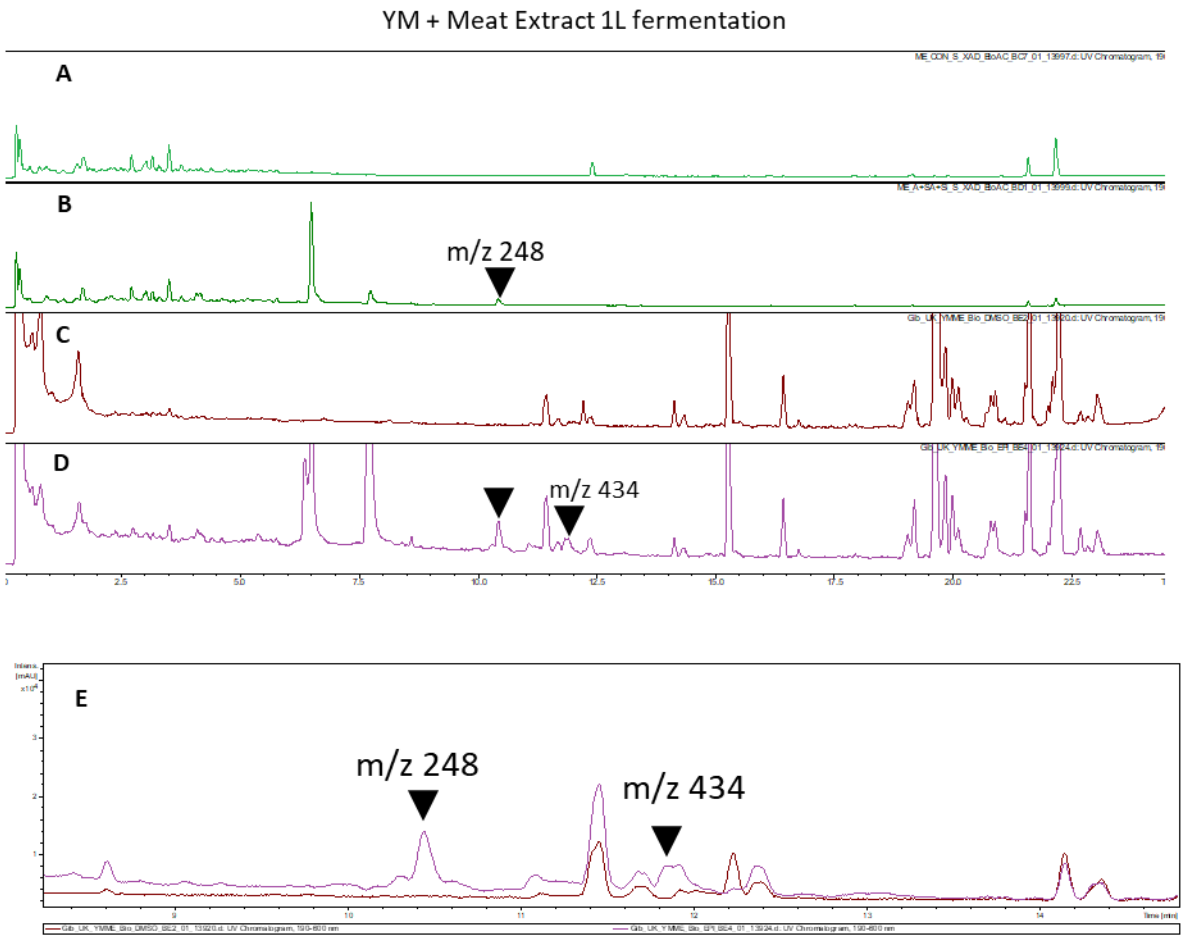


Figure 47. HPLC chromatograms (190-600nm) of supernatant (SN) and biomass (BM) extracts of *Gibellula pulchra* grown with and without azacytidine, SAHA and Sirtinol. **A, C:** DMSO Control SN & BM recpectively. **B, D:** Aza+SAHA+Sirtinol SN & BM respectively. **D:** Several additional peaks can be seen in the biomass extract compared with the supernatant extract. **E:** Several induced peaks are seen in the epigenetic modifier-treated culture **(Purple**) which are absent in the DMSO control (**Brown)**

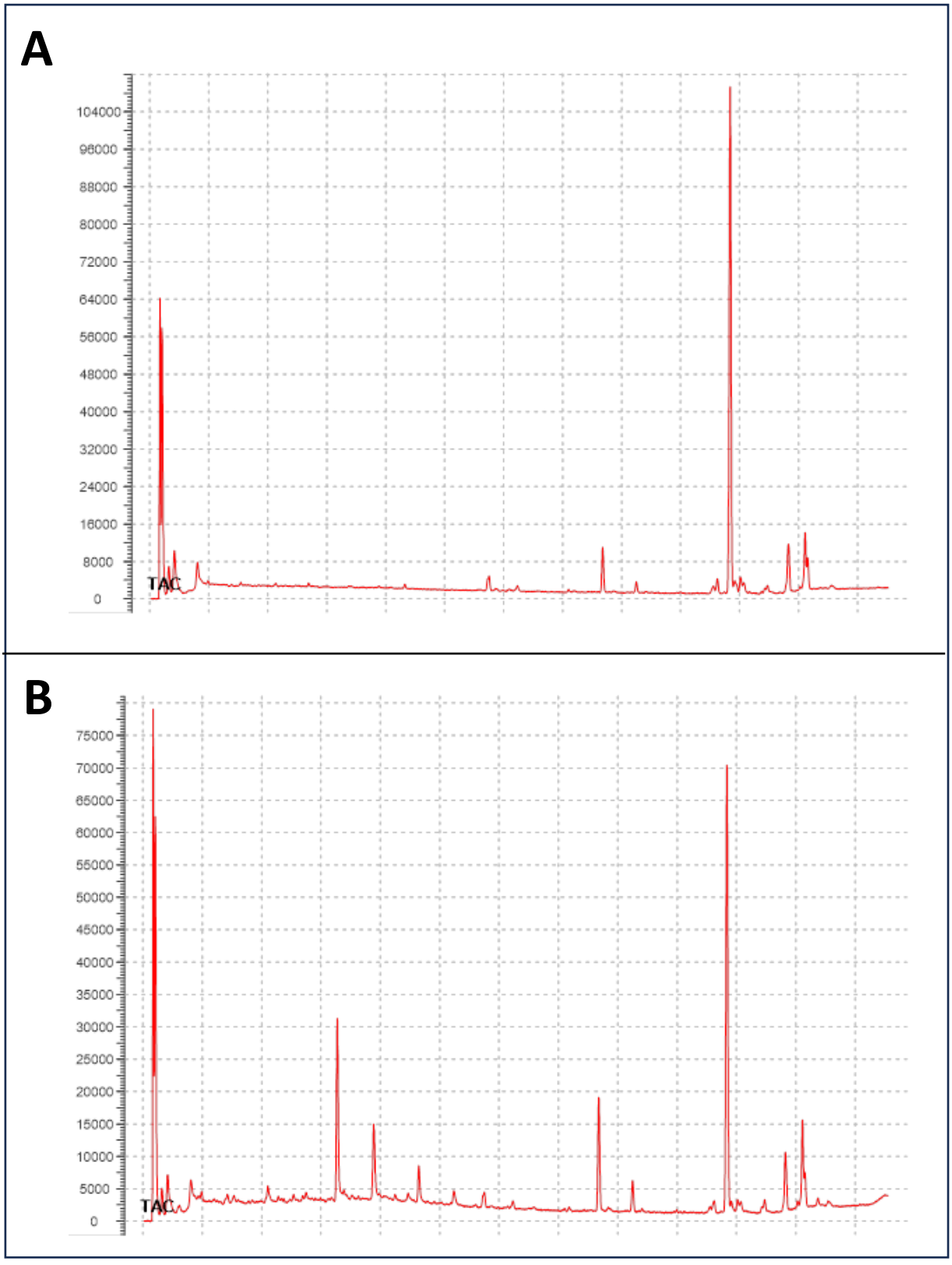


Figure 48. HPLC Total Absorbance Chromatograms (TACs) (190-600nm) of DMSO-control culture extract (A), and epigenetic modifier (Azacytidine 200µM, Vorinostat (100µM) and Sirtinol (50µM)) treated culture (B). In the epigenetic-treated sample, several additional peaks can be seen in the first half of the chromatogram which were not present in the control.

The mass spectrum of the peak at 4.2 minutes in the epigenetic treated sample was averaged, as well as the same time range in the DMSO control, then the mass spectra were displayed in mirror image format (**Figure 36**). The mass spectra suggests that the induced compound has a mass of 264.09, based on the adduct masses *m/z* 265.09833 $[M+H]^+$ and *m/z* 287.07857 $[M+Na]^+$.

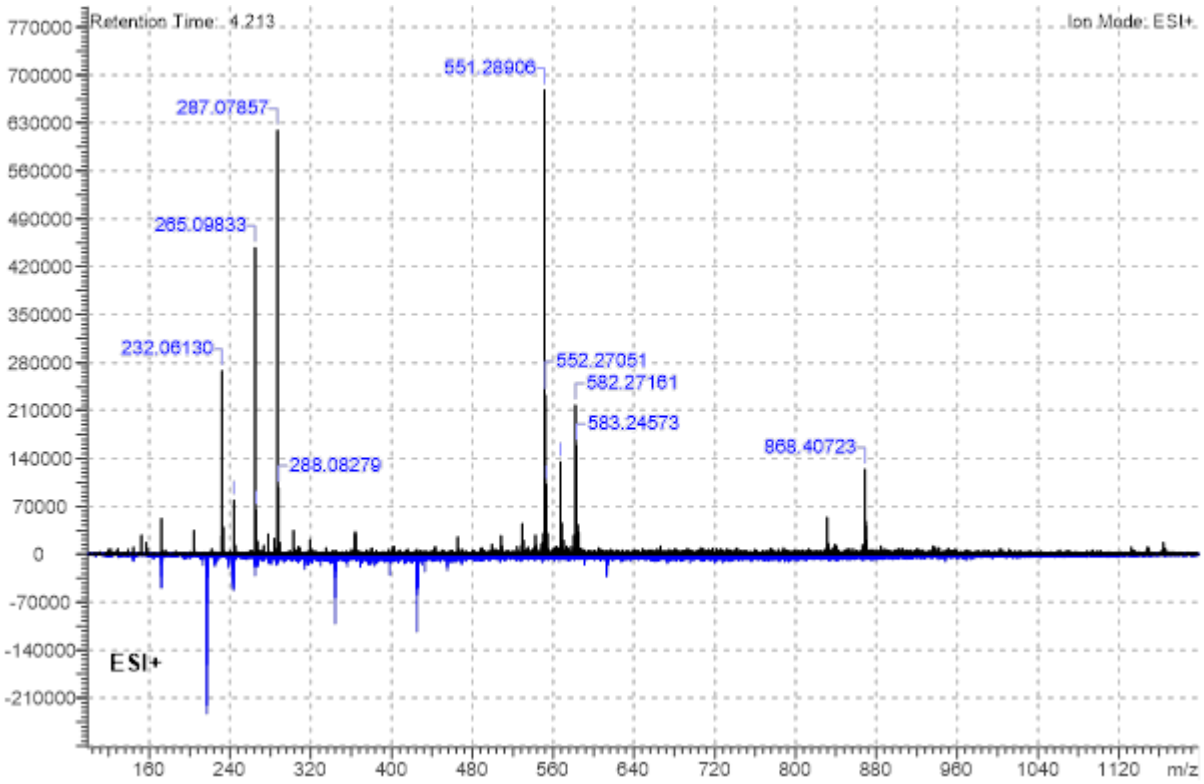


Figure 49. Mass spectrum of an induced peak present in the epigenetic modifier-treated sample (Above, black lines) compared with the DMSO-treated control culture (Below, blue lines). Several ions are present in the treated sample which may correspond to Perlolyrine adducts and fragments

3.7.2 Large-Scale fermentation of *Gibellula pulchra* in ZM Medium

Following the media screening of *Gibellula pulchra* in YM, Q6 and ZM Media, several additional fermentations were conducted in ZM media, as this media resulted in the greatest diversity of metabolites whilst also giving a sufficient yield for potential purifications.

These fermentations were extracted as previously described, separating the biomass and supernatant via centrifugation, extracting the biomass first with Acetone, then the residue with ethyl acetate, and the supernatant with Ethyl Acetate, then partitioning the ethyl acetate extracts between 90% MeOH/10% H_2O and Hexane, in order to separate the highly non-polar constituents such as fatty acids from the more polar metabolites.

Upon HPLC analysis, both the biomass and the supernatant methanol extracts displayed a major peak of medium polarity, surrounded by a number of smaller peaks, including a smaller peak eluting slightly later than the major peak (**Figure 38**). Repeated attempts were made to improve the separation of these peaks by analytical HPLC by varying the mobile phase solvents, additives, pH, gradient program and column chemistry.

Attempts were made to purify the major peak via automated flash chromatography utilising a C18 reverse-phase column, with a shallow gradient method utilising Water and Methanol as mobile phase solvents A and B, respectively. The extract was fractionated, monitoring elution at several wavelengths and collecting the main peak across a series of tubes. HPLC analysis revealed that the major peaks were not completely separated, so a further round of purification was attempted via semi-prep HPLC utilising similar conditions as those employed during the flash chromatography. Initial analytical HPLC revealed that the collected material was fairly pure.

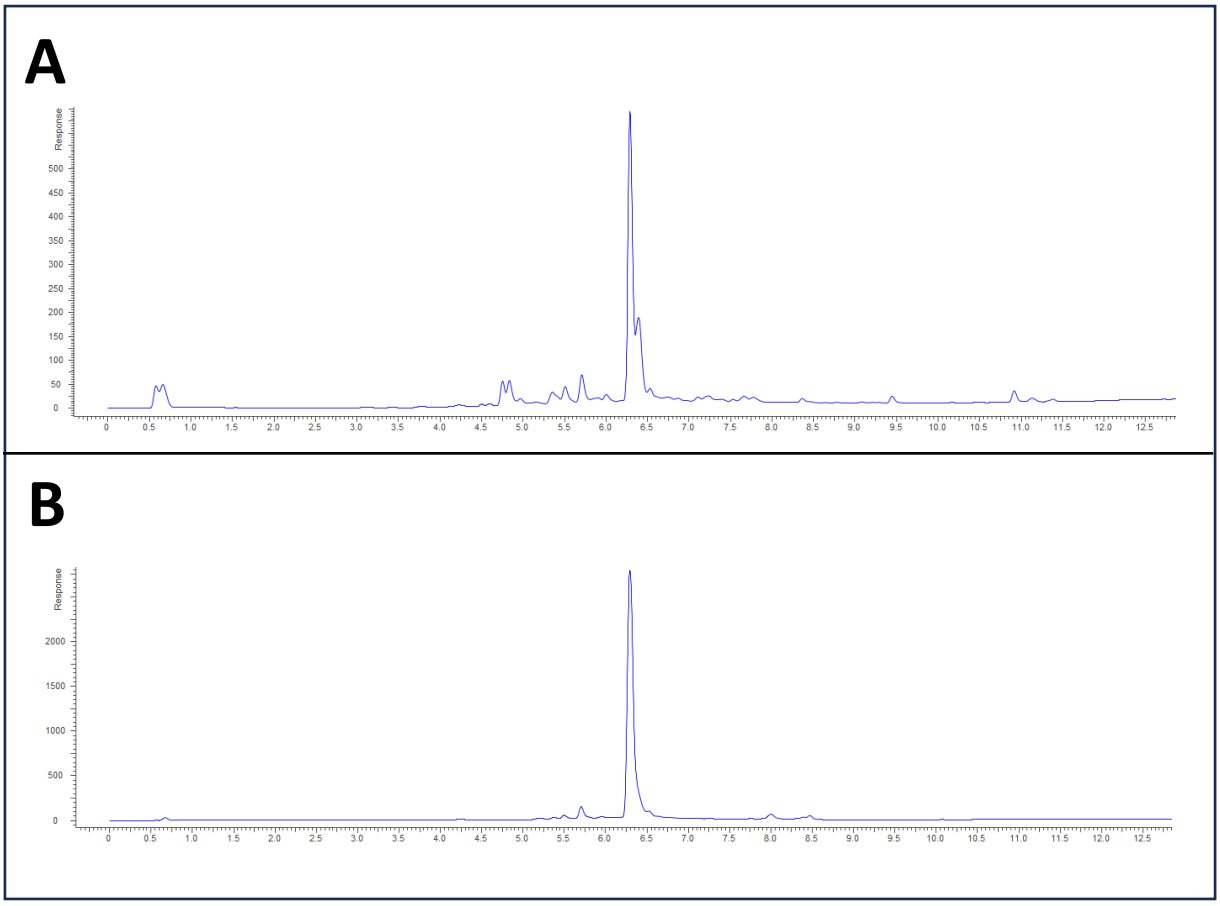


Figure 50. HPLC Chromatograms (254nm) of the crude biomass extract (A) and the material collected following purification via flash chromatography and semi-prep HPLC (B).

The purified fraction was analysed via LCMS and NMR (Proton and Carbon NMR in Appendix 8-9) in efforts to elucidate the structure of the compound. It was revealed that two additional peaks were present in the collected fraction, eluting later than the major peak, and it was assumed that these peaks represented compounds which had formed from the major peak during purification and analysis (**Figure 51**).

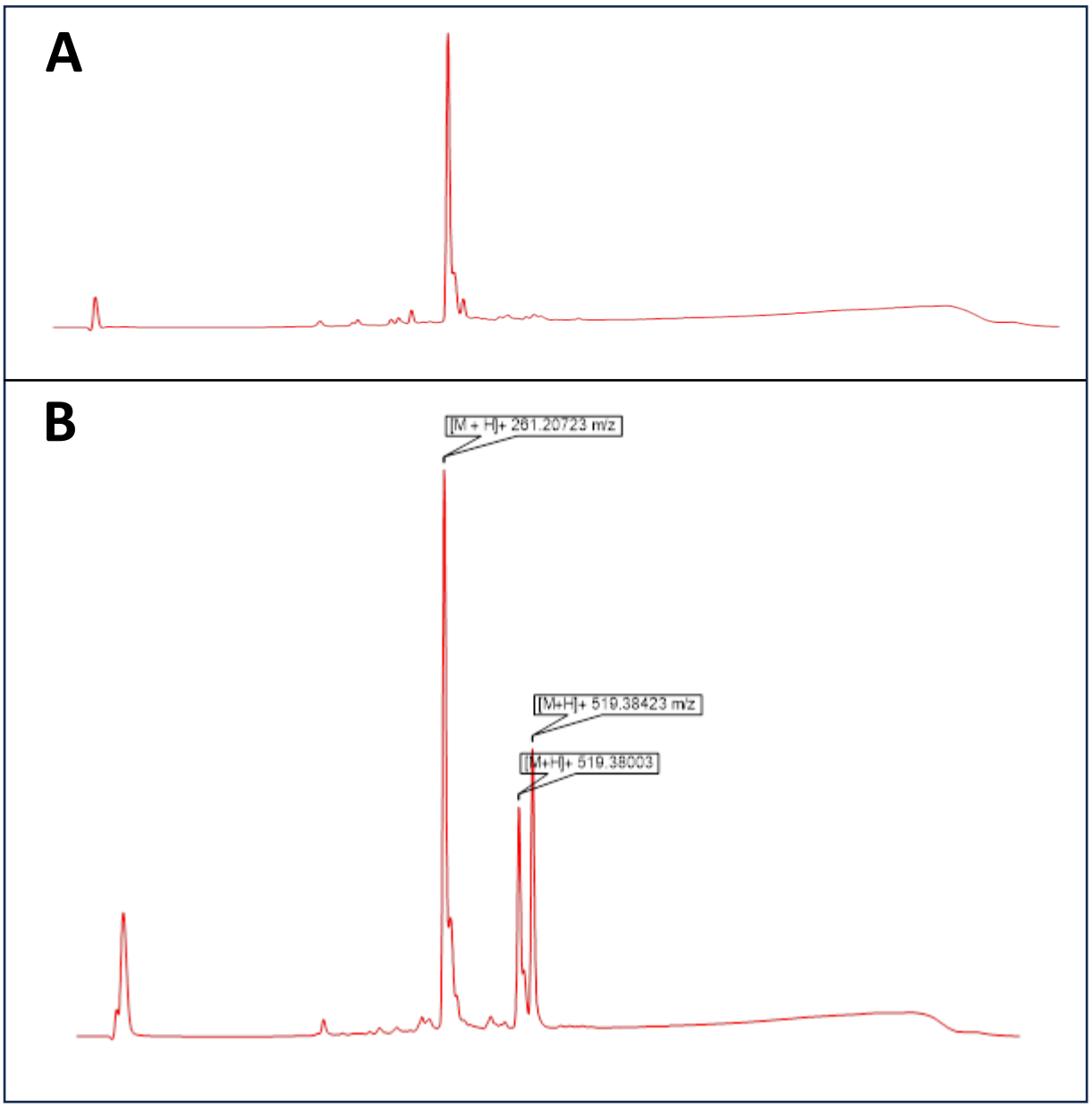


Figure 51. HPLC chromatograms (254nm) of an ethyl acetate extract from *Gibellula pulchra* grown in ZM liquid media (5L) (A), and fraction obtained after purification of the major peak (m/z 261) was attempted via semi-prep HPLC (B).

Analysis of the MS data from the major peak (**Figure 40**) suggests that it has a molecular weight of 260.0608, taking m/z 261.068 and m/z 283.054 as the [M+H] and $[M+Na]^+$ adducts, respectively. The two additional peaks that arose following purification and analysis appear to have *m/z* 519.38 as the [M+H] adduct and therefore an accurate mass of 518.37. As half this mass is 259.18, this suggests that these peaks may be dimers formed from the main purified compound, in which a single bond has formed between two of the monomers, replacing a hydrogen from each. The MS

data also suggests that the compound also contains hydroxyl groups, as loss of hydroxyl groups can be seen in the MS/MS fragment data.

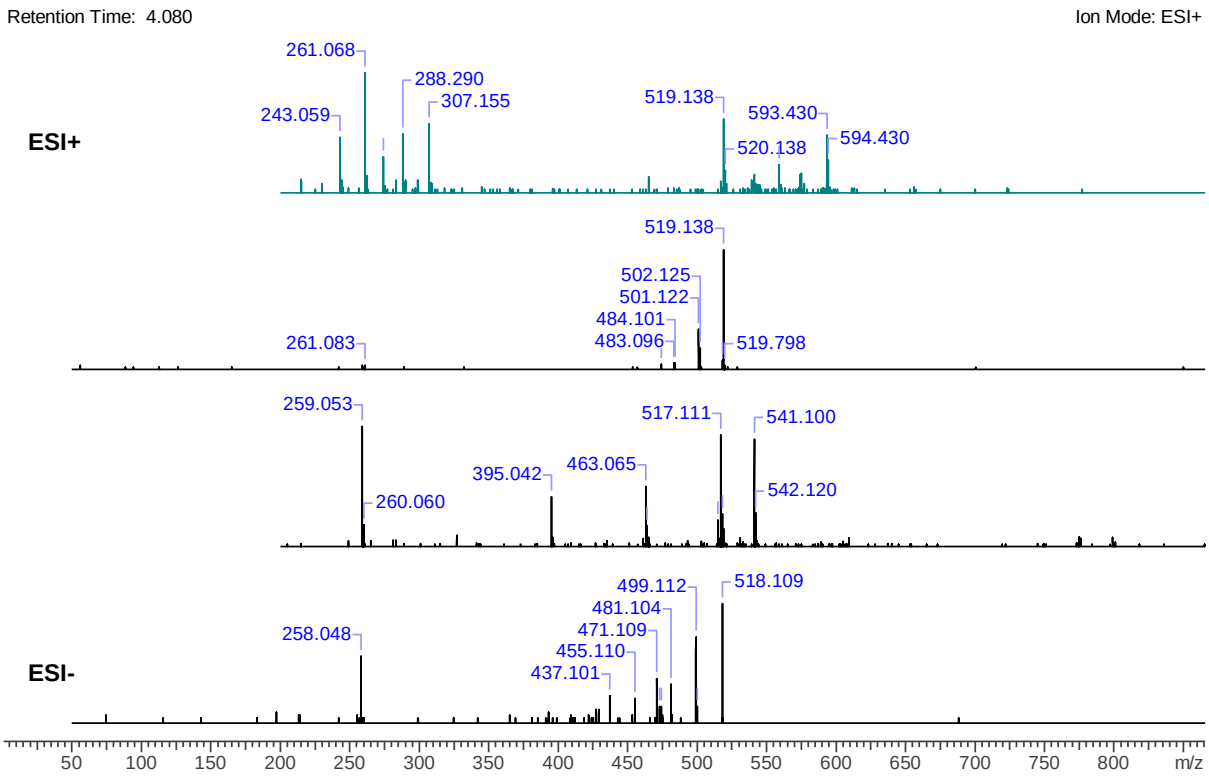


Figure 52. Positive mode (Top) and Negative mode (bottom) MS1 and MS2 mass spectra of the major peak purified from large-scale ZM fermentation of *Gibellula pulchra*.

Gibellula species are known to be capable of producing naphthopyrones including Pigmentosins A and B (**Figure 52**) [113], which have a similar mass to that observed and are also dimeric structures containing multiple hydroxyl groups. They have been found to posess anti-biofilm properties against *Staphylococcus aureus* as well as having some antibacterial efficacy.

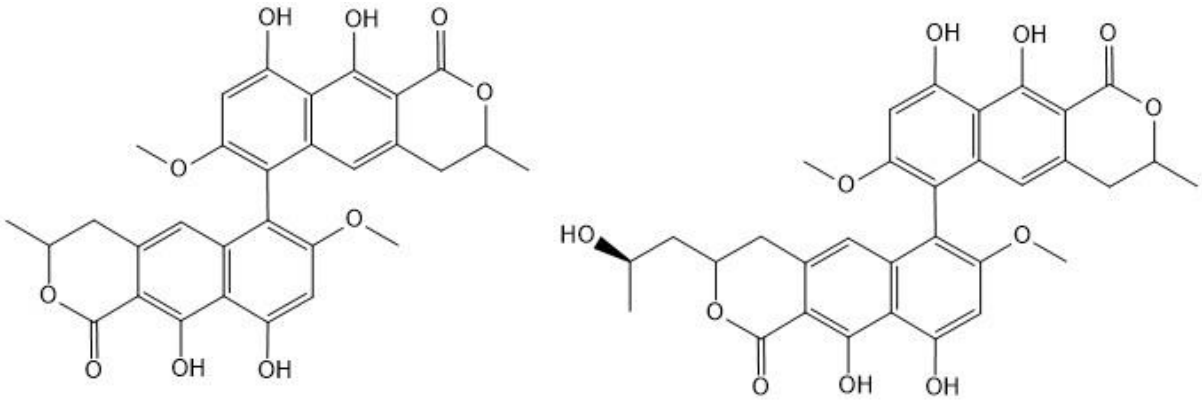
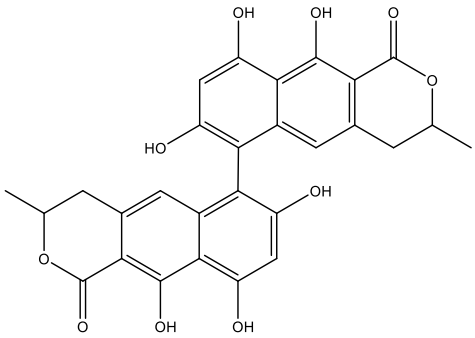


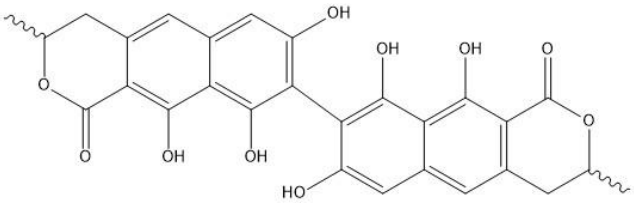
Figure 53. Structures of Pigmentosin A (Left) and Pigmentosin B (Right)

With this in mind, online databases were searched for the observed masses, in an attempt to find likely matches to the MS data which are dimeric structures similar to the pigmentosins known from *Gibellula* sp., as well as containing hydroxyl groups.

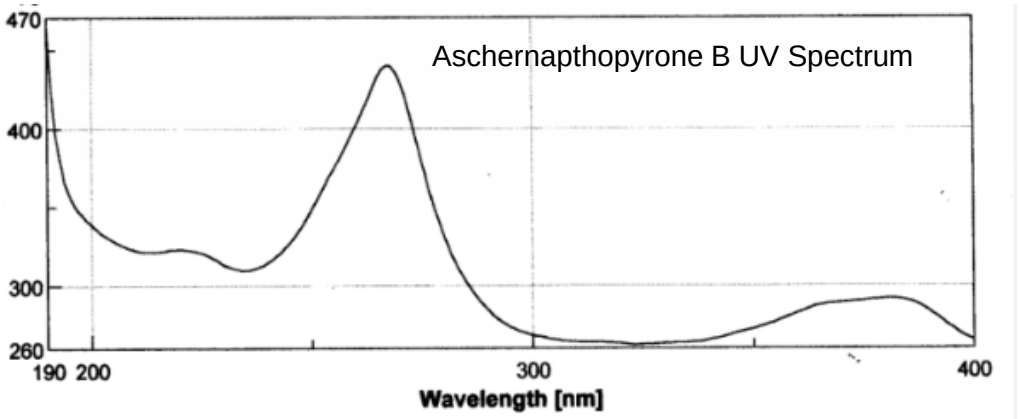
The observed parent and fragment masses are a good match for Aschernapthopyrone A and B (**Figure 42**), two napthopyrones which are closely related to Pigmentosins A and B, that have been isolated from *Aschersonia paraphysata,* another entomopathogenic fungus which is a pathogen of scale insects [114]. The observed UV spectrum of the peak is also very similar to the published spectrum for Aschernapthopyrone B, making it a likely candidate.

However, attempts to obtain NMR spectra from the purified material were not successful, so the unambiguous identification of the metabolite was not possible. Nevertheless, the data as well as the discovery of the Pigmentosins from *Gibellula sp.* and of the Aschernapthopyrones from *Aschersonia* sp. presents a strong argument that the metabolite is a dimeric napthopyrone, perhaps Aschernapthopyrone B, however it is possible that it is a structural analogue or other closely related structure.





Aschernaphthopyrone A **Aschernaphthopyrone B**



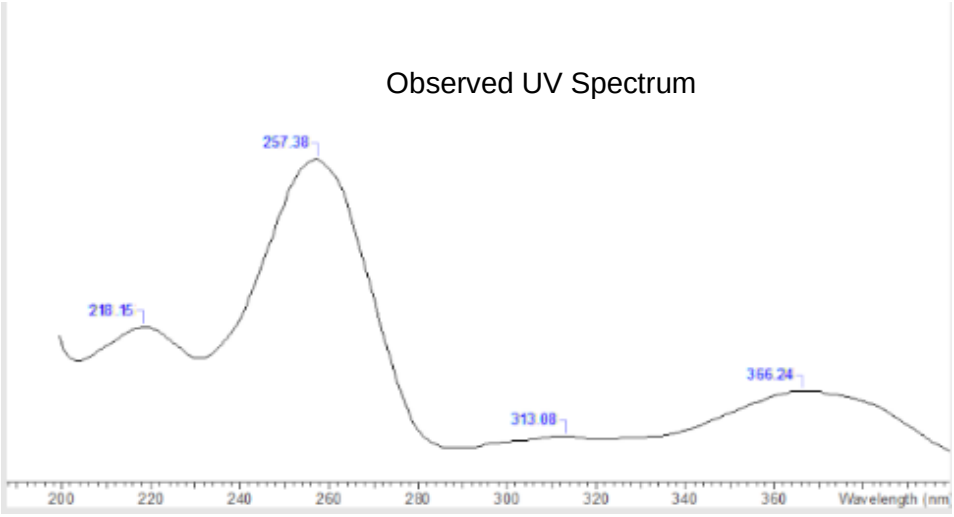


Figure 54. Chemical structures of Aschernapthopyrone A (Top left) and Aschernapthopyrone B (Top right). UV Spectrum of Aschernapthopyrone B, and the UV spectrum of the peak isolated in this work.

3.8 Biotransformation of tryptamine by *Gibellula* sp.

3.8.1 Introduction and background

A number of entomopathogenic fungi have been found to be capable of producing beta-carbolines, such as the wasp-pathogenic species *Ophiocordyceps sphecocephala [115]*, the moth larvae-pathogenic *Ophiocordyceps sinensis [85, 116]*, the ant pathogenic *Ophiocordyceps irangiensis [90]*, and the spider pathogenic species *Gibellula gamsii [111]*, and from these species 17 beta-carboline derivatives have been identified (**Figure 55**).

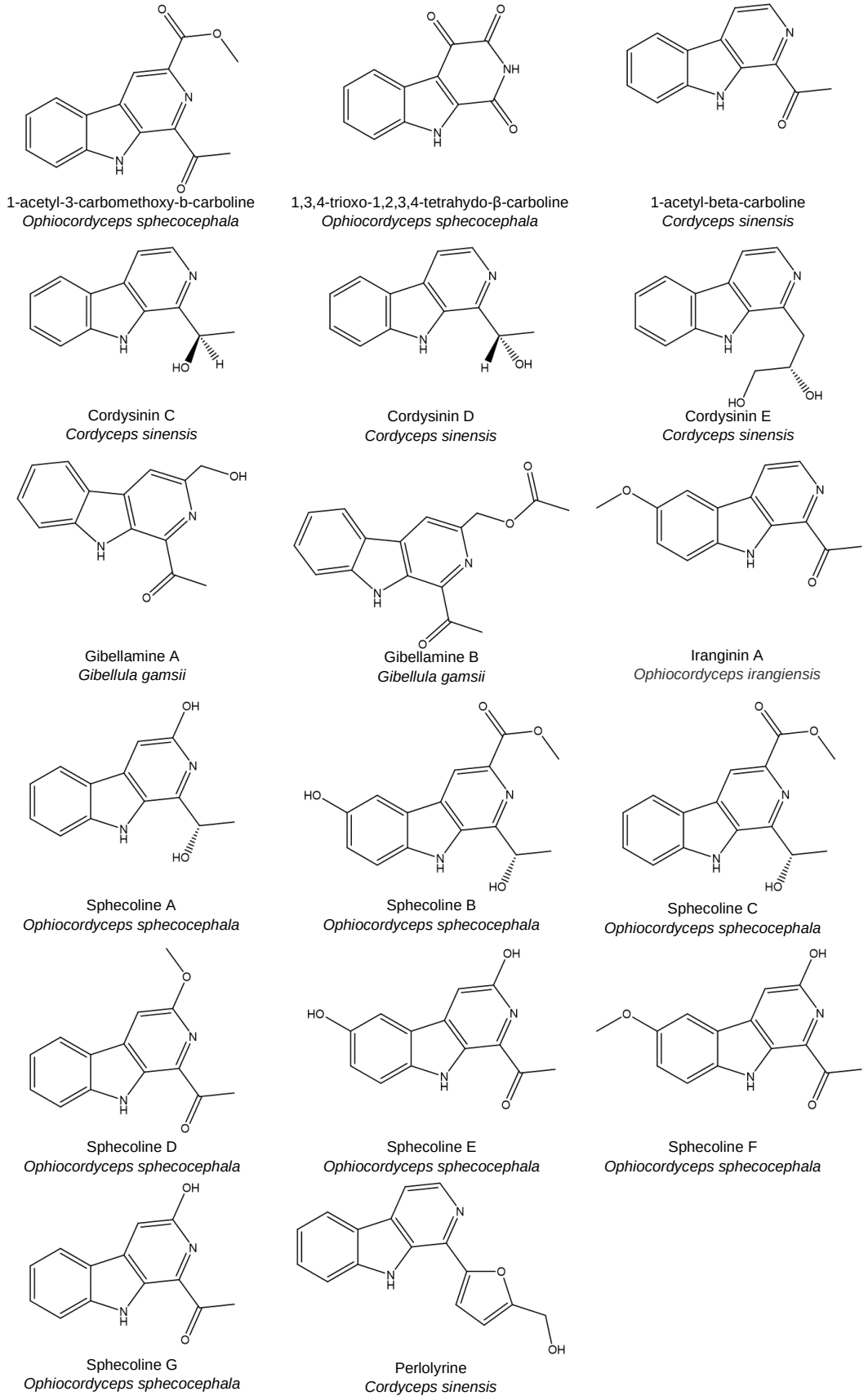


Figure 55. Chemical structures of beta-carbolines that have been previously isolated from entomopathogenic fungi

Many natural alkaloids are biosynthetically-derived from the amino acid l-tryptophan, following its decarboxylation to tryptamine. The β-carbolines are thought to be produced from tryptamine via a pictet-spengler reaction [111, 117]. As the beta-carbolines Gibellamine A and Gibellamine B have been isolated from the spider-pathogenic species *Gibellula gamsii* [111], in this work the addition of several precursor compounds were added to fermentations of *Gibellula* isolate 1 in order to see if their biotransformation resulted in the production of metabolites not previously detected from the strain.

3.9 Initial small-scale screening fermentations

Initially, small-scale fermentations (N=3 per group, 10mL fermentation volume in YM medium, as previously described) were performed with the addition to the media of a variety of indole-containing compounds (100µM final concentration) including l-tryptophan, d-tryptophan, 5-hydroxytryptophan, tryptophol, and tryptamine, which were extracted via ethyl acetate extraction, processed and analysed via HPLC, comparing the fermentation extracts against DMSO (solvent) controls and pure samples of the individual additives. Of these additives, Tryptamine and Tryptophol resulted in the production of new peaks in the chromatograms compared with DMSO-treated controls, with two new major peaks in the tryptamine-treated fermentations, and two new major peaks in the tryptophol-treated fermentations (**Figure 44)**. In both of these groups, there was a new peak at 3.67 min which aligned perfectly with the tryptophol control chromatogram, so this peak was assumed to be tryptophol itself, but in both cases, there were several additional peaks which did not correspond to the tryptophol or tryptamine controls, as well as a greater intensity of peaks present in the controls in the tryptophol group, but only in one replicate. The two major new peaks in the tryptamine-treated cultures did not correspond to the peak of tryptamine itself seen in the control chromatogram, however the peak at 3.67 min aligned with the tryptophol control and was assumed to be tryptophol.

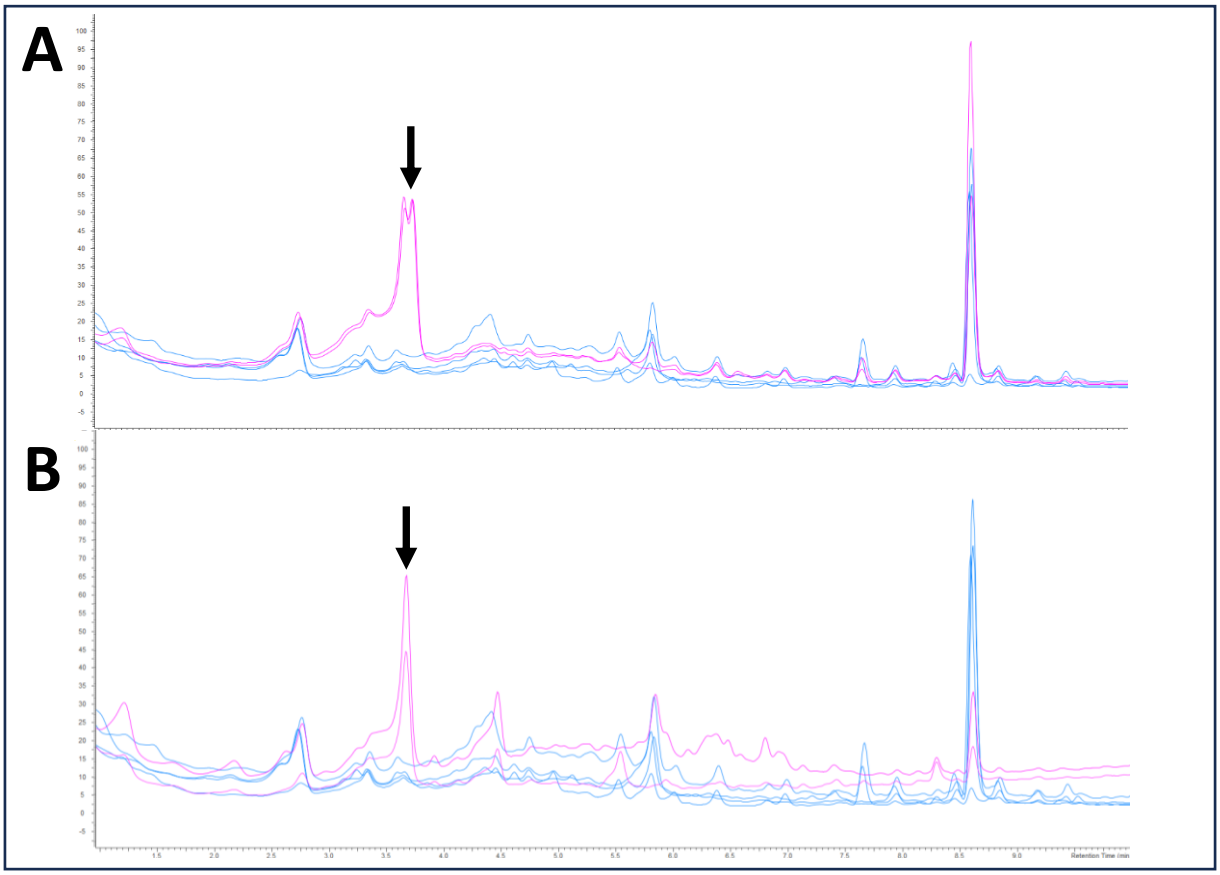


Figure 56. A: HPLC chromatograms (270nm) of Tryptamine-treated culture extracts (Pink) and DMSO-treated control culture extracts (Blue). **B:** HPLC chromatograms (270nm) of Tryptophol-treated culture extracts (Pink) and DMSO-treated control culture extracts (Blue). Arrows show peaks induced by tryptamine and tryptophol

3.9.1 Scale-up of tryptamine-treated fermentations

Following the initial small-scale results, several fermentations were conducted on a larger scale with the addition of tryptamine at a higher concentration of 1mM, in attempts to obtain more of the new metabolites for LCMS analysis, purification, structural elucidation and bioactivity assay attempts. In these larger-scale fermentations, one major peak was observed in the chromatograms, along with several less-abundant peaks (**Figure 45**).

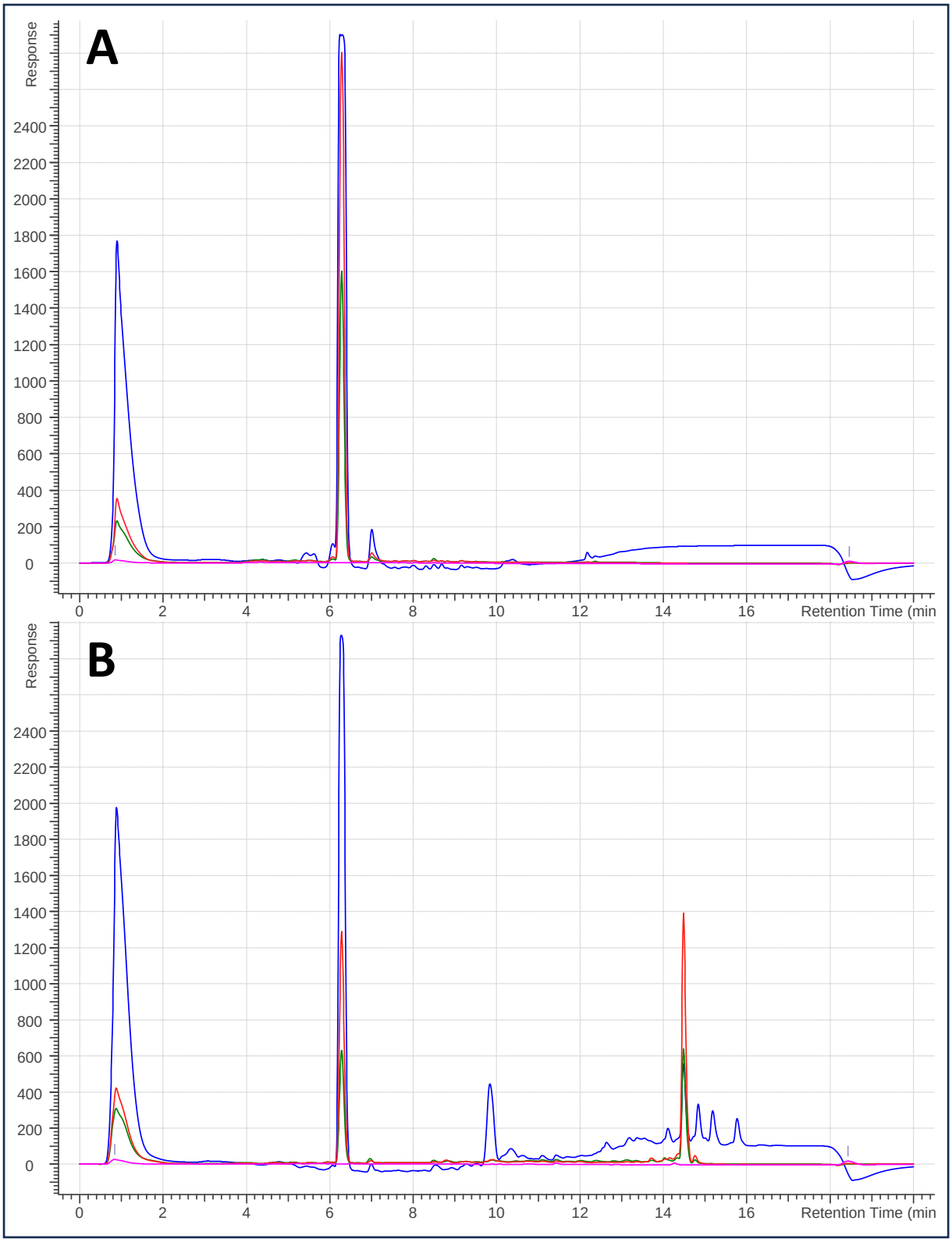


Figure 57. HPLC Chromatograms (Blue: 210nm, Green: 254nm, Red: 270nm, Pink: 360nm) of Supernatant **(A)** and Biomass **(B)** EtoAc extracts from a 2.5L YM +Tryptamine (1mM) fermentation of *Gibellula* isolate 1

The crude extracts were fractionated via reverse-phase flash chromatography (**Figure 58**), yielding several fractions which were analysed via HPLC and LCMS. Tubes 24 from the supernatant extract fractionation and tubes 29-30 from the supernatant extract yielded 31mg of the major peak as a white solid with high purity (accounting for 98.08% of the total peak area when analysed by HPLC at 270nm) (**Figure 59**).

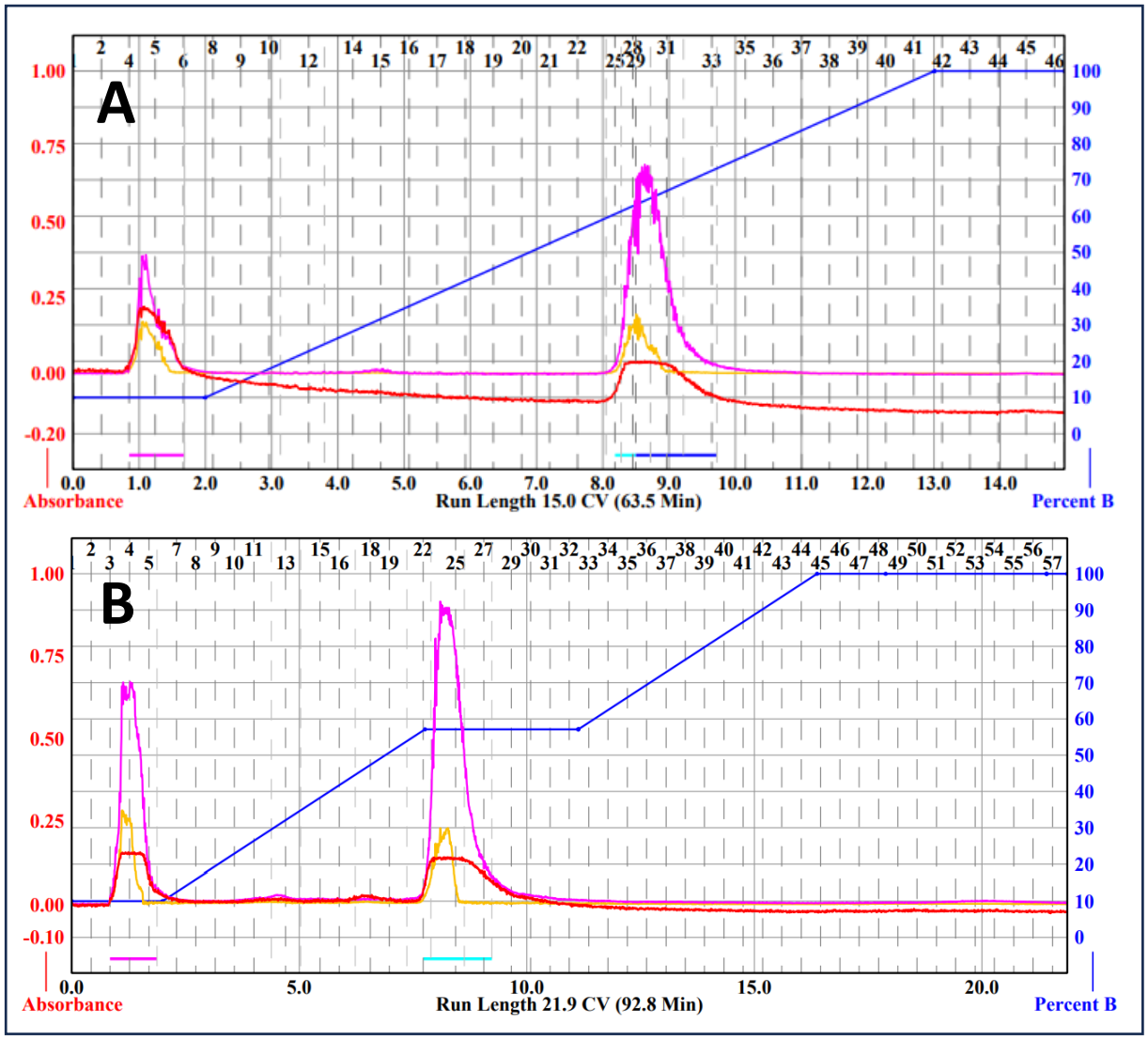
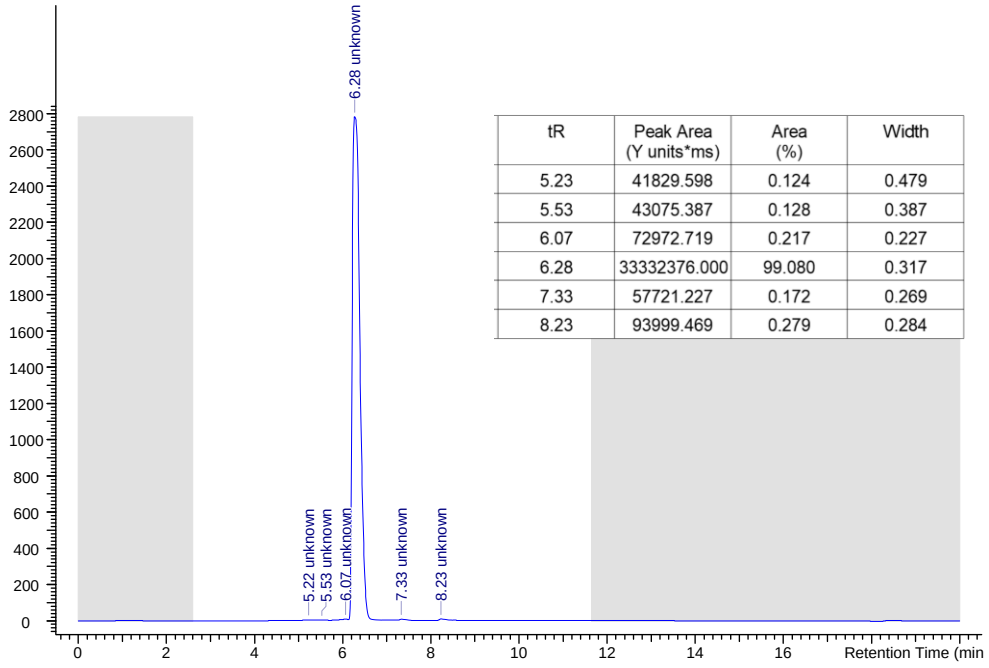


Figure 58. UV chromatograms (Red = 210nm, Pink = 270nm, Orange = 200-360nm) from flash-chromatographic fractionation of **A:** Supernatant extract, and **B:** Pooled supernatant and biomass extracts from YM + 1mM tryptamine *Gibellula* isolate 1 fermentations, showing fractionation of extracts into tubes (Top black numbers, vertical dashed-lines).



tR	Peak Area (Y units*ms)	Area (%)	Width
5.23	41829.598	0.124	0.479
5.53	43075.387	0.128	0.387
6.07	72972.719	0.217	0.227
6.28	33332376.000	99.080	0.317
7.33	57721.227	0.172	0.269
8.23	93999.469	0.279	0.284

Figure 59. HPLC chromatogram (270nm) and percentage peak areas of purified fraction obtained from flash-chromatographic fractionation of the tryptamine-treated fermentation extracts.

3.9.2 Analysis of the collected fractions via LCMS/MS

By using the known and predicted fragment masses alongside natural products database searches, and comparison with mass spectral data published in the literature, the extracts and fractions were analysed via LCMS and attempts were made to identify the metabolites present.

The mass spectrum of the major peak (**Figure 60 A**) had several of the known common fragments produced by MS/MS fragmentation of tryptamine (115, 130, 144 etc) but was not tryptamine itself, which eluted significantly earlier than this peak. Several masses were observed in the mass spectrum and identified as adducts based on their relationship to one another (Table 1), for example $[M+H]^+$ (*m/z* 203.10226), $[M+Na]^+$ (*m/z* 225.08682), $[M+K]^+$ (*m/z* 241.07975). This suggested that the compound had a mass of 202.0951, which is 0.0155 lower than the predicted mass of Acetyltryptamine (202.1106) The UV spectrum of the peak was a close match to the UV spectrum of acetyltryptamine published in a previous study [118].

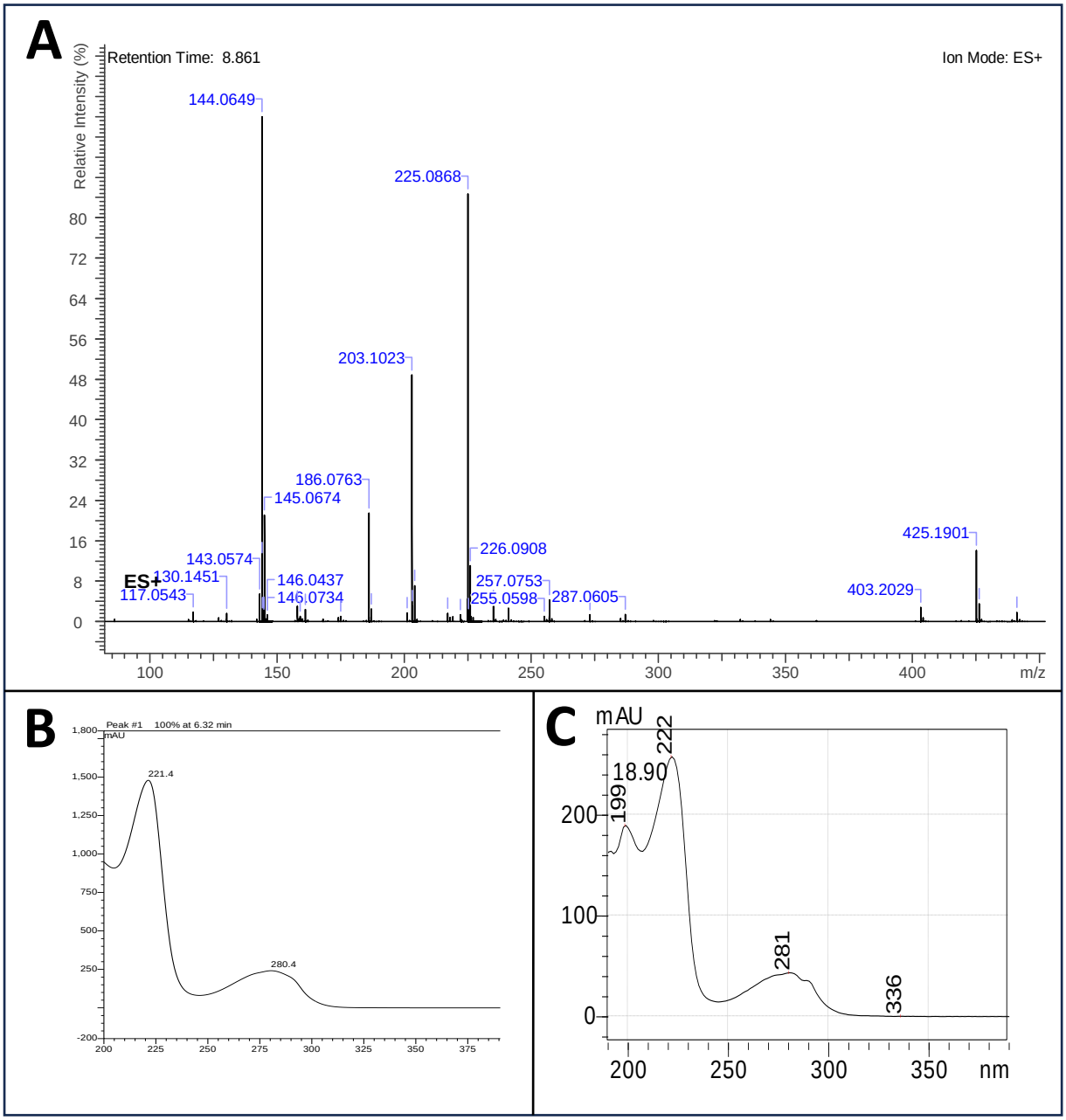


Figure 60. A: Mass Spectrum (MS1, +ve mode) of the major peak present in tryptamine-treated fermentations of *Gibellula* isolate 1, suggesting an apparent molecular mass of 202.0951 based on $[M+H]^+$ 203.1023 and $[M+Na]^+$ 225.0868. B: UV spectrum of the peak showing absorption peaks at 221.4 and 280.4. C: UV spectrum of Acetyltryptamine, published in [118] supplementary material.

Table 1. Predicted Acetyltryptamine adduct masses, observed masses and differences

Adduct	Predicted mass	Observed mass	Difference
[M+2ACN+2H]2+	143.089128	143.05468	-0.034448
[M+H]+	203.117886	203.09956	-0.018326
[M+Na]+	225.099828	225.08672	-0.013108
[M+K]+	241.073768	241.07975	+0.005982
[2M+H]+	405.228496	403.20776	2.020736 (-2H)
[2M+Na]+	427.210438	425.18987	2.020568 (-2H)
[2M+K]+	443.184378	441.17968	2.004698

The identification of the compound as acetyltryptamine was strengthened by the presence of several fragments in the mass spectra (**Table 2**) predicted from *in silico* fragmentation (**Figure 61**).

Table 2. Predicted Acetyltryptamine fragment masses, observed masses and differences

Predicted Fragment mass	Observed mass	Difference
186.11570	185.06645	-1.04925
187.08714	ND	NA
159.09222	159.05098	-0.04124
144.08132	144.04096	-0.04036
130.06567	130.02967	-0.036
116.05002	115.02083	-1.02919
91.04220	91.03727 (MS1)	- 0.00493

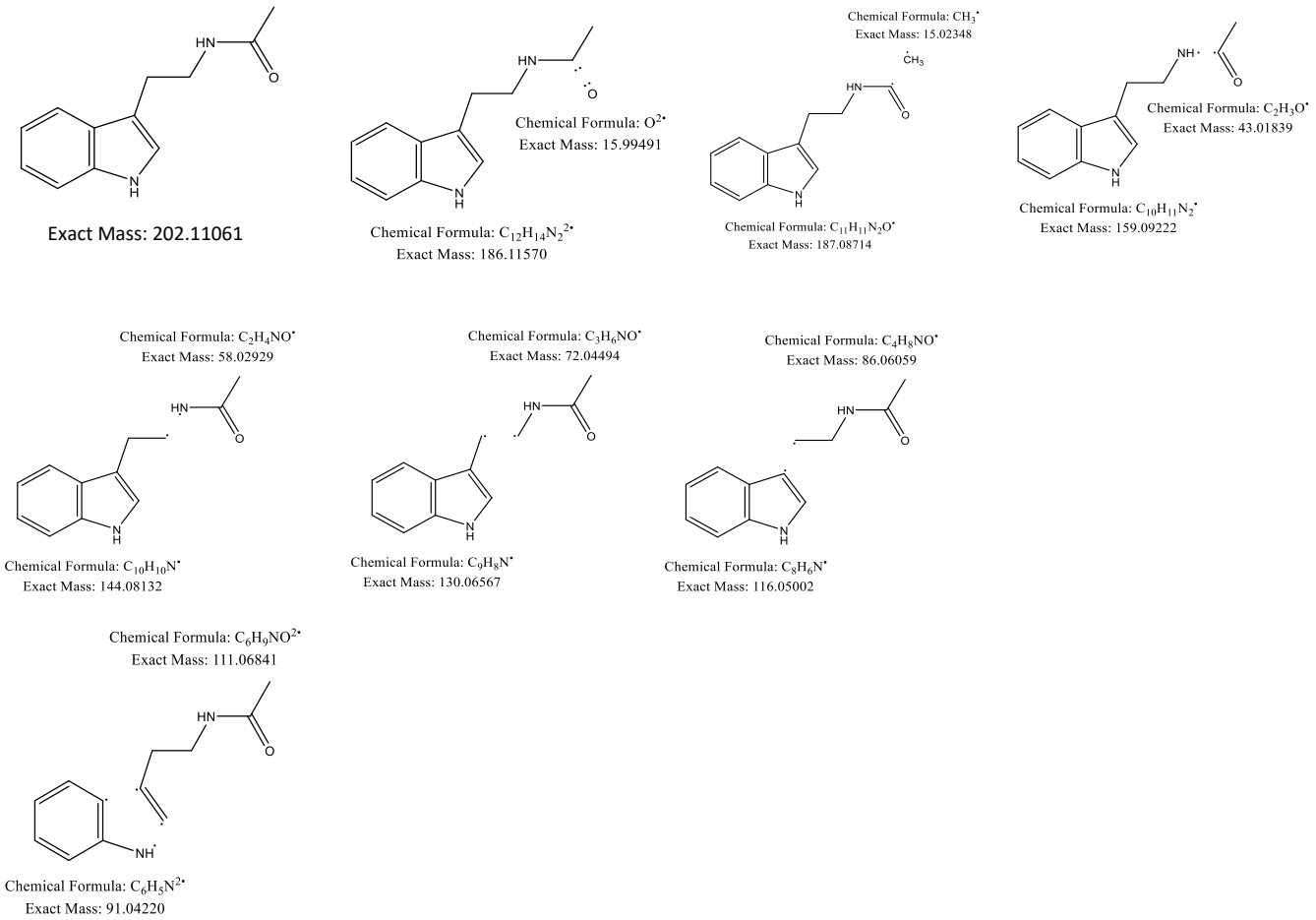


Figure 61. *In-silico* predicted structures and exact masses of fragments produced via LCMS/MS fragmentation of Acetyltryptamine

Finally, the isolated material was analysed via NMR spectroscopy (Proton and Carbon) (**Figures 62-63)** in MeOD, and by comparison with *in silico* predicted spectra the identification of the main peak as acetyltryptamine was confirmed.

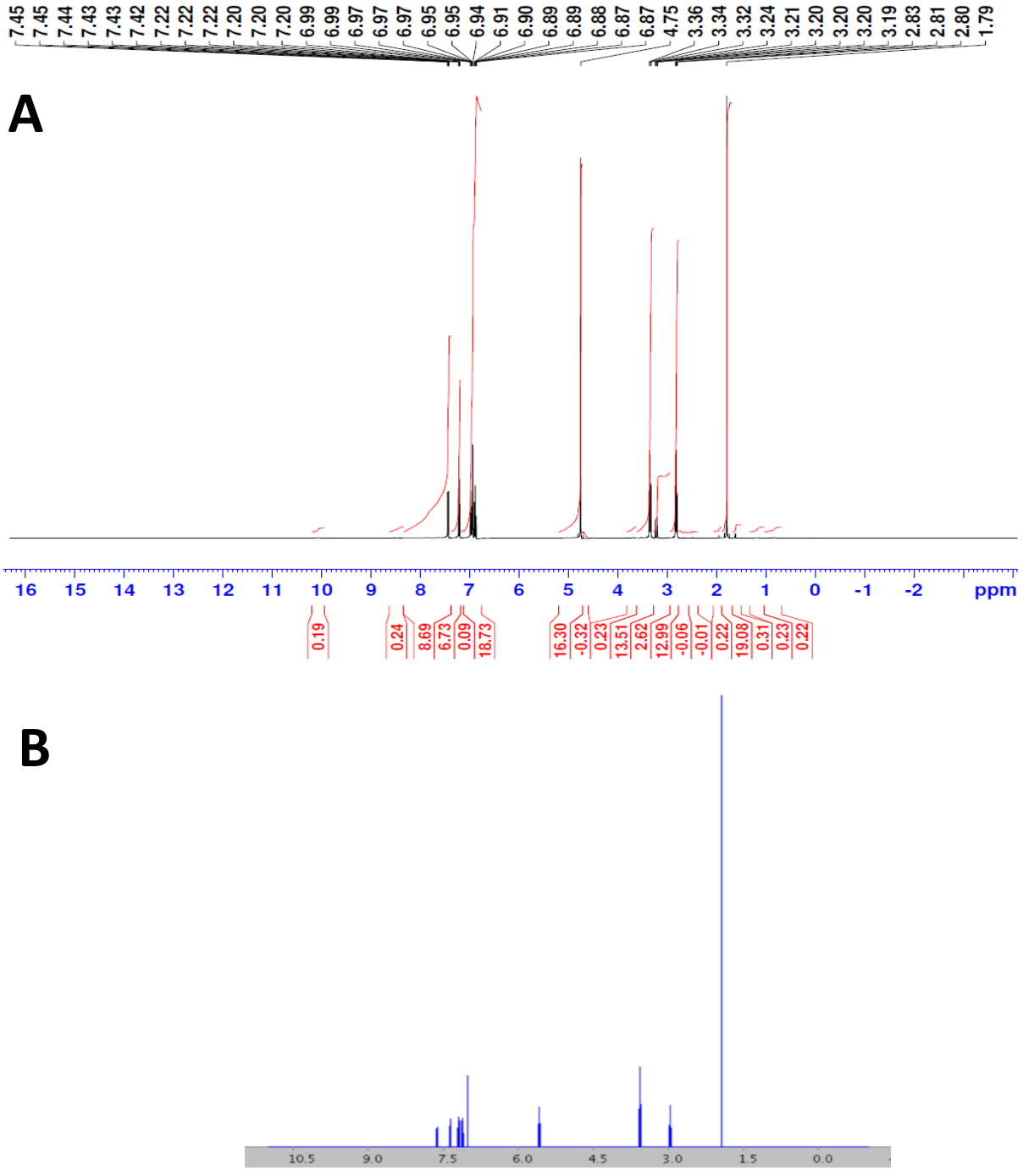


Figure 62. A: Proton NMR obtained from the major isolated peak (MeOD). B: *in silico* predicted Proton NMR spectrum for Acetyltryptamine (Source: SciFinder)

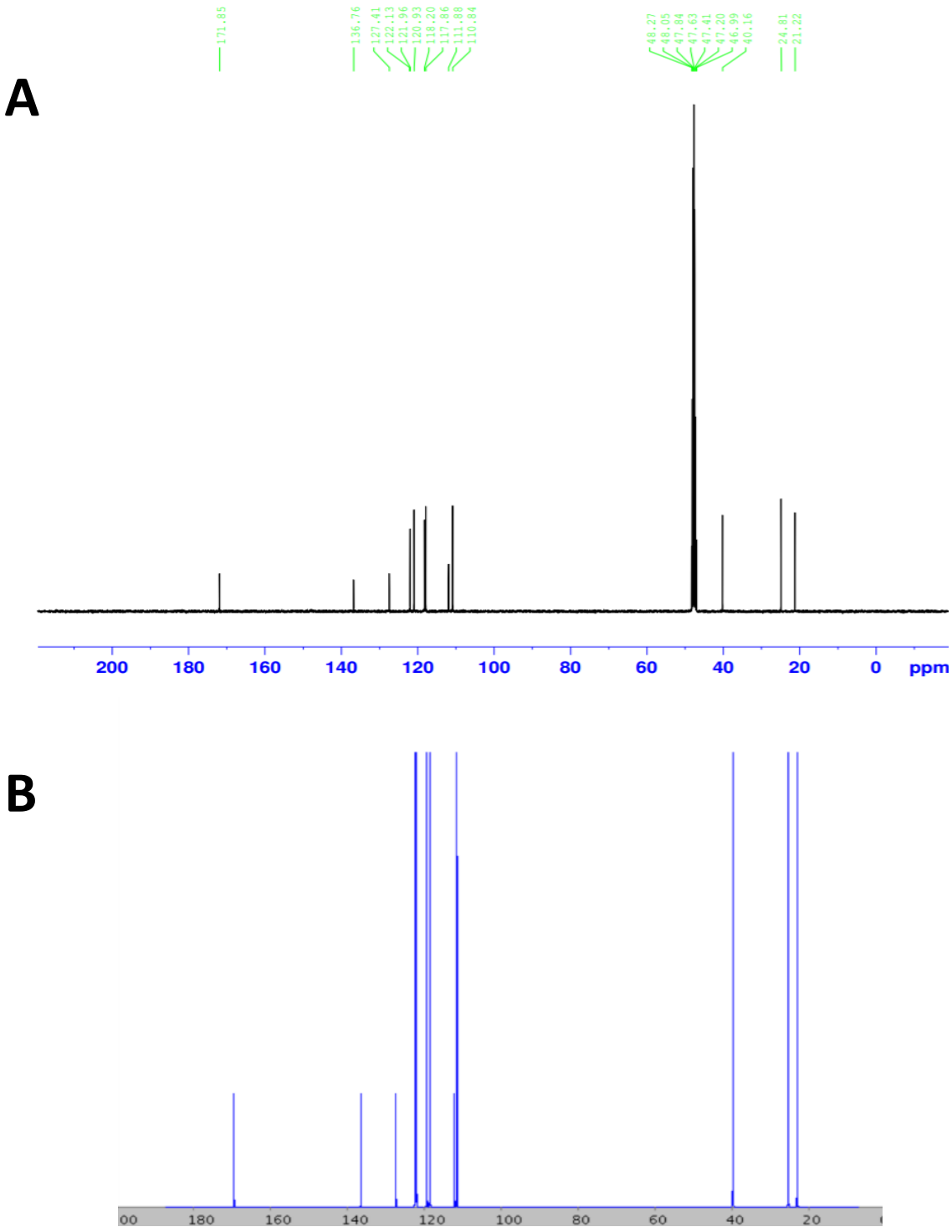


Figure 63. A: Carbon NMR obtained from the major isolated peak (MeOD). B: *in silico* predicted Carbon NMR spectrum for Acetyltryptamine (Source: SciFinder)

3.9.3 LCMS/MS analysis of additional metabolites present in fractionated extracts of tryptamine-treated fermentations of *Gibellula* isolate 1

Analytical methods allowing the detection and identification of a variety of tryptamines via LCMS/MS have been developed, and there is much literature available describing the mass spectral characteristics of these substances [119-121].

Tryptamines are known to commonly fragment in LCMS/MS analysis at particular points in the molecule, producing fragments with characteristic masses, which aids in their structural identification. Using tryptamine itself as an example, various characteristic fragments are often produced **(Figure 64 and 65)**.

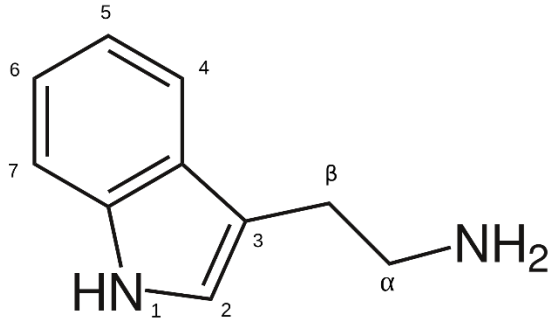
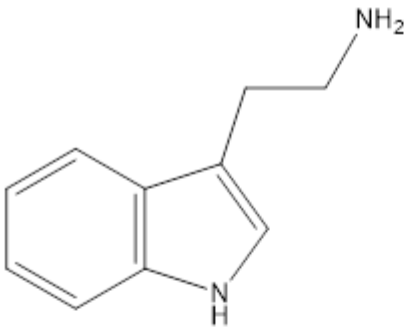
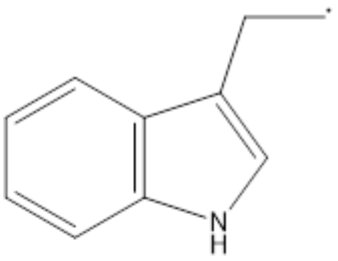


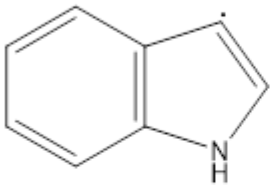
Figure 64. Chemical structure of tryptamine. With LCMS/MS fragmentation, each of the three bonds of the side chain of the molecule can be broken; between the indole ring 3 position and the β carbon, between the α and β carbons, and between the α-carbon and NH_2 group. These fragmentations produce fragments with masses of *m/z* 144, 130 and 115.



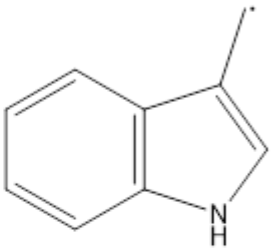
Chemical Formula: $C_{10}H_{12}N_2$
Exact Mass: 160.1000



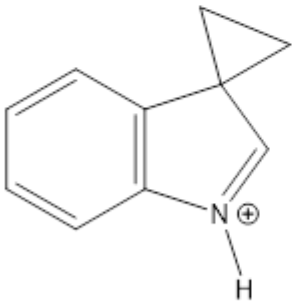
Chemical Formula: $C_{10}H_{10}N^{\bullet}$
Exact Mass: 144.0813



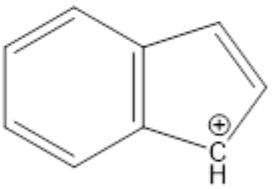
Chemical Formula: $C_8H_6N^{\bullet}$
Exact Mass: 116.0500



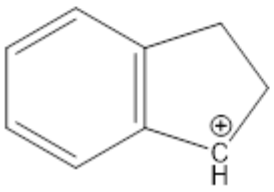
Chemical Formula: $C_9H_8N^{\bullet}$
Exact Mass: 130.0657



Exact Mass: 144.0808



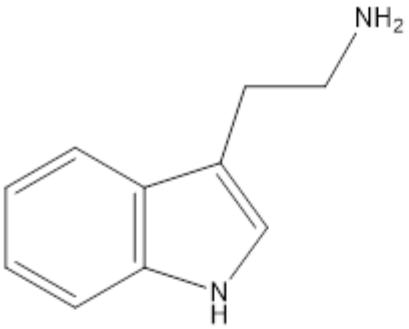
Exact Mass: 115.0542



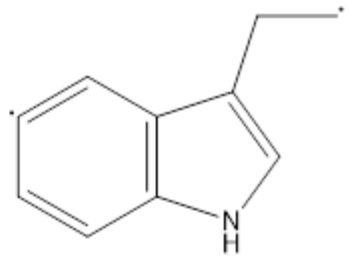
Exact Mass: 117.0699

Figure 65. Structures and *in* silico predicted exact masses of fragments produced via LCMS/MS fragmentation of tryptamine

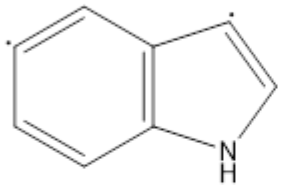
These fragmentations also occur in the case of substituted tryptamine analogues, for example in the case of additional functional groups replacing the hydrogen atoms of the side chain NH_2 group, and functional groups at the 4 and 5 positions of the indole ring, as are seen with serotonin (5-OH-tryptamine), psilocin (4-OH-dimethyltryptamine), and psilocybin (4-phosphoryloxy-dimethyltryptamine) (**Figure 66 and 67**).



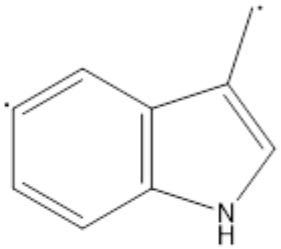
Chemical Formula: $C_{10}H_{12}N_2$
Exact Mass: 160.10005



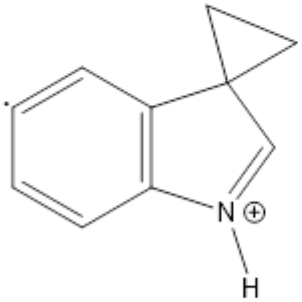
Chemical Formula: $C_{10}H_9N^{2\bullet}$
Exact Mass: 143.07350



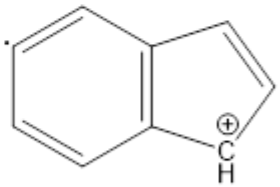
Chemical Formula: $C_8H_5N^{2\bullet}$
Exact Mass: 115.04220



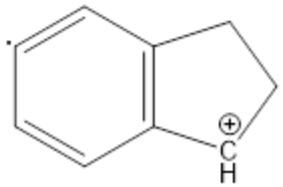
Chemical Formula: $C_9H_7N^{2\bullet}$
Exact Mass: 129.05785



Chemical Formula: $C_{10}H_9N^{\bullet+}$
Exact Mass: 143.07295

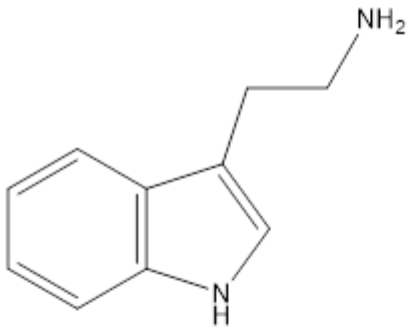


Chemical Formula: $C_9H_6^{\bullet+}$
Exact Mass: 114.04640

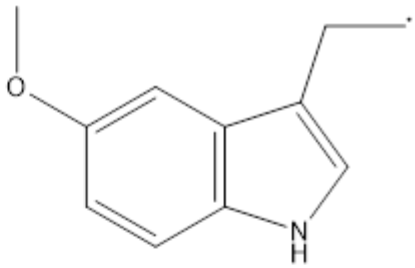


Chemical Formula: $C_9H_8^{\bullet+}$
Exact Mass: 116.06205

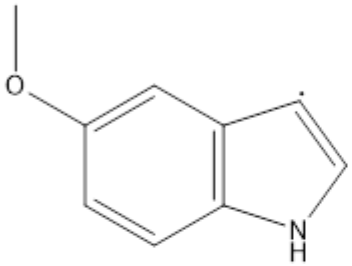
Figure 66. Structures and predicted exact masses of fragments produced via LCMS/MS fragmentation of tryptamine analogues having lost an additional bond



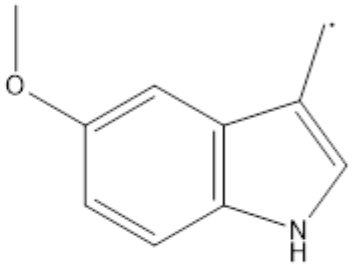
Chemical Formula: $C_{10}H_{12}N_2$
Exact Mass: 160.10005



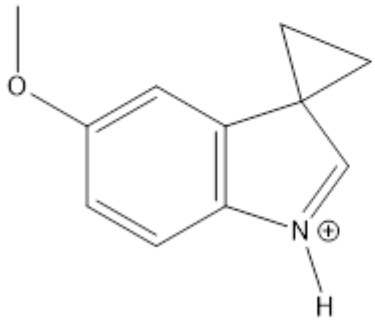
Chemical Formula: $C_{11}H_{12}NO^{\bullet}$
Exact Mass: 174.09189



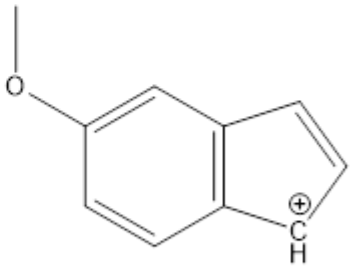
Chemical Formula: $C_9H_8NO^{\bullet}$
Exact Mass: 146.06059



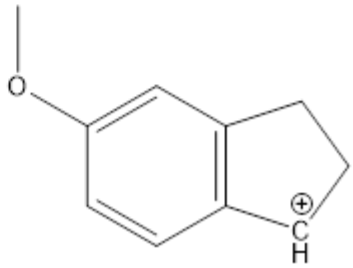
Chemical Formula: $C_{10}H_{10}NO^{\bullet}$
Exact Mass: 160.07624



Exact Mass: 174.09134



Exact Mass: 145.06479



Exact Mass: 147.08044

Figure 67. Structures and predicted exact masses of fragments produced via LCMS/MS fragmentation of tryptamine analogues containing a methoxy group at the 5 position.

Using these known tryptamine MS-MS fragmentation patterns, a series of predicted fragment masses for potential metabolic derivatives of tryptamine was developed in-silico (**Table 3)**, so that the LCMS data obtained from the culture extracts could be searched for these fragments to identify potential biotransformation products from the added tryptamine.

As well as this, natural products databases (Dictionary of Natural Products, Metlin, SciFinder) were searched for the parent masses of these observed fragments, and attempts were made to find potential identification candidates. Results were refined to those containing the structures corresponding to the fragments observed in the data, for example the characteristic tryptamine fragments. Results were further refined to exclude synthetic compounds. Potential matches of fungal origin, particularly from phylogenetically-related species, were given priority.

Table 3. Table of example predicted fragment masses from tryptamines used to interpret the MS data

Structure	Fragment	Predicted exact mass
Unsubstituted tryptamine	α-cleavage	144.08132
	β-cleavage	130.06567
	- side chain	116.05002
	144 rearrangement	144.08078
	-side chain + N>C Double bond	115.05423
	-side chain + N>C Single bond	117.06988
	Indole break + N	91.04220
	Indole break + C (1 or 2 H on C)	89.03858
Loss of functional group from indole ring	α-cleavage	143.07350
	β-cleavage	129.05785
	- side chain	115.04220
	144 rearrangement	143.07295
	-side chain + N>C Double bond	114.04640
	-side chain + N>C Single bond	116.06205
	Indole break + N	90.03437
	Indole break + C (1 or 2 H on C)	88.03075
Fragmented indole-O-R	α-cleavage	159.06841
	β-cleavage	145.05276
	- side chain	131.03711
	144 rearrangement	159.06787
	-side chain + N>C Double bond	130.05697
	-side chain + N>C Single bond	132.05697
	Indole break + N	106.02929
	Indole break + C (1 or 2 H on C)	104.02567
+ indole OH	α-cleavage	160.07624
	β-cleavage	146.06059
	- side chain	132.04494
	144 rearrangement	160.07569
	-side chain + N>C Double bond	131.04914
	-side chain + N>C Single bond	133.04914
	Indole break + N	107.03711
	Indole break + C (1 or 2 H on C)	105.03349
+ indole MeO	α-cleavage	174.09189
	β-cleavage	160.07624
	- side chain	146.06059
	144 rearrangement	174.09134
	-side chain + N>C Double bond	145.06479
	-side chain + N>C Single bond	147.08044
	Indole break + N	121.05276
	Indole break + C (1 or 2 H on C)	119.04914

By generating extracted ion chromatograms (XICs) of the masses of the known tryptamine fragments listed above, it was revealed that the various extracts and fractions analysed contained a complex mixture of apparent tryptamine-derivatives and related compounds other than acetyltryptamine (**Figure 68)**, though they were present in a lower amount than the primary metabolite.

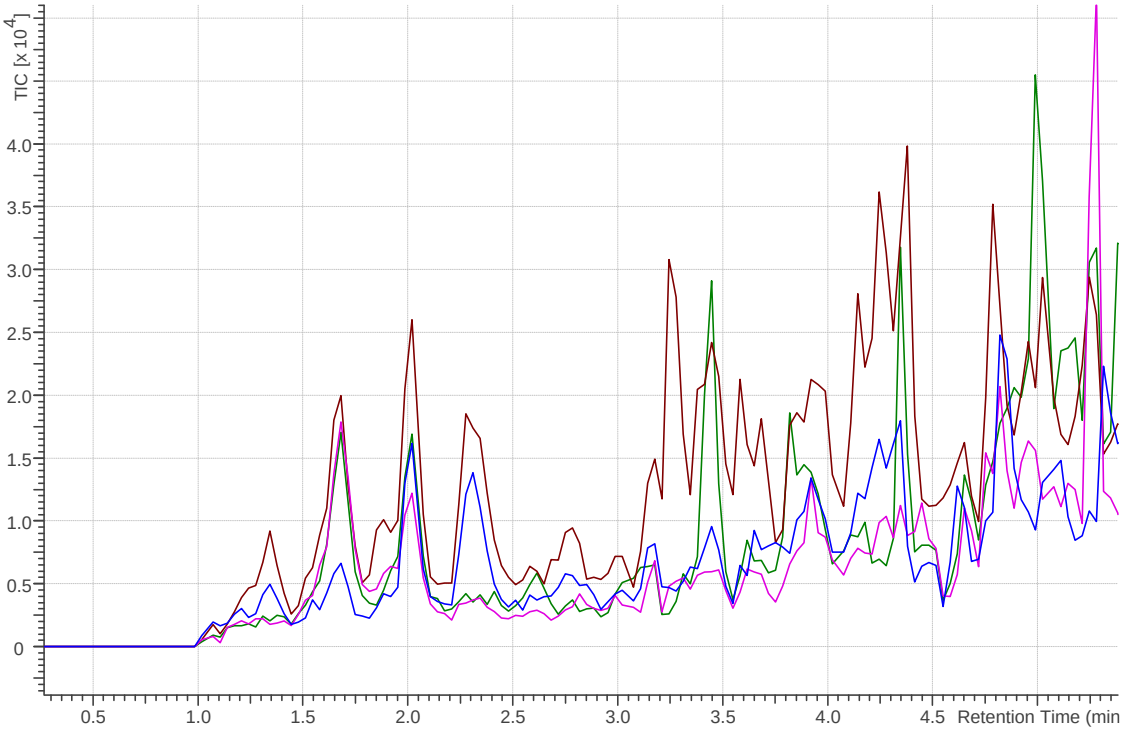
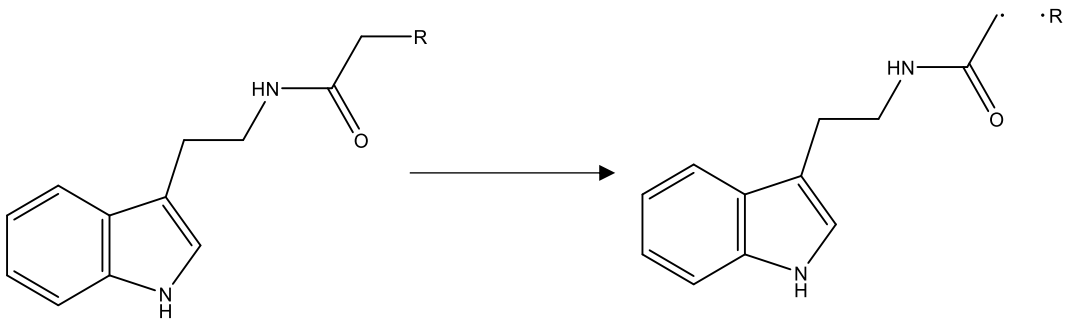


Figure 68. Extracted Ion Chromatograms (MS2, +ve mode) of the common Tryptamine fragments 144 (Green), 130 (Brown), 115 (Blue), and 159 (Pink) observed in the tryptamine-treated fermentation extracts.

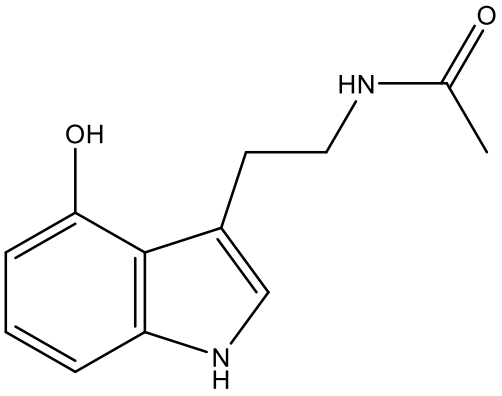
As the major biotransformation product was found to be Acetyltryptamine, the LCMS data were searched for possible acetyltryptamine derivatives by using the predicted fragment mass of *m/*z 201.10279, corresponding to acetyltryptamine – 1.007276 (**Figure 69)**.



Chemical Formula: $C_{12}H_{13}N_2O^{\bullet}$
Exact Mass: 201.10279

Figure 69. Predicted fragment with m/z 201.10279 resulting from the loss of one Hydrogen or the fragmentation of potential acetyltryptamine derivatives.

Another tryptamine derivative eluting earlier than Acetyltryptamine was tentatively identified as a Hydroxylated form of Acetyltryptamine using this approach of identifying adducts and matching the MS/MS fragment masses detected to predicted fragment masses (**Figure 70)**. Fifteen predicted adduct masses for hydroxylated acetyltryptamine were observed in the mass spectra (**Table 4)**, as well as multiple predicted fragment masses of the molecule (**Figure 71, 72 and Table 5)**, supporting the identification of this metabolite as a hydroxylated form of acetyltryptamine. However, it was not possible through this approach alone to determine at which position the hydroxyl group was on the acetyltryptamine structure, though tryptamines in nature are often hydroxylated at the 4 and 5 positions.



Chemical Formula: $C_{12}H_{14}N_2O_2$
Exact Mass: 218.10553
Molecular Weight: 218.25600

Figure 70. Structure of 4-Hydroxy-N-acetyltryptamine, one of the possible identifications

Table 4. Table of predicted adduct m/z values and observed values from the peak tentatively identified as a hydroxylated form of Acetyltryptamine

Adduct	Predicted mass	Observed mass	Difference
[M+H+K]2+	129.037982	129.05018	-0.034448
[M+2Na]2+	132.041983	132.02877	-0.018326
[M+H]+	219.112806	219.09932	-0.013486
[M+Na]+	241.094748	241.08033	-0.014418
[M+CH3OH+H]+	251.139019	251.10176	-0.037259
[M+K]+	257.068688	257.07753	0.008842
[M+ACN+H]+	260.139353	260.11140	-0.027953
[M+2Na-H]+	263.07669	263.08211	0.00542
[M+ACN+Na]+	282.121295	282.10955	-0.011745
[M+2K-H]+	295.02457	295.10567	0.0811
[M+DMSO+H]+	297.12675	297.11629	-0.01046
[M+2ACN+H]+	301.1659	301.11003	-0.05587
[M+IsoProp+Na+H]+	302.16064	302.06812	-0.09252
[2M+H]+	437.218336	437.21068	-0.007656
[2M+Na]+	459.200278	459.18893	-0.011348

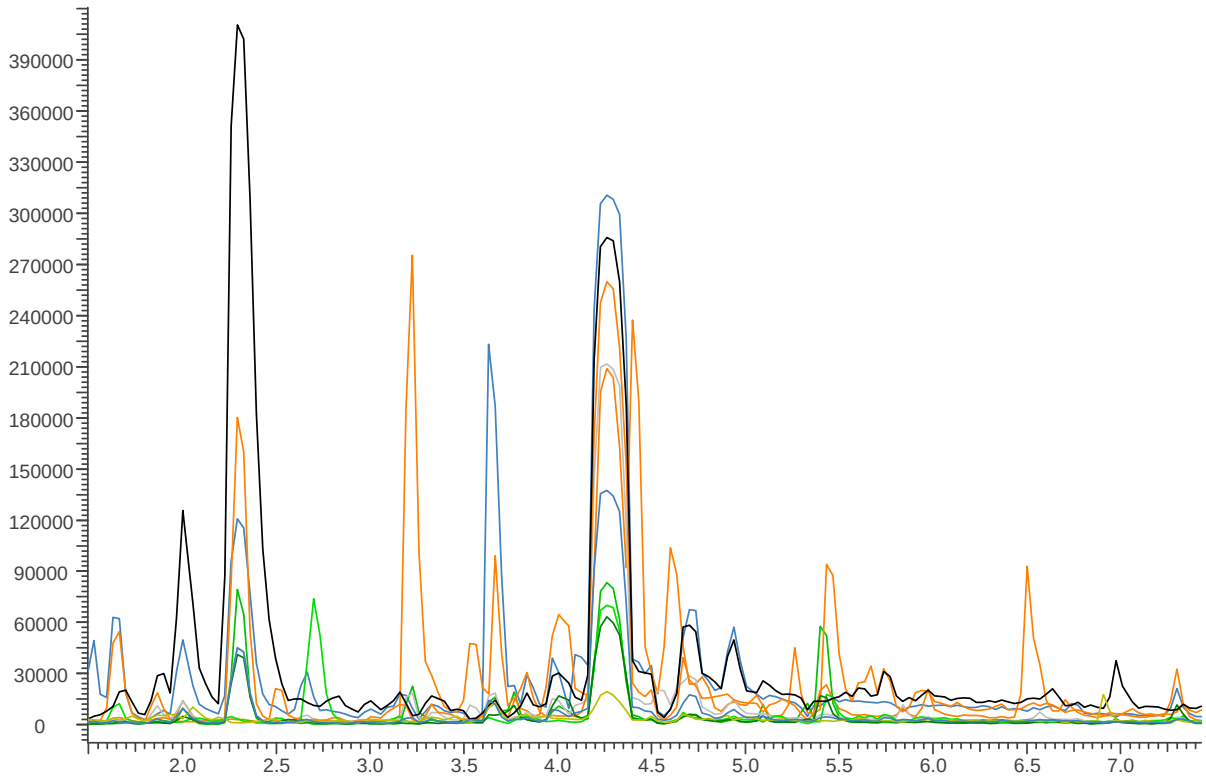


Figure 71. Extracted Ion Chromatograms of predicted 4-hydroxy-N-acetyltryptamine fragment masses. The peaks at Rt 4.25 min are well-aligned, suggesting that the fragments are from the same parent ion.

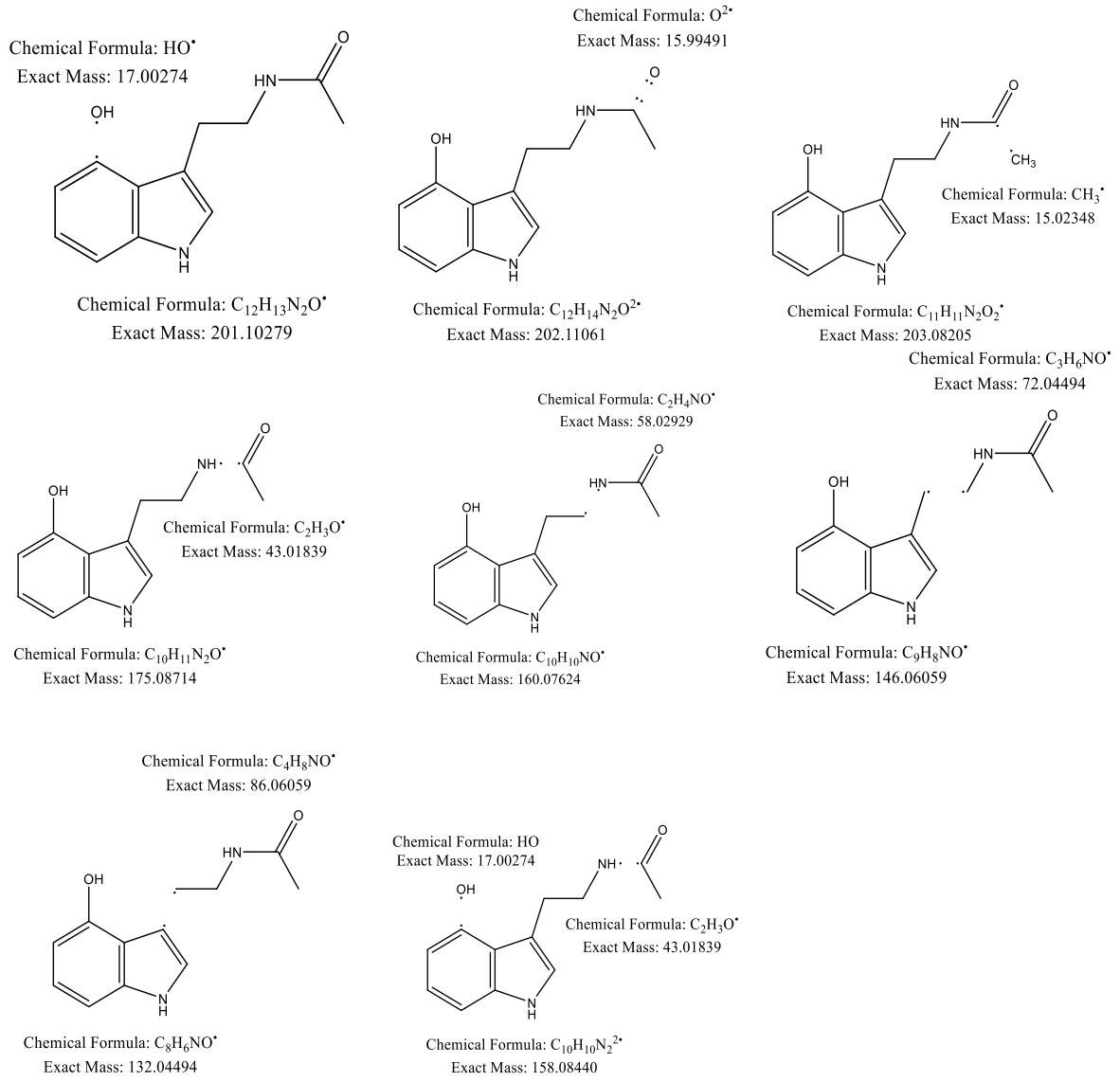


Figure 72. Structures and masses of predicted 4-hydroxy-N-acetyltryptamine fragments searched for in the MS data.

Table 5. Predicted 4-hydroxy-n-acetyltryptamine fragment masses and masses observed (MS1)

Predicted Fragment mass	Observed mass	Difference
201.10279	201.08764	-0.01515
202.11061	202.09058	-0.02003
203.08205	203.08943	0.00738
160.07624	160.06067	-0.01557
132.04494	132.02977 + 132.06353	-0.01517
158.08440	158.06945	-0.01495

3.9.4 Tentative identification of β-carbolines in extracts of tryptamine-treated fermentations via LCMS/MS

As beta-carbolines display UV absorption in the 300-400nm region, this wavelength range was extracted from the total absorption chromatograms of the various fractions from the tryptamine-treated fermentation extracts, which revealed several compounds present in the samples absorbing in this range.

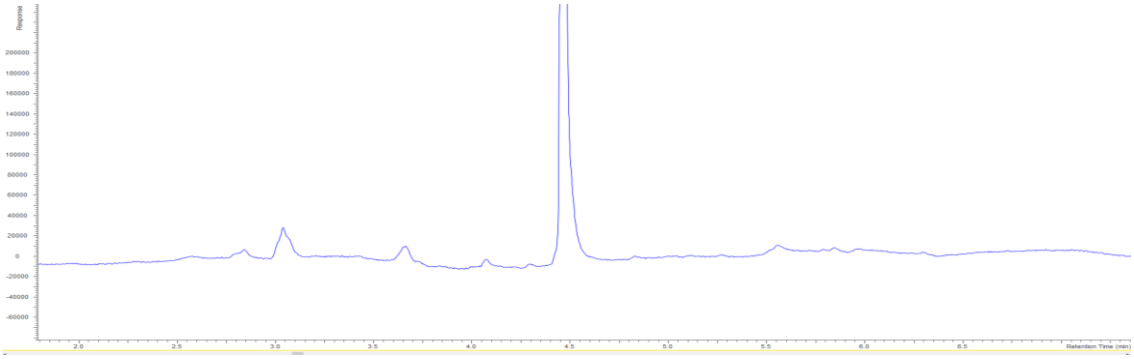


Figure 73. Extracted UV-Vis chromatogram (300-400nm) showing multiple peaks absorbing in this wavelength range in fraction CT14-15

Examination of the UV spectra of these peaks revealed UV spectra with absorption characteristic of β-carbolines.

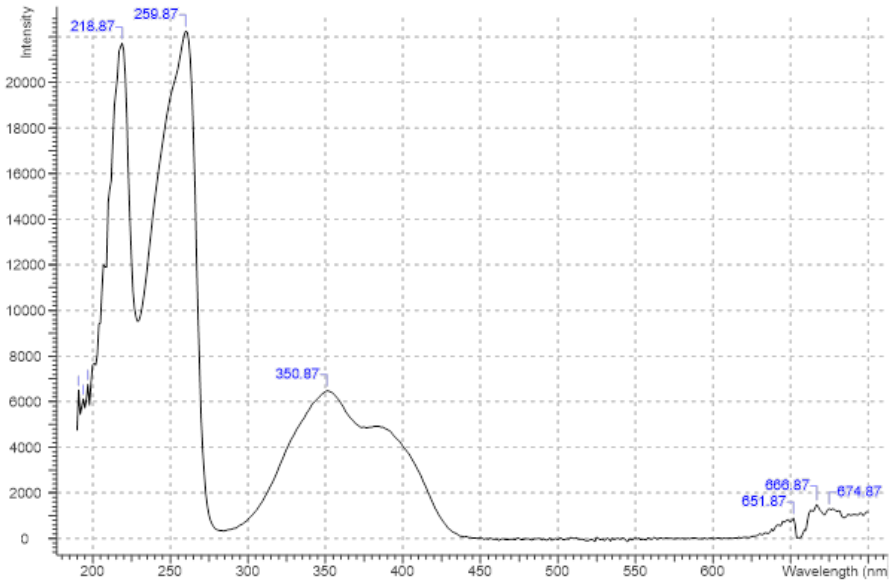


Figure 74. UV-Vis Spectrum of the main peak in fraction CT14+15, showing absorption at 219, 260, 350 and 385nm

Examination of the mass spectra of these peaks revealed ions corresponding to the predicted adducts and fragments of several known beta-carbolines. For example, masses corresponding to multiple predicted masses of Cordysinin C and D (Exact mass: 212.09496) were detected (**Table 6, Figure 75 and 77**). However, there are also masses in the spectrum which could potentially correspond to other related compounds, for example the m/z 287 may represent Perlolyrine (M+Na) or Sphecoline B (M+H), and the m/z 229 peak could correspond to Sphecoline A.

Table 6. Predicted and observed adduct masses (m/z) of Cordysinin C and D

Adduct	Predicted m/z	Detected m/z
[M+H]+	213.102236	213.0887
[M+Na]+	235.084178	235.0692
[M+K]+	251.058118	251.037
[M+ACN+H]+	254.128783	254.115

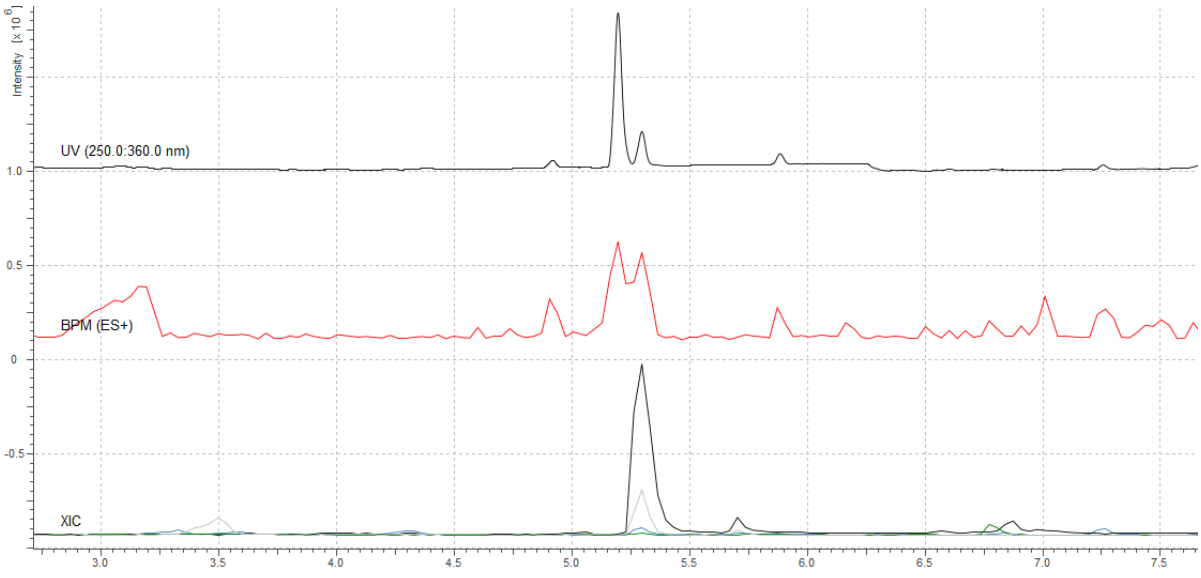


Figure 75. Top: Extracted HPLC chromatogram (250-360nm). **Middle:** BPM trace. **Bottom:** Extracted ion chromatograms of the masses shown in **Table 6**, with two peaks visible at 5.3 min and 5.7 min, corresponding to predicted masses of Cordysinin C and D adducts

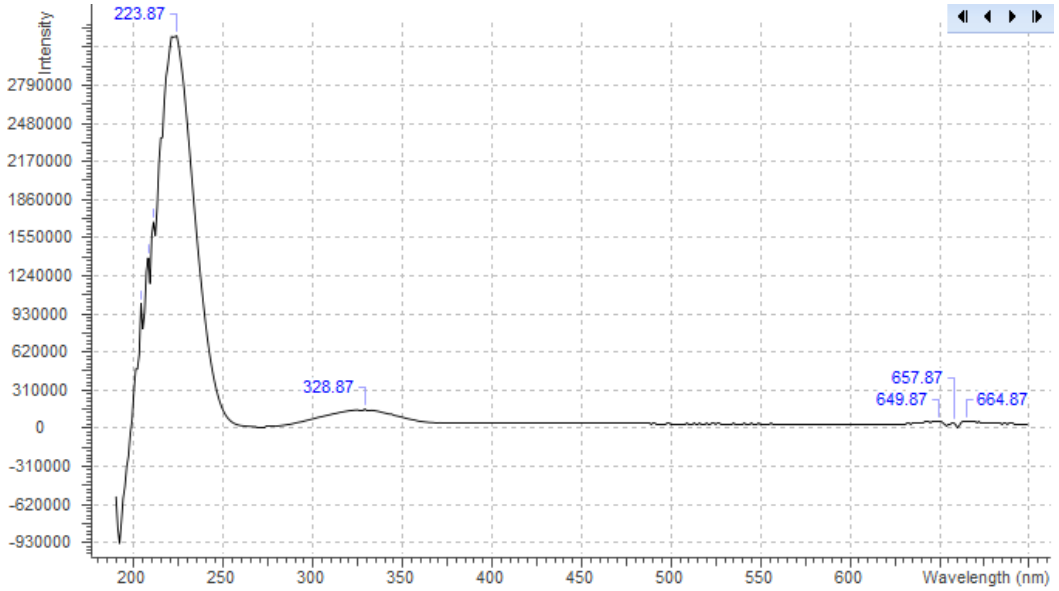


Figure 76. Averaged UV spectrum of the peak at 5.3 min (**Figure 75**) tentatively identified as Cordysinin C or D

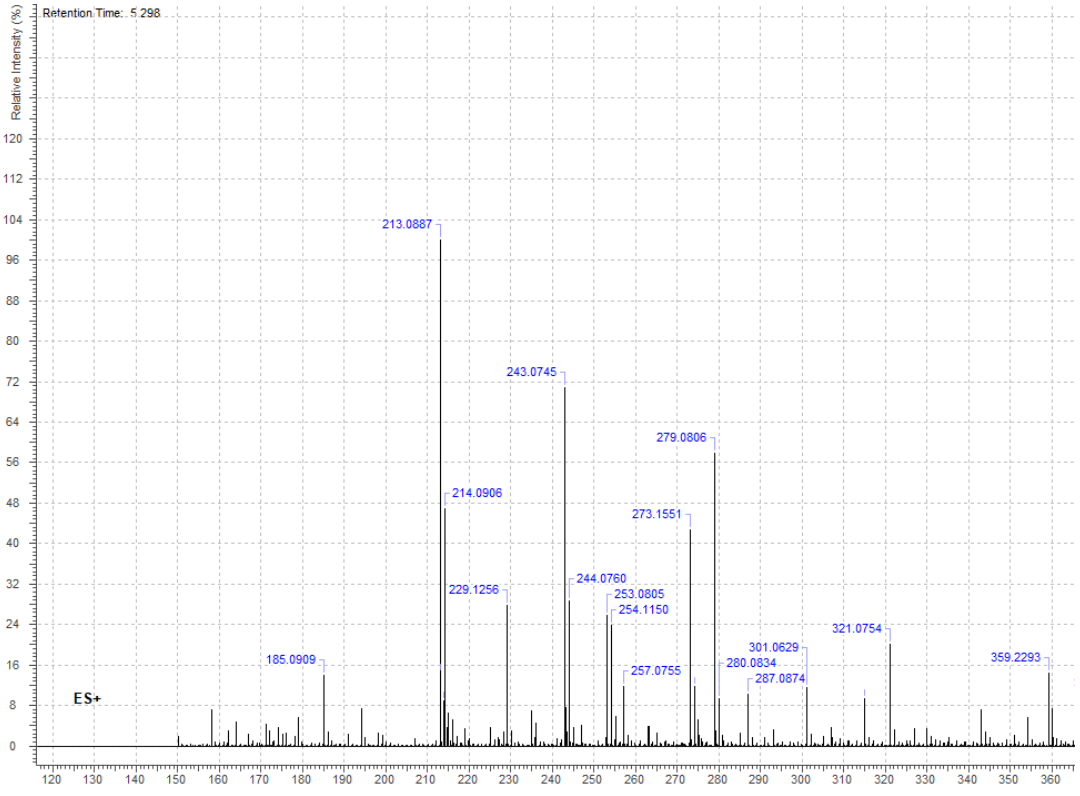


Figure 77. Averaged mass spectrum (MS1, +ve mode) of the peak at 5.3 min (**Figure 75**), showing several masses corresponding to Cordysinin C and D

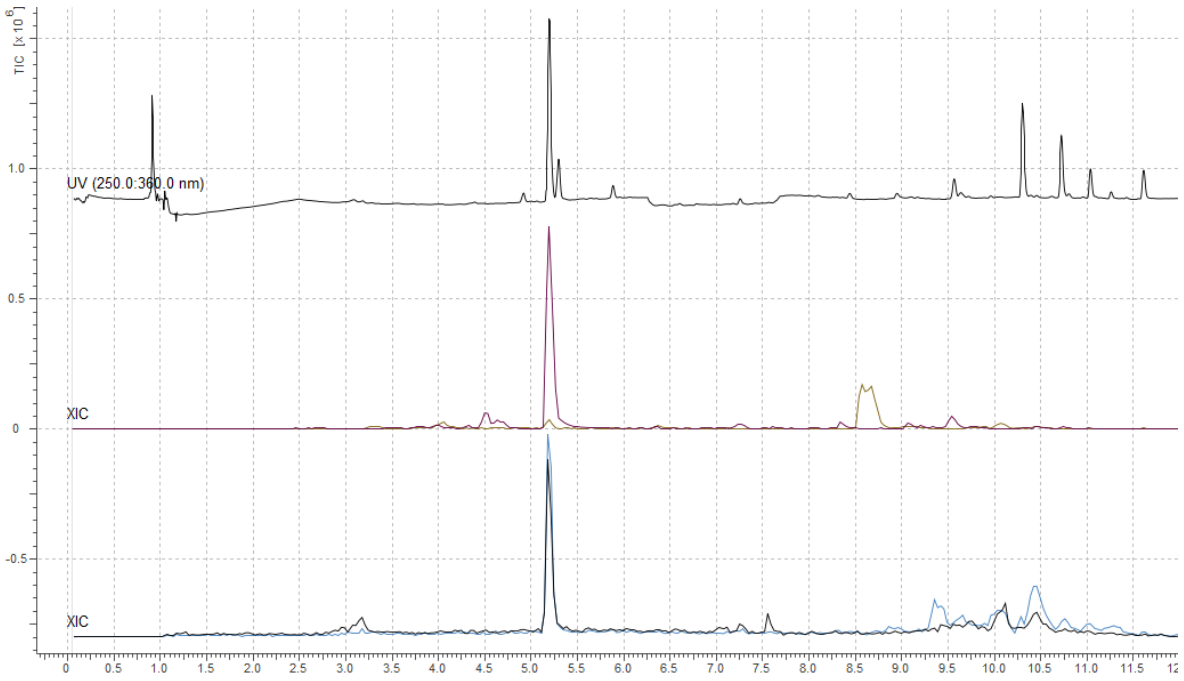


Figure 78. **Top**: UV chromatogram (250-360nm). **Middle**: Extracted Ion Chromatograms of *m/z* 243 and *m/z* 265 (Corresponding to Sphecoline E [M+H]+ and [M+Na]+ respectively), **Bottom**: Extracted Ion Chromatograms of *m/z* 225 (Loss of Hydroxyl), and *m/z* 199 (Loss of Acetyl group).

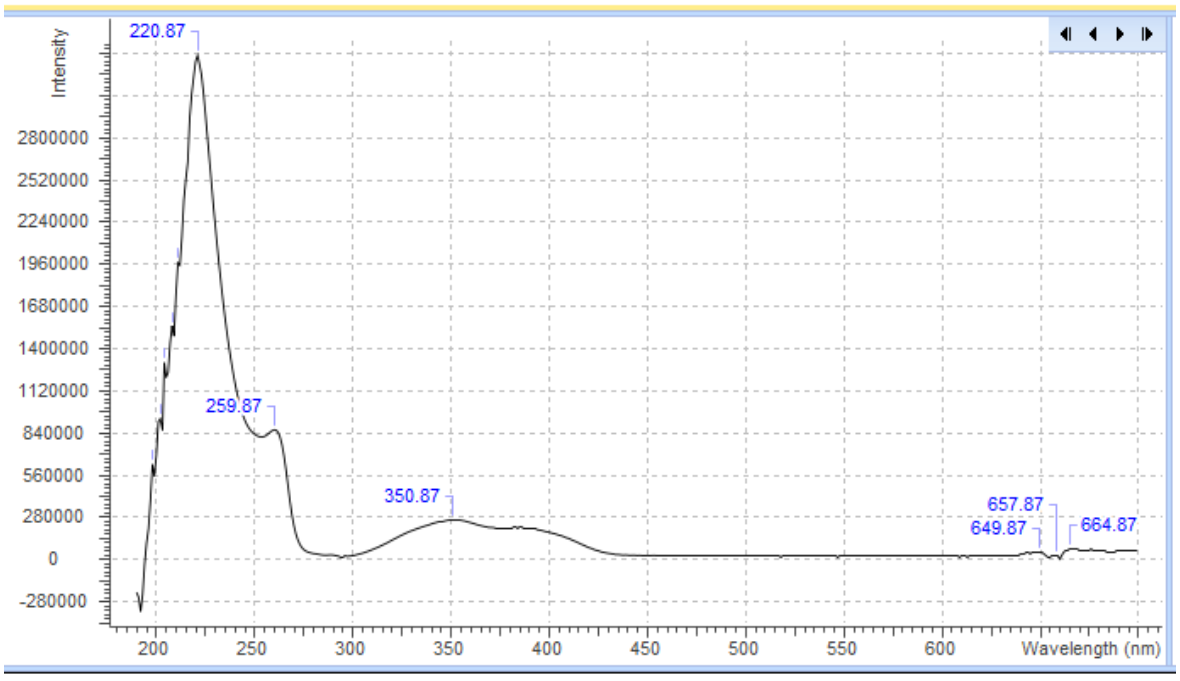


Figure 79. UV profile of the peak tentatively identified as Sphecoline E

Natural products database searches for these masses return several known compounds from various classes including simple substituted tryptamines, ergolines, and several β-carbolines. There are several non-fungal β-carbolines (for example those derived from marine sponges, bacteria, and plant species), fungi-derived β-carbolines, and specifically several known β-carbolines from various entomopathogenic species.

For *m/z* 240.07247 there are 3 compounds all with the predicted exact mass of m/z 240.08988 (+0.01741) and the formula $C_{14}H_{12}N_2O_2$**,** these are Gibellamine A (from the spider-pathogenic fungus *Gibellula gamsii*), Iranginin A (from the ant-pathogenic fungus *Ophiocordyceps irangiensis*) and Sphecoline D (from the wasp-pathogenic fungus *Ophiocordyceps sphecocephala*). All three are analogues of 1-acetyl-beta-carboline, Iranginin A and Sphecoline D are positional isomers, with a methoxy group at positions 6 and 3 respectively, while Gibellamine A is a functional isomer, with a hydroxymethyl group at position 3.

There are 2 known beta-carbolines derived from entomopathogenic fungi with the exact masses 242.06914 and 242.10553, Sphecoline E with the formula $C_{13}H_{10}N_2O_3$ (from the wasp-pathogenic fungus *Ophiocordyceps sphecocephala*) and Cordysinin E with the formula $C_{14}H_{14}N_2O_2$ (from the traditional Chinese medicine *Cordyceps sinensis*) respectively. The observed mass for [M+H]+ (*m/z* 242.09247) is slightly closer to the predicted mass of Cordysinin E (+0.01306) than Sphecoline E (-0.02333).

As these beta-carbolines are structurally similar, likely derived through similar biosynthetic pathways, and all known metabolites of closely related hypocrelean entomopathogenic fungi, it is likely that both the m/z 240 and m/z 242 compounds are present. Their close structural similarity complicates chromatographic separation using standard methods, which adds ambiguity to assignment of source and MS/MS fragments to each parent ion as it is not clear from the data which fragments arise from which parent ion. Nevertheless, there are several source and MS2 fragments which support the identification of these compounds as two of the known beta-carbolines with these masses derived from similar fungal species.

The major MS2 fragments present in the averaged spectrum (**Figure 80, Top**), in order of intensity, are 182.04356, 181.03391, 154.02588, 195.04599, 115.02239, 167.02082, 206.04041. 193.03185. Other significant ions present in lower abundance include 115.02239, 127.02030 and 128.03152. The major MS1 ions presumed to result from in source fragmentation from the proposed β-carbolines (**Figure 80, Bottom**) are, in order of intensity, 159.07521, 225.08588, 160.06185, 201.08517, 169.06069, 181.05983, 132.06403, and 199.06963. Other significant ions in the source spectrum include 154.04794 and 115.03886.

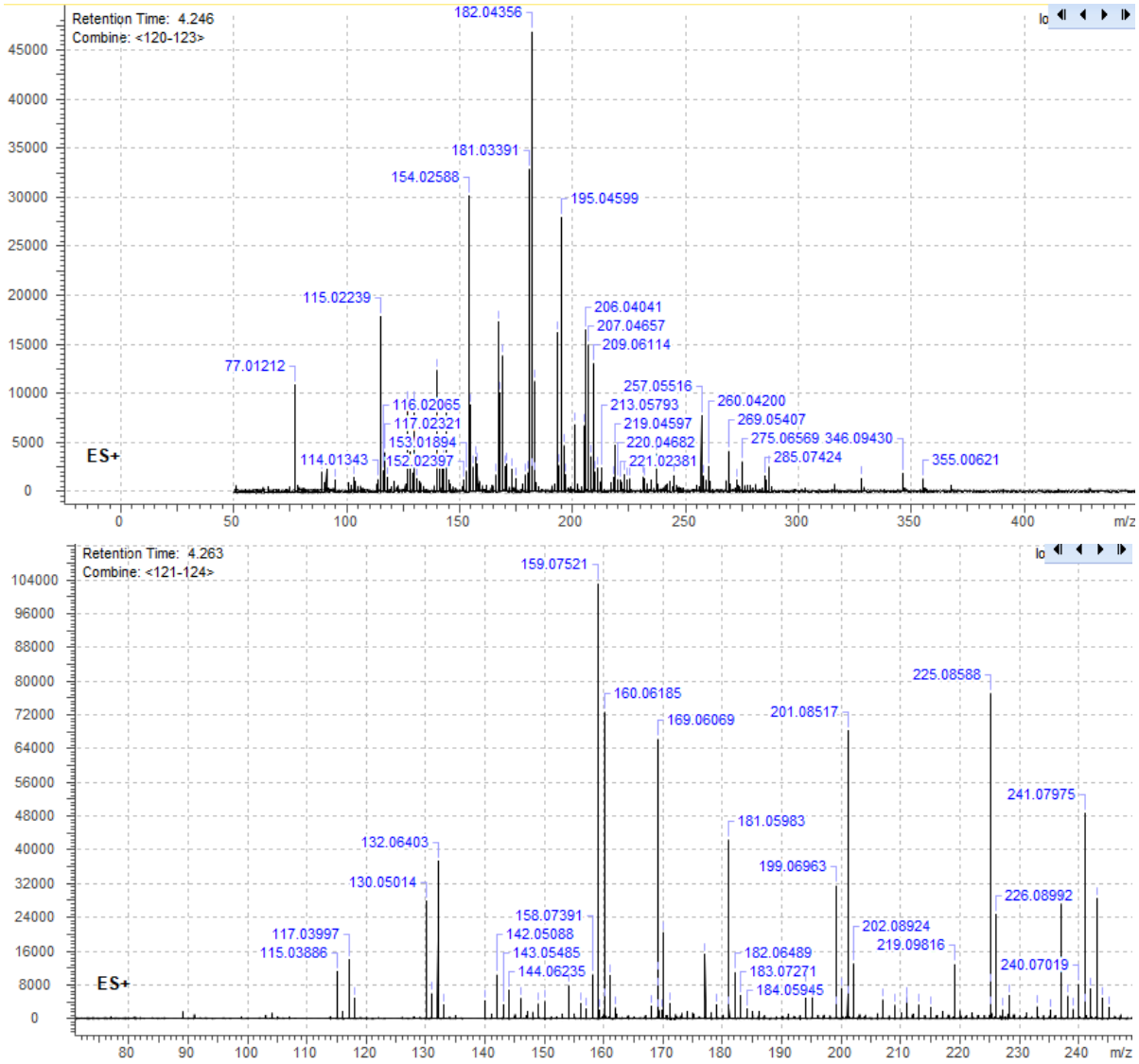


Figure 80. Averaged MS1 spectrum (Top) and averaged MS2 spectrum (Bottom) of a peak showing multiple tryptamine fragments potentially resulting from one of the related β-carbolines

Unfortunately there are not much published data on the fragmentation of these beta-carbolines, however many of these fragment ions can be predicted through *in-silico* fragmentation of the structures of Cordysinin E, Gibellamine A, and Sphecoline E, through removal of functional groups including hydroxyls, carbonyls, methyls, acetyl, cleavage of the side chain in the case of Cordysinin E, removal of CH or C2H2 from the indole ring, and through breakage of the pyridine ring in positions analogous to the alpha and beta cleavage commonly seen with tryptamines. Several of the fragments are also possible through fragmentation of Gibellamine B, or theoretical isomers and analogues of it.

In the MS1 spectrum, m/z 225.08588 is likely to be the result of in-source fragmentation, and all 5 of the possible known entomopathogenic beta-carbolines could fragment to a 225 ion. From Gibellamine A, Iranginin A and Sphecoline D, loss of NH from the indole ring results in 225.07898 (-0.0069). Loss of CH3 from the acetyl group of Gibellamine A, or loss of CH3 from the methoxy or acetyl groups of Iranginin A or Sphecoline D, all result in 225.06640 (-0.01948). From Sphecoline E and Cordysinin E, loss of OH from results in a fragment of 225.10279 (+0.01691). *In-silico* loss of NH from the indole ring of Gibellamine A, Iranginin A or Sphecoline results in the closest mass to that observed, however this fragment is less feasible than loss of OH or CH_3. Several of the possible structures contain two hydroxyl groups, loss of which would result in m/z 208, which is present in low abundance.

Several combined fragmentations could result in m/z 209, being loss of CH_3O (removal of methoxy or CH_3 and O from Acetyl group. It does not appear to be possible for Cordysinin E to produce a 209 fragment through simple fragmentation, though it may be possible through a McLafferty rearrangement. Loss of CH3O from the acetyl group of Gibellamine A, Sphecoline D, Iranginin A, or loss of O and OH from Sphecoline E, all result in 209.07149. Loss of methoxy or CH3O from the acetyl group of Sphecoline D or Iranginin A also results in 209.07149. The observed mass of the fragment (209.06114 in MS2 and 209.09108 in MS1 is a close match to these predicted fragments.

Gibellamine A is unique amongst the 5 known structures as it is the only structure containing a hydroxymethyl group and is the only structure which contains both an

acetyl group and one hydroxyl group. This means that Gibellamine A could produce fragment masses which are possible with the other compounds through unusual fragmentation, but less likely than with Gibellamine A. For example, loss of the OH results in 223.08714 (observed 223.03680), loss of the OH and the carbonyl from the acetyl group results in 207.09222 (observed 207.04657) and loss of CH3 and OH results in 208.06366 (observed 208.05010).

Loss of both hydroxyls from Cordysinin E results in 208.10005, loss of H2O and carbonyl from Sphecoline E results in 208.06366, loss of carbonyl, NH and H from Sphecoline D or Iranginin A results in 208.07624. An ion of m/z 208.05010 is present in the averaged spectrum which likely corresponds to one of these fragments. The 208 fragment from Cordysinin E is more likely to happen than with Sphecoline E, D, or Iranginin A, however the difference between the predicted fragment mass and those observed is greater for Cordysinin E (+0.04995) than it is with Gibellamine A (+0.01356). In the purified sample there is a good match for Sphecoline E (**Figure 81**) at Rt 9.5 min, where *m/z* 243.0713 and *m/z* 265.0575 are observed (**Figure 82**), corresponding to the predicted [M+H] and [M+Na] values of *m/z* 243.076416 (+0.005) and *m/z* 265.058358 (+0.0008). As well as this, multiple fragment masses are observed which can be predicted from fragmentation of the structure, and the averaged UV spectrum of the peak (**Figure 83**) displays absorption in the 300-420nm range, characteristic of a β-carboline.

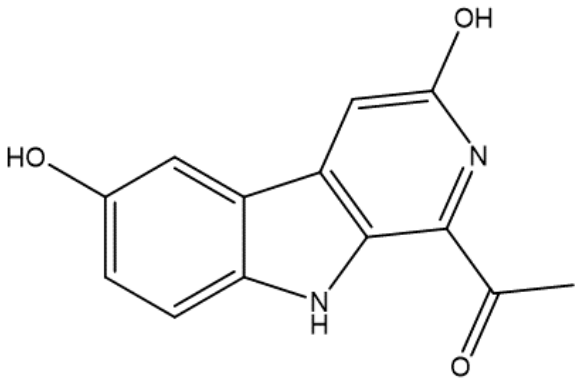
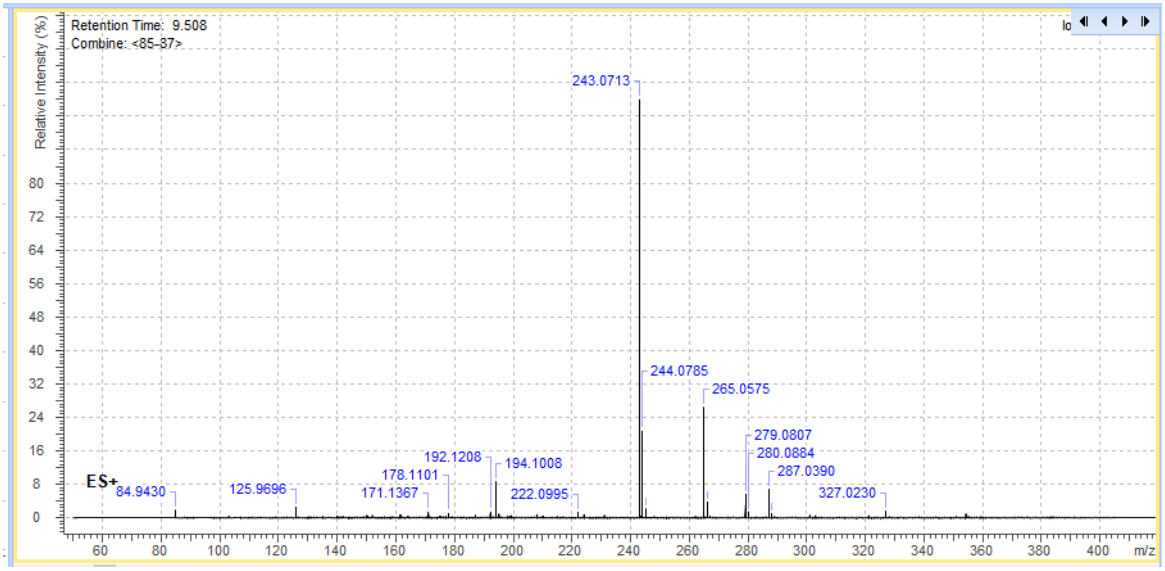


Figure 81. Structure of Sphecoline E, a potential match for one of the compounds detected in the tryptamine-treated fermentation extracts

<u>Parent mass:</u> 243.0719

MS1 spectrum



MS2 spectrum

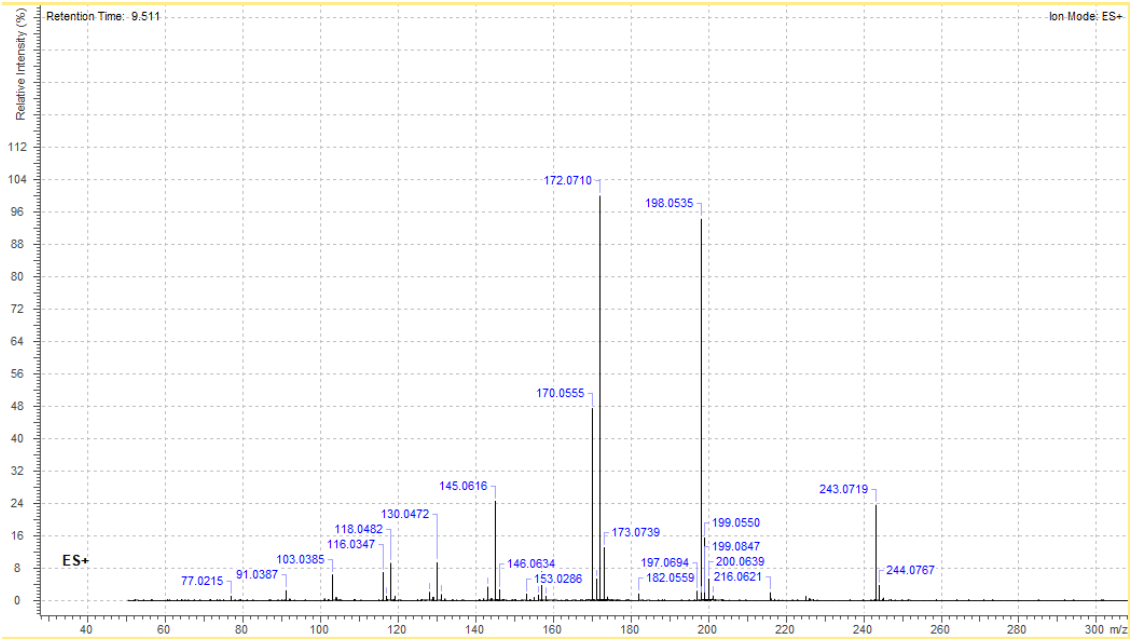


Figure 82. MS1 (+ve) (**Top**) and MS2 (**Bottom**) mass spectra of the peak at Rt 9.5 min, showing masses matching the predicted adduct masses of Sphecoline E, as well as multiple predicted fragment masses .

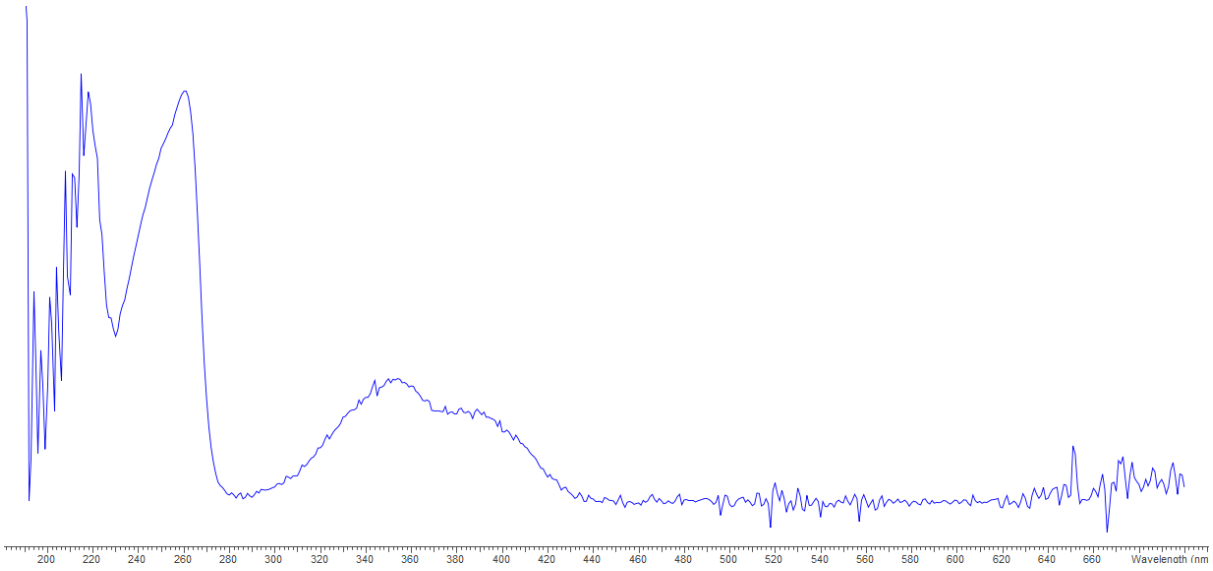


Figure 83. Averaged UV-Vis spectrum of the peak at Rt 9.5 min potentially identified as Sphecoline E, showing characteristic β-carboline absorption in the 300-420nm range

3.10 Combined treatment with tryptamine, vorinostat and sirtinol

The addition of tryptamine (1mM), vorinostat (100µM), and sirtinol (50µM) to the fermentation media resulted in the induction of metabolites not detected in fermentations treated with tryptamine alone and not corresponding to vorinostat of sirtinol (**Figure 84**). Peaks were observed in the Total Absorbance Chromatogram from the tryptamine and epigenetic modifier treated fermentation in the 14-15 minute region, with the major peak eluting at 14.6 minutes (**Figure 84 A**). The averaged UV spectrum of the major peak (**Figure 84 B**) showed absorbance at 300-350nm.

The MeOH blank-subtracted averaged mass spectrum of the major peak suggests a mass of 240.1133 based on the observed $[M+H]^+$ *m/z* 241.1205 and $[M+Na]^+$ *m/z* 263.1031 (**Figure 84 D**).

Extracted ion chromatograms from both treatments revealed the absence of the compound in the tryptamine-only fermentation (**Figure 84 C**), as well as a secondary peak and a (left) shoulder, which may suggest co-elution of several metabolites. The highest abundance ions seen in the MS (*m/z* 137.0548, *m/z* 120.0277 and *m/z* 105.0543) are all in alignment with the $[M+H]^+$ and $[M+Na]^+$ adducts.

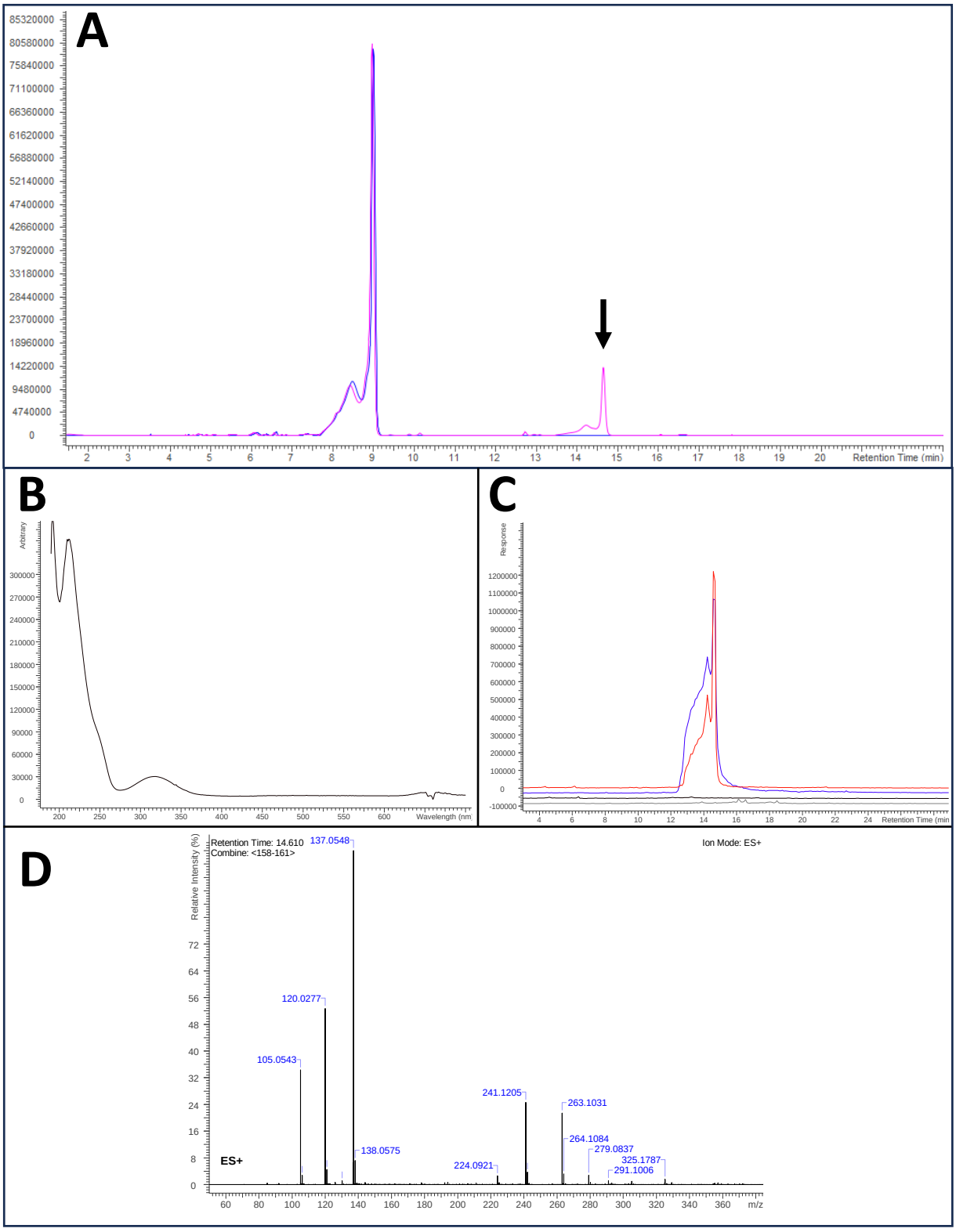


Figure 84. A - HPLC TACs (190-700nm) of *Gibellula* isolate 1 YM fermentation ethyl acetate extracts, treated with 1: tryptamine-only (1mM) (Blue chromatogram), and 2: Tryptamine (1mM), vorinostat (100µM) and sirtinol (50µM) (Pink chromatogram). Additional induced peaks can be seen in the epigenetic modifier treated sample at retention time (RT) 13.5-14.8 minutes (Arrow), with the largest at 14.6 minutes. The major peak in both samples (RT 9min) was identified as Acetyltryptamine. B – Averaged UV spectrum (190-700nm) of the main induced peak in the epigenetic modifier-treated fermentation. C - Extracted Ion Chromatograms (*m/z* 241.1205, m/z 263.1031) from tryptamine-only (Grey and Black, respectively) and epigenetic modifier-treated fermentations (Blue and Red, respectively), showing the absence of both ions in the tryptamine-only fermentation. D – Averaged mass spectrum of the induced peak at 14.6 minutes, after subtraction of all ions detected in the MeOH blank.

Subtraction of the MeOH Blank (background) Total Ion Chromatograms from both the tryptamine-only fermentation and the combined treatment, extraction and comparison of the Base Peak Chromatograms, revealed additional peaks present in the epigenetic modifier treated fermentation not visible in the TAC (**Figure 85**).

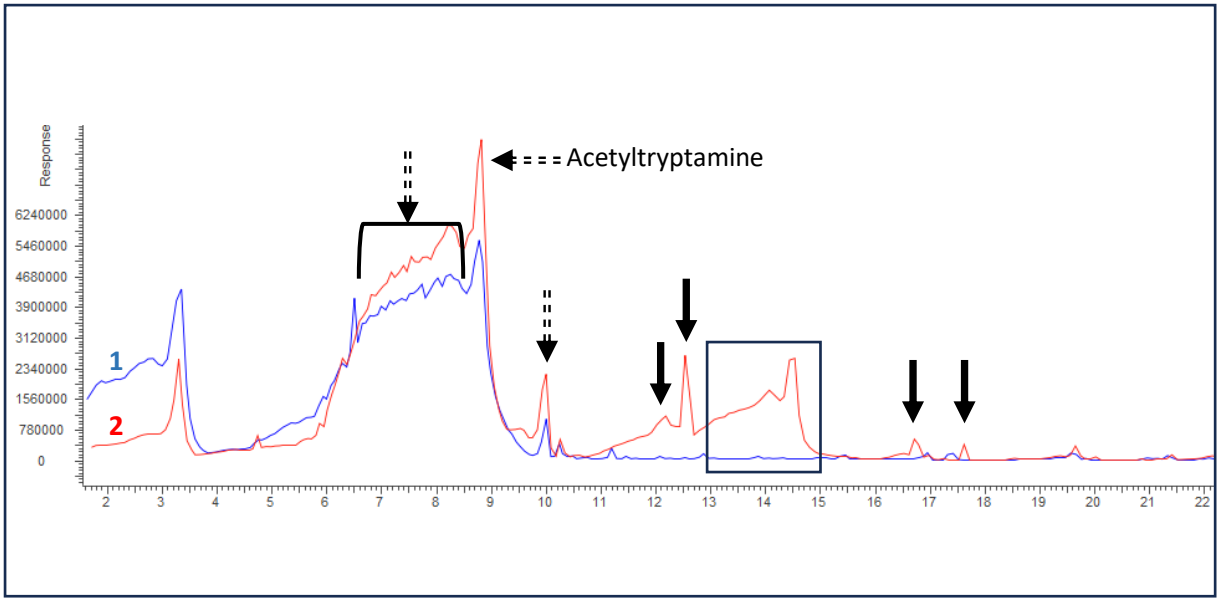


Figure 85. Base Peak Chromatograms (BPCs) (MeOH Blank subtracted) of **1 (Blue):** Tryptamine-only fermentation, and **2 (Red):** Tryptamine, vorinostat and sirtinol-treated fermentation. Additional induced peaks that are not visible in the UV chromatogram **(Figure 84 A)** can be seen in **2 (Arrows),** as well as increased abundances of ions present in **1** (Dashed arrows). Box shows induced peaks seen in **Figure 84.**

3.11 Discussion

Alkaloids represent a large group of natural products present in nature, many of which have diverse pharmacological properties and potential uses in medicine. Throughout history, natural alkaloids have been utilised in medicine, with many still in use. The alkaloids still represent a potential source of novel natural products with medicinal potential.

In this work, *Gibellula* sp. was fermented in the presence of tryptamine added to the culture media, in order to investigate the potential for the fungus to metabolise it into new natural products or ones not currently known to occur in the genus.

The primary product was determined to be acetyltryptamine, which has been previously detected in a variety of microbial species including actinomycetes [122], marine fungi [123], endophytic Fusarium and Aspergillus spp. [56, 124], Penicillium spp. [125]. Acetyltryptamine has also been produced in *Fusarium* sp. lacking a repressive histone modification [126] , as well as via co-culture of different bacterial species [118]. Though it is a known compound, it has not previously been reported from any *Gibellula* species, and there is no currently published on natural products biotransformation from the genus.

The only beta-carbolines (or other indole alkaloids) currently known to occur in *Gibellula* spp. are Gibellamine A and B, though there have been at least 17 related compounds discovered from phylogenetically-related entomopathogenic fungi described previously. In this work, it was hypothesised that addition of tryptamine to the culture medium may result in the production of Gibellamine A and/or B, or related compounds by providing an excess of a plausible precursor in their biosynthesis. The results of the LCMS data interpretation suggest that although the primary product was acetyltryptamine, a number of other indole alkaloids including multiple β-carbolines were also produced. Unfortunately, it was not possible to obtain a great enough yield to unambiguously confirm the identity of the metabolites, and the close structural similarity of the compounds makes it difficult to separate them using standard chromatographic techniques, as well as to determine the position of shared functional groups between the possible compounds. Nevertheless, the data suggests that the biotransformation approach undertaken may be a viable method of producing related

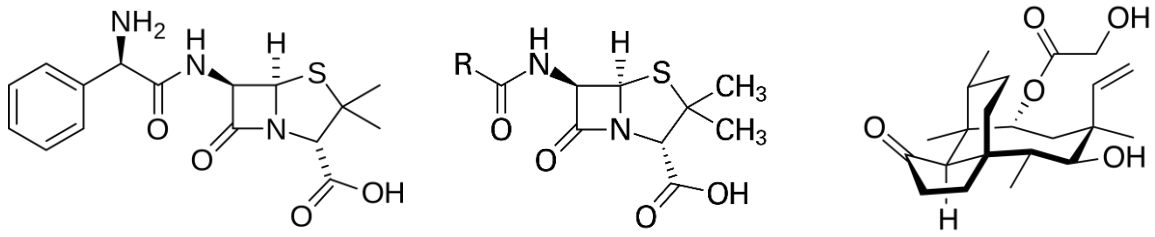
substances, which could potentially be novel or at least not previously found from the species in use.

Chapter 4

Screening wild fungi and fermentation extracts for novel metabolites and bioactivity

4.1 Introduction

Natural products have played an important role in the history of antibiotic drug discovery [127], and the investigation of microbial natural products is responsible for the discovery and development of many of the most important antibiotics still in use today, which have and continue to help save the lives of many people suffering from potentially lethal microbial infections. Fungi in particular have proven to be especially rich sources of new antibiotics (**Figure 86**) which have had a major impact on medicine, the most famous example being the β-lactam class of antibiotics following the discovery of penicillin, which is still the most commonly used class of antibiotics used to combat infection with Gram-negative bacteria [128].



Ampicillin | Penicillin core structure | Pleuromutilin

R = variable group

Figure 86. Examples of antibacterials derived from fungi.

However, the rate of novel antibiotic drug discovery has slowed, while many of the microorganisms affected by them have been becoming resistant to their effects, leading to the phenomenon of antimicrobial resistance, which is considered as a current global crisis in medicine [129]. The mechanisms of antibiotic resistance have been widely researched [130, 131], however there is still an urgent need for novel antibiotic drug discovery in order to tackle the problem. Considering the slowing down of novel antibiotic discovery, many avenues have been explored with this aim in mind

employing more sophisticated techniques such as genome mining [132], the use of gene editing to engineer novel antibiotic biosynthetic machinery [133], and the use of CRISPR and nanotechnology approaches to disrupt genes involved in antimicrobial resistance [134].

Although many of the already known antibiotics have been derived from fungi, a large amount of them have been derived from ascomycetes, with fewer antibiotics discovered from the Basidiomycota, which encompasses most species of mushroom-forming fungi. However, the biological and chemical diversity present within the Basidiomycota still represents a very promising potential source of novel antibiotics, which may be considered as largely untapped [24]. Although less antimicrobial compounds have been discovered from basidiomycete mushroom-forming fungi, there are still many mushroom species which have been investigated and found to produce antimicrobial metabolites or have antimicrobial efficacy [24, 55, 135, 136].

For example, in an antimicrobial screening study, 317 basidiomycete cultures were isolated from 204 species, grown in liquid fermentation and the culture filtrate extracted with methanol. 109 of the crude MeOH extracts had antibacterial and/or antifungal activity.

Another promising reason to continue exploration of fungi is the possibility of discovering bioactive natural products which although not antimicrobial themselves, can restore the efficacy of antibiotics against resistant bacteria [137].

One of the groups of natural products which are potential promising sources of novel antibiotics are the alkaloids [43], and some fungi-derived alkaloids have been shown to have antimicrobial effects, for example some alkaloids derived from marine fungi have shown to have efficacy against gram negative bacterial species [57, 61]. Despite this, it seems that fungal alkaloids from mushroom-forming basidiomycetes is a neglected area of research which may prove fruitful in the search for novel antibiotics.

Considering that microbial species can have silent BGCs when cultivated in laboratory conditions, it could be argued that collection of wild material such as mushrooms may well contain compounds that are not produced and detected if the mycelium of the species is grown in the lab, as in the wild the organism is exposed to vastly different environmental and nutritional conditions, as well as exposed to other microbial, animal and plant species with which it may have complex inter-species interactions affecting

its chemistry. Fungi which grow on multiple tree species may be exposed to different nutritional and biochemical factors which result in biochemical changes within the fungus required to survive on that particular tree host.

Some species which can be grown in laboratory conditions are known to produce different metabolites at different stages of the organism's lifecycle. An example of this can be seen with the *Hericium* genus, in particular *Hericium erinaceus* (Lions mane) is a widely cultivated and consumed edible mushroom known to produce bioactive metabolites differentially in the mycelium and the fruiting body; the erinacines being produced in the mycelium and the hericenones produced in the fruiting body [24, 25].

As well as this, many microbial species cannot be easily cultivated in a laboratory setting, for example the ectomycorrhizal fungi, which is a factor that complicates the investigation of these species as they have to be found from the wild, as well as this many of these species are rare and difficult to obtain in amounts large enough amounts, which combined with the commonly low-yield in metabolite isolation attempts makes it difficult to isolate and elucidate the structures of metabolites from them.

It is also thought that there are a vast number of undiscovered fungal species, which consequently remain completely unresearched. As well as this, even many of the well-known fungi including European mushroom species have still not been chemically examined, or at least there have been few to no metabolites isolated from them to date, and they could be considered as neglected species.

Some of the chemical investigation which has been done on particular mushroom species was conducted decades ago using methodology such as thin layer chromatography, with seemingly little to no additional work published in more modern times. In addition to this, natural products researchers often target the easiest metabolites to isolate, known as the 'low hanging fruit', leaving more difficult to isolate or less abundant metabolites uncharacterised. In the case of these fungi, it is logical to assume that as methodological advances have been made in cultivation, extraction, purification, chemical analysis and biological activity assay technologies, revisiting these species utilising modern methods and equipment could potentially lead to the discovery of metabolites not found in earlier work.

In many of the studies published examining the composition or biological activity of natural materials such as mushrooms and plants, crude extracts such as methanol,

acetone, hexane or ethyl acetate extracts are utilized. This is a limitation which feasibly results in samples in which potentially bioactive metabolites will be present in the extracts, but in amounts that are below the limit of detection of analytical instruments, as well as in concentrations too low to have an effect in biological assays.

More exhaustive and sophisticated multiple solvent extractions and acid-base extractions could obtain more compounds than simple single solvent crude extracts, and additionally partially purify metabolites of interest and remove common metabolites known to cause false positive results in bioassays, such as linoleic acid and other fatty acids.

With these factors in mind, several uncommon and chemically interesting native British mushroom samples were investigated to examine their alkaloidal content, and assess whether they possessed antibacterial components. The species tested are briefly described below (Sample collection details in Appendix 7).

The *Amanita* genus is one of the most famous and also the most feared amongst Britain's native genera, but also around the world, due to the inclusion of several of the most deadly poisonous mushroom species including *Amanita phalloides* (the death cap) and *Amanita virosa* (The Destroying Angel) (**Figure 87**). The genus is also well-known for containing one of the most widely recognised and famous mushroom species, *Amanita muscaria*, which produces iconic large, bright red caps covered with small white 'warts' (remnants of the universal veil which surrounds the mushrooms as they begin to grow), and has been one of the most culturally impactful mushroom species, often depicted in art and a favourite of fungal photographers. The members of the *Amanita* genus are mycorrhizal fungi, often forming mycorrhiza and growing with Birch, Beech, Oak, and Coniferous tree species in the Northern hemisphere.

Figure 87. Examples of several iconic *Amanita* species found in Britain. A – *Amanita virosa* (The Destroying Angel), one of the deadliest toxic mushroom species present in Britain along with *Amanita phalloides*. **B – *Amanita muscaria* (The Fly Agaric),** a mushroom with a long and ongoing history of use as an inebriant, and in folk medicine in Northern Asia. **C – Amanita phalloides (The Death Cap)**, perhaps the most famous of the deadly poisonous mushroom species worldwide, is responsible for the majority of fatal mushroom poisonings.

Although many *Amanita* species are edible, including the highly regarded *Amanita caesarea,* it does contain several potentially deadly toxic species and is responsible for many of the fatalities following mushroom ingestion. The *Amanita* are chemically talented, the most well-known metabolites present in several species within the genus being the amatoxins (**Figure 88**), as well as ibotenic acid and muscimol. The amatoxins are peptides, and cause damage to the liver and kidneys through their inhibition of RNA polymerase II.

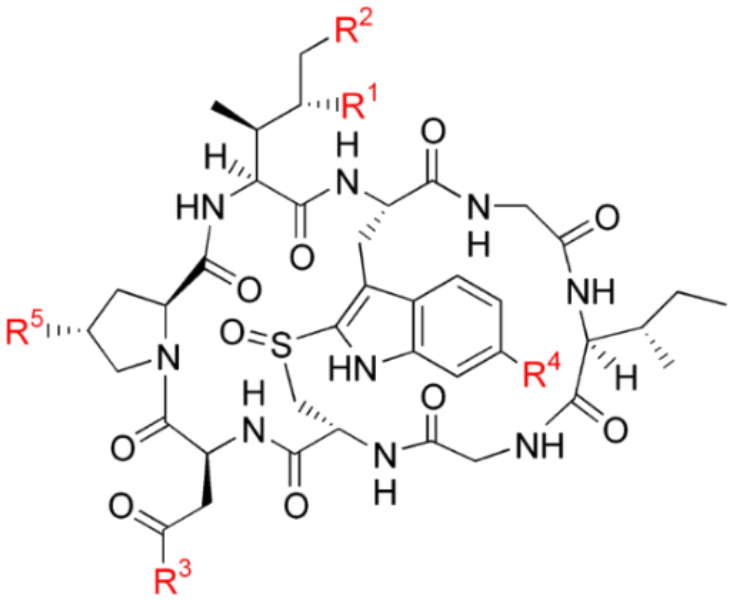


Figure 88. The backbone Structure of the Amatoxins (Black), with the position of the variable side groups marked (R^N).

Though much is known about the chemistry of several *Amanita* species, modern chemical investigation is lacking, and for several species within the genus, particularly those which are less common such as *Amanita strobiliformis*, there are currently no known metabolites.

Amanita pantherina (**Figure 89)** is an uncommon species in the UK though it may be locally common. Several specimens were collected locally to Norwich, as the species is more commonly found in the Southern Norfolk and Sussex regions. Chemically, it is known to be similar to the related species Amanita muscaria, which has a long history of human use as a psychoactive substance in Northern Europe and Northern Asia, particularly regions of Eastern Siberia such as the Kamchatka peninsula, though it is now infrequently used for this purpose and was likely replaced by ethanol in such cultures. Both species are known to contain the toxins ibotenic acid and muscimol, which are responsible for their psychoactive effect and have often led to the species' being described as toxic, inedible, potentially deadly, or psychoactive. A. pantherina it contains greater quantities of ibotenic acid, muscimol, and lesser muscarine than A. muscaria.

Figure 89. *Amanita pantherina* mushrooms collected in Norfolk for chemical and anti-microbial testing

Unlike the much more deadly *Amanita* spp. such as *A. phalloides* and *A. virosa* (The Destroying Angel), neither species has been reported to contain the most famous toxins present in their deadlier relatives, the amatoxins, though it is unclear if they, and other related *Amanita* species, are capable of producing peptides with pharmacological activity.

Though the range of distribution of *Amanita pantherina* has long been considered to be confined to Europe and Western Asia, morphologically similar species in the pantherinoides group in North America were once considered to be the same species, though they have recently been renamed as distinct species. Nevertheless, the North American species are still often misidentified as *Amanita pantherina* and may still be considered as ‘Panther caps’ by many. Since the species complex has diversified, further chemical investigation into the group of fungi should be conducted to ascertain the presence and concentrations of the principle toxins in the different species.

Amanita citrina (**Figure 90**), known as the false death cap due to its superficial similarity to *Amanita phalloides* (the death cap, one of the most toxic mushroom species in the UK), was collected from an area of natural deciduous woodland in Merseyside. The species is considered edible, though it is usually advised that it should not be consumed due to the similar appearance to the highly toxic *A. phalloides*.

In early chemical investigations, it was reported that basic-phase extracts of *Amanita citrina* fruitbodies contained indole alkaloids, including tryptamines such as bufotenine, bufotenine oxide, serotonin, N-methylserotonin, 5-Methoxy-N,N-dimethyltryptamine and N,N-dimethyltryptamine, as well as at least 14 Ehrlich-positive compounds that were at the time unidentifiable [138, 139].

However, to date the presence of these alkaloids has not been confirmed using modern analytical techniques. These early studies identified the compounds using only TLC, staining with Ehrlich's reagent and comparing with reference compounds, and later gas chromatography. To date, it appears that no mass-spectrographic or NMR analyses have been conducted to confirm the suggested compound identifications, or to identify the other unidentified indoles previously reported.

The presence of these compounds could complicate the safety of consuming the mushroom and potentially lead to its use as a source of psychoactive substances. The confirmation of the metabolites, or similar tryptamine derivatives, would mean that there are potential food and drug interactions to consider when assessing the edibility and safety of the species. Most importantly, the species would be potentially psychoactive if it were to be consumed orally alongside monoamine oxidase inhibitors, depending on the medication, the concentration of the alkaloids and the amount consumed. As well as these potential interactions, if the mushrooms do contain such compounds then they, or preparations thereof administered parenterally, may produce psychoactive effects. This would make the mushroom analogous to the various plant and animal species which also contain the psychoactive compounds, which are indeed sometimes sought out for recreational use.

Figure 90. *Amanita citrina* sample collected for use in this study, showing several morphological characteristics of the species.

Data supporting the presence of these biologically active substances in the species is currently lacking, and the species is still considered edible and non-psychoactive. Nevertheless, if the presence of the substance or of any of the similar related N-methylated tryptamines in the species was confirmed, this would have legal implications rendering the species illegal to harvest within many countries, where the substances are controlled, as well as potentially leading to the species being sought out for preparation and consumption as a drug.

Amanita strobiliformis is another uncommon/rare *Amanita* with few records in Britain, though it is locally common in some areas. Several specimens were collected on the University of East Anglia campus and extracted for testing for antibacterial efficacy. It is reported that, unusually for the *Amanita* genus, *A. strobiliformis* is able to grow in the absence of a mycorrhizal association with a tree host, but it is not known whether it is growing as a saprophyte or forming mycorrhizal relationships with the grass in which it is usually found. There are few papers published regarding *A. strobiliformis*, at the time of writing, little is published regarding its chemistry, though it has been

shown to contain ibotenic acid and muscimol [140], and the species is known to hyperaccumulate silver [141, 142].

Gymnopilus junonius (**Figure 91**) is known in Britain by the common name ‘The Spectacular Rustgill’, owing to it’s usually large and visually impressive fruiting bodies, which tend to grow in dense clusters on the stumps of decaying trees. The mushrooms are often various shades of bright yellow and orange, with rusty brown coloured gills and spores. However, in other countries they are sometimes referred to as ‘Big Laughing Gym’, as their consumption can lead to intoxication. This is reportedly due to the presence of Psilocybin, and lactones similar to those found in the psychoactive Polynesian plant *Piper methysticum*, which is consumed for its relaxing properties. The presence of such lactones is uncommon for a mushroom forming fungus.

Although the presence of Psilocybin has been reported in samples originating from Asia, the alkaloid has not been reported in European specimens. It is not known what causes the geographical variation in alkaloid production, or if related alkaloids are present in European specimens, as modern chemical analysis is lacking.

Considering the interesting chemistry of the species, the lack of modern analysis and the known ability of the species to produce bioactive secondary metabolites, further examination of its chemistry and potential bioactivity is warranted.

Figure 91. A cluster of *Gymnopilus junonius* mushrooms growing from the base of a tree, collected for use in chemical and antimicrobial tests.

4.2 Extraction methods

Amanita pantherina and *Gymnopilus junonius* mushrooms were collected and dried at 40°C with fanning in a commercial food dehydrator and subsequently stored desiccated at reduced temperature prior to extraction. A 30g sample of each species was used for extraction. *Amanita citrina* (83.7g) and *Amanita strobiliformis* (100g) samples were frozen fresh and extracted without drying.

Initially the mushrooms were homogenised by blending and extracted several times with MeOH, which was removed under reduced pressure to give a methanolic extract, a sample of which was retained for chemical analysis and bioassay purposes.

Methanolic extracts of the mushrooms were subjected to further purification via acid/base extraction. In brief, HCL solution (pH 3) was added to the MeOH extracts, mixed, and then extracted with hexane and allowed to separate. The layers were separated, and the hexane was dried (MgSO4) and removed under reduced pressure to yield the acidic phase extracts. Subsequently, the mixture of HCL and insoluble material from the first extraction was basified to pH14 via addition of concentrated NH_4OH, and then extracted again sequentially with DCM, Xylene, Toluene and Diethyl Ether to yield several basic phase extracts. All of the extracts were analysed via HPLC and LCMS.

4.3 Anti-microbial assays of extracts and fractions

Crude fungal extracts and semi-purified fractions were tested against MSSA (Meticillin-sensitive *Staphylococcus aureus)*, *Escherichia coli*, MRSA (Methicillin-resistant *Staphylococcus aureus*), *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* for antibacterial activity using the micro broth dilution assay.

Extracts were dissolved in DMSO to give solutions which contained 13mg/mL of extract, which were then serial diluted in LB medium in 96 Well Plates to give a range of final concentrations from 1µg/mL to 1024µg/mL. Ampicillin and DMSO were used as positive and negative controls.

Single colonies of each bacteria were transferred into LB medium using an inoculation loop, and the bacterial concentration was adjusted to give consistent suspensions of colony-forming units/mL between all wells.

Finally, each well of the plates was inoculated with the bacterial solutions, and the plates were incubated overnight at 37°C. The next morning, plates were observed to determine the MIC of each extract, which was taken as the lowest concentration which inhibited the growth of the bacteria.

4.4 Antimicrobial assay test results

Several of the crude extract samples displayed antibacterial efficacy in the assays (**Tables 7-10**, with additional tested samples shown in **Appendix 2**). Most positive results were activity against *Staphylococcus aureus*, with activity against *E. coli* less common and usually requiring a higher concentration. Many of the crude extracts produced in this project were chemically complex, with many distinct components visible in the HPLC and LCMS chromatograms of the samples. The antibacterial efficacy seen with some of the crude extracts may suggest that they contain individual components which would be much stronger than the crude extract if isolated. Alternatively, it is possible that the samples could contain multiple metabolites acting synergistically responsible for the antibacterial effects. Some samples were further fractionated following positive results in initial antimicrobial assays, and the subsequent fractions were then also tested in repeat assays, in a process known as bioassay-guided fractionation. One example of this is the Acid-base extract (acidic stage) of *Amanita strobiliformis* basidiocarps. The initial crude extract contained several non-polar components which proved difficult to separate using traditional methods (reverse phase chromatography). The initial crude extract had an MIC of 128-256µg/mL against *S. aureus* and no activity against *E. coli* up to 1024µg/mL. The extract was further fractionated using a stepwise gradient of water and acetonitrile through a C18 silica cartridge, followed by a propan-2-ol wash of the column. Using this method the extract was split into a further 5 fractions containing components of varying polarity, which were then tested for antimicrobial effects. The first 3 fractions displayed antimicrobial effects against *S. aureus* (512-1024µg/mL, 64-128µg/mL and 64-128µg/mL respectively), with the first two also having efficacy

against *E. coli* (1024µg/mL). Fraction 3 was fractionated again using a similar method as the previous separation, yielding four fractions. All four fractions were active against *S. aureus* and the first three were also active against *E. coli*.

Table 6. Extraction yields (mg) of the various extracts from the mushroom samples tested for antibacterial efficacy.

Species (Sample weight (g))	**Extract**	**Yield (mg)**	**Yield (% of sample weight)**
Gymnopilus junonius (30g (dry))	MeOH	2436	8.12
	Ether	20.65	0.07
	DCM	75	0.25
	Xylene	11	0.04
	MeOH-soluble Aqueous remainder	9	0.03
Amanita citrina (83.7g (wet))	MeOH	677	0.81
	MeOH Freeze-precipitate	238	0.28
	Ether	17	0.02
	DCM	65	0.08
	Xylene	0.6	0.0007
Amanita pantherina (30g (dry))	Ether	39.7	0.13
	DCM	3.55	0.01
	Toluene	1	0.003
Amanita strobiliformis (80g (wet))	MeOH	575	0.72
	DCM	10.31	0.013
	Ether	2	0.0025

Table 7. Antimicrobial assay test results of various mushroom extracts, as well as fermentation extracts from epigenetic modifier-treated fermentations described previously

Sample	MIC (µg/mL)				
	MSSA	**E. coli**	**MRSA**	**KP**	**PA**
Laetiporus sulphureus (Yew) DCM	**256**	>512	**256**	>512	>512
Laetiporus sulphureus (Oak) Chloroform	**128**	>512	**128**	>512	>512
Laetiporus sulphureus (Willow) 1	>512	>512	>512	>512	>512
Laetiporus sulphureus (Willow) 2	>512	>512	>512	>512	>512
Inonotus obliquus (SAHA-treated) 1	>512	>512	>512	>512	>512
Inonotus obliquus (Nicotinamide-treated) 1	>512	128-256	>512	>512	>512
Gymnopilus junonius Xylene extract	**32-64**	**16**	**128**	**32-64**	**32**
Gymnopilus junonius Ether extract	**16**	**4-8**	**32**	**16**	**16**
Ampicillin	**0.25**	**1.0**	**2.0**	**128**	**>128**
DMSO	**ND**	**ND**	**ND**	**ND**	**ND**

Table 8. Minimum inhibitory concentrations (MIC, µg/mL) of the *Amanita citrina* (AC) and *Gymnopilus junonius* (GJ) acid-base extracts tested against a range of bacteria

Sample	**MIC (µg/mL)**				
	MSSA	**E. coli**	**MRSA**	**KP**	**PA**
AC pH14 Xylene	**128**	64	**512**	256	256
AC pH14 Ether	>512	256-512	>512	512	>512
GJ pH 14 DCM	512	256	>512	512	512
GJ Ether	128	64	256	128	128
AC Ether	512	256	>512	512	>512
AC DCM	128	64	512	128	128-256
GJ Xylene	**64**	**32**	**128**	**64-128**	**64-128**
GJ DCM	**128**	**64**	**256-512**	**128**	**128-256**
Ampicillin	**0.5**	**2**	**ND**	>512	>512
DMSO	>512	>512	**ND**	>512	>512

Basic-phase extracts of *Amanita citrina* and *Gymnopilus junonius* showed moderate antibiotic efficacy and were subjected to further attempts at purification with the aid of LCMS, bioactivity-guided fractionation utilising TLC bacterial overlay, automated flash chromatography and preparative TLC

Table 9. Zones of inhibition (mm) of the various mushroom extracts in a disc-diffusion antimicrobial assay. / = full inhibition / partial inhibition zone

Sample ID	**Sample concentration (µg/disc)**	**Zone of inhibition (mm)**	
		E. coli	**S. aureus**
Amanita citrina AB pH14	500	37/40	30
Gymnopilus junonius AB pH3 Hexane		0	0
Amanita citrina pH14 Ether		31	33
Amanita pantherina MeOH		10 (Partial)	0
G. junonius pH14 DCM		38	33
G. junonius pH14 Ether	100	33	23/27
Amanita pantherina MeOH sol. Components from Ether ext.	100	0	0
Amanita pantherina Toluene ext.	10	0	0
Amanita pantherina DCM ext.	10	0	0
Amanita citrina Ether	100	18 (partial)	14 (partial)
GJ aqueous remainder	10	0	0
Amanita citrina MeOH	40	0	0
Amanita citrina DCM	100	33	22/27
GJ Xylene	100	40	23/29
GJ tartrate salts	-	0	0
Amanita strobiliformis ether	10	0	0
GJ Xylene	10	11 (Partial)	0
GJ DCM	**100**	**30**	**20/26**
MeOH Control	10µL	0	0
Ampicillin	130µg/disc	28/34	35/45

Following the results of the disc-diffusion assay of the acid-base extracts from the selected mushrooms, the ph14 DCM extracts of both *Gymnopilus junonius* and *Amanita citrina* were selected for separation via normal-phase prep-TLC in order to further purify the constituents, as these samples displayed both good antimicrobial effect and had a yield large enough for the separation. The *Amanita citrina* and *Gymnopilus junonius* DCM extracts separated into 3 and 7 UV-active bands, respectively, which were scraped from the TLC plates and extracted with methanol. The purified bands were then re-tested in a disc-diffusion assay (**Table 10**).

Table 10. Zones of inhibition (mm) from the disc-diffusion antimicrobial assay test of the most active mushroom extracts after fractionation via Prep-TLC. GJ = *Gymnopilus junonius*, AC = *Amanita citrina.*

Sample	Sample ID	Yield (mg)	Zone of inhibition (mm)	
			S. aureus	E. coli
1	GJ DCM Prep TLC 1	1.58	NA	NA
2	GJ DCM Prep TLC 2	1.67	12	13
3	GJ DCM Prep TLC 3	11.46	15	18
4	GJ DCM Prep TLC 4	7.21	38	42
5	GJ DCM Prep TLC 5	5.09	44	42
6	GJ DCM Prep TLC 6	1.89	20	23
7	GJ DCM Prep TLC 7	0.91	13	11
8	AC DCM Prep TLC 1	5.56	10	16
9	AC DCM Prep TLC 2	23.5	Too large to measure	44
10	AC DCM Prep TLC 3	1.23	20	26
11	MeOH Control	-	0	0
12	Ampicillin 130µg/disc	-	47	32

In a disc-diffusion assay of the DCM extracts, only GJ DCM Prep TLC Band 1 did not have a zone of inhibition. The rest of the Prep-TLC-purified bands at 375µg/disc displayed significant antimicrobial effect against both SA and EC, with a number of the samples having zones of inhibition greater than the Ampicillin control (130µg/disc).

Of the GJ DCM fractions, fractions 4 and 5 proved to be the most potent, and of the AC DCM fractions, fraction 2 was the strongest. Following these promising results, several of the extracts were tested against more bacterial species

The *Amanita citrina* basic-phase Xylene extract was fractionated via automated flash chromatography through a C18 column utilising a typical reverse phase gradient of Acetonitrile/H2O. The extract was collected into 21 fractions, which were then tested for antimicrobial efficacy against *S. aureus* and *E. coli* via disc-diffusion assay, with 300µg extract/disc and 130µg Ampicillin. Of the 21 fractions only fraction 4 displayed antibacterial efficacy, giving zones of inhibition of 20 and 24mm against *S. aureus* and *E. coli*, respectively.

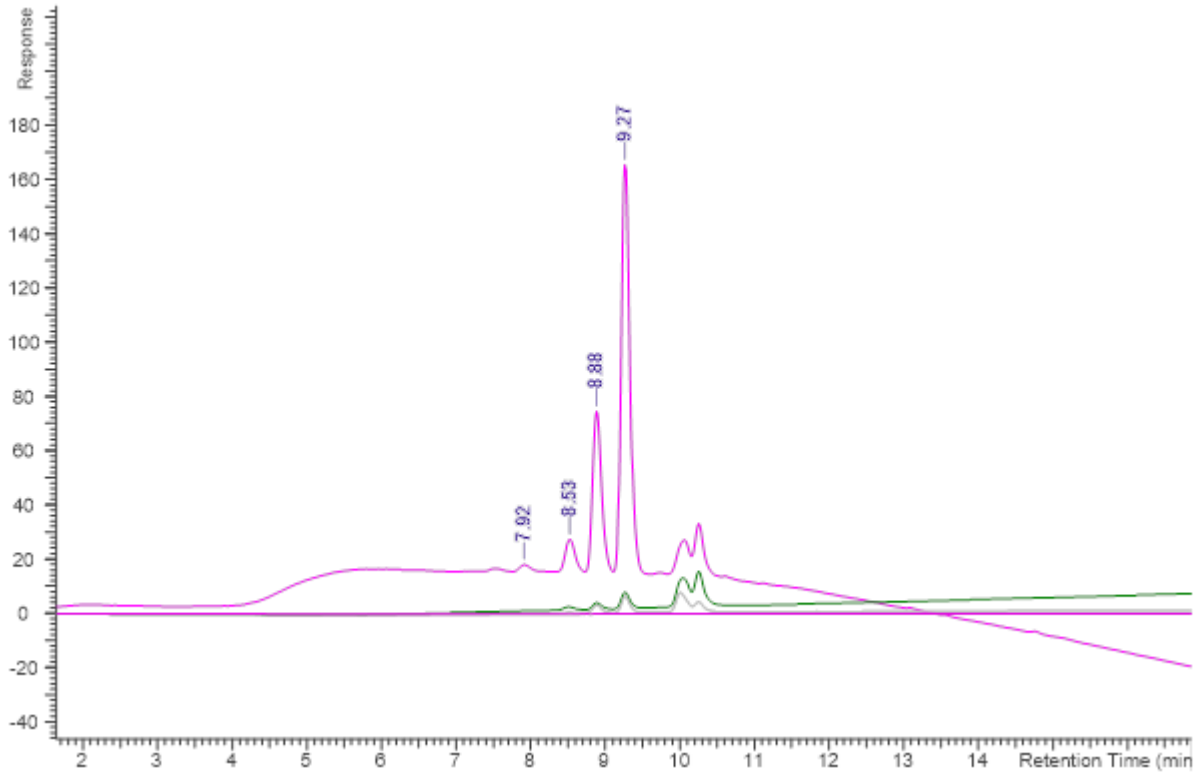


Figure 92. HPLC chromatograms of *Amanita citrina* Xylene extract combiflash fractions, showing components of fraction 4. Pink (with Peaks): 270nm, Green: 254nm, Grey: 210nm, Pink (flat): 360nm.

Comparison with the HPLC chromatogram of the crude *Amanita citrina* MeOH extract (**Figure 93**) shows that the main peaks in the active fraction 4 appear to constitute a small part of the overall initial extract, and are not visible.

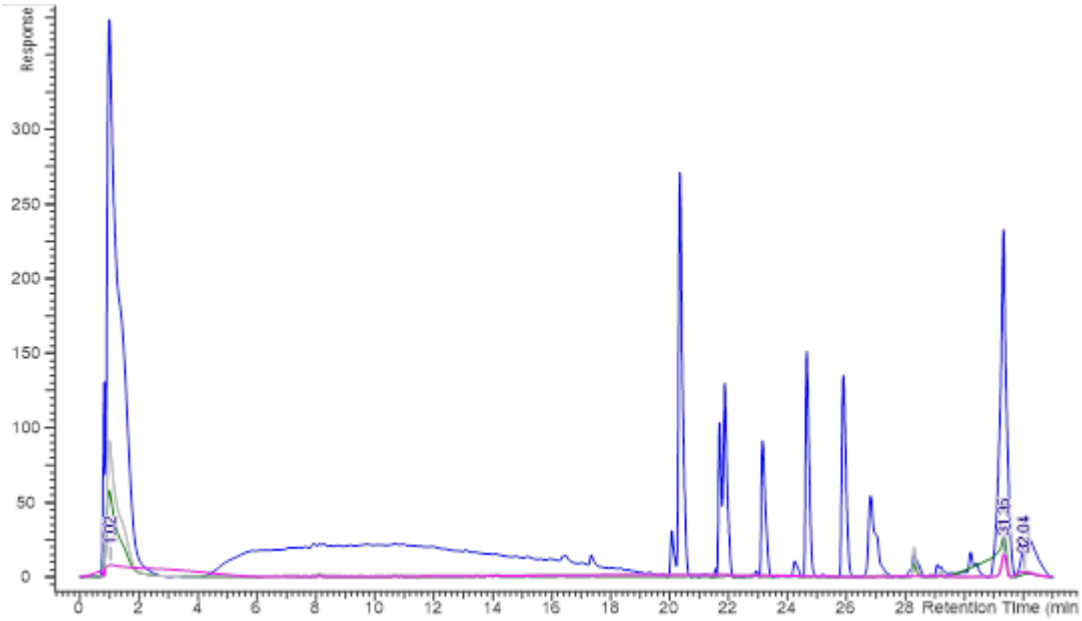


Figure 93. HPLC chromatograms of the *Amanita citrina* crude MeOH extract, showing the extract to contain several large non-polar components eluting after 20 minutes

Figure 94. LCMS Total Ion Chromatogram (TIC, +ve mode) of *Gymnopilus junonius* DCM extract, showing the DCM extract to contain a complex mixture of components difficult to separate using standard chromatographic methods

LCMS analysis appears to show that the active fractions contain a complex mixture of compounds, which based on MS-MS analysis appear to be a series of related compounds of varying molecular weight and polarity. Most of the compounds derived from the two species have common fragments suggesting that they may be a family of related compounds.

After repeated rounds of bioassay-guided fractionation, several fractions reached considerable potency in MIC assays. The most promising fractions were tested against several more bacterial species which are more resistant to antibiotics, including several Gram-positive and Gram-negative species.

The discovery of the antibacterial properties of the basic-phase extract fractions of *A. citrina* and *G. junonius* presented here warrant further work.

Unfortunately, it was not possible to isolate the compound/s responsible for the antibacterial effect in sufficient purity or quantity to perform the analysis required to determine which of the compounds is responsible for the antibacterial properties, or elucidate their structures.

Further attempts to collect wild specimens and extract these compounds may yield novel antibiotics, which appear to be fairly potent, broad-spectrum and active against difficult to treat species of bacteria.

Although attempts to cultivate either species in vitro are attractive prospects, as it is possible that the active constituent/s could be produced on a larger scale through laboratory fermentation, the fact that *G. junonius* is a saprophyte may make it a more attractive option, as mycorrhizal species such as *A. citrina* are often very difficult to cultivate and work with in laboratory conditions, whereas the mycelium of saprophytic mushrooms species is often easier to work with in laboratory fermentation.

Chapter 5

Examining the effect of bacterial co-culture on mycorrhization of Oak by *Tuber aestivum*

5.1 Introduction

5.1.1 The importance of Truffles

The mushroom industry in 2005 was estimated to be worth over £30 billion [143]. Fungi are an important food source and habitat for animals and microbes, they have found a place in the human diet and are used in the production of a number of food and drink products. For example blue cheese, made with *Penicillium* mould, and alcoholic beverages, produced through fermentation by *Saccharomyces cerevisiae*.

Truffles are the spore-bearing hypogeous fruiting bodies of the *Tuber* genus (Ascomycota, Pezizales). They are ectomycorrhizal fungi which form associations (Ectomycorrhizae) with the roots of certain trees and other plants, depending on the species. Although the *Tuber* genus contains the 'true' truffles desired for their gastronomic qualities, there are other genera within the Pezizales which also produce edible truffle-like fruiting bodies, including the desert truffles of the *Terfezia* genus. As well as this there are some truffle-like species outside of the Pezizales, including the *Elaphomyces* spp. There are thought to be 11 clades within the *Tuber* genus containing an estimated 180-220 species, with a worldwide natural distribution apparently limited to the Northern Hemisphere [144].

Figure 95. A wild *Tuber aestivum* truffle in a Beech woodland in England.

Truffles are amongst the most prized of the edible fungi, with black and white truffles being the most valuable [145]. The most expensive, *Tuber magnatum* pico (Piedmont White Truffle), is worth over £260 per 100g [146], the most prized black truffle, *Tuber melanosporum* (Périgord Black Truffle), is worth over £170 per 100g [147]. Large specimens of *Tuber magnatum* have exceeded £40,000 per kg at auction, making them more expensive than gold [144]. The cultivation of truffles has developed significantly since the early 20th century, and since 1973 controlled production of inoculated seedlings has risen from around 9,500 plants to an estimated 500,000 in 2015 in Europe alone [148]. Truffle production has rapidly expanded from central Europe and is currently occurring around the world, now including growing industries in the UK, South Africa, Australia, Chile and North America, and over the next 10 to 20 years truffle sales are expected to reach over £4 billion [147].

Truffles have long attracted the attention of foragers and scientists alike due to their gastronomic qualities and mysterious lifecycle. In attempts to improve the cultivation of truffles researchers have studied the biotic and abiotic factors involved in truffle formation (soil chemistry, climatic conditions etc.) [144], and it is now known that throughout their lifecycle, truffles have an important place within their ecosystems and are involved in a plethora of dynamic interactions within their environments [144]. These include interactions with terrestrial and soil-dwelling animals, the microorganisms of the mycorrhizosphere (the microbial inhabitants of a mycorrhiza) and surrounding soil, the plant host during stages of initial attraction, mycorrhizal morphogenesis and maturation, and interactions with nearby non-host plants. These relationships are multi-directional, affecting all parties involved. They change throughout the truffle lifecycle, and must be better understood in order to fully elucidate their importance. Many of these interactions are known to result in epigenomic regulation of the parties involved, yet the study of epigenetics in mushroom and truffle forming fungi is neglected compared with other organisms.

5.1.2 The Mycorrhizal Biology of Truffles

Mycorrhizal fungi form symbiotic relationships with plant roots and are extremely important. It is thought that they may have been involved in the progression of plants onto the land [149], and today the vast majority of plant species (Over 90%)

form relationships with mycorrhizal fungi [149, 150]. They are classified into 7 different types, which are ectomycorrhiza, endomycorrhiza (Vesicular-Arbuscular, VAM), ectendomycorrhiza, arbutoid, monotropoid, ericoid and orchidoid [149] (**Figure 96**). These sub-types are classified based on their morphological differences, for example ectomycorrhizal species are characterised by the formation of a fungal sheath or 'mantle', and a 'Hartig Net'; fungal cells which grow around root cortex cells. VAM species, which penetrate the cortical cells, are most common and occur on most angiosperms [149]. Many mycorrhizal fungi are obligate mycorrhizal species and cannot complete their lifecycle without a plant symbiont, but there are some species of fungi which have facultative saprotrophic/mycorrhizal lifestyles, for example certain *Morchella* spp., the Morels [151].

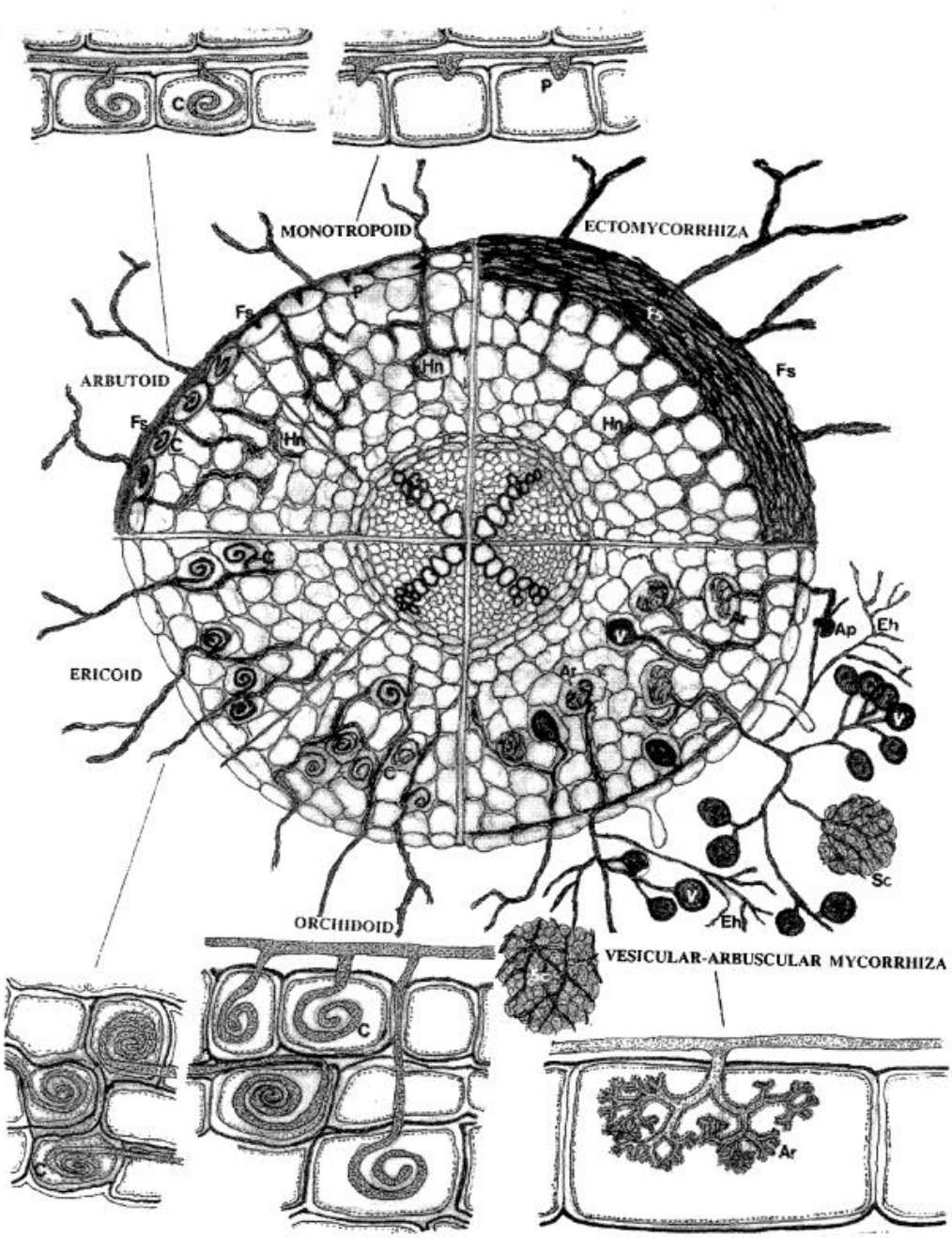


Figure 96. Different types of mycorrhizas have different relationships and morphological characteristics within the plant host root. Taken from [149].

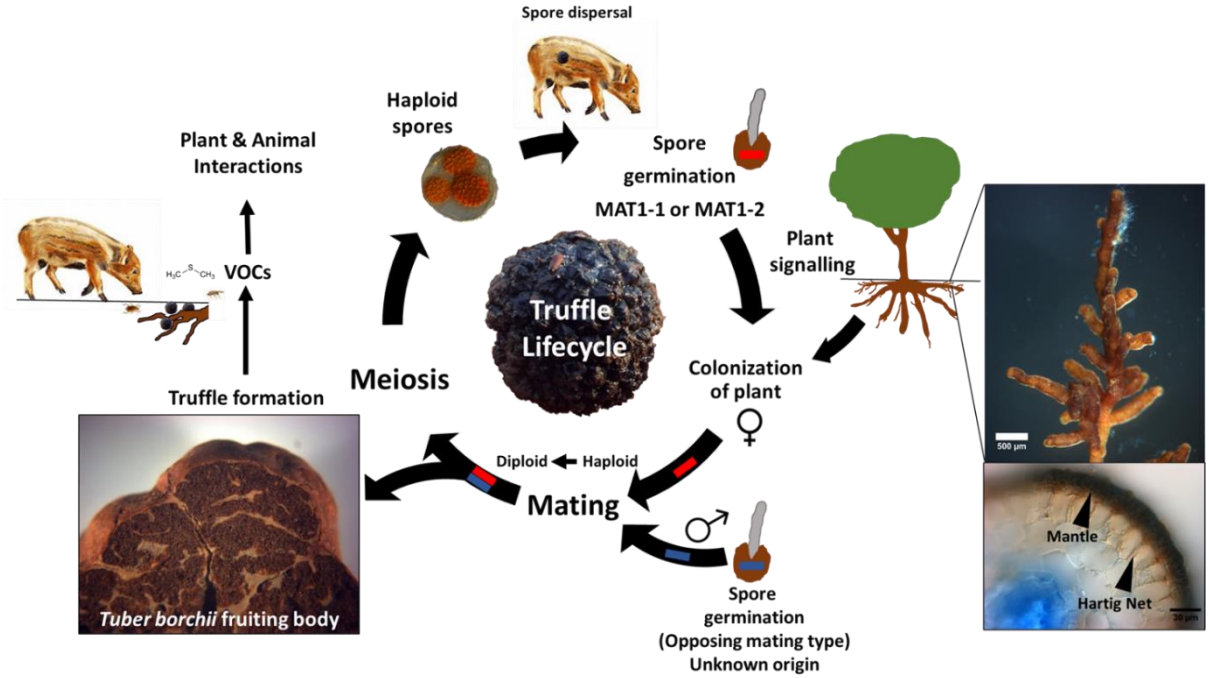


Figure 97. Overview of the truffle lifecycle, including the role of inter-species interactions with animals as a means of improving spore dispersal.

Tuber spp. are ectomycorrhizal (though *Tuber aestivum* has been shown to also form Orchidoid mycorrhizas), requiring a plant host in order to survive and produce truffles [144]. Ectomycorrhizal fungi include several other highly sought after gourmet edible species including members of the *Boletus, Cantharellus* and *Amanita* genera [152]. Commonly, *Tuber* spp. form relationships with deciduous and coniferous trees of the *Quercus* (Oak), *Fagus* (Beech), *Corylus* (Hazel), *Betula* (Birch), *Carpinus* (Hornbeam), *Pinus* (Pine), and *Populus* (Poplar) genera, but also *Castanea* (Chestnut) [153] and *Carya* (Pecan) [154]. Ectomycorrhizal fungi are important components of forest ecosystems, improving the health of host plants and providing a food source for animals including mammals (for truffles, mostly a variety of small mammals, but also the classic example of Wild Boars) and insects. In the symbiosis ectomycorrhizal fungi provide their plant symbionts with an increased access to water and minerals including phosphorus, nitrogen, calcium and zinc [149], protect the plant from heavy metal toxicity [155], improve the plants defence against herbivorous animals through regulation of defensive mechanisms [156] and favourably shape soil microbial communities [157]. In return the plant provides the fungus with a habitat and a supply of photosynthetically derived carbon.

Figure 98: Mycorrhizas (arrows) on *Fagus sylvatica* roots found in association with a *Tuber aestivum* truffle in England.

5.1 Current Truffle Research and Perspectives

5.3.1 Mating in the genus *Tuber*

One of the most groundbreaking discoveries from the sequencing of the *Tuber melanosporum* genome is the confirmation that *Tuber* spp. are heterothallic organisms which require mating with a compatible partner of an opposing mating type in order to reproduce [158, 159]. Ectomycorrhizas can be either of two mating types and function as the maternal partner, providing nutrients from the plant to support truffle development [145]. The paternal partner of the opposing mating type, comes from elsewhere, exactly where is not presently understood but it is thought to arise from other mycorrhizas or germinating ascospores in the surrounding soil [160]. After mating truffle formation can begin. Truffles themselves are composed of material from both parents. The peridium (outside) and supportive hyphae of the gleba (inside tissue) are derived from the maternal partner. Karyogamy occurs between the partners forming dikaryotic hyphae, which form haploid spores within mature asci through meiosis, where both mating types are present [160]. The practical implications of mating types in the context of artificial truffle production are not fully understood [161].

Some authors argue that spatial mating type dominance can occur within plantations and progressively reduce the chance of mating and therefore truffle yields [160]. However this has been disputed by a similar study on an Australian plantation [162]. A more in-depth understanding of how and when *Tuber* species reproduce, and the importance of mating type dominance, may have implications on the design and management of truffle plantations, for example with in-field modifications including the introduction of plants with opposing mating types [161]. Truffle farmers have developed untested methods which may influence mating. For example, some farmers introduce mature truffles into the soil [160]. It is possible that any benefit of this practice is due to an increased chance of mating occurring due to the presence of compatible mating types in the soil, but potential roles of truffle-derived microbes and metabolites have not been determined.

5.3.2 Truffle Traps

Another invention of truffle farmers is the 'truffle trap', which consists of a shallow hole within the brûlé (the productive area around the plant host in which plant growth is inhibited by truffle volatiles), into which vermiculite, compost, honey and blended truffles are introduced. A recent study of truffle traps found that when present, after 2 years 95% of truffles formed selectively within the trap (occupying only 5% of the productive area) as opposed to the normal brûlé soil [163]. Further research is required to elucidate the mechanism behind this phenomenon, for example the investigation of ectomycorrhizal phenotypic differences in the vicinity of the trap, the importance of reintroduced ascospores, and differences between the microbial communities of the trap and the surrounding soil. It is thought that the effect is due to the abundance of ascospores within the trap capable of functioning as the paternal partner, but this is presently unproven. Truffle traps present attractive models for in-field testing of exogenous additives during the mature, productive life-stage of the ectomycorrhizas, and may find use as an alternative to pot-based trials and traditional laboratory methods. Treatments may be chemical or biological; compounds can be added to the truffle traps to determine if there is an effect on the nearby ectomycorrhizas, subsequent truffle yield or quality, including volatile organic compound (VOC) content, or the trap microbiome. Of particular interest would be studies involving the introduction of bacterial species associated with truffles [164] &

the mycorrhrizosphere [165], some of which are considered to be beneficial ‘helper species’ [166] potentially involved in mating and truffle formation, and of insects which could improve truffle spore germination. Study of these factors *in vitro* may not yield data representative of the situation in-field, due to the difficulty of *Tuber* mating research *in vitro*, the lack of environmental interactions and the fact that truffle growth is not currently possible *in vitro*.

5.3.3 Species-species interactions

5.3.3.1 Truffle-animal interactions

As truffles are hypogeous they require mycophagous animals, mostly small mammals for spore dispersal (**Figure 97**) [144, 167], and may have evolved their aromas to attract such animals [168]. Most research has focused on the role of small mammals, with few studies available investigating relationships between truffles and soil fauna [169]. However there is compelling evidence that insects may play important roles in the truffle lifecycle. Truffle VOCs attract a variety of insect species [167]. Several species of insect rely on *Tuber* spp. as their sole food source and/or habitat, including the Truffle Beetle and Truffle Fly [144]. Digestion of truffle ascospores by *Sus scrofa* improves their ability to colonise new plants [168] which adds weight to the historic relationship between the species and the role of Wild Boar as spore dispersal vectors, but potential roles of soil-inhabiting fauna as ‘helper insects’ in truffle spore dispersal are not as clearly defined. Collembola (Springtails) are soil arthropods with a large distribution. They graze on fungi (including hyphae and spores) as a significant dietary component [170]. There is a possible role for Collembola spp. in the lifecycle of *Tuber* spp., as transporters of hyphal fragments and digested ascospores to new substrates and compatible mating partners. *Folsomia candida* present on truffle plantations favour the brûlé of *Tuber aestivum* [171], potentially due to their attraction to *Tuber*-derived 2-methyl propanol, 2-methyl butanol and 3-methyl butanal [167]. *Folsomia candida* grazing on VAMs increases the distance of fungal colonisation to neighbouring plants, but the effect is species-dependant [172, 173]. Nevertheless it is not currently known whether Collembolans and other soil-fauna may serve as spore-dispersal vectors over short distances, and how their digestion affects spore viability.

5.3.3.2 Truffle-microbe interactions

Truffles themselves are now known not to be pure *Tuber* cells, and contain other species of fungi, including yeasts and filamentous fungi [174], as well as mycoviruses, bacteria [164, 175] and insects. Truffles contain substantial amounts of bacteria, for example in *Tuber borchii* 600 bacterial strains have been isolated including 300 different species of Pseudomonas, which produce compounds involved in plant interactions [175]. These microbial communities within truffle ascomata and the mycorrhizosphere are thought to play roles in truffle maturation [176] and ectomycorrhization [177], and are thought to be shaped by *Tuber* spp. themselves [157]. Some are potentially obligate symbionts with *Tuber* mycelia [178], and many can be considered 'mycorrhiza helper bacteria', having positive effects on fungal health [166]. Some *Tuber*-inhabiting microbes are now known to contribute to VOC profiles [179], which typically consist of 30-60 compounds from 300 identified to date [144]. Of these, sulfur containing compounds are the most important for their favourable olfactory properties which are reminiscent of garlic. It is not currently known whether many of the VOCs which give the main truffle species their characteristic aromas are of *Tuber* or other microbial origin, as they have never been detected in pure culture [164].

Species-species interactions between fungi with other fungi and bacteria are known to have effects on the proteomes and metabolomes of fungi, leading to interactions which can be mutualistic, neutralistic or competitive [180]. For example, it has been shown that *Tuber melanosporum* becomes more competitive in the presence of *Tuber brumale* [181], a phenomenon which may be of use in the cultivation of *Tuber melanosporum* outside of its natural habitat range and in laboratory cultures. The communities of ectomycorrhizal fungi present on *Tuber melanosporum* plantations are fairly well documented, and appear to be correlated to the environment (e.g. presence of nearby forests), age of the plantation, speed of growth of the host (e.g. fast growing *Corylus spp.* vs slow-growing *Quercus spp.)* and productivity of the plantation [182]. How *Tuber spp.* Interact with and adapt to other species of fungi, and the functional consequences of such adaptations are poorly understood. Otsing and Tedersoo found that *Tuber melanosporum* remained competitive against native ectomycorrhizal species on *Quercus* in an Estonian plantation, one of the most northern *Tuber*

melanosporum plantations and outside of its normal range [183]. Further study of interactions between *Tuber spp.* with other bacteria and fungi may allow the identification of which species are mutualistic, neutralistic or competitive, and shed light on mechanisms behind positive interactions. For example, in a study investigating the effects of bacterial isolates associated with *T. borchii* ectomycorrhizas on the growth of *T. borchii* mycelial growth, it was determined that 101 of the bacterial species tested had a negative antifungal effect, while only 17 of the isolates had a beneficial effect on mycelial growth [184]. In another study examining the effect of *Pseudomonas fluorescens* CECT 844 on various parameters of plant health, nutrient uptake and mycorrhization by *T. melanosporum*, it was found that co-culture with the bacteria led to significant increase in mycorrhization of the host plant [185].

These inter-species interactions may be especially important for the growth of *Tuber spp.* in non-ideal environments, in strain development, and could have implications on plantation management through the management of fungal and bacterial communities.

5.3.4 *In vitro Tuber* spp. cultivation, challenges and applications

5.3.4.1 Isolation and maintenance of *Tuber* cultures

Tuber spp. are notoriously difficult to isolate and maintain in a laboratory environment [146]. They do not function well as saprotrophs and are very slow growing when traditional methods are used (1.1mm–14mm per week) [186, 187]. Nevertheless, researchers have developed protocols for the isolation and cultivation of mycorrhizal fungi including *Tuber* spp. *in vitro* [152, 186, 187] which have proven essential in morphological and biochemical research. Such cultures are also useful in the study of inter-species interactions with microorganisms including bacteria [178, 188] and fungi, as well as plants and animals, as they allow limitation of the complex variables present on plantations.

Axenic *Tuber* cultures can be isolated from truffles or mycorrhizas though the success rate is generally low, with high rates of bacterial contamination. Sub-cultures also rarely grow after isolation [144]. The high rate of contamination is due to the fact that truffles and mycorrhizas exist within and themselves contain a complex mix of other microorganisms which can easily outcompete them. In brief, procedures for

culture isolation generally involve the aseptic excision of gleba from within a truffle, which is then placed onto a suitable agar medium with or without antibiotics, for example modified Woody Plant Medium (mWPM), modified Melin-Norkrans Medium (MMN), Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA) [187]. Cultures may be isolated from living mycorrhizas, but the mycorrhizas require special treatment due to microbial components of the mycorrhizosphere. Treatment of mycorrhizas with NaClO or H_2O_2 and chloramphenicol in the growth media have been utilized [189, 190]. Truffle spore germination is extremely difficult and is a lengthy process [144]. Once obtained, cultures can be maintained on solid media, in liquid fermentation or in plant-systems.

5.3.4.2 Production of *in vitro* mycorrhizas

A number of *in vitro* plant-systems have been developed to facilitate the laboratory production of mycorrhizas [152], including the use of *Cistus incanus* (Pink Rock Rose), a perennial shrub found on dry or rocky soils throughout the Mediterranean region. *Cistus incanus* can serve as a host species for *Tuber* spp. including *Tuber melanosporum* and *Tuber borchii* [191, 192]. It can be utilised in either whole plant form or as root organ cultures transformed by the gram negative soil bacterium *Agrobacterium rhizogenes* [191], which induces a condition in Dicotyledons known as ‘hairy root disease’ via transfer of tDNA from its root-inducing plasmid into the cells of the plant [191]. These systems improve the growth of the fungus and facilitate the study of physiological and biochemical factors involved in mycorrhizal morphogenesis. They also have the advantage that they can be used to preserve *Tuber* cultures which usually die after sub-culturing [178]. *Tuber melanosporum* ectomycorrhizas in transformed *Cistus incanus* root cultures have much greater longevity than axenic cultures [191]. *Tuber melanosporum* has been shown to grow poorly on the root organ medium, therefore there are possible advantages to utilising multi-compartmental systems in which root organs & mycorrhizas are cultured in one compartment and the mycelium is allowed to grow into a second compartment (which excludes the plant roots), in which the medium is optimised for growth of the fungus. Such a system could potentially improve the nutritional status and growth of both symbionts.

Another potential application of plant systems is the study and propagation of bacterial species associated with the mycorrhizosphere and truffle ascomata. Some of these bacteria are obligate fungal symbionts [178], so these systems may facilitate their isolation from truffle ascomata or mycorrhizas. Transcriptomic, proteomic and metabolomic responses to the bacteria could be studied in such systems, and the bacteria could be grown on to be reintroduced in-field, for example in truffle traps.

It is known that during the initiation of mycorrhization the fungal-plant symbionts release and respond to signals in order to recognise each other [193]. Fungal-plant cultures have proven useful for studying such interactions, for example using a *Tilia Americana*-*Tuber borchii* system in which the two did not make physical contact, Menotta *et al* found using headspace solid-phase microextraction with GC/MS that 29 volatile organic compounds were induced by the interaction and thus possibly involved in pre-contact signalling [194]. In a similar system where contact and mycorrhiza formation is permitted, transcriptional upregulation of many genes occurs, with more plant genes than fungal genes affected [195], though proteomic effects are unknown. The effect of *Tuber*–Plant signalling on non-volatile fungal and plant metabolites also requires further research. As well as the VOCs there may be more non-volatile metabolites induced, which may be involved in signalling and ectomycorrhizal morphogenesis.

5.3.5 Epigenetic Research in Truffles

The genome of *Tuber melanosporum* has been shown to be amongst the most methylated of organisms, with methylation present at over 44% of cytosine residues [196]. This high level of methylation is more so than other fungi, and may be the result of the high abundance of transposable elements (TE) in the *Tuber melanosporum* genome, as cytosine methylation in fungi appears to be important in the silencing of TEs. Treatment of *Tuber melanosporum* cultures with 5-azacytidine (5-aza) displayed no apparent toxicity up to 100µM and induced a visible change in phenotype (Figure 6) [196], though microscopic, proteomic, metabolomic or effects on nutritional composition were not investigated. In this study, 5-aza reduced the level of genome methylation, increased the expression of TEs and altered the expression of 115 genes, several of which are novel transcripts for the species. These transcripts code for proteins involved in oxidation-reduction, transmembrane transport, metabolism,

response to stimuli, and mycelium development [197]. Currently the effect of these transcriptional responses to 5-aza on the metabolome, proteome, phenotypic plasticity and plant-interactions of *Tuber melanosporum* are unknown, but the fact that many of the genes affected are involved in development, metabolism, response to stimuli and transmembrane transport warrants further investigation into the effects on mycorrhizal morphogenesis and interactions with plant hosts, as well as a detailed examination of changes in secondary metabolism in pure culture and at the various stages in the truffle lifecycle. In plant systems, epigenetically modified cultures may shed light into the role of induced secondary metabolites and membrane transporters in mycorrhizal morphogenesis and signalling between symbionts.

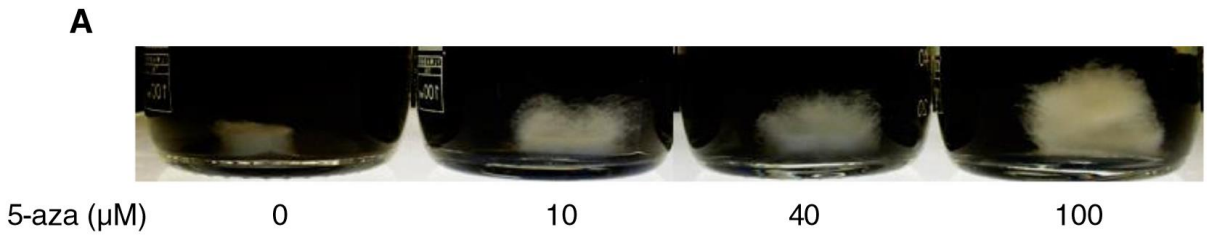


Figure 99. The induction of the 'fluffy' phenotype in *Tuber melanosporum* by 5-azacytidine. Figure from [196].

It has been shown that in the edible Oyster mushroom *Pleurotus ostreatus*, the expression of several DNA methyltransferase (DNMT) genes are temporally dynamic and vary throughout the developmental stages of mushroom formation, and that 5-aza induces morphological changes [70]. Temporal variation of these expression profiles suggests that DNA methylation may play a role in fruiting body development in the Dikarya, and that sensitivity to DNMT inhibitors may vary across lifecycle stages. Epigenomic regulation throughout the truffle lifecycle is poorly understood, however, Montanini *et al* showed that genome methylation differs between fruiting body, free-living mycelium and ectomycorrhizal lifecycle stages [196]. Programmed cell death has been shown to occur during ectomycorrhizal morphogenesis [198] and recently during fruiting body maturation [199]. The expression of 14 genes involved in

programmed cell death are differentially expressed through truffle maturation, therefore epigenetic regulation may be important for autophagic processes involved in truffle maturation, and interference with this may alter truffle morphology. As 5-aza-induced hypomethylation interferes with the balance of sexual and asexual structures in *Neurospora crassa* [200], it would also be interesting to examine the effect of 5-aza on *Tuber* spp. mating utilising truffle traps, and on the formation of ascospores during truffle maturation. Mycorrhizal fungi can help to protect their plant symbiont from detrimental microorganisms through utilisation of excess carbohydrates and production of secondary metabolites including antibiotics [149]. As epigenetic modifiers can increase antibiotic production in fungi, epigenetic modification of mycorrhizas could also influence the communities of the mycorrhizosphere. Whether true truffles have antibacterial properties remains unknown, but methanolic extracts from the desert truffle *Terfezia boudieri* have been shown to have antibacterial efficacy [201].

5.3.6 Volatile organic compounds in the genus *Tuber*

The fact that 5-aza can upregulate the production of microbial secondary metabolites is particularly interesting in the context of truffle development, as truffle-derived VOCs are important factors in their desirability and inter-species interactions (including truffle detectability), and VOC levels could be influenced with epigenetic modification. The plant, fungal, bacterial and animal communities living in soil interact through the production and perception of VOCs. These interactions are important in signalling, and can have repellent or attractant effects on other organisms [6]. Truffle volatiles are important in the formation of the brûlé, an area around the host tree where plant growth is limited. This is thought to be a phenomenon beneficial to the survival of the truffle mycelium [5]. Truffle volatiles affect plant-signalling, mesofauna populations, and manipulate the rhizosphere and wider microbial community in the brûlé [157, 165]. They have allelopathic effects on some plants, affecting seed germination, interfering with root morphogenesis via production of auxin and ethylene [4] and produce an oxidative burst response in *Arabidopsis thaliana* which inhibits growth [4] and affects hormonal function [5].

The VOCs responsible for truffle aromas vary depending on species and are classified as sulfur containing, alcohols, esters, ketones, aldehydes, aromatic compounds and others. Currently it is not known whether some of these compounds are produced by the *Tuber* cells, the truffle microbiome or through species-species interactions, as several of the compounds are only present in the truffle stage of the lifecycle and have not been detected in axenic cultures [164]. It is possible that some of these compounds are the products of genes which are silent under standard laboratory conditions. As non-exhaustive demethylation of the *Tuber melanosporum* genome alters the transcription of enzymes involved in oxidative, reductive and other metabolic processes, it is feasible that 5-aza may lead to the production of metabolites, including truffle volatiles, not-previously produced under laboratory conditions. If so, this will further clarify which VOCs are *Tuber*-derived. If epigenetic modifiers do upregulate VOC biosynthesis, they may have specialised applications in-field, for example to affect the developmental process and communities of the brûlé, increase truffle aromas and facilitate detection by dogs or electronic sensing.

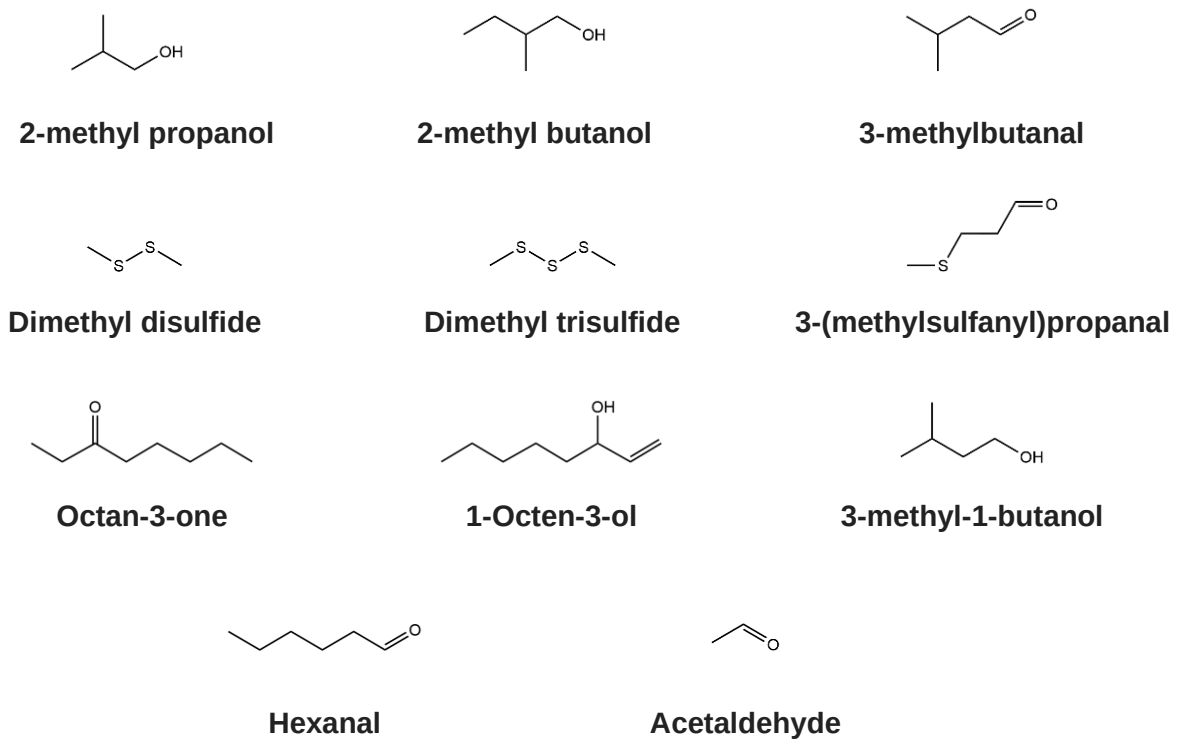


Figure 100. Selected examples of Volatile Organic Compounds (VOCs) produced by *Tuber* spp.. Truffle-derived VOCs are involved in inter-species interactions, and also play a large part in the gastronomical appeal of truffles.

5.3.7 Truffle proteomics

Proteomic methods are currently under-utilised in truffle research, however a number of studies have been conducted to date. In the first analysis of the proteome of *Tuber borchii* in axenic culture, Vallorani *et al* observed over 800 spots using 2D-PAGE, yet were only able to identify 1 by its amino acid sequence [202], highlighting the difference between *Tuber* proteomes and those of other fungi known at the time. Several genes thought to be involved in truffle production were identified via mRNA differential display [203], and proteomic changes occurring through truffle maturation were subsequently investigated [204]. A more metabolically active proteome was observed in immature truffles compared with mature truffles. The publication of the *Tuber melanosporum* genome in 2010 allowed protein identification in *Tuber magnatum* via MALDI-TOF and nLC-ESI-MS/MS using a bioinformatics approach [205]. The authors were able to identify 17 selected proteins which could be used to differentiate between *Tuber magnatum* collected from different geographical areas. In 2013, the *Tuber melanosporum* proteome was functionally annotated. 2587 out of a putative 12771 proteins from the genome were identified via comparison with fungal homologues, and validated via 1D-PAGE and LC-MS/MS [206]. This increase in the available proteomics data remains under-utilised and may lead to improvements in our understanding of proteins involved in responses to environmental factors, adaptation to new climates, inter-species interactions and epigenetic regulation.

5.4 Experimental methods

The main aims of this project were to investigate the effects of epigenetic modification at various stages of the Truffle lifecycle. In order to achieve this, attempts were made to obtain *Tuber* species in axenic culture, produce *in vitro Cistus incanus* seedlings which could be used to produce *in vitro* mycorrhizas, investigate the natural morphology of *Tuber* species through a variety of methods, utilise LCMS/MS to analyse extracts from *Tuber borchii* grown *in vitro* in the presence of various epigenetic modifiers, in order to determine the effect that the epigenetic modifiers had on their metabolic profiles. As well as this, a plant nursery-based study was conducted in order to study the effects of bacterial co-cultures, epigenetic modifiers, and other small molecule elicitors on the mycorrhization of *Quercus robur* seedlings by *Tuber aestivum* A number of secondary aims were pursued, including attempts to investigate the importance of mating type dominance in a UK plantation, and investigate the role of insects in the truffle lifecycle

5.4.1 Isolation of *Tuber* species

Attempts were made to isolate cultures of *Tuber* spp. (*aestivum* and *melanosporum*) from gleba, mycorrhizas and spores. *Tuber aestivum* and *melanosporum* truffles were obtained from the plantations of Mycorrhizal Systems Ltd. Attempts at isolation from gleba and mycorrhizas were conducted following published procedures [187, 189, 190], on a variety of media (mWPM, PDA, MEA). In brief, Mycorrhizas were treated in hydrogen peroxide solution (2.7%, 10s) then rinsed 3x in sterile dH_2O before placing onto agar medium containing chloramphenicol (35µg/mL). Truffle samples were surface sterilised with EtOH (70% v/v), then broken open. Pieces of gleba (1-2mm) were asceptically excised and placed onto agar medium containing chloramphenicol (35µg/mL). Plates were sealed with parafilm and incubated at room temperature in the dark. A culture slant of *Tuber borchii* was kindly provided by Professor Alessandra Zambonelli (University of Bologna). The culture was sub-cultured onto 10 plates of half strength PDA (PDA/2), 3 containing chloramphenicol (35µg/mL), and 4x 50mL sterile centrifuge tubes containing mWPM liquid medium with glucose (10g/L).

Spores were extracted from frozen *Tuber aestivum* and *melanosporum* samples via gentle disruption in PBS + 0.05% Tween-20, filtration (200µm, 40µm and 15µm filters) and centrifugation (1000-5000rcf). Spores were either imaged, placed into PDB, or surface sterilised with Chlorazine T (2% w/v, 20 min), rinsed 3x, treated with 100µM 5-aza or sterile dH_2O (control) then plated onto MYA made with soil extract instead of water, with or without sterile root homogenate (*Fagus* sp.).

5.4.2 Isolation of *Morchella* species

Dried specimens of *Morchella importuna*, *Morchella vulgaris* and *Morchella semilibera* were kindly provided by Jesper Launder. Spores were isolated from dried *Morchella importuna* ascomata via several methods of spore extraction (fractional centrifugation, sucrose gradient centrifugation and tissue disruption, filtering (200µm) and centrifugation (1000-5000rcf). Isolates were examined microscopically to confirm the presence of intact spores. For determination of spore concentrations, a Neubauer haemocytometer was used. Tissue disruption in sterile dH_2O followed by filtration through a 200µm filter and centrifugation (4000-6000rcf) proved adequate for spore isolation. The resulting creamy coloured pellet largely consisted of spores which were washed in either EtOH (70%) or sterile dH2O and then rinsed 3x with sterile dH_2O. Spore suspensions in sterile dH2O were pipetted onto PDYA containing chloramphenicol (35µg/mL) and ampicillin (100µg/mL). Three of the isolates were sub-cultured onto the Water Agar (WA) side of 50/50 PDA/WA plates and PDB. After 7 days, cultures were treated with either 5-aza (100µM, 200µL) or sterile dH2O, dropwise. PDB cultures were treated with 5-aza to a final concentration of 100µM, or sterile dH2O.

5.4.3 Cistus incanus propagation

Cistus incanus seeds (Thompson and Morgan) were washed in sterile dH2O containing Tween-20 (0.05% v/v). Seeds were rinsed 3x in sterile dH2O and placed into NaOCl solution (1% v/v) in sterile dH2O, rinsed again 3x and heat treated (65^{o}C, 5 minutes) in sterile Eppendorf tubes in a water bath. Seeds were then placed onto freshly prepared Murashige and Skoog (MS) medium using sterile forceps, and left in

natural daylight. Seedlings were transferred onto fresh MS medium 1 week after germination.

5.4.4 Morphological examination of *Tuber* species

The morphologies of *Tuber* mycorrhizas, truffles, asci and ascospores were investigated via upright, dissection or confocal microscopy. Spores were prepared by gently disrupting pieces of gleba in sterile dH2O in Eppendorf tubes using a micropestle. The resulting suspensions were then pipetted up and down repeatedly. Some were filtered through 40µm filters then spores were caught on 15µm filters (PluriStrainer) and washed with sterile dH2O.

Soil samples for mycorrhizas were collected from a plantation in Wales with either a dent corer or narrow soil auger, or provided by Mycorrhizal Systems Ltd. Mycorrhizas were collected from soil samples by gently washing the soil from the roots in a sieve under running water. Colonised roots were sonicated in water to further remove soil. Mycorrhizas were either frozen (-20^{o}C), stored in EtOH (50% v/v), glycerol/H_2O (50% v/v) or fixated in formaldehyde-acetic acid-ethanol (FAA, 3.7%, 5% and 50% v/v, respectively). Chlorazol black E (0.03% w/v), lactophenol cotton blue (0.05% w/v) and Melzers reagent were used as stains. Mycorrhizas were cleared by autoclaving (15 min, 121^{o}C) in KOH (10% w/v), then bleached in alkaline hydrogen peroxide (NH_4OH, 0.5% v/v and H_2O_2, 0.5% v/v) until clear before Chlorazol black E staining.

5.4.5 Imaging

For imaging of mycorrhizas, samples were manipulated in petri dishes containing water, using forceps, needles and a scalpel. Samples were placed in-between two microscopy slides and moistened with water from the side, then imaged using a Zeizz SV11 stereo microscope with a colour CCD camera. Mycorrhizal cross-sections were prepared by hand-sectioning mycorrhizas immobilised on microscopy slides under parafilm with a sharp blade. Cross sections and spores were imaged using a Zeiss Axioplan 2ie motorised microscope and monochrome or colour CCD camera. *Tuber borchii* ascomata was prepared by cutting the truffle using a scalpel, and examined with a Zeiss SV11. Mycorrhizas and spores were stained using a

plasma membrane-selective green fluorescent dye and imaged with a Leica TCS SP2 confocal microscope.

5.4.6 DNA extraction and PCR

DNA was extracted from truffle tissue or single mycorrhizas homogenised in eppendorfs with a micropestle, using a QIAGEN Plant Mini kit following the manufacturers instructions. Primers utilised for PCR included species-specific primers for *Tuber borchii* and primers for *Tuber aestivum* mating type 1 (MAT1-1) and mating type 2 (MAT1-2). Tuber aestivum MAT1-1 primers were:

MAT1-1f 5’ CTACATTCTGGTG GGCGATT 3’
MAT1-1r 5’ TCCCGATTTGTCCAACGTAT 3’
MAT1-2f 5’ ATCGTCGGGACTCATCTCAC 3’
MAT1-2r 5’ CGGATATTGGGATTTGATGG 3’

Mating type genes were amplified by multiplex PCR in tubes containing nuclease-free H2O (21µL), Reaction buffer (10µL), Primers (2µL, 10µM), *Taq* polymerase (1µL) and DNA template (10µL). Samples were subjected to the following conditions in a thermal cycler: 3 minutes at 95^{o}C, then 30 cycles of 15s at 95^{o}C, 15s at 57^{o}C, 60s at 72^{o}C, then a final extension of 7 minutes at 72^{o}C.

Tuber borchii primers were:

TBA 5’ TGCCCTATCGGACTCCCAAG 3’
TBB 5’ GCTCAGAACATGACTTGGAG 3’

Tuber borchii PCR was performed in reaction mixtures containing nuclease-free H2O (25µL), Reaction buffer (10µL), Primers (2µL, 10mM), *Taq* polymerase (1µL) and DNA template (10µL). Samples were subjected to the following conditions in a thermal cycler: 5 minutes at 94^{o}C, then 40 cycles of 45s at 94^{o}C, 45s at 65^{o}C, 45s at 72^{o}C, then a final extension of 5 minutes at 72^{o}C.

PCR products were subject to gel electrophoresis (110V, 60min) on 1.5% agarose gels containing 10µL of Safeview (NBS Biologicals) per 50mL. Gels were imaged with an LAS4000 imaging platform set to ethidium bromide.

5.4.7 Chemical extraction and analysis via LCMS

Methanolic extractions were performed on *Tuber aestivum* truffle tissue. Samples were disrupted and placed into conical flasks containing methanol, and stirred magnetically. The suspensions were filtered, then hexane was added to the filtrates, mixed by shaking in a separatory funnel, allowed to separate and the methanol layers were collected and evaporated using a rotary evaporator. Residues were dissolved in MeOH/H2O (90/10% v/v) at 2mg/mL and analysed via LCMS-IT-TOF.

5.5 Results and discussion

5.5.1 Sample collection and morphological investigations

In order to practice techniques in examining the natural morphological features of *Tuber* species, *Tuber aestivum* mycorrhizas on several plant species, as well as *Tuber aestivum* and *melanosporum* spores were obtained and imaged. All methods of sample collection (Dent corer, narrow auger and trowel) were adequate for mycorrhiza collection.

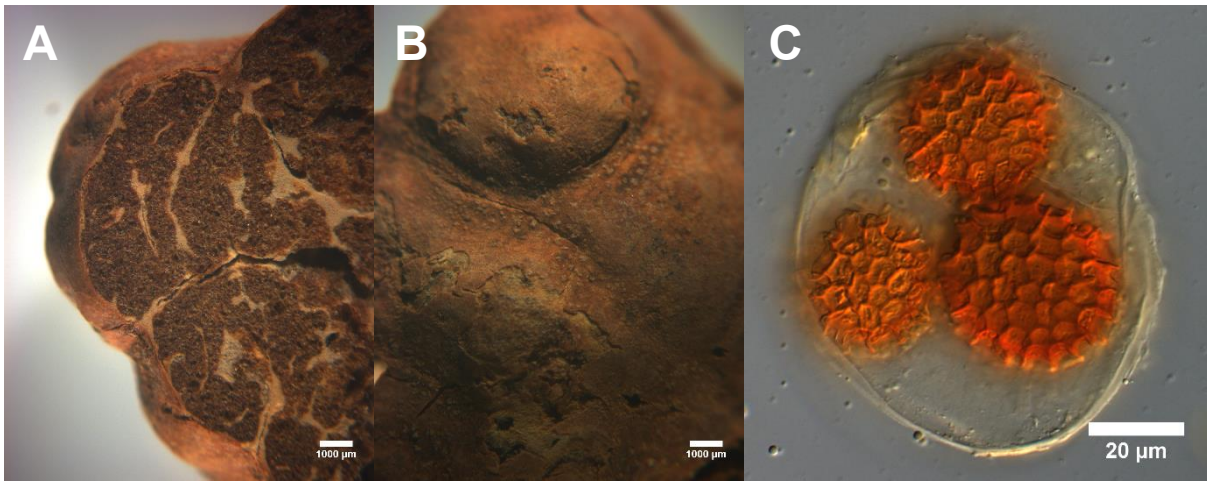


Figure 101. Ascomata, peridium and spores (**A, B, C**) of *Tuber borchii*.

Soil and truffle samples from a *Tuber borchii* plantation in South Africa were provided by Mycorrhizal Systems Ltd for confirmation of their identity via morphological examination and PCR. The truffle sample and extracted spores were imaged and the morphology (vein patterning of the gleba, surface of the peridium, ascospore size and surface ornamentation) matched those of *Tuber borchii* (**Figure 101A, B, and C**, respectively). Root systems with abundant simple and ramified straight or bent mycorrhizas were obtained from the soil samples (**Figure 102**). Several morphological features were observed, including the size and shape of mycorrhizas. Mycorrhizas appeared to be in a desiccated condition upon arrival (evident in Figure 8B, C and E) as the soil samples were quite dry, which may have affected the shape, dimensions and mantle features of mycorrhizas, as well as the prominence of emanating elements such as cystidia. Attempts to visualise the hartig net were unsuccessful as the mycorrhizas were convoluted, with ‘collapsed’ areas (Figure 8C and E). Nevertheless the surface detail of the mycorrhizas including emanating cystidia, ‘jigsaw’ like

pseudoparenchymatous cell pattern of the outer mantle, and cystidia emanating from plectenchymatous areas of the mantle (figure 8D, G and F, respectively) were successfully observed from some of these samples.

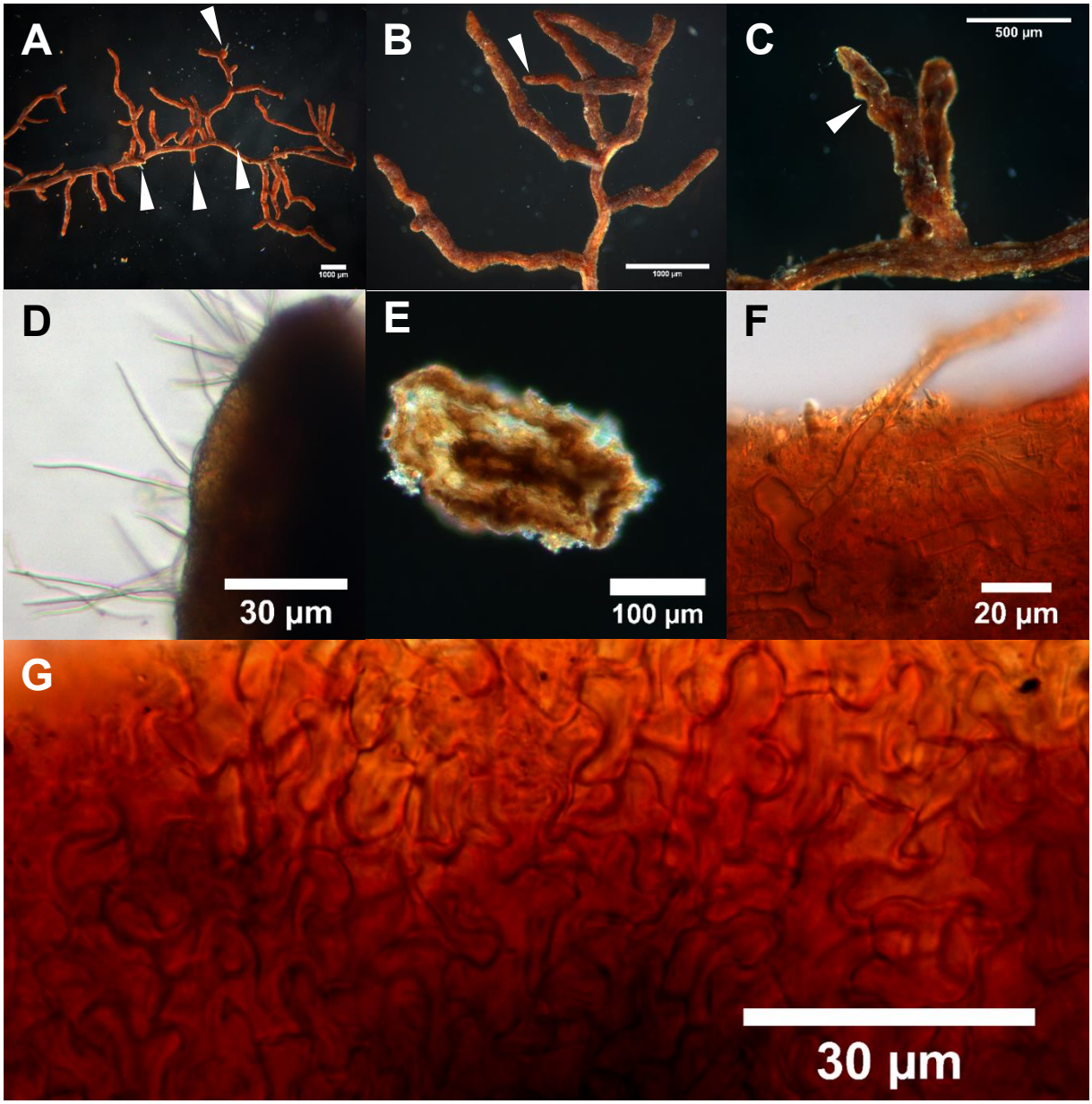


Figure 102. Mycorrhizas on from a South African *Tuber borchii* plantation. **A.** Root system with simple and ramified, straight and bent mycorrhizas, arrows = broken mycorrhizas. **B**. Mycorrhizas showing desiccation, thinning and tapering ends. **C**. Convoluted shape of mycorrhizas. **D**. Awl shaped short, spiny cystidia. **E.** Cross-section showing sample deterioration. **F**. Cystidia emanating from plectenchymatous area. **G**. Jigsaw patterned pseudoparenchymatous mantle surface.

Tuber aestivum mycorrhizas on a *Quercus* sp. were provided by Mycorrhizal systems Ltd and their morphologies were examined (**Figure 103**). The samples

contained root systems with abundant mycorrhizas of simple and monopodial-pyramidal ramification (**Figure 103 A, B**), with cylindrical ends, dense emanating cystidia (**Figure 103 C**) and a pseudoparenchymatous angular outer mantle cell pattern (**Figure 103 D**), which was visible under the stereomicroscope, unlike the *Tuber borchii* samples.

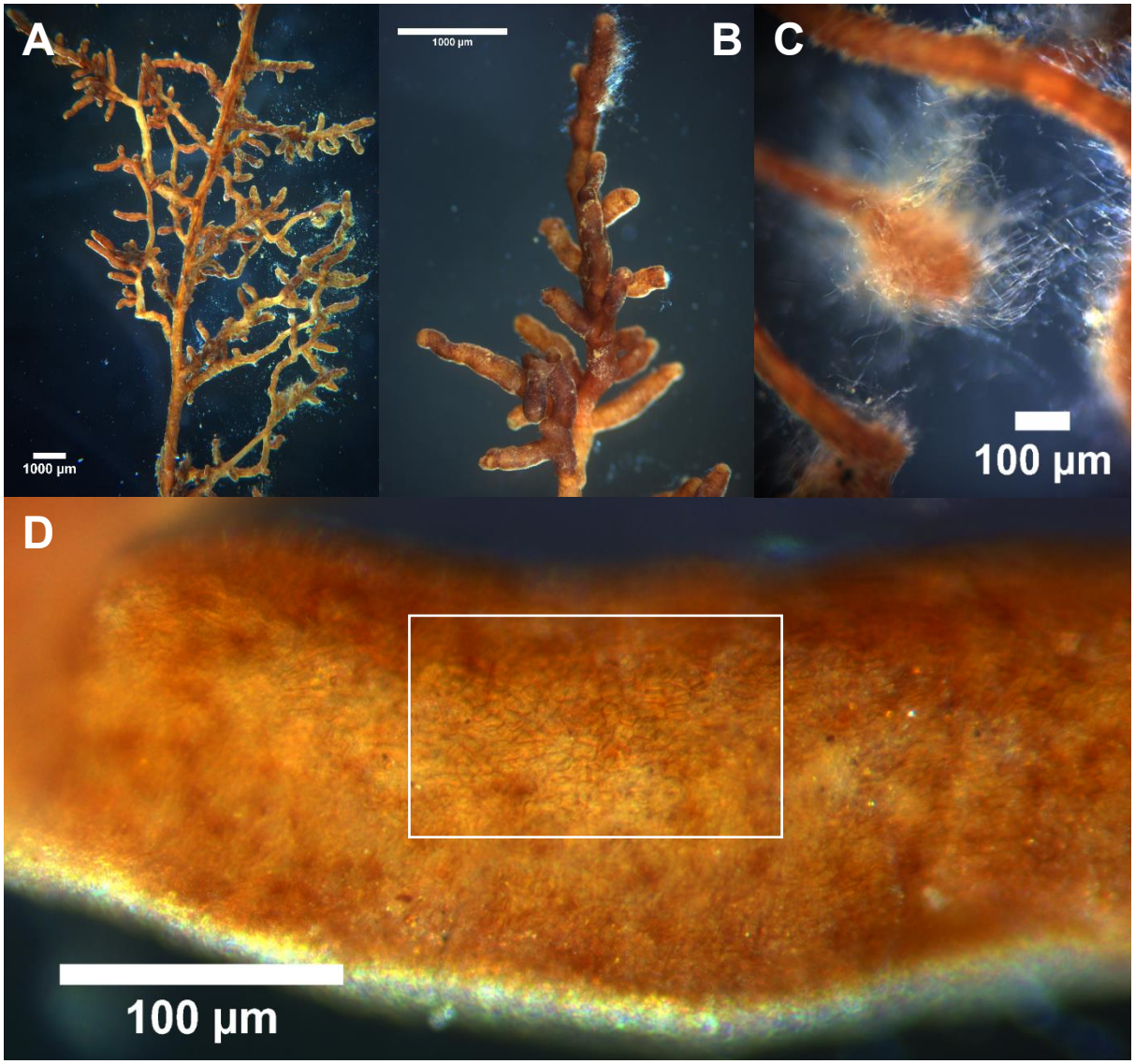


Figure 103. A. *Quercus* root system with abundant simple and ramified *Tuber aestivum* mycorrhizas. **B**. Monopodial-pyramidal ramification with cystidia and cylindric ends. **C.** Dense apical hyphae. **D.** Pseudoparenchymatous angular mantle surface cells (white box).

Attempts made to examine the morphology of the mycorrhizas in cross-section via hand-sectioning and staining for 12 hours in Lactophenol cotton blue proved successful. The staining however appeared somewhat selective to central cortex cells of the root, and some samples were entirely stained leaving little contrast between

fungal and plant cells (not shown). Differential interference contrast (DIC) microscopy greatly improved the clarity of images and contrast between fungal and plant cells compared with brightfield microscopy (**Figure 104 A vs B**). The mantle appeared to have discernible layers with the largest cells in the middle, and the Hartig net varied between 1-3 rows of cortical cells deep, with some areas apparently 1 and a half rows deep (**Figure 104 C**).

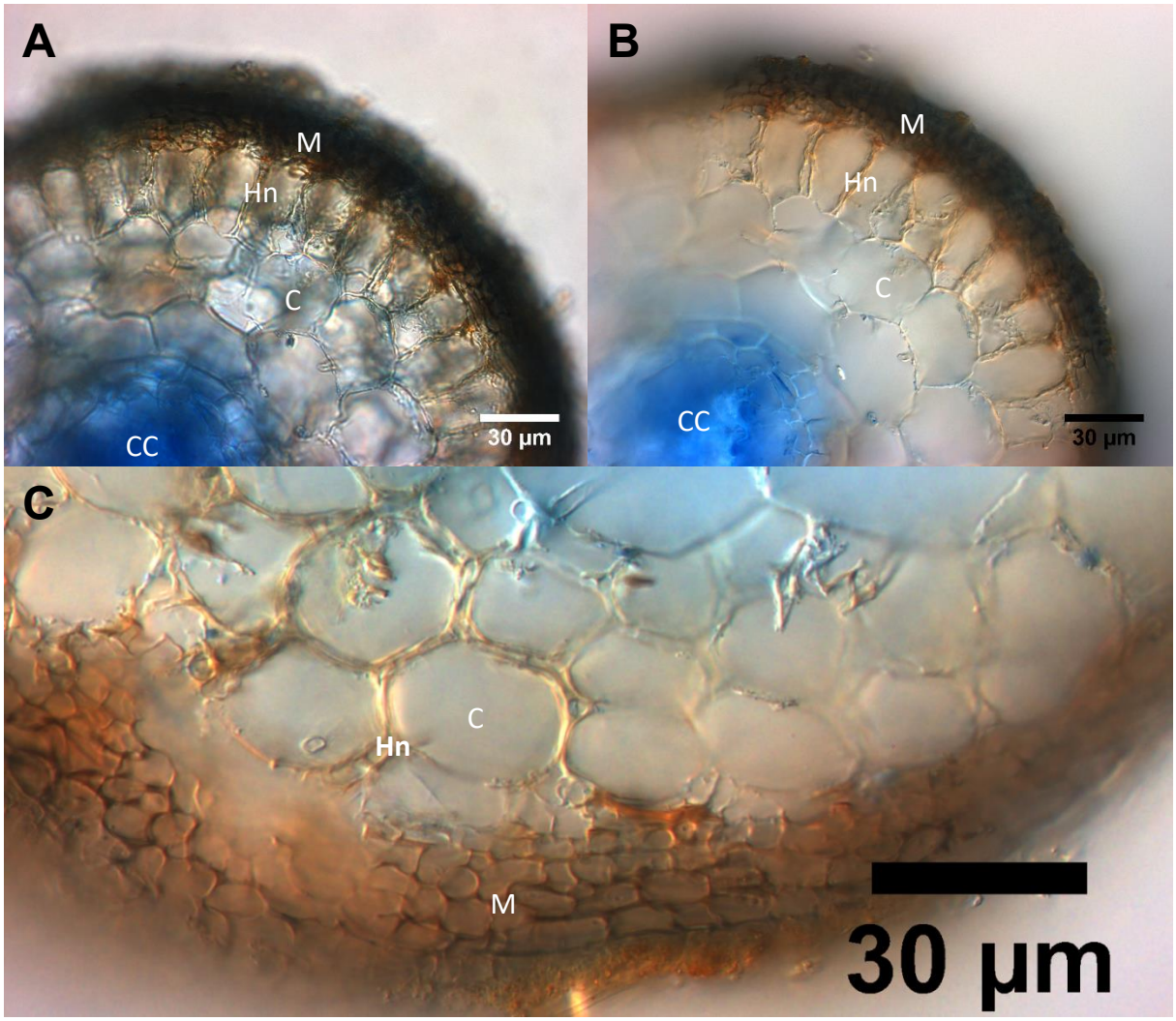


Figure 104. Cross-sections of *Tuber aestivum* mycorrhizas on *Quercus* sp., illuminated via brightfield (**A**) or DIC (**B, C**). **M** = Mantle, **Hn** = Hartig Net, **C** = Cortical cells, **CC** = Central root cortex.

Betula samples provided by Mycorrhizal Systems Ltd contained more sparse mycorrhizas with dense, woolly emanating hyphae with light cylindrical ends and a rough surface texture (**Figure 105 A-C**), compared with the *Quercus* samples. Imaging of the mantle surface was easier than with the *Tuber borchii* samples, and revealed a predominantly angular pseudoparenchymatous cell pattern matching that of *Tuber*

aestivum, but with plectenchymatous regions which are not typical of *Tuber aestivum* **(Figure 105 D and E**, respectively). Closer examination of emanating cystidia and mantle surface hyphae revealed the presence of clamp connections at septae (**Figure 105 F and G**, respectively), meaning that the samples were from a Basidiomycete species and not *Tuber*.

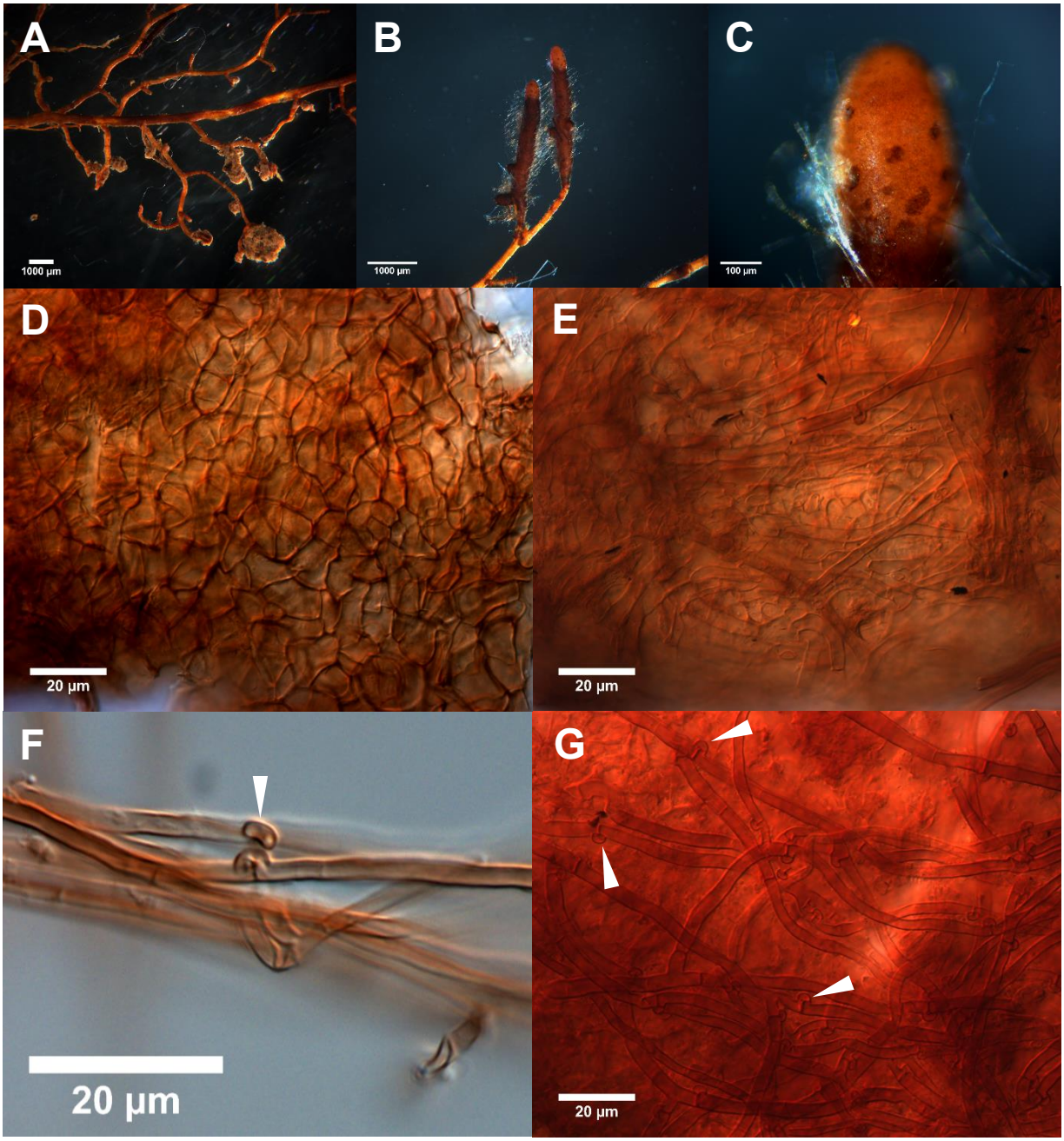


Figure 105. A. *Betula* sp. root system with sparse mycorrhizas with densely woolly emanating hyphae. **B**. Monopodial-pinnate mycorrhizas with densely wooly emanating hyphae and lighter cylindrical ends. **C.** Cylindrical tip with grainy mantle. **D-E**. Pseudoparenchymatous and plectenchymatous outer mantle cells. **F-G**. Emanating & surface hyphae with clamp connections at septae (arrows).

This finding highlights the importance of closely examining specimens under high magnification (or the use of DNA-based methods of identification) before use in further research or culture isolation attempts, as at first glance the mycorrhizas could be assumed to be a *Tuber* sp., but in fact did not even belong to the Ascomycota.

Confocal microscopy proved to be a useful method for the observation of mycorrhiza morphological features (**Figure 106**). The cells of the Hartig net (**Figure 106 A**), were visible around the entire cortex cells, as opposed to just in-between them as in DIC or brightfield microscopy, and the pseudoparenchymatous patterning was also visible. The pseudoparenchymatous angular surface pattern of the outer mantle was visible in good detail (**Figure 106 B**). Spores were imaged both within the ascus, where additional structures were also visible, and removed from the ascus, which provided greater clarity of surface ornamentation (**Figure 106 C and D** respectively).

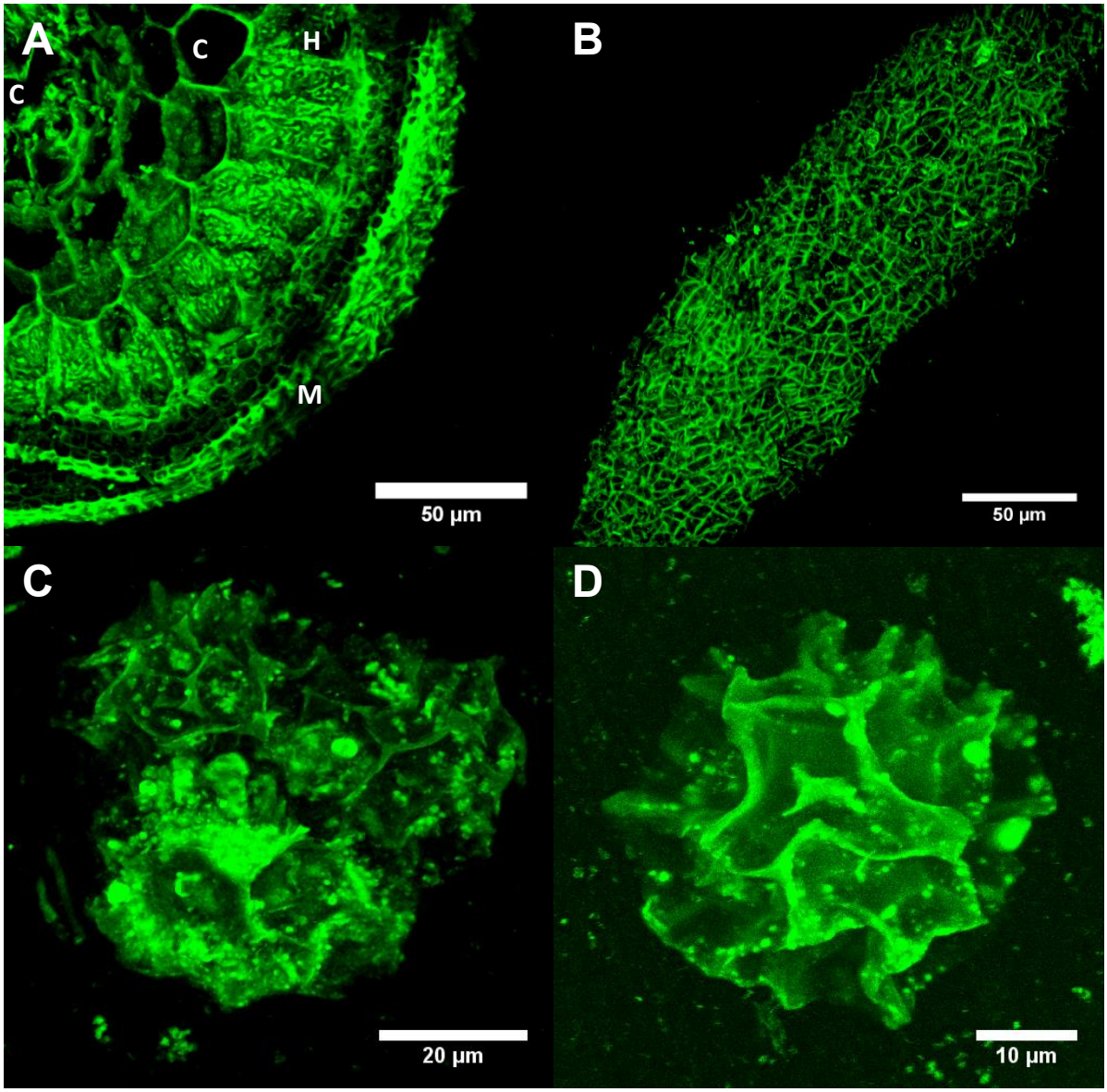


Figure 106. Confocal microscopy images of *Tuber aestivum* mycorrhiza cross-section (**A**), pseudoparenchymatous, angular mantle surface cell patterning (**B**), three spores within ascus (**C**) and single spore liberated from ascus (**D**). **M** = Mantle, **Hn** = Hartig Net, **C** = cortex cell, **CC** = Central Cortex.

5.5.2 Isolation of *Tuber* species from truffles, mycorrhizas and spores

The success rate of the *Tuber* isolation attempts from truffles and mycorrhizas was poor. The contamination rate was high (>25%) due to the fact that the samples contained bacteria, yeasts, and filamentous fungi, which tended to outgrow the *Tuber* mycelium even on antibacterial and selective media. It was also difficult to obtain fresh

material less than a few days old, ideally the starting material should be immature and less than 24h old. The cultures which showed growth all displayed very slow growth, taking several weeks to show initial growth, and mostly growing <5mm in total, where most appeared to stop growing after several weeks. Many samples appeared to grow aerially from the gleba tissue sample but not onto the agar, suggesting poor adaptation to the agar media. All cultures which were sub-cultured died with the exception of 1 culture which was obtained from *Tuber melanosporum* and transferred onto MEA with soil extract (**Figure 107**).

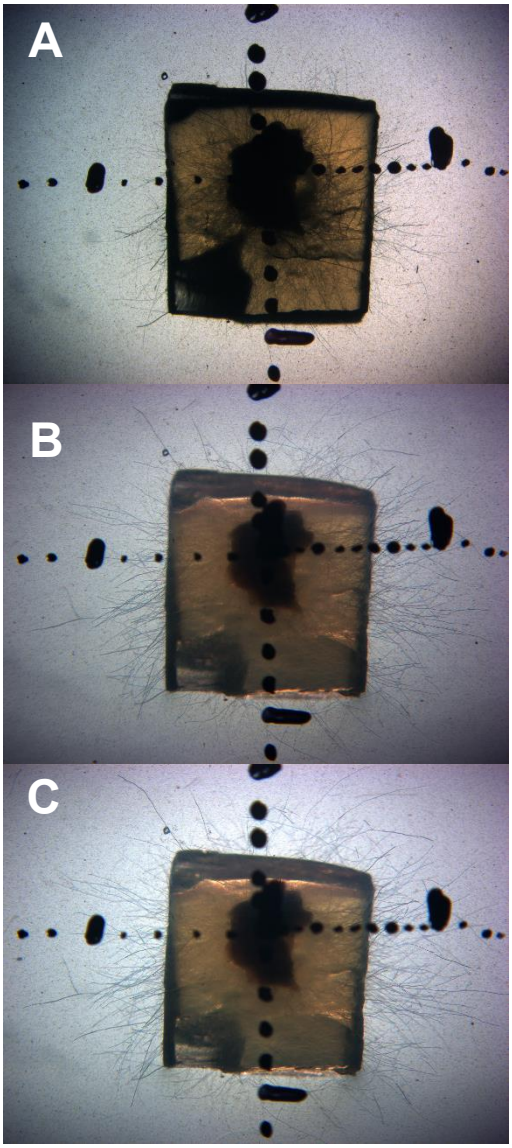


Figure 107. Culture isolated from *Tuber melanosporum* gleba after sub-culturing. **B** and **C** show growth 6 and 8 days after **A**, respectively. Some hyphae appeared to stop growing after some distance, whereas others continued.

Tuber borchii ATCC 96540 TT was the only *Tuber* culture used in *in vitro* testing which was positively identified, and displayed new growth <24h after the first sub-culture from the master culture onto PDA/2 (**Figure 108**). This species is one of the easier and faster species to grow *in vitro*, allowing the study of Tuber spp. in laboratory settings. Unfortunately, the cultures still proved to be slow growing and vulnerable to chemical changes in the medium. Many attempts were made to examine the effects of various epigenetic modifier compounds on the metabolic profile of the species, but the cultures often died in the presence of the modifier compounds, and had severely impacted growth, therefore further attempts to scale up and investigate potential effects of the epigenetic modifiers with this species were abandoned.

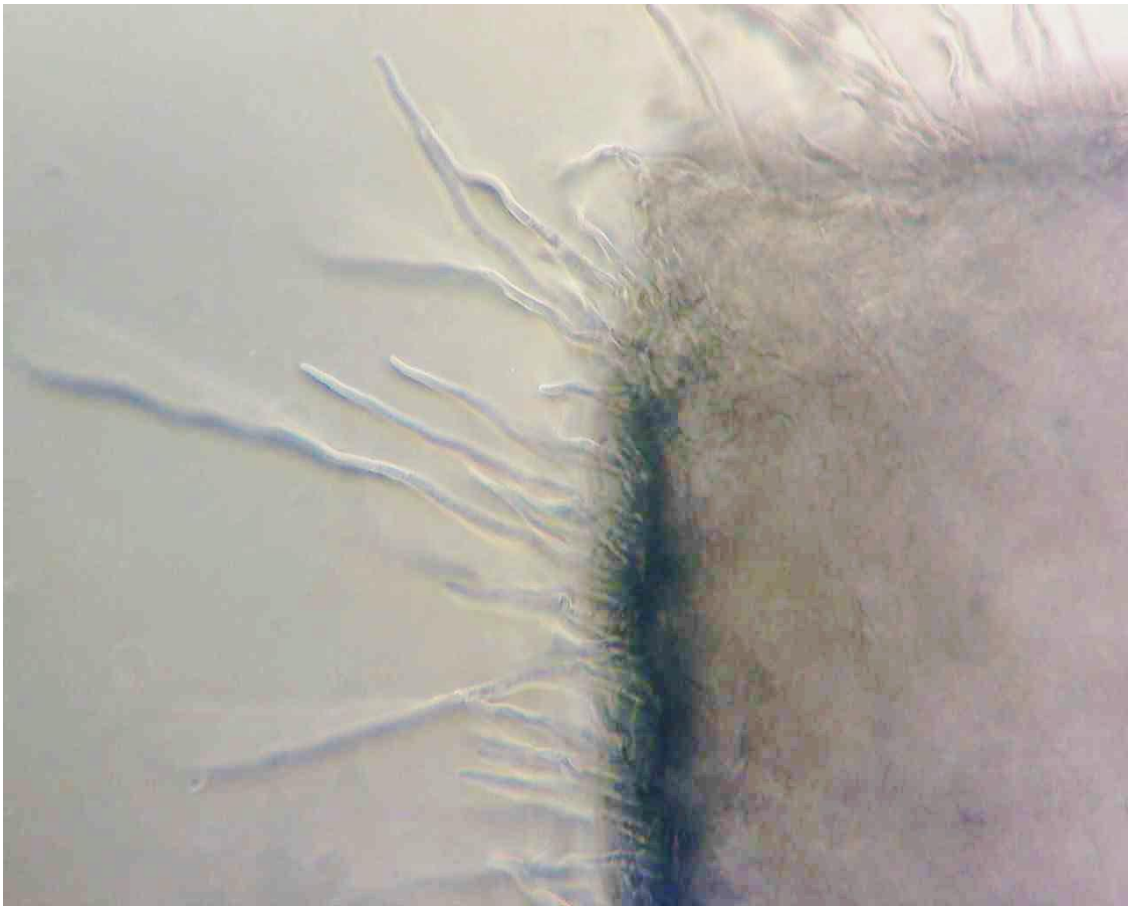


Figure 108. *Tuber borchii* growth <24h after transfer.

5.5.3 Spore germination

Tuber spore germination has only been reported a handful of times in the literature, and there is no reliable method of germination currently available. Therefore attempts were made to germinate *Tuber* spores (*T. aestivum* and *T. melanosporum*). Spores which were incubated in liquid medium (PDB) were found to be contaminated with flagellate protozoa after several weeks (**Figure 109**). The presence of protozoa in truffles is not currently reported in the literature. It is possible that this is a novel finding, which may be unique to the UK climate, but it is also possibly contamination. No germination was evident, and the flagellates eventually consumed the spores when present.

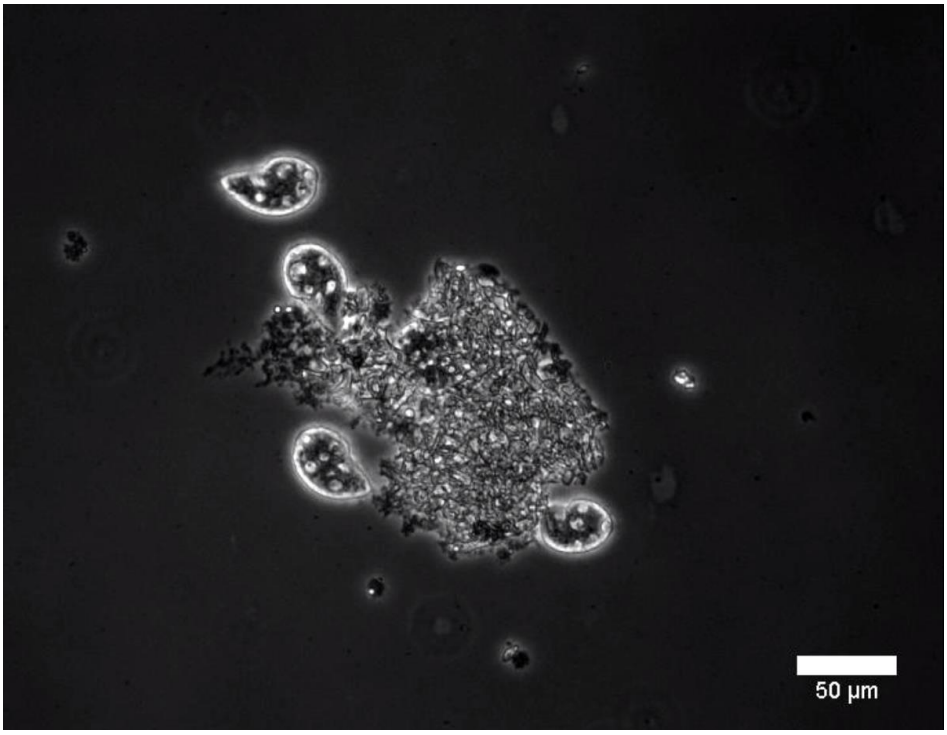


Figure 109. Unidentified protozoa consuming truffle tissue.

An experiment was conducted in order to determine if the presence of root homogenate and soil extract induced spore germination. Spore isolation, surface sterilisation and mixing with root homogenates proved semi-successful in that Chlorazine-T treatment prevented contamination without the inclusion of antibiotics in the media, and the spores were placed in close proximity to root homogenate on the soil agar, however no germination of the spores was observed.

5.5.4 Isolation of *Morchella* cultures

Due to the difficulties encountered isolating *Tuber* cultures, attempts were made to identify an alternative model. *Morchella* has several similarities to *Tuber*, both belonging to the Pezizales. Both genera are heterothallic, possessing two mating types, their ascocarps have high commercial value and some *Morchella* species can form mycorrhizas. *Morchella* mycelium is easier to grow *in vitro* than *Tuber,* however there are still significant difficulties associated with their cultivation, therefore they present an attravctive model for the study of epigenetics in edible Pezizales, and attempts were made to isolate cultures. When dried *Morchella importuna* ascocarps were checked for spores, asci were observed (**Figure 110 A**) however they were not abundant. Tissue disruption, filtering and centrifugation resulted in creamy pellets consisting mainly of spores with minimal tissue fragments (**Figure 110 D**). Spores readily germinated in water or PDB in <24h (**Figure 110 B**). Initially, spreading of isolated spores on PDYA or inoculation into PDB produced colonies within 24 hours (**Figure 110 C),** but the cultures were contaminated with rod-shaped bacteria. When plated onto PDYA containing ampicillin and chloramphenicol, the resultant cultures grew quickly. Washing spores with ethanol did not stop them from germinating, though the resultant cultures initially had a slightly brown colour compared with white mycelium from unwashed spores (not shown).

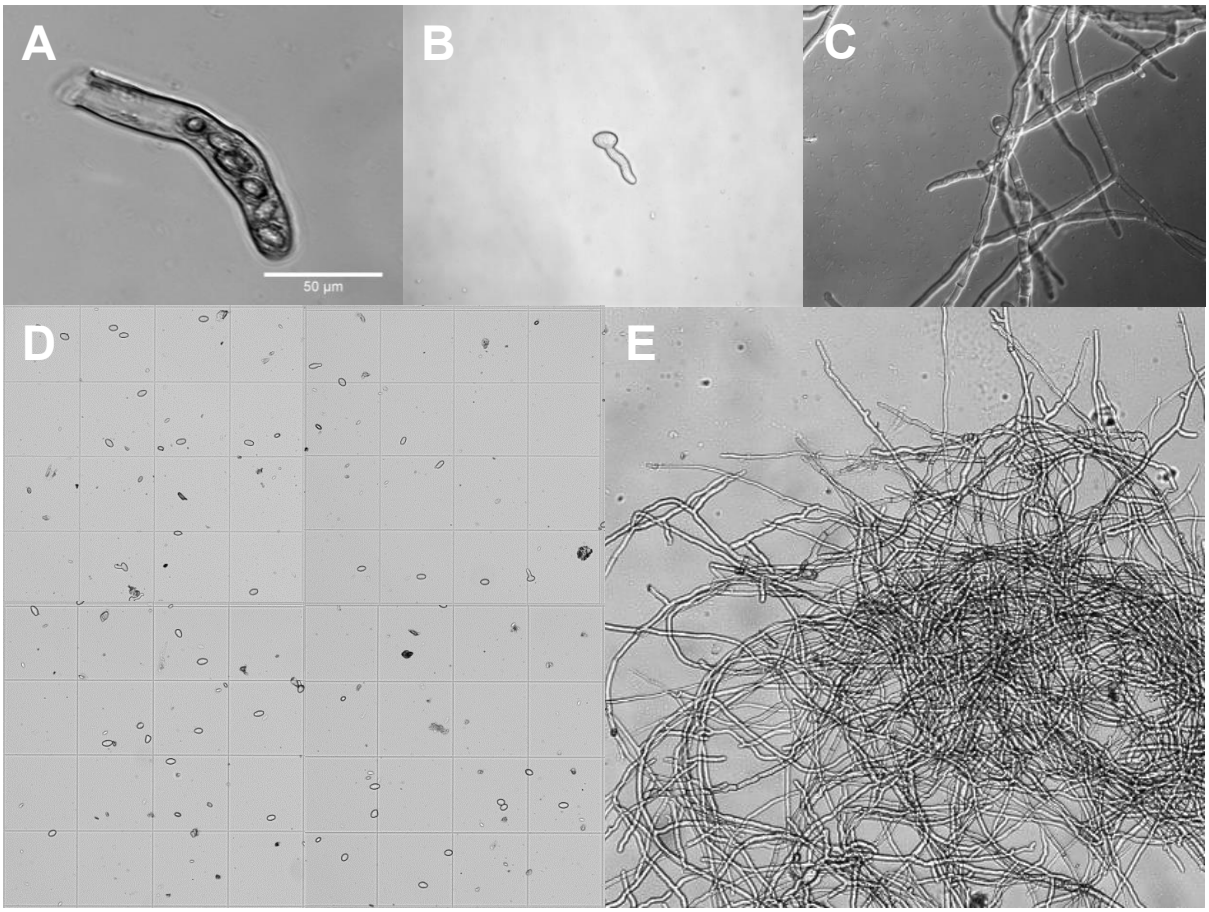


Figure 110. A. *Morchella importuna* ascospores in ascus. **B.** Spore germinating after 24h in water. **C**. Spore with hyphae and rod-shaped bacteria. **D.** Spore isolate sample in Neubauer haemocytometer. **E.** *Morchella importuna* culture after 24h growth in PDB.

Sub-sectioning ~5mm pieces of the initial culture onto fresh PDYA resulted in the isolation of several cultures which grew quickly, uniformly and free of contamination, almost covering the 100mm plate in 72h (**Figure 111 A-C**). Gross microscopic appearance of the mycelium matched those of *Morchella importuna* (**Figure 111 D-E**). The cultures began to develop aerial hyphae after several days and transitioned from a rhizomorphic appearance to a mould-like, fluffy appearance, then slowly began turning brown (**Figure 111 F**), presumably due to cellular ageing and metabolite production. Three isolates were sub-cultured onto 50/50 WA/PDA to check for sclerotia formation and test the effect of 5-azacytidine on this (and assess for toxicity). Only one of the isolates (Culture 1) formed sclerotia (**Figure 111 G-I**, white arrows), therefore the experiment was insufficient to observe any effect of 5-azacytidine. However, 5-azacytadine appeared to produce no obvious toxicity or

change in the macroscopic appearance of the cultures. Liquid cultures in PDB containing 100µM 5-azacytidine appeared to show no difference in speed of growth or colour. All cultures formed a mycelial mat on top of the liquid and began to turn brown (not shown). Although the species proved easier to work with than the *Tuber* genera, further experimentation with the culture was abandoned during early screening experiments following initial results from LCMS analysis, which showed that the culture extracts did not contain as many potential metabolites in high abundance as other strains (Data not shown).

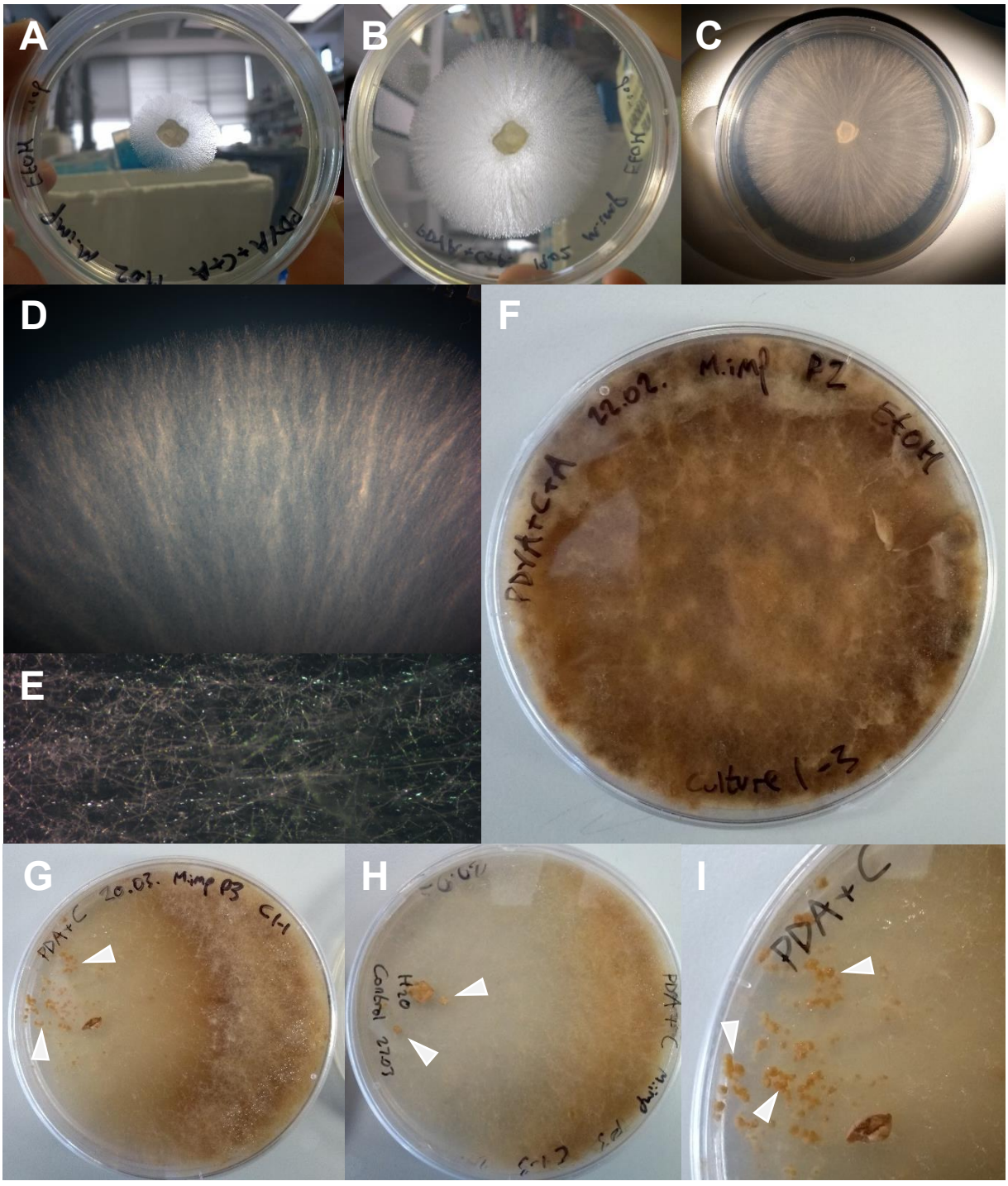


Figure 111. A-C *Morchella importuna* cultures 24, 48 and 72h after transfer on PDYA, respectively. **D.** Uniform rhizomorphic growth after 72h. **E.** Filamentous hyphae. **F.** Browning and matting of aerial hyphae after 2 months. **G-I**. Sclerotia formation (arrows) by isolate 1 on WA side ~1 month after transfer to 50/50 WA/PDA.

5.5.6 Propagation of *Cistus incanus*

A germination rate of 53% was obtained with the seed treatment, however several of the germinated seedlings exhibited slow growth and a dark-leaved phenotype. Several seedlings were obtained which appeared to be healthy and free of contamination, displaying bright green leaves and developing root structures (**Figure 112**). These cultures were intended to be used in attempts to study *Tuber borchii – Cistus incanus* interactions including in the pre-contact signalling stage and mycorrhiza morphogenesis, and also to examine the effects of epigenetic modifier compounds at these stages of the interaction, however, after inoculation of the seedlings with *Tuber borchii* mycelium, no mycorrhization of the plants was observed, and it proved too difficult to maintain suitable controlled conditions for both species using the Petri-dish model.

Figure 112. *Cistus incanus* seedlings on MS medium, before inoculation with *Tuber borchii* mycelium in attempts to create *in vitro* mycorrhizas.

5.5.7 Mating types in *Tuber aestivum*

Mating type distribution has never been reported from a UK plantation, and whether mating-type dominance occurs is still up for debate due to methodological limitations of previous studies and conflicting results. In order to determine the distribution of mating types in a *Tuber aestivum* plantation in Wales, mycorrhizas were collected and attempts were made to genotype them via PCR. Bands of the expected size for MAT1-1 and MAT1-2 (246bp and 348bp, respectively) were obtained from truffle DNA, but only MAT1-1 bands were detected in all mycorrhizas tested (**Figure 113**), except 1 sample in which no band was obtained, which may not have been a *Tuber aestivum* mycorrhiza. The lack of MAT1-2 may be due to mating type dominance occurring in the small area of the plantation from which the samples were obtained. More mycorrhiza samples were obtained to see if MAT1-2 bands could be detected, however subsequent attempts proved unsuccessful.

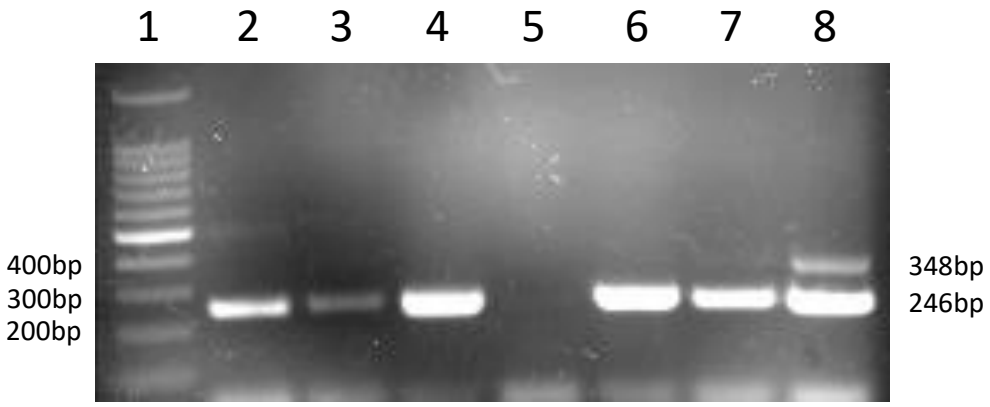


Figure 113. MAT1-1 (246bp) and MAT1-2 (348bp) bands in *Tuber aestivum* ascomata (lane 8) and mycorrhizas (Lanes 2-7). Both MAT 1-1 and MAT 1-2 were detected in ascomata as expected. Only MAT 1-1 has been detected in mycorrhizas. Lane 1 = 100bp increment DNA ladder.

5.5.8 *Tuber borchii* identification via PCR

A band of the expected size (432bp) was obtained from *Tuber borchii* DNA extracted from truffle tissue but not from two mycorrhizas (**Figure 114**, lanes 2 and 3-4 respectively).

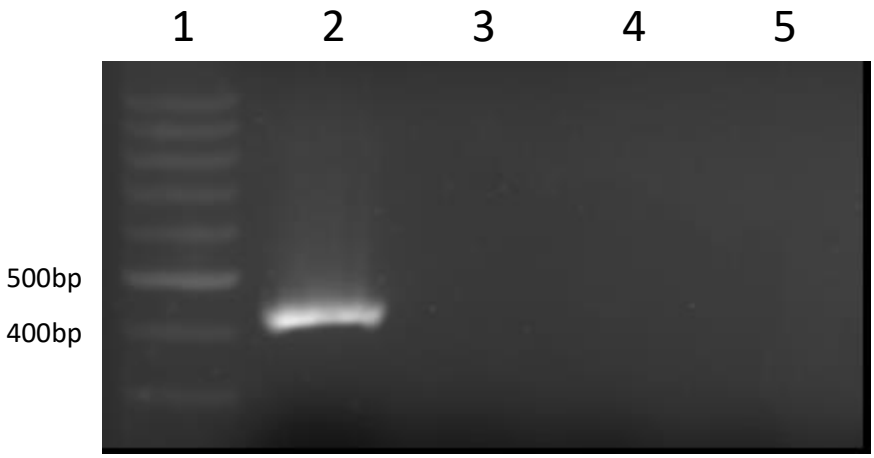


Figure 114. ~432bp band in lane 2 corresponds to the expected amplicon for the *Tuber borchii* specific primers. Lanes 3-4 are Mycorrhizas and Lane 5 is control (no DNA).

5.6 Investigating inter-species interactions and the effect of elicitor compounds in mycorrhizal fungi, utilising *Tuber aestivum* x *Quercus robur,* and several bacterial species

As previously described, truffle species are subject to many inter-species interactions with plants, animals, and other microbial species. Previous studies investigating the effects of bacterial co-culture with Tuber species both *in vitro* and in plant nursery settings have reported mixed results and species-depending effects. In this project, the effect of co-culture of three *Pseudomonas* strains on *Tuber aestivum* – *Quercus robur* mycorrhization was investigated in a nursery setting. At the time of writing, no previous experiments utilising these species combinations had been published. As well as this, a range of small molecule-elicitor compounds including epigenetic modifier compounds, nutritional additives and signalling molecules were included in the experiments to examine the effect of their presence on the *Tuber* – *Quercus* interaction.

5.6.1 Preparation of bacteria

Bacterial stocks of *Pseudomonas fluorescens* (strains SBW25 and F113) and *Pseudomonas protegens* (strain pf-5) were plated out onto LB agar and incubated overnight at 37^{o}C. Single colonies were selected and transferred into vials containing 10mL of LB liquid media, which was incubated for 12 hours (37^{o}C, shaking 120rpm) to create a seed culture. The seed cultures were transferred into 100ml of LB media and incubated for a further 12 hours (37^{o}C, shaking 120rpm), after which the cultures were added to 900ml of LB (creating 1L total) and incubated for an additional 12 hours. The cultures were then stored at 4^{o}C until use the following day.

5.6.2 Inoculation of plants

Young *Quercus robur* (European Oak) seedlings (n = 720) were germinated in wet perlite and selected at random for use in the experiment.

Tuber aestivum inoculum was prepared by blending fresh frozen ascocarps with vermiculite and water shortly before application. The mixture was rolled into small balls

weighing ~1g, which were then smeared onto the plant root system until all of the roots were covered with the inoculum.

The bacterial inocula were added by transferring 10mL of the bacterial suspensions with a syringe onto the root system after application of the *Tuber aestivum* inoculum.

Small molecule additives were added by dissolving in water to create stock solutions, which were then diluted up to 10mL and added the same way as the bacterial inocula to give a final concentration in the tray cells of 100µM. All plants received 10mL of liquid in total, and were regularly watered throughout the study period. Plants were intended to be sampled at 3-month timepoints, however due to the COVID-19 pandemic it was not possible to collect or process samples for an extended period of time, so sampling could only be conducted after 2 years.

Figure 115. A. *Quercus robur* seedling after inoculation with *Tuber aestivum* to the root system. B. Application of bacterial inoculum to a seedling following application of the *Tuber aestivum* inoculum. C. Example of the layout of experimental plants in the nursery environment.

5.6.3 Processing of root system/substrate

The plants were collected from the greenhouse, returned to the laboratory and stored at 4ºC until processing. Plants were removed from the cells with the substrate and root system intact, and measurements were recorded of the substrate depth, plant height from the substrate surface, and stem diameter at the base of the plant. The aerial parts of the plants were cut off at the stem base where it met the substrate, and the substrate was cut into 3 equal zones based on depth measurement. The middle section of the substrate and root systems were collected and cleaned by careful removal of the substrate using slow flowing cold water and gentle agitation. When enough substrate had been removed to reveal the root tips, the samples were stored wet at 4ºC until morphological examination.

5.6.4 Homogenisation of roots

After cleaning the substrate from the root system, the fine roots containing the root tips and mycorrhizas were cut from the larger roots. The fine roots were homogenised by cutting into short sections approximately 0.5-1cm in length. These cut root segments were gently mixed to homogenise.

5.6.5 Preparation of samples for mycorrhizal root tip counts

After processing, root segments were randomly selected and transferred into the lid of a standard petri dish with a $1cm^2$ grid on the outside of the lid. A small amount of water was added to avoid desiccation and improve image quality, and the base of the petri dish was inserted into the lid upside down so that the depth of the root samples were consistent (**Figure 116 A)**.

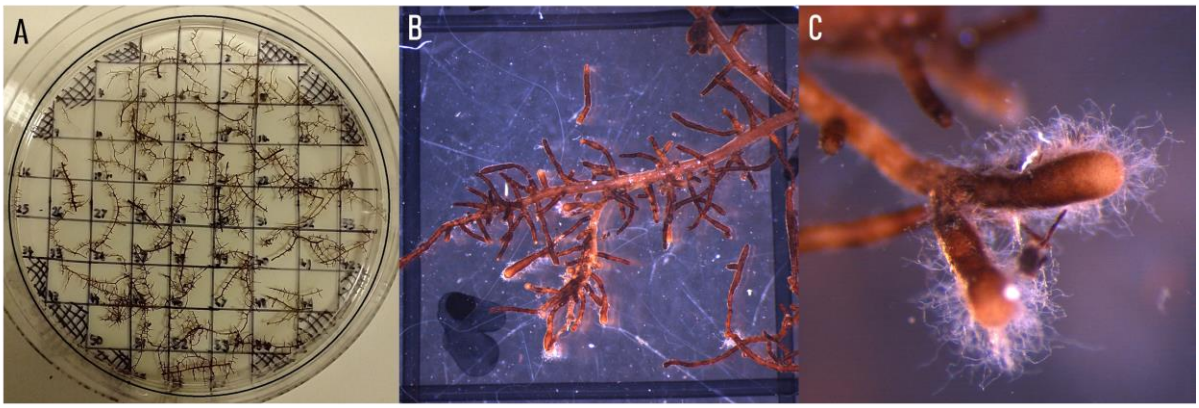


Figure 116. A. Petri dish with 1x1cm gridline filled with randomly selected fine roots for mycorrhizal counts. **B.** Close up of square from **A**, showing several *Tuber aestivum* mycorrhizas amongst non-mycorrhizal root-tips. **C.** *Tuber aestivum* mycorrhizas with characteristic swollen appearance and abundant emanating hyphae

5.6.6 Morphological Identification of Tuber mycorrhizas

In order to assess the percentage colonisation of the root samples by Tuber mycorrhizae, the root tips were examined under a stereo microscope. Identification was performed by morphological analysis; petri dish squares were selected at random and examined, mycorrhizal tips consistent with Tuber aestivum morphology were counted as Tuber aestivum mycorrhizas, mycorrhizas not-consistent with Tuber aestivum morphology were counted as contaminant (or non-Tuber) mycorrhizas. Root tips without mycorrhizas were counted as normal root tips.

In the event of questionable or difficult to identify mycorrhizas, samples were taken and examined under higher magnification to observe the appearance of the mantle. Mantles displaying the characteristic Tuber aestivum grid like morphology and lack of clamp connections were recorded as Tuber, mantles with other morphology were recorded as non-tuber. Any mycorrhizal tips which did not display tuber morphology were counted as non-Tuber mycorrhizas and not identified to species level. Occasionally unusual mycorrhizal tips were observed which displayed two distinct regions of varying morphology (Not shown). It is unclear what the cause of this phenomenon was and not possible to identify the fungal species, so these tips were counted as non-Tuber.

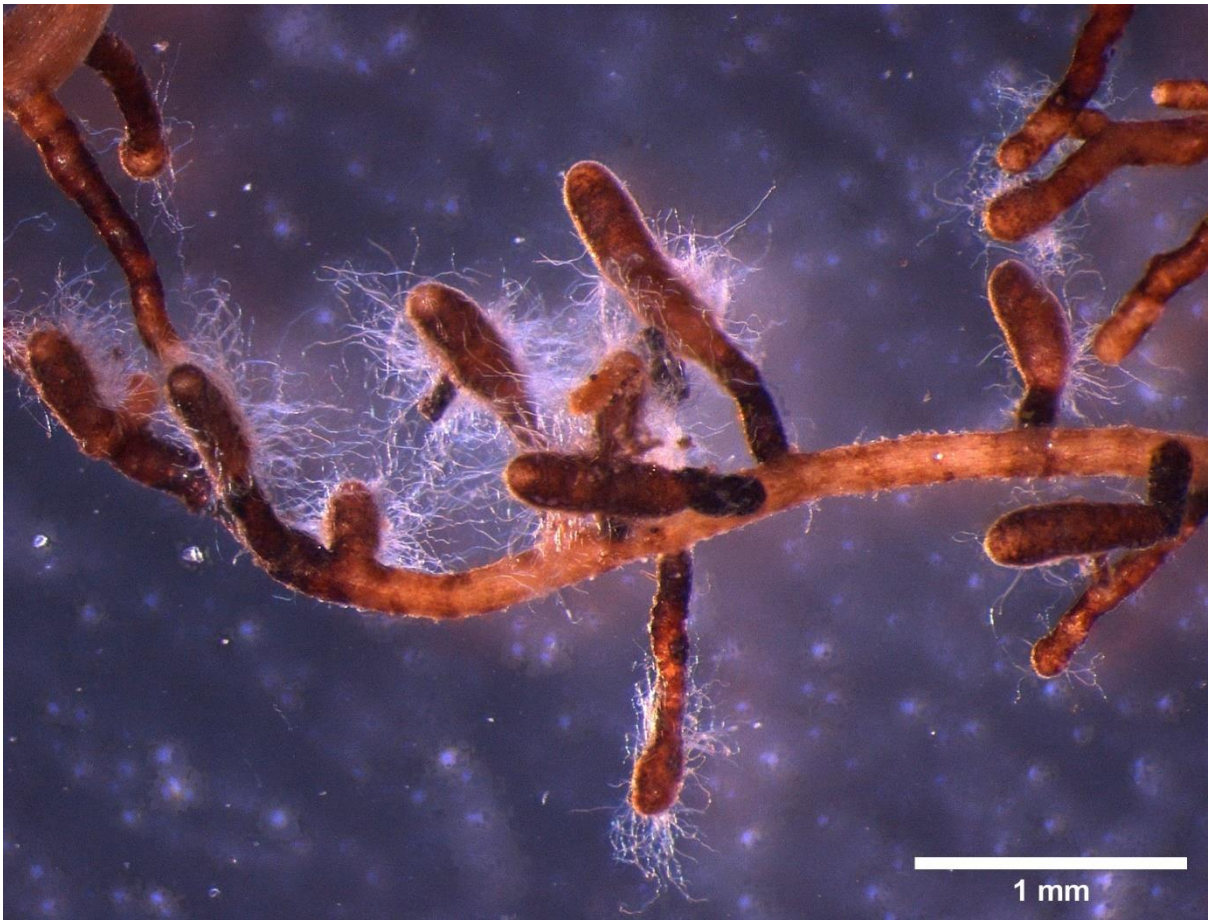


Figure 117. Abundant *Tuber aestivum* mycorrhizae on a root section from a sample co-cultivated with *Pseudomonas protegens*

5.6.7 Effect of bacterial co-culture on mycorrhizal colonisation

Unfortunately due to the restrictions caused by the COVID-19 pandemic, it was not possible to conduct regular sampling of the plants, or to process all of the samples intended, so only certain groups were selected for processing and analysis. Within the processed samples, there was a large amount of variation in mycorrhizal colonisation of the root systems by both *Tuber aestivum* and contaminant mycorrhizas; areas of

heavy colonisation were observed as well as zones with very little or no mycorrhization.

The untreated group of plants had an average percentage colonisation of 19.6%. Most of the treatments did not have a significant effect on the percentage colonisation by *Tuber aestivum* (**Figure 102)**, however two of the bacterial groups did have statistically significant higher percentage colonisations than the control group (*Tuber aestivum* inoculation only) (P = <0.05). *Pseudomonas protegens* pf-5 had the highest percentage of colonised root tips (43.6%), and *Pseudomonas fluorescens* F113 had the second highest (30.0%).

The increased colonisation observed in the bacterial co-culture groups warrants further investigation. As truffle forming species are slow-growing, bacterial additives which improve the rate or total level of colonisation of the host root system by *Tuber* species could lead to commercial benefits, possibly reducing the time that it takes for inoculated trees to start producing truffles. As well as this, it is possible that a greater level of colonisation of the plant root system may give the *Tuber* species a competitive advantage against other mycorrhizal fungi present in the environment.

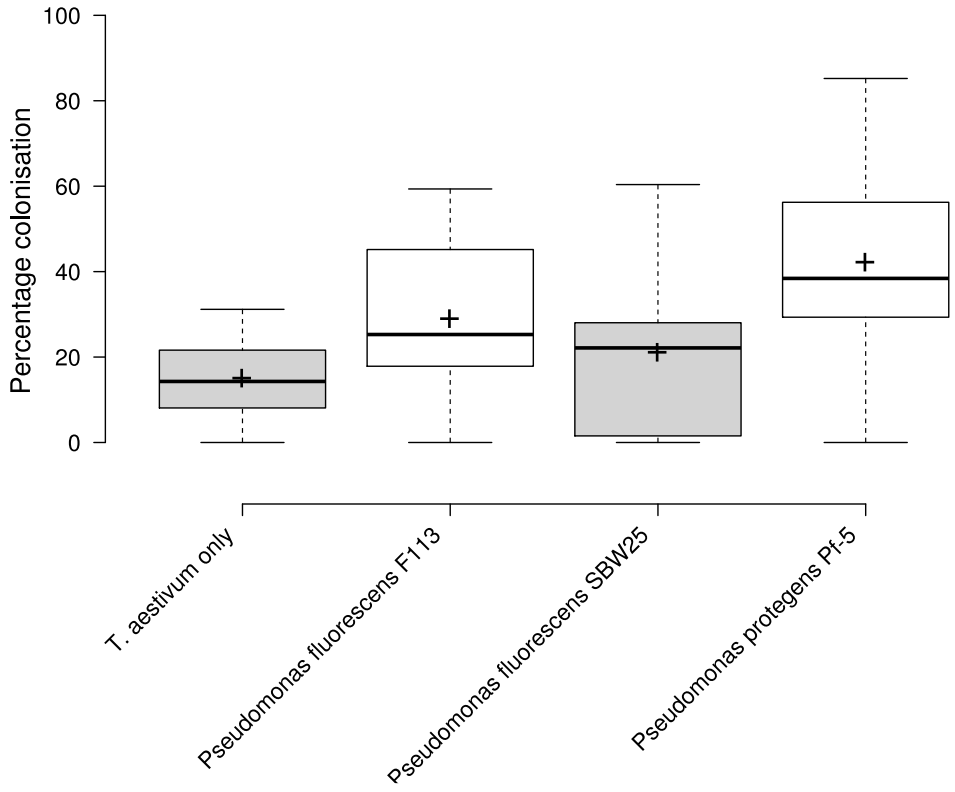


Figure 118. Percentage root tip colonisation by *Tuber aestivum* mycorrhizas between the Tuber-only control and the bacteria-treated groups. Centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots; crosses represent sample means. n = 12 sample points. Generated using BoxPlotR.

Chapter 6

6.1 Overall Conclusions and Discussion

Fungi have historically proven and remain to be an extremely promising source of new natural products with potential varied medicinal applications. The true extent of fungal metabolites discovered appears to be only a fraction of what they are capable of producing, but the problems remain that their biosynthetic gene clusters are often silent or cryptic in laboratory conditions, insufficient yields are often obtained from laboratory fermentation, extraction, and purification, which makes it difficult to determine the chemical structure and potential bioactivity of isolated compounds. The process is also lengthy, difficult and expensive. It is also difficult to obtain novel species, which may exist in inhospitable environments and be difficult or currently impossible to cultivate using standard laboratory methods.

Various different techniques and technologies have been explored and suggested as solutions to these issues, including repressive gene deletions, predominant BGC deletions (resulting in the organism utilising alternative BGCs and producing alternative products), fungal-co-culture systems, bacterial co-culture systems, heterologous gene expression in other organisms, epigenetic modification mechanisms (such as those employed in this research), traditional media variation screening approaches and addition of precursors, and other chemical agents such as enzyme inhibitors. All of these approaches are valid and proven techniques which have yielded fruitful and promising results, which is promising and reassuring considering the rising problem of anti-microbial resistance in human pathogenic microorganisms.

In this work, a great number of fermentation extracts were obtained and chemically investigated, from a diverse range of native British fungi isolated and grown in multiple different media with and without the presence of a variety of epigenetic modifiers and other small molecule additives, alone and in combination. As well as this, a number of mushroom-forming species were collected for extraction, chemical analysis and for use in antimicrobial assays. Many of these extracts were fractionated utilising different

liquid-liquid extraction and chromatographic fractionation methods, creating a library of samples available for further purification and bioactivity screening.

To the knowledge of this author, this work included the first successful laboratory cultivation and chemical investigation of arachnogenous fungi originating from Europe, as well as their morphological examination, their imaging via scanning electron microscopy, and attempts to inoculate spiders using conidiospores and cultures obtained from wild specimens (not shown), as almost all of the previously published work has been conducted using strains originating from South-east Asia, where there is a far greater level of biodiversity.

Morphological examination of the arachnogenous species collected in this project revealed clear morphological differences, suggesting that they are all distinct species. The multi-gene phylogenetic analysis of the primary *Gibellula* isolate used in this work, along with the swollen tips of the synnemata observed on the specimen, suggest that it is potentially a new species. Further genetic testing of the other arachnogenous fungi collected in this work should be conducted in order to ascertain if they too are potentially new species. The finding from the multi-gene phylogenetic analysis that the primary isolate may represent a new species in the *Gibellula pulchra* clade brings into question the validity of previous identifications of spider-parasitic fungi recorded within the UK; it is possible that as identifications have generally been performed based on morphological examination alone, that there is in fact a greater diversity of undiscovered spider-pathogenic fungal species in Europe than is currently known. Regardless, the fact that no previous chemical investigation into the spider-pathogenic fungi of Europe shows that they represent a currently under-researched group of fungi, which may represent an untapped source of novel natural products. The published chemical investigations of species originating in South-East Asia also support this view, as they have proven to be fruitful sources of novel natural products, some with interesting bioactivity. As well as this, metabolic profiles within the same species originating from the same area in Thailand have shown that small differences in collection location can result in differences in the metabolite profiles produced through laboratory fermentation. This may also be the case within other areas of the world, and supports the view that entomopathogenic fungi which have been studied in other geographic locations should still be examined to determine if geographic variation results in metabolic differences.

In this work, several of the other spider-pathogenic fungi isolated and other entomopathogenic fungal isolates were also investigated in screening experiments and shown to have significantly different metabolic profiles (not shown). However most of the experimentation and chemical studies were conducted using the first *Gibellula* isolate. Fermentation of the culture in varied media showed clear differences in the resulting metabolic profiles, validating media variation as a method which can still be successfully utilised to increase the number of metabolites produced via laboratory liquid fermentation.

Following the initial small-scale screening experiments, repeated attempts were made to scale-up fermentations in order to isolate and identify the metabolites seen in the LCMS/MS analyses. Unfortunately, overall the success rate in isolating metabolites in sufficient purity and yield for the HRMS and NMR testing required for structural elucidation was low. The chemical composition of extracts of not just the *Gibellula* isolates but also of the other species examined, for example *Onygena equina* and *Gymnopilus junonius*, was very complex, consisting of components which proved difficult to separate despite repeated attempts utilising various liquid-liquid extraction procedures, chromatographic techniques and rounds of chromatographic method development.

Nevertheless, the studies conducted showed that the primary *Gibellula* strain utilised as well as the other fungal isolates studied produce varied compounds in different media which may represent novel natural products, or known natural products that have not been previously reported from these species. For example, in large-scale fermentation in ZM media, metabolites were produced which based on LCMS/MS analysis appeared to be related to the pigmentosins previously identified from the *Gibellula* genus. MS and UV-Vis spectrographic analyses combined with natural products database searches resulted in the tentative identification of Aschernapthopyrone B, a compound that is structurally similar to the Pigmentosins which has been previously reported from the scale-insect pathogenic fungus *Aschersonia paraphysata*, but not the *Gibellula* genus. However, although the MS and UV data suggest that this compound is a good match to what was observed, it was not possible to unambiguously identify the metabolite. It is possible that the compound observed may be a closely structurally related analogue, of which there are several that can be predicted through repositioning of functional groups on the structure.

The addition of epigenetic modifiers and other small-molecule additives to the culture media often resulted in changes in the fungal morphology, colour of the culture medium and in the metabolic profiles observed in HPLC and LCMS analyses, showing that manipulation of epigenetic mechanisms in *in vitro* fermentation of these species can result in the production of compounds not produced, or produced to a lesser extent in the standard media. A good example of this being the significant upregulation of metabolites seen in *Onygena equina* when fermented in the presence of Vorinostat.

The discovery of Gibellamine A and B from *Gibellula gamsii* and the several other β-carbolines which have been previously reported from other entomopathogenic fungi raised the question of whether the *Gibellula* isolates obtained in this work could also produce β-carbolines in *in vitro* fermentation, despite β-carbolines having not been previously found in other *Gibellula* species. In the media variation screening experiments, β-carbolines were not detected with the exception of the tentative identification of Perlolyrine from epigenetic-treated cultures. It was hypothesised that through addition of tryptamine, tryptophan, or similar compounds, the fungus may utilise the precursors in the biosynthesis of β-carbolines if it was capable. Initial-small scale fermentations with tryptamine, l-tryptophan, d-tryptophan and tryptophol, addition of tryptamine did result in the production of new peaks in the HPLC chromatograms, and the fermentations in the presence of tryptamine were scaled up to investigate further in attempts to identify the new metabolites. In the tryptamine-treated fermentations of *Gibellula pulchra*, the primary biotransformation product was determined via LCMS/MS and NMR analyses to be acetyltryptamine, a compound which has not been previously reported from spider-pathogenic fungi. Although acetyltryptamine was found to be the primary product, LCMS/MS analysis of the other chromatographic fractions collected revealed an apparent abundance of other tryptamine derivatives present in the media, detected via screening the data for known tryptamine MS/MS fragments as well as predicted fragments generated *in silico*. Analysis of the mass spectrographic data combined with the use of natural products database searches allowed the tentative identification of several other varied tryptamine-related compounds, though these compounds were present in a much lower yield than the acetyltryptamine, and again proved difficult to purify in sufficient yield for structural confirmation.

Through chromatographic fractionation, extraction method development and LCMS/MS method development, several metabolites were tentatively identified as β-carbolines which have previously been identified from other related entomopathogenic fungi, based on analysis of the LCMS/MS data, specifically the fragmentation patterns and UV-Vis absorption spectra, which in some cases proved to be characteristic of β-carbolines. Unfortunately it was not possible to obtain sufficient yields or purity in most cases in the attempts made to isolate the compounds from the tryptamine-treated fermentations, many compounds were present which were apparently structurally similar and proved difficult to separate chromatographically, but the data collected suggests that an impressive variety of tryptamine-related compounds were present in the samples. Further work should be conducted investigating the biotransformation of tryptamine in entomopathogenic fungi, as the number of metabolites produced and tentatively identified in this work suggests that the approach may facilitate the discovery of potentially novel alkaloids compared with standard media-screening approaches.

Following the work with tryptamine alone in YM media, the combination of tryptamine and epigenetic modifiers in ZM media, which had previously proved to produce a greater variety of metabolites than YM media, was also trialled in a large scale fermentation utilising a bioreactor setup, as well as an improved acid-base extraction procedure to increase the selectivity of the extraction for alkaloids. The results from this fermentation showed that there was an interaction between the added epigenetic modifiers and the tryptamine, with an apparent upregulation of these tryptamine metabolites compared with non-epigenetic modifier treated fermentations, suggesting that the combination may also have potential utility as a method of discovering novel products in *in vitro* setting (Data not shown).

It is worth noting that little is currently known about the role which the metabolites the spider-pathogenic fungi produce play in its interactions with the infected spider host, but *Gibellula* spp. infected spiders (as well as other entomopathogenic fungi) are often found attached by the fungus or the insect to the underside of leaves, away from the animals usual habitat. As some β-carbolines such as harmine are known to interact with the nervous system as inhibitors of Monoamine oxidases, and serotonin is known to influence the behaviour of spiders (for example in aggressiveness and mating), it is

possible that *Gibellula* spp. may utilise metabolites to interfere with the normal functioning of monoamine neurotransmitters before the death of the infected spider.

Attempts to cultivate *Gibellula pulchra* on spiders *in vitro* were made, as this has not previously been published and may potentially serve as a model to study the effects of infection on spider behaviour, species selectivity of infection, and morphological development of the fungi on different spider hosts. Inoculated spiders were regularly examined under the stereo microscope, and in some cases fungi were observed to colonise the spiders after death, though the morphology of the resulting fungi varied and the species were not identified (not shown).

The synnemata observed growing on agar cultures of *Gibellula pulchra* were an unusual observation which is not commonly seen with *Gibellula* grown *in vitro*. Examination of the lab grown synnemata using standard microscopy methods as well as under the scanning electron microscope revealed morphological differences compared with the synnemata formed on the original spider-colonised specimens (not shown). Through stereo-microscopic examination of the lab-grown synnemata, it was observed that several plates on which synnemata had formed appeared to have a potential contaminant in proximity to where the synnemata formed (not shown). This may suggest that there are inter-species interactions involved in the development of synnemata, and is an area which warrants further investigation.

The screening of wild UK-native mushrooms in this work for novel chemistry and anti-bacterial efficacy has led to the production of a large extract library containing a plethora of metabolites, that likely include many novel compounds as well as known compounds that have not yet been detected in these species. The discovery of potent, broad-spectrum antibacterial extracts from selected interesting native basidiomycetes potentially has implications in the field of antibiotic discovery, and of our understanding of the true chemical complexity and potential of these species. The last new class of antibiotic to be discovered was in 2004, meanwhile overuse and inappropriate use of antibiotics, for example general use in foodstuff for agricultural animals, is still contributing to the issue of rising anti-microbial resistance. The lack of new classes of antibiotics discovered in recent decades, with the rising rate of resistance, is of great concern as it is possible that if new, effective drugs are not discovered, then our ability

to treat serious bacterial infections will be significantly weakened, and mortality from such cases will surely rise.

Most antibiotics derived from fungi have been from Ascomycete species, the most famous examples being the β-lactam class (Penicillin and its analogues) and the cephalosporins. The only class of antibiotic discovered to date from a basidiomycete species is the Pleuromutilins discovered in 1951, which are broad-spectrum and powerful antibiotics. Most fungal alkaloids discovered to date are also derived from Ascomycetes, perhaps the most famous example being *Claviceps purpurea* (Ergot), which led to the discovery of LSD, along with many ergoline-based drugs and semi-synthetic derivatives that are potently pharmacologically active and now used for a variety of medical applications, such as in dementia. Much lower numbers of alkaloids have been discovered to date from basidiomycete species. The most famous example is surely the *Psilocybe* genus, along with certain *Panaeolus* species known for producing simple psychoactive substituted tryptamines that have predominantly psychedelic or hallucinogenic effects. The legal regulations which have controlled these fungal species and natural alkaloids may have hindered our ability to research the true chemical complexity of these species utilising modern instrumentation and potentially uncover their medicinal potential. Recent clinical trials of Hallucinogenic alkaloids derived from fungi and plants (such as Psilocybin, DMT, and LSD) have had initially promising results suggesting potential efficacy in mental disorders such as depression, anxiety, end-of-life anxiety, and PTSD.

The sheer number of metabolites detected in this work from acid-base extractions of selected native basidiomycete mushrooms compared with the number of compounds known from these species suggests that historically, extraction procedures, screening methodology, and the limited capabilities of the analytical instrumentation available at the time were inadequate to extract, detect, elucidate their structures and test these metabolites in bioassays for potential medical application. As our technological capability has increased substantially since many of our native mushroom species were last examined chemically, and the methods used to screen for and detect antibiotics from natural sources has improved, revisiting many of our native mushroom species with more modern methodology and sensitive instrumentation may reveal that we have greatly underestimated the chemical potential of many species, and that they should be revisited using more exhaustive and comprehensive extraction procedures.

For example when *Gymnopilus junonius* has been examined in the past, the extraction techniques utilised were not selective or appropriate for alkaloids, as the methods utilised in this work were.

The initial bioassay results in this work suggest that the basidiomycete derived compounds may have significant potential for bioactivity and applications in medicine, and these fungal species and their metabolites should be examined further. Genome sequencing projects of mushrooms, combined with OMICS approaches to identify potential antibiotic biosynthetic gene clusters in basidiomycetes will surely help to target species of interest that may be potential producers of undiscovered antibiotics.

In the greenhouse project with *Tuber aestivum*, unfortunately it was not possible to sample at various timepoints as was intended, nor was it possible to sample the full set of plants included in the original experiment, including several of the plant groups treated with small molecules. However, co-culture with bacteria was found to significantly increase the percentage mycorrhization of the host plant root-system. There are no previously-published experiments utilising the combinations of species utilised in this study, making it a novel finding which should be explored further. Bacterial co-culture screening-based approaches utilising greater numbers of bacterial species, potentially those isolated from the *Tuber* spp. rhizosphere, may lead to the discovery of additional bacterial species and strains which have a beneficial effect (Mycorrhiza helper bacteria) in promoting greater root-system colonisation by *Tuber aestivum* mycorrhizas. If the co-culture of bacteria in these settings proves to have a consistent beneficial effect, then it may have potential applications in the truffle industry, which could lead to economic benefits if utilised on a large scale.

In summary, this research has highlighted the potential within fungi, showcasing the medicinal potential of underexplored fungal species as well as how their chemical diversity can be accessed and utilised. From the arachnogenous fungi and wild native UK mushrooms to the mycorrhization enhancements observed in Tuber aestivum, the research presented here contributes to the fields of natural products and inter-species interactions between ectomycorrhizal fungi and helper bacteria. Findings made, such as the novel bioactivities in basidiomycetes and the beneficial effects of bacterial co-culture on mycorrhization, offer promising avenues for the development of new

therapeutic agents and agricultural innovations, particularly against a backdrop of rising antimicrobial resistance and agricultural optimisation needs.

Appendices

1. DNA sequences of genes used in the multi-gene phylogenetic analysis of *Gibellula pulchra*

TEF1._Assembly_consensus_sequence

GAACAGCGAAACGGCCCAGAGGGGGGTAGTCGGTGAAAGCCTCAACGCACATGGGCTTGGAGGGAACCATC
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TCTTCTCCTGAAGCTCGGCGAACTTGCAGGCAATGTGGGCGGTGTGGCAGTCAAGAACGGGGGCATAACCGT
TACCAATCTGGCCAGGGTGGTTGATGACAATAACCTGAGCCTGGAAGGAAGCGGCACCCATGGGGGGGTCG
TTCTTGGAGTCACCAGCAACGTTACCACGACGGATTTCCTTGACGGAGACGTTCTTCACGTTGAAACCAACGTT
GTCACCGGGAACACCCTCGGACAGCTGCTCGTGGTGCATCTCGACAGACTTGACTTCAGTGGTGACGTTGGCG
GGAGCGAAGGTGACAACCATGCCGGGCTTGATGATACCAGTCTCGACACGGCCGACAGGCACCGTTCCGATA
CCACCAATCTTGTAGACATCCTGGAGGGGAAGACGGAGAGGCTTGTCGGTGGGACGCTTGGGGGGCTCAAT
GGAGTCGATAGCCTCAAGAAGAGTCTTACCAGTGGACTTGCCAGCCTTGGTCTCCTTCTCCCAGCCCTTGTACC
AGGGGCAGTTGGAGGAAGGCTCAAGCATGTTGTCGCCGTTGAAGCCCGAGATGGGAACGAAGGCAACAGTC
TTGGGGTTGTAGCCGACCTTCTTGATGAAGTTGGAAGTCTCCTTGATGATTTCTTGGAAACGGTTCTCGGACCA
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ITS._Assembly_consensus_sequence

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CCCCATGCCAACCTACCTTTGTTGCCTCGGCGGACGATGTCGGTTTTTTTCCACCGACCCGGCCGCCGTGGACC
ACGCAGACCCACAGCGTCACGGACGCCAGCAGCCCGTCTGAGCCCGCCGCGAGGCAGCGCAAAGCGAGTCA
AAACTTTCAGCAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGCGAAT
TGCAGAATCCAGTGAGCCATCGAATCTTTGAACGCACATTGCGCCCGGCAGCCACTCTGCCGGGCATGCCTGT
TCGAGCGTCACCTCAGCCCTCGAGCCGCCGCCCTCCCAGGCGGCGGCGCGGCGTTGGGGCGCGGCAGCTCCC
CCTGCCAGCCCCGAAATGCAGCGGCGGCCCGCCCGCGGCGACCTGTGCGCAAAGTAGCCATACCTACGCACC
GGGAGCCGGGCGGGACGAACCTCGCACCCTGCTCACCCGCGGGGTGAAGCTGCACAGGCTTCTGTGCAGCA
CACGTTGACCTCGGATCAGGCAGGACCACCCGCTGAACTTAAGCATA

LSU._Assembly_consensus_sequence

CTACTAGAAGGTTCGATTAGTCTTTCGCCCCCATGCCCATGTTTGACGATCGATTCGCACGTCAGAACCGCTGC
GAGCCTCCACCAGAGTTTCCTCTGGCTTCACCCTACACAGGCATAGTTCACCTTCTTTCGGGTCCGGCCCCGTA
TGCTCCTACTCAAATCCATCCGAGAACATCAGGATCGGTCGATGATGCGCCGAGGCTCCGGCTCTCACCTGCG
TTCGCTTTCACTGCGCGTAGGGGTTTGGCACCCCAACACTCGCATACGAGGACGACTCCTTGGTCCGTGTTTCA
AGACGGGTCGCCGGCGACCATTACGCCAGCATCCTTGCGGTGCGCGTGCCTCGGCCCGGCACGGGGCATGGC
GCGGCGGGCTATAAGCACTCCCTGAGGAGCCACGTTCCCGCCGCCTTTGTCCCCCGCGCCGAGCCGATGCTG
GCCTGCGCCCGGCAGAGTGCCCCTGGCCCGAGAGCCTGGGTGACCTGCCGGGCCCAAGTCTGGTCGCGGGC
GCTTCCCTTTCAGCAATTTCACGTGCTCTTTAACCCTCTTTCCAAAGTGCTTTTCATCTTTCGATCACTCTACTTGT
GCGCTATCGGTCTCTGGCCGGTATTTAGCTTTAGAAGACATATACCTCCCACTTTGAGCAGCATTCCCAAACTA
CTCGACTCGTCGAAGAAGCTCTACACTGGCCTGGCGTCCGACCGGACGGGGCTCTCACCCTCTGTGGCGTCCC
GTTCCAGGGAACTCGGAAGGCGCCGCGCCAAGAGCATCCTCTGCAGATCACAACTCGGGCCCCCGCCGGGGG
GCCAGATTTCAAATCTGAGCTGCTGCCGCTTCACTCGCCGTTACTGGGGCAATCCCTGTTGGTTTCTTTTCCTCC
GCTTA

RPB1._Assembly_consensus_sequence

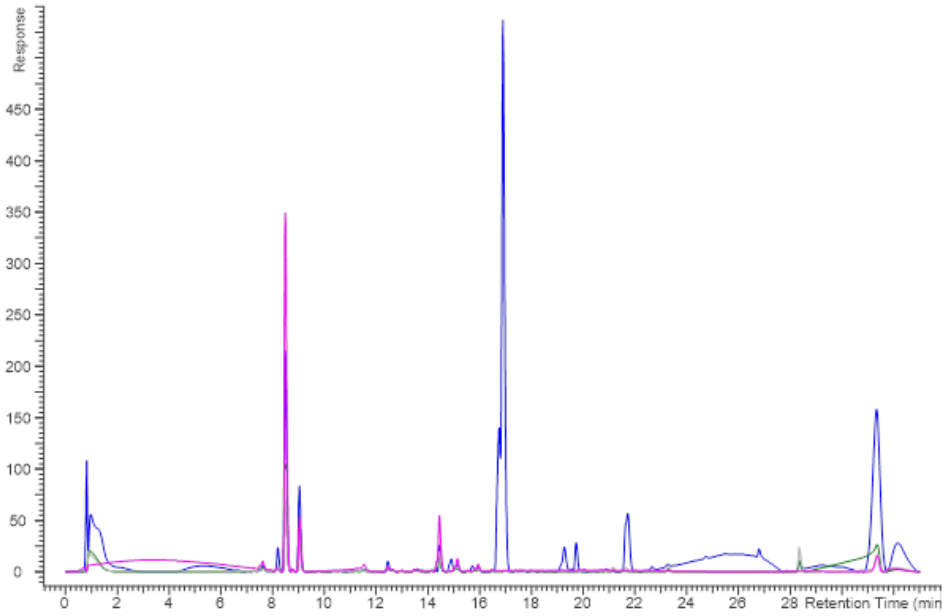
TCCATGTAGGTAGCGACATGATATTGCAAAAGCTCCTCAAAGTCGCGCGCAATGTGCTGCGGTGATCCTTCGC
GAATGGCCTGCTTGACGTTGCCGTTGGCACGAATAATGTCACCCAGCTTGTAGGTCAAATCATCTTCGTTTCTC
ATACCCGTACCAGTGCCATCCATGGAGATACTCGGACGCACAGGAGGCGGCGGCACGGGTAGGACGGTAAC
GATCATCCACTCTGGACGTGCATAGTCCGCGTTGAGGCCAATGTTATGCAAGTCGCTCTCAGAAATACGACGA
AGGATACCGTGAGCCATTTCAGCAGTGATGTTGATGGTTTCTTTTCTCTTGGGCCCTCCATCTTCCGGAACAAC
TTCAAAGGCGGCCTTGAGTTGCAGAGCAGCTTGCCTGACTAGTGGCTGCATGTTACCACATCCGCCGTGACCC
TCGACGACGGGATTCTTTATACCCGGAGCAAAGGCCTCATCGTTGTTCTTGTCCTGTCTATCCTCATTTTCGCAT
TTTCTTTTCTTCTTGCAGACCGCCCAAACGCGCTTGAAACGAACTTTGGGGTCGCGAGTACGCAGGGCTGCGA
CAAAATCTGGATCGGACTGAACAAGTCATGTTAGCATCCACTAAACTTGAGAAAGAGAAGCGTCATGAAGAA
AGGAAAGACTAACTTCATCGGCCAACACTTTACTACAGTTGTGGCAAACAATCTCCAAAACTTTTTTGACTTTTT
TGATAAAACCAGGATGATAGACAGGTTTGGCCAGTTCTATATGACC

2. Results of antimicrobial assays not presented in main text

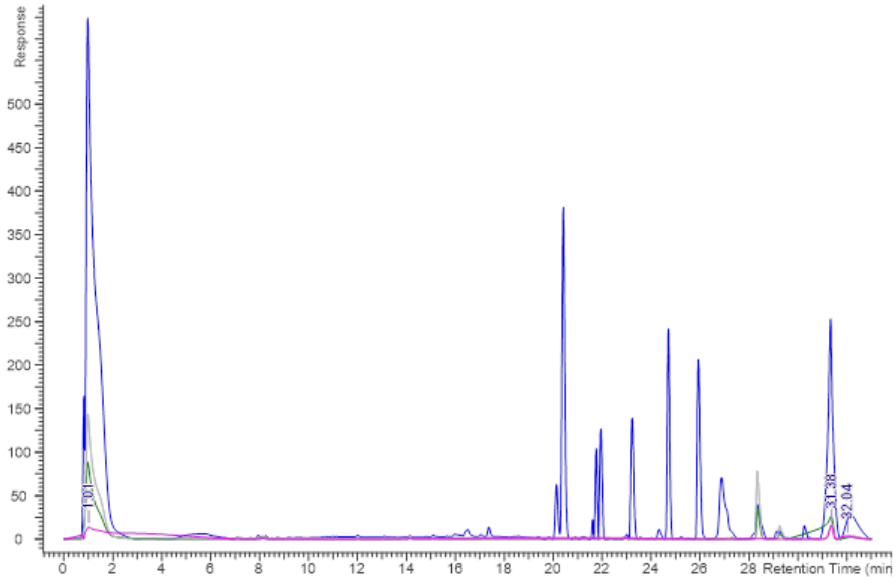
Assay	Concentration range	Sample code	Species	Sample name	MIC (µg/mL)	
					E. coli	S. aureus
1	0-256µg/mL	AM1	Amanita citrina	Amanita citrina MeOH Extract	-	-
		AM2	Amanita strobiliformis	Amanita strobiliformis MeOH Extract		
		AM3	Amanita strobiliformis	Amanita strobiliformis Acid-Base 1 (Acidic stage)		
		AM4	Amanita strobiliformis	Amanita strobiliformis Acid-Base 1 (Basic stage)		
		AM5	Onygena equina	Onygena equina ZM 1.5L Biomass 2 (Felt) MeOH		
		AM6	Onygena equina	Onygena equina ZM 1.5L Biomass 1 (Submerged) MeOH		
		AM7	Onygena equina	Onygena equina ZM 1.5L Biomass 1 (Submerged) Acetone - EtoAc		
		AM8	Onygena equina	Onygena equina ZM S/N		
		AM10	Phaeolepiota aurea	Phaeolepiota aurea MeOH		
		AM11	Phaeolepiota aurea	Phaeolepiota aurea Hexane		
		AM12	Gibellula 1	Gibellula 1 ZM 0.5L Farnesol S/N EtoAc - MeOH		
		AM13	Gibellula 1	Gibellula 1 ZM Biomass Defatted MeOH		
		AM14	Gibellula 1	Giellula 1 2.3L Biomass Hexane		
		AM15	Gibellula 1	Gibellula 1 5L ZM S/N CT 6-8		
2	0-256µg/mL	AM1	Onygena equina	ZM Biomass EtoAc	-	-
		AM2	Gibellula 1	5L Biomass F4 (T9-12)		
		AM3	Gibellula 1	ZM 5L S/N EtoAc	256	256
		AM4	Gibellula 1	5L Biomass F5	128-256	256
		AM5	Gibellula 1	5L Biomass F11 (T26-37)	256	256
		AM6	Gibellula 1	2.3L Biomass Hexane	256	256
		AM7	Gibellula 1	5L S/N CT13-21	256	256
		A	Ganoderma lucidum	Mushroom extract	-	None
		B	Ganoderma resinaceum	Mushroom extract		256
		C	Ganoderma lucidum	Fermentation Biomass extract		-
		D	Ganoderma lucidum	Spore Extract		
		E	Ganoderma resinaceum	Fermentation Biomass extract		
		J1	Stinkbug fungus (Beauvaria bassiana)	Supernatant Extract	256	256
		J2	Stinkbug fungus (Beauvaria bassiana)	Biomass MeOH	-	256
		J3	Stinkbug fungus (Beauvaria bassiana)	Biomass Hexane		256
3	0-1024µg/mL	AM3	Gibellula 1	ZM 5L S/N EtoAc		1024
		AM4	Gibellula 1	5L Biomass F5	1024	-
		AM5	Gibellula 1	5L Biomass F11 (T26-37)	-	
		AM6	Gibellula 1	2.3L Biomass Hexane		
		AM7	Gibellula 1	5L S/N CT13-21		
		J1	Stinkbug fungus (Beauvaria bassiana)	Supernatant Extract	1024	1024
		J2	Stinkbug fungus (Beauvaria bassiana)	Biomass MeOH	-	512-1024
		J3	Stinkbug fungus (Beauvaria bassiana)	Biomass Hexane		512-1024
		B	Ganoderma resinaceum	Mushroom extract		256-512
		Ampicillin			2	1
		DMSO			-	-
4	0-1024µg/mL	AM31	Gibellula 1	6MP EtoAc	1024	-
		AM32	Gibellula 1	HP20 Acetone		
		AM33	Gibellula 1	T9-12 Combi 2 Start and End		
		AM34	Onygena equina	ZM 1.5L S/N MeOH Combi Pooled Tubes		512-1024
		AM35	Onygena equina	Combiflash other tubes		256-512
		AM36	Onygena equina	Mycelial Felt Excretion		-
		AM37	Gibellula 1	Josh Control B S/N		
		AM38	Gibellula 1	Josh 1mM Tryptamine F S/N	-	
		AM39	Gibellula 1	Josh 100uM D S/N		
		AM40	Gibellula 1	Josh 1mM Biomass E+F MeOH		1024
		AM41	Gibellula 1	Josh 1mM Biomass F Hexane		1024
		Ampicillin			1	2
		DMSO			Slight 1024	Slight 1024

5	0-1024µg/mL	AM42	Onygena equina	ZM SN Remaining Tubes		Slight 1024
		AM43		ZM Biomass 2 (Felt) MeOH	Slight 1024	Slight 1024
		AM44		ZM Biomass EtoAc	-	-
		AM45		ZM Biomass DCM Extract		
		AM46		ZM Biomass 1 MeOH Freeze Precip Crystals		
		AM47		ZM Biomass 1 EtoAc CT37-47		~1024
		AM48		ZM SN MeOH CT 14-16	~1024	~1024
		AM49		ZM 1.5L Biomass 1 MeOH	-	-
		AM50		ZM Biomass 1 Acetone - EtoAc - MeOH		Slight 1024
		AM51		ZM S/N		-
		AM52	Gibellula 1	ZM 5L Biomass Aliquot	Slight 1024	~1024
		AM53	Gibellula 1	ZM SN MeOH 80% Hexane Extracted	1024	512-1024
		AM54	Gibellula 1	ZM 2.3L SN Hexane	-	-
		AM55	Onygena equina	ZM Biomass 1 Acetone - EtoAc - Freeze Precipitate		~1024
		AM56	Onygena equina	ZM Biomass EtoAc CT22-27		512-1024
		AM57	Onygena equina	ZM Biomass	~1024	-
		AM58	Gibellula 1	5L SN CT9-12	~1024	256
		AM59	Onygena equina	ZM SAHA 1 SN	-	-
		AM60	Onygena equina	ZM Pooled SN Hexane		
		AM61	Onygena equina	ZM 1.5L Biomass 1 EtoAc CT28-36		256
		AM62	Gibellula 1	Josh - Tryptamine		512-1024
		AM63		Josh - Control A Biomass MeOH	~1024	512-1024
		AM64		Josh - Control A Biomass Hexane	-	512
		AM65		Josh - Fraction C		-
		AM33		T9-12 Combi 2 Start and End	~1024	~1024
		Ampicillin			1 to 2	0-1
		DMSO			Slight 1024	None
6	0-1024µg/mL	AM66	Laetiporus sulphureus Oak	MeOH - MeOH (After Chloroform/Hexane)	-	-
		AM67	Laetiporus sulphureus Oak	MeOH - MeOH Freeze Precipitate		
		AM68	Laetiporus sulphureus Yew	Yew Hexane		128-256
		AM69	Laetiporus sulphureus Oak	Oak Chloroform		128-256
		AM70	Laetiporus sulphureus Oak	Oak H2O - MeOH		-
		AM71	Laetiporus sulphureus Yew	Yew H2O - MeOH		
		AM72	Gibellula 3	Lloyd Q6 Bio MeOH 27/01		
		AM73		Lloyd Q6 S/N EtoAc MeOH		
		AM74	Gibellula 2	Ali ZM Bio Hexane		
		AM75		Ali Q6 S/N 21.10.19	slight 1024	~1024
		AM76		Ali Q6 Bio Hexane	-	-
		AM77		Ali ZM Bio MeOH		
		AM78	Gibellula 1	ZM S/N Hexane		
		AM79	Gibellula 2	Ali Rice Hexane Aliquot		
		AM80		Ali Rice MeOH Aliquot 07.11.19		512-1024
		AM81		Ali Rice Hexane 07.11.19		-
		AM82		Ali YM Bio MeOH		
		AM83	Gibellula 1	YM S/N Farnesol EtoAc - MeOH		
		AM84	Amanita strobiliformis	MeOH - HCL - DCM 30.06.21		128-256
		Ampicillin			1 to 2	0 to 1
		DMSO			-	-
7	0-1024µg/mL	AM84	Amanita strobiliformis	MeOH - HCL - DCM 30.06.21	~1024	64
		AM85		AM84 CombiFlash F1 CT23-27	1024	512
		AM86		AM84 CombiFlash F2 CT28-35	~1024	64-128
		AM87		AM84 CombiFlash F3 CT36-45	-	64-128
		AM88		AM84 CombiFlash F4 CT46-55		-
		AM89		AM84 CombiFlash F5 Propan-2-ol Wash		
		Ampicillin			2	1
		DMSO			-	-
8	0-1024µg/mL	AM90	Amanita strobiliformis	AM87 CT15-20	1024	64
		AM91		AM87 CT22-27	1024	16-32
		AM92		AM87 CT32-34	1024 Partial	8
		AM93		AM87 CT54-58	-	8 (partial)
		AM94	O. equina	4.11.21 F1		~1024
		AM95		4.11.21 F2	1024 Partial	~1024
		AM96	Gibellula 1	22.10.21 Q6 04.03 CFT18-25 F1	None	512-1024 Partial
		AM97		YM 1mM Tryptamine S/N CFT29-30	1024	1024 Partial
		AM98		YM 1mM Tryptamine S/N CFT16-22	1024 Partial	512-1024 Partial
		AM99		YM 1mM Tryptamine S/N CFT23-28	1024 Partial	1024 Partial
		AM100		YM 1mM Tryptamine S/N CFT14-15	1024	1024
		AM101		YM 1mM Tryptamine S/N CFT8-13	-	1024 Partial
		AM102		YM 1mM Tryptamine S/N CFT37-45		-
		AM103	O. equina	11.11.21 FA	1024 Slight	128-256
		AM104		11.11.21 FF	1024	64-128
		AM105		11.11.21 FG	-	-
		AM106		26.10.21 F1		
		AM107		26.10.21 F2		
		AM108	Gibellula 1	25.10.21 F11 CFT10-11		
		Ampicillin			1	1 to 2
		DMSO			-	-

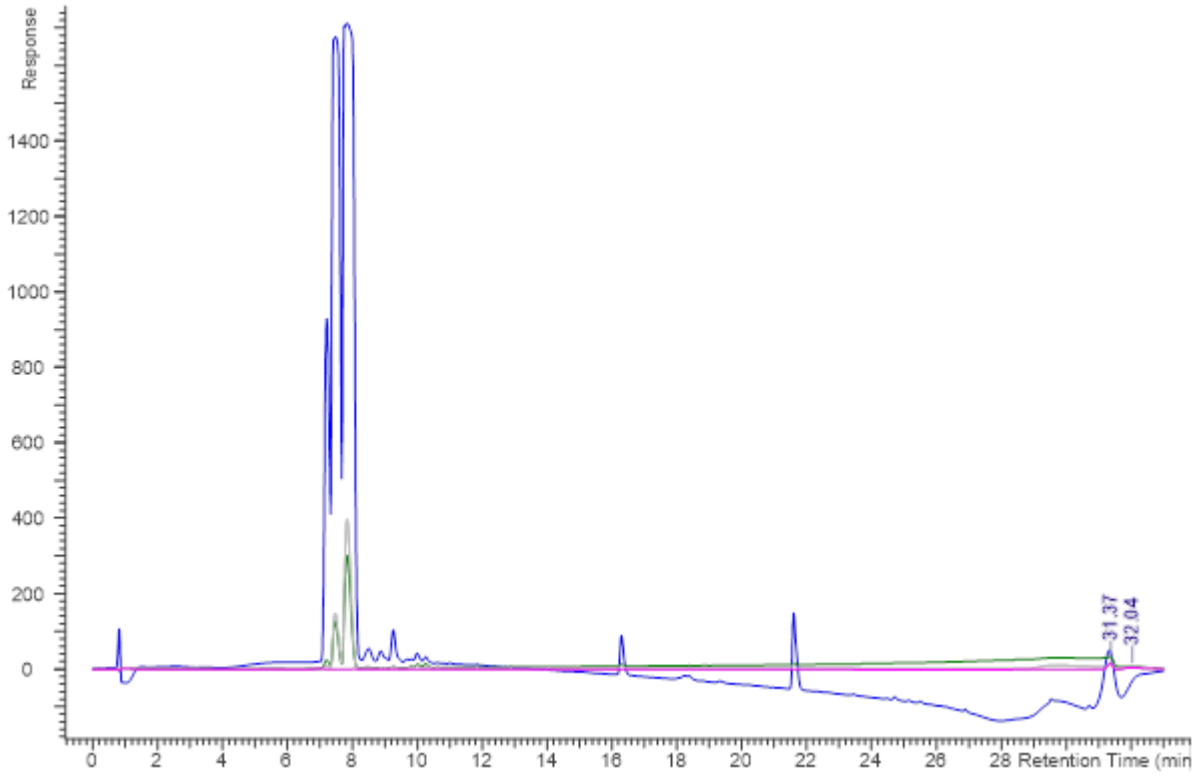
3. **HPLC Chromatograms (Blue: 210nm, Green: 254nm, Grey: 270nm, Pink: 360nm) of various mushroom crude extracts and semi-purified fractions used in antimicrobial assays**



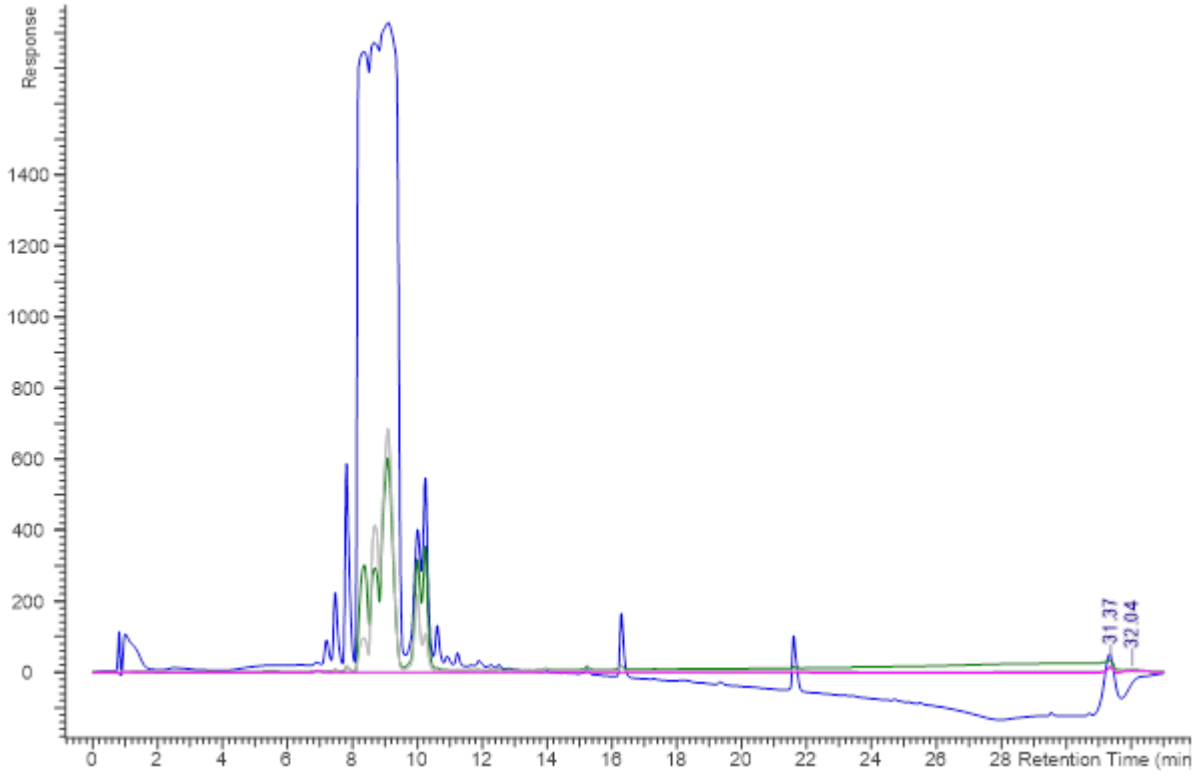
HPLC Chromatograms (Blue: 210nm, Green: 254nm, Grey: 270nm, Pink: 360nm) of *Gymnopilus junonius* crude MeOH extract



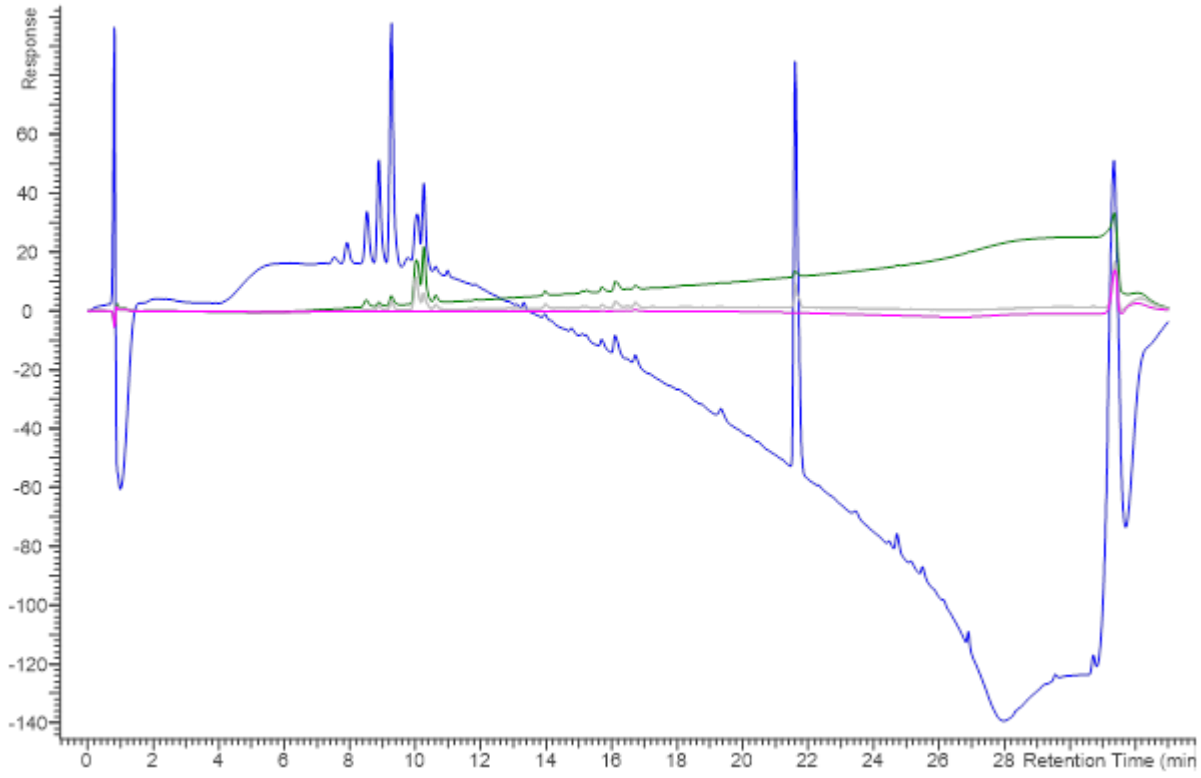
HPLC Chromatograms of *Amanita citrina* crude MeOH extract



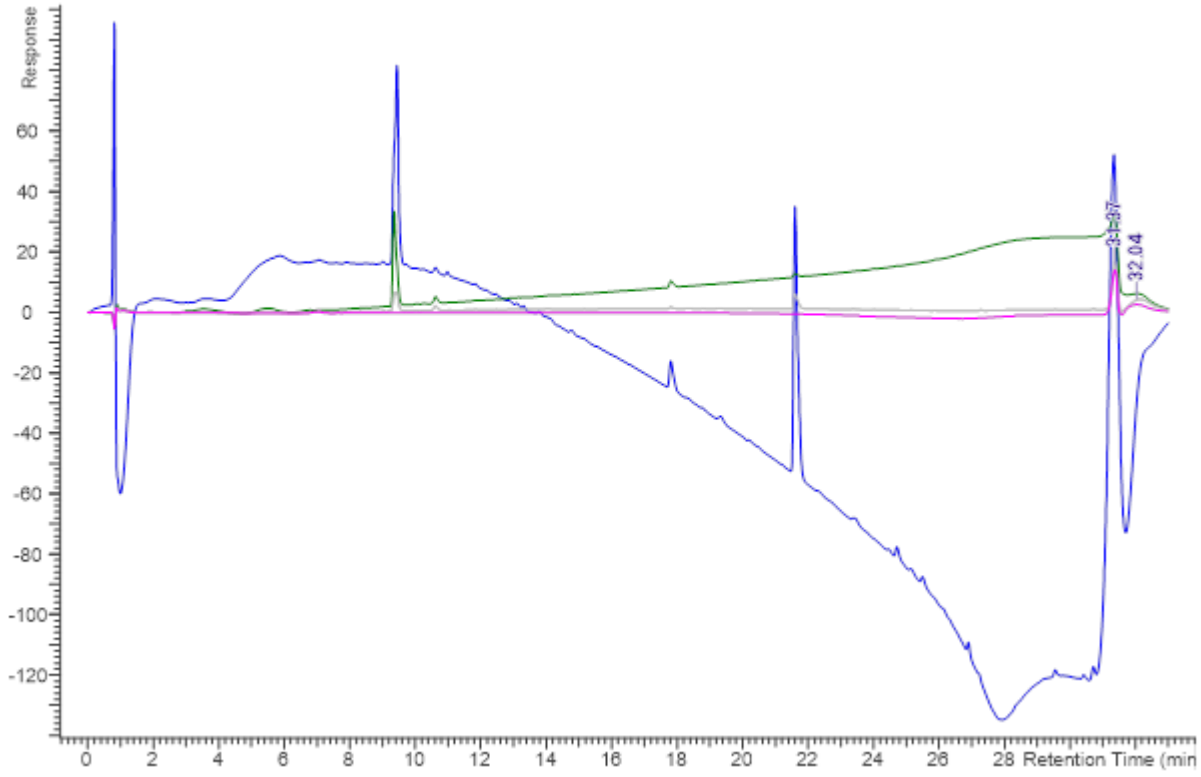
Amanita citrina DCM TLC Band 1



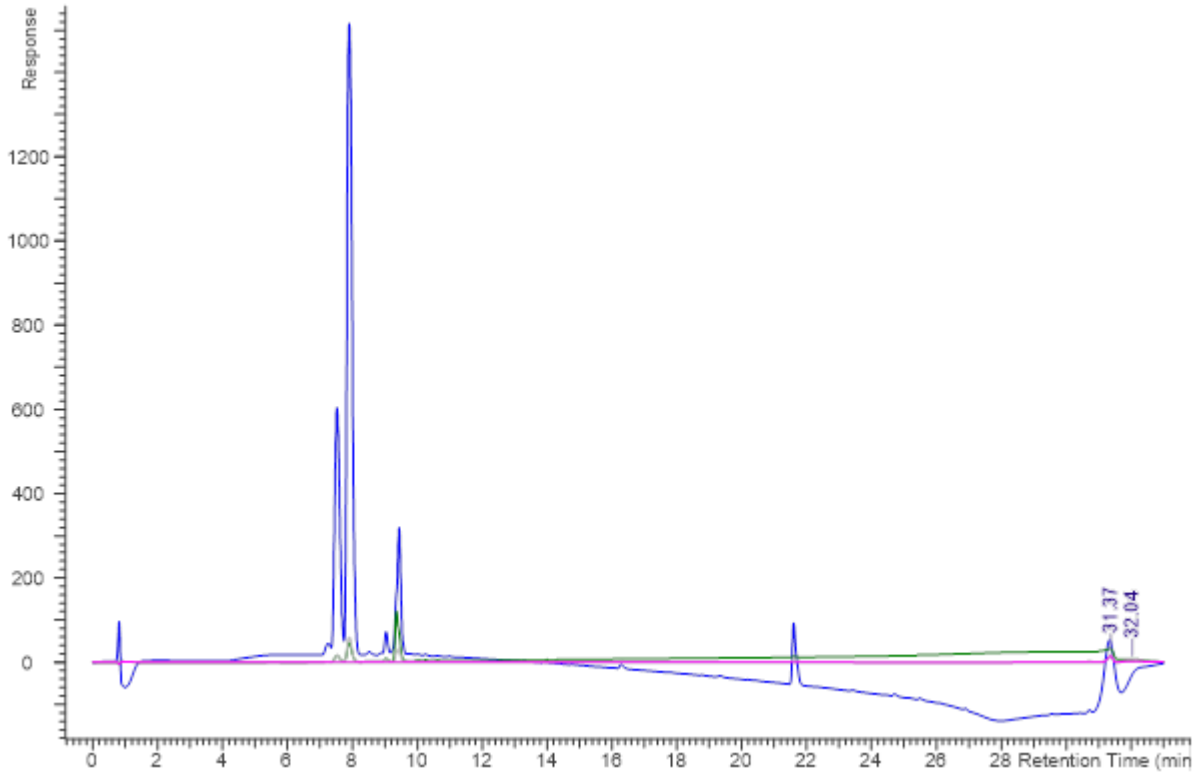
Amanita citrina DCM TLC Band 2



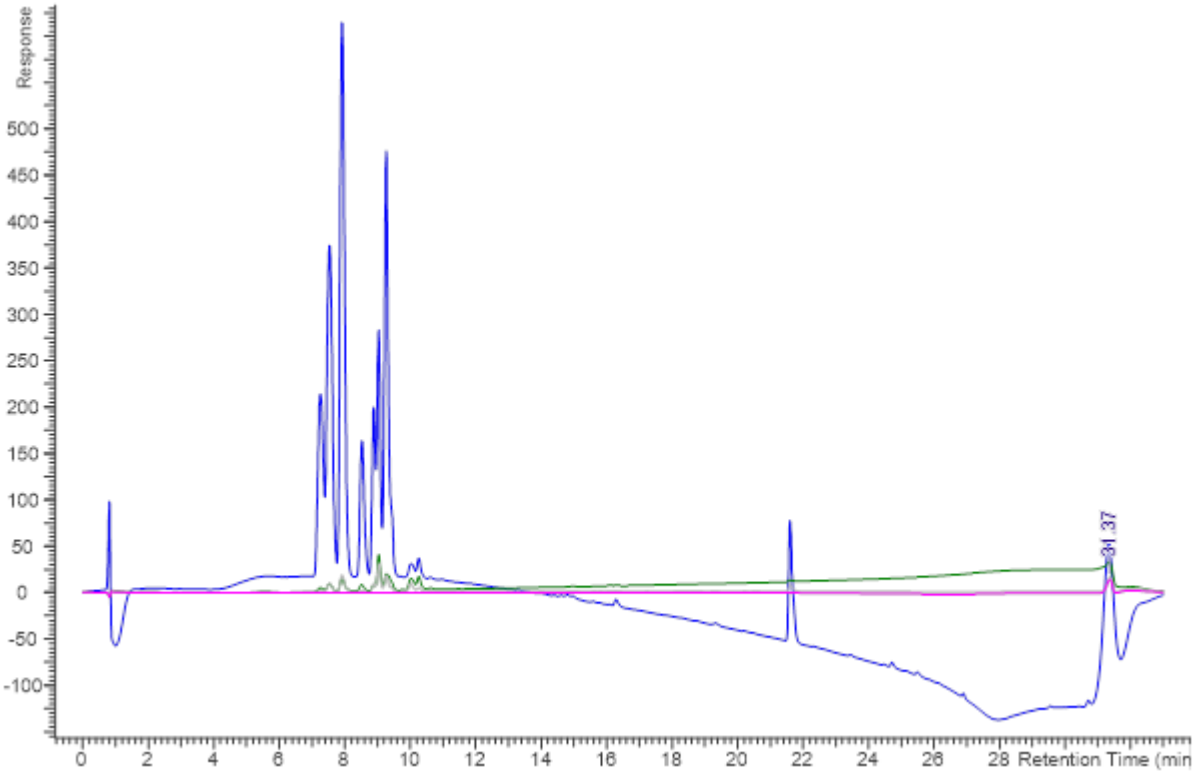
Amanita citrina DCM TLC Band 3



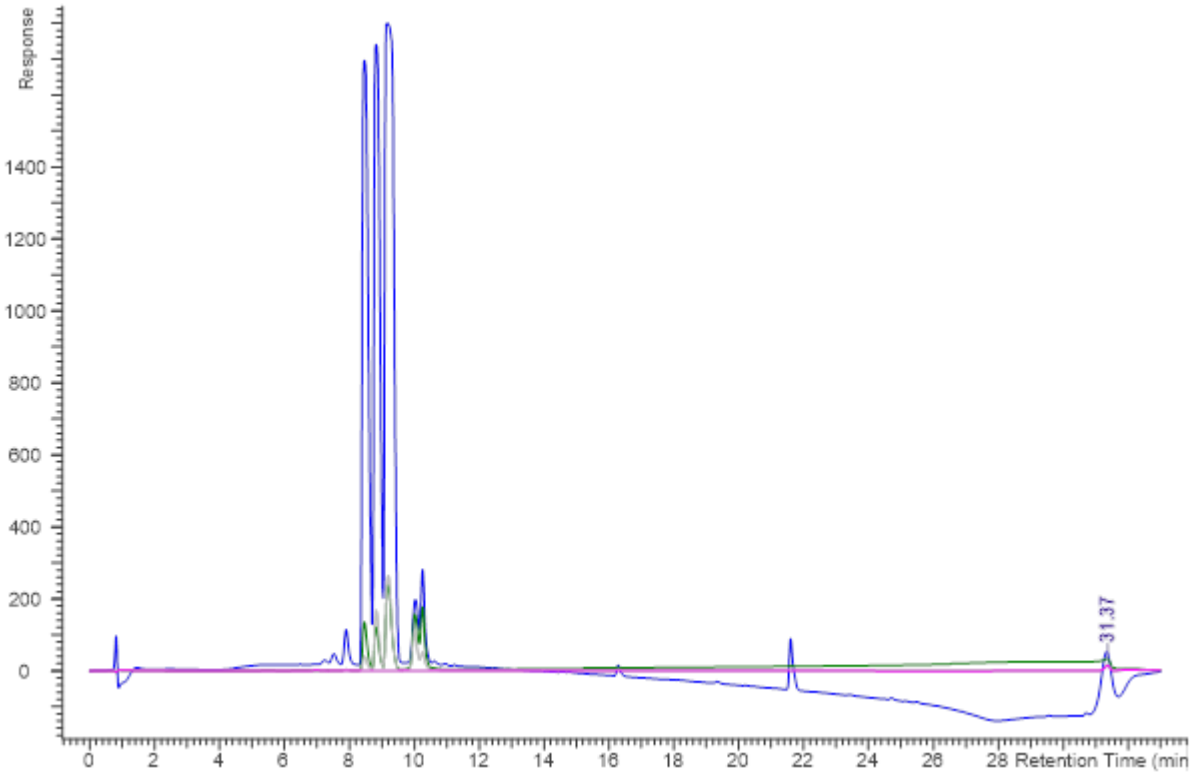
Gymnopilus junonius DCM TLC Band 1



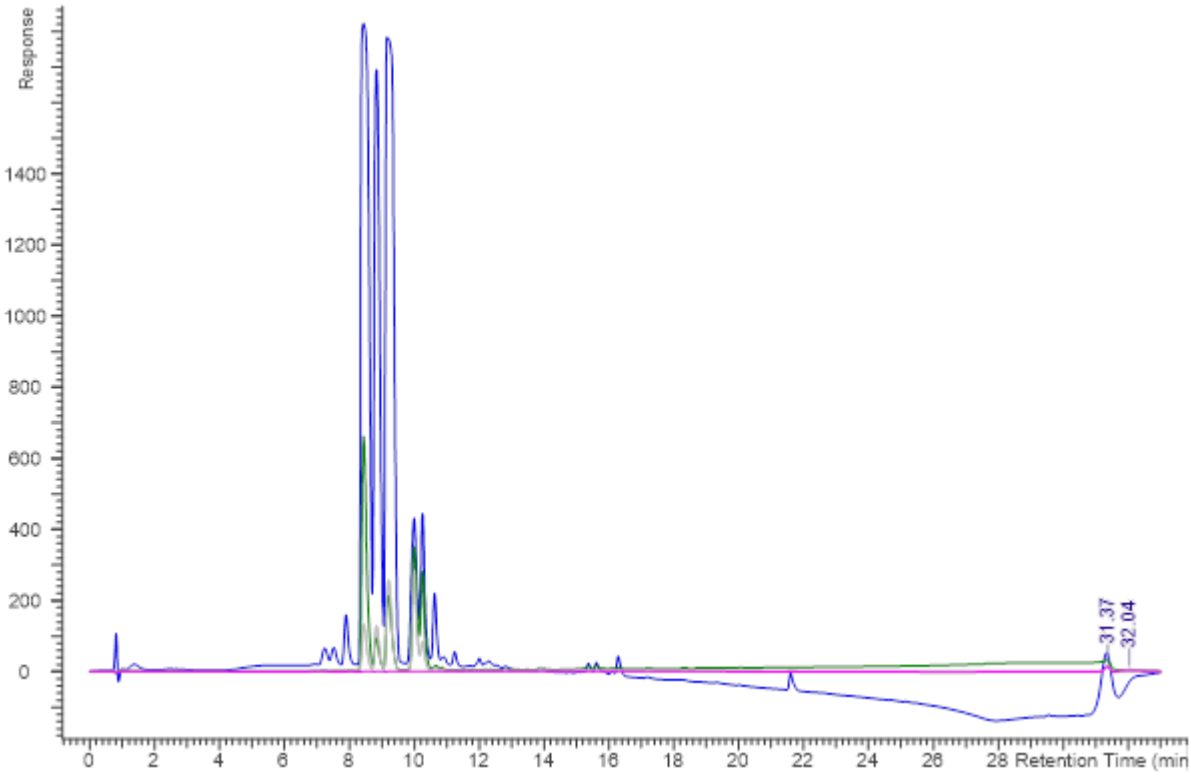
Gymnopilus junonius DCM TLC Band 2



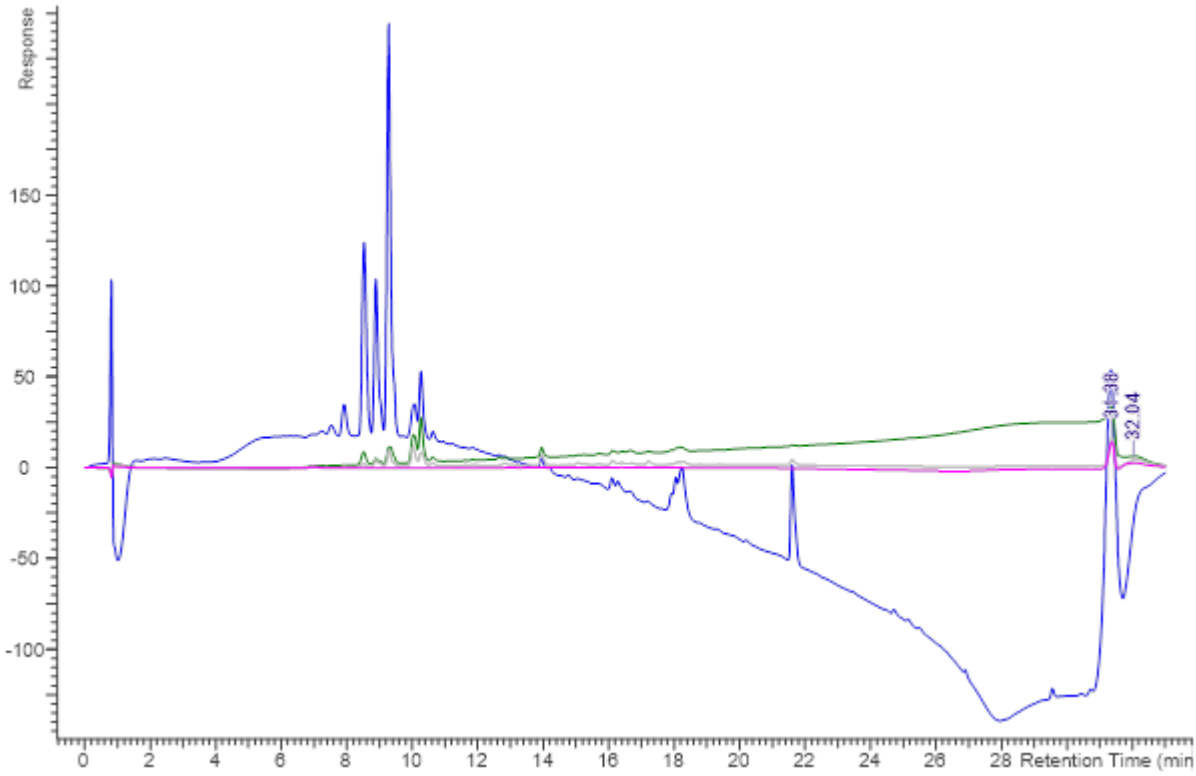
Gymnopilus junonius DCM TLC Band 3



Gymnopilus junonius DCM TLC Band 4



Gymnopilus junonius DCM TLC Band 5



Gymnopilus junonius DCM TLC Band 6

Gymnopilus junonius DCM TLC Band 7 is not displayed as no peaks were detected in the chromatograms

4. Media composition

Media ingredients for the three main media used in the investigation of *Gibellula pulchra* and *Onygena equina*

Potato Dextrose Broth (PDB) or Potato Dextrose Agar (PDA)

- Dextrose 4 g/L
- Malt extract 10 g/L
- Yeast extract 4 g/L
- (Agar 12 g/L for agar plates)

YM (pH 6.3)

- Dextrose 4 g/L
- Malt extract 10 g/L
- Yeast extract 4 g/L
- (Agar 12 g/L for agar plates)

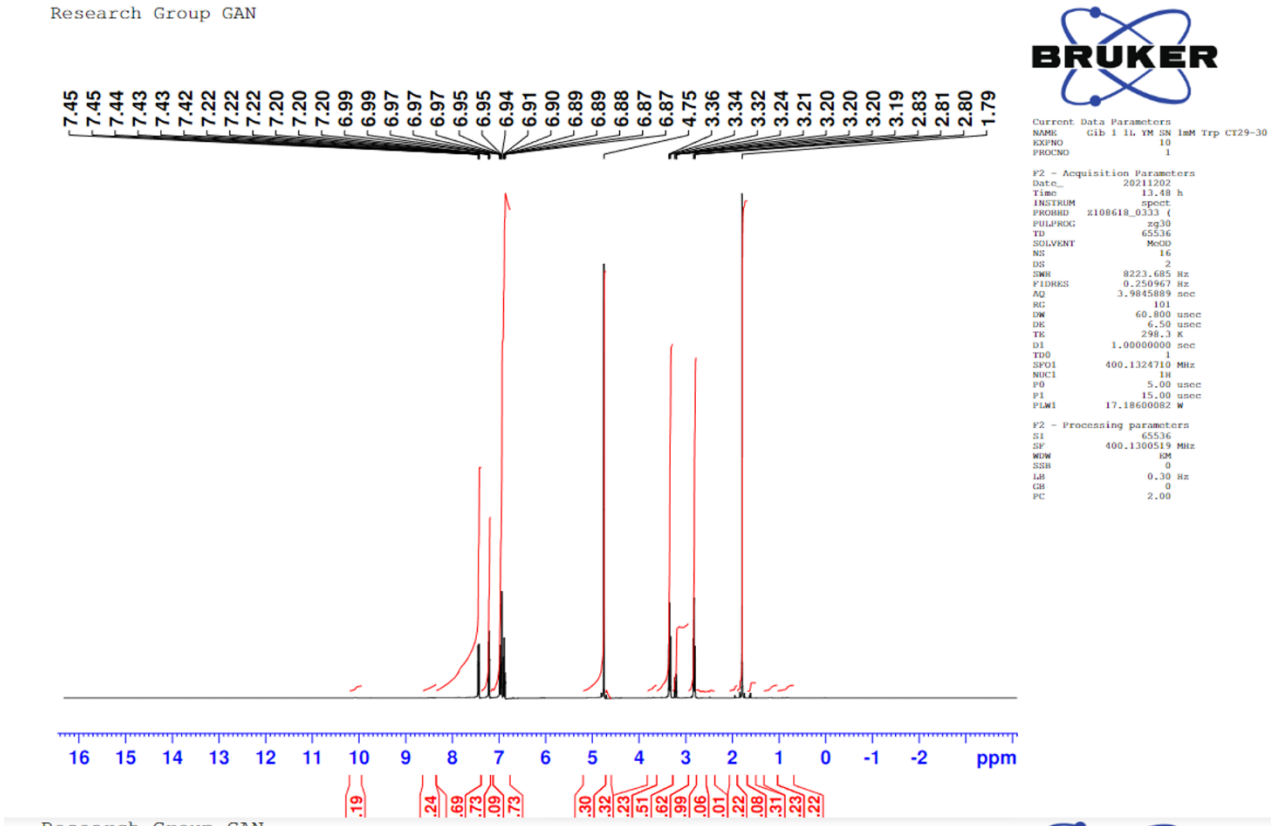
ZM ½ (Sugar(pH 7.2)

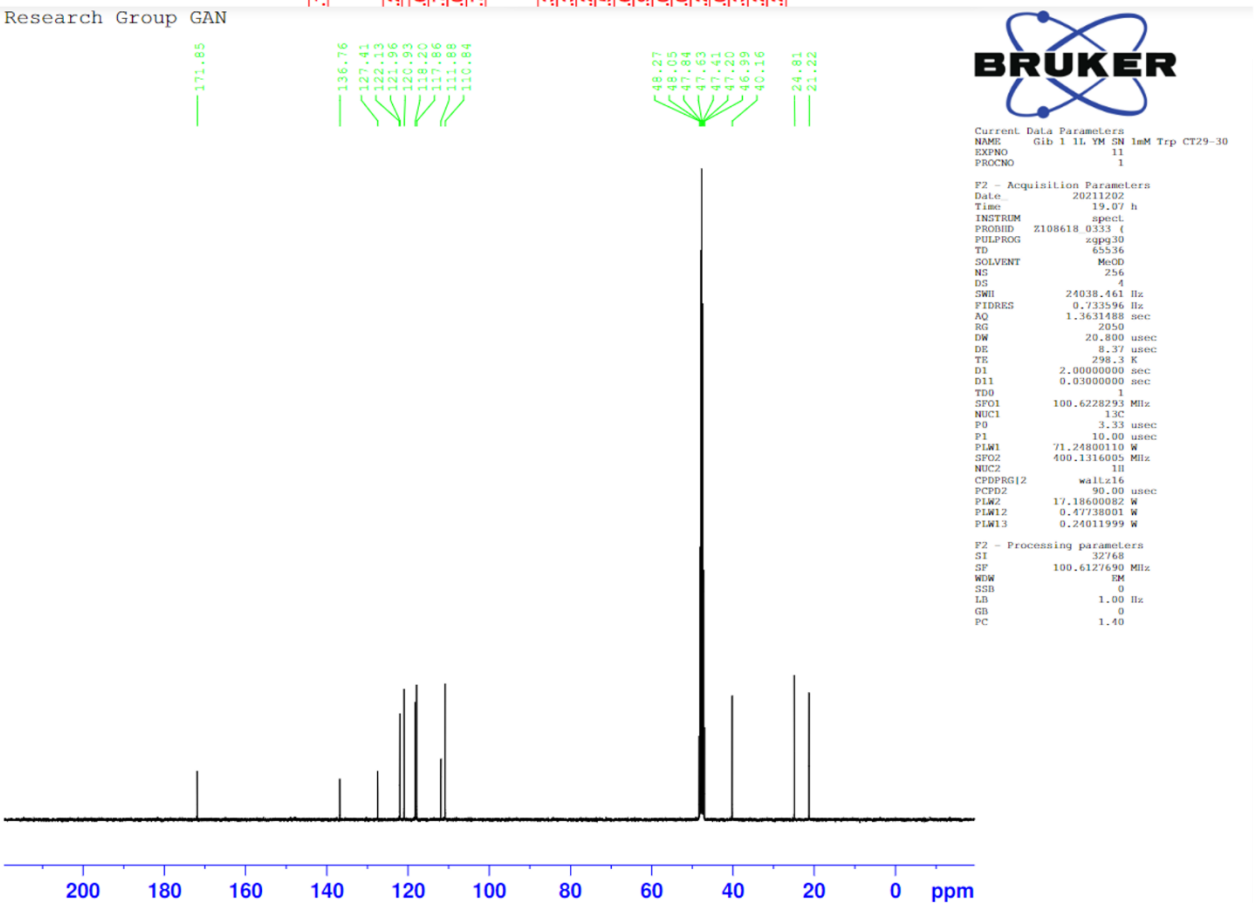
- Dextrose 1.5 g/L
- Molasses 5 g/L
- Oatmeal 5 g/L
- Sucrose 4 g/L
- Mannitol 4 g/L
- Calcium carbonate 1.5 g/L
- Ammonium sulphate 0.5 g/L
- Lactalbumin hydrolysate 0.5 g/L
- (Agar 12 g/L for agar plates)

Q6 ½ (Cottonseed meal medium) (pH 7.2)

- Dextrose 2.5 g/L
- Glycerol 10 g/L
- Cotton seed flour 5 g/L
- (Agar 12 g/L for agar plates)

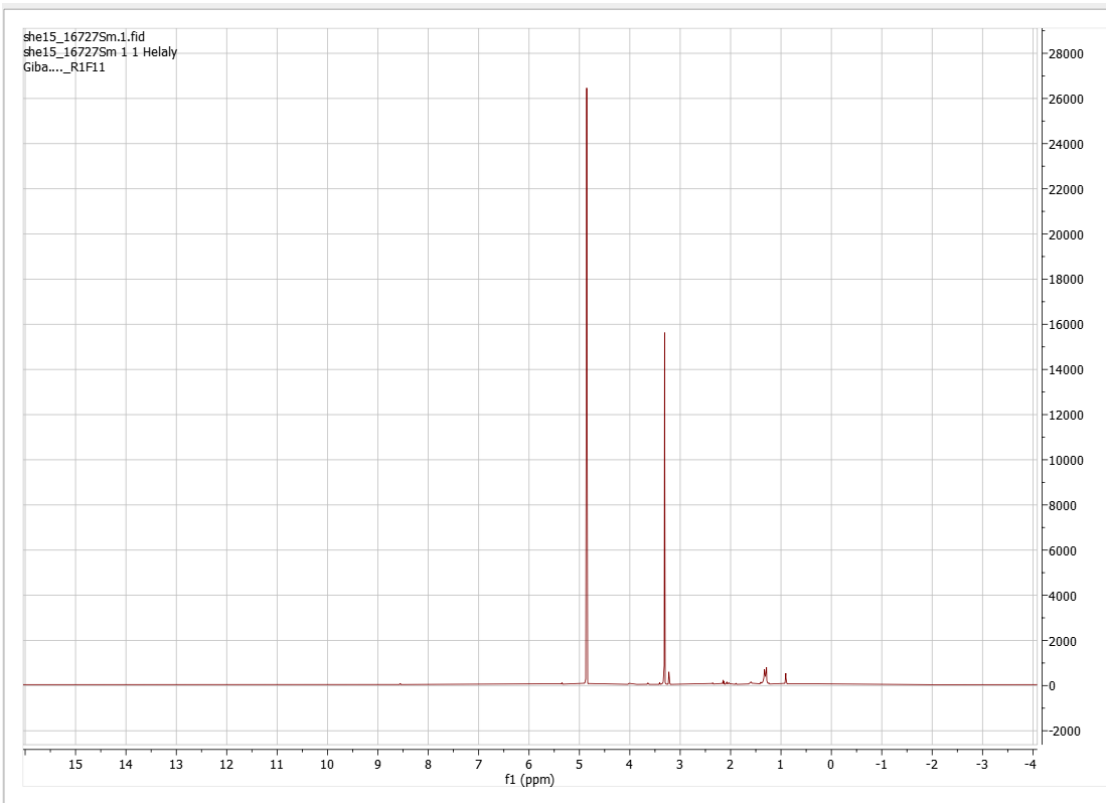
5. Full Acetyltryptamine Proton and Carbon NMR spectra obtained



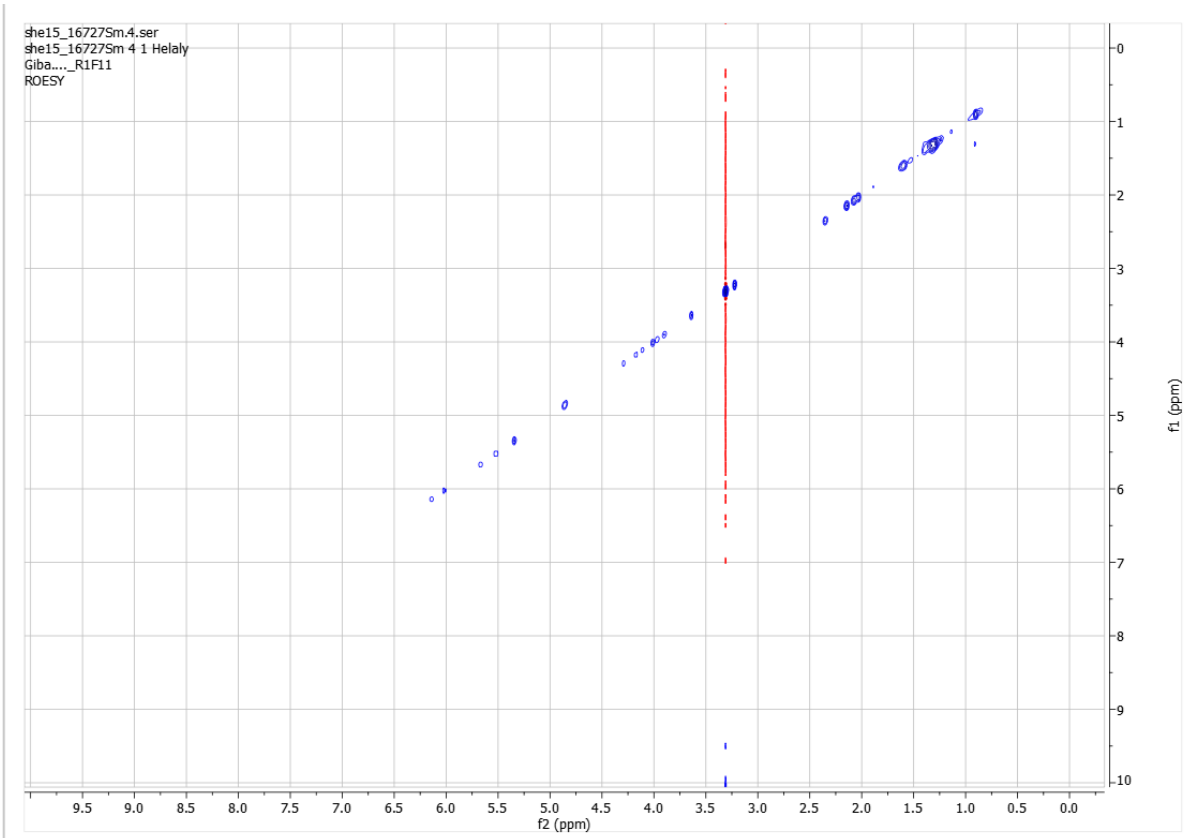


6. NMR spectra of fraction 11 (F11) obtained from *Gibellula pulchra*

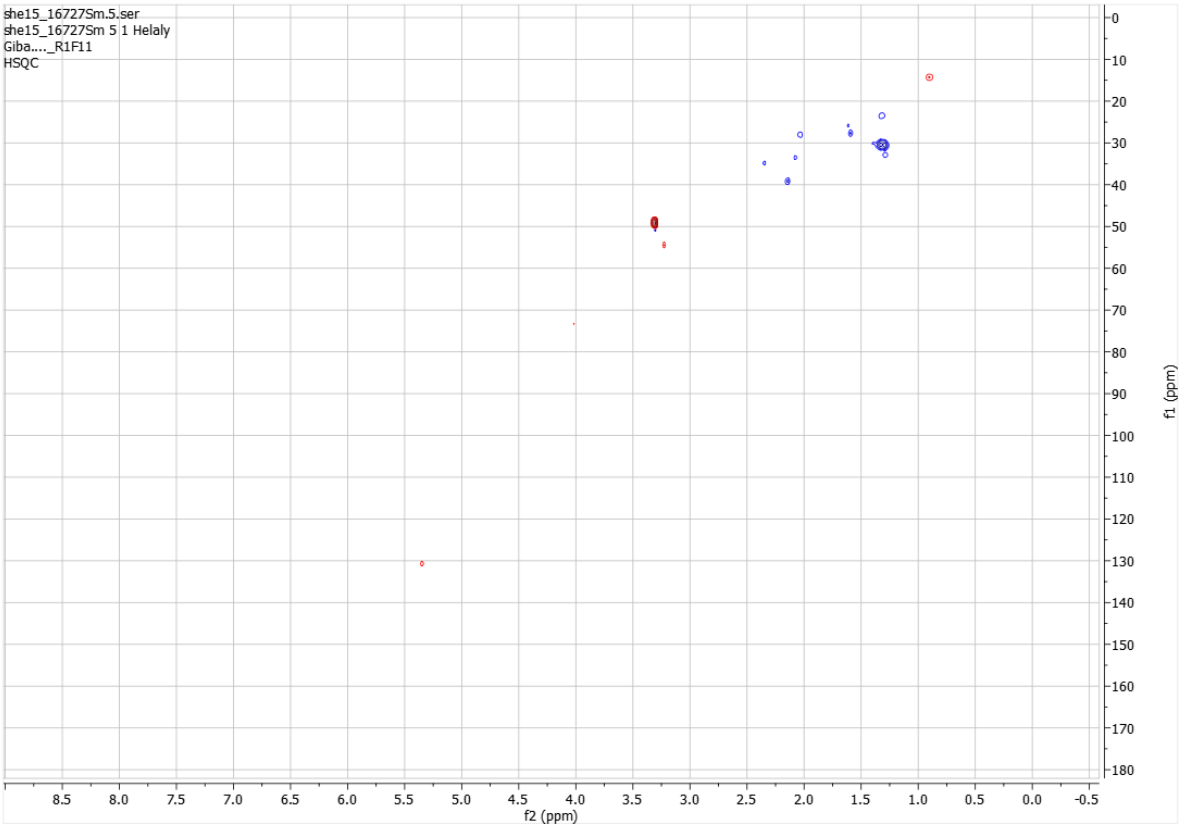
Proton



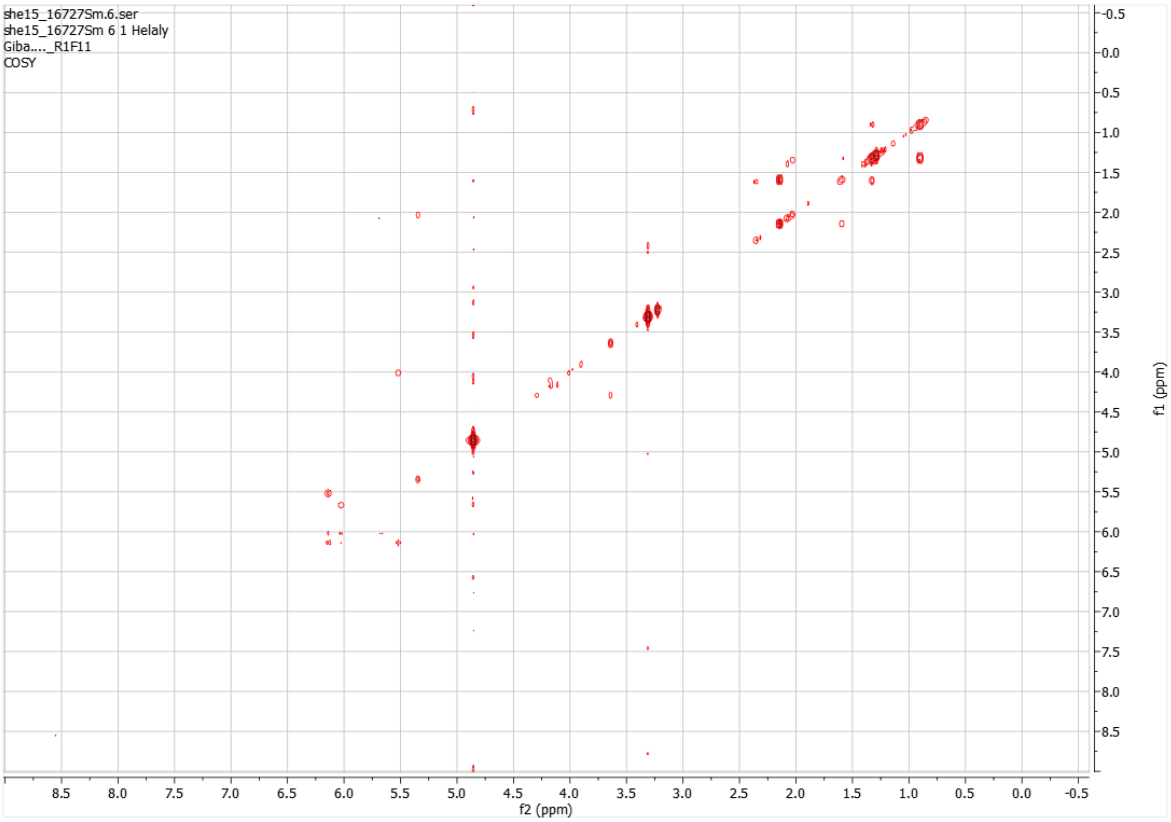
ROESY



HSQC


she15_16727Sm.5.ser
she15_16727Sm 5 1 Helaly
Giba..._R1F11
HSQC
f2 (ppm)
f1 (ppm)

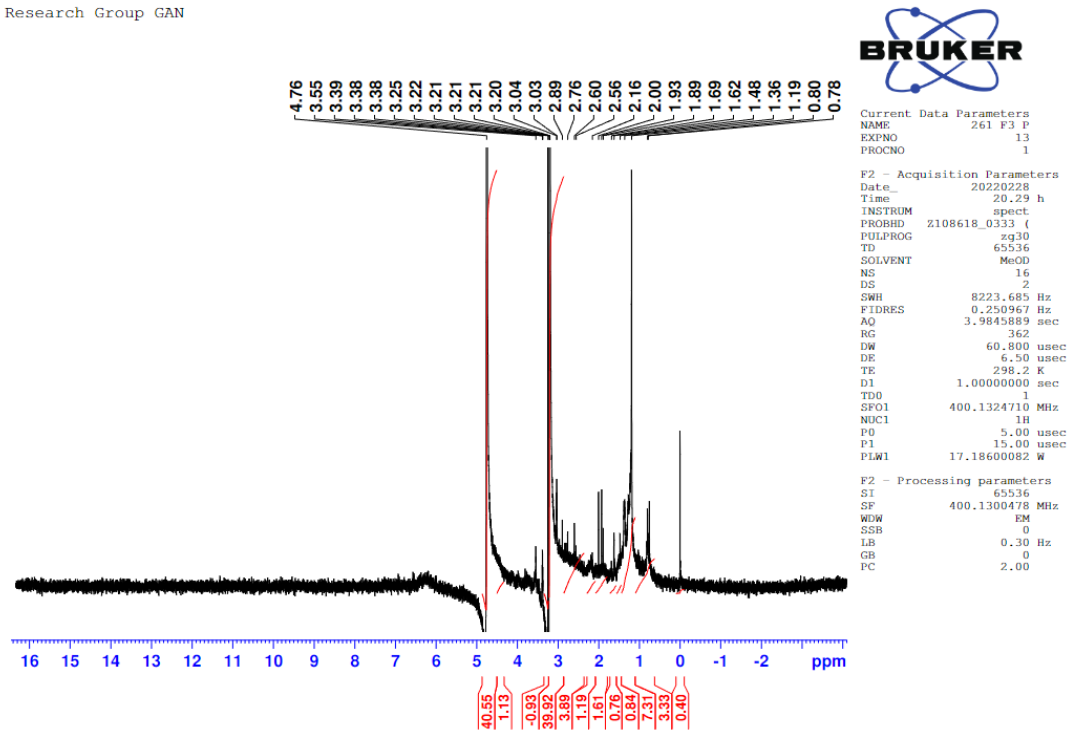
COSY


she15_16727Sm.6.ser
she15_16727Sm 6 1 Helaly
Giba..._R1F11
COSY
f2 (ppm)
f1 (ppm)

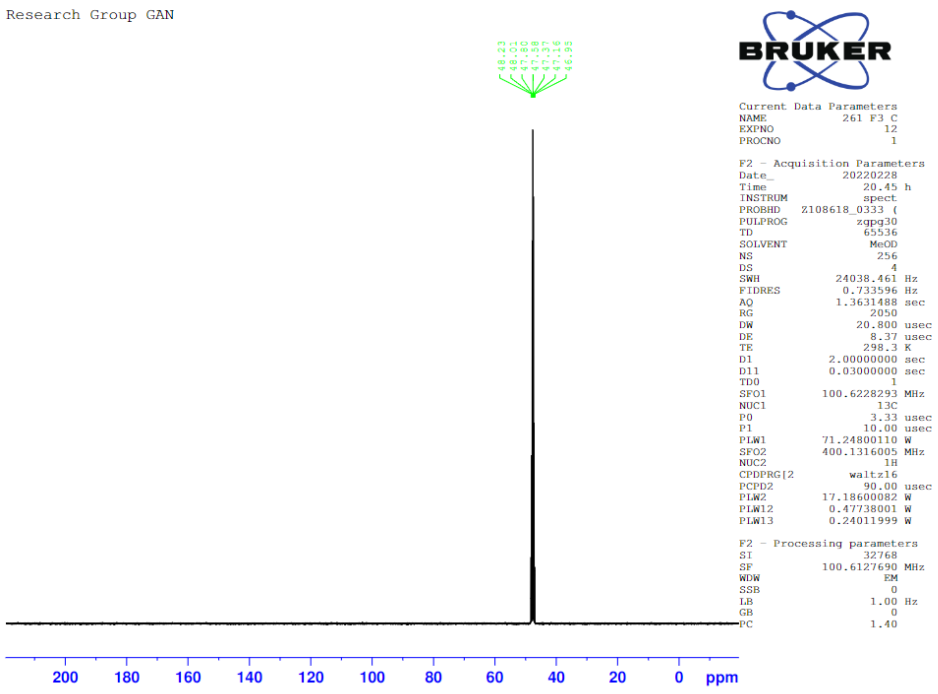
7. Locations of sample collections reported in this research

Sample	Date collected	Location	Grid reference	Lat/Long (decimal)
Onygena equina	15/09/2018	Redisher wood, Bury	SD 77160 15789	53.638113 , -2.346920
Gibellula Sample 1	16/06/2018	Foulden Common, Norfolk	TF 764000	52.569218 , 0.60139936
Ganoderma resinaceum	31/08/2018	Eaton, Norwich	TG 19874 06034	52.607560, 1.245862
Inonotus obliquus	12/08/2018	Sherwood Forest, Mansfield	SK 61444 68113	53.206547 , -1.0815132
Fomitopsis pinicola	12/08/2018	Sherwood Forest, Mansfield	SK 61456 68209	53.207403 , -1.0813200
Trametes versicolor	15/08/2018	UEA Campus, Norwich	TG 19341 07126	52.617576 , 1.2387335
Fomes fomentarius	16/06/2018	Foulden Common, Norwich	TF 764000	52.569218 , 0.60139936
Tremella mesenterica	17/09/2018	UEA Campus, Norwich	TG 19126 07251	52.618784 , 1.2356436
Amanita pantherina	30/10/2021	Broome, Norfolk	TM 34791 91461	52.470557, 1.455452
Amanita citrina	08/08/2019	Formby, Liverpool City Region	SD 28191 06245	53.547906, -3.085235
Amanita strobiliformis	16/10/2020	UEA Campus, Norwich	TG 19607 07704	52.622653 , 1.2430340
Gymnopilus junonius	13/10/2018	Witherslack, Grange-Over-Sands	SD 43634 83923	54.247869, -2.866535
Laetiporus sulphureus (Yew)	03/06/2018	Coldharbour, Surrey	TQ 15228 44067	51.184227 , -0.35270065
Laetiporus sulphureus (Yew)	01/06/2020	Preston, Lancashire	SD 57028 42825	53.879832 , -2.6551580
Laetiporus sulphureus (Willow)	05/06/2018	UEA Campus, Norwich	TG 19009 07067	52.617184, 1.233802
Laetiporus sulphureus (Willow)	05/06/2018	UEA Campus, Norwich	TG 19009 07067	52.617184, 1.233802

8. Proton NMR obtained from isolated compound (m/z 260) referenced in Chapter 3



9. Carbon NMR obtained from isolated compound (m/z 260) referenced in Chapter 3



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